Loss of Individual Electrostatic Interactions between Aminoglycoside Antibiotics and Resistance Enzymes as an Effective Means to Overcoming Bacterial Drug Resistance

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Abstract: Aminoglycoside-modifying enzymes modify the structures of aminoglycoside antibiotics, rendering them ineffective, a process which confers resistance to the antibiotic. Electrostatic interactions (ion pairing and hydrogen bonding) are believed to be significant for both substrate recognition and catalysis by these enzymes. Regiospecific syntheses of seven distinct deaminated analogues of neamine and kanamycin A, two aminoglycoside antibiotics, are described. Each of these compounds would have impaired interaction with a different subsite of the enzyme active sites. All seven molecules were shown to be exceedingly poor substrates for two aminoglycoside-modifying enzymes, aminoglycoside 3′-phosphotransferases types Ia and IIa. The energetic contribution of interactions of the active-site functions with each of these amines on stabilization of the transition-state species has been evaluated to be in the range of 6−11 kcal/mol, the largest energy contribution recorded in the literature for such interactions. The biological activities of these analogues were the same against the resistant organisms harboring aminoglycoside 3′-phosphotransferases types Ia and IIa as those against the background strain without the resistant enzymes. Thus, these compounds are virtually unmodified by those enzymes *in vivo*. The principles described here should be of general interest for circumvention of resistance to other antibiotics, by redesigning the structures to minimize electrostatic interactions with their corresponding resistance enzymes.

Advances in the development of novel antibiotics are being reversed by bacterial populations exhibiting multiple resistance to various antibiotics. The resistance problem has rendered a number of antibiotics obsolete. Treatment of clinical infections caused by such resistant organisms presents a serious challenge.[1] This phenomenon necessitates an intensive search for new antibiotics with novel mechanisms of action. Such undertakings are both time-consuming and financially prohibitive but remain indispensable if we are to provide an acceptable level of care in the immediate future. Meanwhile, it may be less costly in time and money to employ strategies to circumvent existing bacterial resistance mechanisms and thereby to restore usefulness to antibacterials that have become compromised by resistance.

Aminoglycoside antibiotics have been used in the clinic for almost five decades. This prolonged clinical use has resulted in effective selection of resistance to this family of antibacterial agents. Presently, resistance to these agents is widespread among pathogens worldwide.[2] The primary mechanism for resistance to aminoglycosides is the bacterial acquisition of enzymes which modify this family of antibiotics by acetyltransferase, adenylyltransferase, and phosphotransferase activities.[2] Among these enzyme families, aminoglycoside 3′-phosphotransferases [APH(3′)s], of which seven isozymes are known, are widely represented.[2] These enzymes catalyze transfer of the γ-phosphoryl group of ATP to the 3′-hydroxyl of many aminoglycosides, such as kanamycins, neomycins, neamine, paromamine, butirosin, ribostamycin, lividomycin, gentamicin B, geneticin, isepamicin, and amikacin.[3] Such enzymic modification renders aminoglycosides inactive, since the phosphorylated antibiotics no longer bind to the bacterial ribosome—the target of these antibiotics—with high affinity.

Aminoglycoside 3′-phosphotransferases, which are believed to be associated with the inner surface of the plasma membrane,[4] present two logistical problems. First, they may be present in concentrations which constitute as much as 2% of the total protein content of bacteria.[5,6] Furthermore, they often carry out their catalytic function with preferred substrates at or near the diffusion limit.[5−7] Both these factors taken together indicate that resistance to aminoglycosides due to the function of these enzymes is often very serious and difficult to overcome. We recently described the synthesis and evaluation of the first mechanism-based inactivators for these enzymes.[8] These molecules are of considerable interest in reversing the resistance caused by these enzymes. As an extension of our work in this area, we describe herein a new concept in overcoming the function of these enzymes by minimizing electrostatic interactions of aminoglycosides and the resistance enzymes. As will be outlined below, the new type of aminoglycosides disclosed herein benefits not merely from loss of affinity by the enzyme active sites, but also from a profound reduction in the rate of phosphorylation by these enzymes; in effect, these molecules

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circumvent the enzyme effectively, despite the high *in vivo* concentrations of these enzymes and their potent catalytic ability.

Experimental Section

Hydrogen- and carbon-NMR spectra were obtained using a U-500 Varian and a 300-MHz Nicolet spectrometers; chemical shift values (δ) are given in ppm. Mass spectra were recorded on a Kratos MS 80RFA spectrometer. Infrared spectra were obtained on a Nicolet DX instrument. Melting points were taken on an electrothermal melting-point apparatus and are uncorrected. Thin-layer chromatograms were performed on silica-gel plates. Phosphorous oxychloride, tributyl tin hydride, and azobisisobutyronitrile (AIBN) were obtained from the Aldrich Chemical Co. Neomycin, kanamycin A, phosphoenol pyruvate (PEP), ATP, NADH, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1,4-piperazinebis(ethanesulfonic acid) (PIPES), and 4-nitrophenyl formate were purchased from the Sigma Chemical Co. Pyruvate kinase (PK) and lactate dehydrogenase (LD) were purchased from the Worthington Biochemical Co. Neamine hydrochloride was prepared from neomycin sulfate by methanolysis.[9] Neamine and kanamycin A were used in the free-base form, which were prepared from the corresponding ammonium salts by the use of Amberlite IRA 400 (OH^-) strongly basic ion-exchange resin. Kinetic measurements were carried out on a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer. Aminoglycoside 3′-phosphotransferases types Ia [APH(3′)-Ia][6] and IIa [APH(3′)-IIa][5] were purified according to the procedures of Siregar *et al.* Tri-O-silylated derivative of 1,3,6′-tri-*N*-(*tert*-butoxycarbonyl)neamine (compound **22**) was prepared as described by Roestamadji *et al.*[8] *N*-(*tert*-Butoxycarbonyloxy)-5-norbornene-*endo*-2,3-dicarboximide (*N*-*t*BND) was synthesized as described by Grapsas *et al.*[9]

6′-*N*-Carbobenzyloxyneamine (9). *N*-Benzyloxycarbonyloxy-5-norbornene-*endo*-2,3-dicarboximide (*N*-BND, 6.6 g, 21.0 mmol) was dissolved in 250 mL dioxane and was added dropwise to a solution of free-base neamine (**8**, 6.6 g, 21.0 mmol) in 500 mL of dioxane/H_2O (1:1). The mixture was stirred overnight at room temperature. Subsequently, the solvent was evaporated *in vacuo*, and the oily residue was chromatographed on a silica-gel column ($CHCl_3$/MeOH/concentrated ammonia, 2:2:1) to give the desired product (R_f 0.3, 5.66 g, 61% yield). This product gave a poorly resolved ^{1}H-NMR spectrum (DMSO-d_6, 300 MHz); however, the integration indicated a ratio of 1:5 between the anomeric hydrogen $H_{1'}$ (δ 4.77, 1H, d, J = 3.6 Hz) and the aromatic hydrogens of the Cbz group (δ 7.21, 5H, m); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ 38.1 (C_2), 42.4 ($C_{6'}$), 50.6 (C_3), 51.7 (C_1), 56.5 ($C_{2'}$), 65.6 ($CH_2C_6H_5$), 71.8 ($C_{4'}$), 72.5 ($C_{5'}$), 73.6 ($C_{3'}$), 76.4 (C_5), 77.7 (C_6), 91.6 (C_4), 102.3 ($C_{1'}$), 128.1, 128.7, 134.7, 137.7 ($CH_2C_6H_5$), 156.6 (C=O); mp 153 °C dec; IR (KBr) cm^{-1} 3420, 1655; MS-FAB$^+$ 457 (M + H$^+$, 9%).

1,3,2′-Tri-*N*-(*tert*-Butoxycarbonyl)-6′-*N*-carbobenzyloxyneamine (10). Compound **9** (5.7 g, 12.4 mmol) was dissolved in 300 mL of dioxane/H_2O (3:1), followed by the addition of di-*tert*-butyl dicarbonate (8.1 g, 37.2 mmol). The mixture was stirred overnight at 50 °C. The solvent was evaporated *in vacuo* to dryness, and the residue was chromatographed on a silica-gel column ($CHCl_3$/MeOH/concentrated ammonia, 4:1:0.1) to give the desired product (R_f 0.8, 7.6 g, 81% yield). The ^{1}H-NMR spectrum (DMSO-d_6, 300 MHz) showed the presence of the Boc (δ 1.31, 27H, 3s) and the Cbz (δ 7.26, 5H, m) resonances in a 5:1 ratio: ^{13}C-NMR (DMSO-d_6, 75 MHz) δ 28.6, 26.7 [OC(CH_3)$_3$], 35.8 (C_2), 42.3 ($C_{6'}$), 50.0 (C_3), 51.1 (C_1), 55.8 ($C_{2'}$), 65.6 ($CH_2C_6H_5$), 66.7 ($C_{4'}$), 70.9 ($C_{5'}$), 71.7 ($C_{3'}$), 74.1 (C_5), 77.5 (C_6), 77.9, 78.0, 78.1 [$OC(CH_3)_3$], 81.6 (C_4), 98.9 ($C_{1'}$), 128.1, 128.7, 137.6 (C_6H_5), 155.3, 155.6, 156.1, 156.9 (C=O); mp 215 °C dec; IR (KBr) cm^{-1} 3355, 1679; MS-FAB$^+$ 623 [M − $C_6H_5CH_2O(CO)^-$ + 2H$^+$, 2%].

1,3,2′-Tri-*N*-(*tert*-butoxycarbonyl)neamine (11). Compound **10** (7.5 g, 10.0 mmol) was dissolved in 250 mL of dioxane/MeOH/H_2O (2:2:1). The solution was added to a suspension of 15% palladium on carbon (1.13 g) in methanol, which had been saturated with hydrogen gas. The mixture was vigorously stirred in the presence of hydrogen for 5 h. The catalyst was subsequently removed by filtration through a layer of Celite, and the filtrate was evaporated to give a white residue which was chromatographed on a silica-gel column ($CHCl_3$/MeOH/concentrated ammonia, 7:1:0.1). The product (R_f 0.44, 4.84 g) was obtained in 78% yield. The ^{1}H-NMR spectrum (DMSO-d_6, 300 MHz) showed a ratio of 1:27 for the resonances of the $H_{1'}$ anomeric hydrogen (δ 4.61, unresolved doublet) and the hydrogens of the Boc group (δ 1.29, s): ^{13}C-NMR (DMSO-d_6, 75 MHz) δ 28.6, 28.7 [OC(CH_3)$_3$], 35.7 (C_2), 42.9 ($C_{6'}$), 49.9 (C_3), 51.1 (C_1), 56.0 ($C_{2'}$), 71.4 ($C_{4'}$), 72.3 ($C_{5'}$), 72.6 ($C_{3'}$), 74.2 (C_5), 77.6 (C_6), 77.9, 78.0, 78.1 [$OC(CH_3)_3$], 81.6 (C_4), 99.0 ($C_{1'}$), 155.2, 155.6, 156.2 (C=O); mp 235 °C dec; IR (KBr) cm^{-1} 3362, 1685; MS-FAB$^+$ 623 (M + H$^+$, 45%).

1,6′-Di-*N*-(*tert*-butoxycarbonyl)neamine (14). Neamine free base (**8**, 1.0 g, 3.1 mmol) was dissolved in DMSO (60 mL), followed by the addition of zinc(II) acetate (2.7 g, 12.4 mmol), and the mixture was stirred for 30 min at room temperature. Subsequently, *N*-(*tert*-butoxycarbonyloxy)-5-norbornene-*endo*-2,3-dicarboximide (*N*-*t*BND, 1.73 g, 6.20 mmol) was added, and the mixture was stirred overnight. A TLC analysis ($CHCl_3$/MeOH/concentrated ammonia, 4:3:1) showed one major product (R_f 0.58) at this point. The solution was mixed with 600 mL of ethyl ether and was vigorously stirred until a thick oil separated from solution. The ether layer was decanted, and the oily residue was dissolved in methanol/water (200 mL, 4:1). Zinc was precipitated by saturating the solution with hydrogen sulfide gas. The precipitate was filtered through a layer of Celite, and the filtrate was evaporated *in vacuo*. The residue was purified on silica-gel chromatography ($CHCl_3$/MeOH/concentrated ammonia, 2:1:1) to give 0.94 g of the desired product (60% yield): ^{1}H-NMR (DMSO-d_6, 300 MHz) δ 1.31 (unresolved quartet, 1H, H_{2ax}), 1.39 [2s, 18H, OC(CH_3)$_3$), 1.80 (unresolved doublet of triplet, 1H, H_{2eq}), 2.40−3.60 (unresolved multiplets, 19H, various ring hydrogens and hydrogens on heteroatoms), 4.85 (d, 1H, J = 3.3 Hz, $H_{1'}$), 6.55 (br, 1H, $NHCO_2$), 6.56 (br, 1H, $NHCO_2$); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ 28.7 and 28.8 [OC(CH_3)$_3$], 37.1 (C_2), 42.4 ($C_{6'}$), 50.4 (C_3), 51.7 (C_1), 56.6 ($C_{2'}$), 72.1 ($C_{4'}$), 72.7 ($C_{5'}$), 73.7 ($C_{3'}$), 74.4 (C_5), 76.9 (C_6), 77.9 and 78.2 [$OC(CH_3)_3$], 90.4 (C_4), 102.0 ($C_{1'}$), 155.9 and 156.3 (C=O); mp 151 °C dec; IR (KBr) cm^{-1} 3436, 3358, 1686; MS-FAB$^+$ 523 (M + H, 13%).

1,6′-Di-*N*-(*tert*-butoxycarbonyl)-3-*N*-benzyloxycarbonylneamine (15). Compound **14** (1.2 g, 2.3 mmol) was dissolved in 100 mL of dioxane/H_2O (2:1). After the addition of copper(II) acetate (1.8 g, 9.2 mmol), the mixture was stirred for 3 h at room temperature. *N*-Benzyloxycarbonyloxy-5-norbornene-*endo*-2,3-dicarboximide (0.72 g, 2.30 mmol) was added, and the mixture was stirred overnight at room temperature. A major product (R_f 0.3) was observed by TLC analysis ($CHCl_3$/MeOH/concentrated ammonia, 5:1:0.1). The metal was precipitated by saturating the solution with hydrogen sulfide gas and the resultant solid was filtered through a layer of Celite. The filtrate was concentrated under vacuum. The residue was purified on a silica-gel column to give 0.65 g of the desired product (43% yield). The ^{1}H-NMR spectrum (DMSO-d_6, 300 MHz) gave a ratio of 18:5 between the hydrogens of the Boc groups (δ 1.33, 18H, s) and the aromatic hydrogens of the Cbz group (δ 7.30, 5H, m): ^{13}C-NMR (DMSO-d_6, 75 MHz) δ 25.6 [OC(CH_3)$_3$], 35.3 (C_2), 41.7 ($C_{6'}$), 50.1 (C_3), 51.3 (C_1), 55.9 ($C_{2'}$), 65.6 ($CH_2C_6H_5$), 71.0 ($C_{4'}$), 71.5 ($C_{5'}$), 72.7 ($C_{3'}$), 73.6 (C_5), 77.4, 77.9 [$OC(CH_3)_3$], 78.2 (C_6), 83.9 (C_4), 100.7 ($C_{1'}$), 128.9, 128.0, 128.7 (C_6H_5), 155.6, 156.0 (C=O); mp 175 °C dec; IR (KBr) cm^{-1} 3351, 1689; MS-FAB$^+$ 657 (M + H$^+$, 3%).

1,2,6′-Tri-*N*-(*tert*-butoxycarbonyl)-3-*N*-carbobenzyloxyneamine (16). Compound **15** (0.47 g, 0.72 mmol) was dissolved in 50 mL of dioxane/H_2O (3:1). Di-*tert*-butyl dicarbonate (0.16 g, 0.72 mmol) was added to the solution, and the mixture was stirred overnight at room temperature. A TLC analysis ($CHCl_3$/MeOH/concentrated ammonia, 5:1:0.1) showed the presence of one major product (R_f 0.59). Solvent was evaporated *in vacuo*, and the residue was chromatographed on a silica-gel column to give 0.52 g of the desired compound (96% yield). The ^{1}H-NMR spectrum (acetone-d_6, 300 MHz) indicated the presence of three Boc groups (δ 1.35, 1.37, 1.38, 3s, 27H) and one Cbz group (δ 7.29, m, 5H) from the integration ratios: ^{13}C-NMR (acetone-d_6, 75 MHz) δ 27.8 [OC(CH_3)$_3$], 34.8 (C_2), 41.2 ($C_{6'}$), 50.8 (C_3), 51.2 (C_1), 56.1 ($C_{2'}$), 66.0 ($CH_2C_6H_5$), 71.4 ($C_{4'}$), 71.8 ($C_{5'}$), 72.0 ($C_{3'}$), 72.1 (C_5), 75.2 (C_6), 77.3, 77.5, 78.1, 78.4 [$OC(CH_3)_3$], 82.1 (C_4), 99.6 ($C_{1'}$), 127.8, 128.1, 128.4, 137.4 (C_6H_5), 155.9, 157.0, 157.3 (C=O); mp 179 °C dec; IR (KBr) cm^{-1} 3358, 1689; MS-FAB$^+$ 623 (M − Cbz + 2H$^+$, 2%).

1,2′,6′-Tri-*N*-(*tert*-butoxycarbonyl)neamine (17). Compound **16** (0.52 g, 0.69 mmol) was dissolved in 50 mL of MeOH/dioxane/H_2O (2:2:1), and the solution was added to a suspension of 15% palladium

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on carbon (0.08 g) in methanol which had been saturated with hydrogen gas. The mixture was stirred in the presence of hydrogen gas overnight. Subsequently, the catalyst was removed by filtration through a layer of Celite. The filtrate was evaporated *in vacuo* and the resultant residue was purified on a silica-gel column. The product (R_f 0.49) was eluted with $CHCl_3$/MeOH/concentrated ammonia (5:1:0.1) to give 0.3 g of the title compound (70% yield). The ^{1}H-NMR spectrum (DMSO-d_6, 300 MHz) confirmed the presence of three Boc groups (δ 1.28, 27H, s) in comparison with the $H_{1'}$ anomeric hydrogen (δ 4.79, 1H, d, J = 2.7 Hz): ^{13}C-NMR (DMSO-d_6, 75 MHz) δ 28.6 [OC(CH_3)$_3$], 37.0 (C_2), 42.1 ($C_{6'}$), 51.0 (C_3), 51.3 (C_1), 56.2 ($C_{2'}$), 71.2 ($C_{4'}$), 71.6 ($C_{5'}$), 72.9 ($C_{3'}$), 74.7 (C_5), 76.2 (C_6), 77.5, 78.0 [$OC(CH_3)_3$], 88.5 (C_4), 99.2 ($C_{1'}$), 155.6, 156.2 (C=O); mp 204 °C dec; IR (KBr) cm^{-1} 3457, 3520, 3379, 1682, 1661, 1041; MS-FAB$^+$ 623 (M + H$^+$, 20%).

3,6′-Di-*N*-(*tert*-butoxycarbonyl)neamine (18). Neamine free base (**8**, 3.04 g, 9.45 mmol) was dissolved in DMSO (400 mL). Copper(II) acetate (7.55 g, 37.8 mmol) was added, and the mixture was stirred vigorously for 2 h, followed by the addition of di-*tert*-butyl dicarbonate (4.12 g, 18.9 mmol). The mixture was stirred overnight at room temperature. A TLC analysis ($CHCl_3$/MeOH/concentrated ammonia, 2:1:0.25) indicated the formation of one major product (R_f 0.49) and a minor product (R_f 0.77, 1,3,6′-tricarbamoylated product). DMSO was removed by adding 600 mL of ethyl ether to the solution and allowing the mixture to stir vigorously for 10 min, after which the solution separated into two layers. The upper ether layer was decanted, and the process of mixing with a fresh portion of ether was repeated until a thick oily residue was obtained. The oily residue was dissolved in 500 mL of methanol/water (4:1). Copper was precipitated by saturating the solution with hydrogen sulfide gas, and the solid was filtered through a layer of Celite. The filtrate was evaporated to dryness *in vacuo*, and the residue was chromatographed on a silica-gel column using the solvent system $CHCl_3$/MeOH/concentrated ammonia (2:1:0.25). The title compound was obtained as the major product (2.47 g, 50% yield): ^{1}H-NMR (DMSO-d_6) δ 1.27 and 1.29 [s, 19H, OC(CH_3)$_3$ and H_{2ax}], 1.50−5.00 (unresolved multiplets, 21H, various ring hydrogens and hydrogens on heteroatoms), 6.34 (br, 1H, $NHCO_2$), 6.51 (d, 1H, J = 7.5 Hz, $NHCO_2$); ^{13}C-NMR (DMSO-d_6) δ 28.7 and 28.8 [OC(CH_3)$_3$], 36.5 (C_2), 42.2 ($C_{6'}$), 49.7 (C_3), 51.8 (C_1), 56.2 ($C_{2'}$), 70.6 ($C_{4'}$), 71.7 ($C_{5'}$), 73.9 ($C_{3'}$), 76.8 (C_5), 77.4 (C_6), 78.1 and 78.3 [$OC(CH_3)_3$], 84.6 (C_4), 102.0 ($C_{1'}$), 155.5 and 156.8 (C=O); mp 172 °C dec; IR (KBr) cm^{-1} 3358, 1696, 1682; MS-FAB$^+$ 523 (M + H, 13%).

3,6′-Di-*N*-(*tert*-butoxycarbonyl)-1-benzyloxycarbonylneamine (19). Compound **18** (3.75 g, 7.18 mmol) was dissolved in 400 mL dioxane/H_2O (2:1) followed by the addition of zinc(II) acetate (6.3 g, 28.7 mmol). The mixture was stirred for 5 h at room temperature. *N*-Benzyloxycarbonyloxy-5-norbornene-*endo*-2,3-dicarboximide (2.25 g, 7.18 mmol) was added, and the mixture was stirred overnight. A TLC analysis ($CHCl_3$/MeOH/concentrated ammonia, 4:1:0.1) showed the formation of a major product (R_f 0.19). The metal was precipitated by saturating the solution with hydrogen sulfide gas. The precipitate was filtered through a layer of Celite, and the filtrate was evaporated *in vacuo*. The residue was chromatographed on a silica-gel column to give the title compound (3.3 g, 70% yield): The ^{1}H-NMR spectrum (DMSO-d_6, 300 MHz) gave an integration ratio of 5:18 for the Cbz (δ 7.26, 5H, s) and Boc groups (δ 1.32, 1.34, 18H, 2s); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ 28.7, 28.8 [OC(CH_3)$_3$], 35.7 (C_2), 42.7 ($C_{6'}$), 49.4 (C_3), 51.3 (C_1), 55.9 ($C_{2'}$), 65.6 ($CH_2C_6H_5$), 70.6 ($C_{4'}$), 71.6 ($C_{5'}$), 73.1 ($C_{3'}$), 73.8 (C_5), 77.7 (C_6), 78.2, 78.4 [$OC(CH_3)_3$], 83.9 (C_4), 101.1 ($C_{1'}$), 128.3, 128.9, 134.9, 137.8 (C_6H_5), 155.5, 156.4, 156.8 (C=O); mp 180 °C dec; IR (KBr) cm^{-1} 3351, 1689, 1647; MS-FAB$^+$ 657 (M + H$^+$, 1%).

3,2′,6′-Tri-*N*-(*tert*-butoxycarbonyl)-1-benzyloxycarbonylneamine (20). Compound **19** (0.47 g, 0.72 mmol) was dissolved in 50 mL of dioxane/H_2O (3:1). Di-*tert*-butyl dicarbonate (0.16 g, 0.72 mmol) was added, and the mixture was stirred overnight at 50 °C. A TLC analysis ($CHCl_3$/MeOH/concentrated ammonia, 4:1:0.1) indicated the presence of a major product (R_f 0.58). Solvent was evaporated, and the residue was purified on a silica-gel column ($CHCl_3$/MeOH/concentrated ammonia, 4:1:0.1) to give 0.4 g of the title compound (60% yield). The ^{1}H-NMR spectrum (acetone-d_6, 300 MHz) confirmed the presence of one Cbz (δ 7.23, 5H, m) and three Boc groups (δ 1.32, 27H, 3s) from the integration ratio: ^{13}C-NMR (acetone-d_6, 75 MHz) δ 27.7, 27.8 [OC(CH_3)$_3$], 34.9 (C_2), 41.0 ($C_{6'}$), 49.8 (C_3), 51.5 (C_1), 55.6 ($C_{2'}$), 55.7 ($C_{4'}$), 65.6 ($CH_2C_6H_5$), 71.0 ($C_{5'}$), 71.5 ($C_{3'}$), 74.8 (C_5), 77.3 (C_6), 78.4 [$OC(CH_3)_3$], 81.6 (C_4), 99.4 ($C_{1'}$), 127.6, 137.7, 128.2, 137.3 (C_6H_5), 155.1, 156.2, 156.7, 157.2 (C=O); mp 196 °C dec; IR (KBr) cm^{-1} 3357, 1688; MS-FAB$^+$ 779 (M + Na$^+$, 2%).

3,2′,6′-Tri-*N*-(*tert*-butoxycarbonyl)neamine (21). Compound **20** (0.40 g, 0.53 mmol) was dissolved in 60 mL of dioxane/MeOH/H_2O (2:2:1). The solution was added to a suspension of 15% palladium on carbon (0.06 g) in methanol which had been saturated with hydrogen gas. Hydrogenolysis was carried out overnight, followed by the removal of the catalyst through a layer of Celite. The filtrate was evaporated *in vacuo* to give a white solid residue which was purified on a silica column ($CHCl_3$/MeOH/concentrated ammonia, 7:1:0.1) to give the desired product (R_f 0.38, 0.25 g, 75% yield): mp 185 °C dec. The ^{1}H-NMR spectrum (CD_3OD, 300 MHz) indicated an integration ratio of 27:1 for the resonances of the Boc groups (δ 1.38, 27H, s) and the $H_{1'}$ anomeric hydrogen (δ 5.19, 1H, s): ^{13}C-NMR (CD_3OD, 75 MHz) δ 27.3, 27.4 [OC(CH_3)$_3$], 35.3 (C_2), 40.5 ($C_{6'}$), 49.7 (C_3), 50.6 ($C_{1'}$), 55.5 ($C_{2'}$), 70.8 ($C_{4'}$), 71.2 ($C_{5'}$), 71.4 ($C_{3'}$), 77.2 (C_5), 77.4 (C_6), 78.9, 79.2 [$OC(CH_3)_3$], 81.0 (C_4), 98.9 ($C_{1'}$), 156.4, 157.2, 157.8 (C=O); IR (KBr) cm^{-1} 3367, 1692; MS-FAB$^+$ 623 (M + H$^+$, 1%), 645 (M + Na$^+$, 3%).

6′-*N*-Benzyloxycarbonylkanamycin A (24). A solution of free base kanamycin A (**23**, 5.5 g, 11.4 mmol) in 400 mL of dioxane/H_2O (3:1) was allowed to react with *N*-benzyloxycarbonyloxy-5-norbornene-*endo*-2,3-dicarboximide (3.92 g, 12.5 mmol) overnight. A TLC analysis ($CHCl_3$/MeOH/concentrated ammonia, 3:1:0.1) showed a major product (R_f 0.23). The solvent was evaporated to dryness *in vacuo*, and the residue was chromatographed on a silica-gel column ($CHCl_3$/MeOH/concentrated ammonia, 3:1:0.1). The titled compound was obtained as an oily residue (4.56 g, 65% yield). It gave a poorly resolved ^{1}H-NMR spectrum (DMSO-d_6, 500 MHz); however, the integration ratio for the anomeric hydrogens $H_{1'}$ (δ 4.86, 1H, d, J = 3.5 Hz) and $H_{1''}$ (δ 4.89, 1H, d, J = 4 Hz) and the aromatic hydrogens of the Cbz group (δ 7.83, 5H, m) confirmed the presence of one Cbz group: ^{13}C-NMR (DMSO-d_6, 125 MHz) δ 38.6 (C_2), 44.3 ($C_{6'}$), 50.9 (C_3), 51.4 (C_1), 56.2 ($C_{3''}$), 60.9 ($C_{6''}$), 65.6 ($CH_2C_6H_5$), 69.5 ($C_{4''}$), 71.8 ($C_{4'}$), 72.1 ($C_{2''}$), 72.4 ($C_{2'}$), 72.9 ($C_{5''}$), 73.1 ($C_{5'}$), 73.6 ($C_{3'}$), 74.3 (C_5), 87.7 (C_4), 91.2 (C_6), 99.4 ($C_{1'}$), 101.6 ($C_{1''}$), 128.1, 128.2, 128.7, 128.8, 134.8, 137.8 (C_6H_5), 156.7 (C=O); IR (KBr) cm^{-1} 3440, 1653; MS-FAB$^+$ 619 (M + 2H$^+$, 2%).

1,3,3″-Tri-*N*-(*tert*-butoxycarbonyl)-6′-*N*-benzyloxycarbonylkanamycin A (25). Di-*tert*-butyl dicarbonate (5.6 g, 25.5 mmol) was added to a solution of **24** (4.5 g, 7.3 mmol) in 300 mL of dioxane/H_2O (5:1), and the mixture was stirred overnight at 50 °C. A TLC analysis ($CHCl_3$/MeOH/concentrated ammonia, 3:1:0.1) showed the presence of a major product (R_f 0.61). The solvent was evaporated, and the white residue was chromatographed ($CHCl_3$/MeOH/concentrated ammonia, 3:1:0.1) on a silica-gel column to give 6.4 g of the title compound (90% yield). The ^{1}H-NMR spectrum (DMSO-d_6, 500 MHz) showed a ratio of 3:1 for the Boc (δ 1.76, 1.78, 27H, 2s) and the Cbz groups (δ 7.35, 5H, unresolved multiplet): ^{13}C-NMR (DMSO-d_6, 125 MHz) δ 28.6, 28.7, 28.8 [OC(CH_3)$_3$], 35.3 (C_2), 42.3 ($C_{6'}$), 49.4 (C_3), 50.5 (C_1), 56.3 ($C_{3''}$), 60.7 ($C_{6''}$), 56.8 ($CH_2C_6H_5$), 66.8 ($C_{4''}$), 67.8 ($C_{4'}$), 70.6 ($C_{2''}$), 70.8 ($C_{2'}$), 72.5 ($C_{5''}$), 73.1 ($C_{5'}$), 73.3 ($C_{3'}$), 75.5 (C_5), 77.7, 78.2, 78.3 [$OC(CH_3)_3$], 80.7 (C_4), 84.3 (C_6), 98.2 ($C_{1'}$), 101.5 ($C_{1''}$), 128.2, 128.3, 128.8, 134.8, 137.6 (C_6H_5), 155.4, 155.8, 156.8, 157.0 (C=O); mp 201 °C dec; IR (KBr) cm^{-1} 3355, 1681; MS-FAB$^+$ 855 (M − Boc + 3H$^+$, 1%).

1,3,3″-Tri-*N*-(*tert*-butoxycarbonyl)kanamycin A (26). Compound **25** (6.4 g, 6.6 mmol) was dissolved in 300 mL of dioxane/MeOH/H_2O (2:2:1), and the solution was added to a suspension of 15% palladium on carbon (0.96 g) in methanol which had been saturated with hydrogen gas, followed by stirring overnight. The catalyst was removed by filtration through a layer of Celite. A TLC analysis ($CHCl_3$/MeOH/concentrated ammonia, 4:1:0.1) indicated the presence of one major product (R_f 0.24). After evaporating the solvent, the residue was chromatographed on a silica-gel column ($CHCl_3$/MeOH/concentrated ammonia, 4:1:0.1) to give 2.77 g of the desired compound (50% yield). The ^{1}H-NMR spectrum (DMSO-d_6, 500 MHz) showed integration ratio of 27:2 for the resonances for the Boc groups (δ 1.34, 27H, 3s) and the anomeric hydrogens $H_{1'}$ (δ 4.89, 1H, d, J = 3.5 Hz) and $H_{1''}$ (δ 4.91, 1H, d, J = 4 Hz): ^{13}C-NMR (DMSO-d_6, 500 MHz) δ 28.6, 28.7, 28.8 [OC(CH_3)$_3$], 35.1 (C_2), 42.9 ($C_{6'}$), 49.5 (C_3), 50.4 (C_1), 56.3 ($C_{3''}$), 60.7 ($C_{6''}$), 67.8 ($C_{4''}$), 70.8 ($C_{4'}$), 71.3 ($C_{2''}$), 72.7 ($C_{2'}$), 72.8 ($C_{5''}$), 73.3

($C_{5'}$), 73.5 ($C_{3'}$), 75.4 (C_5), 77.6, 78.3 [$OC(CH_3)_3$], 80.8 (C_4), 84.4 (C_6), 98.3 ($C_{1'}$), 101.4 ($C_{1''}$), 155.3, 155.8, 156.8 (C=O); mp 205 °C dec; IR (KBr) cm^{-1} 3365, 1681; MS-FAB$^+$ 785 (M + H$^+$, 14%).

3,6′-Di-*N*-(*tert*-butoxycarbonyl)kanamycin A (27). Free base kanamycin A (**23**, 11.3 g, 23.4 mmol) was dissolved in 400 mL of DMSO followed by the addition of zinc(II) acetate (10.3 g, 46.8 mmol). The mixture was stirred at room temperature for 5 h. Di-*tert*-butyl dicarbonate (10.8 g, 49.1 mmol) was subsequently added, and the reaction was stirred overnight. A TLC analysis ($CHCl_3$/MeOH/concentrated ammonia, 3:1:6 drops) showed one major product (R_f 0.34). DMSO was removed by adding 600 mL of ethyl ether to the solution and allowing the mixture to stir vigorously for 10 min, after which the solution separated into two layers. The upper ether layer was decanted, and the process of mixing with a fresh portion of ether was repeated until a thick oily residue was obtained. The oily residue was dissolved in 500 mL of methanol/water (4:1). Zinc was precipitated by saturating the solution with hydrogen sulfide gas. The precipitate was filtered through a layer of Celite, and the filtrate was evaporated to dryness. The residue was chromatographed on silica gel ($CHCl_3$/MeOH/concentrated ammonia, 3:1:0.1), and 10.5 g of product was obtained (66% yield). The ^{1}H-NMR (DMSO-d_6, 500 MHz) gave a ratio of 9:1 for the hydrogens of the Boc groups (δ 1.34,18H, 2s) and the anomeric hydrogens (δ 4.90, 1H, $H_{1''}$, s; 4.93, 1H, $H_{1'}$, s): ^{13}C-NMR (DMSO-d_6, 125 MHz) δ 28.6, 28.7 [$OC(CH_3)_3$], 35.8 (C_2), 41.8 ($C_{6'}$), 49.7 (C_1), 51.0 (C_3), 56.3 ($C_{3''}$), 60.4 ($C_{6''}$), 67.3 ($C_{4''}$), 70.4 ($C_{4'}$), 70.5 ($C_{5''}$), 71.0 ($C_{2''}$), 72.6 ($C_{2'}$), 73.1 ($C_{3'}$), 75.2 ($C_{5'}$), 78.3 (C_5), 84.4 (C_4), 86.0 (C_6), 99.6 ($C_{1'}$), 101.4 ($C_{1''}$), 155.4, 156.6 (C=O); mp 184 °C dec; IR (KBr) cm^{-1} 3414, 1658; MS-FAB$^+$ 707 (M + Na$^+$, 4%), 685 (M + H$^+$, 1%).

Hexa-O-silylated Derivative of 1,3,6′-Tri-*N*-(*tert*-butoxycarbonyl)-3″-*N*-formylkanamycin A (28).[10] Compound **27** (7.5 g, 10.9 mmol) was dissolved in 400 mL of dioxane/H_2O (3:1) and was stirred at 50 °C. *N*-(*tert*-Butoxycarbonyloxy)-5-norbornene-*endo*-2,3-dicarboximide (3.04 g. 10.95 mmol) was added, and the reaction was stirred overnight at 50 °C. A TLC analysis using $CHCl_3$/MeOH/concentrated ammonia (3:1:6 drops) showed the formation of a new component (R_f 0.64) as an approximately 1:1 mixture with the starting material. 4-Nitrophenyl formate (1.83 g, 11.0 mmol) was added, and the reaction was stirred overnight at 45 °C. A TLC analysis with the above-mentioned system indicated no change in R_f, except for the appearance of a UV-positive, ninhydrin-negative spot (R_f 0.84), indicative of the 4-nitrophenol byproduct. The solvent was evaporated to dryness, and the residue was kept under vacuum overnight. Subsequently, the residue was dissolved in dry pyridine (100 mL), followed by the addition of a mixture of hexamethyldisilazane and chlorotrimethylsilane (3:1, 120 mL). The mixture was stirred overnight at room temperature. The reaction mixture was evaporated *in vacuo*. The oily residue was taken up in ethyl ether (100 mL), and it was washed with water (4 × 200 mL). Subsequently, the organic layer was washed with 10% sodium carbonate until the aqueous layer remained colorless. The ether layer was dried over magnesium sulfate, filtered, and evaporated *in vacuo* to dryness. A TLC analysis with hexane/ethyl ether (1:1) showed three major components (R_f 0.29, 0.62, and 0.82). The desired product was the compound with R_f 0.29. The residue was chromatographed (hexane/ethyl ether, 1:1) on a silica-gel column, and 2.12 g of product was obtained (16% yield). This product gave a poorly resolved ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz). From the integration ratio of the resonances of the Boc (δ 1.37, 27H, 3s) and the trimethylsilyl groups (δ 0.05, 54H, several s) a ratio of 1:2 was obtained. The resonance for the formyl hydrogen was observed as an unresolved doublet at 8.32 ppm: mp 139 °C; IR (KBr) cm^{-1} 3455, 3347, 2965, 1712, 1245; MS-FAB$^+$ 1291 (M + 2Na$^+$, 9%), 1269 (M + Na$^+$, 2%).

Hepta-O-silylated Derivative of 3,6′,3″-Tri-*N*-(*tert*-butoxycarbonyl)-1-*N*-formylkanamycin A (29). Compound **27** (10.0 g, 14.6 mmol) was dissolved in 400 mL of dioxane/H_2O (3:1). 4-Nitrophenyl formate (2.5 g, 14.6 mmol) was added, and the mixture was stirred overnight at room temperature. A TLC analysis with $CHCl_3$/MeOH/concentrated ammonia (4:3:1) showed a component corresponding to the starting material (R_f 0.54) and a UV-positive, ninhydrin-negative spot at R_f 0.7. Di-*tert*-butyl dicarbonate (4.8 g, 21.9 mmol) was added, and the mixture was stirred for 5 h at 50 °C. A TLC analysis with the above-mentioned system showed the formation of a major component (R_f 0.67). A minor spot (R_f 0.54) was still observed. The solvent was evaporated *in vacuo*, and the residue was stored under vacuum overnight. It was then dissolved in dry pyridine (100 mL), followed by the addition of a mixture of hexamethyldisilazane and chlorotrimethylsilane (3:1, 120 mL). The reaction was stirred overnight at room temperature. The solvent was subsequently evaporated, and the residue was dissolved in ethyl ether (100 mL). The ether layer was washed with water (4 × 200 mL). The ether layer was then washed with 10% sodium carbonate until the aqueous layer remained colorless. The ether layer was dried over magnesium sulfate, filtered, and evaporated to dryness. A TLC analysis with hexane/ethyl ether (1:1) showed a major product (R_f 0.28). The residue was chromatographed (hexane/ethyl ether, 1:1), and 0.78 g of product (4% yield) was obtained. From the ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) it was observed that the integration ratio for the resonance of the Boc (δ 1.35, 1.36, 1.37, 27H, 3s) and of the trimethylsilyl groups (δ 0.06, 63H, m) was 3:7. The resonance for the formyl hydrogen was observed as an unresolved doublet at 8.32 ppm: mp 103 °C; IR (KBr) cm^{-1} 2960, 1711, 1698, 1247; MS-FAB$^+$ 1219 (M − Boc + H$^+$, 2%), 1218 (M − Boc, 3%).

Tri-O-silylated Derivative of 1,3,6′-Tri-*N*-(*tert*-butoxycarbonyl)-2′-*N*-formylneamine (30). Compound **22** (2.37 g, 2.82 mmol), prepared as described previously,[9] was dissolved in dry THF (50 mL). 4-Nitrophenyl formate (0.52 g, 3.10 mmol) was added, and the mixture was stirred overnight at 50 °C. The solvent was subsequently evaporated *in vacuo*, and the yellow residue was taken up in ethyl ether. The ether layer was extensively washed with 10% sodium carbonate until the aqueous layer remained colorless. The ether layer was dried over magnesium sulfate, filtered, and evaporated to dryness *in vacuo*. The residue was chromatographed on a silica-gel column. Elution with ethyl ether/hexane (2:1) gave 0.35 g of the desired product (R_f 0.5) as a white solid (14% yield). This product gave a poorly resolved ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz); however, integration of resonances for the Boc (δ 1.44, 27H, 3s) and the trimethylsilyl groups (δ 0.16, 27H, 3s) gave a 1:1 ratio. The resonance for the formyl hydrogen was observed as a doublet at 8.01 ppm (J = 12 Hz): mp 198 °C dec; IR (KBr) cm^{-1} 3365, 2975, 2925, 1682, 1262; MS-FAB$^+$ 767 (M − Boc + H$^+$, 10%).

Tri-O-silylated Derivative of 1,3,6′-Tri-*N*-(*tert*-butoxycarbonyl)-2′-deamino-2′-isocyanoneamine (31). A solution of **30** (0.9 g, 1.0 mmol) in dry methylene chloride (50 mL) was saturated with nitrogen and stirred for 30 min at −78 °C. Subsequently, $POCl_3$ (0.7 mL, 7.2 mmol) was added, followed by dry triethylamine (2.6 mL, 19.0 mmol). The mixture was allowed to warm up slowly to 4 °C, followed by stirring at 4 °C overnight. A portion of a chilled, saturated solution of sodium carbonate (100 mL) was added, and the mixture was stirred at 4 °C for another 30 min. The product was extracted with methylene chloride (3×), and the solution was dried over magnesium sulfate and evaporated *in vacuo* to dryness. The brown residue was chromatographed on a silica-gel column (hexane/ethyl acetate, 3:1) to give 0.23 g of the title compound (R_f 0.58, 27% yield). The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) indicated a ratio of 1:1 for the Boc (δ 1.41, 27H, 3s) and the trimethylsilyl groups (δ 0.16, 27H, 3s). The resonance for the formyl hydrogen of compound **30** was no longer observed: IR (KBr) cm^{-1} 3458, 2973, 2141 (isonitrile), 1717, 1285; mp 142 °C dec; MS-FAB$^+$ 959 [M + thioglycerol (matrix) + 2H$^+$, 1%], 958 [M + thioglycerol (matrix) + H$^+$, 1%], 957 [M + thioglycerol (matrix), 2%].

2′-Deaminoneamine Trifluoroacetic Acid Salt (3). A solution of compound **31** (0.23 g, 0.3 mmol) and azobisisobutyronitrile (AIBN, 16 mg, 7% w/w) in dry benzene was added dropwise to a refluxing solution of tributyl tin hydride (0.83 mL, 3.1 mmol) in benzene. The solution was refluxed for 5 h. The solvent was then evaporated, and the oily residue was chromatographed on a silica-gel column. The column was washed extensively with hexane to remove as much of the tin as possible. The deaminated neamine was eluted with hexane/ethyl acetate (3:1). The compound with R_f 0.6 was collected, and the solvent was removed *in vacuo*. The residue was taken up in dry methylene chloride (20 mL), saturated with nitrogen and stirred at 4 °C. A portion of distilled trifluoroacetic acid (10 mL) was added, and

(10) For both the neamine and kanamycin A analogues, we noted that one hydroxyl group consistently did not undergo silylation, with the exception of those intermediates where the amino group at position 1 underwent selective modifications (compounds **26**, **32**, **35**, and **38**). Molecular modeling on a Silicon Graphics computer suggested that the 5-hydroxyl is the most hindered site in both types of molecules.

the solution was stirred for 3 h at 4 °C. The mixture was warmed to room temperature, and the stirring was continued for 1 h. The solvent was subsequently evaporated to dryness *in vacuo*, and the residue was stirred in ethyl ether (25 mL) until the product triturated as a white solid (76 mg, 43% overall yield): R_f 0.35 (H_2O/acetone/concentrated acetic acid, 7:2:1); ^{1}H-NMR (D_2O, 500 MHz): δ 1.59 (1H, dt, J = 13 Hz, J = 3.5 Hz, $H_{2'ax}$), 1.66 (1H, q, J = 12.5 Hz, H_{2ax}), 2.13 (1H, ddd, J = 14 Hz, J = 5.0 Hz, J = 0.5 Hz, $H_{2'eq}$), 2.30 (1H, dt, J = 13 Hz, J = 4 Hz, H_{2eq}), 3.10 (1H, dd, J = 7.5 Hz, J = 14.0 Hz, $H_{6'R}$), 3.15 (1H, m, H_3), 3.18 (1H, t, J = 9.0 Hz, $H_{4'}$), 3.26 (1H, dd, J = 3.5 Hz, J = 14.0 Hz, $H_{6'S}$), 3.29 (1H, ddd, J = 4.0 Hz, J = 10.0 Hz, J = 13.0 Hz, H_1), 3.33 to 3.50 (2H, unresolved m, H_5 and H_6), 3.59 (1H, t, J = 9.0 Hz, H_4), 3.76 (1H, ddd, J = 3.5 Hz, J = 7.5 Hz, J = 9.0 Hz, $H_{5'}$), 3.82 (1H, ddd, J = 5.0 Hz, J = 9.0 Hz, J = 12.0 Hz, $H_{3'}$), 5.46 (1H, m, J = 3 Hz, J = 0.5 Hz, $H_{1'}$); ^{13}C- and DEPT-NMR (D_2O, 125 MHz): δ 28.1 (C_2), 36.4 ($C_{2'}$), 40.1 ($C_{6'}$), 48.5 (C_3), 49.6 (C_1), 67.0 ($C_{4'}$), 69.2 ($C_{5'}$), 71.8 ($C_{3'}$), 72.5 (C_5), 75.0 (C_6), 78.0 (C_4), 98.3 ($C_{1'}$), 115.1, 117.4, 119.7 (CF_3CO), 162.8, 163.1 (CF_3CO); mp 128 °C dec; IR (KBr) cm^{-1} 3400, 1671, 1523, 1428, 1198, 1127; MS-FAB$^+$ 308 [M − 3(CF_3CO_2)$^-$ − 2H$^+$, 7%], 309 [M − 3(CF_3CO_2)$^-$ − H$^+$, 2%].

Tri-O-silylated Derivative of 1,3,2′-Tri-*N*-(*tert*-butoxycarbonyl)-6′-*N*-formylneamine (12). Compound **11** (1.4 g, 2.3 mmol) was dissolved in dry pyridine (20 mL). A 3:1 mixture of hexamethyldisilazane and chlorotrimethylsilane (48 mL) was added to the solution, and the mixture was stirred at room temperature overnight. The solvent was subsequently evaporated to dryness *in vacuo*. The resultant oily residue was dissolved in methylene chloride (50 mL) and was washed with water (2 × 50 mL). The aqueous layer was washed with methylene chloride (20 mL), and the methylene chloride layer was combined with the organic layer. Solvent was removed *in vacuo* to dryness, and the resultant residue was dissolved in acetone/water (5:1, 60 mL) and was stirred for 5 h at room temperature. Solvent was evaporated, and the residue was taken up in methylene chloride (50 mL). The solution was washed with water (25 mL), dried over magnesium sulfate, filtered, and evaporated to dryness. The silylated compound was isolated as a yellow solid after it was kept under vacuum overnight (1.29 g, 67% yield). The solid was subsequently dissolved in dry THF (100 mL), followed by the addition of 4-nitrophenyl formate (0.42 g, 2.53 mmol). The solution was stirred overnight at 50 °C. The solvent was subsequently evaporated and the yellow residue was taken up in ethyl ether. The ether layer was extensively washed with 10% sodium carbonate until the aqueous layer remained colorless. The ether layer was dried over magnesium sulfate, filtered, and evaporated to dryness. The residue was chromatographed (ethyl ether/hexane, 3:1) on a silica-gel column. The desired product (R_f 0.56, 1.25 g) was obtained in 94% yield. The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) was not well resolved, but from integration, it was observed that the ratio for resonance of the hydrogens for the trimethylsilyl (δ 0.07, 27H, 3s) and those for the Boc groups (δ 1.42, 27H, 3s) was 1:1. The formyl hydrogen was observed at 8.16 ppm as an unresolved doublet: mp 95 °C dec; IR (KBr) cm^{-1} 3363, 2980, 2970, 1687, 1452, 1161; MS-FAB$^+$ 727 (M − NHCHO − Boc + H$^+$ + Na$^+$, 1%).

Compounds **17**, **21**, and **26** were converted to the tri-O-silylated derivatives, and the free amine of each silylated derivative was formylated according to the procedure that was used to prepare compound **12**.

Tri-O-silylated Derivative of 1,2′,6′-Tri-*N*-(*tert*-butoxycarbonyl)-3-*N*-formylneamine (32). The starting material was compound **17**. The reaction mixture was purified using silica-gel column (ethyl ether/hexane, 2:1) to give the title compound (R_f 0.36, 1.48 g, 50% yield). The ^{1}H-NMR ($CDCl_3$, 300 MHz) indicated a 1:1 ratio between the trimethylsilyl groups (0.14, 27H, 3s) and the Boc groups (1.39, 27 H, 3s). The resonance for the formyl hydrogen was observed at 8.05 ppm as an unresolved doublet: mp 88 °C dec; IR (KBr) cm^{-1} 3355, 2975, 2923, 1679, 1452, 1160; MS-FAB$^+$ 976 [M + thioglycerol (matrix) + H$^+$, 2%].

Tetra-O-silylated Derivative of 3,2′,6′-Tri-*N*-(*tert*-butoxycarbonyl)-1-*N*-formylneamine (33). The starting material was compound **21**. The reaction mixture was purified on a silica-gel column (ethyl ether/hexane 2:1) to give the title compound (R_f 0.44, 0.49 g, 55% yield). The ^{1}H-NMR ($CDCl_3$, 500 MHz) spectrum showed a ratio of 4:3 between the trimethylsilyl groups (δ 0.02, 36H, 4s) and the Boc groups (δ 1.33, 27H, 3s). The formyl hydrogen was observed as an unresolved doublet at 8.03 ppm: mp 160 °C dec; IR (KBr) cm^{-1} 3352, 2977, 2925, 1679, 1450, 1253; MS-FAB$^+$ 975 [M + thioglycerol (matrix), 1%].

Hexa-O-silylated Derivative of 1,3,3″-Tri-*N*-(*tert*-butoxycarbonyl)-6′-formylkanamycin A (34). The starting material was compound **26**. The reaction mixture was chromatographed on a silica-gel column (ethyl ether/hexane, 2:1) to afford the title compound (R_f 0.20, 1.84 g, 90% yield). The ^{1}H-NMR ($CDCl_3$, 500 MHz) spectrum showed a ratio of 2:1 between the trimethylsilyl groups (δ 0.09, 54H, several s) and the Boc groups (δ 1.41, 27H, 3s). The formyl hydrogen was observed as an unresolved doublet at 8.2 ppm: mp 190 °C dec; IR (KBr) cm^{-1} 3348, 2971, 2930, 1677, 1378, 1360, 1299, 1245; MS-FAB$^+$ 1026 (M − NHCHO − Boc − TMS + 3H$^+$, 1%).

The preparations of the following isocyanate derivatives were carried out according to the procedure described for **31**.

Hexa-O-silylated Derivative of 1,3,6′-Tri-*N*-(*tert*-butoxycarbonyl)-3″-deamino-3″-isocyanokanamycin A (35). The starting material was compound **28**. The reaction mixture was purified on a silica-gel column (hexane/ethyl acetate, 5:1) to give the title compound (R_f 0.5, 0.3 g, 7% yield). The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) confirmed a ratio of 3:6 between the resonance of the Boc groups (δ 1.36, 1.38, 27H, 2s) and of the trimethylsilyl groups (δ 0.07, 54H, several s). The signal of the formyl hydrogen was no longer observed. The IR spectrum showed a distinctive isonitrile stretch at 2138 cm^{-1}: mp 98 °C; IR (KBr) cm^{-1} 3455, 3368, 2952, 2900, 2138, 1717, 1387, 1359, 1249; MS-FAB$^+$ 1408 [M + thioglycerol (matrix), 2%].

Hepta-O-silylated Derivative of 3,6′,3″-Tri-*N*-(*tert*-butoxycarbonyl)-1-deamino-1-isocyanokanamycin A (36). The starting material was compound **29**. The reaction mixture was chromatographed on a silica-gel column (hexane/ethyl acetate, 7:1) to give the desired compound (R_f 0.43, 0.3 g, 39% yield). The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) showed a ratio of 3:7 for the resonances of the Boc (δ 1.41, 1.42, 27H, 2s) and the trimethylsilyl groups (δ 0.1, 63H, several s). The signal of the formyl hydrogen was no longer observed. The IR spectrum indicated a diagnostic isonitrile stretch at 2133 cm^{-1}: mp 88 °C; IR (KBr) cm^{-1} 3448, 2963, 2898, 2133, 1713, 1385, 1363, 1244; MS-FAB$^+$ 1273 (M − NC, 2%), 1222 (M − Boc + Na$^+$, 1%).

Tri-O-silylated Derivative of 1,3,2′-Tri-*N*-(*tert*-butoxycarbonyl)-6′-deamino-6′-isocyanoneamine (13). The starting material was compound **12**. The reaction mixture was chromatographed on a silica-gel column (hexane/ethyl acetate, 3:1) to give the desired compound (R_f 0.62, 0.37 g, 30% yield). The ^{1}H-NMR ($CDCl_3$, 500 MHz) confirmed the presence of trimethylsilyl (δ 0.15, 27H, 3s) and Boc groups (δ 1.42, 1.44, 27H, 2s) in a 1:1 ratio. The signal of the formyl hydrogen was no longer observed. IR spectrum showed an isonitrile stretch at 2145 cm^{-1}: mp 128−130 °C dec; IR (KBr) cm^{-1} 3448, 3361, 2974, 2903, 2145, 1714, 1387, 1359, 1245; MS-FAB$^+$ 959 [M + thioglycerol (matrix) + 2H$^+$, 1%], 958[(M + thioglycerol (matrix) + H$^+$, 1%], 957[(M + thioglycerol (matrix), 2%].

Tri-O-silylated Derivative of 1,2′,6′-Tri-*N*-(*tert*-butoxycarbonyl)-3-deamino-3-isocyanoneamine (37). The starting material was compound **32**. The reaction mixture was purified on a silica-gel column (hexane/ethyl acetate, 3:1) to afford the title product (R_f 0.72, 0.93 g, 65% yield). The ^{1}H-NMR ($CDCl_3$, 300 MHz) indicated a 1:1 ratio for the resonances of the trimethylsilyl groups (δ 0.14, 27H, 3s) and the Boc groups (δ 1.39, 27 H, 3s). The signal of the formyl hydrogen was no longer observed. IR spectrum confirmed the presence of an isonitrile functionality (2135 cm^{-1}): mp 85 °C dec; IR (KBr) cm^{-1} 2972, 2135, 1714, 1384, 1360, 1244; MS-FAB$^+$ 959 [M + thioglycerol (matrix) + 2H$^+$, 2%].

Tetra-O-silylated Derivative of 3,2′,6′-Tri-*N*-(*tert*-butoxycarbonyl)-1-deamino-1-isocyanoneamine (38). The starting material was compound **33**. The reaction mixture was chromatographed on a silica-gel column (hexane/ethyl acetate 3:1) to give the title compound (R_f 0.66, 0.32 g, 65% yield). The ^{1}H-NMR ($CDCl_3$, 500 MHz) showed a 4:3 ratio for the trimethylsilyl (δ 0.09, 36H, several s) and the Boc groups (δ 1.40, 1.41, 27H, 2s). The resonance of the formyl hydrogen was no longer observed. IR spectrum confirmed the presence of isonitrile moiety with a diagnostic stretch at 2138 cm^{-1}: mp 83 °C dec; IR (KBr) cm^{-1} 3350, 2970, 2138, 1717, 1496, 1270; MS-FAB$^+$ 958 [M + thioglycerol (matrix) + H$^+$, 1%].

Hexa-O-silylated Derivative of 1,3,3″-Tri-*N*-(*tert*-butoxycarbonyl)-6′-deamino-6′-isocyanokanamycin A (39). The starting material

was compound **34**. The reaction mixture was purified on a silica-gel column (hexane/ethyl acetate, 3:1) to give the title compound (R_f 0.58, 0.38 g, 16% yield). The ^{1}H-NMR ($CDCl_3$, 500 MHz) spectrum confirmed the ratio of 2:1 for the resonances of the trimethylsilyl (δ 0.10, 54H, several s) and the Boc groups (δ 1.41, 27H, 3m). The resonance of the formyl hydrogen was no longer observed. IR spectrum showed a diagnostic stretch of isonitrile functionality at 2143 cm^{-1}: mp 196 °C dec; IR (KBr) cm^{-1} 3450, 2965, 2143, 1720, 1490, 1221; MS-FAB$^+$ 1072 (M − NC − Boc − TMS + 2Na$^+$, 4%).

The preparation of the following deaminated compounds were performed by reduction of the isocyano group and deprotection of the protective groups according to the procedure described for **3**.

3″-Deaminokanamycin A Trifluoroacetic Acid Salt (7). The starting material was compound **35**. The reaction mixture was purified on a silica-gel column (hexane/ethyl acetate, 9:1) to afford the desired compound (R_f 0.33) which was subsequently deprotected to give the final product [R_f 0.62 (water/acetone/acetic acid, 7:2:1), 58 mg, 29% yield]: ^{1}H-NMR (D_2O, 500 MHz) δ 1.61 (1H, q, J = 11 Hz, H_{2ax}), 1.74 (1H, q, J = 12 Hz, $H_{3''ax}$), 2.03 (1H, poorly resolved dt, H_{2eq}), 2.38 (1H, poorly resolved dt, $H_{3''eq}$), 2.9−4.0 (16H, poorly resolved, overlapping multiplets of various hydrogens), 4.82 (1H, unresolved doublet, $H_{1''}$), 5.37 (1H, unresolved doublet, $H_{1'}$); ^{13}C- and DEPT-NMR (D_2O, 125 MHz) δ 27.4 (C_2), 34.2 ($C_{3''}$), 40.3 ($C_{6'}$), 47.4 (C_3), 49.8 (C_1), 60.5 ($C_{6''}$), 63.9 ($C_{4''}$), 66.6 ($C_{2''}$), 68.7 ($C_{5'}$), 70.7 ($C_{4'}$), 70.8 ($C_{2'}$), 71.8 ($C_{5''}$), 72.2 ($C_{3'}$), 73.6 (C_5), 78.0 (C_4), 83.5 (C_6), 95.2 ($C_{1'}$), 100.0 ($C_{1''}$), 112.8, 115.1, 117.4, 119.8 (CF_3CO_2), 162.4, 162.7, 162.9, 163.2 (CF_3CO_2); mp 158 °C dec; IR (KBr) cm^{-1} 3463, 2927, 1686, 1524, 1427, 1195, 1130; MS-FAB$^+$ 351 [M − 3(CF_3CO_2)$^-$ + 2Na$^+$, 2%), 307 [M − 3(CF_3CO_2)$^-$, 1%].

1-Deaminokanamycin A Trifluoroacetic Acid Salt (5). Starting material was compound **36**. The reaction mixture was purified on a silica-gel column (hexane/ethyl acetate, 9:1) to give the protected analog of the title compound (R_f 0.53) which was subjected to the deprotection step to afford the final product [R_f 0.66 (H_2O/acetone/acetic acid, 7:2:1), 58 mg, 31% yield]: ^{1}H-NMR (D_2O, 500 MHz) δ 1.23 (1H, poorly resolved dq, H_{2ax}), 1.38 (1H, poorly resolved dq, H_{1ax}), 2.00 (1H, m, H_{2eq}), 2.08 (1H, m, H_{1eq}), 3.0−4.0 (14H, poorly resolved, overlapping multiplets of various hydrogens), 4.98 (1H, d, J = 3.5 Hz, $H_{1''}$), 5.41 (1H, d, J = 3.5 Hz, $H_{1'}$); ^{13}C- and DEPT-NMR (D_2O, 125 MHz) δ 23.4 (C_1), 24.5 (C_2), 40.2 ($C_{6'}$), 50.6 (C_3), 55.0 ($C_{3''}$), 59.8 ($C_{6''}$), 65.6 ($C_{4''}$), 67.5 ($C_{2''}$), 68.6 ($C_{5'}$), 70.7 ($C_{4'}$), 71.6 ($C_{2'}$), 72.2 ($C_{5''}$ and $C_{3'}$), 73.3 (C_5), 76.6 (C_4), 79.3 (C_6), 94.0 ($C_{1'}$), 95.3 ($C_{1''}$), 115.1, 117.4, 128.2, 128.8 (CF_3CO_2), 162.8, 163.1 (CF_3CO_2); mp 160 °C dec; IR (KBr) cm^{-1} 3412, 2945, 1676, 1518, 1426, 1196, 1130; MS-FAB$^+$ 308 [M − 3(CF_3CO_2)$^-$ + H$^+$, 9%], 309 [M − 3(CF_3CO_2)$^-$ + 2H$^+$, 2%].

6′-Deaminoneamine Trifluoroacetic Acid Salt (4). The starting material was compound **13**. The reaction mixture was chromatographed on a silica-gel column (hexane/ethyl acetate, 3:1) to give the protected analog of the title compound (R_f 0.59) which was subjected to the deprotection step to give the final product [R_f 0.79 (water/acetone/concentrated acetic acid, 7:2:1), 0.28 g, 64% yield]: ^{1}H-NMR (D_2O, 500 MHz) δ 1.14 (3H, d, J = 6.5 Hz, $H_{6'}$), 1.66 (1H, q, J = 12.5 Hz, H_{2ax}), 2.32 (1H, dt, J = 13 Hz, H_{2eq}), 3.10 (1H, t, J = 9.5 Hz, $H_{4'}$), 3.15 (1H, m, H_3), 3.26 (1H, dd, J = 4.0 Hz, J = 10 Hz, H_1), 3.34 (1H, unresolved m, H_5), 3.39 (1H, t, J = 10.0 Hz, H_6), 3.47 (1H, t, J = 9 Hz, H_4), 3.64 (1H, dd, J = 6.0 Hz, J = 9.5 Hz, $H_{5'}$), 3.67 (1H, poorly resolved dt, J = 9.5 Hz, $H_{3'}$), 5.52 (1H, d, J = 4 Hz, $H_{1'}$); ^{13}C- and DEPT-NMR (D_2O, 125 MHz) δ 16.4 ($C_{6'}$), 28.1 (C_2), 48.5 (C_3), 49.5 (C_1), 54.0 ($C_{2'}$), 68.6 ($C_{4'}$), 69.5 ($C_{5'}$), 72.3 ($C_{3'}$), 74.4 (C_5), 74.9 (C_6), 78.6 (C_4), 96.4 ($C_{1'}$), 112.7, 115.1, 117.4, 119.7 (CF_3CO), 162.8, 163.1 (C=O); mp 156 °C dec; IR (KBr) cm^{-1} 3414, 2916, 1672, 1526, 1430, 1196, 1130; MS-FAB$^+$ 307 [M − 3(CF_3CO_2)$^-$ − 3H$^+$, 100%), 308 [M − 3(CF_3CO_2)$^-$ − 2H$^+$, 14%], 309 [M − 3(CF_3CO_2)$^-$ − H$^+$, 2%).

3-Deaminoneamine Trifluoroacetic Acid Salt (2). The starting material was compound **37**. The reaction mixture was purified on a silica-gel column (hexane/ethyl acetate, 3:1) to give the protected analog of the desired product (R_f 0.65) which was subsequently deprotected to give the title compound [R_f 0.73 (H_2O/acetone/concentrated acetic acid, 7:2:1), 0.39 g, 55% yield]: ^{1}H-NMR (D_2O, 500 MHz) δ 1.31 (2H, m, H_{2ax}, H_{3ax}), 1.87 (2H, m, H_{2eq}, H_{3eq}), 2.31 (1H, m, H_1), 2.92 (1H, poorly resolved dt, J = 11 Hz, $H_{6'R}$), 3.01 (1H, dd, J = 8 Hz, J = 13.5 Hz, H_6), 3.18 (1H, dd, J = 3.5 Hz, J = 11 Hz, $H_{6'S}$), 3.24 (2H, overlapping t, J = 9 Hz, J = 4.5 Hz, H_4 and $H_{4'}$), 3.31 (1H, t, J = 9 Hz, $H_{2'}$), 3.45 (1H, unresolved m, H_5), 3.71 (1H, t, J = 9.5 Hz, $H_{5'}$), 3.77 (1H, dt, J = 3.0 Hz, J = 9.5 Hz, $H_{3'}$), 5.27 (1H, d, J = 3.5 Hz, $H_{1'}$); ^{13}C- and DEPT- NMR (D_2O, 125 MHz) δ 23.8 (C_3), 27.4 (C_2), 40.1 ($C_{6'}$), 52.8 (C_1), 53.9 ($C_{2'}$), 68.3 ($C_{4'}$), 69.0 ($C_{5'}$), 71.0 ($C_{3'}$), 73.1 (C_5), 76.4 (C_6), 80.0 (C_4), 96.5 ($C_{1'}$), 112.8, 115.1, 117.4, 119.8 (CF_3-CO), 162.3, 162.6, 162.9, 163.1 (C=O); mp 111 °C dec; IR (KBr) cm^{-1} 3418, 2938, 1667, 1528, 1425, 1204, 1138; MS-FAB$^+$ 308 [M − 3(CF_3CO_2)$^-$ − 2H$^+$, 76%), 309 [M − 3(CF_3CO_2)$^-$ − H$^+$, 19%], 310 [M − 3(CF_3CO_2)$^-$, 4%].

1-Deaminoneamine Trifluoroacetic Acid Salt (1). The starting material was compound **38**. The reaction mixture was purified on a silica-gel column (hexane/ethyl acetate, 3:1) to give the protected analog of the desired compound (R_f 0.65) which was subsequently deprotected to give the title compound [R_f 0.68 (H_2O/acetone/concentrated acetic acid, 7:2:1), 0.15 g, 62% yield]: ^{1}H-NMR (D_2O, 500 MHz) δ 1.24 (2H, m, H_{1ax}, H_{2ax}), 1.85 (2H, m, H_{1eq}, H_{2eq}), 3.07 (1H, dd, J = 7 Hz, J = 13.5 Hz, $H_{6'R}$), 3.13 (1H, m, H_3), 3.26 (2H, overlapping m, $H_{4'}$ and $H_{6'S}$), 3.32 (3H, overlapping m, H_1, H_5, H_6), 3.60 (1H, t, J = 9 Hz, H_4), 3.78 (1H, t, J = 9 Hz, $H_{5'}$), 3.82 (1H, overlapping m, $H_{3'}$), 5.71 (1H, d, J = 4 Hz, $H_{1'}$); ^{13}C- and DEPT-NMR (D_2O, 75 MHz) δ 24.2 (C_1), 28.1 (C_2), 40.0 ($C_{6'}$), 51.3 (C_3), 53.4 ($C_{2'}$), 68.3 ($C_{4'}$), 69.0 ($C_{5'}$), 70.6 ($C_{3'}$), 71.4 (C_5), 77.7 (C_6), 79.1 (C_4), 96.0 ($C_{1'}$), 112.8, 115.1, 117.4 (CF_3CO), 162.7, 163.0 (C=O); mp 135 °C dec; IR (KBr) cm^{-1} 3415, 2936, 1668, 1527, 1428, 1203, 1132; MS-FAB$^+$ 308 [M − 3(CF_3CO_2)$^-$ − 2H$^+$, 60%], 309 [M − 3(CF_3CO_2)$^-$ − 1H$^+$, 10%], 310 [M − 3(CF_3CO_2)$^-$, 2%].

6′-Deaminokanamycin A Trifluoroacetic Acid Salt (6). The starting material was compound **39**. The reaction mixture was chromatographed on a silica-gel column (hexane/ethyl acetate, 3:1) to give the protected derivative of the desired product which was subsequently deprotected to give the title compound [R_f 0.59 (H_2O/acetone/concentrated acetic acid, 7:2:1), 85 mg, 39% yield]: ^{1}H-NMR (D_2O, 500 MHz) δ 1.09 (3H, d, J = 6 Hz, $H_{6'}$), 1.69 (1H, q, J = 12.5 Hz, H_{2ax}), 2.33 (1H, dt, J =13.5 Hz, H_{2eq}), 2.9−4.0 (15H, overlapping, unresolved multiplets, various hydrogens), 4.91 (1H, d, J = 3.5 Hz, $H_{1'}$), 5.26 (1H, d, J = 3.5 Hz, $H_{1''}$); ^{13}C- and DEPT-NMR (D_2O, 125 MHz) δ 16.5 ($C_{6'}$), 27.5 (C_2), 48.2 (C_3), 49.6 (C_1), 54.9 ($C_{3''}$), 59.6 ($C_{6''}$), 65.2 ($C_{4''}$), 68.0 ($C_{2''}$), 68.9 ($C_{5'}$), 71.2 ($C_{4'}$), 72.1 ($C_{2'}$), 72.6 ($C_{5''}$), 73.2 ($C_{3'}$), 74.5 (C_5), 79.1 (C_4), 83.6 (C_6), 98.0 ($C_{1'}$), 100.5 ($C_{1''}$), 112.3, 115.0, 117.4, 119.7 (CF_3CO), 162.7, 162.9, 164.7 (C=O); mp 173 °C dec; IR (KBr) cm^{-1} 3418, 2933, 1673, 1524, 1427, 1195, 1130; MS-FAB$^+$ 468 (M, 4%), 469 (M + H$^+$, 2%), 470 (M + 2H$^+$, 100%), 471 (M + 3H$^+$, 24%).

Assay of Enzymic Activity. Enzymic activity was measured by the coupled-spectrophotometric assay of Goldman and Northrop.[11] In this assay, production of ADP during the course of APH(3′) catalysis was monitored. ADP was converted to ATP by pyruvate kinase (PK) and lactate dehydrogenase (LD) in the presence of phosphoenol pyruvate (PEP) and NADH. Conversion of NADH to NAD was monitored at 340 nm. A typical assay mixture contained 200 mM PIPES buffer (pH 7.0), 11 mM magnesium acetate, 22 mM potassium acetate, 1.8 mM PEP, 0.1 mM NADH, 6.1 units of PK, 21 units of LD, 15 μM kanamycin A, and 0.15 mM ATP. The final volume for each assay mixture was 1.0 mL.

Turnover Kinetics. A typical assay mixture contained all the above-described ingredients except for 0.3−600 μM concentrations of the deaminated compounds were substituted for kanamycin A. The K_m and k_{cat} values were obtained from Lineweaver−Burk plots, typically with five to seven concentrations of substrate flanking the K_m value, each recorded in duplicate. For such determinations, the rate of hydrolysis of ATP was subtracted from the rate of phosphorylation of aminoglycosides.

Determination of Minimum Inhibitory Concentrations (MICs). The MICs of neamine, kanamycin A, and compounds **1**−**7** were determined by the broth microdilution method with inocula of 5 × 10^5 colony-forming units per milliliter in Mueller−Hinton broth.[12] The bacterial strains used were *E. coli* HB101 (background strain), *E. coli*

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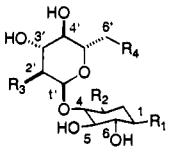
JM83(pTZ18u), which expresses APH(3′)-Ia, and *E. coli* HB101-(pGEME182), which expresses APH(3′)-IIa. The MIC was defined as the lowest concentration of antibiotic that prevented growth, which was determined by the appearance of turbidity after 24 h of incubation at 37 °C.

Results and Discussion

Fersht and colleagues have demonstrated in a series of elegant experiments that electrostatic interactions, such as hydrogen bonding, between an enzyme and a substrate are not merely useful for increased affinity but also would facilitate the rate of the catalyzed reaction by lowering the energy barrier when such interactions are present with the transition-state species.[13] The strategy for overcoming resistance to aminoglycosides which is disclosed here is inspired by this thesis. We reasoned that impairment of electrostatic interaction between these antibiotics and their resistance enzymes would be useful to evade the undesirable modification of the antibiotic. Electrostatic interactions such as hydrogen bonding are strongest between and among charged centers. This view is strengthened by both chemical intuition and the observations of such interactions in protein crystal structures. While such electrostatic interactions are well understood, their quantitative importance is difficult to assess because the interaction energies depend crucially on the dielectric constant of the surrounding medium. The problem of quantifications of these electrostatic effects in proteins is especially difficult because of the heterogeneity of protein structure. The dielectric constant changes throughout the protein and depends on the given microenvironment. An additional complicating factor is that charged moieties are fully solvated in solution, the solvation energies for which are high in aqueous medium.[14] For active-site binding to occur, the substrate should be stripped of waters of hydration and rely on interactions with active-site residues instead. Nonetheless, attempts have been made to quantify specific coulombic contributions in proteins and in protein-small molecule interactions. Such interactions between a carboxylate and an ammonium ion have been attributed binding energies ranging from 0.5−5.0 kcal/mol.[15]

Based on these observations, we expected that the ammonium functions in aminoglycosides should make relatively strong interactions with the active site of the resistance enzymes. The exact nature of these interactions remains unknown at the present. However, we decided to prepare a series of individually deaminated analogues of aminoglycosides to evaluate the importance of these interactions with the active sites of two of the resistance enzymes, namely aminoglycoside 3′-phosphotransferases types Ia and IIa, the first of which is the most commonly found enzyme in resistant organisms. Toward that goal, compounds **1**−**7** were synthesized.[16] Compounds **1**−**4** are based on the structure of neamine and compounds **5**−**7** are based on the structure of kanamycin A.[17] Both neamine and kanamycin A are excellent substrates for the aminoglycoside 3-phosphotransferases (*vide infra*).

The regiospecific syntheses of these compounds presented a challenge because of the seemingly equal reactivity of the various amine groups. In essence, we needed seven different compounds in each of which three amines were protected, leaving the fourth amine available for deamination. We have used temporary selective protection of various amines with transition metals[18] and the use of special sterically encumbered reagents for the introduction of protective groups in these efforts. These syntheses are briefly described here.

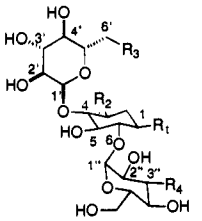


1 $R_1 = H, R_2 = R_3 = R_4 = NH_3^+$

2 $R_2 = H, R_1 = R_3 = R_4 = NH_3^+$

3 $R_3 = H, R_1 = R_2 = R_4 = NH_3^+$

4 $R_4 = H, R_1 = R_2 = R_3 = NH_3^+$



5 $R_1 = H, R_2 = R_3 = R_4 = NH_3^+$

6 $R_3 = H, R_1 = R_2 = R_4 = NH_3^+$

7 $R_4 = H, R_1 = R_2 = R_3 = NH_3^+$

Free-base neamine (**8**) was allowed to react with *N*-benzyloxycarbonyloxy-5-norbornene-*endo*-2,3-dicarboximide (*N*-BND), a sterically encumbered reagent for introduction of the

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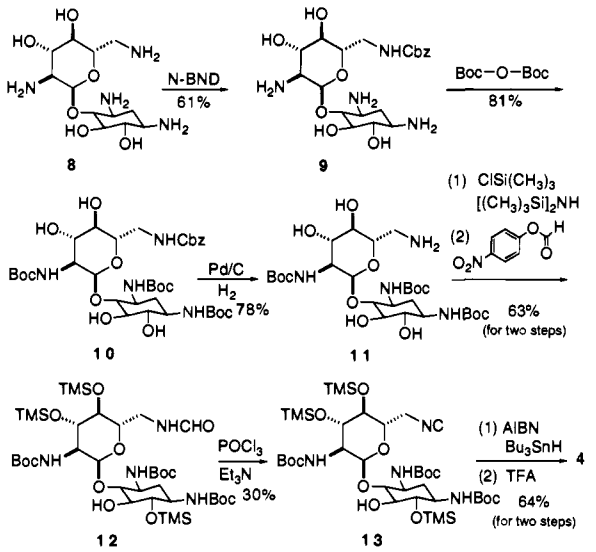
(16) The ^{13}C-NMR assignments for neamine and kanamycin A have been reported in the literature (Naito, T.; Toda, S.; Nakagawa, S.; Kawaguchi, H. In *Aminocyclitol Antibiotics*; Rinehart, K. L., Jr., Suami, T., Eds.; American Chemical Society: Washington, DC, 1980; p 257). We determined the ^{1}H-NMR assignments for neamine and kanamycin A by extensive homonuclear decoupling experiments. These assignments are as follows: *Neamine* (500 MHz, D_2O, pD 9.5): δ 1.03 (q, 1H, J = 12.5 Hz, H_{2ax}), 1.80 (dt, 1H, J = 4.0, J = 12.5 Hz, H_{2eq}), 2.53 (m, 1H, H_1), 2.59 (dd, 1H, J = 7.0, J = 14.0 Hz, $H_{6'R}$), 2.62 (dd, 1H, J = 3.5, J = 10.0 Hz, $H_{2'}$), 2.66 (m, 1H, H_3), 2.81 (dd, 1H, J = 2.5, J = 14.0 Hz, $H_{6'S}$), 2.95 (t, 1H, J = 9.5 Hz, H_6), 3.09 (t, 1H, J = 9.5 Hz, H_4), 3.11 (t, 1H, J = 9.5 Hz, $H_{4'}$), 3.32 (t, 1H, J = 9.5 Hz, H_5), 3.34 (t, 1H, J = 10.0 Hz, $H_{3'}$), 3.57 (m, 1H, $H_{5'}$), 5.10 (d, 1H, J = 3.5 Hz, $H_{1'}$). *Kanamycin A* (500 MHz, D_2O, pD 9.5) δ 1.09 (q, 1H, J = 12.5 Hz, H_{2ax}), 1.83 (dt, 1H, J = 4.0, J = 12.5 Hz, H_{2eq}), 2.69 (dd, 1H, J = 7.5, J = 14.0 Hz, $H_{6'R}$), 2.77 (m, 2H, H_1 and H_3), 2.87 (t, 1H, J = 10.0 Hz, $H_{3''}$), 2.92 (dd, 1H, J = 2.5, J = 14.0 Hz, $H_{6'S}$), 3.12 (t, 1H, J = 9.5 Hz, H_4), 3.18 (t, 1H, J = 9.5 Hz, $H_{4'}$), 3.19 (t, 1H, J = 10.0 Hz, $H_{4''}$), 3.20 (t, 1H, J = 9.5 Hz, H_4), 3.37 (dd, 1H, J = 3.5, J = 10.0 Hz, $H_{2''}$), 3.46 (dd, 1H, J = 4.0, J = 9.5 Hz, $H_{2'}$), 3.53 (t, 1H, J = 9.5 Hz, H_5), 3.57 (t, 1H, J = 9.5 Hz, $H_{3'}$), 3.63 (m, 2H, $H_{6''R}$, $H_{6''S}$), 3.66 (ddd, 1H, J = 2.5, J = 7.5, J = 9.5 Hz, $H_{5'}$), 3.77 (dt, 1H, J = 3.0, J = 9.5 Hz, $H_{5''}$), 4.90 (d, 1H, J = 3.5 Hz, $H_{1''}$), 5.22 (d, 1H, J = 4.0 Hz, $H_{1'}$). The previously reported ^{13}C-NMR assignments and our assignments for the resonances of the ^{1}H-NMR spectra were correlated and mutually verified by ^{1}H−^{13}C heteronuclear-correlated spectroscopy (COSY) experiments. Compounds **1**−**7** were each characterized by ^{1}H-, ^{13}C-, and DEPT-NMR determinations. DEPT-NMR spectra were used to confirm the formation of new methyl or methylene in the deaminated products. The ring substitutions for neamine and kanamycin A are all equatorial. Hence, the preferred conformations for all rings is chair, a fact which is consistent with the reported crystal structure for kanamycin A (Koyama, G.; Iitaka, Y.; Maeda, K.; Umezawa, H. *Tetrahedron Lett.* **1968**, 1875). The conformations of the individual rings do not change for any of the deaminated compounds according to ^{1}H-NMR analysis, as exemplified by the following analysis for compound **3**. The vicinal coupling constants $J_{3',4'}$ and $J_{4',5'}$ are 9.0 Hz for compound **3**, in close accordance with those of neamine, showing a diaxial relationship for $H_{3'}$, $H_{4'}$ and $H_{5'}$. This suggests that the glucopyranosyl portion of both neamine and the deaminated neamine analogue **3** adopt the typical "all-axial"-chair conformation for saccharides in solution (Durette, P. L.; Horton, D. In *Advances in Carbohydrate Chemistry and Biochemistry*; Academic Press: New York, 1971; Vol. 26, pp 49−125). The $J_{2'eq,3'}$ = 5.0 Hz and $J_{2'ax,3'}$ = 12.0 Hz for **3**, values which closely approach those for H_{2ax} and H_{2eq} of neamine ($J_{2eq,1} = J_{2eq,3}$ = 4.0 Hz and $J_{2ax,1} = J_{2ax,3}$ = 12.5 Hz) and support that deamination does not induce any unusual ring distortion around $C_{1'}-C_{2'}$ or $C_{2'}-C_{3'}$ bonds. In addition, the $J_{1,6}$ = 10.0 Hz and $J_{3,4}$ = 9.0 Hz for compound **3** are in good agreement with those for neamine, indicating that there is no conformational distortion induced in the cyclitol ring by deamination either.

(17) Selective preparation of the precursors for deamination at position 3 of kanamycin A proved difficult, so we did not pursue it.

(18) The nature of transition metal-aminoglycoside interactions have been studied in our laboratory (Grapsas and Mobashery, unpublished results). In a series of NMR experiments, we have correlated the effect of certain metal coordination on the aminoglycoside spectra. The picture that emerges indicates that various transition metals show preference for coordination by the amines, hydroxyls, and/or glycosidic oxygens of aminoglycosides in a precise way for each metal under the conditions employed.

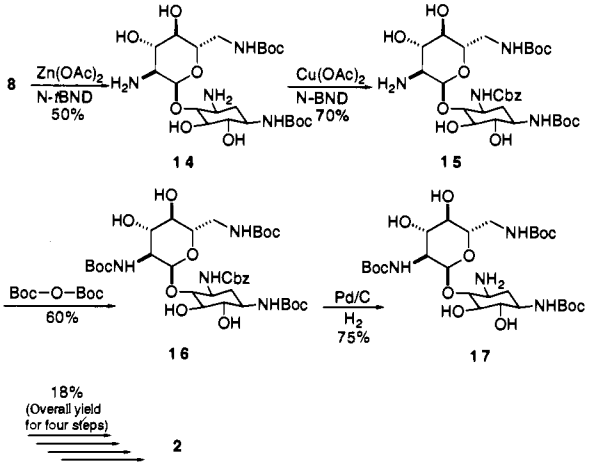
Scheme 1



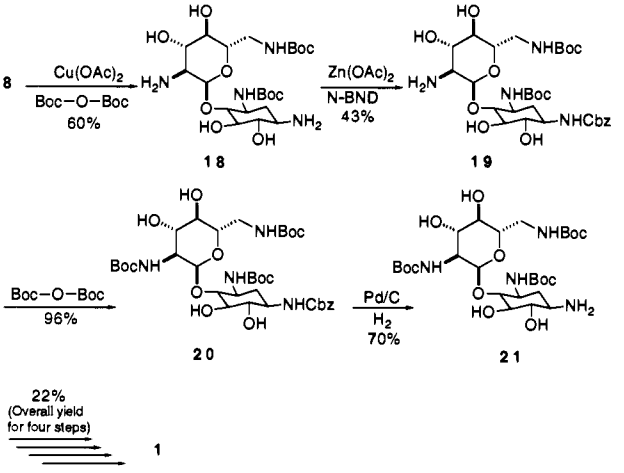
carbobenzyloxy (Cbz) group, to give **9**. Subsequently, the remaining amines in **9** were protected exhaustively by the use of di-*tert*-butyl-dicarbonate to give **10**. The Cbz group was removed from **10** by hydrogenolysis over palladium to afford the tri-Boc neamine analogue **11**. Deamination of the protected neamine (**11**) was carried out according to the reductive deamination method developed by Barton and colleagues.[19] Compound **11** was O-silylated (the silyl group which was introduced at the free amine was selectively removed in the presence of moisture)[20] and subsequently was formylated by *p*-nitrophenyl formate to give **12**. The byproduct of this reaction, *p*-nitrophenol, proved difficult to separate from the product in this and other similar reactions. In general, column chromatography was not useful, hence *p*-nitrophenol was invariably removed by repeated aqueous sodium bicarbonate washes. This procedure removed some of the silyl protective groups contributing to lower yields of **12** (as well as other formylated compounds) after chromatography. The isonitrile **13** was made from **12** by the use of $POCl_3$ in the presence of anhydrous triethylamine. This reaction (and others like it) in general gave low yields in our hands. The subsequent reduction of the isonitrile function and deprotonation of the protective groups afforded the desired **4**.

Reaction of free-base neamine (**8**) with *N*-butoxycarbonyloxy-5-norbornene-*endo*-2,3-dicarboximide (*N*-*t*BND), a sterically encumbered reagent for introduction of the butoxycarbonyl (Boc) group,[9] in the presence of zinc acetate furnished the di-Boc analogue **14** as the major product, along with one minor product. Compound **14** was successfully purified by chromatography and was subsequently protected at N_3 with *N*-BND in the presence of copper(II) acetate to give **15**. A di-Cbz derivative also formed in this reaction as the minor component of the product mixture, which was separated from the desired **15** by chromatography. Protection of $N_{2'}$ of **15** by di-*tert*-butyldicarbonate produced compound **16**, which subsequent to

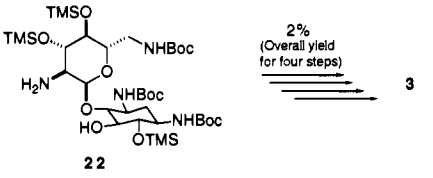
Scheme 2



Scheme 3



Scheme 4



hydrogenolysis yielded **17**. This tri-Boc species was deaminated and deprotected, as described above for the preparation of **4**, to afford compound **2**.

Neamine coordinates the copper(II) ion differently than the zinc ion. Reaction of di-*tert*-butyldicarbonate with neamine (**8**) in the presence of the copper(II) ion gave protection of N_3 and $N_{6'}$ (**18**). Subsequently, reaction of **18** with *N*-BND in the presence of zinc acetate furnished **19** along with a small quantity of the di-Cbz derivative, which was separated by chromatography from **19**. Protection of **19** at $N_{2'}$ with the Boc group (**19** → **20**) and hydrogenolysis of the Cbz group in **20** gave the desired tri-Boc analogue **21**. Deamination at N_1 and deprotection by the usual method furnished compound **1**.

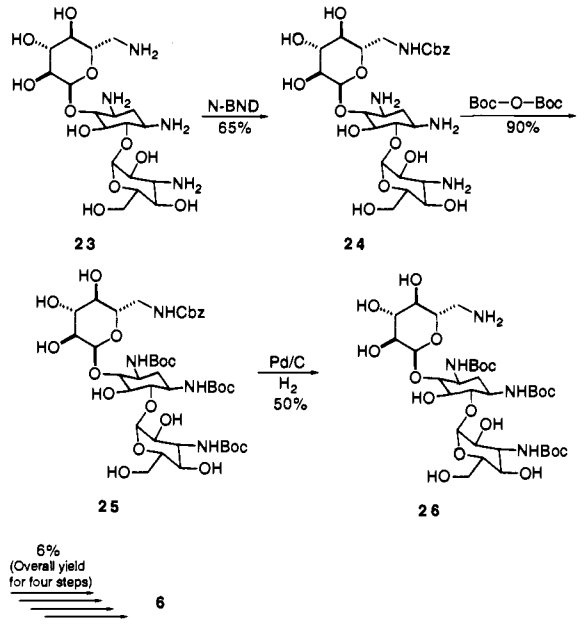
The synthesis of the last tri-Boc neamine derivative, 1,3,6′-triBoc-neamine (**22**), has been reported by us previously.[9] The subsequent steps for deamination and deprotection to give **3** were similar to the already described procedures.

As shown below, the same general synthetic scheme described for the preparation of **11** was applicable for the synthesis of the protected compound **26** and ultimately the deprotected compound **6**, the first molecule in the kanamycin A series. This synthesis started from free-base kanamycin A (**23**). In prepara-

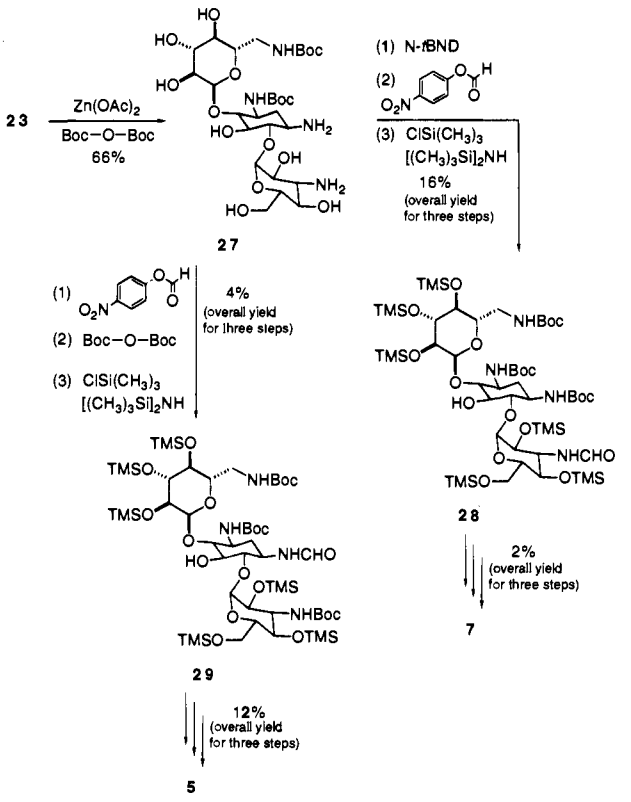
(19) Barton, H. R.; Bringmann, G.; Motherwell, W. B. *J. Chem. Soc., Perkin Trans. 1* **1980**, 2665.

(20) From the analysis of the ^{1}H-NMR spectra of the silylated compounds it is obvious that one hydroxyl group in either neamine or kanamycin A does not undergo silylation. We do not know with certainty which hydroxyl remains unprotected; however, we presume that it is the hydroxyl at position 5 (of neamine or kanamycin A) since molecular modeling suggests that it may be the most hindered hydroxyl in the precursors to the silylated products. We hasten to add that the ease of incorporation of the trimethylsilyl protective group at the 6′-, 3-, and 4-hydroxyls of neamine has been documented previously (Verheyden, J. P. H.; Repke, D. B.; Tompkins, T. C.; Moffatt, J. G. in *Aminocyclitol Antibiotics*; ACS Symposium Series No. 125, Reinhart, K. L., and Suami, T., Ed.; Washington, DC, 1980; p 212).

Scheme 5



Scheme 6



tion of **24**, the formation of a minor di-Cbz derivative was also noted, which was separated from the desired **24** by chromatography.

As was outlined above, prior to deamination, *N*-formyl derivatives of the corresponding amines were prepared. For two of our kanamycin A derivatives, we accessed the *N*-formyl intermediates directly, as shown below. Reaction of kanamycin A (**23**) with di-*tert*-butyldicarbonate in the presence of zinc acetate afforded protection at N_3 and $N_{6'}$ (*i.e.*, **27**). An additional compound—a tri-Boc analogue—was also detected in the product mixture as the minor component, which was removed from the product mixture by chromatography. This intermediate was allowed to react with *N-tert*-butoxycarbonyloxy-5-norbornene-*endo*-2,3-dicarboximide (*N*-*t*BND) to introduce the Boc group at N_1. The resultant compound was formylated at $N_{3''}$, followed by exhaustive silylation to give **28**. We perceived that N_1 in compound **27** was in general more accessible to reagents than $N_{3''}$. Hence, reaction of *p*-nitrophenyl formate with **27** afforded formylation at N_1, which after exhaustive silylation gave the derivative **29**. Deamination and deprotection of derivatives **28** and **29** produced the desired compounds **7** and **5**, respectively.

Turnover of compounds **1**–**7** was studied with purified APH(3′)-Ia and APH(3′)-IIa (Table 1). We were anticipating that some of our compounds might turn out to be poor substrates for the enzymes. As indicated in Table 1, indeed all of compounds **1**–**7** were extremely poor substrates for both phosphotransferases. This was an unexpected finding, which argues for the critical importance of electrostatic interactions with the amino-acid residues in the active site in the course of turnover. In general, the K_m values increased modestly compared to the corresponding values for the parent compounds (neamine and kanamycin A), with the exception of compound **7** with APH(3′)-Ia, for which K_m remained unchanged at 1.2 μM. Insofar as K_m may approximate K_s, this indicates that binding of the compounds to the enzyme active sites has not been affected much. We have not determined the predominant three-dimensional structures for these compounds in solution. Some of the deaminated derivatives of neamine and kanamycin A may have slightly different solution structures, if certain potential inter-ring interactions are disrupted for these compounds. In light of the fact that amines/ammonium ions and hydroxyls in these aminoglycosides should be fully hydrated in aqueous solution, the potential inter-ring interactions cannot be strong, and indeed these aminoglycosides would exist in solution as dynamic structures.[16] On the other hand, interactions with the enzymes would be governed, and even dictated, by the steric and electronic constraints of the active sites, necessitating binding of the deaminated derivatives in an analogous manner to the parent compounds for which the active sites have evolved to near "perfection". This is the rationale that permits measurement of the energetics for the electrostatic contribution of these ammonium groups to catalysis within the enzyme active sites from the kinetics of turnover of compounds **1**–**7** (*vide infra*).

The most significant effects were manifested on k_{cal} values. These values decreased significantly, with the lowest attenuation of 2.4×10^4-fold [for compound **5** with APH(3′)-IIa] and the highest of 6.1×10^6-fold [for compound **7** with APH(3′)-Ia]. The consequence of these effects were such that for these two enzymes, which carry out their catalytic function on neamine and kanamycin A at near the diffusion limit, the k_{cal}/K_m values for compounds **1**–**7** in general did not exceed 168 M^{-1} s^{-1}. The results in Table 1 indicate that the binding energies of the enzyme to the transition-state species are within the 6–11 kcal/mol range.[21] To our knowledge, these examples are the strongest interactions recorded between an ammonium moiety and a counterpart—presumably an acidic amino acid—in an enzyme active site. These data argue for a rather low dielectric constant for the environment of the complex between the transition-state structure and the enzyme, indicative of the specificity and strength of the interactions which would lead to the exclusion of water from the active site. In principle, each ammonium species may accept as many as three hydrogen bonds from the active site. The energetic contributions of these multiple hydrogen bonds are generally not additive, but it is conceivable that some of the deaminated compound may have lost more than one hydrogen bond in the transition-state complexes in the course of catalysis. We add parenthetically here that the kinetic determinations tabulated in Table 1 for compounds **1**–**7** were difficult to evaluate because these

(21) Calculated from $\Delta\Delta G = -RT \ln[(k_{cat}/K_m)_{synth}/(k_{cat}/K_m)_{nonsynth}]$.

Table 1. Kinetic Parameters for Turnover of Neamine, Kanamycin A, and Compounds **1**−**7** with Purified APH(3′)-Ia and APH(3′)-IIa[a]

	APH(3′)-Ia			APH(3′)-IIa		
compd	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/Km (M^{-1} s^{-1})
8 (neamine)[b]	1.3 ± 0.4	4080 ± 1020	5.23×10^7	1.2 ± 0.1	1740 ± 545	2.4×10^7
1	16 ± 4	0.025 ± 0.005	26	6 ± 2	0.045 ± 0.011	124
2	32 ± 21	0.012 ± 0.008	6	112 ± 63	0.357 ± 0.200	53
3	24 ± 4	0.240 ± 0.030	168	13 ± 4	0.013 ± 0.003	17
4	3.6 ± 0.4	0.029 ± 0.002	133	54 ± 20	0.373 ± 0.137	115
20 (kanamycin A)[b]	1.2 ± 0.2	6120 ± 840	8.5×10^7	2.8 ± 0.2	240 ± 45	1.7×10^6
5	73 ± 32	0.002 ± 0.001	0.4	81 ± 25	0.010 ± 0.003	2
6	33 ± 13	0.073 ± 0.030	37	364 ± 52	0.195 ± 0.030	9
7	1.2 ± 0.4	0.001 ± 0.001	11	8 ± 4	0.013 ± 0.006	25

[a] The values for K_m for Mg^{2+}ATP for APH(3′)-Ia and APH(3′)-IIa were 20 ± 3 and 41 ± 7 μM, respectively. [b] Values were obtained from Siregar *et al.*[5,6]

Table 2. Minimum Inhibitory Concentrations of Neamine, Kanamycin A, and Compounds **1**−**7** Against *E. coli* HB101 (Background Strain), *E. coli* JM83(pTZ18u) [Expresses APH(3′)-Ia], and *E. coli* HB101(pGEME182) [Expresses APH(3′)-IIa][a]

compd	*E. coli* HB101 (mM)	*E. coli* JM83 (pTZ18u) (mM)	*E. coli* HB101 (pGEME182) (mM)
8 (neamine)	3.9	15.5	15.5
1	1.6	1.5	1.5
2	3.1	4.4	4.4
3	0.8	0.7	1.4
4	14.7	14.7	14.7
20 (kanamycin A)	0.02	10.3	10.3
5	11.9	11.9	11.9
6	1.2	2.4	2.4
7	>11.5	>23.9	>23.9

[a] An MIC difference of fourfold is considered significant within the limits of these determinations.

molecules were such poor substrates. In fact, turnover of these compounds was so poor that they competed with the basal ATP hydrolase ("ATPase") activity of these enzymes,[22] the contribution of which to turnover of ATP in our assay had to be taken into account in our calculations.

Table 1 summarized the turnover properties of the resistance enzymes with our compounds *in vitro*. We have also evaluated the effect of these compounds *in vivo* by measuring the minimal inhibitory concentrations (MICs) against *E. coli* HB101 [background strain without APH(3′)s], *E. coli* JM83(pTZ18u) [which expresses APH(3′)-Ia], and *E. coli* HB101(pGEME182) [which expresses APH(3′)-IIa](Table 2). When these enzymes are expressed in the cell, the MICs for the parent compounds (*i.e.*, neamine and kanamycin A) are raised. For example, the MIC value for neamine is increased from 3.9 mM for the background strain to 15.5 mM with each of the organisms expressing the enzymes. The same trend, although somewhat more drastic, is seen for kanamycin A. The MIC increased from 20 μM for the background strain to 10.3 mM for the resistant bacteria. If the analogues were to circumvent the harmful enzymic activity *in vivo*, as the *in vitro* results of Table 1 suggest they should, the MIC values for the resistant and background strains should approach each other. That is exactly what is seen, as summarized in Table 2. The MIC for *E. coli* HB101, *E. coli* JM83(pTZ18u), and *E. coli* HB101(pGEME182) are essentially the same for each of the compounds **1**−**7**. In effect, the catalytic function of the enzyme is not realized with these molecules *in vivo*. The antibacterial activities of compounds **1**−**7** (Table 2) do not herald them as promising for clinical use. However, these molecules have been excellent for demonstration of the concepts that we have put forth in this manuscript.

Nature has taken a similar strategy in selecting for resistance to vancomycin. Vancomycin exerts its antibacterial activity by binding to the terminal acyl-DAla-DAla portion of the bacterial peptidoglycan, thereby preventing its participation in cross-linking during cell division. Vancomycin accepts an important hydrogen bond from the amide nitrogen between the two DAla moieties. It has been shown that in the resistant organisms a variant of DAla-DAla ligase is capable of incorporating D-α-hydroxy carboxylates (such as D-lactate) in place of the terminal DAla.[23] Hence, an ester bond is made in place of an amide bond, and vancomycin is no longer capable of binding to this variant form of peptidoglycan because of the absence of the important hydrogen-bond donor. This is a clear example of how a single important hydrogen bond can abolish interactions of two molecules *in vivo*.

A survey of the structures of the known aminoglycosides indicated that one finds at positions 1, 3, 2′, 6′, and 3″ either a hydroxyl group, an amine, or a modified amine (*i.e.*, alkylated, acylated, or carbamoylated).[24] In principle, all of these groups can be involved in hydrogen bonding with functionalities in the enzyme active site, supporting the assertion that these enzymes have evolved to take advantage of these interactions in recognition of their substrates. Of course, an important factor is the strength of these interactions. As we briefly alluded to earlier, such interactions between two charged centers can be quite significant in complexation. Indeed, in instances in which ion-pairing groups may be sheltered from the milieu, or when the dielectric constant is low in the microenvironment, the contribution of ion pairing may approach the overall free energy of protein folding.[25]

The concept outlined here was demonstrated for aminoglycoside 3′-phosphotransferases. However, *the generality of this concept to the principles of catalysis indicates that it should prove useful for other classes of resistance enzymes as well. Furthermore, it is very important to state that the abolished electrostatic interaction need not be at or near the seat of the catalytic reaction in the active site, as the effect of these interactions in lowering of the energy barrier are realized by such interactions even at relatively remote sites in the active site.*

Acknowledgment. This work was supported by the National Institutes of Health. We thank Dr. Mohamad Ksebati for performing the $^1H-^{13}C$ heteronuclear-correlated spectroscopy (COSY) experiments.

JA951703X

(22) In the absence of aminoglycosides, ATP is hydrolyzed by APH(3′)s with the following kinetic parameters: APH(3′)-Ia, $K_m = 49 \pm 1$ μM, $k_{cat} = 0.023 \pm 0.002$ s^{-1}, $k_{cat}/K_m = 6.0 \times 10^2$ M^{-1} s^{-1}; APH(3′)-IIa, $K_m = 52 \pm 19$ μM, $k_{cat} = 0.027 \pm 0.001$ s^{-1}, $k_{cat}/K_m = 5.3 \times 10^2$ M^{-1} s^{-1} (ref 6).

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(24) Hooper, I. R. *In Aminoglycoside Antibiotics*; Umezawa, H., Hooper, I. R., Eds.; Springer-Verlag: New York, 1982; p 1. Yarus, M. In *The RNA World*; Gestland, R. F., Atkins, J. F., Eds.; Cold Spring Harbor Laboratory Press: Plainview, NY, 1993; p 205.

(25) Finney, J. L.; Gellatly, B. J.; Golton, I. C.; Goodfellow, J. *Biophys. J.* **1980**, *32*, 17.