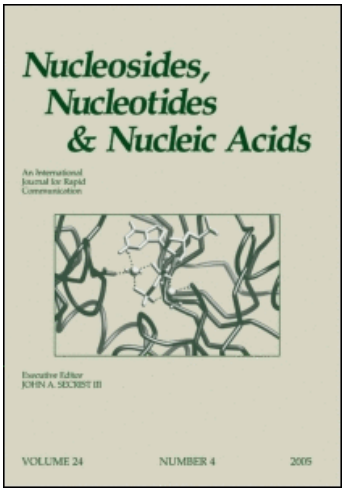

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Nucleoside 3'-*O*-(2-Oxo-"*Spiro*"-4.4-Pentamethylene-1.3.2-Oxathiaphospholane)S: Monomers For Stereocontrolled Synthesis Of Oligo(Nucleoside Phosphorothioate/Phosphate)S

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NUCLEOSIDE 3'-*O*-(2-OXO-"*spiro*"-4.4-PENTAMETHYLENE-1.3.2-OXATHIAPHOSPHOLANE)S: MONOMERS FOR STEREOCONTROLLED SYNTHESIS OF OLIGO(NUCLEOSIDE PHOSPHOROTHIOATE/PHOSPHATE)S

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ABSTRACT: Attempts at synthesis of "chimeric" oligonucleotide constructs (PO/PS-Oligos) possessing phosphate and P-stereodefined phosphorothioate internucleotide linkages *via* combined phosphoramidite/oxathiaphospholane methods were unsuccessful. Therefore, novel monomers for oxathiaphospholane method, namely 5'-*O*-DMT-deoxyribonucleoside 3'-*O*-(2-oxo-*spiro*-4.4-pentamethylene-1.3.2-oxathiaphospholane)s, were prepared and used together with their diastereomerically pure 2-thio analogues for the stereocontrolled synthesis of "chimeric" oligonucleotide constructs (PO/PS-Oligos).

An early concept of minimal alteration of "antisense" [1] or "antigene" [2] oligonucleotides to preserve their natural avidity towards complementary mRNA or double stranded DNA directed efforts of several research establishments towards chimeric constructs composed of phosphodiester core flanked by phosphorothioate segments either at the 3'- end only, or at both 3'- and 5'-ends of the oligonucleotide [3-8]. Phosphorothioate segments enhance the resistance of chimeric constructs towards exonucleases [9], albeit it became apparent only recently that 3'-exonucleases from human plasma, as well as from HUVEC and HeLa cells, are diastereoselective enzymes and preferentially cleave internucleotide phosphorothioate bonds of R_P configuration [10]. Since the routine methods for chemical synthesis of oligo(nucleoside phosphorothioate)s are non-stereospecific [11], the stability of constructs with 3'-terminal internucleotide phosphorothioates in the plasma is reduced due to the presence of those less stable linkages of Rp configuration [10]. Therefore,

it was tempting to test within the antisense strategy the mixed-backbone, or chimeric PO/PS-constructs, with phosphorothioate segments of [S_P]- configuration. The oxathiaphospholane method, elaborated in this laboratory [12,13] is so far the only one allowing for the synthesis of stereoregular either S_P or R_P oligo(nucleoside phosphorothioate) diesters under conditions of automated solid phase synthesis. This method relies upon separation of 5'-O-DMT-nucleoside-3'-O-(2-thiono-1.3.2-oxathiaphospholanes) into pure R_P and S_P diastereomers, followed by DBU assisted 1.3.2-oxathiaphospholane ring opening condensation with 5'-OH nucleosi(ti)des [13]. The condensation process occurs with full stereospecificity and net retention configuration at phosphorus[14]. Very recently modified oxathiaphospholane monomers, namely 5'-*O*-DMT-nucleoside-3'-*O*-(2-thiono-*spiro*-4.4-pentamethylene-1.3.2-oxathiaphospholane)s have been obtained, which are advantageous due to their efficient column chromatography separation into diastereomerically pure species [15].

As the first approach to the synthesis of the stereodefined PO/PS chimeric construct it was tempting to combine the oxathiaphospholane method with phosphoramidite one [16] to elongate the growing oligomer by phosphorothioate unit of desired configuration, or natural phosphate moiety, respectively. We have realized, however, that the oxathiaphospholane method produces phosphorothioate internucleotide diesters, and an extension of oligo(nucleoside phosphorothioate) chain *via* phosphoramidite method is not feasible because pre-formed phosphorothioates would undergo oxidation during treatment of intermediary internucleotide phosphites with the iodine/water/base mixture [17]. Effective protection of the sulfur atom of internucleotide phosphorothioate could not be achieved by standard alkylation (e.g. methylation or benzylation) because this process requires several hours, and if applied to oligomers containing other than thymidine components, it must cause deleterious alkylation of nucleobases. Besides of that the resulting *S*-alkyl substituent is a relatively good leaving group,[18] and final deprotection is accompanied by cleavage of the P-S bond leading to desulfurized product. Therefore, we have tested several other methods including milder than iodine/water oxidants (in order to avoid oxidative destruction of phosphorothioate diester bonds), acylation [19] or alkylation of internucleotide phosphorothioate functions with *S*-2-bromoethylthiobenzoate ($BrCH_2CH_2SCOPh$) before oxidation of intermediary P^{III} internucleotide phosphite.

The ability of *tert*-butyl hydroperoxide to oxidize P^{III} triesters to form corresponding phosphates was reported by Hayakawa *et al.* [20] and Letsinger *et al.* [21]. In order to verify its applicability for the synthesis of chimeric PS/PO oligomers, two samples of the phosphorothioate dinucleotide $d(T_{PS}T)$ anchored to the solid support were subjected to ten elongation cycles *via* phosphoramidite approach. Instead of standard oxidizing reagent we used 0.5 M or 1 M solutions of tert-butyl hydroperoxide (dried over magnesium sulfate) in methylene chloride over 120 or 50 seconds, respectively. HPLC analysis of the reacting mixture showed the presence of fully oxidized T_{12} in the amount of 17-20%, which was unacceptable for our purpose.

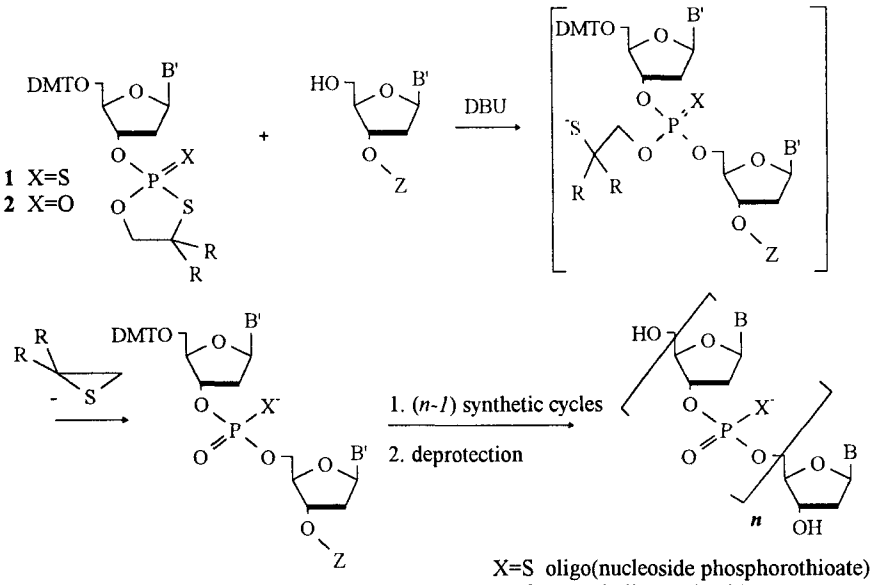
Similar unacceptable results were obtained using 10-camphorsulfonyloxaziridine [22]. Oxaziridines are well known class of reagent that can be used to oxidize phosphites [23] and, as demonstrated recently, are able to oxidize neutral phosphorothioates into corresponding phosphates [24]. In a model experiment two samples of dithymidyl-(2-cyanoethyl)-phosphite were treated with ten-fold molar excess of 10-camphorsulfonyloxaziridine (0.05 M solution in acetonitrile) for five or ten minutes, and then unreacted phosphite was sulfurized with bis(*O,O*-diisopropoxy phosphinothioyl)disulfide (S-Tetra) [25]. HPLC analysis revealed the ratio phosphate/phosphorothioate 91:9 and 95:5, respectively, so even longer time of oxidation is necessary for complete oxidation of phosphite. However, it was found that five minutes exposure of unprotected internucleotide phosphorothioate linkage to this reagent gives 23% of oxidized product.

An approach to protect phosphorothioate linkages by formation of mixed phosphorothioic-carboxylic anhydrides was even more discouraging. In a model experiment phosphorothioate diester (triethylammonium salt of 2-oxo-2-thiono-5.5-dimethyl-1.3.2-dioxaphosphorinane)[26] was quantitatively benzoylated with benzoyl chloride in pyridine in less than five minutes, as judged by ^{31}P NMR spectroscopy. The chemical shift of the product (δ 51.4 ppm) indicated formation of the anhydride of thiono structure, which should be stale against oxidation by standard iodine/water/base mixture. However, an attempt at elongation of $d(T_{PS}T)$ only by one residue *via* phosphoramidite method resulted in detrimental loss of sulfur (28%) from pre-benzoylated phosphorothioate linkage. Most likely, fast hydrolysis of the anhydride exposed unprotected diester towards the oxidizing reagent. When the mixed anhydride

was treated with *tert*-butyl hydroperoxide, the oxidized product was formed in 11% yield, also rendering this approach nonproductive.

Another method for protection was based on alkylation of sulfur atom with *S*-2-bromoethylthiobenzoate. This reagent was originally proposed by Caruthers [27] to convert dialkyl phosphorodithioate into *S*-alkylated triester. Such the protection is reportedly stable during all synthetic steps of the phosphoramidite method leading to the elongation of oligonucleotide. Superiority of this method over methylation or benzylation comes from mild and efficient deprotection of the triester *via* ammonolysis of benzothioate ester followed by elimination of ethylene sulfide. To find the conditions suitable for *S*-alkylation of assembled PS-Oligos a model dinucleotide phosphorothioate diester d(T_{PS}T) attached to the solid support was treated with 30% (v/v) solution of the reagent in pyridine for the time 2-10 h at 45°C, then washed with acetonitrile and treated with standard oxidizing mixture iodine/water/pyridine for ten minutes, which is a period of time equivalent to fifteen standard oxidation steps. After ammoniolytic cleavage from the solid support with simultaneous deprotection of the sulfur atom the resulting mixture was analyzed by HPLC to assess the level of oxidized dinucleotide d(TT). It was found that 10 hour alkylation at 45°C is necessary to reduce the level of the phosphate formed down to 15%, while 2 h alkylation is insufficient and results in 60% of unwanted oxidation. It must be emphasized, that oxidation of non protected T_{PS}T over 5 minutes gives exclusively oxidized dinucleotide. Unfortunately, long alkylation time restricts applicability of this approach, while more reactive alkylating reagent may lead to modification of nucleobases [28].

Since none of presented above attempts was successful with respect to the synthesis of chimeric PS/PO constructs, another approach inferred from the mechanism of the oxathiaphospholane ring opening condensation [12] (see SCHEME 1) was examined. It has been assumed that replacement of the *exocyclic* sulfur (depicted as *X* on SCHEME 1) in oxathiaphospholane monomers **1** (R,R= $-(CH_2)_5-$, X=S) by oxygen gives rise to **2** (R,R= $-(CH_2)_5-$, X=O), which in the condensation reaction should provide an elongated oligomer possessing the natural internucleotide phosphodiester bond, avoiding any oxidation step. In this way phosphorothioate functions present in the already assembled oligomer should be preserved.



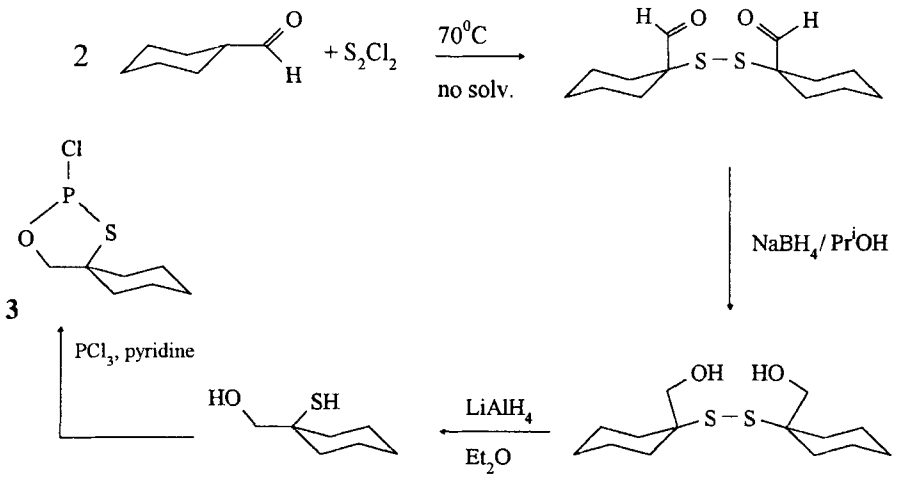
B' = Thy, Ade^{Bz}, Cyt^{Bz}, $Gua^{iBu,(DPC)}$

R,R= - $(CH_2)_5$ -

Z = $C(O)CH_2CH_2C(O)N(CH_3)CH_2C(O)$-LCA-CPG

SCHEME 1

Phosphitylating reagent, 2-chloro-"spiro"-4.4-pentamethylene-1.3.2-oxathiaphospholane (**3**) was obtained as shown on SCHEME 2 and used for phosphitylation of appropriately protected nucleosides at the 3'-*O*- site. 5'-*O*-DMT-Nucleoside-3'-*O*-("*spiro*"-4.4-pentamethylene-1.3.2-oxathiaphospholane)s (the intermediates in the synthesis of monomers **1**) were *in situ* chemoselectively oxidized with selenium dioxide [15] to yield desired 5'-*O*-DMT-nucleoside-3'-*O*-(2-oxo-"*spiro*"-4.4-pentamethylene-1.3.2-oxathiaphospholane)s (**2**). Compounds **2a-d** (B=Thy, Ade^{Bz}, Cyt^{Bz} or Gua^{iBu}) were isolated on a silica gel column with chloroform as an eluent in 41-55% yield, and their characteristic is given in TABLE 1. Applicability of the 2-oxo-monomers **2** to the synthesis of natural oligonucleotides was confirmed by the automated synthesis of "homooctamers" $(N_{PO})_7T$ (N=dA, dG, dC or T) using a protocol similar to that elaborated for synthesis of PS-Oligos.



SCHEME 2

TABLE 1: Characteristics of the oxathiaphospholane monomers **2**.

B'	Yield[a] (%)	δ ^{31}P NMR (ppm, CD_3CN)	TLC[b]
2a Thy	55	44.7, 44.3	0.48
2b Ade^{Bz}	41	45.1, 44.9	0.61
2c Cyt^{Bz}	45	44.6, 44.1	0.57
2d Gua^{iBu}	54	45.3, 44.5	0.54

a) Yield of isolated products.
b) TLC analysis was performed on HP TLC plates (Merck). Developing system: chloroform/methanol 9:1 v/v.

In all syntheses, performed for 1μmol scale, the same solid support LCA CPG-Sar-Thy (23μmol/g) was employed. For each coupling step 20 mg of **2** was used together with 1.0 M DBU in acetonitrile as an activator. The coupling time was 450 s. The resulting oligomers were cleaved from the solid support and RP HPLC analysis confirmed their identity with genuine samples synthesized *via* phosphoramidite method. These encouraging results prompted us to synthesize on this way a mixed sequence PO-d(TAGTGATTCT) (0.75 μmol scale) which was obtained with 56% yield (by RP-HPLC, DMT-ON analysis, FIG.1).

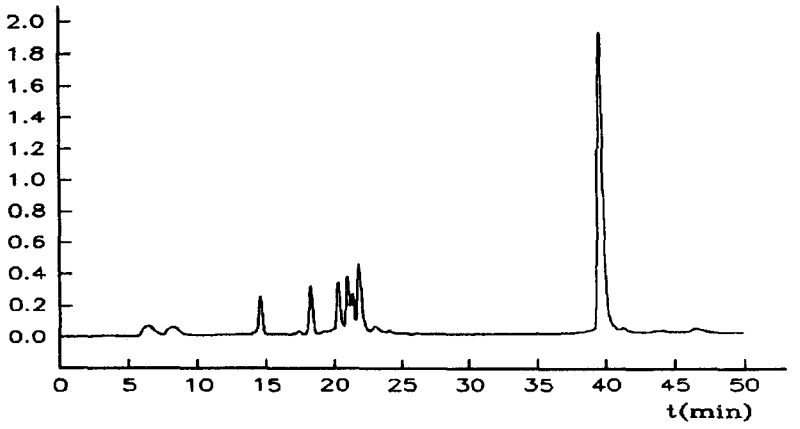


FIG. 1. RP-HPLC trace of crude PO-d[TAGTGATTCT] (DMT-ON) synthesized using monomers **2**.

The repetitive yield of the synthesis (89.4%, based on the DMT cation assay) was relatively low as compared with phosphoramidite or H-phosphonate methods [11], but was considered satisfactory to attempt synthesis of chimeric PO/PS-Oligos using both types of monomers **1** and **2**. Three oligonucleotides, namely [MIX]-$T_{PS}T_{PS}T_{PS}T_{PS}T_{PO}T_{PO}T_{PO}T_{PS}T_{PS}T_{PS}T$ (**4**), [All-R_P]-$T_{PO}T_{PS}T_{PO}T_{PS}T_{PO}T_{PS}T_{PO}T_{PS}T_{PO}T$ (**5**) and [All-S_P]-$T_{PO}T_{PS}T_{PO}T_{PS}T_{PO}T_{PS}T_{PO}T_{PS}T_{PO}T$ (**6**)[29] were synthesized on 1 μmol scale. In these sequences the descriptions [MIX]-, [All-R_P]- and [All-S_P]- refer to the configuration at the phosphorus atoms of phosphorothioate linkages. Repetitive yield, calculated from a DMT cation assay, ranged from 93 to 97%. Compounds **4-6** were isolated by two step HPLC (FIG.2) and analyzed by PAGE.

Compounds **5** and **6** (40 and 27 OD units, respectively) were analyzed by ^{31}P NMR. In each spectrum, as expected, two sets of resonances corresponding to phosphate and phosphorothioate linkages were found. ESI MS spectra of these samples contained molecular ions m/z 3044 confirming their structure. The location and absolute configuration of phosphorothioate centers within oligonucleotide chains of **5** and **6** were confirmed by enzymatic analysis with R_P-specific snake venom phosphodiesterase (svPDE) [30] and S_P-specific Nuclease P1 [31].

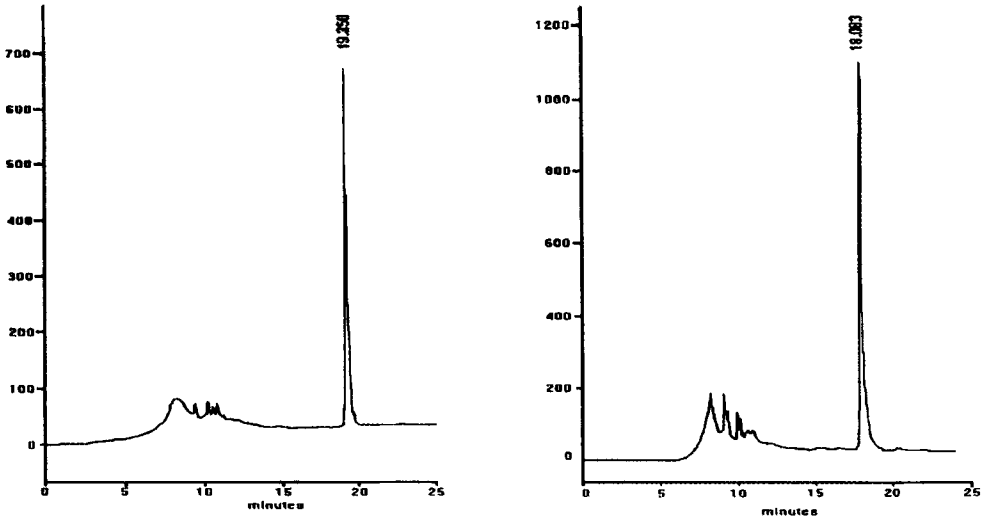


FIG.2. RP-HPLC trace of purified Rp -**5** and Sp -**6** after detritylation

CONCLUSIONS:

None of several methods tested provided sufficient protection for diester internucleotide phosphorothioate linkage to allow for combining of the oxathiaphospholane and phosphoramidite methods. In order to overcome this problem new monomers, 5'-*O*-DMT-deoxyribonucleoside 3'-*O*-(2-oxo-*spiro*-4.4-pentamethylene-1.3.2-oxathiaphospholane)s, were prepared by chemoselective oxidation of corresponding 5'-*O*-DMT-deoxyribonucleoside 3'-*O*-(*spiro*-4.4-pentamethylene-1.3.2-oxathiaphospholane)s with selenium dioxide. They were found to be suitable for the synthesis of stereodefined chimeric PO/PS-Oligos in combination with 5'-*O*-DMT-deoxyribonucleoside 3'-*O*-(2-thio-*spiro*-4.4-pentamethylene-1.3.2-oxathiaphospholane)s. The repetitive yield for those syntheses was *ca.* 90%, which is considerably lower than in standard phosphoramidite method, but this approach offers possibility to synthesize stereodefined chimeras of medium size with phosphorothioate linkages at any selected position within oligonucleotide chain.

EXPERIMENTAL SECTION

^{31}P nuclear magnetic resonance spectra were recorded on a Bruker AC-200 instrument (85% H_3PO_4 as the external standard). Electrospray mass spectrometry analyses were done at Mass Consortium, Corp., San Diego, CA. Ultraviolet (UV) spectra were recorded on a GBC 916 spectrophotometer. HPLC analysis was done on an LDC Analytical gradient system consisting of CM3500 and CM3200 pump, equipped with a UV detector SM5000, using ODS Hypersil column (4.6x250mm) and the gradient 0.1 M TEAB-40% CH_3CN/0.1 M TEAB, 1%/min.

Oxidation of phosphites by tert-*butyl hydroperoxide in the presence of phosphorothioate diesters.* A model dithymidyl phosphorothioate ($T_{PS}T$) was synthesized on the solid support, manually, using oxathiaphospholane method [15]. Then the column was connected to an automatic DNA synthesizer ABI 380B to perform ten standard phosphoramidite synthetic cycles, with the exception that 0.5 M or 1 M solution of *tert*-butyl hydroperoxide in methylene chloride was used for 120 or 50 seconds, respectively, instead of iodine/water/base mixture. The products were cleaved from the solid support, detritylated and analyzed by HPLC. This analysis showed the presence of 17.5 and 19.9% of oxidized oligonucleotide T_{12}. The retention times of desired chimeric $(T_{PO})_{10}T_{PS}T$ and unmodified T_{12} (prepared as the standard) were 15.8 and 15.3 min., respectively.

Oxidation of dithymidyl-2-cyanoethyl phosphite with 10-camphorsulfonyloxaziridine. Dithymidyl-2-cyanoethyl phosphite was synthesized on the solid support using standard phosphoramidite condensation catalyzed by 1-*H*-tetrazole, followed by oxidation with 0.05 M solution of 10-camphorsulfonyloxaziridine in acetonitrile for five or ten minutes. Then the column was washed twice with acetonitrile, dried and treated in standard way with the solution of bis(*O,O*-diisopropoxy phosphinothioyl)disulfide to convert non-oxidized phosphite into more stable phosphorothioate. The product was cleaved from the solid support, detritylated and analyzed by HPLC. This analysis showed the presence of, respectively, 9 and 5% of $T_{PS}T$ - phosphorothioate derivative of non-oxidized substrate.

Oxidation of mixed phosphorothioic-carboxylic anhydride with iodine/pyridine/water mixture. Prepared by oxathiaphospholane method the model dinucleoside phosphorothioate [S_P]-5'-*O*-DMT-$T_{PS}T$ anchored to the solid support was treated with

the acylating mixture (benzoyl chloride-pyridine) for 5 minutes, followed by standard steps of oxidation, capping, coupling and detritylation. The standard sequence of steps i.e. detritylation, coupling, oxidation, capping was altered intentionally to have the substrate not elongated for analytical purpose. The products were released from the solid support and analyzed by HPLC. The HPLC trace showed the presence of 28% of $T_{PO}T$. The R_P-isomer of $T_{PS}T$ was not detected, which means that hydrolysis of mixed anhydride did not cause epimerization of the phosphorothioate centre.

S-2-Bromoethylthiobenzoate. Sodium thiobenzoate was prepared from 0.15 mol of thiobenzoic acid and 3.3 g of sodium) in methanol (70 mL). The solvent was evaporated and the residue was dissolved in benzene (200 mL). Into this solution ethylene bromide (28g, 13 mL, 0.15 mol) was added and the mixture was refluxed for 6 hours. Precipitated sodium bromide was filtered off, the solvent was evaporated and the residue was distilled under reduced pressure (113-118°C/0.3-0.4 mmHg) to yield 9.8g (26%) of pale yellow liquid. FAB MS (positive ions) m/z 245, 247 (M^+, 60%), m/z 105.1 ($PhCO^+$, 100%)

Oxidation of $T_{PS}T$ alkylated with S-2-bromoethylthiobenzoate. The columns containing 5'-*O*-DMT-$T_{PS}T$ (prepared by oxathiaphospholane method) anchored to the solid support were filed up with the alkylating mixture (300 μL of 30% solution of *S*-2-bromoethylthiobenzoate in pyridine) and then thermostated for 2-10 hours at 45°C. The alkylation was followed by washing with acetonitrile and oxidation over ten minutes with the standard iodine/water/base mixture. After detritylation and cleavage from the solid support (accompanied by deprotection of the phosphorothioate linkage) the resulting mixture was analyzed by HPLC and the amount of oxidized $T_{PO}T$ contaminating unchanged $T_{PS}T$ was calculated. The samples alkylated for 2, 4, 6 and 10 hours contained 58, 50, 42 and 15% of $T_{PO}T$, respectively.

2.2'-Dithiobis(cyclohexanecarboxaldehyde) was synthesized from cyclohexanecarboxaldehyde (70% yield) as described by K.Hayashi. [32] The product was crystallized from diethyl ether (m.p. 88-89°C).

1.1'-Dithiobis(1.1-pentamethylene-ethane-2-ol) [33] was obtained by reduction of 2.2'-dithiobis(cyclohexanecarboxaldehyde) (23.5 g, 0.082 mol) with sodium borohydride (62 g, 0.164 mol) in isopropyl alcohol. The crystalline product was precipitated from diethyl ether/hexane to give 23 g of white crystalline material (97.% yield, mp. 49-50°C).

1-Mercapto-1.1-pentamethylene-ethane-2-ol was synthesized by reduction of 1.1'-dithiobis(1.1-pentamethylene-ethane-2-ol) (4.6 g, 0.16 mol) with lithium aluminum hydride (5.9 g, 0.16 mol) in anhydrous diethyl ether, as described elsewhere [15]. The product was obtained (70% yield) as colorless oil (bp. 74-76°C/0.05 mmHg, n^{D}_{20} 1.5188).

2-chloro-"spiro"-4.4-pentamethylene-1.3.2-oxathiaphospholane (3) was obtained from phosphorus trichloride (0.21 mol, 28.2 g) and 1-mercapto-1.1-pentamethylene-ethane-2-ol (0.14 mol, 20 g) in the presence of pyridine (0.27 mol, 22 mL) in benzene. Distillation under reduced pressure gave colorless liquid (76% yield): bp. 82-84°C/0.01 mmHg; δ ^{31}P NMR 217.7 ppm (C_6D_6); EI-MS. (70eV) m/z 210, M^+, 12 %; m/z 175, $[M\text{-}Cl]^+$, 5.8 %; m/z 90, 100%.

5'-O-DMT-deoxyribonucleoside-3'-O-(2-oxo-"spiro"-4.4-pentamethylene-1.3.2-oxathiaphospholane)s **2**. To the magnetically stirred solution of 10 mmol of appropriately protected deoxyribonucleoside (A^{Bz}, G^{iBu}, T, or C^{Bz}) and 0.6 mL (11 mmol) of diisopropylethylamine in 10 mL of dry acetonitrile 2.31g (1.62 mL, 11 mmol) of 2-chloro-"*spiro*"-4.4-pentamethylene-1.3.2-oxathiaphospholane was added dropwise at room temperature. The reaction was complete in 5 minutes and resulting phosphite was *in situ* oxidized by addition of 0.61 g of selenium dioxide (0.55 mmol) at room temperature. After 3 h the mixture was filtered and the solvent was evaporated. The residue was applied to a silica gel column (20x3 cm, 30 g). The column was eluted with chloroform and appropriate fractions were combined and evaporated under reduced pressure to give desired compounds (5'-*O*-DMT-deoxyribonucleoside-3'-*O*-(2-oxo-"*spiro*"-4.4-pentamethylene-1.3.2-oxathiaphospholane)s) in 41-55% yield. The chemical shifts in ^{31}P NMR and TLC parameters for compounds **2a-d** are given in TABLE 1.

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