The synthesis of di- and oligo-nucleotides containing a phosphorodithioate internucleotide linkage with one of the sulfur atoms in a 5′-bridging position†

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Received 27th January 2009, Accepted 3rd March 2009
First published as an Advance Article on the web 30th March 2009
DOI: 10.1039/b901791g

A new type of internucleotide phosphorodithioate linkage is described, wherein one of the sulfur atoms occupies a 5′-bridging position. Representative dinucleotides possessing such a bond were synthesized by S-alkylation of nucleoside-3′-*O*-phosphorodithioates with 5′-halogeno-5′-deoxy-nucleosides. A fully protected dithymidylate containing internucleotide 5′-*S*-phosphorodithioate linkage was converted into a 3′-*O*-phosphoramidite derivative and employed for introduction of a modified dinucleotide into a predetermined position of the oligonucleotide sequence. The 5′-*S*-phosphorodithioate linkage in dinucleotide analogues was found to be resistant toward nucleolytic degradation with snake venom PDE and nuclease P1. However, P-stereoselective degradation was observed for diastereomers of 5′-*S*-phosphorodithioate dithymidine analogs under treatment with calf spleen PDE. The new 5′-*S*-phosphorodithioate linkage was readily degraded by iodine solutions in the presence of water. It was also found that oligothymidylates containing a single 5′-*S*-phosphorodithioate linkage form much weaker duplexes with their complementary sequences.

Introduction

Analogues of nucleotides and oligonucleotides have received much attention due to their use as indispensable tools for studying the structure of nucleic acids and their interactions with other biomolecules, such as DNA, RNA, carbohydrates, lipids or proteins.[1] These compounds are widely employed to investigate the mechanism and stereochemical aspects of various biochemical reactions catalyzed by nucleic acid metabolizing enzymes.[2] Modified oligonucleotides have also been extensively used as rationally designed tools to control the expression of specific gene products and therefore are being explored as potential therapeutics for the treatment of viral infections, cancers or inflammatory disorders.[3]

Besides oligo(nucleoside phosphorothioate)s, among the most intriguing analogues of nucleotides and oligonucleotides are the phosphorodithioate congeners in which two oxygen atoms at an internucleotide phosphate moiety are replaced by sulfur.[4] The vast majority of studies on their synthesis are related to compounds having both sulfur atoms placed in a non-bridging position. Phosphorodithioate analogues of dinucleotides have been prepared in several ways using phosphorodiamidite,[5] phosphorothioamidite,[6] phosphotriester,[7] H-phosphonothioate,[8] H-phosphonodithioate[9] or dithiaphospholane[10] chemistries. Consequently, the synthesis of oligodeoxyribonucleoside phosphorodithioates of various length and sequence was reported[11] as well as examples of phosphorodithioate oligoribonucleotides,[12] including 2′,5′-oligoadenylate analogues.[13] Similarly, oligonucleotides containing a 5′-*O*-phosphorodithioate monoester function were prepared.[14] In addition to the aforementioned di- and oligonucleotide phosphorodithioate congeners, several other phosphorodithioated biomolecules were synthesized, including nucleoside cyclic 2′,3′-*O*,*O*-[15] and 3′,5′-*O*,*O*-phosphorodithioates,[16] nucleoside 5′-*O*-(1,1-dithiotriphosphates),[17] nucleoside dithiophosphate monoesters,[14b,18] nucleoside phosphofluorodithioates,[19] *O*-dithiophosphonopeptides,[20] and phosphorodithioate phospholipid derivatives.[21]

In clear contrast to the aforementioned di- and oligonucleotide phosphorodithioate congeners, very limited information is available in the literature on the synthesis of a phosphorodithioate internucleotide linkage with only one of the two sulfur atoms in a bridging position. In fact, all published examples are related to the preparation of analogous compounds containing an internucleotide 3′-5′-bond with a bridging sulfur atom in the 3′-position.[22] Their syntheses were performed *via* preparation of appropriately protected 3′-mercapto-3′-deoxy-nucleosides, which were 3′-*S*-phosphitylated and then reacted with a 5′-hydroxyl component under the conditions of phosphoramidite synthesis, with subsequent sulfurization of 3′-*S*-phosphorothioite intermediates. By virtue of asymmetry of the phosphorus atom in the 3′-*S*-phosphorodithioate function, a mixture of diastereomers was always formed, which could be separated into individual diastereomerically pure compounds by chromatography. The absolute configuration at the P atom was tentatively assigned by spectroscopic and enzymatic methods.[22] The short oligonucleotide containing a 3′-*S*-phosphorodithioate internucleotide bond in a scissile position was successfully used in studies on the mechanism of action of *Tetrahymena* ribozyme.[22b] An alternative approach for the preparation of dinucleoside 3′-*S*-phosphorodithioate, involving the reaction of 2,3′-anhydrothymidine with *O*,*O*-disubstituted

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† Electronic supplementary information (ESI) available: ^{1}H NMR, ^{31}P NMR, ESI MS, MALDI TOF MS and FAB MS data for **3a–d**, **5**, **6**, **8** and **9**. See DOI: 10.1039/b901791g

phosphorodithioic acid was limited to the synthesis of unnatural 3′-3′-dithymidine analogue.[23]

Here we present our results on the synthesis of di- and oligonucleotides containing the hitherto unknown 5′-*S*-phosphorodithioate internucleotide linkage, possessing one of the two sulfur atoms in a 5′-bridging position. A preliminary communication on this work was published elsewhere.[24]

Results and discussion

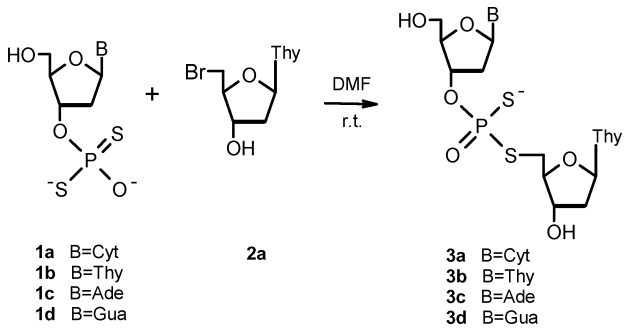
The synthesis and properties of dinucleoside 5′-*S*-phosphorodithioates (3a–d)

Our synthetic strategy was based upon the availability of nucleoside 3′-*O*-phosphorodithioates (**1a–d**) which can be readily prepared by the dithiaphospholane approach.[14b] We assumed that these compounds can be efficiently and selectively *S*-alkylated by 5′-halogeno-5′-deoxy-nucleosides (**2**) at one of the sulfur atoms to yield compounds with a novel 5′-*S*-phosphorodithioate internucleotide linkage, possessing one of the sulfur atoms at a 5′ position (**3a–d**). Our assumption was based on the already known efficient *S*-alkylation of *O*,*O*-dialkylphosphorodithioate anions by alkyl halides.[25] Due to implementation of the HSAB "soft-soft" rule,[26] the alkylation of ambident nucleoside 3′-*O*-phosphorodithioate anions should proceed exclusively at sulfur. Such an assumption was supported by the observation of selective *S*-alkylation of ambident anions of nucleoside 3′-*O*-phosphorothioates by 5′-halogeno-5′-deoxy-nucleosides to yield a 5′-*S*-phosphoromonothioate internucleotide linkage,[27] which was further exploited in a clever so-called chemical ligation of oligonucleotides.[28]

To verify the aforementioned assumption, several attempts of alkylation of deoxycytidine 3′-O-phosphorodithioate (**1a**) with 5′-bromo-5′-deoxythymidine (**2a**)[29] were undertaken in solvents such as ethanol-water (1:1, v/v), pyridine or dimethylformamide (DMF).

Reactions were performed at room temperature on 0.1 mmole scale, and their progress was monitored by ^{31}P NMR. It was found that only in the case of the reaction performed in DMF as the solvent, after 16 hours incubation of substrates, complete conversion of the substrate occurs with the formation of a new product showing in the ^{31}P NMR spectrum two resonance signals at 72.83 and 73.48 ppm (see Table 1).

The observed chemical shift values were found to be similar to those reported by Cosstick and Vyle for diastereomers of the corresponding 3′-*S*-dithymidine analogue (68.7 and 72.4 ppm)[22a], and by Taktakishvili and Caruthers for 5′-*O*-(4,4′-dimethoxytrityl)thymidine-3′-*O*-[*S*-(2,4-dichlorobenzyl)]phosphorodithioate (72 ppm).[30] On this basis we have concluded that the product of alkylation has the desired structure **3a** (see Scheme 1). The modified dinucleotide **3a** was then isolated in 56% yield by ion exchange chromatography on DEAE Sephadex A25 and its elemental composition was confirmed by FAB MS showing the expected M-H ion (*m*/*z* 562.1). The presence in the ^{31}P NMR spectrum of two closely located signals was tentatively assigned to the existence of two diastereomers of **3a** by virtue of a new asymmetric centre at the internucleotide phosphorus atom. The isomer with the lower chemical shift was formed in slight excess (54%) over its downfield counterpart (46%). The presence of two diastereomers was further confirmed by RP HPLC showing two peaks in a 54:46 ratio, with the predominant isomer being slightly less mobile (t_R 16.04 min, SLOW isomer) than the minor one (t_R 15.78 min, FAST isomer). The diastereomeric relationship of this two component mixture was proved by FAB MS after their separation by semi-preparative RP HPLC. Both separated compounds gave almost identical mass spectra with expected M-H molecular ions (*m*/*z* 562.1).



Scheme 1 The synthesis of dinucleoside 5′-S-phosphorodithioates **3a–d**.

This positive result of the reaction of deoxycytidine 3′-*O*-phosphorodithioate (**1a**) with 5′-bromo-5′-deoxy-thymidine (**2a**) prompted us to use this halogen-nucleoside for alkylation of other deoxyribonucleoside 3′-*O*-phosphorodithioates—derivatives of thymine (**1b**), adenine (**1c**) and guanine (**1d**) (see Scheme 1). The reactions leading to the formation of dinucleotides with a novel 5′-*S*-phosphorodithioate linkage were run on 0.1 mmole scale, at room temperature in DMF solution. After 16 hours incubation, ^{31}P NMR inspection showed full disappearance of the corresponding substrates, and the products were isolated by ion exchange chromatography in 44.9–69.8% yield. The physicochemical characteristics of purified products are listed in Table 1. All dinucleoside 5′-*S*-phosphorodithioates were isolated

Table 1 Yields and physicochemical characteristics of dinucleoside 5′-S-phosphorodithioates **3a–d**

Dinucleotide	Isolated yield	λ_{max} (H_2O) [nm]	RP HPLC t_R [min]	δ ^{31}P NMR (D_2O) [ppm][a]	Molecular weight	
					Calculated[b]	Measured[c]
3a	56.4%	267.5	15.78 (FAST) 16.04 (SLOW)	73.48 (46%) 72.83 (54%)	563.54 Da	562.1[d] 562.1[d]
3b	69.8%	266.4	18.49 (FAST) 18.83 (SLOW)	73.36 (48%) 72.59 (52%)	578.55 Da	577.2 [d] 577.3[d]
3c	48.2%	261.0	21.06	73.81 (44%) 72.81 (56%)	587.57 Da	586.0[e]
3d	44.9%	256.0	18.76	73.62 (49%) 72.65 (51%)	603.57 Da	602.0[e]

[a] The percentage of particular diastereomers is given in brackets. [b] Protonated form. [c] FAB MS (m/z, M-H ions). [d] Diastereomers separated by RP HPLC. [e] Mixture of diastereomers.

as a mixture of two isomers which was evidenced by the presence within ^{31}P NMR spectrum of two resonance signals with similar chemical shifts in the range of 72.6–73.8 ppm. In all cases the prevailing isomer (51–56%) had lower chemical shift. The chemical composition of dinucleotides was confirmed by mass spectrometry, and their purity was evidenced by analytical liquid chromatography (RP HPLC). In the case of the dithymidine derivative (**3b**), the observed two isomers with slightly different chemical shifts were also separated by semi preparative RP HPLC showing almost identical FAB MS spectra on which very similar M–H molecular ions were identified (*m/z* 577.2 and 577.3). This observation further supported our assumption that alkylation of deoxyribonucleoside 3′-*O*-phosphorodithioates (**1a–d**) by 5′-bromo-5′-deoxythymidine in DMF solution proceeds exclusively at the sulfur atom and leads to diastereomeric mixtures of dinucleoside 5′-*S*-phosphorodithioates (**3a–d**) with opposite configuration at phosphorus. In the case of compounds **3a** and **3b**, the diastereomers could be separated by preparative RP HPLC; however, for dinucleotides containing one purine base (**3c** and **3d**), no chromatographic separation was feasible under employed conditions.

In order to determine the stability of the 5′-S-phosphorodithioate internucleotide linkage towards the activity of nucleolytic enzymes, modified dinucleotides **3a** and **3b** (mixtures of isomers) were incubated with snake venom phosphodiesterase (svPDE) and nuclease P1 (nP1) under conditions typically used for digestion of DNA. It was found that in buffers recommended by producers, the incubation of either dinucleotide with the enzymes taken in concentration from 1 to 10 μg/ml did not lead to any hydrolytic degradation of substrates during 16 hours at 37 °C, as determined by RP HPLC. Similarly, no change of diastereomeric composition was observed on incubation with either enzyme. In separate experiments it was evidenced that unmodified dinucleotides d(C_PT) and T_PT were completely hydrolyzed by both svPDE and nP1 under aforementioned conditions after one hour of incubation (not shown). The observed resistance of 5′-*S*-phosphorodithioate internucleotide linkage to degradation by nucleolytic enzymes is similar to that described for oligonucleotide phosphorodithioates with both non-bridging oxygen atoms replaced by sulfur.[31] It should be noted, however, that in the case of dithymidine 3′-*S*-phosphorodithioates, containing a bridging sulfur atom in the 3′-position, one isomer (assigned tentatively as S_P) was digested with svPDE while the opposite one (presumably R_P) was very slowly hydrolyzed with nP1.[22a]

The observed stability of dinucleoside 5′-S-phosphorodithioates (**3a** and **3b**) towards svPDE and nP1, which are regarded to be 5′-exonucleases, prompted us to digest those analogues with calf spleen phosphodiesterase (spleen PDE), being typical 3′-exonuclease, *i.e.* degrading DNA and RNA to nucleoside 3′-*O*-phosphates. Since in preliminary attempts it was shown that calf spleen PDE exhibits hydrolytic activity towards **3b** taken as a mixture of diastereomers, in order to draw some conclusions on the stereochemistry of degradation, for further experiments HPLC separated isomers of **3b** (FAST and SLOW) were employed. The digestions were performed under identical conditions in citrate buffer (pH 6.0) at 37 °C and show that both isomers are degraded, however the hydrolysis of SLOW isomer of **3b** proceeds in a much higher rate than that of the FAST one (see Fig. 1).

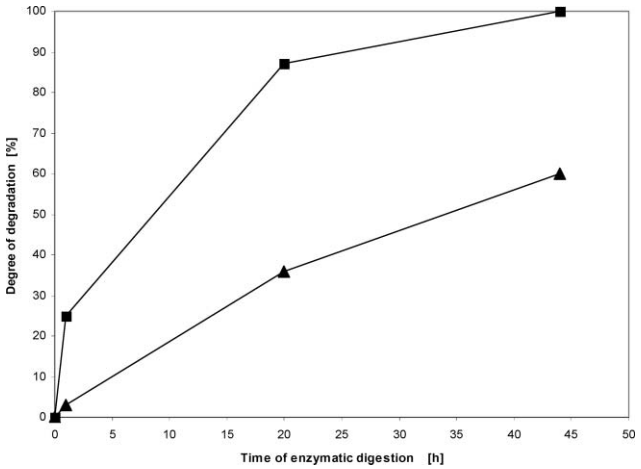
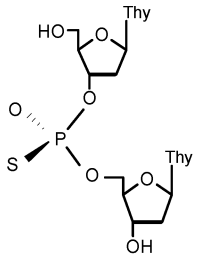


Fig. 1 The time course of digestion of diastereomers of $T_{P(S)S}T$ (**3b**) by calf spleen phosphodiesterase. Isomers of **3b** (1 OD_{260}) were separately incubated with 0.1 unit of enzyme in 200 μl of citrate buffer (pH 6.0) at 37 °C. Aliquots of 20 μl were removed, denatured and analysed by RP HPLC. Isomer FAST (▲), isomer SLOW (■).

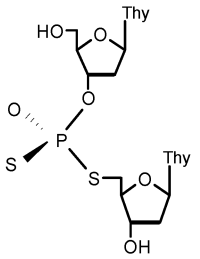
These results were related to those described by Spitzer and Eckstein on digestion of oligodeoxyribonucleotides containing one stereodefined phosphorothioate linkage [R_P or S_P d($A_{P(S)}A$)] by spleen PDE.[32] The authors found that only the linkage of S_P configuration was cleaved by this enzyme.[32] Although in our case the stereodifferentiation between spleen PDE digestion of diastereomers of **3b** was not so dramatic, it prompted us to draw conclusions about the absolute configuration of the dinucleoside 5′-*S*-phosphorodithioate linkage. For such an assignment one can also employ the empirical rules established by Eckstein, linking relative ^{31}P NMR chemical shifts and RP HPLC retention times of diastereomeric dinucleoside phosphorothioates with their absolute configuration.[33] Assuming that these enzymatic and physicochemical criteria may apply for structurally very similar FAST and SLOW diastereomers of $T_{P(S)S}T$ (**3b**), we were able to assign absolute configuration for these isomers as depicted on Fig. 2.

Thus, on the aforementioned basis, S_P configuration has been assigned to the upfield (SLOW) isomer of **3b**, and we suppose that this assignment can also be extended to upfield isomers of **3a**, **3c** and **3d**.

For further confirmation of the structure of dinucleoside 5′-*S*-phosphorodithioates, their chemical cleavage was attempted. It has been known from the work of Cosstick and Vyle that dithymidine-3′-*S*-phosphorothiolate ($T_{SP}T$), containing a single sulfur atom in a 3′-bridging position, was readily cleaved at the phosphorothiolate bond under the action of iodine/acetone/water, iodine/pyridine/water or aqueous silver nitrate, with the formation of thymidine-5′-*O*-phosphate and 3′-mercapto-3′-deoxytymidine (or its derivative).[22a] The application of conditions used by Cosstick and Vyle for dithymidyne-5′-*S*-phosphorodithioate (**3b**, mixture of isomers) resulted in a full degradation of this dinucleotide analogue in iodine/acetone/water at 50 °C or iodine/pyridine/water at room temp (HPLC control). Further studies with ^{31}P NMR and FAB MS control have shown that the degradation of **3b** with aqueous iodine proceeds stepwise. In the first step **3b** reacts with aqueous iodine by exchange of



S_P-$T_{P(S)}T$
(SLOW)



S_P-$T_{P(S)S}T$
(**3b**-SLOW)

Relative position in ^{31}P NMR	Upfield	Upfield
RP HPLC retention time	Longer (SLOW)	Longer (SLOW)
Enzymatic digestion with spleen PDE	Only S_P isomer of d[$A_{P(S)}$A] was found to be digested	Upfield (SLOW) isomer of **3b** was found to be digested at much higher rate than its downfield (FAST) counterpart

Fig. 2 Assignment of absolute configuration to the diastereomers of $T_{P(S)S}T$. Relative ^{31}P NMR chemical shifts, RP HPLC retention times and susceptibility to enzymatic digestion with spleen PDE for S_P-$T_{P(S)}T$ and S_P-$T_{P(S)S}T$ compared to their respective epimers.

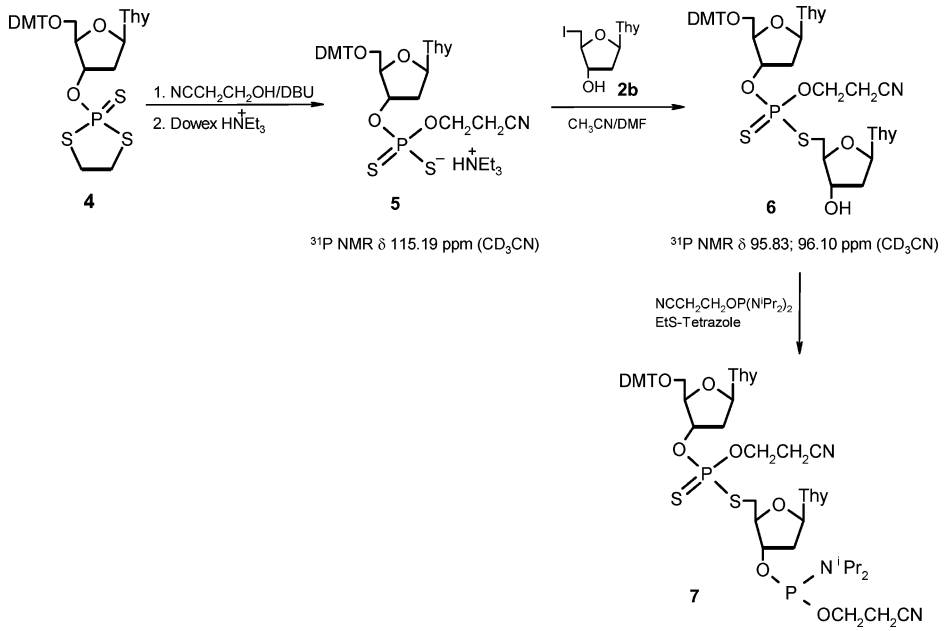
a non-bridging sulfur into oxygen and formation of intermediate dithymidine-5′-*S*-phosphorothiolate ($T_{PS}T$). Thus, partially degraded **3b** in iodine/pyridine/water was found to be a mixture of the aforementioned dithymidine-5′-*S*-phosphorothiolate ($T_{PS}T$; δ 19.58 ppm; M-H, *m*/*z* 560.9, calcd MW 562.50 Da), and thymidine-3′-*O*-phosphate (T_P; δ 0.18 ppm; M-H, *m*/*z* 321.1, calcd MW 322.21 Da). The dithymidine-3′-*S*-phosphorothiolate ($T_{SP}T$), isomeric with respect to the compound $T_{PS}T$ by having a single sulfur atom in a 3′-bridging position was described to have the chemical shift δ ^{31}P NMR 17.4 ppm (D_2O).[22a] The presence in FAB MS of a peak at *m*/*z* 516.0 could also indicate the presence of di(5′-deoxythymidyl) disulfide (calcd MW 514.58 Da), which may result from air-oxidation of 5′-mercapto-5′-deoxythymidine, a possible final product of degradation of **3b**. The aforementioned suggestion regarding oxidative transformation $T_{P(S)S}T \rightarrow T_{PS}T$ was strongly supported by the fact that oxidation of phosphorothioate diesters by aqueous iodine into phosphates was well documented in the literature.[34] In the case of reaction of **3b** with iodine/acetone/water at 50 °C only the presence of final phosphate product was observed (T_P; δ –0.2 ppm; M–H, *m*/*z* 321.0, calcd MW 322.21 Da). No degradation of **3b** by aqueous silver nitrate was observed under conditions used by Cosstick and Vyle.[22a]

The synthesis of oligothymidylates containing 5′-deoxy-5′-S-phosphorodithioate internucleotide linkages

The successful preparation of dinucleoside phosphorodithioates **3a–d** with one of the two sulfur atoms in a 5′-bridging position prompted us to synthesize oligonucleotides containing at least one 5′-*S* phosphorodithioate linkage in a preselected position. For this purpose a dimer block approach was employed,[35] involving the synthesis of a fully protected dinucleotide containing the 5′-*S*-phosphorodithioate modification, and its 3′-*O*-phosphoramidite derivative allowing introduction of such a segment in a preselected position of the oligodeoxyribonucleotide growing chain during its synthesis by the phosphoramidite approach (see Scheme 2).

Thus, the 3′-*O*-dithiaphospholane derivative of 5′-*O*-(4,4′-dimethoxytrityl)thymidine (**4**), prepared as described earlier,[11d] was reacted with an excess of 3-hydroxypropionitrile in the presence of DBU to give quantitatively nucleoside-3′-*O*-phosphorodithioate-*O*-(2-cyanoethyl)diester[14b] which was then transformed into its Et_3NH^+ salt (**5**) by ion exchange and purified by flash column chromatography. The 2-cyanoethyl diester **5** was S-alkylated with 5′-iodo-5′-deoxythymidine (**2b**)[36] in DMF/CH_3CN solution. It has to be mentioned that earlier attempts to alkylate $DBUH^+$ salt of **5** were unsuccessful (not reported). Similarly, the application of 5′-bromo-5′-deoxythymidine (**2a**) for alkylation of diester phosphorodithioate **5** was found to be less efficient than employing iodonucleoside **2b** (not shown). The resulting 5′-*O*-protected-(O3′→S5′)dithymidine-*O*-(2-cyanoethyl) phosphorodithioate (**6**) was isolated by flash column chromatography as an equimolar mixture of two diastereomers which showed two different resonance lines in a ^{31}P NMR spectrum (δ = 95.83 and 96.10 ppm). That mixture could not be chromatographically separated. Compound **6** was *in situ* phosphitylated with *N*,*N*,*N*′,*N*′-bis-(diisopropylamino)-*O*-2-cyanoethylphosphordiamidite in the presence of EtS-tetrazole[35] to give 3′-*O*-phosphoramidite derivative (**7**) which was directly used as a mixture of diastereomers for automated oligonucleotide synthesis.

The application of *dimer block* **7** for introduction of dithymidyl-5′-*S*-phosphorodithioate residue into the selected position of oligodeoxyribonucleotide was demonstrated by the synthesis of two oligothymidylates possessing the modified dinucleotide in the middle of the chain: tetramer **8** (5′-$T_PT_{P(S)S}T_PT$) and decamer



Scheme 2 The synthesis of protected dithymidyl-5′-*S*-phosphorodithioate-3′-*O*-phosphoramidite **7**.

9 (5′-$T_PT_PT_PT_PT_{P(S)S}T_PT_PT_PT_PT$). The syntheses were performed on 1 μmole scale starting with thymidine bound to a LCA CPG support (ABI column) using an automated DNA Synthesizer ABI 394. The protocol of the synthesis was typical for a phosphoramidite method with the time of coupling step of modified phosphoramidite dimer prolonged to 10 min in order to improve the coupling yield. After final detritylation and standard ammoniacal cleavage from the support, the products were isolated by RP HPLC. The structures of **8** and **9** were confirmed by MALDI TOF MS. The application of *dimer block* **7** as a mixture of diastereomers implied that diastereomerism should also be observed for the resulting oligonucleotides **8** and **9**. In fact, only decamer **9** appeared by RP HPLC in the form of two very close, overlapping peaks, most probably related to the presence of diastereomers. No indication of diastereomerism could be seen for tetramer **8**. The amounts of obtained oligonucleotides **8** and **9** were too small for running their ^{31}P NMR spectra in order to confirm the presence of diastereomers. The purity of decamer **9** was additionally checked, after its enzymatic 5′-^{32}P-radiolabeling, by electrophoresis in a 20% polyacrylamide gel under denaturing conditions, with unmodified decathymidylate as a standard (see Fig. 3). It was shown that the presence of one 5′-*S*-phosphorodithioate modification does not influence the electrophoretic mobility of decamer.

In order to check how the presence of 5′-*S*-phosphorodithioate modification influences the oligonucleotide avidity towards its complementary sequence, standard studies of the stability of the duplex formed by **9** with a complementary unmodified matrix d[$(A_P)_{11}$A] were performed. It was found that the melting point of duplex formed by d[$(A_P)_{11}$A] with **9** was considerably lower (Tm = 22.1 °C) than that formed with unmodified decathymidylate under identical conditions (Tm = 27.3 °C). This result clearly demonstrates that the presence of even one 5′-*S*-phosphorodithioate linkage in oligonucleotide strongly destabilizes its duplex with a complementary dodecaadenylate. It has to be mentioned that considerable destabilization was also reported for duplexes formed by oligonucleotides containing P-achiral phosphorodithioate linkages (without bridging sulfur), with their complementary matrices (lowering of Tm by 0.5–14 °C *per* one modified bond).[31]

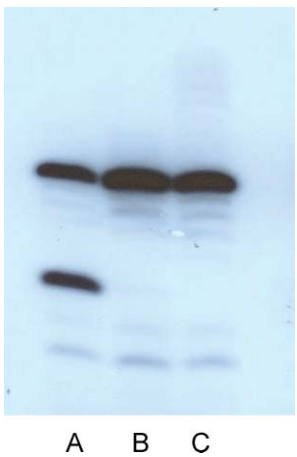


Fig. 3 Electrophoresis of decathymidylate **9** containing one 5′-*S*-phosphorodithioate linkage. Lane A—crude decamer **9**; Lane B—HPLC purified decamer **9**; Lane C—unmodified decathymidylate $(\mathbf{T_F})_9$T.

Conclusions

Easy access to deoxyribonucleoside 3′-*O*-phosphorodithioates *via* the dithiaphospholane approach and known chemoselective S-alkylation of phosphorodithioate ambident ion prompted us to investigate the synthesis of dideoxyribonucleoside (O3′→S5′)-phosphorodithioates and their incorporation into oligodeoxyribonucleotides. The presence of a P-S bond in the bridging position attracted our attention since a single "thiolate" P-S bond in

dinucleoside phosphorothioates can be readily cleaved under the action of iodine/solvent/water[22a] or aqueous silver nitrate reagents.[22a] Indeed, we were able to prove that dinucleoside-5′-*S*-phosphorodithioates undergo chemical cleavage of internucleotide bond with iodine under aqueous conditions. Such opportunity was interesting from the perspective of their possible use in a novel approach to nucleic acids sequencing.[37] Moreover, it has been found that internucleotide (O3′→S5′)-phosphorodithioate linkage is resistant towards phosphodiesterases svPDE and nP1, and rather slowly reacts with calf spleen PDE, which indicates that oligonucleotide congeners can be protected in this way against exo- and endonucleases. On the other hand, the aforementioned modification dramatically influences the avidity of these modified oligonucleotides towards complementary oligodeoxyribonucleotides, and that the strong decrease of *T*m disfavours practical applications of such modification eg. in antisense strategy. However, model compounds as described in this work can be used for mapping of the contact points of oligonucleotides with metal-dependent nucleases and evaluation of the role of 5′-oxygen in the architecture of active site such nucleases and in the studies on metal-ions interactions with various congeners of nucleotides.[38]

Experimental

General procedures

TLC was performed on Kieselgel $60F_{254}$ (Merck) with UV (254 nm) detection. Flash chromatography was run on silica gel 230–400 mesh (Merck). Ion exchange chromatography was performed on a column filled with DEAE Sephadex A25 using a peristaltic pump, gradient mixer, UV detector (260 nm) and fraction collector SuperRac (all from LKB Pharmacia). Reverse Phase High Performance Liquid Chromatography (RP HPLC) was made with a Gilson chromatograph (model 306) using an analytical column 4.6 × 250 mm packed with ODS Hypersil, 5 μ (Alltech) or semi-preparative PRP-1 column 7 × 305 mm (RP-18, 10 μ, Hamilton). The columns were eluted with 0.1M aqueous triethylammonium bicarbonate (TEAB), pH 7.5, supplemented by a linear gradient of acetonitrile from 0% to 30% in 25 min. ^{31}P NMR spectra were recorded on a Bruker AC 200 spectrometer (200.113 MHz for ^{1}H) and referenced to 85% H_3PO_4 used as an external standard. Mass spectra were recorded on Finnigan MAT 95 (FAB) or on Voyager-Elite spectrometers (MALDI TOF). Oligonucleotide syntheses were performed with an automatic ABI 394 DNA Synthesizer on 1 μmole scale.

Materials

Unless otherwise noted, reagents, solvents and materials were obtained from commercial sources. Snake venom phosphodiesterase (svPDE, EC 3.1.16.1) was purchased from Boehringer Mannheim, whereas nuclease P1 (nP1, EC 3.1.30.1) and calf spleen phosphodiesterase (spleen PDE, EC 3.1.16.1) were from Sigma. Nucleoside-3′-O-phosphorodithioates were synthesized exactly as described earlier.[14b] 5′-Bromo-5′-deoxythymidine[29] and 5′-iodo-5′-deoxythymidine[36] were prepared according to literature reports. The unmodified oligonucleotides d[$(A_P)_{11}$A] and T_9T were synthesized by a standard phosphoramidite method.

The S-alkylation of nucleoside-3′-*O*-phosphorodithioates (1a–d) with 5′-bromo-5′-deoxythymidine (2a)

A mixture of one of the nucleoside-3′-*O*-phosphorodithioates (Et_3NH^+ salt, 0.1 mmole) and 5′-bromo-5′-deoxythymidine[29] (0.1 mmole) was dried overnight on a vacuum line and dissolved in dry DMF (2 mL). The solution was stirred magnetically at room temperature until ^{31}P NMR control showed full conversion of substrates (16 h) and the solvent was removed *in vacuo.* The residue was dissolved in water (5 mL) and chromatographed on DEAE Sephadex A25 column eluted with a linear gradient of freshly prepared aqueous TEAB solutions (from 0.05 to 0.5M).

The products **3a–d** were obtained as white powders after evaporation of appropriate fractions. Their yields and spectral as well as chromatographic characteristics are given in Table 1.

5′-*O*-DMT-thymidine-3′-*O*-[-*O*-(2-cyanoethyl) phosphorodithioate] (5)

Into a solution of 600 mg (0.86 mmole) of 5′-*O*-DMT-thymidine-3′-*O*-(2-thio-1,3,2-ditiaphospholane) (**4**)[11d] in 3 mL of CH_3CN (Baker) was added dropwise, with stirring at room temperature, 305 mg (4.3 mmole) of 3-hydroxypropionitrile (Aldrich) followed by 130.5 mg (0.9 mmole) of DBU. After 30 min the solvent was evaporated and the residue was dissolved in 50% aqueous methanol (20 mL). The resulting solution was passed through a column (1.5 × 10 cm) loaded with Dowex 50Wx8 ion exchanger [Et_3NH^+ form, prepared by neutralization of commercial H^+ form (Fluka) with aqueous Et_3N]. After washing of the column with 50% methanol (100 mL) the crude product **5** was obtained by evaporation and was then purified by flash column chromatography with a gradient of methanol in chloroform (from 0 to 20%) as an eluent. The pure diester **5** was obtained after evaporation and drying on a vacuum line as white powder (500 mg, 82.1%). ^{31}P NMR (CD_3CN): δ = 115.19 ppm. TLC (chloroform:methanol, 8:2, v/v): R_f= 0.65.

The S-alkylation of diester 5 with 5′-iodo-5′-deoxythymidine (2b)

The diester **5** (200 mg. 0.28 mmole) was dried overnight on a vacuum line together with 150 mg (0.42 mmole) of 5′-iodo-5′-deoxythymidine (**2b**).[36] The compounds were then dissolved in a mixture of DMF and CH_3CN (1:1, v/v, 4 mL) and the solution was incubated at 50 °C for 36 h. After evaporation, the crude product was purified by flash column chromatography using gradient of methanol in chloroform (from 0 to 6%) as an eluent. The pure dinucleotide **6** was obtained as white powder in 84% yield (220 mg). ^{31}P NMR (CD_3CN): δ = 95.83; 96.10 ppm (*ca* 1:1). FAB MS [M-H]: *m/z* 932.3 (calculated MW 933.97 Da for protonated form). TLC (chloroform:methanol, 9:1, v/v): R_f= 0.55.

The synthesis of modified tetrathymidylate 8

The modified dinucleotide **6** (40 mg, 0.043 mmole) was dried overnight on a vacuum line and dissolved in 100 μL of dry CH_3CN (Baker). Into the resulting solution, 9.5 μL (0.047 mmole) of *N*,*N*,*N*′,*N*′-bis-(diisopropylamino)-O-2-cyanoethylphosphordiamidite was added, followed by dropwise addition of 86 μL of 0.5 M acetonitrile solution of EtS-tetrazole within 15 min. After 1 h stirring at room temp., the resulting

solution of phosphoramidite **7** was reacted with 1 μmole of appropriately protected thymidine bound to LCAA CPG, on an ABI 394 DNA synthesizer under conditions of phosphoramidite synthesis (tetrazole activation, coupling time extended to 10 min). The synthesis was then followed by standard addition of one thymidine phosphoramidite. After detritylation and cleavage from the support with 25% ammonia, the resulting modified tetrathymidylate **8** (5′-$T_PT_{P(S)S}T_PT$) was isolated on a semi-preparative PRP-1 HPLC column (single peak, t_R 17.86 min, 6.8 OD_{260}). Its structure was confirmed by MALDI TOF MS (negative ions): *m/z* 1186 (calculated MW 1186.93 Da for protonated form).

The synthesis of modified decathymidylate 9

The modified dinucleotide **6** (40 mg, 0.043 mmole) was phosphitylated exactly as described in a previous paragraph. The resulting solution of phosphoramidite **7** was reacted with the 5′-OH group of tetrathymidine bound to LCAA CPG, freshly prepared on an ABI 394 DNA Synthesizer (1 μmole scale) under conditions of phosphoramidite synthesis (coupling time of modified dimer extended to 10 min). The synthesis was continued by consecutive addition of four units of thymidine phosphoramidite under standard conditions. After detritylation and cleavage from the support with 25% ammonia, the resulting modified decathymidylate **9** (5′-$T_PT_PT_PT_PT_{P(S)S}T_PT_PT_PT_PT$) was isolated by RP HPLC on a semi-preparative PRP-1 column in the form of two overlapping peaks (t_R 19.88 and 20.01 min). Its structure was confirmed by MALDI TOF MS (negative ions): *m/z* 3012 (calculated MW 3012.07 Da for protonated form).

Enzymatic digestions with svPDE

A sample of dinucleotide **3a** (0.1 OD_{260}) was placed in 100 μL of a buffer containing 100 mM TrisHCl (pH 8.5) and 15 mM $MgCl_2$, and incubated with 0.5 μg of svPDE at 37 °C for 16 h. The solution was then heated at 95 °C for 2 min in order to denature the protein, and centrifuged at 10 000 rpm for 10 min. The extent of digestion was monitored by analytical RP HPLC. The digestion of dinucleotide **3b** with svPDE was performed in an identical manner. In both cases no digestion was observed. The digestion of unmodified dithymidylate (T_PT) performed under identical conditions showed its complete degradation into thymidine and thymidine-5′-*O*-phosphate after 1 h.

Enzymatic digestions with nP1

A sample of dinucleotide **3a** (0.1 OD_{260}) was placed in 100 μL of a buffer containing 100 mM TrisHCl (pH 7.2) and 1 mM $ZnCl_2$, and incubated with 2 μg of nP1 at 37 °C for 16 h. The solution was then heated at 95 °C for 2 min in order to denature the protein, and centrifuged at 10 000 rpm for 10 min. The extent of digestion was monitored by analytical RP HPLC. The digestion of dinucleotide **3b** with nP1 was performed in an identical manner. In both cases no digestion was observed. The digestion of unmodified dithymidylate (T_PT) performed under identical conditions showed its complete degradation into thymidine and thymidine-5′-*O*-phosphate after 1 h.

Enzymatic digestions with calf spleen PDE

A sample of separated FAST or SLOW isomer of dinucleotide **3b** (1 OD_{260}) was placed in 200 μL of 100 mM citrate buffer (pH 6.0) and incubated with 8 μg of spleen PDE (0.1 unit) at 37 °C. Samples of solution were collected after 1, 20 and 44 h, heated at 95 °C for 2 min in order to denature the protein, and centrifuged at 10 000 rpm for 10 min. The extent of digestion was monitored by analytical RP HPLC (see Fig. 1). The digestion of unmodified dithymidylate (T_PT) performed under identical conditions showed its complete degradation into thymidine and thymidine-3′-*O*-phosphate after 1 h.

Chemical degradation of 3b in iodine/solvent/water system

a) Into a solution of **3b** (4.2 OD_{260}, mixture of isomers) in 300 μL of water, 400 μL of 100 mM solution of I_2 in acetone was added. The combined solution was incubated for 1 h at 50 °C, diluted with water (1 mL) and extracted with diethyl ether (5 × 2 mL). A sample of aqueous solution was analysed by RP HPLC to show complete disappearance of peaks of substrate.

b) Into a solution of **3b** (20 mg, mixture of isomers) in 500 μL of water, 500 μL of 200 mM solution of I_2 in acetone was added. The combined solution was incubated for 1 h at 50 °C and evaporated. The residue was extracted with D_2O (500 μL), filtered and analysed by ^{31}P NMR to show complete disappearance of peaks of substrate and the formation of a product at $\delta = -0.2$ ppm.

c) Into a solution of **3b** (4.2 OD_{260}, mixture of isomers) in 300 μL of water, 400 μL of 100 mM solution of I_2 in pyridine was added. The combined solution was incubated for 1 h at 20 °C, diluted with water (1 mL) and extracted with diethyl ether (5 × 2 mL). A sample of aqueous solution was analysed by RP HPLC to show complete disappearance of peaks of substrate.

d) Into a solution of **3b** (20 mg, mixture of isomers) in 500 μL of water, 500 μL of 200 mM solution of I_2 in pyridine was added. The combined solution was incubated for 1 h at 20 °C and evaporated. The residue was extracted with D_2O (500 μL), filtered and analysed by ^{31}P NMR to show complete disappearance of peaks of substrate and the formation of products at $\delta = 19.58$ ppm and 0.18 ppm in *ca.* 45/55 ratio.

When a reaction as above was performed with 50 mM solution of I_2 in pyridine, a presence of *ca.* 60% of undegraded substrate was observed ($\delta = 73.0$ and 73.8 ppm), together with *ca.* 35% of thiolate product at $\delta = 19.6$ ppm and *ca.* 5% of phosphate product at $\delta = 0.2$ ppm.

Thermodynamic stability of duplex formed by 9 with d[$(A_P)_{11}A$]

A solution containing 0.73 μM of **9** and 0.73 μM of d[$(A_P)_{11}A$] was prepared in a buffer of 10 mM TrisHCl (pH 7.4), 100 mM NaCl and 10 mM $MgCl_2$. The *T*m value was calculated on the basis of absorption measurements which were carried out in a 1 cm path-length cell with a UV-VIS 916 spectrophotometer equipped with a Peltier thermocell (GBC, Dandenong, Australia). Melting profile was measured with a temperature gradient of 0.2 °C/min in both directions. The melting temperature (*T*m) was calculated using a first-order derivative method. The *T*m value of duplex formed by d[$(A_P)_{11}A$] with unmodified T_9T was measured in an identical manner.

Acknowledgements

This work was financially assisted by Ministry of Science and Higher Education (Statutory Funds to CMMS PAS, Lodz, Poland). The authors are grateful to Mr M. Deka for the help in RP HPLC analysis.

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