Binding Specificities of Adenosine Aminohydrolase from Calf Intestinal Mucosa with Dialdehydes Derived from Hexofuranosyladenine Nucleosides[1]

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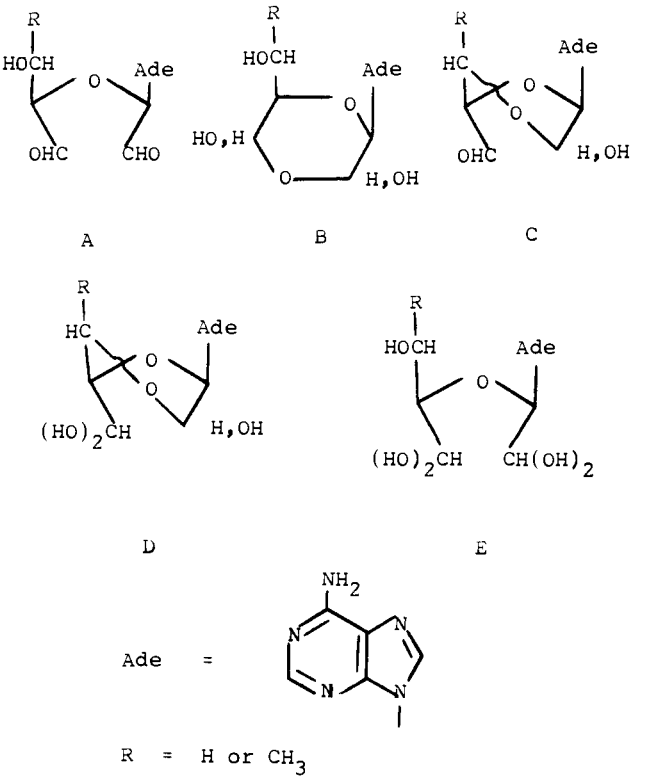
A series of nucleoside dialdehydes has been prepared as powders after treatment of hexofuranosyladenine nucleosides with paraperiodic acid; thus, periodate oxidation and purification of the products yielded dialdehydes derived from 9-(6-deoxy-β-D-gulofuranosyl)adenine (**1**), 9-(6-deoxy-β-L-gulofuranosyl)adenine (**2**), 9-(α-D-rhamnofuranosyl)adenine (**3**), 9-(α-L-rhamnofuranosyl)adenine (**4**), 9-(6-deoxy-α-L-talofuranosyl)adenine (**5**), 9-(5,6-dideoxy-β-L-*ribo*-hex-5-enofuranosyl)adenine (**6**), and 9-(5,6-dideoxy-β-D-*ribo*-hex-5-enofuranosyl)adenine (**7**). Nucleoside dialdehydes 1, **4**, and **5** were weak substrates for adenosine aminohydrolase from calf intestinal mucosa. Dialdehydes **6** and **7** were not substrates for the enzyme but were rather strong competitive inhibitors, with K_i values of 50 and 7 μM, respectively. Dialdehydes **2** and **3** did not bind to the enzyme at all. The dialdehydes did not exhibit time-dependent inhibition, suggesting that they did not form covalent bonds with the protein.

Nucleoside dialdehydes are the products of periodate oxidation of nucleosides. The biological activities of nucleoside dialdehydes as antitumor agents, protein cross-linking agents, and inhibitors of enzymes of nucleic acid metabolism were reviewed in our previous paper.[2] It is commonly recognized that solutions of these compounds are a complex mixture of cyclic and acyclic hydrates and hemiacetals.[3-6] Illustration of these compounds in the dialdehyde form is simply a matter of convenience. They do react with aldehyde reagents and, therefore, can be compared in behavior to free sugars, such as glucose. Chart I illustrates the various forms that can be present at equilibrium. Forms A and C probably do not exist in solution to any great extent because no free aldehyde has been detected in solution or in powdery form.[2,5,6] Because the hydroxyl group in the hydrates can take up either one of two configurations at each carbon atom, structure B can exist in four forms and C and D in two forms. In our previous paper, we proposed that form E, which had been demonstrated earlier[5] to constitute a considerable portion of the equilibrium mixture near pH 7, was the form that was bound to calf intestinal adenosine aminohydrolase either for substrate activity or inhibitor activity.[2] This proposal was supported by data comparing the binding properties of a series of nucleoside dialcohols[7] to the binding properties of the related dialdehydes. A definite preference for the *R* configuration at the carbon atom linked to N^9 of adenine was found in both series of compounds, and there was an excellent correspondence in the values of the inhibitor constants. The present work explores further the stereochemical requirements for binding and the influence of a different series of groups linked to the carbon atom bonded to the oxygen bridge.

Chart I



Results

Chart II illustrates the structures of the parent nucleosides and the dialdehydes **1**-**7** as Fischer projection formulas in the dialdehyde form. Paraperiodic acid was used as the oxidant rather than sodium periodate so as not to introduce salts into the reaction mixtures. Use of an anion-exchange resin in the acetate form to remove iodate ions liberated acetic acid, which was removed during evaporation and lyophilization. The nucleoside dialdehydes were obtained as white powders after lyophilization. These powders contained water of hydration, which was really covalently bound water, i.e., water that was bound to the aldehyde groups so as to form the structures shown in Chart I as well as various polymeric forms.[6] The powders were dried to constant weight at room temperature. Heat was not applied because this could change the amount of water bound in the compound, thereby changing the molecular structure. The calculated elemental analyses represent an attempt to match the determined results with the dialdehyde formulas by correcting for water content. Therefore, the elemental analyses were in best agreement with empirical formulas having the amounts of covalent bound water shown in each case. These are only approximations, and in some cases there are values that are off by amounts that are greater than would be acceptable for defined single, stable compounds, which these are not. However, these data do indicate a fair agreement with the proposed molecular formulas. Ultraviolet spectra were similar to the parent

(1) This work is taken from a thesis of A. J. G. that was submitted to the School of Graduate Studies in partial fulfillment of the requirements for the Ph.D. degree.
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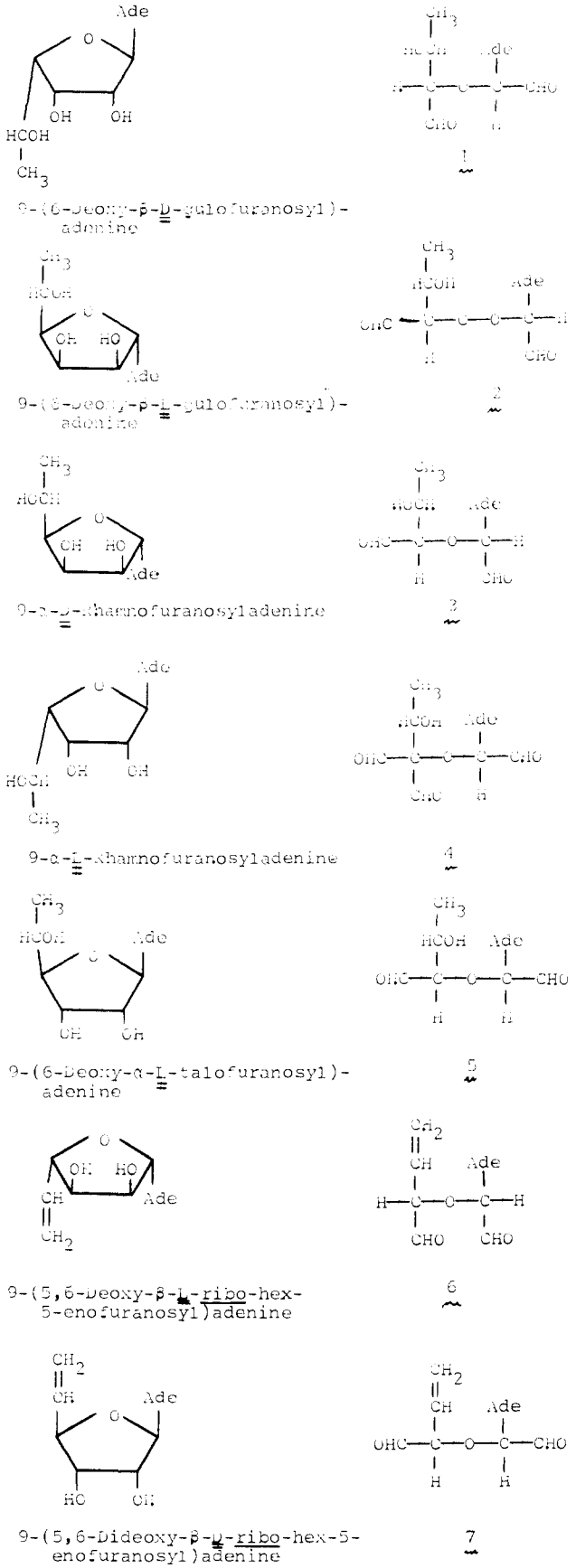
Chart II



nucleosides. The infrared spectra showed that the carbonyl group was missing; hence, this was additional evidence that no free aldehyde groups are present, at least in solid form. Absorption peaks were obtained at 3.0–3.1 (NH, OH), 6.0–7.0 (NH and purine ring), and a very broad peak at 9.0–9.6 μm (C–O–C, C–O–H). The latter is believed to signify polymer formation.[6]

Table I. Kinetic Constants for Nucleoside Dialdehydes with Adenosine Aminohydrolase

nucleoside dialdehyde	configuration proximal	configuration distal	K_m, μM	V_{max}, μmol min^{-1} (mg of protein)$^{-1}$	K_i, μM
1	*R*	*S*	350	0.46	430
2	*S*	*R*	*a*	*a*	*b*
3	*S*	*R*	*a*	*a*	*b*
4	*R*	*S*	200	0.06	150
5	*R*	*R*	140	0.41	370
6	*S*	*S*	*a*	*a*	50
7	*R*	*R*	*a*	*a*	7

[a] Not a substrate. [b] Not an inhibitor.

Kinetic constants obtained with adenosine aminohydrolase are shown in Table I. We have previously called the asymmetric carbon atom linked to N^9 (originally the anomeric carbon of the nucleoside) the *proximal* carbon and the asymmetric carbon atom attached to the oxygen bridge (originally C-4′ of the nucleoside) the *distal* carbon.[2,7] Table I also shows the configurations at these important sites. Nucleoside dialdehydes **1**, **4**, and **5** were weak substrates for the enzyme, with **4** having the lowest V_{max}, but **5** having the best affinity as reflected in the K_m. These compounds exhibited competitive kinetics when assayed with adenosine as the substrate. The unsaturated nucleosides **6** and **7** were not substrates but were fairly good competitive inhibitors, especially **7**.

Dialdehydes **2** and **3** were neither substrates nor inhibitors of adenosine aminohydrolase. Preincubation of these or any of the above compounds with the enzyme failed to alter the results. The K_i values for **1** and **4**–**7** remained the same. These data, plus the substrate activity of several of the dialdehydes, argue against the formation of covalent bonds, such as Schiff base linkages, between the compounds and the enzyme.

Discussion

The present work demonstrates the sensitivity of calf intestinal adenosine aminohydrolase to changes in structure of the dialdehydes at N^9 of adenine. This response to change in structure is dramatized even further upon comparison of the kinetic results obtained here with that obtained previously.[2] Dialdehydes **1** and **4** have the *R* configuration at the proximal carbon and the *S* configuration at the distal carbon. Both are substrates, whereas a related dialdehyde[2] having only a methyl group at the distal carbon was not a substrate despite the favorable *R* configuration at the proximal carbon. Even though the CH_3CHOH– group is bulkier than a methyl group, it does have an hydroxyl group. Placement of the hydroxyl group at the carbon adjacent to the distal carbon confers substrate activity even in the presence of the extra methyl group. Even if it is accepted that the active form of the dialdehyde is as the open-chain dihydrates, those compounds having a real hydroxyl group in this position, as opposed to only an hydrated aldehyde group, tend to be better substrates. Compounds **1** and **4** have an unfavorable *S* configuration at the distal carbon atom when compared to the *R* configuration at C-4′ of hexofuranosyl nucleosides having good substrate activity. However, in the open-chain form the larger group containing the hydroxyl group is free to swing around to bring that group close to a preferred binding site. The configuration at the hydroxyl group is also very important. Hampton et al.[8] found that the ad-

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enine nucleoside containing L-talofuranose was a better substrate than the one containing D-allofuranose, the difference being only in the configuration of the hydroxyl group at C-5′. The present results show that the dialdehyde **1** (derived from the D nucleoside) was a better substrate than **4** (derived from the L nucleoside), although the latter was able to bind better to the enzyme, as shown by both the K_m and K_i values. However, the unfavorable *S* configuration at the distal carbon probably does not allow the most favorable orientation. In comparison, **5**, which has the *R* configuration at both the distal and proximal carbons and is derived from an L sugar, is equal in substrate activity to **1** and has the strongest affinity for the enzyme as reflected in the value of K_m. However, **5** is nowhere as good a substrate as adenosine dialdehyde[2] and may reflect the problem of increasing steric bulk (the methyl group) in a region where the enzyme is most sensitive to size. The analogue of **5** that has a methyl group in place of the hydroxyethyl group is a weak substrate,[2] but this finding may be in part conditioned by the favorable *R* configuration at the distal carbon. The enzyme will not readily accept groups in the *S* configuration at C-4′ in nucleosides either.[9] In comparison, an external double bond, even when oriented in the *R* configuration, such as in **7**, causes the compounds to lose substrate activity; however, they now become excellent inhibitors. This was somewhat surprising because most of the previous data had indicated that the best inhibitors would have the *S* configuration. It would be of interest to prepare an unsaturated dialdehyde analogue having the *R* configuration at the proximal carbon and an *S* configuration at the distal carbon. Such an aldehyde should be a stronger inhibitor than **7**. Dialdehyde **7** is very similar in structure to another unsaturated nucleoside dialdehyde we prepared earlier from 9-(5-deoxy-β-D-*erythro*-pent-4-enofuranosyl)adenine, a compound that has an enolic double bond.[2] The K_i values were very close, 4 μM for the latter compound and 7 μM for **7**. Even though **6** has the *S* configuration at the proximal carbon, it also has the favorable *S* configuration at the distal carbon which favors inhibitory activity. Dialdehyde **6** binds to the enzyme three times better than the corresponding dialdehyde that only has a methyl group at the distal carbon.[2] It appears that an exocyclic double bond in the nucleoside dialdehydes precludes substrate activity but results in good inhibition. This was not found to be the case when the parent nucleosides were examined for enzymatic activity.[9] Finally, **2** and **3** have no substrate or inhibitory activity, probably because of the very unfavorable *S* configuration at the proximal carbon atom. In contrast, the hydroxymethyl and methyl analogues (distal carbon) were very weak substrates[2] and may indicate the disadvantage of extra bulk, especially when the configurations are already less desirable. Unfortunately, we do not have available the related dialdehydes having *S* configurations at both proximal and distal positions. Generally, the data are in excellent agreement with the earlier results of Lerner and Rossi[7] on alcohols derived from nucleosides and supports the view that the open-chain dihydrate (form E in Chart I) is the active form of the dialdehyde.

It has generally been assumed that nucleoside dialdehydes bind to proteins by formation of Schiff-base linkages to amino groups on lysine residues[10-12] and that the reaction is time-dependent. Adenosine aminohydrolase also contains a lysine residue near or at the active site,[13] but despite this the nucleoside dialdehydes do not bind covalently to this enzyme. Presumably, the amino group is masked in the folds of the protein and is not readily available for binding. In any case, the enzyme is responsive to changes in structure and configuration, and it should be most interesting to see if other enzymes react the same way. We have recently found that keeping the substituent at N^9 constant while changing the purine moiety results in dramatic changes in substrate and inhibitor activity.[14] These data also parallel the results obtained with alcohols derived from nucleosides,[15] again supporting the open-chain dihydrate (form E) as the active agent.

The new compounds reported here are undergoing antitumor screening. The results will be reported elsewhere.

Experimental Section

General Methods and Materials. Adenosine was purchased from Sigma Chemical Co. It was recrystallized three times from water and dried under high vacuum at 80 °C over phosphorus pentoxide. Elemental analyses were obtained by the Baron Consulting Co., Orange, Conn. Ultraviolet spectra were recorded with a Beckman DK-2 spectrophotometer in standard 1-cm silica cells. The compounds were dissolved in 0.05 M sodium phosphate buffer, pH 7.0. Infrared spectra were recorded on a Perkin-Elmer Model 21 spectrophotometer. Samples were pressed into potassium bromide pellets. Whatman No. 1 paper was used for paper chromatography by a descending technique. Mobilities were recorded as R_f values. Spots were located with a Mineralight lamp that produced UV radiation at 254 nm. Solvents used were: water (A); 86:14, v/v, 1-butanol–water (B); and 5:1:4, v/v, 1-butanol–ethanol–water, upper phase (C).

General Procedure for Preparation of Nucleoside Dialdehydes. The procedure is based upon that of Khym and Cohn.[4] The nucleosides were oxidized with a slight excess of paraperiodic acid (H_5IO_6) for 1–1.5 h at room temperature, protected from light. Afterward, the solutions were percolated through a column of Bio-Rad AG1-X8 (200–400 mesh, acetate form) anion-exchange resin to remove iodate ion and unreacted periodate ion. The effluents were tested for iodate by a starch–iodide test.[16] Evaporation of the solutions under high vacuum (oil pump) at room temperature were performed on a rotary evaporator. The evaporations were halted before reaching dryness and the small volumes of solution were lyophilized to white powders. These were dried further under high vacuum over phosphorus pentoxide at room temperature. Paper chromatography showed the absence of starting nucleosides.

Nucleoside Dialdehydes. Oxidation of **9-(6-deoxy-β-D-gulofuranosyl)adenine**[17] (0.11 g, 0.4 mmol) with paraperiodic acid (0.11 g, 0.5 mmol) gave 0.08 g (65%) of dialdehyde **1**: R_f 0.57 (A), 0.53 (B), 0.70 (C); UV λ_{max} 258 nm (ε 13 900). Anal. Calcd for $C_{11}H_{13}N_5O_4 \cdot H_2O$: C, 44.44; H, 5.09; N, 23.56. Found: C, 44.14; H, 4.96; N, 22.29.

9-(6-Deoxy-β-L-gulofuranosyl)adenine[17] (0.08 g, 0.29 mmol) was oxidized with paraperiodic acid (0.08 g, 0.37 mmol) to give 0.07 g (87%) of dialdehyde **2**: R_f 0.60 (A), 0.56 (B), 0.70 (C); UV λ_{max} 258 nm (ε 14 200). Anal. Calcd for $C_{11}H_{13}N_5O_4 \cdot 1.5H_2O$: C, 43.13; H, 5.27; N, 22.87. Found: C, 43.26; H, 4.68; N, 22.05.

9-(α-D-Rhamnofuranosyl)adenine[18] (0.14 g, 0.46 mmol) was treated with paraperiodic acid (0.12 g, 0.54 mmol) to give 0.11 g (78%) of dialdehyde **3**: R_f 0.57 (A), 0.60 (B), 0.67 (C); UV λ_{max}

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259 nm (ε 14 400). Anal. Calcd for $C_{11}H_{13}N_5O_4 \cdot 1.5H_2O$: C, 43.13; H, 5.27; N, 22.87. Found: C, 43.84; H, 4.86; N, 22.33.

9-(α-L-Rhamnofuranosyl)adenine[19] (0.23 g, 0.76 mmol) was treated with paraperiodic acid (0.2 g, 0.9 mmol) to yield 0.19 g (81%) of dialdehyde **4**: R_f 0.57 (A), 0.57 (B), 0.70 (C); UV λ_{max} 259 nm (ε 14 200). Anal. Calcd for $C_{11}H_{13}N_5O_4 \cdot H_2O$: C, 44.44; H, 5.09; N, 23.56. Found: C, 44.53; H, 4.87; N, 22.46.

9-(6-Deoxy-α-L-talofuranosyl)adenine[20] (0.12 g, 0.43 mmol) was oxidized with paraperiodic acid (0.13 g, 0.57 mmol) to afford 0.1 g (77%) of dialdehyde **5**: R_f 0.62 (A), 0.54 (B), 0.71 (C); UV λ_{max} 259 nm (ε 14 400). Anal. Calcd for $C_{11}H_{13}N_5O_4 \cdot H_2O$: C, 44.44; H, 5.09; N, 23.56. Found: C, 44.39; H, 5.36; N, 26.11.

9-(5,6-Dideoxy-β-L-*ribo*-hex-5-enofuranosyl)adenine[21] (0.19 g, 0.63 mmol) was treated with paraperiodic acid (0.15 g, 0.68 mmol) to give 0.16 g (91%) of dialdehyde **6**: R_f 0.70 (A), 0.83 (B), 0.72 (C); UV λ_{max} 259 nm (ε 13 400). Anal. Calcd for $C_{11}H_{11}N_5O_3 \cdot H_2O$: C, 47.31; H, 4.69; N, 25.08. Found: C, 47.03; H, 4.75; N, 26.00.

9-(5,6-Deoxy-β-L-*ribo*-hex-5-enofuranosyl)adenine[21] (1.2 g, 4 mmol) was treated with paraperiodic acid (0.93 g, 4.1 mmol) to give 0.91 g (82%) of dialdehyde **7**: R_f 0.69 (A), 0.83 (B), 0.74 (C); UV λ_{max} 259 nm (ε 13 700). Anal. Calcd for $C_{11}H_{11}N_5O_3 \cdot H_2O$: C, 47.31; H, 4.69; N, 25.08. Found: C, 46.53; H, 4.77; N, 26.10.

Enzyme Kinetics. Adenosine aminohydrolase (adenosine deaminase, EC 3.5.4.4) from calf intestinal mucosa (type 1) was purchased from Sigma Chemical Co. The preparation had a specific activity of 220 units/mg of protein. One unit is defined as the amount of enzyme that will deaminate 1 μmol of adenosine to inosine per minute at pH 7.5 and 25 °C. The assay method was that of Kaplan.[22] The rate of change in absorbance at 265 nm was measured on a Beckman DK-2 spectrophotometer. The reactions were run at 25 °C in 0.05 M sodium phosphate buffer. Substrate activity was determined by adding adenosine aminohydrolase (60 units) to 3 mL of buffer containing the nucleoside dialdehyde. The solution was allowed to stand for 24 h at room temperature. A shift in the absorption maximum from about 260 nm to about 249 nm indicated the transformation of an N^9-substituted adenine to the corresponding hypoxanthine dialdehyde. Compounds not deaminated were treated with another 60 units of enzyme for an additional 24 h to be certain that they were not substrates. The double-reciprocal plot method of Lineweaver and Burk[23] was used to determine the values of K_m and V_{max}. A weighted least-square analysis based upon that of Wilkinson[24] was used to obtain the best lines. Initial velocities were measured at concentrations of the dialdehydes ranging from 70 to 200 μM. The quantity of enzyme added to a 3-mL solution ranged from 25 to 50 units, depending on the activity of the compound. The K_m for adenosine was 50 μM; the V_{max} was 212 μmol min^{-1} (mg of protein)$^{-1}$. Inhibitor constants were determined by addition of 0.1 mL of enzyme solution (0.25 units) to 3 mL of buffer solution containing adenosine and the nucleoside dialdehyde. The adenosine concentration ranged from 25 to 75 μM; the concentration of dialdehyde ranged from 3 to 200 μM, depending on the activity of the compound. For each determination of K_i, five substrate concentrations and two inhibitor concentrations were used. The K_i values were also determined by the double-reciprocal plot method and weighted least-square analysis. The slopes of all inhibition lines were statistically different from the line representing no inhibition and the intercepts of all lines on the $1/V$ axis were the same.

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Aminoglycoside Antibiotics. 3. Epimino Derivatives of Neamine, Ribostamycin, and Kanamycin B

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2′,3′-Epimino analogues of neamine, ribostamycin, and kanamycin B possessing little or no intrinsic antimicrobial activity were designed to enhance the activity of kanamycin A against bacterial strains that elaborate aminoglycoside 3′-phosphotransferases. Routes were devised for their synthesis from the parent antibiotics and the 2″,3″-epimino analogue of kanamycin B also was prepared. None of these analogues was active against phosphotransferase-producing bacteria, although the kanamycin B derivatives showed very weak activity against nonresistant strains. At 8 and 32 μg/mL, the 2′,3′-epimino analogue of neamine produced a small synergistic effect on the activity of kanamycin A against a strain of *Escherichia coli* that produces aminoglycoside 3′-phosphotransferase II. The N^3-(carbobenzyloxy) derivative of this analogue produced a small effect against the same strain, and it, as well as the 2″,3″-epimino analogue of kanamycin B, slightly enhanced the activity of kanamycin A against a strain of *Proteus rettgeri* that elaborates a similar enzyme.

Aminoglycosides hold an important place in the treatment of bacterial infections, especially with respect to Gram-negative species that are resistant to other types of antibiotics. The effectiveness of earlier aminoglycosides, such as streptomycin and kanamycin, has become limited by the emergence of resistant strains of bacteria that inactivate these antibiotics through enzymatic reactions. These reactions include the phosphorylation, acetylation, and adenylylation of specific hydroxyl and amino groups.[1-3] The production of the enzymes is mediated by R factors, including plasmids.[4] Certain new aminoglycosides, such

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