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Studies on Deoxynucleic Acids and Related Compounds. VI.[1] Synthesis of Oligodeoxyribonucleotides containing Recognition Sequences for Restriction Endonucleases

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An octanucleotide dACCCGGGT and decanucleotide dCGACCCGGGT have been synthesized by an improved phosphotriester method. The octanucleotide has a self-complementary sequence and the decanucleotide has two protruding nucleotides when it forms a duplex. Thermal properties and circular dichroism spectra of these oligonucleotides have been investigated under various conditions to find differences in the stabilities of these oligonucleotides. A possible concatemer structure of the decanucleotide duplex is suggested on the basis of its circular dichroism spectra in concentrated solution.

Keywords——self-complementary; octadeoxyribonucleotide; partially complementary decadeoxyribonucleotide; Recongnition site for restriction enzyme; circular dichroism (CD) spectra of oligonucleotide; melting temperature of oligonucleotide

Physical properties of short deoxyribonucleic acid (DNA) duplexes have been studied by several investigators using chemically synthesized polydeoxynucleotides with chain lengths of around ten.[2] The phosphotriester method has been improved recently and represents a convenient method to obtain oligonucleotides in large amounts.[3] We have been applying a phosphoranilidate protection method to the synthesis of deoxyribooligonucleotides[4] and have improved aromatic phosphoramidate protection by using phosphoro-*p*-anisidate for the protection of terminal phosphodiesters.[5] In the present paper we wish to report that the octa and decanucleotides dACCCGGGT, and dCGACCCGGGT have been synthesized in high yields by the phosphotriester method involving condensation of oligonucleotide blocks prepared by using phosphoro-*p*-anisidate as protection for the 3′-phospho end. The octamer has a selfcomplementary sequence and the decamer can form a duplex with cohesive ends (Chart 1). The decamer contains sequences of recognition sites of several restriction endonucleases[6] especially when joined to concatemers by means of the DNA ligase reaction.[7] Comparison of properties of these two oligonucleotides is of interest from several points of view since these oligonucleotides contain one or two dCG sequence, which can be assumed to form a unique shape under certain conditions.[2a] Thermal stabilities and circular dichroism (CD) spectra have been investigated in different conditions.

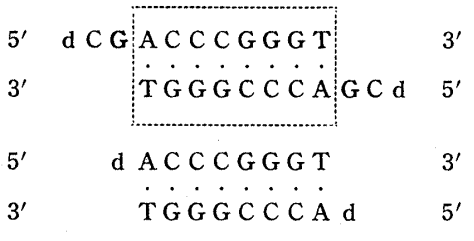


Chart 1

Experimental

General Methods——5′-*O*-Dimethoxytritylthymidine [$(MeO)_2$TrT],[8] d$(MeO)_2$TrbzC, d$(MeO)_2$TrbzA and d$(MeO)_2$TribG (Chart 2) were prepared by published procedures with slight modifications.[4b] The corresponding monomethoxytrityl derivatives were obtained similarly. Mesitylenesulfonyl tetrazolide (MSTe) synthesis[9] and preparation of **3** using *p*-chlorophenyl phosphoro-*p*-anisido chloridate (**2**) were performed as described elsewhere.[5] The 3′-phosphorylation with *p*-chlorophenyl phosphoroditriazolide to yield **4** was performed as described previously.[10]

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

Thin layer chromatography (TLC) was performed on plates of silica gel (Kieselgel HF_{254}, Merck) using a mixture of chloroform and methanol. For reversed phase TLC (RTLC), silanized silica gel (Merck) was used with a mixture of acetone–water. For columns, silica gel (Kieselgel 60 H, Merck) was packed with methylene chloride in a glass funnel and compounds were applied as a concentrated solution. Elution was performed with a mixture of methylene chloride-methanol with suction, unless otherwise specified. For reversed phase column chromatography, silanized silica gel (Merck, 70—230 mesh) was equilibrated with 60—70% acetone and compounds in acetone were applied with addition of water until slight turbidity was apparent. Elution was performed with 60—80% aqueous acetone.

High pressure liquid chromatography (HPLC) was carried out by using an Altex 332 MP apparatus.

For mobility shift analysis,[11] the 5′-end was labelled with ^{32}P and the oligonucleotide was partially digested as described for ribooligonucleotides. Base composition was analyzed by hydrolyzing oligonucleotides (1—2 A_{260}) with nuclease P1 (2 μg) in 0.1 M ammonium acetate (pH 4.5, 25 μl) at 25°C for 2 h followed by high pressure chromatography on anion-exchange resin.[4b] For the analysis of the 5′-terminus, the 5′-labelled oligonucleotide was hydrolyzed with nuclease P1 as above and the labelled mononucleotide was identified by comparison of its mobility in paper electrophoresis (pH 3.5) with those of authentic nucleotides detectable under ultraviolet (UV) light.

Ultraviolet spectra were recorded with a Hitachi 124 or 323 ultraviolet spectrophotometer. Circular dichroism spectra were measured with a Jasco ORD/UV-5 spectropolarimeter equipped with a CD attachment. Calibration of Cotton effect magnitude was effected with *d*-10-camphorsulfonic acid. Solutions of A_{max} *ca.* 1.3 were prepared by using the solvent systems given in the figure legends.

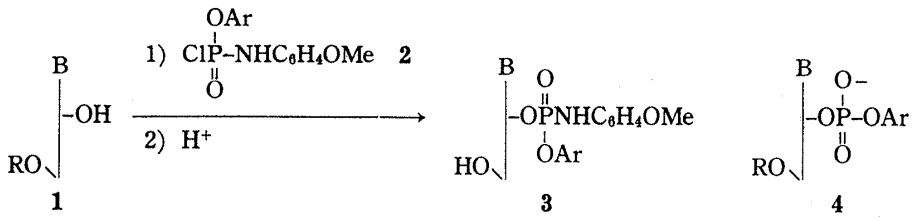
General Methods for Condensation (Table I)——The 3′-phosphodiester component (3.05 mmol) and 5′-hydroxyl component (2.05 mmol) were dried by evaporation of pyridine and dissolved in pyridine (15 ml). The mixture was treated with MSTe (6.10 mmol) at 30°C for 20 min and checked by either TLC (chloroform–methanol) or RTLC (acetone–water) (Table II). After confirmation of disappearance of the 5′-hydroxyl component, the reaction was terminated by addition of aqueous pyridine (50%, 1 ml) with cooling and 0.1 M triethylammonium bicarbonate (40 ml) was added to the mixture. The product was extracted 3 times with methylene chloride (40 ml), washed with 0.1 M triethylammonium bicarbonate 3 times and concentrated with pyridine and then with toluene. The residue was applied to a column of silica gel (40 g). Elution was performed by a stepwise increase of ethanol concentration in methylene chloride. Fractions containing the pure product were combined. In the case of **7**, reversed phase chromatography was used for isolation. After work-up, the residue was dissolved in acetone (6 ml) and water (3 ml) was added to the solution. The slightly turbid solution was applied to a column (2.6×31.5 cm) of silanized silica gel (95 g). Elution was carried out with 60% acetone (300 ml), 65% acetone (150 ml) and acetone (450 ml).

Removal of the 3′- and 5′-Protecting Groups——For example, the monomethoxytrityl group of the fully protected pentamer (**7**) (1.1 mmol) (Chart 3) was removed by dissolving the material in chloroform (70 ml) and adding benzenesulfonic acid (10.5 g) in a mixture of chloroform (70 ml) and methanol at 0°C for 15 min. The reaction was checked by TLC and terminated by addition of 5% sodium bicarbonate. **8** was extracted twice with chloroform (100 ml), washed twice with water, concentrated and precipitated with 2:1 pentane–ether (200 ml) from its solution in methylene chloride (20 ml).

Deamidation of **10** (1.495 g, 0.786 mmol) was performed by treatment with isoamyl nitrite (4.23 ml, 31.4 mmol) in 1:1 pyridine–acetic acid (20 ml) at 30°C for 2 h. The reaction was checked by RTLC and terminated by addition of pyridine (15 ml) and 0.2 M triethylammonium bicarbonate (50 ml) with cooling followed by washing with 1:1 ether–pentane (30 ml) 4 times. The product (**11**) was extracted 3 times with methylene chloride (40 ml) from the aqueous solution, washed 3 times with 0.2 M triethylammonium bicarbonate (30 ml) and precipitated with ether–pentane (120 ml) from its solution in methylene chloride (10 ml). The yield was 1.476 g. **5** and **15** were prepared similarly and purified by chromatography on silanized silica gel.

The Fully Protected Octamer (12)——The trinucleotide (**11**) and the pentamer (**8**) were condensed under the conditions shown in Table I and worked up as described in the general procedure. Chromatography of **12** on a column (φ 3.4×2.5 cm) of silica gel H (15 g) was formed by applying the solution in methylene chloride (8 ml). Methylene chloride and 10% ethanol in the same solvent were used for elution. Fractions containing pure product were combined/and the protected octamer (**12**) was precipitated with hexane (50 ml) from its solution in methylene chloride (3 ml). The yield was 545 mg (0.138 mmol). Impure fractions can be subjected to reversed phase chromatography to yield small amounts of **12**.

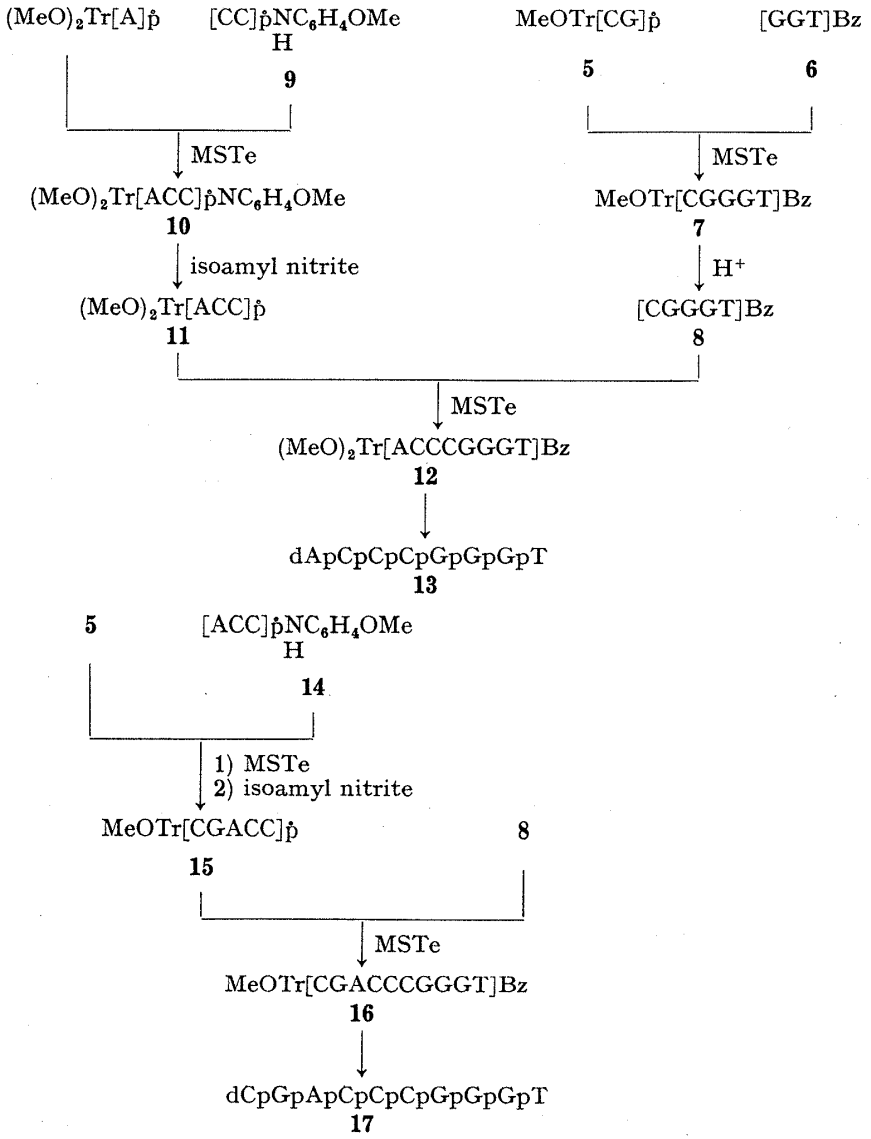
The Fully Protected Decamer (16)——The pentanucleotide (**15**) and the pentamer (**8**) were condensed as shown in Table I. The decamer (**16**) was isolated by chromatography on a column (φ 3.3×3.3 cm) of silica gel H (15 g). The mixture in methylene chloride (8 ml) was applied and **16** was eluted with methylene chloride and then with 12% ethanol. Fractions containing the pure product were combined and the fully protected decamer (**16**) was precipitated with pentane (80 ml) from its solution in methylene chloride (10 ml). The yield was 808 mg (0.155 mmol). Small amounts of the product can be recovered from impure fractions by reversed phase chromatography.



B: a, thymidin-1-yl
b, *N*-benzoylcytosin-1-yl
c, *N*-benzoyladenin-9-yl
d, *N*-isobutyryl-9-yl

Ar = *p*-chlorophenyl
R = dimethoxytrityl or monomethoxytrityl

Chart 2



MeOTr, monomethoxytrityl
$(MeO)_2Tr$, dimethoxytrityl
p̂, *p*-chlorophenyl phosphoryl
[ ], protected deoxynucleotidesexcept for termini

Chart 3

Deblocking of 12 and 16 to yield dACCCGGGT (13) and dCGACCCGGGT (17)——**12** (23 μmol) was dissolved in dioxane (4.85 ml) and stirred with N^1,N^1,N^2,N^2-tetramethylguanidium pyridine-2-carboxaldoximate (1 M, 4.85 ml) at room temperature for 44 h. Volatile materials were removed by evaporation, then the residue was dissolved in pyridine and treated with 28% ammonia (20 ml) at 60°C for 6 h. Ammonia was removed and the solution was passed through a column of Dowex 50×2 (pyridinium form, 30 ml). The

column was washed with 20% pyridine (40 ml). The combined solutions were concentrated by coevaporation with water and the residue was treated with 80% acetic acid (40 ml) for 60 min. Acetic acid was removed by evaporation and the residue was mixed with water (20 ml) and methylene chloride (15 ml). The aqueous phase was washed with methylene chloride (15 ml) and applied to a column of Sephadex G-50. (Fig. 1a) **13** in the main peak was purified by chromatography on DEAE-cellulose (Fig. 1b). The yield was 838 A_{260}, 13 μmol.

The decamer (**17**) was obtained by the same procedure from **16** (10 μmol). Gel filtration and ion-exchange chromatography were performed as shown in Fig. 2. The yield was 539 A_{260}, 7.4 μmol.

Table I. Conditions for Condensations

3′-Phosphodiester component mmol		5′-Hydroxyl component mmol		MSTe mmol	Reaction time min	Product mmol		Yield
MeOTr[C]p̂	3.00	[G]p̂NHPh	1.73	6.00	45	**5**[a]	1.42	82
$(MeO)_2$Tr[G]p̂	4.10	T(Bz)	2.73	8.20	50	[GT](Bz)	2.09	77
$(MeO)_2$Tr[G]p̂	3.00	[GT](Bz)	2.09	6.00	45	**6**	1.62	78
$(MeOTr)_2$[C]p̂	3.07	[C]p̂NHPh	2.05	6.15	60	**9**[b]	1.90	93
5	1.75	**6**	1.22	3.50	40	**7**	1.07	88
$(MeOTr)_2$[A]p̂	2.31	**9**	1.89	4.62	60	**10**	1.59	84
11	0.35	**8**	0.2	0.70	45	**12**	0.138	69
5	1.19	**14**	0.760	2.37	50	**15**[c]	0.61	80
15	0.35	**8**	0.2	0.7	30	**16**	0.155	78

a) Fully protected.
b) 5′-Dimethoxytrityl.
c) 3′-Fully blocked.

Table II. *Rf* Values in TLC and RTLC

	TLC chloroform–methanol (v/v)		RTLC acetone–water (v/v)	
	10:1	8:1	7:3	8:2
[GT](Bz)	0.11		0.51	
[GGT](Bz) (**6**)	0.09		0.50	
MeOTr[CG]p̂NHC_6H_4OMe	0.264 0.30		0.35	
[CC]p̂NHC_6H_4OMe (**9**)	0.34		0.50	
[ACC]p̂NHC_6H_4OMe (**14**)	0.23		0.43	
MeOTr[CG]p̂ (**5**)			0.67	
[CGGGT]Bz (**8**)		0.36	0.22	
(MeO_2)Tr[ACC]p̂ (**11**)	0.294 0.38		0.22	
MeOTr[CGACC]p̂ (**15**)			0.65	
$(MeO)_2$Tr[ACCCGGGT]Bz (**12**)		0.16		0.60

Results and Discussion

Synthesis of the Octamer dApCpCpCpGpGpGpT (13) and Decamer dCpGpApCpCpCpGpGpGpT (17)

The octa- (**13**) and deca- (**17**) nucleotides were synthesized by the triester method using *p*-anisidate as the protecting group for the 3′-ends. Chart 2 shows schemes for the synthesis of mononucleotide units. These nucleotides were used to prepare protected oligonucleotide blocks. The schemes for the condensations of these blocks to yield the octa- and deca-nucleotides are shown in Chart 3. The dinucleotide (**5**) was prepared by condensation of 5′-*O*-monomethoxytrityl-*N*-benzoyldeoxycytidine 3′-*p*-chlorophenyl phosphate (**4b**) (3′-phosphodiester component) and *N*-isobutyryldeoxyguanosine 3′-*p*-chlorophenyl phosphoro-*p*-anisidate (**3d**) (5′-hydroxy component) followed by removal of the *p*-anisidate with isoamyl nitrite. MSTe[6] (2 equivalents with respect to the 3′-phosphodiester component) was used as the condensing reagent. Reaction conditions for mononucleotide condensations are sum-

marized in Table I. To complete the reaction, 1.5 equivalents of the 3′-phosphodiester component with respect to the 5′-hydroxyl component was used. The fully protected oligonucleotides were isolated by chromatography on silica gel. The monomethoxytrityl or dimethoxytrityl group on the 5′-end was removed by treatment with 3.5% or 2% benzenesulfonic acid and the product was purified by silica gel chromatography. For compound **7**, reversed phase chromatography was used to remove impurites which could not be removed by silica gel chromatography. Compounds **5** and **15** were also purified by reversed phase chromatography after removal of the anisidate. The 3′-terminal block (**6**) was synthesized by using 3′-*O*-benzoylthymidine as the starting nucleoside. The pentanucleotide **8** was used as the common intermediate. Condensation of **8** with the trinucleotide **11** gave the fully protected octamer **12** and condensation of **8** with the pentanucleotide **15** yielded the decamer **16** (Table I).

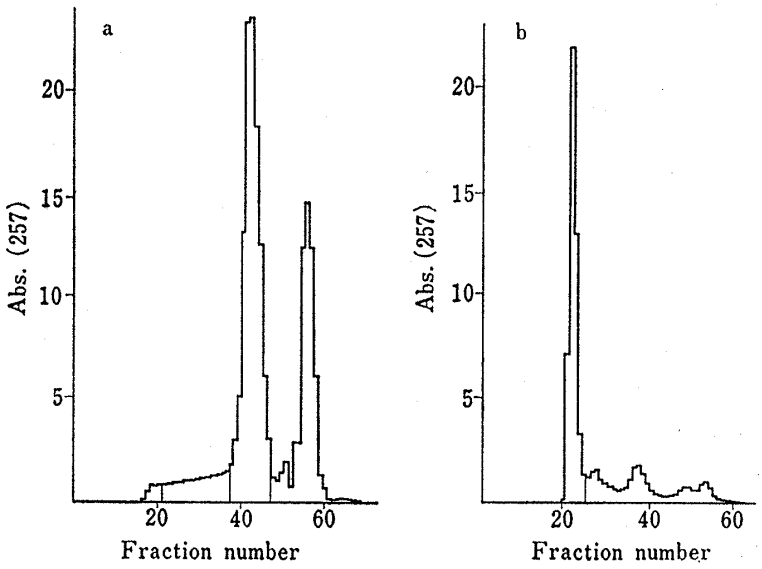


Fig. 1. Gel Filtration of the Octamer dACCCGGGT (a) and the Decamer (b) on a Column (2.8×117 cm) of Sephadex G-50 equilibrated with 0.03M Triethylammonium Bicarbonate

Fractions of 11.6 ml were collected every 7 min.

Deblocking of the products was performed by treatment with pyridine-2-aldoximate followed by treatment with ammonia, and the dimethoxytrityl or monomethoxytrityl group was then removed with 80% acetic acid. The completely deblocked octamer (**13**) was subjected to gel filtration on Sephadex G-50 (Fig. 1a). The product in the main peak was analyzed by high pressure liquid chromatography on C18-silica gel and found to be almost pure. Further purification of 13 was performed by ion-exchange chromatography on diethylaminoethyl (DEAE) cellulose (Fig. 2a). The hypochromicity of **13** was found to be 20% and the overall yield of deblocking on the basis of $\varepsilon_{260}=64400$ was 57%. The decamer (**15**) was isolated similarly (Fig. 1,2,b). The ε_{260} of **17** was 72800 (hypochromicity, 26%) and the overally yield of deblocking of the decamer was 74%. The elution profiles of the purified octamer (**13**) and decamer (**17**) on reverse phase chromatography are shown in Fig. 3.

The structural integrity of these oligonucleotides was confirmed by mobility shift analysis (Fig. 4), 5′-terminal analysis and base composition.

Properties of the Octa- and Decanucleotides

Thermal stabilities of the octamer dACCCGGGT (**13**) and decamer dCGACCCGGGT (**17**) at different strand concentrations were studied by absorption measurement at 260 nm. The midpoint of the absorption-temperature profile (T_m) of the octamer (**13**) in 10^{-5} M solution was 39°C, which is slightly lower than that of the decamer (**17**) (41°C), as shown in Fig. 5. T_m

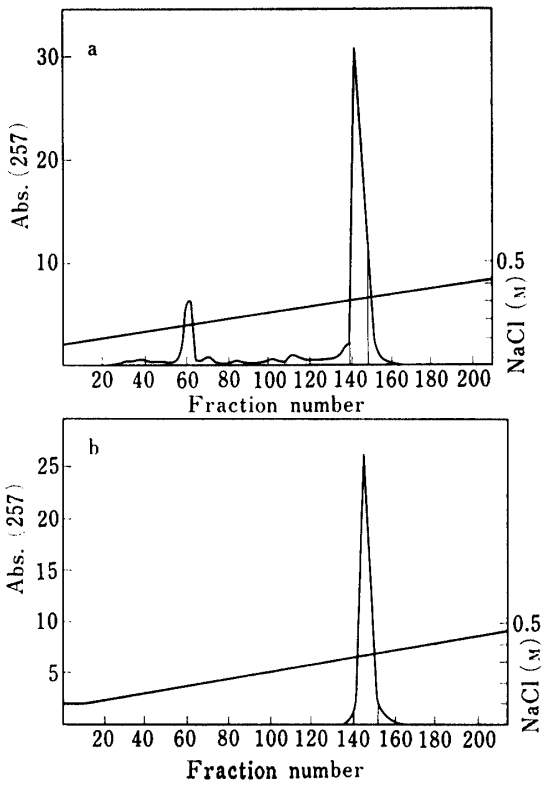


Fig. 2. a: Chromatography of the Octamer dACCCGGGT on a Column (1.3×76 cm) of DEAE-cellulose in the Presence of 7 M Urea in 20 mM Tris–HCl, pH 7.5. b: Chromatography of the Decamer dCGACCCGGGT on a Column (1.3×52 cm) of DEAE-cellulose

a: Fractions of 4.3 ml were collected every 20 min. Elution was performed with a linear gradient of sodium chloride (0.1—0.5 M). b: Fractions of 3.0 ml were collected every 20 min. Other conditions were the same as described for the octamer (a).

values of the octamer in 10^{-4} and 10^{-3} M solutions were 45°C and 53°C, respectively. Those of the decamer were 48°C and 55°C under the same conditions. The enthalpy of these oligonucleotides was calculated from a $1/T_m$ *versus* log c plot[12] (Fig. 6) and found to be −61 kcal/mol. CD spectra of these olionucleotides at 12°C and 76°C are shown in Fig. 7. Fig. 8 shows θ values at 260 nm at different temperatures measured at the same strand concentration as descibed in Fig. 7. The spectrum of the octamer dACCCGGGT at lower temperature suggested that it might have a structure similar to that of A form DNA[13] under the conditions

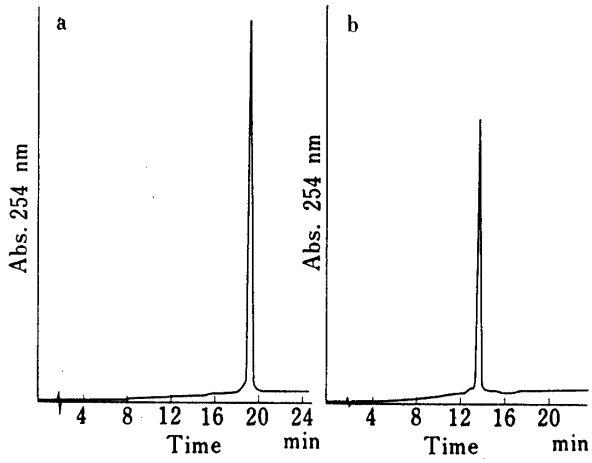


Fig. 3. High pressure Liquid Chromatography of the Octamer (a) and the Decamer (b) on a Column (4.6×250 nm) of Hypersil ODS 5 μ

Elution was performed with a gradient of acetonitrile (5—25%) in 0.1 M triethylammonium acetate (pH 7.0) during 30 min. The flow rate was 2 ml/min.

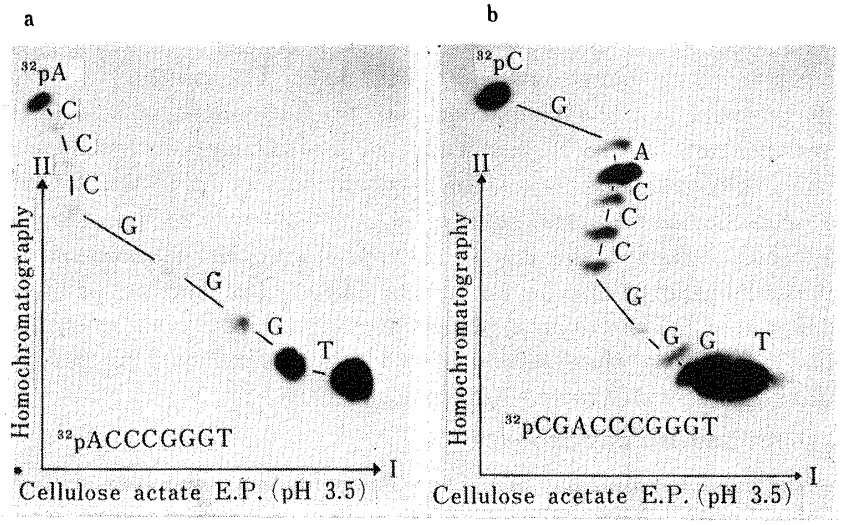


Fig. 4. Mobility Shift Analysis of the Octamer and Decamer

Homochromatography was performed using Homo mix III.[11b]

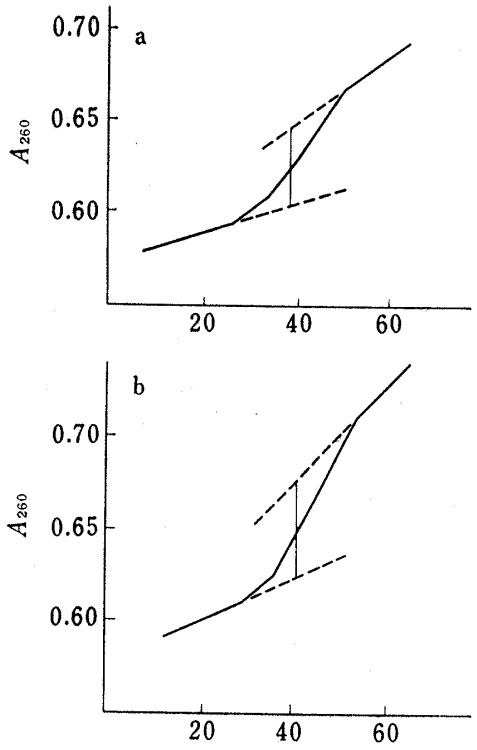


Fig. 5. Absorption-temperature Profile at 260 nm of the Octamer (0.90×10^{-5} M, a) and the Decamer (0.81×10^{-5} M, b) in 0.1 M NaCl, 10 mM Sodium Phosphate, pH 7.0

of measurement. The decamer dCGACCCGGGT could form a concatemer at lower temperature, since the CD spectrum at 12°C resembled that of B form DNA,[14] being quite different from the spectrum of the octamer at 12°C.

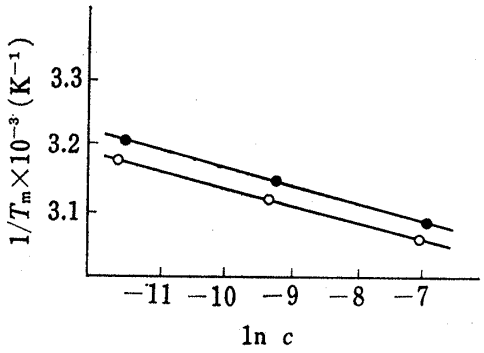


Fig. 6. $1/T_m$ *versus* log *c* Plot of the Octamer (-●-) and the Decamer (-○-)

The conditions were the same as described in the legend to Fig. 5.

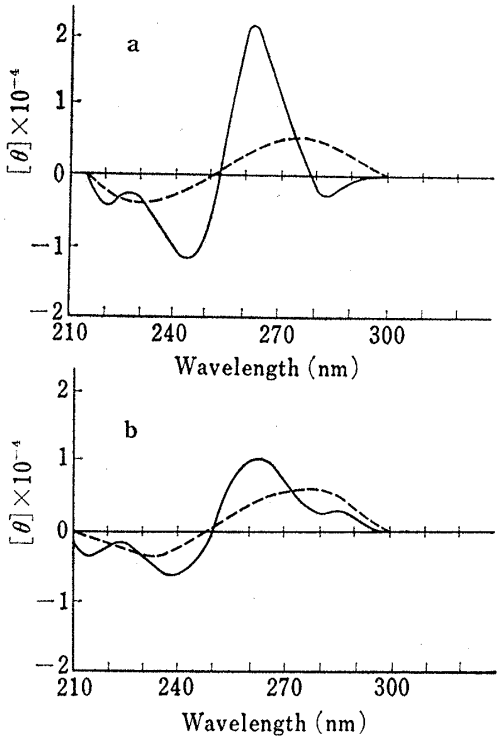


Fig. 7. CD Spectra of the Octamer (0.93×10^{-4} M, a) and the Decamer (0.82×10^{-4} M, b), in 0.1 M NaCl, 10 mM Sodium Phosphate, pH 7.0 at 12°C (——) and 76°C (-------)

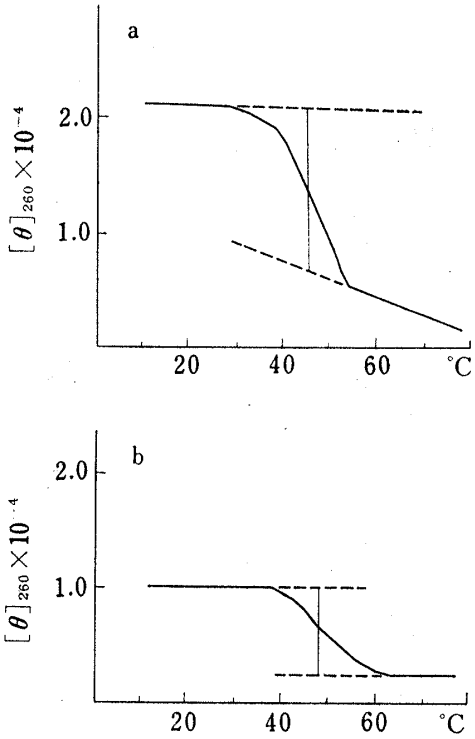


Fig. 8. A_{260} Values at Different Temperatures of the Octamer (a) and the Decamer (b)

Conclusion

The self-complementary octanucleotide dACCCGGGT and partially self-complementary decanucleotide dCGACCCGGGT have been synthesized by the phosphotriester method using *p*-anisidate as protection for the 3′-terminal phosphodiesters. As shown in Chart 3, the monomethoxytrityl group was used as the protecting group for the 5′-hydroxyl end of oligonucleotides which did not contain *N*-benzoyldeoxyadenosine (*e.g.* **7**). The dimethoxytrityl group was employed in the case of deoxyadenosine-containing blocks such as **10**. Removal of the dimethoxytrityl group of **10** was performed by treatment with benzenesulfonic acid under milder conditions[8] than were used for **7**, and gave **14** in a high yield. However, isoamyl nitrite treatment in a mixture of pyridine–acetic acid (1 : 1) to remove the *p*-anisidate of **10** to give **11** had a disadvantage, since the reaction mixture contained acidic materials and isolation of **11** had to be carried out by means of precipitations to prevent dedimethoxytritylation. Reversed phase chromatography essentially solved this problem, since purification of the diester **11** could be performed even if the product was contaminated with a small amount of detritylated compounds.

Fully protected oligonucleotides containing phosphoro-*p*-anisidates (*e.g.* **10**) had generally good stability during column chromatography on silica gel and could be purified almost completely. Purification of the intermediate oligonucleotides is very important to obtain good quality final products. Thus, the present method was found to be suitable for synthesizing pure oligonucleotides. The synthetic scale can be enlarged by using almost the same technique as described here.

The decanucleotide dCGACCCGGGT can be elongated by treatment with DNA ligase. The joined product contains sequences for a variety of restriction endonucleases[6] such as *Sal*I, *Taq*I, *Hind*II, *Ava*I or *Xma*I, *Hpa*II, *Sma*I, *etc.* Studies along these lines will be reported elsewhere.

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