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Published on Web 03/06/2002

Design of Novel Antibiotics that Bind to the Ribosomal Acyltransfer Site

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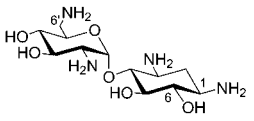
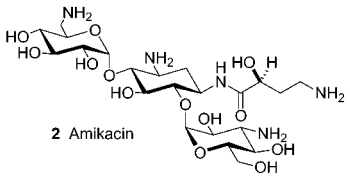
Received July 11, 2001

Abstract: The structure of neamine bound to the A site of the bacterial ribosomal RNA was used in the design of novel aminoglycosides. The design took into account stereo and electronic contributions to interactions between RNA and aminoglycosides, as well as a random search of 273 000 compounds from the Cambridge structural database and the National Cancer Institute 3-D database that would fit in the ribosomal aminoglycoside-binding pocket. A total of seven compounds were designed and subsequently synthesized, with the expectation that they would bind to the A-site RNA. Indeed, all synthetic compounds were found to bind to the target RNA comparably to the parent antibiotic neamine, with dissociation constants in the lower micromolar range. The synthetic compounds were evaluated for antibacterial activity against a set of important pathogenic bacteria. These designer antibiotics showed considerably enhanced antibacterial activities against these pathogens, including organisms that hyperexpressed resistance enzymes to aminoglycosides. Furthermore, analyses of four of the synthetic compounds with two important purified resistance enzymes for aminoglycosides indicated that the compounds were very poor substrates; hence the activity of these synthetic antibiotics does not appear to be compromised by the existing resistance mechanisms, as supported by both in vivo and in vitro experiments. The design principles disclosed herein hold the promise of the generation of a large series of designer antibiotics uncompromised by the existing mechanisms of resistance.

Aminoglycoside antibiotics bind to the bacterial RNA in manifestation of their activity.[1] The structures of two aminoglycoside antibiotics, paromomycin and gentamicin C_{1a}, bound to full length or a fragment of ribosomal RNA (rRNA), have been determined recently by NMR[2,3] and by X-ray crystallography.[4,5] These and other studies show that the neamine class of aminoglycosides binds specifically to the A-site region on the 16S subunit of rRNA. Hence, neamine serves as a minimal structural motif for such binding.[2,6] Neamine (**1**) itself is a poor antibiotic and is not clinically useful. Other clinically useful aminoglycosides, such as gentamicin, amikacin (**2**), or neomycin, face the possibility of clinical obsolescence because of the function of aminoglycoside-modifying enzymes, such as has already happened with kanamycins.[7] Hence, it is desirable to keep the minimal structural motif in aminoglycosides for RNA binding, but to try to deviate away from typical aminoglycoside structures in arriving at new antibiotics. This strategy has the potential for discovery of novel RNA-binding molecules that would conceivably not experience modification by the resistance enzymes. Such an exercise has been carried out successfully in our laboratories and is disclosed herein.



1 Neamine



2 Amikacin

Results and Discussion

Design Strategy. The three-dimensional NMR structure[2] for paromomycin bound to the A-site rRNA template (residues 1404−1412 and 1488−1497 of *Escherichia coli* 16S rRNA, Protein Data Bank code: 1PBR) was the beginning for our

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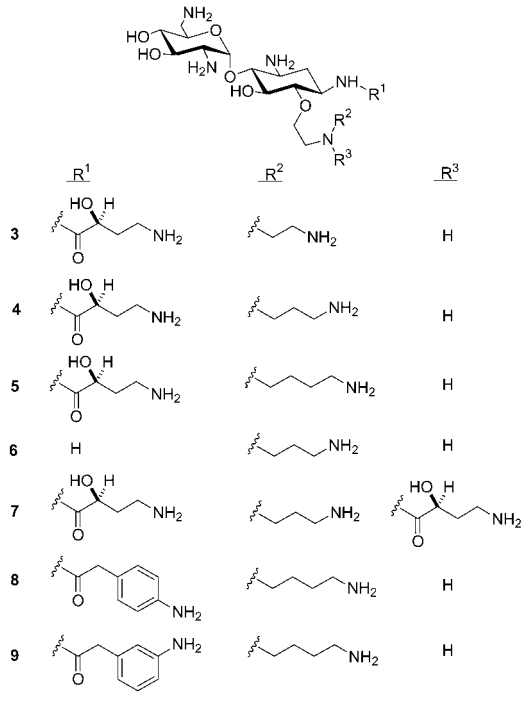
design. The paromamine portion (6′-hydroxyl analogue of neamine) of paromomycin, as a minimal motif for RNA binding, was retained in the A-site template, and the remainder of the paromomycin structure was removed. We defined this complex as the receptor template and performed a DOCK[8] search using the Cambridge Structural Database (CSD) and the National Cancer Institute (NCI) 3-D database for molecules that would bind to this template. The two databases provided a sampling of 273 000 compounds for the three-dimensional search protocol. The list of selected compounds that emerged from the search was reduced according to steric and energetic criteria. A subset of these structures that was predicted to bind near the positions N1 and O6 of paromamine was kept. In addition to this set of compounds, we paid attention to the A-site rRNA-paromamine complex itself. We modified the core moiety of paromamine by attachment of a (*S*)-4-amino-2-hydroxybutyryl group at position N1. At position O6, various amine-containing aliphatic substitutions were made by looking at the distance between O6 and the phosphate backbone of the A site of rRNA, using molecular graphics. The outcome of these protocols was the designed compounds **3**−**9**. The rationale for selecting amino-hydroxybutyryl group at position N1 was that amikacin (**2**) possesses such a substitution in its structure, and this particular substitution is known to impart resistance to modification by a number of aminoglycoside-modifying enzymes.[6,7,9] Neamine, the 6′-amino analogue of paromamine, was used in syntheses because of its ready availability from fragmentation of neomycin. The terminal amine-containing substitutions at position O6 on neamine were selected because of potential ion-pairing interactions between the amine and the phosphate backbone in the target rRNA region.



Syntheses. Neamine hydrochloride **1** was prepared from methanolysis of the commercially available neomycin sulfate, as reported earlier.[10−12] Treatment of this compound with benzyl chloroformate in the presence of sodium carbonate afforded the tetra-*N*-Cbz-protected neamine derivative **10**.[13] This compound was subjected to 1,1-dimethoxycyclohexane to provide the mono-cyclohexylidene-protected compound **11**, along with some 5,6:3′,4′-di-cyclohexylidene-protected derivative, which was fully converted to **11** in the presence of *p*-toluenesulfonic acid and methanol in DMF.[14] Protection of the hydroxyl groups at positions 3′ and 4′ was achieved by treatment of compound **11** with chloromethyl methyl ether in the presence of Hünig's base and tetrabutylammonium iodide to give **12** in good yield. Cyclohexylidene deprotection of this compound with acetic acid afforded intermediate **13**, which was treated with sodium hydride in DMF to furnish the cyclic carbamate **14** in high yield. Protection of the hydroxyl group at position 5 of **14** with triethylsilyl chloride gave the TES-protected derivative **15** (Scheme 1). Treatment of **15** with di-*tert*-butyl dicarbonate in the presence of triethylamine and DMAP afforded compound **16** in high yield. Attempts at opening the oxazolidinone ring under mild basic conditions (0.5 N aqueous LiOH) gave an isomeric mixture of products having the TES group either at position 5 or at position 6 (due to TES migration from position 5 to 6 in the ring-opened product). Several attempts to protect the C5-hydroxyl group with a variety of different types of protective groups, prior to *N*-Boc protection of the cyclic carbamate, failed to give any acceptable result. For instance, attempts at MOM protection resulted in formation of a compound having the MOM groups both at the C5 oxygen and at the N1 of the cyclic carbamate. Therefore, the TES group of **16** was removed by TBAF to give **17**, which was treated with chloromethyl methyl ether to afford the derivative **18** in good yield. Treatment of compound **18** with 0.5 N aqueous lithium hydroxide afforded the advanced intermediate **19**, having the C6 hydroxyl group free for further manipulation (Scheme 1). Compound **19** was allowed to undergo reaction with allyl bromide in the presence of lithium bis(trimethylsilyl)amide and tetrabutylammonium iodide to give the allyl-protected derivative **20**, according to the procedure of Park et al.[15] Ozonolysis of **20**, followed by reductive amination of the aldehyde **21** with the corresponding mono-*N*-Cbz-protected diaminoalkanes, afforded the aminated products **22**−**24**, according to a similar reported methodology (Scheme 2).[15] Efficient deprotection of the Boc and MOM groups with 1.3 N methanolic HCl resulted in the formation of derivatives **25**−**27**, having the N1 group free for the preparation of the corresponding amides. Treatment of these compounds with an activated ester of *N*-Cbz-protected (*S*)-4-amino-2-hydroxybutanoic acid (Scheme 3) furnished **29**−**31** in good yields. Finally, removal of the Cbz groups of **29**−**31** and **26** by catalytic transfer hydrogenation[16] with 1,4-cyclohexadiene over Pd−C afforded the title compounds **3**−**6**

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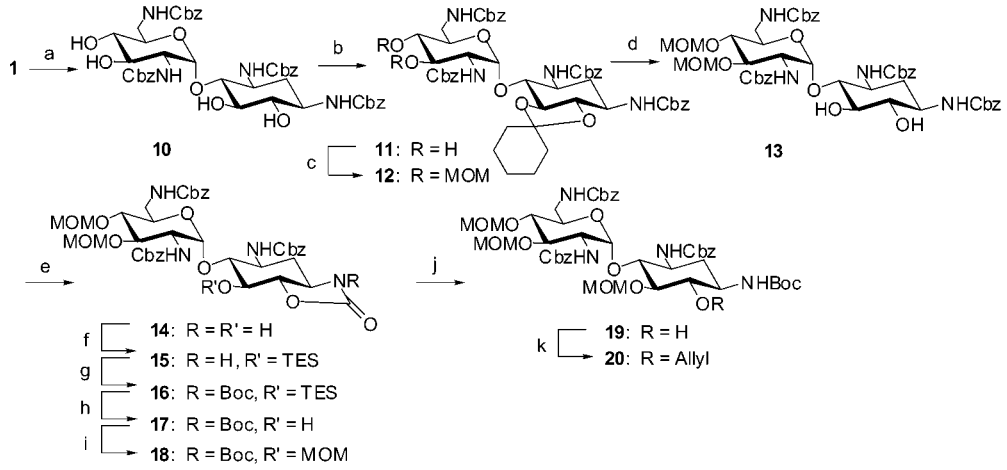
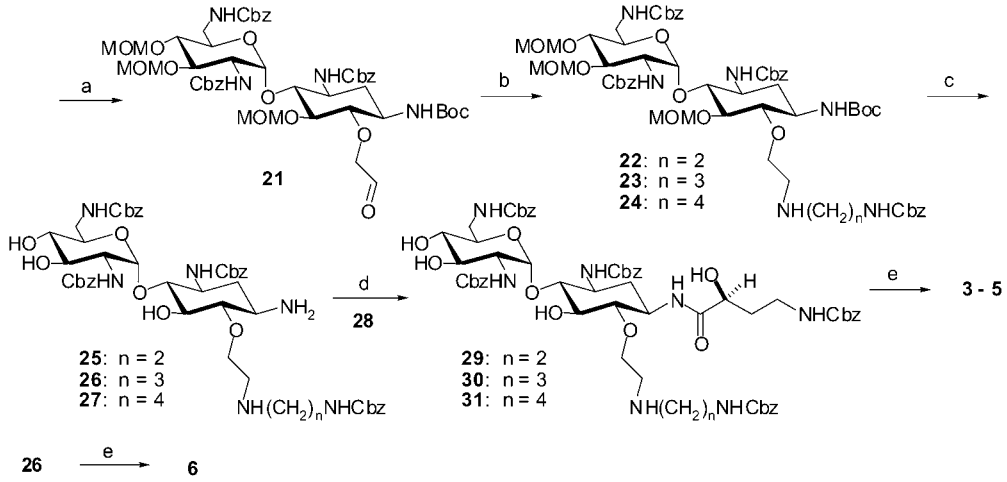
Scheme 1 [a]



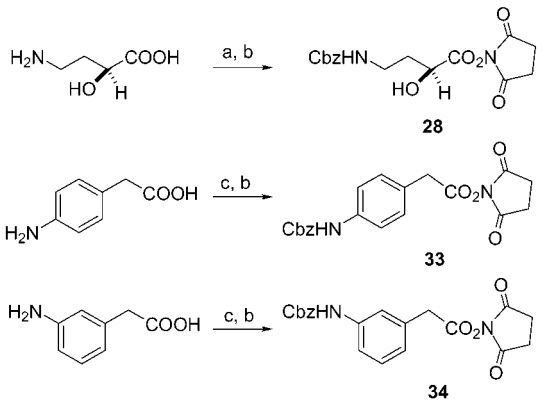
[a] Reagents and conditions: (a) $PhCH_2CO_2Cl$, Na_2CO_3, acetone−water, 95%; (b) 1,1-dimethoxycyclohexane, TsOH, DMF, 87%; (c) MOMCl, Bu_4NI, $(i\text{-Pr})_2NEt$, 32 °C, 79%; (d) AcOH, dioxane−water, 60 °C, 95%; (e) NaH, DMF, 89%; (f) TESCl, imidazole, DMAP, 80%; (g) $(Boc)_2O$, Et_3N, DMAP, 96%; (h) TBAF, THF, 85%; (i) MOMCl, Bu_4NI, $(i\text{-Pr})_2NEt$, 32 °C, 97%; (j) LiOH, dioxane, 88%; (k) $CH_2{=}CHCH_2Br$, Bu_4NI, $(Me_3Si)_2NLi$, 69%.

Scheme 2 [a]



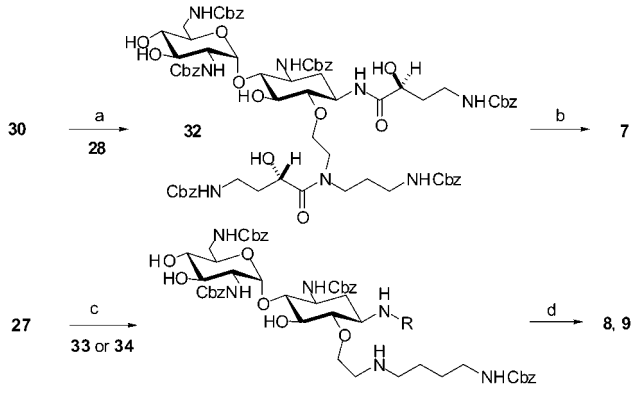
[a] Reagents and conditions: (a) (i) O_3, CH_2Cl_2, (ii) Ph_3P, 81%; (b) $CbzHN(CH_2)_nNH_2$, $NaBH_3CN$, AcOH, MeOH, 55−67%; (c) 1.3 N HCl, MeOH−$CHCl_3$, 73−87%; (d) $NaHCO_3$, THF−water, 46−54%; (e) 1,4-cyclohexadiene, Pd−C, AcOH, 51−68%.

Scheme 3 [a]



[a] Reagents and conditions: (a) $PhCH_2CO_2Cl$, Na_2CO_3, acetone−water, 89%; (b) *N*-hydroxysuccinimide, DCC, THF; (c) $PhCH_2CO_2Cl$, NaOH, acetone−water, 91%.

Scheme 4 [a]



[a] Reagents and conditions: (a) pyridine, 42%; (b) 1,4-cyclohexadiene, Pd−C, AcOH, 52%; (c) $NaHCO_3$, THF−water, 92%; (d) condition b, 56−63%.

in good yields (Scheme 2). Compound **32** was prepared by treatment of **30** with **28** in pyridine, which after removal of the Cbz groups gave compound **7** (Scheme 4).

Compound **35** was prepared by treatment of **27** with 1.0 equiv of an active ester of *N*-Cbz-protected 4-aminophenylacetic acid (**33**). Deprotection of this derivative under the same conditions as described earlier afforded compound **8** in good yield (Scheme 4). Similarly, compound **36** was synthesized by treatment of

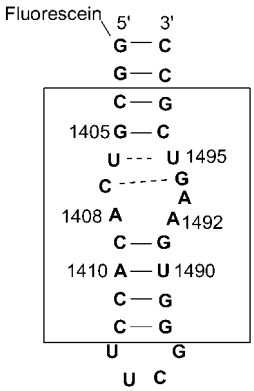


Figure 1. Sequence of the F-AS RNA, representing the A site of the *E. coli* ribosomal RNA. The boxed region shows the highly conserved sequence that is homologous to the A site on 16S rRNA, that was used to elucidate the interactions with paromomycin.[2] The sequence numbering of the nucleic-acid template is according to that for *E. coli* 16S rRNA.

27 with **34**, which after removal of the Cbz groups furnished **9** (Scheme 4).

One of the major difficulties in the synthesis was the preparation of the intermediate **19** from its precursor, cyclic carbamate **14** (Scheme 1). When compound **14** was treated with di-*tert*-butydicarbonate in the presence of Et_3N and DMAP, the product of the reaction was a compound with Boc groups both at C5 oxygen and at N1 nitrogen. Attempts to deprotect the *O*-Boc group under mild basic conditions (Na_2CO_3 or LiOH) resulted in the opening of the oxazolidinone ring.

Another difficulty, encountered in the final step of the synthesis, was deprotection of the Cbz groups, which proved to be difficult under typical conditions. Hydrogenolysis of the Cbz-protected compounds over Pd−C at atmospheric hydrogen pressure required long reaction times and afforded very low yield of the desired compounds. This problem was solved by the application of the catalytic transfer hydrogenation, using 1,4-cyclohexadiene in the presence of activated palladium on carbon and acetic acid as solvent.[16]

Binding of Antibiotics to a Model for the A-Site Region of the *E. coli* 16S Ribosomal RNA. RNA molecules often undergo conformational changes in the presence of specific ligands.[17] Several studies have shown that these ligand-induced changes can be monitored in solution by the use of attached dye molecules.[18] In the present study, we report that a fluorescein molecule attached to the 5′ end of a decoding-region A-site analogue can act as a reporter for binding of antibiotics to RNA. The target RNA, referred to as F-AS (Figure 1), is a tagged version of a small 27-nucleotide fragment of the *E. coli* 16S ribosomal RNA that has been shown previously to bind to aminoglycosides in vitro.[2,19] This RNA serves as a convenient tool in quantitative analyses of aminoglycoside antibiotics that bind to the decoding site of the bacterial ribosomal RNA. Binding of the parent compound neamine (**1**) to F-AS RNA was monitored by a decrease in the fluorescence intensity of

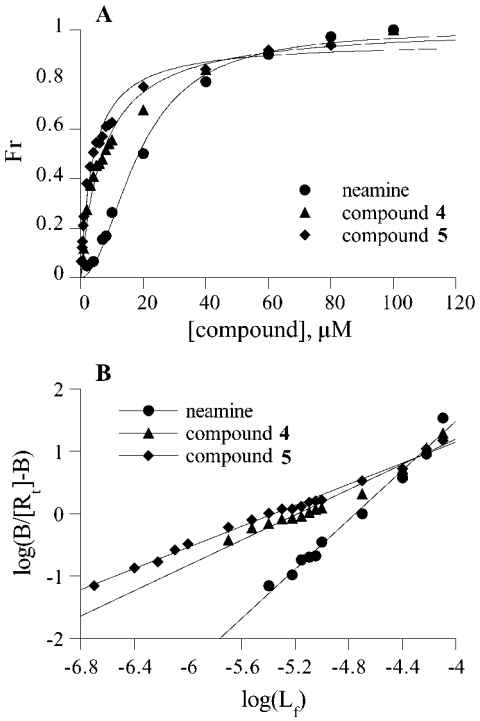


Figure 2. Representations of relative fluorescence (Fr) at 519 nm as a function of ligand concentration (A) and the Hill plots (B) for neamine, compound **4**, and compound **5**. Curves in panel A were fit using eqs 1 (compounds **4** and **5**) and 4 (neamine), respectively. Hill plots in panel B were fit with eq 3 (Supporting Information).

the attached fluorescein dye at 519 nm. A cooperative binding event at low concentration of ligand was observed (Figure 2), in which fitting of the relative fluorescence (Fr) versus the free neamine concentration by Scatchard and Hill equations (eqs 2, 3, and 4 in Supporting Information) gave a K_d of 19 ± 1 μM and a Hill constant of 1.7. This result is in good agreement with the previously reported K_d values for neamine binding by other methods[3a,20,21] that were performed under similar conditions, suggesting that the fluorescein tag does not adversely influence drug binding. A second cooperative binding event for neamine occurs at higher concentrations with a K_d of 3.9 ± 0.1 mM and a Hill constant of 2.0. A similar fluorescence-quenching behavior of F-AS was observed upon titration with neomycin and paromomycin to give K_d values (5 and 87 nM, respectively) that are consistent with previously reported values determined by other methods (data not shown).[2,3a,20,22]

To examine whether the neamine-derived compounds could bind to the A-site RNA, as predicted to be so by design, titrations were carried out with compounds **3**−**9**. In all cases, the fluorescence signal of F-AS decreased upon drug binding, and saturation was reached at relatively low antibiotic concentrations

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(21) In similar buffer conditions, it has been shown that neamine binds as a 2:1 complex to a biotinylated AS-WT RNA with a K_d of 10 μM for each binding site, see: Sucheck, S. J.; Wong, A. L.; Koeller, K. M.; Boehr, D. D.; Draker, K.-A.; Sear, P.; Wright, G. D.; Wong, C.-H. *J. Am. Chem. Soc.* **2000**, *122*, 5230−5231. Possible differences in the temperature of the experiment may explain the slight deviations from our results.

(22) No fluorescence quenching of F-AS was observed upon addition of glucose or spermine up to millimolar concentrations (data not shown), demonstrating that the quenching effect is not due to nonspecific interactions with carbohydrate or cationic compounds.

Table 1. Dissociation Constants for Aminoglycoside Antibiotics and Neamine Derivatives Binding to the Decoding-Region A-Site RNA

compound	K_d (μM)[a]	compound	K_d (μM)[a]
neamine (**1**)	19 ± 1[b,c]	**5**	3.9 ± 0.1
	3900 ± 51[b,d]	**6**	1.6 ± 0.3
3	2.2 ± 0.1[b,e]	**7**	11 ± 2
	1200 ± 2	**8**	24 ± 8
4	5.9 ± 1.3	**9**	24 ± 6

[a] Average of two measurements. [b] Cooperative binding. [c] The average number of interacting sites, n, is 1.7. [d] $n = 2.0$. [e] $n = 1.5$.

($10-100$ μM). Dissociation constants and binding modes are reported in Table 1 for the series of neamine derivatives. Compounds **4**−**9** all exhibited simple behaviors with a single binding site in each case and no cooperativity. For these compounds, the binding curves were fit assuming 1:1 binding (Hill constants = 1; Figure 2, panels A and B, shows the data for **4** and **5**). The binding of derivative **3** was cooperative, similar to the behavior of the parent neamine. In these cases, the Scatchard plots were nonlinear, and the Hill constants were nonunity (Figure 2, panel B, shows the data for **1**). Compound **3** was also unusual in the fact that it did not exhibit saturation behavior at higher drug concentrations. A second nonspecific binding event was observed for compound **3** with a K_d value of 1.2 mM.

The nonlinear Scatchard plots for two of the compounds (**1** and **3**) examined suggested cooperative-binding modes. The nonunity Hill constants for these compounds can be explained by several different models. The binding event may not occur in an "all-or-none" fashion,[23] which is typical of planar and rigid drugs. Cooperativity may occur such that initial contacts (perhaps with a single ring or functional group) make subsequent contacts with the RNA more favorable for entropic reasons. Such a binding event is possible due to the flexible nature of the aminoglycoside structures. Alternatively, we may be observing conformers of drug-RNA complexes. It has been suggested by Fourmy et al.,[3a] on the basis of NMR and dimethyl sulfate probing studies, that neamine may be sampling multiple binding sites within the A-site major groove. A third possibility, as suggested by Sucheck et al.,[21] is that neamine is binding as a 2:1 complex to the A-site RNA. Further experiments are necessary to determine which model is more appropriate.

Previous studies have shown that rings I and II (2-deoxystreptamine and 6′-deoxy-6′-aminoglucose, respectively) of paromomycin are critical for specific recognition within the A-site RNA binding pocket.[4,18a] Further work by Alper et al.[24] and Greenberg et al.[25] revealed that aminoglycoside derivatives with neamine cores were able to bind to the A-site RNA, but with reduced specificity for the wild-type sequence. Interestingly, however, some of their neamine derivatives with lower affinities for the A-site RNA had nearly identical antibiotic activities as the parent neomycin. In the present study, comparison of binding affinities for the neamine derivatives reveals that the presence of amino groups is important for A-site

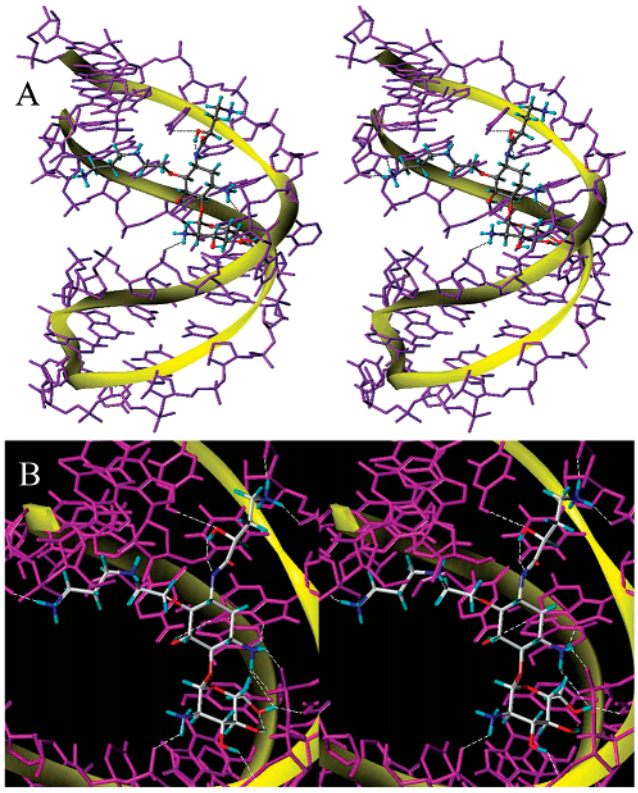

Figure 3. (A) Stereoview of the A-site region of the 16S ribosomal RNA template occupied by compound **4**. Hydrogen-bonding interactions between compound **4** and the nucleic-acid residues are shown as broken lines. The nucleic-acid residues are shown in capped stick representation in violet, and compound **4** is shown in ball-and-stick representation, color-coded according to atom types (C, gray; N, cyan; O, red). (B) Stereoview of the close up of the binding site depicted in panel A.

recognition. Addition of a diaminoalkane group at the O6 position improves binding by 3- to 12-fold relative to the high-affinity binding by neamine (compare compounds **1** and **3**−**6** in Table 1), indicating that the length of the alkyl linker is important. We note that the K_d of **6** is 1.6 ± 0.3 μM, as compared to 5.9 ± 1.3 μM for that of **4**, indicating an approximate 4-fold decrease in binding affinity upon addition of the aminohydroxylbutyryl group at position 1. Similarly, addition of the aminophenylacetyl functional groups at position 1 increases somewhat the dissociation constant for the A-site RNA binding in the two cases examined. A 6-fold increase in dissociation constant is observed for both **8** and **9** relative to **5**. Modification of the secondary amine in **4** to give **7** reduces binding by a mere 2-fold. What is important to note is that *these molecules all clearly bind to the target RNA, and they do so comparably to the parent aminoglycoside neamine*. It would appear that all of these synthetic aminoglycosides bind the target RNA site within less than 2 kcal/mol of each other. In addition, the sequence of the A-site RNA used in this study is highly conserved among bacteria. An inspection of the bacterial A-site RNA sequences revealed that they are identical in the bacterial strains employed for activity studies (following section).

Computational Modeling. Figure 3 shows the energy-minimized structure of compound **4** bound in the A site of the rRNA template. This compound occupies the pocket that is formed by A1492, similar to the case of the other neamine class of compounds, and interacts with the phosphate backbone of the nucleic acid via ion-pairing and hydrogen-bonding interac-

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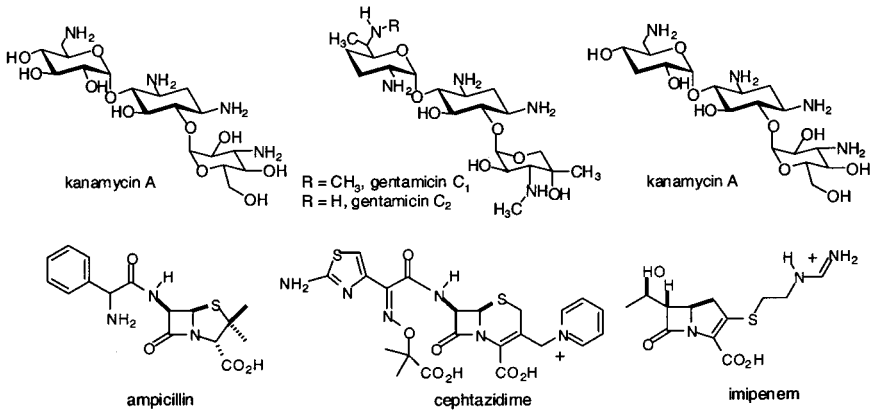
Table 2. Minimum Inhibitory Concentrations (MICs) of Antibiotics against Various Bacterial Strains[a]

	MIC (μg/mL)											
bacterial strains	**1**	KAN	GEN	TOB	**2**	AMP	CAZ	IMI	**3**	**4**	**5**	**6**
Escherichia coli JM83	64	4	<1	<1	1	4	0.03	0.06	8	2	1	32
E. coli JM83 (APH(3′)-I)	8000	2000	<1	1	1	16 000	0.5	0.25	32	8	8	>512
E. coli JM83 (AAC6′/APH2″)	2000	500	64	128	8	8000	0.5	2	32	8	8	>512
Serratia marcescens ATCC13880	16	8	4	16	8	32	0.25	0.5	32	8	4	64
Enterobacter cloacae ATCC 3047	64	8	2	4	4	1000	2	0.5	8	2	2	16
Pseudomonas aeruginosa 66	500	32	>128	2	4	>500	64	0.25	8	2	2	>512
P. aeruginosa C43	4000	1000	16	4	32	>500	128	32	64	4	2	>512
Staphylococcus aureus 3	64	4	<1	<1	<1	4	2	<0.03	2	0.5	0.5	4
Enterococcus faecium 119	1000	64	8	32	64	1	>128	0.5	256	32	32	>512

[a] **1**, neamine; KAN, kanamycin A; GEN, gentamicin; TOB, tobramycin; **2**, amikacin; AMP, ampicillin; CAZ, ceftazidime; IMI, imipenem. Structures are given below:



tions. In addition, the aminohydroxybutyryl moiety forms two hydrogen bonds and one ion-pair interaction with the rRNA template. The O6 side chain has one hydrogen bond with the nucleic acid base (G1405 in Figure 1) and one ion-pairing interaction with the phosphate backbone. This model suggests that the side chains at O6 may be longer than the propylamine (R^2 in compound **4**) due to the presence of more functional groups on the A site and space to accommodate such side chains in this area. These homologous side chains will have similar interactions, such as ionic and hydrogen-bonding interactions as was seen in the case of compound **4**, and indeed compound **5** showed comparable binding affinity to F-AS (vide infra).

Antibiotic Activities of Synthetic Aminoglycosides. The antibiotic activities of the synthetic molecules are shown in Table 2. For the purpose of comparison of activities and demonstration of the properties of the bacterial strains used in this study, we have also performed susceptibility studies with several aminoglycosides (neamine, kanamycin A, gentamicin, tobramycin, and amikacin) and β-lactam [ampicillin (a penicillin), ceftazidime (an expanded-spectrum cephalosporin), and imipenem (a carbapenem)] antibiotics. Neamine (**1**) is a suitable control, since our antibiotics use its structure as the template. Kanamycin A has become clinically obsolete, since it is an excellent substrate for the ubiquitous resistance enzymes aminoglycoside phosphotransferases (APH(3′)s), and it serves as another control. Gentamicin, tobramycin, and amikacin are widely used presently in clinic, either by themselves or in synergistic combinations with β-lactam antibiotics.

Escherichia coli JM83 is an antibiotic-sensitive laboratory strain. *E. coli* JM83 (APH(3′)-I) contains high copies (500−700 copies per cell) of plasmid pUC19 with the cloned kanamycin resistance gene from transposon Tn903 that produces the type I aminoglycoside 3′-phosphotransferase. APH(3′)-I is the most common aminoglycoside phosphotransferase in gram-negative bacteria. *E. coli* JM83 (AAC6′/APH2″) contains a plasmid with the gene for the bifunctional aminoglycoside-modifying enzyme AAC(6′)/APH(2″), which is produced by many cocci. The enzyme is able to phosphorylate at the 2″ position and/or acetylate at the 6′ position of various aminoglycoside antibiotics to manifest resistance for the organisms that express it. *Serratia marcescens* ATCC 13880, *Enterobacter cloacae* ATCC 3047, and *Pseudomonas aeruginosa* 66 and C43 are representative important gram-negative pathogens. Aminoglycosides by themselves or in combination with other antibiotics are often used to combat these microorganisms, especially *P. aeruginosa* that is often very difficult to treat. *Staphylococcus aureus* 3 and *Enterococcus faecium* 119 are representatives of the Gram-positive cocci. Most of these microorganisms are intrinsically resistant to aminoglycosides (poor permeability), but in combination with β-lactam antibiotics are often used to treat infections caused by these microorganisms.

Our results demonstrate that compounds **4** and **5** show good activity against an *E. coli* strain hyperexpressing either APH(3′)-I or AAC(6′)/APH(2″) aminoglycoside-modifying enzymes (Table 2). These activities are superior to those of kanamycin A and neamine (250- to 1000-fold higher activities) against *E. coli* hyper-expressing APH(3′)-I, and merely 8-fold lower than activities of tobramycin and amikacin. The results indicate that **4** and **5** are poorly turned over by APH(3′)-I (supported by enzymology with purified enzyme; vide infra). The activity for the synthetic compounds against *E. coli* hyperexpressing AAC(6′)/APH(2″) is even more impressive. These compounds either equal the activity of clinical aminoglycosides, such as amikacin, or are clearly superior (by 8- to 250-fold) to them (as compared with kanamycin A, gentamicin, or tobramycin). Compounds **4** and **5** demonstrate excellent activities against enterobacteria,

Table 3. Kinetic Parameters for Turnover of Compounds **4**, **5**, **8**, **9**, Neamine, Amikacin, and Kanamycin A by APH(3′)-Ia and the Bifunctional Enzyme AAC(6′)/APH(2″)[a]

	APH(3′)-Ia			BF [APH(2″)]			BF [AAC(6′)]		
compound	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
4	51 ± 4	3.0 ± 0.1	$(5.9 \pm 0.7) \times 10^4$	209 ± 32	$(2.5 \pm 1.2) \times 10^{-3}$	12.0 ± 0.5	563 ± 120	2.1 ± 0.4	$(3.7 \pm 0.3) \times 10^3$
5	40 ± 3	2.8 ± 0.2	$(7.2 \pm 0.9) \times 10^4$	108 ± 20	$(1.6 \pm 0.8) \times 10^{-3}$	14.0 ± 0.5	627 ± 138	4.2 ± 1.7	$(6.7 \pm 0.5) \times 10^3$
8				161 ± 19	$(1.3 \pm 0.6) \times 10^{-3}$	8.1 ± 0.5	489 ± 95	4.0 ± 1.2	$(8.2 \pm 0.4) \times 10^3$
9				108 ± 18	$(1.6 \pm 0.2) \times 10^{-3}$	14.0 ± 0.2	520 ± 37	3.7 ± 0.9	$(7.1 \pm 0.2) \times 10^3$
neamine (**1**)	2.3 ± 0.4	141 ± 35	1.2×10^8	36 ± 2	$(6.8 \pm 0.8) \times 10^{-3}$	189.0 ± 0.1	10 ± 3	4.3 ± 0.7	$(4.3 \pm 0.3) \times 10^5$
amikacin (**2**)	84 ± 44	165 ± 46	2.0×10^6	299 ± 1	105 ± 9	$(3.5 \pm 0.1) \times 10^5$	477 ± 14	4.1 ± 0.2	$(8.6 \pm 0.6) \times 10^3$
kanamycin A	1.2 ± 0.2	102 ± 14	8.5×10^7	24 ± 2	177 ± 19	$(7.4 \pm 0.9) \times 10^6$	4.5 ± 0.1	0.4 ± 0.4	$(8.9 \pm 2.2) \times 10^4$

[a] The turnover data for amikacin, kanamycin A, and neamine with APH(3′)-Ia;[26] those with the bifunctional enzyme[27] have been reported.

such as *S. marcescens*, *E. cloacae*, and also *P. aeruginosa*, that are sensitive or moderately resistant to aminoglycoside antibiotics, including strains also highly resistant to β-lactam antibiotics (ampicillin, ceftazidime, imipenem). In these cases, activities of compounds **4** and **5** are always either equal or superior to those of the most active aminoglycoside(s) used in this study. Compounds **4** and **5** also show good activities against strains of *S. aureus* 3 and *E. faecium* 119 that are moderately resistant to aminoglycosides. Compound **3** (Table 2) showed a spectrum of antimicrobial activity similar to those of compounds **4** and **5**, except that its MICs values were approximately 4-fold higher. Compound **6** (Table 2) gave lower activity than compound **3**. It is not active against *E. coli* strains producing either APH(3′)-I or AAC(6′)/APH(2″) enzymes, although it demonstrates activity against some of the tested organisms (*E. coli* JM83, *S. marcescens* ATCC 13880, *E. cloacae* ATCC 3047, *S. aureus* 3) that are sensitive or moderately resistant to aminoglycoside antibiotics in general. Compound **7** (data not shown) shows moderate activity against *E. coli* strains producing either APH(3′)-I or AAC(6′)/APH(2″) enzymes (MICs 64 and 32 μg/mL, respectively) and also against strains of enterobacteria and *P. aeruginosa*. Finally, compounds **8** and **9** do not show any appreciable activity against any of the microorganisms tested (MICs > 1000 μg/mL), despite the fact that they do bind to the RNA. These molecules may have difficulty being transported into the cytoplasm.

Kinetics of Turnover with Aminoglycoside-Modifying Enzymes. A number of enzymes are known that modify aminoglycosides,[7] but a mere handful are known to be prevalent among pathogens. Compounds **4**, **5**, **8**, and **9** were studied with two of the most important aminoglycoside-modifying enzymes, an aminoglycoside 3′-phosphotransferase type Ia (APH(3′)-Ia)[26] and the bifunctional ("BF") aminoglycoside-modifying enzyme (AAC(6′)/APH(2″)).[27,28] The latter possesses both phosphotransferase (APH(2″)) and acetyltransferase (AAC(6′)) activities. It is conceivable that one or more enzymes may already exist that turn over our synthetic antibiotics well. However, it is interesting to note that these compounds are exceedingly poor substrates for these common resistance enzymes under in vitro conditions (Table 3).

The kinetic parameters for turnover of two known aminoglycosides are of interest here. Neamine is an excellent substrate for APH(3′)-Ia (k_{cat}/K_m of 1.2×10^8 $M^{-1}\,s^{-1}$) and a midrange substrate for the acetyltransferase activity of the bifunctional enzyme (k_{cat}/K_m of 4.3×10^5 $M^{-1}\,s^{-1}$).[27] Despite the fact that neamine does not have the 2″-hydroxyl, it would appear that it accepts phosphate in a reaction catalyzed by the bifunctional enzyme (k_{cat}/K_m of 189 $M^{-1}\,s^{-1}$), but the process is so inefficient that it is irrelevant for manifestation of resistance. On the other hand, the semisynthetic amikacin was included in the present study because of its well-known weak interactions with many resistance enzymes.[29−32] Amikacin is certainly turned over by these enzymes, but the kinetics of turnover are usually sufficiently inefficient that the antibiotic is active against organisms that harbor these enzymes, with a few exceptions. As shown in Table 3, amikacin is turned over by APH(3′)-Ia with a k_{cat}/K_m of 2.0×10^6 $M^{-1}\,s^{-1}$. Similarly, k_{cat}/K_m of 8.6×10^3 and 3.5×10^5 $M^{-1}\,s^{-1}$ are measured for the AAC(6′) and APH(2″) activities, respectively, of the bifunctional enzyme for amikacin. It is important to note that the synthetic compounds (**4**, **5**, **8**, and **9**) are turned over by these enzymes at or below 10^4 $M^{-1}\,s^{-1}$. At best, they are turned over by APH(3′)-Ia with a k_{cat}/K_m of 7.2×10^4 $M^{-1}\,s^{-1}$. The K_m for these substrates is substantially elevated above that for the good substrate neamine (i.e., 2.3 μM), so enzymic catalysis would not reach saturation until a substantially higher concentration of the antibiotic is reached within the bacterial cytoplasm. The same is true for both activities of the bifunctional enzyme with **4**, **5**, **8**, and **9**. The K_m values for these activities were consistently above 100 μM, often substantially so, such that within the organism these concentrations cannot be reached for effective turnover of these antibiotics by the resistance enzymes. Therefore, we conclude that *these antibiotics are not affected by the resistance enzymes in any significant way, consistent with the findings of the biological activity discussed earlier*.

As a parting note, we hasten to add that after the completion of the research presented herein, two reports on X-ray structures of aminoglycosides bound to the A site of bacterial ribosome appeared in the literature.[4,5] These publications revealed that there are some differences between the NMR structures (used for computational studies in this work) and X-ray structures. Nonetheless, we were able to generate molecules, on the basis of the NMR structure, that were shown to bind the ribosomal model in solution, and some showed antibacterial activity.

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Methods

Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance spectra were recorded on either a Varian 400 or a Varian unity-500 MHz 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 performed by the Michigan State University Mass Spectrometry Facility. Melting points were obtained on an Electrothermal melting point apparatus and are uncorrected. 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. All chromatography solvents were 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. *N*-Cbz-protected diaminoalkanes were either prepared[33] or purchased from the Fluka chemical co. as hydrochloride salt. The amine free bases were prepared by treatment of the HCl salts with Amberlite IRA-400 (OH^-) ion-exchange resin. 3-Amino and 4-amino phenylacetic acids were purchased from Fluka chemical co. and converted to the *N*-Cbz-protected derivatives prior to use. 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. Spectrophotometric studies were performed on a Hewlett-Packard 8453 diode array instrument. All calculations were performed by the MS Excel program.

Preparation of F-AS RNA. A 5′-fluorescein-labeled A-site model (5′-F-GGCGUCACACCUUCGGGUGAAGUCGCC-3′) (F-AS) was obtained from Xeragon Oligoribonucleotides (Zurich, Switzerland)) or synthesized by using standard silyl phosphoramidite chemistry with reagents from Glen Research (Sterling, Virginia). The RNA was purified by electrophoresis on denaturing (8 M urea) 15% polyacrylamide gels, followed by electroelution with 1X TBE (90 mM Tris-HCl, 90 mM boric acid, 2.5 mM Na_2EDTA, pH 8.3) in an Amicon centrilutor and Centricon 3's (Amicon). The F-AS RNA was stored at −20 °C in 10 mM HEPES, pH 7.4. RNA concentrations were determined spectrophotometrically using a molar extinction coefficient of 253 300 M^{-1} cm^{-1}.

Fluorescence Measurements. Fluorescence experiments were performed on a Spex Fluoromax luminescence spectrometer. To renature, the F-AS RNA (∼150 μM) was placed in a heating block at 20 °C, heated to 85 °C for 2 min, then slowly cooled back to 20 °C over a 2 h period. The RNA solutions contained 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM Na_2EDTA, and 1 μM F-AS. Fluorescence emission spectra were obtained with an excitation wavelength of 490 nm with a band-pass of 2.9 nm (0.7 mm slit width) over the range of $\lambda_{em} = 500-600$ nm. All measurements were taken at 37 °C. Samples were incubated in a temperature-controlled cuvette holder in the Fluoromax for 2 min before fluorescence intensities were measured. Aliquots of the antibiotic were added sequentially, allowing 2 min of equilibration time before each fluorescence measurement.

Fluorescence intensities were corrected for volume changes using the following equation: $F_{i,corr} = F_{i,obs}*V_i/V_0$, where $F_{i,corr}$ is the corrected intensity for point i of the titration, $F_{i,obs}$ is the measured intensity at point i, V_i is the volume after the i^{th} addition, and V_0 is the initial volume (typically 350 μL).

Determination of Minimum Inhibitory Concentration (MICs). The MICs of all antibiotics, including our synthetic aminoglycosides, were determined by microdilution broth procedure. We performed sequential 2-fold dilutions of antibiotics in 100 μL of Luria-Bertani (LB) broth in sterile 96-well microtiter plates. Overnight cultures of bacteria were diluted 100 times in LB broth and subgrown for several hours, and 10 μL of diluted cultures was transferred into the antibiotic-containing microtiter plates to bring final inoculums to 10^5 CFU/mL (CFU stands for colony forming units). Cultures were incubated overnight at 35 °C, and microtiter plates were checked from below with a reflective viewer. MICs were defined as the lowest concentrations of the drug at which the microorganism did not demonstrate visible growth.

Kinetic Determinations with Resistance Enzymes. Kinetic studies were performed for phosphotransferase activities of the bifunctional enzyme AAC(6′)/APH(2″) and APH(3′)-Ia, as well as the acetyltransferase activity of AAC(6′)/APH(2″), using the methods described by Azucena et al.[27] The assay mixture consisted of 66 mM PIPES, pH 7.5, 11 mM magnesium acetate, 22 mM potassium acetate, 1.76 mM phosphoenol pyruvate, 0.1 mM NADH, 6.1 units of pyruvate kinase, 21 units of lactate dehydrogenase, 100 nM enzyme, the aminoglycoside substrate (at various concentrations), and 150 μM ATP in 500 μL total volume. The components of the assay mixture were mixed in a cuvette in the absence of ATP and enzyme. The solution was allowed to equilibrate at room temperature for 2 min. The reaction was started by the addition of ATP and enzyme. The progress of the reaction was monitored spectrophotometrically at 340 nm. Lineweaver−Burk plots were obtained to determine the K_m and k_{cat} values.

For the acetyltransferase activity assay, the method of Haas and Dowding was employed.[34] The reaction mixture was composed of 58 mM citric acid, 124 mM tripotassium citrate, 18 mM magnesium acetate, 6 mM dithiothreitol, the aminoglycoside substrate (at various concentrations), and 120 μM acetylcoenzyme A (specific activity, 21 mCi/mmol) in a total volume of 30 μL. The reaction was started by the addition of 5 μL of enzyme (final concentration of 100 nM) and was stopped at 1, 2, 3, and 4 min by the addition of 10% tricholoroacetic acid. Kinetic constants were determined from Lineweaver−Burk plots.

Docking and Molecular Modeling. The NMR structure of paromomycin bound to the rRNA A site was used as the starting template.[2] Rings I and II (2-deoxystreptamine and 6′-deoxy-6′-aminoglucose, respectively) in the NMR structure of paromomycin were retained, and the remainder of the structure was removed. These two rings constitute the aminoglycoside paromamine (a structurally similar compound to neamine having a hydroxyl group in place of the amine at position 6′). With this structure at hand, the Connolly surface of the complex (i.e., A-site RNA template bound by paromamine) was computed, which defines the "receptor site". Two ligand databases, the NCI-3D database and Cambridge Structural Database, collectively containing a total of 273 000 compounds, were used to dock the individual compounds into the "receptor site" using the program DOCK version 4.0. The electrostatic and steric counterparts on the receptor site were matched with the docked compounds. This data set was reduced to 40 compounds on the basis of their best fit into the "receptor site". Each compound in the set of 40 was considered in the receptor site individually. These 40 compounds fit in the space near the aminoglycoside-binding site and were scored by the program favorably for their ability to bind to the depressions and niches of the surface of the rRNA structure. We then envisioned neamine analogues that would be covalently tethered to these entities individually. Many of these compounds were predicted to bind the surface such that they were amenable to attachment to neamine at position N1 and O6 (marked in the structure **1**). The tethers were designed such that they themselves would have potential favorable electrostatic interactions with the rRNA A-site. The visualization and structure editing were performed using the Sybyl molecular modeling program.[35] This complex was energy-minimized using the Amber 5.0 package.[36] The point charges on compound **4** were obtained from ESP charges calculated by MOPAC package (PM3 Hamiltonian), and the

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parameters for the carbohydrate rings were according to Woods et al.[37] Sodium ions were added to the complex to neutralize the system using xleap routine as implemented in Amber 5.0, and the complete molecular assembly was solvated with TIP3 waters at least 10 Å from the surface of the assembly. Energy minimization was carried out for 10 000 iterations with a nonbonded cutoff of 12 Å.

Acknowledgment. This work was supported by two NIH grants (to C.S.C. and to S.M.). High-resolution mass spectra were obtained at the Michigan State University Mass Spectrometry Facility, which is supported, in part, by a grant (DRR-00480) from the Biotechnology Research Technology Program, National Center for Research Resources, and National Institutes of Health. We thank Leonard Lamsen for technical assistance.

Supporting Information Available: Synthetic methods and characterizations for all synthetic compounds and procedures for determination of the dissociation constants (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA011695M

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