Tetrahedron Vol 45, No 14, pp 4523 to 4536, 1989
Printed in Great Britain

0040-4020/89 $3 00+.00

A POLYMER-NUCLEOTIDE LINKAGE USEFUL FOR THE SOLID PHASE SYNTHESIS OF CYCLIC OLIGODEOXYRIBONUCLEOTIDES

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(*Received in UK* 17 *April* 1989)

Abstract - 2'-Deoxycytidine-, 2'-deoxyadenosine- and 2'-deoxyguanosine-5'-O-(4,4'-dimethoxytriphenylmethyl)-3'-O-(2-chlorophenyl-2-cyanoethyl)phosphate have been anchored to a solid support (polydimethylacrylamide), through the exocyclic amino group. 2'-Deoxythymidine-5'-O-(4,4'-dimethoxytriphenylmethyl)-3'-O-(2-chlorophenyl-2-cyanoethyl)phosphate has been attached as well, by means of the N^3 and/or O^4 positions of the base moiety. The resulting polymer, which permits chain elongation from either end, can be conveniently used for the solid phase synthesis of cyclic oligodeoxyribonucleotides. The preparation of a number of such molecules containing up to 10 base residues by the phosphotriester approach is described.

The use of cyclic oligonucleotides as tools for conformational studies of DNA fragments had been recognized since 1969[1].

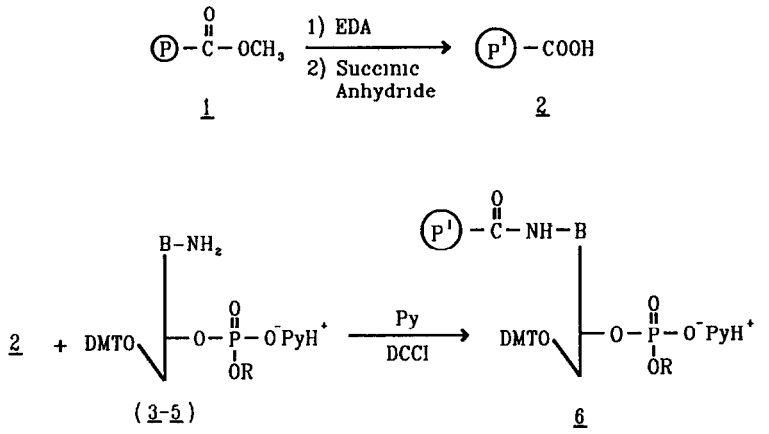
However only a limited number of papers describing the physico-chemical properties of such molecules has appeared in literature[2] from that time, probably due to the lack of a synthetic strategy for the preparation of covalent oligonucleotide circles, which were obtained for the most part as by-product from homopolymerization reactions[3]. The interest for cyclic oligonucleotides has further on increased by the discovery of the role that they may play in biological processes[4].

As a part of our studies on the chemical synthesis of oligonucleotides[5] we recently reported the first solid phase method for the synthesis of cyclic oligodeoxyribonucleotides[6], whose efficiency was checked by the synthesis of oligomers of deoxycytidine containing up to 7 base residues. The idea on the ground of this strategy was to anchor the first nucleotide

through the exocyclic amino group. After the chain assembly, which can be performed from either end, the cyclization of the linear oligonucleotide takes place in the presence of 1-(mesitylensulfonyl)-3-nitro-1,2,4-triazole (MSNT) after removal of the labile protecting groups at both the end. In this way any intermolecular coupling is prevented thus increasing the yields of cyclization which are much higher than those reported for syntheses in solution[7].

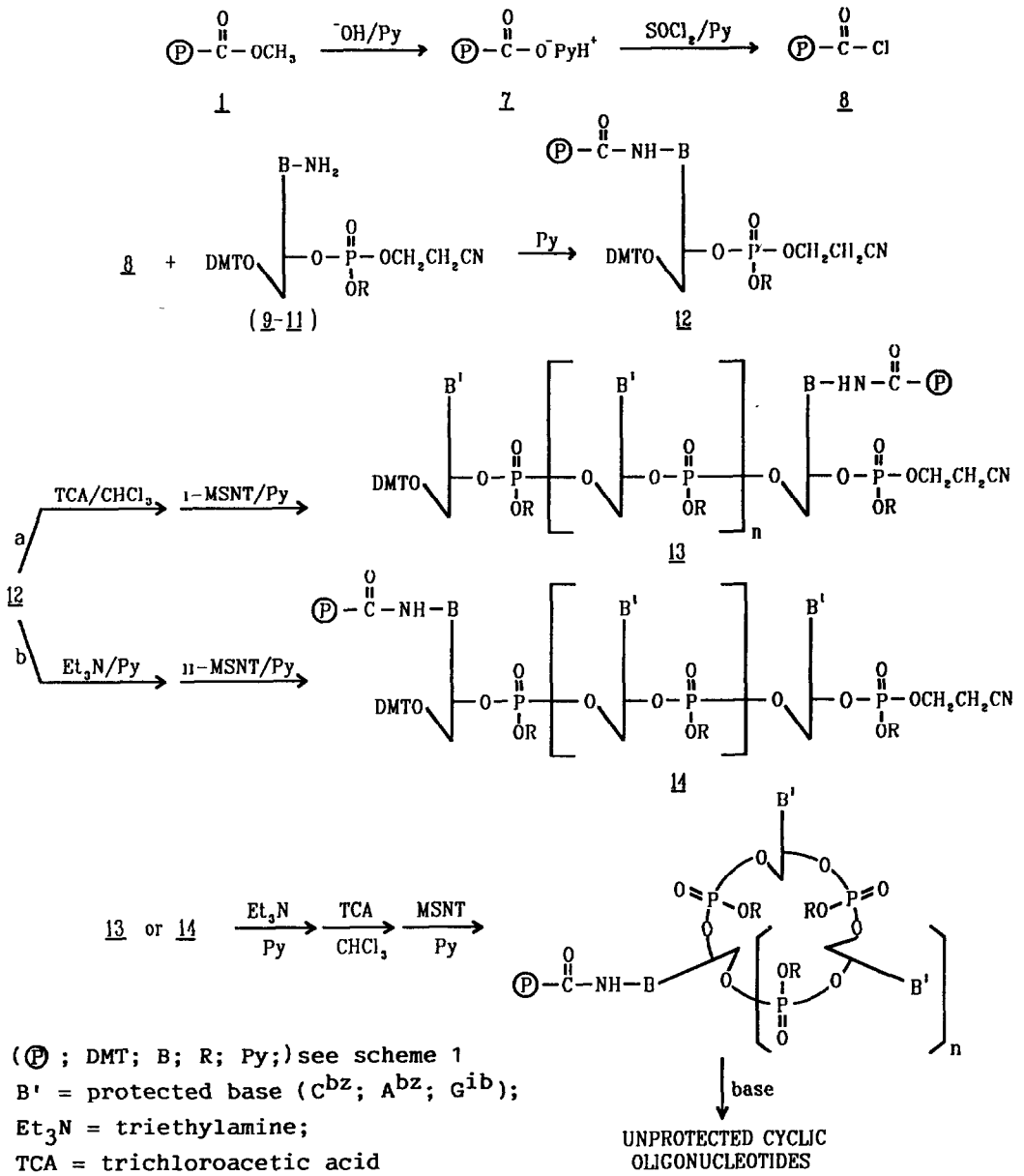
Since cyclic oligonucleotides are devoid of ends, polyacrylamide resin loaded with cytidine derivative 3 is of use for the synthesis of any sequence containing at least a residue of this base[6]. With the aim of further generalizing the method and, particularly, to render it suitable for the preparation of cyclic oligomers of other bases, we tried to attach adenosine and guanosine derivatives (4 and 5) following the described

SCHEME 1



(P)= Polyacrylamide resin; EDA = ethylenediamine;
(P') =(P)-CONH-(CH_2)$_2$-NH-CO(CH_2)$_2$- ; B = base residue;
DMT = 4,4'-dimethoxytriphenylmethyl; R = 2-chlorophenyl;
Py = pyridine; DCCI = N,N'-dicyclohexylcarbodiimide.

SCHEME 2



(Ⓟ ; DMT; B; R; Py;) see scheme 1

B' = protected base (C^{bz}; A^{bz}; G^{ib});

Et_3N = triethylamine;

TCA = trichloroacetic acid

MSNT = 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole

a: chain assembly in 3'-to 5'-end direction

b: " " in 5'-to 3'-end direction

i = 5'-DMT-3'-phosphodiester component

ii = 5'-hydroxy-3'phosphotriester component

procedure[6] (scheme 1). Thus the resin, polyacrylamido acryloylsarcosine methylester, (1, ca. 0.30 meq/g) was prefunctionalized with ethylenediamine and, successively, succinylated by reaction with succinic anhydride. The resulting polymer (2) was left in contact with a solution of 2'-deoxyadenosine- or 2'-deoxyguanosine-5'-O-(4,4'-dimethoxytriphenylmethyl)-3'-O-(2-chlorophenyl)phosphate (4 or 5) in the presence of N,N'-dicyclohexylcarbodiimide (DCCI). Notwithstanding several attempts to force the reaction, only small amount of nucleotidic material (0.005 - 0.010 meq/g) could be incorporated into the resin, due to the reduced nucleophilicity of the exocyclic amino groups of adenine and guanine in comparison with that of cytosine. In order to increase the yields of loading, we eliminated two possible side reactions both stimulated by the presence of the condensing agent DCCI : i) formation of the cyclic imide on the resin; ii) condensation of the carboxy group with the phosphodiester function of the nucleotide. Thus, we introduced a carboxylic function on the resin simply by hydrolising the methylester groups of the polymer. Moreover we masked the phosphodiester function of the nucleotide with the classical labile 2-cyanoethyl protecting group.

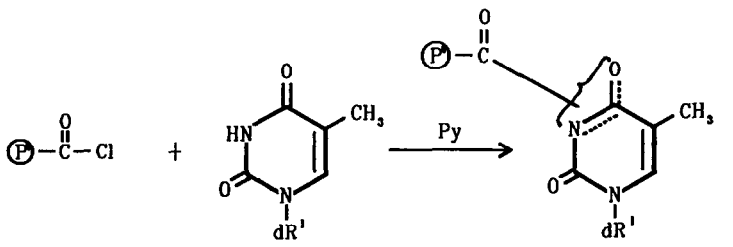
While we obtained higher functionalization with cytidine derivative (0.23 vs. 0.20 meq/g) thanks to these expedients, no sensible benefit was observed for the two purinic nucleotides.

The goal was reached by turning the carboxylic function on the resin obtained as above described, into the corresponding acyl chloride (scheme 2), by treatment with $SOCl_2$ in $CHCl_3$/pyridine. The resulting resin 8, after removal of the excess reagent and solvents *in vacuo* was allowed to react with a solution of 5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxyadenosine-3'-O-(2-chlorophenyl-2-cyanoethyl)phosphate (10) in pyridine for 15 h. After capping the possible unreacted functional groups by treatment with absolute ethanol in the presence of DCCI for 15 h, the resin (12) was exhaustively washed with anhydrous pyridine, $CHCl_3$, Et_2O and dried *in vacuo*. The functionalization was extimated by spectroscopic measurement of the 4,4'-dimethoxytriphenylmethyl cation released by acidic treatment of a weighted sample of the resin 12 and resulted to be 0.22 meq/g.

Functionalities constantly in the range of 0.22 - 0.24 meq/g were also obtained by reaction of the chlorinated resin 8 with analogous derivative of cytidine (9) and guanosine (11), whereas 5'-O-(4,4'-dimethoxytriphenyl-

methyl)-2'-deoxythymidine-3'-O-(2-chlorophenyl-2-cyanoethyl)phosphate (15, scheme 3) was incorporated in the polymer with satisfactory yields (0.04 - 0.05 meq/g) only at higher temperature.

SCHEME 3



8 15 16

(Ⓟ, Py) see scheme 1

dR' = 5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxyribosyl-3'-O-(2-chlorophenyl-2-cyanoethyl)phosphate.

The resulting polymers (12 or 16) were used for the syntheses of a number of a cyclic oligonucleotides containing up to 10 base residues.

The chain assemblies were carried out by the phosphotriester method based on the elongation of the chain in 3'- to 5'-end direction[8], for c(dpCpGpCpG) and c[dpAp(Ap)$_2$A] or 5'- to 3'-end[9] for c(dpCpGpCpG), c[dpGp(Gp)$_2$G], c(dpTpTpT) and c[dpCp(TpCp)$_4$T]. In both cases the overall yields for each cycle were constantly in the order of 96 - 98 % as judged by HPLC profiles.

When the chain had reached the desired length the fully masked oligonucleotide anchored to the polymeric support through the 5' or the 3' terminal nucleoside (depending on the route used for the chain elongation, scheme 2) was deprotected at either end by treatment with Et_3N/pyridine followed by reaction with trichloroacetic acid in $CHCl_3$. Then a solution of coupling agent (MSNT) was added and left in contact with the resin for 2 h

at 50 °C. Appropriate washing steps had to be included. After the complete deprotection of the oligomer and its detachment from the resin by reaction with N^1,N^1,N^2,N^2-tetramethylguanidinium 2-nitrobenzaldoximate and successive treatment with concentrated ammonia, the recovered nucleotidic material was analyzed and purified by HPLC using an anion exchange column (Partisil 10 SAX).

The cyclization yields measured by HPLC patterns, using as a reference a sample of the corresponding linear oligomer detached from a small sample of the resin before the cyclization step, were very close to 100 % (fig 1 a, b).

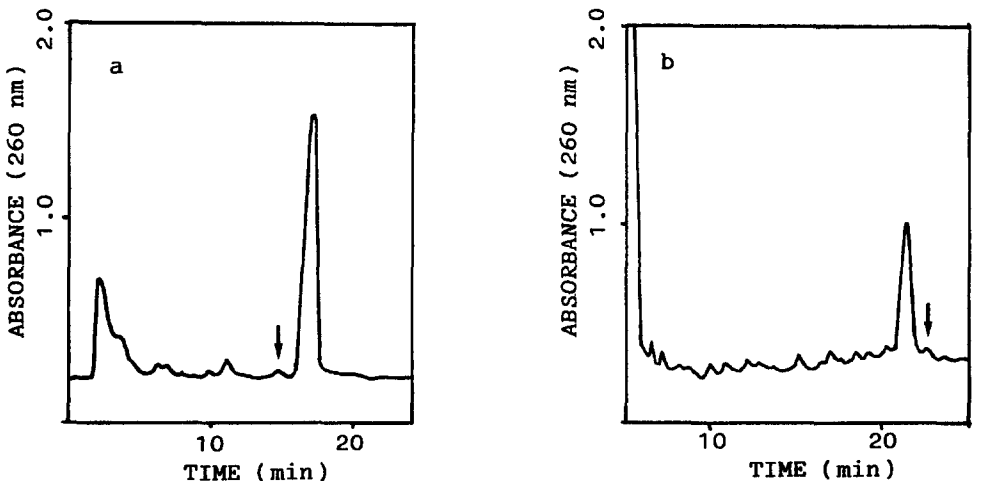


Figure 1. HPLC profiles of crude c[dpAp(Ap)$_2$A] (**a**) and c[dpCp(TpCp)$_4$T] (**b**) on a Partisil 10 SAX column. The arrows indicate the peaks of the residue linear oligomer.

The size of the cyclic oligomer was ensured by the one of the corresponding linear precursor which, in turn, was confirmed by checking the chain elongation step by step.

The cyclic nature of the products was ascertained by their ^{1}H NMR spectra. For c(dpTpTpT), c[dpAp(Ap)$_2$A] and c[dpGp(Gp)$_2$G] ^{1}H NMR spectra (see experimental) showed that all the nucleotides of each cycle are equivalent since only a signal for each type of nucleus was observed. On the other hand in the ^{1}H NMR spectra of the cyclic products c(dpCpGpCpG) and

c[dpCp(TpCp)$_4$T] (fig. 2) only two sets of signals were observed relative to the dimeric repetitive units.

In the case of the decamer, the cyclic nature of the molecule was further confirmed by enzymatic digestion with micrococcal and S1 endonucleases which afforded, as expected dCp and dTp or pdC and pdT, respectively as the sole products.

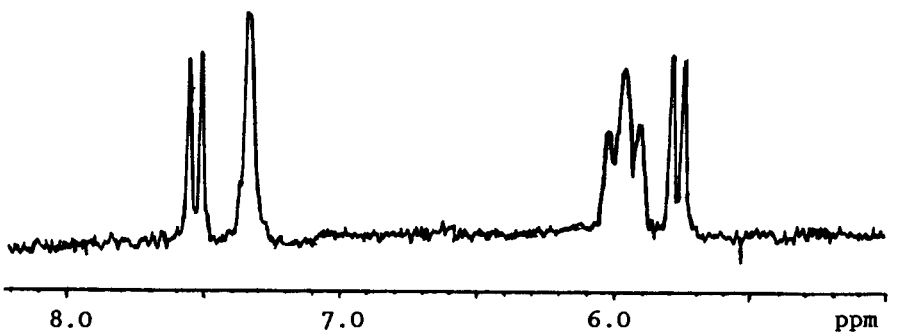


Figure 2. Low-field region of the ^{1}H NMR spectrum (250 MHz) of the decamer c[dpCp(TpCp)$_4$T] showing the resonances of the H-6 and H-5 of the cytosine, H-6 of the thymine and H-1' protons.

EXPERIMENTAL

UV spectra were recorded on a Perkin-Elmer 550S spectrophotometer. ^{1}H NMR spectra were measured on a Bruker WM-250 spectrometer. All chemical shifts were reported with respect to the residual solvent signal.HPLC were performed on a Varian 5000 instrument equipped with a Varichrom UV detector. All the operations on the resin relative to the oligonucleotide assembly were carried out in a short glass column equipped with a sintered-glass filter and a stopcock.

Syntheses of 5'-O-(4,4,-dimethoxytriphenylmethyl)-2-deoxynucleoside-3'-O--(2-chlorophenyl-2-cyanoethyl)phosphates (9-11).

0.5 mmol of the commercially available N-protected-5-O-(4,4'-dimetho-

xytriphenylmethyl)-2'-deoxynucleoside-3'-O-(2-chlorophenyl) phosphate triethylammonium salt, were treated with concentrated ammonia for 20 h at 50 °C under shaking. After removal of the solvent in vacuo, the residue, dissolved in CH_3OH/Et_3N (98:2), was purified by PLC (silica gel, 2 mm, eluent: $CHCl_3/CH_3OH$ (7:3) containing 0.5% pyridine.
The band (UV light) R_f 0.35 (3), 0.3 (4), 0.25 (5) eluted with $CHCl_3/CH_3OH$ (1 : 9) containing 0.5% pyridine afforded the pertinent N-unprotected nucleotide derivative.

5'-O-(4,4'-dimethoxytriphenylmethyl)-2'deoxycytidine-3'-O-(2-chlorophenyl) phosphate (3, 85% yield). ^{1}H NMR (CD_3OD) : δ 7.87 (1H, d, J = 7.4 Hz, H-6); 7.46 - 6.82 (17H, complex signal, aromatic protons); 6.33 (1H, dd, J = 6.7 and 6.7 Hz ,H-1'); 5.61 (1H, d, J = 7.4 Hz, H-5); 5.10 (1H, m, H-3'); 4.26 (1H, m, H-4'); 3.80 (6H, s, 2 OCH_3); 3.79 (2H, m, H_2-5', partially overlapped to OCH_3 signal); 2.71 (1H, m, H_a-2'); 2.38 (1H, m, H_b-2').

5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxyadenosine-3'-O-(2-chlorophenyl) phosphate (4, 80% yield). ^{1}H NMR (CD_3OD): δ 8.25 (1H, s, H-8); 8.17 (1H, s, H-2); 7.40 - 6.73 (17H, complex signal, aromatic protons); 6.48 (1H, dd, J = 6.6 and 6.6 Hz, H-1'); 5.23 (1H, m, H-3'); 4.46 (1H, m, H-4'); 3.76 (2H, m, H_2-5', partially overlapped to OCH_3 signal); 3.78 (6H, s, 2 OCH_3); 2.79 (1H, m, H_a-2'); 2.54 (1H, m, H_b-2').

5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxyguanosine-3'-O-(2-chlorophenyl) phosphate (5, 80% yield). ^{1}H NMR (CD_3OD) : δ 7.80 (1H, s, H-8); 7.39 - 6.69 (17H, complex signal, aromatic protons); 6.27 (1H, dd, J = 6.6 and 6.6 Hz, H-1'); 5.19 (1H, m, H-3'); 4.27 (1H, m, H-4'); 3.76 (6H, s, 2 OCH_3); 3.62 (2H, m, H_2-5'); 2.98 (1H, m, H_a-2'); 2.69 (1H, m, H_b-2').

At 0.4 mmol of the compound 3 (4, 5) coevaporated two times from pyridine/Et_3N (1:1), 4 mmol of 3-hydroxypropionitrile dissolved in 10 ml of pyridine were added and the mixture was evaporated two times from dry pyridine to give a final volume of 5 ml, then MSNT (1.6 mmol) was added. After 40 min at room temperature, excess water was added and the reaction mixture was concentrated to a gum. The residue, dissolved in $CHCl_3$ (15 ml),

washed with water (x 3) and taken to dryness under reduced pressure, was chromatographed on a silica gel column eluted with $CHCl_3$/pyridine (99.8 : 0.2) containing increasing amounts of CH_3OH, thus obtaining the desired product 9 (10, 11) as a mixture of diastereomers.

5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxycytidine-3'-O-(2-chlorophenyl-2-cyanoethyl)phosphate (9, 85% yield). ^1H NMR ($CDCl_3$): δ 7.83 (1H, d, J = 7.4 Hz, H-6); 7.47 -6.83 (17H, complex signal, aromatic protons); 6.29 (1H, dd, J = 6.0 and 6.0 Hz, H-1'); 5.71 (1H, d, J = 7.4 Hz, H-5); 5.27 (1H, m, H-3'); 4.40 and 4.41 (2H, t's, J = 5.7 Hz , OCH_2CH_2CN); 4.29 (1H, m, H-4'); 3.80 (6H, s, $2OCH_3$); 3.46 (2H, m, H_2-5'); 2.91 and 2.90 (2H, t's, J = 5.7 Hz, OCH_2CH_2CN); 2.80 (1H, m, H_a-2'); 2.47 (1H, m, H_b-2').

5'-O-(4,4'-dimethoxytriphenylmetyl)-2'-deoxyadenosine-3'-O-(2-chlorophenyl-2-cyanoethyl)phosphate (10, 80% yield). ^1H NMR ($CDCl_3$): δ 8.26 and 8.22 (1H, singlets, H-8); 8.13 and 8.11 (1H, singlets, H-2); 7.43 - 6.73 (17H, complex signal, aromatic protons); 6.49 (1H, m, H-1'); 5.56 (1H, m,H-3'); 4.41 and 4.40 (2H, t's, J = 5.8 Hz, OCH_2CH_2CN); 4.37 (1H, m, H-4'); 3.78 (6H, s, $2OCH_3$); 3.65 (2H, m, H_2-5'); 2.97 - 2.72 (4H, overlapped signals, OCH_2CH_2CN and H_2-2').

5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxyguanosine-3'-O-(2-chlorophenyl-2-cyanoethyl)phosphate (11, 83% yield). ^1H NMR ($CDCl_3$): δ 7.60 (1H, s, H-8); 7.40 - 6.70 (17H, complex signal, aromatic protons); 6.19 (1H, m, H-1'); 5.42 (1H, m, H-3'); 4.40 - 4.20 (3H, overlapped signals, OCH_2CH_2CN and H-4'); 3.71 (6H, s, 2 OCH_3); 3.65 (2H, m, H_2-5', partially overlapped to OCH_3 signals); 2.81 - 2.59 (4H, overlapped signals, OCH_2CH_2CN and H_2-2').

Preparation of the carboxylic resin 7.

Commercially available polyacrylamido acryloylsarcosine methylester (1, 0.30 meq/g,200 mg), swollen in pyridine, was treated with a solution of 0.1M aqueous NaOH/pyridine (7:3, 30 ml) for 30 min at room temperature under shaking. After filtration the resin was washed with H_2O and then treated with a solution of H_2O/acetic acid (8:2, 5 x 1 min). The resulting polymer

was exhaustively washed with H_2O, pyridine (10 x 2 min), anhydrous $CHCl_3$ (5 x 2 min), dry Et_2O (5 x 2 min) and dried in vacuo.

Chlorination of resin 7 to obtain 8.

100 mg of 7 swollen in a solution of anhydrous chloroform/anhydrous pyridine (9:1, 5 ml) were treated with 0.2 ml of freshly distilled $SOCl_2$ and kept under shaking at room temperature for 2 h and then under reflux for 1 h. After cooling the mixture was taken to dryness and stored in vacuo over P_2O_5.

General procedure for the preparation of the solid support 12.

The freshly prepared resin 8 (100 mg) was treated with a solution of nucleoside derivatives 9 (10 or 11) (0.15 mmol) in dry pyridine (2 ml) with stirring at room temperature for 15 h. The resulting polymer (12), exhaustively washed with dry pyridine, was treated with anhydrous EtOH (0.8 ml) in the presence of DCCI (250 mg) in dry pyridine (3 ml) under shaking at room temperature for 15 h. After washing with dry pyridine (10 x 2 min), dry $CHCl_3$ (5 x 2 min) and dry Et_2O (5 x 2 min), the polymer was dried in vacuo.

The amount of the nucleotide derivative attached to the resin, extimated by spectroscopic measurement (500 nm, ε = 71700) of 4,4'-dimethoxytriphenylmethyl cation released by acidic treatment (70% $HClO_4$/EtOH, 3:2 v/v) on a weighted sample (3-5 mg) of the resin, resulted to be constantly in the range 0.20-0.24 meq/g.

The freshly prepared resin 8 (100 mg) was treated with a solution of 5'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxythymidine-3'-O-(2-chlorophenyl-2-cyanoethyl)phosphate (15, 0.2 mmol) in dry pyridine (2 ml) under shaking at 50°C for 15 h. Following the work up described above for the preparation of 12, we obtained the resin 16 containing 0.04-0.05 meq/g of thymidine derivative.

General procedure for chain assembly and cyclization.

Elongation in the 3'- to 5'- end direction:

The resin 12 (16) (100 mg) washed with dry $CHCl_3$ (10 x 0.5 min) was treated with a solution of 10% trichloroacetic acid in $CHCl_3$ (3 x 2 min). After washing steps with DMF (1 x 1 min), $CHCl_3$ (5 x 1 min), DMF (5 x 0.5 min), pyridine (10 x 0.5 min) the resin was left in contact with a solution of the pertinent N-protected-5'O-(4,4'-dimethoxytriphenylmethyl)-2'- deoxynucleoside-3'-O-(2-chlorophenyl)phosphate triethylammonium salt (0.2 mmol) and MSNT (0.5 mmol) in dry pyridine (2.5 ml) for 20 min at 50°C. After washing with dry pyridine (10 x 0.5 min) the resin was treated with a solution of pyridine /Ac_2O (3:2, 4 ml), and after shaking for 40 min the resin was washed with pyridine.

Cycles were continued until the required lenght was reached.

Elongation in the 5'- to 3'- end direction:

100 mg of the resin 12 (16) were swollen by addition of dry pyridine and then treated with Et_3N /pyridine (1:1) at 50 °C for 30 min . After washing with pyridine (10 x 0.5 min) the resin was left in contact with a solution of N-protected-2'-deoxynucleoside-3'-O-(2-chlorophenyl-2-cyanoethyl)phosphate (0.18 mmol) and MSNT (0.35 mmol) in dry pyridine (2.5 ml) for 20 min at 50°. After washing with dry pyridine (10 x 0.5 min) the second cycle was begun. Cycles were continued until the required lenght was reached.

Cyclization for 100 mg of initial resin 12 (16):

The resin, containing presynthesized oligomer was deprotected at the phosphate 3'-end with pyridine/Et_3N as described above for the elongation in 5'- to 3'- end direction; after appropriate washing steps the 4,4'-dimethoxytriphenylmethyl group was removed by treatment with 10% TCA in $CHCl_3$ as reported above for the elongation in the 3'- to 5'- end direction. After washing steps a solution of MSNT (0.5 mmol) in dry pyridine (2.5 ml) was added and the mixture kept at room temperature for 1 h and succesively for 30 min at 50 °C. After cooling, the resin was exhaustively washed with pyridine.

Cleavage from the solid support, deprotection and purification of cyclic oligodeoxyribonucleotides.

The final resin was treated with a solution of 0.5 M N^1,N^1,N^2,N^2-tetramethylguanidinium 2-nitrobenzaldoxymate in dioxane/water (1:1 v/v, 3 ml) for 20 h at 30 °C. The mixture was taken to dryness and the residue was treated with 8 ml of concentrated ammonia for 6 h at 50°C. The supernatant was filtered and the resin was washed with EtOH-water (1:1, 3 x 3 ml). The combined filtrate and washings were taken to dryness, dissolved in water (6 ml), washed with Et_2O (x 3) and $CHCl_3$ (x 3) and concentrated at 2 ml. Cyclic oligomers c(dpTpTpT), c[dpAp(Ap)$_2$A], c(dpCpGpCpG) and c[dpCp(TpCp)$_4$T] were purified by HPLC on an analitical Partisil 10 SAX column (flow rate 1 ml/min) eluted with a linear gradient from 10 to 60% in 30 min of KH_2PO_4, KCl, 5% EtOH, pH 6.8 from 1 mM to 0.25 M at room temperature. For cyclic products c[dpGp(Gp)$_2$G] and c[dpCp(TpCp)$_4$T] a gradient from 10 to 100% and from 30 to 100% in 30 min respectively was used.

The product peaks were desalted by HPLC on a semipreparative Merck Lichrosorb RP-18 column (250 x 10 mm i.d., 7μm, flow rate 1.5 ml/min) eluted with a linear gradient from 0 to 90% in 45 min of CH_3CN in 0.1 M triethylammonium acetate, pH 7.0. The final products, taken to dryness, were dissolved in water and liophylized.

c[dpCp(TpCp)$_4$T], ^{1}H NMR (D_2O): δ 5.94 (10 H, m, H-1'); 4.03 (10 H, m, H-4'); 3.82 (20 H, m, H_2-5'); 2.21 and 2.02 (10 H each, m's, H_2-2'). H-3' signals were submerged by the residual solvent peak (δ 4.70). Cytosine signals: δ 7.48 (5H, d, J = 7.5 Hz, H-6); 5.75 (5H, d, J = 7.5Hz, H-5); thymine signals: 7.31 (5H, bs, H-6); 1.61 (15H, bs, Me-5).

c[dpAp(Ap)$_2$A], ^{1}H NMR (D_2O): δ 8.09 (4H, s, H-8); 7.93 (4H, s, H-2); 6.25 (4H, dd, J = 7.3 and 7.3 Hz, H-1'); 4.87 (4H, m, H-3'); 4.31 (4H, m, H-4'); 4.08 (8H, m, H_2-5'); 2.60 (8H, m, H_2-2').

c[dpGp(Gp)$_2$G], ^{1}H NMR (D_2O): δ 7.67 (4H, s, H-8); 6.02 (4H, dd, J = 6.0 and 6.0 Hz, H-1'); 4.16 (4H, m, H-4'); 3.97 (8H, m, H_2-5'); 2.67 (4H, m, H_a-2'); 2.42 (4H, m, H_b-2'). H-3' signal was submerged by the residual solvent peak (δ 4.70).

c(dpCpGpCpG), ^{1}H NMR (D_2O): δ 5.98 (4H, m, H-1'); 4.16 and 4.04 (2H each, m's, H-4'); 3.90 and 3.74 (4H, m's, H_2-5'); 2.63, 2.36 and 2.00 (8H, m's,

H_2-2'); H-3' signal were submerged by the residual solvent peak (δ 4.70). Cytosine signals:δ7.52 (2H, d, J = 7.5 Hz, H-6); 5.80 (2H, d, J = 7.5 Hz, H-5); guanine signals:δ7.69 (2H, s, H-8).

c(dpTpTpT), ^{1} NMR (D_2O) : δ 7.63 (3H, bs, H-6); 6.27 (3H, dd, J = 7.1 and 7.1 Hz, H-1'); 4.28 (3H, m, H-4'); 4.00 (3H, m, H_2-5'); 2.51 (3H, m, H_a-2'); 2.29 (3H, m, H_b-2'); 1.81 (9H, bs, CH_3-C-5); H-3' signal was submerged by the residual solvent peak (δ 4.70).

Enzymatic digestion with S1 endonuclease.

The purified c[dpCp(TpCp)$_4$T] (1.8 A_{260} units) was dissolved in 200 μl of a solution 280 mM, NaCl, 50 mM NaOAc (pH 4.6), 4.5 mM $ZnSO_4$ containing 20 μg/ml of DNA carrier and 200 units of S1 nuclease were added. The mixture was incubated at 37 °C for 30 min and heated at 60 °C for 5 min. The analysis of the mixture by HPLC (Partisil 10 SAX column , 250 x 4.6 mm i.d., 10 μm, flow rate 1.0 ml/min; eluted with a linear gradient from 0 to 60% in 60 min of KH_2PO_4, KCl, 5% EtOH, pH 6.8 from 1 mM to 0.25 M at room temperature) showed that the product was completely digested to give pdC and pdT in the ratio 1:1 on the basis of the peak areas and extinction coefficients at 260 nm of the nucleotides.

Enzymatic deigestion with micrococcal endonuclease

The purified c[dpCp(TpCp)$_4$T] (2.0 A_{260} units) was dissolved in 200 μl of a solution 10 mM of $CaCl_2$,8 mM urea (pH 7.5) and 200 units of micrococcal nuclease were added. The mixture was incubated at 37 °C for 40 min. The analysis of the mixture by HPLC (see above) showed that the product was completely digested to give dCp and dTp in the ratio 1:1.

Acknowledgment. This work was supported by "Ministero della Pubblica Istruzione " (Italy) and by C.N.R. (Progetto Finalizzato "Chimica Fine 2").

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