RNA Binding

Targeting RNAs with Tobramycin Analogues**

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There are growing interests in developing small molecules to specifically target RNA because of the potential therapeutic applications.[1] Aminoglycosides and macrolides, for example, have been known to exhibit antibiotic activities by interacting with the bacterial ribosomal RNA.[2] Recent discoveries of other small molecules that control gene expression in living cells by attenuating RNA activities have shed more light on the promising potential of developing RNA-binding molecules as drugs.[3] Many aminoglycosides have been developed to target not only the bacterial ribosomal RNA 16S A-site, but also other RNA sequences, including the regulatory domains of HIV-1 mRNA, the oncogenic Bcr-Abl mRNA sequence, and the group I intron.[4]

From NMR and X-ray crystallographic studies,[2,5] it is clear that the two-ring cores (rings I and II) of both tobramycin (**1**) and paromomycin (**2**), which are 4,5- and 4,6-linked aminoglycosides, respectively (see Figure 1), sit in the bulges of A^{1408}, A^{1492}, and A^{1493} of the A-site and make very similar contacts with the RNA bases and the phosphate backbones. The surface plasmon resonance (SPR) binding studies of the naturally occurring aminoglycosides with the wild-type or mutant 16S A-site RNAs show that the binding affinity and specificity vary when the compositions or the linking positions of the additional sugar moieties change.[6] Previous studies also suggest that both neamine and nebramine are basic cores for binding to various RNA sequences and for cell permeability.[7] It is possible that by keeping the

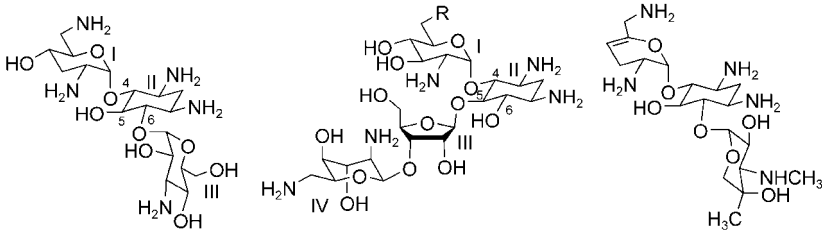

Figure 1. The structures of 4,6- and 4,5-linked aminoglycosides.

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two-ring core and changing the carbohydrate unit attached to position 5 or 6 of the core may affect both affinity and specificity.

Most of the aminoglycoside analogues developed to date have been modified by the attachment of various nonsugar moieties to the original aminoglycosides or to the two-ring core through different linkers. Here, we designed and synthesized a new library of 4,6-linked tobramycin analogues with various mono- or diaminosugars attached to the 6-position of the deoxystreptamine ring (Figure 2). With the relatively rigid conformation of the carbohydrate framework, it was hoped that new aminoglycosides with higher binding affinities and selectivities would be found.

The protected nebramine core **5** was derived from tobramycin (**1**; Scheme 1). The amine groups of tobramycin (**1**) were first converted into azides as protecting groups by the diazo transfer reaction.[8] This was followed by benzylation of the alcohol groups to give the fully protected tobramycin derivative **6**. Cleavage of the glycosidic bond between the nebramine core and the third ring was catalyzed by a Lewis acid in the presence of *p*-thiocresol as nucleophile.[9] The advantage of this method, instead of HCl or copper chloride catalyzed cleavage, is that the resulting cleaved third ring can be recovered as a thioglycoside building block and used in library synthesis.

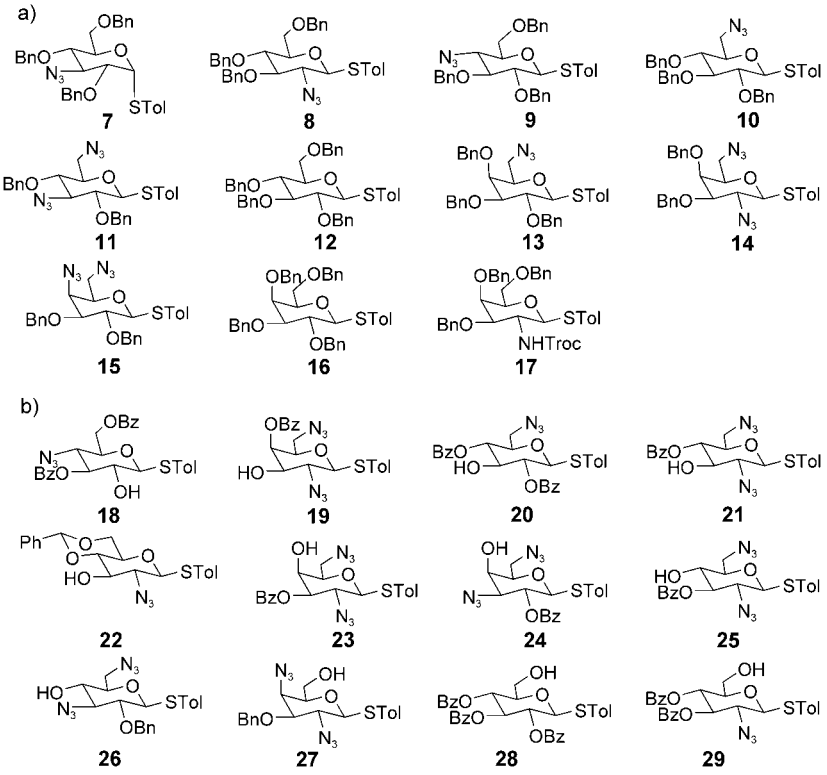


Figure 3. Thioglycoside building blocks as a) the first donors and b) the second donors. Tol = *p*-tolyl, Troc = trichloroethyloxycarbonyl ($CCl_3CH_2OC(O)$-), Bz = benzoyl.

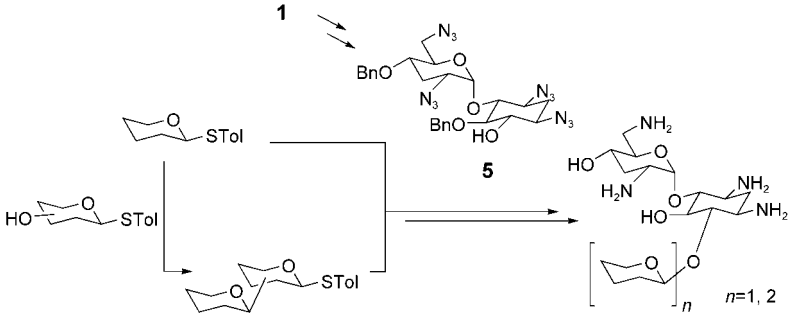
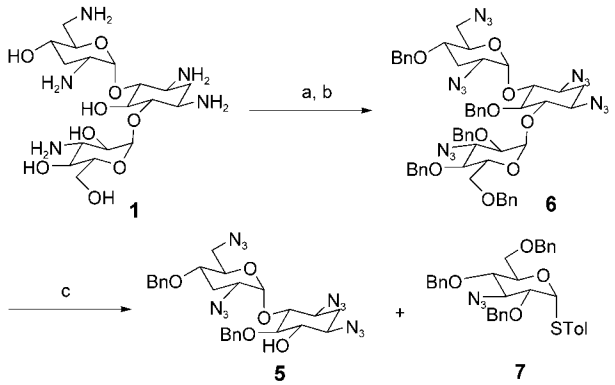
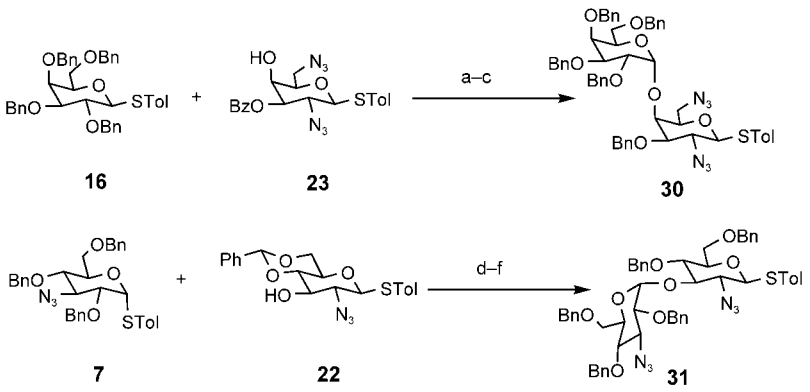


Figure 2. Synthetic strategy of tobramycin analogues.

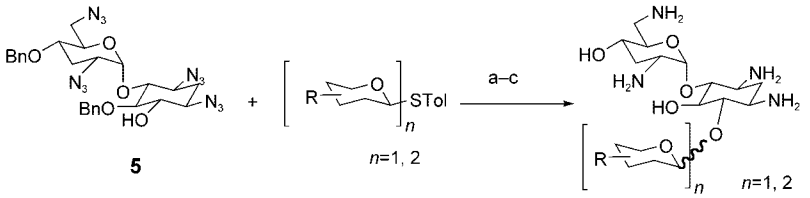


Scheme 1. Synthesis of the protected nebramine core: a) TfN_3, $ZnCl_2$ (cat.), NEt_3, CH_2Cl_2, H_2O, MeOH; b) NaH, BnBr, TBAI, DMF, 82% (two steps); c) *p*-TolSH, $BF_3{\cdot}Et_2O$, CH_2Cl_2, **5** 43%, **7** 47%. Tf = triflate (CF_3SO_2), Bn = benzyl, TBAI = tetra-*n*-butylammonium iodide, DMF = *N*,*N*-dimethylformamide.

A group of protected monosaccharide building blocks, which contained one or more amine groups, was then selected and synthesized from our thioglycoside building block database (Figure 3). Two sets of building blocks were chosen with significant differences in reactivity as glycosylation donors.[10] The first set, which includes compounds **7**–**17**, contains thioglycoside donors with high reactivity which were designed by the introduction of electron-donating protecting groups (Figure 3a). The second set, which includes compounds **18**–**29**, contains thioglycoside donors with lower reactivity, tuned by electron-withdrawing protecting groups (Figure 3b). Thioglycosides **7**–**17** were either directly coupled to the protected nebramine core **5** or were used to assemble disaccharide building blocks with thioglycosides **18**–**29** by *N*-iodosuccinimide (NIS)–TfOH promoted glycosylation (TfOH = trifluorosulfonic acid).[11] After several unsuccessful attempts under different glycosylation conditions, these disaccharide building blocks were found to be relatively inactive. The protecting groups were then changed to benzyl groups to provide higher reactivity toward the nebramine core **5** (Scheme 2). Benzenesulfinylpiperidine (BSP) and Tf_2O were used as promoters for these glycosylation reactions (Scheme 3).[12] Stereoisomers at the anomeric positions (α or β linkage) were usually formed in these glycosylation reactions and these were separated by column chromatog-



Scheme 2. Representative synthesis of disaccharide building blocks. a) NIS, TfOH, CH_2Cl_2, mol. sieves (4 Å), −45 °C; b) NaOMe/MeOH; c) NaH, BnBr, TBAI, DMF, 54% (three steps); d) NIS, TfOH, CH_2Cl_2, mol. sieves (4 Å), −45 °C; e) AcOH, H_2O, 80 °C; f) NaH, BnBr, TBAI, DMF, 41% (three steps). NIS = *N*-iodosuccinimide.



Scheme 3. Synthesis of tobramycin analogues. a) BSP, Tf_2O, CH_2Cl_2, mol. sieves (4 Å), −45 °C, 32–78%; b) Raney Ni, N_2H_4, EtOH, dioxane; c) HCl (0.1 N), H_2, $Pd(OH)_2$ (20%), MeOH/H_2O, 19–65% (two steps). BSP = benzenesulfinylpiperidine.

raphy (silica gel). The number of different protecting groups used was kept to a minimum, with the use of only azido and benzyl groups to simplify the deprotection steps afterward.

Deprotection was completed by first, the reduction of the azido groups with Raney Nickel and anhydrous hydrazine and second, palladium hydroxide catalyzed hydrogenation under acidic conditions to remove the benzyl groups (Scheme 3), to give the tobramycin analogues **32–92** (Figure 4 and Supporting Information). An attempt to remove both groups in one step by hydrogenation in the presence of different palladium catalysts only gave mixtures of incompletely deprotected products probably owing to the poisoning of the catalysts by amines.[13]

The SPR assay was then used to study the binding affinity and specificity of these tobramycin analogues with a number of short RNA sequences (24 to 48 bps), which were identified from sequence-conserved and functionally important regions of several disease-related bacterial, viral, or human RNAs, such as the bacterial ribosomal 16S A-site, *E. coli.* transglycosidase mRNA, Hepatitis C virus (HCV) internal ribosome entry site (IRES) RNAs,[14] HIV frameshift signal,[15] HIV protease mRNA, human oncogenic Bcr-Abl mRNA,[16] and human tyrosine sulfotransferase mRNA (Figure 5). The synthetic compound library as well as commercially available aminoglycosides and macrolides were first screened at 1 μM against the above RNA molecules. By using tobramycin (**1**) and neomycin B (**3**) as standards, compounds with high binding signals were selected for the determination of dissociation constants (K_d).[17] The K_d values of selected compounds from each RNA screening were determined for all RNA sequences in this study to examine the binding specificity (Table 1).

From the results of the binding assay, several molecules displayed an affinity in the nanomolar range to specific RNA sequences. Whereas all of the selected compounds showed some limited affinity toward the RNAs tested, several compounds exhibited higher affinity to specific RNAs. Tobramycin (**1**) showed a higher affinity to the HIV frameshift signal (0.64 μM, 2–100-fold selectivity); neomycin B (**3**) was more specific to the bacterial 16S A-site and the human tyrosine sulfotransferase mRNA (0.2 μM and 0.3 μM, 8–14-fold); sisomycin (**4**) was selective for the HCV IRES IIId domain (0.26 μM, 5 to >100-fold); **33** displayed a relatively high affinity to the HCV IRES IIId (0.7 μM, 1.6–5-fold); **34** also showed a high affinity to the HCV IRES IIId (0.25 μM, 1.7–38-fold); **35** was selective for the HIV frameshift signal (0.32 μM, 5.6–

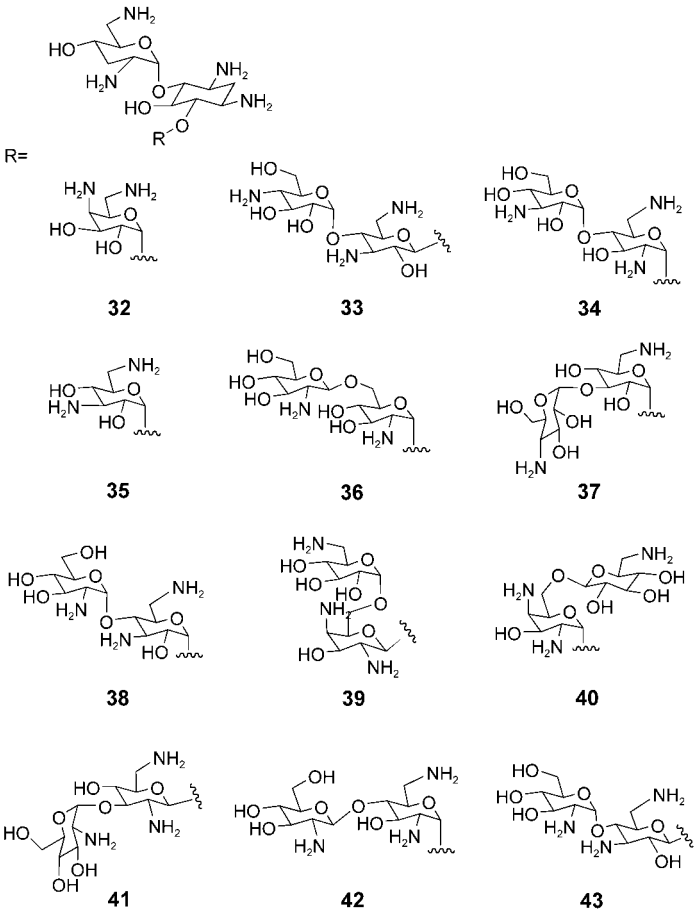


Figure 4. Selected tobramycin analogues (with R groups as shown) which were active in the SPR studies (structures of other synthesized analogues can be found in the Supporting Information).

bacterial ribosomal RNA 16S A-site (16S-AS)
5'-GGCGUCACACCUUCGGGUGAAGUCGCC-3'

E. coli. transglycosidase mRNA (EcTG)
5'-GAAGACAGCCGCUUCUACGAGCAU-3'

HCV IRES domain IIb (HCV2b)
5'-CUGUCUUCACGCAGAAAGCGUCUAGCCAUGGCGUUAGUAUGAGUGUCG-3'

HCV IRES domain IIId (HCV3d)
5'-GGCCGAGUAGUGUUGGGUCGCGAAAGGCC-3'

HIV frameshift signal (HIV-FSS)
5'-UUUUUUAGGGAAGAUCUGGCCUUCCUACAAGGGAAGGCCAGGGAAU-3'

HIV protease active site mRNA (HIV-PAS)
5'-GAAGCUUUAUUAGAUACAGGAGCAGAUGAUACAGUAUUA-3'

human oncogenic Bcr-Abl mRNA (hBcr-Abl)
5'-GGCUGACCAUCAAUAAGGAAGAAGCCCUUCAGCGGCCAGUA-3'

human tyrosine sulfotransferase mRNA (hTSulT)
5'-GCCAACCCACCUAACUACGGAAAACCUGAUCCC-3'

Figure 5. RNA sequences used for the SPR binding assay. All RNA sequences were 5′-biotin-labeled and were heated to 80 °C for 2 min and allowed to cool down slowly to refold into the most stable conformations before immobilization onto a streptavidin-coated sensor chip. SPR experiments were performed according to the procedure reported previously.[17]

Table 1: K_d values [μM] for selected compounds screened against different RNAs.[a, b]

Compound	16S-AS	EcTG	HCV2b	HCV3d	HIV-FSS	HIV-PAS	hBcr-Abl	hTSulT
1	2.1	19	2.7	2.2	0.64	14	1.3	65
3	0.2	2.8	2.9	2.9	1.6	2	2	0.3
4	1.3	12	4.3	0.26	2.7	15	10	37
32	5.9	11	12	6.8	4.7	19	3.2	25
33	1.7	1.8	3	0.7	1.2	1.1	3.5	1.6
34	4.3	0.62	0.42	0.25	3.4	0.83	0.75	9.4
35	3	4.9	1.8	2.7	0.32	6.8	3.8	8.1
36	1.8	5.3	9.4	–	1	5.1	3.2	–
37	3.8	14	0.28	2.3	0.71	3	0.7	52
38	6.6	1	12	2.3	4.6	5	5	14
39	2.6	0.42	2.6	4	1.7	4	4	9.6
40	4.6	8	3.8	3.3	2	14	8.5	13
41	3.6	0.67	3.5	0.53	7.6	1.2	6.4	9.4
42	2.3	2.5	3.1	2	1.6	7	3.5	3.9
43	3.7	1.7	9.5	1	4.5	6	14	5.1

[a] RNA abbreviations used: 16S-AS: Bacterial 16S A-site; EcTG: *E. coli.* transglycosidase mRNA; HCV2b: HCV IRES domain IIb; HCV3d: HCV IRES domain IIId; HIV-FSS: HIV frameshift signal; HIV-PAS: HIV protease active site mRNA; Bcr-Abl: human oncogenic Bcr-Abl mRNA; hTSulT: human tyrosine sulfotransferase mRNA; [b] Errors of K_d values range from ±5% to ±29%.

25-fold); **37** bound better to the HCV IRES IIb domain (0.28 μM, 2.5 to >100-fold); **39** selectively bound the *E. coli.* transglycosidase mRNA (0.42 μM, 4–23-fold); **41** exhibited a high affinity to the HCV IRES IIId domain and the *E. coli.* transglycosidase mRNA (0.53 μM and 0.67 μM, 2–16-fold). The charges of the aminoglycosides tested range from +5 to +7. However, upon comparing the K_d values and the number of charges, no direct correlations were found.

In conclusion, we have developed an efficient method to replace a monosaccharide unit of tobramycin with another monosaccharide or disaccharide moiety to form analogues of tobramycin. The SPR studies of the interactions of these analogues with certain RNA sequences show that new aminoglycosides can be created to target RNA in a sequence-selective manner. Work is in progress to further study the interactions of selected compounds and RNAs to understand the origin of selectivity and to design better RNA-binding molecules as inhibitors of translational processes in cell-based systems.

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