Fluorinated Aminoglycosides and Their Mechanistic Implication for Aminoglycoside 3′-Phosphotransferases from Gram-Negative Bacteria†

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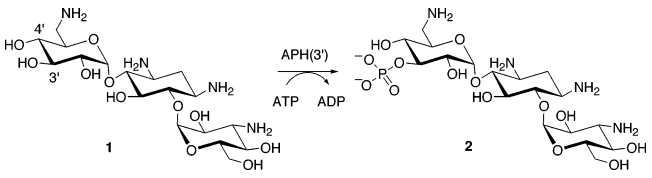
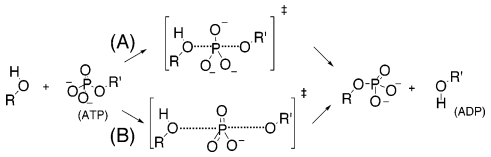
ABSTRACT: Aminoglycoside 3′-phosphotransferases [APH(3′)s] are important bacterial resistance enzymes for aminoglycoside antibiotics. These enzymes phosphorylate the 3′-hydroxyl of these antibiotics, a reaction that inactivates the drug. A series of experiments were carried out to shed light on the details of the turnover chemistry by these enzymes. Quench-flow pre-steady-state kinetic analyses of the reactions of Gram-negative APH(3′) types Ia and IIa with kanamycin A, neamine, and their respective difluorinated analogues 4′-deoxy-4′,4′-difluorokanamycin A and 4′-deoxy-4′,4′-difluoroneamine were carried out, in conjunction with measurements of thio effect and viscosity studies. The fluorinated analogues were shown to be severely impaired as substrates for these enzymes. The magnitude of the effect of the impairment of the fluorinated substrates was in the same range as when the D198A mutant APH(3′)-Ia was studied with nonfluorinated substrates. Residue 198 is the proposed active site base that promotes the aminoglycoside hydroxyl for phosphorylation. These findings collectively argue that the Gram-negative APH(3′)s show significant nucleophilic participation in the transition state for the phosphate transfer reaction.

Structural modification of aminoglycoside antibiotics by aminoglycoside-modifying enzymes is the most common mechanism of resistance to this class of antibiotics (*1*). Aminoglycoside 3′-phosphotransferases [APH(3′)s][1] make up an important family of these resistance enzymes (*2*, *3*). These enzymes catalyze transfer of the γ-phosphoryl of ATP to the 3′-hydroxyl of a wide range of aminoglycoside antibiotics to result in their inactivation. For example, this enzymic activity has caused clinical obsolescence of the previously effective antibiotic kanamycin A (**1** → **2**). Aminoglycoside 3′-phosphotransferase types Ia and IIIa, [APH(3′)-Ia and APH(3′)-IIIa] are the most common members of this family of enzymes in Gram-negative and Gram-positive bacteria, respectively (*1*).



Scheme 1: Transition States of the Associative (A) and Dissociative (B) Mechanisms for the Phophoryl Transfer Reaction



Transfer of phosphate from one molecule to another has been described in terms of a continuum between two extremes, associative (nucleophilic displacement of the γ-phosphoryl of ATP) to dissociative (involving metaphosphate-like species) mechanisms (*4*; Scheme 1). Transfer of phosphate in enzymic reactions is considered "concerted" processes, which do not involve an intermediate, but each would have a single transition state. Terms such as "tight" vs "exploded" or "loose" have been used to describe the transition-state species in enzymic reactions. The Gram-positive APH(3′)-IIIa has been proposed to catalyze its reaction by a dissociative or loose mechanism (*5*, *6*). We describe herein the use of two judiciously fluorinated aminoglycosides (compounds **3** and **4**) as probes, in conjunction with a host of kinetic tools with the wild-type and mutant enzymes in investigation of mechanistic possibilities in the case of APH(3′)-Ia and APH(3′)-IIa (both from Gram-negative bacteria). The use of these molecules, in conjunction with their nonfluorinated parental compounds (**1** and **5**), was useful in documenting that the two Gram-negative APH(3′)s operate by a strong nucleophilic participation in the

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[1] Abbreviations: APH(3′), aminoglycoside 3′-phosphotransferase; Boc, *tert*-butoxycarbonyl; Cbz, carbobenzyloxy; DAST, (diethylamino)-sulfur trifluoride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactoside; LD, lactic dehydrogenase; PEP, phospho(enol)pyruvate; PK, pyruvate kinase; TBSCl, *tert*-butyldimethylsilyl chloride.

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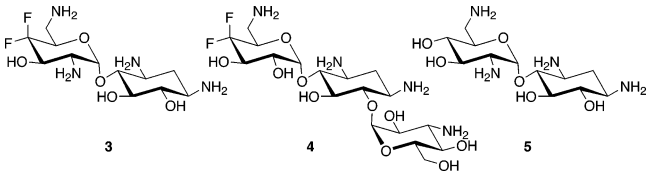
transition state for their mechanisms.



EXPERIMENTAL PROCEDURES

Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance spectra were recorded on either a Varian Mercury 400 or a Varian Unity-500 spectrometer. Chemical shifts are recorded in parts per million (δ) relative to tetramethylsilane (δ 0.00). Infrared (IR) spectra were recorded on a Nicolet 680 DSP FTIR spectrometer. Low-resolution mass spectra (MS) were recorded on a Kratos MS 80RFA spectrometer. High-resolution mass spectra were acquired at the Michigan State University Mass Spectrometry Facility. Thin-layer chromatography (TLC) was performed with Whatman precoated K6F silica gel 60A (0.25 mm thickness plates). The plates were visualized by either ninhydrin spray or immersion in a *p*-anisaldehyde solution and warming on a hot plate. Melting points were obtained on an Electrothermal melting point apparatus and are uncorrected. All chromatography solvents were of reagent grade. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl, and dichloromethane was distilled from calcium hydride. 1,1-Dimethoxycyclohexane was purchased from the TCI America Co. Pyruvate kinase (PK), lactic dehydrogenase (LD), phospho(enol)pyruvate (PEP), ATP, and NADH were purchased from the Sigma Chemical Co. All other reagents were purchased from the Aldrich Chemical Co.

Cloning of the aph(3′)-Ia Gene into the Expression Vector. Plasmid pTZ19-3 (*7*) was used as the source of the gene for APH(3′)-Ia. The *aph(3′)-Ia* gene was PCR-amplified using two custom-synthesized primers, APH3IaNde (5′-ATTACAT**ATG**AGCCATATTCAACGGGAAAC-3′) and APH3IaHind (5′-AATAAAGC**TTA**GAAAAACTCATCGAGCATC-3′). Primer APH3IaNde was designed to incorporate the recognition sequence for the *Nde*I restriction endonuclease (underlined) that includes the ATG start codon (in bold). Primer APH3IaHind was constructed to include the recognition sequence for the *Hind*III restriction endonuclease (underlined) and the stop codon (in bold). The PCR product was digested with restriction endonucleases *Nde*I and *Hind*III and ligated into the corresponding sites of the expression vector pET22b(+), and the ligation mixture was used to transform competent cells of *Escherichia coli* JM83. After selection on agar supplemented with ampicillin (100 μg/mL), the plasmid DNA from several colonies was isolated and checked for the presence of the desired insert by digestion with restriction endonucleases *Nde*I and *Hind*III. Both strands of the cloned fragment were sequenced in order to make certain that no undesired mutations were introduced during PCR. Recombinant plasmid DNA (pET22-APHI) was utilized to transform the competent cell of *E. coli* BL21(DE3).

Site-Directed Mutagenesis. The D198A mutant of APH(3′)-Ia was prepared by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Two mutagenic primers, APH(3′)-ImutD (5′-AGTCGTCACTCATGGTGCTTTCTCACTTGATAACC-3′) and APH(3′)-ImutR (5′-GGTTATCAAGTGAGAAAGCACCATGAGTGACG-ACT-3′), that incorporate the GCT codon (underlined) for alanine instead of the GAT codon for the aspartic acid were utilized to introduce mutation into the *aph(3′)-Ia* gene using the DNA of the pET22-APHI as a template. Following mutagenesis the sequence of the entire mutant gene was confirmed for both DNA strands. The recombinant plasmid harboring the mutant *aph(3′)-Ia* gene was named pET22-APHA.

Protein Purification. The wild-type APH(3′)-Ia, its D198A mutant, and the wild-type APH(3′)-IIa were purified according to the method described by Siregar et al. (*2*) with minor modifications. For purification of the APH(3′)-Ia and its D198A, 3 mL overnight cultures of *E. coli* BL21(DE3) harboring recombinant plasmids were inoculated into 300 mL of LB growth medium, and cells were grown at 37 °C with shaking (180 rpm) to an absorbance of 0.4 at 600 nm (about 1.5 h), followed by the addition of IPTG to a final concentration of 0.4 mM. Bacterial cultures were then incubated at 25 °C for another 20 h. For purification of the APH(3′)-IIa, *E. coli* JM83 harboring plasmid pGEME$_{182}$ (*8*) was incubated for 18 h at 37 °C in Terrific broth supplemented with kanamycin (30 μg/mL). Cells were harvested by centrifugation at 5500*g* for 15 min, washed with buffer A (25 mM HEPES, pH 7.4, and 0.2 mM DTT), and resuspended into 30 mL of buffer A. Cells were disrupted by 18 cycles of sonication (20 s of burst and 20 s of rest for each cycle). The cell debris was discarded after centrifugation at 14000*g* for 30 min. Streptomycin sulfate was slowly added to the supernatant to a final concentration of 1.5%. The solution was stirred for 30 min at 4 °C, followed by centrifugation at 14000*g* for 1 h. The supernatant was diluted to a final volume of 50 mL with buffer A and was loaded at a flow rate of 1.5 mL/min onto the neomycin affinity column [2.5 × 10 cm; 50 mL of Affi-Gel 15 resin (Bio-Rad, Hercules, CA)] coupled with 3.6 mmol of neomycin. The column was washed with buffer A (total volume 150 mL), followed by a linear gradient of 0−1.0 M NaCl in buffer A (500 mL). At the end of the gradients, the column was washed with buffer A supplemented with 1.0 M NaCl (200 mL). The desired protein was finally eluted with buffer A containing 1.5 M NaCl and 3 mM neomycin. The proteins were typically purified to apparent homogeneity for the kinetic studies.

Quench-Flow Analysis. Pre-steady-state kinetic measurements were carried out with a KinTek RQF-3 rapid quench-flow apparatus. The apparatus consists of three syringes driven by a stepping motor. Each reactant is pushed from a sample loop into a reaction line by buffer driven from two of the syringes. The third syringe contains a solution that quenches the reaction, and the quenched reaction is collected in a 1.5 mL centrifuge tube. Varying reaction lines and stepping motor speeds were used to determine the reaction time (from 2 to 1000 ms) (*9*).

Quench-flow experiments were performed by loading the enzyme and ATP into one sample loop and magnesium acetate, potassium acetate, and aminoglycoside into the other. Final concentrations of the reactants were 10 μM APH, 300 μM ATP, 100 μM aminoglycoside, 10 mM magnesium acetate, 20 mM potassium acetate, and a trace (7 nM) of [γ-^{32}P]ATP (10 μCi) (*10*), which was used to follow the progress of reaction, in 100 mM PIPES buffer, pH 7.0. The

reactions were quenched using 500 mM EDTA. The substrates and products were separated by thin-layer chromatography on a silica gel plate. The mobile phase was a chloroform−methanol−ammonia (4:15:12) mixture (*11*, *12*). The radioactivity was quantified by a Molecular Dynamics Storm 820 PhosphorImager.

Data Analysis. The pre-steady-state kinetic data were applied to a nonlinear least-squares fit according to eq 1 to

$$[\text{phosphoaminoglycoside}]/[\text{APH}] = A(1 - e^{-\lambda t}) + k_{cat}t \quad (1)$$

obtain the "burst" amplitude (A), the burst rate constant (λ), and the steady-state rate constant (k_{cat}) for the two phases of the reaction (*10*). The three microscopic rate constants were calculated from eqs 2−4.

$$A = \frac{k_3(k_3 + k_{-3})}{(k_3 + k_{-3} + k_4)^2} \quad (2)$$

$$\lambda = k_3 + k_{-3} + k_4 \quad (3)$$

$$k_{cat} = \frac{k_3k_4}{k_3 + k_{-3} + k_4} \quad (4)$$

Thio Effect. In determining the kinetic parameters for ATP, assays were performed in 100 mM PIPES (pH 7.0) buffer containing 11 mM magnesium acetate, 22 mM potassium acetate, 1.76 mM PEP, 0.1 mM NADH, 2.6 units of PK, and 7 units of LD with varying ATP concentrations (5−160 μM) in the presence of 10 μM aminoglycosides and 20 nM enzymes. ATPγS was substituted for ATP in order to evaluate the thio effects. Since commercial ATPγS can be contaminated with up to 10% ADP (communication by Gerard Wright) and ADP is regenerated to ATP by the action of pyruvate kinase in the coupled assay system, we uncoupled the assay by excluding phosphoenolpyruvate from the assay buffer in order to measure the true rate of ADP production. ATPγS (40, 80, 160, and 320 μM) was incubated in the presence and absence of 20 μM aminoglycosides, and the reaction was initiated by adding the APHs (500 nM). The reaction was allowed to proceed for 10 min at room temperature, and then the competitive inhibitor, tobramycin (1 mM), was added to quench the reaction. One minute after the addition of tobramycin, a 10 μL portion of 176 mM phosphoenolpyruvate was added, and the total change in absorbance at 340 nm was determined. The amount of ADP present in aminoglycoside null controls was subtracted from the amount present in aminoglycoside-containing assays.

Viscosity Effect. Viscosity experiments were carried out with sucrose (0−30%) and PEG 8000 (6.7%). The relative viscosities (η_{rel}) of the solutions were determined per published data (*13*, *14*).

In Vitro Translation Inhibition with Fluorinated and Nonfluorinated Aminoglycosides. In vitro translation levels of β-galactosidase by ribosomes were evaluated using the *E. coli* S30 extract system coupled with the β-galactosidase assay system (Promega, Madison, WI). To determine the degree of translation inhibition of β-galactosidase, several concentrations of aminoglycosides and their fluorinated derivatives (from 40 nM to 360 μM) were tested. Samples were incubated for 2 h at 37 °C, and the reaction was then stopped by placing the tubes on ice for 5 min. The reaction mixture was further incubated with the reagents from the β-galactosidase assay kit for approximately 20 min at 37 °C to develop a faint yellow color. Subsequently, the absorbance at 420 nm was measured, and the activity of β-galactosidase was evaluated. In the absence of any additional compound, the *in vitro* translation measurements were taken as a positive control.

Determination of Minimum Inhibitory Concentrations (MICs). The MICs of kanamycin A, neamine, and compounds **3** and **4** were determined by the microdilution method in Mueller−Hinton broth with inocula of 1×10^5 microorganisms per milliliter. The MICs were defined as the lowest concentration of antibiotic that prevented bacterial growth after overnight incubation at 37 °C (*15*). The bacterial strains used were *E. coli* JM83 (background strain), *E. coli* JM83 (pTZ18u), which expresses APH(3′)-Ia, and *E. coli* JM83 (pGEME$_{182}$), which expresses APH(3′)-IIa. To compare the MICs produced by the wild-type APH(3′)-Ia and its D198A mutant plasmid, pET22-APHI and pET22-APHA were retransformed to *E. coli* BL21(DE3).

*1,3,2′,6′-Tetra-N-tert-butoxycarbonyl-3′-O-tert-butyldimethylsilyl-5,6-O-cyclohexylideneneamine (**9**).* To a mixture of compound **8** (2.25 g, 2.81 mmol) and imidazole (243 mg, 3.57 mmol) in 20 mL of anhydrous *N*,*N*-dimethylformamide (DMF) was added *tert*-butyldimethylsilyl chloride (493 mg, 3.27 mmol) at room temperature under a nitrogen atmosphere, and the mixture was stirred overnight. The reaction was quenched by addition of water (3 mL), extracted with CH_2Cl_2 (3 × 20 mL), back-extracted with water and then brine, dried over $MgSO_4$, and concentrated to dryness *in vacuo*. The residue was purified on a column (SiO_2, 2:1 hexane/ethyl acetate) to furnish compound **9** (1.91 g, 74%) as a pure white solid: mp 140−142 °C; FTIR (film) 3451, 3356, 2977, 1721, 1703, 1507 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.28 (s, 1H, NH), 6.20 (d, J = 5.7 Hz, 1H, NH), 6.02 (d, J = 8.1 Hz, 1H, NH), 5.44 (s, 1H, NH), 5.12 (s, 1H, H1′), 4.88 (d, J = 3.0 Hz, 1H, OH), 3.80−3.46 (m, 9H), 3.17 (d, J = 9.0 Hz, 2H), 2.32 (d, J = 7.5 Hz, 1H, H2$_{eq}$), 1.65−1.28 (m, 47H), 0.86 [s, 9H, SiC(CH$_3$)$_3$], 0.07 [s, 6H, Si(CH$_3$)$_2$]; ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.2, 155.4, 155.1, 111.4, 97.7, 81.1, 79.0, 78.3, 77.9, 77.2, 72.9, 71.8, 71.4, 55.7, 50.7, 48.76, 40.9, 37.1, 36.4, 36.2, 28.1, 27.9, 27.8, 27.8, 25.8, 25.0, 24.0, 23.8, 18.2 [SiC(*C*H$_3$)$_3$], −4.3, −5.4 [Si(*C*H$_3$)$_2$]; MS (FAB, NBA) 917 (MH$^+$); HRMS (FAB, NBA) calcd for $C_{44}H_{81}N_4O_{14}Si$ (MH$^+$) 917.5519, found 917.5521.

*1,3,2′,6′-Tetra-N-tert-butoxycarbonyl-3′-O-tert-butyldimethylsilyl-5,6-O-cyclohexylidene-4′-deoxy-4′-oxoneamine (**10**).* A solution of **9** (270 mg, 0.294 mmol) in 1:1 DMSO/Ac_2O (6 mL) was kept at room temperature under an atmosphere of argon overnight (15 h). The reaction mixture was concentrated to dryness *in vacuo* to remove acetic anhydride and other volatiles. The concentrated solution was quenched by the addition of a saturated solution of $NaHCO_3$ and diluted with water to give a white solid, which was filtered and washed with water. The wet solid was dissolved in diethyl ether, dried (Na_2SO_4) and concentrated *in vacuo* to give the title compound **10** (259 mg, 96%) as a pure white solid. The analytical sample was prepared by purification of the compound on a column (SiO_2, 3:1 hexane/ethyl acetate) to

give **10** as a mixture of ketone and its hydrate form (trace): mp 129−131 °C; R_f 0.30 (2:1 hexane/ethyl acetate); FTIR (film) 3455, 3364, 2978, 2936, 1722, 1506, 1367, 1252, 1167, 1047 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.31 (d, J = 3.0 Hz, 1H, H1′), 5.26 (s, 1H, NH), 5.09 (d, J = 10.0 Hz, 1H, H3′), 4.99 (s, 1H, NH), 4.73 (s, 1H, NH), 4.24 (d, J = 10.5 Hz, 1H), 4.13−4.09 (m, 1H), 3.88 (s, 1H), 3.72−3.65 (m, 2H), 3.54 (t, J = 9.5 Hz, 1H), 3.52 (t, J = 6.5 Hz, 1H), 3.39−3.36 (m, 2H), 2.48 (d, J = 7.0 Hz, 1H, $H2_{eq}$), 1.62−1.54 (m, 11H), 1.42 (s, 21H), 1.34 (s, 9H), 1.33 (s, 6H), 0.87 [s, 9H, $SiC(CH_3)_3$], 0.09 [s, 3H, $Si(CH_3)_2$], 0.01 [s, 3H, $Si(CH_3)_2$]; ^{13}C NMR (125 MHz, $CDCl_3$) δ 203.4, 156.5, 155.2, 154.8, 112.8, 97.8, 80.5, 79.8, 79.6, 79.5, 78.2, 77.3, 77.0, 76.8, 76.1, 57.9, 50.8, 49.1, 39.6, 36.6, 36.4, 36.1, 28.4 (2C), 28.3, 25.9, 25.7, 24.9, 23.9, 23.6, 18.3 [$SiC(CH_3)_3$], −4.5 [$Si(CH_3)_2$], −5.4 [$Si(CH_3)_2$]; MS (FAB, NBA) 915 (MH^+); HRMS (FAB, NBA) calcd for $C_{44}H_{79}N_4O_{14}Si$ (MH^+) 915.5362, found 915.5350.

*1,3,2′,6′-Tetra-N-tert-butoxycarbonyl-3′-O-tert-butyldimethylsilyl-5,6-O-cyclohexylidene-4′-deoxy-4′,4′-difluoroneamine (**11**).* To an ice-cold solution of **10** (183 mg, 0.200 mmol) in dry dichloromethane (10 mL) was added morpholinosulfur trifluoride (366 μL, 3.00 mmol), and the mixture was stirred at ambient temperature under an atmosphere of argon overnight (18 h). The reaction mixture was cooled in an ice bath, quenched by the addition of a saturated solution of $NaHCO_3$, diluted with water, and extracted with CH_2Cl_2. The organic layer was back-extracted with brine, dried (Na_2SO_4), and concentrated *in vacuo* to give the crude product as a yellow residue. The solid was purified on a column (SiO_2, 47:2.5:0.5 CH_2Cl_2/EtOAc/MeOH) to afford **11** (169 mg, 90%) as a pure white solid: mp >140 °C (sublimes); R_f 0.50 (38:1.5:0.5 CH_2Cl_2/EtOAc/MeOH, triple development; cf. **10**, R_f 0.45); FTIR (KBr) 3452, 3376, 2977, 2936, 2859, 1723, 1504, 1367, 1252, 1163, 1043, 1004 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.23 (s, 1H, NH), 5.15 (s, 1H, NH), 5.03 (s, 1H, NH), 4.89 (d, J = 10.0 Hz, 1H, H1′), 4.71 (s, 1H, NH), 3.98 (t, J = 9.0 Hz, 2H), 3.79−3.60 (m, 5H), 3.47 (t, J = 9.5 Hz, 1H), 3.32 (s, 2H), 2.46 (s, 1H, $H2_{eq}$), 1.58−1.52 (m, 10H), 1.43 (s, 9H), 1.41 (s, 9H), 1.39 (s, 9H), 1.38 (s, 9H), 1.34−1.29 (m, 1H, $H2_{ax}$), 0.85 [s, 9H, $SiC(CH_3)_3$], 0.03 [s, 3H, $Si(CH_3)_2$], 0.02 [s, 3H, $Si(CH_3)_2$]; ^{13}C NMR (125 MHz, $CDCl_3$) δ 155.2 (3C), 155.0, 112.8 (t, J = 249.4 Hz, C4′), 97.6 (C1′), 80.4, 79.9, 79.5, 78.3, 70.4 (t, J = 18.3 Hz, C5′), 68.6, 67.8, 67.3, 56.2, 53.8, 50.8, 49.0, 38.3, 36.8, 36.3, 36.1, 29.7, 28.4, 28.3, 25.8, 25.6, 24.9, 23.8, 23.6, 18.1 [$SiC(CH_3)_3$], −5.0 [2C, $Si(CH_3)_2$]; ^{19}F NMR (376 MHz, $CDCl_3$) δ −115.94 (d, J = 247.6 Hz, F_{eq}), δ −136.78 (dt, J = 246.5, 20 Hz, F_{ax}); MS (FAB, NBA) 937 (MH^+); HRMS (FAB, NBA) calcd for $C_{44}H_{79}F_2N_4O_{13}Si$ (MH^+) 937.5302, found 937.5366.

*1,3,2′,6′-Tetra-N-tert-butoxycarbonyl-5,6-O-cyclohexylidene-4′-deoxy-4′,4′-difluoroneamine (**12**).* To a solution of **11** (165 mg, 0.176 mmol) in dry THF (3 mL) was added tetrabutylammonium fluoride (600 μL, 1.0 M solution in THF), and the mixture was kept at room temperature for 1 h. The ice-cold reaction mixture was quenched with water (~2 mL), extracted with CH_2Cl_2 (3 × 20 mL), dried (Na_2SO_4), and concentrated *in vacuo* to give the crude product as a yellow solid. The solid was purified on a column (SiO_2, 20:4.5:0.5 hexane/ethyl acetate/methanol) to furnish **12** (133 mg, 92%) as a pure white solid: mp 168−175 °C; R_f 0.33 (20:9.5:0.5 hexane/ethyl acetate/methanol); FTIR (KBr) 3353, 2978, 2936, 1705, 1511, 1367, 1253, 1165, 1047 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.52 (s, 1H, H1′), 5.39 (s, 1H, NH), 5.17 (s, 2H, NH), 4.68 (s, 1H, NH), 4.06−3.87 (m, 3H), 3.71−3.67 (m, 2H), 3.56−3.53 (m, 2H), 3.49 (t, J = 9.5 Hz, 1H), 3.38−3.35 (m, 2H), 2.42 (s, 1H, $H2_{eq}$), 1.64−1.51 (m, 11H), 1.45 (s, 9H), 1.43 (s, 18H), 1.41 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.2, 156.6, 155.5, 155.2, 118.0 (t, J = 250.0 Hz, C4′), 113.0, 97.3 (C1′), 80.4, 80.3, 80.2, 79.9, 78.2, 71.1 (t, J = 19.4 Hz, C3′), 68.5−67.8 (m, C5′), 54.3, 51.1, 49.0, 38.2, 36.3, 36.1, 28.5, 28.4, 28.3, 24.9, 23.8, 23.7; ^{19}F NMR (376 MHz, $CDCl_3$) δ −119.97 (d, J = 246.4 Hz, F_{eq}), δ −137.57 (dt, J = 245.5, 28.2 Hz, F_{ax}); MS (FAB, NBA) 823 (MH^+); HRMS (FAB, NBA) calcd for $C_{38}H_{65}F_2N_4O_{13}$ (MH^+) 923.4440, found 923.4494.

*4′-Deoxy-4′,4′-difluoroneamine Trifluoroacetic Acid Salt (**3**).* A solution of **12** (70 mg, 0.085 mmol) in CH_2Cl_2 (1.5 mL) was treated with trifluoroacetic acid (1.5 mL) and water (75 μL) at room temperature for 15 min. The mixture was concentrated to dryness *in vacuo*, and the residue was triturated by the addition of ethyl ether. The solid was filtered and washed with ether to give the final compound (14 mg, 94%) as a pure white solid: mp >190 °C (dec); R_f 0.22 (2:1:1:2 MeOH/$CHCl_3$/AcOH/H_2O); FTIR (KBr) 3412, 3383, 3097, 2937, 1679, 1635, 1539, 1437, 1203, 1135, 1052, 843, 799, 725 cm^{-1}; ^{1}H NMR (500 MHz, D_2O) δ 5.92 (s, 1H, H1′), 4.39−3.31 (m, 2H, H3′ and H5′), 3.84 (t, J = 9.5 Hz, 1H, H4), 3.55−3.52 (m, 2H, H2′ and H5), 3.42−3.37 (m, 2H, H6 and H3), 3.35 (d, J = 5.0 Hz, 2H, H6′), 3.16 (m, 1H, H1), 2.33 (dt, J = 12.5, 4.5 Hz, 1H, $H2_{eq}$), 1.72 (q, J = 12.5, 1H, $H2_{ax}$); ^{13}C NMR (125 MHz, D_2O) δ 162.9 (q, J = 35.0 Hz, CF_3COOH), 116.9 (t, J = 250.2 Hz, C4′), 116.3 (q, J = 290.0 Hz, CF_3COOH), 95.7 (C1′), 77.9 (C4), 75.1 (C5), 72.4 (C6), 66.4 (dd, J = 29.0, 23.0 Hz, C3′), 65.2 (t, J = 21.2 Hz, C5′), 52.2 (C2′), 49.6 (C1), 48.2 (C3), 36.0 (C6′), 28.2 (C2); ^{19}F NMR (376 MHz, D_2O) δ −114.32 (d, J = 247.2 Hz, F_{eq}), δ −134.17 (dt, J = 246.3, 21.6 Hz, F_{ax}); MS (FAB, NBA) 343 (MH^+); HRMS (FAB, NBA) calcd for $C_{12}H_{26}F_2N_4O_5$ (MH^+) 343.1793, found 343.1802.

*1,3,3″-Tris(N-benzyloxycarbonyl)-6′-N-tert-butoxycarbonyl-6′-N,4′-O-carbonyl-5,2′:4″,6″-di-O-cyclohexylidene-3′-O-(p-methoxybenzyloxymethyl)-2″-O-methoxymethylkanamycin A (**14**).* To an ice-cold solution of **13** (930 mg, 0.764 mmol), Bu_4NI (562 mg, 1.518 mmol), and Hünig's base (2.65 mL, 15.18 mmol) in dry CH_2Cl_2 (5.5 mL) was added dropwise freshly prepared *p*-methoxybenzyl chloromethyl ether (PMBMCl; 2.0 mL, ~10 mmol), and the mixture was stirred at ambient temperature for 15 h. The reaction mixture was quenched by the addition of a saturated solution of $NaHCO_3$ (5 mL) and stirring the mixture for 10 min. The mixture was diluted with water (10 mL), extracted with CH_2Cl_2 (3 × 30 mL), back-extracted with brine, dried (Na_2SO_4) and concentrated to dryness *in vacuo*. The residue was purified on a column (SiO_2, 1000:10:0.1 $CHCl_3$/MeOH/NH_4OH) to furnish **14** (908 mg, 87%) as a pure white solid: mp 175−177 °C; R_f 0.6 (19:1 $CHCl_3$/MeOH); FTIR (film) 3325, 2938, 1798, 1724, 1515, 1300, 1253, 1152, 1037 cm^{-1}; ^{1}H NMR (500 MHz, acetone-d_6) δ 7.45 (d, J = 8.5 Hz, 2H), 7.36−7.29 (m, 15H), 6.92 (d, J = 8.5 Hz, 2H), 6.75 (s, 1H, NH), 6.43 (s, 1H, NH), 6.28 (s, 1H, NH), 5.60 (s, 1H), 5.46 (s, 1H), 5.13−5.06 (m, 5H), 5.02−4.96 (m, 2H), 4.91 (d, J = 7.5 Hz, 2H), 4.60 (d, J = 7.0 Hz, 1H), 4.51 (d, J = 10.5

Hz, 1H), 4.60 (d, $J = 6.5$ Hz, 1H), 4.18 (s, 1H), 4.08−3.96 (m, 5H), 3.83−3.80 (m, 4H), 3.79 (s, 3H, PMBM), 3.74−3.69 (m, 3H), 3.59 (s, 1H), 3.48 (t, $J = 9.0$ Hz, 1H), 3.33 (t, $J = 9.0$ Hz, 1H), 3.21 (s, 3H, MOM), 2.16−2.06 (m, 3H), 1.91−1.31 (m, 28 H); ^{13}C NMR (125 MHz, acetone-d_6) δ 159.6, 156.3, 156.0, 152.0, 148.5, 138.0, 137.4, 130.6, 130.5, 128.6, 128.5, 128.2 (2C), 128.0, 127.9, 113.7, 103.0, 99.5, 98.6, 97.1,95.5, 95.0, 85.0, 83.0, 76.3, 75.4, 74.9, 74.0, 72.6, 71.0, 69.2, 66.2, 66.0, 65.5, 64.3, 62.0, 55.2, 54.9, 52.6, 52.4, 49.7, 47.8, 38.2, 35.3, 35.1, 31.5, 27.7, 27.5, 25.7, 25.0, 23.5, 22.8, 22.7, 22.6; ESIMS for $C_{71}H_{90}N_4O_{23}Na$ ($M + Na^+$) 1389, 1390 ($MH^+ + Na$).

*1,3,3″-Tris(N-benzyloxycarbonyl)-6′-N-tert-butoxycarbonyl-5,2′:4″,6″-di-O-cyclohexylidene-3′-O-(p-methoxybenzyloxymethyl)-2″-O-methoxymethylkanamycin A (**15**).* To a solution of **14** (770 mg, 0.563 mmol) in 20:3 dioxane/water was added a 0.5 N solution of LiOH (2.25 mL, 1.12 mmol), and the mixture was stirred at room temperature for 4 h. The reaction mixture was then quenched with a 30% aqueous solution of AcOH, concentrated to dryness *in vacuo*, dissolved in CH_2Cl_2, and extracted with water. The organic layer was back-extracted with brine, dried (Na_2SO_4), and concentrated *in vacuo* to furnish a crude solid, which was purified on a column (SiO_2, 1000:10:0.1 $CHCl_3$/MeOH/NH_4OH) to afford **15** (630 mg, 83%) as a white solid: mp 93−95 °C; R_f 0.51 (19:1 $CHCl_3$/MeOH); FTIR (KBr) 3350, 2938, 1718, 1515, 1251, 1157, 1095, 1042 cm^{-1}; ^{1}H NMR (500 MHz, acetone-d_6) δ 7.36−7.33 (m, 15H), 7.29 (d, $J = 7.0$ Hz, 2H), 6.91 (d, $J = 8.5$ Hz, 2H), 6.72 (s, 1H), 6.36 (s, 1H), 6.26 (d, $J = 6.5$ Hz, 1H), 5.78 (s, 1H), 5.48 (d, $J = 3.0$ Hz, 1H, H1′), 5.46 (s, 1H, H1″), 5.18−5.14 (m, 2H), 5.12 (s, 1H), 5.08−4.96 (m, 6H), 4.92 (t, $J = 2.3$ Hz, 1H), 4.81 (d, $J = 11.0$ Hz, 1H), 4.60 (d, $J = 7.0$ Hz, 2H), 4.52 (d, $J = 3.0$ Hz, 1H), 4.46 (d, $J = 6.0$ Hz, 1H), 4.18 (s, 1H), 4.08 (m, 1H), 3.95 (t, $J = 9.0$ Hz, 1H), 3.84−3.79 (m, 3H), 3.78 (s, 3H, PMBM), 3.77−3.62 (m, 6H), 3.50−3.47 (m, 1H), 3.42−3.34 (m, 2H), 3.20 (s, 3H, MOM), 2.11 (m, 3H), 1.87−1.48 (m, 18H), 1.41 (s, 9H, BOC), 1.31 (m, 1H); ^{13}C NMR (125 MHz, acetone-d_6) δ 159.5, 157.3, 156.3 (2C), 156.0, 138.0, 137.8, 130.7, 130.0, 128.6, 128.5, 128.2, 128.0, 127.9, 113.8, 102.7, 99.5, 98.9 (C1″), 98.4 (C1′), 97.1, 95.6, 84.7, 79.3, 78.8, 75.6, 75.2, 74.9, 72.6, 71.0, 70.1, 69.0, 66.2, 66.1, 65.8, 65.5, 64.3, 61.9, 60.5, 55.2, 54.9, 54.8, 52.6, 49.8, 41.4, 38.2, 35.2, 31.6, 28.0, 27.7, 25.7, 25.2, 23.4, 22.8, 22.7, 22.6; MS (FAB, NBA) 1363 ($M + Na^+$); HRMS (FAB, NBA) calcd for $C_{70}H_{92}N_4O_{22}Na$ ($M + Na^+$) 1363.6100, found 1363.6058.

*1,3,3″-Tris(N-benzyloxycarbonyl)-6′-N-tert-butoxycarbonyl-5,2′:4″,6″-di-O-cyclohexylidene-3′-O-(p-methoxybenzyloxymethyl)-2″-O-methoxymethyl-4′-oxokanamycin A (**16**).* A solution of **15** (610 mg, 0.455 mmol) in dry CH_2Cl_2 (3 mL) was added to a solution of Dess−Martin periodinane (481 mg, 1.138) in dry CH_2Cl_2 (7 mL), and the mixture was stirred at room temperature under an atmosphere of argon for 2 h. The reaction mixture was diluted with Et_2O (10 mL) and quenched with a mixture of $Na_2S_2O_3$−$NaHCO_3$ (3 mL of 2.0 M $Na_2S_2O_3$ and 5 mL of a saturated solution of $NaHCO_3$) by allowing the mixture to stir for 15 min. The mixture was extracted with $CHCl_3$, back-extracted with $NaHCO_3$ (saturated solution) and then brine, dried (Na_2SO_4), and concentrated to dryness *in vacuo*. The crude product was purified on a column (SiO_2, 15:3:1 hexane/ethyl acetate/methanol) to give **16** (538 mg, 88%) as a pure white solid: mp 117−119 °C; R_f 0.36 (10:5:1 hexane/ethyl acetate/methanol); FTIR (KBr) 3422, 3351, 2939, 2862, 1720, 1515, 1455, 1260, 1156, 1095, 1042 cm^{-1}; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.36−7.29 (m, 17H), 6.94 (d, $J = 8.8$ Hz, 2H, PMBM), 6.74 (s, 1H, NH), 6.48 (s, 1H, NH), 6.28 (d, $J = 8.0$ Hz, NH), 5.76 (s, 1H, NH), 5.48 (s, 1H, H1′), 5.45 (d, $J = 3.2$ Hz, 1H, H1″), 5.18−5.12 (m, 3H), 5.05−4.97 (m, 4H), 4.92 (dd, $J = 12.0$, 6.4 Hz, 2H), 4.85−4.76 (m, 1H), 4.70−4.65 (m, 1H), 4.60 (d, $J = 6.4$ Hz, 1H), 4.47−4.42 (m, 1H), 4.19 (s, 1H), 4.08−3.98 (m, 2H), 3.90−3.82 (m, 2H), 3.80 (s, 3H, PMBM), 3.76−3.58 (m, 7H), 3.43−3.37 (m, 2H), 3.25 (s, 1H), 3.21 (s, 3H, MOM), 2.15−2.06 (m, 3H), 1.92−1.40 (m, 19H), 1.38 (s, 9H, BOC); ^{13}C NMR (100 MHz, acetone-d_6) δ 201.5, 159.6, 156.4, 156.3, 156.0, 155.7, 138.0, 137.8, 137.4, 130.4, 130.0, 129.9, 129.6, 128.6, 128.5, 128.2, 128.1, 127.9, 113.9, 113.5, 103.0, 99.5, 97.9 (C1″), 97.1 (C1′), 95.5, 94.2, 85.2, 78.3, 75.4, 75.0, 74.7, 71.3, 71.0, 68.9, 66.2, 65.9, 65.5, 64.3, 61.9, 55.2, 54.9, 52.6, 52.4, 49.8, 39.4, 38.2, 35.5, 35.1, 31.6, 28.0, 27.7, 25.7, 25.1, 23.5, 22.8, 22.7, 22.6; MS (FAB, NBA) 1339 (MH^+), 1361 ($M + Na^+$); HRMS (FAB, NBA) calcd for $C_{70}H_{90}N_4O_{22}Na$ ($M + Na^+$) 1361.5940, found 1361.5903.

*1,3,3″-Tris(N-benzyloxycarbonyl)-6′-N-tert-butoxycarbonyl-5,2′:4″,6″-di-O-cyclohexylidene-4′,4′-difluoro-3′-O-(p-methoxybenzyloxymethyl)-2″-O-methoxymethylkanamycin A (**17**).* To an ice-cold solution of **16** (300 mg, 0.224 mmol) in dry CH_2Cl_2 (8 mL) was added morpholinosulfur trifluoride (330 mL, 2.704 mmol), and the mixture was stirred at room temperature under an atmosphere of argon for 15 h. The reaction mixture was cooled in an ice−water bath and quenched with a saturated solution of $NaHCO_3$ (5 mL) by allowing the mixture to stir for 10 min. The mixture was diluted with water (5 mL) and extracted with CH_2Cl_2. The organic layer was back-extracted with brine, dried (Na_2SO_4), and concentrated to dryness *in vacuo*. The crude product was purified on a column (SiO_2, 100:1 $CHCl_3$/MeOH) to afford **17** (197 mg, 65%) as a pure white solid: mp 118−120 °C; R_f of this compound was similar to that of **16** (R_f 0.56, 19:1 $CHCl_3$/MeOH); FTIR (KBr) 3414, 3347, 2939, 2862, 1720, 1515, 1456, 1250, 1157, 1096, 1039 cm^{-1}; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.37−7.29 (m, 17H), 6.93 (d, $J = 9.2$ Hz, 2H), 6.74 (s, 1H), 6.45 (s, 1H), 6.27 (d, $J = 8.0$ Hz, 1H), 5.61 (s, 1H), 5.46 (s, 1H), 5.18−4.91 (m, 8H), 4.75 (d, $J = 11.2$ Hz, 1H), 4.59 (d, $J = 7.2$ Hz, 2H), 4.46 (d, $J = 6.4$ Hz, 1H), 4.17−3.80 (m, 9H), 3.79 (s, 3H, PMBM), 3.75−3.48 (m, 6H), 3.20 (s, 3H, MOM), 2.20−2.08 (m, 3H), 1.89−1.38 (m, 28H); ^{13}C NMR (100 MHz, acetone-d_6) δ 159.6, 156.3 (2C), 156 (2C), 138.0, 137.4, 130.2, 130.0, 129.9, 129.8, 129.6, 128.6, 128.5, 128.2, 128.0, 127.9, 113.9, 113.8, 103.0, 99.5, 97.6, 97.1, 95.6, 95.1, 84.7, 75.4, 74.9, 73.7 (m, C4′), 71.6 (d, $J = 8.2$ Hz), 71.0, 69.1, 66.2, 65.8, 65.5, 64.3, 61.9, 55.2, 54.9, 52.6, 52.3, 49.7, 38.2, 35.3, 35.0, 31.5, 28.0, 27.7, 25.7, 25.0, 23.5, 22.8, 22.6; ^{19}F NMR (376 MHz, acetone-d_6) δ −109.88 (d, $J_{Feq-Fax} = 247.0$ Hz, F_{eq}), −129.06 (dt, $J_{Feq-Fa} = 250.0$ Hz, $J_{Fax-Hax} = 22.0$ Hz, F_{ax}); MS (FAB, NBA) 1361 (MH^+), 1383 ($M + Na^+$); HRMS (FAB, NBA) calcd for $C_{70}H_{90}F_2N_4O_{21}Na$ ($M + Na^+$) 1383.5960, found 1383.5928.

*1,3,3″-Tris(N-benzyloxycarbonyl)-6′-N-tert-butoxycarbonyl-5,2′:4″,6″-di-O-4,4′-difluoro-2″-O-methoxymethylkanamycin A (**18**).* To a solution of **17** (190 mg, 0.140 mmol) in dichloromethane (15 mL) was added 0.77 mL of a solution

of phosphate buffer (prepared by dissolving 50 mg of K_3PO_4 and 43 mg of K_2HPO_4 in 1 mL of distilled water). To this solution was added DDQ (159 mg, 0.700 mmol), the mixture was stirred for 10 min, and then it was kept at room temperature for 8 h. The reaction mixture was quenched with a saturated solution of $NaHCO_3$, diluted with water, and extracted with CH_2Cl_2. The organic layer was extracted with water, back-extracted with brine, dried (Na_2SO_4), and concentrated to dryness *in vacuo*. The resultant residue was purified on a column (SiO_2, 100:1:0.1 $CHCl_3$/MeOH/NH_4OH) to afford the title compound **18** (78 mg, 50%) and some recovered starting compound (25 mg, 13%): mp 144−146 °C; R_f 0.5 (19:1 $CHCl_3$/MeOH); FTIR (KBr) 3433, 3347, 2939, 1718, 1518, 1455, 1253, 1158, 1095, 1066 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ 7.36−7.29 (m, 15H), 6.74 (s, 1H), 6.45 (s, 1H), 6.28 (d, J = 7.2 Hz, 1H), 5.58 (s, 1H), 5.44 (s, 1H), 5.41−5.32 (m, 1H), 5.22−4.98 (m, 7H), 4.60 (d, J = 7.2 Hz, 1H), 4.47 (d, J = 6.4 Hz, 1H), 4.17 (d, J = 4.8 Hz, 2H), 4.04 (t, J = 9.2 Hz, 2H), 3.95 (t, J = 10.0 Hz, 2H), 3.83−3.67 (m, 10H), 3.47 (m, 2H), 3.32−3.28 (m, 1H), 3.21 (s, 3H, MOM), 2.20−2.06 (m, 3H), 1.90−1.40 (m, 18H), 1.38 (s, 9H), 1.35−1.29 (m, 1H); ^{13}C NMR (100 MHz, acetone-d_6) δ 156.3 (2C), 155.8, 138.0, 137.4, 128.6, 128.5, 128.2, 128.0, 127.9, 118.6 (t, J = 246.0 Hz, C4′), 102.8, 99.5, 97.9, 97.2, 95.6, 84.7, 78.4, 75.5, 75.2, 74.8, 71.4, 71.0, 69.7 (t, J = 19.3 Hz, C3′ or C5′), 68.3 (d, J = 20.0 Hz, C3′ or C5′), 66.2, 65.8, 65.5, 64.3, 61.9, 55.2, 52.6, 52.3, 49.8, 38.2, 35.4, 35.1, 28.0, 27.7, 25.7, 25.2, 23.2, 22.8, 22.6; ^{19}F NMR (376 MHz, acetone-d_6) δ −112.15 (d, $J_{Feq-Fax}$ = 247.0 Hz, F_{eq}), −131.93 (d, $J_{Fax-Feq}$ = 245.0 Hz, F_{ax}); MS (FAB, NBA) 1211 (MH^+), 1233 ($M + Na^+$); HRMS (FAB, NBA) calcd for $C_{61}H_{81}F_2N_4O_{19}$ (MH^+) 1211.5460, found 1211.5449.

*1,3,3″-Tris(N-benzyloxycarbonyl)-4′,4′-difluorokanamycin A (**19**).* To a solution of **18** (70 mg, 0.058 mmol) in dry CH_2Cl_2 (1 mL) was added trifluoroacetic acid (1 mL), and the mixture was kept at room temperature for 4 h. The reaction mixture was then concentrated to dryness *in vacuo*, and the residue was washed with a solution of 1:1 petroleum ether/ether (2 mL) to furnish a white solid as a crude product. The solid was purified on a column (SiO_2, 17:3:0.5 $CHCl_3$/MeOH/NH_4OH) to give the product **19** (34 mg, 65%) as a white solid: mp >250 °C; R_f 0.23 (15:4:1 $CHCl_3$/MeOH/NH_4OH); FTIR (KBr) 3546, 3372, 3321, 3091, 3033, 2944, 1689, 1541, 1455, 1267, 1145, 1048 cm^{-1}; ^1H NMR (400 MHz, pyridine-d_5) δ 8.56 (d, J = 8.5 Hz, 1H, NH), 8.52 (s, 1H, NH), 8.46 (s, 1H, NH), 7.52−7.26 (m, 15H), 6.03 (s, 1H, H1′), 5.75 (s, 1H, H1"), 5.42 (d, J = 13.0 Hz, 2H), 5.32 (d, J = 10.5 Hz, 1H), 5.26 (d, J = 12.0 Hz, 1H), 5.17 (s, 2H), 4.96 (s, 1H), 4.82−4.71 (m, 3H), 4.51 (d, J = 10.0 Hz, 1H), 4.44−4.40 (m, 3H), 4.31−4.16 (m, 4H), 4.04 (s, 1H), 3.38−3.32 (m, 2H), 2.73 (d, J = 9.0 Hz, 1H), 2.09 (s, 1H), 1.27 (s, 1H); ^{13}C NMR (100 MHz, pyridine-d_5) δ 158.6, 157.8, 157.2, 138.4, 138.3, 138.1, 129.2, 129.1, 129.0, 128.7, 128.5, 128.4, 128.3, 120.8 (t, J = 250.0 Hz, C4′), 101.3 (C1″), 100.7 (C1′), 84.7, 83.8, 76.2, 75.3, 72.9, 72.2, 72.0, 71.8, 71.6, 70.0, 66.8, 66.7, 66.5, 62.6, 59.1, 52.1, 51.1, 39.6, 35.8, 30.3; ^{19}F NMR (376 MHz, pyridine-d_5) δ −112.44 (d, $J_{Feq-Fax}$ = 245.5 Hz, F_{eq}), −131.71 (dt, $J_{Fax-Feq}$ = 243.6 Hz, $J_{Fax-H3'and5'}$ = 22.0 Hz, F_{ax}); MS (FAB, NBA) 907 (MH^+), 929 ($M + Na^+$); HRMS (FAB, NBA) calcd for $C_{42}H_{53}F_2N_4O_{16}$ (MH^+) 907.3425, found 907.3430.

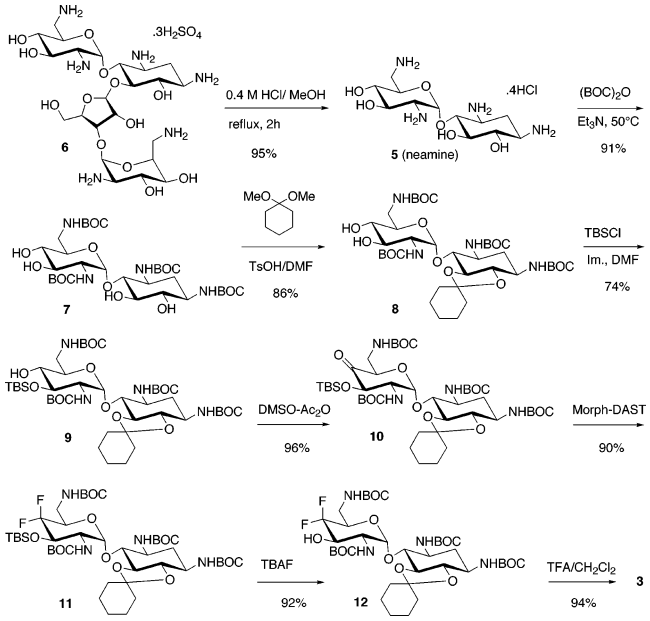
*4′-Deoxy-4′,4′-difluorokanamycin A (**4**).* To a solution of **19** (45 mg, 0.050 mmol) in dioxane/water (1:1, 5 mL) was added palladium on activated carbon (80 mg, 10% Degussa type 101 NE/N, water ∼50%), and the mixture was stirred at room temperature under an atmosphere of hydrogen (hydrogen balloon) overnight. The mixture was passed through a layer of Celite and washed with water, and the filtrate was concentrated to dryness *in vacuo*. The resultant solid was washed with ether, dissolved in D_2O (1 mL), and lyophilized to afford the title compound **4** (24 mg, 96%) as a pure white solid: R_f 0.51 (3:1:1:1 MeOH/NH_4OH/H_2O/$CHCl_3$; cf. kanamycin A, R_f 0.34); FTIR (KBr) 3357, 3300, 2926, 1597, 1351, 1240, 1149, 1050 cm^{-1}; ^1H NMR (400 MHz, D_2O) δ 5.44 (s, 1H, H1′), 4.98 (d, J = 3.2 Hz, 1H, H1″), 4.16 (ddd, J = 24.4, 8.4, 3.2 Hz, 1H, H5′), 4.10−4.01 (m, 1H, H3′), 3.85 (dt, J = 9.6, 3.2 Hz, 1H, H5″), 3.76 (dd, J = 10.4, 3.2 Hz, 1H, H2′), 3.70 (d, J = 2.4 Hz, 2H, H6″), 3.64 (t, J = 8.8 Hz, 1H, H5), 3.46 (dd, J = 10.4, 4.0 Hz, 1H, H2″), 3.34 (t, J = 9.2 Hz, 1H, H4), 3.29 (t, J = 10.0 Hz, 1H, H4″), 3.21 (t, J = 9.2 Hz, 1H, H6), 3.04 (dd, J = 14.0, 3.2 Hz, 1H, H6′), 2.96 (t, J = 10.0 Hz, 1H, H3″), 2.95−2.84 (m, 3H, H1, H3, and H6′), 1.93 (dt, J = 8.8, 4.0 Hz, 1H, $H2_{eq}$), 1.19 (dd, J = 24.4, 12.4 Hz, 1H, $H2_{ax}$); ^{13}C NMR (100 MHz, D_2O) δ 118.5 (t, J = 249.0 Hz, C4′), 100.3 (C1″), 98.4 (C1′), 87.8 (C6), 86.6 (C4), 74.1 (C5), 72.3 (C2″), 71.8 (C5″), 70.5 (d, J = 7.5 Hz, C2′), 70.0 (t, J = 25.2 Hz, C5′), 69.8 (t, J = 19.2 Hz, C3′), 69.2 (C4″), 60.4 (C6″), 54.4 (C3″), 50.7 (C3), 48.8 (C1), 37.3 (d, J = 4.4 Hz, C6′), 35.2 (C2); ^{19}F NMR (376 MHz, D_2O) δ −112.29 (dd, $J_{Feq-Fax}$ = 247.0 Hz, $J_{Feq-H3'orH5'}$ = 4.5 Hz, F_{eq}), −130.37 (dt, $J_{Fax-Feq}$ = 245.5 Hz, $J_{Fax-Hax}$ = 23.0 Hz, F_{ax}); MS (FAB, NBA) 505 (MH^+); HRMS (FAB, NBA) calcd for $C_{18}H_{35}F_2N_4O_{10}$ (MH^+) 505.2321, found 505.2318.

RESULTS AND DISCUSSION

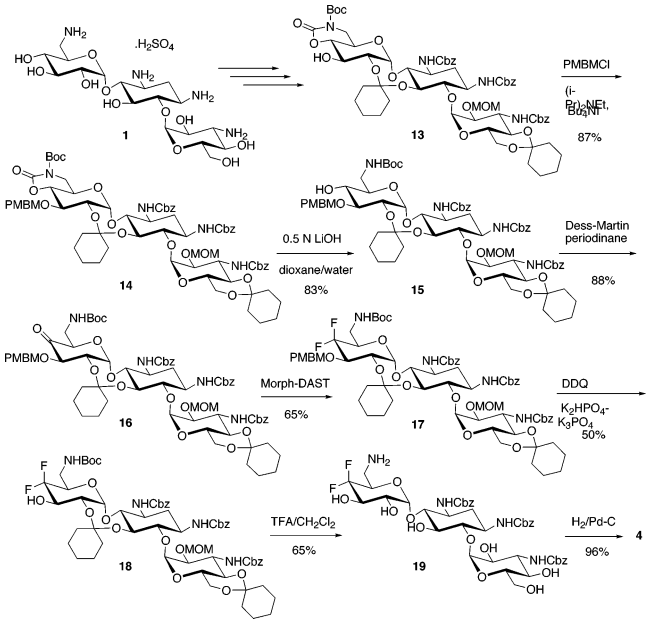
As stated earlier, APH(3′)s transfer the γ-phosphoryl group of ATP to the 3′-hydroxyl of aminoglycosides. It is important to note that the substituents at the 4′ position (the site of fluorination of **3** and **4**) are not in contact with the protein according to the X-ray structure for the ternary complex of APH(3′)-IIIa, ADP, and kanamycin A (*16*). Furthermore, *ab initio* molecular orbital geometry optimizations were carried out on the glucosamine ring of kanamycin A and its fluorinated analogue to assess any potential structural perturbation as a result of difluorination at the 4′ position. The geometry optimizations were carried out with the Gaussian 98 suite at the Hartree−Fock level of theory using the 6-31+G** basis set. The structure of the ring was extracted from the X-ray coordinates of kanamycin A bound to APH(3′)-IIIa (*16*). The root-mean-square deviation fit of the structure of the optimized ring and its difluorinated analogue revealed that difluorination at 4′ introduced no change in the conformation of the ring (rms deviation was merely 0.01 Å).

However, the presence of the fluorine atoms at position 4′ of aminoglycosides **3** and **4** would be expected to reduce the nucleophilicity of the 3′-hydroxyl group (the site of enzymic phosphorylation) substantially. For example, pK_a values of ethanol, α-fluoroethanol, α,α-difluoroethanol, and α,α,α-trifluoroethanol are 16.0, 14.2, 13.3, and 12.4, respectively (*17*, *18*). This reduction of nucleophilicity will be detrimental to the transfer of phosphate when significant

Scheme 2: Synthetic Route for Compound **3**



Scheme 3: Synthetic Route for Compound **4**



nucleophilic participation exists in the transition-state species. However, it should influence the reaction to a lesser degree or none in a dissociative reaction, as the potential intermediary metaphosphate-like species would be an extremely electrophilic species that would be trapped by the 3′-hydroxyl of the antibiotic. We chose neamine (**5**), kanamycin A (**1**), and their respective fluorinated derivatives (compounds **3** and **4**) for this study, because the former two, nonfluorinated molecules, serve as excellent substrates for APH(3′)s.

Syntheses. The syntheses of fluorinated aminoglycosides turned out substantially more complicated and labor-intensive than anticipated. A number of failed synthetic strategies were pursued initially before success was achieved in preparation of the two fluorinated aminoglycosides. 4′,4′-Difluoroneamine (**3**) was prepared from neomycin sulfate (**6**) in 8 steps with an overall yield of 41% (Scheme 2). Starting from kanamycin A (**1**), 4′,4′-difluorokanamycin A (**4**) was obtained in 13 steps with an overall yield of 4% (Scheme 3).

It should be noted that several attempts at protection of the hydroxyl group at position 3′ in intermediate **8** with benzoyl, acetyl, or triethylsilyl chloride resulted in formation of inseparable mixtures of 3′- and 4′-protected compounds. Also, tributyltin oxide-assisted acylation of the 3′ position did not give any satisfactory result. However, treatment with TBSCl yielded a selective protection of the 3′ position along with the formation of a trace of the 4′-protected compound (3−5%), which was chromatographically separable. Another difficulty in the synthesis was fluorination of the oxo derivative **10** in that the application of methyl- or ethyl-DAST resulted in deprotection of the cyclohexylidene group along with the formation of byproducts and low yield of fluorination. On the other hand, morpholino-DAST gave a good yield of the fluorinated product with no complication in deprotection of the acid labile groups.

We encountered several difficulties in the synthesis of difluorokanamycin A. First, protection of the hydroxyl group at position 5, which is extremely hindered, with no precedent report for its protection in the literature, presented serious difficulty. Several attempts at acetyl or benzoyl protection at position 5 of derivatives with Cbz or Boc for amine protection failed to give results. However, by close examination of the crystal structure of kanamycin A, it occurred to us that the hydroxyl groups at positions 2′ and 5 are in close proximity (3−5 Å) of each other. Therefore, it was envisioned that it could be possible to employ hydroxyl (2′) assisted acetal protection of the hydroxyl group at position 5 by forming an eight-membered cyclic acetal. Employment of both dimethoxypropane and dimethoxycyclohexane allowed us to access the corresponding acetals at the aforementioned positions, which was the first reported protection of its type in the chemistries of these aminoglycosides. We opted to use dimethoxycyclohexane for its higher boiling point, which was required under the reduced pressure conditions employed in this transformation.

Another difficulty was selecting an appropriate protecting group for position 3′ such that it could be removed under mild acidic conditions with no harm to the acid-labile cyclohexylidene protecting groups. To that end, *p*-methoxybenzyl chloromethyl ether (PMBMCl) was the reagent of choice, which could survive under basic conditions and could be successfully removed under phosphate buffer-assisted DDQ deprotection with minimal removal of the cyclohexylidene group at the 2′−5 positions. Oxidation of the hydroxyl group at position 4′ was another difficulty, which was ultimately resolved by employing Dess−Martin periodinane in place of other reagents (i.e., oxalyl chloride, ruthenium oxide, or DMSO/Ac_2O), which all failed to give any satisfactory result.

Neamine hydrochloride (**5**) was prepared from methanolysis of commercially available neomycin sulfate (**6**), as reported earlier (*19*−*21*). Treatment of **5** with di-*tert*-butyl dicarbonate in the presence of triethylamine afforded the *N*-Boc-protected compound **7** (Scheme 2). Selective protection of the hydroxyl groups at positions 5 and 6 with 1,1-dimethoxycyclohexane gave derivative **8** in good yield (*22*) (during the course of the reaction some dicyclohexylidene-protected compound was formed, which was fully converted to **8** in the presence of 0.001 mol % TsOH in 10% MeOH/

Table 1: Parameters of Pre-Steady-State Kinetics for APH(3′)-Ia and APH(3′)-IIa

$$\text{APH·ATP·aminoglycosides} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{APH·ADP· phosphoaminoglycosides} \xrightarrow{k_4} \text{APH} + \text{ADP} + \text{phosphoaminoglycosides}$$

	1					**4**	
	k_3 (s^{-1})	k_{-3} (s^{-1})	k_4 (s^{-1})	k_{cat} (s^{-1})	k_3(wt)/k_3(mut)	k_{cat} (s^{-1})	k_3(**1**)/k_{cat}(**4**)
APH(3′)-Ia	28.4 ± 3.6	33.9 ± 1.1	4.0 ± 0.3	1.7 ± 0.1		$(6.7 \pm 0.4) \times 10^{-3}$	$(4.0 \pm 0.5) \times 10^3$
D198A APH(3′)-Ia	$(1.9 \pm 0.2) \times 10^{-2}$	$(1.8 \pm 1.7) \times 10^{-2}$	$(4.0 \pm 1.1) \times 10^{-2}$	$(1.0 \pm 0.1) \times 10^{-2}$	$(1.5 \pm 0.2) \times 10^3$	ND[a]	ND
APH(3′)-IIa	41.4 ± 1.6	30.7 ± 1.4	7.8 ± 0.2	4.0 ± 0.1		$(3.2 \pm 0.3) \times 10^{-4}$	$(1.3 \pm 0.1) \times 10^5$

	5					**3**	
	k_3 (s^{-1})	k_{-3} (s^{-1})	k_4 (s^{-1})	k_{cat} (s^{-1})	k_3(wt)/k_{cat}(mut)	k_{cat} (s^{-1})	k_3(**5**)/k_{cat}(**3**)
APH(3′)-Ia	77.9 ± 3.1	62.4 ± 4.4	11.4 ± 0.8	5.9 ± 0.1		$(4.0 \pm 0.3) \times 10^{-2}$	$(2.0 \pm 0.2) \times 10^3$
D198A APH(3′)-Ia	NB[b]	NB	NB	$(3.6 \pm 0.6) \times 10^{-3}$	$(2.2 \pm 0.3) \times 10^4$	ND	ND
APH(3′)-IIa	73.2 ± 4.0	62.6 ± 1.7	24.4 ± 2.3	11.1 ± 0.7		$(6.3 \pm 0.6) \times 10^{-3}$	$(1.2 \pm 0.1) \times 10^4$

[a] ND: not determined. [b] NB: no burst phase.

DMF overnight). Selective protection of the hydroxyl group at position 3′ was achieved by treatment of compound **8** with *tert*-butyldimethylsilyl chloride to provide **9** in good yield. Oxidation of this compound with DMSO/Ac_2O (Moffatt oxidation) gave the oxo derivative **10** in high yield. This compound was efficiently functionalized to the difluoro derivative **11** by treatment with morpholinosulfur trifluoride (Morph-DAST) in dichloromethane. Finally, deprotection of the TBS group with tetrabutylammonium fluoride afforded **12**, which after treatment with trifluoroacetic acid gave the title compound **3** in good yield (Scheme 2).

Intermediate **13** (Scheme 3) was prepared from kanamycin A as described in a previous report (*23*). Treatment of this compound with freshly prepared *p*-methoxybenzyl chloromethyl ether (PMBMCl) in the presence of Hünig's base and tetrabutylammonium iodide gave compound **14** in high yield. Oxazolidinone ring opening with 0.5 N lithium hydroxide afforded intermediate **15**, which was successfully oxidized by Dess−Martin periodinane to generate the oxo derivative **16** in high yield (Scheme 3). Fluorination of this compound was achieved by treatment of **16** with morpholinosulfur trifluoride in dichloromethane to furnish the difluoro derivative **17**. Deprotection of the PMBM group by DDQ in the presence of phosphate buffer gave **18** in acceptable yield. Removal of the acid-labile groups with TFA generated **19**, which after hydrogenolysis afforded the target compound **4** in excellent yield (Scheme 3).

Kinetic Analyses. To gain a detailed knowledge of the microscopic steps in catalysis by APHs, we attempted quench-flow pre-steady-state kinetics. Pre-steady-state kinetic parameters for phosphorylation of neamine (**5**), kanamycin A (**1**), and their respective fluorinated derivatives **3** and **4** by aminoglycoside 3′-phosphotransferases [APH(3′)-Ia and APH(3′)-IIa] are summarized in Table 1. Let us consider the case of the nonfluorinated compounds first. These compounds exhibited burst kinetics with the two enzymes. The microscopic rate constant for the transfer of the ATP γ-phosphoryl (k_3) was invariably more rapid than k_{cat}, and the release of products (k_4), which may involve a conformation change, was rate-limiting in each case with both enzymes. Whereas invariably the magnitude of the rate constant k_4 was consistently lower than k_3 (Table 1), the effect was a mere 3−7-fold, which accounts for approximately 1 kcal/mol or less of contribution to the energetic profile of the turnover chemistry.

Table 2: Viscosity Effects[a] during Turnover Events of APH(3′)-Ia and APH(3′)-IIa

viscogen	varied substrate	fixed substrate (concn, μM)	$(k_{cat}^0/k_{cat})^\eta$	$[(k_{cat}/K_m)^0/(k_{cat}/K_m)]^\eta$
APH(3′)-Ia				
sucrose	ATP	**1** (20)	0.49 ± 0.11	1.26 ± 0.44
sucrose	ATP	**5** (20)	0.39 ± 0.08	2.47 ± 0.31
sucrose	**1**	ATP (150)	0.70 ± 0.13	0.16 ± 0.08
sucrose	**5**	ATP (150)	0.83 ± 0.04	0.31 ± 0.04
PEG 8000	ATP	**1** (20)	0.08 ± 0.01	-0.35 ± 0.03
PEG 8000	**1**	ATP (150)	0.02 ± 0.05	-0.12 ± 0.07
APH(3′)-IIa				
sucrose	ATP	**1** (20)	1.39 ± 0.08	2.68 ± 0.79
sucrose	ATP	**5** (20)	2.64 ± 0.56	2.92 ± 0.94
sucrose	**1**	ATP (150)	0.59 ± 0.12	0.02 ± 0.16
sucrose	**5**	ATP (150)	0.42 ± 0.04	0.00 ± 0.06
PEG 8000	ATP	**1** (20)	-0.32 ± 0.04	-0.13 ± 0.02
PEG 8000	**1**	ATP (150)	-0.01 ± 0.03	-0.17 ± 0.08

[a] k_{cat}^0 and $(k_{cat}/K_m)^0$ are the rate constants without viscogen. The values of $(k_{cat}^0/k_{cat})^\eta$ and $[(k_{cat}/K_m)^0/(k_{cat}/K_m)]^\eta$ are the slopes of plots with either k_{cat}^0/k_{cat} or $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ vs relative viscosity of the solution.

If the diffusional events for the travel of the substrates into the active site or departure of products away from it were to be rate-limiting, this process can be studied by viscosity studies. Such studies revealed that product release was more rate-limiting for APH(3′)-Ia [$(k_{cat}^0/k_{cat})^\eta = 0.70 \pm 0.13$ for **1** and 0.83 ± 0.03 for **5**], while APH(3′)-IIa was limited to a smaller degree by product release [$(k_{cat}^0/k_{cat})^\eta = 0.59 \pm 0.12$ for **1** and 0.42 ± 0.04 for **5**] when sucrose was used as the viscogen (Table 2). When polymeric PEG 8000 was used as a macroviscogen, which would not affect the diffusion of small molecules, the effects on k_{cat} and k_{cat}/K_m were negligible. Hence, consistent with the measurements of microscopic rate constants, departure of products from the active site is limiting under these conditions.

The X-ray structure of the ternary complex of APH(3′)-IIIa, ADP, and kanamycin A (*16*) indicates that the side chain carboxylate of Asp190, corresponding to Asp198 of APH(3′)-Ia by sequence alignment and generation of a computational homology model, is in hydrogen-bonding contact with the 3′-hydroxyl and, hence, is the likely residue that

Table 3: Thio Effects during Turnover Processes of APH(3′)-Ia and APH(3′)-IIa

	APH(3′)-Ia			APH(3′)-IIa		
	K_m (μM)	k_{cat} (s^{-1})	$k_{cat}^{ATP}/k_{cat}^{ATP\gamma S}$	K_m (μM)	k_{cat} (s^{-1})	$k_{cat}^{ATP}/k_{cat}^{ATP\gamma S}$
compound **1**						
ATP	33.42 ± 2.33	1.71 ± 0.13		48.88 ± 10.45	4.15 ± 0.45	
ATPγS	99.09 ± 2.34	0.02 ± 0.001	100 ± 14	61.55 ± 11.13	0.02 ± 0.001	260 ± 43
compound **5**						
ATP	34.66 ± 4.32	4.46 ± 0.25		40.35 ± 1.63	6.16 ± 0.15	
ATPγS	20.40 ± 4.88	0.04 ± 0.002	124 ± 14	13.75 ± 2.03	0.05 ± 0.001	116 ± 5

would activate the hydroxyl for the nucleophilic displacement of the γ-phosphoryl group of ATP. This residue was mutated to alanine in APH(3′)-Ia. This mutant enzyme was severely impaired in its ability to phosphorylate both kanamycin A and neamine, yet showed burst kinetics, indicating that product dissociation at least in the case of kanamycin A was rate-limiting [k_3(wt)/k_3(mut) of (1.5 ± 0.2) × 10^3 and (2.2 ± 0.3) × 10^4, respectively].

The fluorinated compounds did not show burst kinetics, indicative of product dissociation being more rapid than the phosphoryl transfer step, so the microscopic rate constants could not be evaluated. However, turnover of **3** and **4**, as judged by the k_{cat} values for both enzymes, was substantially impaired. A comparison of the ratios of k_3(wt)/k_3(mut) to the ratios of the rate constants for the nonfluorinated to fluorinated compounds [k_3(**1**)/k_{cat}(**4**) of (4.0 ± 0.5) × 10^3 and k_3(**5**)/k_{cat}(**3**) of (2.0 ± 0.2) × 10^3 for APH(3′)-Ia] revealed them to be very close to one another. *In essence, the magnitude of the degree of activation of the 3′-hydroxyl in nonfluorinated ("normal") substrates by Asp198 carboxylate is the same as that of deactivation of the same hydroxyl by the difluoro moieties of compounds **3** and **4** in evaluations with the wild-type enzyme(s).*

The fluorination of aminoglycosides clearly impaired the molecules as acceptors of phosphate. Alternatively, one may impair the phosphate donor in these studies. One approach has been the use of nucleotides with substitutions of sulfur for oxygen at the point of chemical reaction. If the chemical step contributes to the rate-limiting step, the use of ATPγS in turnover of aminoglycosides should exhibit a contribution, which is referred to as the thio effect (*24*, *25*). Both APH(3′)-Ia and APH(3′)-IIa showed thio effects of 100−260-fold (Table 3) for both kanamycin A and neamine, indicating that the phosphate transfer step was contributing to the overall rate significantly when ATPγS was used. This is consistent with the studies with the fluorinated aminoglycosides in that the rate constant was so affected that the chemical step contributed more significantly to the overall turnover chemistry than did the diffusional step for the departure of products out of the active site.

Antibiotic Susceptibility Studies. Aminoglycosides are inhibitors of bacterial ribosomal translation machinery, since they bind to the acyl transfer site of the ribosome. In an *in vitro* translation assay, the IC_{50} values for **1**, **4**, **5**, and **3** were determined at 0.04, 0.20, 1.0, and 10.0 μM, respectively. Fluorination of these antibiotics works to the detriment of binding to the ribosomal site by as much as 5−10-fold, but the IC_{50} for **4** still remained submicromolar.

It is also important to note that the D198A mutant enzyme would appear not to be a dead enzyme *in vivo*, consistent with similar findings with APH(3′)-IIIa (*5*, *6*). We transformed *E. coli* BL21(DE3) by plasmid pETAPH that expressed either the wild-type APH(3′)-Ia or its mutant variant. We evaluated the minimum inhibitory concentrations (MICs) for kanamycin A with these two strains and for the parental strain with plamid pET22b(+) [that does not have the *aph(3′)-Ia* gene; as a background control]. The MIC values for kanamycin A for the background strain and the strains with wild-type APH(3′)-Ia and with the D198A mutant enzyme were 8, 1000, and 16 μg/mL. The mutant enzyme imparts resistance to kanamycin A by as much as 2-fold. Whereas a 2-fold effect on MIC is generally considered negligible, it was real and reproducible in this case; hence there exists some marginal effect by the mutant enzyme *in vivo*. Since the impairment of activity of the phosphotransferase on mutation at position 198 is severe, it is also conceivable that the *inactive* protein is simply binding to the aminoglycoside without turnover in imparting the 2-fold increase in the MIC data. Such a method for resistance has been documented for another aminoglycoside-modifying enzyme previously (*26*).

Table 4: MICs of Aminoglycosides for APHs

	MIC (μg/mL)			
	1	**4**	**5**	**3**
E. coli JM83	2	64	32	1000
E. coli JM83 [APH(3′)-Ia]	2000	512	>4000	>4000
E. coli JM83 [APH(3′)-IIa]	1000	64	>4000	1000

Table 4 summarizes the antibacterial properties of the two aminoglycosides and their fluorinated derivatives. The background laboratory strain, *E. coli* JM83, is susceptible to kanamycin A (MIC of 2 μg/mL) and less so to neamine (MIC 32 μg/mL). The fluorinated aminoglycosides are apparently not good antibiotics, with MIC values of 64−1000 μg/mL. However, it is most interesting that while the strain transformed by the plamids that express the two resistance enzymes shows substantially elevated MIC values with nonfluorinated antibiotics (1000 to >4000 μg/mL), the MIC values do not change for the fluorinated variants whether the organism expresses APH(3′)-IIa or not. One sees elevations of MIC values for the strain that expresses APH(3′)-Ia by a fewfold. Both of these findings are consistent with results with purified enzyme that show attenuated activity in turnover of fluorinated compounds and with the k_3(**1**)/k_{cat}(**4**) and k_3(**5**)/k_{cat}(**3**) ratios.

Concluding Remarks. The data presented here indicate that the Gram-negative APH(3′)s are somewhat limited by the diffusional step of the departure of the products of the reaction, at least with kanamycin A and neamine as aminoglycoside substrates. However, the mere 3−7-fold higher values for k_3 over k_4 are not sufficient to make these enzymes unequivocally diffusion-limited. Furthermore, when the chemical nature of the substrates, be it the nucleotide or the

aminoglycoside, is changed such that the chemical step is impaired, the chemistry becomes rate-limiting. This was achieved by judicious retailoring of the aminoglycoside structures by the preparation of the fluorinated analogues or by study of ATPγS.

An important finding of the study of the fluorinated aminoglycosides was that they were severely impaired as substrates for these enzymes, consistent with reduced nucleophilicity of the 3′-hydroxyl by the presence of the fluoride atoms adjacent to the site of phosphorylation. Two such molecules, compounds **3** and **4**, were studied with two APH(3′)s. Indeed, it would have been ideal to have prepared additional fluorinated derivatives of aminoglycosides to modulate the degree of nucleophilicity of the aminoglycoside hydroxyl, such as has been done in studies of protein kinases (*27*). Regrettably, the syntheses of these molecules are so difficult that such an undertaking is impractical. However, we find it remarkable that the degree of rate attenuation when the active site base (Asp198) is mutated to alanine [i.e., k_3(wt)/k_3(mut)] with nonfluorinated substrates is within the same range as that for the ratios of the rate constants for the nonfluorinated to fluorinated compounds with the wild-type enzymes (see Table 1). This indicates two things. First, activation of the substrate hydroxyl by Asp198 is important for turnover chemistry, and second, electron-withdrawing entities, such as fluorine atoms, would attenuate nucleophilicity of the site of phosphorylation substantially. Both of these observations are consistent with a mechanism that relies heavily on nucleophilic participation in the transition state for these enzymes. We would like to mention two caveats on these conclusions. First, Asp198 may conceivably play a role also in a potential dissociative mechanism as a residue that interacts with the metaphosphate-like species during the phosphate transfer step. If so, the side chain of Asp198 should be protonated. Hence, if Asp198 would have an important effect on the pH profile of catalysis, it would influence only the basic limb. Since the basic limb of catalysis by APH(3′)-Ia shows a pK_a of approximately 8 (*2*), if this is the case, then the pK_a of Asp198 should be elevated substantially within the active site. Second, despite our argument for the conservative nature of difluorination of the aminoglycosides at the 4′ site, when the fluorinated species bind the active site, they could conceivably do so somewhat differently than the case of the nonfluorinated substrates to explain the kinetic findings. Whereas the definitive answer for this possibility cannot be settled until APH(3′)-Ia is crystallized and ultimately the structures of the ternary complexes with the fluorinated derivatives have to be elucidated, we feel that this is a remote possibility. We are persuaded to conclude so because of the *consistency of the findings with both fluorinated aminoglycosides with two different Gram-negative APH(3′)s*. That the magnitude of the degree of activation of the 3′-hydroxyl in nonfluorinated ("normal") substrates by Asp198 carboxylate is the same as that of deactivation of the same hydroxyl by the difluoro moieties of compounds **3** and **4** in evaluations with the wild-type enzyme(s) is likely not a mere coincidence. We hasten to add that it is tempting to argue that the role of Asp198 could be the removal of the proton from the protonated bridging oxygen in the phosphate ester after the transfer of the metaphosphate-like species to the unactivated 3′-hydroxyl of the antibiotic. We feel that this might only be a remote possibility, as such a potential protonated bridging oxygen in a phosphate ester may reasonably be expected to lose the proton unassisted.

It is noteworthy that a number of phosphotransferases, including APH(3′)-IIIa, are now understood to undergo reaction by a mechanism involving a loose transition state with a small nucleophilic participation ("metaphosphate-like"; *28*−*32*). Our findings with the fluorinated aminoglycosides, their comparison with those of the nonfluorinated analogues, and the study of the mutant enzyme argue that, in the cases of the two Gram-negative APH(3′)s, the enzymes carry out their reactions with a significant nucleophilic participation in the transition-state species.

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