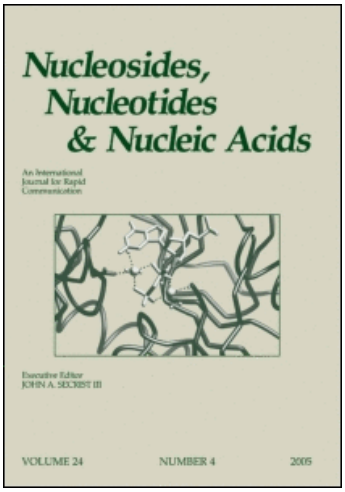

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APPLICATION OF OXATHIAPHOSPHOLANE METHOD FOR THE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDE 5'-O-CONJUGATES

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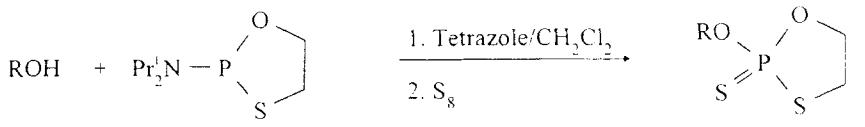
ABSTRACT: 2-Thiono-1,3,2-oxathiaphospholane derivatives of lipophilic alcohols including borneol, cholesterol, menthol and heptadecanol were synthesized and reacted with support-bound oligodeoxyribonucleotides containing free 5'-hydroxyl groups. The reaction is catalyzed by DBU and leads to oligodeoxyribonucleotide conjugates possessing a lipophilic alcohol residue bound at the 5'-end *via* a phosphorothioate linkage.

One of the methods employed recently to improve the efficacy of antisense oligonucleotides involves their conjugation with other organic molecules.[1-3] It was found that covalent attachement of biomolecules such as lipids,[4] cyclodextrins,[5] steroids,[6] porphines,[7] long-chain alcohols[8] or peptides[9-10] to oligonucleotides results is considerable improvement of cellular uptake and has therefore a positive effect upon their activity as sequence-selective inhibitors of expression of target genes.[1-3] In this paper we wish to present our results on application of oxathiaphospholane approach[11,12] to the synthesis of oligodeoxyribonucleotide conjugates bearing at the 5'-end a lipophilic alcohol residue attached *via* a phosphorothioate linkage.

The oxathiaphospholane derivatives of alcohols such as borneol, cholesterol, menthol (all in natural, optically active form), and heptadecanol were chosen for preliminary experiments. Thus, 2-alkoxy-2-thiono-1,3,2-oxathiaphospholanes (**1**-**4**) were prepared according to a procedure described earlier in detail for cholesteryl derivative **2**.[13] Each alcohol was reacted with 2-N,N-diisopropylamino-1,3.2-oxathiaphospholane (**5**) in the presence of tetrazole in CH_2Cl_2 solution, followed by addition of elemental sulfur.



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1, R = Bornyl
2, R = Cholesteryl
3, R = Menthyl
4, R = Heptadecanyl

Crude **1-4** were isolated by a column chromatography on Silicagel 60 (elution system: toluene-chloroform, 3:1) as a mixture of diastereomers (**1-3**) or as a racemate (**4**). Their physicochemical characteristics is presented in Table 1.

The oxathiaphospholane derivatives **1-4** were further reacted with 5'-OH group of nucleosides or oligonucleotides. In preliminary experiments the heptadecanyl derivative **4** was reacted with 3'-O-acetylthymidine in the presence of DBU (1:1:1 molar ratio) in CH_2Cl_2 solution (concentration 0.1 M). The ^{31}P NMR analysis of a reacting solution showed that after 30 min at room temperature 93.8% of a substrate was transformed into 3'-O-acetylthymidine-5'-O-heptadecanyl phosphorothioate (δ ^{31}P NMR 57.9, 58.2 ppm). Similar results were also obtained for oxathiaphospholanes **1-3** albeit the condensation was slower (79.4%, 76.6% and 84.0% of transformation, respectively, reaction conditions as above).

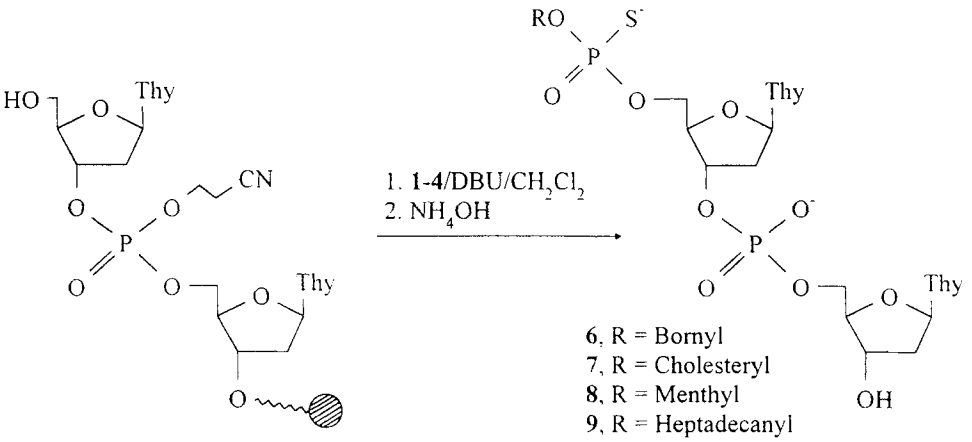
Further experiments on introducting of lipophilic alcohol residue at the 5'-position by oxathiaphospholane approach were performed with a model system involving a thymidine dinucleotide prepared by a standard phosphoramidite method on a solid support, using ABI-Perkin-Elmer DNA synthesis column. The oxathiaphospholane reaction was performed manually, with a column attached to a syringe. The reaction conditions were optimized with regard to the solvent, condensation time (20-60 min), oxathiaphospholane concentration (0.1-0.15M) and DBU concentration (1-3M). Sarcosinylated solid support (LCA CPG SAR) was employed to ensure a stability towards DBU.[14] The most satisfactory yields of dithymidine conjugates were obtained when CH_2Cl_2 solution containing oxathiaphospholane **1-4** (0.15 M) and DBU (2 M) (190 μl, premixed directly before use from more concentrated solutions) was incubated, with occasional manual stirring, with 1 μmol of support bound dinucleotide (with free 5'-OH group) for 40 min. These conditions ensured 30-fold molar excess of oxathiaphospholane and 400-fold molar excess of DBU with respect to support bound dinucleotide. After standard ammonia cleavage from the support the conjugates **6-9** were isolated by preparative RP HPLC. The physicochemical

TABLE 1

Oxathiaphospholane derivative	δ ^{31}P NMR(ppm)[a]	Molecular weight (Da)		Yield (%)
		Calculated	Measured[b]	
1	104.91, 105.23 (CD_3CN)	292.3	293.1	79
2	102.38, 102.39 ($CDCl_3$)	524.7	525.6	74
3	103.43, 104.77 (CD_3CN)	294.3	295.1	73
4	105.09 (CD_3CN)	392.5	395.2	74

[a]85% H_3PO_4 as external reference, positive values when downfield from the standard, Bruker AC 200.
[b]Positive CI Mass Spectrometry, Finnigan MAT 95.

characteristics and yields of dinucleotide conjugates obtained at optimal conditions on an 1 μmol scale are given in Table 2.



The foregoing optimized protocol was further employed for introduction of liphophilic alcohol residue at the 5'-end of oligodeoxyribonucleotides longer than dimers, including dodecamers dA_{12} (phosphodiesters A_{12}-PO, phosphorothioates A_{12}-PS) and hexadecadeoxyribonucleotides complementary to a fragment of mRNA of plasminogen activator inhibitor type 1 (PAI-1)[15] with a sequence d[GAG GGC TGG AGA CAT C], both in phosphodiester (PAI-PO) and phosphorothioate (PAI-PS) form. The parent oligonucleotides were prepared on an automatic DNA Synthesizer (ABI-Perkin Elmer 394)

TABLE 2

Dinucleotide conjugate	HPLC t_R[a]	δ ^{31}P NMR in CD_3CN (ppm)		Molecular weight (Da)		Yield (OD_{260})[c]
		Phosphate	Phosphorothioate	Calculated	Measured[b]	
6	16.79	0.0	56.6, 56.8	776	777.5	3.0
7	28.59 29.26	0.2 0.1	55.7 55.9	1008	1009.4 1009.4	5.0
8	17.65	0.0	55.6, 55.9	778	779.4	4.3
9	29.72	0.0	56.9	876	879.5	3.8

[a]ODS - Hypersil 250x2.1 mm column, linear gradient of 0-80% CH_3CN in 0.1 M ammonium acetate (2%/min), flow 0.3 ml/min.
[b]Negative FAB Mass Spectrometry, Finnigan MAT 95.
[c]Optical density units at 260 nm.

TABLE 3

Oligonucleotide conjugate	HPLC t_R (min)	Molecular weight (Da)		Yield[d] (OD_{260})
		Calculated	Measured[c]	
Chol-A_{12}-PO	22.88[a]	4161	4161	25.3
Chol-A_{12}-PS[e]	23.59[a]			14.8
Chol-PAI-PO	21.51, 22.04[a]	5436	5437	10.0
Chol-PAI-PS	21.83[a]	5677	5676	12.0
Born-PAI-PO	17.77, 18.68[b]	5203.5	5202	22.8
Born-PAI-PS	18.43[b]	5443	5443	27.1
Menth-PAI-PO	19.27, 19.72[b]	5205	5203	11.6
Menth-PAI-PS	19.29[b]	5445.5	5445	18.4
Hept-PAI-PO	26.07[a]	5306	5305	9.0
Hept-PAI-PS	26.36[a]			25.6

[a]2.0% CH_3CN/min.
[b]1.3% CH_3CN/min.
[c]Measured by Electrospray Ionization Mass Spectrometry.
[d]For 1 μmole synthesis.
[e]^{31}P NMR (CD_3CN): δ -0.1 ppm (phosphate) and 55.6 ppm (phosphorothioate), integral ratio 11:1.

with S-TETRA sulfurization for phosphorothioate oligonucleotides.[16] After the condensation with oxathiaphospholane derivative of an appropriate alcohol (**1-4**) the corresponding oligonucleotide conjugate was cleaved from the solid support (LCA CPG SAR) with 25% aq. ammonia (2 hr at rt) and the base protecting groups were removed by further treatment with ammonia (15 hr at 55°C). The conjugates were isolated by preparative RP HPLC on the ODS-Hypersil column with a linear gradient of CH_3CN in 0.1 M ammonium acetate.

The yields and physicochemical characteristics of oligonucleotide conjugates of cholesterol (Chol), borneol (Born), menthol (Menth) and heptadecanol (Hept), obtained by oxathiaphospholane method are given in Table 3.

An analogous series of cojugates was also prepared with phosphodiester and phosphorothioate anti-PAI-1 oligonucleotides (sequences as above) containing at their 5'-end a linker comprising three tetraethylene glycol units connected with each other *via* phosphate or phosphorothioate linkages. The foregoing bioconjugates were used for the studies of antisense inhibition of PAI-1 release from cultured E.A.hy 926 endothelial cells.[15]

ACKNOWLEDGMENT

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