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# Novel Synthesis and RNA-Binding Properties of Aminoglycoside Dimers Conjugated Via a Naphthalene Diimide-based Intercalator

Jeffrey B.-H. Tok[a,*] and Jason Fenker[b]

[a]*Department of Chemistry, York College and Graduate Center, The City University of New York (CUNY), 94-20 Guy R. Brewer Blvd., Jamaica, NY 11451, USA*

[b]*Department of Chemistry, Indiana University-Purdue University, Fort Wayne, IN 46805, USA*

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**Abstract**—The synthesis and RNA-binding properties of naphthalene-based diimide conjugated bis-aminoglycoside antibiotics are reported. Compared to the monomeric aminoglycoside, the conjugated ligands were observed to attain up to 35-fold enhancement in binding affinity towards a novel RNA construct that contained two 16S rRNA A-sites. 

The utilization of small molecules to specifically control important cellular functions has been of major interest in basic medical research. In specific, small molecules capable of controlling the transcription process have been well developed over the last decade. However the successful control of the translation process in vivo via small molecule–mRNA interaction has only been reported recently.[1] The interaction of small molecule with RNA construct has been a well-studied area.[2] A particular class of natural-occurring small molecules, namely the aminoglycoside antibiotics, has been found to bind numerous RNA constructs.[3,4] Aminoglycoside antibiotics have attracted significant attention due to their usage in the treatment of Gram negative infections. The aminoglycoside antibiotics are thought to function in larger part by binding to the decoding region of bacterial 16S ribosomal RNA, thus causing premature termination and mistranslation of proteins and consequently, bacterial death.[5] Besides the 16S rRNA, aminoglycoside antibiotics have also been reported to bind to a variety of natural-occurring RNA species, which includes 16S ribosomal RNA,[6] group I introns,[7] hammerhead ribozyme,[8] the RRE transcriptional activator region from HIV,[9,10] and the site 1 mRNA of thymidylate synthase.[11] However, the binding of aminoglycoside antibiotics to these constructs, measured via the dissociation constant ($K_d$), has been observed to be primarily in the micromolar (μM) range, with neomycin B being the only exception in the high nanomolar (nM) range.[12] As a result, relatively high dosage of aminoglycoside antibiotics is often required in clinical use. To avoid potential cytotoxic side effects such as neptrotoxicity (kidney failure) and ototoxicity (hearing loss),[13,14] it is important to develop higher binding affinity small molecules towards the 16S rRNA.

We have previously studied dimeric aminoglycoside molecules towards the decoding region of the 16S rRNA construct,[15] and have observed that the dimeric aminoglycoside molecules do not exhibit significant improvement in binding affinities towards a simplified 27 nt A-site 16S rRNA construct (Construct **A**, Scheme 1) originally studied by Puglisi and co-workers.[16,17] However in the presence of a novel 46 nt RNA construct that contained two A-site 16S rRNA (Construct **B**), an enhancement of up to 19-fold can be achieved when Construct **B** was studied with a dimeric neomycin molecule **2** (Scheme 2).[15] Such novel RNA constructs are important in designing new antibiotics to better control the translation process using lower dosage of small molecules. In this report, modifications to the previously studied dimeric aminoglycoside ligands were undertaken. Novel ligands involving the conjugation of an intercalator between the two aminoglycosides were synthesized, and their RNA binding properties examined.

This novel class of intercalator-conjugated bis-aminoglycoside ligand was hypothesized to demonstrate a

*Corresponding author. Tel.: +1-718-262-2656; fax: +1-718-262-2652; e-mail: tok@york.cuny.edu

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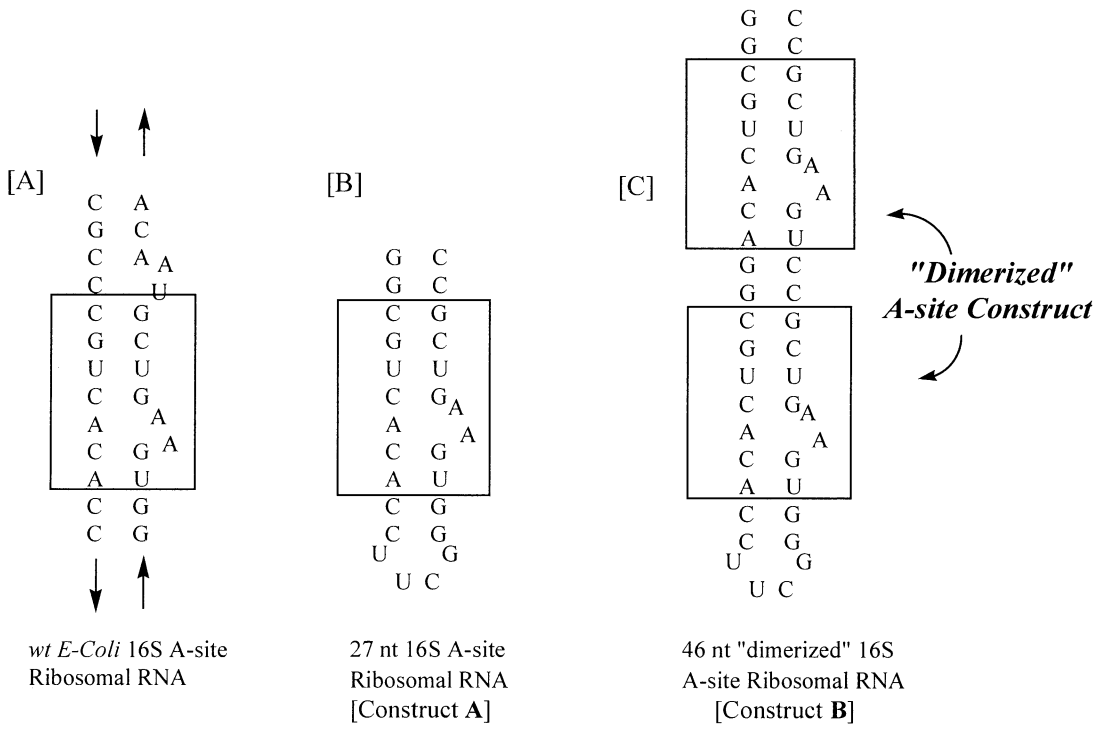



**Scheme 1.** Secondary structures of the RNA constructs used in this study.

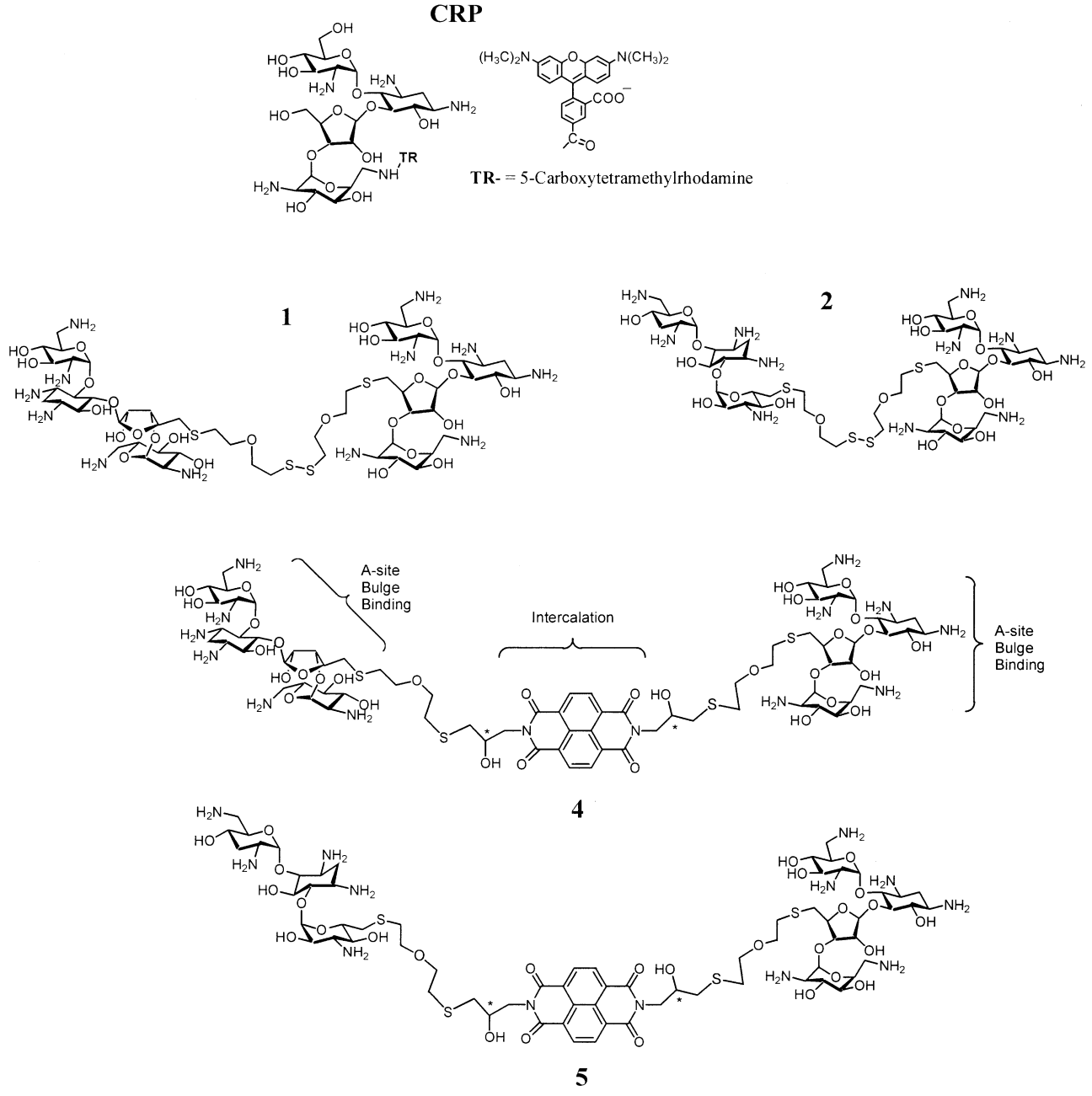
significantly improved binding efficiency against an appropriately designed RNA construct, such as the novel 46-mer RNA construct that contains two A-site 16S rRNA (Construct **B**). The binding mode of the intercalator-conjugated ligand is hypothesized such that the two aminoglycosides would respectively bind the A-site bulges, with the simultaneous binding of the ligand's intercalator on the duplex region between the two A-site bulges of the RNA Construct **B**.

The intercalator in naphthalene diimide (naphthaldiimide) was chosen as it has been widely studied as an efficient intercalator or polyintercalators against DNA duplexes.[18,19] The napthaldiimide moieties have also been studied through linkages with short peptides and oligonucleotides. For example, it has been demonstrated that naphthaldiimide moieties, when linked with short oligonucleotides at both ends, are able to tether to the Watson–Crick and the Hoogsteen strands of DNA triplex.[20–22] It was also demonstrated that when the naphthaldiimide-based moiety was attached to peptides via a head-to-tail arrangement, the resulting conjugated peptide-intercalator ligands are able to bind the DNA through intercalation at the major groove.[23] In addition, the 4,9-disubstituted acridine moiety was also recently demonstrated to be an effective intercalator against DNA and RNA when it is being placed in between two groove binding oligopeptides.[24]

In this present work, the naphthaldiimide-based moiety was inserted as a potential linker in between two aminoglycoside antibiotics. The synthesis of the novel naphthaldiimide bis-aminoglycoside ligand is shown in Scheme 3. The starting material in 1,4,5,8-naphthalenetetracarboxylic dianhydride (**4a**) had been reported to be easily derivatized at both ends. In this report, a procedure recently published by Saito and co-workers was adapted to afford us the important naphthalene intermediate **4c**.[25] First, the starting material in 1,4,5,8-naphthalenetetracarboxylic dianhydride **4a** (Aldrich, WI, USA) was derivatized at both end with 2.5 equiv of (*S*)-4-aminomethyl-2,2-dimethyl-1,3-dioxolane (Synthon Chiragenics Corp., NJ, USA), and was subsequently converted to the di-epoxide derivatized naphthaldiimide intermediate, **4c**.[26] Finally, the intermediate **4c** was converted to a naphthaldiimide-derivatized dimeric aminoglycoside ligands, **4**–**6**, through reaction with the appropriate thiol-derivatized and Boc-protected aminoglycosides **1A** and **1B**, and subsequent deprotection of the Boc groups. The products were then RP-HPLC purified [Waters Spherisorb ODS-1 ($C_{18}$) Columns], and the yields for overall transformation are 21, 22, and 19% for **4**, **5**, and **6**, respectively.[27] The thiol-derivatized and Boc-protected neomycin B (**1A**) and tobramycin (**1B**) aminoglycosides, shown in Scheme 3, have been previously synthesized by Tor and co-workers.[28,29]

To quantify the $K_d$ between the monomeric and various dimeric aminoglycosides to the RNA constructs, we employed the recently developed fluorescence method that enables direct and quantitative binding measurements between aminoglycoside–RNA interactions.[12,30] The basis of this methodology involves the use of fluorescent aminoglycoside conjugates, such as the rhodamine-conjugated paromomycin molecule [CRP, Scheme 2], to first bind the RNA Construct **B**. In this experiment, we hypothesized that two molecules of CRP would bind to both the A-sites within Construct **B**. The binding studies were performed in an incubation buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM

$CaCl_2$, and 20 mM HEPES (pH 7.40, rt). First, 10 nM of the tracer molecule in CRP was titrated with an increasing concentration of the dimeric A-site 16S rRNA Construct **B**. The fluorescence intensity curve gradually when CRP was titrated with an increasing concentration of RNA Construct **B**, reaching saturation at around 550 nM. Interestingly, it should be noted that the titration curve for Construct **B** did not vary significantly from RNA Construct **A**, which contained only a single 16S rRNA A-site. This indicates the mode of binding to the two RNA Constructs **A** and **B** might be similar. Using the previously described curve fitting equation,[12,30] the $K_d$ of CRP was calculated to be $0.28 \pm 0.01$ μM towards Construct **B**, and is in agreement with the observations previously reported utilizing the same fluorescence methodology.[12,15] For the competition experiment, we are interested in monitoring the changes in anisotropy intensity through the displacement of RNA-bounded CRP molecules through an increasing concentration of the intercalator-derivatized dimeric aminoglycoside ligand. To ensure that the method of approach is appropriate for this study, an increasing concentration (0–50 μM) of the well-studied neomycin B was first

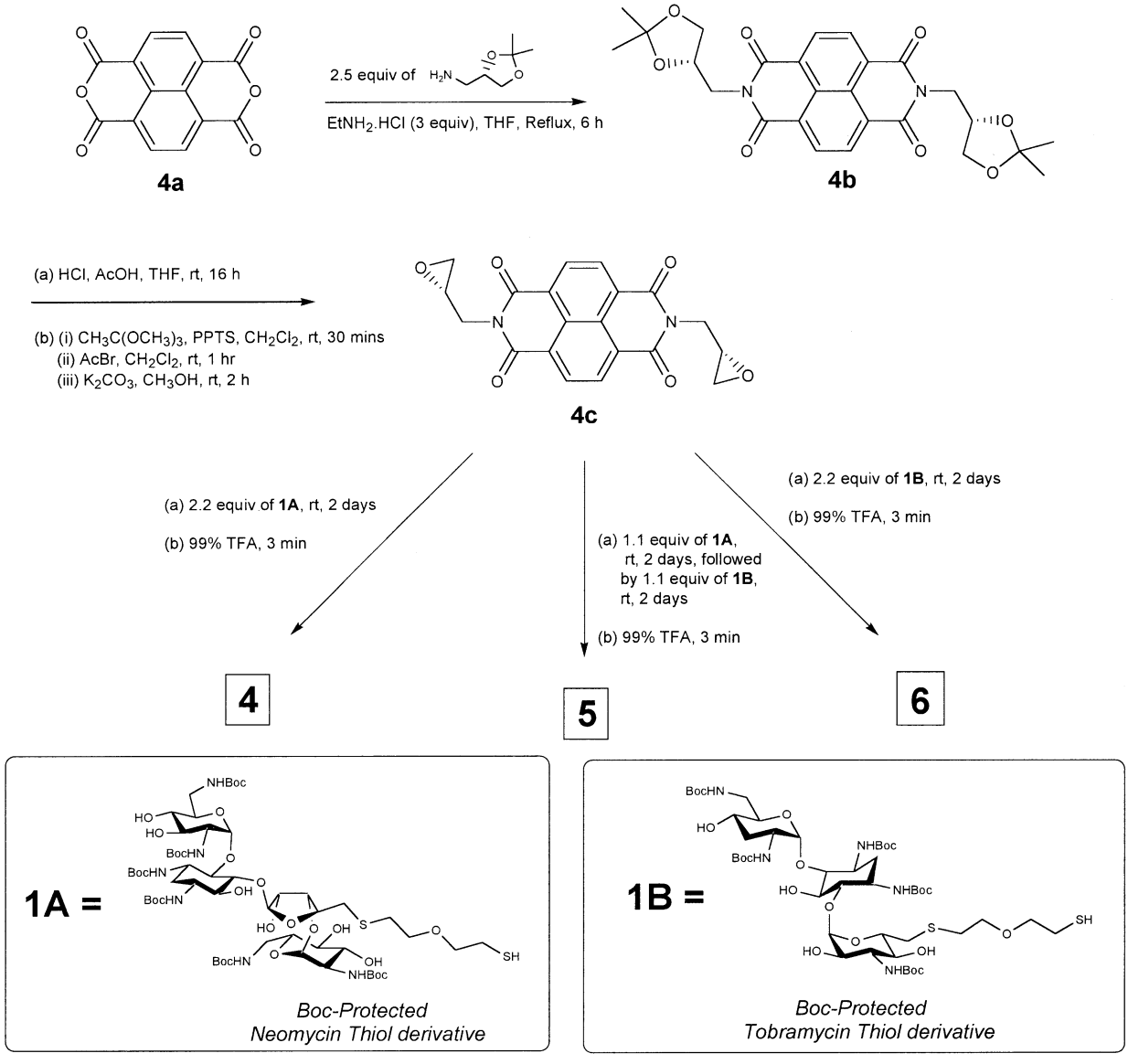



**Scheme 2.** Structures of crp, neomycin–neomycin **1**, neomycin–tobramycin **2**, naphthaliimide–derivatized neomycin–neomycin ligand **4**, naphthaliimide–derivatized neomycin–tobramycin ligand **5** (conjugated ligands tobramycin–tobramycin **3** and naphthaliimide–derivatized tobarmycin–tobramycin ligand **6** not shown).

added to the CRP–Construct **B** complex, which comprised of 10 nM of CRP and 450 nM of Construct **B**. The fluorescence of the complex was observed to quenched gradually in a saturable fashion, and using the described curve fitting equation, the $K_d$ of neomycin B can be calculated. The $K_d$ value was calculated to be $0.35 \pm 0.01$ μM, a value which is again in agreement with the observations previously utilizing the same fluorescence techniques.[12,15]

Subsequently, the various intercalator-derivatized dimeric aminoglycoside ligands **4**–**6** were studied with respect to their abilities to compete with CRP for binding to Construct **B**. Using the same experimental procedures for the neomycin B aminoglycoside, intercalator-derivatized dimeric neomycin aminoglycoside ligand **4** was titrated (0–50 μM) with the CRP–Construct **B** complex, and was observed to exhibit a markedly improvement in binding affinity. The obtained $K_d$ value of $0.01 \pm 0.001$ μM corresponds to approximately 35-fold enhancement when compared to the monomeric neomcyin molecule. Again using the same experimental procedures and conditions, the intercalator-derivatized dimeric neomycin–tobramycin aminoglycoside ligand **5** was observed to have a $K_d$ of $0.15 \pm 0.01$ μM against Construct **B**, and the intercalator-derivatized dimeric tobramycin–tobramycin aminoglycoside ligand **6** has a $K_d$ of $1.11 \pm 0.13$ μM against Construct **B** (data summarized in Table 1).

In summary, the data presented indicates that the simple and widely studied naphthaldiimide moiety can be successfully utilized as a linker between two aminoglycosides to afford a novel dimeric aminoglycoside-intercalator conjugated ligand. The binding characteristics of such intercalator derivatized dimeric aminoglycosides are consistent with previous observations, which have also suggested that through usage of appropriate




**Scheme 3.** Synthetic outlines for napthaliimide-derivatived aminoglycoside ligands **4**–**6**.

**Table 1.** Summary of $K_d$ (μM) of various tested aminoglycoside-conjugated molecules with dimeric 16S rRNA RNA Construct **B**

| CRP | Neomycin (Neo) | Tobramycin (Tob)[a] | Neo–Neo dimer, **1** | Neo–Tob dimer, **2**[a] | Tob–Tob dimer, **3**[a] |
|---|---|---|---|---|---|
| 0.28±0.01 | 0.35±0.01 | 1.68±0.14 | 0.02±0.002 | 0.29±0.02 | 1.81±0.16 |
| Intercalator with Neo–Neo dimer, **4** | Intercalator with Neo–Tob dimer, **5** | Intercalator with Tob–Tob dimer, **6** | | | |
| 0.01±0.001 | 0.15±0.01 | 1.11±0.13 | | | |

[a]Note that the $K_d$ values for tobramycin and dimeric ligands **2** and **3** are from ref 15, and were included here for comparison purposes.

intercalator-conjugated ligands, enhancement of the overall binding affinities to RNA molecular target can be achieved without compromising its specificity.[23,31] Indeed, the naphthaldiimide-dimeric neomycin aminoglycoside conjugated ligand was observed to exhibit a 35-fold enhancement in binding affinity, when studied using the fluorescence binding procedure, towards a novel RNA construct that contained two 16S rRNA A-site. The results described here have extended our concept in which only the bulged regions of RNA constructs are capable of specific binding to small molecules. When the aminoglycosides are tethered to an appropriate intercalator, for example the naphthalene diimide-based intercalator, the duplex region in between the two A-sites of 16S rRNA construct could potentially be utilized to successfully enhance the binding affinity of such novel conjugated small molecules. This concept can potentially be used in conjunction with other structural information to further the exploration of derivatives with improved affinity and specificity towards a wide range of other biologically relevant RNA constructs.

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27. Spectral data for molecule **4**: $^1$H NMR (300 MHz, $D_2O$) δ 1.26 (q, 2H), 2.08 (m, 2H), 2.85 (d, 4H), 2.91 (m, 6H), 2.96 (m, 6H), 3.08–3.119 (m, 12H), 3.34–3.45 (m, 6H), 3.65 (t, 2H), 3.74–3.78 (m, 10H), 3.87 (t, 4H), 3.92 (m, 2H), 4.11 (m, 4H), 4.22 (m, 2H), 4.28–4.30 (m, 4H), 4.42 (m, 4H), 5.12 (s, 2H), 5.44 (s, 2H), 5.71 (d, 2H), 8.64 (s, 4H). ESI MS $m/z$ calcd for $C_{74}H_{125}N_{14}O_{32}S_4$, $[(M+2H)]^{2+}$ 1849.7; found, 1849.3.
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