[Chem. Pharm. Bull. 30(3) 874—880 (1982)]

Studies on Deoxynucleic Acids and Related Compounds. IV. Syntheses of an Octanucleotide containing a Recognition Site for Restriction Enzyme *Eco* RI and of an Arabinosyladenine Analog

Eiko Ohtsuka, Hirokazu Morisawa, and Morio Ikehara*

Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka

(Received August 3, 1981)

An octanucleotide containing a recognition site for *Eco* RI and its arabinosyladenine (araA) analog, dGGAATTCC and dGGaraAATTCC, were synthesized by the phosphotriester approach with phosphoro-*p*-anisidate as the protecting group for 3′-phosphodiesters. araA was converted to 5′-dimethoxytrityl-*N*,2′-*O*-benzoyl 3′-*p*-chlorophenyl phosphate and condensed with *N*-benzoyldeoxyadenosine 3′-*p*-chlorophenyl phosphoro-*p*-anisidate to yield the protected araAdAp. Other deoxynucleotide blocks (dAAp, dGGp) were prepared similarly and condensed with a 5′-deblocked dTTCC block having the 3′-*O*-benzoyl group after removal of the *p*-anisidate group with isoamyl nitrite.

Keywords——*Eco* RI site analog; arabinosyladenine containing deoxy ribooligonucleotide; phosphotriester synthesis; phosphoro-*p*-anisidate; reverse phase high pressure chromatography

Restriction endonucleases have been found in bacteria as protection systems, and they have proved to be convenient tools for recombination studies of deoxyribouncleic acid (DNA).[2] Restriction endonuclease *Eco* RI recognizes a selfcomplementary sequence dGAATTC and cleaves it between the 3′-hydroxy group of deoxyguanosine and deoxyadenosine 5′-phosphate.[3] Methylation at the N^6 position of adenine adjacent to the axis of symmetry prevents cleavage by the enzyme. The mode of action of this enzyme has been investigated by base substitution and by using a virus DNA with known sequences.[4] However, recognition of sugar moieties in the cleavage site by *Eco* RI is not known. One report stated that the DNA strand of DNA: ribonucleic acid (RNA) hybrid was cleaved by some restriction enzymes such as *Eco* RI, *Hind* III, *Sal* I, *Msp* I, *Hha* I, *Alu* I, *Tag* I and *Hae* III.[5] Arabinosyladenine (araA) is well known as an antagonist of deoxyadenosine and it is of interest to know whether or not araA or araA-containing oligonucleotides are recognized by restriction enzymes. We describe in this paper the synthesis of a deoxyoctanucleotide containing a recognition site for *Eco* RI with araA substitution, dGGaraAATTCC. Since this enzyme has been shown to recognize synthetic short duplices,[6] the present octanucleotide should be large enough to be recognized by the enzyme. The synthetic approach involved the phosphotriester method with phosphoro *p*-anisidate protection.[1] The synthesis of the corresponding deoxyoctanucleotide dGGAATTCC is also described.

Protection and Phosphorylation of Arabinofuranosyl Adenine

AraA obtained by transglycosidation[7] was protected as shown in Chart 1 by perbenzoylation of **1** followed by *O*-debenzoylation with strong alkali to give **2** which was treated with monomethoxytrityl chloride to yield bis-substituted compounds. The 3′,5′-substituted isomer (**3**) was separated from the 2′,5′-substituted compound (**4**) and these compounds were benzoylated by treatment with benzoic anhydride in the presence of 4-dimethylaminopyridine[8] to give the 2′-*O*-benzoyl derivatives (**4**) and (**5**), respectively. The assignment of substitution position was performed by nuclear magnetic resonance (NMR) spectroscopy. The NMR data are summarized in Table I. The monomethoxytrityl group of **5** was removed by treatment with acetic acid in chloroform and *N*, 2′-dibenzoyl araA was converted to 5′-*O*-dimethoxytrityl *N*,2′-dibenzoyl araA (**7**) with dimethoxytrityl chloride. **7** was phosphory-

lated by treatment with *p*-chlorophenylphosphoroditriazolide.[9] Hydrolysis of the triazolide on the phosphorylated product yielded the phosphodiester (**8**).

Synthesis of araA-containing Octanucleotide dGGaraAATTCC and Its Prototype dGGAATTCC

The octanucleotides were synthesized by condensation of protected oligonucleotide blocks. Preparations of the 3′-phosphodiester components (**9**) (Chart 2), and 3′-phosphoro-*p*-

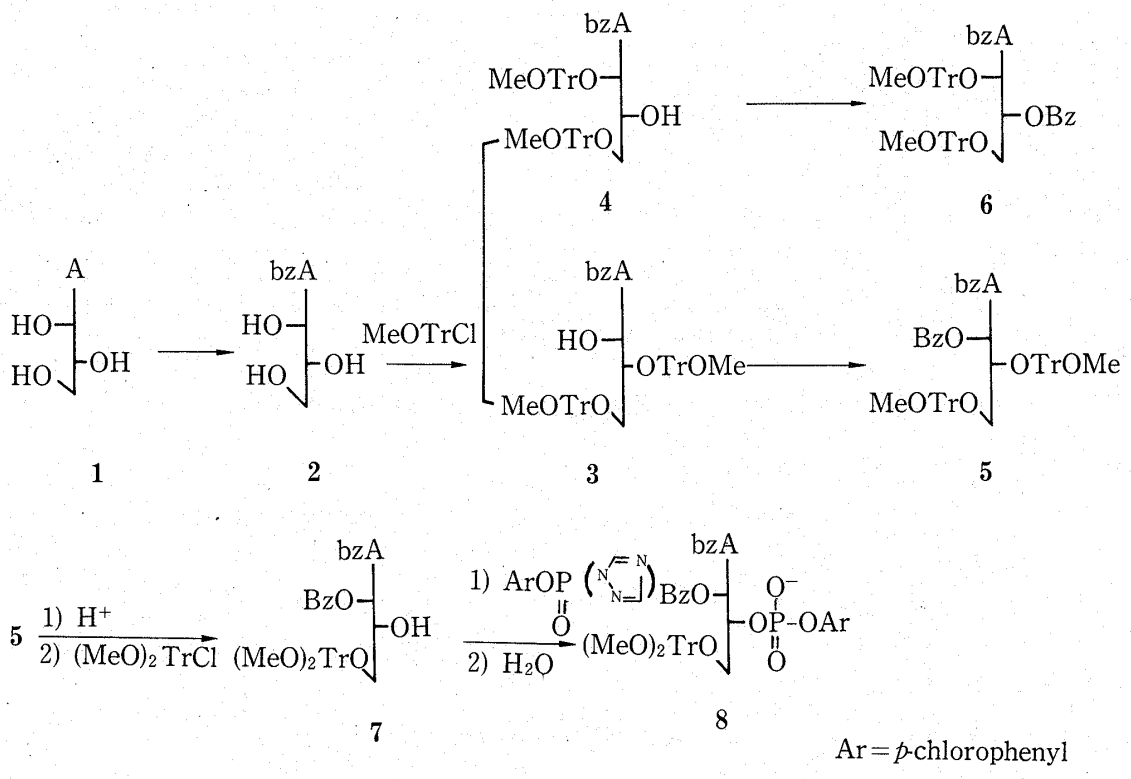
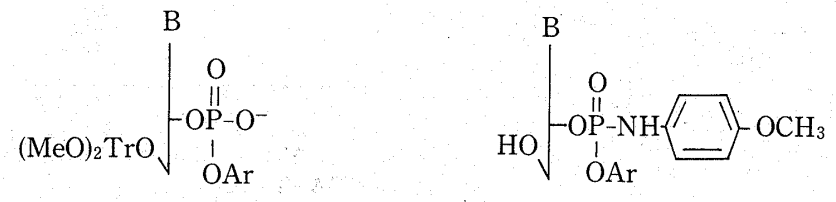


Chart 1

TABLE I. NMR Data for Bis-dimethoxytrityl Compounds (**3**, **4**, **5** and **6**)

Compound	Signal			
	H-1′	H-2′	H-3′	H-4′
3	6.40	4.60[a,b]	4.20	
4	5.95	4.50[b]	4.20	3.92
5	6.90	5.15[b]	4.48	
6	6.04	4.76[b]	5.23	4.10

a) Narrowed by addition of D_2O.
b) Decoupled by irradiation of H-1′ signal.



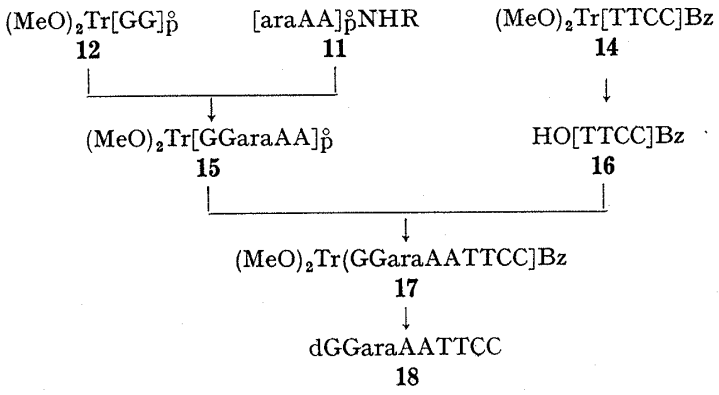
a: thymidin-1-yl
b: *N*-benzoylcytosin-1-yl
c: *N*-benzoyladenin-9-yl
d: *N*-isobutyrylguanin-9-yl

Chart 2

Table II. Reaction Conditions for Condensations

3′-Diester component (mmol)	5′-Hydroxyl component (mmol)	MSTe (mmol)	Py (ml)	Reaction time (min)	Product % (mmol)
8 (1.5)	**10c** (0.75)	1.5	5	75	[araAA]$^{\circ}_{p}$NHC$_6$H$_4$OCH$_3$(**11**) 69
9d (3.6)	**10d** (2.4)	7.2	15	60	(MeO)$_2$Tr[GG]$^{\circ}_{p}$(**12**) 63
9c (1.5)	**10c** (0.75)	1.5	5	75	[AA]$^{\circ}_{p}$NHC$_6$H$_4$OCH$_3$ 66
9d (4.0)	dbzC(Bz) (2.0)	8.0	20	60	[CC]Bz 85
9a (2.5)	[CC]Bz(1.65)	5.0	15	60	[TCC]Bz 67
9a (1.65)	[TCC]Bz(1.11)	3.3	15	60	[TCCC]Bz(**14**) 77
12 (0.35)	**11** (0.415)	0.664	10	60	(MeO)$_2$Tr[GGaraAA]$^{\circ}_{p}$(**15**) 72
15 (0.217)	**16** (0.247)	0.416	10	90	(MeO)$_2$Tr[GGaraAATTCC]Bz(**17**)56
12 (0.33)	**12** (0.396)	0.634	10	60	(MeO)$_2$Tr[GGAA]$^{\circ}_{p}$ 56
(MeO)$_2$Tr-[GGAA]$^{\circ}_{p}$ (0.186)	**16** (0.155)	0.29	10	120	(MeO)$_2$Tr[GGAATTCC]Bz 50

[ ], all protecting groups are omitted except for the terminal ones.



R=*p*-methoxyphenyl
°p=*p*-chlorophenyl phosphate

Chart 3

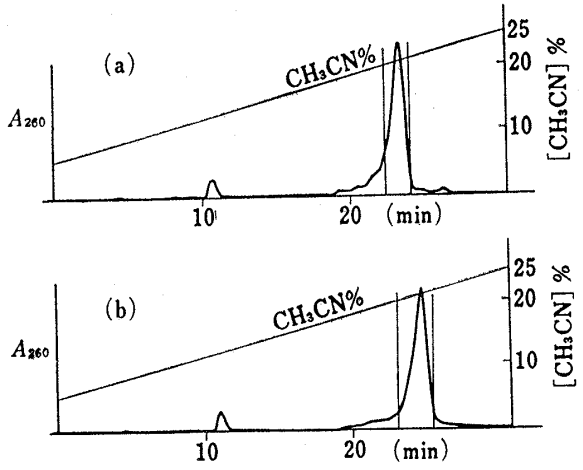


Fig. 1. Preparative HPLC of dGGaraAATTCC (a) and dGGAATTCC(b) on a Column (0.72×25 cm) of Megapaksil C-18

Elution was performed with a gradient starting from 5% acetonitrile in 0.1 M triethylammonium acetate (pH 7) to 25% acetonitrile in the same buffer during 30 min.

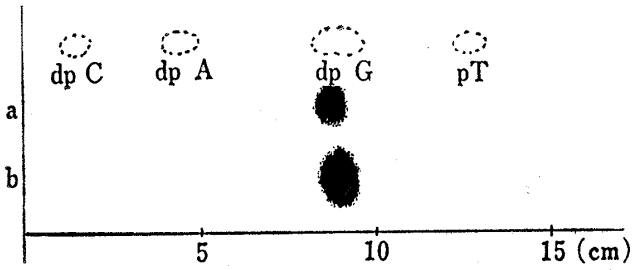


Fig. 2. 5′-End Analysis of dGGaraAATTCC(a) and dGGAATTCC(b)

The 5′-labeled octamers were digested with nuclease P1 and the products were subjected to paper electrophoresis in 0.2 M morpholinium acetate (pH 3.5) at 900 V/40 cm with markers.

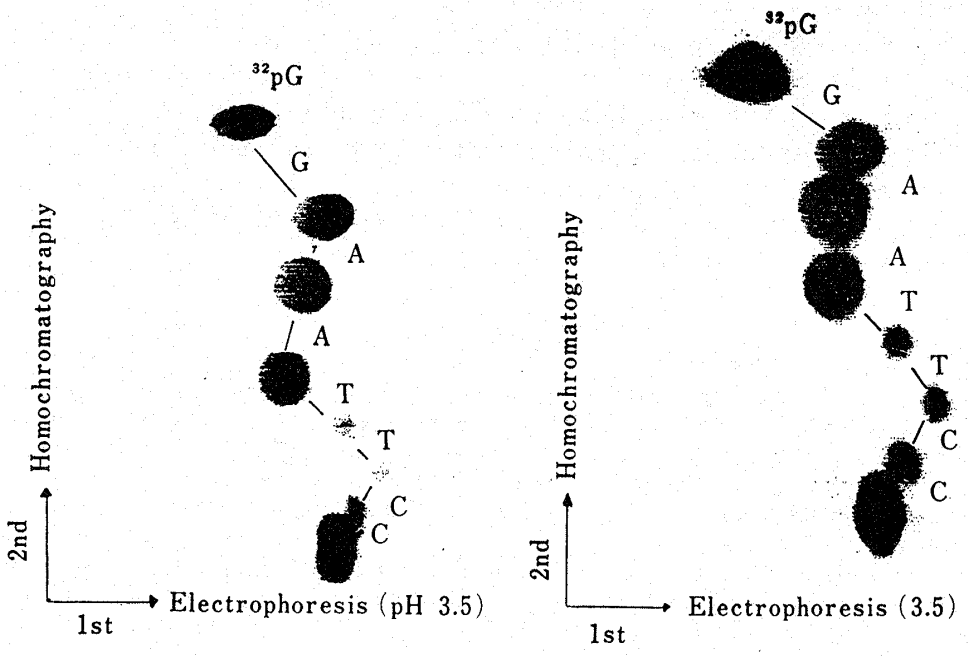


Fig. 3. Two-dimensional Homochromatography of Nuclease P1 Partial Digests of d^{32}pGGaraAATTCC (Left) and d^{32}pGGAATTCC (Right)

anisidate of deoxynucleosides (**10**) were as described previously.[1] The phosphodiester of araA (**8**) was condensed with *N*-benzoyldeoxyadenosine 3′-*p*-chlorophenylphosphoro-*p*-anisidate (**10c**) to yield protected araAAp (**11**). Mesitylenesulfonyl tetrazolide (MSTe)[10] was used as the condensing reagent. Other dimers, protected dGGp (**12**) and dAAp (**13**) were synthesized by the similar procedures. The 3′-terminal block (dTTCC) (**14**) was prepared by condensation of *N*,3′-*O*-dibenzodeoxycytidine[11] with **9b** followed by dedimethoxytritylation and subsequent condensation with **9a**. The reaction conditions for these condensations are summarized in Table I. Dinucleotides thus obtained were also allowed to react to yield the octamers, as shown in Chart 4. The fully protected dGGp was treated with isoamyl nitrite[1] to give the 3′-diesterified dimer **12**, which was condensed with the 5′-deblocked dimer **11** or **13**. The fully protected tetramers thus obtained were treated similarly to give the 3′-diesterified tetramers (*e.g.* **15**). The protected octamer (**17**) was synthesized by condensation of **15** and **16**. **17** was deprotected as described previously[1] to yield the araA-containing octanucleotide dGGaraAATTCC (**18**). The other octamer dGGAATTCC was prepared by essentially the same procedure. The deblocked oligomers were purified by high pressure liquid chromatography (HPLC) on C_{18} silica gel (Fig. 1) and checked for purity by anion-exchange HPLC. The 5′-end analysis was performed by labeling with [γ-^{32}p]ATP and

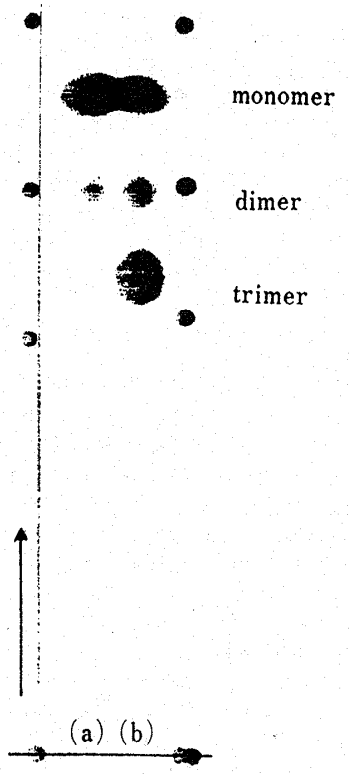


Fig. 4. Homochromatography of Products obtained by Partial Digestion with Venom Phosphodiesterase
(a) d^{32}pGGAATTCC and (b) d^{32}pGGaraAATTCC

polynucleotide kinase followed by complete digestion with nuclease P1[12] as described elsewhere.[11b] The results are shown in Fig. 2. The sequence of these octamers was confirmed by mobility shift analysis[13] using nuclease P1 (Fig. 3). As shown in Fig. 3, araA was hydrolyzed to almost the same extent as dA. Partial digestion with venom phosphodiesterase of the araA-containing octamer accumulated the trimer under conditions where dGGAATTCC was hydrolyzed to the monomer or dimer (Fig. 4). This may indicate that araA is partially resistant to hydrolysis. Hydrolysis of dGGaraAATTCC with *E. cori* will be reported elsewhere.

Experimental

TLC was performed on plates of silica gel (Kieselgel 60 F_{254}, Merck). For RTLC, silanized silica gel (Kieselgel 60 F_{254}, silanisiert, Merck) was used with the indicated solvents. Column chromatography was carried out on silica gel (60H, Merck) under air pressure. For preparative reverse phase liquid chromatography, C_{18} silica gel (35—105 μ, Waters) was used in glass columns. HPLC was performed on a JASCO apparatus with an anion-exchange column (Permaphase AAX 2.1×500 mm) or a reverse phase column (Megapak SIL C-18 7.2×250 mm).

5′-Labeling and nuclease P1 digestion were performed as described previously.[11b,14] For hydrolysis with venom phosphodiesterase, a sample (*ca.* $3A_{260}$ including carrier RNA) was incubated with the enzyme (1 μg, Boehringer) in 0.01 M Tris–HCl (pH 8.0, 15 μl) at 37°C for various periods as described for partial digestion with nuclease P1.[14a]

N-Benzoylarabinosyladenine (2)——araA (1) (6.0 g, 22.5 mmol) was dissolved in pyridine and treated with benzoyl chloride (13.1 ml, 112.5 mmol) at room temperature overnight. Methanol (10 ml) was added with cooling, and the mixture was concentrated. The product was extracted with chloroform (150 ml). The organic layer was washed 3 times with water (50 ml), then concentrated. The residue was dissolved in pyridine (90 ml) and ethanol (45 ml). The mixture was treated with a mixture of 2 N NaOH (70 ml) and ethanol (70 ml) in an ice bath for 50 min. Sodium ions were removed by addition of Dowex 50×2 (pyridinium form) (200 ml) and the solution was concentrated. The crystalline product (7.1 g, 85%) was collected by filtration. UV λ_{max} nm (EtOH): 280. TLC (CH_2Cl_2: MeOH=10: 1, *Rf* 0.16). RTLC (acetone: H_2O= 7: 3, 0.78).

***N*-Benzoyl-3′,5′-*O*-bis-monomethoxytritylarabinosyladenine** (3)——**2** (5.57 g, 15 mmol) was treated with monomethoxytrityl chloride (11.12 g, 36 mmol) in pyridine (50 ml) at room temperature for 18 h. An additional amount (15 mmol) of the reagent was added and the reaction was terminated after 72 h by addition of methanol (10 ml). The mixture was concentrated and the product was extracted with chloroform. The chloroform solution was washed with water, concentrated and coevaporated (4 times) with toluene. The residue was dissolved in chloroform (20 ml) and applied to a column (4.5×30 cm) of silica gel 60. Elution was performed by the use of stepwise increases of ethanol concentration in chloroform (0.5—2%). The product was identified by TLC ($CHCl_3$: EtOH=25: 1, *Rf*, 0.40) and precipitated with hexane from its solution in chloroform (5.2 g, 37%). The 2′,5′-substituted compound (4) was obtained at the same time (*Rf*, 0.25) (7.6 g, 55%). **3**: UV, λ_{max} nm (EtOH): 280. NMR ($CDCl_3$) δ: 9.3 (H–N^6), 8.26 (H-8), 8.22 (H-2), 6.40 (H-1′, $J_{1'.2'}$=2 Hz), 4.60 (H-2′), 4.20 (H-3′, H-4′), 3.75 (OCH_3), 3.70 (OCH_3). **4**: UV λ_{max} nm (EtOH): 280. NMR ($CDCl_3$) δ: 9.38 (H–N^6), 8.70 (H-8), 8.22 (H-2), 5.95 (H-1′, $J_{1'.2'}$=6 Hz), 4.50 (H-2′), 4.20 (H-3′), 3.92 (H-4′).

***N*,2′-*O*-Dibenzoyl-3′,5′-*O*-bis-monomethoxytrityl Arabinosyladenine** (5)——**3** (3.6 g, 4 mmol) was treated with benzoic anhydride (1.53 g, 6.75 mmol) and *p*-dimethylaminopyridine (0.55 g, 4.5 mmol) in chloroform (50 ml) at room temperature for 18 h. Since the reaction was not completed, further amounts of the reagents (0.5 g each) were allowed to react for 6 h and the the mixture was stirred with 0.5 M KH_2PO_4 (20 ml) and chloroform (20 ml) for 10 min. The organic layer was washed 3 times with water (40 ml), then with 0.5 M KH_2PO_4 (40 ml) and water (40 ml). The solution was dried with Na_2SO_4 and concentrated. The product (3.55 g, 87%) was precipitated with hexane from its solution in chloroform. UV λ_{max} nm (EtOH): 280. NMR ($CDCl_3$) δ: 6.90 (H-1′, $J_{1'2'}$=3.6 Hz), 5.15 (H-2′), 4.48 (H-3′), 3.8, 3.65 (OCH_3). TLC (CH_2Cl_2: MeOH=20: 1, *Rf* 0.80), RTLC (acetone: H_2O=7: 3, *Rf* 0.26).

5′-*O*-Dimethoxytrityl-*N*,2′-*O*-dibenzoylarabinosyladenine (7)——**5** (3.55 g, 3.48 mmol) was treated with acetic acid (50 ml) in chloroform (10 ml) at room temperature for 12 h and at 30°C for 5 h. Acetic acid was removed by evaporation and the nucleoside was crystallized by warming it with 95% ethanol (1.1 g) The second crop (300 mg) was obtained by addition of hexane to the concentrated mother liquor (5 ml) The product (1.4 g, 2.94 mmol) was then treated with dimethoxytrityl chloride (1.18 g, 3.5 mmol) in pyridine (15 ml) at room temperature for 1 h. Methanol was added and the solution was concentrated. The residue in chloroform (5 ml) was applied to a column (4×8 cm) of silica gel 60. Elution was performed with stepwise increases of methanol concentration (0—3%) in chloroform containing 0.5% pyridine. The product (7) was precipitated with pentane containing 10% ether (200 ml) from its solution in dichloromethane (15 ml). 1.82 g (80%). UV λ_{max} nm (EtOH): 280.NMR: ($CDCl_3$) δ, 9.30 (H–N^6), 8.70 (H-8), 8.38 (H-2), 6.80 (H-1′, $J_{1'2'}$=

4.2 Hz), 5.60 (H-2′), 5.75 (H-3′), 4.30 (H-4′), 3.54 (H-5′), 3.73 (OCH_3). *Anal.* Calcd for $C_{45}H_{39}N_5O_8$: C, 69.48; H, 5.05; N, 9.00. Found: C, 69.77; H, 4.97; N, 8.60. TLC (CH_2Cl_2: MeOH=20: 1, *Rf* 0.51) RTLC (acetone: H_2O=7: 3, *Rf* 0.40).

Preparation of Phosphodiesters (8 and 9)——**9a, c, d** were prepared as described previously[1] by reaction with *p*-chlorophenyl phosphoroditriazolide for 1 h. Phosphorylation of **7** (583.4 mg, 0.75 mg) by the same procedure required 2 h and **8** was used for condensation without purification.

General Methods for Condensations——The procedures were essentially the same as condensations using mesitylenesulfonyl nitroimidazolide.[1] Phosphodiester components were activated with MSTe at 30°C for various periods of time (Table II). The reaction was terminated by addition of aqueous pyridine (50%) and products were extracted 3 times with chloroform. The chloroform solution of dimers (*e.g.* fully protected dGGp) was washed 3 times with 0.1 M triethylammonium bicarbonate and once with water, and coevaporated with pyridine, then with toluene. The residue in chloroform (10 ml) was applied to a/column (4×4.5 cm) of silica gel. The fully protected dinucleotide was isolated by repeated chromatography (3 times). Dimers thus obtained were subjected to treatment with acid or isoamyl nitrite.[1]

The Octamer dGGaraAATTCC (18)——The fully protected octamer (**17**) was synthesized by condensation of oligomers as shown in Chart 3 under the conditions give in Table II, and isolated by reversed phase chromatography on a column (4×85 cm) of C-18 silica gel. The sample was dissolved in pyridine (0.5 ml), acetone (4 ml) and 0.1 M triethylammonium acetate (1 ml). Stepwise elution was performed with a stepwise gradient of acetone (acetone–0.1 M buffer, 50—90%). The product was eluted with 75—80% acetone and acetone was removed by evaporation. The product was extracted with dichloromethane from the aqueous phase and washed with water. The solvent was removed and the residue was coevaporated with pyridine. The octamer (**17**) was precipitated with pentane (60 ml) from its solution in dichloromethane (10 ml). The yield was 497 mg (56%). TLC ($CHCl_3$–$MeOH_3$ 10: 1, *Rf* 0.49), RTLC (acetone–H_2O, 7: 3, *Rf* 0.08). An aliquot (41 mg, 0.01 mmol)/was treated with 0.5 M tetramethylguanidium pyridine 2-carboxaldoximate[15] in aqueous dioxane (50%, 4.2 ml) at room temperature for 40 h, then aqueous pyridine (20%, 5 ml) was added. The mixture was passed through a column of Dowex 50×2 (pyridinium) (20 ml) and the column was washed with 20% pyridine (80 ml). The combined solution was concentrated and the residue in pyridine (10 ml) was treated with concentrated ammonia (20 ml) at 60°C for 5 h. Volatile materials were removed by evaporation and the residue was mixed with water (150 ml) and ethyl acetate (12 ml×2). The aqueous phase was concentrated and the residue was treated with 80% acetic acid (30 ml) at room temperature for 1 h, then the acetic acid was removed by coevaporation with water. The deblocked product (**18**) was dissolved in water (20 ml) and the solution was washed twice with ethyl acetate (10 ml). The yield was 760 A_{260}. An aliquot (40 A_{260} in 40 μl of water) was subjected to HPLC (Fig. 1, a) and **18** (26 A_{260}) was recovered in a yield of 65%.

The Octamer dGGAATTCC——The synthetic procedure was essentially the same as described above. The fully protected octamer was obtained by using the conditions shown in Table II. An aliquot (40 mg, 0.01 mmol) was deblocked and the product dGGAATTCC (650 A_{260}) was dissolved in water (0.65 ml). An aliquot (35 A_{260}) was subjected to HPLC as shown in Fig. 1, b and the octamer (25 A_{260}) was recovered in a yield of 70%.

References and Notes

1) Part III: E. Ohtsuka, S. Shibahara, and M. Ikehara, *Chem. Pharm. Bull.*, **29**, 3440 (1981).
2) J.R. Roberts, *CRC Crit. Rev. Biochem.*, **3**, 123 (1976); W. Arber, *Angew. Chem. Int. Ed. Engl.*, **17**, 73 (1978).
3) J. Hedgpeth, H.M. Goodman, and H.W. Boyer, *Proc. Acad. Sci. U.S.A.*, **69**, 3448 (1972).
4) *a*) C.P. Woodbury, Jr., O. Hagenbüchle, and P.H. von Hippel, *J. Biol. Chem.*, **255**, 11534 (1980); *b*) M. Goppelt, A. Pingoud, G Maass, H Mayer, H. Köster, and R. Frank, *Eur. J. Biochem.*, **104**, 101 (1980).
5) P.L. Molloy and R.H. Symons, *Nucleic Acids Res.*, **8**, 2939 (1980).
6) *a*) P.L. Greene, M.S. Poonian, A.L. Nussbaum, L. Tobias, D.E. Garfin, H.W. Boyer, and H.M. Goodman, *J. Mol. Biol.*, **99**, 237 (1975); *b*) M. Goppelt, A. Pingoud, G. Maass, H. Mayer, H. Köster, and R. Frank, *Eur. J. Biochem.*, **104**, 101 (1980).
7) T. Utagawa, H. Morisawa, T. Miyoshi, F. Yoshinaga, A. Yamazaki, and K. Mitsugi, *FEBS Lett.*, **109**, 261 (1980).
8) G. Höfle, W. Steglish, and H. Vorbrüggen, *Angew. Chem. Int. Ed. Engl.*, **17**, 569 (1978).
9) *a*) N. Katagiri, K. Itakura, and S. Narang, *J. Am. Chem. Soc.*, **97**, 7332 (1975); *b*) C. Broka, T. Hozumi, R. Arenzene, and K. Itakura, *Nucleic Acids Res.*, **8**, 5461 (1980).
10) J. Stawinski, T. Hozumi, S.A. Narang, C.P. Bahl, and R. Wu, *Nucleic Acids Res.*, **4**, 353 (1977).
11) *a*) H. Schaller, G. Weimann, B. Lerch, and H.G. Khorana, *J. Am. Chem. Soc.*, **85**, 3821 (1963); *b*) E. Ohtsuka, S. Shibahara, T. Ono, T. Fukui, and M. Ikehara, *Heterocycles*, **15**, 395 (1981).
12) M. Silberklang, A.M. Gillum, and U.L. RajBhandary, *Nucleic Acids Res.*, **4**, 4091 (1977).

13) F. Sanger, J.E. Donelson, A.R. Coulson, H. Kössel, and D. Fischer, *Proc. Nat. Acad. Sci. U.S.*, **70**, 1209 (1973).
14) *a*) E. Ohtsuka, S. Tanaka, and M. Ikehara, *J. Am. Chem. Soc.*, **100**, 8210 (1978); *b*) E. Ohtsuka, T. Tanaka, and M. Ikehara, *Nucleic Acids Res.*, **7**, 1283 (1979).
15) C.B. Reese, R.C. Titmas, and L. Yau, *Tetrahedron Lett.*, **1978**, 2727.