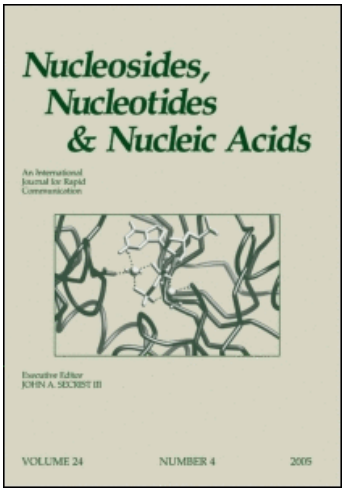


**Nucleosides, Nucleotides and Nucleic Acids**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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M. R. Conte[a]; L. Mayol[a]; D. Montesarchio[a]; G. Piccialli[b]; C. Santacroce[a]
[a] Dipartimento di Chimica delle Sostanze Naturali, Universita'di Napoli "Federico II", Napoli, Italy [b] Dipartimento di Chimica Organica e Biologica, Universita' di Napoli "Federico II", Napoli, Italy

**To cite this Article** Conte, M. R. , Mayol, L. , Montesarchio, D. , Piccialli, G. and Santacroce, C.(1993) 'Automated Synthesis of Cyclic Oligodeoxyribonucleotides via Phosphoramidite Method', Nucleosides, Nucleotides and Nucleic Acids, 12: 3, 351 — 358
**To link to this Article: DOI:** 10.1080/07328319308017831
**URL:** http://dx.doi.org/10.1080/07328319308017831

NUCLEOSIDES & NUCLEOTIDES, 12(3&4), 351-358 (1993)

# AUTOMATED SYNTHESIS OF CYCLIC OLIGODEOXYRIBONUCLEOTIDES VIA PHOSPHORAMIDITE METHOD

M.R.Conte[a], L.Mayol[a], D.Montesarchio[b], G.Piccialli*[,b] and C.Santacroce[b]

a. Dipartimento di Chimica delle Sostanze Naturali, Universita'di Napoli "Federico II", via D.Montesano 49, 80131 Napoli,Italy.

b. Dipartimento di Chimica Organica e Biologica, Universita' di Napoli "Federico II", via Mezzocannone 16, 80134 Napoli, Italy



ABSTRACT. The functionalized polyethylene glycol/polystyrene copolymer support **4** was shown to be suitable for a completely automated synthesis of small- to medium-sized cyclic oligodeoxyribonucleotides. Syntheses of the linear precursors were achieved by the phosphoramidite method, whereas the cyclization reactions were based on the phosphotriester chemistry.

Cyclic oligodeoxyribonucleotides (cyclic ODNs) are ideal models for the study of hairpin structures at high DNA concentration since the interstrand duplex formation is completely avoided[1]. Moreover the favorable entropy of a closed DNA circle ensures a high thermal stability thus allowing a detailed NMR study of hairpin formation in a broad temperature range.

More recently cyclic ODNs have been suggested as promising candidates for an antisense activity[2]. In fact, the absence of free 5'- or 3'-end makes these molecules resistant to exonuclease activity, resulting in a greater stability, *in vivo*, than the corresponding linear ODNs. In addition, cyclic ODNs, as more stable "natural" phosphodiester ODNs, reduce hazards associated

with the potential toxicity and mutagenicity of other classes of chemically modified ODNs, proposed for the antisense technology.

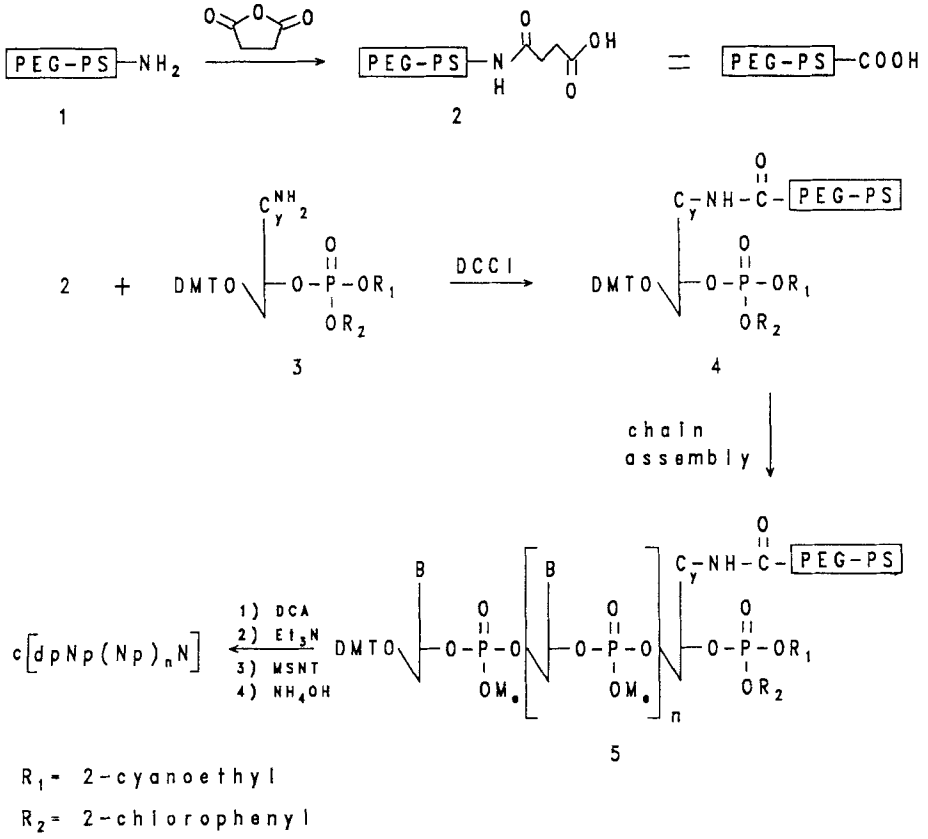
The hypothesized application of cyclic ODNs in medical diagnostics and/or in therapy as well as their importance as tools for investigation of DNA structures both in solution and in the solid state call for the definition of a practical and easy protocol for a fast and efficient large scale production of such compounds.

So far, several methods have been proposed for the synthesis of cyclic ODNs, both in solution[3] and on insoluble[4a-c] or soluble[5] polymeric supports.

While "classical" solution approach described syntheses of only small (2-8 residues) circles, the only reported procedure for the solid phase synthesis[4a-c] afforded oligomers containing up to 18 residues. Such syntheses were performed manually on a polyacrylamide support, following the phosphotriester method. Unfortunately, the above support has two main drawbacks which limit its use: i) it exhibits a poor compatibility with the phosphoramidite chemistry and ii) its low rigidity and its solvent dependent swelling make it unappropriate for automatic synthesis. Thus, according to our experience, this procedure, especially in the case of mixed sequences, produces complex mixtures from which the final cyclic product is not easily isolated, when its size is higher than 10-12 residues. Consequently we have focused our attention on the search for a solid support which could combine the higher coupling efficiency of phosphorus (III) chemistry with an automated process.

In a previous study[4c], we tested the controlled pore glass (CPG), the most widely used support for polynucleotide synthesis, to obtain cyclic ODNs, observing extremely low cyclization yields even in the case of very small oligomers (2-4 residues) and the absence of cyclization products for higher terms.

In this paper, a "tentacle" copolymer of polyethylene glycol and polystyrene (PEG-PS, known as Tentagel), is shown as a suitable solid support for the preparation of cyclic oligomers in




an automated process. Such a support, a graft copolymer of weakly cross-linked polystyrene and linear polyethylene glycol, was reported as a good alternative to CPG[6], allowing a two- or three--fold increase in loading of nucleotide, in conjunction with similar coupling efficiency.

The functionalization of PEG-PS (1, 0.24 meq/g) was carried out through succinylation by reaction with succinic anhydride; the resulting support 2 was treated with a solution of the 2'--deoxycytidine derivative 3, essentially as previously reported[4,5]. The incorporation of nucleotidic material was in the range 0.12-0.16 meq/g, as judged by spectroscopic measurements of the 4,4'-dimethoxytriphenylmethyl (DMT) cation released by acidic treatment of a weighed amount of the support.

Table: Cyclization reaction yields

| OligodC (size) | Cyclization Time (h) | Cyclization Yields (%) |
|---|---|---|
| 4 mer | 4 | 35 |
| 8 " | 6 | 25 |
| 10 " | 15 | 15 |
| 12 " | 15 | 10 |
| 16 " | 15 | 1 |

Using the derivatized support **4**, oligomers containing up to 16 residues were synthesized and their cyclization yields calculated. Chain assembly was performed by the standard phosphoramidite ($OCH_3$) method[7], using an automatic DNA synthesizer. In order to cyclize the linear oligomers still anchored to the polymeric support, deprotection at both ends, carried out by treatment, first, with dichloroacetic acid (DCA, 3 % in $CH_2Cl_2$), then with $CH_3CN$/triethylamine (1:1, v/v), was followed by addition of 1-mesitylenesulfonyl-3-nitro-1,2,4--triazole (MSNT) as the activator of the 3'-phosphodiester end. The given cyclization times insured the disappearance of the linear precursors, as judged by HPLC patterns. Cleavage from the matrix with conc. aqueous ammonia led to mixtures which were purified by HPLC; cyclization yields were calculated by quantitative HPLC analyses of the detached material from a weighed amount of the polymer (see Table).

Using this procedure, cyclic homooligomers of 2'-deoxycytidylic acid could be synthesized up to 16 bases. We furthermore tested this support synthesizing the cyclic decamer with the mixed sequence c[d(pCpGpCpGpT)$_2$] (see Figure a) for which no significant difference of the cyclization yield (ca. 15 %) was observed on the respect to homooligomers of the same size. The size and the cyclic nature of these products were assured by HPLC comparison with authentic samples synthesized according to previously reported procedures[4,5] and by NMR spectra of the isolated ODNs. Particularly in the case of the cyclic

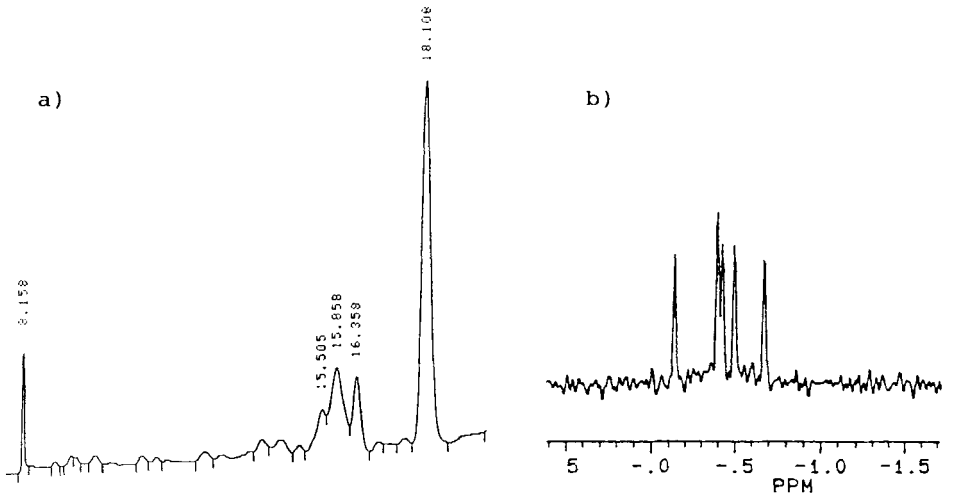



Figure: **a**) HPLC pattern of crude c[d(pCpGpCpGpT)$_2$] on a Partisil 10 SAX column (Whatman, 25 cm) eluted with a linear gradient (from 30 to 100% in 40 min) of $KH_2PO_4$ 350 mM, 20 % $CH_3CN$ (pH 7) in $KH_2PO_4$ 1 mM, 20 % $CH_3CN$ (pH 7), flow rate 1 ml/min. **b**) $^{31}P$ NMR spectrum (proton decoupled) of pure c[d(pCpGpCpGpT)].



homooligomers, all the nucleotides are magnetically equivalent and only a signal for each type of nucleous is observed in the NMR spectra. As for as cyclic decamer of mixed sequence is concerned, it is to be noted that it possesses a two-fold symmetry and consequently $^1H$ and $^{31}P$ NMR spectra display signals for only five different residues (see Figure b). A detailed computer-aided conformational study based on NMR data is in progress and will be published elsewhere.

The cyclization yields observed on Tentagel support showed the same decreasing trend on increasing the size of the circles found for cyclization of linear oligomers anchored on a soluble PEG matrix[5]. These results are consistent with NMR studies[8] asserting similar mobility of polyethylene glycol chains in PEG-PS graft copolymer as in free PEG.

In a further study we also tested a polystyrene matrix in order to verify that the presence of long linear chains of PEG in Tentagel resin was responsible for intermolecular couplings

during the cyclization process. Nevertheless, the use of aminomethyl-polystyrene resin (2% DVB, functionalized as above reported with 0.080 meq/g of **3**), tested for the preparation of the cyclic octamer of 2'-deoxycytidylic acid (following the same procedure), resulted in poor coupling yields, as expected, while no sensible benefit to the cyclization yields was observed.

In conclusion, the use of Tentagel resin seems to be very appealing for the synthesis of cyclic ODNs containing up to 10-12 residues; in fact, its suitability with standard DNA synthesizers and with phosphoramidite chemistry ensures higher final yields of linear precursors in shorter reaction times in comparison with the polyacrylamide support. These items, in our opinion, balance, in the case of small- to medium-sized oligomers, the lower cyclization yields observed, showing this copolymer as the first solid support so far found suitable for an efficient and completely automated synthesis of cyclic ODNs.



## EXPERIMENTAL

### General procedure

UV measurements were performed on a Perkin-Elmer lambda 7 spectrophotometer. NMR spectra were recorded on a Bruker WM-400 spectrometer. All chemical shifts are expressed in p.p.m. with respect to the residual solvent signal. The syntheses on solid support were performed on a Beckman automatic DNA synthesizer system 200A. The resin functionalizations were carried out in a short glass column (7 cm length, 1 cm i.d.) equipped with a sintered glass filter, a stopcock and a cap. For the cyclization procedure, the reactions and the washings were performed directly on the synthesizer. TentaGel Resin was purchased from Rapp-Polymere, Eugenstraβe 38/1 D-7400 Tubingen, Germany. Aminomethyl-polystyrene resin (0.25 meq of amino groups/g) was prepared by standard method[9] from chloromethyl-polystyrene.

### Functionalization of TentaGel Resin

1 g of **1** (0.24 mmol of amino groups) was mixed with succinic anhydride (1 g, 10 mmol) in dry pyridine (7 ml) and the mixture was shaked for 16 h at room temperature. After filtration, the support **2** was exhaustively washed with pyridine, $CHCl_3$, $Et_2O$ and

dried under reduced pressure. Then 0.5 mmol (370 mg) of **3** and 5 mmol (1 g) of N,N'-dicyclohexylcarbodiimide (DCCI) were added to the support keeping the mixture under shaking for 24 h at room temperature. The final support **4** was washed (pyridine, $CHCl_3$ and $Et_2O$)) and then dried under reduced pressure. The amount of the nucleotide derivative attached to the support, extimated by spectroscopic measurement (498 nm, $\epsilon$=71700) of the 4,4'-dimethoxytriphenylmethyl cation released by acidic treatment (70 % $HClO_4$/EtOH, 3:2, v/v) on a weighed sample of the support, was found to be in the range 0.12-0.16 meq/g.

Chain assembly and cyclization on solid support

Oligonucleotide syntheses were carried out using the Beckman automatic DNA synthesizer system 200A. Standard columns were packed with 50 mg (7 $\mu$mol) of the support **4**. Syntheses were performed according to the phosphoramidite ($OCH_3$) method. The steps utilized for each cycle, including capping and time intervals, were those already described in the literature[7]. Coupling yields were found constantly in the range 97-99%, as calculated measuring the amount of the released 4,4'-dimethoxytriphenylmethyl cation.

After washings with $CH_3CN$, the DMT group of **5** was removed by treatment with DCA, then the phosphate 3'-end was deprotected by treatment (2 h, room temp. in stop flow) with a solution of $CH_3CN/Et_3N$ (1:1, v/v). After washings with pyridine, a solution 0.2 M of MSNT in pyridine was left in contact with the resin and the mixture kept (in stop flow) at room temperature for 4-15 h (see table). After washings with pyridine and $CH_3CN$, the final support was dried under reduced pressure.

Deprotection and purification of cyclic oligomers

Deprotection, HPLC analyses and purification of cyclic oligomers were performed as previously described[4c].

In the synthesis of the cyclic decamer c[d(pCpGpCpGpT)$_2$], starting from 50 mg of **4** (7 $\mu$mmol), 2 mg (0.6 $\mu$mmol, 8% overall yield) of the isolated product were obtained.

$^1$H NMR ($D_2O$) significative protons at $\delta$:8.14 (H-8, s, 2H), 7.97 (H-8, s, 2H), 7.59 (H-6, s, 2H), 7.55 and 7.54 (H-6, overlapped doublets, J=7.5 Hz, 4H), 6.25-6.00 (H-1', overlapped signals, 10H), 5.72 (H-5, d, J=7.5 Hz, 2H), 5.52 (H-5, d, J=7.5 Hz, 2H).

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Received 11/2/92
Accepted 1/29/93