Bioorganic & Medicinal Chemistry Letters, Vol.3, No.12, pp. 2765-2770, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00

INTRODUCTION OF A LIPOPHILIC THIOETHER TETHER IN THE MINOR GROOVE OF NUCLEIC ACIDS FOR ANTISENSE APPLICATIONS

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(*Received in USA* 7 *September* 1993; *accepted* 1 *October* 1993)

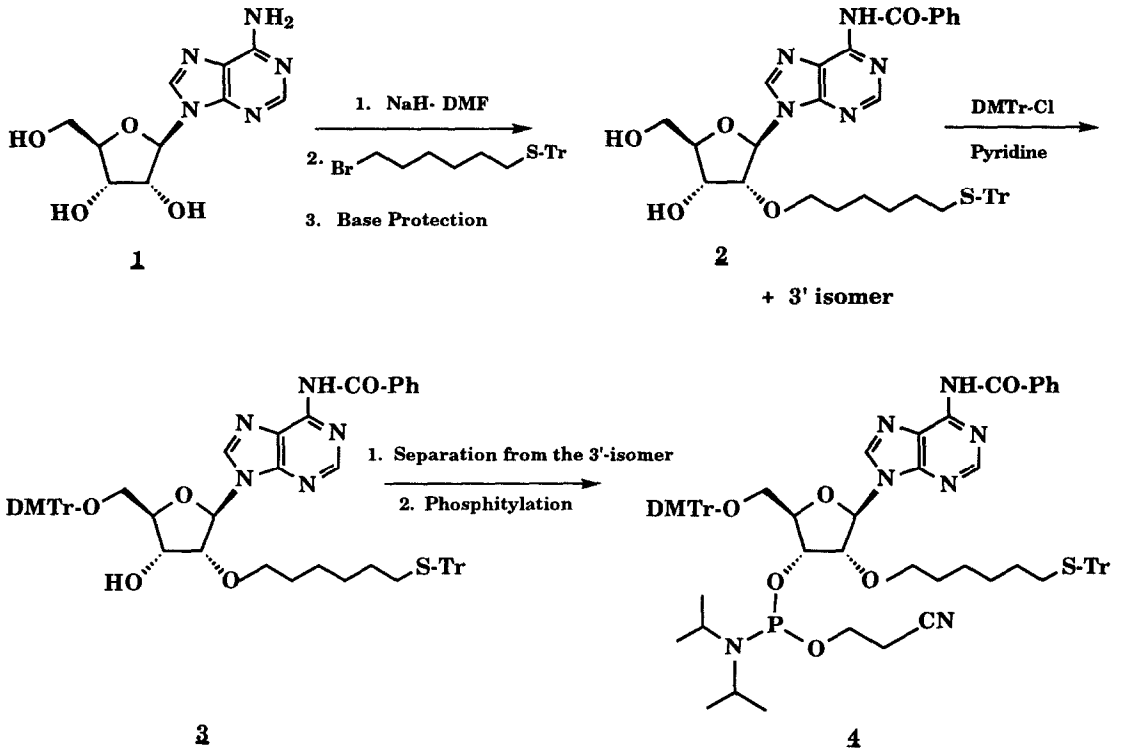
Abstract: A 2'-*O*-hexylthiotrityl adenosine phosphoramidite has been synthesized and incorporated into oligonucleotide phosphodiesters and phosphorothioates. This tether has potential antisense applications, offers a convenient nucleophile for conjugation of various moieties that would reside in the minor groove, and may form novel tertiary structures.

Recently we described[1] the functionalization of oligonucleotides at the 2'-*O*-position using a 2'-*O*-alkylamine. This novel conjugation chemistry[2] (post-oligonucleotide-synthesis attachment of ligands) at the 2'-*O*-position of the carbohydrate moiety offers the possibility of less interference with base pairing and/or stacking interactions compared to conjugations at internucleotide backbone sites or nucleobase sites. Furthermore, chemistry at this site provides technology to perform multiple conjugations in the minor groove. In this report, we describe a method to introduce a cysteine-like thiol tether in the minor groove. Specifically, a hexyl-*S*- tritylthiol linker has been attached at the 2'-*O*-position of adenosine and its phosphoramidite incorporated into oligonucleotides. This particular linker was chosen for the following reasons: **1)** a tritylthioether linkage increases the lipophilicity of antisense oligonucleotides which may enhance their uptake characteristics[3]; **2)** a free thiol group can be conveniently generated and then used for conjugation chemistry[4] to attach intercalators, nucleic acid cleaving agents, cell surface interacting phospholipids, and diagnostic agents such as fluorophores; and **3)** the free thiol group may be used to generate tertiary structures in nucleic acids in the same way that nature uses cysteine in peptides and proteins.

The modified adenosine phosphoramidite **4** was synthesized as shown in Scheme **1**. Treatment of dibromohexane with the sodium salt of triphenylmethanethiol yielded (73% yield) *S*-trityl-6-mercaptohexyl bromide. Unprotected adenosine was alkylated (27%) with this hexyl bromide in the presence of DMF and sodium hydride to yield a 2'-*O*- and 3'-*O*-alkylated mixture in the ratio of 84:16 (as judged by ^{13}C NMR). The mixture was *N*-6-benzoyl protected (95%) using Jones' transient protection[5] and then the 5'-sugar hydroxyl was protected by treatment with dimethoxytrityl chloride/pyridine. The resultant mixture was conveniently separated into the 2'-*O*- and 3'-*O*-isomers on a silica gel column (56%, combined isolated yield). The major 2'-*O*-isomer was phosphitylated to yield (81%) the desired phosphoramidite **4**[6].

A 0.15 M solution of the modified amidite in anhydrous CH_3CN was used in an ABI 380B DNA synthesizer to synthesize oligonucleotides. Oligonucleotides with normal P=O backbones and modified P=S backbones were synthesized. The oligonucleotides containing P=S backbones were synthesized using the

Beaucage reagent[7] and standard synthesis cycles. During the phosphodiester synthesis, oxidation of the tervalent phosphorous was achieved using a 0.5 M solution of *t*-BuOOH in CH_3CN [8] since the tritylthioether group was sensitive to iodine/water solution. With an increased reaction time (10 min), a coupling efficiency of >90% was observed during the modified amidite coupling. For example, a 1 μMol synthesis of oligonucleotide **I**, d(GAA*CT), yielded 36 OD units of purified oligonucleotide (72% overall isolated yield).



Scheme 1

Both P=O and P=S oligonucleotides were dimethoxytrityl-on purified and the dimethoxytrityl group (DMTr) was subsequently removed with 15% acetic acid and then purified again. NMR analysis of oligonucleotides **I**, d(GAA*CT), and **II**, d($G_SA_SA*_SC_ST$), showed the integrity of these compounds (Figure 1). In Figure 1, panel **B**, the trityl group resonance is observed between 7.0 and 7.5 ppm and ^{31}P (panel **A**) showed the expected 4 peaks. It is noteworthy that one of the signals is shifted about 0.5 ppm from the other signals as observed in other RNA/DNA hybrids[9]. In the case of the phosphorothioate oligomer **II**, d($G_SA_SA*_SC_ST$), a total of 2^4 = 16 signals is expected due to the diastereomeric nature of chiral phosphorothioates; the multiplicity observed conforms to this expectation. The oligomer **I** was digested to individual nucleosides with snake venom phosphodiesterase and calf intestinal alkaline phosphatase in order to confirm its nucleoside composition. We observed some resistance to enzymatic digestion (nuclease resistance) compared to unmodified oligonucleotides which may be due to the presence of the 2'-*O*-tether.

To illustrate the conjugation potential of the 2'-*O*-thiol tether, the oligonucleotide **I** was treated with 0.1M $AgNO_3$ in TEAA buffer followed by DTT treatment to generate a free thiol group. At this stage, it was reacted (Scheme **2**) with four classes of compounds, each having either a haloacetamide or a maleimide group and the desired functionality at the other end. The following compounds (Scheme **3**) were employed: **a**) a phospholipid maleimide, which can offer cell signalling and trafficking properties to nucleic acids, **b**) pyrene maleimide, which stabilizes nucleic acid duplexes via intercalation[10], c) fluorescein maleimide,

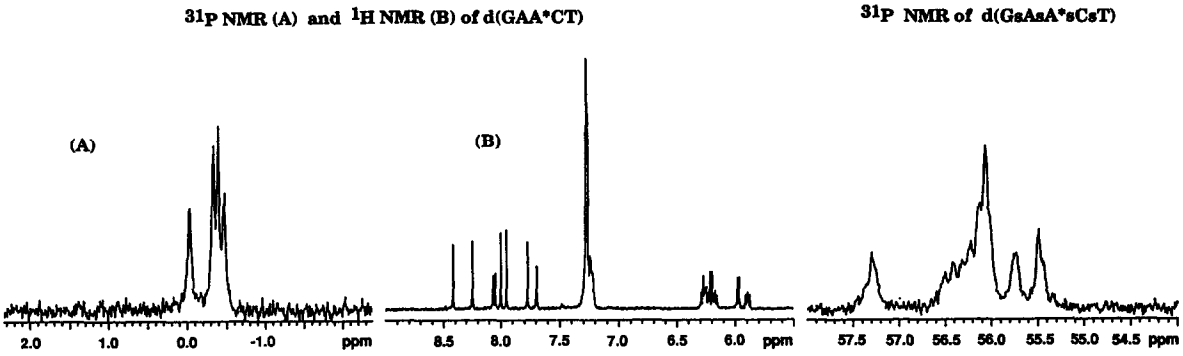
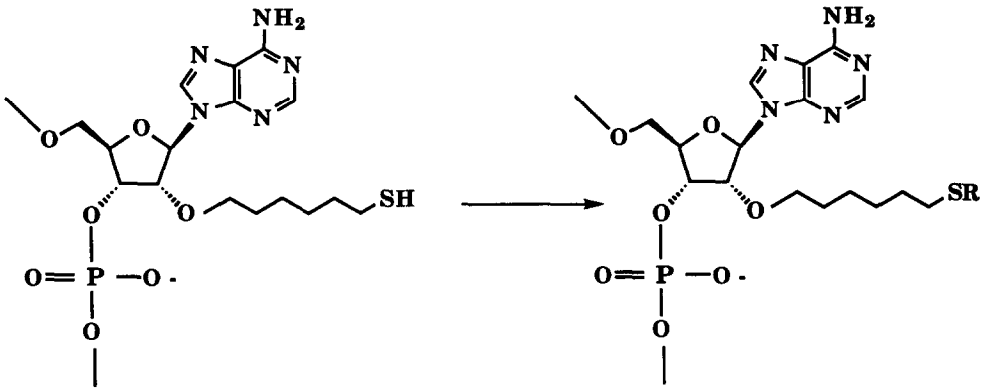
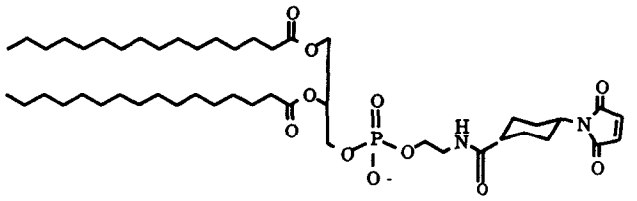


Figure 1

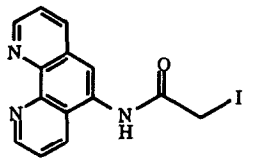
which is used as a general diagnostic tool, serving to follow the uptake of antisense oligonucleotides, and **d**) 5-iodoacetamido-*O*-phenanthroline[11], which is a nucleic acid cleaving agent. This last conjugation offers an added advantage of optimal placement for the cleaving agent: this reagent when complexed to cuprous ion, reacts via a minor groove attack at the C-1' position. All conjugations were carried out in phosphate buffer (pH 8.0) and yields were >95% (no starting oligonucleotide was apparent in the HPLC analysis). The conjugates were easily purified by size exclusion chromatography and reverse phase HPLC and characterized (Figures **2** and **3**) by their UV-VIS spectra (where applicable). The retention times of different oligonucleotides and their conjugates are given in Table **I**.



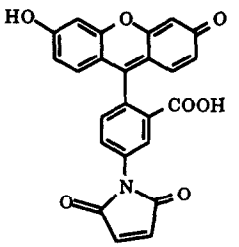
Scheme 2



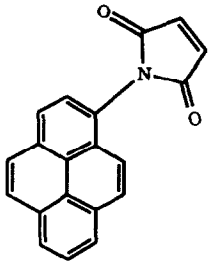
Phospholipid maleimide



o-Phenanthroline-5-iodoacetamide



Fluorescein maleimide



Pyrene maleimide

Scheme 3

Table I. HPLC retention times of oligonucleotides I and II and their derivatives

Entry	Oligonucleotide	Retention Time (min)	Approximate % CH_3CN Needed for Elution[a]
1	d(GAUCT)[b]	17.00	16
2	DMTr-d(GAA*CT)	46.18	45
3	d(GAA*CT), I	39.24	39
4	I-Phospholipid conjugate	22.40	22
5	I-*O*-Phenanthroline conjugate	26.30	26
6	I-Fluorescein conjugate	25.90	26
7	I-Pyrene conjugate	35.5	36
8	DMTr-d($G_SA_SA^*{}_SC_ST$)	46.3	46

[a]Shown as a measure of the lipophilicity of the given oligonucleotide. [b]A normal diester for comparison with entry 3. HPLC conditions: C-18 reverse-phase column; linear 1% increase of CH_3CN concentration for every minute.

Using the same amidite, several full-length antisense oligonucleotide sequences were made as shown below:

III. 5'-TsGsGs GsA*sGs CsCsAs TsAsGs CsGsAs GsGsC-3' (ICAM antisense oligonucleotide with P=S backbone)

IV. 5'-TCT GAG TAG CAG AGG AGC TA*A G-3'
(Sequence in the 5'-cap region of ICAM with P=O backbone)

Oligonucleotide **III** will serve to evaluate the tritylthioether group in uptake experiments to determine its ability to inhibit ICAM (Intracellular Adhesion Molecule) protein expression. The fate of this molecule will be determined with and without an additional fluorescein label at the 5'-position to follow the path of antisense molecules inside and outside the cell membrane. Oligomer **IV** will be conjugated to *O*-phenanthroline and targeted against the 5'-cap-messenger RNA of the ICAM system to cleave the target RNA. Finally, compound **3** was attached to controlled pore glass and the solid support product (30 μMol/gm loading) was used for standard oligonucleotide synthesis. The resultant oligonucleotides have a self-contained 3'-terminal end thiol linker.

In summary, in this report we have demonstrated a simple method of incorporation of a thiol tether in the minor groove of nucleic acids for conjugation purposes. We are exploring the possibility of achieving novel secondary structures of nucleic acids utilizing the present thiol tether via disulfide (-S-S-) crosslinking[12] and other reactions involving thiols.

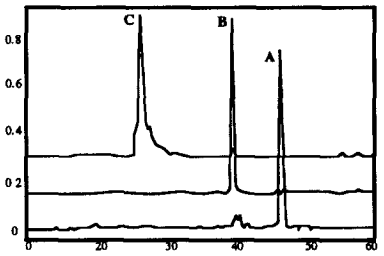


Figure 2. HPLC Analysis of A) DMTr-d(GAA*CT), B) d(GAA*CT) and C) d(GAA*CT)-*O*-Phenanthroline conjugate via the free thiol group.

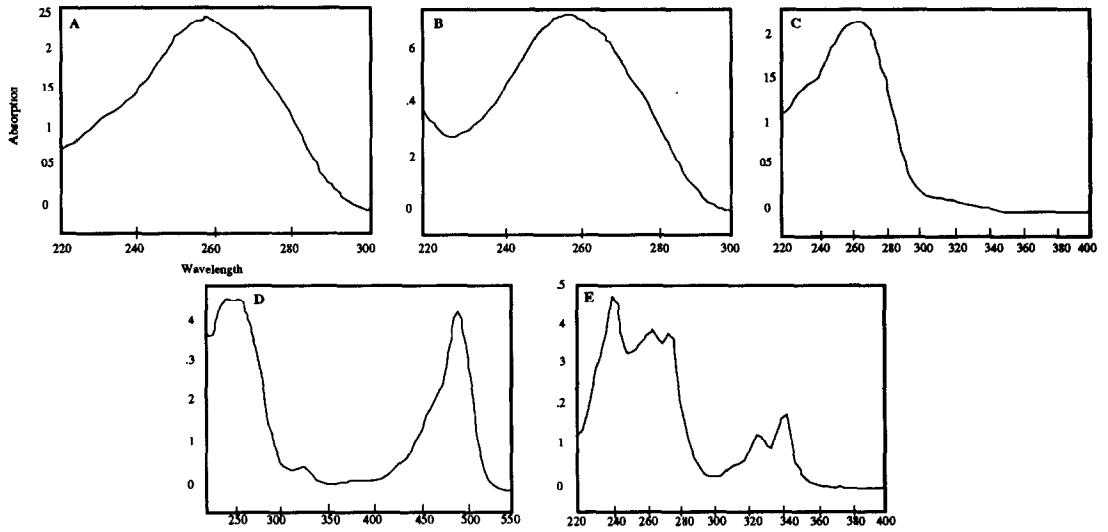


Figure 3. UV-VIS Absorption Spectra of A) d(GAA*CT) and its Conjugates: B) Phospholipid Conjugate, C) *O*-Phenanthroline Conjugate, D) Fluorescein Conjugate, E) Pyrene Conjugate.

Acknowledgements: We are grateful to Bruce Ross, Maryann Zounes, Patrick Wheeler, Len Cummins and Henri Sasmor for their valuable help. We want to thank Andrew Kawasaki and Professor Larry McLaughlin (Boston College) for suggesting the use of *t*-BuOOH oxidation and Professor David Sigman (UCLA) for the *O*-phenanthroline reagent. Julie Walker typed this manuscript.

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