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LIPIDIC NUCLEIC ACIDS

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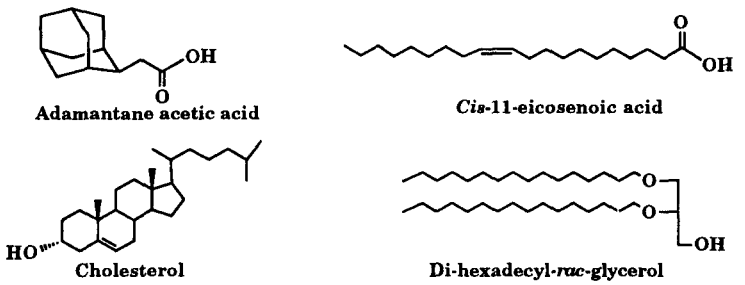
Abstract: Lipophilic nucleosides were synthesized from uridine via either a 2'-*O*-hexylamino tether or the corresponding 3'-linker. These synthons allowed site-specific incorporation of the lipophilic moieties into antisense oligonucleotides. A spectrum of lipophilicity was observed in the oligonucleotides, depending on the pendent group.

The efficiency of antisense oligonucleotides in blocking gene expression requires the maintenance of a high intracellular concentration.[1] In an attempt to increase the cellular concentration of oligonucleotides, we synthesized lipidic nucleoside building blocks and incorporated them into oligonucleotides to yield lipidic nucleic acids. The lipidic pendent groups include adamantane, *cis*-11-eicosenoic acid, cholesterol and a di-hexadecyl-glyceride ether.

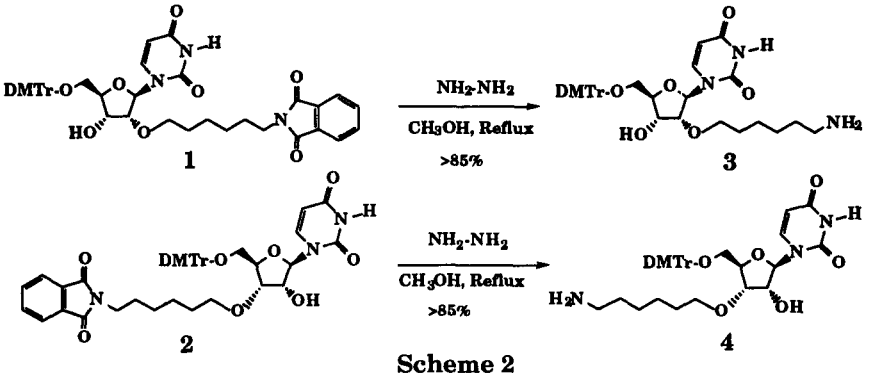
The rationale for lipid conjugation is as follows: 1) Lipidic nucleosides are biosynthetic intermediates. Lipophilic pendent groups are present in therapeutically active nucleoside natural products (e.g., liposidomycin).[2] Lipophilic pendents are employed as uptake-enhancers for synthetic nucleoside drugs like AZT.[3] 2) Lipidic nucleic acids, due to their chimeric nature, should have physical properties of both lipids and nucleic acids; the hydrophobicity, due to their alkyl chains, should reduce the hydrophilicity of oligonucleotides. 3) Lipidic nucleic acids can exploit biological uptake processes: some hydrophobic molecules (e.g., cholesterol) can bind to LDL particles and lipoproteins and activate a delivery process involving these proteins to transport the oligonucleotides;[4] lipoproteins consist of an apolar core of cholesteryl lipids, embedded in triglycerides and apoproteins. 4) Lipidic nucleic acids can also improve the internalization by adsorptive endocytosis. Lipidic conjugates derived from oligonucleotide modifications at the terminal ends, backbone, or base have already shown some promise in improving the efficacy of oligonucleotides.[5]

We report here the synthesis of lipophilic nucleosides employing a functionalization process recently reported from our laboratories.[6] This strategy allows modifications at the 2'- or 3'- position of nucleosides which, after conversion to phosphoramidites, are amenable to automated oligonucleotide synthesis. Conjugation at the 2'-position should minimize interference with hybridization to the target RNA.



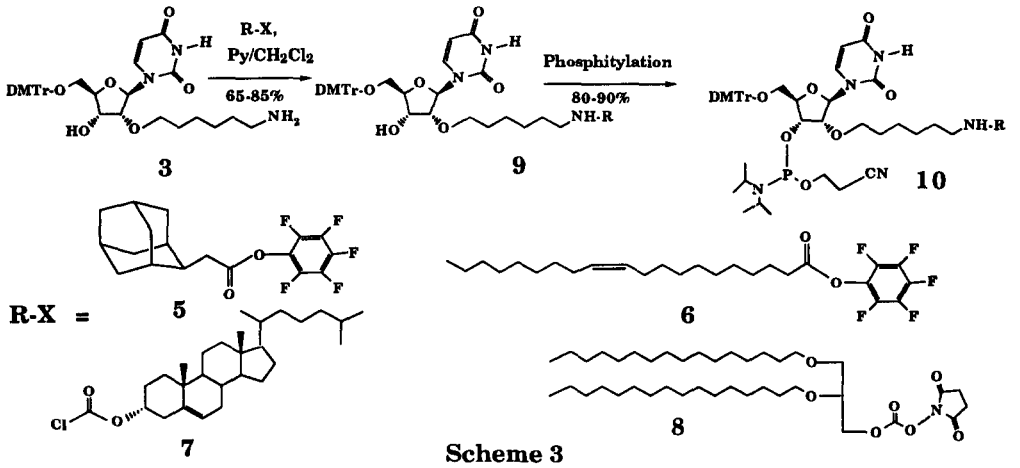
Scheme 1

The hydrophobic ligands used are shown in **Scheme 1**. The nucleoside intermediates needed for the functionalizations are shown in **Scheme 2**. 5'- Dimethoxytrityl-2'-*O*-hexylphthalimidouridine[6] **1** was reacted with hydrazine in refluxing methanol to yield the free amine **3**. The corresponding 3'-isomer[6] **2** yielded the amine **4**. Silica gel purification using ammoniacal methanol yielded crystalline compounds in 85-90% yield.



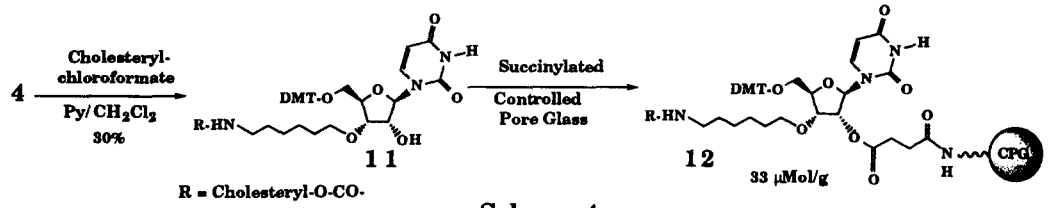
Scheme 2

The lipidic nucleosides were synthesized as follows (**Scheme 3**): Adamantane acetic acid and eicosenoic acid were converted to their pentafluorophenyl esters **5** and **6** (quantitative yield) and condensed with the amine **3** (85% yield). The resultant nucleosides have an amide linkage between the hydrophobic group and the nucleoside. Derivatization with cholesterol was accomplished with cholesteryl chloroformate **7**. 1,2-di-*O*-hexadecyl-*rac*-glycerol was converted to the corresponding carbonate **8** (91% yield) using disuccinimidyl carbonate.[7] Nucleosides **7** and **8** were condensed with the amine **3** to yield (70-75%) the modified nucleosides containing carbamate linkages. The nucleosides, after purification on a silica gel column, were phosphitylated (80-90%) to yield the corresponding phosphoramidites. Amidites were soluble as 0.1 M solutions in either anhydrous CH_3CN (adamantyl and eicosenoyl derivatives) or 1:1 CH_2Cl_2:CH_3CN (cholesterol and the glyceride derivatives). By increasing the reaction time to 10 min and using a double coupling cycle, a coupling efficiency >80% was observed for novel amidites. These amidites are useful in incorporating the lipidic nucleoside at internal or 5' terminal positions of any oligonucleotide.



Scheme 3

The attachment of the cholesterol ligand at the 3'-end of the oligomer is shown in **Scheme 4**. The modified nucleoside was condensed with presuccinylated controlled pore glass (CPG). In this case, two variations are possible: the CPG can be attached via the 3'-OH of the 2'-conjugated nucleoside or the 2'-OH of the 3'-analog. While we speculate that both of these derivatizations will improve nuclease resistance of oligonucleotides, the 3'- lipid derivative will likely be more resistant due to the blocking of 3'-OH group.[8]



Scheme 4

400 MHz NMR analyses of functionalized nucleosides and short modified oligonucleotides (5'-U*T) or d(5'-GAU*CT) confirmed their structures. Several antisense oligonucleotides targeted against C-raf Kinase, Intracellular Adhesion Molecule-1 (ICAM-1) and Cytomegalovirus (CMV) mRNAs were then synthesized using the above synthons. They were analyzed (**Figure 1**) by reverse phase HPLC, capillary gel electrophoresis (CGE) and mass spectrometry. The biophysical data from the ICAM-1 series is summarized in **Table I**. Hybridization experiments of the 2'-conjugated oligonucleotides to their RNA complements reveal that even large lipid modifications are tolerated well at the 2'-position as expected.

Table I. Analytical Data Of Isis 3082 Analogs With 2'-Hydrophobic Groups

U*GCATCCCCCAGGCCACC AT (P=S backbone; ICAM-1 antisense oligonucleotide)

Compound	ISIS #	2'-Modification	Mass Calculated	Mass Observed	HPLC Retention time[a]	Tm °C against RNA
I	3082	None	6288.6	6287.41	18.0	69
II	9759	Adamantane	6565.8	6565.75	22.0, 22.5[b]	69
III	9770	Eicosenoic acid	6682.0	6682.04	38.5, 39.8[b]	68
IV	8005	Cholesterol	6802.0	6802.15	46.1, 47.5[b]	67
V	10826	Glyceride lipid	6956.9	6956.51	57.0, 60.0[b]	c

[a] C-18 RP column; Solvent A: 50 mM Triethylammonium acetate pH = 7.0; B: CH_3CN. Gradient: 10-95% B in 60 min. [b]Two peaks are observed due to diastereomeric grouping of phosphorothioates. [c]Not determined.

As a model for the cell membrane-antisense oligonucleotide interaction, a C-18 reverse phase HPLC column was used to assess the lipophilicity of oligonucleotides. This method was found to be more accurate than the conventional method of evaluating the partition coefficent between 1-octanol and water.[9] As shown in **Figure 1**, the retention time of the oligonucleotide, and hence its lipophilicity, increases with the number of carbon atoms in the ligand. There is a linear correlation between the percentage of acetonitrile needed for elution and the total number of carbon atoms in the 2'- side chain of oligonucleotides (2% increase in CH_3CN concentration for each additional carbon atom). We are presently evaluating this series of antisense conjugates in both *in vivo* and *in vitro* assays.

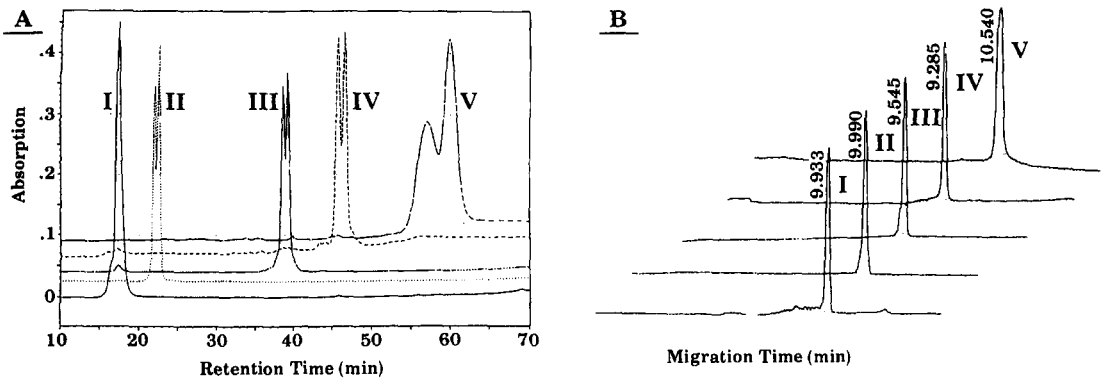


Figure 1. Analysis of Oligonucleotides: **A**) Relative Lipophilicity by RP HPLC and **B**) Purity by CGE.

As illustrated here, the 2'- and 3'-*O*-alkylaminonucleosides are versatile building blocks for the conjugation of activity-enhancing groups to therapeutic nucleosides and antisense oligonucleotides. In addition to the uptake modifiers shown here, reporter groups, intercalators, DNA cleaving agents, metal chelating ligands and other functionalities can be attached and incorporated via automated synthesis. This functionality placement approach is complementary to conjugation chemistry at the oligonucleotide stage that uses an amino group at a preselected position.[6] Finally, at the nucleoside level, several 2'- or 3'-deoxy derivatives can be synthesized for antiviral testing using the deoxygenation protocols of 3'- or 2'- compounds.

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