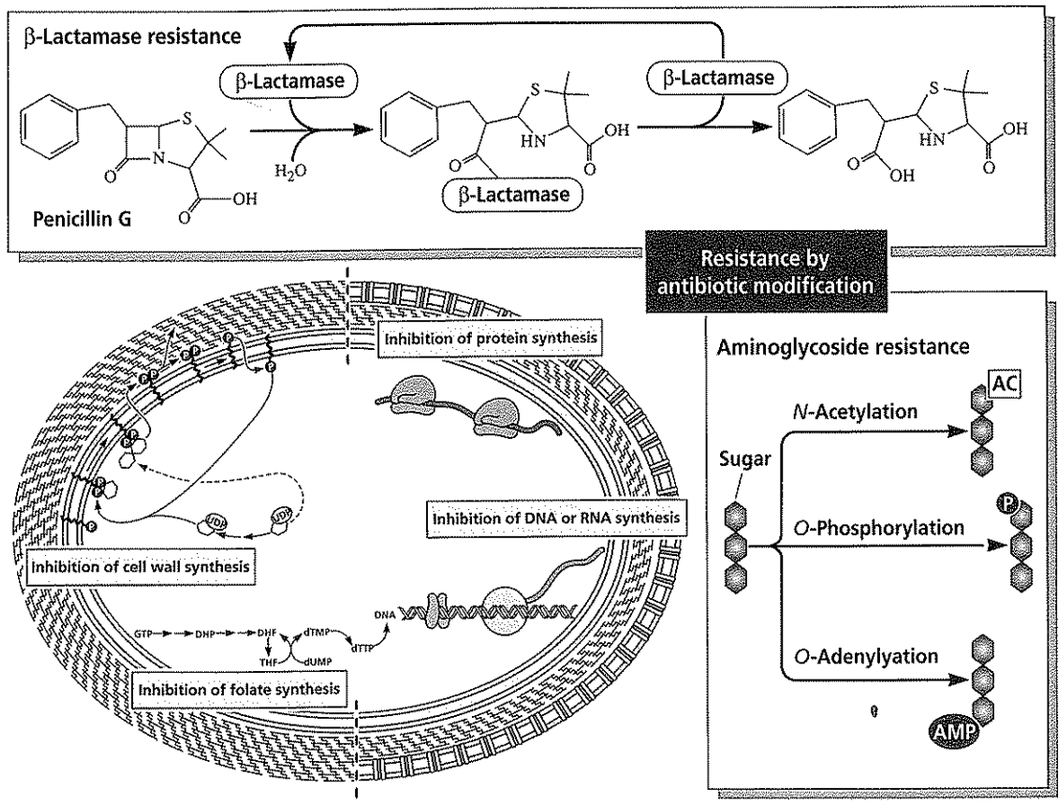


Modification of antibiotics by resistant bacteria.

chapter 8

Enzymatic Destruction or Modification of the Antibiotic by Resistant Bacteria

This chapter is the first of three (chapters 8 to 10) that deal with the three major mechanisms of antibiotic resistance. The chapter opening figure highlights a section of Fig. 2.2 that summarizes resistance by antibiotic modification.

Enzymatic inactivation of antibiotics occurs with several of the natural product antibiotic classes but has not yet been observed as a major route of resistance development for the classes of synthetic antibacterials: the sulfamethoxazole-trimethoprim combination, the fluoroquinolones, or the oxazolidinones. This may reflect the time of exposure of the bacteria to natural products, putatively hundreds of millions of years, versus the 70 years or less for the man-made antibiotics. This criterion might suggest that novel antibiotics made from libraries of synthetic chemicals not found in nature might also be slow to be inactivated by this mechanism. Of course, the other two inactivation routes, discussed in chapters 9 and 10, can still be in effect.

The most widespread mode of clinical resistance development to β-lactam antibiotics is the expression of β-lactamases that hydrolyze the antibiotic (Bush and Mobashery, 1998). An estimate of $30 billion in annual economic loss to the U.S. population from disease caused by lactamase-producing resistant bacteria has been suggested (Palumbi, 2001).

Destruction of β-lactam antibiotics by β-lactamases

Subfamilies of β-lactamases: active-site serine hydrolases

β-Lactamases hydrolyze the four-membered β-lactam ring in both penicillin and cephalosporin classes of antibiotics as well as the carbapenem series (**Fig. 8.1**). They thereby destroy the antibacterial activity by deactivating the chemical warhead in the molecule, the strained β-lactam that is the chemically reactive acylating group for modifying the active-site serine side chains in the penicillin-binding proteins (PBPs) (the transpeptidases and carboxypeptidases in peptidoglycan [PG] cross-linking; see chapter 3). β-Lactamase activity was detected

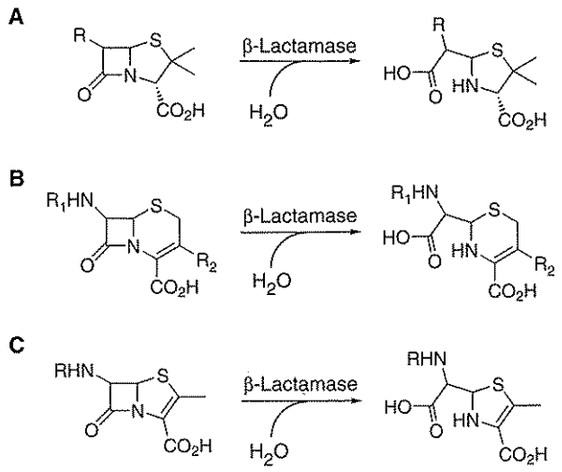


Figure 8.1 Hydrolytic ring opening and deactivation of (A) penicillins, (B) cephalosporins, and (C) carbapenems by β-lactamases.

a few years before clinical use of penicillins in humans, indicating its presence in soil bacteria that combat the natural product penicillins, and by now more than 190 β-lactamases have been described (Bush and Mobashery, 1998; Thomson and Moland, 2000) and categorized into class A, B, C, and D lactamases (Bush and Mobashery, 1998). The A, C, and D classes are active-site serine enzymes, with architectural and mechanistic similarities to the PBPs (Knox, 1995; Knox et al., 1996) (**Fig. 8.2**), suggesting evolution from PBPs.

In the A, C, and D classes of β-lactamases the same type of penicilloyl-*O*-Ser enzyme covalent intermediates are formed as in the catalytic cycle of PBPs that attack and open the β-lactam ring and become self-acylated (chapter 3). There is no such covalent penicilloyl enzyme intermediate in the catalytic cycle of the zinc-dependent, class B β-lactamases (**Fig. 8.3**), which has consequences for the failure of class B lactamases to be inhibited by certain drugs, as discussed below.

It has been argued that PBPs may have evolved into β-lactamases several times independently, to generate the different orientations of the active-site residues in the class A, C, and D lactamases (see Massova and Mobashery, 1998, and references therein). The difference in outcome, turnover for lactamases and suicide for the PBP transpeptidases, arises from the different lifetimes of the acyl-*O*-Ser enzymes. In the penicloyl-PBP acyl enzyme of the transpeptidases, water is excluded from the active site and hydrolysis is exceedingly slow (with a half-time for deacylation of about 90 min, compared to 4 ms for the *N*-acyl-D-Ala-D-Ala acyl enzyme from the normal substrate) (Fisher et al., 1980), and correspondingly the penicilloyl enzyme lifetime is long, the transpeptidase activity is inactivated, and PG cross-linking is halted (see Knox et al., 1996, for review). By contrast, the lactamase activity involves hydrolysis, not capture of the acyl-*O*-Ser enzyme by an amine; water has free access to the penicilloyl-*O*-Ser enzyme active site; and the deacylation rate is fast (**Fig. 8.4**), 2,600 s^{-1} for the TEM-1 β-lactamase. The net difference in deacylation rates of the same penicilloyl-*O*-Ser enzyme intermediate in the lactamase versus PBP is 2.7×10^7. The β-lactamase-producing gram-negative bacteria secrete this surveillance en-

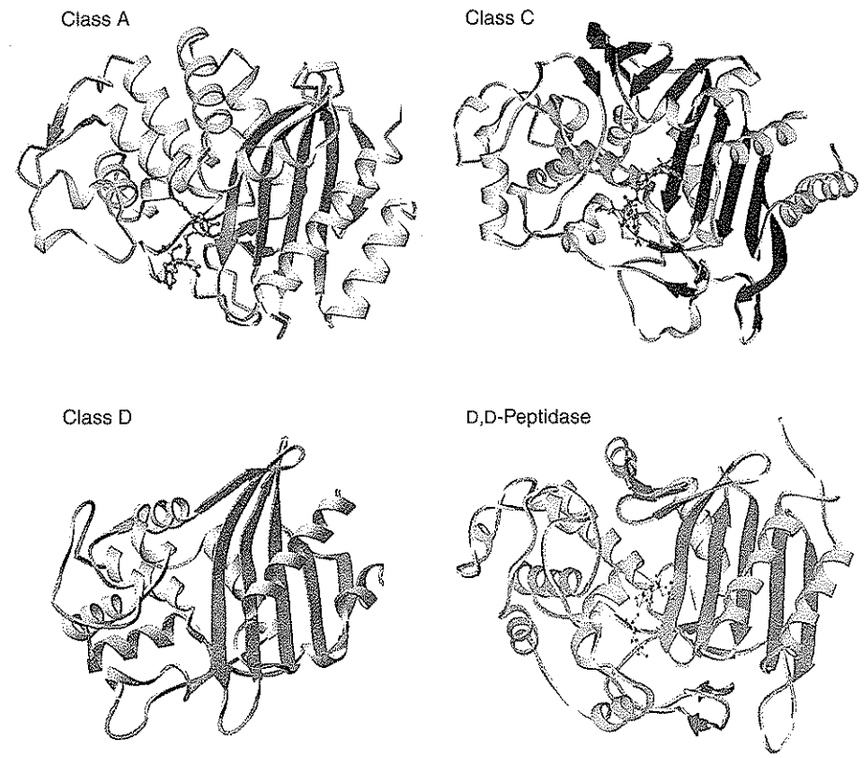
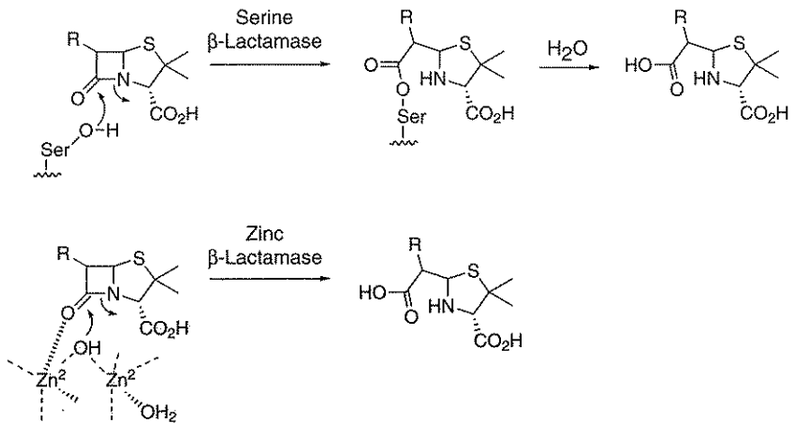


Figure 8.2 Structures of class A, C, and D β-lactamases and homology to the fold of a D,D-peptidase (PBP). (Figure provided courtesy of J. Knox.)

Figure 8.3 Hydrolysis of the β-lactam ring of penicillins by class A, C, and D lactamases involves covalent penicilloyl enzyme intermediates, while the class B zinc-dependent lactamases carry out direct attack by water.



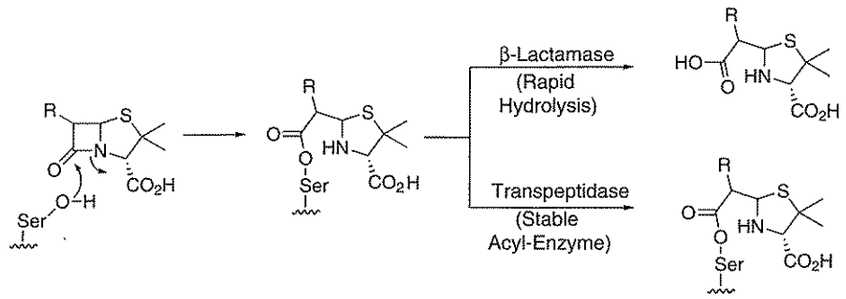
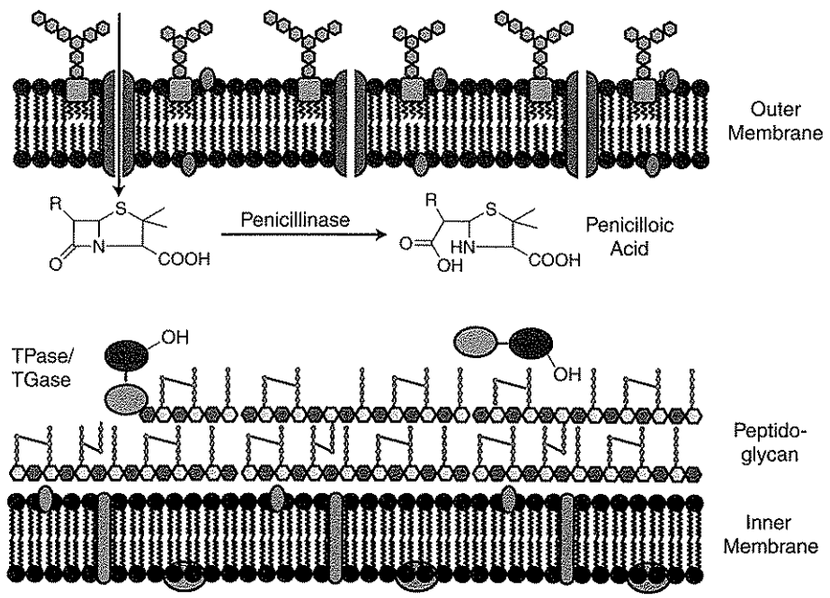


Figure 8.4 Different half-lives for the acyl-*O*-Ser enzyme intermediates control the outcomes with penicilloyl-PBPs versus penicilloyl-β-lactamases.

zyme into the periplasmic space so that β-lactam antibiotics have to run the gauntlet of these hydrolytic enzymes to reach their targets at the surface of the cytoplasmic membrane (**Fig. 8.5**), making it difficult for any intact β-lactam to reach its target PBP.

The TEM-1 (Datta and Kontomichalou, 1965) and related TEM-2 lactamases, prevalent in gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*, are encoded on transposable elements and move rapidly through these populations (Amyes, 2001; Wiedemann et al., 1989). Extended-spectrum cephalosporins such as ceftazidime and cefotaxime (structures in **Fig. 8.6**; also see chapter 3) were developed to combat resistance provided by TEM-1 and

Figure 8.5 β-Lactamases in bacterial periplasms hydrolyze penicillins and cephalosporins before they reach their target PBPs at the outer face of the cytoplasmic membrane. TPase/TGase, bifunctional transpeptidase/transglycosylase.



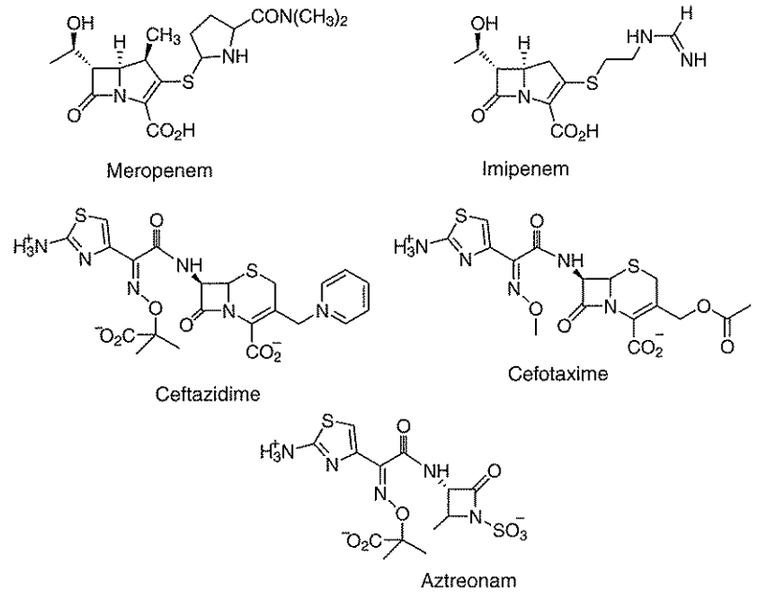


Figure 8.6 Structural modifications in the acyl side chains of β-lactam antibiotics to build in slow processing by β-lactamases. X-ray analysis of extended-spectrum β-lactam antibiotics with β-lactamase cocrystals shows that the bulky side chain provides a severe steric block to proper positioning of water in the deacylation step and accounts for the very low k_{cat}s for enzymatic hydrolysis.

related β-lactamases. In turn, subsequent widespread cephalosporin use is thought to have selected for sequential mutants in the TEM lactamases, producing hydrolytic enzymes that have improved affinity for these lactam scaffolds and consequent extended-spectrum lactam resistance (see chapter 17). Many variants of TEM lactamases have been isolated and sequenced (e.g., Goussard and Courvalin, 1999). For recent progress on determination of X-ray structures of lactams and lactam-derived acyl enzyme intermediates in the active site of lactamases, see Beadle et al. (2002).

Metallo-β-lactamases: zinc-hydrolases

The class B lactamases are zinc enzymes, containing a binuclear zinc cluster in the active site (Toney et al., 1998; Wang et al., 1999). Unlike the class A, C, and D lactamases, which do lactam ring opening via covalent acyl enzyme intermediates, noted above, the class B lactamases use zinc to activate a water molecule and catalyze its direct addition to the β-lactam ring (Fig. 8.3). The metallo-β-lactamases of type B are thought to be the major subclass of hydrolases that destroy the carbapenem antibiotics such as imipenem (thienamycin) and meropenem (Fig. 8.6). The widespread use of carbapenems in Japan (Kurokawa et al., 1999) has probably been instrumental in selecting for the IMP-1 version of the zinc-β-lactamase first seen in *Serratia marcescens* and *Pseudomonas aeruginosa*. The carbapenemases have been described as a clinical problem in waiting for pseudomonal infections (Livermore and Woodford, 2000) but the more acute

carbapenem resistance problems in *P. aeruginosa* are efflux mechanisms, as noted in chapter 9. Many bacteria that produce the type D metallohydrolases also produce a type A, C, or D lactamase (Rasmussen and Bush, 1997); for example, a clinical isolate of *S. marcescens* carries a type A and a type B *bla* gene on a plasmid (Yano et al., 1999).

Strategies to neutralize β-lactamases

Two approaches have been taken in the decades since lactam-resistant clinical isolates began to diminish the efficacy of penicillins and cephalosporins as antibiotics. The first has been to develop semisynthetic β-lactams which were slower substrates for attack by the hydrolytic lactamases. The second approach has been to screen for inhibitors and inactivators of lactamase activity and then combine these molecules with a β-lactam. Both approaches have had their successes (Knowles, 1985).

Slow substrates for the β-lactamases

The search for semisynthetic lactams that would retain antibiotic potency but have increased efficacy against lactamase producers turned up several molecules that made it into clinical therapeutics. Essentially this is a strategy to find substituents on the β-lactam chemical warhead that block β-lactamase binding and/or catalysis but do not interfere with PBP binding and acylation. The monocyclic monobactams fall in this category, aztreonam being an example (Fig. 8.6). Also in this category are the carbapenems, with sulfur in the five ring replaced by carbon (Fig. 8.6); this is the strategy in meropenem and the thienamycin component of imipenem. Much of the effort to find lactams that would not be hydrolytic substrates for lactamases focused on the cephalosporin 4/6 bicyclic scaffold with alteration of the acyl side chain, leading to such drugs as ceftazidime and cefotaxime (Fig. 8.6) that extended the spectrum of antibiotic activity to treat many β-lactamase-producing pathogens (also see chapter 3). The rationale was that the bulky acyl side chains on the 7-amino group of the lactam scaffold permitted formation of the acyl-PBP intermediates but blocked the processing by the lactamases.

The carbapenem thienamycin is a slow substrate for lactamase hydrolysis for a different reason. The initial acyl enzyme intermediate (**Fig. 8.7**) can undergo a double-bond isomerization in the five-membered ring from a Δ^2 to a Δ^1 olefin, an eneamine to an imine, and the latter form of the acyl enzyme is slower to hydrolyze by 50,000-fold (see Massova and Mobashery, 1998). The stereochemistry of the hydroxyethyl side chain, 1R instead of the usual 1S in penicillins, is also an important determinant for blocking water attack in the deacylation of the acyl enzyme. Slow hydrolysis means a long lifetime for the acyl enzyme, so the catalytic destructive power of the lactamase is slowed by orders of magnitude while it is tied up in this covalent adduct. A variant of thienamycin, meropenem (see Mitscher et al., 1999) (Fig. 8.6), has a methyl substituent on the five ring to provide steric hindrance to binding to β-lactamases.

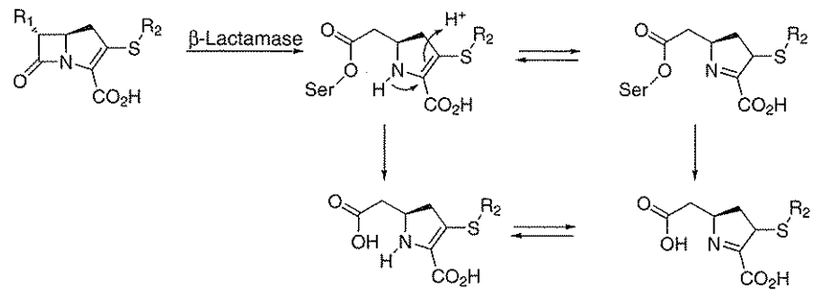
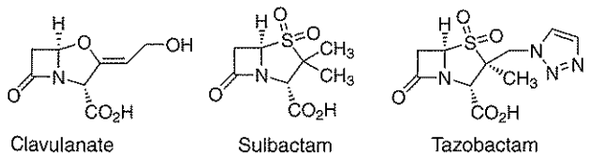


Figure 8.7 Isomerization in the ring-opened acyl enzyme form of the carbapenem thienamycin during destruction by β-lactamase slows net hydrolysis.

Mechanism-based inactivators of β-lactamases

The second approach, screening for mechanism-based inactivators of the β-lactamases, builds philosophically on the pattern seen with the carbapenem-derived acyl enzyme. It relies on a rearrangement of the initial acyl-*O*-lactamase covalent enzyme into an alternate covalent acyl enzyme form that is much slower to hydrolyze. Two types of mechanism-based inactivators, or suicide substrates, for β-lactamases have become clinically successful (Maiti et al., 1998). The first is the natural product clavulanate, an enol ether–β-lactam from *Streptomyces clavuligerus*, and the second class is represented by penicillin sulfone and a substituted congener tazobactam (**Fig. 8.8**). In both clavulanate and the penicillin sulfones, the structural alterations weaken the C-O bond or the C-S bond, respectively, such that the attack by the lactamase active-site serine-OH on the β-lactam carbonyl leads also to fragmentation of the 4/5 ring junction, as indicated in **Fig. 8.9**. Subsequent rearrangements can follow, with further fragmentation and accumulation of a rearranged acyl enzyme (Massova and Mobashery, 1998). The net effect in the clavulanate processing by class A β-lactamases is a conjugated acyl enzyme much less rapidly attacked by water for deacylation and a comparable deactivation of the subsequent ring-opened acyl enzyme derived from the penicillin sulfones. More-stable acyl enzyme forms of lactamase means this antibiotic-destroying catalyst is tied up in knots as long as the acyl enzyme persists. Neither clavulanate nor the sulfones are potent enough as β-lactam antibiotics to be used on their own so they are used in combinations (Fig. 8.8). For example, the combination of amoxicillin and clavulanate, known as Aug-



Clavulanate-Amoxicillin → Augmentin
Clavulanate-Ticarcillin → Timentin
Sulbactam-Ampicillin → Unasyn
Tazobactam-Piperacillin → Zocin

Figure 8.8 Clavulanate, sulbactam, and tazobactam: mechanism-based inactivators of β-lactamases.

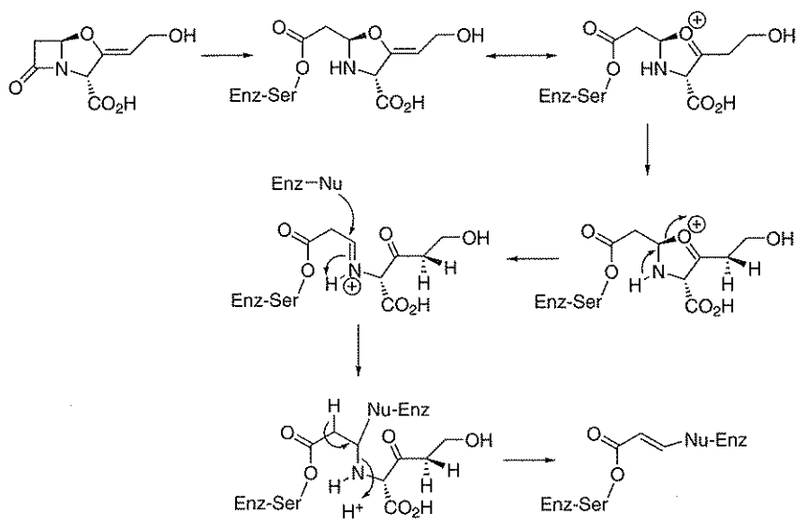


Figure 8.9 Rerouting of the acyl enzyme intermediate by clavulanate and penicillin sulfone to inactivate β-lactamases.

mentin, for the augmenting powers that clavulanate confers to amoxicillin, has been the most widely used form of penicillin in recent years. In the scheme of Fig. 8.5, clavulanate will inactivate enough penicillinase (β-lactamase) molecules to allow amoxicillin to survive in a β-lactamase-producer's periplasm to cross that space intact. Amoxicillin can thus run the gauntlet and reach its PBP targets at the cytoplasmic membrane (e.g., the transpeptidase [TPase] domain of a bifunctional TPase/transglycosylase high-molecular-weight PBP). The corresponding combination of sulbactam and ampicillin is known as Unasyn, and tazobactam and piperacillin are sold as Zosyn. (Fig. 8.8). While it is clear that these mechanism-based inhibitors will target only the serine-based lactamases and not the class D metallolactamases (Fig. 8.3), it is observed that the sulbactams and clavulanates are most active against class A lactamases and lack useful activity against the class C lactamases (Bronson and Barrett, 2001a), so there is room for further development of mechanism-based lactamase inhibitors.

A β-lactamase inhibitory protein (BLIP) has been isolated from *Streptomyces clavuligerus*, the clavulanate producer, where it may serve as an immunity protein to protect the antibiotic-producing organism. BLIP has picomolar to nanomolar K_i values for binding to several β-lactamases (e.g., 0.1 to 0.6 nM for TEM-1) (Rudgers et al., 2001), and the X-ray structure of the complex of BLIP and the TEM-1 β-lactamase has been solved (Strynadka et al., 1996), showing that residues 46-51 of BLIP make a type II′ β-turn in the active-site region of the TEM lactamase, leading to insights into protein-based inhibitors and suggesting that β-turn peptidomimetics would be useful inhibitors (Rudgers et al., 2001). A second protein, BLIP-II, from *Streptomyces exfoliatus*, has also had its structure solved in complex with the TEM-1 β-lactamase, showing a distinct fold from BLIP and a second way to block the active site of TEM-1, as a competitive inhibitor of lactam binding with a remarkably potent 27 pM K_i (Lim et al., 2001). The physiological function of BLIP-II appears to be in streptomycete sporulation, where it may inhibit one or more of the streptomycete PBPs to redirect PG biosynthesis toward spore formation.

Inhibitors of metallo-β-lactamases

The utility of carbapenems in the treatment of both gram-negative and gram-positive infections is becoming compromised by enzymatic hydrolysis and deactivation. While carbapenems as noted above are largely resistant to the class A chromosomal serine-based β-lactamases, they are rapidly hydrolyzed by the class B zinc-β-lactamases (Rasmussen and Bush, 1997), for example, in *Bacteroides fragilis* strains isolated from surgical patient infections. The X-ray structure of the CcrA metallo-β-lactamase from *B. fragilis* has been reported (Toney et al., 1998) and used for structure-based design of biphenyl tetrazole inhibitors that coordinate to the active-site zinc and thereby are specific for this metallolactamase class. A 50% inhibitory concentration of 0.4 μM for the most potent biphenyl tetrazole inhibitor was observed, and this was cocrystallized to confirm active-site binding and zinc ligation by one of the nitrogens of the tetrazole ring. These may be promising leads for molecules to add to carbapenems, much the way clavulanate or sulbactam is added to a β-lactam to get a combination that overcomes class B lactamase-mediated resistance. A series of tricyclic natural products with modest activities toward this subgroup of lactamases has also been described (Payne et al., 2002).

Regulation of β-lactamase gene expression and/or autolysis in the presence of penicillin

β-Lactamase genes can be embedded in bacterial chromosomes, such as the *ampC* gene in enteric bacteria or the *blaZ* gene in *Staphylococcus aureus*, or they can be carried on multiple-copy plasmids or transposons, as is the case for the TEM-1 *bla* gene in a variety of high-level penicillin-resistant gram-negative bacteria found in clinical isolates. *bla* genes are not usually constitutively expressed but get turned on when β-lactam antibiotics show up in the microenvironment. Recently the signaling systems for detecting external β-lactams have been sorted out for *E. coli*, *S. aureus*, and *Streptococcus pneumoniae* and reveal different paths of signal transduction that could be targets for reversing lactamase-mediated resistance.

E. coli

In *E. coli* the *ampG*, *ampD*, and *ampR* genes control expression of the *ampC*-encoding β-lactamase (Jacobs et al., 1997). The *ampG* gene encodes a transmembrane protein thought to act as a permease that imports a cell wall peptidoglycan fragment released by disruption of cell wall–cross-linking enzymatic machinery when a β-lactam begins to acylate PBPs and disrupt the orderly process of PG extension and remodeling. The molecule transported by AmpG is the disaccharyl tripeptide GlcNAc-anhydroMurNAc-L-Ala-D-γ-Glu-*meso*-DAP (**Fig. 8.10**), released by consecutive action of three enzymes. First is endopepti-

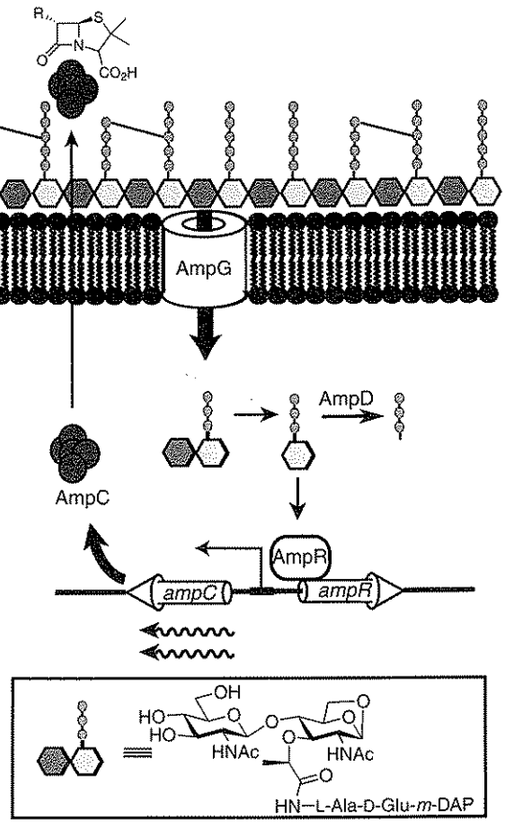


Figure 8.10 The anhydromuramyl tripeptide signaling pathway for induction of *ampC* expression in *E. coli*; transport into the cytoplasm by AmpG and ligation to AmpR to relieve transcriptional repression of *ampC*; and secretion of AmpC into the periplasm.

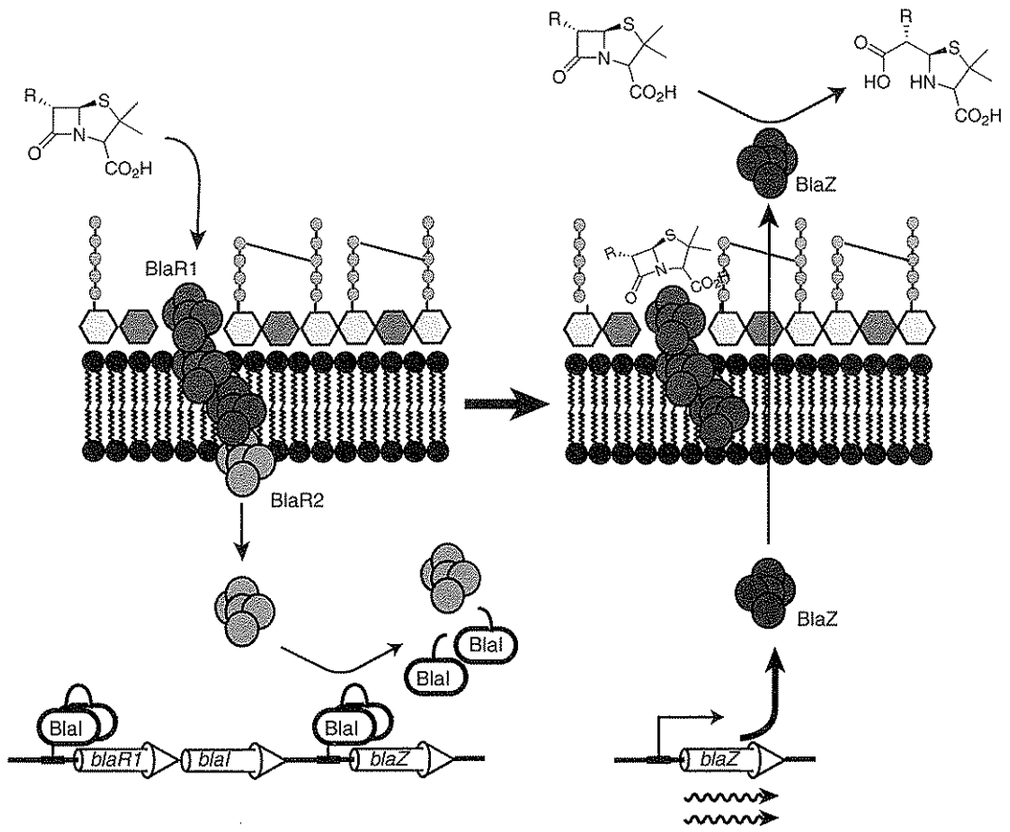
dase action to cleave the cross-links holding peptide strands together. Then comes action of lytic transglycosylases, catalyzing addition of the C_6-OH of a glucosyl group in a glycan strand to the 1-position, cleaving the inter sugar linkage and releasing the disaccharide as the 6,1-internal hemiacetal (the anhydroMurNAc). The tripeptide chain comes from shortening of the tetrapeptide or pentapeptide chain by action of L,D-carboxypeptidase, the third enzyme required to produce the signaling fragment. Now the GlcNAc-anhydroMurNAc tripeptide, a soluble small molecule, can diffuse to the outer domain of AmpG, bind, and be transported into the cytoplasm (**Fig. 8.10**), where it can dissociate from AmpG. The disaccharyl tripeptide released into the cytoplasm can now be recognized as a ligand for AmpR, the transcription factor, and convert it to an activated form that turns on the transcription of *ampC*, and β-lactamase expression begins. The AmpC lactamase is translated with an N-terminal signal sequence that targets it for transport to the cytoplasmic membrane and secretion into the periplasmic space, where it will start to destroy the β-lactam antibiotic hydrolytically (e.g., as in Fig. 8.5). The AmpD protein is also in the cytoplasm and is an amidase enzyme, cleaving the GlcNAc-anhydroMurNAc tripeptide. Thus it contributes at a basal level to the negative regulatory circuit, keeping the disaccharyl tripeptide concentration low and the *ampC* gene off (Jacobs et al., 1997). When the import of the signaling disaccharyl tripeptide is at higher levels

due to antibiotic-induced disruption of PG assembly, presumably the amount in the cytoplasm overwhelms the AmpD capacity and escapes to bind to AmpR.

S. aureus

In *S. aureus* two routes to clinically relevant resistance have been observed. The first is due to expression of the chromosomally encoded BlaZ lactamase, a class A lactamase, discussed below, and the second is the expression, in methicillin-resistant *S. aureus* (MRSA), of a novel, penicillin-insensitive PBP2A, encoded by the *mecA* gene, as will be described in chapter 10. The expression of the BlaZ β-lactamase activity is inducible; expression is off in the absence of penicillins and cephalosporins in the microenvironment and turned on when they arrive at the outside of the cell. The sensing, transduction, and transcriptional activation functions are provided by two proteins encoded by the *blaR1* and *blaI* genes that are found just upstream of the *blaZ* gene (**Fig. 8.11**). All three genes are negatively regulated at rest by the BlaI protein, a 14-kDa protein that dimerizes and binds to promoter regions to repress transcription.

Figure 8.11 The *blaR1-blaI-blaZ* operon of *S. aureus* and the signal transduction pathway for expression of the BlaZ lactamase in *S. aureus*: tandem proteolysis of BlaR1 and BlaI for gene activation.



When a β-lactam antibiotic approaches the outer face of the cytoplasmic membrane in which a few copies of the transmembrane protein BlaR1 are embedded, the antibiotic is detected and the signal transduced. The exo domain of the 66-kDa BlaR1 is a PBP domain and initial signaling is through covalent acylation by the β-lactam, as for any PBP domain (Fig. 8.11). The formation of this penicilloyl enzyme covalent intermediate in the exo domain is sensed (perhaps by conformation change in this PBP domain) and occupancy transduced through the transmembrane domain and read out by the internal 30-kDa domain of BlaR1. The external PBP-type domain of BlaR is most related to class D β-lactamases (Massova and Mobashery, 1998), consistent with evolution of this binding/sensing domain from the class D lactamases (Ghuysen, 1991). This would be the reverse of the evolution of PBPs into lactamases, proposed in an earlier section of this chapter, suggesting protein evolution has gone both ways.

The intracytoplasmic 30-kDa domain of BalR1 has the hallmarks of the proenzyme form of a zinc-dependent protease. When a penicilloyl-*O*-Ser intermediate forms on the extracytoplasmic domain, the 30-kDa domain inside undergoes autocleavage between residues 293 and 294 to liberate the BlaR2 30-kDa cytoplasmic fragment. Precedents for autocleavage of the proenzyme forms of zinc proteases exist and may be mediated by a clustering effect in the penicilloyl BlaR1 exo domain (McKinney et al., 2001; Zhang et al., 2001). Remarkably, one proteolytic signal transduction event generates a second as BlaR1 induces proteolysis of the 14-kDa BlaI to an 11-kDa fragment that has apparently lost ability to dimerize and bind promoter DNA. The net repression of BlaZ and BlaR1 transcription is relieved, and transcriptional upregulation leads to BlaZ lactamase production, transport of BlaZ to the outside of the cell, β-lactam antibiotic hydrolysis, and lactam resistance.

The BlaR1-BlaI two-component system is logically analogous to the two-component sensor/response regulator systems used over and over again by bacteria for gene regulation in antibiotic production (CarR, CarI) and antibiotic resistance (VanS, VanR) (see chapter 15), but those use phosphoryl group transfer (from ATP to His-sensor to Asp-regulator) as the chemical information for state switching from "off" to "on." In the BlaR-BlaI two-component system the switch is proteolytic. Since proteolysis is biologically irreversible switching, while phosphoryl transfers are biologically reversible (by phosphatase action), the BlaR and BlaI proteins must be continually replenished, explaining their autogenous regulation. This system is not unique to *S. aureus* since a BlaR1 homolog has been detected in *B. fragilis* strains that also produce inducible β-lactamase.

Further, it turns out that the regulation of the *mecA* gene expression and the encoded PBP2A production that confers methicillin resistance in MRSA is regulated by entirely parallel logic, with a *mecR1* and *mecI* two-component system, again with gene clustering with *mecA* for coordinate regulation (**Fig. 8.12**). The exo domain of MecR1 is also a PBP, so covalent capture of a lactam as the lactamoyl-PBP acyl enzyme domain initiates transmembrane signaling with the same cascade of an in *cis* proteolysis of the cytoplasmic domain of MecR1 and then an in *trans* proteolysis to cleave the intact MecI and relieve repression of the *mecA* gene. The insertion of PBP2A, the *mecA* gene product, into the membrane allows PG cross-linking that is insensitive to methicillin and other β-lactam antibiotics. It may be that this two-component proteolysis logic will

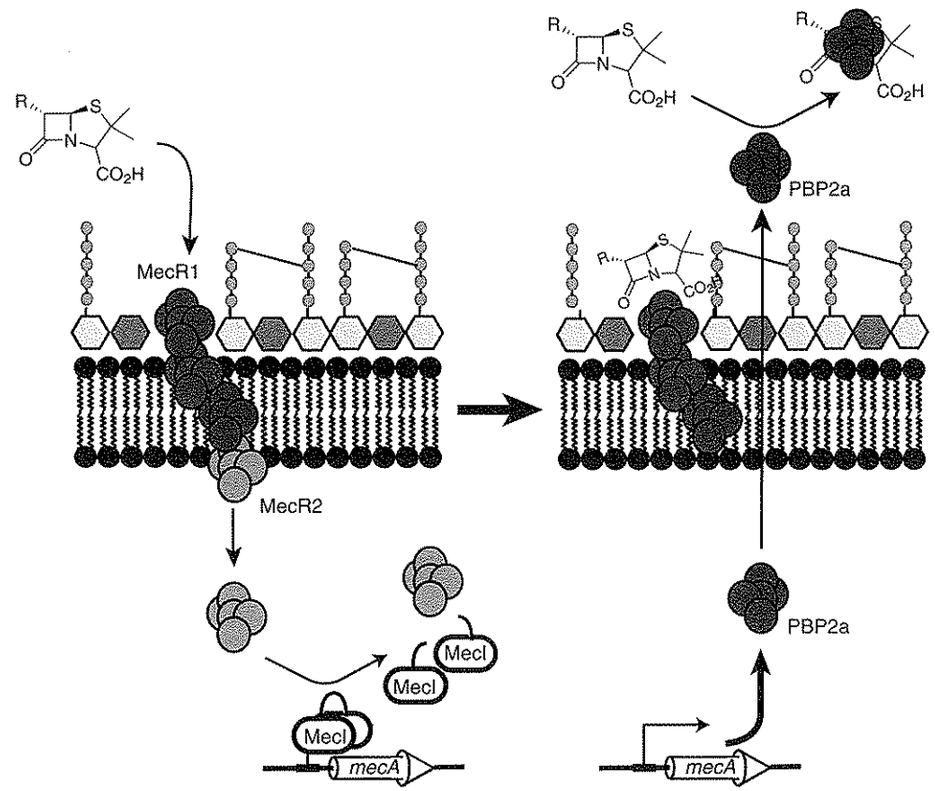


Figure 8.12 Signal transduction logic for regulated expression of the MecA PBP2A to confer methicillin resistance in MRSA.

be more general. In any event it raises the prospect that these signaling pathways for the two routes to inducible lactam resistance in *S. aureus* should be new targets for antibiotic screening and design.

S. pneumoniae

In *S. pneumoniae* external penicillin leads to an increase in autolytic peptidoglycan hydrolase activity and subsequent vulnerability to osmotic lysis and death. Genetic analysis (Novak et al., 2000) has indicated a signal transduction pathway logic distinct from those used by *E. coli* and *S. aureus*, described above.

As will be discussed elsewhere in this book (chapter 15), bacteria use two kinds of signaling systems to relay information from the external environment across the membrane to the cytoplasm for selective gene regulation. One is a two-component protein system, with a transmembrane signaling histidine protein kinase domain and a response regulator which acts as transcription factor. The second signaling system is dependent on the density of the bacterial culture and uses molecules secreted by one cell that diffuse into a neighbor, bind to a receptor/transcription factor, and activate downstream genes. These are known as quorum sensors and in gram-positive bacteria they tend to be small peptides. A two-component system (VncR-VncS) is required for cell killing by both van-

comycin and penicillin (Novak et al., 2000), presumably triggering the upregulation of the gene for the major autolytic enzyme LytA, with VncS sensing derangement in the PG structure and dephosphorylating VncR to relieve repression of gene transcription. Just upstream of the *vncR* and *vncS* genes is an open reading frame encoding a 27-residue peptide, Pep^{27}, and a three-protein ATP-binding cassette-type ATPase pump to pump Pep^{27} out to act as a quorum sensor and a death-inducing peptide on neighboring *S. pneumoniae* cells. The interaction of Pep^{27} and antibiotics (e.g., as ligands for the transmembrane VncS?) to turn on the lytic cascade is not understood but may be a fruitful intersection for new antibiotic targeting in *S. pneumoniae*.

Aminoglycoside-modifying enzymes

The aminoglycoside (aminocyclitol) antibiotics do not have a reactive chemical warhead comparable to the β-lactam that is the core acylating unit embedded in all penicillins, cephalosporins, carbapenems, and monobactams. Instead we have noted that the aminoglycosides read specific regions of the 16S rRNA in the 30S ribosome subunit by a hydrogen bonding network (see chapter 4) through the various hydroxyl and amino substituents on the cyclitol rings to provide a high-affinity docking site for this class of antibiotics. The enzymatic destruction strategy for aminoglycoside-resistant bacteria is to covalently modify those specificity-conferring OH and NH_2 groups in the aminoglycosides and thereby interfere with recognition by the 16S rRNA. In some bacteria such as the gram-negative pathogenic *P. aeruginosa*, the outer membrane can also be a significant initial barrier to entry of aminoglycosides, both by decrease in the number of porin channels in the outer membrane and also by modifications to the lipopolysaccharide outer leaflet (Livermore, 2000; Poole, 2001).

Three kinds of enzymatic modifications of OH and NH_2 groups on aminoglycosides are common determinants of resistance and represent variants of normal electrophilic group transfer enzymes that participate in primary metabolism (see Kotra et al., 2000, and Wright, 1999, for reviews). ATP is one such reactant, used in both *O*-phosphoryl transfers, by attack of the γ-PO_3 group by an OH or NH_2 nucleophile, and *O*-adenylyl transfers, by attack of the aminoglycoside substrate nucleophile on the α-P of ATP to transfer the AMP moiety (**Fig. 8.13**). The second thermodynamically activated but kinetically stable cosubstrate in electrophilic group enzymatic transfers is acetyl-CoA and the NH_2 groups of the aminoglycosides attack the acetyl thioester moiety to transfer the acetyl group. All three reactions—phosphorylation, adenylation, and acetylation—are irreversible, driven by the −7 kcal/mol released and corresponding to a K_{eq} of 10^5 (Walsh, 1979) in favor of aminoglycoside modification.

It is likely that the antibiotic-inactivating enzymes will have evolved from adenyltransferases, phosphotransferases, and *N*-acetyltransferases that had been utilized for normal biosynthetic processes in the bacterial cells. For example, the determination of the X-ray structure of the APH(3′)-IIIa phosphotransferase from enterococci revealed high similarity to eukaryotic serine/threonine protein kinases, consistent with an evolution from protein substrate −OH recognition for attack on the γ-PO_3 of ATP to recognition of the aminocyclitol framework

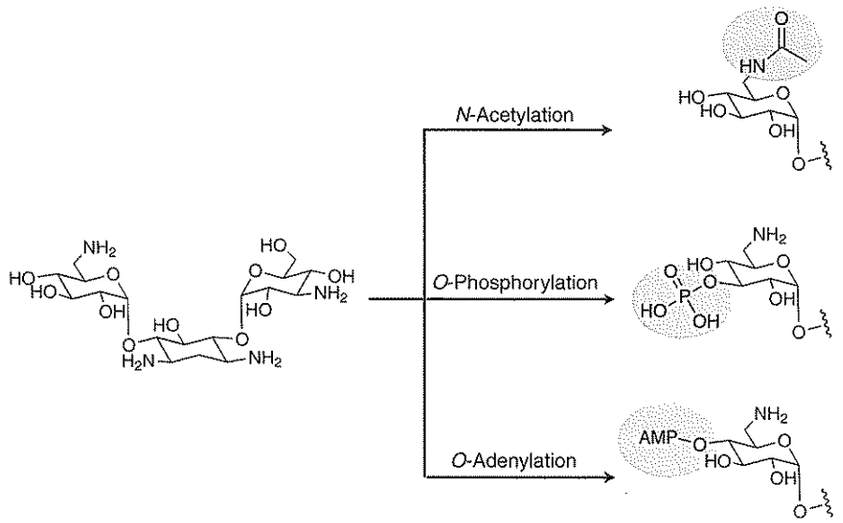


Figure 8.13 Three enzymatic routes to aminoglycoside deactivation: acetylation by acetyl-CoA, phosphorylation by ATP, and adenylation by ATP.

−OH (Hon et al., 1997) under selective pressure to survive. Given the multiplicity of OH and NH_2 groups in the successive generations of aminoglycosides to see clinical use, it is not surprising that modifying enzymes of distinct regiospecificity for acetylation, phosphorylation, and adenylation would arise. A typical pattern for covalent modification of a tricyclic aminoglycoside skeleton is shown in **Fig. 8.14**. Over 30 isoforms of these three kinds of enzymes have been described in aminoglycoside-resistant bacteria, including a fusion protein with two catalytic domains: an adenyltransferase and a phosphotransferase domain (Kotra et al., 2000). The *N*-acetyltransferases are classified according to their regioselectivity for acetylating—N_1, N_2', N_3—or N_6', with N_6' the most common subfamily of the aminoglycoside acetyltransferases, exhibiting broad specificity toward aminoglycoside scaffolds. The presence of the genes for the modifying enzymes on transmissible plasmids helps spread the resistance deter-

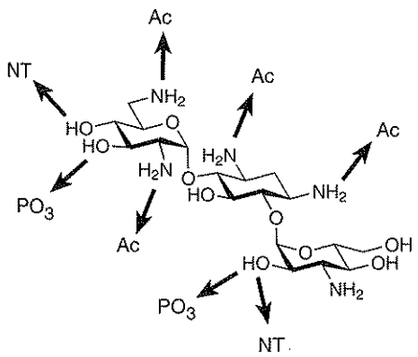


Figure 8.14 Patterns of regioselective enzymatic modification and deactivation of aminoglycoside antibiotics. NT, nucleotidyl transfer; PO_3, phosphoryl transfer; Ac, acetyl transfer.

minants and perhaps speed the evolution of catalytic activities toward newly introduced aminoglycosides.

Some strategies to subvert the enzymatic modifications have been reported by Mobashery and colleagues, studying one of the aminoglycoside O-phosphotransferases. For example, an aminoglycoside mimic once enzymatically phosphorylated rearranges to a reactive species that covalently modifies the O-phosphoryltransferase and takes it out of action (Roestamadji et al., 1995).

Analogous to the aminoglycoside-inactivating acetyltransferases is a family of virginiamycin/streptogramin-inactivating acetyltransferases, designated Vats (for virginiamycin acetyltransferases). We noted in chapter 4 that Synercid is a combination of a streptogramin A and streptogramin B component and that the Erm-mediated methylation of 23S rRNA leads to macrolide-lincosamide-streptogramin B (MLS_B) resistance, blocking binding of the streptogramin B component. The streptogramin A component can be removed by efflux (by mechanisms described in chapter 9) or by *O*-acetylation on the single free hydroxyl group (see Fig. 4.9 for dalfopristin structure). The X-ray structure of the VatD acetyltransferase from *Enterococcus faecium* (Sugantino and Roderick, 2002) has been solved, providing insight into the architecture of this drug-inactivating enzyme for the streptogramin A components.

Fosfomycin enzymatic deactivation

Fosfomycin is analogous to the β-lactam antibiotics in the sense that it also has a reactive chemical warhead, the three-ring epoxide, embedded in its simple chemical structure (**Fig. 8.15**). The epoxide is attacked by a reactive amino acid side chain, in this case the —SH of Cys rather than the —OH of Ser in the PBPs, in its target enzyme MurA at the start of peptidoglycan assembly (see chapter 3). The covalently derivatized active site of MurA leaves the enzyme unable to initiate the enolpyruvyl ether formation in UDP-GlcNAc conversion to UDP-MurNAc. Also analogous to destruction of the β-lactam ring by enzymatic capture of the reactive β-lactam by an alternate nucleophile, water in the case of the β-lactamases, the destruction of fosfomycin is catalyzed by enzymes that capture the epoxide with a soluble cosubstrate nucleophile, in this case the reactive thiolate anion of the tripeptide glutathione (γ-Glu-Cys-Gly). The fosfomycin glutathione *S*-transferase (FosA) is a manganese metalloenzyme (Bernat et al., 1997) (Fig. 8.15) that generates the ring-opened 2-OH thioether adduct in which the epoxide warhead has been deactivated as the glutathione thiol adds to C_1. Glutathione is the most abundant low-molecular-weight cellular thiol in most bacterial and eukaryotic cells, reaching levels of 1 to 10 mM. It is used for

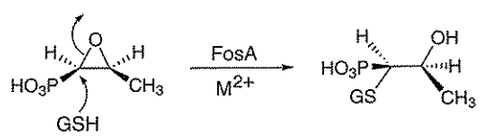


Figure 8.15 Enzymatic deactivation of fosfomycin by epoxide ring opening with glutathione.

protection and detoxification of reactive chemical groups, as in this example, by the high reactivity of its nucleophilic thiolate. The FosA enzyme has homology to both glyoxalase and metal-dependent catechol-cleaving dioxygenases (Bernat et al., 1997), consistent with an evolution from housekeeping enzymes of primary and secondary metabolism.