

# Synthesis of phosphorothioamidites derived from 3'-thio-3'-deoxythymidine and 3'-thio-2',3'-dideoxycytidine and the automated synthesis of oligodeoxynucleotides containing a 3'-S-phosphorothiolate linkage

Ghalia Sabbagh, Kevin J. Fettes, Rajendra Gosain, Ian A. O'Neil and Richard Cosstick\*

Department of Chemistry, University of Liverpool, Crown Street, Liverpool L69 7ZD, UK

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## ABSTRACT

The synthesis of *N*-benzoyl-5'-*O*-dimethoxytrityl-2',3'-dideoxy-3'-thiocytidine and its phosphorothioamidite is described for the first time, together with a shortened procedure for the preparation of 5'-*O*-dimethoxytrityl-3'-deoxy-3'-thiothymidine and its corresponding phosphorothioamidite. The first fully automated coupling procedure for the incorporation of a phosphorothioamidite into a synthetic oligodeoxynucleotide has been developed, which conveniently uses routine activators and reagents. Coupling yields using this protocol were in the range of 85–90% and good yields of singularly modified oligonucleotides were obtained. Coupling yields were also equally good when performed on either a 0.2 or 1  $\mu$ mol reaction column, thus facilitating large scale syntheses required for mechanistic studies.

## INTRODUCTION

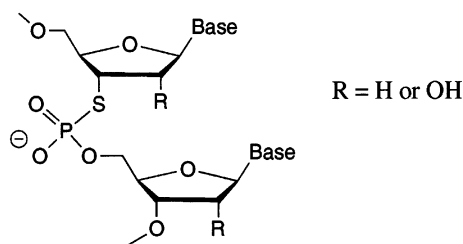
In recent years DNA and RNA analogues have been extensively investigated as potential chemotherapeutic agents (1) and have become essential tools for probing structural and mechanistic aspects of nucleic acid biochemistry (2). Nucleic acid analogues in which the phosphoryl oxygen atoms are replaced with sulfur are amongst the most useful structural modifications. Replacement of one of the non-bridging oxygen atoms, which results in diastereomeric phosphorothioate linkages (3) is readily achieved and these analogues have been widely used to investigate the stereochemistry of phosphoryl transfer reactions (4–8), to identify nucleic acid–protein interactions (9) and to probe for sites of metal ion co-ordination (2,10–12).

Sulfur substitution at one of the bridging oxygen atoms is synthetically more difficult to achieve and has been less extensively investigated. The 3'-*S*-phosphorothiolate linkages

(Fig. 1), in which the 3'-oxygen is replaced by sulfur, have been constructed using a variety of approaches including phosphoramidite chemistry (13–16) and methods based on a Michaelis–Arbusov reaction (17–20). RNA analogues of this type have proved exceptionally useful in detailed mechanistic studies on the role of metal ions (21–26) and hydrogen bonding (27) in RNA cleavage processes. Oligodeoxynucleotides containing this linkage have also been used to investigate a number of processes including: cleavage catalysed by the restriction endonuclease *EcoRV* (28,29); exonucleolytic activity of *Escherichia coli* DNA polymerase I (30,31); and the resolution of Holliday junctions by RuvC (32). NMR studies on oligodeoxynucleotides containing a 3'-*S*-phosphorothiolate linkage have shown that 3'-sulfur substitution results in a sugar conformation that is predominately 3'-*C-endo* (33,34). Very recent thermal melting studies reveal that, when placed in a DNA strand, this linkage stabilises duplex formation with complementary RNA, by up to 2°C per phosphorothiolate linkage (35). Duplex stabilisation results from a locally induced RNA conformation in the DNA strand and maximum effect is derived from an alternate spacing of the phosphorothiolate linkages. The results suggest that these analogues are interesting as potential antisense agents and as conformational mimics of RNA.

Despite the increasingly widespread use of oligonucleotides containing a single 3'-*S*-phosphorothiolate linkage, there is no report of a fully automated synthesis protocol for incorporation of these analogues. We now wish to report significant developments in the synthesis of oligodeoxynucleotides containing a 3'-*S*-phosphorothiolate linkage associated with a pyrimidine nucleoside, including: the synthesis of the phosphorothioamidite derived from 2',3'-dideoxy-3'-thiocytidine; a shortened procedure for the synthesis of phosphorothioamidite derived from 3'-deoxy-3'-thiothymidine; and a fully automated synthesis protocol for incorporation of this thioamidite based on commercially available activators and reagents. Some parts of this study, relating to the incorporation of 3'-thiothymidine into oligodeoxynucleotides, have already been published in a preliminary form (36).

\*To whom correspondence should be addressed. Tel: +44 151 794 3514; Fax: +44 151 794 3588; Email: rcosstic@liverpool.ac.uk



**Figure 1.** 3'-S-Phosphorothiolate linkage.

## MATERIALS AND METHODS

### General methods

FAB mass spectra were recorded on either a VG Analytical 7070E mass spectrometer operating with a PDP 11/250 data system and an Ion Tech FAB ion gun working at 8 kV (Liverpool). High resolution FAB mass spectra were obtained on a VG ZAB/E spectrometer at the EPSRC Mass Spectrometry Service Centre (Swansea, UK). 3-Nitrobenzyl alcohol was used as a matrix. Electrospray mass spectrometry was performed on either a Micromass Quattro II quadrupole mass spectrometer (EPSRC Mass Spectrometry Service Centre) or a Micromass LCT mass spectrometer (Liverpool). Samples in the range of  $10^{-5}$  M were injected in MeOH/H<sub>2</sub>O (1:1). <sup>1</sup>H and <sup>13</sup>C NMR spectra were measured on either a Bruker AMX400 or a Varian Gemini 300 spectrometer and chemical shifts are given in p.p.m. downfield from an internal standard of tetramethylsilane. All DNA synthesis reagents were purchased from Glen Research (Virginia, USA) including reaction columns, standard nucleoside 3'-O-(2-cyanoethyl) phosphoramidites and the activators 5-ethylthiotetrazole (ETT) and 4,5-dicyanoimidazole (DCI).

### Synthesis of nucleoside phosphorothioamidites

**2,3'-O-Anhydronucleosides.** To a stirred solution of the protected nucleoside (**1a/1b**) (1.84 mmol) and triphenylphosphine (2.0 mmol, 0.53g) in ethyl acetate (5 ml), diisopropylazodicarboxylate (2.0 mmol, 0.53g) was added dropwise over a period of 5–10 min, and after stirring for a further 15 min the solvent was concentrated under vacuum. The residue was purified by flash chromatography eluting with a gradient of methanol in ethyl acetate.

**5'-O-(4,4'-dimethoxytrityl)-2,3'-anhydrothymidine (2a).** Eluting solvent for flash chromatography, ethyl acetate followed by a gradient of 0–5% methanol in ethyl acetate, to afford the product as a white solid (93%); <sup>1</sup>H NMR (400 MHz; CDCl<sub>3</sub>) 1.94 (3H, d, *J* = 1.2, 5'-CH<sub>3</sub>), 2.41 (1H, ddd, *J* = 12.8, 3.7, 2.7 Hz, H-2'), 2.58 (1H, dd, *J* = 12.8, 1.2 Hz, H-2''), 3.34 (2H, m, H-5' and 5''), 3.78 (6H, s, 2 × OMe), 4.27 (1H, ddd, *J* = 8.9, 6.5, 2.5 Hz, H-4'), 5.16 (1H, m, H-3'), 5.44 (1H, d, *J* = 3.85 Hz, H-1'), 6.80 (4H, m, DMT), 6.91 (1H, d, *J* = 1.4, H-6), 7.39 (9H, m, DMT); HRMS (ES<sup>+</sup>) 527.2178 [C<sub>31</sub>H<sub>31</sub>O<sub>6</sub>N<sub>2</sub> (M+H)<sup>+</sup> requires 527.2182].

**N4-Benzoyl-5'-O-(4,4'-dimethoxytrityl)-2,3-anhydro-2'-deoxycytidine (2b).** Eluting solvent for flash chromatography, ethyl

acetate followed by EtOAc:MeOH (3:1), to afford the product as a pale-brown, glassy solid (84%); <sup>1</sup>H NMR (400 MHz; CDCl<sub>3</sub>) 2.27–2.32 (1H, dt, *J* = 12.8, 6.4 Hz, H-2'), 2.62–2.65 (1H, d, *J* = 12.8 Hz, H-2'), 3.27–3.31 (1H, dd, *J* = 10.2, 6.14 Hz, H-5'), 3.34–3.39 (1H, dd, *J* = 10.2, 7.3 Hz, H-5'), 3.76 (6H, s, 2 × OMe), 4.17–4.21 (1H, ddd, *J* = 10, 6.9, 2.3 Hz, H-4'), 5.10 (1H, m, H-3'), 5.49–5.50 (1H, m, H-1'), 6.51–6.53 (1H, d, *J* = 7.5 Hz, H-2' cyt), 6.80–6.82 (4H, dd, *J* = 8.8, 1.9 Hz, DMT), 7.00–7.02 (1H, d, *J* = 7.5 Hz, H-1' cyt), 7.11–7.52 (12H, m, Ar-H), 8.09–8.11 (2H, dd, *J* = 8.6, 1.4 Hz, Ar-H); HRMS (FAB<sup>+</sup>) 616.2445 [C<sub>37</sub>H<sub>34</sub>O<sub>6</sub>N<sub>3</sub> (M+H)<sup>+</sup> requires 616.2448].

**3'-S-Benzoyl-3'-deoxy-5'-O-(4,4'-dimethoxytrityl)thymidine (3a).** To 5'-O-(4,4'-dimethoxytrityl)-2,3'-anhydrothymidine (6.90 g, 13.10 mmol) and cesium thiobenzoate (17.7 g, 65.50 mmol) anhydrous DMF (16.35 ml) was added and the solution heated to 110°C. Single equivalents of cesium thiobenzoate (3.54 g, 13.10 mmol) were subsequently added every hour. After 5 h the solvent was removed *in vacuo* and the resultant brown foam resuspended in EtOAc (200 ml) and washed with saturated sodium hydrogen carbonate solution (3 × 100 ml). The organic phase was dried over MgSO<sub>4</sub> and concentrated *in vacuo*. The residue was purified by flash chromatography eluting with a gradient of 0→1% methanol in CH<sub>2</sub>Cl<sub>2</sub> to yield **2** as a light brown foam (7.10 g, 82%). <sup>1</sup>H NMR (400 MHz; CDCl<sub>3</sub>) 1.50 (3H, s, 5'-CH<sub>3</sub>), 2.53 (1H, ddd, *J* = 14.0, 7.0, 7.0 Hz, H2''), 2.76 (1H, ddd, *J* = 13.8, 8.5, 5.4 Hz, H2'), 3.46 (1H, dd, *J* = 11.0, 3.0 Hz, H5''), 3.54 (1H, dd, *J* = 10.7, 2.3 Hz, H5'), 3.75 [6H, s, (OCH<sub>3</sub>)<sub>2</sub>], 4.16 (1H, m, H4'), 4.50 (1H, q, *J* = 7.4 Hz, H3'), 6.30 (1H, t, *J* = 5.9 Hz), 6.81 (4H, dd, *J* = 8.9, 2.6 Hz, DMT), 7.18–7.51 (12H, m, DMT and Bz), 7.71 (1H, d, *J* = 1.1 Hz, H6), 7.90 (2H, dd, *J* = 8.2, 1.6 Hz, Bz); *m/z* (FAB<sup>+</sup>) 665 ([M+H]<sup>+</sup>, 1), 664 (M<sup>+</sup>) and 303 (100%). HRMS (FAB<sup>+</sup>) 664.2230 [C<sub>38</sub>H<sub>36</sub>N<sub>2</sub>O<sub>7</sub>S (M<sup>+</sup>) requires 664.2243].

**N4-Benzoyl-3'-S-benzoyl-2',3'-dideoxy-5'-O-(4,4'-dimethoxytrityl)cytidine (3b).** A suspension of cesium thiobenzoate (750 mg, 2.67 mmol) and 18-crown-6 (200 mg, 0.77 mmol) in anhydrous acetonitrile (5.0 ml) under argon was rapidly dispersed with stirring under reflux for 10 min, by which time the contents had dissolved. The anhydrocytidine derivative (**2b**) (0.48 g, 0.78 mmol) was rapidly added in one portion to the reaction flask and the reaction heated with stirring for a further hour, before it was cooled to room temperature and the contents transferred with ethyl acetate to a separating funnel containing water (20 ml). Following extraction with ethyl acetate (2 × 20 ml), the combined organic solution was dried over sodium sulfate, filtered and concentrated under reduced pressure to yield the crude product which chromatographed (SiO<sub>2</sub>; ethyl acetate) to afford the product as a beige glassy solid (370 mg, 64%); <sup>1</sup>H NMR (400 MHz; CDCl<sub>3</sub>) 2.68–2.70 (1H, m, H-2'), 2.76–2.78 (1H, m, H-2'), 3.52–3.54 (1H, dd, *J* = 10.7, 2.7 Hz, H-5'), 3.57–3.59 (1H, dd, *J* = 10.7, 2.1 Hz, H-5'), 3.75 (6H, s, 2 × OMe), 4.19–4.21 (1H, m, H-4'), 4.46–4.48 (1H, q, *J* = 7.2 Hz, H-3'), 6.23–6.25 (1H, dd, *J* = 6.7, 2.7 Hz, H-1'), 6.81–6.83 (4H, dd, *J* = 8.8, 1.2 Hz, DMT), 7.26–7.52 (17H, m, Ar-H, cyt-H), 7.89–7.92 (4H, m, Ar-H), 9.00 (1H, br, N-H); HRMS (ES<sup>+</sup>) 754.2585 [C<sub>44</sub>H<sub>40</sub>N<sub>3</sub>O<sub>7</sub>S (M+H)<sup>+</sup> requires 754.2587].

*3'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-3'-thiothymidine (4a)*. To ethanol (500 ml) was added 10 M NaOH solution (21.7 ml, 217 mmol) and argon bubbled through for 30 min. To this degassed solution **3a** (4.80 g, 7.22 mmol) was added and the solution stirred whilst argon was bubbled through. After 1 h a solution of 1 M  $\text{KH}_2\text{PO}_4$  (900 ml) was slowly added over 15 min. The resultant precipitate was then filtered and washed with distilled water ( $3 \times 200$  ml). The precipitate was desiccated over  $\text{P}_2\text{O}_5$  for 48 h to yield **4a** as an off-white powder (3.37 g, 83%);  $^1\text{H}$  NMR (400 MHz;  $\text{CDCl}_3$ ) 1.50 (3H, d,  $J = 1.6$  Hz,  $5'-\text{CH}_3$ ), 1.56 (1H, d,  $J = 7.7$  Hz, thiol), 2.36 (1H, ddd,  $J = 13.9, 10.1, 6.8$  Hz,  $\text{H}2'$ ), 2.60 (1H, ddd,  $J = 13.9, 7.7, 3.0$  Hz,  $\text{H}2''$ ), 3.41 (1H, dd,  $J = 11.1, 2.7$  Hz,  $\text{H}5''$ ), 3.56–3.68 (2H, m,  $\text{H}3', \text{H}5'$ ), 3.80 [6H, s,  $(\text{OCH}_3)_2$ ], 3.87 (1H, dt,  $J = 8.4, 2.7, 2.7$  Hz,  $\text{H}4'$ ), 6.15 (1H, dd,  $J = 6.9, 3.0$  Hz,  $\text{H}1'$ ), 6.82 (4H, d,  $J = 8.7$  Hz, DMT), 7.20–7.43 (9H, m, DMT), 7.68 (1H, d,  $J = 1.2$  Hz,  $\text{H}6$ ), 8.30 (1H, br, NH);  $m/z$  ( $\text{ES}^+$ ) 599 ( $[\text{M}+\text{K}]^+$ , 1), 583 ( $\text{M}+\text{Na}^+$ , 3), 560 ( $\text{M}^+$ , 3) and 303 (100%); HRMS ( $\text{ES}^+$ ), 583.1868 [ $\text{C}_{31}\text{H}_{32}\text{N}_2\text{O}_6\text{SNa}$  ( $\text{M}+\text{Na}^+$ ) requires 583.1879].

*N4-benzoyl-5'-O-dimethoxytrityl-2',3'-dideoxy-3'-thiocytidine (4b)*. **3b** (140 mg, 0.18 mmol) was dissolved in a solution of THF (5.0 ml), methanol (4.0 ml) and water (1.0 ml), prior to the contents of the flask being purged with argon and cooled to  $-10^\circ\text{C}$  via an ice–salt–methanol bath. A degassed solution of freshly prepared sodium hydroxide (0.5 M, 1.0 ml) was added, and the contents stirred for 10 min at  $0^\circ\text{C}$ . At this juncture, TLC analysis indicated the complete consumption of the thioester. A cold aqueous solution of citric acid (1.0 M, 3.0 ml) was added via a syringe, and the contents left to stir for a further 10 min at  $0^\circ\text{C}$ . The mixture was transferred with ethyl acetate to a separating funnel containing saturated sodium bicarbonate solution (20 ml), and extracted with ethyl acetate ( $2 \times 20$  ml) and then dried over sodium sulfate. Following concentration under reduced pressure, the crude product obtained was purified by chromatography ( $\text{SiO}_2$ ; ethyl acetate:hexane = 3:1) to furnish the thiol as a white glassy solid (95 mg, 79%);  $^1\text{H}$  NMR (400 MHz;  $\text{CDCl}_3$ ) 1.45 (1H, d,  $J = 7.4$  Hz, S-H), 2.42–2.50 (1H, ddd,  $J = 14, 12, 6.9$  Hz, H-2'), 2.64–2.70 (1H, dd,  $J = 14, 6.4$  Hz, H-2'), 3.45–3.50 (1H, m, H-3'), 3.52–3.55 (1H, dd,  $J = 11.5, 2.5$  Hz, H-5'), 3.64–3.70 (1H, dd,  $J = 11.5, 2.2$  Hz, H-5'), 3.83 (6H, s,  $2 \times \text{OMe}$ ), 3.88–3.91 (1H, dt,  $J = 10, 2.3$  Hz, H-4'), 6.14 (1H, d,  $J = 6.0$  Hz, H-1'), 6.88–6.91 (4H, dd,  $J = 8.7, 1.8$  Hz, Ar-H), 7.26–7.53 (14H, m, Ar-H and cyt-H), 7.87–7.89 (2H, d,  $J = 8.2$  Hz, Ar-H), 8.63–8.65 (1H, br, N-H); HRMS ( $\text{ES}^+$ ) 650.2322 [ $\text{C}_{37}\text{H}_{36}\text{N}_3\text{O}_6\text{S}$  ( $\text{M}+\text{H}^+$ ) requires 650.2325].

### Nucleoside phosphorothioamidites

To a stirred solution of diisopropylammonium tetrazolide (35 mg) and thionucleoside (200 mg, 0.30 mmol) in dichloromethane (5 ml) under argon at room temperature, a solution of 2-cyanoethyl tetraisopropylphosphorodiamidite (95 mg, 0.32 mmol) in dichloromethane was added by syringe. The reaction was left to stir overnight, before the contents were transferred, with dichloromethane, to a separating funnel containing saturated sodium bicarbonate (20 ml). Following extraction with dichloromethane ( $2 \times 15$  ml), the contents were dried over sodium sulfate, filtered and concentrated under reduced pressure to afford the crude residue which was chromatographed on silica gel.

*3'-S-(2-Cyanoethyl-N,N-diisopropylphosphorothioamidite)-3'-deoxy-5'-O-(4,4'-dimethoxytrityl)thymidine (5a)*. Elution with  $\text{CH}_2\text{Cl}_2$ :petroleum ether ( $40-60^\circ\text{C}$ ): $\text{Et}_3\text{N}$  (49.5:49.5:1) initially followed by  $\text{EtOAc}$ :petroleum ether ( $40-60^\circ\text{C}$ ): $\text{Et}_3\text{N}$  (49.5:49.5:1) and subsequently precipitated from  $\text{CH}_2\text{Cl}_2$  and hexane to yield the title compound as a white precipitate (87%).  $^{31}\text{P}$  NMR (122 MHz;  $\text{CDCl}_3$ ) 159.5, 163.6;  $^1\text{H}$  NMR (400 MHz;  $\text{CDCl}_3$ ) 1.18 [12H, m,  $2 \times \text{NCH}(\text{CH}_3)_2$ ], 1.37 and 1.42 (diastereotopic, 3H, d,  $J = 1.1$  Hz), 2.39–2.65 (4H, m,  $\text{H}2', \text{H}2''$  and  $\text{CH}_2\text{CH}_2\text{CN}$ ), 3.38–3.68 [7H, m,  $\text{H}5', \text{H}5'', \text{H}3'$ ,  $2 \times \text{NCH}(\text{CH}_3)_2$  and  $\text{OCH}_2\text{CH}_2\text{CN}$ ], 3.79 and 3.80 (diastereotopic, 6H, ds,  $2 \times \text{OCH}_3$ ), 4.05 (1H, m,  $\text{H}4'$ ), 6.18 (0.5H, m,  $\text{H}1'$ ), 6.23 (0.5H, m,  $\text{H}1'$ ), 6.82 (4H, m, DMT), 7.22–7.48 (9H, m, DMT), 7.66 (0.5H, d,  $J = 1.2$  Hz,  $\text{H}6$ ), 7.72 (0.5H, d,  $J = 1.2$  Hz,  $\text{H}6$ ) and 8.47 (1H, bs, NH);  $m/z$  ( $\text{FAB}^+$ ) 799 ( $[\text{M}+\text{K}]^+$ , 5), 783 ( $\text{M}+\text{Na}^+$ , 20%), 635 (5%), 303 (99%), 201 (100%) and 102 (71%). HRMS ( $\text{ES}^+$ ) 783.2959 [ $\text{C}_{40}\text{H}_{49}\text{N}_4\text{O}_7\text{PSNa}$  ( $\text{M}+\text{Na}^+$ ) requires 783.2957].

*3'-S-(2-Cyanoethyl-N,N-diisopropylphosphorothioamidite)-N4-benzoyl-3'-deoxy-5'-O-(4,4'-dimethoxytrityl)cytidine (5b)*. Elution with  $\text{EtOAc}$  containing 1%  $\text{Et}_3\text{N}$  to afford the product as a white glassy solid (73%) consisting of a diastereomeric mixture;  $^{31}\text{P}$  NMR (162 MHz;  $\text{CDCl}_3$ ) 162.0, 166.2;  $^1\text{H}$  NMR (400 MHz;  $\text{CDCl}_3$ ) 1.18–1.34 (12H, m,  $4 \times \text{Me}$ ), 1.99–2.03 (1H, m, H-2'), 2.35–2.40 (2H, t,  $J = 6.20$  Hz,  $\text{NC}-\text{CH}_2$ ), 2.58–2.62 (3H, m,  $2 \times \text{N}-\text{CH}$  and H-2'), 3.38–3.64 (5H, m,  $2 \times \text{H}-5'$ ; H-4' and P-O- $\text{CH}_2$ ), 3.88 (6H, s,  $2 \times \text{OMe}$ ), 4.01–4.05 (1H, 'd',  $J = 11.50$  Hz, H-3'), 6.10–6.14 (1H, t,  $J = 6.0$  Hz, H-1'), 6.82 (4H, d,  $J = 8.80$  Hz, Ar-H), 7.27–7.49 (8H, m, Ar-H, cyt-H), 7.53–7.56 (1H, t,  $J = 8.0$  Hz, Ar-H), 7.91–7.93 (2H, d,  $J = 7.4$  Hz, Ar-H), 8.74 (1H, br, N-H); HRMS ( $\text{ES}^+$ ) 850.3405 [ $\text{C}_{46}\text{H}_{53}\text{N}_5\text{O}_7\text{PS}$  ( $\text{M}+\text{H}^+$ ) requires 850.3403].

### Synthesis of oligonucleotides

All oligonucleotides were synthesised on an Expedite™ 8909 DNA synthesiser using reagent solutions prepared with DNA synthesis-grade acetonitrile. The synthesis procedure used the standard capping, oxidation and deblocking reagents and protocols. The regular A, T, C and G amidite solutions were used at the recommended concentration (50 mg/ml). The only changes to the synthesis protocol were in the thioamidite coupling cycle. The thymidine 3'-S-phosphorothioamidite was prepared as a solution in acetonitrile at 100 mg/ml and delivered from amidite position 5 on the synthesiser (corresponding to base X in the Expedite synthesis protocol). The activator used was a solution (1 M in acetonitrile) of either ETT or DCI and it was delivered from either the regular activator position on the synthesiser (ACT) or amidite position 6. Optimised coupling times for the thioamidite cycle were either 2.5 min when using ETT as an activator or 15 min when using DCI. When DCI was used on the regular activator position, selection of base X (corresponding to the thioamidite) gave the desired 15 min coupling cycle (see Results and Discussion).

Coupling efficiencies for oligonucleotide synthesis were measured using an on-line dimethoxytrityl cation detector. All oligonucleotides were synthesised 'trityl-on'. Post-synthesis the column was blown dry with helium and the CPG beads were treated with concentrated aqueous ammonia/ethanol, 3:1 v/v, overnight at  $55^\circ\text{C}$ . The crude oligonucleotides were then

**Table 1.** Electrospray HRMS data for phosphorothiolate-containing oligonucleotides

| Sequence            | Calculated mass       | Measured mass               |
|---------------------|-----------------------|-----------------------------|
| d(CCT AAA TTT GCC)  | 3578.639              | 3578.515                    |
| d(CCT AAA TsTT GCC) | 3594.616              | 3594.600                    |
| d(CCT AAA TTsT GCC) | 3594.616              | 3594.512                    |
| d(CCT AAA TTTs GCC) | 3594.616              | 3594.590                    |
| d(TTT TTsT TTT T)   | 2996.000 <sup>a</sup> | 2995.900 ± 0.3 <sup>a</sup> |
| d(CCsT AAA TTT GCC) | 3594.616              | 3594.579                    |
| d(CCT AAA TTT GCsC) | 3594.616              | 3594.601                    |

<sup>a</sup>Nominal mass data obtained.

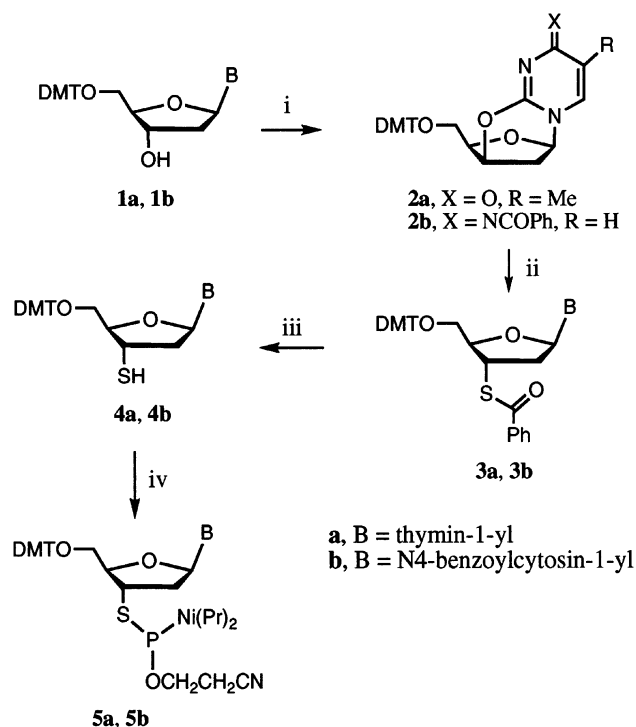
filtered using 0.2 µm syringe filters (Nalgene™) and concentrated to dryness using a rotary concentrator (Savant). The crude oligonucleotides were then reconstituted in 100 mM triethylammonium bicarbonate (TEAB). Reverse-phase HPLC was then carried out using a Hamilton PRP-1 (250 × 4.1 mm) column. Conditions were as follows: Solvent A (0.1 M TEAB) and Solvent B (60% 0.1 M TEAB, 40% MeCN); starting conditions 100% A then a linear gradient to 50% B over 10 min; 100% B over a further 5 min; then hold at 100% B for a further 5 min and returned to starting conditions over 5 min. The purified oligonucleotide was then concentrated to dryness *in vacuo* and treated with 80% acetic acid for 30 min. The detritylated oligonucleotide was then concentrated to dryness *in vacuo* then redissolved in distilled H<sub>2</sub>O and extracted with water-saturated diethyl ether. The aqueous phase was then concentrated to dryness *in vacuo* and reconstituted in distilled H<sub>2</sub>O. Oligonucleotides were re-analysed by HPLC and the incorporation of the phosphorothiolate modification confirmed by electrospray mass spectrometry (Table 1).

## RESULTS AND DISCUSSION

### Preparation of thionucleosides

2'-Deoxy-3'-thionucleosides derived from thymine (13,14) and uracil (21) have previously been prepared and incorporated into oligonucleotides. In both cases the suitably protected 3'-thionucleosides were synthesised from the xylo-configured nucleoside by displacement of the sulfonate ester with sodium thiobenzoate. However, a more immediate route to 3'-deoxy-3'-thiopyrimidine nucleosides was envisaged through direct ring-opening of a 2,3'-anhydronucleoside with thiobenzoate (Scheme 1). Synthesis started from DMT-protected nucleosides; this is in contrast to the originally reported work on 3'-deoxy-3'-thiothymidine that used the more robust MMT group (14). Thus, starting from either 5'-O-(4,4'-dimethoxytrityl)thymidine (**1a**) or N<sup>4</sup>-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine (**1b**) the anhydronucleosides were prepared by a Mitsunobu reaction in ethyl acetate. The Mitsunobu reaction has been most widely performed on unprotected nucleosides in DMF, however, in this case the DMT-protected nucleosides are readily soluble in ethyl acetate and using this solvent the reaction mixture could be concentrated and then applied directly to a flash column for purification.

Conversion of the anhydronucleosides to their corresponding thioesters (**3**), was achieved using the cesium salt of



**Scheme 1.** Reagents and conditions. (i) Triphenylphosphine, diisopropylazo dicarboxylate, ethyl acetate; (ii) CsBz, DMF, 110°C; (iii) aqueous NaOH; (iv) diisopropylammonium tetrazolidate, 2-cyanoethyl tetraisopropylphosphorodiamidite, dichloromethane.

thiobenzoic acid, which gave slightly better yields than the sodium or potassium salts. The thymidine-derived thioester (**3a**) was obtained in 83% yield by reaction of the anhydronucleoside with a large excess of cesium thiobenzoate in DMF at 110°C. Under analogous conditions, ring-opening of the anhydrocytosine proved to be a more capricious reaction and gave significantly lower and less reproducible yields. However, when the reaction was performed in acetonitrile in the presence of 18-crown-6 and only 3.5 equivalents of cesium thiobenzoate, yields between 60–65% were consistently achievable. The <sup>13</sup>C NMR spectrum of both thioesters showed a characteristic signal attributable to the thioester carbonyl group at ~190 p.p.m.

Debenzoylation of the thymidine-derived thioester (**3a**) was accomplished using an argon-saturated solution of sodium hydroxide (0.4 M) in aqueous ethanol. Conveniently, neutralisation of the reaction mixture with aqueous KH<sub>2</sub>PO<sub>4</sub> precipitated the thionucleoside (**4a**, 83%) as an off-white powder which could, after drying, be used without further purification. In the case of corresponding 2'-deoxycytidine thioester, milder conditions were necessary in order to prevent concomitant removal of the N-benzoyl group. Thus, **3b** was treated with an argon-purged solution of sodium hydroxide (0.045 M) in methanol/THF/water at -10°C for 10 min. The reaction was quenched by the addition of citric acid and the thiocytidine derivative purified, after work-up by chromatography. The <sup>1</sup>H NMR spectrum of both thionucleosides showed a characteristic doublet attributable to the mercapto proton (**4a**, 1.56 p.p.m. and **4b**, 1.45 p.p.m.). Both thionucleosides were converted to their phosphorothioamidites (**5a** and

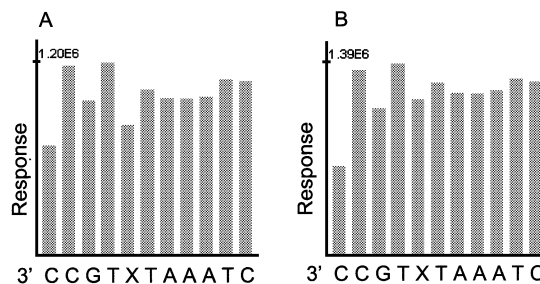
**5b)** by overnight reaction with 2-cyanoethyl tetraisopropylphosphorodiamidite in the presence of diisopropylammonium tetrazolide. After work-up and chromatography the thioamidites gave the expected  $^{31}\text{P}$  NMR spectra.

### Oligonucleotide synthesis

In formerly described procedures for the synthesis of oligonucleotides containing 3'-S-phosphorothiolate linkages the key phosphorothioamidite coupling step has been performed by removing the reaction column from the synthesiser and manually introducing the thioamidite and activator [5-(*p*-nitrophenyl)tetrazole] by syringe (16,21,28). This manual coupling procedure made the rigorous exclusion of moisture difficult and the result was a time-consuming and intricate operation that also gave inconsistent yields. Thus, there was an obvious requirement for a fully automated and relatively simple procedure that would make these analogues more accessible to the nucleic acid community.

Original studies had shown that phosphorothioamidites are much less reactive than the standard amidites and that 5-(*p*-nitrophenyl)tetrazole was a superior reagent to tetrazole for coupling phosphorothioamidites (14). Unfortunately, however, 5-(*p*-nitrophenyl)tetrazole is poorly soluble in acetonitrile and not ideally suited to automated synthesis. Other potential activators were also considered, such as salts derived from pyridines (37), however, these compounds suffer disadvantages in that they are not readily available in the high purity required for DNA synthesis, whereas commercially available activators are reliable and conveniently packaged for use. ETT has been extensively used as an activator in RNA synthesis where it has been shown to increase coupling yields and decrease coupling times (38). Additionally, DCI has been found to increase coupling yields by a similar factor although it is preferred due to its reduced acidity (39).

To assess these commercially available activators and optimise the synthesis cycle, two test sequences [d(TTT TTsT TTT T) and d(CCT AAA TTsT GCC), where Ts = 3'-deoxy-3'-thiothymidine], which contain a single phosphorothiolate residue, were synthesised. When coupling times, activator and thioamidite concentrations were used at their standard values, coupling yields were disappointing. Under these conditions tetrazole failed to catalyse any incorporation of the thioamidite, as determined by the trityl release assay; whereas DCI and ETT gave coupling yields that were detectable, but only around 10%. Such is the low reactivity of the phosphorothioamidite it was necessary to increase both the concentration of the activators and the coupling time. Using the activators at a concentration of 1 M with a protocol that involves a double 15 min coupling cycle, yields were obtained for the introduction of the thioamidite that were in the region of 70–80% for ETT and 65–75% for DCI, as determined by the trityl release assay. However, by increasing the concentration of the thioamidite monomer to 100 mg/ml, the coupling yield with DCI was increased to 85–90% with a single 15 min coupling procedure. Further reduction of the coupling time resulted in a gradual decline in coupling yield. When 1 M ETT was used as the activator with the higher concentration of thioamidite, the optimum coupling time was reduced to 2.5 min which gave a similar 85–90% yield (Fig. 2). When coupling with ETT was extended to 15 min the yield of coupled product was marginally reduced. For both activators, coupling yields



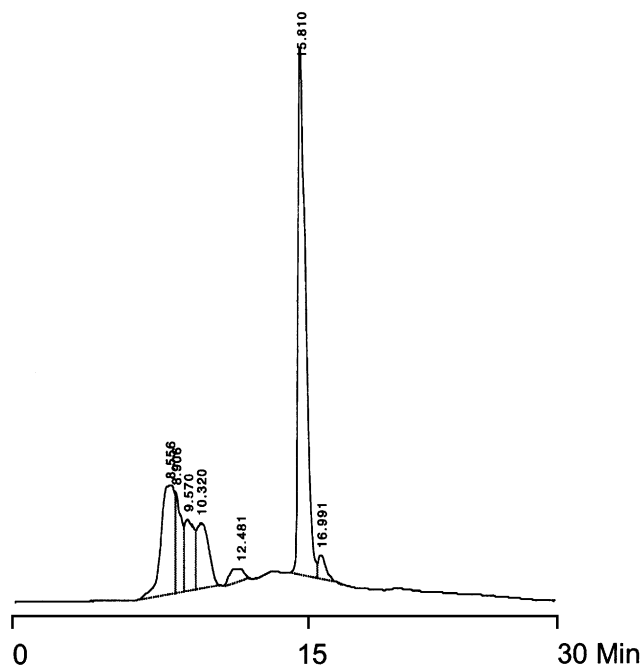
**Figure 2.** Trityl release assay showing coupling efficiencies for the incorporation of thymidine phosphorothioamidite into d(CCT AAA TTsT GCC), using: (A) 1 M solution of ETT (2.5 min coupling time); (B) 1 M solution of DCI (15 min coupling time). The trityl release signature for thiothymidine shows the same intensity dip as observed for guanosine nucleosides, presumably due to relatively rapid detritylation (40). For this reason, yields for the incorporation of the phosphorothioamidite were based on the yield of the subsequent nucleotide incorporation.

were also equally good when performed on either a 0.2 or 1  $\mu\text{mol}$  reaction column.

At the concentration of 1 M, both ETT and DCI show good solubility in acetonitrile and therefore do not present a hazard to the synthesiser. It should be noted that DCI was initially developed to be used at this concentration and is not reported to cause detritylation of the monomer and the resultant unwanted side reactions (39). In the case of ETT there was concern that the greater acidity of this activator may result in a small amount of adventitious detritylation of the monomer. However, it was expected that a significant problem of this type would be apparent from the HPLC analysis of the crude oligonucleotide. Thus, after a standard deprotection treatment, the crude trityl-on oligonucleotides were analysed by reverse-phase HPLC (Fig. 3). Chromatograms obtained using the two activators showed no significant differences, and on this basis both ETT and DCI appeared to be equally suitable activators.

Following HPLC purification and removal of the DMT group, phosphorothiolate-containing oligonucleotides were generally  $\geq 95\%$  pure, as judged by reverse phase HPLC, and gave the expected pseudomolecular ions when analysed by electrospray mass spectrometry (see Materials and Methods). Yields obtained were about 10% lower than those of the unmodified oligomers. Absolute yields are not reported as manufacturers inform us that the amount of support-bound nucleoside is not accurately reproduced from one synthesis column to another and the size of the column (0.2 or 1  $\mu\text{mol}$ ) defines the minimum amount of support-bound nucleoside. In addition, in our experience, significant losses of the oligonucleotide that occur during HPLC purification make absolute yields and their comparison less meaningful.

The standard iodine oxidation solution is used in this protocol, rather than the previously used alternatives such as tetra-*n*-butylammonium periodate (28). There was some apprehension with regard to the use of this oxidant as aqueous iodine solutions have been shown to cleave the P-S bond in a phosphorothiolate diester (14,20). However, the trityl release assay reveals that the coupling yields are consistently maintained subsequent to the introduction of the phosphorothiolate linkage (Fig. 2) and supports an earlier finding (16) that the brief iodine oxidation step is not detrimental to the phosphorothiolate triester to any significant extent.



**Figure 3.** Reverse-phase HPLC trace of crude d(CCT AAA TTsT GCC), synthesised using ETT. See Materials and Methods for elution conditions.

Whilst these results show that equally efficient coupling can be achieved with either DCI or ETT, the use of DCI is particularly attractive on the Expedite instrument. When 1 M DCI replaces the standard activator on the instrument and the phosphorothioamidite is used at amidite position 5, the selection of base X in the sequence editor delivers by default the required 15 min coupling. However, to reduce activator consumption, the 1 M DCI or ETT solution can be attached at position 6 on the synthesiser in addition to the standard activator. Minor alterations in the synthesis protocols can then allow the alternative activator on position 6 to be used only for the phosphorothioamidite coupling step. In the case of ETT this procedure also has the advantage of reducing the possibility of monomer detritylation. The majority of our syntheses were conducted using this two-activator approach.

### Conclusions

Robust procedures are described for the syntheses of the thioamidites derived from *N*4-benzoyl-5'-*O*-dimethoxytrityl-2',3'-dideoxy-3'-thiocytidine and 3'-deoxy-3'-thiothymidine. A fully automated coupling procedure has been developed for the incorporation of the phosphorothioamidite that uses routine activators and reagents together with an extended coupling time. Coupling yields using this protocol and the activator ETT were in the range of 85–90%. Although yields are not currently high enough to support repetitive phosphorothiolate incorporation, good yields of singularly modified oligonucleotides are obtained in high purity. However, given that previously published uses of phosphorothiolate oligonucleotides are as probes to investigate specific nucleic acid processes, and therefore only require a single modification, the procedures reported here represent a very significant improvement in the synthesis of these analogues. Using these procedures d(CCT AAA TTsT GCC) has been obtained in

sufficient purity and quantity for NMR structural studies (34,35).

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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