Synthesis of cyclic oligodeoxyribonucleotides via the 'filtration' approach

M.Vaman Rao and Colin B.Reese

Department of Chemistry, King's College London, Strand, London WC2R 2LS, UK

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ABSTRACT

The synthesis of cyclic di-, tri-, tetra-, penta- and hexa-thymidylic acids (16; n = 2, 3, 4, 5 and 6) and the cyclic hexadeoxyribonucleotide [d($\overline{\text{CpCpTpApGpGp}}^{\text{J}}$)], via the "filtration" approach, is reported. Some of the physical properties of the cyclic oligonucleotides are discussed, and their susceptibility to digestion in the presence of phosphorylytic enzymes has been studied.

INTRODUCTION

Almost all of the work on the chemical synthesis of oligo- and polyribonucleotides reported so far has been concerned with the preparation of *acyclic* systems. In the course of their early work on the synthesis of oligothymidylic acids by the phosphodiester approach in solution, Khorana and his coworkers¹ isolated *cyclic* oligomers, and especially cyclic dithymidylic acid, as by-products. In 1985, in what we believe to be the first report of studies purposely directed towards the synthesis of cyclic oligonucleotides, Hsu *et al.*² described the synthesis of three cyclic diribonucleoside phosphates [r($\overline{\text{ApAp}}^{\text{J}}$), r($\overline{\text{ApUp}}^{\text{J}}$), and r($\overline{\text{UpUp}}^{\text{J}}$)] using the phosphotriester approach in solution. Later van Boom and his coworkers³ synthesized r($\overline{\text{GpGp}}^{\text{J}}$) by a similar approach, and have recently⁴ extended their studies by preparing two cyclic tetraribonucleotides, two cyclic hexadeoxyribonucleotides and a cyclic octadeoxyribonucleotide. Recently, Barbato *et al.*⁵ have developed a solid phase synthesis of cyclic oligodeoxyribonucleotides, based also on the phosphotriester approach.

The recent interest in cyclic oligonucleotides has arisen from their activity² as RNA polymerase inhibitors, and, in the case of r($\overline{\text{GpGp}}^{\text{J}}$) from its properties³ as an activator of cellulose synthase. There are clearly other reasons why it is important that methods should be available for the synthesis

of cyclic oligo- and poly-nucleotides. First, native deoxyribonucleic acid (DNA) sometimes occurs in a circular form. Secondly, intron deletion in eukaryotes and in the primitive organism *Tetrahymena* involves⁶ cyclic polyribonucleotides. In the latter case, the cyclic polyribonucleotides involved display ribozyme activity. In this article, we describe what we regard as a convenient and flexible method for the preparation of cyclic oligodeoxyribonucleotides, based on the rapid "filtration" approach⁷ to the synthesis of acyclic oligonucleotides in solution.

RESULTS AND DISCUSSION

The first really successful rapid synthesis of oligodeoxyribonucleotides on a solid support involved⁸ the use of the phosphotriester approach with aryl protecting groups for the internucleotide linkages. The phosphotriester has now been largely superseded by the phosphoramidite⁹ and *H*-phosphonate approaches¹⁰ in solid phase synthesis not because it is ineffective but largely because the coupling steps proceed more rapidly when either of the latter two approaches is followed. However, the phosphotriester¹¹ has an important advantage over the phosphoramidite approach⁹ in that it involves the use of easily prepared stable monomers; it also has an important advantage over the *H*-phosphonate approach¹⁰ in that it gives rise to stable protected oligonucleotides. Indeed, the phosphotriester appears to be the only approach that can be used effectively in solution as well as in solid phase synthesis, and in any case, it seems likely that it is the method of choice in the synthesis of cyclic oligonucleotides even⁵ on a solid support.

In the present work, adenine, cytosine, guanine and thymine base residues were protected as in (1), (2), (3) and (4), respectively. In our original "filtration" work⁷, the amino functions of the base residues were protected in the same way. Studies with the corresponding simple *N*-acyl-2'-deoxyribonucleosides have shown that pivaloyl, benzoyl and phenylacetyl groups are removed by treatment with concentrated aqueous ammonia at room temperature

from adenine, cytosine and guanine residues in 20, 10 and ca. 8 hr, respectively. Recently, an interest has developed¹² in *N*-acyl protecting groups that are removable from base residues by ammonolysis under very mild conditions indeed. Although they are not exceptionally base-labile, we can recommend the present *N*-acyl protecting groups for use both in the synthesis of polydeoxy-ribo- and polyribo-nucleotides with the proviso that pivaloyl (on adenine residues) should be replaced by a more labile protecting group if it is desirable that the unblocking process should go to completion overnight at room temperature. As in previous studies, thymine residues were protected as their 4-*O*-phenyl derivatives¹³ (8). Guanine residues were, however, protected on *O*-6 with 3-chlorophenyl groups [as in (7)]. Both of these *O*-aryl protecting groups were readily removed by treatment¹³ with *E*-2-nitrobenzaldoximate ions in the first unblocking step at the end of the synthesis. The preparation of the 2'-deoxyadenosine and 2'-deoxyguanosine building blocks [(1; B = 5) and (1; B = 7), respectively] are described in detail in the Experimental Section.

Scheme 1

Reagents: (i) (a) reagent obtained from 2-chlorophenyl phosphorodichloridate (ca. 1.5 mol. equiv.), 1-hydroxybenzotriazole (3.0 mol. equiv.) and triethylamine (3.0 mol. equiv.) in tetrahydrofuran-pyridine (2:1 v/v), RT, 20 min, (b) 2,4-dinitrobenzyl alcohol (2.0 mol. equiv.) in tetrahydrofuran-pyridine (10:7 v/v), RT, 2 hr; (ii) pyrrole (12-15 mol. equiv.) in dichloroacetic acid - dichloromethane (2:98 v/v), RT, 1 min.

While both base-protected 3'-*O*-acyl nucleosides and base-protected nucleoside 3'-(2-chlorophenyl) (2,4-dinitrobenzyl) phosphates¹⁴ (6) have been used as 3'-terminal building blocks in "filtration" synthesis⁷, the latter building blocks (6) are particularly suitable starting materials if cyclic oligonucleotides are required. The 2,4-dinitrobenzyl (Dnb) derivatives (6) can be prepared [Scheme 1 and Experimental] from the corresponding base-protected 5'-*O*-(9-phenylxanthene-9-yl) nucleoside derivatives¹⁵ (5) essentially in 3 steps. Using this procedure, the required thymidine (6; B = 4) and 2'-

deoxyguanosine (6; B = 3) derivatives (see below) were prepared in 80 and 78% overall yields, respectively. The synthetic cycle in the "filtration" approach (Scheme 2) consists⁷ essentially of 4 steps: (i) the coupling step which is carried out in the presence of 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole (MSNT)¹⁶ in pyridine solution, (ii) quenching the coupling reaction and complete removal of the pyridine by extraction with acidic (pH 2) sodium phosphate solution, (iii) the 5'-unblocking step which is effected by treatment with dichloroacetic acid in the presence of pyrrole [added¹⁷ to quench the 9-phenylxanthen-9-yl cations released] in dichloromethane solution, and (iv) the "filtration" step [see Experimental]. The excess of 5'-unblocked mononucleotide building block [e.g. HO-Tp] is removed in the "filtration" step. The success of this step depends on the fact that such 5'-unblocked mononucleotides are charged phosphodiesteres which are consequently more polar than oligonucleotide intermediates with protected internucleotide linkages [e.g. (8) and (10)]. For this reason, they can readily be removed by rapid chromatography [essentially a filtration process] on silica gel with tetrahydrofuran-pyridine (3:1 v/v) as the eluting solvent. The latter solvent system⁷ readily elutes protected oligonucleotides [such as (8) and (10)] but 5'-unblocked mononucleotide 3'-phosphodiesteres [e.g. HO-Tp] remain adsorbed on the column.

Scheme 2

[†]9-Phenylxanthen-9-yl and 2,4-dinitrobenzyl are abbreviated to Px and Dnb, respectively. 6-N-Pivaloyl-2'-deoxyadenosine, 4-N-benzoyl-2'-deoxycytidine, 6-O-(3-chlorophenyl)-2-N-phenylacetyl-2'-deoxyguanosine and 4-O-phenylthymidine residues are represented by A, C, G and T, respectively. Phosphate residues which are protected by 2-chlorophenyl groups are represented by p.

Reagents etc: (i) MSNT, pyridine; (ii) work-up and extraction with ca. 2M aqueous sodium phosphate (pH 2) [see Experimental]; (iii) Cl₂CHCO₂H, pyrrole, CH₂Cl₂; (iv) "filtration" step.

As the "filtration" method is a stepwise approach in which usually one nucleotide residue is added at a time, the opportunity to undertake the synthesis of a series of cyclic oligonucleotides and to compare their properties

Table 1. Coupling reactions^a in the "filtration" synthesis of HO-(Tp)_n-Dnb

Cycle No.	Monomer [Px-Tp] (mmol)	HO-(Tp) _n -Dnb ^b (mmol)	MSNT (mmol)	Pyridine (ml)	Crude yield ^c of HO-(Tp) _{n+1} -Dnb (mmol)
1	0.88	0.80	2.64	7	0.65
2	0.61	0.51	1.69	4.5	0.42
3	0.39	0.32	1.00	3	0.26
4	0.22	0.18	0.61	2	0.15
5	0.10	0.086	0.28	2	0.069

^aThe coupling (condensation) reactions were all allowed to proceed for 20 min before work-up [see Experimental]. ^bThe value of n is equal to the cycle no. ^cThis represents the yield obtained at the end of the cycle.

presented itself. We have therefore prepared d((Tp)₂), d((Tp)₃), d((Tp)₄), d((Tp)₅) and d((Tp)₆), a homologous series of five cyclic oligothymidylic acids and, in order to demonstrate that our procedure was equally suitable for the preparation of cyclic hetero-sequences, we also undertook the synthesis of d(CpCpTpApGpGp). The first and last cycles of the "filtration" synthesis leading to HO-TpTpTpTpTpTp-Dnb (10) are indicated in Scheme 2, and the reaction conditions and yields obtained in all five cycles are summarized in Table 1. The crude yields range from 80-83% per cycle. After each of the first four cycles, some of the product [i.e. HO-Tp-Tp-Dnb, HO-TpTpTp-Dnb, HO-TpTpTpTp-Dnb or HO-TpTpTpTpTpTp-Dnb] was retained for cyclization. For this reason, the synthesis was carried out on a relatively large (0.8 mmolar) scale. The synthesis of HO-CpCpTpApGpGp-Dnb (14) was carried out in five "filtration" cycles, starting from 0.25 mmol of HO-Gp-Dnb (6; B=3); in this case, all the product isolated at the end of each cycle was carried forward to the next. The isolated yield of crude HO-CpCpTpApGpGp-Dnb (14) was 0.28g [32% overall yield based on (6; B = 3), representing an average yield of 79.5% per cycle].

Scheme 3

Reagents: (i) Toluene-4-thiol, triethylamine, pyridine, RT, ca. 1 min; (ii) MSNT, pyridine, RT, 12-14 hr (see Experimental Section).

The procedure used for the conversion of the 5'-unblocked oligothymidylic acids [(11); e.g. the dinucleotide (8), the pentanucleotide (9) and the hexanucleotide (10)] into the corresponding fully-protected cyclic oligothymidylic acids (13) is indicated in Scheme 3(a). The Dnb protecting group¹⁴ was first removed by a very brief treatment with an excess of toluene-4-thiolate ions. The resulting corresponding 3'-terminal phosphodiester (12) were then allowed to react with an excess of MSNT in anhydrous pyridine solution at room temperature. After work-up and chromatography, the fully-protected cyclic di-, tri-, tetra-, penta- and hexa-thymidylic acids [(13), $n = 2, 3, 4, 5$ and 6 , respectively] were isolated in 71, 67, 67, 58 and 59% overall [i.e. for the two steps, starting from (11)] yields. In the same way, the fully-protected cyclic hetero-sequence (15) was obtained [Scheme 3(b)] from (14) in 59% overall yield. These yields have not been optimized. In each cyclization experiment, 0.10g of substrate [(12) or HO-CpCpTpApGpGp] and 0.16g (0.54 mmol) of MSNT were dissolved in 10 ml of anhydrous pyridine, and the reaction was allowed to proceed at room temperature for 12-14 hr. Thus, both the dilution and the excess of MSNT were increased with increasing chain length.

Scheme 4

Reagents: (i) *E*-2-nitrobenzaloxime, N^1, N^1, N^3, N^3 -tetramethylguanidine, acetonitrile-water (9:1 v/v) [see Experimental Section], RT, ca. 24 hr; (ii) concentrated aqueous ammonia (d 0.88), RT, 48 hr.

The fully-protected cyclic oligodeoxynucleotides [(13; $n = 2, 3, 4, 5$ and 6) and (15)] were then unblocked in the usual way. In the case of the cyclic oligothymidylic acids [(13), Scheme 4(a)], all of the protecting groups were removed by treatment with N^1, N^1, N^3, N^3 -tetramethylguanidinium *E*-2-nitrobenzaloximate¹⁸ but, in the case of the cyclic hetero-sequence [(15), Scheme 4(b)], it was necessary to include an additional ammonolysis step in order to remove the *N*-acyl protecting groups from the adenine, cytosine and guanine residues. For each cyclic oligothymidylic acid, 0.05-0.06g of fully-protected material (13) was unblocked and the products were then fractionated by chromatography on DEAE Sephadex A25 to give 500-650 A₂₆₅ units of pure homogeneous

Figure 1 (a) Liquid Chromatogram [Jones APEX 5 μ ODS column] of a mixture of d(L(Tp)₂) (R_t 3.19 min), d(L(Tp)₃) (R_t 7.04 min), d(L(Tp)₄) (R_t 11.65 min), d(L(Tp)₅) (R_t 14.56 min), and d(L(Tp)₆) (R_t 16.31 min), eluted with 0.1M - triethylammonium acetate - acetonitrile (90:10 - 82:18 v/v); (b) liquid chromatogram of d(LCpCpTpApGpGp) (R_t 7.81 min) obtained on the same column by isocratic elution with 0.1M - triethylammonium acetate - acetonitrile (93:7 v/v); (c) polyacrylamide gel electrophoretogram of d(L(Tp)₂) (lane 1), d(L(Tp)₃) (lane 2), d(L(Tp)₄) (lane 3), d(L(Tp)₅) (lane 4), d(L(Tp)₆) (lane 5), and d(LCpCpTpApGpGp) (lane 6).

(by l.c.) cyclic oligothymidylic acids (16); in the same way, 550 A₂₅₇ units of pure d($\overline{\text{CpCpTpApGpGp}}$) (17) were obtained from 0.055g of the fully-protected cyclic hetero-sequence (15).

The five cyclic oligothymidylic acids (16; n = 2-6) are readily distinguishable by reverse phase l.c. and by polyacrylamide gel electrophoresis. An l.c. elution profile of an artificial mixture of d($\overline{\text{Tp}}_2$) (R_t 3.2 min), d($\overline{\text{Tp}}_3$) (R_t 7.0 min), d($\overline{\text{Tp}}_4$) (R_t 11.7 min), d($\overline{\text{Tp}}_5$) (R_t 14.6 min) and d($\overline{\text{Tp}}_6$) (R_t 16.3 min) is illustrated in Fig. 1a, and the homogeneity of d($\overline{\text{CpCpTpApGpGp}}$) (17) is clearly apparent from Fig. 1b. It can be seen from Fig. 1c that the gel electrophoretic mobilities of the series of five cyclic oligothymidylic acids (16; n = 2-6) increase as the number of nucleotide residues (n) increases. However, it is anticipated that larger cyclic oligonucleotides will not behave anomalously in this way, and that their electrophoretic mobilities will decrease as n increases.

The ^1H and the ^{31}P n.m.r. spectra of the synthetic cyclic oligodeoxyribonucleotides have been examined. The n.m.r. spectroscopic data obtained in the case of the cyclic oligothymidylic acids (16) are summarized in Table 2. As expected, each cyclic oligonucleotide displays only one ^{31}P resonance signal, and it is clear from their ^1H n.m.r. spectra that the thymidine residues are in identical magnetic environments in each of the five cyclic oligothymidylic acids (16; n = 2, 3, 4, 5 and 6). This, of course, confirms that they are indeed cyclic oligonucleotides. No useful conclusions can be drawn from a comparison of the ^{31}P resonance signals of the five cyclic oligothymidylic acids. As far as their ^1H n.m.r. spectra are concerned, the spectrum of the cyclic dimer (16; n=2) differs markedly, in certain respects, from the spectra of the larger cyclic oligomers. Thus, in the dimer (16; n = 2), the 4'-proton is more shielded and one especially of the 2'-protons is more deshielded than are the corresponding protons in the other cyclic oligothymidylic acids (16;

Table 2. N.m.r. spectra^a of sodium salts of cyclic oligothymidylic acids d($\overline{\text{Tp}}_n$) (16) in D₂O solution

n	^{31}P	H-6	H-1' ($J_{1',2'}$ Hz)	H-3'	H-4'	H-5'	H-2'	5-CH ₃
2	-0.17	7.71	6.25 (3.7, 7.0)	4.89	4.17	4.11	2.63	1.88
3	-0.70	7.72	6.38 (6.0, 8.3)		4.38	4.10	2.60, 2.39	1.90
4	-0.27	7.71	6.32 (5.8, 8.5)	4.87	4.38	4.11	2.53, 2.31	1.90
5	-0.28	7.71	6.30 (6.0, 8.2)	4.86	4.34	4.10	2.52, 2.33	1.90
6	-0.24	7.66	6.27 (6.0, 8.3)	4.85	4.31	4.11	2.49, 2.30	1.87

^a ^{31}P and ^1H N.m.r. spectra were measured at 101.3 and 360 MHz, respectively.

$n = 3, 4, 5$ and 6). Perhaps even more notably, the coupling constants between the $1'$ - and the two $2'$ -protons are both significantly smaller in the dimer (16; $n = 2$) than in the other cyclic oligomers. It may therefore be concluded that the conformation of the sugar residues is different in the dimer (16; $n = 2$) than in the other cyclic oligothymidylic acids (16; $n = 3, 4, 5$ and 6). The ^{31}P n.m.r. spectrum (D_2O , neutral solution) of $\text{d}(\text{[CpCpTpApGpGp]})$ (17) is illustrated in Fig. 2a: six distinct resonance signals may be seen at -0.23 , -0.18 , -0.07 , -0.01 , 0.02 and 0.10 p.p.m.. The lower field region of the ^1H n.m.r. spectrum (D_2O , neutral solution) of (17) is illustrated in Fig. 2b. The signals at $7.52(\text{s})$, $7.73(\text{d}, J\ 7.5\ \text{Hz})$, $7.79(\text{d}, J\ 7.5\ \text{Hz})$, $7.91(\text{s})$, $7.96(\text{s})$, $8.06(\text{s})$ and $8.24(\text{s})$ may be assigned to $H-6$ of thymine, $H-6$ of cytosine, $H-6$ of cytosine, $H-8$ of guanine, $H-8$ of guanine, $H-2$ (or $H-8$) of adenine and $H-8$ (or $H-2$) of adenine, respectively. This clearly confirms the presence of two differently-situated cytosine, one thymine, one adenine and two differently-situated guanine residues in the cyclic hexanucleotide (17).

Finally, the susceptibility of the cyclic oligonucleotides to digestion by phosphorylytic enzymes was investigated. First, all of the cyclic oligothymidylic acids (16; $n = 2, 3, 4, 5$ and 6) remained completely unaffected after they had been treated with calf spleen phosphodiesterase at 37°C for 20 hr. However, all five substrates underwent digestion to give thymidine $5'$ -

Figure 2 (a) ^{31}P and (b) ^1H (low-field region) n.m.r. spectra of sodium salt of $\text{d}(\text{[CpCpTpApGpGp]})$ in neutral deuterium oxide solution.

phosphate in the presence of nuclease P_1 at 37°C. While the cyclic hexamer (16; $n = 6$) required only 4 hr, the trimer (16; $n = 3$) and dimer (16; $n = 2$) required 6 and 10 hr, respectively, for complete digestion to thymidine 5'-phosphate. When the cyclic hetero-sequence, $d(\overline{CpCpTpApGpGp})$ (17) was treated first with *Crotalus adamanteus* snake venom phosphodiesterase at 37°C for 10 hr and then with calf intestine alkaline phosphatase for 3 hr, it underwent complete digestion to give its four constituent 2'-deoxyribonucleosides in, within the limits of experimental error, the expected proportions.

In conclusion, we believe that the "filtration" approach will prove to be the most convenient and flexible method available for the synthesis of cyclic oligonucleotides. Longer acyclic precursor sequences than hexamers [e.g. (14)] can easily be assembled by a combination of the "filtration" approach together with block condensations⁷ and, with the availability of 2'-acetal protecting groups derived from 1-arylpiperidin-4-ones^{17,19}, it would now seem very likely that cyclic oligoribonucleotides could be prepared in the same way. Studies along these lines, directed towards the extension of the present work, are currently under investigation in our laboratory.

EXPERIMENTAL

¹H N.m.r. spectra were measured at 250 and 360 MHz with Bruker WM 250 and AM 360 spectrometers, respectively; tetramethylsilane was used as an internal standard; ³¹P n.m.r. spectra were measured at 36.4 and 101.3 MHz with Bruker HFX 90 and WM 250 spectrometers, respectively; 85% orthophosphoric acid was used as an external standard. U.v. absorption spectra were measured with a Perkin-Elmer Lambda-3 recording spectrophotometer. Merck silica gel 60 F254 pre-coated plates [Art 5715 and 5642, which were developed mainly in solvent system A [CHCl₃-MeOH (9:1 v/v)], were used for thin layer chromatography (t.l.c.). Liquid chromatography (l.c.) was carried out on a Jones Apex Octadecyl 5μ column which was eluted with 0.1M triethylammonium acetate - acetonitrile mixtures. Polyacrylamide gel electrophoresis was carried out in a Raven Vertical Slab Gel Apparatus connected to an LKB Bromma 2197 power supply. Merck Kieselgel H [Art 7729, 7736 and 9385] was used for short column chromatography. Anion-exchange chromatography on DEAE Sephadex A25 was carried out with linear gradients of triethylammonium hydrogen carbonate buffer (pH 7.5). Acetonitrile, dioxane, pyridine and triethylamine were dried by heating, under reflux, with calcium hydride for 3-5 hr, and were then distilled at atmospheric pressure. Tetrahydrofuran was dried by distillation first from sodium benzophenone and then from lithium aluminium hydride. All solvents were

stored over molecular sieves (no. 4A). Phosphorylytic enzymes were purchased from the Sigma Chemical Company.

6-N-Pivaloyl-2'-deoxyadenosine

Pivaloyl chloride (4.3 ml, 35.0 mmol) was added to a stirred solution of 3',5'-di-O-acetyl-2'-deoxyadenosine (5.34g, 15.93 mmol) in anhydrous pyridine (37 ml) at room temperature. After 4 hr, methanol (3 ml) was added and, after a further period of 30 min, the products were concentrated to ca. one-third volume under reduced pressure and partitioned between chloroform (60 ml) and saturated aqueous sodium hydrogen carbonate (60 ml). The aqueous layer was extracted with chloroform (20 ml), and the combined organic layers were dried (MgSO_4) and evaporated under reduced pressure. The residue was dissolved in pyridine (85 ml) and 5.3M methanolic sodium methoxide (18.3 ml, 96 mmol) was added to the stirred, cooled (ice-water bath) solution. After 45 sec, an excess of Dowex 50 $\times$ 8 (pyridinium form) was added and the neutralized products were filtered. The filtrate was concentrated under reduced pressure and the residue was fractionated by short column chromatography on silica gel. The column was eluted with CHCl_3 -EtOH (97:3 - 85:15 v/v), and the appropriate fractions were combined and concentrated under reduced pressure. Crystallization of the residue from ethyl acetate-methanol gave 6-N-pivaloyl-2'-deoxyadenosine [Found: C, 53.6; H, 6.3; N, 20.85. $\text{C}_{15}\text{H}_{21}\text{N}_5\text{O}_4$ requires: C, 53.7; H, 6.3; N, 20.9%], m.p. 148°C; yield, 4.44g (83%); R_F 0.28 (system A); $\delta_{\text{H}}[(\text{CD}_3)_2\text{SO}, 250 \text{ MHz}]$ 1.28 (9H, s), 2.35 (1H, m), 2.78 (1H, m), 3.58 (2H, m), 3.90 (1H, m), 4.45 (1H, m), 5.03 (1H, t, J 5.5 Hz), 5.36 (1H, d, J 4.1 Hz), 6.47 (1H, t, J 6.7 Hz), 8.65 (1H, s), 8.69 (1H, s), 10.16 (1H, br.s).

6-O-(3-Chlorophenyl)-2-N-phenylacetyl-2'-deoxyguanosine

This compound was prepared by the same method and on the same scale [i.e. from 7.0g (17.0 mmol) of 3',5'-di-O-methoxyacetyl-2'-deoxyguanosine] as was previously described¹³ for the preparation of 6-O-(3,5-dichlorophenyl)-2-N-phenylacetyl-2'-deoxyguanosine except that 3-chlorophenol (6.55g, 50.9 mmol) was used instead of the equivalent quantity of 3,5-dichlorophenol. 6-O-(3-Chlorophenyl)-2-N-phenylacetyl-2'-deoxyguanosine was isolated as a colourless precipitated solid (3.70g, 44% overall yield over 4 steps) following short column chromatography of the products; R_F 0.40 (system A); $\delta_{\text{H}}[(\text{CD}_3)_2\text{SO}, 250 \text{ MHz}]$ 2.30 (1H, m), 2.74 (1H, m), 3.57 (2H, m), 3.72 (2H, s), 3.87 (1H, m), 4.44 (1H, m), 4.92 (1H, t, J 5.4 Hz), 5.34 (1H, d, J 4.1 Hz), 6.36 (1H, t, J 6.8 Hz), 7.2 - 7.6 (9H, m), 8.57 (1H, s), 10.62 (1H, s).

5'-O-(9-Phenylxanthen-9-yl)-6-N-pivaloyl-2'-deoxyadenosine (5; B = 1).

This compound was prepared from 6-N-pivaloyl-2'-deoxyadenosine (2.60g,

7.75 mmol) and 9-chloro-9-phenylxanthene (2.72g, 9.3 mmol) by the procedure previously described¹⁵ for the preparation of 4-*O*-phenyl-(9-phenylxanthen-9-yl)thymidine. After work-up and chromatography of the products, the *title compound* was isolated as a colourless precipitated solid (3.34g, 73%).

Crystallization of this material from ethyl acetate-petroleum ether (b.p. 40-60°C) gave colourless needles [Found: C, 68.9; H, 5.6; N, 11.9%.

C₃₄H₃₃N₅O₅ requires: C, 69.0; H, 5.6; N, 11.8%], m.p. 180-181°C; *R*_F 0.57 (system A); δ_H [(CD₃)₂SO, 250 MHz] 1.29 (9H, s), 2.34 (1H, m), 2.88 (1H, m), 3.04 (1H, dd, *J* 5.7, 10.0 Hz), 3.25 (1H, dd, *J* 4.5, 10.0 Hz), 4.00 (1H, m), 4.51 (1H, m), 5.41 (1H, d, *J* 4.2 Hz), 6.41 (1H, t, *J* 6.5 Hz), 6.88 (1H, m), 7.0 - 7.4 (12H, m), 8.47 (1H, s), 8.54 (1H, s), 10.15 (1H, br.s).

6-*O*-(3-Chlorophenyl)-2-*N*-phenylacetyl-5'-*O*-(9-phenylxanthen-9-yl)-2'-deoxyguanosine (5; R = 3)

This compound was prepared from 6-*O*-(3-chlorophenyl)-2-*N*-phenylacetyl-2'-deoxyguanosine (1.36g, 2.75 mmol) and 9-chloro-9-phenylxanthene (0.99g, 3.4 mmol) by the procedure previously described¹⁵ for the preparation of 4-*O*-phenyl-(9-phenylxanthen-9-yl)thymidine. After work-up and chromatography of the products, the *title compound* was isolated as a pure colourless precipitated solid (1.62g, 78%). Crystallization of this material from methanol gave fine colourless needles [Found: C, 67.4; H, 4.5; N, 9.2; Cl, 4.4.

C₄₃H₃₄ClN₅O₆ . CH₃OH requires: C, 67.4; H, 4.9; N, 8.9; Cl, 4.5%], m.p. 115°C; *R*_F 0.50 [CHCl₃-MeOH (19:1 v/v)]; δ_H [(CD₃)₂SO, 250 MHz] 2.34 (1H, m), 2.79 (1H, m), 3.18 (2H, m), 3.63 (1H, d, *J* 14.8 Hz), 3.74 (1H, d, *J* 14.8 Hz), 3.99 (1H, m), 4.54 (1H, m), 5.33 (1H, d, *J* 4.5 Hz), 6.33 (1H, t, *J* 6.4 Hz), 6.79 (1H, m), 7.0 - 7.6 (21H, m), 8.35 (1H, s), 10.51 (br.s).

Triethylammonium salt of 5'-*O*-(9-phenylxanthen-9-yl)-6-*N*-pivaloyl-2'-deoxyadenosine-3'-(2-chlorophenyl) phosphate (Px-*Ap*)

2-Chlorophenyl phosphorodichloridate (2.205g, 9.0 mmol), 1,2,4-triazole (1.26g, 18.2 mmol), triethylamine (2.76 ml, 19.8 mmol) and tetrahydrofuran (60 ml) were stirred together at room temperature. After 20 min, a solution of 5'-*O*-(9-phenylxanthen-9-yl)-6-*N*-pivaloyl-2'-deoxyadenosine (2.367g, 4.00 mmol) and 1-methylimidazole (0.96 ml, 12.0 mmol) in tetrahydrofuran (30 ml) was added and the resulting mixture was stirred for 20 min. Triethylamine (7.5 ml, 53.8 mmol) and enough water (ca. 4 ml) to give a clear solution were then added. The resulting solution was stirred for 10 min and concentrated (to ca. 4 ml) under reduced pressure. The concentrate was dissolved in chloroform (150 ml), and the solution was extracted with 0.1M - triethylammonium hydrogen carbonate buffer (pH 7.5, 2 × 300 ml). The dried (MgSO₄) organic

layer was concentrated under reduced pressure, redissolved in chloroform (25 ml) and toluene (25 ml) was added. The resulting solution was concentrated, the residue was dissolved in dichloromethane (25 ml) and the solution was evaporated under reduced pressure. This process was repeated and the resulting glass was dissolved in chloroform (ca. 10 ml). The solution obtained was added dropwise with stirring to petroleum ether (b.p. 30-40°C, 400 ml) to give the *title compound* as a colourless precipitate (3.24g, 92%); δ_p [(CD₃)₂SO, 36.4 MHz] -7.02.

The triethylammonium salts of the 3'-(2-chlorophenyl) phosphate esters of 4-*N*-benzoyl-5'-*O*-(9-phenylxanthen-9-yl)-2'-deoxycytidine [Px-Cp, δ_p -7.02], 6-*O*-(3-chlorophenyl)-2-*N*-phenylacetyl-5'-*O*-(9-phenylxanthen-9-yl)-2'-deoxyguanosine [Px-Gp, δ_p -7.02] and 4-*O*-phenyl-5'-*O*-(9-phenylxanthen-9-yl)thymidine [Px-Tp, δ_p -7.02] were prepared on the same scale and in yields of 90, 91, and 94%, respectively.

4-*O*-Phenylthymidine 3'-(2-chlorophenyl) (2,4-dinitrobenzyl) phosphate

[HO-Tp-Dnb (6; B = 4)]

1-Hydroxybenzotriazole (0.405g, 3.0 mmol) and triethylamine (0.42 ml, 3.0 mmol) were added to a stirred solution of 2-chlorophenyl phosphorodichloridate (0.365g, 1.49 mmol) in anhydrous tetrahydrofuran (10 ml), contained in a flask fitted with a rubber septum at 0°C (ice-bath). The ice-bath was removed and, after 20 min, a solution of 4-*O*-phenyl-5'-*O*-(9-phenylxanthen-9-yl)thymidine (0.576g, 1.0 mmol) in pyridine (5 ml) was introduced by means of a syringe. After 20 min, a solution of 2,4-dinitrobenzyl alcohol²⁰ (0.396g, 2.0 mmol) in pyridine (2 ml) was introduced and, after an additional period of 2 hr, half-saturated aqueous sodium hydrogen carbonate (1 ml) was added. After 10 min, chloroform (30 ml) was added and the resulting solution was extracted with saturated aqueous sodium hydrogen carbonate (80 ml). The aqueous layer was back-extracted with chloroform (2 × 100 ml), and the combined, dried (MgSO₄) organic layers were concentrated under reduced pressure to give a residual glass. Freshly distilled pyrrole (1.0 ml, 14.4 mmol) and a 2% solution of dichloroacetic acid in dichloromethane (20 ml) were added to a stirred solution of the latter material in dichloromethane (20 ml). After ca. 1 min, the products were extracted with saturated aqueous sodium hydrogen carbonate (50 ml) and the dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue obtained was fractionated by short column chromatography on silica gel: the appropriate fractions, eluted with chloroform-ethanol (98:2 v/v) were combined and evaporated under reduced pressure. When a solution of this product in chloroform (2 ml) was added dropwise to light

petroleum (b.p. 30-40°C, 150 ml), the title substance was obtained as a colourless solid precipitate (0.55g, 80%); R_F 0.42 (system A).

6-O-(3-Chlorophenyl)-2-N-phenylacetyl-2'-deoxyguanosine 3'-(2-chlorophenyl) (2,4-dinitrobenzyl) phosphate [HO-Gp-Dnb (6; B = 3); yield, 0.34g (78%)] was similarly prepared from 6-O-(3-chlorophenyl)-2-N-phenylacetyl-5'-O-(9-phenylxanthen-9-yl)-2'-deoxyguanosine (0.375g, 0.50 mmol); R_F 0.45 (system A).
Oligonucleotide synthesis by the "filtration" approach: synthetic cycle

(a) Synthesis of HO-(Tp)₂-Dnb, HO-(Tp)₃-Dnb, HO-(Tp)₄-Dnb, HO-(Tp)₅-Dnb and HO-(Tp)₆-Dnb. 4-O-Phenylthymidine 3'-(2-chlorophenyl) (2,4-dinitrobenzyl) phosphate [HO-Tp-Dnb; 0.551g, 0.80 mmol] and triethylammonium 4-O-phenyl-5'-O-(9-phenylxanthen-9-yl)thymidine 3'-(2-chlorophenyl) phosphate [Px-Tp; 0.762g, 0.88 mmol] were dissolved in anhydrous pyridine (30 ml) and the solution was evaporated to dryness under reduced pressure. The residue was redissolved in pyridine (7 ml), a small number (ca. 10-15) of freshly activated no. 4A molecular sieves, followed by 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole^{16,21} [MSNT, 0.781g, 2.64 mmol] were added to the stirred solution at room temperature. After 20 min, saturated aqueous sodium hydrogen carbonate (2.5 ml) was added dropwise to the products and, after a further period of 5 min, the products were partitioned between chloroform (50 ml) and 0.1M - triethylammonium hydrogen carbonate buffer (pH 7.5, 200 ml). The organic layer was separated and extracted with 2M - sodium phosphate buffer (pH 2.0, 200 ml). The dried (MgSO₄) organic layer was concentrated under reduced pressure. The residue was then redissolved in dichloromethane (20 ml), and the solution was re-evaporated.

The material obtained was dissolved in dichloromethane (16 ml), and freshly distilled pyrrole (0.83 ml, 12.0 mmol) followed by a 2% solution of dichloroacetic acid in dichloromethane (16 ml) were added. A fleeting yellow coloration was immediately observed. After ca. 1 min, the products were extracted with saturated aqueous sodium hydrogen carbonate (30 ml). The dried (MgSO₄) organic layer was evaporated under reduced pressure, and a solution of the residue in chloroform (2 x 2 ml) was applied to a short column (2 cm x 5 cm diameter) of Merck Kieselgel 60 [Art 9385]. The column was eluted first with chloroform (40 ml) and then with tetrahydrofuran-pyridine (3:1 v/v; 100-150 ml). The tetrahydrofuran-pyridine eluate was concentrated under reduced pressure to give the required partially-protected oligonucleotide which was either retained (in part or in full) or passed on to the next synthetic cycle. Some details relating to the coupling (condensation) steps in the

preparation of the five partially-protected oligothymidylic acids [i.e. HO-(Tp)_n-Dnb; n = 2-6] are given in Table 1.

(b) Synthesis of HO-CpCpTpApGpGp-Dnb. The first synthetic cycle commenced with the condensation reaction between 6-O-(3-chlorophenyl)-2-N-phenylacetyl-2'-deoxyguanosine 3'-(2-chlorophenyl) (2,4-dinitrobenzyl) phosphate [HO-Gp-Dnb, 0.216g, 0.25 mmol], triethylammonium 6-O-(3-chlorophenyl)-2-N-phenylacetyl-5'-O-(9-phenylxanthen-9-yl)-2'-deoxyguanosine 3'-(2-chlorophenyl) phosphate [Px-Gp, 0.286g, 0.275 mmol], and MSNT (0.267g, 0.90 mmol) in anhydrous pyridine (2 ml). The work-up and treatment with dichloroacetic acid-pyrrole were carried out as above, except that all quantities of reagents and volumes of solvent were scaled down by a factor of ca. one-third. The "filtration" chromatography was carried out on a short column (1.5 cm x 5 cm diameter) of Merck Kieselgel [Art 9385]. The conditions in cycles 2-5 were identical to those followed in the first synthetic cycle except that 0.30 mmol of the phosphodiester component [Px-Ap, Px-Cp or Px-Tp] was used. All the coupling (condensation) reactions were allowed to proceed for 20 min. The yield of crude HO-CpCpTpApGpGp-Dnb obtained was 0.28g (32% overall yield, based on HO-Gp-Dnb).

Preparation of fully-protected cyclic oligonucleotides

(a) [(Tp)₂]. Toluene-4-thiol (0.125g, 1.0 mmol) followed by triethylamine (0.07 ml, 0.5 mmol) were added to a stirred solution of HO-TpTp-Dnb (0.150g, 0.127 mmol) in pyridine (6 ml) at room temperature. A purple coloration, which developed as soon as the triethylamine was added, faded after ca. 1 min. Allyl chloride (0.20 ml, 2.5 mmol) was then added and, after 5 min, the products were concentrated under reduced pressure and the residue was partitioned between chloroform (25 ml) and water (30 ml). The dried (MgSO₄) organic layer was concentrated, and the residue was evaporated with toluene (10 ml), and then dissolved in chloroform (1 ml). The resulting solution was added dropwise with stirring to cyclohexane (100 ml). The colourless precipitate of HO-TpTp obtained was collected by centrifugation, washed with cyclohexane (100 ml) and light petroleum (b.p. 30-40°C; 100 ml) and dried; yield, 0.132g.

The latter material (0.10g, ca. 0.09 mmol) was dissolved in anhydrous pyridine (10 ml), and the solution was evaporated under reduced pressure. After this process had been repeated once more, the residue was dissolved in pyridine (10 ml), and a small number (10-15) of freshly activated no. 4A molecular sieves (10-15) were added to the stirred solution. MSNT (0.16g,

0.54 mmol) was then added and, after 12 hr, saturated aqueous sodium hydrogen carbonate (1 ml) was added to the products. After 5 min, the resulting solution was partitioned between chloroform (25 ml) and 0.1M - triethylammonium bicarbonate (pH 7.5, 75 ml). The aqueous layer was back-extracted with chloroform (20 ml), and the combined organic layers were extracted with 2M - sodium phosphate buffer (pH 2.0, 75 ml), then dried (MgSO_4) and evaporated under reduced pressure. The residue was fractionated on a very short column (0.5 cm $\times$ 5 cm diameter) of Merck Kieselgel 60 [Art 7729] which was eluted with chloroform-ethanol (100:0 - 90:10 v/v). The appropriate fractions were combined and evaporated under reduced pressure. When a solution of the residue in chloroform (1 ml) was added dropwise with stirring to light petroleum (b.p. 30-40°C, 75 ml), $\overline{[(Tp)_2]}$ was obtained as a colourless precipitated solid [0.0066g, 71% overall yield, based on crude HO-*TpTp*-Dnb]; R_f 0.40 (system A).

(b) $\overline{[(Tp)_3]}$, $\overline{[(Tp)_4]}$, $\overline{[(Tp)_5]}$, $\overline{[(Tp)_6]}$ and $\overline{[CpCpTpApGpGp]}$. The procedure followed in each case was closely similar to that described under section (a) above in the preparation of $\overline{[(Tp)_2]}$. The appropriate 3'-(2,4-dinitrobenzyl) esters (0.15g) were treated with toluene-4-thiol (0.125g, 1.0 mmol) and tri-ethylamine (0.07 ml, 0.5 mmol) in pyridine (2.5 - 6.0 ml) solution with an initial substrate concentration of 0.02M, and the dealkylated products were isolated in yields of 90-95%. The latter material (0.10g) was allowed to react with MSNT (0.16g, 0.54 mmol) in anhydrous pyridine (10 ml) solution for 12 hr in the cases of HO-(*Tp*)₃ and HO-(*Tp*)₄, and 14 hr in the cases of the other substrates. After work-up, the pure fully-protected cyclic oligonucleotides were isolated as above by short column chromatography. The isolated yields of $\overline{[(Tp)_3]}$, $\overline{[(Tp)_4]}$, $\overline{[(Tp)_5]}$, $\overline{[(Tp)_6]}$ and $\overline{[CpCpTpApGpGp]}$ were 0.067g (67%), 0.066g (67%), 0.061g (58%), 0.061g (59%) and 0.061g (59%), respectively. The percentage yields are based on the 3'-(2,4-dinitrobenzyl) esters as starting materials.

Removal of N-acyl protecting groups by ammonolysis

N-Acyl-2'-deoxyribonucleosides (0.003g) were dissolved in concentrated aqueous ammonia (d 0.88, 1 ml) at room temperature. After suitable intervals of time, the products were examined by t.l.c. in chloroform-methanol (85:15 v/v). The times required, respectively, for 50 and 100% ammonolysis are indicated in parenthesis: 6-*N*-pivaloyl-2'-deoxyadenosine (120, 1200 min), 4-*N*-benzoyl-2'-deoxycytidine (60, 600 min), 2-*N*-phenylacetyl-2'-deoxyguanosine (50, 500 min).

Unblocking of fully-protected cyclic oligonucleotides

E-2-Nitrobenzaloxime (0.134g, 0.80 mmol) and N^1, N^1, N^3, N^3 -tetramethylguanidine (0.09 ml, 0.72 mmol) were added to a stirred solution of the fully-protected cyclic oligonucleotide (0.05 - 0.06g) in acetonitrile (2.68 ml) at room temperature. After 2 hr, water (0.27 ml) was added and, after a further period of 24 hr, the products were concentrated under reduced pressure. In the case of $[CpCpTpApGpGp]$, the residue was dissolved in concentrated aqueous ammonia (d 0.88, 10 ml) and, after the solution had been allowed to stand at room temperature for 48 hr, it was concentrated under reduced pressure. The residue was dissolved in water (5 ml) and the pH was adjusted to ca. 6.0 by the addition of 0.1M - hydrochloric acid. The aqueous solution was extracted with chloroform (2 $\times$ 10 ml) and ether (2 $\times$ 10 ml), and was then concentrated (to ca. 1 ml) under reduced pressure. The concentrate was applied to a Sephadex G10 column (10 cm $\times$ 2 cm diameter) which was eluted with 0.1M - triethylammonium bicarbonate (pH 7.5, ca. 100-150 ml). The eluate was concentrated under reduced pressure and then applied to a column (9 cm $\times$ 2 cm diameter) of DEAE Sephadex A25. The column was eluted with a linear gradient [0.001 - 1.0M over 1000 ml] of pH 7.5 triethylammonium bicarbonate buffer. The appropriate fractions were combined, concentrated under reduced pressure, redissolved in water (2 $\times$ 10 ml), and reconcentrated. The residue was dissolved in water and the solution was passed through a column (5 cm $\times$ 1.5 cm diameter) of Dowex 50 $\times$ 8 (Na^+ form) cation-exchange resin which was eluted with water-ethanol (4:1 v/v). The eluate was then concentrated under reduced pressure. The quantity of fully-protected cyclic oligonucleotide unblocked, the number of A_{265} [A_{257} in the case of $d([CpCpTpApGpGp])$] units of completely unblocked product obtained and the average molarity of pH 7.5 triethylammonium bicarbonate buffer required to elute it from the DEAE Sephadex A25 column, respectively, were as indicated in parenthesis for each cyclic oligonucleotide: $d([Tp]_2)$ (0.050g, 520, 0.30M); $d([Tp]_3)$ (0.055g, 500, 0.35M); $d([Tp]_4)$ (0.060g, 628, 0.47M); $d([Tp]_5)$ (0.060g, 640, 0.54M); $d([Tp]_6)$ (0.060g, 650, 0.65M); $d([CpCpTpApGpGp])$ (0.055g, 550, 0.60M). Prior to the measurement of its n.m.r. spectrum, each unblocked cyclic oligonucleotide was dissolved in deuterium oxide (2 $\times$ 2 ml), and the solution was lyophilized.

Enzymatic digestion of cyclic oligothymidylates

(a) **Calf spleen phosphodiesterase.** A solution of substrate (ca. 2 A_{265} units) in water (10 μ l) was added to a solution of enzyme (ca. 10 μ g) in

0.1M - tris hydrochloride buffer (pH 7.0, 0.11 ml, 0.1M with respect to MgCl_2), and the reactants were maintained at 37°C. After 20 hr, $d(\overline{\text{C}(\text{Tp})_2})$, $d(\overline{\text{C}(\text{Tp})_3})$, $d(\overline{\text{C}(\text{Tp})_4})$, $d(\overline{\text{C}(\text{Tp})_5})$ and $d(\overline{\text{C}(\text{Tp})_6})$ were all completely unchanged.

(b) Nuclease P₁. A solution of substrate (1-2 A₂₆₅ units) in water (10 μl) was added to a solution of enzyme (ca. 10 μg) in 0.025M - tris hydrochloride buffer (pH 7.5, 0.11 ml), and the reactants were maintained at 37°C. After 10 hr, $d(\overline{\text{C}(\text{Tp})_2})$ was completely digested (as indicated by l.c.) to thymidine 5'-phosphate. The larger oligothymidylic acids underwent digestion more rapidly; thus $d(\overline{\text{C}(\text{Tp})_6})$ and $d(\overline{\text{C}(\text{Tp})_3})$ were completely digested in ca. 4 and 6 hr, respectively.

Enzymatic digestion of $d(\overline{\text{CpCpTpApGpGp})}$

A solution of substrate (ca. 2 A₂₅₇ units) in water (10 μl) was added to a solution of *Crotalus adamanteus* snake venom phosphodiesterase (ca. 10 μg) in 0.1M - tris hydrochloride buffer [pH 9.0 (0.11 ml), 0.1M with respect to MgCl_2], and the reactants were maintained at 37°C. After 10 hr, calf intestine alkaline phosphatase (ca. 3.5 units) was added and, after a further period of 3 hr, the hydrolysate was examined by l.c. and found to contain 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine and thymidine in the relative proportions of 1.0 : 2.0 : 1.86 : 1.0.

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