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Polynucleotides. XXIX.[1] Synthesis of Deoxyribooligonucleotide Blocks by an Extraction Method

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5′-Phosphoryl-N-benzoyldeoxyadenylyl-(3′-5′)-N-anisoyl-3′-O-acetyldeoxycytidine (IVa) and 5′-phosphoryl-N-isobutyryldeoxyguanylyl-(3′-5′)-N-anisoyl-3′-O-acetyldeoxycytidine (IVb) were synthesized by condensation of 5′-O-(N-trityl-*p*-aminophenyl) phosphoryl-N-benzoyldeoxyadenosine (Ia) or 5′-O-(N-trityl-*p*-aminophenyl)phosphoryl-N-isobutyryldeoxyguanosine (Ib) with N-anisoyldeoxycytidine 5′-phosphate using triisopropylbenzenesulfonyl chloride (TPS). The intermediates (III) were isolated by extraction with organic solvents and the N-trityl-*p*-aminophenyl group was removed by oxidative hydrolysis. The dinucleotides (IV) were obtained without ion-exchange chromatography in yields of 50 to 60% and used for synthesis of the oligonucleotides. The tetranucleotide (VI) was synthesized by condensing V with IVa using TPS and was also isolated from the starting materials by the extraction method in a yield of 49%. The tetranucleotide (VI) was further condensed with the dinucleotides IVa and IVb to yield the hexanucleotide (VII) and the octanucleotide (VIII).

Previously we have used N-trityl-*p*-aminophenol for the protection of the phosphomonoester in the synthesis of a thymidine oligonucleotide.[3] This protecting group was thought to be particularly suitable for the synthesis of deoxyribodinucleotide blocks which could be used for elongation of oligonucleotide chains, since dinucleotides bearing N-trityl-*p*-aminophenyl groups could be isolated by extraction with organic solvents. The present paper reports a convenient synthesis of the deoxyoctanucleotide d-CpCpApCpApCpGpC[4] (VIII) using dinucleotide blocks. The octanucleotide (VIII) has a complementary sequence to the two ribohexanucleotides as shown in Fig. 1 and may serve as a template in the enzymatic joining reaction of the ribooligonucleotides with T4 deoxyribonucleic acid (DNA) ligase[5] to yield the 5′-terminal dodecanucleotide of the yeast alanine transfer ribonucleic acid (tRNA).

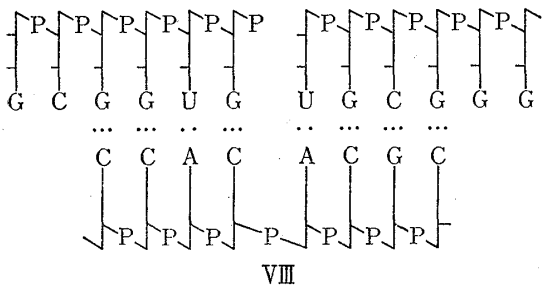


Fig. 1

Synthesis of Two Deoxydinucleotide Blocks (IV)

The N-trityl-*p*-aminophenyl group was introduced into N-benzoyl-3′-O-acetyldeoxyadenosine 5′-phosphate[6a] or N-isobutyryl-3′-O-acetyldeoxyguanosine 5′-phosphate[6b] using dicyclohexylcarbodiimide (DCC) and the 3′-acetyl group was removed by strong alkaline

1) Part XXVIII of this series: E. Ohtsuka, T. Nagura, K. Shimokawa, S. Nishikawa, and M. Ikehara, *Biochim. Biophys. Acta*, **383**, 236, (1975).
2) Location: *133–1 Yamadakami, Suita, Osaka, 565, Japan.*
3) E. Ohtsuka, S. Morioka, and M. Ikehara, *J. Amer. Chem. Soc.*, **95**, 8437 (1973).
4) Abbreviations are as suggested by the IUPAC-IBU combined commission, *J. Biol. Chem.*, **241**, 531 (1966).
5) *a*) K. Kleppe, J.H. van de Sande, and H.G. Khorana, *Proc. Natl. Acad. Sci. U. S.*, **67**, 68 (1970); *b*) G.C. Fareed, E.M. Wilt, and C.C. Richardson, *J. Biol. Chem.*, **246**, 925 (1971).
6) *a*) R.K. Ralph and H.G. Khorana, *J. Amer. Chem. Soc.*, **83**, 2926 (1961); *b*) H. Büchi and H.G. Khorana, *J. Mol. Biol.*, **72**, 251 (1972).

treatment. The yields of Ia and Ib were 62 and 61%, respectively. The 5′-protected nucleotides were condensed with N-anisoyl-3′-O-acetyldeoxycytidine 5′-phosphate[7] as shown in Chart 1 using triisopropylbenzenesulfonyl chloride (TPS) as the condensing reagent. Nucleotides bearing the lipophilic group were extracted with methylene chloride from 50% aqueous pyridine and the dinucleotides (III) were separated from the starting material (I) by precipitation with ether-ethyl acetate. The N-trityl group of III was removed by treatment with 80% acetic acid for 3 min and the 5′-*p*-aminophenyl derivatives became soluble in water. Solubilization of the protected nucleotides was necessary for identification of the nucleotides by paper electrophoresis. *Rf* values and relative mobilities are shown in Table I. The yield of the dinucleotide (III) thus obtained was *ca.* 60% and the protecting group on the 5′-phosphate was removed with iodine in ammonium acetate buffer[3] (pH 7.5) to give the dinucleotides d-pA^{Bz}pC^{An}–OAc (IVa) and d-pG^{iBu}pC^{An}–OAc (IVb). The nucleotides were characterized by enzymatic hydrolysis after removal of the protecting groups.

Table I. Paper Chromatography and Paper Electrophoresis

	Rf values in solvent			Mobilities at	
	A	B	C	pH 7.5	pH 2.7
d-C	4.3		0.7		
d-pC	1.0	0.27	0.45	1.0	1.0
d-pA	1.19	0.24	0.52	0.86	1.28
d-pG	0.54	0.19	0.38	0.91	2.3
d-pC^{An}-OAc		0.56		0.68	
d-pA^{Bz}-OAc		0.51		0.68	
d-pG^{iBu}-OiBu		0.62		0.80	
d-TrNHPhOpA^{Bz}-OAc		0.87			
d-TrNHPhOpA^{Bz}		0.81			
d-TrNHPhOpG^{iBu}-OiBu		0.94			
d-TrNHPhOpG^{iBu}		0.92			
d-TrNHPhOpA^{Bz}pC^{An}-OAc		0.81			
d-TrNHPhOpG^{iBu}pC^{An}-OAc		0.89			
d-pA^{Bz}pC^{An}-OAc		0.41		0.69	
d-pG^{iBu}pC^{An}-OAc		0.50		0.68	
d-pApC	0.54		0.38	1.02	1.34
d-pGpC	0.32		0.30	1.0	2.20
d-MMTrCpC	5.57		0.79	0.29	
d-MMTrCpCpApC	2.08		0.72	0.63	
d-MMTrCpCpApCpApC			0.68	0.78	
d-MMTrCpCpApCpApCpGpC			0.54		
d-CpC			0.62	0.39	
d-CpCpApCp			0.38	0.74	
d-CpCpApCpApC			0.28	0.88	
d-CpCpApCpApCpGpC			0.20	0.93	
d-NH$_2$PhOpA^{Bz}					0.64[a]
d-NH$_2$PhOpA^{Bz}-pC^{An}-OAc					0.81[a]
d-NH$_2$PhOpG^{iBu}					0.77[a]
d-NH$_2$PhOpG^{iBu}-pC^{An}-OAc					0.87[a]

a) Relative mobility to d-pC^{An}-OAc

Synthesis of the Tetranucleotide d-MMTr-C^{An}pC^{An}pA^{Bz}pC^{An} (VI) by the Extraction Method

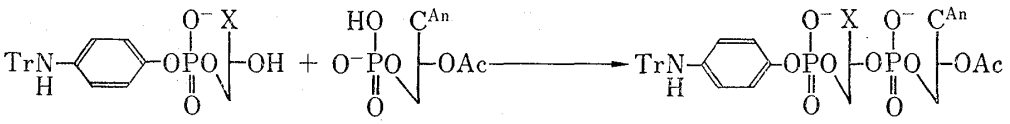
The nucleotide (IVa) was condensed using TPS with the terminal tritylated dimer d-MMTr–C^{An}pC^{An} (V) which is prepared from d-MMTr–C^{An} and d-pC^{An}–OAc as described in the synthesis of MMTr–TpT.[8] After the reaction, the mixture was treated with 50% aqueous

7) H.G. Khorana, A.F. Turner, and J.P. Vizsolyi, *J. Amer. Chem. Soc.*, **83**, 686 (1961).
8) T.M.Jacob and H.G. Khorana, *J. Amer. Chem. Soc.*, **87**, 2971 (1965).

pyridine and the tritylated compounds were extracted with *n*-butanol–ethyl acetate. The product (VI) was separated from the dimer (V) by extracting the mixture with 40% aqueous pyridine. The tetranucleotide (VI) was precipitated with ether-pentane from its solution in anhydrous pyridine, in a yield of 49%. The unprotected tetranucleotide d-CpCpApC was characterized by hydrolysis with venom phosphodiesterase.

Synthesis of the Hexanucleotide d-MMTr–$C^{An}pC^{An}pA^{Bz}pC^{An}pA^{Bz}pC^{An}$ (VII) and the Octanucleotide d-CpCpApCpApCpGpC (VIII)

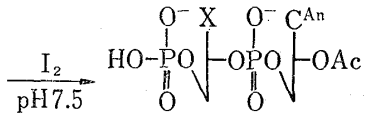
The hexanucleotide (VII) was synthesized by condensing the tetranucleotide (VI) with the dinucleotide (IVa), using TPS and the product was isolated by conventional chromatography on a diethylaminoethyl (DEAE)-cellulose column. The yield of the protected hexanucleotide (VII) was 9% and the unprotected hexanucleotide d-CpCpApCpApC was characterized by digestion with spleen phosphodiesterase. *Rf* values are shown in Table I. The protected hexanucleotide (VII) was allowed to react with the dinucleotide d-pG^{iBu} pC^{An}–OAc (IVb) in the presence of TPS and the reaction mixture was kept in methanolic ammonia for 2 days after treatment with aqueous pyridine. The monomethoxytrityl group was then removed with 80% acetic acid. The mixture was subjected to gel filtration on Sephadex G-50 and the partially excluded fraction was chromatographed on DEAE-cellulose in the presence of 7M



Ia : X=N−benzoyladenine
Ib : X=N−isobutyrylguanine

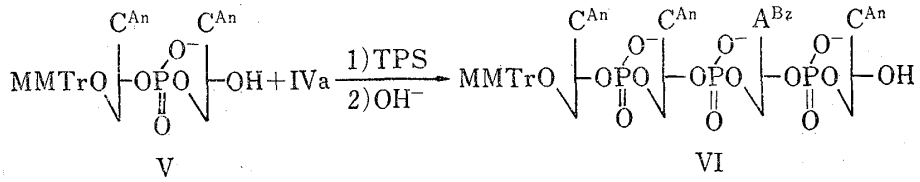
II

IIIa, b



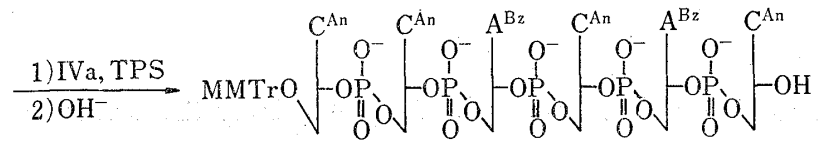
IVa, b

Chart 1

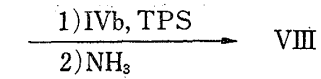


V

VI



VII



VIII

Chart 2

urea. The unprotected octanucleotide, d-CpCpApCpApCpGpC was desalted by gel filtration with Biogel P-2[9] and characterized by enzymatic hydrolysis.

Experimental

General Methods——Paper chromatography was performed using the following solvent systems: A, isopropanol–concentrated ammonia–water (7: 1: 2, v/v); B, ethanol–1 M ammonium acetate (pH 7.5) (7: 3, v/v); C, *n*–propanol–concentrated ammonia–water (55: 10: 35, v/v); D, saturated ammonium sulfate–isopropanol–water (79: 2: 19, v/v). Paper electrophoresis was performed at 900 V/40 cm in 0.05 M triethylammonium bicarbonate (pH 7.5) or in 0.05 M ammonium formate (pH 2.7).

Enzymatic hydrolyses were performed as described previously.[3]

N-Trityl-*p*-aminophenol——*p*-Aminophenol (0.549 g, 5 mmoles) was rendered anhydrous by evaporation with pyridine and trityl chloride (5 mmoles) was added in an ice bath. The mixture was kept at 18° for 2 hr with stirring. Water (10 ml) was added and the product was extracted with chloroform. Chloroform was evaporated and the residue was precipitated with pentane from its solution in anhydrous pyridine. The precipitate was recrystallized from cyclohexane using charcoal. The yield was 1.54 g, 66%, mp 151—153°.

d-TrNHPhOpA^{Bz} (Ia)——Pyridinium N-benzoyl-3′-O-acetyldeoxyadenosine 5′-phosphate (1.57 mmoles) was allowed to react with N-trityl-*p*-aminophenol (3 mmoles) in pyridine (15 ml) for 48 hr using dicyclohexylcarbodiimide (DCC) (5 mmoles). Water (15 ml) and aqueous pyridine (50%, 25 ml) were added and DCC was extracted with hexane. The filtered solution was extracted with methylene chloride and the organic layer was washed with water. Methylene chloride was evaporated and the residue was coevaporated with pyridine. The product was precipitated with ether–pentane (3: 2) from its solution in pyridine. The precipitate was dissolved in aqueous pyridine (60%, 40 ml) and treated with 2 N NaOH (40 ml) in an ice bath for 5 min. Sodium ions were removed with pyridinium Dowex 50×2 (120 ml) and the solution was passed through a column of the same resin. The product (Ia) was precipitated with ether–pentane (3: 2) from its solution in anhydrous pyridine as described above. The yield was 16300 A_{260} (0.97 mmole), 62%. $\lambda_{max}^{H_2O}$ (nm) 232, 282, $\lambda_{max}^{H^+}$ 289 and $\lambda_{max}^{OH^-}$ 297.

d-TrNHPhOpG^{iBz} (Ib)——Ib was prepared by the same method used in the synthesis of Ia. The yield was 61%. $\lambda_{max}^{H_2O}$ (nm) 235, 259, $\lambda_{max}^{H^+}$ 261 and $\lambda_{max}^{OH^-}$ 262.

d-TrNHPhOpA^{Bz}pC^{An}-OAc (IIIa)——Ia (0.47 mmole) and d-pC^{An}-OAc (II) (0.53 mmole) were rendered anhydrous by evaporation with pyridine. The mixture was treated with TPS (1.84 mmoles) at 20° for 5 hr. Tri-*n*-butylamine (0.87 ml), pyridine (6 ml) and water (6 ml) were added and the mixture was kept overnight at room temperature. The solution was extracted with methylene chloride (20 ml) and the organic layer was washed with 50% pyridine until the mononucleotide (II) could not be detected in the organic phase by paper electrophoresis. Methylene chloride was evaporated and the residue was coevaporated with pyridine. The product (IIIa) was precipitated with ether–ethyl acetate (1: 1) (300 ml) from its solution in pyridine (11 ml), to remove Ia. The precipitate was collected by centrifugation. The yield was 0.39 g (0.28 mmole), 60%. $\lambda_{max}^{H_2O}$ (nm) 283, $\lambda_{max}^{H^+}$ 291 and $\lambda_{max}^{OH^-}$ 314.

d-pA^{Bz}pC^{An}-OAc (IVa)——IIIa (0.28 mmole) was dissolved in a mixture of acetone (15 ml) and 1 M ammonium acetate (pH 7.5, 6 ml). The solution was treated with iodine (5.6 mmoles) for 4 hr at room temperature. Volatile materials were removed by evaporation with water and iodine was extracted with ether. Pyridine was added and the solution was passed through a column (1.8×25 cm) of pyridinium Dowex 50×2. The solution was concentrated with added pyridine and poured into ether–ethyl acetate (3: 1, 200 ml). The precipitate was collected by centrifugation and washed with ether. The yield was 5670 A_{260} (0.25 mmole), 89%. The product was characterized by hydrolysis using venom phosphodiesterase after treatment with concentrated ammonia for 2 days. The digested products d-pA and d-pC were separated by paper chromatography in solvent D. The ratio of d-pA to d-pC was 1.00: 0.98.

d-TrNHPhOpG^{iBu}pC^{An}-OAc (IIIb)——The pyridinium salts of d-TrNHPhOpG^{iBu} (Ib) (0.3 mmole) and d-pC^{An}-OAc (II) (0.56 mmole) were allowed to react with TPS (1.7 mmoles) in anhydrous pyridine (3 ml) as described in the case of IVa. The yield was 0.241 g (0.18 mmole), 58%. $\lambda_{max}^{H_2O}$ (nm) 258, 282 $\lambda_{max}^{H^+}$ 259, 280 $\lambda_{max}^{OH^-}$, 260, 315.

d-pG^{iBu}pC^{An}-OAc (IVb)——IIIb (0.18 mmole) was treated with iodine (3.5 mmole) in acetone (9 ml) and 1 M ammonium acetate (pH 7.5, 3.5 ml) for 4 hr. The product (IVb) was isolated as described for IVa. The yield was 4,320 A_{260} (0.15 mmole), 86%. $\lambda_{max}^{H_2O}$ (nm), 262, $\lambda_{max}^{H^+}$ 260, $\lambda_{max}^{OH^-}$, 262, 320.

d-MMTr-C^{An}pC^{An}pA^{Bz}pC^{An} (VI)——d-MMTr-C^{An}pC^{An} (V) (0.19 mmole) was condensed with d-pA^{Bz}pC^{An}-OAc (IVa) (0.13 mmole) using TPS (0.70 mmole) in the presence of tri-*n*-butylamine (0.58 mmole) in anhydrous pyridine (0.6 ml) at 20°. After 12 hr, 50% pyridine (5 ml), water (0.6 ml) and tri-*n*-butylamine (1.4 mmole) were added. The mixture was kept overnight and evaporated with added pyridine. The residue was dissolved in *n*–butanol–ethyl acetate (2: 1, 30 ml) and extracted with 50% pyridine until the dinucleotide

9) M. Uziel and W.E. Cohn, *Biochim. Biophys. Acta*, **103**, 539 (1965).

(IVa) could not be detected in the organic layer. The organic solvents were evaporated and the residue was dissolved in 40% pyridine. The starting material (V) was removed by washing with chloroform (30 ml). The product was back extracted with 40% pyridine and combined with the aqueous layer and evaporated with pyridine. The product (VI) was precipitated with ether–pentane (3: 2) from its solution in pyridine. The yield was 0.139 g (0.064 mmole), 49%. The completely unprotected tetranucleotide d-CpCpApc was characterized by venom phosphodiesterase digestion to give a ratio of d-C: d-pC: d-pA=1.1: 2.1: 1.0.

d-MMTr-$C^{An}pC^{An}pA^{Bz}pC^{An}pA^{Bz}pC^{An}$ (VII)——The tetranucleotide (VI) (6640 A_{280}, 0.093 mmole) and d-$pA^{Bz}pC^{An}$-OAc (IVa) (14760 A_{280}, 0.41 mmole) were allowed to react with TPS (1.51 mmoles) in the presence of tri-*n*-butylamine (0.24 ml), in pyridine (2 ml) at 20° for 24 hr. Aqueous pyridine (50%, 10 ml), tri-*n*-butylamine (0.51 ml) and pyridine (2 ml) were added. The solution was treated with 2 N NaOH (10 ml) for 5 min at 0°, then sodium ions were removed with pyridinium Dowex 50×2. After evaporation and dilution with 70% ethanol (400 ml) the solution was applied to a column (1.7×44.5 cm) of TEAE-cellulose (AcO^-). Elution was carried out using a linear gradient of triethylammonium acetate (0—0.5 M) in 70% ethanol (6 liter). The hexanucleotide (VII) (904 A_{280}) was eluted with 0.365 M salt. The yield was 8.4 μmoles, 9%. The product was characterized by enzymatic hydrolysis with spleen phosphodiesterase after removal of the protecting groups. The degraded products, d-Ap (2.9 A_{260}), d-Cp (1.76 A_{260}) and d-C (0.6 A_{260}) were separated by paper chromatography in solvent D and eluted with 0.05 N HCl for measurement of absorbances. The ratio was d-Ap: d-pC: d-C=2.0: 2.8: 1.0.

d-CpCpApCpApcGpC (VIII)——The hexanucleotide (VII) (8 μmoles), d-$pG^{iBu}pC^{An}$-OAc (2290 A_{260}, 80 μmoles) and tri-*n*-butylamine (80 μl) were treated with TPS (0.33 mmole) in pyridine (0.4 ml) at 18° for 10 hr. Aqueous pyridine (50%, 5 ml) was added in an ice bath, the mixture was kept at room temperature for 2 days and evaporated with pyridine. The residue was kept in methanolic ammonia for 2 days at 20°, concentrated to dryness and treated with 80% acetic acid for 1 hr at 20°. Acetic acid was removed and the mixture was dissolved in 0.05 M triethylammonium bicarbonate (1 ml). The solution was applied to a column (1.7×103 cm) of Sephadex G-50. Fractions of 0.4 ml were collected, fraction 65—110 were combined, evaporated, dissolved in 7 M urea containing 0.02 M Tris-HCl (pH 7.5) (2 ml) and applied to a column (0.6×128 cm) of DEAE-cellulose (Cl^-) equilibrated with 7 M urea, 0.02 M Tris-HCl. Elution was carried out using a linear gradient of sodium chloride (0—0.25 M) in 7 M urea and 0.02 M Tris-HCl. The total volume was 500 ml. Fractions of 3.2 ml were collected every 30 min and peaks were desalted by gel filtration using Biogel P-2. Nucleotides were identified by paper chromatography and electrophoresis. Fraction 114—119 contained the octanucleotide (VIII), which gave the ratio of d-Cp: d-Ap: d-Gp=4.0: 1.9: 1.3 by a nucleic acid analyzer (Varian LSC-1000) after spleen phosphodiesterase digestion.