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Conjugate of neamine and 2-deoxystreptamine mimic connected by an amide bond

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ABSTRACT

An amino-functionalized 2-deoxystreptamine (2-DOS) mimic was conjugated by an amide bond to a neamine moiety containing a carboxylic acid in ring II. A library of A-site RNA and its mutants was prepared to examine RNA binding characteristics of the additional 2-DOS moiety attached to neamine. The 2-DOS mimic conjugated to the neamine increased binding affinity up to 200-folds compared to that of neamine. The conjugate binds to native A-site RNA and its mutants with up to 6-fold difference in sequence selectivity.

RNA is increasingly recognized as a practical drug target due to their important biochemical functions and unique, but well-defined, three-dimensional structures such as loops, bulges, and pseudo-knots.[1–3] However, it remains a formidable challenge to design small molecules to selectively recognize certain RNA sequences or sites.

Aminoglycosides, a large class of clinically important antibiotics, received considerable interest due to their capability to bind to various RNA targets and have been used as a model in understanding the governing principles of RNA recognition by small molecules.[4–17] Unfortunately, they have shown a high degree of binding promiscuity and often lack specificity,[18,19] which is largely attributed to conformational flexibility rendered by glycosidic bonds and multiple amino groups present in aminoglycosides resulting in electrostatically driven binding interaction with target RNAs.

Although aminoglycosides as a whole demonstrate poor binding selectivity, 2-deoxystreptamine (2-DOS), the highly conserved central aminocyclitol scaffold of aminoglycosides, demonstrates sequence-specific recognition capability toward 5′-GU-3′ sequence within the context of various aminoglycosides.[20] Similarly, 2-DOS alone, without an aminosugar attached, was shown to recognize 5′-GU-3′ although its binding affinity was low (~1 mM).[21] These results indicate that 2-DOS binding is not exclusively driven by electrostatic interactions known to promote non-specific interactions, but rather by hydrogen bonding interactions to a certain degree. In fact, two conserved hydrogen bonds were evident in crystal structures between 2-DOS of various aminoglycosides and the 16S ribosomal A-site RNA.[20] Due to this hydrogen bonding capability, it has been speculated that 2-DOS may be utilized to design novel RNA binding ligands capable of sequence selective recognition.

Recently we have reported a 2-DOS mimic capable of amide connectivity in place of glycosidic linkage.[22] Based on this 2-DOS mimic, we report herein preparation of conjugate **1**, a neamine and a 2-DOS mimic connected by an amide bond (Fig. 1). It was hypothesized that the newly introduced amide bond would provide improved binding affinity and sequence selectivity in RNA recognition possibly through additional hydrogen bonding

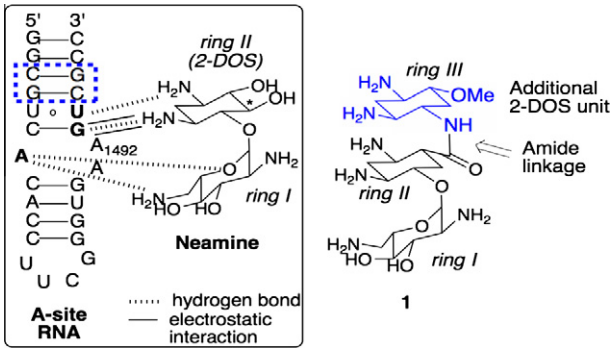

Figure 1. Recognition of A-site RNA by neamine and design of conjugate **1** between neamine and a 2-DOS mimic connected by an amide bond. Sequences inside blue rectangle (will be randomly mutated) are expected to interact with the additional 2-DOS unit (blue) connected by an amide bond. The 5-OH (*) in ring II of neamine is omitted in the conjugate.

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interactions. The neamine moiety was used as a building block for the conjugate preparation so that it may act as an anchor to bring the additional 2-DOS moiety in close proximity to the sequence inside the blue rectangle (Fig. 1).

Compound **1** was prepared starting from asymmetrically protected cyclohexanol **2**[22] following the procedures shown in Scheme 1. 2,5-dideoxystreptamine (2,5-DDOS) was used as the 2-DOS mimic and the 5-OH in ring II of neamine (marked by * in Fig. 1) was omitted in the synthesis of the compound **1** as it projects out of RNA helix and therefore does not interact with any nucleotide according to crystal structure. After protection of the hydroxyl group of **2** to methoxy group and transformation of two azides to NHBoc, the MOM protected hydroxyl group of the cyclohexanol **2** was transformed to NH_2 through two consecutive Mitsunobu reactions. The amino group of **5** was further coupled with **6** to yield **7**. In the coupling reaction, moderate heat was applied to ensure completion of the amide coupling between **5** and **6** using HATU/HOAt as coupling agents. After transformation of the two NHBoc groups to azides, a glycosylation reaction was performed using the procedure developed by Fugedi.[23] The desired α-anomer **9** was obtained as the major product with the approx. ratio of 2:1 for α:β. Conjugate **1** was obtained after deprotection of the acetyl groups and reduction of the azides to amines.

Compound **1** was tested for its RNA binding toward the native A-site RNA sequence (RNA1) and its mutants (RNA2–8) (Fig. 2). The mutants were constituted by replacing the 5′-CG-3′/5′-GC-3′ pair (red in Fig. 1) with random sequences. Neamine interacts with several nucleotides of A-site RNA through four hydrogen bonds and two electrostatic interactions (Fig. 1).[20] All six interactions are conserved when other bulkier aminoglycosides (gentamycin B, kanamycin A, ribostamycin, lividomycin A, and neomycin B, all of which contain neamine as a part of their structures) bind to the same A-site RNA and neamine recognizes the same RNA sequence in the context of these aminglycosides.[20] Based on this observation, the neamine that was used as a base to which to attach the additional 2-DOS mimic was employed to serve as an anchor to bring its attached 2-DOS mimic to a close proximity of the mutated sequences. Therefore, the library of A-site RNA and its mutants will

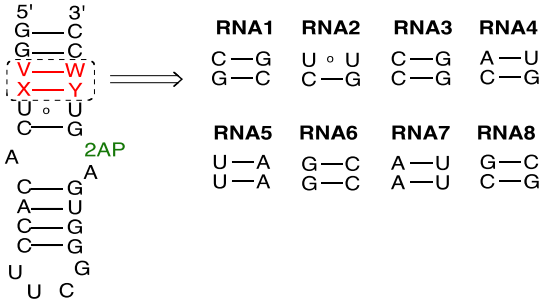
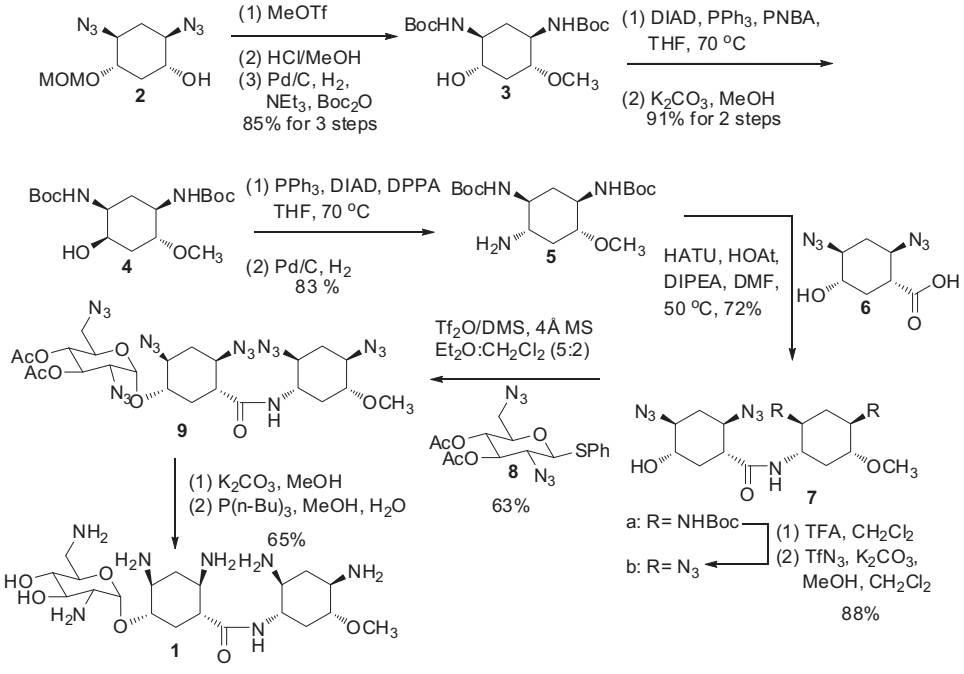


Figure 2. Sequences of RNA targets. RNA1 is the native A-site sequence and RNA2–8 are mutants. A1492 of the native A-site sequence is substituted by 2-AP by 2-aminopurine (2-AP) for each RNA.

serve as a good tool to study preferred sequence by the 2-DOS mimic attached to neamine in the context of fully base-paired dsRNA. For each target RNA, 2AP, a fluorescent adenine analogue, was introduced in place of A1492 to monitor binding of compound **1**.

In order to confirm that the 2-AP modified A-site RNA could be used for the binding assay, we treated RNA1 (the A-site RNA with 2-AP replacement at A1492) with neomycin, which is known to form a 1:1 complex with A-site RNA. The titration exhibited a typical sigmoidal curve (Fig. 3) and its K_d value was estimated to be 1.5 μM by Scatchard analysis. This value is in good agreement with the literature-reported K_d of 0.5 μM for the complex of neomycin and A-site RNA.[24] Then, several select RNAs (RNAs 3, 5, and 7) were then treated with neomycin to ensure that mutation of the stem sequences above the 2-DOS binding region did not cause detrimental effect in neomycin binding. We anticipated that the mutation would have a negligible effect in binding affinity as the mutation was made on the sequences that are not recognized by neomycin. The mutations minimally affected the binding affinity of the RNAs as demonstrated by the measured K_d values found to be between 1.1 and 2.4 μM. All three RNAs demonstrated sigmoidal binding curves.

Then, compound **1** was titrated with each target RNA to assess its binding affinity and sequence selectivity toward different target



Scheme 1. Synthesis of the compound **1**.

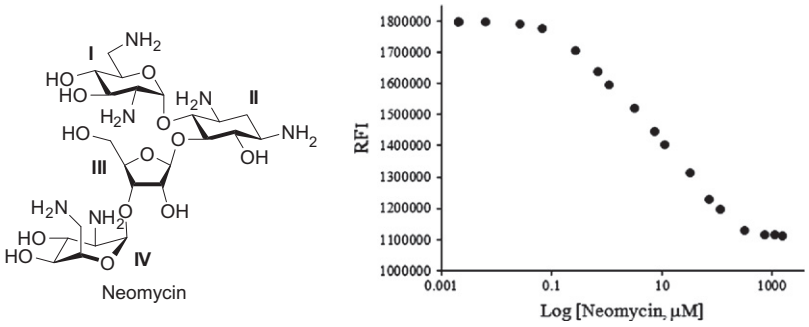
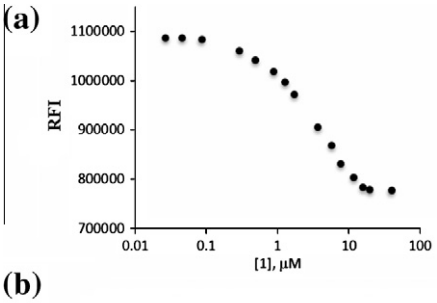


Figure 3. Chemical structure of neomycin and titration curve between RNA1 and neomycin. The four rings of neomycin are indicated as I–IV. Neomycin is known to form a 1:1 complex with A-site RNA and demonstrated a sigmoidal binding curve.



RNA	K_d (μM)	RNA	K_d (μM)
1	2.2	5	0.5
2	1.5	6	1.4
3	2.2	7	2.9
4	1.7	8	1.4

Figure 4. (a) Sample titration curves between RNA2 and compound **1**; (b) Dissociation constant (K_d) values for the complex between RNAs 1–8 and compound **1**.

RNA sequences. All RNAs demonstrated sigmoidal regression curves as shown in a sample binding curve between RNA2 and compound **1** (Fig. 4a). The K_d values were between 0.5 and 2.9 μM (Fig. 4b). While sigmoidal binding curve was observed as the concentration of compound **1** was increased, neamine did not show fluorescence decreases at the given concentrations in the same assay conditions (up to overall 35 μM) (not shown). Based on the dissociation constant (approx. 100 μM) reported for the complex between neamine and the native A-site RNA from a footprint assay, attachment of an additional 2-DOS mimic to neamine increased binding affinity by 35- to 200-fold to sub to low micromolar range, which is predictable as the additional 2-DOS mimic has two amino groups that can interact with the negatively charged phosphate backbone of RNA target. It was found that RNA5 is the preferred target of compound **1** and demonstrated six times stronger binding affinity when compared to RNA7.

In conclusion, we have prepared conjugate **1** by connecting an amino-functionalized 2-DOS analogue with neamine moiety modified to have a carboxylic acid functional group by an amide bond. A library of A-site RNA and its mutants was prepared to examine RNA binding characteristic of the additional 2-DOS moiety attached to neamine. The additional 2-DOS analogue attached to neamine was observed to predictably increase binding affinity up to 200-fold when compared with neamine. The conjugate did not demonstrate exclusive recognition of a single target RNA sequence, which is yet a formidable challenge to achieve for medicinal chemists. It binds to the native RNA sequence and all its mutants with somewhat similar binding affinity. The biggest difference in sequence selectivity was observed to be 6-fold between RNAs 5 and 7.

Supplementary data

Supplementary data (detailed experiment procedures for synthesis and spectral data of all compounds, and fluorescence binding assay condition and procedure) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.06.084.

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