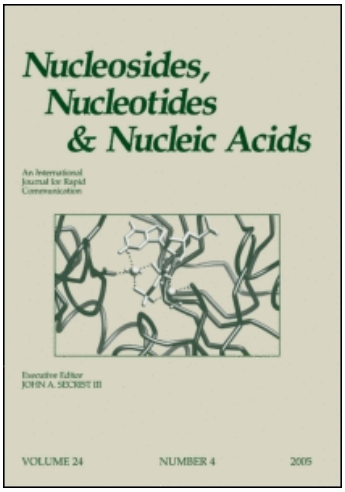

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PEG-Supported Synthesis of Cyclic Oligodeoxyribonucleotides

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PEG-SUPPORTED SYNTHESIS OF CYCLIC OLIGODEOXYRIBONUCLEOTIDES

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ABSTRACT. Cyclic oligodeoxyribonucleotides have been synthesized in satisfactory yields by an easy procedure using polyethylene glycol (PEG) as soluble supporting polymer. The present method is particularly suitable for a medium to large scale synthesis.

Early studies involving biological properties of synthetic[1] and natural[2] cyclic oligonucleotides stimulated a great deal of interest in such kind of molecules.

The recent findings[3] that circular single-stranded oligodeoxyribonucleotides having two runs of pyrimidines can bind tightly and sequence selectively to single-stranded polypurine DNA and RNA by forming triple helical complexes open up new perspectives to the design of more efficient DNA- and RNA-binding molecules.

Our attention is being focused on the synthesis of medium--sized rings, since none of the synthetic procedures described so far proved to be really suitable for the preparation of such molecules on a relatively large scale. Following "classical" solution methods, which in principle should easily allow syntheses

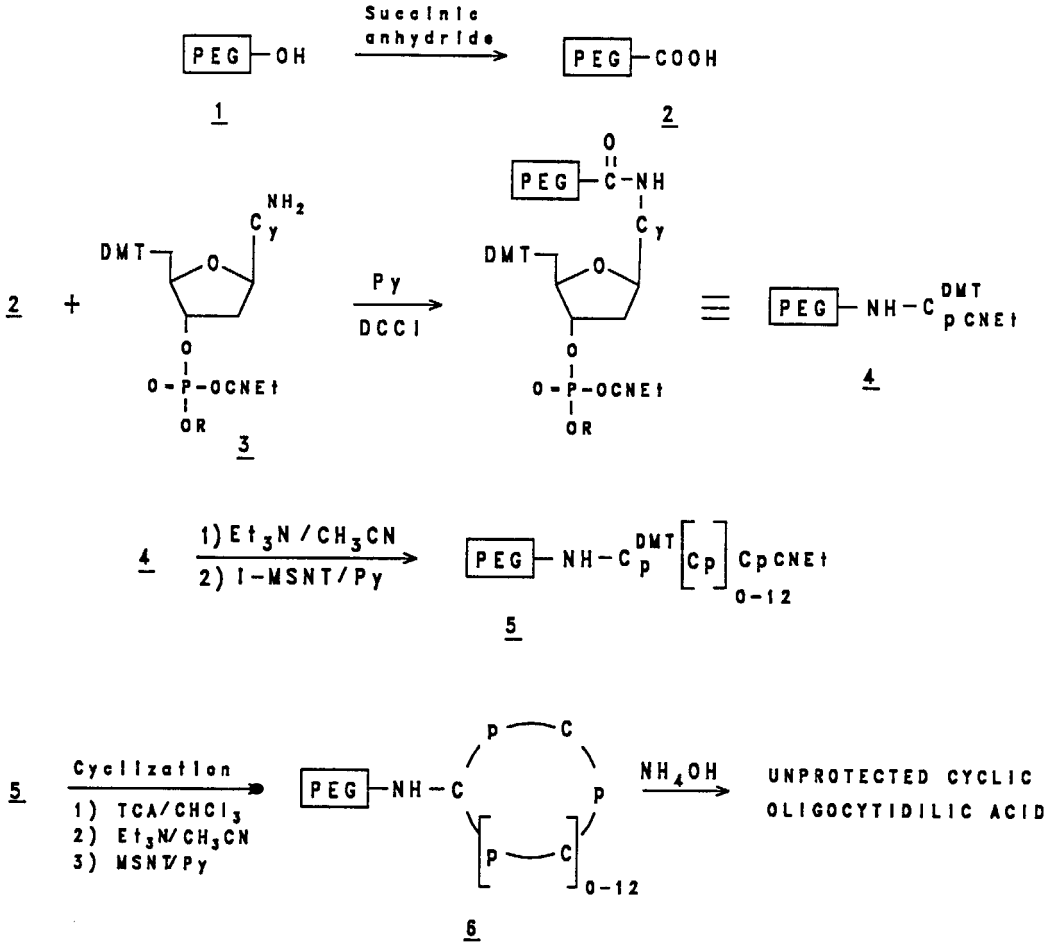
on a preparative scale, oligomers containing from 2 to 8 residues were synthesized[4], with cyclization yields in the range of 80-50 %. On the other hand, for the solid phase synthesis of cyclic oligonucleotides, excellent cyclization yields (very close to 100 %) up to dodecamers were reported[5], using a polyacrylamide support; but the same cyclization yields for higher rings could be obtained only by reducing the level of functionalization of the polyacrylamide support, thus severely limiting the final amounts of nucleotidic material recovered after detachment from the resin.

With the aim of combining some of the advantages of the solution and solid phase methods, we tested the use of polyethylene glycol (PEG) as soluble polymeric support for the synthesis of cyclic oligodeoxyribonucleotides.

The PEG procedure[6] offers some advantages over the "classical" solution methods. While highly soluble in most organic solvents, PEG can be precipitated in microcrystalline forms (by addition of diethylether) which allows the excess of reagent and by-products to be eliminated by simple washings. Moreover purification can be performed by crystallization or diafiltration. Also when compared with the solid phase approach, the so called "liquid phase" method seems to be advantageous: **i**) since both the PEG--oligonucleotide and the coupling monomer are in solution, a lower excess of reagents and coupling agents is required; **ii**) each step of the synthesis can be easily monitored; **iii**) scaling up of the reaction can be more easily performed.

Analogously to the reported solid phase method[5], the first nucleotide was anchored to the PEG through the exocyclic amino group of the cytidine derivative (**3**). Thus PEG (monomethylether, 5000 M.W., hydroxyl number 0.20 meq/g, **1**) was prefunctionalized with succinic anhydride and then reacted with a solution of 5'-O--(4,4'-dimethoxytriphenylmethyl)-2'-deoxycytidine-3'-O-(2-chlorophenyl, 2-cyanoethyl)phosphate (**3**) as a diastereoisomeric mixture in pyridine in the presence of dicyclohexylcarbodiimide (DCCI). Loading of nucleotide was 0.18 meq/g, as estimated by spectroscopic measurements of the 4,4'-dimethoxytriphenylmethyl cation released by acidic treatment of a weighed sample of the support. Using this support, linear homooligomers of 2'-deoxycytidylic acid containing up to 14 residues were synthesized and their cyclization yields calculated.

SCHEME



DMT = 4,4'-dimethoxytriphenylmethyl

$C_y^{NH_2}$ = cytosinyl

CNEt = 2-cyanoethyl

R = 2-chlorophenyl

Py = pyridine

P = 2-chlorophenylphosphoryl in 4, 5 and 6

I = 5'-hydroxy-3'-phosphotriester component

Chain assembly (scheme) was carried out by the phosphotriester method[5] based on the elongation of the chain in the 5'- to 3'-end direction, by addition of monomeric units and/or previously synthesized dimeric building blocks. In both cases the overall yields for each elongation step were constantly in the range 92--96%, as judged by HPLC analyses, performed step by step[7]. The growth of the chain in the 5'-to 3'-end direction was very profitable. In fact only one purification process per elongation step was required (instead of two, as needed for elongation in the opposite direction), while obtaining comparable coupling yields[8]. After each coupling reaction, the PEG-oligonucleotide was purified from excess of reagents and the coupling agent 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT) using a gel filtration column (Fractogel resin, Merck, 100-3000 exclusion limit). This purification was preferred over the previously reported precipitation method[6], since it did not involve losses of PEG--oligomer material in each step. Moreover, the gel filtration procedure allowed recovering, with a high degree of purity, the soluble PEG-oligomer, and, in the same step, the excess addition units.

When the chain had reached the desired length, the fully protected oligonucleotide, anchored to the polymeric support, underwent the usual cyclization procedure, involving deprotection at both ends by treatment first with triethylamine/CH_3CN (1:1), followed by treatment with trichloroacetic acid (TCA) in $CHCl_3$, and successive addition of the condensing agent MSNT, using a concentration of PEG-oligonucleotide of $5x10^{-3}$ M in pyridine. The above concentration value was chosen in order to compare directly our data (see Table 1) to the best ones so far reported in the literature for "classical" solution methods. The cyclization times reported in Table 1 assured the complete disappearance of the linear compounds (by HPLC analysis). The HPLC profiles of crude linear [$l(C)_{14}$] and cyclic [$c(C_{14})$] tetradecacytidylic acids are reported in Figure. The size and the cyclic nature of the final products were unambiguously ascertained by HPLC comparison with authentic samples synthesized according to the previously reported procedure[5] and by ^1H NMR spectra[9] (Table 2) of the isolated products.

Cyclization yields were calculated by quantitative HPLC analyses of the detached material from a weighed amount of the PEG-

Table 1 : Cyclization reaction yields

Oligomers (size)	Cyclization time (h)	Cyclization Yields (%)**
2 mer	2	98
4 "	5	94
6 "	5	72
8 "	15	58
8* "	15	63
10 "	15	40
12 "	15	35
14 "	15	33

* In this case a conc. $5x10^{-4}$ M was used.
** Average values.

-oligomer and are intended as the percentage of linear precursor which was converted into the cyclic product. The obtained data, compared to those referring to solution synthesis, showed higher cyclization yields for dimers and tetramers[10] and only slight improvements for hexamers and octamers. As for oligomers within the range 10-14 residues, the trend to decreasing cyclization yields as the size of cycles increased, was far less pronounced. In this case a direct comparison with solution cyclization data could not be made in the absence of reported experiments.

In all the studied cases, there was a straight evidence that PEG inhibited to some extent intermolecular couplings, even if the effect of the soluble polymeric support on cyclization yields was more marked in the case of small oligomers, as would be expected. In our opinion this method can be considered as a valuable synthetic strategy for an efficient medium to large scale synthesis of linear and cyclic oligodeoxyribonucleotides and, at present, as the only easy and feasible procedure for the obtainment of large rings in high quantities. In addition, in the PEG--procedure, gel filtration could be introduced as a purification method, which is extremely easier and more resolving than silica gel chromatography, usually used in the "classical" solution method.

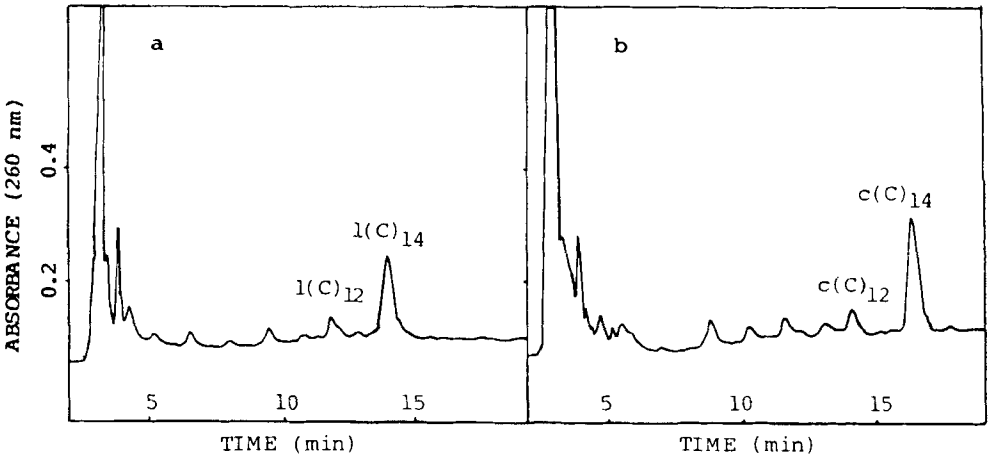


Figure. HPLC profiles of crude LINEAR (a) and CYCLIC (b) tetradecacytidylic acid synthesized using dimeric building blocks. Partisil 10 SAX column (25 cm, 4.6 mm I.D.).

EXPERIMENTAL

General procedure

UV measurements were performed on a Perkin-Elmer Lambda 7 spectrophometer. ^1H NMR spectra were recorded on a Bruker WM-400 spectrometer. All chemical shifts are expressed in p.p.m. from tetramethylsilane. HPLC was carried out on a Beckman System Gold instrument equipped with a UV detector module 166 and a Shimadzu Chromatopac C-R6A integrator. Polyethylene glycol monomethylether (MW 5000) was purchased from Aldrich. Fractogel TSK-HW40 (C) was purchased from Merck.

Functionalization of PEG

2 g of PEG (**1**, 0.20 meq/g) coevaporated three times with pyridine, were left in contact with a solution of succinic anhydride (200 mg, 2.0 mmol) and 4-dimethylaminopyridine (20 mg, 0.164 mmol) in dry pyridine (16 ml) at room temperature. After 48 h the mixture was evaporated to dryness *in vacuo*, dissolved in CH_2Cl_2 and then precipitated by addition of Et_2O at 0°C under vigorous stirring. The solid was washed exhaustively with Et_2O/CH_2Cl_2 (10:1) and dried *in vacuo*. 500 mg of the resulting PEG-succinyl derivative (**2**), coevaporated three times with dry pyridine, were treated with a solution of the cytidine derivative **3** (300 mg, 0.388 mmol) synthesized according to the previously reported procedure[5], in dry pyridine (5 ml) in the presence of DCCI (500 mg, 2.45 mmol). After

Table 2 : ^{1}H NMR (400 MHz) spectra of potassium salts of cyclic oligodeoxycytidylic acids in D_2O solution

Oligomer	H-6 ($J_{5,6}$ Hz)	H-1'($J_{1',2'}$ Hz)	H-5 ($J_{5,6}$ Hz)	H-3'	H-4'	H-5'	H-2'
8 mer	7.78 (7.2)	6.23 (6.9, 6.8)	6.06 (7.2)	4.86	4.30	4.10	2.52, 2.23
10 "	7.77 (7.5)	6.20 (6.8, 6.8)	6.02 (7.5)	4.85	4.29	4.10	2.50, 2.24
12 "	7.78 (7.2)	6.20 (7.3, 7.2)	6.02 (7.2)	4.84	4.28	4.10	2.51, 2.26
14 "	7.78 (7.3)	6.20 (7.2, 7.2)	6.00 (7.3)	4.80	4.27	4.10	2.53, 2.27

48 h at room temperature, 2 ml of water were added and the mixture concentrated to a gum. The residue, dissolved in CH_3OH, was chromatographed on a Fractogel column (150 ml of wet support, 1.5 cm i.d., flow 8 ml/h) eluted with CH_3OH. The column, monitored by TLC (silica gel, 0.25 mm, ethyl acetate/acetone/water, 5:10:1), furnished as totally excluded material the pure PEG-cytidine derivative **4**. The successive fractions allowed the pure unreacted nucleotidic material to be recovered.

The amount of the nucleotide derivative attached to PEG, estimated 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 weighed sample (5-8 mg) of the PEG--nucleotide, was 0.18 meq/g.

4, ^{1}H NMR ($CDCl_3$) significative protons at δ: 8.04 and 8.02 (1H, d's, J=7.5 Hz, H-6); 7.45-7.15 (13H, complex signals, aromatic protons); 7.13 and 7.12 (d's, J=7.5 Hz, H-5); 6.82 (4H, d, J=6.0 Hz, ortho-H to CH_3O- group in DMT); 6.30 and 6.27 (1H, dd's H-1'); 5.30 (1H,m, H-3'); 4.46-4.19 (3H, overlapped signals, OCH_2CH_2CN and H-4'); 3.80 (6H, s, $2OCH_3$); 3.52 (2H, m, H_2-5'); 2.92 (1H,m, H_a-2'); 2.78-2.60 (overlapped signals, OCH_2CH_2CN and H_b--2'); 2.72 (4H, s, succinyl protons).

General procedure for chain assembly

The functionalized PEG **4** (500 mg, 0.09 meq) was treated with 2 ml of a solution of Et_3N/CH_3CN (1:1, v/v) at 50°C. After 1 h the mixture was dried *in vacuo* and then coevaporated four times with dry pyridine. The resulting residue was reacted with a solution of N-benzoyl-5'-hydroxy-3'-O-(2-chlorophenyl, 2-cyanoethyl)phosphate component (**i**, monomer or dimer, 0.36 mmol) and MSNT (0.72 mmol) in dry pyridine (2 ml). After 40 min at 50°C and 1 h at room

temperature, the reaction was quenched by addition of water. The mixture, concentrated to an oil, was dissolved in CH_3OH and chromatographed on a Fractogel column. In a typical purification procedure on Fractogel column (150 ml of wet support, 1.5 cm i.d., flow 8 ml/h) the order of elution is: PEG-oligomer (30-80 ml), condensing agent derivative (100-110 ml) and nucleotide component (160-200 ml). Collecting and drying in vacuo the fractions at Rf=0 (TLC analysis, silica gel 0.25 mm, ethyl acetate/acetone/water, 5:10:1), a quantitative recovery of the pure PEG-oligomer (**5**) was achieved. The elongation cycles were continued until the required length was reached.

General procedure for cyclization

To remove the 5'-DMT group, the PEG-oligomer (**5**, 500 mg) was treated with a solution of 3% TCA in $CHCl_3$ (4 ml). After 30 min pyridine (1 ml) was added and the mixture concentrated to an oil. The residue, dissolved in CH_3OH, was chromatographed on a Fractogel column as described above. The collected fractions containing the 5'-unprotected PEG-oligomer, dried in vacuo, were treated with a solution (4 ml) of Et_3N/CH_3CN (1:1,v/v) to remove the 2--cyanoethyl group at the 3'-phosphate end as described in the chain assembly procedure. After repeated coevaporations with dry pyridine, to the PEG-oligomer, dissolved in pyridine (5×10^{-3} M), MSNT (40 eq with respect to the linear oligomer) was added and the reaction kept at room temperature. After 2-12 h (see Table 1), the mixture, treated with water (0.5 ml), was dried in vacuo and purified on a Fractogel column.

HPLC analyses of linear and cyclic oligodeoxyribonucleotides and purifications

The final PEG-oligomer (**5** or **6**) was treated with 5 ml of concentrated ammonia at 50°C. After 12 h the mixture was concentrated in vacuo, filtered and the solution analyzed by HPLC on a Partisil 10 SAX column (Whatman, 25 cm, 4.6 mm i.d., 10 μm, flow rate 1 ml/min) eluted with a linear gradient of KH_2PO_4, 350 mM, 20 % CH_3CN, pH 7.0 (eluent B) in KH_2PO_4, 1 mM, 20 % CH_3CN, pH 7.0 (eluent A) at room temperature. We used a gradient from 0 to 100 % eluent B in A in 30 min, for oligomers up to 10 base residues and from 30 to 100 % eluent B in A in 30 min, for longer oligomers. Quantitative HPLC analyses were carried out on the detached material of a weighed amount of the linear or the corresponding PEG-cyclic-oligomer using as internal reference cytidine-5'--monophosphate. Preparative purifications were performed by HPLC using a semipreparative column (Partisil M9 SAX, Whatman, 9.4 mm

i.d., 10 μm, flow rate 1.5 ml/min) eluted with the above reported gradients. The isolated products were finally desalted on a Biogel P2 column eluted with water and analyzed by ^{1}H NMR (Table 2). In a typical experiment, starting from 0.5 g of **4**, and performing coupling cycles with monomeric units, 52 mg (0.013 mmol, 14 % overall yield) of pure cyclic tetradecacytidylic acid (potassium salt) were obtained.

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REFERENCES AND NOTES

1) Hsu,C.H. Dennis,D. Nucleic Acids Res. **1982**, 10, 5637.
2) Ross,P.; Weinhouse,H.; Aloni,Y.; Michaeli,D.; Weinberg-Ohana,P.; Mayer,R.; Braun,S.; de Vroom,E.; van der Marel,G.A.; van Boom, J.H.;Benzimann,M. Nature **1987** 325, 279.
3) a) Prakash, G.; Kool,E.T. J. Chem. Soc. Chem. Commun. **1991**, 17, 1161; b) Kool,E.T. J. Am. Chem. Soc. **1991**, 113, 6265.
4) a) de Vroom,E.; Broxterman,H,J.G.; Sliedregt,L.A.J.M.; van der Marel, G.A.; van Boom,J.H. Nucleic Acids Res. **1988**, 16, 4607. b) Capobianco,M.L.; Colonna,F.P.; Garbesi,A. Gazz. Chim. Ital., **1988**,118, 549. c) Vaman Rao.M.; Reese,C.B. Nucleic Acids Res., **1989**, 17, 8221. d) Capobianco,M.L.; Carcuro,A.; Tondelli,L.; Garbesi,A. Bonora,G.M. Nucleic Acids Res., **1990** 18, 2661.
5) a) Barbato,S. De Napoli,L.; Mayol,L.; Piccialli,G.; Santacroce, C. Tetrahedron, **1989**, 45, 4523 and references cited therein; b) De Napoli,L.; Montesarchio,D.; Piccialli,G.; Santacroce,C.; Mayol,L.; Galeone,A.; Messere,A. Gazz. Chim. Ital.,**1991**,121, 505.
6) a) Bonora,G.M. Gazz. Chim. Ital., **1987** 117, 379. b) Bonora, G.M.; Scremin,C.L.; Colonna,F.P.; Garbesi,A. Nucleic Acids Res.,**1990**, 18, 3155. c) Colonna,F.P.; Scremin,C.L. and Bonora, G.M. Tetrahedron Lett., **1991**, 32, 3251.
7) In the case of the first coupling steps, yields could easily be calculated by ^{1}H NMR spectra, on the basis of the ratios between the areas of the H-1' signals and those attributed to the 4 H of the DMT group (ortho-H to CH_3O-group), which in a 100 % reaction yield would correspond to 0.5.
8) The effect of the presence of 2,6-lutidine and N-MeImidazole in the reaction mixture for chain elongation in the 5'- to 3'-end direction was also checked, coupling yield data resulting essentially unaffected unlike as previously reported[6b] for 3'-to 5'-end direction.
9) ^{1}H NMR spectra displayed only one signal for each type of nucleus, confirming the cyclic nature of the synthesized compounds.

10) As a further, more direct reference, a "classical" solution synthesis of c(dpCpCpCpC), using a phosphotriester procedure, under the same conditions adopted in the PEG-supported method, was also performed, affording 82 % cyclization yield.

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