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Studies on Deoxyribonucleic Acids and Related Compounds. II.[1] Synthesis of a Decanucleotide containing a Restriction Enzyme (PstI) Recognition Site

Eiko Ohtsuka, Teiichi Ono,[2] and Morio Ikehara*

Faculty of Pharmaceutical Sciences, Osaka University 133-1 Yamadakami, Suita, Osaka

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A decanucleotide dC–C–C–T–C–G–A–G–G–G was synthesized by phosphotriester block condensation. Three protected blocks, dCCCTp, dCGAp and dGGG were prepared using N-acyl, 5′-O-monomethoxytrityldeoxynucleosides as the starting materials. The protected dGGG which served as the 3′-terminal block was synthesized by condensation of 3′-O-benzoyl-N-isobutyryldeoxyguanosine (5′-hydroxy component) with 5′-O-monomethoxytrityl-N-isobutyryldeoxyguanosine 3′-O-*p*-chlorophenyl phosphate (3′-O-phosphodie ster component) using mesitylenesulfonyl triazolide as the condensing reagent followed by removal of the 5′-monomethoxytrityl group for repeated condensation. The other two blocks were prepared by using the 3′-O-*p*-chlorophenyl phosphoranilidate of N,5′-protected deoxynucleosides as the 3′-end unit (5′-hydroxy component). The phosphoranilidate of the fully protected trimers was removed by treatment with isoamyl nitrite for the condensation with the 5′-hydroxyl group of the growing chain. Fully protected nucleotides were isolated by chromatography on silica gel and the deblocked product was purified by ion-exchange chromatography on DEAE-cellulose. The decanucleotide was characterized by mobility shift analysis and complete enzymatic digestions after labelling the 5′-end with [γ-^{32}P]ATP using polynucleotide kinase.

Keywords——deoxyribo-oligonucleotides; restriction site; nucleotide condensation; phosphoranilidate; ion-exchange chromatography; polynucleotide kinase; homo-chromatography

Recently various approaches to syntheses of deoxyoligonucleotides by the phosphotriester method have been investigated.[3] We have previously used aromatic phosphoramidates as protecting groups for phosphomono-[4] and phosphodi-[5] esters in syntheses of ribooligonucleotides. In this paper we report the use of the anilido group as the protecting group of 3′-phosphotriesters in the triester synthesis of deoxydecanucleotides. *p*-Chlorophenyl N-phenylphosphorochloro amidate[6] (**2**) was used to prepare the fully protected nucleotides (**3**) as shown in Chart 1. The 5′-deblocked nucleotide (**4**) and 3′-phosphodiesterified mononucleo-

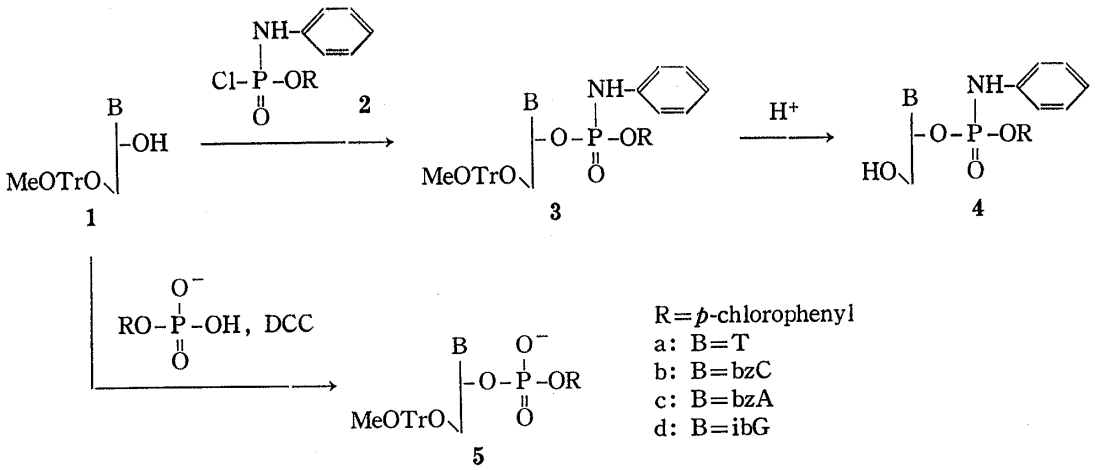

Chart 1

tides[7,8] (**5**) served as the key starting materials. A preliminary account of this approach has been reported.[7] The present decanucleotide dC–C–C–T–G–C–A–G–G–G contains the cleavage sequence of restriction endonuclease from *Providencia stuartii* 164 (*Pst* I),[9] dC–T–G–C–A–G.

Synthesis of Tri- and Tetranucleotide Intermediates

For the synthesis of dC–C–C–T–G–C–A–G–G–G, tri- and tetranucleotide blocks were prepared as intermediates. The synthetic scheme is shown in Chart 2. Since the 3′-terminal block (**8**) did not require the 3′-phosphate, the 3′-hydroxyl group was protected with a benzoyl group. Thus, **8** was synthesized by condensation of 3′-O-benzoyl-N-isobutyryldeoxyguanosine (**6**) with 5′-O-monomethoxytrityl-N-isobutyryldeoxyguanosine 3′-*p*-chlorophenyl phosphate (**5d**) followed by demonomethoxytritylation with benzenesulfonic acid[10] and condensation with the 5′-demonomethoxytritylated dimer (**7**). Mesitylenesulfonyl triazolide (MST)[11] was used as the condensing reagent, and protected oligonucleotides were isolated by chromatography on silica gel. The trinucleotide (**10**) was prepared using the deoxyadenosine 3′-*p*-

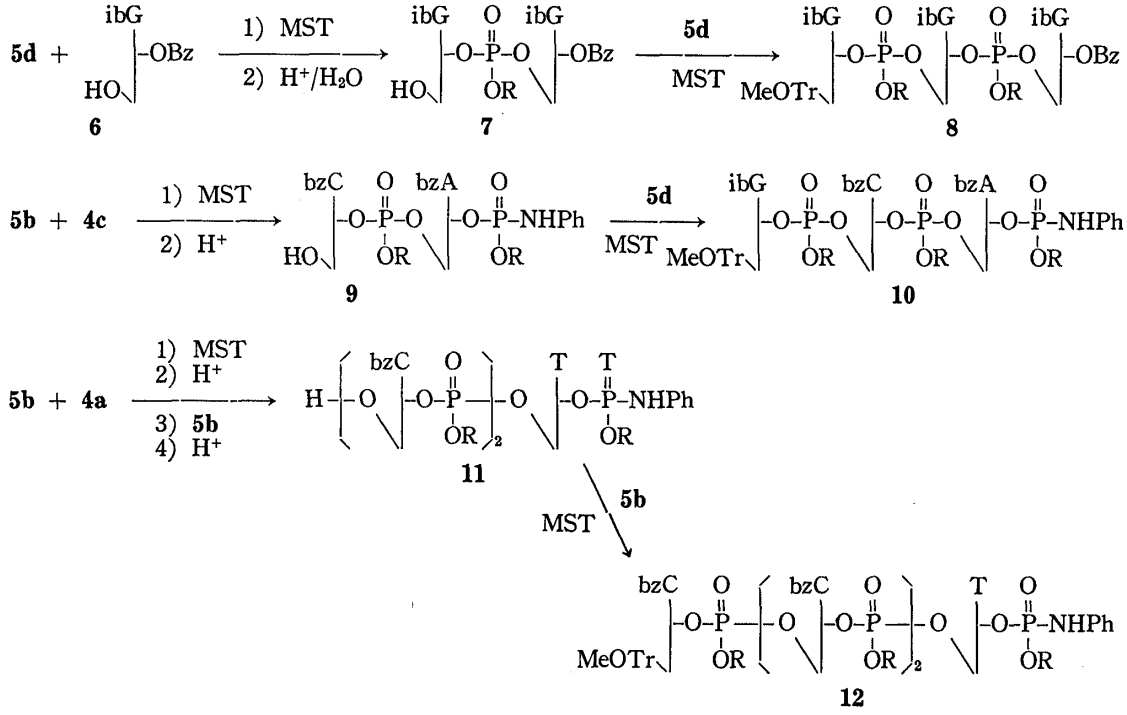

Chart 2

Table I. Reaction Conditions for Condensation

3′-Phosphodiester Component (mmol)		5′-Hydroxy Component (mmol)		MST (mmol)	Reaction time (h)	Product	Yield (%)
5d	1.28	**6**	1	3.84	48	**7**	59
5d	1.2	**7**	0.8	3.6	48	**8**	50
5b	1.2	**4c**	1.0	3.6	48	**9**	81
5d	1.11	**9**	0.7	3.36	72	**10**	77
5b	1.5	**4a**	1.8	5.4	48	Dimer	66
5b	1.35	Dimer	0.9	4.05	48	**11**	42
5b	0.6	**11**	0.35	1.8	72	**12**	41
14	0.23	**13**	0.15	0.69	48	**16**	36
15	0.08	**16**	0.04	MSNI 0.24	23	**17**	50

Table II. *Rf* Values in TLC and RTLC

	TLC (10: 1)	RTLC (7: 3)
6	0.50	0.62
7	0.56 0.64	0.57
8	0.67	0.46
9	0.46	—
10	0.36	0.39
11	0.77	0.17
12	0.68	0.34
13	0.31	0.79
14	0	0.92
15	0	0.68
16[a)]	0.39	0.48
17	0.39	0—0.2

a) with 5′-monomethoxytrityl group.

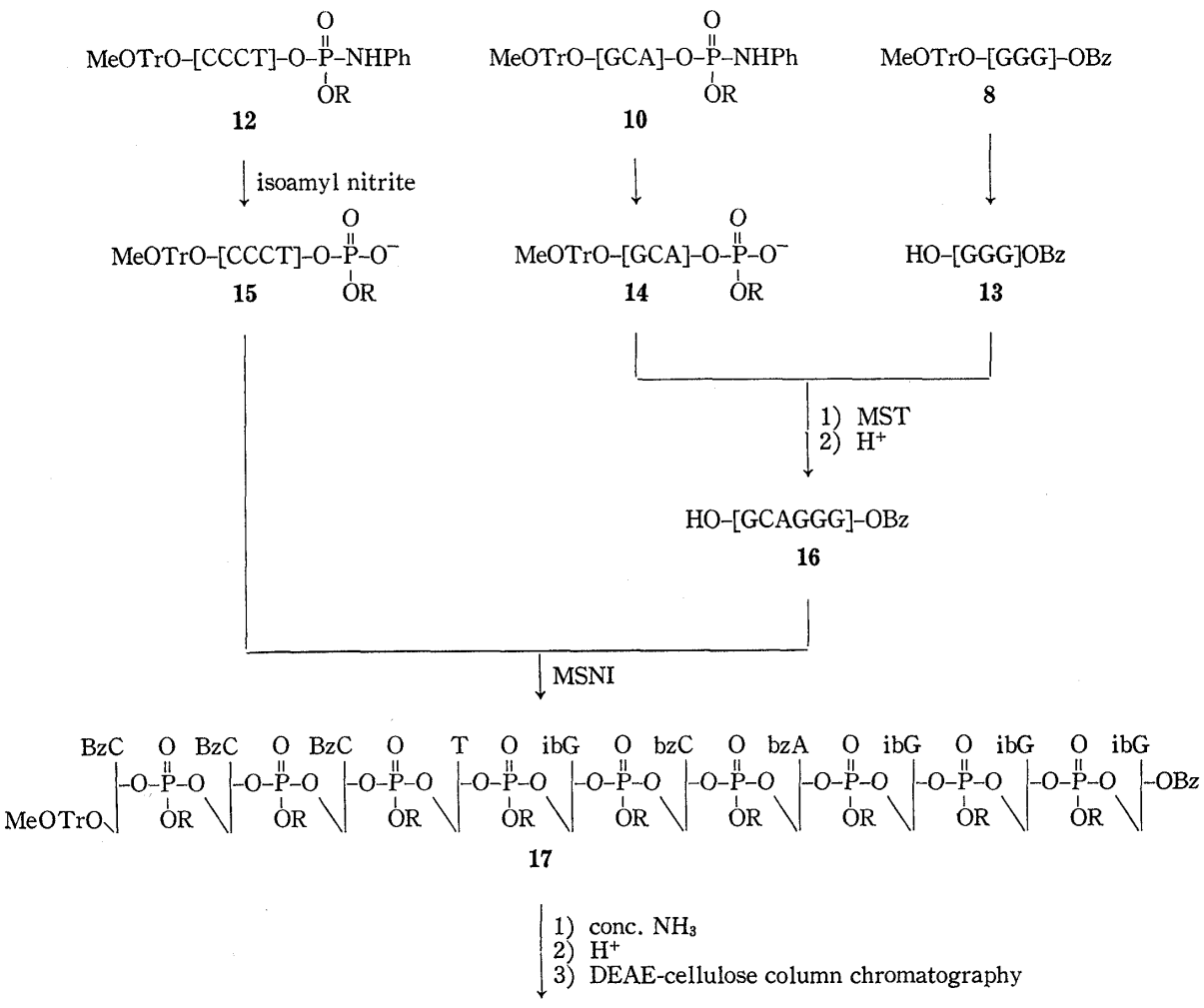

Chart 3

chlorophenyl phosphoranilidate (**4c**) as the terminal unit. Successive condensations with **5b** and **5d** gave the fully protected dGCAp (**10**) in an overall yield of 58%. The tetranucleotide block (**12**) was synthesized by a similar procedure starting from thymidine 3′-*p*-chlorophenyl phosphoranilidate (**4a**). The conditions for these condensing reactions are summarized in Table I.

Condensation of Oligonucleotide Blocks to yield the Decamer dC-C-C-T-G-C-A-G-G-G (18)

The 3′-terminal block (**8**) was demonomethoxytritylated to yield **13** and the trimer (**10**) was treated with isoamyl nitrite to give the phosphodiester (**14**). These trimers were condensed with MST under the conditions shown in Table I. The 5′-protected hexamer was isolated by preparative thin-layer chromatography (TLC) on silica gel and demonomethoxytritylated to give the hexamer (**16**) as shown in Chart 3. The tetranucleotide (**12**) was converted to the phosphodiester (**15**) by treatment with isoamyl nitrite for condensation with **16** (Table I). The fully protected decamer (**17**) was isolated by preparative TLC on silica gel and purified by silica gel column chromatography. The product was deblocked by treatment with concentrated ammonia followed by treatment with 80% acetic acid. The deblocked decamer (**18**) was isolated by ion-exchange chromatography on DEAE-cellulose (Fig. 1) and characterized by mobility shift analysis.[12] The base composition was determined by high pressure ion-exchange column chromatography of the digested materials with nuclease P1 as shown in Fig. 2. The 5′-end analysis was performed by digestion of the 5′-labelled product with nuclease P1 followed by paper electrophoresis at pH 3.5 (Fig. 3), and deoxycytidine was found as the terminal nucleoside.

Thus, the deoxydecanucleotide (**18**) was synthesized by using phosphoroanilidate as the protecting group for the 3′-phospho ends. The monomethoxytrityl group was used in the present study. However, the more acid labile dimethoxytrityl group may be suitable if a chain contains deoxyadenosine at the 3′-terminus.[3] The monomethoxytrityl group of the 5′-terminal block can be removed after deacylation of the heterocyclic bases, and conditions

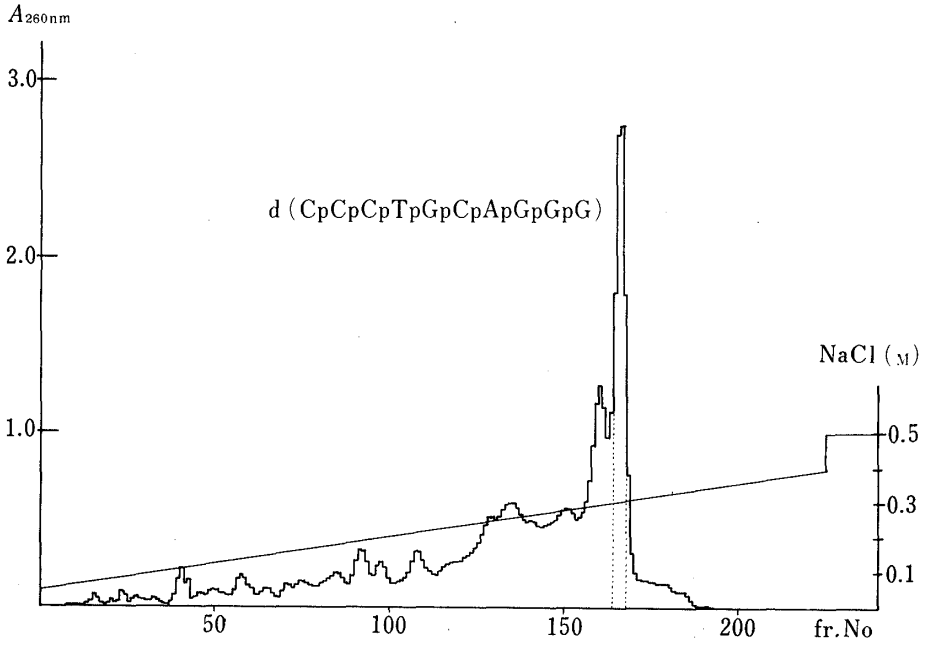

Fig. 1. Chromatography of the Decanucleotide on a Column (1.0×67 cm) of DEAE-cellulose (Chloride Form) in 7 M Urea and 0.02 M Tris-HCl, pH 8.0

Elution was performed with a linear gradient of sodium chloride (0.05—0.5 M, total 1 l). One fraction of 3.4 ml was collected every 23 min.

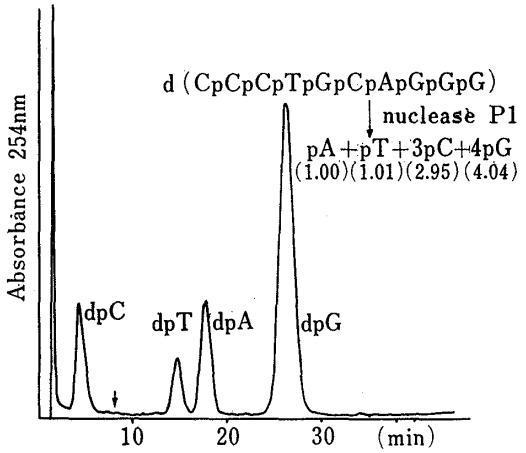

Fig. 2. Base Composition of the Decanucleotide dCCCTGCAGGG by as determined Complete Digestion with Nuclease Pl followed by High Pressure Chromatography of Deoxynucleoside 5′-Phosphates on an Anionexchange Column

Starting buffer, 0.01 M potassium phosphate (pH 3.35); gradient buffer, 0.1 M potassium phosphate (pH 4.5); gradient delay, 9 min. The ratio of dpC: pT: dpA: dpG=2.95: 1.01: 1.00: 4.04.

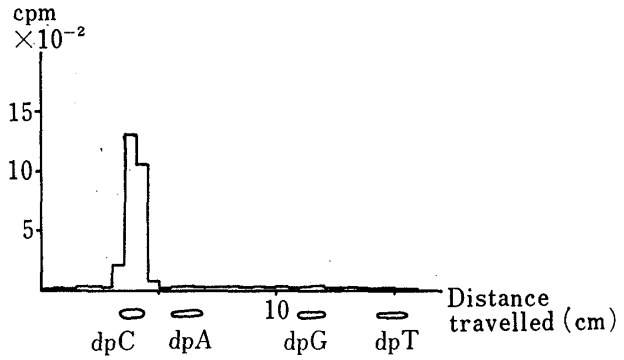

Fig. 3. 5′-End Analysis of the Decanucleotide dCCCTGCAGGG by Paper Electrophoresis of the Digested Products of the 5′-Labeled Compound

Radioactivity was detected by counting 0.5 cm strips cut from the paper.

for the demonomethoxytritylation are tolerable to non-N-acylated deoxyadenosine. Use of a combination of mono- and dimethoxytrityl groups has been employed in the synthesis of gene fragments for bacteriorhodopsin[13] and oxytocin.[14] Isoamyl nitrite treatment for removal of the anilidate group is performed under a slightly acidic conditions. The phosphoro-*p*-anisidate which requires less reaction time, has been introduced instead of the anilidate. The use of this protecting group will be reported elsewhere.

Experimental

TLC was performed on plates of silica gel (Kieselgel 60 F_{254}, Merck) by using a mixture of chloroform and methanol. For columns, silica gel (Kieselgel 60, 70—230 mesh, Merck) was used. For reverse phase TLC (RTLC), silanized silica gel (Kieselgel 60 F_{254}, silanisiert, Merck) was used with aqueous acetone. *Rf* values in TLC and RTLC are listed in Table II.

Paper electrophoresis was performed at 900 V/40 cm using 0.2 M morpholinium acetate (pH 3.5).

Partial digestion with nuclease P1 was carried out as described for oligoribonucleotides.[5b,15] For complete hydrolysis of oligonucleotides (1—2 A_{260}), nuclease P1 (2 μg) was used in 0.1 M ammonium acetate (pH 4.5, 25 μl) at 25°C for 2 h. Base composition was analyzed by a nucleic acid analyzer (Varian LCS 1000). For the analysis of the 5′-terminus, oligonucleotides were labeled as described for substrates of P1 partial digestion and hydrolyzed completely as above. The nucleotides were identified by comparison of their mobilities in paper electrophoresis at pH 3.5 with those of authentic nucleotides (UV detection).

5′-O-Monomethoxytrityldeoxynucleosides (1)——5′-O-Monomethoxytritylthymidine[16] was prepared according to the cited reference with slight modifications by reacting 1.1 equivalents of monomethoxytrityl chloride in pyridine (2 ml/mmol) for 3 h at 25°C. Water was added with cooling and the mixture was concentrated. The product was extracted with methylene chloride, washed 3 times with 0.1 M triethylammonium bicarbonate, and concentrated with pyridine and then toluene. The product was isolated by precipitation with hexane or by chromatography on silica gel.

5′-O-Monomethoxytrityl derivatives of N-benzoyldeoxycytidine,[16] [d(MeOTr)bzC][17] N-benzoyldeoxyadenosine[16] and N-isobutyldeoxyguanosine[18] were prepared similarly from the corresponding N-protected nucleosides using pyridine (4 ml/mmol for dbzC and dbzA, 7.5 ml/mmol for dibG).

The N-protected nucleosides[10,16,18] were synthesized by the reported methods with slight modifications as described below.

a) dbzC: Deoxycytidine HCl (20 mmol) was suspended in dry pyridine (100 ml), stirred with triethylamine (2.8 ml) for 30 min and treated with benzoyl chloride (10 ml, 86.2 mmol) with cooling. The mixture

was stirred at room temperature for 1 h and poured to a mixture of chloroform (500 ml), ice (350 g) and sodium bicarbonate after the completion of the reaction had been checked by TLC (10: 1). The organic layer was washed twice with water and concentrated. The residue was dissolved in THF (100 ml) and methanol (80 ml). The solution was treated with 2 N NaOH (50 ml) at 0°C for 15 min and poured onto ice-cooled Dowex 50×2 (pyridinium form, 150 ml). The mixture was poured onto a column of the same resin (30 ml) and the column was washed with 20% pyridine (600 ml). The solution was concentrated to 3/4 volume (or until an oil or gum appeared). The product was crystallized by concentration of the aqueous solution. The yield was 5.51 g, 83%.

b) dbzA: Deoxyadenosine was benzoylated with benzoyl chloride and treated with 2 N NaOH (3 ml/mmol) in ethanol (4 ml/mmol) and THF (1 ml/mmol) under the same conditions as described for dbzC. The product was crystallized from aqueous pyridine and recrystallized from 5% pyridine. The yield was 90%.

c) dibG: Deoxyguanosine was suspended in pyridine (5 ml/mmol) and treated with isobutyryl chloride (6 eq.) at 0°C for 3 h. The fully acylated product was extracted with chloroform as above and treated with 2 N NaOH (3 ml/mmol) in ethanol (5 ml/mmol) at 0°C for 15 min. The mixture was neutralized with Dowex 50×2 (pyridinium form, 6 ml/mmol) and the resin was washed with three volumes of 10% pyridine. The combined solution was concentrated to dryness and the residue was recrystallized from 5% pyridine. The Yield was *ca.* 90%.

Thymidine 3′-*p*-Chlorophenyl Phosphoranilidate (4a) and (4c)——**1a** (3 mmol) was dried by coevaporation with pyridine and treated with *p*-chlorophenyl phosphoranilido chloridate (**2**) (4.5 mmol) in pyridine (15 ml) at 37°C for 16 h. Ice-water (150 ml) was added and the product was extracted with chloroform. The organic phase was washed with 5% sodium bicarbonate and water. The solution was concentrated with toluene and the residue was treated with benzenesulfonic acid (1.3 g) in chloroform–methanol (7: 3, 150 ml) at 0°C for 15 min. The reaction was checked by TLC and terminated by addition of 5% sodium bicarbonate. **4a** was extracted with chloroform, and the extract was washed with 5% sodium bicarbonate and water, then concentrated. The resulting solution was applied to a column of silica gel. The product was precipitated with hexane from its solution in chloroform. The yield was 1.25 g (66%). *Rf*: TLC (10: 1, 0.46), (20: 1, 0.16). Spots were detected by spraying 3% sulfuric acid followed by heating (violet color). UV: λ_{max} in 50% ethanol 210, 268 and λ_{min} 270 nm. **4c** was prepared by essentially the same method as above. UV: λ_{max} in 95% ethanol 280 nm.

5′-O-Monomethoxytrityl-N-benzoyldeoxycytidine 3′-*p*-Chlorophosphoranilidate (5b) and (5d)——**1c** (6.03 g, 10 mmol) was dissolved in pyridine (150 ml) and treated with DCC (14.83 g, 72 mmol) for 48 h. Aqueous pyridine (50%, 200 ml) was added and the mixture was kept overnight. The filtered solution was extracted with chloroform and the organic layer was washed with 0.2 M triethylammonium bicarbonate. The product was precipitated with ether–pentane (2: 8) from its solution in chloroform. The yield was 8.20 g and was estimated by measuring the absorbance at 304 nm ($\varepsilon=12.1\times10^3$) as 7.19 mmol, 72%. **5d** was prepared similsrly from **1d**. UV: λ_{max} 260, 280 nm. The yield was estimated by using $\varepsilon_{260}=18500$ was 76%.

3′-O-Benzoyl-N-isobutyryldeoxyguanosine (6)——**1d** (1.828 g, 3 mmol) was treated with benzoic anhydride (18 mmol) at 30°C for 72 h and the product (*Rf* 0.84 in TLC, 10: 1) was extracted with chloroform from the reaction mixture after treatment with aqueous pyridine (30 ml). The chloroform solution was washed with 5% sodium bicarbonate and water and concentrated by evaporation with toluene, and the residue was dissolved in chloroform–methanol (7: 3, 90 ml). The solution was treated with benzene-sulfonic acid (3 g, dissolved in chloroform–methanol, 7: 3, 60 ml) for 10 min. Cold 5% sodium bicarbonate (30 ml) was added to the mixture, and the organic layer was washed with 5% sodium bicarbonate and water. **6** was crystallized by concentration of the solution and recrystallized from 95% ethanol. The yield was 1.15 g, 87%. mp 145—146°C, *Rf* (TLC, 10: 1, 0.68). UV: λ_{max} in ethanol 231, 255 and 284 nm.

General Methods for Condensation of Mononucleotides——Condensations were performed as summarized in Table I. For example, **7** was synthesized by condensation of **5d** and **6** in pyridine (5 ml). To terminate the reaction, ice-water was added with cooling, and the product was extracted with chloroform. The organic layer was washed with 0.1 M triethylammonium bicarbonate and concentrated. The residue was applied to a column of silica gel and the eluted product was precipitated with pentane from its solution in chloroform. The *Rf* values of the two diasteroisomers (TLC, 10: 1) were 0.56 and 0.64. The monomethoxytrityl group was removed as described for the synthesis of **6**. The yield of demonomethoxytritylation was 98%.

Fully Protectcd Decanucleotide (17)——The terminal trimer (**8**) was prepared from **7** by removal of the monomethoxytrityl group as described for **6**. The trimer **10** (0.534 mmol) was converted to **14** by treatment with isoamyl nitrite (4.2 ml) in pyridine–acetic acid (3: 2, 24 ml) at 30°C for 48 h. Ice-water was added to the mixture, and **14** was extracted with chloroform. The chloroform solution was washed with 0.1 M triethylammonium bicarbonate and the product was precipitated with pentane from its solution in chloroform. The yield was 991 mg, 96%. The tetramer (**12**) was similarly converted to **15**. Condensation of **14** and **13** was performed in pyridine (1.5 ml) under the conditions shown in Table I. Ice-water was added to terminate the reaction and the product was isolated as described for mononucleotide condensations. The *Rf* values of the fully protected hexanucleotide are shown in Table II. The monomethoxytrityl group was removed by treatment with benzenesulfonic acid under the conditions shown for **6**. The 5′-deblocked hexamer (**16**) was then condensed similarly with **15** to yield the protected decamer (**17**), which was obtained by chro-

matography on a silica gel column. Impurities were removed by preparative TLC. The yield was 35.8 mg.

Deblocking of 17 to give dCCCTGCAGGG——An aliquot of **17** (51 mg) was dissolved in pyridine (5 ml) and concentrated ammonia (50 ml) was added. The mixture was heated at 50°C for 4 h and concentrated to dryness by coevaporation with toluene. The residue was treated with 80% acetic acid (40 ml) at 30°C for 1 h. Volatile materials were removed by evaporation and the residue was mixed with ether and 0.1 M triethylammonium bicarbonate (40 ml). The aqueous layer (792 A_{260}) was applied to a column of DEAE-cellulose as shown in Fig. 1. Fractions (162—165) were combined and desalted by adsorption on DEAE-cellulose (bicarbonate form) followed by stepwise elution with triethylammonium bicarbonate. The yield was 29 A_{260}. UV: λ_{max} 257 (H_2O), 260 (pH *ca.* 2), 265 (pH *ca.* 13); λ_{min} 233 (H_2O), 233 (pH *ca.* 2) 241 (pH *ca.* 13) nm; ε 280/260=0.777 (H_2O), 0.936 (pH *ca.* 2), 0.644 (pH *ca.* 13).

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