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Perspective

New Directions in Antibacterial Research

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Introduction: Emergence of Resistance to Antibiotics

Many of the microorganisms (e.g. viruses, bacteria, fungi, protozoa, etc.) that exist in the environment are able to parasitize animals and humans. When the host−parasite relationship is helpful to both members, it is referred to as symbiotic. For example, the bacteria that reside in the intestine (normal flora) are beneficial: they provide various vitamins and are important in the stimulation of specific immune responses. On the other hand, many of the host−parasite relationships are harmful. Many microorganisms are pathogenic to human or animals and cause various diseases resulting in extensive morbidity and mortality. Common diseases caused by bacteria include upper respiratory tract infections (URTI) such as acute otitis media and sinusitis, lower respiratory tract infections (LRTI) such as pneumonia and bronchitis, skin and soft tissue infections (SSI) surgical wound infections (SWI), urinary tract infections (UTI), gastrointestinal tract infections, sexually transmitted diseases, endocarditis, septicemia and sepsis, and Lyme disease.

Since the discovery of penicillin, pharmaceutical companies have produced more than 100 antibacterial agents/antibiotics to combat a wide variety of bacterial infections. The major classes of antibacterial agents are β-lactams (including penicillins, cephalosporins, monobactams, carbapenems), aminoglycosides, tetracyclines, sulfonamides, macrolides (such as erythromycin), quinolones, and glycopeptides (e.g. vancomycin). By the 1980s, with the use of these antibacterial agents, improved sanitary conditions, and the extensive refrigeration of food, it was believed that industrialized nations had won the war against pathogenic microbes. However, in the past several years, the rapid emergence of bacterial resistance to antibiotics has been observed. The extensive use (and misuse) of antibiotics has provided powerful forces for the selection of microbes that either carried mutations conferring resistance or had the enhanced ability to mutate to resistance in the face of the antibiotic. Bacteria have mutated or have acquired new genes producing novel machinery to overcome the action of many antibiotics. In recent years, many new antibiotic-resistant strains have been isolated from patients throughout the world.

Emergence of bacterial resistance to a number of antimicrobial agents such as β-lactam antibiotics, macrolides, quinolones, and vancomycin is becoming a major worldwide health problem.[1,2] The most significant problem in clinical practice is the increase in the isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) strains. In a recent MRSA surveillance study conducted in Japan, more than 60% of hospital *S. aureus* isolates were found to be methicillin resistant.[3] About 68% of the MRSA in Japan in 1992 were also found to carry multiple drug resistance determinants. In the United States, by the early 1990s, MRSA was detected in 20−40% of all *S. aureus* hospital isolates reported to the National Nosocomial Infections Surveillance (NNIS) System and is also a big problem in long-term care facilities.[4] Other than United States and Japan, the occurrence of epidemic strains of MRSA has also been reported in many countries such as Argentina, Australia, Belgium, Canada, Denmark, France, Germany, Greece, Hong Kong, Italy, Malaysia, Netherlands, New Zealand, Portugal, Spain, Sweden, Taiwan, and the United Kingdom.[5] In addition to resistance to β-lactam antibiotics, multiply resistant MRSA are also resistant

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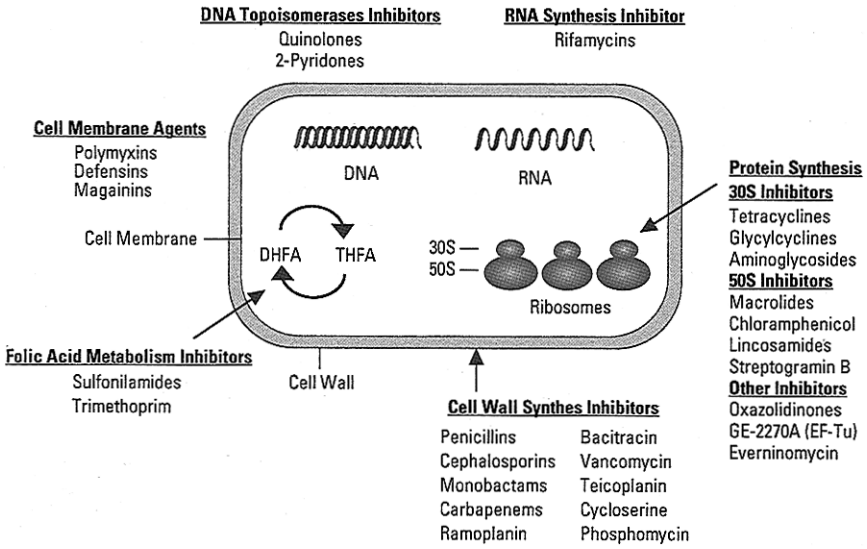


Figure 1. Schematic representation of a bacterial cell showing sites of action of various antibiotics: DHFA, dihydrofolic acid; THFA, tetrahydrofolic acid.

to macrolides, tetracyclines, aminoglycosides, and fluoroquinolones. At present, the only effective treatment for multiply resistant MRSA infections is vancomycin. However, the minimum inhibitory concentration (MIC) for vancomycin against some MRSA isolates has been increasing recently, leading to a situation where standard doses of vancomycin may not be effective for deep-seated infections.[6]

Enterococci are generally fairly resistant to antibiotics such as penicillins, cephalosporins, and aminoglycosides. Current treatment for enterococcal infection is the use either of a combination of two antibiotics or of vancomycin alone. However, with the recent increase use of vancomycin in MRSA infections and colitis due to *Clostridium difficile*, multiply resistant *Enterococcus faecium* has emerged. In a recent report to CDC's NNIS, the percentage of nosocomial enterococci resistant to vancomycin increased from 0.3% in 1989 to 7.9% in 1993. In ICUs, the percentage of enterococcal isolates resistant to vancomycin increased from 0.4% in 1989 to 13.6% in 1993. Because of the fact that not many drugs can be used effectively to treat enterococcal infections, treatment options for patients infected with vancomycin-resistant enterococci are very limited. The transfer of the *vanA* resistance gene from enterococci to *S. aureus* to generate vancomycin-resistant *S. aureus* has been demonstrated in the laboratory.[7] Although a clinical strain of vancomycin-resistant *S. aureus* has not been isolated to date, vancomycin resistance in coagulase-negative staphylococci such as *Staphylococcus hemolyticus* has been reported.[8] Furthermore, the Gram-negative organisms such as *Pseudomonas*, *Klebsiella*, *Proteus*, and *Enterobacter* species were the important antibiotic resistant pathogens in the 1970s; they remain a problem in some hospitals today.[9]

Apart from the nosocomial pathogens described above, the resistance of the important community-acquired pathogen *Streptococcus pneumoniae* to penicillin and other antibacterials is becoming a worldwide problem.[10−12] Multidrug-resistant strains of *Mycobacterium tuberculosis* have emerged in several countries including the United States.[13] The emergence and spread of resistant nosocomial and community-acquired pathogens is generating a great threat to public health worldwide. There is an urgent need to develop new agents to treat patients infected with these multidrug-resistant bacteria. This review summarizes a number of research directions in antibacterial discovery that may be able to address and overcome the problem of emergence of multidrug-resistant bacteria.

Antibacterial Targets

Although antibacterial compounds have been identified to a diverse group of intracellular compounds, the large majority of those that have been developed into drugs are targeted to inhibit the synthesis of one or more components of the bacterial cell wall or to an event involved in macromolecular (DNA, RNA, or protein) biosynthesis. The most prominent exceptions are the sulfonilamides (and other folic acid metabolism inhibitors) which inhibit an enzyme central to metabolism, dihydrofolate reductase. A representative listing of the antibacterial compounds currently in clinical practice or in development along with a schematic overview of their targets is shown in Figure 1. The precise mechanisms of actions of the clinically more important agents are discussed in the sections that follow. Novel targets not shown in Figure 1 are also discussed below and, in particular, in the section that deals with the sequencing of bacterial genomes.

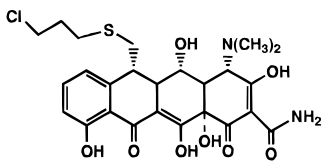
Novel Analogs/Novel Compounds

Classical approaches to the discovery of new antibacterial agents have employed screens to discover bioactive compounds and synthesis programs to chemically modify existing antibacterial agents in order to generate novel molecules with better therapeutic properties. The chemical modification approach normally produces incrementally improved compounds, particularly with respect to pharmacokinetic properties, potency, or spectrum. The question of bacterial resistance has been addressed only modestly with the discoveries of methicillin with greater stability to the action of β-lactamases and clavulanic acid, a β-lactamase inhibitor. Recently, several advances have been made by a chemical approach to tackle bacterial resistance in the areas of tetracyclines, glycopeptides, β-lactams, quino-

lones, and macrolides. New chemical classes have also been identified having activities against clinically important resistant bacteria.

A. Tetracyclines. The tetracyclines are broad-spectrum bacteriostatic antibiotics that imhibit protein synthesis by blocking the binding of aminoacyl-tRNA binding to the acceptor (A) site on the 30S ribosome. The binding of tetracyclines to nucleotide 892 of the 16S rRNA interferes with the correct folding of the 892-1400 region resulting in reduced binding of aminoacyl-tRNA. Though tetracyclines have weak activity on 80S (eukarotic) ribosomes, their selectivity against bacteria is enhanced by their concentration within bacterial but not mammalian cells.[14,15]

The emergence of resistance to tetracyclines has limited their use against a number of Gram-positive bacterial pathogens. Although the rates of resistance among coagulase-positive and -negative staphylococci, pneumococci, and group A streptococci to tetracycline varied, approximately 77% of hospital enterococci cultures isolated in a Boston teaching hospital were recently reported to be resistant to tetracycline.[16] Bacterial resistance to tetracyclines is known to involve two major mechanisms: (1) plasmid or transposon-encoded efflux systems and (2) expression of plasmid or transposon-encoded ribosomal protection factors.[17] A family of 42 kDa inner membrane proteins (Tet efflux proteins) encoded by different tetracycline resistance determinants (e.g. Tn*1721*, Tn*10*, pBR322, pAB124, pTHT15, and pT181) have been identified.[18,19] Expression of Tet proteins (classes A−E in enteric bacteria, classes K and L among Gram-positive bacteria) result in resistance to tetracycline and related analogs through the active transport of the drugs from bacterial cells. In these systems, tetracyline resistance is induced by tetracycline; synthesis of efflux protein TetA in *Escherichia coli* is under the negative control of a Tet repressor, which is inactivated upon the binding of tetracycline. Recently, two other types of plasmid or transposon-encoded ribosomal protection factors (*tetM* and *tetO*) have been described.[20] Although the exact mechanism by which these proteins prevent tetracyclines from interrupting polypeptide chain elongation has not been completely elucidated, it is currently thought that they may act as alternative elongation factors able to bind tetracycline thereby preventing its binding to ribosomes.[21]



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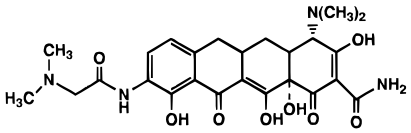

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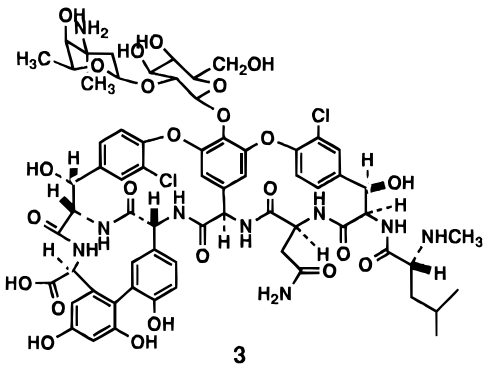
Chemical modification of the tetracycline molecule to generate potent inhibitors of the efflux protein so as to block the active efflux of tetracyclines in whole cells have been reported.[22,23] 13-[(3-Chloropropyl)thio]-5-hydroxy-α-6-deoxytetracycline (**1**) was found to possess good efflux inhibitory activity: it exhibited synergistic activity when tested in combination with tetracycline against a number of tetracycline resistant bacterial strains such as *E. coli* bearing the class A or B Tet protein, *S. aureus* bearing class K and *E. faecalis* bearing the class L determinants. These studies raise the possibility that there are multiple sites on the tetracycline molecule and that chemical modification at one of these sites can generate compounds with potent efflux inhibiting activity that can be used as a potential antibacterial agent either alone or in combination with tetracycline.

Recently, a new class of chemically modified tetracycline compounds called glycylcyclines having potent and broad spectrum antibacterial activity has been synthesized. These compounds are active against both tetracycline-sensitive and -resistant strains containing ribosomal protection factors (*tetM* and *tetO*) or efflux (*tetA*−*E*, *-L*, and *-K*) determinants. These 9-(substituted glycylamido)tetracycline derivatives are derived from minocycline, doxycycline, or sancycline series.[24,25] They are active against both Gram-positive and Gram-negative pathogens including strains resistant to minocycline, vancomycin, and β-lactam antibiotics.[16,26,27] The most promising compound, [(dimethylamino)glycylamido]-6-desmethyl-6-deoxytetracycline (DMG-DMDOT; CL 331,928 [**2**]) is currently undergoing extensive preclinical studies. DMG-DMDOT was found to be active against MRSA with a MIC_{90} of 2 μg/mL while the corresponding value for minocycline was 16 μg/mL. It is active against minocycline-resistant MRSA with TetK determinant.[28] Since minocycline has been used effectively for the treatment of MRSA, DMG-DMDOT, having antibacterial activity against minocycline resistant MRSA, will have an important role for the treatment of infections caused by MRSA. Further chemical modification on the tetracycline molecule to generate potent antibacterials with activity against tetracycline resistant bacteria is being actively pursued by several laboratories.

B. Glycopeptides. The glycopeptides vancomycin (**3**), ristocetin, and teicoplanin (**4**) have been used for the treatment of infections due to Gram-positive pathogens such as *S. aureus* and enterococci. These compounds interfere with the biosynthesis of the cell wall. In enterococci, a peptidoglycan precursor molecule consisting of *N*-acetylmuramic acid linked to the pentapeptide L-Ala-D-Glu-L-Lys-D-Ala-D-Ala is formed which is then linked (through glycosidic linkages) to *N*-acetylglucosomine and the lipophilic carrier undecaprenylpyrophosphate (Figure 2). In this form during cell wall synthesis, the pentapeptide is transported to the extracellular side of the cell membrane.[29] Vancomycin binds to the diglycoside−pentapeptide−phospholipid precursor in the growing cell wall through hydrogen bonding to the terminal D-Ala-D-Ala of the pentapeptide (Figure 3). Such binding blocks the transglycosylation, transpeptidation, and carboxypeptidation reactions which follow, thereby resulting in the inhibition of cell wall biosynthesis.[30]

Vancomycin-resistant *E. faecium* and *E. faecalis* isolates have been found with increasing frequency recently due to the increased use of vancomycin for the treatment of MRSA infections and *C. difficile* induced colitis. Inducible, plasmid-mediated resistance to the



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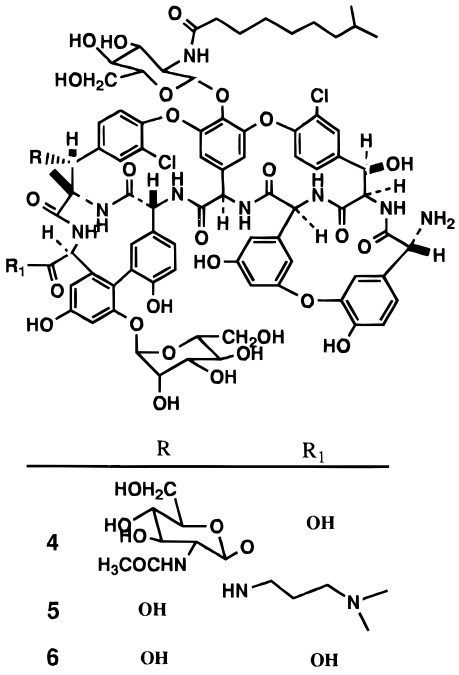
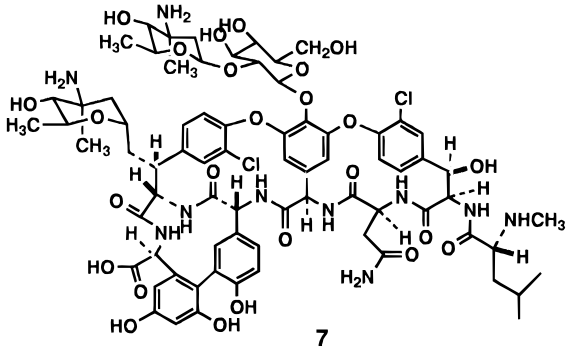


	R	R_1
4		OH
5	OH	
6	OH	OH

glycopeptides in *E. faecium* and *E. faecalis* has recently been reported.[31] Isolation of glycopeptide-resistant strains of enterococci from stool was reported upon oral administration of glycopeptides to patients.[32] This inducible resistance to glycopeptides is due to the acquisition of either a 34- or 38-kilobase plasmid. Although the transfer of resistance to susceptible strain of *E. faecium* was shown to be possible, attempts to transfer resistance to susceptible strains of *E. faecalis* and *S. aureus* were not successful.

Three vancomycin-resistant phentypes have been identified: VanA, which exhibits high-level inducible resistance (MIC > 256 μg/mL) to vancomycin and teicoplanin; VanB, variable inducible vancomycin resistance levels, teicoplanin susceptible; VanC, similar to VanB constitutive resistance.[33] Resistance to glycopeptide antibiotics in enterococci of the VanA, VanB, and VanC phenotypes results from the production of a different terminal dipeptide unit which decreases the antibiotic's binding affinity to the GlcNAc−MurNAc−pentapeptide−pyrophosphate−undecaprenol precursor. The precise mechanisms of vancomycin resistance are described later in this review.

The emergence of resistance to vancomycin and teicoplanin has prompted the search for new glycopeptides with activity against resistant Gram-positive bacteria. This search has proceeded in two directions. One is to search for novel glycopeptides through screening and fermentation. A second approach is to perform chemical modification on existing available glycopeptides to produce novel analogs. Since all naturally occurring glycopeptides exhibit cross-resistance, it seemed unlikely at the outset that novel glycopeptide analogs would possess the desired characteristic of reduced cross-resistance. However, it has been reported recently that certain modifications to glycopeptides have produced novel analogs with enhanced activities against Gram-positive organisms. MDL 62873, produced by the condensation of teicoplanin with 3,3-dimethyl-1-propylamine, increased activity against coagulase-negative staphylococci.[34] MDL 63,246 (**5**), the 3,3-dimethylaminopropyl amide of 6-β-decarboxy-6-β-hydroxymethyl derivative of the natural glycopeptide MDL 62,476 (**6**), was found to be moderately active against strains of *E. faecalis* and *E. faecium* highly resistant to both vancomycin and teicoplanin (with MICs ranging from 4 to 32 μg/mL vs ≥128 μg/mL for vancomycin).[35] In animal studies, this compound was found to possess good efficacy and a good pharmacokinetic profile.[36]



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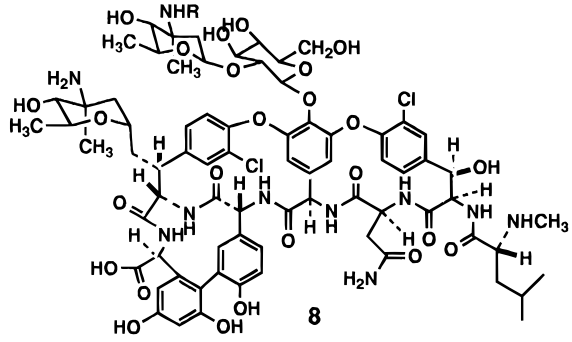

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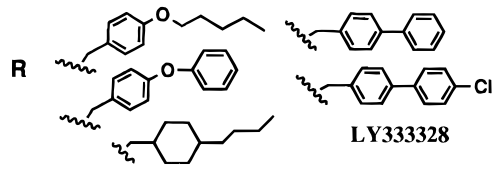


LY333328

Several years ago, scientists al Eli Lilly and Co. prepared the modified vancomycin derivative, LY264826 (**7**), in which the vancosamine sugar is replaced by epivancosamine linked with an additional amino sugar. This compound, while having good activity against vancomycin-sensitive Gram-positive bacteria, exhibited activity against vancomycin-resistant enterococci [MIC (μg/mL): 32 vs 500]. N-Alkylation of epivancosamine in LY264826 produced various potent glycopeptides (**8**)

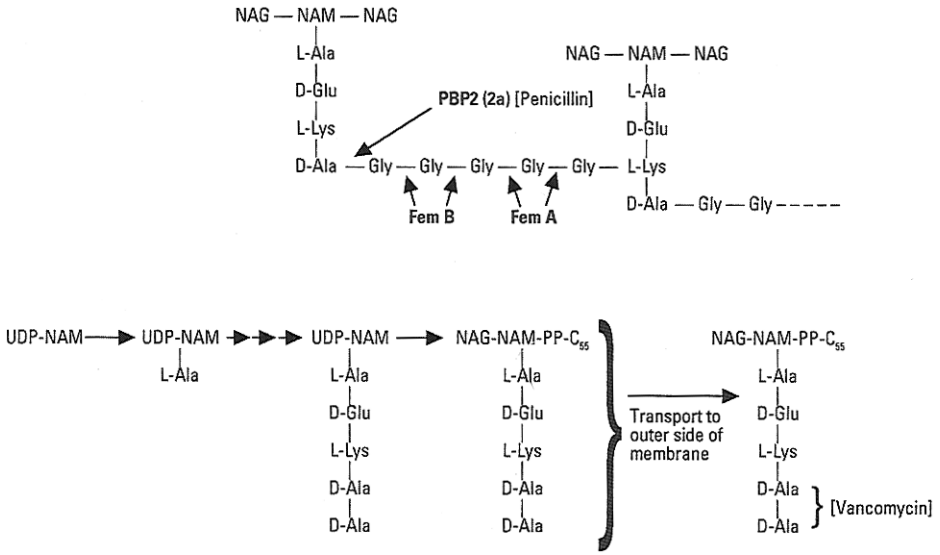


Figure 2. (Top) Schematic representation of chemical structure of the peptidoglycan layer of the cell wall showing cross-linking. (Bottom) Scheme for synthesis of the pentapeptide with respect to binding of vancomycin. The terminal D-alanine is removed to allow formation of the pentaglycine bridge. Enzymes involved in cell wall synthesis are shown in red, and arrows indicate sites where enzymes act. Cell wall synthesis inhibitors are shown in blue at corresponding sites of action. Key: NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid.

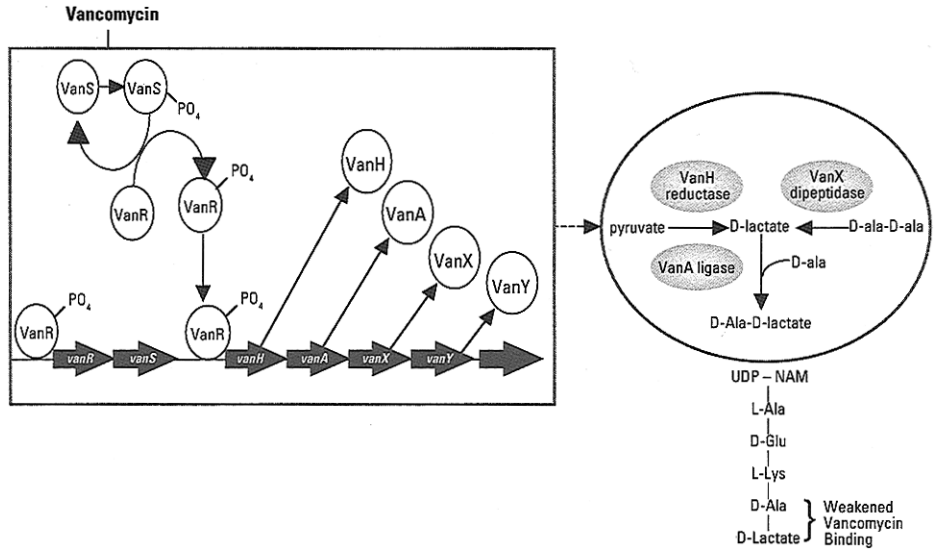
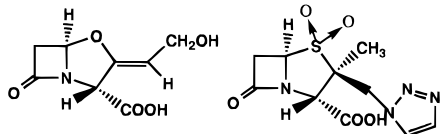


Figure 3. (Left) Scheme for the induction of enzymes conferring vancomycin resistance in VanA *Enterococcus faecium*. Genes of the *van* cluster are shown in red. Gene products are circled in blue. Cascade of phosphorylation of VanR, resulting in expression of *van* genes after vancomycin binds the cell wall is shown. (Right) Pathways for the production of D-Ala-D-lactate and the resulting depsipeptide attached to UDP−NAM through the action of VanH, VanA, and VanX enzymes.

which were very active against glycopeptide-resistant enterococci as well as MRSA, and penicillin-resistant pneumococci.[37−39] Against *E. faecium* and *E. faecalis* VanA strains, the MICs (μg/mL) for these glycopeptides ranged from 0.03 to 2 compared to 64-2108 for vancomycin and 2-64 for LY264826. As for VanB enterococci, they displayed MICs of 0.016−1 μg/mL compared to 16−1054 for vancomycin and were found to have bactericidal activity against enterococci and have excellent activity against MRSA (MICs $\leq$ 0.5 μg/mL) and teicoplanin-resistant coagulase-negative staphylococci. They are also very potent against streptococci with MICs of $\leq$0.06 μg/mL against *S. pyogenes* and $\leq$0.008 against *S. pneumoniae*. These derivatives were also found to be active *in vivo*. Efficacy against vancomycin-resistant enterococci in a mouse colonization model was demonstrated.[40] The pharmacokinetics of LY 333328 in rats has been published.[41] Like vancomycin, these compounds (LY264826 and similar compounds) inhibit transglycosylation by binding to the GlcNAc−MurNAc−pentapeptide−pyrophosphate−undecaprenol precursor. However, they interact with particulate membranes, while no interaction between vancomycin and membranes was observed. A model wherein the dimerization of glycopetides to enhance their interaction with pentapeptide precursor at the cell surface has been proposed.[42] Because they have excellent activity both *in vitro* and *in vivo* against resistant Gram-positive bacteria, these new compounds may prove in the future to have clinical utility. Further chemical modification of glycopeptide antibiotics to generate analogs having activity against vancomycin-resistant bacteria is ongoing in a number of laboratories.

C. β-Lactams. The β-lactams are a family of antibacterials containing a β-lactam ring in their chemical structures. They include penicillins, cephalosporins

(and cefamycins), monobactams, and carbapenems. They act by inhibiting a set of transpeptidases that catalyze the final cross-linking reactions in the synthesis of the cell wall (Figure 2).[43] Penicillin was thought to act as a structural analog of the pentapeptide terminal D-Ala-D-Ala to form an irreversible penicilloyl−enzyme complex during the final transpeptidation reaction. β-Lactams covalently bind to groups of proteins called penicillin-binding proteins (PBPs). In general, there are at least four PBPs found in most bacterial species and are minor components of the cytoplasmic membrane. In *E. coli*, the high molecular mass PBPs 1−3 function as transpeptidases and transglycosylases while low molecular mass PBPs 4−6 as D-alanine carboxypeptidases. Different functions such as bacterial cell elongation, longitudinal growth, or septum formation are associated with different transpeptidation reactions. D-D-Carboxypeptidase activity limits the amount of cross-linking in the cell wall (Figure 2).



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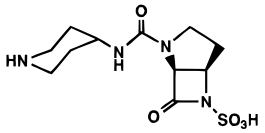

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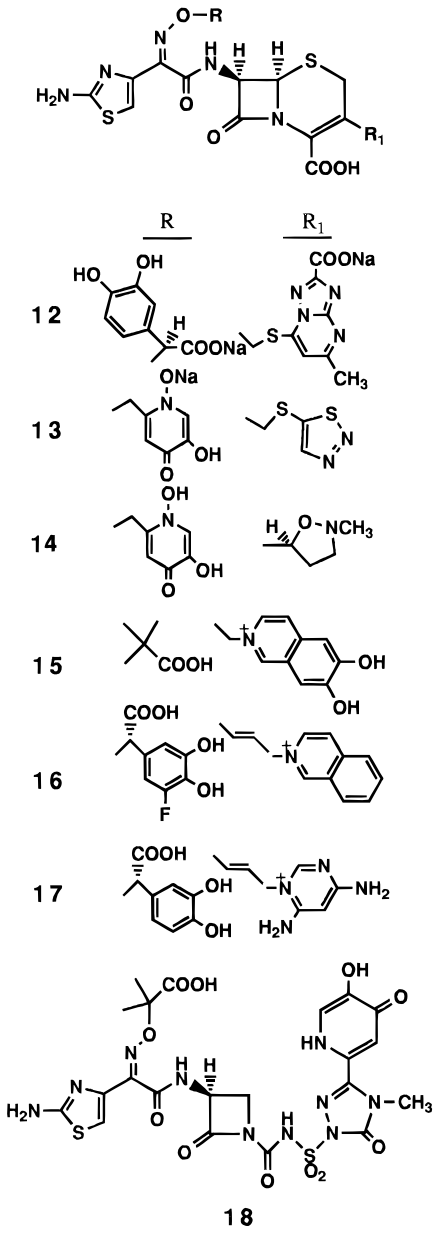
Hydrolysis of the β-lactam ring structure is by far the major cause for bacterial resistance to β-lactam antibiotics.[44] Over the years, chemical modification efforts have produced many semisynthetic β-lactam derivatives that have increased stability to the action of the hydrolyzing enzymes, the β-lactamases. The discovery of the β-lactamase inhibitor clavulanic acid (**9**) has led to amoxicillin−clavulanate combination therapy for the treatment of infections caused by β-lactamase-producing organisms.[45] Another β-lactamase inhibitor, tazobactam (**10**), combined with piperacillin is used clinically for pediatric patients.[46] A new class of β-lactamase inhibitors, the "bridged β-lactams", exemplified by Ro 48-1256 (**11**), is currently undergoing preclinical studies.[47] The successful use of these combinations and other β-lactams for the treatment of various bacterial infections over many years has led to the emergence of β-lactam-resistant organisms employing different resistance mechanisms. These include mutants with decreased outer membrane permeability to β-lactams or strains that produce altered high molecular mass PBPs that have low affinity for the β-lactam antibiotics. Current research efforts are focused on the discovery of β-lactams that are active against these organisms.

Most Gram-negative bacteria have reduced outer membrane porins leading to their decreased susceptibility to a number of antibiotics. To overcome the bacterial resistance due to the loss of outer membrane porins, certain antibiotics use solute-specific transmembrane channels to facilitate their entry into the bacterial periplasm. For example, imipenem, a β-lactam antibiotic that is active against *Pseudomonas aeruginosa* which lacks the high-permeability porins, diffuses across the bacterial cell envelope through a specific channel OprD.[48] However, many *P. aeruginosa* hospital isolates are becoming resistant to imipenem by the loss of the OprD channel.[49] Recent efforts have led to the synthesis of many new semisynthetic catechol-containing β-lactams. These compounds were found to be active against *P. aeruginosa*, other Gram-negative bacteria, and to some extent MRSA. Some of these compounds are cephalosporin derivatives which include M 16459 (**12**),[50] KP 736 (**13**),[51] SPD-391 (**14**),[52] BO-1341 (**15**),[53] RU 59863 (**16**),[54] and LK-10517 (**17**).[55] Examples of monobactam−catechol-containing anti-*Pseudomonas* compounds are U-78608 (**18**),[56] SQ 33,110 (**19**),[57] and BMS-180860 (**20**).[58] This approach for the identification of β-lactams active against *P. aeruginosa* has also been applied to the isocephems exemplified by BOF-12013 (**21**),[59] Interestingly, these compounds enter bacteria via the Fe(III) siderophore and vitamin B12 uptake system that employs the cytoplasmic membrane protein TonB, which also appears to be involved in a catechol-scavenging mechanism.[60−62] The TonB protein couples the cytoplasmic membrane electrochemical potential active transport of the iron siderophore. Though iron is used mainly for the activation of heme required for aerobic growth, it also has been shown to function in the regulation of a number of genes in Gram-negative bacteria.

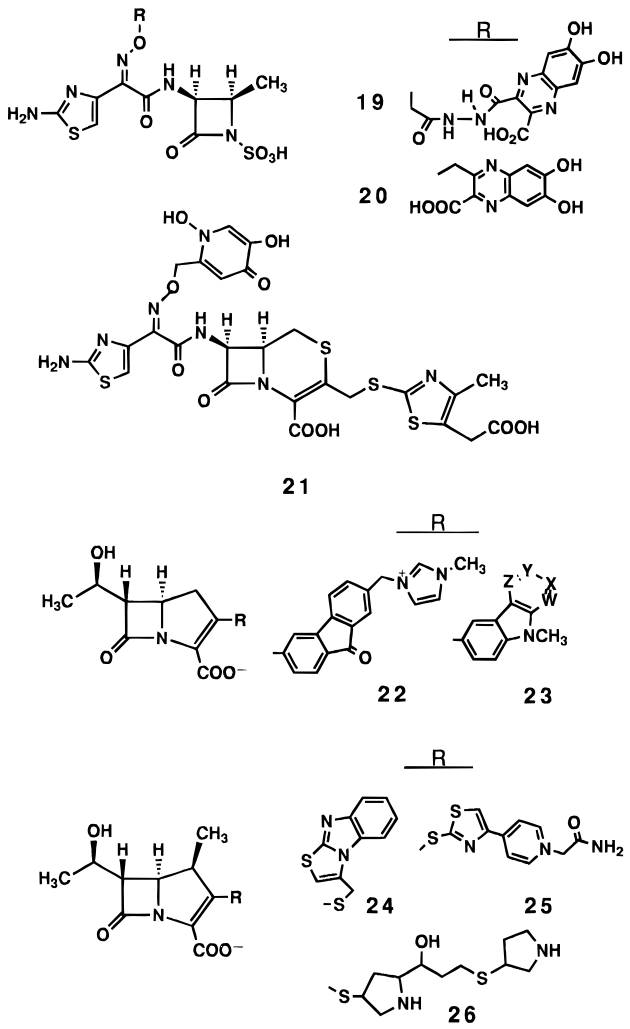
MRSA strains are fairly resistant to a number of antibiotics. Their intrinsic methicillin resistance is due to the production of an additional high molecular weight PBP known as PBP2a (PBP2′) with extremely low affinity for essentially all β-lactam antibiotics.[63] PBP2a is also found in methicillin-resistant coagulase-negative staphylococci (MRCNS) such as *Staphylococcus epidermidis*. Because of the increase in the isolation of MRSA in hospitals, research has been focused on the discovery of β-lactams with higher affinity for PBP 2a, rendering them active against MRSA. L-695,256 (**22**), the first member of a new class of 2-arylcarbapenems, possesses high affinity for the PBP2a and is highly active against MRSA and penicillin-resistant pneumococci.[64] A series of 2-carbolinylcarbapenems (**23**) was recently reported to be active against MRSA And MRCNS.[65] Other anti-MRSA carbapenems belong to 1-β-methylcarbapenems series. 1-β-Methylcarbapenems having good activity against MRSA include **24**,[66] SM-17466 (**25**),[67] and **26**.[68]

D. DNA Gyrase Inhibitors. The fluoroquinolones, such as ciprofloxacin (**27**), are totally synthetic antibacterial agents that have recently gained wide acceptance for use in the treatment of various bacterial infections both in hospital and community settings.[69] Fluoroquinolones enjoy their dominance as major chemotherapeutic agents against a number of bacterial infections such as respiratory tract, skin/soft tissue, sexually transmitted diseases and urniary tract infections. However, they have limited activities against a number of clinically important Gram-positive bacteria such as *S. pneumoniae*, *S. pyogenes*, *S. aureus*, and enterococci. Ciprofloxacin, the market leader, has low potency against anaerobes, however.

Until very recently, it had long been thought that the primary mode of action of the fluoroquinolones is to interfere the activity of DNA gyrase, an essential type II topoisomerase that exists only in bacteria.[70] DNA gyrase introduces negative supercoils into DNA follow-



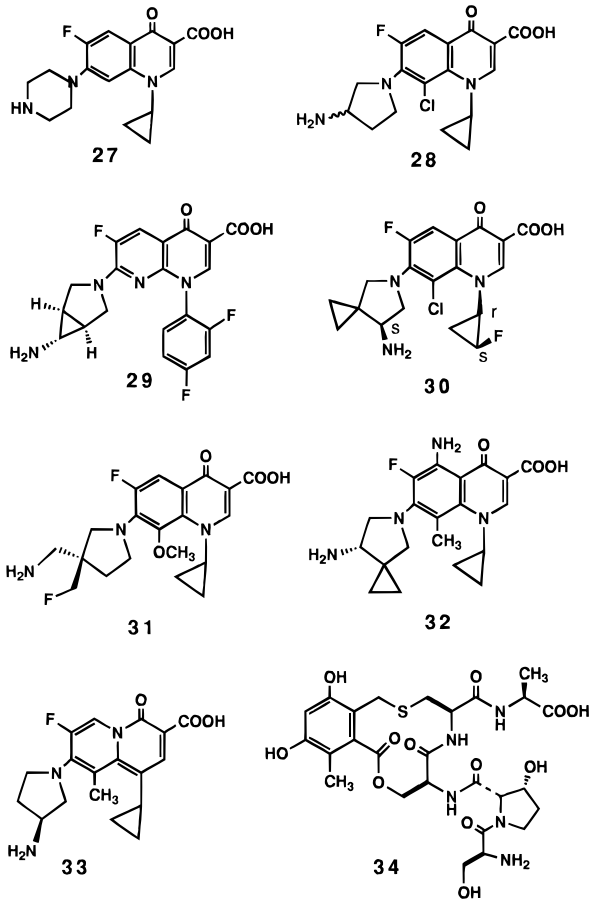
ing the replication process. The action of quinolones on gyrase was determined from enzymatic and binding assays employing the purified *E. coli* enzyme as well as from the examination of the sites in the genome that had undergone mutation when strains of *E. coli* became resistant to fluoroquinolones. After the discovery of topoisomerase IV, an essential enzyme that unlinks daughter DNA catenates that are produced through chromosome replication, it was found, in both Gram-positives and Gram-negatives, that this enzyme is also a target for the action of quinolones.[71] In fact, it is now believed that topo IV is the primary target of quinolones in *S. aureus*. Analysis of the mutations generated in stepwise-selected ciprofloxacin-resistant mutants has pointed to changes first in topoIV (*grlA* gene) to generate low-level resistance and then in the gyrase (*gyrA*) to produce high-level resistance.[72] In *E. coli*, low and moderate level quinolone resistance results from mutations giving rise to altered amino acids (mainly at Ser-83) in the quinolone resistance determining region of *gyrA* only but highly resistant strains carry an additional mutation in *parC*, the gene that determines one of the subunits of topo IV.[73] Therefore, although both



gyrase and topoIV appear to be targets for the action of quinolones, in Gram-positives, the primary target appears to be topoIV, whereas in Gram-negatives, it appears to be gyrase.

Emergence of quinolone resistance during therapy has recently been reported. A recent susceptibility surveillance study of ciprofloxacin showed a substantial increase in the isolation of resistant *S. aureusu S. epidermidis*, *E. faecalis*, *E. faecium*, *P. stuartii*, *P. cepacia*, and *Acinetobacter* sp.[74] The majority of high level quinolone-resistant MRSA clinical isolates have gyrase mutations (*grlA* was not examined).[75] Some quinolone-resistant Gram-positive mutants exhibited increased activity of an efflux system.[76] Additional mutations that reduce the permeability of the outer membrane may also confer resistance.[77]

To overcome the current lack of potency against certain bacteria and the problem of resistance, several novel fluoroquinolones have entered clinical development. These are characterized by having substantially enhanced activities against staphylococci, streptococci, and enterococci as well as activity against anaerobes. Among the newer quinolones, the most promising compounds include clinafloxacin (**28**),[78] trovafloxacin (**29**; CP-99219),[79] and DU-6859a (**30**),[80] Clinafloxacin is very potent *in vitro* with MIC_{90} values of 0.06−0.25 μg/mL against streptococci, 0.016−0.5 μg/mL against the *Enterobacteriaceae*, 0.5 μg/mL against *E. faecalis*, *Providencia* species, *Citrobacter freundii*, and *Serratia marcescens*[81] and 1 μg/mL against ciprofloxacin resistant



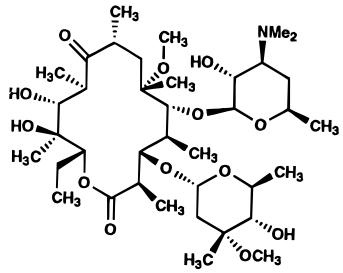
MRSA.[82] Trovafloxacin possesses greater potency against gram-positive cocci and anaerobes than ciprofloxacin. The MIC_{90} values for trovafloxacin are 2−8 μg/mL against ciprofloxacin resistant staphylococci, 0.12−0.25 μg/mL against *S. pneumoniae*, 0.25−2 μg/mL against *E. faecalis*.[83,84] Against acute experimental infections in mice, trovafloxacin was shown to be very effective against *S. pneumoniae* and other Gram-positive pathogens, while maintaining activity comparable to ciprofloxacin against Gram-negative bacteria.[85] The *in vitro* activity of DU-6859a was evaluated and the MIC_{90} values were found to be ≤0.06 μg/mL against staphylococci, ≤0.125 μg/mL against all groups A and B streptococci, 1 μg/mL against *E. faecalis*, 0.5 μg/mL against *E. faecium*, and 1 μg/mL against MRSA.[86] A recent study provided a possible explanation for DU-6859a being more active than other currently available quinolones against quinolone-resistant clinical isolates of *P. aeruginosa*. The authors suggest that this activity is due to strong inhibitory effects against mutant quinolone-resistant DNA gyrases.[87] Several additional novel quinolones such as Y-688[88] (**31**) and HSR-903[89] (**32**) with good activity against MRSA have recently been reported.

Although these new fluoroquinolones possess potential advantages over the existing quinolones used in clinical practice, they still lack activity against quinolone- and methicillin-resistant *S. aureus*, *E. faecium*, and ciprofloxacin-resistant *P. aeruginosa*. A novel structural class of potent DNA gyrase inhibitors named "2-pyridones", exemplified by A-86719.1 (**33**), has recently been reported.[90] A-86719.1 is extremely potent against MRSA, ciprofloxacin-resistant *S. aureus* and *S. epidermidis*, as well as enterococci. Its MIC_{90} values are 0.015 μg/mL against *S. aureus*, 0.03 μg/mL against penicillin-resistant *S. pneumoniae*, 0.25 μg/mL against ciprofloxacin-resistant *S. aureus*, 2 μg/mL against ciprofloxacin-resistant *P. aeruginosa*, 0.5 μg/mL against vancomycin-resistant *E. faecium*, and 0.06 μg/mL against *B. fragilis*.[91,92] It was very efficacious when tested in various *in vivo* experimental animal infection models. When administered orally, it was found to be 4−10-fold more effective than ciprofloxacin against *S. aureus*, *S. pneumoniae*, and *S. pyogenes* infections in normal mice. It was equivalent in efficacy to ciprofloxacin for the treatment of Gram-negative bacterial infections caused by *P. aeruginosa* or *E. coli*. In immunosuppressed mice, A-86719.1 showed good efficacies against *E. faecalis*, and *E. faecium* infections.[93] Investigations on other 2-pyridones were also reported.[94,95] A novel DNA gyrase inhibitor, cyclothialidine (Ro 90-1437; **34**), was reported to have activity against coumarin-resistant DNA gyrase isolated from *E. coli gyrB* mutant.[96] Poor cytoplasmic membrane penetration of cyclothialidine may be the cause of poor activity against aerobic and anaerobic bacteria, however.[97]

E. Macrolides. Macrolide antibiotics have been used for the treatment of various bacterial infections in both in-patient and out-patient settings for more than 40 years and are extensively prescribed for children because of their excellent record of safety. The newer semisynthetic macrolides such as clarithromycin (**35**)[98] and azithromycin (**36**),[99] derivatives of erythromycin, have gained wide acceptance for the treatment of both upper and lower respiratory tract infections. They possess better activity against *Haemophilius influenzae*, increased activities against anaerobes, *Legionella*, *Branhamella* spp., and *Chlamydia* and *Pasteurella multocida*, with retention of the potent activity against streptococci. In addition, clarithromycin exhibits good activity against *Helicobacter pylori* and has been approved in a combination regiment for the treatment of peptic ulcer disease.

Macrolides inhibit protein biosynthesis by binding to the 2058−2062 region of 23S ribosomal RNA of the 50S ribosomal subunit. Macrolides act by stimulating the dissociation of peptidyl-tRNA from ribosome during the translocation process, thereby inhibiting protein synthesis.[100] Recently, 20−25% of clinically isolated pneumococci in France were found to be resistant to macrolides, 18−20% were resistant in Japan, and less than 10% in Spain. High rates of erythromycin resistance among *S. pneumoniae* isolates from blood cultures have recently been reported.[11,12] The incidence of macrolide resistance in methicillin-resistant staphylococci in clinical isolates from South Africa over a period from May 1992 to July 1992 was found to be about 70%.[101] Resistance falls into three major categories: modification of the target (the ribosome), modification of the antibiotic itself, and modification of transport of the antibiotic across the cell membrane. These are described in some detail later in this report.

Several 11,12-carbamate analogs of clarithromycin were found to have activity against both inducibly and constitutively resistant *S. pyogenes*. 11,12-Carbonate analogs of erythromycin with additional modifications at the 4″-position of cladinose were also reported to be active against constitutively resistant *S. pyogenes*.[102] Recently, the 3-descladinosyl-3-oxo-11,12-cyclic carbam-



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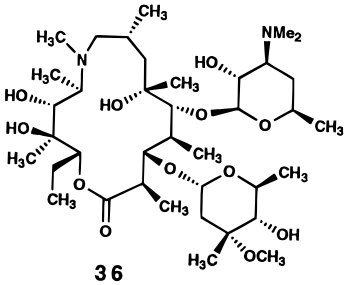

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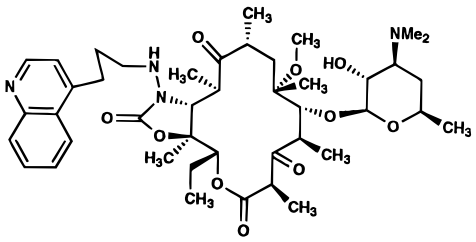

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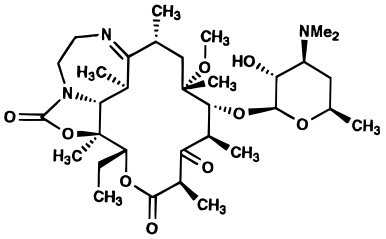

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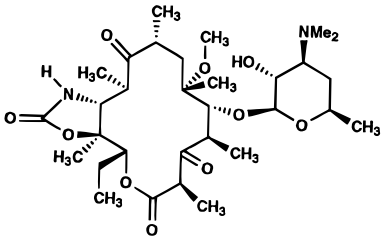

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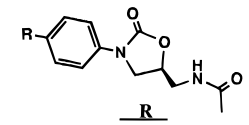
ate clarithromycin derivatives, known as the ketolides, were found to be active against penicillin-resistant and erythromycin-resistant *S. pneumoniae*. The ketolides do not induce MLS_B resistance (see description below).[103] RU 004 (**37**) was found to be highly active against respiratory pathogens including erythromycin-resistant strains.[104] The MIC_{90} value for RU 004 against penicillin- and erythromycin-resistant *S. pneumoniae* is 0.25 μg/mL.[105] RU 004 is active against *H. influenzae* and *Moraxella catarrhalis* with MIC_{90} values (μg/mL) of 2 and 0.25, respectively.[106] It has also been shown to be active *in vivo*.[107] Although RU 004 was found to be active against MLS_B-resistant pneumococci, it did not bind to the methylated ribosomes isolated from the resistant strain.[108] Two additional ketolides, TE-802 (**38**)[109] and TE-810 (**39**),[110] were also reported to have activities against erythromycin resistant *S. aureus* and *S. pneumoniae*. Derivatized ketolides therefore appear to be a promising new class of macrolides targeted to the erythromycin-resistant organisms generated during first-round treatment with the unmodified compound.

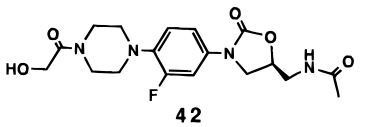
F. Oxazolidinones. A novel class of synthetic protein synthesis inhibitors known as oxazolidinones was first reported in 1987. These compounds have a novel mechanism of action that involves the inhibition of bacterial protein synthesis at a very early stage, prior to chain initiation.[111] The oxazolidinones, exemplified by DuP-721 (**40**) and DuP-105 (**41**), are potent antibacterial agents.[112] DuP-721 possesses potent *in vitro* and *in vivo* activity against Gram-positive pathogens including MRSA and MRSE as well as anaerobes and *M. tuberculosis*; their development was discontinued, however, due to toxicity seen in phase 1 clinical trials. U-100592 (**42**) and U-100766 (**43**) are two new oxazolidinones from Pharmacia & Upjohn currently in clinical development.[113] MIC_{90} values (μg/mL) for U-100592 and U-100766, respectively, against clinical isolates are as follows: MSSA-4,4; MRSA-4,4; methicillin-sensitive *Staphylococcus epidermidis* (MSSE)-1,2; methicillin-resistant *S. epidermidis* MRSE-1,2; *E. faecium*-2,4; *S. pyogenes*-1,2; *S. pneumoniae*-0.5,1; *Corynebacterium* spp.-0.5,0.5; *H. influenzae*-16,16; *M. catarrhalis*-4,4; and *Bacteroides fragilis*-16,4.[114] They are not cross-resistant with vancomycin on enterococci or with penicillin on pneumococci. When tested against staphylococci and enterococci, they exhibit bacteriostatic activity but have bactericidal activity when tested against streptococci. Their *in vivo* activities against *Gram*-positive organisms are similar to vancomycin.[115]



R

40 MeCO

41 MeSO



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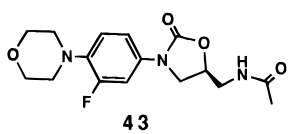

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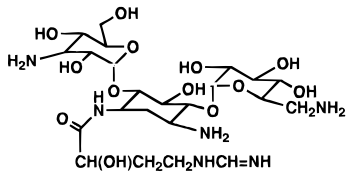

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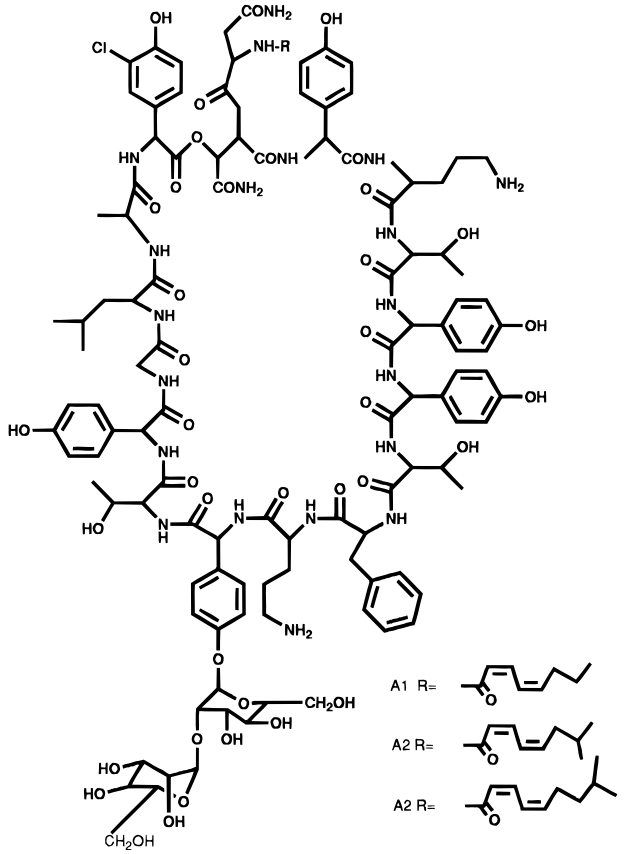
G. Aminoglycosides. Aminoglycoside antibiotics are still effective against certain Gram-negative bacteria. However, development of resistance and potential nephrotoxicity have limited their use. Recent research has identified their possible mode of action to be on the ribosomal RNA rather than ribosomal protein. Resistant mutants that map to the ribosomal RNA were isolated, and the interaction with the specific nucleotides on the ribosomal RNA by certain aminoglycosides was also demonstrated.[116] In an effort to increase the antibacterial activity without increasing nephrotoxicity, the *N*-(4-amidino-2-hydroxybutyl)kanamycin A derivative (**44**) was prepared. It displayed good antibacterial

activity and had renal levels that were 53% less than amikacin at equal doses.[117]



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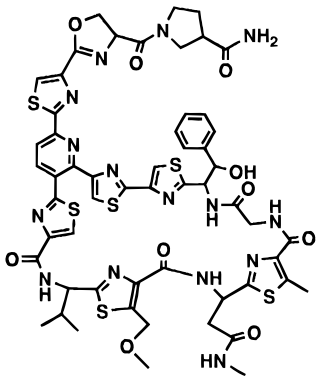
F. Cyclic Peptides/Polypeptides/Natural Products. Ramoplanin (**45**), a depsipeptide complex of three components, is a lipoglycopeptide antibiotic derived from a strain of *Actinoplanes* spp.[118] It is active against a broad range of Gram-positive bacteria including methicillin-susceptible and -resistant staphylococci, streptococci, and enterococci.[119] Since ramoplanin inhibits the peptidoglycan biosynthesis at a step earlier than that of vancomycin, it is not expected to exhibit cross-resistance with vancomycin.[120] It has been found to be active against clinical isolates of vancomycin-resistant enterococci with an MIC_{90} value of 0.5 μg/mL.[121] MIC_{90} values for ramoplanin against *Lactobacillus* spp. and *Leuconostoc* spp. for *Pediococcus* spp. were $\leq$0.25 μg/mL. The high affinity of ramoplanin for plastic substrates[122] led to an investigation on the possible use of ramoplanin in coating catheters, since catheter-related vascular infections are an important clinical problem. Ramoplanin-coated catheter implants produced much reduced *S. aureus* colonization than uncoated implants in mice.[123] Nephrotoxicity limits the clinical utility of ramoplanin by parenteral administration. However, oral ramoplanin administered to healthy volunteers was well tolerated and highly effective in reducing the burden of Gram-positive faecal flora.[124] Thus, this complex or its derivatives may have potential for treatment of infections due to vancomycin-resistant enterococci.

GE-2270A (**46**), a novel thiazolyl peptide antibiotic isolated from the fermentation broth of *Planobispora rosea*, has good activity against aerobic and anaerobic



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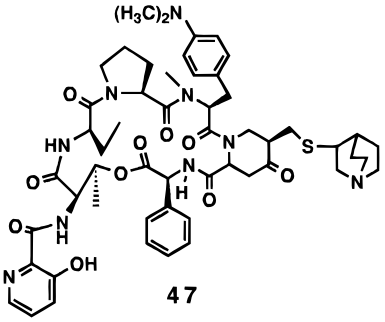

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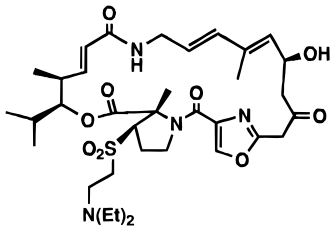

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Gram-positive bacteria and anaerobic Gram-negative bacteria including streptococci, *Propionibacterium acnes*, *M. tuberculosis*, and *B. fragilis*.[125] In experimental septicemia in mouse, the IV ED_{50} values are 1.8, 9.1, and 15.2 mg/kg for *S. aureus*, *S. pyogenes*, and *S. pneumoniae*, respectively. GE-2270A inhibits bacterial protein biosynthesis by interacting with elongation factur Tu, increasing the stability of the EF-Tu/GTP complex and thereby blocking the subsequent binding of aminoacylated tRNA.[126] Other natural antibiotics related to GE-2270A have been isolated.[127] Chemical modification of GE-2270A also led to other interesting potent antibacterial compounds.[128] Due to the difference in mode of action from antibiotics in clinical use, this new class appears to be promising for the treatment of resistant Gram-positive bacterial infections.

Natural streptogramins are complex mixtures of antibiotics possessing macrolactone structures. They usually are bactericidal and do not induce MLS_B resistance. They inhibit protein synthesis by interfering with ribosomal function. RP 59500 is a synergistic, water soluble combination of two semisynthetic pristinamycin derivatives, quinupristin (RP 57669; **47**) and dalfopristin (RP 54476; **48**), in the ratio of 30:70 (w/w).[129] RP 59500 was found to be active against methicillin-resistant and erythromycin-resistant *S. aureus* and *S. epidermidis*, with MIC_{90} values of 1 and 0.5 μg/mL. Its antistreptococci activity is similar to those of clarithromycin and azithromycin.[130] A synergistic effect was

reported for the combination of RP 59500 and vancomycin against vancomycin-resistant *E. faecium*.[131] RP 59500 is also active against Gram-negative cocci including *H. influenzae*. RP 59500 is currently under clinical evaluation for serious enterococcal infections in humans.[132]

Defensins HNP-1

Ala-Cys-Tyr-Cys-Arg-Ile-Pro-Ala-Cys-Ile-Ala-Gly-Glu-Arg-Arg-Tyr-Gly-Thr-Cys-Ile-Tyr-Gln-Gly-Arg-Leu-Trp-Ala-Phe-Cys-Cys

49

Defensins HNP-2

Cys-Tyr-Cys-Arg-Ile-Pro-Ala-Cys-Ile-Ala-Gly-Glu-Arg-Arg-Tyr-Gly-Thr-Cys-Ile-Tyr-Gln-Gly-Arg-Leu-Trp-Ala-Phe-Cys-Cys

50

Defensins HNP-3

Asp-Cys-Tyr-Cys-Arg-Ile-Pro-Ala-Cys-Ile-Ala-Gly-Glu-Arg-Arg-Tyr-Gly-Thr-Cys-Ile-Tyr-Gln-Gly-Arg-Leu-Trp-Ala-Phe-Cys-Cys

51

Magainin 1

Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Gly-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Lys-Ser

52

Magainin 2

Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser

53

Defensins (e.g. **49**−**51**) are microbiocidal and cytotoxic peptides rich in cystine, arginine, and aromatic residues.[133,134] They are found associated with azurophil granules in neutrophils in mammals and amphibians and play a significant role in intraphagosomal killing of Gram-positive and Gram-negative bacteria, fungi, and herpes virus.[135−137] Although the mechanism of action of defensins has not been fully established, it is currently believed that they assemble into complexes in cytoplasmic membranes producing pores that allow extrusion of the contents of the cells resulting in lysis. Recently, two water soluble peptides called magainins (**52**, **53**) with broad-spectrum antimicrobial activity were isolated from the skin of the African frog *Xenopus laevis*.[138] These compounds have activity against many clinically relevant pathogens with MIC values between 5 and 50 μg/mL. Several laboratories are preparing analogs of these peptides as potential clinically effective antimicrobial agents.

The *in vitro* activity of a novel oligosaccharide antibiotic everninomicin SCH 27899, produced by *Micromonospora carbonacea* has been reported. It is active against Gram-positive bacteria including methicillin-resistant staphylococci, streptococci, and vancomycin-resistant enterococci.[139] MICs of SCH 27899 for *E. faecalis* ranged from 0.5 to 1 μg/mL, including multiply-resistant strains; for *S. aureus*, from 0.125 to 0.25 μg/mL including methicillin- and teicoplanin-resistant strains; for *S. hemolyticus* from 0.5 to 4 μg/mL including high-level vancomycin-resistant strains.[140] Since multidrug-resistant Gram-positive cocci are becoming increasingly common causes of nosocomial infections worldwide, further work in this area to identify a safe everninomicin derivative is of interest.

Novel Targets Directly Involved in Antibiotic Resistance: A. Macrolide Resistance. 1. Erm Methyltransferases and MLS Resistance. The most important and best characterized mechanism of erythromycin resistance involves mono- and dimethylation of a specific adenine residue in the 23S ribosomal RNA component of the 50S ribosome. The enzymes that carry out this modification were given the name Erm (erythromycin ribosome methylation).[141] To date, more than two dozen Erm's have been identified (largely by nucleotide sequencing) from a variety of Gram-positive and Gram-negative bacteria, but mainly from organisms which have emerged as clinically resistant to administered erythromycin. The enzymes are similar in size (ca. 29 kDa), act by a common mechanism, and can interchange substrates (rRNA from different bacteria), but share varying degrees of sequence similarity. 23S rRNA extracted from ribosomes isolated from erythromycin resistant cells carrying an *erm* gene carry an N^6-monomethyl or N^6,N^6-dimethyl NH_2 group of A2058.[142] This site is located in the peptidyl transferase loop of the RNA that catalyzes polypeptide chain growth.[143,144] Though erythromycin does not bind free 23S rRNA, footprinting analyses have demonstrated that the binding of the antibiotic to the ribosome is localized to sequences that overlap A2058 in the RNA.[145] Methylation of A2058 dramatically reduces the efficiency of erythromycin binding, hence the failure of erythromycin to inhibit protein synthesis in *erm*-carrying strains.[146]

Methylation of A2058 confers resistance to all known macrolides, the lincosamides (lincomycin, clindamycin, celesticetin), and the streptogramin B class (vernamycin B, pristinomycin I, staphylomycin S), hence the term MLS_B. Since the antibiotics belonging to these classes act competetively with each other in binding to 50S ribosomes, it is thought that they all overlap A2058 and fail to bind ribosomes when A2058 is methylated.[147]

The present group of *erm* genes known are carried on plasmids or transposons and are highly transmissible within (and even between) species.[148] Since the enzymes found in clinically important pathogens resemble those present in the macrolide-producing actinomycetes (for example, *ermE* in the erythromycin-producing *Saccharopolyspora erythraea*[149]) where they are required to provide self-resistance to the antibiotics they produce, it has been proposed that MLS_B resistance spread originally from the macrolide producers. Since actinomycetes are soil organisms that do not occupy the same environments as staphylococci, streptococci, etc., it is difficult to imagine how the rapid spread and diverse sequence evolution of *erm* genes could have taken place only in the last 40 years of widespread use of erythromycin. Yet, through the powerful selection of erythromycin resistance, such spread and rapid evolution must have occurred if ribosome methylation does not confer some other benefit to microorganisms other than resistance to macrolides.

The precise biochemistry of *in vivo* methylation by Erm enzymes has eluded understanding and has confused researchers through a series of contradictory findings relating to mechanism. *In vitro*, the Erm enzymes transfer a methyl group from *S*-adenosylmethionine (AdoMet) to A2085 and use free 23S RNA (or A2058-containing segments thereof) as substrates. Under *in vitro* conditions Erm's do not methylate purified 50S ribosomes. However, free 23S RNA is not present to any measureable extent as such in the cell but is likely assembled into ribosomes during *de novo* synthesis. Thus, the optimal substrate for the enzyme *in vitro* is likely not utilized as the substrate *in vivo*, and the actual *in vivo* substrate for Erm-dependent methylation has not yet been identified.

From the 1950s through the mid 1980s, the greatest majority of MLS_B-resistant clinical strains isolated carried erythromycin-inducible resistance; that is, erythromycin was required to induce MLS_B resistance. For most of the inducibly resistant clinical isolates, only the 14-membered macrolides erythromycin and oleandomycin, and some of their derivatives (e.g. claithromycin, azithromycin) were found to induce resistance. (A notable exception is the synthesis of ErmSF in the tylosin-producing strain *Streptomyces fradiae* which is induced by tylosin.)[150] Strains induced by erythromycin are fully resistant to other macrolides, lincosmides, and streptogramins.

Studies on inducible resistance, most notably by B. Weisblum and D. Dubnau and their associates over the past 20 years (review refs 151, 152), have not only led to a comprehensive understanding of the phenomenon but have also augmented our understanding of a fundamental mechanism of gene regulation. MLS_B resistance in a bacterial cell is dependent upon expression of its resident resistance-conferring gene. In the case of *erm*, expression is regulated by erythromycin at the level of translation. The best studied is *ermC*, first isolated from *S. aureus*. In the absence of erythromycin, the mRNA corresponding to the *erm* coding sequence and an immediately upstream 141 nucleotide leader sequence is made but the segment corresponding to *ermC* is not translated into the ErmC polypeptide. Addition of subinhibitory concentrations of erythromycin somehow enables the mRNA to translate the *ermC* coding region. Mutational analyses of the leader sequence have led to the following translational attenuation model to explain inducible resistance.

As in the case of all procaryotic genes, translation requires the accurate positioning of ribosomes around the ATG start codon which is accomplished by the presence of a ribosome binding site (Shine-Delgarno sequence) [SD-2] in the mRNA a few nucleotides upstream of the starter ATG. In the absence of erythromycin, it has been proposed that the leader assumes a secondary structure such that SD-2 is sequestered and thus inaccessible for ribosome binding; hence no translation of *ermC*. Within the leader there is a coding sequence for a 19 amino acid peptide (with an ATG start and a UAA terminator codon) which contains its own SD sequence, SD-1. It is thought that SD-1 is not sequestered, thus the leader peptide would be produced in the absence of erythromycin. In the presence of subinhibitory levels of erythromycin, it is believed that ribosomes translating through the leader region stall after the ninth amino acid and, in doing so, cause destabilization of the secondary structure of the leader RNA. Destabilization of the leader RNA opens up the region corresponding to SD-2 allowing ribosomes to bind and translate *ermC*. Once ErmC is produced, ribosomes would become methylated and resistant to erythromycin and other macrolides. This model does not yet explain why only erythromycin and oleandomycin, and not other macrolides, can serve as inducers of *erm* gene expression. (It is also curious that the actinomycetes that produce these two macrolides do not carry inducible *erm* genes. ErmE in *Sac. erythraea*, the producer of erythromycin, is made constitutively. *Streptomyces antibioticus*, the producer of oleandomycin does not carry an *erm* gene but contains several other mechanisms to confer self-resistance to the antibiotic.) Furthermore, the sequence or structure of the first nine amino acids in the leader peptide appear to be important in inducibility, although the role of the leader is not yet clear.[152]

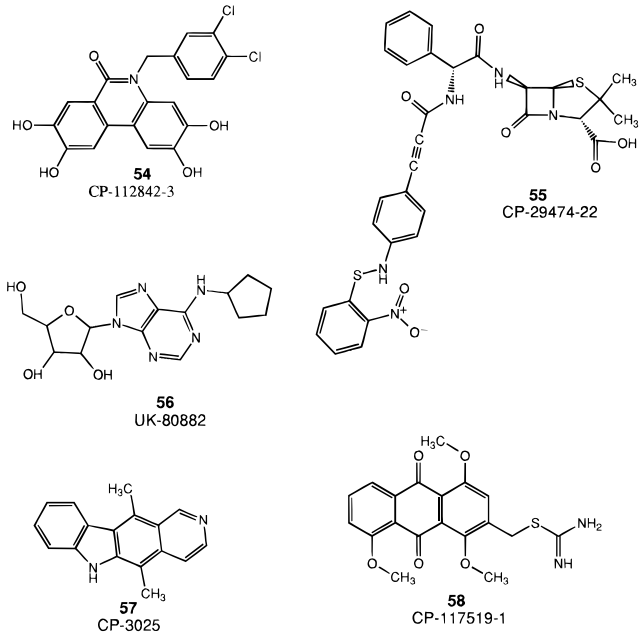
2. Efflux. Strains of staphylococci inducibly resistant to macrolides (14- and 15-membered rings) and the type B streptogramins but not to the lincosamides (MS_B resistance) have been found to carry the gene *msrA*, which encodes an ATP-binding protein.[153] MsrA, a 56 kDa polypeptide, carries two ATP-binding domains and is related to a super-family of the multicomponent ATP-binding cassette (ABC) transporters in Gram-negative bacteria and in eukaryotic cells, including the P-glycoprotein responsible for multidrug resistance. As in the super-family of ABC transporters, MsrA carries the characteristic membrane spanning domains within its sequence and, therefore, is believed to confer resistance through the energy dependent efflux of macrolides and type B streptogramins. MsrA-conferred resistance is inducible by erythromycin, but the mechanism of induction has not yet been reported. The *msrA* gene, or its highly similar homologues, has been found in *S. aureus*, *S. epidermidis*,[154] and other coagulase-negative staphylococci,[155] alone or in combination with an *erm* gene. Though *msrA* can confer MS_B resistance upon introduction into sensitive strains of staphylococci, since ABC transporters usually work in combination with other membrane binding proteins, it is generally thought that the other membrane proteins required for erythromycin efflux are resident in the cell or co-opted for use by MsrA. An efflux system for erythromycin resistance has recently been reported in a number of streptococcal species.[156]

A number of ATP-binding proteins resembling ABC transporters that confer low-level resistance to erythromycin have been found in Gram-negatives. These include a number of genes in the *acr* operon (named for acriflavine resistance) in *E. coli*[157] and the *mexA* and *mexB* genes in *Pseusomonas aeruginosa*.[158] Though they are not similar to MsrA in primary sequence, the *acr* and *mex* genes are believed to participate in active efflux of erythromycin and other drugs; disruption of these genes result in the reduction in the MICs for erythromycin and other antibiotics.

3. Macrolide Modification. A number of mechanisms to modify the structure of erythromycin and other macrolides have been discovered in various bacteria, but only two, hydrolysis of the lactone ring and phosphorylation, have been found in clinically important strains. Two plasmid-borne genes, *ereA* and *ereB*, encoding esterases that irreversibly hydrolyze the macrolactone ring of erythromycin, have been found in clinical strains of *E. coli*.[159,160] The enzymes, specific for erythromycin, differ in size (EreA = 37.8 kDa, EreB = 45 kDa) but are related in sequence (50% overall similarity, 23% identity).[161,162] An erythromycin esterase activity has been reported in a strain of *S. aureus*.[163] A single report of phosphorylation of the 2′-OH of the desosamine of oleandomycin, but not erythromycin, has also been found in *E. coli*.[164]

4. Other Mechanisms. A recent study on macrolide resistance in *Mycobacterium avium* isolated from patients with disseminated disease established a link between mutations within the 23S rRNA gene at position 2274 providing an alteration in free energy

associated with rRNA folding and conformation changes in assembled ribosomes resulting in lowered affinity for macrolides. Thus, this study identified a possible molecular mechanism of resistance at the level of the ribosome.[165] A point mutation in the 23S rRNA gene having a transition A-to-G at position 2063 or 2064 for macrolide resistant *M. pneumoniae* has also been reported.[166] A ribosomal protein gene alternation accounts for the chromosomally mediated, constitutive resistance in *Campylobacter jejuni*.[167]



5. Erm Inhibitors. Since MLS_B resistance (inducible and constitutive) is entirely dependent upon the function of an active Erm, it should be possible to reverse such resistance by inhibiting the enzyme. Since AdoMet-dependent methylation of DNA is a common phenomenon in all cells, selective inhibitors of Erm's would be those that do not compete with AdoMet binding. Furthermore, since all Erm's appear to be structurally related and mechanistically similar (they interchange RNA substrates), it can be envisioned that an agent can be developed that would have broad spectrum anti-Erm activity. The relative simplicity of the Erm assay and its adaptability to a high throughput format for preliminary screening makes it an attractive candidate to search for inhibitors. Such a search through a large chemical library was recently conducted at Pfizer, Inc. employing ErmC.[168] A number of compounds that selectively inhibited ErmC (in contrast to a DNA methylase or a catechol-*O*-methyltransferase) were uncovered (**54**−**58**), the best ones with IC_{50} values in the range of 450 nM to 22 μM. These compounds exhibited poor, if any activity in reversing erythromycin resistance of *ermC*-containing *B. subtilis* on plates and no potentiation of the ability of erythromycin to prevent *ermC*-containing *S. aureus* or *Streptococcus pyogenes* to generate acute infections in mice. Other than their phenolic nature, the structural similarity of these compounds is not obvious and the basis for inhibition of ErmC was not pursued. In addition, it is not known whether any of the compounds inhibited other Erm enzymes. Why these compounds failed *in vitro* and *in vivo* is not understood but may be related to the inherent differences in RNA methylation that take place in bacteria and what is identified as Erm activity in the assays employed (see above). Greater understanding of the intracellular mechanisms of RNA methylation are probably required to enable the development of a successful Erm inhibitor.

B. Methicillin Resistance. Methicillin, a β-lactam antibiotic resistant to hydrolysis by penicillinases, was introduced into clinical practice in the late 1950s to treat penicillin-resistant Gram-positive infections. Methicillin-resistant *S. aureus* (MRSA) was first reported in Europe in 1961[169] and has become a major nosocomial concern today. Initially, all methicillin-resistant *S. aureus* (MRSA) strains exhibited low-level resistance; high-level resistance among hospital indigenous strains began to emerge upon continued use of the antibiotic. Resistance in greater than 90% of MRSA strains is due to the presence of the gene *mecA*.[170] Other, lesser important mechanisms of resistance include the ability to hydrolyze methicillin and the overproduction of constituent penicillin binding proteins.

1. *mecA*. The gene *mecA* encodes a penicillin binding protein designated PBP2a (PBP2′). Though its source is currently not known, it is believed that *mecA* was acquired by one staphylococcal strain from which it spread horizontally among *S. aureus*, *S. epidermidis*, and other *Staphylococcus* species. Hybridization mapping and sequencing has indicated that *mecA* is part of a DNA segment that has been designated the "*mec* core", which consists of *mecA* and the regulator genes *mecI* and *mecR1*.[171] *mecI* is a 15 kDa protein that functions to repress expression of *mecA*. *mecR1* is a 68 kDa protein that serves as co-inducer of *mecA* expression. The core is part of a larger 30−40 kb *mec* element that is present in Mec^+ but absent in Mec^- strains and which occupies a single preferred chromosomal location in all strains.[170] Allelic differences among the three core *mec* genes vary from strain to strain and with individual methicillin resistance levels. In addition, DNA sequences in the *mec* element adjoining the *mec* core may also vary widely from strain to strain. The *mec* element either contains or lies adjacent to insertion sequence (IS) elements in most strains and likely employs them for strain to strain transmission via plasmid vectors.

PBP2a is a 76 kDa protein that has low affinity to methicillin.[63] Like the other large PBPs, PBP2a has both transglycosylase and transpeptidase activity and is believed to function in the methicillin-refractory construction of the peptidoglycan component of the cell wall (Figure 3). However, Mec^+ MRSA strains have altered peptidoglycan layers, indicating that PBP2a cannot carry out all the functions of normal PBP2s; hence it is not known whether PBP2a can build the wall without the participation of other, yet unidentified PBPs. Nonetheless, genetic experiments indicate that *mecA* alone is sufficient to confer methicillin resistance to staphylococcal cells. Retrospective studies and cloning experiments have indicated that acquisition of the "wild type" *mec* element imparts low-level resistance to methicillin. Resistance is also heterogeneous: the majority of the population exhibits low level resistance but a small minority are resistant to high levels of the drug.[171] The basis for heteroresistance is not yet understood. Clinical MRSA isolates exist in which the entire population is resistant to high levels of the drug.

In these strains the gene *mecI* is mutated or deleted, and the strains have undergone mutations at other, uncharacterized chromosomal sites.[172,173] In general, in strains that carry wild type *mecR1* and *mecI*, methicillin resistance is inducible by methicillin. Interestingly, MecR1 and MecI are highly similar to the BlaR1 and BlaI proteins that regulate the plasmid-carried penicillinase (β-lactamase) gene *blaZ*. In MRSA strains that carry penicillinase plasmids, *mecA* can be regulated by the BlaR1−BlaI system.[173]

Corrleations between the amount of PBP2a in the cell wall and the level of resistance to methicillin have been demonstrated up to a resistance level of approximately 16 mg/mL.[174,175] Strains resistant to higher levels do not have additional PBP2a; the basis for increased resistance is not known but is likely due to mutations at chromosome sites that affect binding of the drug to its cellular targets. Whatever its basis, resistance in these strains is lost upon deletion of *mecA*.

2. Auxilliary Genes. Although resistance to methicillin depends upon the presence of *mecA*, five other chromosomal loci, located outside of the *mec* element affect methicillin resistance levels. These have been designated *fem* (for factor essential for methicillin resistance) or aux genes. Inactivation of these genes results in the restoration of methicillin susceptibility regardless of the pre-existing level of resistance. Two *fem* genes, *femA* and *femB*, lie adjacent to each other and appear to form an operon.[176,177] The genes each encode a polypeptide of approximately 50 kDa which share 40% identity and 64% overall similarity with each other but do not appear to have homology with any other proteins presently in the sequence databases. Hybridization experiments indicate that all *S. aureus* strains contain *femA* and *femB*, but the precise roles of the corresponding proteins in cell wall biosynthesis are not yet known but are thought to be involved in the synthesis of the pentaglycine bridge (Figure 2).[178] Deletions in either *femA* or *femB* results in the phenotype of large cellular masses that presumably arises from aberrant formation of the cell septum.[63] *femAB* mutants also have enlarged cell walls but are otherwise normal with respect to growth and survival. Analysis of the muropeptides of the cell wall has indicated a reduction of the intrapeptide glycine bridge but the precise defect in cell wall assembly is not yet understood.[176] *femAB* mutants are sensitive to methicillin regardless of whether they carry *mecA*. The putative roles of FemA and FemB are shown in Figure 2.

A third *fem* locus, *femC*, encodes the repressor (GlnR) of the glutamine synthetase operon (*glnRA*).[179] The *femC* (*glnR*) mutation was found to have a polar effect on expression of *glnA* (glutamine synthetase), resulting in the reduction of isoglutamine residues in the pentapeptide stem of the muropeptide component of the cell wall. This change in the composition of the cell wall results in the loss of resistance to methicillin, analogous to the case of the *femA* and *femB* mutants. The other *fem* loci, *femD* and *femE*, have not been characterized.[180,181] Mutations at these sites all result in the loss of resistance to methicillin, but the bases for this change are not yet understood.

3. Methicillin Resistance Targets. Though sensitivity of β-lactam-resistant staphylococci to methicillin is easily rationalized (the drug resists hydrolysis by β-lactamases yet binds well to normal constituent PBPs 1, 2, 3, and 4 and blocks their action); as described above, resistance to the drug is a complicated process that involves a number of genes and is not yet fully understood. The centerpiece of resistance is PBP2a, a penicillin binding protein with low affinity to methicillin, determined by the *mecA* gene. In highly resistant strains, PBP2a appears to take the entire role of the PBPs in constructing the muropeptide cross links but is likely aided in its duties by the products of the *femA* and *femB* genes. Targeting PBP2a for inhibition would be a rational approach for overcoming methicillin resistance. This could be accomplished in a number of ways. As described above, one would be to design an advanced β-lactam that not only binds efficiently to PBP2a and inhibits its transpeptidase activity, but also which binds to the other constituent PBPs in the cell. This agent would act as a stand-alone antibiotic. A second approach is to discover an agent that inactivates PBP2a but which does not itself kill the host. Such a compound would render cells sensitive to methicillin and would be used in combination therapy with the antibiotic. If such an agent were discovered, it could also be combined chemically with methicillin or another β-lactam and be delivered as a single novel antibacterial compound.

Targeting the polypeptides FemA or FemB for inhibition is also a logical approach to overcoming methicillin resistance. Though *femAB* mutants are seen to undergo morphological changes (yet survive *in vitro*), it cannot be predicted whether the use of FemA/B inhibitors would result in the killing of bacterial cells *in vivo*. At the very least, however, it would be expected that such an agent could be used in combination with methicillin to kill MRSA strains. Since the functions of FemA and FemB are not yet precisely known, screening for inhibitors of these proteins may be difficult at the present time. On the other hand, since the two proteins share very high similarity and appear to each function in the synthesis of the interpeptide cross links, it may be possible to find a single agent to inhibit both functions.

Since *femC* does appear to be directly involved in the synthesis of the cell wall and mutation within it gives rise to methicillin resistance through a fortuitous genetic polar effect, we do not consider FemC as a bonified target for discovery of a methicillin resistance inhibitor. Furthermore, until the roles of *femD* and *femE* are elucidated, their gene products would not presently be considered as targets, as well.

C. Vancomycin Resistance. 1. The VanA Operon and Mechanism of Resistance. A series of genes for resistance to vancomycin in enterococci have been identified. One set that confers inducible resistance is located within the transposon Tn*1546* which itself is carried by the conjugative plasmid pIP816.[182,183] As seen in Figure 3, Tn*1546* carries nine genes, seven *van* genes along with a transposase and a resolvase required for movement of the transposon. Five *van* genes, *S*, *R*, *H*, *A*, and *X* are required for vancomycin resistance.[184] The *van* genes have been cloned, sequenced and characterized for function involved in resistance. *vanH* encodes an enzyme that reduces pyruvate (or α-ketobutyate) to D-lactate (or D-hydroxybutyrate).[185] *vanA* encodes an enzyme similar to the normal ligase that synthesizes D-Ala-D-Ala but which

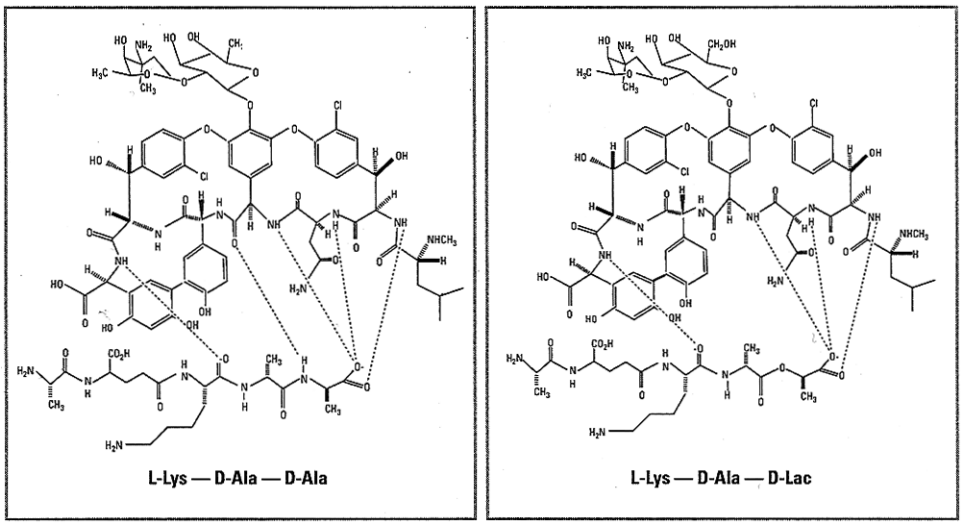


Figure 4. Interaction of vancomycin with the cell wall. (Left) Proposed hydrogen bonds between vacomycin and the D-Ala-D-Ala moiety of the pentapeptide precursor of the peptidoglycan. (Right) Proposed loss of a hydrogen bond when D-lactate is substituted for D-Ala.

produces the depsipeptides D-Ala-D-lactate (or D-Ala-D-hydroxybutyrate).[186,187] The depsipeptide is added to the UDP−MurNac−tripeptide (L-Ala-D-Glu-L-Lys) substrate by the same enzyme (MurF) that normally adds D-Ala-D-Ala. *vanX* encodes a dipeptidase that can break down D-Ala-D-Ala but has only poor activity on D-Ala-D-lactate and D-Ala-D-hydroxybutyrate.[188] VanX activity, therefore, is to ensure quantitative substitution of D-Ala-D-lactate for D-Ala-D-Ala in the peptidoglycan to result in the diminished binding of vancomycin to the cell wall (Figure 4). *vanY* encodes a penicillin-insensitive D-D carboxypeptidase that cleaves the D-Ala-D-Ala dipeptide from the UDP−MurNac−pentapeptide precursor of the peptidoglycan but has low activity on the pentapeptide precursors containing C-terminal D-lactate or D-Ser.[189,190] VanY activity is not required for vancomycin resistance, however. The function of VanZ is not yet known but is not required for resistance to glycopeptides.

Expression of the *van* genes are controlled by *vanS* and *vanR* whose gene products form a two-component regulatory system. VanS is the "sensor" histidine kinase that becomes phosphorylated in response to a signal that arises upon the binding of vancomycin to the cell wall.[184,191] It is not believed that vancomycin itself is the signal but rather a precursor or breakdown product that is produced after the binding of vancomycin takes place. VanS contains a membrane-spanning domain that suggests interaction between the protein and the signal at the membrane. The phosphate group on VanS is then transferred to the responder VanR protein at an aspartate residue that is conserved in members of the response regulator family of proteins. Phospho-VanR then acts as a transcriptional regulator by allowing transcription from the promoters that lie upstream of *vanH* and upstream of *vanS*.[184,192] The VanSR system, therefore, regulates synthesis of the VanH, VanA, VanX, VanY, and VanZ proteins as well as its own synthesis (Figure 3).

2. VanB and VanC. A variant of the VanA system described above has been found in some vancomycin-resistant enterococci. The VanB system carries homologs of each of the seven *van* genes but has two notable differences from the VanA system: the structural genes for the enzymes involved in D-Ala-D-lactate synthesis are in a sequence different from those in VanA yet are within an operon and the VanB system does not confer resistance to teicoplanin.[193−195] VanB is inducible through a cognate VanSR two-component system which responds to vancomycin but not to teicoplanin. *Enterococcus gallinarium* strains carrying the gene *vanC*, which encodes a homolog of the VanA ligase, are constitutively resistant to low levels of vancomycin only, and the D-Ala-D-Ala terminal dipeptide in its peptidoglycan is replaced by D-Ala-D-Ser.[196,197]

3. Vancomycin Resistance Targets. On a strictly theoretical basis, any one of the five proteins required for resistance can be targeted for inhibition to convert cells from the Van^R to Van^S phenotype. One target that is mechanistically unique to vancomycin resistance and which does not have a mammalian counterpart (through current searches of the sequence databases) that could interact with an administered agent is VanX. Mechanistic inhibitors of VanX have been synthesized, but they do not appear to reverse vancomycin resistance to any reasonable degree.[188]

Future Directions: New Targets from Bacterial Genomics

As we have described, a number of bacterial enzymes offer themselves as targets for the development of specific and selective binding agents that will result either in the reversal of drug resistance or, if the enzyme is essential for cell survival, in the death of the bacterial cell. It is expected, however, as has been found in the past, that all targets will undergo change to become resistant to agents developed for their inhibition. Thus, the only certain way to avoid encountering previously generated resistance is to seek new targets against which antagonists have not been previously developed.

Targets are chosen for the search for new antibacterial agents either after exhaustive (and often laborious) efforts at purification of the particular protein has been accomplished and an assay to measure its activity or function has been developed. Alternatively, a whole cell screen can be developed using the corresponding

gene for the target. In general, the number of targets currently available has been limited by our understanding of the functions of genes and proteins. A more traditional approach has been the search for new antibacterial agents in crude screens followed by the determination of the target after the agent was found. These "bottom-up" directions have recently given way to the search for targets through genomic sequencing, a "top-down", systematic approach which rapidly enables the uncovering of previously unidentified genes and their corresponding proteins. Genes (and proteins) found to be essential for survival can be regarded as potential targets. With the recent and future developments in rapid screening technology, many of the new proteins identified will be targeted for the development of novel classes of antibacterial agents.

The past decade has witnessed the generation of large amounts of DNA sequence through advances in rapid gene cloning, PCR-based amplification, and DNA sequencing technologies, along with the development of sophisticated DNA analysis software. At the end of 1995, greater than 25 000 of the total of 240 000 entries in the GenBank and EMBL public data bases contained bacterial sequences from a wide variety of organisms, from small segments of a genes to several kilobases containing many genes. Efforts to sequence entire genomes of microrganisms have also been initiated and completed for *Haemophilus influenzae*[198] and *Mycoplasma genitalium*.[199] The entire genomes of *E. coli* and *B. subtilis* are expected to be fully sequenced by the end of 1996. Sequences of a number of other pathogens (e.g. *S. aureus*, *S. pneumoniae*, *M. tuberculosis*), along with sequencing of the human genome are also under way or have been completed in a number of high throughout DNA sequencing institutions (e.g. The Institute for Genome Research, Human Genome Sciences, Incyte, etc.). It is likely that sequences obtained by companies will not be reported for some time, however.

In general, genes involved in a given biochemical pathway are clustered. Thus, if some of the genes for a pathway are previously known, uncovering the sequences of adjacent DNA will usually reveal more genes in that pathway and, hence, more potential novel targets. By comparing the sequences of a number of proteins among a variety of bacteria it should be possible to find novel targets that are common to either all or most strains as well as those that are unique to a given species. The essentiality of a given target can be determined by attempting to disrupt the corresponding gene, as described below. Cellular locations of proteins and putative functions can also be assessed from examination of the genes themselves (for encoding signal sequences) or of the translation products of genes for membrane-spanning domains, signal sequences for export, etc. and by comparison to like proteins in the databases. Most importantly, the similarity of the target in question to proteins of human origin can be addressed by comparing the target to the more than 150 000 human sequences in accessible databases.

The completed sequence of the *H. influenzae* Rd genome can be used as an example. The 1 830 177 base pair genome contains 1743 open reading frames (genes). The functions of only 1007 genes have been assigned, mainly from sequence comparisons. Sixty-eight genes have been identified as involved in the synthesis of the

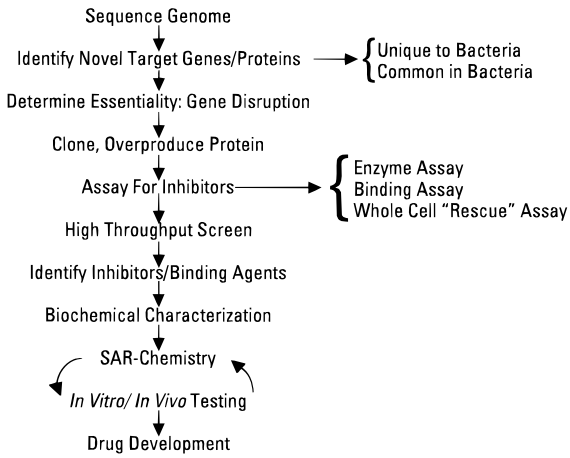


Figure 5. Outline of scheme to use microbial genomics for the development of novel antibacterial agents.

cell wall or of proteins, lipoproteins, or polysaccharides that lie on the cell surface. Since the cell wall is unique to bacteria, many of the proteins encoded by these genes should serve as new, heretofore untouched, antibacterial targets. Furthermore, each of the 736 unassigned genes can be tested for essentiality, commoness among bacteria, and uniqueness to bacteria. It is likely, therefore, that many of them will also encode proteins that are potential new targets.

A general paradigm for employing sequence for target development is outlined in Figure 5. Since the antibacterial agent is usually directed to a bacterial protein, it is first necessary to determine whether the protein in question is essential to the survival of the cell. A disruption experiment employs homologous recombination to introduce an extraneous piece of DNA (generally encoding a drug resistance marker than can be employed for genetic selection) into the gene in question. If disruption leads to bacterial death, the gene would be considered essential. After the gene to be targeted is chosen, it is fairly straightforward to clone and overexpress the gene in order to isolate the corresponding protein. The protein can be purified on the basis of its enzymatic activity or from an antibody-affinity column (if an antibody is available). If an activity is not known for the protein, a common method to obtain highly purified preparations is to introduce (by cloning methods) a segment of 6−10 histidine residues fused to either the N- or C-terminus of the polypeptide and then purify the protein through passage through a nickel−chelate column employing imidazole to release bound material.

If the target is novel or if the new inhibitor sought is not based upon the structure of a known pre-existing compound, screening is required to find leads that have potential activity. In general, high-throughput screens in which thousands of compounds (likely as mixtures) can be screened in a relatively short time are preferred. If the activity of the target protein is known (e.g. MecA, ErmC, VanA, etc.), the screen can consist of a rapid assay for enzyme activity formatted in microtiter plates so that hundreds can be run simultaneously. With the use of robotics, homogeneous assays and rapid methods of measurement, hundreds of thousands of compounds can be examined for activity as an enzyme inhibitor within the period of 1 year or less. On the other hand, it is likely that many of the novel targets chosen in the

future to be screened for inhibition will not be enzymes—it is even possible that the precise function of the protein will not be fully understood. If high-throughput assays that do not depend on known enzyme function can be developed to find binding agents (that can later be developed into inhibitors), all that may be required to initiate a screen is the knowledge of the essentiality of the target and the ability to generate large amounts of the protein in question through standard cloning and expression technology. Rapid screens that can examine the binding of agents in very large numbers to proteins need to be developed. Once done, the union of bacterial genomics and rapid screening should enable the identification of novel inhibitors of yet to be identified novel bacterial targets.

Conclusion

There is worldwide agreement over the present need to develop novel agents to treat bacterial infections that have become increasingly unresponsive to standard antibacterial therapy. Some of the directions being taken to meet this need have been described here. Standard medicinal and combinatorial chemical approaches to the synthesis of novel entities or next generation current drugs are being utilized to produce our next batch of useful antibiotics. It is likely, however, that chemical modification of presently existing drugs will give way in the future to agents discovered first as "hits" from target-directed screens—hits that will turned into drugs through SAR chemistry approaches. Though many potential antibacterial targets known today have yet to be examined, it is likely that they will be exploited as emphasis continues to be placed on screening and advances in screening technology. Through screening, new agents will be discovered for which there no preexisting resistance (and likely no cross resistance). The current and future efforts in bacterial genomic sequencing will greatly amplify the number of targets that can be screened for decades to come in the continuing and necessary search for new antibacterial agents.

Biographies

Daniel T. W. Chu received his B.S. in chemistry from the University of Alberta, and his Ph.D. in organic chemistry with Professor Z. Valenta at the University of New Brunswick. He joined Abbott Laboratories in the antibacterial research area in 1972 where he is currently a Distinguished Research Fellow and Director of Antibacterial Discovery Research.

Leonard Katz received his B.S. in microbiology from McGill University and his Ph.D. in molecular biology with Professor Ellis Englesberg at the University of California, Santa Barbara. After postdoctoral work at the University of California, San Diego, he joined the Biology Department at New York University. He joined Abbott Laboratories in 1979 and is currently a Research Fellow and Senior Project Leader in the Antibacterial Discovery Research Area.

Jacob J. Plattner received his B.S. from the University of Illinois and his Ph.D. in organic chemistry with Professor Henry Rapoport at the University of California, Berkeley. He joined Pfizer in 1972 where he worked on research projects in the areas of analgesics, antipsychotics, and prostaglandins. In 1977, Dr. Plattner joined Abbott Laboratories as a Senior Research Chemist in the Diagnostics Division. He is current Divisional Vice President of Anti-infective Research in the Pharmaceutical Products Division.

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