Mechanism-Based Inactivation of Bacterial Aminoglycoside 3′-Phosphotransferases

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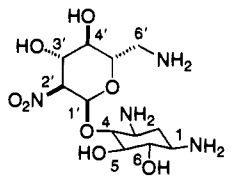
Abstract: The design, synthesis, and properties of the first mechanism-based inactivators for bacterial aminoglycoside 3′-phosphotransferases [APH(3′)s], enzymes which mediate resistance to kanamycins and related aminoglycoside antibiotics, are described. These molecules, whose design was based on the structures of aminoglycosides neamine and kanamycin B, serve as substrates for APH(3′)s. However, as a deviation from the turnover process subsequent to phosphorylation, enzyme inactivation ensues. A mechanism is proposed for the inactivation chemistry. The general chemical principles described for the inactivation strategy herein hold the promise for the development of novel mechanism-based inactivators for other "transferases", such as protein kinases, acetyltransferases, nucleotidyltransferases, sulfotransferases, and the like.

Bacterial resistance to multiple antibacterial agents, which has become common in clinical isolates of bacterial pathogens within the past several years, has created a growing state of crisis in the chemotherapy of such organisms.[1] One currently encounters organisms that cannot be treated with existing antibacterial agents, and this problem is expected to increase in scope in the immediate future.[2] The clinical needs in the 1990s and beyond necessitate the development of new strategies for antimicrobial therapy to continue to provide active agents for the treatment of serious bacterial infections, especially those acquired in the hospital.

Aminoglycoside antibiotics have served as antibacterial agents with potent and rapid bactericidal properties. Although alterations of the ribosomal binding site[3] and a decrease in the rate of permeability into the cells have been implicated for resistance to aminoglycosides,[4] the vast majority of cases of clinical resistance to these drugs arise from bacterial acquisition of aminoglycoside-modifying enzymes.[5] These enzymes are often plasmid-encoded, and the genes for some have been shown to be associated with transposable elements as well; both plasmid exchange and dissemination of transposons may facilitate rapid acquisition of the drug-resistance phenotype among bacterial flora.[5]

Among aminoglycoside-modifying enzymes, aminoglycoside 3′-phosphotransferases [APH(3′)s] are widely present in resistant bacteria.[6-8] These ATP-dependent enzymes phosphorylate the 3′-hydroxyl of a variety of aminoglycosides (including kanamycins, neomycins, paromomycins, neamine, ribostamycin, geneticin, and paromamine). The resulting phosphorylated aminoglycosides fail to bind to their respective ribosomal binding sites with high affinity; hence resistance is conferred to the drugs that are phosphorylated. We have purified recently aminoglycoside 3′-phosphotransferase types Ia [APH(3′)-Ia; unpublished results] and IIa [APH(3′)-IIa] to homogeneity and have reported on the general properties of APH(3′)-IIa.[9] These enzymes serve as good models for this family of enzymes, of which seven variants have been identified to date.[5]

We describe herein the design, synthesis, and characterization of compounds **1** and **2**, the first mechanism-based inactivators for the APH-3′ family of bacterial enzymes. Our interest in mechanism-based inactivators for the APH(3′) family of enzymes stems from the fact that such inactivators are needed to overcome this undesired bacterial activity. The proposed inactivators would be expected to show high selectivity in their inactivation of APH(3′)s, since they utilize the catalytic machinery of these enzymes to inactivate them; the molecules would not be reactive for the inactivation chemistry until the turnover process in the enzyme active site commences such a sequence of events. Furthermore, mechanism-based inactivators would be helpful in delineating the mechanism of action of APH(3′)s, which is currently unknown.



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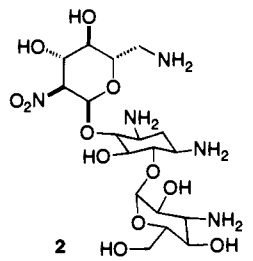

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Experimental Section

Hydrogen- and carbon-NMR spectra were obtained at 500 and 125 MHz, respectively, using a U-500 Varian spectrometer; chemical shift values (δ) are given in parts per million. Mass spectra were recorded on a Kratos MS 80RFA spectrometer. Melting points were taken on a Hoover UniMelt apparatus and are uncorrected. Thin layer chromatograms were made on silica gel. Neomycin sulfate was purchased from the Sigma Chemical Co. Neamine hydrochloride was prepared from neomycin sulfate by methanolysis.[10] Neamine and kanamycin B

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were used in the free-base forms, 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. Phosphoenolpyruvate (PEP), neomycin sulfate, ADP, ATP, NADH, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1,4-piperazinebis(ethanesulfonic acid) (PIPES) were purchased from the Sigma Chemical Co. Magnesium acetate, potassium acetate, sodium phosphate, Amberlite IRA-400, and β-mercaptoethanol were purchased from Aldrich. Pyruvate kinase (PK) and lactate dehydrogenase (LD) were obtained from the Worthington Biochemical Co. Molecular porous membrane tubing was purchased from Spectrum Medical Industry, Inc. Aminoglycoside 3′-phosphotransferase types Ia and IIa were purified according to the procedure developed by Siregar *et al.*[9]

Tri-O-Silylated Derivative of 1,3,6′-Tri-*N*-(*tert*-butoxycarbonyl)-2′-deamino-2′-nitroneamine (4). 1,3,6′-tri-*N*-(*tert*-butoxycarbonyl)-neamine (**3**, 300 mg, 0.48 mmol), prepared as described previously,[10] was dissolved in dry pyridine (6 mL), and a mixture of chlorotrimethylsilane/1,1,1,3,3,3-hexamethyldisilazane (1:3, 12 mL) was added to the solution. The solution was stirred at room temperature overnight, and then the solvent was evaporated to dryness *in vacuo*. The oily residue was dissolved in methylene chloride (50 mL) and was washed with water (2 × 5 mL). The aqueous layer was washed with methylene chloride (5 mL), and the methylene chloride layer was combined with the organic layer. The solvent was removed *in vacuo*, and the residue was dissolved in a mixture of acetone/water (5:1, 60 mL) and stirred for 5 h at room temperature. The solvent was evaporated to dryness, and the residue was redissolved in methylene chloride (50 mL). The solution was washed with water (10 mL) and dried over anhydrous $MgSO_4$, and then it was evaporated *in vacuo* to give the crude poly-O-silylated product as a white solid, which was placed under vacuum for several hours. The resultant compound was dissolved in freshly distilled methylene chloride (15 mL), and the solution was brought to reflux. *m*-Chloroperoxybenzoic acid, 80−90% (413 mg, 4 equiv), was added to the refluxing solution in portions. The addition of the peroxyacid resulted in a blue color, which disappeared with time. The subsequent portions of the peroxy acid were added only when the blue color from the previous addition had disappeared and the solution had regained a slight green color. After the last addition of the peroxy acid, the solution was refluxed until it turned virtually colorless (2 h), by which time some precipitate was observed at the bottom of the flask. The suspension was cooled to room temperature and was filtered. The filtrate was diluted with methylene chloride (80 mL) and was washed successively with solutions of potassium iodide (10%, 10 mL), sodium thiosulfate (10%, 20 mL), and saturated brine (10 mL). All aqueous layers were individually washed with methylene chloride (10 mL), and all organic layers were combined. After drying over anhydrous $MgSO_4$, the combined organic layer was evaporated *in vacuo* to give a yellow solid. The solid material was mixed in hot petroleum ether (30 mL), followed by filtration of the undissolved *m*-chlorobenzoic acid; the filtrate was kept at −20 °C overnight, by which time the remainder of *m*-chlorobenzoic acid precipitated. The precipitate was removed by filtration, the solvent was evaporated, the residual solid was dissolved in methylene chloride (1 mL), and the mixture was chromatographed on a silica-gel column. Elution with hexane/ethyl acetate (3:1) afforded 133 mg of the pure product (R_f 0.34) as a colorless oil (40%). This product gave a poorly resolved ^{1}H-NMR spectrum at 500 MHz; however, from the integration of the signals a 1:1 ratio for Boc and trimethylsilyl groups was determined (signals at 1.44 and 0.14 ppm, respectively).

2′-Deamino-2′-nitroneamine Trifluoroacetic Acid Salt (1). Compound **4** (85 mg, 0.09 mmol) was dissolved in dry methylene chloride (10 mL), and the solution was saturated with argon and was cooled in an ice/water bath. A portion of freshly distilled trifluoroacetic acid (1 mL) was added through a syringe, and the mixture was stirred for 3 h in the ice/water bath. The solution was subsequently evaporated to dryness *in vacuo*, and the residue was stirred with dry ether (20 mL), whereupon the product crystallized. The solid material was collected by centrifugation, was thoroughly washed with ether to remove any traces of remaining acid, and was dried under vacuum to afford 56 mg of the pure product as a white solid (90%): R_f 0.45 (water/acetone/acetic acid, 7:2:1); mp 125 °C dec; ^{1}H-NMR (D_2O, 500 MHz) δ 1.66 (1H, q, J = 13.0 Hz, H_{2ax}), 2.28 (1H, dt, J = 13.0 and 4.5 Hz, H_{2eq}), 3.10−3.70 (8H, overlapping multiplets, various ring hydrogens), 3.88 (1H, ddd, J = 3.5, 7.0, and J = 10.0 Hz, $H_{5'}$), 4.41 (1H, dd, J = 9.0 and 9.5 Hz, $H_{3'}$), 4.79 (1H, dd, J = 4.5 and 11.0 Hz, $H_{2'}$), 6.16 (1H, d, J = 4.5 Hz, $H_{1'}$); ^{13}C-NMR (D_2O, 125 MHz) δ 28.2 (C_2), 40.0 ($C_{6'}$), 48.5 (C_3), 49.4 (C_1), 67.6 ($C_{5'}$), 68.7 ($C_{3'}$), 70.3 ($C_{4'}$), 72.9 (C_6), 75.3 (C_5), 76.6 (C_4), 86.7 ($C_{2'}$), 94.9 ($C_{1'}$), 163.2 (CF_3CO); MS-FAB$^+$ 353 (M + H$^+$, 91).

3,6′-Di-*N*-(*tert*-butoxycarbonyl)kanamycin B Monoacetate Salt (6). A solution of kanamycin B free base (1.45 g, 3.0 mmol) in 90 mL of dimethyl sulfoxide was stirred with copper(II) acetate monohydrate (1.20 g, 6.0 mmol) and zinc acetate dihydrate (1.32 g, 6 mmol) at room temperature for several hours until a clear blue solution resulted. Di-*tert*-butyl dicarbonate was then added (1.31 g, 6 mmol), and the solution was stirred at room temperature for an additional 5 h. A TLC analysis of the reaction mixture at this point revealed the presence of a major product at R_f 0.33 (5:3:1, $CHCl_3$/CH_3OH/concentrated NH_4OH; this turned out to be the desired dicarbamoylated product), a minor product at R_f 0.50 (a tricarbamoylated species), and a trace of the starting material (which remained at the base line of the TLC plate). An additional portion of di-*tert*-butyl dicarbonate (0.13 g, 0.6 mmol) was added to the mixture, and the solution was stirred overnight. The mixture was subsequently poured into ethyl ether (600 mL) and was vigorously stirred until a thick oil separated from the solution. Ether was decanted from the flask, and the residual oil was dissolved in methanol/water (4:1, 300 mL). Hydrogen sulfide was bubbled into the solution for several minutes, and the resultant suspension was filtered through a layer of Celite. The filtrate was evaporated to dryness *in vacuo*, and the residue was redissolved in a small amount of dioxane and chromatographed on a silica-gel column. Elution with $CHCl_3$/CH_3OH/concentrated NH_4OH (5:3:1) afforded a clear separation of both the title dicarbamoylated compound **6** (1.08 g, 49%) and the tricarbamoylated product (0.53 g, 21%; which proved to be 3,2′,6′-tri-*N*-(*tert*-butoxycarbonyl)kanamycin B diacetate) as acetates: mp 153−154 °C dec; ^{1}H-NMR (D_2O, pD 9.4, 500 MHz) δ 1.23 (q, 1H, J = 13.0 Hz, H_{2ax}), 1.33 and 1.34 (2s, 18H, $(CH_3)_3CO$), 1.87 (partially resolved m, 1H, J = 13.0 Hz, H_{2eq}), 2.62 (dd, 1H, J = 4.5 and 10.0 Hz, $H_{2'}$), 2.83 (ddd, 1H, J = 4.0, 9.0, and 13.0 Hz, H_1), 2.92 (t, 1H, J = 10.0 Hz, $H_{3''}$), 3.13 (t, 1H, J = 9.5 Hz, H_4), 3.18 (t, 1H, J = 9.5 Hz, H_6), 3.23 (t, 1H, J = 9.5 Hz, $H_{4'}$), 3.33 (broad, 2H, $H_{6'R,S}$), 3.40 (t, 1H, J = 10.0 Hz, H_5), 3.44 (m, 1H, $H_{4''}$), 3.45 (m, 1H, $H_{2''}$), 3.46 (m, 1H, H3), 3.56 (t, 1H, J = 9.0 Hz, $H_{3'}$), 3.61 (m, 1H, $H_{5'}$), 3.67 (m, 2H, $H_{6''R,S}$), 3.82 (dt, 1H, J = 2.5, 2.5, and 9.5 Hz, $H_{5''}$); ^{13}C (DMSO-d_6, 125 MHz) δ 24.2 (CH_3CO), 28.6 and 28.7 ($(CH_3)_3C$), 35.1 (C_2), 41.9 ($C_{6'}$), 49.3 (C_3), 51.1 (C_1), 55.6 ($C_{2'}$), 56.1 ($C_{3''}$), 60.5 ($C_{6''}$), 70.1 ($C_{4''}$), 70.6 ($C_{2''}$), 71.3 ($C_{4'}$), 72.2 ($C_{5''}$), 72.9 ($C_{5'}$), 73.2 ($C_{3'}$), 75.3 (C_5), 78.2 and 78.3 ($(CH_3)_3CO$), 83.2 (C_4), 85.8 (C_6), 99.8 ($C_{1'}$), 100.0 ($C_{1''}$), 155.4 and 156.7 ($(CH_3)_3CO(C{=}O)$, 175.8 (CH_3CO); MS-FAB$^+$ 685 (M + H, 5), 707 (M + Na, 2).

3,6′-Di-*N*-(*tert*-butoxycarbonyl)-2′-*N*-(benzyloxycarbonyl)kanamycin B Diacetate Salt (7). 3,6′-Di-*N*-(*tert*-butoxycarbonyl)kanamycin B monoacetate salt (**6**, 0.6 g, 0.88 mmol) was dissolved in dimethyl sulfoxide (50 mL), followed by the addition of zinc acetate dihydrate (773 mg, 3.52 mmol). The solution was stirred for 30 min at room temperature followed by the addition of *N*-((benzyloxycarbonyl)oxy)-succinimide (241 mg, 0.97 mmol). The resultant solution was stirred at room temperature overnight, by which time a complete conversion of the starting material to a new species (R_f 0.48; 5:3:1, $CHCl_3$/MeOH/concentrated NH_4OH) was detected by TLC analysis of the reaction mixture. The mixture was subsequently poured into ethyl ether (500 mL) and was vigorously stirred until a thick oil separated from the solution. Ether was decanted from the flask, and the residual oil was dissolved in methanol/water (200 mL, 4:1). Hydrogen sulfide was bubbled into the solution for several minutes, which gave a suspension, which was filtered through a layer of Celite. The filtrate was evaporated to dryness *in vacuo*, and the solution was filtered and evaporated to dryness to give 0.45 g of the title product (63%). The ^{1}H-NMR spectrum of the product at 500 MHz was poorly resolved. However, the integration of the signals for the hydrogens of the Boc and the Cbz groups gave the expected 18:5 ratio (signals at 1.35 and 7.30 ppm, respectively): mp 195 °C dec; ^{13}C-NMR (DMSO-d_6, 125 MHz) δ 23.4

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(CH_3CO), 28.6 and 28.7 ($(CH_3)_3CO$), 36.0 (C_2), 41.9 ($C_{6'}$), 50.0 (C_3), 50.4 (C_1), 56.2 ($C_{3''}$), 56.4 ($C_{2'}$), 60.4 ($C_{6''}$), 65.7 (CH_2Ph), 67.4 ($C_{4''}$), 70.5 ($C_{2''}$), 70.9 ($C_{4'}$), 71.0 ($C_{5''}$), 71.8 ($C_{5'}$), 73.6 ($C_{3'}$), 75.0 (C_5), 78.1 and 78.2 ($(CH_3)_3CO$), 82.1 (C_4), 87.2 (C_6), 99.2 ($C_{1'}$), 99.5 ($C_{1''}$), 128.1, 128.8 and 137.7 (Ph), 155.4, 156.6 and 156.7 (NHCOO), 174.6 (CH_3CO); MS-FAB$^+$ 818 (M + H, 2), 840 (M + Na, 4).

Penta-O-silylated Derivative of 1,3,6′,3″-Tetra-*N*-(*tert*-butoxycarbonyl)-2′-deamino-2′-nitrokanamycin B (8). Compound **7** (150 mg, 0.19 mmol) was dissolved in dioxane/water (4:1, 30 mL), followed by the addition of di-*tert*-butyl dicarbonate (162 mg, 0.52 mmol). The solution was stirred overnight at room temperature and was then evaporated to dryness. The residue was dried under vacuum and was subsequently dissolved in dry pyridine (6 mL). The compound was silylated as described above for compound **1** by the use of a mixture of chlorotrimethylsilane and 1,1,1,3,3,3-hexamethyldisilazane (1:3, 12 mL). The crude polysilylated product gave a poorly resolved ^{1}H-NMR spectrum at 500 MHz, the integration of which showed a 4:5 ratio for the signals of the Boc and the trimethylsilyl groups.[11] The resultant compound was hydrogenolyzed in ethanol (80 mL) in the presence of palladium on carbon (10%, 40 mg) and H_2 gas for 15 min with vigorous stirring. The catalyst was removed by filtration through a layer of Celite. The filtrate was evaporated to dryness to produce 186 mg of a white solid, which appeared as a complex mixture of several ninhydrin-positive spots on a TLC plate, with a major spot at R_f 0.52 (hexane/ethyl acetate, 2:1); the white solid was placed under vacuum for several hours. The compound was dissolved in dry methylene chloride and was oxidized by *m*-chloroperoxybenzoic acid to give the title compound as described above for **1**. Purification of the crude product on a silica-gel column (hexane/ethyl acetate, 3:1; R_f 0.42) afforded 66 mg of the title compound (35%), as a colorless oil.

2′-Deamino-2′-nitrokanamycin B Trifluoroacetic Acid Salt (2). Compound **8** (60 mg, 0.05 mmol) was dissolved in dry methylene chloride (10 mL) and was allowed to react with trifluoroacetic acid (1 mL) as described above for **1**, to afford 41 mg (80%) of the title product: R_f 0.28 (7:2:1, water/acetone/acetic acid); mp 130 °C dec; ^{1}H-NMR (D_2O, 500 MHz) δ 1.60 (1H, q, J = 12.0 Hz, H_{2ax}), 2.28 (1H, broad, H_{2eq}), 3.10−3.80 (14 H, overlapping multiplets, various ring protons), 3.87 (1H, ddd, J = 3.5, 7.0, and 10.0 Hz, $H_{5'}$), 4.38 (1H, dd, J = 9.5 and 9.0 Hz, $H_{3'}$), 4.76 (1H, dd, J = 4.5 and 11.0 Hz, $H_{2'}$), 4.89 (1H, J = 3.5 Hz, $H_{1''}$), 6.09 (1H, J = 4.5 Hz, $H_{1'}$); MS-FAB$^+$ 514 (M + H$^+$, 28).

Assay of Enzymic Activity. The coupled-spectrophotometric assay of Perlin *et al.*[12] was adapted for assay of enzyme activity.[9] In this assay, the production of ADP in the course of catalysis by APH(3′) is monitored. ADP is converted to ATP by the action of pyruvate kinase (PK) and lactate dehydrogenase (LD), whereby the process may be monitored continuously by the disappearance of the chromophore for NADH 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.

Inactivation Experiments. A solution of a given concentration of an inactivator in 500 mM PIPES buffer (pH 7.0), containing 1.7 μM of enzyme [APH(3′)-Ia or APH(3′)-IIa], 50 mM magnesium acetate, and 50 mM ATP (pH 7.0) was incubated at room temperature. At various time intervals, 10-μL aliquots of the mixture were diluted into 990 μL of the assay mixture, and the activity was monitored immediately. To demonstrate that inactivation was active-site directed, the following experiments were performed with the inactivators. A mixture containing 400 mM PIPES buffer (pH 7.0), 1.7 μM enzyme, 50 mM magnesium acetate, 64 μM kanamycin A, and 15 mM inactivator was incubated at room temperature for 2 min, followed by the addition of ATP (pH 7.0) to give a final concentration of 50 mM; the total volume of the mixture was 100 μL. Aliquots (10 μL) were removed at time intervals and diluted into 990 μL of assay mixture, and the activity was monitored immediately. Turnover was studied from initial rates at several concentrations of **1**. A typical assay mixture contained the ingredients described previously, except 50−450 μM concentrations of **1** substituted kanamycin A. The effect of an added nucleophile, β-mercaptoethanol, on the inactivation rate was also investigated. The inactivation experiment outlined above was performed in the presence of β-mercaptoethanol (final concentration 10 mM) and absence of kanamycin A.

Dissociation Constant. The dissociation constants for **1** with both APH(3′)-Ia and APH(3′)-IIa were determined according to the method of Dixon.[13] Two concentrations of the substrate (kanamycin A), 10 and 20 μM, were used. A series of assay mixtures containing both the substrate and various concentrations of **1** were prepared in 200 mM PIPES buffer (pH 7.0). A portion of the enzyme was added to afford a final enzyme concentration of 10 nM in a total volume of 1 mL. The enzyme activity was measured immediately. The rates were calculated from the linear portion corresponding to approximately the first 5% of substrate turnover.

Turnover Kinetics with Inactivators. Since compound **1** turned over many times before inactivation of the enzyme, we were able to measure the turnover kinetics of **1** from initial rates. A typical assay mixture contained the ingredients described for the coupled spectophotometric assay, except 50−450 μM concentrations of **1** were used instead of kanamycin A.

Covalent Modification of Protein. These experiments were carried out to demonstrate whether the enzyme was modified covalently and explore stability of such protein modification. Portions of the enzyme (5 mg) were inactivated to completion with compounds **1** and **2** individually. The mixture was then dialyzed overnight at 4 °C against 10 mM HEPES buffer (pH 7.0) containing 10% glycerol with buffer changes every 3 h. Subsequently, the activity of the protein preparations was measured.

Detection of Inorganic Phosphate in the Course of Enzyme Inactivation. To quantify concentrations of inorganic phosphate, the malachite green colorimetric assay was used.[14] A solution of 11 mM magnesium acetate, 22 mM potassium acetate, 1.8 mM phosphoenolpyruvate, 0.1 mM NADH, 6.1 units of pyruvate kinase, 21 units of lactate dehydrogenase, 160 nM APH(3′)-IIa, 300 μM **1**, and 1.5 mM ATP in 200 mM PIPES buffer (pH 7.0) was incubated at 4 °C. Aliquots (100 μL) were removed at time intervals and were mixed with 400 μL of the colorimetric assay reagent for 10 min, prior to the measurement of the absorbance at 630 nm. Sodium phosphate solutions (pH 7.0) of know concentrations were assayed to establish a standard curve for the color test. As a control, the same experiment was performed for 300 μM neamine, in the absence of **1**.

Results and Discussion

Compounds **1** and **2** were designed in our laboratory as the first mechanism-based inactivators for APH(3′)s. These compounds were synthesized in several steps as shown. Trimethylsilylation of compound **3**, a compound which was prepared according to a published method,[10] followed by selective removal of the trimethylsilyl group from the 2′-amine afforded an intermediate which had three trimethylsilyl groups; NMR analysis suggested that the trimethylsilyl groups were present at the 6, 3′, and 4′ hydroxyl groups.[15] Subsequent oxidation of the 2′-amine produced compound **4**. The desired compound **1** was obtained after removal of the protective groups by treatment with trifluoroacetic acid.[16]

Regioselective introduction of the Boc groups at the 3- and 6′-amines of kanamycin B (**5**) was carried out by the reaction

(11) From the analysis of the ^{1}H-NMR spectrum it is obvious that only five trimethylsilyl groups were incorporated into **8**. We do not know with certainty which hydroxyl remains unprotected; however, we presume that it is the hydroxyl at position 5 since molecular modeling suggests that it may be the most hindered hydroxyl in the precursor to the silylated product.

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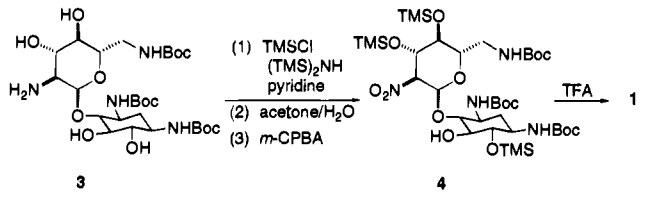
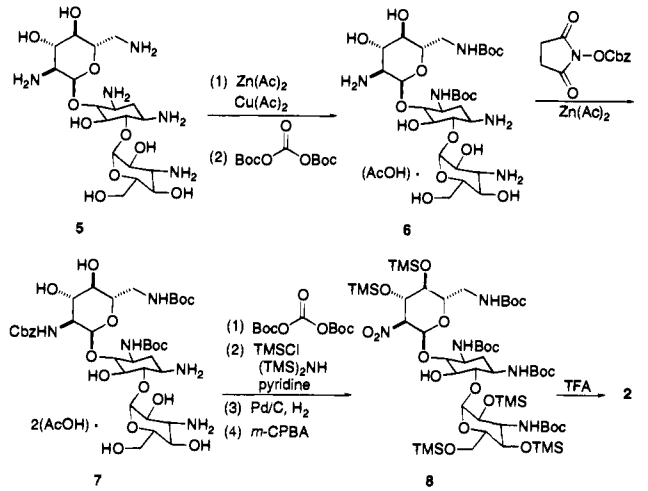
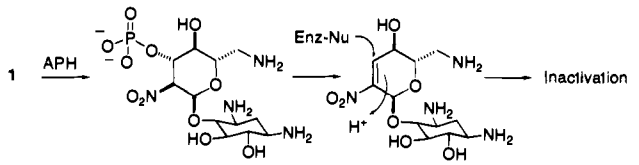
(15) The ease of incorporation of the trimethylsilyl protective group at the 6-, 3′-, and 4′-hydroxyls of neamine has been documented previously (Verheyden, J. P. H.; Repke, D. B.; Tompkins, T. C.; Moffatt, J. G. In *Aminocyclitol Antibiotics*; Reinhart, K. L., Suami, T., Eds.; American Chemical Society: ACS Symposium Series 125; Washington DC, 1980; p 212).



of di-*tert*-butyl dicarbonate in the presence of copper and zinc ions (**5** → **6**). In the presence of only the zinc ion, the Cbz group was introduced selectively at the 2′-amine in 63% yield (**6** → **7**). The remaining amines in compound **7** were exhaustively protected with Boc groups by the use of di-*tert*-butyl dicarbonate. Without isolation of this derivative, the hydroxyl groups were protected as trimethylsilyl ethers,[11] and the Cbz group was hydrogenolyzed, followed by oxidation of the 2′-amine by *m*-chloroperoxybenzoic acid to afford **8** in 35% overall yield. Compound **2** was obtained subsequent to the removal of the protective group in the presence of trifluoroacetic acid (80%).[17]



These two compounds are structural variants of neamine (**11**) and kanamycin B (**5**), which are both excellent substrates for APH(3′)s. We envisioned that these compounds, exemplified by **1** in the scheme **1** → **9** → **10** → inactivation, would serve as substrates for APH(3′)s. For example, **1** would be phosphorylated at the 3′-hydroxyl (**1** → **9**). The mere enzymic phosphorylation would *convert the poor leaving group, the 3′-hydroxyl, into the phosphoryl group, which is an excellent leaving group*. Therefore, the phosphoryl group β to the nitro function in **9** was expected to undergo facile and spontaneous elimination, giving rise to the nitroalkene **10** in the active site. The electrophilic intermediate **10** would serve as a Michael acceptor, trapping an active-site nucleophilic amino acid side chain, resulting in irreversible inactivation of the enzyme. (2-Nitrohex-2-enopyranosides, prepared as intermediates in organic syntheses, have been shown to be excellent Michael acceptors for various nucleophiles.[18,19])



Incubation of compound **1** with APH(3′)-IIa in the presence of Mg^{2+}ATP resulted in a rapid and time-dependent inactivation of the enzyme, which exhibited saturation kinetics. A double-reciprocal plot of the observed rates of inactivation as a function of the concentration of the inactivator furnished $K_I = 15.5 \pm 0.6$ mM and $k_{inact} = 0.030 \pm 0.001$ min^{-1}. The parameter K_I, which is a collection of a number of microscopic rate constants, is similar to K_m for enzyme substrates and should not be confused with the dissociation constant, K_i. The two parameters are not equal to one another for compound **1**, as measured for APH(3′)-IIa; the K_i was evaluated at 40 ± 2 μM. The value of K_i, and not K_I, gives an indication of the affinity of the enzyme for compound **1**. Compound **1** was competitive in its binding to the active site with kanamycin A, as judged by the appearance of the Dixon plot for the determination of the dissociation constant. The observed rate of inactivation by 10 mM **1** was attenuated by 25−30% in the presence of 10 mM β-mercaptoethanol, suggesting that some of the nitroalkene may exist in solution in the course of inactivation, where it is intercepted by β-mercaptoethanol. This could most likely come about by the occasional release of the product of phosphorylation (**9**) from the active site, followed by the spontaneous elimination of phosphate (*vide infra*). In addition, inactivation appears to be irreversible, as extensive dialysis of the inactivated enzyme failed to regenerate any activity.

We noted that compound **1** was turned over by APH(3′)-IIa, and from initial rates the parameters for turnover were evaluated: $K_{m,1} = 108 \pm 9$ μM; $K_{m,MgATP} = 41 \pm 7$ μM; $k_{cat} = 124 \pm 10$ min^{-1}. The value of k_{cat}/k_{inact}, or the partition ratio, was calculated at 4100 for **1** with APH(3′)-IIa. This ratio indicates the number of turnover events of **1** per inactivation of one enzyme molecule.

Compound **1** inactivated APH(3′)-Ia as well in an irreversible process. The following kinetic parameters were determined for both turnover of **1** and inactivation of APH(3′)-Ia by procedures described earlier: $K_{m,1} = 29 \pm 9$ μM; $K_{m,MgATP} = 34 \pm 1$ μM; $k_{cat} = 0.10 \pm 0.03$ min^{-1}; $K_I = 24 \pm 4$ mM; $K_i = 205 \pm 1$ μM; $k_{inact} = 0.06 \pm 0.01$ min^{-1}; $k_{cat}/k_{inact} \sim 2$. The partition ratio (k_{cat}/k_{inact}) of 2 indicates that **1** is an extremely efficient inactivator for APH(3′)-Ia. Distinction between the catalytic

(16) The outcome of each synthetic transformation was characterized by ^{1}H-NMR, ^{13}C-NMR, and MS analysis. However, the definitive structural proof for **1** was obtained from its ^{1}H-NMR spectrum in D_2O. The $H_{1'}$ and $H_{2'}$ hydrogens of **1** appear at 6.16 and 4.79 ppm, respectively, whereas the corresponding resonances for the trifluoroacetic acid salt of neamine in D_2O appear at 5.75 and 3.29 ppm, respectively. The downfield shifts for $H_{2'}$ (1.50 ppm) and $H_{1'}$ (0.41 ppm) for compound **1** clearly indicate that the nitro group is located at the 2′-position. The full ^{1}H-NMR assignments for neamine, kanamycins, and selected other aminoglycosides were determined by selective homonuclear decoupling and NOE-enhancement experiments in conjunction with our work on the three-dimensional solution structures of aminoglycosides. The complete details of these experiments will be communicated at a later time.

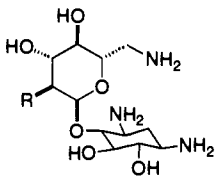
(17) The proof for the structure of compound **6** was obtained by the comparison of the chemical shifts of the H_3 and $H_{6'R,S}$ hydrogens of **6** with the corresponding resonances for kanamycin B free base in D_2O. The chemical shifts for the H_3 and $H_{6'R,S}$ of **6** were recorded at 3.46 and 3.33 ppm, respectively; the corresponding signals for kanamycin B were at 2.74 and 2.66−2.88 ppm, respectively. The downfield shifts for H_3 (0.72 ppm) and $H_{6'R,S}$ (0.45−0.67 ppm) in **6** are due to the deshielding effect of the two Boc groups at the 3- and 6′-amines of kanamycin B. Unfortunately, the poor resolution of the ^{1}H-NMR spectrum of **7** did not permit a similar assignment for its structure. However, the structure of **2**, the desired final product, was unambiguously assigned from its ^{1}H-NMR spectrum in D_2O, as described above for compound **1**. The $H_{1'}$ and $H_{2'}$ hydrogen resonances of compound **2** appeared at 6.09 and 4.76 ppm, respectively, whereas the corresponding signals for kanamycin B (pD 1.0) resonated at 5.82 and 3.35 ppm, respectively. The downfield shifts for $H_{2'}$ (1.41 ppm) and $H_{1'}$ (0.27 ppm) clearly indicated that the nitro function in compound **2** is located at the 2′-position.

(18) Sakakibara, T.; Tachimori, Y.; Sudoh, R. *Carbohydr. Res.* **1984**, *131*, 197.

(19) Holzapfel, C. W.; Marais, C. F.; van Dyk, M. S. *Synth. Commun.* **1988**, *18* (1), 97.

ability of APH(3′)-Ia and APH(3′)-IIa with respect to their substrate profiles is not readily possible. One exception is that in microbiological assays APH(3′)-Ia, in contrast to APH(3′)-IIa, confers resistance to lividomycin.[20] We note that the significant difference for the kinetic parameters measured for compound **1** with APH(3′)-Ia and APH(3′)-IIa is for the k_{cat} values; k_{cat} is higher by 3 orders of magnitude for APH(3′)-IIa, as a consequence of which APH(3′)-IIa is inactivated less efficiently ($k_{cat}/k_{inact} \sim 4100$) than APH(3′)-Ia ($k_{cat}/k_{inact} \sim 2$). This observation presents an additional distinction between the two enzymes.

The presence of the nitro group in **1** is critical for the inactivation chemistry. As noted earlier, neamine (**11**), the parent compound, which possesses an amine moiety instead of the nitro group at the 2′-position, is an excellent substrate for APH(3′)-Ia ($k_{cat} = 68 \pm 17$ s^{-1}; $K_{m,11} = 1.3 \pm 0.4$ μM; $k_{cat}/K_{m,11} = 5.2 \times 10^7$ M^{-1} s^{-1}) and APH(3′)-IIa ($k_{cat} = 29 \pm 1$ s^{-1}; $K_{m,11} = 1.2 \pm 0.1$ μM; $k_{cat}/K_{m,11} = 2.4 \times 10^7$ M^{-1} s^{-1}). Furthermore, 2′-deaminoneamine (**12**) is also merely a substrate—interestingly a very poor one—for APH(3′)-Ia ($k_{cat} = 0.22 \pm 0.04$ s^{-1}; $K_{m,12} = 31 \pm 9$ μM; $k_{cat}/K_{m,12} = 1.2 \times 10^2$ M^{-1} s^{-1}) and APH(3′)-IIa ($k_{cat} = 0.011 \pm 0.002$ min^{-1}; $K_{m,12} = 14 \pm 3$ μM; $k_{cat}/K_{m,12} = 12.7$ M^{-1} s^{-1}).[21]



11 R = NH_2

12 R = H

To provide additional evidence for the proposed mechanism of action, we looked for the generation of inorganic phosphate in the course of incubation of **1** with APH(3′)-IIa; this experiment is feasible in light of the fact that the partition ratio for **1** with APH(3′)-IIa is 4100. On addition of enzyme to a buffered solution of **1** and Mg^{2+}ATP, rapid formation of inorganic phosphate was noted, the rate of which ultimately diminished as the enzyme was inactivated. When the experiment was carried out at room temperature, the phosphate elimination and enzyme inactivation were essentially over before the first data point was measured. Therefore, we slowed down the process by incubation of the inactivation mixture at 4 °C, whereby a somewhat more gradual formation of inorganic phosphate allowed for initial rate measurements (Figure 1). A first-order rate constant of 0.4 min^{-1} was estimated from the initial rate of inorganic phosphate formation at 4 °C. This rate constant and the measured k_{cat} of 124 ± 10 min^{-1} and k_{inact} of 0.030 ± 0.001 min^{-1} (both determined at room temperature) demonstrate that the first two steps of the proposed mechanism are highly favorable processes, and that the likely slow step in the inactivation chemistry is the active-site nucleophile capture. We hasten to add that formation of inorganic phosphate was not noted when the substrate neamine (**11**) was incubated with Mg^{2+}ATP and APH(3′)-IIa under similar conditions.

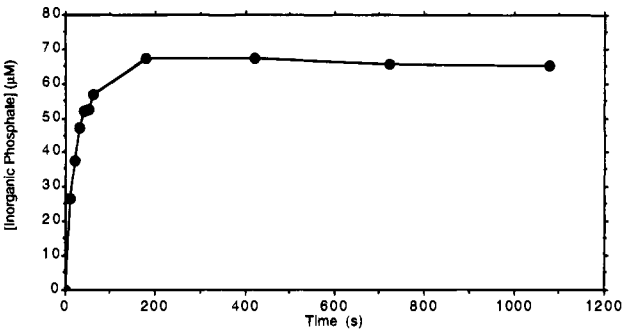


Figure 1. Formation of inorganic phosphate as a function of time in the course of inactivation of APH(3′)-IIa by **1**.

2′-Nitro-2′-deaminokanamycin B (**2**) also inactivated APH(3′)-IIa analogously to **1** ($K_I = 11.7 \pm 5.5$ mM and $k_{inact} = 0.06 \pm 0.02$ min^{-1}). However, two differences were noted in the reaction with **2** compared to that with **1**. Contrary to inactivation by **1**, enzyme inactivated by **2** regains activity over several hours. We attribute this observation to modification of different active-site residues by **1** and **2**, respectively. A second difference is that turnover of **2** could not be studied in a similar manner to turnover of **1**. Our lack of ability to study turnover of **2** suggests that k_{cat}/k_{inact} may be as low as 1 (*i.e.*, one turnover per each inactivation event). As was noted for **1**, inactivation of APH(3′)-IIa by compound **2** was competitive with kanamycin A, and β-mercaptoethanol (10 mM) attenuated the rate of inactivation by 25%.[22]

The inactivation chemistry disclosed here represents the first strategy aimed at inactivation of these bacterial enzymes. Furthermore, the biochemical principles reported herein for mechanism-based inactivation of bacterial aminoglycoside 3′-phosphotransferases hold the promise of novel applications for selective inactivation of a variety of "transferases", potentially including kinases (*e.g.*, protein kinases), acetyltransferases, nucleotidyltransferases, sulfotransferases, and the like. The net effect of each of these "transferase" reactions would be to convert the "functional group" acceptor from a poor leaving group into a good one, which fulfills the central criterion for the inactivation chemistry; namely, the elimination of the leaving group would permit the formation of the electrophilic inactivating species in the active site. In principle, the sole limitation of the method rests in the fact that the catalytic machineries of enzymes are often geared for expulsion of products from active sites, and it is the product of enzymic turnover of the inactivator that serves as an intermediate in the inactivation chemistry. However, should the rate of elimination of the "transferred" moiety be competitive with the diffusion of the product from the active site, as reported here for the APH(3′)s, the strategy may prove applicable for inactivation of a variety of enzymes.

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JA942525V

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(21) The syntheses and properties of **12** and a series of other deaminated aminoglycosides will be communicated later.

(22) The cost of synthesis of **2** was so prohibitively high that we were not able to prepare this compound in quantities necessary to carry out similar experiments with APH(3′)-Ia.