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Solid phase synthesis of oligodeoxyribonucleoside phosphorodithioates from thiophosphoramidites

Kirsten Bjergårde and Otto Dahl*
Department of Chemistry, The HC Ørsted Institute, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark

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ABSTRACT

Oligonucleoside phosphorodithioates 1 are modified DNA sequences with potential use as antisense oligonucleotides. The preparation of up to 20-mers containing all four bases by solid phase synthesis is described, with details on the preparation of the four monomer units (protected nucleoside thiophosphoramidites 2), the conditions used for the assembly of the strands with up to 19 phosphorodithioate linkages, and the purification and characterisation of the products. Full-length homogeneity of HPLC-purified all-phosphorodithioate products is demonstrated by PAGE, but ^{31}P NMR discloses the presence of phosphorothioate impurities (typically 8–9%), the origin of which is discussed. Oligonucleoside phosphorodithioates are freely soluble in water at neutral or basic pH, and are very stable towards oxidation, hydrolysis, and nuclease cleavage. Their ability to hybridise to complementary DNA has been studied by UV melting point (T_m) measurements. The observed depression of T_m, 0.5–2°C per phosphorodithioate linkage, is higher that the 0.4–0.6°C found for phosphorothioates.

INTRODUCTION

Oligodeoxyribonucleotide analogues have been extensively studied in the past decade with the aim to obtain compounds with improved properties for interaction with DNA or RNA under physiological conditions. One major goal is to develop compounds which are effective antisense oligonucleotides.[1, 2] In order to exert an antisense effect *in vivo*, the oligonucleotide analogue must be able to penetrate cell walls, be reasonably resistant to cleavage by nucleases, and be able to hybridize selectively to the target RNA sequence. Other desirable properties are good chemical stability, ease of preparation, and RNase H recognition of the duplex with RNA.

Oligonucleotide analogues modified in the phosphate groups by substitution of one of the nonbridging oxygen atoms with sulphur (phosphorothioates) or a methyl group (methylphosphonates) are currently the best studied and most successful antisense compounds.[1] In contrast to natural DNA, however, these analogues contain chiral phosphorus atoms which normally give rise to diastereomeric mixtures of products that have variable nuclease resistance and form duplexes with RNA of variable stability.

Recently we[3–6] and others[7–24] have examined various methods to prepare di- and oligodeoxyribonucleoside phosphorodithioates **1**. These oligonucleotide analogues are promising antisense oligonucleotides since they are achiral at phosphorus like natural DNA, chemically very stable, and highly resistant to nuclease cleavage. Most reports have been limited to the preparation of deoxynucleoside phosphorodithioate dimers, using phosphorodiamidite,[4, 10, 15, 17] thiophosphoramidite,[3, 8, 10, 14] H-phosphonothioate,[13, 22, 23] H-phosphonodithioate,[9, 16, 20, 21] or phosphorodithioate[5, 24] monomers. The dimers have been further transformed to 3′-*O*-phosphoramidites and used for solid phase synthesis of longer oligonucleotides containing one or several phosphorodithioate groups at alternating positions.[15] Reports on solid phase synthesis of oligonucleotides containing phosphorodithioate groups at all positions are limited to several symposium or conference reports[4,6,7,12,19] and a short communication,[11] without full experimental details.

The present paper describes in detail our studies on the solid phase synthesis of oligodeoxyribonucleoside phosphorodithioates **1** using protected nucleoside *S*-(2-cyanoethyl) *N,N*-dimethylthiophosphoramidites **2** as monomers.

MATERIALS AND METHODS

Acetonitrile (LAB-SCAN C2502), pyridine (LAB-SCAN A3544), and carbon disulphide (Riedel-de Haën 31627) were dried over 4Å molecular sieves (GRACE type 512). Dichloromethane (LAB-SCAN C2510), ethyl acetate (Riedel-de Haën 27227), triethylamine (Fluka 90340), ethyldiisopropylamine (Aldrich D12,580–6), *tert*-butylamine (Fluka 19520), diisopropylamine (Aldrich 11,001–9), and hexane (Riedel-de Haën 15671) were dried by being filtered through basic alumina (ICN Biomedicals Alumina B-Super I) and stored over basic alumina. All solvents had a water content of less than 20 $\mu g\ ml^{-1}$ as determined by Karl Fischer titration (Metrohm 652 KF Coulometer). Elemental sulphur (Aldrich 21,329–2) was dried

* To whom correspondence should be addressed

in vacuo over Sicapent (Merck), and tetrazole (Aldrich 15,569−1) was purified by recrystallization from acetonitrile and dried *in vacuo* over potassium hydroxide pellets. Concentrated aqueous ammonia (32%, Merck 5426), was used as received. Protected deoxyribonucleosides, DMTdA^{bz}, DMTdC^{bz}, DMTdG^{ib}, and DMTdT (Peninsula Laboratories), protected deoxyribonucleoside cyanoethyl diisopropylphosphoramidites (K. J. Ross-Petersen, Hrsholm, Denmark), and derivatized CPG 500 Å support (Milligen, loading ca. 30 μmol g^{-1}) were kept *in vacuo* over potassium hydroxide pellets. Oligonucleoside phosphorodithioates were prepared on an Applied Biosystems 380B DNA synthesizer, using Applied Biosystems columns, standard cycles (Fast Cycle Version 2.00) for the introduction of phosphate linkages, and the cycle given in **Table 2** for the introduction of phosphorodithioate linkages. ^{31}P NMR spectra were obtained on a JEOL FX 90 Q spectrometer at 36.4 MHz in 5 mm tubes; chemical shifts are positive in the low-field direction from the external reference 85% phosphoric acid.

Preparation of the Thiophosphoramidites 2a−d

The 5′-*O*-DMT-*N*-acylnucleoside (4 mmol) was dried by co-evaporation once with dry CH_3CN/CH_2Cl_2 (1:2 v/v, 30 ml) and redissolved in the same solvent mixture (30 ml) under nitrogen. After addition of dry $EtPr^i_2N$ (1.05 ml, 6 mmol) and cooling on ice, $(Me_2N)_2PCl$[25] (0.62 g, 4.0 mmol) was added and the mixture stirred for 10 min at 25°C. Then freshly distilled $HSCH_2CH_2CN$[26] (0.42 g, 4.8 mmol) was added and the stirring continued for 1 h. The clear solution was concentrated on a rotary evaporator (except for **2c** which partly decomposed on concentration), the residue was dissolved in EtOAc (50 ml) (except for **2c**), the solution extracted with saturated, aqueous $NaHCO_3$ (2×25 ml), dried ($MgSO_4$), and the solvent evaporated. The residue was dissolved in dry CH_2Cl_2 (10 ml) and added dropwise to dry, degassed hexane (400 ml) at 0°C with stirring. The precipitate was isolated by filtration, washed once with hexane (20 ml) and finally lyophilized from dry CH_3CN (10 ml) to give the product as a colourless powder. **2a**: Yield 88%, δ_P ($CDCl_3$) 172.0, 171.1, 96% pure according to ^{31}P NMR. **2b**: Yield 89%, δ_P ($CDCl_3$) 172.1, 171.2, 90% pure according to ^{31}P NMR. **2c**: Yield 92%, δ_P ($CDCl_3$) 171.8, 170.0, 83% pure according to ^{31}P NMR. **2d**: Yield 89%, δ_P ($CDCl_3$) 172.6, 170.9, 95% pure according to ^{31}P NMR.

Preparation of Oligonucleoside Phosphorodithioates

Oligonucleotides containing one or a few phosphorodithioate linkages were prepared on the synthesizer using a standard Applied Biosystems cycle (1 μmol scale, DMT on) and normal phosphoramidites up to the point of modification when an interrupt at cycle end was made. The phosphorodithioate cycle was then performed manually with addition of reagents from syringes, and the sequence continued on the synthesizer (the sequence written on the synthesizer was without the modified base(s)). The manual cycle is given in **Table 1** and gave a DMT-efficiency of 94−96%.

Modified oligonucleotides containing many phosphorodithioate linkages were prepared on the synthesizer using the amidite bottles 4−7 for the thiophosphoramidites **2a−d**, bottle 10 for sulphur oxidation solution, and bottle 17 for the pyridine-carbon disulphide wash solution. An abridged version of the automated cycle (1 μmol scale, DMT on) is given in **Table 2** (a printout of the actual machine cycle is available on request; it contains 85 steps and allows for the use of three normal phosphoramidites and four thiophosphoramidites in the same sequence). This cycle gave a thiophosphoramidite DMT-efficiency of 96−98% pr. cycle and a normal phosphoramidite DMT-efficiency (ca. 99%).

The thiophosphoramidite solutions (0.1 M in dry, degassed CH_3CN) were freshly made for each series of syntheses and gave unchanged DMT-efficiencies for 24−36 h. The pyridine-carbon disulphide wash solution (1:1 v/v, degassed) was stable when kept under argon in the presence of molecular sieves, but a crystalline precipitate appeared after a few days on the machine in the absence of sieves. The sulphur oxidation solution (elemental sulphur in pyridine-carbon disulphide-triethylamine 10:10:1 v/v/v, 1.5 M in S, degassed) was freshly made and used within a few days. Bottle 10 containing this solution was manually primed with a pyridine-carbon disulphide wash before and after priming in order to avoid precipitation of sulphur in the lines.

After synthesis the column was opened and the oligonucleotide cleaved from the support and deblocked by treatment with 32% aqueous ammonia (ca. 1 ml) in a tightly stoppered glass at 25°C for 2 h followed by 55°C for 12 h or more. In some experiments the *S*-cyanoethyl groups were removed on the column before the ammonia treatment; this was done with dry *tert*-butylamine/pyridine 1:9 v/v, or neat dry diisopropylamine, at 25°C for 12 h or more.

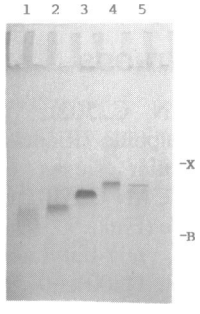


Fig. 1. Denaturing polyacrylamide gel electrophoresis of HPLC purified oligonucleotides, visualised by UV shadowing. Lane 1: 5′-CGC GAA TTC GCG (self complementary sequence, broad band due to a high T_m); lane 2: 5′-CssGssCss GssAssAss TssTssCss GssCssG (ss = phosphorodithioate linkage); lane 3: 5′-CAC CAA CTT CTT CCA CA; lane 4: 5′-CssAssCss CssAssAss CssTssTss CssTssTss CssCssAss CssA; lane 5: 5′-ACA CCC AAT TCT GAA AAT GG; X = xylenecyanol, B = bromophenol blue.

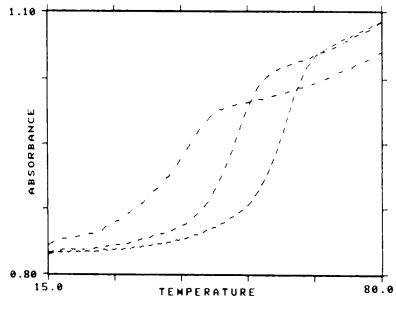


Fig. 2. UV melting curves (medium salt) of - · -: 5′-CAC CAA CTT CTT CCA CA; - · · -: 5′-CsAsCs CsAsAs CsTsTs CsTsTs CsCsAs CsA; - · · · -: 5′-CssAssCss CssAssAss CssTssTss CssTssTss CssCssAss CssA (s = phosphorothioate, ss = phosphorodithioate linkage). All sequences are hybridized to the same DNA sense strand, 5′-TGT GGA AGA AGT TGG TG; the solutions were 3.0 μM in each strand, pH 7.2, and contained 1 mM EDTA, 10 mM Na_2HPO_4, and 0.14 M NaCl.

The 5'-*O*-DMT-oligonucleotides were purified by reverse-phase HPLC (Hamilton PRP-1 column, no. 79425 or 79444), or, for small amounts, on OPC Cartridges (Applied Biosystems 400771) using the procedure given by Applied Biosystems. The eluents for HPLC were: Buffer A, 5% CH_3CN in 0.1 M TEAB, pH 9.0; buffer B, 80% CH_3CN in 0.1 M TEAB, pH 9.0. The gradient was 100% A for 5 min, a linear gradient of 0–100% B for 40 min, and 100% B for 5 min. The product seen as a strong, single peak after 25–30 min was collected, the solvent evaporated, and TEAB removed by co-evaporation twice with water. DMT was removed from the residue, dissolved in water (1.0 ml), by treatment with conc. acetic acid (50 μl) for 30 min, followed by neutralization with conc. aqueous ammonia (oligonucleoside phosphorodithioates are usually poorly soluble in water at pH below 6–7) and extraction with ether (3×0.5 ml). The compounds were isolated from the aqueous phase by lyophilization and kept at −20°C. The yields obtained of purified material were usually 25–35%, calculated from the amount of the first nucleoside bound to the support.

Preparation of Unmodified Oligonucleotides and Oligonucleotide Phosphorothioates

These compounds (seq. 1,2,4, and 5, **Table 3**) were prepared on the Applied Biosystems 380B or a Biosearch 7500 DNA Synthesizer using standard 0.2 or 1 μmol cycles, apart from the oxidation step in case of phosphorothioates, where a sulphurization step was performed before the cap step with benzo-1,2-dithiole-3-one 1,1-dioxide [27] (0.1 M in acetonitrile, 30 s). The products with DMT on were purified on Hamilton PRP-1 columns as described above.

Gel Electrophoresis

PAGE gels were run on a LKB 2001 Vertical Electrophoresis System. The gel was a 20% acrylamide/7 M urea gel of the dimensions 16 cm×16 cm×0.75 mm, and the buffer a standard TBE buffer (90 mM Tris-borate, 2.5 mM Na_2EDTA, pH 8.3). Samples (ca. 1 A_{260} units) were dissolved in 20 μl loading buffer (7M urea/10×TBE buffer 9:1) and heated to 90°C for 5 min just before loading. The gel was run at 400 to 500 V, and the bands visualized by UV shadowing and photographed with a Polaroid CU-5 camera. A representative example is shown in **Fig. 1**.

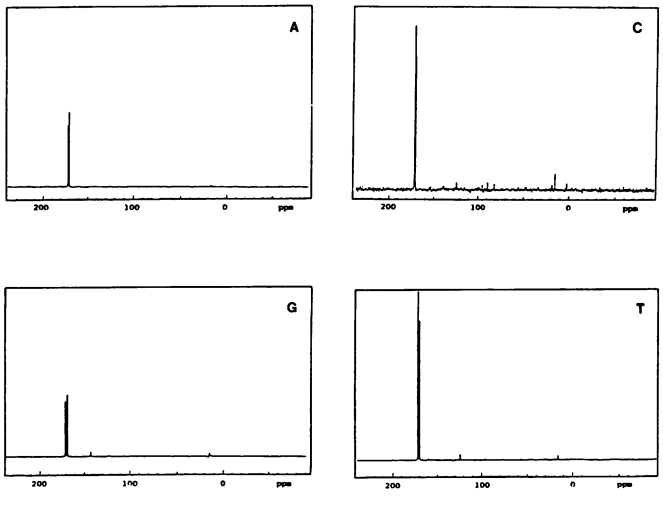


Fig. 3. ^{31}P NMR spectra of protected nucleoside thiophosphoramidites in $CDCl_3$. A = **2a**, C = **2b**, G = **2c**, T = **2d**.

Melting Point Measurements

Melting points, T_m, for an equimolar mixture of the (modified) oligonucleotide and the complementary unmodified oligodeoxyribonucleotide strand were determined on a Gilford Response II UV spectrometer equipped with a Response Thermoset accessory. The absorbance at 260 nm was measured at 0.5°C intervals in 1 cm cuvettes, and T_m determined as the maximum of the first derivative of the melting curve. The solutions were 2.5–3.0 μM in each strand, pH 7.2, and contained 1 mM EDTA, and 10 mM Na_2HPO_4 + 0.14 M NaCl (medium salt), or 10 mM Na_2HPO_4 + 1.00 M NaCl (high salt). Samples were heated to 90°C for 5 min, left at room temperature for 30 min and at 0–5°C for 1 h prior to measurements. Melting point values are given in **Table 3** and some representative melting curves are shown in **Fig. 2**.

RESULTS AND DISCUSSION

When we began our studies on nucleoside phosphorodithioates in february 1988 we decided to use a thiophosphoramidite route to prepare the compounds, since this approach looked the most simple. We prepared first a thymidine *S*-(2-cyanoethyl) *N,N*-diisopropylthiophosphoramidite, but quickly realized that the reactivity of this compound was very much lower than that of the analogous phosphoramidite. We therefore prepared some thioamidites with smaller substituents on nitrogen and found that the *S*-(2-cyanoethyl) *N,N*-dimethylthiophosphoramidite **2d**, or the *S*-(2,4-dichlorobenzyl) analogue **3d**, had a suitable reactivity to couple in a few minutes with 3'-*O*-acetylthymidine and tetrazole as the catalyst.[3,4] Model experiments on dimers showed that a 2-cyanoethyl or a 2,4-dichlorobenzyl protecting group on sulphur could be removed without significant formation of phosphorothioates or cleavage of internucleotide bonds, whereas methyl, benzyl, or 4-chlorobenzyl protecting groups gave unacceptable amounts of internucleotide bond cleavage.[5,28] Since the *S*-(2-cyanoethyl) thiophosphoramidites **2** were easy to prepare and were practically inert to oxidation by air in contrast to the *S*-(2,4-dichlorobenzyl) analogues **3** we selected the thiophosphoramidites **2a–d** for use in solid support syntheses.

Preparation of Protected Nucleoside Thiophosphoramidites

The protected nucleoside thiophosphoramidites **2a–d** were prepared by the method published earlier for **2d**[3] in 88–92% yield after an aqueous sodium hydrogencarbonate wash and precipitation into hexane. The purity was 90–96% according to ^{31}P NMR with the exception of the G derivative **2c** which was only 83% pure (**Fig. 3**). Purification on silica columns was attempted but gave significant losses and did not increase the purity of **2a–d** significantly. The compounds are stable at −20°C under an inert atmosphere for at least 3 months but decompose slowly in acetonitrile at room temperature; solutions (0.1 M) should be used within 24–36 h, after which time a decreased coupling efficiency was observed. Although oxidation by dissolved air is not a serious concern for **2a–d**, degassed solvents were normally used for solid phase syntheses as an extra precaution.

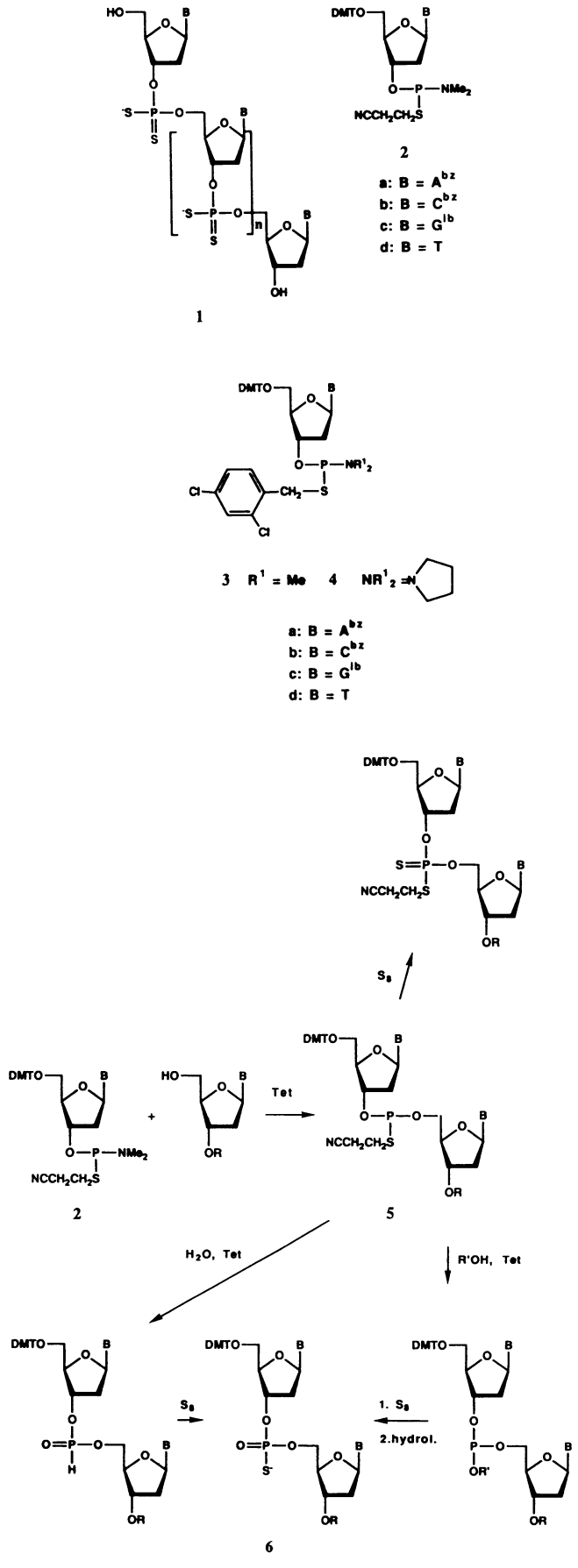
Oligonucleoside Phosphorodithioate Syntheses

Model reactions on the activation of **2d** with tetrazole and subsequent reaction with 3′-*O*-acetylthymidine had shown that the time between mixing of **2d** with tetrazole and addition of 3′-*O*-acetylthymidine should be kept short in order to avoid disproportionation of **2d**, and that the thiophosphite product **5** (**Scheme 1**) should be oxidised with sulphur quickly in order to minimize tetrazole catalyzed reactions of the thiophosphite with nucleophiles.[3] Oligonucleotides containing one or a few phosphorodithioate linkages were prepared using a standard cycle (Applied Biosystems 1 μmol fast cycle, DMT on) and normal amidites, with the thioamidite couplings being performed manually during an interrupt. The manual cycle used is given in **Table 1**. It contains two 1 minute couplings and a 6 minute oxidation with sulphur just after the couplings, with pyridine-carbon disulphide washing steps to avoid precipitation of sulphur in the column. This cycle gave a DMT-efficiency of 94–96%. Oligonucleotides containing phosphorodithioate linkages at many or all positions were prepared using a modified standard cycle (Applied Biosystems 1 μmol fast cycle, DMT on). The cycle was modified to give multiple additions of thioamidite plus tetrazole, to oxidise with sulphur immediately after coupling, to include washing steps with pyridine-carbon disulphide, and to omit oxidation with iodine-water when thioamidites were introduced. The essential steps of the modified cycle is listed in **Table 2** (the full machine cycle is available on request). This cycle gave a thiophosphoramidite DMT-efficiency of 96–98% pr. cycle and a normal phosphoramidite DMT-efficiency (ca. 99%).

The crude oligonucleoside phosphorodithioates (DMT on) were deblocked with conc. aqueous ammonia for 2 h at room temperature (to remove the *S*-(2-cyanoethyl) groups) followed by 12 h at 55°C. The ammonia solutions were concentrated and subjected to ^{31}P NMR analysis, which showed the correct ratio of phosphorodithioate (δ_P ca. 113) to phosphate (δ_P ca. 0) for oligonucleotides containing a few phosphorodithioates (*e.g.* sequence 6–9, **Table 3**). However, ^{31}P NMR analysis of oligonucleotides containing phosphorodithioate groups at many or all positions (*e.g.* sequence 3, 10, **Table 3**) showed repeatedly a phosphorothioate content of 8–9%, with extremes of 4 to 15% being occationally observed. **Fig. 4** is a representative ^{31}P NMR spectrum of a crude product; it shows the absence of significant amounts of phosphates or other impurities apart from phosphorothioates (δ_P ca. 55, 8%). In additon to phosphorothioates, other impurities were sometimes observed as minor peaks in the ^{31}P

Table 1. Manual 1μmol cycle to introduce one phosphorodithioate linkage by solid support synthesis using the thioamidites **2a–d**.

	ml (min)
1. CH_3CN	3×1
2. 20 mmol thioamidite, freshly dissolved in 0.4 M tetrazole in CH_3CN	0.4 (1)
3. Repeat 2.	(1)
4. Pyridine/CS_2 1:1 v/v	2×1
5. 1.5 M S in pyridine/CS_2/Et_3N 10:10:1 v/v/v	1 (6)
6. Pyridine/CS_2 1:1 v/v	3×3
7. CH_3CN	3×3
8. Ac_2O/2,6-lutidine/*N*-methylimidazole/THF 5:5:8:82 v/v/v/v	1 (1)
9. CH_3CN	3×3
10. 3% CCl_3COOH in CH_2C_2	2×1 (1)
11. CH_3CN	3×1



Scheme 1.

NMR spectra of the crude products. These are tentatively assigned to thiophosphortriesters (δ_P ca. 66, < 2%), thiophosphoramidates (δ_P ca. 76, < 1%), H-phosphonates, and phosphates.

The origin of the main impurity, phosphorothioates, is at present unknown, in spite of considerable efforts invested to solve this problem (see below). Caruthers et al. have obtained oligonucleoside phosphorodithioates which contain only 2–3% phosphorothioates,[12, 29] using a cycle very similar to ours, but *S*-(2,4-dichlorobenzyl) *N,N*-dimethyl-**3a–d** or *N,N*-tetramethylenephosphoramidites **4a–d** instead of *S*-(2-cyanoethyl) *N,N*-dimethylphosphoramidites **2a–d**. We have occationally used **3d** or **4d**, prepared according to Caruthers et al.,[11, 12] to prepare homo-T sequences; however, the phosphorothioate content using **3d** or **4d** was in our hands the same, 8–9%, as that obtained from **2d**.

Attempts to Define the Origin of Phosphorothioate Impurities

Phosphorothioate impurities in the crude oligonucleotides are detected by ^{31}P NMR after cleavage from the support and deprotection; the impurities might therefore be created at any step during synthesis or deprotection. The deprotection with 32% aqueous ammonia was originally a concern, since the 2-cyanoethylthio group could be substituted with hydroxide to give a phosphorothioate instead of the intended phosphorodithioate by a β-elimination. Phosphorothioates do form to a degree of 1–2% during deprotection of a TT dimer,[3] but this amount is much less than the 8–9% found in the longer oligonucleotides. Removal of the 2-cyanoethyl group with dry *tert*-butylamine in pyridine[30] or with dry diisopropylamine[31] should reduce the phosphorothioate content considerably (probably to a degree only dependent on the water content) if the deprotection with aqueous ammonia was the origin. However, the same oligonucleoside phosphorodithioate (sequence 3, **Table 3**) deprotected with different reagents to remove the 2-cyanoethyl groups (aqueous ammonia, dry *tert*-butylamine in pyridine, or dry diisopropylamine) gave in all three cases the same result (8–9% phosphorothioate). The 2-cyanoethylthio group is also stable towards capping, detritylation, and oxidation with iodine-water, as shown for a TT-dimer.[3] These results indicate that substitution of a 2-cyanoethylthio group from a protected dithiophosphate linkage is not a significant pathway to the phosphorothioate impurities.

Another pathway to phosphorothioate impurities could be loss

Table 2. Abridged 1μmol cycle to introduce one phosphorodithioate or one phosphate linkage by solid support synthesis on an Applied Biosystems 380B DNA synthesizer. Flow 1.5 ml min^{-1} for amidites and tetrazole, 2.5 ml min^{-1} for other reagents and solvents (measured with CH_3CN in the bottles).

1.	CH_3CN	20
2.	Ar flush	8
3.	0.4 M tetrazole in CH_3CN	3
4.	0.1 M (thio)amidite in CH_3CN + 0.4 M tetrazole in CH_3CN	2
5.	CH_3CN	2
6.	Wait	10
7.	0.4 M tetrazole in CH_3CN	4
8.	0.1 M (thio)amidite in CH_3CN + 0.4 M tetrazole in CH_3CN	4
9.	CH_3CN	1
10.	Wait	60
11.–14.	Repeat 7.–10.	
15.	Ar flush	5
16.	CH_3CN	5
17.	Pyridine/CS_2 1:1 v/v	8*
18.	1.5 M S in pyridine/CS_2/Et_3N 10:10:1 v/v/v	6*
19.	Wait	180*
20.	1.5 M S in pyridine/CS_2/Et_3N 10:10:1 v/v/v	3*
21.	Wait	180*
22.	Pyridine/CS_2 1:1 v/v	30*
23.	CH_3CN	10*
24.	Ar flush	9
25.	Ac_2O/2,6-lutidine/*N*-methylimidazole/THF 5:5:8:82 v/v/v/v	16
26.	Wait	30
27.	CH_3CN	5
28.	Ar flush	9
29.	0.1 M I_2 in H_2O/pyridine/THF 1:2:7 v/v/v	12#
30.	Wait	20#
31.	Ar flush	9#
32.	CH_3CN	10
33.	Ar flush	5
34.–39.	Repeat 32.–33. three times	
40.	3% CCl_3COOH in CH_2Cl_2	10
41.	Ar flush	1
42.–51.	Repeat 40.–41. five times	
52.	Ar flush	10
53.	CH_3CN	20
54.	Ar flush	5

* Thioamidite additions only. # Normal amidite additions only.

Table 3. Melting points T_m of oligodeoxyribonucleoside phosphorodithioates and related oligonucleotides hybridized to their complementary oligodeoxyribonucleotide strand.

	Sequence†	T_m, °C med. salt	T_m, °C high salt #
1.	5′-CAC CAA CTT CTT CCA CA	61	68.5
2.	5′-CsAsCsCsAsAsCsTsTsCsTsTsCsCsAsCsA	51.5	58
3.	5′-CssAssCssCssAssAssCssTssTssCssTssTssCssCssAssCssA	42	46.5
4.	5′-ACA CCC AAT TCT GAA AAT GG	61.5	70.5
5.	5′-AsCsAsCsCsCsAsAsTsTsCsTsGsAsAsAsAsTsGsG	54.5	62.5
6.	5′-ACA CCC AAT TCssT GAA AAT GG	59.5	69
7.	5′-AssCssAss CCC AAT TCT GAA AAT GG	60	69
8.	5′-AssCssA CCC AAT TCT GAA AATss GssG	59.5	66
9.	5′-ACA CCC AATss TssCssTss GAA AAT GG	58	66
10.	5′-AssCssAssCssCssCssAssAssTssTssCssTssGssAssAssAssAssTssGssG	46	54

† Phosphorothioate linkages are abbreviated s, phosphorodithioates ss.
# See Materials and Methods for strand, salt, and buffer concentrations.

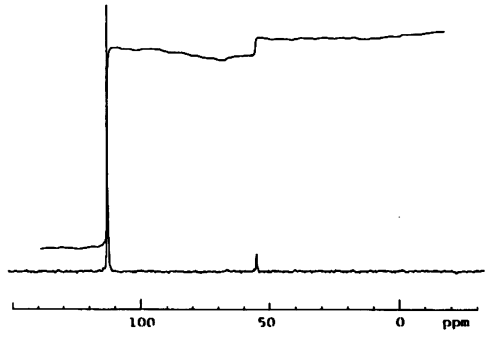


Fig. 4. ^{31}P NMR spectrum of a crude all-phosphorodithioate 17-mer (seq. 3, **Table 3**) in D_2O/aqueous ammonia.

of the 2-cyanoethyl groups during synthesis, followed by exchange of a sulphur atom in an *un*protected phosphorodithioate group with oxygen, *e.g.* during the capping step. Model experiments with a TT dimer however showed that unprotected phosphorodithioate groups neither react with acetic anhydride under capping conditions, nor with the thiophosphoramidite **2d** and tetrazole (^{31}P NMR analysis).

Incomplete oxidation of the intermediate thiophosphite **5** with sulphur, or impurities in the sulphur which can deliver oxygen atoms to the thiophosphite are improbable origins of phosphorothioate impurities. The sulphur used was 99.999% pure, and the reaction time was 2×3 min with 5% triethylamine as a catalyst. Caruthers et al. obtained good products with the same catalyst and a 1 min oxidation period.[12] We have oxidized for 2×3 min without triethylamine and obtained nearly the same coupling efficiencies and amount of phosphorothioate impurities. Without triethylamine a TT dimer thiophosphite reacts with sulphur to give the protected phosphorodithioate in less than 2 min (^{31}P NMR analysis).

Oxidation of the intermediate thiophosphite **5** with dissolved oxygen during the coupling step is another possible route to phosphorothioate impurities. This is unlikely to be a major contributor, since the syntheses are run under argon with degassed solvents. Furthermore, an experiment where air was bobbled through a solution of a thiophosphite dimer (freshly prepared from **2d**, 3′-*O*-acetylthymidine, and tetrazole, 2:1:3 mol/mol/mol in acetonitrile) resulted in some hydrolysis products (due to moisture in the air) but none of the oxidation product, a protected phosphorothioate, was formed (^{31}P NMR analysis).

Hydrolysis of the thiophosphite **5** before oxidation with sulphur is complete would lead to H-phosphonate linkages which with sulphur would be oxidized to phosphorothioate linkages **6** (**Scheme 1**). Model experiments showed that thiophosphite dimers do hydrolyse easily in the presence of tetrazole, but that thiophosphoramidites which are present in large excess during coupling hydrolyse much faster. Hydrolysis of thiophosphites during coupling is therefore improbable although some hydrolysis could occur during the washing steps between coupling and oxidation with sulphur.

The thiophosphite **5** may react with other nucleophiles than water during coupling, e.g. with a hydroxy group from a neighboring strand. This would give rise to thiophosphortriesters after oxidation with sulphur, and phosphorothioates **6** after hydrolysis with aqueous ammonia (**Scheme 1**). Two facts support this hypothesis; thiophosphortriesters are sometimes observed in small amounts in the crude products, and prolonged coupling times (> 2 min) resulted in an increase of impurities.

Phosphorothioate impurities might appear as a result of dismutation of thiophosphoramidites in the presence of tetrazole.[3] When the thiophosphoramidite **2d** in acetonitrile was mixed with tetrazole, a dithiophosphite **7** (δ_P 159) was observed (^{31}P NMR) to form within a few min, and after several min another signal (δ_P 130) appeared as well, probably due to the aminotetrazolide **8** (**Scheme 2**). A similar dismutation occurred for **3d**. The dithiophosphite **7** is unreactive, but the aminotetrazolide **8** is much more reactive under coupling conditions than thiophosphoramidites, and even small amounts would be expected to compete efficiently with **2** and give rise to phosphoramidite linkages **9** in the product (**Scheme 2**). The latter is known to hydrolyse easily and the resulting H-phosphonate linkage would give phosphorothioate linkages **6** upon oxidation with sulphur. Were this pathway to phosphorothioate impurities a major one then premixing of thioamidite and tetrazole, or prolonged coupling times, should give increased amounts of phosphorothioates. Premixing for 4 min, or variations of the coupling time betweeen 10 and 60 sec, however did not change the relative content of phosphorothioate impurities. Only after coupling times longer than about 2 min did we observe increased amounts of impurities.

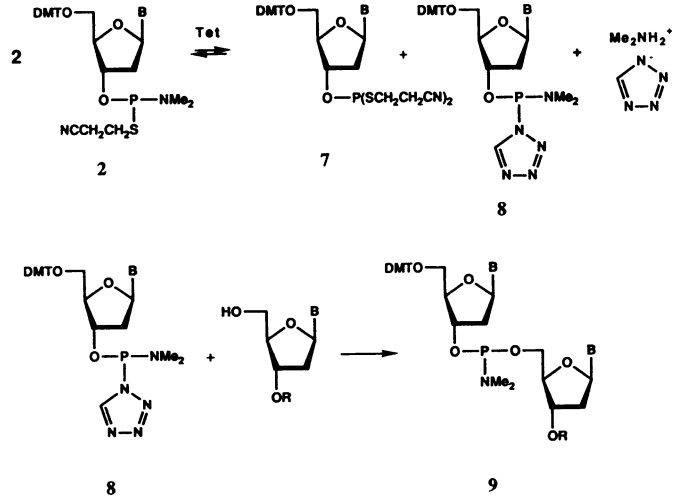
The reactivity of thiophosphites and the dismutation of thioamidites is a consequence of the well known better leaving group ability of RS than of RO at tervalent phosphorus centers[3] and is an inherent weakness of the thiophosphoramidite method to prepare phosphorodithioates. In order to minimise unwanted effects of substitution at the thiophosphites and dismutation of the thiophosphoramidites we deliver tetrazole + thiophosphoramidite in three portions and use a short (10 s) wait for the first delivery; the rather low reactivity of thiophosphoramidites however necessitates a longer wait time (60 s) for the next two deliveries in order to obtain a good coupling efficiency.

Purification of Oligonucleoside Phosphorodithioates

Initial attempts to purify the raw oligonucleoside phosphorodithioates by HPLC on ion exchange (Pharmacia Mono Q, pH 12) or reverse phase (C 18 silica columns, pH ca. 7) columns gave unsatisfactory results. In most cases the all-dithioate oligonucleotides could not be eluted as a distinct band (UV detection at 260 nm) but gave an elevated baseline which necessitated long washings to regenerate the columns. This indicates that oligonucleoside phosphorodithioates are strongly and unspecifically bound to the column materials. Only oligonucleotides with a few phosphorodithioate linkages could be purified in this way. TLC purification on silica plates (Merck 60 F_{250}) eluted with propanol/conc. ammonia/water 55:35:10 could be realised, although the resolving power was rather low.

Purification by polyacrylamide gel electrophoresis (PAGE) and analysis by the same technique was more successful, although some tailing was observed. The phosphorodithioates were somewhat retarded on PAGE gels, e.g. an all-dithioate 17-mer had the same mobility as an unmodified 20-mer (**Fig. 1**).

Fortunately oligonucleotide phosphorodithioates with DMT on behaved normally on Hamilton PRP-1 reverse phase columns,



Scheme 2.

eluted with a triethylammonium bicarbonate/acetonitrile gradient. A large product peak followed failure sequence peaks (**Fig. 5**), and very little tailing was observed. Most of the oligonucleoside phosphorodithioates were purified in this way, and PAGE analysis after removal of DMT showed products with a good full-length homogeneity (**Fig 1**). All-phosphorodithioate oligonucleotides without DMT gave likewise reasonably sharp peaks on PRP-1 columns for sequences up to at least 17-mers.

As expected, ^{31}P NMR analysis run in a few cases on purified products showed that the amount of phosphorothioate impurities was not changed by the above purification. The phosphorothioate groups are probably evenly distributed along the chain, and the resulting family of products are impossible to separate by any of the normal purification methods.

Properties of Oligonucleoside Phosphorodithioates

Oligonucleoside phosphorodithioates, at least up to all-dithioate 27-mers which are the longest we have prepared so far, are freely soluble in water under neutral or basic conditions. At lower pH, e.g. 1 M acetic acid (pH ca. 2.5) during the removal of DMT, they are surprisingly quite insoluble, in contrast to unmodified oligonucleotides. This indicates that oligonucleotide phosphorodithioates are not as well solvated by water as unmodified oligonucleotides, since a diminished charge at lower pH can precipitate them. Protonisation must occur at the bases at lower pH's, since the phosphorodithioate groups are slightly *less* basic (pK_a 1.6–1.8[32]) than phosphate groups.

We have found no indication that oligonucleoside phosphorodithioates are oxidised by air (e.g. to disulphides), neither in solution nor in the solid state. In agreement with a reduction potential for phosphorodithioic acids of about +0.47 V/NHE[32] they are oxidised by iodine/water/*N*-methylimidazole. This reagent has been used by Porritt and Reese to convert a thymidine phosphorodithioate dimer and trimer to the the phosphate dimer and trimer in virtually quantitative yields.[21] We have treated a thymidine phosphorodithioate decamer with the same reagent but observed that the oxidation was accompanied by extensive chain-cleavage (HPLC analysis).

The phosphorodithioate group is hydrolytically very stable. A good example of the stability towards base cleavage is the result of Petersen and Nielsen that a *ribo*nucleoside phosphorodithioate dimer was virtually undegraded after 16 h at 5°C in conc. aqueous ammonia and only partially degraded after 16 h at 55°C.[33] We have shown that an unprotected thymidine phosphorodithioate dimer is unchanged after 24 h at 55°C in conc. aqueous ammonia;[3] after 2.5 years at room temperature the solution of the dimer however contained 14% of the phosphorothioate dimer (d_P 54.3) but less than 1% of other products (^{31}P NMR analysis). This very slow conversion of phosphorodithioate to phosphorothioate could well be light-induced since the solution was kept in a closed tube but not in the dark; a simple hydrolysis would be expected to give a mixture of phosphorothioate and phosphate, unless a phosphorothioate is much more hydrolytically stable than a phosphorodithioate. Data about comparable hydrolysis rates of phosphorothioates and phosphorodithioates are sparse although Cote and Bauer found that bis(2-ethylhexyl) phosphorodithioic acid gave mainly the phosphorothioic acid on acid or neutral hydrolysis,[32] which indicates that phosphorothioates indeed are more stable than phosphorodithioates under those conditions.

The nuclease stability of oligonucleoside phosphorodithioates is very high as anticipated, since two corners of the phosphate tetraeder is occupied by sulphur which should screen it better against nucleases than substitution with only one sulphur, as in phosphorothioates. Caruthers et al. and Porritt and Reese have found that the phosphorodithioate linkage is totally resistant to digestion by snake venom and calf spleen phosphodiesterase, nuclease P1, the 3'-5' exonuclease activity of T4-DNA polymerase, and the nucleases present in Hela cell nuclear extracts.[12, 21] The resistance against digestion by the endonucleases nuclease S1 and DNase I is presently under investigation, as well as the ability of oligonucleoside phosphorodithioates to function as antisense oligonucleotides and to be taken up by cells.[34]

The ability of oligonucleoside phosphorodithioates to hybridise to a complementary DNA or RNA strand is important for their use as antisense compounds. We have measured the melting points (T_m) of a series of oligonucleoside phosphorodithioates, hybridised to their complementary unmodified DNA strand, and compared the values with those for the analogous phosphorothioates and unmodified oligonucleotides (**Table 3**). Sequences 1–3 are an unmodified, an all-phosphorothioate, and an all-phosphorodithioate 17-mer oligonucleotide with the same sequence, and sequence 4,5, and 10 are a similar set of 20-mers. The sequences 6–9 are the same 20-mer with one, three, or four phosphorodithioate linkages. It is seen that T_m is depressed going from unmodified oligonucleotides to phosphorothioates to phosphorodithioates. The depression of T_m per modified linkage, ΔT_m, for a 20-mer is ca. 0.4°C for phosphorothioates and ca. 0.8°C for phosphorodithioates. As expected ΔT_m is larger (1–2°C) for a phosphorodithioate positioned in the middle of a sequence than when the modification is at the end (0.5–1°C). For the 17-mer a larger ΔT_m is found for both the phosphorothioate (0.6°C) and the phosphorodithioate (1.2–1.3°C). Caruthers et al. have published somewhat smaller ΔT_m's (0.2–1.0°C) for some 15- and 20-mers containing alternate phosphate and phosphorodithioate or isolated phosphorodithioate linkages.[12] Whether this discrepancy is due to different conditions of measurement, a sequence dependance

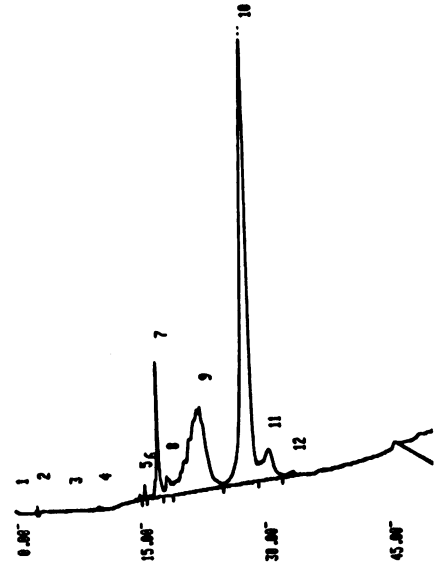


Fig. 5. Reverse phase HPLC chromatogram of a crude all-phosphorodithioate 17-mer (seq. 3, **Table 3**); the product (peak 10) with a retention time of 28.3 min was collected. The running conditions are specified in Materials and Methods.

of ΔT_m, or the result of alternating versus continuous phosphorodithioate linkages, is unclear at the moment.

From a theoretical point of view it is of interest to know the melting point of a double strand containing phosphorodithioates in both strands. Initial experiments with a 12-mer selfcomplementary all-phosphorodithioate (the Dickerson sequence 5'-CGCGAATTCGCG) showed that T_m was further depressed by having phosphorodithioates in both strands; under conditions where the unmodified 12-mer melted at 68°C (high salt, see **MATERIALS AND METHODS**) the T_m for the same dithioate was found to be ca. 20°C.

The reason for the rather large depression of T_m found for phosphorodithioates may be an altered conformation of the backbone due to the large sulphur atoms. Work is in progress to study the conformations of the all-dithioate Dickerson double strand sequence by ^1H NMR and molecular mechanics calculations.[35]

CONCLUSION

The present paper demonstrates that oligonucleoside phosphorodithioates containing all four bases can be prepared by solid phase synthesis using the thiophosphoramidites **2** as the monomers. This thiophosphoramidite method, however, has some limitations which makes it less attractive than the well known, efficient phosphoramidite method. Firstly, thiophosphoramidites couple much more slowly than analogous phosphoramidites, and a sufficient reactivity is only obtained with compounds that have small *N*-substituents like **2a–d**. Such thiophosphoramidites, however, cannot be purified on silica columns and have a limited stability in solution. Secondly, the phosphorodithioate products obtained from **2a-d** (and from **3** or **4**) contain phosphorothioate impurities which cannot be removed by HPLC or PAGE purification. Thirdly, the oxidation step with elemental sulphur is potentially troublesome on a synthesizer because precipitation might block valves and lines; substitution of sulphur with other reagents have so far been unsuccessful.[6] Fourthly, removal of the *S*-protecting groups is more demanding than removal of an analogous *O*-protecting group in the phosphoramidite method; side reactions such as attack on phosphorus (which might give phosphorothioates) or on the 5'-carbon (which gives chain cleavage) must be avoided by proper choice of *S*-protecting groups and deblocking conditions. The 2-cyanoethyl and 2,4-dichlorobenzyl groups however are both *S*-protecting groups which can be efficiently removed.

Despite these limitations the thiophosphoramidite method is at present the only developed method which allows introduction of phosphorodithioate groups at any position in an oligonucleotide. Work is in progress to examine other methods to prepare phosphorodithioates, with special regard to their potential to avoid introduction of phosphorothioates and to function in solid phase syntheses.

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