Drug Design

Dimeric Aminoglycosides as Antibiotics**

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Aminoglycosides are an important class of natural products with antimicrobial activity against Gram-positive and Gram-negative bacteria.[1] They have important clinical applications in cases of opportunistic infections accompanying AIDS, cystic fibrosis, and cancer. They are primarily used against infections caused by Gram-negative bacteria, particularly *Pseudomonas aeruginosa*, which are the leading cause of morbidity and mortality in patients having cystic fibrosis (CF). However, the rapid emergence of aminoglycoside-resistant bacterial strains is limiting their efficacy and making the discovery of structurally novel and more potent classes of semisynthetic antibiotics a pressing necessity.[2]

Aminoglycosides exert their antibacterial action by selectively binding to the prokaryotic ribosome, and thereby impeding the synthesis of bacterial proteins.[3] In particular, 2-deoxystreptamine aminoglycosides (Figure 1) are known to selectively bind to the decoding A site on the 16S subunit of the ribosomal RNA, causing inhibition of translation and thus misreading of the genetic code.[4–10]

Structural studies modeling the interaction between neomycin-class aminoglycosides and the 16S A site of *E. coli* showed that multiple contacts occur between the major groove of the RNA and rings I and II of the antibiotic.[11–15] This conserved pseudodisaccharide motif is the essential pharmacophore responsible for most of the specificity of these interactions, while the rest of the molecule has a less well-defined role. Using surface plasmon resonance (SPR) and an immobilized 27-mer RNA construct (AS-wt), we discovered that neamine (the pseudodisaccharide corresponding to rings I and II of neomycin B, Figure 1) binds the oligonucleotide in a 2:1 stoichiometry, with a K_d of about 10 μM at each binding site. Relying on a favorable divalent interaction with the RNA target, we then constructed

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[**] Supported by the NIH (grants GM 58439 to C.-H.W. and SBIR 1 R43 AI056617-01 to S.J.S.).

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/ange.200353225

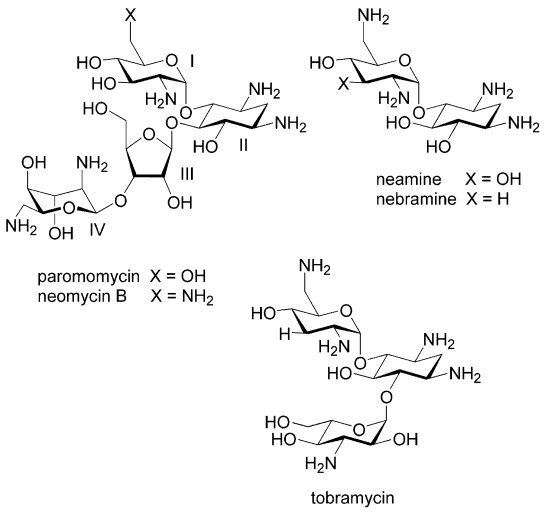


Figure 1. Structures of some representative aminoglycoside antibiotics of the 2-deoxystreptamine class.

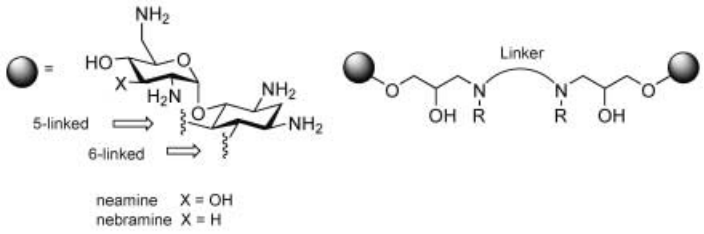
neamine dimers. It was determined that **1** (5-linked with a $(CH_2)_4$ tether) binds to AS-wt with a 1:1 stoichiometry, displaying a K_d of about 40 nM (Figure 2).[16]

Although this binding affinity is better than that of neomycin B, it is still about 250 times lower than the approximate theoretical value.[17] Since no direct structural information about the details of the divalent interaction is available, a rigorous rational-design approach was not possible. In order to improve the affinity of our dimers for RNA, we focused on three possible points of diversity: ring I of the pseudodisaccharide, the linker attachment points, and the linking domain. RNA affinity and antibiotic activity were monitored while structural modifications were introduced into compound **1** without modifying the tether length. At the same time the relative distance between the two neamine cores was systematically probed by using "unobtrusive" polyglycol tethers between 9 and 14 atoms in length, with the expectation that the activity would reach a maximum at the optimal distance between the two sites.

Compounds **5** and **9** were both prepared by an extension of the previously reported procedure,[16] starting from perazido perbenzyl derivatives of neamine and nebramine, respectively (see the Supporting Information for the experimental details). Compound **15** was derived from tobramycin (X = H, Figure 2) but displays a 4,5- (neomycin-type) linking pattern. In this case, after the diazo-transfer reaction, the α-D-glucopyranosyl bond of perazido tobramycin was cleaved by reaction with refluxing 1M H_2SO_4 in methanol (Scheme 1). The perazido nebramine so obtained was dissolved in 1:1 mixture of toluene and acetonitrile, placed on a rotary evaporator, and allowed to stir at 50 °C under reduced pressure with cyclohexanone dimethoxy ketal, in the presence of a catalytic amount of *p*-toluenesulfonic acid, to afford **10**. Subsequent benzylation of the free hydroxy group at C4′ and hydrolysis of the ketal afforded diol **11**. Treatment of the diol with tributyltin oxide followed by alkylation with BOMCl selectively freed the hydroxy group at C5′ for further manipulation. Compound **12** was then alkylated with (2*R*)-(−)-glycidyl tosylate, followed by treatment with 0.5 equivalents of *N*,*N*′-dibenzyl-1,4-diaminobutane. Staudinger reduction and catalytic hydrogenation finally gave deprotected dimer **15**.

The influence of the length and hydrophilicity of the linker was assessed by preparing two series of compounds: one with C_2-symmetric hydrophilic polyglycol tethers and the other with hydrophobic polymethylene tethers, as shown in Figure 3. The polyglycol linkers were obtained starting from the corresponding diallyl ethers, either by ozonolysis/borohydride reduction or hydroboration/oxidation reaction sequences, followed by standard tosylation conditions. The remaining linkers **17c** and **17f** were prepared by tosylation of commercially available tetra- and pentaethylene glycols, respectively. When a solution of the ditosylates **17** and two equivalents of perazido perbenzyl neamine **2** in DMF was treated with an excess of NaH, the reaction smoothly proceeded to afford protected dimers **18a–f** (Scheme 2). Surprisingly, when the corresponding bromides, iodides, and triflates were used, very sluggish reactions ensued. The order of addition of the reagents also proved to be crucial: preformation of the neamine alkoxide, followed by slow addition of the electrophile resulted in a complex mixture of products. A two-step catalytic hydrogenation finally afforded the deprotected dimers **19a–f** in 51–72% yields.[18] The more hydrophobic compounds **19g** and **19h** were obtained by similar routes, starting from commercial 1,10-decenediol and 1,12-dodecenediol, respectively.

When the series of polyglycol dimers **19a–f** was tested by means of SPR against immobilized AS-wt RNA, binding constants in the 1–8 μM range were



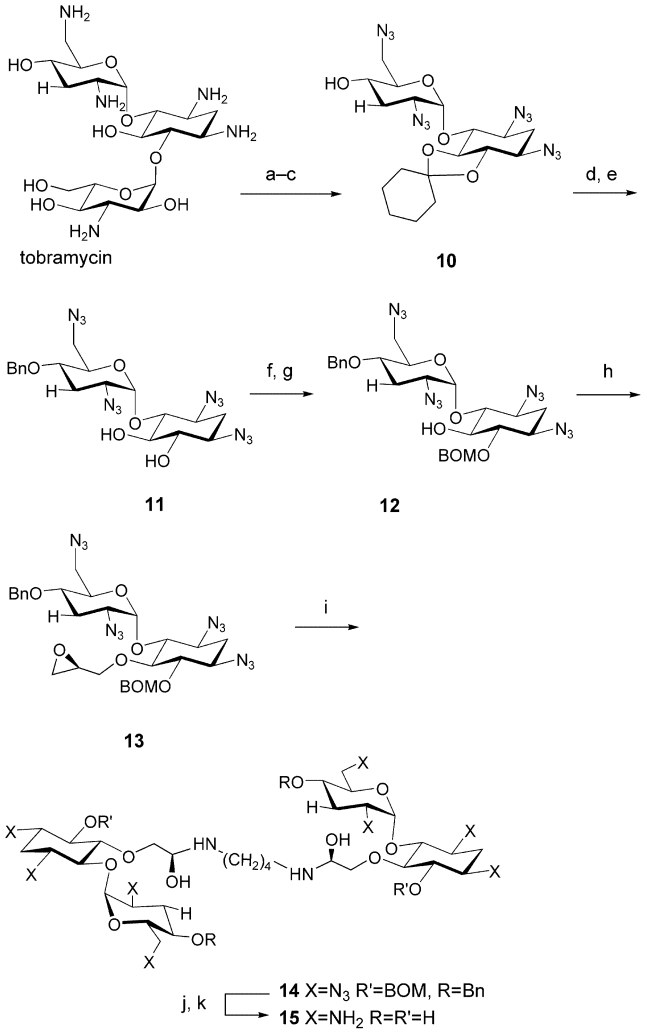
5-Linked Dimers

	Linker	X	R	K_d (nM)	MIC (μM) *S. aureus* ATCC 29213	MIC (μM) *E. coli* ATCC 25922	MIC (μM) *P. aeruginosa* ATCC 27853
1	$(CH_2)_4$	OH	CH_3	42	1.1	4.5	9
5	$(CH_2)_4$	OH	H	141	1.2	4.7	4.7
15	$(CH_2)_4$	H	H	166	2.5	20	5

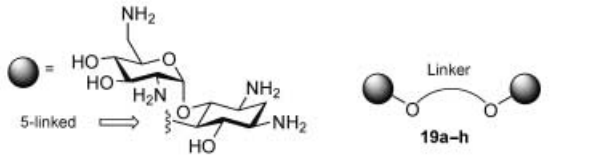
6-Linked Dimers

	Linker	X	R	K_d (nM)	MIC (μM) *S. aureus* ATCC 29213	MIC (μM) *E. coli* ATCC 25922	MIC (μM) *P. aeruginosa* ATCC 27853
9	$(CH_2)_4$	H	H	148	0.6	4.9	2.5

Figure 2. Structures, binding constants (K_d), and minimum inhibitory concentrations (MIC) for the neamine and nebramine dimers.

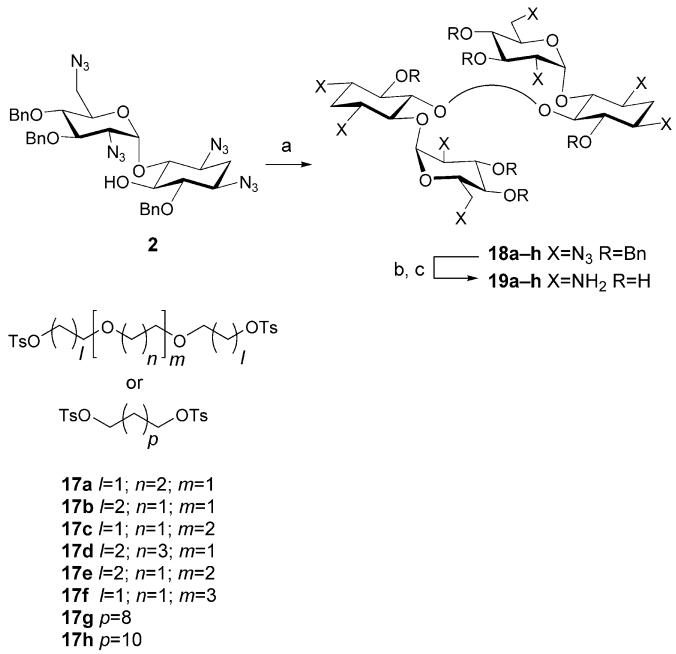


Scheme 1. Reagents and conditions: a) TfN_3, $CuSO_4$, TEA, MeOH, CH_2Cl_2, H_2O; b) H_2SO_4, MeOH, reflux; c) cyclohexanone dimethyl ketal, *p*TsOH, PhMe, MeCN, 50 °C, 20 mm Hg; d) BnBr, NaH, DMF; e) HCl, MeOH; f) SnO_2, PhMe, reflux; g) BOMCl, Bu_4NI; h) (*R*)-(−)-glycidyl tosylate, NaH, DMF; i) *N*,*N*′-dibenzyl-1,4-diaminobutane, EtOH, 70–85 °C; j) PMe_3, NH_4OH, H_2O, THF; k) H_2, $Pd(OH)_2/C$, H_2O/AcOH. BOMCl = benzyloxymethyl chloride, DMF = dimethylformamide, *p*TsOH = *p*-toluenesulfonic acid, TEA = triethylamine, Tf = trifluorosulfonyl.



	Linker	K_d (μM)	MIC (μM) *S. aureus* ATCC 27853	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
19a	$(CH_2)_2O(CH_2)_3O(CH_2)_2$	1.1	31	125	>250
19b	$(CH_2)_3O(CH_2)_2O(CH_2)_3$	3.6	125	>250	>250
19c	$(CH_2)_2[O(CH_2)_2]_2O(CH_2)_2$	1.8	62.5	>250	>250
19d	$(CH_2)_3O(CH_2)_4O(CH_2)_3$	3.3	>250	>250	>250
19e	$(CH_2)_3[O(CH_2)_2]_2O(CH_2)_3$	6.0	>250	>250	>250
19f	$(CH_2)_2[O(CH_2)_2]_3O(CH_2)_2$	1.4	62.5	>250	>250
19g	$(CH_2)_{10}$	5.3	>250	>250	>250
19h	$(CH_2)_{12}$	7.7	>250	>250	>250

Figure 3. Structures, binding constants (K_d), and minimum inhibitory concentrations (MIC) for neamine dimers with hydrophylic (**19a**–**f**) and hydrophobic (**19g**,**h**) tethers of increasing length. No obvious correlation is apparent between tether length and binding affinity.



Scheme 2. Reagents and conditions: a) **17a**–**h** (0.6 equiv), NaH, DMF; b) H_2, $Pd(OH)_2/C$, THF, MeOH; c) H_2, $Pd(OH)_2/C$, aq HCl (1 equiv), MeOH, H_2O.

found, with no obvious dependence of K_d on tether length (Figure 3). The antimicrobial activity was tested versus three standard, antibiotic-sensitive strains: *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853). The activity of the compounds was also weak, in some cases similar to that of neamine, but generally weakening with increasing tether length. A possible explanation for these results might reside in the high hydrophilicity of the linker. In order to test this hypothesis, two representative tether lengths (10 and 12 atoms) were chosen, and the more hydrophobic dimers **19g** and **19h** were synthesized. When tested against AS-wt RNA, they displayed K_d values and activities analogous to those of their polyether-linked counterparts.

The diaminobutane-linked dimers shown in Figure 2 possessed significant affinity and antimicrobial activity, and all displayed a binding stoichiometry of 1:1 for the RNA–antibiotic complex. Surprisingly, compound **5**, lacking the two *N*-methyl groups in the tether with respect to **1**, displayed slightly lower affinities and activities (about threefold) than its predecessor. These data might suggest that this additional hydrogen-bond-donating capability is not a fundamental

feature for binding and that the basicity of the two nitrogen atoms might play a role in determining such properties.

The K_d values of the two nebramine-derived dimers **9** and **15** were comparable to those of the neamine-derived compounds (in the 150 nM range), showing very little contribution of the 3′-hydroxy group to binding. The position of the anchor points for the two dimers (4,6 tobramycin-like for **9** and 4,5 neomycin-like for **15**) seems to play no significant role in the RNA recognition. In spite of these similarities, the antimicrobial activity for the two compounds differed by a factor of four against *E. coli*. These findings, and in particular direct comparison of compounds **1**, **5**, **15**, **19d**, and **19h**, show that the contribution of the linker to binding cannot be neglected and that a more subtle balance of interactions governs the binding affinities of these small ligands.

The antimicrobial activity of the diaminobutane-linked dimers was further investigated against different panels of aminoglycoside-sensitive and -resistant *P. aeruginosa* strains obtained from CF clinical isolates. The neamine-derived compounds **1** and **5** and the nebramine-derived dimer **9** possess activity levels comparable to (and in some cases greater than) tobramycin, which is considered the current gold standard for treatment of CF infections (Table 1). A very

Table 1: MIC values [$\mu g\,mL^{-1}$] of tobramycin and compounds **1** and **5** against *S. aureus* ATCC 33591 (MRSA) and *P. aeruginosa* clinical isolates.

Organism	Resistance	Tobramycin	1	5
S. aureus ATCC 33591 (MRSA)	resistant	>64	16	16
P. aeruginosa (strain 034)[a]	sensitive	8	8	8
P. aeruginosa (strain 609)[a]	resistant	128	8	4
P. aeruginosa (strain 663)[a]	resistant	128	8	8
P. aeruginosa (PAE_NUH01)[b]	resistant	>200	12.5	6.3
P. aeruginosa (PAE_NUH02)[b]	resistant	50	6.3	6.3
P. aeruginosa (PAE_NUH03)[b]	resistant	50	6.3	6.3
P. aeruginosa (PAE_NUH04)[b]	resistant	100	6.3	3.1
P. aeruginosa (PAE_NUH05)[b]	resistant	100	6.3	3.1
P. aeruginosa (PAE_NUH06)[b]	resistant	100	12.5	6.3

[a] Strains obtained from the Foothills Medical Centre, Calgary, AB, Canada. [b] Strains obtained from National University Hospital, National University of Singapore.

common complication in CF-related *P. aeruginosa* infections is biofilm formation. Interestingly, when tested against a panel of biofilm-producing *P. aeruginosa* strains from CF isolates, the tobramycin-derived compound **15** was revealed to be uniquely effective and in many cases more potent than tobramycin itself (Table 2).

Further SPR analysis of **1** and **5** interacting with other RNA targets of interest showed that both compounds are more active than neomycin B against the 16S A site (1.5- to 5-fold, Table 3). A possible explanation of this finding might reside in the increased positive charge density that these molecules bear with respect to neomycin B. However, a

Table 2: MIC [$\mu g\,mL^{-1}$] of compounds **9** and **15** against a panel of biofilm-producing *P. aeruginosa* from CF clinical isolates.

P. aeruginosa	Resistance	Tobramycin	9	15
27 EN	sensitive	4	2	1
64 EN	sensitive	8	32	4
24 EN	sensitive	4	64	2
25 EN	sensitive	8	>64	4
90 EM	resistant	64	>64	4
98 EM	resistant	16	4	0.5
89 EM	resistant	64	>64	4

Table 3: K_d [μM] and selectivity profile of neomycin B and compounds **1** and **5** for the 16S A site (expressed as the ratio of the binding constants, in parentheses) in RNAs of interest.

RNA[a]	Neomycin B	1	5
16S A site	0.2	0.04	0.14
Bcr-Abl	2 (10)	0.55 (14)	0.46 (3)
PAX3-FKHR	0.8 (4)	1.1 (27)	1.4 (10)
HIV-FSS	1.6 (8)	1.1 (27)	1.9 (13)
HIV-PAS mRNA	2 (10)	1.2 (30)	1.7 (12)
HCV-IIb IRES	2.9 (14)	0.58 (14)	0.83 (6)
HCV-IIId IRES	2.9 (14)	1.3 (32)	1.8 (13)
E. coli TG mRNA	2.8 (14)	1.6 (40)	2.7 (19)

[a] Arbitrary abbreviations used. HIV-FSS: HIV frameshift signal; HIV-PAS: HIV protease active site mRNA; HCV-IIb IRES: HCV IRES domain IIb; HCV-IIId IRES: HCV IRES domain IIId; *E. coli* TG: *E. coli* transglycosilase mRNA. See the Supporting Information for RNA sequences.

generally significant increase in selectivity for the 16S A site for compound **1** (and, to a lesser extent, compound **5**) reveals that more subtle factors govern the specificity of the aminoglycoside–RNA interaction. Indeed, design of more potent and selective binders remains the major challenge for future research in the field of aminoglycoside antibiotics.

In conclusion, the neamine and nebramine dimers synthesized in this study represent a new class of aminoglycoside antibiotics that are active against several aminoglycoside-resistant bacterial strains and may show promise for the treatment of *P. aeruginosa* infections. In order to fully elucidate the structural details of the dimeric interaction and further improve the affinity, potency, and specificity of newly designed aminoglycoside antibiotics, additional studies are currently underway.

Received: November 3, 2003 [Z53225]

Keywords: aminoglycosides · antibiotics · carbohydrates · drug design · RNA recognition

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[17] Assuming independent binding events for each site, the total binding energy for a dimerically linked molecule ΔG_T should be the sum of the binding energies for each monomer, ΔG_1 and ΔG_2, plus the contribution of the linker ΔG_L. ΔG_L includes both the free energy of the linker interaction with the RNA and also a term that accounts for the reduced entropy change of binding due to the tethering of the monomers. If the effect of the linker is ignored in a first approximation, the resulting K_d should therefore be in the picomolar range.

[18] A single step deprotection using 20% $Pd(OH)_2/C$ (Degussa type) in 1:1 $AcOH/H_2O$ under H_2 could also be achieved when fresh catalyst was added after the reaction appeared to stop.

