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Preparation of oligodeoxyribonucleoside phosphorodithioates by a triester method

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ABSTRACT

A method to prepare thymidine phosphorodithioate dimers (ref. 1) has been extended to allow the preparation of oligo-2′-deoxyribonucleotide phosphorodithioates containing all four bases. The method is suitable for large-scale synthesis and gives phosphorodithioates without phosphorothioate impurities (^{31}P nmr, detection limit 0.5 to 1%). Oligonucleotides up to octamers which contain -0-(PS_2-)-0- linkages at all positions have been prepared by block synthesis in solution. The phosphorodithioate linkage is introduced by the reaction of a 5′-*O*, *N*-protected nucleoside (or oligonucleotide) with a dithiophosphorylating agent RSP(S)(ODhbt)$_2$, R = 2,4-dichlorobenzyl, Dhbt = 3,4-dihydro-4-oxo-benzotriazin-3-yl, followed by coupling of the product to a 3′-*O*,*N*-protected nucleoside (or oligonucleotide). This method gives pure protected oligodeoxyribonucleoside phosphorodithioates, and phosphorothioate linkages are only introduced if contact with conc. aqueous ammonia during or after deblocking is unduly prolonged.

INTRODUCTION

Oligonucleotides which are modified in a way that decreases nuclease cleavage are of interest as modulators of gene expression *in vivo* (antisense oligonucleotides).[2] Simple modifications of the phosphate linkage, i.e. substitution of one of the non-bridging oxygen atoms with other groups as in methylphosphonates, phosphorothioates, phosphoramidates, and phosphortriesters, have been extensively studied.[3] Such compounds show increased stability *in vivo* compared with unmodified oligonucleotides, but in general the hybridization to DNA and RNA is impaired, and a mixture of diastereomers is usually obtained due to a non-stereospecific synthesis of the chiral modified linkage. Phosphate modified oligonucleotides which are achiral at phosphorus have been less studied; prominent members are 3′- or 5′-*S*-phosphorothioates,[4] 3′- or 5′-*N*-phosphoramidates,[5] and 3′,5′-*O*,*O*-phosphorodithioates (1; abbreviated phosphorodithioates or PS_2 in the following).[1, 6–29] The latter retain the high exonuclease resistance of phosphorothioates, are chemically very stable, and form stable duplexes with DNA, although the additional sulphur atom reduces the binding ability compared to phosphorothioates.[8, 30] The behaviour towards endonucleases seems to be more variable; thus phosphorodithioates were found to be more resistant than phosphorothioates towards S1 nuclease cleavage, but less resistant towards DNase I cleavage and cleavage in some sub-cellular fractions of MCF-7 cells.[30] Phosphorodithioates were also found to be less specific than phosphorothioates in the inhibition of cell-free rabbit β-globin mRNA translation.[30] Apart from their potential for antisense applications, phosphorodithioates strongly inhibit several enzymes which operate on DNA or RNA, e.g. HIV reverse transcriptase,[17, 31] and may find use as stabilised substrate analogues in structural studies of DNA-cleaving enzymes.

The phosphorodithioate linkage can be introduced in oligonucleotides in several ways,[20] but only one method, via thiophosphoramidite monomers, has been applied successfully for the preparation of mixed base oligonucleotides containing contiguous phosphorodithioate linkages.[7, 8, 11] This method, however, gives products which contain varying amounts of phosphorothioate linkages (2–10%), due to as yet poorly defined factors associated with the involvement of trivalent thiophosphorus compounds.[8] Phosphortriester methods which avoid such trivalent compounds should therefore be better routes to pure oligonucleoside phosphorodithioates. Three phosphortriester methods to obtain phosphorodithioates have been published: Caruthers *et al.* [29] prepared dimers from protected nucleoside 3′-phosphorodithioate diesters 2a which coupled with protected 5′-hydroxynucleosides in the presence of suitable coupling agents to give phosphorodithioates that contained only 1% of phosphorothioates under optimal conditions. Dahl *et al.* [1] examined several active phosphorodithioate esters and found that 3a–c could be used to obtain thymidine phosphorodithioate dimers, which were more than 99% pure according to ^{31}P nmr. Stec *et al.* [20] prepared dimers and pyrimidine oligomers from protected nucleoside-3′-phosphorotrithioates 2b and obtained a phosphorodithioate pentamer which contained 1% phosphorothioate linkages after purification.

This paper describes our results using the active ester 3d to prepare phosphorodithioate dimers and oligomers up to octamers containing all four 2′-deoxyribonucleosides. The 2,4-dichlorobenzyl group of 3d can be selectively removed with thiolate ions

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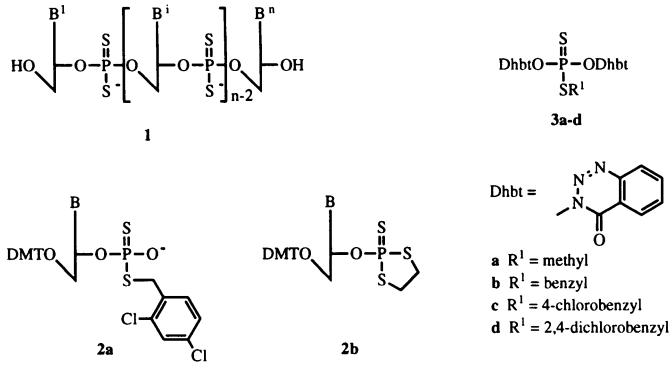


after synthesis, whereas a similar removal of the protecting groups of 3a–c is accompanied by significant cleavage of the phosphorodithioate linkage.[1] The products are shown by ^{31}P nmr to contain less than the detection limit, 0.5 to 1%, of phosphorothioate linkages, and by HPLC and PAGE to be homogeneous.

MATERIALS AND METHODS

The 5′-*O*,*N*-protected 2′-deoxyribonucleosides were from Peninsula Laboratories or Cruachem. 3′-*O*-acetylthymidine was prepared by the method of Horwitz *et al.*.[32] Phenoxyacetic anhydride was prepared by the method of Reese *et al.*,[33] or by refluxing phenoxyacetic acid (0.2 mol) with thionyl chloride (0.1 mol) in benzene (50 ml) overnight (yield 65% after recryst. from diethyl ether, mp 71–72°C). All other chemicals were from Aldrich (usually 99%+). Acetonitrile (LAB-SCAN C2502) and pyridine (LAB-SCAN A3544) were dried over 4Å molecular sieves (GRACE type 512). Dioxane (LAB-SCAN C2512) and *N*-methylimidazole (Aldrich M5,083-4) were purified by filtering through basic alumina (ICN Biomedicals Alumina B-Super I) and dried over 4Å molecular sieves. Methanol (LAB-SCAN C2517), dichloromethane (LAB-SCAN C2510L), and conc. aqueous ammonia (32%, Merck 5426) were used as received. All preparations of dimers and oligomers were performed in predried flasks under nitrogen, and solutions were transferred with polyethylene syringes. TLC (eluent dichloromethane/methanol/pyridine 94:5:1 v/v/v unless otherwise stated) was performed on silica 60 (Merck 5554 aluminium sheet), column chromatography on silica 60 (Merck 9385). ^1H and ^{31}P nmr spectra (in $CDCl_3$ unless otherwise stated) were obtained on a JEOL FX 90 Q spectrometer at 89.6 MHz for ^1H and 36.4 MHz for ^{31}P in 5 mm tubes; chemical shifts are positive in the low-field direction, with internal tetramethylsilane (^1H) or external 85 % phosphoric acid (^{31}P) as references.

2,4-Dichlorophenylmethanethiol. 2,4-Dichlorobenzyl chloride (83.4 ml, 117 g, 0.6 mol) was added dropwise to a solution of disodium 2,2-dicyanoethylene-1,1-dithiolate trihydrate[34] (72.0 g, 0.3 mol) in methanol/water (500 ml, 9:1) at rt with stirring. After stirring for 3 h water was added to complete the precipitation of bis-(2,4-dichlorobenzylthio)methylenemalononitrile, which was isolated and recrystallized from 2-propanol to give yellow crystals (100.5 g, 73%), mp 144–146°C; TLC (trichloromethane/hexane 1:1): R_f 0.52. To a suspension of bis-(2,4-dichlorobenzylthio)methylenemalononitrile (46.2 g, 0.1 mol) in a mixture of methanol/water (150 ml, 9:1) and hexane (150 ml) was added ethylenediamine (6.7 ml, 0.1 mol), and the mixture stirred and refluxed under nitrogen until TLC showed that bis-(2,4-dichlorobenzylthio)methylenemalononitrile was consumed (2–3 h). The mixture was cooled to rt, filtered, the hexane phase separated, and the methanol/water phase extracted with hexane (4×50 ml). The combined hexane phases were dried ($MgSO_4$) and the solvent removed on a rotatory evaporator to give the thiol as a pale yellow liquid (34.4 g, 89%), pure according to TLC and nmr. TLC (trichloromethane/hexane 1:9): R_f 0.67. ^1H n.mr.: δ 7.4–7.2 (3H, m, arom.), 3.77 (2H, d, J 8 Hz, CH_2), 1.92 (1H, t, J 8 Hz, SH).

S-(2,4-Dichlorobenzyl) dithiophosphoric dichloride. This compound was prepared by a method modified from that described for the benzyl analogue.[35] A mixture of redistilled thiophosphoryl chloride (102 ml, 1.0 mol), iodine (0.2 g), DMF (0.4 ml), and 2,4-dichlorophenylmethanethiol (14.2 ml, 19.3 g, 0.10 mol) was heated with stirring under nitrogen to ca. 100°C until evolution of hydrogen chloride decreased. The temperature was then increased to 135–145°C (bath temp.) and kept at that temperature for at least 12 h in order to convert the initially formed mixture of mono-, di-, and tri-(2,4-dichlorobenzyl) thioesters to the monoester. The red-brown mixture was cooled, excess of thiophosphoryl chloride removed *in vacuo* at 20–40°C, and the residue recrystallised twice from a small amount (20–30 ml) of dry diethyl ether. Yield 20–23 g (60–70%) of pale yellow to pale brown crystals, mp 48–49°C, more than 99% pure according to nmr. Nmr: δ_P 68.2, δ_H 7.1–7.5 (3H, m, arom), 4.51 (2H, d, J 20.1 Hz, CH_2).

6-N-Benzoyl-3′-O-(phenoxyacetyl)deoxyadenosine (4a). 6-*N*-Benzoyl-5′-*O*-dimethoxytrityldeoxyadenosine (0.660 g, 1.00 mmol) was dried by co-evaporation once with pyridine and redissolved in dry pyridine (2 ml), and phenoxyacetic anhydride (0.286 g, 1.00 mmol) added. The viscous solution was stirred for 10 min at rt (longer times and excess of phenoxyacetic anhydride gives *N*-acylation) followed by addition of water (0.20 ml). After 20 min the solution was diluted with dichloromethane (20 ml), extracted with aqueous 5% sodium hydrogencarbonate (3×10 ml), dried over magnesium sulphate, the solvent evaporated, and residual pyridine removed by co-evaporation with acetonitrile (3×5 ml). The residue was dissolved in dichloromethane (10 ml) and dichloroacetic acid (0.83 ml, 10 mmol) added. After stirring at rt for 10 min methanol (4 ml) was added, followed by triethylamine (1.40 ml, 10 mmol). The product precipitated upon addition of diethyl ether (30 ml added in portions) and cooling to 4°C overnight. The crystals were washed with diethyl ether and the crude product (0.401 g, 82%) recrystallized from abs. ethanol (10 ml) to give colourless crystals (0.373 g, 76%), mp 132–34°C, pure according to TLC and ^1H nmr. TLC: R_f 0.42. ^1H nmr (DMSO-d_6): δ 11.2 (1H, s, NH), 8.77 (1H, s, H-8), 8.71 (1H, s, H-2), 8.2–6.9 (10H, m, arom.), 6.6–6.4 (1H, m, H-1′), 5.6–5.5 (1H, m, H-3′), 5.20 (1H, t, J 5.4 Hz, OH), 4.89 (2H, s, phenoxyacetyl-CH_2), 4.3–4.1 (1H, m, H-4′), 3.8–3.5 (2H, m, H-5′), 3.3–2.5 (2H, m, H-2′).

The following compounds were similarly prepared: 3′-*O*-(Phenoxyacetyl)thymidine (4b), recrystallized from ethyl acetate, yield 67%, mp 154–154.5°C. TLC: R_f 0.22. ^1H nmr (DMSO-d_6): δ 11.3 (1H, s, br, NH), 7.8–6.9 (6H, m, arom. + H-6), 6.20 (1H, t, J 7.2 Hz, H-1′), 5.5–5.2 (1H, m, H-3′), 4.84 (2H, s, phenoxyacetyl-CH_2), 4.2–3.9 (1H, m, H-4′), 3.8–3.6 (2H, m, H-5′), 2.4–2.2 (2H, m, H-2′), 1.78 (3H, d, J 0.6 Hz, CH_3-T). 2-*N*-Isobutyryl-3′-*O*-(phenoxyacetyl)deoxyguanosine

(4c), purified on a silica column (eluted with a stepwise gradient of dichloromethane/methanol/pyridine 94:5:1 to 89:10:1 to 84:15:1) followed by recrystallization from ethyl acetate. Yield 56%, mp 112–113°C. TLC: R_f 0.16. ^{1}H nmr: δ 12.0 (1H, s, NH), 9.6 (1H, s, NH), 7.7 (1H, s, H-8), 7.3–6.6 (5H, m, arom.), 6.1–5.7 (1H, m, H-1′), 5.5–5.3 (1H, m, H-3′), 4.5 (2H, s, phenoxyacetyl-CH_2), 4.2–3.9 (1H, m, H-4′), 3.7–3.5 (2H, m, H-5′), 3.2–2.1 (2H, m, H-2′), 1.3–1.0 (6H, dd, isobutyryl-CH_3). 4-*N*-Benzoyl-3′-*O*-(phenoxyacetyl)deoxycytidine (4d); the crude product which precipitated on addition of diethyl ether (yield 80%) was pure according to TLC and ^{1}H nmr. TLC (dichloromethane/methanol 85:15): R_f 0.68. ^{1}H nmr (DMSO-d_6): δ 11.3 (1H, br), 8.39 (1H, d, J 7.3 Hz, H-6), 8.1–7.9 (2H, m, arom), 7.7–6.9 (8H, m, arom), 6.3–6.1 (1H, m, H-1′), 5.5–5.3 (1H, m, H-3′), 4.87 (2H, s, phenoxyacetyl-CH_2), 4.3–4.1 (1H, m, H-4′), 3.8–3.5 (2H, m, H-5′), 2.7–2.0 (2H, m, H-2′).

Preparation of dimers

O-[6-N-Benzoyl-5′-O-(dimethoxytrityl)deoxyadenosin-3′-yl] O-[6-N-benzoyl-3′-O-(phenoxyacetyl)deoxyadenosin-5′-yl] S-phosphorodithioate (5a). *S*-(2,4-Dichlorobenzyl) dithiophosphoric dichloride (0.652 g, 2.0 mmol) was added to a solution of 3-hydroxy-3,4-dihydro-4-oxobenzotriazine (Dhbt-OH; 0.684 g, 4.2 mmol) and dry pyridine (0.6 ml, 7 mmol) in dry dioxane (5 ml) under nitrogen. After stirring for 20 min at rt the formation of 3d was complete, and pyridine hydrochloride was removed by filtration under nitrogen. The solution of 3d was added to 6-*N*-benzoyl-5′-*O*-dimethoxytrityldeoxyadenosine (1.19 g, 1.80 mmol) which had first been dried by co-evaporation once with dry pyridine. The solvent was removed *in vacuo* at rt and the residue dissolved in a mixture of dry acetonitrile (5.0 ml) and dry pyridine (1.50 ml, 19 mmol). The formation of the mononucleoside phosphorodithioate 2c was complete (>98% according to ^{31}P nmr, δ_P 104.8 and 103.9) after stirring under nitrogen for 60 min at rt. 6-*N*-Benzoyl-3′-*O*-(phenoxyacetyl)adenosine (1.22 g, 2.50 mmol) was dried by co-evaporation twice with dry pyridine, and the solution of 2c added, followed by dry *N*-methylimidazole (1.4 ml, 18 mmol). The formation of 5a was complete (> 98% according to ^{31}P nmr, δ_P 93.3 and 92.6) after stirring under nitrogen for 90 min. Solvents were removed *in vacuo* and the residue co-evaporated with acetonitrile (2 ×10 ml). The yellow foam was dissolved in dichloromethane (200 ml), extracted with 5% aq. sodium hydrogencarbonate (3 ×50 ml), the organic phase dried over magnesium sulphate, and the dichloromethane removed *in vacuo*. The crude product was purified on a silica column (diam. 4 cm, height 14 cm, eluted with a stepwise gradient of dichloromethane/methanol/pyridine 98:1:1 to 97:2:1) and the fractions containing 5a (TLC, R_f 0.48) pooled, the solvent removed *in vacuo*, and the residue co-evaporated with acetonitrile (3×5 ml). Yield 1.79 g (71%) of 5a as a colourless foam, more than 99% pure according to ^{31}P nmr. Nmr: δ_P 96.5 and 95.1. δ_H 9.3 (2H, s br, NH), 8.76 and 8.73 (2H, 2 s, 2 ×H-8), 8.61 and 8.58 (2H, 2 s, 2 ×H-2), 8.4–6.6 (31H, m, arom), 6.6–6.2 (2H, m, 2 ×H-1′), 5.7–5.1 (2H, m, 2 ×H-3′), 4.71 (2H, s, phenoxyacetyl-CH_2), 4.5–3.8 (6H, m, CH_2S + 2 ×H-4′ + H-5′), 3.74 (6H, s, CH_3O), 3.5–3.2 (2H, m, H-5′), 3.1–2.4 (4H, m, 2 ×H-2′).

The following dimers were similarly prepared: *O*-[6-*N*-Benzoyl-5′-*O*-(dimethoxytrityl)deoxyadenosin-3′-yl] *O*-[3′-*O*-(phenoxyacetyl)thymidin-5′-yl] *S*-(2,4-dichlorobenzyl) phosphorodithioate (5b), yield 77%, more than 99% pure according to ^{31}P nmr. TLC: R_f 0.50. Nmr: δ_P 97.2 and 95.8. δ_H 10.5 and 9.7 (2H, 2 s br, 2 ×NH), 8.7 (1H, s, H-8), 8.2 (1H, s, H-2), 8.2–6.7 (27H, m, arom + H-6), 6.6–6.2 (2H, m, 2 ×H-1′), 5.6–5.3 (1H, m, H-3′-dA), 5.3–5.1 (1H, m, H-3′-T), 4.70 (2H, s, phenoxyacetyl-CH_2), 4.5–3.9 (6H, m, CH_2S + 2 ×H-4′ + H-5′-T), 3.76 (6H, s, CH_3O), 3.6–3.3 (2H, m, H-5′-dA), 3.2–2.1 (4H, m, 2 ×H-2′), 1.94 (3H, s, CH_3-T). *O*-[5′-*O*-(Dimethoxytrityl)-4-*N*-benzoyldeoxycytidin-3′-yl] *O*-[2-*N*-isobutyryl-3′-*O*-(phenoxyacetyl)deoxyguanosin-5′-yl] *S*-(2,4-dichlorobenzyl) phosphorodithioate (5c), yield 61%, contains traces (3%) of the 3′,3′-dimer according to ^{31}P nmr. TLC: R_f 0.45. Nmr: δ_P 94.8 and 94.2. δ_H 12.4, 10.6, 10.3 and 9.7 (3H, 4 s br, 3 ×NH), 8.3–6.7 (29H, m, arom + H-5 + H-6 + H-8), 6.5–6.1 (2H, m, 2 ×H-1′), 5.7–5.1 (2H, m, 2 ×H-3′), 4.74 (2H, s, phenoxyacetyl-CH_2), 4.5–3.9 (6H, m, CH_2S + 2 ×H-4′ + H-5′-dG), 3.76 (6H, s, CH_3O), 3.6–3.2 (2H, m, H-5′-dC), 3.2–2.1 (5H, m, 2 ×H-2′ + isobutyryl-CH), 1.4–1.0 (6H, m, isobutyryl-CH_3). *O*-[5′-*O*-(Dimethoxytrityl)thymidin-3′-yl] *O*-[3′-*O*-(phenoxyacetyl)thymidin-5′-yl] *S*-(2,4-dichlorobenzyl) phosphorodithioate (5e), yield 61%, more than 99% pure according to ^{31}P nmr. TLC: R_f 0.40. Nmr: δ_P 97.3 and 95.8. δ_H 9.2 (2H, s br, 2 ×NH), 7.7–6.8 (23H, m, arom + 2 ×H-6), 6.5–6.1 (2H, m, 2 ×H-1′), 5.5–5.3 (1H, m, H-3′), 5.3–5.1 (1H, m, H-3′), 4.71 (2H, s, phenoxyacetyl-CH_2), 4.5–3.9 (6H, m, CH_2S + 2 ×H-4′ + H-5′), 3.69 (6H, s, CH_3O), 3.5–3.3 (2H, m, H-5′), 2.6–1.9 (4H, m, 2 ×H-2′), 1.91 (3H, s, CH_3-T), 1.47 (3H, s, CH_3-T).

The following dimers were similarly prepared, but with less 4 (1.4 mmol) than 2c (1.8 mmol): *O*-[5′-*O*-(Dimethoxytrityl)-2-*N*-isobutyryldeoxyguanosin-3′-yl] *O*-[4-*N*-benzoyl-3′-*O*-(phenoxyacetyl)deoxycytidin-5′-yl] *S*-(2,4-dichlorobenzyl) phosphorodithioate (5d), yield 63%, more than 97% pure according to ^{31}P nmr. TLC: R_f 0.45. Nmr: δ_P 96.9 and 94.7. δ_H 12.2, 12.1, 10.8, 10.3, and 8.6 (3H, 5 s, 3 ×NH), 8.3–8.2 (1H, m, H-6 or H-8), 7.7–6.7 (28H, m, arom + H-5 + H-6 or H-8), 6.3–5.7 (2H, m, 2 ×H-1′), 5.4–5.0 (2H, m, H-3′), 4.70 and 4.65 (2H, 2 s, phenoxyacetyl-CH_2), 4.6–4.0 (6H, m, CH_2S + 2 ×H-4′ + H-5′-dC), 3.76 (6H, s, DMT-CH_3), 3.4–3.1 (2H, m, H-5′-dG), 3.1–2.1 (5H, m, 2 ×H-2′ + isobutyryl-CH), 1.3–1.1 (6H, m, isobutyryl-CH_3). *O*-[5′-*O*-(Dimethoxytrityl)thymidin-3′-yl] *O*-(3′-*O*-acetylthymidin-5′-yl) *S*-(2,4-dichlorobenzyl) phosphorodithioate (5f), yield 69%, more than 99% pure according to ^{31}P nmr. TLC: R_f 0.53. Nmr: δ_P 96.9 and 95.5. δ_H 9.3 (2H, s br, 2 ×NH), 7.6–6.6 (18H, m, arom + 2 ×H-6), 6.5–6.1 (2H, m, 2 ×H-1′), 5.6–5.2 (1H, m, H-3′), 5.2–4.9 (1H, m, H-3′), 4.4–3.9 (6H, m, CH_2S + 2 ×H-4′ + H-5′), 3.78 (6H, s, CH_3O), 3.5–3.3 (2H, m, H-5′), 3.2–2.2 (4H, m, 2 ×H-2′), 2.1 (3H, s, $COCH_3$), 1.89 (3H, s, CH_3-T), 1.46 (3H, s, CH_3-T).

Removal of DMT (5 → 6). Dichloroacetic acid (0.83 ml, 10 mmol) was added to 5 (1.00 mmol) in dichloromethane (10 ml). The reaction was complete (TLC) after 30 min at rt, and methanol (4.0 ml, 100 mmol) and dichloromethane (40 ml) were added. The solution was extracted with sat. aq. sodium hydrogencarbonate (3 ×50 ml), the organic phase dried over magnesium sulphate, and the dichloromethane removed *in vacuo*. The crude product was purified on a silica column (eluted with a stepwise gradient of dichloromethane/methanol/pyridine 98:1:1 to 96:3:1) and the fractions containing 6 pooled, evaporated, and the residue

co-evaporated with acetonitrile (3×5 ml) to give pure 6. Yield of 6a, 74%, R_f 0.30, δ_P 96.5 and 95.2; 6b, 86%, R_f 0.25, δ_P 96.6 and 95.9; 6f, 73%, R_f 0.28, δ_P 96.6 and 95.6.

Removal of Pac or acetyl (5 → 7). To a solution of 5 (1.00 mmol) in a mixture of methanol (40 ml) and acetonitrile (20 ml) (or dichloromethane (20 ml) in case of 5e), cooled to 0°C, was added *tert*-butylamine (1.05 ml, 10 mmol). The reaction was complete (TLC) after stirring at 0°C for 1 h (6 h in case of 5e, 22 h in case of 5f), and the solution was concentrated *in vacuo* at rt to a foam. The crude product was purified on a silica column (eluted with a stepwise gradient of dichloromethane/methanol/pyridine 97:2:1 to 94:5:1) and the fractions containing 7 pooled, evaporated, and the residue co-evaporated with acetonitrile (2 ×5 ml) to give pure 7. Yield of 7a, 60%, R_f 0.24, δ_P 95.7 and 95.1; 7b, 85%, R_f 0.17, δ_P 96.4 and 95.8; 7c, 75%, R_f 0.08, δ_P 94.0 and 93.6; 7d, 84%, R_f 0.12, δ_P 97.3 and 94.1; 7e, 83% from 5e, 79% from 5f, R_f 0.17, δ_P 96.0 and 95.9.

Preparation of oligomers

Preparation of the protected all-PS_2 tetramer d(ATAA) 10. The protected d(AT) dimer 7b (0.21 g, 0.18 mmol) was dried by co-evaporation once with pyridine, and a solution of 3d in dioxane (0.25 M, 0.80 ml, 0.20 mmol), prepared as described for 5a, added. The solvent was removed *in vacuo*, and the residue dissolved in a mixture of acetonitrile (1.00 ml) and dry pyridine (0.20 ml). The formation of the 3'-dithiophosphorylated dimer 8b was complete after stirring for 100 min at rt (δ_P 105.3, 104.9, 94.0, and 93.3), after which time the solution of 8b was added to a predried sample of the protected d(AA) dimer 6a (0.28 g, 0.25 mmol) and NMI (0.14 ml, 0.9 mmol) added. The formation of the fully protected tetramer 9 was complete after stirring for 90 min at rt (δ_P 94.0−92.3, m). The crude product was purified in a similar way to 5a using for the column chromatography an eluent of dichloromethane/methanol/pyridine 94:5:1, to give 9 (0.36 g, 80%) which still contained some 6a. This contaminant could be removed after the Pac protecting group was removed. To remove Pac, 9 (0.35 g, 0.14 mmol) was dissolved in a mixture of methanol (45 ml), acetonitrile (25 ml), and dichloromethane (10 ml), the solution cooled to 0°C, and *tert*-butylamine (1.25 ml, 12 mmol) added. After stirring for 1.5 h the solution was concentrated *in vacuo* at rt to a foam, which was purified on a silica column, eluted with dichloromethane/methanol/pyridine 89:10:1, to give, after co-evaporation from acetonitrile/dichloromethane 1:1 (2 × 5 ml), 10 (0.17 g, 51% from 9), pure according to TLC and ^{31}P nmr. TLC: R_f 0.21. ^{31}P nmr: δ 96.6−95.0 (m).

Preparation of the fully protected all-PS_2 hexamer d(ATAAAA) 11. The protected tetramer 10 (72 mg, 30 μmol) was dried by co-evaporation once with pyridine, and a solution of 3d in dioxane (0.25 M, 132 μl, 33 μmol), prepared as described for 5a, added. The solvent was removed *in vacuo*, and the residue dissolved in a mixture of acetonitrile (0.20 ml) and dry pyridine (80 μl). After 3 h at rt the solution was added to a predried sample of the protected d(AA) dimer 6a (44 mg, 40 μmol) and NMI (24 μl, 300 μmol) added. The mixture was stirred at rt overnight, concentrated to a foam *in vacuo*, and worked up as described for 5a, including purification on a silica column (diam. 2 cm, height 12 cm, eluted with dichloromethane/methanol/pyridine 95:4:1). Yield 77 mg (69%) of 11, pure according to TLC and ^{31}P nmr. TLC: R_f 0.45. ^{31}P nmr: δ 96.6−94.8 (m).

Deprotection of 11 to the all-PS_2 hexamer d(ATAAAA) 12. The fully protected hexamer 11 (77 mg, 21 μmol) was dissolved in a mixture of thiophenol, triethylamine, and pyridine (1:1:1, 0.30 ml) and kept under nitrogen at rt for 72 h. Then the mixture was concentrated *in vacuo*, conc. aqueous ammonia (32%, 0.4 ml) was added, and the solution heated to 55°C for 6 h in a closed tube. The solvents were removed *in vacuo* and the residue in water (2 ml) extracted with ether (3 × 0.5 ml). The crude DMT-hexamer (1140 OD) was purified on a Hamilton PRP-1 column as previously described [8] to give the purified DMT-hexamer (430 OD), and after removal of DMT, the unprotected hexamer 12 (270 OD, 6.2 mg, 3.2 mmol, 15% from 11), more than 99.5% pure according to ^{31}P nmr. ^{31}P nmr (D_2O): δ_P 112.9 (s, br).

Preparation of the fully protected all-PS_2 tetramer d(GCAT) 13. The protected d(GC) dimer 7d (2.08 g, 1.70 mmol) was dried by co-evaporation once with pyridine, and a solution of 3d (1.89 mmol) in dioxane, prepared as described for 5a, added. The solvent was removed *in vacuo*, and the residue dissolved in a mixture of acetonitrile (5 ml) and dry pyridine (1.5 ml). After 3 h at rt the solution was added to a predried sample of the protected d(AT) dimer 6b (1.28 g, 1.30 mmol) and NMI (1.4 ml) added. The mixture was stirred at rt overnight, concentrated to a foam *in vacuo*, and worked up as described for 5a, including purification of the crude product in two portions on a silica column (diam. 6 cm, height 10 cm, eluted with dichloromethane/methanol/pyridine 94:5:1). Yield 2.36 g (74%) of 13, pure according to TLC and ^{31}P nmr. TLC: R_f 0.25. ^{31}P nmr: δ 97.4−94.1 (m). FAB$^+$ MS: MW calculated by isotopic cluster abundance calculation: 2460 (78%), 2461 (100%), 2462 (85%), 2463 (75%). Found: 2463 ($M+H^+$), 2485 ($M+Na^+$).

Removal of DMT (13 → 16). 13 (0.74 g, 0.30 mmol) was treated in the same way as described for 5 to give 16, yield 0.48 g (74%),

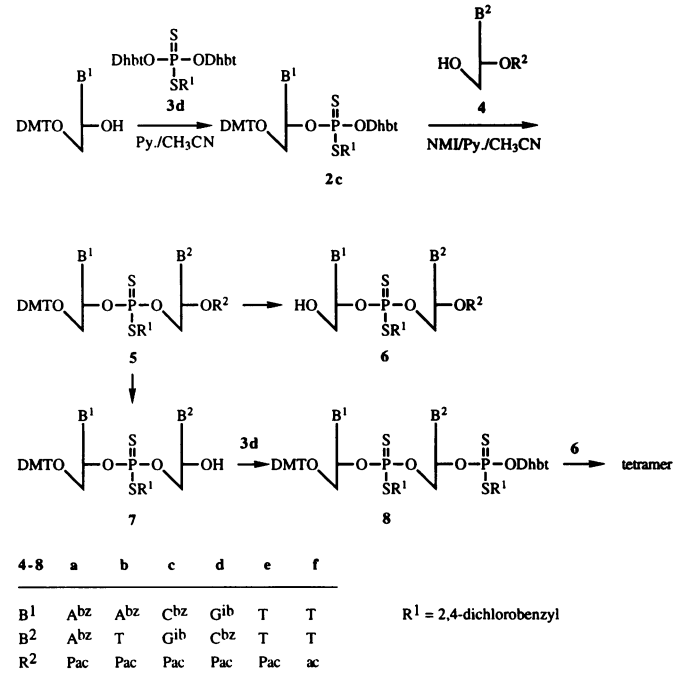


Figure 1. Outline of the triester method and numbering of monomers and dimers.

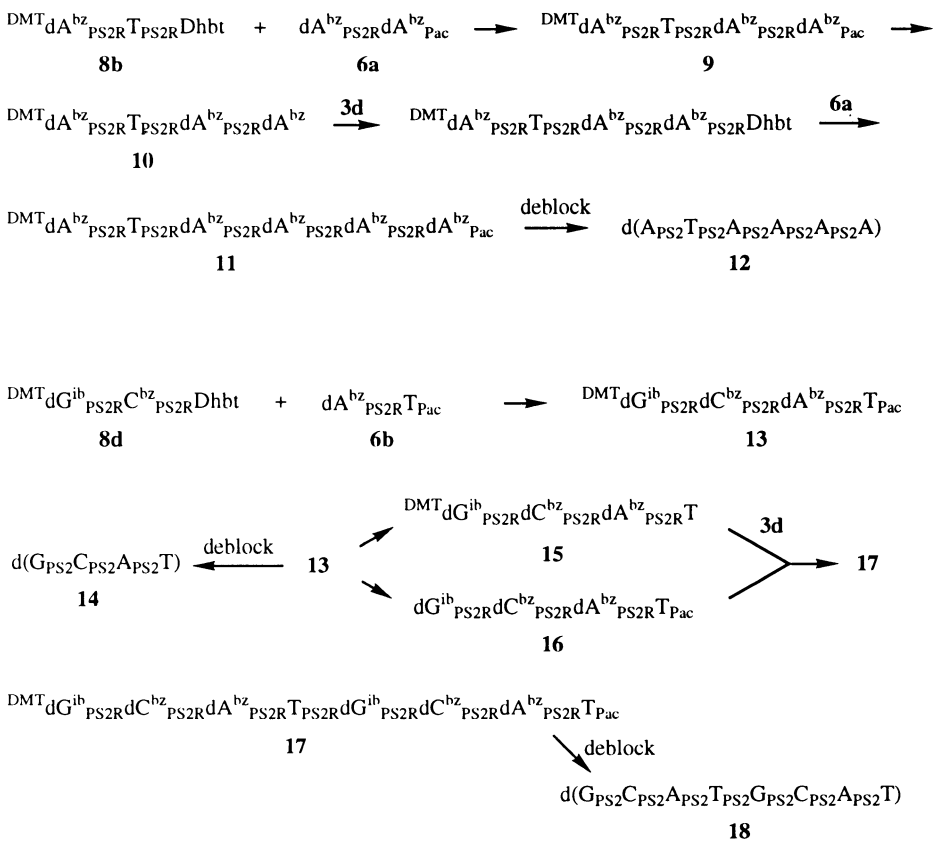
pure according to TLC and ^{31}P nmr. TLC: R_f 0.14. ^{31}P nmr: δ 97.3–94.9 (m).

Removal of Pac (13 → 15). To a solution of 13 (0.98 g, 0.40 mmol) in a mixture of pyridine (5 ml) and methanol (10 ml), cooled to 0°C, was added *tert*-butylamine (0.53 ml, 5 mmol). The removal was nearly complete (TLC) after 2.5 h at 0°C, and the solution was concentrated *in vacuo* at rt to a foam. The crude product was purified on a silica column, eluted with a stepwise gradient of dichloromethane/methanol/pyridine 94:5:1 to 92:7:1, to give, after co-evaporation twice from acetonitrile/dichloromethane 1:1, 15, yield 0.65 g (70 %), pure according to TLC and ^{31}P nmr. TLC (dichloromethane/methanol/pyridine 89:10:1): R_f 0.40 and 0.46. ^{31}P nmr: δ 96.5–94.4 (m).

Deprotection of 13 to the all-PS_2 tetramer d(GCAT) 14. The fully protected tetramer 13 (24.6 mg, 10 μmol) was dissolved in a mixture of thiophenol, triethylamine, and pyridine (1:1:1, 0.30 ml) and kept under nitrogen at rt for 3 h. Then the solution was concentrated *in vacuo*, conc. aqueous ammonia (32%, 1 ml) was added, and the turbid solution heated to 55°C for 6 h in a closed tube. The cooled mixture was diluted with water (3 ml) and, after standing in contact with air for two to three days to allow oxidation of the thiophenolate ions to diphenyl disulphide, extracted with ether (3 × 3 ml). The solution of the crude DMT-tetramer (ca. 410 OD) was purified on a Hamilton PRP-1 column as previously described [8] and DMT removed to give the unprotected tetramer 14, more than 99% pure according to ^{31}P nmr. The yields of two experiments were 3.6 mg (27%) and 8.4 mg (64%). ^{31}P nmr (D_2O): δ_P 113.2 (s, br).

Preparation of the fully protected all-PS_2 octamer d(GCATGCAT) 17. The protected tetramer 15 (355 mg, 0.152 mmol) was dried by co-evaporation once with pyridine, and a solution of 3d in dioxane (0.169 mmol), prepared as described for 5a, added. The solvent was removed *in vacuo*, and the residue dissolved in a mixture of acetonitrile (0.5 ml) and dry pyridine (0.2 ml). After 3 h at rt the solution was added to a predried sample of the protected tetramer 16 (253 mg, 0.117 mmol) and NMI (0.15 ml, 1.9 mmol) added. The mixture was stirred at rt overnight, concentrated to a foam *in vacuo*, and worked up as described for 5a, including purification on a silica column (diam. 4 cm, height 10 cm, eluted with dichloromethane/methanol/pyridine 94:5:1). Yield 330 mg (60%) of 17, pure according to TLC and ^{31}P nmr. TLC: R_f 0.30. ^{31}P nmr: δ 97.9–96.0 (m). FAB^+ MS: MW calculated by isotopic cluster abundance calculation: 4738 (93%), 4739 (98%), 4740 (100%), 4741 (93%), 4742 (83%). Found: 4741 ($M+H^+$), 4763 ($M+Na^+$).

Deprotection of 17 to the all-PS_2 octamer d(GCATGCAT) 18. The fully protected octamer 17 (47.5 mg, 10 μmol) was deprotected in the same way as described for 13 and the crude



DMT = dimethoxytrityl, bz = benzoyl, R = 2,4-dichlorobenzyl, Dhbt = 3,4-dihydro-4-oxo-benzotriazin-3-yl, Pac = phenoxyacetyl, ib = isobutyryl

Figure 2. Synthesis of the the hexamer 12, the tetramer 14, the octamer 18, and numbering of intermediates.

DMT-octamer (ca. 780 OD) purified on a Hamilton PRP-1 column as previously described.[8] The product, after removal of DMT, was more than 99% pure according to ^{31}P nmr. The yields of two experiments were 7.0 mg (25%) and 12.5 mg (46%). ^{31}P nmr (D_2O): δ_P 112.7–113.1 (m).

RESULTS AND DISCUSSION

The triester method described in this paper is outlined in Fig. 1. The dithiophosphorylating reagent 3d was prepared in dioxane/pyridine from DhbtOH and *S*-(2,4-dichlorobenzyl) dithiophosphoric dichloride. The latter compound was obtained from thiophosphoryl chloride and 2,4-dichlorophenylmethanthiol as a stable, non-hygroscopic solid. 3d is somewhat unstable in dioxane towards dealkylation by nucleophiles, as shown by a slow formation of $(DhbtO)_2PSS^-$ (δ_P 131.8 in dioxane). The corresponding HOBT derivative was much more unstable and decomposed during an attempted preparation, as described previously for the 4-chlorobenzyl analogue.[1] 3d is therefore best prepared fresh although after filtration the dioxane solution can be stored frozen for a few days with less than 1% dealkylation per day. Preparations in acetonitrile or pyridine decomposed much faster, probably because pyridinium chloride did not precipitate and chloride ions and/or pyridine dealkylated 3d. Substitution reactions at phosphorus in 3d are 5–6 times faster in acetonitrile than in dioxane, however, so the coupling reactions were performed in acetonitrile in the following way: To the dried, protected 3′-hydroxynucleoside was added a small excess of 3d in dioxane, the solvent was removed *in vacuo*, and acetonitrile and pyridine (a catalyst) added. Complete (>98% by ^{31}P nmr) formation of the activated monomer 2c was observed after 1 h at rt. This solution was then added to the dried, protected 5′-hydroxynucleoside 4, and NMI (a catalyst) added, to give nearly complete (ca. 98% by ^{31}P nmr) formation of the fully protected dimer 5a–f after 1.5–3 h at rt. The coupling rate varied somewhat with the degree of removal of dioxane, the exact amount of pyridine and NMI, and the actual excess of one of the nucleoside components, so often the reactions were allowed to run overnight to secure a complete coupling. The isolated yields of purified dimers 5a–f after an aqueous wash and column chromatography on silica were 61–77%. In some cases (e.g for

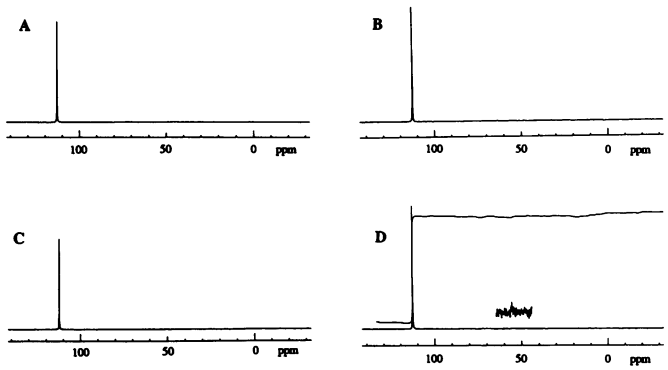


Figure 3. ^{31}P nmr spectra (D_2O/H_2O) of **A**: Crude tetramer 14 with DMT on; **B**: Crude octamer 18 with DMT on; **C**: HPLC-purified hexamer 12; **D**: HPLC-purified octamer 18. The phosphorothioate impurities (ca. 1.5%) seen in D are the result of prolonged contact with aqueous ammonia; crude 18 was kept in four times diluted conc. ammonia for 5 days at 5°C before the HPLC-purification.

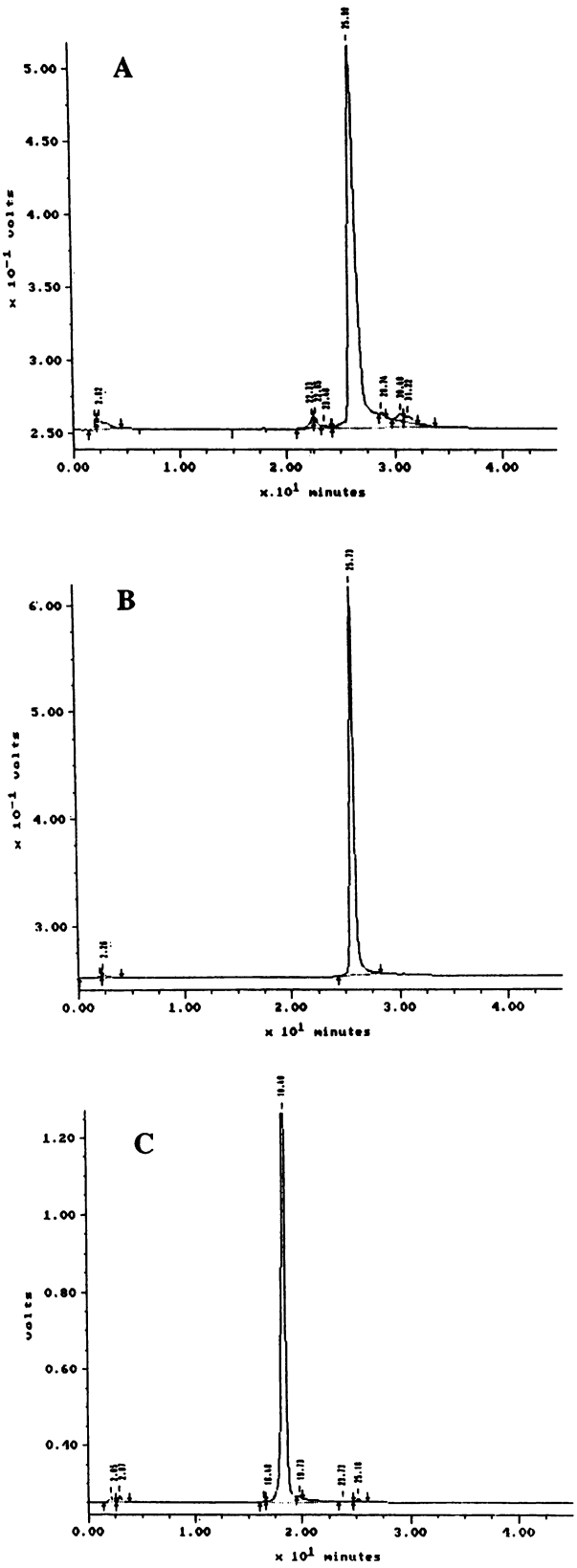


Figure 4. HPLC chromatograms (Hamilton PRP-1 column, rt, eluted with buffer A: 5% acetonitrile, buffer B: 80% acetonitrile in 0.1 M aqueous ammonium hydrogencarbonate, pH 9.0; 100% A for 5 min, 0–100% B for 40 min, 100% B for 5 min). **A**: Crude octamer 18 with DMT on; **B**: Purified octamer 18 with DMT on; **C**: Purified octamer 18.

5d) it was difficult to remove an excess of the protected 5′-hydroxynucleoside 4 by silica column chromatography. In such cases an excess of the activated monomer 2c was used, since excess of the latter was hydrolysed in the washing step to an anion which was easy to remove due to its low mobility on the silica column.

The fully protected dimers 5a−f could be selectively deblocked at the 5′-*O* or the 3′-*O* position to give 6a−f and 7a−e, resp., and coupled to tetramers in the same way as described above for coupling of monomers. The 5′-*O* protecting group was in all cases dimethoxytrityl which could be removed with dichloroacetic acid in dichloromethane, to give the purified 5′-hydroxy dimers 6a, 6b, and 6f in 73−86% yield. The 3′-*O* protecting group was selected to be removable by a mild base treatment (*tert*-butylamine in methanol/acetonitrile, 0°C) without serious cleavage of the base protecting groups, benzoyl at A and C and isobutyryl at G. The phenoxyacetyl (Pac) group could be uniformly used, but for the TT dimer 3′-*O*-acetyl (ac) protection was suitable, if the dimer was used as a 3′-end component or 3′-deblocked before further couplings. The 3′-*O*-protected dimer or tetramer was sometimes poorly soluble in methanol/acetonitrile, and other solvent mixtures had to be used. Methanol/pyridine was acceptable, but in methanol/dichloromethane the removal of the 3′-*O*-protecting group occurred ca. 6 times more slowly than in methanol/acetonitrile, and a significant loss of isobutyryl groups on dG was observed (e.g. 24% for 13). The yields of purified 3′-hydroxy dimers 7a−e were 60−85%.

Two tetramers, 10 and 13, a hexamer 11, and an octamer 17 (Fig. 2) containing PS_2 linkages at all positions were prepared in their protected form and purified by chromatography on silica, in order to show that the chemistry was compatible with the presence of all four bases. The hexamer was prepared in the 5′→3′ direction from dimers using excess of the 3′*O*-protected dimer 6a in each coupling step. The octamer was synthesized from a common tetramer 13 by coupling of the partially deprotected tetramers 15 and 16; an excess of the dimer 8d or a dithiophosphorylated tetramer derived from 15 was used in each coupling step to facilitate the chromatographic separations. In all cases the coupling rates and yields of purified products were similar to those of the monomers.

Fully deblocked oligomers were obtained and purified by a four-step procedure. First the *S*-(2,4-dichlorobenzyl) groups were removed with thiophenol/triethylamine/pyridine. The exchange of the usual solvent dioxane in this mixture with pyridine was necessary for solubility reasons; the cleavage remained fast in pyridine (usually a few h at rt was sufficient, but longer reaction times were not detrimental). Complete removal of the *S*-(2,4-dichlorobenzyl) groups is important since any remaining phosphorodithioate triesters will give phosphorothioate diesters upon the following treatment with aq. ammonia; we therefore checked by ^{31}P nmr that the removal was complete (>99%). Following concentration *in vacuo* the second step was removal of 3′-*O* and base protecting groups with conc. aqueous ammonia at 55°C for 6−8 h. Prolonged contact should be avoided because the dithioate linkage is slowly hydrolysed to monothioate linkages in conc. aqueous ammonia. The hydrolysis is probably most serious at elevated temperatures (approx. 0.5% in 24 h at 55°C), but phosphorothioate impurities also appeared when conc. aqueous ammonia solutions, diluted 2−4 times with water, were kept at 5°C for more than a few days. The cooled conc. aqueous ammonia solution was diluted with water and excess thiophenol oxidised to the disulphide with a slow stream of air. After two to three days, when no more diphenyl disulphide precipitated, the suspension was extracted with ether to give an aqueous solution of the crude oligonucleoside phosphorodithioate with DMT on. The third step was purification by HPLC on a Hamilton PRP-1 column which efficiently separate oligonucleoside phosphorodithioates with DMT on from hydrolysed protecting groups and failure sequences without DMT.[8] The last step was removal of DMT with acetic acid in water followed by extraction with ether.[8]

The oligonucleoside phosphorodithioates prepared by this route were free of phosphorothioate or other phosphorus contaminations, as seen from the ^{31}P nmr spectra of 12, 14, and 18 (Fig. 3). In order to obtain this degree of purity (>99% PS_2) it is important that the *S*-(2,4-dichlorobenzyl) groups are quantitatively removed during the thiophenol treatment, and that contact with aqueous ammonia is not unduly prolonged (see Fig. 3 D). Reverse-phase HPLC chromatograms of crude and purified 18 (Fig. 4) and a denaturing PAGE gel of the fully deprotected oligomers 14 and 18 (Fig. 5) show the homogeneity of the products. The very similar and low PAGE mobility of 14 and 18, compared to the mobility of the unmodified analogue of 18, was surprising and indicates that dithioates sometimes behave anomalously on gels, like they do on ion exchange columns.

The yield of purified oligomers varied with the ease of purification of dimers and oligomers on silica, the loss during deblocking, and the stringency of the HPLC cuts, but representative yields for the individual steps were: Preparation of fully protected dimers and oligomers 60−80%, deblocking of 5′-*O*-DMT and 3′-*O*-acyl 60−85%, deblocking of *S*- and *N*-protecting groups 70−80%, HPLC purification with DMT on plus the final removal of DMT 30−80%. Actual yields found for the hexamer 12, calculated from the amount of starting dimer 7b: 4.5%; for the tetramer GCAT 14, calculated from the amount of starting dimer 6b: 19 or 47% in two separate experiments; for the octamer 18, calculated from the amount of starting dimer 6b: 8 or 15%. These yields are not optimized, and could probably be improved if the syntheses were performed on a larger scale. The substantial loss during HPLC purification is unsatisfactory and indicates a substantial irreversible binding of the compounds

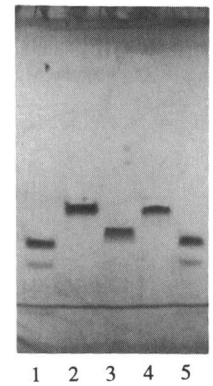


Figure 5. PAGE (20% acrylamide/7 M urea gel, buffer 90 mM Tris−borate, 2.5 mM Na_2EDTA, pH 8.0, samples (0.5 OD) dissolved in 20 μl 7 M urea/10 ×buffer and heated to 90°C for 0.5 h, run at 500 V, visualised by UV shadowing). Lane 1: Bromophenol blue; lane 2: purified dithioate tetramer 14; lane 3: unmodified octamer d(GCATGCAT); lane 4: purified dithioate octamer 18; lane 5: Bromophenol blue.

to the column material. Other column materials (C-18 silica, Pharmacia Mono-Q), however, gave less or no recovery of phosphorodithioates with DMT on.

CONCLUSION

This paper describes in detail the first successful route to pure oligonucleoside phosphorodithioates. The method is based on triester solution chemistry and is quite time consuming, but this is not a serious concern if large amounts of specific sequences are needed, e.g. for physical studies or therapeutic use. The essential chemistry is the coupling of the active phosphorodithioate triester 2c with a 3′-*O*,*N*-protected nucleoside 4 (Fig. 1). This coupling step is rather slow (1.5−3 h at rt) but proceeds cleanly to give protected dinucleoside phosphorodithioates 5, and the dimers can be partially deblocked and coupled to longer oligomers using the same protocol. Although the chemistry appears suitable for solid support syntheses a limited stability of the activated monomers 2c in solution and the slow coupling step makes the method in its present state of development less attractive for automated syntheses.

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