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Synthesis of Aminoglycoside–DNA Conjugates

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Abstract—Development of aminoglycoside-nucleic acid conjugates is presented. Synthesis of a DNA dimer covalently linked to kanamycin and neomycin isothiocyanates has been carried out. The development of such conjugates will help couple the sequence specificity of nucleic acids to the electrostatic/shape complementarity of aminoglycoside antibiotics in binding nucleic acid targets.

Aminoglycoside antibiotics are bactericidal agents that are comprised of two or more amino sugars joined in glycosidic linkage to a hexose nucleus.[1] Though they exhibit a narrow toxic/therapeutic ratio, their broad antimicrobial spectrum, rapid bactericidal action, and ability to act synergistically with other drugs make them highly effective in the treatment of nosocomial (hospital-acquired) infections.[2] They are clinically useful in the treatment of urinary tract infections,[3] lower respiratory infections, bacteremias, and other superinfections by resistant organisms.[2,4] Their greatest potential has been in the combination drug regimens in the treatment of infections that are difficult to cure with single agents and for use in patients who are allergic to other classes of drugs.[5] The bactericidal action of aminoglycosides is attributed to the irreversible inhibition of protein synthesis following their binding to the 30S subunit of the bacterial ribosome. RNA affinity and discrimination by aminoglycosides is modulated by the interplay of nonspecific electrostatic forces, which are critical for affinity and a few specific interactions. The flexible and polycationic nature of the aminoglycoside antibiotics not only allows them to preferentially bind to a prokaryotic ribosomal RNA, but also allows binding to a variety of unrelated RNAs, group I introns,[1] a hammerhead ribozyme,[6] the RRE transcriptional activator region from HIV, (which contains the binding site for the Rev protein),[7–9] the 5′-untranslated region of thymidylate synthase mRNA,[10] a variety of RNA aptamers from in vitro selection,[11,12] and human rRNAs.[13] Thus, aminoglycoside charge is a necessary evil: leading to increased affinity, at the price of increased promiscuity and inefficient cellular uptake.

Our recent finding that aminoglycosides can stabilize DNA/RNA triplexes,[14,15] hybrid duplexes,[16] and that neomycin can even induce hybrid triplex formation[16] suggested that aminoglycoside-DNA conjugates could be effective models for targeting nucleic acids (including rRNA) sequence specifically (via a hybrid duplex or triplex formation). RNA·DNA hybrids are transiently formed in many vital biological processes, including DNA replication,[17] telomere replication by telomerase,[18] and the replication of HIV (and other retroviruses by reverse transcription).[17] In this paper, we report the synthesis of neomycin and kanamycin isothiocyanates as stable reagents that can be coupled to a variety of amines. Their use in the synthesis of a DNA thymidine dimer conjugated to neomycin and kanamycin antibiotics is also presented.

The following recognition elements were kept in mind for the design of the conjugate: The amino groups on rings I, II, and IV (neomycin) and rings I–III (kanamycin) are necessary in stabilizing and in recognizing various nucleic acid forms (aminoglycosides without any of these amines do not stabilize rRNA, DNA/RNA triplexes as efficiently).[14–16,19] The conjugates based on aminoglycosides must then retain these amines. The 5″-OH on ring III (neomycin) and 6″-OH on ring III (kanamycin) were thus chosen to provide the linkage to the nucleic acids. Our retrosynthetic analysis of DNA dimer covalently attached to aminoglycoside (kanamycin and neomycin) is shown in Scheme 1. The scheme was devised to allow its extension to solid-phase DNA synthesis.

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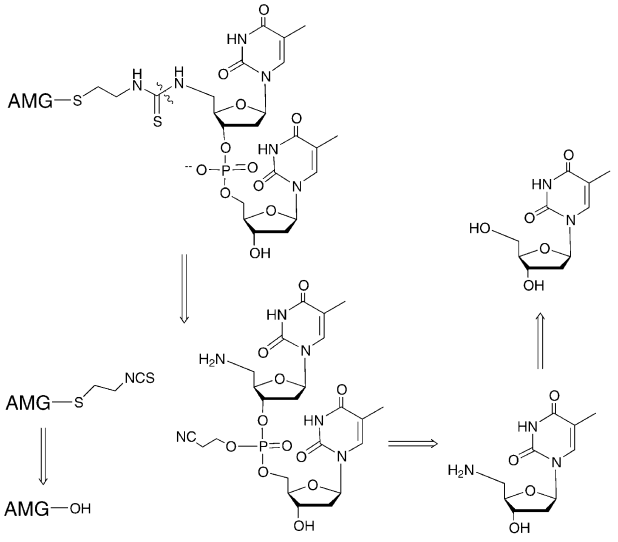
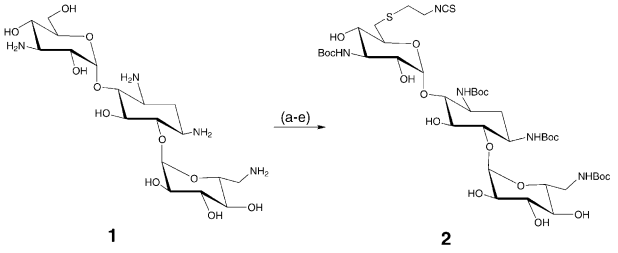
The synthesis involves coupling of kanamycin isothiocyanate **2** (Scheme 2) or neomycin isothiocyanate **11** (Scheme 5) with 5′-amine of a DNA dimer **7** (Scheme 3).

The amino groups of kanamycin were selectively protected with a benzyloxycarbonyl (Cbz) group. Selective protection of the primary hydroxyl group was achieved using triisopropylbenzenesulfonyl chloride (TIBS-Cl) considering the good leaving ability of the sulfonate ester in the presence of a nucleophile such as aminothiolate as described in Scheme 2.[20] Attempts to convert the amino group of modified kanamycin amine to isothiocyanate **2** were unsuccessful using reported reagents such as 1,1′-thiocarbonyldiimidazole[21] and CS_2/HgO/pyridine.[21] The reaction of kanamycin amine with 1 equiv of 1,1′-thiocarbonyldi-2-(1*H*)pyridone,[22] gave the expected isothiocyanate **2** as shown in Scheme 2. Neomycin amine **10** was prepared from neomycin following Tor's reported literature procedure.[20] Synthesis of neomycin isothiocyanate **11** from neomycin amine **10** is shown in Scheme 5. Isothiocyanates **2** and **11** are stable in solution at room temperature. The isothiocyanates were stable (as followed by TLC, IR and reactivity with amines) for 7 days in anhydrous CH_2Cl_2. The colorless solution however turns yellow after 24 h. The reactivity of **2** and **11** with various aliphatic as well as aromatic amines was first examined. The reaction with aliphatic primary amines undergoes completion in less than 4 h. Under similar conditions, even aromatic amines such as aniline and *p*-methoxyaniline react with **2** and **11** to give the corresponding thiourea derivatives in good yields. The reaction times for the aromatic amines are 64 h (aniline) and 48 h (*p*-methoxyaniline), respectively. The isothiocyanates were then used for the synthesis of thymidine dimer–aminoglycoside conjugates.

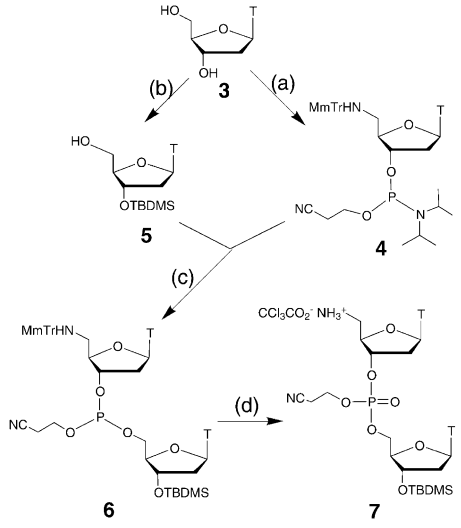
Modeling studies showed that a linker size of 4–6 atoms would allow neomycin/kanamycin to fold back and stabilize a duplex/triplex groove, thus stabilizing a DNA/RNA triplex by bridging the two pyrimidine



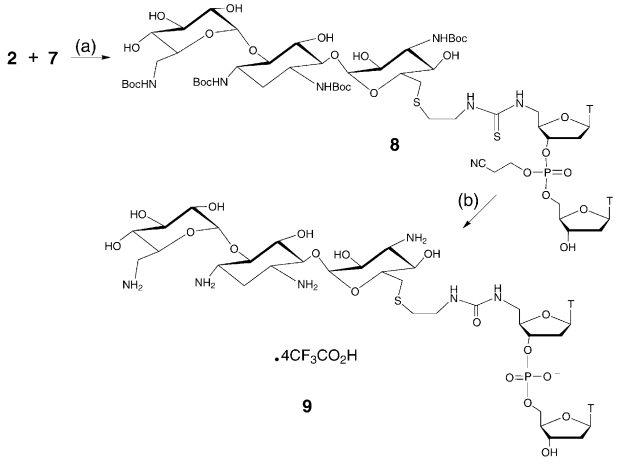
Scheme 1. Retrosynthetic analysis of covalently linked aminoglycoside to DNA dimer.



Scheme 2. Synthesis of kanamycin isothiocyanate. Reagents and conditions: (a) CbzCl, H_2O and Na_2CO_3 (98%); (b) TIPSCl and pyridine (56%); (c) Boc_2O, H_2, Pd/C and MeOH (76%); (d) HCl-$NH_2CH_2CH_2SH$, NaOEt and EtOH (58%); (e) 1,1′-thiocarbonyldi-2(1*H*)pyridone, DMAP and CH_2Cl_2 (55%).

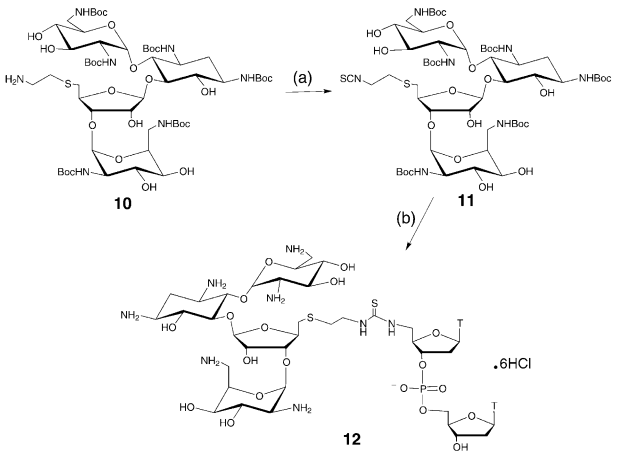


Scheme 3. Synthesis of DNA dimer. Reagents and conditions: (a) (i) tetrachlorophthalimide, PPh_3, DIAD and THF (97%); (ii) ethylenediamine and THF; (iii) MmTrCl, TEA, DMAP and pyridine (60% for two steps); (iv) $CNCH_2CH_2OP[N(iPr)_2]_2$, bis(diisopropylammonium)tetrazolide and CH_2Cl_2 (61%). (b) (i) DMTrCl, DMAP and pyridine (90%); (ii) TBDMSCl, DMAP, Et_3N and DMF (92%); (iii) I_2 and MeOH (83%); (c) (i) 1*H*-tetrazole and CH_3CN (84%); (ii) I_2, H_2O/pyridine/THF (9.0:0.46:90.54 v/v/v %), (79%); (d) CCl_3CO_2H and CH_2Cl_2 (88%).



Scheme 4. Coupling of DNA dimer and kanamycin isothiocyanate **2**. Reagents and conditions: (a) DMAP and pyridine, rt, 9 h (72%); (b) 1,2-ethanedithiol, CF_3CO_2H and CH_2Cl_2 (48%).

strands together. A model of neomycin covalently bound to a triplex pyrimidine strand is shown in the supporting information. The synthesis of DNA dimer **7** from thymidine **3** is presented in Scheme 3. 5′-Amino-5′-deoxythymidine was prepared from thymidine, with regioselective introduction of tetrachlorophthalimide group at the 5′ position under mitsunobu condition, followed by deprotection with ethylenediamine.[23] Protection of 5′-amine by *p*-methoxyphenyldiphenylmethyl (MmTr) group followed by phosphitylation with standard phosphoramidite chemistry gave **4** in good yields.[24]



Scheme 5. Coupling of DNA dimer and neomycin isothiocyanate **11**. Reagents and conditions: (a) 1,1′-Thiocarbonyldi-2(1*H*)pyridone, DMAP and CH_2Cl_2 (60%); (b) (i) compound **7**, DMAP and pyridine, rt, 9 h (43%); (ii) 1,2-ethanedithiol, 4 M HCl/Dioxane (79%).

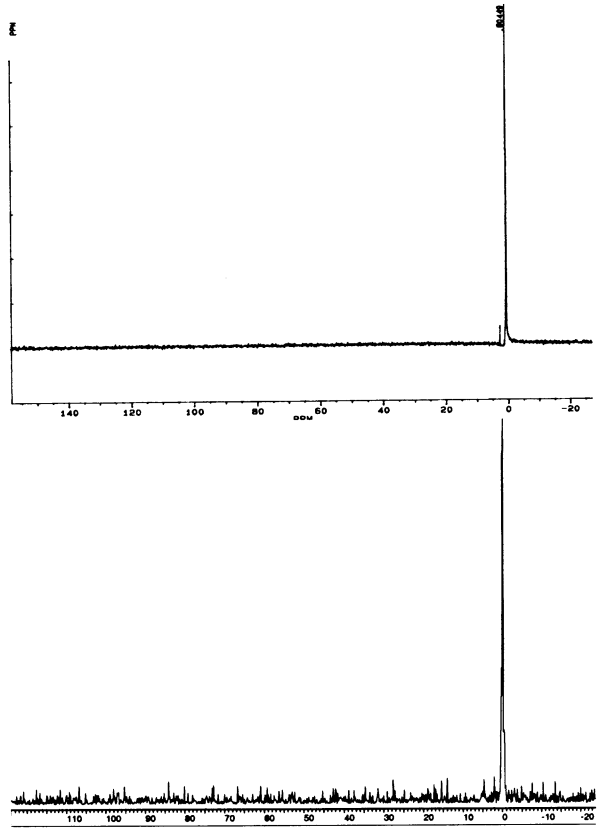


Figure 1. ^{31}P NMR of kanamycin dimer **9** (top) and neomycin dimer **12** (bottom).

3′-TBDMS (*t*-butyldimethylsilyl) protected thymidine **5** was prepared from 5′-*O*-DMTr protected thymidine and TBDMS-Cl in the presence of Et_3N and catalytic amount of 4-*N,N*-dimethylaminopyridine (DMAP).[25] Selective deprotection of di(*p*-methoxyphenyl) phenylmethyl (DMTr) group using I_2 in MeOH[26] gave compound **5** in 83% yield.

Coupling of phosphoramidite **4** and 3′-TBDMS protected thymidine **5** in the presence of 1*H*-tetrazole gave DNA dimer **6** in 84% yield, which was oxidized with I_2 in H_2O/pyridine/THF (9.0:0.46:90.54 v/v/v %) to give **7**. The above conversion was supported by an upfield shift in ^{31}P NMR from 140 to −1.72 ppm. Deprotection of MmTr group was achieved with CCl_3CO_2H in CH_2Cl_2. The final coupling of kanamycin isothiocyanate **2**/neomycin isothiocyanate **11** with 5′-amino DNA dimer **7** was achieved with catalytic amount of DMAP and pyridine as a solvent (Schemes 4 and 5). While the coupling of kanamycin isothiocyanate to the DNA dimer proceeds efficiently and in good yields, neomycin isothiocyanate requires a much longer time to undergo completion, suggesting the importance of crowding/linker size on the electrophilic isothiocyanate carbon.

The ^1H NMR spectra of the conjugates showed Boc to thymidine base methyl protons integration ratio of 9:1 (neomycin) and 6:1 (kanamycin). The ^{31}P NMR spectra of neomycin dimer **12** as well as kanamycin dimer **9** are shown in Figure 1, confirming the presence of a single product.

The mass spectrum of kanamycin dimer shows a *m*/*z* peak at 1173.5 $[M+2H^+ + Na^+]$ (Fig. 2). The MS of neomycin dimer is available in the supporting information.

In conclusion, an efficient synthesis of aminoglycoside isothiocyanates and their DNA dimer conjugate has been achieved. The synthesis is being extended to the development of neomycin/kanamycin–DNA conjugates

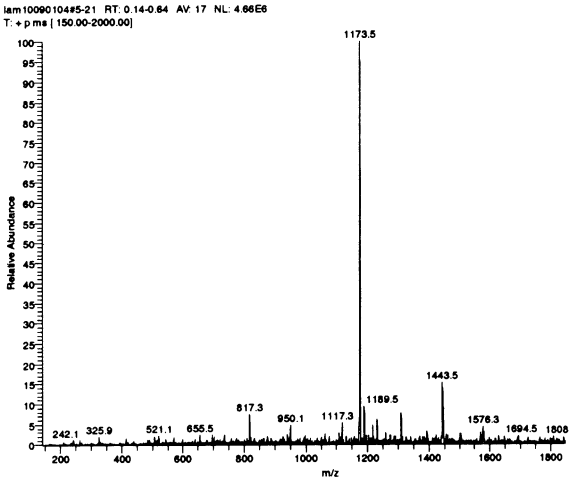


Figure 2. Mass spectra of kanamycin DNA dimer **9**.

using solid-phase DNA chemistry and will be reported in due course. These conjugates can be used for targeting genes of interest via triplex formation, and can also be used to bring sequence specificity to aminoglycoside–rRNA interactions. The potential of these conjugates in targeting RNA remains an attractive option, because of the highly conserved nature of the A-site sequence of the 30S ribosomal subunit aminoglycoside binding site. Additionally, such nucleic acid conjugates can allow us to overcome traditional enzymatic/efflux mediated methods of antibiotic resistance.

Acknowledgements

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Supporting information available: Experimental procedures and characterization data for the compounds prepared.

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