Chapter two

Vulnerable shields—the cell walls of bacteria and fungi

2.1 Functions of the cell wall

In the search for differences between microbial pathogens and animal cells that could provide the basis for selective antimicrobial attack, one evident distinction lies in their general structure. The animal cell is relatively large and has a complex organization; its biochemical processes are compartmentalized and different functions are served by the nucleus with its surrounding membrane, by the mitochondria and by various other organelles. The cytoplasmic membrane is thin and lacks rigidity. The cell exists in an environment controlled in temperature and osmolariy in mammals and birds. It is constantly supplied with nutrients from the extracellular fluid. Bacteria and fungi live in variable and often hostile environments and they must be able to withstand considerable changes in external osmolarity. Some micro-organisms have relatively high concentrations of low molecular weight solutes in their cytoplasm. Such cells suspended in water or in dilute solutions develop a high internal osmotic pressure. This would inevitably disrupt the cytoplasmic membrane unless it were provided with a tough, elastic outer coat. This coat is the cell wall, a characteristic of bacteria and fungi which is entirely lacking in animal cells. It has a protective function but at the same time it is vulnerable to attack, and a number of antibacterial and antifungal drugs owe their action to their ability to disturb the processes by which the walls are synthesized. Since there is no parallel biosynthetic mechanism in animal cells, substances affecting this process may be highly selective in their antimicrobial action.

The term 'wall' will be used to describe all the cell covering which lies outside the cytoplasmic membrane. The structures of the walls of bacteria and fungi are very different from each other, as are the biosynthetic processes involved in their elaboration. This results in susceptibility to quite distinct antimicrobial agents.

2.2 Structure of the bacterial wall

The structure of the bacterial wall not only differs markedly from that of fungi but also varies considerably from one species to another. It nevertheless follows general patterns which are related to the broad morphological classification of bacteria. Classically this has been based on the responses towards the Gram stain, but the well-tried division into Gram-positive and Gram-negative types has a significance far beyond that of an empirical staining reaction. The most evident differences are worth recalling.

Many Gram-negative bacteria are highly adaptable organisms which can use inorganic nitrogen

compounds, mineral salts and a simple carbon source for the synthesis of their whole structure. Their cytoplasm has a relatively low osmolarity. Typical Gram-positive cocci or bacilli tend to be more exacting in their nutritional needs. They are usually cultivated on rich undefined broths or on fairly elaborate synthetic media. In their cytoplasm, Gram-positive bacteria concentrate amino acids, nucleotides and other metabolites of low molecular weight and consequently have a high internal osmolarity. However, not all bacteria fit this neat division. The Gram-negative cocci, the rickettsias, the chlamydias and the spirochetes, for example, are all Gram-negative bacteria with exacting growth requirements. The mycoplasmas lack a rigid wall structure and although technically Gram-negative, they are best treated as a separate group lying outside the usual Gram stain classification.

For many years the bacterial wall was considered to be a rigid structure, largely because when bacteria are disrupted, the isolated walls retain the shape of the intact organisms. More recent evidence, however, shows that this concept of rigidity must be revised. The peptidoglycan sacculus (see later discussion) of the bacterial wall can expand or contract in response to changes in the ionic strength or the pH of the external environment. This responsive flexibility is a property of the wall itself and can even be seen by the unaided eye when salt solutions are added to quantities of walls pelleted by centrifugation. When intact bacteria are subjected to osmotic stress, water moves through the wall and membrane into the cytoplasm. The consequent swelling of the cell, bounded by the membrane, is accommodated to some extent by the limited elasticity of the wall, although even stretchable structures break when sufficiently stressed. The wall breaks and the cell then bursts as a result of the turgor pressure on the thin cytoplasmic membrane. It is important to realize that during cell growth and proliferation, bacterial cell walls are highly dynamic structures, continually undergoing biosynthesis, extension and remodeling. It is this dynamic character which renders bacteria susceptible to antibiotics which attack the biosynthesis and the integrity of cell walls.

Most of the work on wall structure has been done with Gram-positive cocci and bacilli and with enteric bacteria and other Gram-negative rods. The extent to which the structural generalizations apply to groups outside these classes is uncertain.

2.2.1 The Gram-positive wall

The basic structure of the cell walls of Gram-positive bacteria is relatively simple (Figure 2.1), although there are many differences of detail across the species. The wall which lies outside the cytoplasmic membrane is usually beween 15 and 50 nm thick. Bacteria can be broken by shaking with small glass beads and the walls separated from cytoplasmic material by washing and differential centrifugation. In electron micrographs these wall preparations resemble empty envelopes torn in places where the cytoplasmic contents were released. The major part of the Gram-positive wall is a large polymer consisting of two covalently linked components. One of these components, forming at least 50% of the wall mass, is peptidoglycan (sometimes referred to as murein or mucopeptide). Its cross-linked structure provides a tough, fibrous fabric that gives strength and shape to the cell and enables it to withstand a high internal osmotic pressure. The amount of peptidoglycan in the wall shows that it covers the cell in a multilayered fashion, with cross-linking both within and between the layers. Attached to the peptidoglycan is an acidic polymer, accounting for 30–40% of the wall mass, which differs from species to species. Often this is a teichoic acid—a substituted poly(D-ribitol 5-phosphate) (see Figure 2.8)—or a substituted glycerol 3-phosphate (lipoteichoic acid). In some bacteria teichoic acid is replaced by poly(*N*-acetylglucosamine 1-phosphate) or teichuronic acid (a polymer containing uronic acid and *N*-acetylhexosamine units). Bacteria that normally incorporate teichoic acid in their walls can switch to teichuronic acid under conditions of phosphate limitation. The acidic character of the polymer attached to the peptidoglycan ensures that the cell surface is strongly polar and carries a negative charge. This may influence the passage of ions, particularly Mg^{2+} and possibly ionized drugs, into the cell. The teichoic acid or other acidic polymer is readily solubilized and released from the insoluble peptidoglycan by hydrolysis in cold acid or alkali. The nature of the linkage is described later.

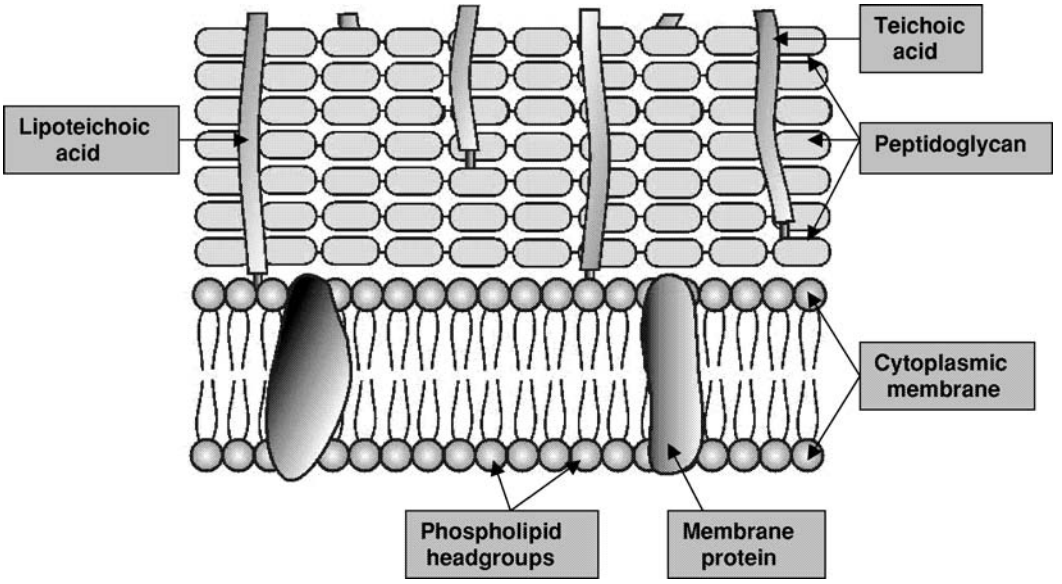


FIGURE 2.1 The arrangement of the cell envelope of Gram-positive bacteria. Note that the term 'cell envelope' includes both the cytoplasmic membrane and the outer layers of the cell. The components are not drawn to scale. (This diagram was kindly provided by Philip Kerkhoff.)

Other components of the Gram-positive wall vary widely from species to species. Protein is often present to the extent of 5–10%, and protein A of *Staphylococcus aureus* is apparently linked covalently to peptidoglycan. Proteins and polysaccharides frequently occur in the outermost layers and provide the main source of the antigenic properties of these bacteria. Mycobacteria and a few related genera differ from other Gram-positive bacteria in having large amounts of complex lipids in their wall structure. The unique features of the mycobacterial cell wall are described later in this chapter.

2.2.2 The Gram-negative wall

The Gram-negative wall is far more complex. Wide-ranging studies of its structure have been concentrated on the Enterobacteriaceae and on *Escherichia coli* in particular. The diagram in Figure 2.2 illustrates the general arrangement of the components of the Gram-negative cell envelope, which includes the cytoplasmic membrane as well as the cell wall. When cells of *Escherichia coli* are fixed, stained with suitable metal salts, sectioned and examined by electron microscopy, the cytoplasmic membrane is readily identified by its 'sandwich' appearance of two electron-dense layers separated by a lighter space. The clear layer immediately outside the cytoplasmic membrane has been described as the periplasmic space. However, techniques in electron microscopy such as freeze-etching and freeze-substitution reveal that a rich, dense periplasm occupies the periplasmic 'space', containing a wealth of biochemicals, including enzymes, transport proteins, secreted materials, components of peptidoglycan and the bacterial outer membrane (see later discussion). The electron-dense layer, about 2 nm thick, immediately outside the periplasm represents the peptidoglycan component of the wall. It is much thinner than in Gram-positive bacteria and may constitute only 5 to 10% of the wall mass. Even so, it contributes substantially to wall strength. Cells rapidly lyse when treated with lysozyme, an enzyme which specifically degrades peptidoglycan. In *Escherichia coli* the peptidoglycan is covalently linked to a lipoprotein which probably projects into the outer regions of the wall. The outer regions of the Gram-negative cell wall have been the most difficult to characterize. The various

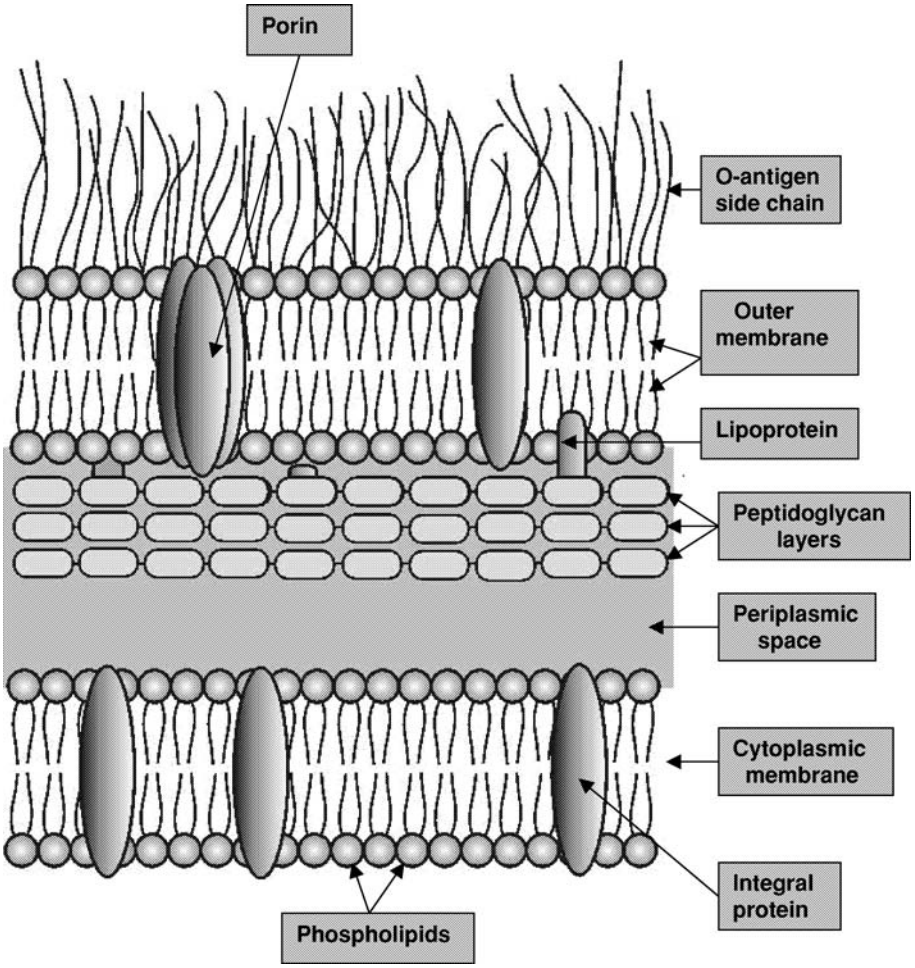


FIGURE 2.2 The arrangement of the various layers of the cell envelope of Gram-negative bacteria. The components are not drawn to scale. (This diagram was kindly provided by Philip Kerhoff.)

components together form a structure 6–10 nm thick, called the outer membrane. Like the cytoplasmic membrane, it is basically a lipid bilayer (giving rise to the two outermost electron-dense bands), hydrophobic in the interior with hydrophilic groups at the outer surfaces. It also has protein components which penetrate the layer partly or completely and form the membrane 'mosaic'.

Despite these broad structural similarities, the outer membrane differs widely in composition and function from the cytoplasmic membrane. Its main constituents are a lipopolysaccharide, phospholipids, fatty acids and proteins. The phospholipids, mainly phosphatidylethanolamine and phosphatidylglycerol, resemble those in the cytoplasmic membrane. The structure of the lipopolysaccharide is complex and varies considerably from one bacterial strain to another. The molecule has three parts (Figure 2.3). The core is built from 3-deoxy-D-*manno*-octulosonic acid (KDO), hexoses, heptoses, ethanolamine and phosphoric acid as structural components. The three KDO residues contribute a structural unit which strongly binds the divalent ions of magnesium and calcium, an important feature that stabilizes the membrane. Removal of these ions by chelating agents leads to release of some of the lipopolysaccharide into the medium; at

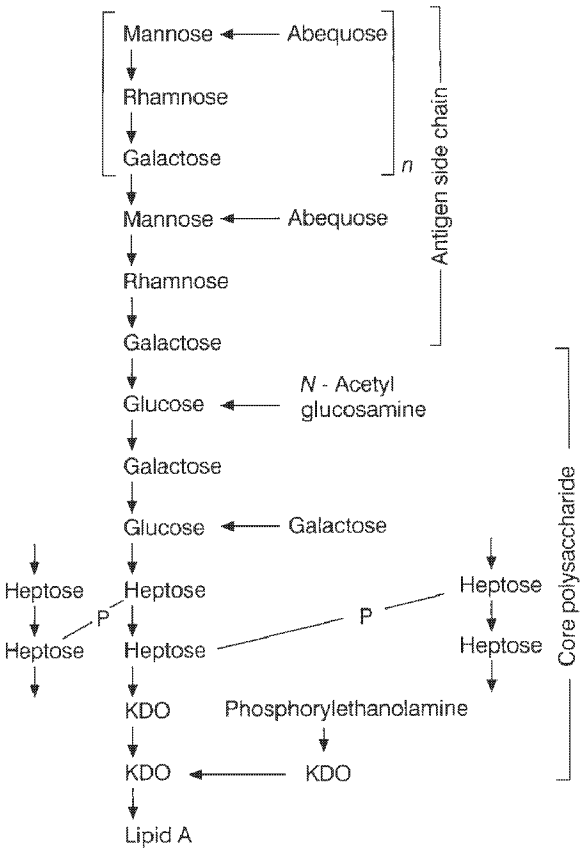


FIGURE 2.3 Structure of the lipopolysaccharide of the cell envelope of *Salmonella typhimurium*. The diagram has been simplified by omitting the configuration of the glycosidic linkages and omitting the *O*-acetyl groups from the abequose units. KDO: 3-deoxy-D-*manno*-octulosonic acid. Lipid A consists of a β-1,6-linked diglucosamine residue to which lauric, myristic, palmitic and 3-D(-)-hydroxymyristic acids are bound. The heptose residues of three lipopolysaccharide polymers are shown linked by phosphate diester bridges. Although there are considerable structural variations in the antigen side chains among *Salmonella* species, the core polysaccharide and lipid A are probably common to all wild-type salmonellae. The core structure in *Escherichia coli* is more variable.

the same time, the membrane becomes permeable to compounds that would otherwise be excluded. The core polysaccharide is linked to the antigenic side chain, a polysaccharide which can vary greatly from one strain to another even within the same bacterial species. Usually it consists of about 30 sugar units, although these can vary in both number and structure. It forms the outermost layer of the cell and is the main source of its antigenic characteristics. At the opposite end, the core of the lipopolysaccharide is attached to a moiety known as lipid A which can be hydrolyzed to glucosamine, long-chain fatty acids, phosphate and ethanolamine. The fatty acid chains of lipid A, along with those of the phospholipids, align themselves to form the hydrophobic interior of the membrane. The outer membrane is therefore asymmetric, with lipopolysaccharide exclusively on the outer surface and phospholipid mainly on the inner surface.

The most abundant proteins of the outer membrane in *Escherichia coli* are the porin proteins and lipoprotein. Electron microscopy of spheroplasts lacking peptidoglycan reveals triplets of indentations in the membrane surface, each 2 nm in diameter and 3 nm apart, through which the stain used in the preparation readily penetrates. This is interpreted as showing that the porin protein molecules stretch across the membrane in groups of three, enclosing pores through which water and small molecules can diffuse. The size of the pores explains the selective permeability of the Gram-negative outer membrane; they freely allow the passage of hydrophilic molecules up to a maximum molecular weight of 600–700. Larger flexible molecules may also diffuse through the pores, although with more difficulty. Artificial vesicles can be made with outer membrane lipids. Without protein, these are impermeable to solutes, but when porins are incorporated, they show permeability characteristics similar to those of the outer membrane itself. The role of porins in influencing the penetration of antibacterial drugs into Gram-negative bacteria is explored in Chapter 7.

Lipoprotein is another major component of the outer membrane proteins. About one-third is linked to peptidoglycan and the remaining two-thirds are unattached but form part of the membrane. The nature of the attachment of lipoprotein to the side chains of peptidoglycan is discussed later. About one in twelve of the peptide side chains is substituted in this way. This arrangement anchors the outer membrane to the peptidoglycan layer. The fatty acid chains of the lipoprotein presumably align themselves in the hydrophobic inner layer of the outer membrane and the protein moiety

may possibly associate with matrix protein, reinforcing the pore structure.

Many other proteins with specialized functions have been identified in the outer membrane. Some of these are transport proteins that allow access to molecules such as vitamin B_{12} or nucleosides which are too large to penetrate the pores of the membrane. Outer membrane proteins that contribute to the function of multidrug efflux pumps are described in Chapter 7.

2.3 Structure and biosynthesis of peptidoglycan

The structure and biosynthesis of peptidoglycan have special significance relative to the action of a number of important antibacterials and have been studied extensively. The biosynthesis of peptidoglycan was first worked out with *Staphylococcus aureus*. Although bacteria show many variations in peptidoglycan structure, the biosynthetic sequence in *Staphylococcus aureus* illustrates the general features of the process. In this description the enzymes involved will be referred to, where appropriate, by their biochemical names and also by the more recent popular abbreviations derived from the genetic nomenclature. The biosynthetic sequence may be conveniently divided into four stages.

2.3.1 Stage 1: Synthesis of UDP-*N*-acetylmuramic acid

The biosynthesis starts in the cytoplasm with two products from the normal metabolic pool, *N*-acetylglucosamine 1-phosphate and UTP (Figure 2.4). UDP-*N*-acetylglucosamine (I) formation is catalyzed by *N*-acetyl-1-phosphate glucosamine uridyl transferase (GlmU) with the elimination of pyrophosphate. This nucleotide reacts with phosphoenol pyruvate catalyzed by UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) to give the corresponding 3-enolpyruvyl ether (II). The pyruvyl group is then converted to lactyl by a reductase (MurB) that requires both flavin NAD and NADPH as cofactors, the product being UDP-*N*-acetylmuramic acid (III, UDPMurNAc). Muramic acid (3-*O*-D-lactyl-D-glucosamine) is a distinctive amino sugar derivative found only in the peptidoglycan of cell walls.

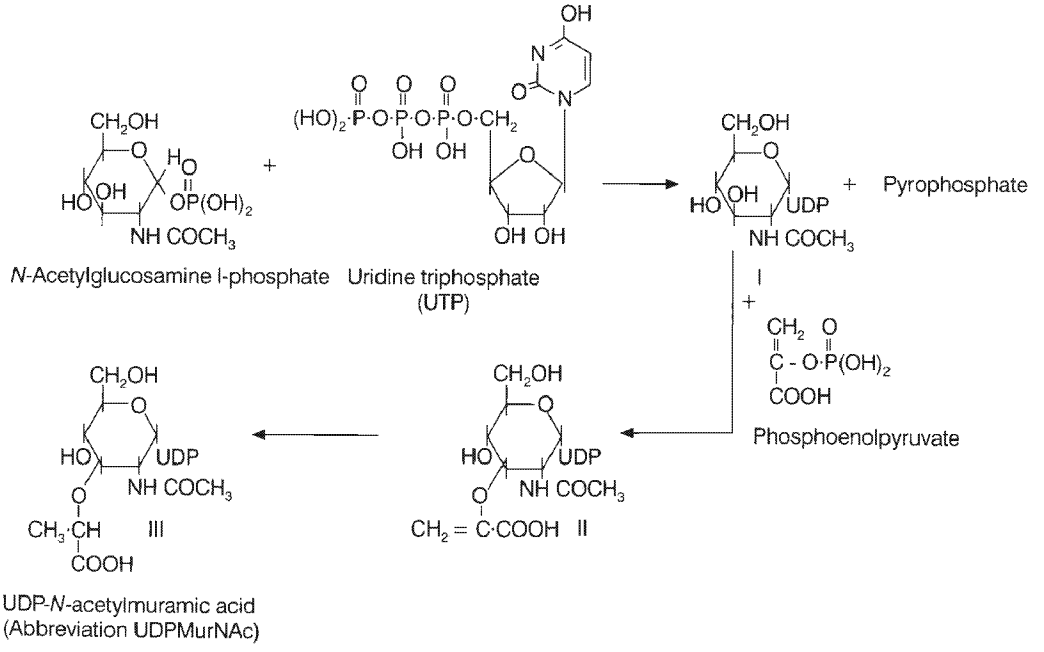


FIGURE 2.4 Peptidoglycan synthesis in *Staphylococcus aureus*. Stage 1: formation of UDP-*N*-acetylmuramic acid.

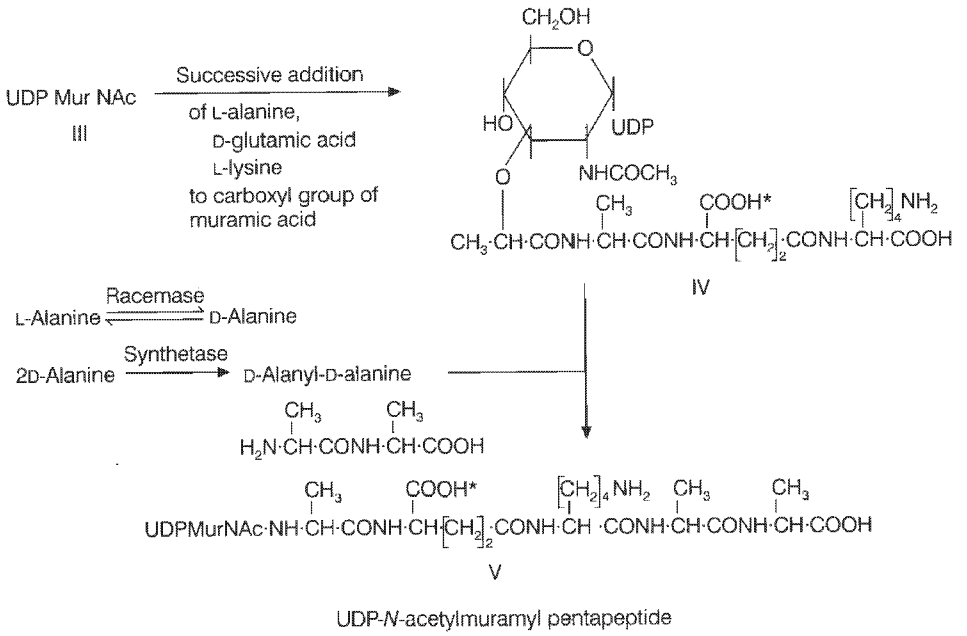


FIGURE 2.5 Peptidoglycan synthesis in *Staphylococcus aureus*. Stage 2: formation of UDP-*N*-acetylmuramyl pentapeptide. Addition of each amino acid and the final dipeptide requires ATP and a specific enzyme. L-Lysine is added to the γ-carboxyl group of D-glutamic acid; the α-carboxyl group (marked *) is amidated at a later stage in the biosynthesis.

2.3.2 Stage 2: Building the pentapeptide side chain

Five amino acid residues are next added to the carboxyl group of the muramic acid nucleotide (Figure 2.5). Each step requires ATP and a specific amino acid ligase. L-Alanine is added first by MurC. The next two residues added are D-glutamic acid catalyzed by MurD and then either L-lysine or *meso*-diaminopimelic acid by amino acid-specific forms of MurE. The incorporation of either L-lysine or *meso*-diaminopimelic acid into the pentapeptide side chain is characteristic of individual bacterial species. These latter amino acids are attached via their α-amino groups to the γ-carboxyl group of the glutamic acid. In *Staphylococcus aureus* and *Streptococcus pneumoniae,* but not in other bacteria, the α-carboxyl group of the glutamic acid is amidated at a later stage in the biosynthesis; this amino acid residue is sometimes referred to as D-isoglutamine. The biosynthesis of the pentapeptide is completed by addition, not of an amino acid, but of a dipeptide, D-alanyl-D-alanine, which is synthesized separately. A racemase acting on L-alanine converts it to D-alanine, and a ligase joins two molecules of D-alanine to give the dipeptide. The linkage of D-alanyl-D-alanine to the tripeptide chain is catalyzed by MurF. The completed UDP-*N*-acetylmuramyl intermediate (V) with its pendant peptide group will be referred to as the 'nucleotide pentapeptide'.

The three-dimensional structures of MurC, MurD, MurE and MurF have all been solved by X-ray crystallography and are generally very similar. Since these enzymes are unique to bacteria, detailed knowledge of the structures may eventually lead to the design of highly specific antibacterial drugs.

2.3.3 Stage 3: Membrane-bound reactions leading to a linear peptidoglycan polymer

The biosynthesis up to this point is cytoplasmic, while the succeeding steps occur on membrane structures. The first membrane-associated step involves the formation of a pyrophosphate link, catalyzed by

UDP-*N*-acetylmuramyl pentapeptide phosphotransferase (MraY), between the nucleotide pentapeptide and undecaprenyl phosphate (the phosphate ester of a C_{55} isoprenoid alcohol), which is a component of the cytoplasmic membrane, to form a complex referred to as lipid I. In this reaction UMP is released and becomes available for reconversion to UTP, which is needed in the first step of peptidoglycan biosynthesis (Figure 2.6). All subsequent reactions occurring while the intermediates are linked to undecaprenyl phosphate take place without release from the membrane. An essential step in this membrane-bound reaction sequence is the addition of a second hexosamine residue through a typical glycosidation by UDP-*N*-acetylglucosamine catalyzed by a glycosyl transferase (MurG) (Figure 2.6). The modified disaccharide known as lipid II is formed by a 1,4-β linkage with liberation of UDP. The involvement of undecaprenyl phosphate is not unique to peptidoglycan biosynthesis. It is also concerned in the biosynthesis of the polysaccharide chain in the *O*-antigen produced by *Salmonella typhimurium* and in the formation of the polysaccharide elements of the lipopolysaccharides of Gram-negative bacteria; in Gram-positive bacteria it fulfils a similar role in the biosynthesis of teichoic acid or polysaccharides of the wall. The structure of MurG has also been solved and active sites identified for inhibitor design studies.

At about this point in the biosynthesis of *Staphylococcus aureus* peptidoglycan and in many other Gram-positive bacteria, an extending group is added to the ε-amino group of the lysine unit in the nucleotide pentapeptide. Glycine and a glycine-specific transfer RNA (tRNA) are involved in this process during which a pentaglycine group is added. This reaction, which is not found in Gram-negative bacteria, is unlike the tRNA reactions in protein biosynthesis because ribosomes are not involved; the five glycine units are added successively to the lysine from the nitrogen end (the reverse direction of protein biosynthesis). The resultant product (VIII, Figure 2.6) with 10 amino acid units is referred to as the disaccharide decapeptide and retains a free terminal amino group. In the biosynthesis of peptidoglycans in certain other bacterial species, for example in *Escherichia coli*, in which no extending group is added, the later reactions involve the ε-amino group of *meso*-diaminopimelic acid (or equivalent diamino acid) instead of the terminal amino group of glycine. During the membrane-bound stage in the biosynthesis of *Staphylococcus aureus* peptidoglycan, the carboxyl group of D-glutamic acid is amidated by a reaction with ammonia and ATP.

The disaccharide decapeptide (VIII) is now attached to an 'acceptor', usually regarded as the growing linear polymer chain. In this reaction the disaccharide with its decapeptide side chain forms a β-linkage from the 1 position of the *N*-acetylmuramic acid residue to the 4-hydroxyl group of the terminal *N*-acetylglucosamine residue in the growing polysaccharide chain. Because this reaction occurs outside the cytoplasmic membrane, the disaccharide-decapeptide linked to the undecaprenyl phosphate (lipid II) first moves across the membrane to gain access to the acceptor on the external face of the membrane. The released undecaprenyl pyrophosphate is reconverted by a specific pyrophosphatase to the corresponding phosphate, ready for another cycle of the membrane-bound part of the synthesis. The extension of the glycan chains thus occurs by successive addition of disaccharide units catalyzed by glycosyl transferases.

2.3.4 Stage 4. Cross-linking

The linear peptidoglycan (IX, Figure 2.6) formed in stage 3 contains many polar groups which make it soluble in water. It lacks mechanical strength and toughness. These attributes are introduced in the final stage of biosynthesis by cross-linking, a process well known in the plastics industry for producing similar results in synthetic linear polymers. The mechanism involved in cross-linking peptidoglycan is a transpeptidation reaction requiring no external supply of ATP or similar compounds. In *Staphylococcus aureus*, the transpeptidation occurs between the terminal amino group of the pentaglycine, side chain and the peptide amino group of the terminal D-alanine residue of another peptide side chain; D-alanine is eliminated and a peptide bond formed (Figure 2.7). In *Staphylococcus aureus* peptidoglycan, the cross-linking is quite extensive and up to 10 peptide side chains may be bound together by bridging groups. Since the linear polymers themselves are very large, it is likely that the whole of the peptidoglycan in a Gram-positive bacterium is made up of

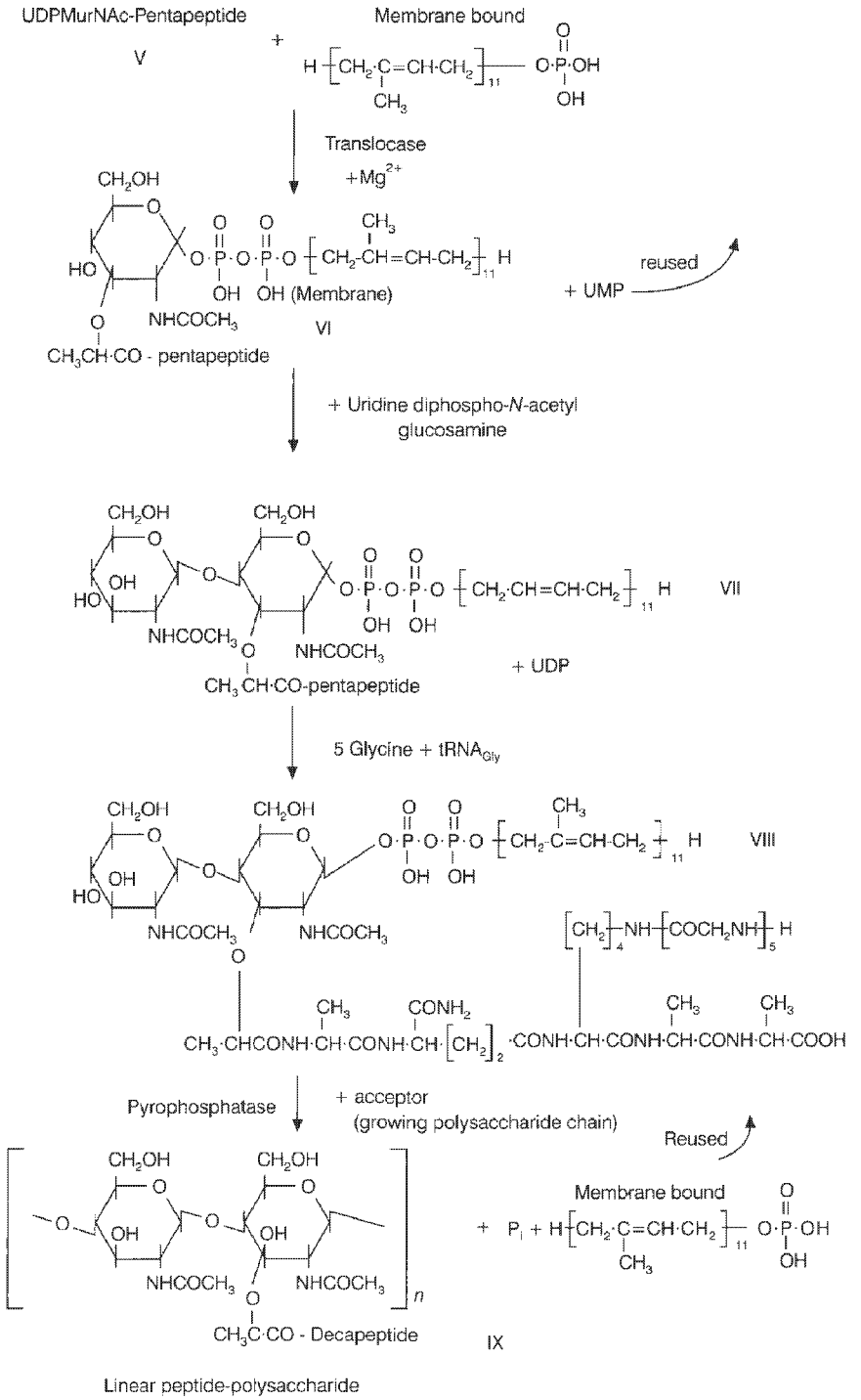


FIGURE 2.6 Peptidoglycan synthesis in *Staphylococcus aureus*. Stage 3: formation of the linear peptidoglycan. The structure of the decapeptide side chain is shown in VIII.

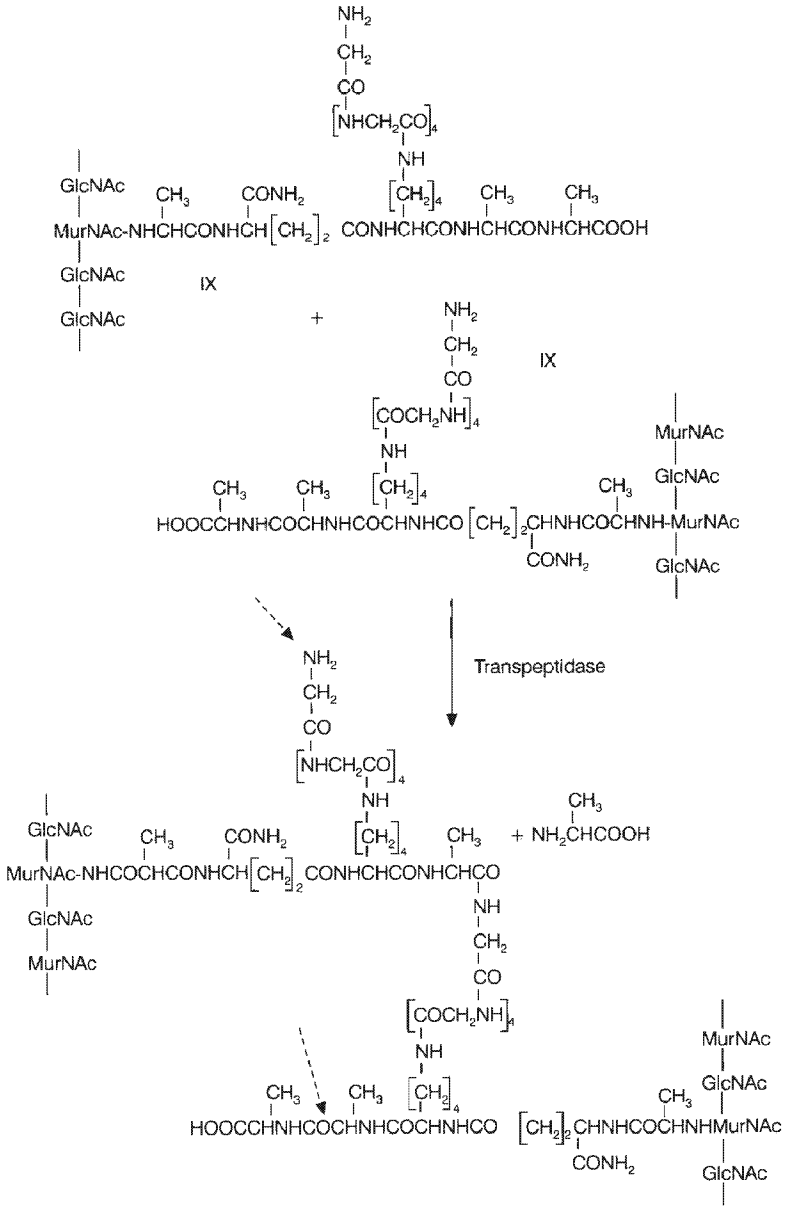


FIGURE 2.7 Peptidoglycan synthesis in *Staphylococcus aureus*. Stage 4: cross-linking of two linear peptidoglycan chains. The linear polymers have the structure IX (Figure 2.6) GlcNac: *N*-acetylglucosaminyl residue. The dashed arrows show points at which further cross-links may be formed with other polymer chains. MurNAc: *N*-acetylmuramyl residue.

units covalently bound together. This gigantic bag-shaped molecule has been called a sacculus. There is also a mechanism for constantly breaking it down and reforming it to allow cell growth and division. Peptidoglycan hydrolases, which hydrolyze the polysaccharide chains of peptidoglycan and others attacking the peptide cross-links, exert this essential catabolic activity during cell growth.

2.3.5 Penicillin-binding proteins

The membrane-bound enzymes involved in linking the disaccharide deca- or pentapeptide to the growing linear peptidoglycan and the subsequent cross-linking reaction are referred to as penicillin-binding proteins or PBPs (Table 2.1). The PBPs are regarded as the specific targets for penicillin and the other β-lactam an-

TABLE 2.1 Properties of penicillin-binding proteins of *Escherichia coli*

Protein no.	*Molecular mass (Kilodaltons)*	*Enzyme activities*	*Function*
1a	91	Transpeptidase Transglycosylase	Peptidoglycan cross-linking
1b	91	Transpeptidase Transglycosylase	Peptidoglycan cross-linking
2	66	Transpeptidase	Peptidoglycan cross-linking
3	60	Transpeptidase	Peptidoglycan cross-linking
4	49	DD-carboxypeptidase	Limitation of peptidoglycan cross-linking
5	41	DD-carboxypeptidase	Limitation of peptidoglycan cross-linking
6	40	DD-carboxypeptidase	Limitation of peptidoglycan cross-linking

tibiotics. As we shall see, the covalent reaction between β-lactam antibiotics and the PBPs, which inactivates their transpeptidase function but not the transglycosylase activity, is central to the antibacterial activity of these drugs. PBPs vary from species to species in number, size, amount and affinity for β-lactams antibiotics. The PBPs fall into two major groups of high (≥ 60 kDa) and low (≤ 49 kDa) molecular mass, respectively. The PBPs with a high molecular mass are essentially two-domain proteins classed as A or B. In both classes the C-terminal domain is responsible for transpeptidation and is the target for penicillin binding and β-lactam action. Class A proteins also catalyze the transglycosylation reactions at the N-terminal domains. PBPs 1a and 1b of *Escherichia coli* exemplify this bifunctional type. The monofunctional class B proteins lack transglycolase activity. Monofunctional glycosyl transferases have been identified in both Gram-positive and Gram-negative bacteria, although not all glycosyl transferases appear to be essential to bacterial viability. In *Escherichia coli,* PBPs 1a and 1b provide the key enzyme activities involved in peptidoglycan synthesis. The synthetic role of PBP2 is specifically involved in cellular elongation and that of PBP3 with the formation of the cell septum during cell division. The low molecular mass PBPs, which include PBPs 4, 5 and 6 in *Escherichia coli,* are monofunctional DD-carboxypeptidases that catalyze transfer reactions from D-alanyl-D-alanine terminated peptides. Although these PBPs are also inactivated by β-lactams, this may not be central to their antibiotic action. Nevertheless, carboxypeptidases of this type are convenient to purify and have been widely used as models for the nature of the interaction between PBPs and penicillin. The most widely studied enzymes are the extracellular DD-carboxypeptidases produced by *Streptomyces* species and carboxypeptidases solubilized from the membranes of *Escherichia coli* and *Bacillus stearothermophilus.* The *Streptomyces* enzymes display some transpeptidase activity besides their high carboxypeptidase activity.

In addition to the seven 'classic' PBPs listed in Table 2.1, a further five have been added to the collection: PBP1c, PBP7, DacD, AmpC and AmpH. An extensive study of deletion mutants reveals that only PBPs 2 and 3 plus PBP1a or 1b are essential for the growth and division of rod-shaped bacteria under laboratory conditions. However, combinations of the activities of the other PBPs may be necessary for growth and viability in more demanding conditions, for example, in an infected host. The possible significance of the 'new' five PBPs in relation to the antibacterial action of β-lactams remains to be explored.

2.3.6 Variations in peptidoglycan structure

Many variations are found in peptidoglycan structure between one species of bacteria and another or even between strains of the same species and only a general

account is possible here. All peptidoglycans have the same glycan chain as in *Staphylococcus aureus* except that the glucosamine residues are sometimes *N*-acylated with a group other than acetyl. *O*-Acetylation of glucosamine residues is also found in some organisms. The peptide side chains always have four amino acid units alternating L-, D-, L-, D- in configuration. The second residue is always D-glutamic acid, linked through its γ-carboxyl group, and the fourth is invariably D-alanine. The peptidoglycan from *Staphylococcus aureus* (type A2) is characteristic of many Gram-positive cocci. Peptidoglycans of this group, and the related types A3 and A4, have similar tetrapeptide side chains but vary in their bridging groups. The amino acids in the bridge are usually glycine, alanine, serine or threonine, and the number of residues can vary from one to five. In type A1 peptidoglycans, the L-lysine of the type II peptide side chain is usually replaced by *meso*-2,6,-diaminopimelic acid, and there is no bridging group. Cross-linking occurs between the D-alanine of one side chain and the 6-amino group of the diaminopimelic acid of another. This peptidoglycan type is characteristic of many rod-shaped bacteria, both the large family of Gram-negative rods and the Gram-positive bacilli. In the less common type B peptidoglycans, cross-linkage occurs between the α-carboxyl group of the D-glutamic acid of one peptide side chain and the D-alanine of another through a bridge containing a basic amino acid.

2.3.7 Cross-linking in Gram-negative bacteria

In contrast to the multiple random cross-linkage of peptidoglycan which is found in the Gram-positive cocci, the peptidoglycan of *Escherichia coli* and similar Gram-negative rods has on average only a single cross-link between one peptide side chain and another. These bacteria contain, besides the transpeptidases concerned in cross-linkage, other enzymes known as DD-carboxypeptidases which specifically remove D-alanine from a pentapeptide side chain. Carboxypeptidase I is specific for the terminal D-alanine of the pentapeptide side chain, whilst carboxypeptidase II acts on the D-alanine at position 4 after the terminal D-alanine has been removed. DD-Carboxypeptidase I therefore limits the extent of cross-linking.

The peptidoglycan sacculus determines the overall shape of the cell, and the peptidoglycan is laid down with a definite orientation in which the polysaccharide chains run perpendicular to the main axis of rod-shaped organisms such as *Escherichia coli*.

2.3.8 Attachments to peptidoglycans

Within the cell wall, the polymeric peptidoglycan is usually only part of a larger polymer. In Gram-positive cocci it is linked to an acidic polymer, often a teichoic acid (Figure 2.8). The point of attachment is through

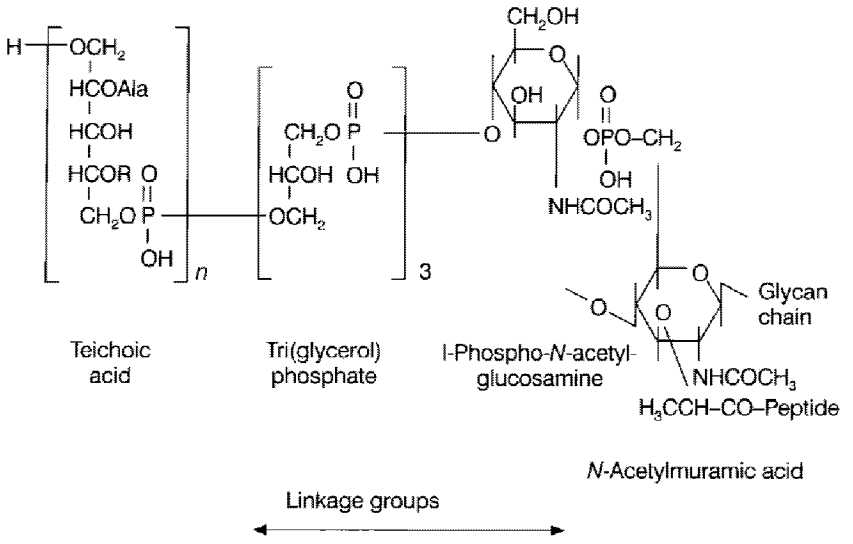


FIGURE 2.8 Teichoic acid and its linkage to peptidoglycan in the wall of *Staphylococcus aureus*.

the 6-hydroxyl group of muramic acid in the glycan chain. Only a small fraction of the muramic acid residues is thus substituted. In *Staphylococcus aureus* cell walls, teichoic acid is joined to peptidoglycan by a linking unit consisting of three glycerol 1-phosphate units attached to the 4 position of *N*-acetylglucosamine which engages through a phosphodiester group at position 1 with the 6-hydroxyl group of muramic acid. This type of linkage seems to occur with polymers other than teichoic acid, e.g. with poly(*N*-acetylglucosamine 1-phosphate) in a *Micrococcus* species. The acid-labile *N*-acetylglucosamine 1-phosphate linkage and the alkali-labile phosphodiester linkage at position 4 explain the ease with which teichoic acid can be split off from peptidoglycan. Within the cell wall, the synthesis of teichoic acid is closely associated with that of peptidoglycan.

In the Gram-positive mycobacteria, the peptidoglycan carries quite a different polymeric attachment. Arabinogalactan is attached to the 6 position of some of the *N*-glycolylmuramic acid residues of the glycan chain through a phosphate ester group. Mycolic acids (complex, very long-chain fatty acids) are attached by ester links to the C-5 position of arabinose residues of the arabinogalactan.The mycobacterial cell wall thus has a high lipid content.

In *Escherichia coli* and related bacteria, the peptidoglycan carries a lipoprotein as a substituent (Figure 2.9). The lipoprotein consists of a polypeptide chain of 58 amino acid units of known sequence with lysine at the C-terminal and cysteine at the N-terminal. This is attached to the 2-carboxyl group of *meso*-2,6-diaminopimelic acid in a peptide side chain of *Escherichia coli* peptidoglycan which has lost both D-alanine groups. Attachment is by an amide link with the ε-amino group in the terminal lysine of the polypeptide. At the opposite end of the polypeptide chain, the cysteine amino group carries a long-chain fatty acid joined as an amide, and its sulfur atom forms a thioether link with a long-chain diacylglycerol.

Lipoprotein occurs in enteric bacteria other than *Escherichia coli,* but it may not be common to all Gram-negative bacteria, although small amounts have been detected in *Proteus mirabilis.*

2.4 Antibiotics that inhibit peptidoglycan biosynthesis

The conclusion that a particular antibiotic owes its antibacterial activity to interference with peptidoglycan biosynthesis rests on several lines of evidence:

1. Bacteria suspended in a medium of high osmotic pressure are protected from concentrations of the antibiotic that would cause lysis and death in a normal medium. Under these conditions the cells lose the shape-determining action of the peptidoglycan and become spherical; they are then known as spheroplasts. These retain an undamaged cytoplasmic membrane, but their wall is deficient or considerably modified. Spheroplasts are in

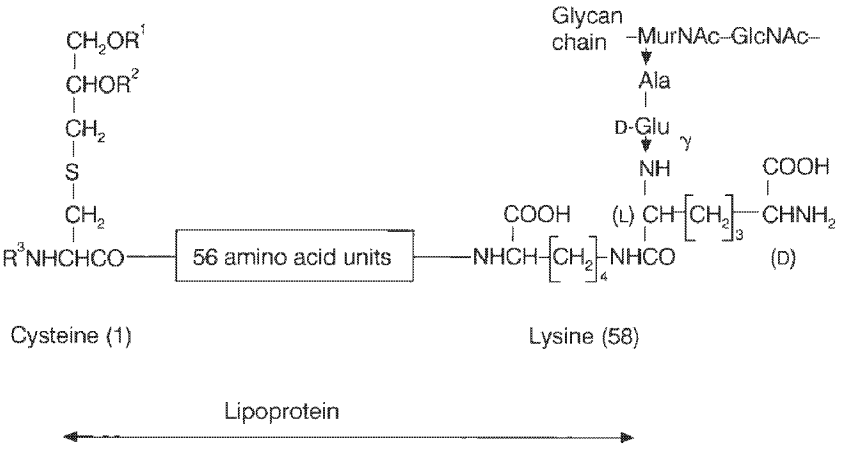


FIGURE 2.9 Lipoprotein and its linkage to peptidoglycan in the envelope of *Escherichia coli.*

principle viable and if the antibiotic is removed, they can divide and produce progeny with normal walls.

2. Several species of bacteria have walls containing no peptidoglycan. These include the mycoplasmas, the halophilic bacteria tolerant of high salt concentrations and bacteria in the L-phase where the normal wall structure is greatly modified. If a compound inhibits the growth of common bacteria but fails to affect bacteria of these special types, it probably owes its activity to interference with peptidoglycan synthesis.
3. Subinhibitory concentrations of these antibiotics often cause accumulation in the bacterial cytoplasm of uridine nucleotides of *N*-acetylmuramic acid, with varying numbers of amino acid residues attached which represent intermediates in the early stages of peptidoglycan biosynthesis. When an antibiotic causes a block at an early point in the reaction sequence, it is not surprising to find an accumulation of the intermediates immediately preceding the block. However, quantities of muramic acid nucleotides are also found in bacteria treated with antibiotics known to affect later stages in peptidoglycan biosynthesis. It seems that all the biosynthetic steps associated with the membrane are closely interlocked, and inhibition of any one of them leads to accumulation of the last water-soluble precursor, UDP-*N*-acetylmuramyl pentapeptide (V, Figure 2.5).

2.4.1 Bacitracin

Bacitracin is a polypeptide antibiotic (Figure 2.10) which is too toxic for systemic administration but is sometimes used topically to kill Gram-positive bacteria by interfering with cell wall biosynthesis. The antibiotic is ineffective against Gram-negative bacteria, probably because its large molecular size hinders penetration through the outer membrane to its target site. Bacitracin inhibits peptidoglycan biosynthesis by binding specifically to the long-chain C_{55}-isoprenol pyrophosphate in the presence of divalent metal ions.

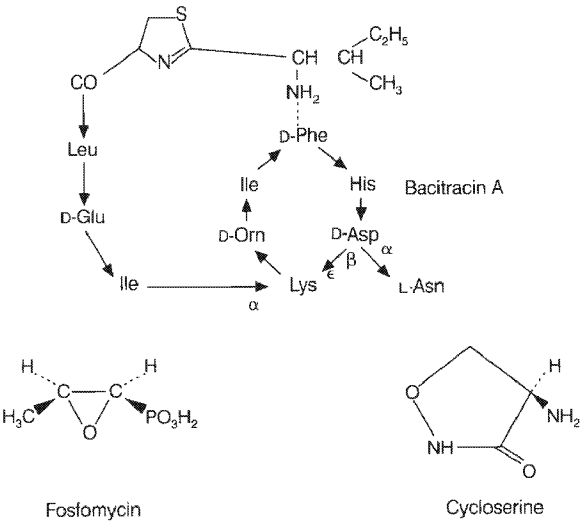


FIGURE 2.10 Antibiotics which inhibit the biosynthesis of the precursors of peptidoglycan.

In the formation of the linear peptidoglycan (IX, Figure 2.7), the membrane-bound isoprenol pyrophosphate is released. Normally this is converted by a pyrophosphatase to the corresponding phosphate which thus becomes available for reaction with another molecule of UDPMur-*N*-Ac-pentapeptide (V, Figure 2.6). Interaction between the lipid pyrophosphate and a metal ion-bacitracin coordination complex blocks this process and eventually halts the synthesis of peptidoglycan. The identity of the divalent metal ion bound to the antibiotic in bacterial cells is uncertain but could well be either Mg^{2+} or Zn^{2+}. Bacitracin forms 1:1 complexes with several divalent metal ions, and investigations employing nuclear magnetic resonance and optical rotary dispersion (ORD) indicate the involvement of the imidazole ring of the histidine residue of the antibiotic in metal ion binding. Additional likely sites of metal ion interaction include the thiazoline moiety and the carboxyl groups of the D-aspartate and D-glutamate residue.

2.4.2 Fosfomycin (phosphonomycin)

This antibiotic has the very simple structure shown in Figure 2.10. It acts on infections caused by both

Gram-positive and Gram-negative bacteria but although its toxicity is low, until recently it achieved only limited use in clinical practice. However, there is a resurgence of interest in fosfomycin for the treatment of serious infections resistant to other antibiotics. Fosfomycin inhibits the first step of peptidoglycan biosynthesis, namely, the condensation of UDP-*N*-acetylglucosamine (I) with phosphoenol pyruvate (PEP) catalyzed by UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), giving the intermediate (II) that subsequently yields UDP-*N*-acetylmuramic acid (III) on reduction (Figure 2.4). Fosfomycin inactivates MurA by reacting covalently with an essential cysteine residue (Cys-115) at the active center of the enzyme to form the thioester illustrated in Figure 2.11. This reaction is time-dependent and is facilitated by UDP-*N*-acetylglucosamine, which appears to 'chase' the other substrate (PEP) from the active site and promotes a conformational change in the enzyme. Both these effects are believed to expose the nucleophilic Cys-115 for reaction with the epoxide moiety of fosfomycin. The three-dimensional structure of MurA (from *Escherichia coli*) complexed with UDP-*N*-acetylglucosamine and fosfomycin has been determined by X-ray crystallography. The analysis confirmed the covalent interaction of the antibiotic with Cys-115 and also revealed that there are hydrogen bonds between the antibiotic and the enzyme and UDP-*N*-acetylglucosamine.

2.4.3 Cycloserine

This antibiotic also has a simple structure (Figure 2.10). Cycloserine is active against several bacterial species, but because of the central nervous system disturbances which are experienced by some patients, clinically it is limited to occasional use in individuals with tuberculosis that is resistant to the more commonly used drugs. Cycloserine produces effects in bacteria that are typical of compounds acting on peptidoglycan biosynthesis. Thus when cultures of *Staphylococcus aureus* are grown with subinhibitory concentrations of cycloserine, the peptidoglycan precursor (IV, Figure 2.5) accumulates in the medium, suggesting a blockage in the biosynthesis immediately beyond this point.

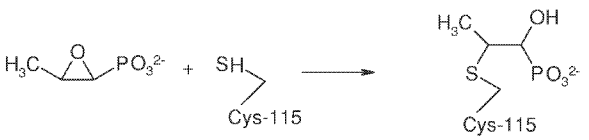


FIGURE 2.11 Fosfomycin inactivates UDP-*N*-acetylglucosamine enolpyruvoyl transferase (MurA) by reacting with the essential cysteine residue (Cys-115) at the active center of the enzyme to form a thioester.

In fact, cycloserine inhibits alanine racemase and D-alanyl-D-alanine ligase, the two enzymes concerned in making the dipeptide for completion of the pentapeptide side chain. Molecular models reveal that cycloserine is structurally related to one possible conformation of D-alanine, so that its inhibitory action on these enzymes appears to be a classic example of isosteric interference. The observation that the action of cycloserine is specifically antagonized by the addition of D-alanine to the growth medium also supports the postulated site of action. The affinity of cycloserine for the ligase is much greater than that of the natural substrate, the ratio of K_m to K_i being about 100. In a compound acting purely as a competitive enzyme inhibitor, this sort of K_m/K_i ratio is probably essential for useful antibacterial activity. The greater affinity of cycloserine for the enzyme may be connected with its rigid structure. This could permit a particularly accurate fit to the active center of the enzyme, either in the state existing when the enzyme is uncombined with its substrate or in a modified conformation which is assumed during the normal enzymic reaction. Rigid structures of narrow molecular specificity are common among antimicrobial agents and similar considerations may apply to other types of action; this theme will recur in later sections. The three-dimensional structures of both alanine racemase and D-alanyl-D-alanine ligase are available and it will be interesting to see whether cycloserine does indeed interact with the active sites of these enzymes according to this concept of inhibition.

Cycloserine enters the bacterial cell by active transport (see Chapter 7). This allows the antibiotic to reach higher concentrations in the cell than in the medium and adds considerably to its antibacterial efficacy.

2.4.4 Glycopeptide antibiotics

Vancomycin (Figure 2.12), which is a member of a group of complex glycopeptide antibiotics, was first isolated in the 1950s, but its real clinical importance only emerged with the inexorable spread of methicillin-resistant staphylococci (MRSA; see Chapters 9 and 10). The use of vancomycin and structurally related glycopeptides has markedly increased because of their value in treating serious infections caused by MRSA and other Gram-positive bacteria. Because of their relatively large molecular size, the glycopeptides are essentially inactive against the more impermeable Gram-negative bacteria. The antibacterial action of glycopeptide antibiotics depends on their ability to bind specifically to the terminal D-alanyl-D-alanine group on the peptide side chain of the membrane-bound intermediates in peptidoglycan synthesis (compounds VI–IX in Figure 2.6). It is important to note that this interaction occurs on the outer face of the cytoplasmic membrane. The glycopeptide antibiotics probably do not enter the bacterial cytoplasm, again because of their molecular size. The complex which is formed between vancomycin and D-alanyl-D-alanine

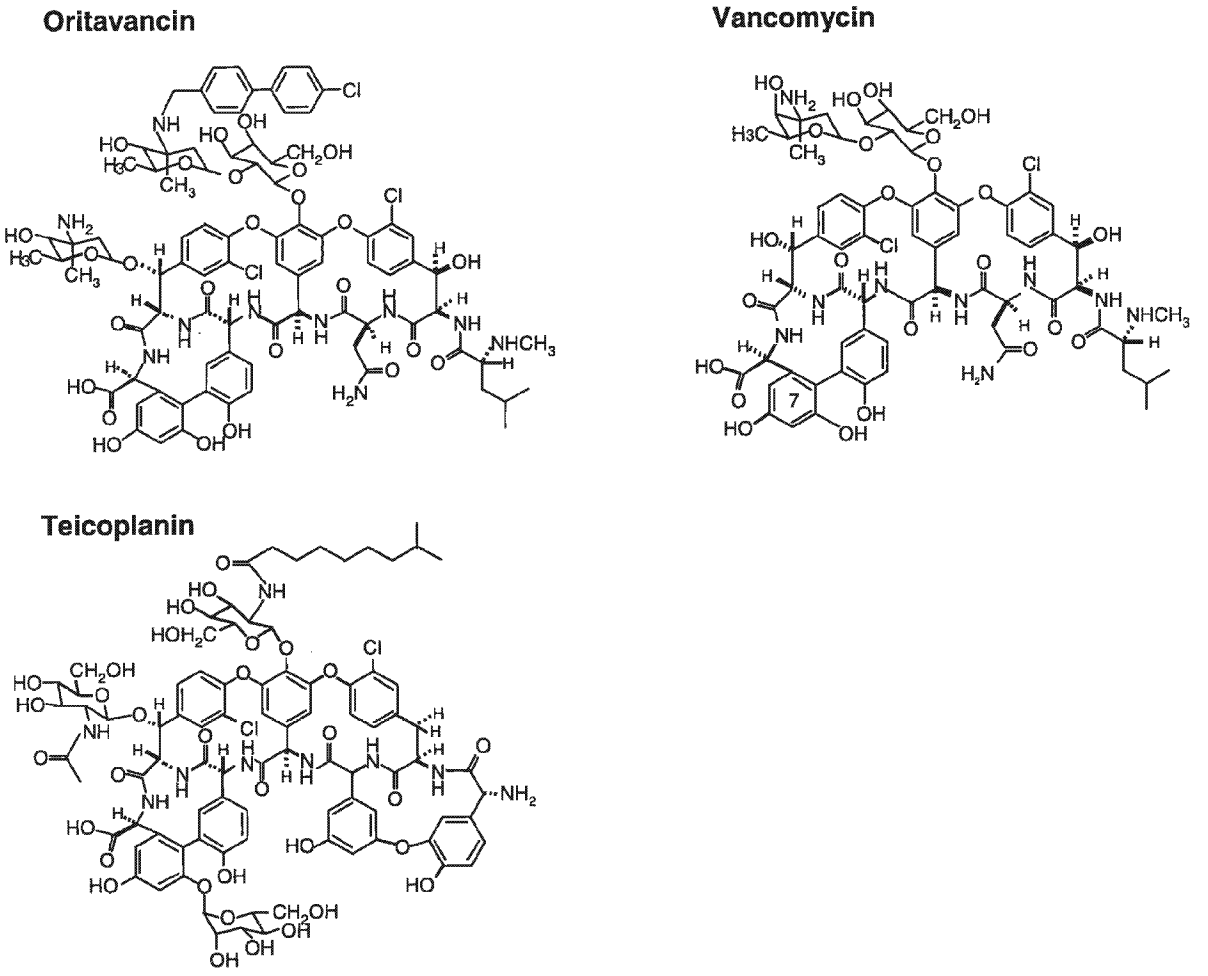


FIGURE 2.12 Glycopeptide antibiotic inhibitors of peptidoglycan synthesis that are increasingly important in the treatment of infections caused by drug-resistant staphylococci.

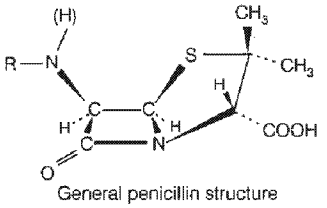
has been studied in considerable detail. The complex blocks the transglycosylase involved in the incorporation of the disaccharide-peptide into the growing peptidoglycan chain and the DD-transpeptidases and DD-carboxypeptidases for which the D-alanyl-D-alanine moiety is a substrate. Both peptidoglycan chain extension and cross-linking are therefore inhibited by glycopeptide antibiotics. This is, in fact, a most unusual mode of inhibition in that the antibiotic prevents the utilization of the substrate rather than directly interacting with the target enzymes.

The side chains of the amino acids of the heptapeptide backbone of vancomycin are extensively cross-linked to form a relatively concave carboxylate cleft into which the D-alanyl-D-alanine entity binds noncovalently via hydrogen bonds and hydrophobic interactions. Furthermore, NMR and X-ray crystallographic studies show that vancomycin spontaneously forms a dimeric structure which enables the antibiotic to bind to two D-alanyl-D-alanine peptide units attached either to the disaccharide-peptide precursor or to adjacent growing peptidoglycan strands. Another glycopeptide antibiotic, teichoplanin (Fig 2.12), is considerably more potent than vancomycin against some important Gram-positive pathogens. It is thought that the *N*-substituted fatty acyl side chain that distinguishes teichoplanin from vancomycin serves to anchor teichoplanin in the cytoplasmic membrane. This localization may facilitate the interaction of the drug with the D-alanyl-D-alanine target site. In contrast with vancomycin, teichoplanin does not form dimers. Thus although the dimerization of vancomycin may in principle facilitate its antibacterial action, the dimerizing potential is relatively weak and it is unclear whether the dimer is indeed a significant contributor to the antibiotic activity of vancomycin *in vivo*. The semisynthetic glycopeptide, oritavancin (Fig 2.12), is strongly dimerized and this may be a factor in the highly potent antibacterial activity of this promising drug.

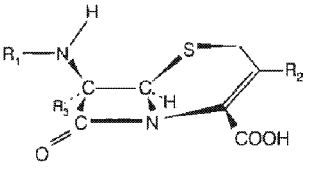
2.4.5 Penicillins, cephalosporins and other β-lactam antibiotics

Penicillin was the first naturally occurring antibiotic to be used for the treatment of bacterial infections, and the story of its discovery and development is one of the most inspiring in the history of medicine. Penicillin is one of a group of compounds known as β-lactam antibiotics which are unrivalled in the treatment of bacterial infections. Their only serious defects include an ability to cause immunologic sensitization in a small proportion of patients, a side effect which prevents their use in those affected, and the frequency of emergence of bacteria resistant to β-lactams. The original penicillins isolated directly from mold fermentations were mixtures of compounds having different side chains. The addition of phenylacetic acid to the fermentation medium improved the yield of penicillin and ensured that the product was substantially a single compound known as penicillin G or benzylpenicillin (Figure 2.13). The first successful variant was obtained by replacing phenylacetic acid by phenoxyacetic acid as the added precursor. This gave phenoxymethylpenicillin or penicillin V (Figure 2.13). The main advantage of this change was an improvement in the stability of the penicillin towards acid. The ready inactivation of penicillin G at low pH limited its usefulness when it was given by mouth since a variable and often considerable fraction of the antibacterial activity was destroyed in the acidic environment of the stomach. Penicillin V thus improved the reliability of oral doses. These early penicillins, produced directly by fermentation, were intensely active against Gram-positive infections and gave excellent results in streptococcal and staphylococcal infections and in pneumonia. They were also very active against Gram-negative infections caused by gonococci and meningococci, but were much less active against the more typical Gram-negative bacilli.

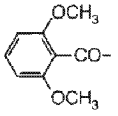
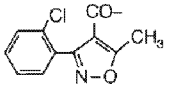
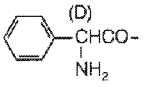
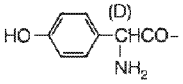
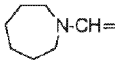
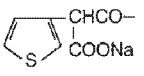
A further advance in the versatility of the penicillins was achieved by workers at the original Beecham company (now part of GlaxoSmithKline) with the development of a method for the chemical modification of the penicillin molecule. Bacterial enzymes were found that remove the benzyl side chain from penicillin G, leaving 6-aminopenicillanic acid, which could be isolated and then acylated by chemical means. This discovery opened the way to the production of an almost unlimited number of penicillin derivatives, some of which have shown important changes in properties compared with the parent penicillin. The value of increased stability has already been mentioned, and some semisynthetic penicillins show this



General penicillin structure



General cephalosporin structure

Side chain	Name
(R)	
CH_2CO-	Benyl penicillin Penicillin G
OCH_2CO-	Phenoxymethyl penicillin Penicillin V
H–	6-Aminopenicillanic acid
	Methicillin
	Cloxacillin
	Ampicillin
	Amoxycillin
	Mecillinam
	Ticarcillin

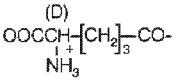
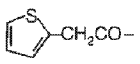
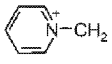
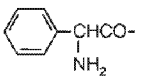
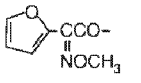
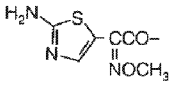
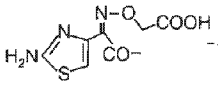
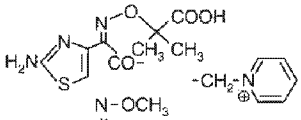
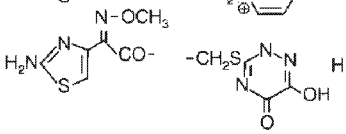
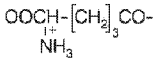
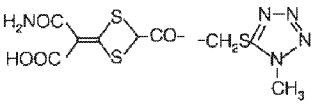
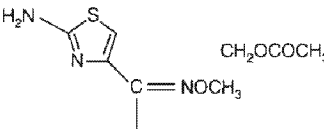
Substituents			Name
R_1	R_2	R_3	
	CH_3COOCH_2	H	Cephalosporin C
H–	CH_3COOCH_2	H	7-Aminocephalosporanic acid
		H	Cephaloridine
	CH_3	H	Cephalexin
	H_2NCOO-	H	Cefuroxime
	CH_3COO-	H	HR 756
	$-CH=CH_2$	H	Cefixime
		H	Ceftazidime
		H	Ceftriaxone
	H_2NCOO-	CH_3O-	Cephamycin C
		CH_3O	Cefotetan
	CH_2OCOCH_3		Cefotaxime

FIGURE 2.13 Representative penicillins and cephalosporins.

property. Other modified penicillins (e.g. methicillin and cloxacillin, Figure 2.13) are much less susceptible to attack by β-lactamase, an enzyme which converts penicillin to the antibacterially inactive penicilloic acid and gives rise to the commonest form of resistance to penicillin (Chapter 9).

The discovery of the β-lactamase inhibitor, clavulanic acid (Figure 2.14), which is a β-lactam itself but without useful antibacterial activity, provided an opportunity to coadminister this agent with β-lactamase-sensitive compounds such as amoxycillin (Figure 2.13) in mixtures such as augmentin (a 1:1 mixture of amoxycillin and clavulanic acid) and timentin (a 1:1 mixture of ticarcillin and clavulanic acid).

Another striking change brought about by chemical modification of the penicillin side chain was an increase in activity against Gram-negative bacteria, a property found in several derivatives, including ampicillin, amoxycillin, carbenicillin and ticarcillin (Figure 2.13). This increase in Gram-negative activity is accompanied by a lessening of activity towards Gram-positive bacteria. Ampicillin is one of the most widely used antibacterial agents. In mecillinam (Figure 2.13), where the side chain is attached by an azomethine link rather than the usual amide bond, the activity spectrum of the original penicillin molecule has been completely reversed. This compound is highly active against Gram-negative bacteria but requires 50 times the concentration for an equal effect on Gram-positive organisms. It can be used in the treatment of typhoid fever, which is caused by the Gram-negative bacterium *Salmonella typhi*.

Cephalosporin C (Figure 2.13), originally isolated from a different organism than that used to produce penicillin, has a structure in its nucleus similar to that in the penicillins. The biogenesis of the nuclei in these two classes of antibiotics is now known to be identical except that in cephalosporin biosynthesis the thiazolidine ring of the penicillin nucleus undergoes a specific ring expansion to form the dihydrothiazine ring of the cephalosporin nucleus. Besides this similarity in structure and biogenesis, cephalosporin C and its derivatives act on peptidoglycan cross-linking in the same way as the penicillins. Cephalosporin C itself is not a useful antibacterial drug, but like the penicillins, it is amenable to chemical modification. Enzymic removal of the side chain gives 7-aminocephalosporanic acid, which can be chemically acylated to give new derivatives. A second change in the molecule can also be made by a chemical modification of the acetoxy group of cephalosporin C. The first successful semisynthetic cephalosporin was cephaloridine. Many others have followed; a selection of some the best known is shown in Figure 2.13. Most are only effective when given by injection, but cephalexin and cefixime can be given by mouth. Cefuroxime is unaffected by many of the common β-lactamases and can be used against bacterial

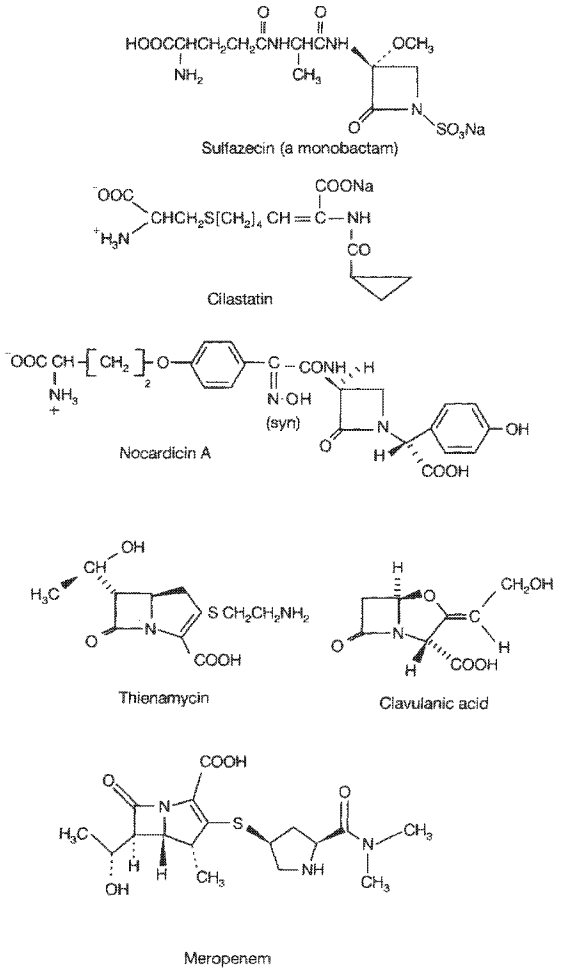


FIGURE 2.14 Additional β-lactam compounds and cilastatin, an inhibitor of mammalian metabolism of thienamycin. Clavulanic acid is an inhibitor of serine-active-site β-lactamases.

strains which are resistant to other β-lactam antibiotics; it can be useful in infections that are due to *Neisseria* or *Haemophilus*. The related compound, cefotaxime, has enjoyed considerable success. Other agents such as ceftazidime and ceftriaxone are useful because of the former's improved antipseudomonal activity and the latter's enhanced half-life in the body, which permits a more convenient dosing schedule, for example, once or twice daily.

The cephamycins resemble the cephalosporins, but have a methoxy group in place of hydrogen at position 7. Cefotetan (Figure 2.13) is a semisynthetic derivative of cephamycin C. The cephamycin derivatives are not readily attacked by β-lactamases and have advantages over the cephalosporin derivatives, with activity against *Proteus* and *Serratia* species.

The enormous success of the penicillins and cephalosporins stimulated a search for other naturally occurring β-lactam compounds. These have been found in a variety of micro-organisms. Some of the most interesting are shown in Figure 2.14. In the carbapenem, thienamycin, the sulfur atom is not part of the ring, but is found in the side chain. This compound is remarkable for its high potency, broad antibacterial spectrum and resistance to β-lactamase attack, but it is both chemically unstable and susceptible to degradation by a dehydropeptidase found in the kidneys. The *N*-formimidoyl derivative of thienamycin is chemically more stable but must administered as a 1:1 mixture with cilastatin (Figure 2.14), an inhibitor of the renal peptidase. A further development in the carbapenem series has been the appearance of the synthetic compound meropenem (Figure 2.14). This drug is not readily degraded by renal peptidase and can therefore be administered as a single agent. Meropenem is active against Gram-positive and Gram-negative pathogens, including many which are resistant to other β-lactams.

Other β-lactam antibiotics include the monobactams (e.g. sulfazecin, Figure 2.14); the name comes from monocyclic bacterial β-lactams) which are derived from bacteria and represent the simplest β-lactam structures with antibacterial activity so far discovered. Many semisynthetic derivatives have been made and exhibit excellent anti-Gram-negative activity, with much weaker activity against Gram-positive bacteria. In contrast, the monocyclic nocardicins (Figure 2.14) appear to offer less activity and are of more historic than clinical interest. Interest in the β-lactam family remains intense and novel drugs with improved properties continue to be developed.

2.4.6 Mode of action of penicillins and cephalosporins

As with many other antibiotics, early attempts to discover the biochemical action of penicillin led to conflicting hypotheses. Gradually it became accepted that the primary site of action lay in the production of cell wall material, and more specifically in the biosynthesis of peptidoglycan.

Evidence for this site of action rests on several different types of experiment. *Staphylococcus aureus* cells were pulse-labelled with [^{14}C]glycine, and peptidoglycan was isolated from their walls after a further period of growth in unlabelled medium. The labelled glycine entered the pentaglycyl 'extending group'. The polysaccharide backbone of the peptidoglycan was then broken down by an *N*-acetylmuramidase, leaving the individual muramyl peptide units linked only by their pentaglycine peptide chains. After the products were separated by gel chromatography, radioactivity was found in a series of peaks of increasing molecular weight representing the distribution of the pulse of [^{14}C]glycine among peptide-linked oligomers of varying size. A parallel experiment done in the presence of penicillin showed the radioactivity to be associated largely with a single peak of low molecular weight, presumably the un-cross-linked muramyl peptide unit, with much less radiolabel in the oligomers. The penicillin had thus inhibited the peptide cross-linking.

In another experiment, 'nucleotide pentapeptide' was prepared with [^{14}C]alanine. This was used as a substrate for an enzyme preparation from *Escherichia coli* in the presence of UDP-*N*-acetylglucosamine. This system carried out the entire biosynthesis of peptidoglycan, including the final stage of cross-linking. Peptidoglycan was obtained as an insoluble product containing [^{14}C] from the penultimate D-alanine of the substrate; the terminal D-[^{14}C]alanine was released into the medium, partly from the transpeptidase cross-linking reaction and partly from a carboxypeptidase

that removed terminal D-alanine residues from cross-linked products. In a parallel experiment, penicillin was added at a concentration that would inhibit growth of *Escherichia coli*. Biosynthesis of peptidoglycan then proceeded only to the stage of the linear polymer (IX, Figure 2.6), which was isolated as a water-soluble product of high molecular weight labelled with [^{14}C]. No D-[^{14}C]alanine was liberated because the penicillin suppressed both the cross-linking transpeptidase reaction and the action of DD-carboxypeptidase.

The understanding of the mechanism of β-lactam action was considerably advanced by the discovery of the penicillin-binding proteins referred to in Section 2.3.5. Of the PBPs in *Escherichia coli* and many other bacteria, PBP1a and PBP1b are the key enzymes involved in peptidoglycan biosynthesis. PBP2 and PBP3 are concerned, respectively, with remodelling of the peptidoglycan sacculus during septation and cell division. All these PBPs are targets of β-lactam antibiotics. Different β-lactams exhibit different affinities for the various PBPs and these can in turn be correlated with different morphological effects. Drugs which bind most strongly to PBPs 1a and 1b cause cell lysis at the lowest antibacterial concentration. Compounds such as the cephalosporin, cephalexin, bind more strongly to PBP3 and inhibit septation, leading to the formation of filaments, which are greatly elongated cells. Another variation is found with mecillinam, which binds almost exclusively to PBP2 and causes cells to assume an abnormal ovoid shape. Cells overproducing PBP2 have enhanced amounts of cross-linked peptidoglycan and are very sensitive to mecillinam.

The interaction of a penicillin or cephalosporin (I) with the enzyme (E) can be represented as:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftarrows}} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E + \text{degraded inhibitor.}$$

The first step is reversible binding to the enzyme. The second stage, involving chemical modification of the inhibitor with covalent binding to the enzyme, is irreversible, as is the final stage of enzyme release. For high antibacterial activity, k_3 should be rapid, preventing release of inhibitor through reversal of the initial binding, and k_4 should be slow to maintain the enzyme in the inactive EI* form and to avoid significant reactivation. Measurements show that the widely used β-lactam antibiotics have just such characteristics, and this scheme goes far to explain their outstanding effectiveness. There is good reason to suppose that the inactivation mechanism is the same with cross-linking transpeptidases as with DD-carboxypeptidases. The nature of the end products of penicillin degradation depends on the enzyme involved. It may be a simple opening of the β-lactam ring to give the penicilloate or there may be more extensive breakdown leading to the production, from benzylpenicillin, of phenylacetyl glycine. Those enzymes which yield penicilloate are equivalent to slow-acting β-lactamases. There is evidence to suggest that active β-lactamases are relatives of carboxypeptidases and transpeptidases in which reaction k_4 is rapid instead of very slow.

The mechanism of action of DD-carboxypeptidases and cross-linking transpeptidases resembles that of certain esterases and amidases. These enzymes possess reactive groups associated with their active centres, which undergo transient acylation in the course of enzymic action. Antibiotics containing a β-lactam ring behave chemically as acylating agents. The action of penicillin on the PBPs thus involves acylation of the enzymically active site in the second reaction to form the inactive complex EI*. This explanation was supported by experiments with purified DD-carboxypeptidases from *Bacillus stearothermophilus* and *Bacillus subtilis*. The enzyme was allowed to react briefly with [^{14}C]benzylpenicillin or with a substrate analogue, [^{14}C]Ac_2L-Lys-D-Ala-D-lactate; D-lactic acid is the exact hydroxyl analogue of D-alanine, and use of this derivative enabled the transient enzyme reaction intermediate to be trapped. In peptide fragments from the *Bacillus stearothermophilus* enzyme, radioactivity was found in a peptide with 40 amino acid residues and the label was shown to be associated with the same specific serine residue, whether the reactant was benzylpenicillin or the substrate analogue. Similar results were found with the *Bacillus subtilis* enzyme from which a labelled 14-unit peptide was isolated. This peptide showed extensive homology with 14 residues of the *Bacillus stearothermophilus* peptide and the label was associated with the corresponding serine residue. It was concluded that penicillin binds to the active site and acylates the same serine as the substrate. Unlike the substrate, the degraded penicillin

was released very slowly (reaction k_4 in the scheme shown) and thus blocked further access of substrate to the site.

How can this action of penicillin be related to its structure? The most widely quoted explanation depends on the similarity of the spatial orientation of the principal atoms and polar groups in the β-lactam nucleus to one particular orientation of the D-alanyl-D-alanine end group of the pentapeptide side chain of peptidoglycan precursors (see Figure 2.15). When the two structures are compared, the peptide bond between the alanine units is seen to correspond in position to the C—N bond in the β-lactam ring which is believed to be responsible for the acylating activity. Such a group bound to the cross-linking transpeptidase close to its active centre could well usurp the acylating function implicit in the normal reaction of the substrate with the enzyme. When the structures (Figure 2.15) are compared more critically, it becomes apparent that the agreement between them is imperfect but can be much improved if the peptide bond of the D-alanyl-D-alanine end group is represented, not in its normal planar form, but twisted nearly 45° out of plane. This may imply that the conformation of the penicillin molecule resembles the transition state of the substrate rather than its resting form. During the enzymic transpeptidation, the peptide bond quite possibly undergoes this sort of distortion.

An alternative model is based on a comparison of certain electrostatic potentials of benzylpenicillin and synthetic *N*-acyl-D-alanyl-D-alanine peptides. Calculation of these potentials reveals a significant similarity in the coplanarity of key electrostatic negative wells of both benzylpenicillin and the dipeptide terminal. The coplanarity of these wells may facilitate the attack of an electrophilic centre in the catalytically active serine of the target PBP on the β-lactam C—N bond. With some modifications this model may be applicable to all

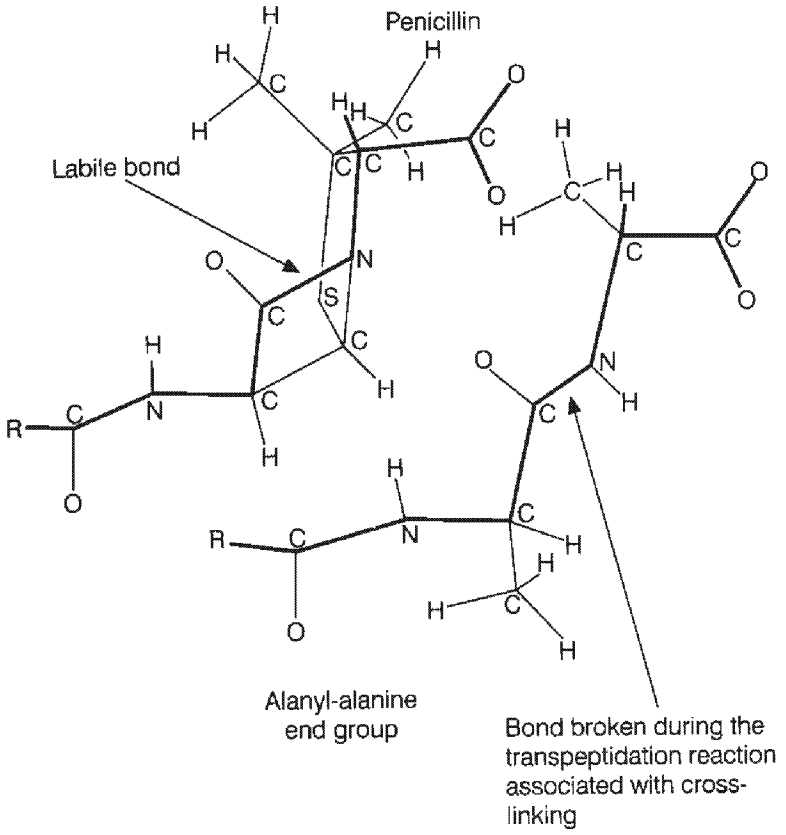


FIGURE 2.15 Comparison of the structures of penicillin with that of the D-alanyl-D-alanine end group of the peptidoglycan precursor. [Reproduced by permission of the Federation of American Societies for Experimental Biology from J. L. Strominger *et al. Fed. Proc.* **26**, 18 (1967).]

types of β-lactam drug. However, the precise details of the interactions between β-lactam and PBPs will have to await data from the various on-going X-ray crystallographic studies of β-lactam–PBP complexes.

2.5 Drugs that interfere with the biosynthesis of the cell wall of mycobacteria

Mycobacteria are responsible for two devastating diseases: tuberculosis (*Mycobacterium tuberculosis*) and leprosy, or Hansen's disease (*Mycobacterium leprae*). The cell wall of mycobacteria is remarkably complex and underlies many of the characteristic properties of these organisms, including their extremely low permeability and intrinsic resistance to commonly used antibiotics. The reader is referred to a review provided in 'Further reading' at the end of this chapter for detailed information on the cell wall of mycobacteria. A key feature of the mycobacterial cell envelope that distinguishes it from most other bacteria is the mycolyl–arabinogalactan–peptidoglycan complex. Arabinogalactan is linked to the peptidoglycan through a phosphodiester link between the C-6 of 10–14% of the muramic acid residues and a disaccharide linker unit attached to the galactan. Arabinogalactan itself is a unique polysaccharide consisting of linear galactan chains composed of alternating 5- and 6-linked β-D-galactofuranose units which in turn are linked though C-5 of some of the 6-linked galactofuranose units to extensively branched chains of D-arabinofuranose (arabinan). Approximately two-thirds of the nonreducing terminals of arabinan are esterified to long-chain mycolic acids. There are other lipids in the mycobacterial cell outer envelope in addition to the mycolic acids, including a range of complex glyco- and peptidolipids. The lipoidal nature of this complex wall is a significant contributor to the impermeability of mycobacteria to many solutes, including some antibiotics. The characteristically slow growth rate of mycobacteria also presents a considerable challenge to the successful chemotherapy of infections caused by these bacteria, which usually requires several months of continuous drug treatment.

2.5.1 Isoniazid

Isoniazid (Figure 2.16) provides one of the foundations of combination therapy for tuberculosis. The relative ease with which *Mycobacterium tuberculosis* becomes resistant to individual drugs led to the concept of combining several chemically distinct drugs with, as it later turned out, different modes of action. In combination variously with rifampicin, ethambutol, pyrazinamide and occasionally streptomycin, isoniazid is an effective antitubercular drug which has been in use since 1952. However, it is only since the 1990s that biochemical and genetic data have revealed the molecular mechanisms underlying the antimycobacterial action of isoniazid.

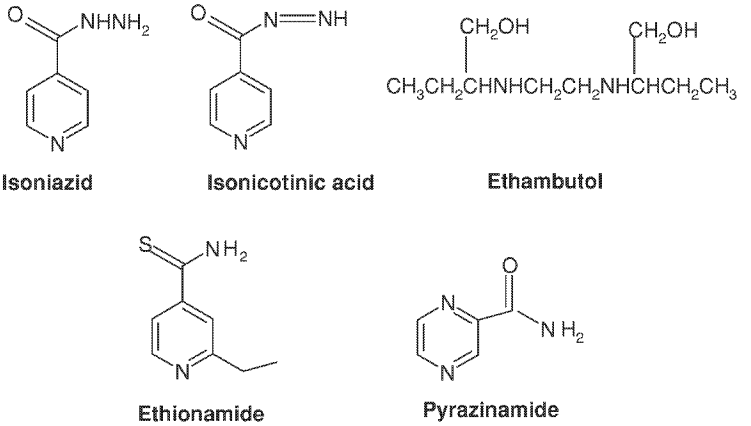


FIGURE 2.16 Structures of synthetic compounds used in combination therapy of tuberculosis. The structure of the microbial metabolite isonicotinic acid can be seen to resemble that of isoniazid.

In the mycobacteria, the *inhA* gene encodes an enzyme that has been identified as a major molecular target for isoniazid and the structurally related drug, ethionamide (Figure 2.16). This enzyme, abbreviated to InhA, catalyzes the NADH-dependent reduction of the 2-*trans*-enoyl-acyl carrier protein (ACP), an essential reaction in the elongation of fatty acids. Long-chain substrates containing between 16 and 18 carbon atoms are preferentially used by InhA, an observation which implicates the reductase in the biosynthesis of the mycolic acids. Inhibition of the biosynthesis of mycolic acids therefore disrupts the assembly of the mycolyl-arabinogalactan-peptidoglycan complex and causes the loss of cell viability. While mutations in *inhA* confer resistance to isoniazid, studies with recombinant InhA show that isoniazid itself is only a weak inhibitor of the enzyme. The drug is in fact first converted by oxidative cellular metabolism to a reactive metabolite which is believed to bind to and inhibit the reductase in the presence of NADH bound to the enzyme. Isoniazid is metabolically unstable in mycobacteria, owing to the activity of a unique mycobacterial catalase–peroxidase encoded by the *katG* gene. Studies with the recombinant form of this enzyme show that it converts isoniazid to several chemically reactive derivatives, isonicotinic acid (Fig 2.16) being the major product. The electrophilic nature of these compounds would enable them to acylate or oxidize vulnerable amino acid residues in the target reductase, although direct evidence for this is lacking. The two-stage concept of the mechanism of action of isoniazid is strengthened by the existence of two forms of mycobacterial resistance to the drug. One type of mutant has a defective *katG* gene that precludes the conversion of the prodrug to its active form. The second resistant phenotype depends on an isoniazid-resistant variant of InhA that is characterized by a markedly lower affinity for NADH, which minimizes the attack of the isoniazid metabolite on the enzyme.

Recently, the product of another gene, *kasA*, has been proposed as an alternative primary site of action for isoniazid. The *kasA* gene encodes the enzyme β-ketoacyl synthase (KasA), which may be involved in the biosynthesis of C_{18}–C_{34} fatty acids required for the elaboration of mycolic acids. The case for the KasA enzyme as a primary target for isonaizid rests largely on isoniazid-resistant clinical isolates of mycobacteria with mutations solely in the *kasA* gene, i.e. with no mutations in either *inhA* or *katG* genes. However, there is contrary evidence of clinical isolates with mutations in *kasA* which retain sensitivity to isoniazid. In summary, the weight of experimental and observational evidence supports the concept of the InhA enzyme as the primary target for isoniazid (and also ethionamide), although a possible contribution from KasA cannot be ruled out at this stage.

2.5.2 Ethambutol

The antibacterial activity of isoniazid is confined to *Mycobacterium tuberculosis*. Ethambutol (Figure 2.16), which has been in clinical use against tuberculosis since 1961, has a broader spectrum of action, including *Mycobacterium avium*, a serious opportunist pathogen in patients with AIDS. Despite many years of use, the molecular basis of the bacteriostatic action of ethambutol was identified only recently. It had long been known that the drug in some way blocked the biosynthesis of the polysaccharide arabinan, but the actual mechanism was not known. The target for ethambutol was eventually established by cloning the genetic elements responsible for resistance to this drug in *Mycobacterium avium*. The structural genes *embA*, *embB* and *embC* all encode arabinosyl transferases which appear to have similar functions in polymerizing arabinose into arabinan. *In vitro* evidence obtained with a crude broken cell preparation from *Mycobacterium smegmatis* indicates that ethambutol inhibits the transfer of a hexa-arabinosfuranosyl unit, from the phospho-decaprenol carrier complex, to arabinan. Most clinical isolates of *Mycobacterium tuberculosis* that are resistant to ethambutol have mutations in *embB*. The molecular target of ethambutol therefore seems to be arabinosyl transferase, with the product of the *embB* gene being the most important. The mechanism of inhibition of the arabinosyl transferases by ethambutol remains to be established and will probably await the purification of the enzymes, which are predicted to be integral membrane proteins with multiple anchoring, transmembrane domains and an external domain.

Disruption of the biosynthesis of the arabinogalactan component of the mycobacterial cell enve-

lope may increase cellular permeability to other drugs. This could account for the valuable clinical synergism that is achieved when ethambutol is combined with a drug of large molecular size such as rifampicin.

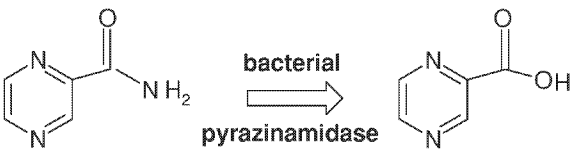
2.5.3 Pyrazinamide

Although first recognized for its substantial antimycobacterial activity in the 1950s, this synthetic drug was not introduced into the combination therapy for tuberculosis until the mid-1980s. Pyrazinamide (Figure 2.16) is a bacteriostatic agent which is especially useful against semidormant populations of *Mycobacterium tuberculosis* located in acidic intracellular compartments such as the phagolysosomes of macrophages.

The active form of pyrazinamide is believed to be pyrazinoic acid (Figure 2.17), formed by the action of an intracellular bacterial amidase, referred to as pyrazinamidase. Some pyrazinamide-resistant strains of *M. tuberculosis* lack pyrazinamidase activity. Genetic and biochemical evidence strongly suggests that the antibacterial activity of pyrazinamide rests upon the inhibition by pyrazinoic acid of a multifunctional fatty acid synthase (type I) encoded by the *fasI* gene, which results in suppression of mycolic acid biosynthesis. By inhibiting the type I fatty acid synthase (FAS I), pyrizinamide blocks the provision of fatty acid precursors for another fatty acid synthase (FAS II) which has an essential role in the elongation of mycolic acids.

2.6 The fungal cell wall as a target for antifungal drugs

Fungal infections (mycoses) pose an ever-increasing threat to health across the world. Immunocompromised individuals, including AIDS patients, those on immunosuppressive drugs following organ transplantation, cancer patients undergoing chemotherapy, people recovering from major surgery and patients receiving prolonged antibacterial treatment, are all at risk from infections caused by a variety of fungal pathogens. Compared with the wealth of drugs available to treat bacterial infections, the current therapeutic options for fungal infections are much more limited.



Pyrazinamide Pyrazinoic acid

FIGURE 2.17 Conversion of the prodrug pyrazinamide to the active molecule by bacterial amidase.

Although it serves functions analogous to those of the bacterial cell wall, the structure of the fungal wall is very different from that of its bacterial counterpart. Critically, fungal walls do not contain peptidoglycan, so neither β-lactam nor glycopeptide antibiotics have any effect on the viability of fungi. The fungal wall is a multilayered structure whose major macromolecular components include chitin, glucan and mannoproteins. Neither chitin nor glucan occurs in mammalian or bacterial cell walls, so the biosynthesis of these materials provides potential targets for specific antifungal drug action. Because glycosylated proteins are found in all eukaryotes, the biosynthesis of fungal mannoproteins may be rather less attractive as a target for chemotherapy. However, the sugar residues of glycosylated proteins are very different in fungi and humans and could conceivably offer opportunities for drug design. The composition and organization of the cell wall vary significantly among the various fungal species and define the identity of the organisms. Chitin is a linear 1,4-β-linked homopolymer of *N*-acetylglucosamine. In yeasts chitin contributes as little as 2% to the cell wall mass, while there can be as much as 60% chitin in some mycelial fungi. Nevertheless, chitin is essential for fungal growth, even in species with very small amounts of the polymer. Glucan is a β-1,3-linked linear glucose homopolymer with varying amounts of β-1,6- and β-1,4-glucose side chains, depending on the species. The mannoproteins make up complex chains of mannose linearly bonded by 1,6-links to which oligomannoside side branches are attached by 1,2- and 1,2-α bonds. The polysaccharide structures are covalently linked to protein via a 1,4-β-disaccharide of *N*-acetylglucosamine residues by either *N*-glycosylation of asparagine or *O*-glycosylation at the free hydroxyl groups of threonine or serine residues. Some idea of the diversity of mannoproteins

may be gauged from the fact that between 40 and 60 different mannoproteins can be isolated from yeast cell walls.

The arrangement of these various polymers in the wall of the important fungal pathogen *Candida albicans* is illustrated in Figure 2.18. The insoluble polymers chitin and glucan confer mechanical strength on the wall. The function of the mannoproteins is less clear but appears to be essential because inhibitors of *N*- and *O*-glycosylation are lethal, although it should be remembered that the effects of inhibition of these reactions are not confined to the biosynthesis of mannoproteins.

2.6.1 Inhibitors of chitin biosynthesis

The enzyme chitin synthase catalyzes a reaction in which an *N*-acetylglucosamine residue is transferred from the donor molecule, UDP-*N*-acetyl glucosamine, to the nonreducing end of the growing chitin chain, with the concomitant release of UDP. Chitin synthase exists in several forms, none of which has been purified so far. Two related groups of antibiotics inhibit chitin synthase, the polyoxins and nikkomycins (Figure 2.19). Both types are analogues of UDP-*N*-acetylglucosamine and presumably inhibit the enzyme by competition with this substrate. In the yeast *Saccharomyces cerevisae,* the gene encoding chitin synthase 1 is essential for repairing damage to the intercellular septum incurred during the separation of daughter cells. The product of the chitin synthase 2 gene is specifically involved in the biosynthesis of the septum itself while that of the chitin synthase 3 gene produces most of the chitin in the bud scar and lateral cell wall. No single synthase appears to be essential for cell viability, but the loss of all three in mutants of *Saccharomyces cerevisiae* is lethal. Chitin synthase exists in multuple isozymic forms in *Candida albicans* and possibly in other pathogenic fungi.

The susceptibility of fungi to polyoxins and nikkomycins varies considerably and may be due to differences in the distribution and sensitivities of the chitin synthase isoforms to these antibiotics. Another factor which determines susceptibility to polyoxins is their transport into fungal cells by a permease that normally carries dipeptides. *Candida albicans* is intrinsically resistant to polyoxins because of the low activity of this permease. Despite these potential problems, it is hoped that the best of the currently available inhibitors of chitin synthase, nikkomycin Z, may eventually find a place in clinical medicine. The essential role of chitin in fungi has encouraged a search for other, more effective inhibitors of the chitin synthases, so far

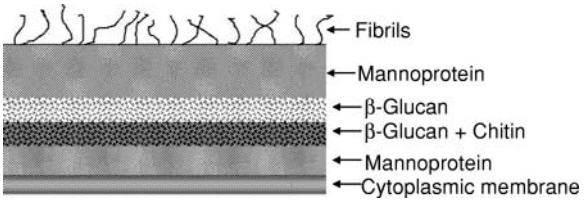


FIGURE 2.18 The general arrangement of layers in the fungal cell envelope. The components are not drawn to scale. It should be remembered that the precise structure of the fungal cell envelope is markedly species-specific.

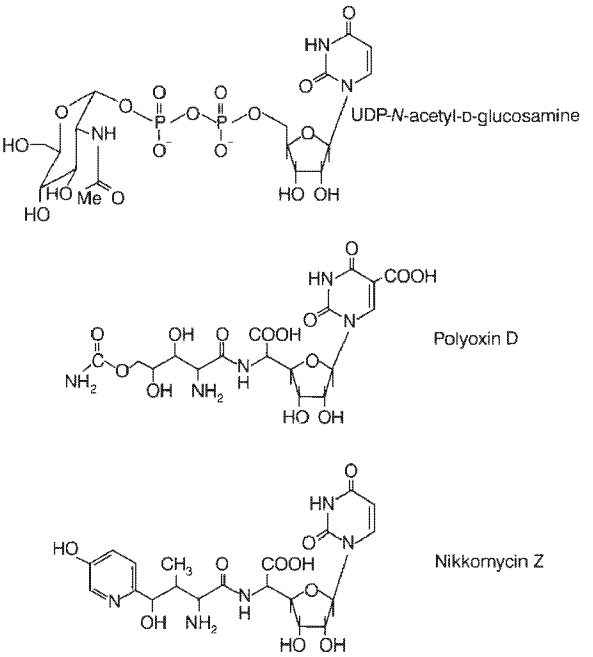


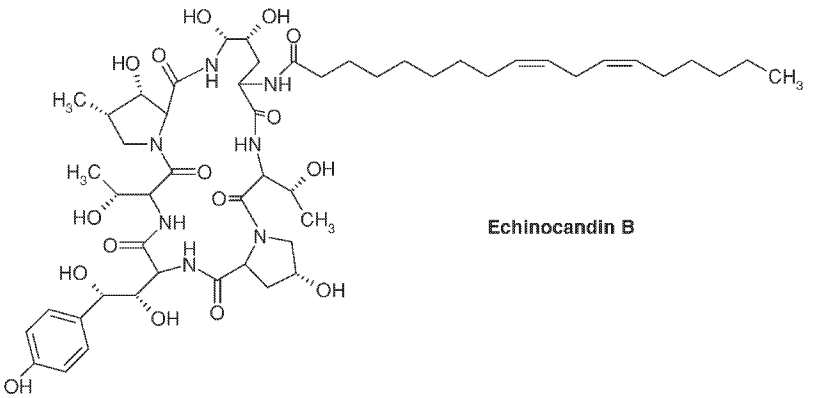
FIGURE 2.19 Antifungal agents that inhibit cell wall chitin synthesis, together with the substrate UDP-*N*-acetylglucosamine.

it must be said, with little success. Purification of the enzymes and the provision of adequate amounts for screening purposes are likely to be prerequisites for further progress.

2.6.2 Inhibitors of glucan biosynthesis

In glucan biosynthesis, the enzyme 1,3-β-glucan synthase catalyzes the transfer of glucose from UDP-glucose to the insoluble, growing glucan polymer. Unlike chitin synthase, glucan synthase has been purified to homogeneity. The enzyme consists of two subunits, one of which is an integral membrane protein, molecular mass 215 kDa, with multiple transmembrane helices. The other subunit is a much smaller protein (20 kDa) that interacts with GTP-binding proteins and is only loosely associated with the cell membrane. The function of the smaller subunit is apparently to activate the catalytic activity of the membrane-bound protein through interaction with the GTP-binding protein complexes. Two closely homologous forms of glucan synthase have been identified in *Saccharomyces cerevisiae*, designated as FKS1 and FKS2. FKS1 is dominant during vegetative growth whereas FKS2 has an essential role in sporulation. Genomic analysis of *Saccharomyces cerevisiae* predicts a third possible glucan synthase, FKS3, although it remains uncharacterized at present. Sequence-related glucan synthases have been found in other yeasts and in filamentous fungi. Five genes apparently encoding glucan synthases have been identified in the genome sequence of *Candida albicans*.

Echinocandin B (Figure 2.20) is a member of a large family of naturally occurring and semisynthetically modified lipopeptide antibiotics which have



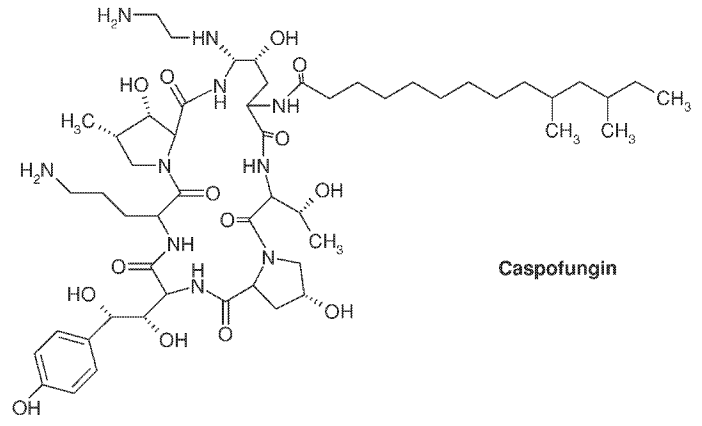


FIGURE 2.20 Inhibitors of the biosynthesis of the glucan polymer in fungal cell walls.

potent activity *in vitro* against *Candida* spp. and against the filamentous *Aspergillus* spp. These compounds are powerful non-competitive inhibitors of 1,3-β-glucan synthase. This specificity may explain the lack of activity against fungi where glucan is not mainly 1,3-β-linked. In *Cryptococcus* spp., for example, a dangerous pathogen affecting the respiratory tract, the glucan is mostly 1,3-α-linked and the organisms are resistant to the cyclic lipopeptide antibiotics. Studies with an echinocandin-resistant mutant of *Saccharomyces cerevisiae* identified the membrane-bound component of 1,3-β-D-glucan synthase as the likely target of the drug. Unfortunately the details of the inhibitory mechanism, including the site of interaction between the antibiotic and the enzyme, are not known at present. The clinical usefulness of echinocandin B is limited by its propensity to cause lysis of red blood cells, which is thought to be due to its extended lipophilic side chain. Another member of the echinocandin group, Caspofungin (Figure 2.20), recently entered clinical practice for the treatment of *Aspergillus* infections unresponsive to other drugs and disseminated *Candida* infections. Caspofungin is likely to have the same mode of action as echinocandin B. Another semisynthetic cyclic lipopeptide, Micafungin, has recently enetered clinical practice in Japan.

2.6.3 Disruption of the function of mannoproteins

Pradimicin A (Figure 2.21) belongs to a unique group of antibiotics originally isolated from *Actinomadura hibisca* and is active against *Candida* spp., *Cryptococcus* spp. and *Aspergillus* spp. The antifungal action involves a change in the permeability of the cell membrane, which may result fom the ability of pradimicin to form an insoluble complex with mannan in the presence of calcium ions. Although this points to some form of interference with mannoprotein function, the biochemistry of the antifungal action of pradimicin requires further investigation. As yet, this drug has not been used to treat fungal infections in human patients although it has shown promise in the treatment of experimental infections.

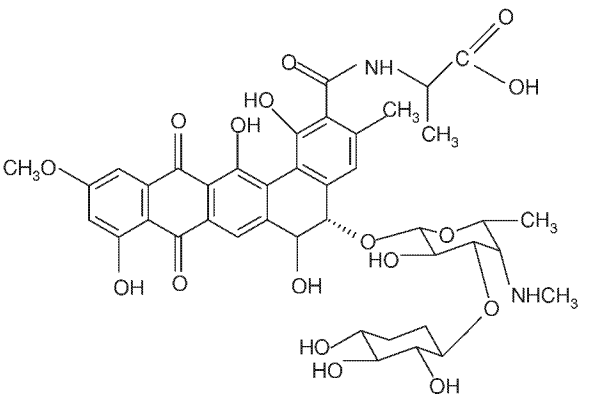


FIGURE 2.21 Pradimicin A, an experimental antibiotic active against several species of yeast pathogens. Its mode of action may depend upon interference with the function of cell wall mannoproteins.

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