The *H*-phosphonate approach to the synthesis of oligonucleotides and their phosphorothioate analogues in solution

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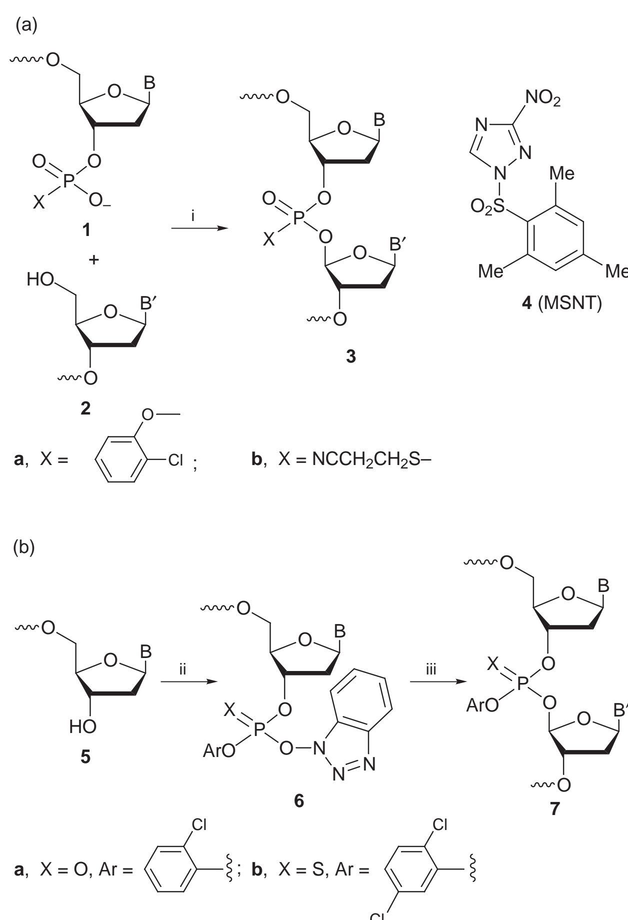
A new approach to the synthesis of oligonucleotides and oligonucleotide phosphorothioates in solution is described; it is based on *H*-phosphonate coupling [with bis(2-chlorophenyl) phosphorochloridate **22** as the coupling agent] at −40 °C, followed by *in situ* sulfur transfer involving either 2-(4-chlorophenylsulfanyl)isoindole-1,3(2*H*)-dione **23a** or 4-[(2-cyanoethyl)sulfanyl]morpholine-3,5-dione **26**. The yields of the coupling and sulfur transfer reactions are virtually quantitative and, following unblocking by previously reported procedures, very pure products (d[ApC], d[TpGpApC], d[TpGp(s)ApC], d[Gp(s)A] and d[Cp(s)Tp(s)Gp(s)A]) are obtained.

Introduction

The phosphotriester,[1] phosphoramidite[2] and *H*-phosphonate[3] approaches have all proved to be effective in the chemical synthesis of oligonucleotides. The phosphotriester approach has been used mainly in solution phase synthesis. On the other hand, the phosphoramidite and *H*-phosphonate approaches have been used almost exclusively in solid phase synthesis. Now that the use of oligonucleotides and their phosphorothioate analogues in chemotherapy[4] is no longer merely a theoretical possibility, the development of a method or methods for their large-scale synthesis has become a matter of considerable importance and indeed of urgency. Although the demand for relatively large quantities of material has so far been met by the scaling up[5] of solid phase synthesis, we believe that, when multikilogram quantities of a specific sequence are required for chemotherapeutic purposes, it is very likely indeed that solution phase synthesis will become the method of choice.

Two distinct strategies have been followed successfully in the synthesis of oligodeoxyribonucleotides and their phosphorothioate analogues by the phosphotriester approach in solution. What is probably the most widely used strategy in solution phase oligonucleotide synthesis involves a coupling reaction between a protected nucleoside or oligonucleotide 3′-(2-chlorophenyl) phosphate[6] **1a** and a protected nucleoside or oligonucleotide **2** with a free 5′-hydroxy function to give a phosphotriester **3a** (Scheme 1a). A coupling agent such as 1-(mesitylsulfonyl)-3-nitro-1,2,4-1*H*-triazole (MSNT)[7] **4** is required. This strategy has also been followed in the synthesis of phosphorothioate analogues of oligodeoxyribonucleotides by, for example, coupling[8,9] a protected nucleoside or oligonucleotide 3′-*S*-(2-cyanoethyl) phosphorothioate **1b** (Scheme 1a) with an appropriate component **2** with a free 5′-hydroxy function. Perhaps the main disadvantages of this 'classical' phosphotriester approach are that some concomitant 5′-sulfonation (usually ≮5%) of the second component **2** occurs,[10] and that coupling reactions are generally not very rapid. The sulfonation side-reaction inevitably leads to a lower coupling yield and also complicates the purification process. The alternative strategy for the solution phase synthesis of oligodeoxyribonucleotides (Scheme 1b) involves the use of a bifunctional reagent,[11] derived from an aryl (usually 2-chlorophenyl) phosphorodichloridate and two molecular equivalents of 1-hydroxybenzotriazole. This coupling reaction is assumed to proceed *via* an intermediate with the general structure **6a**. The corresponding bifunctional reagent, derived from an aryl (*e.g.* 2,5-dichlorophenyl) phosphorodichloridothioate (Scheme 1b),

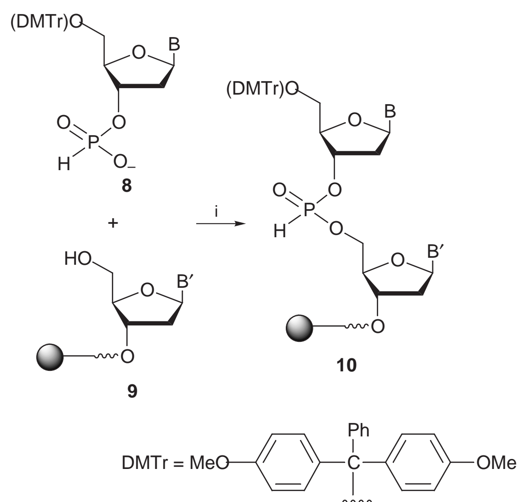


Scheme 1 *Reagents and conditions*: i, MSNT **4**, C_5H_5N, room temp.; ii, reagent prepared from 2-chlorophenyl phosphorodichloridate or 2,5-dichlorophenyl phosphorodichloridothioate, 1-hydroxybenzotriazole, base, 1,4-dioxane or THF, room temp.; iii, **2**, 1-methylimidazole, C_5H_5N.

has been used[12] in the preparation of oligodeoxyribonucleotide phosphorothioates. The coupling reaction is similarly assumed to proceed *via* an intermediate with the general structure **6b**. The main disadvantages of the second strategy result directly from the fact that a bifunctional reagent is involved. Thus, the possibility exists that symmetrical coupling products will be formed. Furthermore, the presence of small quantities of moisture can lead to a significant diminution in coupling yields.

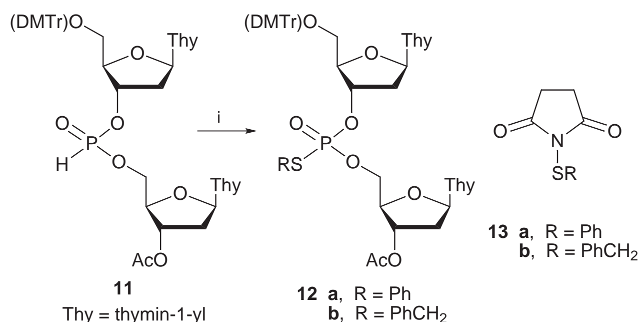
In order to avoid the disadvantages inherent in both of the above strategies, we set out to devise a new coupling procedure for the synthesis of oligonucleotides in solution that (a) is extremely efficient and does not lead to side-reactions, (b) proceeds relatively rapidly, and (c) is equally suitable for the preparation of oligonucleotides, their phosphorothioate analogues, and chimeric oligonucleotides containing both phosphodiester and phosphorothioate diester internucleotide linkages. This study has already been published in a preliminary form.[13]

As indicated above, the *H*-phosphonate approach has been used widely and successfully[3] in solid-phase oligonucleotide synthesis. Chain extension (Scheme 2), leading to an *H*-phosphonate diester **10**, is achieved by coupling a solid-supported 3′-terminal nucleoside residue (or a growing oligomer with *H*-phosphonate internucleoside linkages) **9** with an appropriate 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyribonucleoside 3′-*H*-phosphonate **8** in the presence of a coupling agent (*e.g.* pivaloyl chloride[14]). The coupling reaction is fast, but side-reactions (particularly *P*-acylation[15] by the coupling agent) can occur. After the desired sequence has been fully assembled, the *H*-phosphonate diester linkages are oxidized to natural phosphodiester internucleotide linkages. It is important to recognise that *H*-phosphonate diesters (as in **10**) are very much more susceptible to base-catalyzed hydrolysis[16] than corresponding phosphotriesters (as in **3a**). Therefore, although *H*-phosphonate coupling has also been carried out successfully in solution,[15,17] the purification and isolation of the resulting *H*-phosphonate diesters is likely to be accompanied by some degradation. Nevertheless, simple protected dinucleoside *H*-phosphonates (*e.g.* **11**) have been prepared in solution and then isolated in satisfactory yields.[17]



Scheme 2 *Reagents and conditions*: i, Me_3CCOCl, C_5H_5N, MeCN.

Several years ago, van Boom and his co-workers[18] reported that the fully protected dithymidine *H*-phosphonate derivative **11** reacted with *N*-phenylsulfanyl- and *N*-benzylsulfanyl-succinimide **13a** and **13b** to give the corresponding *S*-phenyl and *S*-benzyl dinucleoside phosphorothioates **12a** and **12b** in high (92 and 95%, respectively) isolated yields (Scheme 3). Like aryl phosphotriesters (as in **3a**), *S*-aryl phosphorothioate triesters (*e.g.* **12a**) are generally much more stable to base-catalyzed hydrolysis than *H*-phosphonate diesters (*e.g.* **11**); thus their purification and isolation would not be expected to be accompanied by degradation and thereby by diminished yields. It has further been shown that such *S*-aryl phosphorothioate triesters (*e.g.* **12a**) can readily be converted into phosphodiesters.[18–21] It then occurred to us that, if conditions could be found such that the *H*-phosphonate coupling reaction (Scheme 2) was virtually quantitative, and if the resulting *H*-phosphonate diesters (*e.g.* **11**) could be converted *in situ* into the corresponding *S*-aryl phosphorothioate triesters (*e.g.* **12a**), again in virtually quantitative yield, a basis would exist for the development of a new approach to the synthesis of oligonucleotides in solution that would meet the criteria outlined above.

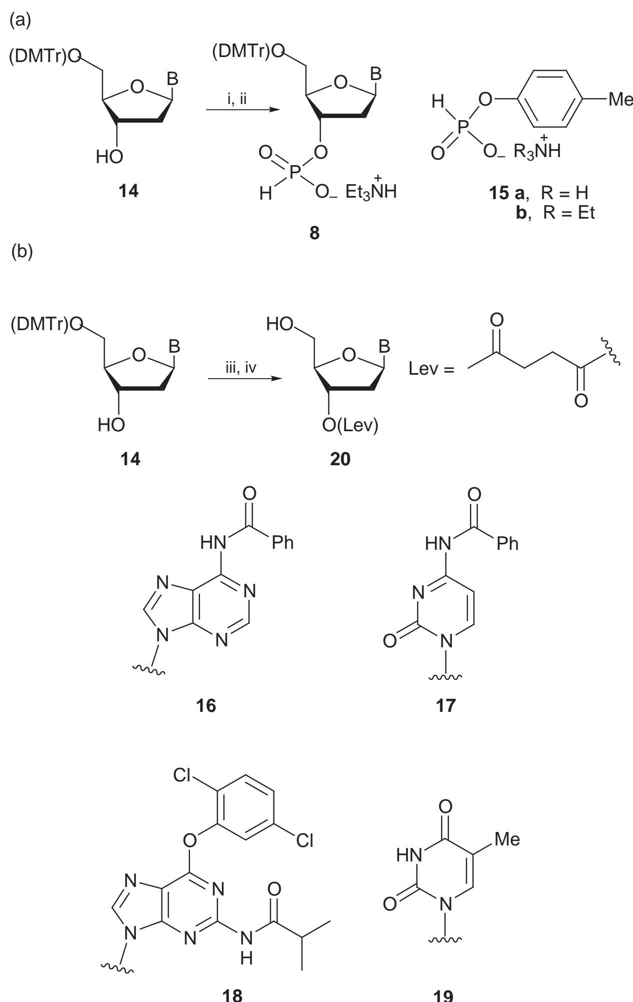


Scheme 3 *Reagents and conditions*: i, **13a** (→**12a**) *or* **13b** (→**12b**), Pr^i_2NEt, CH_2Cl_2.

Results and discussion

Although a number of procedures have been reported[22] for the conversion of 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxynucleoside derivatives **14** into their 3′-*H*-phosphonates **8**, none of them proved to be entirely suitable for the present purposes. Our requirements were particularly demanding in that we needed a completely reliable procedure that was also suitable for the conversion of protected oligonucleotide blocks with free terminal 3′-hydroxy functions into the corresponding 3′-*H*-phosphonates. It was therefore essential that the *H*-phosphonylation conditions should be completely reproducible, and that yields were as nearly quantitative as possible. We have developed[23] what we believe to be a particularly convenient and effective new *H*-phosphonylation procedure (Scheme 4a). The nucleoside derivative **14** (B = **16**, **17**, **18** or **19**) is allowed to react with the putative triethylammonium salt of *p*-tolyl *H*-phosphonate **15b** (prepared by evaporating a solution of the crystalline ammonium salt **15a**, triethylamine and pyridine under reduced pressure) and pivaloyl chloride in dry pyridine solution at −35 °C, and water is then added to the products. Using this procedure, the desired 3′-*H*-phosphonates **8**; B = **16**, **17**, **18** and **19** were obtained and isolated as their triethylammonium salts in very high (97–99%) yields.[23] The products were characterized by ^1H and ^{31}P NMR spectroscopy, and were found to be homogeneous by reversed phase HPLC. As indicated in Scheme 4, the adenine and cytosine residues were protected as their *N*-benzoyl derivatives (as in **16** and **17**, respectively) and thymine residues (as in **19**) were left unprotected. Guanine residues were protected as their 2-*N*-isobutyryl-6-*O*-(2,5-dichlorophenyl) derivatives[9] (as in **18**). As well as providing protection against possible side-reactions, the 6-*O*-(2,5-dichlorophenyl) protecting group improves the solubility properties of protected 2′-deoxyguanosine-containing oligonucleotides and facilitates their chromatographic purification. In addition to the four *H*-phosphonate building blocks **8**; B = **16**–**19**, three 3′-*O*-levulinoyl-2′-deoxyribonucleoside derivatives[24] **20**; B = **16**, **17** and **19** were required. The latter three compounds were prepared by the two-step procedure indicated in Scheme 4b, and were isolated as crystalline solids in 81, 94 and 86% overall yield, respectively. The preparation of 2′-deoxy-6-*O*-(2,5-dichlorophenyl)-2-*N*-isobutyryl-3′-*O*-levulinoylguanosine **8**; B = **18** has been described previously.[9]

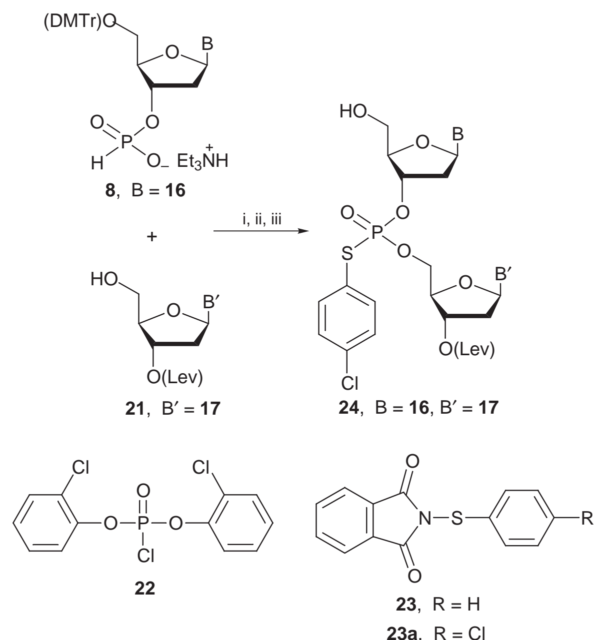
Pivaloyl chloride, which is the most commonly used coupling agent in *H*-phosphonate-based solid phase synthesis[3] (Scheme 2), also promotes rapid coupling in solution at room temperature.[17] Unfortunately, however, the resulting *H*-phosphonate diesters can react further with pivaloyl chloride.[15] One important advantage that solution phase has over automated solid phase synthesis is that the reaction temperature can easily be controlled. We have examined the coupling properties of more



Scheme 4 *Reagents and conditions*: i, **15b**, Me_3CCOCl, C_5H_5N, −35 °C, 30 min; ii, a, water, C_5H_5N, room temp., 1 h, b, pH 7.0 triethylammonium phosphate buffer; iii, levulinic anhydride, Et_3N, DMAP, CH_2Cl_2, room temp., 2 h; iv, HCl/1,4-dioxane, −50 °C, 5 min.

than ten reagents over a range of temperatures, and have found that optimum coupling is obtained with bis(2-chlorophenyl) phosphorochloridate[25] **22** at −40 °C: coupling is usually complete within 5–10 min, and no by-products can be detected. Diphenyl phosphorochloridate, the first reagent ever used for this purpose,[26] is very nearly as effective. We have used bis(2-chlorophenyl) phosphorochloridate exclusively as the coupling agent in this study. In order to facilitate the oximate ions-promoted[7,27] unblocking step, we have used 2-(4-chlorophenylsulfanyl)isoindole-1,3(2*H*)-dione[28] **23a** rather than the unsubstituted phenylsulfanyl derivative **23**; R = H in the conversion of *H*-phosphonate diesters (*e.g.* **11**) into the corresponding *S*-aryl phosphorothioate triesters (*e.g.* **12**; R = aryl).

When triethylammonium 6-*N*-benzoyl-(4,4′-dimethoxytrityl)-2′-deoxyadenosine 3′-*H*-phosphonate[23] **8**; B = **16**, 4-*N*-benzoyl-3′-*O*-levulinoyl-2′-deoxycytidine **21**; B′ = **17** and bis(2-chlorophenyl) phosphorochloridate **22** were allowed to react together in pyridine–dichloromethane solution at −40 °C (Scheme 5), coupling was complete within 5–10 min. No attempt was made to isolate the resulting putative fully protected dinucleoside *H*-phosphonate. Instead, the products were maintained at −40 °C and 2-(4-chlorophenylsulfanyl)-isoindole-1,3(2*H*)-dione **23a** was added. In this way, the fully protected dinucleoside phosphorothioate was obtained in what appeared to be quantitative yield. Again, the latter material was not isolated but was treated with hydrogen chloride at −50 °C to give the partially protected *S*-(4-chlorophenyl) dinucleoside phosphorothioate **24**; B = **16**, B′ = **17**, which was isolated in 98% yield. It is noteworthy that, although only *ca.* 20% excess

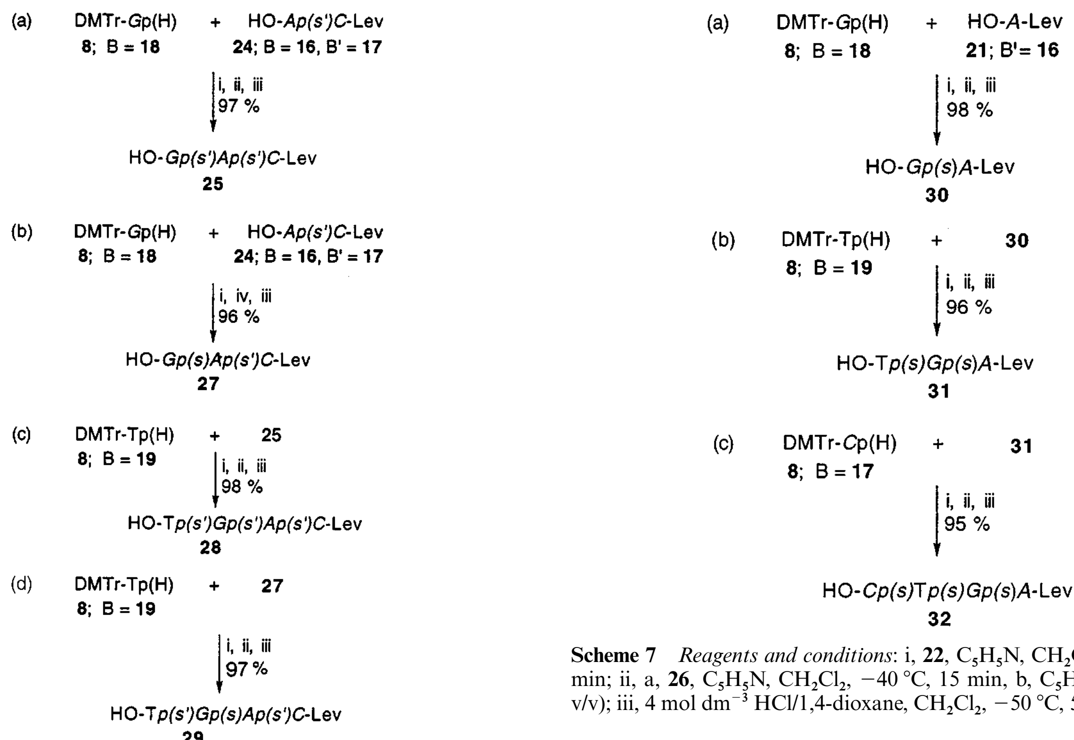


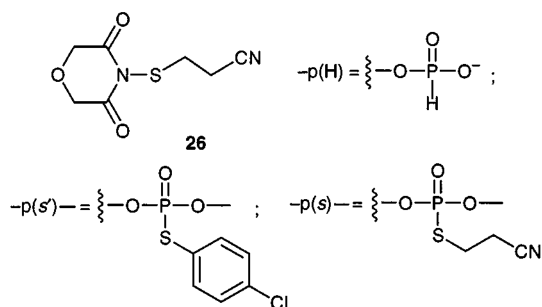
Scheme 5 *Reagents and conditions*: i, **22**, C_5H_5N, CH_2Cl_2, −40 °C, 5–10 min; ii, **23a**, C_5H_5N, CH_2Cl_2, −40 °C, 15 min, b, C_5H_5N–water (1 : 1 v/v), −40 °C to room temp.; iii, 4 mol dm^{-3} HCl/1,4-dioxane, CH_2Cl_2, −50 °C, 5 min.

of *H*-phosphonate **8**; B = **16** was used, coupling proceeded rapidly and quantitatively at −40 °C. The sulfur-transfer reaction also proceeded rapidly and quantitatively at −40 °C. A threefold excess of the coupling agent **22** and a twofold excess of the (4-chlorophenylsulfanyl) derivative **23** were used. A model experiment with 6-*N*-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosine **14**; B = **16** (see Experimental section) confirmed that the removal of the 5′-*O*-(4,4′-dimethoxytrityl) protecting group with hydrogen chloride, under the conditions used above (Scheme 5, step iii), leads to no detectable concomitant depurination.

A number of years ago, we introduced[29] a system of abbreviations for protected oligodeoxyribonucleotides in which nucleoside residues and internucleotide linkages are italicized if they are protected in some defined way. In the present context, *A*, *C*, *G* and T represent 2′-deoxyadenosine protected on N-6 with a benzoyl group (as in **16**), 2′-deoxycytidine protected on N-4 with a benzoyl group (as in **17**), 2′-deoxyguanosine protected on N-2 and on O-6 with isobutyryl and 2,5-dichlorophenyl groups (as in **18**) and unprotected thymidine, respectively; *p(s′)* and *p(s)* represent[13] *S*-(4-chlorophenyl)- and *S*-(2-cyanoethyl)-protected phosphorothioates, respectively, and p(H), which is not italicized, represents an unprotected *H*-phosphonate monoester if it is attached to a monomer or if it is placed at the end of a sequence. Thus 6-*N*-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyadenosine 3′-*H*-phosphonate **8**; B = **16** (Scheme 5) is abbreviated to DMTr-*A*p(H), and the partially-protected dinucleoside phosphorothioate **24**; B = **16**, B′ = **17** is abbreviated to HO-*Ap(s′)C*-Lev.

The partially protected dinucleoside phosphorothioate HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17** was then coupled with DMTr-*G*p(H) **8**; B = **18** (Scheme 6a), and the products were treated first with 2-(4-chlorophenylsulfanyl)isoindole-1,3(2*H*)-dione **23a** and then with hydrogen chloride to give HO-*Gp(s′)Ap(s′)C*-Lev **25** in 97% isolated yield for the three steps. The stoichiometry and the reaction conditions were the same as those indicated above (Scheme 5) in the preparation of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17**. In order to demonstrate that the present methodology can be used to prepare chimeric oligonucleotides containing both natural phosphodiester and phosphorothioate diester internucleotide linkages, this experiment was repeated except that 2-(4-chlorophenylsulfanyl)-





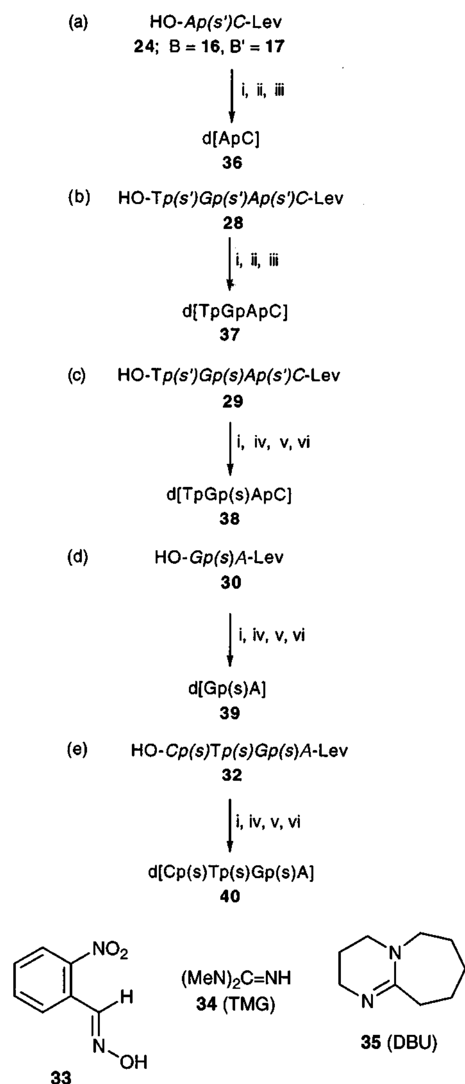
Scheme 6 *Reagents and conditions*: i, **22**, C_5H_5N, CH_2Cl_2, −40 °C, 10 min; ii, a, **23a**, C_5H_5N, CH_2Cl_2, −40 °C, 15 min, b, C_5H_5N–water (1:1 v/v); iii, 4 mol dm^{-3} HCl/1,4-dioxane, CH_2Cl_2, −50 °C, 5 min; iv, **26**, C_5H_5N, CH_2Cl_2, −40 °C, 10 min.

Scheme 7 *Reagents and conditions*: i, **22**, C_5H_5N, CH_2Cl_2, −40 °C, 10 min; ii, a, **26**, C_5H_5N, CH_2Cl_2, −40 °C, 15 min, b, C_5H_5N–water (1:1 v/v); iii, 4 mol dm^{-3} HCl/1,4-dioxane, CH_2Cl_2, −50 °C, 5 min.

isoindole-1,3(2*H*)-dione **23a** was replaced (Scheme 6b) by 4-[(2-cyanoethyl)sulfanyl]morpholine-3,5-dione[30] **26** in the second step. Following treatment with hydrogen chloride, HO-*Gp(s)Ap(s′)C*-Lev **27** was obtained in 96% isolated yield. Each of these partially protected trinucleoside diphosphorothioates **25** and **27** was then coupled with DMTr-Tp(H) **8**; B = **19** (Schemes 6c and 6d, respectively), and the products were treated with 2-(4-chlorophenylsulfanyl)isoindole-1,3(2*H*)-dione **23**. Following the removal of the 4,4′-dimethoxytrityl protecting groups, the partially protected tetranucleoside triphosphorothioates **28** and **29** were obtained and isolated in 98 and 97% overall yield, respectively. 4-[(2-Cyanoethyl)sulfanyl]-morpholine-3,5-dione **26** has an advantage over 2-(2-cyanoethylsulfanyl)isoindole-1,3(2*H*)-dione[8,30] in that the by-product obtained (*i.e.* morpholine-3,5-dione) is more easily removed from the products by aqueous extraction than is phthalimide.

Finally, HO-*Gp(s)A*-Lev **30** and HO-*Cp(s)Tp(s)Gp(s)A*-Lev **32**, the respective precursors of the dinucleoside phosphorothioate, d[Gp(s)A] **39** and the tetranucleoside triphosphorothioate, d[Cp(s)Tp(s)Gp(s)A] **40** (see below), were prepared (Scheme 7) in the same way as HO-*Ap(s′)C*-Lev **24**, B = **16**, B′ = **17** and HO-T*p(s′)Gp(s′)Ap(s′)C*-Lev **28** (Schemes 5 and 6 respectively) except that 4-[(2-cyanoethyl)-sulfanyl]morpholine-3,5-dione **26** was used instead of 2-(4-chlorophenylsulfanyl)isoindole-1,3(2*H*)-dione **23a** in all of the sulfur-transfer steps (step ii). As indicated in Scheme 7, the overall isolated yields for the three steps (*i.e.* coupling, sulfur-transfer and ‘detritylation’) in the preparation of HO-*Gp(s)A*-Lev **30**, HO-T*p(s)Gp(s)A*-Lev **31** and HO-*Cp(s)*-*Tp(s)Gp(s)A*-Lev **32** were 98, 96 and 95%, respectively.

The partially protected oligodeoxyribonucleotides, HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17** and HO-T*p(s′)Gp(s′)Ap(s′)*-*C*-Lev **28**, were unblocked by a three-step process (Schemes 8a and 8b). First, the free 5′-hydroxy functions were acetylated to prevent their possible participation in the subsequent unblocking step. The acetylated material was then treated with *E*-2-nitrobenzaldoxime[27] **33** and N^1,N^1,N^3,N^3-tetramethylguanidine (TMG) **34** in acetonitrile solution at room temperature to convert the *S*-(4-chlorophenyl) phosphorothioate triester [-*p(s′)*-] into standard phosphodiester internucleotide linkages and, in the case of the tetramer **28**, also to remove the 6-*O*-(2,5-dichlorophenyl) protecting group from the guanine residue. The third unblocking step involved treatment with concentrated aqueous ammonia at 50 °C in order to remove the acyl protecting groups from the base residues and from the 3′- and 5′-terminal hydroxy functions. Finally, the fully unblocked dinucleoside phosphate, d[ApC] **36** and tetranucleoside triphosphate, d[TpGpApC] **37** were converted into their sodium salts, which were isolated as colourless solids in *ca.* 90 and 96% yield, respectively. The unblocking of the other three partially protected oligonucleotides **29**, **30** and **32** (Scheme 8c–e), which all contain at least one *S*-(2-cyanoethyl)-protected phosphorothioate linkage [-*p(s)*-], involved an extra step. Following acetylation of the terminal 5′-hydroxy functions, the *S*-2-cyanoethyl protecting group or groups were removed by treatment with an anhydrous solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)[13] **35** in pyridine. Then *E*-2-nitrobenzaldoxime **33** was added to the already basic solution to convert the *S*-(4-chlorophenyl)-protected phosphorothioate triester groups [-*p(s′)*-] in the tetramer **29** into phosphodiester internucleotide linkages, and to remove the 6-*O*-(2,5-dichlorophenyl) protecting group from the guanine residue in each of the three substrates **29**, **30** and **32**. Oximate treatment is, of course, unnecessary when only *S*-(2-cyanoethyl)-protected phosphorothioate triester groups [-*p(s)*-] and no protected guanine residues are present. Finally, in order to avoid partial desulfurization of the resulting phosphorothioate diester groups [-p(s)-], the ammonolysis step was carried out in the presence of 2-mercaptoethanol.[31] The fully-unblocked products (d[TpGp-



Scheme 8 *Reagents and conditions*: i, Ac_2O, C_5H_5N, room temp., 15 h; ii, **33**, **34**, MeCN, room temp., 12 h; iii, a, conc. aq. NH_3 (*d* 0.88), 50 °C, 15 h, b, Amberlite IR-120(plus), Na^+ form, water; iv, **35**, Me_3SiCl, CH_2Cl_2, room temp., 30 min; v, **33**, **35**, MeCN, room temp., 12 h; vi, a, conc. aq. NH_3 (*d* 0.88)–$HSCH_2CH_2OH$ (9:1 v/v), 50 °C, 15 h, b, Amberlite IR-120 (plus), Na^+ form, water.

(s)ApC] **38**, d[Gp(s)A] **39**, and d[Cp(s)Tp(s)Gp(s)A] **40**) were again converted into their sodium salts, which were isolated as colourless solids in *ca.* 91, 89 and 93% yield, respectively.

Apart from being converted into their sodium salts, the target oligonucleotides **36**–**40** were not further purified after unblocking. The reversed phase HPLC profiles of products **36**–**39** are indicated in Fig. 1. It is noteworthy that the two diastereoisomers of d[Gp(s)A] **39** (Fig. 1d) but not those of d[TpGp(s)ApC] **38** (Fig. 1c) are separated by reversed phase HPLC. The ^1H NMR spectrum (D_2O) of d[ApC] **36** (Fig. 2a and Experimental section) clearly reveals resonance signals which can be assigned to the adenine (*δ* 8.0 and 8.15), cytosine (*δ* 5.69 and 7.64) and anomeric protons (*δ* 6.14 and 6.28). It is apparent both from the resonance signals of the three purine protons in the ^1H NMR spectrum of d[Gp(s)A] **39** (Fig. 2b and Experimental section) as well as from HPLC data (Fig. 1d) that both diastereoisomers are present. Integration of the downfield resonance signals in the ^1H NMR spectrum of d[TpGpApC] **37** (Experimental section; not illustrated) confirms the presence of five protons (*δ* 7.26–8.32), which are assignable to H-2 and -8 of the adenine residue, H-8 of the guanine residue, H-6 of the thymine residue and H-6 of the cytosine residue, and five protons (*δ* 5.7–6.2), which are assignable to H-1′ of all four

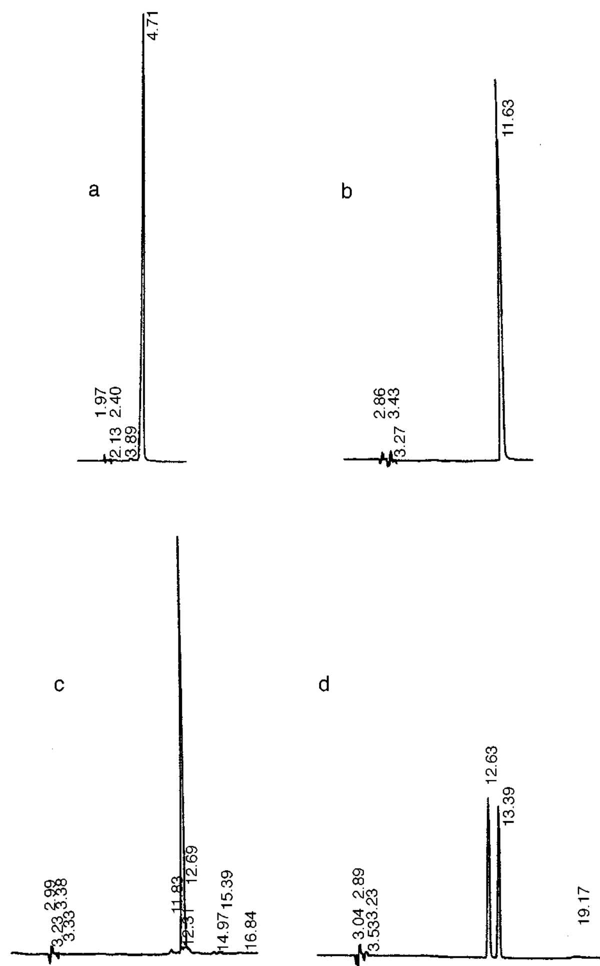


Fig. 1 Reversed-phase HPLC profiles of (a) d[ApC] (programme A), (b) d[TpGpApC] (programme B), (c) d[TpGp(s)ApC] (programme B) and (d) d[Gp(s)A] (programme B).

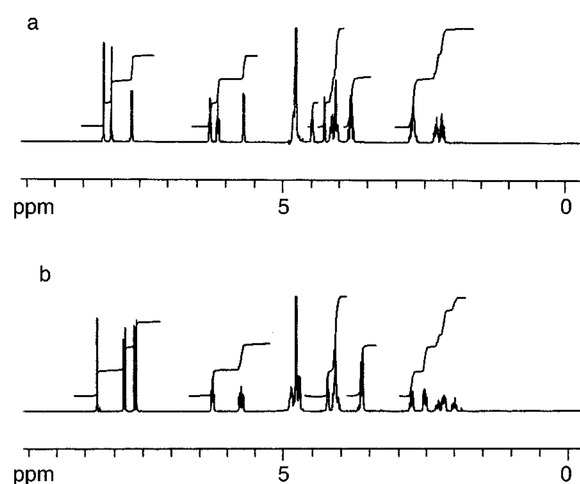


Fig. 2 ^1H NMR spectra (D_2O) of the Na^+ salts of (a) d[ApC] and (b) d[Gp(s)A].

nucleoside residues and to H-5 of the cytosine residue. Notwithstanding the additional complexity due to the presence of the diastereoisomers, similar evidence in support of its structure can be derived from the ^1H NMR spectrum (Experimental section) of d[TpGp(s)ApC] **38**. Further confirmation of their constitutions and homogeneity is provided by the ^{31}P NMR spectra (Fig. 3) of d[ApC] **36**, d[Gp(s)A] **39**, d[TpGpApC] **37**, d[TpGp(s)ApC] **38** and d[Cp(s)Tp(s)Gp(s)A] **40**. The presence of resonance signals that may be assigned to two diastereoisomers is apparent in the ^{31}P NMR spectra of d[Gp(s)A] **39**

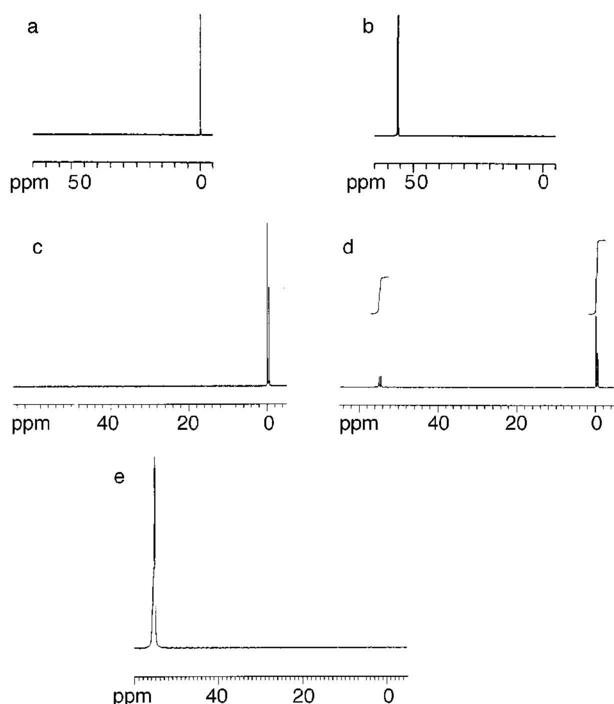


Fig. 3 ^{31}P NMR spectra (D_2O) of Na^+ salts of (a) d[ApC], (b) d[Gp(s)A], (c) d[TpGpApC], (d) d[TpGp(s)ApC] and (e) d[Cp(s)Tp(s)Gp(s)A].

and d[TpGp(s)ApC] **38** (Fig. 3b and 3d, respectively). It is further discernible from Fig. 3d that the integral of the ^{31}P resonance signals at *ca.* 0 ppm is twice as great as that at *ca.* 55 ppm. This is in accord with the presence of two phosphodiester internucleotide linkages and one phosphorothioate diester linkage in d[TpGp(s)ApC] **38**. Finally, it is clear from Fig. 3b and 3e that removal of the acyl protecting groups by concentrated aqueous ammonia in the presence of 2-mercaptoethanol[31] is not accompanied by any detectable desulfurization of the phosphorothioate diester linkages.

In conclusion, we believe that the preliminary results reported here, relating to this new approach to the solution phase synthesis of oligonucleotides and their phosphorothioate analogues, are very promising. It is particularly noteworthy and certainly encouraging that almost quantitative coupling yields are obtained and that side-reactions are not observed. It is also very encouraging that the unblocking of protected sequences, which have been purified only by chromatography on silica gel, generally leads to products that are virtually pure (Figs. 1–3). So far, coupling reactions have been carried out on a relatively small (approximately mmolar) scale, mainly in order to conserve starting materials. However, there is no obvious reason why it should not be possible for this process to be scaled up very considerably indeed. This study has involved the stepwise addition of monomeric *H*-phosphonate building blocks **8**. Other studies have indicated that this *H*-phosphonate approach to solution phase synthesis, which is equally effective in the preparation of oligoribonucleotides (RNA sequences),[32] also lends itself readily to block (*e.g.*, 4 + 4→8) coupling reactions.[33] Studies, involving block coupling and directed towards the synthesis of relatively high molecular weight biologically active oligonucleotides, are now being undertaken in this laboratory.

Experimental

^1H NMR spectra were measured at 360 MHz with a Bruker AM 360 spectrometer; ^{13}C NMR spectra were measured at 90.6 MHz with the same spectrometer. Tetramethylsilane was used as an internal standard, and *J* values are given in Hz. ^{31}P NMR spectra were measured at 145.8 MHz with the same spectrometer; 85% orthophosphoric acid was used as an external standard. High-performance liquid chromatography (HPLC) was carried out on a 250 × 4.6 mm Hypersil ODS 5μ column (for programmes A–C) and on a 250 × 4.6 mm Jones APEX ODS 5μ column (for programme D). The columns were eluted with 0.1 mol dm^{-3} triethylammonium acetate buffer (pH 7.0)–acetonitrile mixtures [programme A: buffer–acetonitrile (92:8 v/v) with isocratic elution; programme B: linear gradient of buffer–acetonitrile (95:5 v/v to 80:20 v/v) over 10 min and then isocratic elution; programme C: linear gradient of buffer–acetonitrile (70:30 to 15:85 v/v) over 10 min and then isocratic elution; programme D: linear gradient of buffer–acetonitrile (70:30 to 20:80 v/v) over 10 min and then isocratic elution]. HPLC peaks were monitored and integrated at 270 nm. Merck Kieselgel (Art 7729 and 9385) was used for short column chromatography (SCC). The general procedure for the chromatography of fully or partially protected oligonucleotide phosphorothioates (see below) was as follows. A suspension of silica gel (*ca.* 10 g/1 g of crude product) in dichloromethane was poured into a column, and the silica gel was packed. The diameter of the column was such that the height of the silica gel was only *ca.* 1–2 cm. A solution of the crude product in dichloromethane was applied to the column, which was eluted first with dichloromethane–methanol (100:0 to 96:4 v/v) and then, if necessary, with ethyl acetate–acetone (40:60 to 30:70 v/v). A higher proportion of acetone was required to elute more polar (higher molecular weight) products. Acetonitrile, triethylamine and pyridine were dried by heating with calcium hydride, under reflux, and were then distilled; 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and N^1,N^1,N^3,N^3-tetramethylguanidine (TMG) were dried by distillation over calcium hydride under reduced pressure; dichloromethane was dried over phosphorus pentaoxide and was then distilled; diethyl ether was dried over sodium wire. All solvents were stored over 4 Å molecular sieves. Protected [*i.e.* *N*-acyl-5′-*O*-(4,4′-dimethoxytrityl)-] 2′-deoxyribonucleosides were supplied by Cruachem Ltd. Petroleum 'ether' refers to the fraction with distillation range 40–60 °C.

General procedure for the preparation of triethylammonium 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)ribonucleoside 3′-*H*-phosphonates 8

A solution of ammonium 4-methylphenyl *H*-phosphonate[23] **15a** (2.84 g, 15 mmol) and triethylamine (4.2 cm^3, 30 mmol) in dry pyridine (20 cm^3) was evaporated under reduced pressure. A solution of the residue and the appropriate 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)ribonucleoside derivative[34] **14** (5.0 mmol) in dry pyridine (2 × 20 cm^3) was then evaporated under reduced pressure. The final residue obtained was dissolved in dry pyridine (40 cm^3), and the solution was cooled to −35 °C [industrial methylated spirits (IMS)–solid CO_2-bath]. Pivaloyl chloride (1.85 cm^3, 15 mmol) was added dropwise to the stirred solution over a period of 1 min, and the reactants were maintained at −35 °C. After 30 min, water (5 cm^3) was added, and the stirred mixture was allowed to warm up to room temperature. After a further period of 1 h, potassium phosphate buffer (1.0 mol dm^{-3}, pH 7.0; 250 cm^3) was added to the products, and the resulting mixture was concentrated under reduced pressure until all of the pyridine had been removed. The residue was partitioned between dichloromethane (250 cm^3) and water (200 cm^3). The organic layer was washed with triethylammonium phosphate buffer (0.5 mol dm^{-3}, pH 7.0; 3 × 50 cm^3), dried ($MgSO_4$) and then evaporated under reduced pressure. The residue was fractionated by SCC on silica gel (25 g). Appropriate fractions, which were eluted with dichloromethane–methanol (95:5 to 90:10 v/v), were evaporated under reduced pressure to give the corresponding triethylammonium 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)ribonucleoside 3′-*H*-phosphonate **8**.

6-*N*-Benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosine

14; B = **16** (3.32 g, 5.0 mmol) was converted into triethylammonium 6-*N*-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-adenosine 3′-*H*-phosphonate **8**; B = **16** (4.10 g, 98%); t_R (programme D) 9.65 min; δ_H[$(CD_3)_2SO + D_2O$] 1.14 (9 H, t, *J* 7.3), 2.58 (1 H, m), 3.00 (6 H, quart, *J* 7.3), 3.08 (1 H, m), 3.22 (2 H, m), 3.69 (3 H, s), 3.70 (3 H, s), 4.19 (1 H, s), 4.89 (1 H, s), 5.83 (0.5 H, s), 6.49 (1 H, t, *J* 6.7), 6.80 (4 H, t, *J* 9.0), 7.19 (7 H, m), 7.32 (2 H, m), 7.47 (0.5 H, s), 7.54 (2 H, m), 7.64 (1 H, m), 8.03 (2 H, m), 8.59 (1 H, s) and 8.63 (1 H, s); δ_P[$(CD_3)_2SO$] 1.2 (dd, *J* 8.9 and 587.6).

4-*N*-Benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)cytidine **14**; B = **17** (3.195 g, 5.0 mmol) was converted into triethylammonium 4-*N*-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)-cytidine 3′-*H*-phosphonate **8**; B = **17** (3.98 g, 98%); t_R (programme D) 10.31 min; δ_H[$(CD_3)_2SO$] 1.11 (9 H, t, *J* 7.3), 2.30 (1 H, m), 2.53 (1 H, m), 2.88 (6 H, quart, *J* 7.1), 3.31 (2 H, m), 3.74 (6 H, s), 4.15 (1 H, m), 4.75 (1 H, m), 5.81 (0.5 H, s), 6.14 (1 H, t, *J* 5.9), 6.91 (4 H, d, *J* 8.9), 7.17–7.41 (10 H, m), 7.46 (0.5 H, s), 7.51 (2 H, m), 7.62 (1 H, m), 8.01 (2 H, m) and 8.22 (1 H, d, *J* 7.6); δ_P[$(CD_3)_2SO$] 1.3 (dd, *J* 8.5 and 588.2).

2′-Deoxy-6-*O*-(2,5-dichlorophenyl)-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-isobutyrylguanosine **14**; B = **18** (3.89 g, 5.0 mmol) was converted into triethylammonium 2′-deoxy-6-*O*-(2,5-dichlorophenyl)-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-isobutyryl-guanosine 3′-*H*-phosphonate **8**; B = **18** (4.62 g, 98%); t_R (programme D) 12.09 min; δ_H[$(CD_3)_2SO$] 0.91 (6 H, d, *J* 6.7), 1.13 (9 H, s), 2.50 (1 H, m), 2.81 (1 H, m), 2.94 (6 H, quart, *J* 7.3), 3.12 (2 H, m), 3.38 (1 H, m), 3.72 (6 H, s), 4.16 (1 H, m), 4.87 (1 H, m), 5.84 (0.5 H, s), 6.42 (1 H, t, *J* 6.8), 6.75 (2 H, d, *J* 9.0), 6.79 (2 H, d, *J* 9.0), 7.19 (7 H, m), 7.31 (2 H, m), 7.45 (0.5 H, s), 7.47 (1 H, m), 7.69 (1 H, d, *J* 8.9), 7.73 (1 H, d, *J* 2.5), 8.48 (1 H, s) and 10.31 (1 H, s); δ_P[$(CD_3)_2SO$] 1.3 (dd, *J* 8.6 and 588.4).

5′-*O*-(4,4′-Dimethoxytrityl)thymidine **14**; B = **19** (2.74 g, 5.0 mmol) was converted into triethylammonium 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-*H*-phosphonate **8**; B = **19** (3.57 g, 99.5%); t_R (programme D) 8.77 min; δ_H[$(CD_3)_2SO$] 1.14 (9 H, t, *J* 7.3), 1.39 (3 H, s), 2.30 (2 H, m), 2.97 (6 H, quart, *J* 7.3), 3.22 (2 H, m), 3.73 (6 H, s), 4.07 (1 H, m), 4.76 (1 H, m), 5.81 (0.5 H, s), 6.21 (1 H, m), 6.89 (4 H, m), 7.21–7.33 (7 H, m), 7.38 (2 H, m), 7.44 (0.5 H, s), 7.51 (1 H, s) and 11.41 (1 H, br); δ_P[$(CD_3)_2SO$] 1.3 (dd, *J* 8.9 and 588.6).

6-*N*-Benzoyl-2′-deoxy-3′-*O*-levulinoyladenosine 20; B = 16

Levulinic anhydride[9] (2.57 g, *ca.* 12 mmol) was added to a stirred solution of 6-*N*-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosine[34] **14**; B = **16** (6.58 g, 10.0 mmol), triethylamine (2.1 cm^3, 15.0 mmol) and 4-(dimethylamino)pyridine (DMAP) (0.10 g, 0.82 mmol) in dry dichloromethane (50 cm^3) at room temperature. After 2 h, the products were poured into saturated aqueous sodium hydrogen carbonate (100 cm^3). The layers were separated and the aqueous layer was extracted with dichloromethane (50 cm^3). The combined organic layers were washed with saturated aqueous sodium hydrogen carbonate (2 × 50 cm^3), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was dissolved in dichloromethane (200 cm^3), and the solution was cooled to −50 °C (IMS–solid CO_2-bath). A solution of hydrogen chloride in 1,4-dioxane (4 mol dm^{-3}; 25 cm^3) was added to the stirred solution which was maintained at −50 °C. After 5 min, pyridine (10 cm^3) and methanol (10 cm^3) were added, and the products were poured into saturated aqueous sodium hydrogen carbonate. The resulting mixture was extracted with dichloromethane (3 × 50 cm^3). The combined extracts were dried ($MgSO_4$) and evaporated under reduced pressure. The residue obtained was fractionated by SCC on silica gel. The appropriate fractions, which were eluted with dichloromethane–methanol (98:2 to 96:4 v/v), were combined, and evaporated under reduced pressure. Crystallization of the residue from ethyl acetate–petroleum ether gave the title compound **20**; B = **16** as a colourless solid (3.67 g, 81%), mp 124–125 °C; t_R (programme C) 4.1 min; δ_H[$(CD_3)_2SO$] 2.15 (3 H, s), 2.56 (3 H, m), 2.79 (2 H, m), 3.06 (1 H, m), 3.65 (2 H, m), 4.12 (1 H, m), 5.23 (1 H, br), 5.41 (1 H, d, *J* 5.9), 6.51 (1 H, dd, *J* 6.0 and 8.4), 7.56 (2 H, m), 7.65 (1 H, m), 8.06 (2 H, m), 8.73 (1 H, s), 8.77 (1 H, s) and 11.24 (1 H, br); δ_C[$(CD_3)_2SO$] 27.79, 29.57, 36.38, 37.46, 61.52, 75.07, 83.79, 85.32, 125.86, 128.48, 128.51, 132.48, 133.34, 142.98, 150.47, 151.61, 151.99, 165.69, 172.04 and 207.0.

4-*N*-Benzoyl-2′-deoxy-3′-*O*-levulinoylcytidine 20; B = 17

4-*N*-Benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)cytidine[34] **14**; B = **17** (6.34 g, 10.0 mmol), levulinic anhydride (4.28 g, *ca.* 20 mmol), triethylamine (3.5 cm^3, 25 mmol) and DMAP (0.10 g, 0.82 mmol) were stirred together in dry dichloromethane (50 cm^3) at room temperature as in the above preparation of 6-*N*-benzoyl-3′-*O*-levulinoyl-2′-deoxyadenosine **20**: B = **16**. After 2 h, the products were worked up and treated, as above, with hydrogen chloride at −50 °C for 5 min. After the addition of pyridine (10 cm^3) and methanol (10 cm^3), the products were poured into saturated aqueous sodium hydrogen carbonate (10 cm^3), and cyclohexane (100 cm^3) was added. The resulting mixture was stirred at room temperature for 2 h and was then filtered to give the title compound **20**; B = **17** as colourless crystals (4.05 g, 94%), mp 184 − 187 °C; t_R (programme C) 5.4 min; δ_H[$(CD_3)_2SO$] 2.13 (3 H, s), 2.26 (1 H, m), 2.49 (3 H, m), 2.76 (2 H, m), 3.66 (2 H, m), 4.11 (1 H, m), 5.23 (2 H, m), 6.18 (1 H, dd, *J* 6.0 and 7.8), 7.37 (1 H, m), 7.52 (2 H, m), 7.63 (1 H, m), 8.01 (2 H, m), 8.38 (1 H, d *J* 7.5) and 11.28 (1 H, br); δ_C[$(CD_3)_2SO$] 27.75, 29.55, 37.44, 38.18, 61.17, 74.83, 85.56, 86.36, 96.35, 128.47, 132.77, 133.18, 144.86, 154.36, 163.16, 172.04 and 207.0.

3′-*O*-Levulinoylthymidine 20; B = 19

5′-*O*-(4,4′-Dimethoxytrityl)thymidine[34] **14**; B = **19** (5.45 g, 10.0 mmol), levulinic anhydride (4.28 g, *ca.* 20 mmol), triethylamine (3.5 cm^3, 25 mmol) and DMAP (0.10 g, 0.82 mmol) were stirred together at room temperature as above in the preparation of 6-*N*-benzoyl-3′-*O*-levulinoyl-2′-deoxyadenosine **20**; B = **16**. After 2 h, the reaction mixture was worked up, treated with hydrogen chloride and the products were then fractionated by chromatography on silica gel, again as in the preparation of the 2′-deoxyadenosine derivative **20**; B = **16**. The appropriate fractions, which were eluted with ethyl acetate, were evaporated under reduced pressure to give the title compound **20**; B = **19** as a pale yellow foam (2.93 g, 86%). Crystallization of this material from ethyl acetate–petroleum ether gave colourless crystals, mp 108–109 °C; t_R (programme C) 2.5 min; δ_H-[$(CD_3)_2SO$] 1.78 (3 H, d, *J* 0.9), 2.12 (3 H, s), 2.25 (2 H, m), 2.51 (2 H, m), 2.75 (2 H, m), 3.62 (2 H, br s), 3.96 (1 H, m), 5.21 (2 H, m), 6.18 (1 H, dd, *J* 5.9 and 8.7), 7.74 (1 H, m) and 11.35 (1 H, br s); δ_C[$(CD_3)_2SO$] 12.21, 27.63, 29.42, 36.32, 37.34, 61.24, 74.77, 83.55, 84.43, 109.66, 135.73, 150.40, 163.60, 171.92 and 206.82.

Removal of the 5′-*O*-(4,4′-dimethoxytrityl) protecting group with hydrogen chloride in dichloromethane solution

A solution of hydrogen chloride in 1,4-dioxane (4 mol dm^{-3}; 2.5 cm^3) was added to a stirred solution of 6-*N*-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosine **14**; B = **16** (0.659 g, 1.0 mmol) in dry dichloromethane (20 cm^3) at −50 °C (IMS–solid CO_2-bath). After 2 min, an aliquot (1.0 cm^3) of the reaction solution was quenched by adding it to pyridine–methanol (1:1 v/v; 1.0 cm^3) at −50 °C. TLC [dichloromethane–methanol (9:1 v/v)] revealed 6-*N*-benzoyl-2′-deoxyadenosine (R_f 0.42) as the sole purine-derived product. No starting material **14**; B = **16** (R_f 0.59) or 6-*N*-benzoyladenine (R_f 0.59) could be detected. Analysis of aliquots that had been removed from the reaction

solution after 1, 2 and 4 h, and quenched in the same way, also revealed 6-*N*-benzoyl-2′-deoxyadenosine as the sole purine-derived product.

Preparation of HO-*Ap(s′)C*-Lev 24; B = 16, B′ = 17

A solution of triethylammonium 6-*N*-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)adenosine 3′-*H*-phosphonate **8**; B = **16** (0.985 g, 1.20 mmol) and 4-*N*-benzoyl-2′-deoxy-3′-*O*-levulinoylcytidine **21**; B′ = **17** (0.429 g, 1.00 mmol) in dry pyridine (5 cm^3) was evaporated under reduced pressure. The residue was dissolved in pyridine (5 cm^3), the solution re-evaporated and then the residue re-dissolved in dry pyridine (10 cm^3). The stirred solution was cooled to −40 °C (IMS–solid CO_2-bath), and a solution of bis(2-chlorophenyl) phosphorochloridate[25] (1.02 g, 3.0 mmol) **22** in dry dichloromethane (1 cm^3) was added dropwise over 5 min. After a further period of 5 min, 2-(4-chlorophenylsulfanyl)isoindole-1,3(2*H*)-dione[28] **23a** (0.579 g, 2.0 mmol) was added to the cooled (−40 °C), stirred reactants. After 15 min, pyridine–water (1 : 1 v/v; 0.2 cm^3) was added. After a further period of 5 min, the products were concentrated under reduced pressure and were then co-evaporated with dry toluene (10 cm^3). The residue was dissolved in dichloromethane (50 cm^3), and the solution was washed first with 1.0 mol dm^{-3} sodium phosphate buffer (pH 2.0; 50 cm^3), and then with saturated aqueous sodium hydrogen carbonate (3 × 50 cm^3). The dried ($MgSO_4$) organic layer was evaporated under reduced pressure and then re-dissolved in dichloromethane (20 cm^3). The stirred solution was cooled to −50 °C (IMS–solid CO_2-bath), and a solution of hydrogen chloride in 1,4-dioxane (4 mol dm^{-3}; 2.5 cm^3) was added. After 5 min, methanol (1 cm^3) and pyridine (1 cm^3) were added, and the products were poured into saturated aqueous sodium hydrogen carbonate (50 cm^3). The layers were separated and the aqueous layer was extracted with dichloromethane (2 × 20 cm^3). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was fractionated by SCC on silica gel (see above for the general procedure for chromatography of fully or partially protected oligonucleotide phosphorothioates): the appropriate fractions, which were eluted with ethyl acetate–acetone (40 : 60 v/v) were evaporated under reduced pressure to give HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17** (0.960 g, 98%) as a colourless solid, t_R (programme C) 10.2, 10.4 min; δ_P[$(CD_3)_2SO$] 23.0, 23.2.

Preparation of HO-T*p(s′)Gp(s′)Ap(s′)C*-Lev 28

A solution of triethylammonium 2′-deoxy-6-*O*-(2,5-dichlorophenyl)-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-isobutyrylguanosine 3′-*H*-phosphonate **8**; B = **18** (0.855 g, 0.90 mmol) and HO-*Ap(s′)C*-Lev **24**, B = **16**, B′ = **17** (0.730 g, 0.75 mmol) in dry pyridine (5 cm^3) was evaporated under reduced pressure. The residue was dissolved in pyridine (5 cm^3), the solution re-evaporated and then re-dissolved in dry pyridine (7.5 cm^3). The solution was cooled to −40 °C (IMS–solid CO_2-bath) and a solution of bis(2-chlorophenyl) phosphorochloridate **22** (0.77 g, 2.3 mmol) in dry dichloromethane (0.75 cm^3) was added over 5 min. After a further period of 5 min, 2-(4-chlorophenylsulfanyl)isoindole-1,3(2*H*)-dione[28] **23a** (0.435 g, 1.5 mmol) was added. The reaction was allowed to proceed for 15 min and the products were worked up and treated with hydrogen chloride (4 mol dm^{-3} solution in 1,4-dioxane; 1.9 cm^3) in dichloromethane (15 cm^3) at −50 °C for 5 min, as in the above preparation of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17**. The ‘detritylated’ products were then worked up as above and fractionated by SCC on silica gel: the appropriate fractions, which were eluted with ethyl acetate–acetone (30 : 70 v/v), were evaporated under reduced pressure to give HO-*Gp(s′)Ap(s′)C*-Lev **25** (1.204 g, 97%) as a colourless solid, t_R (programme C) 14.9, 15.2 min; δ_P($CDCl_3$) 23.50, 23.59, 23.82, 24.11, 24.69, 24.87, 24.92 and 25.17.

A solution of triethylammonium 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-*H*-phosphonate **8**; B = **19** (0.426 g, 0.60 mmol) and HO-*Gp(s′)Ap(s′)C*-Lev **25** (0.822 g, 0.50 mmol) in dry pyridine (5 cm^3) was evaporated under reduced pressure. The residue was dissolved in pyridine (5 cm^3), the solution re-evaporated and then the residue re-dissolved in dry pyridine (5 cm^3). The solution was cooled to −40 °C (IMS–solid CO_2-bath) and a solution of bis(2-chlorophenyl) phosphorochloridate **22** (0.51 g, 1.5 mmol) in dry dichloromethane (0.5 cm^3) was added over 5 min. After a further period of 5 min, 2-(4-chlorophenylsulfanyl)isoindole-1,3(2*H*)-dione **23a** (0.290 g, 1.0 mmol) was added. The reaction was allowed to proceed for 15 min, and the products were worked up and treated with hydrogen chloride (4 mol dm^{-3} solution in 1,4-dioxane; 1.3 cm^3) in dichloromethane (10 cm^3) at −50 °C for 5 min, as in the above preparation of HO-*Ap(s′)C*-Lev **24**, B = **16**, B′ = **17**. The ‘detritylated’ products were then worked up as above and fractionated by SCC on silica gel: the appropriate fractions, which were eluted with ethyl acetate–acetone (30 : 70 v/v), were evaporated under reduced pressure to give HO-T*p(s′)Gp(s′)Ap(s′)C*-Lev **28** (1.018 g, 98%) as a solid, t_R (programme C) 15.4, 15.6 min; δ_P($CDCl_3$) 23.91, 23.99, 24.05, 24.61, 24.72, 24.86, 24.91, 24.96, 25.02, 25.08, 25.22 and 25.25.

Preparation of HO-T*p(s′)Gp(s)Ap(s′)C*-Lev 29

This preparation was carried out on precisely the same scale and in almost exactly the same way as that of HO-T*p(s′)Gp(s′)Ap(s′)C*-Lev **28**. The only difference was that 4-[(2-cyanoethyl)sulfanyl]-morpholine-3,5-dione[30] **26** (0.30 g, 1.5 mmol) was used instead of 2-(4-chlorophenylsulfanyl)isoindole-1,3(2*H*)-dione **23a** after the first coupling step. Following work-up, ‘detritylation’ and chromatography, HO-*Gp(s)Ap(s′)C*-Lev **27** was isolated as a colourless solid (1.144 g, 96%), t_R (programme C) *ca.* 13.7 min (3 unresolved peaks); δ_P($CDCl_3$) 24.60, 24.96, 24.99, 25.21, 26.76, 27.06, 27.46 and 27.48. The combined integrals of the four higher field (δ 24.60–25.21) and the combined integrals of the four lower field (δ 26.76–27.48) resonance signals were virtually equal.

The reaction conditions followed for the conversion of HO-*Gp(s)Ap(s′)C*-Lev **27** (0.793 g, 0.50 mmol) into HO-T*p(s′)Gp(s)Ap(s′)C*-Lev **29** were precisely the same as the conditions [*i.e.* for the coupling reaction with triethylammonium 5′-*O*-(4,4′-dimethoxytrityl)thymidine 3′-*H*-phosphonate **8**; B = **19** and bis(2-chlorophenyl) phosphorochloridate **22**, the reaction with 2-(4-chlorophenylsulfanyl)isoindole-1,3(2*H*)-dione **23a**, ‘detritylation’ and fractionation by SCC] described above for the preparation of HO-T*p(s′)Gp(s′)Ap(s′)C*-Lev **28**. The desired product **29** was isolated as a colourless solid (0.980 g, 97%), t_R (programme C) 11.5 min; δ_P($CDCl_3$) 24.35, 24.53, 24.75, 24.80, 24.88, 24.91, 24.96, 25.06, 25.20, 25.22, 25.36, 25.44, 27.73, 27.88, 27.98 and 28.07. The combined integrals of the twelve higher field (δ 24.35–25.44) were almost exactly equal to twice the combined integrals of the four lower field (δ 27.73–28.07) resonance signals.

Preparation of HO-*Gp(s)A*-Lev 30

A solution of triethylammonium 2′-deoxy-6-*O*-(2,5-dichlorophenyl)-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-isobutyrylguanosine 3′-*H*-phosphonate **8**; B = **18** (1.140 g, 1.2 mmol) and 6-*N*-benzoyl-2′-deoxy-3′-*O*-levulinoyladenosine **21**; B′ = **16** (0.453 g, 1.0 mmol) in dry pyridine (5 cm^3) was evaporated under reduced pressure. The residue was dissolved in pyridine (5 cm^3), the solution re-evaporated and then the residue re-dissolved in dry pyridine (10 cm^3). The solution was cooled to −40 °C (IMS–solid CO_2-bath), and a solution of bis(2-chlorophenyl) phosphorochloridate **22** (1.02 g, 3.0 mmol) in dry dichloromethane (1.0 cm^3) was added over 5 min. After a further period of 5 min, 4-[(2-cyanoethyl)sulfanyl]morpholine-3,5-dione **26** (0.40 g, 2.0 mmol) was added. The reaction was

allowed to proceed and the products were worked up and treated with hydrogen chloride (4 mol dm^{-3} solution in 1,4-dioxane; 2.5 cm^3) in dichloromethane (20 cm^3) at −50 °C for 5 min, as in the above preparation of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17**. The 'detritylated' products were then worked up as above and fractionated by SCC on silica gel: the appropriate fractions, which were eluted with ethyl acetate–acetone (40 : 60 v/v), were evaporated under reduced pressure to give HO-*Gp(s)A*-Lev **30** (1.045 g, 98%) as a solid, t_R (programme C) 11.4 min; δ_P[$(CD_3)_2SO$] 27.39, 27.55.

Preparation of HO-*Cp(s)Tp(s)Gp(s)A*-Lev 32

A solution of triethylammonium 5′-*O*-(4,4′-dimethoxytrityl)-thymidine 3′-*H*-phosphonate **8**; B = **19** (0.639 g, 0.90 mmol) and HO-*Gp(s)A*-Lev **30** (0.800 g, 0.75 mmol) in dry pyridine (5 cm^3) was evaporated under reduced pressure. The residue was dissolved in pyridine (5 cm^3), the solution re-evaporated and then the residue re-dissolved in dry pyridine (7.5 cm^3). The solution was cooled to −40 °C (IMS–solid CO_2-bath), and a solution of bis(2-chlorophenyl) phosphorochloridate **22** (0.77 g, 2.3 mmol) in dry dichloromethane (0.75 cm^3) was added over 5 min. After a further period of 5 min, 4-[(2-cyanoethyl)-sulfanyl]morpholine-3,5-dione **26** (0.30 g, 1.5 mmol) was added. The reaction was allowed to proceed, and the products were worked up and treated with hydrogen chloride (4 mol dm^{-3} solution in 1,4-dioxane; 1.9 cm^3) in dichloromethane (15 cm^3) at −50 °C for 5 min, as in the above preparation of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17**. The 'detritylated' products were then worked up as above and fractionated by SCC on silica gel: the appropriate fractions, which were eluted with ethyl acetate–acetone (30 : 70 v/v), were evaporated under reduced pressure to give HO-T*p(s)Gp(s)A*-Lev **31** (1.041 g, 96%) as a solid, t_R (programme C) 11.2 min (2 unresolved peaks); δ_P($CDCl_3$) 27.50, 27.57, 27.77, 27.99, 28.18 and 28.24.

A solution of triethylammonium 4-*N*-benzoyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)cytidine 3′-*H*-phosphonate **8**; B = **17** (0.479 g, 0.60 mmol) and HO-T*p(s)Gp(s)A*-Lev **31** (0.720 g, 0.50 mmol) in dry pyridine (5 cm^3) was evaporated under reduced pressure. The residue was dissolved in pyridine (5 cm^3), the solution re-evaporated and then the residue re-dissolved in dry pyridine (5 cm^3). The solution was cooled to −40 °C (IMS–solid CO_2-bath), and a solution of bis(2-chlorophenyl) phosphorochloridate **22** (0.51 g, 1.5 mmol) in dry dichloromethane (0.5 cm^3) was added over 5 min. After a further period of 5 min, 4-[(2-cyanoethyl)sulfanyl]morpholine-3,5-dione **26** (0.200 g, 1.0 mmol) was added. The reaction was allowed to proceed, and the products were worked up and treated with hydrogen chloride (4 mol dm^{-3} solution in 1,4-dioxane; 1.3 cm^3) in dichloromethane (10 cm^3) at −50 °C for 5 min, as in the above preparation of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17**. The 'detritylated' products were then worked up and fractionated by SCC: the appropriate fractions, which were eluted with ethyl acetate–acetone (30 : 70 v/v), were evaporated under reduced pressure to give HO-*Cp(s)*T*p(s)Gp(s)A*-Lev **32** (0.910 g, 95%) as a colourless solid, t_R (programme C) 12.55 min; δ_P($CDCl_3$) 27.76, 28.10 and 28.52.

Unblocking of partially protected oligonucleotide *S*-(4-chlorophenyl) phosphorothioates

(a) HO-*Ap(s′)C*-Lev 24; B = 16, B′ = 17. Acetic anhydride (0.38 cm^3, 4.0 mmol) was added to a solution of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17** (0.779 g, 0.80 mmol) in dry pyridine (8 cm^3) at room temperature. After 15 h, methanol (1 cm^3) was added and, after a further period of 30 min, the products were concentrated under reduced pressure. The residue was dissolved in dichloromethane (50 cm^3), and the solution was washed with saturated aqueous sodium hydrogen carbonate (2 × 25 cm^3). The dried ($MgSO_4$) organic layer was evaporated under reduced pressure, and the residue was purified by SCC: the appropriate fractions, which were eluted with ethyl acetate–acetone (30 : 70 v/v), were evaporated under reduced pressure to give a colourless solid (0.805 g). A solution of the latter material (0.10 g), *E*-2-nitrobenzaldoxime **33** (0.166 g, 1.0 mmol) and TMG **34** (0.11 cm^3, 0.9 mmol) in acetonitrile (1 cm^3) was stirred at room temperature. After 12 h, the products were evaporated under reduced pressure, and conc. aqueous ammonia (*d* 0.88; 2 cm^3) was added to the residue. The resulting mixture was heated at 50 °C in a closed vessel for 15 h, and the products were concentrated under reduced pressure. The residue was dissolved in methanol (2 cm^3), and ethyl acetate (40 cm^3) was added. The resulting precipitate was collected by centrifugation, washed with ethyl acetate (2 × 10 cm^3) and dried to give a colourless solid. A solution of this material in water (2 cm^3) was applied to a column (10 cm × 2 cm diameter) of Amberlite IR-120(plus) cation-exchange resin (Na^+ form), which was then eluted with distilled water: concentration of the appropriate fractions gave the sodium salt of d[ApC] **36** as a colourless solid (0.051 g, *ca.* 90%), t_R (programme A) 4.7 min; δ_H(D_2O) 2.19 (1 H, m), 2.31 (1 H, m), 2.71 (2 H, m), 3.76 (1 H, dd, *J* 3.7 and 12.9), 3.82 (1 H, dd, *J* 2.7 and 12. 5), 4.05 (3 H, m), 4.14 (2 H, m), 4.27 (1 H, m), 4.49 (1 H, m), 4.82 (1 H, m), 5.69 (1 H, d, *J* 7.5), 6.14 (1 H, t, *J* 6.5), 6.28 (1 H, t, *J* 6.6), 7.64 (1 H, d, *J* 7.6), 8.00 (1 H, s) and 8.15 (1 H, s); δ_P(D_2O) −0.07.

(b) HO-T*p(s′)Gp(s′)Ap(s′)C*-Lev 28. Acetic anhydride (0.19 cm^3, 2.0 mmol) was added to a solution of HO-T*p(s′)Gp(s′)Ap(s′)C*-Lev **28** (0.83 g, 0.40 mmol) in dry pyridine (5 cm^3) at room temperature. After 15 h, the products were worked up and chromatographed on silica gel as in (**a**) above to give a colourless solid (0.823 g). This material (0.10 g) was subjected to the same two-step unblocking procedure, followed by the cation-exchange process as in (**a**) above, to give the sodium salt of d[TpGpApC] **37** as a colourless solid (0.058 g; *ca.* 96%), t_R (programme B) 11.6 min; δ_H(D_2O) includes the following signals: 5.70 (2 H, m), 5.98 (2 H, m), 6.21 (1 H, m), 7.26 (1 H, s), 7.62 (1 H, d, *J* 7.5), 7.83 (1 H, s), 7.88 (1 H, s) and 8.32 (1 H, s); δ_P(D_2O) −0.53, −0.10 and −0.08.

Unblocking of partially protected oligonucleotide *S*-(2-cyanoethyl) phosphorothioates

(a) HO-*Gp(s)A*-Lev 30. Acetic anhydride (0.38 cm^3, 4.0 mmol) was added to a solution of HO-*Gp(s)A*-Lev **30** (0.853 g, 0.80 mmol) in dry pyridine (8 cm^3) at room temperature. After 15 h, the products were worked up and chromatographed on silica gel as described above in the unblocking of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17** to give a colourless solid (0.870 g). A solution of this material (0.10 g) in dry pyridine (1 cm^3) was evaporated to dryness, and the residue was re-dissolved, in an atmosphere of argon, in a solution of chlorotrimethylsilane (0.025 cm^3, 0.20 mmol) and DBU (0.18 cm^3, 1.2 mmol) in dry dichloromethane (1.8 cm^3). After 30 min, the products were carefully evaporated under reduced pressure to remove the dichloromethane, and then re-dissolved in a solution of *E*-2-nitrobenzaldoxime **33** (0.166 g, 1.0 mmol) in acetonitrile (1 cm^3) at room temperature. After 12 h, the products were evaporated under reduced pressure, and concentrated aqueous ammonia (*d* 0.88, 1.8 cm^3) and 2-mercaptoethanol (0.2 cm^3) were added. The resulting mixture was heated in a closed vessel at 50 °C for 15 h, and the products were evaporated under reduced pressure. The products were then worked up and subjected to the cation-exchange process described above in the unblocking of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17** to give the sodium salt of d[Gp(s)A] **39** as a colourless solid (0.051 g, *ca.* 89%), t_R (programme B) 12.6, 13.4 min; δ_H(D_2O) 1.99 (0.5 H, m), 2.18 (1 H, m), 2.30 (0.5 H, m), 2.52 (1 H, m), 2.76 (1 H, m), 3.64 (2 H, m), 4.08 (3 H, m), 4.23 (1 H, m), 4.70 (1 H, m), 4.86 (1 H, m), 5.75 (1 H, m), 6.26 (1 H, m), 7.61 (0.5 H, s), 7.65 (0.5 H, s),

7.81 (0.5 H, s), 7.84 (0.5 H, s), 8.28 (0.5 H, s) and 8.29 (0.5 H, s); $\delta_P(D_2O)$ 55.77 and 56.10.

(b) HO-*Cp(s)*T*p(s)Gp(s)A*-Lev 32. Acetic anhydride (0.19 cm^3, 2.0 mmol) was added to a solution of HO-*Cp(s)*-T*p(s)Gp(s)A*-Lev **32** (0.761 g, 0.40 mmol) in dry pyridine (5 cm^3) at room temperature. After 15 h, the products were worked up and chromatographed on silica gel as described above in the unblocking of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17** to give a colourless solid (0.750 g). This material (0.10 g) was subjected to the same three-step unblocking procedure as in (**a**) above, followed by the cation-exchange process described above in the unblocking of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17**, to give the sodium salt of d[Cp(s)Tp(s)Gp(s)A] **40** as a colourless solid (0.064 g, *ca*. 93%); $\delta_P(D_2O)$ 55.08–55.74 (numerous signals).

Unblocking of HO-T*p(s′)Gp(s)Ap(s′)C*-Lev 29

Acetic anhydride (0.19 cm^3, 2.0 mmol) was added to a solution of HO-T*p(s′)Gp(s)Ap(s′)C*-Lev **29** (0.807 g, 0.40 mmol) in dry pyridine (5 cm^3) at room temperature. After 15 h, the products were worked up and chromatographed on silica gel as described above in the unblocking of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ =**17** to give a colourless solid (0.792 g). This material (0.10 g) was subjected to the three-step unblocking procedure described above in the unblocking of HO-*Gp(s)A*-Lev **30**, followed by the cation-exchange process described above in the unblocking of HO-*Ap(s′)C*-Lev **24**; B = **16**, B′ = **17** to give the sodium salt of d[TpGp(s)ApC] **38** as a colourless solid (0.058 g, *ca* 91%), t_R (programme B) 12.7 min; $\delta_H(D_2O)$ includes the following signals: 5.66–5.89 (3 H, m), 5.99 (1 H, m), 6.13–6.22 (1 H, m), 7.23 (1 H, s), 7.64 (1 H, d, *J* 7.6), 7.69 (*ca*. 0.5 H, s), 7.75 (*ca* 0.5 H, s), 7.94 (*ca*. 0.5 H, s), 7.95 (*ca*. 0.5 H, s) and 8.43 (1 H, s); $\delta_P(D_2O)$ −0.67, −0.58 and −0.16 (combined integral for the three signals, 2 P), 54.48 and 54.97 (combined integral for the two signals, 1 P).

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