Volume 16 Number 10 1988 Nucleic Acids Research

Synthesis of cyclic oligonucleotides by a modified phosphotriester approach

E.de Vroom, H.J.G.Broxterman, L.A.J.M.Sliedregt, G.A.van der Marel and J.H.van Boom

Gorlaeus Laboratory, PO Box 9502, 2300 RA Leiden, The Netherlands

Received March 4, 1988; Revised and Accepted April 15, 1988

ABSTRACT

Evidence will be presented to show that the allyl group is suitable for the protection of a 3'-terminal phosphodiester function. The latter will be demonstrated by the synthesis, *via* a phosphotriester approach, of two cyclic tetraribonucleotides [r(AAAA) and r($UA^{Me_2}UA^{Me_2}$)], two cyclic hexadeoxyribonucleotides [d(CGCGCG) and d(TAAAAA)] and a cyclic octadeoxyribonucleotide [d(CGTGCGTG)].

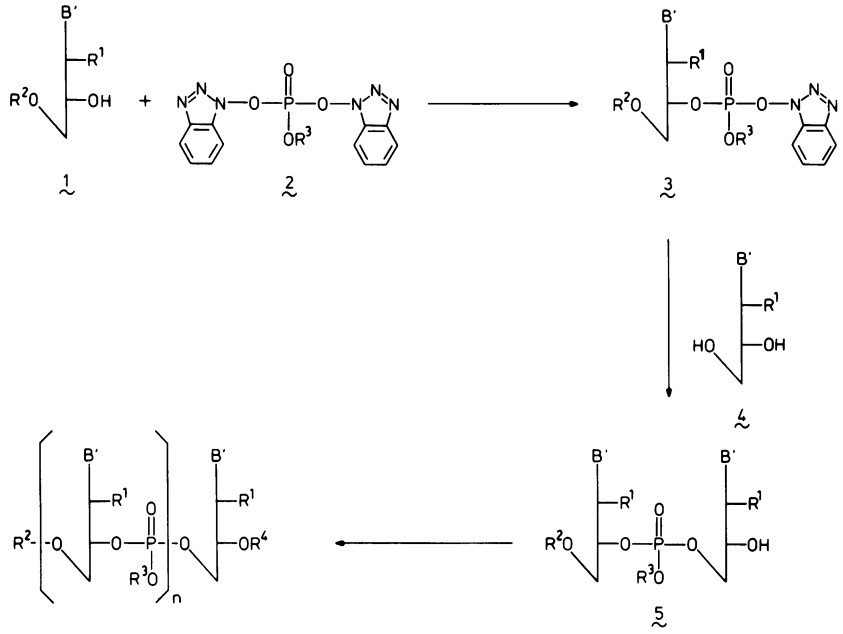
INTRODUCTION

It is well established that cyclic mono- and oligonucleotides, containing solely 3',5'-internucleotidic phosphodiester linkages, play an important role in biological processes. For example, the 3',5'-cyclic monoribonucleotides cAMP[1] and cGMP[2] are biochemical regulators. Further, it was recently demonstrated that some *bis* 3',5'-cyclic diribonucleotides may function as inhibitor of DNA-dependent RNA polymerase (*i.e.* cApAp, cApUp and cUpUp)[3] and as an activator (*i.e.* cGpGp)[4] of cellulose synthase in *Acetobacter Xylinum*. As part of a programme[4,5] to prepare cyclic DNA and RNA fragments with a defined sequence, we report the synthesis of the five cyclic oligonucleotides r(AAAA), r($UA^{Me_2}UA^{Me_2}$)*, d(CGCGCG), d(TAAAAA) and d(CGTGCGTG) by a phosphotriester approach.

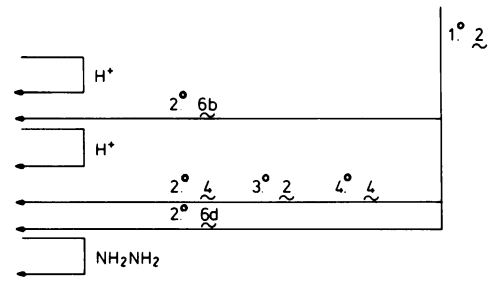
RESULTS AND DISCUSSION

The steps involved in the preparation of the DNA and RNA cyclic oligomers 8 is outlined in Schemes 1 and 2 and consists of the following consecutive steps: (i) preparation (see Scheme 1), *via* a phosphotriester[6,7] approach, of the partially protected linear fragments 6 (R^4 = H) having a free 3'-hydroxyl group; (ii) introduction (see Scheme 2) of a phosphotriester

* A^{Me_2} = N^6,N^6-dimethyladenosine



6	n	R^2	R^4
a	1	DMT	Lev
b	1	H	Lev
c	3	DMT	Lev
d	3	H	Lev
e	3	DMT	H
f	5	DMT	Lev
g	5	DMT	H

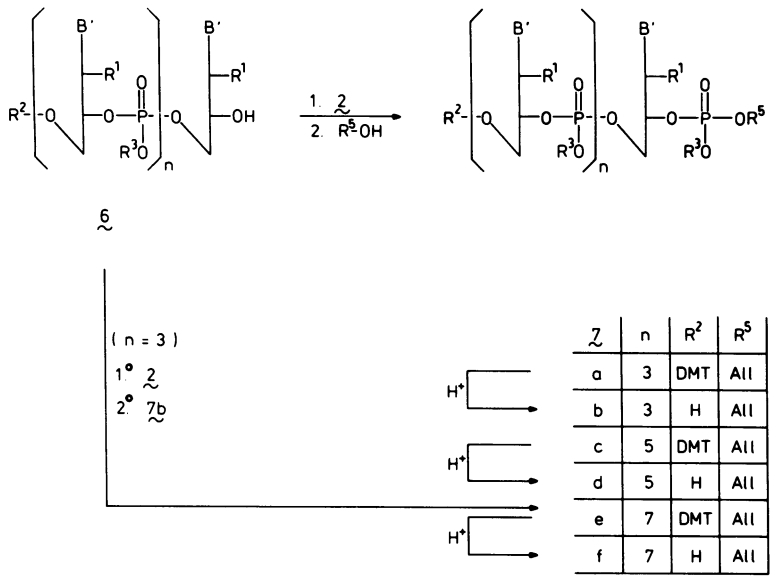


R^1 = H or O-Tetrahydropyranyl (O-THP)
R^2 = 4,4'-Dimethoxytrityl (DMT)
R^3 = 2-Chlorophenyl
R^4 = Levulinoyl (Lev)

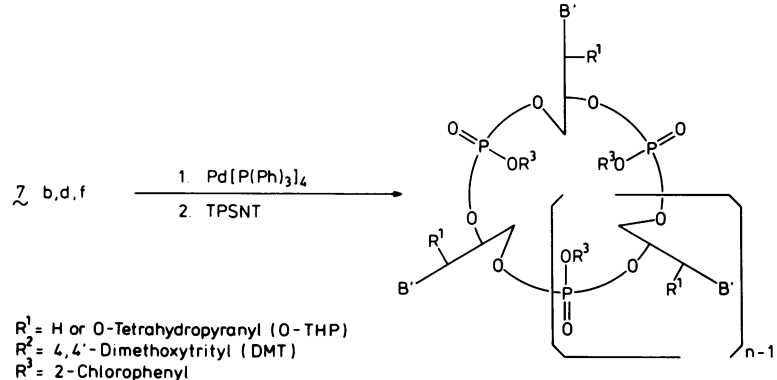
B' = N6, N6-Dimethyladenin-9-yl
N6-Benzoyladenin-9-yl
N4-Anisoylcytosin-1-yl
N2-Diphenylacetylguanin-9-yl
Thymidin-1-yl
Uridin-1-yl

Scheme 1

function at the 3'-end of 6 (*i.e.*, conversion of 6 into fully protected 7); (iii) removal of the protecting group at the 5'-end of 7 to give 7 (R^2 = H); (iv) removal of the protecting group R^5 from the 3'-phosphotriester in 7 to afford the 3'-phosphodiester 7 (R^2 = R^5 = H); (v) cyclisation of 7 (R^2 = R^5 = H) to furnish fully protected cyclic oligomers 8; (vi) deblocking of 8 thus



7	n	R^2	R^5
a	3	DMT	All
b	3	H	All
c	5	DMT	All
d	5	H	All
e	7	DMT	All
f	7	H	All



R^1 = H or O-Tetrahydropyranyl (O-THP)
R^2 = 4,4'-Dimethoxytrityl (DMT)
R^3 = 2-Chlorophenyl

8 a: n=3; R^1=O-THP
b: n=5; R^1=H
c: n=7; R^1=H

Scheme 2

obtained to yield the target cyclic oligonucleotides. A successful outcome of the individual steps mentioned above strongly depends on the choice of the 5'-hydroxyl (R^2) and 3'-phosphodiester (R^5) protecting groups. In this paper we wish to show that the 4,4'-dimethoxytrityl (R^2 = DMT) and the allyl (R^5 = All) protecting groups may be used to achieve our goal.

The preparation of the linear fragments 6 could be accomplished by

applying the earlier described bifunctional phosphorylating reagent 2-chlorophenyl-*bis*-O,O-(1-benzotriazolyl)phosphate (2)[6] which enables regioselective phosphorylation[8]. For example, the linear RNA fragment 6e [sequence r($A^{Bz}A^{Bz}A^{Bz}A^{Bz}$)] was assembled as follows. Phosphorylation of the riboadenosine derivative 1 (B' = A^{Bz}; R^1 = O-THP; R^2 = DMT) gave intermediate 3 which was coupled regioselectively with 4 (B' = A^{Bz}; R^1 = O-THP) to afford the partially protected dimer 5. Repetition (2 x) of this synthetic cycle (see Scheme 1) yielded the linear tetramer 6e (R^1 = O-THP). In a similar way, the preparation of the RNA tetramer 6e [sequence r($UA^{Me_2}UA^{Me_2}$)] could be realised by extending the uridine derivative 1 (B' = U; R^1 = O-THP; R^2 = DMT) with the non-terminal building units of N^6,N^6-dimethyladenosine and uridine 4 (B' = A^{Me_2} or U; R^1 = O-THP).

The linear DNA hexamers 6g [sequences d($C^{An_G}DPAC^{An_G}DPAC^{An_G}DPA$) and d($TA^{Bz}A^{Bz}A^{Bz}A^{Bz}A^{Bz}A^{Bz}$)] having a free 3'-hydroxyl group were obtained by applying the same phosphorylation method described above for the preparation of the RNA fragments. The assemblage of the required hexamers 6g was however performed by block-condensation of dimers 5 (R^1 = H) with tetramers 6d. Thus, in the case of 6g [sequence d($C^{An_G}DPAC^{An_G}DPAC^{An_G}DPA$)], dimer 5 [sequence d($C^{An_G}DPA$)] was first condensed with dimer 6b [sequence d($C^{An_G}DPA$)] which was prepared by levulinoylation[9] of 5 and subsequent acidolysis[10] of the DMT group. Acidolysis of tetramer 6c thus obtained gave 6d which was coupled with 5 [sequence d($C^{An_G}DPA$)] to afford, after work-up and purification by short-column chromatography[11], hexamer 6f in good yield. Hydrazinolysis[12] of 6f then yielded the required hexamer 6g. The other hexamer 6g [sequence d($TA^{Bz}A^{Bz}A^{Bz}A^{Bz}A^{Bz}A^{Bz}$)] was synthesized following the same protocol.

The next stage involves a two-step conversion of the linear fragments 6 into derivatives 7. In the first step 6 is phosphorylated with 2 to afford the intermediate hydroxybenzotriazolyl 3'-phosphotriester 7 (R^2 = DMT; R^3 = 2-chlorophenyl; R^5 = benzotriazolyl) which is then converted by the addition of a hydroxy compound (R^5OH) into the key 3'-phosphotriester 7 carrying a temporary protecting group R^5.

Thus far several protecting groups which proved to be more or less suitable for the 3'-terminal phosphodiester function have been proposed[13]. In this study we show that the allyl group, recently introduced by Hayakawa *et al.*[14] as a permanent protecting group in the preparation of DNA fragments, meets most of the requirements necessary to function as a suitable 3'-terminal protecting group. Thus, the introduction of the allyl group could easily be performed by adding allyl alcohol in the presence of pyridine to

intermediate dimer 7 (B' = A^{Bz}; R^1 = O-THP; R^2 = DMT; R^3 = 2-chlorophenyl; R^5 = benzotriazolyl). Work-up, after 30 min at 20°C, gave the corresponding triester 7 in a yield of 79%. Further, quantitative and selective removal of the allyl-group (R^5) from triester 7 could be effected within 5 min, as monitored by ^{31}P NMR spectroscopy and TLC analysis, by treatment with palladium tetrakistriphenylphosphine/triphenylphosphine/*n*-butylamine[14a].

It was also established that the allyl group was sufficiently stable to pyridine which is usually applied as a solvent in oligonucleotide synthesis. Monitoring of the stability of the 3'-terminal phosphotriester in 7 (n = 0; B' = T, R^1 = H; R^2 = DMT; R^3 = 2-chlorophenyl; R^5 = All) in anhydrous pyridine by ^{31}P NMR spectroscopy showed slow conversion ($t_{½}$ ≈ 20 h) of 7 (R^5 = All; δ_P: -6.31 and -6.55 ppm) into the corresponding phosphodiester 7 (R^5 = H; δ_P: -4.47 ppm). The ^{31}P- and ^1H NMR spectroscopic data of phosphodiester 7 thus obtained were in full accord with 7 prepared by hydrolysis of 3 (B' = T; R^1 = H; R^2 = DMT; R^3 = 2-chlorophenyl). Interestingly, we found that the stability of phosphotriester 7 (n = 0; B' = T, R^1 = H; R^2 = DMT; R^3 = 2-chlorophenyl; R^5 = All) decreased by a factor of ten ($t_{½}$ ≈ 2 h) in a mixture of pyridine and water.

In order to minimize the deleterious effect of pyridine on the stability of the allyl group, we decided to use only two equivalents of this base in the two-step phosphorylation process (*e.g.* conversion of partially protected 6 into fully protected 7). The two RNA tetramers 7a (R^1 = O-THP) and the two DNA hexamers 7c (R^1 = H) were conveniently prepared by coevaporation of the corresponding tetramers 6e and hexamers 6g with dioxane, followed by the addition of pyridine and a slight excess of phosphorylating reagent 2. The mixture was left for 5 min at 20°C and excess allyl alcohol was now added. Work-up, after 30 min, afforded the fully protected compounds 7a and 7c, respectively, in excellent yields.

The stability of the temporary allyl protecting group was further illustrated by the preparation of the DNA octamer 7e [sequence $d(C^{An}G^{DPA}T-G^{DPA}C^{An}G^{DPA}T_G^{DPA})$]. Thus, phosphorylation (see Scheme 2) of tetramer 6e with 2, and subsequently addition of tetramer 7b, carrying at the 3'-end a phosphotriester function (R^3 = 2-chlorophenyl; R^5 = All), gave crude 7e. Work-up and purification furnished octamer 7e in a yield of 69% (based on 7b).

The linear DNA and RNA fragments 7a,c,e were now converted into the corresponding fully protected circular molecules 8a-c. Firstly, the DMT groups were removed from 7a,c,e by acidolysis to afford, after purification, the partially deprotected oligomers 7b,d,f. The allyl group was then de-

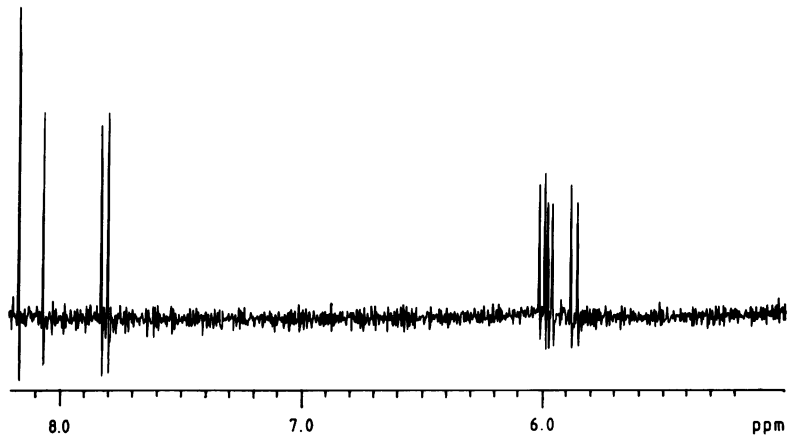


Figure 1. Low-field region of the ^{1}H NMR spectrum (300 MHz) of the cyclic tetramer r($UA^{Me_2}UA^{Me_2}$) showing the resonances of the H-1' and H-2, H-5, H-6, H-8 protons of the ribose and purine/pyrimidine moieties, respectively. Chemical shifts are in ppm relative to TMS.

blocked by treating 7b,d,f with palladium tetrakistriphenylphosphine/triphenylphosphine/*n*-butylamine in tetrahydrofuran for 5 min at 20°C. In all cases, TLC analysis indicated complete conversion of starting materials into compounds having zero mobility.

The cyclisation of the partially deprotected DNA/RNA fragments was effected by replacing the solvent tetrahydrofuran by pyridine (100 mL/mmol) followed by the addition of the activating reagent 2,4,6-triisopropylbenzenesulfonyl-3-nitro-1,2,4-triazole[15]. Work-up of the reaction products, after TLC analysis indicated the cyclisation to be complete (1.5-6 h, depending on the length of the starting fragments), afforded the fully protected cyclic oligomers 8a-c in a yield of 59-73%.

The cyclic oligomers were deblocked by treatment with N^1,N^1,N^3,N^3-tetramethylguanidinium *syn*-pyridine-2-carboxaldimate[16] followed by ammonolysis at 50°C. In the case of unblocking the cyclic RNA derivatives, the last step consisted of acidic hydrolysis (0.01 M HCl; pH 2.00; 20 h)[17] of the 2'-O-THP groups. The completely deblocked cyclic tetramers, hexamers and octamer were purified by gel-filtration and finally converted into the sodium salts. The homogeneity of c[r(AAAA)], c[r($UA^{Me_2}UA^{Me_2}$)], c[d(CGCGCG)], c[d(TAAAAA)] and c[d(CGTGCGTG)] was firmly established by Fast Protein Liquid Chromatography. ^{1}H- and ^{31}P NMR spectroscopy provided further evidence for the cyclic nature of the latter compounds. For example, it can be seen

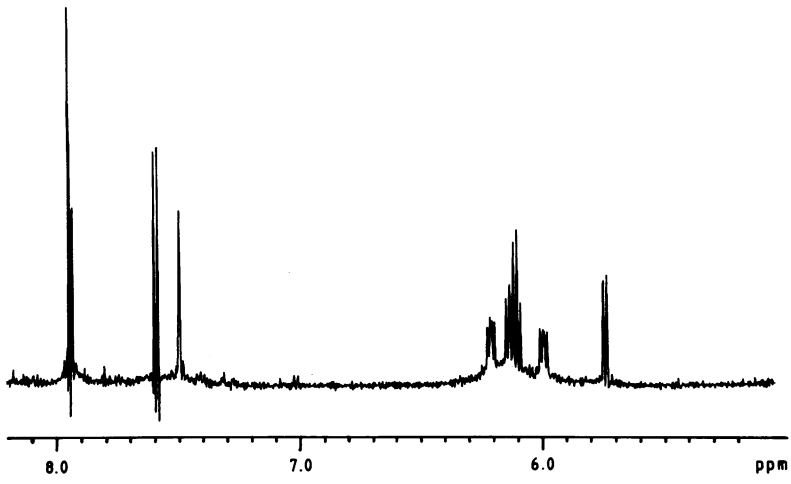


Figure 2. Low-field region of the ^{1}H NMR spectrum (300 MHz) of the cyclic octamer d(CGTGCGTG) showing the resonances of the H-1' and H-5, H-6, H-8 protons of the ribose and purine/pyrimidine moieties, respectively. Chemical shifts are in ppm relative to TMS.

(Fig. 1) that the H-1' protons of the uridine and N^6,N^6-dimethyladenosine nucleosides show-up as two separate doublets having each identical chemical shift and coupling values. The same is also true for the H-5, H-6 and H-2, H-8 protons of the uracil and modified adenine bases, respectively. A similar proton resonance pattern can also be discerned in Fig. 2 for the at low-field resonating protons of the DNA octamer. The above phenomenon, which was also observed for the other fragments, apart from the non-symmetrical compound 8b [sequence d(TAAAAA)], is in complete agreement[4,18] with the proposed structures of the synthetically prepared circular compounds.

The favourable properties (*i.e.* stability and ease of removal) of the allyl function together with its efficient introduction, using the bifunctional phosphorylating reagent 2, makes this easily accessible group particularly suitable to function as a terminal phosphodiester protecting group. It may also be mentioned that the properties of the allyl group are comparable with those of the recently for this purpose introduced 2,4-dinitrobenzyl[13e] group. On the other hand, we believe that the allyl group may be an alternative for other 3'-terminal protecting groups and, especially so, the 2-cyanoethyl group which was recently used by Hsu *et al.*[18] in the preparation in solution of cyclic diribonucleotides and by Barbato *et al.*[19] in the solid phase synthesis of cyclic oligodeoxyribonucleotides.

EXPERIMENTAL

General methods and materials

Dioxane, pyridine and THF were dried by refluxing with CaH_2 for 16 h and then distilled. Pyridine was redistilled from *p*-toluenesulfonyl chloride (60 g per litre) and KOH (25 g per litre). Dioxane and THF were redistilled from $LiAlH_4$ (5 g per litre). N-methylimidazole was distilled under reduced pressure. Allyl alcohol was distilled from K_2CO_3. All liquids were stored under nitrogen. Triethylammonium bicarbonate (TEAB) buffer was prepared by passing CO_2 gas through a cooled (0°C) solution of triethylamine (1 M in water) until a neutral solution was obtained. $(NH_4)_2HPO_4$ buffer: a solution of $(NH_4)_2HPO_4$ (1 M in water) was acidified with H_3PO_4 to pH 5.5.

Schleicher and Schüll DC Fertigfolien F1500 LS254 were used for TLC in CH_2Cl_2/CH_3OH (92:8, v/v). Short column chromatography was performed on Kieselgel 60 (230-400 mesh ASTM) suspended in CH_2Cl_2. Products were eluted with CH_2Cl_2, applying a 0 to 8% gradient of CH_3OH. Sephadex G25 was purchased from Pharmacia (Uppsala, Sweden). Palladium tetrakistriphenylphosphine was purchased from Fluka, 2,4,6-triisopropylbenzenesulfonyl-3-nitro-1,2,4-triazole (TPSNT) and partially protected deoxy-[20] and ribonucleosides[21,22] were prepared as described previously.

Fast Protein Liquid Chromatography was carried out on a Pharmacia LCC-500 liquid chromatograph equipped with a gradient mixing system, UV absorption detector (254 nm), and a photometer output recorder. Pre-packed strong anion-exchange resin Mono Q HR 5/5 (Pharmacia) was used. Gradient elution was performed at 20°C by building up a gradient starting with buffer A (0.01 M NaOH, pH 12.0) and applying buffer B (0.01 M NaOH, 1.2 M NaCl, pH 12.0) with a flow rate of 2.0 mL/min and a pressure of 3.0 kP.

^{31}P NMR spectra were measured at 80.7 MHz, proton-noise decoupled, using a JEOL JNM-FX 200 spectrometer. Chemical shifts are in ppm (δ) relative to 85% H_3PO_4 as external standard. ^1H NMR spectra were measured at 300 MHz, using a Bruker WM-300 spectrometer, equipped with an ASPECT-2000 computer, operating in the Fourier Transform mode. Tetramethylammonium chloride (TMA) was used as internal reference. Chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS; $\delta_{TMA}-\delta_{TMS} = 3.19$ ppm).

Sterile water and glassware were used during the whole deblocking and purification processes. Cation-exchange resin (sodium-form): a solution of NaOH (2 M; 100 mL) was passed over a column packed with cation-exchange resin (Dowex 50W x 8, 100-200 mesh; Fluka, H^+-form, 1.5 x 5 cm) followed by washing of the column with sterile water until pH = 7.0.

Synthesis of dimers 5 [d($A^{Bz}A^{Bz}$), d($C^{An}G^{DPA}$), d(TA^{Bz}), d(TG^{DPA}), r($A^{Bz}A^{Bz}$) and r($UA^{Me_2}UA^{Me_2}$)]

A solution of phosphorylating agent 2 in dioxane (22.0 mL; 4.4 mmol) was added to compound 1 (R^1 = H or O-THP; R^2 = DMT; 4.0 mmol) which had been dried by repeated coevaporation with pyridine (3 x 20 mL). After 5 min at 20°C, TLC analysis indicated complete conversion into intermediate 3. Compound 4 (R^1 = H or O-THP; 4.8 mmol) and N-methylimidazole (0.64 mL; 8.0 mmol) were transferred to the reaction mixture under the exclusion of moisture. After stirring for 30 min at 20°C, TLC analysis showed the absence of 3 and the formation of a new product. The reaction mixture was diluted with CH_2Cl_2 (200 mL) and washed twice with TEAB buffer (1 M; 200 mL, 0.1 M; 200 mL). The organic layer was dried ($MgSO_4$), concentrated to a small volume (15 mL) and triturated with petroleum-ether (40-60°C; 250 mL). The precipitate was collected by filtration and the crude product was purified by short column chromatography. Fractions containing pure 5 were concentrated under reduced pressure to give a colorless foam. Dimers were isolated in an average yield of 84% and ^{31}P NMR spectroscopy ($CDCl_3$) revealed the presence of two resonances (diastereomers) for each of the dimers between -8.0 and -6.9 ppm.

Synthesis of dimers 6b [d($A^{Bz}A^{Bz}$), d($C^{An}G^{DPA}$) and d(TG^{DPA})]

Levulinic acid anhydride (1.0 M in dioxane; 2.0 mL; 2.0 mmol) was added to compound 5 (R^1 = H or O-THP; 1.5 mmol) in pyridine (25 mL). After stirring for 1 h at 20°C, TLC analysis indicated conversion of 5 into 6a (R^1 = H or O-THP) and a few drops of TEAB buffer (1 M) were added. The reaction mixture was evaporated to near dryness, diluted with CH_2Cl_2 (75 mL) and washed twice with TEAB buffer (1 M; 100 mL, 0.1 M; 100 mL). The organic layer was dried over $MgSO_4$, concentrated under reduced pressure to a volume of 10 mL and triturated with petroleum-ether (40-60°C; 3 x 100 mL). The precipitate was collected by filtration and stored *in vacuo* for several hours to remove traces of pyridine. A solution of toluene-*p*-sulfonic acid monohydrate (2.0 g) in CH_2Cl_2/CH_3OH (7:3, v/v; 100 mL) was added and the resulting solution was stirred at 0°C. After 12 min, the reaction mixture was poured into an aqueous solution of $NaHCO_3$ (1 M; 200 mL). The organic layer was separated and washed with water, dried ($MgSO_4$), concentrated to a small volume (10 mL) and triturated with petroleum-ether (40-60°C; 2 x 100 mL). After purification by short column chromatography, 6b was isolated in an average yield of 69%. All dimers thus obtained revealed the presence of two ^{31}P NMR resonances (diastereomers) for each of the dimers between -8.1 and -6.6 ppm.

Synthesis of tetramers 6d [d($A^{Bz}A^{Bz}A^{Bz}A^{Bz}$) and d($C^{An_G}DPAC^{An_G}DPA$)]

A solution of 2 in dioxane (5.5 mL; 1.1 mmol) was added to dimer 5 (1.0 mmol), dried by coevaporation with pyridine (3 x 10 mL). After 5 min at 20°C, dimer 6b (0.9 mmol) and N-methylimidazole (0.16 mL; 2.0 mmol) were added under anhydrous conditions. TLC analysis, after 60 min, revealed the disappearance of 6b and a few drops of TEAB buffer (1 M) were added. The reaction mixture was diluted with CH_2Cl_2 and extracted twice with TEAB buffer (1 M; 75 mL, 0.1 M; 75 mL). The organic layer was dried over $MgSO_4$, concentrated to a small volume and triturated with petroleum-ether (40-60°C; 100 mL). Purification by short column chromatography yielded 6c [d($C^{An_G}DPA$-$C^{An_G}DPA$); 75%, d($TA^{Bz}A^{Bz}A^{Bz}$); 83% as white foams. Compounds 6c thus obtained were detritylated as described above (6b). After purification, tetramers 6d were isolated as colorless foams in an average yield of 83%.

Synthesis of tetramers 6e [d($C^{An_G}DPAT^{G}DPA$), r($A^{Bz}A^{Bz}A^{Bz}A^{Bz}$) and r(UA^{Me_2}-UA^{Me_2})]

After coevaporation of 5 (1.0 mmol) with pyridine (3 x 10 mL), a solution of 2 in dioxane (5.5 mL; 1.1 mmol) was added and the resulting mixture was left for 5 min. TLC analysis showed completion of the first phosphorylation step and 4 (1.25 mmol) and N-methylimidazole (0.15 mL; 2.0 mmol) were added. After 45 min the reaction mixture was diluted with CH_2Cl_2 and washed with TEAB buffer (1 M; 75 mL, 0.1 M; 75 mL). Drying and purification by titration (petroleum-ether 40-60°C) and short column chromatography gave a 3'-unprotected trimer in an average yield of 84%. The latter trimer was coupled with 4 in the same way as described above. Normal work-up and purification yielded pure 6e [d($C^{An_G}DPAT^{G}DPA$): 71%, r($A^{Bz}A^{Bz}A^{Bz}A^{Bz}$): 79%, r($UA^{Me_2}UA^{Me_2}$): 75%].

Synthesis of hexamers 6g [d($C^{An_G}DPAC^{An_G}DPAC^{An_G}DPA$) and d($TA^{Bz}A^{Bz}A^{Bz}A^{Bz}A^{Bz}$)]

Dimer 5 [d($C^{An_G}DPA$) or d(TA^{Bz}); 0.75 mmol] was phosphorylated as described above. After completion of the first phosphorylation step, 6d [d($C^{An_G}DPAC^{An_G}DPA$) or d($A^{Bz}A^{Bz}A^{Bz}A^{Bz}$); 0.67 mmol] and N-methylimidazole were added and the reaction was allowed to proceed for 60 min. TLC analysis indicated the absence of 6d and a few drops of TEAB buffer (1 M) were added. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and extracted with TEAB buffer (1 M; 100 mL, 0.1 M; 100 mL). The organic phase was dried ($MgSO_4$), evaporated under reduced pressure to a volume of 5 mL and triturated with petroleum-ether (40-60°C; 100 mL). The precipitate was collected by filtration and crude 6f thus obtained was purified by short column chromatography. Purified compounds 6f were deprotected as follows. To a stirred solution of

6f (0.50 mmol) in pyridine (5.0 mL) was added a solution of hydrazine hydrate (0.25 mL; 5.0 mmol) and glacial acetic acid (2.0 mL) in pyridine (5.0 mL). After stirring for 10 min at 20°C, the reaction flask was immersed into an ice-water bath and pentane-2,4-dione (1.0 mL; 10 mmol) was added to the reaction mixture. After 3 min, the mixture was diluted with CH_2Cl_2 (50 mL) and washed with TEAB buffer (1 M; 75 mL, 0.1 M; 75 mL). Further work-up and purification as described above yielded pure 6g [d($C^{An}_{G}DPA_{C}A^{n}_{G}DPA_{C}A^{n}_{G}DPA$): 63%, d($TA^{Bz}_{A}Bz_{A}Bz_{A}Bz_{A}Bz_{A}Bz$): 69%, based on 6d] as white foams.

General procedure for the synthesis of compounds 7b [d($C^{An}_{G}DPA_{T}G^{DPA}$), r(A^{Bz}-$A^{Bz}_{A}Bz_{A}Bz_{A}Bz$) and r($UA^{Me_2}UA^{Me_2}$)] and 7d [d($C^{An}_{G}DPA_{C}A^{n}_{G}DPA_{C}A^{n}_{G}DPA$) and d($TA^{Bz}_{A}Bz$-$A^{Bz}_{A}Bz_{A}Bz_{A}Bz$)]

Compound 6e or 6g (0.50 mmol) was coevaporated with dioxane (3 x 10 mL) and pyridine (0.10 mL; 1.3 mmol) was added. The resulting viscous residue was treated with 2-chlorophenyl-*bis*-O,O-(1-benzotriazolyl)phosphate (0.2 M; 2.75 mL; 0.55 mmol). TLC analysis, after 5 min, indicated complete conversion of starting material into a compound having zero mobility. Allyl alcohol (0.05 mL; 0.74 mmol) was added and the mixture was stirred for 30 min. TLC analysis showed the complete disappearance of trityl-positive baseline material. The mixture was diluted with CH_2Cl_2 and washed twice with $(NH_4)_2HPO_4$ buffer (1 M; 75 mL, 0.1 M; 75 mL). The organic layer was dried ($MgSO_4$), concentrated to a small volume (5 mL) and finally triturated with petroleum-ether (40-60°C; 2 x 75 mL). The white precipitate was filtered off and purified by short column chromatography. Compounds 7a and 7c were isolated as white foams in an average yield of 77%. Detritylation of 7a and 7c as described above afforded after work-up [washing with $(NH_4)_2HPO_4$ buffer] and purification pure 7b and 7d in an average yield of 82%.

Synthesis of octamer 7f [d($C^{An}_{G}DPA_{T}G^{DPA}_{T}G^{DPA}_{C}A^{n}_{G}DPA_{T}G^{DPA}$)]

Tetramer 6e [d($C^{An}_{G}DPA_{T}G^{DPA}$); 1.18 g; 0.50 mmol] was coevaporated with dioxane (3 x 10 mL) and pyridine (0.10 mL; 1.3 mmol) was added. A 0.2 M stock solution of 2 in dioxane (2.75 mL; 5.50 mmol) was added, and the reaction was allowed to stand for 5 min. TLC analysis indicated completion of the first phosphorylation step and tetramer 7b [d($C^{An}_{G}DPA_{T}G^{DPA}$-p-allyl); 1.15 g; 0.45 mmol] and N-methylimidazole (0.75 mL; 1.0 mmol) were added under anhydrous conditions. After 90 min, a few drops of $(NH_4)_2HPO_4$ buffer (1 M) were added. Work-up and purification as described for 7b/7d afforded 7e as a colorless foam. Yield: 1.48 g (0.31 mmol; 69%). Detritylation as described above gave 7f as a colorless foam. Yield: 1.08 g (0.24 mmol; 77%).

Synthesis of fully protected cyclic oligonucleotides 8a,b,c

Compounds 7b,d,f (0.30 mmol) was dissolved in THF (7.5 mL) and $Pd[P(C_6H_5)_3]_4$ (15 mg; 15 µmol), $P(C_6H_5)_3$ (24 mg; 90 µmol) and *n*-butylamine (0.60 mL; 6 mmol) were added. After stirring for 5 min at 20°C, TLC analysis revealed the complete formation of baseline material. The reaction mixture was evaporated to near dryness and coevaporated with pyridine (5 x 10 mL). The resulting viscous sirup was dissolved in pyridine (60 mL) and TPSNT (571 mg; 1.5 mmol) was added. TLC analysis indicated completion of the cyclisation of 8a, 8b and 8c in 3 h, 5 h and 12 h, respectively. A few drops of TEAB buffer (1 M) were added and the reaction mixture was evaporated to a small volume (10 mL) and diluted with CH_2Cl_2 (75 mL). The product was washed twice with TEAB buffer (1 M; 100 mL, 0.1 M; 100 mL) and the organic layer was dried ($MgSO_4$) and concentrated to near dryness. Crude 8a-c thus obtained was purified by short column chromatography. Pure cyclic compounds 8a, 8b and 8c were isolated in yields of 73% (average), 69% (average) and 59%, respectively.

Deprotection and purification of compounds 8a,b,c

The fully protected tetramers 8a (90 µmol), hexamers 8b (70 µmol) or decamer 8c (50 µmol) were dissolved in a solution of *syn*-pyridine-2-carboxaldoxime (0.70 g; 5.75 mmol) and N^1,N^1,N^3,N^3-tetramethylguanidine (0.58 g; 5.00 mmol) in anhydrous pyridine (15 mL). After stirring for 16 h at 20°C, concentrated ammonia (14.8 M; 100 mL) was added and the mixture was left in a securely sealed flask for 48 h at 50°C. The reaction mixture was concentrated to a small volume (2 mL) and, in case R^1 = O-THP, acidified with hydrochloric acid to pH = 2.00 (meter reading) and left to stand for 20 h at 20°C. In the latter case, a few drops of ammonia (5 M) were added and the mixture was concentrated to a small volume (2 mL). Crude compounds 8a-c thus obtained were purified by extraction with ether (2 x 75 mL) and gel-filtration [Sephadex G50; 2 x 200 cm; suspended in and eluted with TEAB buffer (0.05 M)]. Fractions were analyzed by FPLC and those containing pure cyclic tetramer, hexamer or octamer were pooled, concentrated to a small volume and evaporated twice with water (50 mL) to remove TEAB buffer. All compounds thus obtained were converted into the sodium salt by passing over a column (1.5 x 5 cm) of Dowex 50W cation exchange resin (sodium-form) and the resulting aqueous solutions were lyophilised and relyophilised from D_2O (3 x 2 mL). ^1H NMR spectra were recorded in D_2O, TMA was used as internal reference, δ-values are given in ppm relative to TMS. ^{31}P NMR spectra were recorded in D_2O, δ-values are given relative to H_3PO_4 (external reference).

8a [r(AAAA), R^1 = OH, R^3 = Na^+]: 69 mg (49 µmol, 54%); ^1H NMR: 8.18 (H-8, s, 4H), 8.10 (H-2, s, 4H), 5.98 (H-1', d, 7.0 Hz, 4H); ^{31}P NMR: 0.27 (s, 4P); Anal. Calcd. (MW 1404.77): P 8.82; Found P 8.78.

8a [r($UA^{Me_2}UA^{Me_2}$), R^1 = OH, R^3 = Na^+]: 78 mg (55 µmol, 61%); ^1H NMR: 8.17 (H-8, s, 2H), 8.07 (H-2, s, 2H), 7.82 (H-6, d, 8.2 Hz, 2H), 6.00 (H-1', d, 7.1 Hz, 2H), 5.97 (H-1', d, 5.9 Hz, 2H), 5.87 (H-5, d, 8.1 Hz, 2H), 3.30 (CH_3, s, 6H); ^{31}P NMR: 2.25 (s, 2P), 0.17 (s, 2P); Anal. Calcd. (MW 1418.83): P 8.73; Found P 8.59.

8b [d(CGCGCG), R^1 = H, R^3 = Na^+]: 80 mg (42 µmol, 60%); ^1H NMR: 7.97 (H-8, s, 3H), 7.70 (H-6, d, 7.6 Hz, 3H), 6.22 (H-1', t, 8.3 Hz/6.3 Hz, 3H), 6.16 (H-1', t, 8.1 Hz/6.4 Hz, 3H), 5.91 (H-5, d, 7.6 Hz, 3H); ^{31}P NMR: -0.98 (s, 3P), -1.10 (s, 3P); Anal. Calcd. (MW 1987.08): P 9.35; Found P 9.30.

8b [d(TAAAAA), R^1 = H, R^3 = Na^+]: 94 mg (47 µmol, 67%); ^1H NMR: 8.13 (H-8, m, 5H), 8.02 (H-2, m, 5H), 7.93 (H-6, s), 6.17 (H-1', m, 6H), 2.32 (CH_3, s, 3H); ^{31}P NMR: 0.23 (s), 0.11 (s, 2P), -0.11 (s), -0.61 (s); Anal. Calcd. (MW 2002.15): P 9.28; Found P 9.16.

8c [d(CGTGCGTG), R^1 = H, R^3 = Na^+]: 75 mg (28 µmol, 56%); ^1H NMR: 7.95 (H-8, s, 2H), 7.94 (H-8, s, 2H), 7.59 (H-6, d, 7.5 Hz, 2H), 7.50 (H-6, s, 2H), 6.21 (H-1', m, 2H), 6.12 (H-1', m, 4H), 6.01 (H-1', m, 2H), 5.74 (H-5, d, 7.5 Hz, 2H), 2.19 (CH_3, s, 6H); ^{31}P NMR: -0.02 (s), -0.09 (s), -0.21 (s), -0.53 (s); Anal. Calcd. (MW 2679.47): P9.25; Found P 9.12.

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