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PHOSPHOROTHIOATE OLIGONUCLEOTIDES: LARGELY REDUCED (N-1)-MER AND PHOSPHODIESTER CONTENT THROUGH THE USE OF DIMERIC PHOSPHORAMIDITE SYNTHONS[a]

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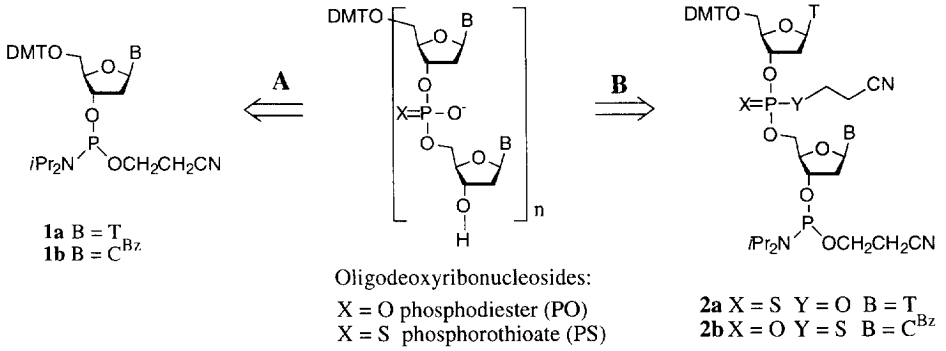
Abstract: Phosphorothioate oligonucleotides synthesized through an assembly of dimeric phosphoramidite synthons on controlled pore glass solid support show a significantly improved impurity profile compared to oligomers synthesized through a coupling of standard monomer phosphoramidites. A greater than 70% reduction of the (n-1)-mer population and a ca 50% reduction of phosphodiester linkages has been achieved.

The enormous potential of modified oligonucleotides for revolutionizing the medical treatment of a variety of diseases through sequence-specific modulation of gene expression has been recognized.[1a] Multiple examples of the first generation of antisense drugs, uniformly modified oligodeoxyribonucleoside phosphorothioates, in which one nonbridging oxygen atom of the internucleotide phosphate group of DNA is replaced by a sulfur atom, are currently in advanced clinical trials.[1b] Recent advances in phosphoramidite coupling chemistry[2] and solid-phase synthesis methodology[3] together with current state-of-the-art large scale synthesizers[4] allow complete synthesis of a phosphorothioate oligonucleotide 20-mer at 0.1 mol scale in 18 h.



Scheme 1. Retrosynthetic analysis of oligodeoxyribonucleosides: (**A**) monomer, (**B**) dimer approach.

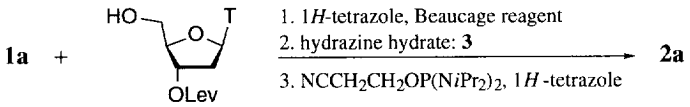
Due to its high efficiency, phosphoramidite coupling[2] followed by stepwise sulfurization of the trialkyl-phosphite linkage is the currently preferred method for phosphorothioate oligonucleotide synthesis, providing +98.5% coupling yields at 1.5-fold molar amidite excess. Problems commonly experienced in automated oligophosphorothioate synthesis via mononucleotide phosphoramidite coupling (Scheme 1, **A**), include formation of a population of shorter deletion sequences ((n-1)-, (n-2)-mers, etc.) within which one or more nucleotides are absent.[5] Several factors, including less than 100% coupling efficiency, incomplete capping or deprotection of hydroxyl groups, reagent quality as well as work-up protocols appear to contribute to the formation of these

[a]Dedicated to Professor Nelson Leonard on the occasion of his 80th birthday.

'shortmers'. While the separation of capped failure sequences and DMT-on full-length oligomer by reversed-phase HPLC is very efficient, the resolution of DMT-on (n-1)-mer and DMT-on (n)-mer is rather poor. The occurrence of phosphodiester (PO) linkages at a low level in the phosphorothioate (PS) oligo-nucleotide product is mainly due to side reactions during the sulfurization step.[6a] Metal ion catalyzed desulfurization of phosphorothioates under the standard deprotection conditions (30% ammonium hydroxide, 55 °C) has also been observed.[6b] The similar chromatographic characteristics of especially DMT-on (n-1)-mers and PO-containing oligomers relative to the full length all-PS (n)-mer (where n is about 20), demand highly selective separation techniques, and these are often associated with a reduced recovery of the desired oligomer due to band overlap.

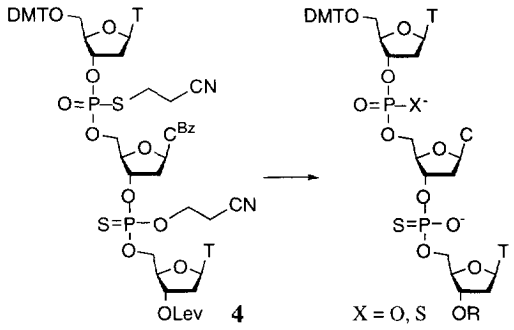
Assuming that coupling and sulfurization inefficiencies are the main causes of (n-1)-mer and phospho-diester linkage formation, a key to reducing both side products could be the use of a blockmer coupling strategy[7] (Scheme 1, **B**). To provide proof of principle, we have investigated this hypothesis by comparing the impurity profiles of model phosphorothioate oligomers T_{19} and $(TdC)_9T$ synthesized on controlled pore glass (CPG) solid support, through monomer and dimer assembly, respectively.[8]

In our study, we used standard amidites **1** as monomeric building units and (*O,O,O*)- and (*O,O,S*)-trialkyl phosphorothioate dimer amidites **2** as dimer building blocks. For protection of the internucleotide linkage in **2a** we chose the *O*-β-cyanoethyl (*O*-CE) group that is selectively removed upon treatment with base (NH_4OH; DBU/CH_2Cl_2 1:19, *v/v*; *t*BuNH_2/pyridine 1:10, *v/v*) through β-elimination without formation of PO linkages.[9] Coupling of 1*H*-tetrazole activated phosphoramidite **1a** with 3′-*O*-levulinyl thymidine,[7f] followed by sulfurization of the resulting phosphite triester with 3*H*-1,2-benzodithiol-3-one-1,1-dioxide[6a] afforded the corresponding protected trialkyl phosphorothioate. Subsequent treatment with hydrazine hydrate in pyridine/glacial acetic acid[7f] followed by phosphitylation with *O*-β-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphordi-amidite afforded dimer phosphoramidite **2a** (Scheme 2, Experimental Procedure).



Scheme 2. Synthesis of a dimeric phosphoramidite building block.

2a allows direct comparison of the impurity profiles of oligomers $(T)_{19}$ and $(\underline{T}_2)_9T$ as the oligonucleotides at the end of the solid-phase synthesis are virtually identical. *S*-CE protected dinucleoside synthons **2b**[10] are readily accessible through phosphotriester chemistry in solution.[11a] In this case, deprotection through β-elimination and hydrolytic deprotection leading to undesired (PO) linkages are competing reaction pathways under aqueous deprotection conditions.[11b] For direct comparison of the deprotection kinetics and chemoselectivity of the decyanoethylation of *O*-CE and *S*-CE protected phosphorothioates, trimer **4** containing both protection schemes in one molecule was synthesized through standard amidite coupling of **2b** and 3′-*O*-levulinyl thymidine in solution. Deprotection of **4** with conc. ammonium hydroxide (60 °C, 10 min, PS/PO = 94:6) or aqueous methylamine (40%, rt, 30 min, PS:PO = 96.5:3.5) yielded a high PO content. A solution of *t*BuNH_2/pyridine (1:10)[11b] effected removal of the *O*-CE group within 15 min whereas complete removal of the *S*-CE group took 3 h at rt without formation of significant amounts of PO linkages (Scheme 3).



reagent	PS/PO ratio[a]
aqu. NH_4OH (30%)	94:6
aqu. $MeNH_2$ (40%)	96.5:3.5
t$BuNH_2$/pyridine	>99.8:0.2

[a] ^{31}P NMR

Scheme 3. Deprotection of (*S*)- and (*O*)-β-cyanoethyl protected phosphorothioates.

Oligomer syntheses using monomer amidites **1** and dimer synthons **2a** or **2b** were performed on a 1 μmol scale on an ABI 394 DNA/RNA Synthesizer.[12] T_{19} and (Tp(*O*-CE)dC^{Bz})$_9$T oligomers were cleaved from the resin and deprotected with 30% NH_4OH at rt. The CPG-bound (Tp(*O*/*S*-CE)dC^{Bz})$_9$T oligomer was first treated with anhydrous *t*$BuNH_2$/pyridine (1:5, *v*/*v*) for 20 h at rt, filtered, rinsed with acetonitrile and then treated with 30% NH_4OH for 1 h at rt, followed by heating of the solution at 60 °C for 2 h. Table 1 summarizes the results of ^{31}P NMR spectroscopy, capillary gel electrophoresis (CGE) and strong ion exchange (SAX) HPLC analyses of several oligomers after DMT removal (80% HOAc). Coupling yields of dimers are approaching the values of monomers eventhough synthesis cycles were not optimized. PS/PO ratios[13] of the DMT-on oligomers were determined by ^{31}P NMR spectroscopy, signal-to-noise ratios were typically greater than 600/1. Consistent with our hypothesis that phosphodiester linkages are formed during the sulfurization on the solid support we observe a reduction of PO linkages of up to 50% (Figure 2). The PO content decreases in the order $(T)_{19}$ > $(\underline{T_2})_5(T)_9 \cong (T)_8(\underline{T_2})_5T$ > $(\underline{T_2})_9T$. SAX HPLC[15b] of the corresponding DMT-off oligomers also shows increasing all-PS and decreasing $(PO)_1(PS)_{17}$ oligomer content. A similar dramatic reduction of phosphodiester linkages was not apparent in oligomers $(TdC)_9T$, $(\underline{TdC})_5(TdC)_4T$, $(TdC)_4(\underline{TdC})_5T$ and $(\underline{TdC})_9T$, possibly due to

Table 1. Analysis of T_{19} and $(TdC)_9T$ phosphorothioates.

oligo	ACE [%][a,b] (per nucl)	n/(n-1)[c,d] [±0.2]	(PO) content [% ±0.1][b,e]	$(PO)_1(PS)_{17}$:$(PS)_{18}$ ratio[b,f]
T_{19}	99.4 (99.4)	97.9:2.1	0.8	20:80
$(TdC)_9T$	98.9 (98.9)	98.0:2.0	0.8	20:80
$T_8(\underline{T_2})_5T$	99.0 (99.3)	99.0:1.0	0.6	12:88
$(TdC)_4(\underline{TdC})_5T$	99.1 (99.3)	99.1:0.9	1.2	20:80
$(\underline{T_2})_5T_9$	98.8 (99.1)	98.8:1.2	0.7	13:87
$(\underline{TdC})_5(TdC)_4T$	99.0 (99.3)	98.8:1.2	0.6	16:84
$(\underline{T_2})_9T$	98.9 (99.5)	>99.3:0.7	0.5	12:88
$(\underline{TdC})_9T$	98.8 (99.4)	>99.4:0.6	1.3	21:79

[a] ACE = (CGE area% of (n)-mer)$^{1/n}$, n = number of couplings (total number of PS linkages), [b] crude oligomer product, [c]ratio of peak areas of CGE, [d] DMT-on HPLC purified oligomer product, [e] ^{31}P NMR, [f] ratio of peak areas of SAX.

a small extent of hydrolysis of the *S*-CE group. Quantitative CGE analysis of the crude oligomer products showed increased full length oligomer content (t_R = 22.6 min) in oligomers synthesized with **2a** or **2b** demonstrating a high coupling efficiency of the dimer synthons. More importantly, the CGE profiles of DMT-on HPLC[15c] purified oligonucleotides show a more than 70% reduced (n-1)/[n+(n-1)] ratio in case of ($\underline{T_2}$)$_9$T and ($\underline{TdC}$)$_9$T (<0.7%)[14] compared to (T)$_{19}$ and (TdC)$_9$T (2.1% and 2.0%), respectively. Two typical CGE traces are shown in Figure 1. These results demonstrate experimentally that most of the (n-1)-mer population is indeed formed during the chain elongation reactions and that only a small portion of it is due to other factors.[5] Oligomers ($\underline{T_2}$)$_5$(T)$_9$ (1.2%) and ($\underline{TdC}$)$_5$(TdC)$_4$T (1.2%) or (T)$_8$($\underline{T_2}$)$_5$T (1.0%) and (TdC)$_4$($\underline{TdC}$)$_5$T (0.9%) have a reduced (n-1)-mer content in proportion to the number of dimer synthons used in the synthesis. No significant preference for the formation of DMT-on (n-1)-mers at either the 3′ or 5′ side of the oligomer is observed for these sequences.[16]

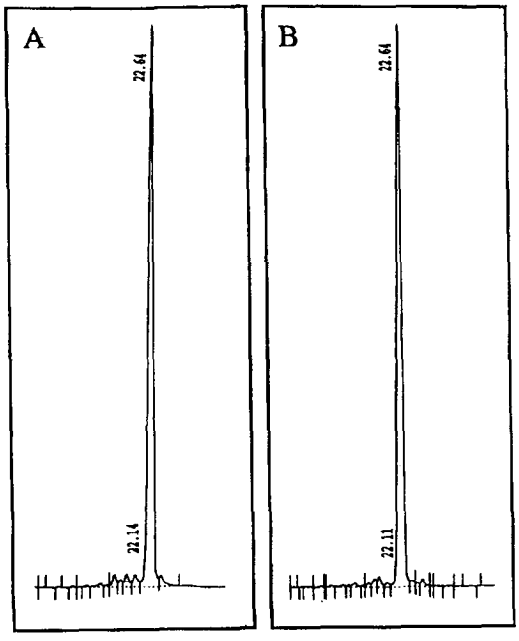


Figure 1. CGE traces of T_{19} (A) and ($\underline{T_2}$)$_9$T (B). Noteworthy is the largely reduced (n-1)-mer content (t_R = 22.1 min). For CGE conditions see ref 15.

In summary, we have shown that the use of dimer building blocks **2a** and **2b** in the solid-phase synthesis of phosphorothioate oligonucleotides leads to largely reduced (n-1) mer content and in case of **2a** also reduces the amount of phosphodiester linkages formed during the sulfurization step by ca 50%. Amidite coupling dependent and, to a much smaller extent, coupling independent factors contribute to the final (n-1)-mer content.

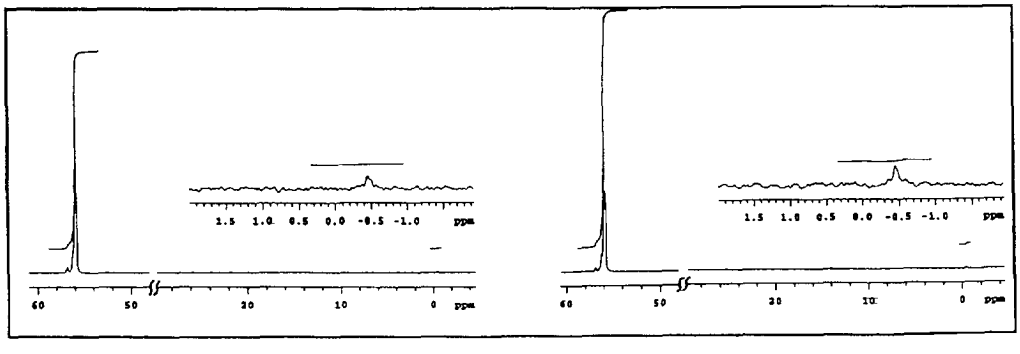


Figure 2. ^{31}P NMR (161.9 MHz, D_2O) spectra: left, T_{19}, phosphodiester content (δ -0.45 ppm) 0.8%; right, ($\underline{T_2}$)$_9$T, phosphodiester content 0.5%, signal-to-noise ratio 700/1.

Experimental Procedure

Phosphoramidite **2a**: Under exclusion of moisture, a mixture of 3′-*O*-levulinyl thymidine (11.0 g, 32.3 mmol), phosphoramidite **1a** (31.3 g, 42 mmol) and 1*H*-tetrazole (11.8 g, 168 mmol) was dissolved in anhydr. CH_3CN (200 mL). After 30 min, the solution was cooled to 0-5 °C (ice-bath). A solution of 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (42.2 g, 211 mmol) in anhydr. CH_3CN (100 mL) was added rapidly. After 15 min, the ice bath was removed and stirring was continued for 30 min at rt. Pyridine (1 mL) was added, and the mixture was filtered. The filtrate was concentrated in vacuo and the oily residue was redissolved in ethyl acetate (400 mL). The solution was extracted three times with $NaHCO_3$ (1 M), dried over Na_2SO_4 and concentrated. 41.0 g of a slightly yellowish solid was obtained. The solid was dissolved in a solution of hydrazine hydrate (7.5 g, 150 mmol) in pyridine (180 mL) and glacial acetic acid (120 mL). After 1 h at rt, the solution was poured onto ice (400 g). The mixture was stirred for 3 h and filtered. The oily residue was washed several times with water until an amorphous solid was obtained, which was dried in vacuo, dissolved in CH_2Cl_2, dried over Na_2SO_4, filtered, and evaporated. Flash chromatography (silica, 20 x 10 cm, gradient elution: 1 to 5% methanol in CH_2Cl_2) afforded the corresponding dimer **3** (24.7 g, 83%). ^{31}P NMR ($CDCl_3$, 81 MHz) δ = 67.2, 67.6 ppm.

A solution of 1*H*-tetrazole (192 mg, 2.7 mmol) and *O*-β-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphorodiamidite (1.81 g, 6.0 mmol) in anhydr. CH_2Cl_2 (20 mL) was added to a solution of **3** (2.76 g, 3.0 mmol) in anhydr. CH_2Cl_2 (10 mL). After 1.5 h, the solution was extracted with $NaHCO_3$ (1 M), dried (Na_2SO_4) and concentrated. The crude product was dissolved in CH_2Cl_2 (30 mL) and hexane (60 mL) was added. A colorless oil separated. After 1 h, the supernatent was decanted. The remaining oil was dissolved in methanol/CH_2Cl_2 (20 mL, 19:1, *v/v*) and filtered through a pad of silica (6 cm). Additional solvent (ca 80 mL) was used to elute **2a** from the silica. The filtrate was concentrated in vacuo, the residue dissolved in CH_2Cl_2 (15 mL) and hexane (40 mL) was added. After 1 h, the supernatent was removed and the remaining oil was rinsed with hexane. After drying at high vacuum **2a** was obtained as a colorless solid (2.9 g, 87%). ^{31}P NMR ($CDCl_3$) δ = 67.5, 67.8, 149.7, 150 ppm.

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8. Nomenclature: T_{19} denotes a nonadecathymidylate phosphorothioate, $(T)_{19}$ and $(TdC)_9T$ indicate monomer assembly, $(\underline{T_2})_9T$, $(\underline{TdC})_9T$ indicate dimer assembly, *TdC* indicates *S*-CE protection.

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12. Columns: dT-CPG (1 μmol) from Glen Research, Sterling,VA. Standard detritylation, capping and 1*H*-tetrazole solutions (Applied Biosystems). Sulfurizing solution was 0.2 M 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (R. I. Chemical, Orange, CA) in anhydr. CH_3CN. Amidites were used as 0.1 M solutions in CH_3CN. Coupling time 200 s, sulfurization time 900 s (not optimized).

13. For a 19mer, a (PS):(PO) ratio of 99.6:0.4 (from ^{31}P NMR) corresponds to an all-PS oligonucleotide content of $(0.996)^{18} = 93\%$.

14. Precise quantitation below 1% is difficult due to lack of baseline resolution. The actual value may be significantly lower.

15. (a) Conditions for CGE: P/ACE System 5000, eCAP ssDNA 100 Gel Capillary (47 cm), tris/borate/7M urea buffer (all Beckman), running voltage 14.1 kV, injection 1 s @ 3 kV, temp 40 °C; (b) conditions for SAX: Waters HPLC 625 LC, 486 Detector (λ = 266 nm), Pharmacia Resource Q column (1 mL), t = 60 °C, 1 M NaCl/100 mM Na_2HPO_4 heptahydrate, pH 11.5 (A), 3 M NaCl/100 mM Na_2HPO_4 heptahydrate, pH 11.5 (B), gradient: 0-5 min: 100% A, 5-40 min: 100% A to 100% B, flow rate 1 mL/min; (c) conditions for RP-HPLC: C_{18} column (Waters Nova Pak) 3.9 x 300 mm, flow rate 0.7 mL/min, CH_3CN (A), triethylammonium acetate (0.125 M), gradient: 0-10 min: 10 to 35% A, 10 to 40 min: 35 to 50% A, $t_R(DMT\text{-}T_{19})$ 22-24 min.

16. Sequencing of the (n-1)-mer population of a 25-mer phosphodiester oligomer has suggested that the formation of (n-1) deletion sequences is more likely at the 3′-terminus.[5a] Electrospray ionization mass spectrometry of phosphorothioate 20- and 24-mer deletion sequences suggested a more uniform distribution of deletions which may be position, base and sequence dependent.[5b]

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