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SELF-COMPLEMENTARY TETRADEOXYRIBONUCLEOSIDE TRIPHOSPHATES CONVENIENT CHEMICAL PREPARATION AND SPECTROSCOPIC STUDIES IN SOLUTION

Jun-ichi Matsuzaki, Hitoshi Hotoda, Mitsuo Sekine, Tsujiaki Hata*,
Shigesada Higuchi[a], Yoshifumi Nishimura[b], and Masamichi Tsuboi[b]

Department of Life Chemistry, Tokyo Institute of Technology,
Nagatsuta, Midori-ku, Yokohama 227, Japan
[a]Mitsubishi-Kasei Institute of Life Sciences,
Minamiooya, Machida, Tokyo 194, Japan
[b]Faculty of Pharmaceutical Sciences, University of Tokyo,
Hongo, Bunkyo-ku, Tokyo 113, Japan

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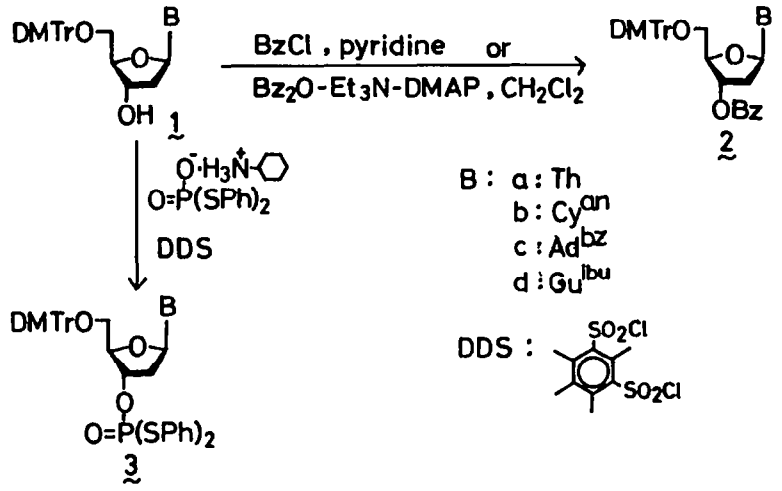
ABSTRACT--Eight kinds of self-complementary tetradeoxyribonucleoside triphosphates were prepared by a simplified method which enabled us to omit purification of synthetic intermediates and provided the tetramers very rapidly and conveniently. The tetramers were characterized by enzyme assay and their conformations were studied by the use of UV and CD spectroscopic methods under various conditions. The detailed analysis of the CD spectra suggested that conformation of the tetramer duplexes was dependent on the sequence.

During the past five years, the conformation of oligodeoxyribonucleotides with unique base sequence has been studied by use of various methods.[1-13] The local conformation of DNA duplex may be an important factor in interactions between nucleic acids and proteins or drugs.[2] Recent X-ray crystallograaphic analysis of several duplex oligomers has suggested that DNA conformations were polymorphic depending on the sequence.[3-7] Compared with the crystalline state, DNA duplex may be more flexible in solution so that subdivided conformers depending on the sequence should be observed even within a duplex of the B-family. Several research groups have tried to obtain the conformations of DNA oligomers with defined sequences in solution by means of spectroscopic methods.[8-13] Particularly, dCGCGCG and its analogues (dCGCG, dmCGmCGmCG, etc.) have been well analyzed on account of the possibility of the Z type structure.[9-13] However, systematic studies have not yet been done in order to elucidate the sequence-dependent conformations at the level of short DNA fragments. Among the shortest GC pairing fragments capable of duplex formation in the usual solution conditions, only dCGCG has been studied by CD[9,10] and NMR[11] spectroscopic methods in order to examine the Z formation. Recently, Thomas and Peticolas[13] studied on possible four kinds of self-complementary tetramers containing cytosine and guanine by laser Raman spectroscopy. However, it is not clear whether the full duplex of the tetramers is formed or not under their conditions. They suggested that the tetramers with GC base pairs, on the whole, tended to form the B duplex in a low salt concentration and that only dCGCG could form the Z duplex in a high salt concentration. In the case of dCCGG, they could not give a clear-cut conclusion because the Raman marker bands for the backbone conformation were very weak to examine the detailed duplex structure. In this paper, we wish to report a convenient method for the synthesis of eight kinds of



Scheme I. Preparation of Monomer Units

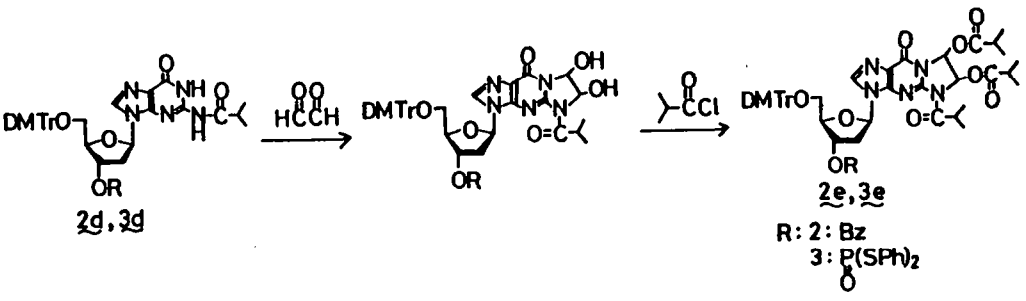
Table I. 3'-O-Benzoylation of Partially Protected Nucleosides (1)

Hydroxyl (1)	Reagents* (equiv.)	time (h)	product	yield (%)
1a	A 1.2	2	2a	86
1b	A 1.2	2	2b	96
1c	A 1.2	4	2c	62
1d	A 1.2	2	2d	94
1c	B 1.5	3	2c	93
1d	B 1.5	3	2d	92

*A: BzCl, B: Bz_2O-Et_3N-DMAP

Table II. Phosphorylation of 1 with PSS and DDS

Hydroxyl (1)	PSS (equiv.)	DDS (equiv.)	time (h)	product	yield (%)
1a	1.2	1.8	4	3a	98
1b	1.2	1.5	5	3b	81
1c	1.2	1.3	6	3c	93
1d	3.0	1.5	2	3d	81



Scheme II. Protection of the Guanine Amide Group

self-complementary tetradeoxyribonucleoside triphosphates, i.e., dGCGC, dGGCC, dCCGG, dCGCG, dATAT, dAATT, dTTAA, and dTATA and also describe a detailed examination of temperature and concentration effects on their duplex formation by the use of UV and CD spectroscopic methods.

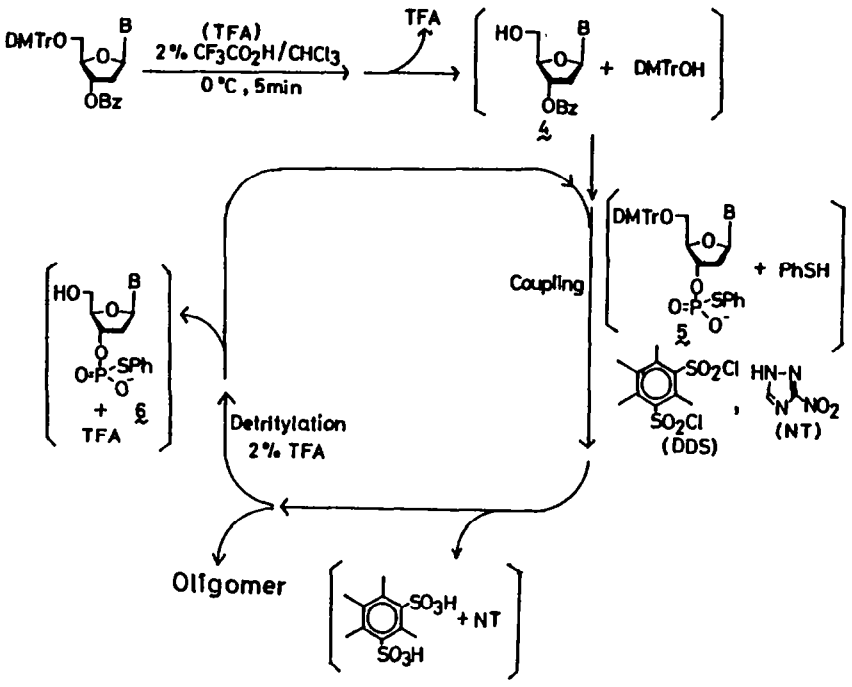
RESULTS AND DISCUSSION

Preparation of Tetramers

The phosphotriester method is now well-established as a useful tool for the chemical synthesis of oligodeoxyribonucleotides. Several modifications of this method have been reported up to date.[14] Application of these approaches to the solid phase synthesis has supplied desired oligomers conveniently but only on a small scale.[15] For our spectroscopic studies, tetradeoxyribonucleotides are required in large quantities with high purity. Therefore, the liquid phase synthesis is more advantageous to prepare the DNA fragments over the solid phase synthesis. Previously, we demonstrated the utility of our phosphotriester method for the synthesis of oligodeoxyribonucleotides by using the phenylthio group as a phosphate protecting group and arenedisulfonyl chlorides as condensing reagents for the liquid phase synthesis.[16,17] We now developed a more improved strategy for the rapid synthesis of tetramers. The tetramers were constructed from 3'-terminal deoxynucleosides in the 5'-direction stepwise without purification of the synthetic intermediates.

The starting mononucleoside units (2) were prepared by 3'-O-benzoylation of partially protected nucleoside (1) in the usual manner (Table I). To obtain pure tetramers, side reactions must be minimized at the synthetic stage. In particular, base modifications during the condensation reaction are most serious. Since the side reaction at the amide function of the guanine moiety was noticed by Reese[18] and us,[19] there have been proposed several protective modes at the reactive sites.[20,21] In this study, the bis(isobutyryloxy)ethylene (Bibe) group[21] was chosen to prevent the side reaction. A new 3'-terminal deoxyguanosine unit (2e) was synthesized as follows. Compound 2d was allowed to react with glyoxal and the resulting diol was separated from the excess glyoxal by extraction. This glyoxal adduct was further protected by the reaction with isobutyryl chloride. The extraction prior to the acylation facilitated separation of the desired unit (2e) from a polymeric byproduct by silica gel column chromatography. If the extractive workup was eliminated, large amounts of polymeric materials derived from glyoxal were formed. Protection of the amide group of a guanylic acid unit (3d) could be performed in a similar way to give 3e. To prepare the mononucleotide units (3a-d), isodurenedisulfonyl chloride (DDS)[17] was used for the 3'-O-phosphorylation. This reagent was found to be more effective than 4,6-dimethoxybenzene-1,3-disulfonyl chloride (DMS)[16] or mesitylenedisulfonyl chroride (MDS)[16] as a condensing reagent (Table II).

Fully protected tetramers were constructed, as in the case of the solid phase synthesis, from 3'-terminal nucleoside in the 5'-direction by stepwise condensation with mononucleotide units. The synthetic procedure was illustrated in Scheme III. A terminal nucleoside was detritylated by treatment with 2% trifluoroacetic acid (TFA) in $CHCl_3$ at 0°C and the resulting hydroxyl component (4) was extracted and used without further purification. Phosphodiester components (5a-c) were conveniently prepared from the nucleotide units (3a-c) by treatment with triethylamine-water-pyridine (2:1:2, v/v/v). The guanosine diester (5e) was prepared by the use of 5 M phosphinic acid--triethylamine (2:1, v/v) at 40 °C followed by extraction. For the synthesis of tetramers, a modified procedure of Itakura[22] was employed and the tetramers were prepared in only one



Scheme III. Elongation Cycle of Simplified Method for the Synthesis of Tetramers.

Table III. Yields of Tetramers

dGCGC	71%	dCGCG	81%	dGGCC	63%
dCCGG	70%	dATAT	73%	dAATT	68%
dTATA	88%	dTTAA	40%		

day from the fully protected monomer units. The unpurified hydroxyl and diester components were mixed, rendered anhydrous, and condensed by DDS and 3-nitro-1,2,4-triazole (NT). After the reaction was completed (usually 40 min), the dimer was extracted and then detritylated as described above. At this stage, the excess detritylated phosphodiester (6) was separated from the hydroxyl dimer by extraction. Elongation of the oligomer chain was repeated without purification of the triester-type intermediates except the desired tetramer which could be purified finally by flush column chromatography. The condensation reactions were much simpler because the excess condensing reagent could be removed as a disulfonic acid by extraction and the last purification was much easier because the sulfonated byproducts could be converted to polar compounds. Thus, eight kinds of self-complementary tetramers including the AT base pairing series and the GC base pairing series were synthesized. The yields of the fully protected products are summarized in Table III.

Complete deprotection of the tetramers were performed as follows:

1) 0.1 M NaOH in pyridine-water (1:1, v/v) at 0°C for 30 min,
2) conc. NH_4OH at room temperature for 36 h,
3) 80% acetic acid for 15 min.

The Bibe group on the guanine moiety could be removed by alkaline treatment of 1). The desired tetramers were purified by anion exchange column chromatography using DEAE-Sephadex A25 (HCO_3^- form) for the AT series or DEAE-Toyopearl 650M (Cl^- form) for the GC series. 500-3000 OD units of the tetramers were obtained in 40-80% yields with high purity. The purities of the tetramers were checked by polyacryl-

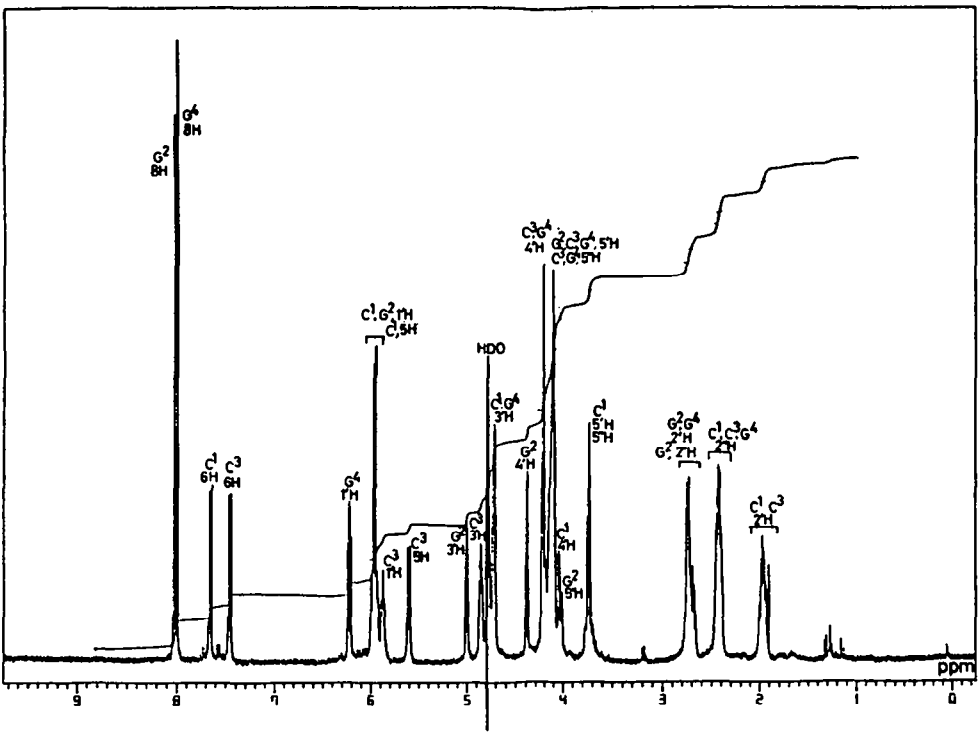


Figure I. ^{1}H NMR Spectrum of dCGCG. 70 OD/ml oligomer in D_2O, 24°C.

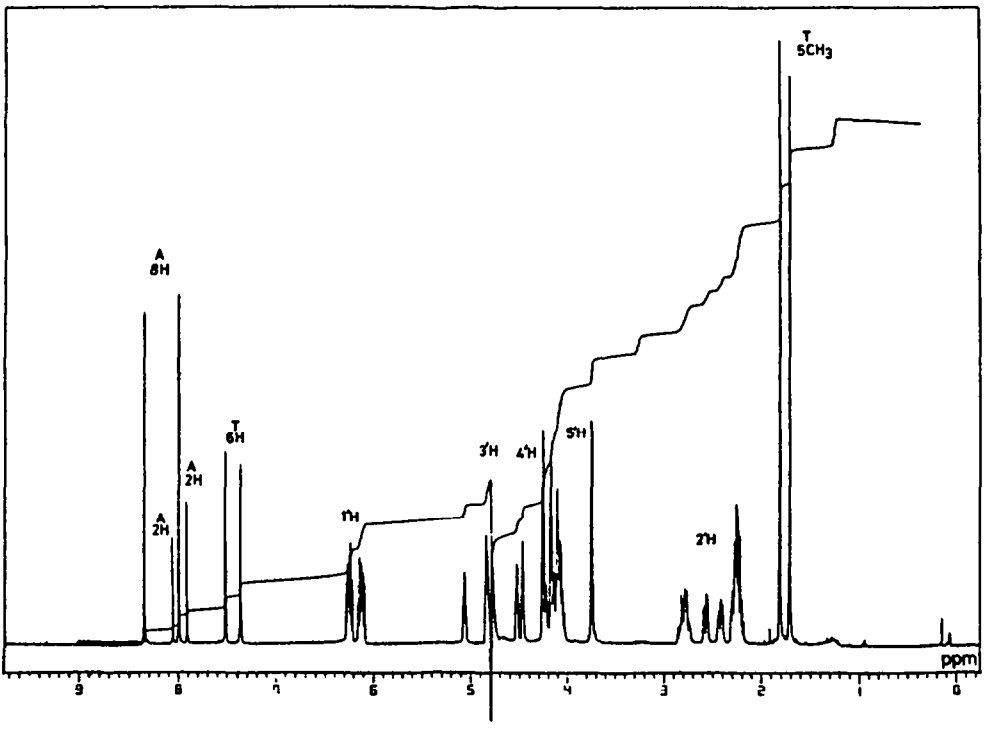


Figure II. ^{1}H NMR Spectrum of dAATT. 70 OD/ml oligomer in D_2O, 24°C.

Table IV. Characteristic NMR Resonance peaks of GC Tetramers. 70 OD/ml oligomer in D_2O, 24°C

	G-8H		C-6H		C-5H	
dGCGC	7.99 (s)	7.96 (s)	7.58 (d)	7.47 (d)	5.55 (d)	5.65 (d)
dGGCC	7.99	7.76	7.82	7.63	5.99	5.75
dCGCG	8.02	7.99	7.64	7.45	5.98	5.58
dCCGG	7.94	7.89	7.77	7.58	6.00	6.87

Table V. UV Data of Tetramers

	pH	λ max	λ min	λmax	λmin
dGCGC	7.0	253 (281)	224	9380	5410
	2.0	275 (258)	231	-	-
dCGCG	7.0	253 (280)	223	9690	5330
	2.0	275 (257)	232	-	-
dGGCC	7.0	253 (278)	223	9410	5340
	2.0	274 (257)	230	-	-
dCCGG	7.0	253 (280)	223	9800	5600
	2.0	276 (258)	231	-	-
dATAT	7.0	260	231	10530	2800
dTATA	7.0	260	230	10610	2840
dAATT	7.0	260	231	10300	2820
dTTAA	7.0	260	232	10430	3020

amide gel electrophoresis (UV illumination) and reversed phase HPLC (μBondapak C_{18}) which showed that they were homogeneous and the structures of the tetramers were confirmed by nuclease P_1 digestion. Furthermore, their 400 MHz ^1H NMR spectra were measured at room temperature in a concentration of 70 OD/ml. The NMR spectra of dCGCG and dAATT are shown in Figure I and II. Among all the tetramers, the resonance peaks of dCGCG have been completely assigned by Cheng et al.[11] and were identical with those of our sample. As shown in Figure I and II, several characteristic resonance peaks gave structural proof of the tetramers. In the case of the GC pairing tetramers, duplex formations were observed to a certain extent under these conditions used by the ^{31}P NMR studies[26]. Chemical shifts of guanine H8 and cytosine H5 and H6 are summarized in Table IV.

UV and CD Spectra of the AT Tetramers

The UV spectra of the AT tetramers at pH 7.0 are shown in Table V. Thermal changes of the spectra were not observed in both low and high concentrations of the tetramers (1.5 OD/ml and 150 OD/ml in 0.5 M NaCl). The CD spectra (Figure III) showed no conformational changes by changing of temperatures. Therefore, we can conclude that the AT tetramers do not prefer the duplex conformations under these conditions.

UV Spectra of the GC Tetramers

The UV spectra of the four GC tetramers in a low concentration at pH 7.0 and pH 2.0 are summarized in Table V. The spectra showed almost the same profiles and

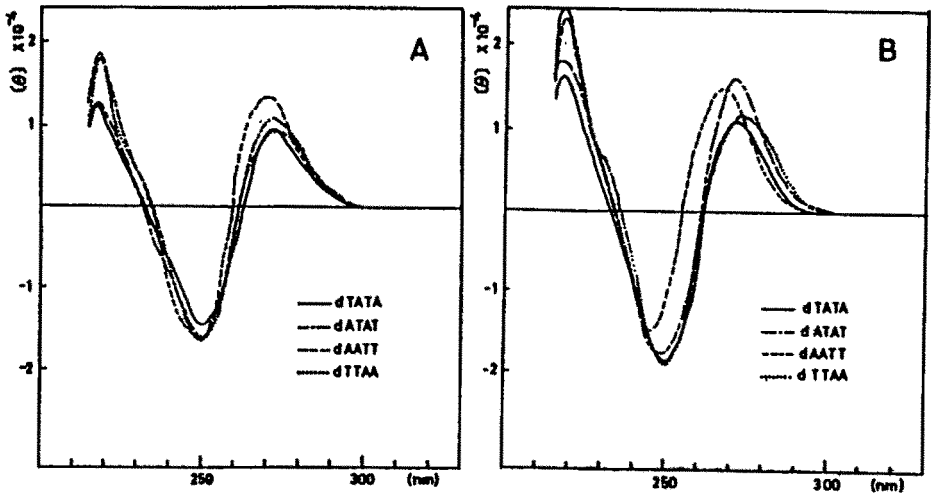


Figure III. CD spectra of AT Tetramers. A; ca. 1.5 OD/ml tetramer, 0.1 M NaCl at 20 °C; B: ca. 130 OD/ml tetramer, 0.5 M NaCl at -5 °C.

the considerable changes were observed between at pH 7.0 and pH 2.0 suggesting that the tetramers contained both cytidine and guanosine residues equally. Their ε values were determined by nuclease P_1 digestion at pH 7.0 and the hypochromicities relative to the monomer mixtures were found to be 11.0, 8.4, 9.0, and 7.6 % for dGCGC, dGGCC, dCGCG, and dCCGG, respectively. In a low concentration of oligomers (ca. 1.5 OD/ml), the UV absorbances were not affected by temperature indicating no duplex formation. In a higher concentration (ca. 130 OD/ml), the duplex formation was clearly observed as judged by the thermal changes of absorbances as shown in Figure IV. The Tm values were obtained from the melting curves in a 0.5 M NaCl concentration at 255 nm: 28 °C for dGCGC, 23 °C for dGGCC, 18 °C for dCCGG, and 0°C for dCGCG. This result may be related to the intramolecular base stacking. At the level of dinucleotides, the stabilizing effect due to the stacking decreases in the order of GC>GG≅CC>CG.[23] Therefore, the order of the duplex stability is expected to be dGCGC>dGGCC>dCGCG>dCCGG. However, in our experiment, dCGCG forms the most unstable duplex from the Tm values. The stacking of the terminal dinucleotide units might be more effective than that of the central dimer units to form the duplex of the tetramers.

CD Spectra of the GC Tetramers

As far as the CD study was concerned, only dCGCG tetramer was studied among all possible GC tetramers.[9,10] Uesugi et al. showed that it could form a duplex of the Z structure in a high concentration of the oligomer under a high salt and low temperature condition.[10] In this study, all of the four GC tetramers which have the equal base compositions have been examined to reveal the conformations under various conditions.

The CD spectra of the four GC pairing tetramers were measured under the conditions of 1.5 OD/ml, 0.1 M NaCl, 25 °C where the UV spectra suggested no duplex formation as described before. As shown in Figure V, it was found that their CD spectra were essentially dependent on the seuquence. The results are in good agreement with the spectral profiles calculated by the nearest neighboring method introduced by Tinoco[24] except for dCCGG (data are not shown). The spectra of all the GC tetramers changed so remarkably in a high concentration (ca. 130 OD/ml) where the tetramers were expected to form duplexes partially or completely as the result of UV studies indicated. From the CD spectra, it was concluded that dGCGC in high concentration existed in the typical B form duplex which gave

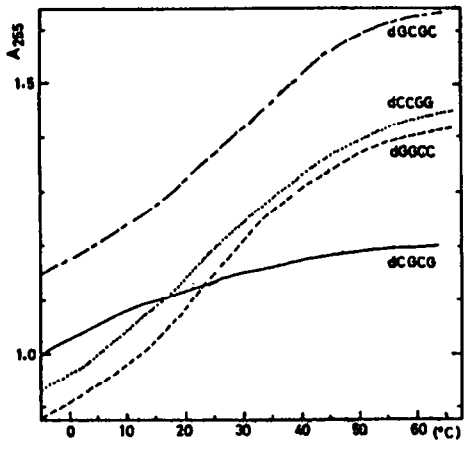
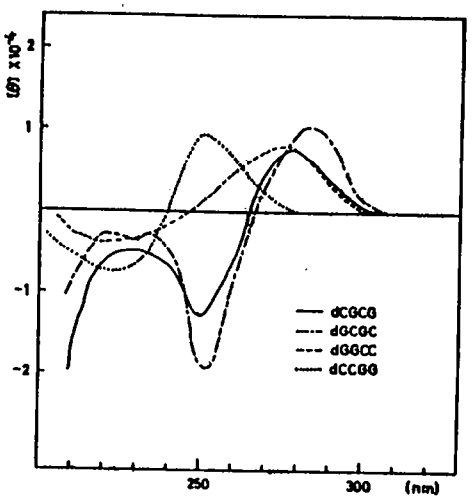


Figure IV. UV Heating Curves of GC Tetramers (ca. 130 OD/ml). 10 mM cacodylate (pH 7.0), 0.5 M NaCl.



FigureV. CD Spectra of GC Tetramer (ca. 1.5 OD/ml). 10 mM cacodylate (pH 7.0), 0.1 M NaCl.

positive bands around 280 nm and negative bands around 250 nm[25] (Figure VI A). This duplex was stabilized at a high salt concentration (0.5 M NaCl) and low temperature.

On the other hand, dCGCGCG and dCGCG were known to form the Z duplex in a high salt concentration (higher than 4 M NaCl) and to form the B duplex in a lower salt concentration.[10,11] As shown in Figure VI B, the CD spectra of dCGCG had weak positive bands at 280 nm and strong negative bands at 250 nm which suggested the B family conformation. Though the duplex would not be formed completely from the UV experimental results, compared with the CD spectra of dGCGC, the intensity of the positive bands around 290 nm remarkably decreased with an increase in salt concentration or with a decrease in temperature. These phenomena should not be explained by the contribution of the Z form, since the strong negative bands around 250 nm suggested the B form character. Thus, we consider that the tetramer dCGCG forms somewhat modified B-type of duplex under these conditions. However, there is still another possibility that this spectral feature is derived from the C form.[25]

The relatively different spectra were observed in the case of dGGCC (Figure VI C). The strong positive and weak negative bands suggested the distortion of the B form duplex probably due to the GG stacking effect. The X-ray analysis showed that self-complementary oligomers containg a GG sequence ($d^{I}CCGG$[5], dGGCCGGCC,[6] dGGTATACC[7]) formed the A form. It is considered that the GG sequence stabilizes the A form duplex rather than the B form. Contrary to these facts, in the case of solution, the confomation of the duplex of dGGCC can be classified in one of the B family because the spectra had the positive bands around 280 nm suggesting the B form character.[25]

However, the duplex of dCCGG gave very interesting results. The CD spectra were dramatically changed in varying concentrations of the oligomer (Figure VI D). The duplex formation led to strong positive bands at 260 nm and new negative bands at 285 nm. These profiles suggested the A form duplex.[25] X-ray analysis of a similar iodonated sample, $d^{I}CCGG$, showed that the duplex formed the A conformation[5] in the crystalline. Recent laser Raman spectroscopic study by Thomas and Peticolas[13] showed that the conformation of the duplex of dCCGG in solution is not the A form,and they described that this conformer might be a flexible form between the A form and the B form. However, our laser Raman

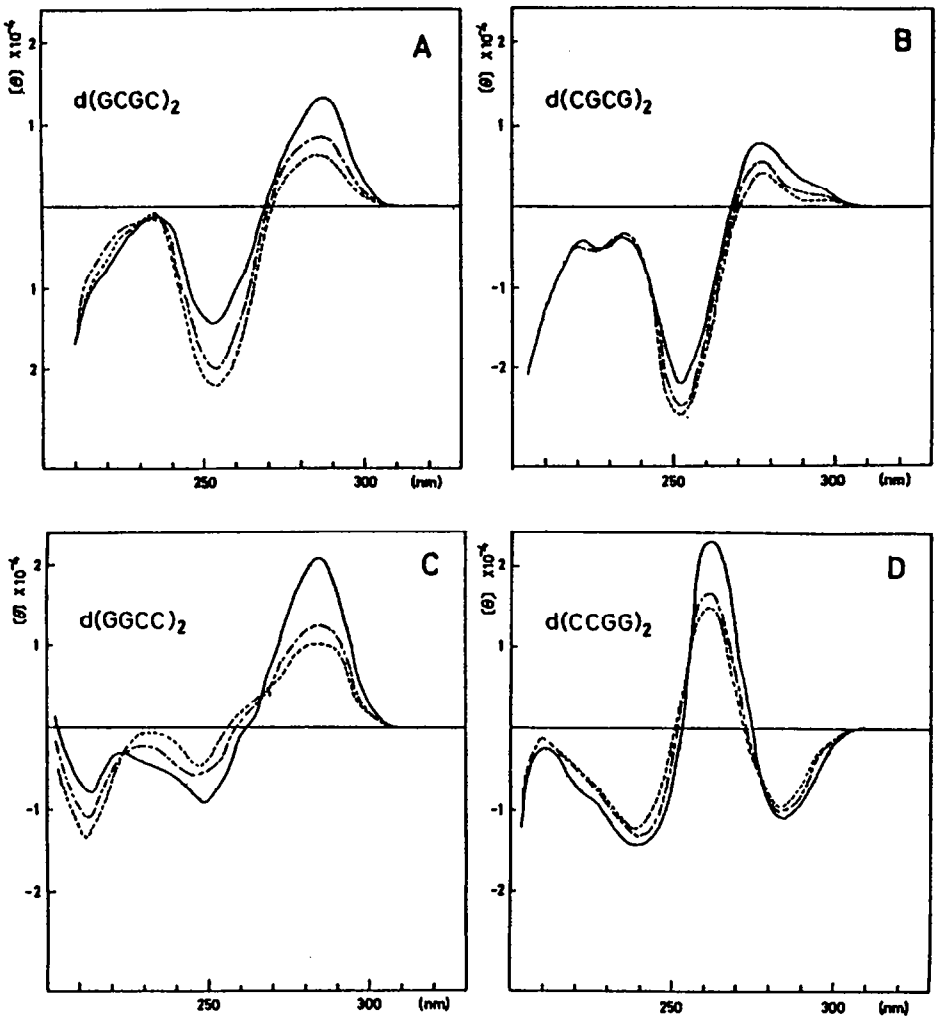


Figure VI. CD Spectra of GC Tetramers (ca. 130 OD/ml). ——:NaCl free at 5 °C; —·—:0.5 M NaCl at 5 °C;-----:0.5 M NaCl at -5 °C.

sepctrum of this tetramer suggested a unigue form rather than the A form or the usual B form[26]. Furthermore, the CD spectra suggested that a two-state conversion should be present because of clear observation of two isosbestic points as shown in Figure VI D. Therefore, it was suggested that dCCGG formed an entirely "new" confomer. It was also very interesting that the similar abnormal CD spectra was reported on dCCAGATCTGG recently.[28]

Moreover, the conformations of the GC tetramers in a much higher salt concentration (4 M NaCl) were determined (Figure VII). Only dCGCG gave the Z duplex while the others resulted in stabilization of the conformations which appeared in low salt concentrations (lower than 0.5 M NaCl). These results are consistent with those obtained by Thomas and Peticolas.[13] In a high salt concentration, the Z duplex of dCGCG was formed not via the B duplex but directly from the single strand because the CD profiles at the temperatures of -5, 5, and 20 °C showed a two-state conversion having an isosbestic point at 280 nm (Figure VIII).

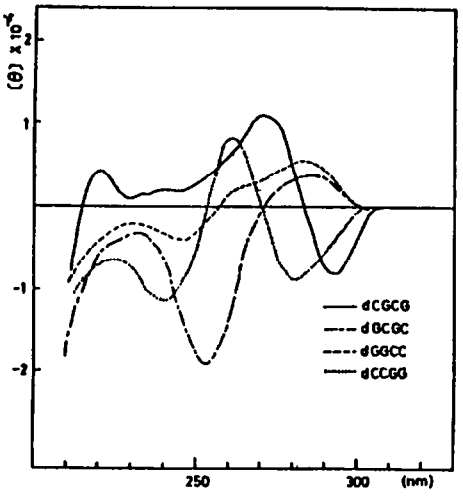


Figure VII. CD Spectra of GC Tetramers (ca. 130 OD/ml) in 4 M NaCl.

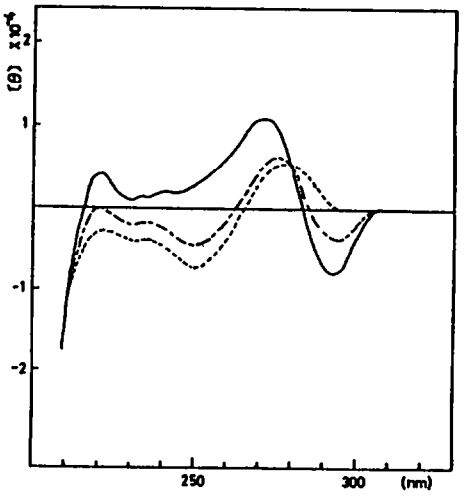


Figure VIII. CD Spectra of dCGCG (ca. 120 OD/ml) in 4 M NaCl. ——:-5 °C; – – –:5 °C; – – – –:20 °C.

CONCLUSIONS

The simplified methods for preparation of short DNA fragments enabled us to obtain the desired oligomers rapidly and conveniently. The amounts of these oligomers were sufficient for several spectroscopic studies and their results revealed the sequence dependent conformations of DNA duplex. Especially, it is noteworthy that dCCGG exists as a abnomal duplex conformer on the basis of the CD patterns in this study. The results of these systematic studies should be concerned with the interactions of proteins and nucleic acids.

EXPERIMENTAL SECTION

General Remarks

^{1}H NMR spectra of the protected compounds were recorded at 100 MHz on a JNM-PS-100 spectrometer using ppm according to tetramethylsilane as an internal reference. Reagent grade pyridine was distilled after being refluxed over p-toluenesulfonyl chloride for several hours, redistilled over calcium hydride after being refluxed for several hours, and stored over molecular sieves 4A. Methylene chloride was dried over phosphorus pentoxide overnight, decanted, distilled from potassium carbonate, and stored over molecular sieves 4A. Column chromatography for the monomer units and fully protected tetramers was performed by using silica gel C-200 purchased from Wako Co. Ltd. under a medium pressure by using a goldfish air pump. Thin layer chromatography for monitoring the reactions was performed on precoated TLC plates (silica gel 60 F-254 Merck, Art. No. 5715) by using the following solvent systems: Solvent A (CH_2Cl_2-MeOH, 12:1, v/v); Solvent B (CH_2Cl_2-MeOH, 9:1, v/v). Elemental analysis was performed by the Microanalytical Laboratory, Tokyo Institute of Technology at Nagatsuta.

2'-Deoxyribonucleosides were purchased from Yoshitomi-seiyaku Co. Ltd. and the partially protected nucleosides (1) were prepared according to the methods reported by Jones.[27] 3-Nitro-1,2,4-triazole was purchased from Dojin-kagaku-yakuhin Co. Ltd. and the other reagents were available from Wako Co. Ltd. and Tokyo Kasei Co. Ltd.

3'-O-Benzoylation of 1

Method A: Partially protected nucleoside (1) (5 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and dissolved in dry pyridine (30 ml). To the solution, benzoyl chloride (703 μl, 6 mmol) was added dropwise and the mixture was stirred at room temperature. After completion of the reaction (usually it took about 2 h), ice blocks (2 g) were added and the mixture was transferred into a separatory funnel with $CHCl_3$ (100 ml). The organic layer was washed three times with 5% $NaHCO_3$ and the combined aqueous layers were backextracted with $CHCl_3$ (100 ml). The latter organic layer was further washed

with 5% $NaHCO_3$, combined with the former organic layer, and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the last traces of pyridine was completely removed by coevaporation with toluene. The residue was applied to a column of silica gel. Chromatography was performed by elution with CH_2Cl_2 containing methanol (0-2%, v/v) to give the desired compounds in the yields described in the text.

Method B: A nucleoside (1b or 1d) (5 mmol) and N,N-dimethylaminopyridine (92 mg, 0.75 mmol) were dissolved in dry CH_2Cl_2 (30 ml). To the mixture was added triethylamine (1.04 ml, 7.5 mmol) and then benzoic anhydride (7.7 g, 7.5 mmol). The mixture was stirred at room temperature for 3 h. The desired products were obtained after the usual workup as described in the above experiment and consistent completely with authentic samples by comparison with their ^1H NMR spectra.

Phosphorylation of 1 with PSS and DDS

Method A: Partially protected nucleoside (1a-c) and cyclohexylammonium S,S-diphenyl phosphorodithioate (PSS) were mixed and rendered anhydrous by repeated coevaporation with dry pyridine. The mixture was dissolved in dry pyridine and DDS was added to the solution. The reaction was stirred at room temperature and monitored by TLC until a spot of 1 had disappeared. The usual workup (washing with 5% $NaHCO_3$, drying over Na_2SO_4, evaporation, coevaporation, and silica gel column) gave mononucleotide unit (3a-c). Equivalent of the reagents, reaction time, and yields are summarised in Table II.

Method B: PSS (5.72 g, 15 mmol) was rendered anhydrous by coevaporation with dry pyridine (3 x 10 ml) and dissolved in dry pyridine (40 ml). DDS (2.48 g, 7.5 mmol) was added and the mixture was stirred at room temperature for 30 min. To the solution, 2d (3.2 g, 5 mmol), pre-dried by coevaporation with dry pyridine, was added with dry pyridine (5 + 5 + 5 ml), and the resulting solution was stirred for 2 h. Then cold aqueous solution of 5% $NaHCO_3$ (10 ml) was added and the solution was stirred further 20 min. The usual workup gave 3d in 81% yield.

These products were identified with authentic units previously reported[17] by comparison with their ^1H NMR spectra.

Protection of the Amide Function of Guanine Moiety

Commercially available glyoxal (40% aqueous soln., 17.5 ml, 100 mmol) was concentrated under reduced pressure to a gum. The gummy material was coevaporated with pyridine several times to remove water. The resulting solution was mixed with a deoxyguanosine derivative (2d or 3d) (5 mmol) and the mixture was further coevaporated several times with dry pyridine for complete conversion to the diol adduct. The mixture was transferred into a separatory funnel with $CHCl_3$ (100 ml) and washed three times with 5% $NaHCO_3$. The washings were backextracted with $CHCl_3$ and the organic layer was further washed with 5% $NaHCO_3$ and combined with the former and then dried over Na_2SO_4. The solvent was removed under reduced pressure and rendered anhydrous by repeated coevaporations with dry pyridine. Isobutyryl chloride (1.57 ml, 15 mmol) was added dropwise and the mixture was kept at room temperature for 3 h. Then 5% $NaHCO_3$ (5 ml) was added and the resulting mixture was stirred for 10 min at 0°C. The usual workup involving washing with 5% $NaHCO_3$, drying over Na_2SO_4, evaporation, and chromatography on a silica gel column gave the completely protected guanosine units 2e and 3e in 94% and 91% yields repectively. The mononucleotide unit (3e) was identified with an authentic sample reported previously[21] by comparison with their ^1H NMR spectra. 2e: NMR δ($CDCl_3$) 1.20 (m, 18H, $CH(CH_3)_2$), 2.64 (m, 5H, $CH(CH_3)_2$ and 2'H), 3.55 (m, 2H, 5'H), 3.78 (s, 6H, CH_3), 4.46 (m, 1H, 4'H), 5.68 (m, 1H, 3'H), 6.36 (t, 1H, 1'H), 6.80-8.08 (m, 21H, ArH and OCHCHO), 7.93 (s, 1H, 8H).

Anal. Calcd for $C_{52}H_{55}O_{12}H_5$: C, 66.30; H, 5.89; N, 7.43. Found: C, 66.66; H, 5.88; N, 7.30.

Phosphodiester Components

Method A: A mononucleotide unit (3a-c) (0.39 mmol) was treated with triethylamine-water-pyridine (2:1:2., v/v/v, 10 ml) at room temperature. After 40-90 min, the reaction mixture was evaporated and coevaporated twice with toluene. The resulting oil was used as a phosphodiester component in coupling reaction without further purification.

Method B: The deoxyguanosine unit (3e) (430 mg, 0.39 mmol) was treated with 5 M pyridine solution of phosphinic acid--triethylamine (2:1, v/v, 9 ml) at 40°C for 30 min. The usual washing procedure with 5% $NaHCO_3$ followed by removal of the solvents gave the unpurified diester component for coupling reaction.

General Procedure for the Synthesis of Fully Protected Tetramer

A 3'-terminal nucleoside unit (0.3 mmol) was dissolved in $CHCl_3$ (12 ml) and cooled in an ice bath. Trifluoroacetic acid (TFA) (240 µl) was added at once and the reaction mixture was kept at 0°C with stirring. After 5 min, a few drops of pyridine was added until the color of dimethoxytrityl cation had disappeared. The colorless solution was transferred into a separatory funnel and washed three times with 5% $NaHCO_3$. The aqueous layers were extracted with $CHCl_3$ and the extract was washed with 5% $NaHCO_3$ again. The organic layers were combined, dried over Na_2SO_4 and mixed with a crude phosphodiester component (0.39 mmol) prepared in the above experiment. The solvent was removed under reduced pressure and 3-nitro-1,2,4-triazole (103 mg, 0.9 mmol) was mixed with the residue. The mixture was rendered

anhydrous by repeated coevaporation several times with dry pyridine and dissolved in dry pyridine (3 ml). DDS (199 mg, 0.6 mmol) was added and the mixture was stirred at room temperature. The reaction was monitored by TLC until a spot of the hydroxyl component had disappeared. The condensation took usually 40-60 min. The usual workup (extraction, drying over $NaSO_4$, evaporation and coevaporation with toluene) gave the crude fully protected dimer. The same operations as described above (TFA treatment, extraction, coupling reaction, and extraction) were repeated twice to gave the crude tetramer which was easily purified by column chromatography (30 g of silica gel, with CH_2Cl_2 containing 0-2% methanol). For the yields of the fully protected tetramers, see the text.

Deprotection and Purification of Tetramers

A typical procedure for dGCGC: The fully protected tetramer (226 mg, 0.1 mmol) was dissolved in pyridine (10 ml) and cooled at 0°C. To the mixture an ice-cooled 0.2 M sodium hydroxide solution (10 ml) was added dropwise and the mixture was stirred at 0°C for 30 min. Then the solution was applied to a column of Dowex 50 W x 8 (pyridinium form, 8 ml) and the column was eluted with pyridine-water (1:1, v/v, 50 ml). All the resulting eluents were collected and evaporated to an oil. The oil was dissolved in conc. NH_4OH (30 ml) and then stirred at room temperature for 36 h. The solvent was carefully removed under reduced pressure and coevaporated with water. The residue was treated with 80% acetic acid (15 ml) at room temperature for 15 min. The reaction mixture was evaporated and coevaporated several times with water and dissolved in 0.05 M triethylammonium bicarbonate (TEAB) buffer (pH 7.0) (10 ml). The solution was washed with ether (3 x 50 ml) and concentrated under reduced pressure again. Purification of the tetramer was performed by one of the two ways as follows;

(a) DEAE Sephadex A25 (2.5 x 25 cm, a liner gradient of 0.05 M- 2.5 M TEAB),
(b) DEAE Toyopearl 650 M (2.5 x 40 cm, 7 M urea, 20 mmol Tris-HCl, pH 8.0, a linear gradient of 0.01 M-0.2 M NaCl), Bio gel P-2 (0.05 M TEAB).

Fractions containing the tetramer were collected and lyophilized several times until triethylamine was completely removed. The powder was dissolved in water and passed through a column of Dowex 50 W x 8 (Na^+ form, 20 ml) to give 3300 A_{254} units of dGCGC which was lyophilized to obtain white fiber-like powder.

^1H NMR Spectra

The measurements were made with JOEL GX-400 Fourier-transform NMR system at 399.65 MHz. The sample solution was placed in a cell of 5 mm diameter. Chemical shift values were measured in ppm by using sodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the reference standard in D_2O.

UV Spectra

UV spectra were recorded on a Hitachi 220A spectrometer at room temperature. Melting curve analysis were performed on a Gilford 2400S spectrometer at 255 nm. The temperature was controlled using circulating ethylene glycol--ethanol from a Haake bath at the rate of temperature increase being approximately 1°C/min. The sample was dissolved in 10 mM sodium cacodylate buffer (10 mM, pH 7.0) containing appropriate amounts of NaCl and placed in a cell (1 cm or 0.01 cm). Absorbance was recorded every 30 sec and a corrected melting curve was obtained by calculation with HP85 (Heulett Packard).

CD Spectra

CD spectra were recorded on a JASCO J40 at controlled temperatures by using a Haake bath circulation. The sample was dissolved in 10 mM sodium cacodylate buffer (pH 7.0) containing NaCl appropriately. Cells used in the experiments were 1 cm x 1 cm for the diluted samples and 0.01 cm x 1 cm (available from Gasukuro Kogyo Co. Ltd.) for the concentrated samples.

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REFERENCES

1) W. Saenger, "Principles of Nucleic Acids Structure", Springer-Verg, New York (1984).
2) C. A. Frederick, J. Grable, M. Melia, C. Smudzi, L. Jen-Jocobson, B.-C. Wang, P. Breene, H. W. Boyer, J. M. Rosenberg, Nature, 309, 327 (1984); K. Bolewska, A. Zielenkiewicz, L. Wierzchowski, Nucleic Acids Res., 12, 3245 (1984); S. Uesugi, T. Shida, M. Ikehara, Y. Kobayashi, Y. Kyogoku, Ibid., 12, 1581 (1984);Y. Nishiyuma, M. Tsuboi, J. Biochem., 96, 1807 (1984).
3) A. H-J. Wang, G. J. Quigley, F. J. Kolpak, J. L. Crowford, J. H. van Boom, G. van der Marel, A. Rich, Nature, 282, 680 (1979); H. R. Drew, T. Takano, S. Tanaka, K. Itakura, R. E. Dickerson, ibid., 286, 567 (1980); J. L. Crowford,

F. J. Kolpak, A. H-J. Wang, G. J. Quigley, J. H. van Boom, G. van der Marel, A. Rich, Proc. Natl. Acad. Sci. USA, 77, 4016 (1980); S. Fujii, A. H-J. Wang, G. van der Marel, J. H. van Boom, A. Rich, Nucleic Acids Res., 10, 7879 (1982).
4) H. R. Drew, R. M. Wing, T. Takano, C. Broka, S. Tanaka, K. Itakura, K. Itano, R. E. Dickerson, Proc. Natl. Acad. Sci. USA, 78, 2179 (1981).
5) B. N. Conner, T. Takano, S. Tanaka, K. Itakura, R. E. Dickerson, Nature, 295, 294 (1982).
6) A. H.-J. Wang, S. Fujii, J. H. van Boom, A. Rich, Proc. Natl. Acad. Sci. USA, 79, 3968 (1982).
7) Z. Shakked, D. Rabinovich, W. B. T. Cruse, E. Egert, O. Kennard, G. Sala, S. A. Salisbury, M. AM. Viswamitra, Proc. Roy. Soc., B213, 479 (1981).
8) J.-R. Mellema, P. N. van Kampen, C. N. Carlson, H. E. Bosshard, C. Altona, Nucleic Acids Res., 11, 2893 (1983); D. B. Reid, S. A. Salisburg, D. H. Williams, ibid., 11, 3779 (1983); J. W. Loweu, C. C. Hanstock, R. C. Bleackley, J.-L. Imbach, B. Rayner, J. J. Vasseur, ibid., 12, 2519 (1984).
9) F. Quadrifoglio, G. Manzini, M. Vasser, K. Dinkespield, R. Crea, ibid., 9, 2195 (1981).
10) S. Uesugi, T. Shida, M. Ikehara, Chem. Pharm. Bull., 29, 3573 (1981).
11) D. M. Cheng, L. Kan, D. Frecht, P. O. P. Ts'o, S. Uesugi, T. Shida, M. Ikehara, Biopolymers, 23, 775 (1984).
12) B. Hartmann, N. T. Thuong, J. Pouyet, M. Ptak, M. Leng, Nucleic Acids Res., 11, 4453 (1983); J. Feigon, A. H.-J. Wang, G. A. van der Marel, J. H. van Boom, A. Rich, ibid., 1243 (1983).
13) G. A. Thomas, W. L. Peticolas, Biochemistry, 23, 3202 (1984).
14) V. Amarnath, A. D. Broom, Chem. Rev., 77, 183 (1977); C. B. Reese, Tetrahedron, 34, 3143 (1978); S. A. Narang, ibid., 39, 3 (1983).
15) M. D. Edge, A. R. Greene, G. R. Heathcliffe, P. A. Meacock, W. Schuch, D. B. Scanlon, T. C. Atkinson, C. R. Newton, A. F. Markham, Nature, 292, 756 (1981); S. Tanaka, T. Shima, K. Ohsuye, T. Ono, K. Mizuno, A. Ueno, H. Nakazato, M. Tsujimoto, N. Higash, T. Noguchi, Nucleic Acids Res., 11, 1707 (1983); J. J. Rossi, R. Kierzek, T. Huang, P. Walker, K. Itakura, J. Biol. Chem., 257, 9226 (1982).
16) M. Sekine, J. Matsuzaki, T. Hata, Tetrahedron Lett., 3209 (1981).
17) M. Sekine, J. Matsuzaki, T. Hata, Tetrahedron, in press.
18) C. B. Reese, A. Ubasawa, Nucleic Acids Res. Symposium Series, 7, 5 (1980).
19) H. P. Daskalov, M. Sekine, T. Hata, Tetrahedron Lett., 3899 (1980).
20) M. Sekine, J. Matsuzaki, M. Satoh, T. Hata, J. Org. Chem., 47, 571 (1982); T. Kamimura, M. Tsuchiya, K. Koura, M. Sekine, T. Hata, Tetrahedron Lett., 2775 (1983); C. B. Reese, P. A. Skone, J. Chem. Soc. Perkin Trans. I, 1263 (1984).
21) M. Sekine, J. Matsuzaki, T. Hata, Tetrahedron Lett., 5287 (1982).
22) C. Broka, T. Hozumi, R. Arentzen, K. Itakura, Nucleic Acids Res., 8, 5461 (1980).
23) R. L. Ornstein, R. Rein, D. L. Breen, R. D. MacElroy, Biopolymers, 17, 2341 (1978); O. Gotoh, Y. Takashira, ibid., 20, 1033 (1981).
24) D. M. Gray, I. Tinoco, Jr., ibid., 9, 223 (1970); C. R. Cantor, M. M. Warshaw, H. Shapiro, ibid., 9, 1059 (1970).
25) D. S. Moore, T. E. Wanger, ibid., 13, 977 (1974).
26) Y. Nishimura et al., in preparation.
27) G. S. Ti, B. L. Gaffney, R. A. Jones, J. Am. Chem. Soc., 104, 1316 (1982).
28) T. Ueda, M. Ono, M. Satoh, H. Ohtani, Abstract Paper 104th Annual Meeting of Pharmaceutical Society of Japan, p255 (1984) Sendai.