Deoxyribonucleoside 3′-*O*-(2-Thio- and 2-Oxo-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane)s: Monomers for Stereocontrolled Synthesis of Oligo(deoxyribonucleoside phosphorothioate)s and Chimeric PS/PO Oligonucleotides[§]

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Abstract: New monomers, 5′-*O*-DMT-deoxyribonucleoside 3′-*O*-(2-thio-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane)s, were prepared and used for the stereocontrolled synthesis of PS−Oligos via the oxathiaphospholane approach. These monomers and their 2-oxo analogues were used for the synthesis of "chimeric" constructs (PS/PO−Oligos) possessing phosphate and P-stereodefined phosphorothioate internucleotide linkages. The yield of a single coupling step is approximately 92−95%, and resulting oligomers are free of nucleobase- and sugar-phosphorothioate backbone modifications. Thermal dissociation studies showed that for heteroduplexes formed by [R_P]-, [S_P]-, or [mix]-PS/PO-T_{10} with dA_{12}, dA_{30}, or poly(dA), for each template, the melting temperatures, as well as free Gibbs' energies of dissociation process, are virtually equal. Stereochemical evidence derived from crystallographic analysis of one of the oxathiaphospholane monomers strongly supports the participation of pentacoordinate intermediates in the mechanism of the oxathiaphospholane ring-opening condensation.

Introduction

Among several analogues of oligonucleotides, which in the main-stream of the "antisense mRNA" strategy[1,2] are tested as potential therapeutics and inhibitors of biosynthesis of unwanted proteins, oligo(deoxyribonucleoside phosphorothioate)s, typically bearing 15−30 nucleobases (PS−Oligos), are widely recognized as the most promising class of DNA congeners. Using several model systems, PS−Oligos were thoroughly tested in vitro[3,4] and convincing results were reported showing their efficacy in vivo.[5] In addition, the clinical trials combating CMV-related retinitis, AIDS, and restenosis in humans have commenced.[6,7] The proposed mechanism of their action involves specific recognition of selected mRNA (or pre-mRNA) by administered PS−Oligos followed by regioselective cleavage of mRNA (involved in a heterodimer mRNA/PS−Oligo) by Ribonuclease H.[8] However, the mechanism of biological activity of PS−Oligos seems to be more complicated and is still a matter of controversy.[9,10]

Considering the biological activities of PS−Oligos, attention should be paid to their polydiastereoisomerism.[11] Replacement of one of two nonbridging oxygens by sulfur at phosphorus in an internucleotide linkage generates asymmetry at the phosphorus atom. Common synthetic methods used to prepare PS−Oligos are nonstereospecific[12] and give a mixture of 2^n diastereomers (where *n* is the number of internucleotide phosphorothioate functions). However, biological properties of PS−Oligo diastereomers, including transport through cell membranes and/or interaction with intracellular biopolymers (e.g., proteins or nucleic acids), may depend on the chirality[13] of PS−Oligos. To verify this assumption, methods for synthesis of PS−Oligos

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§ Abbreviations: Bz, benzoyl; DBU, 1,4-diazabicyclo[5.4.0]undec-7-ene; DCA, dichloroacetic acid; DMAP, 4-(dimethylamino)pyridine; DMT, dimethoxytrityl; ^{i}Bu, isobutyryl; LCA CPG, long-chain alkylamino controlled pore glass; Tris-Cl, tris(hydroxymethyl)aminomethane hydrochloride; DPC, diphenylcarbamoyl; EI, electron impact; ESI MS, electrospray ionization mass spectrometry; FAB, fast atom bombardment; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; RP HPLC, reverse-phase high-performance liquid chromatography; TLC, thin-layer chromatography; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry.

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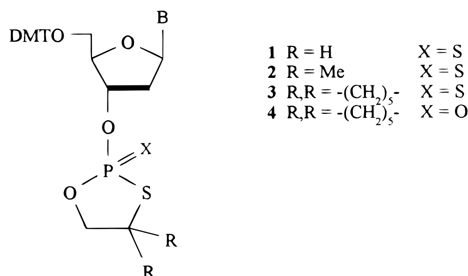
of a predetermined sense of chirality at the phosphorus atoms were sought.[14] In earlier reports[15−17] we presented the oxathiaphospholane method which, for the first time, allowed for stereocontrolled chemical synthesis of PS−Oligos of either S_P or R_P configuration at each preselected phosphorothioate center. The oxathiaphospholane method surpasses the enzymatic one which allows for the stereoselective synthesis of only [all-R_P] PS−Oligos.[18−20] However, broader applicability of the oxathiaphospholane methodology has been hampered by tedious separation of 5′-*O*-DMT-deoxyribonucleoside 3′-*O*-(2-thio-1,3,2-oxathiaphospholane)s (**1**, R = H, X = S), or their 4,4-dimethyl substituted analogues (**2**, R = Me, X = S) into pure diastereomers.



In this report we present new nucleotide monomers, modified within the oxathiaphospholane ring, namely, 5′-*O*-DMT-deoxyribonucleoside-3′-*O*-(2-thio-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane)s (**3**, R,R = $-(CH_2)_5-$, X = S), which like **1** and **2** are prepared in the form of a diastereomeric mixture, but undergo much easier separation into individual diastereomers. We also present here as yet not described nucleotide monomers, 5′-*O*-DMT-deoxyribonucleoside-3′-*O*-(2-oxo-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane)s (**4**, R,R = $-(CH_2)_5-$, X = O), which allow for the synthesis of unmodified oligo(deoxyribonucleoside phosphate)s. The use of both types of oxathiaphospholane monomers **3** and **4** allows for the preparation of "chimeric" oligonucleotides (PS/PO−Oligos) possessing both natural phosphate and stereodefined phosphorothioate internucleotide linkages at the preselected positions in the same molecule. In such constructs, the natural phosphate part is expected to preserve the hybridization properties of natural oligonucleotides, while P-stereodefined phosphorothioate segments (located at the 3′- and/or 5′-ends of the oligomer) would confer significantly higher stability toward nucleases.[21−25]

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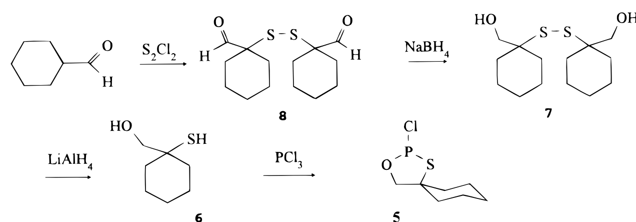
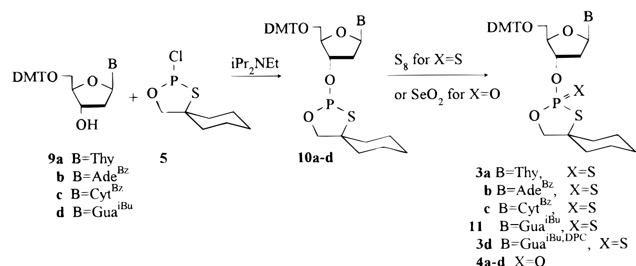
Scheme 1



Scheme 2



Results and Discussion

Oxathiaphospholane Ring-Substituted 4,4-"spiro"-Pentamethylene Monomers. The oxathiaphospholane method of stereocontrolled synthesis of PS−Oligos, depicted in Scheme 3 (Z = 3′-*O*-deoxyribonucleoside, R′O = 5′-*O*-deoxyribonucleoside), was shown to be useful for generation of internucleotide phosphorothioate linkages under conditions compatible with the requirements of solid-phase synthesis. Separation of earlier described monomers **1** and **2** requires tedious silica gel column chromatography[16] or costly preparative HPLC to yield diastereomerically pure substrates. To obtain monomers that afford easier separation, efforts were undertaken to "enhance the asymmetry" of the phosphorus atom involved in the oxathiaphospholane part of the molecule. It was found that "spiro" monomers 5′-*O*-DMT-deoxyribonucleoside 3′-*O*-(2-thio-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane)s (**3**), obtained by introducing a pentamethylene substituent in position 4 of the oxathiaphospholane ring, possess a satisfactory separability of diastereomers. Such modification does not create any new asymmetry center within the substrate molecule. The desired phosphitylating reagent **5** was obtained by the reaction of phosphorus trichloride with mercapto alcohol **6** (Scheme 1). The synthesis of **6** was based on published methods.[26] Our improved synthesis of the intermediate diol-disulfide **7** resulted in 98% yield of the desired product when solid dialdehyde **8**, rather than its solution in a mixture of isopropyl alcohol and benzene, was added to the 2-propanol solution of sodium borohydride in the reduction step. Appropriate 5′-*O*-DMT-base-protected deoxyribonucleosides **9a−d** (B = Thy, Ade^{Bz}, Cyt^{Bz}, Gua^{iBu}) were phosphitylated at room temperature with **5** in acetonitrile in the presence of diisopropylethylamine to yield phosphites **10a−d**, which were further sulfurized with elemental sulfur (Scheme 2). Silica gel column chromatography with chloroform as an eluent afforded 5′-*O*-DMT-deoxyribonucleoside-3′-*O*-(2-thio-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane)s (**3a−c**, **11**) as diastereomeric mixtures in satisfactory yield (78−86%). The guanosine derivative **11** was additionally protected at the O-6 site with diphenylcarbamoyl chloride to yield **3d**. The diastereomers of **3a−d** (B = Thy, Ade^{Bz}, Cyt^{Bz},

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Table 1. Characteristics of the Oxathiaphospholane Monomers **3** and **4**[e]

B′	yield (%)	composition (fast:slow)	δ ^{31}P NMR (ppm, CD_3CN)	TLC
3a Thy	84	50:50	105.3 (fast)	0.61[a](fast)
			105.6 (slow)	0.54[a](slow)
3b Ade^{Bz}	84	49:51	104.7 (fast)	0.54[b](fast)
			105.1 (slow)	0.46[b](slow)
3c Cyt^{Bz}	86	48:52	105.3 (fast)	0.60[b](fast)
			105.6 (slow)	0.40[b](slow)
3d	78	52:48	106.2 (fast)	0.37[a,d] (fast)
Gua^{iBu,DPC}		106.9 (slow)	0.26[a,d] (slow)	
4a Thy	55	nd	44.7, 44.3	0.48[c]
4b Ade^{Bz}	41	nd	45.1, 44.9	0.61[c]
4c Cyt^{Bz}	45	nd	44.6, 44.1	0.57[c]
4d Gua^{iBu}	54	nd	45.3, 44.5	0.54[c]

[a] Developing system: butyl acetate/benzene 1:1 v/v. [b] Developing system ethyl acetate/butyl acetate 1:2 v/v. [c] Developing system: chloroform/methanol 9:1 v/v. [d] R_f values reported for the monomers before protection at *O*-6 site with diphenylcarbamoyl chloride. After protection the R_f's are 0.74 and 0.63 (butyl acetate/benzene 1:1 v/v). [e] TLC analysis was performed on HP TLC plates (Merck).

Gua^{iBu,DPC}) were separated by column chromatography into "fast"- and "slow"-eluting species. Notably, for these monomers, single passage through a silica gel column gave ca. 70% recovery of the applied material in diastereomerically pure forms. Isolated monomers were twice coevaporated with dry toluene (to remove residual water and pyridine) and stored in tightly closed vessels. Separated diastereomers were analyzed by ^{31}P NMR, ^{1}H NMR, MS, and elemental analysis. The diastereomeric purity of separated isomers was determined by integration of the corresponding signals in the ^{31}P NMR spectra. Selected data are presented in Table 1. The stereochemistry of the coupling step utilizing "spiro" oxathiaphospholanes **3a**−**d** has been checked for each of 32 combinations of diastereomeric dinucleotides $N_{PS}N$ (N = dG, dA, dC, or T). It has been found that all four "fast"-eluting diastereomers of **3**, analogous to the monomers **2**,[16] were precursors of the dinucleoside 3′,5′-phosphorothioates of R_P configuration. Consequently, "slow"-eluting isomers of **3** yielded phosphorothioate linkages of S_P configuration. In all cases, the stereoselectivity of condensation was not lower than 98%. Separated diastereomers of **3** are stable at room temperature for at least 6 months when stored in a desiccator.

To elaborate an optimized protocol for automated synthesis of PS−Oligos using monomers **3**, several sets of experiments were performed. In each set, one parameter (such as the coupling time, the monomer concentration, or the DBU concentration) was changed over a considerably wide range. For all syntheses the same support (which possessed the N^2-isobutyryldeoxyguanosine 3′-*O*- attached to LCA CPG via DBU-resistant sarcosinyl-succinoyl linker[27] with loading 29 μmol/g) was used to eliminate any possible effect of support variability. The optimization of monomer concentration was investigated by the syntheses of octamers d[$(A_{PS})_7$G], d[$(G_{PS})_7$G], d[$(C_{PS})_7$G], and d[$(T_{PS})_7$G]. During each synthesis, dimethoxytrityl cation absorbance was monitored, and the crude product was characterized by means of RP HPLC and PAGE to assess the yield and purity. For a 1 μmol scale of synthesis, the monomer amounts tested were 5, 10, 20, and 27 mg per single coupling. Since the total volume of the reacting mixture was 800 μL (530 μL of a DBU solution and 270 μL of a monomer solution), the final concentration of the monomers **3a**−**d** ranged from 6 mM (a dose of 5 mg of **3d**) to 45 mM (27 mg of **3a**).

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Table 2. Synthetic Protocol for the 1 μmol Scale Automated Solid-Phase Synthesis of PS−Oligos Using Monomers **3**, or PO−Oligos Using **4**

step	reagent or solvent	volume (mL)	purpose	time (s)
1	(a) dichloroacetic acid in CH_2Cl_2	2.3	detritylation	50
	(b) acetonitrile	7.0		150
2	(a) activated **3**[a] or **4**[b] in CH_3CN	0.8	coupling	470
	(b) methylene chloride	3.33	wash	200
	(c) acetonitrile	7.0	wash	150
3	(a) DMAP/Ac_2O/lutidine in THF	0.33	capping	20
	(b) acetonitrile	7.0	wash	150

[a] A mixture of 270 μL of a solution of a monomer **3** in CH_3CN (75 mg/mL) and 530 μL of 1.5 M DBU in CH_3CN. [b] A mixture of 270 μL of a solution of a monomer **4** in CH_3CN (75 mg/mL) and 530 μL of 1.0 M DBU in CH_3CN.

The value 20 mg (24−32 mM, 19−26-fold molar excess over nucleoside attached to the support) was found optimal for each of four monomers. Use of 27 mg did not result in higher yield or improved purity of the oligomers. Neither yield nor purity of products was acceptable when 5 or 10 mg of **3** was used in each coupling step. In the search for the optimal concentration of DBU activator, the model phosphorothioate oligomer, PS-d(ACAGTGATCGCTG) (**12**), was synthesized using stock solutions of DBU in acetonitrile at concentrations 0.2, 0.5, 0.75, 1.0 and 1.5 M. Thus, after mixing with a solution of a monomer, the final concentrations ranged from 0.13 to 1.0 M. It was found that the stock solution at concentration 1 M gives significantly better results than 0.5 M solution (the latter was typically used for monomers **1** or **2**), while a 1.5 M concentration did not improve the quality or yield of product. A set of syntheses of **12** using different coupling times (ranging from 150 to 600 s) revealed that the coupling time 450 s gives the best result. There was no improvement when longer reaction time (600 s) or double delivery of the monomer (the second delivery after washing and drying of the support) was applied. The oligomer **12** obtained under all optimized conditions (the protocol is presented in Table 2), after cleavage from the solid support and deprotection, without any purification, was subjected to ^{31}P NMR analysis which showed the absence of detectable amounts of phosphate linkages. Further treatment of the sample with formic acid followed by HPLC analysis of the hydrolysate proved the absence of detectable amounts of modified nucleobases.

Starting from pure diastereomers of **3**, several "stereoregular" [all R_P]- and [all-S_P]-oligonucleotides (10−28 mers) as PS-d[CCTATAATCC], PS-d[AACGTTGAGGGGCAT], PS-d[C-CACACCGACGGCGCCC], PS-d[TTCTTTCCATATTGAATA-TA], and PS-d[CGCTCTCGCACCCATCTCTCTCCTTCTA] have been synthesized for physicochemical studies and biological evaluation. Typically, repetitive yield (based on DMT^+ cation assay) was 94−96% for the [R_P] oligomers, and it was slightly lower (92−94%) for their [S_P] counterparts. The oligonucleotides were purified via two-step RP-HPLC chromatography (DMT-on and DMT-off), and their chain length integrity was assessed by PAGE (Figure 1). The amounts of material isolated after one synthesis at 1 μmol scale ranged from 4 to 10 OD units, which was less than expected from DMT^+ cation assay. It is necessary to mention that in order to achieve satisfactory purity of the products only middle parts of eluted peaks were collected. Six consecutive syntheses at 2 μmol scale yielded total 120 OD units of [all-R_P]-PS-d[CCTATAATCC], and 140 OD units of [all-S_P]-form, allowing for ^{1}H NMR conformational studies (data not shown). The development of "spiro" oxathiaphospholanes **3** can be considered as a significant

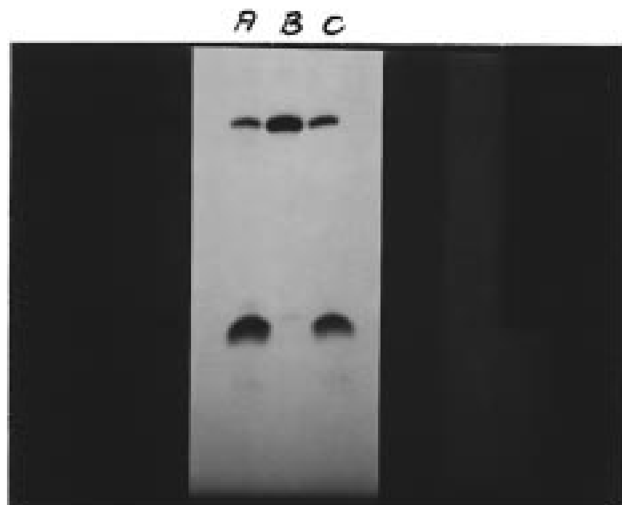
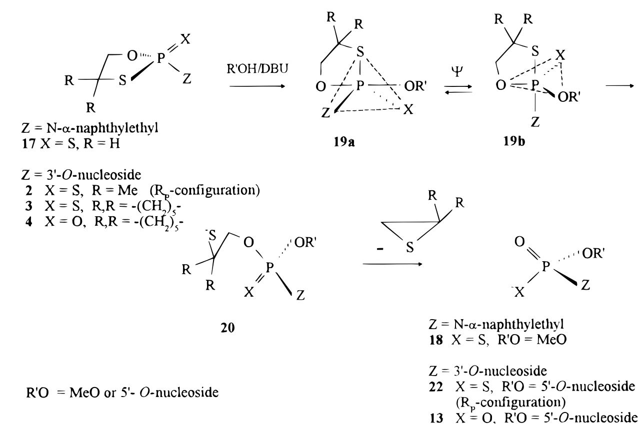


Figure 1. An autoradiogram from PAGE analysis (20% polyacrylamide gel/7 M urea) of phosphorothioate oligonucleotides purified by two-step RP HPLC: lane A, [mix]-PS-d(CCTATAATCC), oligomer synthesized via phosphoramidite/sulfurization approach; lanes B and C, two samples of [all-R_P]-PS-d(CCTATAATCC) synthesized via oxathiaphospholane method (two consecutive batches).

improvement of the oxathiaphospholane method since the diastereomeric purity of substrates used for stereospecific synthesis, especially that involving consecutively repeated cycles, is a crucial factor for the diastereomeric purity of the final product.

Synthesis of "Chimeric" PS/PO Oligonucleotides. It is commonly accepted opinion that PS−Oligos form less stable duplexes with DNA or RNA templates than natural oligonucleotides,[22] although some exceptions have been found.[16] However, since their in vivo stability is superior to that of natural oligonucleotides, several efforts were undertaken aimed at the synthesis of "chimeric" antisense constructs possessing unmodified phosphate and modified phosphorothioate internucleotide linkages in the same molecule (PS/PO−Oligos).[22−25,28,29] Two types of such constructs have been reported. The first one possesses phosphorothioate linkages in alternate positions,[30,31] while the second one contains combined oligophosphate and oligophosphorothioate clusters.[24,28] For the latter type it was assumed that the flanking phosphorothioate parts would provide better in vivo stability against nucleases, while the central unmodified part should enhance the hybridization properties of "chimeric" product (compared to PS−Oligo) and also facilitate the action of RNase H toward the duplexes formed with target RNA. The chimeras prepared via the phosphoramidite method contain phosphorothioate centers of random configuration, while those possessing stereodefined phosphorothioate linkages (of either R_P or S_P configuration) were synthesized using appropriately protected diastereomerically pure dinucleotide building blocks[31−35] In light of our earlier findings that PS−Oligos possessing at the 3′-end phosphorothioate linkages of [S_P] configuration are especially resistant to degradation by 3′-exonucleases,[36] it was tempting to apply the oxathiaphospholane methodology to the synthesis of chimeric PS/PO oligonucleotides of predetermined configuration at P-chiral centers. Unfortunately, the oxathiaphospholane method is not compatible with either the phosphoramidite[37] or the H-phosphonate[38] methods of DNA synthesis because the assembled internucleotide phosphorothioate linkages are easily oxidized during the iodine−base−water oxidation[33] that is unavoidable in both of the aforementioned methods. In our laboratory, several methods for protection of the sulfur atom in PS−Oligos were studied without satisfactory results (data not shown). Therefore, a different approach was examined based on modification of the oxathiaphospholane method. The oxathiaphospholane monomers **1**−**3** possess sulfur atoms in both exo- and endocyclic positions, but the sulfur atom present in the resulting phosphorothioate internucleotide bond originates from exocyclic sulfur depicted as X (Scheme 3, Z = 3′-*O*-deoxyribonucleoside, R′O = 5′-*O*-deoxyribonucleoside). Replacement of that exocyclic sulfur atom by oxygen in **3** gives rise to **4** (R, R = $-(CH_2)_5-$, X = O), which in the condensation reaction should provide compound **13** possessing a natural phosphodiester bond. Consequently, the whole elongation process would not involve any oxidation step, and, therefore, phosphorothioate functions present in the growing oligomer should be preserved. To prove that assumption, efforts toward the synthesis of 5′-*O*-DMT-base-protected nucleoside 3′-*O*-(2-oxo-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane)s were undertaken. Chemoselective oxidation of 5′-*O*-DMT-thymidine-3′-*O*-("spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane) (**10a**, an intermediate in the synthesis of **3a**), to form 5′-*O*-DMT-thymidine-3′-*O*-(2-oxo-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane) (**4a**, see Scheme 2) was not a trivial task, since a reagent to be used

Scheme 3



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must oxidize exclusively the trivalent phosphorus atom leaving untouched the rest of the molecule. Attempts at oxidation using anhydrous *tert*-butyl hydroperoxide[39] in benzene solution gave a mixture of products containing the desired **4a** (a mixture of diastereomers, δ ^{31}P NMR 44.3 and 44.9 ppm, 57%) and a considerable amount of a side-product seen by ^{31}P NMR as a pair of signals at 106.8 and 106.4 ppm (20%). The identity of the side product with **3a** (a mixture of diastereomers) was confirmed by MS analysis. The spectrum showed the presence of the corresponding molecular ion, and a "fingerprint" of the region *m/z* 650−800 was virtually identical with that of genuine material. The origin of this unwanted contamination is obscure and will be studied separately. Chromatographic isolation of **4a** from the reaction mixture was found to be rather difficult. However, oxidation of **10a** with selenium dioxide gave much more pure product **4a** in good yield. Initially the reactants were used in stoichiometric molar ratio (2:1), but it was found that 20% excess of selenium dioxide prevents formation of 5′-*O*-DMT-thymidine-3′-*O*-(2-selena-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane).[40] The progress of oxidation was monitored by TLC, and after 2 h the crude product was analyzed by ^{31}P NMR. The spectrum indicated the presence of two signals corresponding to both diastereomers of desired product **4a** (at 44.3 and 44.7 ppm, 1:1 ratio, 83% of total integral), accompanied by a pair of signals at 100.2 and 100.3 ppm (probably phosphoroselenoates, ca. 4%), and other not identified resonances with a total abundance of 13%. Compound **4a** was isolated in 56% yield using a silica gel column with chloroform as an eluent. Three other monomers **4b−d** (B = Ade^{Bz}, Cyt^{Bz}, and Gua^{iBu}) were prepared in the same way. Their characteristics are given in Table 1.

Applicability of the 2-oxo monomers **4** to the synthesis of natural oligonucleotides was tested by the manual synthesis of four dimers (AT, CT, GT, and TT), and by the automated synthesis of four "homooctamers" $(N_{PO})_7$T (N = dA, dG, dC, or T) using a protocol analogous to that elaborated for synthesis of PS−Oligos. In all syntheses performed on a 1 μmol scale, the same solid support, LCA CPG-Sar-Thy (23 μmol/g), was employed. For each coupling step 20 mg of **4** 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 the phosphoramidite method. These encouraging results prompted us to synthesize similarly a mixed sequence PO-d(TAGTGATTCT) (0.75 μmol scale). An RP-HPLC chromatogram recorded for DMT-on purification showed that the desired product constitutes 56% of the total integrated peak area (Figure 2), while analysis of the detritylated product showed its purity to be better than 92%. MALDI-MS spectrum recorded in the negative mode showed the presence of molecular ion at *m/z* 3031.6. PAGE analysis confirmed satisfactory quality of the product, which was comparable to that of a genuine sample of oligonucleotide prepared via the phosphoramidite method.[41] Approximately 1 OD unit of the crude product was then treated with formic acid.[42] The resulting hydrolysate was dried in a stream of argon and analyzed by means of RP-HPLC. Chromatograms recorded at 255 and 280 nm showed the presence of only four peaks corresponding to cytosine, guanine, thymine, and adenine, indicating that the synthesized oligonucleotide was free of base modification detectable by this method.

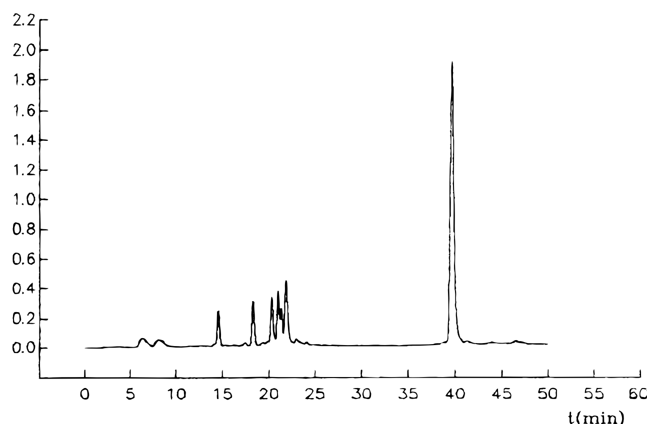

Figure 2. RP HPLC trace of crude PO-d(TAGTGATTCT) (DMT-on) synthesized using the monomers **4**.

Although 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, it was judged to be satisfactory enough to attempt synthesis of chimeric PS/PO−Oligos using both types of monomers **3** and **4**. As the first step, [mix]-TsTsTsTsToToToToTsTsTsT ([mix]-PS/PO-T_{12}) was synthesized, and a MALDI-MS spectrum recorded after two-step purification confirmed the identity of the product (the negative ion mode; *m/z* 3699.2). Repetitive yield, calculated from the DMT cation assay, was 93%. In this sequence as well as in other chimeric sequences **14−16** (vide infra), the letters "s" and "o" stand for phosphorothioate and phosphate internucleotide linkages, respectively, and the descriptors [mix]-, [all-R_P]-, and [all-S_P]- refer to the configuration at phosphorus in the phosphorothioate linkages. Then two oligonucleotides, [all-R_P]-ToTsToTsToTsToTsToT ([R_P]-PS/PO-T_{10}, **14**) and [all-S_P]-ToTsToTsToTsToTsToT ([S_P]-PS/PO-T_{10}, **15**) were synthesized on 1 μmol scale, and repetitive yield 96−97% was found. The reference compound, corresponding to [mix]-ToTsToTsToTs-ToTsToT ([mix]-PS/PO-T_{10}, **16**), was synthesized using standard phosphoramidite method with either oxidation or sulfuration at selected cycles. Compounds **14−16** were isolated by standard two-step HPLC (Econosphere, RP−C_{18}, 220 × 4.6 mm; buffer A, 0.1 M TEAB; buffer B, 40% acetonitrile in 0.1 M TEAB; gradient 1%/min, flow rate 1 mL/min) (Figure 10 in Supporting Information). Surprisingly, PAGE analysis showed that resulting **15** was approximately 25% contaminated with oligomer(s) shorter by one nucleotide. This contamination was substantially removed by rechromatography (Figure 10, panel C) using PTH−C18 column (Brownlee, 220 × 2.1 mm, gradient 0.7%/min, buffers as above, flow rate 0.3 mL/min). Purified **14−16** were labeled at the 5′-end with radioactive [^{32}P]phosphate group and analyzed by PAGE (Figure 3). Densitometry of autoradiograms showed that the purity of **14** was almost 100%, and was not lower than 96% for **15** and **16**. ESI MS analysis of **14** and **15** confirmed expected molecular weight (MW 3043), and ^{31}P NMR analysis showed that each spectrum contained two sets of resonances corresponding to phosphate and phosphorothioate linkages in the correct ratio.

Enzymatic analysis with R_P-specific snake venom phosphodiesterase (svPDE) and S_P-specific Nuclease P1 was used to confirm the location and the absolute configuration of phos-

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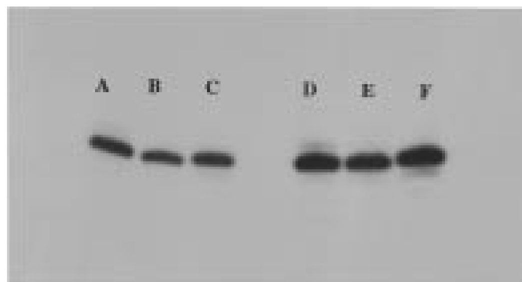

Figure 3. An autoradiogram from PAGE analysis (20% polyacrylamide/7 M urea) of HPLC purified "chimeric" oligonucleotides: lane A, [mix]-PS/PO-T_{10} (**16**); lane B, [R_P]-PS/PO-T_{10} (**14**); lane C, [S_P]-PS/PO-T_{10} (**15**); lanes D−F, as A−C, but at doubled concentration.

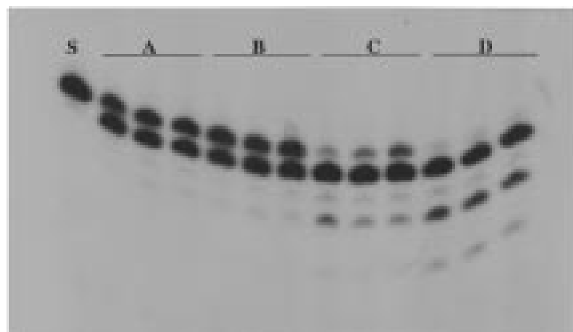

Figure 4. An autoradiogram from PAGE analysis (20% polyacrylamide/7 M urea) after digestion of [R_P]-PS/PO-T_{10} (**14**) with snake venom phosphodiesterase: lane S, the substrate **14**. The sets of lanes A−D correspond to the enzyme amounts 0.002, 0.005, 0.010, and 0.050 μg, respectively. In each set consecutive lanes correspond to the incubation time 5, 10, and 15 min.

phorothioate centers within oligonucleotide chains of **14** and **15**.[16] svPDE exonuclease hydrolyzes oligonucleotides (labeled at the 5′-end with radioactive [^{32}P]phosphate group) starting from the 3′-end. In the autoradiogram (Figure 4), the more abundant bands ($n-1$, $n-3$, $n-5$... nucleotides) correspond to the fragments of **14** possessing at their 3′-ends internucleotide phosphorothioate linkages, because their cleavage by svPDE is relatively slow. The oligomers with natural phosphodiester linkages at the 3′-ends are hydrolyzed fast, thus the relevant bands are of low abundance. Analogous degradation of **14** (not radiolabeled at the 5′-end) with svPDE was followed by MALDI-MS in the negative ions mode. The spectra (Figure 11 in Supporting Information) showed the presence of ions m/z 3043, 2739, 2418, 2114, 1792, 1489, 1168, 864. This pattern corresponds to consecutive removal of thymidine phosphate (m/z 304) or thymidine phosphorothioate moieties (m/z 320). The last recorded ion m/z 864 indicates the presence of a trinucleotide $T_{PO}T_{PS}T$, which was independently confirmed by enzymatic degradation from the 5′-end with spleen phosphodiesterase, as the molecular ion m/z 3042 was accompanied by the ion m/z 2738 (the mass difference of 304 daltons; Figure 12 in Supporting Information).

Nuclease P1 is an endonucleolytic enzyme and the pattern of degradation is different from that observed for svPDE. PAGE analysis revealed (Figure 5), as expected, that **14** is hydrolyzed only at the phosphate sites, because nuclease P1 does not hydrolyze phosphorothioate linkages of R_P configuration. On the other hand, the oligomer **15** is hydrolyzed by Nuclease P1 at each internucleotide bond (either phosphodiester or S_P-phosphorothioate), although the bands corresponding to the truncated oligomers possessing 3′-terminal phosphorothioate internucleotide linkage are slightly more intensive (Figure 6).

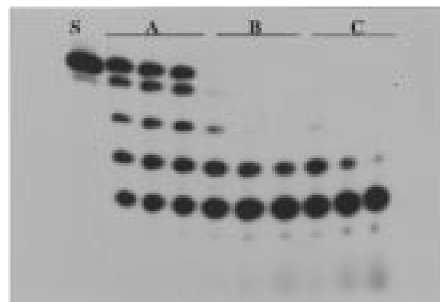

Figure 5. An autoradiogram from PAGE analysis (20% polyacrylamide/7 M urea) after digestion of [R_P]-PS/PO-T_{10} (**14**) with Nuclease P1: lane S, the substrate **14**. The sets of lanes A−C correspond to the enzyme amount 0.01, 0.05, and 0.1 μg, respectively. In each set consecutive lanes correspond to the incubation time 15, 30, and 90 min.

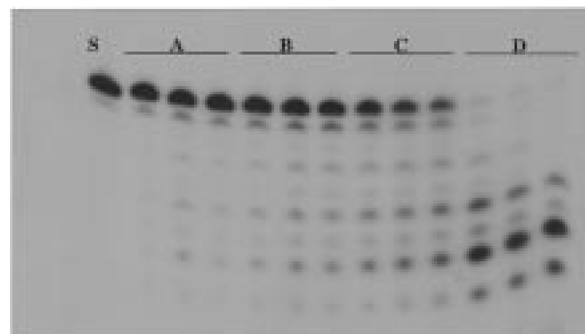

Figure 6. An autoradiogram from PAGE analysis (20% polyacrylamide/7 M urea) after digestion of [S_P]-PS/PO-T_{10} (**15**) with Nuclease P1: lane S, the substrate **15**. The sets of lanes A−D correspond to the enzyme amount 0.001, 0.005, 0.010, and 0.050 μg, respectively. In each set consecutive lanes correspond to the incubation time 15, 30, and 60 min.

The above results thus confirmed the configurations of the phosphorothioate centers in the chimeric oligomers **14** and **15**.

Thermodynamic Studies. For the chimeric constructs **14**, **15**, and **16** the T_m values for duplexes formed with a dA_{12}, dA_{30}, and poly(dA) were measured. It was found that, contrary to a literature report,[31] both stereoregular oligomers [R_P]- and [S_P]-PS/PO-T_{10}, as well as [mix]-PS/PO-T_{10}, form duplexes of practically the same thermal stability (buffer 10 mM Tris, 10 mM $MgCl_2$, 1 M NaCl) within an oligonucleotide concentration range of 1.15 to 6.9 μM (Table 3). The melting temperatures, obtained from the melting curves using first derivative method, allowed for calculation of thermodynamic parameters from an equation $T_m^{-1} = (R/\Delta H°) \ln (C_T) + (\Delta S° - R \ln (4))/\Delta H°$ (representative plots of reciprocal melting temperature vs ln (C_T)[43] are presented in Supporting Information, Figure 13), where C_T is the total strand concentration.

On the other hand, a nonlinear least-squares program (calculating routines developed for the program SigmaPlot, ver. 5.01, Jandel Corp.), based on a model for two-state association process,[44] was used to fit the data from melting curves with a formula $A(T) = A_h(T) + (A_c(T) - A_h(T))\theta$, where $A_h(T) = b_h(1 + m_hT)$ and $A_c(T) = b_c(1 + m_cT)$ represent the absorbance of the pure helical (double strand) and coil (single strand) form, respectively, as the extinction coefficients are assumed to be linear functions of temperature. The parameter $\theta = ((1 +$

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Table 3. Melting Temperatures and Standard Thermodynamic Parameters of Duplex Formation in 10 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$ and 1 M NaCl

duplex	T_m (°C) at concentrations (μM)[a]					T_m^{-1} vs ln c_T				A(T) vs T			
	1.15	3.45	4.6	5.75	6.9	$T_m°$, °C	$\Delta H°$, kcal/mol	$\Delta S°$, cal/molK	$\Delta G°_{37}$ kcal/mol	$T_m°$, °C	$\Delta H°$, kcal/mol	$\Delta S°$, cal/molK	$\Delta G°_{37}$, kcal/mol
[S_P]-PS/PO-T_{10}/dA_{12}	27.4	30.4	30.8	31.3	31.5	26	−77.8 ±5.5	−228.9 ±18	−6.8 ±0.5	23	−63.0 ±0.9	−182.7 ±3.2	−6.4 ±0.1
[R_P]-PS/PO-T_{10}/dA_{12}	26.9	29.8	30.8	31.4	31.8	25	−65.6 ±1.6	−190.1 ±5.3	−6.7 ±0.2	22	−57.1 ±1.7	−163.4 ±5.6	−6.5 ±0.1
[S_P]-PS/PO-T_{10}/dA_{30}	32.8	35.7	36.1	36.2	36.5	32	−89.6 ±10.1	−263.3 ±32.7	−8.0 ±1.0	31	−85.7 ±5.2	−251.7 ±16.8	−7.6 ±0.1
[R_P]-PS/PO-T_{10}/dA_{30}	32.9	35.5	36.3	36.5	37.5	31	−73.1 ±7.3	−209.9 ±23.7	−8.0 ±0.9	31	−81.1 ±3.5	−236.5 ±11.4	−7.8 ±0.1
[mix]-PS/PO-T_{10}/dA_{30}	33.3	36.2	nd	nd	38.2	31	−69.5 ±1.4	−198.4 ±4.5	−8.0 ±0.2	32	−84.9 ±2.2	−248.4 ±6.8	−7.9 ±0.1
[S_P]-PS/PO-T_{10}/poly(dA)	34.5	37.6	38.3	38.7	39.1	34	−73.5 ±3.2	−209 ±10.3	−8.7 ±0.4	35	−114.1 ±2.6	−340.4 ±8.0	−8.5 ±0.1
[R_P]-PS/PO-T_{10}/poly(dA)	35.1	37.7	38.3	38.7	39.3	35	−83.2 ±2.2	−241 ±7.1	−8.8 ±0.2	35	−109.7 ±3.2	−327.4 ±11.5	−8.6 ±0.1
[mix]-PS/PO-T_{10}/poly(dA)	35.4	38.3	nd	nd	40.3	35	−70.0 ±1.4	−198.6 ±4.5	−8.9 ±0.2	35	−107.9 ±4.2	−320.1 ±14.3	−8.7 ±0.4

[a] Error in melting temperatures is estimated as ±1 °C. Concentrations of chimeric PS/PO oligonucleotides are given.

$8E_T)^{1/2} - 1)/4E_T$ is the fraction of strands in the coil form, while E_T is given by formula $\exp((\Delta H°/R)(T_m^{-1} - T^{-1}))$. The molecularity of the association process (a parameter hidden in the formula for θ) was set to 2 independently of the template used. One can argue that for the poly(dA) template a multistate model for association would be more adequate, but we believe that the two-state model is sufficient for comparison of thermodynamic parameters for closely related diastereoisomeric species. The program fits data from melting curves (absorbance versus temperature) with the parameters $\Delta H°$, T_m, m_h, b_h, m_c, and b_c. For each sample the $\Delta H°$'s fitted for consecutive concentrations, and corresponding calculated standard melting temperatures $T_m°$ (i.e., the melting temperature for total strand concentration $C_T = 1$ μM), were used for calculation of standard entropies from the formula $\Delta S° = \Delta H°/T_m° + R \ln (4/C_T)$. Then, standard free Gibbs' energies at 37 °C were derived from the formula $\Delta G° = \Delta H° - T\Delta S°$. The values $\Delta G°$, $\Delta H°$, $\Delta S°$, and $T_m°$ calculated for consecutive concentrations were averaged for each duplex, followed by calculation of standard deviations.

Measured and calculated parameters, obtained from both methods (T_m^{-1} versus ln (C_T) and A(T) vs T), are collected in Table 3. One has to notice that, because of limited range of DNA strands concentrations suitable for UV measurements, standard errors for $\Delta H°$ and $\Delta S°$ parameters calculated from the plots T_m^{-1} versus ln (C_T) are relatively high, thus corresponding data obtained by numeric fitting seem to be more reliable. Nonetheless, both methods gave virtually the same values of free Gibbs' energy for both stereodefined oligomers **14** and **15**, as well as for the corresponding [mix] compound (−6.4/−6.8 kcal/mol for dA_{12} template, −7.6/−8.0 kcal/mol for dA_{30}, −8.4/−8.8 kcal/mol for poly(dA)). In most cases standard enthalpies and entropies are slightly more negative for duplexes formed by [S_P]-PS/PO-T_{10}, but these trends compensate each other and do not result in differences of free Gibbs' energy. This similarity in thermal stability and thermodynamic parameters is also reflected in CD spectra recorded for duplexes formed by [mix]-, [R_P]-, or [S_P]-PS/PO-T_{10} with all three templates. The spectra possess bands characteristic for natural dA_n/T_n duplexes (the B family)[45] and show very little difference between intensities of corresponding bands (Figure 14 in Supporting Information). It should be pointed out that CD spectra recorded for the duplexes [all-R_P]-$(T_{PS})_{11}T/dA_{12}$ and [all-S_P]-$(T_{PS})_{11}T/dA_{12}$ differ much more, especially in the region 265−300 nm (Figure 7), and are arranged in the order (from the top to the bottom) [all-R_P]-PS-T_{12}/-, [all-R_P]-PS/PO-T_{10}/-, unmodified-T_{12}/-, [all-S_P]-PS/PO-T_{10}/-, and finally [all-S_P]-PS-T_{12}/dA_{12}. Since we observed 4 °C difference in thermal stability of duplexes [all-R_P]-$(T_{PS})_{11}T/dA_{12}$ and [all-S_P]-$(T_{PS})_{11}T/dA_{12}$ (18 °C vs 14 °C),[16] discussed above, arrangement of CD spectra suggests that natural phosphate internucleotide linkages present in chimeric compounds in alternate positions allow for effective "relaxation" of tensions or distortions caused by phosphorothioates.

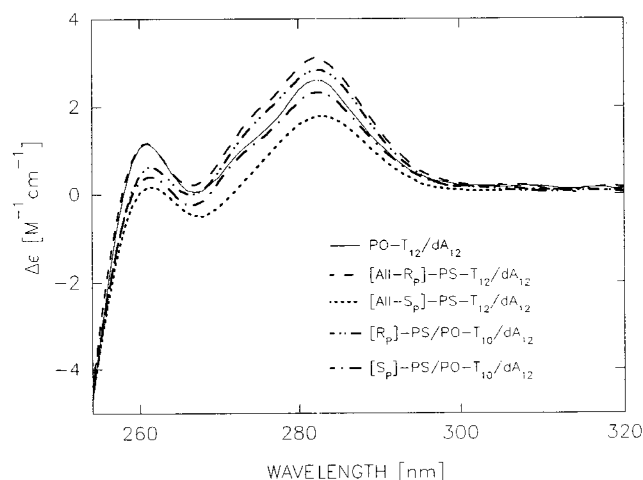

Figure 7. CD spectra (region 255−320 nm) recorded at 5 °C for the duplexes formed by stereoregular [all-R_P]- and [all-S_P]-PS-T_{12}, chimeric [R_P]- and [S_P]-PS/PO-T_{10}, and unmodified PO-T_{12} with dA_{12} matrix. Buffer 10 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 1 M NaCl. Optical path length 5 mm; nine smoothing steps were applied to each spectrum.

Stereochemistry of 1,3,2-Oxathiaphospholane Ring Opening Condensation − Mechanistic Considerations. Earlier model studies indicated that DBU-assisted methanolysis of 2-*N*-(α-naphthylethyl)amino-2-thio-1,3,2-oxathiaphospholane (**17**) yields *O*-methyl-*N*-(α-naphthylethyl)-phosphoramidothioate (**18**) with *net retention of configuration*.[17] The mechanistic proposal (Scheme 3, Z = *N*-α-naphthylethyl, R′O = MeO) involved an attack of a methoxide anion on the phosphorus atom collinearly with the endocyclic P−O bond to form a pentacoordinate

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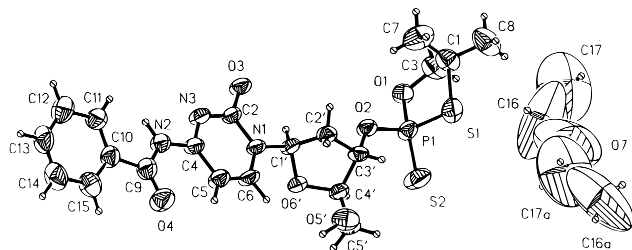

Figure 8. The crystal structure of 5′-OH-*N*[4]−Bz-deoxycytidine-3′-*O*-(2-thio-4,4-dimethyl-1,3,2-oxathiaphospholane) (**21**), aligned with diethyl ether molecule (a molecule of cosolvent present in the lattice), derived from X-ray analysis.

intermediate **19a**. Such a phosphorane, before collapse to **20**, according to Westheimer's pseudorotation theory must undergo a permutational isomerization (marked as Ψ) into **19b**, which places the endocyclic P−S bond in an apical position, thus allowing for its cleavage.[46] The elimination of an episulfide molecule from **20** leads to the final product **18**. This mechanism explained the observed stereochemistry of methanolysis of **17**, but its validity for the oxathiaphospholane method of synthesis of oligonucleotides, where Z is 3′-*O*-deoxyribonucleoside, awaited further confirmation. Such a verification was achieved within the present studies based upon the results of X-ray analysis of *N*[4]-Bz-deoxycytidine-3′-*O*-(2-thio-4,4-dimethyl-1,3,2-oxathiaphospholane) (**21**), which was obtained from the "fast"-eluted diastereomer **2c** (X = S, R = Me, B = Cyt^{Bz}), after detritylation with *p*-toluenesulfonic acid in methylene chloride. Compound **21** was isolated chromatographically on silica gel and dissolved in a mixture of toluene−methylene chloride−diethyl ether (10:2:0.5, v/v). Upon storage at −23 °C crystals suitable for X-ray analysis were formed (mp 119−121 °C, δ ^{31}P NMR 107.57 ppm (CD_3CN), MS FAB^+ 498.2, MS FAB^- 496.1). The bond lengths and valence angles found for the sugar ring and nucleobase (all experimental and calculated data are included in Supporting Information) were similar to those reported in the literature[47] and do not deserve any specific comments. Interestingly, a molecule of compound **21** crystallizes with one molecule of diethyl ether (Figure 8), which was a cosolvent for crystallization. The solvent molecules have relatively high thermal oscillation and are located in a hydrophobic "tunnel" along with molecules of **21** (Figure 9). They do not form any hydrogen bonds with **21** and are immobilized in the structure by van der Waals interactions. The molecules of **21** form a chain due to two intermolecular hydrogen bonds marked in Figure 9. One of them O5′−H′··· O3 is relatively strong (1.86(2) Å), while the second C6−H61··· O3 is weaker (2.34(2) Å). Two intramolecular hydrogen bonds of medium strength C5−H51···O4 (2.22(2) Å) and C1′−H11′··· O3 (2.36(2) Å), as well as several weak interactions (ca. 2.5 Å) stabilize the geometry of the molecule (see Table 4). Two five-membered rings present in **21** have different conformations. The sugar ring adopts an almost perfect open-envelope structure (asymmetry parameter ΔCs(C2′)=O.9(2)) with C2′ as an opening atom, while the oxathiaphospholane ring exists as a half-chair (asymmetry parameters ΔCs(C3) = 10.4(2) and ΔC2-(C3−C1) = 11.1(2), (see Table 11 in Supporting Information). The value −146.0(4)° found for the torsional angle C2−N1−C1′−O6′ indicates that the O3 atom occupies an *exo* position with respect to the sugar ring.

For the mechanistic considerations the most important finding was the assignment of the absolute configuration at the P atom in **21** as R_P (see Figure 8). Since "fast"-**2c** (B = Cyt^{Bz}), a precursor of **21**, when condensed with 5′-OH nucleoside gives phosphorothioate dinucleotides **22** of R_P configuration (as determined by HPLC and enzymatic analysis), the result of crystallographic analysis supports the earlier suggestion[17] that the coupling reaction proceeds via an "adjacent"-type mechanism depicted in Scheme 3 (Z = 3′-*O*-deoxyribonucleoside, R′O = 5′-*O*-deoxyribonucleoside), although recent ab initio calculations suggest that **19b** is not an intermediate product, and the bypiramid **19a** collapses to **20** via tetragonal square pyramid transition state.[48] Nonetheless, within this mechanistic proposal, a DBU molecule acts only as a base and not as a nucleophile.[49] Since we observed that other bases, such as *N*-methylimidazole, 4-(dimethylamino)pyridine, and 2-*tert*-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine, promote the condensation far less effectively than DBU, an attack of DBU on phosphorus atom might be considered[50] as the route to the phosphorane, which upon addition of the hydroxy compound would lead to the hexacoordinate phosphorus intermediate. Departure of the protonated DBU ligand, followed by pseudorotation, cleavage of the P−S bond, and elimination of the episulfide, would give the expected phosphorothioate product, although we must admit that there is limited knowledge about stereochemistry of formation and collapse of such hexacoordinate intermediates. Therefore, to get better insight into the function of DBU in the oxathiaphospholane ring opening condensation, a mixture of the thymidine oxathiaphospholane monomer **3a** and DBU (20-fold molar excess) in acetonitrile was monitored by ^{31}P NMR. The consecutive spectra recorded over the following 30 min showed the formation of only one product (up to 30%) absorbing at 48 ppm, which by means of FAB MS was identified as 5′-*O*-DMT-thymidine 3′-*O*-phosphorothioate, most likely formed by hydrolysis utilizing traces of water acting as a nucleophile. When 5 μL of water (5-fold molar excess over the monomer **3a**) was added to the reaction mixture, the hydrolysis was complete in less than 2 min. Fast formation of dinucleoside phosphorothioate was observed when, instead of water, an equimolar amount of 3′-*O*-acetylated thymidine was added to the mixture containing the monomer **3a** and DBU. If no nucleophile was added, no other resonances were found in the recorded ^{31}P NMR spectrum indicating that, if any intermediate is formed with participation of the oxathiaphospholane monomer and DBU, its lifetime must be relatively short as compared to the NMR time-scale.

The mechanistic proposal invoking the formation of the hexacoordinate intermediate became even less justified because the following experiments demonstrated that a strong nonnucleophilic base such as potassium *tert*-butoxide also catalyzes the condensation process. Equimolar amounts of **3a** and 3′-*O*-acetylated thymidine upon treatment with potassium *tert*-butoxide (1.4 equiv) in a mixture of acetonitrile and dimethylformamide (4:1) after 6 h furnished the corresponding protected dinucleotide in ca. 80% yield (as judged by ^{31}P NMR). The yield was reasonably good taking into account the stoichiometric ratio of both reactants, albeit poor solubility of that catalyst, even in the presence of 18-*crown*-6, and problems encountered

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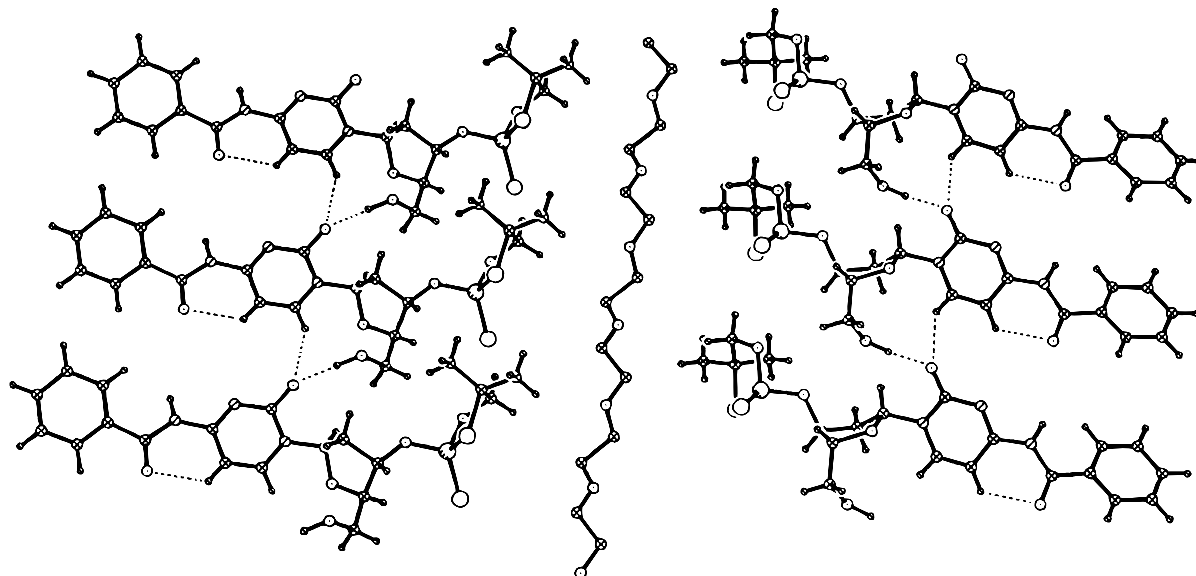

Figure 9. Crystallographic packing in a crystal of 5′-OH-N^4−Bz-deoxycytidine-3′-*O*-(2-thio-4,4-dimethyl-1,3,2-oxathiaphospholane) (**21**). The dotted lines indicate hydrogen bonds (see text).

Table 4. Hydrogen Bonds and Contacts with Distance H···A Not Greater than 2.80 Å

D	H	A	D, −H, Å	H···A (Å)	D···A (Å)	(D−H···A)(°)	symmetry
O5′	H5′	O3	0.99(2)	1.86(2)	2.836(7)	172(2)	*X*, 1 + *Y*, *Z*
N2	H2	C11	0.90(2)	2.49(2)	2.882(8)	107(2)	
C5	H51	O4	0.96(2)	2.22(2)	2.817(7)	119(2)	
C5	H51	C9	0.96(2)	2.77(2)	2.999(7)	94(2)	
C6	H61	O3	0.96(2)	2.34(2)	3.183(6)	147(2)	*X*, 1 + *Y*, *Z*
C6	H61	O6′	0.96(2)	2.44(2)	2.726(6)	97(2)	
C7	H72	O1	0.96(2)	2.68(2)	3.044(9)	103(2)	
C7	H73	O3	0.96(2)	2.63(2)	3.563(9)	165(2)	1 − *X*, *Y*, 2 − *Z*
C8	H82	N3	0.96(2)	2.52(2)	3.456(9)	167(2)	1 − *X*, *Y*, 2 − *Z*
C11	H111	N2	0.96(2)	2.60(2)	2.882(8)	97(2)	
C15	H151	O4	0.96(2)	2.46(2)	2.761(9)	98(2)	
C1′	H11′	O3	0.96(2)	2.36(2)	2.704(6)	101(2)	
C2′	H21′	O5′	0.96(2)	2.70(2)	3.140(7)	109(2)	
C2′	H22′	O5′	0.96(2)	2.67(2)	3.475(7)	142(2)	*X*, −1 + *Y*, *Z*
C3′	H31′	O5′	0.96(2)	2.70(2)	2.887(7)	91(2)	
C5′	H52′	O1	0.96(2)	2.66(2)	3.597(7)	165(2)	*X*, 1 + *Y*, *Z*
C5′	H52′	O2	0.96(2)	2.78(2)	3.374(7)	121(2)	*X*, 1 + *Y*, *Z*

with the stability of the sarcosinylated support[27] make the use of potassium *tert*-butoxide as a catalyst in solid-phase synthesis impractical.

Conclusions

Phosphitylation of appropriately protected deoxyribonucleosides **9a**−**d** at the 3′-*O* site with 2-chloro-“spiro”-4,4-pentamethylene-1,3,2-oxathiaphospholane (**5**), followed by sulfurization, provides compounds **3** in satisfactory yields. These can be efficiently resolved by silica gel column chromatography onto diastereomerically pure species that serve as substrates in the 1,3,2-oxathiaphospholane ring-opening condensation with 5′-OH oligonucleotides. The condensation occurs effectively (90−97% yield) in the presence of DBU with stereospecificity higher than 98%. Independently, 3′-*O*-phosphitylation of protected deoxyribonucleosides with **5**, followed by treatment of the intermediary phosphorothioite **10** with selenium dioxide, provides compounds **4** which in combination with **3** can be used for stereocontrolled synthesis of “chimeric” phosphate/phosphorothioate oligonucleotides. Within the course of this work, the crystal structure of the “fast”-**2c** -related (B = Cyt^{Bz}) N^4-benzoyldeoxycytidine 3′-*O*-(2-thio-4,4-dimethyl-1,3,2-oxathiaphospholane) has been solved providing evidence that the process of DBU-assisted 1,3,2-oxathiaphospholane ring-opening condensation with 5′-OH-deoxyribonucleosides proceeds with *retention of configuration at phosphorus.* This result speaks for an “adjacent”-type A−E mechanism of nucleophilic substitution at phosphorus. Additional evidence has been provided to indicate that DBU does not participate as a nucleophile in that process.

Experimental Section

The nuclear magnetic resonance spectra were recorded on a Bruker AC-200 instrument (200 MHz, TMS internal standard for ^1H and 85% H_3PO_4 as the external standard for ^{31}P). The FAB-MS spectra (13 keV, Cs^+) were recorded on a Finnigan MAT 95 spectrometer, negative ion MALDI mass spectra were recorded on a Voyager-Elite instrument (PerSeptive Biosystems Inc., Framingham, MA), while electrospray mass spectrometry analyses were done at Mass Consortium, Corp., San Diego, CA. Ultraviolet (UV) spectra and melting profiles were recorded on a GBC 916 spectrophotometer equipped with a Thermocell unit. Densitometry of autoradiograms was performed on an LKB Ultroscan XL densitometer. CD spectra were recorded on a CD6 dichrograph (Jobin-Yvon) using cells with 5 mm path length. HPLC analyses were done on an LDC Analytical system (pumps CM3500 and CM3200, SpectroMonitor SM4100). The crystallographic analysis was performed on a CAD4 diffractometer with graphite monochromatized Cu Kα

radiation. The elemental analysis was performed by the Laboratory of Microanalysis of this Centre. Evaporations were carried out at 40 °C (or lower) using an aspirator or oil pump vacuum. Deoxyribonucleosides were purchased from Pharma Waldhof (FRG). Cyclohexanecarboxaldehyde was purchased from Aldrich (USA). Acetonitrile and 1,4-diazabicyclo[5.4.0]undec-7-ene (DBU) were supplied by Merck (FRG). Acetonitrile to be used as solvent for DBU and oxathiaphospholane monomers **1**, **2**, **3**, and **4** was dried over P_2O_5 (5 g/L) and distilled through a 20-cm Vigreux column in an atmosphere of dry argon. At least one-third of the initial volume must be left in the flask. Acetonitrile dried in this way must be transferred using gastight syringes under an atmosphere of dry argon, or by vacuum line. NOTE: *Commercially available acetonitrile marked as "DNA/RNA synthesis grade" supplied even by leading manufacturers is not suitable for that purpose unless dried as above!* Phosphorus trichloride, ethyl acetate, butyl acetate, and selenium dioxide were purchased from POCH (Poland). Snake venom phosphodiesterase (svPDE, EC 3.1.15.1) was obtained from Boehringer Mannheim (FRG). Nuclease P1 (nP1, EC 3.1.30.1), calf spleen phosphodiesterase (EC 3.1.16.1), and poly(dA) were purchased from Sigma. T4 polynucleotide kinase (EC 2.7.1.78) was obtained from Amersham (USA). [γ-^{32}P]ATP was synthesized by Dr. A. Płucienniczak of the Centre of Microbiology and Virology of the Polish Academy of Sciences (Łódź, Poland).

2,2′-Dithiobis(cyclohexanecarboxaldehyde) (8). This compound was synthesized from cyclohexanecarboxaldehyde (70% yield) as described by Hayashi.[26] The product was crystallized from diethyl ether (mp 88−89 °C).

1,1′-Dithiobis(1,1-pentamethylenethan-2-ol) (7). Into a suspension of $NaBH_4$ (62 g, 0.164 mol) in 500 mL of isopropyl alcohol 2,2′-dithiobis(cyclohexanecarboxaldehyde) (23.5 g, 0.082 mol) was added dropwise over 30 min. The mixture was refluxed for 1 h, then evaporated, and a 1.5 M solution of sodium hydroxide (200 mL) was added. The mixture was cautiously neutralized with concentrated hydrochloric acid and extracted with chloroform (2 × 150 mL). The organic layer was dried with magnesium sulfate and the solvent was evaporated. The residue was dissolved in dry benzene (100 mL) and the solvent was evaporated with exclusion of moisture. The crystalline product was precipitated from diethyl ether/hexane; 23 g of colorless crystalline material was collected (97% yield, mp 49−50 °C).

1-Mercapto-1,1-pentamethylenethan-2-ol (6). Into a suspension of lithium aluminum hydride (5.9 g, 0.16 mol) in 500 mL of dry diethyl ether (atmosphere of dry argon, two neck flask equipped with a condenser and a funnel) a solution of 1,1′-dithiobis(1,1-pentamethylenethan-2-ol) (4.6 g, 0.16 mol) in 150 mL of diethyl ether was added, with magnetic stirring, dropwise over 60 min. The reaction was exothermic and mild reflux occurred. The stirring was continued for 1 h and an excess of reducing agent was cautiously decomposed with ethyl acetate, followed by THF containing traces of moisture, and finally with 10% water/THF. Inorganic salts were filtered off and the filtrate was dried with magnesium sulfate. The solvents were evaporated and the residue was distilled under reduced pressure to give 32.4 g (70% yield) of a colorless oil (bp 74−76 °C/0.05 mmHg, n^{20}_D 1.5188).

2-Chloro-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane (5). A solution of PCl_3 (0.21 mol, 28.2 g) in 500 mL of dry benzene was poured into a flask equipped with a thermometer and a separatory funnel. The flask was cooled to 5 °C, and a solution of 1-mercapto-1,1-pentamethylenethan-2-ol (0.14 mol, 20 g) and pyridine (0.27 mol, 22 mL) in 35 mL of benzene was added dropwise over 15 min with magnetic stirring. The temperature of the reaction mixture was kept below 10 °C. Stirring was continued at room temperature for 30 min and pyridine hydrochloride was filtered off. After evaporation of the solvent under reduced pressure, the product was distilled to give 21 g of a colorless liquid (76% yield; bp 82−84 °C/0.01 mmHg; δ ^{31}P NMR 217.7 ppm (C_6D_6); EI-MS (70 eV) *m/z* 210, M^+, 12%; *m/z* 175, $[M - Cl]^+$, 5.8%; *m/z* 90, 100%.

Phosphitylation of Protected Deoxyribonucleosides with 2-Chloro-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane: General Procedure. To the magnetically stirred solution of 10 mmol of appropriately protected deoxyribonucleoside (dA^{Bz}, dG^{iBu}, T, or dC^{Bz}) and 1.91 mL of diisopropylethylamine (11 mmol) in 10 mL of dry acetonitrile, 11 mmol of **5** was added dropwise at room temperature. The reaction was complete in 5 min and then elemental sulfur (15 mmol) was added. Stirring was continued for 12 h and an excess of sulfur was filtered off. After evaporation of the solvent the residue was dissolved in 4 mL of chloroform (distilled with pyridine) and applied to a 230−400 mesh silica gel column (23 × 3 cm, 20 g). The column was eluted with chloroform (250 mL) and then chloroform−methanol (98:2, v/v). Appropriate fractions were combined and the solvents were evaporated under reduced pressure to give desired compounds **3a−c** or 5′-*O*-DMT-N^2-^{i}Bu-deoxyguanosine-3′-*O*-(2-thio-spiro-4,4-pentamethylene-1,3,2-oxathiaphospholane) (**11**, a precursor of **3d**) in 78−86% yield. The diastereomeric composition, chemical shifts in ^{31}P NMR, and TLC parameters of compounds **3a−d** are given in Table 1. Anal. (found/calcd) **3a** (B = Thy) C 61.67/60.79, H 6.15/5.77, N 3.73/3.73, P 4.06/4.13, S 8.00/8.54; **3b** (B = Ade^{Bz}) C 62.84/62.56, H 5.48/5.37, N 7.73/8.11, P 3.41/3.58, S 6.74/7.42; **3c** (B = Cyt^{Bz}) C 62.53/62.92, H 5.47/5.52, N 5.14/5.00, P 3.65/3.69, S 7.26/7.63.

Protection of 5′-O-DMT-N^2-^{i}Bu-deoxyguanosine-3′-O-(2-thio-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane) at the O-6 Site with Diphenylcarbamoyl Chloride.[51] To a solution of 5′-*O*-DMT-N^2-^{i}Bu-deoxyguanosine-3′-*O*-(2-thio-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane) (0.85 g, 1 mmol) in pyridine (5 mL), diisopropylethylamine (0.26 mL, 1.5 mmol) and diphenylcarbamoyl chloride (0.46 g, 2.0 mmol) were added, with stirring, at room temperature. The mixture was stirred for 1 h, concentrated, dissolved in chloroform (1.5 mL), and applied into a silica gel column (ca. 20 g). The column was eluted with chloroform (300 mL) and appropriate fractions (TLC control; silica gel plates, R_f = 0.79, chloroform/methanol 9:1 v/v) were collected and evaporated under reduced pressure to give a pale yellow oil. The pure product **3d** (0.99 g, 95% yield) was evaporated with dry toluene, and stored in a tightly closed vessel. Its diastereomeric composition, chemical shifts in ^{31}P NMR, and TLC parameters are given in Table 1. MS (+FAB) *m/z* 1041.6 (M^+, 1%), *m/z* 1042.6 ($M^+ + 1$, 0.6%), *m/z* 303.2 (DMT^+, 100%). Anal. (found/calcd) C 63.60/63.44, H 5.78/5.52, N 7.77/8.08, P 2.72/2.98, S 5.78/6.15.

Separation of the Diastereomers of 3a−d. A solution of 300 mg of monomer **3** in 1.0 mL of appropriate eluent (vide infra) was applied onto a column (30 × 2 cm) containing 20 g of silica gel (Merck 60H, particle size 5−40 μm). The column was eluted with 300 mL of ethyl acetate−butyl acetate−pyridine (2:1:0.003 v/v/v for the dA and dC derivatives, 1:2:0.003 v/v/v for the dG derivative) or butyl acetate−benzene−pyridine (1:1:0.002 v/v/v for the T monomer), and fractions of 10−12 mL were collected. TLC control of the eluate was performed on HP-TLC plates. Typically for dA, dT, and dC monomers one passage gave 75−80% of separated diastereomers of 96.8−100% diastereomeric purity. For the dG derivative the "fast" isomer was obtained in 28% yield (100% diastereomeric purity) while the "slow" isomer was obtained in 50% yield (90% diastereomeric purity), and had to be rechromatographed.

5′-*O*-DMT-deoxyribonucleoside-3′-*O*-(2-oxo-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane)s (4). Appropriately protected deoxyribonucleosides **9a−d** (1 mmol) were phosphitylated with 2-chloro-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane (vide supra) and oxidized in situ by addition of 0.61 g of selenium dioxide (0.55 mmol) at room temperature. The reaction was complete after 3 h (TLC monitoring, chloroform/methanol 9:1, v/v). The reaction mixture was filtered and the solvent was evaporated. The residue was dissolved in 2 mL of chloroform (distilled with pyridine) and applied to a 230−400 mesh silica gel column (20 × 3 cm, 30 g). The column was eluted with chloroform and appropriate fractions were combined and evaporated under reduced pressure to give the desired compounds (5′-*O*-DMT-deoxyribonucleoside-3′-*O*-(2-oxo-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane)) in 41−55% yield. The diastereomeric composition, chemical shifts in ^{31}P NMR, and TLC parameters of compounds **4a−d** are given in Table 1. Anal. (found/calcd) **4a** (B = Thy) C 59.45/62.11, H 5.95/5,85, N 3.82/3.81, P 4.34/4.22, S 4.08/4.35; **4b** (B = Ade^{Bz}) C 61.12/63.74, H 5.65/5.43, N 7.74/8.26, P 3.87/

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3.66, S 3.76/3.79; **4c** (B = Cyt^{Bz}) C 64.54/64.14, H 5.50/5.58, N 4.62/5.10, P 4.17/3.76, S 4.09/3.88; **4d** (B = G^{iBu}) C 62.38/60.78, H 6.36/5.78, N 7.41/8.43, P 3.67/3.74, S 4.08/3.86.

Solid-Phase Synthesis of PS- and PS/PO−Oligos. The automated synthesis of PS- and PS/PO−Oligos was performed on an ABI 391 synthesizer (Applied Biosystems, Inc., Foster City, CA). Standard solutions of DCA in methylene chloride and DMAP/Ac_2O/lutidine in THF were used for detritylation and capping steps, respectively. A 1.5 M (or 1 M for synthesis of "chimeric" oligomers) solution of DBU in CH_3CN was delivered from the position for 1-*H*-tetrazole. The crucial parameters of the protocol are shown in Table 2. The detailed protocol for the synthesis is available upon request. For manual synthesis, gastight syringes were used while all reagents and solvents were stored under an atmosphere of dry argon, in vials (or bottles) capped with rubber septa. A column containing solid support was equipped with two Teflon adapters: one for insertion of a syringe to deliver reagents, second ended with a needle (or a piece of low diameter Teflon tubing) to allow for safe collecting of wastes. After each wash step the excess of solvent was removed from the column by suction (an aspirator) while dry argon was delivered from the opposite side. Before each coupling step the column was dried under high vacuum (0.1 mmHg) for 5 min. The drying vessel was filled up with dry argon before the column was taken out. An appropriate monomer (20 mg) was dissolved in dry acetonitrile (140 μL) just before each condensation step. To that solution, 30 μL of a solution of DBU in acetonitrile (1:1, v/v) was added and the mixture was applied to the column, followed by intensive swirling. Swirling was continued over whole coupling time, approximately 8 min. The reagents were expelled and the support was washed with dry methylene chloride (4−5 mL) and dry acetonitrile (7 mL). Other steps as detritylation and capping were performed using standard reagent solutions with each step followed by exhaustive washing with 5 mL of dry acetonitrile.

When synthesis was complete, the oligomer was cleaved from the support under standard conditions (25% NH_4OH, 2 h) and the protecting groups from nucleobases were removed at 55 °C over 12 h. The sample was concentrated under reduced pressure in a Speed-Vac concentrator and two-step RP-HPLC (DMT-on and DMT-off) was used to isolate the product (a column Econosphere, RP−C_{18}, 220 × 4.6 mm; buffer A, 0.1 M TEAB; buffer B, 40% acetonitrile in 0.1 M TEAB; gradient 1%/min; flow rate 1 mL/min. Retention times in the range 35−40 or 16−23 min were recorded for DMT-on or DMT-off analyses, respectively. For "chimeric" PS/PO oligonucleotide **15** rechromatography (DMT-off) was necessary (a PTH−C18 column, Brownlee, 220 × 2.1 mm; gradient 0.7%/min, buffers as above, flow rate 0.3 mL/min) to obtain the product of satisfactory purity.

Enzymatic Analysis of PS/PO Oligonucleotides. For 5′-end labeling of PS/PO−Oligos by [γ-^{32}P]ATP and T4 polynucleotide kinase was used the procedure published for labeling of PS−Oligos.[16] Labeled oligonucleotides (1 μg, 0.2 nmol) were hydrolyzed with either svPDE or Nuclease P1, and the hydrolyzates were analyzed by 20% polyacrylamide/7 M urea gel electrophoresis. Because of significant differences in the rates of hydrolysis of phosphate versus phosphorothioate internucleotide bonds by each enzyme, to obtain diagnostic autoradiograms the amounts of nucleases were adjusted experimentally.

Hydrolysis with svPDE. General Procedure. In a typical experiment the reaction mixture (30 μL) containing 0.006−0.15 μg of svPDE, 100 mM Tris-Cl (pH 8.5), 15 mM $MgCl_2$, and an oligomer (0.2 nmol) labeled at the 5′-end with ^{32}P was incubated at 37 °C; 10-μL aliquots, taken after 5, 10, and 15 min, were heat-denaturated and analyzed by PAGE.

Hydrolysis with Nuclease P1. General Procedure. In a typical experiment the reaction mixture (30 μL) containing 0.015−0.15 μg of nuclease P1, 100 mM Tris-Cl (pH 7.2), 1 mM $ZnCl_2$, and labeled oligonucleotide (0.2 nmol) was incubated at 37 °C; 10-μL aliquots, taken after 15, 30, and 90 min, were heat-denaturated and analyzed by PAGE.

Thermodynamic Analysis of Chimeric Oligothymidylates. The chimeric oligothymidylates [R_P]-, [S_P]-, or [mix]-PS/PO-T_{10} at concentrations C_m = 1.15, 2.3, 3.45, 4.6, 5.75, or 6.9 μM, were mixed with equimolar amount of dA_{12}, or equimolar per nucleotide amount of dA_{30} or poly(dA) (MW ∼86 000) matrixes (10 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 70 mM or 1 M NaCl). The samples were incubated at 90 °C for 2 min, slowly cooled to 8 °C, and kept at this temperature for 20 min. Temperature dissociation was carried out with a temperature gradient 0.2 °C/min until final temperature 60 °C was reached. The melting points were determined using the first derivative method. The plots for $1/T_m$ vs ln (C_T), where C_T is a total concentration of oligonucleotides ($C_T = 2C_m$ for dA_{12} template, $4C_m/3$ for dA_{30}, or C_m for poly(dA), were optimized using the least-squares method. The slopes ($R/\Delta H°$) and the intercepts (($\Delta S° - R \ln 4)/\Delta H°$) were used for calculation of $\Delta H°$ and $\Delta S°$.[43] Melting curves were also fit numerically (as described in Results and Discussion) and obtained parameters $\Delta H°$ and $T_m°$ were used for calculation of standard entropy and free Gibbs' energy.

Enzymatic Degradation followed by MALDI-MS. A mixture of a 0.05 M solution of 2,4,6-trihydroxyacetophenone in 50% acetonitrile and 0.2 M solution of diammonium hydrogen citrate in water (8:1, v/v) was used as a matrix for MALDI-MS analyses.

All enzymatic reaction were performed in deionized water without the addition of buffers: (5′→3′ degradation) 4 μL of oligonucleotide solution (0.0005 OD/μL) were mixed with 1 μL of 0.02, 0.03, or 0.07 mU/μL solutions of calf spleen phosphodiesterase and kept for 15 min at 37 °C; (3′→5′ degradation) 4 μL of oligonucleotide solution (0.0005 OD/μL) were mixed with 1 μL of 0.1, 0.2, or 0.3 mU/μL solutions of snake venom phosphodiesterase and kept at 37 °C for 15 min. A 1-μL sample of matrix solution was mixed with 1 μL of enzymatic degradation mixture, applied onto the probe plate, and allowed to crystalize. In the resulting spectra (in each experiment 50−100 spectra were collected in a linear mode and averaged), the negative ions for intact, as well as for partially digested oligomers, were found (*m/z* 3043, 2739, 2418, 2114, 1794, 1489, 1168, 864).

X-ray Analysis of 21. The absolute configuration of crystalline **21** (detritylated "fast"-eluted **2c**; X = S, R = Me, B = Cyt^{Bz})[16] was determined using data collected at room temperature. The compound crystallizes in monoclinic system, space group *C*2. Experimental details as well as crystal data are presented in Tables 5−13 in Supporting Information. Lattice constants were refined by least-squares fit of 25 reflections in Θ range 20.2−30.0°. Decline in intensities of three standard reflections (0,−3,−6; 0,6,−6; −1,−3,−5) was 14.9% during 164.9 h of exposure. Intensity data were corrected by use of the DECAY program.[52] Absorption correction was applied using ψ-scan method (EAC program[52,53]). The observed reflections with $I \geq 3\sigma(I)$ were used to solve the structure by direct methods, and to refine it by full matrix least-squares using F's.[54,55] Hydrogen atoms connected with carbon atoms were placed geometrically at idealized positions, other were found in a difference Fourier map. All hydrogens were set as riding, with fixed isotropic thermal parameters. Anisotropic thermal parameters were applied for all nonhydrogen atoms.

The absolute structure R_P was determined by use of three methods: the Hamilton test,[56] η-refinement,[57] and Flack *x* parameter.[58] The Hamilton test showed (R_{ratio} = 1.088, *N* = 4075) that the probability of opposite configuration $\alpha \ll 10^{-6}$.[55] The Rogers' parameter η refines to η = 1.12(7), and where opposite configuration is assumed, to η_{inv} = −1.12(7). The Flack parameter x = 0.02(4).

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Supporting Information Available: Chromatograms for RP HPLC purification (DMT-off) of **14** and **15**; MALDI spectra for **14** digested with svPDE and calf spleen phosphodiesterase; plots for $1/T_m$ vs ln (C_T) for **14**/dA_{12} and **15**/dA_{12}; CD spectra for duplexes **14**, **15**, or **16** and dA_{12}, dA_{30}, or poly-dA templates; experimental details, crystal data, and atomic coordinates for X-ray analysis of **21** (15 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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