Synthesis and properties of dithymidine phosphate analogues containing 3'-thiothymidine

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ABSTRACT

Dithymidine-3'-S-phosphorothioate (d(TspT)) has been prepared from a 5'-O-monomethoxytritylthymidine-3'-S-phosphorothioamidite (7) by activation with 5-(p-nitrophenyl)tetrazole in the presence of 3'-O-acetylthymidine. The resulting dinucleoside phosphorothioite is readily oxidised to the corresponding 3'-S-phosphorothioate using either tetrabutylammonium (TBA) periodate or TBA oxone and has been deprotected under standard conditions to yield d(TspT). This dithymidine phosphate analogue is comparatively resistant to hydrolysis by nuclease P1, but the P-S bond is readily cleaved by aqueous solutions of either iodine or silver nitrate. Dithymidine-3'-S-phosphorodithioate (d[Tsp(s)T]) was prepared in an analogous fashion using sulphur to oxidise the intermediate dinucleoside phosphorothioite. Absolute stereochemistry has been assigned to the diastereoisomers of d[Tsp(s)T] by comparing their physical and chemical properties to those of the dinucleoside phosphorothioates.

INTRODUCTION

Oligonucleotides containing phosphorothioate linkages have proved useful tools for the study of DNA processing enzymes and DNA structure^{1,2}. In addition their increased resistance to nuclease activity suggests a potential application in the antisense approach to viral chemotherapy. In particular a 28 residue phosphorothioate analogue of oligodeoxycytidine is among the most effective inhibitors of the replication and cytopathic effects of the human immunodeficiency virus³. Although there is a considerable amount of literature relating to phosphorothioate analogues, the isomeric linkages in which a sulphur atom replaces one of the two bridging oxygen atoms in the phosphodiester linkage have received comparatively little attention. Apart from our own work, reports concerning the bridging sulphur analogues have been confined to the isomer in which the 5'-oxygen atom is replaced by sulphur for example the oligo-5'-thiothymidylates^{4,5} (1, Figure 1). Recently we have become interested in the synthesis and properties of dithymidine phosphate analogues in which the 3'-oxygen atom is replaced by sulphur (2, Figure

1). Oligonucleotides containing this linkage are of interest for several reasons: (i) they are isopolar and isosteric with the natural congener; (ii) they are achiral; (iii) the linkage is susceptible to specific cleavage under mild chemical conditions and (iv) they are interesting substrates for nucleases which cleave the 3'-oxygen-phosphorus bond.

We wish to report the preparation of a 5'-O-monomethoxytritylthymidine-3'-S-phosphorothioamidite (7) and its application to the synthesis of dithymidine-3'-S-phosphorothioate (d(TspT), 2) and dithymidine-3'-S-phosphorodithioate (d[Tsp(s)T], 3). Cleavage of the phosphorus-sulphur bond has been studied under enzymatic and chemical conditions and absolute configuration has been assigned to the diastereoisomers of 3. Prior to this work we have published preliminary reports on the synthesis of d(TspT)^{6,7}.

RESULTS AND DISCUSSION

Previous syntheses of nucleotide analogues containing P-S-C(5') linkages have generally relied on the alkylation of a phosphorothioate derivative; this approach is exemplified in the preparation of nucleoside 5'-S-ethyl phosphorothioate⁸ and the oligo-5'-thiothymidylates^{4,5}. In devising a method for the incorporation of 3'-thiothymidine into oligonucleotides we wished to exploit an approach that would be compatible with automated solid phase synthesis and therefore chose to follow the route outlined in Figure 2.

Synthesis of the protected thymidine-3'-S-phosphorothioamidite (7)

5'-O-monomethoxytrityl-3'-O-methanesulphonyl-2'-deoxy-xylo-thymidine (4) was prepared from thymidine according to a literature procedure⁹. Displacement of the sulphonate ester with sodium thiobenzoate in DMF at 100°C gave the thioester (5) in 91% yield. Debenzoylation using an argon saturated solution of sodium hydroxide in aqueous ethanol at 5°C gave 5'-O-monomethoxytrityl-3'-thiothymidine (6) in 95% yield and the corresponding dimeric disulphide (3%). Saturated methanolic ammonia was a less effective reagent for this conversion, the product thiol being obtained in 87% yield; prolonged exposure to these conditions gave the disulphide quantitatively. In earlier

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Figure 1.

Figure 2. Reagents and conditions: i, sodium thiobenzoate/DMF; ii, NaOH/EtOH/H₂O; iii, ClP(OCH₂CH₂CN)NPr₂/EtNP¹₂/CH₂Cl₂; iv, 5-(*p*-nitrophenyl)tetrazole/3'-*O*-acetylthymidine/CH₃CN; v, oxidant; vi, Bu⁴NH₂/pyridine, 80% AcOH, aqueous 35% NH₃; vii, S₈/pyridine.

studies Miller and Fox had attempted the saponification of 3'-*S*-benzoyl-3'-thiothymidine and obtained only the disulphide¹⁰. In support of their results we have noted that 3'-thiothymidine undergoes oxidation to the disulphide more readily than the 5'-*O*-monomethoxytrityl derivative.

Phosphitylation of **6** using 2-cyanoethyl *N,N*-diisopropylaminochlorophosphoramidite under standard conditions¹¹ produced the 3'-*S*-phosphorothioamidite derivative (**7**) in a yield of 87%. The two diastereoisomers were separated by careful column chromatography and characterised independently. Oxidation of **7** with elemental sulphur to the more stable 3'-*S*-phosphorodithioamidate (**11**) and separation of the diastereoisomers provided additional characterisation.

Figure 3.

Synthesis of d(TspT) and d[Tsp(s)T]

Model reactions on the activation of 3'-*O*-phosphoramidites with tetrazole and subsequent reaction with 3'-*O*-acetylthymidine were monitored by ³¹P nmr. Treatment of the 3'-*O*-phosphoramidite with tetrazole (4 equivalents) and 3'-*O*-acetylthymidine (1.1 equivalents) gave quantitative conversion to the dinucleoside phosphite in less than 10 minutes. When these conditions were applied to the activation of **7**, the starting material remained essentially unchanged (less than 5% reaction to unidentified products) over a similar period of time. Variations on this procedure including increased reaction periods and addition of *N,N*-dimethylaminopyridine¹² (DMAP) proved equally unsuccessful for the generation of the required phosphorothioite intermediate (**8**), but two resonances were observed which were attributable to the symmetrical phosphites **12** and **13** (Figure 3). These compounds were identified after oxidation with elemental sulphur and analysis of the purified products by FAB mass spectrometry and ¹H nmr. Activation with the more acidic 5-(*p*-nitrophenyl)tetrazole¹³ gave, in addition to **12** and **13**, resonances (189 and 191 ppm) corresponding to the two diastereoisomers of the intermediate phosphorothioite (**8**, Figure 4). The ³¹P nmr shift values for **8** are consistent with those recently reported for related phosphorothioites¹⁴. The phosphorothioite **8** was also obtained when *N*-methylanilinium trifluoroacetate¹⁵ was used as an activating agent, but the yield was lower than that obtained with 5-(*p*-nitrophenyl)tetrazole.

It is apparent from these results that the phosphorothioamidites are less reactive than the corresponding phosphoramidites and similar conclusions have been made by Caruthers and co-workers based on their studies on S-4(chlorobenzyl)phosphorothioamidites¹⁶. The formation of the symmetrical compounds **12** and **13** appear to result from side reactions that occur on activation of phosphorothioamidite. For example, treatment of **7** with 5-(*p*-nitrophenyl)tetrazole in the absence of 3'-*O*-acetylthymidine gave a significant yield of **6**. This result is consistent with the displacement of the 3'-thionucleoside from the phosphorus centre by the activating agent and would account for the formation of both **12** and **13**.

Our earlier studies had demonstrated that the intermediate phosphorothioite was particularly sensitive to oxidation and could be converted to the fully protected d(TspT) by dissolved air present in the solvents during work-up⁶. However, tetrabutylammonium periodate (TBA periodate) appeared to be a more suitable reagent for this conversion; previous work had established that it effected instantaneous oxidation of dinucleoside phosphites¹⁷, but its oxidation of sulphides to sulfoxides was much slower requiring at least 1h in refluxing chloroform¹⁸. *In situ* oxidation of **8** to the fully protected d(TspT) (**9**) was accomplished by addition of TBA periodate (2 equivalents) in dichloromethane and the product purified by chromatography. The yield for the overall conversion of 3'-*O*-acetylthymidine to **9** was 75%. ³¹P nmr spectra depicting this conversion are shown in Figure 4. Tetrabutylammonium oxone (TBA oxone)¹⁹ was also found to be effective in performing this oxidation and gave

Figure 4. Conversion of the 3'-S-phosphorothioamidite derivative **7** to fully protected d(TspT) (**9**) monitored by ^{31}P nmr spectroscopy. Panel A, **7** in CH_3CN ; panel B, spectrum obtained after the addition of 3'-O-acetylthymidine and 5-(p-nitrophenyl)tetrazole; panel C, spectrum obtained after the addition of TBA periodate (see experimental section for details).

9 in 56% yield. Deprotection of **9** was achieved by successive treatment with *t*-butylamine, 80% aqueous acetic acid and concentrated aqueous ammonia and the crude d(TspT) purified by elution from an ion exchange column with a gradient of triethylammonium bicarbonate (TEAB).

Oxidation of the intermediate phosphorothioite with elemental sulphur gave the fully protected d[Tsp(s)T] (**10**) in 64% yield after chromatography. Following deprotection the two diastereoisomers of d[Tsp(s)T] were separated by elution from an ion exchange column with a shallow gradient of TEAB.

Assignment of configuration to the diastereoisomers of d[Tsp(s)T]

Empirical rules have been established which allow the configuration of dinucleoside phosphorothioates to be assigned on the basis of their: susceptibility to enzymatic digestion with snake venom phosphodiesterase (SVPD) and nuclease P1; relative

	^{31}P nmr	HPLC retention time	Enzymatic digestion with SVPD
 Rp-[Tp(s)T]	down field	shorter	yes
 Sp-[Tsp(s)T]	down field	shorter	yes

Figure 5. Assignment of configuration to the diastereoisomers of d[Tsp(s)T]. Relative ^{31}P nmr shifts, reverse phase hplc retention times and susceptibility to enzymatic digestion with SVPD for Rp-d[Tp(s)T] and Sp-d[Tsp(s)T] compared to their respective epimers.

Table 1. Relative rates for enzymatic hydrolysis of d(TspT) and Rp- and Sp-d[Tsp(s)T].

Compound	Relative rate of cleavage	
	SVPD	Nuclease P1
d(TpT)	100	100
d(TspT)	102	1
Rp-d[Tsp(s)T]	none detected	0.4
Sp-d[Tsp(s)T]	64	none detected

^{31}P nmr chemical shift values and relative retention times on reverse phase hplc². Absolute configuration has been assigned to the diastereoisomers of d[Tsp(s)T] by comparison with these criteria. Thus, the diastereoisomer of **3** which resonated at lower field in the ^{31}P nmr spectra; was more rapidly eluted on reverse phase (ODS) hplc and was digested by SVPD was assigned the Sp-configuration (this corresponds to the Rp-configuration of the dinucleoside phosphorothioates) (Figure 5).

Enzymatic and chemical cleavage of the P-S bond in d(TspT) and d[Tsp(s)T]

d(TspT) and d[Tsp(s)T] provide interesting substrates for phosphodiesterases, particularly those such as SVPD and nuclease P1 which cleave the 3'-oxygen-phosphorus bond. SVPD hydrolysed d(TspT) to thymidine-5'-O-phosphate and 3'-thiothymidine, although the latter was partially oxidised to the disulphide during the incubation. The rate of the hydrolysis of d(TpsT) was almost identical to that observed for the natural substrate d(TpT) (Table 1). In contrast d(TpsT) was comparatively resistant to cleavage by nuclease P1 and these initial studies indicated that its rate of hydrolysis is about 100 fold slower than that of d(TpT). It is worth noting that nuclease P1 is an endonuclease that acts preferentially on relatively low molecular weight polynucleotides (approximately 40 residues)²⁰

and the dinucleoside phosphate analogues used in this study are therefore expected to be inherently poor substrates. Only Sp-d[Tsp(s)T] was cleaved by SVPD and Rp-d[Tsp(s)T] was hydrolysed very slowly by nuclease P1. Studies with enzymes that cleave the 5'-oxygen-phosphorus bond to yield nucleoside-3'-*O*-phosphates have not as yet been undertaken.

The P-S bond of d(TspT) was cleaved slowly (8 hours, 50°C) by a solution of iodine in aqueous acetone to give thymidine-5'-*O*-phosphate and the disulphide of 3'-thiothymidine quantitatively. This reaction has previously been studied with regard to the cleavage of nucleoside 5'-*S*-ethyl phosphorothioates and mechanisms have been proposed that involve either a metaphosphate or a phosphoriodate intermediate²¹. Iodine promoted cleavage was more rapid (2 hours, room temperature) in the presence of pyridine and yielded thymidine-5'-*O*-phosphate and an as yet unidentified derivative of 3'-thiothymidine. Cleavage of the P-S bond could also be achieved by treatment with a solution of dilute aqueous silver nitrate (30 mM) at room temperature for 1 hour and under these conditions the products were thymidine-5'-*O*-phosphate and the silver salt of 3'-thiothymidine. The free thiol could be generated by adding excess dithiothreitol and removing the dithiothreitol-silver complex by centrifugation or filtration. This procedure is analogous to that employed by Connolly and Rider for the removal of *S*-trityl functions from modified oligonucleotides²². The mechanism probably involves coordination of Ag⁺ to the sulphur atom followed by nucleophilic attack by water at phosphorus. Since chemical cleavage of the P-S bond produces a 5'-phosphate the method is complementary to that reported by Bannwarth which enables oligodeoxynucleotides containing 5'-amino-5'-deoxythymidine to be cleaved to yield oligodeoxynucleotide-3'-*O*-phosphates.²³

CONCLUSIONS

Procedures have been developed which enable 3'-thiothymidine to be incorporated into dinucleoside phosphate analogues using phosphoramidite chemistry. The synthetic methods are compatible with automated, solid phase synthesis of oligodeoxynucleotides and in a more recent study, this chemistry has been exploited in the preparation of an 18 residue oligodeoxynucleotide containing a single 3'-thiothymidine substitution. The ease with which the modified linkage is cleaved in the presence of silver ions or iodine solutions suggests that the incorporation of 3'-*S*-phosphorothioate linkages into oligonucleotide primers and their subsequent chemical cleavage may prove a useful technique for the 'nicking' and manipulation of DNA.

EXPERIMENTAL

TBA periodate¹⁸ and TBA oxone¹⁹ were prepared and characterised as previously reported. Pyridine and 2,6-lutidine were refluxed and distilled from ninhydrin and then freshly distilled from potassium hydroxide pellets. Acetonitrile and *N,N*-diisopropylethylamine were refluxed and distilled from calcium hydride. Dichloromethane was refluxed and distilled from phosphorus pentoxide and passed through basic alumina immediately before use. *N,N*-Dimethylformamide (DMF) was stored over barium oxide for at least 3 days and then distilled under reduced pressure (20 mmHg). Enzymes were purchased from Sigma.

Hplc was performed on a Varian 5000 liquid chromatograph

equipped with a variable wavelength Varian UV50 detector. Elution was performed with a linear 20 minute gradient of 0–30% acetonitrile in 50 mM potassium phosphate buffer pH 6.0, at a flow rate of 1 mL/minute. Analytical columns (250×4.6 mm) were packed with 5 μ ODS silica.

Preparation of 5'-*O*-monomethoxytrityl-3'-*S*-benzoyl-thiothymidine (5)

Sodium thiobenzoate was prepared by addition of aqueous sodium hydroxide (10 M) to an ice cooled solution of thiobenzoic acid (10 g) in water (15 mL) until the solution was just alkaline. The solution was adjusted to pH 7 with aqueous thiobenzoic acid, cooled to –5°C and filtered to remove solid impurities. The water was removed by evaporation under vacuo and by repeated coevaporation with ethanol. The resulting yellow solid was stored under vacuum over phosphorus pentoxide. A solution of 5'-*O*-monomethoxytrityl-3'-*O*-methanesulphonyl-2'-deoxy-xylo-thymidine (5.0 g, 8.45 mmol) and sodium thiobenzoate (5.3 g, 33 mmol) in DMF (30 mL) was stirred in an oil bath at 100°C for 4 hours. After cooling to room temperature dichloromethane (300 mL) was added and the solution extracted with saturated sodium bicarbonate (300 mL) and saturated sodium chloride (2×300 mL). The organic phase was dried over sodium sulphate, concentrated under reduced pressure and coevaporated with toluene. The residual gum was dissolved in dichloromethane and subjected to chromatography on Kieselgel 60H eluting with a gradient of 0–3% methanol in dichloromethane. Appropriate fractions were collected, pooled and evaporated to yield the product as a pale yellow foam.

Yield, 4.48 g, 7.07 mmol, 87%; Rf (dichloromethane/methanol 9:1): 0.62; ¹H-nmr (250 MHz, CDCl₃): 1.50 (3H, s, CH₃), 2.54 (1H, m, H2'), 2.76 (1H, m, H2''), 3.47 (1H, m, H5'), 3.52 (1H, m, H5''), 3.75 (3H, s, CH₃O), 4.16 (1H, m, H4'), 4.50 (1H, m, H3'), 6.29 (1H, t, H1'), 6.81 (2H, d, ArH), 7.15–7.92 (18H, m, ArH, H6), 8.42 (1H, s, NH); m/z (FAB⁺) 635 (M+H), 273 (MMT).

Preparation of 5'-*O*-monomethoxytrityl-3'-thiothymidine (6)

To a solution of **5** (1.5 g, 2.4 mmol) in argon saturated ethanol (240 mL) at 5°C aqueous sodium hydroxide (7 mL, 10 M) was added with vigorous shaking to effect proper mixing. The solution was stored at 5°C for 50 minutes and partitioned between dichloromethane (150 mL) and saturated sodium bicarbonate (600 mL). The aqueous layer was washed with a further portion of dichloromethane (150 mL), the combined organic extracts dried over sodium sulphate and evaporated to a foam. The crude material was dissolved in dichloromethane and subjected to column chromatography on Kieselgel 60H eluting with a shallow gradient of methanol in dichloromethane (0–3%). Fractions containing the product were pooled and evaporated to a white foam.

Yield, 1.19 g, 2.4 mmol, 95%; Rf (dichloromethane/methanol 9:1): 0.59; ¹H-nmr (250 MHz, CDCl₃): 1.51 (3H, s, CH₃), 2.37 (1H, m, H2'), 2.59 (1H, m, H2''), 3.41 (1H, m, H5'), 3.61 (2H, m, H3', H5'), 3.79 (3H, s, CH₃O), 3.87 (1H, m, H4'), 6.16 (1H, t, H1'), 6.85 (2H, d, ArH), 7.21–7.46 (12H, m, ArH, H6), 7.68 (1H, d, H6), 9.42 (1H, s, NH); m/z (FAB⁺) 531 (M+H), 273 (MMT).

The corresponding disulphide was also isolated from the column.

Yield, 40 mg, 0.038 mmol, 3%; Rf (dichloromethane/methanol 9:1): 0.54; ¹H-nmr (250 MHz, CDCl₃): 1.47 (3H, s, CH₃),

2.47 (2H, m, H2', H2''), 3.29 (1H, m, H5'), 3.53 (1H, m, H5''), 3.65 (1H, m, H3'), 3.77 (3H, s, CH₃O), 3.99 (1H, m, H4'), 6.17 (1H, t, H1'), 6.82 (2H, d, ArH), 7.23–7.41 (12H, m, ArH, H6), 7.66 (1H, d, H6), 8.50 (1H, s, NH); *m/z* (FAB⁺) 1059 (M+H), 273 (MMT).

Preparation of 5'-*O*-monomethoxytrityl-3'-thiothymidine-3'-*S*-(2-cyanoethyl)-*N,N*-diisopropylphosphorothioamidite (7)

2-Cyanoethyl *N,N*-diisopropylaminochlorophosphoramidite (2.1 mL, 12 mmol) was added dropwise over 5 minutes to a stirred solution of **6** (2.96 g, 5.58 mmol) and *N,N*-diisopropylethylamine (4.85 mL, 27.9 mmol) in dichloromethane (15 mL) at room temperature. The reaction was stirred for a further 50 minutes, quenched with methanol (1 mL) and taken up into ethyl acetate (100 mL). This solution was washed with saturated solutions of sodium bicarbonate (2 × 100 mL) and sodium chloride (2 × 100 mL), dried over sodium sulphate and evaporated. The residual gum was subjected to column chromatography on Kieselgel 60H eluting with dichloromethane/ethyl acetate/triethylamine (60/30/10). Fractions containing product were pooled, evaporated, dissolved in toluene (20 mL) and precipitated into vigorously stirred 40–60 petroleum ether at 5°C. The product was collected by filtration and dried under vacuum to yield a white powder (3.55 g, 4.86 mmol, 87%).

The diastereoisomers were resolved by column chromatography on Kieselgel 60H eluting initially with dichloromethane/triethylamine (9/1) and then with 40–60 petroleum ether/dichloromethane/ethyl acetate/triethylamine (50/25/25/5) and collecting small fractions (5 mL). In order to obtain the slow diastereoisomer uncontaminated with the fast it was necessary to repeat this chromatographic purification on the slow-enriched fractions.

Fast diastereoisomer: *Rf* (ethyl acetate/dichloromethane/triethylamine 45:45:10): 0.63; ¹H-nmr (250 MHz, CDCl₃): 1.19 (12H, m, 2 × (CH₃)₂C), 1.37 (3H, s, CH₃), 2.43 (2H, t, CH₂CN), 2.56 (2H, m, H2' and H2''), 3.42–3.73 (7H, m, CH₂O, (CH₂)N, H3', H5' and H5''), 3.80 (3H, s, CH₃O), 4.05 (1H, m, H4'), 6.18 (1H, m, H1'), 6.85 (2H, d, ArH), 7.24–7.44 (12H, m, ArH), 7.73 (1H, s, H6); ³¹P-nmr (81 MHz, CDCl₃): 164.4; *m/z* (FAB⁺) 731 (M+H).

Slow diastereoisomer: *Rf* (ethyl acetate/dichloromethane/triethylamine 45:45:10): 0.55; ¹H-nmr (250 MHz, CDCl₃): 1.07 (6H, d, (CH₃)₂C), 1.18 (6H, d, (CH₃)₂C), 1.42 (3H, s, CH₃), 2.62 (2H, t, CH₂CN), 2.50–2.78 (2H, m, H2' and H2''), 3.38–3.75 (7H, m, CH₂O, (CH₂)N, H3', H5' and H5''), 3.79 (3H, s, CH₃O), 4.07 (1H, m, H4'), 6.35 (1H, m, H1'), 6.83 (2H, d, ArH), 7.23–7.44 (12H, m, ArH), 7.67 (1H, s, H6); ³¹P-nmr (81 MHz, CDCl₃): 159.9; *m/z* (FAB⁺) 731 (M+H).

Preparation of 5'-*O*-monomethoxytrityl-3'-thiothymidine-3'-*S*-(2-cyanoethyl)-*N,N*-diisopropylphosphorodithioamidate (11)

To a solution of **7** (unresolved diastereoisomers) (120 mg, 0.164 mmol) in chloroform (2.5 mL) and 2,6-lutidine (0.2 mL) elemental sulphur (158 mg, 4.9 mmol) was added and the reaction mixture stirred at room temperature. After 4 hours the solvents were evaporated and residual 2,6-lutidine removed by coevaporation with toluene. The residue was dissolved in dichloromethane and subjected to chromatography on Kieselgel 60H eluting with a gradient of methanol in dichloromethane

(0–2%). Fractions containing a pure diastereoisomer were pooled and evaporated.

Fast diastereoisomer: Yield, 53 mg, 0.073 mmol, 45%; *Rf* (dichloromethane/methanol 95:5): 0.48; ¹H-nmr (250 MHz, CDCl₃): 1.31 (12H, m, 2 × (CH₃)₂C), 1.44 (3H, s, CH₃), 2.36–2.64 (3H, t, CH₂CN and H2'), 3.30 (1H, m, H2''), 3.42 (1H, m, H5'), 3.58 (1H, m, H5''), 3.75 (2H, m, (CH₂)N), 3.80 (3H, s, CH₃O), 3.81–4.10 (2H, m, CH₂O), 4.13–4.27 (2H, m, H3' and H4'), 6.29 (1H, t, H1'), 6.85 (2H, d, ArH), 7.22–7.51 (12H, m, ArH), 7.65 (1H, s, H6), 8.51 (1H, s, NH); ³¹P-nmr (81 MHz, CDCl₃): 87.8; *m/z* (FAB⁺) 763 (M+H).

Slow diastereoisomer: Yield, 45 mg, 0.062 mmol, 37%; *Rf* (dichloromethane/methanol 95:5): 0.37; ¹H-nmr (250 MHz, CDCl₃): 1.26 (12H, m, 2 × (CH₃)₂C), 1.38 (3H, s, CH₃), 2.60 (1H, m, H2'), 2.72 (1H, m, H2''), 2.76 (2H, t, CH₂CN), 3.45 (1H, m, H5'), 3.52 (1H, m, H5''), 3.71 (2H, m, (CH₂)N), 3.79 (3H, s, CH₃O), 4.33 (4H, m, H3', H4' and CH₂O), 6.29 (1H, t, H1'), 6.84 (2H, d, ArH), 7.23–7.47 (12H, m, ArH), 7.64 (1H, s, H6), 8.40 (1H, s, NH). ³¹P-nmr (81 MHz, CDCl₃): 87.4; *m/z* (FAB⁺) 763 (M+H).

Synthesis of the dithymidine phosphorothioite intermediate (8)

Acetonitrile (0.9 mL) was added to a mixture of 5-(*p*-nitrophenyl)tetrazole (100 mg, 0.52 mmol) and **7** (250 mg, 0.342 mmol) and the suspension stirred for 1.5 minutes at room temperature. To this mixture a solution of 3'-*O*-acetylthymidine (35 mg, 0.12 mmol) in acetonitrile (1.6 mL) was added slowly over a period of 20 minutes. After stirring for an additional 5 minutes 2,6-lutidine (0.1 mL) was added and the intermediate phosphorothioite oxidised *in situ* by one of the following procedures.

Synthesis of fully protected d(TspT) (9)

(i). *Oxidation with tetrabutylammonium periodate*. A solution of tetrabutylammonium periodate (295 mg, 0.68 mmol) in dichloromethane (2 mL) was added to the intermediate phosphorothioite and the mixture stirred at room temperature. After 7 minutes dichloromethane was added and the solution washed with aqueous solutions of sodium metabisulphite (5%, 50 mL), saturated sodium bicarbonate (30 mL) and saturated sodium chloride (50 mL). The organic layer was dried over sodium sulphate and evaporated. The crude product was redissolved in dichloromethane and subjected to column chromatography on Kieselgel 60H eluting with a shallow gradient of methanol in dichloromethane (0–3%). Fractions containing the product (mixture of diastereoisomers) were pooled and evaporated to a white foam.

Yield, 86.7 mg, 0.092 mmol, 75%; *Rf* (dichloromethane/methanol 9:1): 0.39; ¹H-nmr (250 MHz, CDCl₃): 1.45 (1H, s, CH₃-dTsp-), 1.90 (3H, s, CH₃-spdT), 2.10 (3H, s, CH₃CO), 2.24 (1H, m, H2' -spdT), 2.40 (1H, m, H2'' -spdT), 2.55–2.82 (4H, m, CH₂CN, H2', H2'' dTsp-), 3.43 (1H, m, H5' dTsp-), 3.59 (1H, m, H5'' dTsp-), 3.79 (3H, s, CH₃O), 4.00–4.50 (7H, m, CH₂O, H4', H5', H5'' -spdT and H3', H4' dTsp-), 5.28 (1H, m, H3' -spdT), 6.22–6.43 (2H, m, H1' dTsp- and H1' -spdT), 6.85 (2H, d, ArH), 7.20–7.57 (13H, m, ArH, H6), 7.56 (1H, s, H6), 9.68 (2H, s, NH); ³¹P-nmr (81 MHz, CDCl₃) 26.0, 26.4; *m/z* (FAB⁺) 930 (M+H), 952 (M+Na).

(ii). *Oxidation with tetrabutylammonium oxone*. A solution of tetrabutylammonium oxone (650 mg, 0.68 mmol) in dichloromethane (2 mL) was added to the intermediate

phosphorothioite and the mixture stirred at room temperature for 10 minutes. The reaction mixture was then evaporated and residual solvents removed by coevaporation with toluene. The residue was dissolved in dichloromethane and purified by chromatography as described above.

Yield, 64.7 mg, 0.069 mmol, 56%. The material was identical to that described above.

Synthesis of fully protected d[Tsp(s)T] (10). Dichloromethane (2 mL) and elemental sulphur (220 mg, 6.85 mmol) was added to the intermediate phosphorothioite and the suspension stirred at room temperature. After 4 hours dichloromethane (40 mL) was added, the solution washed with saturated sodium bicarbonate (2×40 mL) and saturated sodium chloride (2×40 mL), the organic layer dried over sodium sulphate and the solvent evaporated. The residue was redissolved in dichloromethane and subjected to column chromatography on Kieselgel 60H eluting with a shallow gradient of methanol in dichloromethane (0–3%). Fractions containing the product (mixture of diastereoisomers) were pooled and evaporated to a white foam.

Yield, 74.5 mg, 0.079 mmol, 64%; R_f (dichloromethane/methanol 9:1): 0.43; ¹H-nmr (250 MHz, CDCl₃): 1.45 (3H, s, CH₃-dTsp-), 1.92 (3H, s, CH₃-p(s)dT), 2.11 (3H, s, CH₃CO), 2.21 (1H, m, H2'-p(s)dT), 2.41 (1H, m, H2''-p(s)dT), 2.50–2.83 (4H, m, CH₂CN, H2', H2''dTsp-), 3.43 (1H, m, H5' dTsp-), 3.56 (1H, m, H5'' dTsp-), 3.80 (3H, s, CH₃O), 4.00–4.50 (7H, m, CH₂O, H4', H5', H5''-p(s)dT and H3', H4' dTsp-), 5.27 (1H, m, H3'-p(s)dT), 6.24 (1H, t, H1'), 6.26 (1H, t, H1'), 6.85 (2H, d, ArH), 7.23–7.52 (13H, m, ArH, H6), 7.60 (1H, s, H6), 8.94 (1H, d, NH), 9.03 (1H, d, NH); ³¹P-nmr (81 MHz, CDCl₃) 94.6, 94.5; m/z (FAB⁺) 946 (M+H), 968 (M+Na).

Deprotection and isolation of d(TspT) and d[Tsp(s)T]

A solution of **9** or **10** (approximately 0.15 mmol) in pyridine/*t*-butylamine (9:1, 4.5 mL) was left at room temperature for 1 hour. The solvents were evaporated, residual pyridine removed by repeated coevaporation with toluene/dichloromethane (2:1) and the residue dissolved in acetic acid/water (8:2). After 5 hours at room temperature the solvents were evaporated and traces of acetic acid removed by coevaporation with water. The detritylated product was dissolved in water (5 mL) and extracted with *n*-pentane (3×5 mL). The water was evaporated and the residue dissolved in concentrated aqueous ammonia (8 mL) and heated at 50°C for 3 hours. The solution was evaporated once more, dissolved in water and subjected to ion exchange chromatography on DEAE Sephadex eluting with a gradient of TEAB (0.0–1.0 M, over 1L). Fractions containing product were pooled, evaporated, coevaporated with water and stored as frozen aqueous solutions. Yields in the range of 75–80% were generally obtained.

d(TspT) (2). ¹H-nmr (250 MHz, D₂O): 1.84 (6H, s, CH₃), 2.37 (2H, t, H2', H2''-pdT), 2.65 (2H, m, H2', H2'' dTsp-), 3.52 (1H, m, H3' dTsp-), 3.97 (3H, m, H5', H5'', H4' dTsp-), 4.13 (2H, m, H5'', H4'-pdT), 4.30 (1H, m, H5'-pdT), 4.57 (1H, m, H3'-pdT), 6.01 (1H, m, H1' dTsp-), 6.31 (1H, t, H1'-pdT), 7.79 (1H, s, H6), 7.84 (1H, s, H6); ³¹P-nmr (81 MHz, D₂O): 17.40; (FAB⁻) 561 (M⁻); hplc retention time 9.9 minutes.

Rp- and Sp-d[Tsp(s)T] (3). d[Tsp(s)T] was obtained as a mixture of diastereoisomers (Rp/Sp, 43:57) which were separated by further passage through DEAE Sephadex eluting with a very shallow gradient of TEAB (0.0–0.15 M, over 5 L).

Rp-d[Tsp(s)T] (3): ¹H-nmr (250 MHz, D₂O): 1.85 (6H, s, CH₃), 2.36 (2H, m, H2' and H2''-p(s)dT), 2.67 (2H, m, -H2' and H2'' dTsp-), 3.58 (1H, m, H3' dTsp-), 3.96 (3H, m, H5', H5'' and H4' dTsp-), 4.16 (1H, m, H4'-p(s)dT), 4.27 (2H, m, H5' and H5''-p(s)dT), 4.57 (1H, m, H3'-p(s)dT), 5.96 (1H, m, H1' dTsp-), 6.28 (1H, t, H1'-p(s)dT), 7.81 (1H, s, H6), 7.86 (1H, s, H6); ³¹P-nmr (18 MHz, D₂O): 68.7, (FAB⁻) 577 (M⁻); hplc retention time 12.6 minutes.

Sp-d[Tsp(s)T] (3): ¹H-nmr (250 MHz, D₂O): 1.87 (3H, s, CH₃), 1.91 (3H, s, CH₃), 2.37 (2H, m, H2' and H2''-p(s)dT), 2.62 (2H, m, -H2' and H2'' dTsp-), 3.68 (1H, m, H3' dTsp-), 3.84 (1H, m, H5' dTsp-), 3.90 (1H, m, H5' dTsp-), 4.08 (1H, m, H4' dTsp-), 4.01–4.16 (2H, m, H5' and H4'-p(s)dT), 4.28 (1H, m, H5'-p(s)dT), 4.59 (1H, m, H3'-p(s)dT), 6.07 (1H, t, H1' dTsp-), 6.32 (1H, t, H1'-p(s)dT), 7.72 (1H, s, H6), 7.76 (1H, s, H6); ³¹P-nmr (81 MHz, D₂O): 72.4, (FAB⁻) 577 (M⁻); hplc retention time 12.1 minutes.

Enzymatic digestion of dithymidine phosphate analogues

A solution of the dinucleoside phosphate analogue (0.25 A₂₆₀ unit) in tris(hydroxymethyl)aminomethane·hydrochloride (pH 7.4, 25 mM, 25 μL) containing either SVPD (2 μg) or nuclease P1 (1 or 6 units) was incubated at room temperature. After 10 and 30 minutes reactions were analysed by hplc and products identified by coinjection with standards.

Chemical cleavage of d(TspT)

(i). **Iodine in aqueous acetone.** To a solution of d(TspT) (2 A₂₆₀ units) in water (200 μL) a solution of iodine in acetone (200 μL, 100 mM) was added, the mixture stoppered and heated at 50°C for 8 hours. The reaction mixture was diluted by the addition of water (0.5 mL) and extracted with diethyl ether (5×1 mL). The cleavage products (thymidine-5'-*O*-phosphate and 3'-thiothymidine dimeric disulphide) were identified by hplc analysis of the aqueous solution.

(ii). **Iodine in aqueous pyridine.** To a solution of d(TspT) (2 A₂₆₀ units) in water (200 μL) a solution of iodine in pyridine (200 μL, 100 mM) was added and the mixture set aside at room temperature for 2 hours. The reaction mixture was extracted and analysed as described above. The products of cleavage were thymidine-5'-*O*-phosphate and an unidentified derivative of 3'-thiothymidine.

(iii). **Aqueous silver nitrate.** A solution of d(TspT) (2 A₂₆₀ units) in water (200 μL) containing silver nitrate (20 mM) was maintained at room temperature for 1 hour. The reaction was quenched by the addition of a solution (100 μL) containing dithiothreitol (100 mM) and sodium hydrogen carbonate (100 mM) and the suspension centrifuged. The supernatant was analysed by hplc and the cleavage products were identified as thymidine-5'-*O*-phosphate and 3'-thiothymidine.

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