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DIMERIC AMINOGLYCOSIDES: DESIGN, SYNTHESIS AND RNA BINDING

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Abstract: The design and synthesis of symmetrical and non-symmetrical dimeric aminoglycosides derived from kanamycin A, tobramycin, and neomycin B are described. The covalently-linked dimeric aminoglycosides show enhanced activity as ribozyme inhibitors when compared to their natural counterparts.

Aminoglycoside antibiotics are known to interact with a variety of RNA molecules, including ribosomal RNA,[1,2] group I introns,[3] and the hammerhead ribozyme.[4] These low molecular weight antibiotics have also been found to block the binding of the HIV-1 Rev protein to its viral RNA recognition site, thereby inhibiting the production of the virus.[5] Recently, aminoglycoside antibiotics have been shown to inhibit the binding of a Tat-derived peptide to TAR RNA.[6] While this family of molecules emerge as an intriguing group of RNA binders, the molecular details of aminoglycoside-RNA interactions are still obscured.[7]

How do aminoglycoside antibiotics bind to RNA? Since no sequence homology between the different RNA families known to interact with aminoglycosides exists, such small organic molecules are likely to recognize similar structural motifs.[8] The antibiotics may bind to existing binding sites within the folded RNA or induce a conformational change, thus altering the three-dimensional structure of the host RNA. An important feature of both models is that, in principle, several sites with different affinities may coexist in a given RNA molecule.[9] We have sought to explore this possibility by synthesizing covalently-linked dimeric aminoglycosides. In this contribution we report on the design, synthesis, and hammerhead ribozyme inhibitory activity of dimeric aminoglycoside antibiotics derived from kanamycin A, tobramycin, and neomycin B (Figure 1). We demonstrate that certain covalently-linked dimers are approximately tenfold more efficient than their natural counterparts in inhibiting the hammerhead ribozyme.

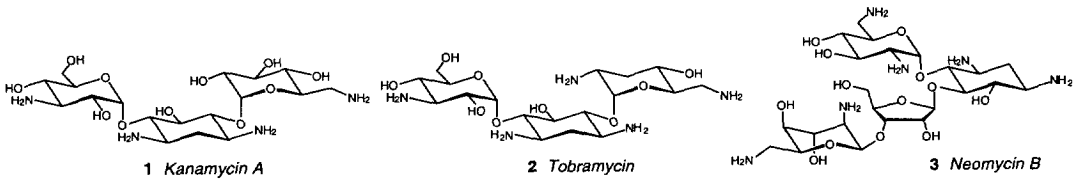
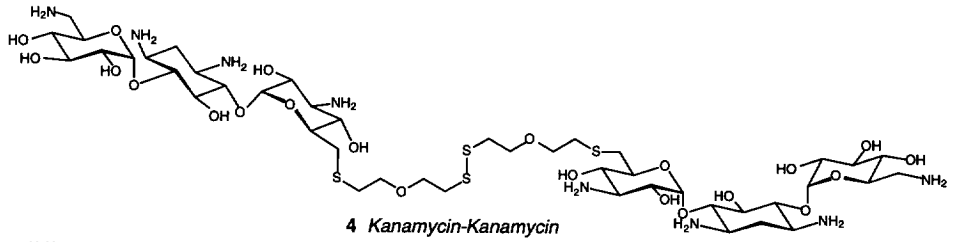
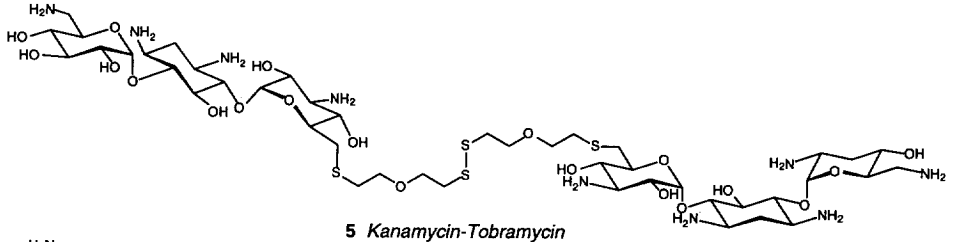


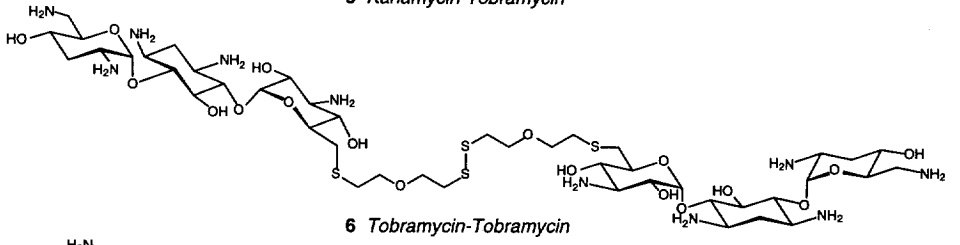
Figure 1. Natural aminoglycoside antibiotics.



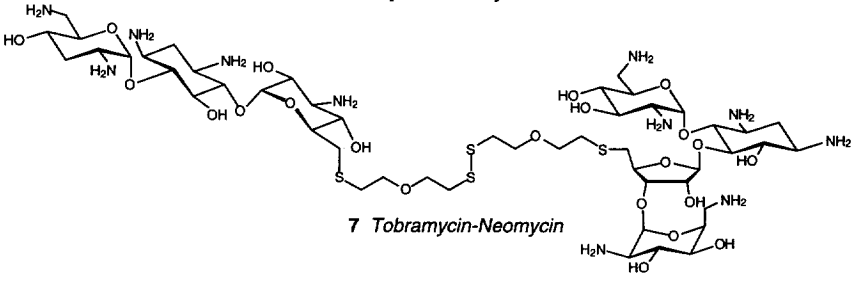
4 *Kanamycin-Kanamycin*



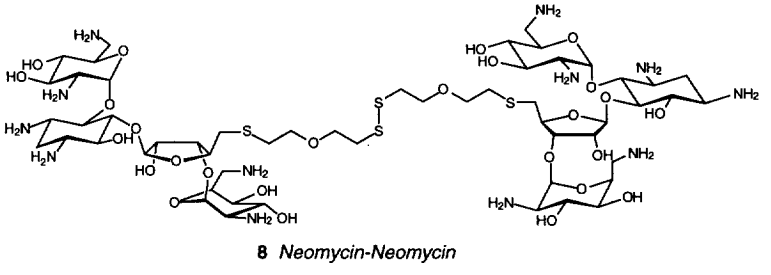
5 *Kanamycin-Tobramycin*



6 *Tobramycin-Tobramycin*



7 *Tobramycin-Neomycin*

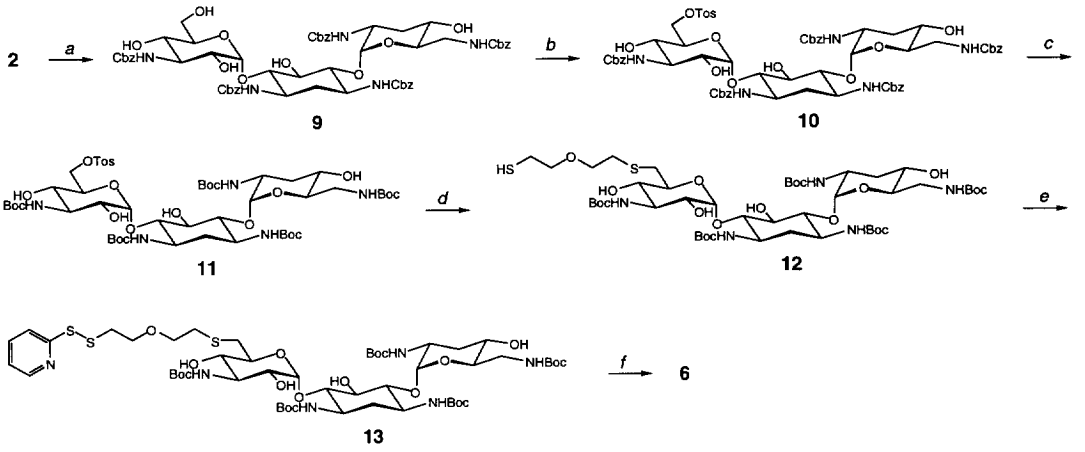


8 *Neomycin-Neomycin*

Figure 2. Dimeric aminoglycosides.

In designing dimeric aminoglycosides, three major factors had to be considered: (1) the tethering position, (2) the nature of the linker, and (3) synthetic accessibility and versatility. We have recently synthesized and studied a series of systematically deoxygenated analogs of tobramycin. Preliminary results have indicated that 6"-deoxytobramycin is comparable to tobramycin in its affinity to the hammerhead ribozyme, indicating that the 6"-OH is not essential for RNA binding.[10] This primary position has therefore been selected as the tethering point (Figure 2). We have initially chosen a relatively long and conformationally flexible linker in order to allow the two aminoglycosidic portions to "scan" the conformational space in the search for an additional RNA binding site, thus increasing the chances to observe cooperativity and enhanced inhibitory activity. In addition, selecting a disulfide bridge as the central linkage introduces synthetic flexibility, making both symmetrical as well as non-symmetrical dimers readily accessible.

We have developed a general synthetic strategy that allows us to readily synthesize symmetrical as well as non-symmetrical dimeric aminoglycosides. The approach is illustrated in Scheme 1 for the tobramycin–tobramycin dimer **6**. Tobramycin is first protected as its Cbz derivative to give **9**, followed by tosylation of the primary 6"-hydroxyl to give **10** in 52% yield. The fully Cbz-protected monotosylate derivative **10** is converted in 93% yield to the Boc-protected derivative **11** in a one-pot "deprotection-protection" step. Treatment of **11** with five equivalents of 2-mercaptoethyl ether in DMF in the presence of one equivalent of cesium carbonate affords the monothiol **12** in 93% yield. Treatment with Aldrithiol™ affords the protected thiol **13** in 85% yield. This derivative can be treated under mild conditions with another molecule of the free thiol **12** to give the protected tobramycin–tobramycin dimer in 65% yield. Mild acidic deprotection affords the desired dimer **6** in 95% yield.[11] The other tobramycin-containing dimers have been obtained according to a similar procedure by treating the protected thiol **13** with the appropriate thiol-linked aminoglycosides. All other dimers have been synthesized by using the same approach.[12]



Scheme 1. The synthesis of dimer **6**. Reagents: (a) $PhCH_2OCOCl$, H_2O, Na_2CO_3; (b) TosCl, pyridine; (c) Boc_2O, H_2, Pd/C, MeOH; (d) $HSCH_2CH_2OCH_2CH_2SH$, Cs_2CO_3, DMF; (e) Aldrithiol™, MeOH; (f) (i) **12**, MeOH, (ii) 10% TFA/CH_2Cl_2.

The natural and dimeric aminoglycosides have been tested for their ability to inhibit the hammerhead ribozyme, a well-studied small RNA enzyme.[13,14] The effect of adding dimeric aminoglycoside derivatives on the rate of the cleavage reaction of the hammerhead ribozyme has been studied at 21 °C under single turnover conditions in the presence of subsaturating Mg^{2+} concentration and subsaturating ribozyme.[15] Figure 3 illustrates a typical experiment, and Table 1 summarizes the pseudo first-order rate constants obtained at different aminoglycoside concentrations.

Under our conditions, the ribozyme cleaves its substrate with a pseudo first-order rate constant of 0.075 min^{-1}. At a 100 μM concentration, kanamycin A (**1**) slows the ribozyme down to about 75% of its rate, while at 10 μM, kanamycin A does not inhibit the ribozyme at all. In contrast, kanamycin–kanamycin dimer **4** at 10 μM, decreases the cleavage rate fivefold (Figure 3). This observation indicates that covalently linking two aminoglycosides can convert a very poor RNA binder to a potent one. Linking kanamycin A to a stronger RNA binder such as tobramycin further enhances the affinity to RNA. Thus, the non-symmetrical dimer kanamycin–tobramycin **5** is a better inhibitor than kanamycin–kanamycin **4**. On the other hand, tobramycin–tobramycin dimer **6** is a strong inhibitor at 10 μM, slowing down the ribozyme by a factor of seven. Since the parent tobramycin (**2**) is a relatively weak ribozyme inhibitor under these conditions, this observation demonstrates that "dimerizing" a moderate ribozyme binder can substantially improve its RNA binding capability.

Neomycin B (**3**) is one of the strongest RNA binders. At 10 μM it shows strong inhibitory activity that is comparable to the kanamycin–kanamycin dimer **4**. Even at 1 μM, neomycin B shows some observable inhibition (ca. 20%). Covalently attaching neomycin B to tobramycin or to itself, yields the nonsymmetrical dimer **7** and the symmetrical one **8**, respectively. At 10 μM dimers **7** and **8** are extremely potent inhibitors, slowing down the ribozyme by a factor of ten and twenty-five, respectively. These observations indicate that even the RNA binding capability of the strongest binders can still be enhanced. However, it appears that with this relatively small RNA enzyme, we may have reached an upper limit with dimers **7** and **8**, since at 1 μM these dimers slow down the hammerhead ribozyme by a factor of two at the most.

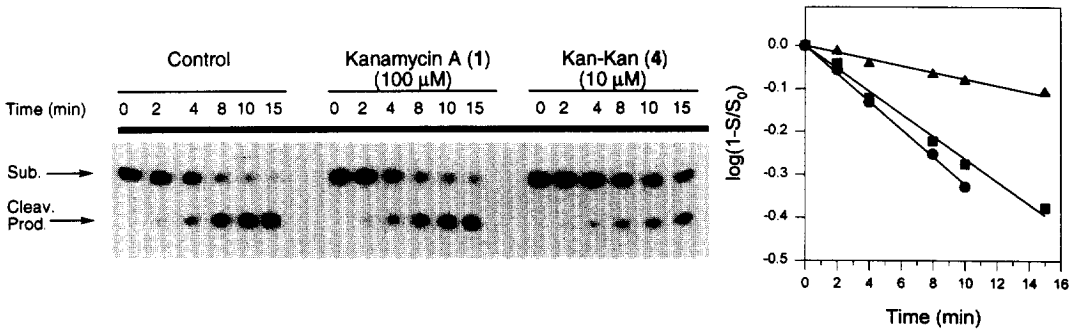


Figure 3. *Left:* an autoradiogram of a 20% polyacrylamide gel used to separate the products of the time-dependent hammerhead ribozyme cleavage reactions in the absence (control) and the presence of the kanamycin A **1** and kanamycin–kanamycin dimer **4**. *Right:* kinetic analysis of the quantified gel. Control (●), 100 μM kanamycin A (■), 10 μM kanamycin–kanamycin dimer **4** (▲).

Table 1. Pseudo first-order rate constants for the inhibition of the cleavage step (k_2) of the hammerhead ribozyme by aminoglycoside antibiotics and their dimeric derivatives[a]

Aminoglycoside Derivative	k_2 (min^{-1}) 100 μM	10 μM	1 μM
None (Control)		**0.075**	
Kanamycin A (**1**)	0.058	-[b]	-[b]
Tobramycin (**2**)	0.012	0.051	-[b]
Neomycin B (**3**)	-[c]	0.018	0.062
Kanamycin–Kanamycin (**4**)	-[c]	0.017	-[b]
Kanamycin–Tobramycin (**5**)	-[c]	0.014	-[b]
Tobramycin–Tobramycin (**6**)	-[c]	0.011	-[b]
Tobramycin–Neomycin (**7**)	-[c]	0.008	0.046
Neomycin–Neomycin (**8**)	-[c]	0.003	0.041

[a] See footnote 15 for reaction conditions and kinetic analysis. The values given are the average of three independent experiments, and the error is estimated to be 10%. [b] No inhibition is observed at this concentration. [c] Under these conditions, the derivatives are too active and complete inhibition is observed.

In summary, we have demonstrated that symmetrical as well as non-symmetrical dimeric aminoglycosides can be simply synthesized. These highly charged aminoglycoside derivatives show enhanced affinity to RNA when compared to their "monomeric" counterparts. In general, covalently linking two moderate or good RNA binders (e.g., tobramycin, neomycin B) provides novel derivatives with an inhibitory activity surpassing any natural aminoglycoside antibiotic. Experiments exploring the biophysical aspects of aminoglycosides dimerization as well as the RNA binding of these dimers to larger RNA enzymes (e.g., group I introns) are underway in our laboratory.

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11. Selected data for dimer **6**: UV (H_2O) 250 nm, ε 427 (S-S). ESI MS (MeOH/H_2O) Calcd for $C_{44}H_{89}N_{10}O_{18}S_4$ $[M+H]^+$ 1174.5, Found 1174.1; Calcd for $C_{44}H_{88}N_{10}NaO_{18}S_4$ $[M+Na]^+$ 1196.5, Found 1196.2. ^1H NMR (500 MHz, D_2O, 298K), δ 5.37 (d, J = 2.5 Hz, 2H, H1'), 5.05 (d, J = 4.0 Hz, 2H, H1''), 4.02 (m, 2H, H5''), 3.84 (t, J = 5.6 Hz, 4H, S-S-CH_2CH_2O), 3.81 (m, 2H, H5), 3.74 (t, J = 5.6 Hz, 4H, 6''-S-CH_2CH_2O), 3.72 (m, 2H, H5'), 3.58 (m, 4H, H4', H2''), 3.44 (t, J = 10.0 Hz, 2H, H4''), 3.34 (m, 4H, H4, H6), 3.29 (m, 2H, H3''), 3.12-3.04 (m, 10H, H1, H3, H2', S-S-CH_2CH_2O), 2.96 (m, 6H, one of H6', 6''-S-CH_2CH_2O), 2.83 (m, 4H, H6''), 2,78 (d×d, J_1 = 7.5 Hz, J_2 = 15.0 Hz, 1H, H6'), 2.10 (m, 2H, H3eq), 2.02 (m, 2H, H2'eq), 1.78 (d×d, $J_1 = J_2$ = 12.0 Hz, H3ax), 1.31 (d×d, $J_1 = J_2$ = 12.5 Hz, H2'ax). ^{13}C NMR (125 MHz, D_2O, 298K), 99.8, 97.9, 87.6, 83.8, 74.7, 71.8, 71.6, 71.2, 70.3, 69.3, 68.0, 65.9, 54.0, 50.0, 49.1, 48.7, 40.6, 37.3, 34.3, 33.5, 33.2, 31.8.
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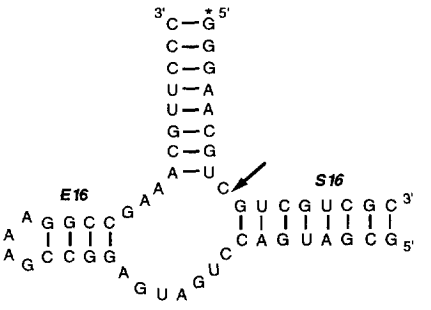
14. Secondary structure of the hammerhead ribozyme (HH16), showing the enzyme E16 and the ^{32}P 5'-labeled substrate S16 (see ref 4). The arrow shows the cleavage site.



Previous studies have shown that aminoglycoside antibiotics interact preferentially with the enzyme-substrate complex and inhibit the cleavage step by displacing critical Mg^{2+} ions. See ref 4 and: Clouet-d'Orval, B., Stage, T. K.; Uhlenbeck O. C. *Biochemistry* **34**, 11186.
Both E16 and S16 were prepared by in vitro transcription reactions of the corresponding synthetic DNA templates using T7 RNA polymerase. The crude transcripts were purified by preparative electrophoresis on a 20% polyacrylamide gels. The gel was UV-shadowed; the bands were excised, crushed and extracted with 200 mM KOAc, 1 mM EDTA, pH 5.4 for 20 h at 4 °C. The RNA was precipitated, dried, resuspended in DEPC water, and kept at -80 °C.
15. Reaction conditions: 20 nM E16, trace ^{32}P 5'-labeled S16 (≤1 nM), 7.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.3, with varying concentrations of aminoglycosides. Reactions were initiated by mixing equal volumes of preincubated solutions of the enzyme E16 and the labeled substrate S16 (see scheme of HH16 in footnote 14). Aliquots were removed at various times and quenched by adding excess stop solution/loading buffer (8 M urea, 50 mM Na_2-EDTA, pH 7.3, bromophenol blue and xylene cyanol). The substrate and product were resolved on 20% polyacryamide/7M urea gels, and the fraction cleaved was quantified using a Molecular Dynamics Phosphorimager and ImagQuant™ software. Plotting log(1-S/S_0) vs. time (min), gives a straight line with a slope of $-k_2$.

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