Facile Preparation of Cyclic Oligoribonucleotides

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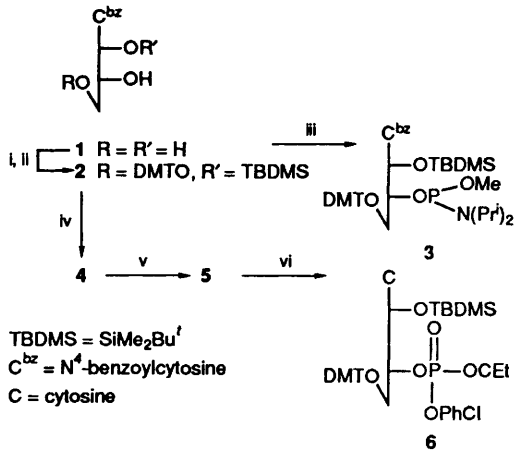
A convenient solid-phase synthesis of small cyclic oligoribonucleotides based on elongation of the chain by phosphoramidite chemistry, and successive cyclization of the linear fragments by a phosphotriester approach is described.

The potential of cyclic oligodeoxyribonucleotides (cODNs) and cyclic oligoribonucleotides (cORNs) in a variety of biological connections[1-3] justifies work on improving their synthesis. Whilst both solid phase[4] and solution[5] methods have been described for the preparation of cODNs, for cORNs the only available procedure is based on a modified phosphotriester approach in solution.[5a] Here we report the first solid-phase synthesis of small cyclic RNAs. Previously we have described a number of solid supports for the synthesis of cODNs evaluating the cyclization yields on the basis of the linear precursors still anchored to the support through the exocyclic amino function of a base residue.[4] In this work we found that decreasing yields accompanied increasing size of the starting linear oligomers, steric factors affecting the intermolecular couplings and, further, that, with an equal number of residues, the highest product yields were obtained with a polyacrylamide resin solid support. Unfortunately, such a resin is not only incompatible with phosphoramidite chemistry but its poor mechanical properties preclude its use in an automated process. Chain elongation for synthesis of the linear precursor on polyacrylamide resin must be performed, therefore, by a manual process based on phosphotriester chemistry, whose relatively low efficiency (*ca.* 92% average yield per cycle), limits the size of cODNs of mixed sequences which can be prepared. Recently we have found that a copolymer of polyethylene glycol and polystyrene (PEG-PS, commercially known as Tentagel[6]) is a suitable solid support for an efficient and completely automated synthesis of mixed sequences of cODNs containing up to 12 residues.[7] The observed lower cyclization yields, in comparison with the polyacrylamide resin, are balanced by the compatibility of Tentagel with DNA synthesizers and with phosphoramidite chemistry which ensures higher final yields of the linear precursor through a standard, fully automated, process.

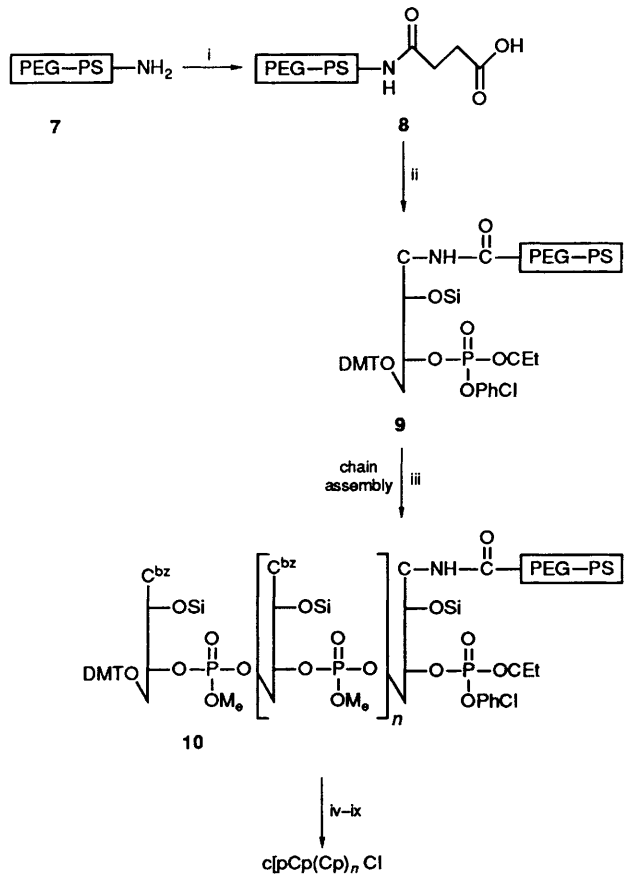
Here we describe the successful synthesis of cyclic homo-oligomers of cytidine containing up to 8 residues using Tentagel as solid support. The most attractive feature of this approach for the synthesis of cORNs is that both the elongation process (*via* phosphoramidite chemistry) and the cyclization reaction (*via* phosphotriester chemistry) are performed on the solid support thus avoiding the unconvenience associated with the syntheses in solution.

The preparative route for cORNs is outlined in Schemes 1 and 2 and consists of the following: (a) synthesis of the intermediate **2**, useful for the preparation of the building block **3** and of the phosphotriester intermediate **6**: (b) incorporation of **6** into the resin **8** thus obtaining the functionalized support **9**; (c) elongation of the chain; (d) removal of the protecting groups from both the ends of the chain and cyclization; (e) detachment of the cyclic ORNs from the resin and deprotection. The



Scheme 1 *Reagent and conditions:* i, DMT-Cl, pyridine, 10 h (90%); ii, TBDMSi-Cl, $AgNO_3$, pyridine, 6 h (65%); iii, $MeOPr^i{}_2NPCl$, dimethylaminopyridine, $EtNPr^i{}_2$, THF, 3 h (85%); iv, $ClC_6H_4OPOCl_2$, pyridine, 0 °C, 1 h (84%); v, aq. NH_3 (32%)/EtOH (3:1), 55 °C, 2 h (89%); vi, HOCEt, MSNT, pyridine, 3 h (60%)

intermediate **2** was obtained from N^4-benzoylcytidine **1** which was protected as a 5'-dimethoxytrityl derivative and, successively, 2'-silylated as previously described.[8] 3'-Phosphitylation of **2**, with *N*,*N*-diisopropylmethylphosphonamidic chloride, by a standard method[9] afforded the phosphoramidite intermediate **3**. Treatment of **2** with 2-chlorophenyl phosphorodichloridate gave the phosphodiester derivative **4** which underwent debenzoylation, using aq. NH_3 (32%) in EtOH, and was then fully protected at the 3'-phosphate by reaction with β-hydroxypropionitrile (HOCEt) and mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT) in pyridine to yield the intermediate **6**. The PEG-PS **7** (0.24 mequiv. g^{-1} of amino groups) was prefunctionalized[4b] with succinic anhydride, thus giving **8** which was then treated with a solution of **6** in pyridine in the presence of *N*,*N*'-dicyclohexylcarbodiimide (DCCI). The incorporation of the nucleotidic material was in the range 0.10–0.14 mequiv. g^{-1}, as estimated by quantitative spectroscopic measurement of the 4,4'-dimethoxytriphenylmethyl (DMT) cation released from resin **9**. The support **9** was used for the preparation of linear homooligomers of cytidine (tetramer, hexamer and octamer) by standard phosphoramidite (OMe) chemistry[10] on an automated synthesizer. In order to allow the cyclization of the linear fragments, **10** was deprotected at both ends by treatment with dichloroacetic acid (DCA; 3% in CH_2Cl_2) followed by reaction with triethylamine–pyridine (1:1). Successively, a solution of MSNT in pyridine was left in contact



Scheme 2 *Reagent and conditions:* i, succinic anhydride, pyridine 16 h; ii, **6** (2.5 equiv.), DCCI (10 equiv.), pyridine 16 h; iii, ref. 10; iv, DCA, CH_2Cl_2 (5 min); v, Et_3N/pyridine (1 : 1), 0.5 h; vi, MSNT, pyridine, 12 h; vii, PhSH/Et_3N/dioxane (1 : 2 : 2), 1 h; viii, aq. NH_3 (32%)/EtOH (3 : 1), 4 h, 55 °C; ix, Bu_4NF, THF

with the resin for 12 h. This cyclization time assured complete disappearance of the linear precursors, as judged by HPLC profiles. The cyclic oligomers were removed from the resin and deprotected in three steps[9] (vii–ix, Scheme 2). The completely deblocked cyclic oligomers were purified by ion exchange HPLC. The results of analyses of the products allowed the cyclization yields to be calculated; these were 40% (tetramer), 38% (hexamer) and 28% (octamer). It is to be noted that the decreasing trend of the cyclic yields on increasing the size of the oligomers matches that observed in the cyclization of deoxyoligomers. After desalting, the products were analysed by ^1H NMR spectrscopy which established their cyclic nature, showing the complete equivalence of each nucleotide (one signal for each type of nucleus was observed).

In conclusion, Tentagel has been shown to be a suitable polymeric support for a completely automated solid-phase synthesis of small RNA cyclic compounds. Furthermore, the coupling yields observed (>98%) in conjunction with short reaction time (2 min) in the chain elongation steps, suggest that this support is a valid alternative to the widely used controlled pore glass (CPG) resin for the synthesis of linear ORNs; further, it results in a two–three fold higher degree of functionalization for Tentagel over CPG.

Experimental

Synthesis of Intermediate **3**.—Compound **2** was converted into the phosphoramidite **3** essentially as previously reported.[9] Purification was performed by HPLC using a silica gel column (Merck, LiChrosorb Si 60, 25 mm i.d.) eluted with increasing amounts (0–100%) of AcOEt in hexane, flow rate 13 cm^3 min^{-1}. The fraction eluted with AcOEt afforded pure **3** (as a diastereoisomeric mixture) the structure for which was confirmed on the basis of NMR data.

Synthesis of Intermediate **6**.—A solution of 2-chlorophenyl phosphorodichloridate (0.7 cm^3, 4 mmol) in pyridine (4 cm^3), was added to a stirred solution of **2** (380 mg, 0.5 mmol) in pyridine (5 cm^3) at 0 °C. After 40 min the mixture was diluted with water (1 cm^3) and then concentrated. The residue was dissolved in $CHCl_3$ and the solution washed with water (×3). The organic layer was separated, concentrated and purified on a silica gel column eluting with increasing amounts of MeOH in $CHCl_3$ (5–20%) to give pure **4** (84%). The latter was treated with aq. NH_3 (32%)/EtOH (3 : 1), 2.5 h, 50 °C, to afford the debenzoylated crude product **5** (90%). After purification (essentially as above), to a solution of **5** (340 mg, 0.4 mmol) in pyridine (5 cm^3), was added HOCEt (284 mg, 4 mmol) and then MSNT (474 mg, 1.6 mmol). After 2 h, water (2 cm^3) was added to the mixture which was then concentrated. The residue was dissolved in $CHCl_3$ (15 cm^3) and the solution washed with water (×3), taken to dryness and finally purified on a silica gel column using increasing amounts of MeOH in $CHCl_3$ (0–5%), to afford pure **6** (60%), the structure of which (mixture of diastereoisomers) was confirmed by NMR data.

Chain Assembly and Cyclization on Solid Support.—Syntheses were performed using support **8** according to the phosphoramidite method by a Beckman 200A automatic synthesizer. The steps utilized for each cycle have already been described in the literature.[10] For a coupling reaction with intermediate **3** we used a longer time (30 min). After deprotection of **10** at both ends (steps iv–v, Scheme 2) and washing with pyridine, a solution of 0.2 mol of MSNT in pyridine was left in contact with the resin, 12 h, room temp. After washing with pyridine and MeCN, the final support was dried under reduced pressure.

Deprotection and Purification of Cyclic Oligomers.—Detachment and deprotection of cyclic oligomers were performed as previously described.[9] HPLC analyses, purifications, and desalting of the linear and cyclic products were carried out as previously reported.[4a–c] In the synthesis of cyclic octamer c-[pCp(Cp)$_6$C] starting from 50 mg (6 μmol) of **8**, 2.2 mg (0.9 μmol, 15% overall yield) of the isolated product was obtained; δ_H(400 Mz, D_2O, protons at lowfield region) 7.42 (8 H, d, *J* 3.2, 1′-H); 5.56 (8 H, d, *J* 7.0, 5-H) and 5.45 (8 H, d, *J* 3.2, 2′-H).

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