Pergamon

Bioorganic & Medicinal Chemistry, Vol. 3, No. 10, pp. 1325–1329, 1995

0968-0896/95 $9.50 + .00

0968-0896(95)00090-9

Automated Solid Phase Synthesis of Cyclic Oligonucleotides: a Further Improvement

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Abstract—The solid phase approach for the preparation of cyclic oligodeoxy- and oligo-ribonucleotides was improved thus allowing the fully automated synthesis of larger DNA and RNA circles, using commercially available amidite building blocks.

Introduction

Small cyclic oligonucleotides are useful models for NMR conformational studies of unusual DNA structures, especially as far as hairpin forms are concerned;[1] nevertheless, their importance is also related to a variety of biological activities.[2,3] Furthermore, one of the major interests in circular oligonucleotides arises from their great potential as antisense agents,[4,5] selective inhibitors of gene expression, as a result of the marked increase in resistance to cellular nucleases when compared to linear oligomers. Recently, circular polypyrimidine oligodeoxyribonucleotides were shown to bind to polypurine single-stranded DNA fragments by forming a triple helical complex,[6,7] assuring a very high and sequence-selective affinity to the target. For these promising applications in medical diagnostics, as well as in therapy, circular oligonucleotides of at least 20–25 bases are desirable and this justifies further efforts aimed at improving the synthetic methodologies for their preparation.

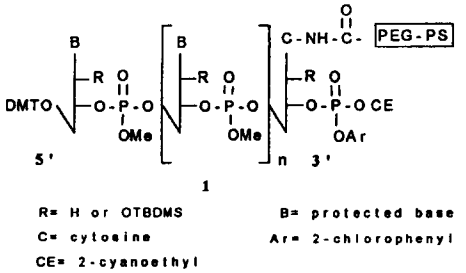
Enzymatic methods,[8] although very efficient for the cyclization of oligonucleotide chains, can only be used for analytical purposes, not being feasible for the preparation of large quantities needed for *in vivo* or *in vitro* experiments. On the other hand, the chemical ligation approach[9] seems to be very appealing as far as cyclization yields and scale-up are concerned, but its applicability is not universal, its success being strongly dependent upon the secondary structure adopted by the oligomers and so ultimately, upon their sequence.

In principle, chemical synthesis can be regarded as the method of choice when amounts of materials in the orders of micromoles are required. Therefore, several chemical methods have been so far proposed for the preparation of cyclic oligonucleotides, both in solution[10] and on insoluble[11–15] or soluble[16] polymeric supports. A common finding in all the reported procedures is the complete independence of cyclization yields from the sequence synthesized.

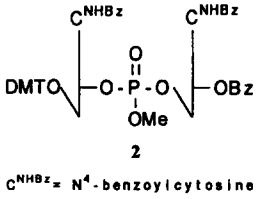
Results and Discussion

On pursuing our original studies on the solid phase synthesis of cyclic oligodeoxyribonucleotides,[11–13] we found that a complete automation of the synthetic process could be achieved using as a solid support a graft copolymer of polyethylene glycol (PEG) and polystyrene (PS), commercially known as Tentagel.[14,15] Oligomers of both deoxyribo- and ribo- series could be successfully synthesized and circularized in acceptable overall yields up to 10–12 bases being still anchored to the matrix. The intrinsic limit of such a methodology is essentially connected to the size of the molecules of interest, since cyclization yields rapidly decrease as the oligonucleotide chain grows, most likely due to the polymerization which competes with the desired intramolecular reaction.

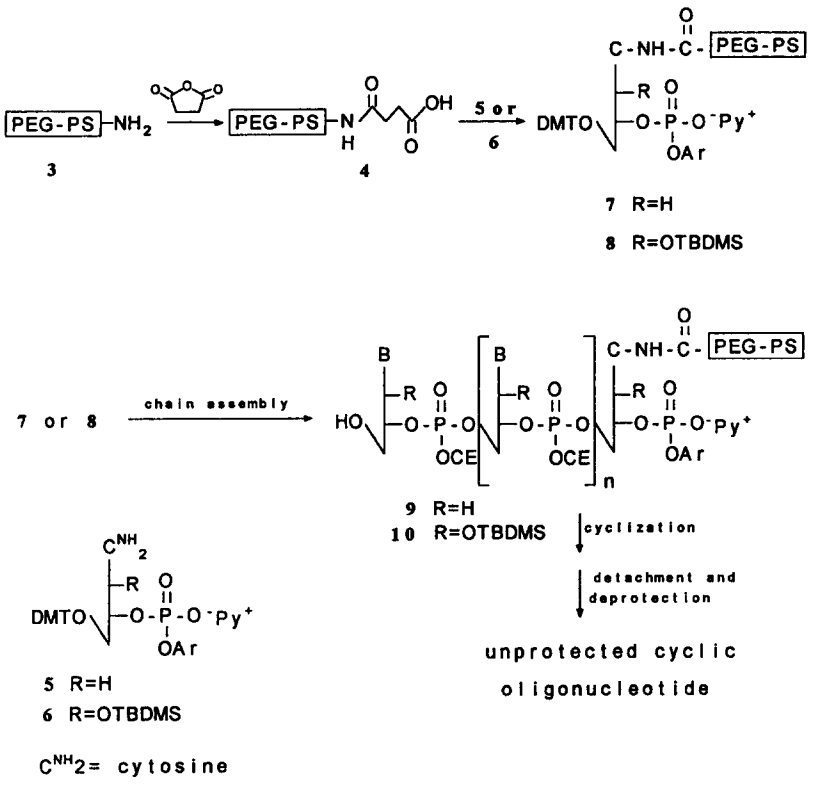
We re-examined the whole methodology with the aim of rendering it suitable to the preparation of larger circles. The previously described procedure is very simple in its general scheme.[14,15] A 5'-dimethoxytrityl (DMT) 2'-deoxycytidine-3'-fully protected phosphate was attached to the resin, through the NH_2 function of the base. The functionalized support could be used in the synthesis of the linear precursor **1** by the standard automatic phosphoramidite method.[17] A cyclization procedure was carried out, deprotecting the assembled linear oligomer at both the 5' and 3' ends by treatment with trichloroacetic acid and triethylamine, respectively, and successively activating, by addition of 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT),[18] the 3'-phosphodiester function to the nucleophilic attack of the 5'-OH terminus of the chain.



In order to improve the efficiency of the automated synthesis we wondered whether the competition of intermolecular versus intramolecular coupling was enough to justify the rapidly decreasing trend of the yields in the circular oligomer on increasing the size of the linear precursor. In other words, we did not exclude that some other side-reactions, whose prominence increased with the number of the nucleotides, interfered with the formation of the circle. Thus we hypothesized, among other things, that a possible cause of the dramatic fall of the cyclization yields for larger sized molecules resided in the incomplete resistence of the methyl protecting group of the phosphotriester functions to the basic treatment with triethylamine required to remove the 3'-cyanoethyl phosphate protecting group. To check the stability of the *O*-methyl phosphotriester functions to triethylamine, the dimer model **2**, synthesized according to amidite solution method,[17] was reacted with anhydrous triethylamine:acetonitrile (1:1, v/v, 1 h, rt) and underwent a partial loss (5–10%) of the above protecting group as clearly shown by TLC and ^{1}H NMR analyses. The search for an alternative to triethylamine deblocking reagents, such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or 1,5-diazabicyclo-[4.3.0]non-5-ene (DBN), was unsuccessful, leading to similar results. The partial deblocking of *O*-methyl phosphotriester functions during this basic, not hydrolytic treatment, might evidently result in particularly adverse side-reactions during the cyclization step, whose damage increases with the number of reactive sites, i.e. the deprotected phosphates generated in the oligomers.



To overcome this drawback we tested the feasibility of a solid support functionalized with the first nucleotide carrying only one protecting group at the 3'-phosphate end (**7**, Scheme 1). The advantages of this approach would be immediately evident: the triethylamine treatment in the cyclization procedure could be eliminated, and, consequently both the first monomer to be attached to the support and the amidite monomer building blocks should not be synthesized *ad hoc* anymore, since no incompatibility emerged with the usage of commercially available 3'-*O*-(2-cyanoethyl)-phosphoramidite nucleosides.



Scheme 1.

The PEG-PS **3**, (0.24 meq g^{-1} amino groups) was prefunctionalized[14] by treatment with succinic anhydride thus giving **4** which was then treated with a solution of **5** in the presence of *N,N'*-dicyclohexylcarbodiimide (DCCI). Incorporation of nucleotidic material resulted to be in the range 0.08–0.12 meq g^{-1}, calculated on the basis of spectroscopic measurement of 4,4'-dimethoxytrityl cation released by acidic treatment of a weighed amount of support **7**. Using support **7**, a number of oligodeoxyribonucleotides (Table 1) were assembled according to standard phosphoramidite protocols.[17] HPLC analyses of the crude linear oligomers and quantitative trityl tests carried out at the end of each coupling step showed coupling efficiencies in the range usually observed in a phosphoramidite procedure (98–99%), thus confirming that no interference, such as parasite growing chains, was produced by the unprotected 3'-phosphodiester ends.[19] In order to cyclize the linear oligomers **9** (or **10**) still anchored to the polymeric support, the resin, washed with pyridine, was left in contact (15 h, rt) with a solution 0.3 M of MSNT in pyridine. Cleavage from the support with conc ammonia led to mixtures which were purified by HPLC. Quantitative HPLC analyses of the cyclic oligonucleotide and its linear precursor detached from weighed amounts of resins allowed cyclization yields to be calculated as percentages of linear precursor circularized. The data obtained (Table 1) showed a marked increase in the yields, if compared to the previously reported ones.[14,15] Consequently, following this procedure cyclic oligodeoxyribonucleotides containing up to 30 residues could be synthesized in satisfactory overall yields.

Table 1. Cyclization reaction yields

Oligomers (size)	Sequence 5'–3'	Cyclization yields (%)*
8	$d(C)_8$	37
8	$r(C)_8$	24
12	$d(C)_{12}$	24
14	$r(C)_{14}$	12
16	$d(C)_{16}$	23
20	$d(C)_{20}$	17
21	d(AAAAATGCCCTC--CATAGAGC)	16
24	$dT(CT)_{11}C$	13
24	$d(TGAC)_6$	12
30	$d(C)_{30}$	8

*Average values calculated by HPLC comparison of the cyclic oligonucleotides and its linear precursors released from a weighed amount of resins.

Similar results were obtained for the preparation of cyclic oligoribonucleotides using the support **8** obtained by incorporation of 2'-*O*-(*t*-butyldimethyl)silyl derivative **6** on the support **4**. However, in this case a general reduction of cyclization yields was observed when compared with oligomers of DNA series of corresponding length, thus lowering the limit of size for circular RNA obtainable in satisfactory yields.

It should be noted that cyclization yields both for DNA and RNA fragments seem not to be particularly affected by the sequence. In a typical experiment, starting from 5 μmol of functionalized support **7**, 2.7 mg of cyclic 24-mer $c[d(TC)_{12}]$ and 2.2 mg of $c[d(TGAC)_6]$ were obtained after purification by HPLC. In the case of RNA products 100 mg of support **8** furnished, after purification, 1.9 mg of $c[r(C)_{14}]$.

The cyclic nature of the synthesized products was ascertained by comparison with authentic samples[12–14] (up to 16 residues) and by ^1H NMR spectra, which, in the case of cyclic homooligomers, showed only one signal for each type of proton, clearly indicating that all the nucleotides in the molecule are equivalent. As for the 24-mer $c[d(CpTp)_{12}]$, the ^1H NMR spectrum showed only two sets of signals, thus confirming a structure with a two-fold symmetry. On the contrary no symmetry was found in the 24-mer $c[d(TGAC)_6]$, most likely due to a particular secondary structure adopted by the oligomer. However, in this case, as well as for all the synthesized cyclic oligodeoxynucleosides, enzymatic tests definitively confirmed the cyclic nature of the substrates. Particularly, the cyclic products were found to be unaffected by treatment with phosphodiesterase II, which, as expected, showed a very high 5'-exonuclease activity towards the corresponding linear fragments.

In conclusion, this paper shows that cyclic oligonucleotides containing up to 30 or 15 residues for DNA and RNA series, respectively, can be prepared using an automatic solid phase approach. Significantly, the lack of any interference of the phosphodiester function present on the first monomer anchored to the solid support during the assembly of the linear precursors besides leading to significant improvements in cyclization yields, allowed the usage of commercially available nucleoside building blocks.

Experimental

General procedure

UV measurements were performed on a Perkin Elmer Lambda 7 spectrophotometer. NMR spectra were recorded on a Bruker WM-400 and WM 270 spectrometers. All chemical shifts are expressed in ppm with respect to the residual solvent signal. HPLC was carried out on a Beckman System Gold instrument equipped with a UV detector module 166 and a Shimadzu Chromatopac C-R6A integrator. The syntheses on solid support were performed on a Cyclone Plus Millipore automatic DNA synthesizer. 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. Tentagel Resin was purchased from Rapp Polymere, Tubingen, Germany. The phosphodiesterase II was purchased from Sigma.

Synthesis of intermediate 5

One gram (1.12 mmol) of the commercially available N^4-benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxycytidine-3'-*O*-(2-chlorophenyl)phosphate, triethylammonium salt, was treated with concentrated ammonia for 20 h at 50 °C under stirring. TLC analysis (silica gel, eluent $CHCl_3$:CH_3OH, 8:2) showed the complete conversion of the starting product into the base deprotected derivative **5** (R_f 0.25). After removal of the solvent, *in vacuo*, the residue, dissolved in $CHCl_3$:CH_3OH (9:1, v/v) containing 0.5% pyridine, was chromatographed on a silica gel column, eluted with increasing amounts of CH_3OH (from 10 to 30%) in $CHCl_3$ containing 0.5% pyridine. The appropriate fractions gave 815 mg (1.06 mmol, 95% yield) of the pure N-deprotected deoxyribonucleotide **5**[12] as a pyridinium salt. (^{1}H NMR see Ref. 12.)

Synthesis of intermediate 6

Five hundred milligrams (0.62 mmol) of 5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-(*t*-butyldimethyl)silyl-N^4-benzoylcytidine, synthesized according to the previously reported procedure,[20] dissolved in 4 mL of dry pyridine, were treated with 0.5 mL (3 mmol) of a solution of 2-chlorophenylphosphorodichloridate in 4 mL of dry pyridine at 0 °C under stirring. After 1 h, the reaction was quenched by addition of H_2O and the resulting mixture was concentrated under reduced pressure. The residue was dissolved in $CHCl_3$ and the resulting solution washed with H_2O (× 3). The organic layer was separated, concentrated under reduced pressure, then treated with aq. NH_3:CH_3OH (2:3, v/v) for 4.5 h at 50 °C. The concentrated mixture was chromatographed on a silica gel column eluted with increasing amounts of CH_3OH (from 5 to 20%) in $CHCl_3$ containing 0.5% pyridine, affording 420 mg (0.45 mmol, 75% overall yield) of the pure N-deprotected ribonucleotide **6** as a pyridinium salt. (R_f 0.5, TLC silica, eluent $CHCl_3$:CH_3OH, 7:3). ^{1}H NMR (270 MHz, CD_3OD, almost equimolecular mixture of diastereomeric pyridinium salts): δ 7.89 and 7.86 (1H, *d*'s, J = 5.8 Hz, H-6); 7.58–6.75 (22H, complex signals, aromatics protons and pyridinium protons); 6.03 and 5.92 (1H, *d*'s, J = 5.8 Hz, H-6); 5.93 (1H, *dd*, H-1'); 4.95 (H, *m*, H-3'); 4.58 (2H, *m*, H_2-2'); 4.45 (1H, *m*, H-4'); 3.78 (6H, *s*'s, OCH_3 of DMT); 3.53 (2H, complex signal, H_2-5'); 0.9 (9H, *s*'s, *t*-butyl protons); 0.10–0.13 [6H, *s*'s, $Si(CH_3)_2$ protons].

Functionalization of Tentagel resin

One gram of support **3** (0.24 mmol of amino groups per g), mixed with 1 g (10 mmol) of succinic anhydride in 7 mL of dry pyridine, was shaken for 16 h at room temperature. The resulting support **4** was filtered and exhaustively washed with pyridine, $CHCl_3$, Et_2O and dried under reduced pressure. Compound **4** was then converted into the desired support **7** by reaction with 385 mg (0.5 mmol) of **5** and 1 g (5 mmol) of DCCI in 10 mL of dry pyridine for 24 h at room temperature under shaking.

Analogous reaction conditions afforded the functionalized support **8**, obtained treating 400 mg of **4** with 420 mg (0.45 mmol) of **6** and 900 mg (4.5 mmol) of DCCI in 4 mL of dry pyridine. The final supports **7** and **8**, washed with pyridine, $CHCl_3$ and Et_2O, were dried under reduced pressure.

The amount of nucleotide derivative attached to the support, estimated by spectroscopic measurements (λ_{max} 498 nm , ε = 71,700) 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, resulted to be, in both cases, in the range 0.08–0.12 meq g^{-1}.

Chain assembly and cyclization on solid support

Syntheses of linear DNA and RNA oligomers were performed using supports **7** and **8** on a Cyclone Plus Millipore automatic synthesizer following the standard phosphoramidite method.[17,21] The obtained support **9** (or **10**) was washed with pyridine and then left in contact with a solution 0.3 M of MSNT in pyridine (1 mL/50 mg resin) at room temperature for 15 h. After washings with pyridine and CH_3CN the support was dried under reduced pressure. Such reactions conditions assured the complete disappearance of the linear precursors, as judged by HPLC analyses of the crude detached material.

Deprotection and purification of cyclic oligomers

DNA products. The final resin (weighed amount for linear and cyclic products) was treated with conc NH_3 at 50 °C for 6 h. The supernatant was filtered and the resin washed with H_2O. The combined filtrate and washings were taken to dryness, redissolved in water and analyzed by HPLC on an analytical Partisil 10 SAX column. Analyses and purifications were performed using linear gradients of KH_2PO_4 (20% CH_3CN, pH 7) from 1 to 350 mM. The polymeric materials formed as by products in the cyclization step were generally strongly retained on column and high ionic strength was required to elute them. The collected product peak, desalted on a Biogel P2 column eluted in H_2O:EtOH (8:2, v/v) was lyophilyzed and analyzed by ^{1}H NMR and by enzymatic digestion. c[d$(TC)_{12}$]: δ_H (400 MHz, D_2O, protons at lowfield region): 7.89 (12H, *d*, J = 8.0 Hz, H-6 Cyt.; 7.70 (12H, *s*, H-6 Thy.); 6.31 (24H, *m*, 2H-1' Cyt. and Thy.); 6.11 (12H, *d*, J = 8.0 Hz, H-5 Cyt.). c[dC_{30}]: δ_H (400 MHz, D_2O, protons at lowfield region) 7.93 (30H, *d*, J = 7.9 Hz, H-6); 6.36 (30H, *dd*, H-1'); 6.15 (30H, *d*, J = 7.9 Hz, H-5).

RNA products. The final resin (weighed amount for linear and cyclic products) was treated with a solution of aq. NH_3 (32%):EtOH (3:1,v/v), 4 h, 55 °C. The supernatant was filtered and the resin washed with EtOH:H_2O (8:2,v/v). The combined filtrate and washings taken to dryness were treated with a solution of 1.1 M of tetrabutylammoniumfluoride in THF (1 mL/50 mg of support) for 16 h at room temperature. The reaction

was quenched by the addition of 0.1 M triethylammonium acetate (1 mL/50 mg of support) and then applied on a Sephadex G-25 column (30 × 1.5 i.d.). The column eluted with H_2O:EtOH (8:2, v/v) furnished desalted crude product which was purified by HPLC on a Partisil 10 SAX column as described above.

c[rC_{14}]: δ_H (400 MHz, D_2O, protons at lowfield region) 7.92 (14H, *d*, *J* = 7.1 Hz, H-6); 5.96 (14H, *d*, *J* = 7.1 Hz, H-5); 5.82 (14H, *bs*, H-1').

Enzymatic digestion

The purified cyclic (or linear) product (*ca.* 1.0 A_{260} units) was dissolved in 1 mL of a solution of 0.1 M sodium succinate and 0.5 units of calf spleen phosphodiesterase II were added. The mixture incubated at 37 °C was analyzed at fixed times, in a 72-h period, by HPLC on a Partisil 10 SAX column. The linear products were completely digested in 24–48 h to give the expected nucleotides 3'-phosphate while the cyclic compounds after 72 h were unaffected.

Acknowledgements

We are grateful to MURST and CNR—"Progetto strategico: Nucleotidi antisenso come strumento di nuove strategie terapeutiche" for grants in support of this investigation and to "Centro di Metodologie Chimico-Fisiche" dell' Università di Napoli "Federico II" for NMR facilities.

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(*Received in U.S.A.* 13 *March* 1995; *accepted* 24 *April* 1995)