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Chemical synthesis of a tridecanucleoside dodecaphosphate sequence of SV40 DNA

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ABSTRACT

The preparation, by the phosphotriester approach, of d[C-T-A-T-T-C-C-A-G-A-A-G-T] from one tetranucleoside triphosphate and three trinucleoside diphosphate blocks is described. The use of the *o*-dibromomethylbenzoyl (DBMB) protecting group in oligodeoxyribonucleotide synthesis is described for the first time. Internucleotide linkages are protected by *o*-chlorophenyl groups which are finally removed by treatment with the N^1,N^1,N^3,N^3-tetramethylguanidinium salt of *syn*-4-nitrobenzaldoxime. The first phosphorylation step (leading to phosphodiester intermediates) is carried out by treatment with *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) followed by treatment with water and triethylamine. 1-Mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) is used as the activating agent in the second phosphorylation step in which 5'-protected mono- and di-nucleotides are condensed with nucleoside building blocks containing unprotected 3'-hydroxy functions.

INTRODUCTION

The synthesis of oligodeoxyribonucleotides by the phosphotriester approach[1] with aryl protecting groups has recently been investigated in a number of laboratories[2-7] and, at present, appears to be the method of choice. Soon after we had originally reported[8] that phenyl groups could be used for the protection of internucleotide linkages, we showed[9] that substituted aryl groups including *o*-chloro-, *o*-fluoro- and *p*-chlorophenyl could also be used for this purpose. We now favour the use of *o*-chlorophenyl protecting groups which are easily removable at the end of the synthesis by treatment of the fully-protected oligonucleotide with N^1,N^1,N^3,N^3-tetramethylguanidinium *syn*-*p*-nitrobenzaldoxime (or a related oxime)[10] in aqueous dioxan solution at room temperature. Under these conditions, concomitant cleavage of the internucleotide linkages appears to occur only to a negligible extent. While phenyl and presumably other aryl protecting groups derived from phenols with significantly higher pK_a's than that of *o*-chlorophenol (pK_a 8.47)[11] are removed inconveniently slowly[10] by the

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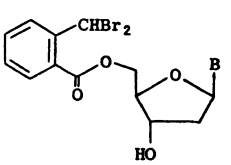
oximate ion procedure, aryl groups derived from phenols with pK_a's close to that of *o*-chlorophenol would most probably also be easily removable.

What is perhaps at present the most widely used phosphotriester approach is based on mono- or oligo-nucleotide blocks which may be de-protected at their 5'-ends to liberate hydroxy functions and at their 3'-ends to liberate phosphodiester groups. This approach was originally adopted by Catlin and Cramer[12]. An alternative synthetic strategy differs from the Catlin and Cramer approach in that it does not involve the use of building blocks which contain phosphotriester groups at their 3'-ends. We now report the synthesis of a tridecanucleoside dodecaphosphate (d[C-T-A-T-T-C-C-A-G-A-A-G-T]) sequence of SV40 DNA[13] by the latter approach.

RESULTS AND DISCUSSION

Nucleoside and mononucleotide building blocks



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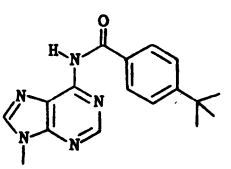

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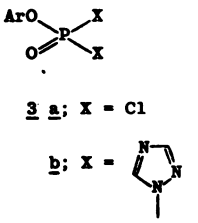
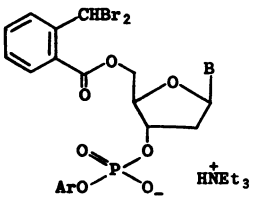
We recently introduced[14] the *o*-dibromomethylbenzoyl (DBMB) protecting group especially for use in oligonucleotide synthesis. *o*-Dibromomethylbenzoyl chloride[14], which may easily be prepared from *o*-dibromomethylbenzoic acid[15] (see Experimental), reacts regioselectively with thymidine and *N*-acyl-2'-deoxyribonucleosides in acetonitrile-pyridine solution to give the corresponding 5'-DBMB derivatives (1). Thus the 5'-DBMB derivatives of 6-*N*-(*p*-t-butylbenzoyl)-2'-deoxyadenosine, 4-*N*-benzoyl-2'-deoxycytidine and thymidine (1; B = 2, 4-*N*-benzoylcytosin-1-yl and thymin-1-yl, respectively) were isolated from the acylation products in yields of 71.5, 64 and 77%, respectively. Each of the latter compounds was obtained as a pure crystalline solid. We have previously found[2] that the polarity of oligonucleotide phosphotriester intermediates increases and hence that their solubility in organic solvents (e.g. chloroform) decreases with increasing molecular weight. This leads to difficulties with extraction and chromatography. For this reason we came to the conclusion that it is desirable to use lipophilic protecting groups in the phosphotriester approach[16]. In the present work, we have used the *p*-t-butylbenzoyl group[17] to protect adenine residues (as in 2).



3 a; X = Cl

b; X =



4

Narang and his coworkers[18] have developed a method for converting 5'-protected 2'-deoxyribonucleosides and their *N*-acyl derivatives into the corresponding 3'-(*p*-chlorophenyl) 2-cyanoethyl phosphates in good yields by reaction first with *p*-chlorophenyl phosphorodi-(1,2,4-triazolide) (3b; Ar = 4-ClC_6H_4) and then with 2-cyanoethanol. Agarwal and Riftina later reported[6] that the reaction of 3b (Ar = 4-ClC_6H_4) with 5'-protected 2'-deoxyribonucleoside building blocks is not accompanied by the formation of symmetrical phosphotriester products and thus that this apparently bifunctional phosphorylating agent can behave as though it were monofunctional. Very recently, Gough *et al.* have reported[19] that when 5'-protected 2'-deoxyribonucleoside building blocks are treated with a threefold excess of 3b (Ar = 4-ClC_6H_4), the corresponding 3'-(*p*-chlorophenyl) phosphates are obtained and may be isolated, in high yields, as their barium salts, presumably free from barium *p*-chlorophenyl phosphate. We have similarly reported[20] that when the 5'-DBMB derivatives of thymidine and appropriate *N*-acyl-2'-deoxyribonucleosides (1) are treated, in acetonitrile-pyridine solution, with 2-3 molecular equivalents of *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) (3b; Ar = 2-ClC_6H_4), generated from *o*-chlorophenyl phosphorodichloridate (3a; Ar = 2-ClC_6H_4), 1,2,4-triazole and triethylamine, the corresponding 3'-(*o*-chlorophenyl) phosphates are obtained and may be isolated as pure solid triethylammonium salts (4; Ar = 2-ClC_6H_4) in high (90-95%) yields. The success of our method depends on the fact (see Experimental) that the phosphodiester salts (4), unlike the triethylammonium salt of *o*-chlorophenyl phosphate, is not extracted from chloroform solution by aqueous sodium hydrogen carbonate. Our phosphorylation procedure is equally suitable (see below and Experimental) for converting oligonucleotide phosphotriester intermediates with free 3'-hydroxy functions into the corresponding 3'-(*o*-chlorophenyl) phosphates.

Preparation of dimer, trimer and tetramer blocks

We recently reported[20] that when a solution of a phosphodiester inter-

Table 1. Condensation reactions involving 1-Mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT, 7).

Expt. No.	5'-Protected Component[a] (mmol)	5'-Hydroxy Component (mmol)	MSNT (mmol)	Reaction Time (min)	Product (% isolated yield)
1.	d[Dbmb-*Ap*] (1.53)	d[HO-*G*-OH] (1.84)	7.65	25	d[Dbmb-*ApG*-OH] (83)
2.	d[Dbmb-*Cp*] (1.0)	d[HO-*C*-OH] (1.3)	6.5	20	d[Dbmb-*CpC*-OH] (81)
3.	d[Dbmb-*Cp*] (1.0)	d[HO-T-OH] (1.3)	6.5	20	d[Dbmb-*Cp*T-OH] (84)
4.	d[Dbmb-Tp] (2.0)	d[HO-T-OH] (2.5)	12.5	20	d[Dbmb-TpT-OH] (79)
5.	d[Dbmb-*ApGp*] (0.58)	d[HO-*A*-OH] (0.70)	3.0	23	d[Dbmb-*ApGpA*-OH] (73)
6.	d[Dbmb-*ApGp*] (0.58)	d[HO-T-Bz] (0.47)	2.9	60	d[Dbmb-*ApGp*T-Bz] (91)
7.	d[Dbmb-*Cp*Tp] (0.61)	d[HO-*A*-OH] (0.74)	3.1	20	d[Dbmb-*Cp*Tp*A*-OH] (69)
8.	d[Dbmb-TpTp] (0.46)	d[HO-*CpC*-Px] (0.46)	2.3	40	d[Dbmb-TpTp*CpC*-Px] (85)
9.	d[Dbmb-*ApGpAp*] (0.38)	d[HO-*ApGp*T-Bz] (0.30)	2.7	90	d[Dbmb-*ApGpApApGp*T-Bz] (81)
10.	d[Dbmb-*Cp*Tp*Ap*] (0.40)	d[HO-TpTp*CpC*-Px] (0.25)	2.4	100	d[Dbmb-*Cp*Tp*Ap*Tp*Tp*CpC*-Px] (83)
11.	d[Dbmb-*Cp*Tp*Ap*Tp*Tp*CpCp*] (0.113)	d[HO-*ApGpApApGp*T-Bz] (0.086)	1.15	45	d[Dbmb-*Cp*Tp*Ap*Tp*Tp*CpCpApGpApApGp*T-Bz] (71)

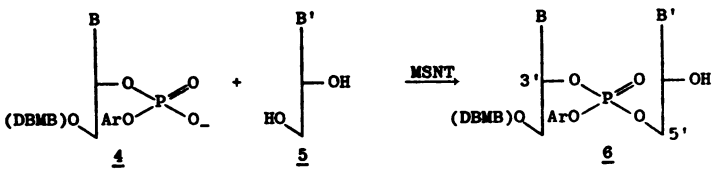
[a] *o*-Dibromomethylbenzoyl and 9-phenylxanthen-9-yl are abbreviated to Dbmb and Px, respectively. 6-*N*-(p-t-Butylbenzoyl-2'-deoxyadenosine, 4-*N*-benzoyl-2'-deoxycytidine and 2-*N*-benzoyl-2'-deoxyguanosine residues are represented by *A*,*C* and *G*, respectively; phosphate residues which are protected by *o*-chlorophenyl groups are represented by *p*.

mediate (4) and thymidine or an *N*-acyl-2'-deoxyribonucleoside derivative (5, Scheme 1) in anhydrous pyridine is treated with an excess of 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole[10,21] (MSNT, 7) for *ca.* 20 min at room temperature, the corresponding partially-protected 3'→5'-dinucleoside phosphate (6) is obtained usually in good yield. Thus phosphorylation occurs with a high degree of regioselectivity on the 5'-hydroxy function of 5. Small quantities of isomeric 3'→3'-dinucleoside phosphates may be obtained but the latter[20] are generally less polar than the desired products (6) and may be removed by adsorption chromatography on silica gel. Four such dinucleoside phosphates (d[Dbmb-*ApG*-OH]*, d[Dbmb-*CpC*-OH], d(Dbmb-*Cp*T-OH] and d[Dbmb-T*p*T-OH]) were required in the present synthesis and were obtained, free from their 3'→3'-isomers, in 83, 81, 84 and 79% isolated yields (see Table 1, experiments nos. 1-4 and Experimental), respectively.

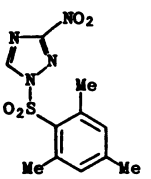
Three of the latter partially-protected dinucleoside phosphates (d[Dbmb-*ApG*-OH], d[Dbmb-*Cp*T-OH] and d[Dbmb-T*p*T-OH]) were converted into the triethylammonium salts of their 3'-(*o*-chlorophenyl) phosphates (i.e. d[Dbmb-*ApGp*], d[Dbmb-*Cp*T*p*] and d[Dbmb-T*p*T*p*]) in 92, 91 and 94% yields, respectively, by treatment with 2-3 molecular equivalents of *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) (3b; Ar = 2-ClC_6H_4) in acetonitrile-pyridine solution, according to the procedure[20] described above in the preparation of the mononucleotide building blocks (4). The fourth partially-protected dinucleoside phosphate (d[Dbmb-*CpC*-OH]) was treated (see Experimental) with an excess of 9-phenyl-9-xanthenyl (pixyl) chloride[17] (8) in pyridine solution

*While the abbreviations suggested by the 1970 IUPAC-IUB Commission on Biochemical Nomenclature [Biochemistry 9, 4022-4027 (1970)] are very clear for unprotected oligonucleotides, they are much less suitable for protected oligonucleotides. This is especially the case for phosphotriester intermediates: the sequences then become particularly difficult to read as they are dominated by abbreviated protecting groups rather than by nucleoside (base) residues. This situation is likely to become even more complicated with the introduction of new protecting groups. We therefore propose a new and, in our opinion, more convenient notation for fully- and partially-protected oligodeoxyribonucleotides in which the *N*-protected nucleoside residues and protected internucleotide linkages are indicated simply by the appropriate italicized letters (i.e. *A*, *C*, *G* and *p*, respectively). Terminal phosphodiester groups are also represented by *p*. It then remains necessary only to provide a key indicating which protecting groups are being used. We have provided such a key in Table 1 (footnote[a]). Occasionally, a particular base residue (or the internucleotide linkages) might be protected in more than one way in a particular synthesis. Such a complication could be dealt with by the use of asterisks: thus, for example, 6-*N*-benzoyl- and 6-*N*(*p*-t-butylbenzoyl)-2'-deoxycytidine residues could be represented by *C* and *C**, respectively.

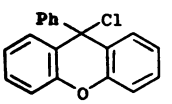
Scheme 1



4 5 6



7(MSNT)



8(PxCl)

to give its 3'-*O*-pixyl derivative (d[Dbmb-*CpC*-Px]) which was isolated in 89% yield. The latter fully-protected dinucleoside phosphate was then treated with nearly 10 molecular equivalents of *ca.* 0.4 *M*-silver perchlorate in acetone-water (98:2 v/v) in the presence of *ca.* 6 molecular equivalents of 2,4,6-collidine at room temperature for 1 hr and, after removal of the silver salts, the products were treated with morpholine for 5 min. The 5'-DBMB protecting group[14] was thereby removed and d[HO-*CpC*-Px] was obtained in 92% isolated yield.

The tridecanucleoside dodecaphosphate was assembled from three trimers and one tetramer. The dinucleotides (d[Dbmb-*ApGp*] and d[Dbmb-*CpTp*]) were converted (Table 1, experiments nos. 5 and 7) into the partially-protected trinucleoside diphosphates (d[Dbmb-*ApGpA*-OH] and d[Dbmb-*CpTpA*-OH]) in satisfactory yields; the dinucleotide (d[Dbmb-*ApGp*]) was converted (experiment no. 6) into d[Dbmb-*ApGp*T-Bz] in excellent yield. The above two partially-protected trinucleoside diphosphates were converted by treatment with *o*-chlorophenyl phosphorodi-(1,2,4-triazolide) (3b; Ar = 2-ClC_6H_4) into the corresponding trinucleotide derivatives (d[Dbmb-*ApGpAp*] and d[Dbmb-*CpTpAp*]) in 88 and 94% isolated yields, respectively. The required tetramer, d[Dbmb-T*p*T*pCpC*-Px] was obtained (Table 1, experiment no. 8) from d[Dbmb-T*p*T*p*] and d[HO-*CpC*-Px] in good yield. The 5'-DBMB protecting groups were then removed from d[Dbmb-*ApGp*T-Bz] and d[Dbmb-T*p*T*pCpC*-Px] to give d[HO-*ApGp*T-Bz] and d[HO-T*p*T*pCpC*-Px] in 78 and 77% isolated yields, respec-

tively.

Preparation of the fully-protected tridecanucleoside dodecaphosphate

As indicated in Table 1 (experiment no. 11), the desired fully-protected tridecanucleoside dodecaphosphate was obtained in 71% isolated yield following the condensation between *ca.* 1.3 molecular equivalents of the heptanucleotide derivative (d[Dbmb-*Cp*T*pAp*T*p*T*pCpCp*]) and the hexanucleoside pentaphosphate derivative (d[HO-*ApGpApApGp*T-Bz]) in the presence of a large excess of MSNT(7). The required heptamer component was obtained as indicated in Scheme 2(a). Following the condensation between the appropriate trimer and tetramer blocks [step (i); Table 1, experiment no. 10], the 3'-terminal pixyl protecting group was removed [step (ii), 90% isolated yield] and the product was phosphorylated [step (iii), 87% isolated yield]. Finally, the required hexamer component was obtained as indicated in Scheme 2(b). Following the condensation between the appropriate trimer blocks [step (i); see Table 1, experiment no. 9], the 5'-terminal DBMB protecting group was removed [step (iv), 75% isolated yield].

Scheme 2

(a) d[Dbmb-*Cp*T*pAp*] + d[HO-T*p*T*pCpC*-Px] $\xrightarrow{\text{(i),(ii),(iii)}}$ d[Dbmb-*Cp*T*pAp*T*p*T*pCpCp*]

(b) d[Dbmb-*ApGpAp*] + d[HO-*ApGp*T-Bz] $\xrightarrow{\text{(i),(iv)}}$ d[HO-*ApGpApApGp*T-Bz]

Reagents: (i) MSNT(7)/C_5H_5N; (ii) TsOH/$CHCl_3$-MeOH (95:5 v/v); (iii) (a) 3b (Ar = 2-ClC_6H_4)/MeCN-C_5H_5N, (b) Et_3N-H_2O; (iv) (a) $AgClO_4$ - 2,4,6-collidine/Me_2CO-H_2O (98:2 v/v), (b) morpholine.

Unblocking of the fully-protected tridecanucleoside dodecaphosphate

The unblocking of the fully-protected tridecanucleoside dodecaphosphate was effected by the two step procedure indicated in Scheme 3. An independent study[22] has indicated that partial removal of the 5'-DBMB (which is less stable to base than the 3'-benzoyl) protecting group before the completion of the oximate ion-promoted unblocking[14] of the internucleotide linkages leads to the occurrence of only a very small amount (< 0.5%) of terminal phosphoryl migration. Although this side-reaction could be completely prevented by replacement of the DBMB by a base-stable (e.g. pixyl) protecting group before step (i) (Scheme 3) is carried out, we believe that the at least 99.5% integrity of the internucleotide linkage at the 5'-end of the molecule is acceptable.

The unblocked products were then fractionated by chromatography on DEAE-

Sephadex A25 (Figure 1) and the main peak was found to contain *ca.* 93% of the total number of A_{260} units eluted from the column. The latter material was completely digested to its monomeric components in the presence both of snake venom and calf spleen phosphodiesterases. In each case, the digest was analyzed by h.p.l.c. and the results (see Experimental) were, within the limits of experimental error, in accordance with the structure assigned to the synthetic tridecanucleoside dodecaphosphate. The assigned structure was further confirmed by nucleotide sequence analysis.

The synthetic approach described in this paper leads to satisfactory yields of high quality oligonucleotides. The methods which have been

Scheme 3

d[Dbmb-*Cp*T*p*A*p*T*p*T*p*C*p*C*p*A*p*G*p*A*p*A*p*G*p*T-Bz]

↓ (i),(ii)

d[C-T-A-T-T-C-C-A-G-A-A-G-T]

Reagents: (i) N^1,N^1,N^3,N^3-Tetramethylguanidinium *syn*-4-nitrobenzaldoximate in dioxan–water (1:1 v/v), 20°C, 24 hr;
(ii) aqueous ammonia (*d* 0.88), 50°C, 24 hr.

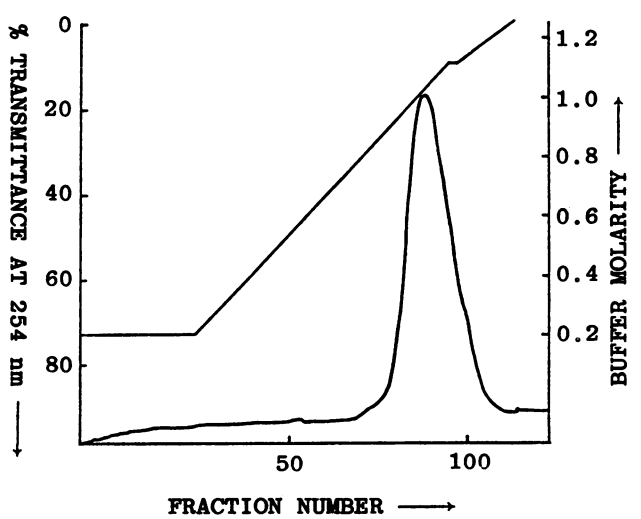


Figure 1. DEAE-Sephadex chromatography of the products obtained after the complete unblocking [by the procedure indicated in Scheme 3] of the fully-protected d[C-T-A-T-T-C-C-A-G-A-A-G-T]. Triethylammonium bicarbonate buffer (pH 7.5) was used as the eluting agent.

developed[20] for the preparation of 5'-protected mononucleotides (4) and for their conversion into partially-protected dinucleoside phosphates (6) and the corresponding dinucleotides (such as d[Dbmb-*ApGp*]) are relatively convenient and not particularly time-consuming. Since we have completed this work, we have found[23] that it is unnecessary and indeed undesirable to use more than *ca.* 2-3 molecular equivalents of activating agent (MSNT, 7) in condensation reactions. We have further found[23] that significant excesses of the 3'-phosphodiester component and long reaction times can lead to side-reactions involving the guanine residues. As well as taking these observations into account, we intend in the future to use more lipophilic protecting groups for cytosine and guanine residues and possibly also for the internucleotide linkages. As we have suggested above, the latter modifications should facilitate the work-up and chromatography of condensation reactions.

EXPERIMENTAL

U.v. absorption spectra were measured with a Perkin-Elmer 402 spectrophotometer. ^{1}H N.m.r. spectra were measured at 60 MHz with a Perkin-Elmer R12B spectrometer and at 90 MHz with a Bruker HFX 90 spectrometer; tetramethylsilane was used as an internal standard. I.r. spectra were measured with a Perkin-Elmer 257 spectrometer.

Merck silica gel 60 F_{254} pre-coated plates and DC-Alufolien cellulose F_{254} sheets were used for t.l.c. Paper electrophoresis was carried out in a Savant tank on Whatman No.1 paper in 0.05 *M*-sodium phosphate buffer (pH 7.0). H.p.l.c. was carried out on a Partisil PXS 10/25 SAX column which was eluted isocratically with 0.05 *M*-potassium phosphate buffer (pH 3.35). Merck Kieselgel H and Reeve Angel silica gel CT were used for short column chromatography[24]. Anion-exchange chromatography on DEAE-Sephadex A-25 was carried out with linear gradients of triethylammonium hydrogen carbonate buffer (pH 7.5).

Dioxan, acetonitrile and pyridine were dried by heating, under reflux, with CaH_2 for 3-5 hr; these solvents were then distilled at atmospheric pressure and stored over molecular sieves (no. 4A). Dimethylformamide was stirred with CaH_2 at 20°C for 16 hr, then distilled under reduced pressure (at *ca.* 14 mmHg) and stored over molecular sieves (no. 4A).

o-Dibromomethylbenzoyl chloride (carried out by Mr. S. Sibanda).

A suspension of *o*-dibromomethylbenzoic acid[15] (5.0g, 17.0 mmol, m.p. 173°C; prepared by the bromination of *o*-toluic acid and recrystallized from ethanol-water (9:1 v/v)), thionyl chloride (6 ml, 9.8g, 82.3 mmol) and

dimethylformamide (2 drops) was heated, under gentle reflux, for 15 min. The cooled products were evaporated, under reduced pressure and the residue recrystallized from petroleum ether (b.p. 30-40°C, 15 ml) to give *o*-dibromomethylbenzoyl chloride [Found: C, 31.0; H, 1.6. $C_8H_5Br_2ClO$ requires: C, 30.8, H, 1.6%] as colourless crystals, m.p. 53.5 - 54.5°C; yield 4.6g (87%); $\nu_{max}^{CHCl_3}$ 1740, 1775 cm^{-1}; $(M-Cl)^+$ at *m/e* = 276, 278; ^{13}C n.m.r. ($CDCl_3$): δ 36.5, 128.0, 129.8, 131.8, 132.8, 135.2, 142.8, 167.9.

5'-*O*-(*o*-Dibromomethylbenzoyl)thymidine

A solution of *o*-dibromomethylbenzoyl chloride (7.117g, 22.8 mmol) in anhydrous acetonitrile (20 ml) was added dropwise over a period of 2 hr to a magnetically-stirred, anhydrous solution of thymidine (5.017g, 20.7 mmol) in pyridine (75 ml) at room temperature. After a further period of 14 hr, saturated aqueous sodium hydrogen carbonate (250 ml) was added and the products were extracted with chloroform (6 x 150 ml). The combined chloroform extracts were dried ($MgSO_4$), filtered through hyflo-supercel and concentrated under reduced pressure to a pale yellow solid. Short column chromatography on silica gel, evaporation of the appropriate fractions [eluted with $CHCl_3$-EtOH (95:5 v/v)] and crystallization of the residue from ethyl acetate gave 5'-*O*-(*o*-dibromomethylbenzoyl)thymidine; yield 8.2g (77%). After recrystallization from methanol, the product had m.p. 158°C [Found: C, 42.0; H, 3.5; N, 5.4. $C_{18}H_{18}Br_2N_2O$ requires: C, 41.7; H, 3.5; N, 5.4%]; ^1H n.m.r. (90 MHz, in $(CD_3)_2SO$-D_2O): δ 1.62 (3H, s), 2.26 (2H, m), 4.14 (1H, m), 4.25 - 4.65 (3H, m), 6.26 (1H, t, *J* = 7 Hz), 7.3 - 8.2 (6H, m).

4-*N*-Benzoyl-5'-*O*-(*o*-dibromomethylbenzoyl)-2'-deoxycytidine

This compound was prepared in the same way from 4-*N*-benzoyl-2'-deoxycytidine; after recrystallization from ethyl acetate, it was isolated in 64% yield [Found: C, 47.6; H, 3.6; N, 6.9; Br, 26.5. $C_{24}H_{21}Br_2N_3O_6$ requires: C, 47.5; H, 3.6; N, 6.9; Br, 26.3%], m.p. 159 - 160°C dec.

6-*N*-t-Butylbenzoyl-5'-*O*-(*o*-dibromomethylbenzoyl)-2'-deoxyadenosine

This compound was similarly prepared from 6-*N*-t-butylbenzoyl-2'-deoxyadenosine; after recrystallization from ethyl acetate-cyclohexane, it was isolated in 71.5% yield [Found: C, 50.9; H, 4.3; N, 10.1; Br, 23.1. $C_{29}H_{29}Br_2N_2O_5$ requires: C, 50.7; H, 4.25; N, 10.2; Br, 23.25%], m.p. 143-144°C.

Triethylammonium 5'-*O*-(*o*-dibromomethylbenzoyl)-2'-deoxyribonucleoside 3'-(*o*-chlorophenyl) phosphates

To a solution of *o*-chlorophenyl phosphorodichloridate (0.60g, 2.44 mmol) in acetonitrile (2.5 ml) is added 1,2,4-triazole (0.44g, 6.36 mmol), followed by more acetonitrile (2.5 ml). Triethylamine (0.50g, 0.68 ml, 4.9 mmol) is

then added and the reactants are stirred at room temperature. After 15 min, the 5'-*O*-(*o*-dibromomethylbenzoyl)-2'-deoxyribonucleoside (1.0 mmol) is added, followed by pyridine (5 ml). After a further period of *ca.* 30 min a solution of triethylamine (0.50g, 0.68 ml, 4.9 mmol) and water (0.2 ml) in pyridine (2 ml) is added and, 10 min later, the products are poured into a separating funnel containing saturated aqueous sodium hydrogen carbonate (100 ml). After thorough shaking, the resulting cloudy solution is extracted with chloroform (2-5 x 35 ml). The combined chloroform extracts are washed with saturated aqueous sodium hydrogen carbonate (2-5 x 80 ml), dried ($MgSO_4$) and concentrated under reduced pressure. A solution of the residual glass in chloroform (2.5 - 4 ml) is added dropwise to stirred petroleum ether (b.p. 30-40°C, 200 ml). The resulting precipitate is collected by filtration and dried in a desiccator. Pure products, in yields of 90 - 95%, are usually obtained.

The above procedure is equally suitable for the conversion of oligonucleotide intermediates, in which the terminal 3'-hydroxy functions only are unprotected, into the triethylammonium salts of the corresponding 3'-(*o*-chlorophenyl) phosphates. Again, high yields (often 90% or greater) are obtained.

General procedure for the preparation of partially-protected dinucleoside phosphates with free 3'-hydroxy functions and for all condensations involving phosphodiester intermediates.

To a magnetically-stirred, anhydrous solution of the appropriate triethylammonium 5'-*O*-(*o*-dibromomethylbenzoyl)-2'-deoxyribonucleoside 3'-(*o*-chlorophenyl) phosphate (1 mmol) and thymidine or the appropriate *N*-acyl-2'-deoxyribonucleoside (1.25 mmol) in pyridine (20 ml) is added 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT, 6 mmol) at room temperature. After 20 min, saturated aqueous sodium hydrogen carbonate (1.5 ml) is added dropwise and, after a further period of 10 min, the products are poured slowly, with swirling, into saturated aqueous sodium hydrogen carbonate (75 ml) contained in a separating funnel. The resulting mixture is extracted with chloroform (6 x 20 ml) and the dried ($MgSO_4$) chloroform extracts are concentrated under reduced pressure to give a glass. T.l.c. [silica gel, $CHCl_3$-MeOH (9:1 v/v)] examination of the latter material usually reveals the desired product as the major component [$R_F \sim 0.3$] and higher R_F minor components which may include the isomeric 3'→3'-dinucleoside phosphate. The products are fractionated by short column chromatography on silica gel. The appropriate fractions are evaporated and a solution of the residue in

chloroform (*ca.* 2 ml) is added dropwise to petroleum ether (b.p. 30 - 40°C, *ca.* 100 ml). The precipitated partially-protected 3'→5'-dinucleoside phosphate, which is free from its 3'→3'-isomer, is collected by centrifugation. Yields in the region of 75 - 80% are usually obtained.

The above procedure may also be used in the condensation between the 3'-(*o*-chlorophenyl) phosphate of an otherwise protected dinucleoside phosphate and an *N*-acyl-2'-deoxyribonucleoside or thymidine to give a trinucleoside diphosphate which is unprotected on its 3'-terminal hydroxy group. This procedure is also at least as suitable for condensations between 5'-protected nucleosides or oligonucleotide 3'-(*o*-chlorophenyl) phosphates and nucleosides or oligonucleotides in which all the functions except the terminal 5'-hydroxy functions are protected. In the latter condensations, the component with the free 3'-hydroxy function is not normally used in excess.

Reaction between a partially-protected dinucleoside phosphate with a free hydroxy function [d(Dbmb)-*CpC*-(OH)] and 9-phenyl-9-xanthenyl(pixyl) chloride.

A solution of the partially-protected dinucleoside phosphate (0.50g, 0.44 mmol) and pixyl chloride[17] (0.216g, 0.74 mmol) in anhydrous pyridine (4 ml) was stirred at room temperature. After 1 hr, methanol (2 ml) was added and, after a further period of 20 min, the products were partitioned between chloroform (40 ml) and saturated aqueous sodium hydrogen carbonate (35 ml). The layers were separated and the aqueous layer was extracted with chloroform (4 x 20 ml). The combined chloroform extracts were dried ($MgSO_4$) and concentrated under reduced pressure to give a glass. The latter material was purified by short column chromatography on silica gel. The appropriate fractions [eluted with $CHCl_3$-EtOH (96.5:3.5 v/v)] were evaporated to give d[(Dbmb)-*CpC*-(Px)] which was isolated as a solid (0.548g, 89%) by precipitation from petroleum ether (see above).

Removal of the 5'-terminal *o*-dibromomethylbenzoyl (Dbmb) group from a fully-protected oligonucleotide.

(a) A fully-protected dinucleoside phosphate. The suspension obtained by adding 2,4,6-collidine (0.623g, 5.1 mmol) to a solution of silver perchlorate (1.776g, 8.6 mmol) in acetone-water (98:2 v/v; 7 ml) was added to a magnetically-stirred solution of the substrate (1.20g, 0.87 mmol) in the same solvent (13 ml) at room temperature. After 1 hr, a solution of lithium bromide (0.74g) in acetone-water (90:10 v/v; 20 ml) was added and, after a further period of 10 min, the products were filtered. Morpholine (5 ml, 57 mmol) was added to the filtrate and, after 5 min, chloroform (50 ml) was

added and the mixture was poured into saturated aqueous sodium hydrogen carbonate (150 ml), contained in a separating funnel. The layers were separated and the aqueous layer was extracted with chloroform (5 x 35 ml). The combined chloroform layers were concentrated under reduced pressure. Toluene was then added and the evaporation of solvent was continued. The product was isolated as a pure (t.l.c.) solid by precipitation from petroleum ether (see above); yield 0.87g (92%).

(b) A fully-protected hexanucleoside pentaphosphate. The suspension obtained by adding 2,4,6-collidine (0.177g, 1.46 mmol) to a solution of silver perchlorate (1.0g, 4.9 mmol) in acetone-water (98:2 v/v; 8 ml) was added to a magnetically-stirred solution of the substrate (0.861g, 0.24 mmol) in acetone-water (98:2 v/v; 15 ml) at room temperature. After the reaction had been allowed to proceed in subdued light for 2.5 hr, a solution of lithium bromide (0.844g, 9.72 mmol) in acetone-water (90:10 v/v, 20 ml) was added. After 10 min, the products were filtered and the residue was washed several times with acetone (total volume, *ca.* 70 ml). Morpholine (11 ml) was added to the combined filtrate and washings and, after 5 min, the products were worked-up as above and then purified by short column chromatography. The desired product was isolated by precipitation (see above); yield, 0.601g (75%).

Removal of the 3'-terminal pixyl group from a fully-protected heptanucleoside hexaphosphate.

A solution of toluene-*p*-sulphonic acid monohydrate (0.101g, 0.53 mmol) in chloroform-methanol (95:5 v/v; 10 ml) was added to a magnetically-stirred solution of substrate (0.793g, 0.2 mmol) in the same solvent (35 ml) at room temperature. After 7 min, the products were poured, with swirling, into a separating funnel containing saturated aqueous sodium hydrogen carbonate (130 ml). The resulting mixture was extracted with chloroform (45 ml + 6 x 30 ml). The chloroform extracts were combined, toluene (*ca.* 20 ml) was added and then the solvents were evaporated under reduced pressure to give a glass. The latter material was purified by short column chromatography on silica gel. The appropriate fractions [eluted with $CHCl_3$-EtOH (92:8 v/v)] were evaporated and the residual material isolated by precipitation from petroleum ether (see above); yield, 0.669g (90%).

Unblocking of fully-protected tridecanucleoside dodecaphosphate

To a magnetically-stirred solution of the substrate (0.018g, 0.0025 mmol) in dioxan (0.3 ml) was added *syn*-*p*-nitrobenzaldoxime (0.050g, 0.3 mmol), N^1,N^1,N^3,N^3-tetramethylguanidine (0.034g, 0.3 mmol) and water (0.3 ml)

at room temperature. A homogeneous solution was obtained after a short period. After 24 hr, aqueous NH_3 (*d* 0.88, 3 ml) was added and the reactants were heated at 50°C in a screw-cap pressure bottle. After 24 hr, the products were concentrated under reduced pressure, dissolved in acetic acid-water (1:1 v/v; 12 ml) and the solution immediately extracted with chloroform (27 x 15 ml). The aqueous layer was then concentrated under reduced pressure and the residue chromatographed on a column (9 cm x 2 cm diameter) of DEAE-Sephadex A25. The column was eluted with triethylammonium bicarbonate buffer (pH 7.5; 0.2 *M*, followed by a linear gradient from 0.2 - 1.1 *M*). The main component (246 A_{260} units) was eluted (Figure 1) with *ca.* 0.95 *M*-buffer. It had λ_{max} 255 nm.

Enzymatic digestion of unprotected tridecanucleoside dodecaphosphate

(a) with *crotalus adamanteus* snake venom phosphodiesterase. To a solution of the substrate (*ca.* 0.003g) in 0.1 *M*-tris hydrochloride buffer (pH 8.9, 0.01 *M* with respect to magnesium chloride, 0.085 ml) was added stock enzyme solution (0.022 ml, containing 0.001g/ml of the same tris hycrochloride buffer). The resulting solution was maintained at 37°C for 18 hr. H.p.l.c. analysis of the hydrolysate revealed: 2'-deoxycytidine and other nucleosides (R_T 3.0 min, *ca.* 1.6 parts), 2'-deoxycytidine 5'-phosphate (R_T 4.2 min, 2.1 parts), 2'-deoxyadenosine 5'-phosphate (R_T 5.6 min, 3.9 parts), thymidine 5'-phosphate (R_T 6.0 min, 4.1 parts) and 2'-deoxyguanosine 5'-phosphate (R_T 9.35 min, 2.0 parts).

(b) with calf spleen phosphodiesterase. To a solution of the substrate (12 A_{260} units) in 0.1 *M*-ammonium acetate buffer (pH 7.0, 0.002 *M* with respect to EDTA and containing 0.05% Tween 80, 0.08 ml) was added stock enzyme solution (0.02 ml, containing 0.001g enzyme/ml of the same buffer). The resulting solution was maintained at 37°C for 18 hr. H.p.l.c. analysis of the hydrolysate revealed: thymidine (R_T 3.45 min, 1.2 parts), 2'-deoxycytidine 3'-phosphate (R_T 4.15 min, 3.1 parts), 2'-deoxyadenosine 3'-phosphate (R_T 5.5 min, 4.0 parts), thymidine 3'-phosphate (R_T 5.9 min, 3.4 parts) and 2'-deoxyguanosine 3'-phosphate (R_T 9.2 min, 2.0 parts).

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