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SYNTHESIS OF DNA FRAGMENTS BY THE HYDROXYBENZOTRIAZOLE PHOSPHOTRIESTER APPROACH

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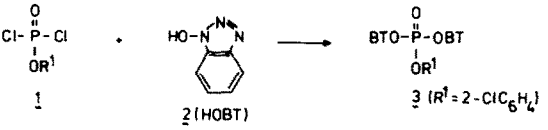
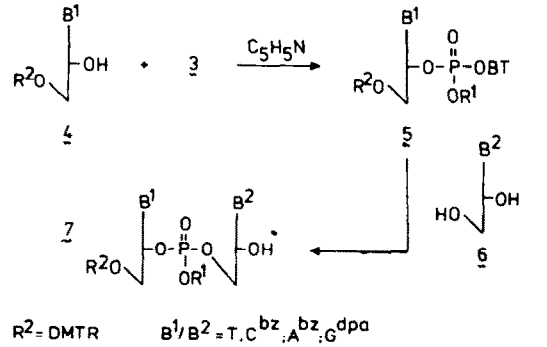
Abstract—The application of the phosphorylating agent 2-chlorophenyl-o,o-bis-[1-benzotriazolyl]-phosphate (HOBT-approach) for the introduction of 3'-5'-phosphotriester linkages between a properly 5'-protected (4,4'-dimethoxytrityl) deoxy-nucleoside and a deoxy-nucleoside having free OH groups is described. In this way, all sixteen partially-protected DNA dimers having a free 3'-OH group have been synthesized. The dimers thus obtained have been applied, using the HOBT-approach, to the synthesis of the DNA fragments d-CGCGCG, d-CGTACG and d-ATATTAATAAC.

In earlier studies we reported on the use of the bifunctional phosphorylating agent **3** (R^1=2-chlorophenyl; Scheme I) in nucleic acid chemistry.



Scheme I.

Thus we demonstrated that reagent **3** could be applied for: (i) the synthesis of 3'-phosphotriesters[1,2] of properly protected deoxy- or ribo-nucleosides; (ii) the formation of 3'-5'-internucleotide linkages[1,3] between deoxy- or ribo-nucleosides having 3'- or 5'-unprotected OH groups; (iii) synthesis of DNA fragments on a solid support.[4] We also showed[5] that reagent **3**, in which OR^1 was replaced by a morpholino group, could be used for the synthesis of 5'-phosphorylated DNA fragments. Furthermore, we recently reported,[6] that the phosphorylating agent **3** could be applied for the selective introduction of a 3'-5'-phosphotriester linkage between a ribonucleoside having a free 3'-OH and another ribonucleoside with unprotected 3'- and 5'-OH groups. We now wish to report that 2-chlorophenyl-o,o-bis-[1-benzotriazolyl]-phosphate (i.e. reagent **3**) can also be used for the synthesis of dimers **7** (Scheme II)



Scheme II.

having a free 3'-OH group and fully protected DNA oligonucleotides.

RESULTS AND DISCUSSION

The selective introduction of a 3'-5'-phosphotriester linkage which was accomplished by reacting reagent **3** with two ribonucleosides of which the incoming ribonucleoside carried free 3'- and 5'-OH groups, urged us to find out if this phosphorylation was also applicable to the synthesis of DNA dimers in which the incoming deoxynucleoside has unprotected OH groups (i.e. deoxynucleoside **6** in Scheme II).

We therefore treated the partially protected deoxynucleoside **4** (R^2=4,4'-dimethoxytrityl; 1 mmole) with a small excess of **3** (R^1=2-chlorophenyl; 1.15 mmole). TLC-analysis (Experimental), of the mixture after 15 min, revealed that starting compound **4** was completely converted into a product with zero mobility. An excess of deoxynucleoside **6** (1.25 mmole) in pyridine was now added to the reaction mixture. TLC-analysis after 1 hr, showed the absence of **5**. The presence of a main product together with some minor products having the highest R_f-values, and the deoxynucleoside **6**. Furthermore, the main product and also the products with the highest mobilities gave a positive 4,4'-dimethoxytrityl (DMTR)-test. Work-up of the mixture, followed by short-column chromatography,[7] afforded a homogeneous main product and a mixture consisting of the minor impurities. ^{31}P NMR spectroscopy of the main product showed, in most cases (Table 1), the presence of two P resonances. The identity of the main product was further corroborated as follows. Benzoylation of the product, followed by complete removal of all protective groups (see later), gave a DNA dimer which was completely degraded by enzymatic digestion with venom phosphodiesterase. The above data are in complete agreement with the formation of dimer **7** (i.e. a dimer having a 3'-5'-phosphotriester linkage and a free 3'-OH group).

In order to get a better insight into the nature of the previously mentioned minor impurities, we analyzed the mixture obtained by preparing dimer **7** (B^1 = T; B^2 = C^{bz}) as follows. ^{31}P-NMR spectroscopy of the crude product obtained after work-up and rapid purification over a small bed of silica-gel

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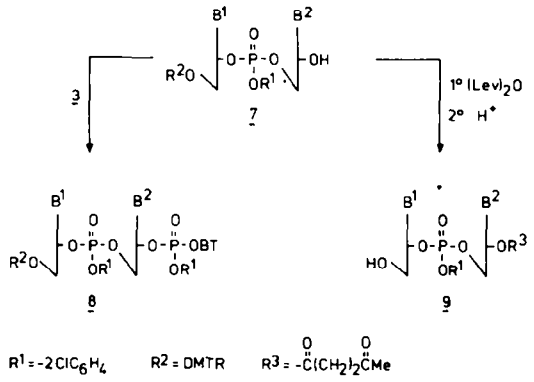
Table 1. Yields and ^{31}P NMR data of dimers **7** prepared according to Scheme II

B^1	B^2	Yield[a]	^{31}P-NMR[b]	B^1	B^2	Yield[a]	^{31}P-NMR[b]
T	T	74	-7.3:-8.4	A	T	65	-7.6:-7.9
T	C	57	-7.2:-8.4	A	C	55	-7.3:-7.7
T	A	74	-7.8:-8.2	A	A	64	-7.8
T	G	71	-8.4:-8.7	A	G	77	-8.0:-8.4
C	T	60	-7.0:-8.5	G	T	76	-6.6:-7.8
C	C	50	-7.1:-8.1	G	C	50	-6.6:-8.7
C	A	77	-7.6:-8.4	G	A	65	-7.3:-8.2
C	G	71	-7.9:-8.5	G	G	72	-7.8:-8.6

a) Yields (%) are based on compound **4**.
b) δ-Values (ppm) relative to the internal standard H_3PO_4 (85%)

showed: (i) two resonances at −7.2 and −8.4 ppm which are in accordance with **7** ($B^1 = T$; $B^2 = C^{bz}$) having a 3′-5′-triester linkage; (ii) two resonances at −8.2 and −9.3 ppm which indicates the presence of **7** ($B^1 = T$; $B^2 = C^{bz}$) having a 3′-3′-phosphotriester linkage; (iii) other resonances which we tentatively assigned to the presence of a trimer which was formed by the reaction of **7** with **5**. The formation of this trimer was based on the following evidence. Purification (Sephadex A-25 ion-exchange chromatography) of the minor products obtained, after benzoylation with benzoic anhydride, followed by *syn*-pyridine-2-carboxaldoximate,[8] aqueous ammonia and acetic acid treatment, afforded mainly two products having different mobilities as followed from ion-exchange HPLC-analysis.[9] We also observed that both compounds were resistant to enzymatic digestion with venom phosphodiesterase. The latter finding is in accordance with the absence of a free 3′-OH function in these products. The yields and ^{31}P NMR data of all sixteen dimers prepared according to Scheme II are recorded in Table 1.

It can be seen from these data that the yields of dimers **7** in which B^2 is a cytosine residue are rather low. The main reason for these low yields, in comparison with dimers **7** in which $B^2 = A^{bz}$; G^{dpa}; or T, is that a greater amount, as judged from TLC-analysis, of the above mentioned impurities are been formed. The dimers **7** could easily be converted (Scheme III) into terminal (i.e. **9**) and non-terminal (i.e. **8**) dimers.



Scheme III.

Thus **7** was quantitatively (TLC-analysis) converted with **3**, under the same conditions as used for the phosphorylation of **4** with **3**, into the 3′-phosphorylated dimer **8**.

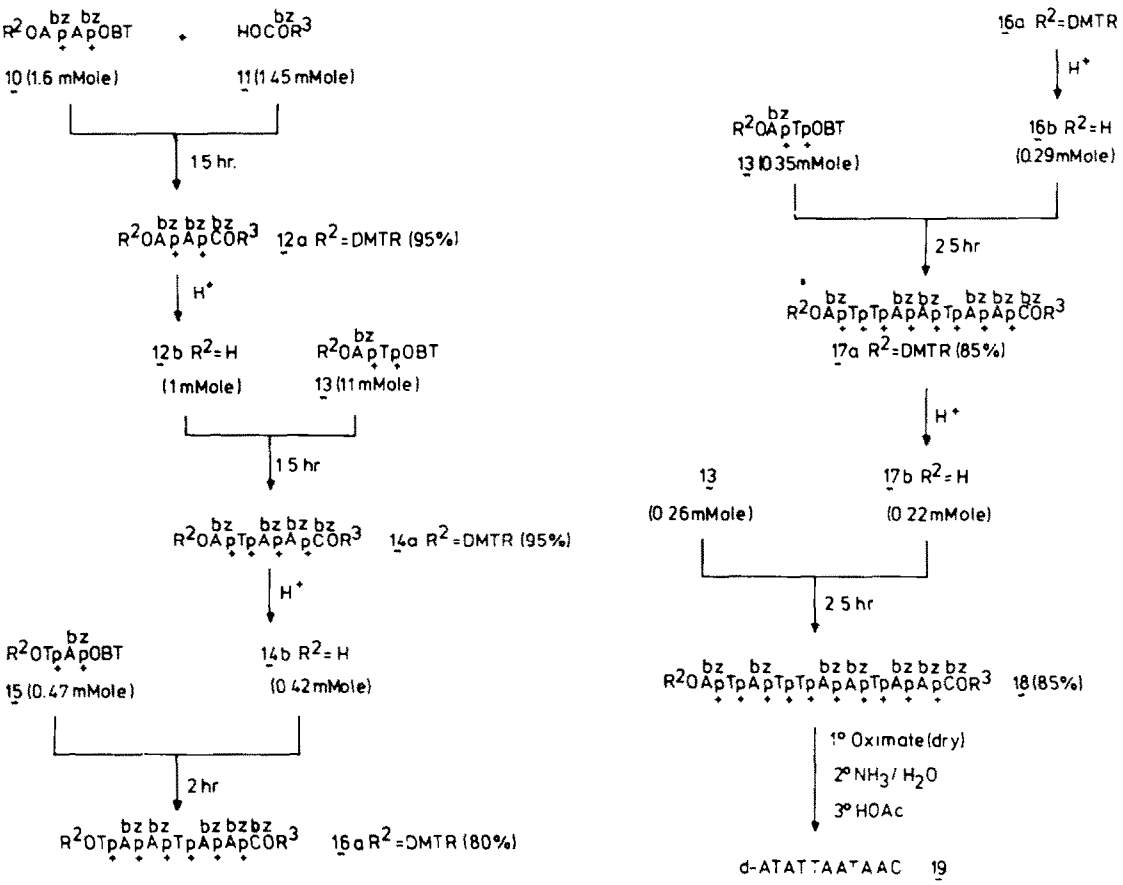
The other type, dimer **9**, was easily accessible by the well-known sequence of blocking (levulinoylation[10]) and deblocking (benzenesulfonic acid[11]) procedures as illustrated in Scheme III. The application of the non-terminal dimers **8** will now be demonstrated (Scheme IV) in the synthesis of the naturally occurring[12] undecamer **19**.

Close inspection of the assemblage route in Scheme IV reveals that in every coupling step a small excess of the dimers (i.e. **10**; **13**; **15**) has been used. It can also be seen that the yields (based upon the 5′-OH components) of the isolated fully protected DNA fragments (i.e. **12a**; **14a**; **16a**; **17a** and **18**) are very satisfactory, and that the yields do not fall-off dramatically with increasing length of the incoming partially protected (5′-OH free) DNA fragments.

Another noteworthy feature which emerges from Scheme IV is that the difference in coupling time of a dimer with a monomer (i.e. **10** with **11**) and a dimer with a nonamer (i.e. **13** with **17b**), is 60 min. In order to get information about the efficiency of the second coupling processes involved in the synthesis of **19**, we completely deblocked most of the intermediate fully protected DNA fragments in Scheme IV and analyzed the crude products thus obtained by ion-exchange HPLC-analysis. The results of this analytical study are illustrated in Fig. 1.

From the data presented in Fig. 1 we may conclude that the hydroxybenzotriazole phosphotriester approach (HOBT-approach) affords DNA fragments of rather high quality. The required DNA fragment **19** obtained by deblocking fully protected **18** was purified by Sephadex G50 column-chromatography,[10] to afford homogeneous **19** which was completely digested by venom phosphodiesterase.

In the same way as illustrated in Scheme IV for the synthesis of the undecamer **19**, we prepared the fully-protected hexamer d-CGCGCG by condensing dimer **9** ($B^1 = C^{bz}$; $B^2 = G^{dpa}$) with dimer **8** ($B^1 = C^{bz}$; $B^2 = G^{dpa}$) to afford a fully-protected tetramer. The latter, after removal of the DMTR-group, was condensed with dimer **8** ($B^1 = C^{bz}$; $B^2 = G^{dpa}$), to give the fully-protected hexamer. We also prepared the hexamer d-CGTACG starting from the terminal dimer **9**



Scheme IV.

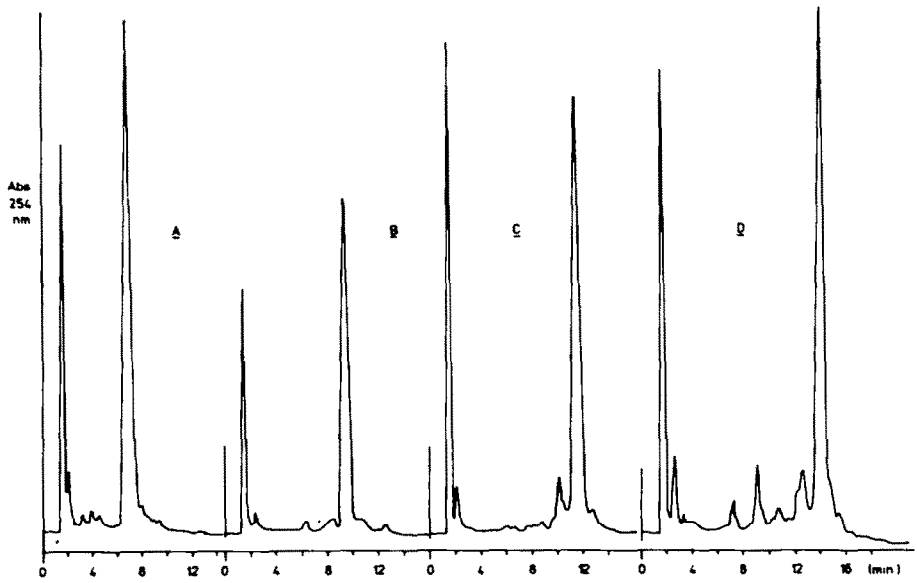


Fig. 1. HPLC-analysis (system I) of the crude DNA fragments obtained after complete deblocking. (a) Fully deblocked pentamer **14**, (b) fully deblocked heptamer **16**, (c) fully deblocked nonamer **17**, (d) fully deblocked undecamer **19**.

($B^1 = C^{bz}$; $B^2 = G^{dpe}$) and the two non-terminal dimers **8** having the sequence $B^1 = T$; $B^2 = A^{bz}$ and $B^1 = C^{bz}$; $B^2 = G^{dpe}$, respectively. In this respect, it is interesting to note that all fully-protected DNA intermediates were isolated in an average yield of 90%. Furthermore, complete deblocking of the two fully-protected hexamers, followed by purification on Sephadex G50 column chromatography, afforded the two hexamers, which were in every aspect—^{1}H- and ^{31}P-NMR spectroscopy, chromatographic behaviour —identical with the hexamers d-CGCGCG and d-CGTACG which have been synthesized[13] earlier by

us and applied successfully to serve as tools for biophysical studies.[14,15]

The synthesis of dimers **7** starting from a 5′-protected and a deoxynucleoside having free OH functions (i.e. **6**) has been reported earlier by several groups. A common feature of this earlier work was the use of the bifunctional phosphorylating agent 2-chlorophenylphosphorobis-(1,2,4-triazolide)[16] which was generated from 2-chlorophenyl phosphorodichloridate, 1,2,4-triazole and triethylamine. In one approach, dimers **7**[17] (R^1 = 2-chlorophenyl; R^2 = 2-dibromomethylbenzoyl) or dimers **7**[18] (R^1 = 2-chlorophenyl; R^2 = pixyl) were obtained by treating the appropriate d-nucleosides **4** with this particular agent, followed by hydrolysis with triethylamine-water, to afford the triethylammonium salts of the 3′-(2-chlorophenyl) phosphate of **4**, which were then coupled in the presence of an activating agent (i.e. 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole[19]), with the deoxynucleosides **6** (Scheme II).

In another approach[20a–d] to the synthesis of dimers **7**, the deoxynucleosides **6** were coupled, in the presence of 4-dimethylaminopyridine or 1-methylimidazole with the 3′-(2-chlorophenyl)-phosphoromono-(1,2,4-triazolide) derivatives of **4** obtained by treating **4** (R^2 = DMTR or pixyl) with 2-chlorophenylphosphorobis-(1,2,4-triazolide).

Our results show that 2-chlorophenyl-o,o-bis-[1-benzotrizolyl]-phosphate **3** presents an alternative for the above mentioned methods for the synthesis of dimers **7** having unprotected 3′-OH functions. With respect to the phosphorylating agent **3**, it is interesting to note that the phosphorylating agent benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate[21] has been used for the synthesis of dimers **7**, and that the rate-determining step was the formation of the intermediate **5** (Scheme II). Furthermore, we showed that the HOBT-approach could be applied successfully for the preparation of DNA fragments in solution. The synthesis of DNA in solution using 2-chlorophenylphosphorobis-(1,2,4-triazolide) in the absence of an activating agent, has been recently demonstrated,[22] however, the formation of the 3′-5′-phosphotriester linkages was rather slow.

In conclusion, the data presented in this paper indicate that the phosphorylating agent **3** (Scheme I) presents an attractive alternative for the existing methods which have been developed for the introduction of 3′-5′-phosphotriester bonds. The advantage of this approach in comparison with, *inter alia*, others phosphotriester approaches is that no activating agents, which may lead to modification of the thymine or guanine bases,[23,24] are required.

EXPERIMENTAL

General methods and materials. Pyridine and dioxane were dried by refluxing with CaH_2 for 16 hr and then distilled. Pyridine was redistilled from *p*-toluenesulfonyl chloride (50 g/l.) and stored over molecular sieves 4 Å. Dioxane was redistilled from $LiAlH_4$ (5 g/l.) and stored over molecular sieves 4 Å. 1-Methylimidazole was distilled under reduced pressure and stored over molecular sieves 4 Å. 1-Hydroxybenzotriazole (purchased from Aldrich) was dried *in vacuo* (P_2O_5) for 70 hr at 50°. Schleicher and Schüll DC Fertigfolien F 1500 LS 254 were used for TLC analysis in the solvent system CH_2Cl_2–MeOH; 85:15, v/v. The phosphorylation and coupling reactions were quenched on TLC-sheets with a mixture of pyridine–water (1:1, v/v). Short column chromatography was performed on Kieselgel 60 (230–400 mesh ASTM) suspended in CH_2Cl_2. ^1H NMR spectra were measured at 100 MHz with a Jeol-JNMPS-100 spectrometer; shifts are given in ppm (δ) relative to TMS as internal standard.

^{13}C NMR and ^{31}P NMR spectra were measured at 25.15 MHz and 40.48 MHz, respectively, with a Jeol-JNMFT-100 spectrometer equipped with an EC-100 computer, operating in the Fourier transform mode; proton noise decoupling was used. ^{13}C-chemical shifts are given in ppm (δ) relative to TMS as internal standard. ^{31}P-chemical shifts in ppm (δ) relative to 85% H_3PO_4 as external standard.

High performance liquid chromatography was carried out on a Micromeritics liquid chromatograph 7000B. UV absorption (254 nm) was monitored with a Spectra Physics SP8200 detector.

High performance anion-exchange chromatography (system I) was performed as described earlier[9] using the anion-exchange resin Permaphase AAX (Dupont, USA) dry-packed into a stainless-steel column (1 m × 2.1 mm). The column was eluted with a linear gradient, starting with buffer A (0.005 M KH_2PO_4, 10% acentonitrile, pH = 4.5) and applying 3% buffer B (0.05 M KH_2PO_4, 0.5 M KCl, 10% acetonitrile, pH = 4.5) per min. A flow of 1 ml/min at 50° was standard. Reverse-phase HPLC (system II) was performed on a Zorbax ODS column (25 cm × 4.6 mm). The column was eluted with a linear gradient of acetonitrile (1–20% in 20 min) in 0.1 M ammonium acetate buffer pH = 5.5, and with a flow-rate of 1 ml/min at 20°.

Synthesis of N-acyl protected deoxynucleosides **6**. 4-N-benzoyldeoxycytidine and 6-N-benzoyldeoxyadenosine were obtained according to the procedure described by G. S. Ti *et al.*[25] 2-N-diphenyl-acetyldeoxyguanosine was prepared by slightly modifying this procedure.

Synthesis of 2-N-*diphenylacetyldeoxyguanosine* **6** ($B^2 = G^{dpa}$). To a suspension of deoxyguanosine (10 mmole) in anhyd pyridine (40 ml) was added chlorotrimethylsilane (6.4 ml). The mixture was stirred for 30 min at 20° then, a stock soln of diphenylacetyl chloride in dioxan (1 M; 12 ml) was added.

The reaction was monitored by TLC. The soln was stirred for 60 min at room temp, cooled in an ice-water bath and water (10 ml) was then added. After 5 min, 20 ml aqueous ammonia (25%) was added and the mixture was stirred for 30 min at 20°. The mixture was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (150 ml) and the insoluble material was filtered off. The CH_2Cl_2 layer was washed with water, dried with $MgSO_4$ and concerntrated under reduced pressure. The oil was triturated with petroleum ether (40–60°)–ether (2:1, v/v) and the crude product was applied to a short kieselgel column (20 g). The column was eluted with CH_2Cl_2–MeOH (95:5, v/v). The appropriate fractions were collected and concentrated to give pure 2-N-diphenylacetyldeoxyguanosine (3.2 g, 70%) as a foam. ^1H NMR ($CDCl_3/CD_3OD$): δ = 8.14 (s, H8), 7.28 (m, phenyl), 6.21 (t, H_1'), 5.19 (s, CH). ^{13}C-NMR ($CDCl_3/CD_3OD$): δ = 87.9, 40.5, 71.4, 84.9, 62.2, (s, C_1'-C_5' β-D-2-deoxyribofuranose); 147.9, 148.4, 156.0, 121.1, 138.9 (s, C_2, C_4, C_5, C_6, C_8 guanine); 127.7, 128.8, 137.9 (s, phenyl); 175.0 (s, C=O diphenylacetyl)).

Synthesis of diphenylacetyl chloride. To a soln of diphenylacetic acid (21.2 g, 100 mmole) in anhyd dioxane (100 ml) was added 17 ml of distilled oxalyl chloride. The mixture was refluxed for 3 hr. The soln was concentrated to an oil under reduced pressure. The residue was diluted with anhydrous dioxan to give a 1 M stock soln of diphenylacetyl chloride. This soln could be stored for several weeks at 0–5°.

General procedure for synthesis of dimers **7**

Step 1: *preparation of intermediate* **5**. Compound **4** (1.0 mmole; B = T, A^{bz}, C^{bz} or G^{dpa}) was dissolved in anhyd pyridine and evaporated to dryness. To the dried residue was added 5.75 ml of a 0.2 M soln of the phosphorylating

agent **3** (1.15 mmole; R_1=2-ClC_6H_4) in dioxan and the mixture was stirred for 15 min at room temp. TLC analysis of the mixture showed that the starting compound **4** was completely converted into intermediate **5** ($R_f = 0$). The thus obtained soln of **5** was immediately used for the synthesis of dimers **7**.

Step 2: preparation of dimers **7**. The nucleoside **6** (1.25 mmole; B = T, C^{bz}, A^{bz} or G^{dpa}) was dissolved in anhyd pyridine (18 ml) and concentrated to one third of the initial volume. To this soln was added **5** (1.0 mmole; see above). The pyridine–dioxan mixture was stirred for 1 hr at 20°. TLC analysis of the mixture showed the absence of **5**. The mixture was diluted with CH_2Cl_2 (100 ml), washed with 1 M TEAB (triethylammonium bicarbonate, 30 ml) and with water (30 ml). The CH_2Cl_2 layer was dried with $MgSO_4$ and concentrated under reduced pressure to an oil. The crude product was triturated with petroleum ether (40–60°) and applied to a column of Kieselgel (10 g). The column was eluted with CH_2Cl_2–MeOH (98–90:2–10, v/v). The appropriate fractions were concentrated and precipitated from petroleum ether (40–60°). The ppt was filtered off and stored *in vacuo* (KOH-pellets). The yields together with the ^{31}P-chemical shifts of the pure dimers thus obtained, are recorded in Table 1. All 16 dimers recorded in Table 1 were completely deblocked by the following procedure. The dimers were first benzoylated by treating with benzoic acid anhydride. The fully protected dimers were deblocked by first treating them with N^1,N^1,N^3,N^3-tetramethylguanidinium *syn*-pyridine-2-carboxaldoximate, and then with aqueous ammonia and AcOH. The crude products thus obtained were purified by anion-exchange chromatography on a column of DEAE Sephadex A25. All purified dimers were completely degraded with venom phosphodiesterase; this followed from reverse-phase HPLC analysis (system II) of the digested products.

Preparation of dimer **8** (B^1/B^2 = T; C^{bz}, A^{bz}; G^{dpa}). The preparation of dimer **8** was performed in the same way as described in step 1 of the general procedure for the synthesis of dimers. Thus, dimer **7** (1.0 mmole) was treated with a slight excess of **3** (1.15 mmole). After 30 min, TLC analysis of the mixture showed the reaction to be complete: compound **7** was completely converted into dimer **8** ($R_f = 0$).

Preparation of dimer **9** ($B^1 = C^{bz}$; $B^2 = G^{dpa}$). Levulinoylation of the 3'-OH group of dimer **7** ($B^1 = C^{bz}$; $B^2 = G^{dpa}$) was performed according to the same procedure as described previously. The 5'-dimethoxytrityl group was removed by treating the fully protected dimer (1 mmole) with a soln (50 ml) of benzenesulfonic acid (2% by weight) in CH_2Cl_2–MeOH (7:3, v/v) during 5 min. The reaction was quenched by adding $NaHCO_3$ AQ (10% by weight, 25 ml). The organic layer was separated and washed with water, dried with $MgSO_4$ end concentrated to an oil under reduced pressure. The crude product was applied to a short Kieselgel column (10 g). Elution of the column was effected with CH_2Cl_2–MeOH (99–90:1–10, v/v). The fractions containing dimer **9** were collected and evaporated. The resulting oil was precipitated from petroleum–ether (40–60°). The ppt was filtered off and dried *in vacuo* (KOH-pellets). Pure dimer **9** was obtained in a yield of 80–85%.

Synthesis of fully protected undecamer **18** (Scheme IV)

Synthesis of trimer **12a**. A soln of **10** (1.5 mmole) was added to **11** (1.45 mmole) which was previously dried by coevaporating with anhyd pyridine. 1-Methylimidazole (7 mmole) was now added and the reaction was stirred for 1.5 hr at 20°. TLC analysis of the mixture showed the reaction to be complete. The excess of **10** was hydrolysed by adding pyridine–water (1:1, v/v, 1 ml) to the mixture. The mixture was then diluted with CH_2Cl_2(100 ml) and washed with 1 M TEAB (30 ml) and water (30 ml).

The organic layer was dried with $MgSO_4$ and concentrated to an oil. The crude product was first triturated with petroleum–ether (40–60°) and then applied to a short Kieselgel column (8 g). The column was eluted with CH_2Cl_2–MeOH (99–90:1–10, v/v). The appropriate fractions were concentrated to give a foam. Pure **12a** (R^2 = DMTR) was obtained in a yield of 95% based on **11**. Subsequent treatment of **12a** (R^2 = DMTR) with benzenesulfonic acid, as described previously, provided **12b** (R^2 = H) in a yield of 85%.

Synthesis of pentamer **14a**. A soln of **13** (1.1 mmole) in dioxan containing 1-methylimidazole (5 mmole) was added to **12b** (1 mmole) which was previously dried by coevaporating with pyridine. The mixture was then stirred for 1.5 hr at 20°. The reaction was monitored by TLC and the product was isolated and purified as described above. Pure **14a** (R^2 = DMTR) was obtained in a yield of 95%.

Chain extension of pentamer **14a** *to obtain undecamer* **19**. Pentamer **14a** was extended, as illustrated in Scheme IV, using the same experimental conditions as described for the synthesis of **12a**. The fully protected oligomers **14a**, **16a**, **17a** and **18** were deblocked by treating with N^1,N^1,N^3,N^3-tetramethylguanidinium *syn*-pyridine-2-carboxaldoximate followed by aqueous ammonia and AcOH.

For example: the fully protected undecamer **18** (27 μmol) was dissolved in a soln of *syn*-pyridine-2-carboxaldoximate (3.0 mmole) in 8 ml dioxan-acetonitrile (1:1, v/v).

N^1,N^1,N^3,N^3-tetramethylguanidine (2.7 mmole) was added and the reaction mixture was stirred at 20°.

After 16 hr, the mixture was diluted with aqueous ammonia (25%, 50 ml). The reaction vessel was sealed and kept at 50° for 48 hr. The mixture was concentrated and treated with aqueous AcOH (80% by volume, 20 ml). After stirring for 30 min at 20° the soln was diluted with water (20 ml) and extracted twice with ether (20 ml). The aqueous layer was concentrated and coevaporated three thime with water. The curde product 19 thus obtained was analyzed (system I) by anion-exchange HPLC (Fig. 1).

Purification of fully deprotected DNA-fragments. The fully deprotected DNA-fragments obtained above were purified by Sephadex G-50 chromatography (column; 2m × 3 cm^2). Elution was performed with 0.05 M TEAB at a flow-rate of 14 ml per hr. Fractions of 3 ml were collected and analyzed by HPLC (system I). The appropriate fractions were collected and lyophilized.

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