RNA – Aminoglycoside Interactions: Design, Synthesis, and Binding of "Amino-aminoglycosides" to RNA**

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At the frontiers of RNA chemistry and biology stands the intriguing and largely unexplored area of RNA recognition. Despite the central role RNA plays in numerous biological processes, little is known about the interactions between small organic molecules and RNA biomolecules.[1, 2] The discovery that aminoglycoside antibiotics interact specifically with diverse RNA molecules—such as 16S ribosomal RNA,[3, 4] introns of group I,[5] and the hammerhead ribozymes[6]—and block the binding of the Rev protein[7] and Tat peptide[8] to their viral RNA targets attracted considerable interest and stimulated studies attempting to identify the elements involved in these recognition phenomena.[9, 10] The elucidation of general "recognition rules" for RNA – ligand interactions is essential for developing RNA binders of low molecular weight that target pivotal bacterial and viral RNA sites.

As aminoglycosides are highly functionalized polycationic oligosaccharides, interactions between their polar residues (amino and hydroxyl groups) and the RNA backbone and/or heterocyclic bases are likely to occur.[11] Since the amino groups are predominantly protonated at pH 7.0,[12] the overall charge density presented by the aminoglycosides toward the RNA host is likely to be important for RNA binding. The reported structure – activity relationships for natural aminoglycosides support this general view. Hence, aminoglycosides containing four amino groups show very little ability to bind RNA, whereas the most active derivatives contain five or six amino groups.[5–8] Although rather general, this view does not explain marked differences in RNA binding among related aminoglycoside antibiotics.[5, 7]

Here we focus on the importance of electrostatic interactions for binding between RNA and aminoglycoside, and address the following questions: 1. Will the ability of poor RNA binders (e. g. kanamycin A) to bind RNA be increased by incorporating an additional amino group into their structures? 2 Will the ability of the most active antibiotics (tobramycin and neomycin) to bind RNA be further increased by adding more amino groups? 3. Can a common recognition pattern be identified? We report here the design, synthesis, and ribozyme inhibitory activity of novel "amino-aminoglycosides" derived from kanamycin A, tobramycin, and neomycin B. We provide experimental evidence for the critical role of the overall charge of aminoglycosides in RNA binding and propose a general recognition model emphasizing the stereochemical display of the charged ammonium groups.

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In designing amino-aminoglycosides three major factors had to be considered: 1. the modification site, 2. the degree of substitution of the amine and its basicity, and 3. synthetic accessibility. We recently synthesized and studied a series of systematically deoxygenated analogues of tobramycin. Preliminary results indicated that 6″-deoxytobramycin is comparable, yet slightly inferior, to tobramycin in its affinity to the hammerhead ribozyme.[13] This observation suggests that while the primary 6″-OH group might not be essential for RNA binding it is likely to be involved in RNA contact. Regarding the basicity of the amino groups, the primary aminomethyl groups ($-CH_2NH_2$) in various aminoglycosides are more basic (pK_a 8.6 – 9.0) than the other primary amines (pK_a 6.2 – 8.1).[12] Taken together with the relative ease of derivatizing a primary alcohol, we selected position 6″ in kanamycin A and tobramycin, and position 5″ in neomycin B as the modification sites.

The derivatives studied (Figure 1) include kanamycin A (**1**), 6″-amino-6″-deoxykanamycin A (**2**), kanamycin B (**3**), tobramycin (**4**), 6″-amino-6″-deoxytobramycin (**5**), neomycin B (**6**), and 5″-amino-5″-deoxyneomycin B (**7**). These represent aminoglycoside derivatives in which a single primary hydroxyl group is replaced by a primary amine while all other functional groups remain intact. A representative synthesis is illustrated in Scheme 1 for **5**. Tobramycin (**4**) was first protected as its Cbz derivative **8** and then tosylated at the primary 6″-hydroxyl group. The fully Cbz-protected monotosylate **9** was treated with sodium azide to afford **10**, which was then reduced to amine **11** with triphenylphosphane and an aqueous solution of sodium hydroxide. Catalytic hydrogenation afforded **5**. Similar transformations were utilized for synthesizing **2**[14] and **7**.[15]

The aminoglycosides and their amino derivatives were tested for their ability to inhibit the hammerhead ribozyme, a well-studied, small RNA enzyme.[6, 16] The effect of aminoglycoside derivatives on the cleavage rate of the hammerhead ribozyme was investigated at pH 7.3 under single-turnover conditions in the presence of Mg^{2+} and ribozyme at subsaturating concentrations. Figure 2 illustrates the results of 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 at a rate of 0.075 min^{-1}. At a concentration of 100 μM, kanamycin A (**1**) slows the ribozyme down to about 75 % of this rate, whereas at 10 μM **1** does not inhibit the ribozyme at all. In contrast, at a concentration of 100 μM 6″-amino-6″-deoxykanamycin A (**2**) decreases the cleavage rate fivefold, which is similar to the effect induced by kanamycin B (**3**). This observation indicates that adding an amino group can convert a very poor RNA binder into a reasonably strong one. Modifying a stronger RNA binder such as tobramycin further enhances its affinity to RNA. Thus, at a concentration of 10 μM 6″-amino-6″-deoxytobramycin (**5**) decreases the ribozyme-cleavage rate fivefold more effectively than the parent tobramycin (**4**).

Neomycin B (**6**) is one of the strongest RNA binders. At a concentratin of 10 μM it shows strong inhibitory activity that is comparable to that of **2** at 100 μM. Replacing the 5″- primary

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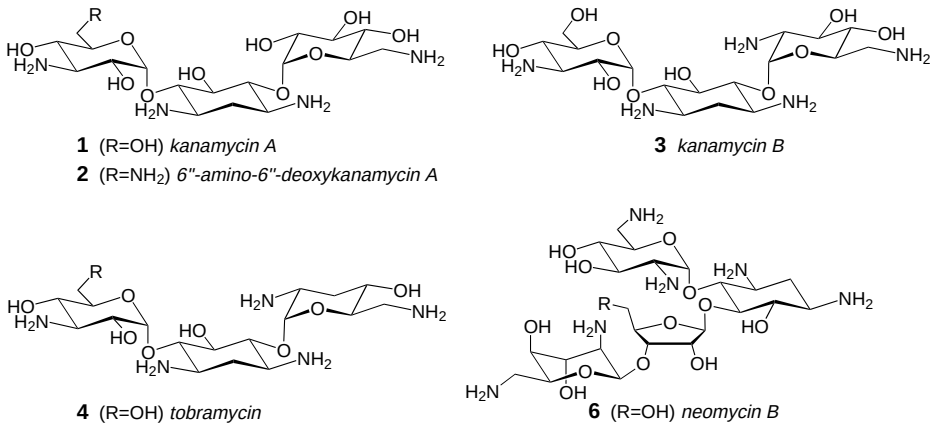
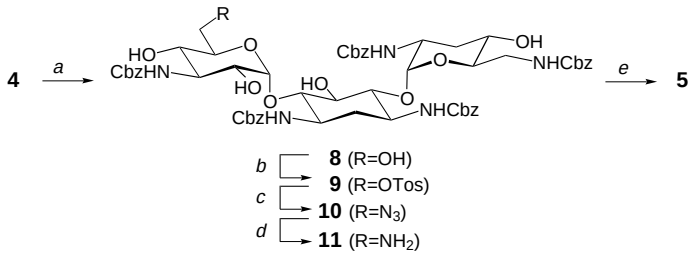


Figure 1. The structural formulas of the natural aminoglycosides and their amino derivatives studied in this work.

OH group with a primary NH_2 group leads to a novel aminoglycoside derivative **7** containing seven amino groups. At 10 μM **7** is an extremely potent inhibitor, and slows down the ribozyme by a factor of twenty-five. At 1 μM **7** slows down the hammerhead ribozyme twice as effectively as the parent neomycin B (**6**). These observations indicate that even the ability of the most active aminoglycosides to bind RNA can be further enhanced by increasing their overall charge.

The comparable ribozyme inhibitory activity of 6″-amino-6″-deoxykanamycin A (**2**) and kanamycin B (**3**) may not be coincidental. Both derivatives have five amino groups, albeit in different positions of the aminoglycosidic skeleton, and are likely to have a similar overall positive charge at a given pH. We propose that these aminoglycosides present a similar stereochemical array of positive charges. Thus, **2** is rotated around an imaginary pseudo-C_2 axis going through positions 2 and 5 in the central 2-deoxystreptamine ring so that the two central rings "overlap" (Figure 3). Amino groups 1 and 3 of **2** can overlap with amino groups 3 and 1 of **3**. The 3″-NH_2 group of **2** can take the position of the flexible 6′-NH_2 group in **3**, and the new 6″-NH_2 group in **2** can assume the position of the 2′-NH_2 group in **3**. In addition, 6′-NH_2 of **2** can occupy the position of 3″-NH_2 in **3**.[17] Taken together with the spherical shape of an ammonium group and its geometrical degeneracy, both derivatives can display a similar array of ammonium groups and charge densities toward the RNA skeleton (Figure 3).



Scheme 1. Synthesis of 6″-amino-6″-deoxytobramycin (**5**). Reagents and conditions: a) $PhCH_2OCOCl$, H_2O, Na_2CO_3, 90%; b) toluenesulfonyl chloride, pyridine, 4 °C, 52%; c) NaN_3, DMF, 80%; d) Ph_3P, THF, NaOH/H_2O; e) H_2, Pd/C, dioxane, H_2O, AcOH, 60% (for d+e).

Table 1. Inhibition of the hammerhead ribozyme by aminoglycoside derivatives.[a]

Derivative	k_2 [min^{-1}] 100 μM	10 μM	1 μM
none (control)		0.075	
kanamycin A (**1**)	0.058	–[b]	–[b]
6″-amino-6″-deoxykanamycin A (**2**)	0.018	0.050	–[b]
kanamycin B (**3**)	0.018	0.060	–[b]
tobramycin (**4**)	0.012	0.051	–[b]
6″-amino-6″-deoxytobramycin (**5**)	–[c]	0.011	–[b]
neomycin B (**6**)	–[c]	0.018	0.062
5″-amino-5″-deoxyneomycin B (**7**)	–[c]	0.003	0.030

[a] See Experimental Section for conditions. 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.

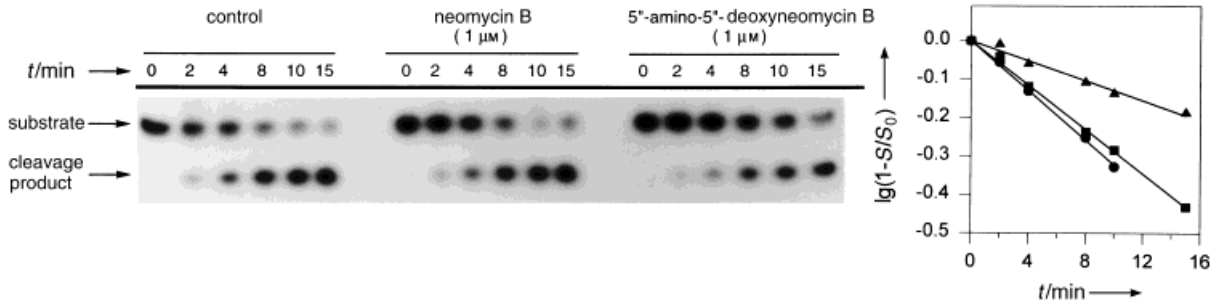


Figure 2. Left: autoradiograms of a 20% polyacrylamide gel used to separate the products of the time-dependent hammerhead ribozyme cleavage reactions in the absence (control) and the presence of **6** and **7**. Right: kinetic analysis of the quantified gel. Control (●), 1 μM **6** (■), and 1 μM **7** (▲).

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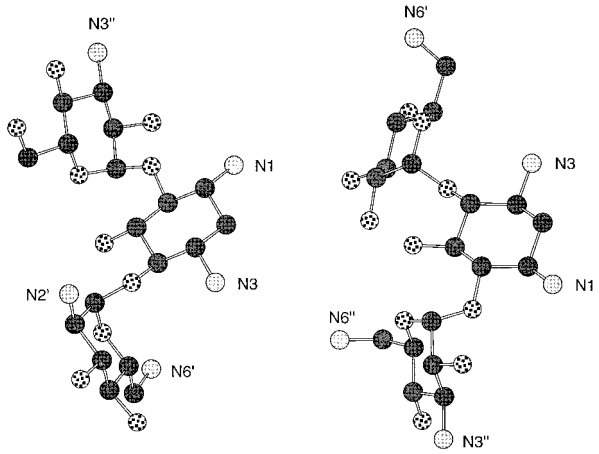

2 *kanamycin B* **3** *6''-amino-6''-deoxykanamycin A*

Figure 3. Minimized conformations (Spartan program) of the fully protonated 6″-amino-6″-deoxykanamycin A (**2**) and the rotated kanamycin B (**3**) showing the similar position occupied by the ammonium groups in space. C: dark gray, N: light gray, O: dotted.

Previous studies showed that aminoglycoside antibiotics interact preferentially with the enzyme–substrate complex of the hammerhead ribozyme and inhibit the cleavage step.[6] The positively charged ammonium groups were suggested to displace critical Mg^{2+} ions.[18] Our results support this view and clearly demonstrate that the total number of amino groups in the aminoglycoside is correlated with inhibitory activity and RNA binding. Our aminoglycoside–RNA recognition model proposes a specific yet versatile three-dimensional projection of positively charged ammonium groups towards acceptor groups on the RNA binding site. This is in agreement with the results of recent NMR studies[10] and MD simulations[19] that suggest a three-dimensional electrostatic complementarity rather than highly specific contacts between aminoglycoside antibiotics and their RNA hosts.

Our studies have important implications for the design of RNA binders.[20] Such molecules will have to possess sufficient positive charges to bind electrostatically to the RNA target site. Competition with bound Mg^{2+} can be achieved by a strategic incorporation of ammonium groups with predetermined N–N distances and three-dimensional projection, and possibly by the incorporation of functional groups capable of coordinating Mg^{2+}. RNA binders of low molecular weight with higher efficacy and lower toxicity than aminoglycoside antibiotics may replace these drugs in clinical uses and may find novel applications as anti-viral agents.

Experimental Section

The syntheses of the aminoglycoside derivatives will be reported elsewhere.

The hammerhead ribozyme used was HH16, which contains the enzyme E16 and the ^{32}P-5′-labeled substrate S16.[6] Both E16 and S16 were prepared by in vitro transcription reactions of the corresponding synthetic DNA templates using T7 RNA polymerase. Ribozyme reaction conditions: 20 nM E16, trace ^{32}P-5′-labeled S16 ($\leq$ 1 nM), 7.5 mM $MgCl_2$, 50 mM Tris-HCl, and pH 7.3 with varying concentrations of aminoglycosides. The reactions were initiated by mixing equal volumes of preincubated solutions of the enzyme E16 and the labeled substrate S16. Aliquots were removed at various times and quenched by adding excess stop solution/loading buffer (8 M urea, 50 mM disodium ethylenediaminetetraactate (Na_2EDTA), pH 7.3, bromophenol blue, and xylene cyanol). The substrate and product were resolved on 20% polyacryamide/7 M urea gels, and the fraction cleaved was quantified using a Molecular Dynamics Phosphorimager with ImagQuant software. Plotting $\lg(1 - S/S_0)$ vs. time gives a straight line with a slope of $-k_2$ (Figure 2, right).

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