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2'-*O*- AND 3'-*O*- PYRIMIDINE AMINOTETHER-CONTAINING OLIGONUCLEOTIDES: SYNTHESIS AND CONJUGATION CHEMISTRY

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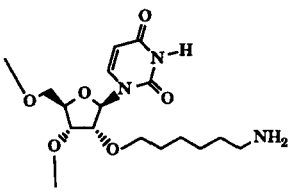
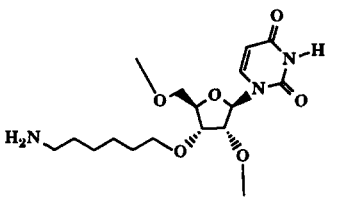
Abstract: Nucleoside synthons carrying an aminohexyl moiety tethered at the 2'-*O*- or 3'-*O*- positions of uridine have been synthesized and incorporated into oligonucleotides. The aminohexyl tether was used to conjugate functionalities such as intercalators, nucleic acid alkylators and absorption-modifying agents. The 2'-modification allows conjugations in the minor groove in the normal 3'-5'-linked oligomers; the 3'-modification allows synthesis of 2'-5'-connected oligonucleotides with a built-in nucleophile. This structural motif has the potential to build novel lariat RNAs and ribozymes.



2'-*O*-Hexylamine, 3'-5' Linkage

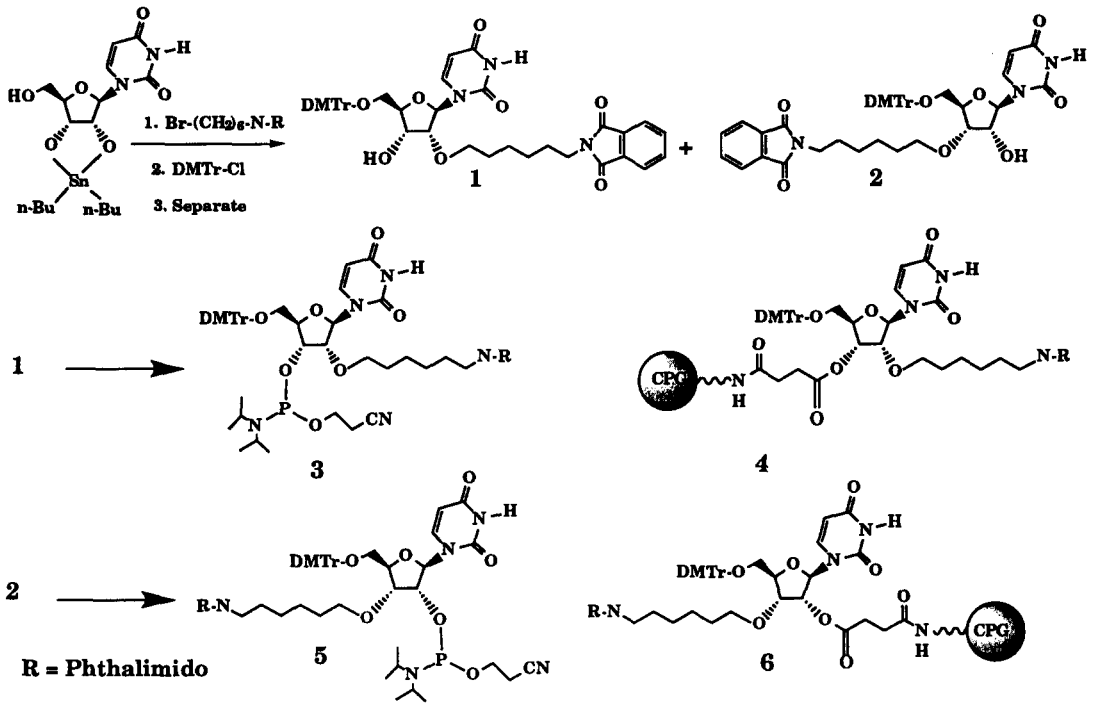


3'-*O*-Hexylamine, 2'-5' Linkag

The drug properties of antisense and antigene oligonucleotides can be enhanced by strategic positioning of ligands capable of ameliorating these properties.[1,2] Certain ligands may improve the cellular delivery of oligomers and increase their affinity for the target gene and resistance to nucleases. The 2'-*O*-position is an attractive modification site.[3] Oligonucleotides possessing the 2'-*O*-alkyl modifications exhibit higher chemical stability under depurination conditions, higher stability to enzymatic cleavage by both endo- and exonucleases, and increased affinity for target mRNA. In addition, they form highly stable triple helices. Thus they promise to be versatile compounds in controlling gene expression by antisense and antigene technologies.

Conjugations at the 2'-position of the carbohydrate moiety may interfere less with base pairing and/or stacking interactions than do conjugations at the internucleotide backbone sites or nucleobase sites. Secondly, this site provides a way to perform multiple conjugations in the minor groove. Thirdly, one can augment the desired properties of the 2'-*O*-alkyls by 2'-*O*-alkyl-ligand conjugates.

Recently, we described[4] a new approach to functionalize oligonucleotides at the 2'-*O*- carbohydrate site via an alkyl aminolinker and an alkyl thiol tether placed at the adenosine nucleoside. In this report we describe the synthesis and incorporation into oligonucleotides of nucleosides containing an *O*-aminohexyl moiety either at the 2' or 3' position of uridine. The *O*-aminohexyl moiety can be used as such to produce amphipathic oligonucleotides.[5] The resulting aminotether[4] has also been used as a site of conjugation.[6] Incorporation of a 3'-*O*-alkylamine-nucleoside gives novel oligonucleotides having a 2'-5' linkage with a built-in amino group for conjugation chemistry. The terminal 3'-*O*-alkylamine-containing oligonucleotide and its pyrene conjugate exhibit resistance to 3'-exonucleases.



Scheme 1

The chemistry used to construct the synthons is summarized in the **Scheme 1**. 2', 3'-*O*-Dibutylstannyleneuridine was synthesized according to the procedure of Wagner *et al.*[7] and alkylated with 6-bromohexylphthalimide and sodium iodide in DMF at 130 °C. The resulting mixture of 2' and 3'-alkylated products was purified (46 % yield, 55:45 ratio of 2' and 3' isomers as confirmed by ^{13}C NMR) and treated with dimethoxytritylchloride/pyridine to protect the 5'-*O*-hydroxyl (80% yield). At this stage, isomers **1** and **2** were easily separated by silica gel chromatography. Compounds **1** and **2** were phosphitylated[8] in approximately 85% yield using reported protocols to give **3** and **5** respectively. The structures of all nucleosides were confirmed by ^{13}C and ^{1}H NMR and by 2D-TOCSY (2'-H to 2'-OH or 3'-H to 3'-OH correlation). Long-chain alkylamino controlled pore glass (CPG) was succinylated and loaded with nucleosides **1** and **2** according to the general procedure of Damha *et al.*[9] The solid support products **4** and **6** (30 μmol/g loading) and the phosphoramidites **3** and **5** were used in an automated DNA synthesizer under standard conditions. During the coupling steps involving these compounds, a 0.15 M solution of the amidite and an extended reaction time of 10 min were used. Coupling efficiency of 90 to 95% was observed. The oligonucleotides were deprotected in the standard manner[10], purified by reverse-phase HPLC followed by size-exclusion chromatography, and analyzed by HPLC, NMR and PAGE. Table I summarizes the oligomers synthesized and the corresponding precursors.

The 400 MHz ^{1}H spectrum of **I**, [d(GAU$_2$*CT), where U$_2$*=2'-*O*-aminohexyluridine], showed 8 signals between 7.5 and 9.0 ppm corresponding to the 8 aromatic protons. In addition, the anomeric proton of U* appears as a doublet at 5.9 ppm with $J_{1',2'}$=7.5 Hz, indicative of C2'-endo sugar puckering. The corresponding 2'-5' linked isomer **V** shows a similar structure with a $J_{1',2'}$=3.85 Hz at 5.75 ppm, indicating a move towards an RNA type sugar puckering at the modification site favorable for hybridization to an mRNA target. The proton

spectrum of oligomer **VII**, [d(GACU$_3$*), where U$_3$*=3'-*O*-hexylamine], showed the expected 7 aromatic proton signals between 7.5 and 9.0 ppm and the anomeric proton doublet at 5.9 ppm with $J_{1',2'}$=4.4 Hz. This suggests a greater C3'-endo puckering for the 3'-*O*-alkylamino compounds compared to their 2' analogs. ^{31}P NMR of these oligonucleotides showed the expected 4 and 3 signals from the internucleotide phosphate linkages for d(GAU*CT) and d(GACU*), respectively. The isomers **II** and **VI** have different retention times in RP HPLC and hence different lipophilicities, implying potentially different interactions with cell membranes (**Fig. 1 A**).

TABLE I. Representative Oligonucleotides Synthesized

Oligo	Sequence (5' to 3')	Precursor	Features of the Sequence
I	GAU$_2$*CT	3	P=O; 3'-5' linkage
II	GCG TGU$_2$* CTG CG	3	P=O; 3'-5' linkage
III	GACU$_2$*	4	P=O; 3'-5' linkage; terminal 2'-tether
IV	GsCsG TGU$_2$* CTGs CsG	3	P=O/P=S (diastereomeric mixture) chimera; 3'-5' linkage
V	GAU$_3$*CT	5	P=O; 2'-5' linkage
VI	GCG TGU$_3$* CTG CG	5	P=O; 2'-5' linkage
VII	GACU$_3$*	6	P=O; 3'-5' linkage; terminal 3'-tether
VIII	GCCTTTCGCGACCCAACACU$_3$*	6	P=O; HCV antisense; 3'-5' linkage; terminal 3'-tether

The oligonucleotides were conjugated to pyrene via the aminohexyl linker using pyrene butyric acid-N-hydroxy-succinimidyl ester . Oligonucleotide **II** was further conjugated to two other novel ligands: an intrastrand nucleic acid alkylator using bromoacetic acid-N-hydroxy-succinimidyl ester and a poly(ethylene glycol)-propionic acid-N-hydroxy-succinimidyl ester (PEG)[11] of molecular weight 5000 (which has been shown to modify the pharmacokinetic properties of therapeutic proteins). These conjugates were obtained in 80-90% yield. The HPLC retention time of the oligonucleotide is substantially increased by conjugation of the pyrenyl group or the PEG polymer (**Fig. 1**). However, as the hydrophobicity of conjugated oligonucleotides is now dictated solely by the functional groups (pyrene or PEG), and not by the nature of the linkage, there was no difference in retention times between conjugates derived from **II** and **VI**.

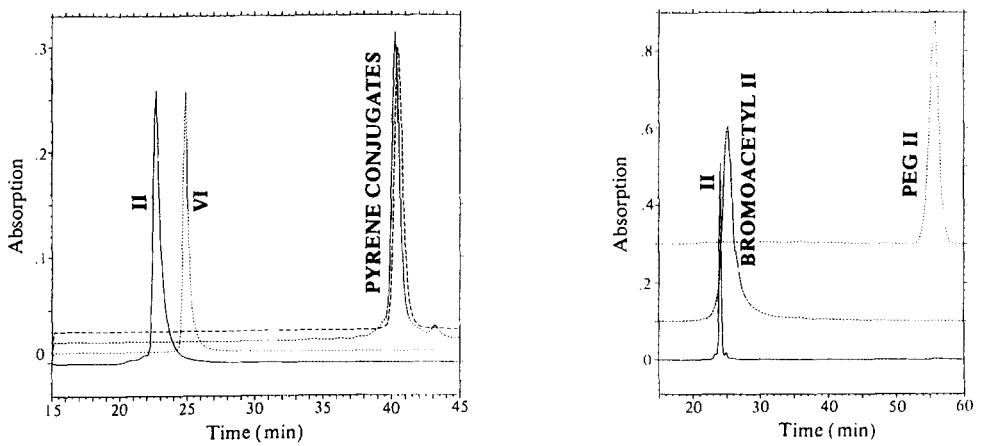


Figure 1. RP HPLC Analysis of **A) II, VI** and their pyrene conjugates **B) II** and other conjugates.

Since 3'-exonucleases need the 3'-hydroxyl group of the 3'-terminal nucleotide for their mechanism of action,[12] blocking the 3'-OH group with the hexylamino group is expected to improve the nuclease resistance of the oligonucleotide.[13] In accordance with this expectation, the 3'-*O*-alkylamine-containing phosphodiester HCV antisense oligonucleotide **VIII** and its pyrene conjugate had 3-4 times longer half-lives than the parent 3'-hydroxy compound in nuclear and cytoplasmic extracts (data not shown).

In conclusion, conjugation chemistry has been carried out from both the normal 3'-5'- and the 2'-5'-phosphodiester linked oligomers using the novel amino tethers. Experiments using the alkylator conjugate to target nucleophilic sites in the minor groove of the complementary strand[14] and PEG conjugates to improve the pharmacokinetics of oligonucleotides are underway. The less well-known 2'-5' analogs[15] already have a favorable RNA-like sugar pucker, are nuclease resistant and known to activate RNase L.[16] Conjugations of novel ligands to these analogs have promise for therapeutic applications, design of novel ribozymes or construction of new lariat structures.[17] Furthermore, the nucleosides **1** and **2** can be further derivatized at the monomer stage for functionality placement via the amino groups.

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