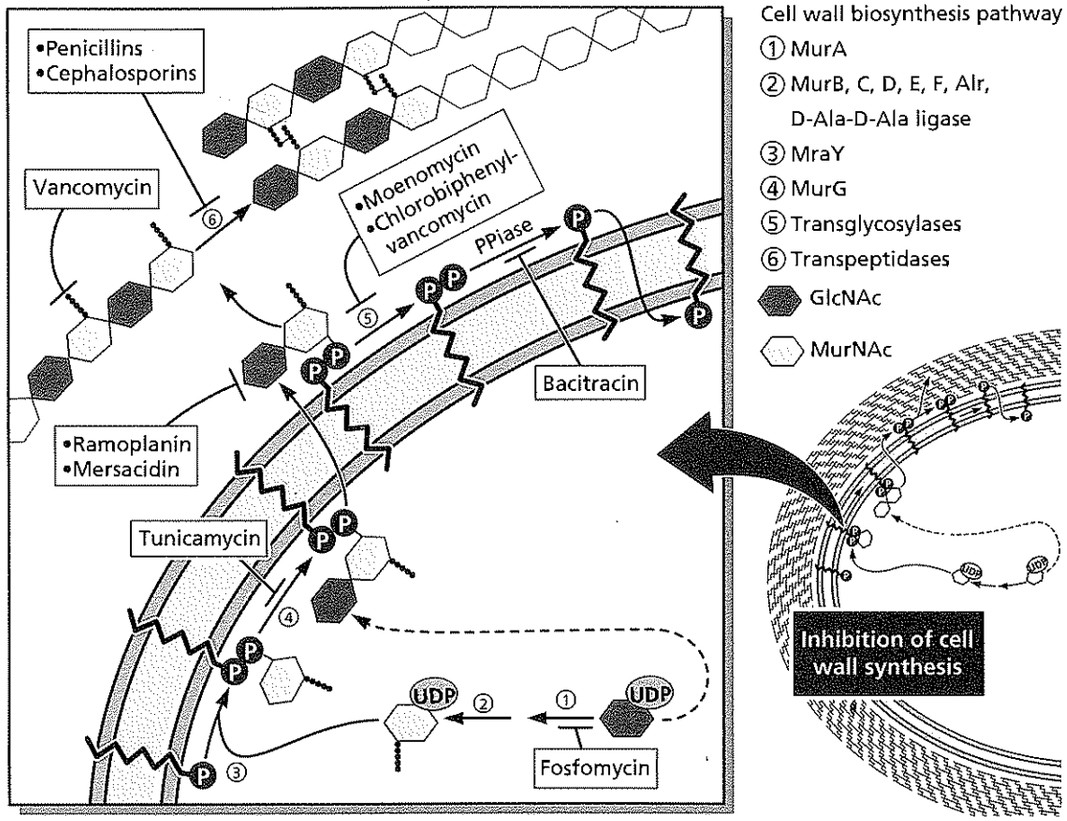


Antibiotics that act on cell wall biosynthesis.

chapter 3

Antibiotics That Act on Cell Wall Biosynthesis

This chapter deals with antibiotics that interdict any of the several steps in bacterial cell wall assembly, from biogenesis of the dedicated monomers to the specialized assembly, membrane translocation, and extracellular cross-linking and strengthening of the exoskeletal peptidoglycan layers. The figure on the facing page shows a blowup of a section of Fig. 2.2 and emphasizes the reactions of cell wall biosynthesis and the antibiotics that block them.

Similarities and differences in gram-negative and gram-positive cell wall structure affect susceptibility to antibiotics

Bacteria such as *Escherichia coli, Salmonella, Pseudomonas*, and *Yersinia* are negative in Gram staining, while staphylococci, streptococci, and enterococci are gram positive. The difference in retention of the stain, crystal violet in an ethanol solution, depends on the extent to which the outer membrane of bacteria is intact and a significant permeability barrier (gram-negative organisms) or is incomplete and fragmentary (gram-positive organisms) (Fig. 3.1A) (Lee and Schneewind, 2001; Navarre and Schneewind, 1999; Nikaido, 1994). Gram-negative and gram-positive bacteria both have a peptidoglycan (PG) layer as part of their cell wall structure. The PG layer is generally substantially thicker and multilayered in the gram-positive bacteria (Fig. 3.1A). The PG, with orthogonal glycan and peptide strands (Fig. 3.1B), undergoes enzymatic cross-linking of the glycan strands, by transglycosylase action, and of the peptide strands, by transpeptidase action (Fig. 3.1C). The peptide cross-links introduce covalent connectivity to the meshwork, impart mechanical strength, and provide the major structural barrier to osmotic pressure forces that could kill the bacteria. Many of the antibiotics that affect bacterial cell walls inhibit enzymes or sequester substrates involved in PG assembly and cross-linking, as we will note in the subsequent sections of this chapter.

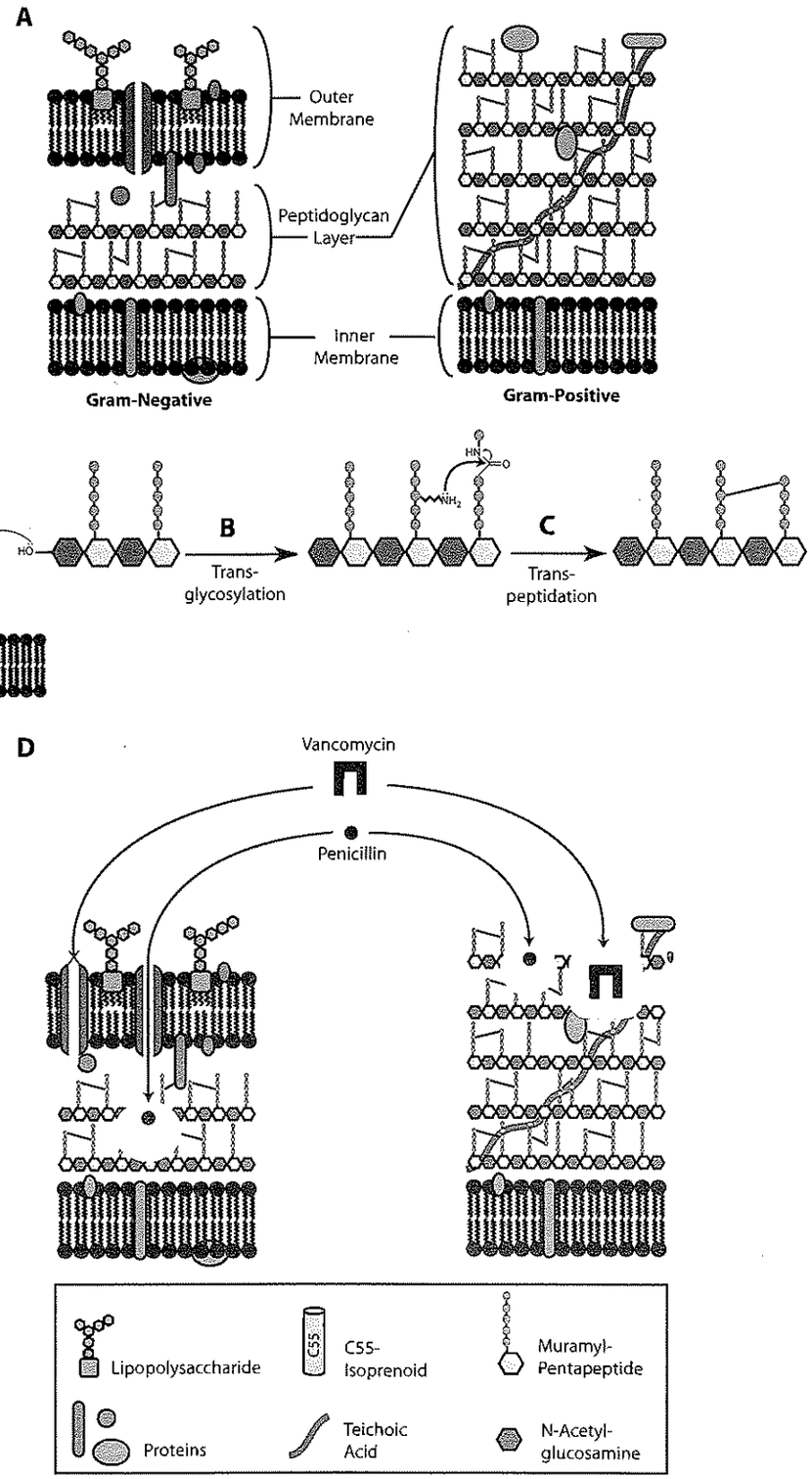


Table 3.1 Proteins covalently linked to peptidoglycan

Functional category	Example protein	Mechanism
Protection from immune system	M-family proteins	Antigenic shift, antiphagocytic
	Proteins A	Antigenic shift, antiphagocytic
	C5a peptidases	Destroy chemoattractant
Structural	Lipoproteins	Link to outer membrane
	Fimbriae	Assemble to form filaments
Infection/virulence	MSCRAMMs	Bind components of extracellular matrix
	Invasins	Bind to β1-integrin, tissue invasion
	Internalin	Facilitate host cell invasion
Nutrient acquisition	Glycosidases	Cleave saccharides
	Peptidases	Cleave peptides
	Nucleotidases	Cleave oligonucleotides?
Bacterial cell adhesion	Aggregation substance	Bind to enterococcal binding substance
	Surface exclusion protein	Prevent mating between bacteria with identical plasmid by unknown mechanism

The thick PG layer of gram-positive bacteria has been described as a surface organelle, for display of carbohydrates and proteins, while the outer membrane is the equivalent surface organelle in gram-negative organisms (Lee and Schneewind, 2001). Both gram-negative and gram-positive bacteria have proteins that are covalently linked to peptide chains of the PG layer (Table 3.1) (Braun and Hantke, 1974). Some of these outer membrane proteins act as adhesions for specific proteins on vertebrate cell membranes, such as the protein invasin from *Yersinia pseudotuberculosis*, which binds to β1-integrin proteins displayed on host cells, an interaction required for bacterial penetration into intestinal lymphoid tissue (Isberg and Leong, 1990). Surface proteins tethered to the thick PG layer of gram-positive bacteria are connected during biosynthesis by the action of the enzyme sortase, discussed in chapter 15 as a potential antibacterial target. The outer membrane of gram-negative bacteria is asymmetric in its lipid composition, with phospholipids in the inner leaflet and lipid A as the predominant lipid in the outer leaflet (Raetz, 1987), with variable O-antigen chains covalently attached and facing tbe external environment as highly antigenic surface carbohydrates (Fig. 3.1A). The thick PG layer in gram-positive bacteria also has polymers of teichoic acids (Fig. 3.1A) associated with it. The surface carbohydrates and proteins can serve many roles, including protection against host-cell killing, providing specific ligands for attachment to biotic and abiotic surfaces, and facilitating interconversion between singe cell (planktonic) forms and biofilm communities of bacteria.

Figure 3.1 Cell wall structures of gram-positive and gram-negative bacteria: (A) differences in outer membrane permeability barriers; (B) peptidoglycan elongation by transglycosylase action; (C) peptidoglycan cross-linking by transpeptidase action; (D) penetration of antibiotics to the cytoplasmic membrane in gram-positive bacteria.

Gram-positive bacteria are susceptible to some antibiotics that do not work or work poorly (e.g., against pseudomonads) against gram-negative baceria, and this difference is related to the ability of antibiotics to be blocked by the limiting pore sizes of the porin proteins (Fig. 3.1A and D) (Koebnik et al., 2000) of the gram-negative organisms' outer membranes. There is no such barrier to diffusion in gram-positive bacteria. Vancomycin, for example, cannot penetrate the outer membrane and so is effective as an antibiotic only against gram-positive pathogens. In gram-negative bacteria the space between the inner and outer membranes is the periplasmic space (Fig. 3.1D). In addition to the strands of the PG layer, the periplasm has hydrolytic enzymes to convert oligomeric and polymeric nucleotides, peptides, and saccharides to monomers that are then bound by periplasmic carrier proteins, presented to membrane transport proteins, and internalized. There are also protein chaperones to help proteins being secreted to the outer membrane to fold and transit the periplasmic space.

Each of these cell wall structures is a potential target for interruption by antibiotics. Distinct features of outer membranes even among gram-negative bacteria can lead to differences in permeability to antibiotics. For example, *Pseudomonas aeruginosa* outer membranes show about 100-fold lower permeability to cephalosporins such as cephaloridine (Nikaido, 1998) than other gram-negative bacteria, in part because of porins with small pores to reduce inward passage of the antibiotics into the periplasmic space.

The distinctive appearances of the cell walls of gram-negative and gram-positive bacteria can be discerned in both transmission electron micrographs and in scanning electron micrographs. In Fig. 3.2A and B, cell wall schematics are mirrored by the photograph of the gram-positive *Arthrobacter crystallopoietes* (Fig. 3.2C) and of the gram-negative *Leucothrix mucor* (Fig. 3D). The scanning micrographs of the gram-positive *Bacillus subtilis* (Fig. 3.2E) and of the gram-negative *E. coli* (Fig. 3.2F) show different surface textures.

Three phases of peptidoglycan enzymatic assembly: cytoplasmic, membrane-associated, and extracytoplasmic

Enzymes in the cytoplasmic phase of the Mur pathway: MurA-F

As bacteria grow and divide, PG layer(s) have to be laid down both transversely and laterally (for septum formation) (Holtje, 1998). The PG unit that is added to the expanding PG layers is a disaccharyl pentapeptide, presented at the membrane surface while attached to a C_{55} (undecaprenyl) lipid (lipid II) in phosphodiester linkage that gets cleaved in the enzymatic transglycosylation step (Fig. 3.3). The lipid, sugars, and pentapeptide moieties are each provided by enzymes committed to PG assembly. The PG layer is also known as murein (from the Greek for "wall") and the genes for the early steps in assembly are named *murA-G* (van Heijenoort, 2001a).

The cytoplasmic phase of murein assembly is accomplished by the six enzymes MurA-F, starting from the nucleotide diphosphosugar UDP-*N*-acetylglucosamine (UDP-GlcNAc) and proceeding to the UDP-muramyl pentapeptide, UDP-muramyl-L-Ala-D-γ-Glu-*meso*-diaminopimelate-D-Ala-D-Ala (Fig. 3.4). The UDP-GlcNAc is itself made by a bifunctional enzyme, GlmU

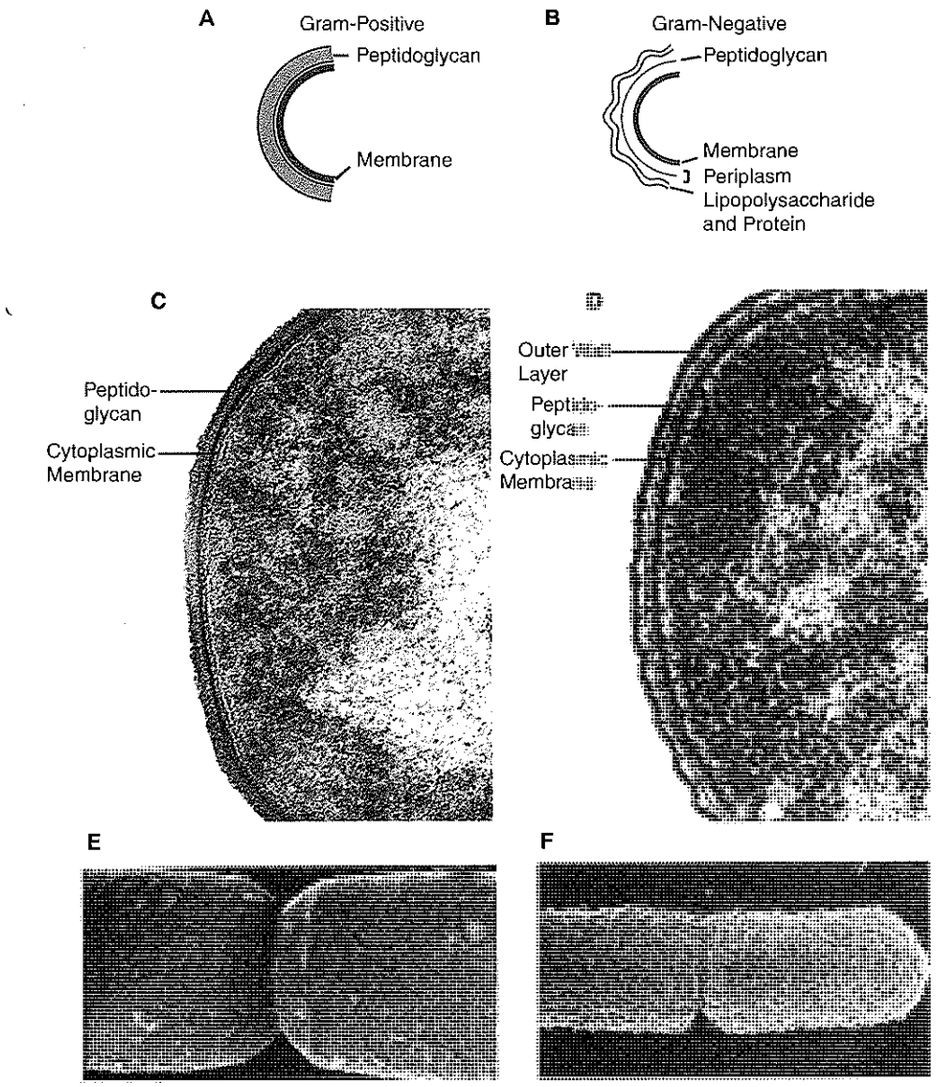


Figure 3.2 Cell walls of bacteria. (A and B) Schematic diagrams of gram-positive (A) and gram-negative (B) cell walls. (C and D) Electron micrographs showing the cell walls of a gram-positive bacterium, *Arthrobacter crystallopoietes* (C), and a gram-negative bacterium, *Leucothrix mucor* (D). (E and F) Scanning electron micrographs of gram-positive (*Bacillus subtilis*) (E) and gram-negative (*Escherichia coli*) (F) bacteria. Note the surface texture in the cells shown in panels E and F. A single cell of *B. subtilis* or *E. coli* is about 1 μm in diameter.

(Gehring et al., 1996; Mengin-Lecreulx and van Heijenoort, 1994), that acetylates glucosamine-1P and then uridylylates it.

The conversion of the GlcNAc to the muramyl moiety involves construction of the 3′-*O*-lactyl ether of the GlcNAc residue and is accomplished by two enzymes, MurA and MurB (Fig. 3.5A). MurA uses phosphoenolpyruvate (PEP) as cosubstrate and installs the 3′-*O*-enolpyruvyl ether linkage by an unusual

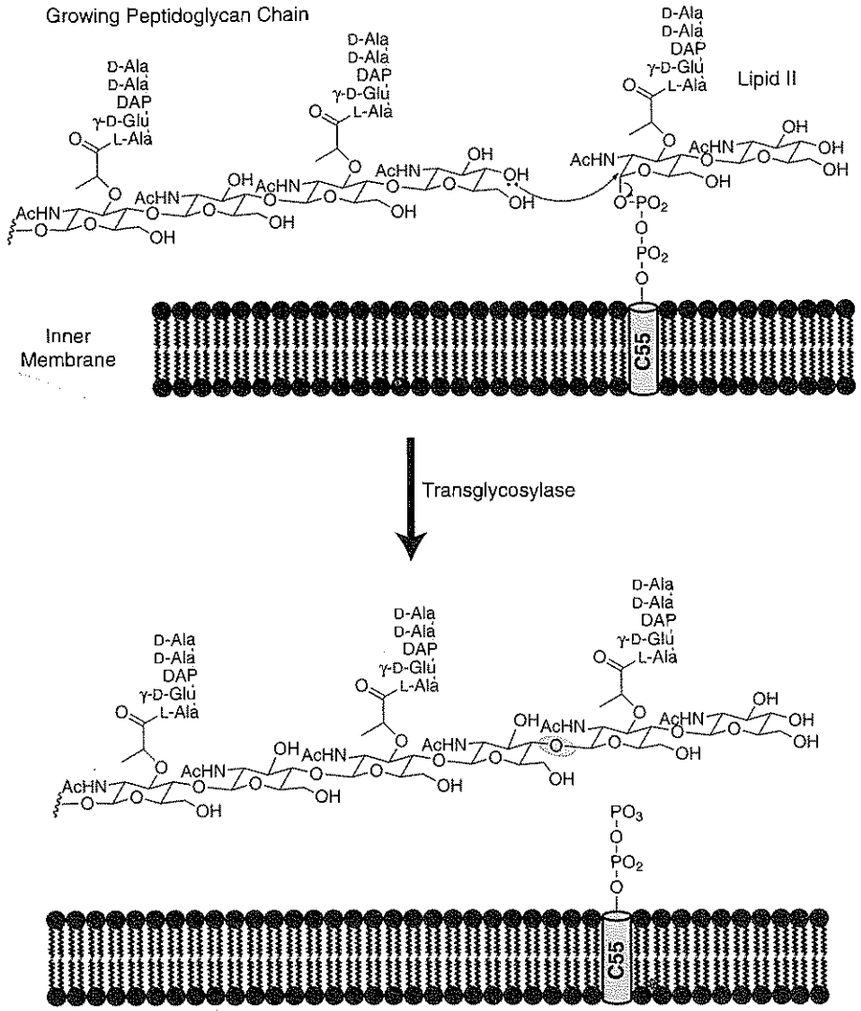


Figure 3.3 Action of cell wall transglycosylases on the C_{55}-lipid-linked *N*-acetyl-muramyl (MurNAc) pentapeptide substrate.

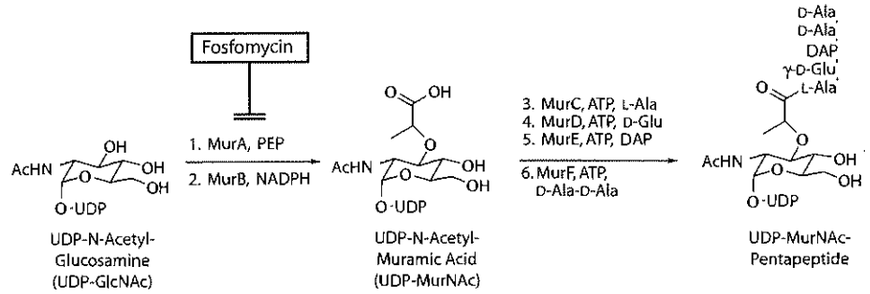


Figure 3.4 Assembly of UDP-MurNAc pentapeptide by the six enzymes MurA-F.

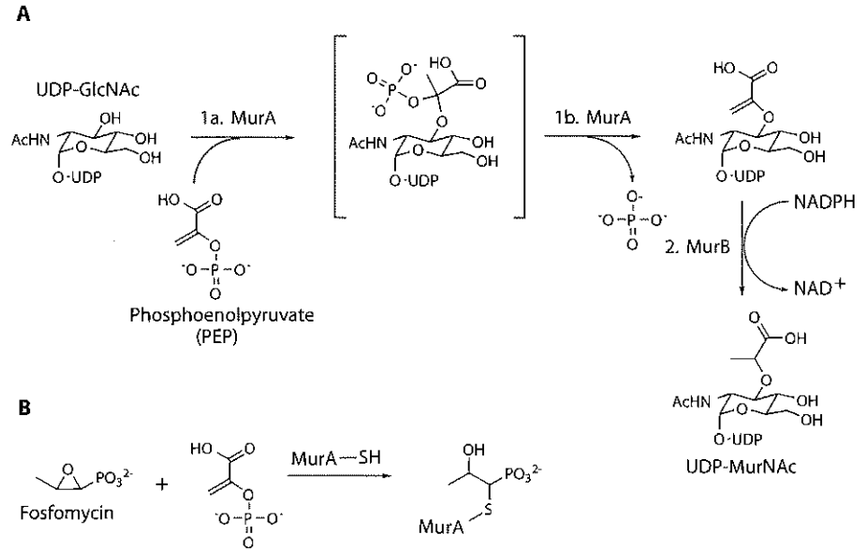


Figure 3.5 (A) Sequential action of MurA and MurB to convert UDP-*N*-acetyl-glucosamine (GlcNAc) to UDP-*N*-MurNAc. (B) Inactivation of MurA by the antibiotic fosfomycin.

addition/elimination sequence, where the 3′ oxygen of the GlcNAc is added into the PEP double bond, regio- and stereospecifically at C_2, and C_3 transiently becomes a methyl group (Cassidy and Kahan, 1973; Walsh et al., 1996a). The second step is enzyme-catalyzed elimination of H^+ and P_i to generate the enol ether. MurB is an NADPH-oxidizing flavoprotein that adds a hydride to C_3 and protonates C_2 to generate the lactyl ether and produce UDP-muramic acid (Benson et al., 1993). The carboxylate of the lactyl ether is tbe locus for subsequent peptide chain building by MurC, D, E, and F.

The X-ray structures of MurA and MurB have been determined (Benson et al., 1995; Schonbrunn et al., 1996; Skarzynski et al., 1996) and corroborate the above mechanistic observations architecturally. MurA is the target of the antibiotic fosfomycin (see Table 2.1 for its recommended use in treating urinary tract infections), a simple three-carbon epoxy propyl phosphonate metabolite from streptomycetes (Seto, 1997) that acts as an inactivating analog of PEP (**Fig. 3.5B**). There is an active-site cysteine in MurA, Cys-115 in the *E. coli* MurA, whose thiolate side chain opens the reactive epoxide of bound fosfomycin, producing a stable covalent tether, blocking the active site and preventing subsequent catalytic turnover. An X-ray structure of the fosfomycin-inactivated MurA has been determined (Skarzynski et al., 1998), which should be an aid to design of successors to fosfomycin. Thiazolidinone inhibitors of MurB have recently been reported (Andres et al., 2000).

MurC, D, and E carry out homologous reactions and belong to the same superfamily (van Heijenoort, 2001a), as they sequentially make amide bonds adding L-Ala, D-Glu, and *meso*-DAP (lysine instead of DAP in some gram-positive bacteria) to the growing UDP-muramyl chain, resulting in the UDP-muramyl tripeptide (**Fig. 3.6**) at the end of the MurE step. MurD makes the

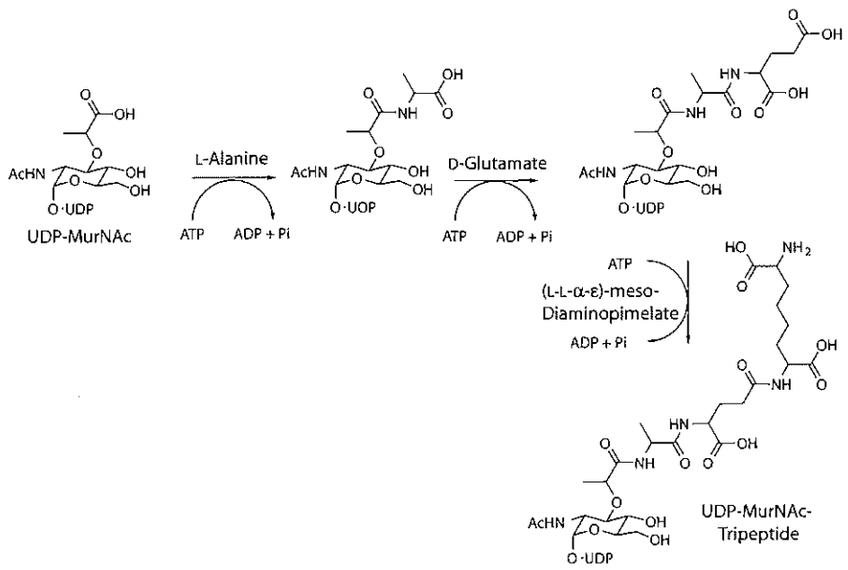


Figure 3.6 Conversion of UDP-MurNAc to UDP-MurNAc tripeptide by action of MurC, D, and E.

γ-glutamyl isopeptide bond rather than the standard peptide bond to the α-carboxylate of D-Glu. ATP is the cosubstrate cleaved by each of these three enzymes, to ADP and P_i, with the intermediacy of acyl phosphates as the donors in the amide-forming steps to each of the three amino acids (Fig. 3.7A). For example, the UDP-muramyl phosphate is the presumed mixed anhydride intermediate generated in the active site of MurC and captured by L-Ala (Fig. 3.7B). X-ray structures of several of these amino acid ligases are available (van Heijenoort, 2001b) to enable structure-based inhibitor design, and nanomolar inhibitors of both MurC (Marmor et al., 2001) and MurD have been described (Gegnas et al., 1998).

MurF completes the pentapeptidyl chain by adding the D-Ala-D-Ala dipeptide as a unit, again cleaving ATP to ADP and P_i, and presumably involving the UDP-muramyl tripeptidyl-phosphoric anhydride as intermediate (Fig. 3.7C). This concludes the classically defined cytoplasmic phase of PG assembly. Aminoalkylphosphinate inhibitors of MurF are weak (K_i values of 200 to 700 μM) but might be starting points for optimization (Miller et al., 1998) of more-potent drug candidates. Teichmann et al. (2001) have noted that the whole collection of Mur pathway enzymes may have arisen by gene self-duplication.

Enzymes that convert L-Ala to D-Ala-D-Ala: racemase and D-D-ligase

The D-Ala-D-Ala cosubstrate for MurF is in turn provided by a pair of enzymes acting sequentially: the first is alanine racemase, the second D-alanyl-D-alanine ligase (Fig. 3.8A). The racemase is a pyridoxal phosphate-dependent catalyst, taking the normal cellular metabolite L-alanine and equilibrating its configuration to make D-Ala with an equilibrium constant of 1 (Walsh, 1988). The D,D-ligase is the fifth enzyme in the Mur pathway to spend an ATP to make an amide

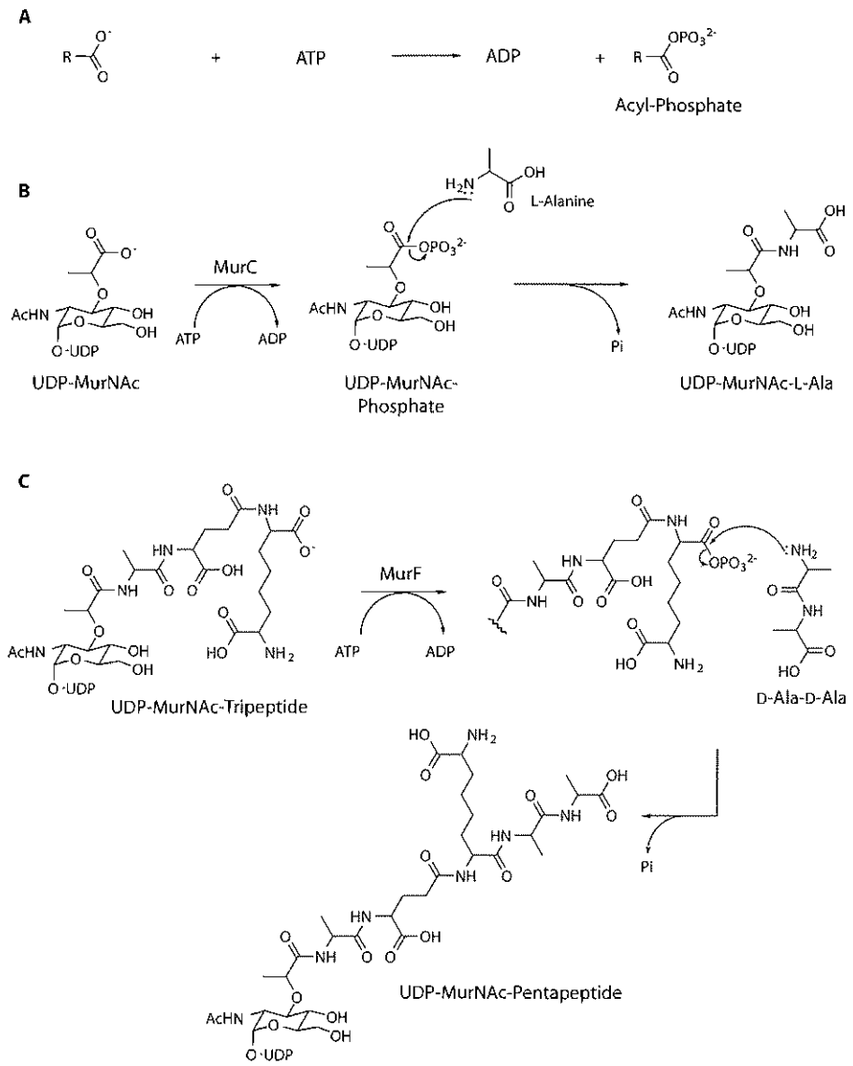


Figure 3.7 (A) Aminoacyl-phosphate generation. (B) MurC example with UDP-MurNAc-P as a bound intermediate attacked by the amino group of cosubstrate L-Ala. (C) UDP-tripeptidyl acyl-P intermediate in MurF catalysis: attack by D-Ala-D-Ala.

bond, with cleavage to ADP and an acyl phosphate, in this instance D-alanyl-PO_3, to be captured by the second D-alanine (Fig. 3.8B). X-ray structures are available for both the racemase and the ligase (Fan et al., 1994; Shaw et al., 1997). The racemase is inhibited by a phosphonate analog of L- and D-Ala, Ala-PO_3, which had promise as an antibiotic (reviewed in Bugg and Walsh, 1992; Neuhaus and Hammes, 1981), but a high frequency of mutation of the transporter systems for uptake of Ala-P rendered bacteria resistant. The D-Ala-D-Ala ligase and the racemase both are inhibited by cycloserine (Neuhaus and Hammes, 1981), a natural product, but the activity is weak and relatively unselective,

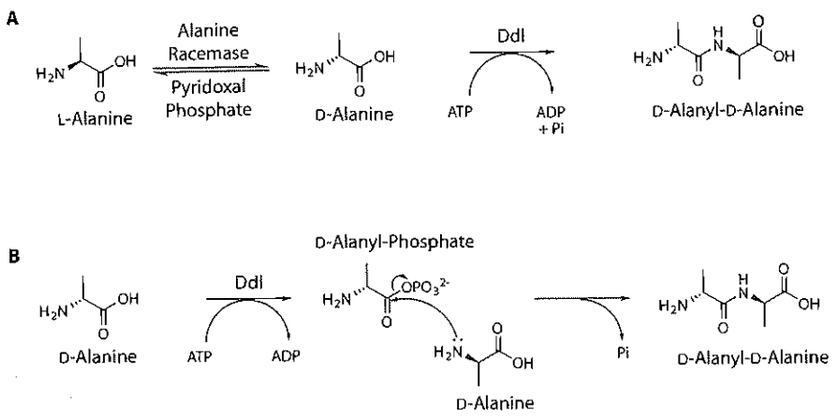


Figure 3.8 (A) Sequential action of alanine racemase and D-Ala-D-Ala ligase (Ddl) to generate D-Ala-D-Ala. (B) D-Ala-P intermediate in Ddl catalysis.

creating toxicity. These examples suggest that all eight enzymes noted above are in principle good targets for new antibiotic development. We will return to the D,D-ligase story when we discuss vancomycin resistance in chapter 10 and observe that glycopeptide antibiotic resistance centers around D,D-ligase specificity.

Enzymes that provide D-glutamate and *meso*-DAP for MurD and MurE

The pathways to D-glutamate have been reviewed recently (van Heijenoort, 2001b) and involve either a glutamate racemase encoded by the *murI* gene or a D-amino acid transaminase pathway. In bacteria using the MurI route, *murI* is an essential gene, and the racemase has been well characterized structurally and mechanistically as a cofactor-independent racemase acting by a two-base mechanism. No useful inhibitors with antibacterial activity have been described. In gram-positive bacteria such as bacilli that use D-alanine as the donor to α-ketoglutarate, the transaminase is pyridoxal phosphate (PLP) dependent; the structure is known but no antibacterial leads have been described.

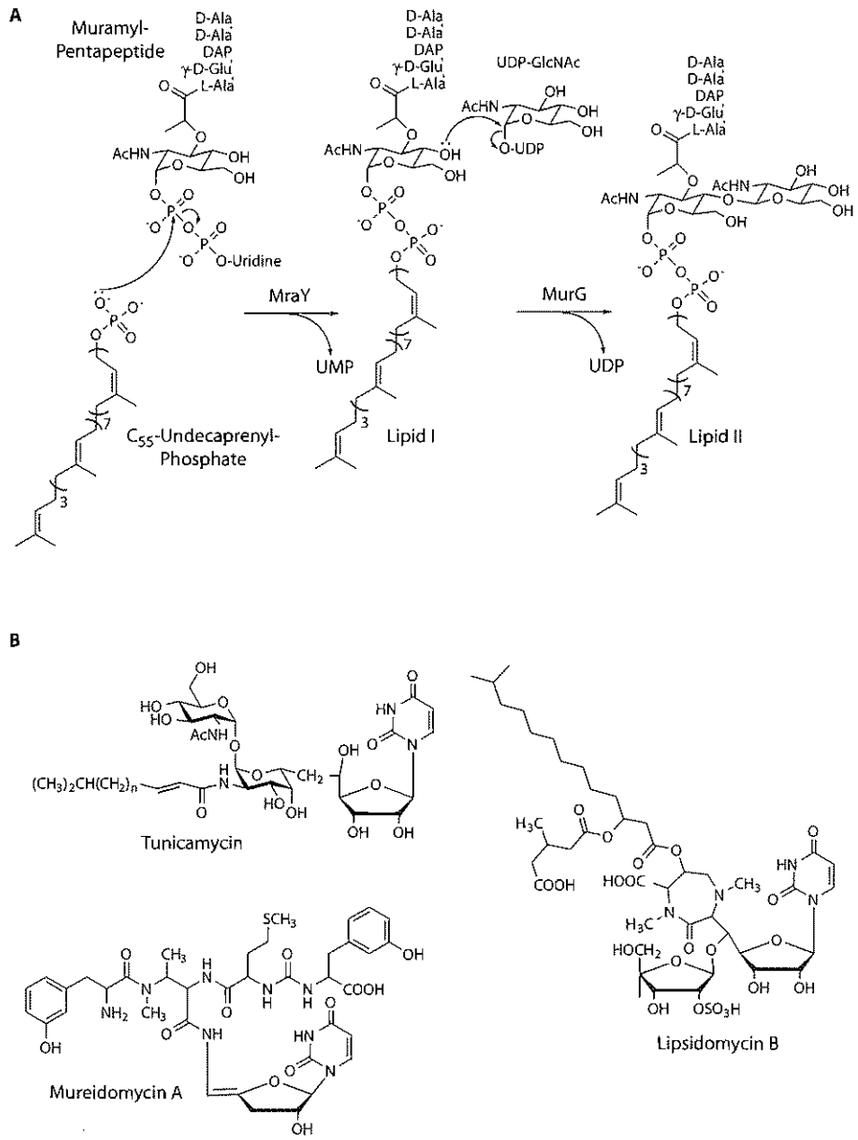
The biosynthesis of *meso*-DAP in gram-negative bacteria is a multistep pathway, well characterized mechanistically and structurally with recent X-ray structures of almost every enzyme in the pathway (Born and Blanchard, 1999), but to date no inhibitors that are effective antibacterial agents have been found by design or screening.

Lipid attachment and addition of the second sugar: the lipid I and lipid II intermediates and actions of ramoplanin and bacitracin

The membrane-associated stage two of murein assembly starts with the enzyme MraY, which transfers the muramyl pentapeptide from its UDP water-soluble anchor to an unusual membrane component, the C_{55} undecaprenyl phosphate on the cytoplasmic surface of the membrane. The C_{55} lipid phosphate oxygen

attacks the pyrophosphate linkage of the UDP moiety, releases UMP, and produces a new pyrophosphate bridge between the membrane-anchored C_{55} lipid and the muramyl pentapeptide (Fig. 3.9A). This is the first lipid intermediate, known as lipid I. MraY has also been called translocase, but there is no direct experimental evidence that the pentapeptidyl chain is translocated at this step; indeed, the subsequent conversion of lipid I to lipid II described below utilizes a cytoplasmic cosubstrate, UDP-GlcNAc, consistent with the active site of MraY and MurG being accessible to the cytoplasmic face of the membrane. Natural

Figure 3.9 (A) Enzymatic formation of the lipid I and lipid II intermediates in the membrane phase of peptidoglycan assembly. (B) Nucleoside-peptide inhibitors of MraY.



products that inhibit MraY action include mureidomycins A to F (Fig. 3.9B) (Lee and Hecker, 1999), liposidomycins, and tunicamycin, all uridyl peptide antibiotics that are thought to compete with the UDP-muramyl pentapeptide substrate for MraY. While tunicamycin also inhibits the eukaryotic biosynthesis of dolichol-PP-GlcNAc in glycoprotein biosynthesis, the liposidomycins and mureidomycins are selective for inhibition of the prokaryotic MraY (Lee and Hecker, 1999). They may offer starting points for semisynthetic antibiotic development.

At this point the second sugar is added to the C_4-OH of the muramyl group of lipid I by the enzyme MurG via UDP-GlcNAc as cosubstrate. The product is the lipid-disaccharyl pentapeptide known as lipid II (Fig. 3.9A). The MurG glycosyltransferase, associated with the membrane but not deeply buried in it, has been solubilized, purified, and crystallized (Ha et al., 2000). Because the substrates and products of MurG have been difficult to obtain in quantities, recent progress in synthesis of reasonable quantities of substrate and substrate analogs has facilitated both assay and screening for inhibitors (Ha et al., 1999; Liu et al., 2001; Men et al., 1998) and, as noted below, suggests a mechanism of action of the lipoglycopeptide antibiotic ramoplanin.

The translocation and extracellular reactions to complete PG unit synthesis and assembly

Subsequent to its formation by MurG, lipid II is translocated from the internal face of the cytoplasmic membrane to the periplasmic/external face. No definitive evidence for or identification of a translocase protein is yet available. Once facing outside and presumably anchored at the membrane surface by the C_{55} lipid tail, the disaccharyl pentapeptide unit is substrate for transglycosylases and transpeptidases that are also membrane bound (Fig. 3.3). There are multiple transglycosylases (four known in the *E. coli* genome and two in the *Staphylococcus aureus* and *Streptococcus pneumoniae* genomes) and multiple transpeptidases. Some of them are bifunctional with discrete transglycosylase and transpeptidase domains (Spratt, 1994), and members of this subset are of particular importance as killing targets of β-lactam antibiotics, as will be noted below. The transglycosylase activities cleave the muramyl-C_1-O-PO_3 bond by attack of the 4′-OH of a terminal GlcNAc moiety of an elongating glycan chain in a PG layer onto the PG unit to be incorporated (Fig. 3.3), releasing the C_{55} lipid pyrophosphate. For the C_{55} lipid carrier to cycle back to the cytoplasmic face of the membrane, the pyrophosphate linkage of the C_{55} lipid pyrophosphate must be hydrolyzed to the starting C_{55} lipid phosphate by a membrane-bound phosphatase. The C_{55} lipid phosphate can then be available for another round of lipid I synthesis, conversion to lipid II, and translocation (Fig. 3.10).

This lipid carrier cycle is susceptible at several points to inhibition by antibiotics. The cyclic lipodepsipeptide ramoplanin (Fig. 3.11A) has been shown in in vitro studies (Lo et al., 2000) to complex with both lipid I and lipid II. Sequestration of these lipo-sugar-pentapeptides away from transglycosylases could block subsequent enzymatic maturation of the PG units. The three-dimensional structure of this cyclic 17-residue nonribosomal depsipeptide has

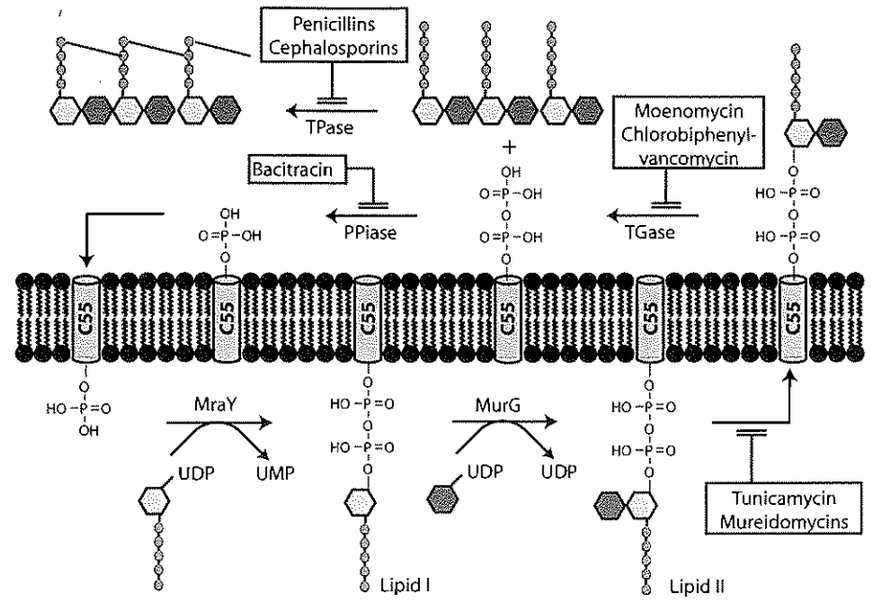


Figure 3.10 The lipid carrier cycle in peptidoglycan assembly. TGase, transglycosylase; PPiase, pyrophosphatase.

been determined by nuclear magnetic resonance (NMR) imaging (Kurz and Guba, 1996) and resembles that of the lantibiotic peptide mersacidin (Fig. 3.11B) (McCafferty et al., 1999; Prasch et al., 1997) which also complexes with lipid II, in 1:1 stoichiometry and thereby inhibits PG biosynthesis (Brotz et al., 1998). The molecular details of complexation of mersacidin with lipid II are not yet known, while progress has been made with ramoplanin (Cudic and Otvos, 2002; Cudic et al., 2002). Mersacidin and a related peptide antibiotic, actigardin (Zimmermann and Jung, 1997), are ribosomally synthesized as inactive precursor peptides, which are then posttranslationally cross-linked by four methyl lanthionine thioether bridges and finally proteolyzed to release a signal peptide (see chapter 6). The resultant peptide is globular, highly constrained, and thought to interact with the sugar-pyrophosphate and lipid moieties of lipid II. It has been reported (Breukink et al., 1999) that the lantibiotic nisin Z also complexes with lipid II as well as being a pore former. The large size (1.8 to 4.6 kDa) of lantibiotics restricts their passage across the outer membranes of gram-negative organisms; they are primarily active at killing gram-positive bacteria (Sahl and Bierbaum, 1998).

The nonribosomal decapeptide antibiotic bacitracin (Fig. 3.12) also interdicts the C_{55} lipid carrier cycle, at the stage of the C_{55} lipid phosphate (Brotz et al., 1998), with a cation-dependent complexation between the thiazoline ring at residue 2 of bacitracin and the phosphate moiety of the C_{55}-O-PO_3 likely.

In the steady state there has to be a balance between PG polymerases and PG hydrolases to allow orderly insertion of new PG units into existing walls during PG enlargement as bacteria grow and to initiate septum formation at cell division (Holtje, 1998). As will be noted below, the cell wall transpeptidases (PG

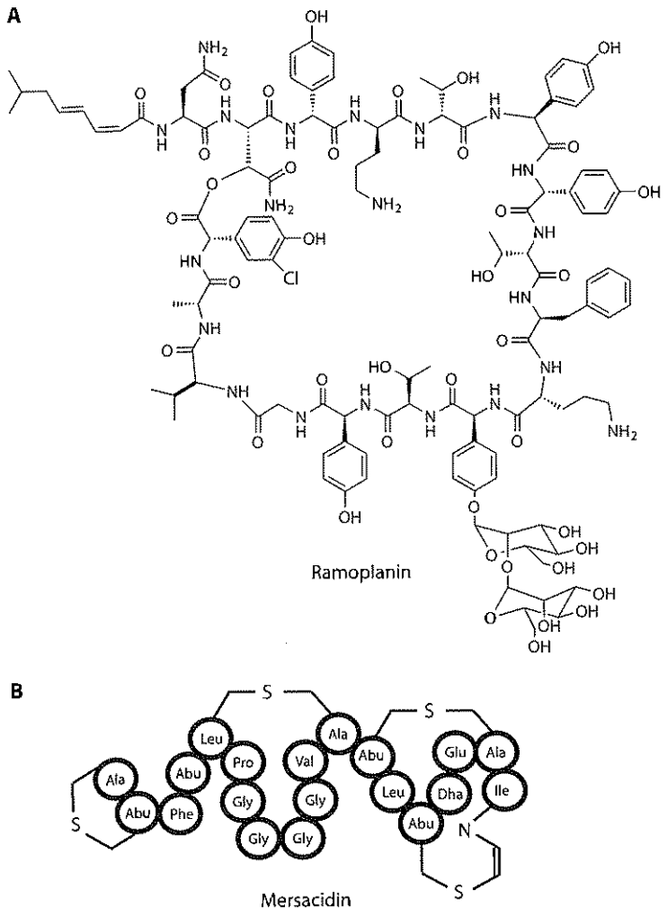


Figure 3.11 Structures of two antibiotics that form stoichiol metric complexes with lipid II: (A) ramoplanin; (B) mersacidin.

polymerases) are covalently acylated by penicillins and were identified historically as penicillin-binding proteins (PBPs). Complexes between the high-molecular-weight PBP1B, a lytic transglycosylase, MltA, and a scaffolding protein, MipA, have been described from *E. coli* and are thought to be fragments of a larger protein machine involved in the growth of the PG meshwork, also known as the sacculus (Holtje, 1998). Such a complex is proposed to contain PBP2 and PBP3 for transpeptidase and endopeptidase action in PG chain growth, with attachments to both the outer membrane (MltA) and the inner membrane (PBP1B) (Fig. 3.13).

β-Lactam antibiotics; penems, cephems, carbapenems, and the plethora of PBPs

The most celebrated of the antibiotics that kill bacteria by blocking the crucial transpeptidations that lead to mechanically strong PG through the covalent

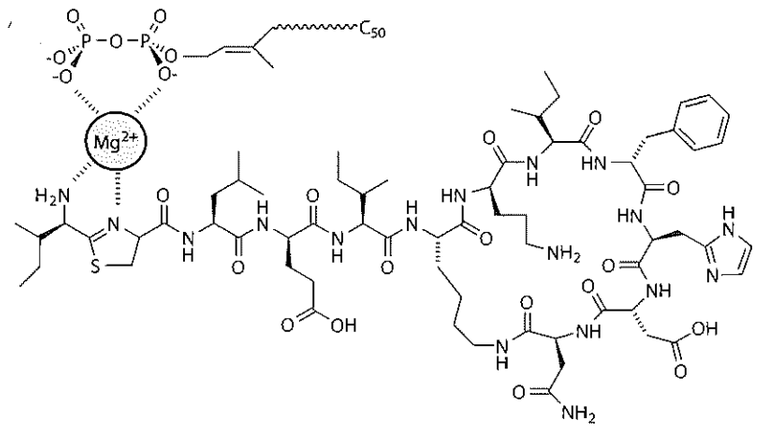


Figure 3.12 Bacitracin and a model for complexation of the C_{55} lipid phosphate to block the lipid cycle.

cross-links of peptide strands are the β-lactam antibiotics (Fig. 3.14). These include the penicillins, where the chemical warhead, the four-membered β-lactam ring, is fused to a five-membered sulfur ring system, and the cephalosporins, where the β-lactam is fused to a sulfur-containing ring-expanded system. Penicillins are converted enzymatically to the cephalosporins by a ring expandase enzyme, as we shall note in chapter 13. Both are fungal secondary metabolites, with *Penicillium chrysogenum* an important producer organism for the penicillin two-ring system and *Acremonium chrysogenum* for the cephalosporin nucleus (O'Sullivan and Ball, 1983). The antibacterial drug imipenem is a slight variant of a naturally occurring carbapenem, thienamycin (Fig. 3.14), and is administered along with an inhibitor of a renal dipeptidase, cilastatin, to enhance the carbapenem in vivo lifetime by blockade of β-lactam hydrolysis. Thienamycin was first isolated from the bacterium *Streptomyces cattleya* (Kahan et al., 1979), and the epimeric side chain alcohol olivanic acid has been isolated from *S. flavogriseus*. The simplest carbapenem is the unsubstituted bis ring system with the 2,3-double bond, produced by the plant-pathogenic *Erwinia* bacteria (see chapter 11) (Bycroft et al., 1988). Two other variants of β-lactam natural products are known: the monobactams, represented by nocardicins and the synthetic aztreonam, and the clavams, represented by clavulanate, not in

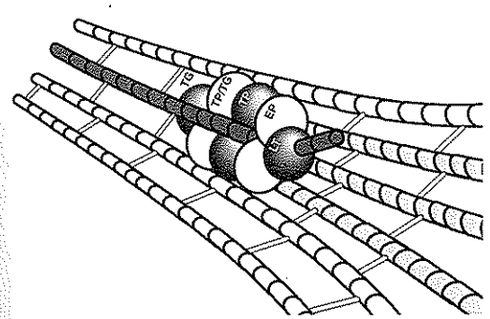


Figure 3.13 Schematic of a multienzyme complex involved in traveling along the peptidoglycan scaffold during elongation. TG, transglycosylase; TP, transpeptidase; TP/TG, bifunctional transpeptidase/transglycosylase; EP, endopeptidase; LT, lytic transglycosylase. (Adapted from Holtje [1998], with permission.)

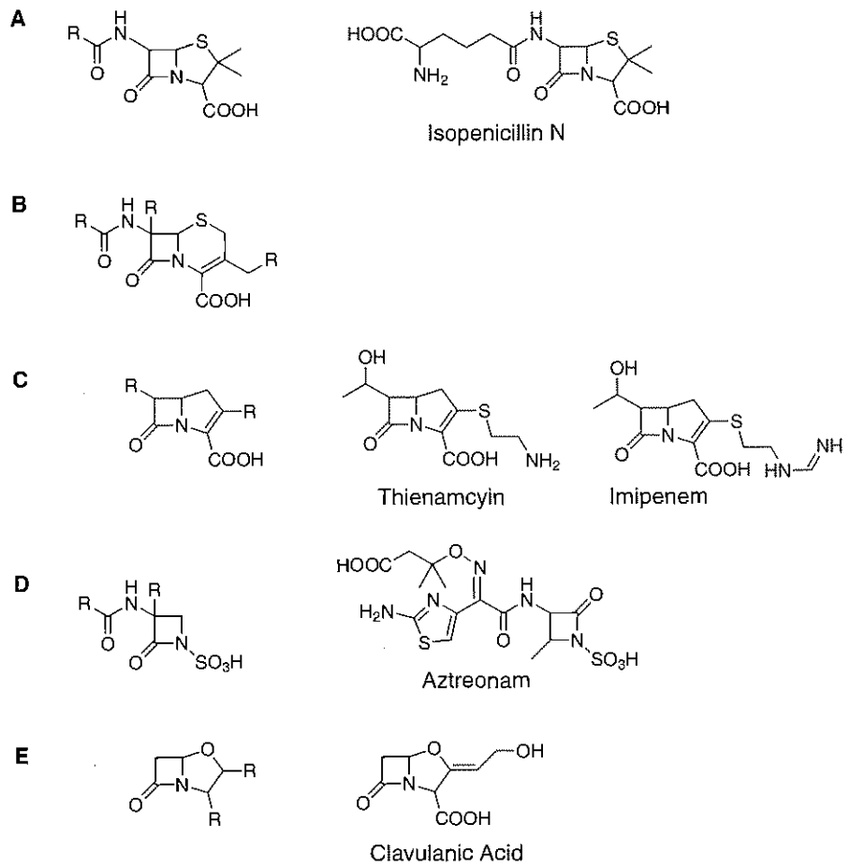


Figure 3.14 β-Lactam antibiotics: (A) penicillins, (B) cephalosporins, (C) carbapenems, (D) monobactams, and (E) clavams.

itself an antibiotic but a mechanism-based inactivator of β-lactamases (discussed in chapter 8).

To understand how penicillins inactivate the PG-cross-linking transpeptidases requires a brief analysis of the catalytic mechanism followed by all the transpeptidase isoforms. As the name of this enzyme family implies, the cross-linking steps are transpeptidations with no net peptide bonds being formed. One Lys-D-Ala or DAP-D-Ala isopeptide bond is formed and one D-Ala-D-Ala peptide bond is cleaved in each catalytic cycle, releasing free D-Ala (Fig. 3.15), which is recycled to the cytoplasm or oxidized at the cytoplasmic membrane. There is no requirement for energy input, in accord with the fact that these enzymes work outside the cell on the periplasmic face of the membrane where ATP and other energy sources are not routinely available. The transpeptidases are all variants of active-site "serine" hydrolases, with an active-site serine as nucleophile and another side chain that functions as a general base (Bush and Mobashery, 1998). The first half-reaction involves attack of the active-site serine on the amide bond joining the D-Ala$_4$ to D-Ala$_5$. The tetrahedral adduct collapses to an acyl-*O*-Ser

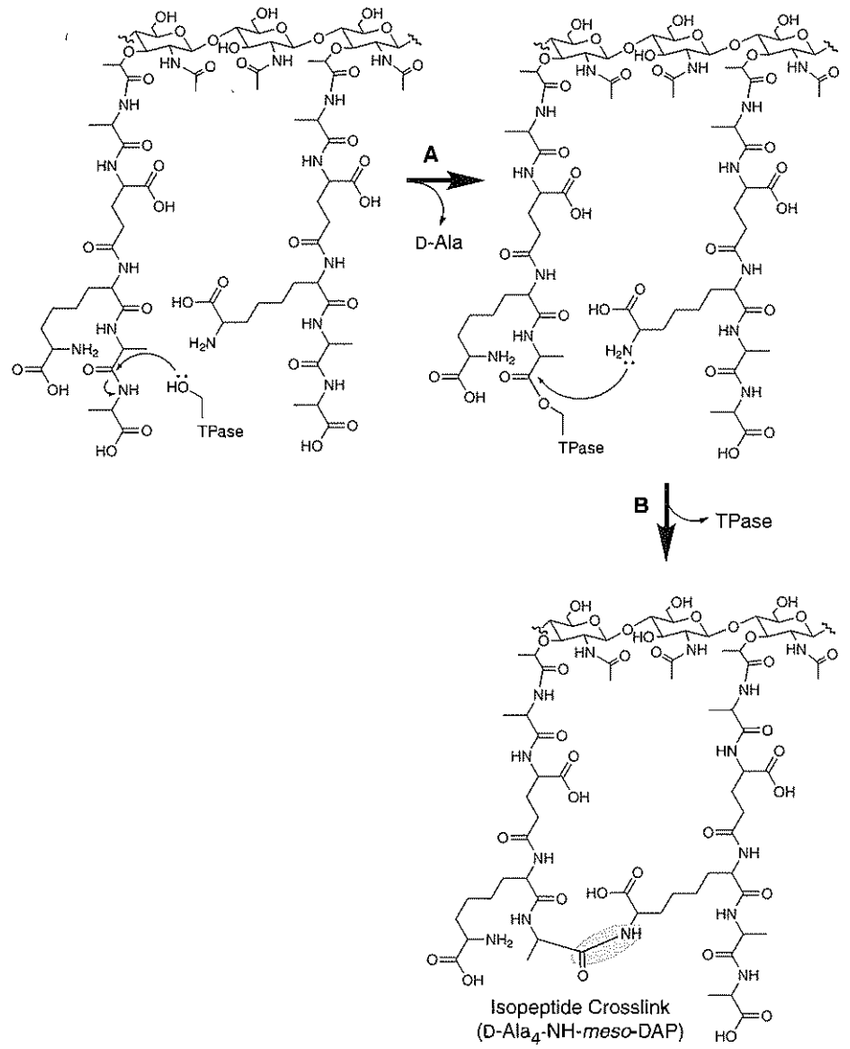


Figure 3.15 Mechanism of the PG transpeptidation reaction to create the DAP-D-Ala isopeptide bond: acyl enzyme intermediate in transpeptidase action. (A) Acyl enzyme formation; (B) acyl enzyme deacylation and capture by the amine nucleophile of a neighboring chain.

enzyme with release of D-Ala$_5$ as the free amino acid. The acyl-*O*-transpeptidase intermediate has the glycan-tetrapeptidyl moiety as the transiently tethered acyl group (Fig. 3.15A). In most "serine" enzyme family members, a water molecule is productively bound in the active site and acyl transfer to water ensues, with regeneration of the starting form of the enzyme for another catalytic cycle. This is the fate in the PBP forms that act as D-,D-carboxypeptidases. But in these transpeptidases, water is excluded and the only kinetically competent nucleophile is the amine group of C_6 of DAP$_3$ or Lys$_3$ of an adjacent PG chain. Acyl transfer to this nucleophile in the second half-reaction (Fig. 3.15B) completes the catalytic

cycle, regenerating free enzyme as the isopeptide bond introduces a meshwork-strengthening cross-link. The X-ray structures for several transpeptidase catalytic domains have been solved (Knox, 1995; Knox et al., 1996; Pares et al., 1996) and support this mechanistic view. In some gram-positive bacteria such as *S. aureus*, the cross-link between peptide chains does not occur directly via the ε-NH_2 of a Lys or DAP side chain but involves peptide cross-bridges. Such a pentaglycine bridge (Gly_5) is built up on the Lys before cross-linking occurs in *S. aureus*. The transpeptidation is then between the NH_2 group of Gly_5 and the D-Ala_4 carbonyl on an adjacent peptide chain.

The transpeptidases commit suicide when they start a catalytic cycle with β-lactam antibiotics as substrates, mistaking them for a yet to be cross-linked PG chain terminating in D-Ala-D-Ala. The active-site serine adds into the strained four-ring lactam carbonyl (**Fig. 3.16**) and generates an acyl enzyme intermediate in which the β-lactam ring has opened. Now the enzyme gets stuck in mid-catalytic cycle. These transpeptidases are designed to exclude water from intercepting the normal acyl enzyme intermediates and, analogously, the penicilloyl enzyme forms are very slow to hydrolyze (half-lives of many hours to days). The enzyme piles up as the covalent penicilloyl enzyme and is effectively dead until slow hydrolysis allows it to recover. As we shall note in chapter 8, the long lifetimes of these structurally variant acyl enzyme intermediates account for β-lactam killing of bacteria.

With radioactive penicillins or cephalosporins it is easy to demonstrate long-lived covalently labeled acyl transpeptidases by sodium dodecyl sulfate gel electrophoresis, which resolves denatured proteins by size. Bacteria typically show multiple radiolabeled protein bands (**Fig. 3.17**), of which there are four in *S. pneumoniae* and up to eight in *E. coli* (Denome et al., 1999; Spratt, 1977). This approach gave the first historical evidence of multiple transpeptidases and established the full catalytic inventory of these enzyme families. It was then possible to identify each labeled PBP and prove their enzymatic activity after the penicilloyl enzymes finally hydrolyzed. Low-molecular-weight PBPs tend to be *N*-acyl-D-Ala_4-D-Ala_5 carboxypeptidases, hydrolases that generate un-cross-linkable tetrapeptide stems, while the high-molecular-weight PBPs (PBP1A, B, and C) are bifunctional transglycosylases/transpeptidases (Holtje, 1998; Schiffer and Holtje, 1999). Mutational analysis could determine which PBPs represented major targets to particular antibiotics and pinpoint their physiologic roles in PG maturation and assembly (for a recent example in *S. pneumoniae*, see Paik et al., 1999). Different lactam antibiotics can induce characteristic changes in phenotype, such as long chains or rounded appearances, reflecting preferential blockade of a subset of PBPs (Greenwood, 2000). Mutations to penicillin resis-

Figure 3.16 Reaction of penicillin as a suicide substrate for PG transpeptidases.

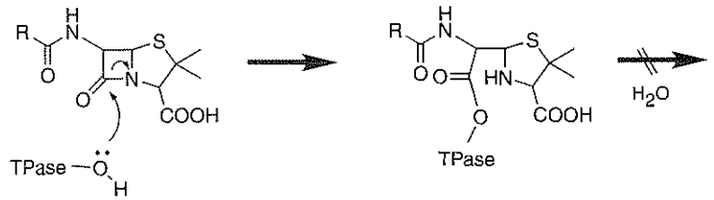


Figure 3.17 Multiple penicillin-binding proteins in *E. coli*: autoradiographs of ^{14}C-penicilloyl-proteins of *E. coli* separated on denaturing gel electrophoresis. (From Dougherty et al. [1996], with permission.)

tance could also be correlated with decreased affinity of a given PBP to a particular β-lactam antibiotic, and this helped further define the physiologic role of PBPs.

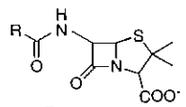
In *E. coli* both the bifunctional transpeptidase/transglycosylase PBP1A and 1B forms, when inhibited, e.g., by acylation with the cephalosporin cephaloridine, lead to spheroplast formation and rapid lysis. They are clearly prime killing targets for β-lactam antibiotics. Selective inhibition of PBP2 by acylation with low concentrations of the penicillin mecillinam produces spherical forms, relative osmotic stability, and slow lysis. Penicillin G at low concentrations acylates PBP3 first, generating chains of bacteria in filaments. It is not clear if PBP3 has much significance as a target for lactam antibiotics. At higher levels of penicillin G, PBP1 isoforms are acylated and lysis occurs. A quantitative estimate from radiolabeled penicillin binding yields about 2,500 PBP molecules per *E. coli* cell (Dougherty et al., 1996), with about 220 PBP1A and 125 PBP1B molecules as major killing targets and about 1,500 (two-thirds of the total) low-molecular-weight PBP4-7, which are not killing targets (Scholar and Pratt, 2000).

How the acylation and inhibition of PBPs by β-lactam antibiotics is translated into cell wall degradation and cell death by inappropriate or excess murein hydrolase activity has been under study for decades (see Bayles, 2000). One current hypothesis is that the hydrolases are normally constrained in their access to the PG substrates and that antibiotic encourages oligomerization of certain proteins, forming channels in the cytoplasmic membrane that allow passage of the PG hydrolases to reach their substrate. In bacteriophage λ infections of *E. coli* there are such channel proteins, holins and antiholins (Young et al., 2000), that control the timing of access of bacteriophage-encoded murein hydrolases to the PG. These may serve as a precedent for the action of related holin-antiholin systems in organisms such as *S. aureus* (Bayles, 2000).

Side chain modifications in penicillins

The natural side chain in the initial β-lactam antibiotic natural product after biosynthetic cyclization (chapter 13) is L-aminoadipoyl (Fig. 3.13). This is then

A



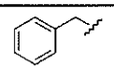
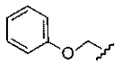
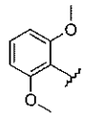
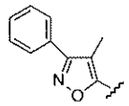
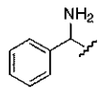
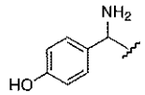
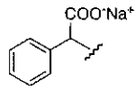
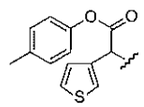
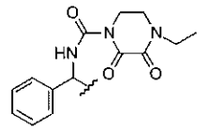
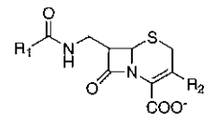
Category		R	Property
1. Narrow Spectrum Penicillinase-Sensitive	PenG		Poor acid stability
	PenV		Good acid stability
2. Narrow Spectrum Penicillin Resistant	Methicillin		Penicillinase resistant due to bulky side chains
	Oxacillin		
3. Broad Spectrum Aminopenicillins	Ampicillin		Orally active, penicillinase sensitive; active against *H.influenzae*, *E.coli*
	Amoxicillin		
4. Broad Spectrum Antipseudomonal	Carbenicillin		I.V. administration, active against *P.aeruginosa*
	Ticarcillin		
5. Extended Spectrum	Piperacillin		Active against *P.aeruginosa*. Increased activity against *Enterobacteriaceae*

Figure 3.18 Different generations of (A) penicillins and (B) cephalosporins. (Adapted from Scholar and Pratt [2000], with permission.)

epimerized to the D-form and the ring-expanding dioxygenase generates cephalosporins with an olefin in the six ring and the same D-adipoyl side chain, followed by hydroxylation and acetylation at C_3 to yield cephalosporin C (O'Sullivan and Ball, 1983). As medicinal chemists tried to broaden the spectrum of antibacterial activity of the original β-lactams and to gain potency and combat developing resistance (discussed in chapter 8), they made many side chain variants by semisynthetic modifications, using the deacylated 6-aminopenicillanic acid or 7-aminocephem for reacylation with a variety of different kinds of side chains, screening for gain or optimization of the desired activity.

B



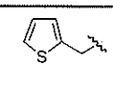
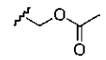
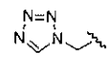
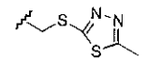
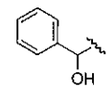
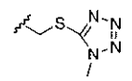
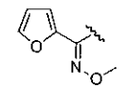
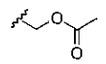
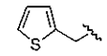
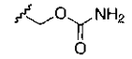
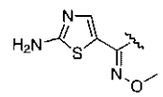
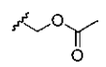
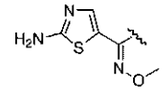
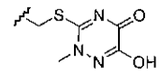
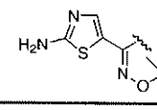
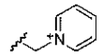
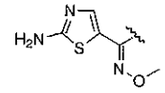
Category		R1	R2
1. First Generation	Cephalothin		
	Cephazolin		
2. Second Generation	Cefamandole		
	Cefuroxime		
	Cefoxitin		
3. Third Generation	Cefotaxime		
	Ceftriaxone		
	Ceftazidime		
4. Fourth Generation	Cefipime		

Figure 3.18 *Continued.*

This has led to multiple waves of semisynthetic β-lactams over the 50 years of their clinical use. Scholar and Pratt (2000) note five categories of penicillins (**Fig. 3.18A**) based on narrow- versus broad-spectrum activities and whether there is antipseudomonal activity. The early synthetic side chains, phenylacetyl in penicillin G and phenoxyacetyl in penicillin V, gave narrow-spectrum drugs, active for example against streptococci and neisseria, and were β-lactamase sensitive. Replacement of the unsubstituted aryl groups with the 2,6-dimethoxy substituents in methicillin, the napthyl moiety in nafcillin, and the phenyloxazolyl group in oxacillin created misalignment in β-lactamase active sites and consequent resistance to hydrolysis. For example, with the β-lactamase from *S.*

aureus the K_m values for hydrolysis for penicillin G and penicillin V were in the range of 2 to 4 μM, while the dimethoxy groups in methicillin raised the K_m value by 10^4 to 28,000 μM (Novick, 1962), making methicillin useful against staphylococcal infections. A broader spectrum of activity resulted when the phenylacetyl side chain was converted to the phenylglycyl chain by introduction of an amino group (ampicillin) or by the *p*-OH-phenylglycyl of amoxicillin, generating orally active penicillins with good bioavailability. These aminopenicillins are active against such gram-negative bacteria as *E. coli* and *Haemophilus influenzae*. To obtain antipseudomonal activity, requiring increased penetration through the restrictive porins of the pseudomonal outer membranes, further side chain modifications led to the development of such drugs as ticarcillin, a carboxyl ester derivative of a thiazolyl side chain, which is active by intramuscular and intravenous routes for hospital uses. Finally, for *P. aeruginosa* bacteremias in nosocomial (in-hospital) settings, the ureido derivative of ampicillin with a piperazino group, piperacillin, is an extended-spectrum intravenous penicillin.

Side chain modifications in cephalosporins: multiple generations

Cephalosporins are the most widely prescribed and largest-selling class of the β-lactam antibiotics. Side chain modifications have led to differential penetration through the porins in cell envelope structures and provide varied antibacterial and pharmacokinetic properties (see Scholar and Pratt, 2000). **Figure 3.18B** lists examples of first- to fourth-generation cephalosporins. Narrow-spectrum ("first-generation") examples include intravenous and oral drugs, with cephalothin a prototype. The narrow-spectrum drugs have the best activity against gram-positive pathogens, except methicillin-resistant *S. aureus* (MRSA), and are active against some gram-negative organisms, such as *E. coli* and klebsiella strains. The expanded-spectrum ("second-generation") cephalosporins, represented by intravenous drugs cefoxitin and cefamandole and oral drugs such as cefaclor and loracarbacef, are somewhat less effective against gram-positive but have a broader spectrum against gram-negative pathogens including *Bacteroides fragilis* and *H. influenzae*. The increased gram-negative activity stems from a combination of better penetration, increased affinity for binding to PBP targets, and lowered catalytic efficiency toward hydrolysis by β-lactamases. The side chains in both narrow- and expanded-spectrum cephalosporins built on the experience with penicillins, including thiazolyl and phenylglycyl side chains. The side chains in expanded- and broad-spectrum ("second-" and "third-generation") cephalosporins typically provide a couple of logs of resistance to β-lactamases. Also varied is the 3′ substituent on the hydroxyl of the six ring. In the broad-spectrum cephalosporins, gram-negative activity has been optimized and extended to cover *P. aeruginosa*, while retaining sufficient activity against gram-positive bacteria (e.g., methicillin-sensitive *S. aureus*), except for ceftazidime (Scholar and Pratt, 2000), such that they are useful for surgical prophylaxis. The "fourth-generation" cephalosporin molecule, cefepime, approved for use in the United States, has properties akin to the broad-spectrum cephems, but gains increased resistance to many β-lactamases. The favored acyl side chains on the β-lactam in the third and fourth generation of cephalosporins are aminothiazole oximes, some of which have charged carboxylates (e.g., ceftazidime, cefixime), which allow good penetration through the porins of gram-negative outer membranes while retaining high affinities against PBP targets. The 3′ substituents differ more widely, some with positively charged amines, which also affect intrinsic antibacterial activity and pharmacokinetics and distribution. For example, many of them penetrate well into the cerebrospinal fluid when meninges are inflamed (Scholar and Pratt, 2000) and are effective for treating meningitis.

Overall, the semisynthetic manipulation of cephalosporin side chains shows the ability for optimization against different subsets of pathogens, accounting for the dominant role in many infections where β-lactam antibiotics are prescribed. The cephalosporins also have excellent safety profiles, leading to broad use in hospitals in both preoperative and postoperative contexts. On the other hand, the success of cephalosporins may in the end have selected for bacteria with resistance determinants (see chapter 17).

Carbapenems and monobactams

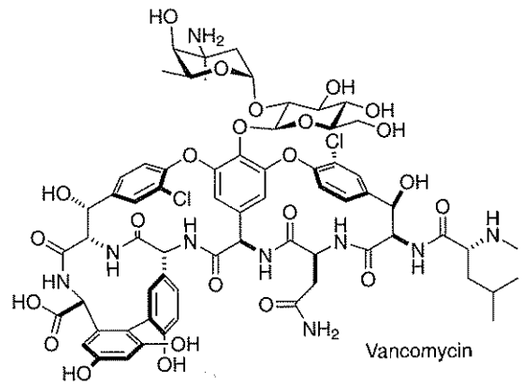
Two carbapenems, imipenem and meropenem (Fig. 3.14), are approved for clinical use in the United States, with a third, ertapenem (MK-0826), in clinical development with the intent of once-daily dosage (see Fuchs et al., 2001). Imipenem and meropenem are water soluble, have low oral bioavailability, and are utilized in hospital settings against antibiotic-resistant infectious organisms, where they show broad-spectrum activity (see Table 4-9 in Scholar and Pratt, 2000). They tend to be resistant to most serine-based β-lactamases but are sensitive to hydrolysis by metallo-β-lactamases, as examined in chapter 8. Although imipenem is resistant to bacterial enzyme β-lactam ring-mediated hydrolysis, in vertebrates dehydropeptidase I in renal epithelial cells hydrolyzes the lactam. Cilastatin, a dehydropeptide mimic that inhibits the renal hydrolase, is thus given with the carbapenem. Meropenem with a C-methyl substituent is not susceptible to the renal enzyme. Ertapenem has a prolonged half-life compared to the earlier carbapenems, probably due to serum protein binding, and is proposed for once-a-day dosing. Both carbapenems have useful antipseudomonal activity (Livermore and Woodford, 2000).

One monobactam antibiotic, aztreonam (Fig. 3.14), is in human clinical use. The acyl side chain is the same as in ceftazidine, while the lactam has an N-sulfonate substituent on the other side. The spectrum of antibacterial activity (Scholar and Pratt, 2000) is such that it is useful only against gram-negative pathogens, with good activity against *P. aeruginosa*. It appears to target PBP3 at low concentrations and has low susceptibility to the lactamases of the gram-negative target pathogens.

The clavam scaffold is found in clavulanate (Fig. 3.13) (see also chapter 13). Clavulanate on its own is a poor substrate for PBP and so is not considered an antibiotic. Its utility derives from its "suicide substrate" properties with β-lactamases, discussed further in chapter 8.

Glycopeptide antibiotics act by complexing the un-cross-linked peptide strands and blocking transpeptidation

Two glycopeptide antibiotics in the vancomycin family have been approved for human clinical use, vancomycin itself (**Fig. 3.19**) and, outside of the United States, teicoplanin.



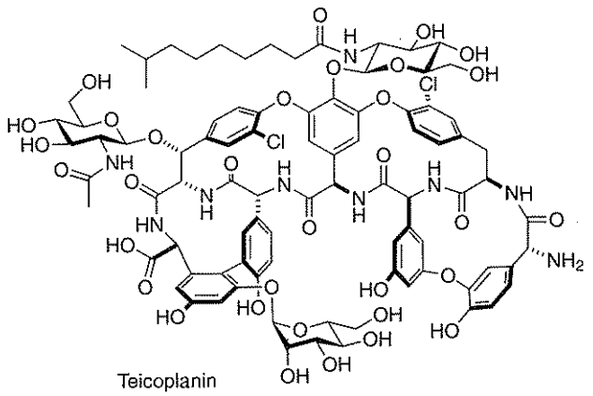
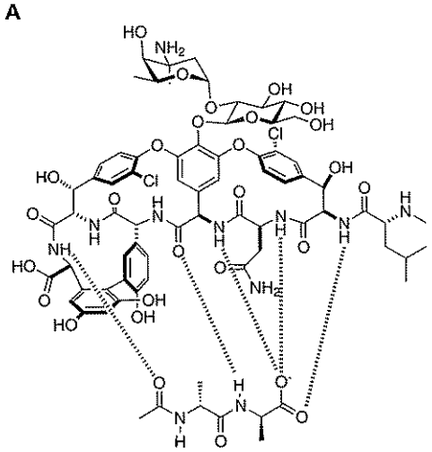


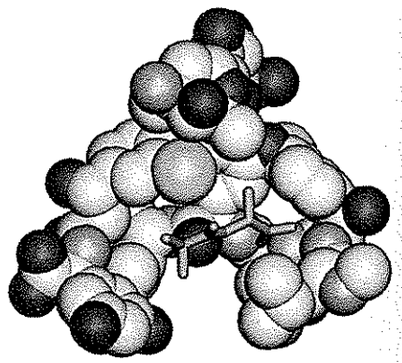
Figure 3.19 Structures of the glycopeptide antibiotics vancomycin and teicoplanin.

Figure 3.20 Sequestration of PG-D-Ala-D-Ala termini by vancomycin. (A) Five hydrogen bonds between the antibiotic and PG terminus; (B) space-filling model of the antibiotic and PG terminus.

A



B

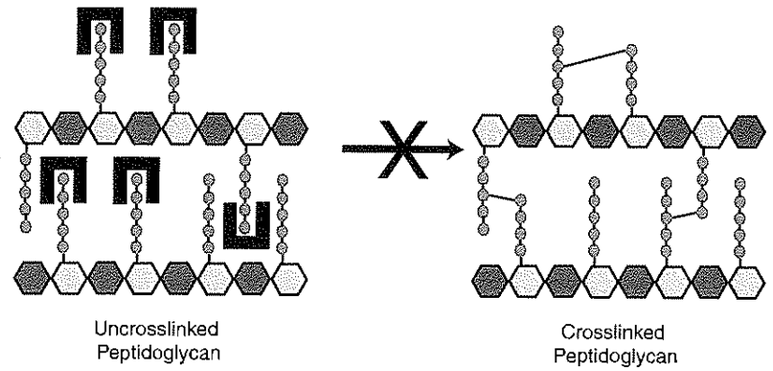


Teicoplanin differs in three ways from vancomycin: (i) the glycosylation number and placement is distinct; (ii) teicoplanin has a long-chain fatty acid substituent in amide linkage to the GlcNAc sugar attached to $PheGly_4$; and (iii) the cross-linked heptapeptide scaffold is different at residues 1 and 3, allowing four side chain cross-links (1-3, 2-4, 4-6, 5-7) in contrast to the three in vancomycin (see Hubbard and Walsh, 2002; Williams and Bardsley, 1999; and references therein). As noted in chapter 2, vancomycin and teicoplanin cannot penetrate the pores of the gram-negative outer membranes so are restricted to treating infections of life-threatening gram-positive pathogens such as staphylococcal, streptococcal, and enterococcal infections.

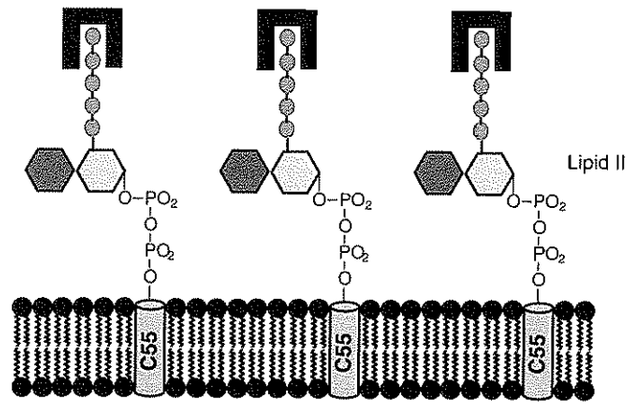
Both these antibiotics act not by inhibiting the transglycosylases or transpeptidases per se, but rather by complexation of the substrate PG units that have

Figure 3.21 PG termini interacting with vancomycin and teicoplanin: (A) un-cross-linked strands on preexisting PG; (B) the lipid II substrate before polymerization into PG.

A



B



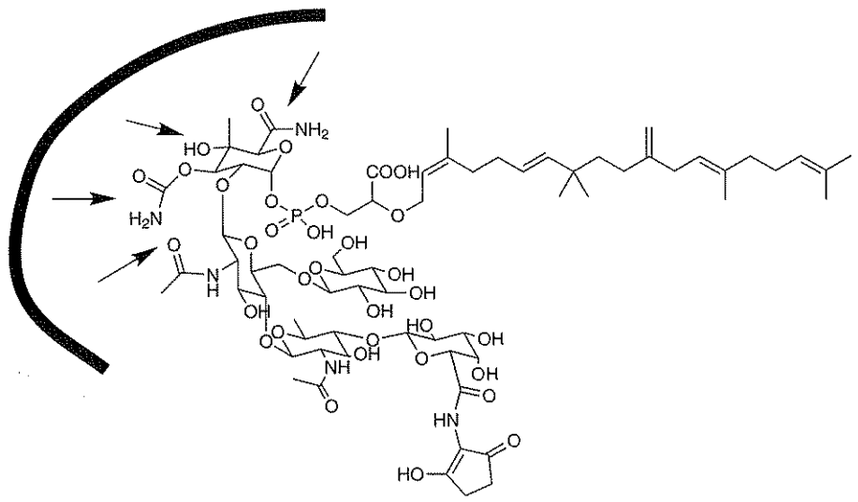


Figure 3.22 Model for moenomycin interaction with target transglycosylases. (From Kurz et al. [1998], with permission.)

the pentapeptidyl tails terminating in D-Ala$_4$-D-Ala$_5$. This substrate sequestration effectively shuts down transpeptidation by making the N-acyl-D-Ala-D-Ala acceptor unavailable to the transpeptidases (Fig. 3.20). In this sense the sequestration of substrate is analogous to the sequestration proposed for lipid II by ramoplanin (also a lipoglycopeptide antibiotic) (Lo et al., 2000). The complex has been characterized first by NMR and then by X-ray (see Williams and Bardsley, 1999) to involve molecular recognition of a not yet cross-linked N-acyl-D-Ala-D-Ala terminus of a PG-pentapeptidyl strand by the underside of the rigid, cup-shaped vancomycin via a series of five hydrogen bonds (see Walsh et al., 1996b; Williams and Bardsley, 1999). The space-filling model shows the optimized tightness of fit of antibiotic for its target. There are two kinds of PG units that have the intact pentapeptide strand, the lipid II molecules at the periplasmic face of the membrane, and also strands as yet un-cross-linked in polymerized PG (Fig. 3.21). The steric blockade of the transpeptidation can also have effects on transglycosylases, especially in the bifunctional, high-molecular-weight PBPs. Different members of the vancomycin glycopeptide antibiotic family have different tendencies to dimerize, and this may enable an enhanced avidity for complexation with PG termini (Williams, 1996). We will return to these mechanisms in chapter 10 with a discussion of the molecular mechanisms of glycopeptide antibiotic resistance in one type of opportunistic human pathogens, vancomycin-resistant enterococci (VRE). Given that vancomycins and penicillins work on two different aspects of the PG cross-linking, one might expect to, and does, observe synergy of antibacterial effects in combination.

Moenomycin as an inhibitor of the transglycosylase activity of PBP1B

In contrast to the many β-lactam antibiotics that inhibit the transpeptidase activity of the bifunctional transglycosylase/transpeptidase activities of the high-

molecular-weight PBPs, there are very few natural product antibiotics that target the transglycosylase active site. Moenomycin A (Kurz et al., 1998) is one such compound (Fig. 3.22), used as a growth promoter in animal feed (Ritter and Wong, 2001).

Moenomycin has a 25-carbon lipid alcohol, moecinol, linked via a phosphoglycerate to a pentasaccharide tail in phosphodiester linkage. NMR analysis has provided a model for the three-dimensional structure with the proposal that the E and F rings of the carbohydrate moiety interact, as a substrate analog, with the target transglycosylase to shut down addition of the disaccharyl pentapeptide units in PG layer growth. The moecinol tail is probably a membrane anchor that preconcentrates the antibiotic at the external side of the cytoplasmic membrane where PBP1 enzyme molecules are located. Libraries of constituent disaccharides have been made, but so far without retention of useful activity (see Ritter and Wong, 2001, for review).