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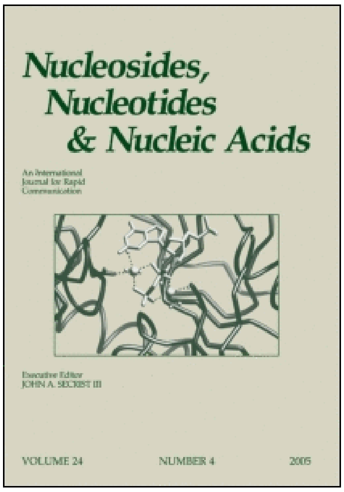

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NUCLEOSIDES & NUCLEOTIDES, 17(1-3), 451-470 (1998)

PREPARATION OF AN OCTADEOXYRIBONUCLEOSIDE HEPTAPHOSPHOROTHIOATE BY THE PHOSPHOTRIESTER APPROACH IN SOLUTION†

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ABSTRACT: The synthesis of the octadeoxyribonucleoside heptaphosphorothioate, d[Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T] by the phosphotriester approach in solution is described. The phosphorothioate internucleotide linkages are protected by the *S*-(2-cyanoethyl) group and 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-1*H*-triazole (MSNT) is used as the coupling agent. A block synthesis strategy (2 + 2 → 4 and 4 + 4 → 8) is followed.

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INTRODUCTION

In the past few years, there has been considerable interest in the large scale synthesis of phosphorothioate analogues[1,2] of oligodeoxyribonucleotides. A number of specific sequences have shown promise as chemotherapeutic agents, and several are at present undergoing clinical trials[3]. It appears from the literature that the consequent need for relatively large (certainly of the order of hundreds of grams and possibly considerably more) quantities of specific oligodeoxyribonucleotide phosphorothioates has almost exclusively led to the scaling-up of phosphoramidite-based solid-phase synthesis[4].

The particular advantage of solid-phase synthesis[1,2], especially on a small (say, 10^{-6} molar) scale are that it can readily be automated and coupling reactions are usually very fast and efficient. It is also very flexible. Generally only one nucleotide residue is added at a time, and the target sequence can easily be changed even in mid-synthesis. However, there

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†This article is dedicated to the memory of Professor Tsujiaki Hata
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are a number of drawbacks to the use of solid-phase synthesis in the large scale preparation of a specific nucleotide sequence. First, while machines have been developed that are reputedly suitable for the solid-phase synthesis of hundreds of grams of oligonucleotides at a time, it is perhaps unlikely that such scaling-up could be extended to accommodate multikilogram quantities in one run. Secondly, it may be necessary to use a relatively large excess[4] of phosphoramidite building block in each synthetic cycle if coupling efficiencies are to be maintained. Thirdly, the addition of more than one nucleotide residue at a time is likely to be more costly and possibly not particularly advantageous. For these reasons, synthesis in homogeneous solution involving, for example, the phosphotriester approach[5] might well prove to be superior to solid-phase synthesis in the preparation of really large quantities of specific oligonucleotides and their analogues (including phosphorothioates). Among the obvious merits of the phosphotriester approach are (i) that scaling-up should not be a problem even if multikilogram quantities are required, (ii) that only a relatively small (probably $\ngtr$ 25%) excess of building block is likely to be needed in each coupling step, and (iii) that the addition of two or more nucleotide residues at a time (i.e. block synthesis) is likely to be a routine operation.

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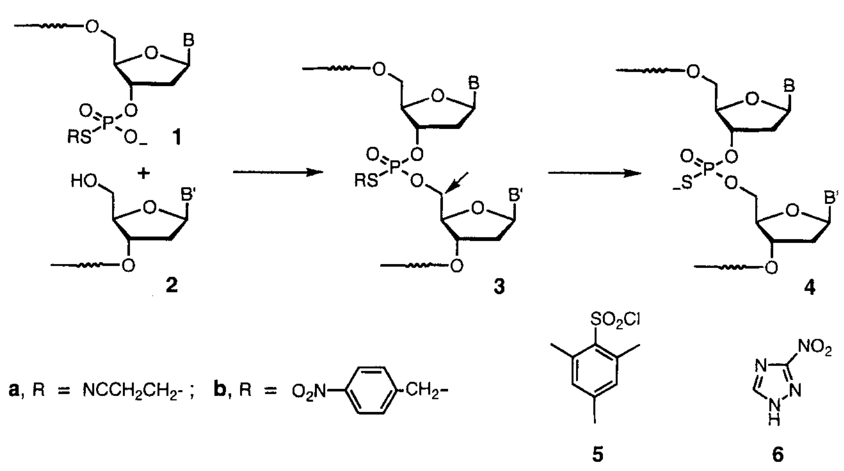
We recently suggested[6] an approach to the synthesis of phosphorothioate analogues of oligonucleotides by the phosphotriester approach in solution which is indicated in outline in SCHEME 1. The key reaction involved coupling between a protected nucleoside or oligonucleotide terminating in a 3'-(*S*-2-cyanoethyl) phosphorothioate **1a** and a protected nucleoside or oligonucleotide **2** terminating in a free 5'-hydroxy function, in pyridine solution. The coupling reaction was promoted by mesitylene-2-sulfonyl chloride **5** in the presence of 3-nitro-1,2,4-1*H*-triazole[7] **6** to give the intermediate phosphorothioate triester **3**. The *S*-(2-cyanoethyl) protecting group(s) were removed from the internucleotide linkage(s) by treatment with *tert*-butylamine[8] in dry pyridine solution. The other protecting groups were then removed from the base residues in an appropriate manner to give the fully-unblocked oligonucleotide phosphorothioate **4**. In the course of our previous study[6], we rejected the use of 4-nitrobenzyl (as in **3b**) as an alternative protecting group for phosphorothioate internucleotide linkages. We estimated that removal of *S*-(4-nitrobenzyl) protecting groups with toluene-4-thiolate ions would lead to *ca.* 2% cleavage per internucleotide linkage. This is due to competitive nucleophilic attack[9] on the 5'-carbon atoms (marked by an arrow in **3**) vicinal to the internucleotide linkages. Barber *et al.*[10] recently found that the thiophenate ions-promoted removal of *S*-(2,4-dichlorobenzyl) protecting groups similarly leads to *ca.* 1.8% concomitant cleavage per internucleotide linkage. Very recently, Püschl *et al.*[11] have shown that such cleavage can be decreased to *ca.* 0.3% per internucleotide linkage if *S*-(4-chloro-2-nitrobenzyl) protecting groups are used. In the same article, Püschl *et al.*[11] stated, without any experimental evidence, that



SCHEME 1

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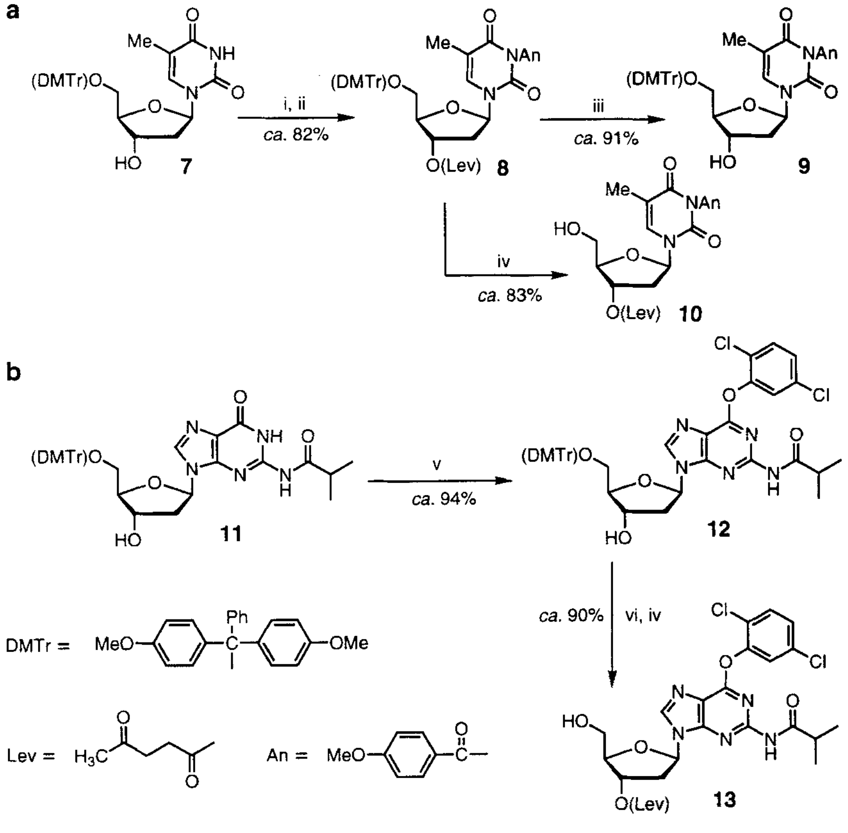
'the cyanoethyl *S*-protecting group is too labile for normal block synthesis'. We now report the synthesis of d[Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T] by the phosphotriester approach in solution and by a strategy involving block condensation reactions. We confirm our view[6] that the *S*-(2-cyanoethyl) group is perfectly suitable for the protection of phosphorothioate internucleotide linkages. *S*-(2-Cyanoethyl) [albeit less readily than *O*-(2-cyanoethyl)] protecting groups are quantitively removed by base-catalysed elimination without the occurrence of internucleotide cleavage or any other undesirable side reaction.

RESULTS AND DISCUSSION

Four nucleoside building blocks **9**, **10**, **12** and **13** (SCHEME 2) were required in the synthesis of the octadeoxynucleoside heptaphosphorothioate (d[Tp(s)Tp(s)Gp(s)Gp(s)-Gp(s)Gp(s)Tp(s)T]). In order to suppress possible side reactions in the coupling steps and also to facilitate the chromatographic purification of the products, it was decided to protect the thymine residues with the 3-*N*-(*p*-anisoyl) group[12] and to protect the guanine residues on *O*-6[13] with the 2,5-dichlorophenyl group as well as on *N*-2 with the commonly used isobutyryl group[14]. 5'-*O*-(4,4'-Dimethoxytrityl)thymidine[15] **7** was converted, *via* its 3-*N*-(*p*-anisoyl)-3'-*O*-levulinyl derivative **8**, into its 3-*N*-(*p*-anisoyl) derivative **9** (*ca.* 74% overall yield) and into 3-*N*-(*p*-anisoyl)-3'-*O*-levulinylthymidine **10** (*ca.* 68% overall yield). The commercially available 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-2-*N*-isobutyrylguanosine **11** was converted directly into its 6-*O*-(2,5-dichlorophenyl) derivative **12** in 94% overall



SCHEME 2 *Reagents and conditions*: i, levulinic anhydride, Et_3N, 4-(dimethylamino)pyridine, CH_2Cl_2, room temp., 2h; ii, AnCl, diisopropylethylamine, C_5H_5N, room temp., 15h; iii, a, $N_2H_4 \cdot H_2O$, C_5H_5N - AcOH (1:1 v/v), 0°C, 10 min, b, pentan-2,4-dione, C_5H_5N, 0°C, 10 min; iv, $Cl_2CHCOOH$, pyrrole, CH_2Cl_2; v, a, Me_3SiCl, C_5H_5N, room temp., 30 min, b, mesitylene-2-sulfonyl chloride **5**, 1-methylpyrrolidine, C_5H_5N, 0°C, 15 min, c, 2,5-dichlorophenol, C_5H_5N, 0°C, 3h; vi, levulinic anhydride, C_5H_5N, room temp., 16h.

yield. Following 3'-*O*-(trimethylsilylation) and activation with mesitylene-2-sulfonyl chloride **5**, 1-methylpyrrolidine-catalyzed[13] nucleophilic substitution at *C*-6 was effected with the conjugate base of 2,5-dichlorophenol. Finally, the 3'-*O*-levulinyl derivative **13** was prepared in two steps and in *ca.* 90% yield from compound **12**.

As indicated in SCHEME 3, the two required protected 2'-deoxyribonucleoside 3'-*S*-(2-cyanoethyl) phosphorothioates **16a** and **16b** were prepared from the corresponding 5'-*O*-(4,4'-dimethoxytrityl)nucleoside derivatives **9** and **12** by the previously reported procedure[6] involving intermediate 3'-*H*-phosphonates (i.e. **15a** and **15b**).

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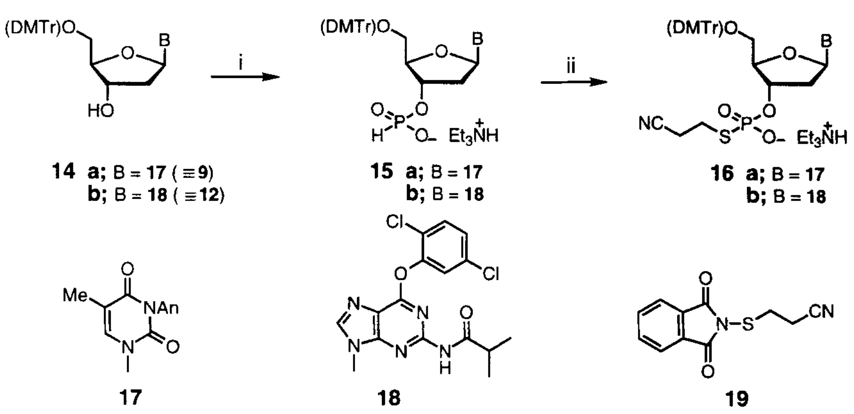

SCHEME 3 *Reagents and conditions*: i, a, [reagent prepared from PCl_3, Et_3N, 1,2,4-1*H*-triazole, THF, -50°C, 15 min], -50°C, 30 min, b, aq. triethylammonium phosphate buffer (pH 7.0); ii, **19**[6], 4-methylmorpholine, Me_3SiCl, CH_2Cl_2, room temp., 1h.

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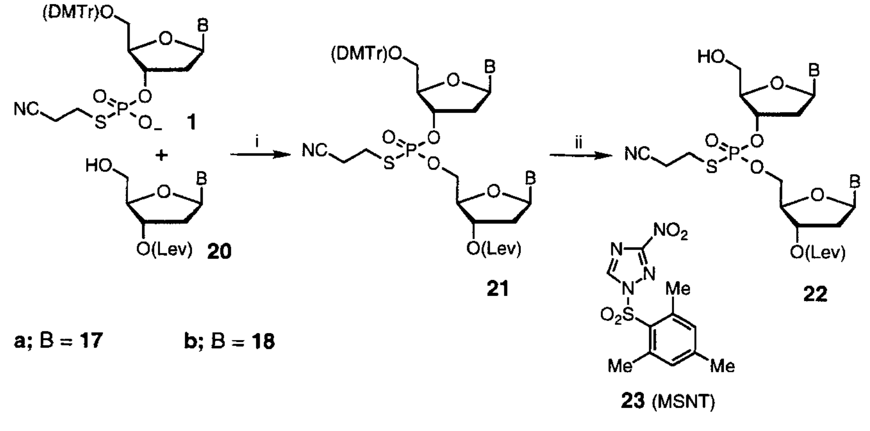
Following short column chromatography on silica gel, the monomeric building blocks **16a** and **16b** were isolated as colourless precipitated solids in high overall yields (*ca.* 94 and 92%, respectively). These protected 2'-deoxyribonucleoside 3'-*S*-(2-cyanoethyl) phosphorothioates **16a** and **16b** were then coupled (SCHEME 4) with the corresponding 3'-*O*-levulinyl derivatives **20a** (≡ **10**) and **20b** (≡ **13**), respectively, following the phosphotriester approach in solution[5] to give the fully-protected dinucleoside phosphorothioates **21a** and **21b**. These products were isolated in *ca.* 96 and 94% yield, respectively. The coupling agent used was 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-1*H*-triazole (MSNT)[7] **23** rather than a mixture of mesitylene-2-sulfonyl chloride **5** and 3-nitro-1,2,4-1*H*-triazole **6** (SCHEME 1). The fully-protected dinucleoside phosphorothioates **21a** and **21b** were then treated with dichloroacetic acid and pyrrole in dichloromethane solution[16] to give the partially-protected dimer blocks **22a** and **22b** in *ca.* 98 and 96% isolated yield, respectively.

A number of years ago, we introduced[17] a system of abbreviations for protected oligonucleotides in which nucleoside residues and internucleotide linkages are italicised if they are protected in some defined way. In the present context, *T* and *G* represent thymidine protected on *N*-3 with a *p*-anisoyl group and 2'-deoxyguanosine protected on *N*-2 and on *O*-6 with isobutyryl and 2,5-dichlorophenyl groups, respectively, and *p(s)* represents an *S*-(2-cyanoethyl)-protected phosphorothioate. If *p(s)* occurs at the 3'- or 5'-end of a sequence, it represents a phosphorothioate diester; if *p(s)* occurs elsewhere in a sequence, it represents a phosphorothioate triester. Thus DMTr-*Tp(s)* and HO-*Gp(s)G*-Lev are abbreviations for **16a** and **22b**, respectively. The preparation of the two tetramer



SCHEME 4 *Reagents and conditions*: i, MSNT **23**, C_5H_5N, room temp., 30 min; ii, Cl_2CHCO_2H, pyrrole, CH_2Cl_2, 0°C, 10min.

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blocks (Ac-*Tp(s)Tp(s)Gp(s)G*-Lev **28** and DMTr-*Gp(s)Gp(s)Tp(s)T*-Lev **29**) required in the synthesis of the octanucleoside heptaphosphorothioate d[Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)-Gp(s)Tp(s)T] is indicated in SCHEME 5. It was first necessary to prepare the two protected dinucleotide analogues Ac-*Tp(s)Tp(s)* **25** and DMTr-*Gp(s)Gp(s)* **27** (SCHEME 5a,b). Following acetylation of HO-*Tp(s)T*-Lev **22a**, the 3'-*O*-levulinyl protecting group was removed by hydrazinolysis by a slight modification of the literature procedure[18]. The resulting partially-protected dinucleoside phosphorothioate **24** was then converted into Ac-*Tp(s)Tp(s)* **25** by the procedure used above (SCHEME 3) in the conversion of 3-*N*-(*p*-anisoyl)-5'-*O*-(4,4'-dimethoxytrityl)thymidine **9** into its 3'-*S*-(2-cyanoethyl) phosphorothioate **16a**. The triethylammonium salt of the protected dinucleotide analogue **25** was isolated as a colourless solid precipitate in *ca.* 85% overall yield, based on HO-*Tp(s)T*-Lev **22a**. By a closely related procedure (SCHEME 5b), DMTr-*Gp(s)G*-Lev **21b** was converted into DMTr-*Gp(s)Gp(s)* **27** in *ca.* 83% isolated yield. The dinucleotide analogues **25** and **27** were coupled, in the presence of MSNT **23** in dry pyridine solution, with the partially-protected dinucleoside phosphorothioates **22b** and **22a** (SCHEME 5c,d), respectively, to give the fully-protected tetranucleoside triphosphorothioates **28** and **29**. The latter products were isolated in *ca.* 78 and 84% yield, respectively. The 5'-terminal tetramer **28** was prepared on a *ca.* 1.3 mmolar scale using a *ca.* 1.3-fold excess of the 3'-phosphorothioate **25**, and the 3'-terminal tetramer **29** was prepared on a 0.7 mmolar scale using a *ca.* 1.2-fold excess of the 3'-phosphorothioate **27**. It is relevant to add (see Experimental) that the 5'-terminal tetramer **28** was purified by standard short column

(a) HO-*Tp(s)T*-Lev **22a** $\xrightarrow{\text{i, ii}}$ Ac-*Tp(s)T*-OH **24** $\xrightarrow{\text{iii, iv}}$ Ac-*Tp(s)Tp(s)* **25** (*ca.* 85%)

(b) DMTr-*Gp(s)G*-Lev **21b** $\xrightarrow{\text{ii}}$ DMTr-*Gp(s)G*-OH **26** $\xrightarrow{\text{iii, iv}}$ DMTr-*Gp(s)Gp(s)* **27** (ca. 83%)

(c) Ac-*Tp(s)Tp(s)* **25** + HO*Gp(s)G*-Lev **22b** $\xrightarrow{\text{v}}$ Ac-*Tp(s)Tp(s)Gp(s)G*-Lev **28** (*ca.* 78%)

(d) DMTr-*Gp(s)Gp(s)* **27** + HO-*Tp(s)T*-Lev **22a** $\xrightarrow{\text{v}}$ DMTr-*Gp(s)Gp(s)Tp(s)T*-Lev **29** (*ca.* 84%)

SCHEME 5 *Reagents and conditions*: i, Ac_2O, C_5H_5N, room temp., 16h; ii, a, $N_2H_4 \cdot H_2O$, C_5H_5N - AcOH (1:1 v/v), 0°C, 10 min, b, pentan-2,4-dione, C_5H_5N, 0°C, 10 min; iii, a, [reagent prepared from PCl_3, Et_3N, 1,2,4-1*H*-triazole, THF, -50°C, 15 min], -50°C, 30 min, b, aq. triethylammonium phosphate buffer (pH 7.0); iv, **19**, 4-methylmorpholine, Me_3SiCl, room temp., 1h; v, MSNT **23**, C_5H_5N, room temp., 2h.

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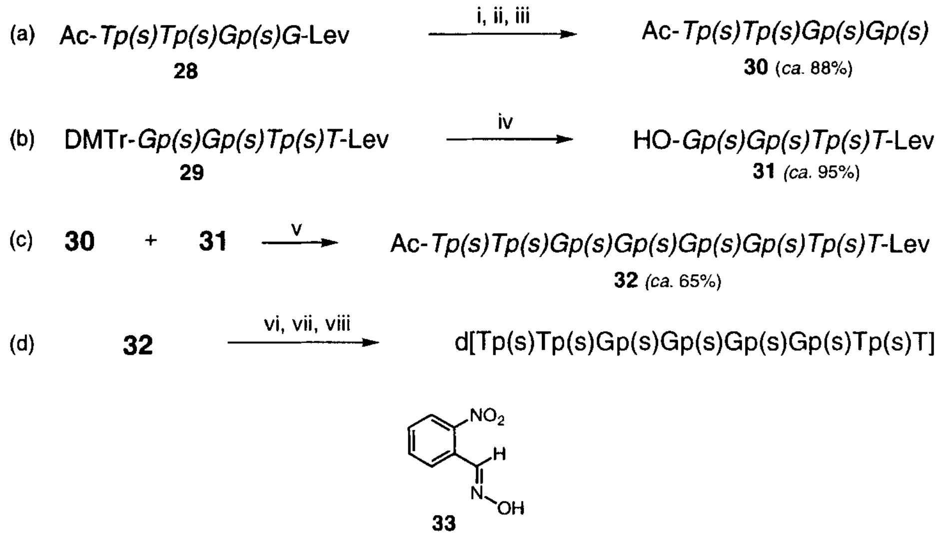
chromatography whereas the 3'-terminal tetramer **29** was purified by chromatography on silanised silica gel. Neither of the coupling reactions has been optimised.

The fully-protected tetranucleoside triphosphorothioates **28** and **29** were converted (SCHEME 6a,b) into the tetramer blocks **30** and **31**, respectively, required for the preparation of the target octadeoxyribonucleoside heptaphosphorothioate d[Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T], following procedures indicated above. Thus the procedure used for the conversion of Ac-*Tp(s)Tp(s)Gp(s)G*-Lev **28** into Ac-*Tp(s)Tp(s)Gp(s)Gp(s)* **30** (obtained in *ca.* 88% isolated yield; SCHEME 6a) was closely similar to that used for the conversion (SCHEME 5b) of DMTr-*Gp(s)G*-Lev **21b** into DMTr-*Gp(s)Gp(s)* **27**, and the standard 'detritylation' procedure[16] was used in the conversion of DMTr-*Gp(s)Gp(s)Tp(s)T*-Lev **29** into HO-*Gp(s)Gp(s)Tp(s)T*-Lev **31** (obtained in *ca.* 95% yield; SCHEME 6b). The two tetramers **30** (*ca.* 0.37 mmol) and **31** (0.30 mmol) were then coupled together in the presence of MSNT **23** in dry pyridine solution (SCHEME 6c) to give the fully-protected octamer **32**. The latter product **32** was isolated in *ca.* 65% yield, following chromatography on silanised silica gel. This coupling reaction has not been optimised. Indeed, it is anticipated that the isolated yield would be considerably increased if the reaction were carried out on a large scale.

The fully-protected octamer **32** was unblocked by the three step procedure indicated in SCHEME 6d. First, the 2-cyanoethyl protecting groups were removed from the internucleotide linkages by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene(DBU) in very dry (chlorotrimethylsilane was added to remove traces of moisture) pyridine solution.



(a) Ac-*Tp(s)Tp(s)Gp(s)G*-Lev **28** $\xrightarrow{\text{i, ii, iii}}$ Ac-*Tp(s)Tp(s)Gp(s)Gp(s)* **30** (*ca.* 88%)

(b) DMTr-*Gp(s)Gp(s)Tp(s)T*-Lev **29** $\xrightarrow{\text{iv}}$ HO-*Gp(s)Gp(s)Tp(s)T*-Lev **31** (*ca.* 95%)

(c) **30** + **31** $\xrightarrow{\text{v}}$ Ac-*Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T*-Lev **32** (*ca.* 65%)

(d) **32** $\xrightarrow{\text{vi, vii, viii}}$ d[Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T]

SCHEME 6 *Reagents and conditions*: i, a, $N_2H_4 \cdot H_2O$, C_5H_5N - AcOH (1:1 v/v), 0°C, 10 min, b, pentan-2,4-dione, C_5H_5N, 0°C, 10 min; ii, a, [reagent prepared from PCl_3, Et_3N, 1,2,4-1*H*-triazole, THF, -50°C, 15 min], -50°C, 30 min, b, aq. triethylammonium phosphate buffer (pH 7.0); iii, **19**, 4-methylmorpholine, Me_3SiCl, CH_2Cl_2, room temp., 1h; iv, Cl_2CHCO_2H, pyrrole, CH_2Cl_2, 0°C, 10 min; v, MSNT **23**, C_5H_5N, room temp., 3h; vi, Me_3SiCl, DBU, CH_2Cl_2, room temp., 30 min; vii, *E*-2-nitrobenzaldoxime **33**, DBU, MeCN, room temp., 12h; viii, conc. aq. NH_3 (*d* 0.88) - $HOCH_2CH_2SH$ (9:1 v/v), 50°C, 15h.

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Since our earlier study[6], we have found that DBU is a superior reagent to *tert*-butylamine[8] for this purpose. Secondly, the 6-*O*-(2,5-dichlorophenyl) protecting groups were removed from the guanine residues by treatment with *E*-2-nitrobenzaldoxime[13] **33** and DBU in acetonitrile solution. Thirdly, the acyl protecting groups were removed from the base residues and from the 3'- and 5'-terminal hydroxy functions by treatment with concentrated aqueous ammonia (*d* 0.88) - 2-mercaptoethanol (9 : 1 v/v) at 50°C. We have very recently found[19] that the presence of 2-mercaptoethanol completely suppresses sulfur loss in this unblocking step. We and other workers have found[10,19,20] that when this final unblocking step is carried out in the usual way just with concentrated aqueous ammonia, the conversion of phosphorothioate diester to phosphodiester internucleotide linkages can occur to a small (say, *ca.* 1%) extent. The ^{31}P NMR spectrum (in D_2O) of fully-unblocked d[Tp(s)Tp(s)-Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T] is illustrated in FIG. 1, and it is clear that no resonance signal is observable in the region of 0 ppm.

Finally, d[Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T] was prepared on a 15 μmolar scale by solid phase synthesis, using standard DNA phosphoramidites and 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent[21]) as the sulfur transfer agent. HPLC

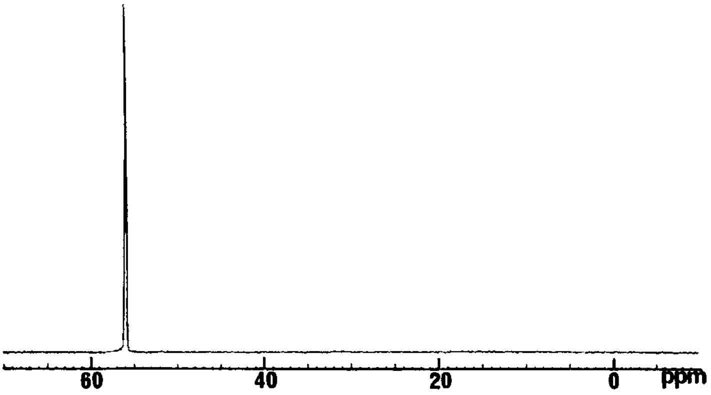

FIG. 1. ^{31}P NMR Spectrum (D_2O) of d[Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)-Gp(s)Tp(s)T], prepared by the phosphotriester approach in solution.

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traces of d[Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T], prepared as above by the phosphotriester approach in solution and by solid phase synthesis, are illustrated in FIG. 2. No further purification steps were carried out on either sample prior to HPLC analysis. The preparation of d[Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T] by the phosphotriester approach in solution has previously been reported in a conference abstract[22] but the synthetic methodology used was not revealed. As indicated above, we believe that solution synthesis may well prove to be superior to solid-phase synthesis in the large scale preparation of oligonucleotides and oligonucleotide phosphorothioates of moderate size. Studies directed towards the improvement of solution phase coupling procedures and purification methods are currently being undertaken in our laboratories.

EXPERIMENTAL

^{1}H NMR Spectra were measured at 360 MHz with a Bruker AM 360 spectrometer; ^{13}C and ^{31}P NMR spectra were measured at 90.6 and 145.8 MHz, respectively, with the same spectrometer. J values are given in Hz. Infrared spectra were measured with a Perkin Elmer Paragon 1000 FT spectrometer. Merck silica gel 60 F_{254} TLC plates (Art 5715 and 5642) were developed in solvent systems A [dichloromethane - methanol (90 : 10 v/v)] and B [dichloromethane - methanol (95 : 5 v/v)]. Merck silica gel 60 (Art 7729 and 9385) was used for short column chromatography. Preparative reverse phase chromatography was carried out on silanised silica gel 60 (Art 7719) columns which were eluted with acetonitrile - 0.1M-potassium phosphate buffer (pH 7.0) mixtures. Acetonitrile, 4-methylmorpholine, 1-methylpyrrolidine, pyridine and triethylamine were dried by heating with calcium hydride, under reflux, and were then distilled. 1,8-Diazabicyclo[5.4.0]undec-7-ene

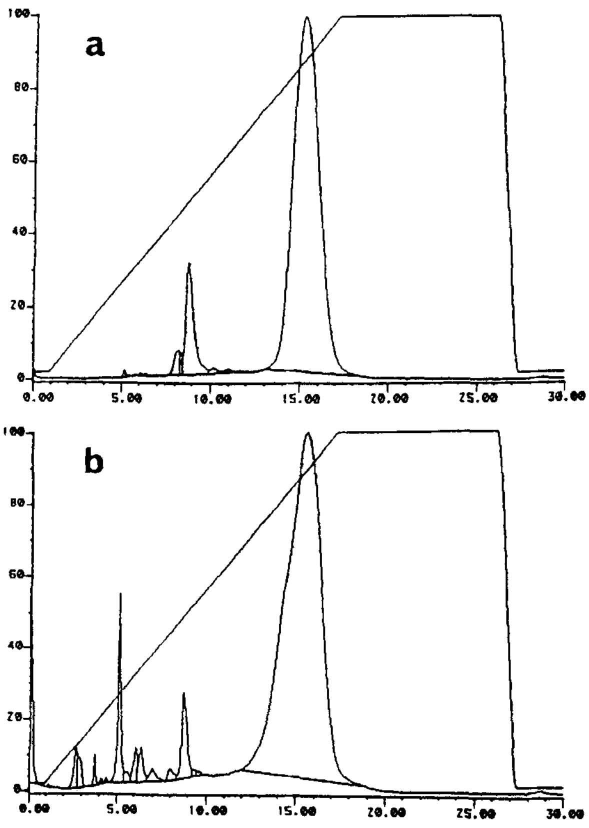

FIG. 2. Anion-exchange HPLC (NucleoPac PA-100 column - see Experimental) profiles of d[Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T], prepared (a) by the phosphorotriester approach in solution, and (b) by solid phase synthesis.

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(DBU) was dried by heating with calcium hydride and was then distilled under reduced pressure. Diethyl ether, tetrahydrofuran (THF) and dichloromethane were dried over sodium wire, sodium benzophenone and phosphorus pentaoxide, respectively, and were then distilled. HPLC was carried out on (i) a NucleoPac PA-100 (25 cm x 4 mm) anion-exchange column which was gradient eluted with 0.02 M tris HCl (pH 8.0) buffer, 0.4 M with respect to $NaClO_4$ - acetonitrile (99.5 : 0.5 v/v) and (ii) a Hypersil 5 μ ODS (25 cm x 4 mm) column which was eluted with acetonitrile - 0.1 M - aqueous triethylammonium acetate mixtures[5].

Levulinic anhydride. Levulinic acid (23.2 g, 0.20 mol), *N,N'*-dicyclohexylcarbodiimide (20.6 g, 0.10 mol) and dry diethyl ether (200 mL) were stirred together at room temperature. After 1.5 h, the products were filtered. The filtrate was evaporated under reduced pressure to give crude levulinic anhydride as a pale yellow viscous oil (*ca.* 22 g) which solidified on cooling to 4 °C; υ_{max} (Nujol) 1717, 1747, 1820 cm^{-1}.

3-*N*-(*p*-Anisoyl)-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-levulinylthymidine (8). Crude levulinic anhydride (4.28 g, *ca.* 20 mmol) was added to a stirred solution of 5'-*O*-(4,4'-dimethoxytrityl)thymidine (7) (5.45 g, 10.0 mmol), triethylamine (3.5 mL, 25 mmol) and 4-(dimethylamino)pyridine (0.10 g, 0.8 mmol) in dry dichloromethane (50 mL) at room temperature. After 2 h, the products were poured into saturated aqueous sodium hydrogen carbonate (100 mL). The aqueous layer was extracted with dichloromethane (50 mL). The combined organic layers were washed with saturated aqueous sodium hydrogen carbonate (2 x 50 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: the appropriate fractions, which were eluted with ethyl acetate, were combined and evaporated under reduced pressure to give a pale yellow foam (5.80 g); R_f 0.72 (system B).

p-Anisoyl chloride (2.56 g, 15 mmol) was added to a stirred solution of the latter material (4.82 g) and di-isopropylethylamine (3.1 mL, 17.8 mmol) in dry pyridine (40 mL) at room temperature. After 15 h, the products were poured into saturated aqueous sodium hydrogen carbonate (75 mL). The resulting mixture was extracted with chloroform (3 x 30 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: the appropriate fractions, which were eluted with ethyl acetate - petroleum ether (b.p. 40 - 60 °C) (40 : 60 to 60 : 40 v/v), were combined and evaporated under reduced pressure. A solution of the residue in chloroform (12 mL) was added dropwise with stirring to petroleum ether (b.p. 30 - 40 °C, 250 mL) to give 3-*N*-(*p*-anisoyl)-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-levulinylthymidine (8) as a pale yellow solid (5.32 g, *ca.* 82% yield for the two steps starting from 5'-*O*-(4,4'-dimethoxytrityl)thymidine 7); R_f 0.86 (system B); δ_H [$(CD_3)_2SO$] 1.51 (3 H, s), 2.09 (3 H, s), 2.40 (1 H, m), 2.48 (2 H, m), 2.60 (1 H, m), 2.72 (2 H, m), 3.27 (1 H, m), 3.36 (1 H, m), 3.75 (6 H, s), 3.87 (3 H, s), 4.11 (1 H, m), 5.34 (1 H, m), 6.22 (1 H, m), 6.92 (4 H, d, *J* 8.4), 7.09 (2 H, d, *J* 8.9), 7.2 - 7.4 (9 H, m), 7.75 (1 H, s), 7.95 (2 H, d, *J* 8.7).

3-*N*-(*p*-Anisoyl)-5'-*O*-(4,4'-dimethoxytrityl)thymidine (9). A solution of hydrazine monohydrate (7.27 mL, 0.15 mol) in pyridine (75 mL) and glacial acetic acid (75 mL) was added to a stirred, cooled (ice-water bath) solution of 3-*N*-(*p*-anisoyl)-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-levulinylthymidine (8) (12.75 g, *ca.* 16.4 mmol) in pyridine (150 mL). After 10 min, pentan-2,4-dione (30 mL) was added. After a further period of 10 min, the products were partitioned between dichloromethane (600 mL) and water (600 mL). The organic layer was separated, washed with saturated aqueous sodium hydrogen carbonate (3 x 300 mL), dried ($MgSO_4$) and evaporated under reduced pressure. A solution of the

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residue in dichloromethane (30 mL) was added dropwise with stirring to petroleum ether (b.p. 30 - 40 °C, 300 mL) to give 3-*N*-(*p*-anisoyl)-5'-*O*-(4,4'-dimethoxytrityl)thymidine (**9**) as a pale yellow solid (10.2 g, *ca.* 91%); R_f 0.58 (system B); $\delta_H[(CD_3)_2SO]$ 1.52 (3 H, d, *J* 0.8), 2.24 (1 H, m), 2.35 (1 H, m), 3.24 (2 H, m), 3.74 (6 H, s), 3.87 (3 H, s), 3.93 (1 H, m), 4.37 (1 H, m), 5.37 (1 H, d, *J* 4.6), 6.18 (1 H, m), 6.92 (4 H, d, *J* 8.8), 7.09 (2 H, m), 7.25 - 7.35 (7 H, m), 7.42 (2 H, m), 7.72 (1 H, m), 7.92 (2 H, d, *J* 8.8).

3-*N*-(*p*-Anisoyl)-3'-*O*-levulinylthymidine (10). A solution of dichloroacetic acid (3.1 mL, 37.5 mmol) in dichloromethane (25 mL) was added to a stirred solution of 3-*N*-(*p*-anisoyl)-5'-*O*-(4,4-dimethoxytrityl)-3'-*O*-levulinylthymidine (**8**) (12.75 g, *ca.* 16.4 mmol) and pyrrole (7.8 mL, 0.11 mol) in dichloromethane (75 mL) at room temperature. After 10 min, the products were poured into saturated aqueous sodium hydrogen carbonate (150 mL). The resulting mixture was separated and the aqueous layer was extracted with dichloromethane (3 x 150 mL). The combined organic layers were dried ($MgSO_4$) and then evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel: the appropriate fractions, which were eluted with dichloromethane - methanol (99 : 1 to 97 : 3 v/v), were evaporated under reduced pressure to give 3-*N*-(*p*-anisoyl)-3'-*O*-levulinylthymidine (**10**) (6.48 g, *ca.* 83%) as a pale yellow glass; R_f 0.47 (system B); $\delta_H[(CD_3)_2SO]$ 1.88 (3 H, s), 2.10 (3 H, s), 2.31 (1 H, m), 2.41 (1 H, m), 2.50 (2 H, m), 2.74 (2 H, m), 3.69 (2 H, m), 3.87 (3 H, m), 4.04 (1 H, m), 5.27 (1 H, m), 5.32 (1 H, t, *J* 5.1), 6.20 (1 H, m), 7.10 (2 H, d, *J* 8.9), 7.95 (2 H, m), 8.01 (1 H, s).

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2'-Deoxy-6-*O*-(2,5-dichlorophenyl)-5'-*O*-(4,4'-dimethoxytrityl)-2-*N*-isobutyrylguanosine (12). Chlorotrimethylsilane (3.2 mL, 25 mmol) was added to a stirred solution of 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-2-*N*-isobutyrylguanosine (**11**) (6.08 g, *ca.* 9.5 mmol) in dry pyridine (15 mL) at room temperature. After 30 min, the products were concentrated under reduced pressure to *ca.* one-half volume and more dry pyridine (10 mL) was added. Mesitylene-2-sulfonyl chloride (3.28 g, 15 mmol) and 1-methylpyrrolidine (10 mL, 96 mmol) were then added to the cooled (ice-water bath), stirred solution. After 15 min, 2,5-dichlorophenol (4.89 g, 30 mmol) was added and, after a further period of 3 h, triethylamine (5 mL, 36 mmol) and water (5 mL) were added. The stirred reactants were then allowed to warm up to room temperature. After 16 h, the products were poured into saturated aqueous sodium hydrogen carbonate solution (200 mL). The resulting mixture was extracted with dichloromethane (3 x 100 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel : the appropriate fractions, which were eluted with dichloromethane - methanol (99 : 1 v/v) were evaporated under reduced pressure to give 2'-deoxy-6-*O*-(2,5-dichlorophenyl)-5'-*O*-(4,4'-dimethoxytrityl)-2-*N*-isobutyrylguanosine (**12**) as a colourless glass (7.06 g, *ca.* 94%); *R*f 0.57 (system B); δ_H [$(CD_3)_2SO$] 0.92 (6 H, d, *J* 6.4), 2.38 (1 H, m), 2.82 (1 H, m), 2.91 (1 H, m), 3.12 (1 H, dd, *J* 2.9 and 10.2), 3.38 (1 H, m), 3.72 (6 H, 2s), 4.01 (1 H, m), 4.57 (1 H, m), 5.35 (1 H, d, *J* 4.6), 6.43 (1 H, t, *J* 6.4), 6.74 (2 H, d, *J* 8.9), 6.79 (2 H, d, *J* 8.9), 7.18 (7 H, m), 7.32 (2 H, m), 7.47 (1 H, dd, *J* 2.4 and 8.7), 7.70 (2 H, m), 8.49 (1 H, s), 10.27 (1 H, s).

2'-Deoxy-6-*O*-(2,5-dichlorophenyl)-2-*N*-isobutyryl-3'-*O*-levulinylguanosine (13). A solution of 2'-deoxy-6-*O*-(2,5-dichlorophenyl)-5'-*O*-(4,4'-dimethoxytrityl)-2-*N*-isobutyrylguanosine (**12**) (3.76 g, *ca.* 4.8 mmol) and crude levulinic anhydride (2.14 g, *ca.* 10 mmol) in dry pyridine (15 mL) was stirred at room temperature. After 16 h, the products were concentrated under reduced pressure and the residue was partitioned between dichloromethane (50 mL) and saturated aqueous sodium hydrogen carbonate (50 mL). The organic layer was separated, washed with saturated aqueous sodium hydrogen carbonate (2 x 50 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was co-evaporated with toluene (2 x 20 mL) and then dissolved in dichloromethane (150 mL). Pyrrole (5 mL, 72 mmol) and then a solution of dichloroacetic acid (4.14 mL, 50 mmol) in dichloromethane (50 mL) were added to the cooled (ice-water bath), stirred solution. After 10 min, the products were extracted with saturated aqueous sodium hydrogen carbonate (100 mL). The aqueous layer was back-extracted with dichloromethane (3 x 50 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel : the appropriate fractions, which were eluted with dichloromethane - methanol (98 : 2 v/v), were evaporated under reduced pressure to give 2'-deoxy-6-*O*-(2,5-dichlorophenyl)-2-*N*-isobutyryl-3'-*O*-levulinylguanosine (**13**) as a colourless glass (2.52 g, *ca.* 90%); *R*f 0.48 (system B); δ_H [$(CD_3)_2SO$] 0.94 (6 H, d, *J* 6.7), 2.13 (3 H, s), 2.47 - 2.56 (3 H, m), 2.75 - 2.84 (3 H, m), 3.06 (1 H, m), 3.61 (2 H, m), 4.07 (1 H, m), 5.37 (1 H, m), 6.38 (1 H, dd, *J* 5.9 and 8.6), 7.46 (1 H, dd, *J* 2.5 and 8.6), 7.69 (1 H, d, *J* 8.7), 7.77 (1 H, d, *J* 2.5), 8.63 (1 H, s), 10. 38 (1 H, s).

Triethylammonium salts of base-protected 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)ribonucleoside-3'-*S*-(2-cyanoethyl) phosphorothioates (16). Triethylamine (18 mL, 0.13 mol) and phosphorus trichloride (3.5 mL, 40 mmol) were added to a stirred solution of 1,2,4-1*H*-triazole (9.11 g, 0.132 mol) in dry THF (330 mL) at *ca.* -50 °C (methanol - dry ice bath). After 15 min, a solution of base-protected 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)ribonucleoside (**14**) (10.0 mmol) in dry THF (165 mL) was added with continued cooling (to *ca.* -50 °C). After a further period of 30 min, the cooling bath was removed and 1.0 M-potassium phosphate buffer (pH 7.0, 1000 mL) was added. The resulting mixture was concentrated to small volume, and then partitioned between

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chloroform (400 mL) and 0.5 M-aqueous triethylammonium phosphate buffer (pH 7.0, 100 mL). The chloroform layer was separated, washed again with triethylammonium phosphate buffer (100 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was co-evaporated with acetonitrile (3 x 35 mL) and then dissolved in dry dichloromethane. 3-(Phthalimidosulfanyl)propanonitrile[6] (**19**) (3.1 g, 13.3 mmol) followed by 4-methylmorpholine (6.7 mL, 61 mmol) and chlorotrimethylsilane (5.0 mL, 39 mmol) were then added, and the reactants were stirred together at room temperature. After 1 h, the products were poured into 0.5 M - aqueous triethylammonium phosphate buffer (pH 7.0, 200 mL). The layers were separated and the aqueous layer was back-extracted with dichloromethane (2 x 100 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel : the appropriate fractions, which were eluted with dichloromethane -methanol (90 : 10 to 85 : 15 v/v), were combined and evaporated under reduced pressure. A solution of the residue in chloroform (30 mL) was added dropwise with stirring to petroleum ether (b.p. 30 - 40 °C, 300 mL) to give triethylammonium 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)ribonucleoside 3'-*S*-(2-cyanoethyl) phosphorothioate (**16**) as a colourless solid.

3-*N*-(*p*-Anisoyl)-5'-*O*-(4,4'-dimethoxytrityl)thymidine (**9**) (6.79 g, *ca.* 10.0 mmol) was converted into the corresponding triethylammonium *S*-(2-cyanoethyl) phosphorothioate (**16a**) (8.74 g, *ca.* 94%); δ_P [$(CD_3)_2SO$] 13.6.

2'-Deoxy-6-*O*-(2,5-dichlorophenyl)-5'-*O*-(4,4'-dimethoxytrityl)-2-*N*-isobutyrylguanosine (**12**) (7.85 g, *ca.* 10.0 mmol) was converted into the corresponding *S*-(2-cyanoethyl) phosphorothioate (**16b**) (9.55 g, *ca.* 92%); δ_P [$(CD_3)_2SO$] 13.2.

Preparation of the fully-protected dinucleoside phosphorothioates (DMTr-*Tp(s)T*-Lev 21a and DMTr-*Gp(s)G*-Lev 21b). (a) A solution of triethylammonium 3-*N*-(*p*-anisoyl)-5'-*O*-(4,4'-dimethoxytrityl)thymidine 3'-*S*-(2-cyanoethyl) phosphorothioate (**16a**) (11.61 g, *ca.* 12.5 mmol) and 3-*N*-(*p*-anisoyl)-3'-*O*-(levulinyl)thymidine (**10**) (4.74 g, *ca.* 10 mmol) in dry pyridine (50 mL) was concentrated under reduced pressure to very small volume. Evaporation from pyridine (50 mL) solution was repeated two more times. The residue was then dissolved in dry pyridine (50 mL) at room temperature and 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-1*H*-triazole (MSNT **23**) (8.89 g, 30 mmol) was added. After the reactants had been stirred for 30 min, saturated aqueous sodium hydrogen carbonate (2.5 mL) was added, and the products were partitioned between chloroform (250 mL) and saturated aqueous sodium hydrogen carbonate (250 mL). The layers were separated, and the aqueous layer was back-extracted with dichloromethane (2 x 100 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was evaporated with toluene and then

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fractionated by short column chromatography on silica gel : the appropriate fractions, which were eluted with ethyl acetate - petroleum ether (b.p. 40 - 60 °C) (30 : 70 to 60 : 40 v/v), were evaporated under reduced pressure. A solution of the residue in chloroform (30 mL) was added dropwise with stirring to petroleum ether (b.p. 30 - 40 °C, 600 mL) to give DMTr-*Tp(s)T*-Lev (**21a**) as a colourless solid (12.40 g, *ca.* 96%); R_f 0.74 (system A); δ_P [$(CD_3)_2SO$] 27.94, 28.18.

(b) In the same way, DMTr-*Gp(s)G*-Lev (**21b**) (5.36 g, *ca.* 94%) was obtained as a colourless solid from triethylammonium 2'-deoxy-6-*O*-(2,5-dichlorophenyl)-5'-*O*-(4,4'-dimethoxytrityl-2-*N*-isobutyrylguanosine 3'-*S*-(2-cyanoethyl) phosphorothioate (**16b**) (5.07 g, *ca.* 4.89 mmol), 2'-deoxy-6-*O*-(2,5-dichlorophenyl)-2-*N*-isobutyryl-3'-*O*-levulinylguanosine (**13**) (2.20 g, *ca.* 3.80 mmol) and MSNT (3.56 g, 12.0 mmol); R_f 0.68 (system A); δ_P [$(CD_3)_2SO$] 27.16, 27.26.

Detritylation of fully-protected dinucleoside phosphorothioates (DMTr-*Tp(s)T*-Lev 21a and DMTr-*Gp(s)G*-Lev 21b). (a) A solution of dichloroacetic acid (3.3 mL, 40 mmol) in dichloromethane (40 mL) was added to a cooled (ice-water bath) stirred solution of DMTr-*Tp(s)T*-Lev (**21a**) (5.14 g, 4.00 mmol) and pyrrole (4.0 mL, 58 mmol) in dichloromethane (120 mL). After 10 min, the products were poured into saturated aqueous sodium hydrogen carbonate (150 mL). The layers were separated and the aqueous layer was extracted with dichloromethane (3 x 50 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel : the appropriate fractions, which were eluted with dichloromethane - methanol (99 : 1 to 97 : 3 v/v) were evaporated under reduced pressure to give HO-*Tp(s)T*-Lev (**22a**) as a colourless glass (3.88g, *ca.* 98%); R_f 0.57 (system A); δ_P[$(CD_3)_2SO$] 27.73, 27.86.

(b) Using the same procedure but scaling down the quantities of reagents and solvents, DMTr-*Gp(s)G*-Lev (**21b**) (2.12 g, *ca.* 1.41 mmol) was converted into HO-*Gp(s)G*-Lev (**22b**) (1.63 g, *ca.* 96%); R_f 0.58 (system A); δ_P [$(CD_3)_2SO$] 27.30.

Preparation of dimeric triethylammonium 3'-*S*-(2-cyanoethyl) phosphorothioates (Ac-*Tp(s)Tp(s)* 25 and DMTr-*Gp(s)Gp(s)* 27). (a) A solution of HO-*Tp(s)T*-Lev (**22a**) (2.45 g, *ca* 2.5 mmol) and acetic anhydride (1.18 mL, 12.5 mmol) in dry pyridine (15 mL) was stirred at room temperature. After 16 h, the products were concentrated under reduced pressure and partitioned between dichloromethane (50 mL) and saturated aqueous sodium hydrogen carbonate (50 mL). The aqueous layer was back-extracted with dichloromethane (2 x 25 mL). The combined organic extracts were dried ($MgSO_4$) and evaporated under reduced pressure. A solution of the residue in dichloromethane (10 mL) was added dropwise with stirring to petroleum ether (b.p. 30 - 40 °C, 100 mL) to give a colourless solid precipitate (2.52 g). A solution of hydrazine

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monohydrate (1.12 mL, 23 mmol) in pyridine (11.5 mL), glacial acetic acid (11.5 mL) and water (2.3 mL) was added to a cooled (ice-water-bath), stirred solution of the above acetylated material (2.35 g) in pyridine (23 mL). After 10 min, pentan-2,4-dione (4.6 mL) was added and, after a further period of 10 min, the products were partitioned between dichloromethane (100 mL) and water (100 mL). The organic layer was separated, washed with saturated aqueous sodium hydrogen carbonate (3 x 50 mL), dried ($MgSO_4$) and evaporated under reduced pressure. A solution of the residue in dichloromethane (10 mL) was added with stirring to petroleum ether (b.p. 30 - 40 °C, 100 mL) to give a colourless solid precipitate (1.95 g).

A solution of the latter material (1.85 g) in dry THF (100 mL) was added over a period of 30 min to the stirred products of the reaction (15 min at *ca.* -50 °C) between phosphorus trichloride (1.05 mL, 12.0 mmol), 1,2,4-1*H*-triazole (2.48 g, 36 mmol) and triethylamine (5.5 mL, 40 mmol) in dry THF (100 mL), maintained at *ca.* -50 °C (methanol - dry ice bath). After a further period of 30 min, the cooling bath was removed and 1.0 M-potassium phosphate buffer (pH 7.0, 300 mL) was added. The resulting mixture was concentrated to small volume and then partitioned between chloroform (100 mL) and 0.5 M - triethylammonium phosphate buffer (pH 7.0, 50 mL). The chloroform layer was separated, washed again with triethylammonium phosphate buffer (50 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was co-evaporated with acetonitrile (3 x 20 mL) and then dissolved in dry dichloromethane (36 mL). 3-(Phthalimidosulfanyl)propanonitrile (**19**) (0.93 g, 4.0 mmol), followed by 4-methylmorpholine (1.3 mL, 12 mmol) and chlorotrimethylsilane (1.0 mL, 7.9 mmol), were added and the reactants allowed to stand at room temperature. After 1 h, the products were poured into 0.5 M - aqueous triethylammonium phosphate buffer (pH 7.0, 50 mL). The layers were separated and the aqueous layer was back-extracted with dichloromethane (2 x 20 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel : the appropriate fractions, which were eluted with dichloromethane - methanol (90 : 10 to 85 : 15 v/v), were combined and evaporated under reduced pressure. A solution of the residue in chloroform (10 mL) was added dropwise with stirring to petroleum ether (b.p. 30 - 40 °C, 100 mL) to give triethylammonium Ac-*Tp(s)Tp(s)* (**25**) as a colourless solid (2.37 g, *ca.* 85% overall yield, based on HO-*Tp(s)T*-Lev **22a**); δ_P [$(CD_3)_2SO$] 12.90 (1 P, s), 27.82, 27.96 (1 P, 2s).

(b) The levulinyl protecting group was removed from DMTr-*Gp(s)G*-Lev (**21b**) (2.16 g, *ca.* 1.44 mmol) with hydrazine monohydrate (0.73 mL, 15 mmol) in pyridine (15 mL), glacial acetic acid (15 mL) and water (1.5 mL), and the products were isolated as a colourless solid (1.89 g) according to the procedure described in (a) above. This material

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(1.88 g) was then converted, also by the procedure described in (a) above [the phosphitylating agent was prepared from phosphorus trichloride (0.74 mL, 8.5 mmol), 1,2,4-1*H*-triazole (1.74 g, 25.2 mmol) and triethylamine (3.86 mL, 28 mmol) in THF (70 mL) at *ca.* -50°C (methanol - dry ice bath), and sulfur-transfer was effected with 3-(phthalimidosulfanyl)propanonitrile (**19**) (0.64 g, 2.8 mmol) in the presence of 4-methylmorpholine (0.93 mL, 8.5 mmol) and chlorotrimethylsilane (0.71 mL, 5.6 mmol) in dichloromethane (25 mL)] into the triethylammonium salt of DMTr-*Gp(s)Gp(s)* (**27**) (1.98 g, *ca.* 83%, based on DMTr-*Gp(s)G*-Lev (**21b**) as starting material); δ_P [$(CD_3)_2SO$] 12.55 (1 P, s), 27.26 (1 P, s).

Preparation of fully-protected tetranucleoside triphosphorothioates (Ac-*Tp(s)Tp(s)Gp(s)G*-Lev 28 and DMTr-*Gp(s)Gp(s)Tp(s)T*-Lev 29). (a) A solution of the triethylammonium salt of Ac-*Tp(s)Tp(s)* (**25**) (2.00 g, *ca.* 1.7 mmol) and HO-*Gp(s)G*-Lev (**22b**) (1.54 g, 1.29 mmol) in dry pyridine (6 mL) was evaporated to dryness under reduced pressure. Evaporation from pyridine (6 mL) solution was repeated twice more. The residue was dissolved in dry pyridine (6 mL) at room temperature and MSNT (**23**) (1.20 g, 4.05 mmol) was added. After the reactants had been stirred for 2 h, saturated aqueous sodium hydrogen carbonate (1 mL) was added and the products were partitioned between chloroform (50 mL) and saturated aqueous sodium hydrogen carbonate (50 mL). The organic layer was separated and the aqueous layer was back-extracted with dichloromethane (2 x 25 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel : the appropriate fractions, which were eluted with acetone - toluene (30 : 70 to 60 : 40 v/v), were combined and evaporated under reduced pressure. A solution of the residue in chloroform (10 mL) was added dropwise with stirring to petroleum ether (b.p. 30 - 40 °C, 100 mL) to give Ac-*Tp(s)Tp(s)Gp(s)G*-Lev (**28**) as a colourless solid (2.26 g, *ca.* 78%); R_f 0.61 (system A); δ_P [$(CD_3)_2SO$] 27.64, 27.77, 27.93, 28.22.

(b) In the same way, the triethylammonium salt of DMTr-*Gp(s)Gp(s)* (**27**) (1.404 g, *ca.* 0.85 mmol), HO-*Tp(s)T*-Lev (**22a**) (0.687 g, *ca.* 0.70 mmol) and MSNT (**23**) (0.622 g, 2.1 mmol) were allowed to react together in dry pyridine (6 mL) solution at room temperature. After 2 h, the products were worked up as in (a) above, and then fractionated on a column (15 cm x 3 cm diameter) of Merck 7719 silanised silica gel. The column was eluted with acetonitrile - 0.1 M-potassium phosphate buffer (pH 7.0) : the appropriate fractions, which were eluted with acetonitrile - phosphate buffer (50 : 50 to 60 : 40, v/v), were combined and the acetonitrile was removed by evaporation under reduced pressure. The remaining mixture was extracted with dichloromethane (3 x 50 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. A solution of

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the residue in chloroform (5 mL) was added dropwise with stirring to diethyl ether (100 mL) to give DMTr-*Gp(s)Gp(s)Tp(s)T*-Lev (**29**) as a colourless solid (1.488 g, *ca.* 84%); R_f 0.64 (system A); δ_P [$(CD_3)_2SO$] 27.21, 27.33, 27.68, 28.16, 28.30.

Preparation of fully-protected octanucleoside heptaphosphorothioate (Ac-*Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T*-Lev 32). (a) The levulinyl protecting group was removed from Ac-*Tp(s)Tp(s)Gp(s)G*-Lev (**28**) (1.77 g, *ca.* 0.78 mmol) with hydrazine monohydrate (0.40 mL, 8.2 mmol) in pyridine (8 mL), glacial acetic acid (8 mL) and water (0.8 mL), and the product was isolated as a pale yellow precipitated solid (1.59 g) by the procedure described above for the removal of the 3'-*O*-levulinyl group from Ac-*Tp(s)T*-Lev. This material (1.58 g) was then converted [the phosphitylating agent was prepared from PCl_3 (0.26 mL, 3.0 mmol), 1,2,4-1*H*-triazole (0.93 g, 13.5 mmol) and triethylamine (2.1 mL, 15 mmol) in THF (35 mL) at *ca.* -50 °C (methanol - dry ice bath), and sulfur-transfer was effected with 3-(phthalimidosulfanyl)propanonitrile (**19**) (0.35 g, 1.5 mmol) in the presence of 4-methylmorpholine (0.49 mL, 4.5 mmol) and chlorotrimethylsilane (0.38 mL, 3.0 mmol) in dichloromethane (15 mL)] by the procedure described above (see preparation of Ac-*Tp(s)Tp(s)* **25**) into the triethylammonium salt of Ac-*Tp(s)Tp(s)Gp(s)Gp(s)* (**30**) (1.66 g, *ca.* 88% based on Ac-*Tp(s)Tp(s)Gp(s)T*-Lev **28** as starting material); δ_P [$(CD_3)_2SO$] 13.40 (1 P, s), 27.54, 27.84, 28.05 (3 P, 3s).

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(b) A solution of dichloroacetic acid (0.41 mL, 5 mmol) in dichloromethane (5 mL) was added to a stirred solution of DMTr-*Gp(s)Gp(s)Tp(s)T*-Lev (**29**) (1.234 g, *ca.* 0.49 mmol) and pyrrole (0.5 mL, 7.2 mmol) in dry dichloromethane (15 mL) at 0 °C (ice-water-bath). After 10 min, the products were poured into saturated aqueous sodium hydrogen carbonate (100 mL). The layers were separated and the aqueous layer was back-extracted with dichloromethane (3 x 50 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. A solution of the residue in chloroform (5 mL) was added dropwise with stirring to diethyl ether (100 mL) to give HO-*Gp(s)Gp(s)Tp(s)T*-Lev (**31**) as a colourless solid (1.037 g, *ca.* 95%); R_f 0.55 (system A); δ_P [$(CD_3)_2SO$] 27.31, 28.12, 28.30.

(c) A solution of the triethylammonium salt of Ac-*Tp(s)Tp(s)Gp(s)Gp(s)* (**30**) (0.888 g, *ca.* 0.37 mmol) and HO-*Gp(s)Gp(s)Tp(s)T*-Lev (**31**) (0.649 g, *ca.* 0.3 mmol) in dry pyridine (5 mL) was evaporated to dryness under reduced pressure. Evaporation from pyridine (5 mL) solution was repeated twice more. The residue was dissolved in dry pyridine (1.5 mL) and MSNT (**23**) (0.264 g, 0.89 mmol) was added. The reactants were stirred at room temperature for 3 h and were then worked up as in the above preparation of the fully-protected tetranucleoside triphosphorothioates (**28** and **29**). The crude products were fractionated on a column (15 cm x 3 cm diameter) of Merck 7719 silanised silica gel. The column was eluted with acetonitrile - 0.1 M-potassium phosphate buffer (pH 7.0) : The appropriate fractions, which were eluted with acetonitrile - phosphate buffer (60 : 40 to 70 : 30 v/v), were combined and the acetonitrile was removed by evaporation under reduced pressure. A solution of the residue in chloroform (5 mL) was added dropwise with stirring to diethyl ether (100 mL) to give Ac-*Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T*-Lev (**32**) as a colourless solid (0.866 g, *ca.* 65%); R_f 0.57 (system A); $\delta_P[(CD_3)_2SO]$ 27.18, 27.33, 27.41, 27.55, 27.80, 28.06, 28.21.

Removal of protecting groups from fully-protected octanucleoside heptaphosphorothioate (Ac-*Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T*-Lev 32). Fully-protected octamer (**32**) (0.10 g, *ca.* 0.022 mmol) was co-evaporated with anhydrous pyridine (1 mL) and the residue was dissolved, in an atmosphere of argon, in a solution of chlorotrimethylsilane (0.025 mL, 0.2 mmol) and DBU (0.18 mL, 1.2 mmol) in dry dichloromethane (1.8 mL). After 30 min, the solution was evaporated to small volume under reduced pressure and the residue was redissolved in a solution of *E*-2-nitrobenzaldoxime (**33**) (0.166 g, 1.0 mmol) in dry acetonitrile (1 mL) at room temperature. After 12 h, the products were evaporated under reduced pressure, and concentrated aqueous ammonia (*d* 0.88, 1.8 mL) and 2-mercaptoethanol (0.2 mL) were added. The resulting mixture was heated at 50°C for 15 h and was then evaporated under reduced pressure. The residue was dissolved in methanol (2 mL) and ethyl acetate (30 mL) was added. The resulting precipitate was collected by centrifugation, washed with diethyl ether (2 x 10 mL) and dried to give d[Tp(s)Tp(s)Gp(s)Gp(s)Gp(s)Gp(s)Tp(s)T] as a colourless solid (0.050 g); δ_P $[D_2O]$ 55.81, 55.95, 56.05.

Solid phase synthesis. The solid phase synthesis of d[Tp(s)Tp(s)Gp(s)Gp(s)-Gp(s)Gp(s)Tp(s)T] was carried out on a 15 μmolar scale in a Cruachem PS 250 synthesizer. Standard DNA phosphoramidites and high-loaded controlled pore glass (100 μmol/g) were used. The manufacturer's 15 μmol synthesis protocol was followed, and 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent[21]) was used as the sulfur-transfer reagent.

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