Glycopeptide and Lipoglycopeptide Antibiotics

Dan Kahne,*,† Catherine Leimkuhler,† Wei Lu,‡ and Christopher Walsh*,‡

The Department of Chemistry, Princeton University, Princeton, New Jersey 08544, and the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

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* To whom correspondence should be addressed. C.W.: phone, 617-432-1715; fax, 617-432-0438; e-mail, Christopher_walsh@hms.harvard.edu. D.K.: phone, 617-496-0208; fax, 617-496-0215; e-mail, kahne@chemistry.harvard.edu.
† Current address: The Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138.
‡ Harvard Medical School.

1. Introduction

Vancomycin and teicoplanin are the two glycopeptide antibiotics that are used clinically, and the emergence of resistance to them poses a serious threat to human health. Some microorganisms are resistant to both vancomycin and teicoplanin, but some resistant strains remain sensitive to teicoplanin when resistance to vancomycin develops. Given the apparent structural and mechanistic similarities of these two drugs, one longstanding question is why they have different effects on some microorganisms. This review will summarize what is known about the structure and function of the glycopeptide and lipoglycopeptide antibiotics, how glycopeptide resistance develops, and how natural and semisynthetic lipoglycopeptide derivatives are being used to learn how to overcome glycopeptide resistance and for development as novel therapeutic agents.

Vancomycin and teicoplanin are used to treat serious Gram-positive bacterial infections that are resistant to other antibiotics, e.g. β-lactams. The frequency of resistance to the glycopeptide antibiotics has increased significantly over the past decade and now represents a serious threat to public health.[1] Moreover, multiple genera, including Staphylococcus, have developed resistance to these drugs.[2] It is hoped that research into the molecular basis for glycopeptide resistance will lead to the ability to design new glycopeptide antibiotics with activity against both sensitive and resistant bacterial strains. In this review we will describe the structures of a set of important natural glycopeptide antibiotics and outline their biosynthetic pathways. We will then discuss what is known about the mode of action of the glycopeptides and how structural differences influence biological activity. Next, we will analyze how resistance to the glycopeptides develops and summarize the efforts to develop glycopeptides that can overcome resistance. Finally, we will discuss how access to unnatural glycopeptides has provided tools that have been used to identify new targets of the glycopeptide class and activity differences observed for natural glycopeptides against different bacterial pathogens.

Antibiotics can be classified on several axes. One is by the nature of the targets in susceptible bacteria, for example blockade of bacterial cell wall biosynthesis or bacterial protein synthesis, or DNA and RNA replication.[3] A second axis is whether the antibiotics derive from natural product scaffolds or

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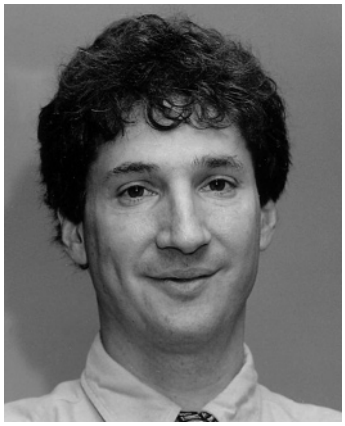
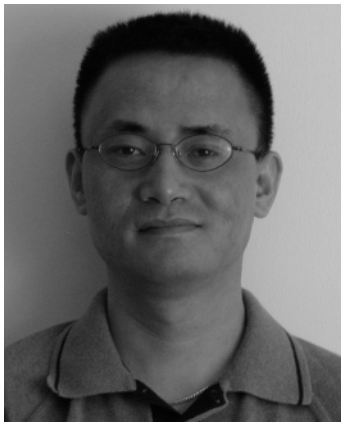


Daniel Kahne recently moved to Harvard University from Princeton University, where he was on the faculty for 16 years. He holds appointments in the departments of Chemistry and Chemical Biology (CCB) and Biological Chemistry and Molecular Pharmacology (BCMP). He trained as a synthetic organic chemist with Gilbert Stork and continued postdoctoral training at Columbia with Clark Still. He has longstanding interests in the chemistry and biology of natural products, and in recent years become interested in how natural products can be used to probe cellular pathways. Professor Kahne's research group is divided into students who develop synthetic methods to make and modify complex natural products and students who combine some chemistry with molecular and cellular biology to address questions relating to how various natural products function. In the last five years, the Kahne group has become interested in antibiotic resistance and has made significant contributions to understanding the mechanisms of action of glycopeptide antibiotics and derivatives that overcome glycopeptide resistance. The Kahne group has also been using glycopeptide derivatives and other antibiotics in conjunction with genetics to probe pathways involving cell wall biosynthesis and outer membrane biogenesis.

Catherine E. Leimkuhler attended Villanova University, where she received her B.S. in Chemistry in 2001. Upon graduation, she attended Princeton University, where she received her M.A. under the direction of Daniel Kahne. She has worked on a chemoenyzmatic synthesis of novel glycopeptide antibiotics. She is currently pursuing her Ph.D. with Daniel Kahne at Harvard University.



Wei Lu received his B.E. degree from East China University of Chemical Technology in 1992. He did his Ph.D. in Philip Cole's lab at Johns Hopkins University. His Ph.D. research focused on the regulation of protein tyrosine phosphatases. Since 2002, he has been pursuing his postdoctoral research in Christopher Walsh's group at Harvard Medical School. His current research interest lies in functional analysis of glycosyltransferases involved in natural product biosynthesis. He is currently a Ruth L. Kirschstein Nation Institute of Health postdoctoral fellow.

Christopher T. Walsh is the Hamilton Kuhn Professor of Biological Chemistry and Molecular Pharmacology (BCMP) at Harvard Medical School. He has had extensive experience in academic administration, including Chairmanship of the MIT Chemistry Dept (1982–1987) and the HMS Biological Chemistry & Molecular Pharmacology Department (1987–1995) as well as serving as President and CEO of the Dana Farber Cancer Institute (1992–1995). His research has focused on enzymes and enzyme inhibitors, with recent specialization on antibiotics. He and his group have authored over 590 research papers; a text, *Enzymatic Reaction Mechanisms*; and a book, *Antibiotics: Origins, Actions, Resistance*. He is a member of the National Academy of Sciences, the Institute of Medicine, and the American Philosophical Society.

are synthetic antibacterial drugs.[4] The glycopeptide antibiotics of the vancomycin (**1**) and teicoplanin (**2**) (Figure 1) class block steps in the biosynthesis of the peptidoglycan layer of bacterial cell walls. They are natural products elaborated by actinomycete soil bacteria, with vancomycin being isolated in the 1950s from *Amycolatopsis orientalis* and teicoplanin around 1980 from *Actinoplanes teichomyceticus*.[5] Each of these antibiotics is effective against Gram-positive bacteria but not Gram-negative ones, due to the permeability barrier of the intact outer membrane in Gram-negative bacteria that keeps the glycopeptides from reaching their targets at the periplasmic face of the cytoplasmic membrane.

Vancomycin has been approved for human use in many countries, while teicoplanin, widely prescribed in Europe, was never been brought successfully through the FDA approval process in the US. The drugs have been front line agents for treating endocarditis caused by enterococci, opportunistic pathogens, and have become mainstays in the treatment of life-threatening infections due to methicillin resistant *Staphylococcus aureus* (MRSA).[1]

2. Structures of Vancomycin and Teicoplanin

In this review, the term lipopeptide is used for acylated peptide natural products (such as daptomy-

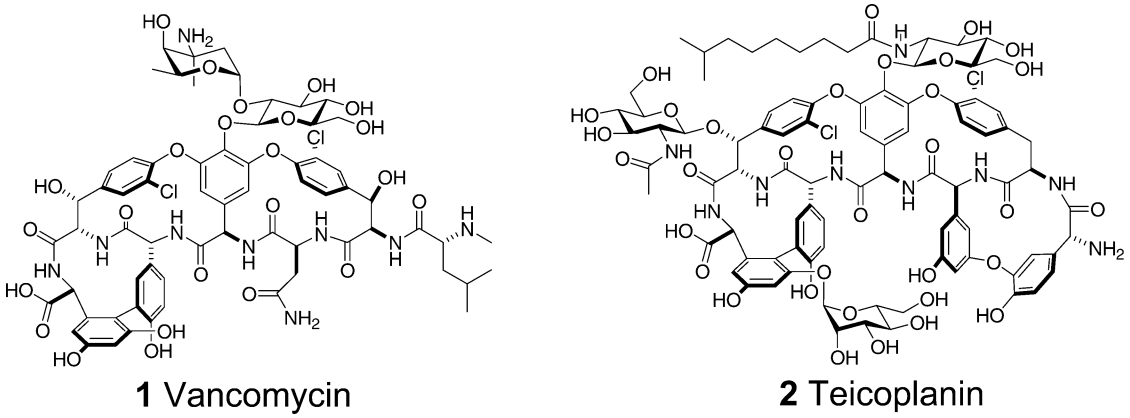


Figure 1. Structures of vancomycin (**1**) and teicoplanin (**2**), glycopeptide and lipoglycopeptide antibiotics approved for human therapy.

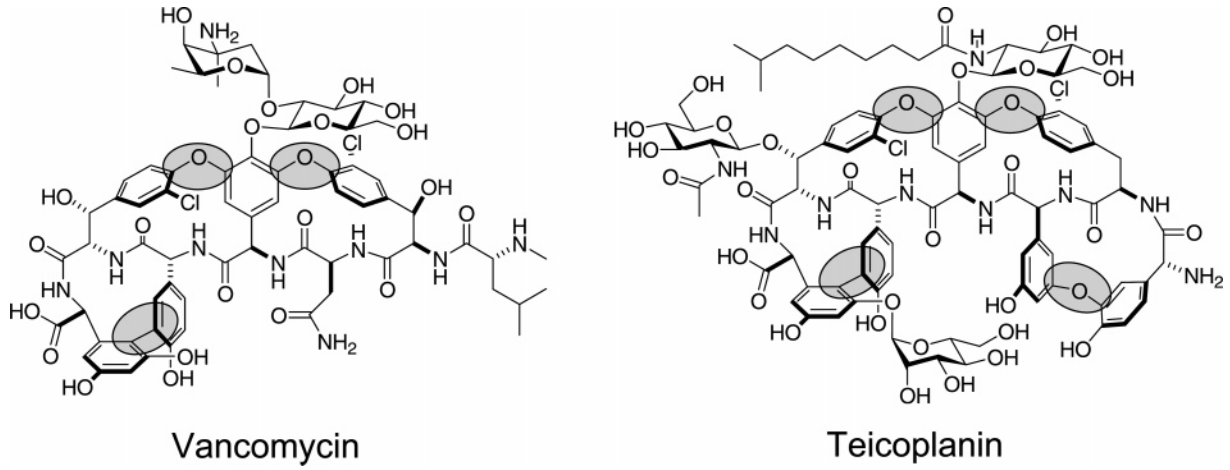


Figure 2. Oxidative cross-linking by hemeprotein enzymes of the nascent heptapeptides to yield the oxidized, cross-linked heptapeptide scaffolds: three cross-links at residues 2−4, 4−6, and 5−7 in the vancomycin scaffold and a fourth cross-link at residues 1−3 in the teicoplanin scaffold.

cin); the term glycopeptide is used for molecules such as vancomycin. Since teicoplanin has a peptide scaffold and both covalently attached glycosyl and long chain acyl groups, the term lipoglycopoeptide is used for such natural products.

Vancomycin and teicoplanin are closely related structures, containing a homologous heptapeptide scaffold that has undergone extensive oxidative cross-linking.[6] Five of the seven residues in vancomycin are aromatic, while all seven are in teicoplanin: Leu_1 and Asn_3 of vancomycin are replaced by hydroxyphenylglycine regioisomers in teicoplanin. There are five such nonproteinogenic phenylglycines in teicoplanin and three in vancomycin. The phenylglycines are either 4-hydroxyphenylglycine (HPG) (residues 4 and 5 in **1** and **2** and also residue 1 in teicoplanin) or 3,5-dihydroxyphenylglycine (DPG) (residue 7 in **1** and residues 3 and 7 in **2**). In vancomycin, the remaining two residues are modified tyrosines, with chlorine at the meta position of the aromatic ring and an OH substituent at the benzylic carbon of the side chain. In teicoplanin, only Tyr_6 has been converted to the β-OH-Tyr.

The electron-rich side chains of these aromatic amino acid residues facilitate the oxidative cross-linking (see below) that sets the rigid architecture of the heptapeptide scaffolds of the two drugs. In vancomycin, three cross-links are effected, joining the aromatic rings of 2−4 and 4−6 in aryl ether linkages and of 5−7 in a direct C−C coupling (Figure 2). In teicoplanin, residues 1 and 3 are now hydroxyphenylglycines in the un-cross-linked precursor heptapeptide, and a fourth cross link, 1−3, is produced by the teicoplanin tailoring enzymes (Figure 2). The four cross-links of the teicoplanin scaffold involve all seven side chains to produce the rigid, cup-shaped aglycon.

These are *glyco*peptide antibiotics, so subsequently, the peptide framework is glycosylated, by a disaccharide chain on residue 4 in vancomycin or by three monosaccharides at residues 4, 6, and 7 in teicoplanin. The disaccharide in vancomycin is a D-glucosyl-2,1-D-vancosamine, linked through C_1 of glucose to the phenolic oxygen of OH−$Phegly_4$ of the cross-linked peptide. The vancosamine sugar is a 2,3,6-trideoxy-L-hexose, with methyl and amino substituents at C_3. Such deoxyhexoses are typical in antibiotics and mediate some of the recognition of target molecules.[7−11] In teicoplanin, there is a glucosamine at the corresponding OH−$Phegly_4$ residue, a GlcNAc at the β-hydroxyl substituent of β-OH-Tyr_6, and a mannose on the cross-linked Dpg_7. The *N*-decanoyl moiety on the glucosamine installed at residue 4 blocks the elongation to the disaccharide chain observed in the vancomycin family.

Teicoplanin, but not vancomycin, is a *lipo*glycopeptide with a C_{10} fatty acyl chain in an amide linkage to the amino group of the glucosamine sugar moiety (a series of fatty acyl variants are found in the producer organism, but control of the feedstock during fermentation yields the C_{10} acylated form of teicoplanin). The hydrophobic acyl chain alters the physical properties and presumably the partitioning of lipoglycopeptide vs glycopeptide. This is likely a determining factor not only in the differential activity of **2** vs **1** against some forms of vancomycin-resistant enterococci (VRE) noted below in section 5 but also in the second-generation semisynthetic lipoglycopeptides oritavancin, dalbavancin, and TD-6424 discussed in section 6.

2.1. Variations in Vancomycin and Teicoplanin Natural Analogues

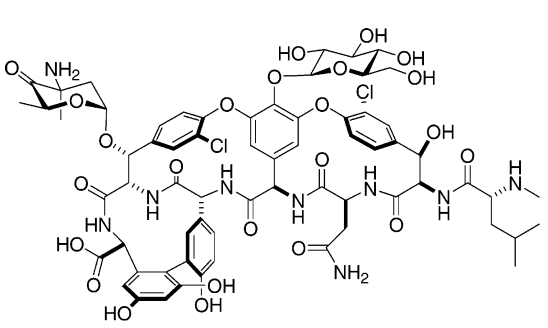
After the clinical success of vancomycin and teicoplanin, dozens of additional glycopeptide congeners have been isolated from strains of actinomycetes.[6] Congeners with the vancomycin heptapeptide scaffold include balhimycin (**3**) and chloroeremomycin (**4**) (Figure 3, structures **3**−**6**). Both have the identical heptapeptide scaffold of vancomycin. Balhimycin differs in having a vancosamine derivative, 4-oxovancosamine, at residue 6. Chloroeremomycin has almost the same disaccharide appended to OH−Phegly$_4$. The difference is in the terminal 2,3,6-trideoxy-3-amino-3-methyl hexose. In **4**, the trideoxyhexose-4-OH is equatorial rather than the 4-axial OH of vancosamine. This is epivancosamine, and an additional epivancosamine residue is found at the benzylic oxygen of β-OH-Tyr$_6$. The biosynthetic gene clusters for **3** and **4**[12−14] have been reported and have revealed much about the molecular logic for assembly of these antibiotics.

In the teicoplanin subfamily, we note two congeners, A47934 (**5**), a sulfated aglycon form produced by *Streptomyces toyocaensis*, and A40926 (**6**), from a *Nonomuraea* species. In section 6, we shall note that chloroeremomycin is the starting point for oritavancin and A40926 the starting point for dalbavancin, the two most clinically advanced second-generation, semisynthetic lipoglycopeptides. The DNA sequence for the biosynthetic clusters of **5** and **6** have also been reported.[15−17]

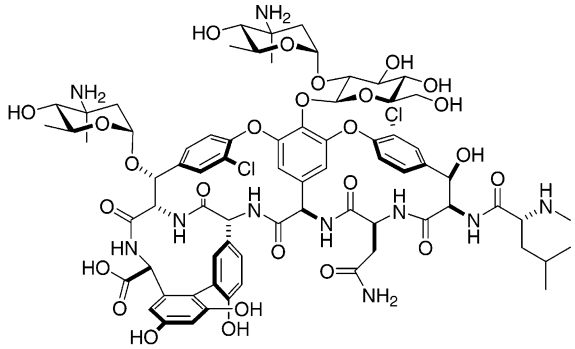
3. Biosynthetic Strategies—Enzymatic Assembly Lines and Tailoring Enzymes

Although the total synthesis of the vancomycin and teicoplanin family of glycopeptides and lipoglycopeptides have been accomplished [6,18,19] with substantial invention of new chemistry, the complexity of these natural products makes fermentative routes the only viable route to bulk production. Semisynthetic chemical tailoring of the scaffolds has been the predominant route to structure activity variation to produce second-generation clinical development candidates, as will be noted in section 6.[20]

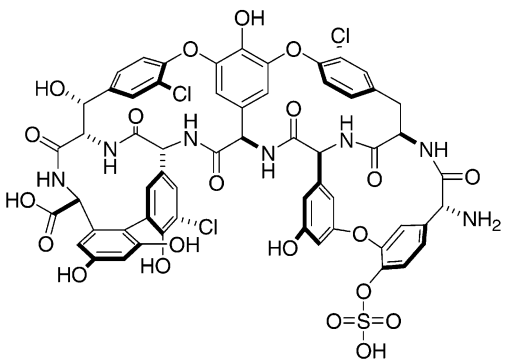
Thus, understanding the biosynthetic logic for construction of the heptapeptide core and its subsequent oxidations, glycosylations, and acylations is of both basic and applied interest. It had been clear



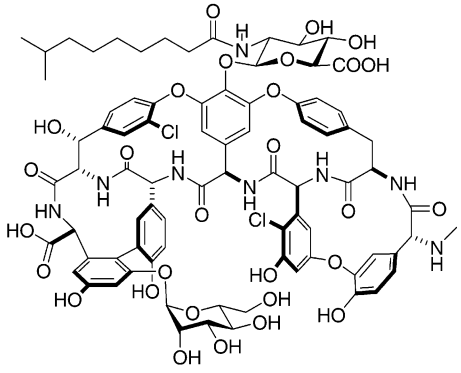
3 Bahlimycin



4 Chloroeremomycin



5 A47934



6 A40926

Figure 3. Balhimycin (**3**) and chloroeremomycin (**4**) in the vancomycin family and A47934 (**5**) and A40926 (**6**) in the teicoplanin family.

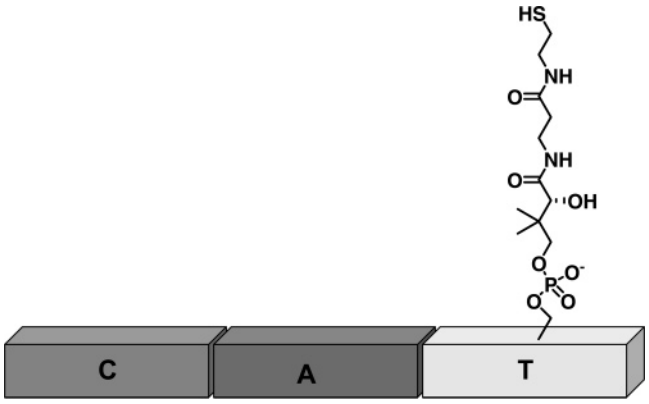


Figure 4. Core domains: C-A-T, in the elongation modules of the NRPS assembly line for vancomcyin and teicoplanin family members: C = condensation domain, A = adenylation domain, and T = thiolation domain. Amino acids get selected and activated by the A domain and installed as thioester on the T domain. The C domain catalyzes peptide bond formation.

from the prevalence of the nonproteinogenic phenylglycines in the peptide backbones that vancomycin and teicoplanin family members must be assembled by nonribosomal peptide synthetase (NRPS) enzymatic machinery.[5] The DNA sequence for the chloroeremomycin gene cluster[12] revealed 30 adjacent genes attributable to the pathway and validated the existence of seven NRPS modules, distributed over three subunits, Cep ABC, in a 3/3/1 distribution.[21] Each of the seven modules selects and activates one amino acid, and the order of the modules mandates the heptapeptide sequence (Leu-Tyr-Asn-Hpg-Hpg-Tyr-Dpg). In the teicoplanin subfamily, the catalytic domains in modules one and three of the NRPS assembly lines instead select and activate Hpg and Dpg, respectively.[16,22] In addition to a catalytic domain for amino acid selection and activation as the aminoacyl-AMP, each module has a thiolation domain modified with phosphopantetheine[23] to provide a thiol for covalent aminoacyl-S-enzyme formation. The third domain in each module is the condensation domain, responsible for amide bond formation and directional peptide chain growth and translocation from N-terminal to C-terminal modules (Figure 4).

The source of the nonproteinogenic amino acids in the microbial cell at the time antibiotic production is switched on is relevant for yields and coordination. Among the 30 clustered Cep biosynthetic genes are four that encode enzymes that generate L-Hpg from the common bacterial metabolic intermediate chorismate.[21,24] There are another four encoded enzymes in the cluster that channel four molecules of malonyl CoA to the eight-carbon intermediate dihydroxyphenylacetyl CoA on the way to L-Dpg.[25−27] Residues 2 and 6 in **1** and **4** differ from the proteinogenic amino acid L-tyrosine by chlorine at the meta position of the aromatic ring and the β-OH that is the site of glycosylation in **2**−**4**. The Cep cluster contains a gene encoding a flavoprotein halogenase[12] as well as a pair of enzymes[28] that hydroxylate tyrosine while installed as a tyrosyl-S-enzyme. Thus, 11 enzymes are coordinately induced to enable generation of residues 2, 4, 5, 6, and 7 required for the vancomycin-type NRPS assembly lines and for generation of six of the seven residues in teicoplanin NRPS multimodular enzymes.

One additional feature of these NRPS assembly lines is worth note. Although the biosynthetic pathways generate the L-forms of modified tyrosines, L-Hpg, and L-Dpg, the heptapeptide released by the NRPS assembly line has the configuration D-D-L-L-D-D-L. Inspection of the CepABC subunit domain composition predicts epimerase (E) domains in module 2, 4, and 5 to convert L-aminoacyl-/peptidyl-S-enzyme intermediates to the D-isomers. The first module lacks an epimerase domain but can select and activate D-Leu directly.[29] All told there are 24 domains in the seven-module CepABC assembly line, each with a well-defined function. Note that condensation domains downstream of E domains must be chiral peptide synthase catalysts that recognize upstream D-aminoacyl-/peptidyl donors. Depending on the timing of the epimerization, the downstream acceptors can be L- or D-amino acids.[26,30]

Following release from the last module of CepC in the NRPS assembly line, the nascent heptapeptide **7** undergoes three kinds of postassembly tailoring reactions to yield the glycopeptides **1** and **4** (Figure 5). The first reaction is *N*-methylation of the amino terminal D-Leu_1 residue carried out by orf 16 in the Cep biosynthetic cluster.[31]

Then the oxidative cross-links are introduced. In both the Cep and balhimycin gene clusters there are three tandem heme proteins, termed OxyABC,[32,33] that act *ad seriatim* to generate the fully cross-linked aglycon **8**. Genetic analysis in the balhimycin system has defined the regiospecificity and timing of cross-linking, one bond each introduced by catalytic action of the hemeproteins[33] as determined by gene knockouts and structure determination of the accumulating intermediates (OxyB > OxyA > OxyC) (Figure 6). The coupling chemistry is probably via one-electron pathways and regioselectivity and atropisomer selectivity may be imposed by the folding and orientation of acyclic and partially cross-linked substrates in each hemeprotein active site. Although there are X-ray structures of two of the three bahlimycin Oxy proteins, OxyB and OxyC,[34,35] *in vitro* activity of the purified enzymes has not yet been reconstituted so the possible promiscuity toward other substrates is not yet known.

In teicoplanin heptapeptide scaffolds, a fourth cross-link, connecting Hpg_1 and Dpg_3, needs to be introduced. In the A47934, A40926, and teicoplanin clusters,[15,16,22] there is indeed a fourth tandem hemeprotein (OxyD) that is the obvious catalytic candidate. The timing of the 1−3 cross-link compared to 2−4, 4−6, and 5−7 is not yet known.

The last phase of tailoring involves the glycosyl transferases (Gtfs) (Figure 7). There are three Gtfs, GtfABC, embedded in the Cep cluster[12] and corresponding two Gtfs, GtfDE, in the vancomycin biosynthetic gene cluster.[36] GtfB (=GtfE) transfers D-glucose from the nucleoside diphosphosugar dTDP-D-glucose to the phenolic-OH at $PheGly_4$ in the cross-linked aglycon peptide. This yields the monoglycosylated heptapeptide scaffold, **9**, known historically as desvancosaminyl vancomycin (DVV). The other two Gtfs in the Cep cluster, GtfA and GtfC, transfer

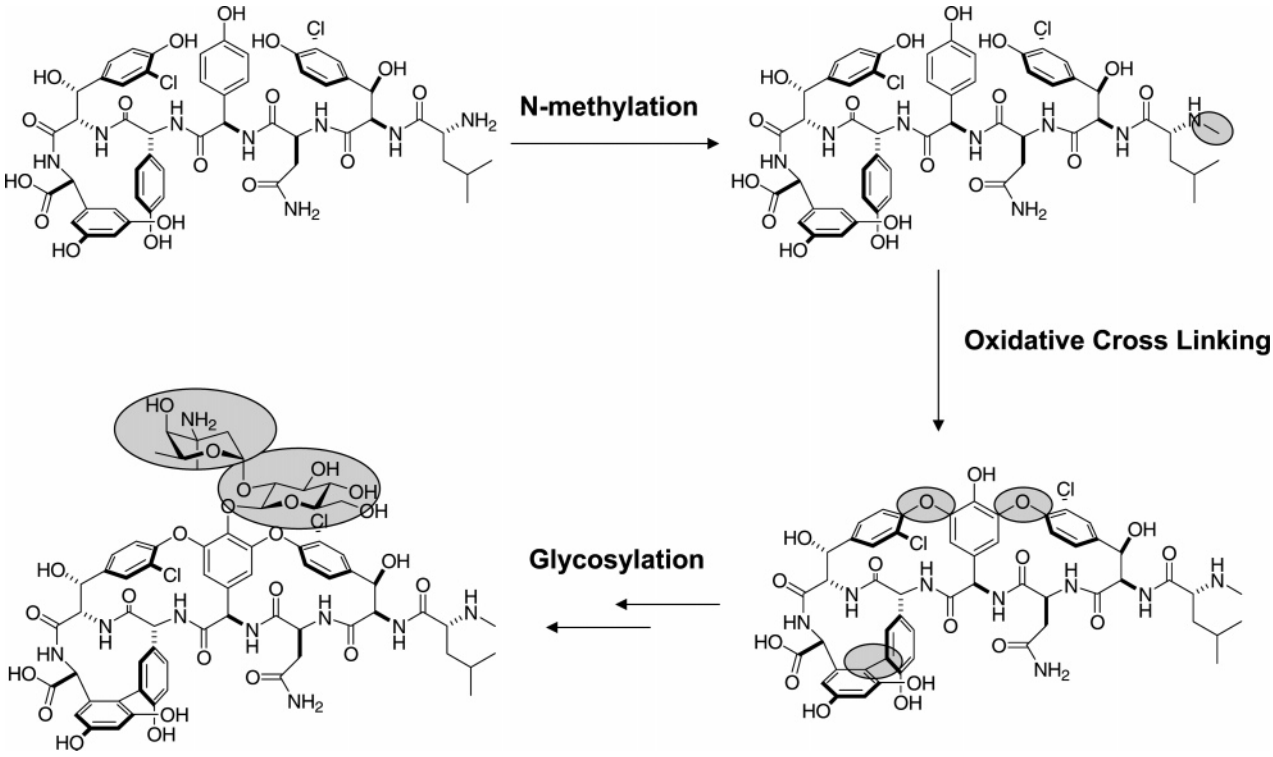


Figure 5. Post-NRPS assembly line tailoring enzymatic modifications to convert the heptapeptides to vancomycin and teicoplanin: *N*-methylation, oxidative cross-linking, and glycosylations.

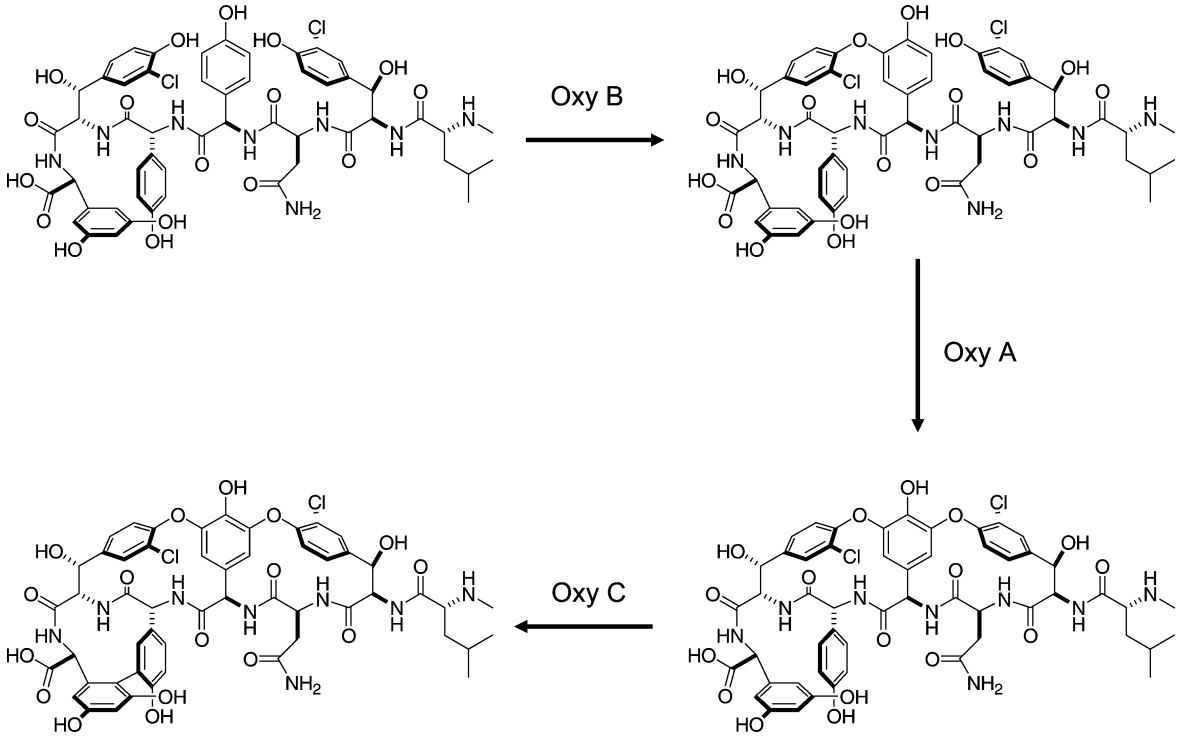


Figure 6. Sequential action of OxyB, OxyA, OxyC to introduce the 2−4, 4−6, and 5−7 cross-links in the bahlimycin scaffold.

L-epivancosamine to the β-OH-Tyr$_6$ and the C$_2$ of the glucosyl moiety of DVV, respectively[37,38] accounting for seven post-assembly line tailoring enzymes in the Cep cluster.

The donor substrate for GtfA and GtfC is dTDP-L-β-epivancosamine **10**. It is not a standard primary metabolite and is also made with just-in-time inventory control logic like the nonproteinogenic amino acids. In particular, there are five genes, *EvaA-E*, in the Cep cluster that channel the common NDP sugar dTDP-D-glucose to dTDP-L-epivancosamine.[39] These enzymes effect remarkable changes of the hexose attached to dTDP, deoxygenating C$_6$ and then C$_2$ and reductively aminating a C$_3$ keto intermediate and then C-methylating it. This yields the dTDP-4-oxovancosamine, the sugar donor to residue 6 in

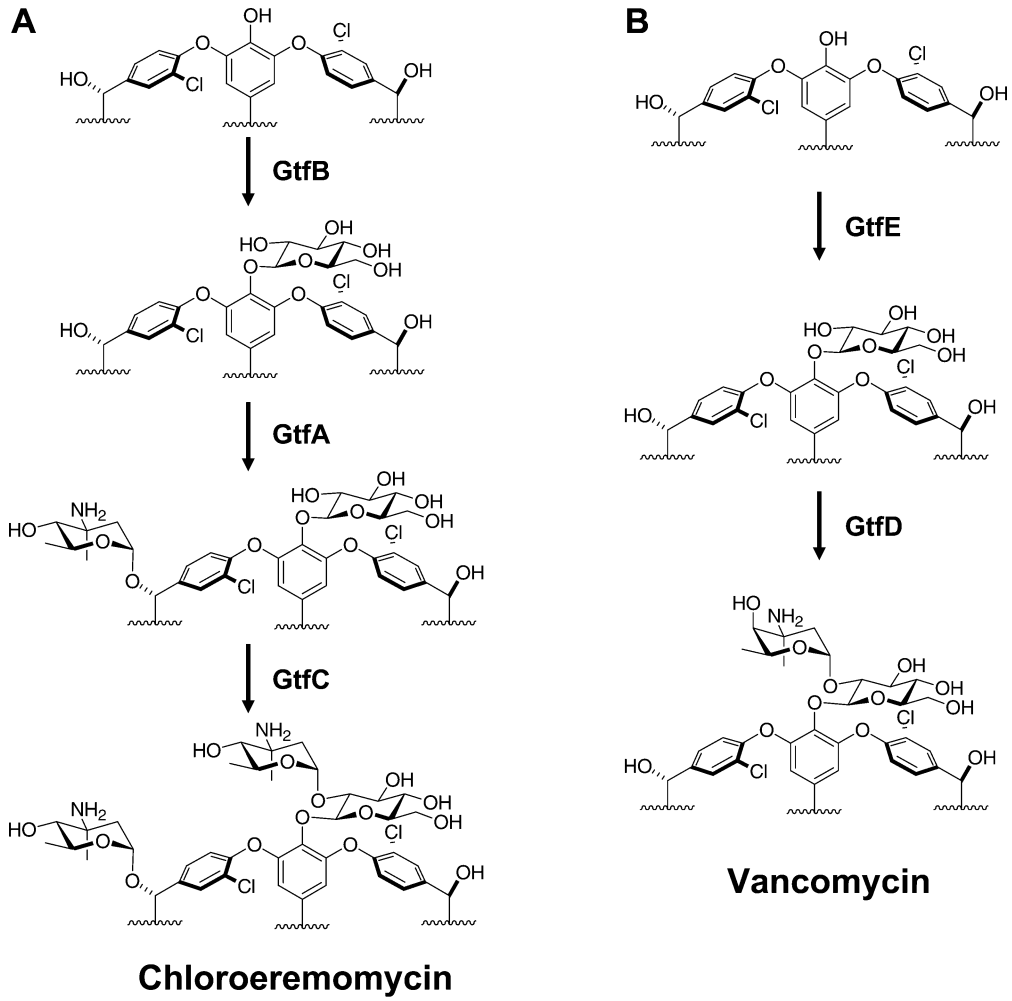


Figure 7. Action of three glycosyltransferases, GtfABC, to add the three sugars in chloroeremycin maturation and of two Gtfs, GtfDE, in vancomycin maturation.

bahlimycin tailoring. The C_4 ketone in dTDP-4-oxovancosamine can be reduced with chirality control to yield the 4-equatorial OH to finish the biosynthesis of dTDP-L-epivancosamine for GtfC.[39] Alternatively, stereospecific enzymatic reduction to the other carbonyl face yields the 4-axial-OH and dTDP-L-vancosamine in vancomycin-producing actinomycetes, presumably used by GtfD to build the D-glucosyl-2,1-l-vancosamine disaccharide chain as the last step in formation of **1**.

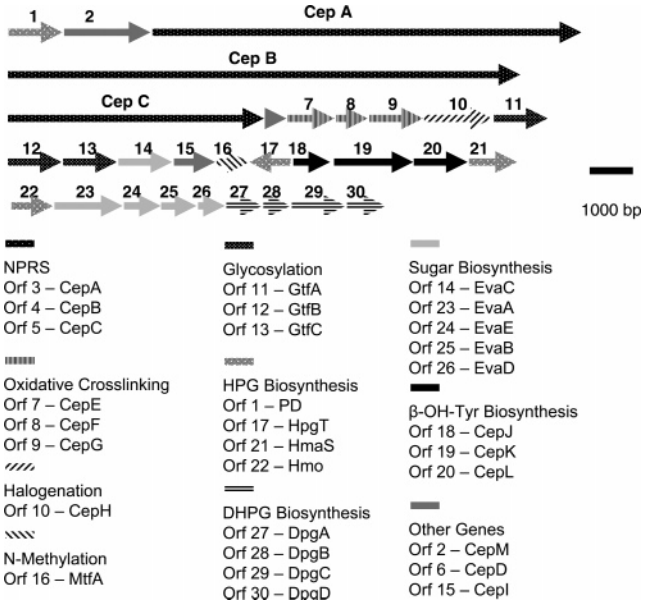


Figure 8. Twenty-four open reading frames (Orfs) in the chloroeremomycin biosynthetic cluster.

Including the EvaA-E enzymes in the list of dedicated monomer generation and tailoring enzymes yields a total of 24 proteins, over and above the three NRPS assembly line subunits needed to make chloroeremomycin (one Gtf less to make vancomycin) (Figure 8). The biosynthetic gene clusters for A40926 and for teicoplanin reveal the anticipated comparable logic with a few variations, including a putative mannosyl transferase for glycosylation of Dpg_7 and an acyl transferase that is the catalyst for the C_{10} fatty acyl amide formation to the glucosamine moiety in **2** and **6**.[17]

4. Mechanism of Action of Glycopeptide Antibiotics

4.1. Cellular Targets of Antibiotics

The majority of antibacterial agents inhibit the synthesis of DNA, RNA, proteins, or peptidoglycan. These are all macromolecules containing different monomer building blocks. Therefore, it is possible to

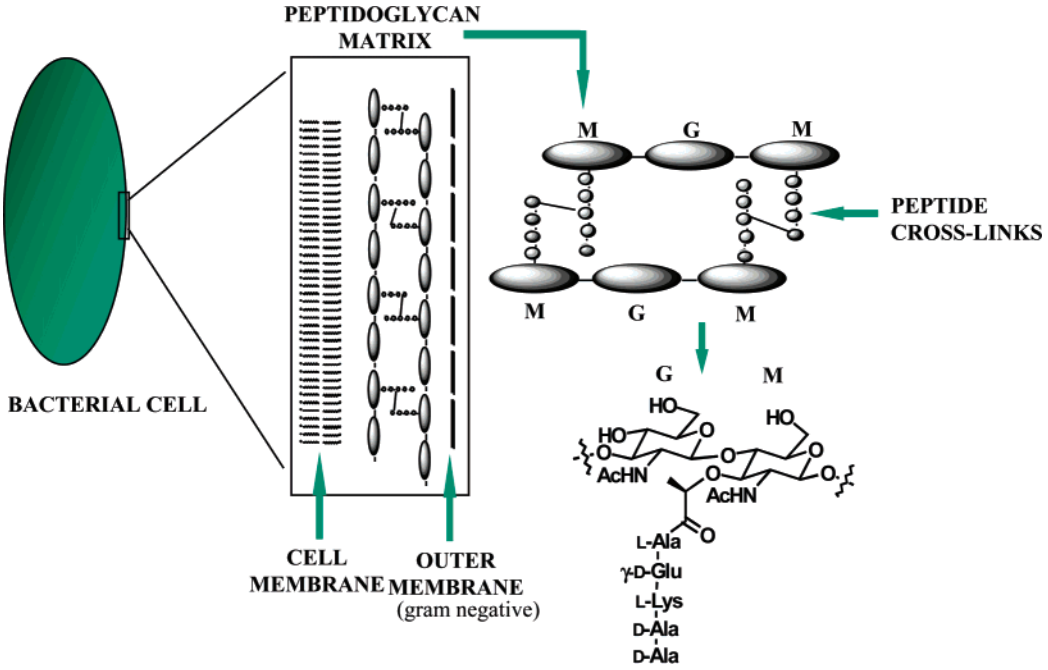


Figure 9. The peptidoglycan layer (PG) surrounding bacterial cells is a giant macromolecular meshwork with peptide cross-bridges connecting glycan strands.

ascertain generally how an antibiotic functions by examining whether it affects the incorporation of radiolabeled monomer units into one of these four different types of macromolecules. Vancomycin, the first glycopeptide antibiotic discovered, was shown to inhibit the incoporation of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc into bacterial peptidoglycan and was therefore classified as a cell wall active antibiotic.[40,41] An overview of the structure and biosynthesis of bacterial peptidoglycan is provided in the following section as a prelude to a detailed discussion of the mechanism of action of vancomycin and other glycopeptide antibiotics.

4.2. Three Stages of Peptidoglycan Biosynthesis

The internal osmotic pressure inside a typical bacterial cell fluctuates significantly, depending on environmental conditions. For bacteria to survive, their cell membranes must be able to withstand osmotic pressures in excess of 5−15 atm without rupturing. Bacterial cells are surrounded by layers of peptidoglycan, a mesh-like carbohydrate polymer that provides the mechanical support necessary to prevent the cells from lysing as the osmotic pressure fluctuates.[42] Peptidoglycan is composed of linear chains of β-(1,4)-*N*-acetyl hexosamine units joined by peptide cross-links (Figure 9).

The biosynthesis of peptidoglycan takes place in three distinct stages (for a review on peptidoglycan biosynthesis, see Bugg et al.[43]) (Figure 10). The first stage takes place in the cytoplasm and involves the conversion of UDP-GlcNAc to UDP-*N*-acetylmuramyl-pentapepide.[4] The muramyl group is a D-2-*N*-acetyl-3-*O*-lactylglucose, assembled from UDP-GlcNAc and PEP by MurA and MurB catalysis. Mur C, D, E sequentially add L-Ala-D-γ-Gln and Lys (Gram positives) or meso diaminopimelate (DAP in Gram negatives), respectively, in ATP-dependent amide-forming steps to create the UDP muramyl-tripeptide. MurF adds preformed D-Ala-D-Ala in the fourth amide-forming step to create the UDP-muramyl-L-Ala-γ-D-Gln-L-Lys-D-Ala-D-Ala (pentapeptide) and complete stage I.

Stage II involves the transfer of the muramyl-pentapeptide from UDP to a C_{55} isoprenol-P (bactoprenol) carrier. The first reaction (MraY) moves the muramyl pentapeptide to the membrane interface and creates lipid I. The second reaction, catalyzed by MurG, adds GlcNAc in a β-1,4-linkage to the muramyl moiety, generating the disaccharyl-pentapeptide (Figure 10) attached to bactoprenol-PP.[44−47] This is lipid II, which is the donor for the peptidoglycan elongation reactions that occur on the external surface of the bacterial membrane. Once lipid II is formed, the disaccharide−pentapeptide is somehow flipped from the cytoplasmic phase of the membrane to the external face (Figure 11). That flipping, still mysterious in whether an enzyme accelerates the facial equilibration, completes phase II of PG assembly.

The third stage of PG synthesis involves lipid II molecules presenting disaccharyl-pentapeptide as donor, on the outer face of membranes, to the 4-OH of the GlcNAc termini of existing PG glycan strands as acceptors. This is a net disaccharide transfer in each elongation step, catalyzed by enzymes known as transglycosylases (TGases) (Figure 12). These newly elongated glycan strands are immature, or nascent, in the sense that the pentapeptide units are not cross-linked, so this portion of the PG layer will be mechanically weak until transpeptidation between adjacent peptide strands has occurred. This is the task of a family of transpeptidases (TPases), using the ϵ-NH_2 of Lys_3 on one strand to attack D-Ala_4 on an adjacent strand, liberating D-Ala_5 as the free amino acid (Figure 12). The Lys_3-D-Ala_4 interstrand

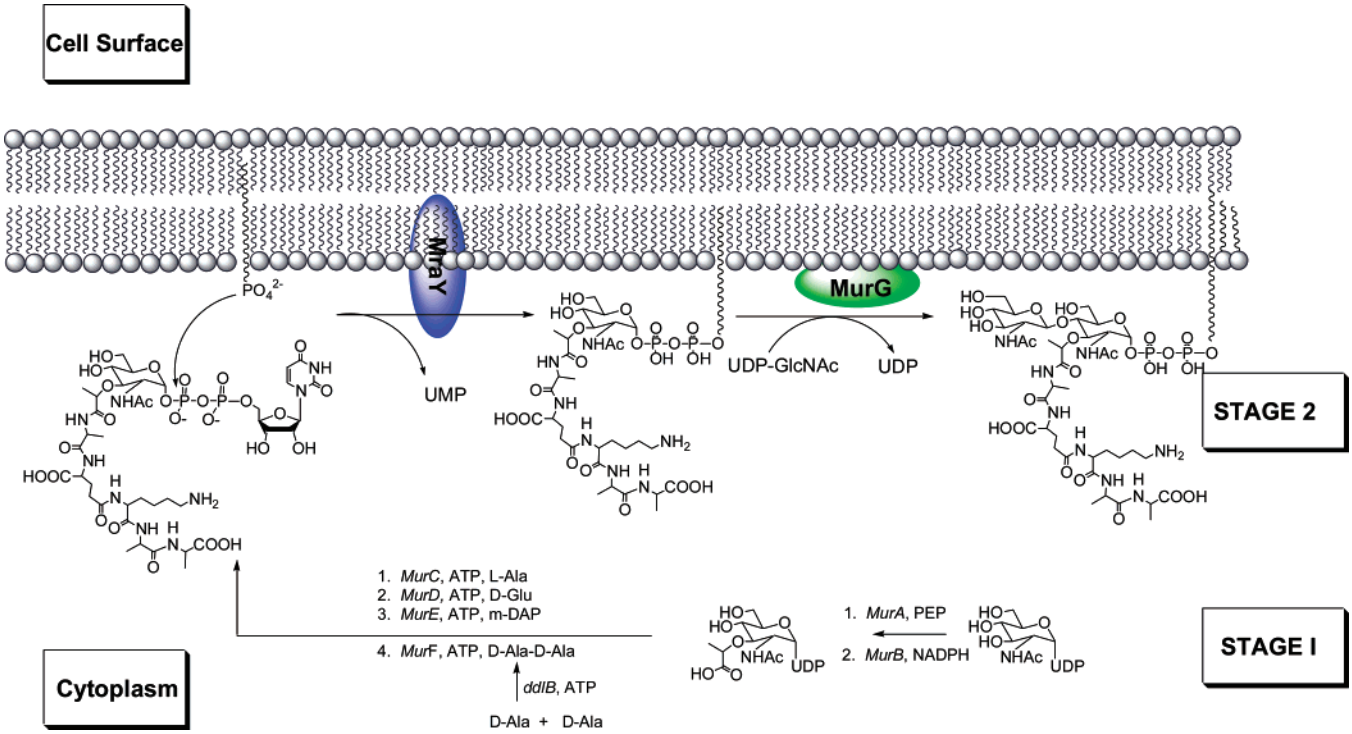


Figure 10. Cytoplasmic phases of peptidoglycan assembly. Phase I culminates in UDP murmamyl pentapeptide assembly in the bacterial cytoplasm; phase II involves enzymatic transfer of muramyl pentapeptide to C_{55} bactoprnol-P to make lipid I, followed by MurG-mediated GlcNAc transfer to make the C_{55}-P-P-disaccharly pentapeptide, lipid II.

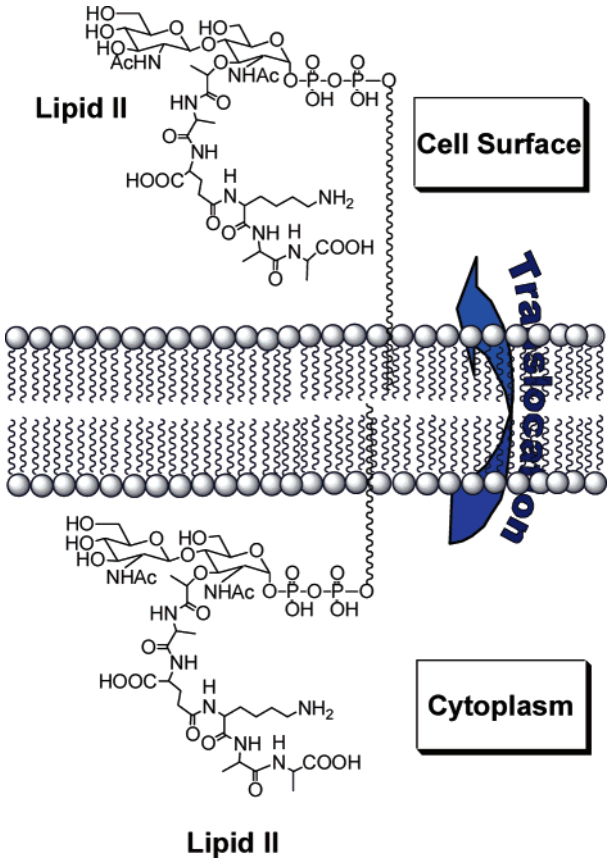


Figure 11. Translocation of lipid II. Lipid II is flipped between the inner (cytoplasmic) and the external face of the bacterial membranes via an unknown mechanism.

isopeptide bond thus generated is a mechanically strengthening covalent cross-link. The crucial role of transpeptidation in PG maturation is borne out by the fact that the active site serine side chains that function as catalytic nucleophiles in the transpeptidases are covalently acylated by penicillins (and cephalosporins).[3]

The first two stages of peptidoglycan biosynthesis, leading to the production of lipid II, are well-understood at this point. Most of the enzymes involved in this part of the pathway have been studied extensively, and crystal structures are available for all of the essential enzymes in stage I and stage II, except MraY.[47−55] The final stage of peptidoglycan biosynthesis, involving glycan polymerization and cross-linking, is not nearly as well-understood. From a chemical perspective, there are only two different reactions involved in this final stage of peptidoglycan synthesis—a glycosidic bond-forming reaction and a transpeptidation reaction; however, the resulting product is a complex polymer, and there is presumed to be considerable structural heterogeneity at the molecular level in both the final product and the various intermediates. Furthermore, there are several different transglycosylase domains and an even larger number of transpeptidase domains involved in the biosynthesis of this polymer, as noted in the section below.

4.3. Transglycosylase and Transpeptidase Isoforms

There are multiple genes encoding TGases and TPases in bacterial cells.[42,56] These genes play different roles in cell growth and division.

Historically, transpeptidases were classified into high and low molecular weight forms, collectively known as penicillin-binding proteins (PBPs). Some

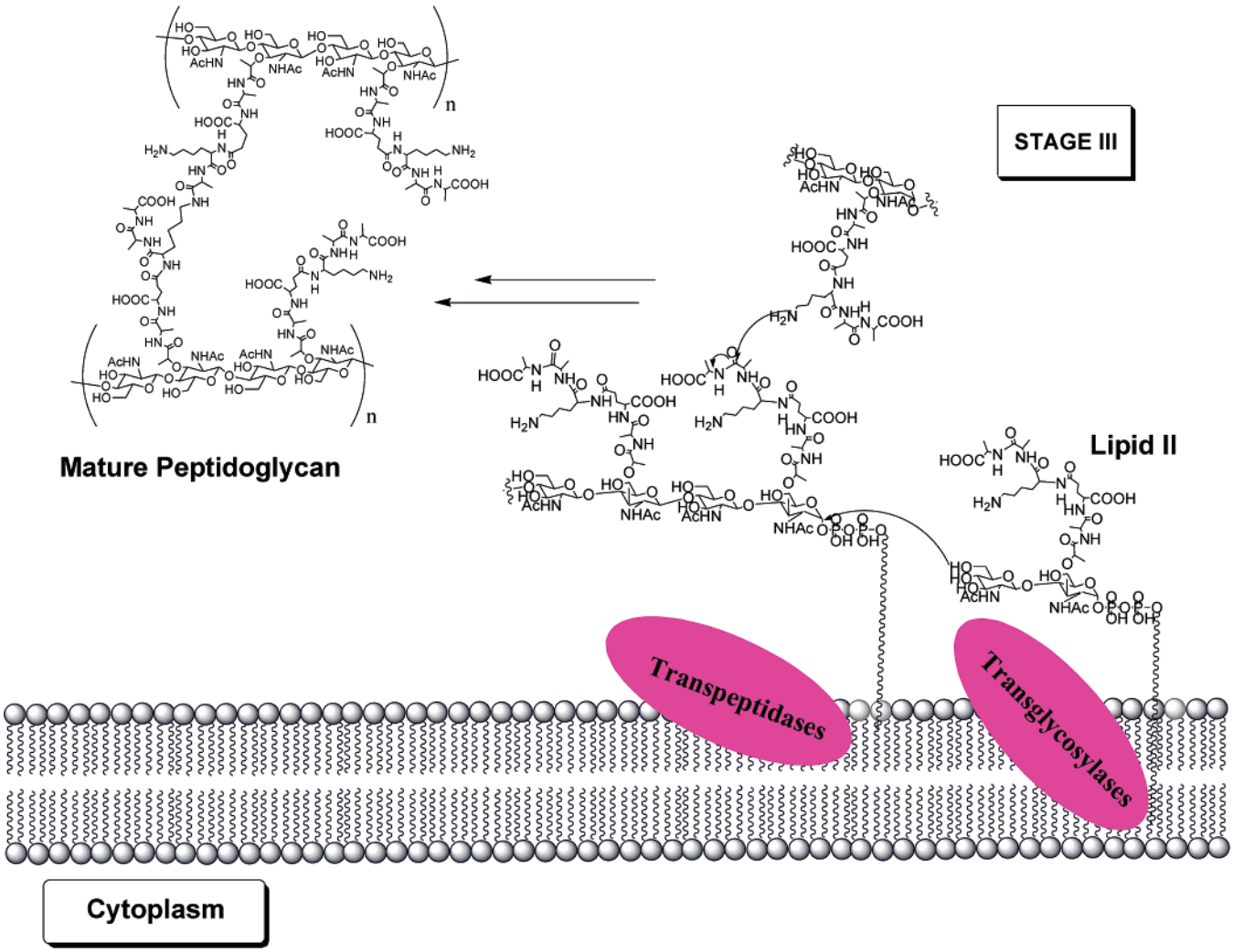


Figure 12. External phases of peptidoglycan assembly. Phase III involves lipid II as disaccharyl pentapeptide donor to the 4-OH of GlcNAc at the ends of PG glycan chains undergoing elongation (transglycosylases) and then isopeptide bond formation between Lys_3 on one peptide strand and D-Ala_4 on a neighboring peptide strand (transpeptidases).

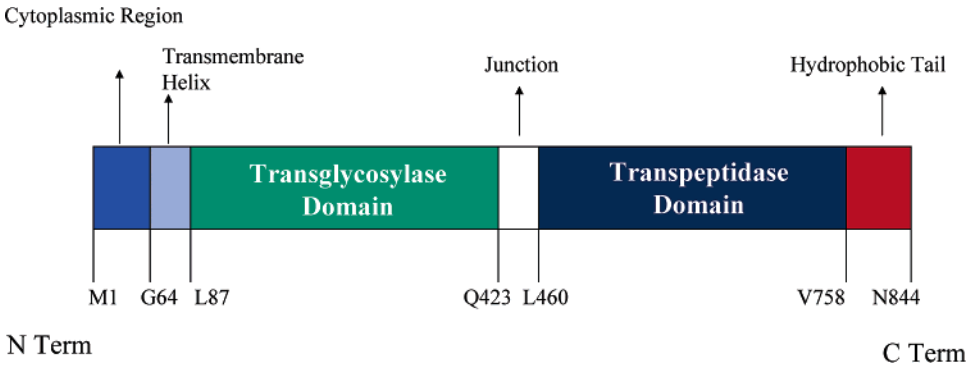


Figure 13. Schematic of bifunctional TGase/Tpase domains in high molecular weight PBPs.

of the high molecular weight PBPs contain transglycosylase domains in addition to transpeptidase domains. In *Escherichia coli*, for example, PBP1A, -1B, and -1C are bifunctional TGase/TPase enzymes (Figure 13).[57−59] *E. coli* PBP1B has been proposed to carry out 85% of PG elongation in that organism.[60−62] The remaining 15% of peptidoglycan is made by some combination of other transglycosylases and transpeptidases. Other high molecular weight PBPs, such as PBP2 and PBP3 in *E. coli*, have domains that appear to be vestigial transglycosylase domains.[56] Genetics experiments have shown that PBP2 is involved in cell elongation/maintaining cell shape and PBP3 in cell division.[63−65] In addition to the high molecular weight PBPs, there are several low molecular weight PBPs containing only transpeptidase or carboxypeptidase activities. These enzymes are thought to be important in maintaining the shape of the cell.[56,65]

Table 1. List of Predicted Bifunctional TGase/TPases in Gram-Positive Bacterial Pathogens

	bifunctional PBPs	
bacteria	enzyme	gene
E. faecalis	unnamed	ponA
	unnamed	pbpF
	unnamed	pbpZ
E. faecium	unnamed	ponA
	unnamed	pbpF
	unnamed	pbpZ
S. aureus	PBP2	pbpB
S. pneumoniae	PBP1a	pbp1a
	PBP2a	pbp2a
	PBP1b	pbp1b
B. subtilis	PBP1	ponA
	PBP2c	pbpF
	PBP4	pbpd
	PBP2d	ywhE

Several high molecular weight TGase/TPase enzyme forms have been identified in other bacterial strains, including Gram-positive organisms such as *S. aureus, Streptococcus pneumoniae*, and *Enterococcus faecalis* (Table 1).[59,66] The putative functions of most of these enzymes are based on what has been learned about the corresponding enzymes in *E. coli* from a combination of genetic and biochemical studies.

Although genetics has provided considerable insight into the general roles of different PBPs in cell growth and division, only limited biochemical work on either the transglycosylase or transpeptidase domains of the high molecular weight PBPs has been carried out. In large part, this is because assays to monitor the activity of the Tgase and Tpase domains of high molecular weight PBPs were not available until recently. The lack of good assays for the high molecular weight PBPs was related to difficulties in obtaining adequate quantities of lipid II for study. Both chemical and enzymatic methods to produce quantities of lipid II have recently enabled the study of high molecular weight PBPs, and kinetic characterizations of *E. coli* PBP1b and *S. pneumoniae* PBP2a have been reported.[10,67−71,136]

The historical lack of methods to study and deconvolute the roles of individual transglycosylase and transpeptidase domains in vitro and in bacterial cells has made it difficult to understand the detailed mechanisms of antibiotics that inhibit the final steps of peptidoglycan biosynthesis. Nevertheless, it is clear from studying the glycopeptides that some of the differences in activity between antibiotics with ostensibly similar mechanisms of action are related to differential inhibition of related targets (i.e., PBPs). Now that better biochemical and genetic tools to probe the function of different PBPs have become available, it should be possible to learn more about why different glycopeptides have different effects on cells. This knowledge, in turn, may lead to the development of better antibiotics.

4.4. Peptidoglycan Biosynthesis as an Antibiotic Target

Peptidoglycan biosynthesis is a good pathway to target for antimicrobial chemotherapy, because it is essential for survival and highly conserved even in disparate microorganisms. In fact, many antibiotics that inhibit the biosynthesis of bacterial peptidoglycan are derived from natural products produced by microorganisms themselves.[3] For reasons that are not clear, a disproportionate number of natural product-based, cell wall-active antibiotics inhibit the final steps of peptidoglycan biosynthesis of peptidoglycan biosynthesis. It is possible that these steps are preferentially targeted because the enzymes involved are extracellular and are thus accessible to compounds that would not penetrate the cell membranes. It has also been argued, however, that it is advantageous to inhibit metabolic processes that involve multiple, related enzymes because spontaneous mutations in single enzymes do not result in resistance.[72]

Antibiotics that inhibit the final stages of peptidoglycan biosynthesis fall into three major classes with respect to mechanism of action (Figure 14).[73] The first class, which includes the β-lactams, carbapenems, and cephalosporins, comprises those antibiotics that prevent glycan cross-linking by inhibiting the active sites of enzymes catalyzing transpeptidation. The second class includes those antibiotics that inhibit glycan polymerization by binding to bacterial transglycosylases. Moenomycin, which is used commercially in animal feed, is the prototypical member of this class. It is notable for its outstanding potency, but is not used in humans because it has poor physical properties. The third class includes antibiotics that can inhibit glycan polymerization and/or cross-linking by binding to the substrates of transglycosylases and transpeptidases. The glycopeptides are the best known of these substrate-binding antibiotics, but there are many others, including ramoplanin and members of the lantibiotic family of antibiotics, both of which are reviewed in this issue in the reviews by Walker and Boger and by Van der Donk et al. The glycopeptides present special challenges for mechanistic analysis, because they are potentially able to inhibit multiple enzymes involved in two distinct processes—i.e., glycan polymerization *and* cross-linking—at the same time.

Vancomycin is the prototypical member of the glycopeptide family of antibiotics and has served as the model system for many mechanistic investigations of glycopeptides. It is commonly assumed that teicoplanin and other glycopeptides kill bacterial cells by the same mechanism of action as vancomycin, because they recognize the same dipeptide motif on peptidoglycan precursors (see below). However, there is increasing evidence that different glycopeptides preferentially target different enzymatic steps. A major goal of this review is to draw attention to the differences between vancomycin, teicoplanin, and other glycopeptide antibiotics and to address possible mechanistic explanations for these differences. In the following sections, we provide an overview of the general mechanism of action and the molecular basis for resistance of the glycopeptide class of antibiotics as a prelude to discussing the differences between compounds.

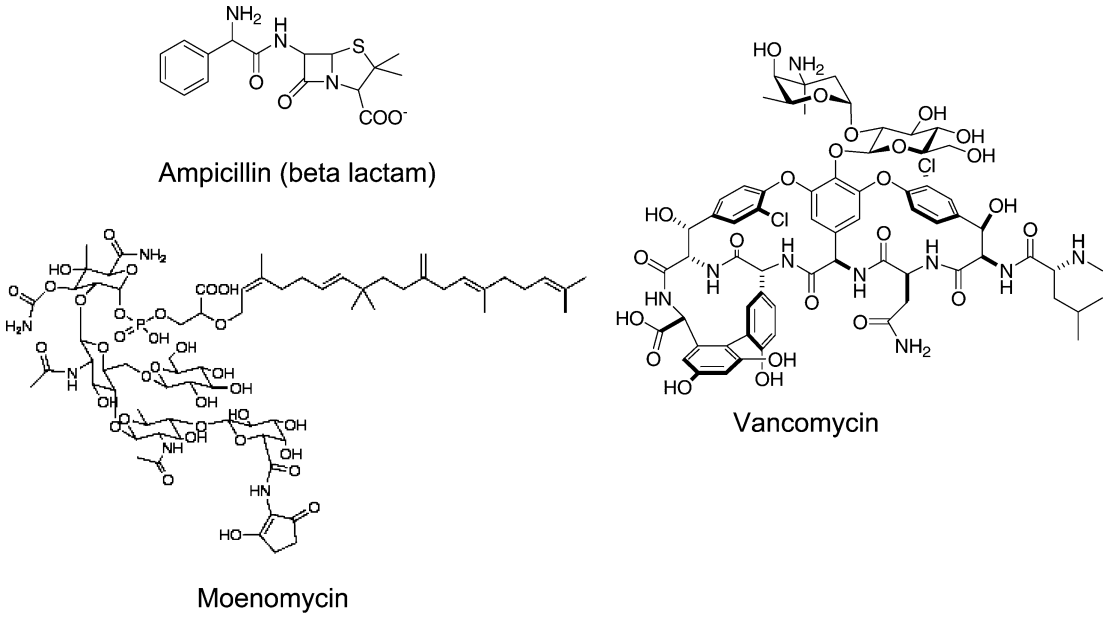


Figure 14. Three classes of antibiotics inhibiting stage III of peptidoglycan assembly: β-lactams, moenomycin, and glycopeptides.

4.5. Early Studies on the Mechanism of Action of Glycopeptide Antibiotics

Vancomycin was discovered in 1956 and its general mechanism of action was elucidated in the 1960s.[41,74] In 1965, Strominger and co-workers reported the first studies on the mechanism of action of vancomycin and the related glycopeptide ristocetin.[40] These authors carried out a series of studies using crude bacterial membranes containing peptidoglycan-synthesizing enzymes supplemented with radiolabeled UDP-MurNAc pentapeptide and UDP-GlcNAc in which they showed that the glycopeptide antibiotics block peptidoglycan biosynthesis at either transglycosylation or transpeptidation. This work represented a tour de force at the time, because key steps in the biosynthetic pathway to peptidoglycan were not understood. Subsequent work by Perkins showed that vancomycin actually binds to peptidoglycan precursors, specifically to the D-Ala-D-Ala terminus, leading to the hypothesis that enzyme inhibition is related to this phenomenon.[75,76] Stepwise degradation of UDP-MurNAc-pentapeptide indicated that vancomycin interacts with the D-Ala-D-Ala terminus of this precursor. In addition to UDP-MurNAc-pentapeptide, other peptidoglycan intermediates that contain the D-Ala-D-Ala dipeptide include (inward facing) lipid I, (inward and outward facing) lipid II, and nascent (uncross-linked) peptidoglycan (Figures 10 and 12). Since experiments with radioactive vancomycin derivatives showed that vancomycin does not enter cells, it was concluded that vancomycin and other glycopeptides affect the extracellular enzymes that utilize intermediates downstream of lipid I, such as lipid II.[77]

Many of the structural features of vancomycin were determined in 1978, when Sheldrick and Williams determined the structure of a degradation product, CDP-1, through X-ray analysis.[78] However, the structure of vancomycin was damaged during the degradation process and the correct structure was finally put forth in 1982, based on important contributions from the Harris and Williams labs.[79,80] Shortly after the structure of vancomycin was solved, the Williams lab used NMR to show the binding interaction between vancomycin and the D-Ala-D-Ala dipeptide.[81] Binding was shown to occur through a set of backbone contacts between the D-Ala-D-Ala dipeptide and the amides that line a cleft formed by the cross-linked heptapeptide of the glycopeptide. (Figure 15) All glycopeptides have a similar binding pocket and are believed to make the same contacts.[82] Through binding to D-Ala-D-Ala, the bound glycopeptide acts as a steric impediment that prevents lipid II and/or the

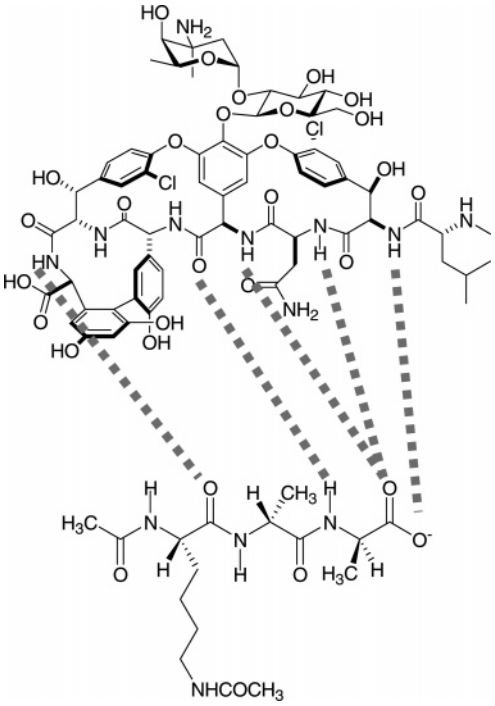


Figure 15. Complexation of vancomycin with *N*-acyl-D-Ala$_4$-D-Ala$_5$ termini: five hydrogen bonds between the underside of the glycopeptide and the acyl-D,D-dipeptide moiety.

nascent glycan chain from being processed further.[83] The net result is inhibition of the transglycosylation and/or transpeptidation steps of peptidoglycan synthesis, which overall weakens the peptidoglycan layers and leaves the cell susceptible to lysis due to changes in osmotic pressure.

5. Mechanisms of Resistance

Scientists have been intrigued by the unusual mechanism of action and behavior of vancomycin since its discovery. Researchers noticed early on that it is difficult to induce resistance to vancomycin. Ziegler et al., for example, compared penicillin and vancomycin, both of which inhibit stage III of peptidoglycan synthesis, and found that the MICs of penicillin against a range of *S. aureus* strains increased by more than 100 000-fold after 25 serial passages in antibiotic-containing media.[84] In contrast, the MICs of vancomycin increased by only 8-fold. Furthermore, whereas resistance to the β-lactams appeared almost immediately upon the introduction of penicillin into clinical use, glycopeptide resistance was not observed for a very long time. Resistance to antibiotics typically develops when either an antibiotic itself or its target is altered in some way.[11] Unlike the β-lactams, the glycopeptides did not appear to be susceptible to modifications that rendered them inactive. Furthermore, it was widely believed that microorganisms could not readily alter the target of the glycopeptides, the D-Ala-D-Ala peptide terminus, because that would entail simultaneous, coordinated changes to multiple enzymes in the pathway to peptidoglycan synthesis. In 1986, Cooper and Given noted that "during the three decades in which vancomycin has been in clinical use, there has been no trend toward resistance among organisms usually susceptible", and speculated that the mode of action of glycopeptide antibiotics made the development of high-level resistance virtually impossible.[85] One year later, vancomycin-resistant enterococcal strains began to appear in hospitals, and 15 years after that the incidence of VRE in hospitalized patients with enterococcal infections in the US had spread to 30%.[1]

In retrospect, the appearance of significant glycopeptide resistance is not surprising, because the widespread use of an antibiotic virtually guarantees the emergence of resistance.[86] However, high-level resistance to the glycopeptides in enterococci is not the result of spontaneous mutations in clinically relevant microorganisms. Instead, the genes conferring glycopeptide resistance in the organisms producing glycopeptide antibiotics appear to have been transferred to pathogenic microorganisms. The mechanism of glycopeptide resistance in enterococci was elucidated by Courvalin, Walsh, and their co-workers in the 1990s. Subsequent work on glycopeptide resistance in producer organisms has revealed that they contain the same sets of resistance genes as the resistant enterococcal strains. The mechanism of glycopeptide resistance in enterococci is described below.

5.1. Vancomycin Reistant Enterococci (VRE)

VRE has been categorized into distinct clinical phenotypes, originally VanA and VanB[87,88] (but now including additional variants of these classes[89]). The VanA and VanB phenotypes were initially distinguished by differential susceptibility to vancomycin vs teicoplanin. The VanA phenotype shows 1000-fold increased resistance to both drugs, while VanB VRE isolates have equivalent resistance to vancomycin but remain susceptible to teicoplanin.[4] Additionally, a VanC VRE phenotype has been observed: it confers about 1 log increase in resistance to vancomycin and has not been a widespread problem in humans.[88] VanA and VanB isolates of VRE contain five *van* genes, *VanRSHAX*, necessary and sufficient to cause high-level resistance (Figure 16).

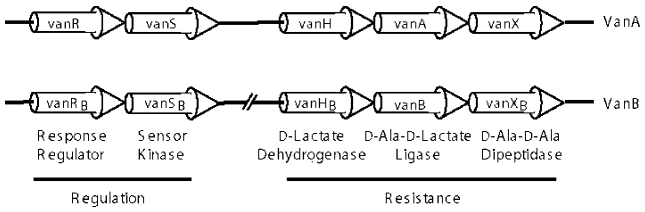


Figure 16. Schematic of the Five Gene Van RSHAX cassette in VanA and VanB phenotypes of VRE.

The encoded five proteins sort into two categories. The VanR and VanS pair up to function as a two-component regulatory system.[90] VanS is a transmembrane receptor histidine kinase. The extramembrane domain, directly or indirectly, senses vancomycin on the outside and transmits that information to the cytoplasmic domain, which autophosphorylates on the His side chain. The phospho-VanS then transfers the PO_3 group to an aspartyl side chain in the N-terminal domain of VanR in the enterococcal cytoplasm. The C-terminal domain of phospho-VanR now acts as a transcriptional regulator[91,92] to activate the transcription of the *VanHAX genes*.

The VanHAX proteins comprise the second category. All three are enzymes, coordinately acting to reprogram the peptidoglycan termini from *N*-acyl-D-Ala-D-Ala, a high-affinity target of vancomycin and teicoplanin, as noted above in section 4, to *N*-acyl-D-Ala-D-lactate. VanH reduces the common metabolite pyruvate to D-lactate. VanA is a D,D-depsipeptide ligase, making D-Ala-D-lactate. VanX is a D-Ala-D-Ala dipeptidase, removing the normal D-D-dipeptide intermediate hydrolytically while sparing D-Ala-D-lactate. The D-Ala-D-Lac accumulates and gets added by the MurF ligase to UDP muramyltripeptide to generate the UDP muramyl-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Lac that can serve as cross-linking substrate for transpeptidase action (Figure 17).

The 1000-fold resistance in VanA and -B VRE phenotypes results from the reprogramming of the peptidoglycan termini from D-Ala-D-Ala to D-Ala-D-lactate.[90,93] The absence of the amide NH bond and the ground-state repulsion of the oxygen lone pair cause a 1000-fold loss of binding affinity for vancomycin and teicoplanin to *N*-acyl-D-Ala-D-lactate, correlating genotype with phenotype (Figure 18).[93,94]

5.2. VRE Genotypes: VanB Resistance and How To Overcome It

One of the interesting aspects of glycopeptide resistance is that the VanB genotype remains sus-

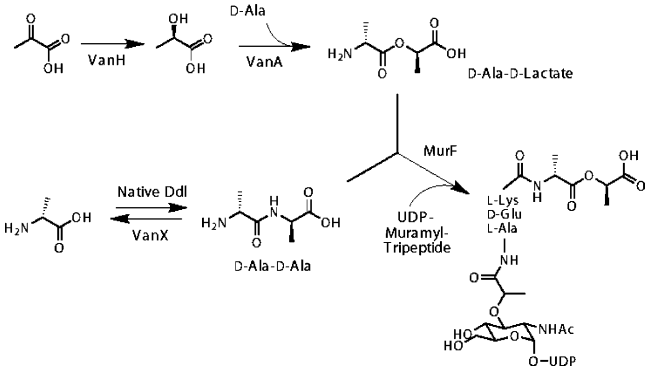


Figure 17. Action of VanHAX to make D-Ala-D-Lac and destroy competing D-Ala-D-Ala as a substrate for MurF.

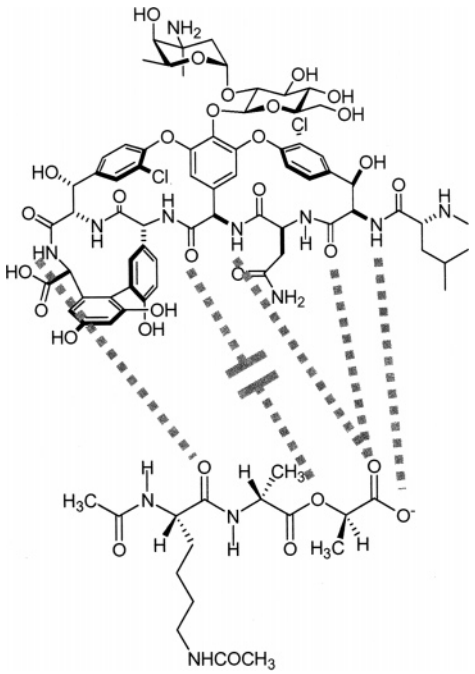


Figure 18. Loss of the one H-bond in the vancomycin complex with D-Ala-D-Lac vs D-Ala-D-Ala and ground-state repulsion by the ester oxygen accounts for a 1000-fold drop in affinity.

ceptible to teicoplanin. VanB strains of VRE have *vanRSHAX* genes and can reprogram PG termini to produce D-Ala-D-Lac. However, only vancomycin induces these strains to make altered peptidoglycan precursors.[87] The difference in behavior between vancomycin and teicoplanin with respect to VanB strains is puzzling, because vancomycin and teicoplanin have remarkably similar structures (Figure 1). There are many hypotheses that attempt to explain these observations.

Williams and co-workers found that lipidated glycopeptides are anchored to the bacterial cell membrane, whereas nonlipidated glycopeptides are distributed more broadly in the peptidoglycan layers.[95,96] They reasoned that teicoplanin inserts itself into the membrane, where the peptidoglycan precursors terminating in D-Ala-D-Lac are located. Thus, the two binding partners are located in close proximity to each other and the interaction between teicoplanin and D-Ala-D-Lac becomes intramolecular, which is more favorable than an intermolecular interaction.[97,98] Williams and co-workers concluded that it is the restriction of motion of intermediates and the intramolecular bond formation that allow teicoplanin to overcome VanB resistance.[31]

In 1996, Courvalin and co-workers found that VanB VRE remain susceptible to teicoplanin, because this antibiotic does not induce the cell wall reprogramming machinery. They showed that vancomycin, but not teicoplanin, activates the $VanS_b$ sensor kinase.[99] Furthermore, they found that mutations in the $VanS_b$ sensor kinase render cells resistant to teicoplanin.[100] From these findings, they reasoned that activation of $VanS_b$ required direct interaction with the glycopeptide.[100,101]

To determine which structural features of teicoplanin and vancomycin correlate with induction of resistance, Dong et al. undertook a direct comparison of pairs of teicoplanin and vancomycin analogues. The compounds examined included the teicoplanin and vancomycin aglycons, the monoglucosylated scaffolds bearing the same disaccharyl chain, and the two scaffolds bearing a C_{10}-acyl glucosamine group (Figure 19).[102]

The teicoplanin and vancomycin aglycons and the monoglucosylated scaffolds induced resistance, but the lipoglycopeptides did not (as judged by susceptibility of VanB VRE strains to the test compounds). Thus, adding a simple C_{10} acyl chain to vancomycin abrogated its ability to activate the resistance genes.

More recently, Boger and co-workers have investigated the activity of methyl ester derivatives of vancomycin and teicoplanin, replacing the carbohydrate portion of the glycopeptide with a methyl group.[103,104] They found that these compounds were still active against VanB VRE strains and attributed their activity to the hydorophobic nature of these compounds. Thus, they reasoned that it might not be necessary to add a lipid chain to the glycopeptide to overcome VanB resistance; rather, a simple methyl group will prove effective.

Two hypotheses to explain the differences between lipoglycopeptides and the corresponding glycopeptides *vis-à-vis* the induction of resistance have been put forth.[102] Both hypotheses attribute the differences between lipidated/hydrophobic and nonlipidated glycopeptides to differential partitioning among available targets on bacterial cell surfaces. Williams and co-workers showed that the lipid chain of teicoplanin localizes the molecule to the membrane. Therefore, it has been suggested that membrane localization renders teicoplanin and other lipidated glycopeptides inaccessible to the sensor kinase, so that induction does not occur. This hypothesis presumes that induction of resistance involves a direct interaction between the sensor kinase and the antibiotic.[100,101] Alternatively, it has been suggested that the sensor kinase interacts not with the glycopeptide itself but with peptidoglycan intermediates or degradation products produced by the metabolic blockade.[102] Teicoplanin does not induce resistance according to this hypothesis, because it blocks a different enzymatic step than vancomycin, which means that different intermediates and degradation products buildup.

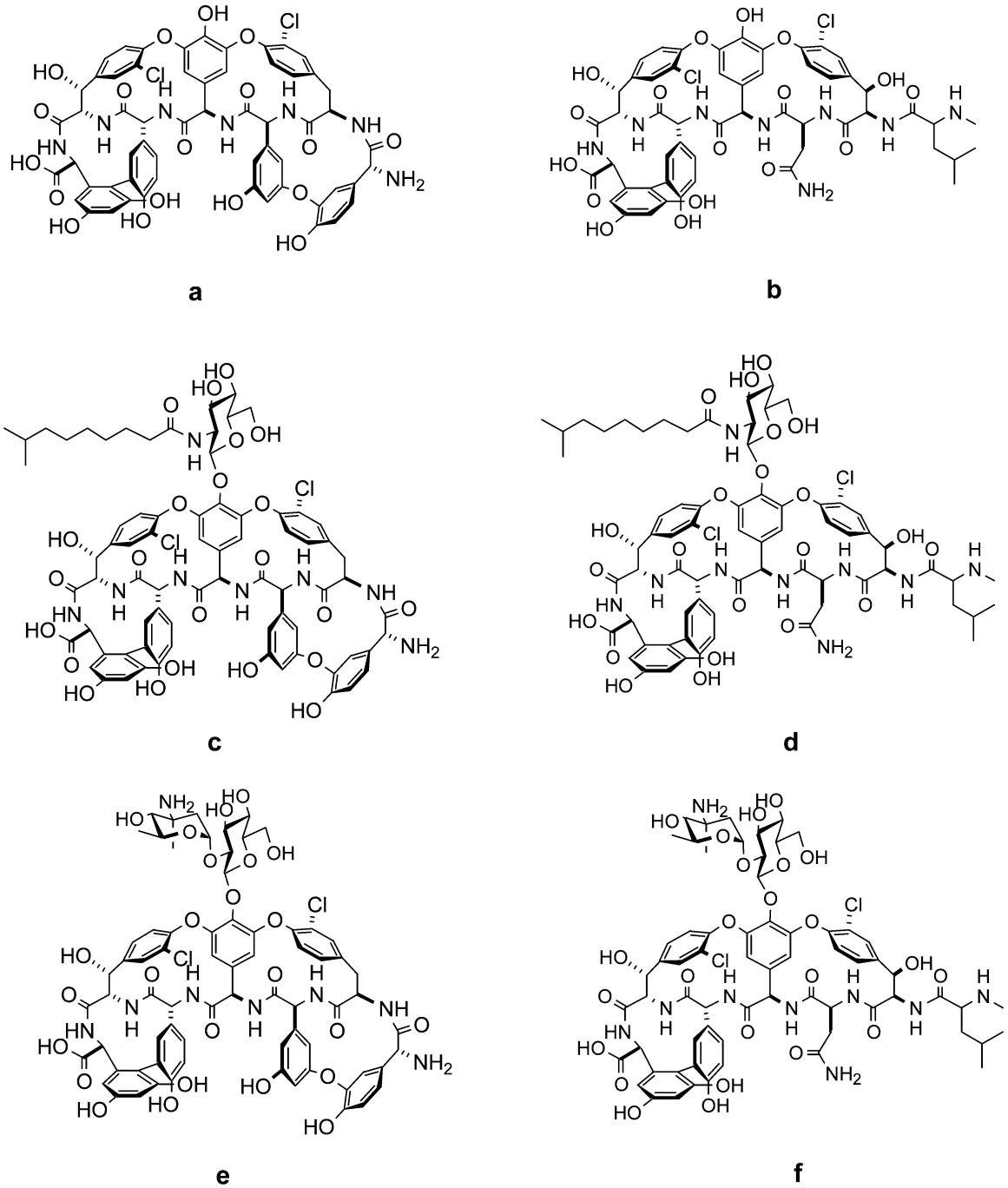


Figure 19. Teicoplanin and vancomycin analogues. A set of matched pairs of vancomycin and teicoplanin derivatives (**a**−**f**) were used to probe what triggers the sensor kinase. It was found that **C** and **D** did not induce resistance, whereas all other compounds did.

There is no direct evidence about how the sensor kinase is activated. However, using an *E. coli* model system, Kahne and co-workers have shown that lipidated glycopeptides preferentially inhibit the transglycosylation step of peptidoglycan biosynthesis, whereas nonlipidated glycopeptides inhibit the transpeptidation step.[8,105] Membrane-anchored glycopeptides evidently inhibit transglycosylation, because they bind preferentially to lipid II, the membrane-anchored substrate for the transglycosylases, rather than to nascent peptidoglycan (Figure 20). Thus, relatively small structural changes in glycopeptides can lead to differential partitioning among available binding sites and thus, in turn, can have functional consequences in terms of the metabolic steps that are affected. While it is still not known how the $VanS_B$ sensor kinases are activated, mechanistic investigations aimed at understanding the molecular basis for glycopeptide resistance have already led to a clear prescription for how to avoid reprogramming the bacterial cell wall, namely, use lipoglycopeptides.[74,102]

5.3. VRE Genotypes: VanA Resistance and the Compounds That Overcome It

VanA-resistant enterococcal strains contain similar sets of genes for sensing glycopeptide activity and for reprogramming peptidoglycan precursors as VanB strains. Unlike the latter, however, VanA strains are

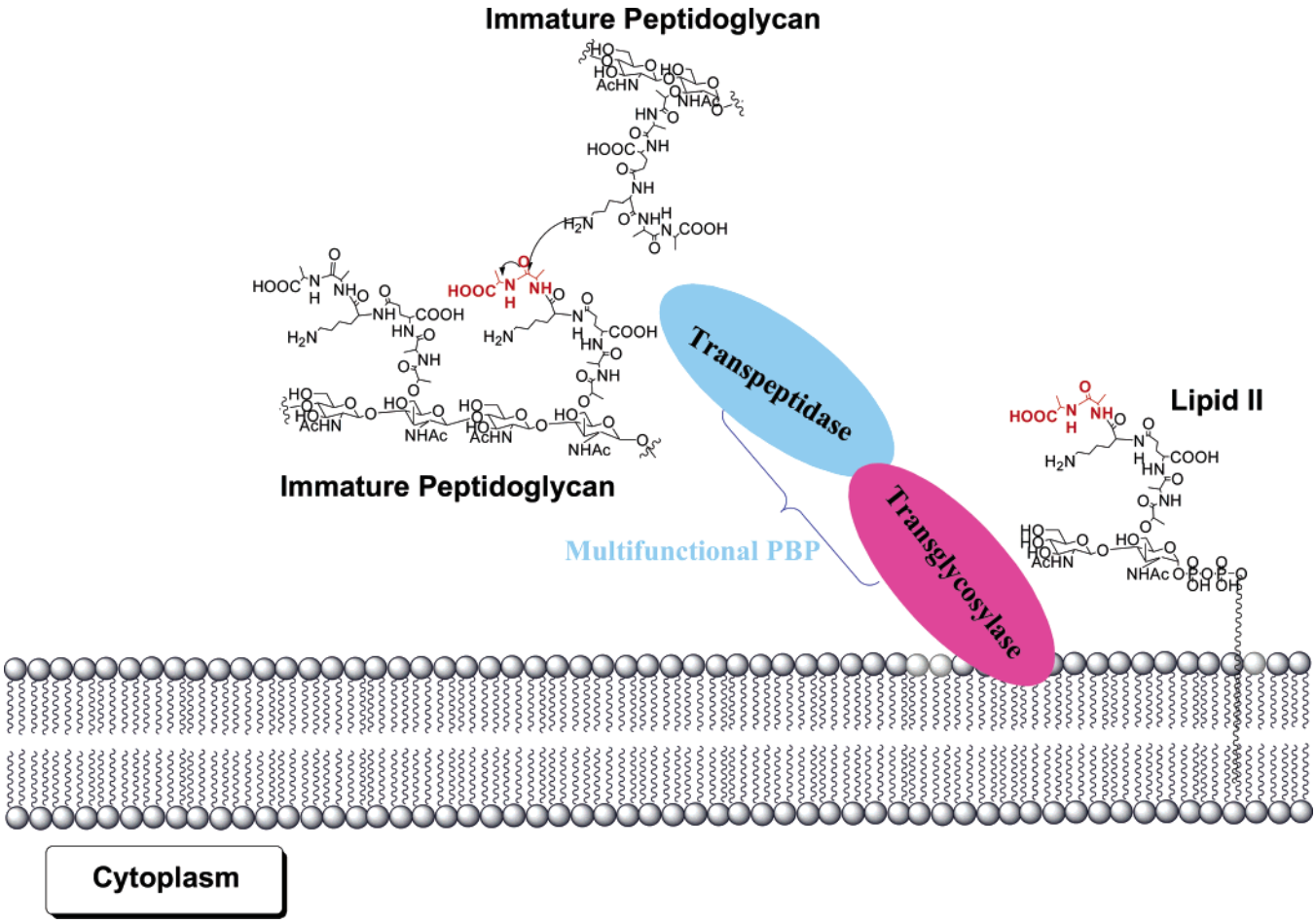
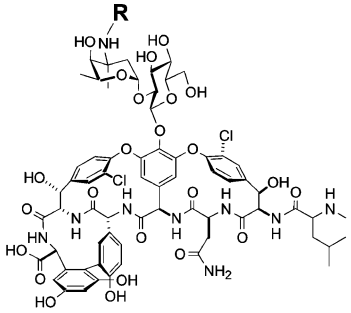


Figure 20. Two types of TPase substrates containing -D-Ala-D-Ala termini (in red) that can be complexed with vancomycin. lipid II are molecules embedded in the bacterial membrane via the C_{55} anchor. Immature PG chains that have been elongated by TGase action and are now ready for TPase action.

resistant to all natural glycopeptides that have been examined, including teicoplanin. Thus, irrespective of differences in how they are distributed among the possible binding sites or which steps they preferentially block, natural glycopeptides induce VanA strains to make altered cell wall precursors. Nevertheless, some semisynthetic glycopeptide derivatives overcome VanA resistance, and studies on these compounds are beginning to provide clues as to how this form of resistance can be overcome.

Researchers at Eli Lilly reported the first glycopeptide derivatives capable of overcoming both VanA and VanB resistance. These compounds, which were vancomycin derivatives containing lipid substituents attached to the nitrogen of the vancosamino sugar (Figure 21), showed good activity against a broad panel of vancomycin-resistant strains as well as outstanding activity against sensitive strains.[106,107] The vancosamino nitrogen of vancomycin can be readily modified without extensive protecting group manipulations and was, therefore, a logical position to explore. The motivation for attaching hydrophobic substituents was not made clear in the reports on these compounds, but it is possible that teicoplanin's activity against VanB strains provided the inspiration. It would have been reasonable to speculate that activity against VanB strains could be recovered by attaching a hydrophobic substituent to the vancomycin carbohydrate, because the teicoplanin carbohydrate, which is attached to the same amino acid, contains a hydrophobic substituent. Remarkably,



Vancomycin Derivative	Sensitive Enterococci MIC (μg/mL)	Resistant Enterococci MIC (μg/mL)
R= H	1.0	263
R= *N*-decyl	0.56	4.8
R = N-*p*-octylbenzyl	0.74	5.6
R = N-*p*-octyloxybenzyl	0.62	4.6

Figure 21. Lipoglycopeptide derivatives found at Eli Lilly to reverse VanA phenotypic resistance.

some of the glycolipid derivatives of vancomycin proved to be active not *only* against VanB strains but also against VanA strains, and the Lilly group ultimately took a lipid derivative of chloroeremomycin, now known as oritavancin, into the clinic (vide infra). Understanding the mechanism by which VanA resistance is overcome is crucial to developing better second-generation glycolipid derivatives. Two different models for how compounds such as oritavancin overcome VanA resistance have been proposed and are discussed below.

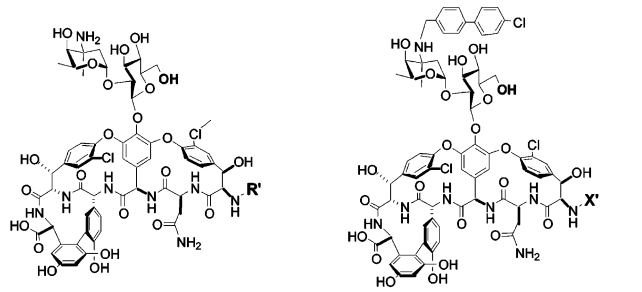
5.4. Models for How Vancomycin Analogues Overcome Vancomycin Resistance

5.4.1. Dimerization and Membrane Anchoring

Williams and co-workers proposed the first model which holds that vancomycin analogues kill resistant bacterial strains by the same mechanism that they kill sensitive strains, i.e., these analogues still bind to peptide termini of peptidoglycan precursors such as lipid II, but they have acquired the ability to bind to D-Ala-D-Lac better than vancomycin itself can (for a review, see Williams et al.).[31] Williams has proposed that the lipid substituent on vancomycin facilitates dimerization and membrane localization. It is these characteristics that promote better binding to D-Ala-D-Lac peptides that are presented in multiple copies in close proximity on the cell surface.[108] Williams argues that the second binding event is entropically favored to the extent that it compensates for the loss of the hydrogen bond between the depsipeptide ligand and the glycopeptide. There is considerable evidence to support the proposal that dimerization can enhance binding avidity to ligands that are presented in multiple copies on a surface of vesicles.[109] Moreover, it has been shown that covalent dimers and covalent trimers of vancomycin bind with high avidity to polyvalent peptide ligands.[110,111] However, there is no evidence that improved biological activity is related to the ability of glycopeptide analogues to dimerize. Furthermore, there is no evidence that these glycopeptides kill resistant bacteria by binding to D-Ala-D-Lac.

Membrane anchoring has also been suggested as a factor contributing to enhanced activity against resistant strains. It has been proposed that localizing the glycopeptide near the membrane partially compensates for the weak binding to D-Ala-D-Lac. Again, there is both theoretical and experimental support for the hypothesis that anchoring to membranes should increase avidity of glycopeptide analogues for membrane-anchored precursors presenting D-Ala-D-Ala or D-Ala-D-Lac.[95,108] Williams argues that it is a combination of membrane anchoring and dimerization that allows lipidated derivatives of vancomycin to bind to D-Ala-D-lactate. To show the effectiveness of the dimerization/membrane localization combination, Allen and co-workers damaged the lipidated derivative of vancomycin so that it could no longer bind D-Ala-D-Ala.[112] It has been shown that if the N-terminal amino acid is removed from vancomycin, it can no longer bind to peptidoglycan precursors.[113] Allen and co-workers showed that the damaged compounds still formed dimers and that these dimers were able to bind D-Ala-D-Ala, albeit very weakly.[112] Allen and co-workers attributed the biological activity of these damaged compounds to their ability to dimerize, which allows them to weakly bind to precursors. However, it is interesting to note that Ellman and co-workers have published data showing that covalent dimers of damaged vancomycin derivatives, nevertheless, have biological activity.[114,115] The following section describes a second mechanism of action that explains how these lipidated compounds kill resistant bacteria, despite being unable to bind peptidoglycan precursors.

Table 2. MICs for a Series of Glycopeptides Derivatives against Sensitive and Resistant Strains[a]



	MIC (μg/mL)	
glycopeptide	sensitive *E. faecium*	resistant *E. faecium*
vancomycin	1	2048
chlorobiphenyl vancomycin	0.03	16
damaged vancomycin	no activity	no activity
damaged chlorobiphenyl vancomycin	10	40

[a] Compounds lacking the *N*-terminal methylleucine amino acid were used to evaluate which component of the activity derives from a peptide-binding-independent mechanism.

5.4.2. A Second Mechanism of Action—Direct Interaction with the Transglycosylase

Biophysical studies of binding using model systems are at best suggestive with respect to mechanism of action. Kahne and co-workers, therefore, designed experiments to test the hypothesis that activity against resistant bacterial strains depends on binding to D-Ala-D-Lac. To address this issue, vancomycin analogues in which the peptide binding pocket was damaged were prepared (Table 2). These analogues lack the *N*-methylleucine moiety of the aglycon, which makes both hydrogen bonding and electrostatic contacts to D-Ala-D-Ala. It has been established that these damaged vancomycin analogues are unable to bind *N*-acyl-D-Ala-D-Ala.[113] The damaged vancomycin analogues were tested for activity against both sensitive and vancomycin-resistant strains. The chlorobiphenyl derivative lost considerable activity against sensitive strains, as expected; however, its activity against resistant strains did not change significantly.

While it is conceivable that membrane-anchoring or dimerization could compensate for the loss of a single hydrogen bond, it would not be reasonable to

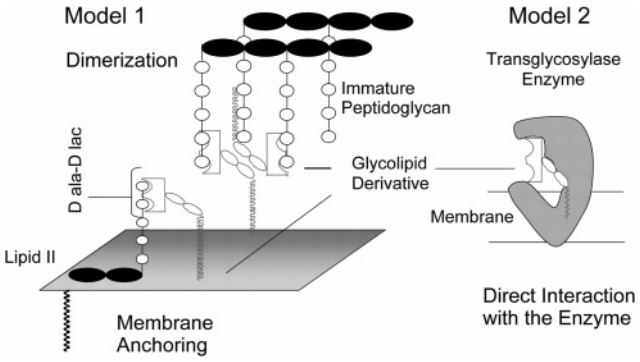


Figure 22. Two mechanisms of action for glycopeptides: (a) inhibition of the transpeptidase step by binding to the D-Ala-D-Ala terminus and (b) direct inhibition of the transglycosylases.

conclude that these phenomena compensate for the loss of two hydrogen bonds and an electrostatic contact. Kahne and co-workers therefore concluded that depsipeptide binding does not play a role in the mechanism of action by which chlorobiphenyl vancomycin analogues kill VanA resistant bacteria. They proposed, instead, that there must be a second mechanism of action that explains the activity against resistant strains. Studies aimed at probing the site of inhibition of the damaged chlorobiphenyl vancomycin analogues established that the damaged compound, like the intact parent, blocks peptidoglycan biosynthesis[8] at the transglycosylation step (Figure 22).

On the basis of these experiments, they suggested that chlorobiphenyl vancomycin analogues might interact directly with a key component of the transglycosylation machinery.

Goldman and co-workers provided additional evidence for a second mechanism of action for chlorobiphenyl derivatives of vancomycin when they showed that both chlorobiphenyl vancomycin and damaged chlorobiphenyl vancomycin are able to inhibit formation of nascent peptidoglycan when UDP-MurNac-tetrapeptide (L-Ala-γ-D-Gln-L-Lys-D-Ala) is provided to bacterial cells as a substrate. Vancomycin, in contrast, does not inhibit the incorporation of this substrate into peptidoglycan.[116] The following year, Chapman and co-workers showed through affinity chromatography of bacterial cell extracts that chlorobiphenyl derivatives of vancomycin retained multiple PBPs,[117] including *E. coli* PBP1b, whereas vancomycin was unable to retain PBPs. These experiments provided support for the hypothesis that chlorobiphenyl derivatives of vancomycin interact directly with PBPs involved in peptidoglycan biosynthesis. Chen et al. subsequently developed an assay to monitor the activity of *E. coli* PBP1b and found that chlorobiphenyl vancomycin analogues can inhibit this enzyme without binding substrate.[10] Inhibition by vancomycin, in contrast, depends on peptide binding. Taken together, these studies provide strong evidence for a second mechanism of action. At this point, however, it is essential to develop methods to study Gram-positive transglycosylases found in relevant microorganisms in order to determine whether chlorobiphenyl vancomycin analogues directly inhibit these enzymes.[137] If, in fact, vancomycin analogues with activity against resistant microorganisms function because the derivatized carbohydrate moiety facilitates a direct and inhibitory interaction with transglycosylases, then it should be possible to design improved antibiotics by attaching to the vancomycin aglycon structural elements that have better activity against relevant lipid II-utilizing transglycosylases.[8,20,46,118] The design of better antibiotics is important not only for overcoming VanA resistance but also for tackling the problem of the newly emerging vancomycin-resistant staphylococcus.

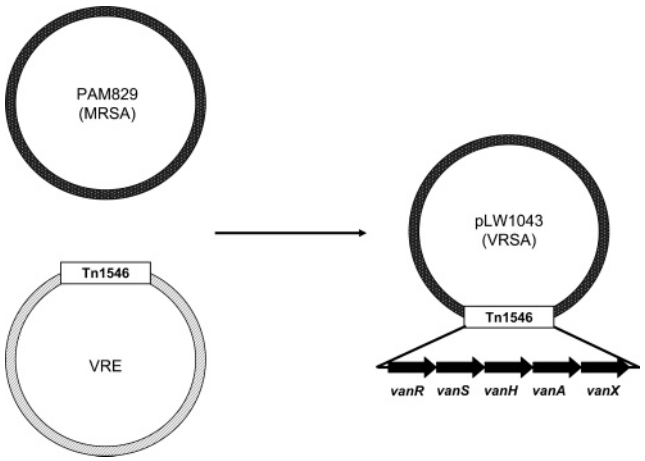


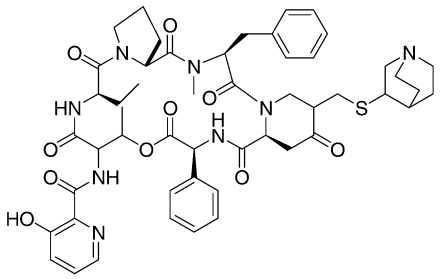
Figure 23. Proposal for recombination in a patient infected with VRE and MRSA allowing TN1546 to move to MRSA and create VRSA.

5.5. Vancomycin-Resistant *S. aureus* (VRSA)

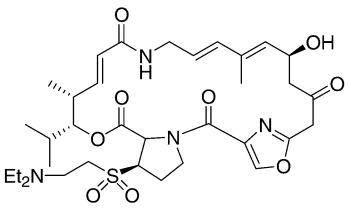
For some years the levels of vancomcyin resistance among clinical isolates of *Staphylococci* were low. *S. aureus* with such diminished susceptibility to vancomycin were termed VISA (vancomycin intermediate *S. aureus*).[119,120] The genotypes of these isolates were not of the *VanHAX* type noted above for VRE. Rather, they typically generated multilayered, thickened cell walls as though more sites for stoichiometric binding of drug were the cause of lessened susceptibility. But in 2003, VRSA were isolated from a dialysis patient who also had a chronic infection with VRE.[121] Genotyping of the VRSA showed the same five *VanRSHAX* genes found also on transposons in the VRE (Figure 23). Every indication is that the transposon hopped from the enterococcal host to the *S. aureus*.[122] It remains to be seen what the rate of spread of the *VanRSHAX* genes will be into MRSA strains, but there is no doubt that this only hastens the need for second- and third-generation forms of glycopeptides and lipoglycopeptides that can combat various phenotypes of VRE and VRSA.

6. New Directions for Treating VRE and VRSA

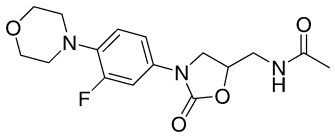
The long time frame and progressively broader clinical use of the first-generation natural product glycopeptides vancomycin and teicoplanin as front line agents has eventually led to widespread incidence of VRE (up to 30% of enterocccal infections in US hospitals in 2002) and the recent emergence of VRSA. Over the past decade there has been keen interest in the discovery and development of new treatment regimes for these life-threatening bacterial



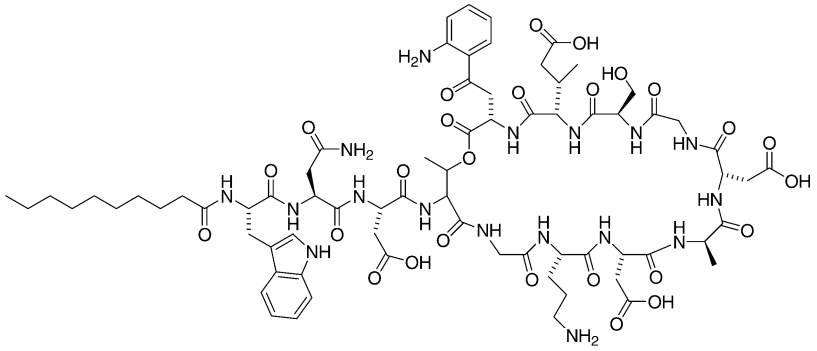
Quinupristin



Dalfopristin

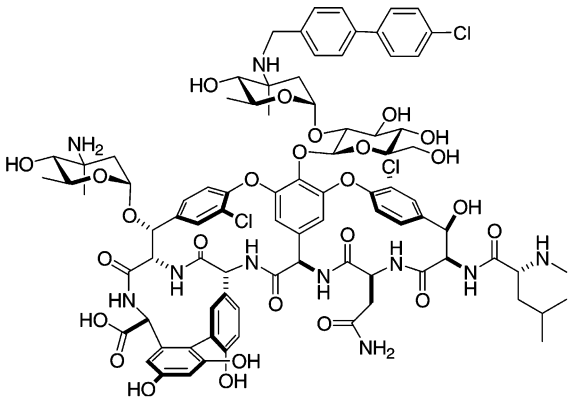


12 Linezolid

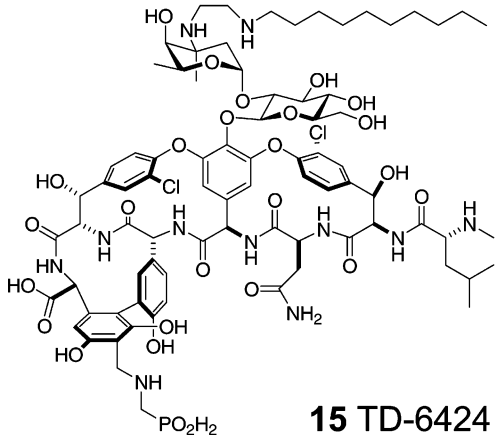


13 Daptomycin

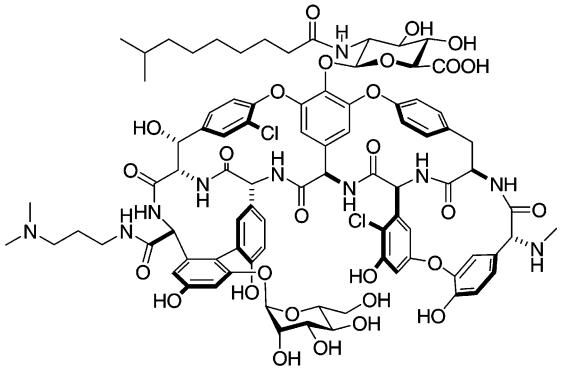
Figure 24. Three nonglycopeptide classes of antibiotics recently approved for treatment of VRE infections: Synercid (**11**) combination of Quinupristin and Dalfopristin blocks protein synthesis; the oxazolidinone Linezolid (**12**) blocks the first peptide-bond-forming step at bacterial ribosomes; the lipopeptide daptomycin (**13**) damages membranes and causes ion leaks in bacteria.



14 Oritavancin



15 TD-6424



16 Dalbavancin

Figure 25. Lipoglycopeptide antibiotics in clinical development: oritavancin (**14**) and TD-624 (**15**) are *N*-aryl and *N*-alkyl hydrophobic derivatives modified on the vancomycin-type heptapeptide scaffold; dalbavancin (**16**) is a lipoglycopeptide modified in the amide linkage on a teicoplanin-type scaffold.

pathogens. Two parallel lines of discovery and development have ensued. One has been the search for a replacement to vancomycin and teicoplanin and has yielded newly approved drugs. The other line began

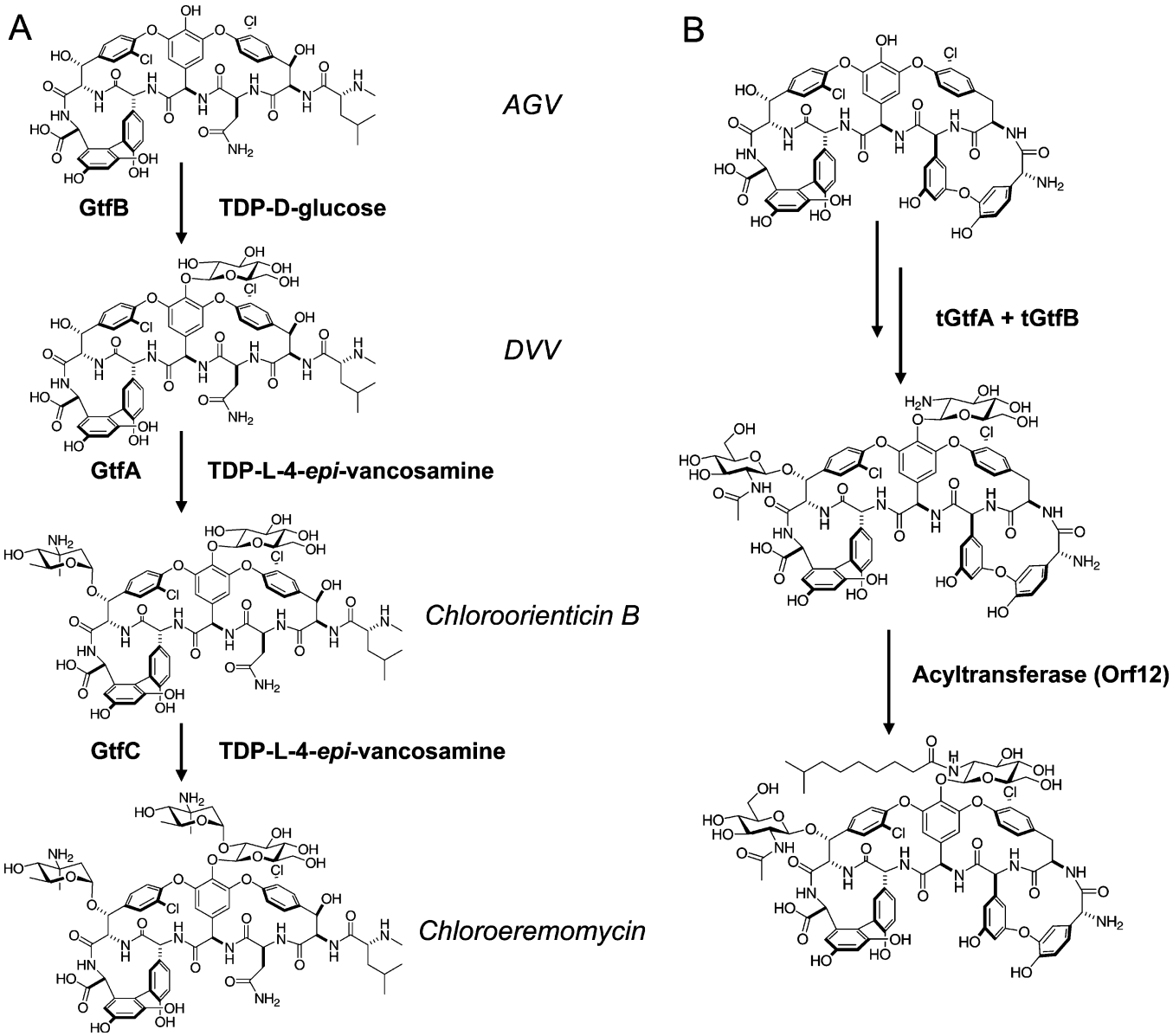


Figure 26. (a) Reconstitution of chloroermomycin from the aglycon scaffold by consecutive action of Gtfs B, A, and C. (b) Decoration of the teicoplanin aglycon with GlcNAc at residue 6, with glucosamine at residue 4 by tGtfA and tGtfB, and *N*-acylation of the glucosamine residue by acyl transferase action.

with semisynthetic improvements to the vancomcyin and teicoplanin subfamily scaffolds.

6.1. Recently Approved Drugs for VRE

Over the past 5 years three new classes of antibiotics have been approved in the US with efficacy against enterococcal infections (Figure 24).[3] None of the drugs are glycopeptides, so they do not induce the VanRSHAX enzymes and therefore are active against all VRE strains. Synercid (**11**), approved in 1998, is a mixture of two natural product cyclic lactones, pristinamycins I and II. They block bacterial protein synthesis in the elongation phase.[4] Linezolid (**12**), a synthetic antibacterial agent with an oxazolidinone backbone approved in 2000, blocks the first peptide-bond-forming step at the peptidyl transferase center of bacterial ribosomes. The third molecule, daptomycin (**13**), approved in 2003 under the trade name Cubicin, is also a natural product. It is a lipopeptidolactone, made nonribosomally,[4] that disrupts bacterial membrane integrity, causing ions to leak out and thus bacterial death. All three of these drugs then act by mechanisms independent of those used by vancomycin and teicoplanin and will not be subject to cross-resistance with the glycopeptides.

6.2. Second-Generation Semisynthetic Lipoglycopeptides in Clinical Development

Given the structural complexity of the glycopeptide antibiotic class, in particular, the oxidatively cross-linked heptapeptide scaffolds that are variant in vancomcyin and teicoplanin family members, efforts have been undertaken to create semisynthetic drugs by chemical modification of the natural products.

One successful route has involved the single-step reductive alkylation of the amino group of the vancosamine sugar in the vancomycin/chloroeremomycin scaffold and led to oritavancin (**14**). Use of an *N*-alkyl rather than *N*-aryl group has generated TD-624 (**15**) (Figure 25). A comparable single-step chemical modi-

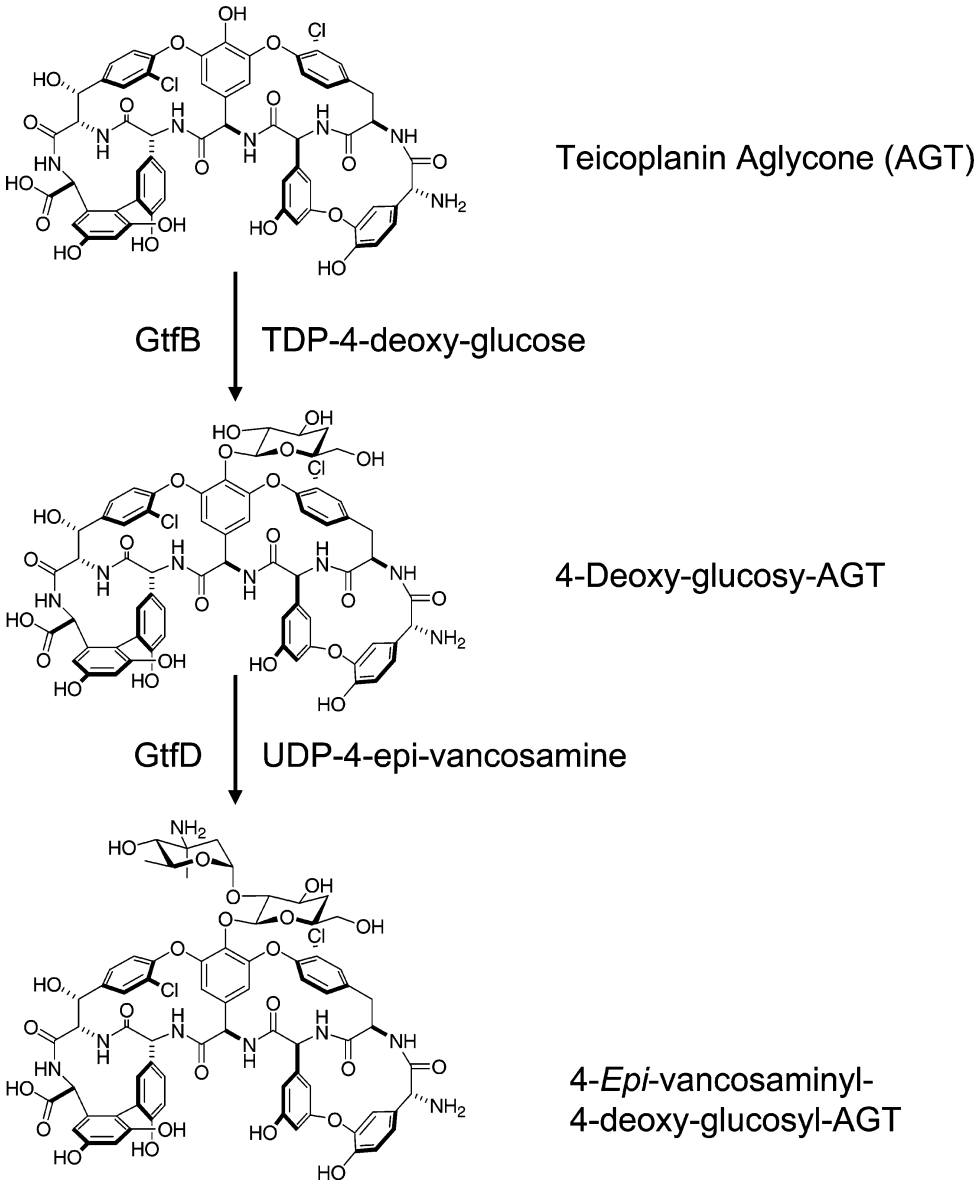


Figure 27. Enzymatic synthesis of a variant glycopeptide, starting from the teicoplanin aglycon, UDP-4-deoxyglucose and GtfB, and TDP-l-epivancosamine and GtfC.

fication by amidation of the carboxy terminus of the heptapeptide scaffold of the teicoplanin family member A40926 has generated dalbavancin (**16**).

6.2.1. Oritavancin

Screening of the natural and semisynthetic glycopeptide collection at Lilly led to the choice of the chloroeremomycin scaffold and a series of *N*-alkyl or *N*-acyl derivatives of the terminal aminosugar on the glucosyl-epivancosamine chain.[123] The oritavancin molecule, originally known as LY333328, is a simple *N*-aryl derivative of the natural product chloroeremomycin **4**. The epivancosamine moiety in the disaccharide chain could be selectively derivatized at the amino group by imine formation with aryl aldehydes, followed by reduction to the stable secondary amine. The chlorobiphenyl group was one of several lipophilic groups that conferred activity against VRE, allowing a regain of about 2 of the 3 logs of activity lost in VanA type VRE.[123] Arylamines have been used elsewhere to decorate natural product scaffolds, notably in the antifungal echinocandins as replacements for fatty acyl amide groups,[124] and they may mimic the *N*-acyl substituents found in the teicoplanin series of natural lipoglycopeptides. Oritavancin is active against both VanA and VanB phenotypes of VRE and also MRSA and VRSA at MIC values of $<1\ \mu g/mL$.[123] Once-a-day administration is proposed. Oritavancin shows strong bacteriocidal properties under conditions where vancomycin is bacteriostatic. Oritavancin has shown clinical effectiveness in complicated skin and soft tissue infections caused by Gram-positive bacteria, including MRSA.[125]

6.2.2. Dalbavancin

Dalbavancin is a semisynthetic variant of the teicoplanin family member A40926.[22,126,137] Like A40926 and teicoplanin, it has a long fatty acyl moiety, in this case a C_{12} terminally branched chain, in amide linkage to the glucosamine. The mannose at residue 7 is present but not the GlcNAc at residue 6. The synthetic modification is amidation of the

C-terminal carboxylic acid of the cross-linked heptapeptide scaffold by a *N*,*N*-dimethyl propylamide group.

This lipoglycopeptide is active against VanB type VRE at 0.1 μg/mL but is ineffective against VanA phenotypes. It has enhanced potency over vancomycin and teicoplanin against susceptible enterococci and against MRSA.[126] Dalbavancin also has bacteriocidal activity. A once weekly administration is being planned, given the long lifetime.[127] Dalbavancin has also shown efficacy in complicated skin and skin structure infections.[128]

6.2.3. TD-6424

This is the third of the second-generation, semisynthetic *lipo*glycopeptide variants to enter clinical development. In some analogy to oritavnacin, TD-6424 utilizes a vancomycin-type cross-linked heptapeptide scaffold. There is one modification in that scaffold at residue 7. The Dpg_7 has been replaced with a 4-aminoethyl side chain, in turn bearing a $CH_2PO_3^{2-}$ substituent, to enhance solubility of the scaffold. The other modification maintains the lipoglycopeptide character of these second-generation compounds. The vancosamine sugar is alkylated by a long alkyl chain, with an NH at the two positions. The reductive alkylation is again a presumptive mimic of the normal fatty acyl chain attached to the single sugar of teicoplanin. TD-6424 is rapdly bacteriocidal[129] and more potent than teicoplanin or vancomycin against MSSA and MRSA.

6.3. In Discovery: Chemoenzymatic Routes To Modify Sugars and Acyl Groups on Heptapeptide Scaffolds

The second-generation lipoglycopeptides in development noted above represent different one-step chemical manipulations of chloroeremomycin or A40926, and multiple-step chemical manipulation of both scaffold and aminosugar *N*-alkylation in TD-624 on the natural products derived from fermentation. The current knowledge of the biosynthetic gene clusters for both vancomycin and teicoplanin family members noted in section 3 and the importance of the sugars and acyl groups decorating the heptapeptide scaffold noted in section 5 have focused attention on using the tailoring enzymes to embellish the scaffolds in new combinations.

For example, three glycosyltransferases, GtfA, -B, and -C, convert the vancomycin aglycon to chloroeremomycin, a pathway that has been reconstituted *in vitro* with the purified enzymes (Figure 26a)[38] Analogously, the two homologous Gtfs from the teicoplanin cluster, tGtfA and tGtfB, have been overproduced and shown to transfer GlcNAc to residues 6 and 4 of the teicoplanin aglycon.[16,17] Further, tGtfB will transfer glucosamine, which can be N-acylated by an acyl transferase also encoded in the biosynthetic cluster (Figure 26b).[17] Different sugars and scaffolds can be used by some of the Gtfs such that novel glycopeptides can be assembled. Thus, GtfB will take several UDP-glucose derivatives,[130,131] some of which can be elongated by GtfC or by GtfD from the vancomycin biosynthetic cluster. It is possible, for example, to build a variant disaccharide, e.g. 4-deoxy-D-glucose-2,1-L-epivancosamine, on an altered scaffold, the teicoplanin aglycon, introducing three variations via the action of GtfB and -D (Figure 27).[132] X-ray structures of GtfA, -B, and -D[133−135] have been determined to allow for molecular insights into Gtf specificity for NDP sugar donor and scaffold acceptor and aid in structure-based Gtf evolution.

Finally, it is possible to *N*-alkylate and *N*-arylate variant aminosugars introduced by Gtfs on the heptapeptide scaffolds to effect chemoenzymatic manipulations that enable multistep variations of structure in glycopeptide and lipoglycopeptide antibiotics.[102] This approach should allow construction of focused libraries of novel lipoglycopeptide structures to optimize properties desired in a third-generation glycopeptide antibiotic, including broad spectrum, rapid cidality, oral activity, high potency, good pharmacokinetic, and therapeutic ratio.

7. Acknowledgments

The authors are indebted to many colleagues in the Kahne and Walsh research groups for contributions to glycopeptide and lipoglycopeptide projects, some of which are acknowledged in the references cited. Research in the Walsh group was supported in part by NIH Grant GM49338 and in the Kahne group by NIH Grant 66174**.** C.W. is a member of the board of directors of Vicuron Pharmaceuticals.

8. Note Added after ASAP Publication

This review was posted ASAP on January 22, 2005. The spelling of transglycosylase was corrected in Figures 12 and 13, and refs 136 and 137 were added. The review was reposted January 27, 2005.

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