CHAPTER ELEVEN

Rationale of Design of Anti-HIV Drugs

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Contents

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1 INTRODUCTION

Acquired immunodeficiency syndrome or AIDS was first reported in the United States in 1981. The disease has killed more than 400,000 people in the United States and almost 21.8 million men, women, and children worldwide. Estimates by the Joint United Nations Program on HIV/AIDS (UNAIDS) and World Health Organization (WHO) indicate that, by the end of the last decade, over 36 million people were infected with the human immunodeficiency virus (HIV) that causes AIDS. Epidemiologists have shortened the name acquired immune deficiency syndrome to the chilling abbreviation AIDS. The disease is considered as the first catastrophic pandemic of the second half of the twentieth century and it was once described as the modern plague. Despite the fact that more is known now than ever before about how to prevent the spread of the epidemic, 5.3 million new HIV infections were reported during the year 2001 alone. Unless a cure is found or a life-prolonging therapy can be made more available throughout the world, the majority of those now infected with the HIV will die within a decade. These deaths will not be the last because the virus continues to spread, causing 16,000 new infections a day.

Recently, Western countries have recorded a decrease in the death rate imputed to AIDS. This success has been largely attributed to the development and availability of chemotherapeutic agents that inhibit the infectivity of the HIV-1 virus, the causative agent of AIDS. The advancement in HIV research had led to unveiling of the details of the virus life cycle and the mechanism of its replication inside the immune system T-cells. The understanding of functions and molecular structures of the viral enzymes have contributed to the discovery of a large number of anti HIV drugs through rationale drug design. Several HIV inhibitors have been successfully introduced to the physician's arsenal to combat AIDS infections. All clinically approved anti-HIV drugs interfere with the virus life cycle by inhibiting one of two enzymes: the transcriptase or the protease. The two enzymes play a vital role in the process of viral replication inside the T-cell. Unfortunately, the efficacy of available drugs

is always challenged by the development of drug resistance within short periods of clinical use (1). The development of drug resistance is attributed to the continuous evolution of mutant HIV strains. The problem of drug resistance has been solved, in part, by the combination therapy approach. Combination therapy requires administering more than one drug, each of which works by a different mechanism to suppress the virus ability to mutate. However, even with this approach, new viral resistant strains still emerge during therapy. The increasing development of resistance and cross-resistance to the currently available anti-HIV medications prompted researchers to seek alternative anti-HIV drug classes beyond the reverse transcriptase and protease inhibitors.

Efforts are directed now toward developing drugs capable of interfering with other steps of HIV life cycle including its adsorption, entry, fusion, uncoating, or integration with the T-cell lymphocytes. To understand the medicinal chemistry of anti-AIDS drugs and the rationale of their design, it is important for the reader to be acquainted with some information about both AIDS (the disease) and HIV (its causative organism). The present chapter covers various anti-HIV drug classes, the rationale of their design, and both the site and the molecular mechanism of action of each class. The clinical and cellular aspects of AIDS, together with its molecular biology, genetics nature, structure, and life cycle of the HIV, are also addressed.

2 HUMAN RETROVIRUSES AND AIDS EPIDEMIOLOGY

AIDS is a disease state that arises from infection with the human immune deficiency virus (abbreviated HIV). The virus belongs to a family of viruses called retroviruses. This group of viruses is characterized by having RNA as its genetic material. Retroviruses are capable of constructing DNA from RNA, a reversed process of the normal replication schemes, hence the classification of this group as retroviruses. Only three retroviruses are known to infect humans: HTLV I (Human T Leukemia Virus I), HTLV II (Human T Leukemia Virus II), and HTLV III (Human T Lymphotropic Virus). Although HLTV I (first isolated in 1978) (2, 3) and HTLV II (first isolated in 1982) (4) are both known to cause leukemia, HTLV III was confirmed to be the etiological virus for the acquired immunodeficiency syndrome (AIDS). To distinguish HTLV III from the other two human retroviruses, HTLV I and HTLV II, it was given the name human immunodeficiency virus (HIV). HIV is further subcategorized into HIV-1 and HIV-2. The first causes AIDS, whereas the second causes lymphadenopathy (or swelling of the lymphatic glands). HIV-2 is sometimes called "lymphadenopathy associated virus," or LAV, to avoid name confusion with HIV-1, the most detrimental subtype to the immune system.

3 HIV INFECTION AND ITS PATHOLOGICAL EFFECTS

3.1 Mode of Viral Transmission

HIV is transmitted by several infection mechanisms, including unprotected sex, sharing of hypodermic needles for injection for drug use, from HIV-infected mother to her baby, human breast milk, or blood transfusion and coagulation products. The disease was found to be common among certain social groups who share a common lifestyle such as homosexuals and drug users.

3.2 Cellular Picture of the Infection

Once the virus is inside the body, it targets a certain type of cell called a T4 lymphocyte, a white blood cell that has a central role in regulating the immune system, specifically the CD4 helper T-cells (5). After the virus entry to the T4 cell, it may remain latent until the lymphocyte is immunologically stimulated by a secondary infection. Then the virus bursts into action, reproducing itself so furiously that the new virus particles escaping from the cell riddle the cellular membrane with holes and the lymphocyte ultimately dies. HIV starts its replication cycle in the host cell with the help of a viral enzyme called reverse transcriptase. The enzyme uses viral RNA as a template to assemble a double strand of viral DNA. The latter travels to the cell nucleus and inserts

itself among the host's chromosomes, which provide the machinery for HIV-1 transcription and translation.

3.3 Clinical Picture of HIV Infection

3.3.1 Immunosuppressive Effects. HIV infection results in a progressive decrease in the helper T-cell count, the hallmark of AIDS, from a normal value of 800–1300 cells/cm^2 of blood to below 200, which may give rise to a life-threatening illness **(6).** The suppressed immune system leaves the patient vulnerable to the so-called opportunistic infections by agents that would not harm a healthy person. The most common of such infections is pneumonia caused by Pneumocystis carinii, a widespread but generally harmless protozoan. Most of the clinical complications of AIDS patients result from such infections.

3.3.2 Neurological Effects. Although most of the attention given to the HIV virus has gone to suppression of the immune system or AIDS, the virus is associated also with brain diseases and several types of cancer. The brain and spinal cord disease caused by HIV was first detected in brain and spinal cord tissues from AIDS patients in 1984. The chief pathologies observed in the brain, which appears to be independent of the immune deficiency, are an abnormal proliferation of the glial cells that surround the neurons and lesions resulting from loss of white matter (which is, along with gray matter, one of the two main types of brain tissue). This can ultimately give rise to a wide range of neurological symptoms such as dementia and multiple sclerosis.

3.3.3 Carcinogenic Effects. Cancer is the third main type of HIV pathological manifestations. Whereas the neurological effects of HIV are distinct from the immune deficiency, cancer has a more ambiguous relation to the crippling process of the immune system. People infected with the virus have an increased risk of at least three types of human tumor. One is known as Kaposi's sarcoma, a rare tumor of blood vessel tissue in the skin or internal organs and had been known to exist mainly among older Italian and Jewish men and in Africa. The second type of cancer is carcinomas, including skin cancers, which are often seen in the mouth or rectum of infected homosexuals. The third major type of cancers observed with HIV infection is B-cell lymphomas (tumor originating in B-lymphocytes). The appearance of the same types of cancer among certain groups of young white middle-class males provided the first basis for how HIV infection is transmitted because this group turned out to have a history of homosexuality.

4 HIV STRUCTURE AND MOLECULAR BIOLOGY

4.1 Virus Structure (7)

The AIDS virus exists as a small particle called HIV virion. The particle is spherical in shape, with a diameter of roughly 1000-Å units (one ten thousandth of a millimeter). The particle is covered by a membrane, made up of lipid (fatty) bilayer material that is derived from the outer membrane of the host cell. Studding the membrane are glycoproteins (proteins with sugar chains attached). Each glycoprotein has two components: gp41 spans the bilayer membrane and gp120 extends beyond it. The combination of the bilayer membrane and its glycoproteins is called the envelope, which covers the virus core made up of proteins designated p24 and p18. The viral **RNA** is carried in the core, along with several copies of the enzyme reverse transcriptase, which catalyzes the assembly of the viral **DNA.** The envelope glycoproteins have an important role in HIV's entry to its host cell and also in the death of the host cell. The envelope proteins include some regions that are constant in all HIV strains, some of which are highly variable and others that are intermediate. The virus entry to the cell seems to depend on an interaction between one or more of the constant regions and certain molecules in the cell membrane. The envelope proteins are also involved in the process of budding of a new virus particle from the host cell, which may leave a hole in the cell's surface. On the other hand, T4 cells, which are the most significant target in HIV infection, selectively interact with the outer envelope of the virus, a process that sets up the mechanism for the virus entry into the

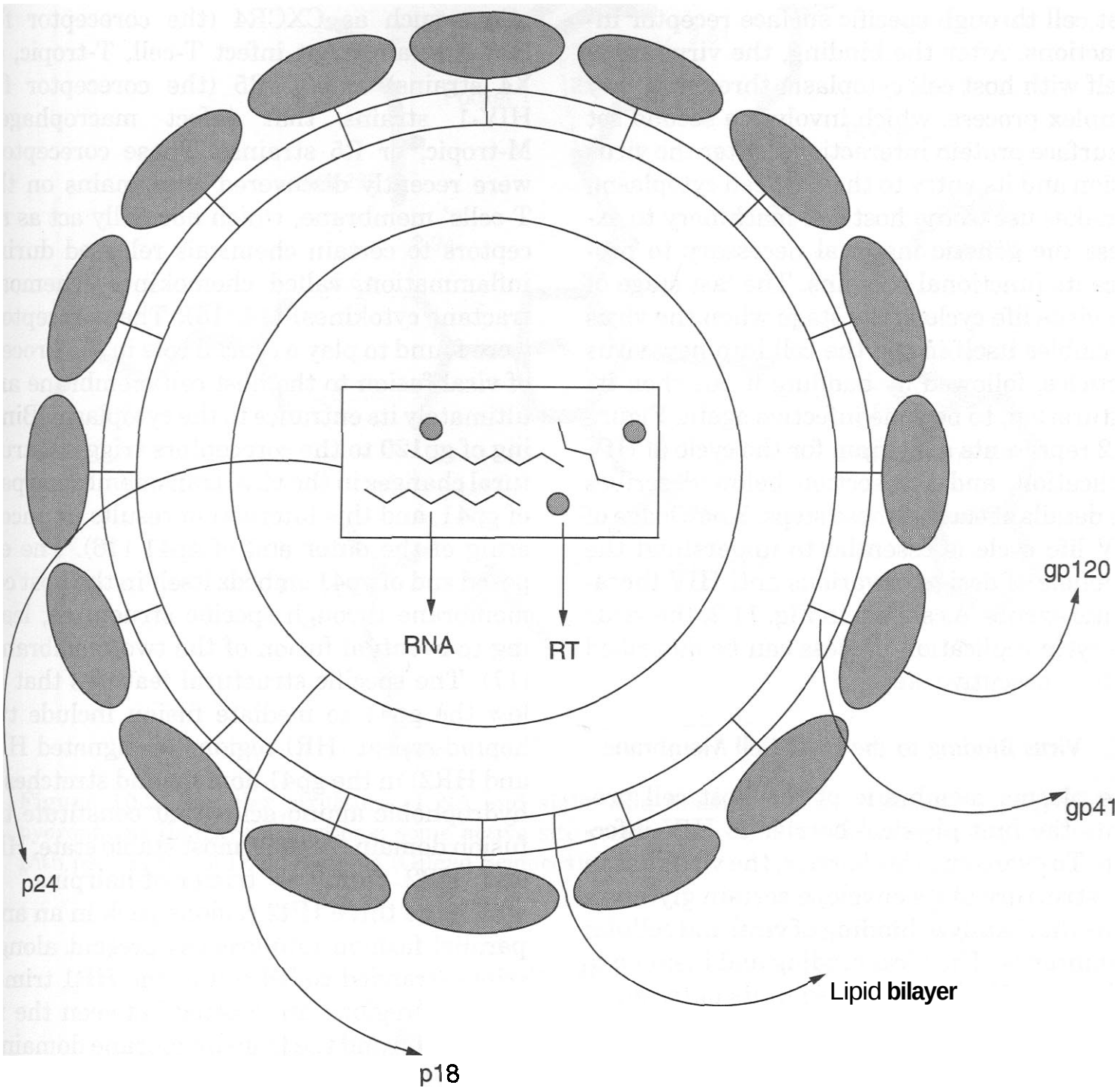


Figure 11.1. Schematic structure for the AIDS virus.

cell. Figure 11.1 depicts a schematic representation for HIV-1 virus structure.

4.2 HIV Genome

HIV is perhaps the most complicated retrovirus studied. In common with other retroviruses, it contains genes encoding the core structural protein (gag), the outer envelope glycoprotein (env), and viral DNA polymerase (pol) (the reverse transcriptase). It also contains sequences at either end of the genome called the long terminal repeats (LTRs), which contain regulatory elements associated with the virus replication (8). HIV is quite unusual, however, in containing five nonstructural genes, tat-III, artltrs, 3'-orf, sor, andr, some of which are known to have important regulatory functions for viral replication (9). These unusual genes encode proteins that help to control the expression of viral genes to form its RNA. The latter is spliced to yield an array of messenger RNAs (mRNAs), from which all viral proteins are synthesized. The core proteins and reverse transcriptase are made from an mRNA corresponding to the entire genome. One splice yields the envelope protein mRNA and a second, the small mRNA from which the tat and trs proteins are made.

5 HIV LIFE CYCLE (10)

The overall process of HIV life cycle and replication starts with the virus binding to the

host cell through specific surface receptor interactions. After the binding, the virus fuses itself with host cell cytoplasm through a very complex process, which involves a second set of surface protein interactions. After the virus fusion and its entry to the host cell cytoplasm, it makes use of the host cell machinery to express the genetic material necessary to produce its functional proteins. The last stage of the virus life cycle is the stage when the virus assembles itself inside the cell into new virus particles, followed by budding it out then its maturation, to become infective again. Figure 11.2 represents a diagram for the cycle of HIV replication, and the section below describes the details of each of these steps. Knowledge of HIV life cycle is essential to understand the rationale of design of various anti-HIV therapeutic agents. As shown in Fig. 11.2, the virus life cycle replication process can be described in 10 consecutive steps.

5.1 Virus Binding to the Host Cell Membrane

The plasma membrane of the host cell presents the first physical barrier to HIV infection. To overcome this barrier, the virus has in the structure of its envelope certain glycoproteins that catalyze binding of viral and cellular membranes. The viral binding and fusion machinery in HIV is contained in its outer envelope glycoproteins gp120 and gp41. Both glycoproteins are generated by proteolysis of the160-kDa precursor, gp160 (11–13). The glycoprotein gp120 binds with high affinity to another specific glycoprotein on the T-cell surface, called the CD4 receptor. This interaction represents the first step in the process of HIV infection.

5.2 Virus Fusion with Host Cell Cytoplasm and its Uncoating

Membrane fusion is another important step in the process of HIV infection. The fusion process allows HIV entry into the host cell cytoplasm. The process is mediated by the second envelope viral glycoprotein gp41. However, engagement of gp41 with the host cell membrane requires gp120, to bind, in addition to the CD4 receptors, to a second set of receptors on the T-cell surface, called coreceptors. These coreceptors are designated by certain abbreviations such as CXCR4 (the coreceptor for HIV-1 strains that infect T-cell, T-tropic, or X4 strains) and CCR5 (the coreceptor for HIV-1 strains that infect macrophages, M-tropic, or R5 strains). These coreceptors were recently discovered as domains on the T-cells' membrane, which normally act as receptors to certain chemicals released during inflammation, called chemokines (chemoattractant cytokines) (14, 15). The coreceptors were found to play a crucial role in the process of viral fusion to the host cell membrane and ultimately its entrance to the cytoplasm. Binding of gp120 to the coreceptors triggers structural changes in the viral transmembrane part of gp41, and this interaction results in uncovering of the outer end of gp41 (16). The exposed end of gp41 embeds itself in the host cell membrane through specific structures, leading to eventual fusion of the two membranes (17). The specific structural features that allow the gp41 to mediate fusion include two heptad-repeat (HR) regions (designated HR1 and HR2) in the gp41 domain and stretches of hydrophobic amino acids that constitute the fusion domain. In their most stable state, HR1 and HR2 form a "trimer-of-hairpins," in which the three HR2 regions pack in an antiparallel fashion into grooves present along a triple-stranded coiled-coil of the HR1 trimer. The HR regions are located between the fusion region and the transmembrane domain of gp41. Structural reorganization of HR1 and HR2 thus brings the fusion site and the transmembrane domains together and promotes mixing of the viral and cellular membranes. After HIV enters the cell, the virus loses its outer envelope in a process known as uncoating, then releases its RNA genome (encapsulated with the p24; see Fig. 11.1) into the cytoplasm.

5.3 Viral DNA Formation by the Reverse Transcriptase

After uncoating, a complementary strand of DNA is copied from viral RNA by HIV-DNA polymerase (reverse transcriptase). Subsequently, a second copy (positive strand) of DNA is made so that the genetic information is encoded in a double-strand form of DNA (18). The viral RNA (now bound to the newly formed DNA) is degraded by another ribonu-

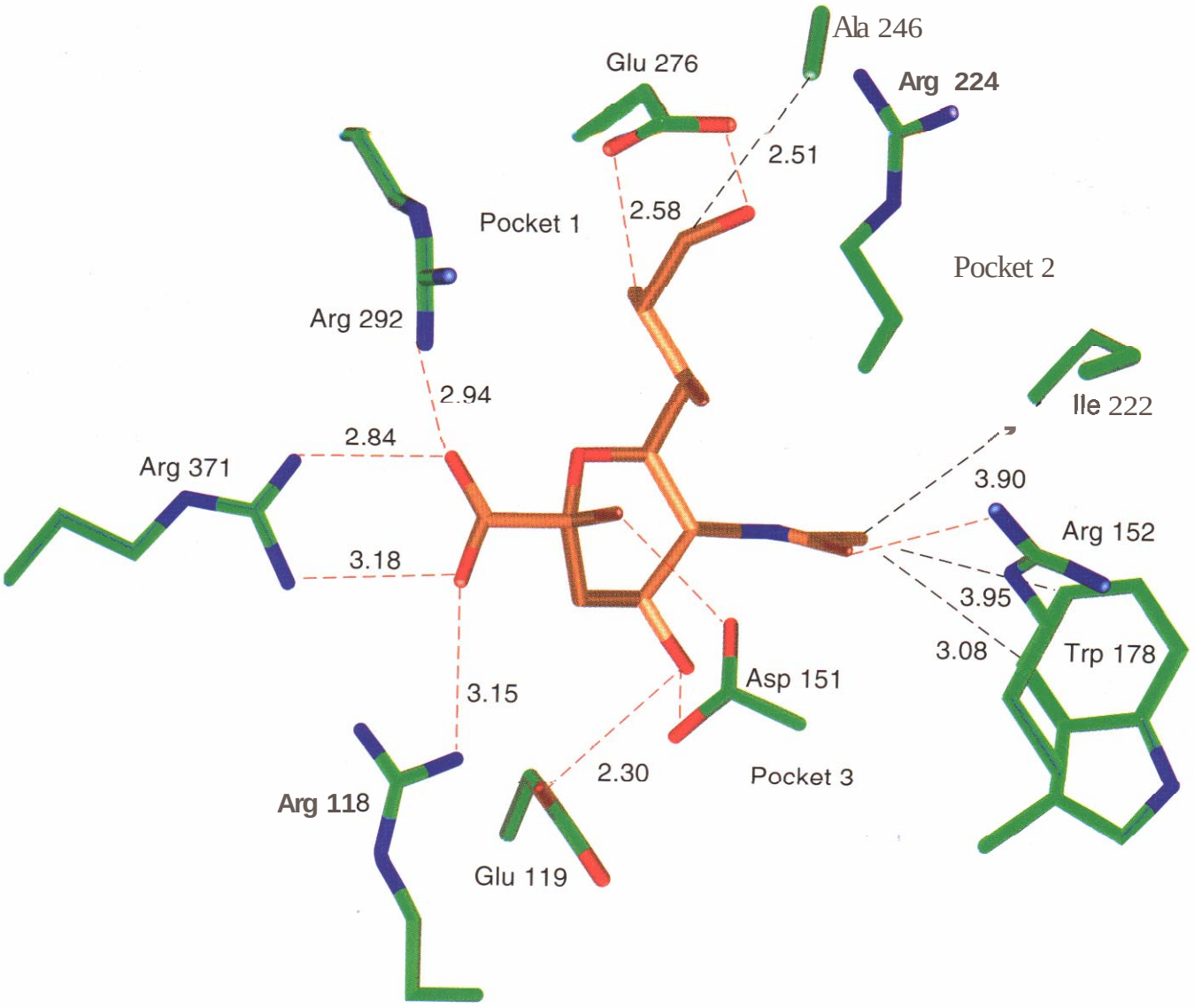


Figure 10.2. Complex structure of NA and sialic acid (dashes indicate H-bonding [red] and hydrophobic [black] interactions; some active site residues are omitted for clarity). Reproduced with permission of Dr. C. U. Kim (Gilead Sciences).

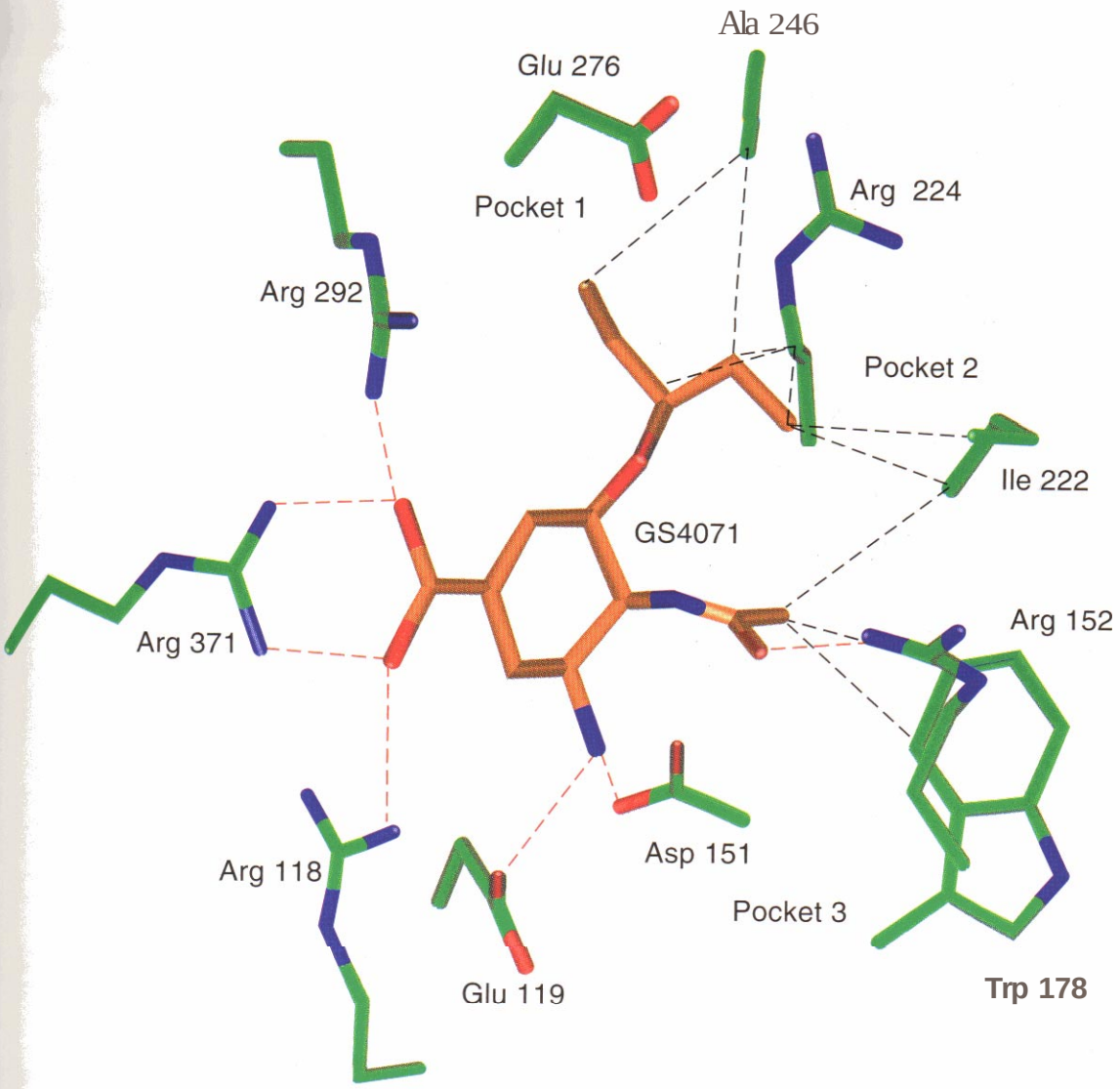


Figure 10.4. Complex structure of NA and GS 4071 (dashes indicate H-bonding [red] and hydrophobic [black] interactions; some active site residues are omitted for clarity). Reproduced with permission from Dr. C. U. Kim (Gilead Sciences).

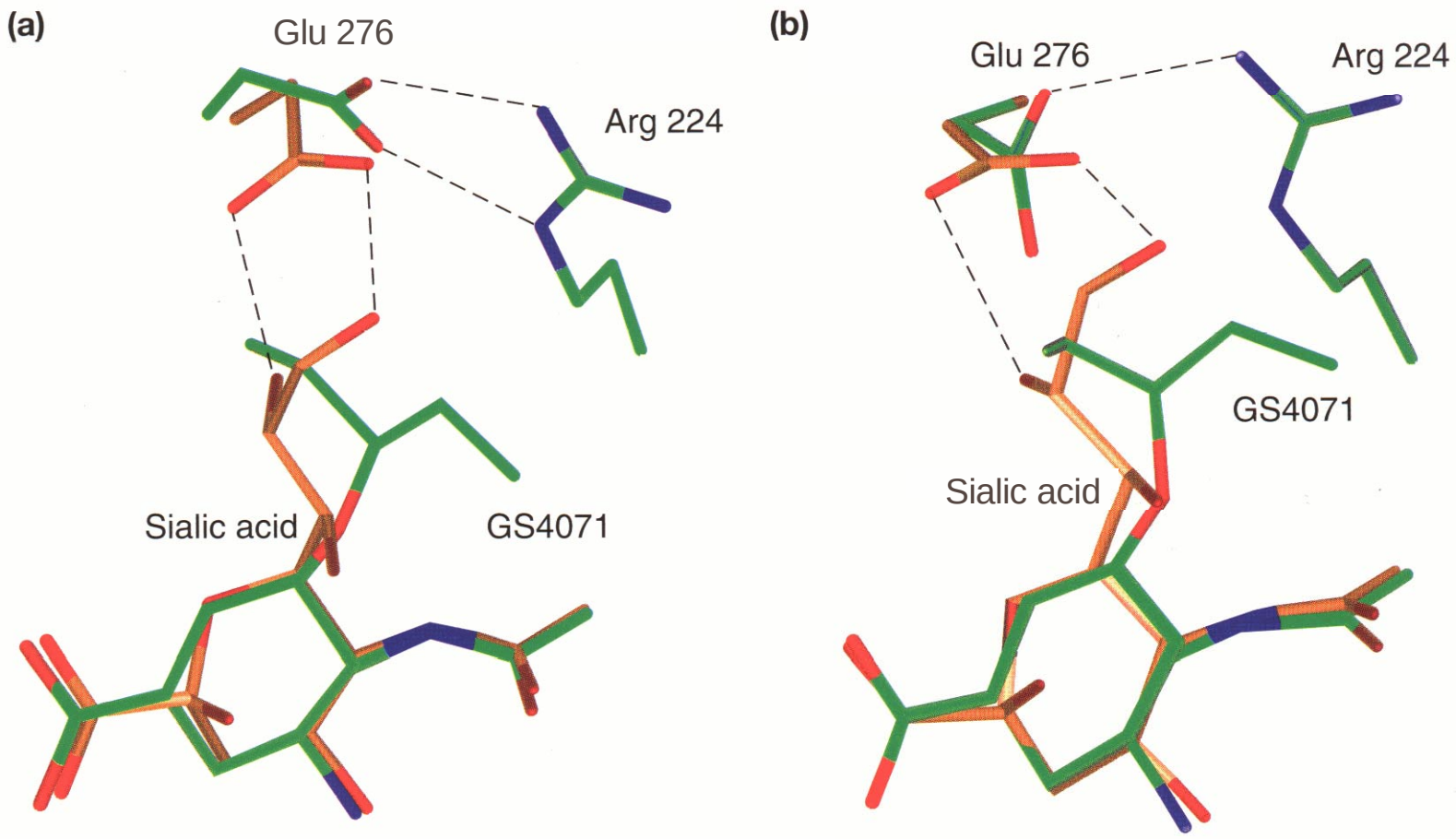


Figure 10.5. NA Glu-276 side-chain (a for type A neuraminidase complex; b for type B neuraminidase complex) can adopt alternative conformations on binding of sialic acid or GS4071 (dashes indicate H-bond; atoms are colored as following: blue for nitrogen, red for oxygen, brown for carbon in sialic acid complex, and green for carbon in GS4071 complex). Reproduced with permission from Dr. C. U. Kim (Gilead Sciences).

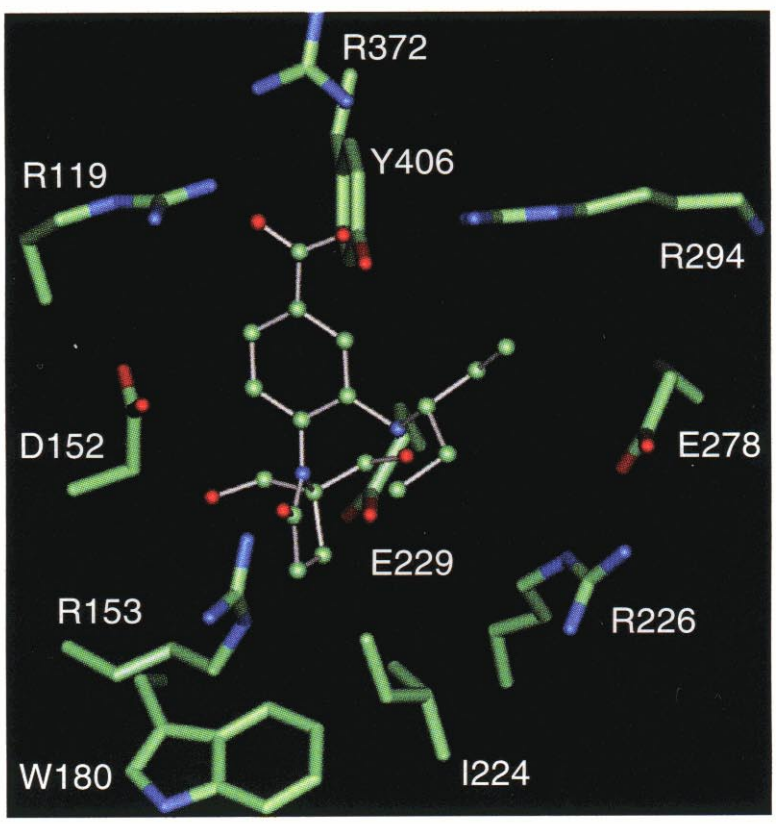


Figure 10.6. Complex structure of NA and BANA-206. Reproduced with permission from Dr. W. Brouillette (University of Alabama at Birmingham).

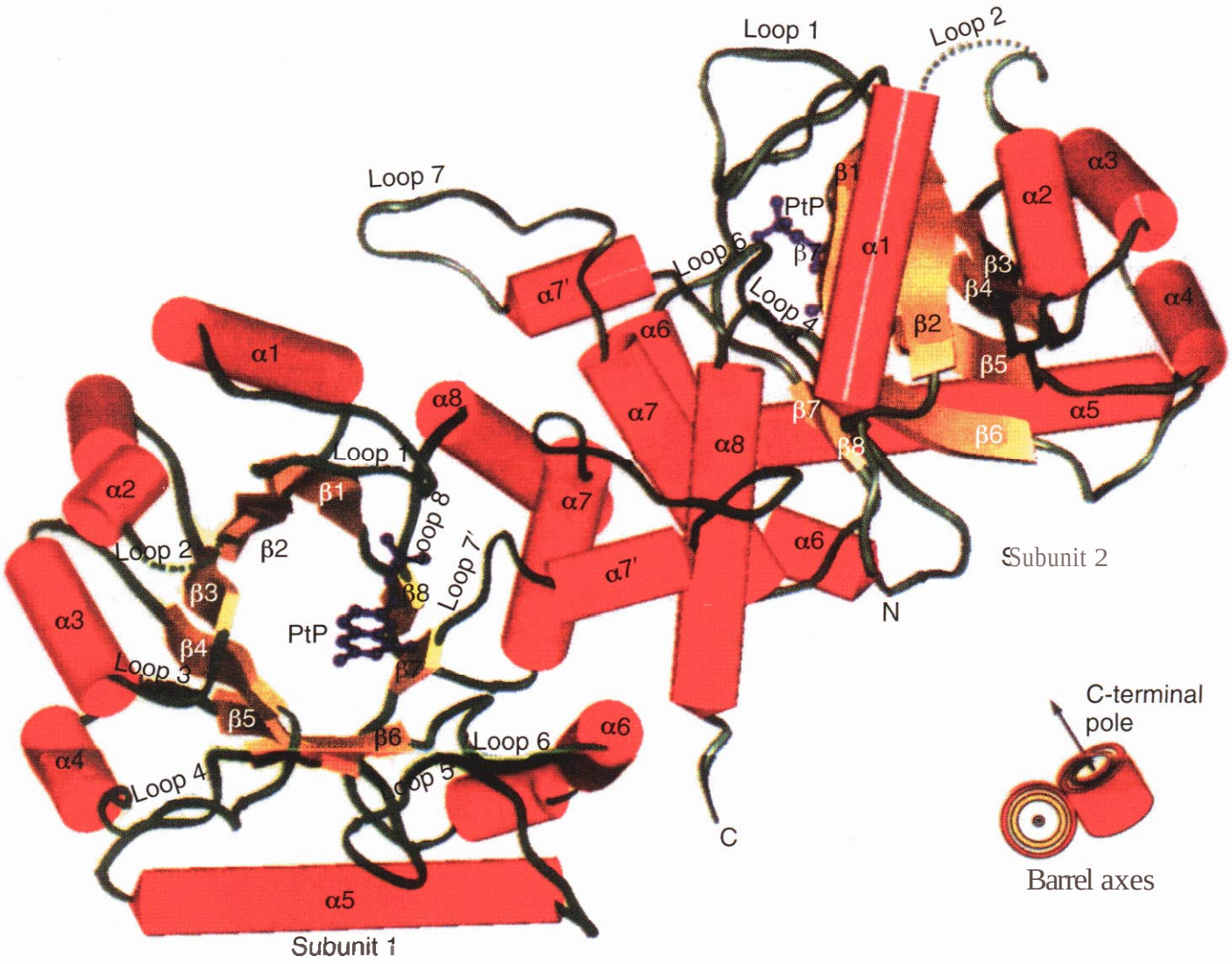


Figure 13.2. Overall folded structure of M. tuberculosis DHPS dimer (from Ref. 203).

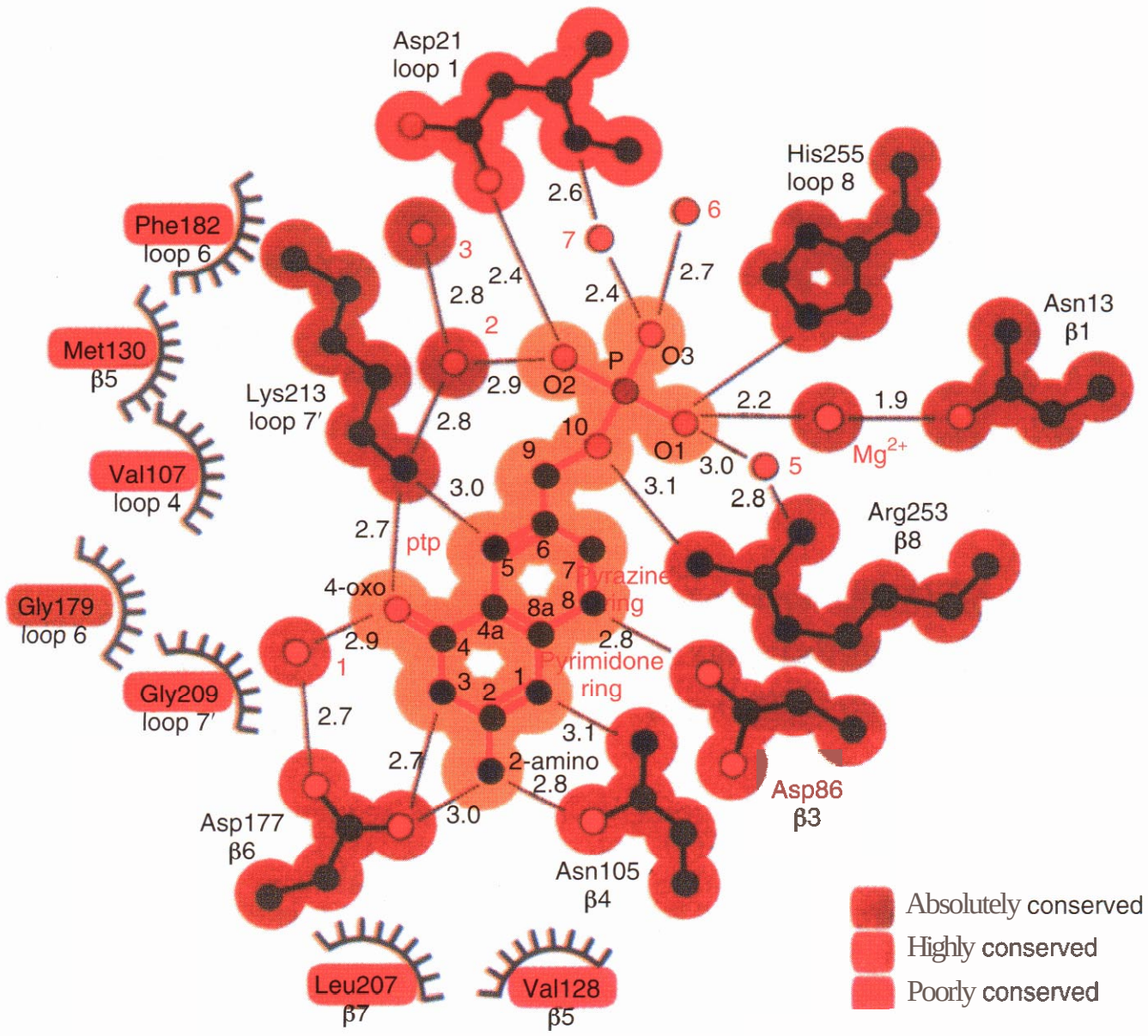


Figure 13.3. The pterin monophosphate binding site in M. *tuberculosis* DHPS (from Ref. 203).

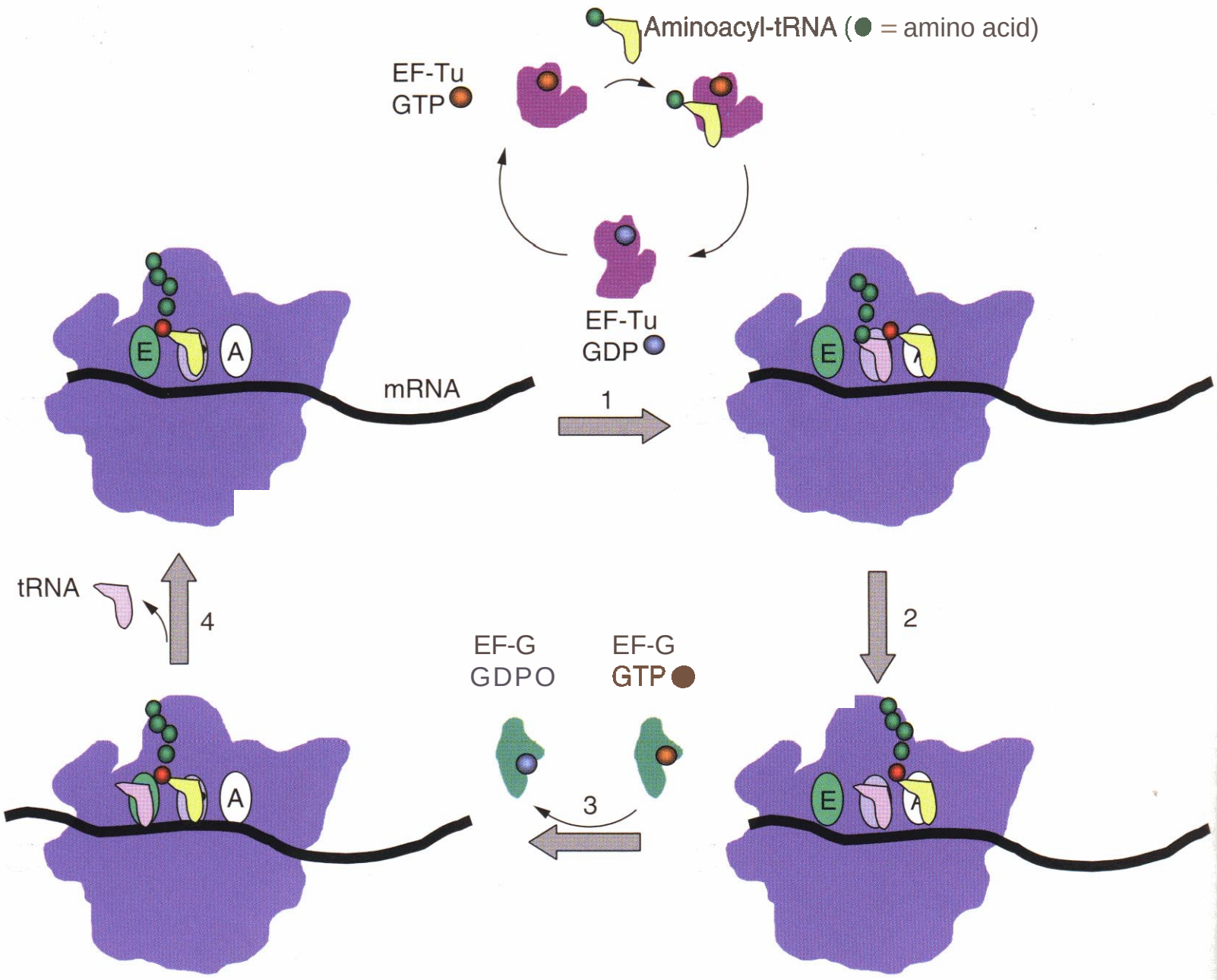


Figure 15.1. The bacterial protein synthesis elongation cycle. Step **1:** Recognition and binding of the cognate **aminoacyl-tRNA** to the anticodon in the A-site. Step 2: Peptidyl transfer resulting in elongation of the **peptide** by one amino acid residue. Step **3:** Translocation of the **peptidyl-tRNA** from the A-site to the P-site with concomitant movement of the unchanged **tRNA** to the E-site. Step 4: Exit of the unchanged **tRNA** completes the cycle.

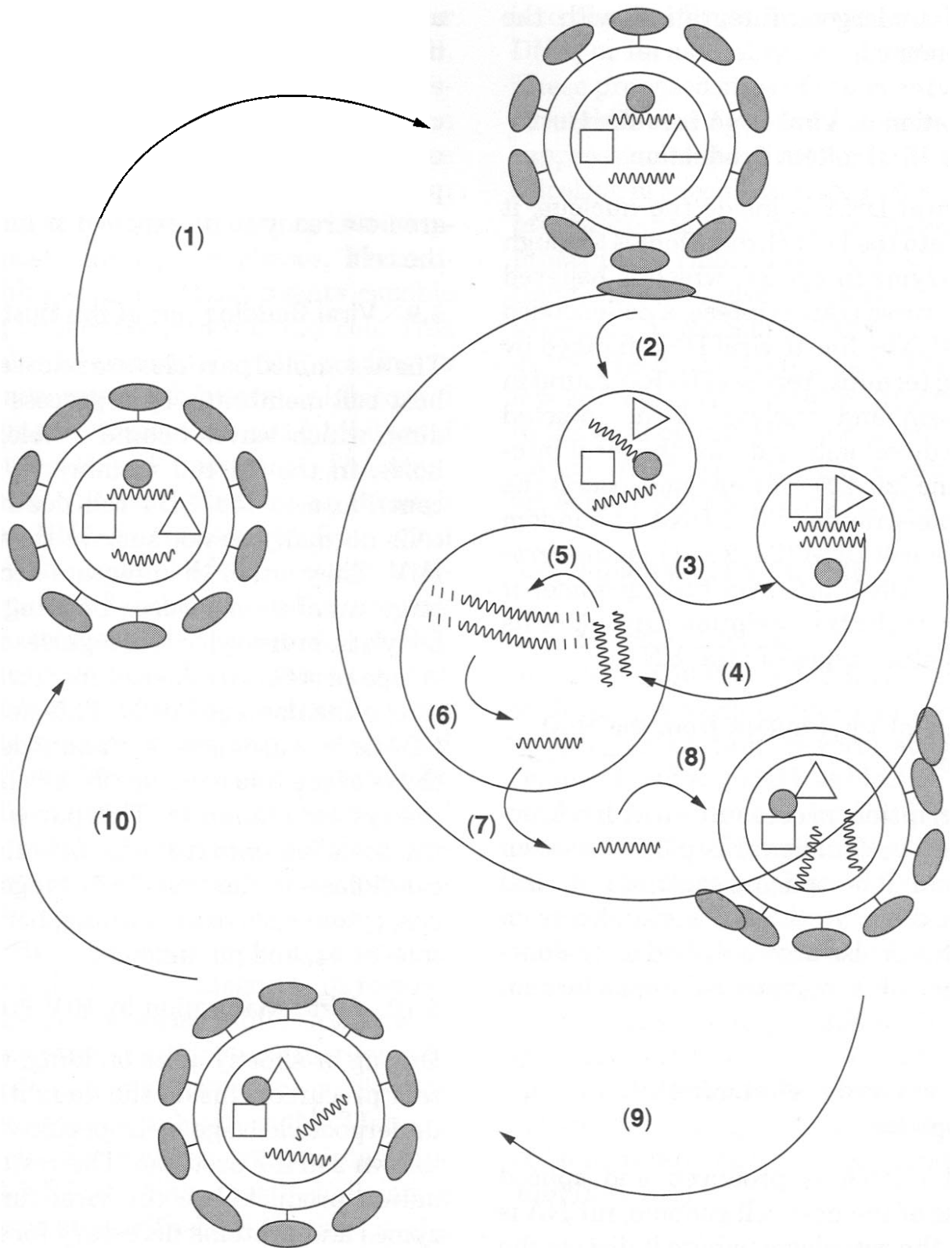


Figure 11.2. Schematic representation for HIV life cycle. **(1)** Binding of the virus to the T-cell through the gp120 and **CD4** receptors. (2) Fusion through viral gp41 and loss of its envelope, the uncoating. (3) Viral DNA formation by reverse transcriptase followed by RNase. (4) Viral DNA entry to the host cell nucleus through its nuclear pores. **(5)** Viral DNA integration into host cell DNA by integrase. (6)Splicing of viral RNA by host RNA polymerase to produce viral mRNA. (7) Migration of viral RNA to the cytoplasm as mRNA to encode the synthesis of viral proteins. (8) Assembly of the virion containing the viral proteins as a single chain. (9) Viral budding through the host cell membrane with proteins as single chain. (10) Breakdown of the polyprotein precursor by the protease to give structural proteins and enzymes.

clease enzyme (RnaseH), which cleaves RNA from the hybrid DNA, leading to the release of the viral DNA in the host cell cytoplasm. The newly formed viral DNA is called the provirus.

5.4 Viral DNA Entry to the Host Cell Nucleus

In the host cell HIV DNA, or the provirus, may stay in a free form or may enter the host cell nucleus, through pores in the nuclear mem-

brane, and undergoes integration with the host cell genome.

5.5 Integration of Viral DNA into the Host Cenome for Viral mRNA Production

Once the viral DNA is inside the nucleus, it integrates into the host chromosomes through the viral enzyme integrase, which is believed to be, like reverse transcriptase, a pol-encoded product (19). The linear viral DNA flanked by the two long terminal repeats (LTRs), found in the cytoplasm and nucleus of the infected cells, is the direct substrate for the viral integrase enzyme (20, 21). The enzyme inserts the linear double-stranded viral DNA at random into the host cell DNA (22, 23). Upon integration of viral DNA into the host genome, it starts to direct the transcription to produce its mRNA and other types of viral RNA.

5.6 Splicing of Viral mRNA from the Host Cell Cenome

The transcription process of viral RNA occurs in the T-cell nucleus, as part of its own transcription. The splicing (cutting) of viral mRNA and other viral RNA molecules from the host DNA is also accomplished by the normal host cell RNA polymerase to produce an array of viral mRNA.

5.7 Migration of the Viral mRNA to the Cytoplasm

After viral mRNA is produced and spliced through use of the host cell genome, mRNA is released to the cytoplasm, where it directs the manufacture of various viral proteins, including core proteins, envelope proteins, regulatory proteins, and enzymes. Although the virus exploits the biochemical machinery of the host cell to produce its functional components, the synthesis of such products is regulated by the virus genome (24, 25).

5.8 Assembly of Viral Proteins to Form the Virion

The resulting viral proteins are produced as a single large polyprotein precursor, which is then transported, with the help of viral RNA, to the host cell membrane in preparation for its assembly into daughter particles. The assembly process occurs at the host cell membrane where the original uncoated viral envelope proteins exist. Each polyprotein precursor is enveloped with gp120 and gp41 glycoproteins after being encapsulated with the p18 and p24 viral core proteins. The particles are now ready to be expelled or budded out of the cell.

5.9 Viral Budding out of the Host Cell

The assembled particles are released out of the host cell membrane by a process called budding, which leaves behind a hole, or several holes, in the T4 cell membrane, which may contribute to the CD4 cell death. The host cells normally do not survive the invasion by HIV. They either disintegrate because of the large number of viruses budding off, or the body's immune system recognizes the viral envelope proteins in the cell membrane and destroys the damaged cells. This destruction of CD4 cells causes severe immunodeficiency because of the role of helper T-cells in mediating the system immunity. This paves the way for the so-called opportunistic infections such as candidiasis in the bronchi or lungs, cryptococcus, cytomegalovirus retinitis, herpes simplex infections, and pneumonia.

5.10 Virus Maturation by HIV Protease (PR)

During or shortly after budding, the polyprotein precursor, inside the daughter particles, undergoes cleavage by a specific viral enzyme known as HIV protease. The resulting degradation products are the viral functional enzymes and proteins necessary for its survival. The viral particles at this stage are called virions. The virus particles after the protease action have all the necessary constituents of mature virus and are capable of invading other T4 cells and repeating the life cycle *(26,* 27). The enzyme HIV protease constitutes, with the reverse transcriptase enzyme, the most extensively targeted steps in the virus life cycle to design anti-HIV agents.

6 TARGETS FOR DRUG DESIGN OF ANTI-HIV AGENTS

6.1 An Overview

The information available on HIV life cycle and its details provided medicinal chemists

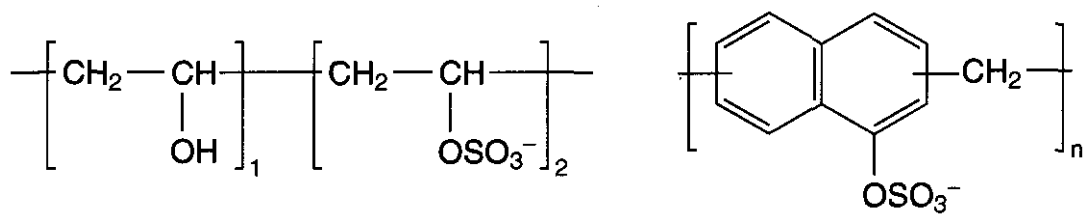
with several molecular and cellular sites for intervention to inhibit HIV replication (28). Virtually every step of the cycle has been targeted for drug design. Anti-HIV agents can be grouped into three groups based on the phase of their interference with the virus replication cycle, that is, the pretranscription, transcription, and posttranscription phases. Pretranscription inhibitors are those agents capable of blocking viral entry into the host cell. This class includes inhibitors of gp120 binding to CD4 (viral adsorption inhibitors), inhibitors of gp120 binding to coreceptors, and inhibitors of viral fusion and viral uncoating. The transcription phase is the central stage, through which viral DNA is transcribed from viral RNA under the control of the reverse transcriptase enzyme. Inhibitors for this enzyme represent a very important class of anti-HIV agents. The posttranscription inhibitors are agents that inhibit viral DNA integration into the host cell genome (integrase inhibitors) and agents that interfere with virus maturation by blocking the crucial step of producing viral functional proteins from the polyprotein precursor (protease inhibitors or viral maturation inhibitors).

All anti-HIV drugs currently approved for clinical use fall into the categories of reverse transcriptase and protease inhibitors. The reverse transcriptase inhibitors are further subclassified into two subclasses, the nucleoside reverse transcriptase inhibitors (NRTIs) and the nonnucleoside reverse transcriptase inhibitors (NNRTIs). The most challenging problem associated with HIV therapy is the development of drug resistance. The combination therapy approach has been broadly practiced in HIV treatment as a strategy to avoid such problem. However, despite the overall success of combination therapy in reducing viral load, morbidity, and mortality, it is mitigated by a variety of issues. The long-lived nature of the infection and the genetic plasticity inherent to this virus has led to the spread of multidrug-resistant variants of the virus. Therefore, the need for new treatment options beyond reverse transcriptase and protease inhibitors has emerged. Current efforts to develop new anti-HIV drugs are now directed more toward developing inhibitors for other steps in the virus life cycle, such as viral entry into the T-cell, its integration with the host DNA, or its assembly into daughter particles. These proposed sites of intervention may provide additional options to maintain long-term suppression of the virus replication. The identification of specific inhibitors for each of these processes has recently validated these approaches as viable alternatives for the traditional classes of reverse transcriptase and protease inhibitors. Some of these new agents are in the preclinical trial phase. The section below addresses the latest in drug development in the area of anti-HIV therapeutics. The section is arranged in the same sequence of the virus life cycle shown in Fig. 11.2.

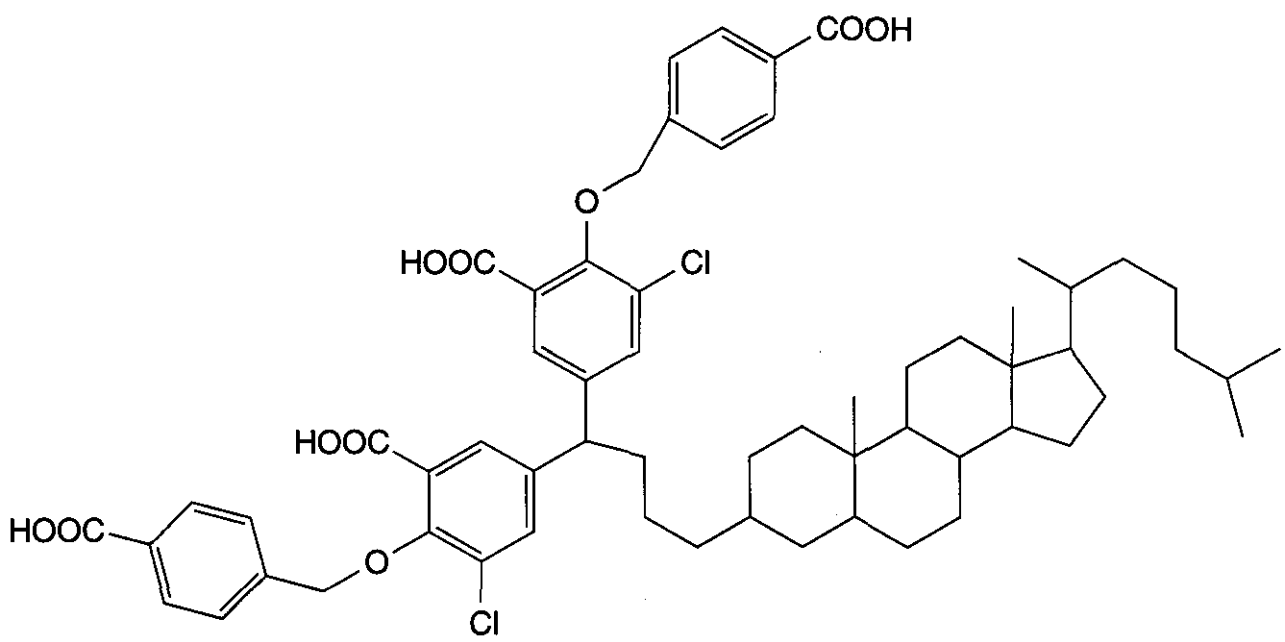
6.2 Inhibitors of Viral Entry

6.2.1 HIV Vaccines. The most ideal approach to inhibit HIV binding to the T-cells is to develop a vaccine that can neutralize the virus in circulation. Several attempts to develop antibodies to HIV as blocker for the viral binding to prevent infection have been reported (29, 30). However, because of the nature of the disease, people infected with HIV develop only low titers of neutralizing antibodies and that presents a problem for vaccine development. Knowledge of the detailed steps of the virus binding to the T-cell, together with discovery of the molecular structure of gp120, CD4, and the chemokine as coreceptors for gp120 binding, have directed vaccine development into more selective viral adsorption inhibitors (31).

6.2.2 Viral Adsorption Inhibitors. The recognition of the role of viral gp120 and gp41 glycoproteins in the processes of HIV binding and fusion with the T-cell surface flagged these sites as an attractive molecular target for intervention. In principle, agents affecting any of the viral entry events would be effective as inhibitors of HIV-1 replication. Targets of viral entry inhibition and fusion include the process of gp120 binding to CD4 receptors and other coreceptors (CCR5, CXCR4) on the T-cell membrane. The development of viral adsorption inhibitors and other anti-HIV agents together with molecular details of gp120 structure have been recently reviewed (32, 33) and are summarized below.



PVAS (1) Naphthalene sulfonate (2)

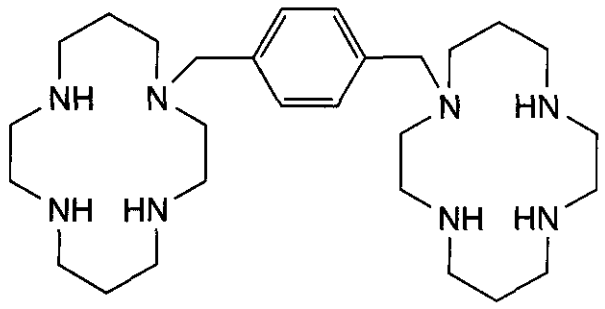


Cosalane (3)

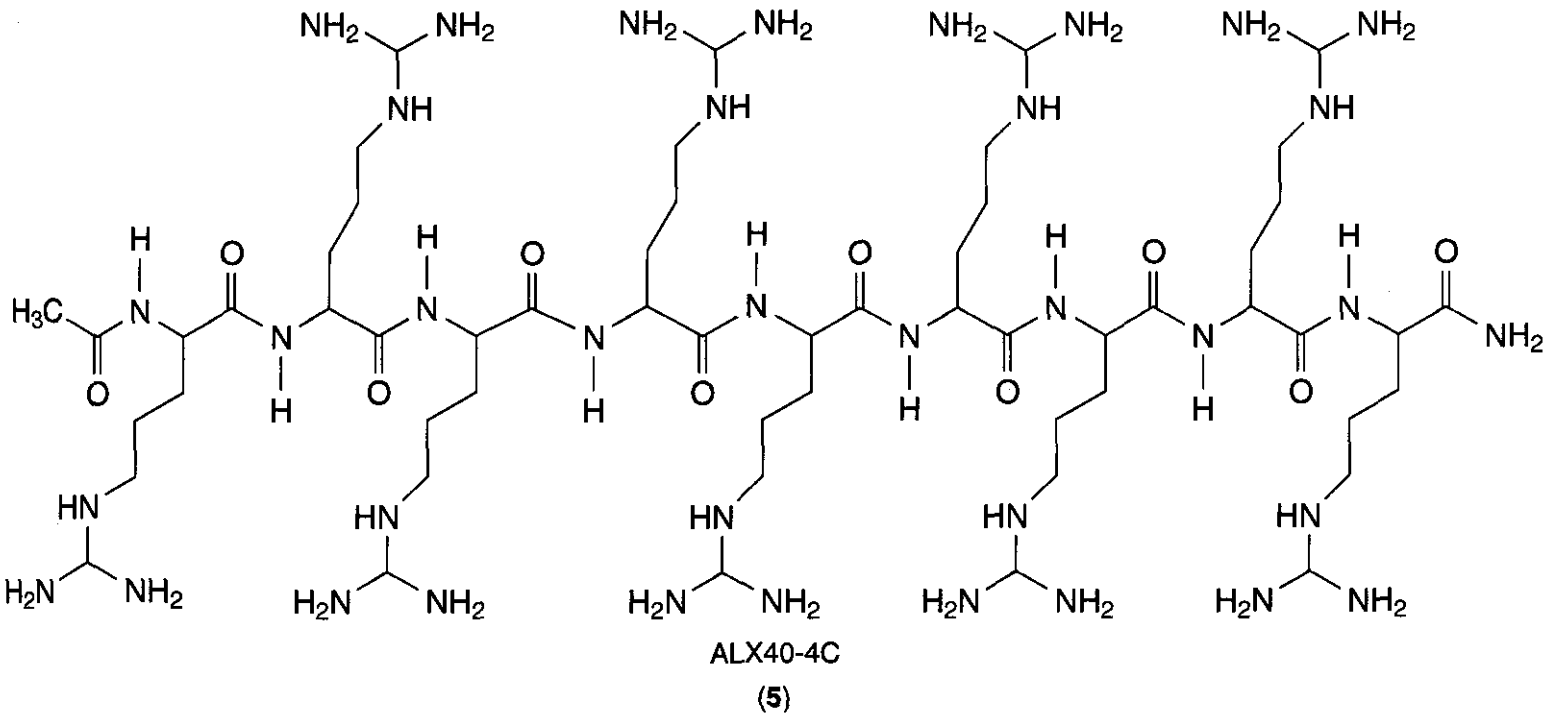
Figure **11.3.** Promising adsorption inhibitors.

6.2.2.1 Polyanionic Compounds. Early reports showed that small peptide sequences for the env gp120 glycoprotein inhibit viral binding and replication (34). The polysulfated carbohydrate dextran sulfate was also found to inhibit HIV replication at a micromolar concentration range with a mechanism related to blocking the virus binding to the target cells (35, 36). Several other polyanionic compounds of natural and synthetic sources have been reported to inhibit gp120 binding to the cell membrane. Among these anionic compounds are polysulfates, polysulfonates, and polycaroxylates. All are believed to exert their antiviral activity through inhibiting gp120 binding to the CD4 receptor. The major molecular mechanism of action of these polyanionic substances in inhibiting gp120 binding is by shielding the positively charged sites on the V3 loop of the viral envelope glycoprotein gp120 (32). The V3 loop is one of five polypeptide loops that constitute the backbone of the glycoprotein gp120, found to be necessary for the initial viral attachment to the cell surface. Figure 11.3 depicts the structures of these promising viral adsorption inhibitors. The most promising of the polyanionic substances described as gp120 binding inhibitors are polyvinyl alcohol sulfate (PVAS, 1), polynaphthalene sulfonate (2) and the polycarboxylate analog cosalane (3). The major role of polyanionic substances in general in the management of HIV infections may reside in the prevention of the sexual transmission of HIV. These compounds viewed as vaginal formulations may block HIV infection through both virus-to-cell and cell-to-cell contacts. Therefore, the merit of using these compounds as anti-HIV agents can be achieved if they are applied as vaginal microbicides (32).

6.2.2.2 ***Soluble CD4 Pepfide Fragmenfs.*** Shortly after the discovery of CD4 as the primary receptor for HIV-1 binding on the T-cell membrane, soluble forms of CD4 (sCD4) were isolated and evaluated for clinical efficacy to suppress HIV infection (37–39). Although laboratory experiments had validated the overall approach, results from the initial clinical trials proved disappointing (38, 39). Recent efforts to use soluble CD4 forms have focused on CD4



AMD3100
(4)



ALX40-4C
(5)

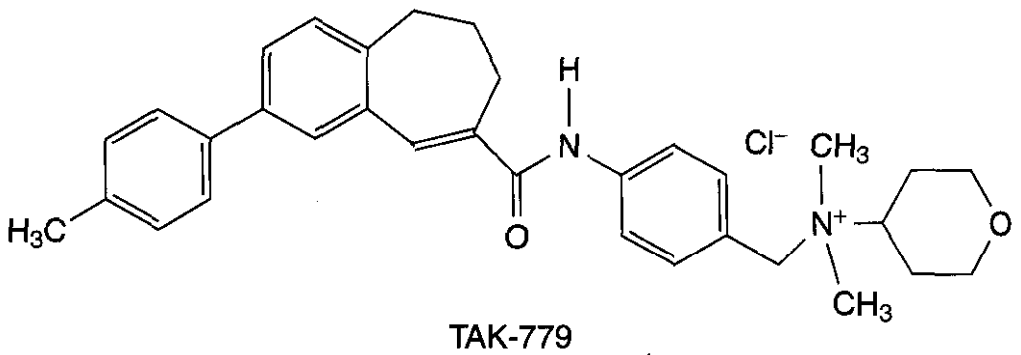
Figure 11.4. CXCR4 antagonists.

conjugates with toxins (40–42). Pro542 is a conjugation product of the CD4 soluble receptors (first two domains of CD4) with the constant regions (both heavy and light chains) of the human IgG2 (43,441. Pro542 was found to be more effective than the soluble CD4 alone in blocking HIV transmission in the scid-hu-PBL mouse model (45). The results of clinical trials of Pro452 in HIV-infected adults and pediatric patients have demonstrated that the CD4 conjugate is both safe and effective (46).

6.2.3 Inhibitors of Gp120 Binding to the T-Cell Coreceptors

6.2.3.1 ***Gp120*** **Coreceptors.** As explained under the virus entry part of the HIV life cycle, the coreceptors CXCR4 and CCR5 are newly discovered binding sites for gp120 on the T-cell membrane surface. These coreceptors naturally act as receptors for internal ligands released normally in cases of inflammation or immune responses. These ligands are generally designated as chemokines (*chemo*attractant cyto*kines*). Whereas CXCR4 has only one natural chemokine ligand called SDF-1 (stroma-cell-derived factor), CCR5 has two natural ligands named RANTES (regulated upon activation, normal T-cell expressed and secreted) and MIP-1 (macrophage inflammatory protein). MIP-1 is further classified into two subtypes, MIP-1α and MIP-1β. The importance of inhibiting gp120 binding to these coreceptors emerges from their role in the process of activating the second viral envelope protein gp41 binding to the T-cell surface, which ultimately leads to the virus entry.

6.2.3.2 ***Gp120-CXCR4 Binding Inhibitors.*** The most interesting member of this class is the compound bicyclam (AMD3100, **4**, Fig. 11.4). Bicyclam showed an in vitro anti-HN-1 activity before the discovery of the HIV-1 coreceptors (47). Originally thought to inhibit a postentry step, however, AMD3100 was subsequently found to bind selectively to CXCR4 (48, 49). Biochemical evidence for this proposed mechanism of bicyclam action in-



TAK-779
(6)

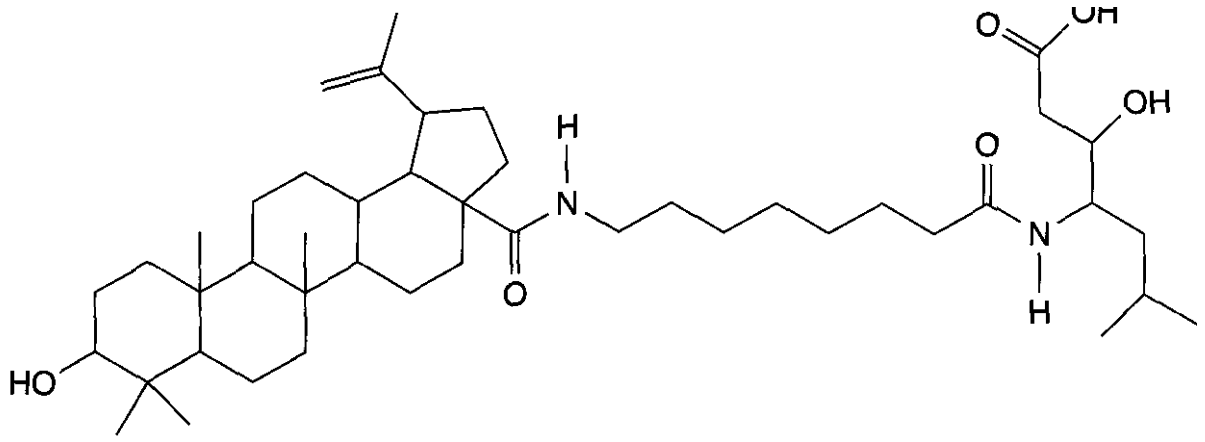
Figure 11.5. **TAK-779,** a potent nonpeptide **CCR5** antagonist.

cludes competition binding studies with both the natural ligand of CXCR4 (SDF-1) and the anti-CXCR4 monoclonal antibody 12G5. Bicyclam has shown modest antiviral activity in the mouse model (50). The compound is reported to be the most specific and most potent CXCR4 antagonist. Bicyclam is currently undergoing clinical evaluation. However, data with respect to safety and efficacy are not yet available. Additional CXCR4 inhibitors in preclinical studies include the polypeptide derivatives ALX40–4C (5, Fig. 11.4) (51), T22 (52), T134, and T140 (53). There is a concern with all of these compounds that long-term antagonism of CXCR4 function could be detrimental to the immune function, particularly in the B-cell and granulocyte compartments (54–56). Other agents that have been evaluated to block gp120-CXCR4 binding included SDF-1, the natural ligand for the CXCR4 receptor, which was found to block a certain HIV strain called T-tropic HIV (57). Figure 11.4 shows the chemical structures for some of the CXCR4 antagonists.

6.2.3.3 Gp120-CCR5 Binding Inhibitors. Several small molecules have been reported to inhibit binding of gp120 to CCR5. Some of these agents are in preclinical development. These CCR5 antagonists include TAK-779 (58, 59); the 2-aryl-1-{*N*-(methyl)-*N*-(phenylsulfonyl) amino}-4-(piperidin-1-yl)butanes (60); 1-amino-2-phenyl-4-(piperidin-1-y1)butanes (61); 1,3,4-trisubstituted pyrrolidines (62); and PRO440, an anti-CCR5 monoclonal antibody (63). All of these agents were found to block the binding of chemokines (e.g., MIP-1) to CCR5. Consequently, they have the potential to block HIV binding to CCR5 and inhibit replication of CCR5-dependent virus strains. Although a promising class, an unresolved issue regarding the clinical utility of CCR5 inhibitors is the variability of gp120 structure among different viral strains. The most attractive member of this class undergoing clinical evaluation is the quaternary ammonium derivative TAK-779 (6, Fig. 11.5) (58, 59). It is the first nonpeptide molecule that has been described to block the replication of M-tropic R5 HIV-1 strains at the CCR5 level. Other agents undergoing evaluation as blockers for gp120-CCR5 binding is the chemokine MIP-1α, which has been identified as the most potent chemokine for inhibiting HIV infection (64, 65). Figure 11.5 depicts the structure of CCR5 antagonist TAK-779.

6.3 Inhibitors of Viral Fusion

The fusion of the virus envelope with the T-cell membrane commences after the binding of gp120 to the coreceptors CXCR4 and CCR5. This binding triggers a spring-loaded action of the glycoprotein gp41, which is normally covered by the larger gp120. The gp41 anchors itself to the T-cell membrane through the hairpin structures HR1 and HR2 (see virus life cycle, fusion step). This initiates the fusion of the two lipid bilayers of the virus and cell membranes (66). Before the knowledge of structural information of gp41, synthetic peptides overlapping the two HR regions HR1 [e.g., DP-107 peptide (67)] and HR2 [e.g., DP-178 peptide (68)] were found to block HIV-1 replication in cell culture. The discovery that these two regions constituted the fusion trigger subsequently provided a mechanistic basis for their antiviral affects. The peptides act in a dominant-negative fashion to prevent formation of the trimer-of-hairpins arrangement. The first clinical trial of pentafuside (or DP-178 or T-20 in the trial) was reported in 1998 (69). Pentafuside is a synthetic 36 amino acid peptide that corresponds to the 127–162 resi-



RPR103611
(7)

Figure **11.6.** The nonpeptide fusion inhibitor betulinic acid derivative RPR103611.

dues of the of gp41 domain structure. Subsequent clinical trials have confirmed the overall safety and efficacy of T-20. Another polypeptide that is undergoing evaluation and believed to interact in a manner similar to that of T-20 is the antibiotic siamycin (a tricyclic 21 amino acid peptide isolated from Streptomyces) (70, 71). The betulinic acid derivative RPR103611 (7, Fig. 11.6) represents a nonpeptide inhibitor for gp41 fusion (72). Peptide and nonpeptide HIV fusion inhibitors have recently been reviewed (73). Figure 11.6 depicts the structure of the fusion inhibitor betulinic acid derivative RPR103611.

6.4 Inhibitors for Viral Uncoating

The viral uncoating process (loss of its nucleoprotein outer coat after fusion) results in the release of its RNA genome into the cytoplasm. Uncoating is controlled by the p7 nucleocapsid protein (NCp7), which is a peptide segment of the p18 protein. NCp7 is a zinc-containing protein, and zinc-displacing compounds were found to inhibit the virus uncoating process (74). The anti-influenza drug amantadine (8, Fig. 11.7) is an example that is reported to work by such a mechanism (75). Several other agents are reported to interfere with this step of viral nucleo-protein disassembly (i.e., uncoating). Among these agents are NOBA (3-nitrosobenzamide, 9, Fig. 11.7) (76, 77), the dithiobenzamide-sulfonamide derivative DIBA (10, Fig. 11.7) (78, 79), SRR-SB3 (cyclic 2,2′-dithiobisbenzamide, 11, Fig. 11.7) (80), dithiane (1,2-dithiane-4,5-diol,1,1-dioxide, 12, Fig. 11.7) (81), and ADA (azadicarbonamide) (82, 83). Figure 11.7 depicts the structures of viral uncoating inhibitors.

6.5 Inhibitors of HIV Reverse Transcription

The reverse transcription process has been one of the most thoroughly investigated steps in the virus life cycle. The transcription of HIV genetic material is initiated and mediated by the reverse transcriptase enzyme (RT). The enzyme is multifunctional, presenting both RNA and DNA polymerase as well as RnaseH activities. The reverse transcriptase is responsible for converting the single-strand RNA into the double-strand DNA. In addition, the enzyme has a catalytic unit for activating the RnaseH enzyme, which liberates the proviral DNA from RNA after transcription. Considering the crucial and complex role it plays in the synthesis of viral DNA, its inhibition has proved to be an effective approach in interrupting the HIV replication cycle. The enzyme has captivated the attention of many investigators striving to develop new anti-HIV drugs to overcome the continuously evolving HIV mutant strains.

6.5.1 Molecular Aspects of HIV Reverse Transcriptase. The mature HIV-1 RT is a heterodimeric enzyme composed of two subunits, p66 and p51 (84). X-ray crystal structures of the enzyme complexes with different inhibitors have been successively refined at 7 Å (85), 3.5 Å (86), 3.0 Å (87), and 2.2 Å (88). These crystal structure studies show that the polymerase regions of p66 and p51 are divided into four subdomains denoted *finger*, *palm*, thumb,

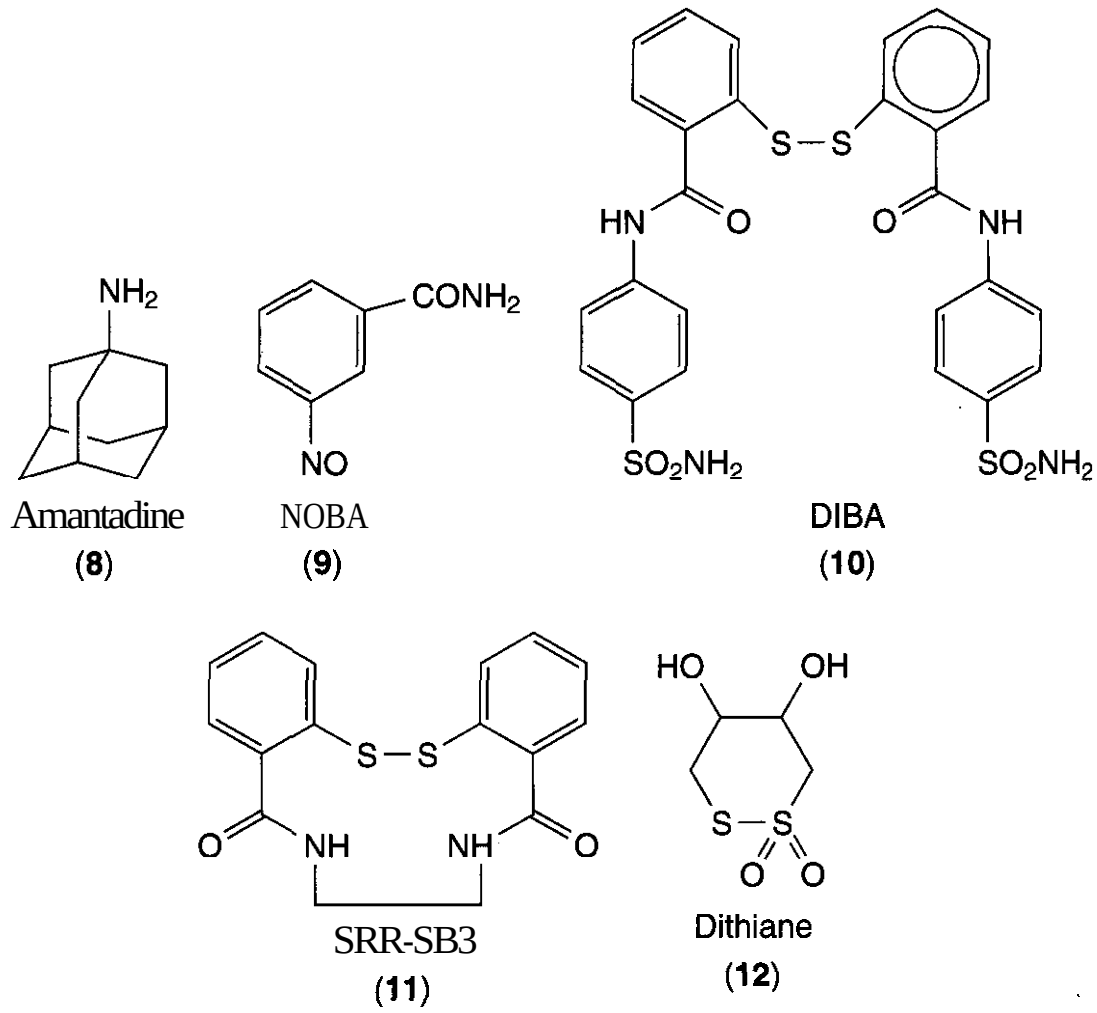
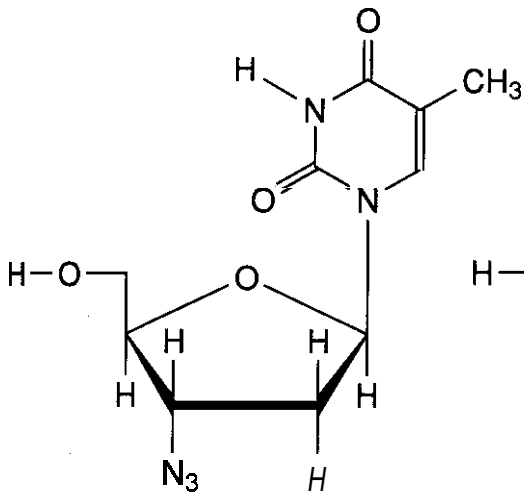


Figure 11.7. Viral uncoating inhibitors.

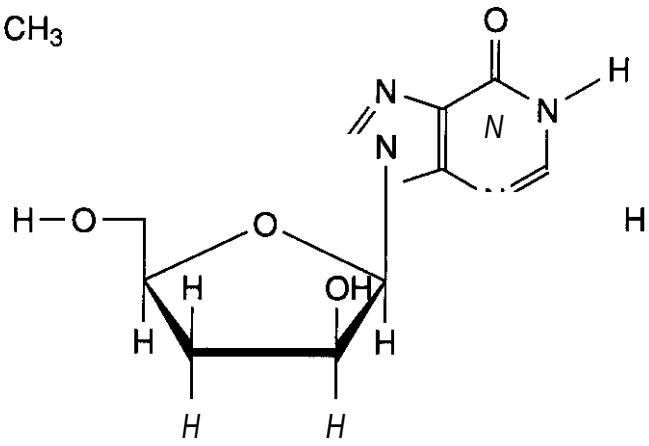
and connection. The p66 palm domain contains the polymerase active site. Three aspartic acid residues at positions 110,185, and 186 constitute the catalytic triad (86, 87). A feature of HIV-1 RT that makes it such a difficult target for therapeutic intervention is the high degree of nucleotide sequence variation between different viral strains. The HIV-1 RT is highly error prone, with a predicted error rate of 5 to 10 per HIV-1 genome per round of replication in *vivo*. The mutations incorporated by retroviral RT include amino acid substitution and deletions of nucleotide sequence (89, 90).

6.5.2 HIV ***Reverse Transcriptase Inhibitors.*** Reverse transcriptase inhibitors are designed to inhibit viral DNA synthesis from viral RNA. The process of DNA synthesis involves incorporation, sequentially and one at a time, of activated nucleotides (as triphosphate) to the template RNA. The enzyme reverse transcriptase catalyzes this process by simultaneous binding to both the nucleotide and RNA at its catalytic active site. Inhibitors of HIV transcriptase act by impeding the nucleotide binding to the active site. The inhibition is achieved by two distinct mechanisms, either competitively or noncompetitively. The competitive inhibitors are nucleoside analogs or have nucleoside-like structures. The nucleoside inhibitors are further classified into purine and pyrimidine nucleoside inhibitors based on the nucleic acid base existing in the molecule. On the other hand, the noncompetitive inhibitors are not structurally related to the nucleotides and are referred to as allosteric inhibitors.

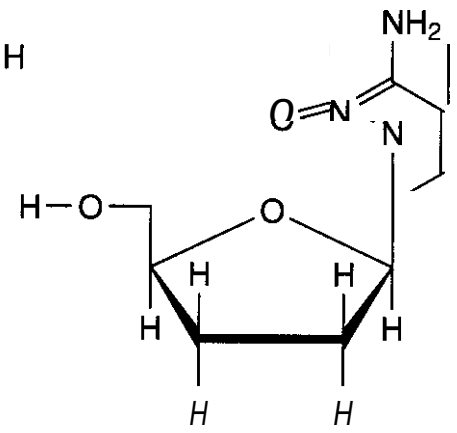
6.5.2.1 Nucleoside Reverse Transcriptase Inhibitors (NRTIs). Members of this class act as irreversible, competitive inhibitors for the HIV RT. Their description as competitive indicates the mechanism of action, given that they compete with normal substrates at the enzyme catalytic site. These normal substrates are different types of deoxyribonucleosides triphosphate (dNTP) or the deoxynucleotides. Therefore, members of this class, being nucleoside in nature (i.e., contain only the base and the sugar), require intracellular activation to the nucleoside triphosphate form (the nucleotide). This activation process requires three phosphorylation steps, whereby the compounds are converted successively to



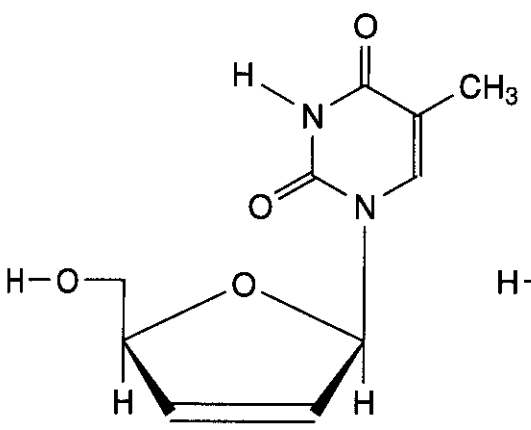
Zidovudine
(13)



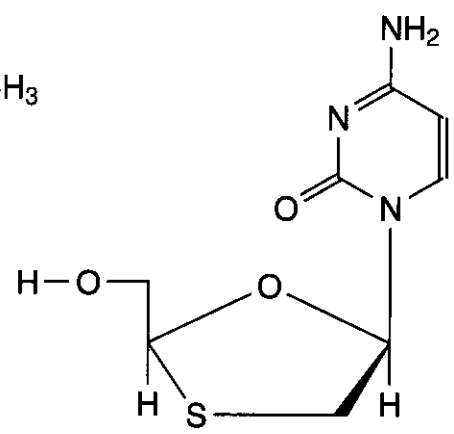
Didanosine
(14)



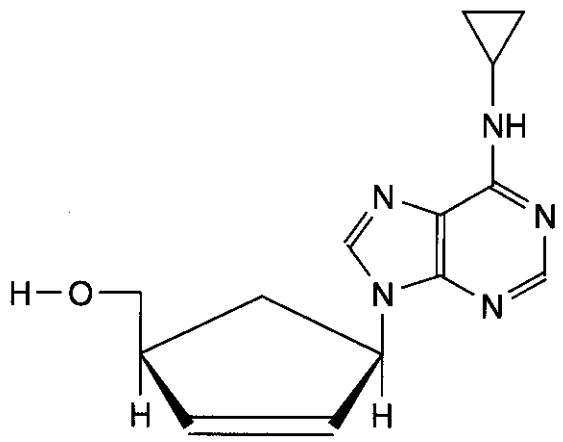
Zalcitabine
(15)



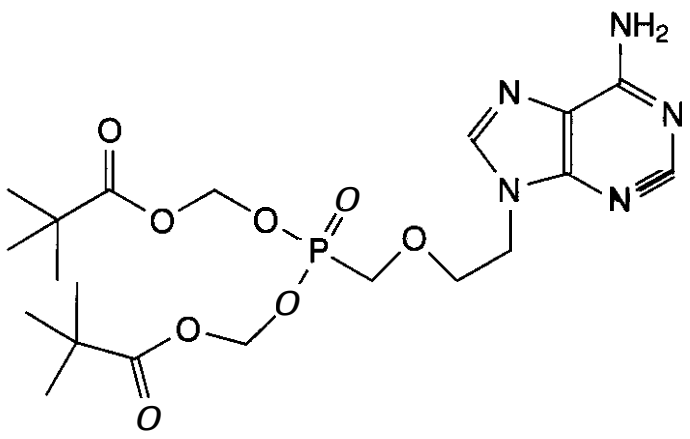
Stavudine
(16)



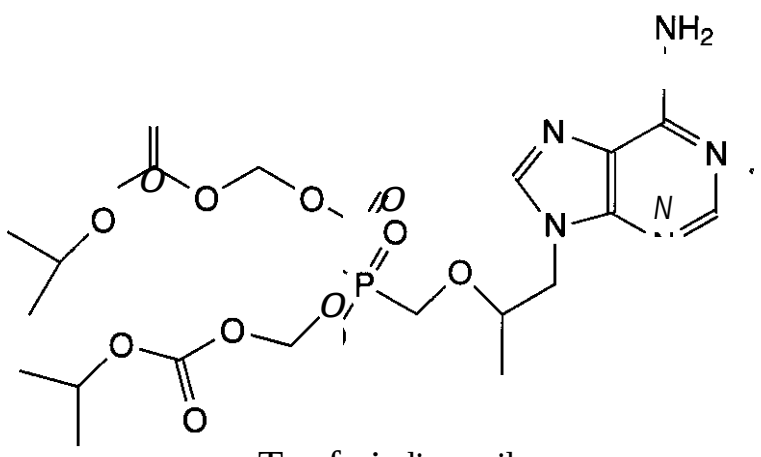
Lamivudine
(17)



Abacavir
(18)



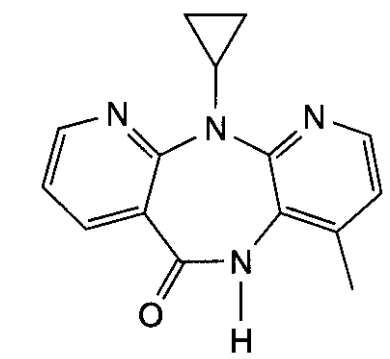
Adefovir dipivoxil
(19)



Tenofovir disproxil
(20)

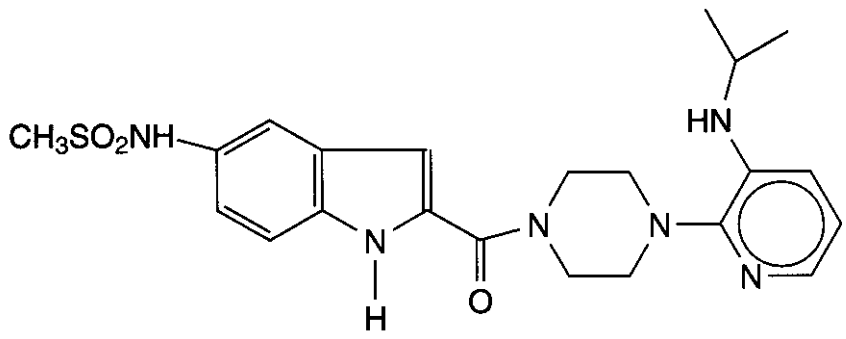
Figure 11.8. Clinically approved and phase III clinical trial NRTI.

mono-, di-, and triphosphate by cellular kinases. The irreversible description of the mechanism of action of NRTIs is attributed to the fact that they are incorporated into the viral DNA through an irreversible covalent bond. The irreversible bond occurs through the activated 5-hydroxyl of the sugar. On the other hand, the structures of all NRTIs indicate their lack of the 3-hydroxyl group of the sugar. The absence of the 3-hydroxyl group results in a DNA intermediate product that does not elongate further through position 3. This process of blocking DNA elongation is commonly referred to as chain termination. Most drugs approved for treatment of HIV infections belong to the class of nucleoside inhibitors. These drugs include zidovudine (AZT, 13, Fig. 11.8), which was the first member of



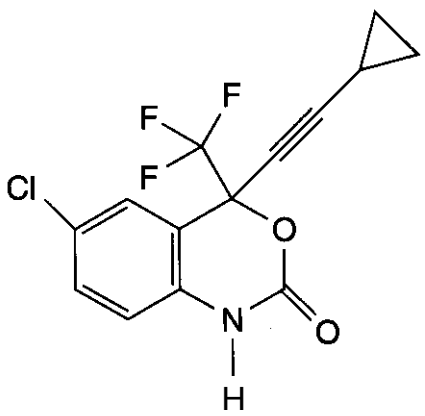
Nevirapine

(21)



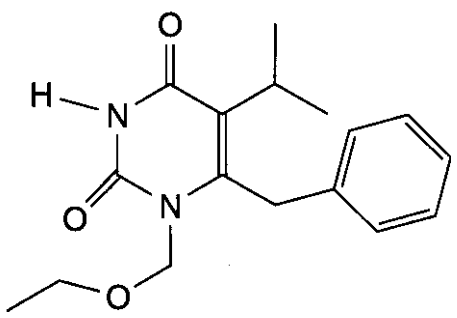
Delaviridine

(22)



Efavirenz

(23)



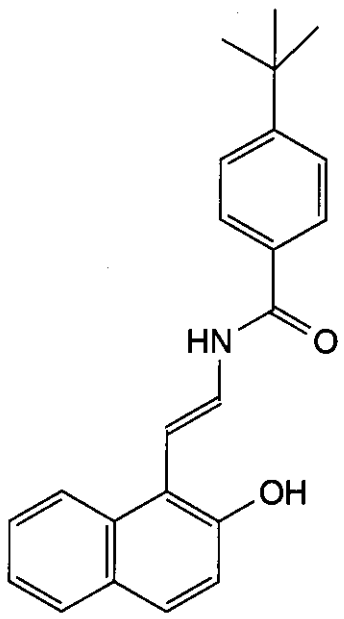
Emivirine

(24)

Figure 11.9. Clinically approved and phase III clinical trial NNRTI.

the class to be approved (91–93); didanosine (DDI, 14, Fig. 11.8), zalcitabine (ddC, 15, Fig. 11.8), stavudine (D4T, 16, Fig. 11.8), lamivudine (3TC, 17, Fig. 11.8), and abacavir (ABC, 18, Fig. 11.8). In addition to acting as irreversible competitive inhibitors, NRTIs act as chain terminators by preventing DNA elongation. Some newer (second-generation) NRTIs have been developed and are undergoing preclinical trial phases, such as adefovir dipivoxil [bis(POC)-PMEA, 19, Fig. 11.81 and tenofovir [bis(POC)-PMPA, 20, Fig. 11.81, which is undergoing phase III clinical trials for the treatment of HIV infections (94). Trends in the design of nucleoside analogs such as anti-HIV drugs were recently reviewed (95). Figure 11.8 represents the structures of clinically approved nucleoside inhibitors and the phase III clinical trial inhibitors adefovir and tenofovir.

6.5.2.2 Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs). In contrast to the NRTIs, the nonnucleoside reverse transcriptase inhibitors (NNRTIs) target the allosteric nonsubstrate binding sites. It has been suggested that the NNRTI binding sites may be functionally, and possibly also spatially, related to the substrate binding site (96, 97). Members of this class are described as noncompetitive, reversible inhibitors. The dissimilarity of their chemical structures to the natural substrate implies different binding sites, hence the classification as noncompetitive inhibitors. Furthermore, unlike NRTIs, members of this class are highly specific to HIV-1 RT without affecting the host DNA polymerases, which explains the low toxicity and side effects of these drugs. Compounds belonging to this class are classified as first-generation NNRTIs, which belong to more than 30 different classes of compounds (98) or second-generation NNRT inhibitors. Newer nonnucleoside reverse transcriptase inhibitors have recently been reviewed (99). Only three drugs belonging to the NNRTI class have been approved for clinical use: nevirapine (21, Fig. 11.9), delavirdine (22), and efavirenz (23, Fig. 11.9). The second-generation NNRTI emivirine (MK-442, 24, Fig. 11.9) is in Phase III clinical trial. Figure 11.9 shows the structures of clinically approved NNRTIs and the Phase III clinical trial inhibitor emivirine.



BBNH
(25)

Figure 11.10. The RNaseH inhibitor BBNH.

6.6 Inhibitors of HIV Ribonuclease (RnaseH)

The transcription of viral RNA copy to form the DNA molecule (under the control of RT) is followed by a second transcription of a positive strand of DNA to form a double helix. The tristranded product requires cleavage of the original RNA strand from the double-helix DNA molecule (the provirus). RnaseH (a viral ribonuclease) is the enzyme responsible for this step of removal of viral RNA from DNA. The activity of viral RNase is believed to be mediated by a special domain on the reverse transcriptase enzyme (100). Very little is known about inhibitors for this RT-associated ribonuclease H enzyme and research in this area is limited. This class of inhibitors is exemplified by the compound N-(4-t-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH, 25, Fig. 11.10), which was reported to be a very potent inhibitor for RNase H (101). Figure 11.10 shows the structure of the Rnase H inhibitor BBNH.

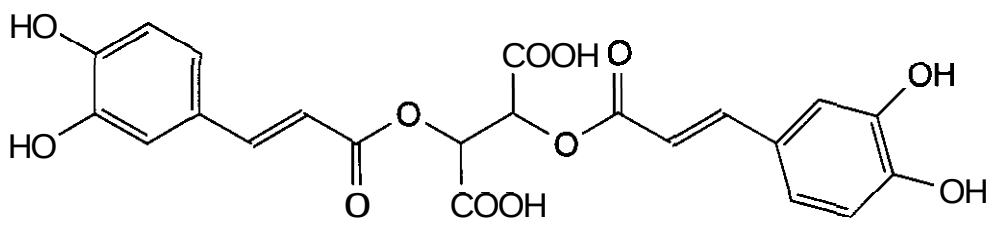
6.7 HIV Integrase Inhibitors

6.7.1 HIV Integrase (IN). Integrase is one of the enzymes encoded by HIV-1. It catalyzes the insertion of the HIV-1 DNA into the host cell genome. Integration is required for stable maintenance of the viral genome and viral gene expression. The integration process requires that the IN protein recognizes linear viral DNA ends through its long terminal repeats (LTRs), followed by removal of two base pairs from the 3' ends of each provirus dinucleotide (102). These 3' processed viral DNA ends are then joined to the 5' ends of the cleaved host genome target site DNA by a one-step transesterification (103). Both the crystal structure of the IN catalytic domain and the solution structure of the DNA binding domain of HIV-1 IN have been determined (104,105). The structural information has been helpful in the design of inhibitors at this crucial step in the HIV-1 replication cycle (106).

6.7.2 Integrase inhibitors. Like other retroviruses, HIV cannot replicate without integration into a host chromosome. Accordingly, HIV integrase has been considered as an attractive target in the design of anti-HIV drugs. Numerous compounds have been described as inhibitors of HIV-1 integrase (107). The most promising examples of these inhibitors are L-chicoric acid (26, Fig. 11.11) and the diketo acid derivatives L-731,988 (27) and L-708,906 (28); all have been described as potent integrase inhibitors. The aspects of drug design for integrase inhibitors were the subject of a recent review as emerging therapeutic approaches to design inhibitors for HIV-1 infections (108). Figure 11.11 shows the structures of representative examples of the integrase inhibitors.

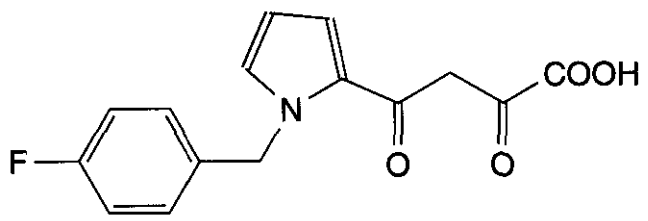
6.8 Inhibitors of HIV Gene Expression (Transactivation Inhibitors)

Agents belonging to this class inhibit the virus transcription phase at the host genome level after viral DNA integration. HIV uses the host genome to express its env, gag, and pol genes. These genes translate the production of the virus structural protein, for example, the envelope and functional proteins such as the reverse transcriptase, the integrase and, the protease. HIV gene expression occurs after the promotor LTR (long terminal repeat) ends of the gene are activated by binding to an activating viral protein called tat (transactivating). The activated LTR amplifies the process of gene expression. A number of compounds have been reported to inhibit HIV-1 replication through inhibiting this process of the gene transactivation through inhibiting tat



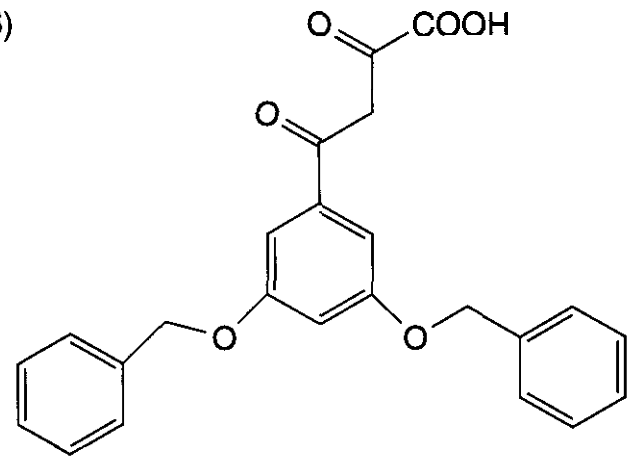
L. Chicoric acid

(**26**)



L.731,988

(**27**)



L708,906

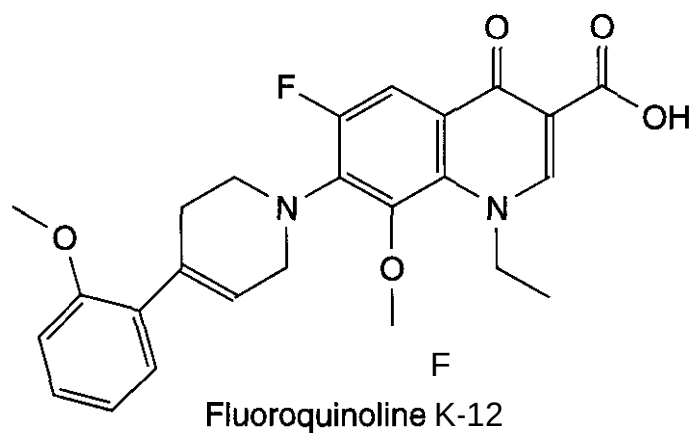
(**28**)

Figure 11.11. Integrase inhibitors.

binding to the promoter LTR ends. Among these compounds are **fluoroquinoline** K-12 (29, Fig. 11.12) (109) and **temacrazine** (bistriazoloacridone, **30** Fig. 11.12). The latter was found to block HIV-1 RNA formation without interfering with the transcription of any cellular genes (110). Figure 11.12 shows representative examples of HIV transcription transactivation inhibitors.

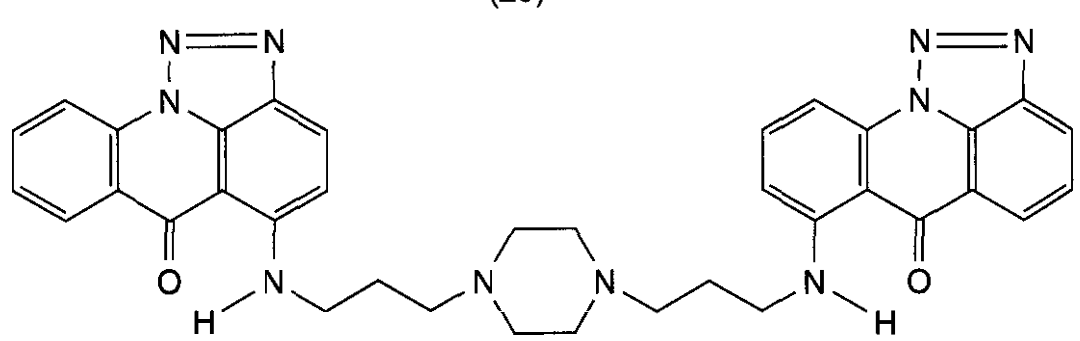
6.9 Inhibitors of Virion Assembly

The tripeptide Gly-Pro-Gly- NH_2 was found to have anti-HIV activity. This was originally attributed to an entry-inhibitory mechanism



Fluoroquinoline K-12

(**29**)



Temacrazine

(**30**)

Figure 11.12. HIV transcription transactivation inhibitors.

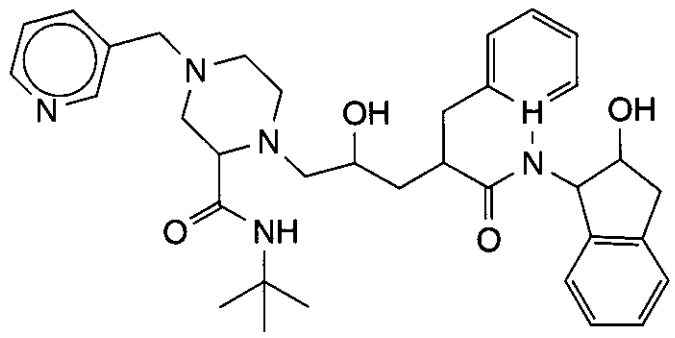
on the basis of the observation that this sequence is a highly conserved motif in the V3 loop of gp120 (111). However, recent studies have shown that **GPG** does not inhibit the early events in HIV-1 replication (because it affects the virus production from cells chronically infected with HIV-1; see Ref. 112). This suggested a novel mechanism of action that targets viral assembly and/or maturation. The mechanism of **GPG** is not yet fully understood, although this tripeptide may represent the first proof that small molecules can affect HIV replication by inhibiting the process of virion assembly.

6.10 Inhibition of Viral Maturation (HIV-1 Protease Inhibitors)

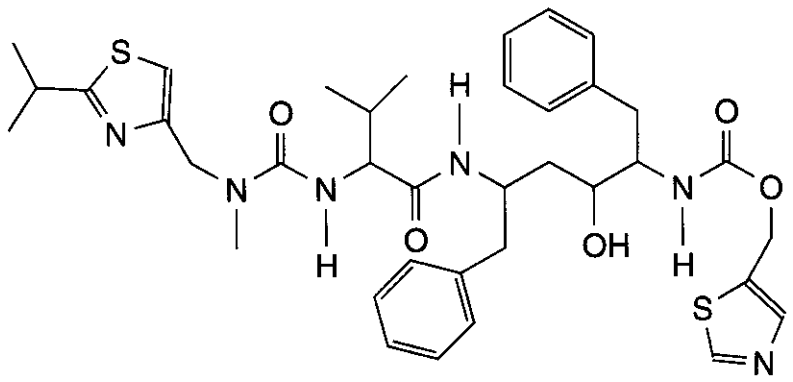
6.10.1 HIV Protease. HIV protease represents the second enzyme in the virus life cycle, after the reverse transcriptase, that has been extensively targeted for drug design. HIV protease is a proteolytic enzyme responsible for cleaving the large polyprotein precursor into biologically active protein products. HIV polyprotein precursor is encoded by the *gag* and *gag-pol* genes. These genes encode the precursor with HIV structural core proteins and various viral enzymes, including the reverse transcriptase, the RnaseH, the integrase, and the protease. HIV protease cleaves this polyprotein precursor during or shortly after viral budding to produce the mature virion. Therefore, inhibition of this posttranslational step leads to total arrest of viral maturation, thereby blocking infectivity of the virions (113). Structurally, the enzyme is classified as an aspartic protease. The X-ray crystal structure of HIV-1 protease (114–116) has revealed that the enzyme is a C2 symmetric homodimer of two identical subunits. Each of the two subunits is a 99 amino acid chain and each contributes a single aspartic acid residue to the active catalytic site, which exists in a cleft enclosed between two projecting loops from each subunit. The active site of the enzyme is a triad aspartyl-threonine-glycine residue, which is located within a loop whose structure is stabilized by hydrogen bonds. The loops of each monomer are interlinked by four hydrogen bonds that contribute to the stabilization the two subunits around the symmetry axis. These hydrogen bonds and the axiomatic structure of the enzyme have served as a foundation for designing inhibitors for the enzyme.

6.10.2 HIV Protease Inhibitors (PI). Three different approaches have been taken in the design of protease inhibitors. One is based on the transition-state mimetic approach. A large number of inhibitors were developed as competitive inhibitors for the natural substrate (the polyprotein precursor) binding to the enzyme. Eventually, all these competitive inhibitors are peptide in nature. The second approach of inhibitor design is based on disrupting the enzyme's twofold rotational C2-symmetry axis by forming specific hydrogen bonds and hydrophobic interactions with the amino acid residues involved in stabilizing the dimer. Agents belonging to this class are also peptide in nature. To improve the poor pharmacokinetic properties exhibited by peptide protease inhibitors, a third approach has emerged to develop inhibitors with fewer peptide characteristics. Accordingly, HIV protease inhibitors are classified as peptide inhibitors (first generation) and nonpeptide inhibitors (second generation). Members of both classes act by one of the above-mentioned mechanisms (i.e., competitive inhibition or dimer destabilizer). HIV protease inhibitors, peptides, and nonpeptides were previously (117) and recently (118) reviewed. At the molecular level of drug design, all first- or second-generation protease inhibitors are tailored after the target peptide linkages in the natural substrate (polyprotein precursor). The cleavage region has a phenylalanine-proline sequence at positions 167 and 168 of the gag-pol polyprotein. Inhibitors are designed with chemical functional groups that resist the cleavage. This is generally achieved by replacement of the scissile peptide bond of the substrate with a nonscissile structural moiety such as the hydroxyethylene group in the inhibitor.

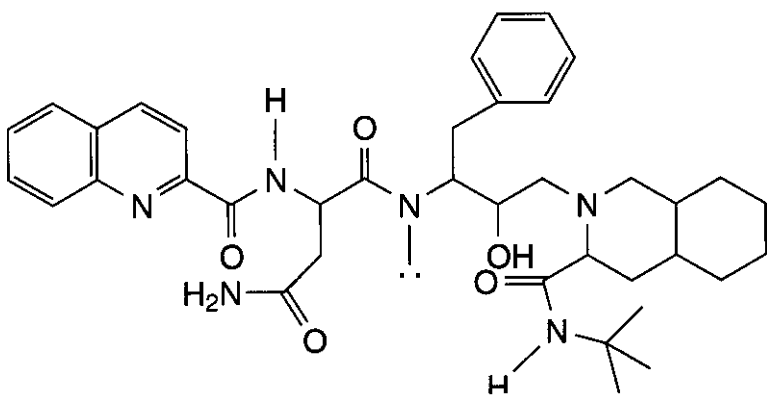
All currently licensed protease inhibitors for the treatment of HIV infection (Fig. 11.13), that is, indinavir (**31**), ritonavir (**32**), saquinavir (**33**), neflinavir (**34**), and amprenavir (35) (118, 119), share the same structural determinant, a hydroxyethylene group replacing the normal peptide bond. The hydroxyethlene



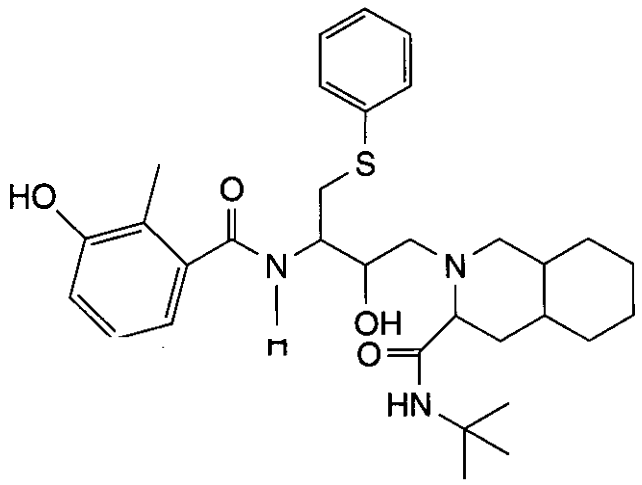
Indinavir
(**31**)



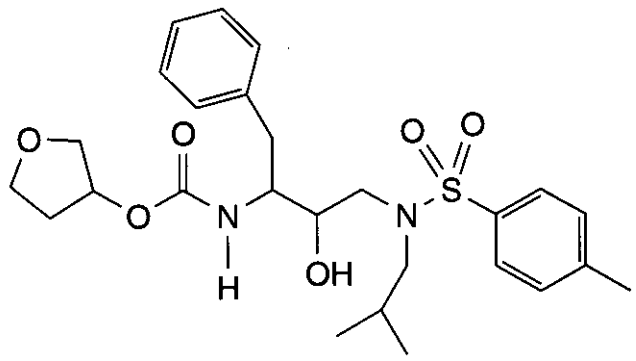
Ritonavir
(**32**)



Saquinavir
(**33**)



Neflinavir
(**34**)

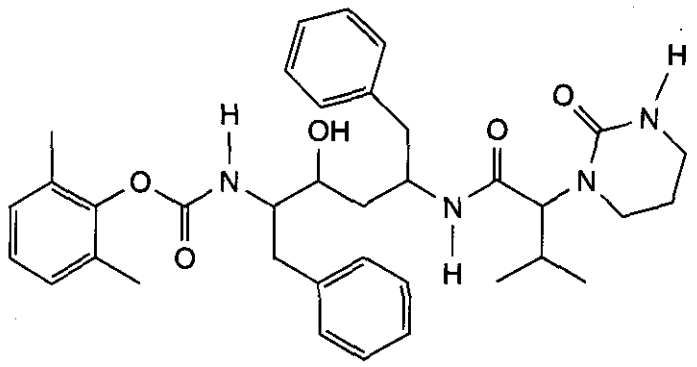


Amprenavir
(**35**)

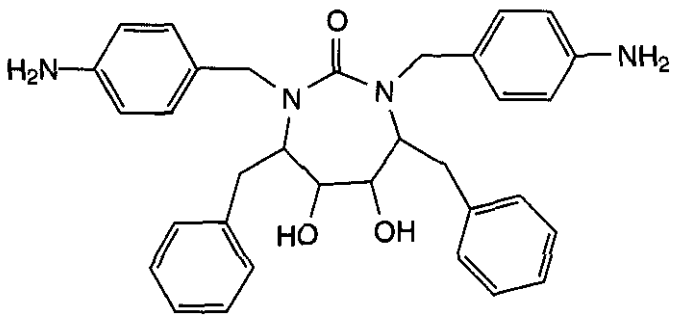
Figure 11.13. Clinically approved protease inhibitors.

group has a dual purpose. In addition to making the molecule nonscissile, it allows the molecule to bind to the catalytic site by hydrogen bonding, which can destabilize the enzyme dimer. Other small size peptide inhibitors are reported to contain hydrophilic carboxyl groups as the hydrogen bonding destabilizer (120). Reports have appeared for peptidomimetic inhibitors with the sessile segment of the peptide substrate replaced with a 15-member macrocylic peptide with stable conformation (121). Certain monoclonal antibodies were also studied as noncompetitive inhibitors for the enzyme acting through suppressing the dimerization process (122). In general, protease inhibitors are designed with the aim of driving the molecule to the active site, where it acts either as a competitive inhibitor with stronger hydrophobic and or hydrogen bonding or as a destabilizer to the enzyme dimeric structure through similar forces of interaction. Figure 11.13 depicts the structure of the clinically approved protease inhibitors.

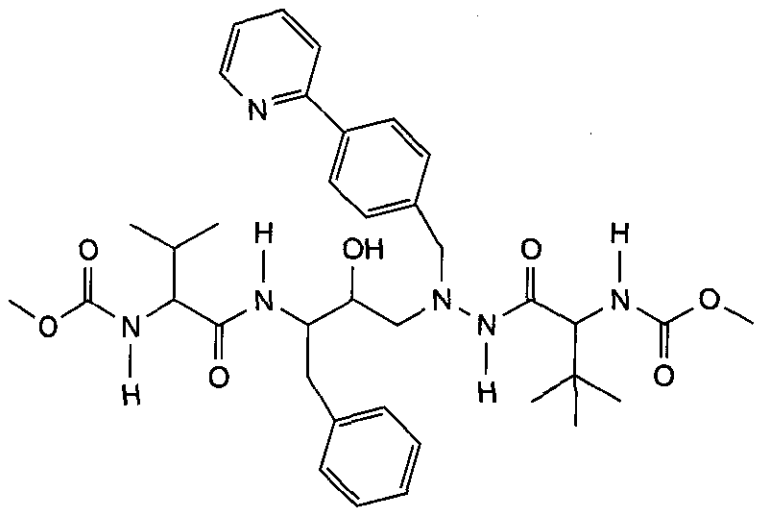
Some newer inhibitors with nonpeptide structure have been developed, such as lopinavir (ABT-378, **36,** Fig. 11.14) (119), the cyclic urea mozinavir (DMP 450, 37, Fig. 11.14) (123), atazanavir (BMS-232632, 38, Fig. 11.14) (124), tipranavir (PNU 140690, **39**, Fig. 11.14) (125), and the C2-symmetric protease



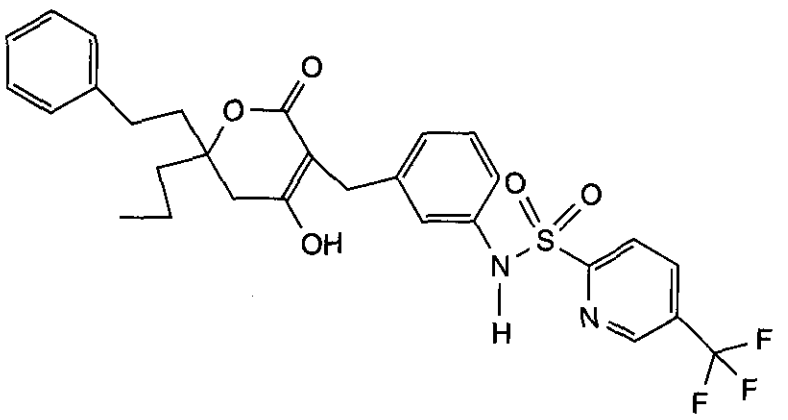
Lopinavir
(**36**)



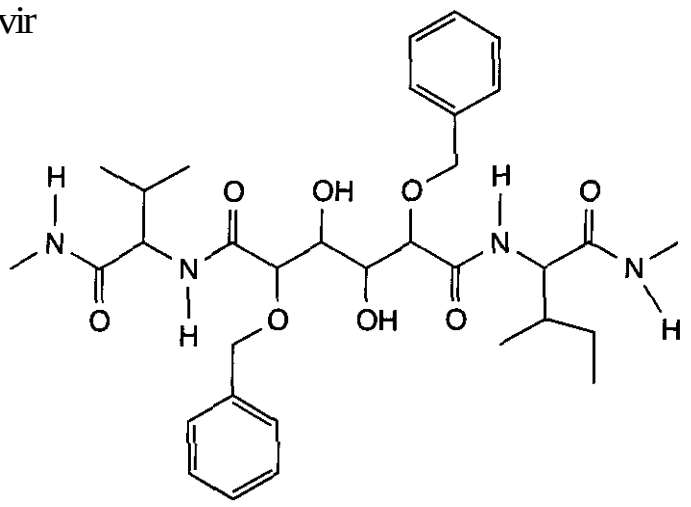
Mozenavir
(**37**)



Atazanavir
(**38**)



Tipranavir
(**39**)



L-Mannaric acid
(**40**)

Figure 11.14. Newer protease inhibitors.

inhibitor L-mannaric acid (40, Fig. 11.14) (126). Figure 11.14 depicts the structures of these newer protease inhibitors. Recently, other potential sites were targeted for HIV protease inactivation by peptides and peptidomimetics based on the terminal sequence of the enzyme. These terminal sequences exist at the enzyme surface and are believed to be less prone to mutations. Cupric ion was described to bind to such surface sequences rich in histidine and cysteine amino acid residues, leading to enzyme inhibition (127). Computer-aided drug design and molecular modeling are used to analyze binding of the inhibitors to the enzyme and to develop new agents based on a rationale drug design approach (128–132).

7 HIV DRUGS IN CLINICAL USE

All currently available drugs for HIV therapy belong to one of three classes of inhibitors: the nucleoside reverse transcriptase inhibitors (NRTIs), the nonnucleoside reverse transcriptase inhibitors (NNRTIs), and the protease inhibitors (PIs). These drugs have gained a def-

Table 11.1 HIV Approved Drugs for the Treatment of AIDS

Generic Name	Brand Name	Firm	Class
Zidovudine (AZT)	Retrovir	Glaxo Wellcome	NRTI
Didanosine (ddI)	Videx	Bristol-Myers Squibb	NRTI
Zalcitabine (ddC)	Hivid	Hoffman-La Roche	NRTI
Stavudine (d4T)	Zerit	Bristol-Myers Squibb	NRTI
Lamivudine (3TC)	Epivir	Glaxo Wellcome	NRTI
Abacavir (ABC)	Ziagen	Glaxo Wellcome	NRTI
Neveirapine	Viramune	Boehringer Ingelheim	NNRTI
Delavirdine	Rescriptor	Pharmacia	NNRTI
Efavirenz	Sustiva	Hoffman-La Roche	NNRTI
Idinavir	Crixivan	Mercke	PI
Ritonavir	Norvir	Abbott	PI
Saquinavir	Invirase	Hoffman-La Roche	PI
Nelfinavir	Viracept	Agouron Pharma	PI
Amprenavir	Agenerase	Glaxo Wellcome	PI

inite place in the treatment of HIV-1 infections because they interfere with crucial events in the HIV replication cycle. NRTIs, which target the substrate binding site, include six drugs: zidovudine, didanosine, zalcitabine, stavudine, lamivudine, and abacavir. NNRTIs, which target nonsubstrate binding sites, include three drugs: nevirapine, delavirdine, and efavirenz. Protease inhibitors bind to the active site and act as either enzyme inhibitors or dimer-destabilizing factors; these include five drugs: indinavir, ritonavir, saquinavir, neflinavir, and amprenavir. Table 11.1lists for each compound the generic name, brand name, the pharmaceutical firm that manufactures it, and its mechanistic classification.

8 THE NEED FOR NEW ANTI-HIV DRUGS

8.1 Development of Drug Resistance

In spite of having such an arsenal of drugs available for treatment of HIV infections, millions of dollars are being spent on AIDS research for developing new drugs. The development of drug-resistant HIV strains is a compelling reason for more efforts to develop newer inhibitors. Resistance arises from mutations in the viral genome, specifically in the regions that encode the molecular targets of therapy, HIV-1 RT and HIV-1 PR enzymes. These mutations alter the viral enzymes in such a way that the drug no longer inhibits the enzyme functions and the virus restores its free replication power. In the case of reverse transcriptase inhibitors, drug resistance to nucleoside analogs develops after prolonged treatment and has been exhibited for all the nucleoside inhibitors (133–135). However, NNRTIs are notorious for rapidly eliciting resistance (136) because of the mutations that are clustered around the putative hydrophobic binding site of these drugs (137).

8.2 Approaches to Overcome Drug Resistance (138–140)

8.2.1 Switching the Drug Class. Switching from one class of inhibitors to another (or, even within each class, from one compound to another) has proved to be effective in downplaying the resistance issue. Different NNRTIs do not necessarily lead to *cross-resistance*. For example, TSAO-resistant HIV-1 mutant strain containing ^{138}Glu to Lys mutation can be completely suppressed by another HIV-1-specific RT inhibitor like TIBO or nevirapine (141). Also a-APA derivative R89439 is very active against the ^{100}Leu to Ile mutant, which is highly resistant to the TIBO derivative (142).

8.2.2 The Virus "Knock-Out" Strategy. This approach requires starting therapy with sufficiently high drug concentrations so as to "knock out" the virus and prevent breakthrough of any virus, whether resistant or nonresistant (143). Total knock-out can also be achieved by beginning with drug combina-

tions that allow cells to clear from the virus at much lower drug concentrations than if the compounds were to be used individually.

8.2.3 Combination Therapy. HIV makes new copies of itself inside the infected cells at a very fast rate. Every day billions of new copies of HIV are made and millions of new cells die. One drug, by itself, can slow down the fast rate of infection. Two or more drugs can slow it down even more efficiently. Anti-HIV drugs from different drug groups attack the virus in different ways. For example, RT and protease are two different molecular targets. RT inhibitor will stop the HIV just after it enters the cell and a protease inhibitor stops it immediately before leaving it. Hitting at the different targets increases the chances of stopping HIV and protecting other cells from infection. In addition, different anti-HIV drugs can attack the virus in different cell types and different parts of the body. Combination therapy can reduce drug resistance to the virus, especially if the mutations counteract each other. A classic example is the ^{181}Tyr to Cys mutation, which causes resistance to most of the NNRTIs. This mutation suppresses the ^{215}Thr to Tyr mutation that causes resistance to AZT (144). In addition, the use of multidrug therapy allows low dosage of each drug. This minimizes the toxic effects exerted by individual drugs than if they were administered individually.

8.2.4 Officially Approved Drug Combinations. Among the formally recognized combination therapies are zidovudine and lamivudine (NRTI + NRTI); lopinavir and ritonavir (PI + PI); and abacavir, zidovudine, and lamivudine (NRTI + NRTI + NRTI). These combinations have been reported to achieve 1 to 3 log reductions in viral loads. Today, quadruple drug combinations are in vogue. These therapies have been reported to reduce the viral load to "undetectable levels." Such combinations are called highly active antiretroviral therapy (HAART). However, recent studies have shown that even after 30 months of HAART and undetectable viral load, patient-derived lymphocytes that were actively producing virus could be cultured in *vitro* (145). HIV remains a "sleeping giant."

8.2.5 Prodrug Conjugates: A New Trend in Combination Therapy. The recent trend in combination therapy has shifted lately into a new approach that combines the concepts of prodrug design as well as the combination therapy benefits. The approach involves the design of prodrug conjugates consisting of two of the desired inhibitors chemically conjoined though a linker molecule such as an amino acid or succinic acid. The prodrug is designed to spontaneously release the two drug components in the body. The prodrug conjugates not only provide single-dosing regimens, but have also proved to be of better anti-HIV activity than that of the individual drugs, probably because of the improved cell penetration properties (146–148).

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CHAPTER TWELVE

Organ Transplant Drugs

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1 INTRODUCTION

The immune response protects the body from potentially harmful substances (antigens) such as microorganisms, toxins, and cancer cells. The immune system distinguishes "self' from "foreign" by reacting to proteins on the surfaces of cells. The presence of foreign blood or tissue in the body triggers an immune response that can result in blood transfusion reactions and transplant rejection when antibodies are formed against foreign antigens on the transplanted or transfused material.

No two people (exceptidentical twins) have identical tissue antigens. Therefore, in the absence of immunosuppressive drugs, organ and tissue transplantation would almost always cause an immune response against the foreign tissue (rejection), which would result in destruction of the transplant. Though tissue typing ensures that the organ or tissue is as similar as possible to the tissues of the recipient, unless the donor is an identical twin, no match is perfect and the possibility of organ/tissue rejection remains. Immunosuppressive therapy is used to prevent organ rejection.

Blocking the activated cells, which attack a transplanted organ without shutting down the rest of the immune system, allows the body to learn to tolerate the transplant and still defend itself against germs. Thus, the holy grail of transplantation biology is how to prevent organ-specific graft rejection in transplant recipients without compromising their ability to fight infections. Currently, transplant patients face a life-long battle to control their immune system; this requires that they use strong immunosuppressive drugs that

may leave them unable to fight off infection by viruses or bacteria.

Historically, organ transplantation involving the kidney had been performed sporadically during the first half of the century (1, 2); however, planned programs for human organ transplantation started only in the late 1940s. The organs that are most commonly transplanted currently are kidney, liver, heart, lung, intestine, and pancreas.

1.1 Role of T-Cells in Organ Rejection

In experimental animals with congenital or induced T-cell deficiency, organ or tissue grafts survive indefinitely, indicating that T-cells play a central role in the specific immune response of allograft rejection. When a graft is introduced, it is accompanied by rejection because T-lymphocytes of the donor recognize the T-lymphocytes from the recipient as "foreign" bodies and react. Several studies have demonstrated that binding of alloantigen presented in the context of the major histocompatibility complex molecule (MHC) to the T cell receptor (TCR) on the surface of T-lymphocytes through direct and indirect recognition pathways (3) is the starting signal for T-cell activation (Fig. 12.1). Engagement of TCR with alloantigens recruits and activates a series of tyrosine kinases, including p561ck, p59fyn, and ZAP-70, followed by phosphorylation and activation of phospholipase C, and ultimately a rise in intracellular calcium (4). This leads to the activation of calcineurin enzyme, a serine-threonine phosphatase, that transduces signals to the nucleus to transcribe genes encoding cytokines relevant for the transition of the T-cells from the resting to the activated state.

Many studies have found that TCR stimulation alone is not eriough to sustain full activation and that additional signals are required (5). Thus, antigen-presenting cells (APC) are able to deliver costimulatory signals to T-cells that, through independent intracellular pathways, synergize with TCR stimulation, leading to cytokine production and T-cell activation (6). The best-characterized costimulatory signal is that driven by CD28, expressed as a homodimer on the surface of T-cells (7). Engagement of TCR and activation of costimulatory signals allow transcription of genes for cytokines that induce T-cells to move from the resting GO to the activated G1 state. G1 to S phase is regulated through a variety of receptors present on the T-cell surface, including IL-2 receptor, all acting on common intracellular elements in a pathway that controls key enzymes for the induction of cell division, such as cyclin/cyclin-dependent kinases. In this pathway *de novo* purine and pyrimidine synthesis is needed before lymphocytes can complete cell division. Thus, strategies to prevent T-cell activation or effector function can be potentially useful for antirejection therapy.

1.2 Classification of Organ Transplant Rejections

Clinically, organ transplant rejections can be classified into three categories and their management varies in accordance with the classification. Hyperacute rejection usually occurs within hours and is caused by antibodies already present in the recipient. Acute rejection is primarily T-cell based and can be controlled in most patients by the use of immunosuppressants. Chronic rejection is probably T-cell based and appears to be related to poor control of drug therapy, and usually requires another transplant. It is a slow, progressive process that usually begins inside the transplant organ's blood vessels, which are lined by donor cells that interact with host white blood cells in the bloodstream. Over time, as a result of inflammation and rejection reactions, scar tissue can accumulate inside these vessels, thus reducing or preventing blood flow into the filter and chemical plant portions of the kidney. If blockages become widespread, the organ becomes compromised because of the lack of oxygen and nutrients.

1.3 Current Trend in the Management of Organ Transplantation

In recent years, a greater understanding of the immunobiology of graft rejection, coupled with progressive improvements in the surgical and medical management, has revolutionized the field of clinical transplantation. Newer immunosuppressive schedules continue to be designed, not only to reduce the risk of rejection, but also to obtain a better therapeutic index that allows a further improvement of the graft

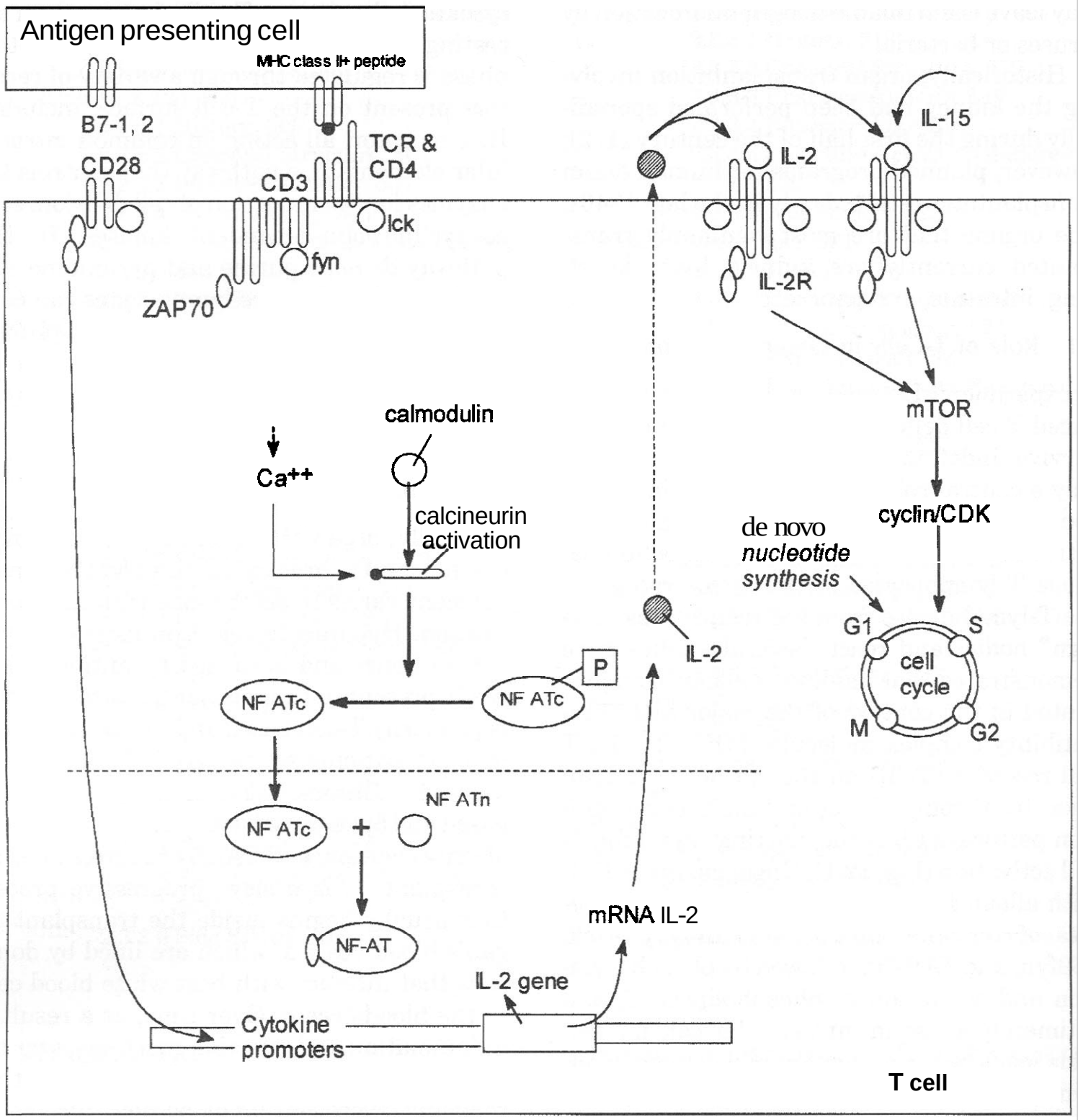


Figure 12.1. Principal pathways of T-cell activation that follow engagement of the alloantigen, presented by an APC in the context of an MHC molecule, with TCR on the surface of T-lymphocytes. APC, antigen-presenting cell; CD, cluster determinant; G1/G2, G1/G2 phases of cell cycle; Fyn, tyrosine kinase; lck, tyrosine kinase; IL, interleukin; IL-2R, interleukin receptor; M, M phase of cell cycle; MHC, major histocompatibility complex; mTOR, target for sirolimus protein; NF-AT, nuclear factor activating T-cell; NF-Atc, cytoplasmic subunit of NF-AT; NF-Atn, nuclear subunit of NF-AT; P, phosphate; S, S phase of cell cycle; TCR, T-cell receptor; ZAP 70, tyrosine kinase.

survival while minimizing the morbidity and drug-related toxicity. Presently, the optimal immunosuppressive strategy uses combinations of agents that act in synergistic fashion to provide the potency, freedom from toxic reactions, convenience of administration, and cost appropriate for the individual patient. This in turn has resulted in the significant increase in the overall survival rates for transplant recipients, which is at an all-time high. Kidney recipients lead the 1-year patient survival rate with **94.3%** followed by pancreas recipients at 91.1% (8). However, along with the success of organ transplantation, also comes the need for organs, which has grown at a much faster rate than the number of donated

organs. In the United States alone the wait list is now more than 75,000, with approximately 5000 dying every year waiting for the organ transplantation (9). Nevertheless, an increase in the number of patients with end-stage kidney disease has also been observed at the rate of 7–8% each year. Currently, 12,000–14,000 kidney transplants alone are performed annually (10). Overall, organ transplants have risen by 5.4% in 2000 compared to 1999, according to preliminary data on U.S. organ donors released by the U.S. Department of Health and Human Services' Health Resources and Services Administration and the United Network for Organ Sharing. Organ transplants in 2000 totaled 22,827, an increase of 1172 over that of 21,655 transplants that occurred in 1999 (11). Thus, although multiple thousands of lives are being saved by the use of various immunosuppressive regimens, serious complications still occur as a result of treatment. This necessitates the ongoing search for novel, clinically efficacious, and nontoxic organ transplant drugs.

During the past 50 years, a large number of organ transplant drugs have been described. They have been successfully used under both allograft and xenograft conditions. These therapeutic agents interfere at different stages of T-cell activation and proliferation and can be classified into two main groups:

1. Agents that specifically block T-cell function, such as cyclosporine, tacrolimus, sirolimus, 15-deoxyspergualin, FTY 720, and monoclonal antibodies.
2. Agents that block nucleotide synthesis, such as azathioprine, brequinar, mycophenolate mofetil, mizoribine, and leflunomide.

The oldest class of immunosuppressants is the corticosteroids, which are extremely potent inhibitors of inflammation but when used by themselves do not adequately prevent organ rejection. The purpose of this review is to give an account of the organ transplant drugs, which are clinically used for inhibiting the rejection of allografts. Xenotransplantation using pig's organs, however, is gaining momentum, although its use under clinical conditions has yet to be established (12). Many new experimental prototypes being studied for their antirejection properties in laboratory animals and in preclinical trials have been reviewed earlier **(13)** and will not be discussed here.

2 CLINICAL USE OF AGENTS

2.1 Current Drugs on the Market

Although cyclosporine, FK 506, and azathioprine, just to name a few of the most commonly used agents, are clinically available for treating organ transplants, their long-term use is associated with a high incidence of clinical complications, such as nephrotoxicity, hepatotoxicity, neurotoxicity, and gastrotoxicity. There is thus an ongoing requirement to develop immunosuppressive drugs with novel modes of action, with an improved ratio of desired activity to toxic effects that not only is exhibited by clinically used agents but also has an ability to prevent acute rejection and improve long-term graft function. Since the appearance of azathioprine in the 1960s, a large number of agents with immunosuppressive activity have been reported in the literature. Some of them are undergoing clinical trials and may soon enter into routine clinical use, whereas other pharmacological agents are currently being evaluated in laboratory animals. These compounds interfere at different stages of T-cell activation and proliferation and can be identified as inhibitors of nucleotide synthesis, growth factor signal transduction, and differentiation. In the last 5 years several new immunosuppressive drugs have been introduced in the market, out of which many new agents offer better therapeutic indexes and show promise in the treatment of allograft transplantation. **A** variety of structurally diverse immunosuppressive drugs used currently are summarized in Table 12.1.

2.2 Agents that Specifically Block T-Cell Function

2.2.1 Cyclosporine

2.2.1.1 ***History.*** Cyclosporine (Novartis, Basel, Switzerland) is a cyclic undecapeptide isolated from the extracts of the fungus *Tolypocladium inflatum* by Jean-Francois Borel,

Table 12.1 Current Drugs on Market

Drug Name	Code Numbers1 Other Names	Commercial Name/ Launching Year	Company Name	Injectable Formulation	Oral Capsule/Formulation
Cyclosporine	Ciclosporine	Sandimmun, 1983	Novartis, Switzerland	50 mg/mL	Soft gelatin capsules 25, 50, and 100 mg; oral solution 100 mg/mL
		Neoral, 1995	Novartis, Switzerland		Soft gelatin capsules in 25 and 100 mg strengths; oral solution available in 100 mg/mL alcohol concentration
Tacrolimus	FK 506	Prograf, 1994	Fujisawa, USA	5 mg/mL	Capsules of 0.5, 1, or 5 mg
Sirolimus	Rapamycin	Rapamune, 1999	Wyeth-Ayerst Pharmaceuticals, USA		Oral solution with 1 mg/mL,
Deoxyspergualin	NSC 356894 and Gusperimus	Spanidin, 1994	Nippon kayaku, Japan		Tablet 100 mg
Monoclonal antibodies	Muromonab CD3	Orthoclone (OKT3), 1986		Solution 5 mg/5 mL	
	Basiliximab	Simulect, 1998	Novartis, Switzerland	20 mg lyophilized powder	
	Daclizumab	Zenepax, 1997	Hoffman Laroche, USA	25 mg/5 mL	
Azathioprine	B-W 322	Imuran, 1968	Burroughs Wellcome Lab	10 mg/mL	50 mg tablets
Mycophenolate	RS 61443	Cell Cept, 1995	Roche, USA	6 mg/mL	500 mg, 250 mg tablets or liquid 200 mg/mL

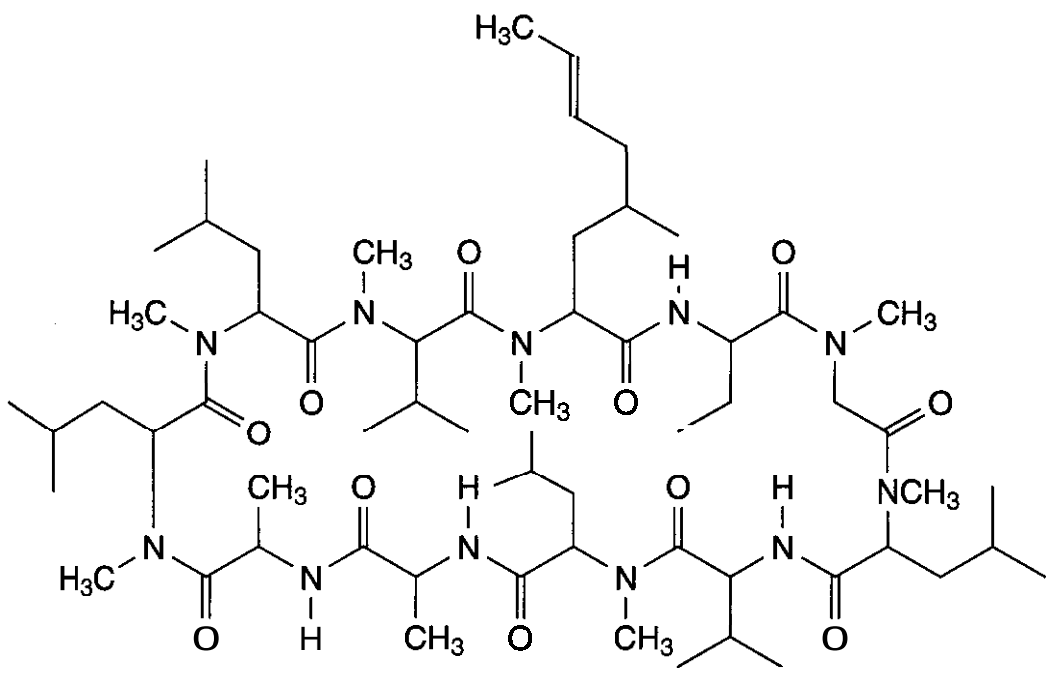


Figure 12.2. Structure of cyclosporine (1).

who worked in Switzerland for Novartis (formerly known as Sandoz) Pharmaceuticals. He discovered the immunosuppressant agent that ultimately moved transplantation from the realm of curiosity into routine therapy. Borel chose to examine a weak compound that was isolated from the soil fungus *Tolypocladium inflatum Gams* (subsequently renamed Beauveria *nivea*). The compound was thought to have little practical value, yet Novartis chemists continued to study and purify the compound because of its "interesting" chemical properties. Borel discovered that, unlike immunosuppressants that acted indiscriminately, this compound selectively suppressed the T-cells of the immune system (14). Excited by these characteristics, Borel continued his study and, in 1973, purified the compound called cyclosporine (also known as cyclosporin, ciclosporin). Cyclosporine was tested in humans for the first time in 1978 by Calne et al. (15) for kidney and by Powles et al. (16) for bone marrow. The results were startling: rejection was effectively inhibited in five of the seven patients receiving kidneys from mismatched deceased donors. Following extensive patient clinical trials, the U.S. Food and Drug Administration (U.S. FDA) granted clearance to Novartis to market cyclosporine under the brand name Sandimmune in November of 1983. This revolutionary therapy is indicated today for the prevention of organ rejection in kidney, liver, and heart transplant patients. Sandimmune (17) thus became the lifeline for thousands of transplant recipients throughout the 1980s and into the next decade. However, the absorption of cyclosporine during chronic administration of Sandimmune soft gelatin capsules and oral solution was found to be erratic. Hence, continuous efforts were made to improve its bioavailability so as to minimize the toxicity and improve the risk-to-benefit ratio. Subsequently in 1995, a new oral formulation of cyclosporine in the form of cyclosporine microemulsion was introduced under the trade name Neoral by Novartis (18). With this formulation cyclosporine was found to disperse faster in the gut, thereby enhancing its absorption.

2.2.1.2 Chemical Structure. Chemically, cyclosporine (1) is a cyclic undecapeptide and designated as [*R*-[*R**,*R**-(*E*)]]-cyclic-(L-alanyl-D-alanyl-N-methyl-L-leucyl-N-methyl-L-leucyl-N-methyl-L-valyl-3-hydroxy-N,4-dimethyl-L-2-amino-6-octenoyl-L-aminobutyryl-N-methylglycyl-N-methyl-L-leucyl-L-valyl-N-methyl-L-leucyl). The structure is shown in Fig. 12.2.

2.2.1.3 Pharmacokinetics. Absorption of cyclosporine during chronic administration of Sandimmune soft gelatin capsules and oral solution was found to be erratic. However, Neoral was found to have increased bioavailability over that of sandimmune. The extent of absorption of cyclosporine was dependent on the individual patient, the patient population, and the formulation. The relationship between administered dose and exposure [area under the concentration vs. time curve (AUC)] was linear within the therapeutic dose range.

Intrasubject variability of AUC in renal transplant recipients was 9–21% for Neoral and 19–26% for Sandimmune. The disposition of cyclosporine from blood is generally biphasic, with a terminal half-life of approximately 8.4 h (range 5–18 h). After intravenous administration, the blood clearance of cyclosporine (assay: HPLC) was approximately 5–7 mL/min/kg in adult recipients of renal or liver allografts. After oral administration of Neoral, the time to peak blood cyclosporine concentrations (T_{max}) ranged from 1.5 to 2.0 h. Cyclosporine is distributed largely outside the blood volume. The steady-state volume of distribution during intravenous dosing has been reported as 3–5 L/kg in solid organ transplant recipients. In blood, the distribution is concentration dependent: approximately 33–47% is in plasma, 4–9% in lymphocytes, 5–12% in granulocytes, and 41–58% in erythrocytes. Cyclosporine is extensively metabolized by the cytochrome P450 111-A enzyme system in the liver, to a lesser degree in the gastrointestinal tract, and the kidney. At least 25 metabolites have been identified from human bile, feces, blood, and urine. The biological activity of the metabolites and their contributions to toxicity are considerably less than those of the parent compound. Blood cyclosporine clearance appears to be slightly slower in cardiac transplant patients (18). Cyclosporine blood concentration needs to be monitored in transplant and rheumatoid arthritis patients taking Neoral, to avoid toxicity resulting from high concentrations. Dose adjustments also need to be made in transplant patients to minimize the possibility of organ rejection attributed to low concentration (18). Only 0.1% of a cyclosporine dose is excreted unchanged in the urine. Elimination is primarily biliary, with only 6% of the dose (parent drug and metabolites) excreted in the urine (18).

2.2.1.4 ***Pharmacology.*** It has been indicated in transplantation for the prophylaxis of organ rejection in kidney, liver, and heart transplants, and continues to be a cornerstone of immunosuppressive therapy. The immunosuppressive effect of cyclosporine depends on its inhibition of calcium-dependent T-cell activation (19). After entering cells, cyclosporine binds to an intracellular receptor, cyclophilin (20). The crystal structure of a complex between recombinant human cyclophilin A and CsA has been determined from a novel orthorhombic crystal form that contains only one monomer complex per asymmetric unit. The structure has been refined at 2.1-Å resolution to a crystallographic R-factor of 16.7%. The binding pocket of cyclophilin is a hydrophobic crevice defined by 13 residues that are within 4 Å of the bound CsA. In this high resolution structure, five direct hydrogen bonds and a network of water-mediated contacts stabilize the interactions between CsA and cyclophilin (Fig. 12.3). One of the water molecules plays a pivotal role and it is within a hydrogen bond distance of four atoms (MeBmt 1 O, W19, Gln63 NE2, and His54 NE2). The solvent molecule W19 is hydrogen bonded to this water molecule and to the carbonyl oxygen atom Asn71. The structure of the cyclophilin/CsA complex is consistent with a two-domain description of CsA. The cyclophilin binding domain composed of residues 1, 2, 3, 9, 10, and 11, and an exposed "effector domain" composed of residues 4, 5, 6, 7, and 8, which has higher flexibility and may be interacting with calcineurin. The accessible surface area of these five residues in this crystal complex is about 460 $Å^2$, compared with 230 $Å^2$ for the six residues making contact with cyclophilin. Many CsA analogs have shown a good agreement between cyclophilin binding and immunosuppressive activity based on the two-domain description of CsA. For example, substitution of $MeVal^{11}$ to $MeAla^{11}$ results in a 91.5% decrease in the immunosuppressive activity.

Once complexed with cyclophilin, the cyclosporine-cyclophilin complex inhibits the calcium- and calmodulin-dependent serine-threonine phosphatase activity of the enzyme calcineurin (21, 22), which in turn prevents the generation of the potent nuclear factor of activated T-cells. In fact, it acts in two different ways to inhibit the activation and proliferation of T-cytotoxic lymphocytes (white blood cells), the specific mediators of organ rejection. First, cyclosporine impedes the production and release of a protein IL-2 by T-helper white blood cells. Second, cyclosporine inhibits interleukin 2 receptor (IL-2R) expression on both T-helper and T-cytotoxic white blood

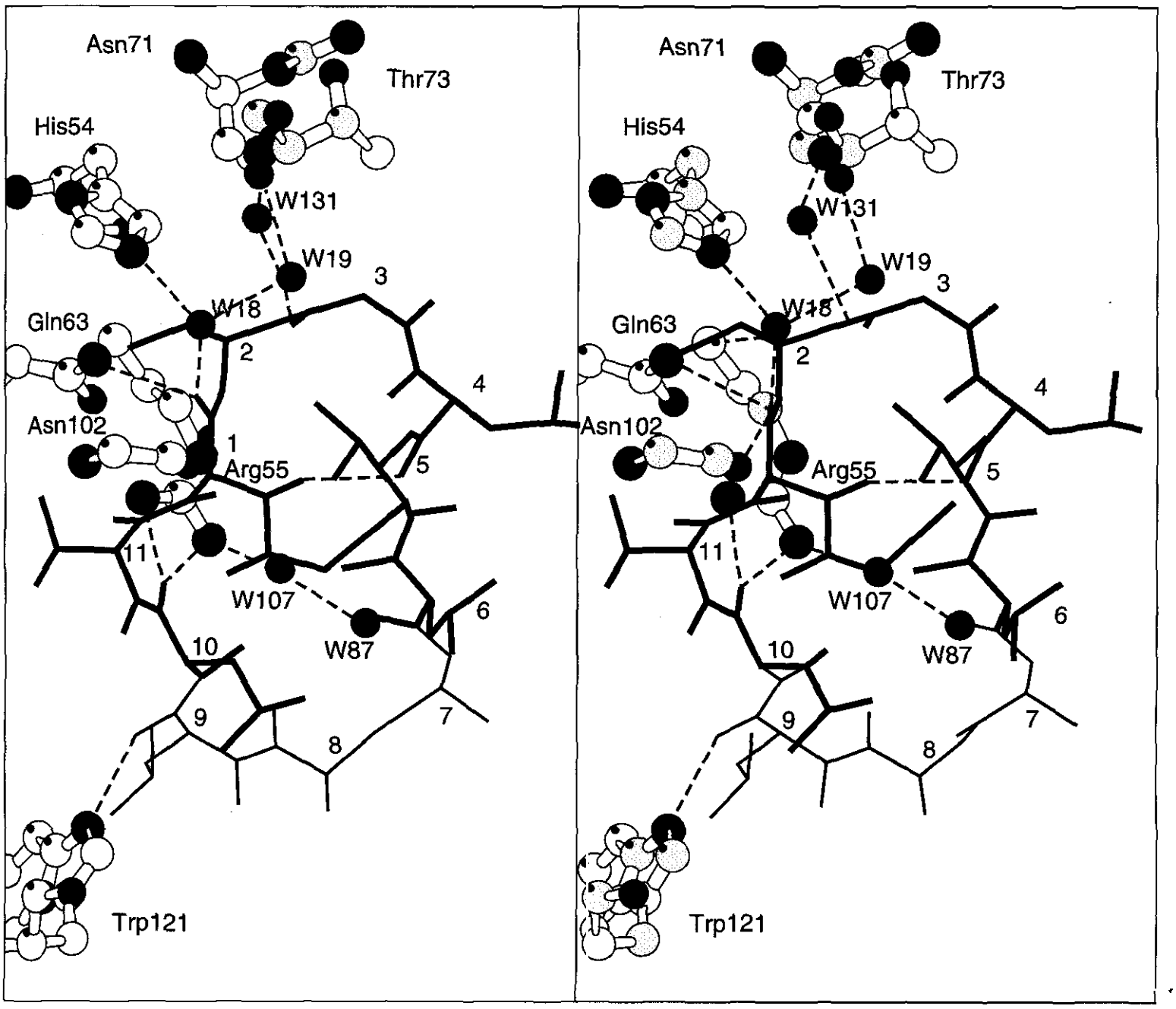


Figure 12.3. Stereoview of contacts between CsA and cyclophilin.

cells. These two actions effectively and selectively limit the differentiation and proliferation of T-cytotoxic white blood cells (22).

The superior selectivity and efficacy of cyclosporine compared with those of azathioprine were demonstrated by an increase in 1-year survival rates of renal allografts from cadaver donors to 80% for cyclosporine-based regimens and only 51% for azathioprine-based regimens (23). Furthermore, in contradistinction to the azathioprine-prednisone regimen, cyclosporine-prednisone therapy facilitated the successful clinical transplantation of hearts (24), livers (25), lungs (26), and combined heart and lung (24) allografts. Reports suggest that cyclosporine therapy may have an impact on chronic rejection and allograft coronary artery disease. The half-life for heart transplants improved from 5.4 years in the 1980–1985 period to 8.7 years in the 1986–1990 period (27). Similarly, an increase in renal allograft half-life from 16.6 to 23.04 years was observed when maintenance therapy was changed from azathioprine with high doses of steroids to cyclosporine and low dose steroids (28). In a prospective randomized study, the frequency of clinically defined chronic rejection after 4 years was 25% for the patients on azathioprine and steroids and only 9% for the patients on triple therapy including cyclosporine (29).

Cyclosporine dose and trough levels have been related to the development of chronic rejection in liver, kidney, and heart transplantation. Liver grafts in patients maintained on median cyclosporine levels (whole blood, trough level) of more than 175 μg/L in the first 28 days posttransplant had a significantly

lower incidence of chronic rejection [2 of 49 versus 22 of 97 ($P = 0.002$)] (30). In a multivariate analysis of 587 kidney-alone transplants performed over a 5-year period, patients on less than 5 mg/kg of cyclosporine at 1 year were more prone to develop chronic rejection ($P = 0.007$) (31). Similarly, results from 225 heart transplant patients who had survived for 1 year after transplant demonstrated that actuarial 5-year survival in patients whose average cyclosporine dose was less than 3 mg/kg/day, was 60% compared to 77% in patients whose average dose was more than 3 mg/kg/day (32). Cyclosporine-based therapy significantly reduced patient morbidity and overcame at least some of the negative effects of the risk factors that were associated with azathioprine therapy. The risk factors included pretransplant blood transfusions, the possibility of retransplantation, and the requirement for matching of alleles of the human leukocyte antigen (HLA) system (33).

2.2.1.5 Structure-Activity Relationship. Cyclosporine is the cornerstone of immunosuppressive therapy to prevent rejection of transplanted organs but it also causes kidney and liver damage. Thus there is a clear need for an alternative to cyclosporine with a reduced toxicity profile. This led researchers to carry out structure-activity relationship studies, to reduce or remove the toxic component. Studies with both naturally occurring modifications (34, 35) as well as with synthetically modified analogs of cyclosporine (36, 37) have been carried out. In the literature more than 3000 analogs of the drug have been synthesized, although none was found to be more potent than cyclosporine and was as toxic as cyclosporine. A strong correlation was observed between the immunosuppressive activity of cyclosporine analogs and the ability of the receptor-analog complex to bind to calcineurin in ***vitro*** and to inhibit its phosphatase activity against an artificial substrate ***in vitro.*** For example, even though the nonimmunosuppressive cyclosporine analog MeAla^6-cyclosporine binds to cyclophilin with an affinity similar to that of cyclosporine, the cyclophilin/MeAla^6-cyclosporine complex failed to inhibit calcineurin phosphatase activity (38). Recently, Isotechnika Biotech identified a novel analog ISAtx247 (the exact structure is currently confidential), which provided better organ transplant survival and a superior toxicity profile to that of cyclosporine on a mg-for-mg basis in all animal models studied to date. Initial phase I single-dose human studies exhibited no significant adverse effects. Isotechnika has received approval from Canada's Health Protection Branch to begin phase I human clinical trials. An application for conducting human trials in the United States has also been filed (39).

2.2.1.6 ***Side Effects.*** The major limitations of cyclosporine are its various toxicities; its nephrotoxicity, especially, is a serious one because several cell types, including those of the brain, heart, and kidney, require calcineurin to function. The nephrotoxicity of cyclosporine is associated with mucoid deposits in arterioles, focal fibrosis potentiated by vasoconstriction, a disturbed balance of ionic calcium, an altered balance of prostacyclin to thromboxane, and prorennin activation.

The other major limitation of cyclosporine is its inability to prevent chronic transplant nephropathy. The most common adverse events reported with Neoral in transplantation include renal dysfunction, tremor, hirsutism, hypertension, and gum hyperplasia (18). The risk increases with increasing doses of cyclosporine. High cyclosporine levels result in acute toxic symptoms such as nausea, headache, acute sensitivity of the skin, flushing, gum pain and bleeding, and a sensation of increased stomach size. High levels may also cause liver or kidney failure but this is temporary and no permanent failure is known (40). No clinically significant pharmacokinetic interactions occurred between cyclosporine and aspirin, ketoprofen, piroxicam, or indomethacin (18).

2.2.2 FK 506

2.2.2.1 History. In 1984 scientists from Fujisawa isolated the macrolide FK 506 (tacrolimus) from a fermentation broth of ***Streptomyces tsukubadensis*** (soil sample, Tsukuba Japan) (41). The discovery was part of an extensive, ongoing screening for agents that can suppress or heighten the body's immune responses. Further tests conducted in laboratory animals by Ochiai at Chiba University in Chibashi, Japan, proved its ability to suppress

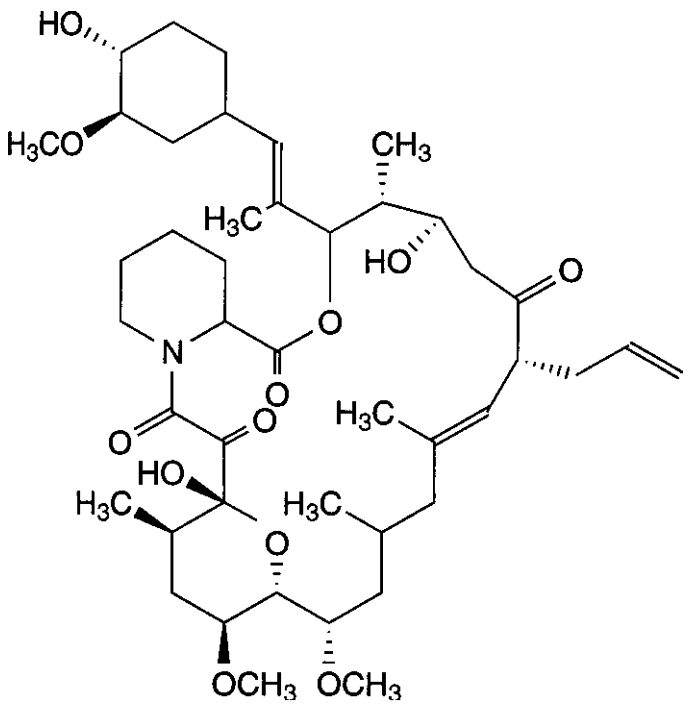


Figure 12.4. Structure of **FK 506 (2).**

the immune system. Unfortunately, further progress with this drug in Japan could not be pursued because of the cultural prohibition of cadaver transplantation. Subsequently, Starzl and his colleague carried out the drug's clinical development at the University of Pittsburgh Medical Center (Pittsburgh, PA). They found that the use of FK 506 decreased the incidence of rejection episodes while allowing a much lower dosage of steroids.

2.2.2.2 ***Chemical Structure.*** FK 506 is a 23-member macrolide designated as *[3S-[3R*[E(1S*, 3S*, 4S*)], 4S*, 5R*, 8S*, 9E, 12R*, 14R*, 15S*, 16R*, 18S*, 19S*, 26aR*]]-5,-6,-8,-11,-12,-13,-14,-15,-16,-17,-18,-19,-24,-25,-26,-26a-hexa-deca-hydro-5,19-di-hydroxy-*3-[2-(4-hy-droxy-3-meth-oxy-cyclo-hexy1)-1-methyl-ethenyl]-14,16-di-meth-oxy-4,-10,-12,-18-tetra-methyl-8-(2-propeny1)-15,-19-epoxy-3*H*-pyrido[2,1-*c*][1,4]-oxa-azacyclo-tricosine-1,-7,-20,-21*(4H,-23H)*-tetrone monohydrate (42). The structure (2; Fig. 12.4) was deduced on the basis of extensive chemical degradation and spectroscopic studies. The first report of total synthesis was published in 1989 by chemists at the Merck, Sharp & Dohme laboratories (43, 44).

2.2.2.3 ***Pharmacokinetics.*** Absorption of tacrolimus from the gastrointestinal tract after oral administration is incomplete and variable. The oral bioavailability in humans is variable and ranges from 5% to 67% in recipients of liver, small bowel, and kidney transplantation. The absolute bioavailability of tacrolimus was 17 rt 10% in adult kidney transplant patients ($n = 26$), 22 ± 6% in adult liver transplant patients ($n = 17$), and 18 ± 5% in healthy volunteers ($n = 16$) (42). The tacrolimus maximum blood concentrations (C_{max}) were found to vary in different organ transplant settings: 29.7 ± 7.2 in normal healthy volunteers, 19.2 ± 10.3 in kidney transplant patients, and 68.5 ± 30.0 in liver transplant patients. In 11 liver transplant patients, Prograf administered 15 min after a high fat (400 kcal, 34% fat) breakfast, resulted in a decreased AUC (27 ± 18%) and C, (50 ± 19%), compared to that of a fasted state. The $t_{1/2}$ (h) of tacrolimus has been determined after intravenous (i.v.) and oral (p.o.) administration in healthy volunteers and both liver and kidney transplant patients. In healthy volunteers, $t_{1/2}$ is 34.8 + 11.4 (p.o. 5 mg), in liver patients $t_{1/2}$ is 11.7 ± 3.9 (i.v. 0.05 mg/kg/12 h), and in kidney transplant patients $t_{1/2}$ is 18.8 ± 16.7 (i.v. 0.02 mg/kg/12 h) (42). The plasma protein binding of tacrolimus is approximately 99% and is independent of concentration over a range of 5–50 ng/mL. The distribution of tacrolimus between whole blood and plasma depends on several factors, such as hematocrit, temperature at the time of plasma separation, drug concentration, and plasma protein concentration. The mean clearance following i.v. administration of tacrolimus is 0.040, 0.083, and 0.053 L/h/kg in healthy volunteers, adult kidney transplant patients, and adult liver transplant patients, respectively. In humans, less than 1% of the dose administered is excreted unchanged in urine (42).

2.2.2.4 Pharmacology. Prograf has been used for the prophylaxis of rejection in liver transplants (since 1994) as well as for kidney transplants (since 1997). Tacrolimus was found to be at least 10–100 times more potent than cyclosporine in the in vitro models of immune suppression (45, 46). It binds competitively and with high affinity to a cytosolic receptor (immunophilin) termed as the FK binding protein (FKBP-12) (47). Studies have shown that FK 506 elicits its immunosuppressive activity by inhibiting the cis-trans peptidyl-prolyl isomerase activity of FKBP. The structure of the human FKBP complexed with

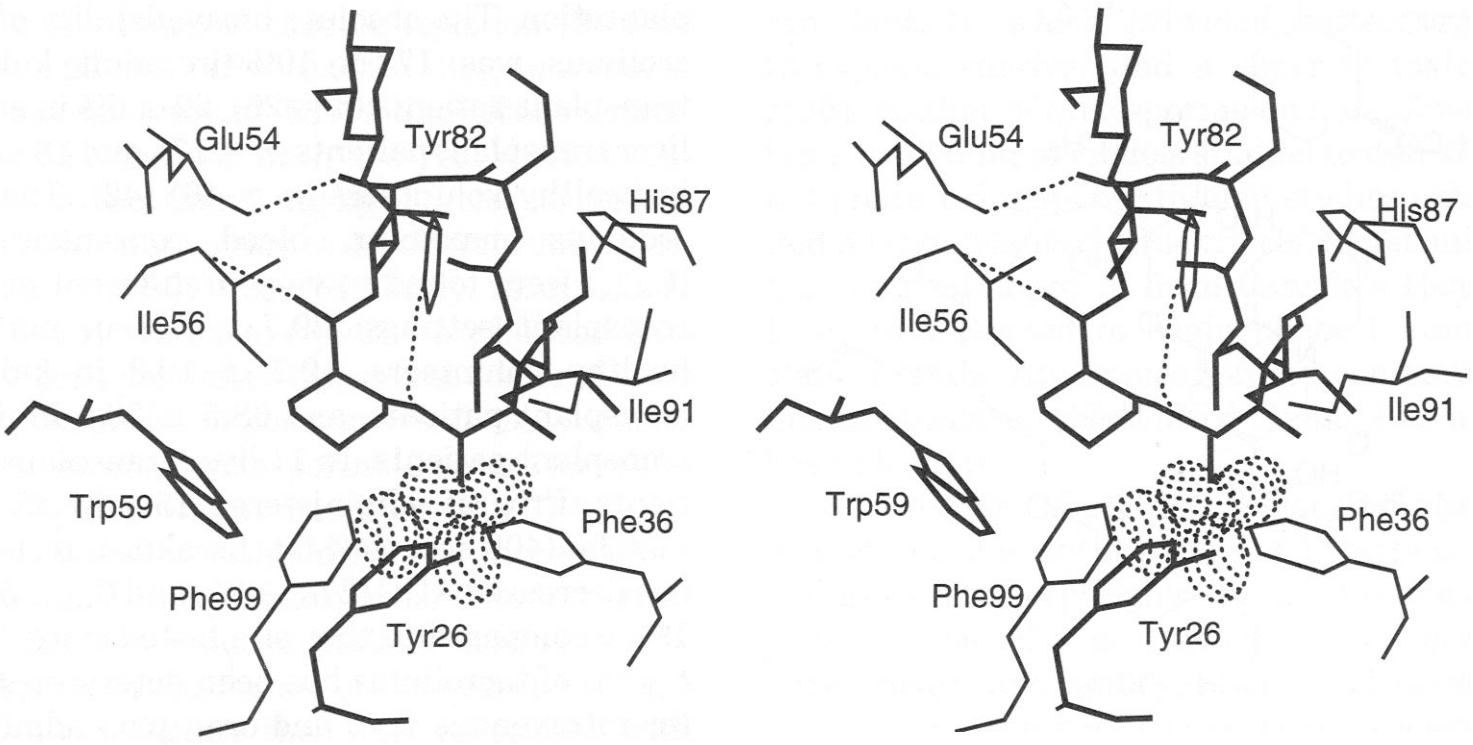


Figure 12.5. Stereoview of the binding region of FKBP-FK506 and selected residues of the hydrophobic pocket. Hydrogen bonds are shown between the C-1 ester carbonyl and the NH of Ile^{56}, the C-24 hydroxyl and the main-chain carbonyl of Glu^{54}, and the C-8 amide and the phenolic-OH of Tyr^{82}. The C-9 carbonyl binding pocket is illustrated with van der Waals dot surfaces for the ϵ-CHs of Tyr^{26}, Phe^{36}, and Phe^{99}. The side chains from residues Tyr^{26}, Phe^{36}, Asp^{37}, Arg^{42}, Phe^{46}, Glu^{54}, Val^{55}, Ile^{56}, Trp^{59}, Tyr^{82}, His^{87}, Ile^{91}, and Phe^{99} are within 4 Å of FK506.

immunosuppressant FK 506 has been determined to 1.7-Å resolution by X-ray crystallography (47). The conformation of the protein changes little upon complexation, but the conformation of FK 506 is markedly different in the bound and unbound forms. It binds in a shallow cavity between the a-helix and the β-sheet with roughly 430 $Å^2$ (50%) of the ligand surface being buried at the protein-ligand interface and the remainder, encompassing the region around the allyl and the cyclohexyl groups, being exposed to the solvent (Fig. 12.5). Loops composed of residues 39–46, 50–56, and 82–95 flank the binding pocket, which is lined with conserved, aromatic residues. The side chain of Tyr^{26}, Phe^{46}, Phe^{55}, and Val^{55}-Ile^{56} make up the sides of the pocket, whereas the indole of Trp^{59}, in the a-helix, is at the end of the pocket and serves as a platform for the pipecolinyl ring, the most deeply buried part of FK 506. There are five hydrogen bonds between FKBP and FK 506, out of which the fifth hydrogen bond, involving the C8 amide, is the most conspicuous because it is nearly orthogonal to the carbonyl plane and thus may be relevant to the mechanism of rotamase activity. Notably, complexed FK 506 more closely resembles free rapamycin than it does free FK 506, which suggests that the higher affinity of rapamycin to FKBP (K_d = 0.2 nM for rapamycin versus 0.4 nM for FK 506) reflects its greater preorganization. These investigations provide a structural framework to improve on the high affinity interactions of a clinically promising immunosuppressant with its predominant cytosolic receptor in the T-cell.

After binding with FKBP, the FK 506-FKBP complex binds with the catalytic A subunit of calcineurin and in turn inhibits protein phophatase activity of calcineurin, thereby blocking the activity of the cytoplasmic component of the nuclear factor of activated T-cells (48). This in turn prevents dephosphorylation of the cytoplasmic subunit of a transcription factor, nuclear factor of activated T-cells, which otherwise enters the nucleus and activates expression of T-cell activation lymphokine genes (49–51). Tacrolimus has been found to inhibit IL-2 production by T-cells in a fashion that is similar to that of cyclosporine (41). It prolonged the survival of the host and transplanted graft in animal transplant models of liver, kidney, heart, bone marrow, small bowel and pancreas, lung and trachea, skin, cornea, and limb, thereby sug-

gesting its potential for clinical application to organ transplantation (52–54). In fact, in animals, tacrolimus has been demonstrated to suppress some humoral immunity and, to a greater extent, cell-mediated reactions such as allograft rejection, delayed-type hypersensitivity, collagen-induced arthritis, experimental allergic encephalomyelitis, and graft-vs.-host disease (GVHD).

In one of the first clinical trial studies carried out in liver transplant recipients (55), primary prevention with tacrolimus led to a significant improvement in both patient and graft survival. Rejection episodes and corticosteroid requirements were also reduced compared to those of the control receiving cyclosporine. In another trial performed by the same group (56), no significant difference was observed in patient survival between the tacrolimus and cyclosporine arms; however, freedom from rejection for patients treated with tacrolimus (61%) was much higher than that for patients treated with cyclosporine (18%). This was followed by extensive multicenter trials performed worldwide in liver and kidney transplant recipients, to compare the efficacy and tolerability of tacrolimus-based immunosuppressants with those of cyclosporine. In one of the trials (57) conducted in 529 recipients of liver transplants, the regimens based on tacrolimus and cyclosporine were comparable in terms of patient and graft survival. Similarly, in a European multicenter clinical trial involving 545 liver transplant recipients, the tacrolimus-treated group displayed an acute rejection episode rate of 43.4%, compared to 53.6% for the cyclosporine-treated group (58). In this study, both renal toxicity and neurological complications were more common among those patients who had been treated with tacrolimus. In the kidney transplant recipients, a European multicenter study failed to provide any evidence that the tacrolimus provides better immunosuppression than cyclosporine (59). Similarly, the 1-year analysis of the tacrolimus U.S. kidney transplant multicenter study demonstrated that immunosuppressive therapy with tacrolimus compared to cyclosporine led to statistically fewer rejections without affecting patient and graft survival and without major morbidity (60). Interestingly, in both liver and kidney transplant settings, tacrolimus unlike cyclosporine is effective as a rescue therapy for acute rejection episodes resistant to conventional corticosteroid pulses (61, 62). Similar rescues after refractory rejection episodes have been reported for cardiac and combined pancreas-kidney transplants (63–65).

The efficacy of tacrolimus for the salvage treatment of chronic GVHD was evaluated in a single-arm, open-label phase II study. A total of 39 evaluable patients with chronic GVHD who failed previous immunosuppressive therapy with cyclosporine and prednisone were treated with tacrolimus, starting at a median of 20 months (range, 3–68 months) after transplantation (66). The Kaplan-Meier estimate of survival was 64% (95% confidence interval, 49–79%) at 3 years posttransplantation and the response rate was consistent with the earlier reports of salvage treatment for chronic GVHD. Further, because the elimination by *ex vivo* irradiation of mature lymphoid elements from the bowel allografts was known to eliminate the GVHD risk, the Pittsburgh group hypothesized that the infusion of donor bone marrow cells (BMC) in recipients of irradiated intestine may improve tolerogenesis without increasing the risk of GVHD. Their studies showed that, although recipients were protected from GVHD by irradiating intestinal allografts, the resulting leukocyte depletion led to chronic rejection of the transplanted bowel (67).

The safety and potential of tacrolimus are further evident by a growing number of pregnancies occurring in mothers receiving tacrolimus systemically. One hundred pregnancies in 84 mothers were recorded, out of which 71 progressed to delivery (68 live births, 2 neonatal deaths, and 1 stillbirth), 24 were terminated (12 spontaneous and 12 induced), and 3 were lost to follow-up. The most common complications in the neonate were hypoxia, hyperkalemia, and renal dysfunction, although they were transient in nature. Four neonates presented with malformations, without any consistent pattern of affected organ (68).

Recently, the efficacy of tacrolimus as monotherapy has also been evaluated in adult cardiac transplants. Forty-three patients received tacrolimus and prednisone as primary immunosuppression agents, without azathio-

Table 12.2 Conversion to Tacrolimus from Cyclosporine in Various Organ Transplant Settings

Organ Transplant	Number of Patients	Results	Reference
Lung or heart–lung transplantation	11	Conversion to tacrolimus slows the decline of lung function in bronchiolitis obliterans syndrome.	71
Liver transplant	21	Conversion to tacrolimus improves hyperlipidemic states in stable liver transplant recipients.	72
Heart transplant	10 Japanese	Postoperative follow-up after cardiac transplantation appears to be satisfactory with conversion to tacrolimus.	73
Renal transplant	17	Conversion from Cyclosporine to tacrolimus in stable renal transplant recipients may lead to attenuation of cardiovascular morbidity and chronic transplant nephropathy in the long term.	74
Heart transplant	85	Tacrolimus seems safe and effective for preventing rejection during the first year after heart transplant. It causes less high blood pressure and less high cholesterol, and has no more side effects in areas other than cyclosporine.	75

prine or mycophenolate mofetil. Thirty-two of the 43 patients started on tacrolimus were weaned off steroids and maintained on monotherapy. Their results suggest that use of tacrolimus alone after steroid weaning provides effective immunosuppression with low incidence of rejection, cytomegalovirus infection, transplant arteriopathy, or posttransplant lymphoproliferative disease (69). In another study in heart transplant recipients, monotherapy with tacrolimus has been found to significantly reduce blood clot formation compared to that with cyclosporine, thereby suggesting that the drug may help prevent heart transplant vasculopathy (70).

In recent years a number of groups have recommended tacrolimus conversion as a means to antilymphocyte preparations for corticosteroid-resistant rejection in cyclosporine-based regimens. Some of the studies pertaining to tacrolimus conversions in a variety of organ transplant settings and their outcomes are summarized in Table 12.2. These findings suggest that conversion from a cyclosporine-based to a tacrolimus-based maintenance immunosuppression appears to be an effective and safe approach to the management of patients with persistent or recurrent allograft rejection or those with cyclosporine intolerance.

In liver transplant patients, early chronic rejection episodes can be reversed by shifting from a cyclosporine-based protocol to a tacrolimus-based immunosuppressive regimen. This has been attributed to the fact that tacrolimus and transforming growth factor β-1 share the same binding site, the immunonophilin FKBP-12 (76). Thus, tacrolimus may interfere with the TGF β-1 signaling through competitive binding, and by antagonizing the fibrosis-promoting effect of this growth factor. Given the similar effect on T-lymphocytes, tacrolimus has also been proposed as an alternative to cyclosporine in the triple- or double-immunosuppressive regimen. Several groups have evaluated the efficacy of tacrolimus in combination with other clinically used immunosuppressants for the prevention of organ transplant rejections. In one of the studies, a randomized three-arm, parallel group, open label prospective study was performed at 15 North American centers to compare the three immunosuppressive regimens: *(1)* tacrolimus

+ azathioprine, (2) cyclosporine (Neoral) + mycophenolate mofetil, and (3) tacrolimus + mycophenolate mofetil. All patients were first cadaveric kidney transplants receiving the same maintenance corticosteroid regimen. All regimens yielded similar acute rejection rates and graft survival, but the tacrolimus + mycophenolate mofetil regimen was associated with the lowest rate of steroid-resistant rejection requiring antilymphocyte therapy (77). Tacrolimus plus mycophenolate mofetil has also been found to be effective in patients seeking second transplants. Low dose tacrolimus administered intravenously followed by oral tacrolimus, mycophenolate mofetil, and steroids provided effective immunosuppression in recipients (n = 10) of simultaneous pancreas-kidney transplants who had undergone previous transplants.

In this study, the investigators sought to decrease the morbidity associated with antibody therapy without increasing acute rejection rates. Two patients experienced episodes of acute rejection that responded to steroid treatment. After a mean follow-up of 20.4 months, the kidney and pancreas were found to be functioning in all patients. This is a new induction treatment for patients with previous transplants, sparing them of antibody induction therapy (78). Another group studied the efficacy of combined therapy involving tacrolimus plus mycophenolate mofetil in heart transplant recipients. The researchers enrolled 45 patients, 15 into phase I and 30 into phase II of the study. Intravenous tacrolimus was given for 2–3 days before switching to oral drugs. Target blood levels were 10–15 ng/mL. Treatment also included steroids and mycophenolate mofetil. During phase I, a 2 glday dose of mycophenolate mofetil was given, whereas in phase II, doses were adjusted according to mycophenolic acid levels in the blood, with target levels of 2.5–4.5 μg/mL. Average follow-up was for 696 days in phase I and for 436 days in phase II. During phase I, patient survival was 100% and rejection was diagnosed in 67% of the patients. Data analysis suggests that an average mycophenolic acid plasma level of 3 μg/mL or more prevented rejections. Interestingly, during phase II, one patient died but rejection could be seen in only 10% of the patients. Steroids were successfully withdrawn from all patients who completed 6 months of treatment.

Thus, although combination therapy with tacrolimus and mycophenolate mofetil is effective for preventing rejection in heart transplant patients, routine drug level monitoring is critical (79). Similarly, in cadaveric kidney transplant recipients randomized to receive tacrolimus in combination with either azathioprine (n = 59) or mycophenolate mofetil 1 g/day (n = 59), or mycophenolate mofetil 2 g/day group (n = 58), tacrolimus in combination with an initial dose of mycophenolate mofetil 2 g/day was found to be a very effective and a safe regimen. The incidence of biopsy-confirmed acute rejection at 1 year was 32.2, 32.2, and 8.6% in the azathioprine, mycophenolate mofetil 1 g/day, and mycophenolate mofetil 2 g/day groups, respectively ($P < 0.01$). The overall incidence of posttransplant diabetes mellitus was 11.9%, with the lowest rate observed in the mycophenolate mofetil 2 g/day group (4.7%), and was reversible in 40% of the patients (80). In yet another study, the efficacy of double-drug protocol has also been evaluated in the hamster-to-rat xenotransplantation model involving heart and liver. Survival of heart and liver xenografts in the rats was 48 ± 4 and 63 ± 8 days, respectively, and after cessation of all immunosuppression, hearts were rejected after 18 ± 4 days and livers after 33 ± 8 days (81). Thus, the studies demonstrate improved graft survival and reduced rejection rates with the combined use of tacrolimus and mycophenolate mofetil in both allograft and xenograft settings.

2.2.2.5 Structure-Activity Relationship. Several structurally related compounds of FK 506 have been isolated from Streptomyces tsukubaensis: methyl (FR900425), ethyl (FR900520), and proline (FR900525) analogs of FK 506. Because the FK 506 is the most active in this series of molecules, it has been studied most exclusively (82, 83). A novel analog of tacrolimus, L732531, a 32-*O*-1(1-hydroxyethylindol-5-yl) ascomycin derivative has been reported with potent immunosuppressive activity. It exhibited an improved therapeutic index compared to that of FK 506 in rodent models. Moreover, its biochemical properties were also found to be distinct from

those of FK 506. These data suggest L732531 to be a potential candidate for treating organ transplant rejection (84).

2.2.2.6 Side Effects. Adverse effects associated with Prograf include tremor, hypertension, hypophosphatemia, infection, creatinine increase, headache, and diarrhea. Other toxicities include hyperglycemia, nephrotoxicity, chest pain, chest discomfort, palpitations, abnormal electrocardiogram, and abnormal distension (85). Prograf can cause neurotoxicity and nephrotoxicity, particularly when used in high doses. Nephrotoxicity has been reported in approximately 52% of kidney transplantation patients and in 40 and 36% of the liver transplant patients receiving Prograf in the U.S. and European randomized studies, respectively (42). New onset posttransplant diabetes mellitus (PTDM) was seen in 20% of the Prograf-treated kidney transplant patients and median time to the onset of PTDM was 68 days. Insulin dependency was reversible in 15% of these patients at 1 year and in 50% at 2 years posttransplantation (86). Prograf injection is contraindicated in patients with a hypersensitivity to HCO-60 (polyoxyl 60 hydrogenated castor oil).

2.2.3 Sirolimus

2.2.3.1 History. Another interesting immunosuppressant with a unique mechanism of action is sirolimus (rapamycin), which has been under development for more than 20 years before it gained FDA approval on September 15, 1999. It is also a microbial natural product and is produced by the actinomycete Streptomyces hygroscopicus isolated from Easter Island (Rapanui to its natives) soil samples in 1975. It emerged from an antifungal drug discovery program directed by Sehgal (87) at Ayerst Research in Montreal, Canada. The antifungal properties of the drug were not pursued when it became apparent that the drug caused involution of lymphoid tissue. Martel subsequently demonstrated that rapamycin suppresses experimental allergic encephalomyelitis and passive cutaneous anaphylaxis in the rat (88). It was not until the newly discovered structure of tacrolimus was found to be remarkably similar to that of sirolimus that led Cambridge group (89) to uncover the potential of the drug as an immunosuppressant.

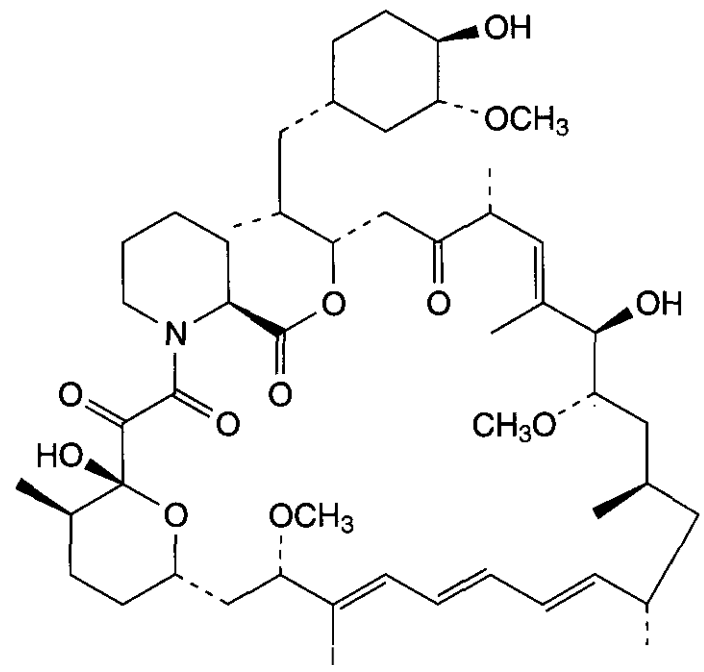


Figure 12.6. Structure of Rapamune (3).

2.2.3.2 Chemical Structure. Structural analysis of rapamycin (3; Fig. 12.6) revealed a macrocyclic triene lactone with strong structural similarity to FK 506.

2.2.3.3 Pharmacokinetics. For Rapamune the adult loading dose is 6 mg (equivalent to 3 × the maintenance dose). A loading dose is recommended because of the drug's long half-life; without a loading dose it may take ≥2 weeks to reach the steady state. Whereas the adult maintenance dose is 2 mg/day, the maintenance dose for children ≥13 years old is 1 mg/m^2/day, with a maximum dose of 2 mg/day. Though the oral absorption of sirolimus is rapid, with time-to-peak concentration of 2 h, the oral bioavailability is approximately 14%. Sirolimus is extensively metabolized in the liver by *O*-demethylation and/or by hydroxylation. Sirolimus is the major component in human whole blood and contributes to more than 90% of the immunosuppressive activity. The terminal elimination half-life ($t_{1/2}$) is 72 h in males and 61 h in females. The sirolimus trough concentrations (whole blood; steady-state; ng/mL) is 8.59 + 4.01 for the 2 mg/day dose and 17.3 ± 7.4 for the 5 mg/day dose (90).

2.2.3.4 Pharmacology. Rapamycin, is a novel immunosuppressive agent developed for the prevention of organ rejection after renal transplantation. It allows doctors to eliminate cyclosporine, a standard component of typical multidrug regimens, at an early treatment stage. Like FK 506, rapamycin binds to and inhibits the isomerase activity of FKBPs and it

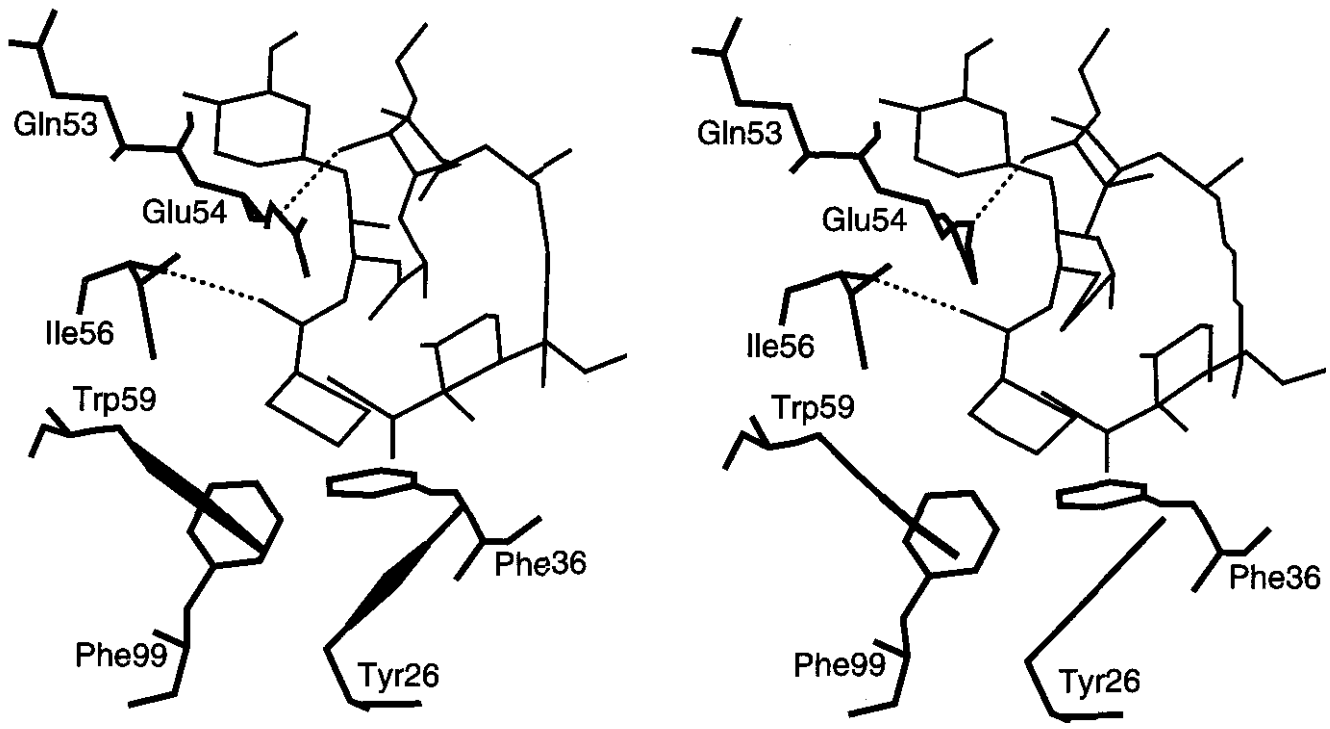


Figure 12.7. A stereodrawing of the binding pocket showing all of the bound rapamycin molecule and selected **FKBP-12** residues.

is this rapamycin-FKBP complex that is responsible for eliciting immunosuppressive activity (91–93). Although it binds to the same intracellular binding protein or immunophilin known as FKBP-12 as FK 506, its unique mechanism of action and superior potency in blocking allograft rejection made it an entirely new class of immunosuppressants. The three-dimensional structure of the complex of human FKBP-12 and raparnycin, determined to 1.7-Å resolution by X-ray crystallographic techniques, provides a framework to interpret the effects of structural perturbation of either rapamycin or human FKBP-12 on signal transduction pathways (94). The protein component in the complex forms a five-stranded antiparallel β-sheet wrapping with a right-handed twist around a short a-helix, the same folding topology found in the complex of FKBP-12 with FK 506 and in uncomplexed FKBP-12. Rapamycin binds in a cavity between the β-sheet and a-helix with the pipecolinyl ring deeply buried in the protein (Fig. 12.7). The protein-ligand interface involves atoms from the pyranose ring through the C28 hydroxyl, with the remainder, including the C17-C22 triene exposed. The C1 ester, the pipecolinyl ring, the C8 and C9 carbonyls, and the pyranose ring adopt a conformation that is superimposable with the same groups in the FKBP-12/FK 506 complex. Three hydrogen bonds between this region and FKBP-12 (Ile-56 NH to C1 carbonyl, Tyr-82 hydroxyl to C8 carbonyl, and Asp-37 carboxylate to C10 hydroxyl) and a C9 carbonyl binding pocket involving C—H—O interactions with ← hydrogens from Tyr-26, Phe-36, and Phe-99 are also identical with those found in the complex with FK 506, thus confirming the identical binding roles of the common structural elements in the two immunosuppressant ligands. Two additional hydrogen bonds are involved in rapamycin binding to FKBP-12. The first is from the Glu-54 main-chain carbonyl to C28 hydroxyl, which along the Ile-56 NH to C1 carbonyl-hydrogen bond may mimic the interaction of the dipeptide portion of a natural substrate with FKBP-12. The sirolimus-FKBP-12 complex, which has no effect on calcineurin activity, binds to and inhibits the activation of the mammalian target of rapamycin (mTOR), a key regulatory kinase in the signal transduction in T-cells.

The effect of rapamycin on immune cells in vitro markedly differs from the actions of other known immunosuppressants. Sirolimus was found to be an extremely potent inhibitor of both murine and human thymocyte proliferation induced by phytohemagglutinin and IL-2 and was at least 10–1000 times more potent than cyclosporine (95, 96). It strongly inhibits murine, porcine, and human T-lympho-

cyte proliferation when induced by antigens and cytokines (IL-2, IL-4, and IL-15), whereas cyclosporine and FK 506 block T-cell activation induced only by stimuli employing Ca-dependent pathways (97–100). Nevertheless, the effect of rapamycin could be seen even when the drug was added up to 12 h after the stimulation of T-cells, in contrast to cyclosporine and FK 506, which lose their effect after 2 h. These findings suggest that unlike cyclosporine and FK 506, which block Ca^2+-dependent cytokine transcription early in the G1 phase, rapamycin inhibits cytokine-mediated signal transduction pathways later in the G1 phase (97, 101).

Sirolimus inhibits the proliferation of B-cells induced by lipopolysaccharide through a Ca-independent pathway that is resistant to cyclosporine and FK 506 (101, 102). Further, its effect on the proliferation of purified peripheral blood mononuclear cells (PBMC) was also investigated ***in vitro*** and it was found to be at least 50–500 times more potent than cyclosporine in inhibiting PBMC proliferation induced by 0.1% PHA. Other biochemical events that are inhibited by rapamycin involve activation of p70S6kinase (103–105) and activation of the cdk2/cyclin E complex phosphorylation of retinoblastoma protein (106). In some animal studies, sirolimus-induced tolerization and the immunosuppressive effect lasted up to 6 months, even after the discontinuation of the therapy. Whether sirolimus can induce significant tolerization in humans is yet to be established.

Based on rapamycin's ability to inhibit both T- and B-cell proliferation ***in vitro,*** it was expected to exhibit potent inhibition in allograft rejection. This was evident in several animal models where rapamycin significantly prolonged survival of heart, kidney, and pancreas allografts in rats (107); nonvascularized fetal cardiac grafts in murine (108); and kidney allografts in pig (109) and primate (110). In addition it was also found to be effective in reversing advanced allograft rejection. Thus, rapamycin is a potent immunosuppressant with remarkable antirejection activity in animal models of organ transplantation. Further, because rapamycin and cyclosporine interfere at different stages and levels of immune response, combined use of these agents has been found to be very promising. In ***in vitro*** assays, the sirolimus-cyclosporine combination produced an inhibitory effect that was between 10- and 1000-fold greater than that produced by either agent alone (111). This was further evident in the phase 1/11 clinical trial studies when addition of sirolimus (0.5–7 mg/m^2/day) was found to reduce overall incidence of rejection episodes from 32% to 7.5%, in patients who were treated with cyclosporine and prednisone alone (112). Similarly, a multicenter trial involving 149 renal transplant patients who had been treated with cyclosporine and prednisone confirmed that the addition of sirolimus (at 1, 3, or 5 mg/m^2/day) reduced the incidence of acute rejection episodes (113); although within 6 months the cyclosporine-prednisone group showed a 40% incidence of rejection episodes, patients treated adjunctively with 1 or 3 mg/m^2/day of sirolimus experienced only 10% incidence of rejection. The studies also showed that 1, 3, or 5 mg/m^2 of sirolimus did not reduce the number of rejection episodes in African-American patients who were treated with reduced cyclosporine doses. This finding was in contrast to what was found when this regimen was used to treat patients of non–African-American origin. However, the most recent clinical trial revealed that the adjustment of the cyclosporine-sirolimus regimen in African-American patients (using higher doses than those given to non–African-Americans) improved the 1-year survival rate of kidney allografts. The survival rate was increased to 97% from only 70% for those recipients who were treated with the cyclosporine-prednisolone regimen (114).

Finally, two phase III trials evaluating the efficacy of sirolimus have provided data indicating that sirolimus is safe and effective. The Rapamune U.S. Multicenter Study (115) included 719 renal transplant recipients who were randomized to receive either sirolimus (either 2 or 5 mg/day) or azathioprine (2–3 mg/kg/day). All patients also received cyclosporine and prednisone. Both sirolimus regimens were found to be significantly more effective than azathioprine at reducing the incidence of acute rejection. Graft and patient survival were similar in all treatment groups. In the Rapamune Global Study (116), conducted in

Table 12.3 Results of phase III Clinical Trial Studies

	Sirolimus		Azathioprine/ Placebo
	2 mg	5 mg	
Rapamune U.S. multicenter studies (719 renal transplant recipients)			
Incidence of biopsy-confirmed acute rejection at 6 months	16.5% (47/284)	11.3% (31/274)	29.2% (47/161)
Graft survival at 12 months	94.7% (269/284)	92.7% (254/274)	94.0% (151/161)
Patient survival at 12 months	97.1% (276/284)	96.0% (263/274)	98.1% (158/161)
Rapamune worldwide studies (576 renal transplant recipients)			
Incidence of biopsy-confirmed acute rejection at 6 months	24.7% (56/227)	19.2% (42/219)	41.5% (54/130)
Graft survival at 12 months	89.9% (204/227)	90.9% (199/219)	87.7% (114/130)
Patient survival at 12 months	96.5% (219/227)	95.0% (208/219)	94.6% (123/130)

Australia, Canada, Europe, and the United States, 576 renal transplant recipients were stratified to receive either sirolimus (either 2 or 5 mg/day) or placebo. All patients also received cyclosporine and prednisone. As in the U.S. study, sirolimus was found to be superior to placebo at reducing the incidence of acute rejection within the first 6 months after transplantation. Graft and patient survival were similar in all treatment groups. The results of both phase III clinical trials are summarized in Table 12.3.

2.2.3.5 Structure-Activity Relationship. Structure-activity relationship studies with sirolimus led to the identification of SDZ-RAD (40-*O*-[2-hydroxyethyl]-rapamycin) as a new, orally active rapamycin derivative with potent immunosuppressive activity (117). Because of its synergistic interaction, SDZ-RAD is under clinical investigation as an immunosuppressant in combination with cyclosporine after organ transplantation (118). Coadministration of microemulsion cyclosporine (neoral) and the novel immunosuppressant SDZ-RAD potentiates the immunosuppressive efficacies of both drugs to suppress allograft rejection (119, 120). SDZ-RAD effectively ameliorates chronic renal allograft rejection in rats, probably mediated through the suppression of growth factors (121). SDZ-RAD exerts its pharmacological effect by binding to a different effector protein, inhibits the S6p 70-kinase, and interrupts a different signal transduction pathway in contrast to tacrolimus. The pharmacokinetic data of the SDZ-RAD dose, normalized to 1 mg SDZ-RAD, were as follows: AUC (0, 24 h): 35.4 ± 13.1 μg/L/h; C_{max}: 7.9 ± 2.7 μg/L/h; and t_{max}: 1.5 ± 0.9 h (122). SDZ has also been evaluated in combination with FTY 720 and was found to be effective in maintaining heterotopic cardiac graft throughout the treatment period (123). Presently it is undergoing phase III clinical trials.

2.2.3.6 Side Effects. The most common side effects associated with Rapamune are hypercholesterolemia and hypertrigliyceridemia (hyperlipemia), thrombocytopenia, anemia, acne, abdominal pain, urinary tract infection, arthralgia, diarrhea, hypokalemia, lymphocele, and increased lactic dehydrogenase. Increased susceptibility to infection and the possible development of lymphoma may also result from immunosuppression (90). Specific adverse reactions associated with Rapamune administration occurring at a significantly higher frequency vs. controls were observed for both 2 and 5 mg/day schedules: hypercholesterolemia, hyperlipemia, hypertension, and rash in patients on the 5 mg/day schedule;

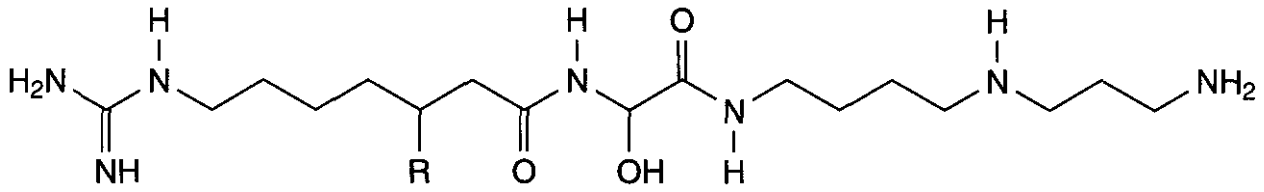


Figure 12.8. Structure of spergualin (**4a**) and deoxyspergualin (**4b**).

anemia, arthralgia, diarrhea, hypokalemia, thrombocytopenia, and acne for patients on the 2 mglday schedule. Elevations of triglycerides and cholesterol, and decreases in platelets and hemoglobin occurred in a dose-related manner (124). No significant interactions were observed with acyclovir, digoxin, glyburide, prednisolone, oral contraceptives, and co-trimoxazole (90).

2.2.4 15-Deoxyspergualin

2.2.4.1 History. In 1981 Umezawa isolated an antitumor and immunosuppressive antibiotic from the culture filtrates of Bacillus *laterosporus* (125, 126) called spergualin. Although it was originally identified because of its antitumor and antibiotic activity, the immunosuppressive activity was found to be more pronounced. Its structural instability, attributed to the presence of an α-hydroxyglycine moiety, led to the synthesis of several hundred analogs in different laboratories, to identify a stable analog with potent immunosuppressive activity (127–129). These studies culminated in the identification of 15-deoxyspergualin (DSG), with strong immunosuppressive activity both in vitro and in *vivo* (130).

2.2.4.2 Chemical Structure. Spergualin, 1-amino-19-guanitido-11,15-dihydroxy-4,9,12-triazathioprinenonadecane-10,13-dione (**4a**; Fig. 12.7) is a water-soluble peptide with a molecular weight of 496 Da. It is synthetically dehydroxylated to produce 15-deoxyspergualin (**4b**; Fig. 12.8).

2.2.4.3 Pharmacokinetics. DSG is a highly polar molecule because of which it has a poor (3–6%) oral bioavailability (131). When administered by intravenous bolus injection, it is rapidly cleared with a biphasic half-life in mice of about 1.9 and 11.6 min (132). The clearance rate was independent of dose and the predominant route of elimination of DSG was renal. The maximum tolerated dose (MTD) in mice was 10.5 mg/kg when administered by intravenous bolus route. In mice, the peak plasma concentration after a single LD_{10} dose was approximately 30 μg/mL.

Because the MTD of DSG is attributable to the peak plasma concentration, the generally accepted mode of administration to humans has been a 3-h intravenous infusion. Human pharmacokinetic studies of DSG given intravenously over 3 h for 5 consecutive days revealed rapid plasma clearance with $t_{1/2\alpha}$ = 37 min and $t_{1/2\beta}$ = 9.2 h. There was no accumulation of DSG in the plasma after each 3-h infusion for 5 days (132). Six metabolites of DSG have been identified using HPLC, but none of these metabolites exhibits the antitumor or immunosuppressive activity of the parent component. The area under the curve of 15-deoxyspergualin matched well with the administered dose (132, 133).

2.2.4.4 Pharmacology. In vitro, DSG moderately inhibits both the mitogen-stimulated proliferation of T-cells and the generation of cytotoxic T-cells (134, 135), without affecting IL-2 production. DSG inhibited growth of mouse EL-4 lymphoma cells with an IC_{50} value of 0.02 μg/mL. Even though the cells were treated with DSG for only 4 h and then washed, the antiproliferative effect was long lasting, with an IC_{50} value of 0.4 μg/mL. Studies have shown that DSG binds to the cells through its spermidine moiety instead of guanidine moiety and thus exerts its long-lasting antiproliferative effect (136). Because DSG blocks T-cell function in vitro when added 3–4 days after the stimulation of T-cells (134), it is believed to act at a late stage of the cell cycle of T-cells. However, this inhibitory effect can be reversed by the exogenous supplementation of IFN-γ, but not by IL-2 (137).

Although the molecular mode of action of DSG is not yet clearly understood, it binds spe-

cifically to two heat-shock proteins (Hsp), Hsp70 and Hsp90, which represent two members of a class of immunophilins. Although the exact binding site on Hsp70 for protein substrates is not yet known, a recent study has shown that the C-terminal four amino acids ^{647}EEVD^{650} might be playing a role in regulating ATPase activity and substrate binding. These four amino acids are also found at the C-terminus of Hsp90 and may be involved in a similar function (138). However, the role of the EEVD motif in biological processes has not yet been investigated.

Other effects of **DSG** include liposomal enzyme release, superoxide production, major histocompatibility antigen (MHC) class II upregulation, and IL-1 production (139). **DSG** also reduced the production of antibodies in immunotoxin-treated mice (140), and in vitro reduced the expression of IgM on the surface of B-cells after the administration of lipopolysaccharide or IFN-y to a mouse cell line of pre-B-cells (141). These studies may account for the immunosuppressive and antitumor properties of **DSG** and the pathway of immune suppression for **DSG** appears to be entirely different from that of other clinically used immunosuppressants.

The immunosuppressive activity of **DSG** has been demonstrated in many animal models of transplant rejection such as heart (142), liver (143), pancreas (144), pancreatic islet (144), and bone marrow (145). In one of the experiments, treatment of baboons with 4 mg/kg/day of 15-deoxyspergualin improved the survival of heart, but not renal, allografts (146). Similarly, treatment with **DSG** slightly delayed the rejection of heart allografts in rat recipients that had been presensitized with skin allografts (147). In preclinical models, DSG was found to be active both in prolonging allograft survival and in the reversal of graft rejection (148,149). This is in contrast to clinically used cyclosporine, which is ineffective in reversing the ongoing rejection. **DSG** also demonstrated a rescue effect when intraperitoneal alginate-poly-L-lysine-alginate microencapsulated xenoislets induced cellular overgrowth that threatened the survival of the graft in streptozotocin-induced diabetic mice (150). Experiments in dogs showed that **DSG** treatment reversed acute rejection episode (151), but caused significant gastrointestinal disturbances. In another study, **DSG** (40–220 mg/m^2) was highly effective in reversing rejection episodes in 27 of 34 (79.4%) recipients of renal transplants when used alone (152), and even more effective when administered in combination with methylprednisolone (87.5%; (153)). Similarly when **DSG** (3–5 mg/kg) was administered in daily 3-h intravenous infusions for 7 days, it reversed 76% of the rejection episodes in 260 patients within the first 6 months after transplantation (154). In addition, **DSG** also reversed 70% of steroid-resistant rejection episodes.

Its efficacy has been further demonstrated in xenograft models as well, where it prolonged the survival of xenogeneic kidney grafts in mongrel dogs receiving kidney of silver foxes (155). In fact, its activity was found to be markedly superior to that of cyclosporine and FK 506 in prolonging xenograft survival (156). Recently, several groups evaluated the efficacy of **DSG** alone or in combination with either T-cell monoclonal antibody or cyclosporine or FK 506 for the prolonged survival of xenografts in animal models (157–161). Coadministration of **DSG** and cyclosporine was effective and nontoxic and prolonged heart grafts in primate models (162). Further, it has also been reported that the use of **DSG** permits lowering of cyclosporine doses and reduction of nephrotoxicity when coadministered together (163).

In human transplantation, Amemiya et al. studied a variety of techniques for reversing rejection, using **DSG** alone, along with the use of **DSG** in rejections unresponsive to steroids (rescue therapy) (152,164,165). High rates of 70–90% of overall reversal were achieved in primary treatment, with an average rate of 81% for all patients studied. The rescue therapy similarly gave high rates of reversal with an average of 66% in these groups. Tanabe et al. showed that **DSG** was effective in allograft prolongation when used with a donor-specific transfusion protocol (166). These results suggest that **DSG** may be valuable as a prophylaxis for clinical human transplantation.

2.2.4.5 ***Structure-Activity Relationship.*** The structural instability of **DSG** resulting from the presence of an α-hydroxyglycine moiety led to the synthesis of several hundred an-

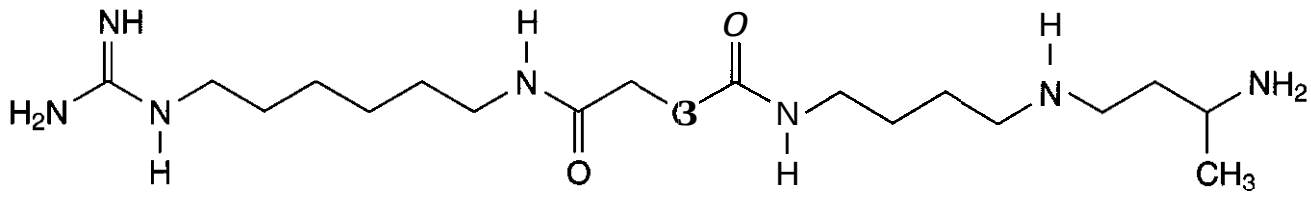


Figure 12.9. Structure of DSG analogue (5).

alogs in different laboratories, to identify a stable analog with potent immunosuppressive activity. Modifications were introduced in all three regions—spermidine moiety, α-hydroxyglycine moiety, and guanidinoheptanoic acid moiety—to clarify the roles of various functional moieties. Earlier structure-activity relationship studies carried out with DSG led to the conclusions that (1) the molecular length of the guanidino fattyacylaminoacyl moiety plays an important role in the expression of biological activity; (2) modification around the spermidine moiety plays an important role in biological activity; and (3) the 5-hydroxyl group is not essential for activity. None of the congeners was found to have a better activity profile than that of DSG; however, further optimization led to the identification of six novel analogs with strong immunosuppressive activity but all of them exhibited activity at a dose higher than 3 mg/kg, which is an effective dose for DSG (167). Later, Lebreton et al. (168, 169) carried out structure-activity relationship studies by synthesizing a series of novel analogs of DSG. They too introduced structural modifications in the hydroxyglycine region and in the spermidine region. The congeners were synthesized and tested in a graft-vs.-host disease (GVHD) model in mice to determine its optimum structure in terms of in *vivo* immunosuppressive activity. The studies led to the identification of a novel analog (5; Fig. 12.9), which demonstrated powerful activity at a dose of 0.3 mg/kg in the GVHD model and was much more potent than DSG in the demanding heart allotransplantation model in rats. The improvement of in *vivo* activity was related to an increase of the metabolic stability of the methylated analogs compared to that of the parent molecules. Because of its very low active dose, compatibility with subcutaneous administration in humans, and its favorable pharmacological and toxicological profile, it has been selected as a candidate for clinical evaluation.

2.2.4.6 Side Effects. Adverse effects associated with the treatment of 15-deoxyspergualin for allograft rejection includes numbness of the face, lips, and limbs (in 14% of patients), gastrointestinal toxicity (in 9%), and bone marrow suppression (in 54%). Because of its low bioavailability, 15-deoxyspergualin needs to be administered intravenously for only a short duration, thereby limiting its widespread application in organ transplantation. In rats treated with DSG at the active period, bone marrow suppression and damage of small intestine were significantly severe. The toxicity of DSG varied with the dosing time, whereas its efficacy did not (164).

2.2.5 FTY 720

2.2.5.1 History. In the 1990s, Yoshotomi Pharmaceuticals Ltd., Japan, in collaboration with Fujita of Kyoto University, began research on immunosuppressive substances from the products of vegetative wasp. From the culture broth of *Isaria sinclairii*, they isolated a potent immunosuppressant, ISP-I. It was found to be at least 10–100 times more potent than cyclosporine as an immunosuppressant of the immune response both in *vitro* and in *vivo* (170). Structurally, ISP-I was identical to myriocin (171, 172) and thermozymocidin (173), previously isolated from M. *albo*-myces (ATCC 16425) and M. *sterillia* (ATCC 20349), respectively, as an antifungal agent. During structure-activity relationship studies of myriocin, it was observed that reduction of the ketone at C14 to methylene led to a congener with a 10-fold increase in the immunosuppressive activity (174), thereby suggesting that there is a strong possibility for the presence of compounds more potent than myriocin in the culture broth of myriocin-producing microorganisms. A search for new compounds having more potent activity than that of ISP-I led to the isolation of mycestericins D, F, E, and G (175–177) as minor analogs. These compounds were evaluated for their immunosup-

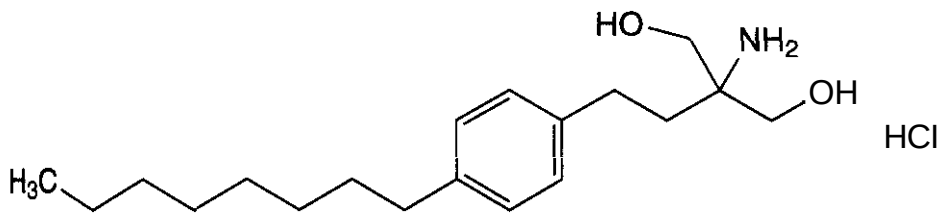


Figure 12.10. Structure of FTY 20 (6).

pressive activity in mouse allogenic MLR assay, where mycestericins D and E were found to be more or less equipotent to myriocin, whereas F and G were less active. A structure-activity relationship study on these natural variants by Fujita et al. led to the identification of 2-amino-2-tetradecylpropane-1,3-diol hydrochloride (ISP-1-55) as a promising lead for the development of potent organ transplant drugs (178). Finally, it was Adachi in 1995 who optimized these leads obtained from the synthetic as well as natural variants of ISP-I that led to the design and synthesis of the potent compound FTY 720 as a clinical candidate (179).

2.2.5.2 Chemical Structure. The compound 2-amino-2[2-(4-octylphenyl)ethyl]-1,3-propane diol (6; Fig. 12.10) was first synthesized in 1995 in an effort to minimize the toxic in *vivo* properties of ISP-I.

2.2.5.3 Pharmacokinetics. No data have yet been reported on the pharmacokinetic parameters of FTY 720.

2.2.5.4 Pharmacology. FTY 720 exerts its immunosuppressive effect by inducing a drastic and selective decrease in blood lymphocytes, especially T-cells both in vitro and in *vivo*. *In vitro*, it causes cell death in lymphocytes and leukemia cells, whereas in vivo (rats and mice), a marked decrease in the number of blood lymphocytes is observed within 1 h after a single oral administration of FTY 720 at 5–10 mg/kg doses (180). It is presumed to be acting by altering lymphocyte trafficking/homing patterns through modulation of cell surface adhesion receptors and ligands in a manner that has yet to be elucidated. In the thymus, long-term daily administration of FTY 720 caused a three- to fourfold increase in the proportion of mature medullary thymocytes [CD4(+)CD8(−) and CD4(−)CD8(+)] as well as a slight decrease in the double-positive cell [CD4(+)CD8(+)] ratio, thereby suggesting that the immunosuppressive action of FTY 720, at least in part, could be attributable to its inhibitory effect on T-cell emigration from the thymus to the periphery (181).

Thus, because of its distinct mechanism of action to reversibly alter lymphocyte homing patterns away from the graft, FTY 720 offers a unique reagent for both induction and maintenance of immunosuppression. FTY 720, when given i.v. or orally at 0.03 mg/kg or more, significantly prolonged skin allograft survival in a dose-dependent manner and showed more potent immunosuppressive activity than that of either cyclosporine or tacrolimus (182). To elucidate the mechanisms of the remarkable synergistic effect, mRNA expressions of Il-2 and γIFN and that of CD3, which reflects T-cell infiltration in allograft, were analyzed. The results suggest that the synergistic effect on the prolongation of allograft survival may be the result of the respective inhibitors of T-cell infiltration and cytokine production in grafts (183). The same group studied the significance of the timing of FTY 720 administration on the immunosuppressive effect to prolong rat skin allograft survival and found that it should be administered before the increase in T-cell infiltration into grafts so as to inhibit acute allograft rejection (184). Its clinical potential as an immunosuppressant has been demonstrated in a variety of allograft and xenograft transplantation models. It prolonged cardiac (185), pancreas (186), islet (187), liver (188), kidney (189), and renal (190, 191) allograft survival in laboratory animals without any drug-induced toxic side effects. Similarly, it was also able to prolong concordant xenograft survival in rats (192).

Although much research has yet to be done to unravel the nature of the mechanism of action of FTY 720. its efficacy has been sufficiently proved in numerous rodents and subhuman primate models, especially when administered in combination with one of the conventional immunosuppressants, such as cyclosporine, tacrolimus, or sacrolimus. FTY 720 potentiates the immunosuppressive ef-

fects of cyclosporine and/or sacrolimus both *in vitro* (by inhibiting of T-cell proliferative response) and *in vivo* (by inhibiting graft rejection).

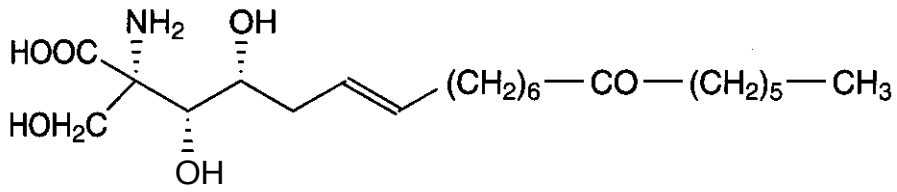
Several groups have studied its efficacy in adjunct therapy with either cyclosporineor sacrolimus both in experimental allograft models involving renal (193), heart (194), liver (195, 196), and kidney (197) transplantation, and in a xenograft model involving islet transplantation in the pig-to-rat model (198).In one of the experiments carried out by Kahan et al. (199) a 14-day course of FTY 720 (0.05–8.0 mg/kg/day) by oral gavage prolonged heart allograft survival in a dose-dependent fashion. Although a 14-day oral course of cyclosporine (1.0 mg/kg/day) alone was ineffective (mean survival time = 7.0 ± 0.7 versus 6.4 ± 0.6 days in treated vs. untreated hosts), treatment with a combination of 1.0 mg/kg/day cyclosporine and 0.1 mg/kg/day FTY 720 extended allograft survival to 62.4 ± 15.6 days. Similarly, a 14-day oral course of 0.08 mg/kg/day sirolimus alone was ineffective (6.8 ± 0.6 days; NS), but the combination of sirolimus with 0.5 mg/kg/day FTY 720 extended the mean survival time to 34.4 ± 8.8 days. The cyclosporine/sirolimus (0.5/0.08 mg/kg/day) combination acted synergistically with FTY 720 (0.1 mg/kg/day) to prolong heart survivals to >60 days (CI = 0.18). In experiments with other combinations, an attempt was made to define an effective range of FTY 720 doses that could be combined with a suboptimal dose (10 mg/kg) of cyclosporine for canine kidney allograft recipients. FTY 720, at a dose ranging from 0.1 to 3.0 mg/kg, significantly prolonged allograft survival in all groups receiving FTY 720 in combination with cyclosporine. None of the recipients died from the notable side effects of the drug, thereby suggesting that FTY 720 has a potent effect at an extremely low dose and a wide therapeutic window when combined with cyclosporine (197).

Recently, Fururkawa et al. (200) compared a single-dose study with FTY 720 at various doses and a combined-dosestudy with the conventional immunosuppressant cyclosporine in a canine liver allograft model. The median survival of untreated control animals was 9 days, whereas treatment with FTY 720 at a dose of 0.1 mg/kg/day prolonged graft survival to 49.5 days. FTY 720 at 1 mg/kg/day showed a slight but insignificant prolongation to 16 days, but when the dose was increased to 5 mg/kg/day, the graft was rejected at 10 days. The combination of FTY 720 (0.1 mg/kg/day) with a subtherapeutic dose of cyclosporine (5 mg/kg/day) prolonged median animal survival from 40 days with cyclosporine alone to 74 days.

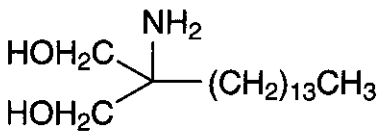
FTY 720 thus exhibits potent immunosuppressive activity and can be used synergistically with either cyclosporine, sacrolimus, or tacrolimus for antirejection therapy without enhancing their side effects. Although there is no information about the toxicity of the drug in humans, FTY 720 is metabolized by lysosomal and membrane enzymes as well as possibly by CYP 4A, but not by CYP 3A4, the enzyme that primarily biotransforms cyclosporine and sirolimus. These findings suggest that FTY 720 offers possibilities for the design of immunosuppressive drug combinations. Not only should the agent allow a further reduction in cyclosporine and/or sirolimus doses, but it should also facilitate the elimination of steroids from the immunosuppressive regimen. It is likely that in the near future FTY 720 will eventually prove to be an efficacious new weapon in the immunosuppressive armamentarium.

Recently, an antibody against FTY 720 has been raised by immunizing rabbits with an ovalbumin conjugate of 2-amino-2-(4-(4-mercaptobutyl)phenyl)ethylpropane-1,3-diol HCl (AMPD-4), which contains the essential structure of the novel immunosuppressant FTY 720. As the antibody reacted not only to AMPD-4 but also to FTY 720, it should be useful for the immunoassay of FTY 720 in body fluids, tissues, and cells (201).

2.2.5.5 Structure-Activity Relationship. To investigate the structure-activity relationship, extensive modifications of ISP-I (7; Fig. 12.8) were conducted, and it was established that the fundamental structure possessing the immunosuppressive activity is a symmetrical 2-alkyl-2-aminopropane-1,3-diol. The tetradecyl, pentadecyl, and hexadecyl derivatives prolonged rat skin allograft survival in combination of LEW donor and F344 recipient and were more effective than cyclosporine. Among them, 2-amino-2-tetradecylpro-



7: SP-I



8: ISP-55

Figure 12.11. Structure of ISP-I (7) and ISP-55 (8).

pane-1,3-diol hydrochloride (ISP-1-55, 8; Fig. 12.11) showed the lowest toxicity (178). Interestingly, each of the 2-amino-1,3-diol hydrochloride compounds is composed of a hydrophilic part (amino alcohol) and a lipophilic part (hydrocarbon chain), thereby suggesting that amphiphilicity should be one of the most important features of this class of compounds. Moreover, given that the lipophilic side chain contains a number of rotatable bonds, Adachi et al. hypothesized that by imposing restriction in the conformational flexibility, the activity could be improved. Accordingly, they introduced a phenyl ring in the lipophilic side chain because it is considered to be an effective template for introducing conformational constraints. Thus, eight compounds possessing a phenyl ring on a variety of positions within the side chain of ISP-1-55 were synthesized (179). The compounds displayed moderate to potent inhibitory activity; however, FTY 720 was the most potent compound and was at least three times more potent than ISP-1-55.

2.2.5.6 ***Side Effects.*** Administration of FTY 720 at an oral dose of 10 mg/kg for 14 days resulted in a prolongation of graft survival in rodents with a median mean time of 27.0 days without renal toxicity or other toxic sign. However, no information about the toxicity of the drug in humans is yet available.

2.2.6 Monoclonal Antibodies

2.2.6.1 History. Monoclonal antibodies offer enormous potential as saturation immunosuppressants because they can be used in prophylactic regimens to prevent early acute rejection. They are directed against various levels and stages of T-cell activation or effector function such as against CD4, adhesion molecules, cytokine receptor, transferrin receptor, portion of T-cell receptor (CD3), and other targets as a means to alter the immune response to alloantigen in a more selective manner. Out of these, the most attractive approach is to target IL-2R with monoclonal antibodies, such as those raised in mice and reacting with the p55 light chain of the receptor, which show promise as immunosuppressive agents in experimental allograft models. Besides IL-2R, another interesting approach is to target CD3 antigen with anti-CD3 monoclonal antibody. Thus the drug works by targeting and blocking a specific region of immune system T-cells, thereby preventing the cells from multiplying in the transplanted organ. In other words, instead of attacking all of the immune cells and making the patient susceptible to infections, monoclonal antibodies can selectively target the cells that have recognized the foreign graft and have started to react to it.

Monoclonal IL-2R antibodies have been successful in pilot studies in preventing solid organ transplantation and in treating GVHD bone marrow transplantation. The monoclonal antibody directed against the p55 chain of the human IL-2R (BT 563) given for 10 days to 19 renal transplant recipients with corticosteroid-resistant graft rejection rescued 13 of 19 patients from rejection, and all had a functioning graft 43 months after treatment (202). Given the human anti-mouse antibody response, however, these antibodies were less effective in human transplantation than those in rodents (203, 204) because humans make antibodies of their own that neutralize these mouse proteins. As a consequence, mouse antibodies typically can be given only once, which is rarely enough to prevent or treat disease. Humanized monoclonals attempt to solve this problem. Using computer modeling

and genetic engineering, the binding site of a mouse antibody, the small part of the antibody that attaches to its target, is combined with 90% of a human antibody to develop humanized antibodies. Although their availability was originally limited to murine antibodies produced by hybridoma technology, advances in the past few years have enabled production of "humanized" antibodies by the DNA method. They are reported to have decreased antigenicity and a prolonged plasma half-life, and can be used repeatedly for a longer period of time.

In contrast to monoclonal IL-2R antibodies, the anti-CD3 monoclonal antibody recognizes one of the seven protein chains that constitute the antigen receptor of T-cells and, by binding to this molecule, inhibits the function of T-cells (205). The target specificity of anti-CD3 provides considerable improvement over the use of polyclonal antibodies. Muromonab CD3 (trade name: orthoclone OKT3) has been introduced as an induction treatment in the attempt to block the initial insult to the target organ by T-lymphocytes, as well as to attenuate the frequency and severity of rejection episodes that threaten allograft survival (206). This product was developed at Ortho Biotech Inc., and was approved by the FDA in 1986. Commercially, 0KT3 is available as an injectable solution (5 mg/5 mL).

Besides OKT3, two anti-IL-2R monoclonal antibodies, daclizumab and basiliximab (daclizumab, 10% murine and 90% human; basiliximab 40% murine and 60% human), have also been approved by the FDA for the prevention of acute renal transplant rejection (207, 208). Both are engineered human IgG monoclonal antibodies directed against the alpha-subunit (CD25) of the interleukin 2 receptor (IL-2R). Because they are specific for the alpha-subunit (Tac/CD25) of the interleukin 2 (IL-2) receptor on activated T-cells, they achieve immunosuppression by competitive antagonism of IL-2-induced T-cell proliferation. Basiliximab (trade name: Simulect) gained FDA approval in May 1998 and is marketed by Novartis Pharmaceutical Corporation (207). Daclizumab (trade name: Zenepax), developed and patented by Protein Design Laboratories, gained FDA approval in December 1997 and is marketed by Hoffman LaRoche (208). Commercially, daclizumab is available in solution form (25 mg/5-mL vials) for injection, whereas basiliximab is available as a lyophilisate powder for injection (20 mg/vial).

2.2.6.2 Pharmacokinetics. The limited data available on the pharmacokinetics of intravenous daclizumab in renal transplant recipients indicate that a dosage of 1 mg/kg every 14 days for a total of five doses can maintain sufficient serum concentrations to provide immunosuppression for 3 months after transplantation. The first dose is given within 24 h before the transplant surgery. Daclizumab has a small volume of distribution (about 5.3 L), and systemic clearance is low (—15.1 mL/h, with interindividual variation of about 20%). The drug has a long terminal half-life (harmonic mean about 480 h), which is similar to that reported for human immunoglobulin G (207). With basiliximab, both single- and multiple-dose pharmacokinetic studies (ranging from 15 to 150 mg) have been carried out in kidney transplant recipients. The recommended regimen consists of two 20-mg doses given i.v. The first dose is given within 2 h before the transplant surgery. The second dose is given 4 days after the transplant. Pediatric (2–15 years of age) protocol is similar to that for adults except that the recommended dose is 12 mg/m^2 BSA, up to a maximum of 20 mg. Following i.v. infusion of 20 mg for 30 min, the peak mean ± SD serum concentration was 7.1 ± 5.1 mg/L. A dose-dependent increase in the C_{max} and AUC value was observed up to the maximum tested single dose studied (i.e., 60 mg). The value of distribution is 8.6 ± 4.1 L, terminal half-life is 7.2 ± 3.2 days, and total body clearance is 41 ± 19 mL/h (208,209).

For OKT3, each dose must be filtered through a low protein-binding 0.22-μ filter before administration, and is given by i.v. push over 20–40 s. The dose schedule for children less than 12 years old is 0.1 mg/kg/day for 10–14 days, whereas for children over 12 years and for adults it is 5 mg/day for 10–14 days. Immunosuppressive therapy should be either discontinued or substantially reduced during the course of 0KT3 therapy. An adequate level of immunosuppression should be restored before the 0KT3 course ends (206).

2.2.6.3 Pharmacology. In a randomized prospective trial conducted in 101 renal trans-

plant recipients, induction with 2.5 mg OKT3 provided excellent rejection prophylaxis with fewer but persistent side effects. The use of mycophenolate diminishes the number of acute rejection episodes and the formation of anti-KT3 xenoantibody (210). Azathioprine, corticosteroids, and delayed addition of cyclosporine have also been used in the immunosuppressive regimen using prophylactic OKT3 to avoid overimmunosuppression and possible cyclosporine-related nephrotoxicity (211–213). As prophylaxis against renal allograft rejection, muromonab CD3 has been used in transplant recipients with delayed (214) and immediate graft function (215). Although OKT3 is used mainly in renal transplant patients, it is also effective in the treatment of heart, liver, and bone marrow transplants.

Daclizumab was studied in adult and pediatric renal allograft recipients, liver allograft recipients, and calcineurin-sparing protocols in renal transplant recipients (207). It directly and specifically interferes with IL-2 signaling at the receptor level by inhibiting the association and subsequent phosphorylation of the ILSR beta- and gamma-chains induced by ligand binding (216). Daclizumab, when added to standard cyclosporine-based immunosuppressive therapy with or without azathioprine, significantly reduced the 6-month rate of acute rejection compared with that of placebo in two multicenter placebo-controlled phase III studies in renal transplant recipients. The mean number of rejection episodes was significantly reduced and the time to first acute rejection significantly increased in daclizumab vs. placebo recipients. Patient survival at 1 year after transplantation was significantly higher with daclizumab than with placebo in one study and showed a trend in favor of the drug in the other study (217). In a phase II study, acute rejection rates in patients treated with both daclizumab and mycophenolate mofetil (plus standard cyclosporine-based immunosuppression) were lower than those achieved with mycophenolate mofetil alone (218). A pooled analysis of two randomized, double-blind studies was performed on the efficacy and safety of daclizumab in renal transplant patients when combined with standard immunosuppression. Patients receiving their first cadaveric renal allograft were randomized to receive five doses of daclizumab (n = 267) or placebo (n = 268), starting preoperatively. Acute rejection at 1 year occurred less frequently with daclizumab (n = 74, 27.7%) than with placebo (n = 116, 43.3%) (P = 0.0001). Mean cumulative doses of corticosteroids were lower with daclizumab (4133 mg) than with placebo (4562 mg). One-year graft survival was 91.4% with daclizumab compared with 86.6% on placebo (P = 0.065), with patient survival of 98.5 and 95.1% for daclizumab and placebo, respectively (P = 0.022). Thus, therapy with daclizumab significantly reduces acute rejection in renal transplantation and improves patient survival without any increase in morbidity (219). Daclizumab was also found to exhibit substantial activity in patients (n = 43) with ongoing acute GVHD. There were no infusion-related reactions and no serious side effects related to daclizumab (220).

Basiliximab was studied in renal allograft recipients and subgroups of recipients of living-related and cadaveric transplants, and in patients with diabetes mellitus. Immunoprophylaxis with basiliximab has been demonstrated to significantly reduce the incidence of acute cellular rejection in adult renal allograft recipients (32% versus placebo, $P < 0.01$) (221). In a study of 732 patients treated with basiliximab, the percentage of biopsy-confirmed acute rejection episodes was decreased from 48% in those patients treated with placebo to 33% in the basiliximab group, which was statistically significant ($P < 0.0001$) (222). Although the risk of acute rejection was reduced, patients who received basiliximab had no adverse reactions and no increase in infectious complications or cancers compared to the placebo group.

Clinically, both daclizumab and basiliximab are used as part of an immunosuppression regimen that may include tacrolimus, cyclosporine, MMF, or corticosteroids. The results of some of the triple-drug regimens used under different organ transplant settings are summarized in Table 12.4. Although both new anti-IL-2R monoclonal antibodies, basiliximab and daclizumab, are additional agents for the prevention of acute renal transplant rejection, their general applicability and long-term therapeutic success remain to be proved.

Table 12.4 Examples of Some Triple Drug Regimens Involving Monoclonal Antibodies

Monoclonal Antibody + Immunosuppressant Drug	Organ Transplantation Model (n = number of patients)	Remarks	Reference
Daclizumab + MMF + steroids	Liver (n = 25)	Daclizumab-based initial immunosuppression can be used safely to reduce the risk for infection with improved long-term graft and patient survival.	223
Tacrolimus + MMF + steroids + induction with monoclonal antibody	Solitary pancreas (n = 23)	Improved graft survival and reduced rejection rates with the use of monoclonal antibody has been observed.	224
Daclizumab + sirolimus + MMF	Kidney transplantation (n = 14)	A calcineurin inhibitor-sparing regimen appears to provide effective nonnephrotoxic immunosuppression for kidney transplantation without the need for a lymphocyte-depleting regimen.	225
Daclizumab + cyclosporine + MMF + prednisone	African–American and high risk Hispanic renal patients (n = 49)	The addition of Daclizumab to an immunosuppressive regimen decreases acute rejection episodes in a high risk group of African–American and Hispanic renal transplant recipients.	226
Daclizumab + MMF + steroids	Renal transplantation (n = 45)	Most of the rejections were moderate and easily reversible. Interestingly the actuarial 1-year graft survival was 95% with 100% patient survival without using cyclosporine.	227
Daclizumab + cyclosporine + MMF + prednisone	Cardiac transplantation (n = 55)	Induction therapy with daclizumab safely reduces the frequency and severity of cardiac-allograft rejection during the induction period.	228
Tacrolimus + sirolimus + daclizumab	Islet transplantation in patients with type I diabetes mellitus (n = 7)	Islet transplantation can result in insulin independence with excellent metabolic control.	229
Basiliximab + early initiation of cyclosporine therapy	Cadaveric or living-related donor renal transplants (n = 138)	Basiliximab combined with early initiation of cyclosporine therapy resulted in low acute rejection rates similar to those achieved with ATG combined with delayed cyclosporine. Basiliximab therapy showed an excellent safety profile, with no increases in malignancies, infections, or deaths.	230

2.2.6.4 Side Effects. Clinical experience from 2 phase III studies conducted to date indicates that daclizumab does not increase the incidence of adverse events when administered with standard cyclosporine-based dual or triple therapy to renal transplant recipients. The incidence of overall adverse events considered to be possibly or probably related to treatment is similar in the daclizumab and placebo treatment groups in both studies (222). For Simulect, severe acute (onset within 24 h) hypersensitivity reactions including anaphylaxis have been observed both on initial exposure and/or after reexposure after several months. In the case of severe hypersensitivity reaction, therapy with Simulect is permanently discontinued (208). The side effects of OKT3 may be life threatening, given that it is associated with markedly increased susceptibility to infection and leads to hypertension, hypotension, chest pain, dizziness, fainting, trembling, headache, and stiff neck (231).

2.3 Agents that Block Nucleotide Synthesis

2.3.1 Azathioprine

2.3.1.1 History. Several antimetabolites synthesized for cancer therapy were incidentally found to have immunosuppressive activity. In 1951 Elion for the first time synthesized 6-mercaptopurine (6-MP) as an inhibitor of nucleic acid base metabolism that significantly increased the life expectancy of leukemic children (232). To increase its efficacy, a large number of derivatives of 6-MP were synthesized and examined for their activities and metabolic fate. One of these was azathioprine, a prodrug of 6-MP, which temporarily protects 6-MP from catabolism and releases 6-MP inside leukemic cells. Though initially used in leukemia, 6-MP was found to suppress antibody formation and allograft rejection. The breakthrough came in 1959 when Schwartz and Dameshek (233) could prevent rabbits from producing antibodies to human serum albumin by treating them for 2 weeks with the antimetabolite 6-MP. Next, Calne et al. observed that bilaterally nephrotectomized dogs living on solitary renal allograft survived for years by treating them with derivatives related to 6-MP (234). Among several drugs provided by Hitchings and Elion, azathioprine was found to have the best therapeutic index. The first renal transplant recipient to receive azathioprine was an adult transplanted with an unrelated kidney in March 1961 (235). Finally, its clinical use was recommended in 1968 and since then it has remained the keystone of immunosuppressive treatment for renal transplantation. It is also used to treat severe cases of rheumatoid arthritis, systemic lupus, polymyositis, Crohn's disease, ulcerative colitis, and other autoimmune disorders.

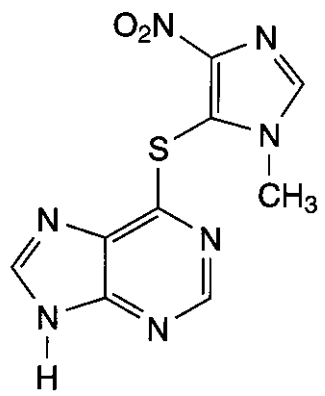


Figure 12.12. Structure of azathioprine (9).

2.3.1.2 Chemical Structure. Chemically, azathioprine is 6-(1-methyl-4-nitro-5-imidazyl) thiopurine (9; Fig. 12.12).

2.3.1.3 Pharmacokinetics. Oral azathioprine is well absorbed, and both azathioprine and its metabolite 6-mercaptopurine (6-MP) distribute throughout the body and are able to cross the placenta. Azathioprine is converted by hepatic xanthine oxidase to 6-MP, which is further metabolized to several compounds including 6-thiourate. These metabolites are excreted in the urine. The plasma half-life of azathioprine is <15 min, whereas the half-life of its active derivative 6-MP is 1–3 h.

2.3.1.4 Pharmacology. Azathioprine exerts its immunosuppressive and toxic properties through the release of 6-MP as the main metabolite *in vivo* (235, 236). The metabolic studies of azathioprine revealed that, after absorption, it is nonenzymatically cleaved by sulfhydryl-containing compounds (e.g., cysteine, red blood cells glutathione, etc.) to 6-MP. The latter is then enzymatically converted to ribonucleotide and thioinosinic acid. Interestingly, it is this thioinosinic acid that eventually interferes with the conversion of inosinic acid to guanylic and adenylic acids and gets itself converted to thioguanylic acid,

which in turn affects the synthesis of DNA and polyadenylate-containing RNA (237, 238). The antiproliferative activity of azathioprine allows the drug to affect the dividing B- and T-lymphocytes during their proliferation cycle. Because T-cell stimulation by antigens causes cell proliferation, the predominant immunosuppressive activity of azathioprine is to block mitosis of activated cells by interfering with the nucleotide synthesis.

Earlier clinical transplant experiments demonstrated that the drug alone was relatively successful in preventing rejection, but soon it became evident that adjunctive maintenance corticosteroids were more effective. Studies suggested that low dose maintenance corticosteroids when used in combination with azathioprine could be as effective as the more generally accepted higher dose (239). Later randomized trials in renal transplant recipients of high dose vs. low dose corticosteroids in combination with azathioprine supported the concept, in that the low dose group exhibited fewer corticosteroid-related problems. Thus, low dose corticosteroids in combination with azathioprine became the common maintenance immunosuppressive regimen for kidney transplant patients (240). In fact, this was the breakthrough that allowed kidney transplantation to become a routine clinical approach.

However, after the introduction of cyclosporine in 1978, triple-drug therapy with cyclosporine, corticosteroids, and azathioprine became the most frequently used regimen for cadaver kidney recipients (241, 242). These three drugs are believed to complement each other in preventing graft rejection. One advantage of triple-drug therapy is that it allows more flexible immunosuppression, with the possibility of adjusting the dosage of individual components to minimize adverse effects. while maintaining adequate overall immunosuppression. The superior immunosuppressive efficacy of the azathioprine-based triple-drug regimen led to clinical trials with other triple-drug regimens consisting of recently introduced immunosuppressants, to identify the optimal combination of immunosuppressants for a variety of solid organ transplantation settings. Recently, several groups from the United States and Europe reported results of their single center/multicenter randomized trials by replacing azathioprine with other potent immunosuppressants: MMF, tacrolimus, and sirolimus in a variety of organ transplantation models. The results of comparative studies involving an azathioprine-based regimen with other regimens are summarized in Table 12.5. Substituting azathioprine with either MMF or sirolimus or tacrolimus resulted in an improved survival of graft and reduced occurrence and severity of acute rejection episodes in many organ transplantation models. Nevertheless, in another interesting experiment, conversion from azathioprine plus cyclosporine to MMF (20 mg/day) with consecutive reduction of cyclosporine in heart transplant recipients with cyclosporine-impaired renal function improved renal function to a significant extent (251). Thus, although recent studies point toward superior immunosuppression by use of a nonazathioprine-based triple-drug regimen, azathioprine still remains the keystone of immunosuppressive treatment.

2.3.1.5 Structure-Activity Relationship. Though it is evident that controlled release of 6-MP plays an important role in azathioprine's activity, studies from different laboratories suggest that the immunosuppressive effects of azathioprine may not be ascribed to the 6-MP alone. Crawford et al. (252) proposed that the secondary immunosuppressive effects of azathioprine might be attributable to the action of the methylnitroimidazolyl substituent. Based on this hypothesis, several analogs of azathioprine were designed and synthesized by replacing the 6-MP component with nontoxic thiols. In all, 24 such congeners were synthesized, out of which two compounds, IXa and IXb (Fig. 12.13), were found to be more effective than azathioprine in prolonging graft survival in mice. Toxicity studies with these two compounds showed that these analogs had no toxic effects at doses equivalent to that of azathioprine, which caused severe bone marrow depression. Biological effects that have been attributed to the methylnitroimidazolyl moiety of azathioprine involve interference with the processes such as antigen recognition, adherence, and cell-mediated cytotoxicity (253). These findings suggest that azathioprine may further inhibit cell proliferation by mechanisms

Table 12.5 Comparison of Azathioprine (ha)-Based Regimen with Other Potent Immunosuppressive Drugs in Different Organ Transplantation Settings

Organ Transplantation Model	Azathioprine-Based Treatment	Other Immunosuppressants	Results	Reference
Renal transplantation	Aza + cyclosporine + steroids (n = 161)	Sirolimus + cyclosporine + steroids (n = 558)	Use of sirolimus reduced occurrence and severity of acute rejection episodes with no increase in complications.	114
Pediatric renal transplant recipients	Cyclosporine + Aza + corticosteroids (n = 6)	Cyclosporine + MMF + corticosteroids (n = 16)	MMF leads to an improvement in the immunosuppression and renal function in children with ongoing rejection.	243
Renal recipients	Aza + cyclosporine + prednisone (n = 50)	MMF + cyclosporine + prednisone (n = 62)	MMF-based triple drug regimen results in fewer rejection episodes.	244
Renal allograft recipients	Aza + cyclosporine + prednisone (n = 26)	MMF + cyclosporine + prednisone (n = 22)	Graft function was excellent and similar in both groups during the first 6-month observation period.	245
Simultaneous kidney–pancreas recipients	Aza (n = 76)	MMF (n = 74)	Trends for most efficacy parameters favored MMF over Aza, and time to renal allograft rejection or treatment failure was statistically significantly longer for MMF. The use of MMF in the treatment of SPK recipients is a useful advance.	246
Liver allograft recipients	Aza + neoral + lymphocyte antibodies + steroid (n = 29)	MMF + neoral + lymphocyte antibodies + steroid (n = 28)	Primary immunosuppression with MMF is advantageous over Aza with regard to safety and efficacy.	247
Liver transplantation	Tacrolimus + Aza + antilymphocyte globulin + prednisolone (n = 56)	Tacrolimus + prednisone (n = 61)	Both tacrolimus-based dual and quadruple immunosuppressive induction regimens yield similar safety and effectiveness after liver transplantation.	248
Kidney transplantation	Aza + cyclosporine + steroid (n = 26)	MMF + cyclosporine + steroid (n = 25)	Graft survival demonstrated 12.5% graft losses in the Aza group vs. no kidney transplant losses in MMF group.	249
Pancreas transplantation	Aza + cyclosporine + steroid + antilymphocyte globulin (n = 13)	MMF + cyclosporine + steroid + antilymphocyte globulin (n = 12)	Patients treated with MMF required less frequent and less intensive treatment for acute rejection. However, its short- and long-term side effects should be further investigated.	250

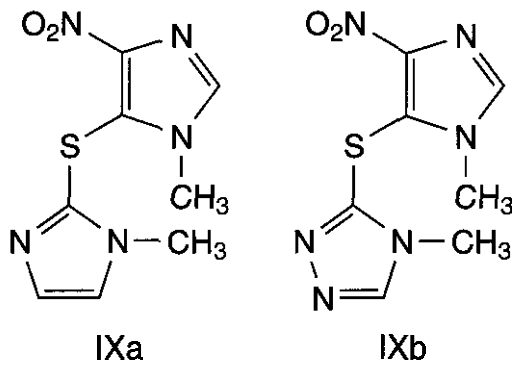


Figure **12.13.** Structure of azathioprine analogues (**9a** & **9b**).

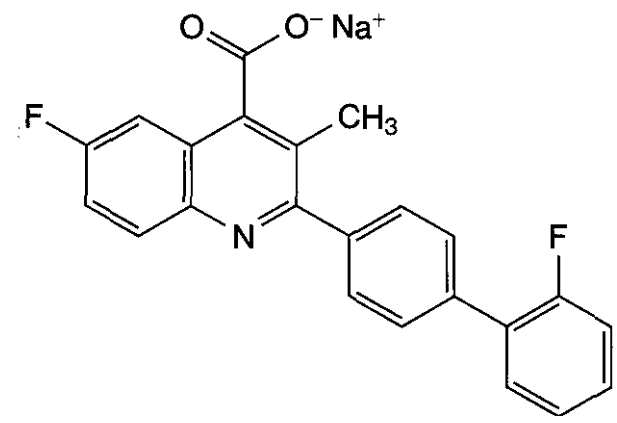


Figure 12.14. Structure of brequinar sodium (**10**).

independent of its effect on purine synthesis (254, 255). The drug inhibits the primary immune response with little effect on secondary responses, and is thus useful in preventing acute rejection but not in reversing the process, once started.

2.3.1.6 Side Effects. Major side effects associated with azathioprine are nausea, vomiting, mouth ulcers, or anorexia (60%). More serious. but less common. is bone marrow suppression, with reversible leucopenia occurring in up to 20% of the patients. This adverse reaction is usually dose dependent and can lead to further complications including infections and bleeding. Other unwanted side effects include headache, muscle aches, rash, pancreatitis, and, rarely, hepatotoxicity. The reported increased risk of lymphoproliferative disease and skin and urogenital cancers has not been confirmed but may be up to 4%. Azathioprine, however, does not appear to be excreted into breast milk but, in principle, its introduction during lactation should be avoided. Hepatotoxicity occurs in 2–10% of transplant patients receiving azathioprine. Metabolism of azathioprine is inhibited by Allopurinol, which potentiates the effect of azathioprine and increases the risk of myelosuppression. Combining it with other myelosuppressives may also increase the risk (256, 257).

2.3.2 Brequinar

2.3.2.1 History. Like other antiproliferative agents, brequinar was originally developed in 1985 as an antitumor agent (258). Later in 1993 it was found to exhibit potent and selective immunosuppressive activity (259).

2.3.2.2 Chemical Structure. The chemical structure of synthetic brequinar 6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt **(10)** is shown in Fig. 12.14.

2.3.2.3 Pharmacokinetics. Brequinar is a water-soluble derivative with an oral bioavailability of 90%, and reaches peak concentrations in the plasma within 2–4 h of oral administration (260). The drug circulates in the peripheral blood tightly bound to serum proteins, with a half-life of approximately 15 h in humans and 17 h in rats. Once the drug is absorbed, it is distributed rapidly to peripheral organs, including liver and kidney (261). The areas under the curve (AUCs) for plasma levels of the drug increase linearly with the dose of the drug, and the plasma clearance is 19.2 ± 7.7 mL/min/mm^2 (260, 262). The extended half-life and the low plasma clearance allow its administration at an interval of 24–48 h. Brequinar is metabolized in the liver by the P450 cytochrome oxidase system, and is excreted primarily in feces (66%) and, to a lesser extent, in urine (23%). The oral administration of brequinar to rats for 30 days did not affect cyclosporine pharmacokinetics (263). The levels of the compound in the blood can be directly measured using high pressure liquid chromatography (264). The high level of bioavailability, the relative ease with which the compound can be administered, and the prolonged half-life are all features of the pharmacokinetics of brequinar that make the compound attractive for use in the clinical setting.

2.3.2.4 Pharmacology. Brequinar inhibits pyrimidine biosynthesis and noncompetitively blocks the activity of the enzyme dihydroorotate dehydrogenase (DHODH) (265, 266).

This enzyme is critical for the formation of uridine and cytidine, which are required for the synthesis of DNA and RNA (267). Studies have shown that brequinar sodium also has the ability to inhibit protein tyrosine phosphorylation and src-related protein tyrosine kinases, thereby suggesting that the activity of brequinar sodium may not be solely the result of the inhibition of pyrimidine nucleotide synthesis; inhibition of protein tyrosine phosphorylation may also be involved (268).

Although brequinar displays antitumor effects over a wide range of concentrations, it displays antilymphocyte effects over only a relatively narrow range (22–185 nmol/L) (269).In vitro, brequinar not only inhibits the proliferation of lymphocytes in a mixed lymphocyte culture but also a wide variety of cellular immune responses including alloantigen and mitogen-induced proliferation (270,271).

In *vivo*, brequinar sodium has been found to be effective in suppressing graft-vs.-host responses and allograft rejections, which can be attributed to the potent inhibitory effect on T- and B-cell-mediated responses. Its efficacy as a primary immunosuppressive agent is evident by the effective inhibition of rejections in several models of vascular allografts in rodents. In rat transplant models, brequinar monotherapy has been shown to prolong the allograft survival of hearts, livers, and kidneys. A dose of 12 mg/kg brequinar administered three times a week for 30 days prolonged the survival of heart allografts to 45.5 ± 12.26 days, compared with a survival rate of 7.0 ± 0.69 days for untreated controls. The same brequinar protocol produced long-term survival (>230 days) in 12 out of 26 rat recipients of orthotopic liver (272). Furthermore, the oral administration of 4 mg/kg brequinar three times per week prolonged the survival of heterotopic cardiac allografts in nonhuman primates to 20.0 ± 21.5 days, compared to a survival rate of 8.0 ± 0.5 days for the controls (272).

Brequinar is also capable of suppressing xenograft rejection because it effectively prolonged survival of hamster-to-rat heart xenograft. In one experiment, brequinar (3 mg/kg/day for 90 days) prolonged the mean survival time of hamster heart xenotransplants in LEW recipients (an inbred strain of rats). The survival time was increased to 24.5 ± 42.2 days (from 4.0 ± 0.48 days in the control animals); furthermore, four of the hearts continued to beat for more than 90 days (272). The hamster-to-rat heart xenograft is an example of an accelerated xenograft reaction and brequinar is the only agent capable of prolonging survival for this type of xenograft.

One of the most striking features of the immunosuppressive activity of brequinar 'sodium is its ability to synergistically interact with a number of other agents to prevent allograft and xenograft rejection. The combination of brequinar with cyclosporine and/or sirolimus was synergistic, as shown by the median effect analysis (273, 274). In primates, the brequinar-cyclosporine combination was able to prolong graft survival to a significant extent (275). Brequinar in combination with leflunomide or tacrolimus exhibited prolonged graft survival in a heterotopic rat cardiac allotransplantation model (276). Administration of BQR (3 mg/kg) with leflunomide (5 mg/kg) or FK 506 (0.5 mg/kg) exhibited prolonged graft survival in both drug combination groups, with a median survival time of 14 days compared to 5 days for controls. Similarly, brequinar in combination with cyclosporine also inhibited islet xenograft rejection in the pig-to-rat model (277). Thus the drug exhibits a number of characteristics that are considered desirable for inclusion in multidrug antirejection protocols.

In a phase I safety and pharmacokinetic study the efficacy of BQR in combination with cyclosporine was examined for the treatment and prophylaxis of rejection in organ transplant patients. The studies were performed in stable renal, hepatic, and cardiac transplant patients receiving cyclosporine and prednisone maintenance therapy for immunosuppression. In all three patient populations, the pharmacokinetics of BQR were characterized by a lower oral clearance (12–19 mL/min) than that seen in patients with cancer (approximately 30 mL/min at similar doses) and a long terminal half-life (13–18 h). This slower oral clearance for brequinar could be attributed either to a drug interaction between brequinar and cyclosporine or to altered clearance or metabolic processes in patients with transplants. Steady-state cyclosporine trough levels

and the oral clearance of cyclosporine were not affected by brequinar coadministration. Among the three transplant populations, the cardiac transplant patients had lower oral clearance values for brequinar and of cyclosporine; however, the cause of this lower clearance is not yet clear. Safety results indicate that brequinar was well tolerated by this patient population (278).

2.3.2.5 Structure-Activity Relationship. Structure-activity relationship studies of substituted cinchonic acid (279) and of some tetracyclic heterocycles (280) related to brequinar were studied to determine the structural features required for good activity and an optimal pharmacokinetic profile. In the first instance, compounds with various substituents at the 2-, 3-, 4-, and 6-position of the quinoline ring system were synthesized. The compounds were evaluated for their DHODase inhibitory activity as well as in the MLR assay, a standard model of cell-based immunity indicative of potential allograft rejection. The cinchonic acid core was found to be essential for activity, with only small lipophilic, electron-deficient substitution allowed on the benzo ring. The methyl group was optimal at position 3, although bridging with the 2-biphenyl retained potency. Among tetracyclic heterocycles series, a correlation between DHODase and MLR was observed. Compounds with an ethylene bridge or compounds with a thiomethylene moiety represent the best of the tetracyclic compounds synthesized. Molecular modeling showed the topology of all the tetracyclic ring systems to be similar. The differences in the activity observed with these topologically similar compounds have been in some cases attributed to changes in lipohilicity or basicity, or to the projection angle of the pendant aryl ring. Several of the compounds exhibited activity in DHODase and MLR assays comparable to that of brequinar, warranting further investigations for the development of potent and clinically useful immunosuppressants.

2.3.2.6 Side Effects. In a phase I safety trial. 45 cancer yatients were administered brequinar by a single daily intravenous infusion for 5 days at a dose range of 36–300 mg/m^2/day; several side effects including transaminase elevations, thrombocytopenia, mucositis, phlebitis, and dermatitis were recorded (262). Subsequently, in a phase II efficacy study of brequinar, doses as high as 1800 mg/m^2 failed to reduce tumor growth in cancer patients (281). Once the adverse side effect appears, the noncompetitive nature of the enzyme inhibition provides the opportunity to reduce or withdraw treatment with the drug with rapid reversal of the drug-related effects.

2.3.3 Mycophenolate Mofetil

2.3.3.1 History. Mycophenolate mofetil (MMF; RS 61443) is a semisynthetic derivative of the antimetabolite mycophenolic acid. Mycophenolic acid (MPA) was initially derived from the cultures of the *Penicillium* species by Gosio in 1896 (282) and purified in 1913. Its antibacterial and antifungal activities were recognized in the 1940s. It was not until the 1980s that Nelson, Eugui, and Allison of Syntex, USA considered MPA for use as an immunosuppressant, as part of their search for selective immunosuppressants with a novel mode of action. They searched for a metabolic pathway more susceptible to inhibition in human lymphocytes than that in other cell types and their choice ultimately fell on two major pathways of purine synthesis. They postulated that depletion of GMP by inhibiting ionosine monophosphate dehydrogenase (IMPD) might result in an antiproliferative effect on lymphocytes to a greater extent than that on other cell types, given that lymphocytes use *de novo* purine synthesis, whereas other cells depend more on purine salvage. Thus, from several possible inhibitors, an antimetabolite mycophenolic acid was selected because it was found to be a potent noncompetitive reversible inhibitor of eukaryotic but not of prokaryotic IMP dehydrogenases (283, 284). In subsequent studies morpholinoethyl ester of MPA, mycophenolate mofetil, was selected from a number of derivatives on the basis of its structure, its ability to inhibit lymphocyte proliferation *in vitro*, its ability to inhibit antibody synthesis in mice, and its greater bioavailability when compared to MPA. Finally in 1987, Morris and colleagues at Stanford University (285) decided to evaluate MMF for use in transplantation.

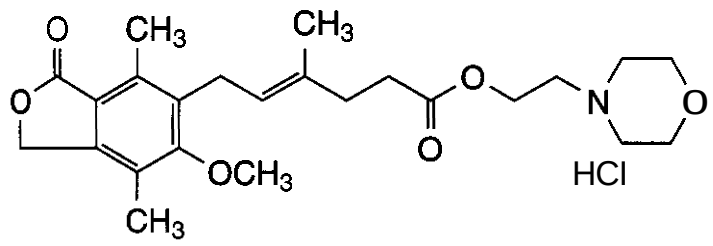


Figure 12.15. Structure of mycophenolate mofetil (**11**).

2.3.3.2 Chemical Structure. Mycophenolate mofetil (trade name: CellCept, 11; Fig. 12.15) is a morpholinoethyl ester of mycophenolic acid.

2.3.3.3 Pharmacokinetics. The bioavailability of MPA is only 43% to that of the ester, which is highly soluble at the lower pH in the upper GI tract and is absorbed more rapidly. The liver is the primary location for esterase-mediated hydrolysis of MMF into MPA. The liver is also the site of conversion of MPA to its primary metabolite, mycophenolate glucuronide (MPAG), a significant amount of which is secreted into the bile only to be recycled to the liver (enterohepatic recirculation), where it may be converted back to MPA. Thus, enterohepatic recirculation is thought to contribute significantly to the MPA serum level. The high concentration of drug in the gut may account for the gastrointestinal side effects. Oral bioavailability (of MPA) varies from approximately 95% in healthy volunteers to approximately 50% in newly transplanted patients. The pharmacokinetic profiles of recipients of cadaveric-kidney transplants who had been treated with escalating doses of MMF (100–1750 mg, every second day) exhibited C_{max} in the blood, 1.0–3.2 μg/mL, and the area under the concentration-time curve over a 24-h period (AUC_{0-24}), 6.4–37.6 μg/h/mL. The terminal half-life of the drug in the blood was 2.3–9.6 h (286).

2.3.3.4 Pharmacology. Mycophenolate mofetil (MMF; RS 61443) is a semisynthetic derivative of the antimetabolite mycophenolic acid, with improved oral bioavailability and is rapidly deesterified in vivo to its active metabolite, mycophenolic acid (MPA) (287). It was first introduced in 1995 for its clinical use in renal transplant patients. Later in 1998 it was approved for other solid organ transplant settings involving heart patients and recently as a non-nephrotoxic immunosuppressant, for pediatric renal transplant recipients with chronic cyclosporine nephrotoxicity (288).

Both MMF and MPA inhibited proliferation of T-cells and B-cells by blocking the production of guanosine nucleotides required for DNA synthesis. Mycophenolic acid noncompetitively and reversibly inhibited the enzyme inosine monophosphate dehydegenase (IMPDH), the rate-limiting enzyme for de novo purine synthesis during cell division. Transfer of fucose and mannose to glycoproteins was also found to be inhibited by mycophenolic acid-mediated depletion of GTP, which in turn may decrease the recruitment of lymphocytes into the sites of vascularized organ graft rejection and inhibit the ongoing rejection (289).

Measurement of intracellular pools of GTP and dGTP in mitogen-activated PBMC and human T-lymphocytes in the presence or absence of mycophenolic acid supports the hypothesis that antiproliferative effects of mycophenolic acid mainly result from the depletion of GTP or dGTP. It was also found to completely and reversibly suppress DNA synthesis in phytohemagglutinin-stimulated peripheral blood cells, thereby suggesting selectivity in the action of mycophenolic acid by not acting on other enzymes or metabolic functions or on thymidine transport (289).

Studies in vivo supported the in vitro findings with regard to the selectivity of MMF for lymphocytes than for other cell types. It inhibited generation of cytotoxic T-cells and rejection of allogenic cells (290) and completely suppressed the formation of antibodies against xenogenic cells in rats (290,291). The studies also provided theoretical justification for use of the drug in organ transplantation and autoimmune diseases.

In European phase III trials, 491 recipients of kidney transplants were randomized to receive a placebo ($n = 166$) or 2 g/day MMF ($n = 165$) or 3 g/day MMF ($n = 160$), in combination with cyclosporine and prednisone therapy (292). The incidence of biopsy-proved rejection episodes was reduced from 46.4% among placebo-treated patients to 17.0% for the 2 g/day MMF group and to 13.8% for the 3 g/day MMF group. However, 6 months after the transplant, the incidence of graft loss was 6.7% for the 2 glday MMF group and 8.8% for

the 3 g/day MMF group, compared to 10.2% for the placebo group. Although MMF reduced the thickening of the coronary vessels in a rat model, to date there is no clinical evidence that MMF affects the progression of graft-vessel disease or chronic rejection (293). These clinical studies show that MMF decreases the incidence and severity of acute rejection episodes, but has little effect on the overall long-term rate of graft survival.

In organ transplantation models MMF, when given in combination with corticosteroids or cyclosporine, reduces the frequency and severity of acute rejection episodes in kidney and heart transplants, improves patient and graft survival in heart allograft recipients, and increases renal allograft survival up to 3 years (294). The ability of MMF to facilitate sparing of other immunosuppressive agents, particularly in cyclosporine-related nephrotoxicity, is also promising. By permitting reduction in cyclosporine doses, MMF may stabilize or improve renal graft functions in patients with cyclosporine-related nephrotoxicity or chronic allograft nephropathy. Because MMF has a different mechanism of action to cyclosporine and corticosteroids, its immunosuppressive effect appears to be at least additive. The drug has therefore been proposed as an alternative to azathioprine for the prevention of graft rejection in kidney transplant patients. In patients shifted from cyclosporine to either azathioprine (2 mg/kg) or MMF (1g twice daily), 1 year after transplantation, significantly fewer rejections occurred in patients converted to MMF than in patients converted to AZA (295). Conversion from azathioprine to MMF in pediatric renal transplant recipients with chronic rejection also led to significant improvement in the immunosuppression and renal function in children (243, 296). When used in combination with steroids and cyclosporine in heart transplant trials, CellCept reduced acute rejection and death better than did azathioprine. Thus, CellCept is considered to be a better replacement for azathioprine. Its use has been also favored in the prevention of renal rejections after primary simultaneous kidney-pancreas transplantation (246,297).

Recently, daclizumab (monoclonal antibody) and MMF have been used as part of an immunosuppressive protocol, with the aim of inducing acceptance of ABO-incompatible mismatched liver allografts in humans (296). It has also been safely and effectively used for the treatment of GVDH in hematopoieticstem cell transplantation; however, the optimal dosage needs further investigation (297). Diabetic rats transplanted with adult porcine islets and immunosuppressed with MMF, cyclosporine, and leflunomide remained normoglycemic for up to 100 days (298).

Interestingly, CellCept has also been effective in reversing acute and resistant rejection episodes in liver and combined pancreas/kidney recipients. Early results of phase I and II clinical trials evaluating MMF therapy in liver, and in combined pancreas/kidney transplant recipients are encouraging (294). The main adverse effects associated with oral or intravenous MMF are gastrointestinal and hematologic in nature. Although the direct costs of using MMF versus azathioprine are higher, the decreased incidence and treatment of acute rejection in patients treated with MMF support its use as a cost-effective option during the first year after transplantation. Thus, MMF has become an important therapeutic tool in the transplant clinician's armamentarium.

2.3.3.5 ***Structure-Activity Relationship.*** Over a considerable period of time several research groups have attempted to obtain better therapeutic agents based on MPA by means of chemical modification (299), microbiological modification (300), or by latentiation (301). Nelson carried out synthesis and immunosuppressive activity of 12 side-chain variants of MPA (302). The compounds were synthesized either from MPA itself or from 5-(chloromethyl)-1,3-dihydroxy-6-methoxy-7-methyl-3-oxoisobenzofuran, a versatile intermediate for the synthesis of diverse side-chain variants. Replacement of the methylated E-bond of the natural product with a triple bond or a sulfur atom, with overall chain lengths equal to or greater than that of MPA led to compounds devoid of significant activity. Replacement of side-chain double bonds with difluoro, dobromo, or unsubstituted cyclopropane rings also removed most of the activity. Replacement of the double bond with an allenic link-

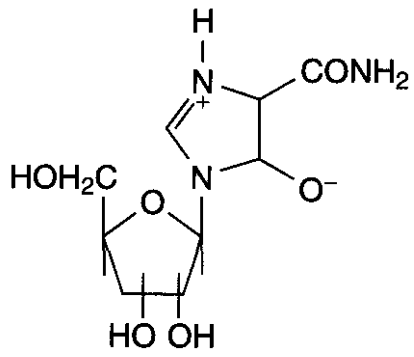


Figure 12.16. Structure of mizoribine **(12).**

age yielded a compound with about one-fifth of the immunosuppressive activity of MPA.

2.3.3.6 Side Effects. The side effects seen with CellCept use include diarrhea, leukopenia (reduction of white blood cells), sepsis, nausea, and vomiting (303). Allergic reactions to MMF have been observed; therefore, it is contraindicated in patients with a hypersensitivity to MMF, MPA, or any component of the drug product. Intravenous MMF is contraindicated in patients who are allergic to Polysorbate 80 (Tween). Antacids and oral Mg supplements markedly reduce MMF absorption. The administration of antibiotics such as metronidazole and fluoroquinolones and the resultant elimination of intestinal flora are associated with a 35–45% reduction in MPA bioavailability (303). In a phase I study of 48 recipients of cadaveric kidneys, MMF at doses of 100–3500 mg/day caused dose-dependent gastrointestinal toxicity (including gastritis and mild ileus) without any evidence of bone marrow suppression. In another trial, 21 recipients of renal transplants from cadaveric donors were administered 0.25–3.5 g/day of MMF in combination with a cyclosporine-prednisone regimen, and experienced limited side effects such as diarrhea, nausea, elevated liver enzymes, and an increased rate of infections with cytomegalovirus (304).

2.3.4 Mizoribine

2.3.4.1 History. Mizoribine is a naturally occurring molecule isolated from the filtrate of culture medium of *Eupenicillium brefeldianum* (M-2166) from the soil in 1974 by Mizuno et al. (305).

2.3.4.2 Chemical Structure. Mizoribine (MIZ) is a novel imidazole nucleoside (12; Fig. 12.16), with a strong immunosuppressive activity.

2.3.4.3 Pharmacokinetics. Mizoribine is mainly excreted by the kidney (306); therefore its plasma concentration depends greatly on renal function. Dosage adjustment is thus required when MIZ is given to patients with poor renal function (307). After absorption MIZ is metabolized in the liver into its active component mizoribine-5-P by adenosine kinase (308).

2.3.4.4 Pharmacology. Mizoribine has been approved in Japan and recommended as combination therapy along with glucocorticosteroids for renal transplant recipients. Like azathioprine, mizoribine is also a prodrug, which undergoes intracellular phosphorylation, affecting the synthesis of nucleic acid by antagonizing IMPDH and guanosine monophosphate (GMP) synthase, thus blocking one of the essential pathways of *de novo* purine synthesis in lymphocytes (309). It exerts its immunosuppressive activity by inhibiting DNA synthesis in the S phase of the cell cycle, thus preventing T-cell proliferation (308). Another possibility for the therapeutic effect of MIZ has been attributed to the regulation of glucocorticoid receptor function by binding to 14–3-3 proteins (310). It has also been found to suppress the expression of cyclin A mRNA in human B-cells by downregulating its stability, and thus downregulating their response (311). Studies to investigate specific binding proteins by use of the MIZ affinity column led to the identification of mammalian HSP 60 as a major target (312).

As an immunosuppressant, the drug is more potent than azathioprine with less bone marrow suppression (313). In experimental allograft models, mizoribine was found to exhibit a potent synergistic effect with cyclosporine. Therefore its use as an alternative to azathioprine has been recommended in the triple-drug combination therapy. The clinical efficacy of MIZ has been established by comparing standard triple therapy: cyclosporine + prednisone + azathioprine with a MIZ-based regimen in kidney transplant recipients (314–316). The MIZ group exhibited better renal function and graft survival than did the azathioprine group. MIZ also exhibited fewer adverse effects than did azathioprine. Therefore,

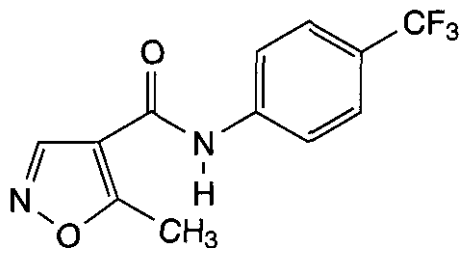


Figure 12.17. Structure of leflunomide **(13).**

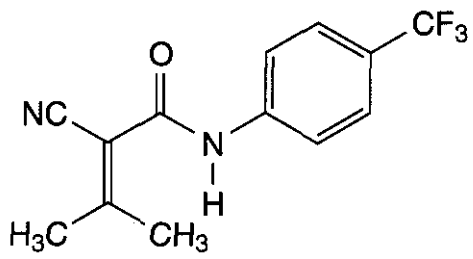


Figure 12.18. Structure of leflunomide analog **A771726 (13a).**

MIZ seems to be a much more useful immunosuppressive agent than azathioprine for renal transplantation.

2.3.4.5 Structure-Activity Relationship. No studies have been carried in this direction.

2.3.4.6 Side Effects. MIZ has been shown in animal experiments to lack oncogenicity, and has been shown clinically to be associated with a low incidence of severe adverse reactions. An increased incidence of hepatic dysfunction and diabetes are some of the side effects observed in humans.

2.3.5 Leflunomide

2.3.5.1 History. Leflunomide (LF), a derivative of isoxazole (HWA 486; Hoechst, Basel, Switzerland), inhibits various T-lymphocyte functions (317,318) and was first described as an inhibitor of the T-cell-dependent antibody production by B-cells (319–321). In recent years several studies have demonstrated that the effect of LF is mediated through inhibition of de *novo* pyrimidine synthesis and tyrosine phosphorylation (322–324).

2.3.5.2 Chemical Structure. Leflunomide (13; Fig. 12.17) is an isoxazole derivative [*N*-(4-trifluoro-methylphenyl)-5-methylisoxazol-4-carboxamide], with potent immunosuppresive, anti-inflammatory, and anticancer activity (SU 101, Sugen Inc., USA).

2.3.5.3 Pharmacokinetics. Although leflunomide has been extensively studied in animal models of transplantation, its clinical use has been initially approved as a disease-modifying antirheumatic drug (Arava) for the treatment of rheumatoid arthritis. This has been attributed to the long half-life (14 days) of leflunomide in humans, which can lead to a difficult situation with regard to its dose adjustment. In rodents, A771726 an active metabolite of LF (**13a**; Fig. 12.18), however, had a half-life of 10–30 h, which is 10 times shorter than its half-life in humans. Because the absorption of leflunomide varies significantly between individuals and produces severe adverse effects, less-toxic analogs of leflunomide are needed.

2.3.5.4 Pharmacology. After its administration, leflunomide is metabolized quickly to form an active metabolite, A771726, which has been identified as N-(4-trifluoromethylphenyl-2-cyano-3-hydroxy crotoamide. *In vitro,* A771726 is a potent inhibitor of protein tyrosine kinases (325) and human DHODH, an enzyme involved in pyrimidine biosynthesis (326). This in turn inhibits the proliferation of several immune and nonimmune cell lines and cell cycle progression (327,328). Another important effect of A771726 is its ability to inhibit humoral-mediated responses by directly blocking T-cell-dependent and T-cell-independent B-cell proliferation and antibody production (298).

In spite of the chemical potential of leflunomide, its mechanism of action is not yet clear. It has been hypothesized that leflunomide inhibits T-cell activation by blocking the lck and *fyn* families of tyrosine kinases. These enzymes are known to be associated with the transduction of such growth factor receptor signals as IL-2, interleukin 3 (IL-3) and tumor necrosis factor alpha (TNF-a) but not interleukin 1 (329). However, the most recent data show that leflunomide inhibits signal transduction after binding of interleukin 4 (IL-4) to the IL-4 receptor (330).

In vivo, leflunomide was found to prevent GVHD and prolonged allograft and xenograft survival in animal models. Moreover, leflunomide also suppressed antibody production in several animal models with allo and xenograft transplantation. In a rat model of GVHD, leflunomide not only was a powerful agent to prevent this otherwise terminal disorder, but was also effective when used as a therapy in an established GVHD. Its efficacy in preventing

GVHD has been attributed to the inhibition of uridine biosynthesis by leflunomide, which leads to a dual antiproliferative effect on both lymphocytes and smooth muscle cells (331, 332). It significantly prolonged survival of allografts: heart (333), pulmonary (334), and islet (335) in rat recipients. In the hamster-to-rat pulmonary model, although leflunomide displayed significant inhibition in xenograft rejection, it was accompanied by severe side effects (334,335). However, subsequent studies with combination therapy using a variety of clinically used immunosuppressants such as leflunomide plus MMF plus cyclosporine (336), leflunomide plus DSG (337), leflunomide plus cyclosporine (338, 339), leflunomide plus tacrolimus (340), and leflunomide plus brequinar (276) have been reported to be very effective in preventing several xenograft and allograft rejections in animal models. In one of the experiments, the combination of cyclosporine (20 mg/kg/day by gavage) and leflunomide (5 mg/kg/day by gavage for 14–21 days) continuously from the day of transplant was able to completely inhibit the rejection of kidney, spleen, and pancreas xenografts in a hamster-to-rat xenotransplantation model. Only a transient treatment with leflunomide was necessary, and long-term graft survival could be maintained by cyclosporine alone. Histological examination of these grafts at >80 days posttransplantation indicated minimal signs of rejection (341). Leflunomide is currently undergoing clinical evaluation in a phase 1/11 clinical trial, to test its efficacy for preventing the rejection of kidney allografts.

2.3.5.5 **Structure-Activity *Relationship.*** Extensive structure-activity relationship studies with A771726 have been carried out to develop suitable analogs that will be clinically acceptable as immunosuppressants. A large number of compounds have been synthesized by different laboratories (342, 343), which suggests that substitution at position 4 of the aromatic ring results in potent congeners with enhanced activity compared to compounds with substitution at positions 2 and 3. Few malononitrilamide analogs (e.g., 279 and 715) have been identified that inhibit T- and B-cell proliferation, suppress immunoglobulin production, and interfere with cell adhesion.

The effects of these agents have been demonstrated in rat skin and cardiac allo- and xenotransplant models. The combined effect of MNA and tacrolimus in a high responder rat cardiac allotransplant model has also been investigated. Optimal doses of MNA or tacrolimus were found to have comparative effects on graft survival and histological changes, whereas a combination of the two drugs,was beneficial with respect to both these parameters. Histological analysis of grafts have confirmed the benefit of the drug combination and no additional toxicity has been observed with combined therapy (344). Interestingly, a novel leflunomide analog, HMR 279, has been found to potentiate the immunosuppressive efficacy of microemulsion cyclosporine (Neoral) in rodent heart transplantation and also in a stringent allogeneic rodent lung transplant model. Combination therapies of Neoral (7.5 mg/kg/day) plus HMR 279 (10 mg/kg/day) or Neoral plus LFM were found to be most successful in preventing histologic allograft rejection compared to that of LFM monotherapy (345). Malononitrilamide analogs of A771726 are also being evaluated for immunosuppressive efficacy in preclinical models of transplantation.

2.3.5.6 **Side Effects.** In a phase II clinical trial, leflunomide showed high tolerability and efficacy in patients with advanced rheumatoid arthritis.

3 RECENT DEVELOPMENTS

3.1 Stem Cells

Stem cells are primitive cells that are capable of forming many different types of body cells. At the time of delivery, the blood in the baby's umbilical cord is quite rich in stem cells; however, as the child ages, these stem cells become less abundant and harder to find. Stem cells, induced to transform themselves into pancreatic cells, could overcome the shortage of donor pancreases and could possibly, when necessary, be genetically engineered to resist being rejected by the immune system. Liver tissue, damaged by infection or toxins such as alcohol, can similarly be replaced by stem cells differentiated into liver cells. Because the cells come from the patient's own body, there is no problem of rejection by the immune system,

and no risk of introducing an infection that might have been present in a donor. However, the use of adult stem cells for therapy could certainly reduce, or even avoid, the practice of using stem cells obtained from human embryos or human fetal tissue. Adult stem cells, although present in only minute quantities, can be found throughout the body and are used to repair and regenerate certain types of tissue (346, 347).

Recently, exciting work has been performed in which cells exposed to appropriate inducing agents have been made to differentiate into a far larger number of cell types than was previously thought possible. Thus, bone marrow cells have been used to make nerve cells and nerve cells have been used to make liver cells.

Scientists from Stem Cells, Inc., demonstrated the production of mature liver cells from rigorously purified hematopoietic (blood) stem cells in mice. The study provides the first evidence that liver function can be restored from bone marrow cells in mice with a virulent form of liver failure and that highly purified blood stem cells can efficiently give rise to normal liver cells. Bone marrow is known to contain many cell types, including both mesenchymal (bone- and tissue-forming) and hematopoietic (blood-forming) stem cells. Different subsets of bone marrow cells were purified and each subset was tested by transplantation into mice. Only the subsets containing blood stem cells were able to produce hepatocytes. Furthermore, normal liver cells could be produced from as few as 50 of these highly purified hematopoietic stem cells. These remarkable results indicate that the hematopoietic stem cells are the only cells in the bone marrow responsible for the restoration of liver functions. This exciting development could greatly increase our ability to use adult stem cells therapeutically for the restoration of organs with end-stage disease instead of going for transplantation (348,349).

3.2 Donor Bone Marrow as Antirejection Therapy

Organ rejection occurs less often and is less severe in patients who receive infusions of bone marrow from the same donor. A study of patients who received the extra boost of donor immune system cells indicates the procedure is safe and augments the cellular environment that is necessary for long-term acceptance of a transplanted organ. In other words, the presence of immune system cells from the donor help condition the recipient's system not to attack the new organ. The effect was found to be most dramatic in recipients of new hearts and lungs. The study included 268 patients who received donor bone marrow along with transplants of livers, pancreases, pancreatic islet cells, kidneys, intestines, hearts, and lungs. Of these, 229 received the bone marrow during their transplant operations. The remaining 39 received one infusion during surgery and two more infusions the first and second days after transplantation. The results were compared to 131 patients who received organ transplants without bone marrow. For heart transplant patients, acute cellular rejection occurred in 38% of those who received bone marrow, compared to 82% of those who did not. In lung transplant patients, the most significant result was a reduced incidence of chronic rejection. Obliterative bronchiolitis, the telltale marker for chronic rejection that prevents air exchange in the lung's bronchioles, occurred in 4.7% of the bone marrow patients and 31% in those who did not receive bone marrow. Similarly, the incidence of acute cellular rejection was much lower in the study kidney/pancreas recipients (350). Biopsies taken from the transplanted organs, lymph nodes, skin, and other tissues revealed that donor leukocytes, or white blood cells, had migrated from the transplanted organs to recipient tissues, where they persisted for years—in one case, for 29 years—after transplantation. Thus, simultaneous bone marrow infusion with solid organ transplantation significantly boosts the number of donor immune cells circulating with recipient cells, creating conditions considered essential for whole organ graft acceptance and the development of tolerance (351).

3.3 Antisense Technology

Antisense technology offers one of the most effective methods of drug design for selectively inhibiting the expression of a specific mRNA (352) involved during the process of organ rejection. It is hypothesized that the metabolic

stages of a specific mRNA can be blocked by allowing an antisense oligonucleotide with a matching sequence to bind to a particular region of the specific mRNA. Despite several limitations, antisense technology offers a clear advantage over all of the previously described drugs. The advantages include: (1) a possibility to target any mRNA; (2) higher affinity and specificity compared with those of classical drugs; and (3) improved drug design, given that both the antisense oligo and the specific mRNA share the same chemistry. Thus, it is possible to target those molecules that are important in the rejection process or those that are used exclusively by T-cells or B-cells, thereby avoiding several nonspecific toxic effects. PS-oligos that are more stable than PD-oligos have been extensively used for antirejection therapy. Many studies have shown that antisense PS-oligos might inhibit protein expression by various mechanisms, that is, degradation of the targeted RNA, promotion of translational arrest, and/or inhibition of RNA processing. In one of the experiments, mouse antisense PS-oligos to ICAM-1 (IP-3082), which targets the 3' end of ICAM-1 mRNA, when delivered intravenously for 7 days, prolonged the survival of heart allografts. Similarly, a combination therapy of anti-ICAM-1 monoclonal antibody and anti-LFA-1 monoclonal antibody delivered intraperitoneally for 7 days induced tolerance to the transplantation of heart allograft (353). In another experiment, survival of pancreatic islets was prolonged to 78.3 + 16.5 days by treating with combined IP-3082 and anti-LFA monoclonal antibody therapy.

Interestingly, the injection of donors intravenously with 10 mg/kg of an antisense oligo to rat ICAM-1 (IP-9125, which targets the 3′-untreated region of rat ICAM-1 mRNA) prolonged the survival of kidney allografts compared with that of controls (354). In yet another experiment, *ex vivo* perfusion of kidney allografts with 5, 10, or 20 mg of IP-0125 also prolonged the survival of kidney allografts. Thus, the PS-oligo IP-9125 blocks the rejection of organ allografts when used (1) for the postoperative treatment of a recipient, (2) for the pretransplant treatment of a donor, or (3) for the perfusion of grafts. Although these studies successfully demonstrate the efficacy of antisense technology, further studies are needed to increase the resistance of oligos to degradation by nucleases and to improve the delivery of the oligos to the cells.

3.4 Molecules Capable of Inhibiting Costimulatory Signals

Induction of antigen-specific unresponsiveness to grafts is the ultimate goal for organ transplantation. In recent years blockade of the costimulatory signals has been reported to significantly prolong allograft survival. In the costimulation paradigm, the accessory signals generated by antigen-presenting cells are interrupted by distinct agents: the receptor conjugate CTLA4-immunoglobulin and anti-B7 or anti-CD40 ligand mAbs.

CTLA4-Ig is a genetically engineered fusion protein of human CTLA4 and the IgG 1 Fc region. It prevents T-cell activation by binding to human B7, which costimulates T-cells through CD28. Administration of CTLA4-Ig completely inhibited CD11a(−/−) T-cell proliferation in response to alloantigens and significantly improved skin allograft survival in CD11a(−/−) mice. Prolonged treatment of wild-type recipient mice with CTLA4-Ig and anti-LFA-1 increased median survival time to 45.5 days compared with 16 days after induction therapy, but it was not sufficient to induce indefinite allograft survival in this model (355). Repligen Corporation has entered into a clinical trial agreement with the National Cancer Institute (NCI) for a phase II trial of CTLA4-Ig.

Monoclonal antibodies to B7–1 and B7–2 have been used *in vivo* to examine the mechanisms underlying these processes and to evaluate costimulation antagonism as an approach to treatment of chronic autoimmune diseases. Administering monoclonal antibodies specific for these B7 ligands was able to delay the onset of acute renal allograft rejection in rhesus monkeys. The most durable effect resulted from simultaneous administration of both anti-B7 antibodies. The mechanism of action does not involve global depletion of T- or B-cells. Despite *in vitro* and *in vivo* evidence demonstrating the effectiveness of the anti-B7 antibodies in suppressing T-cell responsiveness to alloantigen, their use does not result in durable tolerance. Prolonged therapy with murine

anti-B7 antibodies is limited by the development of neutralizing antibodies, but that problem was avoided when humanized anti-B7 reagents were used. Thus, anti-B7 antibody therapy may have use as an adjunctive agent for clinical allotransplantation (356).

Blockade of CD154-CD40 pathway with anti-CD154 antibodies has also been reported to prolong allograft survival in experimental transplantation models and to induce tolerance in some instances. However, anti-CD154 monotherapy is unable to induce tolerance in "stringent" models such as skin and islet transplantation and is not sufficient to prevent chronic graft vasculopathy in vascularized organ transplantation. Therefore, combined therapies of anti-CD154 antibodies plus donor-specific transfusion, bone marrow infusion, or B7 blockade by CTLA4-Ig have been tried, and synergistic effects for tolerance induction have been reported. Furthermore, the efficacy of CD154 blockade in primate models has been confirmed for islet and kidney transplantation. The mechanisms of CD154 blockade in *vivo* include CTLA4-dependent anergy or regulation, T-cell apoptosis, and induction of regulatory cells (357).

4 THINGS TO COME

In the near future, the continued development of organ transplantation will be shaped by at least four issues that have emerged with the recent maturation of this medical specialty.

1. The need to develop novel immunosuppressants that will offer more effective remedy for antirejection, especially xenograft rejection, without exacerbating the problems of toxic side effects or susceptibility to infection.
2. An emphasis on broadening the application of transplantation as a treatment modality to include patients, such as those presensitized to potential donors, that are not considered good candidates for transplantation.
3. The necessity to work out strategies to use organs from other species (xenograft) to provide a solution to an acute and growing shortage of human donor organs.
4. Development of artificial organs.

5 WEB SITE ADDRESSES FOR FURTHER INFORMATION

1. http://www.novartis-transplant.com/
2. http://www.upmc.edu
3. http://www.prohostonline.com
4. http://www.docguide.com
5. http://www.healthlink.mcw.edu
6. http://www.isotechnika.com
7. http://www.fujisawa.com
8. http://home.eznet.net
9. http://www.fp.com.pl/jkc/spanidin.html

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CHAPTER THIRTEEN

Synthetic Antibacterial Agents

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1 INTRODUCTION

Synthetic antibacterial compounds are divided into two major classes: topical agents and systemic agents. The topical agents are termed disinfectants, antiseptics, and preservatives, depending on how they are used. Antiseptics and disinfectants differ from systemic agents in that they show little selective toxicity between the microbes and the host. Furthermore, most of them do not aid wound healing and may even impair it. Nevertheless, there are indispensable uses for disinfectants in hospital sanitation, including sterilization of surgical instruments, public health methods, and in the home. Antiseptics have important applications in the preoperative preparation of both surgeons and patients. They also are used in treating local infections caused by microorganisms refractive to systemic antimicrobial agents.

The development of systemic antibacterials had a strong dye-drug connection. Ehrlich in his classic studies on the selective uptake of chemicals by cells and tissues used dyes because they could be followed visually. His development of selective staining methods for the identification of microorganisms led him to propose that dyes may also have selective toxicities for microbes. In fact many dyes were found to have antimicrobial activity and some are still used as germicides and disinfectants. This was the backdrop in which Ehrlich coined the term chemotherapy for selective killing of pathogenic microbes by chemicals. Discovery of the antibacterial activity of prontosil rubrum, a sulfonamide-azo dye, in a streptococcal infection in mice, led to the first effective chemotherapeutic agent employed for the treatment of systemic bacterial infections. The identification of sulfanilamide as the active component of prontosil led to the synthesis of a number of analogs called sulfonamides with improved activity.

The discovery of antibiotics and the development of bacterial resistance to sulfonamides reduced the use of sulfonamides. Nevertheless, they remain important for specific bacterial infections. The structurally related sulfones are the mainstay for treating leprosy. Other synthetic antibacterial agents have gained increasing attention in recent years.

This is especially true for the fluoroquinolones, which are highly effective systemically against Gram-positive and Gram-negative bacteria resistant to other agents. Very recently, the oxazolidinones have become another very promising class of synthetic antibacterial agents.

There are also some synthetic agents useful specifically for urinary tract infections. They include the nitrofurans and methenamine.

Systemic antibacterial agents are described in two different places in this volume. The antimycobacterial agents are discussed elsewhere, whereas the sulfonamides, sulfones, quinolones, oxazolidinones, and other compounds are discussed in the present chapter.

2 TOPICAL SYNTHETIC ANTIBACTERIALS

Table **13.1** summarizes the topical synthetic antibacterial agents in common use today. These agents possess a wide variety of chemical structures and properties, and they act by many different mechanisms to produce their antibacterial effects. Some of them are extremely toxic, which restricts their use to sterilizing surgical instruments and fumigating structures. Others are powerful agents that are irritating to skin. They are used to disinfect dairy barns, hospital areas, and the like. Less irritating agents are used as surgical scrubs. Still milder agents that possess potent antibacterial activity are used as antiseptics for treating wounds, or as mouthwashes. Very mild and nonirritating compounds may be used to kill bacteria on contact lenses. Thus there is a broad spectrum of needs for topical antibacterial agents, and a large number of chemical agents have been developed to meet these needs.

2.1 Terminology

The terms describing topical antibacterial agents are used rather loosely in everyday language, which sometimes creates confusion. Some of these terms have strict definitions by the U.S. Food and Drug Administration. Perhaps the two most important terms are disinfectant and antiseptic. A disinfectant is defined as a substance that destroys harmful microorganisms, although it may not kill bacterial spores. It is used when referring to agents applied to inanimate objects. Physical agents such as X-rays and ultraviolet light also are considered to disinfect. The term antiseptic is used for agents that kill or prevent the growth of microorganisms when used on living tissues. Antiseptics are used in soaps, mouthwashes, douches, and preparations for minor wounds and burns.

The ending *-cide* is used to denote killing action. Thus, a bactericide kills bacteria, a fungicide kills fungi, a virucide destroys viruses, a germicide kills various kinds of microorganisms, and a biocide kills all living organisms. Similarly, the ending -stat is used to describe an agent that prevents the growth of organisms, but does not necessarily kill them. The corresponding terms are bacteriostat, fungistat, and so forth.

Other commonly used terms include antimicrobial, which refers to an agent that kills or suppresses the growth of microorganisms, and sanitizer, which refers to an agent that reduces the number of harmful bacteria to an established safe limit. The criterion for sanitization is killing of 99.999% of specific test bacteria in **30** s, and it is commonly applied to eating and drinking implements and dairy equipment (1).

Terms used to describe yrocesses include. asepsis, which means the prevention of contamination by microorganisms; decontamination, which refers to the disinfection or sterilization of infected things; sterilization, which refers to killing all forms of life, especially microorganisms; and fumigation, which is the exposure of an area or object to disinfecting fumes.

2.2 Principles of Topical Antimicrobial Activity

2.2.1 Selective Toxicity. The ideal disinfectant would exert a rapidly lethal action against every pathogenic microorganism or spore and it would be inexpensive, stable, odorless, and nonstaining. Requirements for an ideal antiseptic are rapid and sustained lethality to microorganisms, activity in the presence of skin and bodily fluids, lack of irritation and allergenicity, lack of systemic toxicity when applied to skin and mucous membranes,

Table 13.1 Topical Synthetic Antibacterials

Generic Name	Formula	Other Names	Use
	Chlorine and chlorophores		
Chlorine	Cl_2		Drinking water; disinfection
Sodium chlorite	$NaClO_2$		Germicide
Sodium hypochlorite	NaOCl	Dakin's solution	Wound disinfectant
Calcium hypochlorite dihydrate	$Ca(OCl)_2 \cdot 2H_2O$		Germicide
Lithium hypochlorite	LiOCl		Germicide
Chlorinated trisodium phosphate	$NaOCl \cdot 4Na_3PO_4$		Germicide
Chloramine T	**1**	Chlorazone, etc.	Germicide
Halazone	**2**	Pantocid	Drinking water; disinfection
Dichloromethylhydantoin	**3**	Halane, Dactin	Sanitizer
Trichloroisocyanuric acid	**4**	Symcolsine	Disinfect swimming pools
Dichlorocyanuric acid	**5**		Wound disinfectant
Chlorazodin	**6**	Azochloramide	Wound disinfectant
	Iodine		
Iodine	I_2 + KI	Lugol's solution	Wound disinfectant
Iodine	I_2 + ethanol	Iodine tincture	Wound disinfectant
Povidone–iodine	I_2 + **7**		Surgical antiseptic
	Alcohols		
Ethanol	C_2H_5OH		Antiseptic
Isopropanol	$(CH_3)_2CHOH$		Antiseptic
Benzyl alcohol	$C_6H_5CH_2OH$		Pharmaceutical preservative
Phenethyl alcohol	**8**		Pharmaceutical preservative
Chlorobutanol	**9**	Chloretone, etc.	Bacteriostatic for ophthalmology
Octoxynol	**10**	Triton X, etc.	Lens disinfectant
	Phenols		
Phenol	C_6H_5OH	Carbolic acid	Disinfectant
Cresols	$CH_3C_6H_5OH$		Disinfectant
2-n-Amyl-5-methylphenol	**11**		Oral antiseptic
Eugenol	**12**		Mouthwash
2-Benzyl-4-chlorophenol	**13**	Chlorophene	Disinfectant
2-Phenylphenol	**14**	Dowicide 1	Disinfectant
Triclosan	**15**	Irgasan	Disinfectant for cosmetics
Resorcinol	**16**		Keratolytic

Hexylresorcinol	**17**	Crystoids, etc.	Mouthwash
Parabens	$HOC_6H_4CO_2alkyl$		Pharmaceutical preservative
	Bisphenols		
Hexachlorophene	**18**	PHisohex, etc	Topical antiseptic
Anthralin	**19**	Anthraderm, etc.	Keratolytic
	Epoxides and Aldehydes		
Ethylene oxide	**20**		Sterilization
Formaldehyde solution	HCHO + water	Formalin	Sterilization
Glutaraldehyde	**22**		Sterilization
	Acids		
Acetic acid	CH_3CO_2H		Irrigation
Benzoic acid	$C_6H_5CO_2H$		Pharmaceutical preservative
	Oxidizing agents		
Hydrogen peroxide	H_2O_2		Sterilization
Urea hydrogen peroxide	$H_2NCONH_2.H_2O_2$	Hydrogen peroxide carbamide	Sterilization
Benzoyl peroxide	**23**		Acne
Peracetic acid	CH_3CO_3H		Bactericide
Potassium permanganate	$KMnO_4$		Skin lesions
	Heavy metals		
Ammoniated mercuric chloride	$Hg(NH_2)Cl$	Ammoniated mercury	Skin infections
Mercuric oxide	HgO		Eye infections
Nitromersol	**24**	Metaphen	Bacterial antiseptic
Thiomersol	**25**		Bacterial antiseptic
Phenylmercuric acetate	**26**	PMA, etc.	Bacterial antiseptic
Phenylmercuric nitrate	**27**+**28**	Phermernite	Bacterial antiseptic
Merbromin	**29**	Mercurichrome	Bacterial antiseptic
	Silver salts		
Silver nitrate	$AgNO_3$		Burns, ophthalmic
Toughened silver nitrate	$AgNO_3$	Lunar caustic	Wounds

Table 13.1 ***(Continued)***

Generic Name	Formula	Other Names	Use
Colloidal silver	Ag + protein	Mild silver protein	Wounds
Silver sulfadiazine	**30**		Burns
	Dyes		
Gentian violet	**31**	Methylrosaniline chloride, crystal violet, etc.	Topical antiseptic
Methylene blue	**32**	Urolene blue	Cystitis and urethritis, antiseptic
	Diarylureas, amidines, and biguanides		
Triclocarban	**33**	Solubacter	Soaps and cosmetics
Propamidine	**34**		Soaps and cosmetics
Dihromopropamidine	**35**		Soaps and cosmetics
Chlorhexidine digluconate	**36**	Nolvasan, Sterilon	Wound cleansing, burns, surgical scrub
Polyhexamethylene higuanide	**37**	Polyaminopropyl biguanide	Contact lenses
Polyquaternium I	**38**	Polyquad	Contact lenses
	Cationic surfactants		
Benzalkonium chloride	$[C_6H_5CH_2N(CH_3)_2\text{-alkyl}]^+/Cl^-$	Zephiran chloride, etc.	Skin disinfectant
Benzethonium chloride	**39**	Quatrachlor, etc.	Skin disinfectant
Methylbenzethonium chloride	**40**	Diaperine chloride, Hyamine 10X	Diaper rash preventative
Cetylpyridiniun chloride	**41**	Cepachol, etc.	Mouthwash and lozenges
Alkylbenzyldimethyl-ammonium chloride + alkyldimethyl(ethyl-benzyl)ammonium chloride		BTC 2125M, Dual Quat	Biocide
Dimethyldioctyl-ammonium bromide		Deciquam	Food preservative
Polyinones	**42**	Onamer M	Contact lenses
	Anionic surfactants		
Sodium dodecylbenzene-sulfonate	**43**	Conoco C-50, etc.	Dairy disinfectant
Oxychlorosene	**43** + HClO		Skin disinfectant
	Amphoteric surfactants		
Dodecyl + tetradecyl di(aminoethyl)-glycines + tetradecyl-aminoglycines		Tego 51	Surgical disinfectant

and no detrimental effect on wound healing. This ideal has not been realized completely (2). The need for selective toxicity increases from disinfectants, which are used on inanimate objects, through antiseptics, which are applied to skin and mucous membranes, to systemic antimicrobial agents. For antiseptics, a major concern is the *therapeutic* index, which is the ratio of the concentration that produces harmful effects to the concentration that is effective against microorganisms. The harmful effects include local tissue irritation and interference with the wound healing process. Hypersensitivity reactions and systemic toxicity resulting from absorption of the drug can be serious problems, as was the case when infants were commonly washed with hexachlorophene. A high degree of selective toxicity may be associated with a narrow antimicrobial spectrum and the emergence of resistance.

2.2.2 Cellular Targets. Chemical antimicrobial agents have a variety of cellular targets and, in some cases, multiple targets. Although the precise mechanisms of many agents remain unclear, there are a number of known interactions (3). For aldehydes, the target is the cell wall and the chemical mechanism is interaction with amino groups. The cell wall also is the target of anionic surfactants and the mechanism of action is lysis. Certain chelating agents such as EDTA form chelates with cations in the outer membrane of the cell wall and this process induces the release of lipopolysaccharides.

Phenols, quaternary ammonium compounds, biguanides, parabens, and hexachlorophene cause the leakage of low molecular weight compounds from cells and interfere with the normal proton flux. Phenol produces leakage, possible cell lysis, and proton flux changes; quaternary ammonium compounds cause leakage and protoplast lysis, and they interact with membrane phospholipids; chlorhexidine induces leakage and protoplast and spheroplast lysis; parabens cause leakage, transport inhibition, and selective inhibition of proton flux; and hexachlorophene causes leakage, protoplast lysis, and inhibition of respiration.

A variety of agents interfere with nucleic acid function. They include alkylation of DNA and RNA (and proteins) by ethylene oxide and formaldehyde, DNA intercalation by acridines and certain dyes, and inhibition of bacterial DNA gyrase (topoisomerase I) by quinolones.

Metal ions bind with sulfhydryl groups on enzymes or other proteins that may be associated with membranes.

2.2.3 Mechanisms of Bacterial Resistance. The outer membrane of Gram-negative bacteria is composed of lipopolysaccharide, proteins, and lipids (4). It presents a barrier to many chemical agents, including quaternary ammonium compounds and triphenylmethane dyes. In contrast, the cytoplasmic membrane of Gram-positive bacteria, excepting spores and mycobacteria, has greater permeability to most agents (5). Spores have a special coat and/or cortex that may not be permeable to hydrogen peroxide and chlorine disinfectants. Bacterial cells may also have efflux mechanisms that extrude agents such as quaternary ammonium compounds, dyes, and mercury compounds from their interiors *(6)*.

2.2.4 Kinetics and Other Factors. Antibacterial action follows approximately first-order kinetics. The rate depends on concentration, pH, and the vehicle in which the agent is applied. The kinetics are most important when time is critical. An example of a rapid kinetics is the action of 70% ethanol on skin, which results in a 50% reduction in bacterial count in about 36 s. This ethanol concentration does not afford complete bactericidal action; only about 90% reduction in the bacterial count is obtained. Determination of kinetics is complicated by many factors, including diffusion, penetration, binding, and redistribution (7). Consequently, the rate of action often is not directly related to concentration and there is an optimal concentration. Thus, increasing the concentration of ethanol above 70% does not result in increased antibacterial activity. There is a general correlation of antibacterial activity and thermodynamic activity. The latter is related to the proportional saturation of the drug in the medium. This correlation breaks down when the capacity of the medium for the drug becomes a limiting factor because the amount of agent in the medium becomes

depleted rapidly (8). One way to increase the capacity of water for poorly soluble agents such as iodine or hexachlorophene is to add a surfactant.

2.3 Evaluation of Antimicrobial Activity

2.3.1 Methods of Testing Disinfectants. Disinfectants are required to have rapid and lethal antimicrobial effects, which means that bactericidal rather than bacteriostatic test methods are used. The test methods must be precise and reproducible, use standardized microbial strains, and have clearly defined inoculum and culture conditions. Standard bacterial strains are obtained from the American Type Culture Collection (ATCC, Manassas, VA). The activity of disinfectants is affected by pH, chelators and other metal ions, macromolecules and other organic matter, and detergent residues. Disinfectant activity depends on concentration and contact time according to the relationship $C^n t = k$, where C is the disinfectant concentration, t is the time required for lethal action, n is the concentration exponent, and k is a constant. Disinfectants with high exponents have a poor safety margin and require significantly increased contact time when they are diluted (9).

In the United States, the principal methods for testing disinfectants are published in the 16th edition of the *Official* Methods of Analysis of the Association of *Official* Analytical Chemists International (AOAC) (10). Detailed outlines may also be found in the 4th edition of Disinfection, Sterilization, and Preservation (11). Other countries have their own compendia of official methods. Three representative AOAC test methods are described briefly below. The literature indicated should be consulted for more details and further examples.

The phenol coefficient method compares bacterial activity of a disinfectant with that of phenol by a suspension test. A phenol coefficient number is the ratio of the greatest dilution killing test organisms in 10 rnin but not in 5 rnin to the greatest dilution of phenol giving the same result. The assay is run in broth culture at 20°C with contact times of 5, 10, and 15 min. It is used with specific strains of Salmonella typhi, Staphylococcus aureus, and Pseudomonas aeruginosa.

Available chlorine in disinfectants is measured by a capacity test in which disinfectant activity of a compound is compared with that of a standard sodium hypochlorite solution. The test organisms are S. typhi and S. aureus. Ten additions of inoculum are made at 1.5-rnin intervals and a subculture is made 1 rnin after each addition.

A carrier test is used for germicidal spray products. Broth cultures of test bacteria are spread on slides and dried 30–40 rnin at 37°C. The disinfectant is then sprayed onto the slides under standard conditions. After 10 rnin contact time at room temperature, material is transferred from the slides to subculture broth using suture loops or penicylinders. For a successful test, organisms must be killed in 10 out of 10 trials. The test organisms include S. aureus, P. aeruginosa, and Salmonella choleraesuis.

2.3.2 Methods for Testing Antiseptics. The most important tests for disinfectants involve surgicalhand scrubs. These tests incorporate the cup scrubbing procedure, in which a small area of skin is delineated by a glass cup. A wash solution (1mL containing Triton X-100 in pH 7.9 phosphate buffer) is added by pipette and the skin area is scrubbed for 1 min. The procedure is repeated and an aliquot of the pooled sample is diluted and plated on agar. The plates are incubated 48 h at 37°C and colonies are counted (12).

The glove juice test is required by the FDA for surgical hand scrubs. In this test, loose gloves are placed on each hand and a sampling solution containing buffer and surfactant is added to one hand. This hand is massaged for 1 min and the sample is removed, plated on agar, and incubated to determine microbial growth. The opposite hand serves as a control and the test involves a group of people and statistical analysis (12).

In the modified Cade procedure, used for antimicrobial soaps, repeated hand washing under standard conditions is made in a series of basins and the bacteria in selected basins are plated on agar and incubated (12, 13). Hands are washed three times daily for 10 days for at least 10 consecutive days. Reductions in bacterial counts from baseline to the first basin and to the fourth and/or fifth basin

are measured. At least 35 subjects are used in the test. Other surgical scrub tests are described in the literature (11).

2.4 History

As described in the interesting historical review by Block (14), the first disinfectant reported in the literature was in Homer's *The* Odyssey. Upon his return from the Trojan Wars, Odysseus burned sulfur to fumigate his house. The burning of sulfur (to sulfur dioxide) was used during the great plagues of the Middle Ages. Sulfur dioxide is still used as a disinfectant and preservative for fruit, fruit juices, and wine.

In the early 1500s, Paracelsus reformed the pharmacopeia and introduced compounds of mercury, lead, arsenic, copper, iron, and sulfur as disinfectants and antiseptics. Acidulated water, an antiseptic preparation containing wine or cider vinegar and cream of tartar, was used in the eighteenth century, as was mercuric chloride. Labarraque reported the use of calcium hypochlorite for wound dressings and general sanitation in 1825 (15) and Alcock recommended the use of chlorine to disinfect drinking water in 1827 (16). Tincture of iodine was admitted to the United States Pharmacopeia as an antiseptic in 1830. Richardson discovered the disinfectant activity of hydrogen peroxide in 1858. In 1887 an emulsion of coal tar creosote and soap was patented. A version of it was sold under the name Lysol.

Following the development of the principle of contagion by Holmes and Semmelweiss (17, 18), who independently reported the benefit of physicians washing their hands with calcium hypochlorite before conducting examinations and other procedures, Lister made a great advance in medicine by introducing antiseptic surgery (19). His techniques included the liberal use of phenol. Pasteur's pioneering studies in microbiology were followed by Koch's conclusive demonstration that bacteria invaded tissue and caused disease. In a comprehensive paper in 1881 he evaluated the ability of many chemicals to kill anthrax spores, reporting that halogens, mercuric chloride, and potassium permanganate were highly effective, but phenol was not (20).

Kronig and Paul established the basis for modern chemical disinfection in 1897 (21). They reported that chemical agents killed bacteria at a rate determined by concentration of the chemical and the temperature and they noted that disinfectants can be compared accurately only when they are tested under carefully controlled conditions. Rideal and Walker introduced the important phenol coefficient method for comparing disinfectants in 1903 (22). It is still used in standard assays.

The development of organic chemistry provided the basis for the synthesis of antibacterial agents of increasing potency and selectivity. Bechold and Ehrlich reported the high antibacterial activity of halogenated bisphenols in 1906 (23) and alkylresorcinols appeared in 1921. In 1935 Domagk showed that the addition of long alkyl chains to quaternary ammonium compounds greatly increased their disinfectant activity (24). Clorhexidine, a biguanide, was developed in 1965 by Rose and Swain through the structural modification of the antimalarial biguanides (25).

2.5 Halogens and Halophores

2.5.1 Chlorine and Chlorophores. Chlorine has been used since 1827 to disinfect drinking water and it remains the leading agent for this purpose because it is cheap and effective. Nevertheless, a variety of chlorine-generating compounds (chlorophores) and related compounds have been used in drinking water. Chlorine dioxide (ClO_2) has been used in recent years for drinking water disinfection and wastewater treatment. This highly reactive compound cannot be manufactured and shipped in bulk, but it is prepared at the site of consumption by treating a sodium chlorite solution with chlorine as indicated (Equation 13.1). Inorganic chloramines were used in the 1930s and early 1940s to improve the taste of drinking water, but their use was largely discontinued because of their inferior disinfectant properties. Combining chlorine and ammonia in water results in a mixture of species including $ClNH_2$, Cl_2NH, and Cl_3N. Approximately 25 times as much of these species is needed as is chlorine and the contact time required is 100 times as long (26). More recently, the addition of inorganic chloramines to water

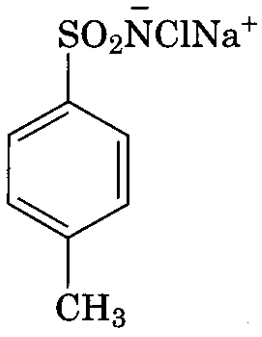
is being reconsidered because they prevent the formation of carcinogenic trihalomethanes from pollutants (27).

$$Cl_2 + 2NaClO_2 \rightarrow 2ClO_2 + 2NaCl \quad (13.1)$$

The germicidal species formed in water solutions of chlorine is hypochlorous acid (Equation 13.2). Based on this finding, a variety of inorganic hypochlorites were introduced as disinfectants. They include sodium hypochlorite, calcium hypochlorite dihydrate, lithium hypochlorite, and chlorinated trisodium phosphate [$NaOCl \cdot 4(Na_3PO_4)$]. The water solutions of sodium hypochlorite are available in different concentrations of available chlorine including the following: 0.4–0.5% (Dakin's solution used on wounds); 5.25% (Chlorox and related preparations); and 12–15% (liquid bleach).

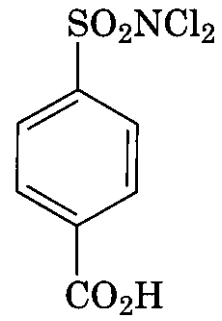
$$Cl_2 + HOH \rightarrow HOCl + HCl \quad (13.2)$$

Organic compounds such as amides, sulfonamides, imides, and amidines form reasonably stable N-chloro derivatives, which may be produced in bulk quantities by treating appropriate nitrogen compounds with HOCl. In water they slowly release HOCl and regenerate the parent nitrogen compound (Equation 13.3). Among the *N*-chlorosulfonamides, chloramine-T (sodium *p*-toluenesulfonchloramide, **1**) has been used for



(1)

disinfecting wounds. It is permitted for use as a sanitizer in dairies and restaurants by the U.S. Public Health Service. Halazone (p-dichlorosulfamoylbenzoic acid, 2) is only slightly soluble in water, but its sodium salt is very soluble and has been used to disinfect drinking water (28). Heterocyclic chloramines include dichlorodimethylhydantoin (halane, 3), which is used in some commercial sanitizing products and trichloroisocyanuric acid (trichloro-s-triazinetrione, 4),



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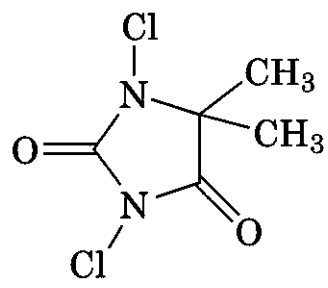

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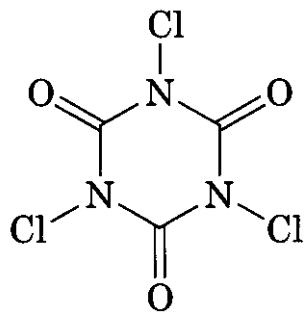
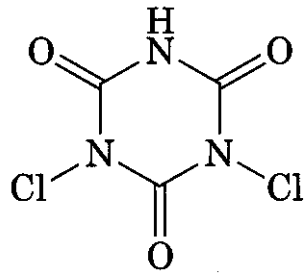


(4)

which is widely used to disinfect swimming pools. The sodium and potassium salts of dichloroisocyanuric acid (5) are used in laundry bleaches, dish-washing compounds, scouring powders, and industrial sanitizing products (29). Chloroazodin (*N,N'*-dichloroazodicarbonamidine, **6**) is used for wound



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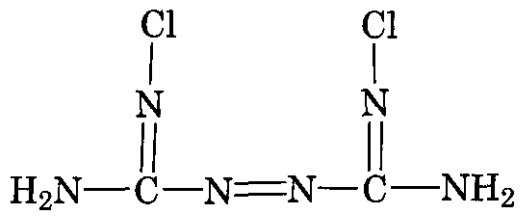


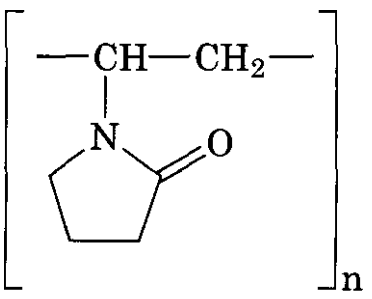
(6)

disinfection, packing for cavities, and lavage and irrigation. It has prolonged antiseptic action because of its relatively slow reaction with water.

$$RCONCl_2 + HOH \rightarrow RCONHCl + HOCl \qquad (13.3)$$

Chlorine in minute amounts results in a destructive permeability of bacterial cell walls (30). Two different mechanisms have been proposed for the germicidal effects of hypochlorous acid. Both of them involve reactions with proteins: one is oxidation of sulfhydryl groups in the proteins and the other is chlorination of amide nitrogens (31). Although early workers considered that the germicidal action of N-chloramines was caused solely by hydrolysis to HOCl, it was suggested more recently that there is direct transfer of positive chlorine from *N*-chloramines to receptors in the bacterial cells (32).

2.5.2 Iodine. Molecular iodine (I_2) is one of the oldest germicides and still one of the most useful. It is highly effective, economical, and minimally toxic at the usual concentrations. Water solutions of iodine are complex systems containing at least seven species, of which I_2 and HIO are considered to be the main disinfectants (33). Iodine preparations include the tincture (2% solution of I_2 in 50% ethanol containing iodide), Lugol's solution (5% I_2 in water with KI), and iodine solution (2% I_2 in water with NaI). The iodide salts solubilize I_2 and decrease its volatility. Preparations containing iodide ion have the triiodide ion (I_3^-) as a major species. This ion is a weak disinfectant compared with I_2. Iodine as I_2 readily penetrates the cell walls of microorganisms. Suggested mechanisms of action include oxidation of the SH groups in proteins, iodination of amino groups, iodination of tyrosine residues, and addition to the double bonds of unsaturated fatty acids (34). Povidone-iodine is a complex of I_2 with polyvinylpyrrolidone (7), a non-



(7)

ionic surfactant polymer. This water-soluble complex contains approximately 10% of available I_2 (35), which it releases slowly. Povidone-iodine is nontoxic, nonirritating, and nonstaining. It is used as an antiseptic before surgery and injections and for treating wounds and lacerations. Available products include surgical scrubs, aerosols, ointments, antiseptic gauze pads, and mouthwashes.

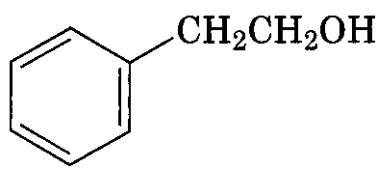
2.6 Alcohols

Alcohols have desirable properties as disinfectants and antiseptics. They are bactericidal, inexpensive, and relatively nontoxic when used topically; however, they are more active against vegetative forms than against spores. Structure-activity relationships (SARs) among aliphatic alcohols have been established (36). Bactericidal activity increases as the homologous series of normal alcohols is ascended from methanol through ethanol, propanol, and so on to octanol. The phenol coefficients range from 0.026 for methanol to 21.0 for *n*-octanol when Salmonella typhosa is the organism. Against Pseudomonas aeruginosa, bactericidal activity of alcohols decreased in the order n-primary > i-primary > n-secondary > tertiary (36).

Ethanol and isopropanol are the only alcohols used routinely as antiseptics and disinfectants. They are most effective as 60–90% solutions in water and their mode of action is gross protein denaturation. The main use is skin disinfection, and an aerosol preparation is used to disinfect air.

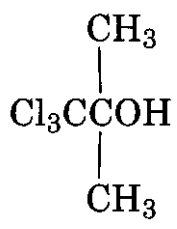
Benzyl alcohol is commonly used as a preservative in vials of injectable drugs in concentrations of 1–4%. Its mild local anesthetic ac-

tivity also is useful for injection. Further antiseptic uses for benzyl alcohol are in ointments and lotions. Phenylethyl alcohol **(8)** is



(8)

used primarily in perfumery. Among alcohols it has the unique property of greater potency against Gram-negative bacteria than against Gram-positive bacteria (37). It has been found to inhibit mRNA synthesis and DNA repair (38). Chlorobutanol (1,1,1-trichloro-2-methyl-2-propanol, 9) is used as a bacteriostatic agent

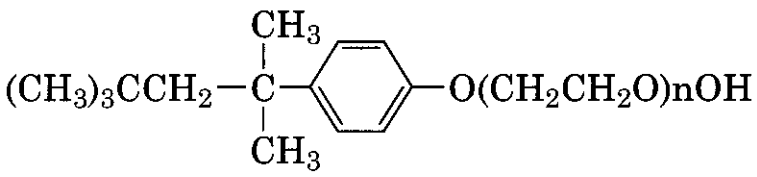


(9)

in pharmaceutical preparations for injection and ophthalmic and intranasal administration.

Ethylene glycol, propylene glycol, and other glycols were used in the past as vapors to disinfect air. Good antimicrobial activity in this use depends on precise control of humidity.

Octoxynol (octylphenoxy polyethoxyethanol, 10) consists of a mixture of compounds



(10)

containing 4–14 ethoxy units in addition to the terminal hydroxyethyl residue. Solutions containing this mixture are used to disinfect contact lenses. A single member of this set of compounds, octynol-9 ($n = 8$) is used as a spermatocide (39).

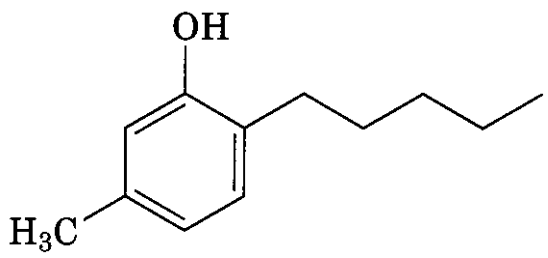
2.7 Phenols

2.7.1 Monophenols. Phenol was introduced by Lister as a surgical anesthetic in 1867. Originally, it was used undiluted and was toxic to tissue; however, it was considered to be less harmful than potential infections. Lister subsequently found that dilutions as low as 1:40 still gave effective antisepsis. The development of substituted phenols and bisphenols with superior antiseptic activity and lower toxicity has led to phenol being replaced as an antiseptic. It has limited use as a preservative for pharmaceuticals and a disinfectant for inanimate objects and excreta. Liquified phenol is phenol containing 10% water. This form is convenient for use in pharmaceutical preparations. Phenol and its derivatives act as gross cellular poisons at higher concentrations, penetrating cells and denaturating proteins. At lower concentrations, they inactivate essential enzymes. In 1% solution, phenol causes a pronounced leak of glutamic acid from cells. This finding suggests that the bactericidal activity of phenol results from physical damage to the permeability barrier in the bacterial cell wall (40).

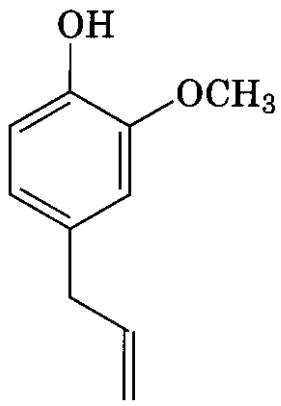
Mixtures of the three isomeric methylphenols (cresols) are obtained from coal tar or petrolatum by alkaline extraction, acidification, and distillation. Cresol, NF is an inexpensive disinfectant with a phenol coefficient of 2.5. Compound cresol solution contains 50% cresol in saponified linseed or other suitable oil. It is soluble in water and is widely used for disinfecting inanimate objects and excreta.

The antibacterial potency of alkylphenols increases with increasing size of the alkyl group, which suggests that lipophilicity may be an important physical property for these compounds. para-Substituted alkylphenols increase in potency up to a chain length of six, then potency declines because of poor water solubility. Among the alkylphenols, 2-n-amyl-5-methylphenol **(11)** is used as an antiseptic in mouthwashes, gargles, and lozenges (41).

Eugenol **(4-allyl-2-methoxyphenol,** 12) has a phenol coefficient of 14.4. It has local anesthetic activity in addition to antiseptic activity and these properties account for its use in mouthwashes.



(11)



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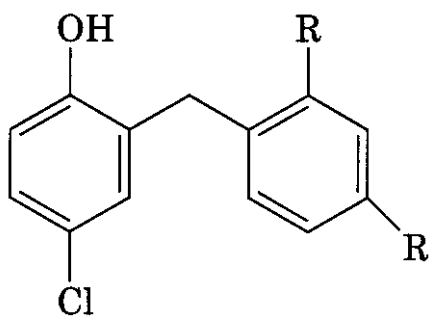
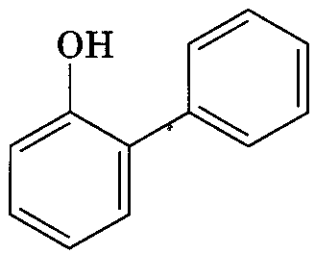


(13) R = H
(15) R = Cl

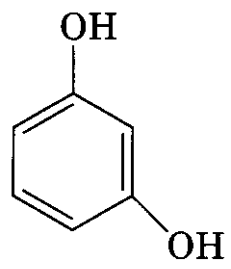


(14)

The antibacterial potency of phenols is increased by halogenation. para-Substituents are more effective than *ortho*-substituents. Addition of alkyl chains further increases potency and straight chains are more effective than branched ones. It is more effective to have the halogenpara and the alkyl group *ortho* than in the reverse orientation. Increasing the molecular weight of the alkyl group usually increases the antibacterial potency (depending on the species) and decreases the toxicity (42, 43). A free hydroxyl group is required for a ntibacterial activity. Chloro and alkyl groups enhance potency by increasing lipophilicity and consequently reducing surface tension. Electron-withdrawing groups such as halogens increase the acidity of the phenol. Nitration increases antibacterial potency but also increases toxicity to higher species. Nitrophenols uncouple oxidative phosphorylation (44). The most widely used and effective substituted alkylhalophenols are chlorophene (2-benzyl-4-chlorophenol, **13**) and 2-phenylphenol (**14**). They are used against a broad spectrum of Gram-positive and Gram-negative bacteria as environmental disinfection in places such as hospitals, dairies, barns, poultry houses, and rest rooms. Triclosan (15)is a diphenylether substituted with one phenolic hydroxyl group and three chlorines. Its bacteriostatic action on a broad spectrum of organisms makes it a useful disinfectant for cosmetic and detergent preparations.

The methyl, ethyl, propyl, and butyl esters of p-hydroxybenzoic acid (parabens) are used as preservatives for liquid dosage forms of pharmaceuticals and in cosmetics and industrial products. They are active against bacteria, yeasts and molds. Parabens are effective in low concentrations (0.1–0.3%)that are devoid of systemic effects, but as constituents of antibacterial ointments they can cause severe contact dermatitis.

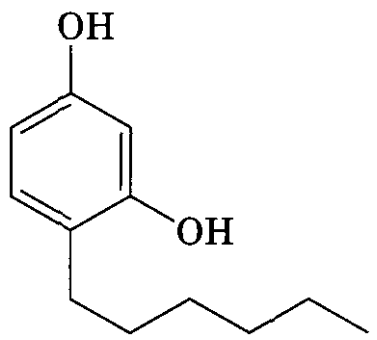
2.7.2 Bisphenols. The parent bisphenol, resorcinol (**16**), has antibacterial and antifun-



(16)

gal activity, but it is less potent than phenol. Nevertheless, its keratolytic properties make it useful in treating conditions such as acne, ringworm, eczema, and psoriasis. Resorcinol monoacetate is a prodrug that slowly liberates resorcinol. It has weaker but more prolonged

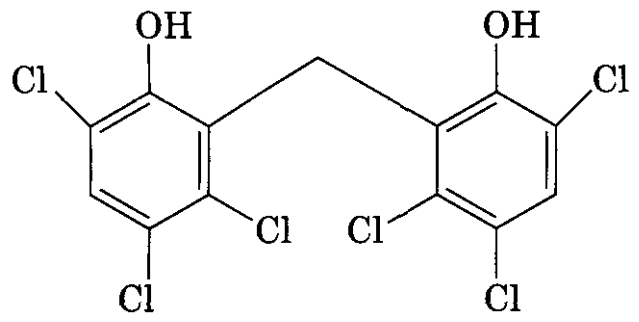
action. As found with monophenols, the addition of alkyl substituents to bisphenols significantly increases antibacterial potency. Hexylresorcinol (4-n-hexylresorcinol, 17) is an



(17)

effective bactericidal agent that has a phenol coefficient of 313 against ***Staphylococcus aureus*** (45). It also has local anesthetic activity. Hexylresorcinol is used in 1:1000 water solution or glycerite in mouthwashes or pharyngeal antiseptic preparations.

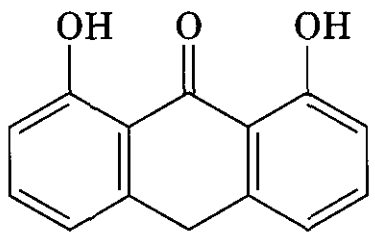
Hexachlorophene is 2,2′-methylenebis-(3,4,6-trichlorophenol) (18). It was synthe-



(18)

sized in 1941 by condensing 2 mol of trichlorophenol with 1 mol of formaldehyde in the presence of sulfuric acid (46). Hexachlorophene has high bacteriostatic activity, especially against Gram-positive bacteria: a 3% solution kills S. ***aureus*** in 15–30 s. It is less potent against Gram-negative bacteria and bactericidal activity requires longer contact time (24 h for some Gram-negative bacteria). Single applications of hexachlorophene are not more effective than ordinary soaps, but daily use results in a layer on the skin that confers prolonged bacteriostatic action. This property led to its widespread use as a topical antiseptic and in the late 1950s it successfully combatted virulent ***Staphylococcus*** infections in hospital nurseries throughout the world. Unfortunately, systemic toxicity can develop from topical use, especially in infants, and more than 30 infants in France died from neurotoxicity resulting from exposure to baby powder containing 6% of hexachlorophene. In 1972 the FDA banned it from all over-the-counter (OTC) and cosmetic preparations, except at preservative levels of 0.1%. Prescription products bear warning labels concerning absorption and potential neurotoxicity (47). Hexachlorophene is still used for surgical scrubs, hand washing as part of patient care, and control of outbreaks of Gram-positive infections where other procedures have failed.

Anthralin (1,8-dihydroxyanthrone, 19) is



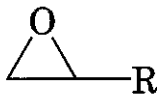
(19)

used against psoriasis and other chronic skin conditions because of its antiseptic, irritant, and keratolytic properties.

2.8 Epoxides and Aldehydes

Epoxides and simple aldehydes are highly reactive functionalities that readily alkylate nucleophilic groups such as amino, hydroxyl, and thiol on proteins and nitrogens on nucleic acids. Because of their high toxicity to higher organisms, they are used mainly to disinfect inanimate objects.

2.8.1 Epoxides. Ethylene oxide (oxirane, 20) is a colorless flammable gas. It readily dif-



(20) X · R = H
(21) X · R = CH_3

fuses through porous materials and destroys all forms of microorganisms at room temperature (48). Ethylene oxide is very toxic and possibly carcinogenic. In concentrations of 3 to 80% v/v it forms explosive mixtures with air. This hazard may be eliminated by mixing it

with carbon dioxide or fluorocarbons. For example, the product carboxide has 10% ethylene oxide and 90% carbon dioxide. Ethylene oxide has been used to sterilize temperature-sensitive medical equipment and certain pharmaceuticals that cannot be autoclaved. Propylene oxide is a liquid that also has been used as a sterilizing agent (49).

2.8.2 Aldehydes. Formaldehyde is a gas that readily undergoes oxidation to formic acid and polymerization to paraformaldehyde. It usually is used as formalin, an aqueous solution containing not less than 37% formaldehyde and 10–15% methanol to prevent polymerization. Formaldehyde exerts a slow but potent germicidal action thought to involve direct nonspecific alkylation of nucleophilic groups on proteins to form carbinol derivatives (Equation 13.4). It is used to disinfect surgical instruments. Formaldehyde is highly allergenic and a cancer suspect agent.

$$\text{Rnu} + \text{HCHO} \rightarrow \text{RnuCH}_2\text{OH} \quad (13.4)$$

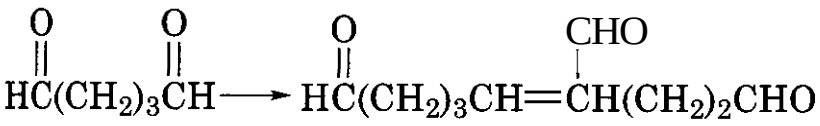
Glutaraldehyde (1,5-pentanedial, 22) is a reactive dialdehyde that readily undergoes self-condensation to form α,β-unsaturated polymers (Equation 13.5). The commercial product is a stabilized solution containing 2% glutaraldehyde buffered to pH 7.5–8.0. Polymerization occurs above pH 8.5 (50). Glutaraldehyde Disinfectant Solution, NF is used to sterilize instruments and equipment that cannot be autoclaved. One special use is in disinfection of fiber-optic endoscopy equipment, especially to prevent the transmission of *Mycobacterium tuberculosis* between patients (51). Glutaraldehyde is effective against all microorganisms and its advantages over formaldehyde include less irritation and odor, although it can cause contact dermatitis. The mode of action of glutaraldehyde is based on its strong binding to outer cell layers, especially involving the ε-amino groups of proteins, rendering them impermeable (52). It may also inactivate cellular enzymes and alkylate nucleic acids (53).

2.9 Acids

Acetic acid in 1% water solution has been used in surgical dressings as a topical antimicrobial agent. In 0.25 to 2% solutions it is useful for infections of the external ear and for irrigation of the lower urinary tract. It is especially effective against *Pseudomonas* and other aerobic Gram-negative bacteria (54). Benzoic acid is employed externally as an antiseptic in lotions, ointments, and mouthwashes. As a preservative in food and pharmaceuticals, its effect depends on both pharmacokinetics and distribution between phases (55). It is more active when the pH is below its pK_a value of 4.2.

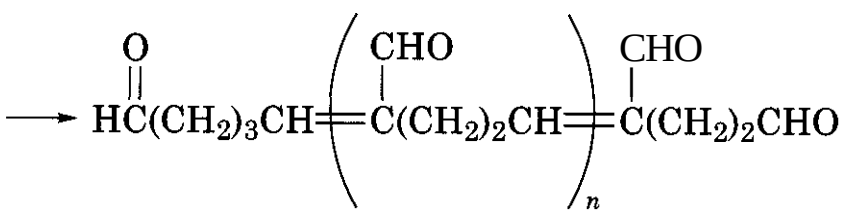
2.10 Oxidizing Agents

Hydrogen peroxide (H_2O_2) is stable when it is pure, but small amounts of impurities promote rapid decomposition to water and oxygen. It is stabile in 3% water solution if deionized water and clean equipment are used. This solution is used for topical disinfection. Although it has potent activity against bacteria including anaerobes, it penetrates tissue poorly and the amount that does penetrate is rapidly decomposed by catalase. Thus, its antibacterial action in tissue is weak and brief. Hydrogen peroxide is more effective where living tissue is not present. It finds use in sterilizing milk, hospital water, food containers, and in the ultrasonic disinfection of dental and medical instruments. The lethal effect of hydrogen peroxide on microorganisms is thought to be attack on membrane lipids and



(22)

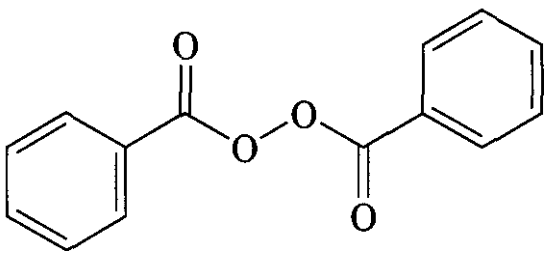
(13.5)



DNA. Decomposition to the highly reactive hydroxyl radical may be important in these processes (56). Concentrations of hydrogen peroxide used in sterilization (3% and higher) overcome the protective effect of catalase in bacterial cells.

Carbamide peroxide is a stable 1:1 complex of urea and hydrogen peroxide, which is provided as a 12.6% solution in anhydrous glycerine. It releases hydrogen peroxide when it is mixed with water.

Benzoyl peroxide (23) is chemically unstable and decomposes when heated. Its safety is improved by dilution with 30% water (Hydrous Benzoyl Peroxide, USP). Lotions contain 5–10% of hydrous benzoyl peroxide and they are stabilized by addition of dicalcium



(23)

phosphate. Nonstabilized water solutions slowly decompose to hydrogen peroxide and benzoic acid. The main use of benzoyl peroxide is in treatment of acne, where it kills *Propionibacterium acnes*, decreases production of irritating fatty acids in sebum, and induces cell proliferation by its keratolytic action.

Peracetic acid is used widely in food processing and beverages. It is bactericidal at 0.001% and has the advantage of decomposing only to water, oxygen, and acetic acid (Equation 13.6).

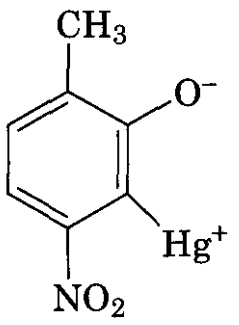
$$2CH_3COOOH \rightarrow 2CH_3COOH + O_2 + H_2O \qquad (13.6)$$

Potassium permanganate ($KMnO_4$) kills many microorganisms at a dilution of 1:10,000; however, this concentration is irritating to tissues and causes stains. It finds limited use in weeping skin lesions.

2.11 Heavy Metals

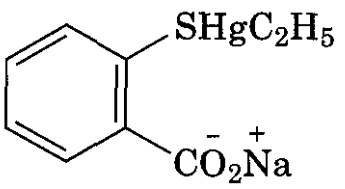
2.11.1 Mercury Compounds. In the past, mercuric chloride was used widely as an antiseptic, but its present use is limited to disinfecting instruments and occasional application to unabraided skin. The most significant inorganic mercury compound is ammoniated mercury ($Hg(NH_2)Cl$), which is used for skin infections such as impetigo. It is formulated as ammoniated mercury ointment, which contains 5 or 10% of the compound in liquid petrolatum and white ointment. Mercuric oxide (HgO) is used sometimes for inflammation of the eye.

There are two basic types of organomercurials: those in which the mercury is covalently bonded to carbon and those in which the mercury is bonded to a heteroatom such as oxygen, sulfur, or nitrogen. The latter type dissociates more readily than the former. Organomercurials are more bacteriostatic, less toxic, and less irritating than inorganic mercury compounds (57). Their mechanism of action is thought to result from binding with thiols in enzymes and other proteins. Thiols such as cysteine reverse their toxicity. Organomercurials are bacteriostatic and their potency is reduced substantially in serum because of the proteins present. They are not effective against spores. Among many organomercurials, nitromersol (24,



(24)

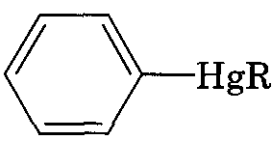
designated as the anhydride of 4-nitro-3-mercuri-2-methylphenol by the USP); thimerosal (merthiolate, 25); phenyhnercuric acetate (26); and



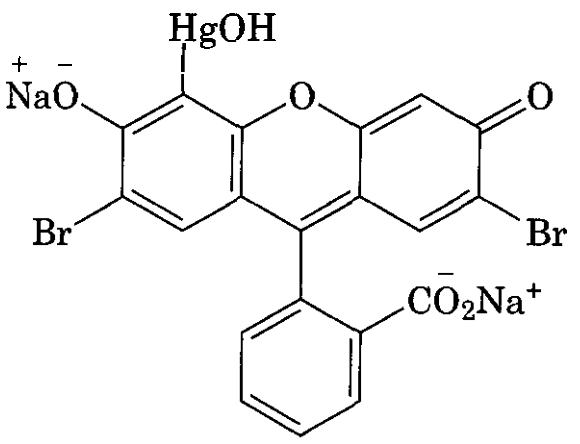
(25)

phenyhnercuric nitrate [a mixture of phenylmercuric nitrate (27) and phenyhnercuric hydroxide (28)] are marketed in many liquid and solid forms as bacteriostatic antiseptics. They also are used in biological products to prevent contamination (58).

Merbromin (mercurochrome, 29) is used despite its very weak bacteriostatic action. Its brilliant red color may account for its popularity.



(26) R = $OCOCH_3$
(27) R = NO_2
(28) R = OH



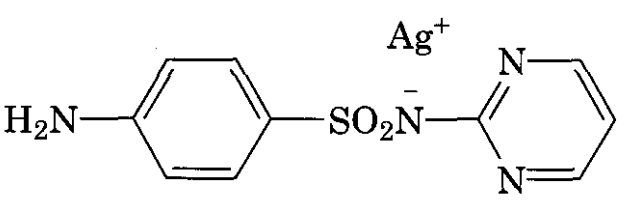
(29)

2.11.2 Silver Salts. Silver ions bind readily with biologically important functional groups including thiol, amine, phosphate, and carboxylate. Some of these interactions can alter the properties of bacterial proteins and cause them to precipitate. Other interactions may cause alterations in the bacterial cell wall and cytoplasmic membrane. These drastic changes result in an immediate bactericidal effect, and small amounts of silver ions subsequently liberated from silver-protein complexes provide sustained bacteriostatic action (56). Silver nitrate ($AgNO_3$) solutions are highly germicidal, destroying most microorganisms at 0.1% concentration. Lower concentrations are bacteriostatic. Silver nitrate is particularly effective against gonococci and 1% solutions are used for the prophylaxis of *ophthalmia neonatorum*. In 5% solutions, silver nitrate is used, usually in conjunction with antibiotics, to treat extensive burns.

A solid form of silver nitrate known as lunar caustic (Toughened Silver Nitrate, USP) has been used to cauterize wounds.

Colloidal silver preparations retain substantial antibacterial activity and they are less injurious to tissues. One of these preparations, Mild Silver Protein, contains about 20% of elemental silver.

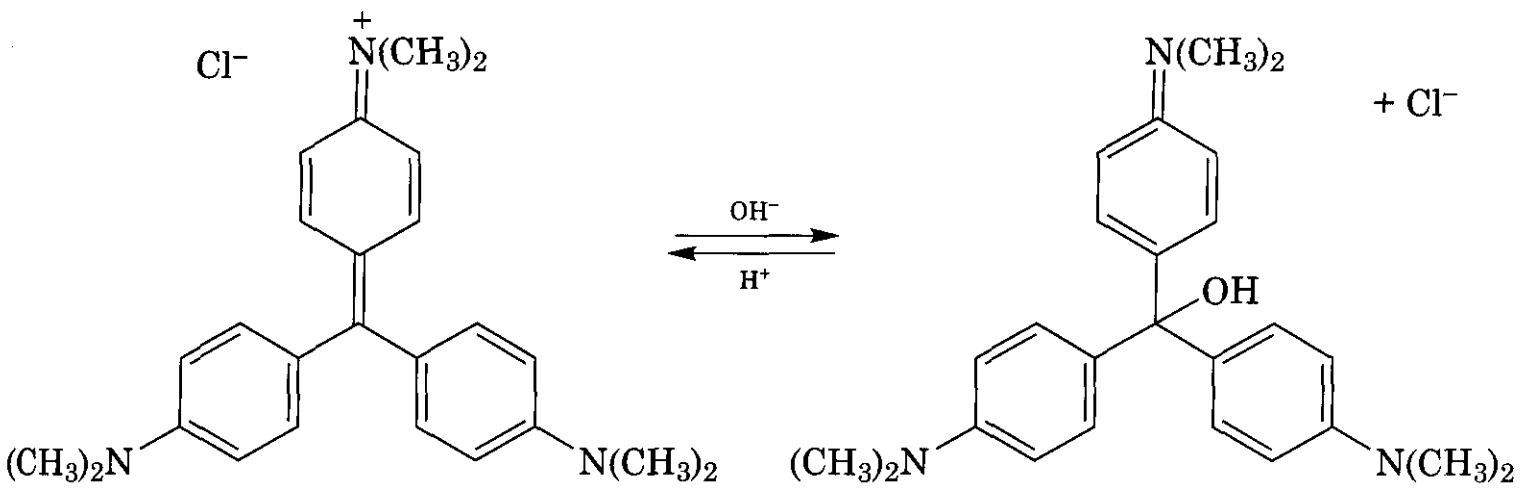
Silver sulfadiazine **(30)** is used in the topical treatment of extensive burns. It readily penetrates the eschar and the solubility is low enough that insufficient silver is released to precipitate proteins or chloride ions. It is effective against *Pseudomonas aeruginosa*.



(30)

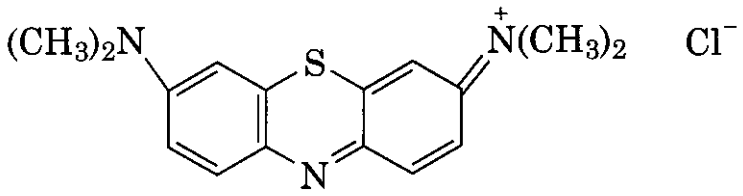
2.12 Dyes

Organic dyes were used extensively as antimicrobial agents before the development of sulfonamides and antibiotics. Now only a few dyes such as gentian violet and methylene blue are used. Gentian violet (hexamethyl-*p*-rosaniline chloride, 31) is a triphenylmethane



(31) Leucobase

dye that is converted into a colorless form (leucobase) in alkaline solution (Equation 13.7). Cationic dyes such as gentian violet generally are active against Gram-positive bacteria and fungi; however, acid-fast and Gram-negative bacteria are resistant. Gentian violet has been used as a topical antibacterial agent, but its main use is as a topical agent for fungal infections. Gentian violet topical solution contains 1% of the agent and 10% of ethanol. Methylene blue (32) is a bacteriostatic agent that has

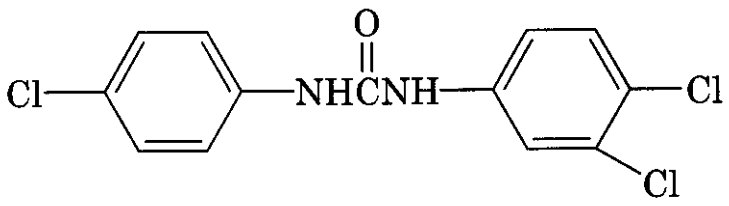


(32)

been used for cystitis and urethritis, infections associated with E. *coli* and ***Neisseria gonorrhea,*** respectively. Its redox properties make it useful for treatment of cyanide poisoning.

2.13 Diarylureas, Amidines, and Biguanides

Diarylureas (carbanilides) are potent antibacterial agents (60). Among a large number of these compounds, triclocarban (33) was chosen for commercial development. It is used mainly in detergents, toilet soaps, and medicated cosmetics. Propamidine (34) and dibromopropamidine (35) are diamidines with activity against Gram-positive bacteria (61, 62). Their mode of action is not known.

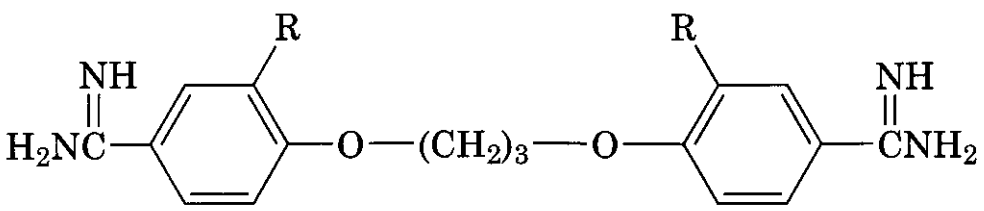


(33)

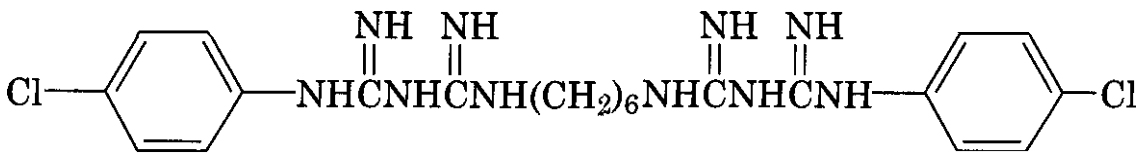
The antibacterial properties of biguanides were discovered by structural manipulation of earlier biguanides with antimalarial activity (25). They are strongly basic compounds that exist as dications at physiological pH. Their physical and antimicrobial properties resemble those of cationic surfactants, but they are not inactivated by anionic detergents unless the counter ions cause precipitation. The most important antibacterial biguanide is chlorhexidine (**36**), which is active against a broad spectrum of bacteria, except acid-fast bacteria and spores. It is not absorbed through skin or mucous membranes and it has no systemic toxicity or teratogenicity (63). The commercial product is chlorhexidine digluconate, which is highly water soluble. A 4% emulsion of it is used in wound cleansing (64), treatment of burns (65), and surgical scrub preparation of skin (66). In 0.2% solution it is used as a mouthwash to combat plaque-inducing bacteria (67).

Chlorhexidine acts by a sequence of events involving attraction to the bacterial cell, strong binding to certain phosphate-containing compounds on the bacterial surface, overcoming bacterial cell wall exclusion mechanisms, attraction to the cytoplasmic membrane, leakage of low molecular weight cytoplasmic components, and complexation with phosphated molecules such as ATP and nucleic acids (68).

Polymeric analogs of chlorhexidine, such as polyhexamethylene biguanide (also called polyaminopropyl biguanide, **37**), wherein the molecular weight is in the range 1000–3000 Da are used in disinfecting contact lenses because they have high antimicrobial potency, low binding to the lenses, and very low ocular toxicity (69). Another important polymeric quaternary compound is polyquaternium 1 (Polyquad, **38**), which has 2-butenyl chains



(34) R = H
(35) R = Br



(36)

separating the quaternary nitrogens, and triethanolammonium groups at the chain ends (70).

2.14 Surface-Active Agents

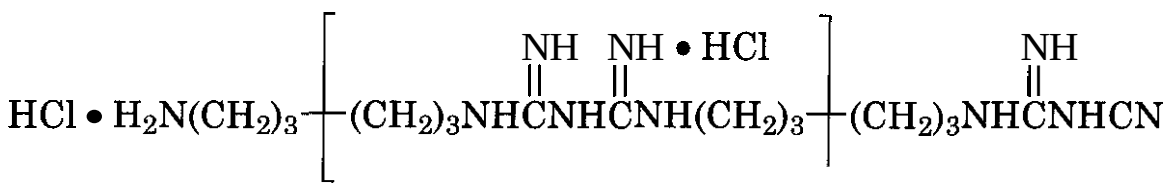
2.14.1 Cationic Surfactants. Cationic surfactants are quaternary ammonium or pyridinium salts that are ionic in water and have surface-active properties. These properties are associated with the cationic head, which has high affinity for water, and a long hydrocarbon tail, which has high affinity for lipids. Cationic surfactants show potent activity against Gram-positive bacteria and lower activity against Gram-negative bacteria (71). Pseudomonas species and Mycobacterium tuberculosis are resistant.

The mechanism of action of cationic surfactants is association with cell wall protein followed by penetration and disruption of the cell membrane. The resistance of Gram-negative bacteria is attributed to difficulty in penetrating the outer membrane (72). There is no activity against bacterial spores. Desirable features of cationic surfactants include water solubility, low toxicity, relatively good tissue penetration, and freedom from stains and corrosion. Disadvantages include inactivation by anionic surfactants (all traces of soap must be removed from skin), reduced effectiveness in the presence of blood serum and pus, strong adsorption on fibrous material such as cotton, occasional allergic responses on prolonged use, and resistant organisms.

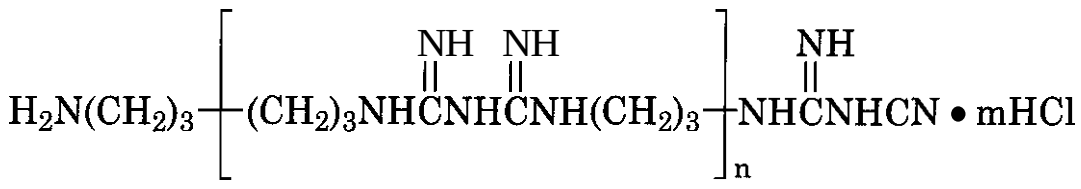
The important surfactants are benzalkonium chloride, benzethonium chloride, methybenzethonium chloride, and cetylpyridinium chloride. Benzalkonium chloride is a mixture of alkylbenzyldimethylammonium chlorides having the general formula $[C_6H_5CH_2N(CH_3)_2R]^+ Cl^-$, where R is a mixture of alkyl groups of which $C_{12}H_{25}$, $C_{14}H_{29}$, and $C_{16}H_{33}$ are the main components. It is used as an antiseptic for skin and mucosa in concentrations of 1:75 to 1:20,000. Other uses include irrigation and disinfection of surgical instruments. Benzethonium chloride **(39)** is also is used as a skin disinfectant and irrigant of mucous membranes. Methylbenzethonium chloride (Diaperene, 40) is used specifically for control of diaper rash in infants caused by Bacterium ammoniagenes, a species that liberates ammonia.

Cetylpyridinium chloride (1-hexadecylpyridinium chloride, 41) has its positively charged nitrogen as part of a pyridine ring. It finds use as a general anesthetic, irrigant for mucous membranes, and as a component of mouthwashes and lozenges.

Structure-activity relationships have been



(37)



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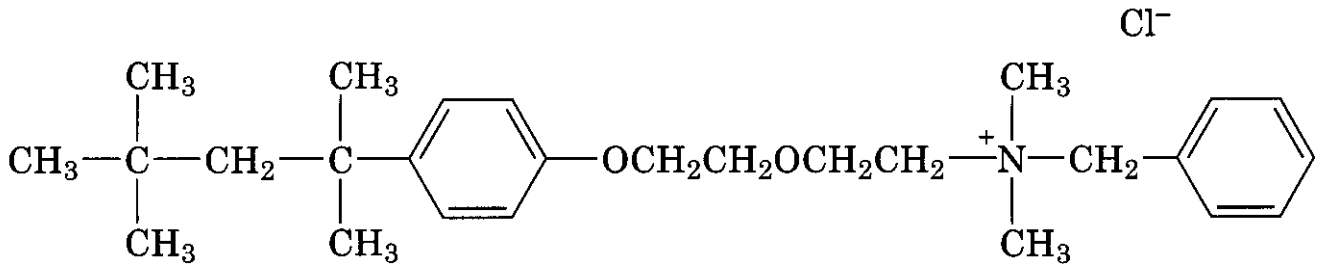
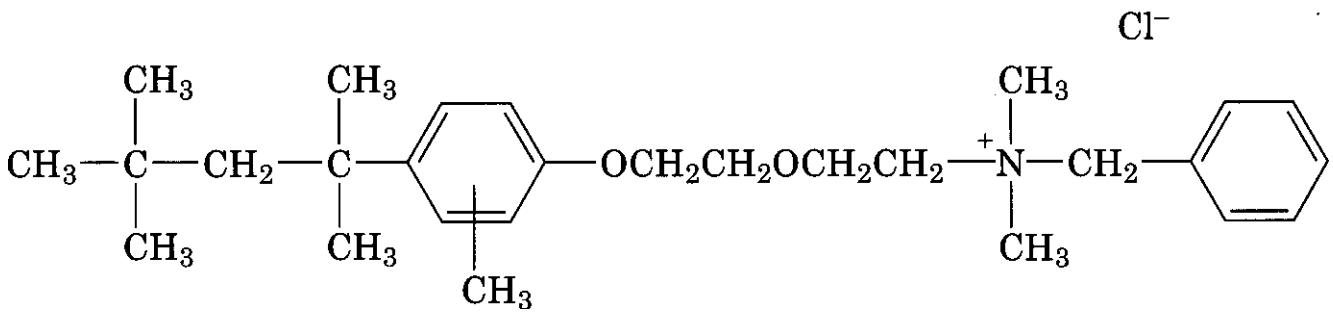


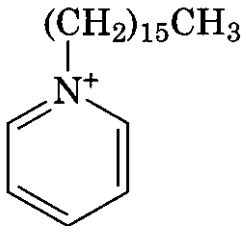
(39)



(40)

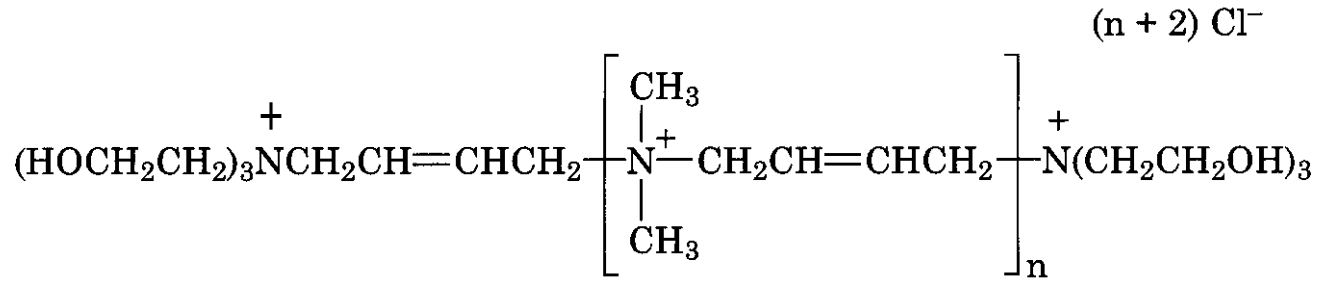
defined for cationic surfactants. For laurylpyridinium chlorides, antibacterial potency depends on the electronegativity of substituents on the pyridine ring with electron-releasing groups affording the highest potencies. There is a linear relationship between potency and partial charge on the pyridine nitrogen (73). Some highly potent substituted pyridines have not been commercialized because of their potential cost. Octanol-water partition coefficients were measured for a series of alkylbenzyldimethylammonium chlorides and used as the independent variable in a correlation with antibacterial potency. The parabolic relationship of the form $\log(1/C) = a + b \log P + c(\log P)^2$ was obtained, wherein P is the partition coefficient and C is the minimum inhibitory concentration (MIC). Maximum potency was found for the compound with the $C_{14}H_{29}$ side chain. Potency depended on the organism, with the MIC being 10 times as great for Gram-negative bacteria as for Gram-positive bacteria (74).

Mixtures of equal proportions of alkylbenzyldimethylammonium chloride and alkyldimethyl(ethylbenzyl)ammonium chloride (alkyl = C_{12} to C_{18}) are known as "dual quats." An example of these mixtures, BTC 2125M, has better biocidal activity than that of the individual species (75). Quaternary ammonium compounds having two long alkyl chains were made possible when catalytic amination of long-chain alcohols to give dialkylmethylamines became a commercial process. Quaternization of these compounds with methyl chloride provides products known as "twin-chain quats." An example of these compounds, dimethyldioctylammonium bromide (DECIQUAM) is used in the British food industry because of its low toxicity and good antibacterial potency (76). Polymeric quaternary ammonium compounds, named polyionenes, are milder and safer than monomeric quaternary ammonium salts (77). Among these compounds, Onamer M (**42**), is used as a preservative for contact lens solutions. It is less irritating than chlorhexidine.

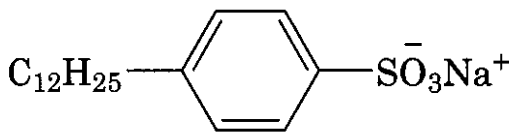


(41)

2.14.2 Anionic Surfactants. Mixtures of anionic surfactants with acids to lower the pH to 2–3 show rapid germicidal activity (78). Alkyarylsulfonates such as dodecylbenzenesulfonic acid (43) are the most effective surfactants and phosphoric acid is frequently used in the mixture. Possible modes of action are disorganization of the cellular membrane, denaturation of key enzymes and other proteins, and



(42)



(43)

interruption of cellular transport (79). Produds have been developed for disinfecting equipment in the food and dairy processing industry.

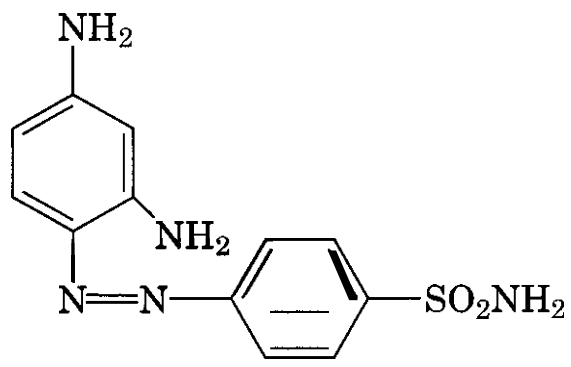
Oxychlorosene is a complex of sodium dodecylbenzenesulfonate and hypochlorous acid. It has a markedly rapid and complete cidal action against both Gram-positive and Gram-negative bacteria, fungi, yeasts, molds, viruses, and spores. It is applied as a disinfectant by irrigation, instillation, sprays, soaks, or wet compresses.

2.14.3 Amphoteric Surfactants. These preparations, known as ampholites, have been used as biocides in Europe for more than 40 years. They are based on mixtures of alkyldi(aminoethyl)glycines and other diaminoglycine derivatives. For example, Tego 51 contains dodecyl and tetradecyl di(aminoethyl)glycines plus dodecyl and tetradecyl aminoethylglycines (80). A 1% solution of this product kills many Gram-positive and Gram-negative bacteria within 1 min (81). Specific uses are in hand disinfection before surgery, disinfection of surgical instruments, and disinfection of rooms in hospitals and food-processing facilities.

3 SYSTEMIC SYNTHETIC ANTIBACTERIALS

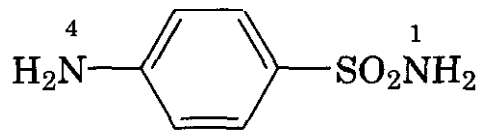
3.1 Sulfonamides and Sulfones

3.1.1 introduction. Prontosil rubrum (**44**), a sulfonamido-azo dye, was the first clinically useful systemic antibacterial agent to be discovered. This discovery in the early 1930s and the development of sulfonamides and sulfones as a class of antibacterial agents, which followed, forms a fascinating chapter in the annals of medicinal chemistry. Their broad antimicrobial spectrum provided, for the first time, drugs for the cure and prevention of a variety of bacterial infections; their widespread clinical use brought about a sharp decline in morbidity and mortality of treatable infectious diseases, and thus proved of great medical and public health importance. Recognition of the inhibition of the action of sulfonamides by yeast extracts, which was shown to be attributed to the presence of *p*-aminobenzoic acid (PABA), required in folate biosynthesis, was the first clear demonstration of metabolite antagonism as a mechanism of drug action; this provided the long sought after mechanistic basis for drug action.



(44)

This led Fildes (1940) to propose his classic *theory of antimetabolites* as an approach to chemotherapy. The development of dihydrofolate reductase inhibitors as antimicrobial agents and their synergistic use in combination with sulfonamides, providing a unique rationale for the use of drug combinations, was a direct result of this interest generated in antimetabolites. That sulfanilamide (45) formed *in vivo* was responsible for the antibacterial action of prontosil focused attention on the



(45)

importance of drug metabolism and blood levels of the active species for drug action. Pharmacokinetic studies thus became an internal part of drug development. Carefully observed side effects in pharmacological and clinical studies of the early sulfonamides revealed new and unanticipated activities; successful exploitation of these leads opened up new areas in chemotherapy, such as oral antidiabetics, carbonic anhydrase inhibitors, and diuretics. This also highlighted the importance of side effects of drugs as a source of new leads in drug design. The rapidity with which new developments took place between 1933 and 1940, from the discovery of antibacterial activity of prontosil to the enunciation of the theory of antimetabolites by Fildes, indicates that the time was just ripe for major developments in drug research and needed only a catalyst, which was provided by the discovery of the antibacterial activity of sulfonamides. The discovery of sulfonamides thus was not only the beginning of the modern era of systemic synthetic antibacterials but also had a strong impact on developments in medicinal chemistry, which influenced later work in drug research in general and chemotherapy in particular.

The interest in sulfonamides and sulfones continues even seven decades after their discovery. Although no major new drug in this class has been added in the last three decades, and the addition of new classes of antibacterials has diminished the clinical use of the existing sulfonamides/sulfones, they still occupy a distinct place in the therapeutic armamentarium; for some conditions alone or in combination with trimethoprim they are still the drugs of choice. One major recent development in sulfonamides is the elucidation of their mode of action at the molecular level. With the identification of the pterin, PABA, and sulfonamide binding sites on the dihydropteroate synthase (DHPS) of the different classes of microbes, the stage is set for the design of a new generation of DHPS inhibitors with broad-based antimicrobial activity including prokaryotes and lower eukaryotes, as well as of agents with selective action against specific classes of microbes.

3.1.2 Clinical Use of Sulfonamides and Sulfones. About 20 sulfonamides and sulfones have been commonly used in clinical practice (Table 13.2). These vary widely in their absorption, distribution, and excretion patterns. Some remain largely unabsorbed after oral administration and are thus considered useful for gastrointestinal (GI) tract infections. Sulfonamides of another group characterized by high solubility, quick absorption, and rapid excretion, mainly in the unaltered form, are widely used in urinary tract infections. Those belonging to yet another group are absorbed rapidly but excreted slowly, or reabsorbed, resulting in maintenance of high blood levels for long periods; these sulfonamides require less frequent administration and are particularly useful for chronic conditions and for prophylaxis.

Sulfonamides and sulfones are widely used in clinical practice, even six decades after their discovery, because of the wide choice that provide of agents with greatly differing half-life and pharmacokinetic characteristics meeting the requirements of varied clinical situations, their wide antimicrobial spectrum, the benefits of synergistic action that their combination with dihydrofolate reductase inhibitors (DHFRI) provides, their highly selective action on the microbes with minimal effects on the host, their relative freedom from problems of superinfection, ease of administration, and favorable pharmacoeconomics. Furthermore, *p,p'*-diaminodiphenylsulfone (dapsone), remains the mainstay for the treatment of all forms of leprosy.

3.1.2.1 Present Status in Therapeutics. The number of conditions for which sulfonamides are drugs of first choice has declined because of a gradual increase in resistance to them and the addition of new classes of antimicrobials, although they still have a distinct and significant place in therapeutics (82–86).

Sulfonamides combined with trimethoyrim are of value in the treatment of urinary tract infections, bacillary dysentery (particularly that caused by Shigella), salmonellosis, and chronic bronchitis. In meningococcal infec-

tions, sulfonamides are of value when the strains of N. meningitides or H. *influenzae* are sensitive to them. Sulfonamides are commonly used in preventing streptococcal infections and recurrence of rheumatic fever among susceptible subjects, especially in patients who are hypersensitive to penicillins. In methicillin-resistant staphylococcal and streptococcal infections and vancomycin-resistant enterococcal infections co-trimoxazole, a fixed-dose combination of sulfamethoxazole and trimethoprim is often considered as one of the treatment options.

Sulfacetamide sodium eye drops are employed extensively for the management of ophthalmic infections; a combination of topical and systemic application is of value in some conditions. Topical sulfonamides such as silver sulfadiazine and mafenide inhibit enterobacteriacease, P. aeruginosa, staphylococci and streptococci, and they are extensively used to reduce the bacterial load in burn eschars.

Sulfonamides have been found useful for the treatment of infections resulting from *Listeria* monocytogenes, especially in penicillin-allergic patients. They are commonly used for the prophylaxis and treatment of otitis media in children.

Sulfonamides, alone or combined with trimethoprim, are the drugs of choice in the treatment of infections resulting from *Nocardia* species, including cerebral nocardiosis; sulfisoxazole, sulfamethoxazole, and sulfadiazine are the commonly used drugs (87). Some clinicians prefer to use sulfonamides alone to avoid the greater risk of hemolytic toxicity observed more commonly with combination therapy. A pyrimethamine-sulfadiazine combination is commonly used for the treatment of all forms of toxoplasmosis, including maternofetal toxoplasmosis. Co-trimoxazole and fansidar (fixed-dose combinations of sulfadeoxine and pyrimethamine) are commonly used for the prophylaxis and treatment of Pneumocystis carinii infection, a common sequelae in patients with AIDS.

Sulfamethoxine, sulfamethoxypyridazine, and dapsone combined with pyrimethamine find use for the prophylaxis and treatment of chloroquine-resistant falciparum malaria.

Dapsone remains the of drug choice for all forms of leprosy and is an essential component of all multidrug therapy regimens. Dapsone has also been reported to cure some cases of Crohn's disease, which may have a mycobacterial origin. Though sulfonamides in general are not very effective against tuberculosis, some are active against nontuberculous mycobacterial infections, which have acquired importance in immunocompromised subjects such as in cases of AIDS; co-trimoxazole has been found effective in patients with *Mycobacterium marinum* infection, whereas sulfisoxazole has been used successfully for M. *fortuitum* infections (88).

3.1.2.1.1 Other Conditions. Shortly after the introduction of sulfa drugs, sulfapyridine was found to have a unique beneficial effects on some inflammatory conditions, especially dermatologic, unrelated to their antibacterial activity (89). Later, dapsone was found to share the same properties at a much lower dose and with improved therapeutic index (90). The disorders that respond are dermatitis herpetiformis (DH), pyoderma gangrenosum, subcorneal pustular dermatosis, acrodermatitis continua, impetigo herpetiformis, ulcerative colitis, and cutaneous lesions of patients with lupus erythematosus. Dapsone is the drug of choice for the treatment of DH and other similar inflammatory conditions that are characterized by neutrophil infiltration. These disorders are characterized by edema followed by granulocytic inflammation or by vesicle or bullae formation (91). Coleman et al. described useful in vitro test systems for the study of inhibition of neutrophil function, which could help in picking up more leads and active compounds in this area (92). The mechanism of action is not fully understood, but it has been proposed that these drugs enter or influence the protein moiety of glycosaminoglycans and decrease tissue viscosity, resulting in prevention of edema, dilution of tissue fluid, and decrease in inflammation and vesicle and bullae formation. It is likely that this additional action of DDS may in part account for the extraordinary sensitivity of lepra bacilli to it. Salicylazosulfapyridine is the treatment of choice for ulcerative colitis.

3.1.2.2 Adverse Reactions. The sulfonamides are generally safe drugs, even though

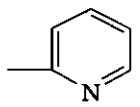
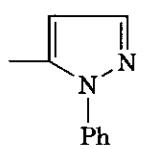
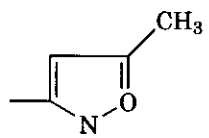
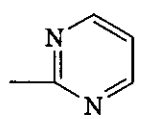
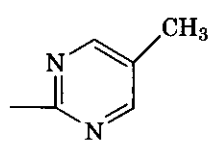
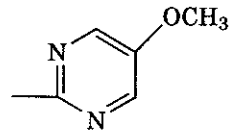
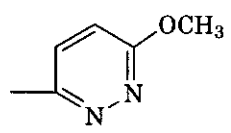
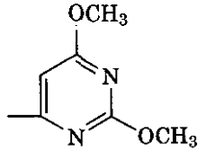
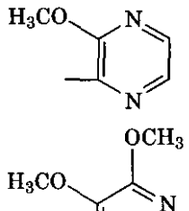
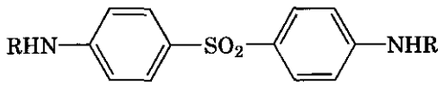
Sulfacetamide	-$COCH_3$	**Albucid**	**2.3**	**670**	**5.4**	**2.0**	**9.5**	**7**	**5**
Sulfapyridine		Eubasin Dagenan MB693	4.8	30	8.4	14	70	9	30
Sulfanilamide	H	Prontalbin	128	750	10.5	71	9	9	
			Readily absorbed, medium rate of excretion						
Sulfaphenazole		Orisul	1.0	150	6.09	69	87.5	10	20 (80)
Sulfamethoxazole		Ganthanol	0.8	Sparingly soluble	6.0	20.5	60	11	60 (14)
Sulfadiazine		Debenal Pyrimal	0.9	8	6.52	26.4	37.8	17	25
			Readily absorbed, slowly excreted						
Sulfamethyldiazine		Pallidin	1.0	40 (pH 5.5)	6.7	69.6	74.0	35	
Sulfamethoxydiazine Sulfamonomethazine		Sulfameter Durenat	2.0	Very sparingly soluble	7.0	64.0	74.2	37	20[c] (30)
Sulfamethoxypyridazine		Lederkyn Kynex	1.0	147 (pH 6.5)	7.2	70.4	77	37	50 (15)

Table 13.2 *(Continued)*

Generic Name	R	Common Proprietary Names	In *Vivo* Activity[b] Against E. coli (μmol/L)	Water Solubility (mg/100 mL at 25°C)	pK_a	Liposolubility[d] (%)	Protien Binding at (1.0 μmol/ml, % bound)	Plasma Half-Lifekt (human)	% N^4-Metabolite in Urine[e] (human)
Sulfadimethoxine		Madribon	0.7	29.5 (pH 6.7)	6.1	78.7	92.3	40	15 (70)
Sulfamethoxpyrazine Sulfametopyrazine		Sulfalene Kelfizina	1.85	Very sparingly soluble	6.1		65	65	65
Sulformethoxine		Sulfadoxine Fanasil	0.8	—	6.1	5	95	150	60° (10)
Diaminodiphenylsulfone	 H	Dapsone Avolo-sulphon	44	14	Base	13	50.[c] ii	20	
Diacetamidodiphenyl-sulfone	$COCH_3$	Acedapsone		0.3				43 days, i.m.	

[a] Arranged in order of increasing plasma half-life. Unless otherwise stated the data are from Rieder (115).
[b] From Struller (296).
[c] From Ref. 297.
[d] Determined by partition between ethylene dichloride and sodium phosphate buffer.
[e] From William and Park (100).
[f] N^4-Phthalyl.

the list of possible adverse reactions is long (85, 86). The most common side effects are related to allergic skin reactions, which vary from relatively minor skin rashes, maculopapular rashes, and urticarial reactions to severe, even life-threatening reactions such as erythema multiform, Stevens-Johnson syndrome, and toxic epidermal necrolysis (TEN). The severe hypersensitivity reactions occur most commonly after treatment with long-acting sulfonamides, whether used alone or in combination with pyrimethamine as for malaria prophylaxis or treatment. The skin eruption is also frequent in patients with AIDS being treated with pyrimethamine-sulfadoxine for P. *carinii* pneumonia, and is associated with pancytopenia in some patients, and may be severe enough to require discontinuation of drugs (90). Individuals seropositive for HIV are more susceptible to developing adverse reactions to sulfonamides/sulfones: 40–80% in patients with AIDS compared to 5% in patients with other immune deficiencies (93, 94). Photosensitivity reactions are also relatively common with sulfonamides.

Hemolytic adverse reactions may occur occasionally, and when they occur, drug administration may need to be discontinued. These include hematologic reactions such as methemoglobinemia, agranulocytosis, thrombocytopenia, kernicterus in the newborn, and hemolytic anemia in patients with G6PD deficiency. Kernicterus can result from administration of sulfonamides to the mother or to the newborn because sulfonamides displace bilirubin from albumin in the newborn. Therefore pregnant women near term or newborns should not be given sulfonamides. Hemolytic anemia is relatively more common with sulfone therapy in leprosy patients, and most often is related to the undernourished status of these patients; discontinuation of treatment is often not necessary and only supplemental therapy is required.

The minor adverse reactions reported include GI reactions such as nausea, vomiting, and diarrhea; and neurologic effects, such as peripheral neuritis, insomnia, and headache. Crystalluria, one of the earliest serious toxic reactions reported with sulfonamides, has been more or less overcome with the discovery of agents that are highly soluble at the pH of urine, or are excreted mainly as water-soluble metabolites.

By binding to albumin sites, sulfonamides may displace drugs such as warfarin, methotrexate, and hypoglycemic sulfonylurea drugs, and may thus potentiate the action of these drugs. Sulfonamide concentrations are increased by indomethacin, salicylates, and probenecid.

It has been suggested that adverse reactions of sulfonamides may be attributable to the formation of reactive hydroxylamine metabolites, together with a deficient glutathione system needed for scavenging these reactive molecules (95). It has been suggested that the covalent adducts formed by the N^4-hydroxylamine metabolites with human epidermal keratinocytes are very likely responsible for the initiation and propagation of the cutaneous hypersensitivity reaction observed with these drugs (96). This is supported by *in vitro* experiments in which sulfamethoxazole hydroxylamine has been found to be cytotoxic for lymphocytes, whereas the parent compound was not (97, 98). With dapsone it has been shown that its hydroxylamine metabolite seems to be responsible for methemoglobinemia; when dapsone is combined with cimetidine, an inhibitor of N-oxidation, the increase of methemoglobinemia is reduced (99).

3.1.2.3 Pharmacokinetics and Metabolism

3.1.2.3.1 Sulfonamides. The sulfonamide drugs vary widely in their pharmacokinetic properties (Table 13.2). Those that are highly ionized are not absorbed from the GI tract after oral administration, leading to a high local concentration of the drug, and were thus considered useful for enteric infections. A majority of the sulfonamides, however, are well absorbed, mainly from the small intestine, and insignificantly from the stomach. Absorption occurs of the un-ionized part, related to their lipid solubility. In rate and extent of absorption most sulfonamides behave similarly within the pK_a range 4.5–10.5. After absorption they are fairly evenly distributed in all the body tissues. High levels are achieved in pleural, peritoneal, synovial, and ocular fluids that approximate 80% of serum levels; CSF levels are effective in meningeal infections. Those that are highly soluble do not, in general, at-

tain a high tissue concentration, show no tendency to crystallize in the kidney, are more readily excreted, and are useful in treating genitourinary infections. The relatively less soluble ones build up high levels in blood, tissues, and extravascular fluids and are useful for treating systemic infections. This wide range of solubilities and pharmacokinetic characteristics of different sulfonamides permits the access of one or the other member of the group to almost any site in the body, thus adding greatly to their usefulness as chemotherapeutic agents. The free, non-protein-bound drugs and their metabolic products are ultrafiltered in the glomeruli, then partly reabsorbed. Tubular secretion also plays an important role in the excretion of sulfonamides and their metabolites. The structural features of the compounds have a marked effect on these processes and determine the rate of excretion. The renal clearance rates of the metabolites are generally higher than those of the parent drugs.

Metabolism of sulfonamides takes place primarily in the liver and involves mainly N^4-acetylation, to a lesser extent glucuronidation, and to a very small degree, C-hydroxylation of phenyl and heterocyclic rings and of alkyl substituents and O- and ring N-dealkylation. Variation of the substituents markedly influences the metabolic fate of the sulfonamides (Table 13.2); the metabolism also differs markedly in different animal species (100–105). Some of the sulfonamides, such as sulfisomidine, are excreted almost unchanged; in most of them N^4-acetylation occurs to a substantial degree; but some of the newer sulfonamides, such as sulfadimethoxine and sulfphenazole, are excreted mainly as the glucuronide. The metabolites in human urine of the commonly used sulfonamides reveal the wide variation in their metabolic disposition (106).

Fujita (107) has carried out regression analyses on the rates of metabolism and renal excretion of sulfonamides in terms of their substituent constants. Equations showing the best correlation indicate that the most important factor governing the rate-determining step of the hepatic acetylation is the hydrophobicity of the drug and that pK_a does not play a significant role. The excretion pattern seems to be more complex and would have to take into consideration additional parameters to give an acceptable correlation.

3.1.2.3.2 Sulfones. Dapsone is well-absorbed after oral administration and is evenly distributed in almost all the body tissues. It is excreted mainly through the kidneys; less than 5% is excreted unchanged, very little N-acetylation takes place, and most of it is present as the mono-N-glucuronide (108–110). It has been shown that there are marked animal species differences in the metabolism of dapsone; humans are relatively slow acetylators compared to rhesus monkey (111, 112). Dapsone has a half-life of about 20 h. Acedapsone following intramuscular injection is very slowly absorbed and deacetylated. It has a half-life of about 42.6 days. There are marked animal species differences in the metabolism of acedapsone also; mice deacetylate acedapsone efficiently, but rats do not (113).

3.1.2.3.3 Half-Life. The half-lives of sulfonamides is of importance because the dosage regimen is related to it; dose schedule is a function of the pharmacokinetic parameters. Kruger-Thiemer and his associates have reported a mathematical model for correlating these parameters with dose schedules (114).

The half-life of different sulfonamides in clinical use vary widely, from 2.5 to 150 h (Table 13.2) and also show marked differences in different animal species. Reider (115) correlated the pK_a, liposolubility, surface activity, and protein binding of a group of 21 sulfonamides with their half-life in humans. It was reported that long-acting sulfonamides were, in general, more lipid soluble than were the short-acting compounds, but no clear-cut relationship could be established; factors such as tubular secretion and tubular reabsorption are also involved. In 2-sulfapyrimidines; a 4-CH, group increases the half-life, 4,6-$(CH_3)_2$ reduces it to less than one-half, the corresponding methoxy derivatives have a much longer half-life, and both 5-CH, and 5-OCH, prolong half-life to the same extent. Similarly, in 4-sulfapyrimidines, the 2,6-$(OCH_3)_2$ analog is the most persistent sulfonamide known; sulfamethoxypyridazine has a half-life about twice as long as that of sulfapyrazine. Thus although no clear-cut pattern of relationship between structure and half-life is discernible,

Table 13.3 Antimicrobial Spectrum of Sulfonamides and Sulfones

Gram-Positive/Acid *Fast*	*Gram-Negative*	*Others*
	Highly sensitive	
Bacillus anthracis (some strains)	***Calymnzatobacterium granulomatis***	***Actinomyces bovis***
Corynebacterium diphtheria (some strains)	***Hemophilus ducreyi***	***Chlamyia trachomatis***
Mycobacterium leprae (to sulfones)	***H. influenzae***	***Coccidia***
Staphylococcus aureus	***Listeria monocytogenes***	***Lymphogranuloma venereusm virus***
Streptococcus pneumoniae	***Neisseria gonorrheae***	***Plasmodium falciparum***
S. pyogenes (Group A)	***N. Meningitidis***	***P. malariae***
	Pasteurella pestis	***Pneumocystis carinii***
	Proteus mirabilis	***Nocardia species***
	Shigella flexneri	***Toxoplasma***
	S. sonnei	***Trachoma viruses***
	Vibrio cholerae	
	Weakly susceptible	
Clostridium welchii	***Aerobacter aerogenes***	***Plasmodium vivax***
Mycobacterium tuberculosis	***Brucella abortus***	
Mycobacterium avium	***Escherichia coli***	
Mycobacterium intracellulare	***Klebsiella pneumoniae***	
Streptococcus viridans	***Pseudornonas aeruginosa***	
	Salmonella	

the methoxy and methyl groups in general seem to prolong half-life.

3.1.3 Antimicrobial Spectrum. After the initial dramatic results obtained with sulfonamides in the treatment of streptococcal infections, studies with these drugs were extended to other microorganisms including bacteria, viruses, protozoa, and fungi. It was found that many Gram-positive and Gram-negative bacteria, mycobacteria, some large viruses, protozoa, and fungi are susceptible to the action of sulfonamides and sulfones (Table 13.3). In almost all cases their action is related to PABA antagonism.

The sulfonamides and sulfones have a relatively broad antibacterial spectrum. Individual sulfonamides do differ in their antibacterial spectrum, but these differences are more quantitative than qualitative. The bacteria most susceptible to sulfonamides include pneumococci, streptococci, meningococci, staphylococci, some coliform bacteria, and shigellae. Lepra bacilli are susceptible to sulfones. One limitation of sulfonamides is their weak activity against bacteria responsible for typhoid fever, diphtheria, and subacute bacterial endocarditis. They have practically no activity against P. aeruginosa. Another limitation with sulfonamides is the rising incidence of resistant isolates in the community. Synergism of their action by dihydrofolate reductase inhibitors, and their introduction to combination therapy has to some extent helped to remedy this situation. Sulfamethoxazole has been shown to have impressive in vitro activity against Mycobacterium avium and M. *intracellulare* (116).

Sulfonamides and sulfones are also active against malarial parasites, although parasites vary greatly in their sensitivity to them; P. falciparum is very sensitive and P. *vivax* is less so. Their effect is potentiated by pyrimethamine (117). The action of sulfones and sulfonamides is mainly against the blood forms, with marginal activity against primary (preerythrocytic) tissue forms and no activity against sexual forms and latent tissue forms.

Sulfonamides have been shown to be highly active against Eimeria (118–120), Toxoplasma (121, 122), and Nocardia spp. (123, 124), and in combination with pyrimethamine are widely used for coccidiosis (125), toxoplasmosis (126), and nocardiosis (87, 127, 128).

McCallum and Findlay (129) showed that

experimental Lymphogranuloma *venereum* virus infection in mice was cured by sulfonamides. Later, other Chlamydiae were also found to be inhibited by sulfonamides, which led to the successful clinical use of these drugs in the treatment of trachoma (130).

Sulfonamides have also high activity against *Pneumoystis* carinii (131) and combined with trimethoprim are largely used for the treatment of P. carinii pneumonia in AIDS patients.

3.1.4 Mechanism of Action

3.1.4.1 Site of Folate Inhibition. The antimicrobial action of sulfonamides is characterized by a competitive antagonism with *p*-aminobenzoic acid (PABA), an essential growth factor vital to the metabolism of the microorganisms. Evidence for this antagonism started coming soon after the discovery of sulfonamides. It was found that substances antagonizing the action of sulfonamides were present in peptones (132), various body tissues, and fluids, especially after autolysis or acid hydrolysis (133), pus (134), bacteria (135, 136), and yeast extract (137,138). Woods (137) obtained evidence that PABA is the probable antagonistic agent in yeast extract, and showed that synthetic PABA could completely reverse the bacteriostatic activity of sulfanilamide against various bacteria in *vitro*. Selbie (139) and Findlay (140) soon after found that PABA could antagonize the action of sulfonamides in *vivo* as well. Blanchard (141), McIllwain (142), and Rubbo et al. (143) finally isolated PABA from these sources. This led Woods (137) to suggest that, because of its similarity of structure with that of PABA, sulfanilamide interfered with the utilization of PABA by the enzyme system necessary for the growth of bacteria. Based on these observations, a more general and clear enunciation of the theory of metabolite antagonism to explain the action of chemotherapeutic agents was given by Fildes in 1940 (144) in his classic paper entitled "A rational approach in chemotherapy."

Further studies showed that the inhibition of growth by sulfonamides in simple media can be reversed not only competitively by PABA, but also noncompetitively by a number of compounds not structurally related to PABA, such as L-methionine, L-serine, glycine, adenine, guanine, and thymine (145, 146). The relationship of sulfonamides to purine was uncovered by the finding that sulfonamide-inhibited cultures accumulated 4-amino-5-imidazolecarboxamide ribotide (147), a compound later shown by Shive et al. (148) and Gots (149) to be a precursor of purine biosynthesis.

With the concurrent knowledge gained in the field of bacterial physiology and metabolism, these isolated facts could be gradually fit into a pattern. The determination of the structure of folic acid by Angier et al. (150) and Mowat et al. (151) revealed that PABA was an integral part of its structure. After this, Tschesche (152) made the suggestion that folic acid is formed by the condensation of PABA or p-aminobenzoylglutamic acid (PABG) with a pteridine and that sulfonamides compete in this condensation. Soon the structure of the active coenzyme form of folic acid, leucovorin (folinic acid, citrovorum factor), was established and its involvement in biosynthetic steps where one-carbon units are added was elucidated (153, 154); the amino acids, purines, and pyrimidines that are able to replace or spare PABA are precisely those whose formation requires one-carbon addition catalyzed by folic acid.

Direct evidence of the inhibition of folic acid synthesis by sulfonamides was soon obtained by studies on bacterial cultures. It was already known that a number of organisms could use PABA and folic acid as alternative essential growth factors (155). Lampen and Jones (156, 157) found that the growth of some strains of Lactobacillus arabinosus and L. *plantarum* in media containing PABA was inhibited competitively by sulfonamides, whereas folic acid caused a noncompetitive type of reversal of this inhibition, suggestive of its being the product of the inhibited reaction. Nimmo-Smith et al. (158) reported a similar inhibition of folic acid synthesis by sulfonamides and its competitive reversal by PABA in nongrowing suspensions of L. plantarum. Inhibition of folic acid synthesis by sulfonamides was also demonstrated in a PABA-requiring mutant, in the parent wild strain of *E.* coli (159, 160), and in cultures of Staphylococcus aureus.

The demonstration of the enzymic synthesis of dihydropteroate (DHP) and dihydrofo-

late (DHF) (Fig. 13.1) in cell-free extracts of a number of organisms (161–165) set the stage for examining the action of sulfonamides at the enzyme level. It was soon demonstrated that the synthesis of DHP from PABA is sensitive to inhibition by sulfonamides, and that the relation between a sulfonamide and PABA remained strictly competitive as long as the two compounds were added simultaneously. If the enzyme and sulfonamide are preincubated with a low concentration of pteridine, subsequent addition of PABA failed to reverse the inhibition; if, however, a high pteridine concentration is used, preincubation results in a much lesser degree of inhibition. Brown (166) showed that the enzyme was not irreversibly inactivated. These results were suggestive of sulfonamide incorporation. It was soon realized that sulfonamides could act as alternate substrates for the enzymes (165–168), resulting in the formation of sulfa-pteroates. Roland et al. (169), however, showed that dihydropterinsulfonamide thus formed did not inhibit DHPS or other folate enzymes. Consequently, this incorporation was not of physiological significance.

Brown (166) observed that the enzymic synthesis was much more sensitive to inhibition by sulfonamides than to bacterial growth, suggestive of impeded permeability of the intact organisms to sulfonamides compared to that of PABA. The more potent inhibitors of folate biosynthesis were, in general, better growth inhibitors also. Hotchkiss and Evans (170) suggested that differences in the response of various organisms to sulfonamides may be attributed to quantitative differences in the ability of individual isoenzymes to produce folic acid from PABA in the presence of sulfonamides.

In a more recent study of the enzymic mechanism and sulfonamide inhibition of DHPS from Streptococcus *pneumoniae* (219), it has been shown that the sulfonamides were capable of displacing PABA in a competetive manner, with equilibrium binding constants that were significantly higher than the equivalent K_i values deduced from steady-state kinetic measurements, indicating that the target for sulfonamide inhibition of S. *pneumoniae* DHPS is the enzyme-DHPP binary complex, rather than the apoprotein form of the enzyme.

Richey and Brown (171) purified dyhydropteroate synthetase/synthase (H_2-pteroate synthase; DHPS) from E. coli, and showed that it could use p-aminobenzoylglutamate (PABG) also as the substrate to form dihydrofolate directly (Fig. 13.1). PABA is, however, not the natural substrate for this enzyme except in a few bacteria such as M. tuberculosis, which forms dihydrofolate directly from dihydropterin pyrophosphate. Shiota et al. (172) and Ortiz and Hotchkiss (173) have shown that the utilization of both substrates, PABA and PABG, is competitively inhibited by sulfonamides.

The cell-free H_2-pteroate synthesizing system isolated from E. coli has become a very useful tool for studying structure-activity correlations among agonists and antagonists of PABA and the inhibitory effect of sulfonamides (173–176).

The mechanism of action of dapsone (and other sulfones) is similar to that of sulfonamides, in that the action is antagonized by PABA in mycobacteria (175–177), other bacteria (178), and protozoa (179). The exceptionally high antibacterial activity of DDS against M. leprae has attracted special attention (180). There is evidence that, as with sulfonamides (167), DDS is also incorporated to form an analog of dihydropteroate, although this also may be of no physiological importance. In M. *kansasii*, Panitch and Levy found a 14- to 15-fold accumulation of DDS within the bacterial cells after 8 days of treatment (181); there may also be similar accumulation within the M. leprae bacilli. Additional sites of action outside the folate-synthesizing enzyme system have also been proposed. DDS has unique beneficial effects on some dermal inflammatory conditions (loc. cit.), and it is likely that this action may contribute to its activity against M. leprae.

A similar mode of action of sulfonamides and sulfones has been demonstrated in most of the other classes of microbes tested that are susceptible to their action. In the case of chlamydia it has been shown that the sulfonamide-sensitive members of this group, such as trachoma inclusion conjunctivitis viruses, have a folic acid metabolism similar to that of bacte-

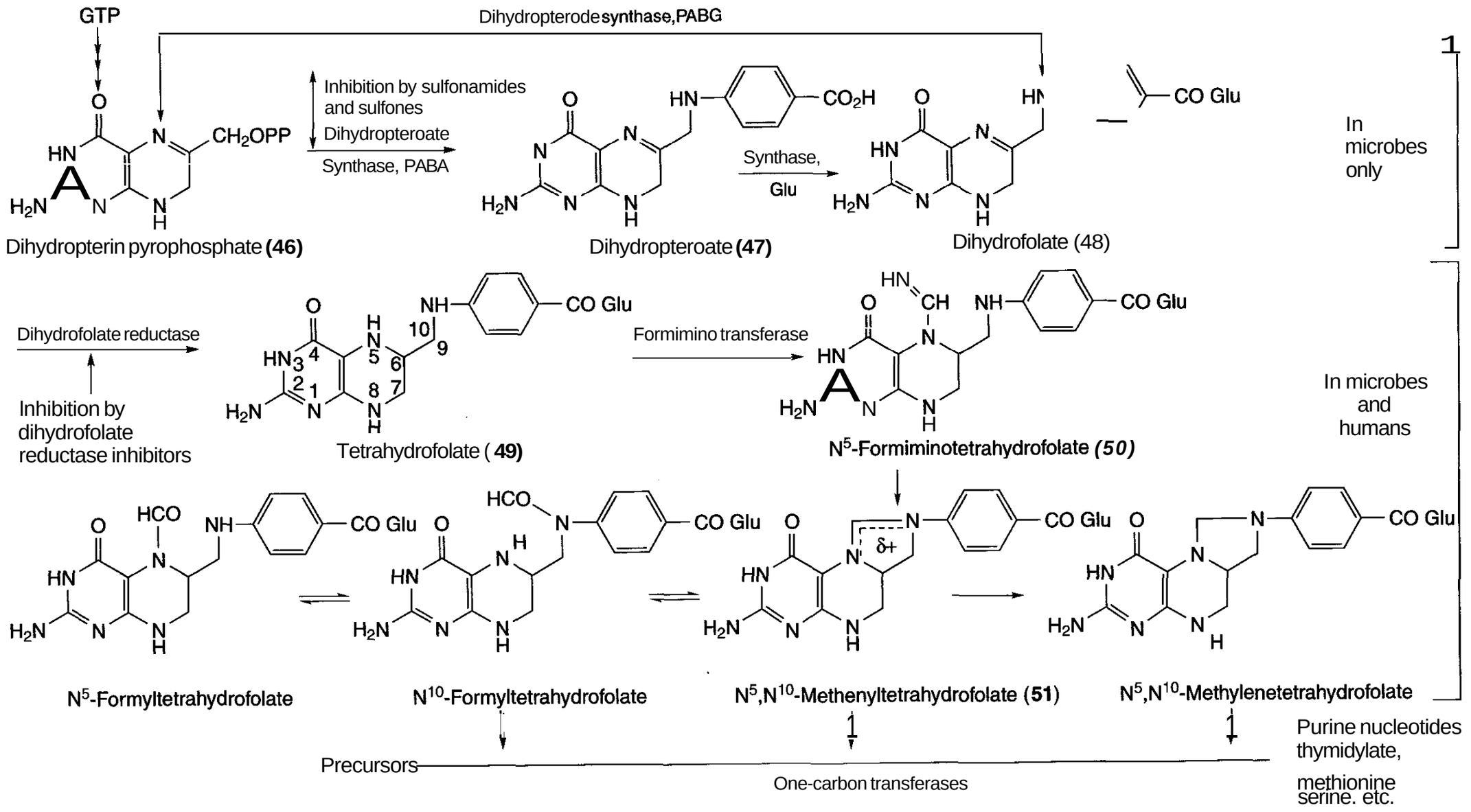


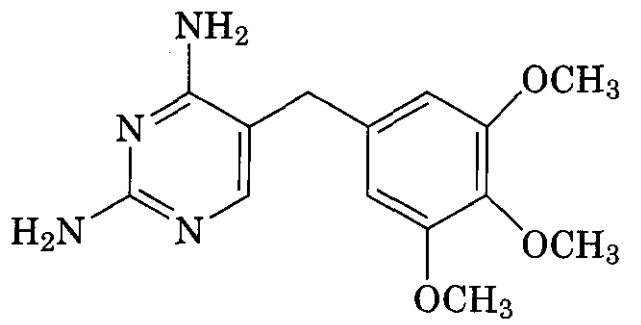
Figure 13.1. Folate metabolism. Sites of action of sulfonamides/sulfones and dihydrofolate reductase inhibitors.

ria, and that the action of sulfonamides against them is competitively antagonized by PABA (182–184).

3.1.4.1.1 Selectivity of Action. The presence of the folate-synthesizing system has been demonstrated in a variety of bacteria (164,167,173, 175, 185), protozoa (186–188), yeasts (189), and plants (190–192), and this serves to explain the broad spectrum of action of sulfonamides. Higher organisms (e.g., mammals) do not possess this biosynthesis system and require preformed folic acid and are thus unaffected by sulfonamides. This selective action on the parasite based on the difference in the metabolic pathway between the microbes and humans makes sulfonamides "ideal" chemotherapeutic agents.

3.1.4.2 Synergism with Dihydrofolate Reductase Inhibitors. The discovery by Hitchings et al. in 1948 (193) of certain diaminopyrimidines showing good antimicrobial activity through antifolate mechanism, and the reports that antifolates acted synergistically with sulfonamides (194–196), added a new dimension to the therapeutic use of sulfonamides (197). The elucidation of the folic acid pathway and the demonstration of its inhibition by both sulfonamides and dihydrofolate reductase inhibitors (Fig. 13.1) elucidated the mechanism of this synergism. It is a consequence of the sequential occurrence of the twin loci of inhibition in the de *novo* folic acid biosynthesis. Factors resulting from this combination that contribute to its usefulness include a severalfold increase in chemotherapeutic indices, better tolerance of the drugs, ability to delay development of resistance, and ability to produce cures where the curative effects of the individual drugs are minimal (198).

Recent crystallographic studies of DHPSs and DHFRs from different organisms have greatly helped to understand the structure of the ligand-binding sites on these enzymes and the molecular basis of their action and synergism (loc. cit.). The choice of the individual drugs used in the combinations is based on the best pharmacokinetic fit (199). For example, trimethoprim with sulfamethoxazole, both having a half-life of about 11 h, is a commonly used antibacterial combination. Dihydrofolate reductases from various sources differ markedly in their binding ability to various inhibitors; pyrimethamine is bound much more strongly to the enzyme from plasmodia than from bacteria, and the converse is true for trimethoprim (200, 201). This explains the choice of trimethoprim for bacterial infections and of pyrimethamine for antimalarial chemotherapy. Thus, although pronounced differences exist in the affinity of DHF inhibitors for dihydrofolate reductases of different origins, the structural requirements for inhibitors of the dihydropteroate synthases for the various species studied are somewhat similar (198). Co-trimoxazole, a fixed-dose combination of trimethoprim and sulfamethoxazole (TMP/SMX), is a very commonly used drug for a variety of bacterial infections.



(52)

3.1.4.2.1 Dihydropteroate Synthase (DHPS). The gene encoding the DHPS from a number of organisms has been cloned, sequenced, and expressed (203–211). Although DHPS is a monofunctional enzyme in prokaryotes, including Mycobacterial spp. in plants (210) and protozoa (208), it is part of a bifunctional enzyme and in yeasts (209), it is part of a trifunctional enzyme combining the preceding one and two steps, respectively, of the folate biosynthetic pathway (203). DHPS is reported to be a homodimer in most prokaryotes, including *E.* coli (205), S. aureus (207), and M. *tuberculosis/leprae* (203), whereas eukaryotic bifunctional DHPS is reported to be either a dimer or a trimer. The DHPSs from E. coli (205), S. aureus (207), and M. tuberculosis (203) have now been crystallized and their high resolution crystal structures determined. Based on the information available from the crystal-structure studies and that of the distribution of known sulfonamide/sulfone resistance mutations, the binding sites for the substrates could be located, and the possible

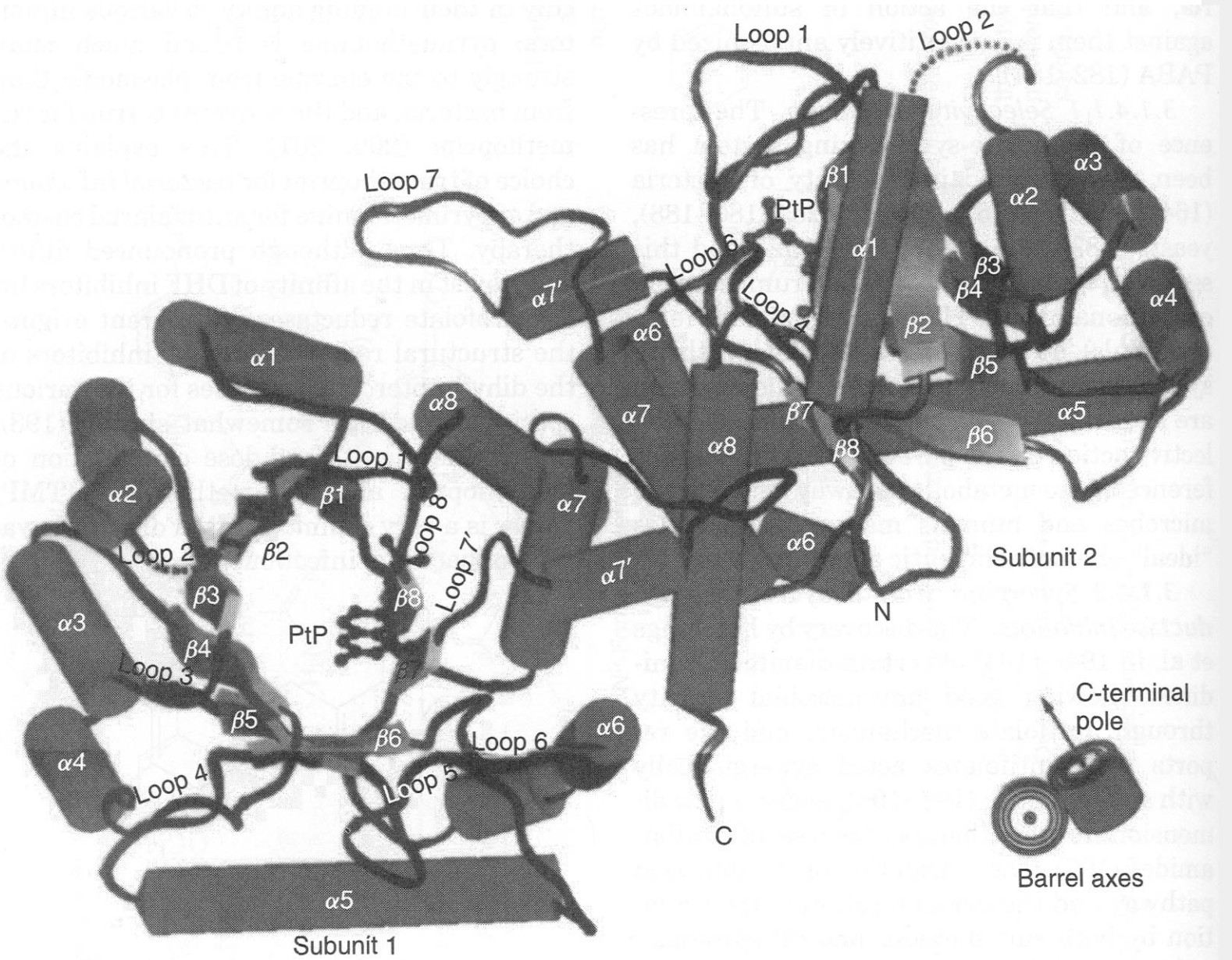


Figure 13.2. Overall folded structure of *M. tuberculosis* DHPS dimer (from Ref. 203). See color insert.

mechanism of action of PABA, sulfonamides, and sulfones proposed *(203)*.The implications of this model for the catalytic mechanisms and the likely geometry of the transition state have also been proposed (203).

There are individual variations in the structure of the DHPSs from different organisms, but overall there are many common structural features and a unified picture of the site and mode of binding of the substrates and the inhibitors has emerged. The DHPS consists of *282* amino acids in the case of E. *coli* (205, 206), of 267 amino acids in *S. aureus,* and of *280* amino acids in *M. tuberculosis* (203). The DHPSs belong to the TIM-barrel class of protein structures.

The polypeptide chain is folded into an eight-loop alp-barrel with a distorted cylindrical shape. It has eight a-helices stacked around the outside of an inner cylinder of parallel β-strands. The residues constituting the outer eight helices, the inner parallel β-sheets, and the α,β-connections have been identified. The intermolecular contacts within the crystal structure suggest a dimeric structure for the enzyme, the interface deriving from the proximity of extensive shallow concave areas of each monomer.

The overall TIM-barrel fold and dimerization interface of DHPSs of *M. tuberculosis (Mtb),E. coli,* and *S. aureus* are similar, with *38%* sequence identity *(203)*.The folded *Mtb* DHPS dimer structure as obtained from crystal structure studies is shown in Fig. *13.2.*

It has been shown that the pterin-binding pocket of DHPS of *Mtb* is formed by the side chains of *12* amino acid residues (Fig. *13.3*), and that this binding pocket occurs in a deep cleft in the barrel *(203)*.Each hydrogen-bond donor/acceptor group of the pterin moiety is

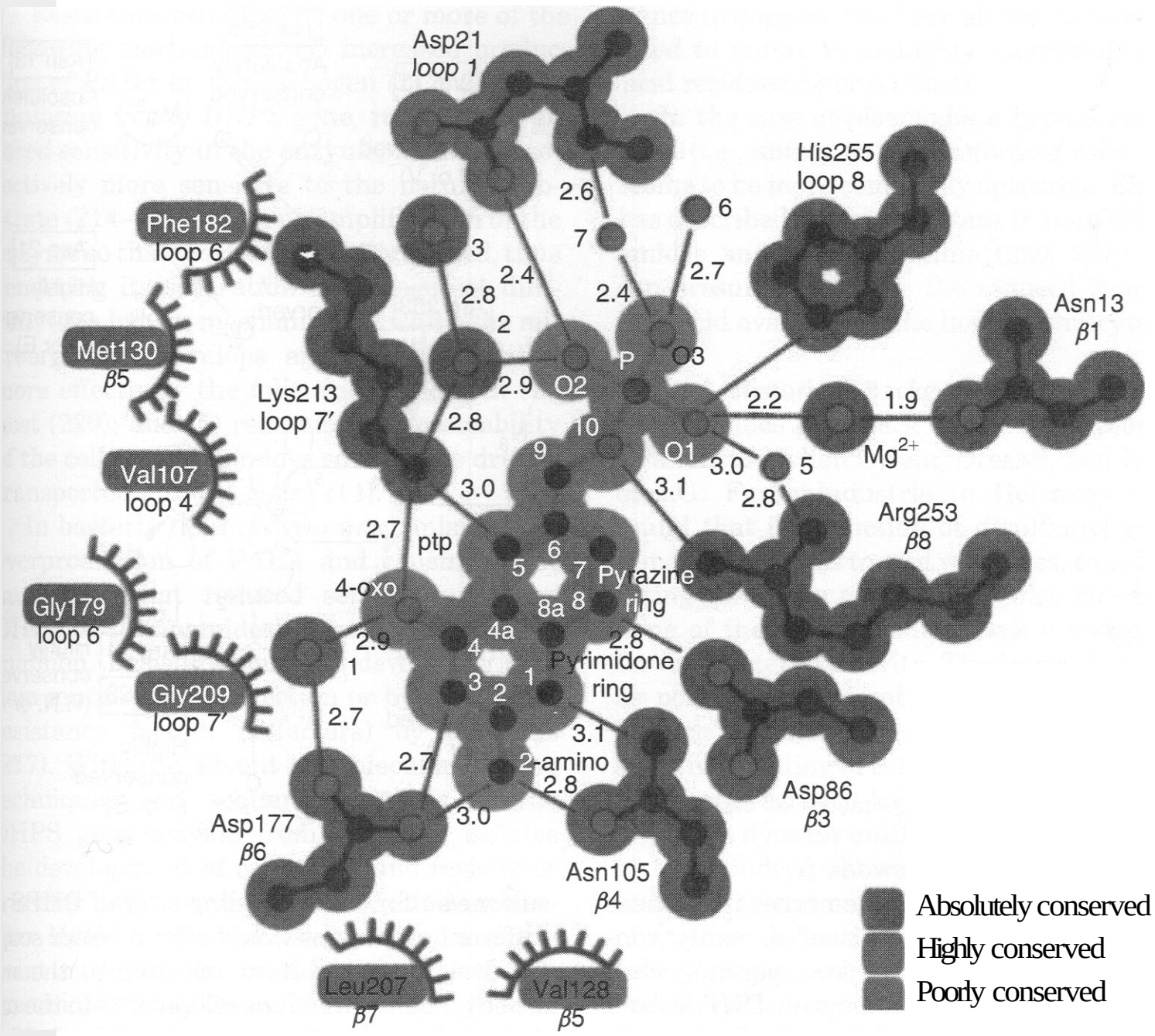


Figure 13.3. The pterin monophosphate binding site in M. tuberculosis DHPS (from Ref. **203).** See color insert.

engaged in interactions with hydrogen-bond donors/acceptors provided by the DHPS amino acid side chains. The residues involved in forming polar bonds and hydrophobic interactions have also been identified. The residues that form the DHP binding site are highly conserved in a number of bacterial and even fungal species, and the residues involved in forming the binding site of the phosphate moiety are somewhat different between different organisms. This has been attributed to different conformations the enzyme assumes during catalysis. The loop 1 containing the C-terminal pole of the β-barrel has been suggested to play a crucial and dynamic function during the catalytic action of the enzyme, and it undergoes extensive conformational changes to place the functionally relevant residues, such as Asp21, in the proper position for catalysis. It thus appears that a high level of sequence conservation with relative conformational flexibility of loop 1 seem to be important for the catalytic reaction mechanism of DHPS. It has been suggested that the loops of DHPS, which serve an important functional role, are flexible and can assume different conformations (203).

Baca et al. (203) proposed a mechanism in which both loops 1 and 2 play an important role in catalysis by shielding the active site from bulk solvent and allowing PP transfer to occur. Based on these data the transition state geometry, as shown in Fig. 13.4, has been proposed for the catalytic site in which the 4-amino group of the attacking nucleophile displaces the pyrophosphate from the opposite side of the 6-methyl carbon atom.

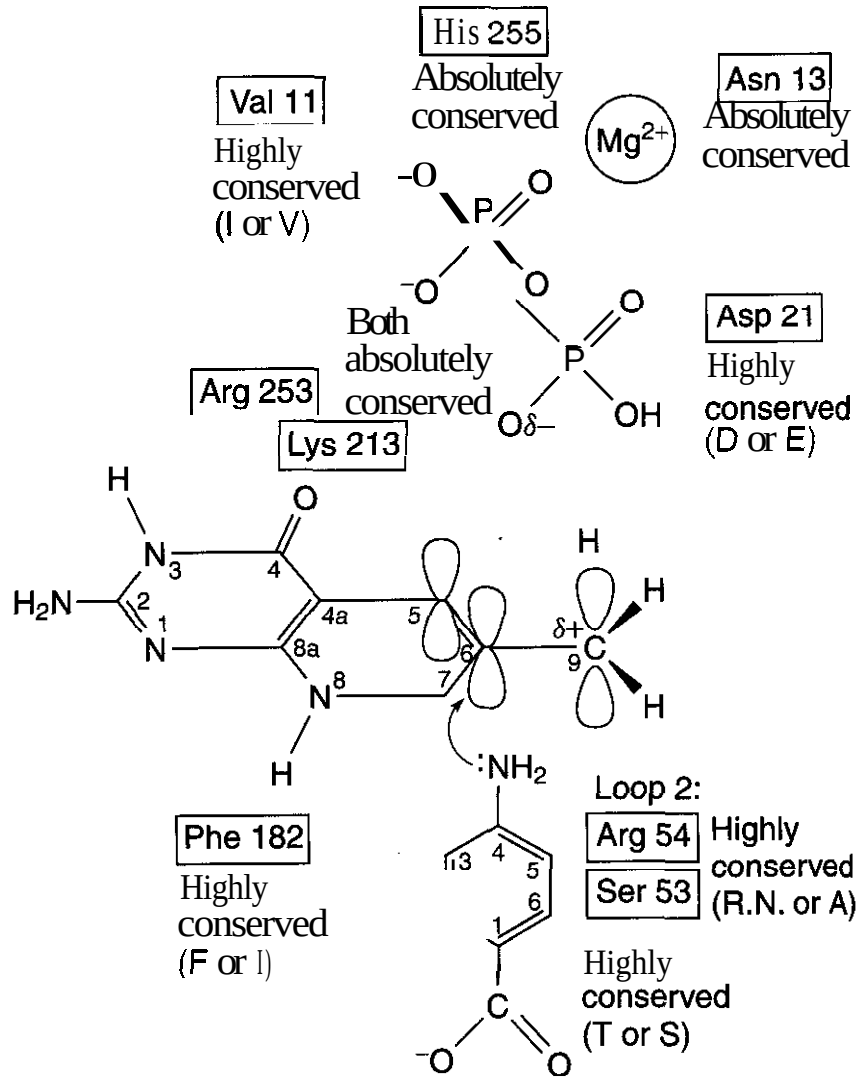


Figure 13.4. Possible geometry of catalytic transition state.

The main picture that emerges from these crystallographic studies is of a highly conserved pterin-binding pocket spanning both prokaryotic and lower enkaryotic DHPSs from a wide range of species. Inhibitors designed to fit this site should have a broad spectrum of activity against a variety of microbial pathogens. The fact that this site is highly conserved would imply that drug-resistance mutations are less likely to occur, which would minimize the resistance development problems in inhibitors based on this target. Further, having no counterpart in the mammalian host specificity would not be a concern in the design of inhibitors of this class and selectivity is ensured. This target is not very well exploited and offers good scope for design of antimicrobial agents, including against mycobacteria. The only concern may be overlap with dihydrofolate reductase inhibitors, which have a mammalian counterpart. Sulfones/sulfonamides (and PABA) bind in a less conserved site, which offers greater scope for selectivity of action for different classes of microbes. Most of the sulfonamides so far designed belong to this class. The knowledge gained about the PABA/sulfone/sulfonamide binding sites of DHPS of different organisms would offer a better scope for design of inhibitors selective to the microbes. These current developments in the understanding of molecular mechanism of action of sulfonamides/sulfones offers a suitable scope for design of a second generation of dihydropteroate synthase inhibitors.

3.1.4.3 ***Drug Resistance.*** Emergence of drug-resistant strains is a serious problem with sulfonamides, as with many other antimicrobials. Because of the long-time use of this group of drugs, the incidence of drug-resistant isolates in the community has become quite alarming. The majority of isolates of *N. **meningitides*** of serogroups ***B*** and C in the United States and of group A isolated from other countries are now resistant. A similar situation prevails with respect to Shigella and strains of E. *coli* isolated from patients. Given that the mode of action of all sulfonamide/sulfone antimicrobials involves the same basic mechanisms, different sulfonamides usually show cross-resistance, but not to antimicrobials of other classes acting by different mechanisms.

Resistance can arise by one or more of the following mechanisms: (1)increased production of PABA by the pathogen (212, 213); (2) mutation in the DHPS gene, resulting in altered sensitivity of the enzyme, making it selectively more sensitive to the natural substrate (214–219); (3)gene amplification of the enzyme so that more enzyme is produced, thus rendering its saturation by antagonist difficult; (4)a bypass mechanism by which the microorganism develops an ability to utilize more effectively the folic acid present in the host (220); and (5)reduction in permeability of the cell to sulfonamides so that less drug is transported into the cells (214).

In bacteria the first two mechanisms (i.e., overproduction of PABA and causing point mutations that reduced sensitivity of the DHPS to sulfonamides) seem to be the most common. Resistant mutants develop by random mutation and selection or by transfer of resistance factors (R-factors) by plasmids (217). With the advent of molecular cloning techniques and sequence analysis of the DHPS gene/enzyme from resistant isolates the development of resistance and resistance transfer have now been studied at the molecular level. It has been shown that a single point mutation can confer resistance to sulfonamides and sulfones. Most of the high level sulfonamide resistance in Gram-negative bacteria appears to be accounted for by only two plasmid-borne genes, Sul-I and *Sul-II* (221). It has been known for many years that multiple drug resistance involving streptomycin, chloramphenicol, tetracycline, and sulfonamides could be transferred between Shigella and E. coli in mixed cultivation in the host (222). Drug resistance acquired in this manner is usually persistent and irreversible and can be transferred to other sensitive strains indefinitely. However, in meningococci the sulfonamide resistance has been reported to be chromosomally located. Similarly with S. aureus, it has been reported that of the nine resistant clinical isolates from different geographicallocations analyzed, all had mutations in their chromosomal gene leading to an altered DHPS (207). These isolates differed in 15 amino acid residues from the wild-type sequence, and in this way DHPS appears to be different from DHFR (223). In M. leprae resistance to dapsone has been shown to be attributed to mutation at highly conserved amino acid residues 53 or 55 (224).

In the case of plasmodia a bypass mechanism (i.e., ability to use preformed folic acid) seems to be more commonly operative. Bishop has described strains resistant to both sulfonamides and pyrimethamine (220, 225) that can presumably utilize the reduced forms of folic acid available in the host erythrocytes.

3.1.5 Historical Background. The story of sulfonamides goes back to the early years of this century when Orlein, Dressel, and Kethe of I.G. Farbenindustrie in Germany (226) found that introduction of a sulfamyl group imparted fastness to acid wool dyes, thus indicating affinity for protein molecules. However, none of these sulfonamides was investigated for antibacterial activity. The interest in dyes as possible antimicrobials was prompted by Ehrlich's studies on the relationship between selective staining of cells by dyes and their antiprotozoal activity, which also led to the testing of azo dyes for antibacterial activity; some of them indeed showed such activity. In an attempt to improve the antimicrobial activity of quinine derivatives, Heidelberger and Jacobs (227) prepared dyes based on dihydrocupreine, which included p-arninobenzenesulfonamido-hydrocupreine. Although the latter was reported to have bactericidal activity, it did not arouse much interest because the activity, having been tested in vitro, was of a low order and no further work was published on these compounds.

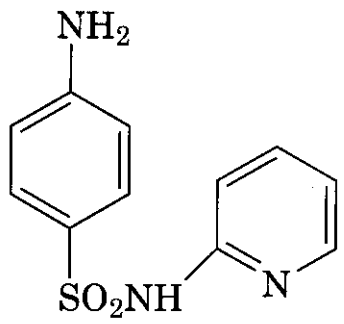
Mietzchand Klarer at I.G. Farbenindustrie synthesized a variety of azo dyes, a continuation of Ehrlich's interest in imparting to azo dyes the property of specific binding to bacterial proteins, comparable to the binding to wool proteins. Mietzsch and Klarer (228) synthesized a group of such dyes containing a sulfonamide radical, which included prontosil rubrum (1). Domagk, also at I.G. Farbenindustrie, carried out the antibacterial testing of these dyes. Realizing the lack of correlation between in vitro and in vivo screening, Domagk decided to do the testing in mice, a very fortunate decision, because otherwise the fate of sulfonamides might have been different. Domagk (229)observed in 1932 that pron-

tosil protected mice against streptococcal infections and rabbits against staphylococcal infections, though it was without action in vitro on bacteria. Anecdotal reports say that the first patient to be successfully treated with prontosil was Hildegarde Domagk, the daughter of its discoverer, who had septicemia attributed to a stitching needle prick. Foerster (230) published the first clinical success with prontosil in a case of staphylococcal septicemia in 1933.

These studies aroused worldwide interest and further developments took place at a very fast rate. One of the earliest systematic investigations on sulfonamides was by Trefouel, Nitti, and Bovet (231) working at the Pasteur Institute in Paris. Under a program of structural modification of this class of compounds, they prepared a series of azo dyes by coupling diazotized sulfanilamides with phenols with or without amino or alkyl groups. They observed that variation in the structure of the phenolic moiety had very little effect on antibacterial activity, whereas even small changes in the sulfanilamide component abolished the activity. These observations pointed to the benzenesulfonamide residue as the active structural unit, and led to considering *p*-aminobenzenesulfonamide (sulfanilamide, 45) as the putative metabolite responsible for the antibacterial activity. They suggested that prontosil was converted to sulfanilamide in animals and showed that sulfanilamide was as effective as the parent dyestuff in protecting mice infected with streptococci. They also showed that sulfanilamide exerted a bacteriostatic effect in vitro on susceptible organisms. Soon after, Colebrook and Kenny (232) observed that although prontosil was inactive in vitro, the blood of patients treated with it had bacteriostatic activity. They also reported the dramatic cure of 64 cases of puerperal sepsis by prontosil, whereas Buttle, Gray, and Stephenson (233) showed that sulfanilamide could cure streptococcal and meningococcal infections in mice. Fuller's (234) demonstration of the presence of sulfanilamide in the blood and its isolation from urine of patients (and mice) under treatment with prontosil firmly established that prontosil is reduced in the body to form sulfanilamide (64), a compound synthesized as early as 1909 by Gelmo (235). Fuller concluded that the therapeutic action of prontosil was very likely the result of its reducton in vivo to sulfanilamide. Among the early patients to be treated with sulfanilamide was Franklin D. Roosevelt Jr., the son of the President of the United States. His recovery from a streptococcal throat infection helped to overcome early reservations on the medicinal value of antibacterial chemotherapy with sulfonamides. Ehrlich's concept of a relationship between the affinity of dyes for a parasite and their antimicrobial activity, which focused attention on sulfonamide azo dye, was found to be irrelevant to the activity of the latter. Nevertheless, sulfanilamide proved to be the "magic bullet" of Ehrlich and heralded the era of chemotherapy, a term coined by Ehrlich to emphasize the concept of selective action of chemicals on microbes as opposed to the action on host cells. The era of modern chemotherapy had now begun. Domagk was awarded the Nobel Prize for Medicine in 1939, primarily for the discovery of the antibacterial activity of sulfonamides.

Earlier Sulfonamides. These discoveries had a tremendous impact not only on the development of sulfonamides as antimicrobials, but also on developments in chemotherapy in general. Sulfanilamide, being easy to prepare, cheap, and not covered by patents, became available for widespread use and brought a new hope for the treatment of microbial infections. Recognizing the potential of sulfonamides, almost all major research organizations the world over initiated research programs for the synthesis and study of analogs and derivatives of sulfanilamide, particularly to improve its antimicrobial spectrum, therapeutic ratio, and pharmacokinetic properties. New sulfonamides were introduced in quick succession. Sulfapyridine (M&B 693, 66) (236), reported in 1938, was one of the earliest of the new sulfonamides to be used in clinical practice for the treatment of pneumonia and remained the drug of choice until it was replaced by sulfathiazole. Sulfapyridine was used on Winston Churchill to cure pneumonia in 1943 during his trip to Africa.

Sulfathiazole (67) (237) was the second sulfonamide to be introduced in clinical practice. It replaced sulfapyridine because of its wider antibacterial spectrum and higher therapeutic



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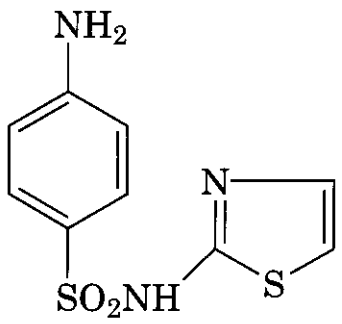


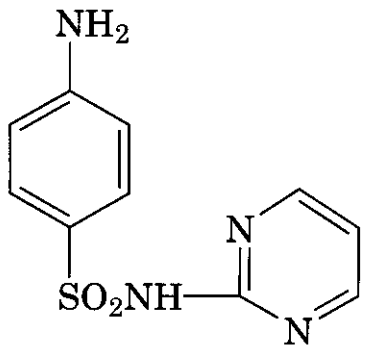
(54)

index. Substitution of the thiazole ring by alkyl groups did not improve the activity, whereas a 4-phenyl residue enhanced both the activity and the toxicity.

Some of the other important sulfonamides introduced in clinical practice during this period were sulfacetamide, the corresponding N^4-pthallyl and succinyl derivatives, sulfadiazine, sulfamerazine, sulfamethazine, sulfisomidine, sulfamethizole, and sulfisoxazole (Table 13.2). These compounds differed widely in their pharmacokinetic profile and helped in enlarging the scope of therapeutic use of sulfonamides. This work was described in 1948 in a very exhaustive monograph by Northey, which may be consulted for research work of this period (238).

Later Sulfonamides. This widespread interest in new sulfonamides continued until about 1945, when interest gradually shifted to antibiotics after the introduction of penicillin. However, after about a decade of the use of penicillins, problems encountered with antibiotics, such as emergence of resistant strains, superinfection, and allergic reactions, brought about a revival of interest in sulfonamides. The knowledge gained during this period about the selectivity of action of sulfonamides on the pathogens, the relationship between their solubility and toxicity, and their pharmacokinetics gave a new direction to further developments and a second generation of sulfonamides began to appear with improved characteristics.

A major advance in sulfonamide therapy came with the proper appreciation of the role of pharmacokinetic studies in determining the dosage schedule of these drugs. It was realized that some of the "earlier" sulfonamides such as sulfadiazine (55) and sulfamerazine (56)



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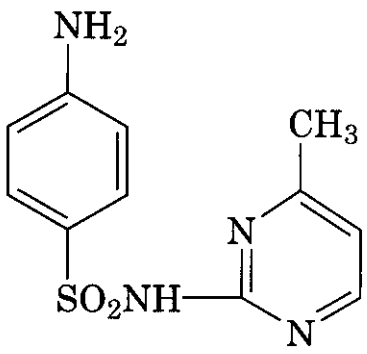

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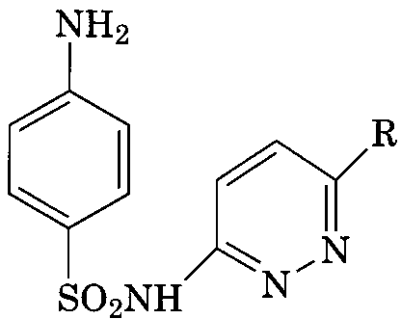
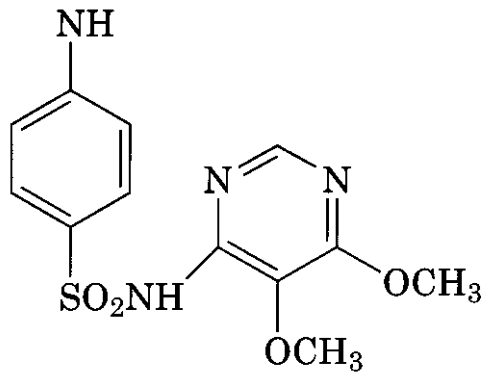


(57)

had a long half-life (17 and 24 h, respectively) and required less frequent administration than was normally prescribed. The era of newer, long-acting sulfonamides started in 1956 with the introduction of sulfamethoxypyridazine (57) (239) having a half-life of around 37 h, the longest known at that time, which needed to be administered only once a day (240). In 1959 sulfadimethoxine was introduced with a half-life of approximately 40 h

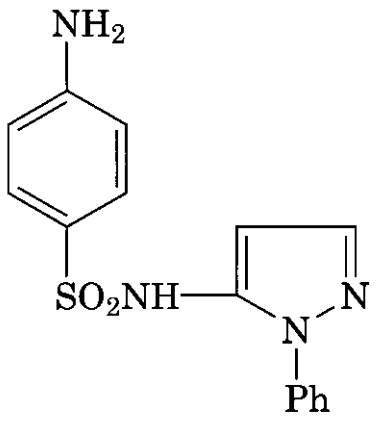
(241–243). A related 4-sulfonamidopyrimidine, sulfomethoxine (**58**) (244, 245), having the two methoxyl radicals in the 5,6-positions,



(58)

was soon introduced. It has by far the longest half-life, about 150 h, and needs administration only once a week.

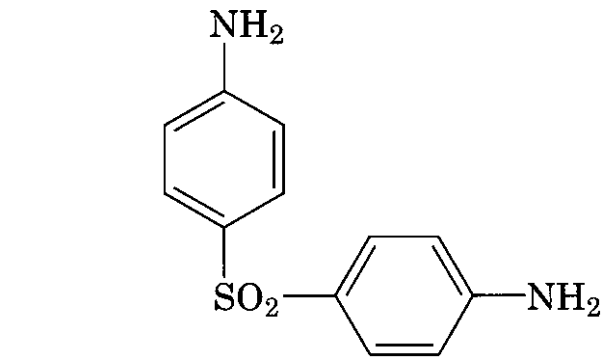
Some of the other sulfonamides introduced in clinical practice in this period were sulfamethyldiazine, sulfaphenazole, sulfamoxol, and sulfamethoxazole (**59**)(246) (Table 13.2).



(59)

Sulfamethoxazole is a particularly important sulfonamide of this period in view of its well-matched half-life (~11 h) with that of trimethoprim, and a fixed-dose combination of the two, co-trimoxazole is widely used in clinical practice.

Sulfones. The demonstration that experimental tuberculosis could be controlled by 4,4′-diaminodiphenylsulfone (dapsone, **60**)(247) and disodium 4,4′-diaminodiphenylsulfone-*N*,*N*′-didextrose sulfonate (promin, **61**)(248) was a major advance in the chemotherapy of mycobacterial infections. Although dapsone and promin proved disappointing in the therapy of human tuberculosis, the interest aroused in



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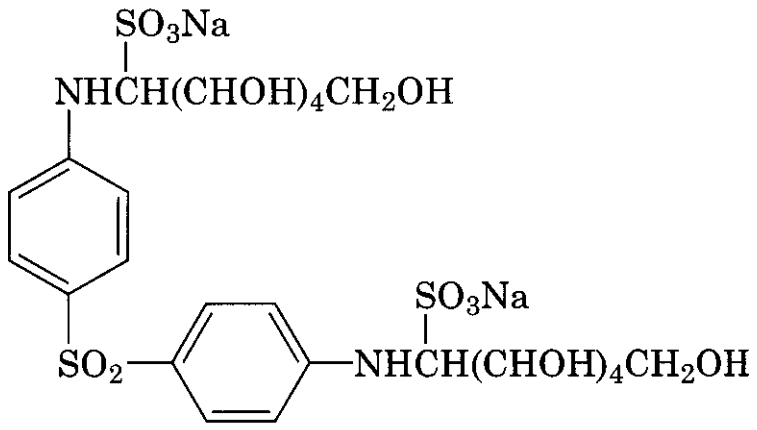
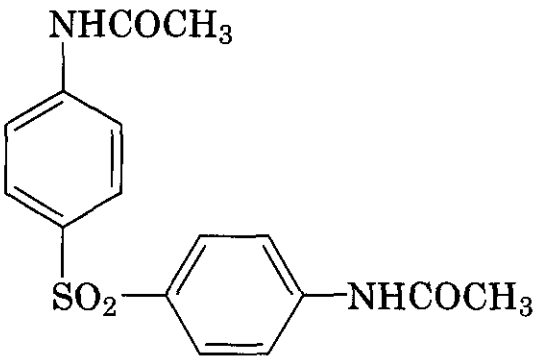


(61)

the possibility of treatment of mycobacterial infections with sulfones led to the demonstration of the favorable effect of promin in rat leprosy (249). This was soon followed by the successful treatment of leprosy patients, first with promin and later with dapsone itself. Since then dapsone has remained the mainstay for the treatment of all forms of human leprosy (250). It has now been shown that *Mycobacterium leprae* is unusually sensitive to dapsone (251) and that its growth can be inhibited by very low concentration of the latter.

An important advance in the use of dapsone took place with the demonstration that *N*,*N*′-diacyl derivatives and certain Schiff bases of dapsone are prodrugs and have a repository effect and release dapsone slowly; *N*,*N*′-diacetamidodiphenylsulfone (acedapsone, 62) and the Schiff base DSBA (63) are particularly useful as repository forms (252). After a single intramuscular injection of 225 mg of acedapsone, a therapeutic level of DDS (20–25 μg/mL) is maintained in the blood for as long as 60–68 days, and it is useful in the prophylaxis and treatment of leprosy.

To improve on the antimycobacterial and antiprotozoal (especially antimalarial) activi-



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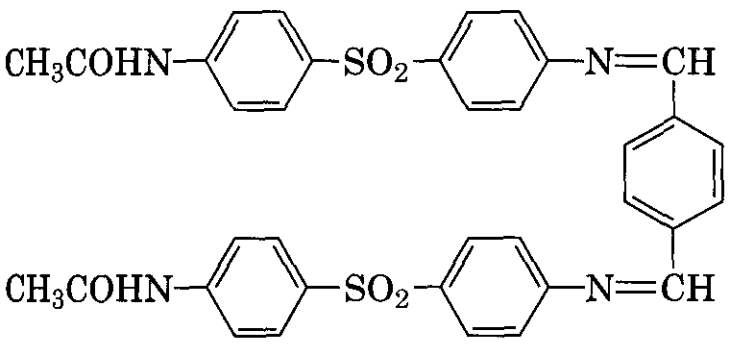


(63)

ties of dapsone a variety of substituted sulfones have been prepared.

Overall, none of the substituted diaminodiphenylsulfones was significantly more active *in* vivo than DDS to offer much advantage **(253).** Even after almost 50 years of use in clinical practice, DDS alone or in combination with other drugs (as in multidrug therapy) continues to be the mainstay of chemotherapy of leprosy.

3.1.6 Structure-Activity Relationship

3.1.6.1 Structure and Biological Activity. Although the story of sulfonamides started with the discovery of their antimicrobial action, susequent studies established their usefulness as carbonic anhydrase inhibitors, diuretics (saluretics), and antidiabetics (insulin-releasers), and more recently also as endothelin antagonists. Compounds with each type of action possess certain specific common structural features. The present discussion, however, is restricted only to the antimicrobial sulfonamides and sulfones, characterized by their ability to inhibit the de novo biosynthesis of folic acid by competing with PABA for 7,8-dihydro-6-hydroxymethylpterin pyrophosphate at the active site of dihydropteroate synthase (254–256).

3.1.6.1.1 Sulfonamides. Because sulfanilamide (45) is a rather small molecule and there are not too many variations that can be carried out without changing the basic nucleus, the following generalizations regarding structure-activity relationships were arrived at quite early in the development of sulfonamides, which guided the subsequent work on molecular modification, and these generalizations still hold:

1. The amino and sulfonyl radicals on the benzene ring should be in 1,4-disposition for activity; the amino group should be unsubstituted or have a substituent that is removed readily in vivo.
2. Replacement of the benzene ring by other ring systems, or the introduction of additional substituents on it, decreases or abolishes the activity.
3. Exchange of the SO_2NH_2 by $SO_2C_6H_4$-*p*-NH_2 retains the activity, whereas exchange by $CONH_2$ by COC_6H_4-*p*-NH_2 markedly reduces the activity.
4. N^1-Monosubstitution results in more active compounds with greatly modified pharmacokinetic properties. N^1-Disubstitution in general leads to inactive compounds.
5. The N^1-substitution should be such that its pK_a value would approximate the physiological pH.

The presence of a *p*-aminobenzensulfonyl radical thus seems inviolate for maintaining good activity and practically all the attention was focused on N^1-substituents. These substituents seem to affect mainly the physicochemical and the pharmacokinetic characteristics of the drugs.

3.1.6.1.2 Sulfones. The following broad generalizations hold for the SAR of sulfones:

1. One *p*-aminophenylsulfonyl residue is essential for activity; the amino group in this moiety should be unsubstituted or have a substituent that is removed readily in vivo.
2. The second benzene ring should preferably have small substituents that will make this

ring electron rich (such as CH_3, OCH_3, OH, NH_2, NHC_2H_5); p-substitution is most favorable for activity.

3. Replacement of the second phenyl ring by heterocycles does not improve the activity.

Diaminodiphenyl sulfone (DDS) has retained its preeminent position, even after 50 years of use.

3.1.6.2 Quantitative Sar. Studies to find a correlation between physicochemical properties and bacteriostatic activity of sulfonamides have been pursued almost since their discovery. The substituents that attracted the attention of investigators quite early were the amino and the sulfonamido groups in the molecule, and several groups of investigators almost simultaneously noted a correlation between the bacteriostalic activity and degree of ionization of sulfonamides. The primary amino group in sulfonamides apparently plays a vital part in producing bacteriostasis, given that any substituent on it causes complete loss of activity. Seydel et al. (257, 258), from a study of infrared (IR) spectra and activity of a number of sulfonamides, concluded that the amount of negative charge on the aromatic amino group is important for the activity. However, variation in activity within a series of compounds could not be related to a change in base strength, given that all the active sulfonamides (and sulfones) have a basic dissociation constant of about 2, which is close to that of PABA. Foernzler and Martin (259) computed the electronic characteristics of a series of 50 sulfonamides by the combination of linear atomic and molecular orbital methods (LCAO-MO methods) and found that the electronic charge on the p-amino group did not vary significantly with a change in the N^1-substituent.

Thus attention was focused mainly on the N^1-acidic dissociation values, which vary widely from about 3 to 11. Fox and Rose (260) noted that sulfathiazole and sulfadiazine were about 600 times as active as sulfanilamide against a variety of microorganisms, and that approximately 600 times as much PABA was required to antagonize their action as to antagonize the action of sulfanilamide; however, the same amount of PABA was required to antagonize the MIC of each drug. This suggested that the active species in both cases were similar, and that the increase in bacteriostatic activity was ascribed to the presence of a larger proportion of the drug in an active (ionized)form. They found that the concentration of the ionized form of each drug at the minimum effective concentration was of the same order. Thus if only the ionized fraction at pH 7 was considered instead of the total concentration, the PABA/drug ratio was reduced to 1:1.6–6.4. They also observed that with a 10-fold increase in ionization of sulfanilamide on altering the pH from 6.8 to 7.8, there was an eightfold increase in bacteriostatic activity. On the basis of these observations, Fox and Rose suggested that only the ionized fraction of the MIC is responsible for the antibacterial action. Schmelkes et al. (261) also noted the effect of pH of the culture medium on the MIC of sulfonamides and suggested that the active species in a sulfonamide solution is anionic.

Bell and Roblin (262), in an extensive study of the relationship between the pK_a of a series of sulfonamides and their in vitro antibacterial activity against E. coli, found that the plot of log 1/MIC against pK_a was a parabolic curve, and that the highest point of the curve lay between pK_a values of 6 and 7.4; the maximal activity was thus observed in compounds whose pK_a approximated the physiological pH. Because the pK_a values are related to the nature of the N^1-substituent, the investigators emphasized the value of this relationship for predicting the MIC of new sulfonamides. The pK_a of most of the active sulfonamides discovered since then, and particularly of the long-acting ones, falls in this range (Table 13.2). Bell and Roblin correlated Woods and Fildes's hypothesis regarding the structural similarity of a metabolite and its antagonist with the observed facts of ionization. They emphasized the need of polarization of the sulfonyl group of active sulfonamides, so as to resemble as closely as possible the geometric and electronic characteristics of the *p*-aminobenzoate ion, and postulated the "the more negative the SO_2 group of N^1-substituted sulfonamides, the more bacteriostatic the compound will be." The acid dissociation constants were considered to be an indirect measure of the negative character of the SO_2 group. The hy-

pothesis of Bell and Roblin stated that the un-ionized molecules had a bacteriostatic activity, too, although weaker than that of the ionized form. Furthermore, it was supposed that increasing the acidity of a compound decreased the negativity of the SO_2 group, thus reducing the bacteriostatic activity of the charged and uncharged molecules.

Cowles (263) and Brueckner (264), in a study of the effect of pH of the medium on the antibacterial activity of sulfonamides, found that the activity increased with increase in pH of the medium only up to the point at which the ionization of the drug was about 50%, and then it decreased. Brueckner assumed different intra- and extracellular pH values to explain the observed effects. Cowles suggested that the sulfonamides penetrate the bacterial cell in the un-ionized form, but once inside the cell, the bacteriostatic action is attributed to the ionized form. Hence for maximum activity, the compounds should have a pK_a value that gives the proper balance between the intrinsic activity and penetration; the half-dissociated state appeared to present the best compromise between transport and activity. This provided an alternative explanation for the parabolic relationship observed by Bell and Roblin between pK_a and MIC.

Seydel et al. (257, 265, 266) and Cammarata and Allen (267) have cited examples of active sulfonamides whose pK_a values lie outside the optimal limits given by Bell and Roblin, and showed that if a small homologous series is used, a linear relationship of the pK_a to the MIC is obtained.

Seydel and associates in a study of sulfanilides and N^1-(3-pyridyl)-sulfanilamides extrapolated the electron density on the 1-NH group from the study of IR and NMR data and Hammett sigma values of the parent anilines and have correlated the data with the MIC against E. *coli*. Anilines were used for studying the IR spectra because they could be dissolved in nonpolar solvents, thus giving more valid data; this was not possible with sulfanilamides because of low solubility in such solvents. Seydel (268) and Garrett et al. (269) found an approximately linear relationship between bacteriostatic activity, Hammett sigma value, and electron density of the N^1-nitrogen of a group of m- and p-substituted sulfanilides and emphasized the possibility of predicting the in vitro antibacterial activity of sulfanilamides by use of this relationship. Later, Seydel (270) included in this study 3-sulfapyridines, carried out regression analyses of the data, and obtained a very acceptable correlation coefficient.

The functional relationship between acid dissociation constant and the activity of sulfonamides has not been questioned since the investigations just cited were published. This, however, does not imply that the ions of different sulfonamides are equally active; other factors also have an influence and account for the observed differences in activity of different sulfonamides, such as affinity for the concerned enzyme. The pK_a value is related to solubility, distribution and partition coefficients, permeability across membranes, protein binding, tubular secretion, and reabsorption in the kidneys.

Fujita and Hansch (271), in a multiparameter linear free-energy approach, correlated the pK_a, hydrophobicity constant, and Hammett sigma values of a series of sulfanilides and N^1-benzoyl and N^1-heterocyclic sulfanilamides with their MIC data against Gram-positive and Gram-negative organisms and their protein-binding capacity. They devised suitable equations by regression analyses for the meta- and para-substituted compounds; the correlation for the para-substituted compounds was rather poor. The hydrophobicity of the compounds was found to play a definite role in the activity. It was shown that keeping the lipophilicity of the substituents unchanged, the logarithmic plot of activity against the dissociation constant gives two straight lines with opposite slopes, the point of intersection of which corresponds to the maximal activity for a series of sulfanilamides. They suggested the optimal values of the dissociation constant and hydrophobicity for maximum activity against the organisms studied.

Yamazaki et al. (272), in their study of the relationship between antibacterial activity and pK_a of 14 N^1-heterocyclic sulfanilamides, considered separately the activities of the compounds in terms of the concentrations of their ionized and un-ionized forms and their total concentrations in the culture medium.

They found that, when the relationship between pK_a and ions is considered, it is linear for ionized and un-ionized states, giving two lines having opposite slopes and intersecting each other. The point of intersection corresponds to the pH of the culture medium. They found the pK_a for optimal activity to be between *6.6* and 7.4.

In these studies it was noticed that some of the sulfonamides had lower antibacterial activity than expected, possibly because of their poor permeation. To define the role of permeability in the antibacterial activity of sulfonamides, Seydel et al. (273) extended these investigations to cell-free folate synthesizing systems and correlated the inhibitory activity of these compounds on this enzyme system and on the intact organisms to their pK_a, Hammett sigma, chemical shift, and π values. The rate-determining steps for sulfonamide action in the cell-free system and a whole-cell system were found to have similar substituent dependencies. From a comparison of the linear free-energy relationships obtained in the two systems, they suggested that the observed parabolic dependency of the antibacterial activity indicates that it is not the extracellular ionic concentration, which, in turn, is limited by the permeation of un-ionized compounds, thus supporting Cowles and Brueckner's postulates (loc. cit.). They concluded that the lipophilic factors are not important in the cell-free system or for in vitro antibacterial activity when permeability is not limited by ionization.

Intensive subsequent work in this field over the last four decades has fully supported the views expressed quite early in the development of sulfonamides of the predominant role of ionization for their antibacterial activity, and that the degree of ionization determines the antibacterial activity, given that the ionized form is more potent than the un-ionized form.

3.1.6.2.1 Sulfones. Ever since the discovery of the antimycobacterial activity of sulfones in the 1940s and that they share a common biological mode of action with sulfonamides (competitive antagonism of PABA), the question of their structural similarity with sulfonamides, which enables them to inhibit dihydropteroate synthase, has attracted much attention. It was realized quite early in these studies that as with sulfonamides, $4\text{-}NH_2\text{-}C_6H_4\text{-}SO_2$ was inviolate for optimal activity and the substituents in the second phenyl could modulate the activity. A number of QSAR studies have been reported (274–282) on 4-aminodiphenylsulfones to analyze the contribution of these substituents to the biological activity using linear free-energy, molecular modeling, and conformational analysis methods. It has been shown that electronic and steric effects have the decisive role both on binding to the enzyme and the overall biological activity. The electronic effects were rationalized in terms of electronic charge perturbations that are transmitted from the multisubstituted aryl ring to the essential structural moiety, $4\text{-}NH_2\text{-}C_6H_4\text{-}SO_2$, through the SO_2 group mainly through hyperconjugation (279). In conformational analysis using the MINDO semiemperical molecular orbital method, it was found that 4-aminodiphenylsulfones show multiple conformational energy minima, mainly attributed to the torsional freedom of the sulfur-carbon bond of the substituted aryl ring with the $4\text{-}NH_2\text{-}C_6H_4\text{-}SO_2$. The highly active derivatives were in general shown to be less flexible and inhibition potency increased as entropy decreased (280, 281); a butterfly-type structure (Fig. 13.5) was considered to best represent the active conformations.

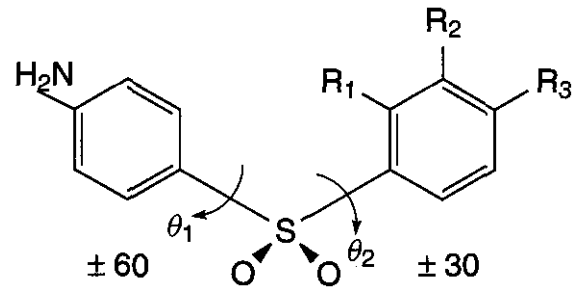


Figure 13.5. Proposed active conformation of 4-aminodiphenylsulfones.

3.1.6.3 Water/Lipid ***Solubility.*** The clinically used sulfonamides being weak acids are, in general, soluble in basic aqueous solutions. *As* the pH is lowered, the solubility of their N^1-substituted sulfonamides decreases, usually reaching a minimum in the pH range of 3 to 5. This minimum corresponds to the solubility of the molecular species in water (Table

13.2). With a further decrease in pH corresponding to that of a moderately strong acid, the sulfa drugs dissolve as cations.

The solubility of sulfonamides is of clinical and toxicological significance because damage to kidneys is caused by crystallization of sulfonamides or their N^4-acetyl derivatives. Their solubility in the pH range of urine (i.e., pH 5.5–6.5) is thus of practical interest. One of the significant advances in the first phase of sulfonamide research was the development of compounds with greater water solubility, such as sulfisoxazole, which helped to overcome the problem of crystallization in the kidney of earlier sulfonamides. However, apart from the solubility of the parent compounds, the solubility of their N^4-acetyl derivatives, which are the main metabolic products, is of great importance because these are generally less soluble than the parent compounds. For example, sulfathiazole, which itself is unlikely to be precipitated, is metabolized to its N^1-acetyl derivative, which has a poor solubility that is likely to lead to its crystallization in the kidney. The solubility of sulfonamides and their principal metabolites in aqueous media, particularly in buffered solutions and body fluids, therefore, has been the subject of many studies aimed at enhancing our understanding of their behavior in clinical situations (284,285).

An important factor affecting the chemotherapeutic activity of sulfonamides and their in *vivo* transport is lipophilicity of the undissociated molecule. The partition coefficients measured in solvents of different dielectric constants have been used to determine the lipid solubility and hydrophobicity constant (269–284). Chromatographic R_1 values in a number of thin-layer chromatography systems have also been used as an expression of the lipophilic character of sulfonamides and found to correspond well with the Hansch values in an isobutyl alcohol-water system (286).

Table 13.2 gives the percentage of various sulfonamides passing from aqueous phase into ethylene chloride as determined by Rieder (284). Lipid solubility of different sulfonamides varies over a considerable range. These differences unquestionably influence their pharmacokinetics and antibacterial activity. It has been noted by Rieder (284) that long-acting sulfonamides with a high tubular reabsorption are generally distinguished by a high degree of lipid solubility. The antibacterial activity and the half-life are also related to lipid solubility. Although a precise relationship between these factors has not been established, it has been shown in general, that as the lipid solubility increases, so does the half-life and in *vitro* activity against E. *coli*.

3.1.6.4 Protein Binding. A particularly important role in the action of sulfonamides is played by their binding to proteins. Protein binding, in general, blocks the availability of sulfonamides as of many other drugs (the bound drug is chemotherapeutically inactive), and reduces their metabolism by the liver. The binding is reversible; thus the active free form is liberated as its level in the blood is gradually lowered. The sulfonamide concentration in other body fluids is also dependent on its protein binding. Thus the unbound fraction of the drug in the plasma seems to be significant for activity, toxicity, and metabolism, whereas protein binding appears to modulate the availability of the drug and its half-life. The manner and extent of binding of sulfonamides has been the subject of many studies (284, 287–289), and the important characteristics of the binding are now reasonably clear. The binding affinity of different sulfonamides varies widely with their structure (Table 13.2) as also with. the animal species and the physiological status of the animal (284, 290). In plasma the drug binds predominantly to the albumin fraction. The binding is weak (4–5 kcal) and is easily reversed by dilution. It appears to be predominantly hydrophobic, with ionic binding being relatively less significant (271,289). Thus the structural features that favor binding are the same as those that increase lipophilicity, such as the presence of alkyl, alkoxy, or arly groups (261, 287, 291). N^4-Acetyl derivatives are more strongly bound than the parent drugs. Introduction of hydroxyl or amino groups decreases protein binding, and glucuronidation almost abolishes it. Seydel (292), in a study of the effect of the nature and position of substituents on protein binding and lipid solubility, has shown that among isomers, *ortho*-substituted compounds have the lowest protein binding. This would indicate that steric factors have a role in protein binding and that N^1-nitrogen atom of the sulfonamide

is involved. The binding seems to take place with the basic centers of arginine, lysine, and histidine in the proteins (284). The locus of binding of several sulfonamides to serum albumin has been shown by high resolution NMR spectral studies to involve the benzene ring more than the heterocycle (293).

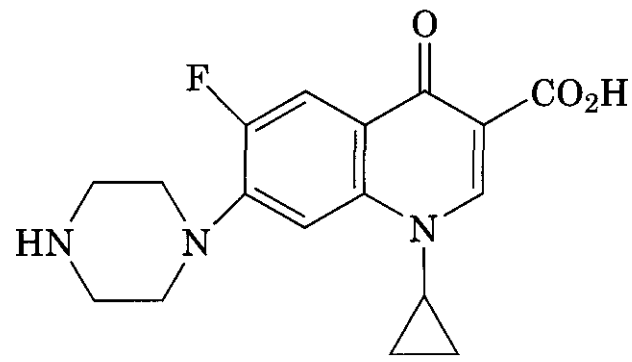
There have been attempts to establish correlations between physicochemical properties of sulfonamides, their protein binding, and their biological activity. Martin (294) established a functional relationship between excretion and distribution and binding to albumin, and Kruger-Thiemer et al. (288) derived a mathematical relationship. Moriguchi et al. (295) observed a parabolic relationship between protein binding and in vitro bacteriostatic activity in a series of sulfonamides, and suggested that too strong an affinity between sulfonamides and proteins would prevent them from reaching their site of action in bacteria; with too low an affinity, they would not be able to bind effectively with enzyme proteins to cause bacteriostasis, assuming that affinity for enzyme proteins is paralleled by affinity to bacterial proteins. In a multiparameter study of a series of N^1-heterocyclicsulfonamides, Fujita and Hansch (271) considered that in the free state, sulfonamides exist as two different species, neutral and ionized, whereas in the bound state they exist in only one form. They developed suitable equations by regression analysis and showed that for the series of sulfonamides of closely related structure, whose pK_a value does not vary appreciably, the binding is governed mainly by the N^1-substituent, which supported the earlier results (287).

The implications of protein binding for chemotherapeutic activity are not fully understood. The factors favoring protein binding are also those that would favor transport across membrances, tubular reabsoption, and increased binding to enzyme protein. N^1-Acetyl derivatives are more strongly bound to proteins and yet are better excreted. No universally applicable relationship has been found between half-life of sulfonamides and protein binding, although it has been established in general that protein binding modulates bioavailability and prolongs the half-life of sulfonamides, as of other drugs.

3.2 Quinolones

The 4-quinolones have a number of advantages over other classes of antibacterial agents. They are effective against many organisms, well-absorbed orally, well-distributed in tissues, and they have relatively long serum half-lives and minimal toxicity. Because of deep-tissue and cell penetration, they are useful for urinary tract infections, prostatitis, infections of the skin and bones, and penicillan-resistant sexually transmitted diseases.

3.2.1 introduction. The first quinolone of commercial importance, nalidixic acid (**64**), was prepared in 1962 by Lesher (298). Norfloxacin, a fluoroquinolone with a broad spectrum of antibacterial activity, was patented in 1978 by Irikura (299). Chemical modifications based on their structures have since led to thousands of new analogs, some of which have significantly improved effectiveness. The quinolone antibacterial group contains 4-quinoline-3-carboxylic acids such as ciprofloxacin (**65**), lomefloxacin (**66**), norfloxacin



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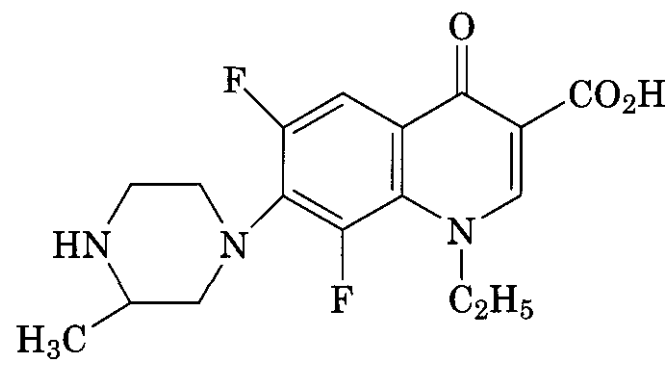

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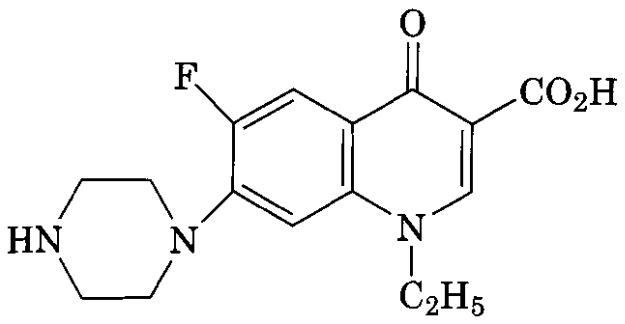
(**67**), pefloxacin (**68**), sparfloxacin (**69**), gatifloxacin (**70**), and moxifloxacin (**71**); and the tricyclic analog ofloxacin (**72**), for which the



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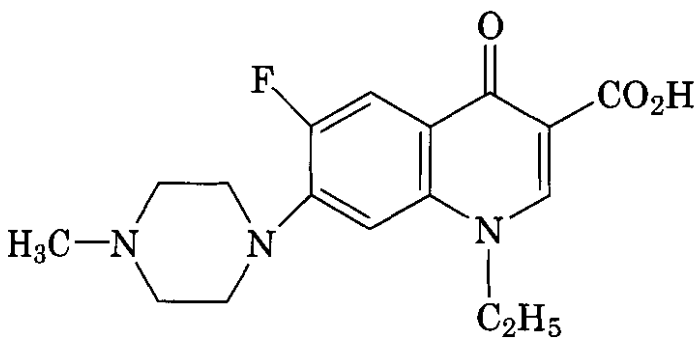

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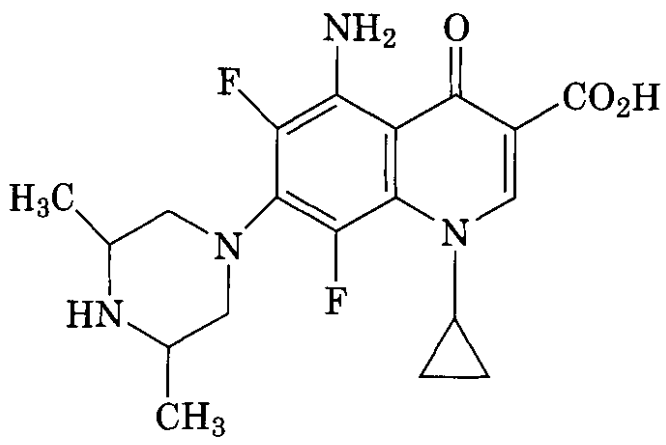

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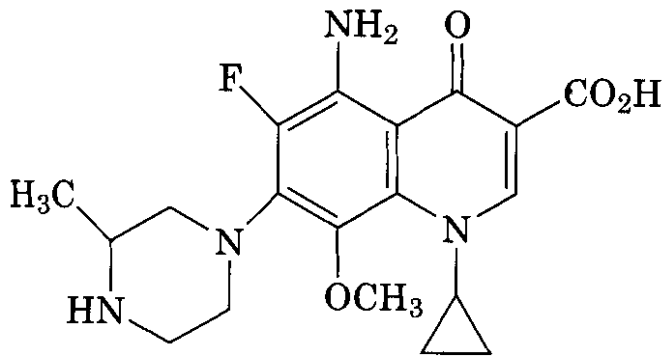

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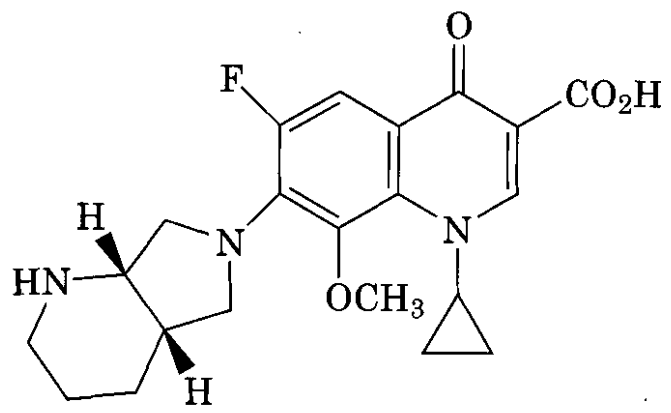
(*S*)-enantiomer (levofloxacin)is marketed separately. It also contains 1,8-naphthyridine-4-one-3-carboxylic acids such as nalidixic acid (enoxacin, **73**), and trovafloxacin/alatrofloxacin (**74**), as well as cinnolin-4-one-3-carboxylic acid (cinoxacin, 75).



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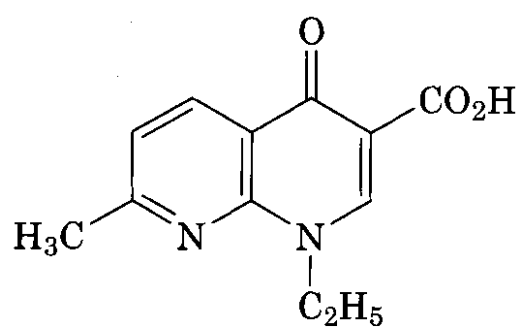
3.2.2 Antibacterial Activity. Fluoroquinolones are important broad-spectrum antibacterial agents. All of them are active against species such as *Enterobacter cloacea, Proteus*



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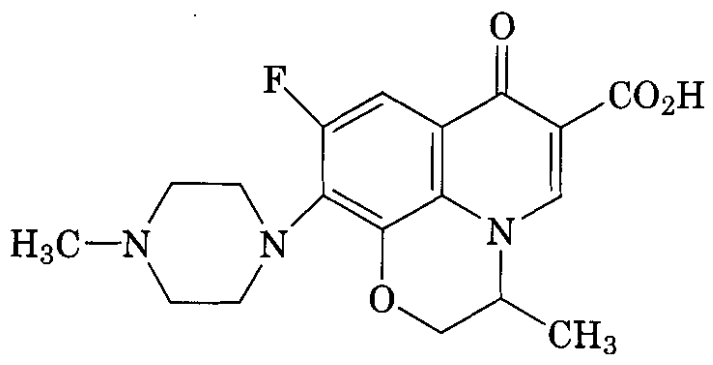

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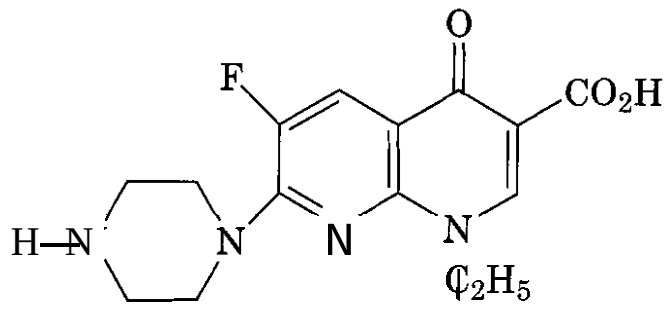

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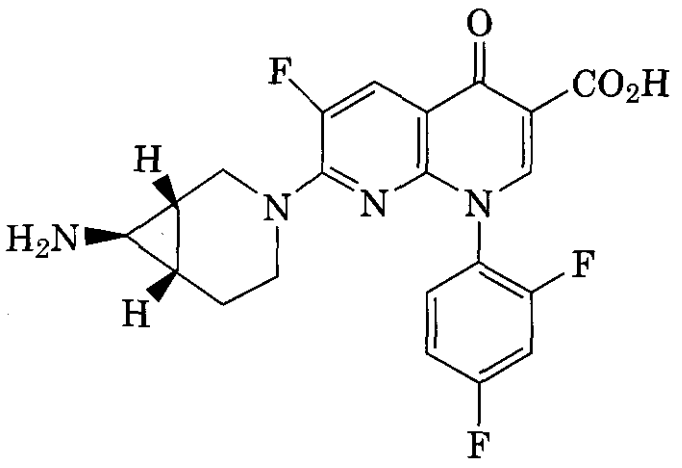
mirabilis, Morganella morganii, and *Staphylococcus epidermis.* Furthermore, most of



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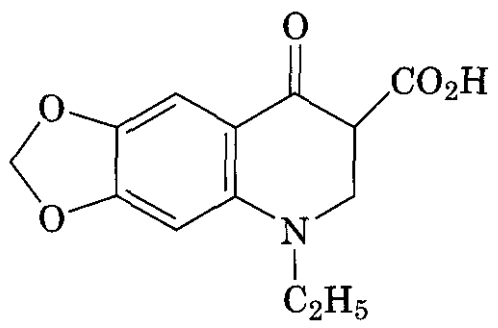


(75)

them are active against Hemophilus *influenzae*, Providencia rettgeri, Pseudomonas aeruginosa, Serratia marcesans, Staphylococcus aureus, Enterococcus fecalis, Mycoplasma pneumonia, Chlamidia pneumonia, and *Nis*seria gonorrhoea. When anthrax infections resulting from terrorist activities in the United States emerged in October of 2001, ciprofloxacin became the principal drug for treating this bacterium. Although other antibacterial agents such as penicillin and doxycycline are active against anthrax infections, ciprofloxacin is effective against more strains of anthrax. Clinical indications for the quinolones and fluoroquinolones are given in Table 13.4.

3.2.3 Structure-Activity Relationship. The minimum pharmacophore required for significant antibacterial activity consists of the 4-pyridone ring with a 3-carboxylic acid group (Fig. 13.6). Reduction of the 2,3-double bond eliminates activity. Most of the highly active quinolones have a fluorine atom at C6 (fluoroquinolones) because it increases lipophilicity, which facilitates penetration into cells. Many analogs have piperazino groups on C7 because they broaden the spectrum, especially to include Gram-negative organisms such as Pseudomonas aeruginosa; however, they also increase affinity for the GABA receptor, which contributes to CNS side effects. This receptor binding affinity can be reduced by adding a methyl or ethyl group to the piperazine or by placing a bulky substituent on N1 (300). Substitutents on the piperazine ring can shift excretion of the compound from kidney to liver and they extend its half-life. Quinolones with greater amounts of liver metabolism and biliary excretion are useful in patients with impaired renal function (301). Replacement of C8 by nitrogen, to give a naphthyridine (e.g., enoxacin and trovafloxacin) increases bioavilability (302), whereas a methoxy group in place of hydrogen or fluorine on C8 provides greater stability to ultraviolet light and less phototoxicity in mice (303). Compounds such

Table 13.4 Therapeutic Indications for Quinolones

Compound	Indications
Nalidixic acid	Urinary tract infections
Cinoxacin	Urinary tract infections
Ciprofloxacin	Acute sinusitis, lower respiratory tract infections, nosocomial pneumonia, skin infections, bone/joint infections, urinary tract infections, gonorrhea, anthrax
Enoxacin	Gonorrhea, urinary tract infections
Gatifloxacin	Chronic bronchitis, acute sinusitis, urinary tract infections, pyelonephritis
Levofloxacin	Chronic bronchitis, acute sinusitis, urinary tract infections, pneumonia, skin infections
Norfloxacin	Urinary tract infections, gonorrhoea, chronic bacterial prostatitis
Ofloxacin	Liver cirrhosis, epididmytis, gonorrhea, chlamydia
Sparfloxacin	Pneumonia, chronic bronchitis
Trovofloxacin/alatrofloxacin	Gynecological and pelvic infections

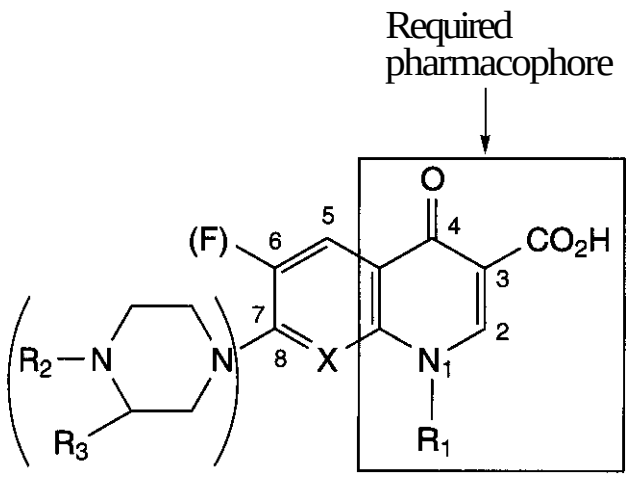


Figure 13.6. Quinolone pharmacophore and substituents.

as sparfloxacin (**69**), with an amino group at C5, also have reduced phototoxicity.

Isosteric replacement of nitrogen for C2 provides cinnolines, such as cinoxacin (**75**), which have good antibacterial activity and pharmacokinetic properties. Isomeric naphthyridines including the 1,5 and 1,6 isomers retain antibacterial activity. Compounds with ring fusions at 1,8 (ofloxacin, 72); 5,6; and 7,8 also are effective antibacterials.

The stereochemistry of the methyl group in the third ring of ofloxacin is important to antibacterial activity. The (*S*)-enantiomer (marketed separately as levofloxacin) is 10-fold more potent than the (*R*)-enantiomer and it is less selective for topoisomerase II (304, 305). Interestingly, the corresponding methylene analog (which flattens the ring to allow stronger intercalation between base pairs) has 20-fold greater topoisomerase activity than that of either of the methyl enantiomers (306).

Fluorine at C6 enhances inhibition of DNA gyrase and provides activity against Staphylococci, whereas a piperazine substituent at C7 affords the best activity against Gram-negative bacteria. Addition of a second fluorine at C8 increases absorption and half-life. Ring alkylation improves gram-posotive potency and half-life. A number of newer quinolones such as ciprofloxacin, gatifloxacin (307), and moxifloxacin (308) have cyclopropyl sunstituents at N1. This substituent, or the combination of an amine at C5 and fluorine at C8 (sparfloxacin), increases potency against mycoplasma and chlamydia species.

Earlier antibacterial quinolones such as nalidixic acid and cinoxacin have only the 3-carboxylic acid as an ionizable group. Its relatively high pK_a in the range of 5.4 to 6.4 is thought to result from an acid-weakening hydrogen bond with the 4-carbonyl group (309). More recent quinolones with a piperazine or other substituent with a basic nitrogen at C7 have a second pK_a in the range of 8.1 to 9.3. Consequently, significant fractions of these compounds exist as zwitterions at physiological pH values. Decreased solubility in urine of higher pH presents a potential problem for these compounds.

The 4-carbonyl group and 3-carboxylic acid functionalities of quinolones provide an excellent site for chelation with divalent of trivalent metals. Quinolones can form 1:1, 2:1, or 3:1 chelates, depending on the particular metal ion, relative concentration of the quinalone, and the pH. The relative insolubility of these chelates causes incompatibilities with antacids (Ca^{2+}, Mg^{2+}, and Bi^{3+}), hematinics (Fe^{2+}), and mineral supplements (Zn^{2+}).

It has been noted that, if just 20 different substituents are taken two at a time for the seven available positions on quinolones, there would be 84,000 possible compounds to synthesize and test (310). The use of computer-assisted quantitative structure-activity relationships has helped narrow the search for new compounds of this type. Koga and coworkers developed an equation that related the potency of compounds against E. coli (MIC) to the length of the substituent on N1, the size of substituents on C8, an enhancement factor for C7 substituents, and a factor for the detrimental effect of attachment of C7 substituents by an NCO function (311).

3.2.4 Mechanism of Action. The mechanism of action of quinolones is prevention of the detachment of gyrase from DNA. This enzyme is the bacterial form of topoisomerase, which allows the relaxation of supercoiled DNA that is required for normal transcription. Bacterial gyrase is different enough from mammalian topoisomerase so that 78 of 90 quinolones tested were selective only to bacteria. Those compounds with poorer selectivity had a propyl group at N1 and two fluorines at C6 and C8 (312). The antibacterial activity of quinolones is antagonized by chloramphenicol and rifampin, which suggests that protein synthesis is required for killing (313). This

Table 13.5 Pharmacokinetic Properties of Quinolones[a]

Compound	Single Oral Dose (mg)	Peak Serum Concentration, (μg/mL)	Half-Life (h)	Protein Binding (%)	Urinary Recovery (% unchanged)
Nalidixic acid	1000	20–40	6	93–97	3
Ciprofloxacin	200	0.8	4–6	40–50	20–40
Enoxacin	200	1.0	5	40	20–40
Lomefloxacin	200	0.7	3–4	10	65
Norfloxacin	100	1.0	5	10–15	26–32
Gatifloxacin	200	2.0	7.8	20	74
Ofloxacin	200	1.5	9	32	65–80
Sparfloxacin	400	1.3	20	46	10
Trovafloxacidalatrofloxacin	100	1.0	9.1	76	6

[a]Abstracted from *Drug Facts and Comparisons.*

mechanism is consistent with apoptosis rather than necrosis. Fluoroquinolines also inhibit topoisomerase IV, an enzyme that functions in partitioning of the chromosomal DNA during bacterial cell division.

3.2.5 Microbial Resistance. Drug resistance was observed in the early clinical trials with nalidixic acid (314). One mechanism of resistance is by mutation at various locations on the gyrase gene. These mutations confer cross-resistance to all other quinlones. Another mechanism is by mutation in the genes that code for porins, which are membrane proteins by which quinolones enter Gram-negative cells. These mutations raise tolerance fourfold. Other mutations of serious concern are those that reduce membrane lipopolysaccharides to afford cross-resistance with antibacterial agents of other chemical classes (315). Considering the problem of resistance to quinolones, Moellering has concluded that, "The future viability of the quinolones will in large part depend on the ability of the medical community to use them wisely" (314). In particular, the increasing use of quinolones for oral periodental treatment could bring about an expansion of resistance to quinolones that would limit their value in treating deep-tissue infections.

3.2.6 Pharmacokinetic Properties. Some phamacokinetic properties of quinolones are listed in Table 13.5. Bioavalibility after oral administration is good, but it is substantially reduced by magnesium or aluminum antacids. Distribution to tissues is superior to that of most other drugs because there is little binding to plasma proteins. Quinolones achieve tissue-to-serum ratios of over 2 to 1, in contrast to less than one-half for β-lactams and aminoglycosides (316). Clearance is by kidneys (ofloxacin), or by liver (pefloxacin and difloxacin), or by both (norfloxacin, ciprofloxacin, enoxacin, and fleroxacin). Renal clearance correlates with creatinine clearance; consequently, reduced drug dosage is appropriate for patients having creatinine clearance under 30 mL/min. The renal clearance rates of ciprofloxacin and norfloxacin exceed the glomerular filtration rates, indicating net renal tubular secretion. Lomafloxacin has the advantage of a relatively long half-life and so can be administered once daily.

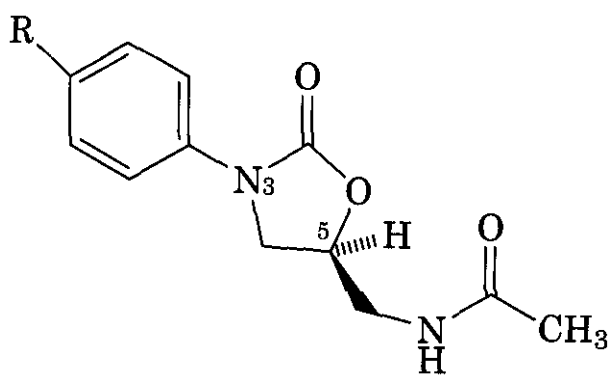
Metabolism of quinolones is primarily by glucuronide conjugation at the 3-carboxyl group and is inactivating. The piperazine ring is readily metabolized in the compounds that have this functionality, and this metabolism reduces antimicrobial activity (316). Approximately one-eighth of enoxacin is cleared as the oxo metabolite. Six metabolites with modifications in the piperazine ring were found for norfloxacin (301).

3.2.7 Adverse Reactions. Adverse reactions, which are mostly mild and reversible, include headache, dizziness, joint swelling, and leukopenia. Lomefloxacin, sparfloxacin, ofloxacin, and trovafloxacin/alatrofloxacin cause photosensitization. Quinolones can affect the central nervous system by two mechanisms: (1) accumulation of ingested xanthines, including caffeine and theophylline

(317); and (2) blockade of GABA receptors, which can cause convulsions (318). A study on proconvulsant effects of quinolones in a strain of DBA/2 mice susceptible to sound-induced seizures showed an incidence in the order pefloxacin > enoxacin > rufloxacin > norfloxacin > cinoxacin > ciprofloxacin > nalidixic acid (319). Another study based on the induction of fatal convulsions by a combination of nonsteroidal anti-inflammatory drugs and quinolones gave the following order of potency when fenbufen was the anti-inflammatory agent: enoxacin > lomefloxacin > norfloxacin, with ofloxacin and ciprofloxacin causing no deaths (320).

3.3 Oxazolidinones

3.3.1 Introduction. The oxazolidinones are a new class of synthetic antibacterial agents with activity against a broad spectrum of Gram-positive pathogens, including those resistant to currently used antibacterials (321). Following the lead of activity discovered in a series of 5-(halomethyl)-3-aryl-2-oxazolidinones against plant pathogens described in a patent issued to them in 1978, scientists at E. I. DuPout de Nemours (Wilmington, DE) observed antibacterial activity in (*R*)-5-hydroxymethyl-3-aryl-2-oxazolidinone (S-6123) against human pathogens (322). Further optimization led to the emergence of two highly active antibacterial drug candidates (322): (*S*)-[(3-(4-methylsulfinylphenyl)-2-oxo-5-oxazolidinyl)methyl]acetamide (DUP-105, 76)



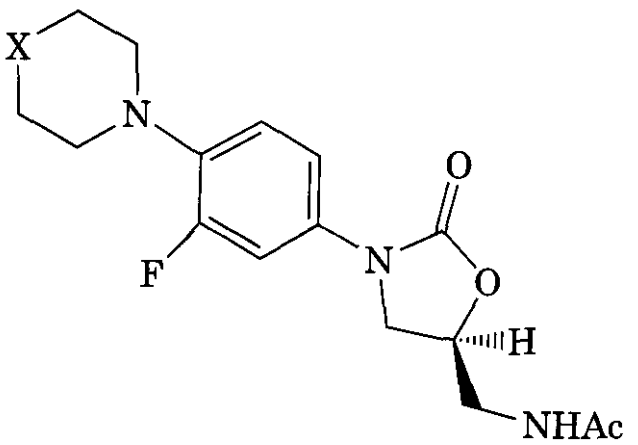
(76) DUP 105, R = CH_3SO
(77) DUP 721, R = CH_3CO

and (*S*)-[(3-(4-acetylphenyl)-2-oxo-5-oxazolidinyl)methyl]acetamide (DUP-721, 77) (323, 324), with a number of special features, including:

- activity against a number of therapeutically important multidrug resistant Gram-positive organisms (322–326)
- equally active when administered by oral or parenteral routes, showing good oral absorption (323, 327)
- a novel mechanism of action, and consequently less likely to have cross-resistance with existing antimicrobials (322, 324, 328, 329).

Furthermore, the prototype structure consisting of distinct structural units offered much scope for molecular modification.

Although the development of these two agents was subsequently discontinued consequent upon DUP-721 exhibiting toxicity in rodents (330), the special features of their antibacterial activity attracted much attention and prompted studies on oxazolidinones in a number of laboratories. Pharmacia-Upjohn (Piscataway, NJ) scientists were able to identify two drug candidates for human studies, eperezolid (PNU-100592, 78) and linezolid



(78) PNU 100592, X = $NCOCH_2OH$
(79) PNU 10076, X = O

(PNU-100766, 79) (330–332). Both compounds were active in vitro and in vivo (experimental mouse models) against methicillin-resistant S. aureus (MRSA) and S. epidermidis (MRSE), against penicillin and cephalosporin-resistant S. pneumoniae, comparing favorably with vancomycin activity, and against vancomycin-sensitive and -resistant Enterococcus spp. (VSE and VRE). Both eperezolid and linezolid went successfully through phase I human trials without any significant safety con-

cerns. Linezolid has undergone more extensive phase II and III clinical evaluation (333).

3.3.2 Linezolid. Linezolid (Zyvox, Zyvoxam, 79) is the first of this new class of oxazolidinone antibacterials to be approved in the United States, the United Kingdom, and Canada for the treatment of Gram-positive infections. Specific indications include complicated and uncomplicated skin and soft-tissue infections (SSTIs), community and clinically acquired pneumonia, and vancomycin-resistant enterococcal infections (334–337).

Synthesis. Linezolid and related oxazolidinones have been prepared through a novel asymmetric synthesis involving the reactions of N-lithiocarbamates of the appropriate aniline with (*R*)-glycidylbutyrate as the key step, and the resultant (*R*)-5-(hydroxymethyl)oxazolidinone converted to linezolid in a few steps in excellent yield and high enantiomeric purity (330, 334).

3.3.2.1 Antibacterial Activity. Linezolid has inhibitory activity against a broad range of Gram-positive bacteria, including methicillin-resistant Staphylococcus aureus (MRSA), glycopeptide-intermediate S. aureus (GISA), vancomycin-resistant enterococci (VRE), and penicillin/cephalosporin-sensitive, -intermediate, and -resistant streptococci and pneumococci (332). Linezolid is in general bacteriostatic and displays bactericidal activity only against some strains, which include some pneumococci Bacteroides fragilis and *Clostridium* perfringens (332, 333). Initial breakpoint criteria established for MICs of linezolid are ≤4 μg/mL for susceptibility and ≥16 μg/mL for resistance (334,335, 338).

3.3.2.1.1 Gram-Negative Bacteria. Linezolid is significantly less active against most Gram-negative organisms. It has only moderate activity against *Moraxella* catarrhalis, *Haemophilus influenzae*, Legionella spp., and Bordatella pertussis and practically no activity against enterobacteriaceae, Klebsiella, Proteus, and Pseudomonas aeruginosa. The few Gram-negative organisms against which linezolid has good activity are Flavobacterium meningosepticum and Pasteurella multicida with MIC values of 2 and 4 μg/mL, respectively (332,336, 337).

3.3.2.1.2 Activity Against Anaerobes. Linezolid demonstrated activity comparable to that of vancomycin (MIC: 1–2 μg/mL) against Clostridium *difficile* and *C.* perfringens. It also showed good activity against Gram-negative anaerobes including Bacteroids spp. (MIC: 4 μg/mL), Fusobacterium *nucleatum* (MIC: 0.5 μg/mL), F. meningosepticum (MIC: 2–4 μg/mL), and Prevotella spp. (MIC:1–2 μg/mL) (336, 338, 339).

3.3.2.1.3 In Vivo Antibacterial Activity. In murine bacteremia models linezolid was more active than vancomycin against methicillin-sensitive Staphylococcus aureus (MSSA), and displayed comparable activity against MRSA, though less active for MRSE. In addition linezolid displayed consistent in vivo activity against pneumococci, including against multidrug-resistant strains and vancomycin-resistant E. *faecium* but was less active than vancomycin against arninoglycoside-resistant E. *faecalis* (340).

Of particular interest is the report by Cynamon et al. (341) that oral linezolid (25, 50, and 100 mg/kg) demonstrated efficacy against M tuberculosis in a murine model, though it was somewhat less active than isoniazid. Subsequently, PNU-100480, the thiomorpholine analog of linezolid, has been reported to be as active as INH against M. tuberculosis. This provides a new lead for the design of antimycobacterial agents. Linezolid also showed promising activity in a rat experimental endocarditis model (342) and in an experimental model of acute otitis media (343) produced by a multidrug-resistant pneumococcal isolate.

3.3.2.2 Mechanism of Action. It has been established that linezolid (and related oxazolidininones) act through inhibition of the initiation phase of bacterial protein synthesis. Although the exact mode of action at the molecular level is not fully elucidated, linezolid has been reported to bind directly to a site on 23S ribosomal RNA of the bacterial 50S ribosomal subunit, thereby preventing the formation of the functional 70s-initiation complex (344, 345), formed with 30S ribosomal subunit, mRNA, initiation factors, fMet-tRNA, and 50S ribosome, which is an essential step of the bacterial translation process (Fig. 13.7). In a subsequent study it has been reported that mutations in the central loop of domain V of

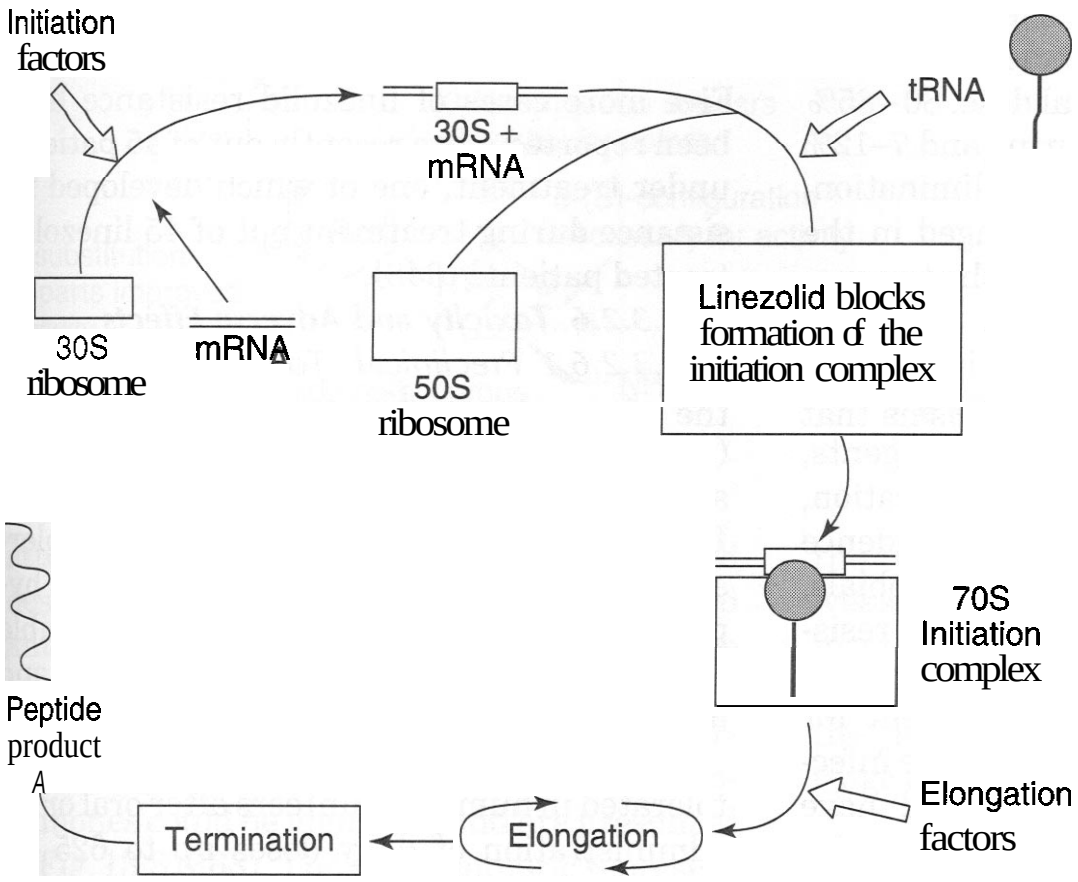


Figure 13.7. Oxazolidinones: schematic representation of the proposed mechanism of action.

23S rRNA, a component of the ribosomal *peptidyl* transferase center, conferred resistance to linezolid (346). It would thus appear that linezolid and other oxazolidinones disrupt the interaction of fMet-tRNA with the 50S subunit during formation of the preinitiation complex, and affect the translocation step.

The mechanism for the poor activity against Gram-negative bacteria is not clear. One possible reason is the operation of the selective efflux mechanism of Gram-negative bacteria.

This inhibition of the protein synthesis at an early stage is a new site/mechanism of action, and therefore there is little chance of development of cross-resistance between linezolid and other existing antibacterial agents; in fact, none has been reported so far (347).

3.3.2.2.*1 Effect On Virulence Factors.* Linezolid inhibited the expression of virulence factors from S. aureus and Streptococcus *pyogenes* in *vitro* at concentrations ranging from 12.5 to 50% of the MIC for the organism; production of a-hemolysin and coagulase by S. *aureus* and of streptolysin O and DNAase by *S. pyogenes* was markedly inhibited at these concentrations (348).

3.3.2.3 Pharmacokinefics and Metabolism. Linezolid follows a similar pharmacokinetic (PK) profile by both oral and intravenous routes of administration. It is rapidly and completely absorbed in humans *after oral* administration, with a mean absolute availability of almost 100%; the peak plasma concentration was reached in 1–2 h, with elimination half-life of 5.5 h and volume of distribution of 45 L (349,350). The circulating drug is moderately (31%) bound to plasma proteins (351). At steady state after 15 days of twice-daily administration of 375 and 625 mg of linezolid in 24 volunteers, C_{max} values were 12 and 18 μg/mL, respectively, whereas the MIC_{90} for susceptible pathogens were ≥4 μg/mL (349). With intravenous linezolid (500 or 625 mg b.i.d. for 16 doses) the minimum plasma concentrations were 3.5 and 3.8 μg/mL, with plasma concentration exceeding 4 μg/mL for ≥75% of the dosage interval, above the MIC of most of the pathogens targeted (350).

The pharmacokinetic properties of linezolid do not seem to be influenced by age. The clearance was somewhat lower in females, both young and old, but this was not of much significance and dose adjustments are not warranted on the grounds of age or gender (351).

3.3.2.3. I *Metabolism.* Linezolid appears to be metabolized by oxidation of the morpholine ring to form two inactive carboxylic acid metabolites (352). In volunteers, unchanged lin-

ezolid accounted for 90% of the circulating dose, with the major metabolite accounting for <6%. After a single 500 mg oral dose, 80–85% of linezolid was recovered in urine and 7–12% in feces over a 7-day period. In elimination, 35% of the drug appeared unchanged in the urine and 50% appeared as one of the two major inactive metabolites (352, 353).

3.3.2.4 Therapeutic Uses. The impressive antimicrobial activity against organisms that are resistant to other antimicrobial agents, high bioavailability after oral administration, favorable metabolic stability, low/no incidence of cross-resistance with other antimicrobials, low propensity to developing microbial resistance, and favorable safety profile make linezolid an attractive antimicrobial agent for the treatment of problem Gram-positive infections. Oral and intravenous linezolid have been reported to be equally effective in the treatment of plain or complicated SSTIs (354, 355), urinary tract infections (UTIs) caused by methicillin-sensitive or -resistant *Staphylococcus* spp., and pneumonia, including nosocomial and community-acquired pneumonia, which require hospitalization (356). The clinical response had a parallel favorable microbiological response. In these studies, linezolid was as effective as established treatments, including third-generation cephalosphorins, oxacillin, flucloxacillin, and clarithromycin.

3.3.2.4.1 Other Serious Gram-Positive Bacterial infections. Linezolid has also been evaluated in a variety of other infections (e.g., bacteremia, intra-abdominal abscesses, osteomyelitis, lower and upper respiratory tract infections) caused by vancomycin-resistant E. faeciumlfaecalis, MRSA or MRSE, with clinical and microbiological cures ranging from 70% to 90% (357,358). Linezolid thus appears an effective treatment option for a variety of serious, multidrug-resistant Gram-positive bacterial infections.

3.3.2.5 Microbial Resistance. There are as yet very few definitive reports of the development of resistance to linezolid, but these are bound to appear as the use increases. Development of resistance to linezolid was reported first in two patients who had E. faecium bacteremia with long-standing indwelling devices that could not be removed and had more than 4 weeks of i.v. linezolid; the MIC, which was initially 2 μg/mL dose, rose to 16 and 32 μg/mL (347), respectively, after treatment. Five more cases of linezolid resistance have been reported more recently out of 45 patients under treatment, one of which developed resistance during treatment out of 45 linezolid-treated patients (348).

3.3.2.6 Toxicity and Adverse Effects

3.3.2.6.1 Preclinical Toxicology. In dogs, the "no observed adverse effect. level" (NOAEL) of linezolid was 20 mg/kg for both sexes when administered orally for 28 days. Doses of 40 and 50 mg/kg/day were well tolerated with only mild effects. At toxic levels hypocellularity of the bone marrow (reversible decrease in white blood cells and platelets) and atrophy of the lymphoid tissue were observed.

3.3.2.6.2 Human Safety. Linezolid was well tolerated in human volunteers after oral or i.v. administration of daily doses up to 625 mg b.i.d. (349, 350, 360, 361). The most common adverse effects were nausea (5.4%), diarrhea (5.2%), or oral cavity symptoms (tongue discoloration, 2.5%; oral monilia, 2.2%). Serious drug-related adverse events (e.g., elevated liver enzymes, atrial fibrillation, or worsening renal failure) occurred in <1% of cases. In the linezolid compassionate-use trial of patients with significant, resistant Gram-positive infections, the overall adverse event rate was about 33%, of which approximately 6% were considered serious events (360). The most frequent adverse events reported were thrombocytopenia (2.6%) and dermatological reaction (2.5%) (361). Overall, at the end of therapy, linezolid was well tolerated by about 78% of patients (361).

Myelosuppression (including anemia, leukopenia, pancytopenia, and thrombocytopenia) has been reported in three patients receiving linezolid for 2 and 6 weeks, and 4 months, respectively. With discontinuation of linezolid treatment, the affected hematological parameters rose toward pretreatment levels (362). The U.S. FDA has recommended that complete blood counts should be monitored weekly, particularly in patients who receive linezolid for longer than 2 weeks.

3.3.3 New Oxazolidinones. The special features of the antibacterial activity of oxazolidinones highlighted earlier attracted much

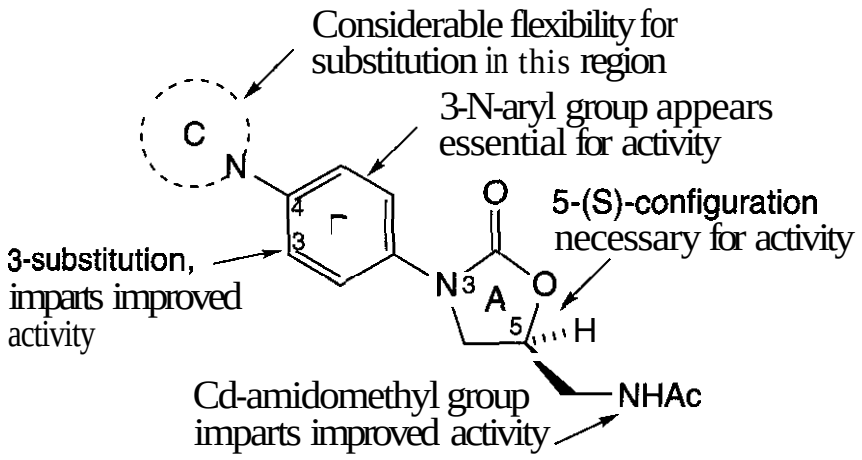
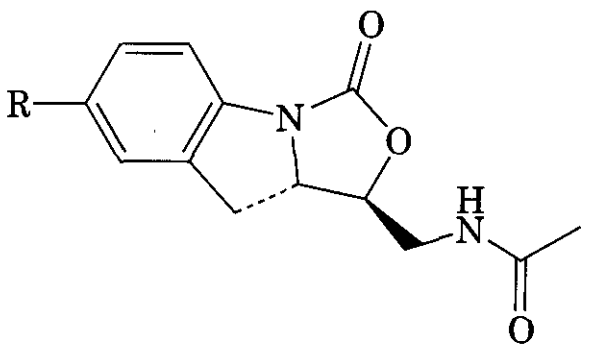


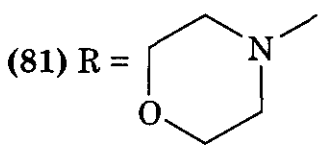
Figure 13.8. Oxazolidinones as antibacterials: important structural features.

attention. A number of reports have appeared describing new oxazolodinones with improved antibacterial activities. On the basis of the early structure-activity studies **(321,** 330), some structural features that appeared important for good antibacterial activity in oxazolidinones could be identified and are presented in Fig. **13.8(363).** The diagrammatic representation in Fig. **13.8** acted as a useful guide for subsequent studies and broadly still holds. The structure-activity relationship of the reported oxazolidinones **(364)** is presented in the context of this picture of the pharmacophore.

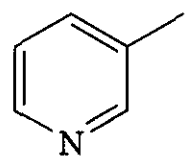
A-Ring Modification. The oxazolidinone ring structure seems essential for good antibacterial activity and relatively few successful modification of the A-ring have been reported **(364).** The tricyclic analogs having a methylene bridge between rings A and B have been reported, with the transfused *trans* homolog (80)having good activity (365, 366), although



(80) R = $COCH_3$



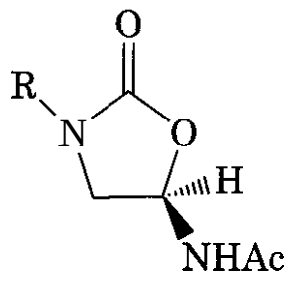
(81) R =



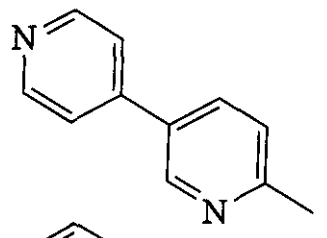
(82) R =

the exact linezolid analog **(81)** was only weakly active. The 3-pyridyl analog **(82)** is the most active compound in this series (365).

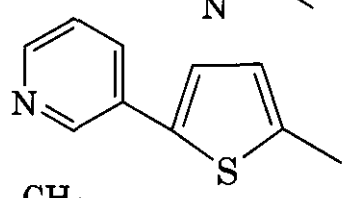
B-Ring Modification. The replacement of the B-aromatic residue by heteroaromatic rings as in **(83-87)** gave compounds that had



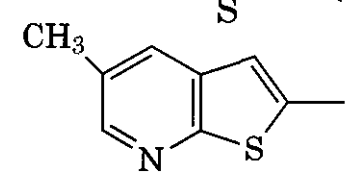
(83) R =



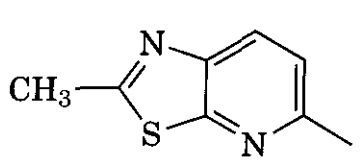
(84) R =



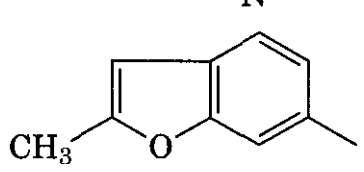
(85) R =



(86) R =



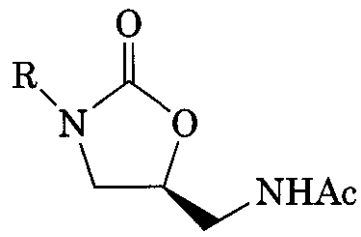
(87) R =

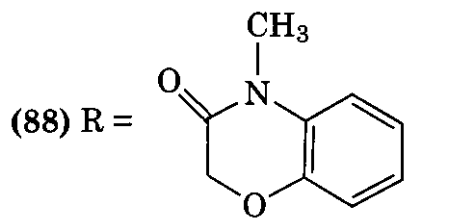


modest to good activity by subcutaneous administration **(367);** only the benzofuran analog **(87)** had good Gram-positive antibacterial activity **(368).** Thus the replacement of ring B

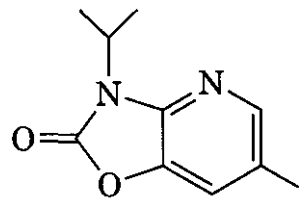
did not seem to result in any significant improvement in the antibacterial profile.

Analogs that incorporate a fused hetero ring on aromatic ring B with a lactam residue, as in (**88–92**), have been reported to exhibit good an-

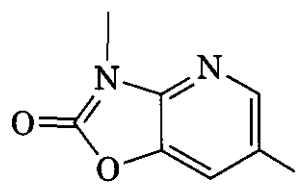




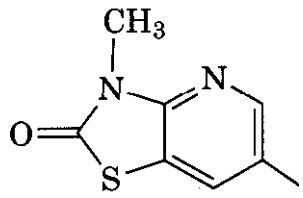
(88) R =



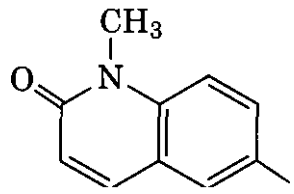
(89) R =



(90) R =



(91) R =



(92) R =

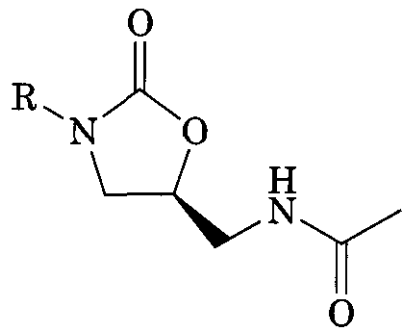
tibacterial activity. The benzoxazinone **(88)** has in vitro activity comparable to that of linezolid, with better i.v. PK parameters **(369, 370).** The benzoxazolone **(89)** and benzothiazolone **(91)** exhibit greater potency than that of linezolid (MIC range 0.25–2 μg/mL) **(371).** Other active compounds reported in this group include the oxazolopyridine analog **(15)** and the quinolone analog **(92) (372,373).**

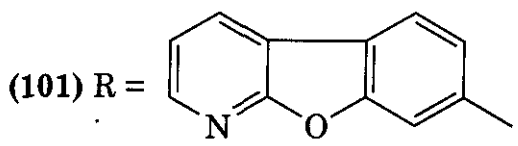
C-Ring Modification. A great deal of flexibility exists around the site C. A series of oxazolidinones in which the distal nitrogen on the aromatic ring is replaced by a five-membered heteroaromatic residue were reported to have good activity. This included the pyrrole analog **PNU-107922 (93),** the cyanopyrrole analog **(94),** and the cyanopyrazole analog **PNU-172576 (95) (374).** These analogs, apart from being more active than linezolid against Gram-positive pathogens, are also active against fastidious Gram-negative organisms; **(94)** and **(95)** showed significant activity against H. *influenzae* and M. catarrahalsis and represent the first oxazolidinoneswith potentially useful activity against Gram-negative pathogens; MIC values of <0.125–0.5 μg/mL against Gram-positive organisms and of **1–4** μg/mL against Gram-negative bacteria have been reported. These compounds are also orally active in mice. The cyanothiazole **(96)** and 5-cyanothiophene **(97)** analogs were even more potent against both Gram-positive and fastidious Gram-negative organisms (MIC > **0.125** μg/mL) and were orally active (375). Similar activity is reported for cyanoethylthiadiazole **(98) (376).** The pyrazoles **(99)** and **(100)** were also active but were less potent than other members of this group (375).

B- and C-Ring Fused Analogs. A number of analogs have been reported wherein the B and C rings are bridged by one or two atoms to form a rigid tricyclic system **(377–379).** The bridged pyrido-benzofuran **(101),** the imidazobenzoxazinyl **(102),** the pyrazinoindolyl **(103),** and pyrazinobenzoxazinyl analogs **(104)** have been reported and shown to have potent in vitro activity against both Gram-positive and Gram-negative bacteria, but have poor oral activity attributed to unfavorable pharmacokinetics.

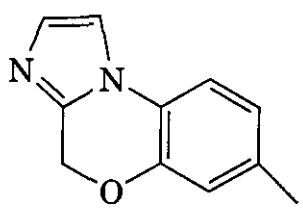
D-Ring Analogs. These oxazolidinones are quite flexible for substitution around ring C and can tolerate a ring on the distal nitrogen of the piperazinyl ring. The analogs **(105–111)** have been reported to have good in vitro activity against Gram-positive organisms and are also orally active in vivo mouse model **(380).** The isooxazolylpiperazines **(110)** have even better activity **(381).** A QSAR analysis of these compounds has been carried out and suggests that steric factors are the most important determinants of activity in this class (382).

Acetamide Side-Chain Modification. The tolerance limits for change around the acetamide side chain are rather limited. A study with the benzthiazolones **(112)** and the

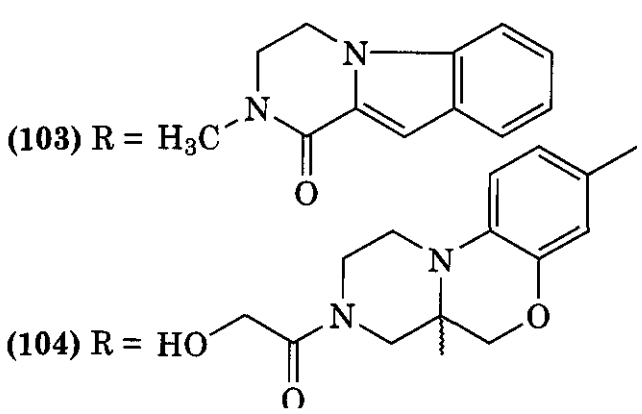




(101) R =



(102) R =

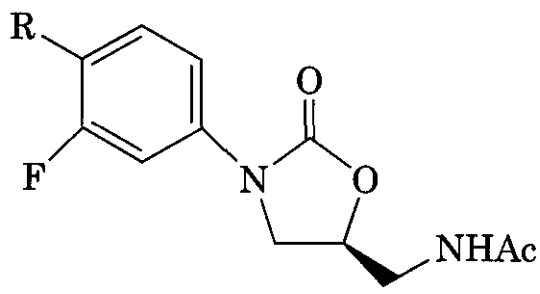


(103) R =

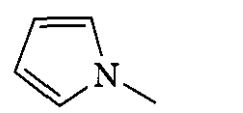
(104) R =

piperazinoindoles (**113**) showed that good antimicrobial activity was retained when R was small alkyl or alkoxy, or corresponding thioamide or thiourea derivatives; the thioamides in some cases resulted in improvement of activity. The thioamide **(113)** is fourfold more active than the corresponding carboxamide, with MIC values against staphylococci, streptococci, and enterecocci ranging from **0.12** to 0.5 μg/mL and with good activity against H. *influenzae* with MIC of 0.5–1 μg/mL **(370, 383).** Tokuyama et al., in a recent SAR study on 5-substituted oxazolidinones, have also shown that elongation of the methylene chain and conversion of the acetamido moiety into a guanidino group decreased the antibacterial activity **(384).** However, replacement of carbonyl by thiocarbonyl or thiocarbamate groups greatly enhanced the *in vitro* antibacterial activity, and some of the compounds have stronger activity than that of linezolid (384).

3.3.4 Perspective. Oxazolidinones as a new class of antibacterial agents offer some special features; their unique mode of action provides low likelihood of developing cross-resistance with existing antibacterials; excellent oral bioavailability offers good flexibility in patient



(93) R =



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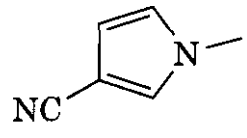

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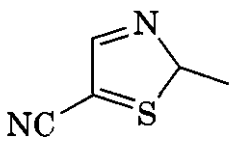

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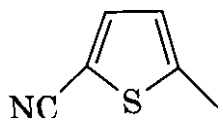

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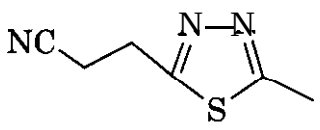

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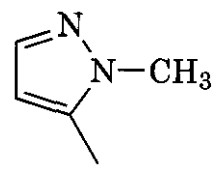

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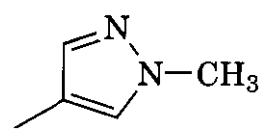
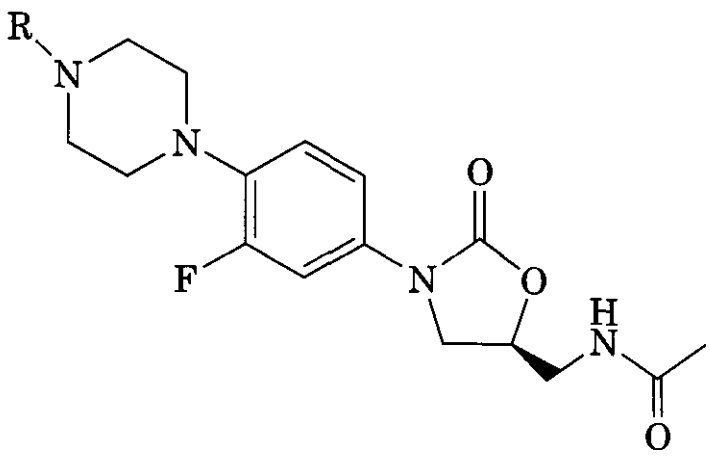
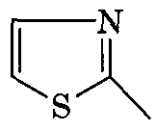


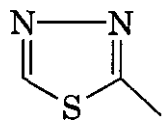
(100) R =

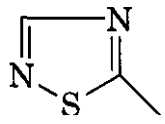
management from hospital to ambulatory setting; their chemical structure offers good scope for molecular modification to improve their therapeutic profile, reduce toxicity, and improve safety. The structure-activity studies carried out so far indicate the promise that this class holds.

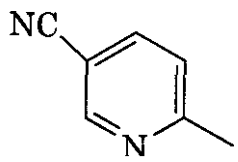
Linezolid is the first of this new class of antibacterial drugs. Its broad spectrum of activity

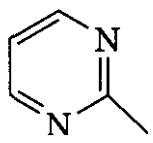


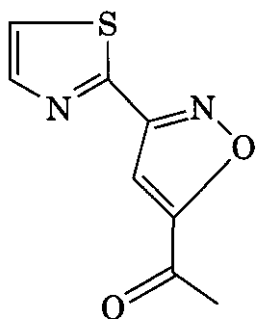
(105) R = 

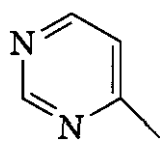
(106) R = 

(107) R = 

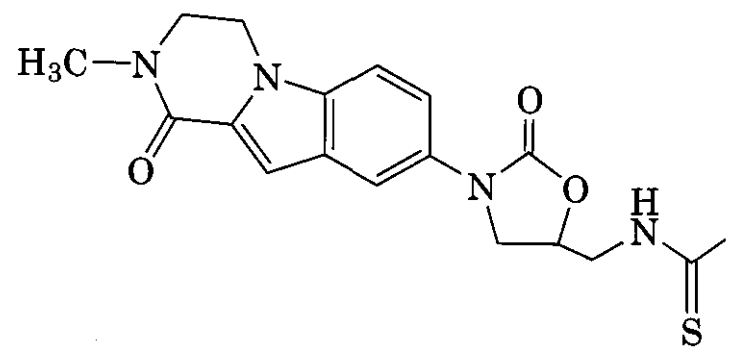
(108) R = 

(109) R = 

(110) R = 

(111) R = 

against many problem Gram-positive infections makes it a useful addition to therapeutics. It may also find use in combination therapy with other antibacterials, including in mycobacterial infections. However, there have been some reports of myelosuppression in persons treated for longer than 2 weeks, which calls for continuous monitoring during treatment.



(113)

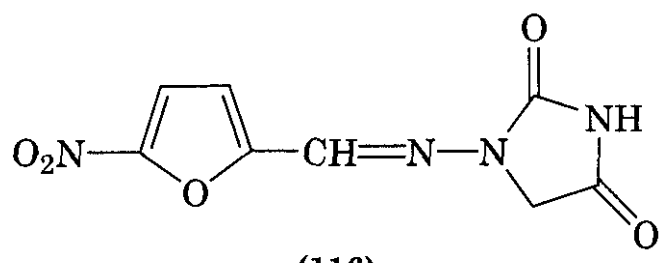
3.4 Other Systemic Synthetic Antibacterials

3.4.1 Nitrofurans. Thousands of nitrofurans have been synthesized and tested for antibacterial activity, but only nitrofurazone, nitrofurazolidone, and nitrofurantoin have been approved for human use in the United States. Nitrofurazone was described by Dodd and Stillman in 1944 (385) and nitrofurantoin was patented in 1952. Nihydrazone and furaltadone are used in animal feeds.

Nitrofurazone (114) is the semicarbazone of 5-nitro-2-furaldehyde (386). It has a broad spectrum of antibacterial activity in the range of 1:100,000 to 1:200,000, although it is not effective against Pseudomonas aeruginosa or fungi. It is used topically in the treatment of burns and in prevention of bacterial infection in skin graft procedures.

Furazolidone (115) is the hydrazone formed from 5-nitro-2-furaldehyde and 3-amino-2-oxazolidinone (387). It has a broad spectrum of bactericidal activity against intestinal pathogens, including various species of Salmonella, Shigella, *Proteus*, and Enterobacter, as well as E. coli and Vibrio cholerae. It is useful for the treatment of bacterial or protozoal diarrhea because it is effective and only a small fraction of an oral dose is absorbed.

The hydrazone prepared from 5-nitro-2-furaldehyde and 1-aminohydantoin is known as nitrofurantoin (116). It is active against

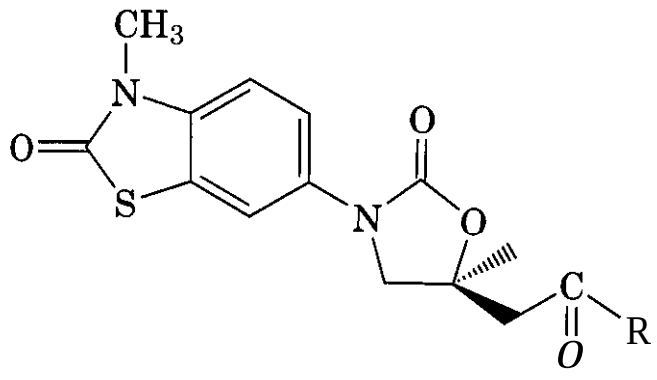


(116)

many Gram-positive and Gram-negative bac-

teria at concentrations of 5–10 μg/mL. Upon oral administration, it is rapidly absorbed, but cleared so rapidly that concentrations adequate for an antibacterial effect in plasma cannot be obtained; however, it accumulates in urine in sufficient concentration for treatment of urinary tract infections.

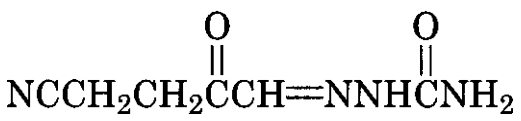
Structure-activity relationships for nitrofuran derivatives indicate that a 5-nitro group



(112)

is required for antibacterial activity. Substitutents at position 2 can be varied widely, with the most potent compounds having azomethine (C=N), vinyl, or heterocyclic groups. Few compounds with 3 or 4 substituents have been prepared (388).

Products found in the urine of rats fed nitrofurazone included 4-cyano-2-oxobutyraldehyde semicarbazone (117) and other compo-

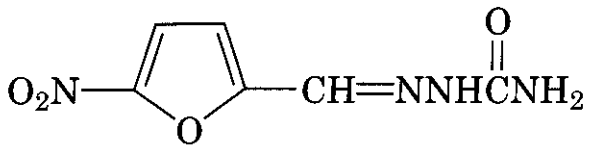


(117)

nents thought to include amino or hydroxylamino-substituted furaldehyde semicarbazone (389). 4-Cyano-2-oxobutyraldehyde semicarbazone also was found when nitrofurazone was incubated with small intestinal mucosa or liver homogenates from rats (390). Chickens and rats fed nitrofurantoin produced metabolites in urine from which a small amount of the corresponding 4-hydroxy derivative (118) was isolated (391).

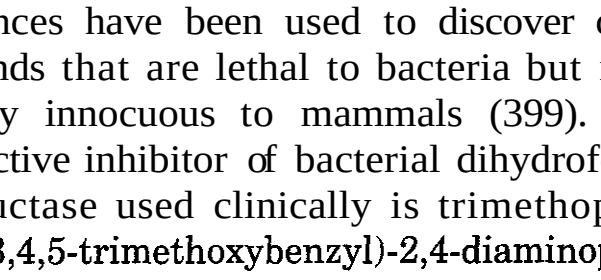
It has been suggested that nucleic acids, especially tRNA, are the primary target in nitrofuran mutagenesis and carcinogenesis (392). Thus, incubation of labeled 2-amino-4-(5-nitro-2-furyl)-thiazole with rodent liver preparations led to covalent attachment of metabolites to added yeast tRNA. Enzymatic hydrolysis of the product gave two covalent adducts (393). Another factor in mutagenesis is thought to be protein binding. When nitrofurazone-sensitive strains if E. *coli* were exposed to labeled nitrofurazone, radioactive species were tightly bound to proteins in the trichloroacetic acid-insoluble fraction, whereas very little radioactivity was found in proteins from resistant mutants (394). A similar result was obtained when labeled nitrofurylthiazole derivatives were incubated with mammalian tissues. Addition of thiols to the incubation mixtures substantially decreased protein binding (395). The structure of the carcinogen-protein adducts is not known; however, the reactive form of nitrofurazone is thought to be a N-hydroxylamine based on an experiment in which albumen binding was observed when nitrofurazone was reduced electrochemically at −0.8 V. This potential reduces nitro groups, but not hydroxylamines (394).

3.4.2 Methenamine. Methenamine (hexamethylenetetramine, 119) is prepared by evaporating a mixture of formaldehyde and strong ammonia water. It was patented by Missner and Schwiedessen in 1956 (396). The free base has almost no antibacterial activity, but acidification results in the liberation of formaldehyde, which is strongly bactericidal (see Section 2.7.2). Acidification is provided by formulating methenamine as a mandelate or hippurate salt, or by administering ammonium chloride or sodium biphosphate to acidify the urine. Methenamine is sometimes used in long-term suppression of bacterial urinary tract infections, although it is not a treatment of choice for corresponding acute infections (397). Certain bacteria are resistant to methenamine because they liberate urease, an enzyme that hydrolyzes urea to ammonia and thereby raises urinary pH. This problem can



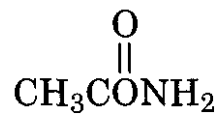
(114)

be overcome by giving acetohydroxamic acid (**120**), which inhibits urease.



(115)

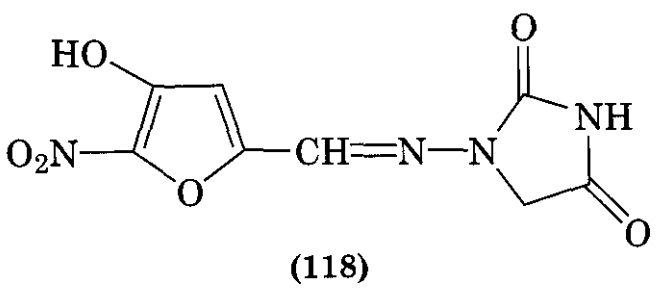
3.4.3 Cotrimoxazole. Reduced folates are carriers of one-carbon fragments in the biosynthesis of purines and pyrimidines. Agents that inhibit dihydrofolate reductase prevent the formation of important tetrahydrofolates, which results in decreased nucleotide synthesis and cell death. The use of dihydrofolate reductase inhibitors in chemotherapy of cancer and malaria is discussed in the appropriate chapters. There are species differences in dihydrofolate reductases (398), and these differences have been used to discover compounds that are lethal to bacteria but relatively innocuous to mammals (399). The selective inhibitor of bacterial dihydrofolate reductase used clinically is trimethoprim [5-(3,4,5-trimethoxybenzyl)-2,4-diaminopyrimidine, (**52**)](400). It is potent against a broad spectrum of Gram-positive and Gram-negative bacteria, although P. aeruginosa is resistant and resistant strains of E. coli have been isolated from patients (401). Trimethoprim in not usually used alone, but in combination with sulfamethoxazole, a sulfonamide with a similar half-life. This combination, called cotrimazole, has a synergistic effect that prevents the development of bacterial resistance (402). Cotrimazole is used mainly in the treatment of urinary tract infections, but it has been effective against gonorrhea, chest infections caused by pneumococci and Hemophilis *influenzae,* and enteric Salmonella infections. Side effects occur mainly in patients with impaired folate metabolism and include megaloblastosis, leukopenia, and thrombocytopenia (403).



(120)

3.5 The Challenge of Antibacterial Chemotherapy

There is a dramatic increase in the incidence of bacterial infections resistant to most commonly used antibacterials. In a few reports, infections in some patients were not susceptible to any known antibacterial, and the patients eventually died. These tragedies sound an alarm bell for antibacterial chemotherapy.

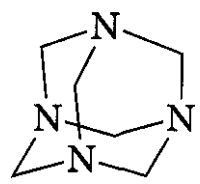


(118)

A certain propensity to resistance development could be expected in bacteria exposed to antibiotics because of their rapid rate of multiplication, which would promote the selection of less susceptible (resistant) mutants continuously from a population with mixed sensitivities. This effect, coupled with overprescription and indiscriminate use of antibacterial agents, is to a large measure responsible for the emergence of this alarming situation.

This situation was aggravated further by a decline in antibacterial research, which resulted in very few new classes of antibacterial agents being discovered over the last few decades. The big successes achieved in antibacterial chemotherapy by sulfonamides and antibiotics created the feeling that bacterial infections were conquered. Complacency set in, and for many years little attention was paid to new agents for antibacterial chemotherapy, despite the inherent nature of infectious agents to develop resistance, and the fact that the developing world still had significant morbidity and mortality from bacterial infections. The serious challenge to public health caused by the emergence of bacterial resistance has highlighted the need for a continuous effort to develop new antibacterial agents. Recent ad-

vances in molecular biology, genomics, and drug design approaches offer many new opportunities to discover antibacterial agents. The recent introduction of linezolid, a synthetic antibacterial with a new mechanism and activity against multidrug-resistant Gram-positive organisms, demonstrates that this can be achieved. Folate antagonists, fluoroquinolines and related prototypes, and oxazolidinones offer good prospects for development of further new analogs, as discussed above, and they need to be fully explored more fully. Even more important is the discovery of new classes of antibacterial agents with novel modes of action, which would be less likely to have cross-resistance to known antibacterials. There is a need to maintain constant pressure in the effort to develop new antibacterial agents.



(119)

The activity of linezolid has aroused worldwide interest, and many research laboratories around the world have been working in this class of compounds. The compounds that deserve special mention are AZD 2563 and RBx 7644. Clinical trials of AZD 2563, developed by AstraZeneca, have started; whereas RBx 7644, developed by Ranbaxy Laboratories, India, is awaiting clinical trial permission. AZD 2563 has a novel heterocyclic replacement for the C5 N-acetate group. Worldwide data presented (404) show that it is active against Gram-positive bacteria, including multidrug-resistant strains, and may have the added convenience of potential once-daily administration. RBx 7644 (405) is a novel compound that retains linezolid's excellent activity against both sensitive and resistant Gram-positive pathogens; moreover, it is distinctly more active against all anaerobes (Gram-positive or Gram-negative) and has significant, improved inhibitory activity on slime-producing and glass-adherent bacteria, thus extending the indications for its use and decreasing the chances of resistance development.

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CHAPTER FOURTEEN

β-Lactam Antibiotics

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Contents

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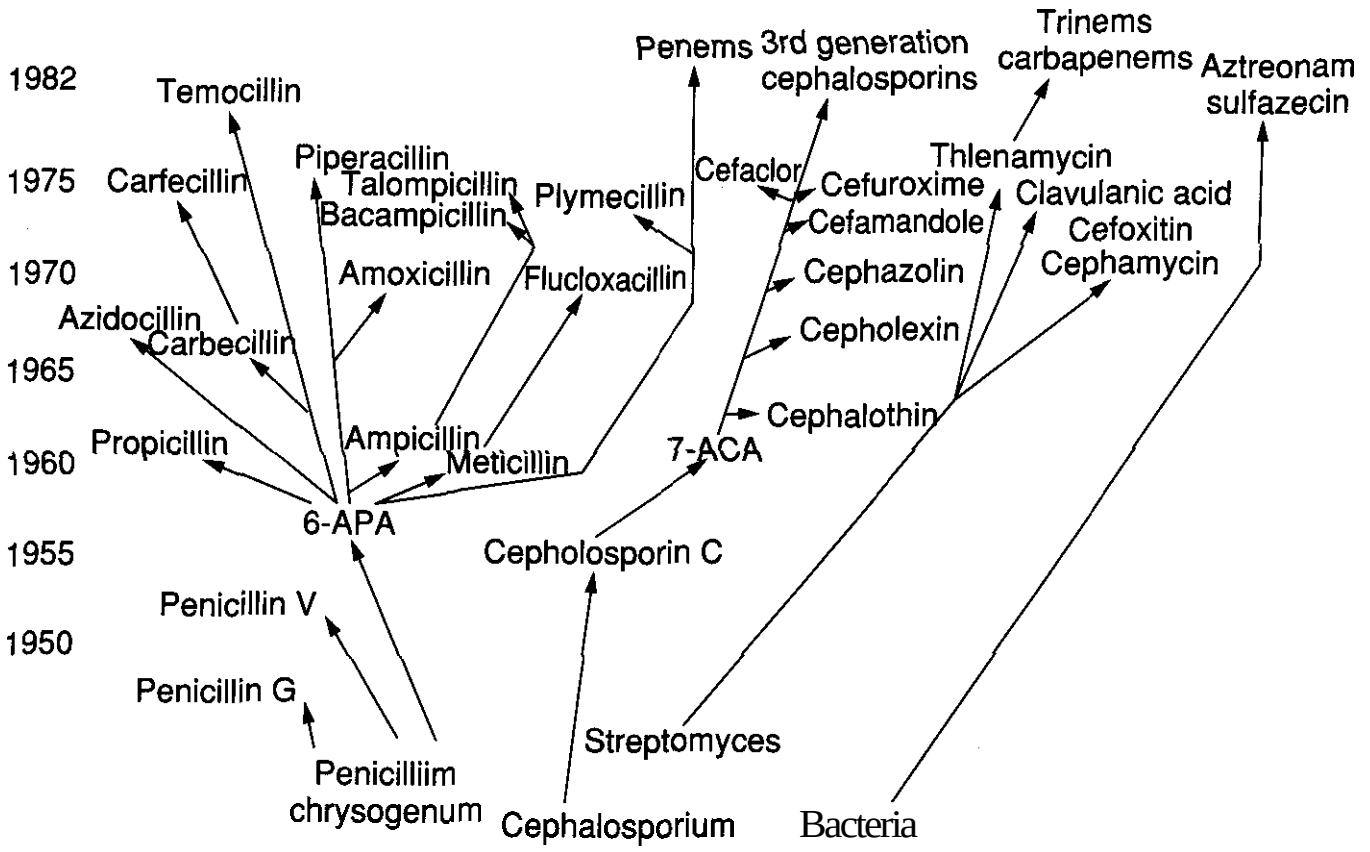
1 INTRODUCTION

The history of β-lactam antibiotics is generally considered to begin with Alexander Fleming and his observation in 1928 that a strain of the mould ***Penicillium*** produced an antibacterial agent that was named penicillin **(1).**

β-Lactam antibiotics have been in clinical use for over 50 years. These pharmaceuticals remain to the present the most commonly used antibiotics, and their introduction for the clinical use is one of the most important, if not the most important, medical developments of the century.

The success of penicillin G had an unexpected effect: an increasing number of ***Staphylococcus aureus*** isolates resistant to penicillin G in London hospitals spread progressively all around the world. It was discovered that certain bacterial enzymes, the β-lactamases, had the ability to hydrolyze the β-lactam ring of these antibiotics, thus diminishing their effectiveness as antibacterial agents **(2).** Based on the rapidity of the widespread resistance to penicillins, it became obvious that the hydrolytic enzymes could potentially destroy the utility of this potent class of antibiotics. The pharmaceutical industry has invested to iden-

Evolution of the β-lactam class



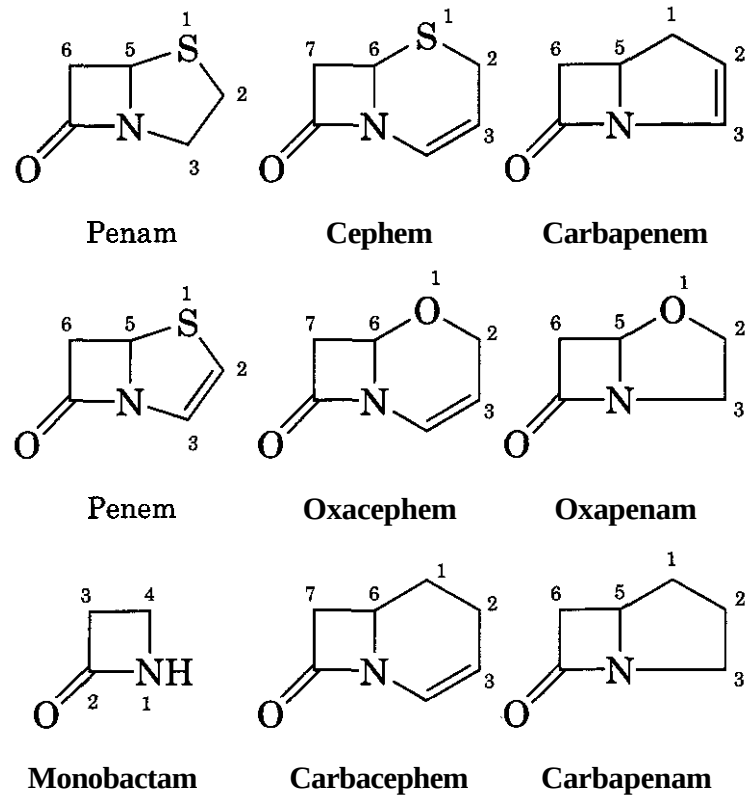
tify novel β-lactams over the past 40 years in an attempt to keep ahead of the continuous evolution of new β-lactamases with altered hydrolytic properties.

Two approaches were undertaken: development of agents stable to hydrolysis by the major β-lactamases, and identification of potent inhibitors for these enzymes. The number of compounds, even when limited to therapeutically important compounds, based on the β-lactam ring is now huge. As new compounds have been discovered and developed they have been described by their trivial names, penicillins, cephalosporins, clavulanic acid, and so on, although the situation has become so complicated that it has become necessary to group them according to their chemical structures.

The agents having current or potential therapeutic use can be represented by seven principal chemical skeletons:

- *Penams*. Penicillins are bicyclic structures where the β-lactam ring is fused with a five-membered thiazolidine ring.
- Penems. These differ from penams by the presence of a double bond between the C2 and C3 positions. No natural penems have so far been described, but many have been synthesized in the expectation that they would combine desirable properties of penicillins and carbapenems.
- *Carbapenams* and carbapenems. These compounds differ from penams and penems by the presence of a carbon atom at position 1. Many natural and synthetic members of the group have been described, and some of them are currently used in the clinics.
- Cephems, oxacephems, and carbacephems. These compounds are characterized by the presence of a β-lactam ring fused with a six-membered unsaturated ring, having at position 1 a sulfur, an oxygen, or a carbon atom, respectively. In particular, the Cephem class has been very prolific in generating good antibiotics, which have found extensive application in the treatment of bacterial infections.
- Oxapenams. This class is generally characterized by a very weak antibacterial activity but has found therapeutic applications as inhibitors of bacterial β-lactamases. Its skeleton differs from that of penams by the presence of an oxygen atom at position 1.
- Monobactams. The monocyclic β-lactam is the simplest structure still retaining antibacterial activity.

β-Lactam skeletons



Scheme 14.1.

2 MECHANISM OF ACTION

2.1 Bacterial Cell Wall of Cram-Positive and Cram-Negative Strains

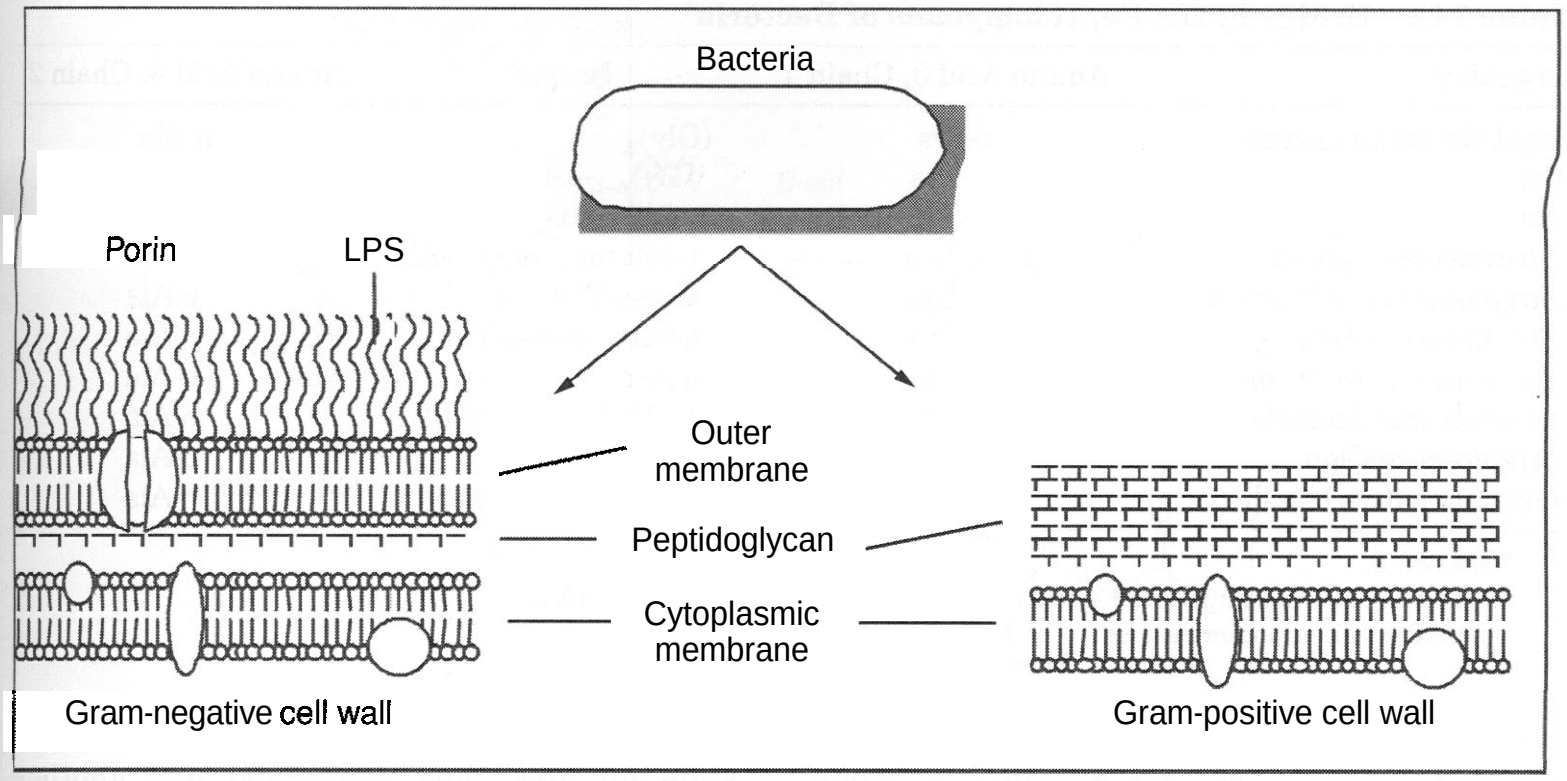
β-Lactams inhibit the synthesis of peptidoglycan (3, 4), which is the major polymer of the bacterial cell wall. Peptidoglycan maintains the cell shape and protects against osmotic forces. That capability depends on its netlike structure, composed of long sugar chains crosslinked by peptides.

In Gram-positive organisms, the peptidoglycan typically forms a thick layer external to the cytoplasmic membrane and accounts for 50% of the dry weight of the bacterium, whereas in Gram-negative bacteria and mycobacteria, peptidoglycan is a thinner layer (5), or hydrated gel (6), sandwiched between the outer and cytoplasmic membranes (Fig. 14.1). To manufacture peptidoglycan, bacteria first synthesize precursor molecules of uridine diphosphate (UDP) linked to *N*-acetylmuramic acid pentapeptide (7). The sequence of the pentapeptide varies among species, but the two terminal residues are D-alanine, and the third amino acid, usually L-lysine or *m*-diaminopimelic acid, bears a free amino group, which may be substituted with a "bridge" of additional amino acids (Table 14.1).

These precursors that are produced in the cytoplasm, are then transferred from UDP to an isopropenoid carrier located in the cytoplasmic membrane. An N-acetylglucosamine residue is added to give "disaccharide pentapeptide," which is transported across the membrane and inserted into the existing sacculus by transglycosylation and transpeptidation. Transglycosylation extends sugar chains by attaching the muramyl residue of a new precursor to a free N-acetylglucosamine residue on the existing peptidoglycan. Transpeptidation crosslinks adjacent sugar chains through their pentapeptides (Fig. 14.1). Peptidoglycan transglycosylase and D-alanyl-D-alanine transpeptidase activities often reside at separate sites on the same proteins, and most bacteria possess multiple peptidoglycan transglycosylases/transpeptidases, each with a different role (9, 10). These enzymes contain lipophilic sequences, which anchor them to the cytoplasmic membrane and allow particular activities to be localized. Their interplay ensures the maintenance of cell shape (10, 11) and their relative amounts may vary with the growth rate (12). Additional enzymes hydrolyze crosslinked peptidoglycan and are collectively called autolysins or peptidoglycan hydrolases (13). D-Alanyl-D-alanine carboxypeptidases hydrolyze D-alanine groups from pentapeptides that have served as amino donors (Fig. 14.2) and probably have a regulatory role in peptidoglycan synthesis.

More drastic hydrolysis of peptidoglycan are undertaken by (1) D,D-endopeptidases, which cleave the crosslink synthesized by D-alanyl-D-alanine transpeptidases; (2) N-acetylmuramyl-L-alanine amidase, which cleaves peptides from muramyl residue; and (3) lytic glycosylase, β-*N*-acetylglucosaminidases, and β-*N*-acetylmuraminidases, all of which hydrolyze the sugar backbone of the peptidoglycan (Fig. 14.2).

Many species have multiple autolysins (14, 15); for example, *Escherichia coli* has at least 11 different enzymes (16), catalyzing five modes of cleavage. The transglycosylases, transpeptidases, and autolysins are often (but not always) membrane bound, allowing their activities to be localized. Their main role is to



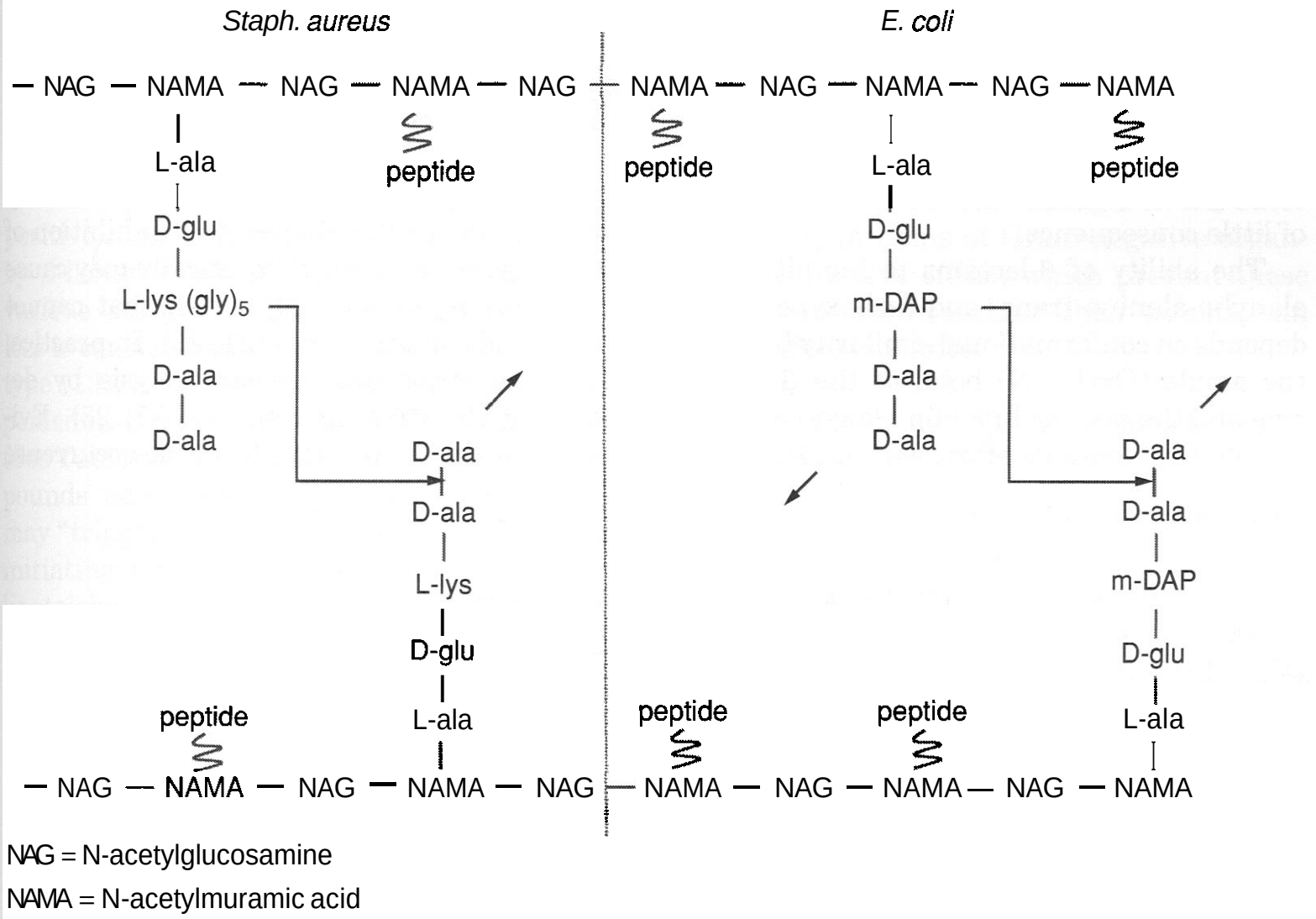


Figure **14.1.** Bacterial cell wall of Gram-positive and Gram-negative strains.

provide sites for insertion of new peptidoglycans (17), but they may also assist in daughter cell cleavage, autolysis, and flagellar extrusion. Degraded peptidoglycan fragments are absorbed and recycled.

2.2 Action of β-Lactams

β-Lactams inhibit D-alanyl-D-alanine transpeptidase activity by acylation, forming stable esters with the opened lactam ring attached to

Table 14.1 Bridge in the Peptidoglycans of Bacteria[a]

Organism	Amino Acid 3, Chain 1[b]	Bridge[b]	Amino Acid 4, Chain 2[b]
Staphylococcus *aureus*	L-Lys	$(Gly)_5$	D-Ala
Staphylococcus epidermidis	L-Lys	$(Gly)_{4-5}$–L-Ala	D-Ala
Streptococcus agalacticae	L-Lys	L-Ala (or L-Ser)–L-Ala	D-Ala
Streptococcus group G	L-Lys	L-Ala (or L-Ser)–L-Ala	D-Ala
Streptococcus *salivarius*	L-Lys	Gly–L-Thr	D-Ala
Streptococcus *bovis*	L-Lys	L-Ser–L-Ala–L-Thr	D-Ala
Enterococcus faecium	L-Lys	D-Asn	D-Ala
Enterococcus faecalis	L-Lys	$(L\text{-Ala})_{2-3}$	D-Ala
Streptococcus spp.	L-Lys	Gly–L-Ala	D-Ala
Gram-negative bacilli	m-Dap[c]	Direct; no bridge	D-Ala

[a] See Ref. 8.
[b] Positions of amino acids 3 and 4 and of the bridge are indicated in Fig 14.1.
[c] *m*-DAP, m-diaminopimelic acid

the hydroxyl group of the enzyme's active site (Fig. 14.3). These esters mostly have hydrolysis half-lives of several hours, so inactivation is effectively permanent (18). β-Lactams have no effect on transglycosylation. They do inhibit the D-alanyl-D-alanine carboxypeptidases and some peptidoglycan endopeptidases, but these are regulatory enzymes whose inactivation is of little consequence.

The ability of β-lactams to inhibit the D-alanyl-D-alanine trans- and carboxypeptidase depends on conformational similarity between the amide (O═C—N) bond of the β-lactam ring and the peptide link of D-alanyl-D-alanine (3, 19). Considerable effort was put into comparing the bond angles of these structures and showing how they resembled one another. However, D-alanyl-D-alanine transpeptidases are more versatile than was once thought, and attempts to find conformational identity now seem misplaced. In particular, many D-alanyl esters, as well D-alanyl-D-alanine, are substrates, despite the fact that these have very different molecular shapes (20). Inhibition of transpeptidases alone theoretically may cause bacteriolysis, by yielding a wall that cannot withstand osmotic forces (21, 22). In practice, however, autolysins accelerate lysis by destroying the existing wall (13, 17, 23). Evidence for this view comes from the occurrence

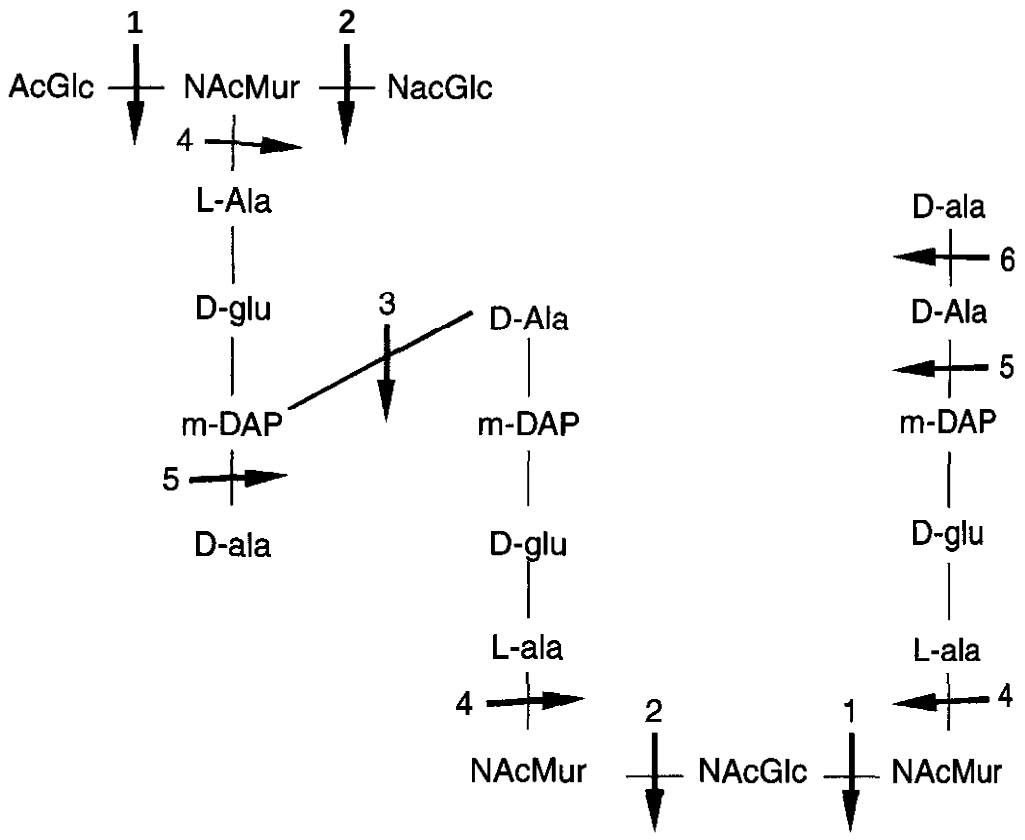


Figure 14.2. Action of autolysins. 1, β-*N*-acetylglucosaminidase; 2, lytic transglycosylase and β-*N*-acetylmuraminidase (lysozyme); 3, D,D-endopeptidase; 4, *N*-acetylmuramyl-L-alanine amidase; 5, L,D-carboxypeptidase; 6, D,D-carboxypeptidase. Although lytic transglycosylase and β-*N*-acetylmuraminidase (lysozyme) cleave the same target bond, the former enzyme additionally catalyzes transfer of the glycosyl bond to the 6'-hydroxyl group of the same muramic acid, yielding 1,6-anhydromuramic acid. NacGlc, *N*-acetylglucosamine; NacMur, *N*-acetylmuramic acid; *m*-DAP, m-diaminopimelic acid.

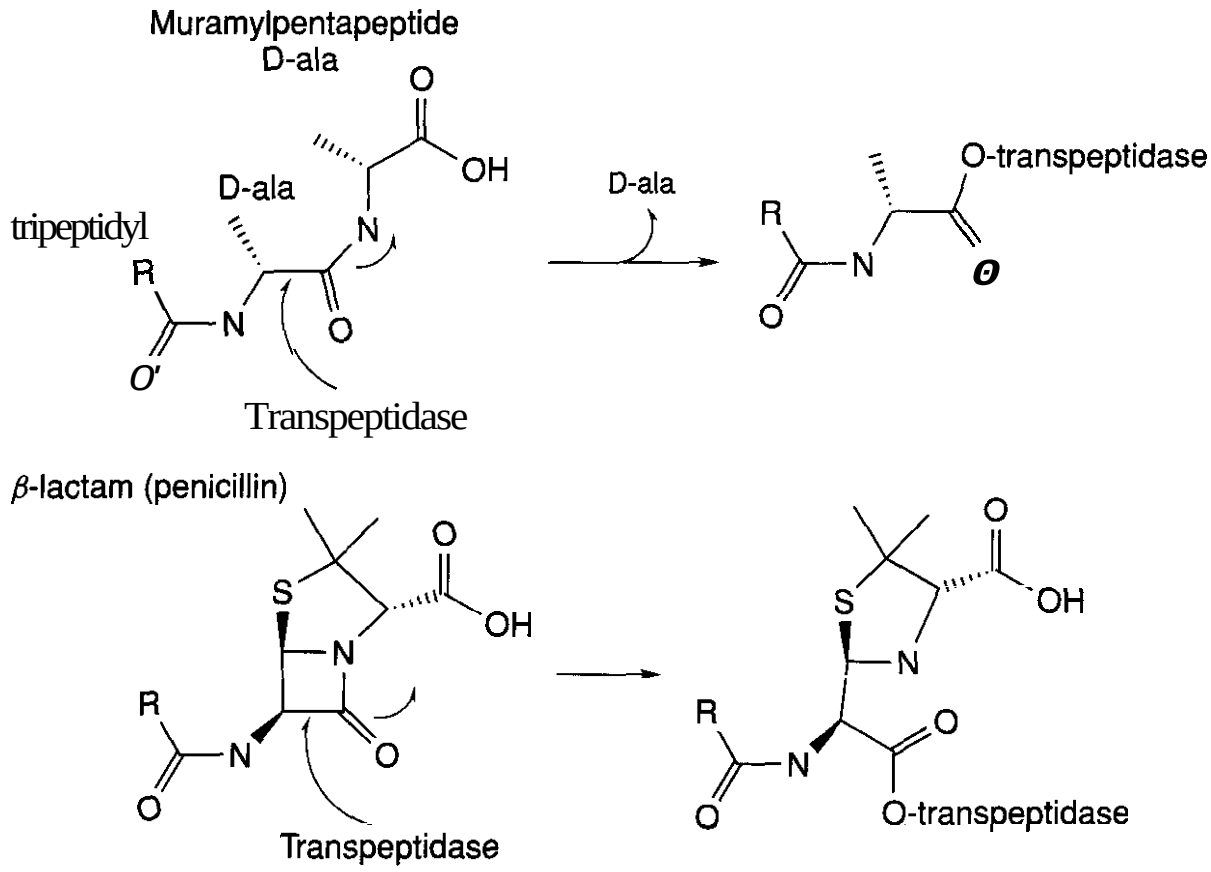


Figure 14.3. Mechanism of action of β-lactams.

of "tolerant" mutants, particularly of Gram-positive cocci that are inhibited but not killed by β-lactams (24–28). These organisms lack various autolysins (29), although no single loss is consistently associated with the phenotype (13); a few tolerant isolates have minor penicillin-binding protein (PBP) changes (30) (See Tables 14.2 and 14.3). β-Lactams, or compounds accumulating through their action, may "trigger" deregulation of autolysins, thus initiating lysis. Tomasz (13) suggested that lipoteichoic acid was the trigger in pneumococci, but the general relevance of this observation is uncertain. Insight into the complexity of the relationships that arise is provided by the realization that mutational loss of cytoplasmic *N*-acetylmuramyl-L-alanine amidase autolysins is the cause of β-lactamase de-repression in *C.* freundii (31).

3 MECHANISM OF RESISTANCE TO β-LACTAMS

Among the various mechanisms of acquired resistance to β-lactam antibiotics, resistance attributed to production of β-lactamases by the cell is the most prevalent. Alteration in the preexisting PBPs; acquisition of a novel PBP insensitive to β-lactams; changes in the outer membrane proteins of Gram-negative organisms; and active efflux, which prevent these compounds from reaching their targets, can also confer resistance.

3.1 Acquisition and Spread of Resistance to β-Lactams

Bacteria can become resistant to some antibiotics mutating existing genes, but in many cases a new gene must be acquired.

Although bacteria can acquire new genes by bacteriophage transduction or by transformation (uptake of DNA from the external environment), these types of transfer tend to occur mainly between members of the same species. Such narrow host range resistance transfer can be important clinically. For example, transformation is probably spreading PBP-type resistance genes between clinical isolates of Streptococcus *pneumoniae*, a common cause of bacterial pneumonia. A major clinical problem is the transfer of resistance genes across genus and species lines. Such broad host range transfer is most likely to be mediated by conjugation (transfer of DNA through a pore formed in the fused membranes of two bacteria). There are two type of

Table 14.2 PBPs of Gram-Negative Bacteria

PBP	Molecular Mass (kDa)[a]	Activity[b]	Essentiality	Reference(s)
Gram-negative rods				18, 34
1a	88–118	Tg/Tp	Yes	35
1b	81–103	Tg/Tp	Yes	35–37
2	63–78	Tg/Tp	Yes	38, 39
3	59–66	Tg/Tp	Yes	35
4	44–51	D,D-Cp	No	40, 41
5	40–44	D,D-Cp	No	40, 42
6	38–41	D,D-Cp	No	42, 43
***Neisseria* spp.**				44–47
1	90–108	—[c]	Yes	
2	59–63	—	Yes	
3	44–48	—	No	
Haemophilus influenzae				32–34
$1^d = \mathbf{1a}^e$	90	—	—	
$2^d = 1b^e$	84	—	—	
$3^d = 2^e$	75	—	—	
$4^d = \mathbf{3a}^e$	68	—	Yes	
$5^d = \mathbf{3b}^e$	64	—	Yes	
$6^d = 4^e$	48	—	—	
$7^d = 5^e$	41	—	—	
$8^d = 6^e$	27	—	—	
Acinetobacter baumannii				34, 48
1	94	—	—	
2	64	—	—	
3	57	—	—	
4	51	—	—	
5	38	—	—	

"Ranges indicate values found by different workers and for different species within the group.

[b]Activity: Tg/Tp, transglycosylase/D-alanyl-D-alanine transpeptidase, with these activities residing at different sites on the protein; D,D-Cp, D-alanyl-D-alanine carboxypeptidase. Catalytic activities of these enzymes are shown in Figs. 14.2 and 14.3.

[c]—, Not determined.

[d]Nomenclature of Ref. 32.

'Nomenclature of Ref. 33.

conjugative elements, **plasmids** and chromosomal elements known as conjugative transposons.

Plasmids. The best-studied type of conjugative element is the **plasmid. Plasmids** that transfer themselves by conjugation must carry a number of genes encoding proteins needed for the conjugation process itself ***(tra*** genes). Thus, self-transmissible **plasmids** are usually at least **25** kb (kilobases). Some plasmids that cannot transfer themselves can still be transferred by conjugation because they are mobilized by self-transmissible plasmids. Such **plasmids** are called mobilizable **plasmids** and can be much smaller than self-transmissible **plasmids** because they need only one or two genes ***(mob*** genes) that allow to them to take advantage of the transfer machinery provided by the other plasmids. Self-transmissible or mobilizable **plasmids** can acquire and transmit multiple antibiotic resistance genes. There are two ways **plasmids** can acquire multiple resistance genes. One way is to acquire sequential transposon insertions. However, most multiresistance **plasmids** apparently did not arise in this way. **A** newly discovered type of integrating element, called integron, is probably responsible for evolution of many of the **plasmids** that carry multiple resistance genes (Fig. 14.4).

Integrons, like transposons, are linear **DNA** segments that insert into **DNA.** Unlike

Table 14.3 PBPs of Gram-Positive Bacteria

PBP	Molecular Mass (kDa)	Activity[a]	Essentiality	Notes	Reference(s)
Enterococcus faecalis					49
1	115	—[b]	—	Overproduced in resistant mutants	
2	—	—	—		
3	—	—	—		
4	—	—	—		
5	74	—	—		
6	43	D,D-Cp	—		
Enterococcus *faecium/Enterococcus hirae*					49
1	126	—		Overproduction causes resistance to penicillins in clinical isolates	50–53
2	92.5	—			
3	90	Tg/Tp	Yes		
4	86	—	—		
5	79–81[c]	Tg/Tp	Yes[d]		
6	40		—		
Rhodococcus equi					54
1	59	—	—	Replaced by 3' in imipenem mutants	54
2	56	—	—		
3	43	—	—		
4	26	—	—		
Staphylococci					55–57
1	85		Debated		57
2	81	Tg/Tp	Yes	Resolves into two components	56, 57
3	75	Tg/Tp	Yes		56
4	45	D,D-Cp	No		58–60
2' = 2a (MRS only)	76.4	Tg/Tp	—[d]	Not related to PBPS	61–63
Streptococcuspneumoniae					47, 64–66
1a	98[e]	—	Yes	PBP-1C is low affinity form of PBP-1A	
1b	95	—	Yes		
2a	81[e]	—	Yes		
2b	79[e]	—	Yes		
2x	85[e]	—	Yes		67, 68
3	43	D,D-Cp	No		69
Streptococcuspyogenes					30, 70
1	95	—	—		
2	83	—	—		
3	76	—	—		
4	48	—	—		

[a]Activity: Tg/Tp, transglycosylase/D-alanyl-D-alanine transpeptidase, with these activities residing at different sites on the protein, D,D-Cp, D-alanyl-D-alanine carboxypeptidase. Catalytic activities of these enzymes are shown in Figs. 14.2 and 14.3.

[b]—, Not determined.

[c]Range of values reported by different authors.

[d]PBP-2′ of MRS and PBP-5 of E. *faecium* can functionally replace all other PBPs in these species.

[e]Molecular weights vary in resistant isolates where the PBPs are encoded by mosaic genes.

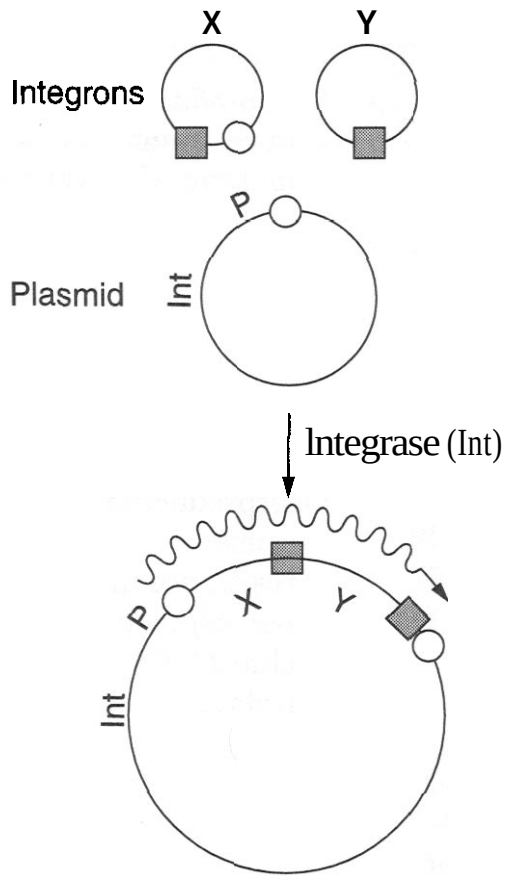


Figure 14.4. Integration of two integrons, carrying promoterless resistance genes X and Y, into a plasmid. The plasmid supplies the promoter (P) and integrase (Int). ○, 8-bp consensus site; ■, 59- to 60-bp shared sequence.

transposons, however, integrons integrate at a single site and do not encode a transposase. The first integron inserted into a plasmid is inserted at an 8-bp consensus site on the plasmid. Integration is mediated by an integrase encoded on plasmid that receives the integron. The plasmid must also provide a promoter because most integrons contain promoterless antibiotic resistance genes.

Conjugative Transposons. A second type of conjugative element has been discovered and characterized only within the past decade: the conjugative transposon. Conjugative transposons are usually located in the bacterial chromosome and can transfer themselves from the chromosome of the donor to the chromosome of the recipient. They can also integrate into plasmids. Their mechanism of transfer is different from that of other known gene transfer elements. They excise themselves from the donor genome to form a covalently closed circle that does not replicate. This circular intermediate transfers with a mechanism similar to that of plasmids. In the recipient, the circular intermediate integrates in the chromosome by a mechanism that does not duplicate the target site. Conjugative transposons are probably responsible for at least as much resistance gene transfer as plasmids, especially among Gram-positive bacteria, and they have a very broad host range. Conjugative transposons can transfer not only among species within the Gram-positive group or within the Gram-negative group but also between Gram-positive and Gram-negative bacteria. Conjugative transposons were overlooked for a long time because they are located in the chromosome and thus cannot be detected as easily as plasmids. Moreover, one of the few groups of bacteria in which conjugative transposons have not been found is the group of species closely related to E. ***coli.*** Like self-transmissible plasmids, conjugative transposons can mediate the transfer of the other DNA. They can mobilize coresident plasmids, and some can also mediate the transfer of unlinked segments of chromosomal DNA.

3.2 Modification of Native PBPs: Mosaic Gene Formation

Modification of the normal PBPs is the sole cause of resistance to β-lactams in pneumococci (71–73) and other a-hemolytic streptococci (74) and, together with impermeability, is a major component of non-β-lactamase-mediated "intrinsic" resistance in ***Neisseria*** spp. (44, 45, 47) and ***Haemophilus*** *influenzae* (32, 75). Target modification may contribute to β-lactam resistance in some ***Acinetobacter*** isolates (76, 77), but its general importance is uncertain in this genus, where β-lactamases and impermeability are also involved (78). Although modifications of normal PBPs are very rare, in some instances they have been thought to cause resistance in Staphylococci, enterococci, enterobacteria, and pseudomonas (79, 80). Critically, the organisms in which PBP modification has proved important (i.e., a-hemolitic streptococci, hemophili, and ***Neisseria*** spp.) are transformable with naked DNA (47). They can acquire fragments of PBP genes from other organisms with inherent or acquired β-lactam resistance and insert this into their own PBP genes. The resulting "mosaic" gene encodes β-lactam-resistant PBPs. The transformation can occur when sequence divergence between the incoming DNA and

native material is less than 25–305 bp, permitting interspecies recombination (47). Restriction and mismatch repair systems do not limit exchange as much as might be anticipated and may even serve to correct errors during replication. Mosaic gene formation is understood for ***Neisseria meningitidis,*** where penicillin-insensitive strains have alteration only in PBP-2. In particular, the PBP-2 gene has regions of normal meningococcal sequence interspersed with inserts with 14 to 23% divergences (81, 82). These inserts closely resemble the corresponding section of the PBP-2 genes of ***Neisseria flavescens*** and ***Neisseria cinerea,*** which are throat commensals that are inherently unsusceptible to penicillin.

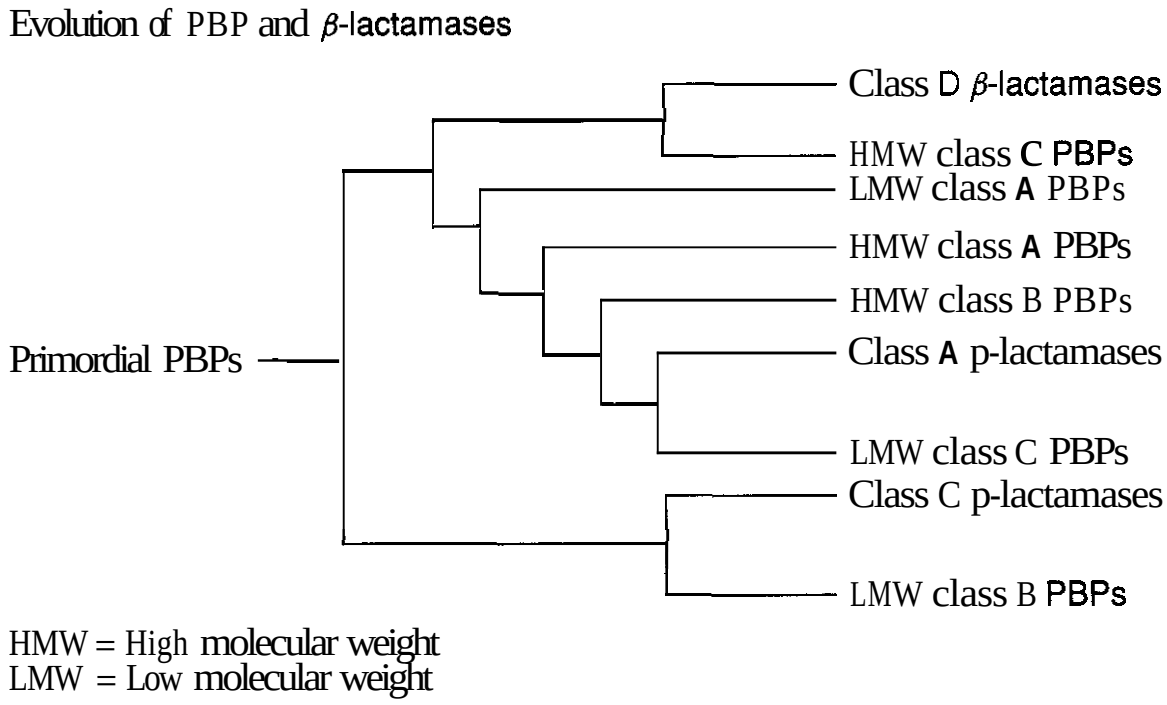
Penicillin-resistant gonococci have mosaic PBP-2 genes with inserts identical to those in resistant meningococci (46, 82). In addition, however, gonococci with high level penicillin resistance commonly have modifications of PBP-1 and reduced permeability (44, 45, 83, 84). Mosaic gene formation has also been extensively studied in pneumococci (47). High level benzylpenicillin resistance [minimum inhibitory concentration (MIC) values of more than 1 μg/mL, compared to MIC values of ≤0.06 μg/mL for "normal" isolates] requires modification, through mosaic gene formation, of each PBP-1a, -2x, -2a, and -2b, although low level resistance (MIC values of 0.12 to 1 μg/mL) arise when only three of these are altered (47). Mosaic PBP-1a, -2x, and -2b genes of different resistant isolates have diverse inserts, whereas corresponding PBP gene sequences from susceptible isolates are highly conserved (65, 67, 68, 85). It is concluded that resistance has evolved on many separate occasions. The source of the foreign DNA is uncertain. Recombination events between penicillin-resistant ***Streptococcus pneumaniae*** and ***Streptococcus mitis*** are implicated, but their direction is uncertain (71). Mosaic gene formation has not yet been proved in H. ***influenzae*** isolates with non-β-lactamase-mediated ampicillin resistance but seems likely, inasmuch as the resistance is associated with reduced PBP affinity (32, 75, 86, 87) and is readily transformable in the laboratory (88). The PBPs affected are 68- and 63-kDa proteins, numbered 3a and 3b or 4 and 5, respectively.

Alternative routes of peptidoglycan synthesis allow a different form of target-mediated resistance, compared with that described above, and are important in Staphylococci and E. *faecium*.

3.2.1 Methicillin-Resistant Staphylococci (MRS): Mechanism and Genetics. Staphylococci normally have two essential PBPs, 2 and 3 (55). These components are inhibited by many β-lactams, including methicillin. MRS retain β-lactam-sensitive PBP-1 to PBP-3 but manufacture an additional transglycosylasel transpeptidase, PBP-2' (PBP-2a), encoded by the ***mecA*** gene (61, 62, 89). PBP-2' continues to function when PBP-1, -2, and -3 have been inactivated and, by itself, can yield a stable peptidoglycan, albeit with many fewer crosslinks than that of the normal wall (90). ***mecA*** by a chromosomal insert called the ***mec*** determinant (91, 92) is probably originated outside Staphylococci, perhaps through fusion between a β-lactam gene and a PBP gene (93). The ***mec*** determinant is not readily self-transmissible but has spread within S. ***aureus*** and several coagulase-negative species, indicating that some horizontal transfer is possible (94–96).

Expression of ***mecA*** and, consequently, of resistance varies among and within staphylococcal strains (63).

Many strains show "hetero-resistance," with only a small proportion of the cell being obviously resistant, whereas the huge majority remain apparently susceptible (97–99). This behavior reflects the genic background of the host staphylococcus, not genes on the ***mec*** determinant itself (100). Several chromosomal genes have been implicated in the control of *mecA*, including those in the ***fem*** cluster, particularly *femA* (101) as well as a recently described determinant, ***chr**** (102). Curiously, loss of β-lactamase from MRS causes ***mecA*** to be homogeneously expressed; the relation is unclear but implies a linkage in the control of the two-resistance determinant (103). Expression of ***mecA*** and of resistance is induced by β-lactams and is also influenced by the environmental conditions, being promoted by high osmolality, low temperature, and neutral to alkaline pH (104–106). The latter points bear on laboratory detection, which



is best achieved by testing with methicillin or oxacillin at 30°C on Mueller-Hinton or IsoSensitest agar supplemented with 5–7% NaCl. Tests with other p-lactams, at 37°C, or media with normal osmolality often fail to detect resistance, especially in coagulase-negative species.

PBP-5 in Enterococcus Faeciurn. Bypass resistance is also important in E. ***faecium,*** although rare in E. ***faecalis.*** E. ***faecium*** has six PBPs, with PBP-3 serving as the main D-alanyl-D-alanine transpeptidase. This protein is inhibited by penicillins and carbapenems, not by cephalosporins and monobactams. At low temperature (32°C), or following mutation, E. ***faecium*** can switch to using PBP5, a transpeptidase that has minimal affinity for any β-lactam. Acting alone, PBP-5 can manufacture a stable peptidoglycan (51, 107). Unlike PBP-2' of MRS, PBP-5 is universal in E. ***faecium*** and its permanent expression requires only a regulatory mutation, not aquisition of foreign DNA. Many isolates of E. ***faecium*** have undergone this mutation and are consequently (and obviously) resistant to all β-lactams (52, 108).

3.3 β-Lactamase Enzymes: Biological Evolution of PBP and β-Lactamases

Study (109) of the evolution of β-lactamases and PBPs based on the multiple amino acid sequence alignment resulted in a scheme that revealed the relationship among these enzymes. The diagram "Evolution of PBP and p-lactamases" shows that, among various PBPs, the low molecular weight enzymes are the most related to p-lactamases.

The diverse classes of p-lactamases are more closely akin to the different PBP classes from which they arose than they are to each other. This is a further clear indication that p-lactamases originated from different groups of PBPs rather than from an immediate shared common ancestor. Class A β-lactamases clustered together with the class C low molecular weight PBPs and the class C β-lactamases group with the class B low molecular weight PBPs. It would appear that the PBW with the simpler domain structure (low molecular weight) were used as templates for β-lactamase evolution. On the other hand, class D for p-lactamases represents an exception to this observation. They cluster together with the high molecular weight class C PBPs. The unique role of these PBPs in bacteria is in signal transduction, to initiate the synthesis of PBPs with low affinity for β-lactams and class A p-lactamases. Therefore, the functional and structural relationships of these PBPs to the other classes of PBPs are quite distant, as was also demonstrated by multiple-sequence analysis (109).

3.4 Classification and Relevance of β-Lactamases in Clinics

From a clinical perspective, the most important p-lactamases are those that threaten the use of the most beneficial β-lactam–containing antimicrobial agents. These agents argu-

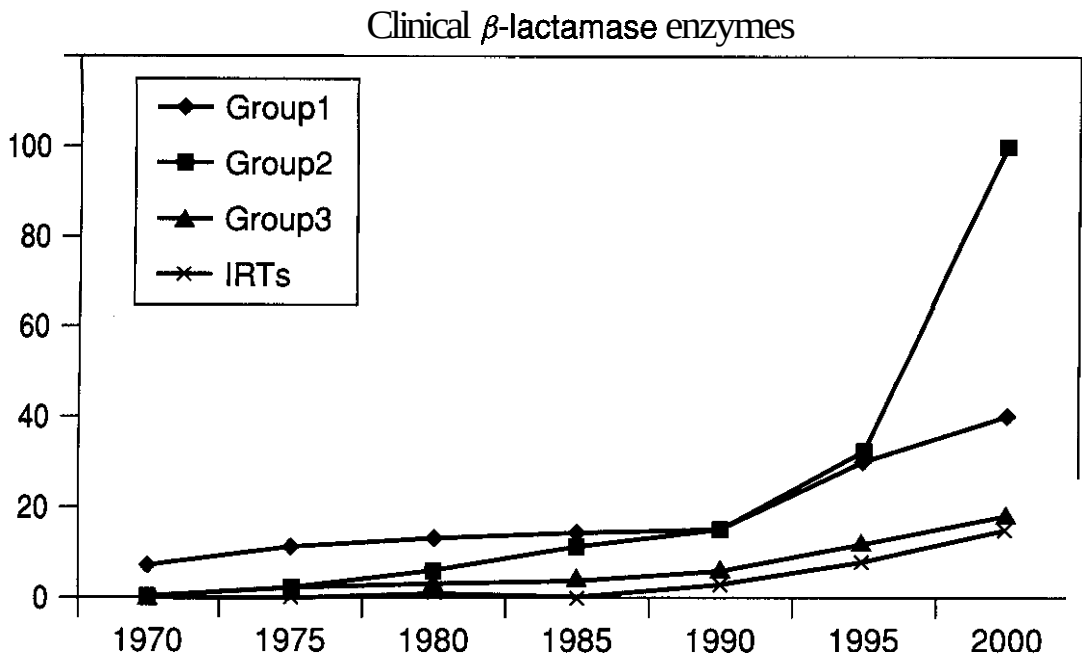


Figure 14.5. Frequency of occurrence of β-lactamases in clinical isolates.

ably include the oral cephalosporins and the β-lactamase inhibitor combination, amoxicillin-clavulanic acid, that are used frequently for community infections such as otitis media, and the more potent agents such as the expanded-spectrum cephalosporins and carbapenems that are used extensively for nosocomial infections. Most of the β-lactam agents introduced after 1980 were selected for pharmaceutical development, in part, because they were stable to hydrolysis by the major β-lactamases. However, enzymes that hydrolyzed these agents emerged shortly after introduction of new members of these classes of agents. Among the more relevant groups of enzymes recently identified are the ESBLs (extended-spectrum p-lactamases) group 1, plasmid-mediated cephalosporines group 2, the inhibitor-resistant TEM (IRT) and SHV variants, and the group 3 (class B) metallo-β-lactamases. The frequency with which they have appeared is shown in Fig. 14.5, again emphasizing the almost exponential growth of ESBLs.

3.4.1 Hydrolysis of Different Classes of β-Lactams. Many attempts have been made to classify β-lactamases. Most have been based on phenotypic properties, with great weight being placed on whether cephaloridine is hydrolyzed more rapidly than benzylpenicillinor vice versa; whether enzyme inhibition is achieved by cloxacillin, clavulanate, aztreonam, or *p*-chloro-mercuribenzoate; and whether the enzyme is chromosomally encoded or plasmid-mediated. Phenotypic features are useful and convenient, in that they can be related to antibiogram data from clinical laboratories, because they permit all p-lactamases to be classified, and because they allow "weighting" of minor structural changes that critically alter the hydrolytic spectrum. The first phenotypic classification that achieved wide acceptance was proposed by Richmond and Sykes in 1973 (110) (Table 14.4).

Superimposed on this there grew to be a convention whereby plasmid-mediated β-lactamases (which fell into classes III and V of the Richmond-Sykes scheme) were given a three-letters and one-number code (111). By 1984, these plasmid-mediated types could be clustered into three groups, according to whether they were most active against benzylpenicillin, oxacillin, or carbenicillin (112). Broad-spectrum enzymes corresponded to Richmond-Sykes class III, but the oxacillinases and carbenicillinases, although very different from each other, both fell into class V. Other problems also emerged in the Richmond-Sykes scheme (113). Class I, it was realized, included two different classes of cephalosporinases: (1) the clavulanate-insensitive, molecular weight 40-kDa, chromosomally encoded enzymes from most enterobacteria; and (2) the 30-kDa cefuroxime, hydrolyzing β-lactamases from ***Proteus*** *vulgaris* (114, 115). Finally, many enzymes placed in class II were proved to be transposon-mediated and accordingly were transferred to classes III and V. These deficiencies prompted Bush to propose a major organization in 1989 (113), with a revision in 1995 (116).

The Bush numbering classifies β-lactamases based on their relative hydrolysis of penicillin, oxacillin, carbenicillin, cephaloridine, expanded-spectrum cephalosporins, and imipenem and on their susceptibility to inhibition by clavulanate, aztreonam, and ethylenediaminetetraacetic acid (EDTA) (Table 14.4). Four "functional groups" are recognized, with group 2 enzymes (clavulanate-sensitive, aztreonam-resistant penicillinases) being divided into six subgroups and with the enzymes in group 4 only partially characterized. This scheme is now to be preferred over the Richmond-Sykes classification. Increasingly, however, p-lactamases are classified by primary structure and sequence homology, as first proposed by Ambler (117), rather than by their phenotypic properties (Table 14.4).

Sequence-based classifications reflect fundamental relationships, unlike phenotypic classification, and cannot be distorted by mutations that alter substrate specificity and inhibitor susceptibility. Only four molecular classes, A to D, are recognized, each with distinct sequence motifs (118).Classes A, C, and D constitute serine active-site enzymes, and class B has the zinc types. The distinction in chromosomally encoded and plasmid-mediated p-lactamases is dubious to biochemists because each molecular class and functional group contains plasmidic enzymes as well as those normally encoded by chromosomes (116). Nevertheless, the distinction between chromosomal p-lactamases and those coded by plasmids (or other inserts) remains fundamental to the clinical microbiologist; chromosomal p-lactamases are ubiquitous in species, whereas plasmid-mediated types are less universal but cross interspecies lines. In Fig. 14.6 is reported a schematic representation of the mechanism of catalysis of serine p-lactamases.

3.5 β-Lactam Resistance Attributed to Decreased Outer Membrane Permeability and Active Efflux

In contrast to Gram-positive organisms, in which β-lactams have unhindered access to their PBP targets, in Gram-negative bacteria the outer membrane acts as a barrier to these compounds. The balance between antibiotic influx and clearance, whether ascribed to hydrolysis or trapping, determines the susceptibility or resistance of the cell, given that permeability barriers alone rarely produce significant levels of resistance. In E. coli and other Gram-negative bacteria, β-lactams diffuse across the outer membrane primarily through water channels consisting of a specific class of proteins, termed porins. E. coli produces at least two porin types, OmpF and OmpC. Mutations that cause reduced expression or alternation of OmpF and/or OmpC result in decreased susceptibility to many p-lactams (119).

3.5.1 Porin Loss in Enterobacteria. Permeability mutants of enterobacteria can easily be isolated in the laboratory and are found to lack one or more porins (120–124). For E. coli, MICs of penicillins, cefoxitin as well as narrow- and extended-spectrum cephalosporins, increase in a stepwise manner as first OmpC and OmpF are lost (125). Loss of OmpF has the greater effect because it forms larger pores than OmpC (126). MICs of carbapenems aminothiazolyl cephalosporins are only scarcely affected by these losses, probably because disruption in the periplasm is minimal. Despite this ease of selection, porin-deficient enterobacteria are rarely found in clinical settings, probably because they are nutritionally disadvantaged (122). Nevertheless, such mutants have been selected during cefoxitin therapy of infections caused by Klebsiella pneumoniae, producing ESBLs (127, 128) and during imipenem therapy of an infection caused by a β-lactamase-depressed Enterobacter cloacae strain (129).

In some cases, the combination of impermeability with weak β-lactamase production confers clinical resistance, whereas the β-lactamase alone fails to do so (128, 130, 131). Porin-deficient mutants of Serratia *marcescens*, lacking a 40-kDa protein, seem to be selected more often than analogous mutants of other enterobacteria (122).

3.5.2 Intrinsic Resistance *to* β-Lactams in *Pseudomonas aeruginosa*. Impermeability-mediated resistance is widely perceived as a greater problem in P. aeruginosa than in enterobacteria but is not completely understood. Most P. aeruginosa isolates are 32- to 48-fold less susceptible to penicillins and cephalosporins than hy-

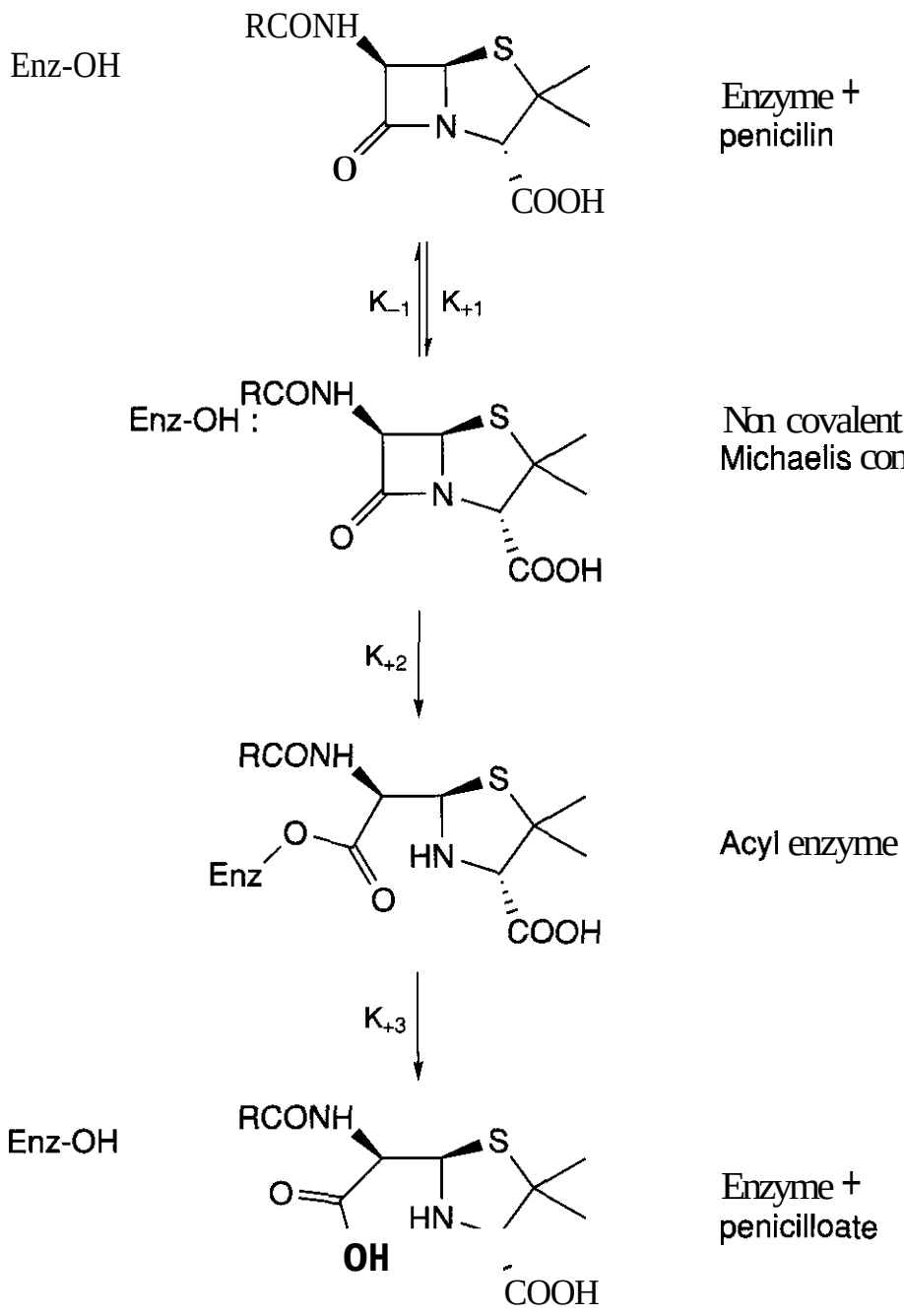


Figure 14.6. Mechanism of action of serine β-lactamases.

K_{+1}, K_{-1}, K_{+2}, K_{+3} are the rate costants; K_{+3} is extremely slow. Release of the β-lactam, is often in a fragmented form, as shown. Enz-OH = serine-enzyme.

persusceptible laboratory mutants, which are inferred, from their lack of other mechanisms, to be hyperpermeable (132,133).

P. *aeruginosa* is characterized by an intrinsic resistance to a wide variety of antimicrobial agents, including β-lactams, β-lactam inhibitors (134), tetracyclines, quinolones, and chloramphenicol. Although this resistance has most often been caused by a highly impermeable outer membrane, it is now recognized to result from the synergy between a unique tripartite energy-driven efflux system (MexAB-OprM) with wide substrate specificity (135–137) and low outer membrane permeability. Recently, it has been shown that MexAB-OprM directly contributes to β-lactam resistance through efflux of this class of antibiotics (138). The emergence in P. *aeruginosa* of resistance specific to imipenem is associated with the loss of the specific channel OprD (139).It appears that imipenem preferentially uses this channel to cross the outer membrane, which under normal circumstances probably transports basic amino acids (140).

The carbapenem uptake is reduced irrespective of whether OprD porin of P. *aeruginosa* is lost. This causes an increase of the MIC values of imipenem from 1–2 to 8–32 μg/mL and those of meropenem from 0.25–0.5 to 2–4 μg/mL (141). This loss has no effect on resistance to β-lactam classes (142). The greater

effect on imipenem than on meropenem reflects the slight liability of imipenem to the class C β-lactamase of P. *aeruginosa*. This enzyme gives greater protection when permeability is reduced by porin loss (143). Meropenem has the capacity to escape the β-lactamase (141, 144) and so is less affected by the porin loss. Selection of OprD-deficient mutants is a problem in imipenem therapy, occurring in 15 to 20% of patients treated for *Pseudomonas* infections (145, 146).

4 CLINICAL APPLICATION (HOSPITAL AND COMMUNITY)

4.1 Pharmacokinetic Properties

Penicillins. For most penicillins, renal excretion is the major route of elimination. With few exceptions, renal clearance tends to exceed the glomerular filtration rate. In all cases studied, there is evidence of active renal tubular secretion, in that the coadministration of probenecid invariably causes a decrease in renal clearance, an increase in serum concentrations, and/or a prolongation in serum-life. By the same token, renal dysfunction has a profound effect on elimination rate, serum levels, and drug accumulation. Although homodialysis or peritoneal dialysis can be effective in some patients with end-stage renal failure, the rate of drug removal is not simply related to serum protein binding. Even though antimicrobial activity in bile is usually high compared to that in serum, biliary excretion contributes only insignificantly to the elimination of most penicillins. Notable exceptions are metampicillin, nafcillin, and the ureidopenicillins. β-Lactam cleavage appears to be a common metabolic pathway among penicillins. Penicilloic acid formations, although variable, may represent as much as 50% of total clearance of a given agent. Part of this may occur in the gastrointestinal lumen or during initial transit through the gut wall and liver after oral administration. Although there is evidence of N-deacylation for some penicillins, only traces of 6-aminopenicillanic acid or penicilloic acid are found in human urine.

Cephalosporins. In general, the cephalosporin and cephamycin antibiotics are a family of parenterally administered antimicrobial agents. Notable orally absorbed cephalosporins include cephaloglycine, cephalexin, cephradine, cefadroxil, cefaclor, and cefratrizine. Urinary excretion is the primary route of elimination for all cephalosporins, although biliary excretion and metabolism contribute to the elimination of several. For most, urinary excretion involves both glomerular filtration and renal tubular secretion. As might be expected, kidney dysfunction or the coadministration of probenecid causes dramatic changes in drug elimination. Cephalosporin antibiotics are remarkably stable in *vivo*, with few exceptions. The metabolism of cephalosporins has generally not been studied with discriminating assay technique. Those that have been studied in this fashion have shown insignificant metabolism.

The pharmacological properties of the parenteral penicillins and cephalosporins are summarized in Table 14.5 (147–149). In general, β-lactam antibiotics penetrate most areas of the body except the eye, prostate (except aztreonam), and uninflamed meninges. Although entry into the cerebrospinal fluid (CSF) is satisfactory with most of the penicillins and carbapenems, only certain cephalosporins reach therapeutic levels in the CSF. Protein binding varies from 2% to 98%. The serum half-life of penicillins is short, ranging from 0.5 to 1.5 h. The half-life of cephalosporins, and especially the third-generation cephalosporins, tends to be longer. The half-life of ceftriaxone, the longest of all these agents, is 8 h. Most penicillins, cephalosporins, and aztreonam are removed intact by renal excretion, and those require dose modification in patients with renal impairment. However, the isoxazolyl penicillins (nafcillin and oxacillin), the ureidopenicillins, and a few cephalosporins (ceftriaxone and cefoperazone) have partial biliary excretion. Dose modification is needed less often for the ureidopenicillins and these cephalosporins, and is not needed at all for the isoxazolyl penicillins.

Carbapenems. Imipenem is hydrolyzed in the renal tubules, producing nephrotoxic metabolites, and requires coadministration of cilastain, an inhibitor of the dehydropeptidase enzyme (DHP-1). This prolongs its half-life and preserves renal function. Meropenem is

Table 14.5 Pharmacokinetic Properties of the β-Lactams

Antimicrobial	Protein Binding	$t_{1/2}$ (h)	Cerebrospinal Fluid Penetration	Route of Excretion
Penicillin G	55	0.5	Yes	Renal
Nafcillin	87	0.5	Yes	Biliary and renal
Oxacillin	93	0.5	Yes	Biliary and renal
Ampicillin	17	1	Yes	Renal
Carbenicillin	50	1.1	Yes	Renal
Ticarcillin	50	1.2	Yes	Renal
Piperacillin	50	1.3	Yes	Renal and biliary
Mezlocillin	50	1.1	Yes	Renal and biliary
Azlocillin	20	0.8	Yes	Renal and biliary
Aztreonam	56	1.8	Yes	Renal
Imipenem/cilastatin	20/40	1	Yes	Renal
Meropenem	2	~1	Yes	Renal
Cephalothin	71	0.6		Renal
Cefazolin	80	1.8		Renal
Cephradine	10	0.7		Renal
Cefamandole	75	0.8		Renal
Cefonicid	98	4.5		Renal
Cefuroxime	35	1.3	Yes	Renal
Cefoxitin	70	0.8		Renal
Cefotetan	90	3.5		Renal
Cefotaxime	35	1	Yes	Renal
Ceftizoxime	30	1.7	Yes	Renal
Ceftriaxone	90	8	Yes	Renal and biliary
Cefoperazone	90	2		Renal and biliary
Ceftazidime	17	1.8	Yes	Renal
Cefepime	20	2.1	Yes[a]	Renal

[a] Animal studies only.

not susceptible to renal hydrolysis and therefore does not require coadministration of an inhibitor of hydrolysis (150). Both carbapenems require dose reductions in patients with renal impairment.

4.2 Side Effects and Drug Interactions

A remarkably favorable chemotherapeutic index has been one of the most important qualities of the animal and human pharmacology of the first penicillins. Aside from problems of local irritation and of neurotoxicity with extremely large doses, these antibiotics have minimal direct toxicity. For nearly 20 years, allergic or immune-mediated reactions represented the only common complications of the use of penicillins. However, the development of newer penicillins and cephalosporins, although providing some of the safest and most important antibiotics currently used, has introduced new direct toxicity problems.

Pain and sterile inflammatory reactions at the site of intramuscular injection are among the most common local effects of therapy with the penicillins and cephalosporins. With benzylpenicillin these reactions appear to be related to the concentration of the antibiotic, and this also may be the case for the other penicillins (151).

Phlebitis develops in many patients receiving intravenous penicillins or cephalosporins (152). The relative risk of phlebitis among the different cephalosporins is unsettled (153–155).

Gastrointestinal side effects are among the most common adverse reactions to oral treatment with the β-lactam antibiotics. Some degree of gastric irritation is reported in 2–7% of patients (156). The discomfort occasionally results in discontinuation of the drug, although serious consequences are rare. Diarrhea is also a relatively common problem, which may

be of mild or life-threatening severity. At least three types are distinguishable: *(1)* nonspecific diarrhea, (2) pseudomembranous colitis, and **(3)** presumed ischemic colitis.

Some of the most important reactions to β-lactam antibiotics result from the involvement of humoral or cellular immunity. Almost all patients receiving benzylpenicillin develop antibodies to it (151, 157). The frequency of allergic reactions to penicillins has been reported as 0.7–10%, and that to cephalosporins has been estimated to be 0.8–7.5% (151, 158, 159). Humoral hypersensivity produces some of the most serious adverse effects, including urticaria, angioedema, bronchospasm, and anaphylaxis. The frequency of anaphylactic reactions to penicillin has been estimated to be 0.045–0.15% (151, 160). A history of previous exposure to penicillin is common but not invariable (151). Skin testing may be helpful in distinguishing those patients at risk for anaphylaxis, especially if testing is conducted with both major and minor antigenic determinants of the penicillin (158, 161).

Skin rashes during the administration of a penicillin or cephalosporin are relatively common, occurring in approximately 2% of cases (156,162,163).

Aztreonam, however, is felt to be safe for administration to penicillin-allergic patients. Imipenem produces seizures in 0.4–1.5% of patients, predominantly affected by renal insufficiency with underlying central nervous system (CNS) disease. Meropenem, however, appears to have a much lower potential to cause seizures (164–167).

4.3 indications for Use

Table 14.6 summarizes some of the more important reasons for using p-lactam agents. In addition to being the drugs of choice for treatment of a variety of infections caused by specific bacteria, the β-lactam drugs are the stalwart agents for empirical treatment of febrile neutropenic patients (168). Unfortunately, the choice of empirical therapy today is less straightforward than in the past, and no obviously best regime exists. Important factors influencing the choice of empirical therapy for febrile neutropenia include the type of cancer chemotherapy being used, expected severity and duration of neurotropenia, the presence of an indwelling long-term catheter, previous use of prophylactic antibiotics or gut decontamination, the patients' symptoms, and the bacterial resistance pattern of the hospital.

Although there has been a striking increase in Gram-positive infections, the morbidity and mortality caused by Gram-negative infections (especially Pseudomonas) suggest that empirical therapy continues to cover these organisms. Supportive evidence exists for the empiric use of β-lactam drugs in combination with an aminoglycoside. Piperacillin, mezlocillin, azlocillin, ticarcillin, and ceftazidine have shown similar efficacy, with response rates of 55–88% (169–177). Also, a convenient regimen of once-daily dosing of ceftriaxone and amikacin proved as effective as multiple daily-dosing regimens (178). Toxicity for aminoglycoside use led to trials with combinations of two β-lactam agents. Good results were achieved with multiple regimens, including carbenicillin plus cephalotin, cefoperazone plus aztreonam, cefoperazone plus mezlocillin, ceftazidime plus piperacillin, and ceftazidime plus ticarcillin/clavulanate (169–171). However, double β-lactam therapy remains controversial because of the possibilities of increased selection of resistant organisms, drug antagonism, prolongation of neutropenia, and potentiation of bleeding disorders.

β-Lactam monotherapy has also been investigated. Supportive data exist for the use of ceftazidime and imipenem (170–172,179–183). However, the increase in frequency of resistant Gram-negative infections, and the finding by some workers that ceftazidime treatment is inadequate, suggest cautions in using ceftazidime monotherapy (170–174). Aztreonam in combination with an agent covering Gram-positive organisms (e.g., vancomycin) is of great utility in patients with penicillin allergy (184, 185). p-Lactam antibiotics have played a key role in improving the care of both immunocompromised and nonimmunocompromised patients. These agents are bactericidal, well tolerated, widely distributed throughout the body, and, most important, clinically effective.

Table 14.6 Recommendations for the Use of β-Lactams

β-Lactam	Recommendation	Organism or Condition	Notes
Penicillin G	Primary indication	***Streptococcus pyogenes, Streptococcus pneumoniae,*** and enterococcal infections	
	Drug of choice	Treponemal infection, prevention of rheumatic fever.	
	Can be used	Puerperal infection: anaerobic streptococci, ***Streptomyces agalactiae,*** clostridial infection, and infection attributed to mouth flora: Gram-positive cocci, Gram-negative cocci, and ***Actiomyces***	
Penicillinase-resistant penicillins	Can be used	Susceptible ***Staphylococcus aureus*** infection	
Arninopenicillins	Recommended	Prevention of endocarditis	
	Can be used	Infection of respiratory tract in areas with low prevalence of β-lactamase and ***Haemophilus influenzae***.	
		Urinary tract infection	
Extended-spectrum	Primary indication	***Pseudomonas*** spp.	
	Can be used	Infection of urinary tract, respiratory tract, and bone with Gram-negative bacilli and mixed aerobic/anaerobic infections	
Combination drugs	Can be used	Mixed bacterial infection: community and hospital acquired pneumonia, especially if aspiration, intra-abdominal and gynecological infections, osteomyelitis, and skin-structure infection	
Timentin (ticarcillin and clavulanic acid)			
Unasyn (ampicillin and sulbactam)	Can be used	Mixed bacterial infection: intra-abdominal: obstetric, gynecologic, soft-tissue, bone infection	
Zosyn (Piperacillin and Tazobactam)	Can be used	Mixed bacterial infection: lower respiratory tract, intra-abdominal, skin, and soft tissue infection	
Monobactams	Can be used	Urinary tract, lower respiratory tract, skin-structure, and intra-abdominal infections, patients with penicillin allergy	
Aztreonam			
Carbapenems	Can be used	Resistant Gram-negative bacilli infection with ESBL.	
Imipenem	Drug of choice	Nosocomial infection, when multiresistant Gram-negative bacilli or mixed infections are suspected	
Meropenem	Can be used	Nosocomial infection when multiresistant Gram-negative bacilli or mixed infections are suspected.	
		Meningitis in pediatric population older than 3 months	

First-generation cephalosporins	Drug of choice	Prophylaxis of surgical procedures	
	Can be used	Infection attributed to S. aureus or nonenterococcal streptococci (e.g, skin and soft tissue infections, pharyngitis)	
Second-generation cefuroxime	Can be used	Respiratory tract infections: pneumonia epiglottis, complicated sinusitis, soft-tissue infections, bacteremia	Good H *influenzae* coverage
Cefoxitin	Drug of choice	Pelvic inflammatory disease (+ doxycicline)	
	Recommended	Prophylaxis of colorectal surgery	
	Can be used	Mixed aerobic/anaerobic infections: intra-abdominal infections, skin and soft-tissue infections, including diabetic foot infections and decubitus ulcers	
Cefotetan		Mixed aerobic/anaerobic infections: intra-abdominal infections, skin and soft-tissue infections, including diabetic foot infections and decubitus ulcers	Slightly less anaerobic coverage but better Gram-negative coverage than cefoxitin
Third-generation Ceftriaxone	Drug of choice	Neisseria gonhorroea chancroid, Lyme disease if neurological involvement, carditis, arthritis, or refractory late constitutional symptoms	Once-daily dosing, good CSF penetration
	Recommended	Meningitis attributed to H. *influenzae,* Neisseria meningitis, and penicillin-resistant S. pneumoniae	
	Can be used	Nosocomial infection caused by a sensitive Gram-negative baccilli: pneumoniae, wound, and complicated urinary tract infections	
		Home treatment of chronic infections	
Ceftazidime	Should be used	Infections that are likely attributable to Pseudomonas *aeruginosa*	
	Can be used		
	Drug of choice	Empiric treatment of febrile neutropenia	
		Meningitis attributed to P. *aeruginosa*	
Cefoperazone	Can be used	Empiric treatment of febrile neutropenia	Moderate antipseudomonal activity
Cefepime	Can be used	Infections of lower respiratory tract, urinary tract, skin and soft tissue, and in female reproductive tract	

5 HISTORY AND DISCOVERY OF β-LACTAMS

One of the fundamental milestones in medicinal chemistry is represented by the knowledge acquired during the studies carried out on a small and only apparently simple four-membered ring, the β-lactam. The history of β-lactam can be compared to a sky full of stars and the following paragraphs describe the discovery of the most important β-lactam classes that have had a remarkable impact in the antibacterial field, either as antibacterials or as β-lactamase inhibitors.

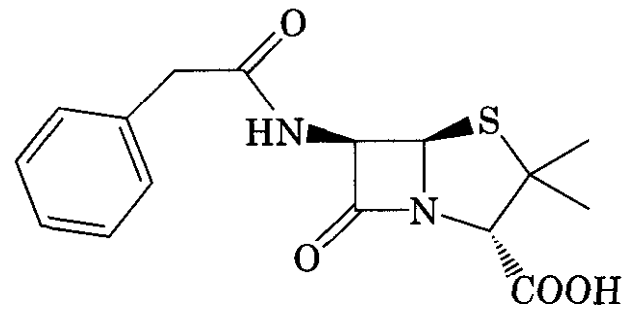
5.1 Penicillin

The antibiotic era began in 1940 with the demonstration made by Florey and his colleagues (186) that penicillin, the substance discovered by Fleming in 1929, was a chemotherapeutic agent of unprecedented potency (187). This story has been recounted many times (188–191) and is here presented only as a brief overview. The observation by Alexander Fleming that partial lysis of Staphylococci colonies occurred on plates contaminated with Penicillium notatum was made in 1928 (192).

Although Fleming cultured the mould and named the active "mould broth filtrate" penicillin, its therapeutic potential was not recognized at that time.

The life-saving capacity of penicillin was first demonstrated in animals (186), and in humans the following year, when the small quantities of penicillin available necessitated the recovery of the antibiotic from the urine of the patient for reuse (193). Once penicillin had been shown effective in humans, the search for improved production was initiated. War-torn Britain was thought unsuitable for such research, so key scientists moved to the United States to continue the effort alongside their American colleagues. Here, replacement of the surface cultures of the low yielding P. notatum with an aerated deep fermentation of a high yielding strain of Penicillium *chrysogenus*, and the use of corn-steep liquor as growth promoter, led to greatly improved penicillin production. This provided sufficient material for the treatment of infections associated with serious battle casualties during the end of World War II and so confirmed the important role of penicillin in the saving of life.

Soon after the demonstration of the extraordinary efficacy in treating infections, penicillin was the subject of in-depth studies by groups on both sides of the Atlantic to solve the structure of penicillin. The correct structure of benzylpenicillin (Pen-G) was eventually identified as the fused β-lactam-thiazolidine bicyclic structure (**1**), as opposed to an



(1) Penicillin G (1929–1940)

oxazolone alternative, by an X-ray crystallographic analysis in 1945 (194).

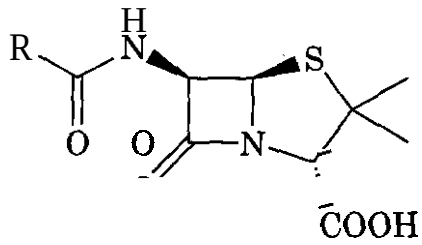
Isolation of reasonably pure samples of penicillin revealed differences in chemical behavior and biological properties between the materials studied by different groups. This arose as a consequence of the different acyl side-chains (RCO) attached to the C6-amino group.

The source of the acyl side chains is the carboxylic acids present in the culture media producing that penicillin; thus, the phenylacetic acid in corn-steep liquor resulted in the phenylacetamido group ($RCO = PhCH_2CO$) of penicillin *G* (1). From among the many penicillins that have been isolated from Penicil*lium* spp. fermentations, either unaided or resulting from the incorporation of added precursor acids (RCO_2H), several of the better-known examples are shown (Table 14.7) with their British and U.S. designations.

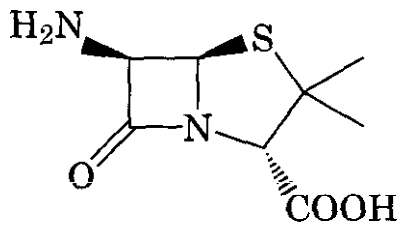
The next major advance was the isolation and characterization of the penicillin nucleus, 6-amino-penicillanic acid (6-APA) (**2**), in 1957 by the Beecham group at Brockham Park (203). This major breakthrough provided a source of chiral material for the preparation of semisynthetic penicillins.

Concurrently with identification of 6-APA (**2**) from biochemical investigations, Sheehan and Henerey-Logan concluded their total syn-

Table 14.7 Biosynthetic Penicillins



(1)	Name	British Name	U.S. Name	R	Ref.
a	2-Pentenyl penicillin	I	F	$CH_3CH_2CH{=}CHCH_2$–	195,196
b	Pentyl penicillin	Dihydro I	Dihydro F	$CH_3(CH_2)_4$–	196,197
c	Heptyl penicillin	IV	K	$CH_3(CH_2)_6$–	196
d	Benzyl penicillin	II	G	$PhCH_2$–	198
e	p-Hydroxybenzyl penicillin	III	X	4-HO-$PhCH_2$–	198
f	(D)-4-Amino-4-carboxybutyl penicillin		N	(D)-HO_2C-$CH(NH_2)(CH_2)_3$–	199
g	(L)-4-Amino-4-carboxybutyl penicillin		iso-N	(L)-HO_2C-$CH(NH_2)(CH_2)_3$–	200
h	Phenoxymethyl penicillin		V	$PhOCH_2$–	197
i	Butylthiomethyl penicillin	BT		$C_4H_9SCH_2$–	201
j	Allylthiomethyl penicillin	AT	O	$CH_2{=}CHCH_2SCH_2$–	201
k	p-Aminobenzyl penicillin		T	4-H_2N-$PhCH_2$–	202
1	p-Nitrobenzyl penicillin			4-O_2N-$PhCH_2$–	202

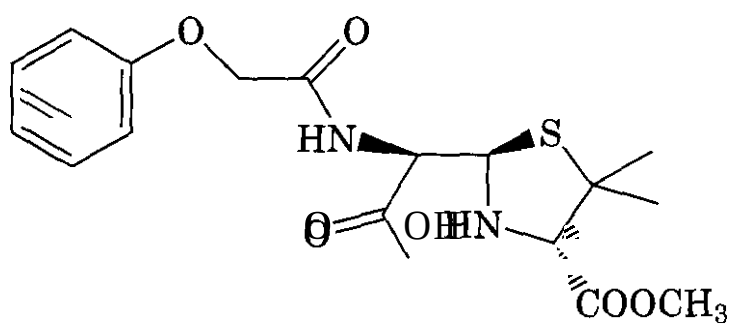


(2) 6-amino-penicillanic acid (6-APA)

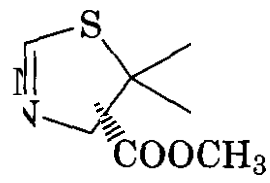
thesis of penicillin **(1)**(204, 205) (see Section 5.1.1) and 6-APA (206, 207). However, this synthesis gave a low overall yield and lacked stereospecificity, so it could not compete with the biochemical route as a practical source of material for chemical modifications. Today, **6-APA** (2), obtained from penicillin *G* by enzymatic cleavage of the phenylacetyl side chain, is a readly available bulk chemical that is used for the preparation of semisynthetic penicillins and related antibiotics.

5.1.1 Synthesis of Penicillin. From the considerable synthetic effort of many groups that began in the 1940s, three major synthetic approaches to penicillin have been reported. The firstone resulted from the already mentioned pioneering work of Sheehan, in which the β-lactam ring was formed by cyclization in very low yield of the penicilloic methyl ester **(3)** with dicyclohexylcarbodiimide (DCCI) (204,205).

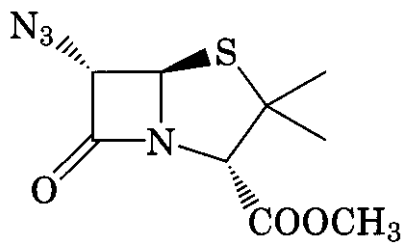
In the Bose approach, formation of the β-lactam ring through cycloaddition of the dihydro-thiazoline (4) and the ketene derived from azidoacetyl chloride gave the thermodynamically favored, unnatural *trans* β-lactam penicillin structure (5)(208).



(3)

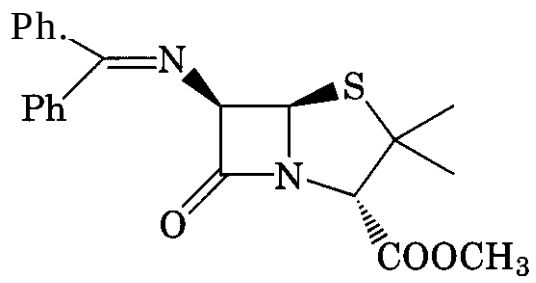


(4)



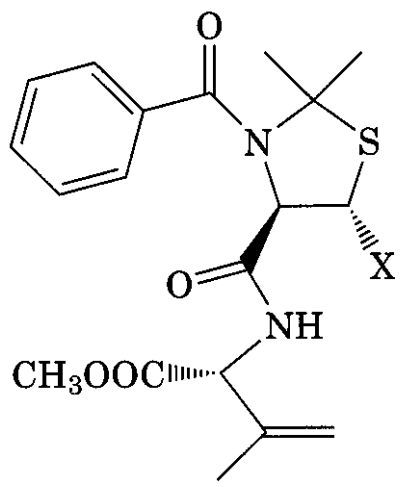
(5)

Subsequently, however, a method was reported for equilibration of the C6-stereochemistry, to give some of the cis β-lactam possessing the natural stereochemistry, by kinetic quenching of the anion generated from the derived Schiff base (6)(209).



(6)

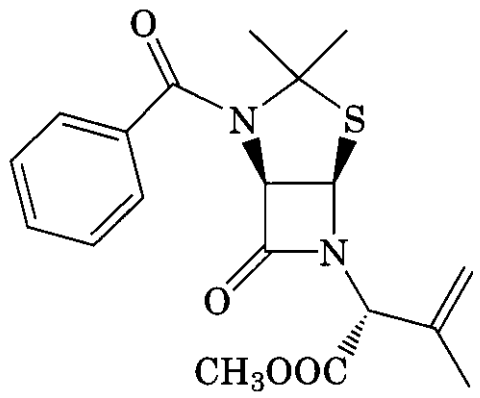
It was not until 1976 that the only stereoselective synthesis of penicillin was described by Baldwin (210). Thus, the peptide precursor (7), derived from cysteine and D-isodehydrova-



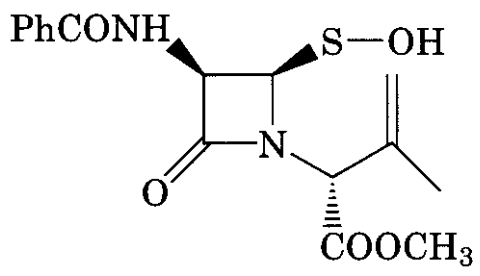
(7) X = H
(8) X = Cl

line methyl ester, was cyclized through the chloride (8)to the β-lactam (9).

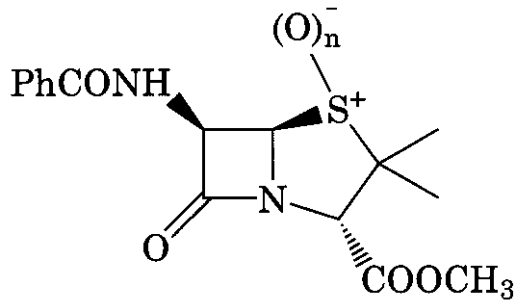
Conversion of the latter by a multistep process then generated the sulfenic acid (10), which cyclized to provide the sulfoxide (11).



(9)



(10)

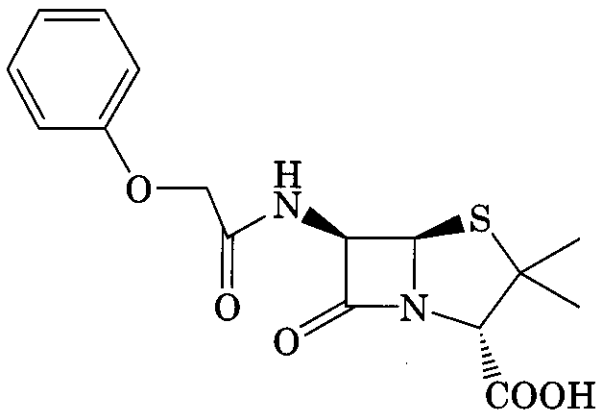


(11) n = 1
(12) n = 0

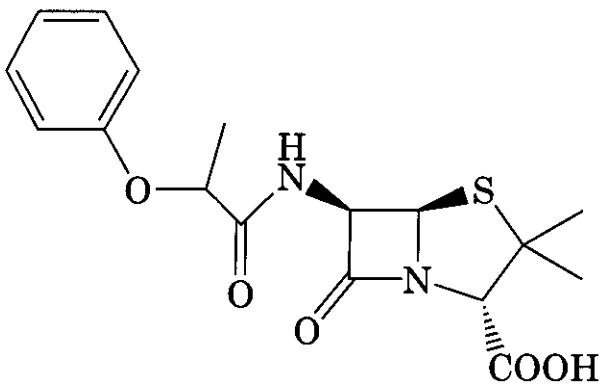
Finally, deoxygentation gave the penicillin ester (12).

5.1.2 Derivatives of 6-Amino-Penicillanic Acid (6-APA): Biological Activity and Structure-Activity Relationship (SAR). The availability of 6-APA (2)led to the preparation of many thousands of semisynthetic penicillins, through N-acylation of the C6-amino group. Most, if not all, of the methods used in peptide chemistry have been employed for this process (211). This increase in the scope of side-chain variation allowed significant improvements in the biological properties of the derived analogs, including greater stability to penicillinase (β-lactamase) and an expanded spectrum of antibacterial activity. Some early examples of

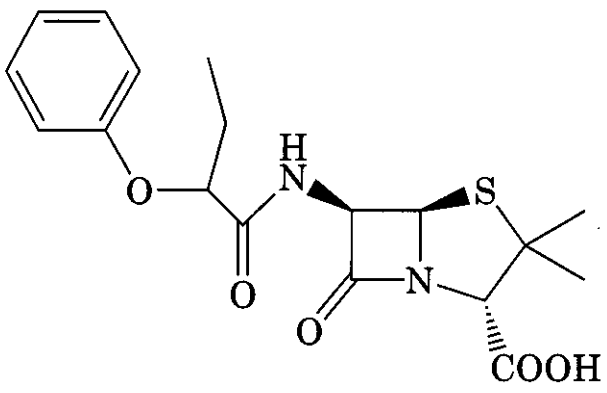
semisynthetic penicillins to be studied in humans were substituted derivatives of the acid-stable penicillin V (**13**), such as phenethicillin (14) and propicillin (**15**), which, despite being



(13) Penicillin V



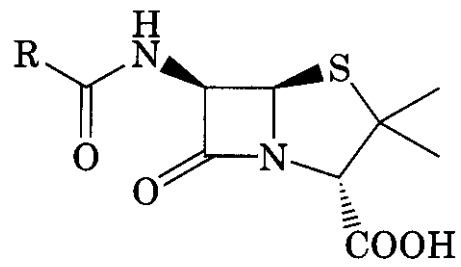
(14) Phenethicillin

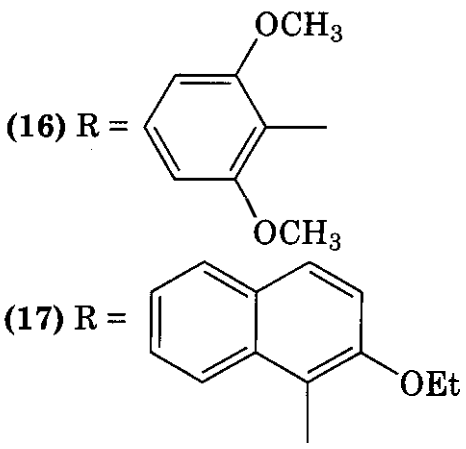


(15) Propicillin

mixtures of diastereoisomers, offered increased oral absorption, thus giving higher and more prolonged blood levels than those of penicillin V (212).

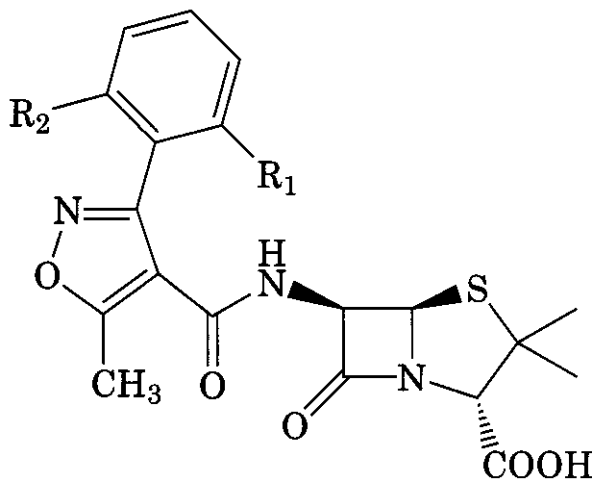
The fast widespread use of penicillin G and probably the misuse or abuse of this drug had already resulted in an increase in the occurrence of penicillinase (β-lactamases)-producing strains of S. ***aureus.*** By 1960 this was becoming a worldwide clinical problem (213). Often these strains of S. ***aureus*** were virulent and resistant to most other antibiotics, thus making them difficult to eradicate. Much early research was therefore directed at identifying new penicillins resistant to inactivation by these penicillinases. Methicillin (16) was introduced into clinical usage in 1960 (214), followed by nafcillin (17) (**215**) and the isoxazole penicillins, oxacillin (**18a**) (216), cloxacillin (**18b**) (217), dicloxacillin (**18c**) (218), and flucloxacillin (**18d**) (219). In all these cases, the steric bulk of the side-chain group adjacent to





(16) R =

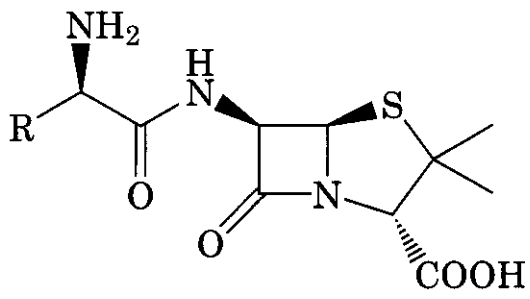
(17) R =



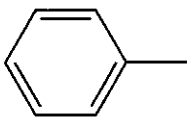
(18a) $R_1 = R_2 = H$
(18b) $R_1 = H$, $R_2 = Cl$
(18c) $R_1 = R_2 = Cl$
(18d) $R_1 = F$, $R_2 = Cl$

the amide carbonyl group is believed to protect the β-lactam ring from unwanted hydrolysis by the bacterial enzyme penicillinase.

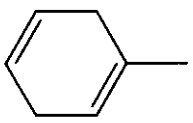
The aminopenicillins such as ampicillin **(19)** (220), epicillin **(20)** (221), amoxycillin



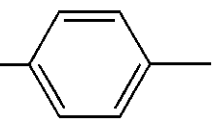
(19) R =

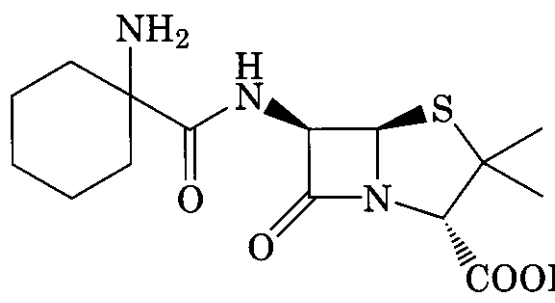


(20) R =



(21) R = HO



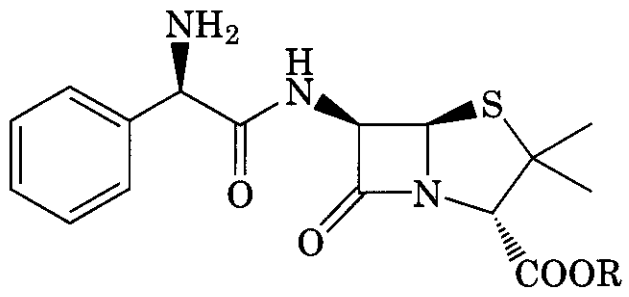


(22)

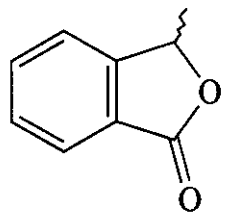
(21) (222), and cyclacillin **(22) (223)** introduced into the clinic between **1961** and **1972,** are characterized by their broad spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria and good oral absorption.

Subsequently the prodrug esters talampicillin **(23)** (224), bacampicillin **(24)** (225), pivampicillin **(25)** (226), and lenampicillin **(26)** (227), which all release ampicillin **(19)** once absorbed, have been developed as agents that improve the oral absorption of ampicillin (**19**). With these esters, the oral absorption of ampicillin approaches the excellent level achieved by amoxycillin **(21).**

One of the next challenges to be addressed by researchers was to extend the spectrum of antibacterial acitivity to cover the opportunistic pathogen *Pseudomonas aeruginosa.* Car-



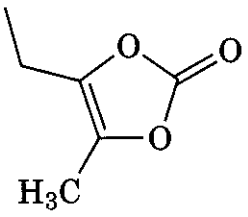
(23) R =

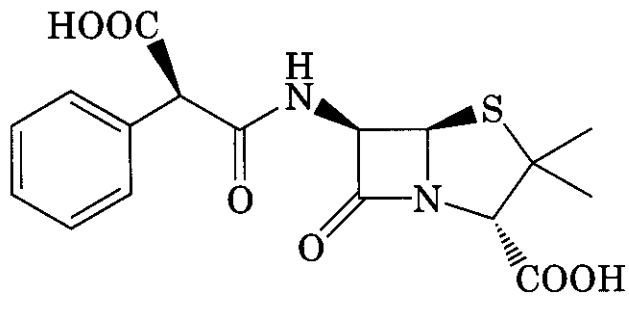


(24) R = $CH(CH_3)OCO_2Et$

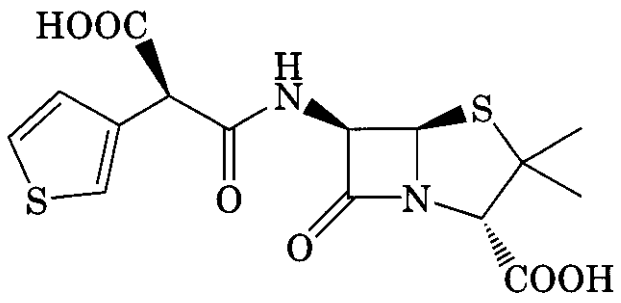
(25) R = $CH_2OCOC(CH_3)_3$

(26) R =





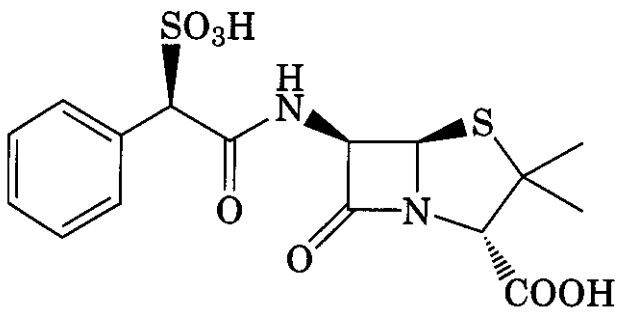
(27)



(28)

benicillin **(27)** (228), ticarcillin **(28)** (230), and sulbenicillin **(29)** (229), all derivatives with an acidic group in the acylamino side chain, are parenteral agents with adequate antipseudomonas potency combined with good safety tolerance that have found clinical use.

A second generation of penicillins active against *Pseudomonas* species was obtained by acylation of the side-chain amino group of ampicillin **(19)** or amoxycillin **(21).** A selection from the many N-acylated aminopenicillin an-

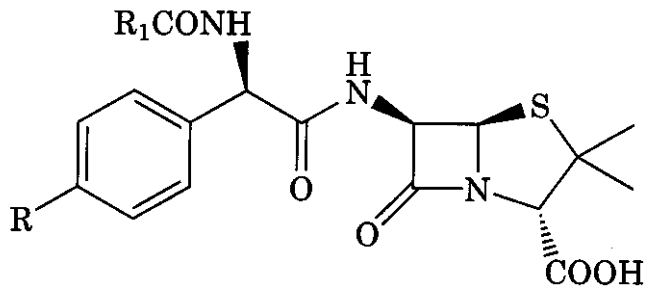


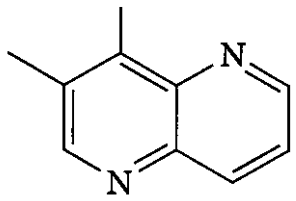
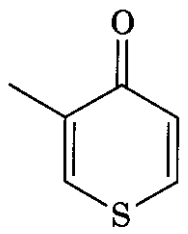
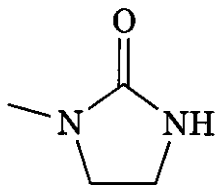
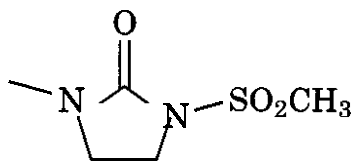
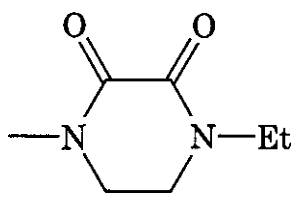
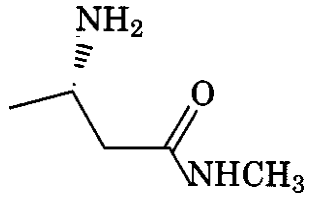
(29)

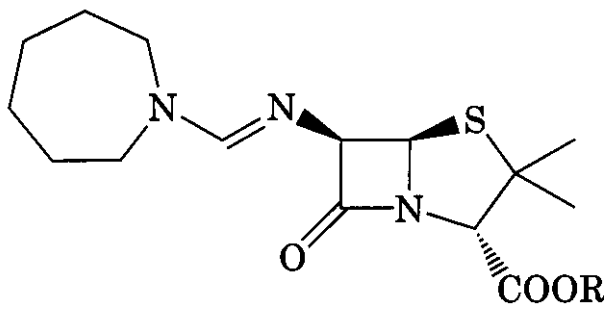
alogs (**30–35**) that have been considered for development as antipseudomonas agents is shown in Table 14.8 (**30–35**). Of these the most successful has been piperacillin (**34**), which together with ticarcillin (**28**), remains the penicillin of choice for treating infections caused by this organism.

In the 1970s the amidino penicillin, **mecillinam** (**36**), which is unusual in that it has a nonacyl side chain and antibacterial activity

Table 14.8 N-Acylated Aminopenicillins with Activity Against *Pseudomonas aeruginosa*



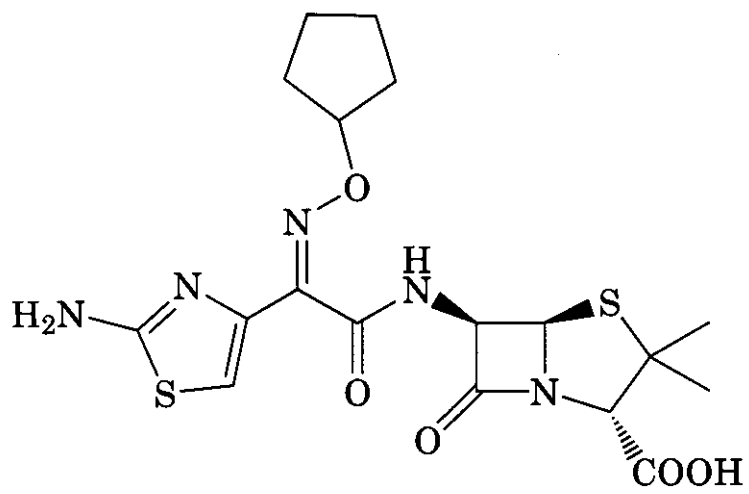
Compound	Name	R	R_1	Reference
(**30**)	Apalcillin	H		**231**
(**31**)	Timoxicillin	OH		232
(**32**)	Azlocillin	H		233
(**33**)	Mezlocillin	H		234
(**34**)	Piperacillin	H		**235**
(**35**)	(D)-Asparagine derivative	OH		**236**



(36) R = H
(37) R = $CH_2OCOC(CH_3)_3$
(38) R = $CH(CH_3)OCO_2Et$

limited to Gram-negative organisms, was reported (237). Also, the oral pivaloyloxymethyl (238) and carbonate prodrug esters have been described (239).

A recent publication describes a further range of C6-acylamino β-lactamase stable penicillins from which BRL-44154 (39) was



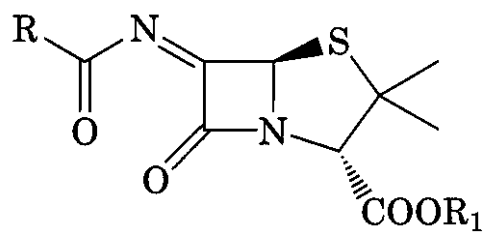
(39) BRL-44154

identified as exhibiting high activity against Gram-positive organisms including methicillin-resistant Staphylococci (MRSA) (240).

Table 14.9 shows a comparison of the in *vitro* antibacterial activities of representative penicillins, including C6-a-substituted derivatives described below (see Section 5.1.2.1). Further information on the penicillin described above (and many more that have not been included) is available from the references in the data review of β-lactam antibiotics by Rolinson and more recently by Wright (241, 242).

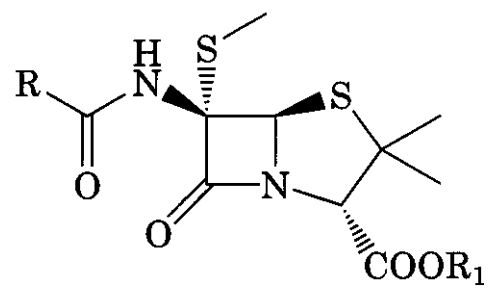
5.1.2.1 ***C6-Substituted Penicillins.*** Early interest in C6-substituted penicillins arose from the idea that the presence of a C6-α-methyl group in a penicillin molecule would provide a closer mimic of the D-alanyl-D-alanine component of the cell wall (244, 245). Although this was not shown to be the case (246, 247), the discovery of the naturally occurring C7-α-methoxy cephalosporin derivatives (cephamycin, Section 6.2), turned attention to the synthesis of penicillins having this and other substituents in the C6-position.

In many cases the methodology for the introduction of the methoxy substituent parallels that developed for the cephalosporins (248). A method for direct methoxylation of a C6-acylaminopenicillin relies on the addition of methoxide to an acylimine (40) (249, 250),



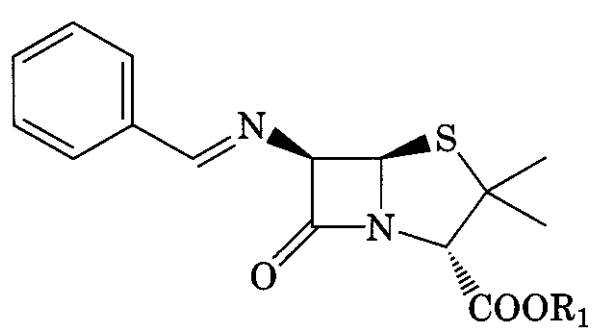
(40)

whereas an indirect procedure involves the addition of methanol to the putative acylamine formed from the C6-a-methylthiopenicillin (41) in the presence of a mercuric salt (251).



(41)

The C6-α-methylthio substituent, as well as alkyl and substituted alkyl, is available from the anion of the C6-Schiff base (42) with a suitable reagent (247, 251, 252). The C6-α-stereochemistry of the new substituents is en-



(42)

Table 14.9 In *Vitro* Antibacterial Activity of Penicillins[a,b]

Organism	Pen G	Meth	*Clox*	Amox	Ticar	Pip	Mecill	BRL-44154	6a-Substituted	
									Temo	*Form*
E. coli	50–>100	>100	>100	2–>100	0.8–>100	0.06–>64	<0.4–50	1–>64	2–16	<0.03–0.5
K. ***pneumoniae***	0.5–100	>100	>100	16–100	25–>100	1–>64	<0.4–>100	8–>64	1–>100	<0.03–2
Enterobacter sp.	50–>100	>100	>100	12.5-100	0.8–>100	1–>64	<0.4–>100	16–>64	1–32	<0.03–0.12
Citrobacter sp.	>100	>100	>100	5–> 100	<1.6–>100	1–>64	0.5–>100	8–>32	2–16	<0.03–16
S. marcescens	>100	>100	>100	>100	4–>100	2–>64	0.8–>100	16–>64	8–>100	<0.03–8
P. ***mirabilis***	16–32	>100	>100	0.8–>100	<1.6–>100	0.06–64	3.2–>100	2–>64	0.5–16	0.06–0.25
Proteus sp (In. +)	0.4–>100	6.3–>100	50–>100	0.4–>100	<1.6–100	0.06–>64	1.6–>100	2–>64	1–4	<0.03–2
P. ***aeruginosa***	>100	>100	>100	>64	3.2–>100	1–>64	12.5>100	16–>64	>100	0.12–16
H. ***influenzae***	0.08–>100	—	2–>100	0.05–>64	0.25–>100	<0.03–2	16–>100	0.06–2	0.5–4	0.06–0.5
B. ***fragilis***	0.08–>25	>100	>100	0.12–>32	<1.6–>100	3–100	>100	8–>64	16–128	32
S. ***aureus***	<0.005–>100	0.4–2.5	0.12–2	0.05–>100	0.4–25	0.8–100	1.6–>100	0.25–1	>100	>100
S. pyogenes	0.005-50	0.1–0.8	<0.03–0.06	<0.03–0.12	0.2–0.8	<0.015–0.12	0.8–6.3	0.03–0.06	>100	4
S. ***pneumoniae***	0.006–2	0.1–0.2	0.12	<0.03–4	1.25–2	0.015–0.06	0.8–12.5	<0.03–8	>100	4
N. ***gonorrhoeae***	0.003–>100	0.05–2	0.12–>64	0.01–>16	0.02-16	0.015–1	0.03–8	—	0.03–8	<0.008–0.06

[a]Minimum inhibitory concentrations (range mg/L). Refs. 241,265,243, and GSK in house data.

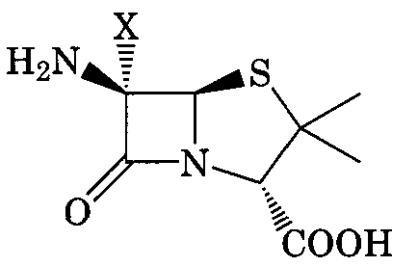
[b]Abbreviations: Pen G, benzylpenicillin; Meth, methicillin; Clox, cloxacillin; Amox, amoxicillin; Ticar, ticacillin; Pip, piperacillin; Mecill, mecillinam; Temo, temocillin; Form, Formidacillin.

sured, given that the incoming group always approaches from the less-hindered α-face of the β-lactam ring (252).

Other methods for introduction of methoxy and a variety of alkyl or substituted alkyl variations make use of diazo-intermediates (253) or isonitrile chemistry (254).

Alternative reactive intermediates for the insertion of a C6-α-substituent have been generated by way of keteneimines (255), sulfenimines (256), and quinolone methide intermediates (257).

After the methoxy series a wide variety of the other C6-α-substituents were investigated on the penicillin ring system, and it was found that a C6-a-formamidino group with an appropiate side chain provided a series of highly active antibiotics (258).At the time of this discovery naturally occurring β-lactams possessing this substituent were not known. Introduction of the C6-a-formamido substituent followed a similar pattern to that of the methoxy group. Initially, this was obtained by displacement of the methylthio group from (41)by ammonia in the presence of a mercury salt, followed by formylation of the C6-α-amino group. Subsequently, a more direct addition used *N,N*-bis trimethylsilylformamide (BSF) (258,259).In both the methoxy and formamido series it is also possible to generate the C6-amino nucleus (43), which can be ac-

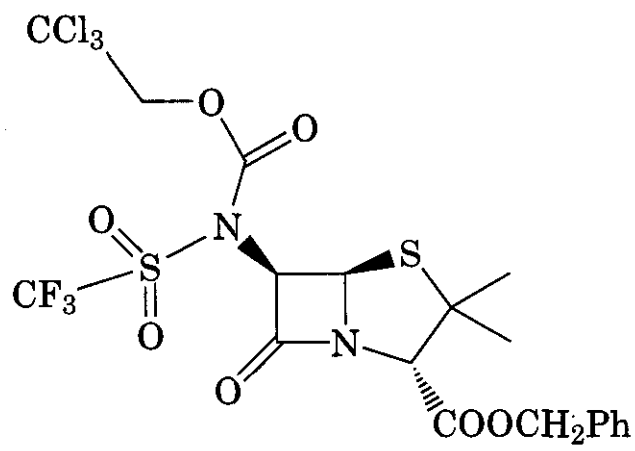


(43) X = OCH_3, NHCHO

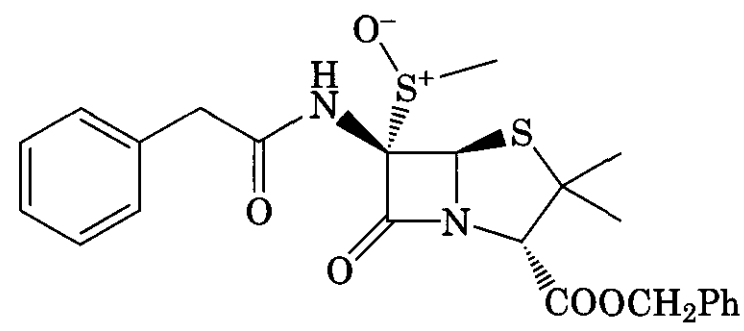
ylated with the appropriate side-chain acid as required (259).

Other methods for the introduction of the formamido group make use of the *N*-trifluoromethylsulfonylamino penicillin (44) (260) or the C6-α-methylsulfinyl penicillin (45) (261)to generate the acylimine, which is then trapped with BSF.

A feature of the 6,6-disubstituted penicillins is the stability of the β-lactam ring to hydrolysis by β-lactamases, but usually the

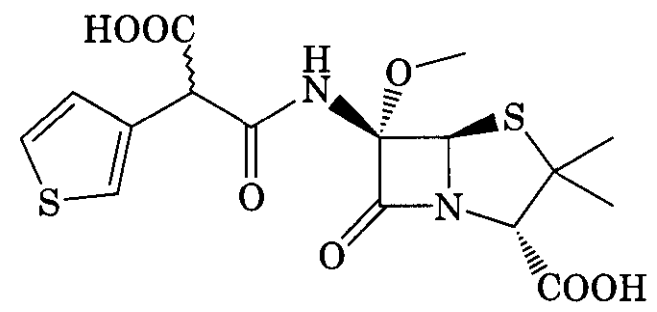


(44)



(45)

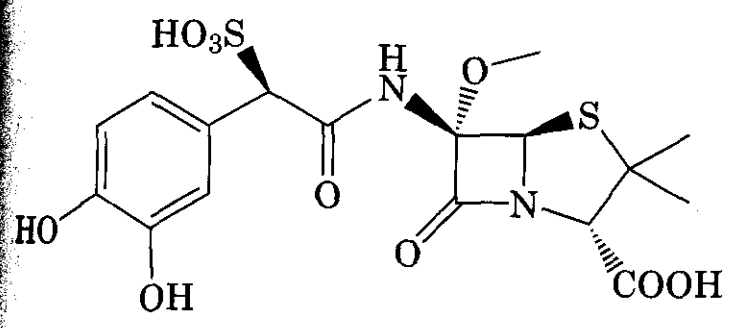
antibacterial potency is compromised in comparison to the C6-a-hydrogen analogs. Only one derivative, temocillin (46) (262), has pro-



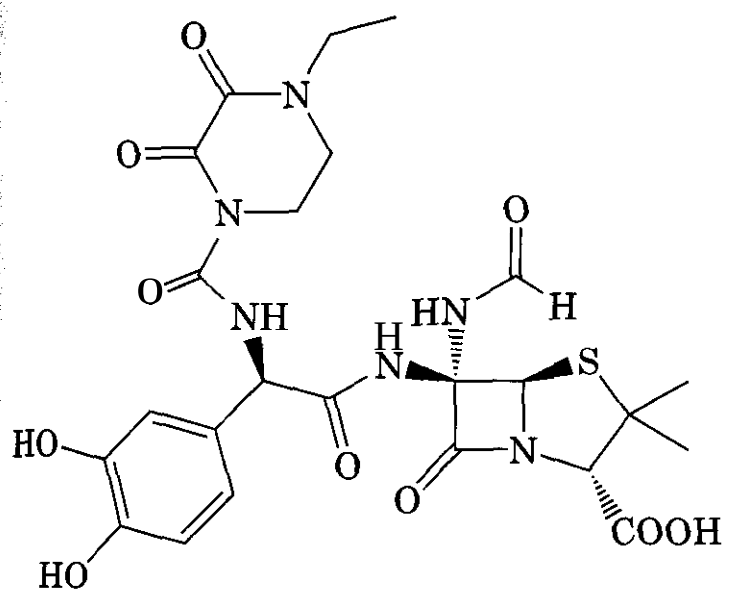
(46)

gressed to the clinic but, in spite of the prolonged blood levels in humans ($t_{1/2}$ = 4.5–5 h), has found limited utility because its spectrum of antibacterial activity is restricted to Gram-negative organisms only. When the thienyl carboxylic acid moiety was replaced by a catecholic sulfonic acid, to give (47), the spectrum of antibacterial activity was expanded to include P. *aeruginosa* but not the Gram-positive organisms (263).

After an extensive research program (264) the combination of the N-acylated dihydroxyphenylglycyl C6-a-formamidino group produced formidacillin (BRL-36650, 48) (265). This derivative was highly potent against



(47)



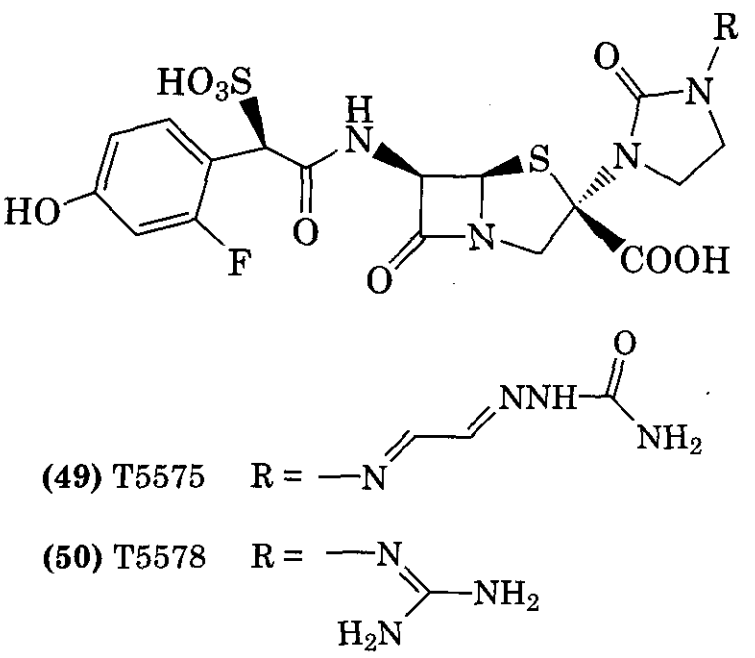
(48) BRL-36650

Gram-negative organisms, including *Pseudomonas* species, and possessed some activity against Streptococci but not against the Staphylococci.

5.1.3 Other Modified Penicillins. Chemical modification of most of the other positions (S1, C2, C3, and C6) of the penicillin molecule have been reported and summarized elsewhere (211). More recent reports have described novel 2-carboxypenam analogs T5575 (**49**) and T5578 (**50**), in which both the C2- and C3-substituents have been changed from those of the natural penicillins (266–269). These compounds displayed potent antibacterial activity against Gram-negative organisms, including *Pseudomonas* aeruginosa, with stability to β-lactamases generally greater than that observed for the natural penicillin structure.

5.2 Cephalosporins and Synthetic Analogs: Oxacephems, Carbacephems

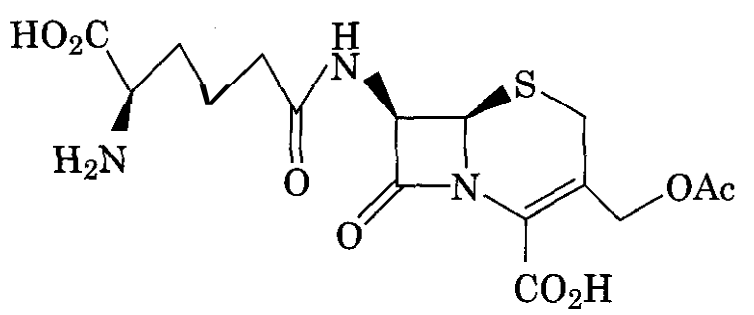
After penicillins, cephalosporins represent the best-known β-lactam class. The discovery of



(49) T5575 R =

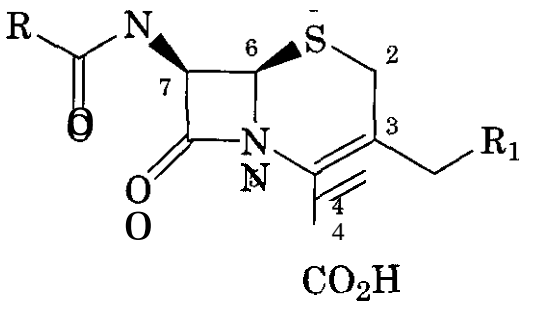
(50) T5578 R =

this class followed a similar story. In fact, the antibacterial activity of a metabolite produced by Cephalosporium *acremonium* was originally detected in 1945 by Brotzu, an Italian scientist in Sardinia who isolated a strain from sewage outfall (270). Subsequent work by Abraham at Oxford resulted in the isolation and characterization of the active drug, in particular cephalosporin C (**51**) (271, 272). Confirmation of its structure was later obtained by X-ray crystallography (273).



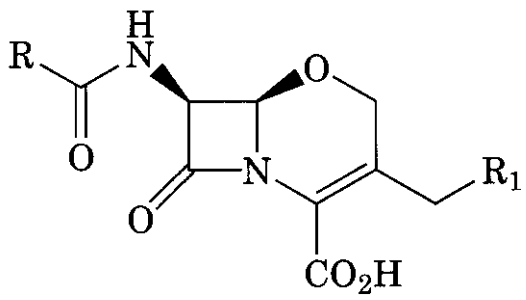
(51) Cephalosporin C (1948)

Cephalosporin C (51) attracted the attention of scientists mainly because of its stability to penicillinases. Cephalosporins (**52**) are characterized by a bicyclic ring system, in which the β-lactam is fused to a six-membered

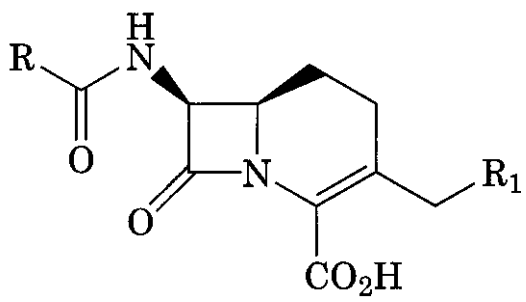


(52) cephalosporins

ring bearing at position 1 a sulfur atom; oxacephalosporins (**53**), an oxygen atom; and carbacephalosporins (**54**), a carbon atom.



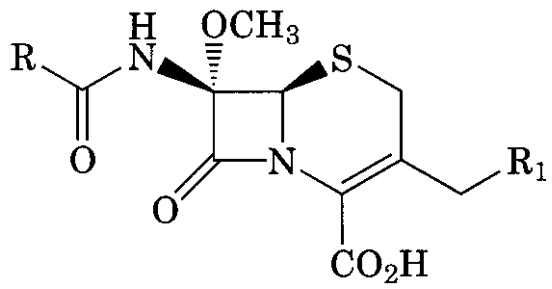
(53) oxacephalosporins



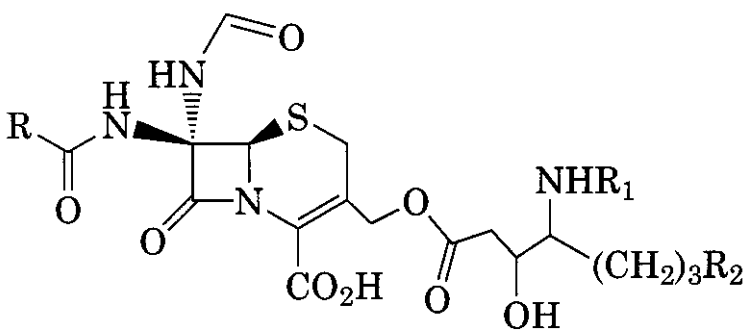
(54) carbacephalosporins

The isolation of the first cephalosporin was followed by the discovery of other natural metabolites from fungi and actinomycetes, including those with a C7-α-methoxy substituent (55) (274) and a C7-α-formamido group (56) (275).

5.2.1 Derivatives of C7-Aminocephalosporanic Acid. The structure of naturally occurring members of this series is characterized by



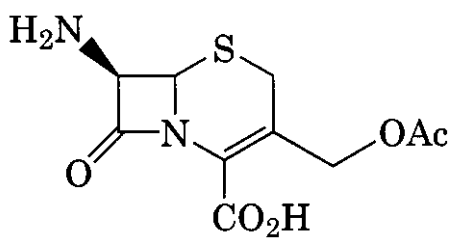
(55) cephamycins



(56) cephabacins

the a-aminoadipic side chain at C7 (271, 272, 274,276–280). Some variations have been isolated that have the side chain derived from glutaric acid (281) or 5-hydroxy-5-carboxy-valeric acid (282,283).

The natural products exhibit a low level of antibacterial activity. Cleavage of the amide bond of the aminoadipoyl side chain of cephalosporin C (51) is a high yielding process (284) that affords 7-amino-cephalosporanic acid (7-ACA, **57**), ideally suited for the synthesis of a

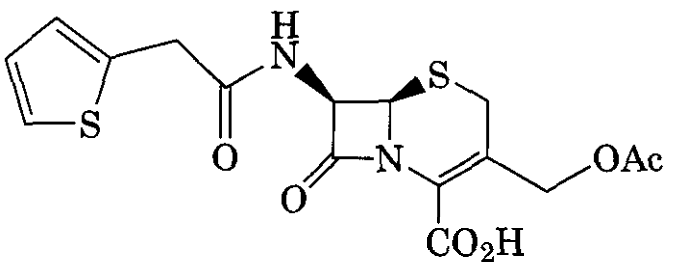


(57) 7-amino-cephalosporanic acid (7-ACA)

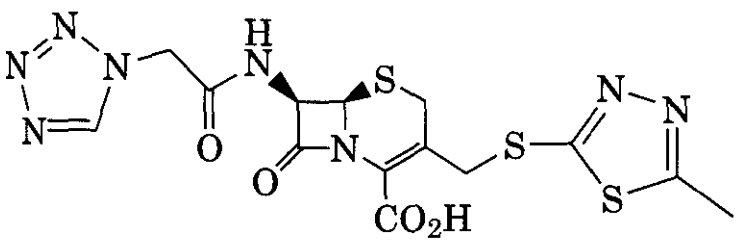
wide range of semisynthetic cephalosporins by acylation of the C7 amino group.

In cephalosporins there are two positions available for chemical manipulation, C3 and C7. A wide variety of amine acylation methods have been used for the production of C7-acylamino derivatives by the use of acyl chlorides, mixed anhydrides, active esters, and carbodiimides (285). To improve the chemical reactivity of 7-ACA (**57**), the solubility in organic solvents is increased by conversion of the carboxylic acid at C4 of 7-ACA (57) into an ester such as tert-butyl dimethylsilyl, benzhydryl, *p*-nitrobenzyl, orp-methoxybenzyl. However, ester derivatives of 7-ACA (57) become very sensitive to basic conditions and the Δ^3 double bond isomerizes to the unwanted Δ^2 isomer (286).

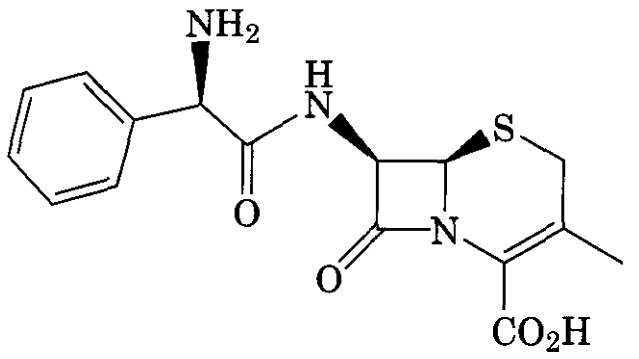
Reactions at C3 have mainly involved displacement of 3'-acetate, to give substitution with heteroatoms, especially sulfur and nitrogen (287), whereas elimination followed by catalytic hydrogenation (288) or acid-catalyzed reduction by use of trialkylsilanes leads to 3-methyl cephalosporins (285). An alternative to the displacement of the 3'-acetoxy for the synthesis of 3'-heteroatom cephalosporins is through functionalization of a 3-methyl derivative of a cephalosporin sulfoxide by allylic bromination to the 3'-bromomethyl compound suitable for displacement reactions (286). Hydrolytic deacylation by base or ester-



(58) Cephalothin (first generation)



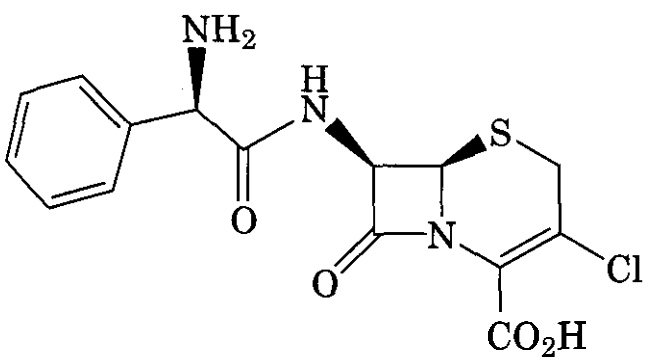
(59) Cefazolin (fist generation)



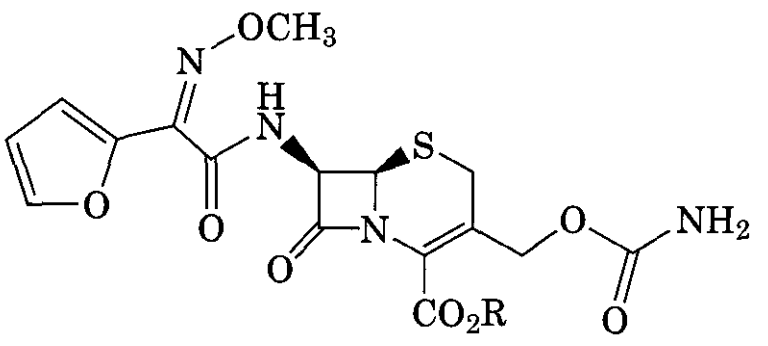
(60) Cephalexin (first generation)

ase gives 3'-hydroxymethyl cephalosporins (289), which can be converted into carbamoyl compounds such as cefuroxime (**62a**) (290).

Other important intermediates are 3-exomethylene derivatives that, by ozonolysis of the exocyclic double bond, provide an entry to cephalosporins having a heteroatom attached directly at C3 as in the commercially important cefaclor (**61**) (291). The exomethylene compounds are also easily converted to 3'-bromomethyl derivatives with bromine and base



(61) Cefaclor (second generation)

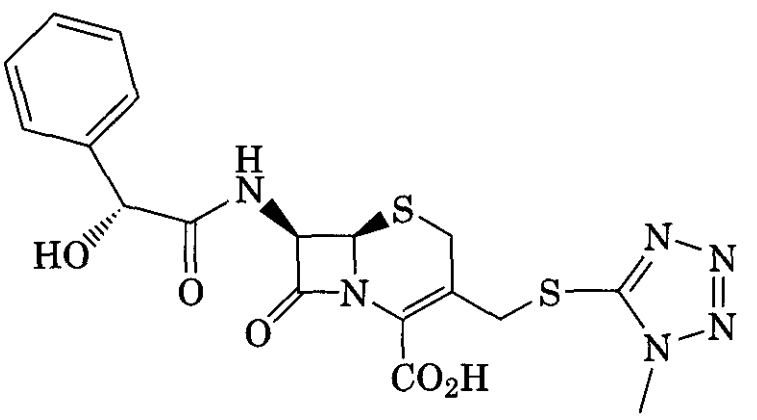


(62a) Cefuroxime (second generation) R = H
(62b) Cefuroxime axetil R = $CH(CH_3)OAc$

(292). A summary of many of the common reactions and derivatives of 7-ACA (57) is given in Fig. 14.7.

5.2.2 Classification, Biological Activity, and Structure-Activity Relationship of Cephalosporins. Some 50 different cephalosporins are in clinical use or at an advanced stage of development (241, 285, 293, 294) and many attempts have been made to classify these based on stability to β-lactamases, potency, antibacterial spectrum, and pharmacological properties. The most common approach has been to divide the group into various generations primarily based on their antibacterial spectrum, with both parenteral and oral agents being covered (285,295,2961,and also on their level of chemical sophistication. First-generation derivatives such as cephalothin (58)(297), cefazolin (59) (298), and the orally absorbed cephalexin (60) (299) possess activity against Gram-positive bacteria, but a relatively narrow spectrum against Gram-negative strains attributed in part to their susceptibility to β-lactamases.

Second-generation compounds such as cefamandole (63) (300) and cefuroxime (**62a**) (301) have a broader spectrum of activity, with enhanced activity against H. *influenzae* and



(63) Cefamandole (second generation)

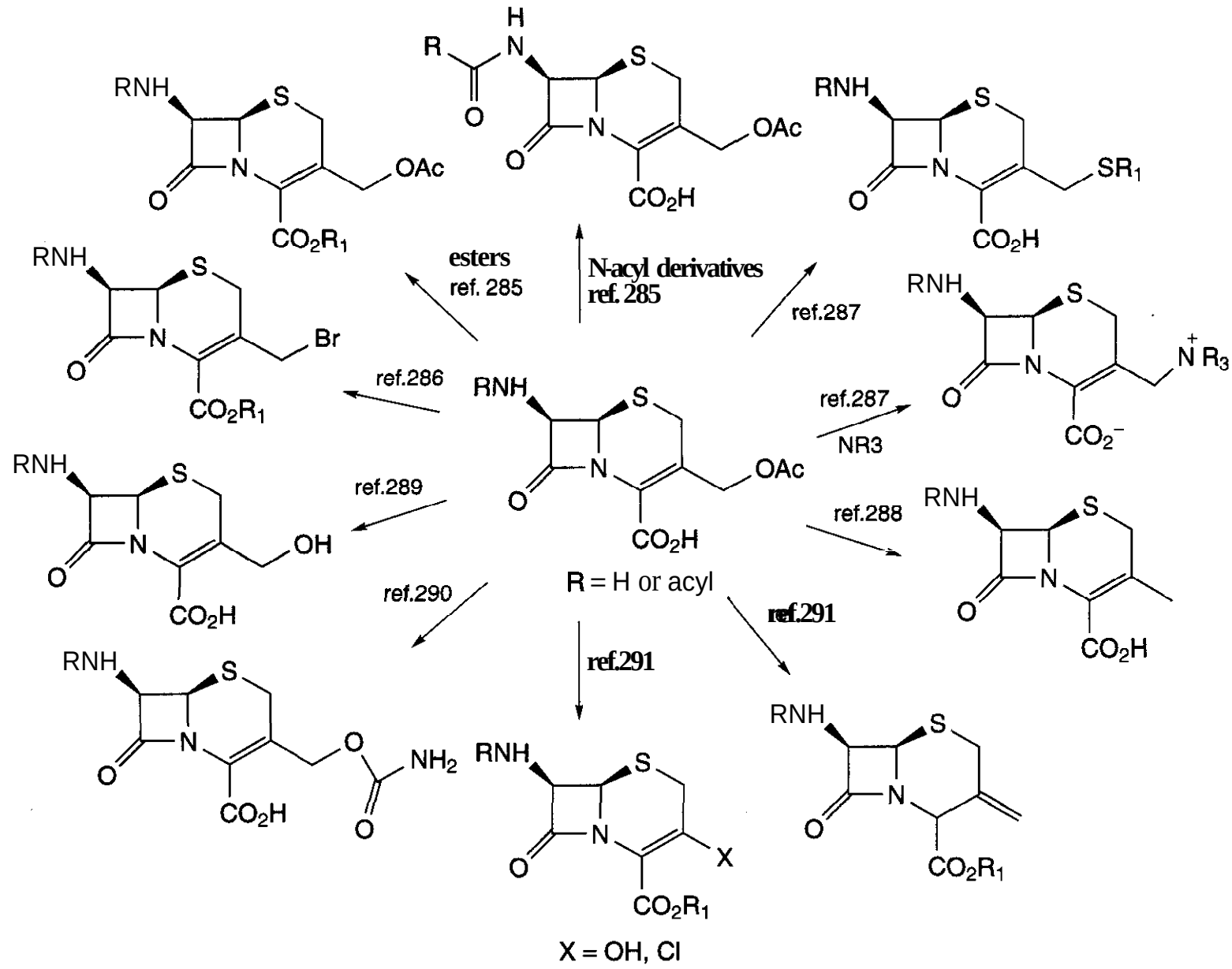
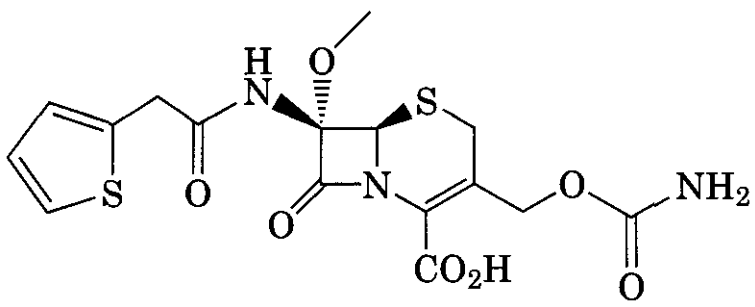


Figure 14.7. Summary of some common reactions and derivatives of 7-ACA.

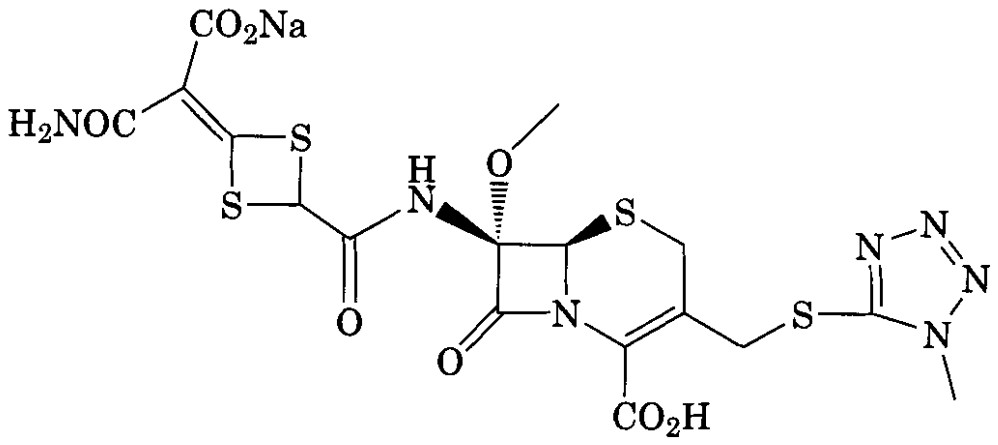
the Enterobacteriaceae resulting from an increased stability to β-lactamases. Oral agents in this group are cefaclor (**61**) (302) and the axetil ester of cefuroxime (**62b**) (303). The highly β-lactamase stable cephamycins such as cefoxitin (64) and cefotetan (**65**) are also



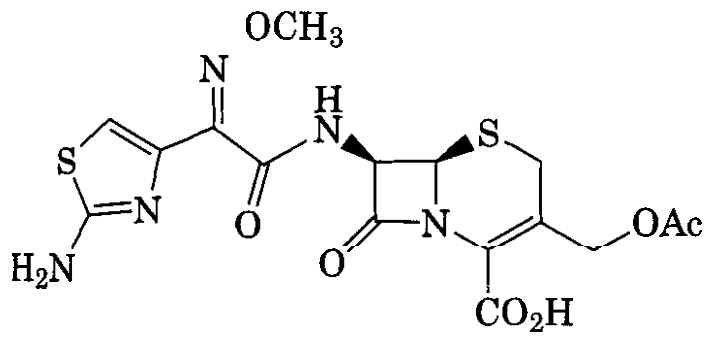
(64) Cefoxitin **(second generation)**

included in this group and show excellent activity against Bacteroides *fragilis* (300,304).

The third-generation cephalosporin compounds, which originated with cefotaxime (66) (305, 306), have a broader spectrum of activity, especially against the Enterobacteriaceae. They show several important advantages over the first- and second-generation compounds, including increased resistance to many plasmid and chromosomally mediated β-lactamases (296). Ceftriaxone (67) (305, 307) is an example that has a very prolonged half-life ($t_{1/2}$ = 7–8 h in humans), making it suitable for once-daily administration. Compounds such as cefoperazone (68) (308) and ceftazidime (69) (309) show improved activity against Pseudomonas aeruginosa, which is also a predominant feature of cefsulodin (70) (305,310). The only oral agent of this group is cefixime (71) (311). The third-generation cephalosporins show potent anti-Gram-negative but modest anti-Gram-positive activity, being inferior to the first-generation agents in this respect, and like the majority of cephalo-



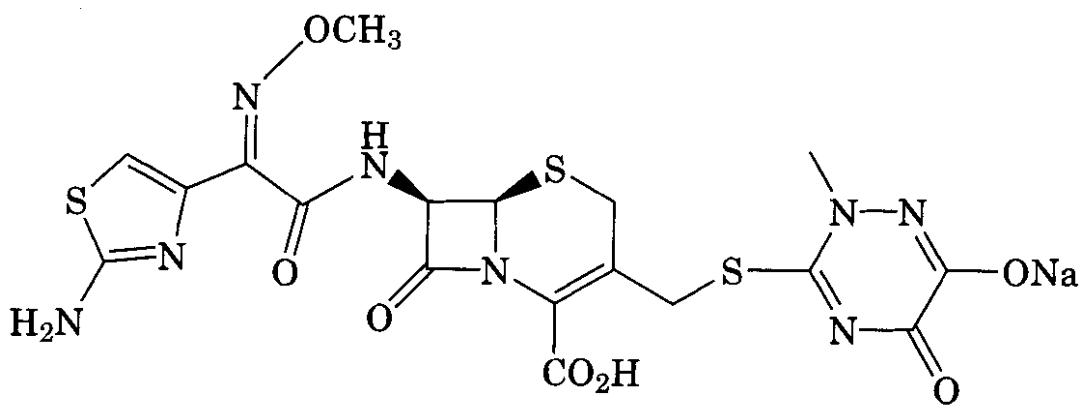
(65) Cefotetan (second generation)



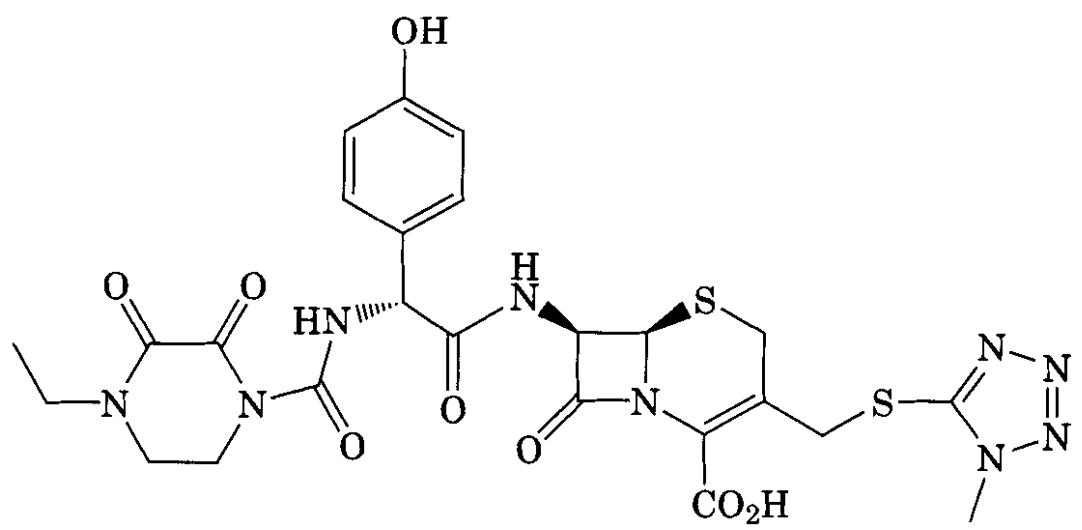
(66) Cefotaxime (third generation)

sporins described so far, show no activity against methicillin-resistant Staphylococci (MRS) (296).

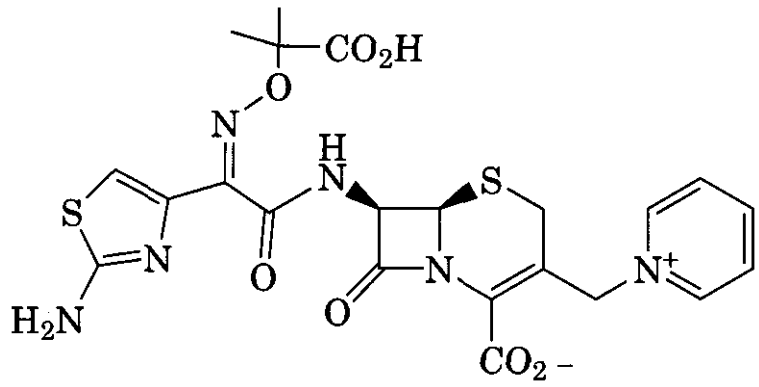
Compounds such as cefpirome (**72**) (312) and cefepime (73) (313), and others undergoing clinical development (314, 315), constitute examples of the fourth-generation cephalosporins (285), which show some slight further advantages. However, although recent research effort in the cephalosporin field has identified new derivatives able to treat methicillin-resistant *Staphylococcus aureus* strains (see Sec-



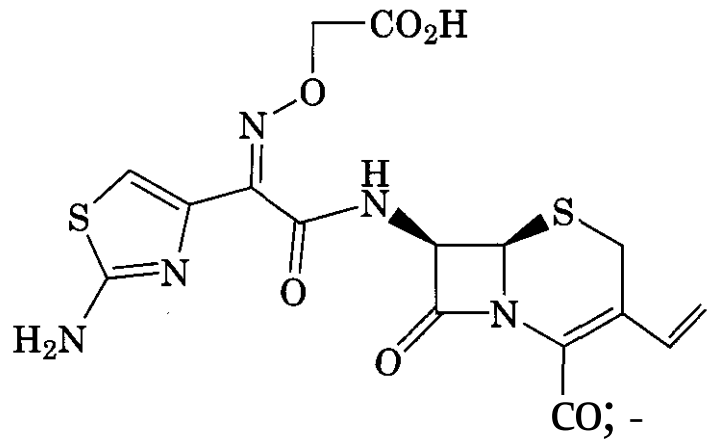
(67) Ceftriaxone (third generation)



(68) Cefoperazone (third generation)



(69) Ceftazidime (third generation)



(71) Cefixime (third generation)

tion 6.1.1), problems with extended-spectrum β-lactamases (ESBLs) still undermine their potential use as broad-spectrum agents.

Table 14.10 shows a comparison of the in vitro antibacterial activities of representative cephalosporins of these groups.

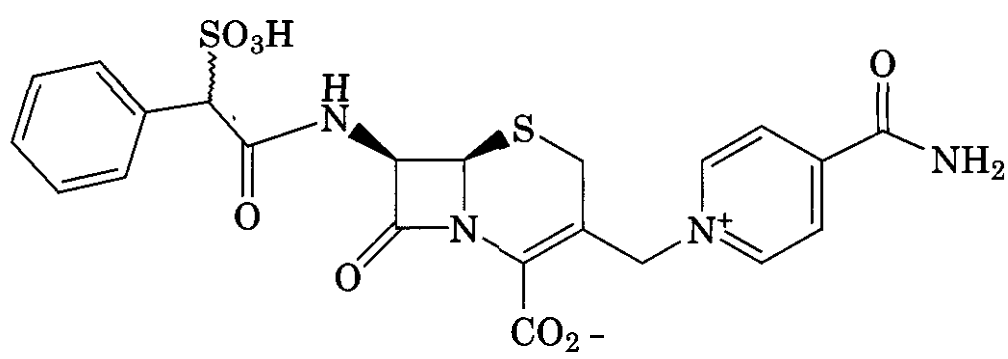
Ultimately, the biological activity of the cephalosporins depends on their affinity and interaction with the target enzymes (PBPs). However, many other factors such as penetration to the target site, β-lactamase stability, pharmacokinetic parameters, and metabolic stability will all influence the final antibacterial effectiveness of these agents. Most structure-activity relationships (SARs) have been derived from in vitro activities against whole cells by use of MIC values to compare structural variations.

Major factors influencing these properties result from changes in the C7-acylamino substituent or C3 variations, although virtually all positions of the cephalosporin nucleus have been modified (296), as illustrated in Fig. 14.8.

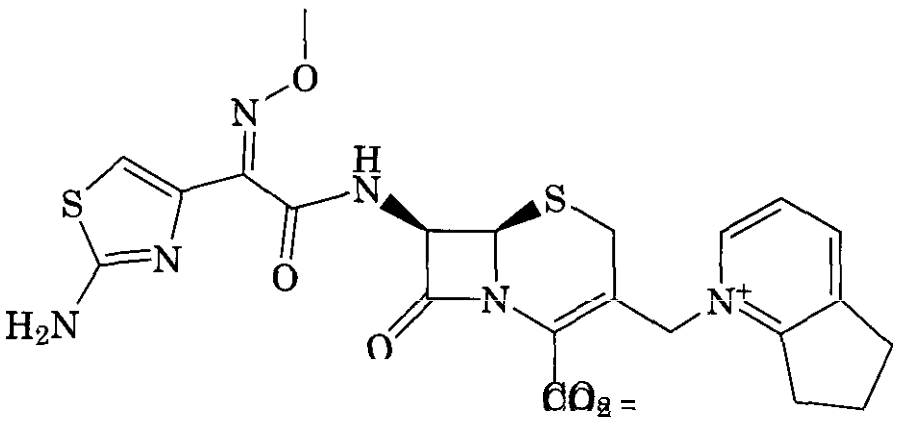
Several highly active derivatives have resulted from replacement of the sulfur atom with oxygen or carbon or by transposition of the heteroatom from position 1 to position 2 ("isocephem" derivatives), whereas substitution at C7 strongly affects β-lactamase stability.

A large number of acyl groups have been introduced at C7 and showed significant changes in both potency and spectrum of activity (285). Introduction of the aminothiazole group improves the activity against Gram-negative strains (316), and in combination with the syn oximino grouping confers resistance to β-lactamases (317). The nature of the C3-substituent predominantly influences the pharmacokinetic and pharmacological properties but also the antibacterial activity. Thus C3 methyl compounds with a C7 phenylglycine side chain are orally absorbed, whereas C3 vinyl derivatives, even with the C7 oximino side chain, also show promise as oral agents (285). Some interesting synthetic methodologies have been developed for these vinyl compounds by use of allenyl azetidinone intermediates and a variety of organometallic reagents (318, 319).

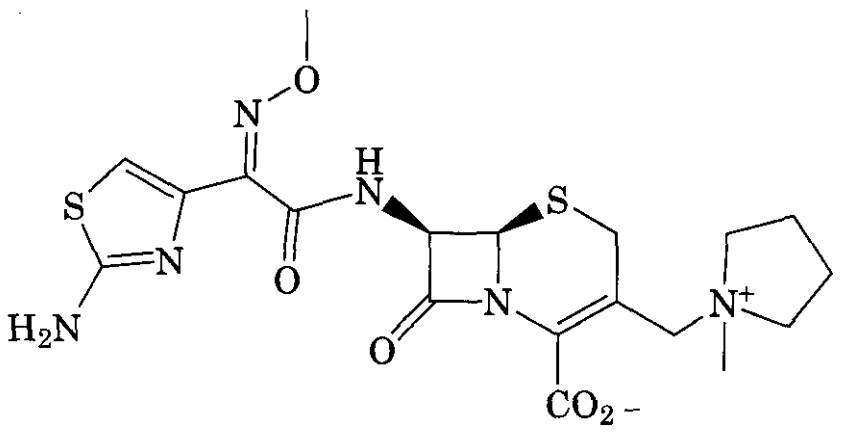
The synthesis of potent antipseudomonal β-lactam derivatives can be achieved by introducing siderophore-like moieties that allow the antibiotic to use the *tonB* transport system, overcoming in that way the mechanism of resistance caused by a reduced permeation barrier. In general, siderophores are high affinity iron-binding compounds that the bacte-



(70) Cefsulodin (third generation)



(72) Cefpirome (fourth generation)

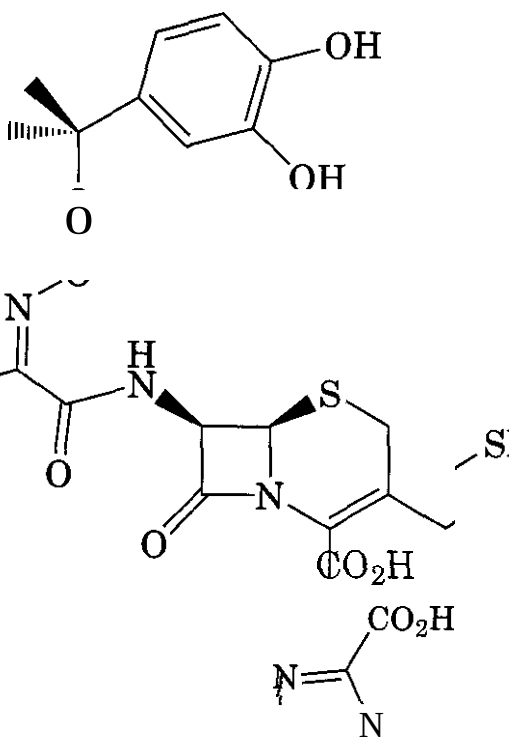


(73) Cefepime (fourth generation)

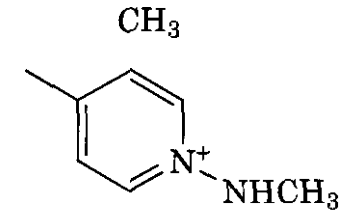
ria release in their surroundings (320, 321). These bacterial products, which are capable of chelating ferric iron, are actively transported into the bacteria.

Catechol groups have been used to enhance the activity, particularly against *Pseudomonas* aeruginosa, and many examples have been described (294). The effect of the catechol group seems to be similar irrespective of whether it is incorporated in the C7 acylamino side chain, as in M-14659 (74) (322), or the C3 position, as illustrated by (76) (323). Noteworthily, BRL-57342 (75) has been described (324), which not only exhibits antipseudomonal activity but also retains some anti-Gram-positive activity that is an unusual feature for cephalosporins bearing a catechol group. Evidence has been presented to show that these compounds penetrate into the cell through use of the bacterial *ton*B-dependent iron-transport system (325,326).

Other examples of cephalosporin derivatives bearing a catechol moiety are GR69153 (77)(327) and KP-736 (78) (328). However, it is very common that bacteria can very rapidly develop resistance to siderophore-like drugs.



(74) M-14659 R =



(75) BRL 57342 R =

Table 14.10 Antibacterial Activity (mg/L) of Some Representative Cephalosporins[a,b]

	1st			2nd			3rd				4th	
Organism	CET	CEZ	CEX (os)	CMD	CXM	CFL (os)	CTX	CTR	CAZ	CFM (os)	CPR	CPM
E. coli	4–>64	2–>128	4–>128	1–32	1–8	1–>128	0.03–1	0.12–1	0.12–1	0.4–32	0.03–0.25	0.02–1
K. pneumoniae	4–>128	2–>128	4–>128	0.5–>128	24	1–>128	0.03-0.12	0.06–0.12	0.06-0.25	0.05–0.4	0.06–NA	0.01–2
Enterobacter sp.	>128	>128	>128	32–>128	16–>128	>128	0.12->64	0.25–NA	0.25–>64	64–>100	0.124	0.01–32
Citrobacter sp.	64	>128	>128	8	8	>128	0.25	0.5	0.5	64–>100	0.12	0.01–0.5
S. *marcescens*	>128	>128	>128	>64	64–>128	>128	0.12–>64	0.25–NA	0.12–2	2–100	0.12–4	0.03–3.2
P. mirabilis	24	4-8	8	1–2	1–2	1	0.03	0.03–NA	0.06	0.05	0.03–NA	0.02–0.25
Proteus sp. (In +)	>128	>128	>128	8	8	>128	0.12	0.12	0.12	NA	0.25	NA
P. aeruginosa	>128	>128	>128	>128	>128	>128	32–>128	32–64	2–16	NA	4–16	0.5–16
H. *influenzae*	4	8	8	1–>81–>8	0.5	1–2	0.03–0.06	0.03–0.06	0.12	0.12	0.03	0.02-0.06
B. *fragilis*	64	64	64	64	32	>128	32	64	64	NA	32	8–>125
S. *aureus*	0.25–0.5	0.25–1	2 4	0.5 1	1	1 4	2	4	4–8	8–>100	0.5	1 4
S. *pyogenes*	0.12	0.12	0.5	0.06	0.03	0.25	0.03	0.03	0.12	0.2	0.03	<0.01–0.06
S. *pneumoniae*	0.12	0.12	2	0.25	0.12	1	0.12	0.25	0.25	0.4	0.12	0.01–0.25
N. *gonorrhoeae*	0.5	0.5–2	2	0.5	0.06	0.12–0.5	<0.01–0.03	<0.01–0.01	0.06–0.12	NA	<0.01	<0.01–0.06

[a]Refs. 241, 311, 313.

[b]Abbreviations: CET, cephalotin; CEZ, cefazolin; CEX, cephalexin; CMD, cefamandole; CXM, cefuroxime; CFL, cefaclor; CTX, cefotaxime; CTR, ceftriaxone; CAZ, ceftazidime; CFM, cefixime; CPR, cefpirome; CPM, cefepime.

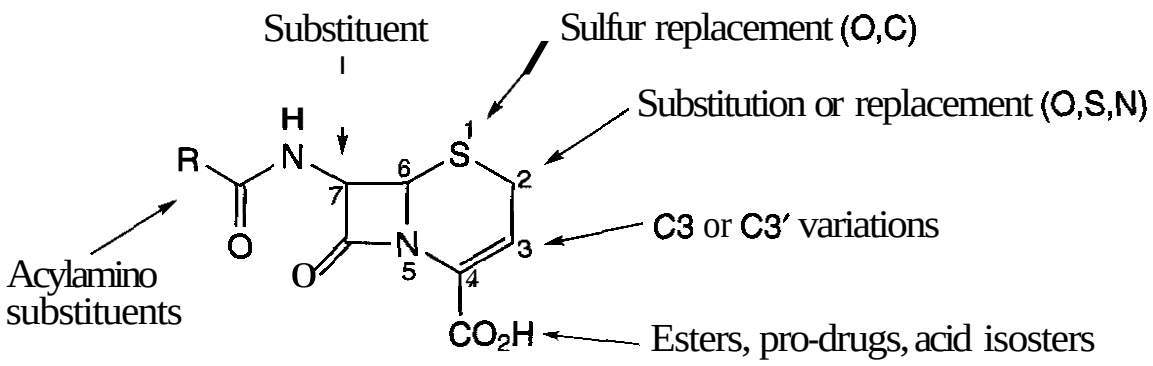
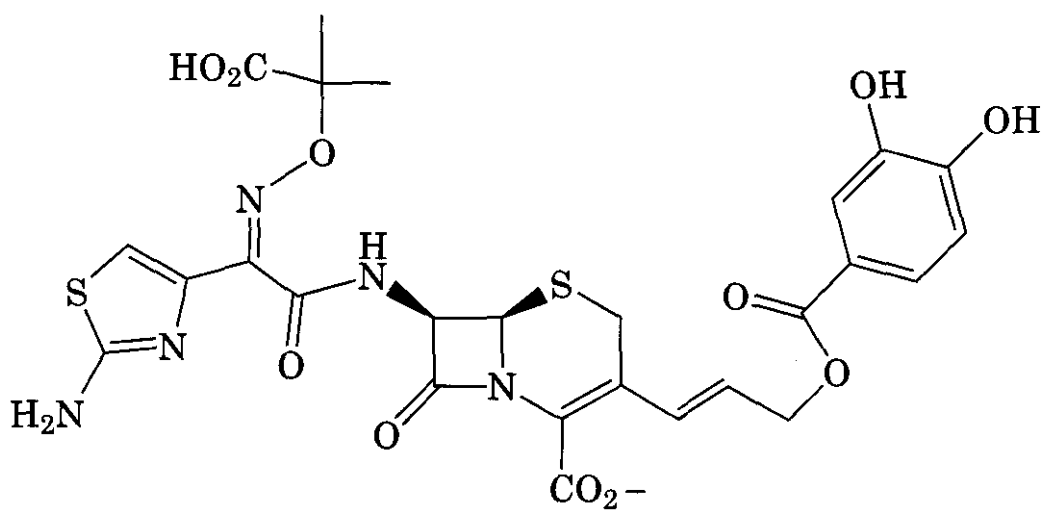


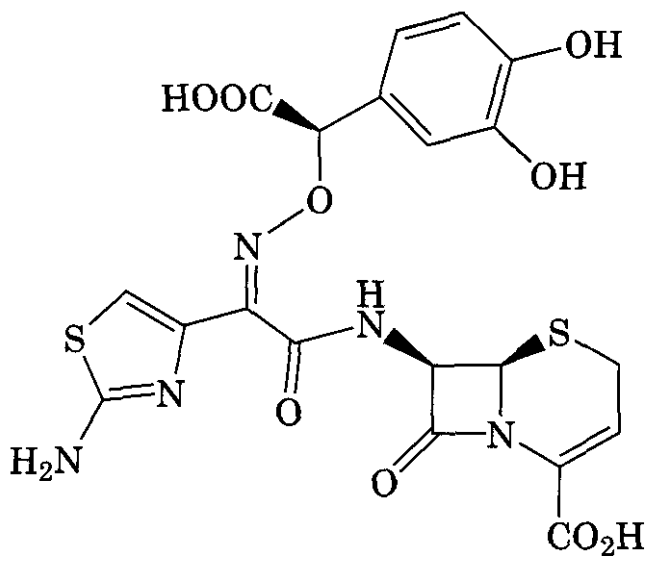
Figure 14.8. Structural variations of the cephalosporin ring system.



(76)

5.2.3 The Total Synthesis of Cephalosporin C. Intermediates such as (79) (329,330) have been used to provide the cephalosporin lactone ring system (80) and deacetylcephalothin (331). Later, by use of a [2 + 2] cycloaddition reaction between the thiazine (81) and the ketene derived from azidoacetyl chloride, the total racemic synthesis of cephalothin was achieved (332–334).

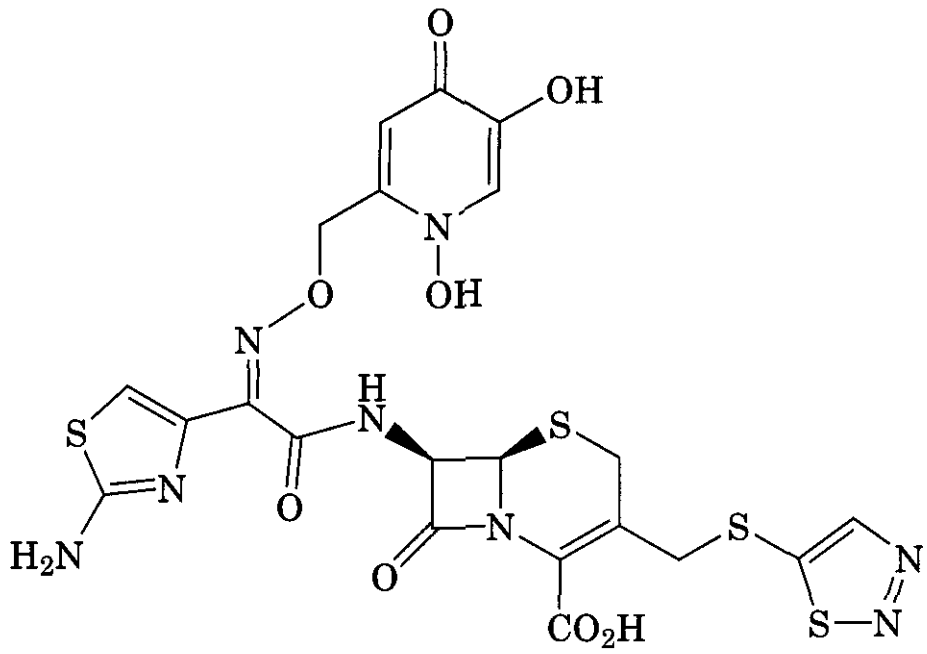
To date, however, the only complete synthesis of cephalosporin C is that described by



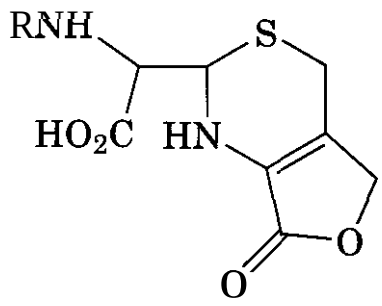
(77) GR 69153

Woodward in his Nobel lecture of 1965 and published in 1966 (335, 336) (Figs. 14.9 and 14.10). Protection of the nitrogen, sulfur, and carboxylic acid of L(+) cysteine (82) provided the cyclic intermediate (83). Introduction of the hydrazino group gave (84) that, by oxidation with lead tetracetate and treatment with sodium acetate, was converted into the ***trans***-hydroxy ester (**85**). Formation of the mesylate, inversion of the stereochemistry by displacement with azide, and reduction provided the cis-amino ester (86) that gave the key β-lactam intermediate (**87**), of which, on cyclization, the absolute stereochemistry was confirmed by X-ray crystallography.

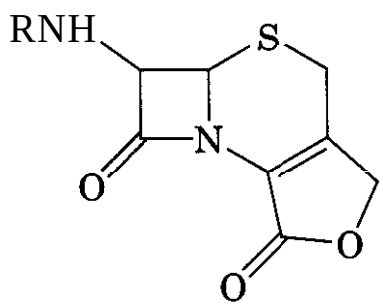
This β-lactam derivative was reacted with dialdehyde (88) in a Michael addition manner to yield (89). Treatment with trifluoroacetic acid removed both nitrogen- and sulfur-protecting groups and resulted in cyclization to the cephalosporin precursor (**90**), in which the amino group was then acylated with the suitably protected D-α-aminoadipic acid side chain, forming (91). Reduction, acetylation, and equilibration provided the cephalosporin C ester (**92**), from which the protecting groups



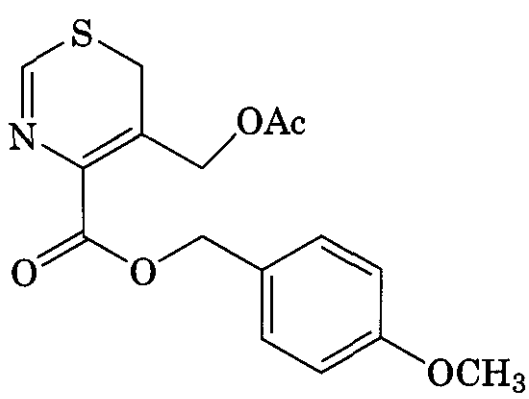
(78) KP-736



(79)



(80)

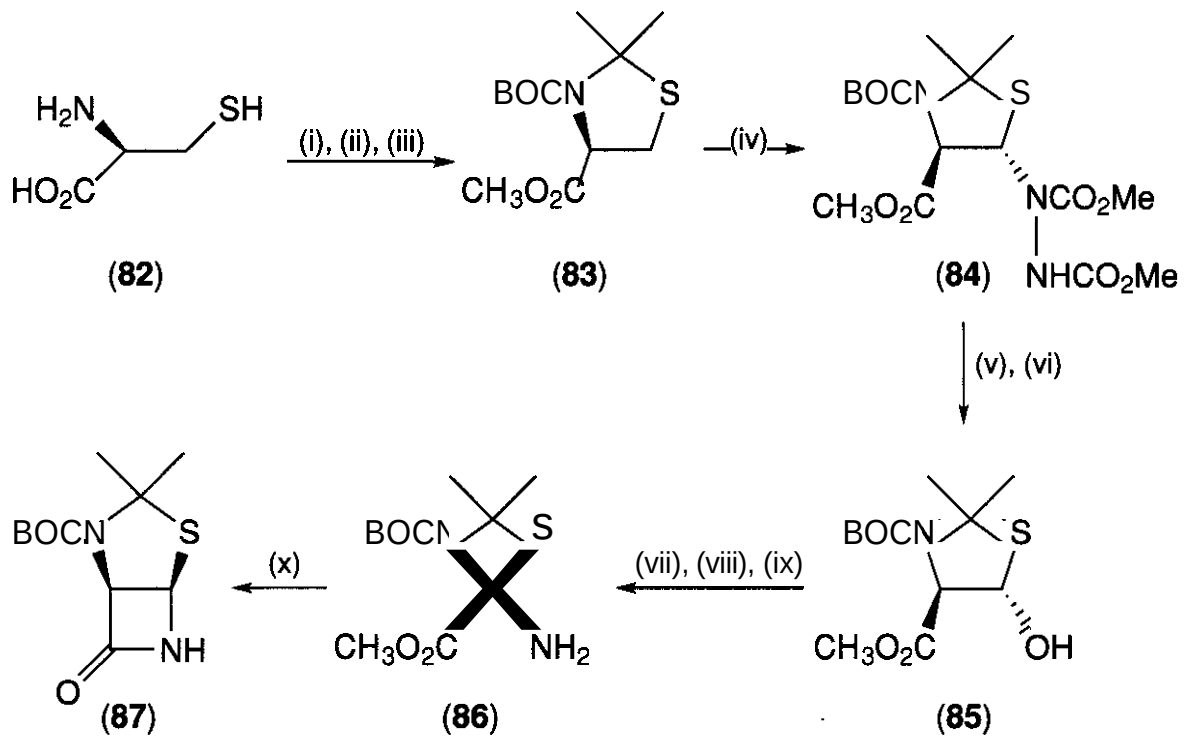


(81)

were removed with zinc and acetic acid to give the free acid (**93**), which **was** identical to a sample of authentic material.

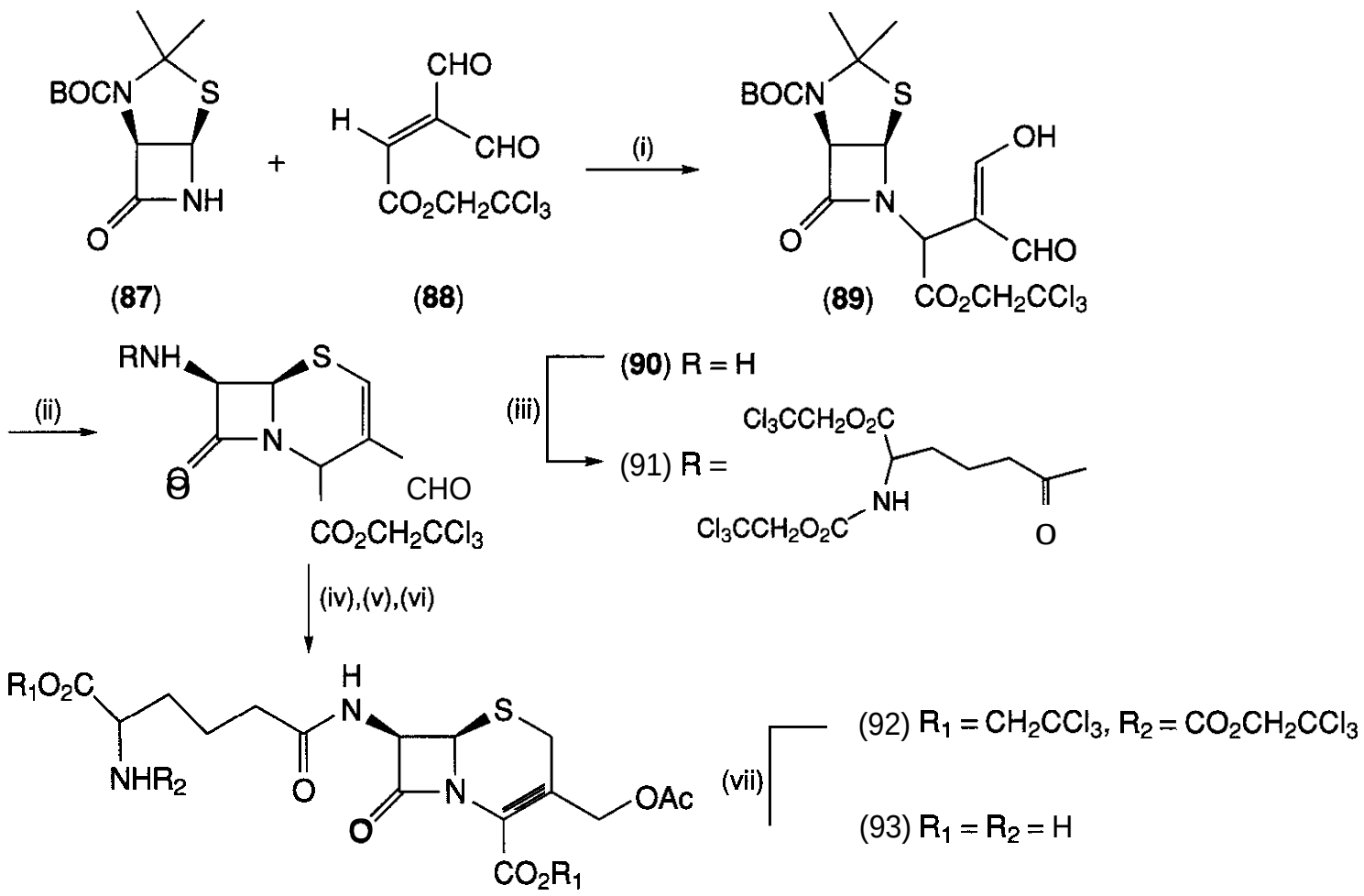
5.2.3.1 ***The Penicillin Sulfoxide-Cephalosporin Conversion.*** Early work on the chemistry of penicillin sulfoxide by Morin (337) demonstrated the thermal rearrangement of (94) in the presence of acid to the **deacetoxycephalosporin** ring system (96). Speculation that this transformation occurred through the sulfenic acid intermediate (95) was later confirmed by its isolation in a crystalline form (338). The rearrangement of (94) to (**96**) afforded an attractive route to cephalosporins lacking the C3-acetoxymethyl group of the natural derivative, starting from a relatively cheap chiral starting material while retaining the stereochemical integrity of the β-lactam ring. The chemistry of the penicillin sulfoxides and their use for the interconversion of β-lactam antibiotics have been extensively reviewed by Cooper and coworkers (339–341). Here is reported only the process leading to the commercially available cephalexin (**60**) and cefaclor (61) (Fig. 14.11).

In the case of cephalexin (62) the **trichloroethyl** ester of penicillin V sulfoxide (94) was successfully rearranged to the **deacetoxycephalosporin**, followed by cleavage of the C7 side chain and acylation with a suitably protected D-α-phenylglycine. Removal of the amine- and **acid**-protecting groups gave a synthesis that could be adapted for the production of **cephalexin** (62) on a multikilogram scale (342). The



Reagents: (i) acetone; (ii) BOCCl, pyridine; (iii) CH_2N_2; (iv) $MeO_2CN=NCO_2Me$; (v) $Pb(OAc)_4$; (vi) NaOAc, MeOH; (vii) $MeSO_2Cl$, DIPEA; (viii) NaN_3; (ix) Al/Hg; (x) triisobutylaluminium.

Figure 14.9. Woodward's synthesis of cephalosporin C.



Reagents: (i) 80°C; (ii) CF_3CO_2H; (iii) acylation; (iv) diborane; (v) Ac_2O, pyridine; (vi) pyridine; (vii) Zn, CH_3CO_2H.

Figure 14.10. Woodward's synthesis of cephalosporin C.

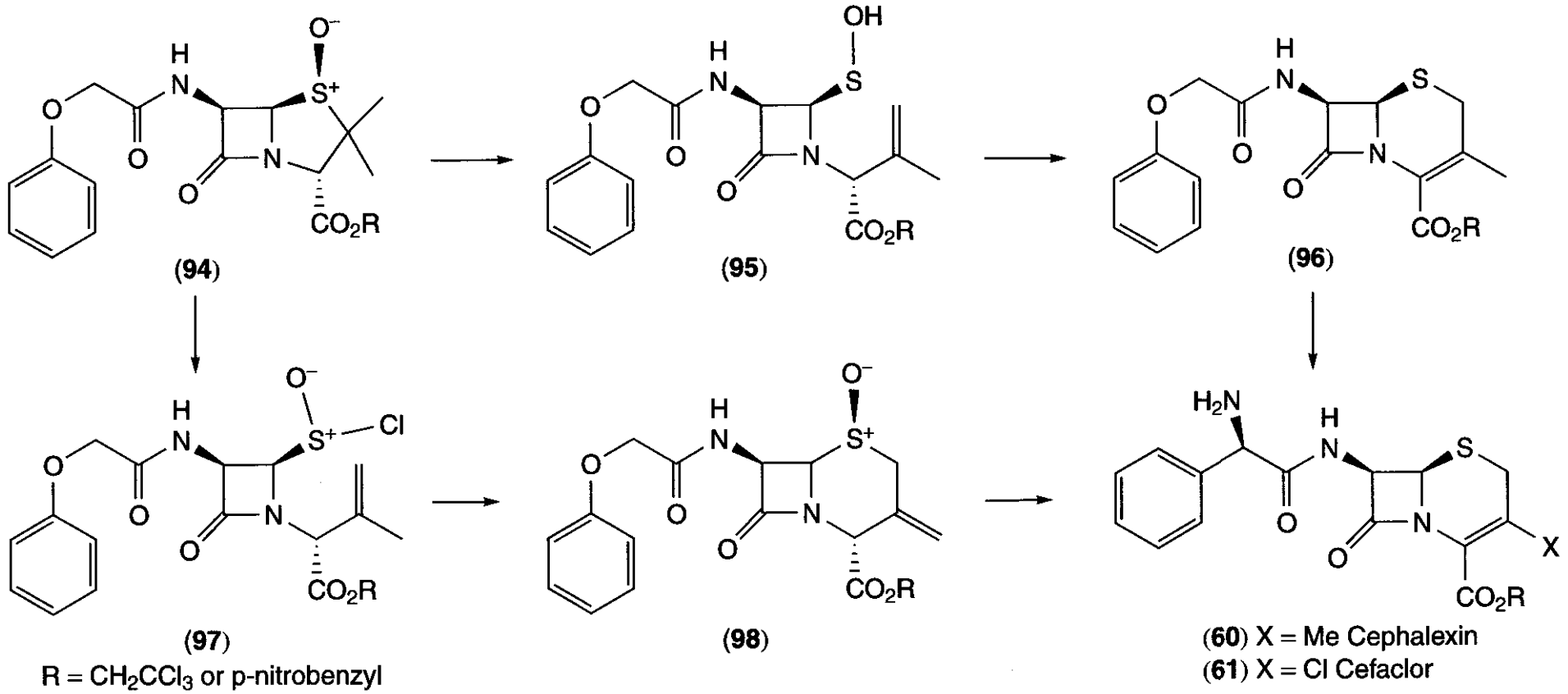
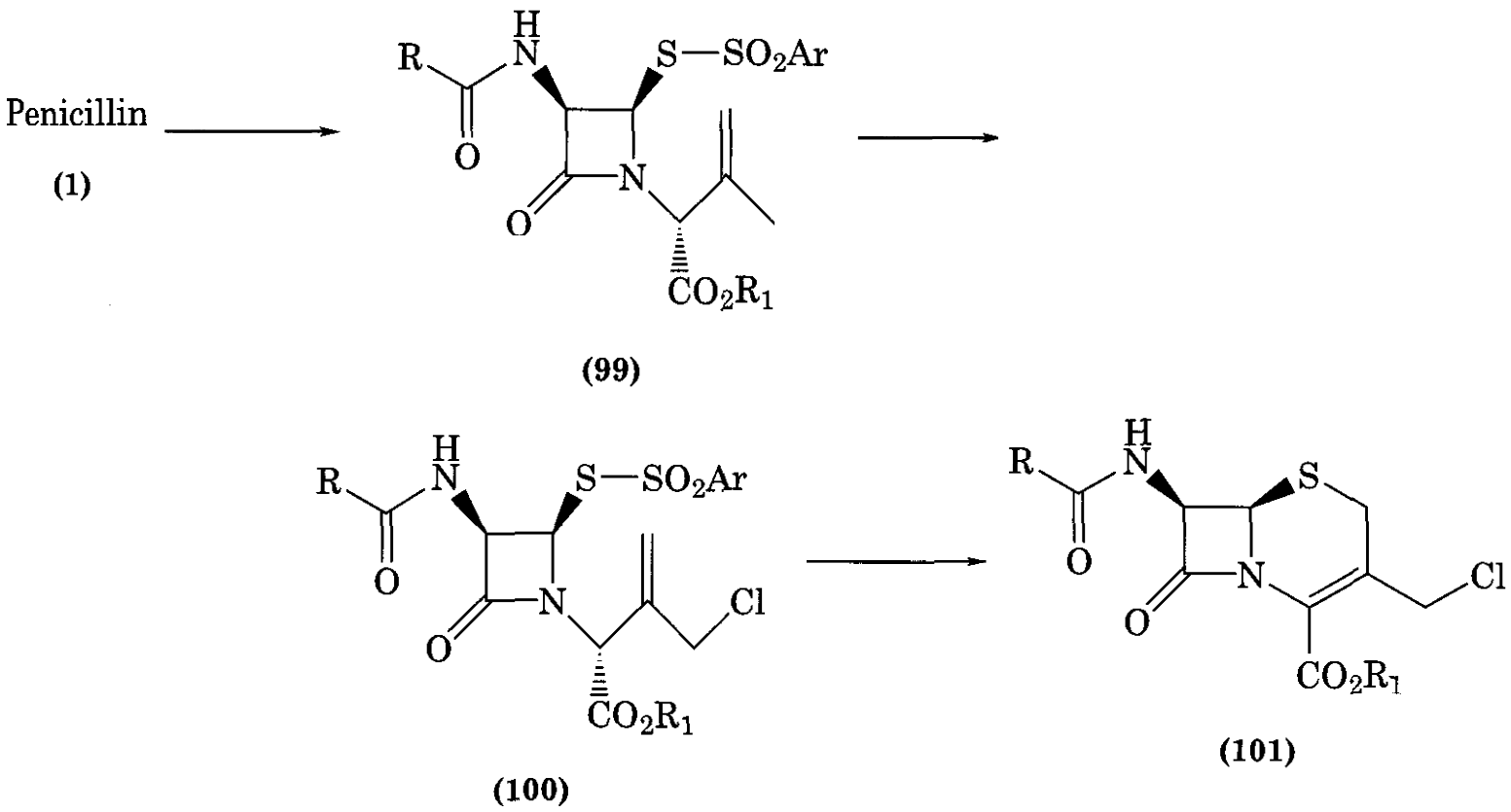


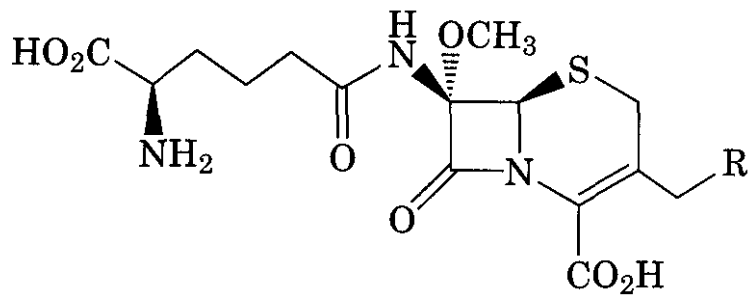
Figure 14.11. Rearrangement of penicillin sulfoxide to cephalosporins.



synthesis of cefaclor (59) stems from work that showed that penicillin sulfinyl chlorides (97) generated from (94) afforded 3-exomethylene cepham sulfoxides (98) on treatment with tin chloride (IV) or other Lewis acids (343). Reduction of the sulfoxide and ozonolysis of the double bond gave the C3-hydroxycephem, which was converted to the corresponding chloride. Side-chain exchange and deprotection provided a viable synthesis of cefaclor (59) (291). 4-Arylsulfonylthioazetidin-4-ones (**99**) have been efficiently converted (yields of 70–90%) to 3-chloromethyl cephalosporins (101) through an electrolytic ene-type chlorination to (100) and subsequent base-catalyzed ring closure (344). This procedure has provided the basis for the successful development of a commercially viable process for the synthesis of the key cephalosporin intermediate 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid, p-methoxybenzyl ester (ACLE), as well as other derivatives, by the Otsuka Chemical Company of Japan. Variations of the process have also afforded 3-hydroxy cephalosporins (345).

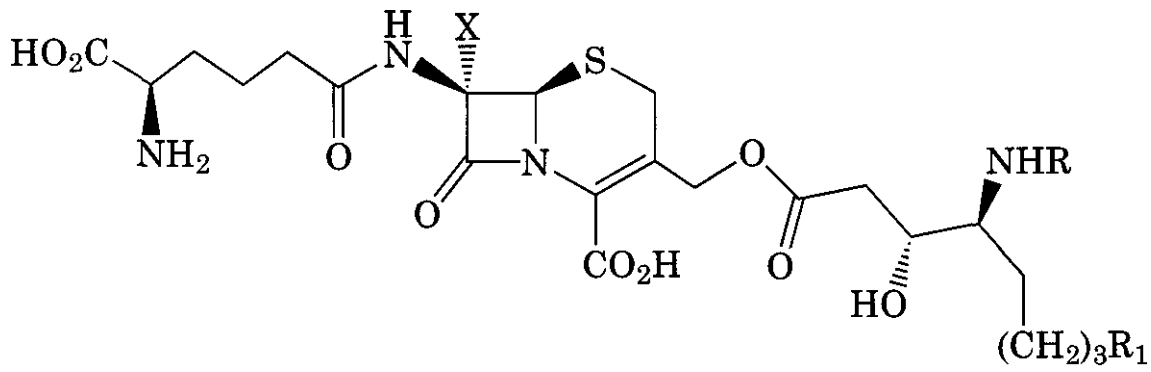
5.2.4 C7-α-Substituted Cephalosporins. In 1971 two naturally occurring cephalosporins possessing a C7-α-methoxy substituent were isolated from a Streptomyces strain, and shown to be C7-a-methoxycephalosporin C (**102**) and the C3-carbamate (103) (274).

Subsequently, several further examples of this type of natural product were discovered and characterized (285, 346). All possess the



(102) R = $OCOCH_3$
(103) R = $OCONH_2$

(D)-α-aminoadipic acid side chain but differ in the nature of the C3-substituent. Interest in this class of compounds arises primarily from their intrinsically higher resistance to hydrolysis by β-lactamases. Collectively, they have been called the cephamycins and their chemistry and biology have been comprehensively reviewed (248). Later, a range of C7-α-methoxylated cephalosporins (**104**), of bacterial origin possessing oligopeptide side chains at C3 and known as the cephabacins-M, have been reported (347). In addition, a new structural class incorporating a C7-α-formylamino sub-

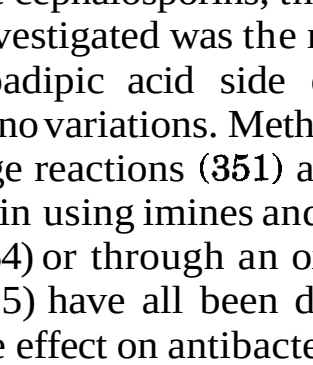


(104) Cephabacins-M X = OCH_3
(105) Cephabacins-F X = NHCHO

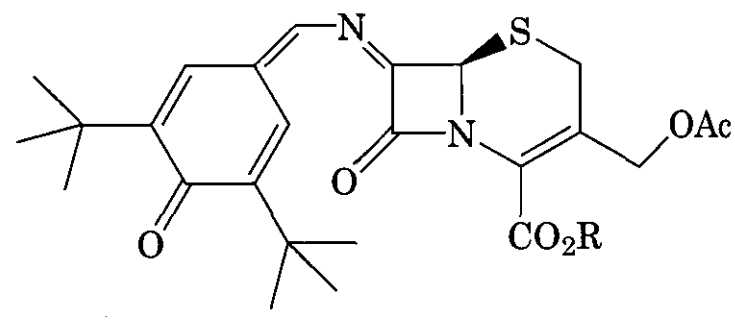
stituent (**105**) has been isolated (275, 348, 349). These have been named as the cephabacins-F or alternatively as the chitinovorins A-D (275, 349,350).

5.2.4.1 C7-α-Methoxylated Derivatives. As with the cephalosporins, the first area of SAR to be investigated was the replacement of the a-aminoadipic acid side chain with other acylamino variations. Methods involving acyl-exchange reactions (351) and removal of the side chain using imines and imidoyl chlorides (352–354) or through an oxamic acid derivative (355) have all been described, together with the effect on antibacterial activity (248). Numerous approaches have been directed toward the chemical introduction of the 7α-methoxy, and other substituents into the cephalosporin ring system (248). Many have also found application in the penicillin field (Section 5.1). Of the range of groups introduced, however, it is the methoxy and recently the formamido group that have been found to provide the best combination of stability to β-lactamases and antimicrobial potency (356,357).

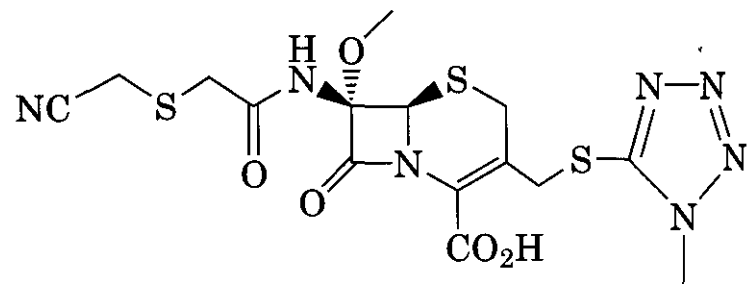
Although initial approaches to methoxylation used C7-diazo cephalosporins (253), as in the penicillins, the most widely used methods rely on addition of methoxide to acylimine intermediates such as (**106**) (358), sulfenimines (107) (256), or ketenimines (359). Addition to the quinone methide derivative (108) is a method that has found application in the synthesis of cefmetazole (**109**) (360, 361), a semisynthetic cephamycin.



(106) R_1 = RCO
(107) R_1 = RS

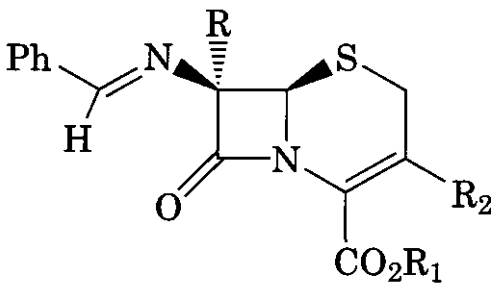


(108)



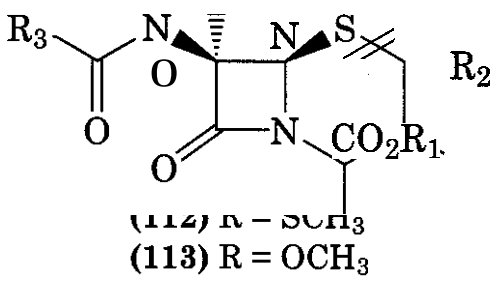
(109) Cefmetazole

Again, in a manner similar to that of the penicillins, an alternative approach to acylimine formation uses carbanion formation α to the β-lactam carbonyl followed by reaction with an electrophile, to give a substrate suitable for the introduction of the methoxy group. Thus, generation of the anion from the Schiff base (110) and reaction with methyl methanethiosulfate gave the corresponding C7-α-thiomethyl cephalosporin (**111**), which can be converted to the acylamino derivative (112). Solvolysis in methanol in the presence of mercury salts then leads to C7-α-methoxy cephalosporin (251).



(110) R = H
(111) R = SCH_3

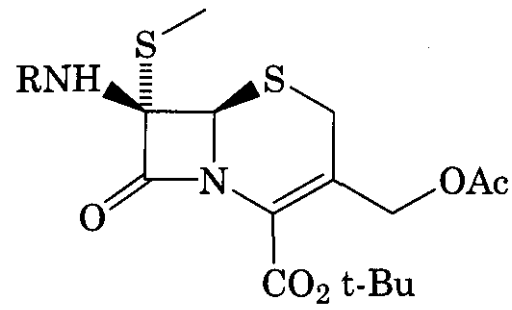
H R



(112) R = SCH_3
(113) R = OCH_3

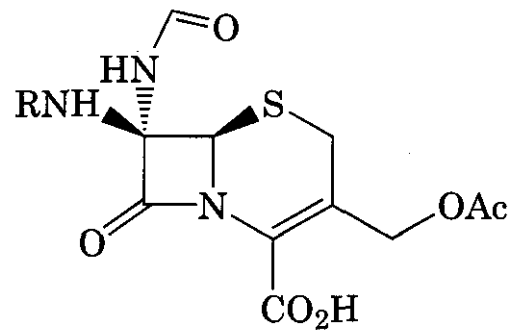
Factors related to the methylthiolation of cephalosporins have been extensively studied, particularly by workers at Squibb (362). Approaches to the total synthesis of methoxylated cephalosporins have been described by Merck researchers (332–334) and Kishi (363). The first member of this group to be used clinically was cefoxitin (**64**), which is very stable to β-lactamases, whereas the carbamoyl group provides stability to esterases (300). Cefoxitin (64) has a fairly broad spectrum of activity, including anaerobic bacteria such as *Bacteroides fragilis*, a common pathogen resulting from abdominal surgery (364). Examples of newer cephamycins having a C3-N-methyltetrazolylthiomethyl substituent are cefmetazole (109) (361, 365) and cefotetan (**65**) (304), reported as showing improved Gram-positive activity or pharmacokinetic properties over those of cefoxitin (64).

5.2.4.2 C7-α-Formamido Derivatives. Before the discovery of the natural products, the Beecham group had already shown the utility of the formamido substituent with the synthesis of several highly active, β-lactamase stable C6/C7-α-substituted penicillins and cephalosporins (258, 259). One of the initial approaches to the conversion of the unsubstituted cephalosporin ring system into the formamido nucleus was by way of (**114**), readily available from work on the methoxy



(114) R = H
(115) R = R_1CO

series. Acylation provides (**115**), from which the methylthio group can be displaced with ammonia in the presence of a mercury salt followed by formylation and acid deprotection, to give the appropriate C7-a-formamido cephalosporin acid (116). Alternatively, the form-

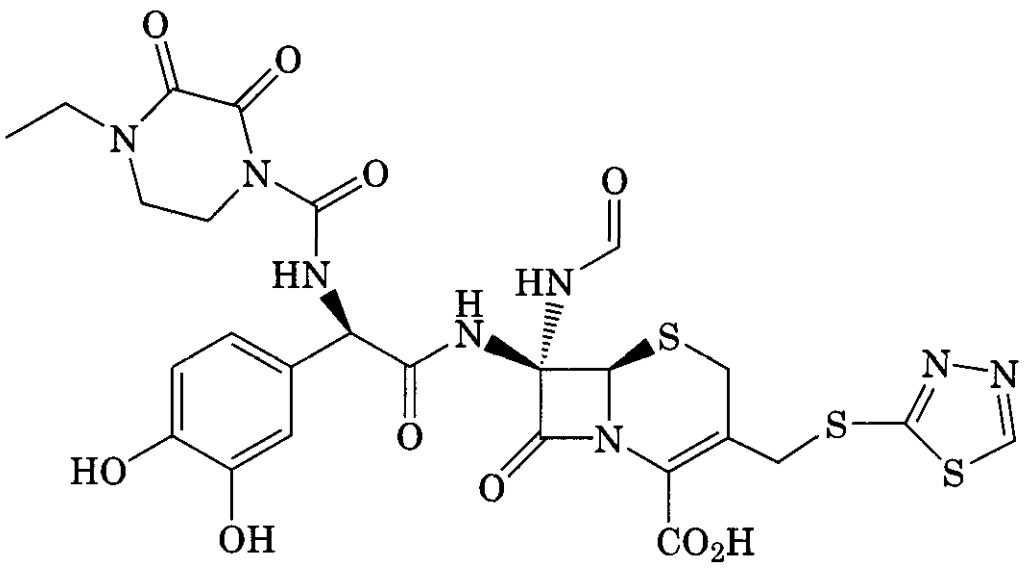


(116) R = R_1CO
(117) R = H

amido group can be introduced directly by treatment of (115) with *N,N*-bis(trimethylsilyl)formamide in the presence of mercuric acetate (259, 366).

Other methods for incorporating the formamido substituent have been reported (260, 261), as well as high yielding "one-pot" large-scale preparation of the formamido nucleus by use of silyl protection and quinone methide methodology (367).

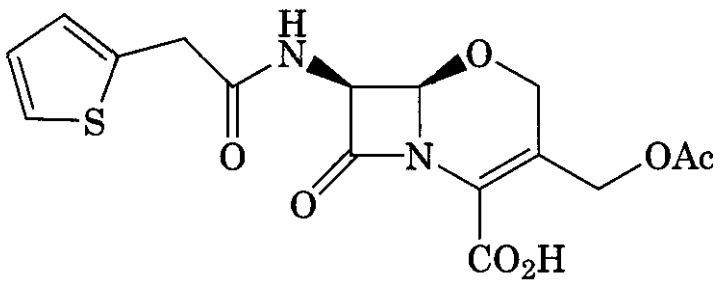
In a series of structure-activity studies varying both the C7-acylamino side chain and C3-substituent it was demonstrated that the formamido cephalosporins in many cases showed advantages over the cephamycins in conferring high β-lactamase stability without compromising antimicrobial activity (368, 369). In particular the catecholic derivative (**118**, BRL 41897) was identified as a broad spectrum agent active against both Gram-negative and Gram-positive bacteria with high potency against Pseudomonas aeruginosa, and which reached the stage of single dose volun-



(118) BRL-41897

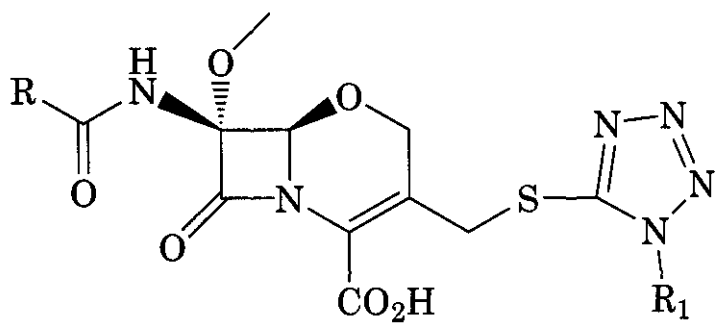
teer studies, before adverse toxicological effects caused its withdrawal from development (370).

5.2.5 Oxacephalosporins (1-Oxadethiacephems). The synthesis of 1-oxadethiaceph-3-em-4-carboxylic acids (**1-oxacephems**) was initially carried out by the Merck group in 1974 with the synthesis of racemic **1-oxa**acephalothin (119) (371). This showed activity comparable to cephalothin (58) itself.

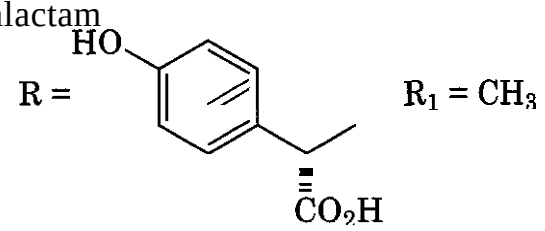


(119)

Subsequent examples also demonstrated the potential utility of these nonnatural **1-oxa** analogs over the natural cephalosporins (372–375). Extensive **SAR** studies by the Shionogi group led to the identification and development of moxalactam (latamoxef, 120) as a broad-spectrum clinical agent equal to or better than many third generation **cephalosporins** (376). However, moxalactam possesses poor activity against *S.* aureus, while the manifestation of side effects, including problems associated with blood clotting due to decreased vitamin K synthesis, have considerably restricted its clinical use (377, 378). **Fur-**



(120) Moxalactam

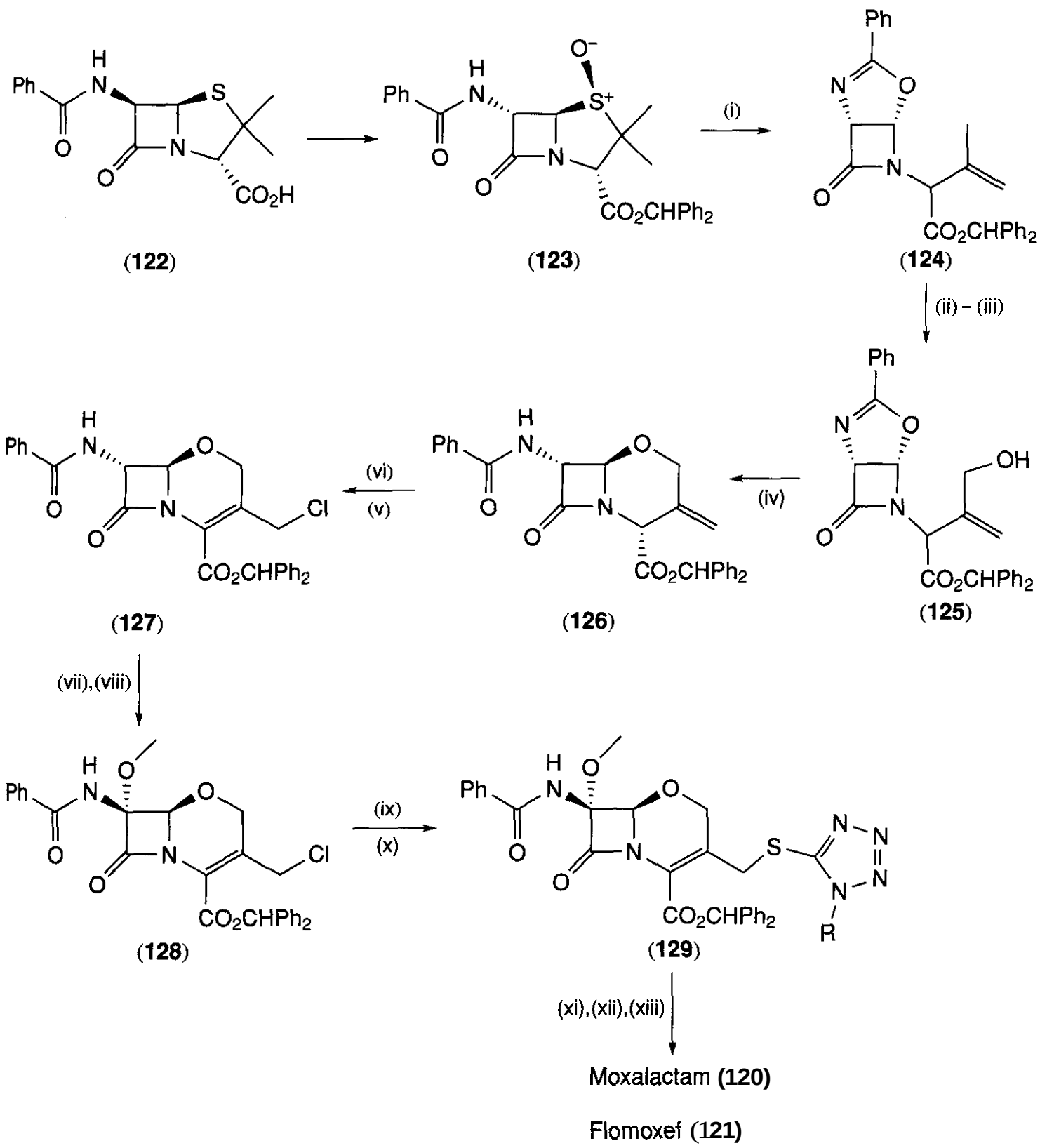


(121) Flomoxef **R** = F_2CHSCH_2
$R_1 = CH_2CH_2OH$

ther studies identified flomoxef (121) with improved Gram-positive activity as an agent to overcome these problems (379).

The identification of moxalactam (120) and flomoxef (121) as clinical candidates led to an intensive effort by Shionogi to establish a **stereocontrolled**, commercially viable process for the production of the compound, and other **1-oxacephems**. The culmination of this effort can be illustrated (Fig. 14.12) by the conversion of the readily available penicillin nucleus (6-APA) to the **1-oxacephem** nucleus (129) used for the production of both moxalactam (120) and flomoxef (121) (378,380).

Protection, oxidation, and epimerization of the benzoyl derivative of 6-APA (**122**) readily gave the epipenicillin **β-sulfoxide** (**123**), which was converted to the key epi-oxazolinone **azetidinone** intermediate (124). Transformation

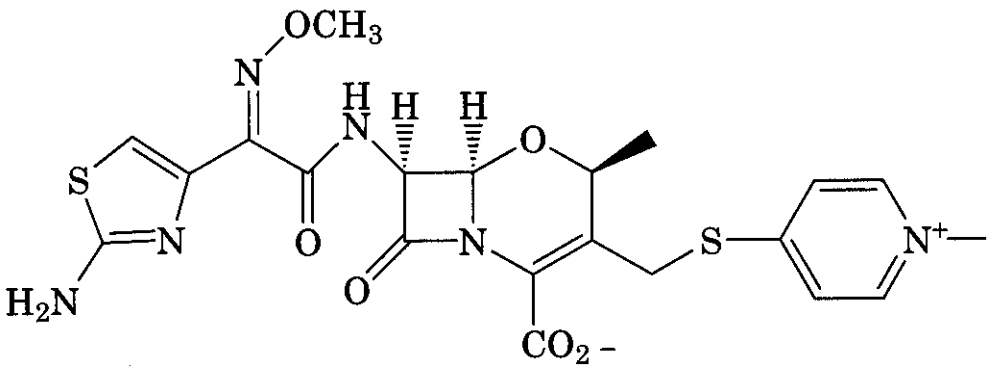


Reagents: (i) Δ/Ph_3P; (ii) Cl_2/Base, (iii) $I_2/CuO/DMSO/H_2O$; (iv) BF_3-Et_2O; (v) $Cl_2/h\nu$; (vi) DBN; (vii) t-BuOCl/LiOMe; (viii) H^+; (ix) $Na_2S_2O_3$; (x) NaS-Tetrazole; (xi) PCl_5/Pyridine; (xii) MeOH; (xiii) Acylation.

Figure 14.12. Conversion of penicillin to 1-oxacephems.

to the allylic alcohol **(125)** was followed by Lewis acid catalyzed cyclization to the oxacepham ring system (**126**), and then photochemical halogenation to the chloromethyl 1-oxo-cephem ester **(127).** Introduction of the methoxy group and appropriate tetrazole substituent afforded **(128).** Deacylation then gave in high overall yield the ester (**129**), which is a suitable substrate for conversion to moxalactam **(120)** or flomoxef **(121)** on a commercially viable scale. A range of 2-methyl analogs (e.g., **130)** have been reported but no clinical candidates were selected **(381).**

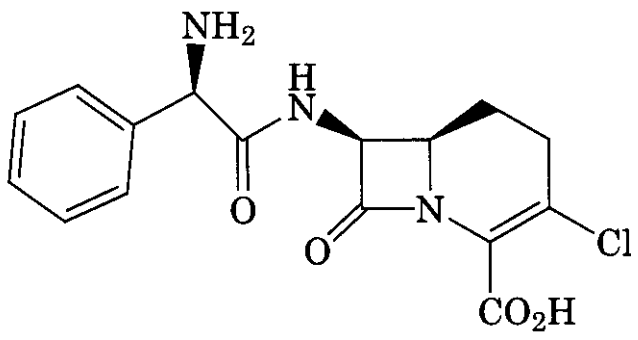
Subsequent to the discovery of the C7-α-formamido-substituted cephalosporins the synthesis of a series of analogous 1-oxacephem antibiotics was described **(370).** Many of these show a level of antibacterial activity and β-lac-



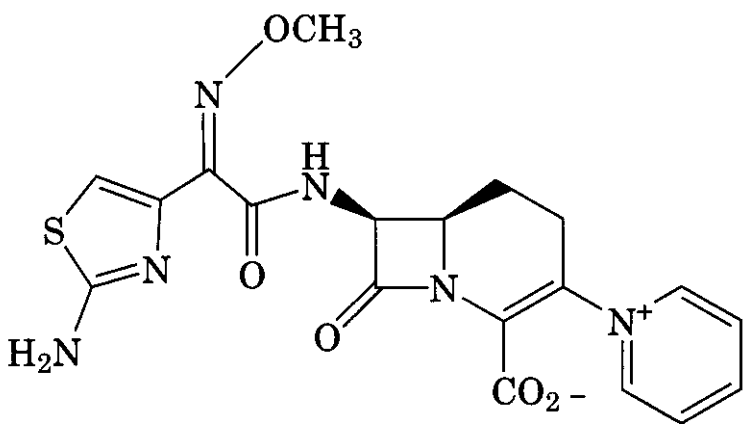
(130)

tamase stability comparable to that of moxalactam or cephalosporins such as ceftazidime (23) (382).

5.2.6 ***Carbacephalosporins (Carbacephems).*** Another series of nonnatural cephalosporins of recent interest are the 1-carbacephems, particularly with the discovery of the oral compound loracarbef (**131**), which has a spectrum of activity similar to that of cefaclor, but with greater chemical stability, a longer half-life, and better oral absorption (383,384). The additional chemical stability conferred by the carbacephem nucleus has been further demonstrated by the synthesis of directly linked quaternary derivatives such as (**132**), which have not been reported in the cephalosporin series (385). The Lilly group has also described detailed SAR leading to the identification of agents, exemplified by (**133**), with activity against methicillin-resistant Staphylococci (386).



(131) **Loracarbef**



(132)

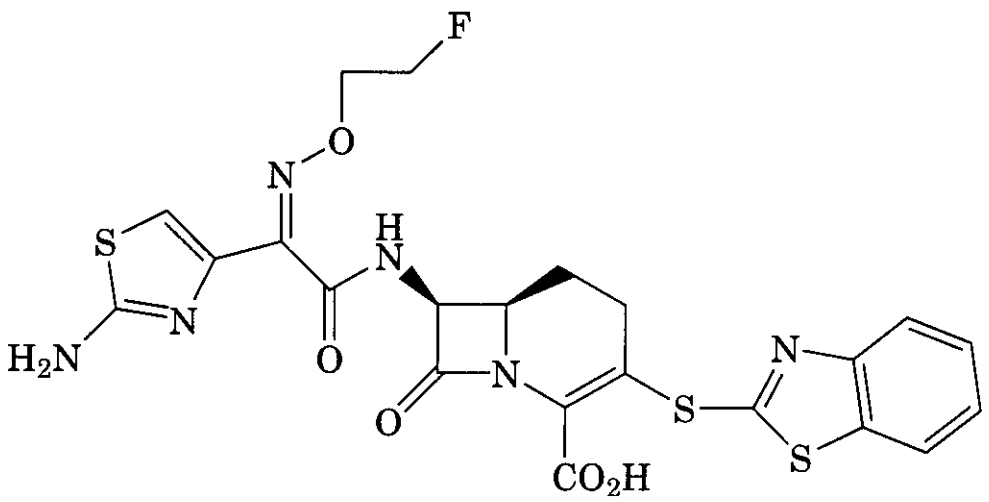
5.3 Penems

Penems, unlike penams, carbapenems, and cephalosporins (Section 5.5), have not been found in nature. However, penem development has clearly been influenced by the structures of closely related β-lactam natural products. In fact, when first described by Woodward, penems were viewed as hybrid molecules that combined the chemical structural features responsible not only for β-lactam reactivity of penams and cephalosporins (387) but also, so they hoped, for their biological properties.

5.3.1 *Synthesis*

5.3.1.1 Woodward's Phosphorane Route. The first penem synthesis to be described was that of the 6-phenoxyacetamido penem (140) (Fig. 14.13) (387, 388). The strategy adopted involved a semisynthetic approach based on the penicillin derived Kamiya disulfide (134) and employed an intramolecular Wittig reaction as the key step (135to 139)in the formation of the fused thiazoline ring system. The demonstration of antibacterial activity for the penem (**140**), despite its limited stability, led to a proliferation of studies on this new β-lactam system.

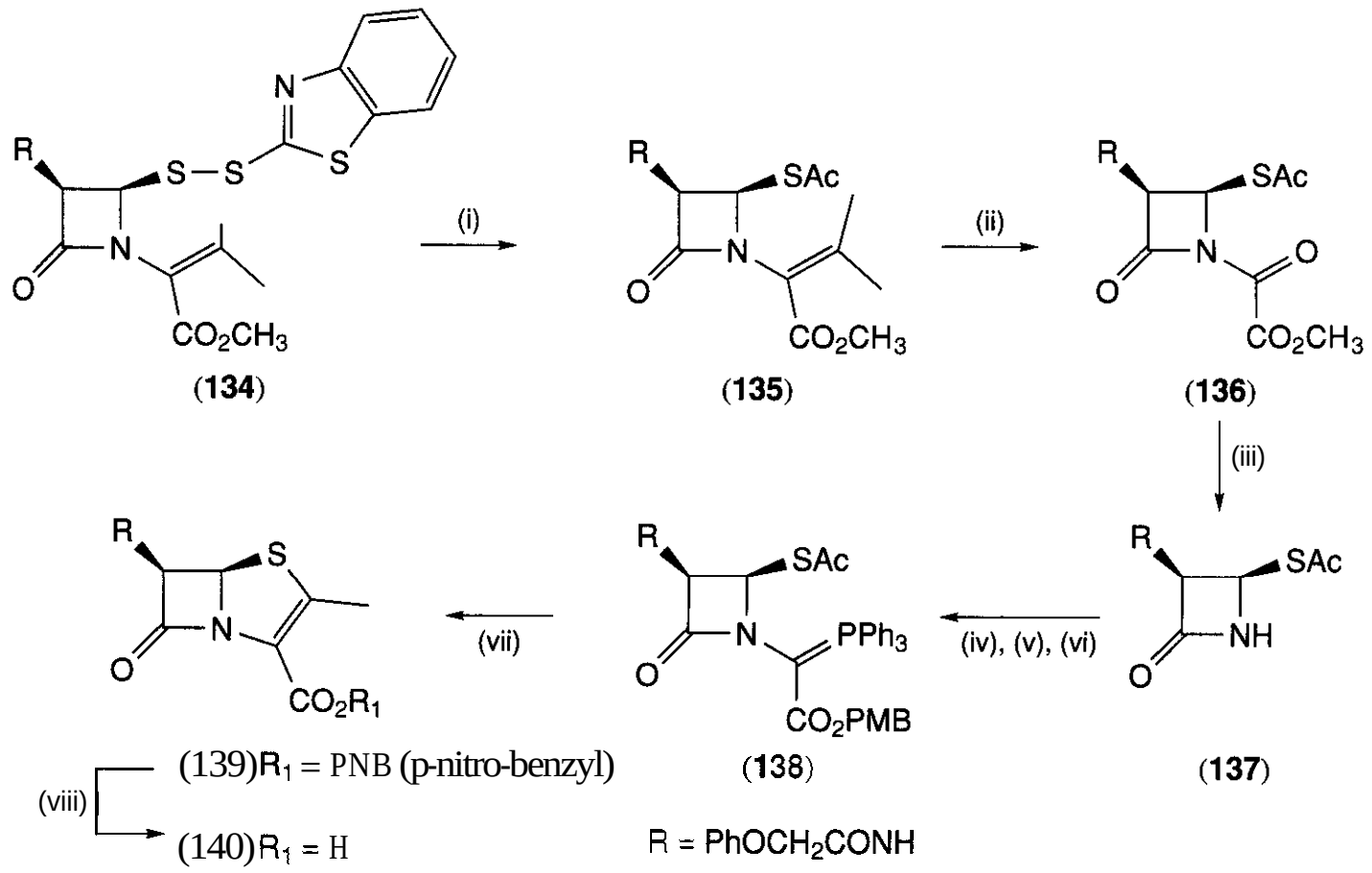
5.3.1.2 Extension of the Phosphorane Route. Although alternative routes have been developed (Section 5.3.1.3), the most versatile routes to penems remain those involving the phosphorane strategy or closely related methods involving 2,3-double bond formation. To-



(133)

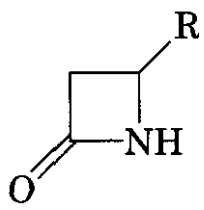
tal synthesis embodying this strategy has been used to prepare a range of 2- and 6-substituted racemic penems. Nucleophilic displacement of the acetate function of 4-acetoxyazetidinone (**141**) (389) provided azetidinones (142–144) that were elaborated to give the penems (145) (390), (**146**) (391), and (147) (392).

Similar treatment of 3-substituted (or disubstituted) acetoxyazetidinones provided examples of 2-substituted 6-alkyl and 6,6-dialkylpenems (393). Acetoxyazetidinone (**141**) also provided the starting point for the preparation of the racemic mixture (**148**) of a penem containing the l-hydroxyethyl substituent found in the naturally occurring carbapenems (394). The demonstration that, in common with the carbapenems, maximal antibacterial activity resided in the racemate with the *trans*-6-[1(*R*)-hydroxyethyll group together with the earlier indication that it was also associated with 5(*R*)-stereochemistry (395), provided the impetus for the development of routes to chiral penerns.

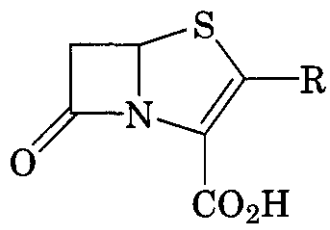


Reagents: (i) Ph_3P, Ac_2O, AcOH, Pyridine; (ii) O_3; (iii) MeOH; (iv) p-nitro-benzyl glyoxylate ethyl hemiacetal; (v) $SOCl_2$, base; (vi) Ph_3P, base; (vii) A, Toluene; (viii) H_2, Pd/C.

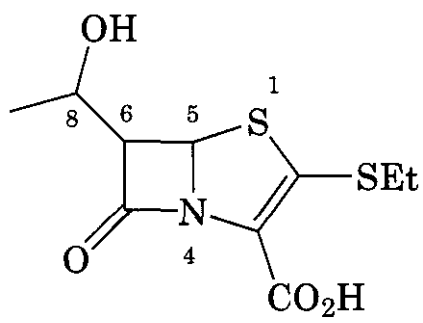
Figure 14.13. Woodward's synthesis of penems.



(141) R = OAc
(142) R = $SCOCH_3$
(143) R = SC(=S)SEt
(144) R = SC(=S)OEt



(145) R = CH_3
(146) R = SEt
(147) R = OEt



(148)

A publication describing the stereochemical outcome of alcohol condensations of the readily available 6,6-dibromopenicillanates (149) with acetaldehyde (396), made them attractive starting materials for the synthesis of chiral 6-(1-hydroxyethyl) penems (Fig. 14.14).

Reductive removal of the halogen atom of the major product (**150**), having the desired 8(*R*) stereochemistry, provided the (5*R*,6*S*,8*R*) penam (151). A number of strategies have been developed for opening the thiazolidine ring of the suitably protected penams (151) to give intermediates suitable for elaboration to penems. For example, chlorinolysis of (151) provided access to the chloro azetidinone (**152**) (397), whereas cleavage using mercuric acetate gave the acetoxy azetidinone (153) after oxidative removal of the nitrogen substituent (398). Reaction of either (**152**) (397) or (**153**) (399) with sodium trithiocarbonate proceeded with retention of configuration to give azetidinones (**154**), which were converted to the desired *trans*-8(*R*)-penems (155). In these studies it was noted that the high temperatures required to cyclize the intermediate phosphoranes was also responsible for a novel C5-epimerization of the penem, possibly involving the intermediacy of a betaine leading to the unwanted cis-penems (156) (399).

Other methods for the cleavage of the thiazolidine ring of penicillanates retaining the stereochemistry of the carbon bearing the sulfur atom, to produce suitable intermediates for penem synthesis, have been described. Silver-assisted cleavage afforded mercaptides (400), whereas the sulfenic acids generated by the thermolysis of penicillanate sulfoxides have been trapped by acetylenes (401) and thiols (402). Most of the more efficient synthesis reported use the commerciallv available *tert*-butyldimethylsilyl protected acetoxy azetidinone (**153**), which is prepared by total synthesis.

5.3.1.3 Alternative Methods for the Synthesis of Penems. Figure 14.15 summarizes the more widely applicable alternative strategies, which have been used to synthesize penems (163). Although demonstrated, syntheses involving S-C2 and N-C3 ring closures have been little used. The diazoketone/carbene insertion route widely used for the synthesis of carbapenems has not been demonstrated for penems (see Section 5.5) (403). In fact, lack of reactivity appears related to the interaction of the sulfur atom with the carbenoid derived from the diazo species.

The reactivity of the oxalimide carbonyl group provides two useful alternatives to the original phosphorane route for the formation of the 2,3-double bond. Treatment of thioesters (**160a**) (404,205) and trithiocarbonates (**161a**) (405, 406) with trialkylphosphites at high temperatures gave the corresponding carbon- and sulfur-substituted penems (163). In a detailed study (407) it was demonstrated that for the thioesters, carbene generation and interception gave the intermediate trialkoxyphosphoranes (**160b**), which underwent *Wittig-type* cyclization. In contrast, it has been proposed that cyclization of the trithiocarbonates proceeds through insertion of the carbene into the more reactive thiocarbonyl group, to form a tricyclic episulfide

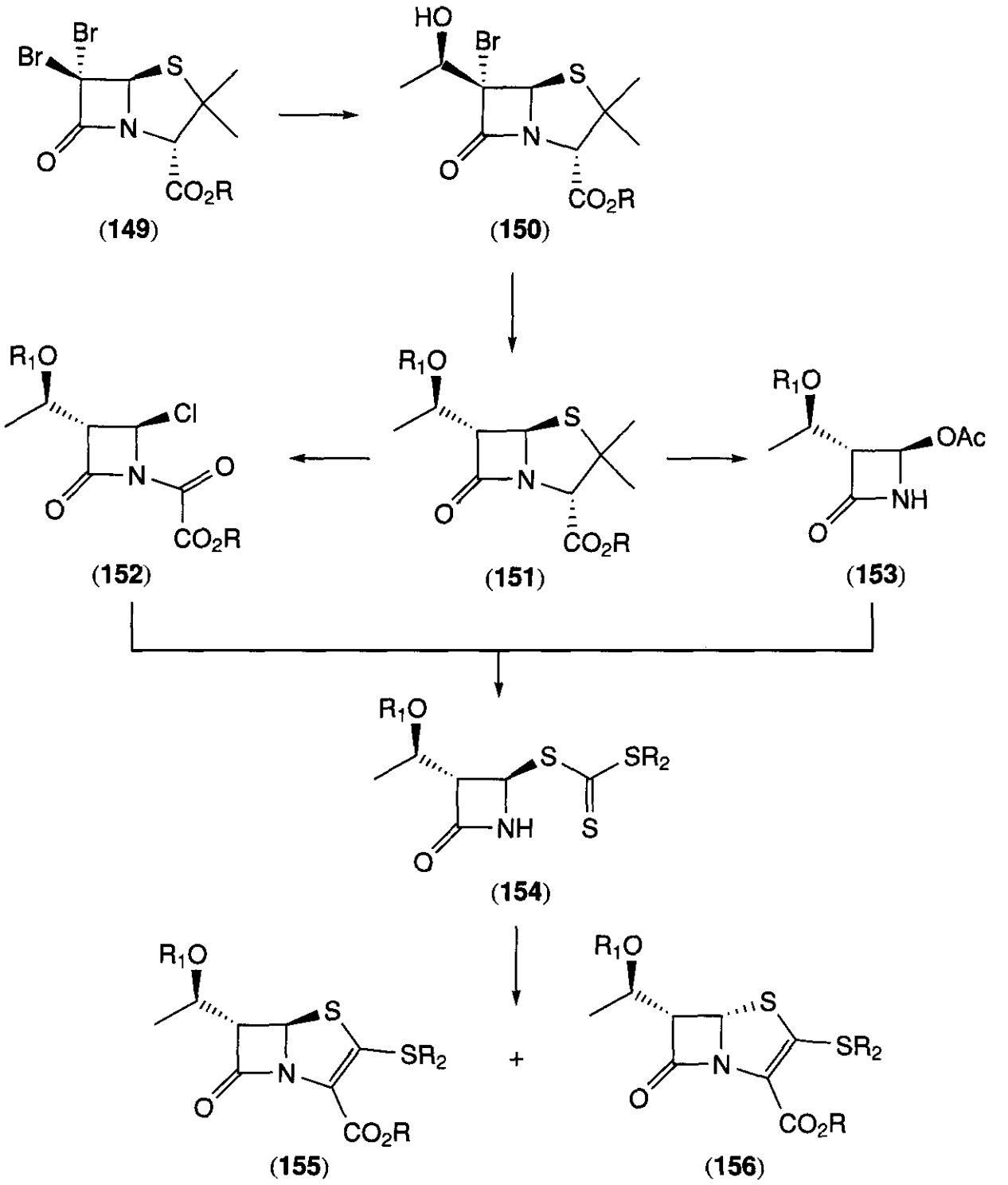


Figure 14.14. Synthesis of chiral penems.

(**162**), which is readily desulfurized to give the penem (**163**) (405, 406). The lower temperatures and shorter reaction times required for cyclization of trithiocarbonates, by use of this procedure, avoided the C5 epimerization experienced with the conventional phosphorane route. The high reactivity of the oxalimide carbonyl has also been exploited in the cyclization of "inverse phosphoranes" of the type (**161b**) (408). Sulfur-C5 bond formation was employed for the synthesis of the first examples of 2-aryloxy penems (163, Y = OAryl) (409).

Stereospecific cyclization of an intermediate of the type (**158**), in which the leaving group (Z) was chlorine, proceeded with inversion of configuration to give the 5 (*R*)-penem (163). Ring contraction of 2-thiacephems of the type (**157**) has been the subject of extensive studies (410). The unpredictable stereoselectivity of desulfurizations that use phosphorus (III) reagents was overcome by oxidation to the 1,1-dioxides, which underwent stereospecific thermal desulfonylation to give the corresponding 5-(*R*)-penems. Despite this, it was concluded that the route offered no practical advantage over routes that used 2,3-double bond-forming strategies. Penems possessing leaving groups at the two positions have

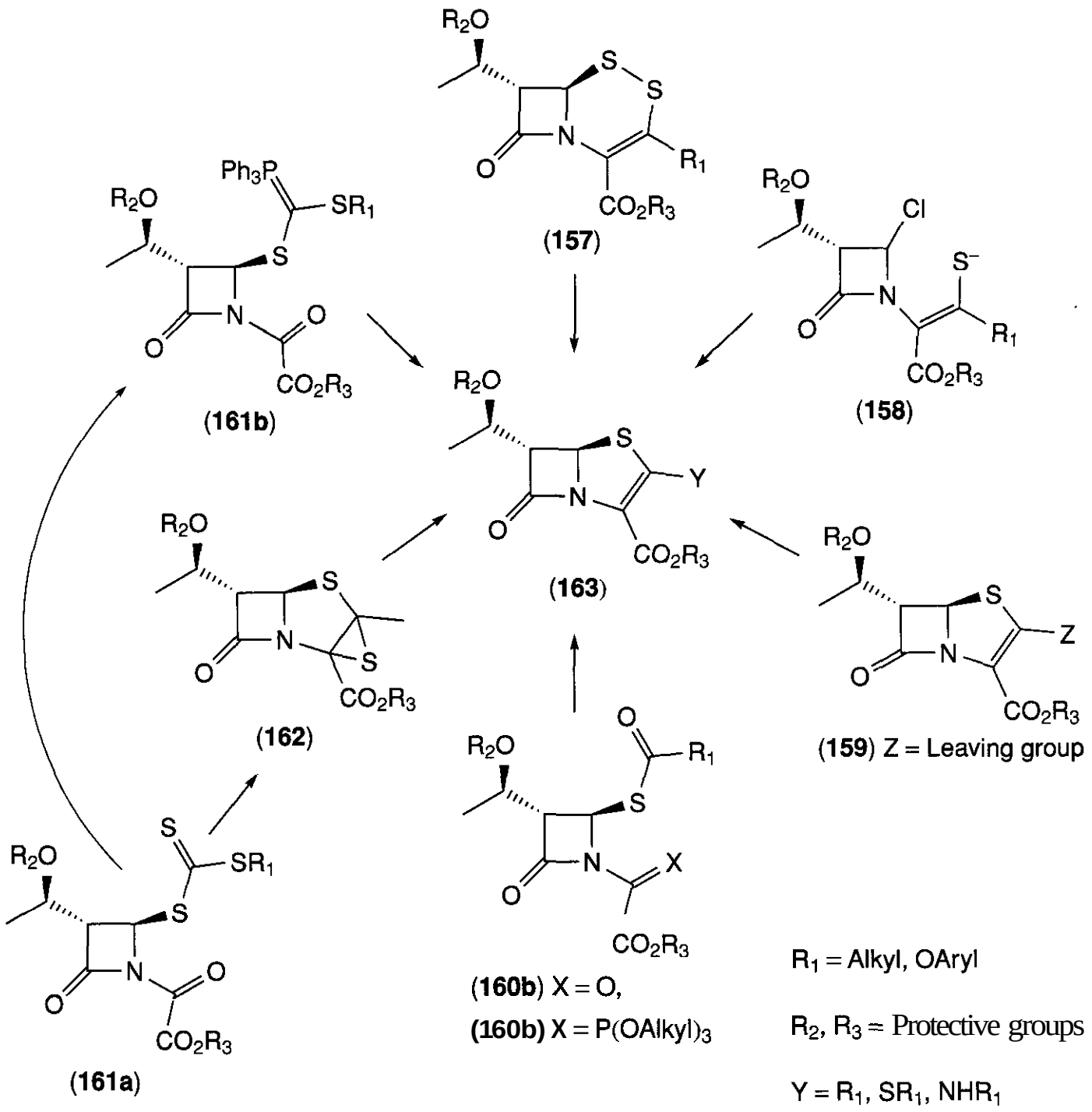


Figure 14.15. Alternative methods for the synthesis of penems.

proved to be useful intermediates for the synthesis of a large number of analogs. Regioselective oxidation of the sulfide provided 2-ethylsulfinylpenems [(**159**), Z = S(O)Et], which underwent displacement reactions with thiolates, to give a range of thiosubstituted penems (411). In a conceptually similar approach, amines have been shown to displace phenolic-leaving groups from penems of the type (**159**) (Z = OAr) to provide a general route to 2-substituted aminopenems (163, Y = NHR_1) (412).

Later, the generality of this approach has been realized with the demonstration of displacement of the triflate group of penems (159, Z = OSO_2CF_3) by thiolates and cuprates, to give both sulfur- and carbon-substituted penems (413).

5.3.2 Biological Properties. Unlike the antibacterially inactive penicillanic and cephalosporanic acids, the 6-unsubstituted penems exhibited good activity against Gram-positive bacteria but displayed more modest potency against Gram-negative strains (390). Penems such as (**140**), incorporating a 6β-acylamino side chain, were of limited stability and showed only weak antibacterial properties (388). In contrast to methoxylation, 6α-meth-

ylation of (140) improved stability, although the compound still exhibited only weak antibacterial activity (414). Similarly, 6α-methylpenems were also reported to be more stable than the methoxy analogs, whereas 6,6-dialkylpenems have been shown to be extremely stable but devoid of antibacterial activity (415).

Incorporation of the 1(*R*)-hydroxyethyl moiety found in the naturally occurring carbapenem thienamycin proved crucial for providing penems with broad-spectrum of activity and stability to β-lactamases. As with the carbapenems, activity was markedly affected by the relative stereochemistry at the three chiral centers. Further evaluation of the components within the racemic mixture of penems (148)revealed that the *trans*-8(*R*)-isomer was 20- to 30-fold more potent than the *trans*-8(*S*)-isomer, with the two cis-isomers being of intermediate potency (416). The same study showed that removal or alkylation of the 8-hydroxy group resulted in loss of p-lactamase stability and reduced potency against non-β-lactamase-producing bacteria. Increased bulk resulting from an additional methyl group or substitution of the C8-methyl group was not tolerated; 6-hydroxy-isopropyl-, 6-(1-hydroxy-1-propyl)-, and 6-(1-hydroxy-2-phenylethyl)-penems were all devoid of useful antibacterial activity (417). With the exception of the 6-(substituted) methylenepenems, which showed potent p-lactamase inhibitory properties (Section 5.7), all the penems that have progressed have 5(*R*),6(*S*),8(*R*)-stereochemistry. The relatively less demanding nature of the 2-substituent is amply demonstrated by the wide range of 6-(1-hydroxyethyl) penems, which have been described as having potent antibacterial activity. Typically, these compounds show excellent activity against Gram-positive bacteria including β-lactamase-producing strains, whereas activity against Gram-negative organisms is more modest and generally not as good as that seen with carbapenems. Except for compounds possessing a basic amino function in the 2-substituent, penems are devoid of useful activity against *Pseudomonas* species (418). Changes in 2-substituents are often accompanied by changes in susceptibility to β-lactamase and human renal dehydropeptidase-1 enzymes as well as pharmacokinetics (e.g., oral absorption); these properties have proved important in the selection of candidates for progression.

Table 14.11 lists some of the compounds that have been selected for development as potential antibacterial agents. Some of these penems [e.g., SCH 29482 (164) and faropenems (169, SUN 5555)] have oral absorption properties as acids, whereas others [e.g., ritipenem (166, FCE 22101)] have required the use of the prodrug approach to give good blood levels by this route.

However, development of the orally active β-lactam antibiotic SCH 29482 (**164**) has been discontinued by Schering-Plough because of an odor problem (427). This compound was in Phase II studies for treatment of gonorrhea, pneumonia, and UTI in the United States, United Kingdom, Ireland, and other European markets. In humans, it was rapidly absorbed when administered orally, with half-life of 1.5–2 h. No significant clinical or biochemical changes were seen. It was highly active (MIC < 8 μg/mL) against Gram-positive and Gram-negative bacteria, including some strains resistant to third-generation cephalosporins, but had no antipseudomonal activity.

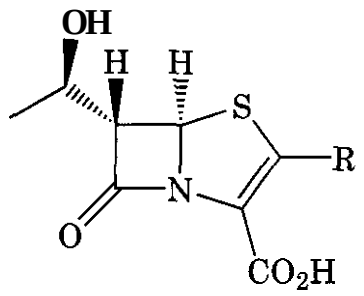
Faropenem (**169**) is another example of an oral and injectable penem antibacterial agent with a unique spectrum of activity against aerobic and anaerobic Gram-positive and Gram-negative bacteria, excluding Pseudomonas aeruginosa. It is also highly stable against various β-lactamases. Faropenem was launched in Japan in 1997.

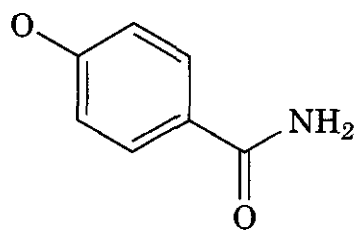
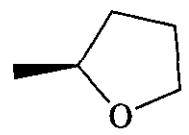
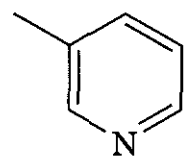
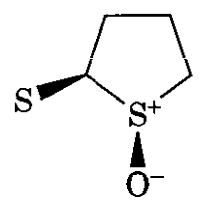
Ritipenem acoxil (FCE 22891) is the oral acetoxymethyl ester of ritipenem (166, FCE 22101), an injectable penem derivative for the treatment of urinary tract and lower respiratory tract infections.

5.4 Monobactams and Nocardicins

The isolation of the nocardicins in 1976 (428) and the monobactams in 1981 (429, 430) revealed for the first time the potential for antimicrobial activity in simple monocyclic β-lactam structures as opposed to the fused-ring systems of the penicillins and cephalosporins. This provided great impetus for new ideas on the structural features necessary for β-lactam

Table 14.11 Penems Selected for Detailed Investigation



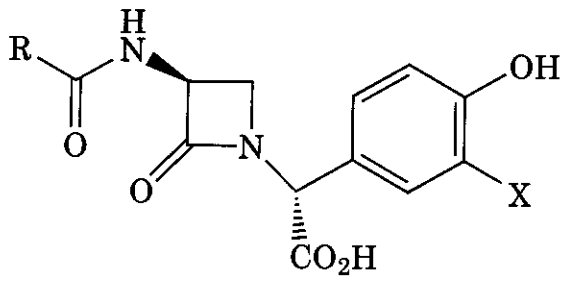
Compound	Name/Code	R	Reference
(**164**)	SCH 29482	SCH_2CH_3	419
(**165**)	SCH 34343	$S(CH_2)_2OCONH_2$	420
(**166**)	FCE 22101	CH_2OCONH_2	421
(**167**)	FCE 29464	CH_2OCH_3	422
(**168**)	HRE 664		423
(**169**)	SUN 5555		424
(**170**)	CGP 37697		425
(**171**)	CP 70429		426

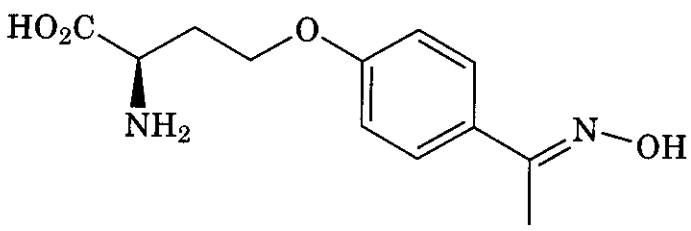
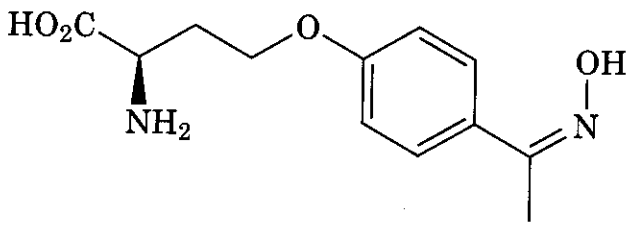
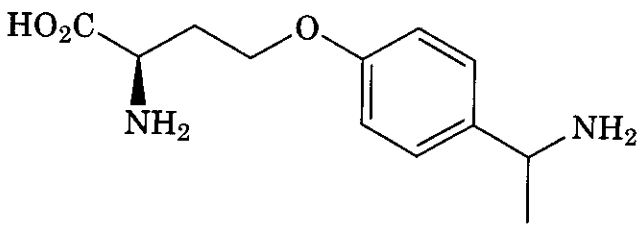
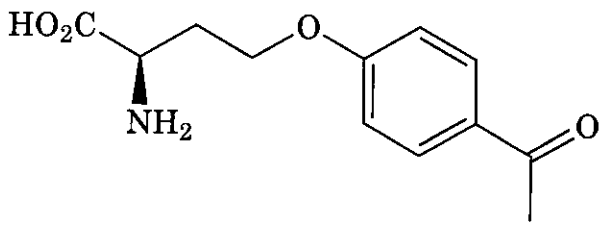
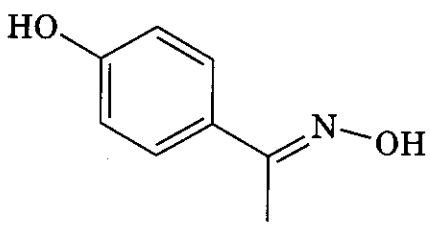
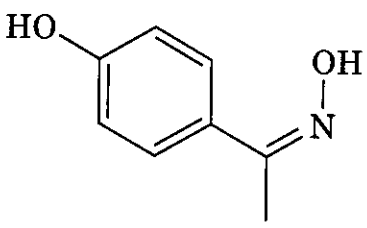
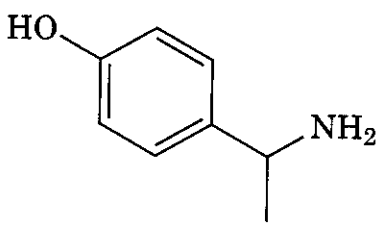
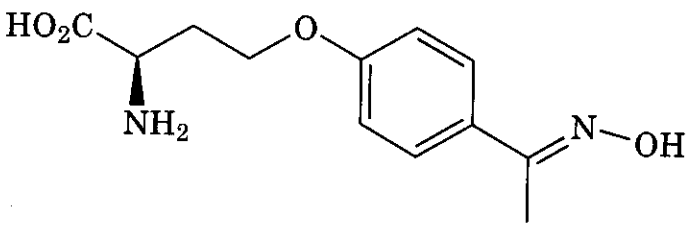
recognition and activation, together with the development of a wide range of synthetic methods for the construction of both natural products and analogs (431).

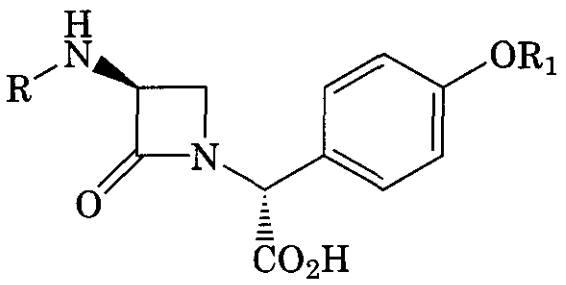
5.4.1 **Nocardicins.** The nocardicins were detected in a fermentation broth of a strain of *Nocardia uniformis* by a screening procedure through the use of a mutant strain of E. *coli* supersensitive to β-lactam antibiotics. The first compound to be characterized was nocardicin A, which shows a modest level of antibacterial activity *in vitro* against Gram-negative bacteria (428). Structural determination was from spectroscopic evidence and by degradation experiments (432,433). Several related compounds (nocardicins B-G) have also been characterized (432,4341, whereas the most recent variation is the chloro-substituted derivative from a Streptomyces species (435). The structures of the natural nocardicins are shown in Table 14.12.

5.4.1.1 Derivatives of 3-Amino Nocardicinic Acid (3-ANA) and the Synthesis of Nocardicins. As with the penicillins and cephalosporins initial approaches to improve the potency of the natural products revolved around changing the acylamino side chain by way of the 3-amino norcardicinic acid (3-ANA) nucleus (**180**). Initially, this was prepared by deacylation of nocardicin C by use of microbial amidases or chemical methods (436, 437). A more practical approach made use of the reaction of the oxime group of nocardicin A with a large excess of di-t-butyl dicarbonate, to give

Table 14.12 Structures of Natural Nocardicins



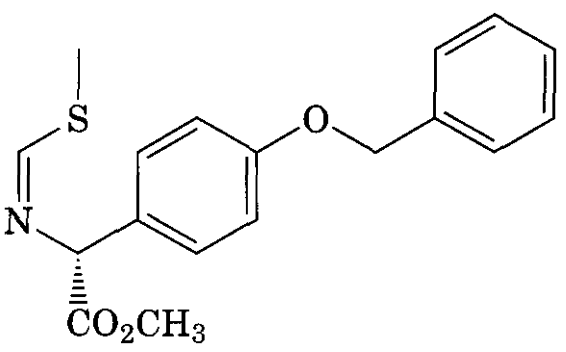
Compound	Nocardicin	X	R	Reference(s)
(172)	A	H		432, 433, 435
(173)	B	H		432, 435
(174)	C	H		432, 435
(175)	D	H		432, 435
(176)	E	H		432, 435
(177)	F	H		432, 435
(178)	G	H		432, 435
(179)	Chlorocardicin	Cl		436



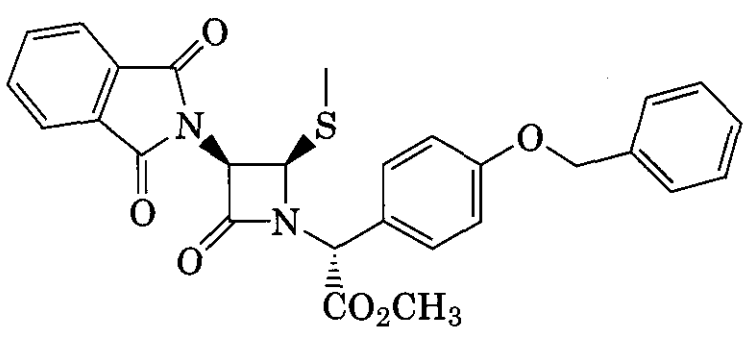
(180) R = R_1 = H (3-ANA)
(181) R = R_1 = CO_2 t-But

(**181**), which on treatment with trifluoroacetic acid afforded 3-ANA in excellent yield (438).

Although semisynthetic approaches to the nocardicins from penicillin-derived β-lactams have been reported (437, 439), by far the greatest effort has been directed toward totally synthetic methods that afford a versatile range of structural types. One early approach made use of the cycloaddition reaction of the thioimidate (**182**) with the ketene generated



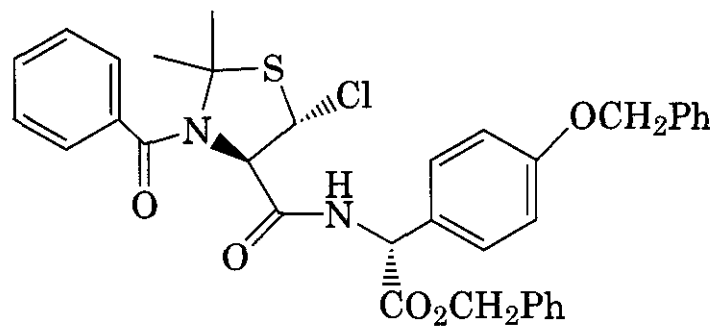
(182)



(183)

from phthalimidoacetyl chloride. This gave the cis-substituted β-lactam (**183**), which was readily converted to 3-ANA, and then nocardicin A and other natural side-chain derivatives (440).

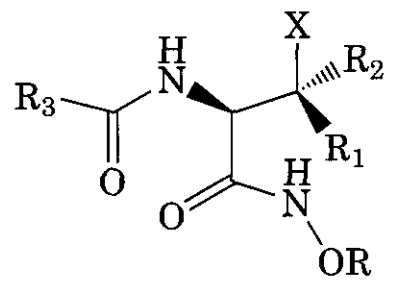
Another synthesis of nocardicin A reported by the Lilly group used the L-cysteine-derived thiazolidine (**184**), where intramolecular displacement of the chlorine provided one of the



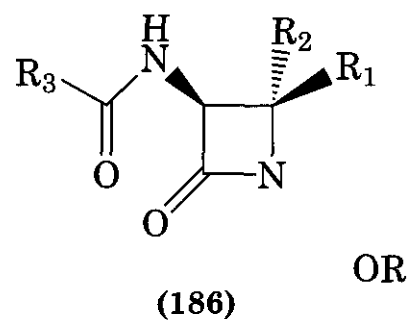
(184)

first examples of N1-C4 ring closure to form the β-lactam ring (441).

Undoubtedly, however, the most widely used approach involving cyclizations of this type is the hydroxamate method developed by Miller, which provides the opportunity to use readily available amino acids in a high yielding process to afford chiral β-lactams of virtually any description without racemization or elimination taking place (442). Thus, use of the Mitsunobu procedure or base-catalyzed cyclization of β-halo hydroxamates (**185**) provides good yields of the cyclic product (**186**),



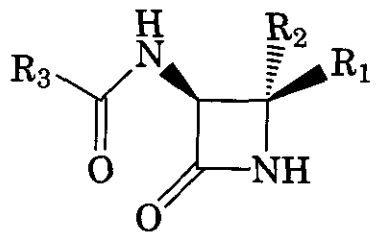
(185)



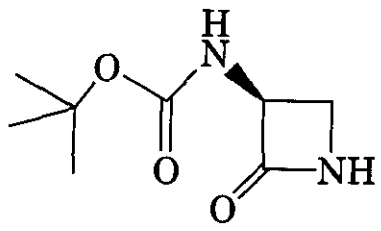
(186)

from which the β-lactam (187) is obtained by reduction of the free N-hydroxy β-lactam (186; R = H) with titanium trichloride (443).

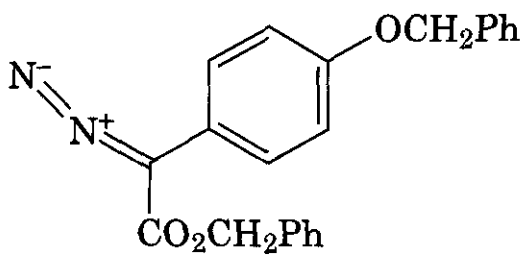
Miller used this method for the synthesis of the nocardicin ring system starting from tBoc-L-serine, to give the β-lactam (188). This was followed by introduction of the phenylglycine residue by alkylation or diazo insertion using



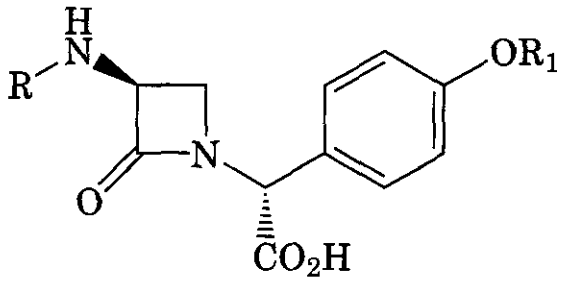
(187)



(188)



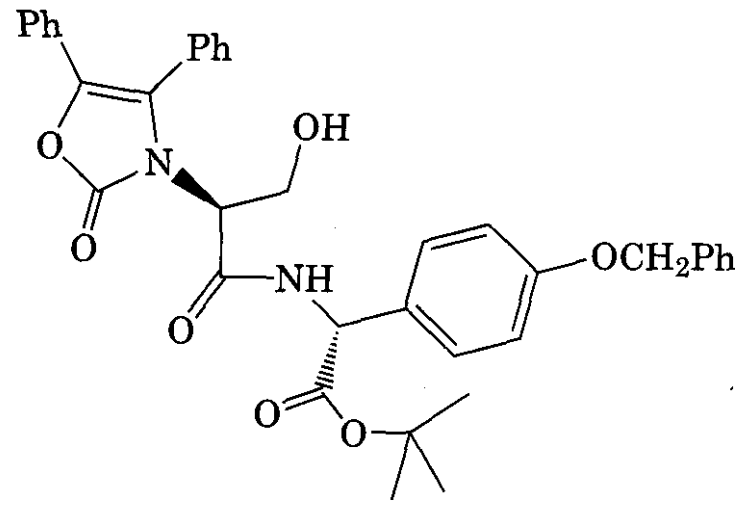
(189)



(190)

(189), giving an overall yield of 45% of (190), a fully protected version of 3-ANA (444).

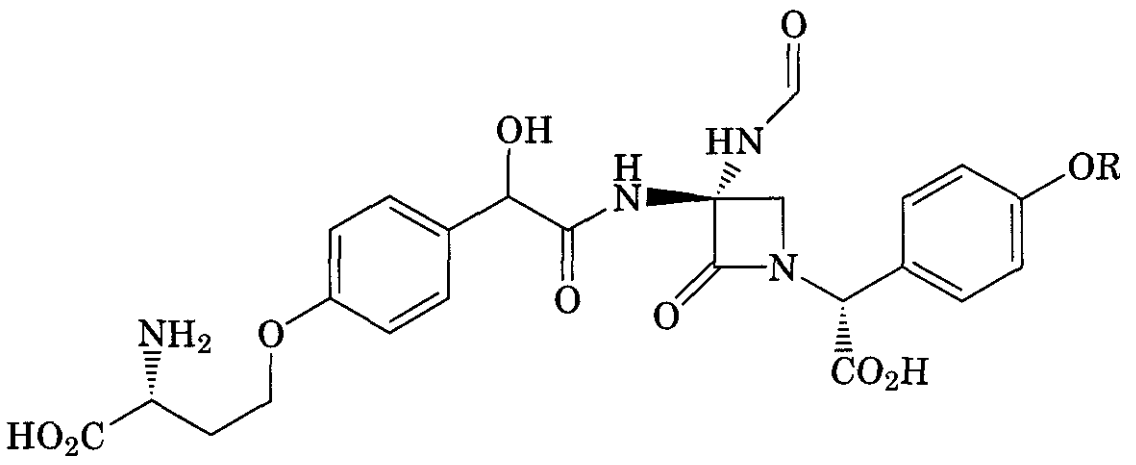
This biomimetic-like ring closure has also been used by Townsend (446). Intermediate (191) was cyclized in a modified cyclodehydration procedure by use of triethylphosphite rather than triphenylphosphine, which avoided any problems of epimerization at C5. After suitable deprotection and acylation with the appropriate side chains, nocardicins A-G were synthesized in good yields; for example, nocardicin A was produced in an overall yield of 22% from L-serine and D-(*p*-hydroxyphenyl)-glycine. Other approaches to the nocardicin skeleton have made use of a-methylene β-lactams (446) as well as novel β-lactam-forming



(191)

reactions (447), whereas general aspects of the synthesis of monocyclic β-lactams and 3-amino-2-azetidinones have been reviewed (431, 448,449).

5.4.1.2 Biological Activity and SAR. Nocardicin A shows in vitro activity (3.13–50 μg/mL) against several Gram-negative organisms, including Pseudomonas aeruginosa, Proteus species, Serratia *marcescens*, and strains of Neisseria. No activity of significance is seen against Staphylococci or E. coli (450). Nocardicins C-E show weak activity, whereas nocardicins F and G are inactive, illustrating the importance of the *syn*-oxime function and the homoserine residue for activity in these natural products (451). Functional group modifications of the side chain (oxime, ketone, amine) of nocardicins A, C, and D have been carried out, but almost all the compounds show reduced activity. Acetamido derivatives of 3-ANA show weak activity, but replacement of the p-hydroxyphenyl residue by other aromatic or heteroaromatic groups generally does not reduce potency. After extensive SAR studies involving the preparation of several hundred compounds, it was concluded that the only useful antibiotic in the series was nocardicin A and, to maintain an effective level of activity, only limited modification of the natural product is possible (451). The most interesting property of nocardicin A relates to its mode of action in *vivo*, where it was found to act synergistically with serum bactericidal factors against P. aeruginosa and with polymorphonuclear leukocytes (PMNs) against P. aeruginosa, E. coli, and P. vulgaris. Unlike most antibiotics the bactericidal activity of nocardicin A increased markedly against these

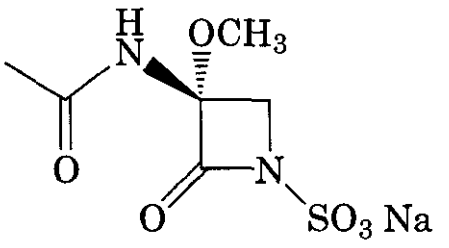


(192) R = D-glucuronic acid; Formadicin **A**
(193) R = H; Formadicin C

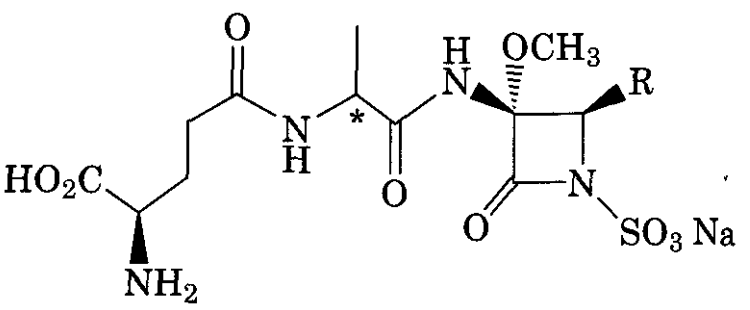
organisms in the presence of fresh serum and PMNs, an effect that was reflected in a more potent *in vivo* activity than would have been anticipated from *in vitro* MIC values (450,451).

5.4.1.3 ***Formadicins.*** The most recently described natural products having the nocardicin skeleton are the formacidins A-D, isolated from a species of *Flexibacter* (452).

Structural determination showed that formadicins **A** (192) and C (193) possessed the formamido substituent in the C3-position of the β-lactam ring (453). These natural products show a fairly narrow spectrum of activity that is similar to that of nocardicin **A,** of which those with the formamido substituent were significantly more stable to hydrolysis by β-lactamases. No synthetic analogs have yet been reported.

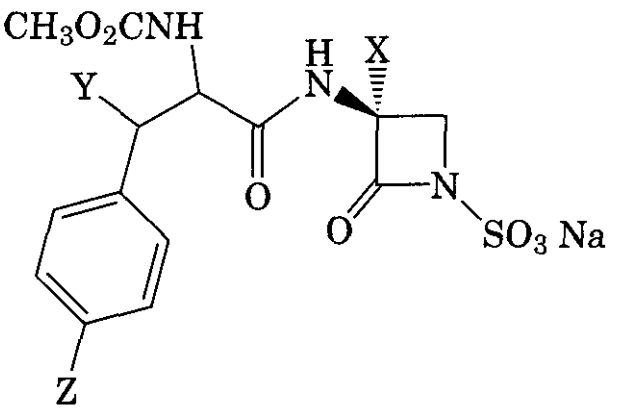


(194) SQ 26180



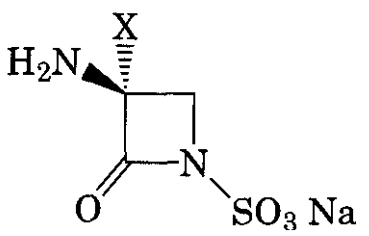
(195) R = H; Sulfazecin; * = D
(196) R = H; Isosulfazecin; * = L
(197) R = CH_3; * = D

5.4.2 ***Monobactams.*** In 1980 groups at Takeda and Squibb reported on the isolation of **a** new class of monocyclic β-lactams from bacteria (429, 430). Up to that time, the only producers of β-lactam antibiotics were the fungi and actinomycetes. Characterized by the presence of an N1-sulfonic acid grouping in the β-lactam ring, they have collectively become known as the monobactams (454). The simplest member of the family is the acetamido derivative SQ 26180 (194) (455), whereas other members of the series are sulfazecin (**195**) (456), isosulfazecin (196) (457), and several related compounds based on structure (**197**) (458), together with other examples having oligopeptide side chains (459–461). One natural compound (**198**) has been reported to have a C4-methyl group (462).



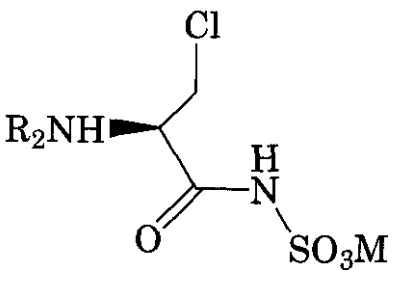
(198) X = OCH_3
Y = OSO_3M
Z = H, OH, OSO_3M

5.4.2.1 Structure Determination and Synthesis. The structure of SQ 26180 (**194**) was readily determined from spectroscopic data. Confirmation was made by degradation of the thiazine ring of an appropriately substituted methoxylated cephalosporin to a simple acetamido β-lactam. N-Sulfonation then gave material identical with the natural product, thus establishing that the stereochemistry at the C3 position of SQ 26180 (**194**) was the same as found in the cephalosporins and cephamycins (455). The structure and stereochemistry of sulfazecin (195) was unambiguously established by X-ray crystallography (**463**), whereas that of the other natural monobactams was obtained by a combination of spectroscopic properties and hydrolytic experiments to determine the nature of the peptide side chains. Isolation of the monobactams has led to the synthesis of many acylamino derivatives of 3-amino monobactamic acid (3-AMA) (**199**) or



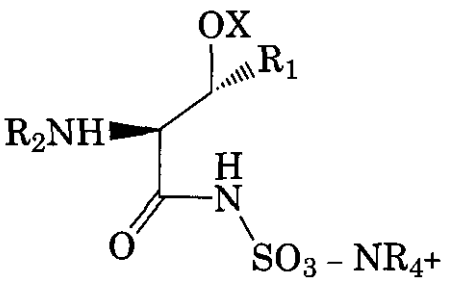
(199) X = H
(200) X = OCH_3

the methoxy nucleus (200). Because deacylation of the natural products was not satisfactory, 0-lactams (199) and (200) were initially prepared by degradation of 6-APA (2) followed by sulfonation of the derived β-lactam; in the case of the methoxy β-lactam (**200**), only racemic material was obtainable (454,464). Total synthesis has also been extensively used and is now almost always the method of choice. This was first achieved by direct base-catalyzed cyclization of the acyl sulfamate (**201**) (465).

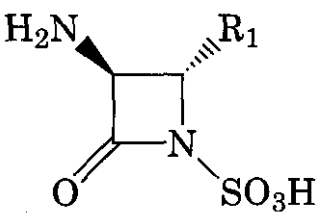


(201) R_1 = H, CH_3
R_2 = Acyl derivative

Subsequently, a wide range of methods have been developed, starting from β-hydroxy amino acids such as serine to intermediate (202) (R_1 = H) or threonine, giving (203) (R_1



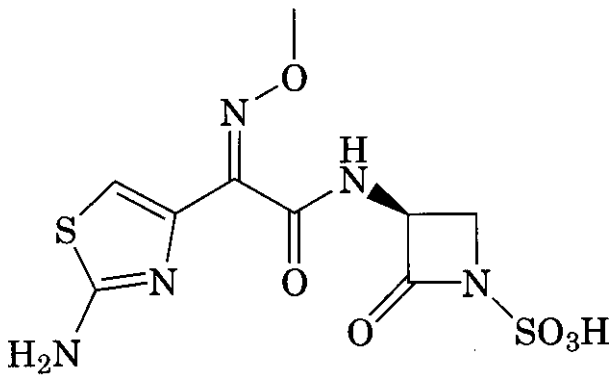
(202)



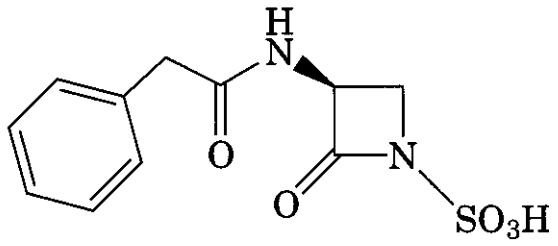
(203)

= Me), to provide a ready route to the nucleus (203) (R_1 = H or Me), ideally suitable for derivatization by acylation of the C3 amino group (466).

5.4.2.2 ***Biological Activity of Natural Products and Synthetic Derivatives.*** Like the penicillins, cephalosporins, and other β-lactam antibiotics, the monobactams interfere with the synthesis of bacterial cell walls by binding to PBPs. Aztreonam (see below), one of the clinically efficacious agents, binds specifically to PBP-3 (467). All of the natural products exhibit poor activity, but extensive modification of the C3-amido side chain by acylation of 3-AMA has provided several highly potent compounds. In addition, structural modification at C4 and N1 of the β-lactam ring can profoundly affect antibacterial activity and β-lactamase stability (468). The importance of the C3-stereochemistry was demonstrated by the synthesis of the enantiomers of (204). Only the S-isomer (MIC < 0.05 μg/mL against P. rettgeri; R-isomer > 100 μg/mL) corresponding to that found in the penicillin and cephalosporin family of natural products was active (466). Compared to benzyl penicillin the analogous monobactam (**205**) shows a similar pattern of activity, but with reduced potency. With the carbenicillin, side-chain activity was reduced, but with ureido derivatives activity

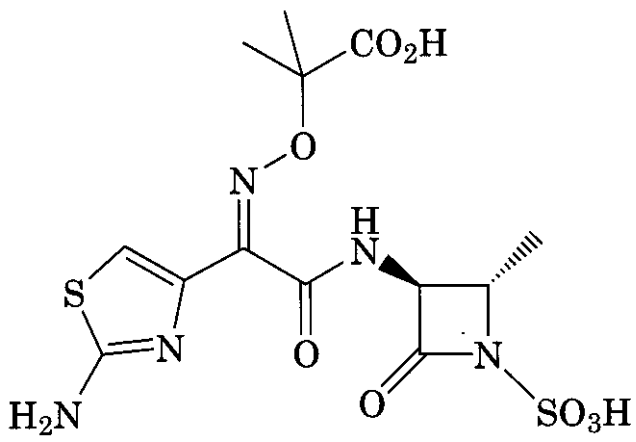


(204)



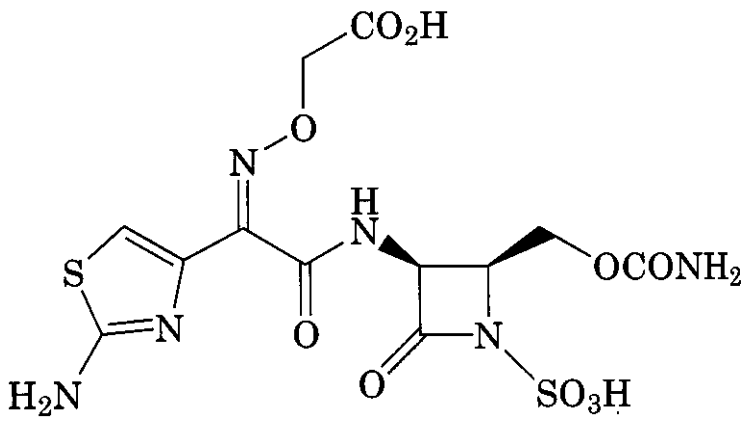
(205)

was similar to that of the penicillins. A similar pattern was apparent with the corresponding analogs of the cephalosporins (468). One early observation revealed that C4-alkyl substituents could considerably influence both antimicrobial potency and β-lactamase stability. Extensive SAR studies resulted in the identification of the totally synthetic derivative aztreonam (206) as a product with a potent and



(206) Aztreonam

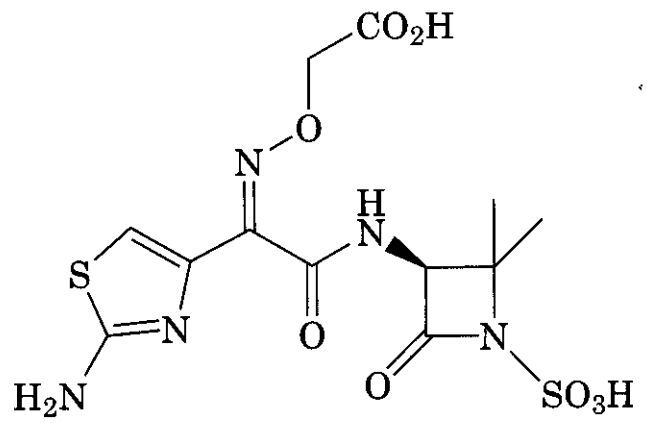
useful spectrum of clinical activity against Gram-negative bacteria, but with virtually no activity against Gram-positive pathogens (466,469, 470). A second synthetic monobactam to reach the clinic is carumonam (**207**), which shows some improvement in activity over that of aztreonam against the Enterobacteriaceae family of bacteria (471). Table 14.13



(207) Carumonam

shows some representative activities of a number of monobactams and related analogs.

5.4.2.3 Alternative N1-Activating Croups. The sulfamate residue found in the monobactams is considered both to activate the β-lactam ring for interaction with the active site serine of a PBP and to provide the anionic charge required for binding, as postulated by Knox (466, 473). This has stimulated the effort to synthesize monocyclic derivatives that have new N1-activating substituents, which could act in the same way. Several of them have been discovered that exhibit the properties necessary to afford antibacterial activity, and are exemplified by structures (**208–211**),



(208) Tigemonam

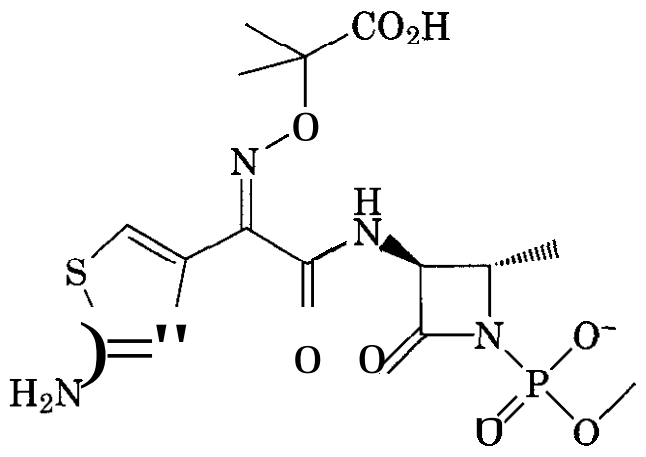
all of which exhibit various levels of antibacterial activity (Table 14.12) and β-lactamase stability (466). Tigemonam (208) is a potent anti-Gram-negative agent with good stability to β-lactamases. It is also orally absorbed, which is unusual among the monobactams, and encouraging results have been obtained in phase I and phase II clinical studies (466). Another example of heteroatom activation is seen in the oxamazin series exemplified by oximonam (211). The t-butyl glycolate ester prodrug (gloximonam) of (211) is also absorbed by the

Table 14.13 In *Vitro* Antibacterial Activity of Monobactams and Analogs[a,b]

Organism	Pen G	CAZ	(204)	AZTR (205)	CAR (206)	TIG (207)	(208)	(209)	OXIM (210)
E. coli	>100	0.4	50–>100	0.1–0.2	0.1–0.2	0.4–0.8	0.4–0.8	0.4–0.8	0.25–0.5
K. *aerogenes*	12.5–>100	0.1–1.6	50	0.2–>100		0.4–0.8	0.4–0.8	0.4–>100	
E. cloacae	25–>100	0.2–>100	>100	0.01–50	0.25–16	0.8–50	0.8–>100	0.8–50	1–>64
P. rettgeri	3.1	<0.05	25	0.05	0.06–0.12	<0.05	0.2	<0.05–0.4	0.12
P. *aeruginosa*	>100	1.6	>100	3.1	1 4	1.6–>100	0.3–>100	0.8–12.5	>64
S. aureus	0.05–3.1	12.5	3.1–6.3	>100	>128				>64

[a] Minimum inhibitory concentrations (range mg/L). Refs. 466,468,471,472.

[b] Abbreviations: Pen G, benzyl penicillin; CAZ, ceftazidime; AZTR, aztreonam; CAR, carumonam; TIG, tigemonam; OXIM, oximonam.



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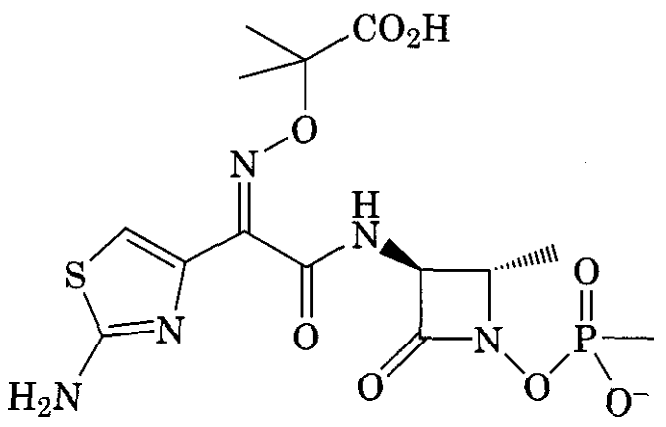

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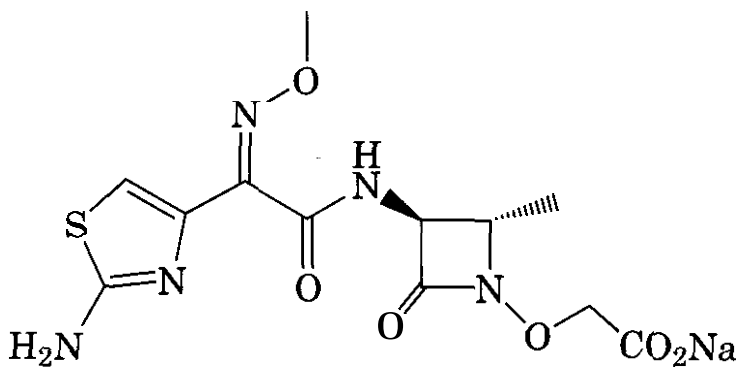


(211) Oximonam

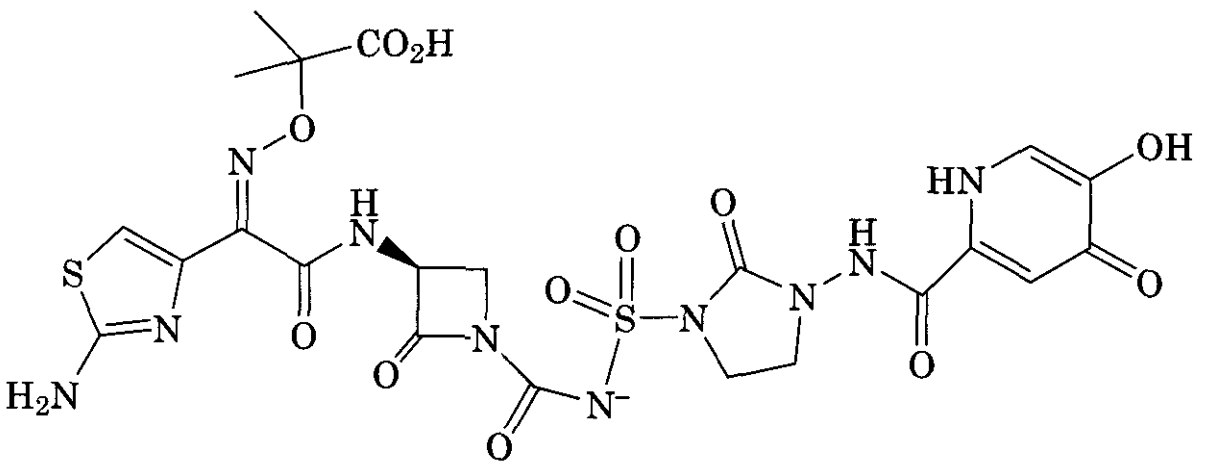
oral route, giving good therapeutic levels of the parent drug (466). Efforts to improve potency against Pseudomonas aeruginosa led to the discovery of SQ 83360 (**212**), having the 3-hydroxy-4-pyridone residue, which acts as a catechol bioisoter, leading to uptake by the bacterial *ton*B-dependent iron transport pathway in a manner similar to that of the catecholic β-lactams (474). A somewhat different series is that in which a tetrazole has replaced the sulfonate group to give, for example, RU 44790 (**213**), which has been shown to compare favorably in activity with aztreonam (475).

In contrast, N1-sulfonated monobactams havingthe (*R*)-1-hydroxyethyl of the carbapenems (Section 5.4) at the C3-position showed only weak activity (476). The most recent class of compounds reported as being active are C3-alkylidene derivatives (214) with, surprisingly, a neutral N-acyl activating group on the N1-position of the β-lactam ring (477).

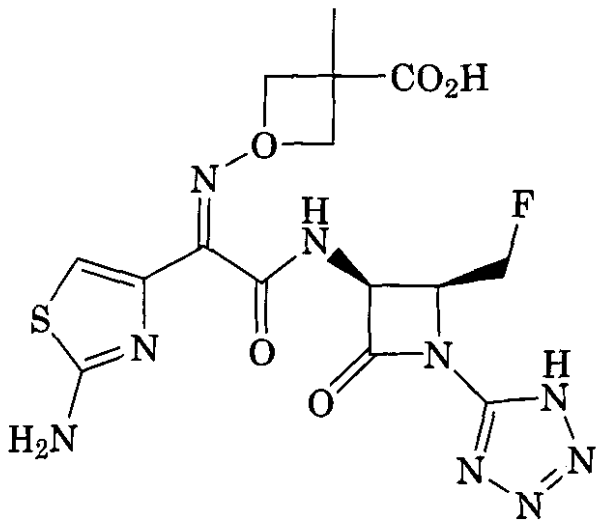
5.5 Carbapenems and Trinems

Although structurally related, carbapenems and trinems have different origins. The carbapenems were discovered as natural products during the screening of microbial metabolites, whereas the trinems were conceived as synthetic analogs designed to improve the biological properties of existing classes of β-lactam antibiotics.

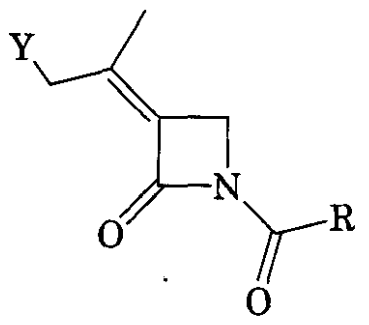
5.5.1 Discovery of Carbapenems. Imipenem (MK 0787, 215) and meropenem (SM 7338, 216) can be considered as the most significant examples of β-lactams. The rationale by which it has been possible to discover these agents



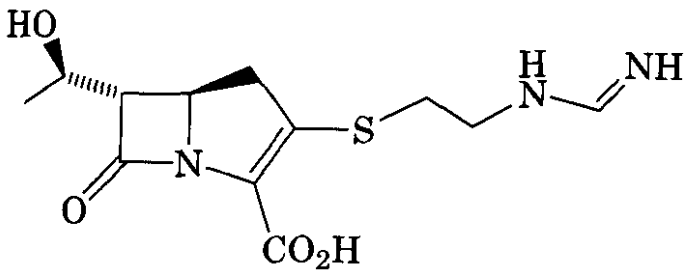
(212) SQ 83360



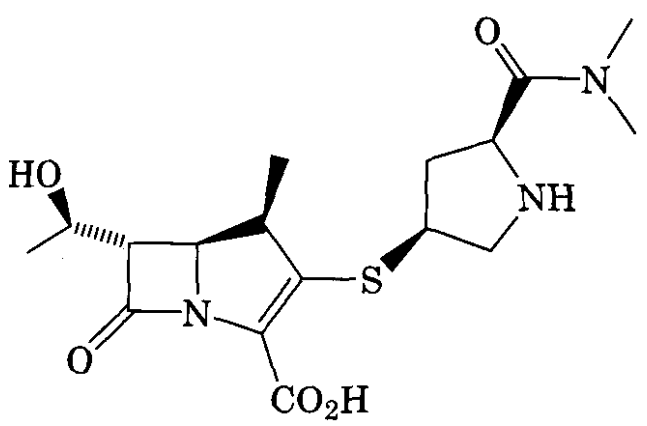
(213) Ru 44790



(214)

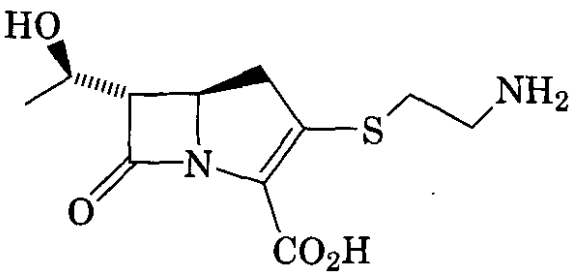


(215) Imipenem MK 0787 (1982)



(216) Meropenem SM7338 (1987)

can be described as an elegant and successful example of applied medicinal chemistry. The research has faced and resolved several issues related to chemical and biological properties of these molecules optimizing the key stuctural elements, to adjust the spectrum of activity and the enzymatic and chemical stability. Direct comparison of thienamycin (**217**) with

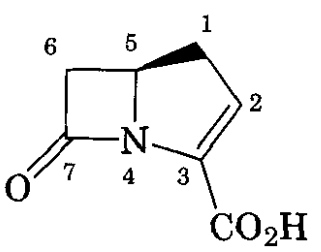


(217) Thienamycin (1977)

meropenem (216) clearly shows this process. Introduction of a 1-β-methyl group and formation of a conformationally constrained $SCH_2CH_2NH_2$ system resulted in one of most potent and effective β-lactam antibiotics currently used.

In the mid-1970s during the course of screening for inhibitors of cell wall biosynthesis the Merck group isolated thienamycin (**217**) from Streptomyces *cattleya*, followed by other members of the family of carbapenems (478).

At the same time a Beecham group, while screening for inhibitors of β-lactamases (Section 5.7), isolated from Streptomyces *olivaceus* a group of interrelated metabolites that they called the olivanic acids (479, 480). All these compounds were characterized by the presence of the 5-(*R*)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid ring system, generally referred to as 1-carbapen-2-em-3-carboxylic acid (**218**), and which has been isolated as a natural



(218)

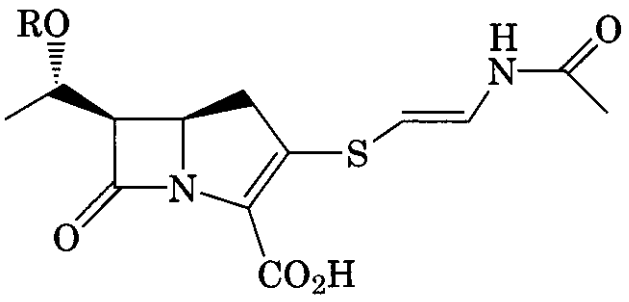
product from certain species of *Serratia* and *Erwinia* (481).

All the other natural products possess substituents at C2 and C6 of this nucleus. Over 50 variations are known and many of them have been listed (346, 482). The discovery of this novel series of highly active bicyclic compounds, so markedly different from the peni-

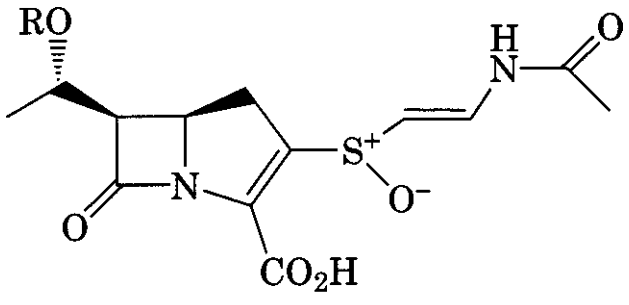
cillins and cephalosporins, revitalized ideas concerning the structural and stereochemical features required for biological activity and stability among the β-lactams.

5.5.1.1 Natural Products: Occurrence, Structural Variations, and Chemistry. While a variety of Streptomyces spp. produce the carbapenems, only S. cattleya and S. penemfaciens have been reported to produce thienamycin (**217**) (470,482). Several research groups have been involved in the search for new carbapenems (483–487). Often the natural products are found as a mixture of stereoisomers, usually in low yield (1–20 μg/L), and much effort has been expended on strain improvement and the optimization of fermentation conditions (488). Thienamycin (217) has the (*R*)-configuration at the C8-hydroxy group with a trans arrangement of protons about the β-lactam ring (489). The history of the discovery, structural elucidation, and chemistry of thienamycin (217) and related carbapenem antibiotics has been well documented (490). The first metabolites isolated from S. olivaceus were a series of sulfated derivatives such as MM13902 (**219a**; R = SO_3H), the corresponding sulfoxide MM4550 (**220a**), and the side-chain-saturated compound MM17880 (**221**) (480,486,491).

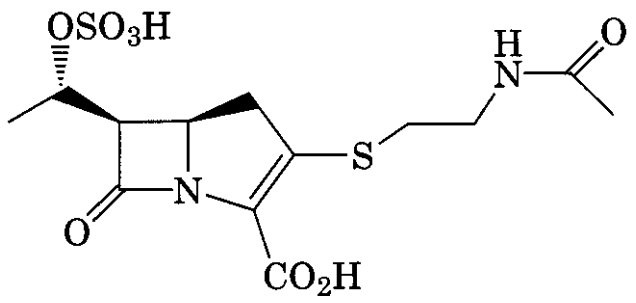
Later, several nonsulfated members (e.g., **219b**, **220b**, 221,222 and 223) of this group



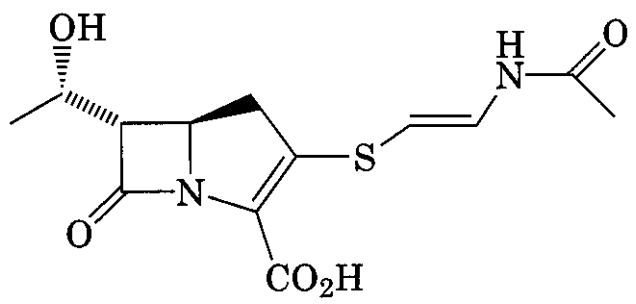
(219a) MM13902 R = SO_3H
(219b) MM22380 R = H



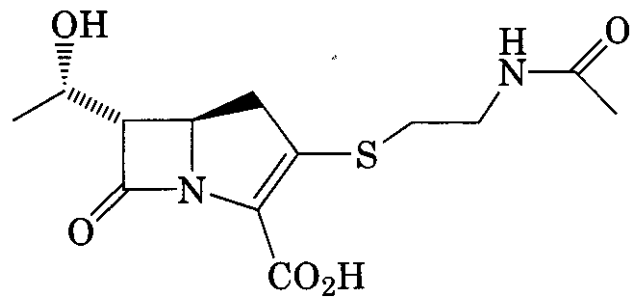
(220a) MM4550 R = SO_3H
(220b) MM22382 R = H



(221) MM17880



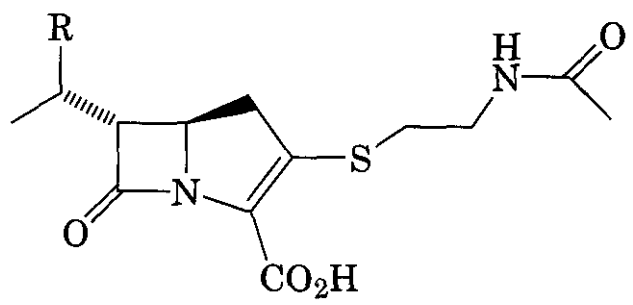
(222) MM22383



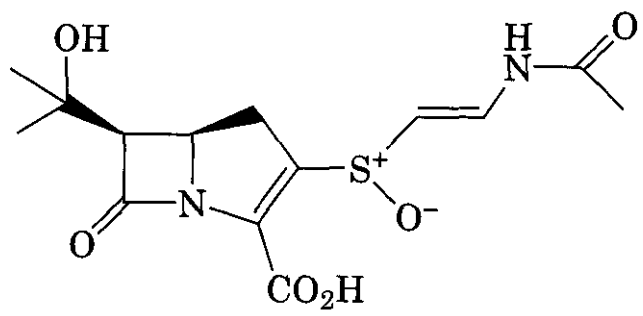
(223) MM22381

were also obtained from S. olivaceus (**492,** 493) and have shown to correspond to a series of thienamycins and epi-thienamycins analogs isolated from S. parvogriseus (490). In the olivanic acids the stereochemistry is 8(*S*) with a cis-substituted β-lactam in the sulfated series, whereas both cis- and *trans*-β-lactams are found in the case of the C8-hydroxy compounds (494,495). Following reports of thienamycin (217) and the olivanic acids (219–223) a number of structurally related carbapenems having differing C2- and C6-substituents were reported. Representative examples of these compounds are PS-5 (**224**), PS-6 (**225**) (496), carpetimycin A (226) (497), asparenomycin A (227) (290), and pluracidomycin A (**228**) (487), isolated from a wide variety of sources.

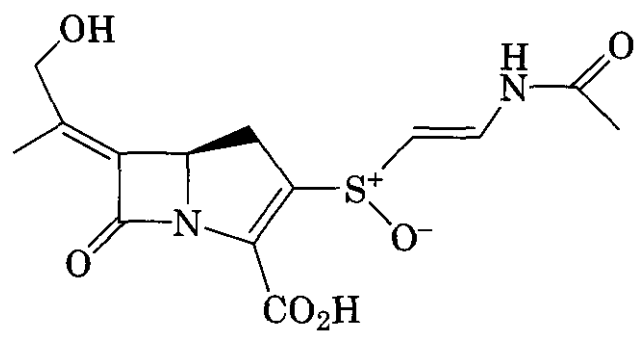
The carbapenems often show chemical instability and can be extremely sensitive to reaction conditions. Nevertheless, a wide range of functional group modifications have been carried out on the natural products. Many de-



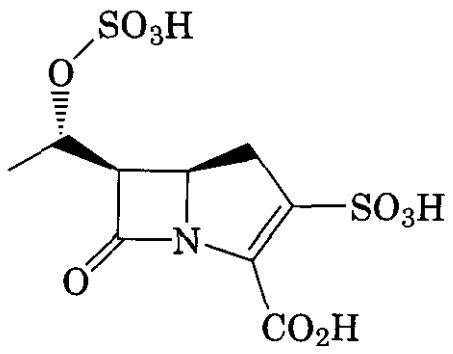
(224) PS-5 R = H
(225) PS-6 R = CH_3



(226) Carpetimycin A



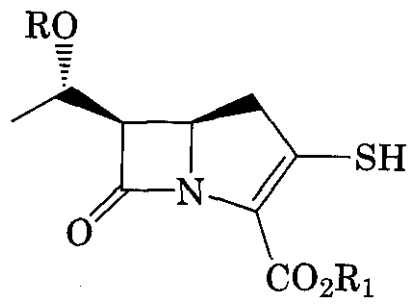
(227) Asparenomycin A



(228) Pluracidomycin A

rivatives of the amino, hydroxy, and carboxy groups of thienamycin have been obtained for SAR studies; other reactions cover oxidation or removal of the cysteaminyl side chain and isomerization of the double bond (490, 499). The largest class of derivatives are those obtained by modification of the amino group, particularly amidines, which greatly improve chemical stability without compromising potency. It was this series that gave *N*-formimidoyl thienamycin (imipenem, 215) (500), which eventually became the first clinically used member of the carbapenem family (501).

In the olivanic acids the side-chain double bond can be isomerized to the (2)-isomer (502). It also reacts readily with hypobromous acid to produce a bromohydrin, which readily breaks down to the key thiol intermediate (**229**), ideally suited for the synthesis of new C2-alkenyl or alkyl analogs (503).



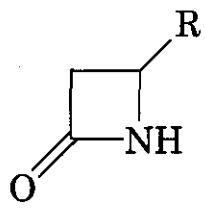
(229)

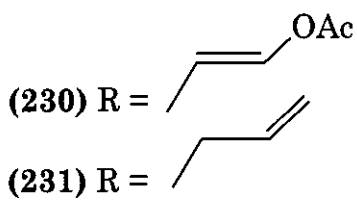
With the C8-hydroxyolivanic acids, inversion of the (*S*)-stereochemistry affords an entry into the 8(*R*) thienamycin series (504). Another useful reaction for introducing C2-sulfur substituents was developed with PS-5 (**224**) by displacement of the *S*-oxide with other thiols (505).

Many wide-ranging and detailed reviews of the early and most recent chemistry of the carbapenems have been published (370,482,490, 499,502,506–513).

5.5.1.2 Synthesis of Natural Carbapenems. The novelty and potent biological properties of the carbapenems, together with low fermentation yields, resulted in an intensive synthetic effort at a relatively early stage, to produce not only the natural products but also, particularly, synthetic analogs having improved chemical and metabolic stability (see Section 5.5.1.3) over that of the natural materials (489, 514–516). The most common approach has been to construct an appropriately functionalized and stereochemically correct β-lactam, followed by cyclization, to form the more highly strained bicyclic system in the first steps of the synthesis. Variations of this approach have been applied to produce virtually all of the natural products (517). The unsubstituted ring system (218) was synthesized,

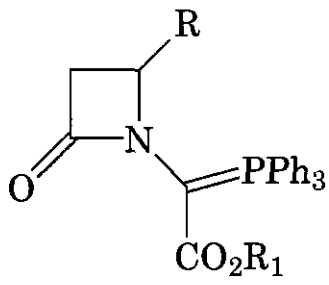
before the discovery of the natural material, in racemic or chiral form by several groups, starting from azetidinone (230) or (**231**), ob-

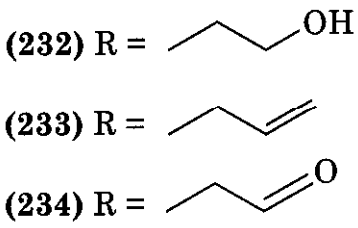




tained from chlorosulfonyl isocyanate (CSI) and an appropriate olefin (518–521).

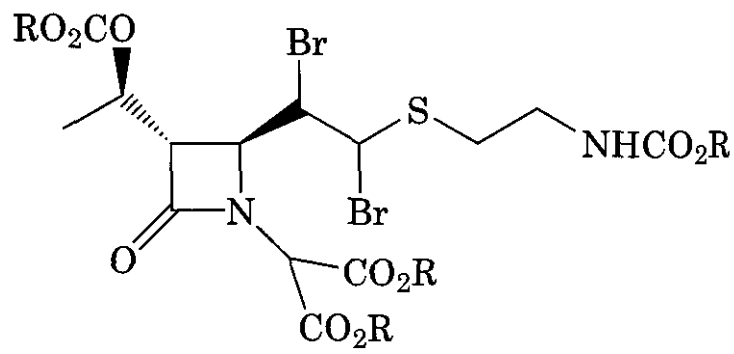
Progression to the phosphorane (232 or 233) was followed by oxidation to the aldehyde





(**234**), which readily cyclized by an intramolecular Wittig reaction. Removal of the acid-protecting group provided the unstable sodium salt of (218). The formation of the [2,3] double bond by this procedure has been extensively used both for analogs and for natural product synthesis (522). Since its discovery, thienamycin (217) has been the focal point for innumerable synthetic studies reported in the carbapenem field (490, 512, **517**). Many of the methods originated from the Merck group are still widely used. Their first synthesis of racemic thienamycin (217) also made use of (**230**), introduction of the hydroxyethyl side chain being by way of an aldol reaction giving a *trans* β-lactam, which was elaborated by a lengthy process to the dibromide (**235**).

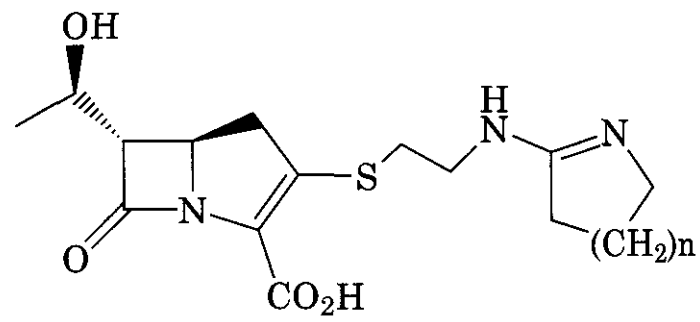
Cyclization and decarboxylation, followed by elimination and deprotection, then gave



(235) R = p-nitro-benzyl

(±)-thienamycin (523). A chiral synthesis involving [3,4] bond formation and, starting from the L-aspartate-derived β-lactam, (**236**) soon followed (524). Stereocontrolled introduction of the side chain and elaboration to the dithiane derivative (**237**) was followed by introduction of the hydroxyethyl side chain (**238**) and then the diazo intermediate (**239**) (Fig. 14.16).

Through the use of a catalytic amount of dirhodium tetra acetate, cyclization proceeded extremely efficiently to the bicylic keto-ester (**240**), which has become the essential intermediate for the synthesis of a wide range of analogs of thienamicyn. Introduction of the C2-cysteaminyl side chain through the enol phosphate (**241**) and final deprotection afforded thienamycin (**217**), whereas using a protected amidine side chain imipenem (**215**) is directly accessible (525). By following a similar methodology, several 2-thio-substituted derivatives were obtained, including cyclic amidines (**242**), aryl and heteroarylthio (**243**)

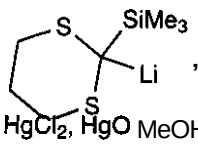


(242)

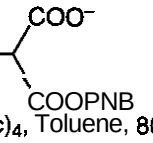
carbapenems (526), and compounds bearing a quaternary ammonium group (**244**) (527).

A similar approach that used the stable enol triflate (**245**) allowed the synthesis of aryl and vinyl carbapenems such as (**246–248**) through the use of an organometallic reagent-mediated coupling methodology (528).

Reagents: (i) $NaBH_4$, MeOH; (ii) $MeSO_2Cl$, TEA, DCM 0°C; (iii) NaI, Acetone, A; (iv) TBSCl, TEA, DMF

(v) 

, THF, –78°C; (vi) LDA, N Acethyl imidazole, THF, –78°C; (vii) K selectride. KI, Ether;

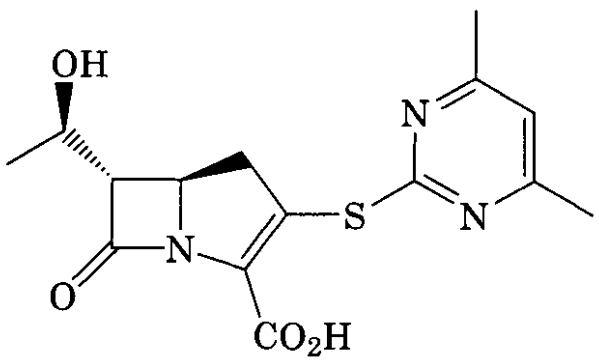
(viii) $HgCl_2$, HgO MeOH, H_2O, A; (ix) H_2O_2, MeOH, H_2O; (x) CDI, THF, then Mg 

(xi) HCl, MeOH; (xii) p-carboxy benzene sufonyl azide, TEA, CH_3CN; (xiii) $Rh(OAc)_4$, Toluene, 80°C;

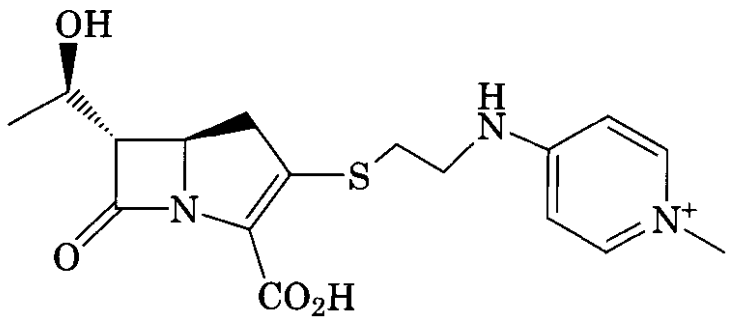
(xiv) $ClPO(OPh)_2$, DIPEA, CH_3CN, 0°C; (xv) $HSCH_2CH_2NHCOOPNB$, DIPEA, CH_3CN, –5°C;

(xvi) H_2, Pd/C, 3.5 atm.

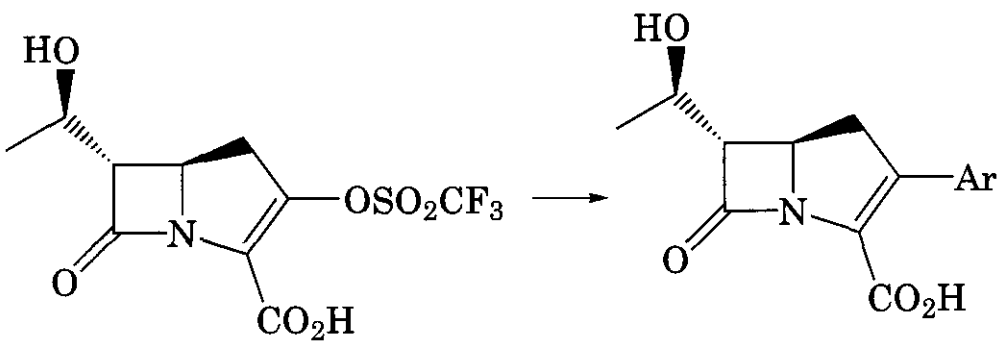
Figure 14.16. A stereocontrolled synthesis of thienamycin.



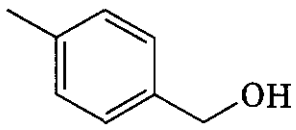
(243)

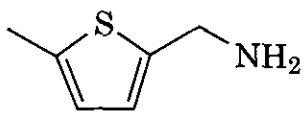


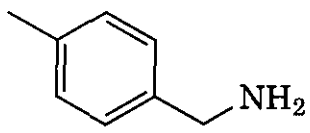
(244)

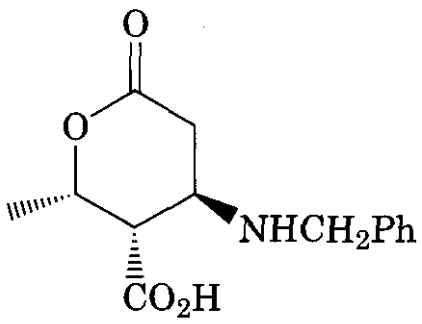


(245)

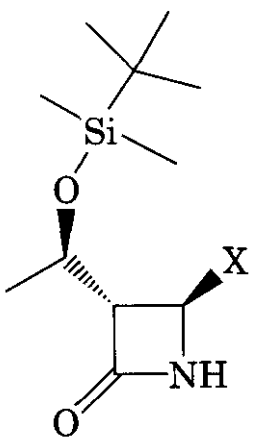
(246) Ar = 

(247) Ar = 

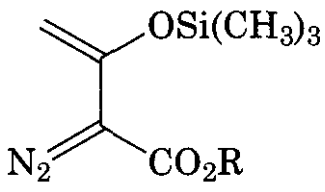
(248) Ar = 



(249)



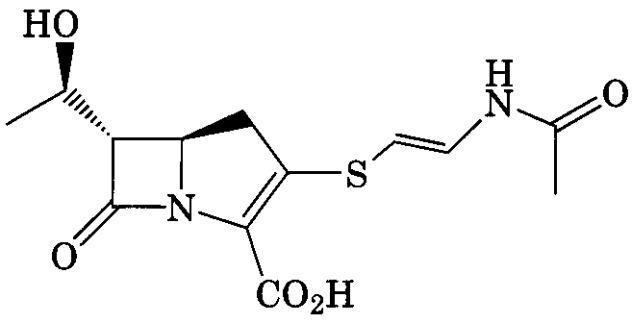
(250) X = Cl
(251) X = OAc



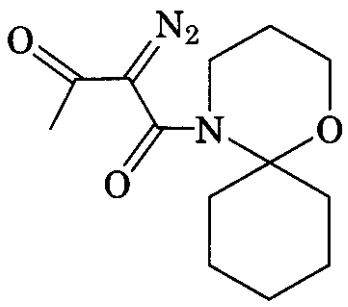
(252)

Another approach starts from the (+)-lactone (249) obtainable from acetone dicarboxylic acid (516). Initially carried out in the racemic series, this route afforded a practical synthesis of thienamycin, although conversion of the side-chain stereochemistry from (*S*) to (*R*) was required. Subsequently, an enantioselective route was developed from enantiomerically pure lactone (249) (529). Displacement of the 4-acetoxy or chloro group from azetidinones such as (**250**) and (**251**), respectively, with the trimethylsilyl enol ether (**252**) provided a further improvement in refining the synthesis of thienamycin (217) and its analogs (530, 531).

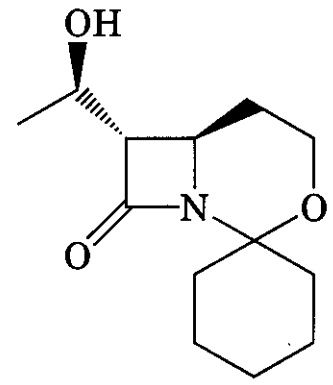
The success of this approach has been recognized by the commercial development of 4-acetoxy azetidinone (251) for penem, carbapenem, and trinem synthesis. Of several reported syntheses, the two most successful use simple chiral starting materials derived from 3-hydroxybutyric or lactic acid and a cycloaddition procedure to construct the β-lactam ring (532,533). Other syntheses in the thienamycin series using β-lactams derived from penicillin (530,534, 535), carbohydrates (536, 537), amino acids (538, 539), isoxazolidines (540), and organo-iron or cobalt complexes (541, 542) have been described together with alternative methods for bicyclic ring construction by use of the Dieckman cyclization (543) or an intramolecular Michael reaction (544).



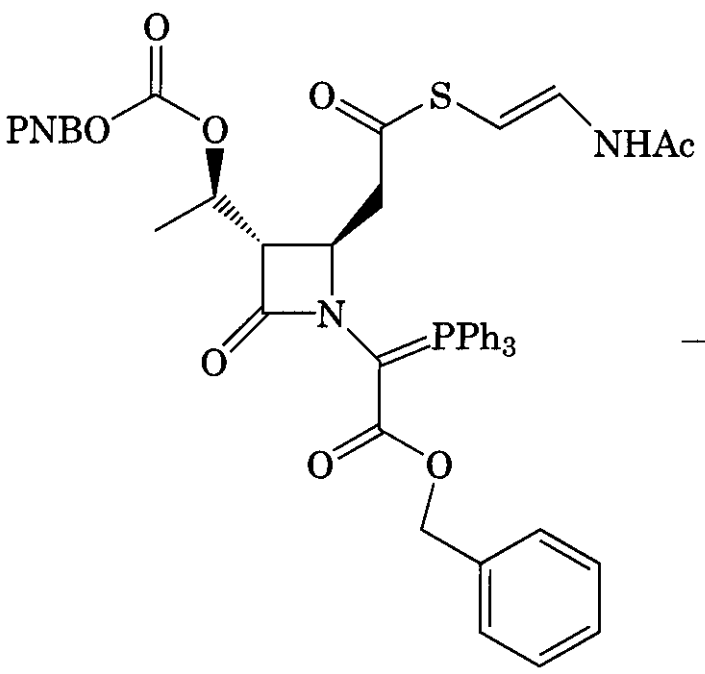
(253)



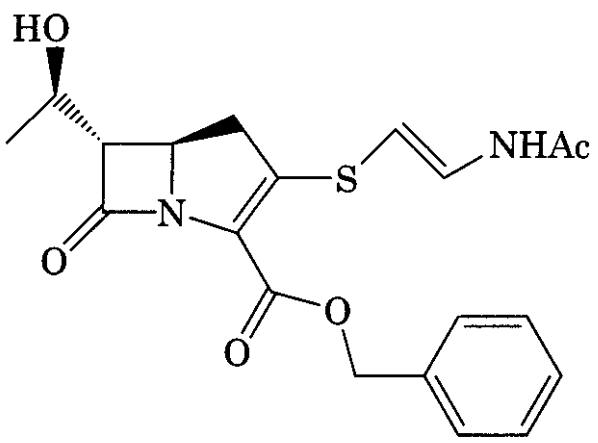
(254)



(255)



(256)



(257)

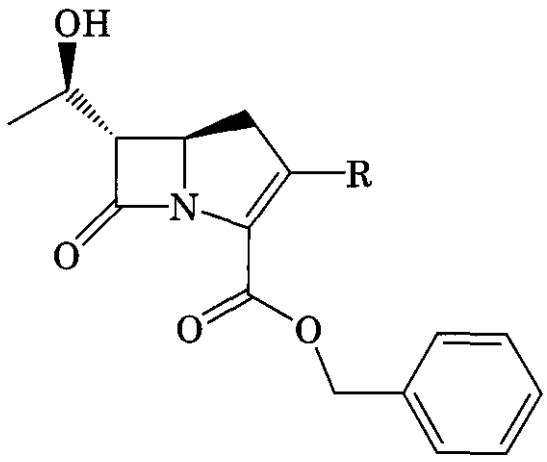
The discovery that thiol esters could participate in the Wittig cyclization procedure to give C2-substituted derivatives provided the basis for the total synthesis of the olivanic acid MM22383 (222) (493) and *N*-acetyldehydrothienamycin (253) (490).

In this case, formation of the β-lactam ring was obtained by means of dirhodium tetra acetate-catalyzed cyclization of the diazo-intermediate (**254**), to give the *trans*-β-lactam (**255**), as a mixture of hydroxy epimers after reduction of the carbonyl group.

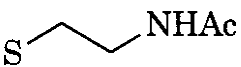
Progression to the thio-ester (256) was followed by cyclization in boiling toluene to give the two epimers of the cyclic product (**257**). These were separated and deprotected to afford the racemic natural products (522,545).

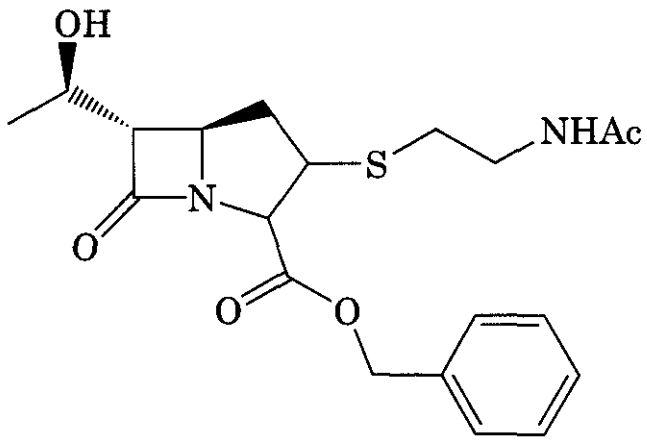
Another route to the olivanic acids was by way of (**258**) (546), which readily reacted with acetamidoethanethiol to form the carbapenam (**259**). Reintroduction of the double bond gave the ester of (+) MM22381 (**260**) (547).

This method has also been used for the synthesis of PS-5 (**224**), although most approaches toward this natural product have used the chiral (3*R*,4*R*) 3-ethyl, 4-acetoxy azetidinone (261) and the diazo-ketone (262). In the case of the carpetimycins such as (**226**), the major problem is to obtain the thermodynamically less favored cis-arrangement of protons around the p-lactam ring in precursors such as (263). One attractive method involved a directed aldol condensation using (264) and

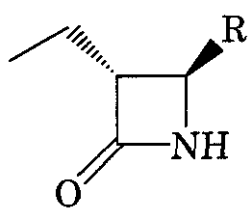


(258) R = H

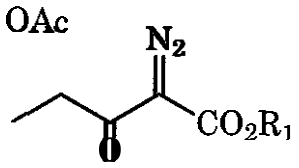
(260) R = 

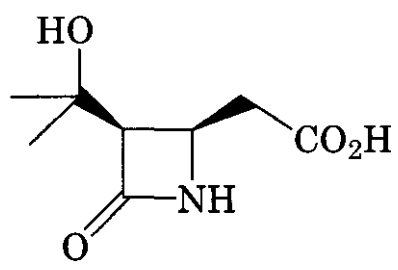


(259)



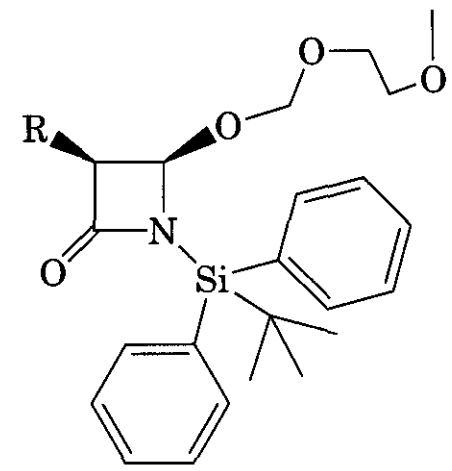
(261) R = OAc

(262) R = 

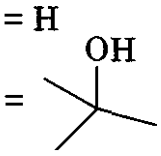


(263)

acetone, where metal ion chelation of the β-lactam enolate with the neighboring methoxyethoxymethoxy (MEM) group in the pres-



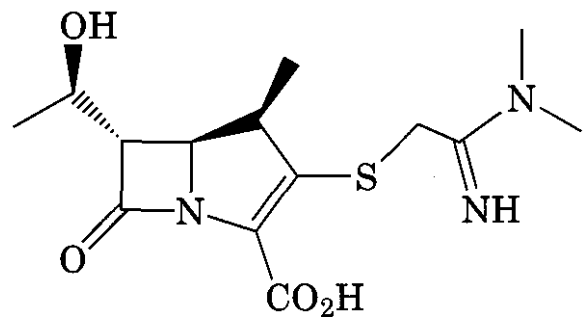
(264) R = H

(265) R = 

ence of the bulky silyl residue resulted predominantly in addition on the p-face, giving the cis-product (265) (548).

The asparenomycin natural products such as (227) have an alkylidene substituent at the C6-position. Synthesis in this series has followed a similar pattern by way of a bicyclic keto-ester derived from an appropriately functionalized monocyclic precursor. Many elegant routes to all these natural products and synthetic intermediates have been reported and reviewed (513, 517).

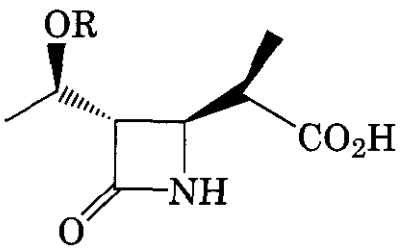
5.5.1.3 Synthesis of ***1β-Methylcarbapenems.*** In 1984 the Merck group reported the synthesis of the 1β-methyl-substituted carbapenem (**266**), which showed improved chemi-



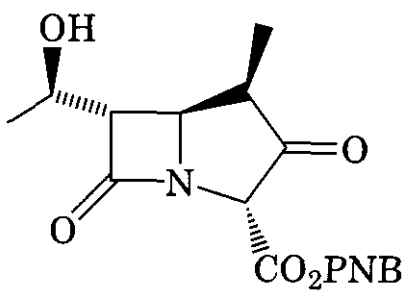
(266)

cal and metabolic stability while retaining the antimicrobial activity of imipenem (215) (549). The synthesis was achieved by alkylation of the methyl ester of (235) (R = TBS), followed by elaboration of the acid (**267**) to the keto-ester (**268**), although little stereocontrol was achievable by this route.

The advantages offered by the 1β-methyl series has led to a proliferation of methods for

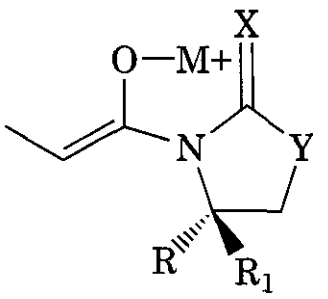


(267)



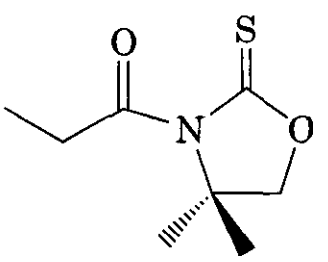
(268)

the stereoselective synthesis of (262) and other intermediates leading to (263). Most often, this method has made use of displacement of the acetoxy group from the chiral 4-acetoxy azetidinone (**251**) with tin or boron enolate of general structure (**269**), the product being



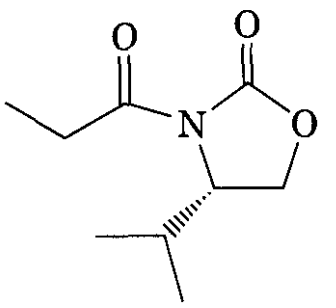
(269) X, Y, = O, S
R, R_1 = alkyl

readily convertible to the acid. Yields are generally good, with a ratio of β:α isomers ranging from 24:1 with (**270**) (550) to greater than



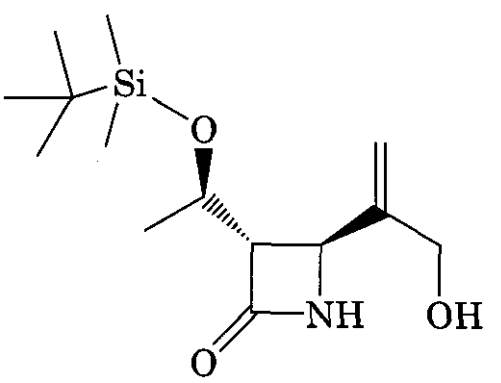
(270)

90:1 with (**271**) (551); many other variations have been reported (506). Direct incorporation of the diazo side chain is also possible (552).



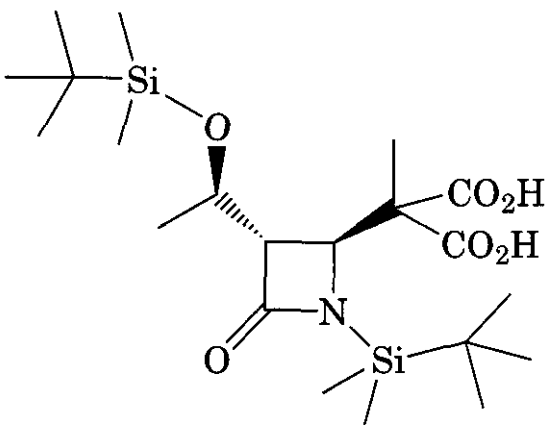
(271)

Alternative variations for constructing the 1β-methyl carbapenem ring system have made use of (*R*)- or (*S*)-methyl 2-methylhydroxypropionate (553, 554), the alcohol (272)

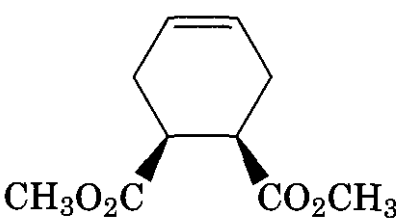


(272)

(555), malonic acid derivative (**273**) (556), and an enzymatic approach starting from (274) (557). Numerous synthetic efforts have fo-

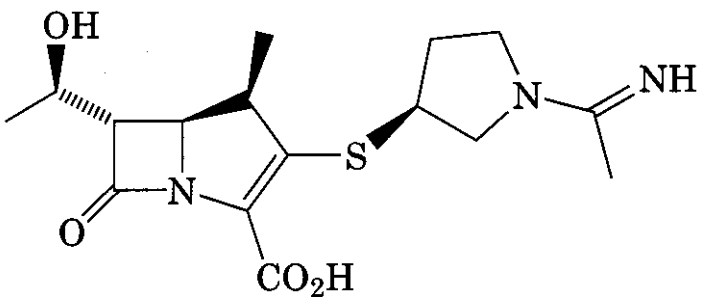


(273)

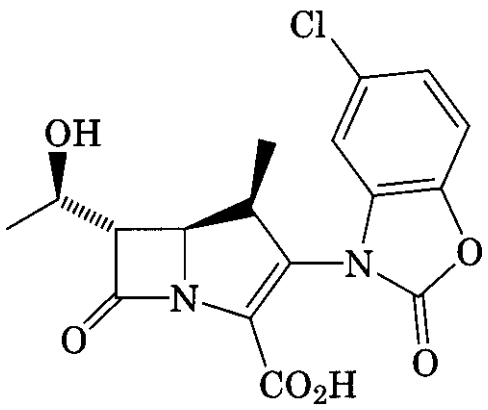


(274)

cused on the synthesis of 1β-methyl carbapenems in the search for a broad-spectrum agent suitable to treat severe infections. Compounds **(275–286)** are representative examples taken from the literature **(558–563).**



(275) Panipenem



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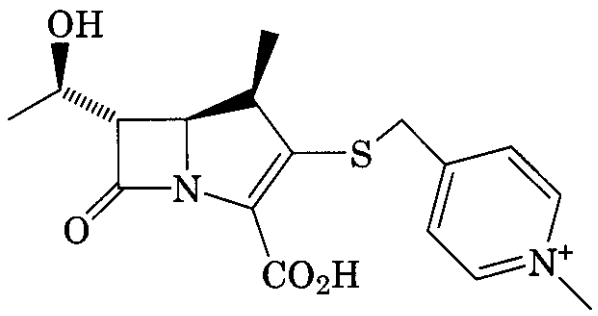

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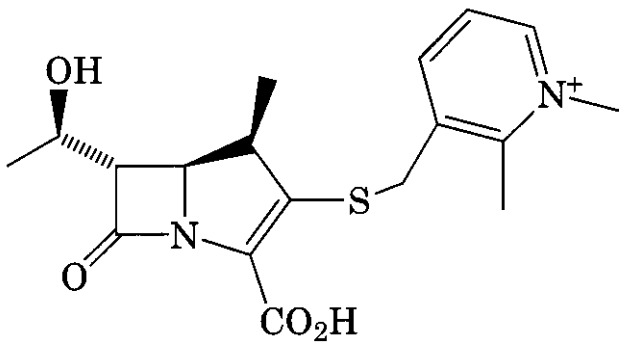
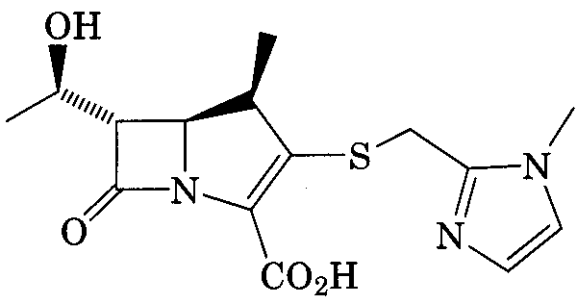
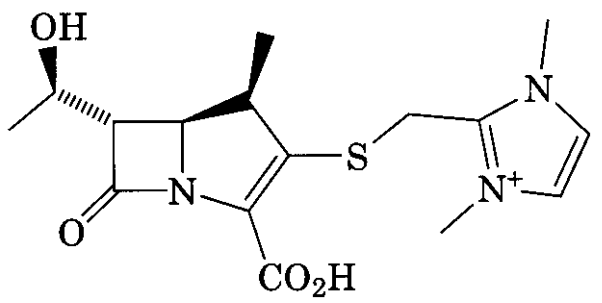


(278)

Effective substituents replacing the 1β methyl were extensively explored by several research groups, although compounds having



(279)



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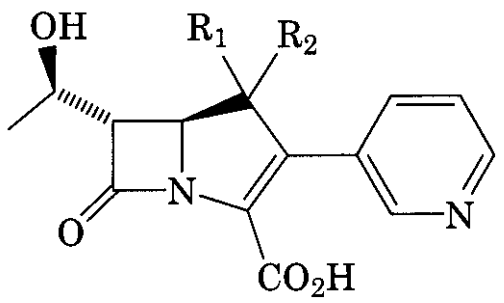
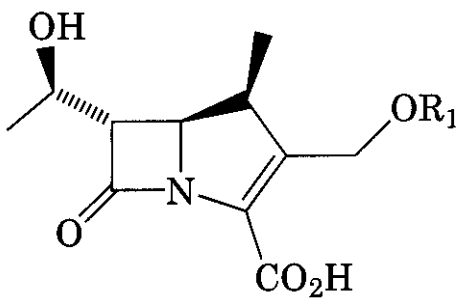
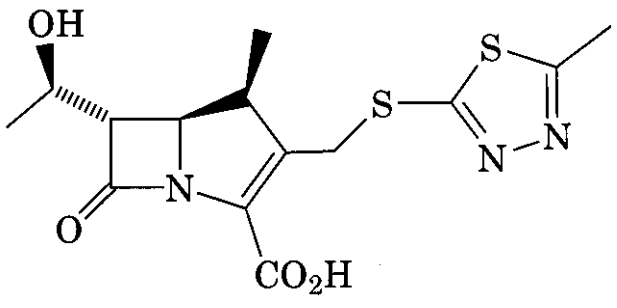


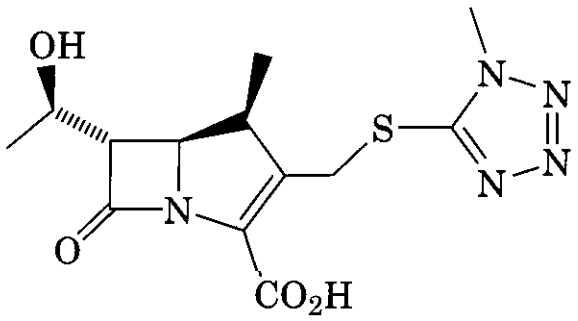
(281) R_1, R_2 = H or CH_3



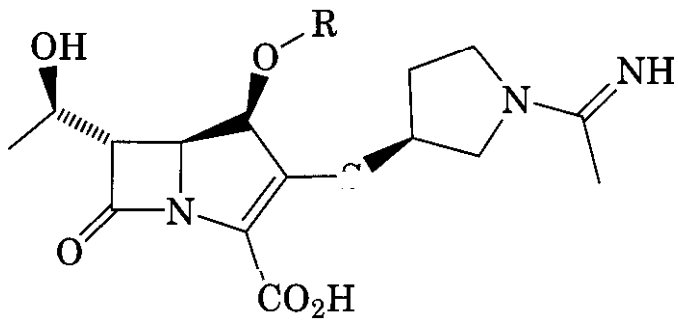
(282) R_1 = H, Ac, $CONH_2$, $CON(CH_3)_2$, CONHAr, $CONH(CH_2)_2Py+$



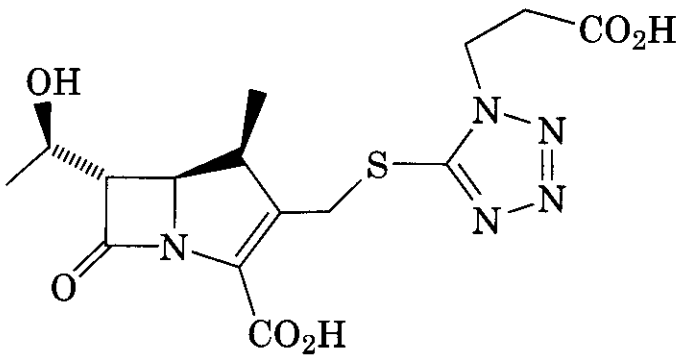
(283)



(284)



(288) R = H
(289) R = CH_3



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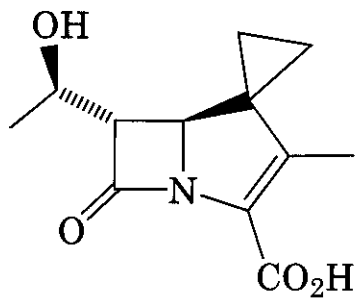

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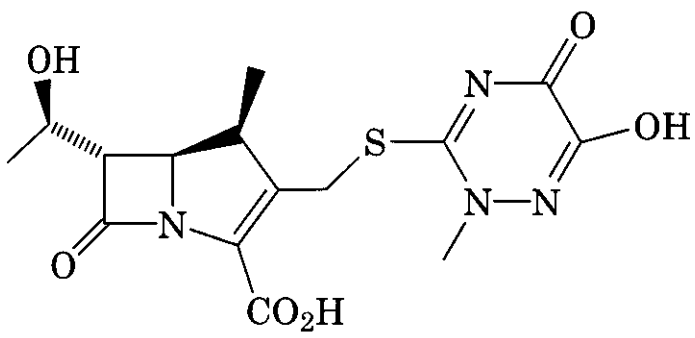

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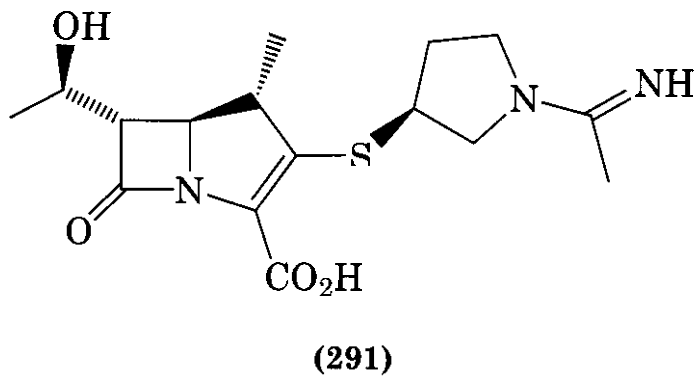

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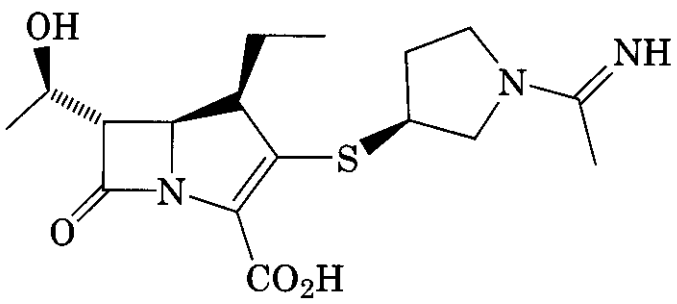

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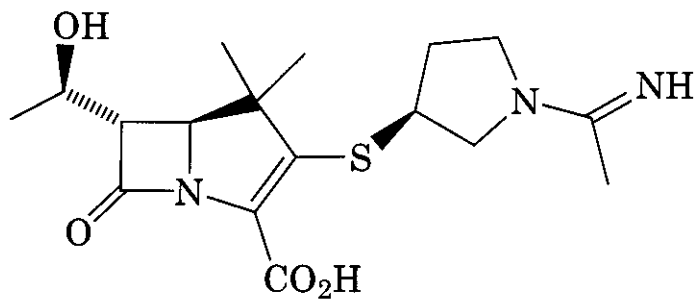
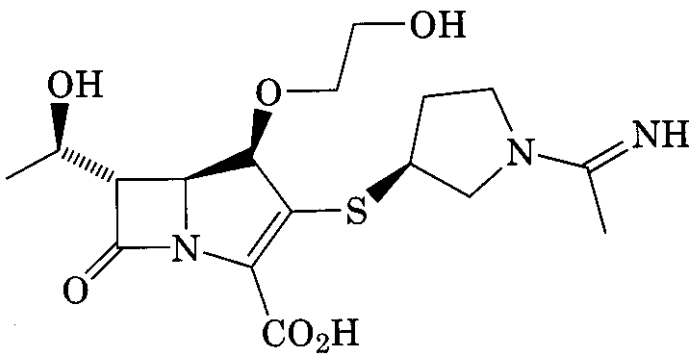


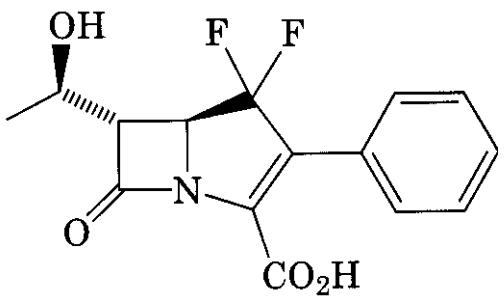
(292)

ethyl (**287**), hydroxy (**288**), methoxy (**289**), or 1–1′-*spiro*-cyclopropane (**290**) in place of methyl are less active (564, 565). Other compounds prepared by Sankyo include la-methyl and 1,1-dimethyl panipenem derivatives (291) and (292) (566–569). Further modifications were studied by Roche, which eventually obtained Ro 19–8928 (293) (567), a compound with good activity against Pseudomonas aeruginosa. Moreover, fluorination at position 1, reported by Merck, resulted in very unstable compounds such as (**294**) (570).

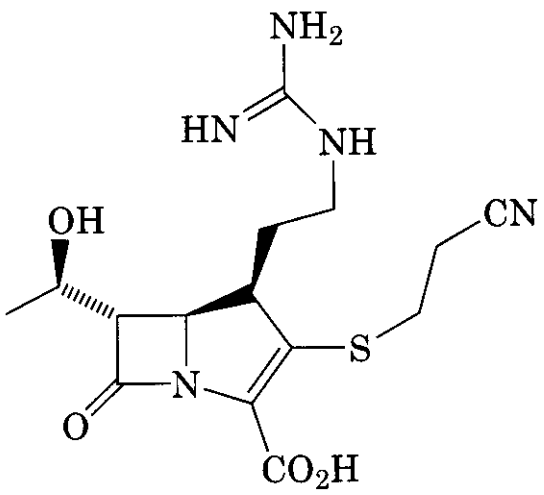
A series having 1β-aminoalkyl substituents has aroused some interest (571). The Bristol-Myers Squibb group extensively explored this class of derivatives and compounds (295) and (**296**) are examples of such modifications (572–577).



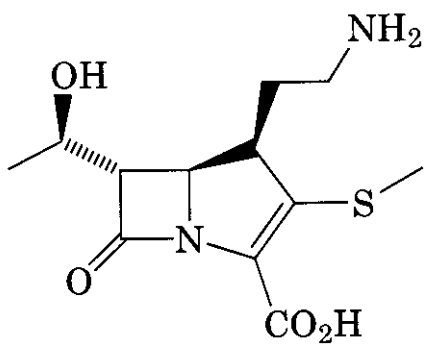
(293) Ro 19-8928



(294)

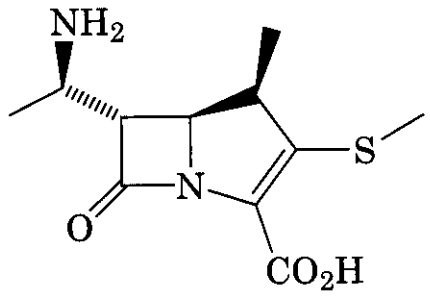


(295) BMS-181139

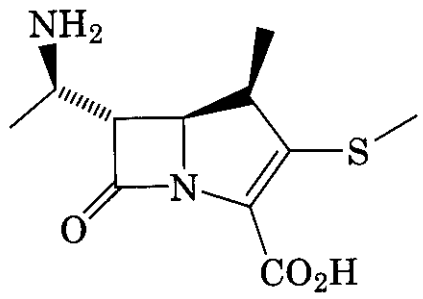


(296) BMS-40383

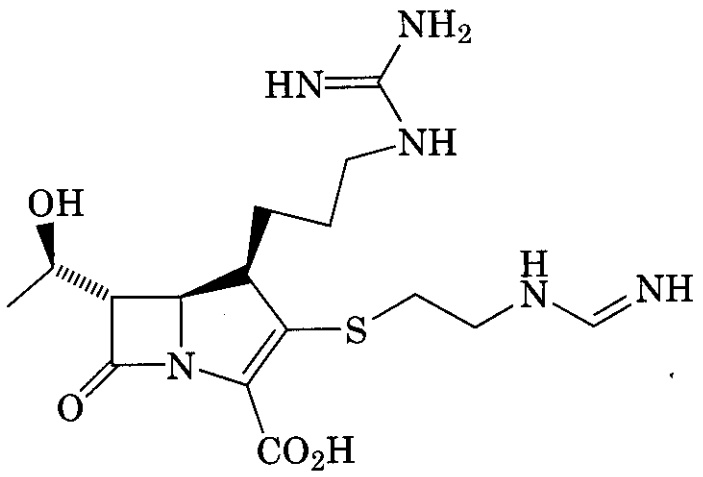
They also synthesized carbapenems (**297–302)** that bore the amino moiety either at *C6* or at the C1 position and made a comparison with other derivatives that bore the hydroxy-



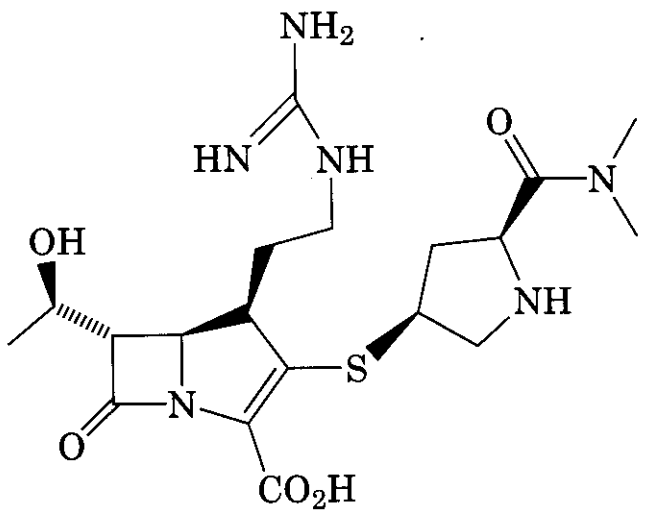
(297) BMS-40383



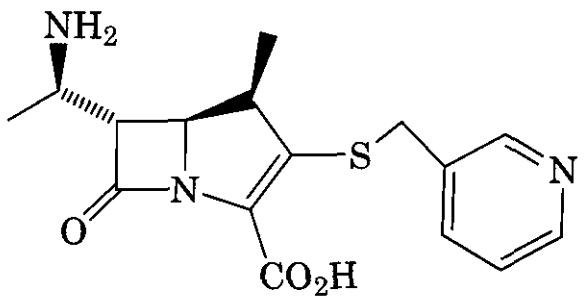
(298) BMS-40591
Carbapenems bearing a basic group at C6 position



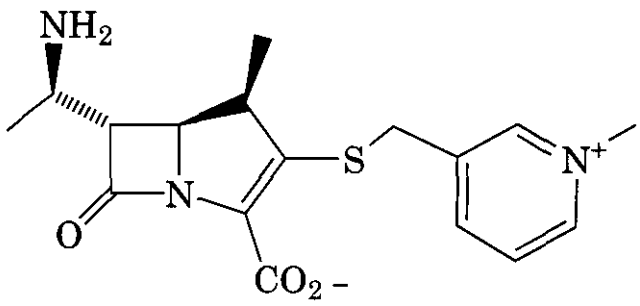
(299) BMS-45742



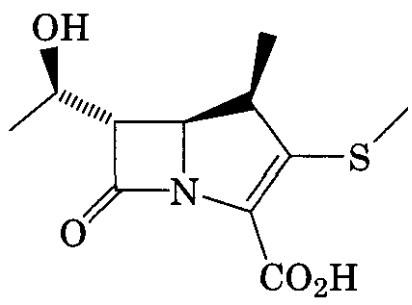
(300) BMS-45047
Carapenems bearing a basic group at C1 and C2 positions



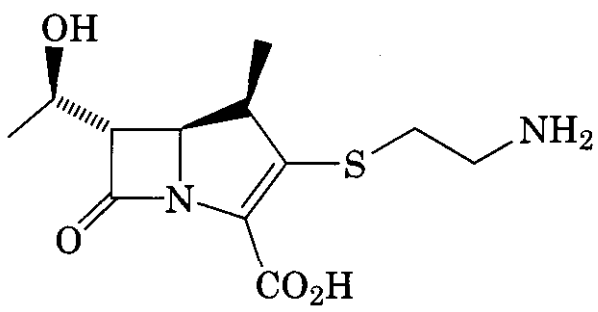
(301) BMY-40732



(302) BMY-40886
Carbapenems bearing a cationic or basic group at C2 and C6 position



(303) BMY-27946

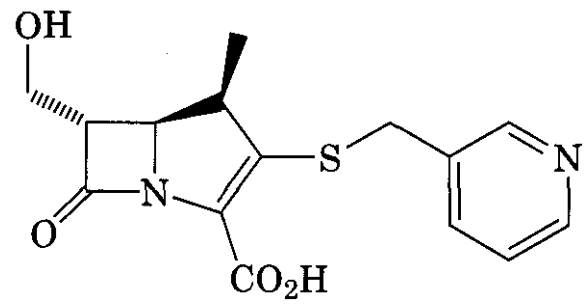


(304) BMS-182880

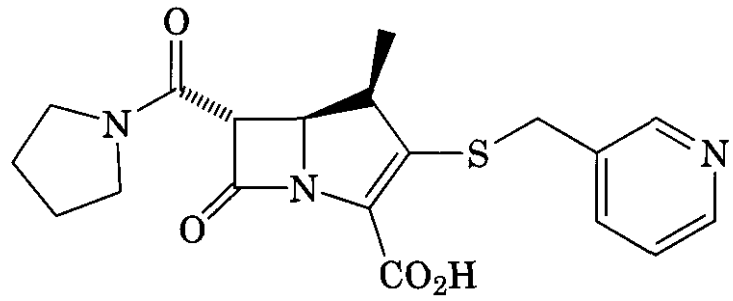
ethyl side chain (303 and **304**), reporting three major findings (571,578,579):

1. The presence of a cationic group was reconfirmed as essential to retain antipseudomonal activity.
2. The antipseudomonal activity was observed, regardless of the position of the cationic group at C1, C2, or C6.
3. The presence of a second basic group at the C1 or C6 position of a carbapenem already bearing a cationic center at C2 position allows the molecule to exert its antipseudomonal activity without being taken up through porin protein D2, thereby overcoming one of the mechanisms of resistance acquired by *Pseudomonas aeruginosa*.

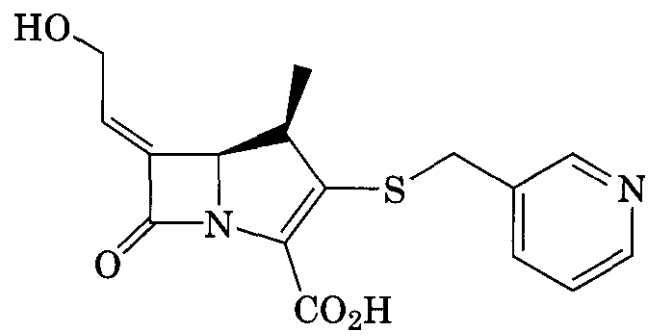
Besides the introduction of a basic functional group, Bristol-Myers Squibb also investigated other modifications of the hydroxyethyl side chain (**580**). From their studies it was reconfirmed that the presence of an electron-withdrawing group at C6 was difficult because of the low chemical stability of the resulting molecules (**305–307**) and that the



(305)



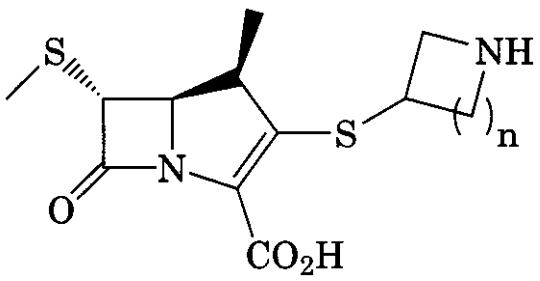
(306)



(307)

hydroxyethyl side chain is the best compromise between chemical stability and microbiological activity (**581–584**).

Another interesting example regarding modification at C6 has been reported by Nagao (585), where the synthesis of 6-methylthiocarbapenems (308)and (**309**), by reaction of the



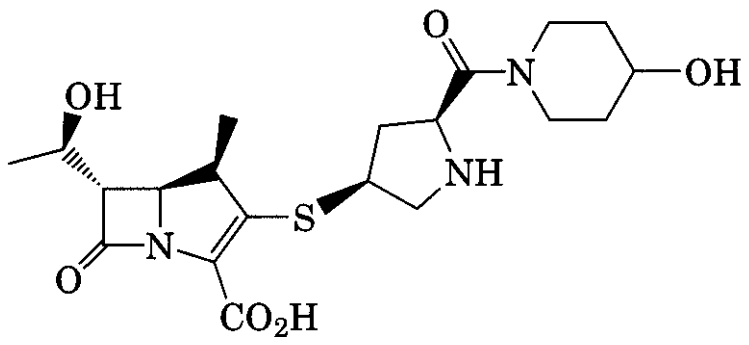
(308) n = 1
(309) n = 2

anion at position C6 of the carbapenem scaffold, is described.

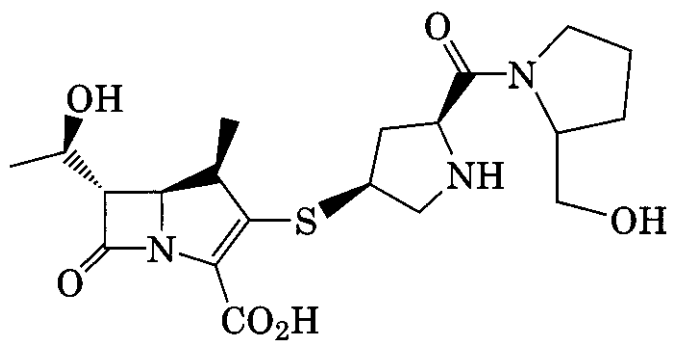
In 1987 meropenem (216) was presented by Sumitomo as a drug candidate (586), featuring improved efficacy and safety over those of other types of carbapenem antibiotics. As soon as it became clear that meropenem was endowed with very potent antibacterial activity and a wide spectrum of action against Gram-positive and Gram-negative organisms including *Pseudomonas* spp., synthesis of a variety of analogs having the 1β-methylcarbapenem skeleton and 2,4-disubstituted pyrrolidine moiety at C2 was reported by many research groups (587–590).

Methylation of the nitrogen atom in the pyrrolidine ring and modification of the carboxamide group enhanced the DHP-I stability (591) and, in particular, the introduction of hydrophilic groups (310, 311) on the amido moiety increased the antipseudomonal activity (592).

Addition of a second cationic (basic) center was explored to improve the biological profile



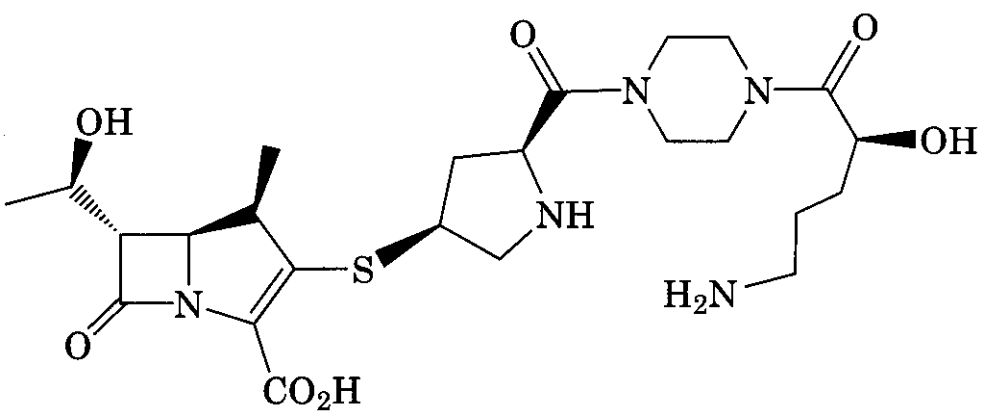
(310)



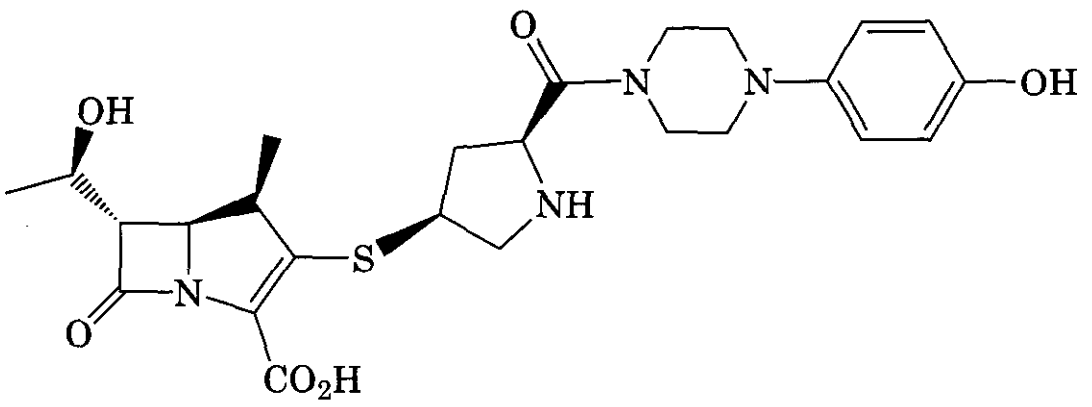
(311)

of the drug. Following this line of research, Nishi reported (593) the piperazine derivative (**312**), which retained the antipseudomonal activity.

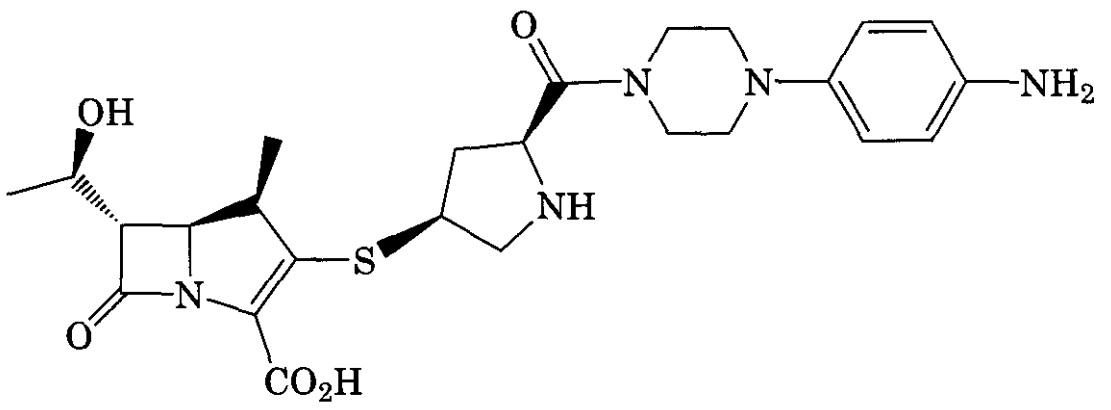
Subsequently, other piperazine derviatives (313, 314) were reported by Lee (594); interestingly, only these examples showed good activity against *Pseudomonas aueruginosa*. Introduction of a quaternary ammonium moiety in the C2 proline side chain was particularly efficacious at improving the half-life of the drug by reduction of the renal clearance (**315**) (595). Alternatively, it has been shown by Oh (596) that the use of a sulfonium moiety can also provide the extra cationic center (316). Zeneca and Merck groups achieved a remarkable improvement in terms of half-life with



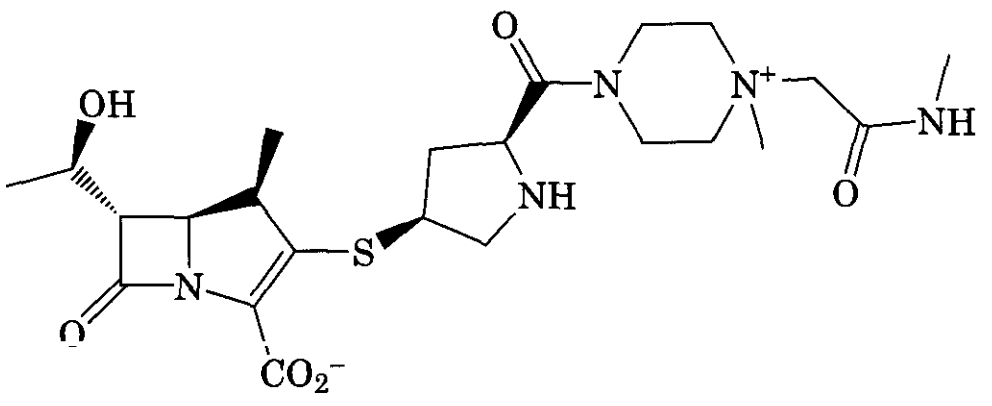
(312) DX-8739



(313)

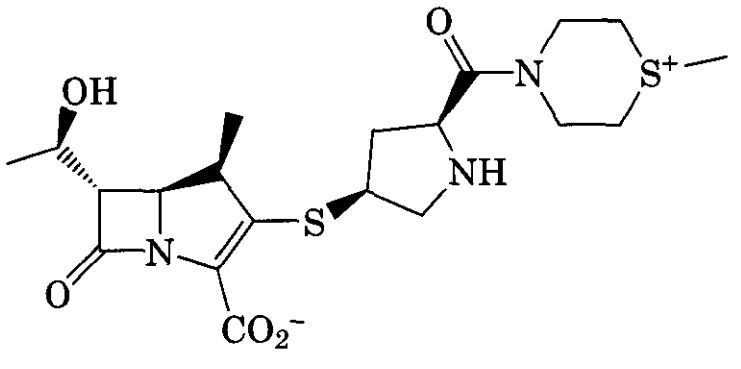


(314)



(315)

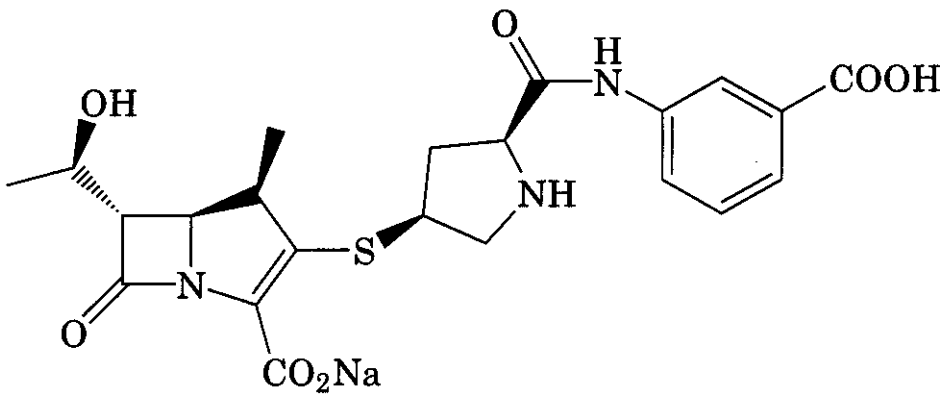
the synthesis of MK-826 (**317**), a result that has been confirmed in humans. The compound is highly serum bound, approximately 95%, and has a mean terminal $t_{1/2}$ of about 4.5 h after intravenous administration (597, 598). However, the presence of an overall negative charge on the molecule has limited the broadness of spectrum of action of the antibiotic. In fact, it lacks activity against P. ***aueruginosa*** and also against penicillin-resistant ***S. pneumoniae*** (599).



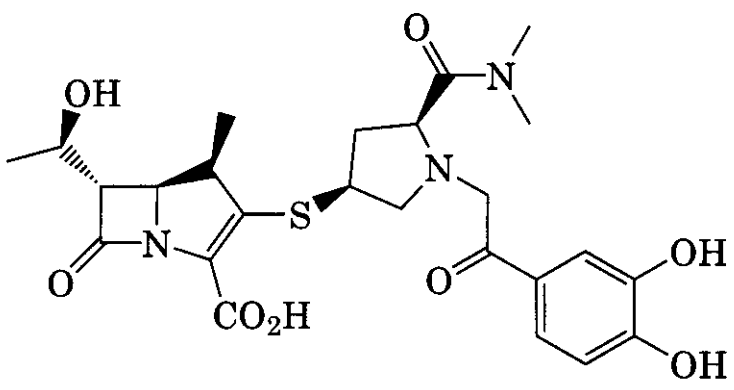
(316)

Another approach to prepare meropenem mimics was undertaken by introduction of a catechol moiety in a manner similar to what has been done in cephalosporin chemistry (see Section 5.2.2). This modification confirmed that it is possible to improve the ***in vivo*** antipseudomonal activity (318)(600).

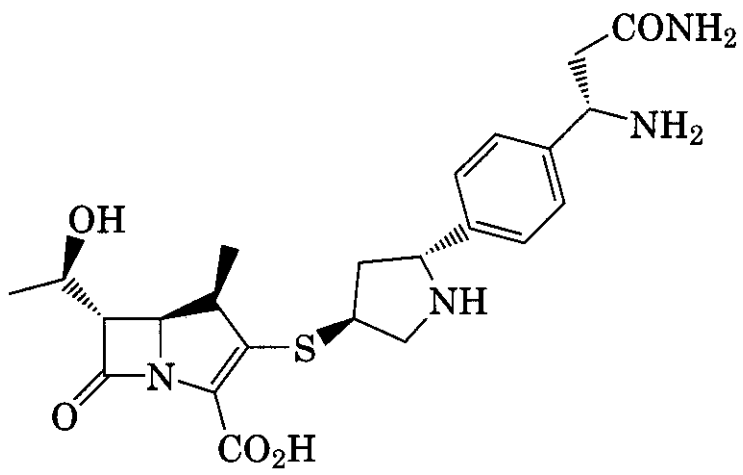
Although most compounds exhibited an antibacterial profile similar to that of meropenem, Banyu, first with J-111,347 (319)and



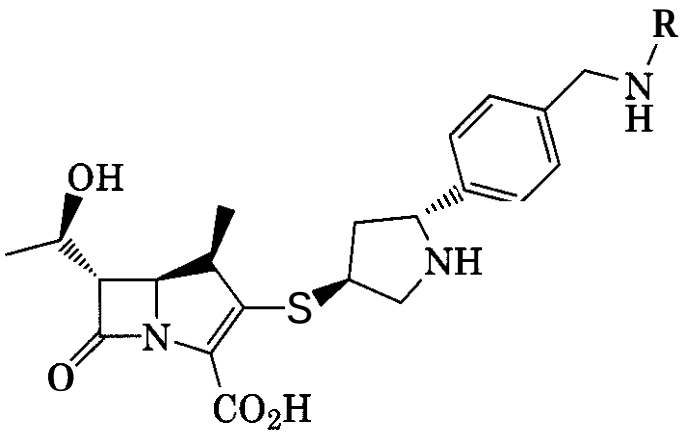
(317) MK-826



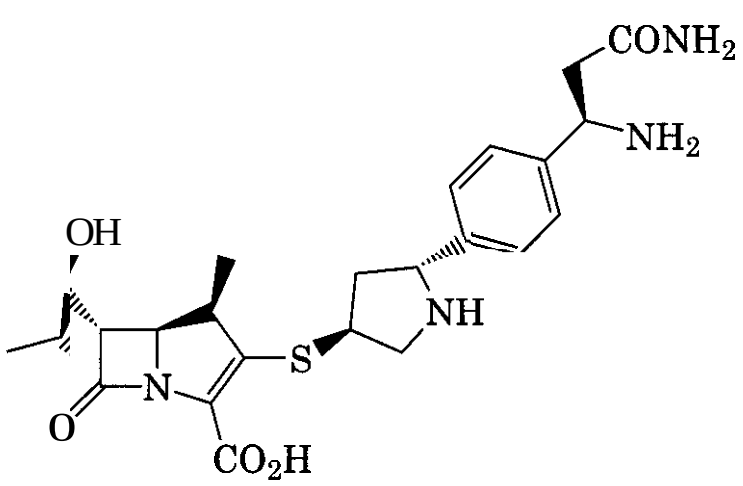
(318)



(322) J-114,871



(319) J-111,347 R = H
(320) J-111,225 R = CH_3



(321) J-114,870

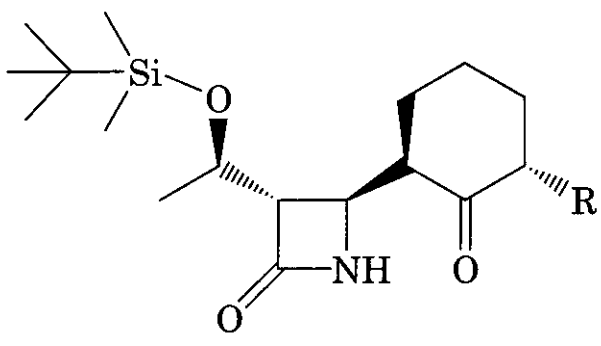
then with J-111,225 (**320**), J-114,870 (**321**), and J-114,871 (**322**), demonstrated that it is possible to further expand the antibacterial spectrum of meropenem mimics, including anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) activity (601–604).

Further evaluation of the prototype compound J-111,347 (319) has been suspended because of its epileptogenicity. This has been eliminated by N-methylation (320) or carbamoylmethyl substitutions (321,322) in the other three compounds. In vitro and in vivo data are reported in Tables 14.14 and 14.15, respectively (604).

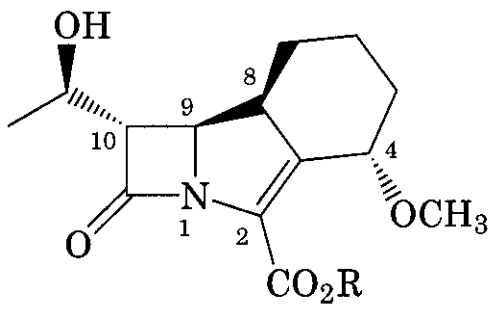
5.5.2 Trinems and Policyclic Carbapenems. Further developments in seeking to advance the properties of the carbapenems have seen the synthesis of both tricyclic and polycyclic analogs. Starting from azetidinone (**323**) and using [illegible] group prepared a series of so-called tribactams (and lately renamed trinems). Belonging to this class the 4-methoxy trinem, GV104326 (sanfetrinem, 324) (605, 606), showed a very potent microbiological activity. Its conversion to

Table 14.14 MIC 90 (μg/mL) of Imipenem Derivatives

Organism	J-111,225	J-114,870	J-114,871	*Imipenem*
S. aureus (MSSA)	0.016	0.016	0.016	0.016
S. aureus (MRSA)	*4*	4	4	128
Coagulase-negative staphylococci MR	4	*4*	4	128
S. pyogenes	50.008	50.008	50.008	50.008
S. pneumoniae (incl. PenR *strains)*	0.25	0.25	0.25	0.25
E. *faecalis*	8	*8*	8	4
Enterobacteriaceae	0.032–1	0.016–2	0.032–1	0.1254
H. influenzae	0.125	0.125	0.125	1
P. aeruginosa Imi-S	*4*	4		*4*
P. aeruginosa Imi-R	16	16	16	32
Acinetobacter spp.	0.125	0.25	0.125	0.125
C. difficile	2	2	2	8

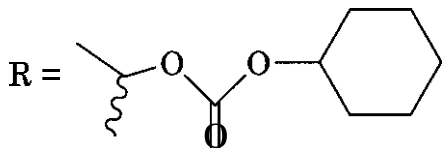


(323)



(324) ***Sanfetrinem*** ***R = Na***

(325) ***Sanfetrinemcilexetil***



the corresponding metabolically labile prodrug ester (325)demonstrated a safety profile and pharmaceutical stability to warrant further progression into the clinics **(607–610).** The biological properties of trinems include a broad spectrum of activity versus Gram-positives and Gram-negatives, either aerobic and anaerobic, and stability to clinically relevant β-lactamases and human dehydropeptidases **(DHP·I)(611).**

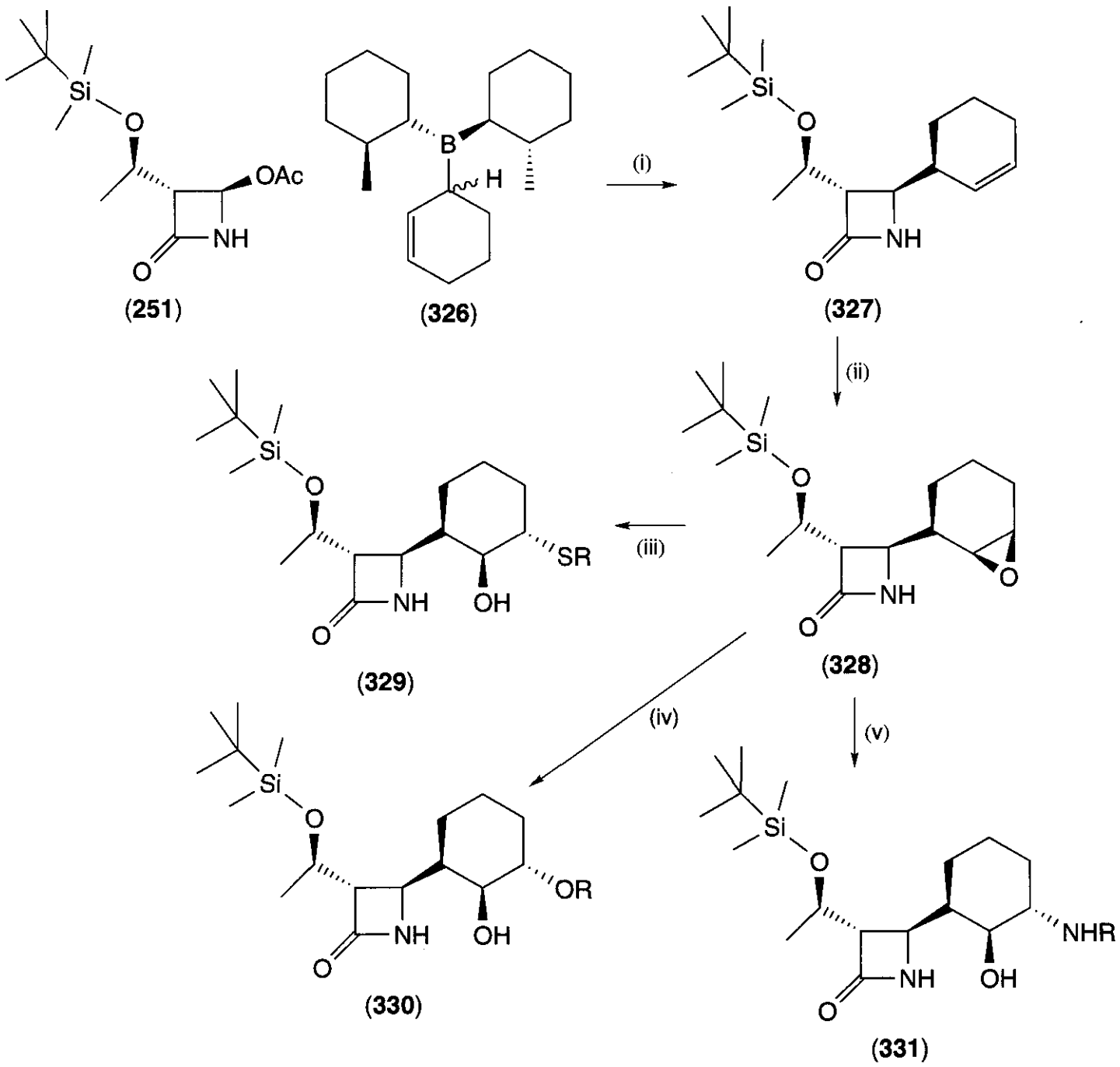
Structure-activity relationship studies showed that the best biological profile was observed in those compounds bearing a heteroatom attached at C4 and having absolute configuration (4*S*,8*S*). The construction of the trinem backbone is very challenging because it contains five stereogenic centers that need to be built in a stereospecific manner. **A** great deal of work has involved the stereoselective

Table 14.15 *In* Vivo Efficacy in Systemic Murine Infections Expressed as ED_{50} (mg/kg)[a,b]

Systemic infection	J-111,225	*Imipenem*	*Vancomycin*
MS S. aureus	0.040	0.060	0.523
MRS. aureus BB6221 *normal mice*	5.42	94.73	2.56
MRS. aureus BB6221 *immunosuppressed mice*	8.53	93.76	8.83
Pseudomonas aeruginosa BB5746	0.483	0.242	—

[a]MS, methicillin-susceptible;MR, methicillin-resistant; PR, penicillin-resistant.

[b]In a model of thigh infection caused by MR S. *aureus* BB6294 in immunosuppressed mice, bacterial counts were significantly decreased with J-111,225 versus controls and imipenem- or vancomycin-treated mice ($P < 0.01$) at 4 h post-therapy.



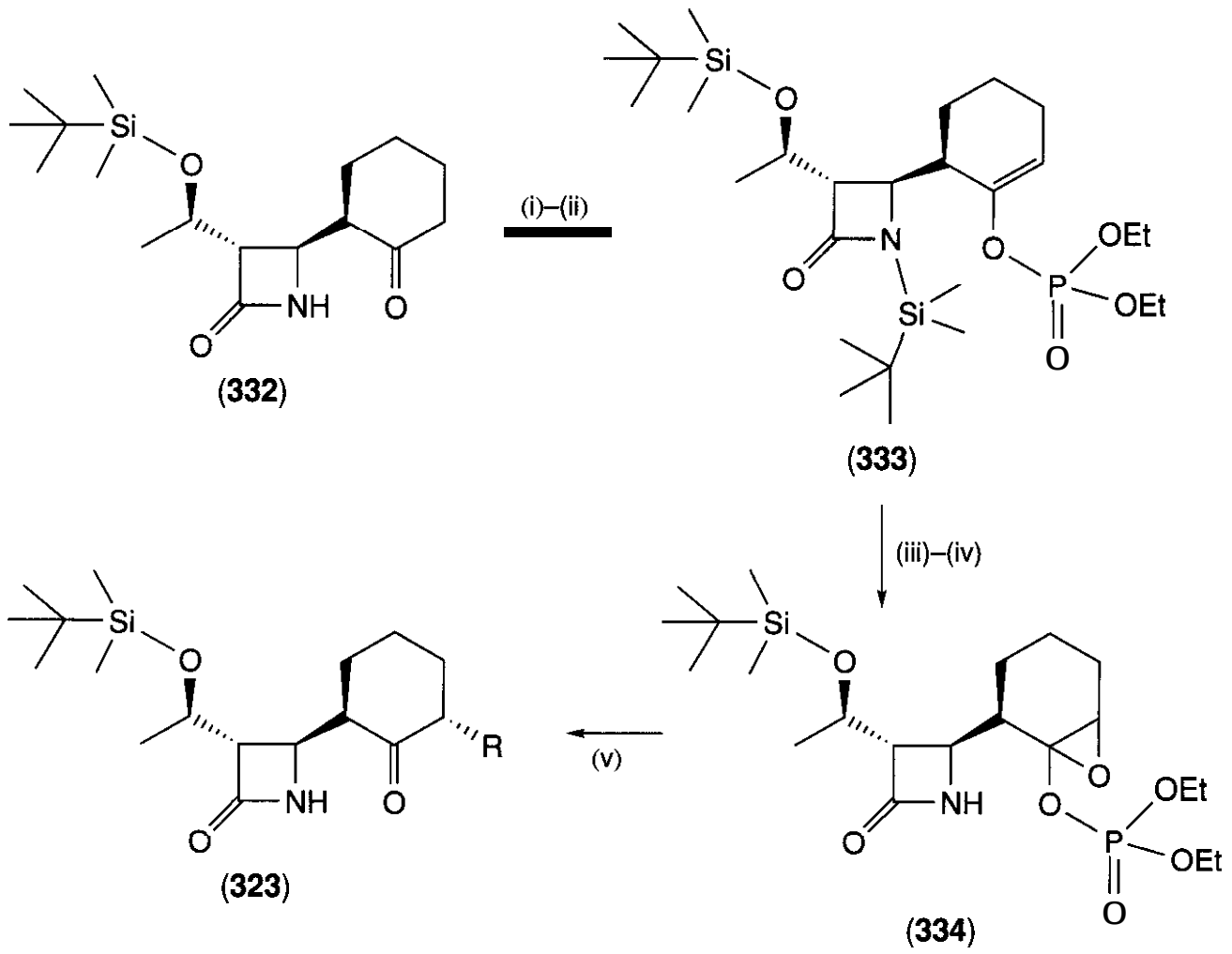
Reagents: (i) $ZnEt_2$, THF, 25C; (ii) Magnesium monoperoxyphthalate, CH_2Cl_2; (iii) RSH, H+; (iv) ROH, H+; (v) RNH_2, H+.

Figure 14.17. Highly diastereoselective synthesis of intermediates to trinems.

synthesis of advanced intermediates such as epoxide (328) and epoxyphosphate (**334**) that have allowed the introduction at C4 of sulfur-, oxygen-, and nitrogen-containing functional groups, as shown in Figs. 14.17 and 14.18. Reaction of 4-acetoxy azetidinone (251) with 2-cyclohexenylborane (326) gave cyclohexenyl derivative (327) in high yield and selectivity (612). This intermediate was converted into epoxide (328) that underwent nucleophilic addition, to afford sulfides (**329**), alkoxides (**330**), and amines (331) that were further progressed to derivatives of general formula (323).

Another *diastereoselective* synthesis involved the ketoazetidinone (332) that was protected at the nitrogen of the p-lactam ring and reacted with diethylchlorophosphate to give (333). Epoxidation gave the advanced intermediate (**334**) that, upon reaction with nucleophiles, was converted into azetidinone (323) (613, 614).

A synthetic improvement has been introduced with the direct condensation methodology (615–617), in which the enantiomerically enriched silyl enol ether of 2-methoxycyclohexanone (**335**) (618,619) is reacted with the 4-acetoxy azetidinone (**251**), to yield the 6′-methoxy ketoazetidinone (**336**) with high stereoselectively. After introduction of the cyclo-



Reagents: (i) TBDMSCI, TEA, DMF; (ii) LHMDSA, $ClP(O)(OEt)_2$, -70C; (iii) KF, MeOH; (iv) MCPBA, CH_2Cl_2; (v) Nuclophile.

Figure 14.18. Synthesis and use of epoxyphosphate 334.

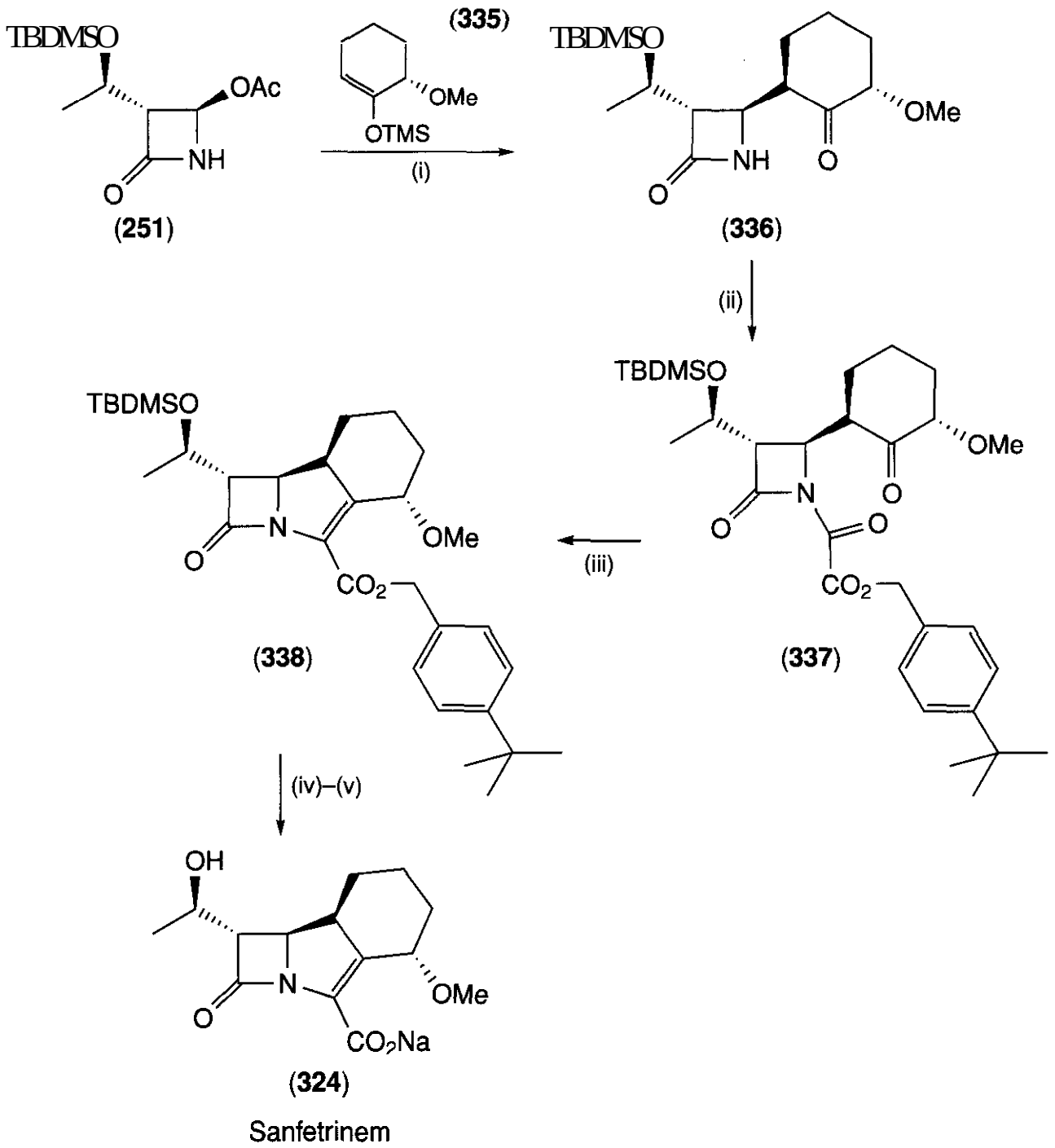
hexyl ring, intramolecular cyclization of an oxalimido derivative (**337**) produced the tricyclic system (**338**), and removal of the protecting groups gave sanfetrinem (**324**). A highly diastereoselective and practical synthesis of sanfetrinem (**324**), in which a diasterometic mixture of 6'-methoxy ketoazetidinone is enolized and the zinc enolate protonated with diethylmalonate, to give diastereomerically pure (**336**), has been reported by Hanessian (620). Figure 14.19 shows a route that has been applied on large scale to produce multikilogram quantities of sanfetrinem (324).

Furthermore, the synthesis of 4-N-substituted trinems such as (**339**) has allowed the preparation of ureas (340) (621), amides (341) (622), and, most important, 4-N-methylformamidine compound GV129606X (**342**) (623–625). GV129606X (342) is an injectable trinem with a broad spectrum of activity, including Pseudomonas spp., whose development has been terminated after preclinical studies showed toxicity problems.

Through use of similar chemistry, both, Hoechst and Bayer described tetracyclic analogs (626, 627). Thus, compound (343) was elaborated to afford the tetracyclic carbapenem (344) (627). Ring size, stereochemistry, and heteroatom position were modified (**345–348**), although these studies were not fruitful from the viewpoint of antibacterial activity.

Introduction of an aryl or heteroaryl substituted 4-exomethylenyl substituent (628) provided a series of compounds that showed high potency against resistant Gram-positive strains such as penicillin-resistant Pneumococci and methicillin-resistant Staphylococci (MRS). In particular, GV143253X (**349**) showed a broad spectrum, including vancomycin-resistant Enterococci and *H. influenzae*, good in *vivo* efficacy against MRSA in septicemia, and thigh infections in the mouse.

Sankyo Laboratories synthesized a series of 4-substituted trinems (350–352) bearing a



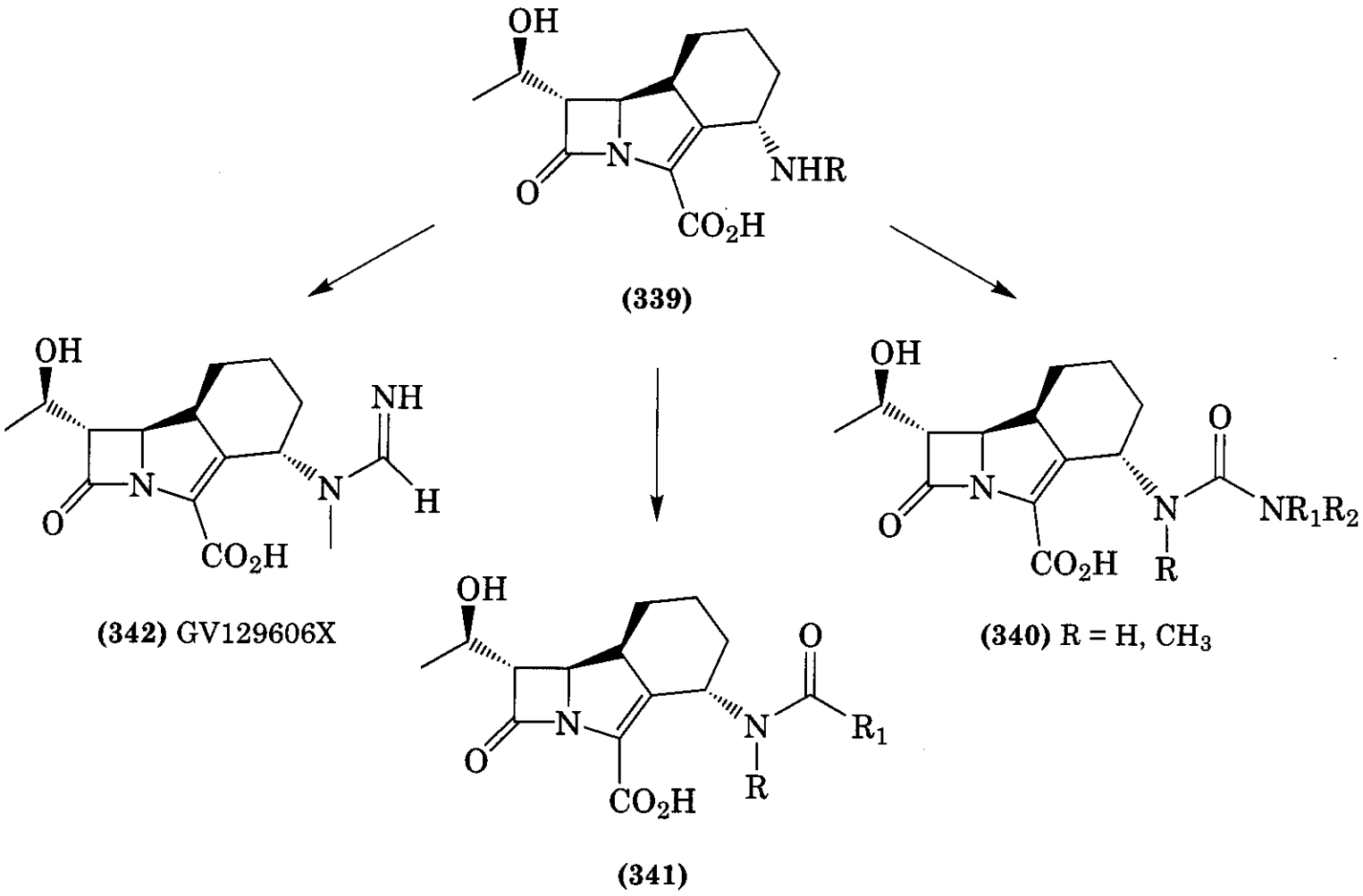
Reagents (i) $SnCl_4$; (ii) $ClCOCO_2R$, Pyridine, CH_2Cl_2; (iii) $P(OEt)_3$, xylene, reflux; (iv) TBAF, AcOH, THF; (v) H_2, Pd/C.

gure 14.19. Highly diastereoselective and practical synthesis of sanfetrinem.

heteroaryl substituent with an antibacterial spectrum similar to that of 4-exomethylenyl trinems (629).

GlaxoWellcome also reported the synthesis of pentacyclic β-lactams (**353**) that were conceived as conformationally constrained analogs of 4-exomethylenyl trinems (630). This class is characterized by antibacterial activity and affinity to PBP2a similar to those of 4-exomethylenyl trinems but with an inferior pharmacokinetic profile (631).

Another interesting series of anti-MRSA trinems bearing a pyrrolidinyl moiety was recently studied by Sankyo (632, 633), who prepared a series of derivatives starting from epoxide (328) (Fig. 14.20), or hydroxymethyl intermediate (358) (Fig. 14.21). Specifically, epoxide (328) was converted in two steps into ketoazetidinones (354) and (**355**), which upon standard cyclization and deprotection procedures gave trinems (356) and (**357**). Alcohol (358) was mesylated to give (**359**), which was reacted with thiol (**360**), giving rise to both 6′ isomers (**362**), possibly through elimination of mesylate to form intermediate (**361**) and subsequent Michael addition. Standard procedures resulted in the formation of the corresponding trinems (**363**) and (**364**), which are

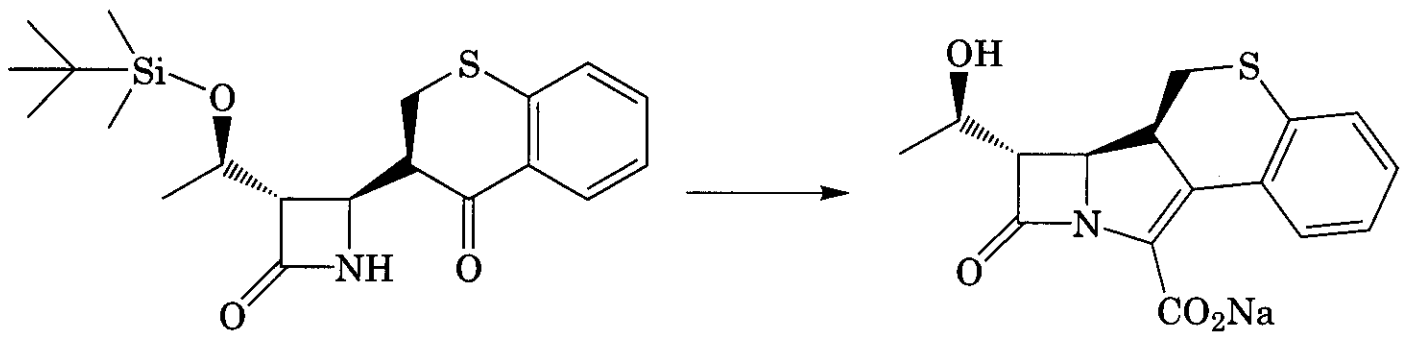


(339)

(342) GV129606X

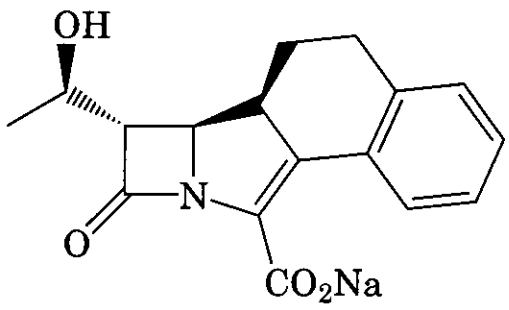
(340) R = H, CH_3

(341)



(343)

(344)



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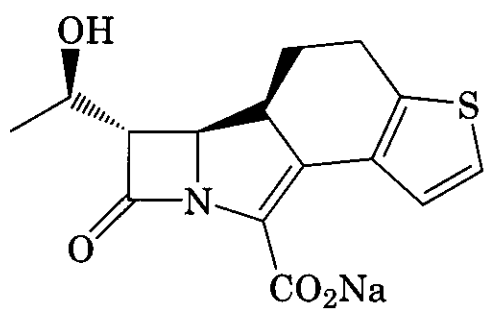

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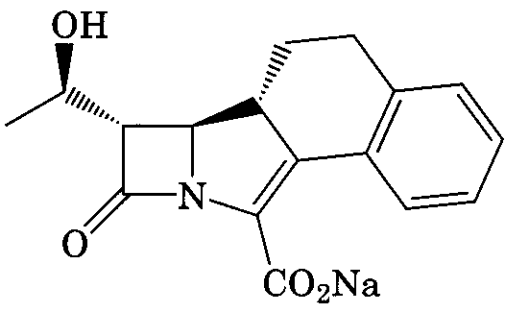
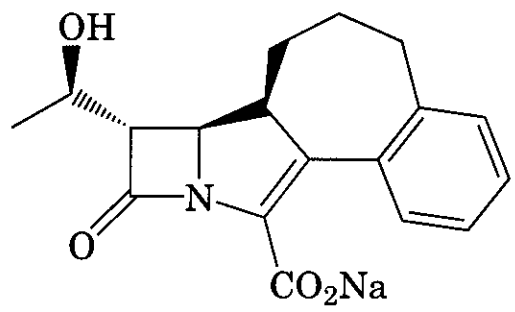
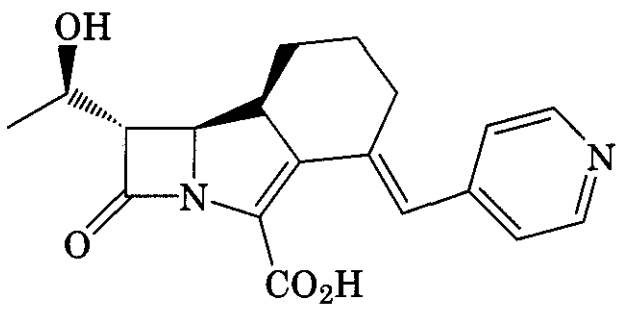


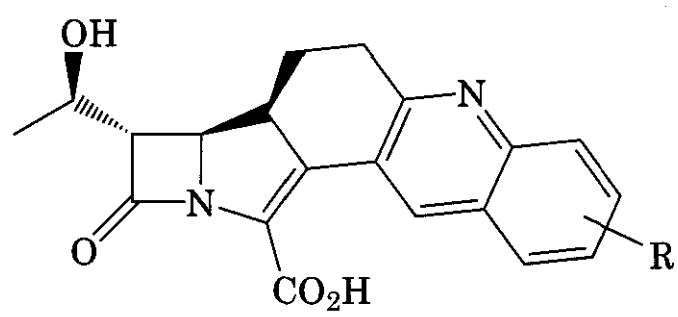
(346)



(348)

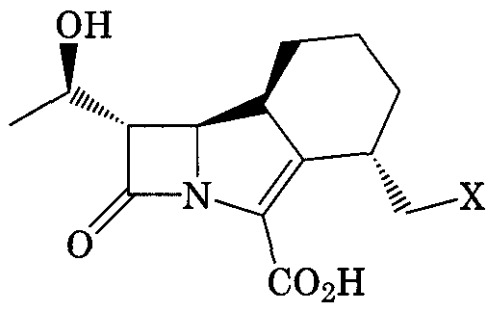


(349) GV143253X

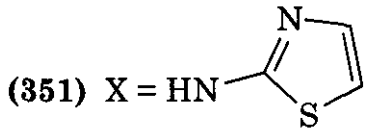


(353)

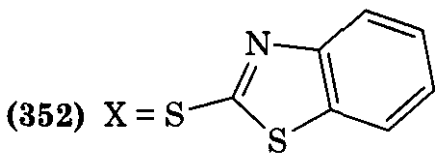
sulfur regioisomeric variants of their counterparts, (**356**) and (**357**). Interestingly, they found out that the pyrrolidinyl group properly oriented could result in a good antibacterial activity against Gram-positive strains including MRSA. Best results were obtained with compound (**364**), which was comparable to vancomycin.



(350) X = NH_2

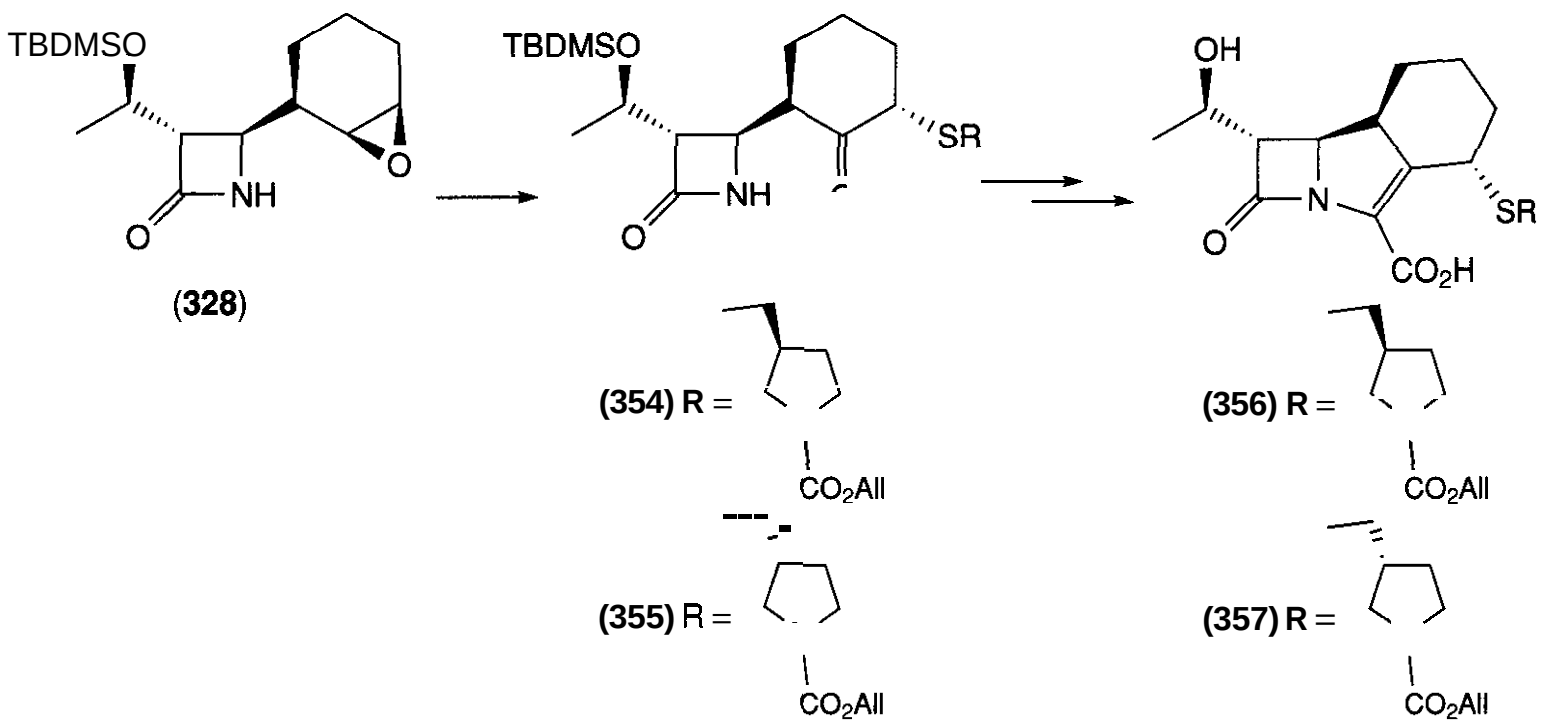


(351) X = HN



(352) X = S

5.5.3 Biological Properties of Carbapenems and Trinems. Thienamycin (**217**), the olivanic acids, and the majority of carbapenems are broad-spectrum antibacterial agents showing good stability to β-lactamases. Thienamycin (**217**) is the most potent of the natural products having activity against a wide range of Gram-positive and Gram-negative bacteria including *Pseudomonas aeruginosa* (**634**). Structure-activity studies indicate that the latter results derive from the presence of the basic C2-cysteaminyl residue, given that N-acylated derivatives show much reduced activity (499). The olivanic acid MM13902 (**219a**; R = SO_3H) is also a broad-spectrum. agent, but lacks significant activity against *Pseudomonas* spp. (**635**). In many cases, the carbapenems, particularly the olivanic acid



(328)

(354) R =

(355) R =

(356) R =

(357) R =

Figure 14.20. Preparation of derivatives starting from epoxide (328).

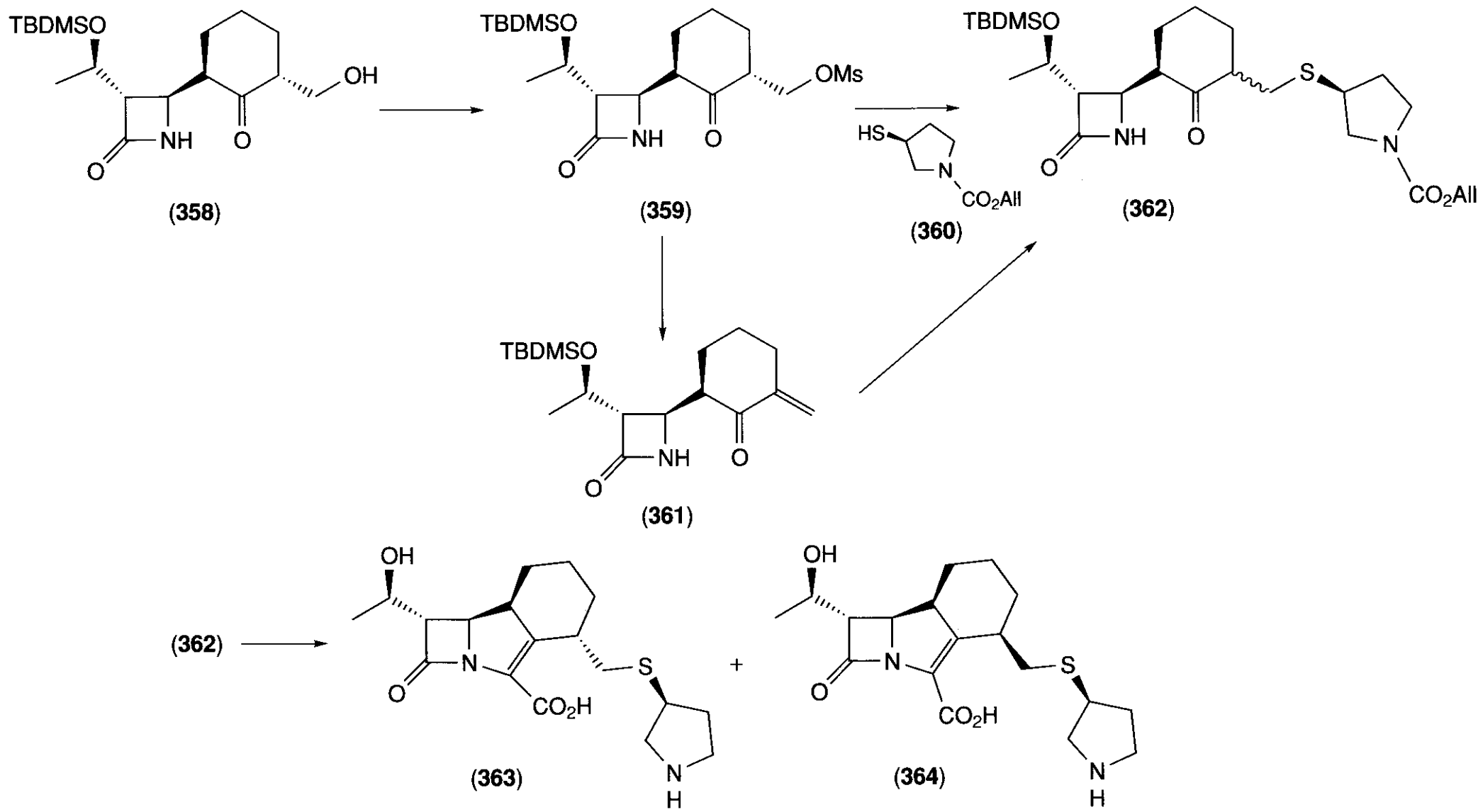


Figure 14.21. Preparation of derivatives from hydroxymethyl intermediate (**358**).

Table 14.16 ***In Vitro*** **Antibacterial Activity of Carbapenems [MIC (μg/mL)][a]**

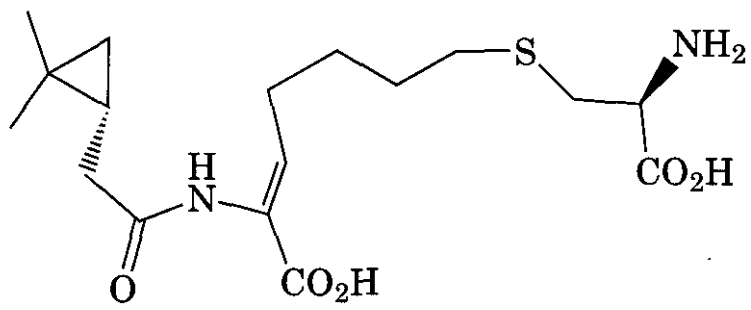
Organism	Thienamycin (213)	Imipenem (224)	MM13902 (216a)	PS-5 (219)	Asparenomycin A (222)	Pluracidomycin A (223)	Meropenem (214)	Biapenem (361)	Sanfetrinem (319a)
E. coli	0.2–0.4	0.25	0.2–1.6	1.56	0.39–3.13	6.3	0.03	0.25	0.5
K. pneumoniae	0.4	0.25	0.4–3.1	3.13	0.78	6.3	0.06	0.25	2
Enterobacter spp.	1.6	0.5–1				12.5	0.12	0.25	2
S. marcescens	1.6	0.5–2				12.5	0.12	2	16
P. mirabilis	3.1	2	0.2	6.25	3.13	12.5	0.12	2	2
P. aeruginosa	3.1	2–4	25–50	50–100	25	>100	8	4	>32
H. influenzae	—	0.25–0.5	0.1	—	—	—	0.12	1	0.25
B. fragilis	0.4	0.25	0.4	—	—	—	1	0.5	—
S. aureus	0.04	0.03–0.06	1.6	0.025–0.39	1.56	25–50	0.12	0.06	0.12
S. pyogenes	0.01	0.01	0.2	0.08	1.56	25–50	0.12	0.008	0.015
S. pneumoniae	1.6	0.03	6.2	0.02	—	—	0.06	0.03	0.007

[a] Refs. 241, 487, 506, 605, 635, 636, 637.

sulfoxide MM4550 (**220a**; R = SO_3H) (480), are good inhibitors of isolated β-lactamases, (Section5.7). The in vitro activity of some representative natural products is shown in Table 14.16.

Unlike the penicillins and cephalosporins a rigid adherence to the cis-stereochemistry of substituents around the β-lactam ring is not necessary for activity. In the olivanic acids the des-sulfate derivatives are not as active as the corresponding sulfates, and in this series the *cis*-isomers are better than the trans-isomers (635). Thienamycin (**217**) is the most stable to β-lactamases, which is attributed to the presence of the 8(*R*)-hydroxyethyl side chain, given that synthetic examples lacking the C6-substituent are much more susceptible to hydrolysis by these enzymes (638). Overall, in the nonsulfated hydroxyethyl, series SAR would indicate the order of potency as trans-&lactam with 8(*R*) stereochemistry > cis 8(*S*) > trans 8(*S*) (499). Binding studies indicate that in E. coli thienamycin has the greatest affinity for PBPS, whereas most of the newer cephalosporins bind to PBP-3 (639). Although thienamycin (217) was not suitable for further development because of its chemical instabiity both in concentrated solution and in the solid state, the corresponding *N*-formimidoyl derivative, imipenem (215) was a crystalline product with much improved stability and suitable for progression (500).

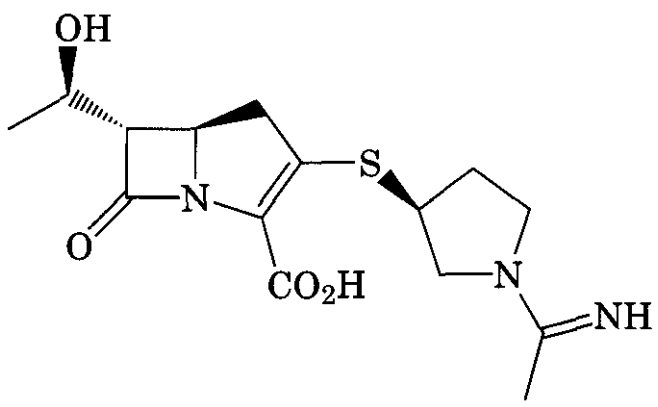
Imipenem (215) exhibits an outstanding spectrum of activity against aerobic and anaerobic Gram-positive and Gram-negative bacteria, showing a potent bactericidal effect against Pseudomonas aeruginosa, Serratia, Bacteroides fragilis, Enterococci, and many other species (640). Against some 800 clinical isolates, imipenem inhibited the majority of organisms at concentrations below 1 μg/mL and was not hydrolyzed by plasmid or chromosomal β-lactamases, although both *Pseudomonas* maltophilia and P. cepacia were resistant (641). The plasma half-life of imipenem (1 h) in humans was considered satisfactory, although urinary recoveries were quite variable (6–40%)(642). This was attributed to extensive metabolism by the renal tubular brush border dipeptidase (DHP-1), to which all of the natural carbapenems are susceptible (643). To overcome this obstacle, Merck researchers developed imipenem in combination with an inhibitor (cilastatin, 365) of DHP-1, to give an acceptable urinary recovery of the antibiotic



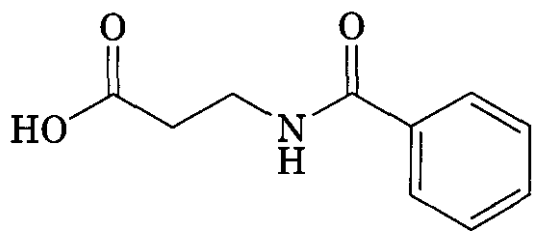
(365) Cilastatin

(644); the formation of any potential nephrotoxic degradation products was also reduced. After successful clinical trials it was this combination that was developed as the broad-spectrum parenteral agent primaxin (640,645).

Although large numbers of analogs of imipenem (215) have been synthesized, particularly with modified C2-sulfur-linked substituents, the only other compound that has been developed in this series is panipenem (**275**), which, although a broad-spectrum agent, still requires coadministration with betamipron (**366**), a DHP-I inhibitor (646). One series of



(275) Panipenem

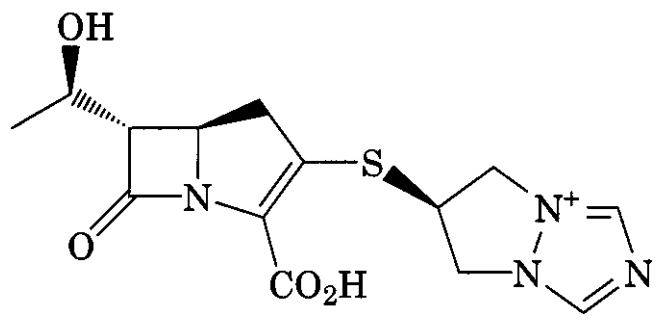


(366) Betamipron

thienamycin analogs of some interest are those with an aryl group directly attached at C2. These have been reported as having good activity and stability (647) and have evolved

into a very interesting class of anti-MRSA agents (see Section 6.1).

The lp-methyl series has seen the development of meropenem (**216**), a broad-spectrum agent comparable to imipenem (215) (Table 14.16), and which is sufficiently stable to DHP-I to allow administration without the necessity of an inhibitor (587, 648). Another compound that has been considered as a clinical candidate is the triazolium derivative **biapenem** (367) (506). Sanfetrinem (324) is not



(367) Biapenem

active against *Pseudomonas* spp. but it has a broad spectrum of antimicrobial activity (Table 14.16) and is stable to DHP-I. Of particular interest is the cilexetil **prodrug** ester (**325**), which is orally absorbed in humans (506). Oral absorbption was also demonstrated with the pivaloyloxymethyl ester of the tetracyclic β-lactam derivative (344) (627).

The antibacterial activity of trinems can be highly influenced by the kind of substituent at C4 (649), and could be targeted toward broad-spectrum agents including *Pseudomonas* spp. (623), as shown by GV129606X (342) or highly resistant Gram-positive bacteria like MRSA and penicillin-resistant Streptococci like GV143253X (**349**).

5.6 β-Lactamase Inhibitors

Widespread use of β-lactams, the largest family of antibiotics in current clinical use, has inevitably led to the emergence of resistant bacteria. The most commonly encountered mechanism of resistance is that attributable to the production of β-lactamases, a group of enzymes capable of catalyzing the hydrolysis of the β-lactam ring. The existence of these enzymes was recognized as early as 1940 soon after the isolation of penicillin, and fears relating to their plasmid-mediated spread throughout the bacterial population have been fully realized (2, 650).

The β-lactamases constitute a large and diverse family of enzymes, classification of which has been the subject of a number of publications. As stated earlier (Section 3) the most recent and comprehensive of these and its relationship to previous classification schemes (651–655) have been described by Bush and coworkers (656). However, for simplicity, the earlier classification originally proposed by Ambler, which is based on amino acid sequence homology, will be used in this review (657). In this classification the enzymes are divided into three groups (classes A, C, and D), which are active-site serine β-lactamases and one group (class B) of metallo β-lactamases containing at least one Zn^{2+} ion per active subunit.

Class A contains many of the clinically important enzymes, including those from Gram-positive bacteria and the widely found TEM-type enzymes from Gram-negative organisms. The majority of them are transmissible, plasmid-mediated enzymes, often referred to as penicillinases because their preferred substrates are the penicillins.

Class B contains the broad-spectrum metallo-enzymes, which are capable of hydrolyzing most classes of β-lactams including the carbapenems. The earliest reported metallo-enzymes were confined to a small group of organisms with little clinical relevance. However, recent isolation of plasmid-mediated metallo β-lactamases has led to concern that there is potential for rapid dissemination of these enzymes, which are not susceptible to any of the clinically used inhibitors (658).

Class C contains predominantly chromosomally mediated enzymes from Gram-negative bacteria whose preferred substrates are the cephalosporins and are thus often referred to as cephalosporinases. Many producers of this class of enzymes are regulated by an inducible mechanism of control in response to the presence of antibiotics (659). Class D includes enzymes capable of hydrolyzing the more β-lactamase stable isoxazolyl penicillins.

Two strategies have evolved to combat β-lactamase-mediated resistance. The first of these, involving the development of classes of β-lactam antibiotic with improved stability is

Table 14.17 **β-Lactamase Inhibitory Activity (IC_{50} μg/mL)**[a]

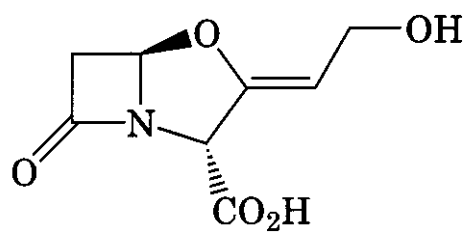
Organism	Enzyme class	Clavulanic acid (**362**)	Sulbactam (**369**)	Tazobactam (**372**)	BRL 42715 (**380**)
S. aureus	A	0.063	1.4	0.27	0.016
E. coli (TEM-1)	A	0.055	1.7	0.028	0.002
E. coli (SHV-1)	A	0.035	13.0	0.14	0.001
Enterobacter cloacae (P99)	C	>50	5.0	0.93	0.002
E. coli (OXA-1)	D	0.71	2.2	1.1	0.001

[a] IC_{50} determined after 5-min preincubation of enzyme and inhibitor.

dealt with in the appropriate sections of this chapter. The second approach has been the identification of β-lactamase inhibitors for coadministration with the antibiotic, thereby protecting it from hydrolysis by β-lactamase-producing organisms.

The search for inhibitors has involved both natural product screening and the creative ingenuity of the medicinal chemist. The early developments in the field of β-lactamase inhibitor research have been reviewed by Cole (660)and Cartwright and Waley (661). In this section we cover the clinically used p-lactamase inhibitors and the important developments since these early reviews were written. None of the reported non-β-lactam inhibitors is likely to have any clinical impact and will not be discussed because they fall outside the scope of this chapter.

5.6.1 Discovery of Clavulanic Acid. Clavulanic acid (368) can be considered as the most important and representative among the inhibitors of p-lactamases (662).



(368) Clavulanic acid (1976)

Clavulanic acid (**368**), the first clinically useful β-lactamase inhibitor, was identified as a natural product from a strain of *Streptomyces clavuligerus* through use of an assay designed to detect inhibitors by their ability to protect penicillin G from hydrolysis by a β-lactamase-producing strain of *Klebsiella aerogenes* (663).

X-ray analysis of the 4-nitrobenzyl ester revealed a novel fused p-lactam containing an oxygen atom instead of sulfur and lacking the acylamino side chain present in penicillins and cephalosporins (662). Clavulanic acid is a potent inhibitor of a wide range of clinically important class A β-lactamases, with more modest activity against class D and none against the class B and C enzymes (664). Comparative inhibition data for clavulanic acid and other inhibitors, which will be discussed in detail, are shown in Table 14.17 (665).

Clavulanic acid (368)possesses only a poor level of antibacterial activity in its own right, but is capable of synergizing the activity of a number of β-lactam antibiotics against a range of β-lactamase-producing strains of both Gram-positive and Gram-negative bacteria (666). Comparative synergy data from a study in which low inhibitor concentrations

Table 14.18 **Amoxycillin MIC (μg/mL) in the Presence of μg/mL of Inhibitor**

Organism	Enzyme class	No. inhibitor	Clavulanic acid (**362**)	Sulbactam (**369**)	Tazobactam (**372**)	BRL 42715 (**380**)
Proteus mirabilis	A	>512	16	64	16	2
E. *coli* (TEM-1)	A	>512	8	128	8	2
K. pneumoniae	A	256	4	64	16	2
Enterobacter cloacae	C	512	>512	256	256	1
E. *coli* (OXA-1)	D	>512	>512	>512	>512	2

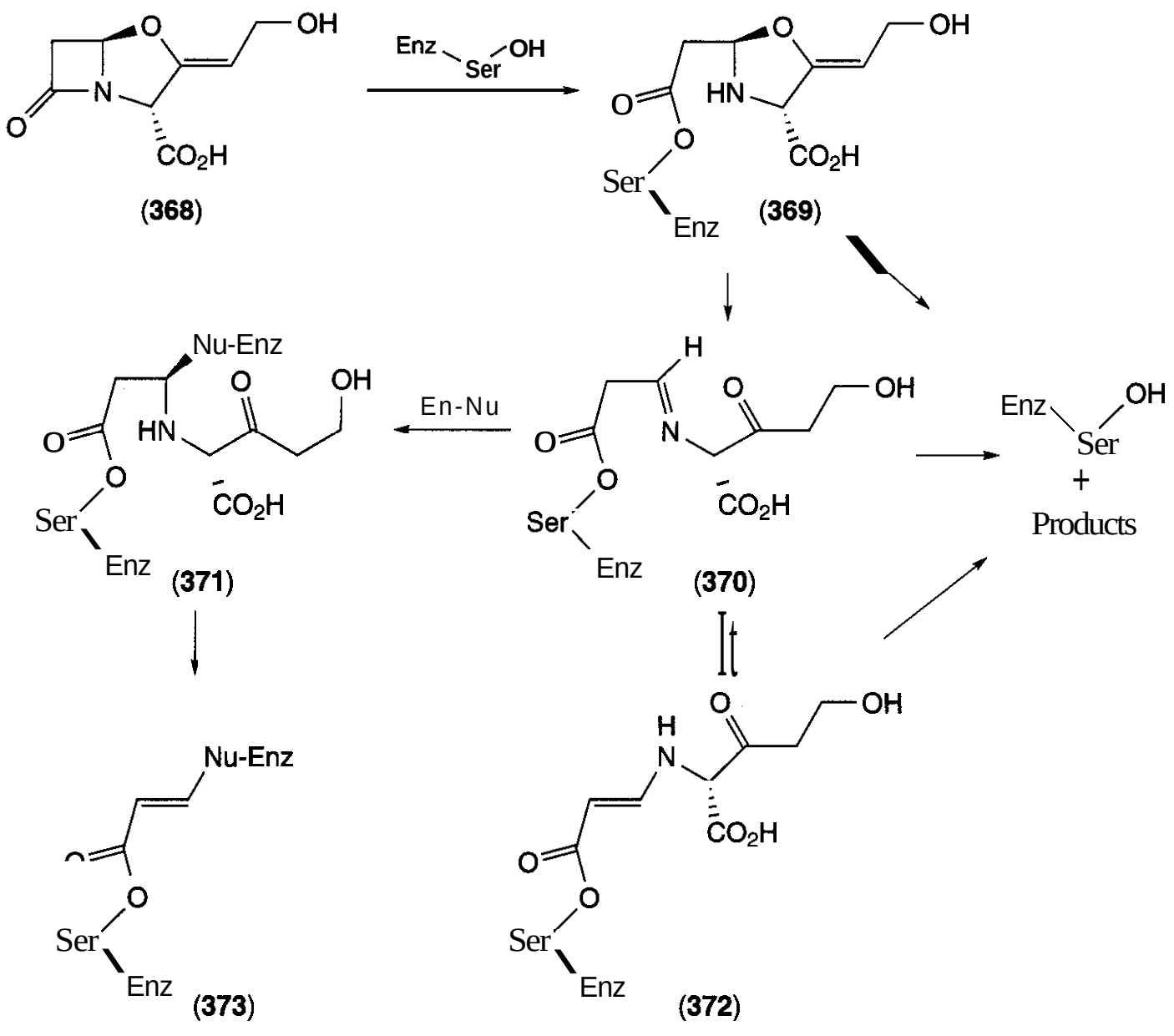


Figure 14.22. Mechanism of action of clavulanic acid.

were used are shown in Table 14.18 (667). Although greater synergy can be demonstrated at higher levels these data serve to illustrate the differences in spectrum and potency of the four inhibitors featured in this review. Clavulanic acid (**368**) markedly reduces the MIC of amoxycillin (21) against the organisms producing class A p-lactamase. However, at this inhibitor level, its more modest potency against the class D enzyme is not translated into whole-cell activity.

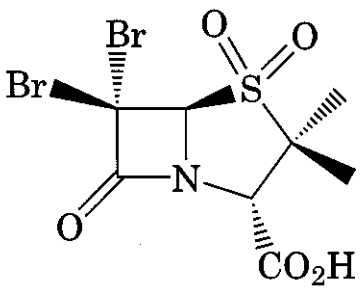
Marketed as Augmentin (potassium clavulanate plus amoxycillin) and Timentin (potassium clavulanate plus ticarcillin), clavulanic acid has found widespread use, particularly as the former formulation, with sales in excess of $1500 million in 2000. The pharmacokinetic and early clinical trial data for amoxycillin/clavulanic acid combinations were summarized by Cole (660) and further clinical experience was reviewed in 1989 (668).

Despite a general increase in the frequency of β-lactamase-producing strains observed in the period since its introduction, two recent and extensive reviews of published clinical data concluded that there had been no significant increase in resistance to Augmentin (669, 670). Clavulanic acid (368) belongs to a class of enzyme inhibitors often referred to as "suicide" or "mechanism-based" inhibitors and, because of its clinical importance, has been the subject of many kinetic and mechanistic studies (671–674). These studies have led to a complex and still incomplete model for the interaction of clavulanic acid with β-lactamases, the salient features of which are shown in Fig. 14.22.

Acylation of the active site serine followed by ring opening of the intermediate oxazolidine (369) provides the imine (**370**), which may undergo tautomerization to the enarnine derivative (372). Although all three of these intermediates may undergo hydrolysis, lead-

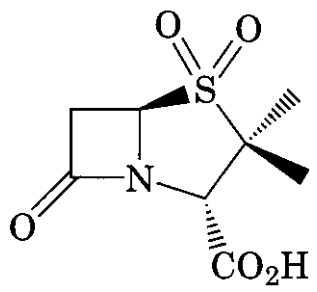
ing to turnover and release of active enzyme, the anticipated hydrolytic stability of (372) (a stable β-aminoacrylate) led to its assignment as the structure for the so-called transiently inhibited species (673). It has been proposed that the irreversible inhibition results from the trapping of a second nucleophile of the enzyme by the imine species (**370**) to afford (**371**), followed by p-elimination to give (**373**). Although it has been suggested that the second nucleophilic group might be provided by a lysine, some modeling studies based on enzyme X-ray crystal structure led to the hypothesis that it is a second and conserved serine that is captured by the imine species (**370**) (674). The biosynthesis (675) and chemistry (370, 676) of clavulanic acid have also received much attention. Despite extensive modifications, especially those involving derivatization of the allylic alcohol, no inhibitors derived from clavulanic acid have progressed to the clinic.

5.6.2 Sulbactam. Diazotization/bromination of 6-APA (**2**), followed by oxidation, gave the 6,6-dibromopenicillanic acid sulfone (**374**), which on catalytic hydrogenation provided sulbactam (**375**) (677).



(374)

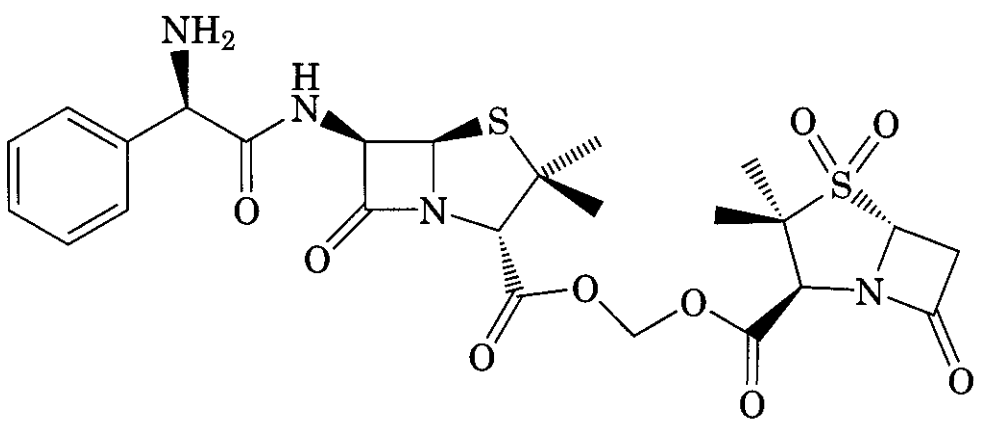
Sulbactam (**375**) was shown to be an irreversible inhibitor of several β-lactamases



(375) Sulbactam

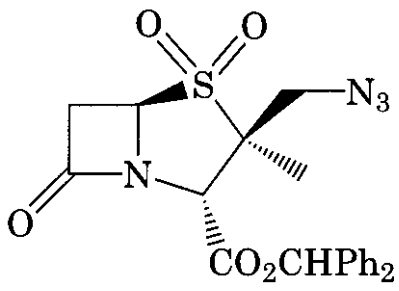
(678, 679). Compared with clavulanic acid (Table 14.17) sulbactam is a modest inhibitor of the class A enzymes but shows improved potency against those of class C, although at a level considered to be of little clinical use. The levels of synergy achieved in whole cells with sulbactam (375) (Table 14.18) reflect its poorer potency against the class A enzymes. Its mode of action is believed to be essentially similar to that of clavulanic acid (**368**), with a cascade following β-lactam ring opening, eventually leading to the same type of inactivated species (**373**) (673). Demonstration of synergy in combination with ampicillin (19) led to the development of a 1:1 formulation, which is marketed as Unasyn for parenteral use. Poor absorption of sulbactam (375) precluded the use of this combination by the oral route and led to the development of the mutual prodrug sultamicillin (376). After absorption from the gut this double ester undergoes rapid cleavage by nonspecific esterases, to give good serum levels of both ampicillin (19) and sulbactam (**375**) (680).

5.6.3 Tazobactam. Renewed interest in penicillanic acid sulfones stimulated by the discovery of the properties of sulbactam (**375**) led to the investigation of the effects of substitution in the 2-methyl group. Exploitation of the reactivity of the azido function of the 2-β-

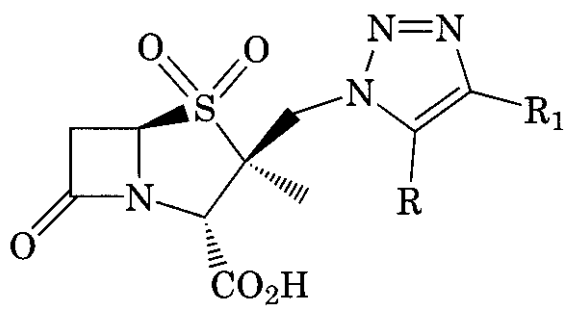


(376) Sultamicillin

azidomethylpenam (**377**) to cycloaddition reactions with acetylenes provided, after deprotection, a series of 2β-(triazolyl)methylpenams (378) with potent β-lactamase inhibitory activity (681).



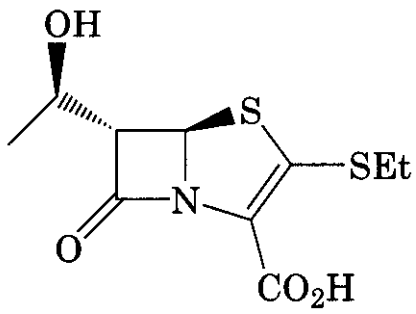
(377)



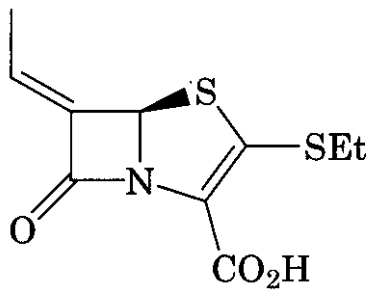
(378) Tazobactam

The unsubstituted triazole (378; R = R_1 = H), which was also available through the reaction of (374) with vinyl acetate, originally selected for its potency and ease of preparation, has found clinical use as tazobactam. Tazobactam (378) is more potent than sulbactam (Table 14.17), with good activity against the clinically important TEM-1 enzymes. It also shows a modest level of activity against the class C enzymes, which are not inhibited by clavulanic acid (368). In combination with amoxycillin levels of synergism similar to those of clavulanic acid are observed for organisms producing class A β-lactamases (Table 14.18). At higher inhibitor levels synergy against class C-producing organisms has been demonstrated for tazobactam (**378**) as well as its ability to synergize the activity of a range of β-lactam antibiotics (682, 683). Among these, piperacillin (34) was selected as the partner antibiotic for the marketed parenteral formulation; experience of its clinical use was reviewed in 1993 (684). The degradation of tazobactam (**378**) has been studied extensively and its mechanism of action was considered similar to that of sulbactam (375) (685,686).

5.6.4 6-Heterocyclylmethylene Penems. Sch-29482 (379) has been reported to be an inhibitor of class C and class D β-lactamases (687). However, fewer additional data are available for penems bearing the 6-(1-hydroxyethyl) substituent, which have been studied widely for their antibacterial activity. Dehydration of (379) gave the E- and 2-isomers of the 6-ethylidenepenem (**380**), both of which were



(379)



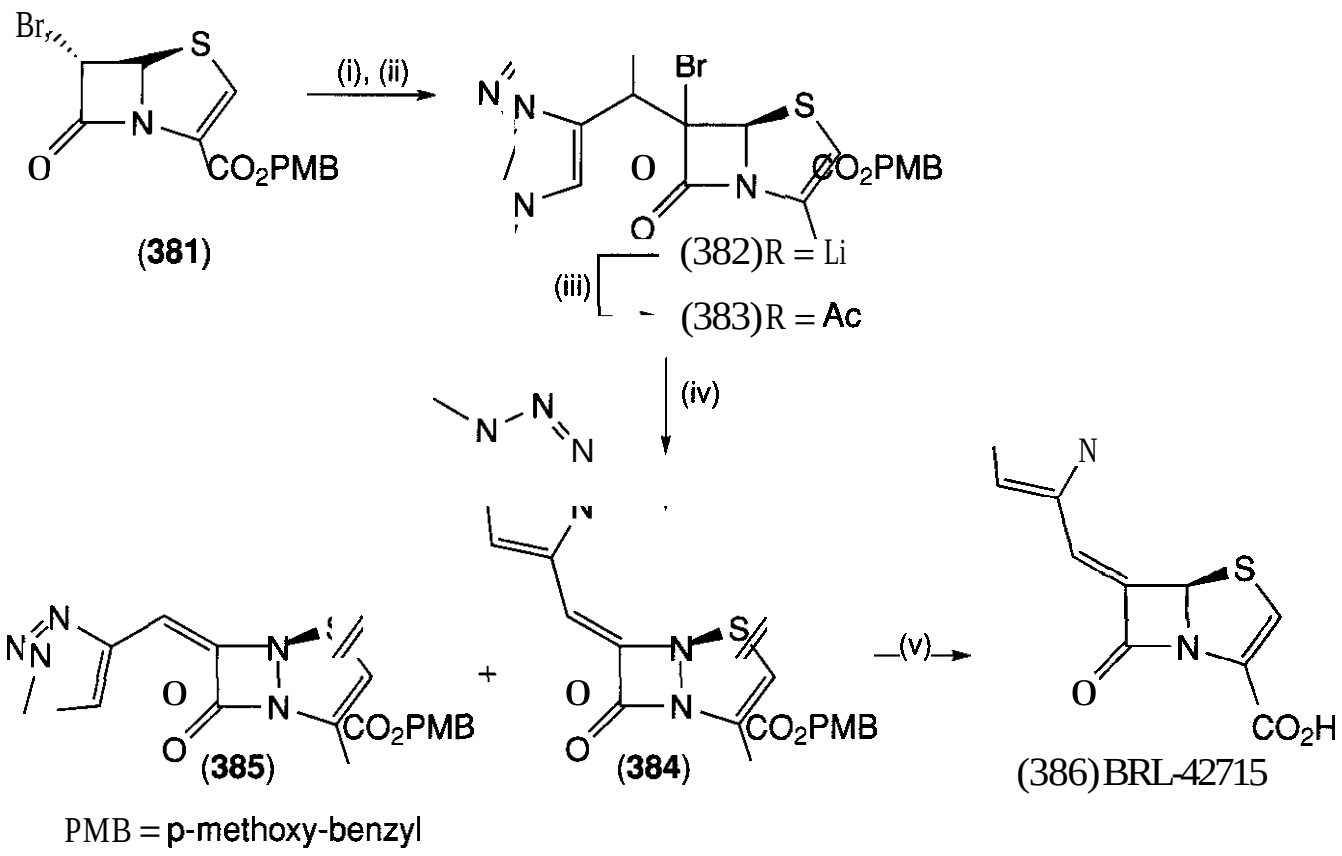
(380)

shown to be potent broad-spectrum β-lactamase inhibitors with much weaker antibacterial activity than that of the parent penem (688).

Extensive structure-activity relationship studies involving modification at both the C2 and C8 positions have been described (689).

From these studies the *Z*-triazolylmethylenepenem (386, BRL-42715) was selected for further evaluation based on its overall activity and stability to human renal dehydropeptidase (DHP-I), which has the ability to degrade penems in a manner similar to that of the carbapenems (690). An atom-efficient, stereocontrolled synthesis from 6-amino penicillanic acid (2) has been described, the key steps of which are shown in Fig. 14.23 (691).

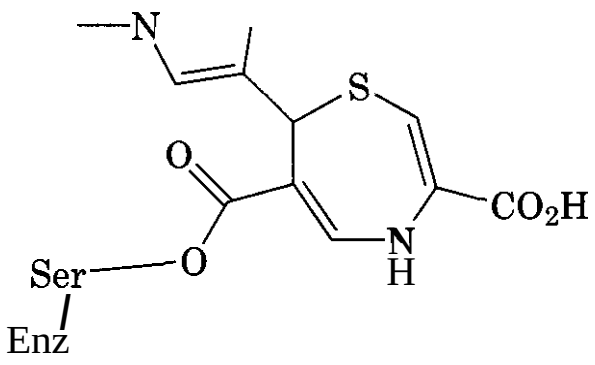
Reaction of the anion derived from the bromopenem (381) with 1-methyl-1,2,3-triazole-4-carboxaldehyde provided a mixture of the lithium salts (**382**), *in situ* acetylation of which gave a mixture of the bromoacetates (383). Reductive elimination of this mixture



Reagents: (i) Ph_2NLi, THF, -78°C; (ii) 1-methyl-1,2,3-triazole-4-carboxaldehyde; (iii) Ac_2O, -78°C to 20°C; (iv) Zn, N,N,N′,N′-tetramethylethylethylenediammine dihydrochloride, NH_4Cl, DMF; (v) $AlCl_3$, anisole.

Figure 14.23. Synthesis of **BRL-42715.**

gave the desired *Z*-triazolylmethylenepenem ester **(384)** as the major product, which was deprotected to provide the acid (**386**). **BRL-42715 (386)** is a potent broad-spectrum inhibitor capable of synergizing the activity of β-lactam antibiotics against a wide range of β-lactamase-producing organisms **(665).** Tables **14.17** and **14.18** reveal that **BRL-42715** (**386**) is a more potent inhibitor than clavulanic acid (**368**), sulbactam (**375**), and tazobactam (**378**) against class A, C, and D enzymes and that this potency is translated into more impressive synergism in whole-cell assays. Detailed kinetics confirming the efficiency of inactivation have been described for a number of p-lactamases **(692, 693),** and a mechanism of action involving a novel rearrangement to a dihydrothiazepine (**387**) proposed (694).

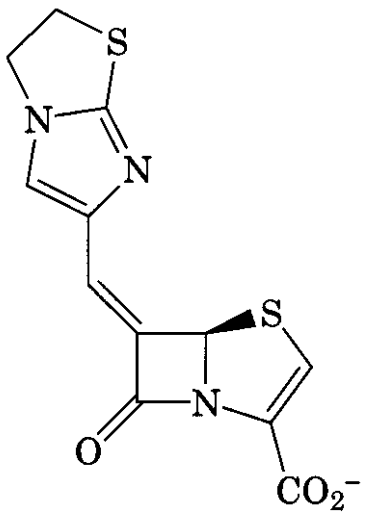


(387)

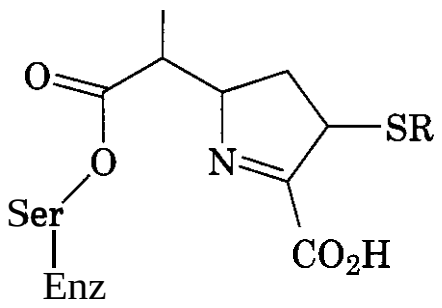
Despite its impressive inhibitor profile, development of BRL-42715 (**386**) was terminated because of its failure to fulfill other desired technological features. A second inhibitor, SB-206999 (**388**), possessing an activity profile similar to that of **BRL-42715,** is currently under development (695).

5.6.5 Carbapenems. Many of the carbapenems described in Section **5.5** are potent β-lactamase inhibitors with a spectrum that includes the class C enzymes not inhibited by clavulanic acid **(665).**

The mechanism of action of carbapenems has been studied **(673)** and the rearrangement to a Δ^1-pyrroline **(389)** after β-lactam ring opening was proposed to explain stabilization



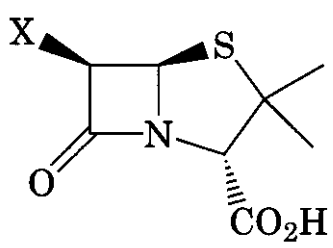
(388) SB-206999



(389)

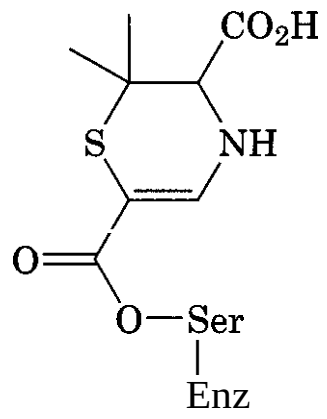
of the enzyme-bound species. Limitations on the level of carbapenem used, imposed by their potent antibacterial activities, have often precluded the demonstration of significant synergy and no compound of this class has progressed as an inhibitor.

5.6.6 **Penams and Penam Sulfones.** 6β-Bromopenicillanic acid (**390**), often referred to as



(390) X = Br
(391) X = I
(392) X = Cl

brobactam, was described as a p-lactamase inhibitor as early as 1978 (696). Extensive studies of its mechanism of action have provided powerful evidence for an active-site serine-bound dihydrothiazine (393) in the inactivated enzyme (697).



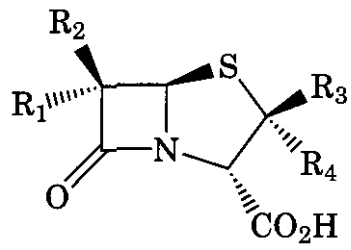
(393)

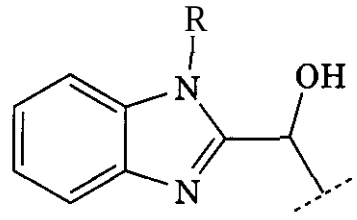
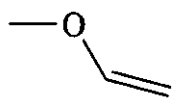
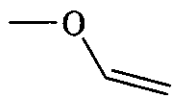
It has been noted that this dihydrothiazine (393) and the dihydrothiazepine (387) share the same enamine moiety seen in the proposed structure of the transiently inhibited species (**372**) for clavulanic acid (694, 698). Despite the demonstration of good synergistic properties and a favorable pharmacokinetic profile in humans, brobactam (390) has not found clinical application (699, 700). Comparable activity has been reported for 6β-iodopenicillanic acid (391) (701), whereas the chloro analog is less potent (392) (702). Stimulated by the discoveries of sulbactam (**375**), tazobactam (**378**), and brobactam (**390**), modification of the penam nucleus has provided a large number of β-lactamase inhibitors. Tables 14.19 and 14.20, which are by no means comprehensive, illustrate the structural types of penam and penam sulfones that have been reported to possess these properties.

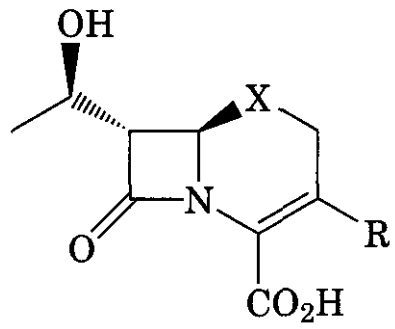
5.6.7 **Miscellaneous β-Lactams.** β-Lactamase inhibitory properties have been reported for a large number of other β-lactam-containing structures including cephems and monocyclic β-lactams (660,661). Among the more recent reports are those of activity for 7α-(1-hydroxyethyl)-cephems (including their sulfoxides and sulfones) and oxacephems (394) bearing electron-withdrawing groups at the 3-position (721). Activity has also been reported for other cephems bearing nonclassical 7-substituents [e.g., the 7-allene functionality and alkylidene cephems (**395**) and (**396**) (722)].

Stabilization of the oxapenem ring system by the introduction of bulky 2-substituents has allowed the demonstration of β-lac-

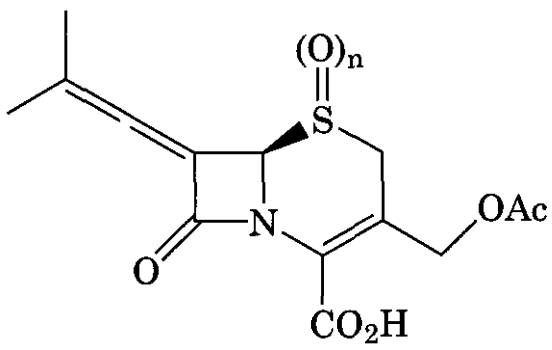
Table 14.19 Penam Inhibitors of β-Lactamases



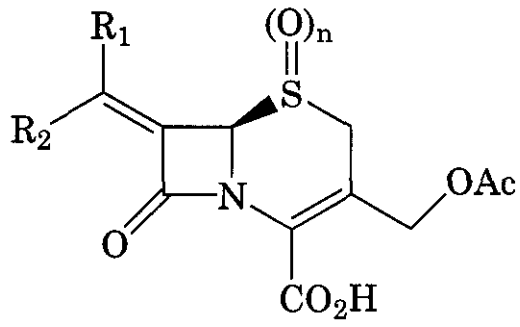
R_1	R_2	R_3	R_4	Reference
H	Br	CH_2X X = F, N,, OAc, OMe, SCN	Me	**703**
H	$(CF_3SO_2)_2N$	Me	Me	**704**
H	$HOSO_2NH$	Me	Me	**705**
H		Me	Me	**706**
		Me	Me	707
		Me	Me	708



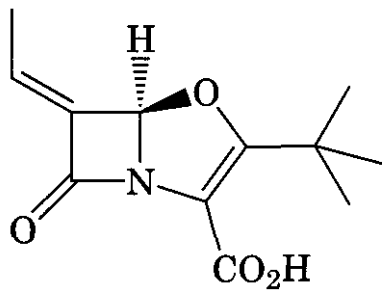
(394) X = S, SO, SO_2, *O*
R = electron withdrawing group



(395) n = **0, 2**



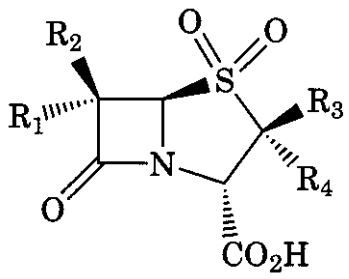
(396) n = 0, 2

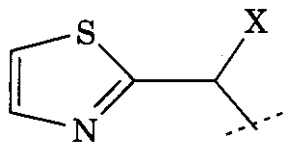
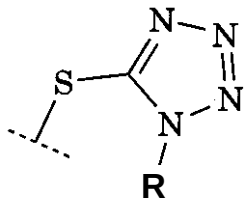
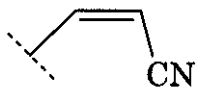
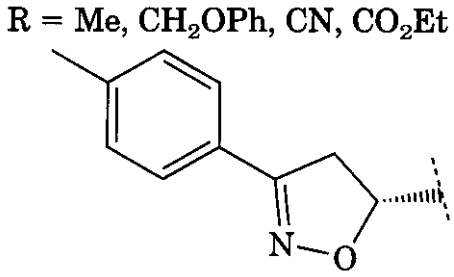
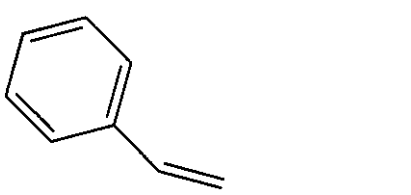
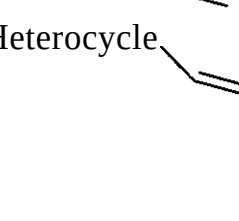
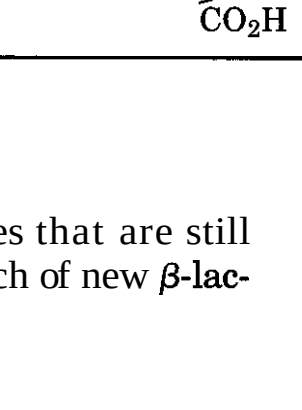


(397)

tamase inhibition for members of this highly ring strained class of β-lactam derivatives. Activity has been demonstrated for the 2-*tert*-butyloxapenems [(**397**) (723) and (398) (**724**)] and the 2-isopropyloxapenem (399)(**725**), the latter in particular showing potent inhibition of class C enzymes. Recently, selective and potent inhibitors of class C enzymes have been reported for a series of bridged monobactams (**400**) (726).

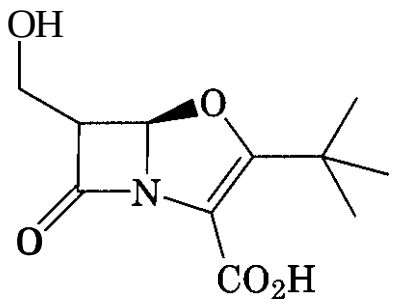
Table 14.20 Penam Sulfone Inhibitors of β-Lactamases



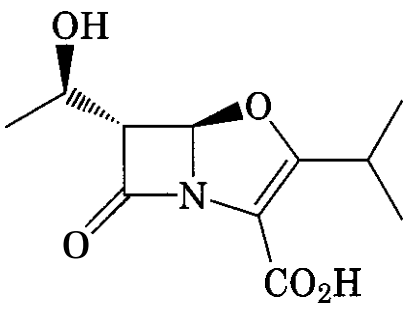
R_1	R_2	R_3	R_4	Ref.
H	H	CH_2Cl	Me	709
H	CF_3SO_2NH	Me	Me	710
H	PhCH(OH)	Me	Me	711
H	X = OAc, F, OH 	Me	Me	712
H	H	 R = Me, Ph	Me	713
H	H	CH_2SCN	Me	713
H	H		Me	714
H	RCOCH$_2$  R = Me, CH_2OPh, CN, CO_2Et	Me	Me	715
H		Me	Me	716
		Me	Me	717
	Heterocycle	Me	Me	718, 719
				720

6 RECENT DEVELOPMENTS IN β-LACTAMS

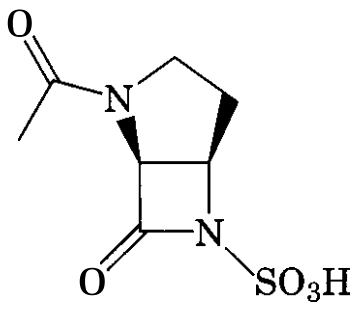
The pharmaceutical companies that are still actively involved in the research of new β-lactam antibiotics after almost 60 years are encouraged by the safety profile that has been proved by extensive use throughout the years and by the continuous launch of new agents. In



(398)



(399)



(400)

particular, the main efforts are directed toward the discovery of agents active against dangerous emerging resistant strains, and a more favorable pharmacodynamic and tolerability, reducing the dosage, number of daily administrations, length of treatment, and side effects.

6.1 Anti–Cram-Positive β-Lactams

MRSA (methicillin-resistant Staphylococcus aureus) was first described in the early 1960s and has since become one of the major nosocomial infections in the hospital setting (727). MRSA still remains as an important target for the development of anti-infective agents. It has been shown that these strains are resistant to penicillins, cephalosporins, and also carbapenems. Although effective, therapy with glycopeptides such as vancomycin or teicoplanin is relatively limited because of side effects shown by this class of antibacterial agents. Therefore, potent anti-MRSA agents with low levels of side effects would be highly desirable. Nearly all strains of MRSA share a common feature of carrying the *mecA* gene encoding for an additional, but with low affinity for, β-lactam penicillin binding protein, PBP2a (728, 729).

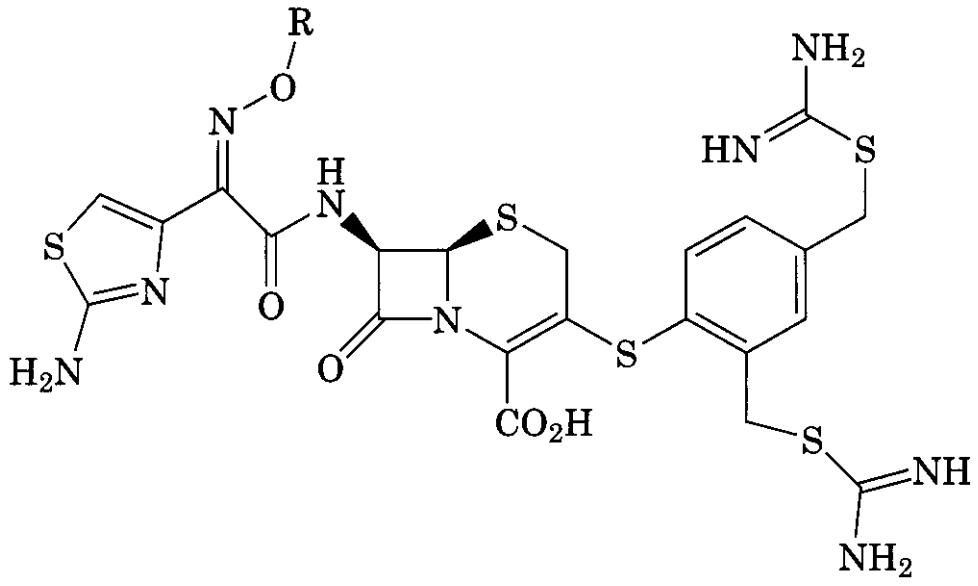
6.1.1 Cephalosporins. A new generation of cephalosporins with enhanced affinity for PBP2a was identified during the late 1990s. The most interesting derivatives are characterized by the presence of a highly basic or ionized moiety at the C3-position bearing at C7 the amino thiazole ring or a closely related structure.

Microcide was particularly active in this field with the identification of several products: MC-02331 (**401**), MC-02363 (**402**), and MC-02479 (403); RWJ-54428 (404); RWJ-333441 and its prodrugs (730) (Microcide Pharmaceuticals/RW Johnson Pharmaceutical Research Institute). Other cephalosporin derivatives of particular interest were also identified: Ro-63–9141 (405, F. Hoffmann-La Roche), TOC-39 (406, Taiho) and TOC 50 (407, Taiho). All these compounds have shown a promising anti-MRSA activity with the potential to overcome the recent issues associated with the development of bacterial resistance.

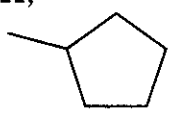
6.1.2 **Carbapenems and Trinems.** Several pharmaceutical companies have reported a series of carbapenems and trinems showing excellent activity against MRSA and vancomycin-resistant Enterococci (731–735). These compounds (**408–411**) showed very good affinity for PBP2a, typical of MRSA and PBP5, which is overproduced by resistant Enterococci.

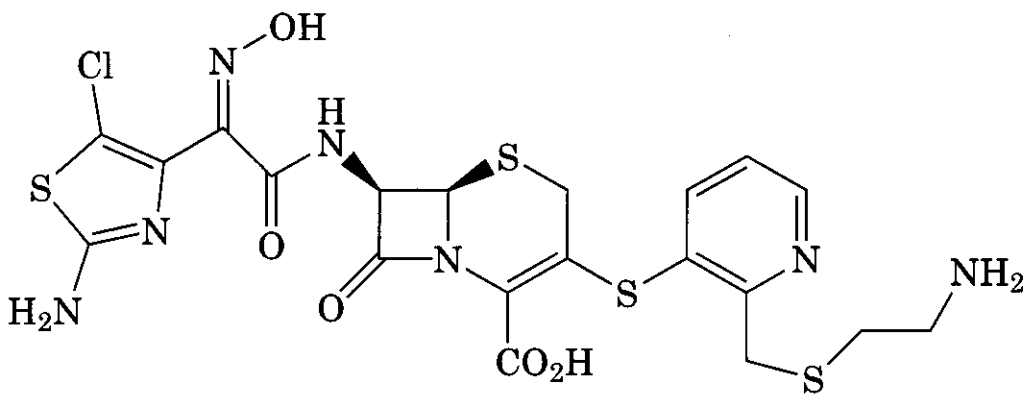
The presence of an aromatic moiety, as in the 2-arylcarbapenems or aryl-4-exomethylenyl trinems, was recognized as a necessary key feature in the most active β-lactam anti-MRSA agents. Most of these carbapenems were derivatives in which the C2 side chain was linked by a C-C bond instead of a C-S bond. Moreover, the presence of a quaternary nitrogen on the side chain conferred satisfactory water solubility, an essential property for an injectable drug.

The carbapenem class was extensively investigated and several potent compounds

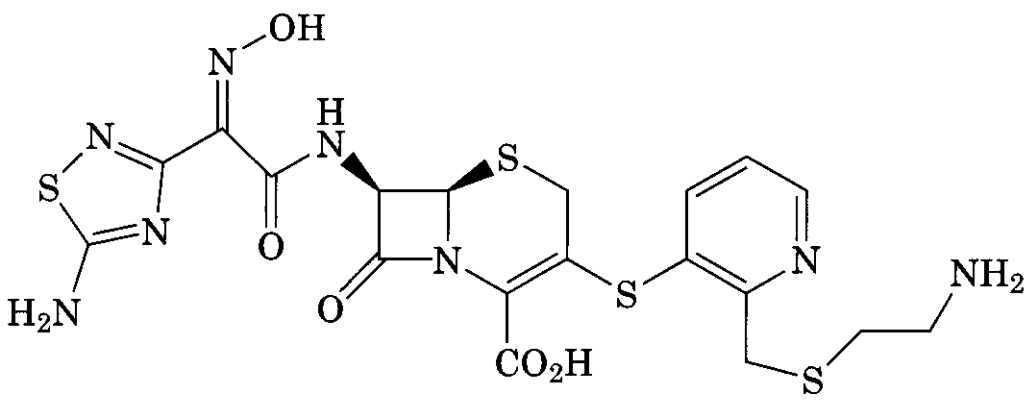


(401) MC-02331 R = H,

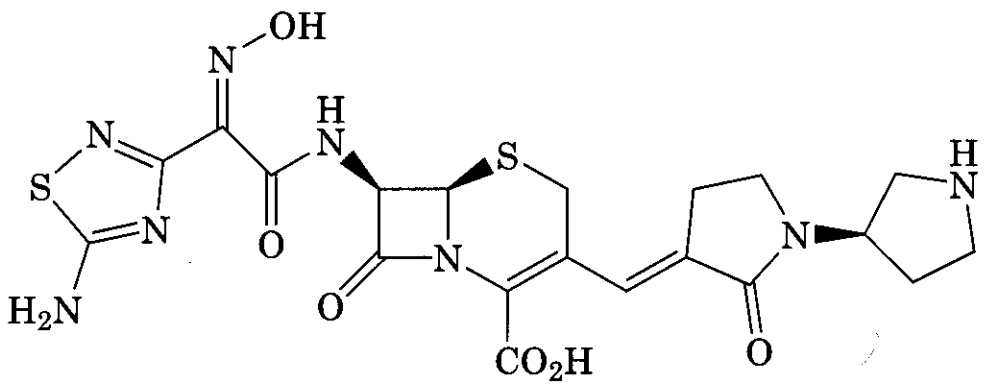
(402) MC-02363 R = 



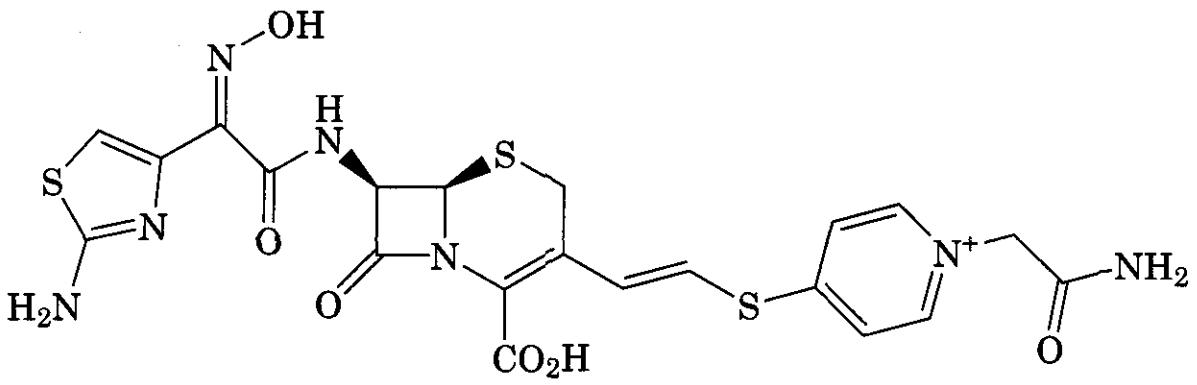
(403) MC-02479 or RWJ-54428



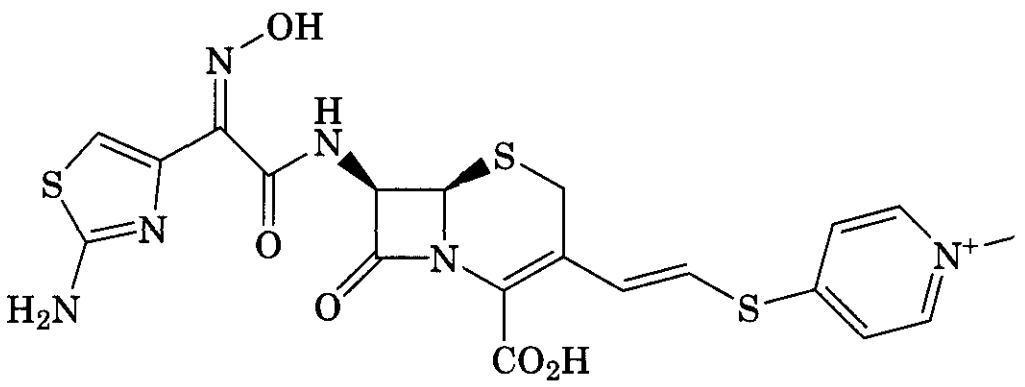
(404) RWJ-333441



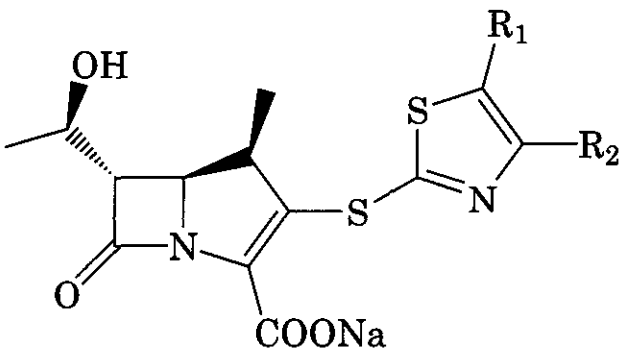
(405) Ro-63-9141



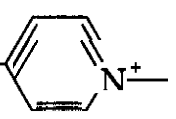
(406) TOC-39



(407) TOC-50



Sumitomo

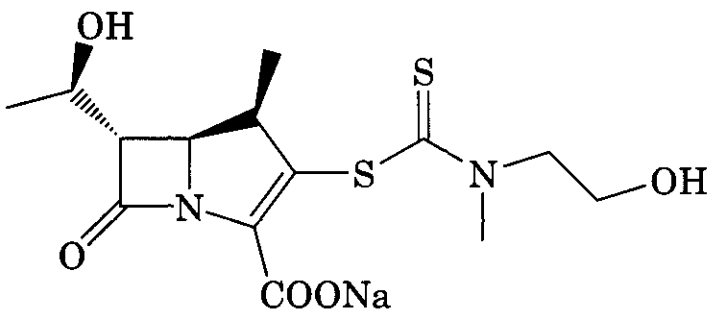
(408) R_1 = H, R_2 = 

(409) $R_1 = CH_3$, $R_2 = CH_3$

(410) R_1 =  R_2 = H

were identified: L-741–462 (**412**), L-696–256 (413), L-742–728 (**414**), and L-786–392 (**415**). All these compounds underwent clinical trials but for different reasons their clinical development was discontinued.

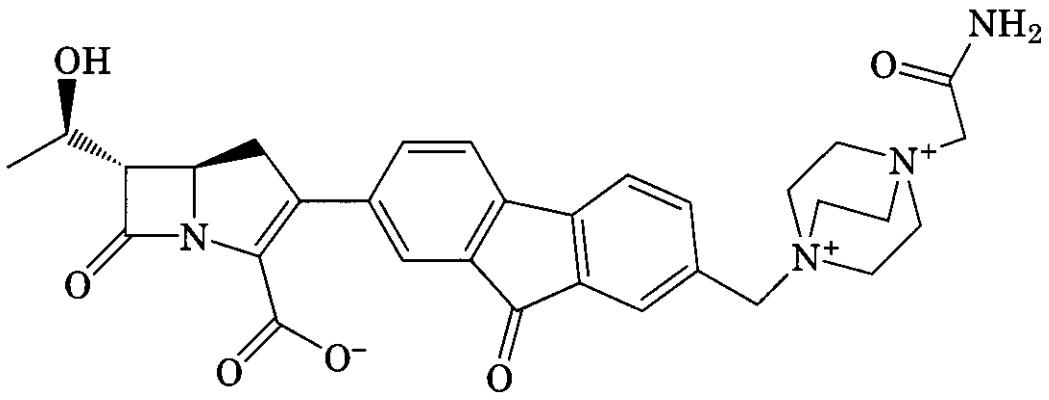
Clinical development of L-741–462 (412) was discontinued because of an unexpected immunotoxic reaction observed in Rhesus monkeys (736), which was thought to be the result of a specific acylation of lysine residues



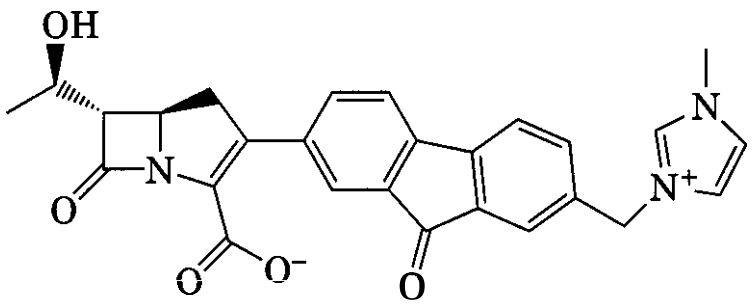
(411) BO-3482 Banyu

on the cell surface followed by recognition of the appended hapten (Fig. 14.24). The carbapenem L-786–392 (**415**) was designed to overcome this problem, given that acylation of lysine by β-lactam would release the sulphonamido moiety, thereby avoiding its recognition as hapten. However, despite its excellent *in vitro* and *in vivo* activity, this compound failed to meet the required safety profile.

GV143253X (349, GlaxoWellcome) was the first reported example of trinem with a very interesting *in vitro* and *in vivo* antimicrobial profile that is comparable to that of vancomycin. This compound belongs to a relatively novel class of antibiotics and in this class the presence of the double bond has been shown to be important for the anti-MRSA activity.



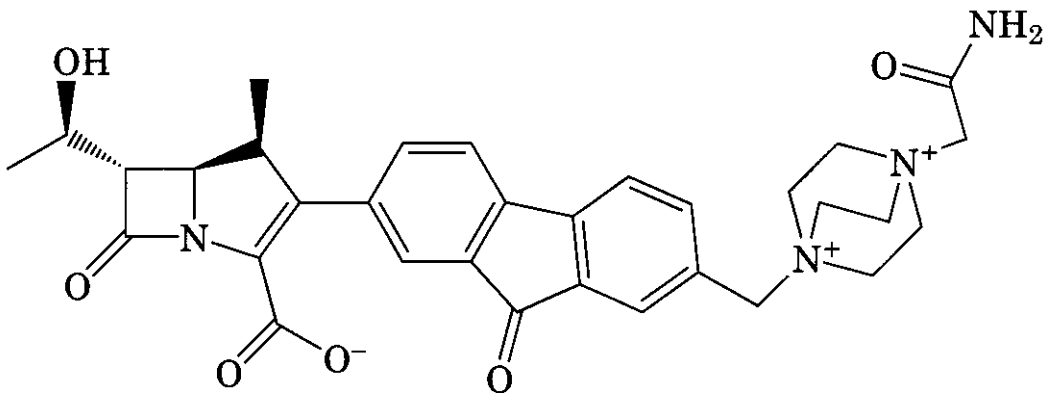
(412) L-741-462



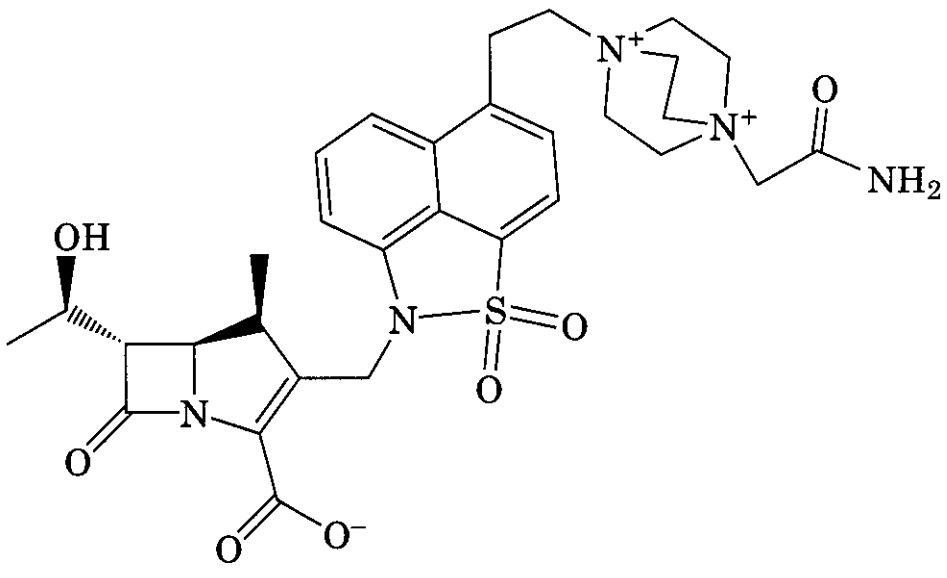
(413) L-696-256

To substantiate the hypothesis, the cyclopropanation of the exo-double bond resulted in a compound (420) still microbiologically active, but lacking anti-MRSA activity. Additionally, inclusion of the double bond into an aromatic ring (419) retained the anti-MRSA activity (630, 631).

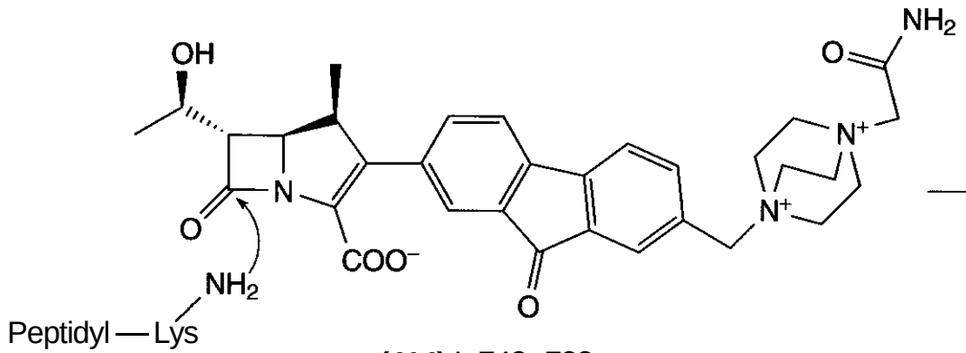
However, the unsatisfactory pharmacokinetic profile shown by GV143253X (**349**) in healthy volunteers suggested discontinuing further studies on this compound.



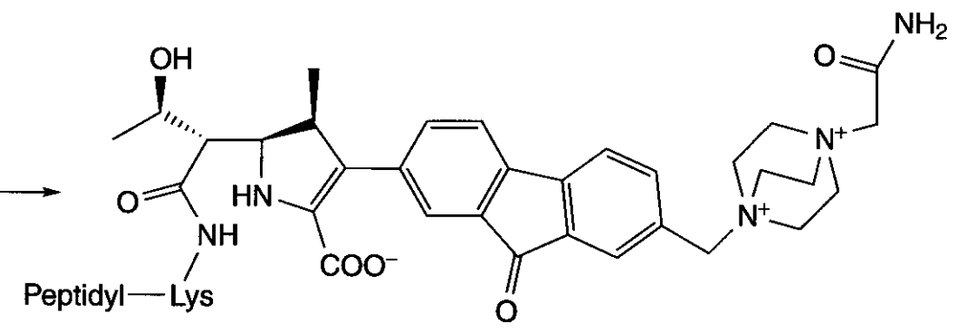
(414) L-742-728



(415) L-786-392

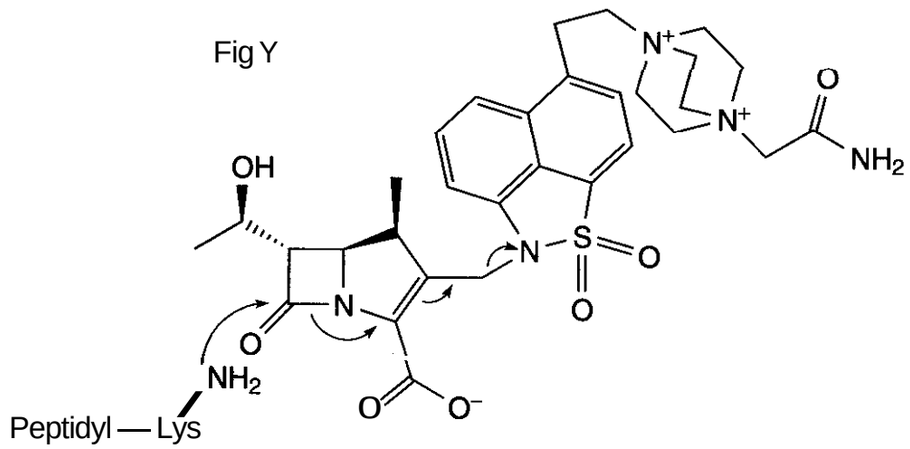


(414) L-742–728

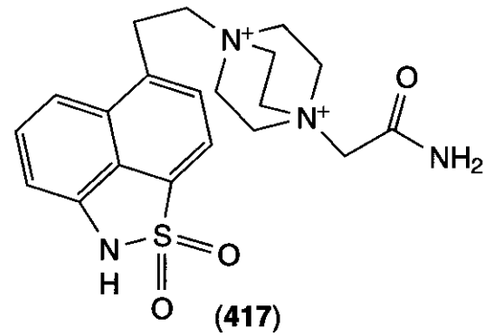


(416) Immunogenic reaction in C. Monkey

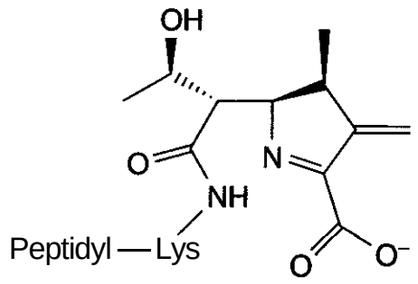
Fig Y



(415) L-786–392

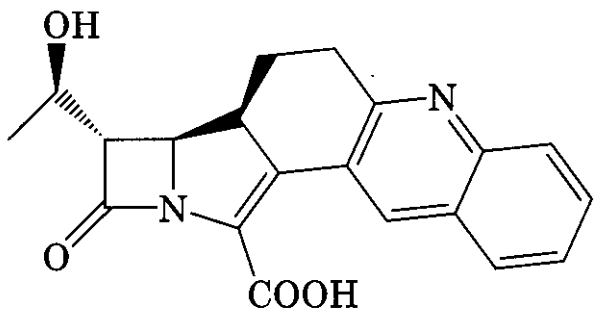


(417)

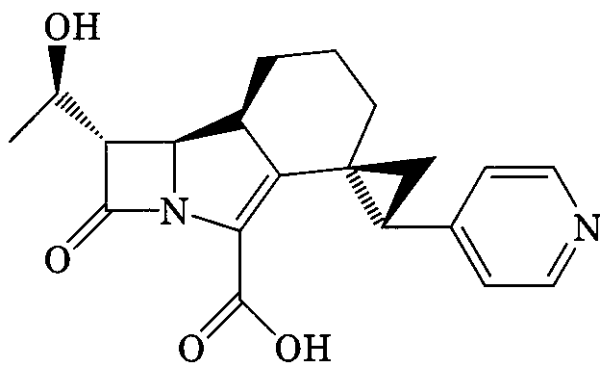


(418)

Figure 14.24. Acylation of cell surface protein by L-741–762 and L-786–392.



(419)

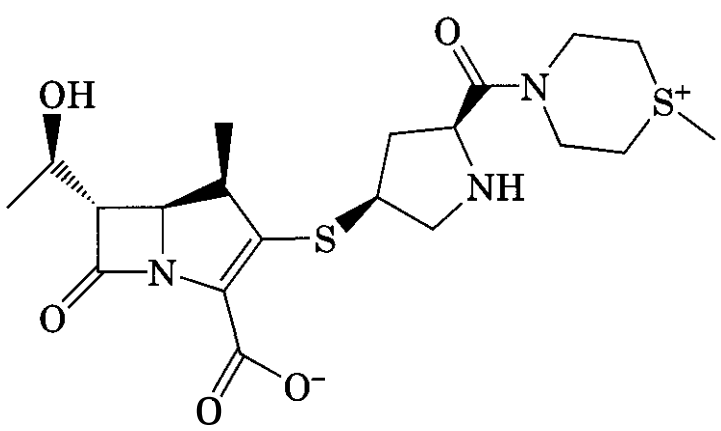


(420)

6.2 Antibacterial Broad-Spectrum β-Lactams

The intrinsic potency of carbapenems and trinems has encouraged pharmaceutical companies to investigate these classes of β-lactams to find broad-spectrum agents for the treatment of severe hospital infections.

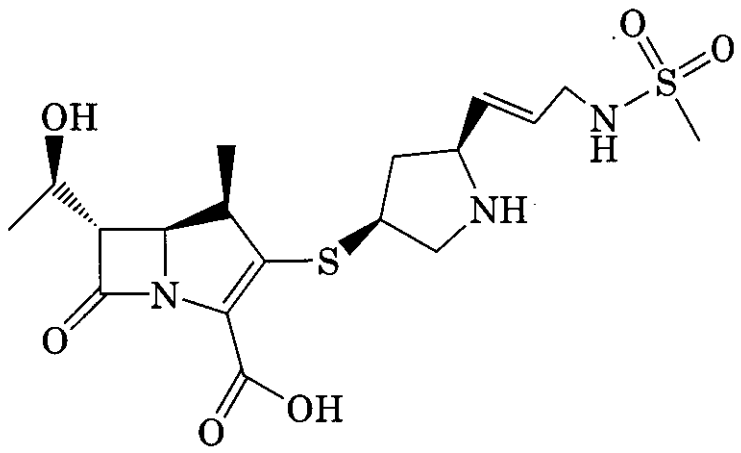
A series of compounds that are characterized by the presence of the 3-thiopyrrolidine group, also present in meropenem (**216**), have been reported. The Korea Institute of Science and Technology (KIST) has synthesized DK-35C (421) (737, 738), a compound that exhib-



(421) DK-35C

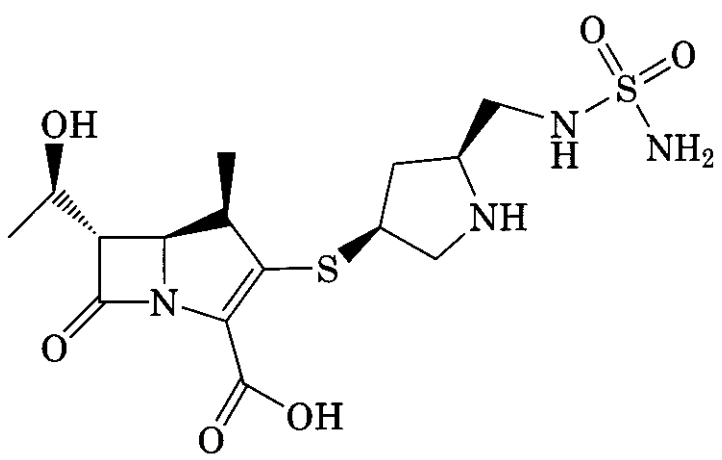
ited greater potency than that of imipenem (215) or meropenem (216). Intracerebroventricular injection in male rats (739) showed a proconvulsive activity greater than that of meropenem but weaker than that of imipenem or cefazolin (59); however, it is not known whether DK-35C (421) possesses a greater capability than that of other carbapenems to penetrate the blood-brain barrier.

Another interesting broad-spectrum compound is DA-1131 (422) (740–744) that, similarly to panipenem [(**275**), marketed by Sankyo in combination with betamipron (366) under the trade name Carbenin], needs to be coadministered with a nephroprotector to avoid renal impairment caused by drug accumulation in the renal cortex (742).



(422) DA-1131

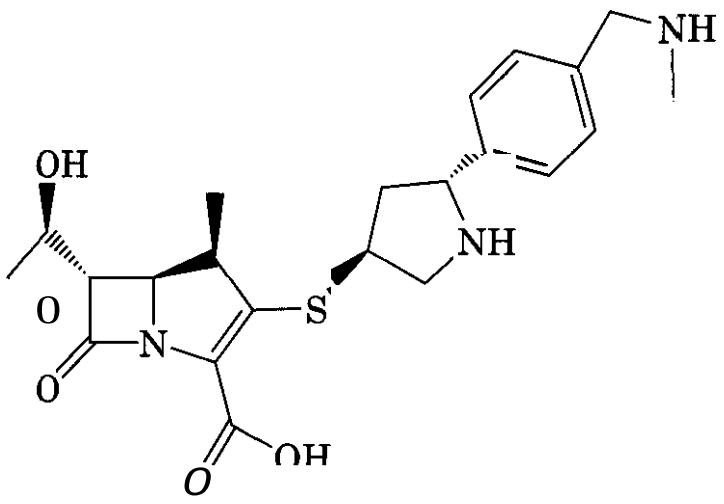
Shionogi has reported S-4661 (**423**) (745, 746), a carbapenem with an antibacterial activity comparable to that of meropenem (**216**)



(423) S-4661

but with a lower frequency of antibiotic-induced resistance. Clinical trials have shown its good efficacy for urinary tract infections (UTI) and respiratory tract infections (RTI), but there was evidence also of an increase in the levels.of hepatic enzymes (GOT/GPT) (747, 748).

Other recent compounds include MK-826 (317) (Merck and Zeneca, Daloxate), a compound with a prolonged half-life and excellent activity against β-lactamase producer strains (749–752), and J-111,225 (424) (Banyu Pharmaceutical Co.) (753), which is an ultra-broad-spectrum agent including MRSA and *Pseudomonas* species.

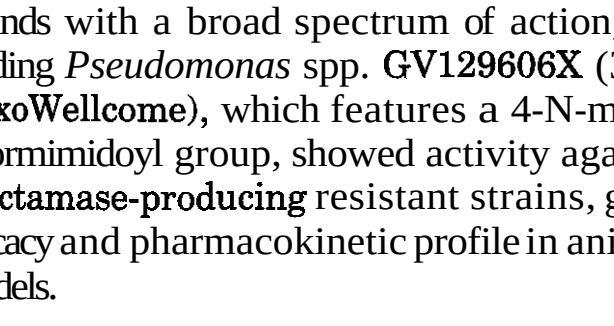


(424) J-111,225

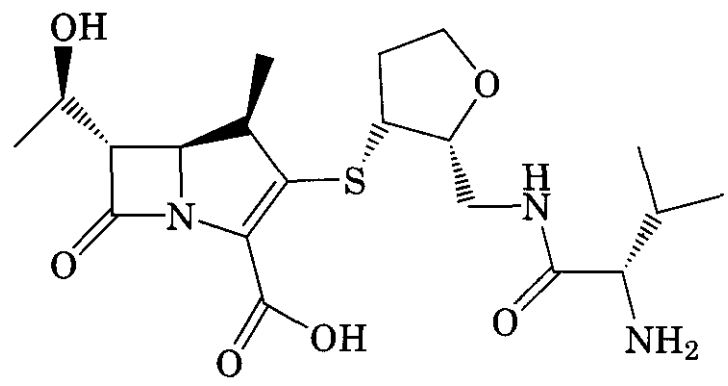
Trinems were also studied to obtain compounds with a broad spectrum of action, including *Pseudomonas* spp. GV129606X (342, GlaxoWellcome), which features a 4-N-methylformimidoyl group, showed activity against β-lactamase-producing resistant strains, good efficacy and pharmacokinetic profile in animal models.

6.3 Orally Active Carbapenems/Trinems

Pharmaceutical companies have put an intensive effort to produce orally active 1β-methyl carbapenems and trinems for the treatment of community-aquired infections. Wyeth-Ayerst has published a study in which CL-191121 (425), an aminomethyl tetrahydrofuranyl carbapenem, was reacted with amino acids to give the corresponding peptidoyl derivatives (754, 755). This work led to the identification of OCA-983 (426) (756), which is a valinyl prodrug of CL-191121 (425) with improved oral bioavailability and *in vivo* efficacy against Gram-positive and Gram-negative bacteria (757,758). OCA-983 (426) represents the first example of a peptidic prodrug of carbapenems orally absorbed by active transport.

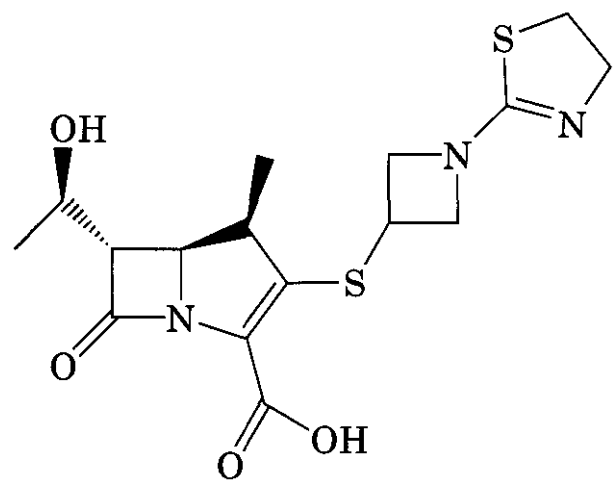


(425) CL-191121



(426) OCA-983

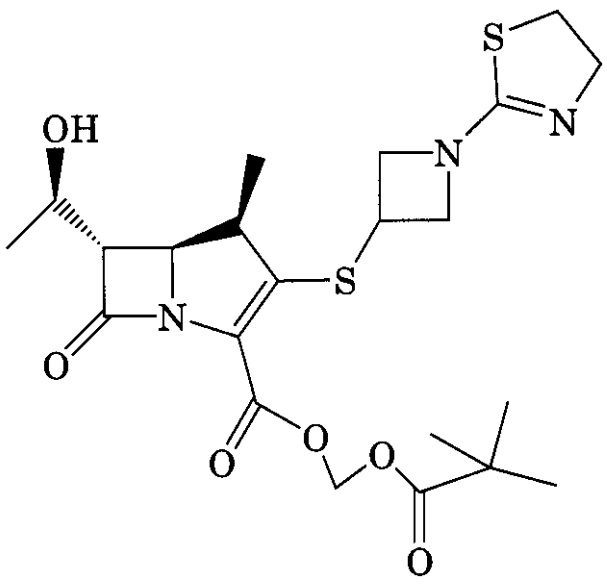
Other examples of broad-spectrum oral carbapenems for the community are reported by Lederle, with L-084 (428) (759,7601, a prodrug of LJC-11036 (427) and by Sankyo, with CS-834 (430) (761–763), a prodrug of R-95867 (429).



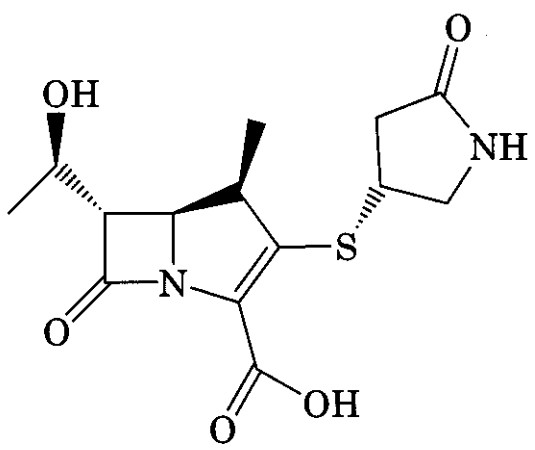
(427) LJC-11036

OCA-983 (426), L-084 (428), and CS-834 (430) showed very similar antimicrobial profiles and are suitable for the treatment of respiratory tract infections, with good stability to ESBL and broad-spectrum β-lactamases. Two major drawbacks could be envisaged for such compounds:

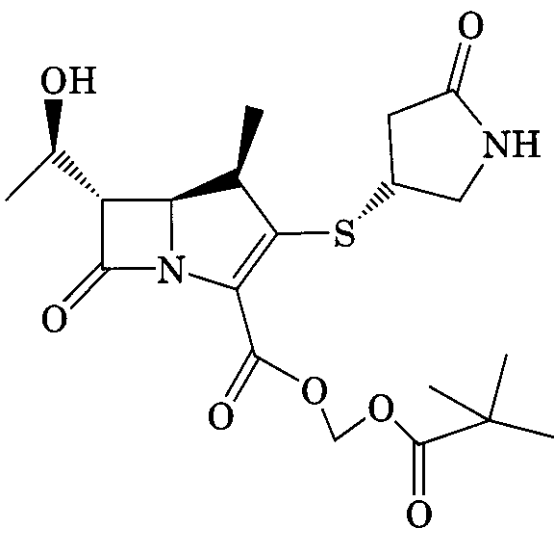
- The dosing regimen (t.i.d. for L-084 and CS-834) required maintenance of the concen-



(428) L-084



(429) R-95867



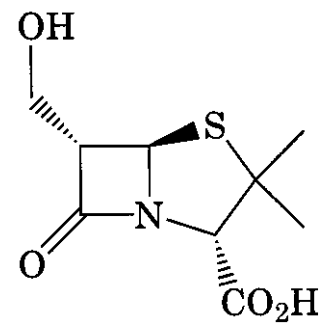
(430) CS-834

tration of the circulating compound above the MIC value for a sufficient time in the 24 h.

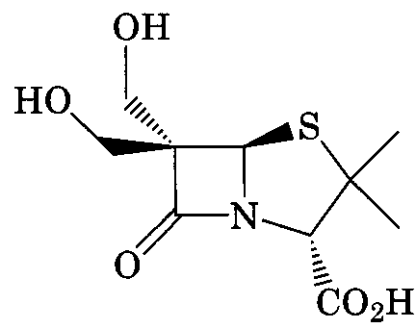
- The relatively high cost of treatment compared to that of other drugs already available in this overcrowded market.

6.4 β-Lactamase Inhibitors

Most recently, the problem of inactivation of β-lactams by β-lactamases has been carefully investigated, as it is evident from the literature (764–767). Several sulfone derivatives such as compound (**375**), obtained from chemical modification of 6-APA (**2**), have been obtained (768). In addition, mechanistic and crystallographic studies with mono and bis penicillanic acids (432,433)and TEM-1-β-lac-



(432)

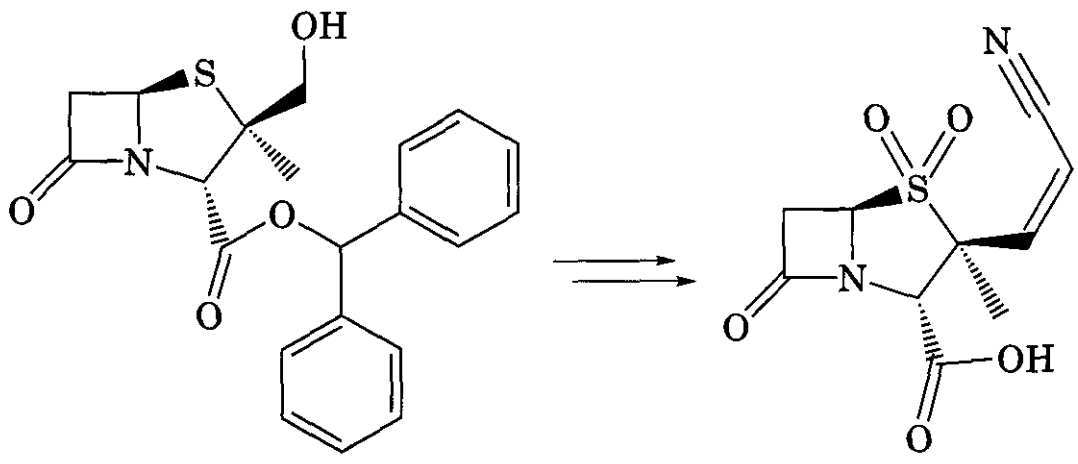


(433)

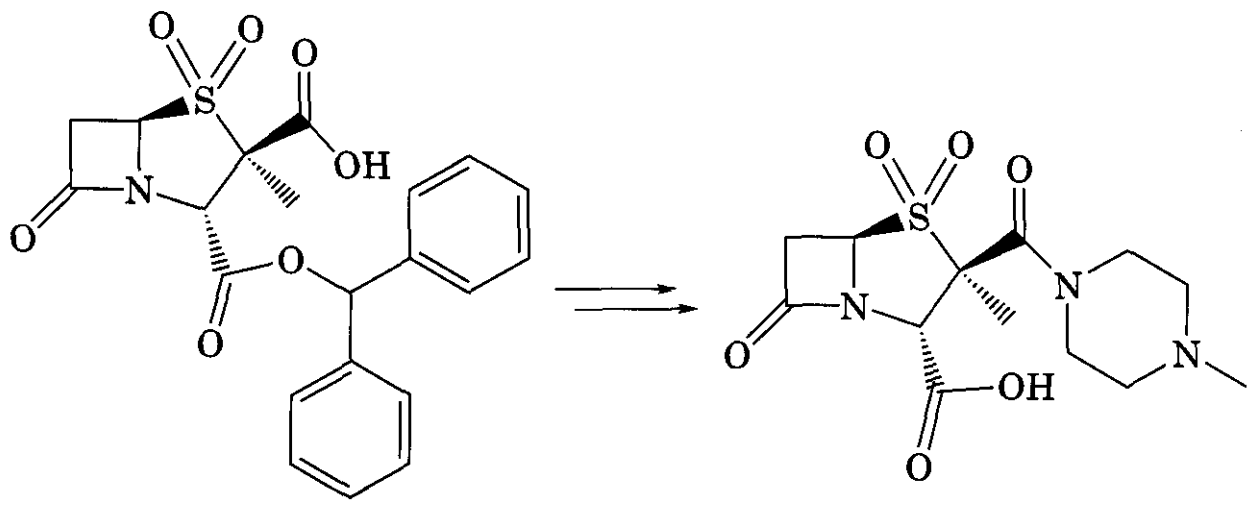
tamase from E. *coli*, have been reported (769–771). In particular, bis hydroxymethyl penicillanic acid (433) showed inhibitory activity on both class A and class C β-lactamases.

Scientists from Hoffmann-La Roche used the β-hydroxymethyl derivative (434) to prepare a series of alkenyl penam sulfones (772) that inhibited TEM and SHV, among which Ro 48–1220 (435) showed the best inhibitory properties (773), comparable to those of clavulanic acid (368) and tazobactam (**378**). Through use of a similar procedure, Taiho prepared a series of amides, starting from the carboxylic acid (**436**), among which compound (437) showed better synergy than that of tazobactam (378) in *vitro* against class C β-lactamases (774).

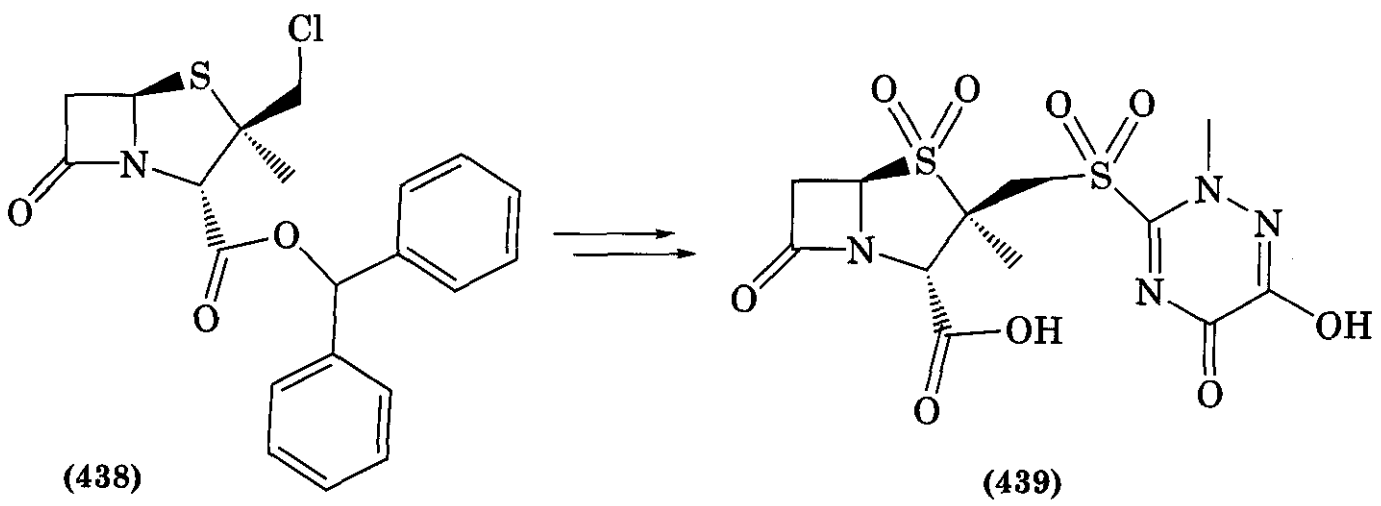
Starting from the chloro methyl (**438**), Taiho also reported the synthesis of an inter-



(434) (435) Ro 48-1220



(436) (437)

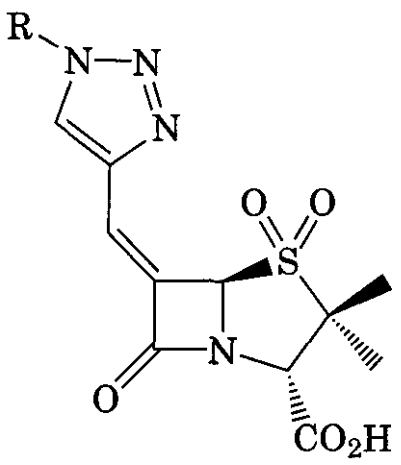


(438) (439)

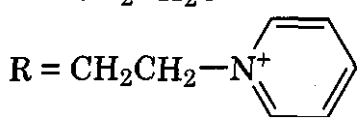
esting compound (439) bearing the same heteroaromatic moiety of ceftriaxone. This compound possesses an excellent *in vitro* activity against cephalosporinases (775).

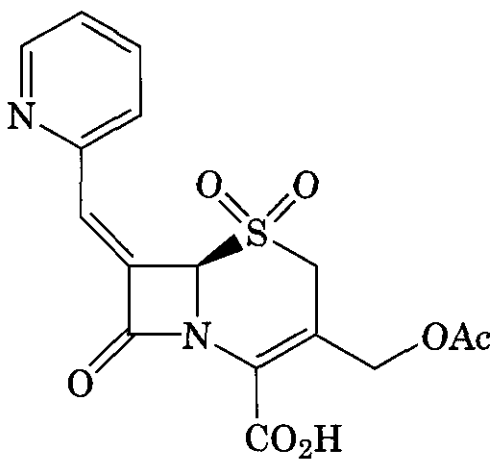
Introduction of a double bond conjugated with the carbonyl groups of the β-lactam ring (alkylidene derivatives) has been extensively studied in several classes of β-lactams other than penems (see Section 5.6.4), producing potent inhibitors. (*Z*)-Triazol-4-yl-methylene penicillanic acid sulfones (440–442) showed very good inhibitory activity against TEM p-lactamases. Compounds (**440–442**) were 2–3 times more active than tazobactam (378) against cephalosporinases from ***Enterobacter cloacae*** (776).

Inhibition of class C β-lactamases has been observed for 7-alkylidene cephalosporanic acid sulfones such as compound (**443**). This gave excellent inhibitory activity against ***Enterobacter cloacae*** P99 and ***Enterobacter cloacae*** SC-12368 (722).



(440) R = CH_3
(441) R = CH_2CH_2OH





(443)

7 CURRENT TRENDS DRIVING INDUSTRY

Almost 300 antibacterial products are available on the world market, but successful treatment of bacterial disease is becoming increasingly problematic as the number of elderly and immunocompromised patients increases, the pathogens undergo changes, and resistance to current agents become more widespread. These factors are driving the pharmaceutical industry to develop new and more powerful agents from existing classes of antibacterial drugs and to reevaluate their approaches to antibacterial drug discovery. Bacterial genomics, supported by fundamental research on bacterial physiology, environmental adaptation, and host-pathogen interactions are expected to supply the drug targets of the future. Although products of this research are some way off, new and improved agents continue to emerge from established screening and chemical modification programs.

The antibacterial market is increasingly well supplied with effective oral and parenteral agents that can provide treatment for the majority of infectious diseases. The criteria of novelty and competitive advantage for new agents therefore become increasingly difficult to attain. The relentless spread of antibacterial resistance to agents that were initially thought to have overcome all resistance problems is now offering necessary scope for novelty and innovation. The message coming from many opinion leaders in the medical profession is also one of strong encouragement for the pharmaceutical industry to pursue the quest for new and better antibacterials. Drug resistance is seen as a serious potential threat to the continuing effectiveness of current antibacterial agents, and a new compound that can address an important resistance problem is likely to find a place in the crowded market.

The most problematic forms of resistance being encountered are penicillin resistance in *S.* pneumoniae, glycopeptide resistance in enterococci, methicillin and multidrug resistance in Staphylococci, extended-spectrum β-lactamase resistance in MTB, and metronidazole resistance in Helicobacterpylori.

Other potential areas for innovation relate to the changing pattern of infectious disease brought about by the AIDS epidemic and the increasing frequency of organ transplantation, implanted prosthetic devices, and other developments in invasive medical procedures, together with a demographic shift to a more elderly and institutionalized population in the developed world. Antibiotics are now increasingly required to be effective in patients who are immunocompromised or debilitated through age-underlying disease.

7.1 Inhibitors of Protein Export in Bacteria

Signal peptidase enzymes represent a novel class of antibacterial targets (777) involved in the export of proteins across the cytoplasmic membrane in bacteria (778, 779). Proteins that are processed through this pathway are synthesized as preproteins with an extra domain, the signal sequence, attached to the amino terminus (780). This signal sequence allows the preprotein to cross the cytoplasmic

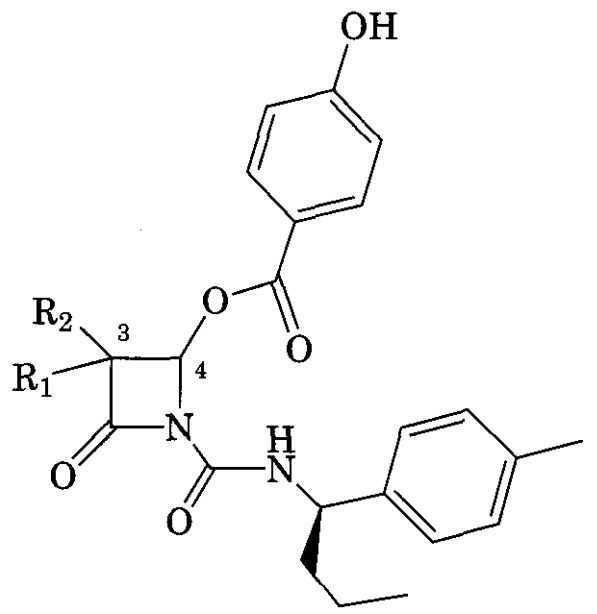
Current medical needs

	Organism	Infection	Resistance
Nosocomial	MRSA/E	Bacteremia, Pneumonia Sugical wound infections, Endocarditis	Quinolones, β-Lactams, Rifampin, Tetracyclines
	Enterococci	Bacteremia, UTI, Surgical wound infections	Vancomycin, Quinolones Aminoglycosides; β-Lactams
	Pseudomonas spp	Bacteremia, Pneumonia UTI, Burns	Quinolones, Aminoglycosides Tetracyclines, β-Lactams
Community	*S* pneumoniae	Meningitis, Pneumonia, Arthritis, Bacteremia	Macrolides, β-Lactams
Emerging pathogens	Stenotrophomonas malt. Burkholderia cepacia	Cystic fibrosis, burns	Carbapenems
	Mycobacterium tuberc.	Tuberculosis	lsonyazide

membrane by means of a multiprotein complex, the Sec proteins. When it is outside, the signal peptidase enzyme **(781)** cleaves the signal sequence, thus liberating the protein, which is now able to fold into its active conformation. It is believed that by inhibiting this pathway, the proteins remain functionally inactive and eventually should accumulate in the cytoplasmic membrane, compromising its integrity and leading to cell death. A weak inhibition of signal peptidases was obtained with polysubstituted azetidinones **(444–446)** (782), in which was observed a stereochemical preference for (4*S*) isomers.

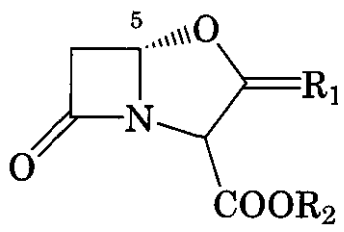
A screening of serine protease inhibitors against the signal peptidase from E. coli LP1 allowed identification of several classes of active bicyclic β-lactams. Esters of clavulanic acid **(447)** confirmed this preference for (5*S*) stereochemistry and were more active than the corresponding thioclavam analogs (**448**). Oxidation to the corresponding sulphone regained the activity in the decarboxylated series (R_2 = H).

The best compounds were obtained in the 5(*S*) penem series, allowing identification of benzyl ester (**449**), which has the opposite stereochemistry to that required for inhibition of bacterial PBPs and showed an IC_{50} value of **10** μ*M* **(783,784).** The hit compound was studied in depth and led to the synthesis of the penem

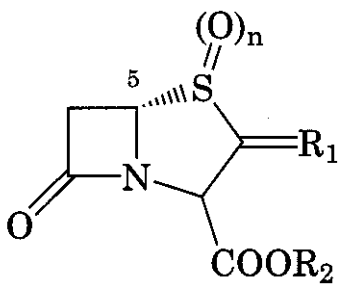


(444) (4S) R_1 = Et, R_2 = Et
(445) (4S) $R_1 = CH_3$, $R_2 = CH_3$
(446) (4R) $R_1 = CH_3$, $R_2 = CH_3$

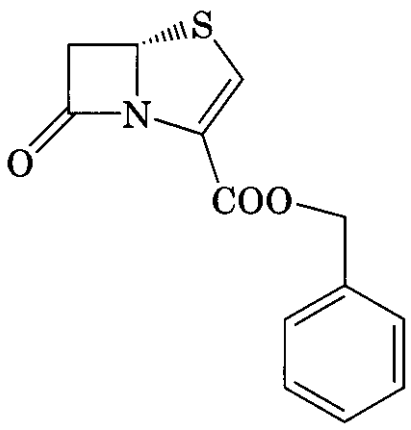
allyl ester derivative (**450**), which showed complete inhibition of processing in a permeable strain of E. coli, with an IC_{50} value of 0.07 μ*M*. Although the activity could not be man-



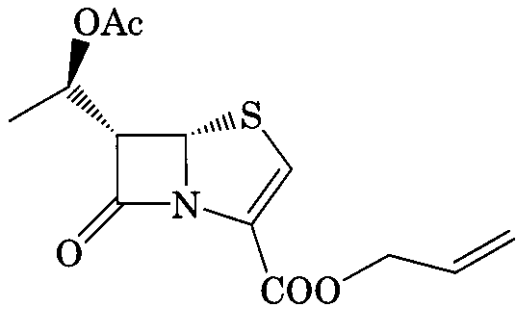
(447)



(448)



(449)



(450)

tained in standard and clinical strains, this work has highlighted a new valuable target for the discovery of novel β-lactam antibiotics (785).

8 WEB SITE ADDRESSES AND RECOMMENDED READING FOR FURTHER INFORMATION

If someone just needs quick information related to the p-lactam antibiotics and their use, nowadays it is sufficient to use a popular research engine searching for "lactam antibiotics" and retrieve thousands of Web pages. Here are listed only a few of them that contain interesting and precise specific information:

- The links http://www.microbe.org, http://www.idlinx.com, http://www.bact.wisc.edu/bact330/330Lecturetopics.htm, http://www.ches.siu.edu/fix/medmicro/index.htm can be consulted to get an overview of the infection disease treatments, bacteria morphology, mechanism of action of antibiotics, and mechanism of resistance to the current antibacterial agents.
- The link http://wizard.pharm.wayne.edu/medchem/betalactam.htm leads to the Web page entitled: Chemistry of Beta Lactam Antibiotics PHA 421—Infectious Disease Module, a Medicinal Chemistry Tutorial on Beta Lactam Antibiotics.
- An interesting course of Chemotherapy can be found at the following address: http://www.vet.purdue.edu/depts/bms/courses/chmrx/bms45 96.htm, which leads to Drug Groups, in which the major antibacterial classes are extensively discussed.
- At http://www.grunenthal.com/knowledgeBase/kb_start.htm there is the Grünenthal Knowledge Base, a library of Knowledge-Modules in which are reported the antibiotics, including Beta-Lactam Antibiotics. A fast entry-level reference guide.
- A very good microbiology textbook can be found at the following link: http://www.bact.wisc.edu/microtextbook/index.html, in which, by clicking on http://www.bact.wisc.edu/microtextbook/ControlGrowth/antibiotic.html, a chapter on antibiotics is reported.

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CHAPTER FIFTEEN

Tetracycline, Aminoglycoside, Macrolide, and Miscellaneous Antibiotics

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Contents

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1 INTRODUCTION

Infections caused by bacteria are among the leading causes of death worldwide and antibiotics have made a dramatic impact in our capacity to intervene in these diseases. The tetracycline, aminoglycoside, and macrolide antibiotics were among the first classes of antibiotics discovered and have been mainstays of antibacterial therapy for half a century. These antibiotics all target the bacterial ribosome and interfere in the process of translation of the messenger RNA into protein and thus block a fundamental process in bacterial metabolism. For the most part, these compounds have little effect on the translational machinery of eukaryotic organisms, and thus this differentiation is the basis for the selectivity and consequent lower toxicity of these natural products. These antibiotics continue to be essential for the treatment of bacterial infections and in many cases are drugs of choice.

The bacterial ribosome consists of both a large (50S) and small (30S) subunits that come together to form the intact, 70S ribosome. Each subunit consists of both protein and RNA (rRNA) components: 31 proteins, 23S and 5S rRNAs for the 50S subunit, and 21 proteins and 16S rRNA for the small subunit. Although a complete description of the translational process is beyond the scope of this chapter, a brief outline of the key steps is presented in Fig. 15.1. The active site of the ribosome is composed of three subsites: the aminoacyl site (A), the peptidyltransfer site (P), and the exit site (E). Briefly, translation is initiated with the recognition and binding of messenger RNA (mRNA), which encodes a specific protein, to the 30S subunit in the presence of an initiator transfer RNA (tRNA) and of initiation factor 2 (IF2). Binding of the initiator tRNA in the P-site is followed by binding of the 50S subunit, generating a translation competent ribosome particle. The elongation cycle then consists of rounds of presentation of specific tRNAs charged with their cognate amino acids by elongation factor Tu and binding of these aminoacyl-tRNAs to the A-site with synchronized hydrolysis of GTP (Step 1, Fig. 15.1). Once in place on the ribosome, the aminoacyl-tRNA in the A-site participates in peptide bond formation by nucleophilic attack of the amino acid a-amino group at the activated carboxyl group of the growing peptide chain linked to the tRNA in the P-site (Step 2, Fig. 15.1). The result is the elongation of the peptide by one amino acid residue in the A-site followed subsequently by a translocation step consisting of a shift in the mRNA register in an elongation factor G–dependent manner (Step 3, Fig. 15.1). This includes binding of the now uncharged tRNA in the E-site followed by its ejection, and binding of the peptidyl-tRNA to the P-site, leaving the A-site

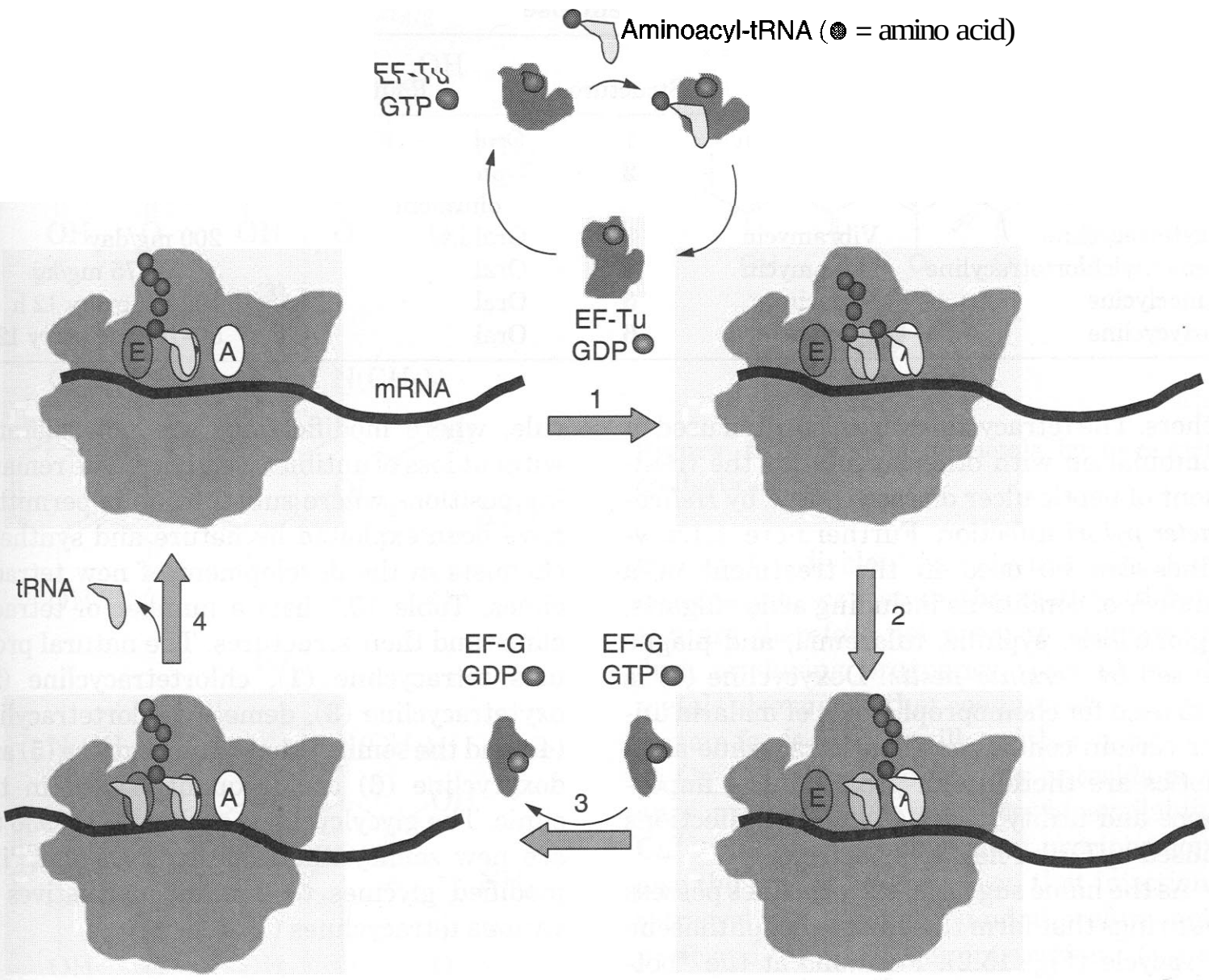


Figure 15.1. The bacterial protein synthesis elongation cycle. Step 1: Recognition and binding of the cognate aminoacyl-tRNA to the anticodon in the A-site. Step 2: Peptidyl transfer resulting in elongation of the peptide by one amino acid residue. Step 3: Translocation of the peptidyl-tRNA from the A-site to the P-site with concomitant movement of the uncharged tRNA to the E-site. Step 4: Exit of the uncharged tRNA completes the cycle. See color insert.

free again to bind an appropriate aminoacyl-tRNA (Step 4, Fig. 15.1). The complexities of the binding and enzymatic events as well as the multitude of proteins and RNAs involved in the translation process have been fertile ground for the evolution of antimicrobial agents, and all of the antibiotics discussed in this chapter exert an impact directly on critical aspects of translation.

2 TETRACYCLINES

2.1 Clinical Use of Tetracycline Antibiotics and Currently Used Drugs

The tetracycline antibiotics (see Table 15.1) were first introduced over 50 years ago as the first of the broad-spectrum antibiotics, efficacious against aerobic and anaerobic Gram-positive and Gram-negative bacteria. They are generally orally administered and have found extensive use in the treatment of infectious diseases and continue to be widely used, but are now being supplanted by other agents such as the quinolones. Nonetheless, the tetracyclines remain first-line drugs in the treatment of infections caused by pathogens of the family Rickettsiae (causative agents of Rocky Mountain spotted fever, typhus, Q fever, ehrlichiosis), Chlamydia pneumoniae, *Mycoplasma* pneumoniae, Chlamydia trachomatis, *Borrelia* burgdorferi (Lyme disease), members of the genus Brucella that cause brucellosis, Calymmatobacterium granulomatis (granuloma inguinale), Vibrio cholera (cholera), and

Table 15.1 Tetracycline Antibiotics in Current Use

Generic Name	Trade Name	Structure	Route	Dose
Tetracycline	Achromycin	1	Oral	250 mg, 4 times daily
Chlortetracycline	Aureomycin	2	Ophthalmic or topical ointment	
Oxytetracycline	Vibramycin	3	Oral i.v.	200 mg/day
Demethylchlortetracyline	Declomycin	4	Oral	3.25–3.75 mg/kg
Minoclycine	Minocin	5	Oral	100 mg every 12 h
Doxycycline	Doxycin	6	Oral	50–100 mg every 12 h

others. The tetracyclines are also often used in combination with other agents for the treatment of peptic ulcer disease caused by *Helicobacter* pylori infection. Furthermore, tetracyclines can be used in the treatment of a number of conditions including acne vulgaris, legionellosis, syphilis, tularemia, and plague caused by Yersinia *pestis*. Doxycycline (6) is also used for chemoprophylaxis of malaria under certain conditions. The tetracycline antibiotics are therefore of great clinical importance and utility, especially aginst infections caused by Gram-negative bacteria.

As the name suggests, tetracyclines possess four rings that form the minimal naphthacene tetracycle (Fig. 15.2). Positions at the "bottom" of the molecule (10, 11, 1) and most of ring A (positions 2, 3, and 4) represent the invariant pharmacophore region of the molecule, where modifications are not tolerated without loss of antibiotic activity. The remaining positions where substitution is permitted have been exploited by nature and synthetic chemists in the development of new tetracyclines. Table 15.1 lists a number of tetracyclines and their structures. The natural products tetracycline (1), chlortetracycline (2), oxytetracycline (3), demethylchlortetracyline (4), and the semisynthetic minocycline (5) and doxycycline (6) are frequently used in the clinic. The glycylcyclines such as GAR-936 (7) are new semisynthetic derivatives that link modified glycines to 9-amino derivatives of various tetracyclines (1, 2).

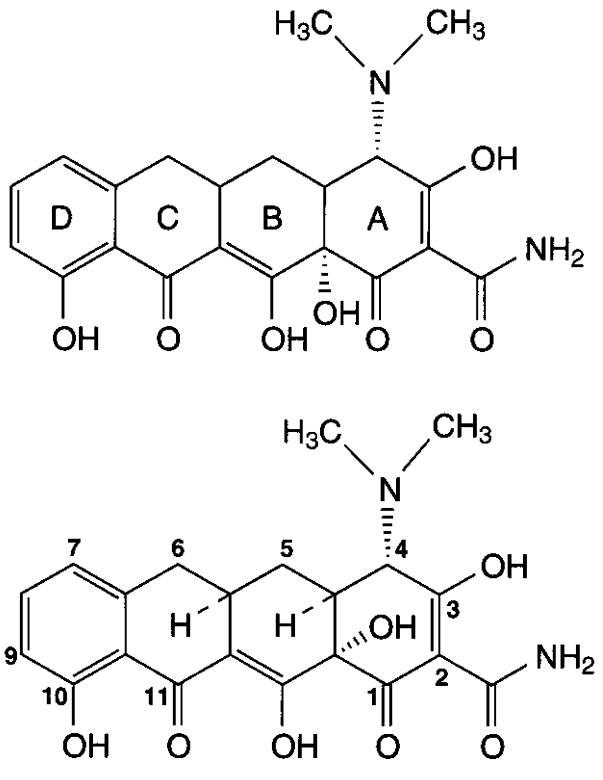
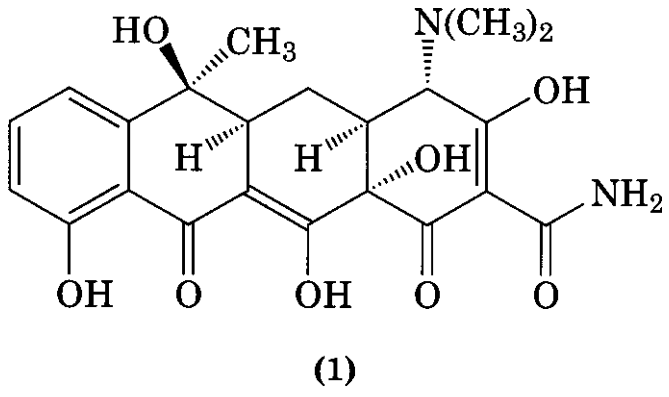


Figure 15.2. Tetracycline pharmacophore and numbering.



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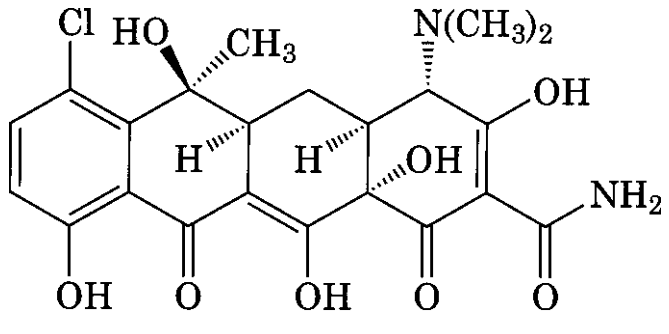
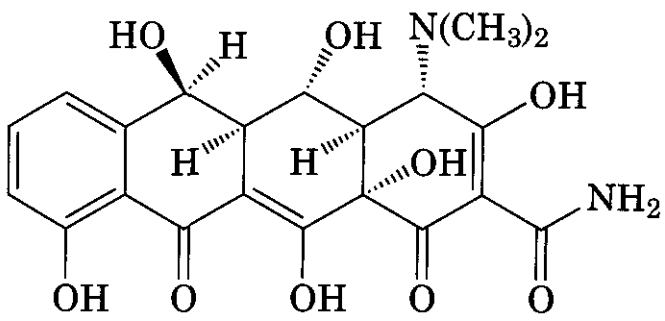


(2)

The tetracyclines avidly bind metals through the hydroxyl and carbonyl groups of



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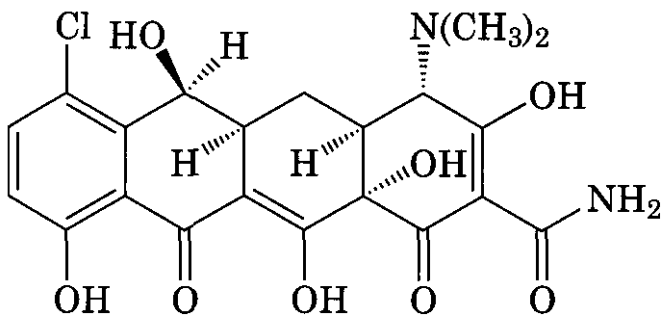

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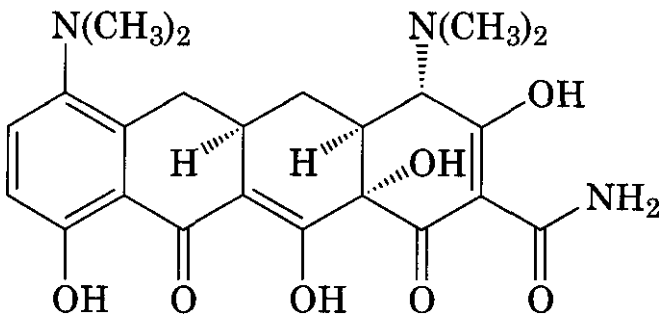

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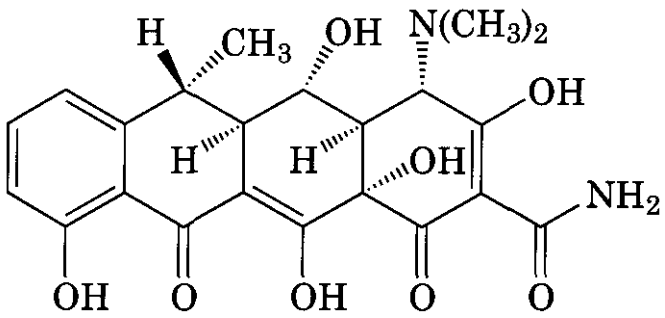


(6)

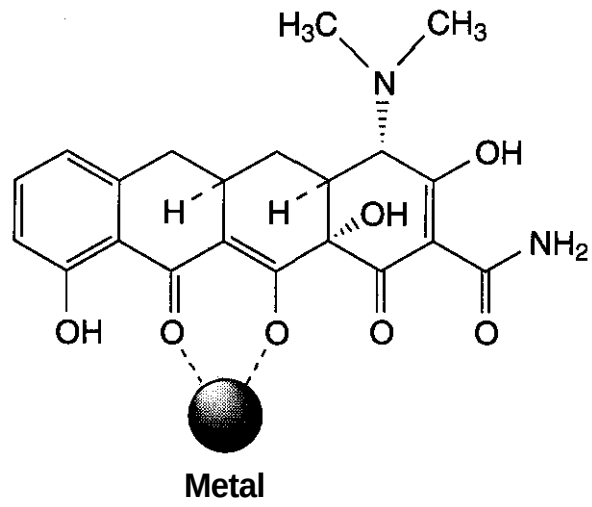


Figure 15.3. Binding of metals by tetracycline antibiotics.

the B and C rings (Fig. **15.3)** and this appears to be the biologically active form of the molecules (see Section **2.3).**

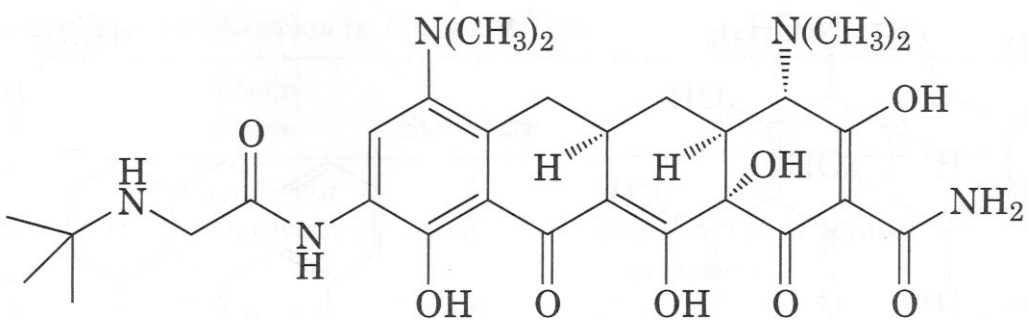
2.2 Side Effects, Toxicity, and Contraindications of Tetracycline Antibiotics

The tetracyclines are in general quite safe and well tolerated. They are all associated with photosensitivity, and patients should avoid direct exposure to the **sun. As** a result of their affinity for divalent metals including Ca^{2+}, tetracyclines can stain the teeth and bones and are therefore not given to younger children or during pregnancy. The affinity for metals also means that coadministration with certain foods such as milk or other agents such as Ca^{2+}- or Mg^{2+}-containing antacids is not appropriate because it reduces bioavailability. Ca^{2+} binding and subsequent precipitation is also thought to be the reason that injection of tetracyclines is painful and therefore not a preferred route of administration. Tetracyclines can also affect the usefulness of oral contraceptives.

Side effects tend to be minor, including dizziness or lightheadedness for minocycline, some gastrointestinal discomfort, and candidal overgrowth, which is common to virtually all antibiotics, and under rare conditions esophageal ulceration. High levels of tetracycline have been associated with hepatotoxicity, which is exacerbated by preexisting renal impairment or coadministration with other hepatotoxic agents.

2.3 Pharmacology and Mode of Action of the Tetracycline Antibiotics

Tetracyclines are readily absorbed in the gastrointestinal tract and are subsequently widely distributed in most tissues. These antibiotics also cross the placenta and are present in breast milk and therefore are not recommended for pregnant or lactating women. Most tetracycline antibiotics are eliminated through the kidney and are therefore not recommended for patients with renal problems



(7)

because it may lead to toxicity. The exception is doxycycline (**6**), which is excreted through the bile. Serum half-lives are long (6 to >20 h, depending on the agents) and thus once or twice daily dosing is recommended on the order of 100 mg per dose (Table 15.1). Metabolism of these antibiotics is minimal.

The tetracycline antibiotics in current clinical use are generally bacteriostatic, inhibiting cell growth but not actually killing the cell. Thus, if the antibiotic is removed from the medium, the cells recover and resume growth. Tetracycline freely passes through lipid bilayers and enters the cell in a diffusion-controlled manner and no transport proteins appear to be necessary (3, 4). The mechanism of antibiotic action involves binding to the bacterial ribosome and arresting translation. Various methods have determined (1) that the 30S subunit is the preferred site of binding and (2) that there is one high affinity binding site, located in the A-site of the ribosome, and several lower affinity sites. The crystal structure of tetracycline bound to the 30S subunit of the *Thermus thermophilus* 30S subunit has been determined to 3.4 Å (5). In this structure, two binding sites for the antibiotic were determined, one in the A-site as predicted and another in the body of the ribosome. The first site is likely the more clinically relevant site, in that it agrees well with several decades of binding and biochemical data describing the tetracycline–ribosome interaction. The key features of the binding of tetracycline to the A-site are shown in Fig. 15.4. All of the interactions between the antibiotic and the ribosome occur with the 16S rRNA, and no contacts with proteins are observed. Tetracycline binds in a 20 × 7-Å pocket above the A-site binding pocket for the aminoacyl tRNA. As predicted by structure–activity studies, all four rings of tetracycline participate in binding to the ribosome and, in particular, Ring D stacks with the pyrimidine ring of C1054. The entire pharmocophore region of the molecule (see Fig. 15.2) is involved in specific interactions with the 16S rRNA. Not surprisingly, a Mg^{2+} ion is chelated by the antibiotic and the rRNA phosphate backbone as predicted by the affinity of tetracycline for divalent ions and the requirement for Mg^{2+} ion in tetracycline binding. This Mg^{2+} is also present in the structure of the ribosome in the absence of the antibiotic and may represent a key conserved binding element. The tetracycline-binding region is poorly conserved between eukaryotes and bacteria and helps to explain the low toxicity and specificity of these antibiotics.

Modeling of a tRNA molecule in the structure of the tetracycline–30s complex reveals a steric clash between the tRNA and the antibiotic. Furthermore, because tetracycline binds on the opposite side of the codon–anticodon binding pocket, it is possible that tetracycline does permit presentation of the aminoacyl-tRNA by EF-Tu, which would trigger GTP hydrolysis (see Fig. 15.1). Therefore, tetracyclines may act in two ways, first by preventing occupancy of the A-site by the aminoacyl-tRNA and thus arresting translation, and second in a catalytic fashion, depleting GTP stores in the cell.

2.4 History and Biosynthesis of the Tetracycline Antibiotics

The first tetracycline antibiotic to be isolated was chlortetracycline (2) from cultures of *Streptomyces aureofaciens* in 1948, which was followed in the same year by oxytetracycline (3) from *Streptomyces rimosus*. Chemical deoxygenation of oxytetracycline at C6 yielded doxycycline (6); minocycline (5) was prepared

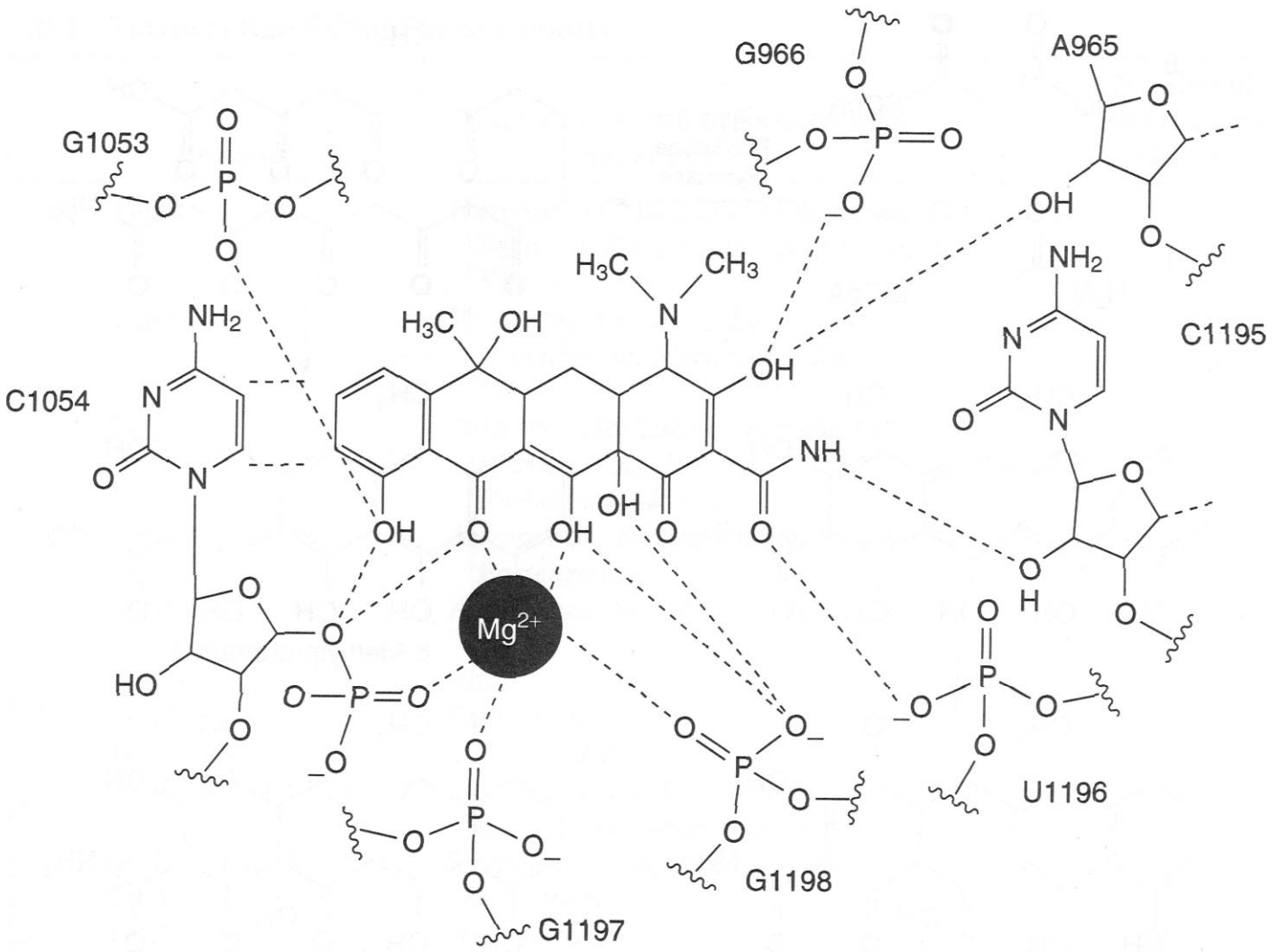


Figure 15.4. Interaction of tetracycline with the 30S ribosomal subunit (redrawn from Ref. 5). Base numbers are from E. *coli*.

from demethylchlortetracycline in the mid 1960s. The synthesis of the newer glycylcyclines was first described in the early 1990s.

The tetracycline antibiotics are polyketide natural products and therefore derived from the sequential linkage of acetyl units that form the core polyketide. The biosynthetic clusters encoding the genes necessary for the biosynthesis of chlortetracycline and oxytetracycline have been cloned and sequenced (6–8). From these gene sequences and chemical intuition, a plausible biosynthetic pathway can be constructed, beginning with the starter unit malonamyl-CoA, which provides the requisite amide at C2 (Fig. 15.5). Eight successive condensations with malonyl CoA, each providing a C2 unit, generate the linear polyketide. This is followed by cyclization to yield 6-methylpretetramid [possibly by the *otcD1* gene product of S. *rimosus* (9)], hydroxylation, oxidation, and reductive transamination at C4a as well as hydroxylation at C12a to generate 4-amino-anhydrotetracyline. *S*-Adenosylmethionine–dependent methylation followed by two successive hydroxylation events and a reduction generates oxytetracycline. The *otcC* gene product of S. *aureofaciens* has been shown to be the anhydrotetracyline oxidase, hydroxylating anhydrotetracycline at position 6 (10). The gene encoding the enzyme required for chlorination of tetracycline at position 7 to give chlortetracycline has also been cloned (11). The precise gene products for the remainder of the biosynthetic steps have not yet been fully characterized and therefore the details of this pathway remain probable, but speculative.

2.5 Tetracycline Resistance

Tetracycline antibiotics are no longer used for empirical treatment of many Gram-negative and Gram-positive infections as a result of the dissemination and prevalence of resistance, which has played an important role in decreased use of these otherwise potent antibiotics over the past years. Clinically relevant resistance occurs primarily by two mechanisms: active efflux and ribosomal protection,

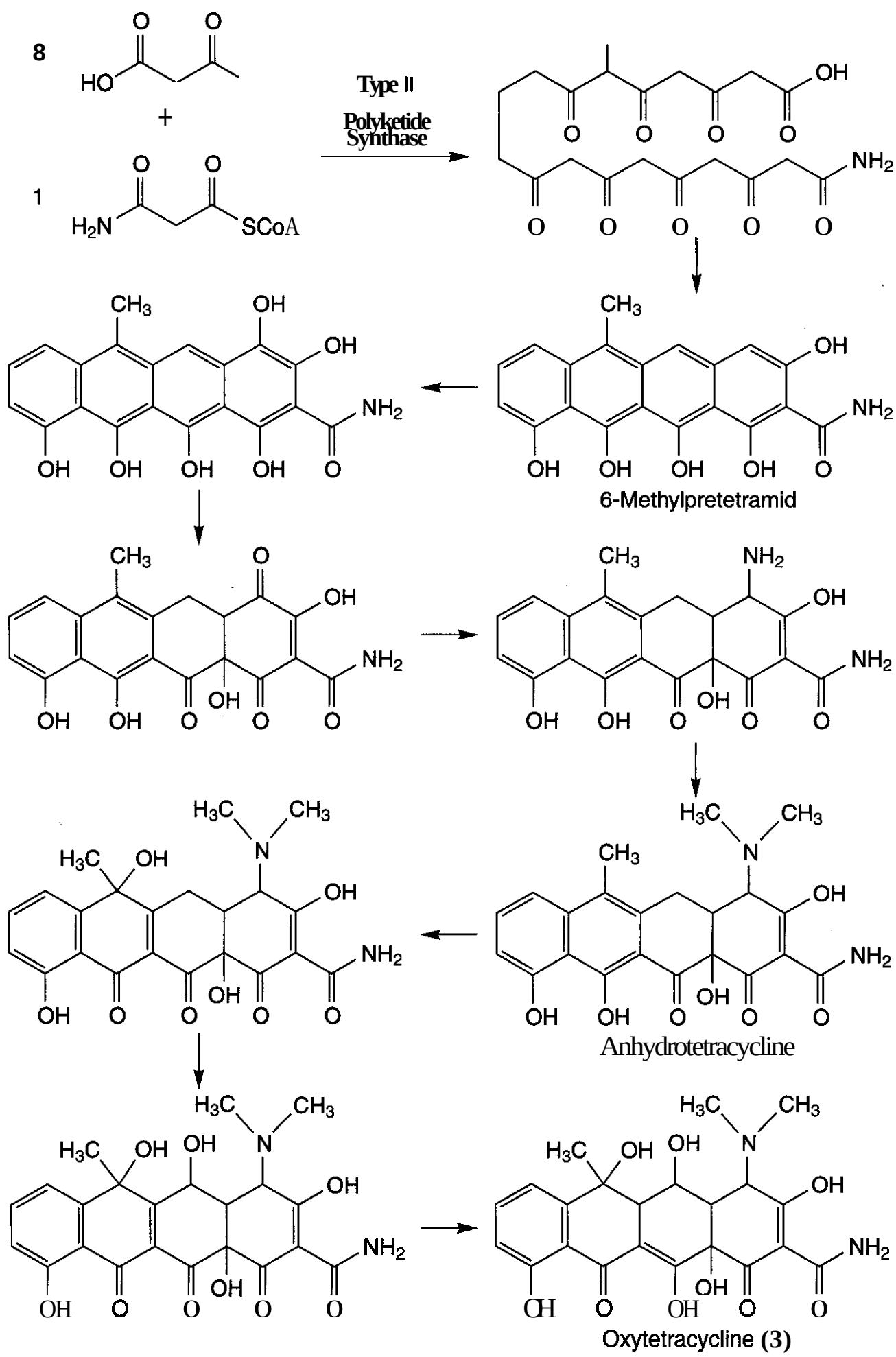


Figure 15.5. Predicted oxytetracycline biosynthetic pathway.

although enzymatic modification and other unknown mechanisms have been documented (see reviews in Refs. 12–14). Ribosomal mutations, a major source of resistance for other inhibitors of translation, are rarely implicated in tetracycline resistance, though mutation at position 1058 of the 16S rRNA (*Escherichia* coli numbering) has been associated with tet-

Table 15.2 Tetracycline Efflux Determinants

Group	Proteins	Bacterial Genera Associated with Resistance Type	Number of Transmembrane Helices
1	Tet(A)	Numerous including Pseudomonas, Serratia, Escherichia, Klebsiella, Vibrio	12
	Tet(B)	Numerous including Escherichia, Providencia, Moraxella, Shigella, Vibrio	
	Tet(C)	Numerous including Salmonella, Proteus, Pseudomonas, Escherichia, Vibrio	
	Tet(D)	Aeromonas, Yersinia, Enterobacter, Pasteurella	
	Tet(E)	Aeromonas, Serratia, Escherichia, Vibrio	
	Tet(G)	Vibrio	
	Tet(H)	Pasteurella	
	Tet(Z)	Corynebacterium	
2	Tet(K) and Tet(L)	Staphylococcus, Clostridium, Enterococcus, Streptococcus	14
3	OtrB and Tcr3	Streptomyces, Mycobacterium	14
4	TetA(P)	Clostridium	12
5	Tet(V)	Mycobacterium	10–11
6	TetAB	Corynebacterium	ATP binding cassette transporter

racycline resistance in Propionibacterium *acnes* (15, 16). The efflux proteins all appear to be members of the major facilitator superfamily of efflux proteins that export small molecules in a proton-dependent fashion (17). They are integral membrane proteins of approximate molecular mass of 46 kDa and can be subdivided into six groups based on amino acid sequence similarity (Table 15.2) (18).

At present there is no available three-dimensional (3D) structure available for a tetracycline efflux protein, but there has been a significant amount of biochemical research describing the topology and antibiotic specificity of some of these proteins. The preponderance of work has come from the Yamaguchi laboratory describing the group 1 protein Tet(A) (19–22). The work from this lab has suggested that the 12 helices form a water-filled channel flanked by transmembrane helices TM1, TM2, TM4, TM5, TM8, TM10, and TM11 with a flexible intracellular loop between TM6 and TM7. This channel presumably facilitates the export of tetracycline from inside the cell, resulting in resistance.

The second prevalent mechanism of tetracycline resistance is ribosomal protection (23). There are a number of genes encoding these proteins of approximately 70 kDa (Table 15.3), and the Tet(M) and Tet(O) proteins have been the best studied (24, 25). Amino acid sequence analysis shows similarity of these proteins with elongation factors G and Tu, especially in the N-terminal GTP binding domain. GTP binding and perhaps hydrolysis are necessary for binding of these tetracycline resistance determinants to the ribosome, which triggers release of tetracycline from the A-site. In the presence of nonhydrolyzable GTP analogs, stable complexes between this class of resistance protein and the ribosome can be formed. Cryoelectron microscopic studies have been used to study the Tet(O)–ribosome complex, and the 3D structure was determined to be 16 Å (26). This structure demonstrates that Tet(O) has a shape similar to that of EF-G and binds analogously to the A-site region of the ribosome. However, unlike EF-G, which causes a significant structural change in the ribosome (27), binding of Tet(O) does not sig-

Table 15.3 Ribosomal Protection Tetracycline Resistance Determinants

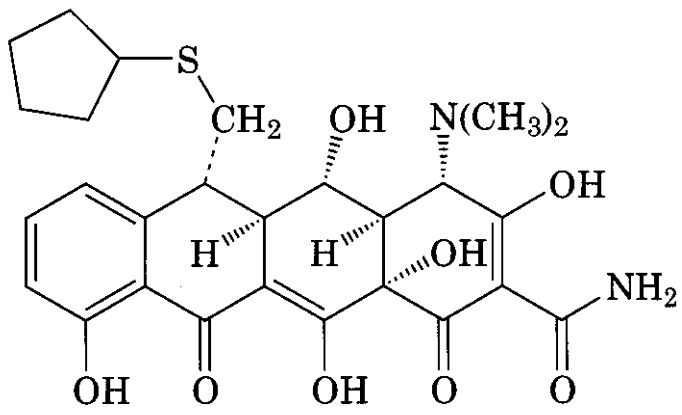
Protein	*Bacterial Genera Associated with Resistance Type*
Tet(M)	*Numerous including Enterococcus, Streptococcus, Staphylococcus, Neisseria*
Tet(O)	*Campylobacter, Enterococcus, Lactobacillus*
Tet(Q)	*Bacteroides, Streptococcus, Lactobacillus*
Tet(S)	*Enterococcus, Lactococcus, Listeria*
Tet(T)	*Streptococcus*
Tet(W)	*Butyrivibrio*
TetP(B)	*Clostridium*
Otr(A)	*Streptomyces, Mycobacterium*

nificantly affect the ribosome conformation. These studies provide a possible mechanism of action, in which Tet(O)–GTP binding decreases the affinity of tetracycline for the ribosome and subsequent GTP hydrolysis releases Tet(O), generating a translation competent ribosome.

A final mechanism of tetracycline resistance is enzymatic degradation. The only enzyme described with this activity is TetX, an 0,-dependent oxidase that paradoxically was identified in the anaerobe Bacteroides fragilis (28, 29). Under aerobic conditions in E. coli, TetX modifies tetracycline in an unknown fashion to generate inactive antibiotics. This resistance element has not yet been identified as a clinical problem.

2.6 Recent Developments in the Tetracycline Antibiotic Field

Although the use of tetracycline antibiotics has diminished over the past years, they continue to be of great importance in the treatment of infectious disease. The broad dissemination of resistance throughout both Gram-negative and Gram-positive populations has had a significant impact on their clinical utility. The glycylcyclines, tetracyclines substituted on position 9 [e.g., GAR-936 ***(7)*** (1)], do not appear to be susceptible to the common ribosome protection or efflux resistance (30). Some molecules are in advanced clinical trials for the treatment of drug-resistant infections (31, 32). Modified tetracyclines [e.g., 13-cyclopentylthio-5-hydroxy-6-deoxytetracycline (**8**)] have also been shown to block some tetracycline efflux pumps such as TetA and TetB, resulting in a reversal of antibiotic resistance (33–35).



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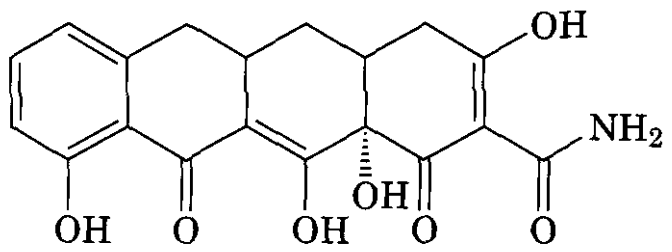
Tetracyclines and chemically modified tetracyclines have been investigated for properties other than their antimicrobial activity **(36).** For example, tetracyclines are known to have anti-inflammatory properties. These molecules have therefore been pursued as chemotherapeutic agents with promise in the treatment of inflammatory disease such as osteo- and rheumatoid arthritis. Tetracyclines exert a number of effects related to inflammation. These include the reduction of levels of mRNA encoding NO synthase (37), increasing the levels of cyclooxygenase-2 and subsequent increase in prostaglandin E2 levels (38), and the inhibition of enzymes important to connective tissue biology such as matrix metalloproteinases (MMPs) and gelatinases. There is a rich literature on the inhibition of MMPs by antimicrobial and chemically modified tetracyclines [e.g., CMT-3 (**9**)] and this property could have application in osteoarthritis (39), periodontitis (40, 41), corneal diseases (42, 43), and cancer (44).

Thus, in addition to the well-established antimicrobial activity of tetracyclines, there are several new therapeutic areas on which



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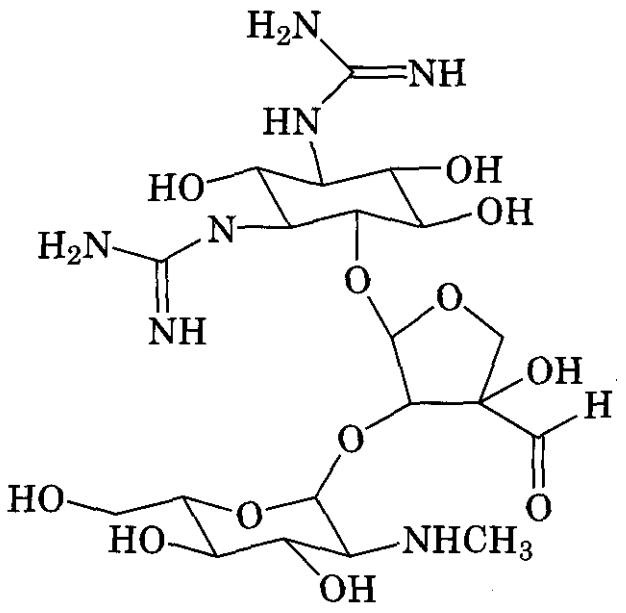
these compounds, or their derivatives, may prove to have a profound impact.

3 AMINOGLYCOSIDE ANTIBIOTICS

3.1 Clinical Use of Aminoglycoside Antibiotics and Currently Used Drugs

The aminoglycoside antibiotics find use as broad-spectrum agents for the treatment of infections caused by aerobic Gram-negative and Gram-positive bacteria including *Klebsiella pneumoniae, Pseudomonas aeruginosa, E. coli, Proteus* sp., *Serratia marcescens,* and *Staphylococci* (45). Arninoglycosides are also used in combination therapy with penicillins for the treatment of enterococcal infections. Some compounds such as paromomycin have been employed in treatment of protozoal infections **(46).** Streptomycin (10) was the first aminoglycoside isolated and the first antibiotic with potent activity against *Mycobacterium tuberculosis* and this antibiotic continues to be used to treat tuberculosis, but as a result of the development of resistance, now in combination therapy with other antibiotics (45). Streptomycin can also be used for the treatment of tularemia, plague and leprosy. The aminoglycosides are highly water soluble and poorly absorbed orally. These antibiotics are therefore primarily delivered by intramuscular injection or intravenously. Topical use of aminoglycosides also is frequently used including gentamicin (11), a complex of gentamicin C1, gentamicin C1a, and gentamicin C2, in drops to treat ophthalmic infections, neomycin (12) in topical formulation with corticosteroids, and tobramycin (13), aerosolized to treat respiratory infections.

The aminoglycoside antibiotics are more properly termed aminoglycoside-aminocyclitols because they not only incorporate aminosugars, but also six-membered aminocyclitol



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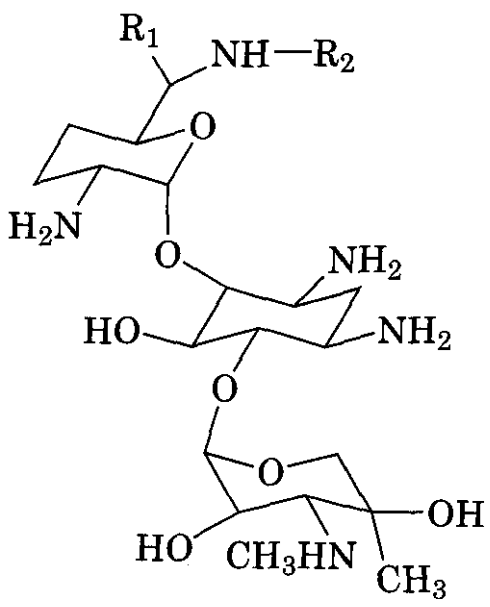
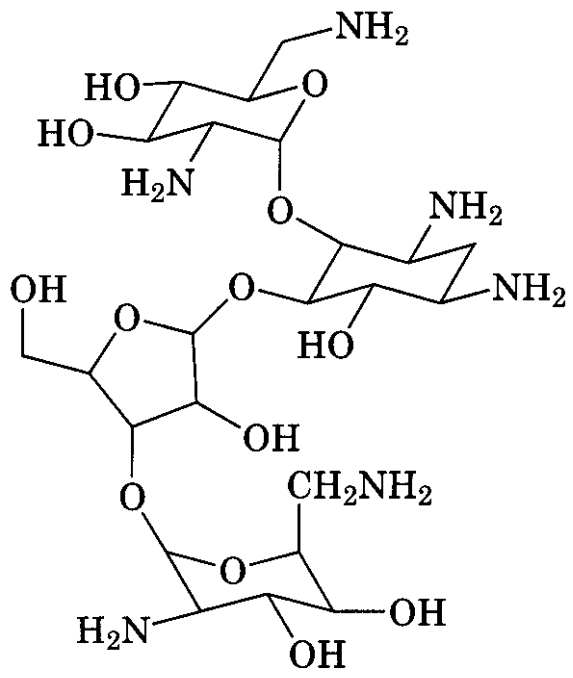


Gentamicin **(11)**
C1 R1 = CH_3 R2 = CH_3
C1a R1 = H R2 = H
C2 R1 = CH_3 R2 = H

rings, cyclic carbon rings functionalized with amino and hydroxyl groups (47). However, the name aminoglycoside is more frequently used, despite the fact that some members of the group such as spectinomycin do not have any sugar component. The clinically important aminoglycosides have been generally subdivided into two broad groups based on structure: compounds that contain a 2-deoxystreptamine aminocyclitol ring and those that do not. The 2-deoxystreptamine–containing antibiotics are further divided into two groups based on whether the aminocyclitol is substituted with amino sugars at positions 4 and 5 or **4** and **6.** The former are also characterized by the presence of a pentose at position 5 [e.g.,



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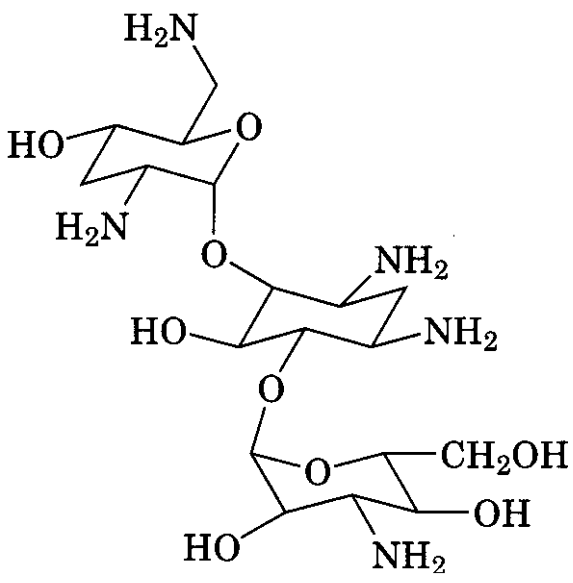


(13)

neomycin (**12**)]. The preponderance of the best-tolerated antibiotics are the **4,6-disubsti**tuted 2-deoxystreptamine compounds such as gentamicin (**11**).The second broad class of antibiotics includes the clinically used antibiotics streptomycin (**10**) and spectinomycin, which differ in their modes of action (see Section 3.3).

The characteristics of the most commonly used aminoglycosides can be found in Table 15.4. The presence of numerous **amine** groups in all compounds, positively charged at neutral pH, ensures an overall **cationic** nature and substantial water solubility.

3.2 Side Effects, Toxicity, and Contraindications of the Aminoglycoside Antibiotics

Most aminoglycosides show oto- and **nephro**toxic effects and the risk of damage is higher in patients with impaired renal function or after high doses of these antibiotics. They are therefore contraindicated with other drugs that can impair renal function such as some diuretics and cephalosporins. Ototoxicity is the result of damage to the sensory hair cells and the eighth cranial nerve and is irreversible (48). Nephrotoxicity is the result of accumulation of the antibiotics in the proximal renal tubule and this has a number of effects including **hydroxyl** radical formation, inhibition of **phos**pholipases, increases in thomboxane synthesis, and others (reviewed in Ref. 49). **As** a result of this ubiquitous problem, once-daily regimens have been established that account for patient body weight; close monitoring of serum concentration is recommended (45). Aminoglycosides cross the placenta and are thus used in treatment of pregnant women only in life threatening circumstances. Small amounts of the antibiotics are present in breast milk and therefore have the potential to harm the infant; however, this must be balanced with the knowledge that oral absorption

Table 15.4 Aminoglycoside Antibiotics in Clinical Use

Generic Name	Trade Name	Structure	Route	Dose[a]
Streptomycin	Streptomycin	**10**	i.m.	**15** mg/kg/day
Amikacin	Amikin	**14**	i.v. or i.m.	15 mg/kg
Tobramycin	TOBI	**13**	Inhalation	**300** mg/12 h
Gentamicin C	Garamycin Parenteral	**11**	i.v. or i.m.	**3** mg/kg/day in three **1** mg/kg doses
Gentamicin C	Garamycin Ophthalmic	**11**	Drops	
Netilmicin	Netromycin	**15**	i.v. or i.m.	4 mg/kg/day in two **2** mg/kg doses
Neomycin	Neosporin (and others)	**12**	Topical	

"Adult patients with normal renal function.

is poor. Rapid infusion by i.v. and coadministration with muscle relaxants can lead to neuromuscular blockade.

3.3 Pharmacology and Mode of Action of the Aminoglycoside Antibiotics

As noted above, as a consequence of the poor oral availability of aminoglycosides, parenteral administration is required. This poor absorption through the gastrointestinal tract has been taken advantage of for the sterilization of the gut before abdominal surgery. Upon injection, the serum half-life of aminoglycosides in patients is on the order of 2 h, but this is highly variable, especially in cases of renal failure (45). There is little metabolism of the arninoglycosides, which are excreted by glomerular filtration. However, there have been reports of the generation of an uncharacterized metabolite of gentamicin and other aminoglycosides after incubation with hepatic microsomes that is toxic to outer hair cells (50, 51). The details of the structure and mechanism of these metabolites and their relationship with clinical toxicity remain to be explored.

The aminoglycosides are generally bactericidal antibiotics and the main site of action of these antibiotics is the ribosome. Identification of the ribosome as the primary target is supported by the observation that high level resistance to aminoglycosides can occur through ribosomal modification by specific methyltransferases or by point mutation in rRNA and certain ribosomal proteins. Chemical footprinting studies have identified the 16S rRNA as the primary site of binding for the aminoglycoside antibiotics, particularly within the mRNA decoding A-site (52, 53). However, simple interference with translation is rarely sufficient to achieve cell death, and the mechanism of bactericidal activity of aminoglycosides has not been completely resolved. Aminoglycosides enter the cell in a multiphasic process (54), consisting of first an energy-independent accumulation of antibiotic on the cell surface, likely as a result of interaction of the cationic drugs with the anionic components of the cell wall (teichoic acids in Gram-positive bacteria), outer membrane (lipopolysaccharides in Gram-negative bacteria), and cellular membrane (phospholipids). This is followed by an energy-dependent transport of the antibiotic across the cell membrane. This transport requires the $\Delta\psi$ portion of the membrane potential and explains why anaerobic bacteria are less sensitive to these antibiotics and how mutations in electron-transport components or the presence of inhibitors of this process interfere with antibiotic access to the cell (55, 56). The initial entry phase is followed by a second energy-dependent accumulation of antibiotic within the cell. This has numerous effects other than on translation, including membrane damage resulting in membrane "leakiness" and inhibition of DNA synthesis (57–59).

A key element in the process of cell death appears to reside in the fact that bactericidal aminoglycosides cause mistranslation of the genetic code (60–62). This results in the biosynthesis of miscoded proteins. Davis has proposed that these aberrant proteins, with associated unpredictable folding, play a role in membrane damage, which precipitates cell death (63, 64). Miscoding associated with aminoglycosides has long been recognized and in fact has been used to suppress point mutations (65).

The specific details of the interactions of aminoglycosides with the target 16S rRNA are now being elucidated. A series of elegant model studies using NMR techniques by the group of Puglisi have suggested that binding of antibiotics such as paromomycin and gentamicin results in a conformational change in the rRNA in this region (66–68). Recently, the structures of the 30S ribosomal subunit of T. ***thermophilus*** in complex with streptomycin, spedinomycin, and paromomycin have been solved at a resolution of 3 Å, providing unprecedented molecular insight into their mode of action (69). Although all these aminoglycoside antibiotics interfere with translation, they have different effects. Both streptomycin and 2-deoxystreptamine aminoglycosides such as paromomycin, which is prototypical of this group, cause mistranslation and cell death but footprint to different areas of the 16S rRNA. Spectinomycin, on the other hand, does not cause mistranslation but blocks the translocation of the tRNA from the A-site to the P-site after peptidyltransfer, by freezing the ribosome in an inactive conformation, and is

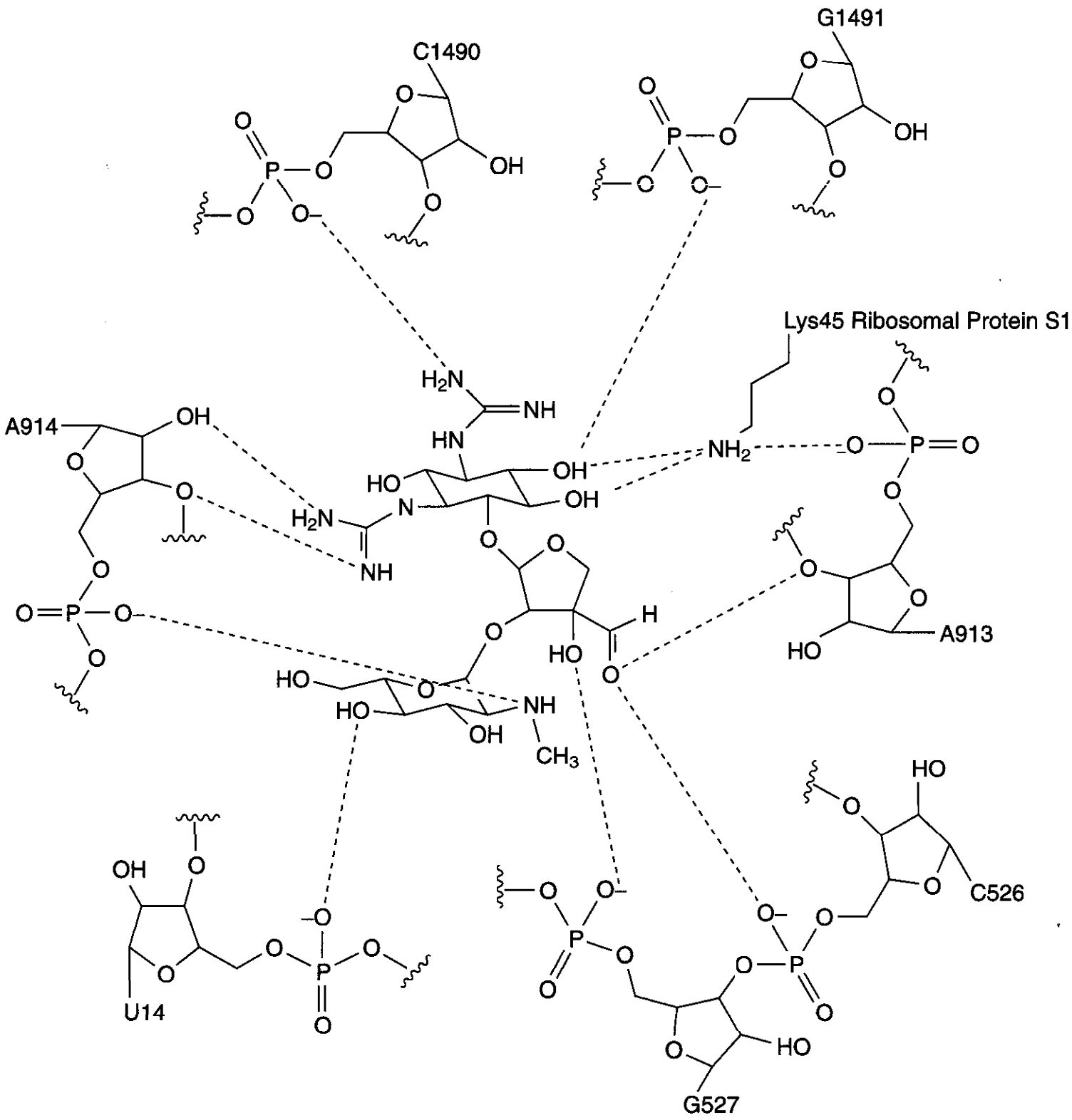


Figure 15.6. Interaction of streptomycin with the 30S ribosomal subunit (redrawn from Ref. 69). E. *coli* numbering of 16S rRNA is shown.

bacteriostatic (70–72). The structure of the spectinomycin–30S complex confirms the predicted (70) direct interactions with G1064 and C1192 of the 16S rRNA and places the rigid spectinomycin molecule at the juncture of 16S rRNA helices H34 and H35. This interaction prevents either movement or a conformation change in H34 during translocation.

Streptomycin, on the other hand, binds not only to the 16S rRNA through a number of contacts between H27, H44, and H18 but also directly with ribosomal protein S12 (Fig. 15.6). This interaction stabilizes a conformation of the ribosome, termed ***ram*** (ribosomal ambiguity), that increases tRNA affinity in the A-site. Stabilization of this conformation likely results in the loss of tRNA selectivity in the A-site and consequently increases translation errors, which contribute to cell death. The paromomycin–30S complex (Fig. 15.7) reveals that the antibiotic binds to a pocket formed by the major grove of H44 and forms direct interactions between a number of A-site 16S bases including A1408 and G1494 (69). Importantly,

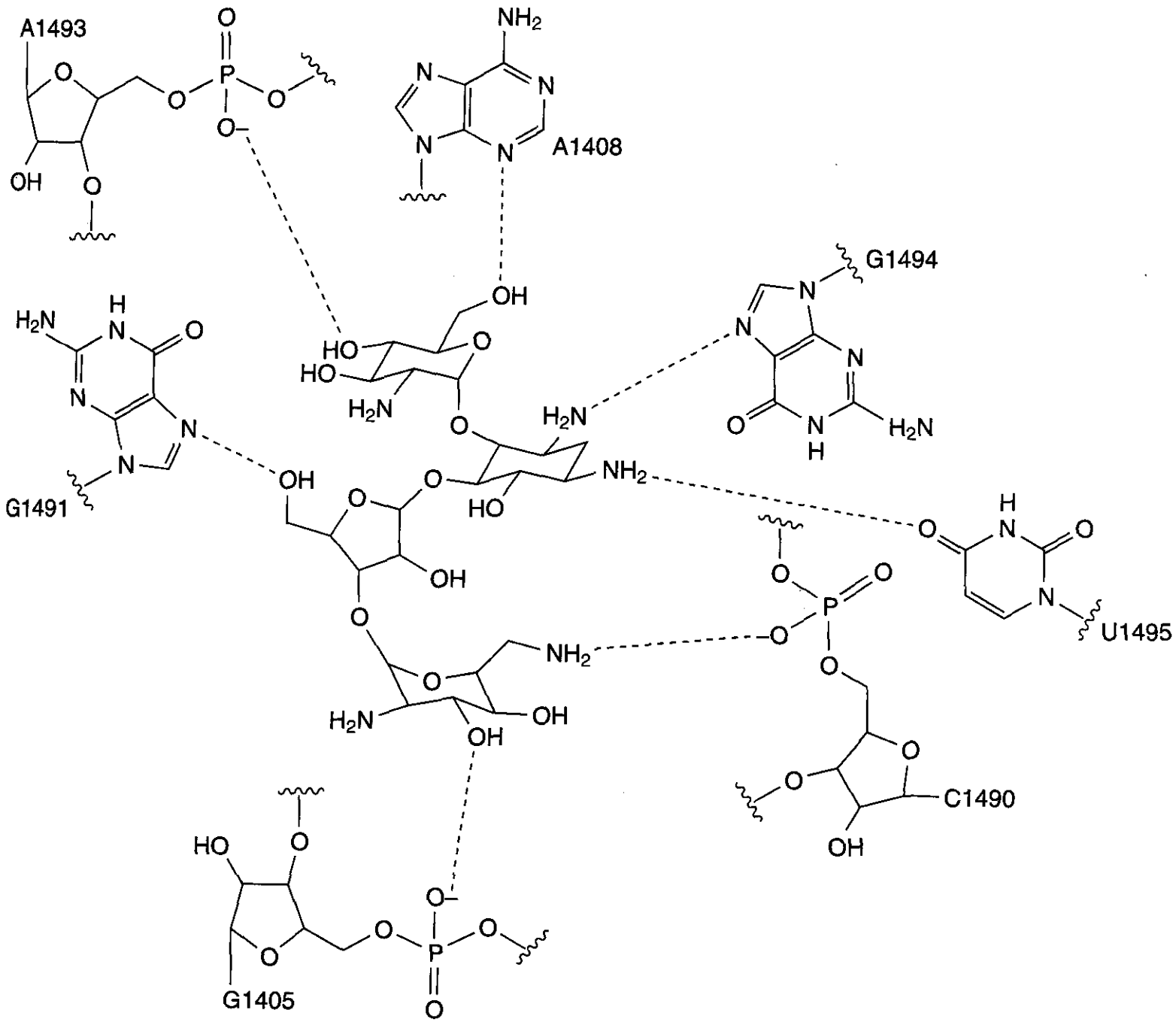


Figure 15.7. Interaction of paromomycin with the 30S ribosomal subunit (redrawn from Ref. **69).** E. *coli* numbering of 16S rRNA is shown.

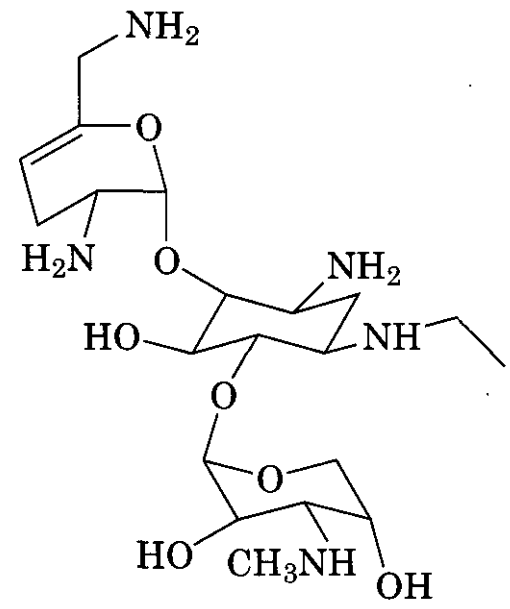
bases A1492 and A1493 of H44 are flipped out toward the mRNA binding region upon binding of paromomycin. The structure of the paromomycin–30S complex is now complemented by the structure of the complex of the 30S subunit with the anticodon loop of $tRNA^{Phe}$ and a U_6 hexanucleotide (73). In this structure, 16S rRNA bases A1492 and A1493 are also flipped out toward the mRNA, interacting with the minor grove of the codon–anticodon helix. Paromomycin therefore induces a similar conformational change in the absence of a cognate codon–anticodon interaction, and this may be the molecular basis for mRNA misreading caused by binding of the 2-deoxystreptamine aminoglycosides to the ribosomal A-site.

3.4 History and Biosynthesis of the Aminoglycoside Antibiotics

Walksman and colleagues discovered the first aminoglycoside antibiotics, streptomycin in 1944 and neomycin in 1949, using systematic searches for antimicrobial compounds derived from soil organisms (74, 75). These antibiotics were rapidly incorporated into clinical use, streptomycin being the first antibiotic with potent antimycobacterial activity used for the treatment of tuberculosis. The discovery of these antibiotics was followed by the identification of numerous additional members of the group including kanamycin in 1957 by Umezawa's group in Japan (76), and gentami-

cin (1963) and sisomicin (1970) both from Schering-Plough in the United States (77, 78).

Although new aminoglycosides continued to be identified and characterized from natural sources after this initial phase of discovery, clinically directed drug discovery in the 1970s saw a focus on the semisynthetic derivatization of existing antibiotics. This effort was guided by known natural derivatives that showed less sensitivity to aminoglycoside resistance by chemical modification, which was rapidly emerging as a grave clinical problem (see Section 3.5). For example, modification of N1 through amination by an α-hydroxy-4-aminobutyryl (AHB) group naturally found in the aminoglycoside butirosin was mimicked in the synthesis of amikacin (N1-AHB-kanamycin A) (**14**), netilimicin (N1-ethylsisomicin) (**15**), isepamicin (N1-α-hydroxy-γ-aminopropionyl-gentamicin B), and arbekacin [N1-AHB-3'-4'-dideoxykanamycin B (dibekacin, **16**)], all of which found, and continue to find, clinical use. Despite the identification of a number of aminoglycosides derived from both natural sources and through semisynthesis, there has not been a new antibiotic of this class introduced into clinical practice for over 2 decades, with the exception of arbekacin (16) in Japan. The reasons for this may reflect the increased availability of newer broad-spectrum agents such as the fluoroquinolones, which lack the general toxicity of aminoglycosides.



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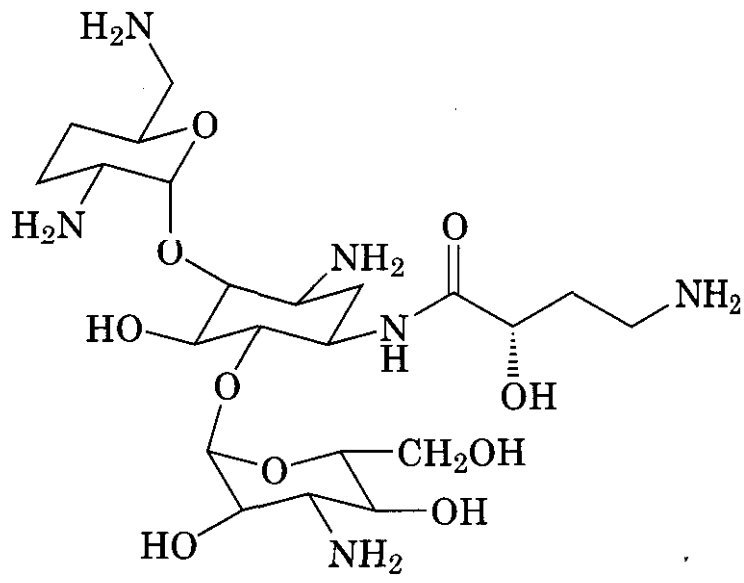

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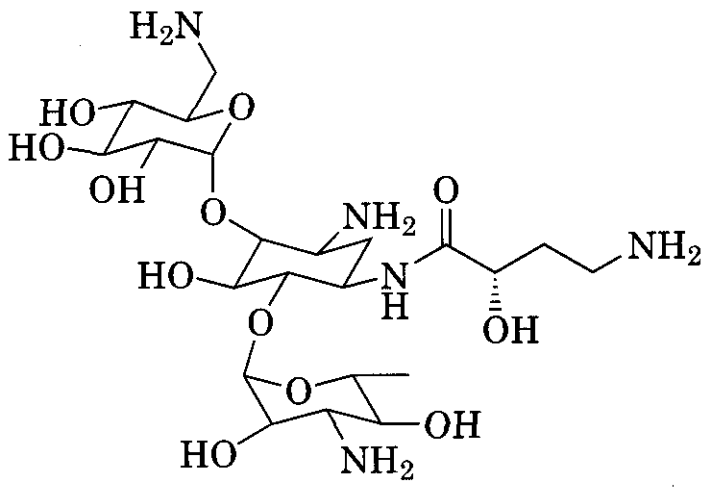


(14)

The aminoglycoside antibiotics are synthesized primarily by actinomycete bacteria from the genera Streptomyces, Micromonospora, and Saccharopolyspora, which produce a number of antibiotics, but also from bacteria of the genera Bacillus and Pseudomonas (79). The distinguishing aminocyclitol ring is derived from inositide biosynthesis from glucose-6-phosphate, which, although essential to eukaryotic membrane structure and signal transduction, is generally thought to be rare within the prokaryotes. Nonetheless, it has been shown that actinomycetes and a few other bacteria have inositol-containing lipids in their membranes and cell walls (see references in ref. 80). Piepersburg has noted that the available biosynthetic data point to two major routes for the synthesis of the aminocyclitol rings: (*1*) through a D-myo-inositol-3-phosphate synthase generating *scyllo*-inosamine, which is necessary in the formation of streptomycin and spectinomycin; and (**2**) through a dehydroquinate synthaselike path-

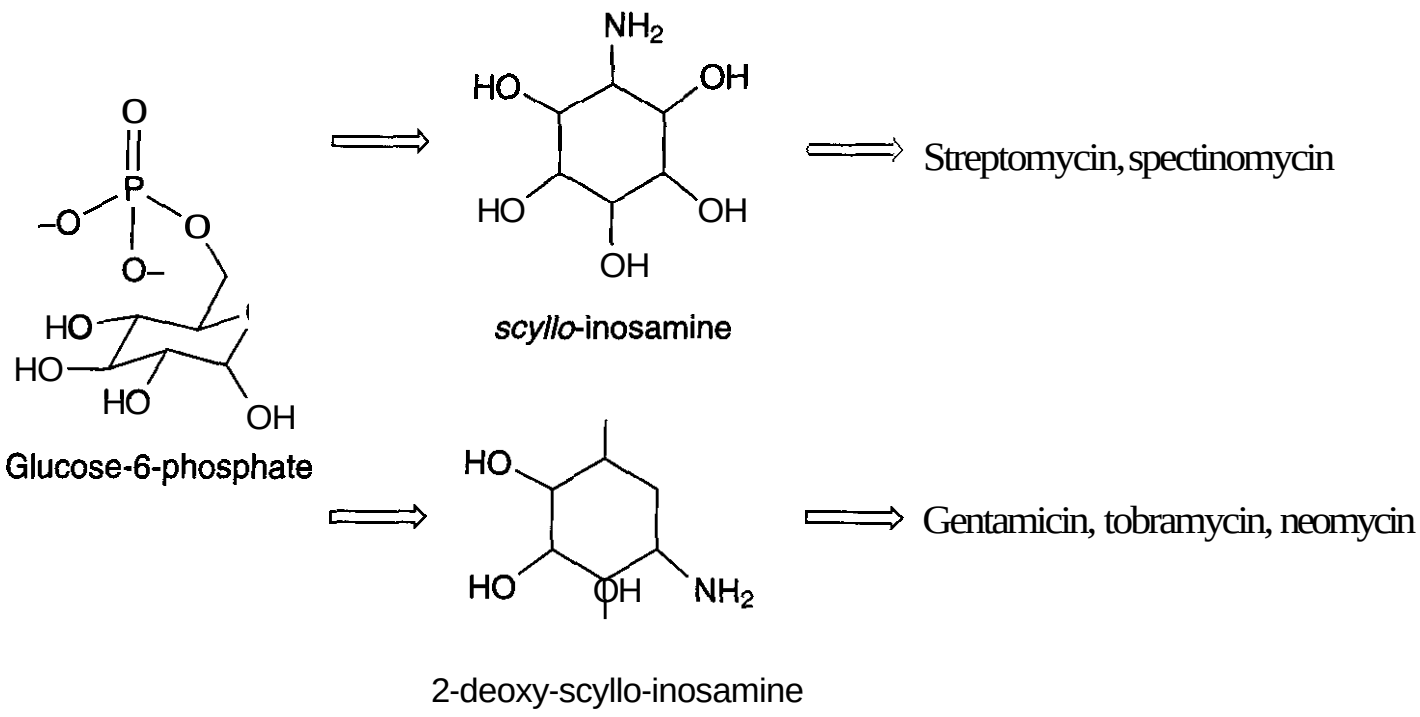


Figure 15.8. Biosynthetic strategies for the generation of arninocyclitol rings.

way generating 2-deoxy-scyllo-inosamine, which is essential for the formation of all the important 2-deoxystreptamine antibiotics such as kanamycin and gentamicin (Fig. 15.8) (79). These then are the fundamental steps in aminoglycoside biosynthesis. The specific sugar components that are characteristic of each individual aminoglycoside arise both by modification before and after transfer to the aminocyclitol rings, and the precise pathways appear to be unique to individual antibiotics with few common intermediate steps. An exception is the biosynthesis of paromamine, which is a central element in the formation of the majority of 2-deoxystreptamine antibiotics (Fig. 15.9).

The precise details of aminoglycoside biosynthesis await complete sequencing of biosynthetic genes and the accompanying detailed biochemical analysis of gene product function, which are lacking for most important arninoglycosides, with the exception of streptomycin and fortimicin (reviewed in Ref. 79) and more recently butirosin (81). Research on the biosynthesis of streptomycin has formed the basis of our understanding of aminoglycoside biosynthesis and has been essential in deciphering the molecular strategy used by the microorganisms to generate the antibiotic. Of particular interest is the fact that streptomycin is prepared as the 6-phospho-derivative, which does not have antimicrobial activity. This therefore protects the cell against suicide during antibiotic production (82). A 6-phospho-streptomycin–specific exporter, StrV/StrW, facilitates passage from the cytosol (83). Once on the outside of the cell, an extracellular 6-phospho-streptomycin phosphatase, StrK, removes the phosphate, revealing the active streptomycin antibiotic in the extracellular medium (84). This clever approach of biosynthesizing antibiotic precursors that are activated only once outside the cell may prove to be a general approach in avoiding toxicity in this class of antibiotics.

3.5 Aminoglycoside Antibiotic Resistance

Resistance to the aminoglycosides occurs primarily through the production of enzymes that modify the antibiotics in either an ATP- or acetylCoA-dependent fashion, although other resistance mechanisms are known including drug efflux, alteration of ribosome components, and altered uptake of the drugs. Efflux has thus far been of minor clinical importance and restricted to nonspecific pumps in only a few organisms [e.g., P. aeruginosa (85, 86), Burkhoderia pseudomallei (87), Mycobacterium fortuitum (88), and E. coli (89, 90)]. Importantly, the presence of the active MexXY–OprM efflux system in P. aeruginosa has recently been linked to suppression of aminoglycoside activity in this organism by cations such as Mg^{2+}, suggesting that inhibitors of efflux pumps may potentiate aminoglycoside action in this organism (91). Altered up-

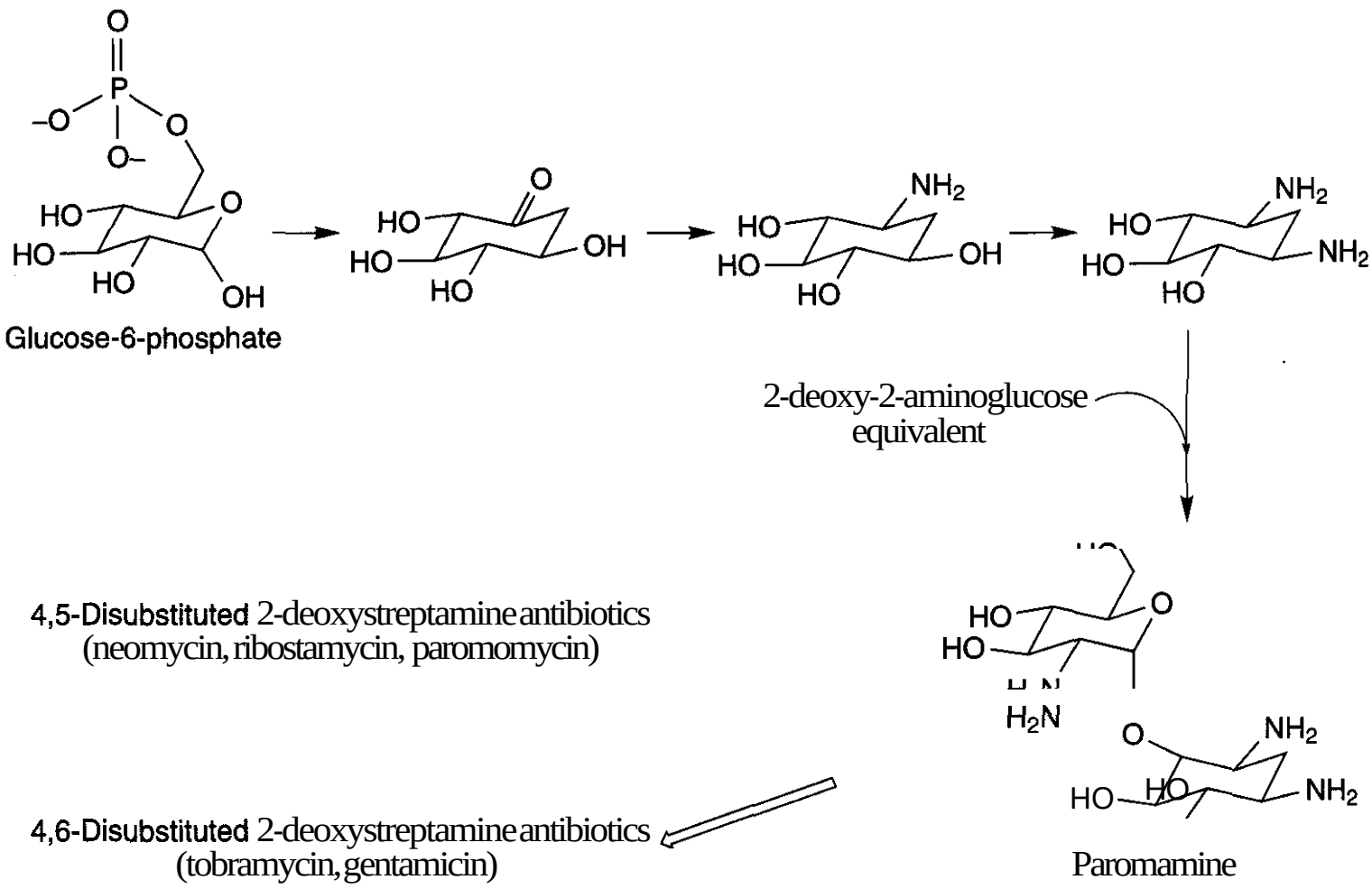


Figure 15.9. Paromamine is a common biosynthetic intermediate for several 2-deoxystreptamine arninoglycoside antibiotics.

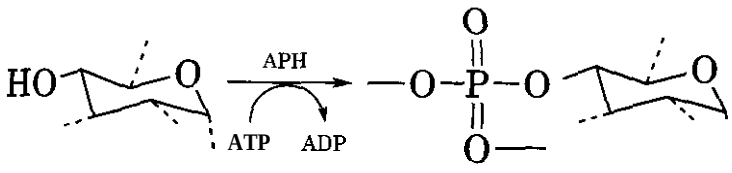
take of aminoglycosides is also infrequently associated with clinical resistance, though as noted above, anaerobic conditions and mutations that affect the membrane potential necessary for aminoglycoside entry into the cell are known.

Target modification by mutation or chemical modification of the ribosome also results in aminoglycoside resistance. Point mutations in the target 16S rRNA are not uncommon in some clinically important bacteria such as M. tuberculosis, where streptomycin resistance is associated with a variety of mutations in the 16S rRNA gene rrs (92). The structure of the streptomycin–30s subunit complex (69) clarifies the molecular basis of resistance, demonstrating ionic and H-bond interactions between the antibiotic and mutation-susceptible bases. Resistance to streptomycin can also arise frequently from point mutation of Lys43 of the ribosomal protein S12 (92), which in the crystal structure of the streptomycin–30s subunit complex binds directly to streptomycin (69). Resistance to the 2-deoxystreptamine aminoglycosides, which do not bind in the same area of the A-site as streptomycin, can also arise from 16S rRNA mutations, though these are generally less frequently associated with clinical resistance. Modification of the 16S rRNA target by methylation is a prevalent mechanism of high level resistance in aminoglycoside-producingbacteria,where S-adenosylmethionine–dependentmethylation of N7 of G1405 and/or A1408 results in resistance (93). These bases interact directly with paromomycin in the crystal structure of the paromomycin–30S subunit complex (see Fig. 15.7) (69).

The most frequent means of aminoglycoside resistance is through enzyme-catalyzed detoxification of the antibiotics. Unlike other antibiotics such as the β-lactams, where enzymatic destruction is also the most relevant mechanism of resistance, aminoglycoside resistance does not occur through cleavage of the molecule. Rather, the aminoglycosides are covalently modified by phosphorylation, adenylation, or acetylation on key hydroxyl or amine groups that result in steric blocking of the antibiotic binding site on the ribosome.

These group transfer reactions require ATP or acetylCoA co-substrates and thus antibiotic inactivation occurs only inside the cell and consequently aminoglycoside modifying enzymes are intracellular. There are numerous individual aminoglycoside resistance enzymes and these are classified by the type of modification: APH for aminoglycoside phosphotransferases, ANT for aminoglycoside adenyltransferases, and AAC for acetyltransferases. The standard nomenclature used to describe aminoglycoside resistance enzymes consists first of designation of the type of transfer (APH, ANT, or AAC) followed by the site of modification in parentheses (e.g., position 3, 3′, *2*), followed by a designation of the resistance profile by a Roman numeral (I, II, III, ...), and finally a lowercase letter designating the specific gene, which is assigned in incremental fashion as it is reported (94). For example, spectinomycin is modified by ANT(9)-Ia, an adenyltransferase (ANT) that modifies spectinomycin only (I) at position 9, and the gene was cloned from Staphylococcus aureus (a); AAC(6′)-Ic is an acetyltransferase(AAC) that acetylates kanamycin, tobramycin, amikacin, and neomycin (I) at position 6′, and the gene was cloned from Serratia marcescens. Figure 15.10 shows the structures of gentamicin C2 and streptomycin and the predominant sites of modification by resistance enzymes typically found in clinical isolates. (For a more comprehensive description of aminoglycoside resistance genes and the associated regiospecificity of group transfer see Refs. 47, 94, and 95.) Three-dimensional structures of members of each class of resistance enzyme (APH, ANT. and AAC) are now known and have served to illuminate the molecular basis of resistance.

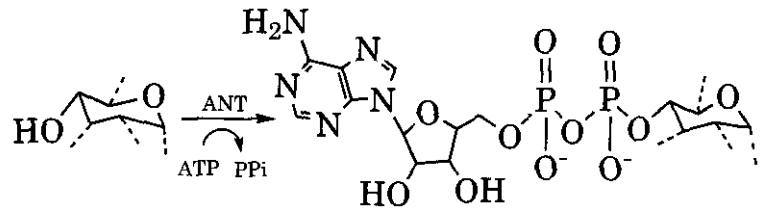
Aminoglycoside phosphotransferases constitute a large family of aminoglycoside resistance enzymes that require ATP for activity (96). APHs modify aminoglycosides by phosphorylation of hydroxyl groups with release of ADP:



(15.1)

Enzymes of this family share significant primary sequence homology in the C-terminus, which is now known to contain many of the residues important to catalysis. The 3D structure of one aminoglycoside kinase, APH(3′)-IIIa, has been determined in the absence of bound nucleotides and in the presence of ADP and the nonhydrolyzable ATP analog AMPPNP (97, 98). These structures revealed close similarity to the fold of the Ser/Thr/Tyr protein kinase superfamily, despite the fact that only 5 of 264 amino acids are conserved between APH(3′)-IIIa and protein kinases. Not surprisingly, these residues play important roles in ATP binding and phosphoryl transfer, which is common to both groups of enzymes (97, 99). The structural relationship between both aminoglycoside and protein kinases has been expanded by studies indicating that these enzyme families share sensitivity to inhibitors (100,101) and by the fact that aminoglycoside kinases do have weak, but demonstrable protein kinase activity (102).

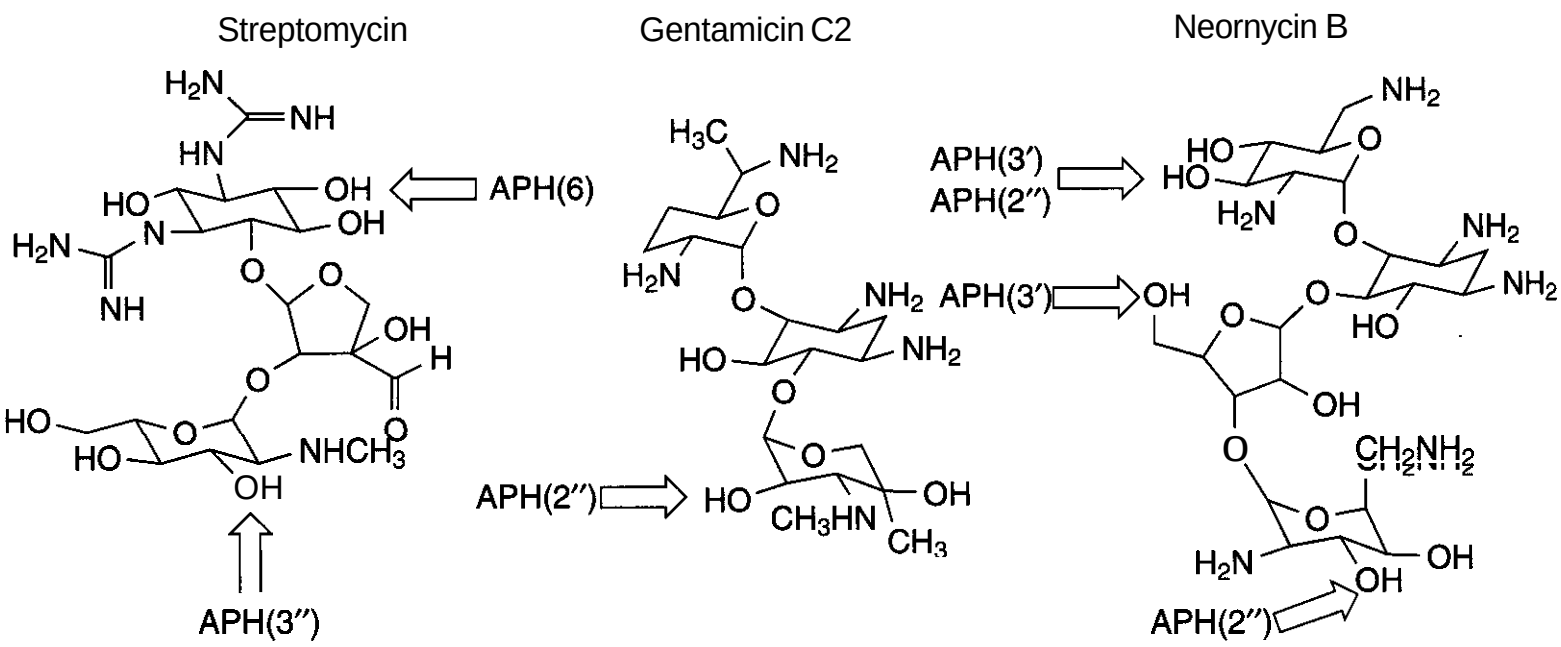
There are fewer known aminoglycoside adenyltransferases than either APHs or AACs; nonetheless, two of these, ANT(4′)-Ia from S. aureus and ANT(2″)-Ia from *Enterobacteriaceae* are significant clinical problems conferring resistance to most clinically important 2-deoxystreptamine aminoglycosides including tobramycin, gentamicin C, and amikacin. These enzymes catalyze the transfer of an AMP group to aminoglycoside hydroxyls with release of pyrophosphate:



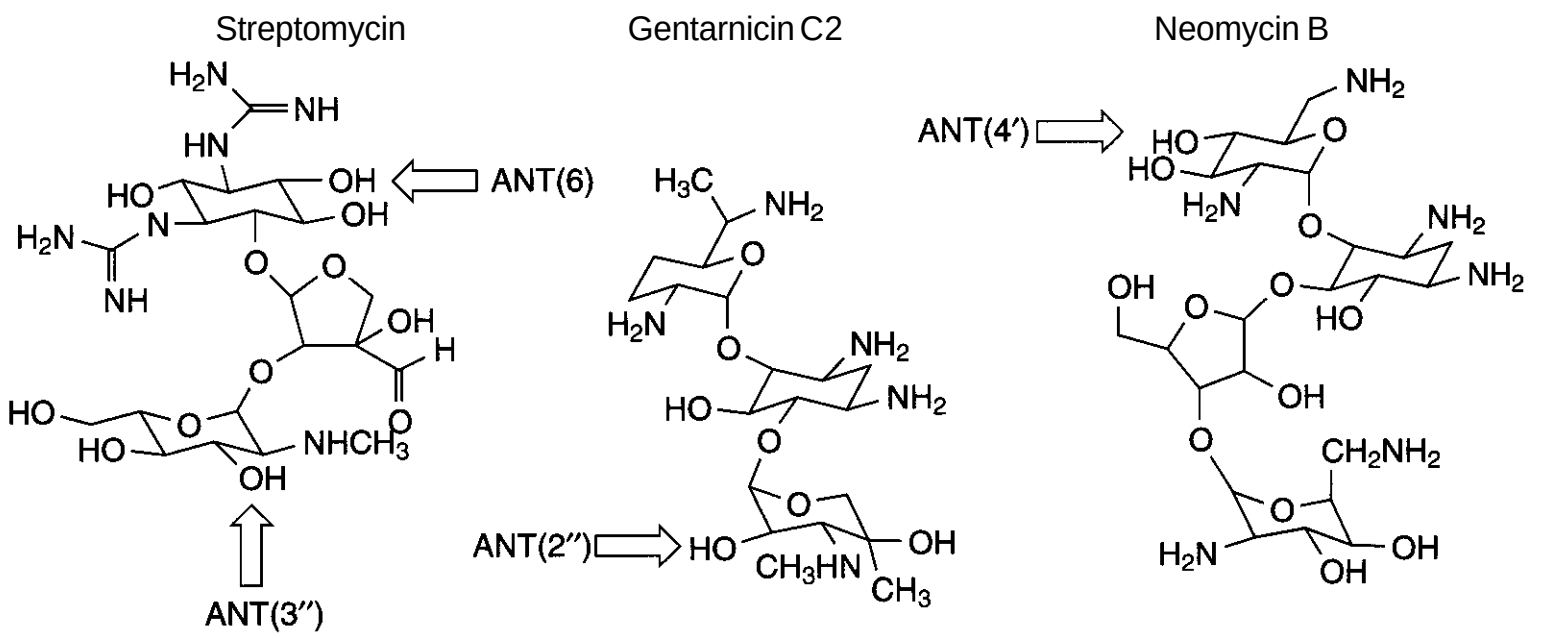
(15.2)

The 3D structure of dimeric ANT(4′)-Ia in the absence of substrates and in the presence of kanamycin and the nonhydrolyzable ATP analog AMPCPP have been determined (103, 104). The enzyme active site is at the interface of the dimer with residues from both subunits contributing to substrate binding and catalysis. Although there is minimal primary sequence homology among the ANTs, several key residues are conserved, indicating that the

A. Sites of aminoglycoside phosphoryation by APHs



B. Sites of aminoglycoside adenylation by ANTs



C. Sites of aminoglycoside acetylation by AACs

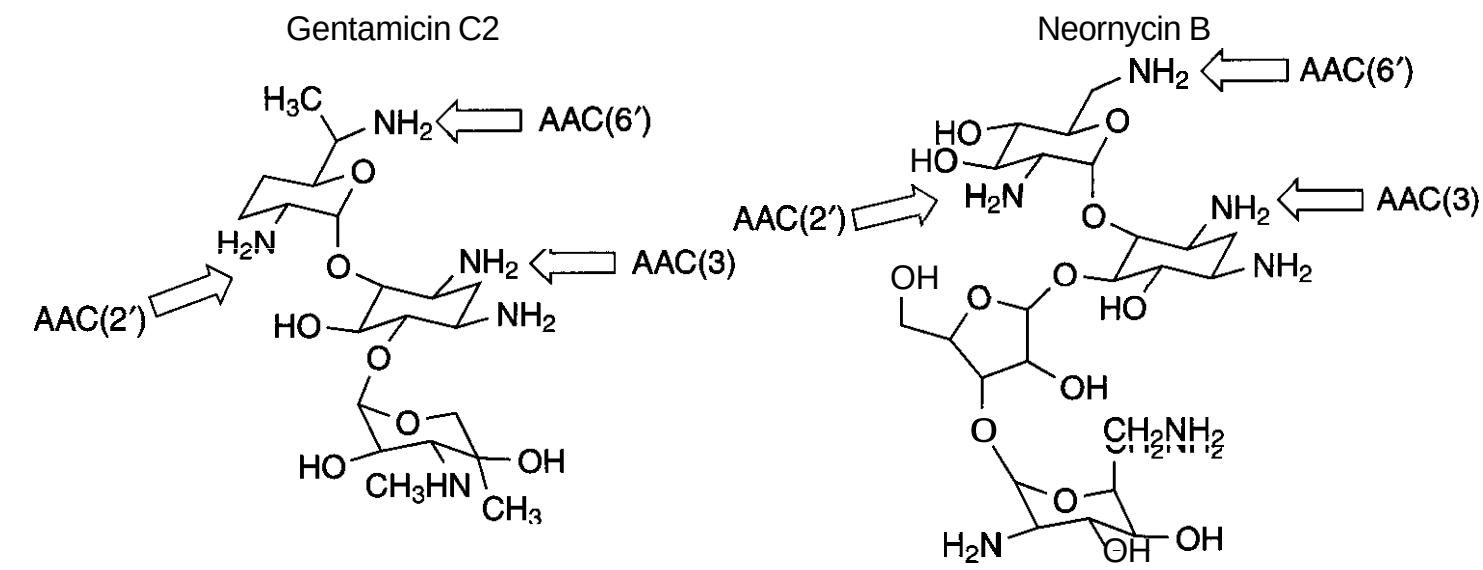
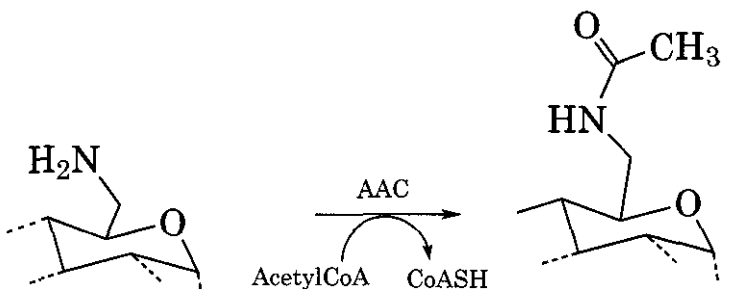


Figure 15.10. Major sites of aminoglycoside modification catalyzed by resistance enzymes.

general structure and active site are largely conserved among the enzymes, though the specific details of aminoglycoside recognition are divergent.

The aminoglycoside acetyltransferases represent the largest group of resistance aminoglycoside enzymes. These enzymes *N*-acetylate positions 2′, 6′, 3, or, less frequently, position 1 of 2-deoxystreptamine aminoglycosides:

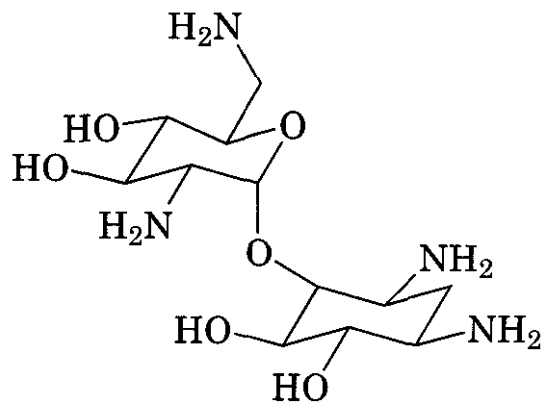


(15.3)

Whereas N-acetylation is clearly predominant, low level O-acetylation can occur with at least one enzyme (105). AACs are widely distributed in both Gram-positive and Gram-negative bacteria and are important causes of aminoglycoside resistance in the clinic, where AAC(3) isozymes are frequently associated with gentamicin resistance and AAC(6′) with resistance to amikacin and tobramycin. The 3D structures of members of both of these families have been characterized: AAC(6′)-Ii, a chromosomally encoded enzyme from *Enterococcus faecium* (106), and the plasmid-encoded AAC(3)-Ia from *S.* marcescens (107). Although these enzymes show little amino acid homology, the fold of the proteins is conserved and shared with other enzymes that belong to the GCN5 superfamily of acyltransferases (108).

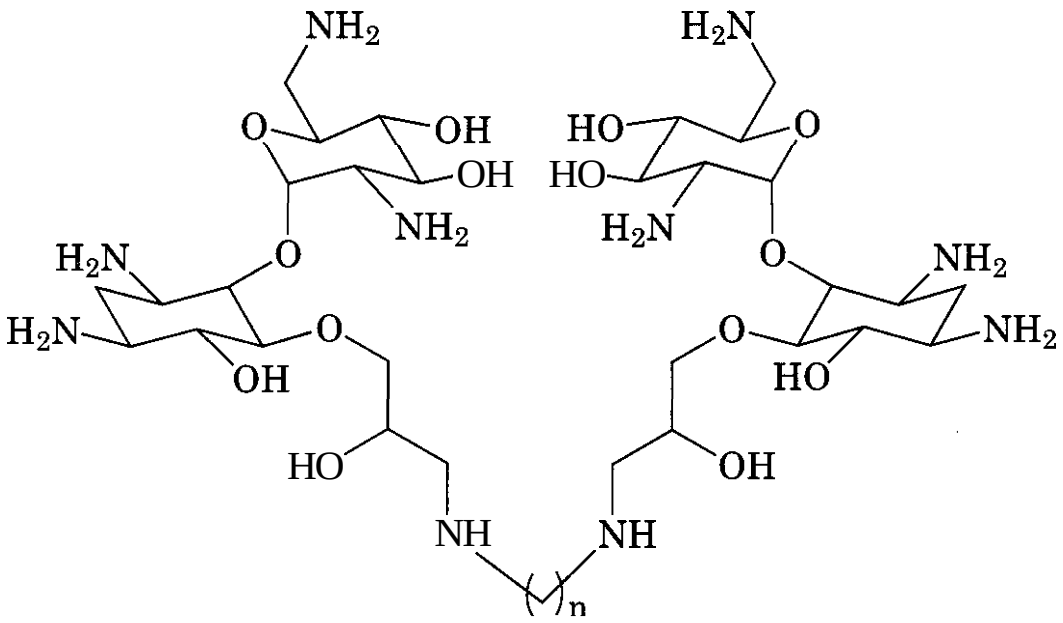
3.6 Recent Developments in the Aminoglycoside Antibiotic Field

Research in the development of new antimicrobial aminoglycosides has not been extensive over the past decade but recently several new reports have appeared that examine these antibiotics and their derivatives in a creative fashion. The group of Chi-Huey Wong at the Scripps Research Institute has prepared a series of *O*-acyl and dimeric aminoglycosides based on the minimal neamine (17) core structure (109, 110). Some of these compounds such as (18) possess not only good antimicrobial activity but also inhibit some aminoglycoside resistance enzymes (111). A series of modified aminoglycosides, in which the amino groups at positions 1, 3, 2′, 6′ of neamine and kanamycin were replaced by hydrogen atoms, have been synthesized by Mobashery and colleagues at Wayne State University (112). Some of these compounds retain antibacterial activity and are not substrates for a subset of aminoglycoside resistance enzymes (112,113). The same group also reported the synthesis and characterization of mechanism-based inactivators of aminoglycoside 3′-phosphotransferases by incorporating a nitro group at position 2′, such as compound (19), that generates an alkylating species upon enzymatic phosphorylation at position 3′ (114).

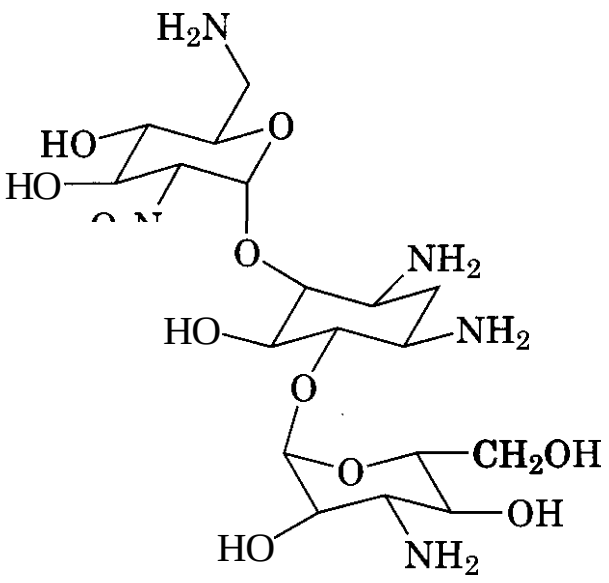


(17)

The affinity of aminoglycosides for RNA has proven useful in numerous studies (reviewed in Ref. 115). This includes selection of RNA aptamers with high and specific affinity for aminoglycoside using methods such as SELEX (systematic evolution of ligands by exponential enrichment). Aminoglycosides have also been shown to be inhibitors of ribozyme action such as Group I intron splicing (123–125), hammerhead (123–125), human hepatitis deltavirus (126), ribonuclease P (127,1281, and others. These experiments raise the possibility of using aminoglycoside antibiotics and their derivatives to target specific RNA molecules directly in a therapeutically useful fashion.



(18)



(19)

highly substituted lactone ring (termed an aglycone) functionalized with various carbohydrate residues. This chapter covers antibacterial macrolides of clinical significance having aglycone of 12–16 atoms, with one or more sugars (or aminosugars) attached to the lactone core. The currently marketed macrolide antibiotics play a very important role in treating bacterial infections and have gained wide acceptance for the treatment of both upper and lower respiratory tract infections, as well as cutaneous infections. Because of the extensive medical use of this class of antibiotics, the term macrolides is now generally synonymous with macrolide antibiotics in the medical-related scientific communities. A book on the chemistry, pharmacology, and clinical uses of approved macrolides was published in 1993 (130); this chapter therefore presents a brief summary of information on these three areas.

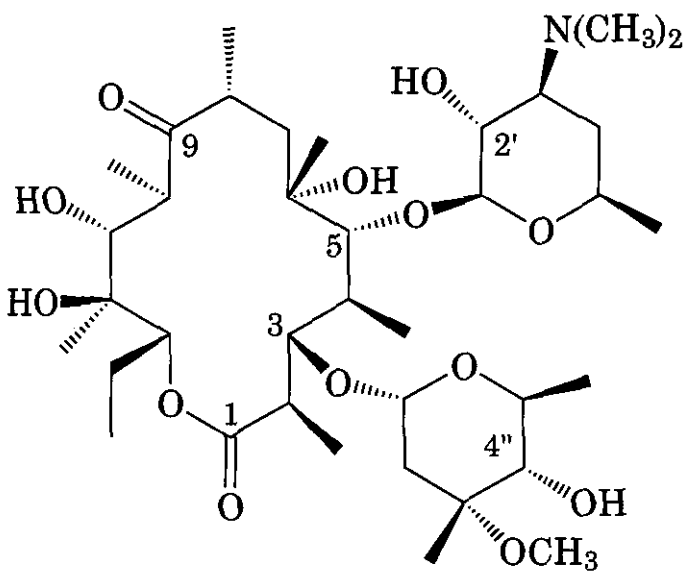
4 MACROLIDE ANTIBIOTICS

4.1 Clinical Use of Macrolide Antibiotics and Currently Used Drugs

Macrolide antibiotics belong to the family of macrocyclic antibiotics and are a well-established class of antibacterial agents for both human and veterinary applications. They have been used for the treatment of various bacterial infections in both out-patient and in-patient settings for more than 40 years, and as a result of their very good safety profile, they are extensively prescribed to children. The term macrolide means large macrocyclic lactone and was first used by R. B. Woodward for the class of natural products produced by *Streptomyces* species (129). Macrolide antibiotics are generally lipophilic and consist of a central

Naturally occurring macrolide antibiotics are grouped into three major groups of 12-, 14-, and 16-membered macrolides with the aglycone consisting of 12-, 14-, and 16-atom cyclic lactone rings, respectively. For example, erythromycin A (20)is a 14-membered macrolide (a 14-atom cyclic lactone ring) and possesses desosamine and cladinose glycosidically linked to C-5 and C-3, respectively.

In addition to natural macrolides, many semisynthetic macrolides have been made and developed for clinical use. A 15-membered macrolide, the azalide azithromycin (21), that incorporates an additional nitrogen atom in



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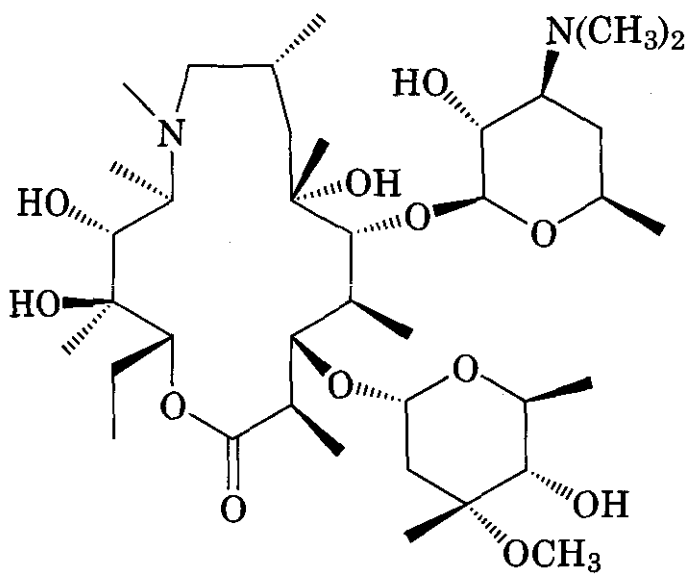
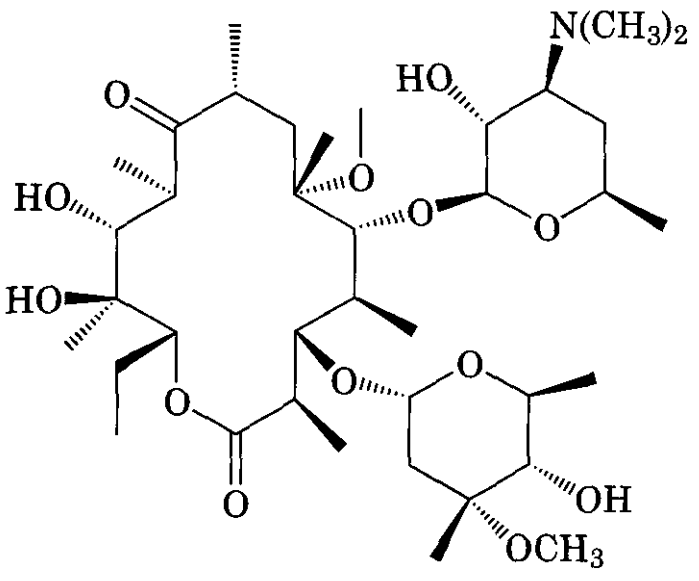


(21)

the aglycone has been successfully synthesized and developed for human use.

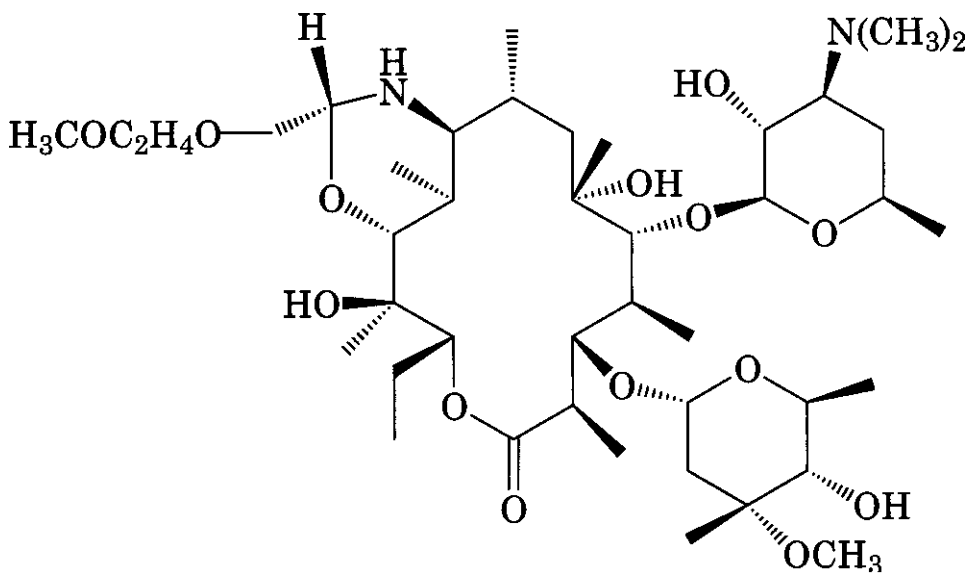
Among the 14-membered naturally occurring macrolides, erythromycin A is by far the most useful and possesses excellent antibacterial activity against Gram-positive bacteria and mycoplasmas. Erythromycin was developed initially for the treatment of staphylococcal infections for patients allergic to penicillin; however, after more than 40 years of use, most of the staphylococci isolated in hospitals are erythromycin resistant. Erythromycin and its semisynthetic derivatives are now used to treat lower and upper respiratory infections as well as skin and soft tissue infections, and are administered either orally or parenterally. Some of the disadvantages of erythromycin A are low bioavailability, a narrow spectrum of activity, and high gastrointestinal side effects. These liabilities have prompted research laboratories throughout the world to search for better semisynthetic erythromycin derivatives. These derivatives possess improved therapeutic properties such as enhanced antibacterial activity and/or broadened spectrum (including Gram-negative bacteria such as Haemophilus *influenzae*), or improved pharmacokinetic properties as well as reducing gastrointestinal side effects.

Several semisynthetic derivatives of erythromycin A, including clarithromycin (**22**), dirithromycin (**23**), flurithromycin (**24**), and roxithromycin (**25**), have been successfully developed and are currently in clinical use. These semisynthetic newer macrolides are used for the following indications: community acquired pneumonia, acute bacterial exacerbation of chronic bronchitis, acute bacterial sinusitis, tonsillitis/pharyngitis, otitis media, skin and soft-tissue infections, and ophthalmologic infections. They possess good antibacterial activity against the common respiratory pathogens such as Streptococcus pyogenes, Streptococcus pneumoniae, H. *influenzae*, and M. catarrhalis as well as atypical bacteria such as L. pneumophila, M. pneumoniae, and *C.* pneumoniae. In addition to these derivatives of erythromycin A, an oleandomycin (**26**) analog called triacetyloleandomycin (27) has also been developed for limited clinical use.



(22)

The 16-membered macrolides possess a wide diversity of structures and are usually subdivided into the leucomycin- and tylosin-related groups, with derivatives from the first



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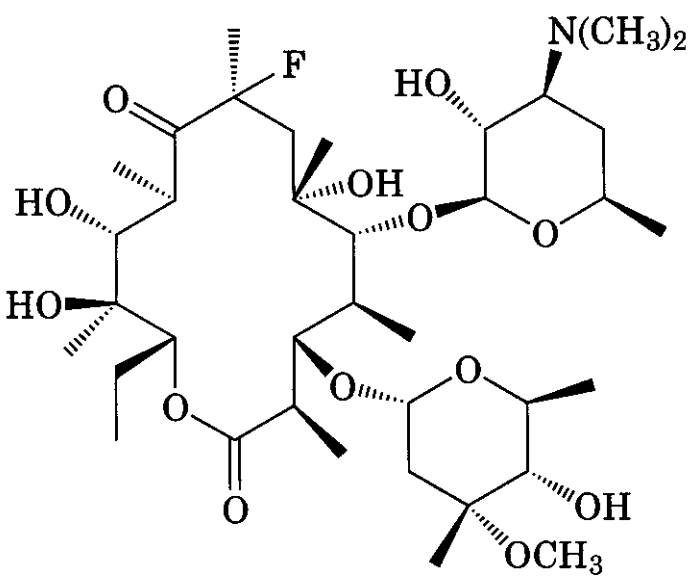

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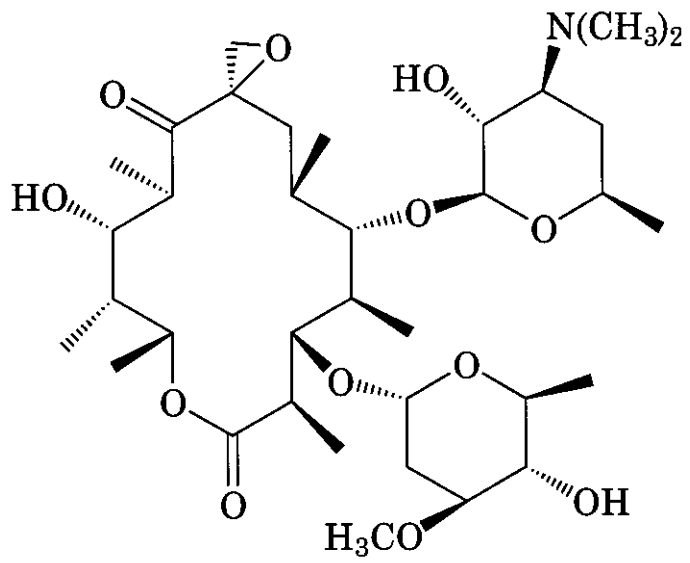

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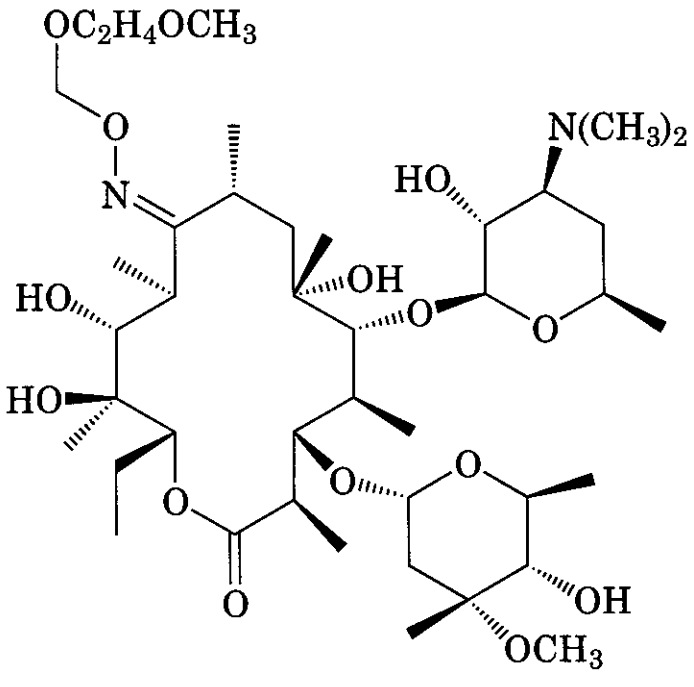

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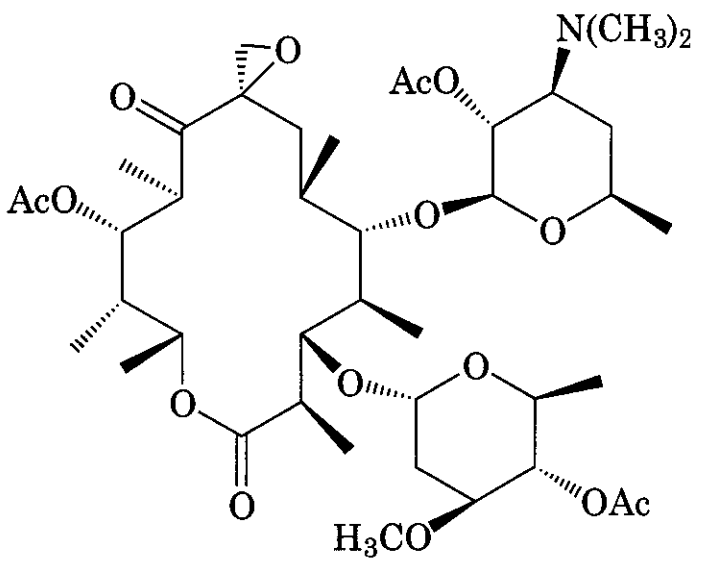

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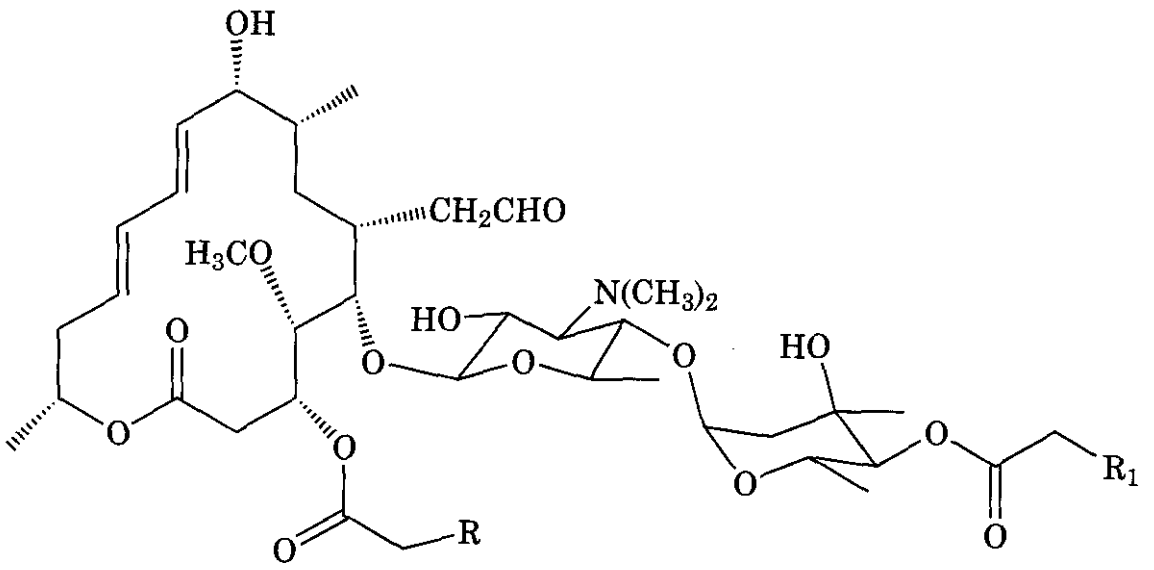


(28) R = H R_1 = **isopropyl**
(29) R = CH_3 R_1 = CH_3

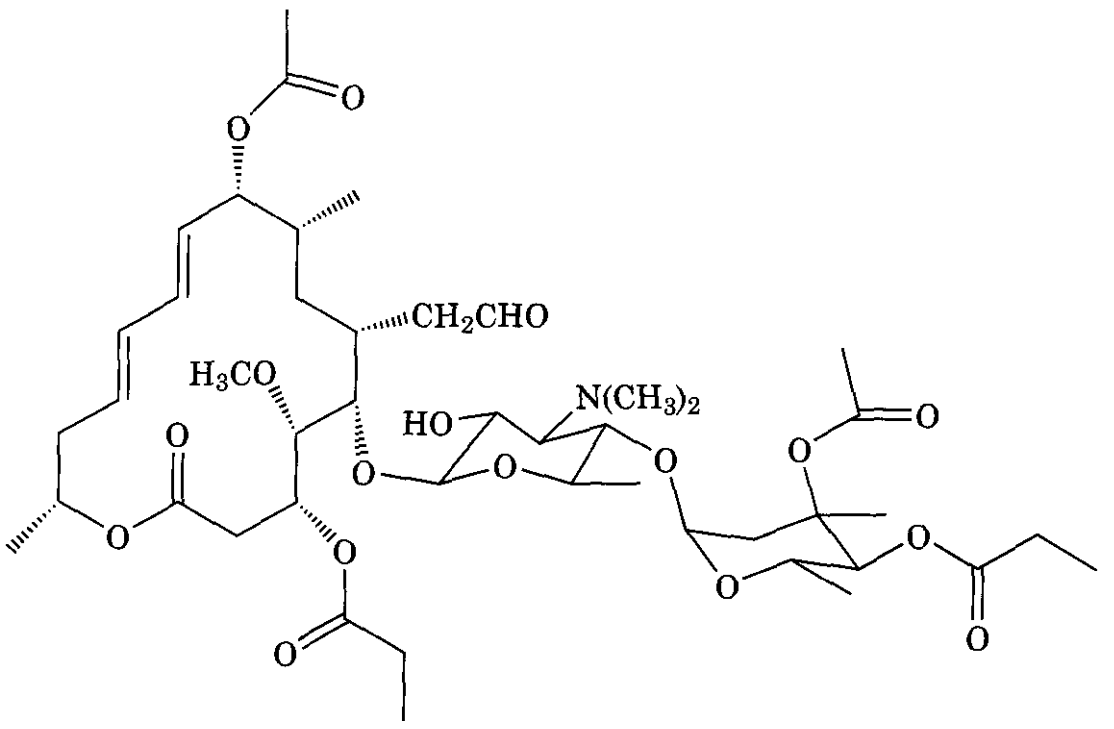
group developed for human use and the latter group for veterinary purposes. These compounds, natural or semisynthetic, have achieved limited success in human medicine and include josamycin (**28**), midecamycin (**29**), miokamycin (**30**), **rokitamycin** (**31**), and **spiramycin** (32).

A list of macrolides currently on the market for various clinical uses is summarized in Table 15.5.

4.2 Side Effects, Toxicity, and Contraindications of the Macrolide Antibiotics

The majority of side effects associated with macrolide antibiotics are mild and transient in nature. The most frequently reported events in adults are diarrhea, nausea, abnormal taste, dyspepsia, abdominal pain (substantially less with newer macrolides), and headache. Like other kinds of antibiotics, pseudomembranous colitis has been reported with macrolide use, ranging in severity from mild to life-threatening. There have been isolated reports of transient central nervous system side effects such as confusion, hallucinations, seizures, and vertigo associated with erythromycin use.

Macrolides are known to interact with cytochrome P450-dependent monooxygenases that affect the metabolism and elimination of



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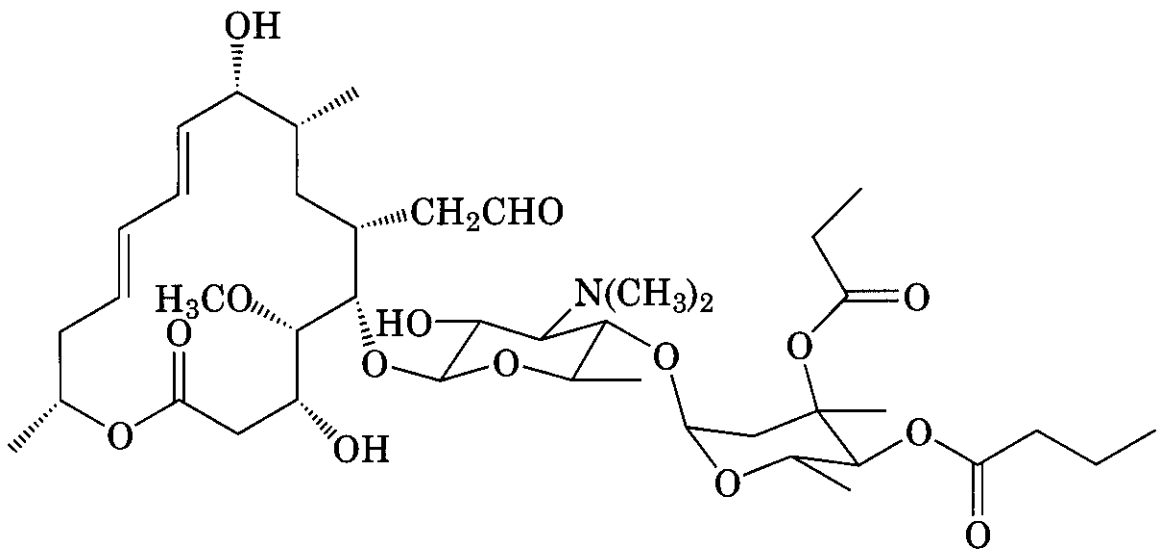
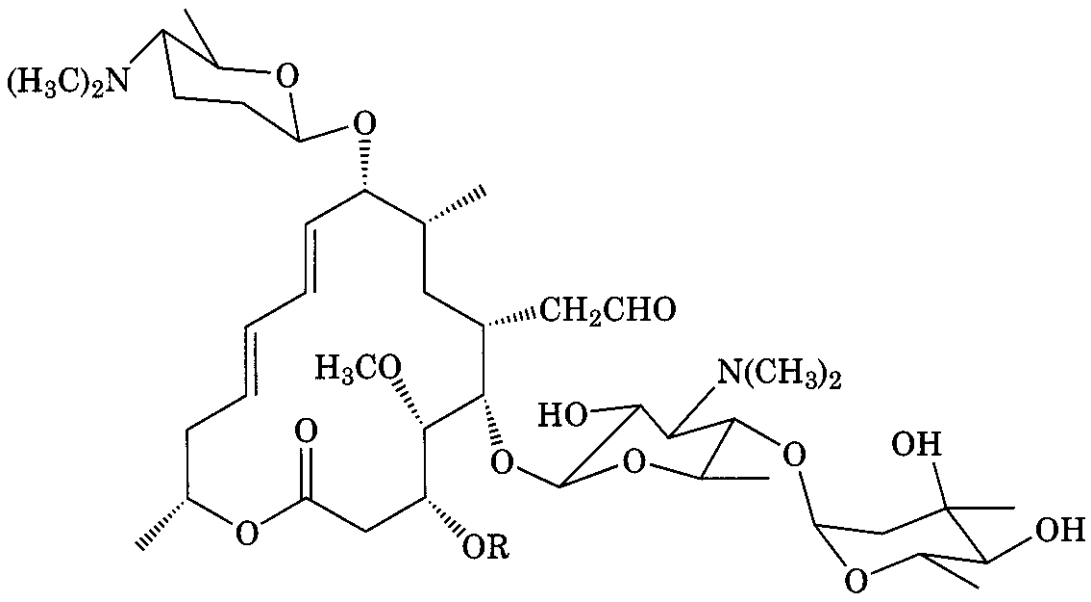


(31)

other drugs (131). Thus, macrolides may have an impact on the drug level of other drugs using these enzymes for drug metabolism. For example, combined therapy with a macrolide and theophylline (a bronchodilator) or carbamazepine (a psychotropic drug) causes an increase in the serum levels of the latter drugs. Cardiac arrhythmias such as ventricular tachycardia have been reported in patients receiving erythromycin A therapy. Thus, macrolides are contraindicated in patients receiving terfenadine therapy who have preexisting cardiac abnormalities (arrhythmia, bradycardia, QT interval prolongation, ischemic heart disease and congestive heart failure). Hepatotoxicity (132), ototoxicity (133), dermatologic effects (134), pancreatitis (135), cardiovascular toxicity such as QT prolongation (136) and induced hypotension (137), and hemolytic anemia (138) have been reported less commonly for some earlier macrolides.

4.3 Pharmacology and Mode of Action of the Macrolide Antibiotics

Macrolide antibiotics are primarily administered orally. They are readily absorbed from the gastrointestinal tract (139, 140). Because macrolides are weakly basic, they are predominantly absorbed in the alkaline intestinal environment. Erythromycin A is acid unstable. Thus, its absorption among different patients is highly variable. The drug degrades differently during its passage through the acid environment of the stomach of different patients. The lower bioavailability of earlier macrolides is the result of acidic instability, incomplete absorption, and a first-pass effect. Many different water-insoluble salts or esters



(32)

I R = H; II R = acetyl; III R = propionyl

Table 15.5 Selected Commercial Clinical Macrolides

Generic Name	Trade Name	Originator	Class	Route
Erythromycin (enteric-coated tablet)	Ery-Tab	Abbott	14	Oral
Erythromycin (polymer-coated particles)	PEC Dispertab	Abbott	14	Oral
Erythromycin topical gel	Emgel	Glaxo Wellcome	14	Topical
Erythromycin ophthalmic ointment	Ilotycin ointment	Dista	14	Topical
Erythromycin + benzoyl peroxide gel	Benzamycin	Dermik	14	Topical
Erythromycin acistrate	Erasis (Finland)	Orion	14	Oral
Erythromycin ethylsuccinate	E.E.S.	Abbott	14	Oral
Erythromycin ethylsuccinate + sulfisoxazole acetyl	Erythromycin ethylsuccinate + sulfisoxazole acetyl	Lederle	14	Oral
Erythromycin estolate	Ilosone	Dista	14	Oral
Erythromycin latobionate	Erythrocin lactobionate	Abbott	14	i.v.
Erythromycin gluceptate	Ilotycin glucoheptonate	Dista	14	i.v.
Erythromycin stearate	Erythrocin stearate	Abbott	14	Oral
Erythromycin stinoprate	Eritrocist (Italy)	Edmond Pharma	14	Oral
Erythromycin-11,12-carbonate	Davercin (Poland)	Tarchomin	14	Oral
Azithromycin	Zithromax Sunamed (Yugoslavia)	Pfizer Pliva	15	Oral
Clarithromycin	Biaxin	Abbott	14	Oral
Dirithromycin	Dynabac	Sanofi	14	Oral
Flurithromycin ethylsuccinate	Flurizic (Italy)	Pierre1	14	Oral
Roxithromycin	Rulid (France)	HMR	14	Oral
Triacetyloleandomycin	TAO	Roerig	14	Oral
Josamycin	Josamycin (Japan)	Yamanouchi	16	Oral
Midecamycin	Medemycin (Japan)	Meiji Seika	16	Oral
Miokamycin	Miocamycin (Japan)	Meiji Seika	16	Oral
Rokitamycin	Ricamycin (Japan)	Toyo Jozo	16	Oral
Spiramycin	Rovamycine (France)	RPR	16	Oral
Telithromycin (pending)	Ketek	Aventis	Ketolide	Oral

of erythromycin A have been prepared to protect it from acid degradation. A polymeric enteric-coated erythromycin A has also been made. The enteric coating can protect it from the degradation by the acidic environment of the stomach. Newer semisynthetic macrolides are more acid stable and retain good oral bioavailability.

The pharmacokinetics of a single-dose administration of roxithromycin was found to be nonlinear in the dose range of 150 to 450 mg given orally (141). The C_{max} values were 7.9 and 12.4 mg/L, respectively. The C_{max} and AUC values were decreased with food intake. The oral absorption of dirithromycin was rapid, having an absolute bioavailability of 10% (142). Upon oral administration of 500 mg of dirithromycin, the C_{max} values ranged from 0.1 to 0.5 μg/mL, with an elimination serum half-life of 44 h and the oral bioavailability ranged from 6 to 14% (143). The absorption is not significantly affected by food (144). The oral bioavailability of azithromycin is 37%. A regimen of 2 $\times$ 500 mg on the first day followed by the maintenance dose of 500 mg daily gave a mean C_{max} of 0.62 μg/mL (145). The apparent half-life between 8 and 24 h was 11–14 h. In humans, clarithromycin was nearly completely absorbed. Over a dose range of 100–1000 mg administered orally, the pharmacokinetics of clarithromycin was dose dependent, with a terminal half-life ranging from 2.3 to 6 h (146).

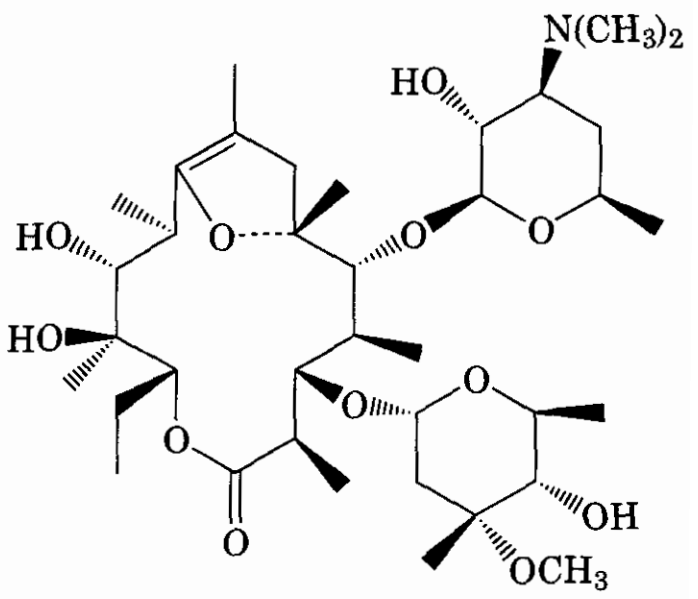
Macrolides bind to plasma and interstitial proteins and binding to plasma proteins varies

widely from 10 to 93% at therapeutic concentrations. Erythromycin A and roxithromycin bind specifically to a-l-acid glycoprotein (AGP) and, to a minor extent, nonspecifically to albumin (147). Roxithromycin (300 mg daily), clarithromycin (500 mg daily), and azithromycin (500 mg daily) for 12 days provided steady-state maximum and trough plasma concentration values of approximately 10 and 1.9 μg/mL; 2.1 and 0.4 μg/mL; and 0.19 and 0.05 μg/mL, respectively (148). The terminal elimination half-life for roxithromycin was significantly prolonged in patients with severely impaired renal function (15.5 h) compared with that of the group with normal renal function (7.9 h) (149). Clarithromycin and its 14-OH active metabolite were found to be extensively distributed into human lung tissue of patients undergoing lung resection. A mean calculated ratio of concentrations of 11.3 in lung tissue and 2.4 in plasma were found (150). The pharmacokinetics of clarithromycin suspension in infants and children has been reported (151). The drug absorption was rapid, reaching a mean peak plasma concentration within 3 h. The mean C_{max} of 4.58 and 1.26 μg/mL for its metabolite were obtained upon a single dose of 7.5 mg/kg clarithromycin suspension to children. After a single oral dose of 500 mg, concentrations of azithromycin in all tissues were higher than that in serum (152). The pharmacokinetics of a 3-day (500 mglday) and 5-day (500 mg on day 1, followed by 250 mg/day on days 2–5) regimens of azithromycin were found to be similar, having identical plasma profiles (C_{max} 0.37 versus 0.31 μg/mL) on day 1. The accumulation of azithromycin in plasma was higher with the 3-day regimen than that with the 5-day regimen (0.31 versus 0.18 μg/mL) on the last day (153). Once-a-week azithromycin in AIDS patients on zidovudine did not alter the disposition of zidovudine (154). The concentration of azithromycin was elevated in middle ear effusion in children, with effusion concentration of 1.02 μg/mL at 12 h after the oral administration of 10 mg/kg (155). Concentrations of azithromycin in gallbladder and liver were usually within twofold of each other and ≥20-fold greater than those in serum (156).

As a result of their appropriate antibacterial spectrum as well as good tissue distribution profiles, macrolides are excellent therapeutic agents for upper and lower respiratory infections. A high lung-to-plasma ratio is found with newer macrolides such as azithromycin, clarithromycin, and roxithromycin. The ability of macrolides to concentrate in phagocytes and alveolar macrophages (9 to >20 times extracellular levels) accounts for the high tissue concentrations. Clarithromycin attained high and balanced concentrations both intracellularly (to levels severalfold higher than that of serum) and extracellularly (to levels approximately equal to or higher than that of serum). The peak concentration in μg/mL or μg/g of clarithromycin after oral dose of 250 mg bid for bronchial secretion, lung (500 mg bid), tonsil, nasal mucosa, and saliva were 3.98, 13.5, 5.34, 5.92, and 2.22, respectively (157).

The primary site of metabolism of macrolides is the liver and, to a lesser degree, the kidneys and lungs (139). The common metabolic pathways for 14-membered macrolides include the *N*-demethylation of the desosamine by the P450 3A and, to a lesser extent, the hydrolysis of the neutral sugar cladinose. Roxithromycin does not undergo extensive metabolism, with the parent compound found in both urine and feces. Minor metabolites, the mono- and di-*N*-demethyl, and the dew cladinosyl derivatives were found in the urine (158). Transformations specific for individual macrolides are given below. Erythromycin A undergoes an intramolecular cyclization reaction under acidic conditions to form the 8,9-anhydroerythromycin-6,9-hemiketal (**33**) and subsequently the erythromycin-6,9;9,12-spiroketal (**34**) (159).

Erythromycylamine (35) is the principal metabolite of dirithromycin found in both urine and feces (160). Azithromycin does not metabolize extensively, with the parent compound accounting for 75% of the excreted drug-related substances (161). Minor metabolites are derivatives resulting from various reactions or combinations thereof, such as 3′-N-demethylation, 9a-*N*-demethylation, hydrolysis of the cladinose, 3″-*O*-demethylation, hydroxylation, and hydrolysis of the lactone core. 14-(*R*)-Hydroxyclarithromycin (36) was the major metabolite found in the plasma in humans together with as many as eight minor



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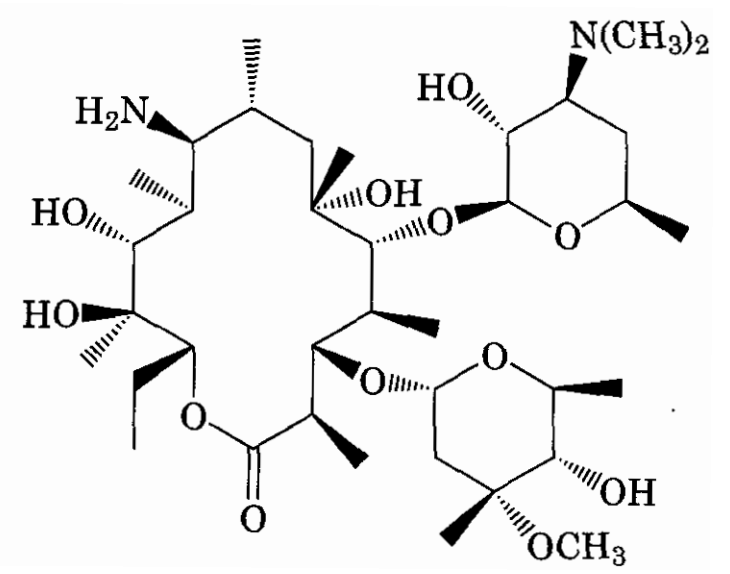

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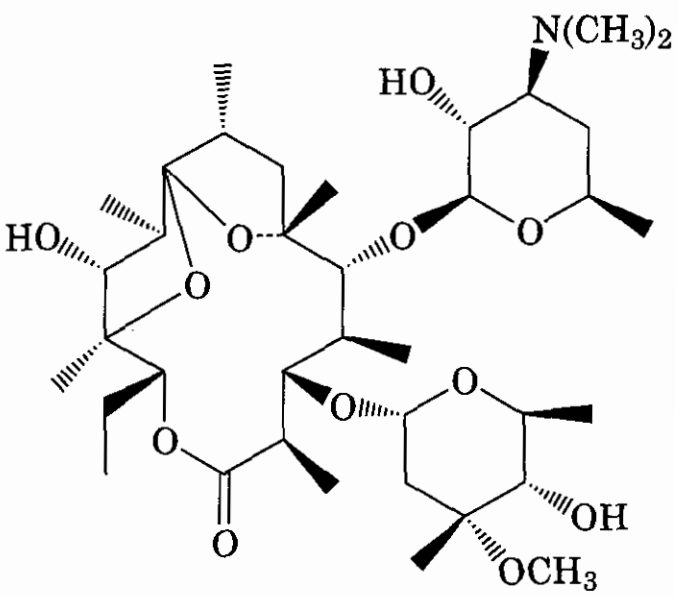

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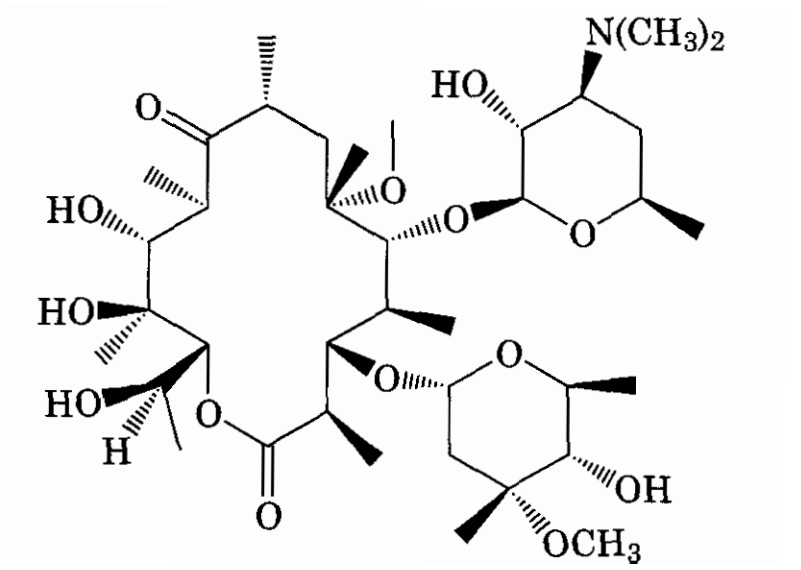


(36)

metabolites (162). Combination of the 14-(*R*)-hydroxyclarithromycinand the parent clarithromycin produced a synergetic effect against H. *influenzae* (163). As for the metabolism of 16-membered macrolides, deacylation of the esters and hydroxylation by enzymatic oxidative reaction are more important than *N*-demethylation (164).

Macrolide antibiotics inhibit protein biosynthesis through interaction with the 50S subunit of the bacterial ribosome. Erythromycin A possesses high specificity and affinity for the bacterial 50S subunit, having a dissociation constant of approximately 10^{-8} M, as measured by equilibrium dialysis (165) and footprinting (166). Specifically, this class of antibiotics binds to the ribosomal P-site. The exit of this site includes a tunnel through which the newly formed peptide emerges after peptide bond formation (167,168). The atomic structures of the macrolides erythromycin, clarithromycin, and roxithromycin bound to the 50S ribosomal subunit of Deinococcus *radiodurans* have been determined and have revealed that these antibiotics block the entrance to the peptide elongation tunnel, thereby providing the molecular rationale for inhibition of translation by these antibiotics (Fig. 15.11) (169).

4.4 History, Biosynthesis, and Structure-Activity Relationships of the Macrolide Antibiotics

The most widely used macrolide antibiotic, erythromycin A was first identified in the fermentation products of a strain of *Saccharopolyspora erythraea* (formerly known as

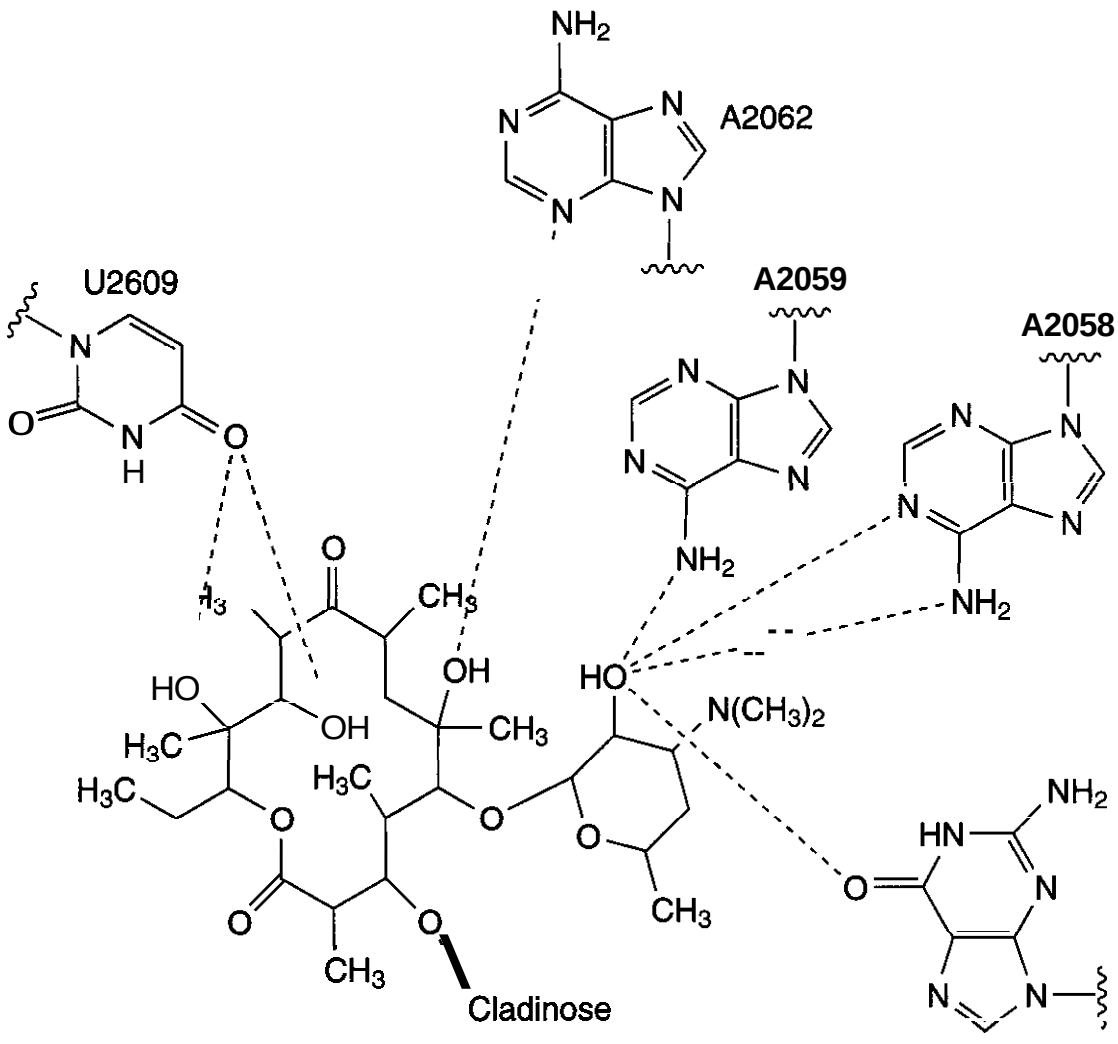


Figure **15.11.** Interaction of erythromycin with the 50S ribosomal subunit of D. ***radiodurans*** (redrawn from Ref. 169). The equivalent E. *coli* numbering is shown and the interaction with G2057 is predicted based on the interaction with **A2040** for the D. ***radiodurans*** 23S rRNA.

Streptomyces erythreus) isolated from a soil sample from the Philippines (170). The various aglycones (12-, 14-, and 16-monocyclic lactones) are produced through polyketide biosynthetic pathways. Thus, many macrolides with their core lactones share similar substituents and stereochemistry predicted by the polyketide biosynthetic mechanisms (170, 171). The biosynthesis of the macrolides can best be illustrated by the synthesis of erythromycin A (Fig. 15.12). Erythromycin A is composed of a 14-membered aglycone, to which are attached 6-deoxysugars, D-desosamine at C-5, and L-cladinose at C-3. The aglycone deoxyerythronolide (37) is assembled by a polyketide synthase complex, deoxyerythronolide B synthase (DEBS) encoded by the *eryAI*, *eryAII*, and *eryAIII* genes corresponding to DEBS1, DEBS2, and DEBS3, respectively (172). After the completion of the synthesis of (37) by DEBS, it is hydroxylated at C-6 by the P450 hydroxylase EryF to produce erythronolide B (38) (173, 174). Addition of L-mycarose by the gene products of the EryB locus yields 3-α-mycarosylerythronolide B (39). Subsequent addition of D-desosamine by EryC-associated enzymes gives the first bioactive macrolide in the biosynthetic pathway erythromycin D (40). Conversion of (40) to erythromycin A is accomplished by two enzymatic actions: (1) a P450 hydroxylase encoded by *eryK* gene hydroxylates the C-12 of erythromycin D [to give erythromycin C (**41**)] (175) and (2) an O-methyltransferase encoded by *eryG* gene methylates the hydroxyl at C″-3 on the mycarose moiety of erythromycin C [to yield erythromycin A (**20**)] (176).

The 14-membered macrolides are in general more potent, having lower MIC values than those of the 12- or 16-membered macrolides (177). Currently, the 14- and 15-membered macrolides are by far the most useful in terms of use as human therapeutics. Their comparative in vitro antibacterial activities are given in Table 15.6.

Many chemical modifications of erythromycin have been performed over the years. The orientation of the sugars relative to the

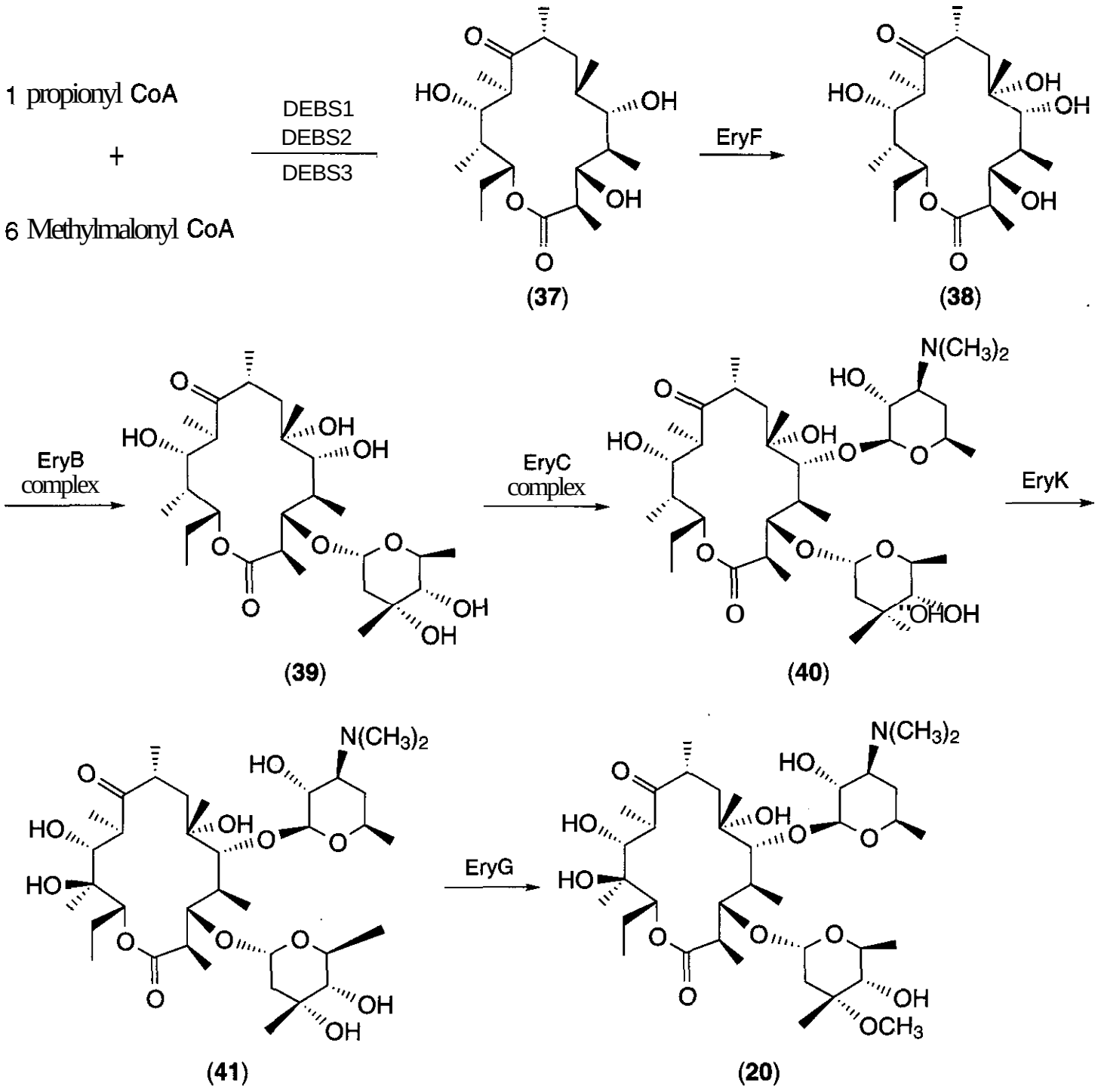


Figure 15.12. Biosynthesis of erythromycin by S. *erythraea.*

aglycone macrolactone in erythromycin **A** is considered to be important to ribosomal binding and hence antibacterial activity. The conformation of the erythronolide is therefore important because of its influence on the interaction of erythromycin **A** with the bacterial ribosome (178). Erythromycin **A** is acid labile, making it readily susceptible to degradation in the stomach. The acid-conversion products (33) and (34) lack antibacterial activity. Research efforts in the past several years were focused on strategies to prevent this undesirable acid degradation.

In the early years, relatively water-insoluble, acid stable salts, esters, and formulations were developed to protect erythromycin A's passage through the stomach. **An** additional benefit from this approach was that these compounds mask the bitter taste associated with macrolides. Ester derivatives of erythromycin **A** were prepared by the acylation of the 2'-hydroxyl group. These esters (such as erythromycin propionate, acetate, and ethyl succinate) are **prodrugs** and are converted back to the active parent erythromycin **A** by hydrolysis in the body. Triacetyloleandomycin (27) possesses improved oral bioavailability and taste over that of oleandomycin.

The initial erythromycin **A** acid-degradation product (33) is formed by the intramolecular cyclization of the 9-ketone and C-6-hydroxyl groups. The subsequent production of a

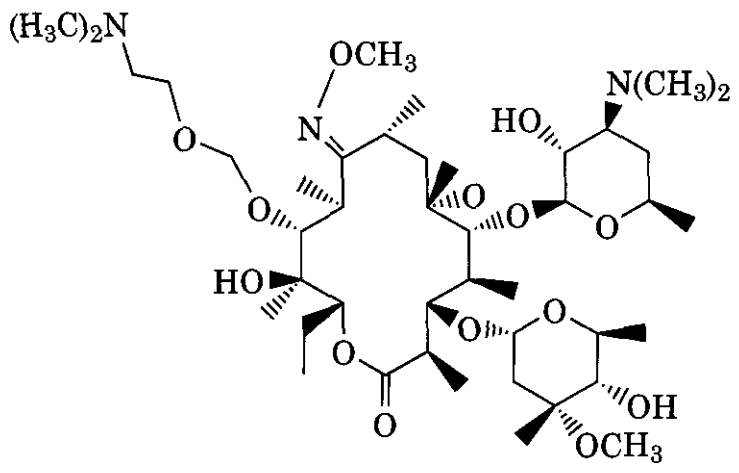
Table 15.6 Comparative *In* Vitro Activities of Selected Macrolides[a]

Organism	MIC_{90} (μg/mL) of							
	E	C	A	R	EA	D	F	AZ
S. aureus	>128	>128	>128	>128	>128	>128	>128	>128
MRSA	>128	>128	>128	>128	>128	>128	>128	>128
S. epidermidis	>128	>128	>128	>128	>128	>128	>128	>128
S. pyogenes	0.03	0.015	0.03	0.06	0.12	0.12	0.06	0.12
S. pneumoniae	0.03	0.015	0.015	0.03	0.06	0.12	0.06	0.12
S. agalactiae	0.06	0.06	0.06	0.25	0.12	0.25	0.12	0.12
Corynebacterium spp.	16	4	8	16	8	8	16	128
L. monocytogenes	0.5	0.25	0.5	1	1	2	0.5	2
B. catarrhalis	0.25	0.25	0.12	1	0.25	0.25	0.25	0.06
N. gonorrhoeae	0.5	0.5	0.5	1	2	4	1	0.06
C. jejuni	1	2	2	4	1	0.25	1	0.12
L. pneumophila	2	0.25	0.5	0.5	8	16	2	2
H. influenzae	4	8	4	8	8	8	8	0.5
B. pertussis	0.03	0.03	0.06	0.25	0.03	0.03	0.06	0.06
B. fragilis	4	2	1	32	32	>128	8	2
C. perfringens	1	0.5	0.5	2	1	4	2	0.25
P. acnes	0.03	0.03	0.06	0.06	0.5	0.5	1	0.03
P. streptococcus spp.	4	4	4	32	>128	>128	16	2
Enterococcus spp.	>128	>128	>128	>128	>128	>128	>128	>128

[a]Data taken from Ref. 177. E, erythromycin; C, clarithromycin; A, A-62671; R, roxithromycin; EA, erythromycylamine; D, dirithromycin; F, flurithromycin; AZ, azithromycin; MRSA, methicillin-resistant Staphylococcus aureus.

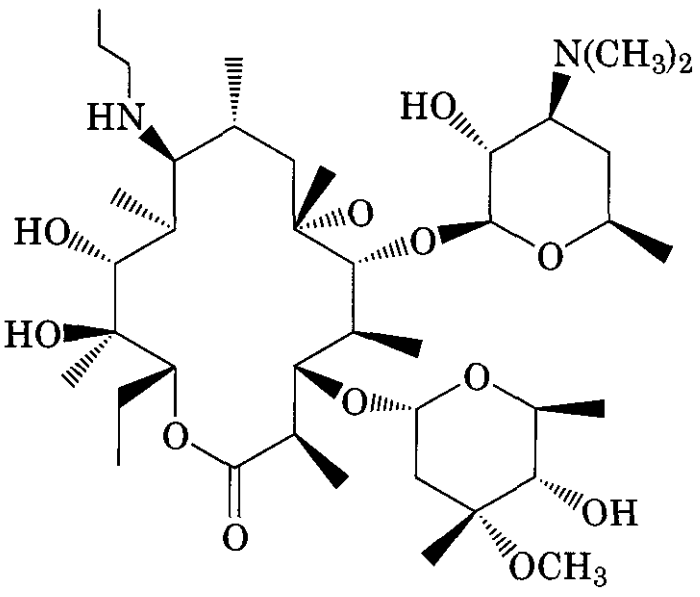
6,9;9,12-spiroketal (34) involves the action of C-12 hydroxyl group on the C-8–C-9 olefin. Chemical modification aimed at these functional groups (i.e., at positions C-9, C-8, C-6, and C-12) was attempted in search of stable derivatives with antibacterial activity.

4.4.1 C-9 Ketone Modification. Reduction of erythromycin gave 9-dihydroerythromycin, which is more stable than erythromycin A but has less potent antibacterial activity (179). However, reduction of the oxime or hydrazone of erythromycin yielded 9-(*S*)-erythromycylamine (35) with antibacterial activity similar to that of erythromycin A but now with decreased oral bioavailability (180). Five series of 9-oximino-ether derivatives of erythromycin A have been prepared and evaluated (181). These are aliphatic-, aromatic-, oximino-ethers containing nitrogen, oxygen, or sulfur atoms. The oxime with E stereochemistry is more active than the isomer with *Z* configuration. The more important of these derivatives is roxithromycin (25), a derivative having a methoxyethoxyether side-chain (182). It possesses slightly less antibacterial activity than that of erythromycin A, with the exception of *Ureaplasma urealyticum*, against which it is more potent. Its antibacterial spectrum is similar to that of erythromycin A (183). The 11-*O*-alkyl-9-oximino ether ER 42859 (42) was also prepared and is more acid stable than erythromycin (184).



(42)

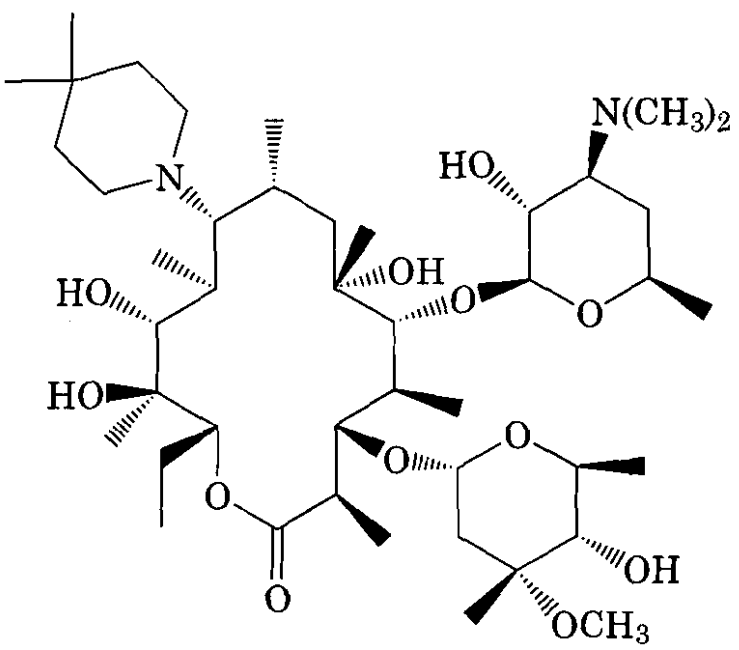
The 9-(*S*)-erythromycylamine was found to be poorly absorbed after oral administration in phase I clinical study (185). To improve its oral bioavailability, a series of 9-N-alkyl derivatives of erythromycylamine were prepared and were found to have potent in vitro and in *vivo* antibacterial activity. The *N*-(1-propyl)-9-(*S*)-erythromycylamine (LY 281389) (43) has in vitro activity similar to that of erythromycin A with onefold better activity against H. *influenzae*. Its in vivo activity is substantially better and it is more acid stable with superior oral bioavailability, achieving higher plasma and tissue levels (186). Although the *S*-isomer is more active in the monosubstituted 9-amino erythromycylamine, for the dialkylamino series, the 9-(R) isomer is more active. A-69991 (44) and A-70310 (45) are the two 9-(*R*) azacyclic erythromycin derivatives with superior in vivo efficacy and pharmacokinetic parameters than those of erythromycin A (187). They are as active as erythromycin A in vitro (188).



(43)

Another approach to improve the oral bioavailability of (35) is to search for a prodrug of erythromycylamine. Dirithromycin rapidly nonenzymatically hydrolyzes under acid condition back to erythromycylamine. When given subcutaneously in mice and rats, the serum levels of dirithromycin and its major metabolite erythromycylamine were found to be higher than the corresponding values obtained for erythromycin A (189).

A series of 9-deoxo-12-deoxy-9,12-epoxy erythromycin derivatives were prepared. A-69334 [(9*S*,11*S*)-11-amino-9-deoxo-11,12-dideoxy-9,12-epoxyerythromycin A (46)] possesses antibacterial activity similar to that of



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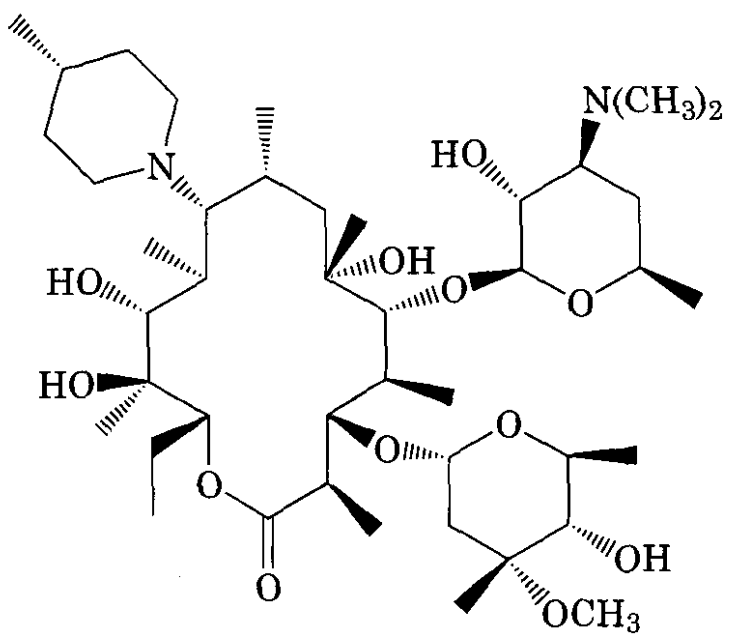

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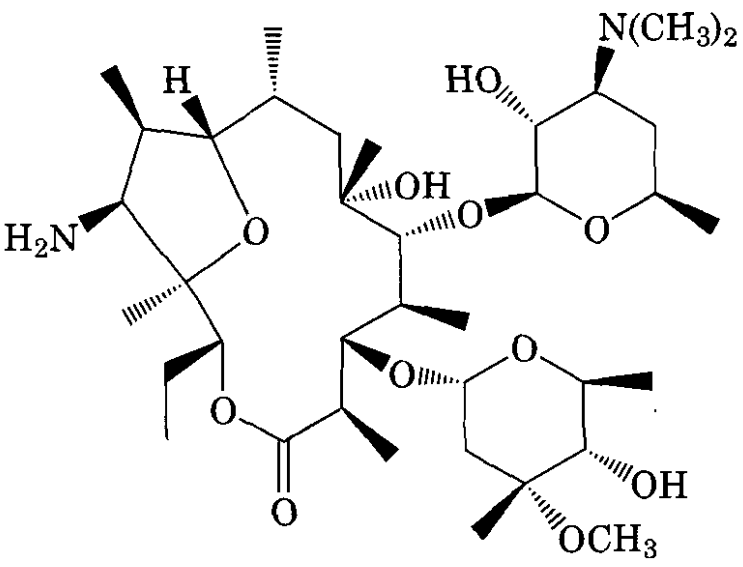

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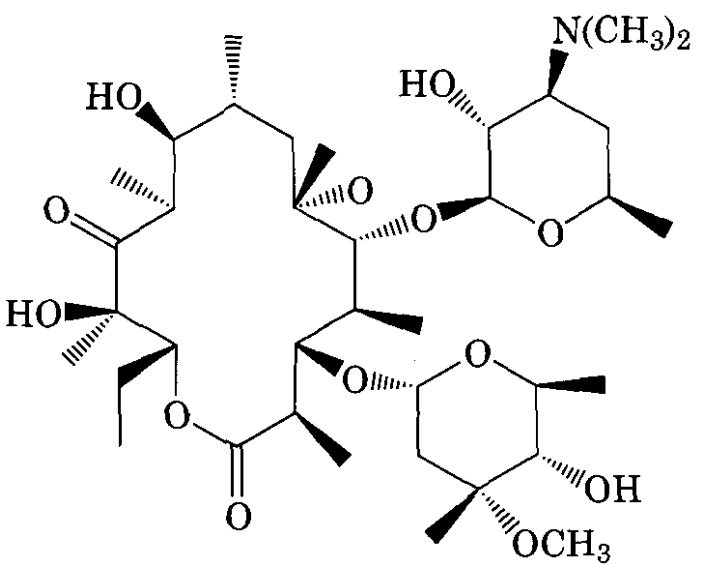


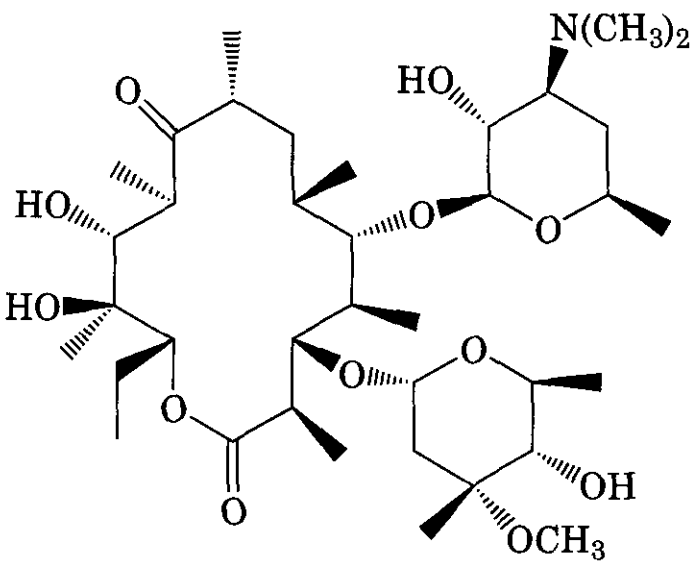
(47)

erythromycin A, with twofold more potent activity in vitro against *S.* pyogenes C203 and S. pneumoniae 6303 (190). It showed superior oral efficacy over that of erythromycin in H. influenzae–induced otitis media in a gerbil model (191). An erythromycin derivative with 9,11 functional groups interchanged, (9S)-11-dehydroxy-9-deoxo-9-hydroxy-11-oxo-erythromycin A (**47**), was made and found to be slightly less active than erythromycin A (192).

4.4.2 C-8 Modification. To prevent the formation of anhydrohemiketal erythromycin (**33**), the 8-fluoro-erythromycin A, flurithromycin, was prepared by the use of both chemical and biotransformation procedures (193, 194). Although its activity against staphylococci, streptococci, *H. influenzae*, and M. *catarrhalis* was similar to that of erythromycin **A,** it was more potent against anaerobes (195).

4.4.3 C-6 Modification. A family of 6-deoxyerythromycins was prepared by using a genetically engineered strain of S. *erythraea*, in which the *eryF* gene (which encodes the P450, C-6–specific hydroxylase) had been inactivated (196). These 6-deoxy derivatives are less potent than their corresponding erythromycins against bacteria in vitro. However, 6-deoxyerythromycin A (48) is as potent as erythromycin A in mouse protection tests against *S.* aureus, S. pyogenes, and S. pneumoniae (197). 6-Deoxyerythromycin A loses antibacterial activity slowly, although there is no C-6 hydroxyl group participation in the degradation process. A loss of the cladinose moiety was ob-

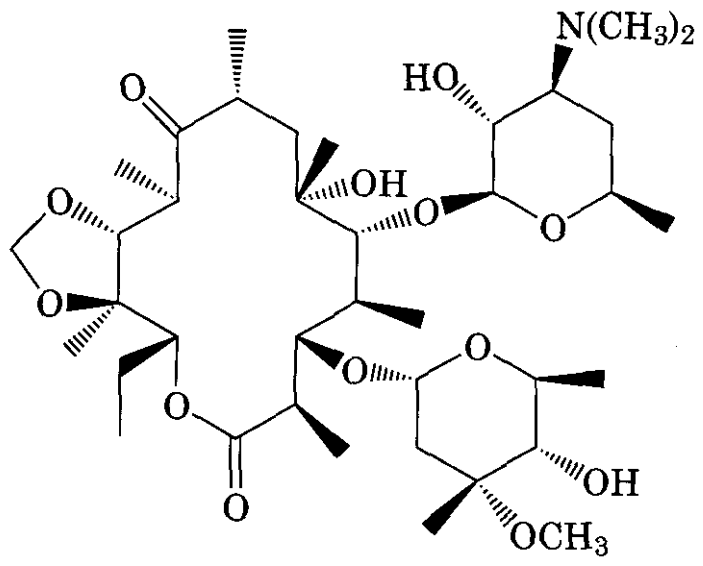
served when it was treated with 10% acetic acid (198). These results suggest that the C-6 hydroxyl group (or another substituent at this position) may be important for biological activity. This hypothesis has been confimed with the availability of the 3D structure of erythromycin bound to the 50S ribosomal subunit, where this hydroxyl is within hydrogen-bonding distance with N of A2062 (E. *coli* numbering) (Fig. 15.11) (169).



(48)

To define the importance of the 6-hydroxyl group on the biological activity of the 14-membered macrolide, a series of 6-*O*-alkyl derivatives of erythromycin have been synthesized (199). Alkylation by small alkyl groups at the 6-hydroxyl was found to have a minimal impact on the biological activity. However, this modification makes the molecule more acid stable, improving its pharmacokinetic profiles. 6-0-Methylerythromycin A, known as clarithromycin, has been developed and is now marketed worldwide (200). 6-0-Methyl derivatives of erythromycin A are much more acid stable than 6-deoxyerythromycin A. Although clarithromycin's antibacterial spectrum is similar to that of erythromycin A (201), it is about half as active as erythromycin A against H. influenzae. Its 14-hydroxy human metabolite, 14-(*R*)-hydroxyclarithromycin, possesses the same MIC against *H.* influenzae as erythromycin (163). Combining it with clarithromycin produced a synergistic effect against H. influenzae both in vitro (202) and in vivo (203). Clarithromycin was found to be safe and effective upon combination with other antimycobacterial agents for the treatment of disseminated M. avium complex infection in AIDS patients (204). Clarithromycin was also found to be effective when combined with a proton pump inhibitor for the treatment of metronidazole-resistant H. pylori–positive gastric ulcer (205).

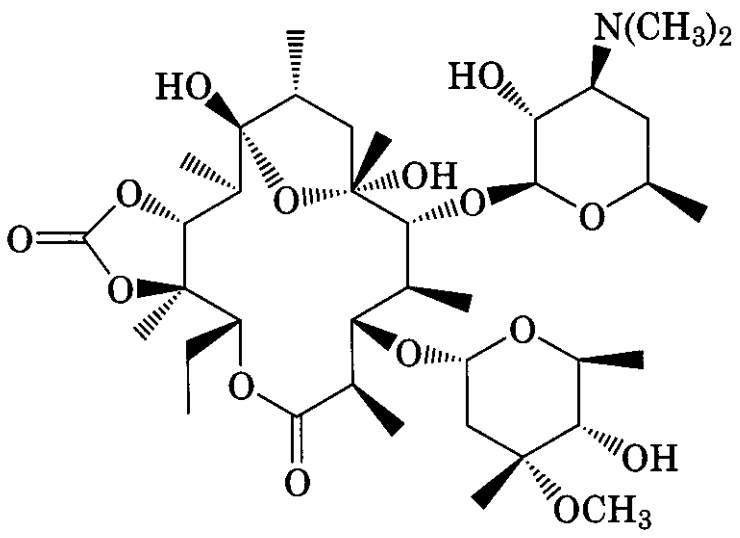
4.4.4 C-11 and C-12 Modification. A series of erythromycin-11,12-methylene cyclic acetals was prepared to test for antibacterial activity. Although the erythromycin-11,12-methyleneacetal (49) is more active than erythromycin A in vitro, it possesses similar efficacy to that of erythromycin A in treating experimental infections in mice (206).



(49)

Erythromycin-11,12-carbonate (50) was prepared to avoid the spiroketal formation during the degradation process of erythromycin A in acid medium (207). It is twice as active in vitro and more stable, with better pharmacokinetics than that of erythromycin A (208). However, it has higher hepatotoxicity potential and its development was discontinued (209, 210). The 3″-fluoro-11,12-carbonate derivatives of erythromycins (51) and (52) possess about half the in vitro activity as that of erythromycin A (211).

A series of 11-deoxy-11-(carboxyamino)-6-0-methylerythromycin A 11,12-cyclic carbamate derivatives (53) have been synthesized (212). They possess in vitro antibacterial activity comparable to that of clarithromycin.



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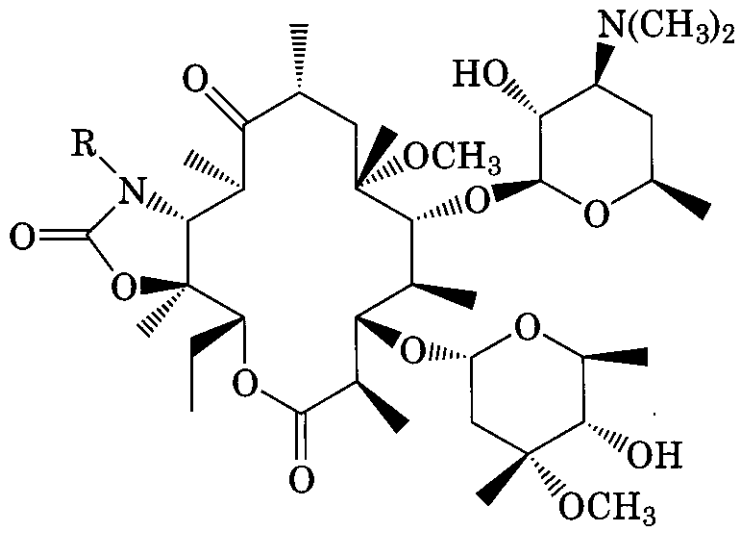

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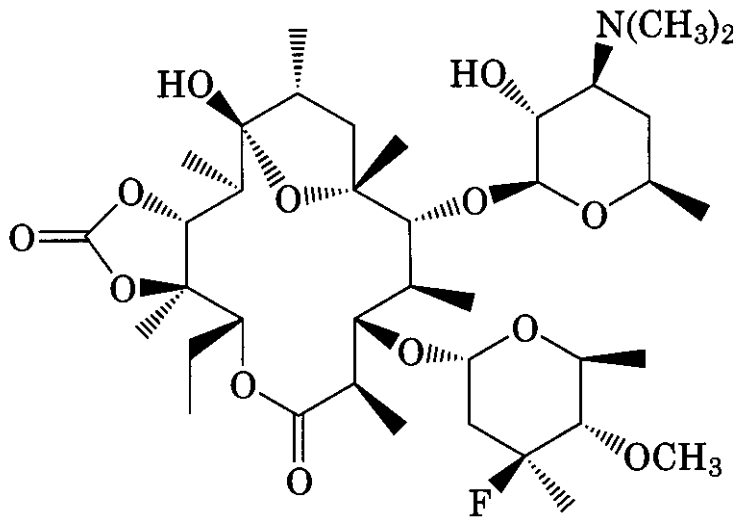

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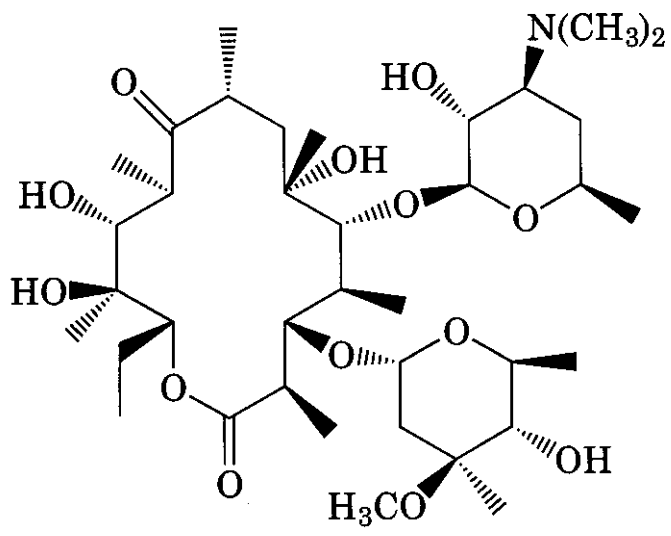

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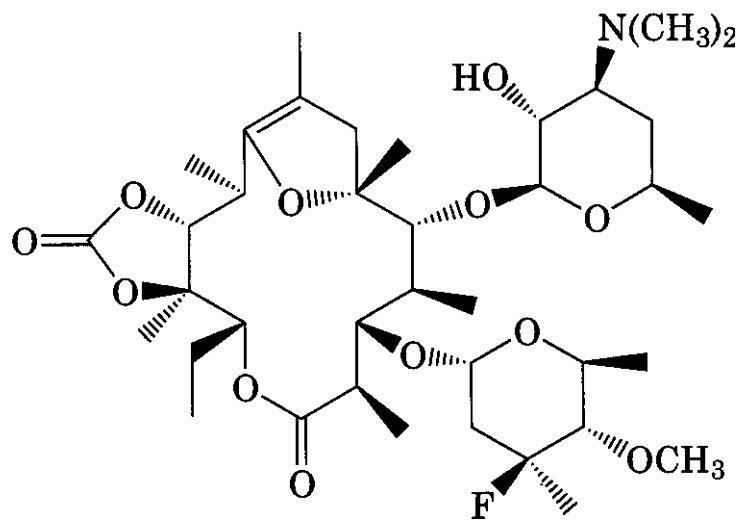


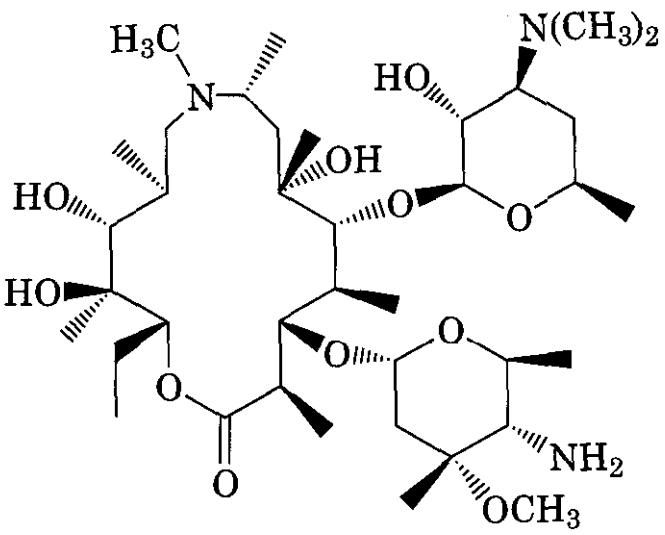
(52)

4.4.5 Cladinose Modification. Modification on the cladinose sugar has been attempted. The 3"-epi-erythromycin **A** (54) has *in vitro* antibacterial activity comparable to that of erythromycin **A,** indicating that the stereochemistry at the **3** position does not have great influence on the antibacterial activity (192).

4.4.6 Core–Skeleton Modification. Natural antibacterial macrolides have 12-, 14-, and 16-membered ring core structures by virtue of their biosynthetic process. The 15-membered macrolides, however, are made synthetically by a Beckmann rearrangement of erythromycin-9-oxime, to produce the ring-expanded derivatives in which an extra amino group is embedded in the 14-membered ring skeleton. These derivatives are named azalides. Azithromycin (21) is made by a Beckmann rearrangement of erythromycin-9-(*E*)-oxime, followed by reduction of the imino ether and subsequent N-methylation (213), and has antibacterial activity comparable to that of erythromycin **A.** Even though it has a 15-membered ring structure, erythromycin-resis-

tant staphylococci show complete cross-resistance to it (214). Azithromycin is more acid stable and the bioavailability in animals was found to be two- or threefold higher than that of erythromycin A (213).

A series of 8a-azalides (9-deoxo-8a-aza-8a-homoerythromycins) was prepared to compare their activities with the 9a-azalide, azithromycin, by Beckmann rearrangement of erythromycin-9-(Z)-oxime followed by reduction of the imino ether and subsequent N-methylation (215). Simple 8a-alkyl derivatives exhibited good antibacterial activity similar to that of azithromycin. Polar 8a–side chain 8a–azalide derivatives, however, have less antibacterial activity. Replacement of the 4″-hydroxyl group with an amino group resulted in a two- to eightfold increase in activity against Gram-negative bacteria but two- to eightfold decrease in activity against Gram-positive bacteria. 9-Deoxo-8a-methyl-8-aza-8a-homoerythromycin A (55) and 4''-deoxy-4''-amino-9-deoxo-8a-methyl-8a-aza-8a-homoerythromycin A (56) were found to be more efficacious than azithromycin and clarithromycin in mouse protection tests because they were more acid stable and had better pharmacokinetic profiles (216,217).



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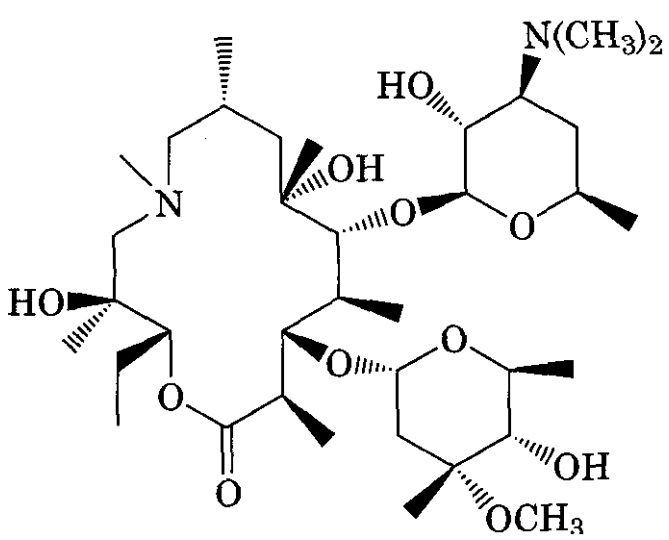

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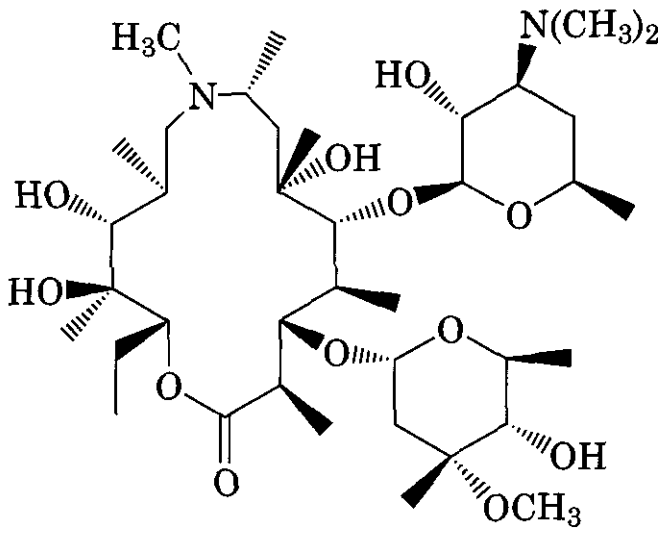

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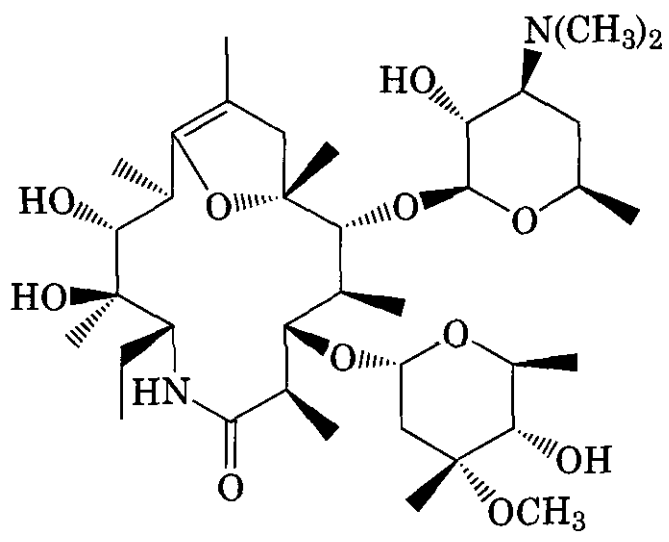


(58)

A series of novel 14-membered azalides was prepared. The 10-aza-10-methyl-9-deoxo-11-deoxyerythromycin (57) has an antibacterial spectrum similar to that of erythromycin A but was less active than either erythromycin A or azithromycin (218,219). The 14-membered lactam (58) was prepared but found to have minimal antibacterial activity but good gastrointestinal motor-stimulating activity (220).

4.5 Resistance to Macrolide Antibiotics

Bacterial resistance to macrolide antibiotics is emerging as a significant problem. Thirty-seven percent of 1113 S. pneumoniae clinical isolates collected between 1996 and 1997 in Spain were found to be resistant to macrolides (221). Based on the susceptibility study on 302 S. pneumoniae isolates from central Italian patients, the erythromycin A resistance rate increased from 7.1% in 1993 to 32.8% in 1997 (222). In a 1996–1997 winter U.S. surveillance study, among 1276 S. pneumoniae clinical isolates, 23% were resistant to erythromycin A, azithromycin, and clarithromycin (223). Although the S. pneumoniae resistance to macrolides is high in the United States as well as in most of Europe, the resistance rates in Norway and Canada are rather low (about 3–5%) (224,225).

With respect to S. pyogenes, 14.5% clinical isolates identified in a Finnish surveillance study were erythromycin resistant (226). In Spain, 27% of the β-hemolytic group A and 12% of group C streptococci isolates collected between May 1996 and April 1997 were erythromycin resistant (221). In France, the erythromycin resistance rate in viridans group streptococci was 40% (227), whereas in Taiwan, 55% of S. *oralis* isolates were erythromycin resistant (228). Antimicrobial susceptibility data from the SENTRY antimicrobial surveillance program conducted in the United States and Canada indicated that M. *catarrhalis* and H. *influenzae* isolates remain highly susceptible to macrolides with less than 1% and 5% resistance, respectively (223,229).

The molecular mechanisms of resistance to macrolides in pathogenic bacteria has been summarized and reviewed (230). Resistance to macrolides is found to occur by one of the three mechanisms: target-site modification, active efflux, and enzyme-catalyzed antibiotic inactivation.

Target-site modification is by far the most clinically significant resistance mechanism for macrolides. Two types of target-site modification resistance for macrolides have been described: (1) posttranscriptional modification of the 23S rRNA by adenine-$N^{6,6}$-dimethyltransferase (MLS_B resistance), and (2) site-specific mutations in the 23 rRNA gene. The MLS,- resistance confers resistance to macrolide, lincosamide, and streptogramin B classes of antibiotics. This resistance is mediated by plasmid or transposon-encoded genes called ermn (erythromycin-resistant methylases), encoding enzymes that catalyze the $N^{6,6}$-dimethylation of the adenosine-2058 residue of bacterial 23S rRNA (E. coli numbering) (231, 232). The determination of the 3D structures of macrolide antibiotics bound to the' ribosomal 50S subunit provides insight into the molecular basis of Erm-mediated resistance (169). In these structures, the hydroxyl group of the macrolide desosamine sugar forms hydrogen bounds with N1 and N6 of A2058; methylation of N6 therefore generates a steric block of this interaction, resulting in resistance (Fig. 15.13).

The $N^{6,6}$-dimethyltransferases have been found in numerous organisms including staphylococci, streptococci, enterococci, *Clostridium difficile*, E. coli, and Bacillus strains. The erm-mediated resistance phenotype can be constitutive or induced (233,234). Enzyme induction is affected by exposure of the organism to both 14- and 15-membered, but not 16-membered, macrolides. Over two dozen erm genes have been identified encoding proteins of about 29 kDa in size (235). These genes are widely distributed in Gram-positive bacterig and, paradoxically, in many Gram-negative bacteria, which are generally not susceptible to macrolides as a result of the impermeable outer membrane. The presence of erm genes also confers reduced susceptibility to azithromycin (236).

The 3D structures of ErmC′ and ErmAM have been determined by X-ray crystallography and NMR methods, respectively (237, 238). These enzymes are approximately 50% identical and, not surprisingly, then show very similar folds consisting of an amino-terminal S-adenosyl-methionine binding domain and a helical C-terminal RNA binding domain. The structures of ErmC′, in complex with the substrate S-adenosyl-methionine, the product S-adenosyl-homocysteine,and the methyl transferase inhibitor sinefungin, have also been reported (239). These structures therefore provide the opportunity to develop inhibitors of Erm activity that could be used to block resistance. Several Erm inhibitors have in fact

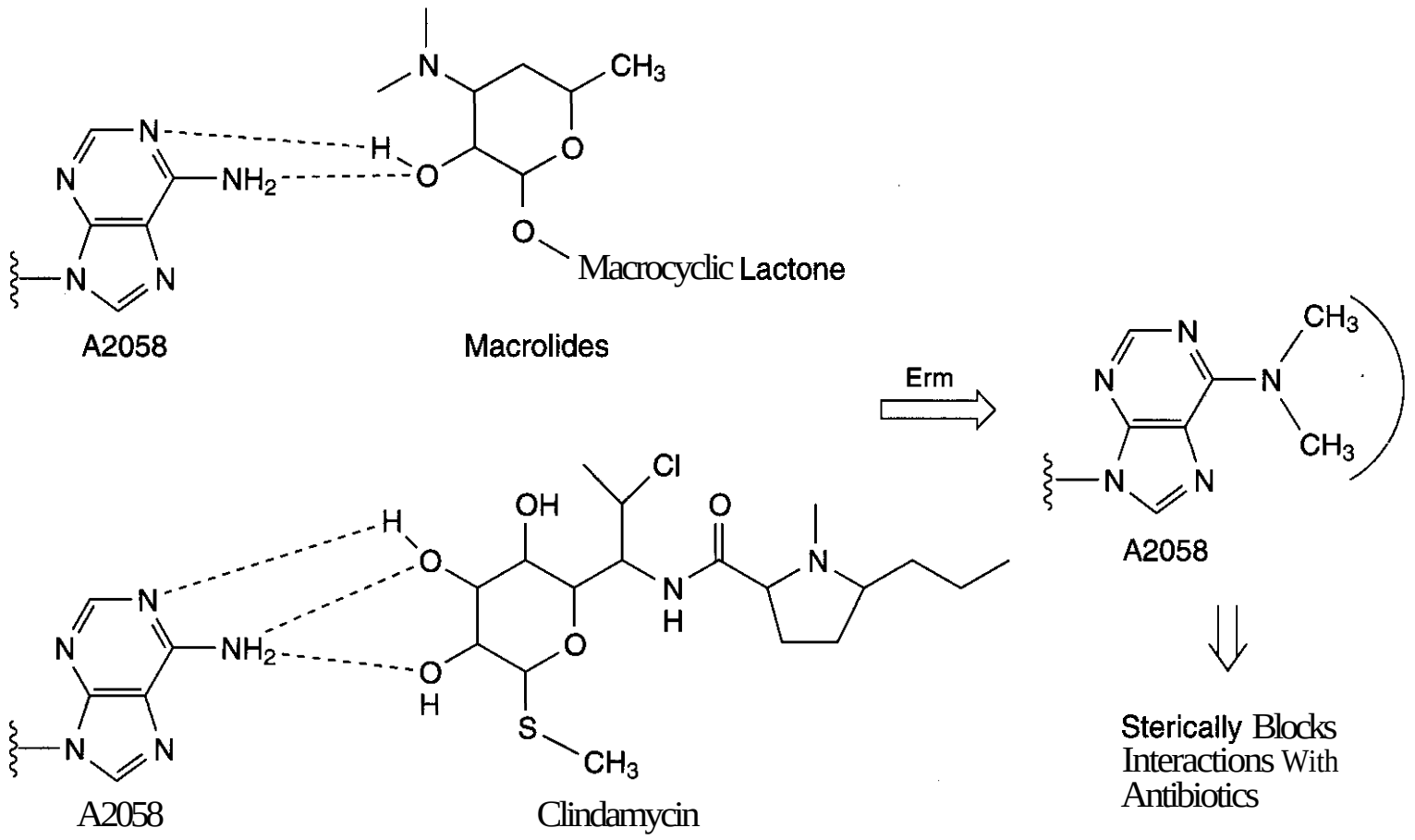


Figure 15.13. Outcome of methylation of A2058 by Erm resistance enzymes.

been identified by high throughput screening (240) and the SAR by NMR (241) methods, and phage display has also been used to discover inhibitory peptides (242). These studies indicate that it may be possible to identify molecules that block Erm-catalyzed methylation of the ribosomes.

The second target-site modification mechanism conferring macrolide resistance is through site-specific mutation in the 23S rRNA gene (reviewed in ref. 243). In H. pylori, A2142G mutation was linked with high level cross-resistance to all MLS, antibiotics. The A2143G mutation gave rise to an intermediate level of resistance to clarithromycin and clindamycin, but not streptogramin B (244, 245). Other examples of site-specific mutations include A2063G or A2064G in M. pneumoniae; A2059G or C2611A and G in S. *pneumoniae*; A2058C, G, and U in M. *avium*; and A2048G, G2057A, or A2059G in Propionibacteria (243). The number used here corresponds to the position in E. *coli* 23S rRNA. Novel mutations in either 23S rRNA alleles or ribosomal protein L4 were recently found to be responsible for macrolide resistance in S. pneumoniae isolates passaged with azithromycin (246).

Active efflux of macrolides is a fairly common mechanism for erythromycin A resistance in S. pyogenes and S. *pneumoniae* (247). In some of these bacteria, this type of resistance is attributed to the presence of the *mefA* gene, which. encodes a membrane-associated efflux protein that confers resistance to macrolides but not to lincosamides or streptogramin B (248). The *mefE* gene (90%sequence identity to *mefA* in S. *pyogenes*) was found to be present in erythromycin-resistant M phenotype (macrolide resistant but clindamycin and streptogramin B susceptible) in S. *pneumoniae* (249). The macrolide efflux gene *mreA*, distinct from the *mefA* in S. pneumoniae and S. pyogenes and the multicomponent *mrsA* in S. *aureus*, was found in a strain of S. agalactiae, which displayed resistance to 14-, 15-, and 16-membered macrolides (250). Several S. agalactiae clinical isolates with the M phenotype harbor *mefA* and *mefE* genes (251). The efflux system is the major contributor to the macrolide resistance in *Burkholderia pseudomallei* (252). The mefgene has been identified in clinical isolates of *Acinetobacter* junii and Neisseria gonorrhoeae. These strains could transfer the *mef* gene into one or more of the following recipients: Gram-negative M. *ca-*

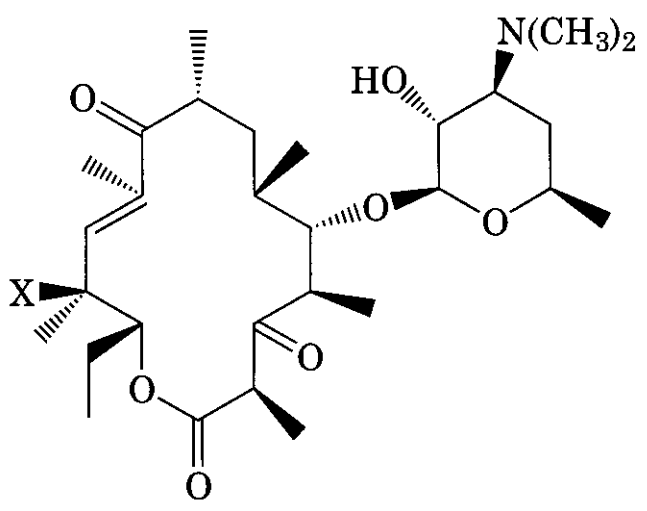
tarrhalis, N. *perflava*, and N. mucosa and Gram-positive E. faecalis (253). The *mtrCDE*-encoded efflux pump has been suggested to be one of the resistant mechanisms in N. gonorrhoeae. The *mtrCDE* genes constitute a single transcriptional unit that is negatively regulated by the adjacent *mtrR* gene product. N. gonorrhoeae clinical isolates with mutation in *mtrR* transcriptional repressor gene possessed reduced azithromycin susceptibility (253). The *mrsA* and *mrsB* genes are also efflux systems that are mostly isolated from S. aureus and S. *epidermidis* (254); a homolog *mrsC* was isolated from *Enterococcus* faecium (255).

Macrolide resistance by antibiotic inactivation can also occur through phosphorylation (256, 257), deesterification of the lactone ring (256, 257), and glycosylation (258–260). Two glycosyltransferases and a glycosidase are involved in oleandomycin modification (261). A glycosyl transferase inactivating macrolides encoded by *gimA* from S. ambofaciens was found to locate downstream of *srmA*, a gene that confers resistance to spiramycin and has a high degree of similarity to S. lividans glycosyl transferase, which inactivates macrolides (262). The E. coli clinical isolate BM2506 is highly resistant to macrolides by having macrolide 2′phosphotransferase II [MPH(2′)II]. This strain is found to harbor two plasmids, pTZ3721 (84kb) and pTZ3723 (24kb). It appears that the *mphB* gene is located on these two plasmids in BM2506 and can be transferred to other strains of E. coli by conjugation or mobilization (263).

A minireview on new nomenclature for macrolide and macrolide–lincosamide–streptogramin B (MLS_B) resistance determinants was recently published (264). The common macrolide phenotypes are: MLS_B for strains that carry Erm methylase; M for strains resistant to macrolide but are clindamycin and streptogramin B susceptible; ML for strains resistant to 14-, 15-, and 16-membered macrolide and lincosamides; and MS for strains resistant to macrolide and streptogramins.

4.6 Recent Developments in the Macrolide Antibiotic Field

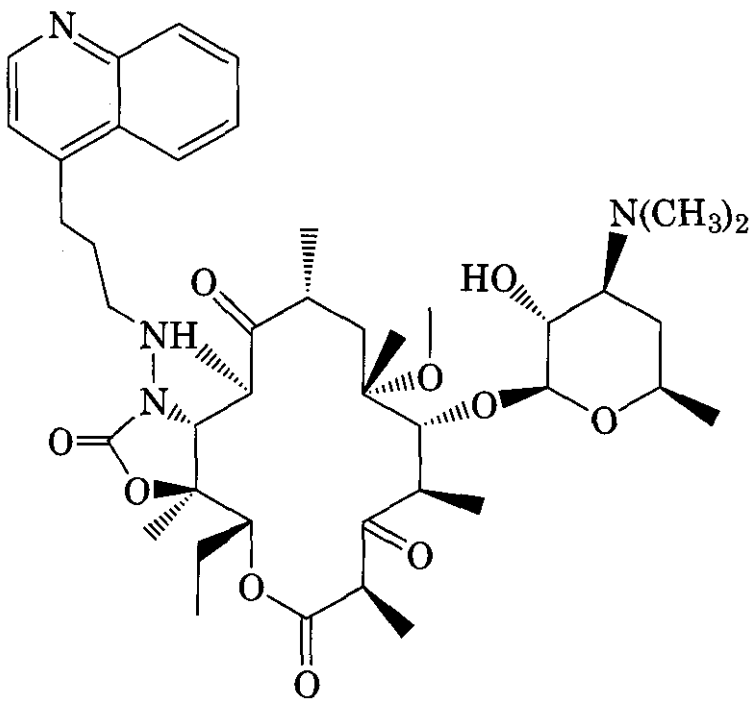
4.6.1 Novel Macrolides to Overcome Bacterial Resistance. Several macrolide derivatives having potent antibacterial activity against erythromycin-sensitive strains and at the same time with moderate activity against erythromycin-resistant S. pyogenes were first reported in 1989 (265). The 11,12-carbamate clarithromycin analogs and the 11,12-carbonate erythromycin analogs with additional modifications at the 4' position of the cladinose were found to be active against both inducible and constitutive resistant S. pyogenes. The two naturally occurring descladinosyl 14-membered macrolide derivatives, narbomycin (59) and picromycin (**60**), with the presence of a 3-keto group, were isolated in the early 1950s (266, 267). Although these two compounds are poorly active, they are not inducers of macrolide resistance. With these new insights provided by the above information, recent chemical modifications of erythromycin have generated several new classes of macrolide derivatives having potent activity against erythromycin-sensitive and -resistant bacteria.



(59) X = H
(60) X = OH

4.6.1.1 Ketolides. Apart from the natural products narbomycin and picromycin, certain recently synthesized 3-keto macrolide derivatives were found to be active against both penicillin-resistant and erythromycin-resistant S. pneumoniae. Like narbomycin and picromycin, these derivatives do not induce MLS, resistance in staphylococci and streptococci (268). These 3-descladinoxyl-3-oxo-11,12-cyclic carbarnate derivatives of erythromycin or clarithromycin are called ketolides. So far, three ketolides have undergone clinical development: HMR 3004 (**61**), HMR 3647 (telithro-

mycin, (**62**), and ABT-773 (**63**). The ketolides are very active against respiratory pathogens, including erythromycin-resistant strains (269, 270).



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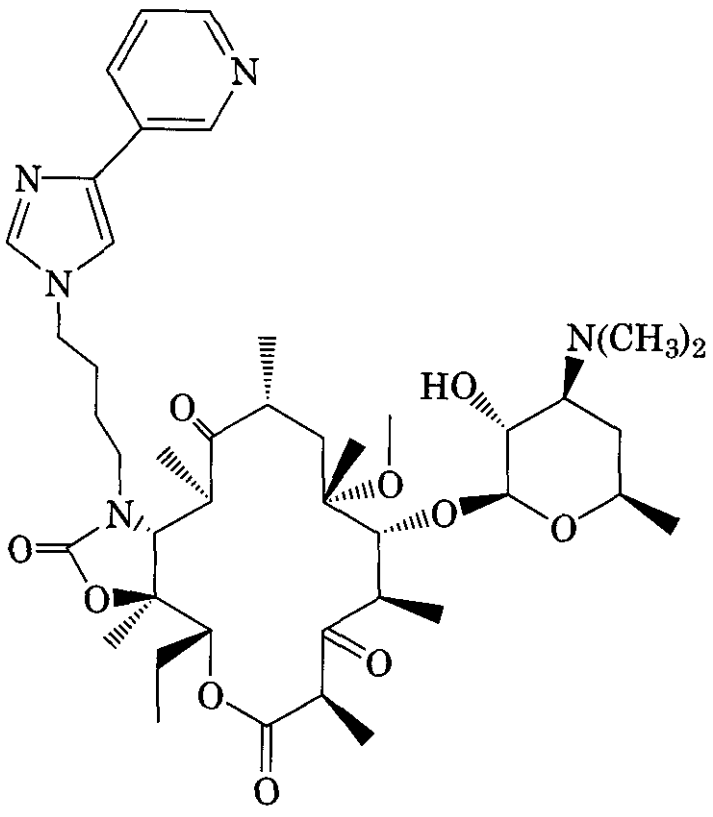
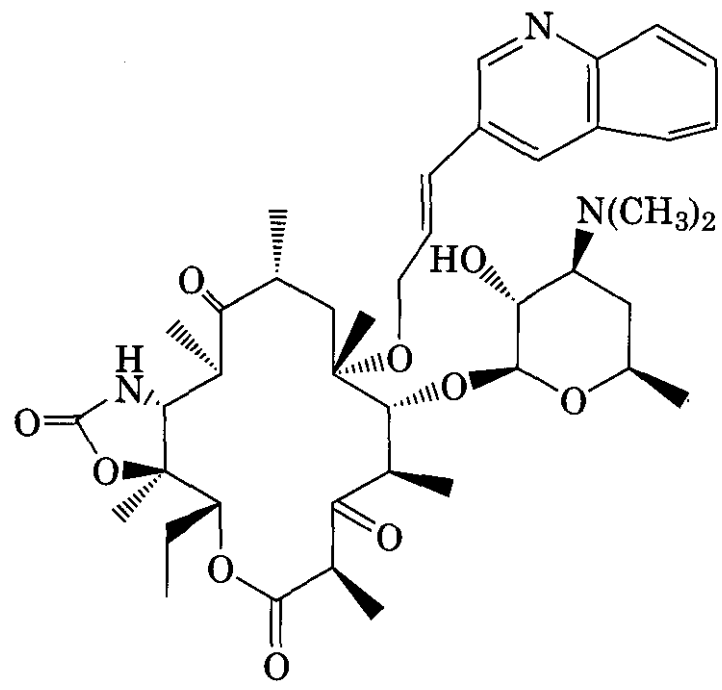


(62)

HMR 3004 is very active against penicillin- and erythromycin-resistant pneumococci with a MIC_{90} value of 0.25 μg/mL (271, 272) and is very efficacious against respiratory infections in animal studies (273). It was found to be



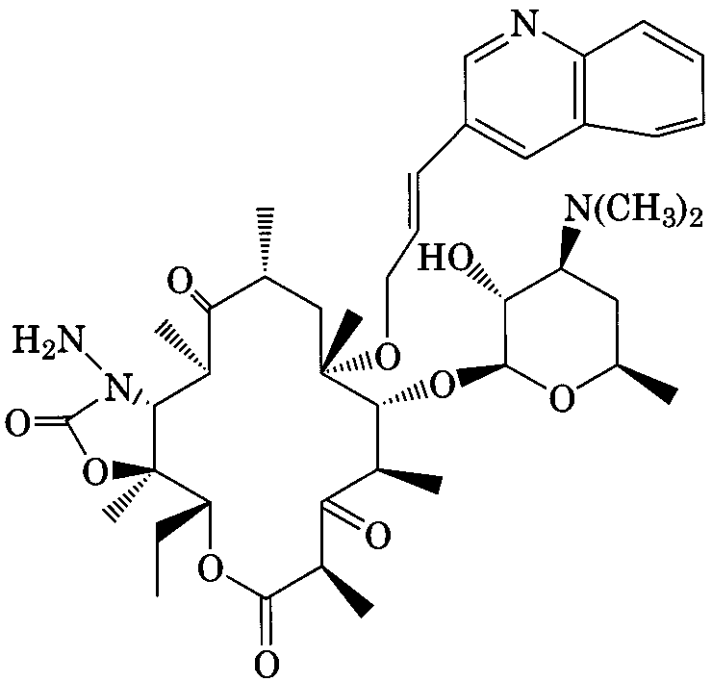
(63)

active against β-lactamase–producing H. *influenzae* in a murine model of experimental pneumonia and more active than azithromycin, ciprofloxacin, clarithromycin, erythromycin, and pristinamycin (274).

Another ketolide being developed is telithromycin (HMR 3647). The U.S. FDA issued an approval letter for telithromycin in 2001 for the following indications: community-acquired pneumonia, acute bacterial exacerbation of chronic bronchitis, and acute bacterial sinusitis. Against H. influenzae, telithromycin is as active as azithromycin, with a MIC_{90} value of 4 μg/mL (275). When tested against M. catarrhalis, it has MIC_{50}/MIC_{90} values of 0.06/0.125 μg/mL (276). It has MIC_{90} and MBC_{90} values of 0.25 μg/mL (277). The pharmacodynamic properties of telithromycin demonstrated by time-kill kinetics and post-antibiotic effect on enterococci and *Bacteroides fragilis* were found to be similar to those obtained with macrolides (278). Although it is found to be active against MLS_B-resistant pneumococci, telithromycin did not bind to the methylated ribosomes isolated from the MLS_B-resistant strain (279). In a murine model of experimental pneumonia, telithromycin was effective against β-lactamase–producing H. *influenzae* (274). It is also effective for the treatment of L. *pneumophila* in a guinea pig pneumonia experimental model (280). In a mouse peritonitis model of enterococci infection, telithromycin was found

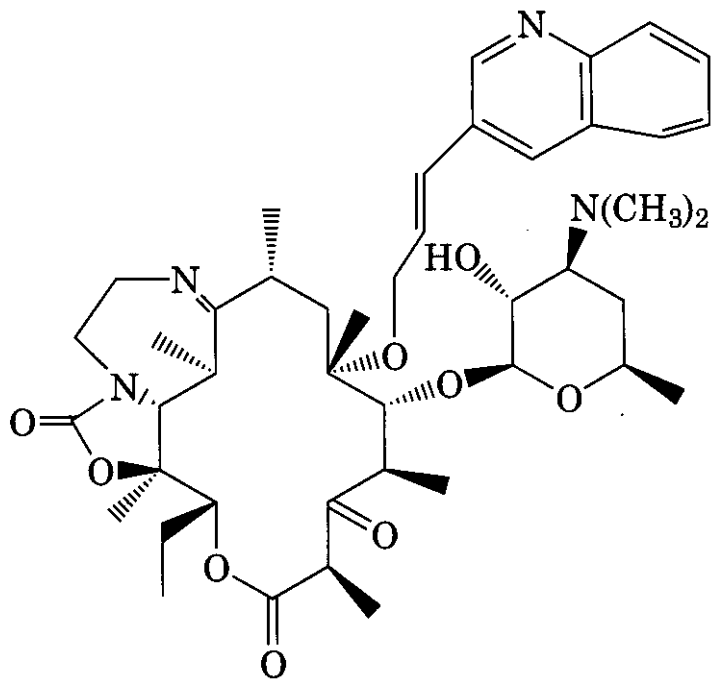
to be highly active against erythromycin-susceptible and -intermediate strains (281). HMR 3004 and telithromycin were found to be highly active ***in vitro*** against ***M. pneumoniae*** (282), ***E. faecalis*** (283), and *Bordetella pertussis* (284). The oral antibacterial activities of telithromycin in different infections induced in mice by ***S. aureus, S. pneumoniae,*** streptococci, enterococci, and H. ***influenzae*** were found to be excellent (285).

A series of 6-0-substituted ketolides was reported to possess excellent activity against inducible resistant S. ***aureus*** and ***S. pneumoniae*** as well as constitutively resistant *S. **pneumoniae*** (286). Derivatives with an aryl group at the 6-0 position with a propenyl spacer together with an 11,12-cyclic carbamate and a 3-keto group exhibited the best activity. In this study, the unsubstituted 11,12-cyclic carbamate analogs were more active than either the carbazate or tricyclic analogs [ABT-773 (63)versus A-201316 (64) versus 197579 (**65**)].



(64)

ABT-773 is currently in late-stage clinical trials. It has a potent antibacterial spectrum including activity against penicillin- and macrolide-resistant Gram-positive bacteria (270, 287). ABT-773 was found to be the most active compound tested among other macrolides against ***S. pneumoniae*** with MIC_{90} value of 0.03 μg/mL. It is as potent as azithromycin but



(65)

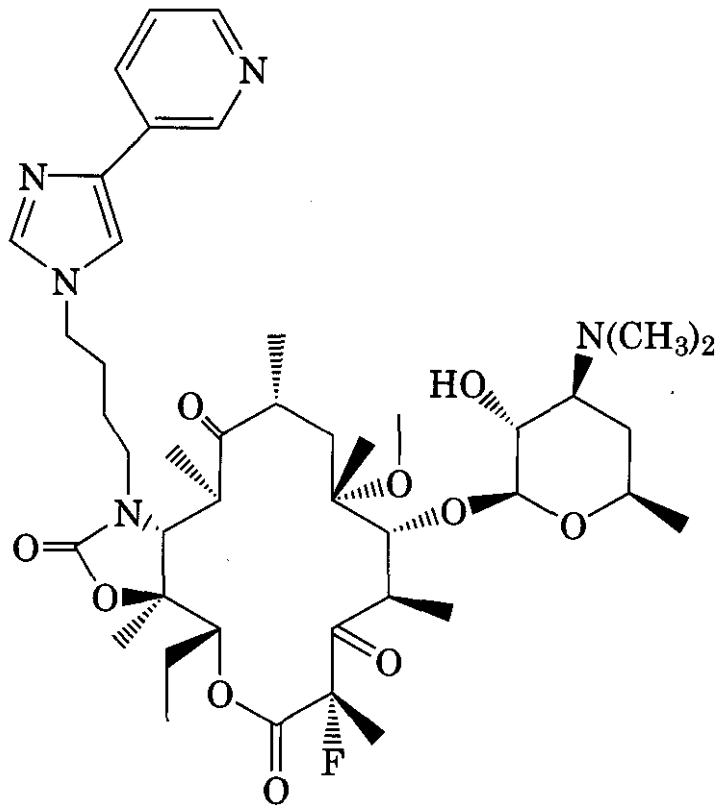
more potent than clarithromycin and erythromycin A against H. ***influenzae*** (MIC_{90} value = 4 μg/mL) and M. ***catarrhalis*** (MIC_{90} value = 0.06 μg/mL). It also has good activity against Gram-negative and atypical respiratory tract pathogens and H. ***pylori*** (MIC_{90} value = 0.06 μg/mL against macrolide-susceptible strain) (288). ABT-773 is active against anaerobic bacteria. For ***B. fragilis,*** telithromycin is one to two dilution levels less active than ABT-773. For all anaerobic tested strains, ABT-773 was found to be more active than erythromycin A by four or more dilution levels (289). A comparative study on the ***in vitro*** antibacterial activity of ABT-773 against 207 aerobic and 162 anaerobic antral sinus puncture isolates showed that erythromycin-resistant pneumococci strains were susceptible to ABT-773 with MIC_{90} value of 0.125 μg/mL (290). ABT-773 had superior activity against macrolide-resistant ***S. pneumoniae*** than that of telithromycin with MIC_{90} value in μg/mL: erm strain 0.015 versus >0.12 and mef strain 0.12 versus 1. Against different S. ***pyogenes*** strains ABT versus telithromycin has the following MIC_{90} values in μg/mL: erm strain 0.5 versus >8 and mef strain 0.12 versus 1 (291–294). The high activity of ABT-773 against macrolide-resistant ***S. pneumoniae*** is probably the result of several factors. For example, ABT-773 was demonstrated to bind tighter than erythromycin to the ribosome target (295). ABT-773 was also shown (1) to accumulate in macrolide-

sensitive *S.* pneumoniae at a higher rate than that of erythromycin A, (2)to bind to methylated ribosomes, although at lower affinities than those of wild-type ribosomes; and (3) to accumulate in S. pneumoniae strains with the efflux-resistant phenotype.

ABT-773 possesses a favorable pharmacokinetic profile, with plasma elimination half-lives averaging 1.6, 4.5, 3.0, and 5.9 h after i.v. dosing in mouse, rat, monkey, and dog, respectively. Peak plasma concentrations averaged 1.47, 0.52, 0.56, and 0.84 μg/mL, having bioavailabilities of 49.5, 60.0, 35.8, and 44.1% after oral dosing in the same animal species, respectively. The lung concentration of ABT-773 was >25-fold higher than plasma concentration after oral dosing in rat (296). In animal studies, ABT-773 was found to be fivefold more efficacious than azithromycin against me@-bearing strains and had excellent efficacy against *ermAM*-bearing strains, whereas azithromycin was inactive (297). ABT-773 was shown to have 3- to 16-fold improved efficacy over that of telithromycin against macrolide-resistant S. pneumoniae (both *ermAM* and *mefE*) in rat pulmonary infection (298).

HMR 3562 (66) and HMR 3787 (67) are two 2-fluoro ketolides having potent in vitro antibacterial activity against inducible resistant S. aureus and S. pneumoniae as well as constitutively resistant *S.* pneumoniae. They are more potent than their corresponding nonfluorinated parent ketolides against H. influenzae (299, 300). Both compounds display high therapeutic efficacy in mice infected by different common respiratory pathogens, such as multidrug-resistant pneumococci and H. *influenzae* (301). A series of C-2 halo-substituted tricyclic ketolides were prepared, showing that the 2-fluoro-ketolide analog is more potent than the 2-bromo derivative. A-241550 (2-F analog of TE-802) (68) was found to be more potent and efficacious than TE-802 (the C2-H parent), particularly against H. influenzae (302).

A C-2 fluorinated ketolide with the 9-keto group replaced by an oxime, designated as CP-654,743 (**69**), was shown to be potent in vitro against macrolide-resistant respiratory pathogens, including S. pneumoniae, S. pyogenes, and H. influenzae (303). The MIC_{90} values in



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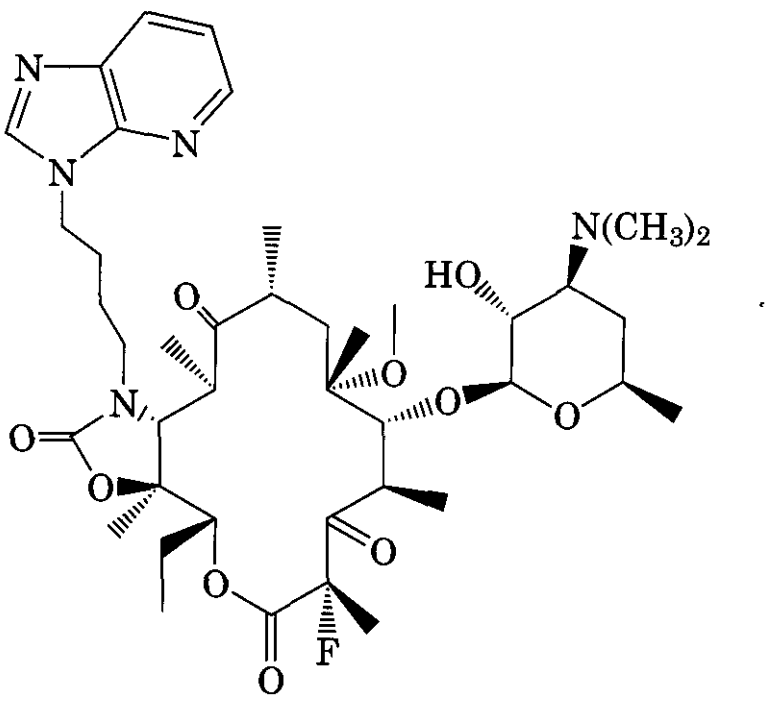
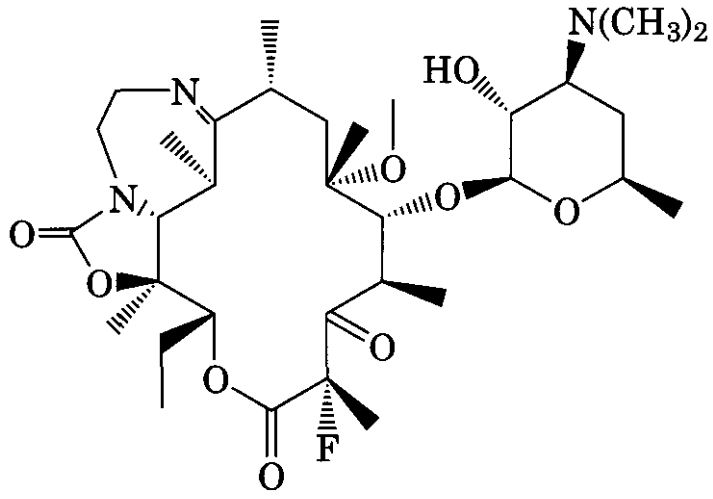


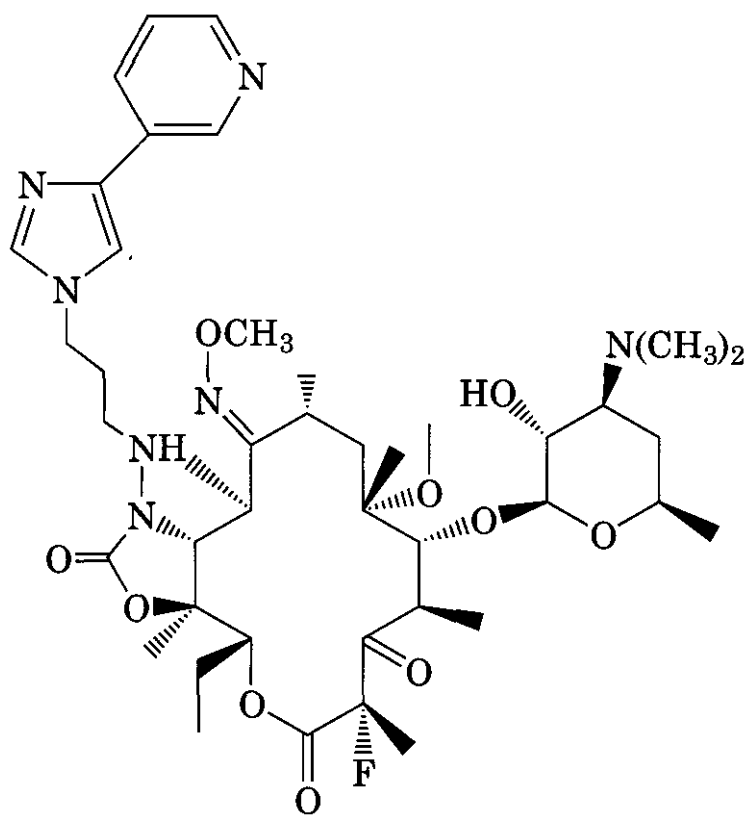
(67)

μg/mL against S. pneumoniae, S. pneumoniae (*ermB*$^+$), S. pneumoniae (*mefA*$^+$), H. influenzae, *S.* pyogenes (*ermB*/*ermA*$^+$), and *S. pyogenes* (*mefA*$^+$) for CP-654,743 are 0.004, 0.125, 0.25, 2.0, 6.3, and 1.0, respectively. This improved in vitro potency appeared to translate into improved in vivo activity, especially with improved activity against MLS_B pneumococci (304). CP-654,743 has a large volume of



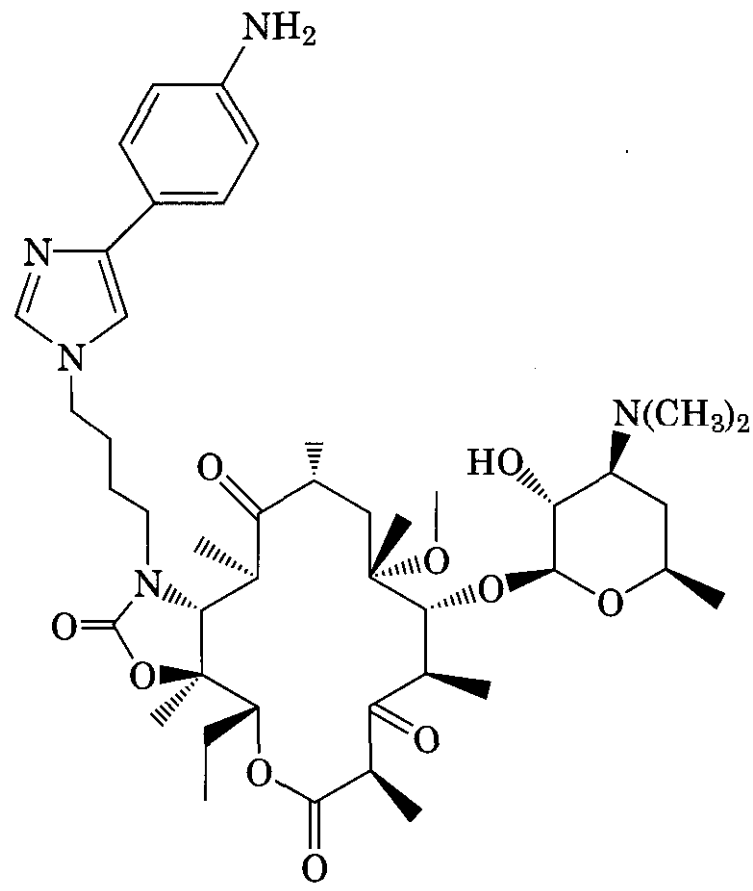
(68)

distribution and moderately high clearance in rats, dogs, and monkeys and the liver microsomal data predict moderate plasma hepatic clearance in humans. It is moderately bound to serum proteins (305).



(69)

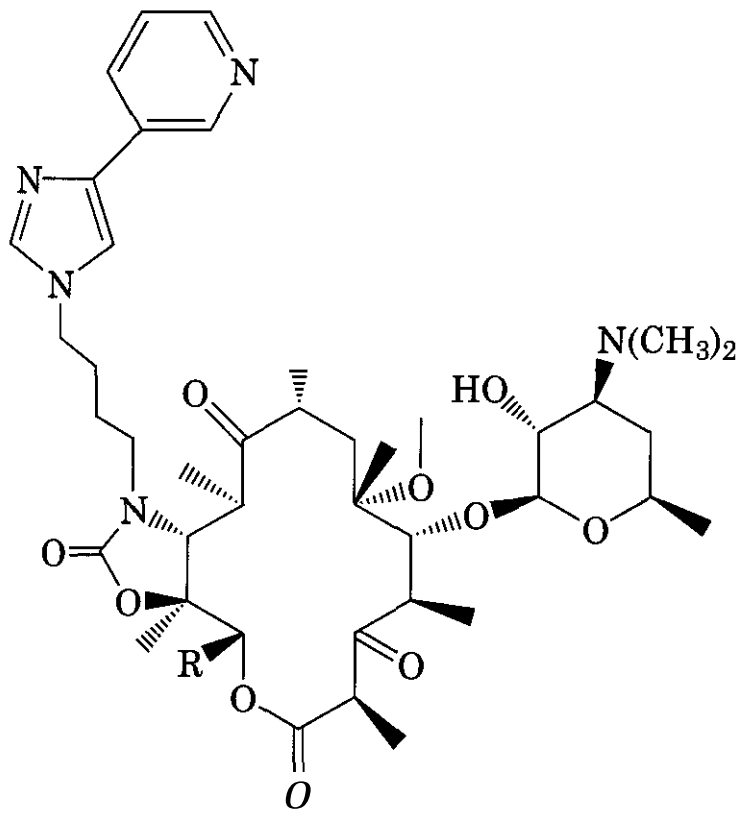
The in vitro activity of the ketolide HMR3832 (**70**), with a butyl imidazoyl anilino moiety substituted on the N-carbamate position, was reported (306). This compound is about one to two times less active than clarithromycin against macrolide susceptible S. pneumoniae, but at the same time retains activity against strains harboring macrolide-resistant determinants. The MIC_{90} values in $\mu g/mL$ against H. *influenzae*, M. catarrhalis, N. meningitis, B. pertussis, C. pneumoniae, and M. pneumoniae are 2–4, 0.12, 0.12, 0.12, 0.25, and 0.0005, respectively.



(70)

Apart from the synthesis of ketolides from erythromycin A or its analog clarithromycin, a series of C-13–modified ketolides (**71**), in which the C-13 ethyl group is replaced by other alkyl or vinyl groups (R = alkyl or vinyl), were prepared. These were synthesized from a modified erythromycin A template produced in a genetically engineered Streptomyces *coelicolor* strain (307). The S. erythraea polyketide synthase genes were engineered to contain an inactive ketosynthase 1 domain ($KS1^0$) and then expressed in *S.* coelicolor. The strain was fermented with the appropriate diketide thioester precursor, to yield C-13–modified 6-deoxyerythronolide analogs with C-13 being a methyl or vinyl group. These derivatives were then purified and fermented with a $KS1^0$ S. erythraea to glycosylate at the 0-3 and 0-5 positions and hydroxylate at the C-6 and C-12. Further chemical modification of these novel core templates produced the C-13–modified ketolide (71). The in vitro activity of the C-13–modified ketolides compared to that of te-

lithromycin (R = ethyl) is given in Table 15.7. They have activity slightly less than that of telithromycin, showing that certain C-13–modified ketolides retain good activity against macrolide-resistant bacteria. Thus, this biosynthetic strategy may offer an opportunity to generate potent and useful novel macrolide derivatives.



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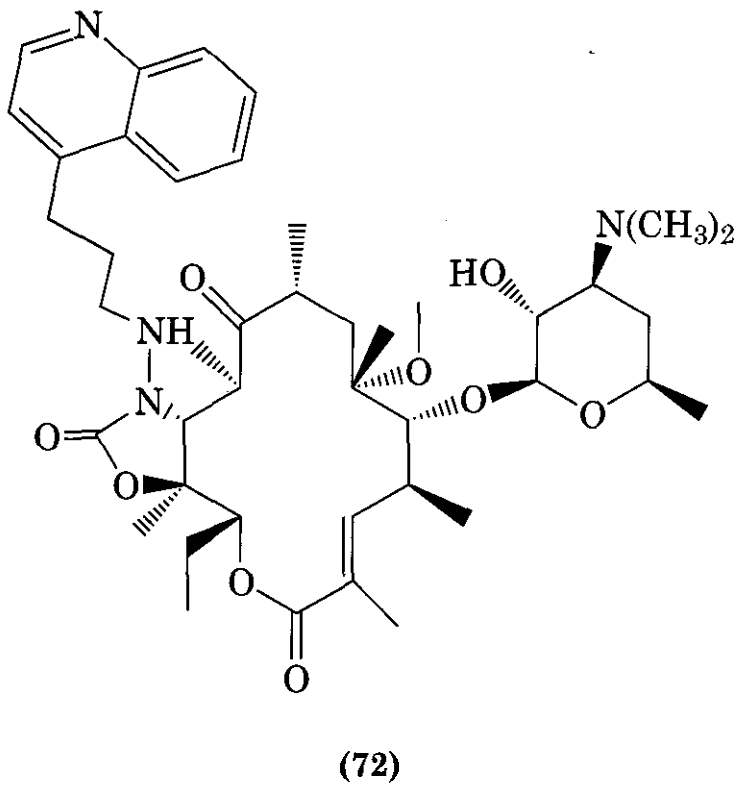
4.6.1.2 Anhydrolides and Acylides. A series of 2,3-anhydroerythromycin derivatives, termed anhydrolides, were synthesized. They possess a carbon–carbon double bond at the C-2–C-3 position, having an sp^2 carbon at position C-3 (the same position as the 3-keto group in the ketolides) (308,309). A-179461 (**72**), an anhydrolide, having a structure similar to that of HMR 3004, was found to have similar antibacterial activity in vitro to that of HMR 3004 against Gram-positive organisms. Thus, functionality other than a keto group at the C-3 position can be used as cladinosyl group replacement to produce potent novel macrolide derivatives (310).



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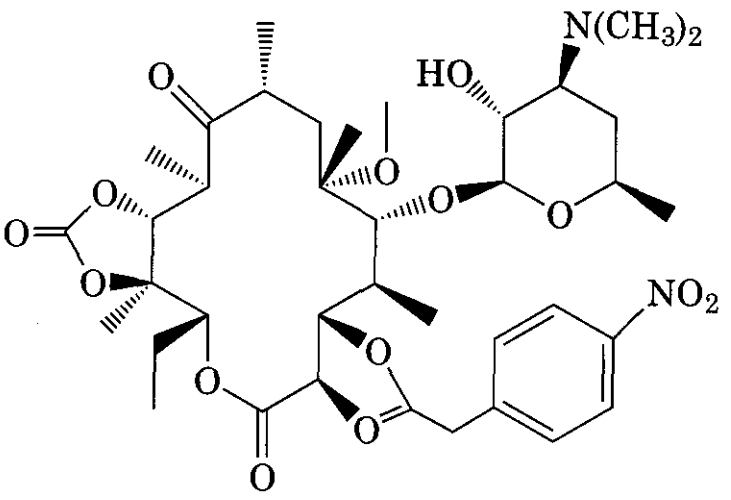
A series of 3-descladinosyl-3-acylated macrolide derivatives with potent antibacterial activity were prepared and called acylides. The 3-*O*-acyl-5-desosaminyl-erythronolide 11,12-carbonate TEA 0769 (73) and 3-*O*-acyl-5-desosaminyl-erythronolide 6,9;11,12-biscarbonate FMA 122 (74) have potent in vitro

Table 15.7 Comparative *In* Vitro Activity of C-13–Modified Ketolides (71)

Organism	Resistance Mechanism	N	Median MIC (μg/mL) Telithromycin Derivatives R = Ethyl	R = Methyl	R = Vinyl
M. catarrhalis		3	0.25	8	0.25
H. influenzae		12	4	8	8
S. aureus (MSSA, MRSA)		3	0.25	0.5	0.5
Staphylococci	erm_j	5	0.5	16	4
	erm_c	5	≥16	≥16	≥16
	msr	2	1	2	2
Enterococci (VRE)		5	0.06	0.12	0.12
Enterococci		11	≤0.015	0.03	0.03
S. *pneumoniae*	*erm*	5	0.03	0.12	0.12
	mef	8	0.25	0.5	



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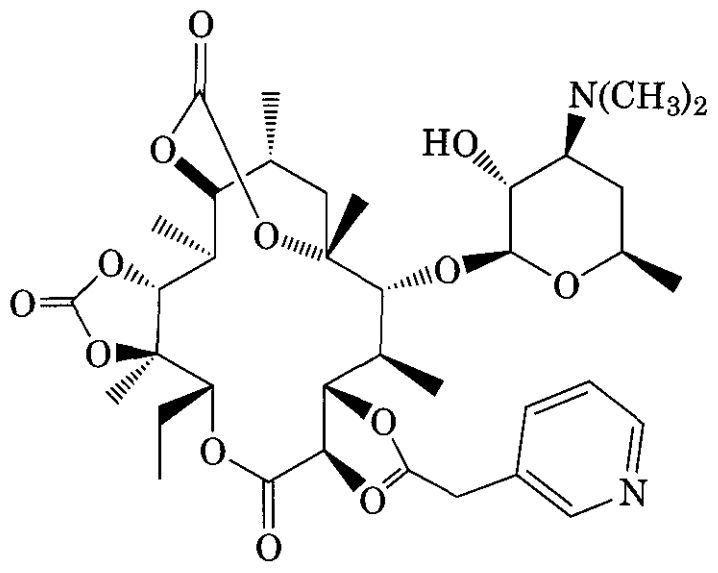
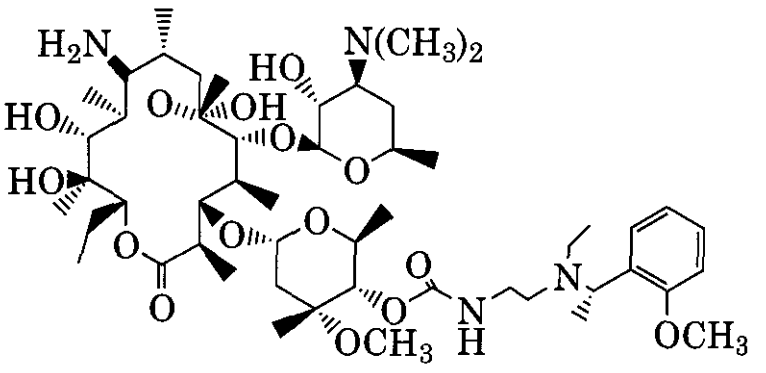


(74)

antibacterial activity against Gram-positive organisms similar to that of telithromycin (311–313). TEA 0769 was found to be twofold more active against staphylococci (MIC, S. *aureus* 209P: 0.05 μg/mL) and 16-fold more active against enterococci (MIC, E. *faecalis*: 0.05 μg/mL) than was clarithromycin. It is also active against erythromycin-resistant streptococci. The antibacterial activity of FMA 122 compared with that of azithromycin given in μg/mL against H. influenzae, S. aureus 209P, S. pneumoniae IID533 (erythromycin sensitive), S. pneumoniae 210 (erythromycin-resistant efflux), and S. pneumoniae 205 (erythromycin-resistant-erm) is as follows: 0.78 versus 1.56, 0.10 versus 0.39, 0.025 versus 0.10, 0.10 versus 0.78, and 6.25 versus 100, respectively. Thus, the acylides represent a potent macrolide series with good anti–H. influenzae activity while having activity against macrolide-resistant bacteria

4.6.1.3 4'-Carbarnate Macrolides. *A* series of 4″-carbamates of 14- and 15-membered macrolides were prepared, in which the 4" hydroxyl group was replaced by a carbamate group. They have potent in *vitro* activity against Gram-positive and Gram-negative respiratory pathogens including the macrolide-resistant S. pneumoniae (314). CP-544,372 (75) possesses good in uitro antibacterial activity comparable to that of telithromycin. Its MICs in μg/mL against S. pyogenes *mefA*, S. pneumoniae *ermB*, and S. pneumoniae *mefE* are 0.5, 0.16, and 0.08, respectively (315). Its maximum drug concentration (C_{max}) in the lung is higher than that in serum. In mice, CP-544,372, given at 1.6–100 mg/kg orally, produced the serum C_{max} of 0.001–0.66 μg/mL, AUC of 0.13–5.5 $\mu g\ h^{-1}\ mL^{-1}$, and a mean terminal half-life of 6.5 h (316). In murine acute pneumonia models of infection induced by macrolide-sensitive and -resistant strains of pneumococci or H. influenzae, CP-544,372 was shown to be orally active (317). It has either more potent or similar in *vivo* efficacy against macrolide-resistant S. *pneumoniae* compared to telithromycin or HMR 3004. Thus, modification at the 4"-position of erythromycincylamine led to 4-carbamate macrolide derivatives with increased in *vitro* and oral in *vivo* antibacterial activity against macrolide-resistant S. pneumoniae, while maintaining potent in *vitro* and in *vivo* activity against macrolide-sensitive S. pneumoniae and H. influenzae.



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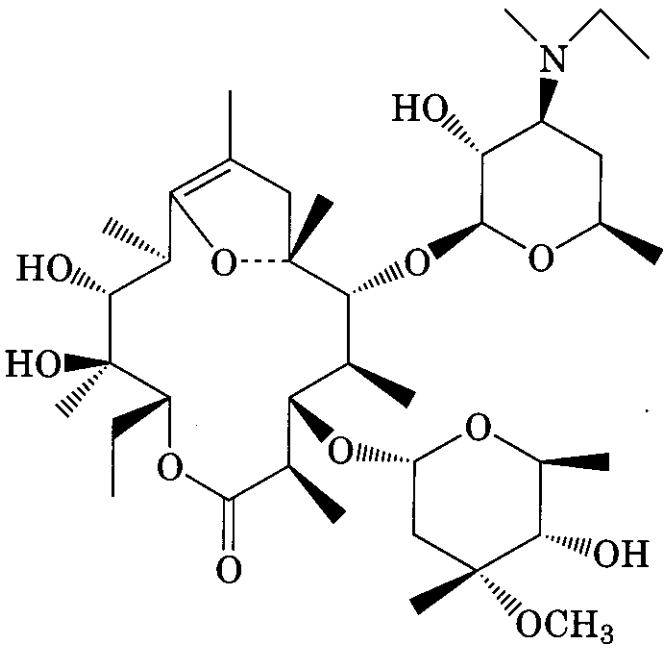
4.6.2 Non-Antibacterial Activity *of* Macrolides. Other than having antibacterial activity, macrolide antibiotics also have diverse non-antibiotic properties. They have a broad range of biological response-modifying effects on in-

flammation, tumor cells, airway secretions, and host defense. Other effects such as their gastrokinetic effect are also described below.

4.6.2.1 Gastrokinetic Effects. The gastrokinetic effect of macrolides such as erythromycin A and oleandomycin has been known for some time. They induced strong macular contraction in the gastrointestinal tract of dogs. Erythromycin A, when given at 0.03 mg/kg intravenously to dogs, induces a pattern of migrating contractions in the gastrointestinal tract, mimicking the motilin effect on gastrointestinal contractile activity (318). At an intravenous dose of 7 mg/kg, an immediate increase in contractile activity in the whole length of the intestinal tract was observed (319). Thus, erythromycin A acts as a nonpeptide agonist of motilin (320, 321). Erythromycin A administered intravenously to normal volunteers at a dose of 200 mg after a meal induced powerful peristaltic contractions, improved antroduodenal coordination, and phase three–like activity (322).

Motilides are macrolide derivatives with reduced antibacterial activity and have the activity to induce gastrointestinal contractions acting as a motilin mimic (323). A detailed study of macrolide derivatives on their potency in displacing motilin and contractility identified EM-523 (76) for clinical development as a gastrointestinal tract prokinetic agent (324, 325). 8,9-Anhydro-4″-deoxy-3′-*N*-desmethyl-3'-ethylerythromycin B-6,9-hemiacetal, ABT-229 (77) is another potent prokinetic agent (326), with >300,000 times more potency than that of erythromycin A. It is also 400-fold more active than its 4″,12-dihydroxy congener EM-523. It has good oral bioavailability with 39% compared to 1.4% for EM-523. Thus the 4"-hydroql group is not a major contributor to the motilin agonistic activity, whereas the 2'-hydroxyl group seems necessary (327).



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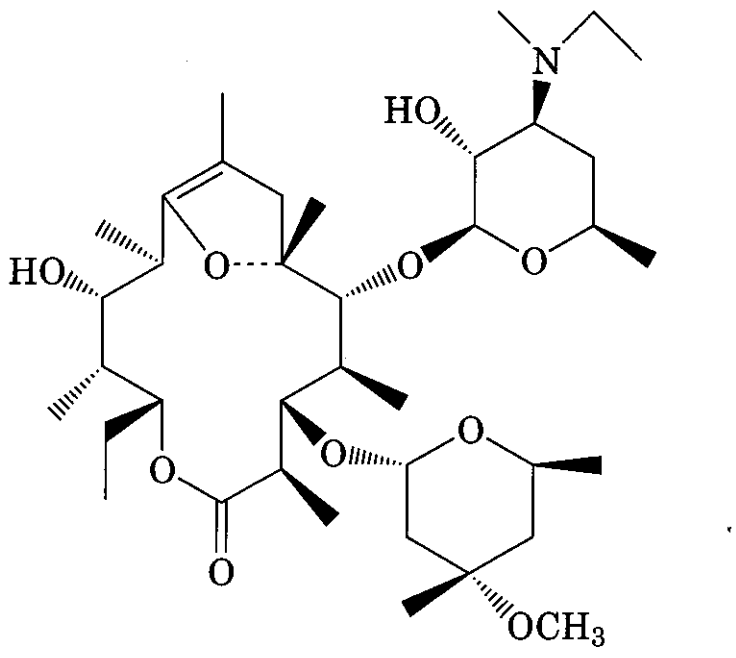

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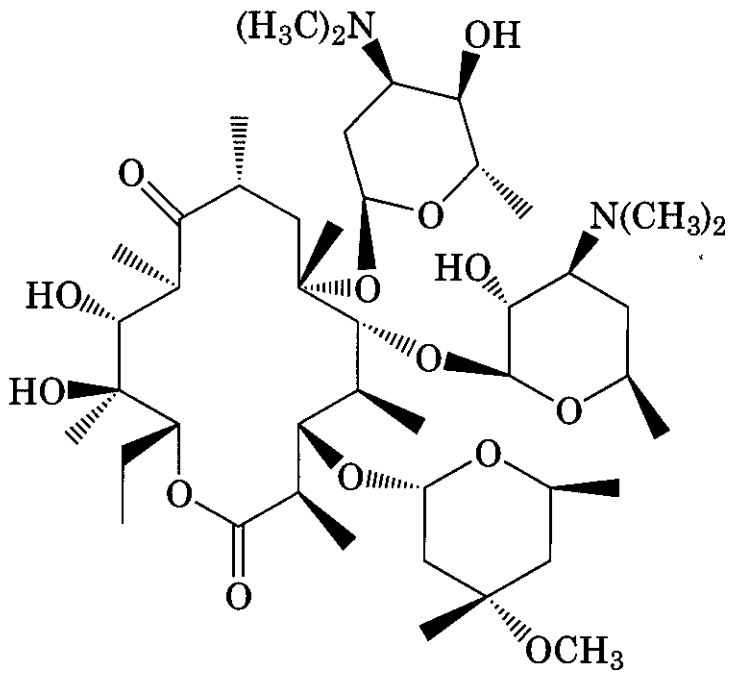
4.6.2.2 Effects on ***Cytokines.*** Macrolides have the ability to inhibit the production of proinflammatory cytokines and reactive oxygen species by airway neutrophils. In whole blood, erythromycin A was found to cause a dose-dependent decrease in heat-killed S. pneumoniae–induced production of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) in vitro (328). In patients with chronic airway diseases, administration of erythromycin had an inhibitory effect on IL-8 release (329, 330). In mice, HMR 3004 was found to downregulate the pneumococcus-induced IL-6 and IL-1β and nitric oxide in bronchoalveolar lavage fluid. It limited the neutrophil recruitment to the lung tissue and alveoli without interference on phagocytosis. It also abrogated lung edema (331, 332). Roxithromycin was also found to suppress the production of IL-8, IL-6, and granulocyte-macrophage colony-stimulating factor in vitro in human bronchial epithelial cells. It also inhibited neutrophil adhesion to epithelial cells. Roxithromycin's efficacy in airway disease may be attributable to its effects in modulating local

recruitment of inflammatory cells (333). Clarithromycin was also reported to suppress the IL-8 release in human bronchial epithelial cell line BRAS-2B (334) and inhibit NF-κB activation in human peripheral blood mononuclear cells and pulmonary cells (335). Azithromycin, clarithromycin, and roxithromycin were reported to suppress the edema production in carrageenin-injected rats (336). Macrolides are also shown to inhibit mRNA expression of IL-1 (337), IL-8 (338), endothelin-1 (339), and inducible NO synthase (340).

4.6.2.3 Immunomodulatory Effects. Macrolide antibiotics such as clarithromycin decrease neutrophil oxidative burst and proinflammatory cytokine generation and release. Thus they are shown to possess immunomodulatory effects and can be used as biological response modifiers. Macrolides may protect airway epithelium against oxidative damage by phospholipid-sensitized phagocytes (341). They can decrease mucus hypersecretion both *in vitro* (342) and *in vivo* (343). Clinical efficacy of macrolides is high upon administration of macrolides to patients with diffuse panbronchiolitis (DPB), a disease with airflow limitation, sinusitis, sputum expectoration, dyspnea, as well as infection with *P. aeruginosa* (344, 345). The 5-year survival rate for patients with DPB was 26% in 1984. However, after the introduction of macrolide therapy, the 10-year survival rate has increased to 94%, an impressive improvement (346). Erythromycin A, clarithromycin, and azithromycin were found to suppress the expression of *P. aeruginosa* and *P. mirabilis* flagelin dose dependently (347). Flagella are among the virulence factors of Gram-negative rods and have a role in the initiation of biofilm formation. Macrolide therapy is found to be beneficial for patients with macrolide-resistant *P. aeruginosa* (DPB) and cystic fibrosis, although the improvement was not associated with the disappearance of *Pseudomonas* (348–350). A beneficial effect was also seen for patients on macrolide therapy for chronic sinusitis (351).

4.6.2.4 Antitumor Effects. Macrolides have been reported to have antitumor effects in mice (352). Clarithromycin prolonged the survival of patients with nonresectable, non-small cell lung cancer (353). It inhibited tumor angiogenesis, growth, and metastasis of mouse B16 melanoma cells. Although clarithromycin showed no direct cytotoxicity to the tumor cell *in vitro,* spleen cells obtained from the tumor-bearing rats receiving this compound showed a stronger tumor-neutralizing activity (354). Using a mouse dorsal air sac model, roxithromycin was found to inhibit tumor angiogenesis in a dose-dependent manner (355).

4.6.2.5 Antiparasitic Activity. Megalomicin ***(78)*** is a 14-membered macrolide with three sugar substituents (one neutral sugar and two aminosugars) isolated from *Micromonospora megalomicea* (356). It was found to have antiparasitic activity. It inhibits vesicular transport between the medial- and *trans-Golgi*, resulting in the undersialylation of the cellular protein (357). It was found to be active in an *in vivo* animal model with complete protection of the BALA/c mice from death caused by acute *Trypanosoma brucei* infection and significantly reducing the parasitema.



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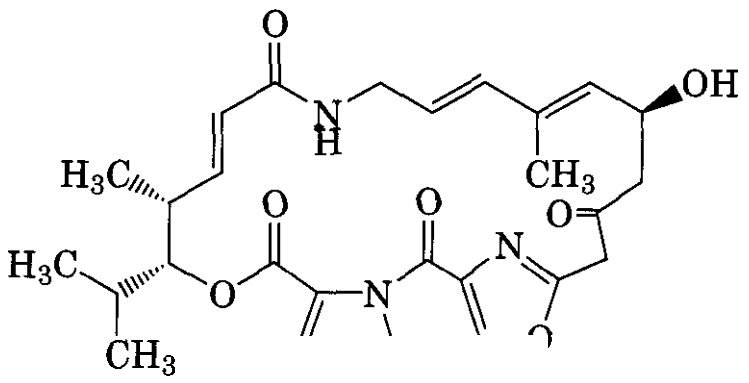
4.7 Future Prospects in the Macrolide Field

Novel macrolides can now be prepared by either chemical modification of the natural macrolides or gene manipulation of the producing organisms. The current novel macrolides have activity against macrolide-resistant bacteria. Telithromycin, a ketolide, has been successfully developed for the treatment of bacterial infections. Further clinical study shall no

doubt show its efficacy for the treatment of respiratory infections caused by macrolide-resistant pneumococci. Additional ketolides are expected to come to market in the near future. This will provide physicians with additional tools to combat the threat of bacterial resistance. The recently expanding knowledge of mechanisms of bacterial resistance to macrolides will accelerate the discovery of new and useful macrolide derivatives other than the ketolide class. The new findings on many nonantibacterialactivities of macrolide will open doors for the design and development of novel agents for the treatment of inflammatory diseases, parasitic diseases, cancers, and gastrointestinal motility disorders. Investigation directed toward study of the mechanism of action of these nonbacterial effects will no doubt be intensified.



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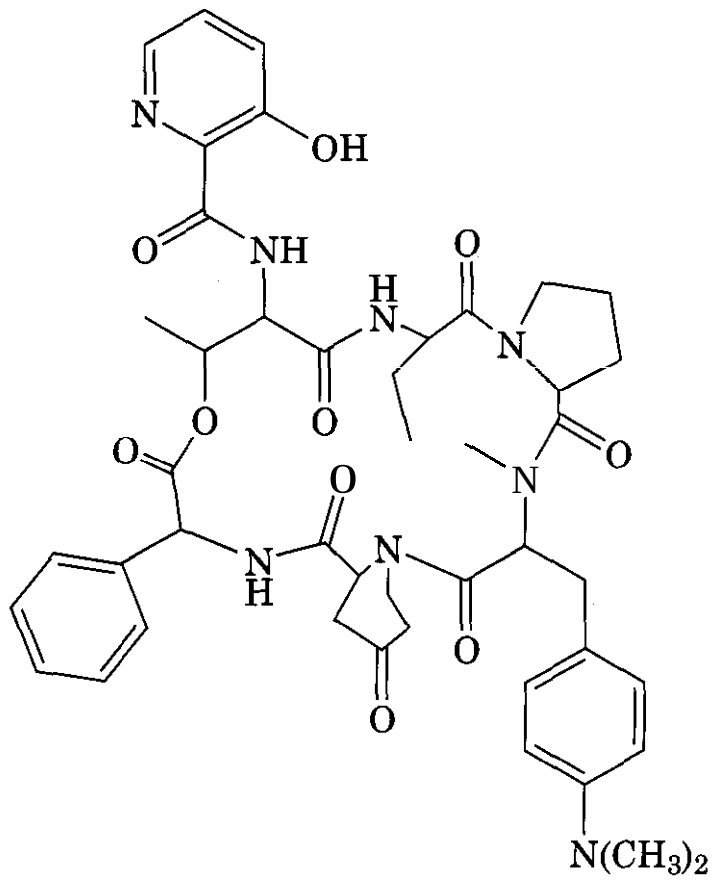


(80)

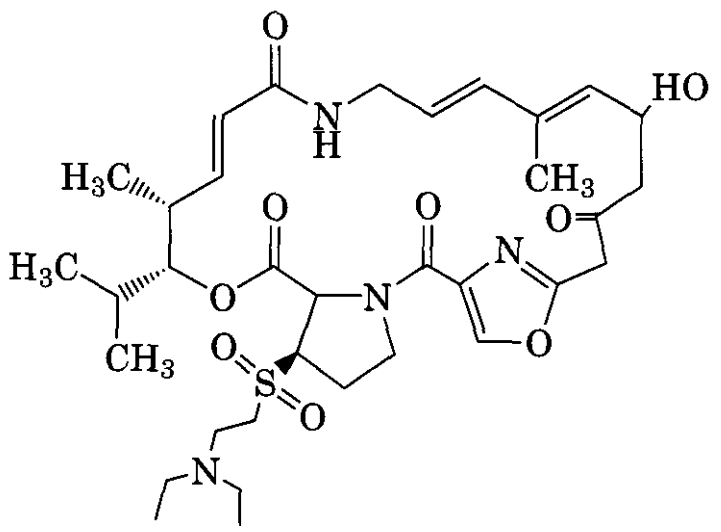
5 OTHER ANTIBIOTICS THAT TARGET THE BACTERIAL RIBOSOME

5.1 Streptogramins

The streptogramin antibiotics are natural products, derived from soil bacteria, that inhibit bacteria protein synthesis, thus resulting in cell death. These antibiotics were discovered in the 1950s but did not find extensive clinical use as a result of poor water solubility. Recently, the preparation of semisynthetic water-soluble derivatives of streptogramins has facilitated their use in the treatment of infections caused primarily by Gram-positive bacteria such as staphylococci, streptococci, and enterococci as well as some Gram-negative organisms including Neisseria and *Legionella* (358,359). Streptogramins are composed of two distinct chemical classes, group A and group B. Group A streptogramins are polyunsaturated macrolactones derived from acetate (polyketide synthesis) and amino acids [e.g., pristinamycin-IIA (**79**)]. Group B streptogramins are cyclic hexadepsipeptides [e.g., pristinamycin-IA (**80**)], cyclized through an ester linkage between the C-terminal carboxylate and the hydroxyl of an N-terminal Thr. The streptogramin-producing bacteria, such as the pristinamycin producer Streptomyces *pristinaespiralis*, simultaneously generate both a group A and a group B antibiotic in a molar ratio of approximately 60:40.

Individually, the group A and B streptogramins are generally bacteriostatic, but become bactericidal when coadministered. This synergistic effect has been exploited in the use of the semisynthetic compounds dalfopristin (**81**)and quinupristin (**82**), water-soluble derivatives of pristinamycin IIA and pristinamycin IA, respectively, that together in a 7:3 ratio form the drug Synercid. This formulation was recently approved in North America for the treatment of serious infections caused by antibiotic-resistant Gram-positive pathogens. The route of administration is by i.v. in a dose of 7.5 mg/kg every 8–12 h. Synercid inhibits

cytochrome P450 3A4 activity, which is implicated in the metabolism of numerous other drugs including cyclosporine, midazolam, and others (for reviews, see refs. 360 and 361), and thus caution should be used when administering Synercid with other drugs metabolized by this route.



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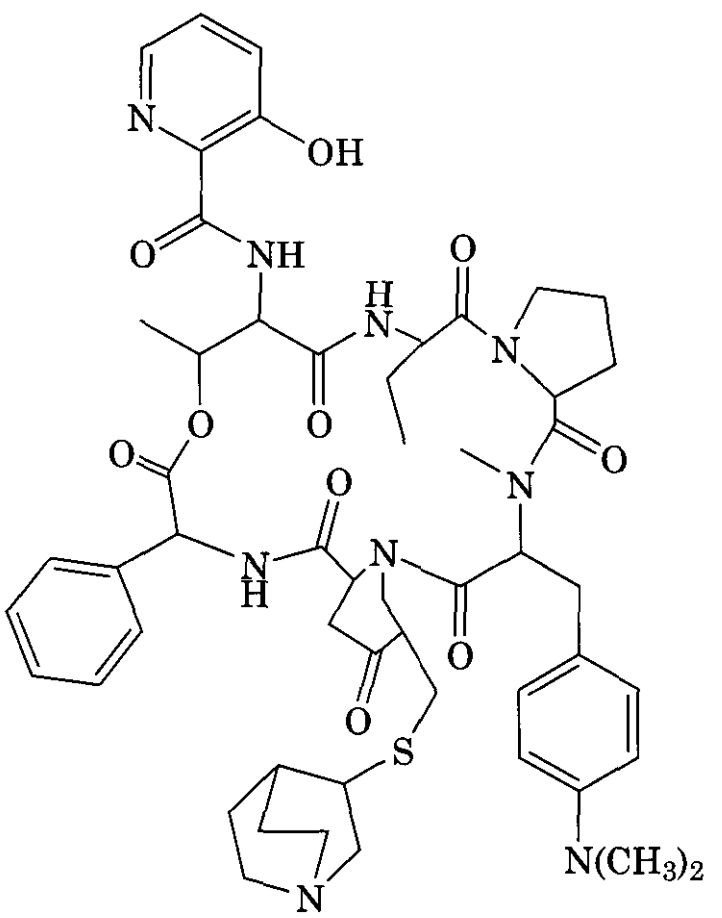

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Both group A and group B streptogramins bind to the 50S ribosomal subunit, but in distinct sites (362). Group A antibiotics inhibit both aminoacyl-tRNA binding to the A-site and peptide bond formation by binding tightly to 50S and free 70S ribosomal particles (363, 364). This tight interaction is also accompanied by a conformational change in the 50S subunit (365, 366). Group B streptogramins bind to the 50S subunit, which blocks peptide elongation and induces premature chain termination (367, 368). The B streptogramins overlap the macrolide–lincosamide binding sites (369), which explains the cross-resistance observed by Erm ribosomal .methyltransferases (232). Binding of group A streptogramins to the ribosome facilitates binding of a group B streptogramin, which is the likely basis for synergy between the molecules (369–371).

Resistance to the streptogramins can occur by way of distinct efflux, chemical modification, or target protection/alteration mechanisms for both A and B group streptogramins. On the other hand, resistance to the synergistic mixture of A and B group streptogramins requires an A group resistance determinant, not necessarily coupled with one for group B streptogramins (372).

Ribosomal protection by Erm methyltransferases at position A2058 of the 23S rRNA, which also confers resistance to the macrolide and lincosamide antibiotics (see section above), provides resistance to the group B streptogramins. This results in the NILS,-resistance phenotype. In the case of inducible erm gene expression, group B streptogramins are not inducers, unlike the 14-membered macrolides. The presence of an Erm methyltransferase does not confer resistance to the group A streptogramins and thus Synercid is still effective against these strains (359, 373, 374). Point mutations in the 23rRNA can also result in resistance to the group B streptogramins [e.g., C2611U (375), C2611A, C2611G, and A2058G (246)].

Active efflux mechanisms specific to either the A or B group streptogramins have been identified. The Vga proteins from *S.* aureus, members of the ABC (ATP-binding cassette) family of efflux proteins, are associated with group A streptogramin resistance (376–378). Efflux-mediated resistance to the group B streptogramins has not been associated thus far with clinically relevant resistance. Recently, a virginiamycin S (group B streptogramin) resistance gene, *varS*, was cloned

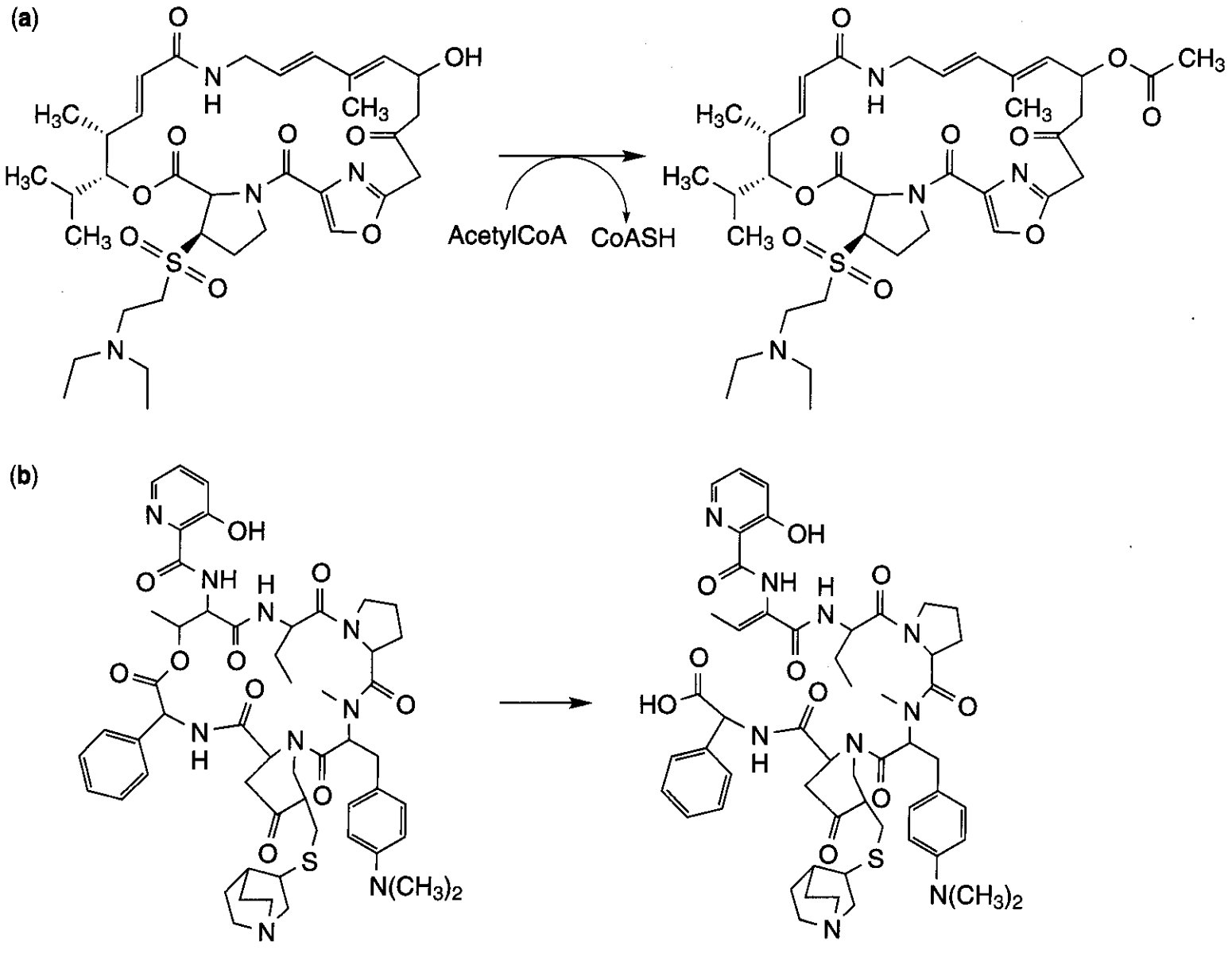


Figure 15.14. Modification of streptogramin by resistance enzymes. (a) Group A streptogramin acetyltransferase modification of dalfopristin. (b) Group B streptogramin lysase inactivation of quinupristin.

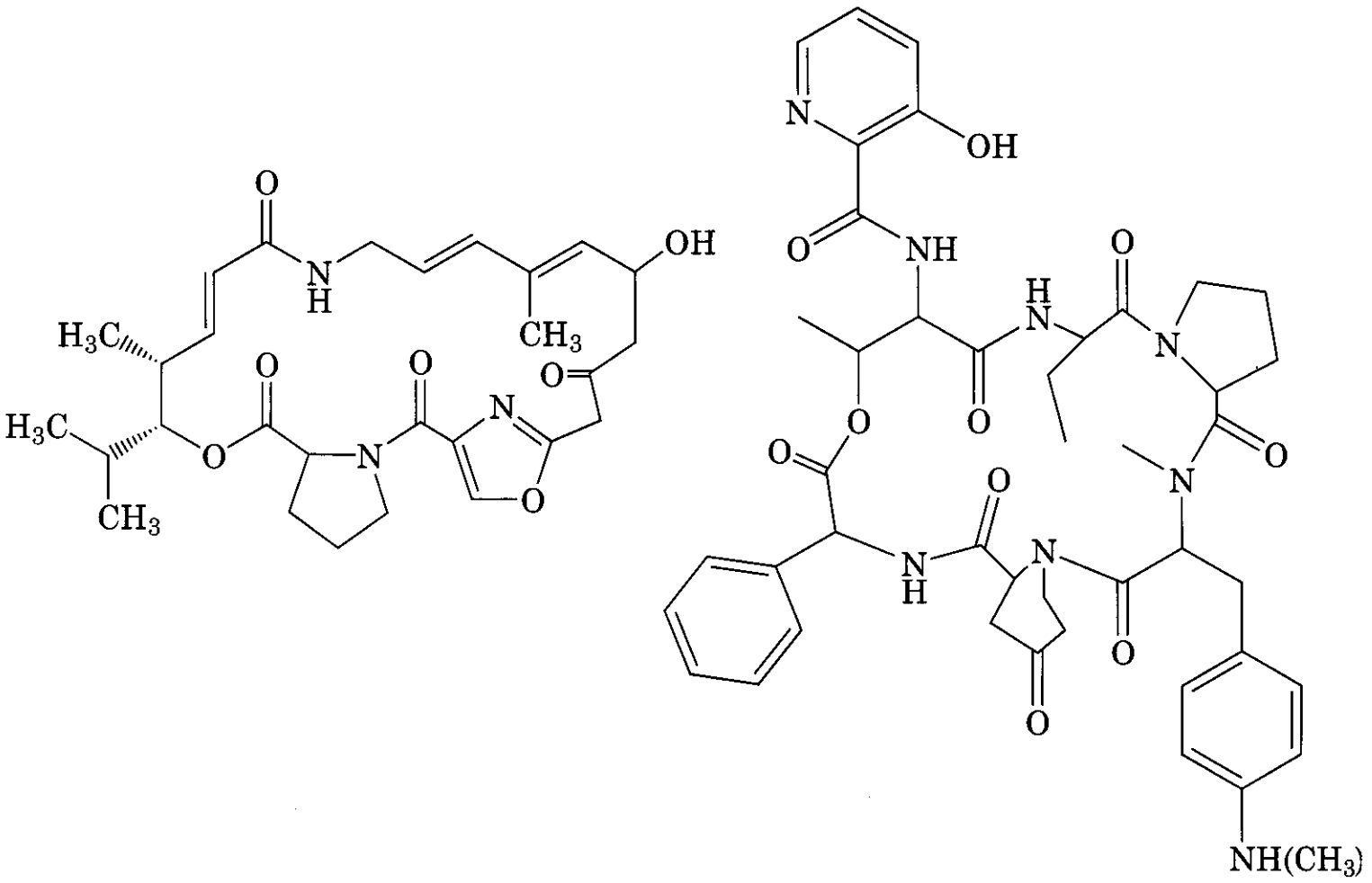
from the virginiamycin producer ***Streptomyces virginiae*** (379). The VarS protein is a homolog of other drug-resistant ABC transporters and heterologous expression in ***Streptomyces lividans*** resulted in virginiamycin S resistance.

Enzymatic inactivation of the streptogramins is a predominant mechanism of resistance. Group A streptogramins are inactivated by O-acetylation catalyzed by acetyl-CoA-dependent acetyltransferases (Fig. 15.14a). Several acetyltransferases termed Sat or Vat have been identified both in group A streptogramin-resistant Gram-positive clinical isolates (e.g., 380–382) and in the chromosomes of a number of bacteria (383). The crystal structure of VatD from E. ***faecium*** has been determined in the presence and absence of virginiamycin M1 and CoA (384). Inactivation of group B streptogramins occurs by linearization of the cyclic depsipeptide by the enzyme Vgb. The mechanism of ring opening had been thought to occur by hydrolysis; thus, Vgb has been termed a hydrolase or lactonase (385–387). However, recent results indicate that this enzyme does not use water to open the ring and in fact operates by an elimination reaction (Fig. 15.14b) (388). Homologs of this enzyme are also found in the chromosomes of numerous bacteria (388), and thus potential streptogramin inactivating enzymes are widespread.

A new orally active streptogramin, RPR-106972 (**83**), has been reported with an activity profile similar to that of Synercid but with increased activity toward the respiratory pathogens H. *influenzae* and ***M. catarrhalis*** (389).

5.2 Lincosamides

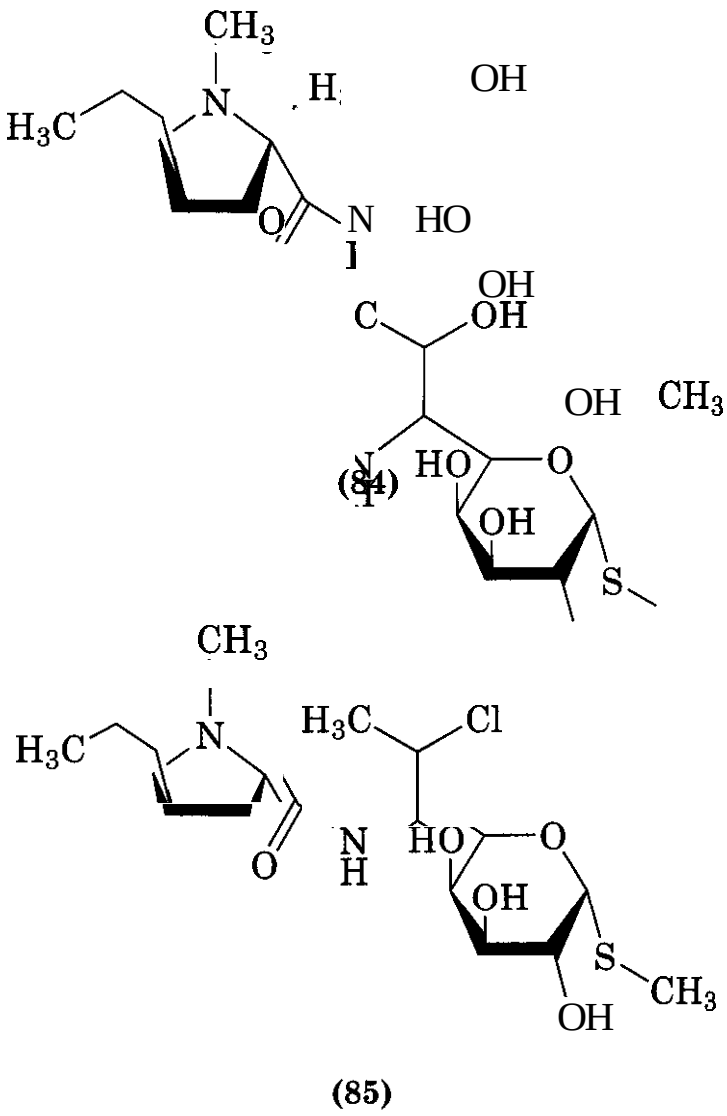
Lincomycin (**84**), a lincosamide, is produced by ***Streptomyces lincolnensis*** and was first dis-



(83)

covered in 1962 (390). This antibiotic is composed of an amino acid portion, trans-N-methyl-4-N-propyl-L-proline, linked through an amide bond to an unusual sugar, 6-amino-6,8-dideoxy-1-thio-D-erythro-α-D-galactooctopyranoside. Clindamycin (85), a chlorinated semisynthetic derivative of lincomycin, shows better absorption and antimicrobial activity than that of the parent compound. These antibiotics are useful for the treatment of non-CNS infections caused by Gram-positive bacteria such as streptococci, staphylococci, and enterococci. Clindamycin is especially important in the treatment of infections caused by susceptible anaerobes, including species of *Bacteroides* and *Clostridium* (391) [but not *C.* difficile, where clindamycin treatment can cause C. difficile–associated colitis (392)]. Clindamycin is also useful in combination with primaquine for the treatment of *Pneumocystis* carinii infection in AIDS patients (393, 394).

Clindamycin is orally active and efficiently absorbed with an adult dose of 140–150 mg every 4 h. The mode of action of the lincosamide antibiotics includes binding to the 50S



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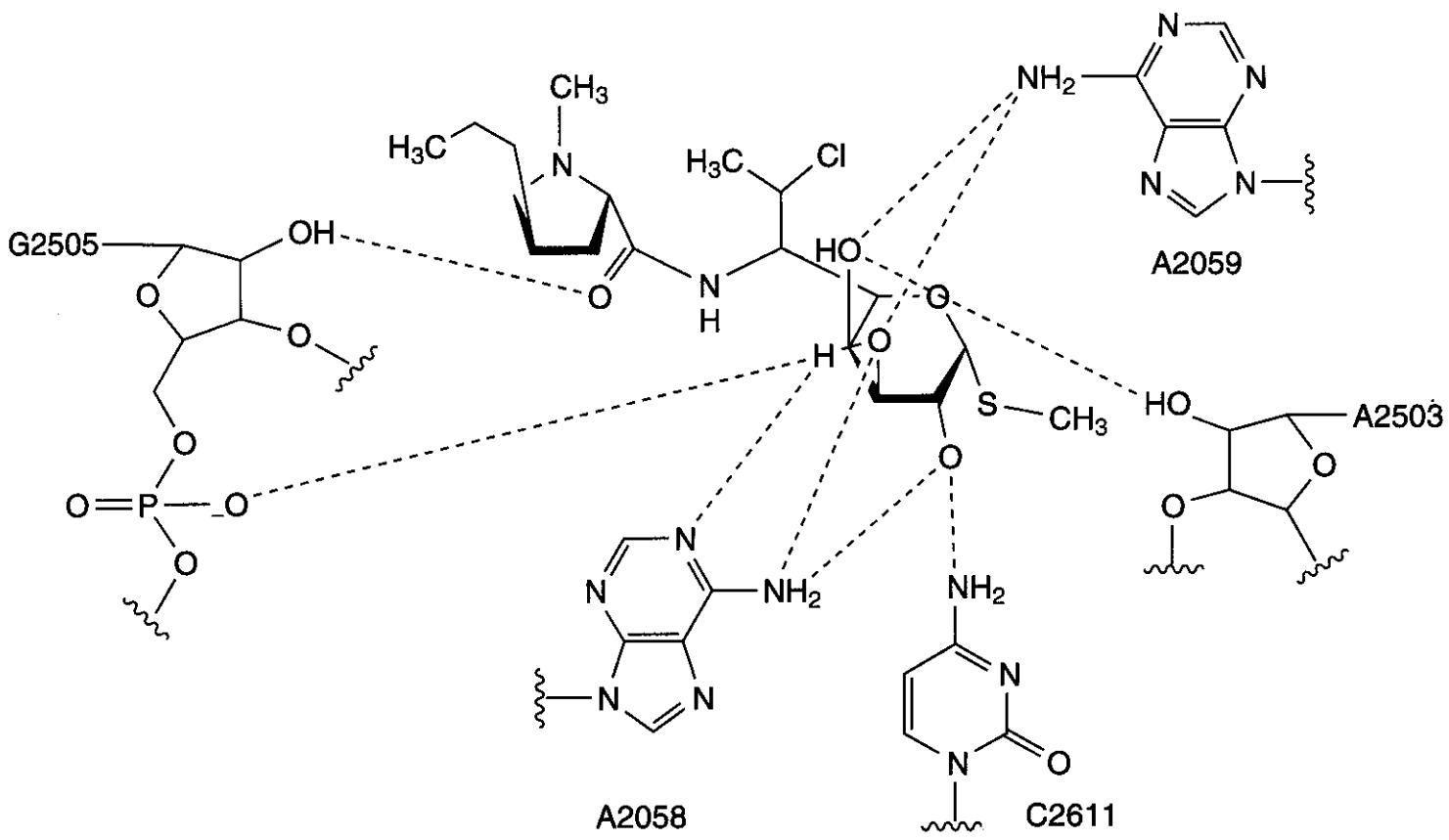


Figure 15.15. Interaction of clindamycin with the 50S ribosomal subunit of D. radiodurans (redrawn from Ref. 169). The equivalent E. coli numbering is shown and the interaction with C2611 is predicted based on the interaction with U2590 for the D. radiodurans 23S rRNA.

ribosomal subunit, which blocks aminoacyl-tRNA binding, thereby inhibiting protein synthesis (395). The 3D structure of clindamycin bound to the 50S ribosomal subunit has been reported (169). The molecular structure demonstrates that the antibiotic binds in a region overlapping the A- and P-sites of the ribosome exclusively through contacts with the 23S rRNA (Fig. 15.15), resulting in blocking of the peptide elongation tunnel and interfering with peptidyltransfer.

The complete lincosomycin biosynthetic gene cluster from S. lincolnensis has been cloned by the group of Piepersburg (396). Biosynthetic studies have indicated that the propyl proline moiety is derived from tyrosine (397) and that the sugar is derived either from glucose 1-phosphate or octulose-8-phosphate (395).

Clindamycin resistance can occur through ribosomal methylation by the erm gene products (MLS, phenotype), thereby confirming the site of action of the antibiotic and suggesting overlapping binding sites on the ribosome with the macrolide and group B streptogramins (232). Lincosamide resistance can also occur through mutations in the 23S rRNA (e.g., at positions A2058 and A2059) (246, 398). Chemical modification of lincosamides by phosphorylation and adenylation has also been detected, with the latter mechanism being associated with resistance in S. aureus, S. haemolyticus, and E. faecium (399–401). Surprisingly, the *linA* and *linA'* gene products from S. haemolyticus and S. aureus, respectively, modify lincomycin at position 3 of the sugar moiety and modify clindamycin at position 4 (Fig. 15.16) (400). The LinB enzyme from E. faecium, on the other hand, modifies lincosamides exclusively at position 3 (399).

5.3 Chloramphenicol

Chloramphenicol **(86)** is an antibiotic produced by Streptomyces *venezuelae* and other soil bacteria that was first discovered in 1947 (402) and is now exclusively produced synthetically. Chloramphenicol has a broad spectrum of activity against both aerobic and anaerobic Gram-positive and Gram-negative bacteria but is now infrequently used because of toxicity (see below). Nonetheless, chloramphenicol (or its prodrugs, the palmitate or succinate esters) is still indicated for the treatment of acute typhoid fever (Salmonella *typhi*) and as an alternative choice in the treatment of bacterial meningitis (*H. influenzae*, S. *pneu-*

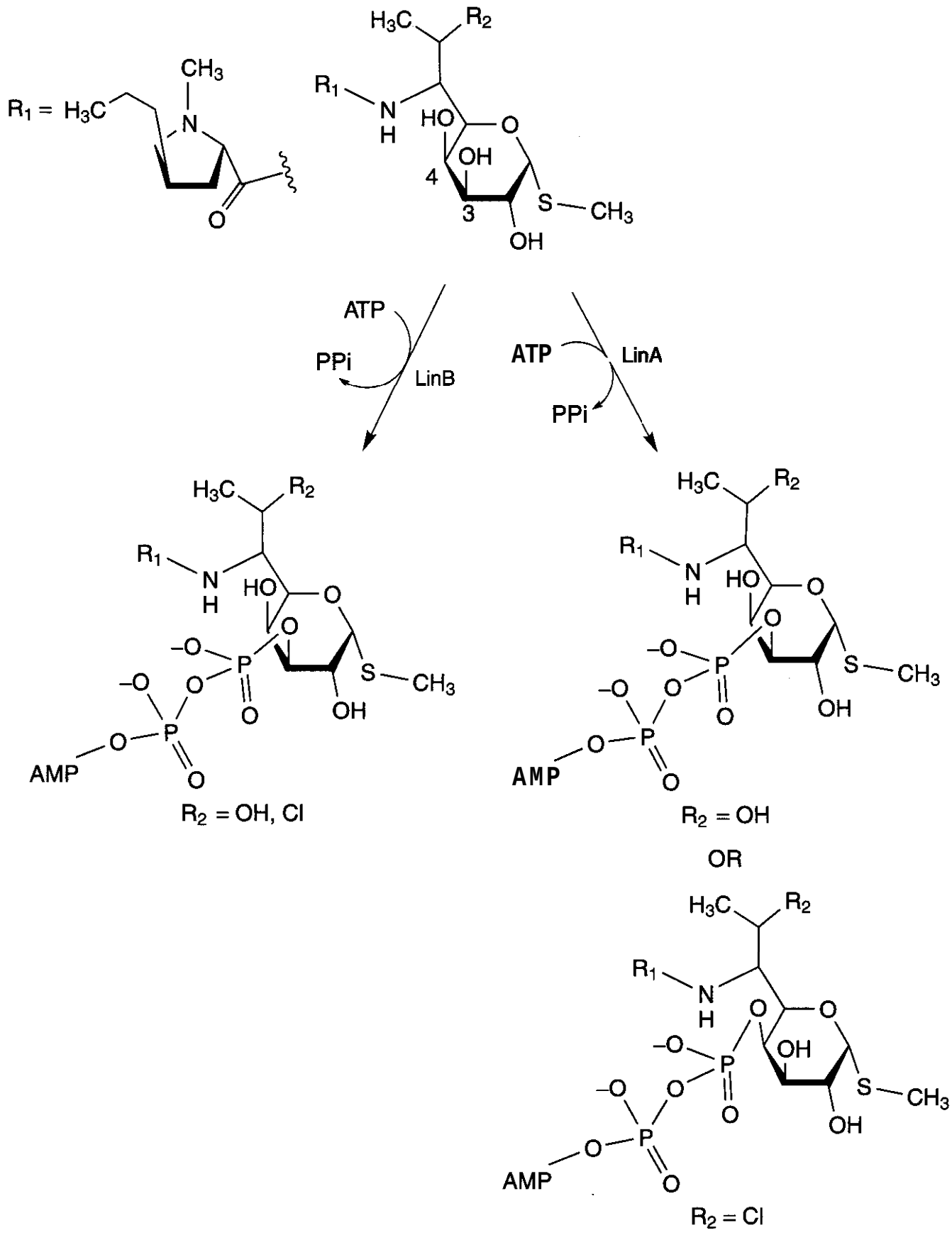


Figure 15.16. Modification of lincosamide antibiotics by adenyltransferases.

moniae, N. menigitidis) and other serious infections where β-lactam allergy or resistance is a problem. The antibiotic is well distributed in all tissues including the brain, which enables its use in the treatment of meningitis ***(391).*** Dosage is typically **50 mg/kg/day** orally administered at 6-h intervals and peak serum levels are achieved ***1–3*** h after ***an*** oral dose.

(86)

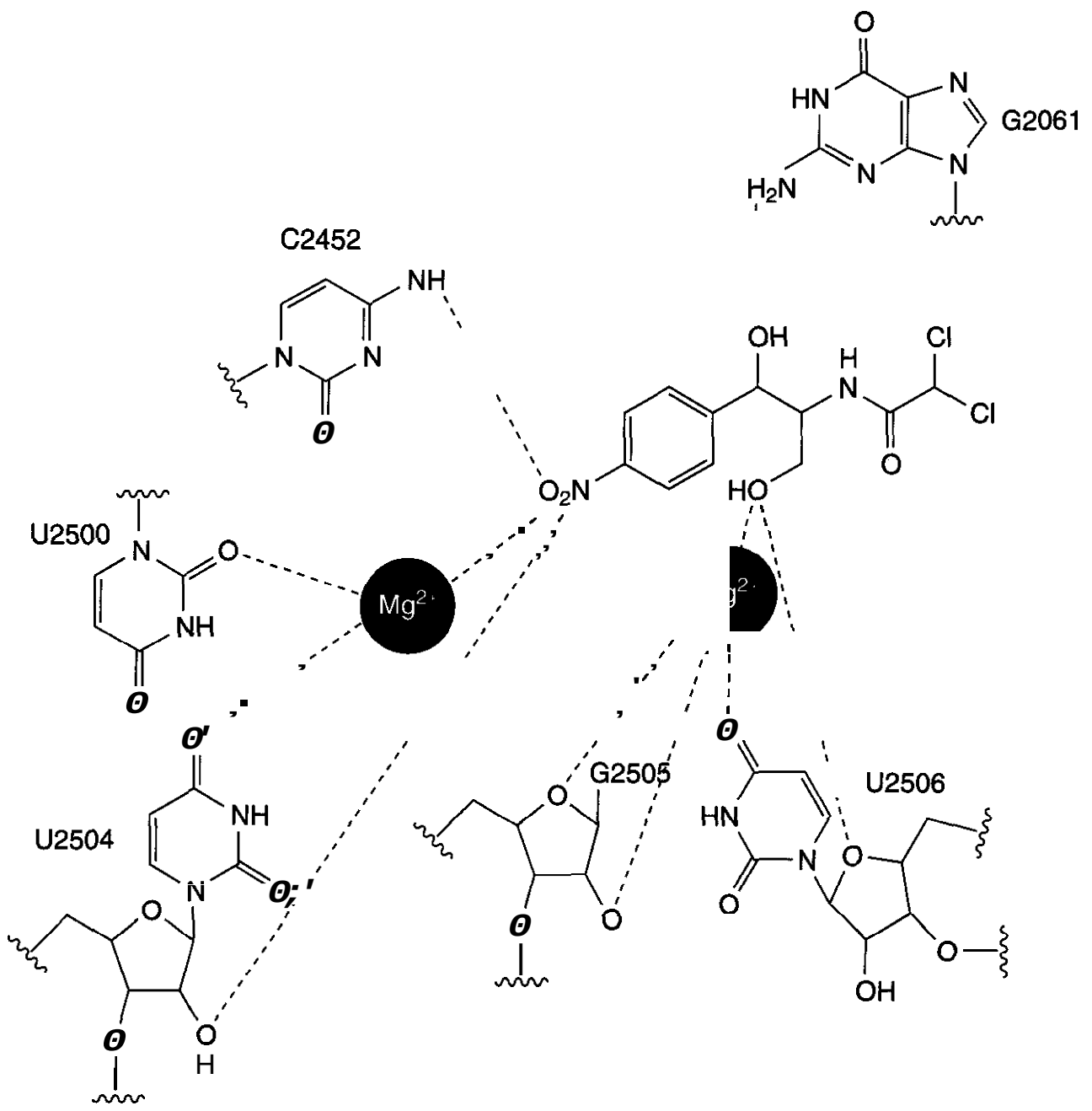


Figure 15.17. Interaction of chloramphenicol with the 50S ribosomal subunit of D. ***radiodurans*** (redrawn from Ref. 169). The equivalent E. ***coli*** numbering of the 23S rRNA is shown.

Chloramphenicol is generally bacteriostatic and binds to the P-site of the 50S ribosomal subunit, thus inhibiting translation (403). Affinity labeling has indicated that chloramphenicol binds to ribosomal protein L16 (404) and chemical footprinting studies have shown the 23S rRNA to also be a binding site (405). The 3D structure of chloramphenicol bound to the 50S ribosomal subunit has been reported, confirming the interaction with the 23S rRNA, although not the predicted contact with L16 (169). Interestingly, key contacts between the primary alcohol at C3 and the nitro group with the rRNA are mediated through contacts with putative Mg^{2+} ions (Fig. 15.17).

The major adverse effect of chloramphenicol is a risk of fatal irreversible aplastic anemia that occurs after therapy and does not appear to be related to dose or administration route (406–408). Large doses in premature and neonatal infants can result in "gray baby syndrome," which is associated with vomiting, failure to feed, distended abdomen, and blue-gray skin color and can be rapidly fatal (391, 409). Reversible bone marrow suppression and several other adverse effects including gastrointestinal problems, headache, and mild depression have also been noted (391,406).

Resistance to chloramphenicol can be the result of mutation in the 23S rRNA [e.g., G2032 (410) or C2452 (411) (*E. coli* numbering), altered permeability (412), and efflux (413–417)], but is generally the result of modifying enzymes that acetylate the antibiotic at the hydroxyl at position 3 in an acetylCoA-

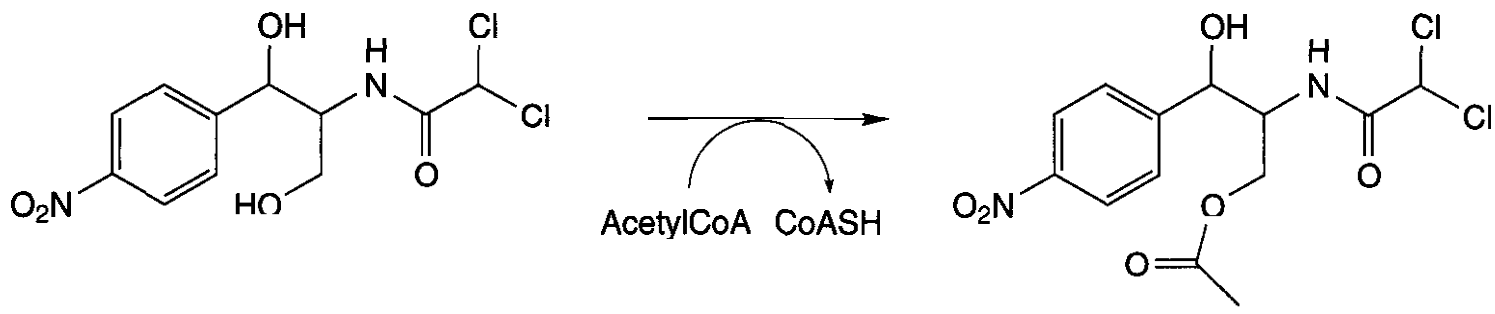


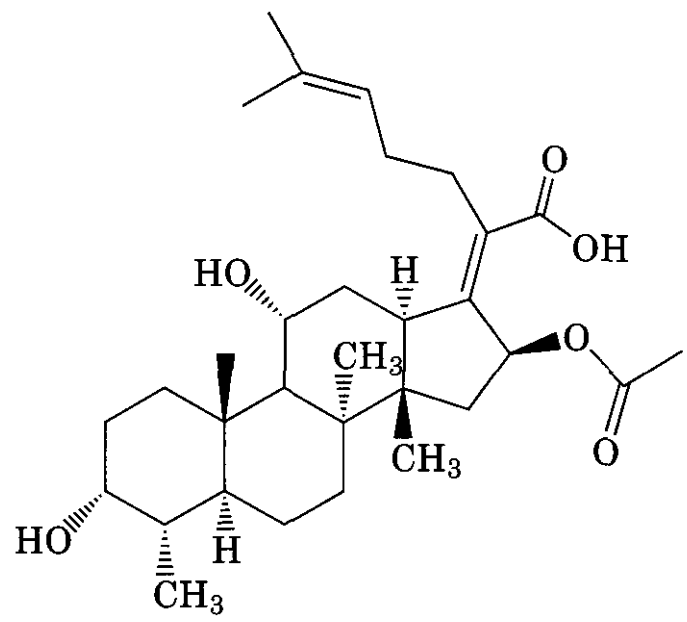
Figure 15.18. Action of chloramphenicol acetyltransferases.

dependent fashion (Fig. 15.18) (418,419). The 3D structure of chloramphenicol bound to the 50S ribosomal subunit reveals contacts between the the cytosine base at position 2452 and the antibiotic nitro group, and the primary hydroxyl at position 3 with nucleotides, both direct and through a Mg^{2+} ion, demonstrating the molecular basis of resistance (169).

There are more than 20 chloramphenicol acetyltransferase (CAT) isozymes known, all of which are trimers composed of monomers of approximately 25 kDa. These enzymes form part of a larger acetyltransferase family that also includes the streptogramin A acetyltransferases (418). The CAT_{III} isozyme from E. *coli* has been studied most extensively and the 3D structure has been observed at high resolution (1.75 Å) (420). The structure and subsequent enzyme studies have revealed that the enzyme active site lies at the interface of the subunits of the trimer, with amino acid side chains from each partner contributing to substrate binding and catalysis (reviewed in Ref. 419). The CAT, isozyme is the most prevalent in clinical settings and also confers resistance to the antibiotic fusidic acid (421).

5.4 Fusidic Acid

Fusidic acid (87) is a steroidal antibiotic produced by the fungus *Fusidium coccineum* that finds use largely as an ophthalmic and topical agent for the treatment of wound infections caused by staphylococci and streptococci. The antibiotic can also be introduced by the i.v. route in cases of staphylococcal infection that has not responded to other therapies. Fusidic acid has no effect on P. *aeruginosa* and other Gram-negative bacteria because of the lack of outer membrane permeability (422). This antibiotic acts by binding to EF-G on the ribosome after GTP hydrolysis (see Fig. 15.1). EF-G undergoes a dramatic conformational change upon hydrolysis of GTP (423, 424), and fusidic acid stabilizes the complex, thus preventing dissociation of the elongation factor (425, 426). Resistance to fusidic acid is predominantly the result of point mutations in EF-G (427, 428), although binding to chloramphenicol acetyltransferase I can also contribute to resistance (421,429).



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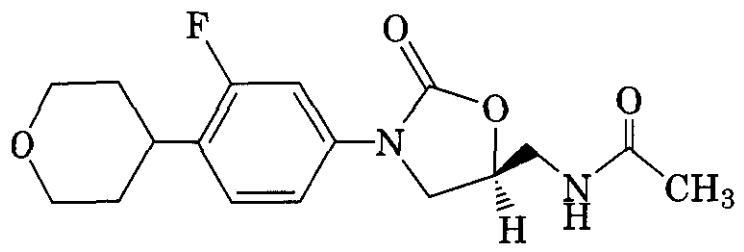
5.5 Oxazolidinones

The oxazolidinones are a relatively new class of antibiotics that inhibit bacterial protein synthesis. These compounds are not derived from natural products and represent one of the few completely synthetic antibiotics available, along with the sulfonamides, trimethroprim, and the fluoroquinolones. The oxazolidinones were first reported in the late 1980s (430) and one member of this class, linezolid (Zyvox) (88), is now approved for use in the treatment of drug-resistant and life-threatening infections caused by the Gram-positive

staphylococci, streptococci, and enterococci (431–433). These antibiotics are bacteriostatic against enterococci and staphylococci but bactericidal against streptococci. Linezolid can be administered either orally or by i.v. injection in a dose of 600 mg every 12 h. Oral absorption is rapid with a T_{max} of 1–1.5 h, generally. The oxazolidinones bind to the 50S ribosomal subunit with micromolar affinity and impede protein synthesis, possibly by interfering with the initiation step (434), although a consensus mechanism of action has not yet been established; recent evidence indicates that the 23S rRNA is the main site of interaction (435). Resistance to the oxazolidinones has been observed in the laboratory associated with mutations in the 23S rRNA [e.g., G2447U and G2576U (436,43711.



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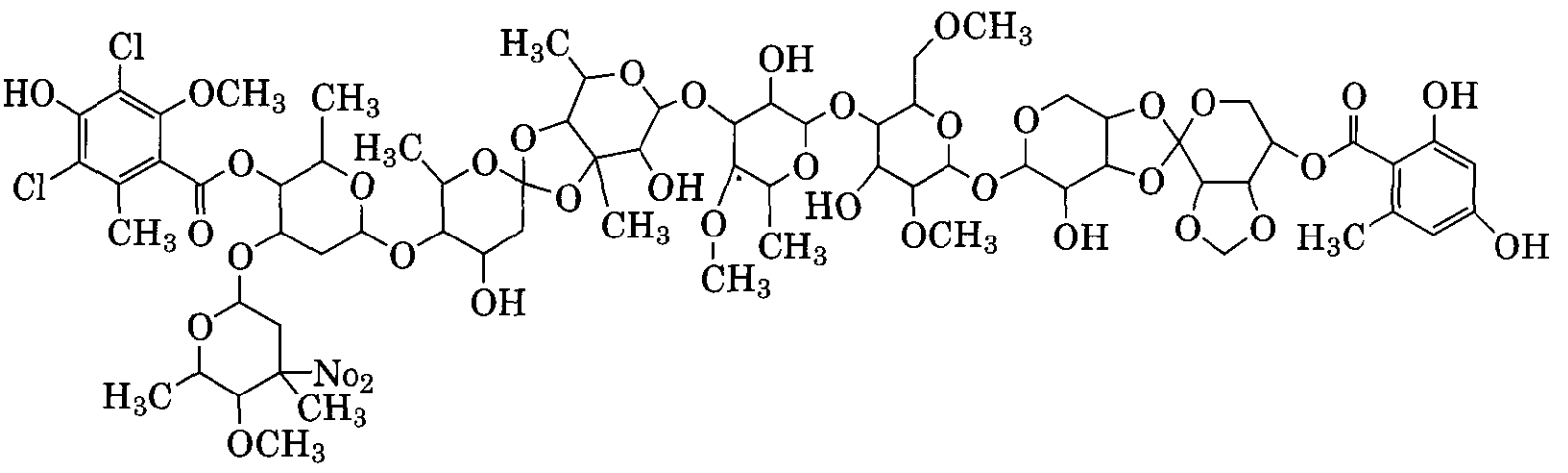
6 FUTURE PROSPECTS

Inhibition of the bacterial protein synthesis remains, along with DNA synthesis and cell wall synthesis, a favorite and proven target for antibacterial agents. The past 50 years have seen the discovery and clinical use of numerous antibacterial agents that inhibit translation and the highly conserved 16S and 23S rRNAs serve as the molecular target for most of these agents. The need for new antibacterial agents in the face of a growing antibiotic-resistance problem means that derivatives of known antibiotics that evade resistance or the synthesis of inhibitors of resistance mechanisms that can be coadministered with an "old" antibiotic are routes that must be explored (438). New antibiotics that inhibit translation at sites that are not susceptible to existing resistance are also being discovered. For example, evernimycin (89) is an oligosaccharide antibiotic active against Gram-positive bacteria (439) that acts at the initiation factor 2 binding site (440, 441).

The example of Synercid and other streptogramin antibiotics provides precedent for a reexamination of known compounds with antimicrobial activity as starting places for new drug development programs. Other antibiotics that interfere with translation have not been exploited, such as the peptide edeine [for which there is now a structure of the antibiotic bound to the 30S ribosome (442)], and the compound sparsomycin, largely attributed to indiscriminant inhibition of eukaryotic and bacterial translation, may therefore serve as templates for further compound screening and development. Other strategies such as the catalytic ribotoxins produced by aspergilli that specifically cleave rRNAs may also prove useful under some conditions (443).

The increasing number of high resolution crystal structures of ribosomes and their complexes with antibiotics now provides an unprecedented opportunity to rationally undertake structure and function analyses on inhibitors of translation and to leverage this information in the development of new antibiotics. The proven utility of bacterial protein



(89)

synthesis as an outstanding target for antibiotics will no doubt continue to be exploited in the future.

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