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THE USE OF NEAMINE AS A MOLECULAR TEMPLATE: INACTIVATION OF BACTERIAL ANTIBIOTIC RESISTANCE ENZYME AMINOGLYCOSIDE 3'-PHOSPHOTRANSFERASE TYPE IIa

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Abstract: Aminoglycoside 3'-phosphotransferase type IIa [APH(3')-IIa] is a member of the family of bacterial aminoglycoside-modifying enzymes. Bacteria that harbor these enzymes are resistant to aminoglycoside antibiotics. Four aminoglycoside-based affinity inactivators were synthesized and were shown to be both substrates and inactivators for APH(3')-IIa. These affinity inactivators are *N*-bromoacetylated derivatives of neamine, an aminoglycoside antibiotic, where the bromoacetyl moiety in each was introduced regiospecifically at a different amine of the parent compound.

Bacterial populations produce enzymes that modify the structures of aminoglycoside antibiotics by their acetyltransferase, nucleotidyltransferase, or phosphotransferase activities.[1-3] By so doing, resistance to the lethal action of these antibiotics is conferred, as the modified aminoglycosides do not bind to the bacterial ribosome—the target binding site for these antibiotics—with high affinity anymore. In light of the fact that the genes for these enzymes are either plasmid-borne or are situated on transposable elements, acquisition of the resistance phenotype by non-resistant organisms has been rapid and widespread.[1,3] The resistance problem has created a state of crisis in chemotherapy, as there exist presently infectious organisms that cannot be treated with any existing antibiotic.[4-12]

We have undertaken a systematic study of the functions of aminoglycoside-modifying enzymes. Towards that goal, we have reported recently on the purification and characterization of two important members of this family of enzymes, aminoglycosides 3'-phosphotransferases types Ia [APH(3')-Ia][13] and IIa [APH(3')-IIa].[14] We have also investigated potential strategies for overcoming resistance to organisms which express these enzymes.[15,16] In this manuscript, we report our use of four affinity inactivators for APH(3')-IIa based on the structure of the antibiotic neamine. These inactivators, compounds **1-4**, were synthesized regiospecifically such that each has been incorporated with a bromoacetyl group attached to a different amine of neamine. As such, they

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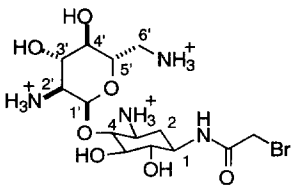
were expected to serve as inactivators that would covalently modify different nucleophilic residues in the active site and map a different subsite in the active site of the targeted enzyme.



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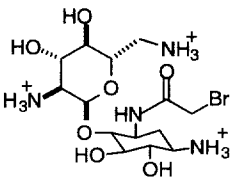

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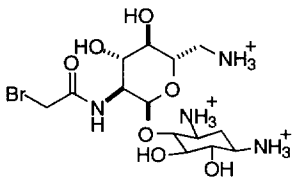

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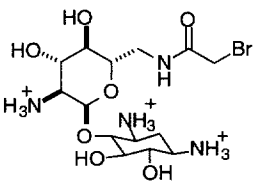

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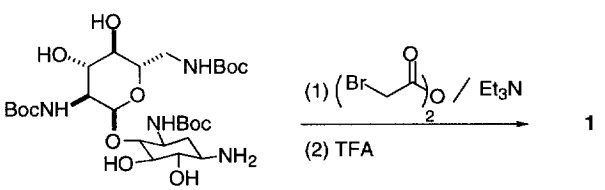
Compounds **1-4** were synthesized from individually protected neamine analogues that had three of the amines protected, leaving the fourth available for modification with bromoacetic anhydride. The syntheses for the protected precursor neamine derivatives have been reported in our earlier work on the preparation of a series of deaminated aminoglycosides.[16] The two-step preparation of compound **1** via the protected precursor **5** is given below as an example. The bromoacetylation in each case proceeded to give the corresponding protected *N*-bromoacetylated amino sugars. These molecules underwent facile removal of all the *t*-butoxycarbonyl (Boc) groups in one step in the presence of trifluoroacetic acid and anisole to give the desired compounds **1-4**.[17]

Bromoacetylation of various amines in the neamine structure renders the resultant compounds poorer



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substrates for APH(3')-IIa compared to neamine itself (Table 1). However, phosphorylation of compounds **1-4** did take place in the presence of Mg^{2+}ATP and the enzyme well within detectable limits of the assay. Insofar as K_m may approximate K_s, the effect of structural modification is manifested both on binding and the turnover numbers (i.e., k_{cat}). On the other hand, in the absence of Mg^{2+}ATP, all four compounds inactivate the enzyme in

Table 1. Kinetic parameters for phosphorylation of neamine and of compounds **1-4** by the native and cyanylated aminoglycoside 3'-phosphotransferase type IIa, along with the parameters for inactivation of the same enzyme by the said compounds.[a]

	Native APH(3')-IIa					Cyanylated[b] APH(3')-IIa				
	k_{inact} (min^{-1})	K_I (mM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{inact} (min^{-1})	K_I (mM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
neamine			1740 ± 545	1.2 ± 1.0	(2.4 ± 2.0) x 10^7			596 ± 54	7.9 ± 0.7	(1.3 ± 0.2) x 10^6
1	0.14 ± 0.02	12 ± 3	624 ± 338	71 ± 39	(1.5 ± 1.2) x 10^5	0.17 ± 0.05	17 ± 6	170 ± 69	58 ± 25	(4.9 ± 2.9) x 10^4
2	0.06 ± 0.03	363 ± 150	12 ± 3	340 ± 77	(5.7 ± 1.9) x 10^2					
3	0.04 ± 0.01	7 ± 1	68 ± 9	36 ± 5	(3.2 ± 0.6) x 10^4	0.04 ± 0.01	16 ± 2	17 ± 2	19 ± 2	(1.5 ± 0.2) x 10^4
4	0.20 ± 0.01	15 ± 1	142 ± 30	14 ± 3	(1.7 ± 0.5) x 10^5	0.17 ± .06	18 ± 7	117 ± 17	31 ± 5	(6.4 ± 1.4) x 10^4

[a]A typical assay mixture contained the ingredients mentioned above except for 5-400 μM concentration of the bromoacetylated compounds were substituted for kanamycin A. The K_m and k_{cat} values were obtained from Lineweaver-Burk plots, typically with five to seven concentrations of substrate flanking the K_m value, each recorded in duplicate. Inactivation assays were performed in the following manner. A solution of a given inactivator was placed in 150 mM PIPES (pH 7.0) buffer containing 1.6 μM of enzyme. At various time intervals, 10-μL aliquots of inactivation mixture were removed and added to 990 μL of assay mixture containing the ingredients specified above and the activity was monitored immediately. [b]The enzyme (670 μg) in 220 mM HEPES buffer (pH 7.5) containing 10% glycerol was cyanylated with NTCB (10 μL of 7.3 mM stock solution in absolute ethanol), at 4 °C in buffer mentioned above (final volume of 1 mL). Due to the relative instability of NTCB in solution, a fresh portion of NTCB (10 μL of 7.3 mM stock solution) was added to the reaction mixture every 3-4 h. After five additions, the excess NTCB was removed by continuous washing on an Amicon concentrator (YM 10 membrane). The resultant protein was diluted again with the 220 mM buffer and cyanylation was resumed for an additional period (5 portions of NTCB), followed by dialysis in the Amicon apparatus. The protein solution was titrated with 0.34 mM DTNB, which demonstrated that 93% of the thiols were cyanylated. The activity of the cyanylated enzyme was monitored by the spectrophotometricassay.

a time-dependent and saturable manner. The K_I values for inactivators are in the millimolar range. A series of protection experiments were carried out with either β-γ-CH_2-ATP or a mixture of β-γ-CH_2-ATP and 32 μM kanamycin A serving as active-site protecting agents. These agents typically attenuated the observed rate of inactivation two- to ten-fold. Hence, the inactivation process was active-site directed in each case. The inactivated enzyme in each case did not regain activity on extensive dialysis overnight, indicative of covalent bond formation between the enzyme and the inactivator. Furthermore, we demonstrated the incorporation of the amino sugar in each case into the protein structure by detection of protein glycosylation.[18]

Since the enzyme contains five cysteines and no disulfide bonds,[13] there was the potential that our compounds would modify these cysteines preferentially, whereby modification of other active site amino-acid residues would be prevented. Therefore, we prepared the cyanylated enzyme, which has all the cysteine thiols protected by the nitrile group. We had shown earlier that the activity of the enzyme is largely unaffected on cyanylation (see Table 1).[13] The parameters for inactivation of the enzyme and turnover of the inactivators were highly similar for the two enzymes, as listed in Table 1. The only exception was compound **2**, which could not be studied with the cyanylated enzyme; this compound was the worst substrate and inactivator for the native enzyme as well. Furthermore, inactivation was irreversible, saturable, and active-site directed (i.e, protection was seen with substrate analogues) also with the cyanylated enzyme.

Binding of aminoglycosides in the active sites of aminoglycoside-modifying enzymes should rely heavily on electrostatic interactions (e.g., ion pairing and hydrogen bonding). We have recently evaluated the energetic contributions of each of the amines in the structures of neamine and kanamycin A to the stabilization of the transition-state species for the phosphotransferase activity by APH(3') types Ia and IIa.[16] These results indicated that each of the amines in the structure of substrates is quite important. These findings are consistent with our analyses of turnover of the *N*-bromoacetylated inactivators disclosed in this report, each of which has been rendered a poorer substrate because of the acylation of the given amine of neamine. The same effect is seen with certain acylated derivatives of aminoglycosides, which are resistant to the action of the resistance enzymes since their affinities for the enzyme active site are reduced.[3,19,20]

Compounds **1**-**4**, which are disclosed in this report, are binding the active site of APH(3')-IIa and serve as substrates. This fact indicates that the binding mode has not been impaired by acylation of the various amines. Furthermore, since the bromoacetyl moiety is electrophilic, these molecules modify the enzyme active site, and they do so at sites other than the five cysteine residues. In light of the fact that the four amines are sequestered through out the neamine template at various positions, these enzyme inactivators are useful in mapping the active site of this enzyme for which the structure is not known. This effort is described in the ensuing manuscript.

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17. Synthesis of 6'-*N*-Bromoacetyl-neaminetrifluoroaceticacid salt (1): Compound **5** (2.76 g, 4.42 mmol) was dissolved in 200 mL dioxane/H_2O (4:1), followed by the addition of Et_3N (0.68 mL, 4.86 mmol). Bromoacetic anhydride (**4**, 1.15 g, 4.86 mmol) was added and the mixture was stirred in the dark at room temperature. After 2 h, the TLC analysis ($CHCl_3$/MeOH/acetic acid, 4/1/0.1) showed the formation of a product (R_f 0.79), however, some starting material was still present (R_f 0.44). An additional portion of Et_3N (0.68 mL) was added, followed by 1.15 g of bromoacetic anhydride. The mixture was stirred in the dark at room temperature for an additional h. A tlc analysis at this point indicated that no starting material remained. The solvent was evaporated to dryness and the residue was chromatographed on a silica-gel column ($CHCl_3$/MeOH/acetic acid, 4/1/0.1) to give the desired intermediate (1.68 g) in 51% yield. The solid residue was then dissolved in dry methylene chloride (30 mL), was saturated with nitrogen at 4 °C. A portion of distilled trifluoroacetic acid (15 mL) was added and the mixture was stirred at 4 °C for 3 h. Subsequently, the solution was warmed to room temperature and the stirring was continued for 1 h. The solvent was evaporated to dryness in vacuo, and the residue was stirred in ethyl ether (30 mL) until the product was triturated as a white solid. The solid was washed 5 times with ethyl ether (20 mL) to remove any trace of trifluoroacetic acid. The title compound was obtained in 45% yield (0.80 g). R_f 0.56 (acetone/H_2O/acetic acid, 2/1/1). ^1H NMR (D_2O, 300 MHz): δ 1.13 (t, 1H, J = 7.5 Hz, H_{2ax}), 1.70 (q, 1H, J = 12.6 Hz, H_{2eq}), 2.58 (s, 2H, -C$\underline{H}_2$Br), 3.10 to 4.10 (unresolved multiplets, 25H, various ring protons), 5.62 (d, 1H, J = 3.0 Hz, $H_{1'}$). ^{13}C NMR (D_2O, 75 MHz): δ 28.3 (-$\underline{C}H_2$Br), 28.4 (C_2), 39.0 ($C_{6'}$), 48.8 (C_3), 49.9 (C_1), 54.0 ($C_{2'}$), 68.8 ($C_{4'}$), 70.6 ($C_{5'}$), 71.8 ($C_{3'}$), 72.6 (C_5), 75.2 (C_6), 78.9 (C_4), 96.8 ($C_{1'}$), 114.6, 118.5 ($\underline{C}F_3CO_2$), 170.8 (C=O). m.p. 162 °C (dec.). IR (cm^{-1}): 3278, 1673, 1530, 1194, 1124. MS-FAB$^+$: 447 (M-3$CF_3CO_2^-$, 1.2%), 446 (M-3$CF_3CO_2^-$, 7%).
Compounds **2-4** were synthesized similarly. Their characteristics are given below:
2'-*N*-Bromoacetyl-neamine trifluoroacetic acid salt (**2**): After purification on a silica-gel column ($CHCl_3$/MeOH/acetic acid, 6/1/0.1), the desired bromoacetylated intermediate was obtained in 58% yield (R_f 0.56, 0.68 g). The desired product was obtained after the deprotection step in 58% yield (0.39 g). R_f 0.61 (acetone/H_2O/acetic acid, 2/1/1). ^1H NMR (D_2O, 300 MHz): δ 1.78 (q, 1H, J = 12.6 Hz, H_{2ax}), 2.36 (dt, 1H, J = 3.3 Hz, J = 12.3 Hz, H_{2eq}) 2.00 to 4.00 (unresolved multiplets, 27H, various ring protons and -C$\underline{H}_2$Br), 5.72 (d, 1H, J=3.9 Hz, $H_{1'}$). ^{13}C NMR (D_2O, 75 MHz): δ 27.9 (-$\underline{C}H_2$Br), 28.2 (C_2), 40.1 ($C_{6'}$), 48.8 (C_3), 49.6 (C_1), 52.5 ($C_{2'}$), 68.8 ($C_{4'}$), 69.4 ($C_{5'}$), 71.0 ($C_{3'}$), 72.9 (C_5), 75.6 (C_6), 76.5 (C_4), 96.5 ($C_{1'}$), 170.3 (C=O). m.p. 147°C (dec.). IR (KBr) cm^{-1}: 1675, 1531. MS-FAB$^+$: 447 (M-3$CF_3CO_2^-$, 1.8%), 446 (M-3$CF_3CO_2^-$, 6.8%).

3-*N*-Bromoacetyl-neamine trifluoroacetic acid salt (**3**): After silica-gel column chromatography ($CHCl_3$/MeOH/acetic acid, 5/1/0.1), 0.14 g (R_f 0.67) of the bromoacetylated intermediate was obtained (65% yield). The subsequent deprotection step afforded 80 mg of the title compound (55% yield). R_f 0.67 (acetone/H_2O/acetic acid, 2/1/1). ^1H NMR (D_2O, 300 MHz): δ 1.65 (q, 1H, J = 12.3 Hz, H_{2ax}), 2.13 (unresolved dt, 1H, H_{2eq}), 2.50 to 4.20 (unresolved multiplets, 25H, various ring protons and -CH_2Br), 5.76 (d, 1H, J=3.3 Hz, $H_{1'}$). ^{13}C NMR (D_2O, 75 MHz): δ 28.4 (C_2), 30.2 (-CH_2Br), 39.7 ($C_{6'}$), 48.4 (C_3), 50.3 (C_1), 54.0 ($C_{2'}$), 68.6 ($C_{4'}$), 69.0 ($C_{5'}$), 70.2 ($C_{3'}$), 72.8 (C_5), 76.2 (C_6), 78.3 (C_4), 95.5 ($C_{1'}$), 114.6 and 118.5 (CF_3CO_2), 169.8 (C=O). m.p. 105 (dec.). IR (KBr) cm^{-1}: 3278, 1672, 1535, 1196, 1125, 1041. MS-FAB$^+$: 446 (M-3$CF_3CO_2^-$, 2.1%), 447 (M-3$CF_3CO_2^-$, 14.4%).

1-*N*-Bromoacetyl-neamine trifluoroacetic acid salt (**4**): The desired bromoacetylated intermediate was obtained in 20% yield (60 mg, R_f 0.73) after column chromatography ($CHCl_3$/MeOH/acetic acid, 5/1/0.1). The subsequent deprotection step afforded the title compound in 58% yield (37 mg). R_f 0.70 (acetone/H_2O/acetic acid, 2/1/1). ^1H NMR (D_2O, 300 MHz): δ 1.54 (q, 1H, J = 12.6 Hz, H_{2ax}), 2.18 (dt, 1H, J = 12.9 Hz, J = 4 Hz, H_{2eq}), 2.12 (s, 2H, -CH_2Br), 3.0 to 4.0 (unresolved multiplets, 25H, various ring protons), 5.80 (d, 1H, J = 3.9 Hz, $H_{1'}$). ^{13}C NMR (D_2O, 75 MHz): δ 26.7 (-CH_2Br), 28.6 (C_2), 38.8 ($C_{6'}$), 47.4 (C_3), 48.2 (C_1), 52.2 ($C_{2'}$), 67.0 ($C_{4'}$), 67.8 ($C_{5'}$), 69.3 ($C_{3'}$), 72.4 (C_5), 74.5 (C_6), 77.2 (C_4), 94.8 ($C_{2'}$), 109.2, 113.1, 115.4, and 117.0 ($CF_3CO_2^-$), 168.8 (C=O). m.p. 171°C (dec.). IR (KBr) cm-1: 3412, 1674, 1530, 1190, 1128. MS-FAB$^+$: 44.6 (M-3$CF_3CO_2^-$, 6.4%), 447 (M-3$CF_3CO_2^-$, 1.0%).

(18) Glycosylation of APH(3')-IIa was detected by spotting a portion of the inactivated enzyme to Immobilon-P^{SQ} PVDF (polyvinylidene difluoride) membrane (approximately 10 cm x 10 cm and subjecting the membrane to the detection protocol described for GlycoTract^{TM} by Oxford Glycosystems. The membrane was washed in 20 mL of PBS buffer (575 mg Na_2HPO_4, 100 mg NaH_2PO_4 and 800 mg of NaCl in 500 mL of water, pH 7.2) by agitating it at room temperature for 10 minutes. The membrane was then immersed in 20 mL solution of 10 mM sodium periodate (prepared in 200 mM sodium acetate, pH 5.5, containing 5 mM EDTA) and agitated at room temperature in the dark for 20 min. Subsequently, the membrane was washed 3 times, each in 20 mL of fresh PBS buffer for 10 min. A portion (4 μL) of hydrazide solution (supplied in the Glyco Tract^{TM} kit) was dissolved in 20 mL of 200 mM sodium acetate/EDTA solution mentioned above. The membrane was submerged in this solution with gentle agitation at room temperature for 1 h. Subsequently, the membrane was washed 3 times, each in 20 mL fresh TBS buffer (6.05 g Trisma base, 1.6 g NaCl dissolved in water to make 1000 mL, pH 7.2) for 10 minutes. The membrane was then submerged in 20 mL of the blocking agent (provided in the Glyco Tract^{TM} kit) which was dissolved in the TBS buffer (0.5 g blocking reagent in 100 mL, heated at 60 °C for 45 min) with gentle agitation at 4 °C overnight. The membrane was washed 3 times, each with 20 mL of fresh TBS buffer for 10 min. at room temperature. A portion [10 μL of S-AP (streptavidine-alkaline phosphatase] conjugate (provided in the kit) was dissolved in 20 mL of TBS buffer. The membrane was incubated in this solution for 1 hr at room temperature with gentle agitation. The membrane was washed 3 times, each with 20 mL of fresh TBS buffer. The staining solution was prepared immediately prior to use by combining 10 mL of TBS-Mg buffer (1.21 g Tris, 0.58 g NaCl, 1.01 g $MgCl.6H_20$ dissolved in H_2O to make 100 mL, pH 0.5), 50 μL NBT (nitroblue tetrazolium) and 37.5 μL BCIP (5-bromo-4-chloro-3-indolylphosphate). This solution was used to immerse the membrane without agitation at room temperature to allow the blue-brown color to develop in the desired intensity (approximately 3-60 minutes), and then the membrane was rinsed several times in water and allowed to air dry. Ovalbumin was used as a positive control in this test.

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