Molecular Insights into Aminoglycoside Action and Resistance

Sophie Magnet and John S. Blanchard*

Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Received April 20, 2004

Contents

1. Introduction 477
2. Aminoglycosides 478
   2.1. Chemical Structures 478
   2.2. Ribosome Binding Site and Translation Effects 478
   2.3. Secondary and Bactericidal Effects 482
   2.4. Properties and Clinical Use 482
3. Aminoglycoside Resistance 482
   3.1. Decreased Intracellular Concentration of the Drug 482
   3.2. Target Modification 485
      3.2.1. 16S rRNA Methylation 485
      3.2.2. Ribosomal Mutations 485
   3.3. Enzymatic Drug Modification 486
      3.3.1. Aminoglycoside Adenylyltransferases 486
      3.3.2. Aminoglycoside Phosphotransferases 487
      3.3.3. Aminoglycoside Acetyltransferases 489
   3.4. Origin and Prevalence 493
4. Resisting Resistance 494
5. Acklnowledgment 495
6. References 495

1. Introduction

Following the discovery of penicillin, which was inactive against *Mycobacterium tuberculosis*, Waksman discovered the first antituberculosis agent, streptomycin, by systematic screening of bacterial culture supernatants for the presence of *M. tuberculosis* inhibitory activity.[1] Several years after the introduction of this aminoglycoside in human antibacterial chemotherapy, organisms resistant to streptomycin began to appear. Subsequently, other antituberculosis agents, including isoniazid, rifampicin, and ethambutol, were discovered and replaced streptomycin in the treatment of tuberculosis. However, resistance to these drugs also appeared rapidly. To reduce the emergence of resistant organisms, a six-month-long, multidrug chemotherapy regimen was adopted. More recently, streptomycin has regained interest and significance for the treatment of multidrug-resistant (isoniazid- and rifampicin-resistant) *M. tuberculosis* infections. In this interval, a large number of other aminoglycosides were isolated from various Actinomycetes producers. Among them, gentamicin, isolated from *Micromonospora* in 1963, constituted a significant advance in the treatment of Gram-negative bacterial infections. Several other molecules, such as dibekacin and amikacin, were semisynthesized by modification of natural compounds with the aims of extending their antibacterial spectrum and potency, reducing their nephro- and ototoxicity, and evading the resistance mechanisms. The most recent aminoglycoside introduced into human antibacterial therapy was arbekacin, a kanamycin B derivative used in Japan since 1990. Today, the family of aminoglycosides includes a large number of compounds. However, the number of resistance mechanisms developed by microorganisms has increased in parallel with the number of drugs available and the frequency of their use.

The mechanisms of bacterial resistance to aminoglycosides have been the subject of numerous genetic and biochemical studies and have been the focus of many recent reviews.[2−4] In some species, broad spectrum and low level resistance can be achieved by decreasing the intracellular drug concentration. However, decreased affinity of the drug for its target, the bacterial ribosome, by modification of the drug or ribosome is the major cause of aminoglycoside resistance, and among these mechanisms the enzymatic inactivation of the drug is by far the most clinically significant. There are three classes of aminoglycoside-modifying enzymes: the aminoglycoside nucleotidyltransferases (ANTs), the aminoglycoside phosphotransferases (APHs), and the aminoglycoside acetyltransferases (AACs). The reactions catalyzed by these enzymes are usually regioselective, and the site of modification is indicated in parentheses. A roman numeral and a letter are then added to the nomenclature to distinguish the enzymes according to the pattern of aminoglycoside resistance that they confer and to their primary sequence, respectively. Nevertheless, in some bacterial species other mechanisms of resistance that involve unique features of the bacterium are predominant. This is the case for aminoglycoside resistance due to impermeability in *Pseudomonas aeruginosa* or due to ribosomal modification in *M. tuberculosis*.

In the past decade, several high-resolution structural studies have been performed to identify the molecular nature of the interactions between aminoglycosides and the ribosome or the proteins involved in their inactivation via enzymatic modification. This work has brought considerable new insight

* Corresponding author. Phone: (718) 430-3096. Fax: (718) 430-8565. E-mail: blanchar@aecom.yu.edu.

10.1021/cr0301088 CCC: $53.50
Published on Web 12/08/2004

Sophie Magnet was born in Montélimar, France, in 1973. She studied Biology at the Pierre et Marie Curie University in Paris and received her Master's degree in Microbiology in 1997. She then did her graduate research on aminoglycoside resistance in the Unité des Agents Antibacteriens at the Institut Pasteur in Paris. After receiving her Ph.D. in 2001, she joined the laboratory of Dr. John S. Blanchard at the Albert Einstein College of Medicine, New York, for a Postdoctoral fellowship in Biochemistry, during which time she performed mechanistic and kinetic studies of aminoglycoside acetyltransferases and DNA ligases. She is returning to France to work on enzymes involved in cell wall synthesis in Gram-positive bacteria at INSERM.

John S. Blanchard was born in Waterbury, CN. He received his B.S. in Chemistry from Lake Forest College and did his graduate research in biochemistry in the laboratory of Dr. W. W. Cleland at the University of Wisconsin. After a three-year Postdoctoral NIH-supported Fellowship, he was appointed Assistant Professor of Biochemistry at the Albert Einstein College of Medicine, New York, in 1983 and became the Dan Dancinger Professor of Biochemistry in 1998. His early interests in enzyme kinetics and isotope effects focused on flavoenzyme catalyzed reactions. His research efforts into the mechanism of action of isoniazid in the human bacterial pathogen *Mycobacterium tuberculosis* led to his current interest in antibiotic resistance. He was supported in 2000 by a Fogarty International fellowship in the laboratory of Dr. Patrice Courvalin at the Institut Pasteur in Paris, where he studied aminoglycoside *N*-acetyltransferases and met Dr. Magnet. He is the author of over 100 peer-reviewed manuscripts and 20 reviews and has been awarded six United States patents. His work has been generously supported by the National Institutes of Health for the last 21 years.

into the mechanism of action of various compounds and into the mechanisms by which bacteria become resistant to them.

In this review we will describe the current molecular understanding of aminoglycoside action and resistance, focusing on recent structural advances. The emergence of new mechanisms of resistance and the evolution of the distribution of resistance determinants will be presented in parallel with changes in antibiotic use. Finally, we will present the different strategies used to design inhibitors of the resistant determinants or new compounds that can escape from known mechanisms of resistance.

2. Aminoglycosides

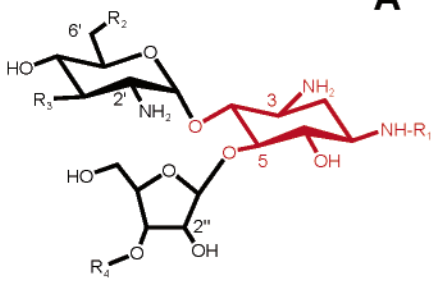
2.1. Chemical Structures

Aminoglycosides are hydrophilic molecules, consisting of a characteristic, central aminocyclitol linked to one or more amino sugars by pseudoglycosidic bond(s). For the majority of clinically useful compounds, referred to in this review as typical aminoglycosides, the aminocyclitol is the 2-deoxystreptamine ring, and it can be monosubstituted in position 4, as is the case for neamine, or disubstituted in positions 4 and 5, or 4 and 6. The accepted nomenclature usually used refers to ring I as the primed ring and corresponds to the amino sugar at position 4 of the deoxystreptamine ring. Ring II is unnumbered and corresponds to the central aminocyclitol, while ring III is referred to as the doubly primed ring and has the substituent in position 5 or 6 of the deoxystreptamine ring. Ring IV (triply primed numbering) corresponds to any additional ring attached to ring III (Figure 1). However, a number of active aminoglycosides are structurally atypical according to the above description. For instance, streptomycin possesses a streptidine ring as the central aminocyclitol, and spectinomycin consists of three fused rings. The structures of the more common atypical aminoglycosides are shown in Figure 2.

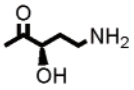
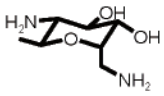
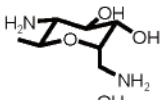
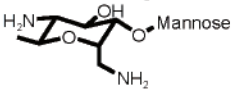
2.2. Ribosome Binding Site and Translation Effects

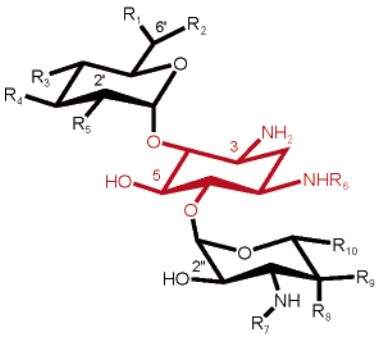
The primary target of aminoglycosides is the bacterial small ribosomal subunit. Aminoglycoside binding to the 16S rRNA, at the tRNA acceptor A site (Aminoacyl site), inhibits the translation process by causing misreading and/or hindering the translocation step. Two crystal structures of the 30S ribosomal subunit of *Thermus thermophilus* were solved by X-ray diffraction methods in 2000.[5,6] These studies brought considerable insights into the components and the function of the ribosomal A site. It is believed that the fidelity of translation depends on two steps, an initial recognition between the codon of the mRNA and the anticodon of a charged tRNA, and subsequent proofreading. The A site includes portions of the 530 loop, helix 34, and the base of helix 44. The tRNA anticodons bind within a cleft formed between the individual domains, and relative movements of these domains are likely to be involved in both the decoding and translocation processes.

The earliest structural studies of complexes containing aminoglycosides were performed using a 27-nucleotide-long RNA stem loop which mimicked the conserved helix 44 moiety of the 16S rRNA A site that was shown to bind the 2-deoxystreptamine-containing aminoglycosides. A stoichiometric 1:1 complex was generated with the 4,5-disubstituted 2-deoxystreptamine, paromomycin, and its three-

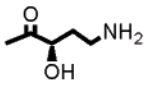
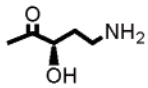
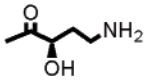


A

Antibiotic	R_1	R_2	R_3	R_4
Ribostamycin	H	NH_2	OH	H
Butirosin		NH_2	OH	H
Paromomycin	H	OH	OH	
NeomycinB	H	NH_2	OH	
Lividomycin A	H	OH	H	



B

Antibiotic	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}
Kanamycin A	H	NH_2	OH	OH	OH	H	H	H	OH	CH_2OH
Kanamycin B	H	NH_2	OH	OH	NH_2	H	H	H	OH	CH_2OH
Tobramycin	H	NH_2	OH	H	NH_2	H	H	H	OH	CH_2OH
Dibekacin	H	NH_2	H	H	NH_2	H	H	H	OH	CH_2OH
Gentamicin B	H	NH_2	OH	OH	OH	H	CH_3	OH	CH_3	H
Gentmicin C1	CH_3	$NHCH_3$	H	H	NH_2	H	CH_3	OH	CH_3	H
Gentmicin C1A	H	NH_2	H	H	NH_2	H	CH_3	OH	CH_3	H
Gentmicin C2	CH_3	NH_2	H	H	NH_2	H	CH_3	OH	CH_3	H
Sisomicin*	H	NH_2	H	H	NH_2	H	CH_3	OH	CH_3	H
Netilimicin*	H	NH_2	H	H	NH_2	CH_2CH_3	CH_3	OH	CH_3	H
Isepamicin	H	NH_2	OH	OH	OH		CH_3	OH	CH_3	H
Arbekacin	H	NH_2	H	H	NH_2		H	H	OH	CH_2OH
Amikacin	H	NH_2	OH	OH	OH		H	H	OH	CH_2OH

*(Δ5'>4' unsaturation)

Figure 1. Structures of clinically useful typical aminoglycosides: A, 4,5-disubstituted deoxystreptamine aminoglycosides; B, 4,6-disubstituted deoxystreptamine aminoglycosides. The deoxystreptamine ring is shown in red.

dimensional structure was solved using transfer nuclear Overhauser effect-derived distance constraints and simulated annealing methods.[7] The data revealed that the antibiotic binds in the major groove of the RNA, where it adopts an L-shaped conformation. The primed ring was bound in a pocket formed by the non-Watson−Crick A1408−A1493 base pair and the unpaired A1492, which generates a bulge in the helical structure. The majority of the intermolecular contacts were made with the central 2-deoxystreptamine ring and the primed 2,6-dideoxy-2,6-diamino-glucose ring substituents. Comparison of the unliganded RNA oligonucleotide with the drug−RNA complex structure showed that the most dramatic change occurred at the totally conserved triplet adenine pocket (A1408, A1492, A1493), which is displaced toward the minor groove upon paromomycin binding.[8] In another report, the same authors described the structures of three additional aminoglycoside−RNA complexes,[9] one containing the structurally simplest aminoglycoside, neamine, and two others containing the 4,5-disubstituted 2-deoxystreptamine ribostamycin or neomycin. The conformation of the bound drug, the binding site, and the intermolecular contacts made with the RNA were similar for ribostamycin and neomycin to those observed in the paromomycin complex. In the case of neamine, two possible orientations were proposed involving opposite interactions of the 1- and 3-amino groups of the deoxystreptamine ring. In 2000, Carter

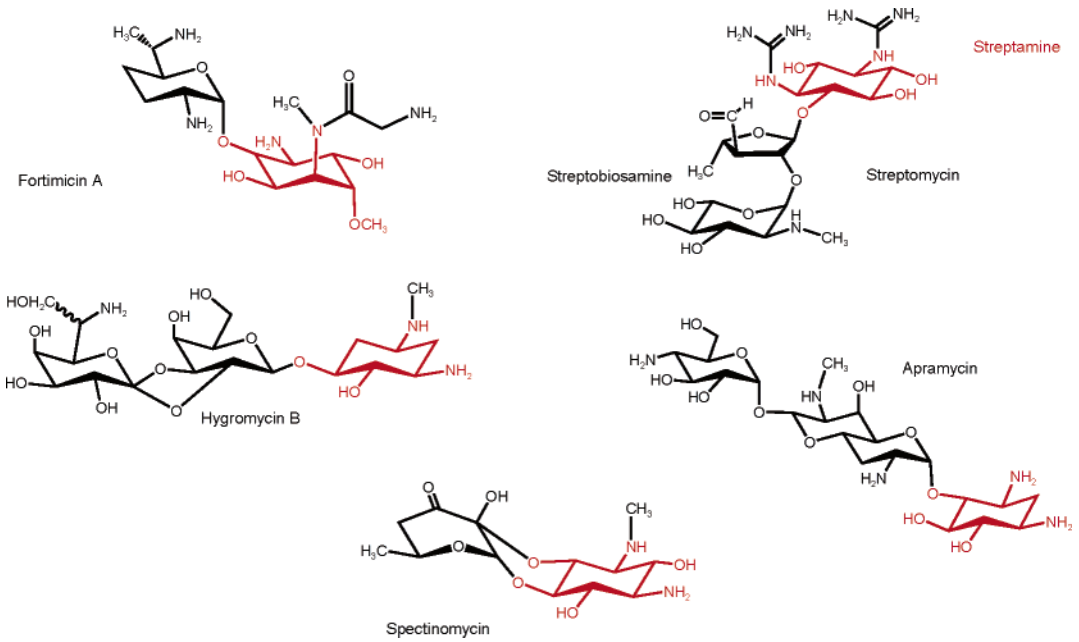


Figure 2. Structures of clinically useful atypical aminoglycosides. The aminocycitol ring is shown in red.

et al. described the crystal structures of various complexes of aminoglycoside bound to the intact 30S ribosomal subunit.[10] The structure of the paromomycin complex revealed, as expected, that the drug binds at the A site in the major groove of helix 44. The interactions reported in the NMR structures were largely confirmed. In addition to a stacking interaction between the primed ring and G1491, the hydroxyl groups in positions 4′ and 6′ make hydrogen bonds with the N1 of A1408 and the phosphate oxygen of A1493, respectively (Figure 3, top). The two amino groups of the deoxystreptamine ring in positions 1 and 3 interact directly with N7 of G1494 and O4 of U1495, respectively. Besides an intramolecular hydrogen bond observed between the 2′-amino group and the 5″-hydroxyl group, the double primed ribose ring makes a single interaction with the N7 group of G1491. Finally, the triply primed 2,6-dideoxy-2,6-diamino-glucose ring, which was disordered in the NMR structure, was clearly observed in the 30S subunit structure interacting with backbone phosphates from both sides of helix 44, including C1490 and G1405. In the 30S−paromomycin complex, A1492 and A1493 are flipped out compared to the free 30S subunit and to a greater extent than previously observed in the NMR structure. The structures of two 4,6-disubstituted aminoglycosides, gentamicin C1a[11] and tobramycin,[12] bound to a single or a dimeric synthetic A site RNA, respectively, were also solved by X-ray crystallography. Both complexes were very similar, and only small differences were observed in the position of the double primed ring. The interactions with specific nucleotides as well as with phosphate backbone oxygen atoms and the central and primed rings were, as expected, very similar to those observed for the 4,5-disubstituted deoxystreptamines. The 2″-hydroxyl group and 3″-amino group of ring III make additional specific contacts with O6 and N7 of G1405, respectively, compared to the 4,5-disubstituted aminoglycosides (Figure 3, bottom). Two additional intramolecular hydrogen bonds were also

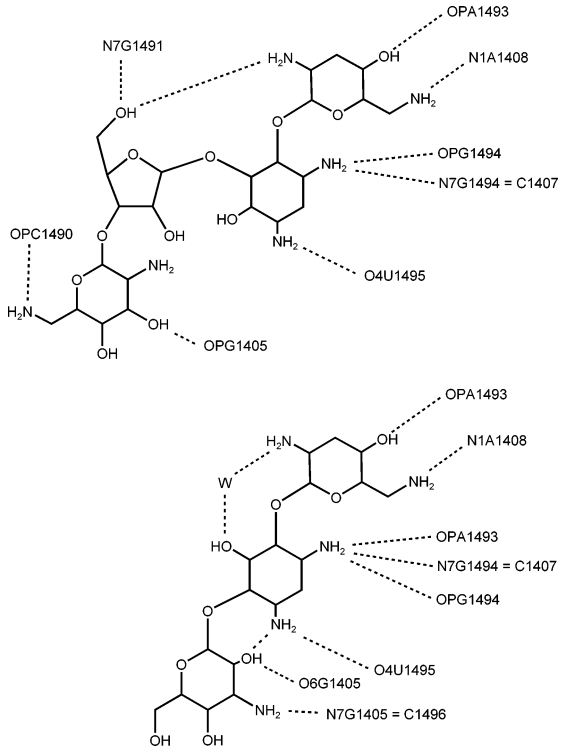


Figure 3. Interactions between typical aminoglycosides and the 30S ribosome. Top: Interactions between paromomycin and the 30S ribosome (Reprinted with permission from *Nature* (http://wwww.nature.com), ref 10. Copyright 2000 Nature Publishing Group.). Bottom: Interactions between tobramycin and the 30S ribosome (Reprinted with permission from ref 12. Copyright 2002 Elsevier). The superscripts refer to the nucleotide functional group that interacts with the bound aminoglycoside. The nucleotide numbering is for the homologous *E. coli* ribosome.

revealed between the 5-hydroxyl group and the ring III oxygen and between the 1-amino and the 2″-hydroxyl groups of the drug.

The selection of aminoacyl-tRNA during translation occurs by formation of a minihelix between the

codon of the mRNA and the anticodon of the cognate tRNA. When a tRNA−mRNA complex is formed, A1492 and A1493 form a hydrogen-bonding network with the ribose 2′-hydroxyl groups of the two first bases of both the codon and the anticodon, allowing discrimination between different base pairing geometries. When a cognate mRNA−tRNA complex is so recognized, the two adenines flip out from the helix with the net effect of increasing the affinity for the cognate tRNA and so stabilizing the complex. The structural data presented above showed that the binding of a disubstituted 2-deoxystreptamine in the decoding center causes a similar conformational change to the formation of a cognate mRNA−tRNA complex. These data confirm biochemical results that show that this class of aminoglycosides both decreases the rate of A site tRNA dissociation[13] and increases the tRNA binding affinity.[14] As a consequence, the affinity of the A site for a noncognate mRNA−tRNA complex is increased upon drug binding, preventing the ribosome from efficiently discriminating between noncognate and cognate complexes. This provides a dramatic atomic level rationalization of the well-known miscoding effect of the disusbtituted 2-deoxystreptamines antibiotics.

The structures of three structurally atypical aminoglycosides, streptomycin, spectinomycin, and hygromycin B, bound to the 30S subunit, have also been reported. The structure of the streptomycin−30S complex revealed that the drug binds tightly to the A site, but its binding site is adjacent to the one of the disubstituted deoxystreptamines.[10] In contrast to paromomycin, which interacts only with residues contained within helix 44, streptomycin makes interactions with the backbone phosphates and ribose hydroxyl groups of residues from four different domains of the 16S rRNA molecule, including U14 in helix 1, C526 and G527 of the 530 loop in helix 18, A913 and A914 from helix 27 and 28, respectively, and C1490 and G1491 from helix 44 (Figure 4, top). In addition, streptomycin is the only aminoglycoside that interacts with ribosomal protein side chains. Indeed, the structure of the complex showed that the ϵ-amino group of K45 of the S12 ribosomal protein contacts both the 4- and 5-hydroxyl groups of the streptamine ring. This structure has provided considerable insights into the dynamic functions that occur during aminoacyl-tRNA binding to the A site. It was proposed that H27, which interacts with H44, can have two alternative base pairing schemes during translation—one which leads to a ribosomal ambiguity (ram) conformation of the decoding center, with high affinity for tRNA which results in increased miscoding, and a second that leads at the opposite to a restrictive state with low tRNA affinity—and the balance of theses two states could be involved in the proofreading process.[15] The binding site of streptomycin revealed by the structural data suggests that the antibiotic affects the dynamic equilibrium of the two ribosomal conformations by stabilizing the ram state, providing an explanation for the error prone effect of this drug.[10] However, a confirmation of this model would require an atomic resolution structure of the restrictive form of the ribosome.

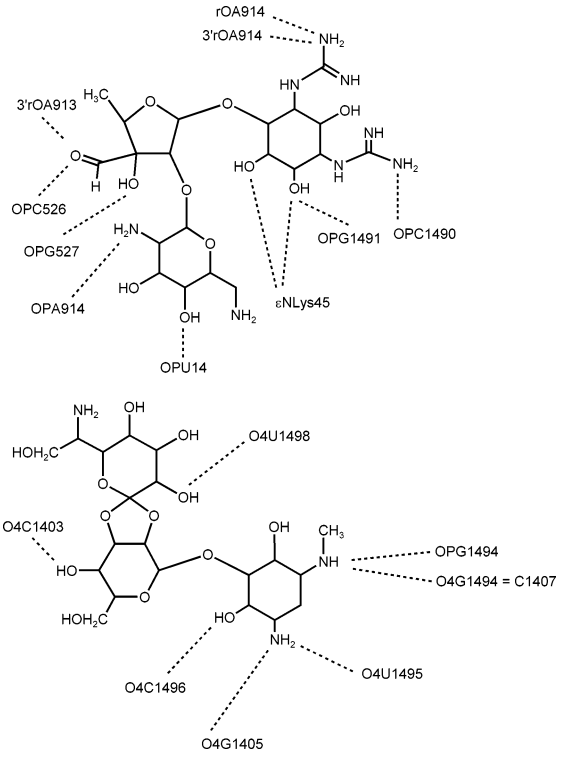


Figure 4. Interactions between atypical aminoglycosides and the 30S ribosome. Top: Interactions between streptomycin and the 30S ribosome (Reprinted with permission from *Nature* (http://wwww.nature.com), ref 10. Copyright 2000 Nature Publishing Group.). Bottom: Interactions between hygromycin B and the 30S ribosome (Reprinted with permission from ref 16. Copyright 2000 Elsevier). The superscripts refer to the nucleotide functional group that interacts with the bound aminoglycoside. The nucleotide numbering is for the homologous *E. coli* ribosome.

The structure of spectinomycin bound to the 30S subunit was also described in the same paper.[10] The rigid molecule binds in the minor groove of the 16S rRNA, at the end of helix 34 near to the pivot point of the head region. The drug makes four hydrogen bonds with the bases G1064, C1066, and C1192 of helix 34 in addition to the interaction with the 2′-ribose hydroxyl group of G1068 contained within helix 36. These observations lead the authors to propose that the binding of spectinomycin, known to inhibit the EF-G catalyzed GTP-dependent translocation of the peptidyl-tRNA from the A site to the P site (Peptidyl site), prevents the movement of the head region and helix rearrangements necessary for the translocation step.

Finally, the structure of the 30S−hygromycin B complex has been reported by the same group in a separate paper.[16] Hygromycin B is active against both prokaryotic and eucaryotic ribosomes. It acts primarily by inhibiting the translocation step but also, to a lesser extent than streptomycin or the disubstituted 2-deoxystreptamines, causes miscoding. Hygromycin B binds just above the binding site of paromomycin at the very top of helix 44. The ring I substituents interact with both the backbone phosphate oxygen atoms of G1494 and the bases G1405 G1494, U1495, and C1496 (Figure 4, bottom). The rest of the molecule makes only weak base specific hydrogen bonds with C1403 and U1498. Ring IV is positioned

very close to the P site. The binding of hygromycin B between the A site and the P site explains its dual effect on translation accuracy and translocation. Moreover, the absence of specific contact between hygromycin B and A1408, a residue totally conserved among eubacteria, can certainly account for the affinity of this molecule for eucaryotic rRNA, which possesses a G at the corresponding position.[17]

2.3. Secondary and Bactericidal Effects

Whereas spectinomycin and kasugamycin, two inhibitors of the translocation step, act bacteriostatically, streptomycin and the disubstituted 2-deoxystreptamines are bactericidal antibiotics. Although the mechanism of action of aminoglycosides at the translational level was substantially clarified by the structural data detailed above, the origin of the bactericidal effect of most of these compounds remains enigmatic. Comparison of the effect of aminoglycosides on translation and on cell viability suggests that lethality is correlated with the production of mistranslated proteins, which could have fatal secondary effects. It was shown that aminoglycosides that cause misreading, like streptomycin, increase the passive permeability of the cell membranes to several small molecules, as originally probed by the release of K^+ ions.[18] This permeabilization presumably occurs as a result of the incorporation of truncated or incorrectly folded proteins in the membranes, since it has not been observed in mutant strains in which protein synthesis is insensitive to streptomycin by mutation in the *rpsL* gene.[19−21] This phenomenon results in an increased aminoglycoside uptake that leads to an irreversible accumulation of the drug inside the cell. It was proposed that following the rapid degradation of the membrane-associated mistranslated proteins the drug becomes trapped inside the cell.[18] The resulting high intracellular drug concentration is believed to play a critical role in the bactericidal effect.[21] The saturation of the ribosomes with aminoglycosides, potentially coupled to the inhibition of new ribosome synthesis and assembly,[22] could result in the complete inhibition of protein synthesis, leading to bacterial death. Alternatively, the production of mistranslated proteins might affect another vital cellular processes. For instance, it has been reported that aminoglycosides that cause misreading inhibit the initiation of DNA replication,[20] but this finding requires more detailed investigations that have not been pursued.

2.4. Properties and Clinical Use

Because of their poor oral absorption, aminoglycosides are most often administered parenterally in order to achieve therapeutically adequate serum concentrations. As a consequence of their polar structure, the drugs inefficiently cross biological membranes and thus their intracellular tissue concentration is low except in the proximal renal tubule, where they are concentrated.[23,24] Whereas the conventional dosing for gentamicin, tobramycin, or netilmicin is around 5 (mg/kg)/day, 3-fold higher doses are used for other compounds such as streptomycin or amikacin.[23,25] For patients with normal renal activity, a single daily dosing was shown to be more favorable than the traditionally recommended multiple daily dosing regimens. Because of the concentration-dependent activity and postantibiotic effect of aminoglycosides, a similar efficacy can be obtained by single daily dosing with a lower cost, reduced toxicity, and reduced emergence of an adaptive resistant population.[25−31] In addition, aerosolized or liposome-encapsulated aminoglycosides have been shown to be advantageous for the treatment of respiratory tract infections[32−34] or against intracellular bacteria like *Mycobacterium avium*,[35−38] respectively. Finally, therapy strategies such as alternating between different classes of antibiotics, "switch therapy", or the rotational use of different aminoglycosides are increasingly encouraged to prevent therapeutic failure due to resistance.[31,39,40]

Aminoglycosides are active against a wide range of aerobic Gram-negative bacilli, many staphylococci, mycobacteria, and some streptococci.[4,23,24,41,42] They are particularly useful for the treatment of neutropenic patients and serious infections caused by aerobic Gram-negative bacteria. Gentamicin continues to be the aminoglycoside of choice to treat hospital acquired enterobacteriaceae and *P. aeruginosa* infections. Most often it is used in combination with a β-lactam, which results in a synergistic bactericidal effect due to an enhanced uptake of aminoglycosides.[43] Other inhibitors of bacterial cell wall synthesis can also be coadministered with aminoglycosides to treat infections due to bacterial species that are naturally resistant to aminoglycosides because of impaired uptake, including the enterococci.[41,42] For the treatment of urinary tract infections, aminoglycosides can be used alone in monotherapy. More recently introduced aminoglycosides, such as amikacin or arbekacin, which are not substrates for a number of aminoglycoside-modifying enzymes[23,44] or which retain their antibacterial activity after modification,[45] are used to treat infections due to gentamicin-resistant organisms, including methicillin-resistant *Staphylococcus aureus*.[46−50] Finally, streptomycin is still used in multidrug chemotherapy to treat multidrug-resistant *M. tuberculosis*.[23,39]

3. Aminoglycoside Resistance

3.1. Decreased Intracellular Concentration of the Drug

Decreased aminoglycoside concentration inside a target cell, by reduction of drug uptake, activation of drug efflux, or both, will affect the susceptibility of the strain to the whole class of aminoglycoside compounds and can be the cause of intrinsic or acquired resistance. Although the exact mechanism of aminoglycoside uptake remains unknown, it is accepted that the process consists of three consecutive steps.[51,52] The first step is the adsorption of the cationic compounds to the surface of bacteria by electrostatic interactions with the negatively charged lipopolysaccharides of the outer membrane of Gram-negative bacteria. The next two steps are dependent

on the transmembrane potential generated by the respiratory chain, with the second being characterized by a faster rate of uptake. As a result, anaerobic bacteria are intrinsically resistant to aminoglycosides due to impermeability.[53] Similarly, respiratory chain mutants or strains containing functional mutations in their ATP synthases were shown to exhibit decreased susceptibility to aminoglycosides.[51,54] Such mutants have been isolated from clinical or experimental endocarditis caused by infection with *Escherichia coli*, *S. aureus*, or *P. aeruginosa*.[55] Changes in membrane components involved in the initial electrostatic binding of aminoglycosides have also been associated with increased levels of resistance, especially in the case of *P. aeruginosa*.[56] Clinical strains exhibiting low level resistance to gentamicin were shown to have a modified, less negatively charged, lipopolysaccharide that exhibits a lower affinity for gentamicin.[57] In addition, the extracellular alginate produced by mucoid strains of *P. aeruginosa*, which both inhibits phagocytosis by monocytes and neutrophils and enhances bacterial adherence to the respiratory epithelia, was shown to decrease the uptake and early bactericidal effect of aminoglycosides. It was proposed that the viscous polyanionic alginate gel acts as a physical and ionic trap for the drug.[58]

Energy-dependent bacterial efflux is now recognized as a major cause of antibiotic resistance. This is particularly true for the multidrug-resistant opportunist pathogens responsible for nosocomial infections, which have to counter the environmental pressure exerted by the constant presence of antibiotics. Bacterial species constitutively expressing such transporters are intrinsically resistant to low levels of various antibiotics. Moreover, mutations in the regulatory genes of the pumps, or induction of expression in the presence of substrate, can lead to the overexpression of the originally constitutive, or silent, pump genes. It had been thought that multidrug transporters were specific for hydrophobic or amphiphilic compounds and, thus, that aminoglycosides would not be affected by this mechanism of resistance. However, in the last several years, aminoglycosides have been shown to be substrates for a number of multidrug efflux pumps, including members of the five superfamilies of bacterial transporters.[59]

Recently, crystallographic structural studies on different components of the tripartite transporters of the RND (resistance nodulation cell division) superfamily, which play a particularly important role in Gram-negative bacteria, have been revealing.[60,61] The transporters of the RND superfamily use the membrane proton-motive force as energy source. They are localized in the cytoplasmic membrane, and in Gram-negative bacteria they interact with a membrane fusion protein (MFP), located in the periplasmic space, and an outer membrane protein (OMP) to form a continuous, tripartite channel able to export substrates directly out of the cell. *E. coli* AcrB, which exhibits broad substrate specificity but does not efflux aminoglycosides, has served as the structural prototype of a bacterial RND protein involved in antibiotic resistance. AcrB interacts with the MFP, AcrA, and the OMP, TolC. The structures of the AcrB apoprotein,[60] AcrB in complex with four substrates (rhodamine 6G, ethidium bromide, dequalinium, and ciprofloxacin),[62] and TolC[63] have been solved. Both membrane proteins exist as homotrimers. Trimeric TolC forms a barrel composed of 12 β-strands that span the outer membrane and 12 α-helices that extend into the periplasmic space over 100 Å. The internal cavity is open to the external medium and provides solvent access. Each monomer of AcrB contains 12 transmembrane domains and two large periplasmic domains. The transmembrane domains of the three protomers are arranged in a ringlike manner, creating a ~30 Å diameter cavity, where substrates bind. This cavity is connected to the periplasmic funnel by a very narrow pore, and the dimensions of the funnel are compatible with a direct interaction with TolC. Between the AcrB monomers, an opening formed by residues of the periplasmic domaims is observed that links the central channel to the periplasm. It was proposed that these openings ("vestibules") provide a way for substrates selected from the outer leaflet of the cell membrane or from the periplasmic space to gain access to the channel and be exported[60] (Figure 5). Such a model explains the structurally broad substrate specificity of this type of transporter, since both amphiphilic compounds, which can partially penetrate the lipid bilayer, and polycationic molecules such as aminoglycosides, which interact electrostatically with the phospholipids of the membrane, can be captured at the entrance of the vestibule.[61] The selection of efflux substrates would then be determined by the nature of the residues at the entrance of the openings. This is supported by domain-swapping experiments between two RND proteins of *P. aeruginosa*, MexY and MexB,[64] and two RND proteins of *E. coli*, AcrB and AcrD,[65] indicating that the periplasmic domains are responsible for efflux specificity. The presence of many more acidic residues at the entrance of the vestibules of AcrD, which does export aminoglycosides, compared to AcrB, which does not, also supports this model.[61] The structures of AcrB in complex with various substrates have shown than once they reach the central cavity, the structurally distinct ligands bind to different sites, with a stoichiometry of one per protomer.[62] Three conserved charged residues, located in transmembrane helices 4 and 10, might constitute the transmembrane proton translocation site.[60] Protonation of these residues would disrupt the ion pair and trigger a conformational change leading to the pore opening. A preliminary structure of AcrA by electron crystallography reveals that the protein has an elongated shape of 100−200 Å overall length,[66] but the nature of the contacts made with AcrB and TolC, as well as its role in the system, is still very poorly understood.

Several RND proteins have been shown to be involved in intrinsic and/or acquired, proton motive force-dependent, aminoglycoside resistance in various Gram-negative pathogens, including *P. aeruginosa*, *Burkholderia pseudomallei*, *Acinetobacter baumannii*, and *E. coli*. The disruption or deletion of the

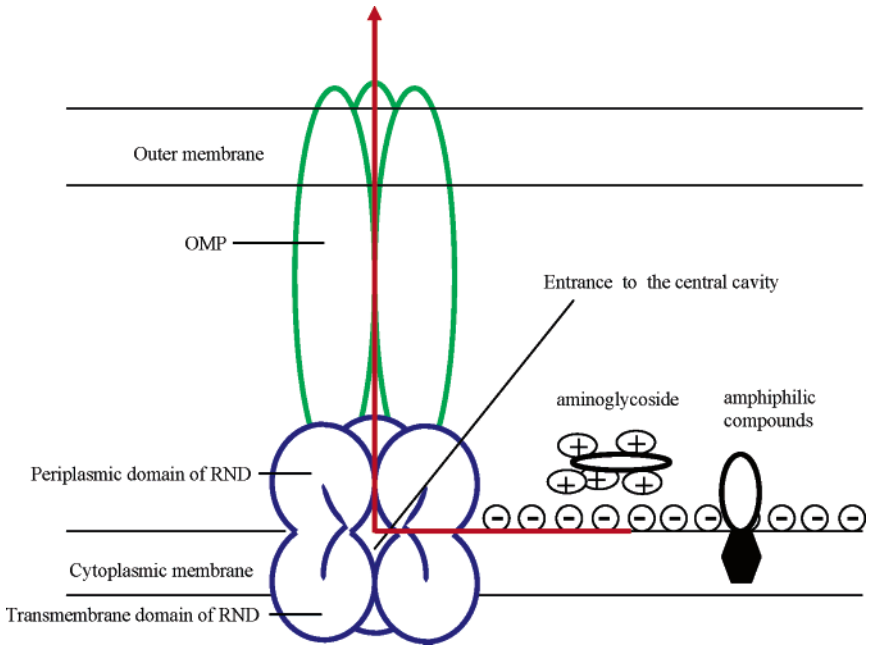


Figure 5. Schematic representation of a model proposed for drug capture at the outer leaflet of the cytoplasmic membrane by a trimeric RND protein. Hydrophilic and positively charged compounds such as aminoglycosides may bind to the acidic outer surface of the membrane, and amphiphilic compounds such as fluoroquinolones or chloramphenicol may partially diffuse into the lipid bilayer before being recognized by specific interactions at the periplasmic vestibules of the RND and drawn into the central cavity: OMP, outer membrane protein; RND, resistance nodulation cell division.

genes encoding the RND proteins MexY from *P. aeruginosa*,[67,68] AmrB from *B. pseudomallei*,[69] and AcrD from *E. coli*[70] resulted in the hypersusceptibility of the strains to aminoglycosides, indicating that these proteins contribute to the intrinsic resistance of these species to aminoglycosides. Whereas the AmrAB system seems to be specific for aminoglycosides and macrolides, others exhibit a very broad spectrum of antibacterial substrate specificity. Although no clear correlation has been made between the level of expression of MexXY and the MIC values of aminoglycosides in clinical isolates of *P. aeruginosa*, overexpression of the *mexXY* structural genes has been shown to be responsible for high-level acquired resistance in some cases.[71] The MexXY system also appears to be involved, in combination with membrane impermeability, in the ability of *P. aeruginosa* to develop adaptative resistance in response to exposure to inhibitory concentrations of aminoglycosides.[72] The AdeABC system of *A. baumannii* is another example of an RND pump mediating acquired aminoglycoside resistance. It was shown that the inactivation of the *adeB* gene from a multidrug-resistant clinical isolate of *A. baumannii* restored the susceptibility of the strain to various drugs, including aminoglycosides (from 2- to 32-fold), fluoroquinolones, cefotaxime, erythromycin, tetracycline, chloramphenicol, and trimethoprim.[73]

Gene(s) encoding putative transcriptional regulator(s) are frequently present in the immediate environment of the structural genes of the efflux system. The genes *amrR*[69] and *mexZ*,[68] and the two component regulatory gene homologues *adeRS*,[73] have been identified upstream from the corresponding efflux genes. While the regulator proteins of some efflux systems belonging to distinct superfamilies of transporters, such as BmR from *Bacillus subtilis* or QacR from *S. aureus*, have been the subject of detailed studies showing that they modulate the transcription of the adjacent genes following drug binding,[74,75] very little is known about the regulators of the RND family transporters. Although mutations in *mexZ* have been associated with increased expression of *mexXY*, other results suggest that the regulation of the system is a more complex process involving various components, including yet uncharacterized factors.[71] On another hand, the expression of the OMP component could play an important role in the functional expression of these efflux systems. In the case of *P. aeruginosa*, no gene encoding a cognate OMP was found in the closed environment of *mexXY*, suggesting that the pump can use independently encoded protein(s) to form an efficient tripartite system. Although it has been shown that MexXY can act together with OprM, a more recent report identified two other OMPs, OpmG and OpmI, that can contribute to aminoglycoside resistance in this species.[76]

Members of the major facilitator superfamily (MFS) of transporters have also been shown to decrease aminoglycoside susceptibility in strains harboring the structural gene on a multicopy plasmid. The MFS proteins are implicated in the active transport of both sugars and antibiotics. They contain 12 or 14 transmembrane segments and use the proton motive force as energy source. MdfA from *E. coli* was the first putative MFS protein reported to have an effect on aminoglycoside resistance.[77] Althought this effect, observed in a strain of *E. coli* harboring the gene on a plasmid, was very modest and not clinically relevant (2- to 3-fold increases in the MIC values of kanamycin and neomycin), this finding led to other investigations on the role of MFS proteins in other species. The *tap* and *P55* genes, isolated from *Myco-*

bacterium fortuitum[78] and *Mycobacterium bovis*,[79] respectively, encode putative MFS proteins and conferred resistance to aminoglycosides, including streptomycin, when cloned in *Mycobacterium smegmatis*. Genes and proteins homologous to *P55*/P55 were detected by polymerase chain reaction (PCR) or by western blot analysis, in many *Mycobacterium* species, including *M. tuberculosis* (Rv1410c). These observations, together with the identification of a total of 16 open reading frames that encode putative MFS proteins in the genome of *M. tuberculosis*,[80] may account for streptomycin-resistant clinical isolates of *M. tuberculosis* that cannot be assigned to mutations in *rpsL* or *rrs* genes or enzymatic modification of the drug.

3.2. Target Modification

3.2.1. 16S rRNA Methylation

The lack of post-transcriptional methylation of A1518 and A1519 16S rRNA nucleotides, as a result of mutations in the *ksgA* gene, which encodes an *S*-adenosylmethionine (SAM)-dependent RNA methylase, was associated with resistance to kasugamycin, an inhibitor of the initiation step of translation, in *E. coli* and *Bacillus stearothermophilus*.[81−83] Although this example is not clinically relevant, it illustrates the influence of the 16S rRNA methylation pattern on the interactions with aminoglycosides.

Members of the Actinomycetes produce inactive aminoglycosides, including acetylated- or phosphorylated-forms, which are cleaved during or after their export out from the cell by specific enzymes, to form the active antibiotics.[84−86] To further resist the secreted active compounds, many aminoglycoside-producing organisms also express rRNA methylases capable of modifying the 16S rRNA molecule at specific positions critical for the tight binding of the drug.[87] A number of genes encoding such enzymes have been identified from several aminoglycoside producers.[88−95] The corresponding rRNA methyltransferases form the Agr family of methyltransferases (for aminoglycoside resistance).[96] Some of these enzymes have been characterized. KamA from *Streptomyces tenjimariensis* and KamB from *Streptomyces tenebrarius* catalyze the modification of A1408 at the N_1 position and confer high-level resistance to kanamycin, tobramycin, sisomicin, and apramycin but not gentamicin.[87,88] GmrA from the gentamicin producer *Micromonospora purpurea* and KgmB from *S. tenebrarius* catalyze the modification of G1405 at the N_7 position, conferring high-level resistance only to the 4,6-disubstituted deoxystreptamines, including gentamicin.[87,89] Methylation of these nucleotides presumably abolishes the intermolecular contacts that they make with the drug (discussed previously in section 2.2 and Figure 3). The specific interaction observed between ring III of the 4,6-disubstituted deoxystreptamines and G1405 is consistent with the resistance pattern conferred by G1405 methylation. The presence of a methyl group at the 6′ position of some compounds of the gentamicin mixture could modify the interaction with A1408 compared to those observed with tobramycin, explaining why modification of this residue does not confer resistance to gentamicin.

Until recently, genes encoding a 16S rRNA methyltransferase had been restricted to the aminoglycoside producers, but three reports in 2003 and 2004 described the characterization of similar genes in clinical isolates of human Gram-negative pathogens. The *rmtA* and *rmtB* genes, located on plasmid-borne transposons, were found in clinical isolates of *P. aeruginosa* and *Serratia marcescens*, respectively.[97,98] These strains were all isolated in Japan, where arbekacin has been used extensively since 1990. The two genes share 82% sequence identity, and the encoded Rmt enzymes confer high-level resistance (MICs > 1024 μg/mL) to almost all clinically useful aminoglycosides, including arbekacin, but not streptomycin. The considerable primary sequence similarity observed between the Rmt proteins and the 16S rRNA methylases of Actinomycetes, as well as the high G−C content of the gene (55%), suggests a possible gene transfer from the producing organisms to Gram-negative pathogens. Another 16S rRNA methylase was characterized from *Klebsiella pneumoniae*. The structural gene, *armA*, was located on a plasmid containing several other resistant genes, including those conferring resistance to β-lactams, trimethoprim, sulfonamides, and other aminoglycoside resistance determinants. In contrast to *rmtA* and *rmtB*, the low G−C content of *armA* (30%) does not suggest a direct or recent acquisition from the *Actinomycetes*. However, the ArmA methylase is able to confer high-level resistance to essentially all aminoglycosides except streptomycin.[99] The site specificity of the modification catalyzed by these two enzymes was not determined, but the pattern of resistance associated with ArmA, which includes all 4,6-disubstituted deoxystreptamines but not apramycin, led the authors to propose that the N_7 of G1405 is the locus of modification. The *armA* gene has been detected by PCR in several other *Enterobacteriaceae* isolated from different European countries.[99] Because these methylases can modify all copies of the 16S rRNA and lead to high-level resistance to an extremely wide range of compounds, the emergence of this resistance mechanism in human pathogens is of concern for the future, especially considering that the structural genes can apparently be easily disseminated.

3.2.2. Ribosomal Mutations

Resistance to aminoglycosides by mutation of the ribosomal target is clinically relevant only for streptomycin in *M. tuberculosis*. *Mycobacterium* is the only genus of eubacteria with species that contain a single copy of the ribosomal operon, which implies that a single mutation can lead to the production of a homogeneous population of mutant ribosomes and thus can result in resistance regardless of the recessive nature of the mutation involved. The mutations in the *rrs* gene, encoding the 16S rRNA and associated with streptomycin resistance in *M. tuberculosis*, affect two highly conserved regions, the 530 loop and the region around nucleotide 912, according to *E. coli* numbering,[100−105] and result in decreased affinity for

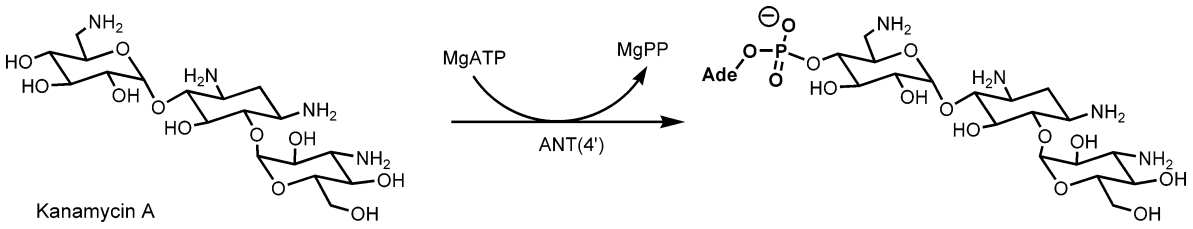


Figure 6. Reaction catalyzed by aminoglycoside O-adenylyltransferases. The reaction shown is that catalyzed by ANT(4′) catalyzing the MgATP-dependent 4′-O-adenylylation of kanamycin A.

streptomycin.[106] The structure of the streptomycin−30S ribosomal subunit complex revealed specific interactions between the drug and the backbone phosphate or ribose hydroxyl groups of nucleotides C526 and G527 of helix 18 and A913 and A914 of helixes 27 and 28, respectively,[10] providing a rationale for the location of mutations previously identified and their effect on streptomycin binding. Apart from clinically significant streptomycin resistance in *M. tuberculosis*, a few reports have described 16S rRNA mutations associated with aminoglycoside resistance in clinical isolates of microorganisms containing a single or low copy number of *rrs* genes. Mutations at positions 1400 and 1401 were found in kanamycin-resistant isolates of *M. tuberculosis*,[107] the mutation A1408G (corresponding to the eucaryotic allele of this position) was identified in the unique rRNA operon of *Mycobacterium abscessus* and *Mycobacterium chelonae* isolates resistant to 2-deoxystreptamine-containing aminoglycosides, and mutations affecting the base pair G1064−C1192 of helix 34 were found in the three copies of the 16S rRNA of spectinomycin-resistant isolates of *Neisseria*.[108]

The introduction of a single *rrs* gene on a multicopy plasmid has been used for many years to study the effect of 16S rRNA mutants on the activity of aminoglycosides in a heterogeneous population of ribosomes or homogeneous purified mutant ribosomes. These studies have shown that at least half of the ribosomes must be in the mutant form to confer aminoglycoside resistance.[106,109,110] More recently, to circumvent the problem of the recessive nature of ribosomal mutations, strains containing a single copy of the *rrs* gene have been genetically engineered.[111−113] Synthetic oligonucleotides mimicking the A site have also been used for similar in vitro studies.[114] These studies have confirmed the effect of the naturally occurring mutations previously shown to affect streptomycin activity,[115] and they revealed other mutations associated with spectinomycin,[116,117] hygromycin B,[118,119] or 4,6-disubstitued 2-deoxystreptamine[113,120−122] resistance. These studies have shown that mutations leading to a steric or allosteric change in the drug-binding pocket can be more deleterious for antibiotic activity than mutations abolishing direct contacts between the drug and the 16S rRNA.

Mutations in genes encoding ribosomal proteins can also alter the activity of aminoglycosides. Notably, mutations in protein S12 are the other major cause of streptomycin resistance in *M. tuberculosis*[100,101,105,123] and other species.[124,125] Mutations occurred in two regions, located around residues 42 and 87 (*E. coli* numbering), that contact helixes 18, 27, and 44 of the 16S rRNA. The most frequent substitutions were found to occur at residues P41, K42, K87, and P90. Although structural data showed that S12 makes direct contact with streptomycin,[10] the effect of such mutations appears likely to be conformational changes in the rRNA that prevent drug binding. This conclusion, highlighted by structural data and chemical protection experiments, was first predicted by the observation that different mutations in S12 lead to different phenotypes: streptomycin resistance or streptomycin dependence.[115,126−129] In addition, S12 mutations can be compensated for by other mutations in the rRNA or in other ribosomal proteins.[128−131] With the exception of K42R, mutations in S12 are associated with a hyperaccurate phenotype that can lead to dependence on streptomycin, which stabilizes the ram state of the A site. The streptomycin-dependent phenotype can be relieved by ram mutations in proteins S4 and S5 and located at the interface of the two proteins.[127−129,132] Other mutations altering rRNA, EF-Tu, or S12 itself can also compensate for streptomycin dependence,[128] indicating that all three components are involved in the conformational stability of the A site. Restrictive mutations located in 50S subunit components, including truncation of the C terminus of L6 or substitution of G2661, have also been associated with resistance to various aminoglycosides.[126,131] Such mutations do not affect the binding of aminoglycosides but likely compensate for the ram phenotypic effect of these drugs by increasing the affinity of the EF-Tu for 50S. Finally, mutations in the N-terminal half of S5, which contact helix 34, can confer resistance to spectinomycin by destabilizing the network of interactions in the 30S subunit, allowing the head region to move even in the presence of the drug.[10,133]

3.3. Enzymatic Drug Modification

3.3.1. Aminoglycoside Adenylyltransferases

Aminoglycoside adenylyltransferases, with only 10 enzymes identified to date, include examples of both chromosomally encoded and plasmid-encoded enzymes. In Gram-negative organisms, the *ant(2″)* and *ant(3″)* genes encoding adenylyltransferases are often identified on mobile genetic elements in resistant organisms. In Gram-positive organisms, the *ant(4′)*, *ant(6)*, and *ant(9)* genes are also found on plasmids or integrated into transposons.[134] These enzymes catalyze the reaction between Mg-ATP and aminoglycoside to form the O-adenylylated aminoglycoside and the magnesium chelate of inorganic pyrophosphate (Figure 6). Enzymes that regioselectively adenylylate the 6 and 3″ positions and the 9 and 3″ positions, of the atypical aminoglycosides streptomy-

cin and spectinomycin, respectively, have been identified. From a clinical perspective, the reactions catalyzed by the ANT(2″) and ANT(4′) are of most significance and have been the most thoroughly mechanistically and structurally studied.

The earliest mechanistic studies of the adenylyltransferases were those of Lombardini[135] and Northrop[136−139] on the *E. coli* ANT(2″). Both found the kinetic mechanism to be sequential, requiring both substrates to be present before catalysis could take place, and Lombardini suggested an ordered mechanism of substrate binding, with nucleotide (ATP) binding before aminoglycoside. In a detailed series of kinetic investigations, Northrop argued for this same order of substrate addition but added the ordered release of inorganic pyrophosphate followed by the rate-limiting release of the adenylylated aminoglycoside. The slow release of this final product makes the kinetic mechanism Theorell−Chance. The enzyme exhibits comparable activity with all nucleotide triphosphates and even their deoxy derivatives. It also shows activity with a broad array of 4,6-disubstituted substrates, but the relative *V*/*K* values for these substrates vary by a factor of 4000. Interestingly, there is a significant positive correlation between the enzymatic *V*/*K* values for aminoglycoside substrates and the MIC values for these aminoglycosides in strains expressing ANT(2″).[140]

The *S. aureus* ANT(4′) has also been studied in significant detail. The enzyme was initially identified from a kanamycin- and gentamicin-resistant clinical strain[141,142] and subsequently shown to have activity with a large number of aminoglycosides possessing either 4′- or 4″-hydroxyl substituents.[143] The kinetic mechanism is sequential, and the stereochemistry at the α-phosphorus atom of ATP has been shown to undergo inversion during turnover,[144] suggesting that adenylyl transfer occurred via a direct displacement of the leaving group, inorganic pyrophosphate, by the nucleophilic hydroxyl group of the aminoglycoside. In 1993, Holden reported the three-dimensional crystal structure of the enzyme[145] and subsequently reported the structure of the ANT(4′)−kanamycinA−Mg-AMPCPP (α,*β*-methylene-ATP) ternary complex.[146] These structures were determined using a thermostable mutant of the wild-type enzyme, and this was both the first structure of an aminoglycoside-modifying enzyme and the first structure of an aminoglycoside in complex with a modifying enzyme.

The structure of the complex revealed an unusual dimeric arrangement of monomers with obvious N- and C-terminal domains of approximately equal size (Figure 7). In general, residues from the N-terminal domain interact with the nucleotide, while those of the C-terminal domain interact with the aminoglycoside. The two bound nucleotides are far apart, and the majority of interactions are between the triphosphate moiety and the enzyme, suggesting a structural basis for the lack of nucleotide triphosphate specificity. The two bound kanamycin A molecules are as close as 3.5 Å. There are at least four negatively charged side chains of aspartate and glutamate residues that interact with the aminoglycoside, and one of these, glutamate 145, appears

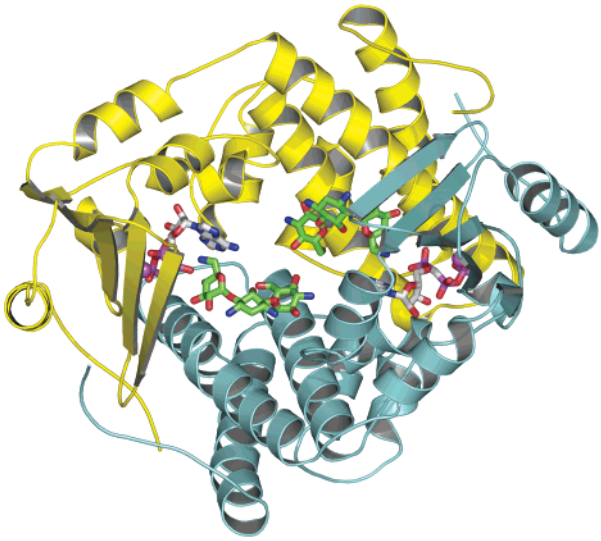


Figure 7. Structure of the *Staphylococcus aureus* ANT(4′)−AMP-PNP−kanamycin A complex. The monomers making up the active dimer are shown as blue and yellow ribbons. AMP-PNP is shown in stick representation and colored by atom type (C, gray; N, blue; O, red; P, pink). Kanamycin is shown in stick representation and colored by a different atom type (C, green; N, blue; O, red). Coordinates were obtained from the Protein Data Bank (1KNY).

positioned to act as a general base to deprotonate the 4′-hydroxyl group. The distance from the 4′-hydroxyl to the α-phosphorus atom of AMPCPP is 5.0 Å, suggesting to these authors that a precatalytic conformational change must occur to allow for the nucleophilic attack on the nucleotide. Alternatively, the use of the nonreactive nucleotide might not allow for the appropriate positioning of the nucleotide, or contacts in the crystal do not allow this positioning to be obtained. The 4′-hydroxyl group and pyrophosphate moiety are positioned for a direct, "in-line" attack at the α-phosphorus atom of AMPCPP. Due to the symmetry of kanamycin A and the resolution at which the structure was determined (2.5 Å), it was not possible to unambiguously distinguish between two conformations in which either the 4′- or 4″-hydroxyl groups would be adenylylated.

This last problem was recently solved by the assignment of the regioselectivity of the reaction using NMR.[147] In this report, the exclusive monoadenylylation of kanamycin A was demonstrated, as was the exclusive 4′-regiospecificity. A priori, both the 4′- and 4″-hydroxyl groups in this symmetric molecule could have been adenylylated, and there are examples of ANT(4′,4″) isozymes that, when presented with a substrate lacking the 4′-hydroxyl group (dibekacin), adenylylate at the 4″ position.[148] Finally, using sensitive ^{18}O kinetic isotope effect methods and a poor substrate, *m*-nitrobenzyl triphosphate, the transition state for the enzymatic 4′-adenylylation of kanamycin A was shown to be associative.[149]

3.3.2. Aminoglycoside Phosphotransferases

Aminoglycoside phosphotransferases catalyze the regiospecific transfer of the *γ*-phosphoryl group of ATP to one of the hydroxyl substituents present on aminoglycoside (Figure 8). They represent a large

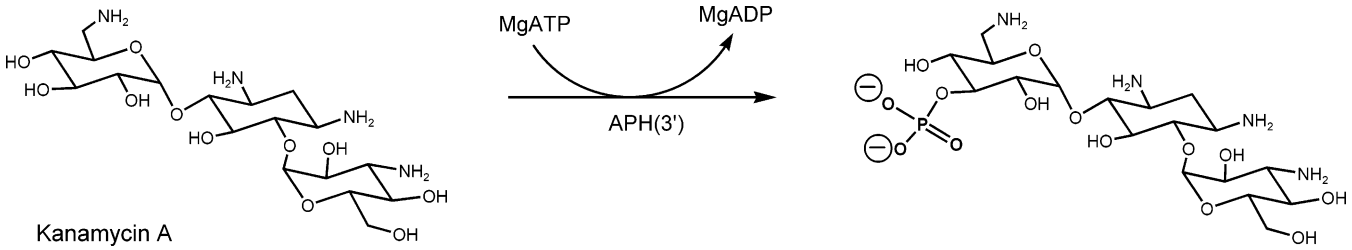


Figure 8. Reaction catalyzed by aminoglycoside O-phosphotransferases. The reaction shown is that catalyzed by APH(3′) catalyzing the MgATP-dependent 4′-O-phosphorylation of kanamycin A.

class of aminoglycoside-modifying enzymes and are particularly relevant to clinical resistance to aminoglycosides in Enterococcal and Staphylococcal species. The vast majority of the biochemical and structural work on this family has focused on the plasmid-encoded APH(3′)-IIIa enzyme from *Enterococcus faecalis*, as will this discussion. The 264 amino acid protein was overexpressed, purified, and shown to catalyze phosphorylation of a broad spectrum of aminoglycosides,[150] as expected by the broad range of aminoglycosides to which the original clinical strain was resistant (tobramycin, a 4,6-disubstituted aminoglycoside lacking a 3′-hydroxyl substituent, is not a substrate for the enzyme, but rather a potent inhibitor). The enzyme binds substrates tightly, with steady-state affinities in the low micromolar range, and the kinetic parameter for aminoglycosides which correlated with the MIC values was *V*/*K*. Enzyme-catalyzed generation of phosphorylated kanamycin A followed by one- and two-dimensional NMR analysis revealed that the proposed 3′-regioselectivity, based on the resistance phenotype, was correct. Several extensions of these regioselectivity studies have been performed. On the basis of the resistance phenotypes observed for a battery of aminoglycosides, it was proposed that for aminoglycosides lacking a 3′-hydroxyl substituent, such as the 4,5-substituted lividomycin A, phosphorylation could occur on the 5″-hydroxyl substituents of the ribose ring.[151] Using β,γ-bidentate Cr-ATP as a paramagnetic probe, the conformations of amikacin, a 4,6-disubtituted aminoglycoside, and butirosin A, a 4,5-disubstituted aminoglycoside, bound to the APH(3′)-IIIa were probed.[152] The conformations of the two types of aminoglycosides were quite different, and the modeled conformations allowed only the 3′-hydroxyl substituents of amikacin to approach the γ-phosphate of ATP, whereas the conformation of enzyme-bound butirosin allowed both the 3′- and 5″-hydroxyl substituents of butirosin to approach the γ-phosphate of ATP. These results were confirmed and extended, and the exclusive monophosphorylation of the 3′-hydroxyl substituents of 4,6-disubstituted aminoglycosides was demonstrated. Those 4,5-disubstituted aminoglycosides containing both 3′- and 5″-hydroxyl substituents were shown to be rapidly monophosphorylated and subsequently bis-phosphorylated.[153] These results reveal that the APH(3′)-IIIa has a remarkable ability to bind a large number of structurally distinct aminoglycosides and catalyze their phosphorylation with a range of regioselectivities.

The steady-state kinetic mechanism was shown to be sequential on the basis of the intersecting pattern of lines observed in the reciprocal plot, arguing against mechanisms invoking a phosphoenzyme intermediate.[154] ATP binds first to the enzyme followed by aminoglycoside, since tobramycin, a potent dead-end inhibitor, exhibits linear uncompetitive inhibition versus ATP and linear competitive inhibition versus kanamycin A. On the basis of product inhibition studies and the uncompetitive substrate inhibition exhibited by aminoglycosides versus ATP, the ordered release of phosphorylated drug followed by the slow rate-limiting release of ADP was proposed. This corresponds to a Theorell−Chance-type kinetic mechanism, where the binding of the aminoglycoside to the E-ADP complex results in the observed uncompetitive substrate inhibition. Strong support for this Theorell−Chance kinetic mechanism came from a subsequent study, employing viscosity variation, solvent kinetic isotope effect measurements, and the use of γ-thio-ATP.[155]

The three-dimensional structure of the APH(3′)-IIIa−ADP complex was reported in 1997.[156] Although the selenomethionine substituted enzyme had been crystallized in the presence of Mg-ATP, the electron density due to the bound nucleotide was only compatible with a bis-magnesium-chelated-ADP complex. This suggests that, during the month-long crystallization, hydrolysis of ATP occurred and that the more tightly bound ADP product complex was observed. The enzyme existed as a doubly disulfide-bonded dimer in the crystal, confirming earlier results showing the enzyme to be active as both a monomer and dimer, and with this equilibrium being affected by the presence of disulfide reducing agents.[150] Each 263 amino acid monomer, lacking only the N-terminal methionine residue, folded into a 94-residue N-terminal domain and a 157 residue C-terminal domain connected by a flexible 12-residue linker (Figure 9). The bound ADP molecule was observed in the cleft between the two domains. The overall architecture of the APH(3′)-IIIa, and in particular the predominantly β-strand N-terminal domain, was similar to the structures previously reported for serine/threonine protein kinases, including the catalytic subunit of the cAMP-dependent protein kinase. Residues at the active site included two conserved lysine residues, K33 and K44, E60, D190, N195, and D208. K33 and D190 make no interactions with bound ADP, but all others make direct or water-mediated interactions with the pyrophosphate moiety of the nucleotide. These results drove a series of site-directed mutagenesis studies that confirmed an important role for K44 in nucleotide binding and an essential role for D190, which

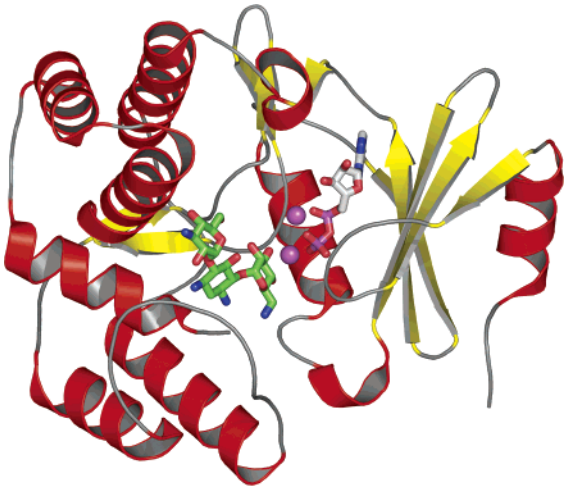


Figure 9. Structure of the *Enterococcus faecalis* APH(3′)−Mg_2-ADP complex. The monomer is shown composed of red α-helices and yellow β-strands. ADP is shown in stick representation and colored by atom type (C, gray; N, blue; O, red; P, pink), and the two magnesium atoms are shown as purple spheres. Kanamycin is shown in stick representation and colored by a different atom type (C, green; N, blue; O, red). Coordinates were obtained from the Protein Data Bank (1L87).

was proposed to function as the general base in the deprotonation of the 3′- (or 5″-) hydroxyl group of bound aminoglycoside.

The three-dimensional structure of the APH(3′)-IIIa in complex with ADP and either kanamycin A or neomycin B[157] provided a molecular explanation for many of the above-referenced studies and others which probed the importance of residues in the carboxyl terminal domain using site-directed mutagenesis.[158] As expected, both aminoglycosides make the majority of interactions with residues of the C-terminal domain and the overwhelming majority of these interactions are electrostatic. Thus, the positively charged aminoglycosides interact with E157, E160, D190, E230, D262, E262, and the carboxyl terminus of F264. Aspartate 190 interacts with the 3′-hydroxyl, supporting its role as the general base responsible for initiating catalysis by deprotonating the 3′-hydroxyl substituent. In the neomycin B complex, the ribose 5″-hydroxyl is pointed toward D190, but the distance between the two is not consistent with D190 acting as a base, in catalysis. The majority of the interactions are between the enzyme and the central deoxystreptamine and the primed, 6-deoxy-6-aminoglucose ring. Two additional subsites were identified that provide interactions to the doubly primed rings of 4,6-substituted and 4,5-substituted aminoglycosides. Although there are no large domain movements accompanying aminoglycoside binding, residues 147−170 close over the bound aminoglycoside and constitute a binding loop that firmly fixes the conformation of the aminoglycoside. The conformations of the bound aminoglycosides were compared to those observed for structurally related aminoglycosides bound to the A site and to the entire 30S ribosomal subunit. Remarkably for these conformationally flexible tricyclic compounds, the structures were quite similar, with root mean squared (rms) deviations of 1.7 Å between neomycin B bound to APH(3′)-IIIa and paromomycin bound to the 16S rRNA of the 30S subunit. The authors suggest that these are low energy conformations and that, while of obviously different character, the kinase active site has evolved to "mimic" the rRNA binding site and tempt the drug into this binding pocket before it can find its true target-binding site.

While eukaryotic protein kinases and APH(3′)-IIIa do not exhibit high degrees of overall sequence homology, the presence of highly conserved residues at the nucleotide binding site and the structural similarity of the proteins suggested that the two proteins were evolutionarily related. Whether these similarities extended to the functional ability of the bacterial APH(3′)-IIIa to phosphorylate proteins was demonstrated in 1999.[159] APH(3′)-IIIa was shown to be capable of phosphorylating several, but not all, basic peptides, including the MARCKS (myristolated alanine-rich C-kinase substrates) K and R peptides as well as protamine and myelin basic protein at rates that were 10−20% that of kanamycin A phosphorylation. The specificity of the activity was limited, and the enzyme was incapable of phosphorylating kemptide, histone 1, and peptide substrates for tyrosine protein kinases. Phosphoamino acid analysis of the product phosphopeptides revealed that the phosphorylation occurred on the serine residue of the MARCKS K peptide, although this peptide contains neither a threonine nor tyrosine residue (Ac-FKKS-FKL-NH_2).

Although this section has focused on the *E. faecalis* APH(3′)-IIIa, in *S. aureus* a bifunctional enzyme containing both a kinase domain and an acetyltransferase domain (see below) is of major clinical significance. This enzyme is termed AAC(6′)-Ie-APH(2″)-Ia and, because of its dual kinase−acetyltransferase activities, can provide resistance to the majority of clinically useful aminoglycosides. The kinetic mechanism of the kinase reaction of the bifunctional enzyme differs from that of APH(3′)-IIIA, being rapid equilibrium random.[160] APH(2″) is inactivated by the lipid kinase inhibitor, wortmannin, but contrary to APH(3′)-IIIa is not inactivated by the ATP analogue, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine.[161,162] However, like APH(3′)-IIIa, the enzyme can use protein kinase substrates to perform phosphorylation on serine residues.[159]

3.3.3. Aminoglycoside Acetyltransferases

Well over four dozen unique sequences exist for aminoglycoside acetyltransferases. These enzymes catalyze the acetyl-CoA-dependent N-acetylation of one of the four amino groups of typical aminoglycosides (Figure 10). They include enzymes that acetylate the 1- and 3-amino groups of the central deoxystreptamine ring and enzymes that acetylate the 2′- and 6′-amino groups of the primed, 6-deoxy-6-aminoglucose ring. Two distinct AAC(1) activities have been identified in *E. coli* and *Actinomycete* strains,[163,164] but their importance is minor, because the *E. coli* enzyme does not modify the clinically useful aminoglycosides and the other, which exhibits a broader substrate specificity, is not found in human pathogens.

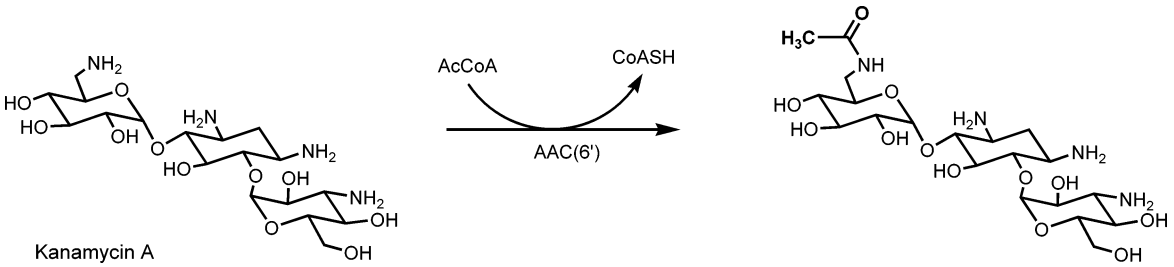


Figure 10. Reaction catalyzed by aminoglycoside *N*-acetyltransferases. The reaction shown is that catalyzed by AAC(6′) catalyzing the acetyl-CoA-dependent 6′-*N*-acetylation of kanamycin A.

There are five aminoglycoside acetyltransferases that catalyze regioselective acetylation of the 2′-amino group, and they all are chromosomally encoded and species specific. The first to be identified was the 178 amino acid AAC(2′)-Ia from *Providencia stuartii*.[165] In wild-type strains, the low level of expression of the *aac(2′)-Ia* gene is not sufficient to confer aminoglycoside resistance, but the transcription of the structural gene was shown to be subject to a complex regulation.[166] Mutations in the *aac(2′)-Ia* gene resulted in altered levels of peptidoglycan O-acetylation and cell morphology,[167] suggesting that peptidoglycan acetylation might be a physiologic function of the enzyme. The expressed and purified enzyme was shown biochemically to exhibit broad specificity for aminoglycosides containing 2′-amino substituents. The relative *V*/*K* values for aminoglycosides did not correlate with in vivo MIC values,[168] supporting the idea that the enzyme functions physiologically in other reactions, possibly peptidoglycan acetylation. All other members of the AAC(2′) family have been identified in mycobacterial species. They were originally identified in rapidly growing species of mycobacteria.[169] The *aac(2′)-Ib* gene identified in *M. fortuitum* appeared to be transcriptionally silent in its natural host, but its expression in *M. smegmatis* resulted in increased MIC values for all 2′-amino-substituted aminoglycosides.[170] Subsequently, other *aac(2′)* genes were identified in both *M. smegmatis* and *M. tuberculosis*. The corresponding enzymes were shown to bear 60−70% sequence identity with the *M. fortuitum* enzyme but only 30−40% identity with the *P. stuartii* AAC(2′)-Ia. None of the encoded proteins bore significant sequence homology (<10%) to other proteins, including other aminoglycoside acetyltransferases.

The *M. tuberculosis* AAC(2′)-Ic, chromosomally encoded by the Rv0262 gene, was expressed and purified and shown to catalyze the acetyl-CoA-dependent acetylation of a broad range of aminoglycoside substrates. In contrast to several other aminoglycoside acetyltransferases, the AAC(2′)-Ic activity, even though highest with aminoglycosides containing a 2′-amino substitutent, could also be detected with kanamycin A and amikacin, both of which contain a 2′-hydroxyl substituent,[171] suggesting that this enzyme can catalyze O-acetylation. Both standard steady-state and alternative substrate kinetics supported the ordered addition of acetyl-CoA followed by aminoglycoside to generate a ternary complex from which acetyltransferase chemistry ensued. Very modest solvent kinetic isotope effects were observed, suggesting that chemistry was not rate limiting. The appearance of "substrate activation" at high aminoglycoside concentrations suggested that acetylated aminoglycoside dissociated first, followed by the slow release of CoA, and that binding of aminoglycoside to the CoA binary complex could increase this product's rate of dissociation.

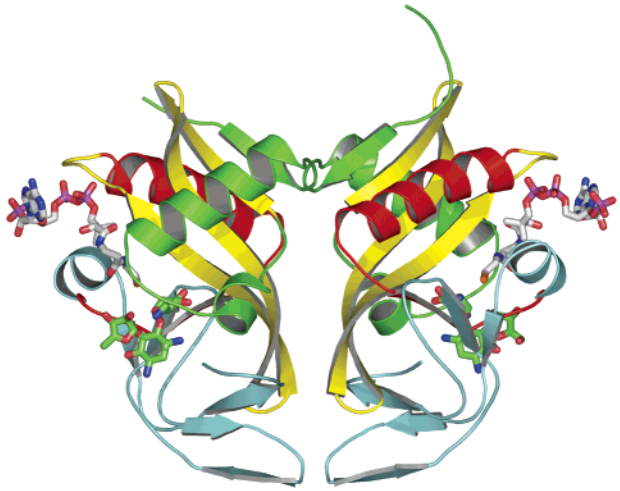


Figure 11. Structure of the *Mycobacterium tuberculosis* AAC(2′)−CoA−ribostamycin complex. The monomers making up the active dimer are shown as ribbons that are colored by sequence position (N-terminal, green > yellow > red > blue, C-terminal). CoA is shown in stick representation and colored by atom type (C, gray; N, blue; O, red; P, pink). Ribostamycin is shown in stick representation and colored by atom type (C, green; N, blue; O, red). Coordinates were obtained from the Protein Data Bank (1M4G).

The three-dimensional structures of the full-length 181 amino acid apo form of the enzyme, plus three different ternary complexes containing bound CoA and tobramycin, kanamycin A, or ribostamycin, were recently reported at resolutions between 1.5 and 1.8 Å.[172] Although lacking any sequence homology to other aminoglycoside *N*-acetyltransferases, the monomer fold was nearly identical to those of the *S. marcescens* AAC(3′) and *E. faecalis* AAC(6′)-Ii, identifying AAC(2′)-Ic as a member of the Gcn5-related *N*-acetyltransferase (GNAT) superfamily.[173] The conserved features of this fold are an N-terminal α-helix, a central, antiparallel three-stranded β-sheet, and a helix−sheet−helix at the C-terminus of the fold (Figure 11). This fold serves to bind and orient the phosphopantothenoyl arm of acetyl-CoA, while very few interactions are made between the enzyme and the adenine ring. The majority of interactions between the enzyme and aminoglycoside occur between acidic residues in the active site (D35, D40, E82,

D152, D179, and the carboxyl terminus of W181) and the hydroxyl and amino substituents of the central deoxystreptamine and primed rings, either directly or via intervening water molecules. The 2′-amino group of the bound substrate is positioned 3.8 Å from the sulfur atom of bound CoA, confirming the regioselective nature of the acetylation. The C-terminal carboxylate of W181 is hydrogen-bonded through a series of water molecules to the 2′-amino group of bound ribostamycin, suggesting that this residue functions as the general base responsible for activating the amine and deprotonating the zwitterionic tetrahedral intermediate. V84 and G83 are positioned in the "beta bulge" that is conserved in GNAT superfamily members, but a model of the complex containing acetyl-CoA suggests that a single hydrogen bond between the thioester carbonyl of the substrate and the backbone amide nitrogen of V84 can be accommodated. The phenolic hydroxyl group of Y126 is appropriately positioned to function as a general acid, protonating the leaving thiolate of CoA. Together, these structures suggest that the enzyme binds the two substrates tightly, in an orientation that facilitates catalysis, and provides enzyme side chains that can function as both general base (W131) and general acid (Y126). Thus, in spite of the strongly favorable thermodynamics of the reaction, the enzyme assists in carbonyl polarization, base-assisted amine protonation, and general acid-assisted breakdown of the tetrahedral intermediate.

The AAC(3) family of aminoglycoside acetyltransferases is one of the largest and includes four major types, I−IV, based on the pattern of aminoglycoside resistance that they confer. The first aminoglycoside-modifying enzyme to be purified to homogeneity was the *E. coli* R-plasmid-encoded gentamicin acetyltransferase.[174] This allowed for the first studies of the substrate specificity of these enzymes and the establishment of a correlation between measured MIC values and the kinetic parameter *V/K*, indicative of the efficiency of substrate utilization. These early studies revealed that the kinetic mechanism was sequential but was dependent on the identity of the substrate: with good substrates such as sisomicin, the mechanism was random, while, with poor substrates such as tobramycin, the kinetic mechanism was rapid equilibrium random, with chemistry being rate-limiting for the overall reaction.[175] The demonstration of uncompetitive substrate inhibition suggested that aminoglycosides could bind to the product binary E−CoA complex. A subsequent detailed structure−activity relationship allowed important interactions involving the 2′-, 6′-, 3-, and 3″-amino groups to be defined.[140] Finally, these studies made it clear that the kinetic parameter *V/K* for the aminoglycoside was positively correlated with in vivo activity against strains expressing the enzyme.

The AAC(3)-I from *S. marcescens*, originally identified in 1991,[176] was the first aminoglycoside acetyltransferase whose three-dimensional structure was determined.[177] The enzyme−CoA complex structure was determined at a resolution of 2.3 Å, allowing the interactions between the enzyme and the product to be identified. The monomer fold was typical of the

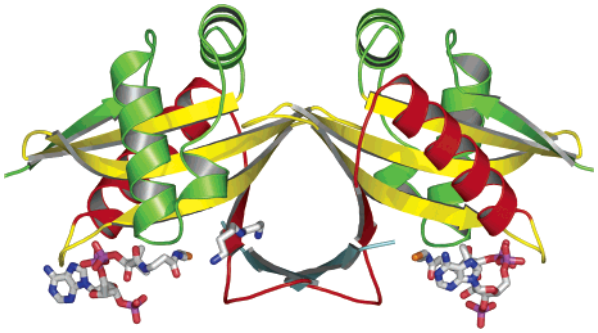


Figure 12. Structure of the *Serratia marcescens* AAC(3)−CoA complex. The monomers making up the active dimer are shown as ribbons that are colored by sequence position (N-terminal, green > yellow > red > blue, C-terminal). CoA is shown in stick representation and colored by atom type (C, gray; N, blue; O, red; P, pink). Coordinates were obtained from the Protein Data Bank (1BO4).

GNAT superfamily, with the characteristic central antiparallel β-sheet covered by the two amino terminal helices on one side and the two carboxy terminal helices on the other (Figure 12). While the oligomeric property of the enzyme in solution was not discussed, the enzyme crystallizes with a dimer in the asymmetric unit, suggesting that, like most GNAT superfamily members, the dimer is functionally active. Unfortunately, no structures containing bound aminoglycosides were reported, leaving the discussion of how these enzymes regioselectively catalyze acetylation open. The recent studies of the AAC(6′) subgroup of acetyltransferases have allowed this issue to be resolved.

As probed by the structural data of bound aminoglycosides to the 30S ribosomal subunit,[5,7,9,10] the 6′-amino group plays an important role in target binding and the subsequent antibacterial activity of the drug. This substituent is thus not surprisingly the target of one of the major classes of aminoglycoside-modifying enzymes, the AAC(6′) class, which includes more than 25 members. The most common pattern of resistance associated with the production of these enzymes (type I) includes the majority of the useful aminoglycosides except the mixture of gentamicins. Among this subclass, three enzymes have been extensively studied, the two species specific and chromosomally encoded AAC(6′)-Ii and AAC(6′)-Iy from *E. faecium* and S*almonella enterica*, respectively, and the widespread, plasmid-encoded, bifunctional AAC(6′)-Ie-APH(2″).

In *E. faecium* the chromosomally encoded AAC(6′)-Ii is, in part, responsible for the intrinsic low-level resistance to aminoglycosides of the species. The first structural studies of aminoglycoside binding to AAC(6′)-Ii involved the use of NMR spectroscopy and molecular modeling to determine the conformations of two aminoglycosides, the 4,6-disubstituted deoxystreptamine isepamicin and the 4,5-disubstituted butirosin A, in the active site of the enzyme.[178] These data showed that, in the ternary AAC(6′)-Ii−CoA−isepamicin complex, the drug can adopt two distinct conformations, which are able to interconvert. The structure of AAC(6′)-Ii with either the substrate acetyl-CoA[179] or the product CoA[180] bound at the active site was subsequently reported. The overall

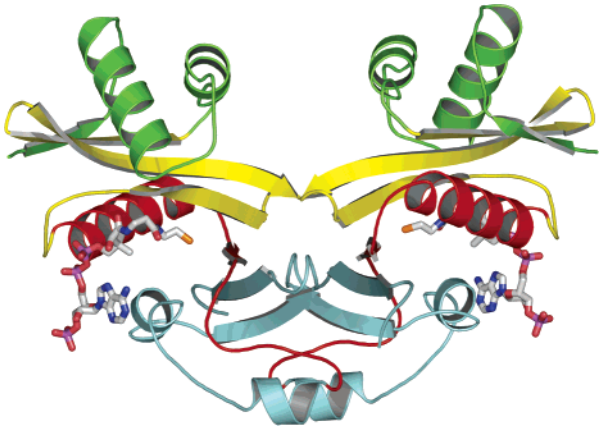


Figure 13. Structure of the *Enterococcus feacium* AAC(6′)−CoA complex. The monomers making up the active dimer are shown as ribbons that are colored by sequence position (N-terminal, green > yellow > red > blue, C-terminal). CoA is shown in stick representation and colored by atom type (C, gray; N, blue; O, red; P, pink). Coordinates were obtained from the Protein Data Bank (1N71).

fold of the AAC(6′)-Ii monomer revealed that the enzyme was a member of the GNAT superfamily characterized by a structurally conserved core (see above). The shape of the monomer can be likened to a V, with the acetyl-CoA binding site positioned between the two arms. Most of the interactions formed between the enzyme and acetyl-CoA involved the main chain atoms, and only the side chains of K149 and T89 interact with the substrate. Although the monomer fold was quite similar to those of other GNAT superfamily members, the structure of the AAC(6′)-Ii dimer revealed a significant diversity in the dimer assembly (Figure 13). The dimeric enzyme exhibits broad substrate specificity for acyl donors and aminoglycosides containing a 6′-amino group.[181] Initial velocity and inhibition studies performed with desulfo-CoA are consistent with an ordered sequential kinetic mechanism with acetyl-CoA binding first and CoA released last.[182] This result is consistent with the location of the acetyl-CoA binding site in the bottom of the active site. The rate-limiting steps of the reaction were explored by solvent viscosity and solvent isotope effects. The results showed that diffusion-controlled events (substrate binding and/or product release) were the rate-limiting steps of the reaction rather than chemistry.[182] The potential catalytic roles of several residues located in the AAC(6′)-Ii active site were investigated kinetically using different mutant forms of the enzyme.[183] These studies showed that none of the residues mutagenized (Q72, H74, L76, and Y147) perturb the structural integrity of the enzyme and none are involved in general base or acid catalysis. However, the results suggest that Q72 may play a role in aminoglycoside recognition and orientation in the active site and that the amide group of L76 is involved in transition state stabilization. Another mutant form of AAC(6′)-Ii, W104A, which does not form a dimer in solution, was also produced to investigate subunit cooperativity in the AAC(6′)-Ii dimer that was suggested by the partial and mixed inhibition kinetic patterns.[182] The unusual inhibition patterns were alleviated when the mutant monomeric form of the enzyme was used, and isothermal calorimetry (ITC) analysis of aminoglycoside binding to WT AAC(6′)-Ii revealed that two nonequivalent binding sites exist in the dimer, supporting a subunit cooperativity. The structural homology between eucaryotic histone acetyltransferases and AAC(6′)-Ii, as well as the relatively inefficient aminoglycoside acetyltransferase activity displayed by AAC(6′)-Ii ($k_{cat}/K_m \sim 10^4$ M^{-1} s^{-1}) and the lack of correlation between V/K values for aminoglycosides and MIC values, has led the authors to investigate the ability of the enzyme to acetylate other substrates. AAC(6′)-Ii is capable of acetylating small basic proteins such as calf histones, myelin basic protein, or ribonuclease A.[179]

The *S. enterica* AAC(6′)-Iy has been shown to confer broad aminoglycoside resistance to a clinical isolate in which a chromosomal deletion lead to the expression of the usually silent structural gene.[184] The purified recombinant AAC(6′)-Iy was expressed in *E. coli* and shown to exist as a dimer in solution. The enzyme exhibits a high preference for acetyl-CoA as the acyl donor ($K_m = 10$ μM) and a strict specificity for the aminoglycosides containing a 6′-amino group; lividomycin A, which possess a 6′- hydroxyl substituent, is a powerful inhibitor of the reaction. On the basis of their kinetic behavior, the aminoglycoside substrates can be divided into two classes, one that exhibits Michaelis−Menten kinetics and a second that displays "substrate activation".[185] The thermodynamic parameters of substrate binding, obtained from both fluorescence spectroscopy and ITC, showed that both aminoglycosides and acyl-CoA's can bind to the free enzyme and that aminoglycoside binding to AAC(6′)-Iy is strongly enthalpically driven.[186] Kinetic and thermodynamic studies performed using the wild type or mutant forms of the enzyme suggest that C70 is essential for drug binding at the active site. Steady-state kinetics and alternative antibiotic kinetics indicated that the enzyme displays a sequential kinetic mechanism. Dead-end inhibition performed with desulfo-CoA and lividomycin A together with the dependence of V and $V/K_{acetyl-CoA}$ on the identity of the aminoglycoside used argued for the random order of substrate binding to the enzyme. The inequality of the solvent isotope effects on ^{D_2O}V and $^{D_2O}V/K$ suggests that release of CoA is rate-limiting.[185]

The three-dimensional structure of the enzyme, solved at 2.4 Å resolution by multiwavelength anomalous diffraction methods, placed AAC(6′)-Iy in the Gcn5-related *N*-acetyltransferase (GNAT) superfamily.[187] While the tertiary structure of the monomer is very similar to those observed for other members of the superfamily, the structure of the active dimer consists of a continuous 12-stranded β-sheet characterized by a carboxyl terminal strand exchange (Figure 14). This exchange has not been previously observed with aminoglycoside *N*-acetyltransferases but has been observed in the dimeric yeast histone acetyltransferase Hpa2, which is the closest structural homologue of AAC(6′)-Iy. Although not added in the crystallization solution, CoA was found in both active sites of the enzyme formed by a negatively

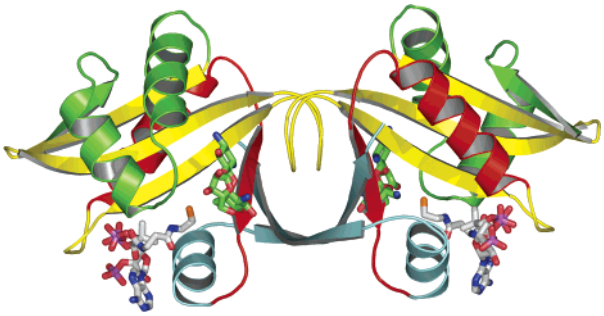


Figure 14. Structure of the *Salmonella enterica* AAC(6′)−CoA−ribostamycin complex. The monomers making up the active dimer are shown as ribbons that are colored by sequence position (N-terminal, green > yellow > red > blue, C-terminal). CoA is shown in stick representation and colored by atom type (C, gray; N, blue; O, red; P, pink). Kanamycin is shown in stick representation and colored by atom type (C, green; N, blue; O, red). Coordinates were obtained from the Protein Data Bank (1S3Z).

charged channel at the dimer interface. In the AAC(6′)-Iy−CoA−ribostamycin ternary complex, described in the same paper, ribostamycin is stacked between the aromatic rings of W22 and Y66, and the primed and central rings make contacts with the side chains of E79, D115, and E136. The 6′-amine of the antibiotic is 3.4 Å away from the sulfur atom of CoA, consistent with a direct nucleophilic attack of the amine on the thioester. D115 was proposed to function as the general base via an intervening water molecule between D115 and the 6′-amino group. Similarly, it was proposed that the protonation of the thiolate after collapse of the tetrahedral intermediate occurs using a water molecule accessible to bulk solvent. The comparison of the two ternary complexes of CoA and ribostamycin bound to AAC(6′)-Iy or AAC(2′)-Ic provided a molecular basis for the regioselective activity of these enzymes. In the AAC(6′)-Iy−ribostamycin complex, the central and primed rings make contacts with N-terminal elements of the enzyme and the 2′-amine makes an intramolecular hydrogen bond with the 5″-hydroxyl group of the ribose ring orientating the 6′-amino group toward acetyl-CoA. In the AAC(2′)-Ic complex, the primed and central rings make contacts with C-terminal elements of the enzyme and the 6′-amino group makes an intramolecular hydrogen bond with the 2″-hydroxyl group of the ribose ring, which is rotated almost 180° relative to that of the AAC(6′)-Iy complex. This change results in the positioning of the 2′-amino group toward acetyl-CoA. Finally, intimate contacts between two dimers of recombinant AAC(6′)-Iy were seen in the crystal structure, such that the extended affinity tag-containing N-terminal extension of one dimer is bound into the active site of an adjacent dimer. This observation led to the demonstration that AAC(6′)-Iy is also capable of autoacetylation, acetylation of eucaryotic histones and the human histone H3 N-terminal peptide in a regioselective manner.[187]

The bifunctional AAC(6′)-Ie−APH(2″)-Ia, which confers broad spectrum, high-level aminoglycoside resistance in Enterococci and Staphylococci, differs from the two AAC(6′)'s described above in its genetic localization and catalytic mechanism.[188] The structural gene of the enzyme is generally found on transposable elements, frequently carried on R plasmids. These mobile supports account for the intergenus transfer of the resistant determinant, originally isolated from *E. faecalis*. The enzyme is monomeric in solution, and the acetyltransferase activity exhibits exceptionally broad substrate specificity for aminoglycosides including fortimcin A and aminoglycosides possessing a hydroxyl group at the 6′-position. The O-acetylated paromomycin product was identified on the basis of its lability under basic conditions and infrared spectrometry analysis.[188] The ability of AAC(6′)-Ie to catalyze both N- and O-acetylation was attributed to the presence of a general base that would assist in hydroxyl deprotonation. The solvent kinetic isotope effect, pH studies, and site-directed mutagenesis identified D99 as the active site base required for aminoglycoside O-acetylation and *N*-acetylation of fortimicin A.[189] Finally, a mutant form of this enzyme, exhibiting an arbekacin 4‴-*N*-acetyltransferase activity, was identified in a methicillin-resistant strain of *S. aureus* in Japan. It was proposed that the G80D mutation affects the conformation of the protein and leads to a change in its enzymatic regiospecificity from 6′- to 4‴-acetylation.[190] The bifunctional AAC(6′)−APH(2″) enzyme has been proposed to arise by gene fusion to confer resistance to a wider spectrum of aminoglycosides to its bacterial hosts than either enzyme alone, and it can develop an AAC(4‴) activity to more efficiently modify arbekacin, which is widely used in Japan. This example illustrates the remarkable ability of bacteria to rapidly adapt to changes in antibiotic use and selective pressure.

3.4. Origin and Prevalence

Acquired aminoglycoside resistance is primarily due to the expression of enzymes that catalyze the chemical modification of the drug, thus preventing, or substantially weakening, their interaction with the ribosome. Whereas some enzymes are encoded by chromosomal genes specific for a bacterial genus or species, the majority of the structural genes encoding inactivating enzymes, both in Gram-negative and Gram-positive bacteria, are located on transferable genetic elements including plasmids, transposons, and integrons, which allowed their dissemination.[191−195] For these modifying enzymes, a positive correlation between the *V*/*K* values for aminoglycosides and the MIC values obtained in vivo for the same aminoglycosides in strains expressing one of these enzyme is observed. Northrop has argued that, for the greatest efficiency, an aminoglycoside-modifying enzyme would be expected to exhibit such a correlation.[140] On the contrary, there is usually no correlation between the *V*/*K* values for aminoglycosides and the MIC values for strains expressing a originally chromosomally encoded modifying enzyme. This observation suggests that enzymes carried on mobile genetic elements have evolved to be efficient aminoglycoside-modifying enzymes and have been disseminated for this purpose. Their presence may thus be the result of the dissemination of the resistant determinants of producer microorganisms. On

another hand, chromosomally encoded and species specific enzymes likely have other physiological functions in their bacterial host and are selectively recruited to counter antibacterial agents. The possible physiological functions of some AAC(2′) and AAC(6′) acetyltransferases have been discussed in the literature;[167,172,179,187] however, the physiological substrates for such bacterial GNAT proteins have not been unambiguously identified.

As a consequence of the influence of selective pressure on the acquisition of transferable resistance genes, the distribution of specific inactivating enzymes varies depending on the geographic area and on specific aminoglycoside use.[196,197] For example, the incidence of resistance due to expression of AAC(6′)-I enzymes capable of inactivating amikacin is significantly higher in countries where this antibiotic is used extensively, including France, Belgium, and Greece, but is less frequent in other European countries or in the United States.[196] Because mobile genetic supports usually harbor several antibiotic-resistant genes, their acquisition often results in a multidrug resistance phenotype. Besides this genetic linkage, the simultaneous selective pressure of various antibiotics can also be the origin of multidrug resistance acquisition, illustrated by the higher frequency of aminoglycoside resistance in organisms simultaneously resistant to another class of drugs. For example, a study performed under the European SENTRY program indicated that while the percentage of gentamicin resistance was only 7% in strains of methicillin-susceptible *S. aureus*, it reached 80% in methicillin-resistant strains.[198] The emergence of multidrug-resistant organisms expressing efflux pumps and responsible for nosocomial infections is another consequence of the multidrug pressure.

Finally, mechanisms of resistance such as permeability alteration and target mutation are not horizontally transferable, but they remain important and can account for a high percentage of the resistant population of a particular species.[197] The recent characterization of genes encoding methyltransferases that catalyze the modification of the 16S rRNA and are located on transferable elements portends a wide and rapid dissemination of these enzymes, responsible for broad spectrum and high-level aminoglycoside resistance, soon.

4. Resisting Resistance

On the basis of the therapeutic revival of the β-lactam class of antibacterials upon the introduction of formulations containing a β-lactamase inhibitor and the parent β-lactam, interest in the development of inhibitors of aminoglycoside-modifying enzymes has increased sharply. The first report of such an inhibitor was by Northrop, who semisynthetically prepared the bisubstrate analogue of kanamycin B and CoA (Figure 15).[199] This exerted powerful inhibition versus the aminoglycoside acetyltransferase, exhibiting a K_i value of 9 nM, and did not restore sensitivity to aminoglycosides in strains expressing the *N*-acetyltransferase, undoubtedly because the compound was incapable of penetrating the bacterial envelope. The ability of the natural product 7-hydroxytropolone to inhibit the ANT(2″) enzyme and to restore sensitivity to aminoglycosides in *E. coli* strains harboring the *ant(2″)* gene was reported in 1982.[200] A more recent report has attempted to synthesize compounds that have high affinity for the bacterial ribosomal A site but are only poorly recognized and acetylated by aminoglycoside-modifying enzymes. An example of a successful approach used variably cross-linked dimers of the bicyclic aminoglycoside neamine.[201] This series of compounds were shown to bind to an A site rRNA oligoribonucleotide with high affinity (40 nM for the diaminobutane cross-linked compound, Figure 16) and displayed antibacterial activity (MIC = 6.25 μM). This compound was a poor substrate for both acetyltransferases AAC(6′)-Ii and AAC(6′)-APH(2″), and in fact, it was a powerful inhibitor of the kinase activity (K_i = 0.7 μM) of the bifunctional enzyme. Another approach that found some success was based on the highly anionic nature of the aminoglycoside binding site in the structure of the AAC(6′)-Ii. Reasoning that cationic peptides that by themselves exhibited antibacterial properties might bind to this enzyme, a series of relatively short (12−24 residues) and highly cationic (charge +4 to +9) peptides were synthesized and tested as inhibitors. These peptides did indeed inhibit the AAC(6′)-Ii and both the APH (3″)-IIa and APH(2″)-Ia with micromolar affinity.[189]

Mobashery and colleagues have synthesized a number of aminoglycoside analogues with the potential to be poor substrates for aminoglycoside phosphotransferases, to inactivate the modifying enzyme,

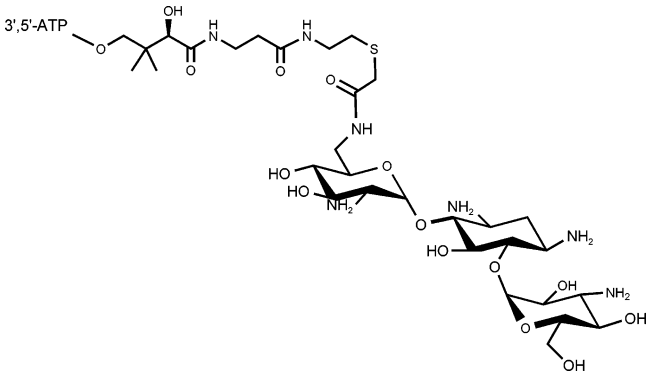


Figure 15. Structure of the bisubstrate analogue described by Williams and Northrop.

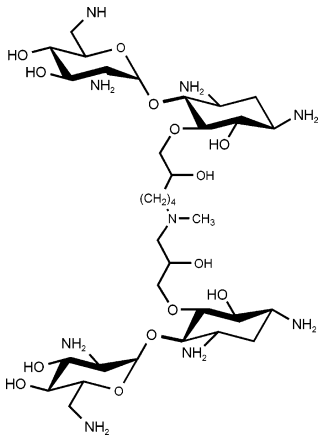


Figure 16. Structure of the bis-neamine dimer described by Wong et al.

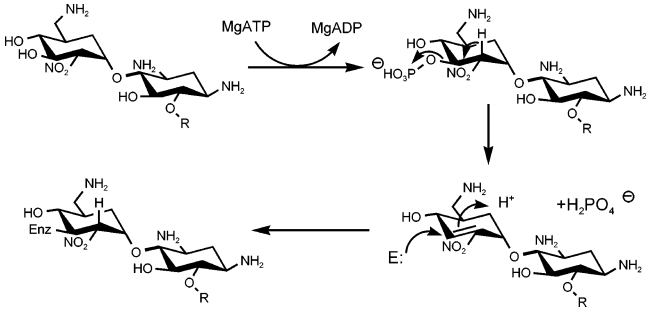


Figure 17. Proposed reactions of 2-nitrokanamycin with the APH(3′) phosphotransferase. (Reprinted with permission from ref 202. Copyright 1995 American Chemical Society).

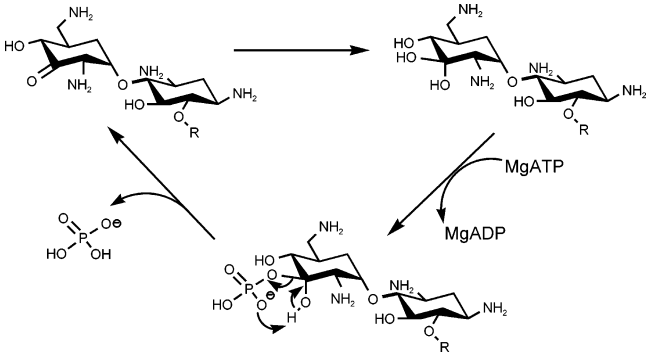


Figure 18. Proposed reaction of 3-ketokanamycin with the APH(3′) phosphotransferase. (Reprinted with permission from ref 203. Copyright 1999 American Chemical Society.)

or to regenerate themselves after enzymatic modification. The first of these was a 2′-nitro-substituted aminoglycoside. Upon phosphorylation by APH(3′), the adjacent nitro group reduces the pK of the 2′-proton sufficiently that elimination of the 3′-phosphate occurs. The vinylogous product is a Michael acceptor that can be captured by an enzymic nucleophile, resulting in a novel mechanism-based inhibition of the kinase (Figure 17).[202] A second strategy involved the synthesis of a 3′-keto aminoglycoside derivative.[203] In solution, the ketone is hydrated and mimics the 3′-equatorial hydroxyl group that is the locus of phosphorylation. In fact, the compound is phosphorylated by APH(3′) but decomposes with the elimination of phosphate to regenerate the 3-keto group (Figure 18). The compound itself exhibits weak antibiotic activity, as determined by its MIC value (250 μM), and does modestly sensitize bacteria harboring APH(3′) to other aminoglycosides (4−8-fold decreases in MIC values). Finally, the synthesis and evaluation of 4′,4′-difluorokanamycin A and neamine derivatives have recently been reported.[204] Because of the presence of the highly electron withdrawing fluorine substituent adjacent to the 3′-hydroxy group, the nucleophilicity of the hydroxy group is significantly diminished. The turnover numbers for the 3′-phosphorylation of the difluoroaminoglycosides are decreased by almost 3 orders of magnitude. While the MIC values of these difluoroaminoglycosides are not impressive, the compounds are as effective against strains expressing APH(3′) as against wild-type strains. This is a clear demonstration that the concept of synthesizing aminoglycoside derivatives that are slowly modified, or incapable of being modified, but yet exhibit good antibacterial properties, either alone or in combination with extant aminoglycosides, is a good one. Another example illustrating this concept are the neamine derivatives, substituted in positions 1 and 6, that exhibit a higher antibacterial activity against both neamine-sensitive and neamine-resistant strains than the parental compounds.[205]

5. Acknowledgment

The authors would like to thank Dr. Matt W. Vetting for assistance in preparing Figures 7, 9, 11, 12, 13, and 14. This work has been supported by the Unites States National Institutes of Health.

6. References

(1) Schatz, A.; Bugie, E.; Waksman, S. A. *Proc. Soc. Exp. Biol. Med.* **1944**, *55*, 66−69.
(2) Kotra, L. P.; Haddad, J.; Mobashery, S. *Antimicrob. Agents Chemother.* **2000**, *44*, 3249−56.
(3) Vicens, Q.; Westhof, E. *Biopolymers* **2003**, *70*, 42−57.
(4) Vakulenko, S. B.; Mobashery, S. *Clin. Microbiol. Rev.* **2003**, *16*, 430−50.
(5) Wimberly, B. T.; Brodersen, D. E.; Clemons, W. M., Jr.; Morgan-Warren, R. J.; Carter, A. P.; Vonrhein, C.; Hartsch, T.; Ramakrishnan, V. *Nature* **2000**, *407*, 327−39.
(6) Schluenzen, F.; Tocilj, A.; Zarivach, R.; Harms, J.; Gluehmann, M.; Janell, D.; Bashan, A.; Bartels, H.; Agmon, I.; Franceschi, F.; Yonath, A. *Cell* **2000**, *102*, 615−23.
(7) Fourmy, D.; Recht, M. I.; Blanchard, S. C.; Puglisi, J. D. *Science* **1996**, *274*, 1367−71.
(8) Fourmy, D.; Yoshizawa, S.; Puglisi, J. D. *J. Mol. Biol.* **1998**, *277*, 333−45.
(9) Fourmy, D.; Recht, M. I.; Puglisi, J. D. *J. Mol. Biol.* **1998**, *277*, 347−62.
(10) Carter, A. P.; Clemons, W. M.; Brodersen, D. E.; Morgan-Warren, R. J.; Wimberly, B. T.; Ramakrishnan, V. *Nature* **2000**, *407*, 340−8.
(11) Yoshizawa, S.; Fourmy, D.; Puglisi, J. D. *EMBO J.* **1998**, *17*, 6437−48.
(12) Vicens, Q.; Westhof, E. *Chem. Biol.* **2002**, *9*, 747−55.
(13) Karimi, R.; Ehrenberg, M. *Eur. J. Biochem.* **1994**, *226*, 355−60.
(14) Pape, T.; Wintermeyer, W.; Rodnina, M. V. *Nat. Struct. Biol.* **2000**, *7*, 104−7.
(15) Puglisi, J. D.; Blanchard, S. C.; Green, R. *Nat. Struct. Biol.* **2000**, *7*, 855−61.
(16) Brodersen, D. E.; Clemons, W. M., Jr.; Carter, A. P.; Morgan-Warren, R. J.; Wimberly, B. T.; Ramakrishnan, V. *Cell* **2000**, *103*, 1143−54.
(17) Lynch, S. R.; Puglisi, J. D. *J. Mol. Biol.* **2001**, *306*, 1037−58.
(18) Busse, H. J.; Wostmann, C.; Bakker, E. P. *J. Gen. Microbiol.* **1992**, *138* (Pt 3), 551−61.
(19) Yoneyama, H.; Sato, K.; Nakae, T. *Chemotherapy* **1991**, *37*, 239−45.
(20) Bakker, E. P. *J. Gen. Microbiol.* **1992**, *138* (Pt 3), 563−9.
(21) Davis, B. D. *Microbiol. Rev.* **1987**, *51*, 341−50.
(22) Mehta, R.; Champney, W. S. *Antimicrob. Agents Chemother.* **2002**, *46*, 1546−9.
(23) Edson, R. S.; Terrell, C. L. *Mayo Clin. Proc.* **1999**, *74*, 519−28.
(24) Gonzalez, L. S., 3rd.; Spencer, J. P. *Am. Fam. Physician* **1998**, *58*, 1811−20.
(25) Freeman, C. D.; Nicolau, D. P.; Belliveau, P. P.; Nightingale, C. H. *J. Antimicrob. Chemother.* **1997**, *39*, 677−86.
(26) Craig, W. A.; Vogelman, B. *Ann. Intern. Med.* **1987**, *106*, 900−2.
(27) Zhanel, G. G.; Hoban, D. J.; Harding, G. K. *DICP, Ann. Pharmocother.* **1991**, *25*, 153−63.
(28) Gilbert, D. N. *Antimicrob. Agents Chemother.* **1991**, *35*, 399−405.
(29) Karlowsky, J. A.; Zhanel, G. G.; Davidson, R. J.; Hoban, D. J. *J. Antimicrob. Chemother.* **1994**, *33*, 937−47.
(30) Preston, S. L.; Briceland, L. L. *Pharmacotherapy* **1995**, *15*, 297−316.
(31) Santucci, R. A.; Krieger, J. N. *J. Urol.* **2000**, *163*, 1076−84.
(32) Ratjen, F.; Doring, G.; Nikolaizik, W. H. *Lancet* **2001**, *358*, 983−4.
(33) Moss, R. B. *Chest* **2002**, *121*, 55−63.
(34) Couch, L. A. *Chest* **2001**, *120*, 114S−117S.
(35) Schiffelers, R.; Storm, G.; Bakker-Woudenberg, I. *J. Antimicrob. Chemother.* **2001**, *48*, 333−44.

(36) Ashtekar, D.; Duzgunes, N.; Gangadharam, P. R. *J. Antimicrob. Chemother.* **1991**, *28*, 615−7.
(37) Gangadharam, P. R.; Ashtekar, D. R.; Flasher, D. L.; Duzgunes, N. *Antimicrob. Agents Chemother.* **1995**, *39*, 725−30.
(38) Majumdar, S.; Flasher, D.; Friend, D. S.; Nassos, P.; Yajko, D.; Hadley, W. K.; Duzgunes, N. *Antimicrob. Agents Chemother.* **1992**, *36*, 2808−15.
(39) Gillespie, S. H. *Antimicrob. Agents Chemother.* **2002**, *46*, 267−74.
(40) Gerding, D. N. *Infect. Control Hosp. Epidemiol.* **2000**, *21*, S12−7.
(41) Graham, J. C.; Gould, F. K. *J. Antimicrob. Chemother.* **2002**, *49*, 437−44.
(42) Shanson, D. C. *J. Antimicrob. Chemother.* **1998**, *42*, 292−6.
(43) Nakamura, A.; Hosoda, M.; Kato, T.; Yamada, Y.; Itoh, M.; Kanazawa, K.; Nouda, H. *J. Antimicrob. Chemother.* **2000**, *46*, 901−4.
(44) Inoue, M.; Nonoyama, M.; Okamoto, R.; Ida, T. *Drugs Exp. Clin. Res.* **1994**, *20*, 233−9.
(45) Hotta, K.; Sunada, A.; Ikeda, Y.; Kondo, S. *J. Antibiot. (Tokyo)* **2000**, *53*, 1168−74.
(46) Cordeiro, J. C.; Reis, A. O.; Miranda, E. A.; Sader, H. S. *Braz. J. Infect. Dis.* **2001**, *5*, 130−5.
(47) Kono, K.; Takeda, S.; Tatara, I.; Arakawa, K. *Jpn. J. Antibiot.* **1994**, *47*, 710−9.
(48) Suzuki, K. *Pediatr. Int.* **2003**, *45*, 301−6.
(49) Hamilton-Miller, J. M.; Shah, S. *J. Antimicrob. Chemother.* **1995**, *35*, 865−8.
(50) Ida, T.; Okamoto, R.; Shimauchi, C.; Okubo, T.; Kuga, A.; Inoue, M. *J. Clin. Microbiol.* **2001**, *39*, 3115−21.
(51) Taber, H. W.; Mueller, J. P.; Miller, P. F.; Arrow, A. S. *Microbiol. Rev.* **1987**, *51*, 439−57.
(52) Hancock, R. E. *Annu. Rev. Microbiol.* **1984**, *38*, 237−64.
(53) Bryan, L. E.; Kowand, S. K.; Van Den Elzen, H. M. *Antimicrob. Agents Chemother.* **1979**, *15*, 7−13.
(54) Miller, M. H.; Edberg, S. C.; Mandel, L. J.; Behar, C. F.; Steigbigel, N. H. *Antimicrob. Agents Chemother.* **1980**, *18*, 722−9.
(55) Balwit, J. M.; van Langevelde, P.; Vann, J. M.; Proctor, R. A. *J. Infect. Dis.* **1994**, *170*, 1033−7.
(56) Bryan, L. E.; O'Hara, K.; Wong, S. *Antimicrob. Agents Chemother.* **1984**, *26*, 250−5.
(57) Kadurugamuwa, J. L.; Lam, J. S.; Beveridge, T. J. *Antimicrob. Agents Chemother.* **1993**, *37*, 715−21.
(58) Hatch, R. A.; Schiller, N. L. *Antimicrob. Agents Chemother.* **1998**, *42*, 974−7.
(59) Putman, M.; van Veen, H. W.; Konings, W. N. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 672−93.
(60) Murakami, S.; Nakashima, R.; Yamashita, E.; Yamaguchi, A. *Nature* **2002**, *419*, 587−93.
(61) Yu, E. W.; Aires, J. R.; Nikaido, H. *J. Bacteriol.* **2003**, *185*, 5657−64.
(62) Yu, E. W.; McDermott, G.; Zgurskaya, H. I.; Nikaido, H.; Koshland, D. E., Jr. *Science* **2003**, *300*, 976−80.
(63) Koronakis, V.; Sharff, A.; Koronakis, E.; Luisi, B.; Hughes, C. *Nature* **2000**, *405*, 914−9.
(64) Eda, S.; Maseda, H.; Nakae, T. *J. Biol. Chem.* **2003**, *278*, 2085−8.
(65) Elkins, C. A.; Nikaido, H. *J. Bacteriol.* **2002**, *184*, 6490−8.
(66) Avila-Sakar, A. J.; Misaghi, S.; Wilson-Kubalek, E. M.; Downing, K. H.; Zgurskaya, H.; Nikaido, H.; Nogales, E. *J. Struct. Biol.* **2001**, *136*, 81−8.
(67) Westbrock-Wadman, S.; Sherman, D. R.; Hickey, M. J.; Coulter, S. N.; Zhu, Y. Q.; Warrener, P.; Nguyen, L. Y.; Shawar, R. M.; Folger, K. R.; Stover, C. K. *Antimicrob. Agents Chemother.* **1999**, *43*, 2975−83.
(68) Aires, J. R.; Kohler, T.; Nikaido, H.; Plesiat, P. *Antimicrob. Agents Chemother.* **1999**, *43*, 2624−8.
(69) Moore, R. A.; DeShazer, D.; Reckseidler, S.; Weissman, A.; Woods, D. E. *Antimicrob. Agents Chemother.* **1999**, *43*, 465−70.
(70) Rosenberg, E. Y.; Ma, D.; Nikaido, H. *J. Bacteriol.* **2000**, *182*, 1754−6.
(71) Sobel, M. L.; McKay, G. A.; Poole, K. *Antimicrob. Agents Chemother.* **2003**, *47*, 3202−7.
(72) Hocquet, D.; Vogne, C.; El Garch, F.; Vejux, A.; Gotoh, N.; Lee, A.; Lomovskaya, O.; Plesiat, P. *Antimicrob. Agents Chemother.* **2003**, *47*, 1371−5.
(73) Magnet, S.; Courvalin, P.; Lambert, T. *Antimicrob. Agents Chemother.* **2001**, *45*, 3375−80.
(74) Grkovic, S.; Brown, M. H.; Skurray, R. A. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 671−701, table of contents.
(75) Schumacher, M. A.; Brennan, R. G. *Mol. Microbiol.* **2002**, *45*, 885−93.
(76) Jo, J. T.; Brinkman, F. S.; Hancock, R. E. *Antimicrob. Agents Chemother.* **2003**, *47*, 1101−11.
(77) Edgar, R.; Bibi, E. *J. Bacteriol.* **1997**, *179*, 2274−80.
(78) Silva, P. E.; Bigi, F.; de la Paz Santangelo, M.; Romano, M. I.; Martin, C.; Cataldi, A.; Ainsa, J. A. *Antimicrob. Agents Chemother.* **2001**, *45*, 800−4.
(79) Ainsa, J. A.; Blokpoel, M. C.; Otal, I.; Young, D. B.; De Smet, K. A.; Martin, C. *J. Bacteriol.* **1998**, *180*, 5836−43.
(80) De Rossi, E.; Arrigo, P.; Bellinzoni, M.; Silva, P. A.; Martin, C.; Ainsa, J. A.; Guglierame, P.; Riccardi, G. *Mol. Med.* **2002**, *8*, 714−24.
(81) Van Buul, C. P.; Damm, J. B.; Van Knippenberg, P. H. *Mol. Gen. Genet.* **1983**, *189*, 475−8.
(82) van Buul, C. P.; van Knippenberg, P. H. *Gene* **1985**, *38*, 65−72.
(83) Vila-Sanjurjo, A.; Squires, C. L.; Dahlberg, A. E. *J. Mol. Biol.* **1999**, *293*, 1−8.
(84) Walsh, C. *Antibiotics: actions, origins, resistance*; ASM Press: Washington, DC, 2003.
(85) Lacalle, R. A.; Tercero, J. A.; Vara, J.; Jimenez, A. *J. Bacteriol.* **1993**, *175*, 7474−8.
(86) Tercero, J. A.; Espinosa, J. C.; Lacalle, R. A.; Jimenez, A. *J. Biol. Chem.* **1996**, *271*, 1579−90.
(87) Cundliffe, E. *Annu. Rev. Microbiol.* **1989**, *43*, 207−33.
(88) Skeggs, P. A.; Thompson, J.; Cundliffe, E. *Mol. Gen. Genet.* **1985**, *200*, 415−21.
(89) Thompson, J.; Skeggs, P. A.; Cundliffe, E. *Mol. Gen. Genet.* **1985**, *201*, 168−73.
(90) Kelemen, G. H.; Cundliffe, E.; Financsek, I. *Gene* **1991**, *98*, 53−60.
(91) Holmes, D. J.; Cundliffe, E. *Mol. Gen. Genet.* **1991**, *229*, 229−37.
(92) Demydchuk, J.; Oliynyk, Z.; Fedorenko, V. *J. Basic Microbiol.* **1998**, *38*, 231−9.
(93) Kojic, M.; Topisirovic, L.; Vasiljevic, B. *J. Bacteriol.* **1992**, *174*, 7868−72.
(94) Holmes, D. J.; Drocourt, D.; Tiraby, G.; Cundliffe, E. *Gene* **1991**, *102*, 19−26.
(95) Ohta, T.; Hasegawa, M. *Gene* **1993**, *127*, 63−9.
(96) Bujnicki, J. M.; Rychlewski, L. *Acta Microbiol. Pol.* **2001**, *50*, 7−17.
(97) Yokoyama, K.; Doi, Y.; Yamane, K.; Kurokawa, H.; Shibata, N.; Shibayama, K.; Yagi, T.; Kato, H.; Arakawa, Y. *Lancet* **2003**, *362*, 1888−93.
(98) Doi, Y.; Yokoyama, K.; Yamane, K.; Wachino, J.; Shibata, N.; Yagi, T.; Shibayama, K.; Kato, H.; Arakawa, Y. *Antimicrob. Agents Chemother.* **2004**, *48*, 491−6.
(99) Galimand, M.; Courvalin, P.; Lambert, T. *Antimicrob. Agents Chemother.* **2003**, *47*, 2565−71.
(100) Finken, M.; Kirschner, P.; Meier, A.; Wrede, A.; Bottger, E. C. *Mol. Microbiol.* **1993**, *9*, 1239−46.
(101) Honore, N.; Cole, S. T. *Antimicrob. Agents Chemother.* **1994**, *38*, 238−42.
(102) Honore, N.; Marchal, G.; Cole, S. T. *Antimicrob. Agents Chemother.* **1995**, *39*, 769−70.
(103) Bottger, E. C. *Trends Microbiol.* **1994**, *2*, 416−21.
(104) Musser, J. M. *Clin. Microbiol. Rev.* **1995**, *8*, 496−514.
(105) Meier, A.; Kirschner, P.; Bange, F. C.; Vogel, U.; Bottger, E. C. *Antimicrob. Agents Chemother.* **1994**, *38*, 228−33.
(106) Powers, T.; Noller, H. F. *EMBO J.* **1991**, *10*, 2203−14.
(107) Suzuki, Y.; Katsukawa, C.; Tamaru, A.; Abe, C.; Makino, M.; Mizuguchi, Y.; Taniguchi, H. *J. Clin. Microbiol.* **1998**, *36*, 1220−5.
(108) Galimand, M.; Gerbaud, G.; Courvalin, P. *Antimicrob. Agents Chemother.* **2000**, *44*, 1365−6.
(109) Sigmund, C. D.; Ettayebi, M.; Borden, A.; Morgan, E. A. *Methods Enzymol.* **1988**, *164*, 673−90.
(110) Sparling, P. F.; Davis, B. D. *Antimicrob. Agents Chemother.* **1972**, *1*, 252−8.
(111) Asai, T.; Zaporojets, D.; Squires, C.; Squires, C. L. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1971−6.
(112) Pfister, P.; Hobbie, S.; Vicens, Q.; Bottger, E. C.; Westhof, E. *ChemBioChem* **2003**, *4*, 1078−88.
(113) Sander, P.; Prammananan, T.; Bottger, E. C. *Mol. Microbiol.* **1996**, *22*, 841−8.
(114) Recht, M. I.; Douthwaite, S.; Puglisi, J. D. *EMBO J.* **1999**, *18*, 3133−8.
(115) Springer, B.; Kidan, Y. G.; Prammananan, T.; Ellrott, K.; Bottger, E. C.; Sander, P. *Antimicrob. Agents Chemother.* **2001**, *45*, 2877−84.
(116) Brink, M. F.; Brink, G.; Verbeet, M. P.; de Boer, H. A. *Nucleic Acids Res.* **1994**, *22*, 325−31.
(117) Johanson, U.; Hughes, D. *Nucleic Acids Res.* **1995**, *23*, 464−6.
(118) Pfister, P.; Risch, M.; Brodersen, D. E.; Bottger, E. C. *Antimicrob. Agents Chemother.* **2003**, *47*, 1496−502.
(119) De Stasio, E. A.; Dahlberg, A. E. *J. Mol. Biol.* **1990**, *212*, 127−33.
(120) Recht, M. I.; Douthwaite, S.; Dahlquist, K. D.; Puglisi, J. D. *J. Mol. Biol.* **1999**, *286*, 33−43.
(121) Recht, M. I.; Puglisi, J. D. *Antimicrob. Agents Chemother.* **2001**, *45*, 2414−9.
(122) Prammananan, T.; Sander, P.; Brown, B. A.; Frischkorn, K.; Onyi, G. O.; Zhang, Y.; Bottger, E. C.; Wallace, R. J., Jr. *J. Infect. Dis.* **1998**, *177*, 1573−81.
(123) Dobner, P.; Bretzel, G.; Rusch-Gerdes, S.; Feldmann, K.; Rifai, M.; Loscher, T.; Rinder, H. *Mol. Cell Probes* **1997**, *11*, 123−6.

(124) Stuy, J. H.; Walter, R. B. *J. Bacteriol.* **1992**, *174*, 5604−8.
(125) Bonny, C.; Montandon, P. E.; Marc-Martin, S.; Stutz, E. *Biochim. Biophys. Acta* **1991**, *1089*, 213−9.
(126) Toivonen, J. M.; Boocock, M. R.; Jacobs, H. T. *Mol. Microbiol.* **1999**, *31*, 1735−46.
(127) Allen, P. N.; Noller, H. F. *J. Mol. Biol.* **1989**, *208*, 457−68.
(128) Gregory, S. T.; Cate, J. H.; Dahlberg, A. E. *J. Mol. Biol.* **2001**, *309*, 333−8.
(129) Bjorkman, J.; Samuelsson, P.; Andersson, D. I.; Hughes, D. *Mol. Microbiol.* **1999**, *31*, 53−8.
(130) Ramakrishnan, V.; White, S. W. *Nature* **1992**, *358*, 768−71.
(131) Melancon, P.; Tapprich, W. E.; Brakier-Gingras, L. *J. Bacteriol.* **1992**, *174*, 7896−901.
(132) Zuurmond, A. M.; Zeef, L. A.; Kraal, B. *Microbiology* **1998**, *144* (Pt 12), 3309−16.
(133) Davies, C.; Bussiere, D. E.; Golden, B. L.; Porter, S. J.; Ramakrishnan, V.; White, S. W. *J. Mol. Biol.* **1998**, *279*, 873−88.
(134) Shaw, K. J.; Rather, P. N.; Hare, R. S.; Miller, G. H. *Microbiol. Rev.* **1993**, *57*, 138−63.
(135) Lombardini, J. B.; Cheng-Chu, M. *Int. J. Biochem.* **1980**, *12*, 427−31.
(136) Van Pelt, J. E.; Northrop, D. B. *Arch. Biochem. Biophys.* **1984**, *230*, 250−63.
(137) Gates, C. A.; Northrop, D. B. *Biochemistry* **1988**, *27*, 3834−42.
(138) Gates, C. A.; Northrop, D. B. *Biochemistry* **1988**, *27*, 3826−33.
(139) Gates, C. A.; Northrop, D. B. *Biochemistry* **1988**, *27*, 3820−5.
(140) Williams, J. W.; Northrop, D. B. *J. Biol. Chem*. **1978**, *253*, 5908−14.
(141) Le Goffic, F.; Martel, A.; Capmau, M. L.; Baca, B.; Goebel, P.; Chardon, H.; Soussy, C. J.; Duval, J.; Bouanchaud, D. H. *Antimicrob. Agents Chemother.* **1976**, *10*, 258−64.
(142) Le Goffic, F.; Baca, B.; Soussy, C. J.; Dublanchet, A.; Duval, J. *Ann. Microbiol. (Paris)* **1976**, *127*, 391−9.
(143) Davies, J.; Smith, D. I. *Annu. Rev. Microbiol.* **1978**, *32*, 469−518.
(144) Van Pelt, J. E.; Iyengar, R.; Frey, P. A. *J. Biol. Chem*. **1986**, *261*, 15995−9.
(145) Sakon, J.; Liao, H. H.; Kanikula, A. M.; Benning, M. M.; Rayment, I.; Holden, H. M. *Biochemistry* **1993**, *32*, 11977−84.
(146) Pedersen, L. C.; Benning, M. M.; Holden, H. M. *Biochemistry* **1995**, *34*, 13305−11.
(147) Gerratana, B.; Cleland, W. W.; Reinhardt, L. A. *Biochemistry* **2001**, *40*, 2964−71.
(148) Schwotzer, U.; Kayser, F. H.; Schwotzer, W. *FEMS Microbiol. Lett.* **1978**, *3*, 29−33.
(149) Gerratana, B.; Frey, P. A.; Cleland, W. W. *Biochemistry* **2001**, *40*, 2972−7.
(150) McKay, G. A.; Thompson, P. R.; Wright, G. D. *Biochemistry* **1994**, *33*, 6936−44.
(151) Trieu-Cuot, P.; Courvalin, P. *Gene* **1983**, *23*, 331−41.
(152) Cox, J. R.; McKay, G. A.; Wright, G. D.; Serpersu, E. H. *J. Am. Chem. Soc.* **1996**, *118*, 1295−1301.
(153) Thompson, P. R.; Hughes, D. W.; Wright, G. D. *Biochemistry* **1996**, *35*, 8686−95.
(154) McKay, G. A.; Wright, G. D. *J. Biol. Chem*. **1995**, *270*, 24686−92.
(155) McKay, G. A.; Wright, G. D. *Biochemistry* **1996**, *35*, 8680−5.
(156) Hon, W. C.; McKay, G. A.; Thompson, P. R.; Sweet, R. M.; Yang, D. S.; Wright, G. D.; Berghuis, A. M. *Cell* **1997**, *89*, 887−95.
(157) Fong, D. H.; Berghuis, A. M. *EMBO J*. **2002**, *21*, 2323−31.
(158) Thompson, P. R.; Schwartzenhauer, J.; Hughes, D. W.; Berghuis, A. M.; Wright, G. D. *J. Biol. Chem*. **1999**, *274*, 30697−706.
(159) Daigle, D. M.; McKay, G. A.; Thompson, P. R.; Wright, G. D. *Chem. Biol.* **1999**, *6*, 11−8.
(160) Martel, A.; Masson, M.; Moreau, N.; Le Goffic, F. *Eur. J. Biochem.* **1983**, *133*, 515−21.
(161) McKay, G. A.; Robinson, R. A.; Lane, W. S.; Wright, G. D. *Biochemistry* **1994**, *33*, 14115−20.
(162) Boehr, D. D.; Lane, W. S.; Wright, G. D. *Chem. Biol.* **2001**, *8*, 791−800.
(163) Lovering, A. M.; White, L. O.; Reeves, D. S. *J. Antimicrob. Chemother.* **1987**, *20*, 803−13.
(164) Sunada, A.; Nakajima, M.; Ikeda, Y.; Kondo, S.; Hotta, K. *J. Antibiot. (Tokyo)* **1999**, *52*, 809−14.
(165) Rather, P. N.; Orosz, E.; Shaw, K. J.; Hare, R.; Miller, G. *J. Bacteriol.* **1993**, *175*, 6492−8.
(166) Macinga, D. R.; Rather, P. N. *Front. Biosci.* **1999**, *4*, D132−40.
(167) Payie, K. G.; Rather, P. N.; Clarke, A. J. *J. Bacteriol.* **1995**, *177*, 4303−10.
(168) Franklin, K.; Clarke, A. J. *Antimicrob. Agents Chemother*. **2001**, *45*, 2238−44.
(169) Udou, T.; Mizuguchi, Y.; Wallace, R. J., Jr. *Am. Rev. Respir. Dis.* **1987**, *136*, 338−43.
(170) Ainsa, J. A.; Martin, C.; Gicquel, B.; Gomez-Lus, R. *Antimicrob. Agents Chemother.* **1996**, *40*, 2350−5.
(171) Hegde, S. S.; Javid-Majd, F.; Blanchard, J. S. *J. Biol. Chem*. **2001**, *276*, 45876−81.
(172) Vetting, M. W.; Hegde, S. S.; Javid-Majd, F.; Blanchard, J. S.; Roderick, S. L. *Nat. Struct. Biol.* **2002**, *9*, 653−8.
(173) Dyda, F.; Klein, D. C.; Hickman, A. B. *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 81−103.
(174) Williams, J. W.; Northrop, D. B. *Biochemistry* **1976**, *15*, 125−31.
(175) Williams, J. W.; Northrop, D. B. *J. Biol. Chem*. **1978**, *253*, 5902−7.
(176) Javier Teran, F.; Alvarez, M.; Suarez, J. E.; Mendoza, M. C. *J. Antimicrob. Chemother.* **1991**, *28*, 333.
(177) Wolf, E.; Vassilev, A.; Makino, Y.; Sali, A.; Nakatani, Y.; Burley, S. K. *Cell* **1998**, *94*, 439−49.
(178) DiGiammarino, E. L.; Draker, K. A.; Wright, G. D.; Serpersu, E. H. *Biochemistry* **1998**, *37*, 3638−44.
(179) Wybenga-Groot, L. E.; Draker, K.; Wright, G. D.; Berghuis, A. M. *Structure Fold Des.* **1999**, *7*, 497−507.
(180) Burk, D. L.; Ghuman, N.; Wybenga-Groot, L. E.; Berghuis, A. M. *Protein Sci.* **2003**, *12*, 426−37.
(181) Wright, G. D.; Ladak, P. *Antimicrob. Agents Chemother.* **1997**, *41*, 956−60.
(182) Draker, K. A.; Northrop, D. B.; Wright, G. D. *Biochemistry* **2003**, *42*, 6565−74.
(183) Draker, K. A.; Wright, G. D. *Biochemistry* **2004**, *43*, 446−54.
(184) Magnet, S.; Courvalin, P.; Lambert, T. *J. Bacteriol.* **1999**, *181*, 6650−5.
(185) Magnet, S.; Lambert, T.; Courvalin, P.; Blanchard, J. S. *Biochemistry* **2001**, *40*, 3700−9.
(186) Hegde, S. S.; Dam, T. K.; Brewer, C. F.; Blanchard, J. S. *Biochemistry* **2002**, *41*, 7519−27.
(187) Vetting, M. W.; Magnet, S.; Nieves, E.; Roderick, S. L.; Blanchard, J. S. *Chem. Biol.*, in press.
(188) Daigle, D. M.; Hughes, D. W.; Wright, G. D. *Chem. Biol.* **1999**, *6*, 99−110.
(189) Boehr, D. D.; Jenkins, S. I.; Wright, G. D. *J. Biol. Chem*. **2003**, *278*, 12873−80.
(190) Fujimura, S.; Tokue, Y.; Takahashi, H.; Kobayashi, T.; Gomi, K.; Abe, T.; Nukiwa, T.; Watanabe, A. *FEMS Microbiol. Lett.* **2000**, *190*, 299−303.
(191) Deverstein-van Hall, M. A.; Blok, H. E.; Donders, A. R.; Paauw, A.; Fluit, A. C.; Verhoef, J. *J. Infect. Dis.* **2003**, *187*, 251−9.
(192) White, P. A.; McIver, C. J.; Rawlinson, W. D. *Antimicrob. Agents Chemother.* **2001**, *45*, 2658−61.
(193) Culebras, E.; Martinez, J. L. *Front. Biosci*. **1999**, *4*, D1−8.
(194) Derbise, A.; Dyke, K. G.; el Solh, N. *Plasmid* **1996**, *35*, 174−88.
(195) Simjee, S.; Gill, M. J. *J. Hosp. Infect.* **1997**, *36*, 249−59.
(196) Miller, G. H.; Sabatelli, F. J.; Hare, R. S.; Glupczynski, Y.; Mackey, P.; Shlaes, D.; Shimizu, K.; Shaw, K. J. *Clin. Infect. Dis.* **1997**, *24 Suppl 1*, S46−62.
(197) Schmitz, F. J.; Verhoef, J.; Fluit, A. C. *Eur. J. Clin. Microbiol. Infect. Dis*. **1999**, *18*, 414−21.
(198) Schmitz, F. J.; Fluit, A. C.; Gondolf, M.; Beyrau, R.; Lindenlauf, E.; Verhoef, J.; Heinz, H. P.; Jones, M. E. *J. Antimicrob. Chemother.* **1999**, *43*, 253−9.
(199) Williams, J. W.; Northrop, D. B. *J. Antibiot. (Tokyo)* **1979**, *32*, 1147−54.
(200) Allen, N. E.; Alborn, W. E., Jr.; Hobbs, J. N., Jr.; Kirst, H. A. *Antimicrob. Agents Chemother*. **1982**, *22*, 824−31.
(201) Sucheck, S. J.; Wong, A. L.; Koeller, K. M.; Boehr, D. D.; Draker, K.; Sears, P.; Wright, G. D. *J. Am. Chem. Soc.* **2000**, *122*, 5230−5231.
(202) Roestamadji, J.; Grapsas, I. L.; Mobashery, S. *J. Am. Chem. Soc.* **1995**, *117*, 80−84.
(203) Haddad, J.; Vakulenko, S.; Mobashery, S. *J. Am. Chem. Soc.* **1999**, *121*, 11922−11923.
(204) Kim, C.; Haddad, J.; Vakulenko, S. B.; Meroueh, S. O.; Wu, Y.; Yan, H.; Mobashery, S. *Biochemistry* **2004**, *43*, 2373−83.
(205) Haddad, J.; Kotra, L. P.; Llano-Sotelo, B.; Kim, C.; Azucena, E. F.; Liu, M.; Vakulenko, S. B.; Chow, C. S.; Mobashery, S. *J. Am. Chem. Soc.* **2002**, *124*, 3229−37.

CR0301088