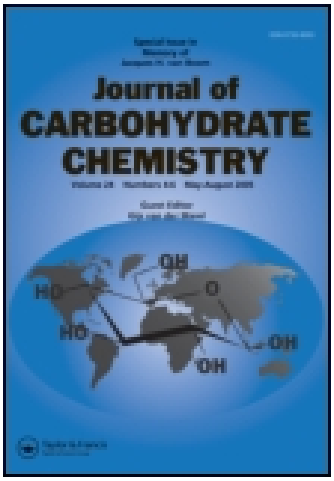

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Synthesis of Neomycin-DNA/Peptide Nucleic Acid Conjugates

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Syntheses of neomycin conjugates of DNA and peptide nucleic acid (PNA) is reported. The DNA and PNA conjugates were prepared by coupling neomycin isothiocyanate with the amino group of DNA/PNA in order to form a thiourea linkage. The use of neomycin isothiocyanate as an electrophilic reagent for a general route to aminoglycoside-coupled nucleic acid biopolymers is described.

Keywords Neomycin, PNA, DNA, Neomycin isothiocyanate

INTRODUCTION

Aminoglycoside antibiotics (Figs. 1 and 2), containing a unique polyamine/carbohydrate structure, have attracted considerable attention because of their specific interactions with RNA. Their antibacterial activity is mainly exerted by their binding to the RNA of the bacterial ribosome,[1] and thus interfering with the mRNA translation process. The miscoding causes membrane damage, which eventually disrupts cell integrity, leading to bacterial cell death.[2–5]

Aminoglycosides bind to various RNA molecules such as ribosomal RNA, 5′-untranslated region of thymidylate synthase mRNA,[6] both the Rev response element and the transactivating response element RNA motifs[7–9] of HIV-1, and a variety of catalytic RNA molecules such as group I introns,[10] ribonuclease P RNA,[11] hairpin ribozyme,[12] hammerhead ribozyme,[13] and hepatitis delta virus ribozyme.[14,15]

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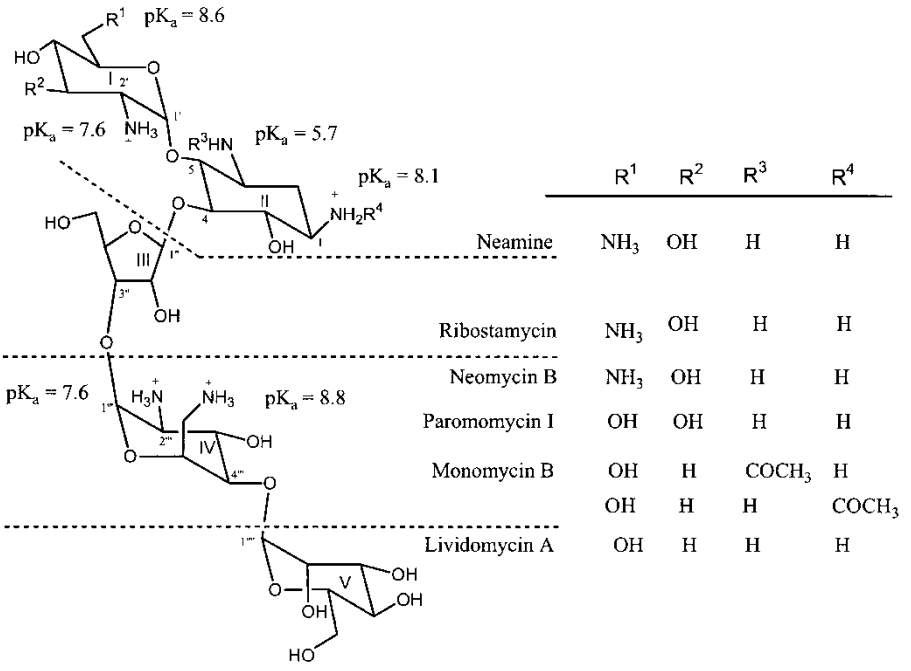
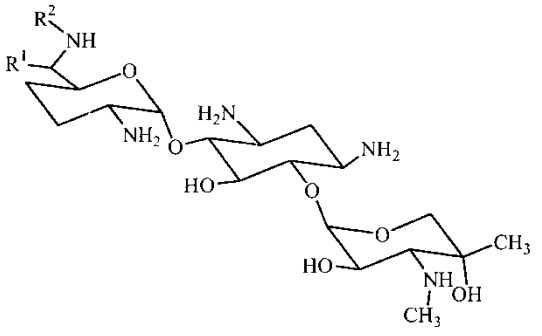


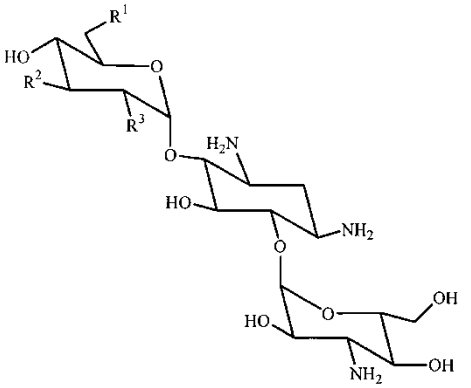
Figure 1: Structures/pK_as of aminoglycosides with a central ribose.

Aminoglycosides binding to HIV-1 RNA molecules prevents binding of the cognate viral proteins Tat and Rev to TAR[16] and RRE[7] respectively.[17,18] While neomycin stabilizes DNA triplex structures,[19] it (Fig. 1) does not affect DNA duplex stability (under physiologic ionic conditions).[19] Our previous work has also suggested that aminoglycoside specificity (neomycin in high nM-low μM range) may be for nucleic acid forms that show some features characteristic of an A-type conformation rather than for naturally occurring RNA.[20] This finding suggested that aminoglycoside-DNA conjugates could be effective models for targeting nucleic acids' sequence specifically (via a hybrid duplex or triplex formation). Covalent attachment of a ligand to an oligonucleotide (ODN) increases the stabilization induced by other nucleic acid-stabilizing agents such as spermine,[21,22] acridine,[23] Hoechst 33258,[24] and psoralen.[25] Conjugation of an aminoglycoside to an ODN can assist in the following processes: (1) delivery of aminoglycoside to a specific DNA/RNA site; (2) increased stabilization induced by this hybrid conjugate; and (3) increased cellular permeability/site-specific delivery of the ODN.

Additionally, since the polyanionic nature of the DNA/RNA backbone is a major deterrent in forming duplexes and triplexes, peptide nucleic acid (PNA), a neutral backbone containing oligonucleotide, has been developed.[26–28] The hybrid complexes formed with PNA and DNA/RNA exhibit extraordinary



	R^1	R^2
Gentamicin C1	CH_3	CH_3
Gentamicin C2	CH_3	H
Gentamicin C1a	H	H



	R^1	R^2	R^3
Kanamycin A	NH_2	OH	OH
Kanamycin B	NH_2	OH	NH_2
Kanamycin C	OH	OH	NH_2
Tobramycin	NH_2	H	NH_2

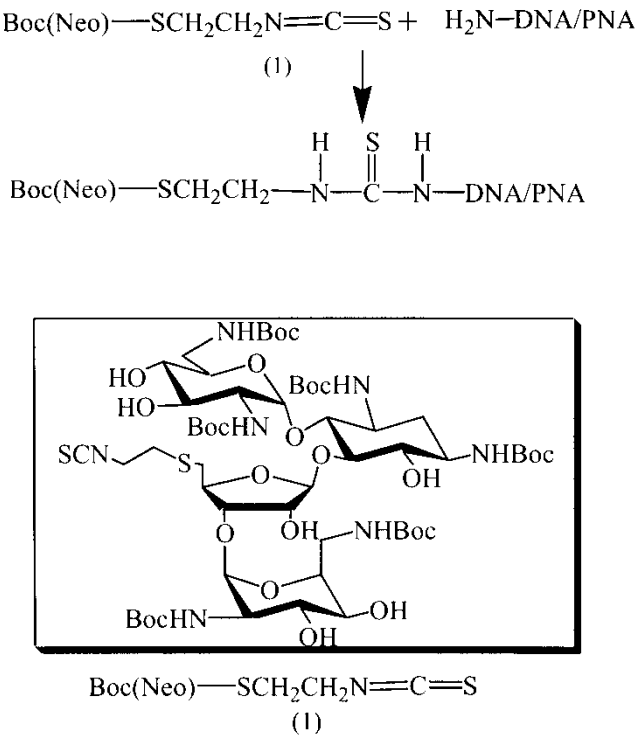
Figure 2: Structures of aminoglycosides (kanamycin and gentamicin families).

thermal stability and are independent of ionic strength.[26] PNA is not susceptible to hydrolytic (enzymatic) cleavage,[29,30] which displays its potential in antisense therapeutics. The effective delivery[31,32] of these oligomers to their respective targets has been achieved with the help of their neutral and lipophilic nature. Because of their significant effects on replication, transcription, and translation processes as well as their high affinity, high specificity, and high stability with DNA/RNA observed in *in vitro* experiments,[33] PNA oligomers are strong candidates for effective antigene and antisense drug design. Herein, we describe a general strategy for the solid phase synthesis of covalently attaching aminoglycosides (neomycin) to nucleic acids and analogs (DNA and PNA).

RESULTS AND DISCUSSION

(1) Covalent Attachment of Neomycin to a DNA Oligomer

The following recognition elements were kept in mind for the design of the conjugates: The amino groups on rings I, II, and IV (neomycin, Fig. 1, Sch. 1a)



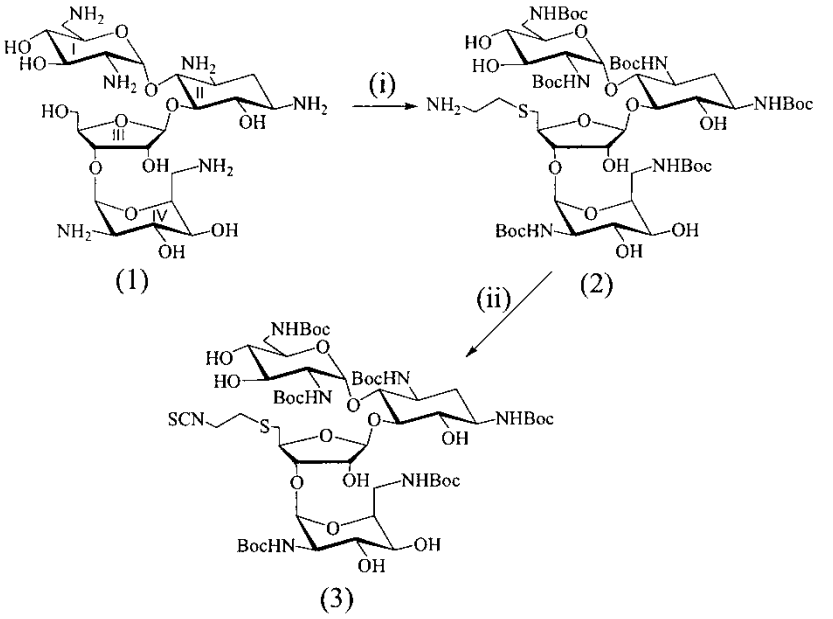
Scheme1a: Synthesis of neomycin-DNA conjugate.

are necessary in stabilizing and recognizing various nucleic acid forms (aminoglycosides without any of these amines do not stabilize rRNA, DNA/RNA triplexes as efficiently).[19,34–37] The conjugates based on aminoglycosides must then retain these amines. The 5″-OH on ring III (neomycin) was thus chosen to provide the linkage to the nucleic acids. The key synthetic step involves coupling of neomycin isothiocyanate with 5′-amino group of the modified DNA to give the isothiourea linkage (Sch. 1a).

Synthesis of neomycin isothiocyanate[36] **3** (Sch. 1b) was initiated by conversion of the natural product-neomycin to 5″-deoxy-5″-amino neomycin **2** (Sch. 1b). Reaction of this amine and 1,1′-thiocarbonyldi-2(1*H*)pyridone (TCDP) in the presence of 4-*N*,*N*-dimethylaminopyridine (DMAP) leads to the formation of isothiocyanate, which can be easily followed by IR spectroscopy (Fig. 3).

The IR of neomycin isothiocyanate shows the appearance of a new peak at $1/\lambda = 2305.50\,\mathrm{cm}^{-1}$, which corresponds to the isothiocyanate group.

The DNA oligomer was modified at the 5′-end by introducing 5′-amino-5′-deoxythymidine 5 (Sch. 2) at the end of the oligomer synthesis. The synthesis of 5′-amino-5′-deoxythymidine **5** from thymidine **4** is described in Scheme. 2. The amino group was protected with 4-methoxyphenyldiphenylmethyl



Scheme1b: Reaction conditions: (i) (a) $(Boc)_2O$, DMF, H_2O, Et_3N, 60°C, 5 hr 60%; (b) 2,4,6-triisopropylbenzenesulfonyl chloride, pyridine, rt, 40 hr, 50%; (c) $H_2NCH_2CH_2SH$, NaOEt/EtOH, rt, 18 hr, 50%; (ii) 1,1′-thiocarbonyldi-2(1*H*)pyridone, 4-*N*,*N*-dimethylaminopyridine and CH_2Cl_2 (12 hr, rt 60%).

(MmTr) group, which was followed by phosphitylation using standard phosphoramidite chemistry to get **6** (Sch. 2) in good yields.

The synthesis of dT_{15} **7** was carried out on a CPG column using standard phosphoramidite chemistry. After oligomer synthesis, the di(p-methoxyphenyl)

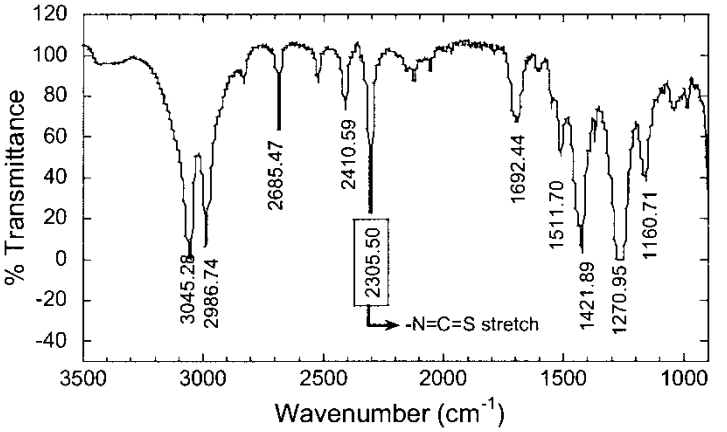
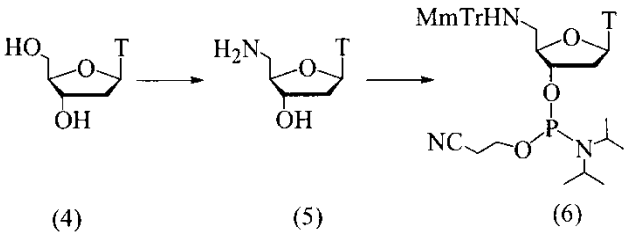


Figure 3: IR of neomycin isothiocyanate (recorded as a solution in CCl_4, and then manually subtracting the CCl_4 peaks to get neomycin isothiocyanate **3** spectrum).



Scheme 2: Reaction conditions: (i) tetrachlorophthalimide, PPh_3, DIAD, and THF (97%); (ii) ethylenediamine and THF; (iii) 4-methoxyphenyldiphenylmethyl (MmTr) chloride, triethylamine, 4-*N,N*-dimethylaminopyridine, and pyridine (60% for two steps); (iv) $CNCH_2CH_2OP(N(iPr)_2)_2$, bis(diisopropylammonium)tetrazolide, and CH_2Cl_2 (61%).

phenylmethyl (DMTr) group on the 5′-hydroxyl group of dT_{15} was deprotected with 4% CCl_3CO_2H/CH_2Cl_2. MmTr-protected 5′-amino-5′-deoxythymidine phosphoramidite **6** was coupled with the 5′-hydroxyl group of dT_{15} **7** in the presence of 1*H*-tetrazole as a coupling reagent with extended coupling time (20 min). After oxidation and capping steps, 5′-MmTr group was deprotected with 4% CCl_3CO_2H/CH_2Cl_2 to give **8**. The CPG column containing the oligomer **8** was then dried with argon gas.

The 5′-amino group of the oligomer was reacted with a 0.1 M solution of neomycin isothiocyanate **3** and 10 mol% DMAP. After 12 hr, the column was washed with 5 × 1 mL of CH_2Cl_2 and dried by flushing with argon gas. The conjugate **9** was then detached from the solid support using NH_4OH and purified by preparative reverse phase HPLC (RP-HPLC) using a triethylammonium acetate buffer system. The dried sample was treated with a 1,4-dioxane solution containing 5% CF_3CO_2H and 1% *m*-cresol (v/v/v %). After 30 min, the deprotected conjugate **10** was precipitated and washed with excess diethyl ether. The deprotected conjugate **10** was finally purified by preparative anion exchange HPLC using a Tris.HCl buffer system (Fig. 4).

Conjugate **10** elutes with a retention time of 6.07 min, whereas the nonconjugated dT_{16} elutes at 7.22 min. All major fractions were pooled together and checked for their identity using MALDI-TOF mass spectrometry (Fig. 5). The expected mass for neomycin-DNA conjugate is m/z 6202.30 Da and the MALDI-TOF mass spectrum showed a peak at m/z 6202.90 Da, confirming the identity of the desired compound.

(2) COVALENT ATTACHMENT OF AMINOGLYCOSIDE TO PNA OLIGOMER

The 5′-amino group of T_{10} PNA **11** (Sch. 4) was similarly reacted with neomycin isothiocyanate in the presence of DMAP and pyridine to give PNA-neomycin conjugate connected through a thiourea linkage. T_{10} PNA **11** was first synthesized using the standard PNA chemistry protocols and deprotected from solid support.

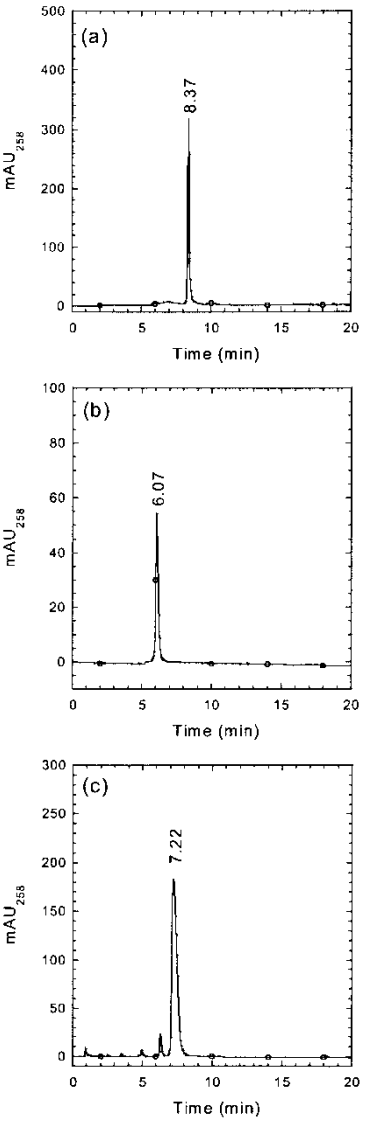


Figure 4: HPLC profiles for dT_{16}-neomycin conjugate with Boc protected neomycin (**9**, plot (a)), dT_{16}-neomycin conjugate (**10**, plot (b)), and dT_{16} (plot (c)); HPLC conditions for plot (a): buffer A: 100 mM of triethylammonium acetate in 10% of acetonitrile, pH = 7.0; buffer A in 90% of acetonitrile; 10–50% of buffer B over buffer A during 20 min; flow rate was 1.5 mL/min; conditions for plot (b) and (c): buffer A: 25 mM of tris-HCl and 1 mM of EDTA, and pH = 8.0; buffer B: buffer A + 1M of NaCl, and pH = 8.0; 30% of buffer B in buffer A for 2 min; 30–50% during 18 min; flow rate was 0.75 mL/min.

After precipitation, the sample was dried and stirred with a pyridine solution containing neomycin isothiocyanate and DMAP for 12 hr. The solution was evaporated and washed with diethyl ether. Boc groups on neomycin were deprotected with trifluoroacetic acid/*m*-cresol (4 : 1) in methylene chloride

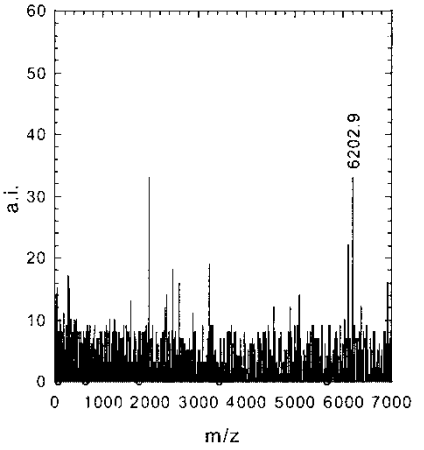
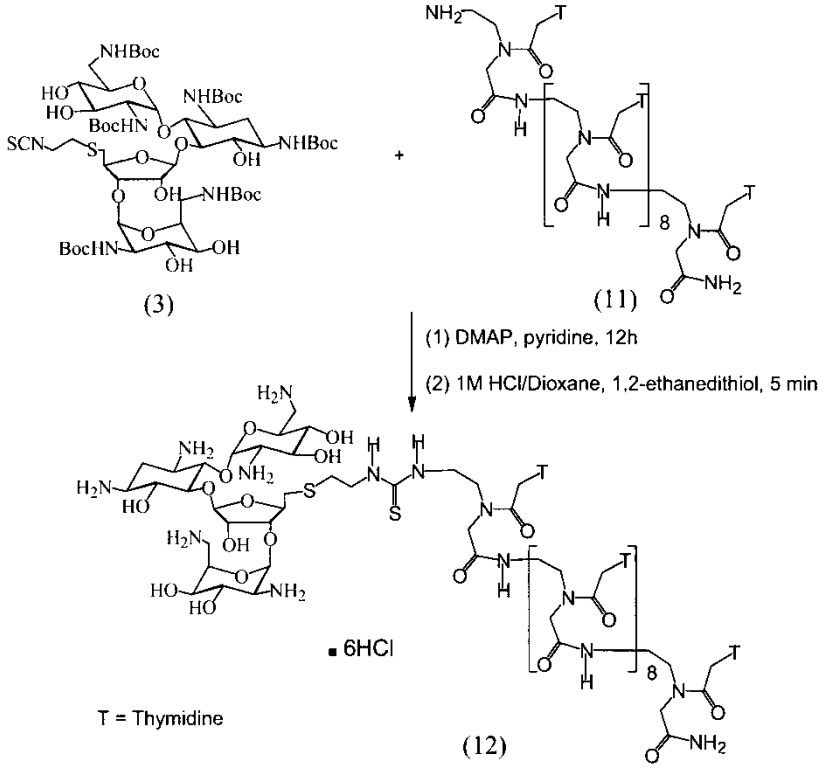


Figure 5: MALDI-TOF profiles for dT_{16}-neomycin conjugate. MALDI-TOF mass was recorded with picolinic acid as a matrix.



Scheme 3: Synthesis of PNA T_{10}-neomycin conjucate.

(v/v%). Conjugate **12** (Sch. 3) was then purified with RP-HPLC using a trifluoroacetic acid buffer system (Fig. 6).

HPLC purification and characterization of T_{10} PNA **11** (Sch. 3) has been found to be difficult because of poor solubility in water and self-aggregation even at lower PNA concentrations (50 μM). After introducing one lysine residue at the 3′-end, T_{10} PNA solubility increases and self-aggregation also decreases.[26,38–42] Conjugation of T_{10} PNA with neomycin remarkably increased the solubility, and conjugate 12 (Sch. 3) did not self-aggregate even at high PNA concentrations.

This is most likely due to the introduction of six amino groups in the conjugate. The identity of the molecule was confirmed with MALDI-TOF (Fig. 7). The mass spectrum of the conjugate **12** (Sch. 3) showed a molecular ion peak at m/z 3398.37 Da (calculated $m/z = 3398.22$ Da).

A few other PNA sequences that are complementary to biologically important DNA/RNA sequences were prepared, and their neomycin conjugates were also successfully prepared.[43–48] The synthesis and purification were carried out in a manner similar to that of PNA T_{10}-neomycin conjugate **12**. The HPLC retention times of these PNAs with and without neomycin conjugation are listed in Table 1.

EXPERIMENTAL

All commercial reagents were used without further purification. Pyridine, methylene chloride, and *N*,*N*-dimethylformamide (DMF) were refluxed over

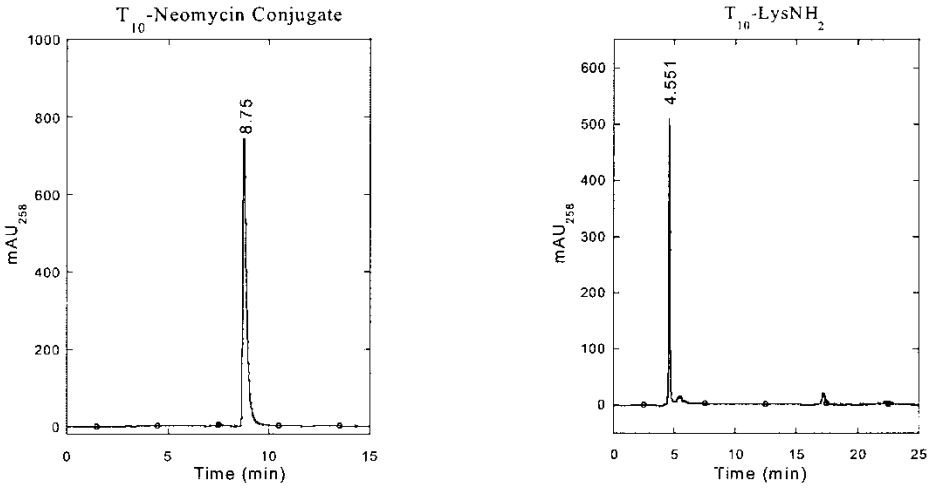


Figure 6: HPLC profile for T_{10}-Neo (**12**) and T_{10}-LysNH_2; buffer conditions for HPLC: buffer A, 0.1% of trifluoroacetic acid in water; buffer B, 0.08% of trifluoroacetic acid in acetonitrile: for PNA, 0–100% buffer B during 7 min; for PNA conjugate, 0–30% of buffer B over buffer A during 13 min; 30–100% buffer B during 2 min.

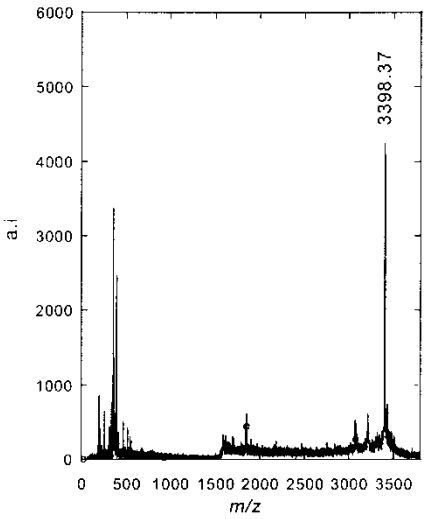


Figure 7: MALDI-TOF profile for PNA T_{10}-neomycin conjugate **12**. α-Cyano-4-hydroxycinnamic acid was used as a matrix.

calcium hydride and distilled. All reactions were carried out in oven-dried glassware under N_2/argon atmosphere. All reactions were monitored by thin-layer chromatography (TLC) with precoated silica gel on glass plates. Visualization of TLC plates was achieved with either UV light or staining solutions such as *p*-anisaldehyde or phosphomolybdic acid followed by heating the plate with a heating gun. ICN silica gel 32–63 (60 Å) was used for column chromatography. ^{1}H NMR spectra were recorded either using a 300 MHz or a 500 MHz NMR. IR was recorded on a Nicolet Magna-IR^{TM} spectrometer-550 either as a solution of 1,2-dichloroethane or CCl_4, and the peaks corresponding to the solvent were subtracted manually. MALDI-TOF mass was recorded on a Bruker Daltonics OmniFLEX^{TM} Bench-Top MALDI-TOF mass spectrometer.

Table 1: HPLC retention times of PNA and PNA-neomycin conjugates.

Serial No.	PNA Sequence	Retention Time (min)	
		PNA	Conjugate
1	HTCTCCCTCTCLysNH$_2^{(43)}$	4.6	8.83
2	HAGCGTGCGCCATCCCLysNH$_2^{(44)}$	4.62	8.98
3	HAGATCTTGGAGTGCGLysNH$_2^{(45)}$	4.64	8.97
4	HTAAACLysNH$_2^{(46)}$	4.52	8.65
5	HCCTAGGAGGAATLysNH$_2^{(46)}$	4.51	8.98
6	HCCGGCNH$_2^{(47)}$	4.50	8.82

Buffer conditions: buffer A: 0.1% of TFA in water; buffer B: 0.1% of TFA in acetonitrile; for PNA, 0–100% buffer B during 7 min; 50°C; for PNA conjugate, 0–30% of buffer B over buffer A during 13 min; 30–100% buffer B during 2 min, 65°C.

Preparative anion-exchange HPLC was carried out on a Phenomenex SAX 80 Å column (10 × 250 mm, 5 μ), and for analytical anion-exchange HPLC, a Waters Gen-Pak FAX (4.6 × 100 mm) column was used. Preparative and analytical RP-HPLC was carried out with Alltima C8 100 Å (250 × 4.6 mm, 10 μ) column.

Preparation of neomycin isothiocyanate (3, Sch. 1b):[36] To a stirred solution of neomycin amine **2** (Sch. 1b) (60 mg, 0.05 mmol) in anhydrous CH_2Cl_2 was added 1,1′-thiocarbonyldi-2(1*H*)pyridone (12 mg, 0.05 mmol) and DMAP (1 mg, 0.008 mmol) under an argon atmosphere. After 13 hr, the solution was concentrated and loaded on a silica gel column. Flash chromatography with EtOAc as the eluent gave the required compound neomycin isothiocyanate **3** (Sch. 1b) in 48% yield (30 mg); $R_f = 0.55$ (10% CH_3OH in CH_2Cl_2); ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 1.4 (m, 54H), 1.89 (2H), 2.85–2.9 (4H), 3.0–3.3 (9H), 3.40–3.52 (6H), 3.76 (2H), 3.89 (2H), 4.15 (2H), 4.23 (1H), 4.92 (1H), 5.13 (1H), 5.3 (br, 1H); IR (as a solution in CCl_4 followed by subtraction of solvent peaks) ν cm^{-1}; 1160.71, 1270.95, 1421.9, 1511.7, 1692.44 (>C═O stretch), 2305.5 (>N═C═S stretch), 2986.74, 3045.28. Maldi MS: 1315.60; found: 1334.55 ($M + H_2O$).

Covalent attachment of neomycin to dT_{16}: The oligonucleotide synthesis was carried out on an Expedite Nucleic Acid Synthesis System (8909) using standard phosphoramidite chemistry. The 5′-amino-5′-deoxythymidine 5 (Sch. 2) was synthesized from thymidine using the literature procedure.[49,50] The 5′-amino deoxythymidine was added at the 5′-end of the dT_{15}(7) with the same phosphoramidite chemistry with 20 min coupling time.

After the oligomer synthesis, the CPG column was dried using argon gas. A syringe containing 0.1 M pyridine solution of neomycin isothiocyanate **3** (Sch. 1b) and 10 mol% of DMAP was attached to one end of the expedite CPG column containing the oligomer **8**, and another end was attached to an empty syringe. The solution was transferred from one syringe to another and left undisturbed for 30 min. Again, the solution was pushed back to the other syringe and this push-and-pull procedure was repeated every 30 min. After 12 hr, the column was detached from both syringes, washed with 5 × 1 mL of CH_2Cl_2, and dried by flushing with argon gas. Then, conjugate **9** was detached from the solid support using NH_4OH, and the resulting solution was evaporated. RP-HPLC purification using triethylammonium acetate buffer gave the conjugate **9**. The dried sample was treated with 1 mL of 1,4-dioxane solution containing 5% CF_3CO_2H and 1% *m*-cresol (v/v/v %). After 30 min, the conjugate **10** was precipitated with 10 mL of diethyl ether, and the precipitate was washed with 3 × 10 mL of diethyl ether.

Conjugate **10** was purified by preparative anion exchange HPLC using a tris buffer (buffer A: 25 mM of tris.HCl and 1 mM of EDTA, buffer B; buffer A and 1 M NaCl; 0–60% of buffer B over buffer A during 60 min). The fractions

were collected and concentrated. The major fractions were checked for their identity using MALDI-TOF mass spectrometry.

Covalent attachment of neomycin to T_{10} PNA oligomer (11): The PNA oligomer synthesis was carried out on Expedite Nucleic Synthesis System (8909) using standard PNA chemistry on PAL resin (5-(4′-Aminomethyl-3′,5′-dimethoxyphenoxy)-valeric acid resin). At the end of the synthesis, the Fmoc-free 5′-amino group containing PNA oligomer **11** was dried by flushing with argon gas. A portion of the sample was removed and deprotected from the column by the following procedure. A syringe containing a solution of CF_3CO_2H (TFA) and *m*-cresol (4 : 1, v/v%) was attached to one end of the PAL resin column and another end was attached to an empty syringe. The solution was transferred from one syringe to another and left undisturbed for 30 min. Again, the solution was transferred back to the other syringe and this push-and-pull procedure was repeated every 30 min. After 90 min, the syringes were detached from the column, and the column was rinsed with 5×0.3 mL of TFA. T_{10} PNA **11** (Sch. 3) was then precipitated with ethyl ether and centrifuged. The precipitate was washed with ethyl ether and dried. Another portion of the T_{10} PNA **11** (Sch. 3) was stirred with a pyridine solution containing 0.1 M solution of neomycin isothiocyanate **3** (Sch. 1b) and 10% of DMAP. After 12 hr, the conjugate was precipitated with 10 mL of diethyl ether and the precipitate was further washed with 3×10 mL of diethyl ether. The Boc groups on neomycin amines were deprotected with 1M HCl/dioxane in the presence of 1,2-ethanedithiol to give **12** (Sch. 3).

Conjugate **12** (Sch. 3) was purified by preparative RP-HPLC using a TFA buffer system (buffer A: 0.1% of TFA in water, buffer B: 0.1% TFA in acetonitrile). The fractions were collected, concentrated, and pooled together after checking their identity using HPLC and MALDI-TOF mass spectrometry.

CONCLUSION

In conclusion, a novel synthesis of nucleic acid (PNA/DNA)-neomycin conjugate is reported. In view of the increased hybrid duplex/triplex stability observed with neomycin, this synthetic methodology opens up new avenues in sequence-specific drug interaction with nucleic acids. These conjugates can be used for targeting DNA/RNA of interest via triplex/duplex formation, and can be used to bring sequence specificity to aminogycoside–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 may assist in overcoming traditional enzymatic/efflux-mediated methods of antibiotic resistance.

ACKNOWLEDGEMENTS

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