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Studies on Transfer Ribonucleic Acids and Related Compounds. X.[1]
Synthesis of the Yeast Tyrosine tRNA 5′-Terminal Oligonucleotides

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The pentanucleotide which has the sequence of 8th to 12th base of the 5′-terminus of yeast tyrosine transfer ribonucleic acid (tRNA), UpApm^{2}GpCpC (1) has been synthesized by stepwise addition of properly protected mononucleotides using dicyclohexylcarbodiimide. 5′-O-Monomethoxytrityl-N^2-methylguanosine 3′-phosphate was prepared by phosphorylation of the 5′-protected nucleoside to give the 2′,3′-cyclic phosphate and successive hydrolysis with ribonuclease (RNase) St. The isolated yields of the protected di-, tri, tetra-, and penta-nucleotides were 75, 38, 31, and 21%, respectively. The 5′-terminal tetranucleotide CpUpCpUp (3) corresponding to 1st to 4th nucleotides, was synthesized by condensation of the dinucleotide, Bz-C^{Bz}(OBz)-pU(OBz)-p (14) and the anilidate, C^{Bz}(OBz)-pU-(OBz)-pNHPh (**16b**) in a yield of 22%. The trinucleotide CpGpGp (2) spanning 5th to 7th nucleotides from the 5′-end was also prepared by the amidate method from the dinucleotide, MMTrC^{Bz}(OBz)-pG^{iBu}(OiBu)-p(18) and G^{iBu} (OiBu)-pNHPhOCH$_3$ (**19b**) in a yield of 17%.

We have synthesized nona- and hexa-ribonucleotides which have sequences corresponding to the 3′- and 5′-termini of yeast alanine transfer ribonucleic acid (tRNA) by condensing properly protected oligonucleotide blocks.[3] These synthetic fragments were reconstituted with larger natural fragments of the tRNA and shown to be active in accepting alanine.[4] The acceptor stem of tRNA's could be recognized by amino acyl-tRNA synthetase[5] and was found to be the most thermally stable stem in some instances.[6] It is of interest to synthesize terminal fragments of other tRNA's for further studies on synthetic methodology and properties of ribopolynucleotides. In this paper we report the synthesis of the 5′-terminal penta-, tetra-, and tri-nucleotides of yeast tyrosine tRNA[7] as shown in Fig. 1. The pentanucleotide UpApm^2-GpCpC[8] (1), which corresponds to the sequence of 12th to 8th nucleotide was synthesized by stepwise condensation of mononucleotides. One reason to employ the stepwise synthesis was to find how high a yield could be obtained in condensation of ribomononucleotides to a growing chain. The protection of the trinucleotide CpGpGp (2) was designed to allow condensation to the pentanucleotide (1) and deprotection at the 5′-hydroxyl group for further condensation with the terminal tetranucleotide block, CpUpCpUp (3). The pentanucleotide contained a minor nucleoside, N^2-methylguanosine which could be synthesized from 5-amino-

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[1] Part IX: E. Ohtsuka, S. Tanaka, and M. Ikehara, *Nucleic Acid Res.*, 1, 1351 (1974).
[2] Location: *133-1 Yamadakami, Suita, Osaka.*
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[8] Abbreviations are as suggested by IUPAC-IUB combined commision, *J. Biol. Chem.*, 241, 531 (1966). For the protected ribonucleotides see ref. 14.

1-β-D-ribofuranosyl-4-imidazolecarboxamide (AICA-riboside) in larger amount.[9] 5′-O-Monomethoxytrityl-2′-O-benzoyl-N^2-methylguanosine 3′-phosphate (**7**) was prepared from the nucleoside as shown in Chart 1 and used for the stepwise synthesis of the protected pentanucleotide (**11**). The 5′-terminal tetranucleotide bearing the 3′-phosphate (**17**) was synthesized by condensation of the dinucleotide blocks as illustrated in Chart 2. The 3′-phosphate of one of the dinucleotides was protected as the anilidate.[10] Condensation of oligonucleotides having a phosphoramidate has been tried in the ribo-series and been proved to give a reasonable yield as discussed later in this paper. The trinucleotide (**20**) was synthesized also by the aromatic amidate method[10] as shown in Chart 3.

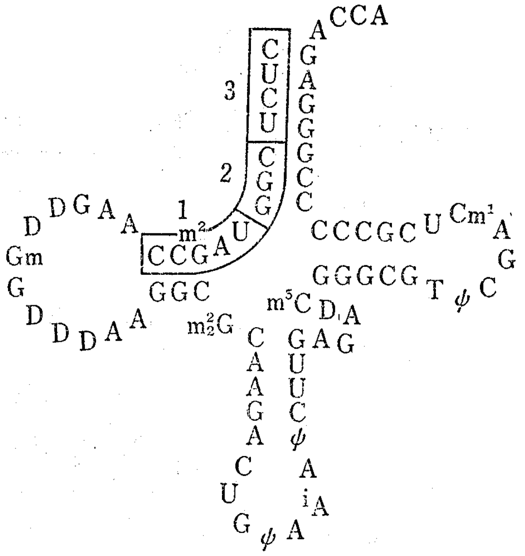

Fig. 1. Yeast Tyrosine tRNA

Synthesis of 5′-O-Monomethoxytrityl-2′-O-benzoyl-N^2-methylguanosine 3′-Phosphate (7) and the Protected Pentanucleotide U(OBz)p-A^{Bz}(OBz)-p-m^{2}G(OBz)-p-C^{Bz}OBz-p-C^{Bz}(OBz)2 (11)

N^2-Methylguanosine was converted to 5′-O-monomethoxytrityl N^2-methylguanosine (**4**) in a yield of 66% and phosphorylated with morpholinophosphorodichloridate.[11] The resulting 2′(3′)-phosphate was cyclized with dicyclohexylcarbodiimide (DCC) and the cyclic phosphate (**5**) was hydrolyzed with ribonuclease (RNase) St,[12] which had higher affinity than RNase T$_1$ for substituted guanosine cyclic phosphate, to give the 3′-phosphate (**6**).[13] The 2′-hydroxyl group of **6** was benzoylated with benzoic anhydride in the presence of tetraethylammonium benzoate.[14] The yield of the fully protected mononucleotide (**7**) from the cyclic phosphate (**5**) was 47%. The minor nucleotide thus protected was condensed with the 3′-terminal dinucleoside monophosphate, C^{Bz}(OBz)-pC^{Bz}(OBz)2 (**8**), which was synthesized by a method similar to that used in the synthesis of C^{Bz}(OBz)-p-A^{Bz2}(OBz)2.[15] DCC was used as the activating reagent for the mononucleotide throughout these stepwise condensations to yield the pentanucleotide (**11**). The trinucleotide m^{2}G(OBz)-p-C^{Bz}(OBz)-pC^{Bz}(OBz)2 (**9**) was isolated by chromatography on triethylaminoethyl (TEAE)-cellulose after removal of the monomethoxytrityl group and the yield was 38%. The trinucleotide (**9**) was then condensed with a 5 fold excess of MMTr-A^{Bz}(OBz)-p and the tetranucleotide (**10**) was isolated by the same chromatographic procedure. The elution pattern and conditions are shown in Fig. 2. The yield of the tetranucleotide obtained in peak (VI) was 31%. The identity of oligonucleotides in the peaks are summarized in Table I. The pentanucleotide U(OBz)-p-A^{Bz}(OBz)-p-m^{2}G(OBz)-p-C^{Bz}(OBz)-p-C^{Bz}(OBz)2 (**11**) was synthesized by condensing the tetranucleotide (**10**) with 4 fold excess of the mononucleotide, MMTrU(OBz)-p in a yield of 21%. As shown in Figure 3 the amount of the pentanucleotide was larger than the recovered tetranucleotide. The yield, however, was lower than in the case of Figure 2, in which a larger amount of the starting trinucleotide was recovered. This might mean that chain cleavage occurred more extensively

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[10] *a*) E. Ohtsuka, K. Murao, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, **91**, 1537 (1969); *b*) *Idem*, *ibid.*, **92**, 3441 (1970).

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[13] N. Yoshida and H. Otsuka, *Biochim. Biophys. Acta*, **228**, 648 (1971).

[14] D.H. Rammler, Y. Lapidot, and H.G. Khorana, *J. Amer. Chem. Soc.*, **85**, 1989 (1963).

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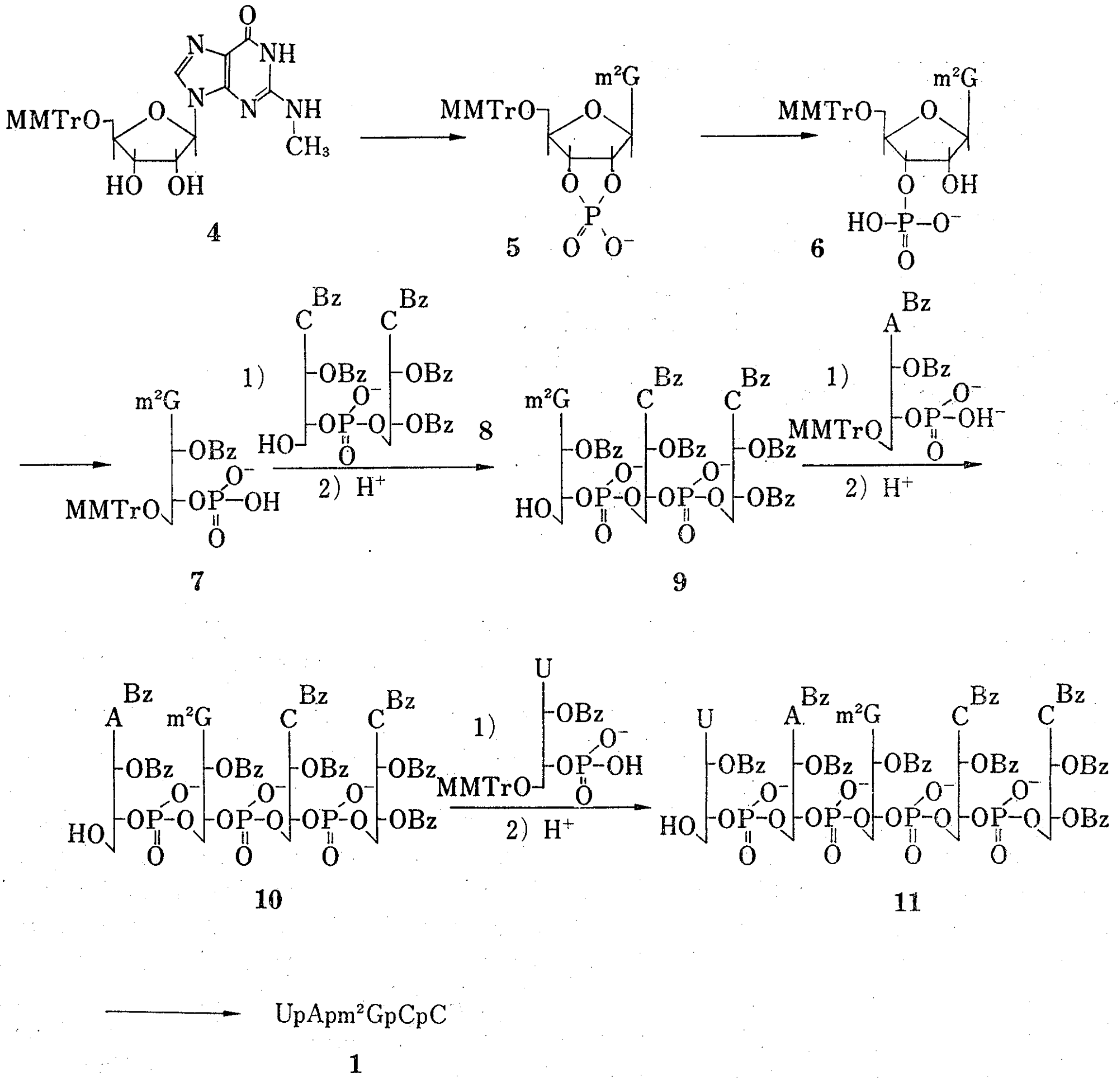

Chart 1

Table I

Peak	Fractions pooled	A_{260}	Identification
I	21— 26	637	A^{Bz}p cyclic
II	27— 40	1540	mainly A^{Bz}p cyclic
III	51— 57	822	mainly A^{Bz}(OBz)-p
IV	58— 70	678	A^{Bz}(OBz)-p
V	94—135	2045	m^2G(OBz)-p-C^{Bz}(OBz)-p-$C^{Bz}(OBz)_2$
VI	231—270	1890	A^{Bz}(OBz)-p-m^2G(OBz)-p-C^{Bz}(OBz)-p-$C^{Bz}(OBz)_2$

in the longer oligonucleotide. These oligonucleotides were characterized by enzymatic hydrolysis with base-nonspecific RNase M[16] and venom phosphodiesterase. *Rf* values in paper chromatography and relative mobilities in paper electrophoresis are shown in Table II.

Synthesis of the Tetranucleotide, Bz-C^{Bz}(OBz)-p-U(OBz)-p-C^{Bz}(OBz)-p-U(OBz)-p (17)

The 5′-terminal tetranucleotide was synthesized by condensing two dinucleotides. The dinucleotide Bz-C^{Bz}(OBz)-p-U(OBz)-p (**14**) was synthesized using phosphoramidate protection[10]

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[16] M. Irie, *J. Biochem.* (Tokyo), **62**, 509 (1967).

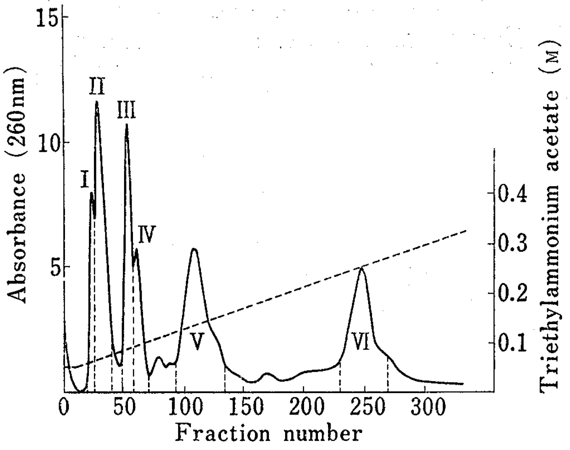

Fig. 2.

Chromatography of the products obtained in the synthesis of the tetranucleotide (**10**) on a column (3.0×55 cm) of TEAE-cellulose (acetate) equilibrated with 80% ethanol. Elution was carried out using a linear gradient of triethylammonium acetate in 80% ethanol. The mixing chamber contained 0.05 M salt (4 liter) and the reservoir contained 0.4 M salt (4 liter). Identification of peaks is shown in Table I.

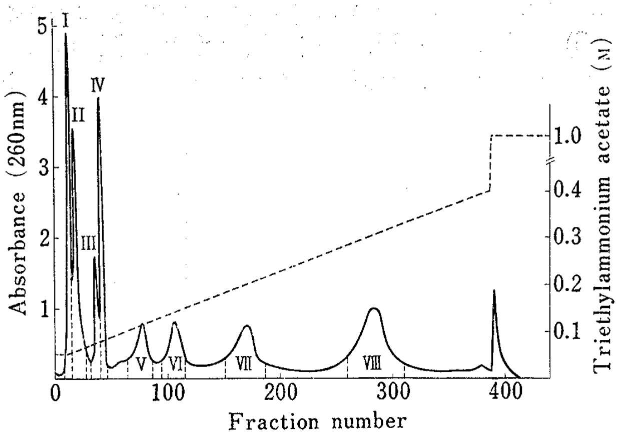

Fig. 3

Chromatography of the products obtained the synthesis of the pentanucleotide (**11**) on a column (2.0×55 cm) of TEAE-cellulose (acetate) equilibrated with 80% ethanol. Elution was carried out using a linear gradient of triethylammonium acetate in 80% ethanol. The mixing chamber contained 0.05 M salt (4 liter) and the reservoir contained 0.45 M salt (4 liter). The product (**11**) was obtained in peak VIII and the starting material (**10**) as recovered in peak VII.

Table II. Paper Chromatography and Electrophoresis

	Rf solvent			Relative mobility	
	A	B	C	pH 7.5	pH 2.7
m^2Gp	0.12	0.34	1.00	0.94	
m^2G cyclic p	0.39	0.59	1.32	0.54	
$MMTrm^2Gp$				0.76	
$MMTrm^2G$ cyclic p		0.73		0.36	
$MMTrm^2G(OBz)p$		0.82			
$MMTrm^2G$		0.83			
m^2G		0.68			
CpC	0.25	1.15		0.47	
m^2GpCpC	0.09	0.87		0.60	
Apm^2GpCpC	0.04	0.73		0.64	
$UpApm^2GpCpC$	0.01	0.52		0.75	
Up				1.00	1.00
U cyclic p				0.74	
Cp		0.18	0.38	0.93	0.37
Gp			0.24	1.00	
UpNHPh			0.66	0.57	
CpUpNHPh			0.48	0.76	
CpUp		0.07	0.24	1.01	
CpUpCpUp			0.08		
CpGp			0.19	0.97	
CpGpGp			0.08		

as shown in Chart 2. The yield of the condensation of Bz-C^{Bz}(OBz)-p (**12**) (2 fold excess) and the anilidate (**13a**) using DCC, successive isoamyl nitrite treatment and column chromatography on TEAE-cellulose was 55%. The elution pattern and conditions are shown in Fig. 4. The recovered mononucleotide in peak I was mainly the cytidine derivative (**12**). The condensation of the phosphoranisidate (**13a**) proceeded nearly completely and relatively small amounts of side products were formed in this case. The product (**14**) was identified by paper chromatography and electrophoresis after removal of the protecting groups (Table II). The 3′-protected dinucleotide (**16b**) was prepared by condensation of the monomethoxytritylated nucleotide (**15**) with the anilidate (**13b**) using DCC as the activating reagent and the monomethoxytrityl group was removed by treatment with a mixture of acetic acid–methylene chloride (8:2).

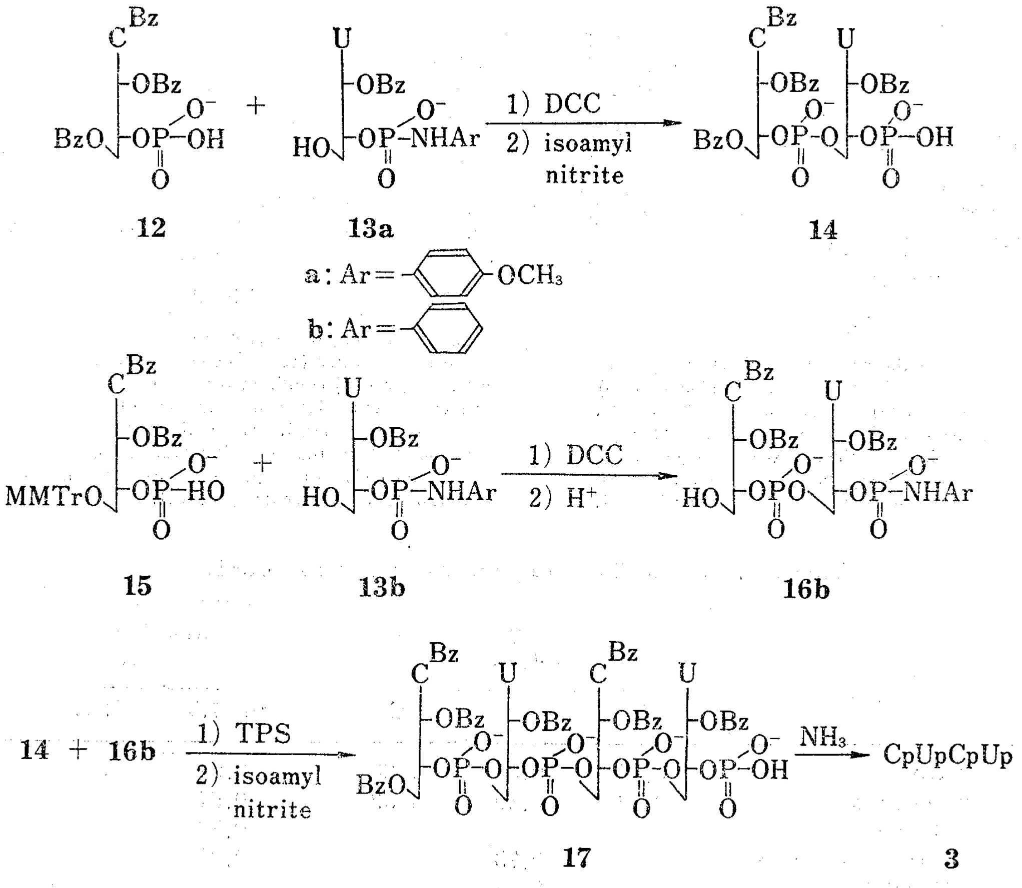

Chart 2

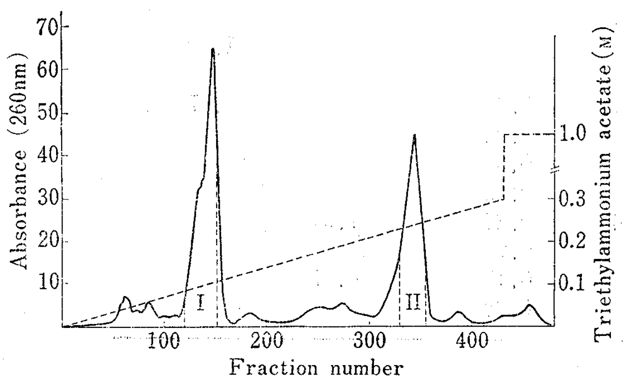

Fig. 4

Chromatography of the products obtained in the synthesis of the dinucleotide (**14**) on a column (3.5×60 cm) of the TEAE-cellulose equilibrated with 70% ethanol. Elution was carried out using a linear gradient of triethylammonium acetate (0—0.2 M) in 70% ethanol (6 liter). Peak I contained mainly BzOC^{Bz}(OBz)-p and peak II contained the pure dinucleotide (**14**).

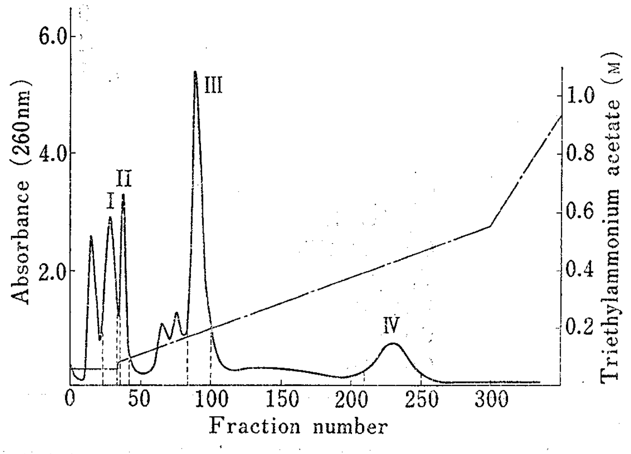

Fig. 5

Chromatography of the product obtained in the synthesis of the tetranucleotide (**17**) on a column (2.5×38) of TEAE-cellulose equilibrated with 75% ethanol. The column was washed with 75% ethanol (200 ml) and 0.05 M triethylammonium acetate (400 ml). Elution was carried out using a linear gradient of the salt (0.08—0.5 M) in 75% ethanol (5.2 liter). Peak III contained the dinucleotide and peak IV contained the tetranucleotide (**17**).

Aromatic phosphoramidates were found to be more stable in acetic acid-methylene chloride than in aqueous acetic acid.[17] The anilidate (**13b**) was chosen because it was found to be more stable than the corresponding anisidate during acid treatment and ion exchange chromatography.[18] The dinucleotide bearing a 3′-phosphoranilidate (**16b**) was isolated by ion-exchange chromatography on a TEAE-cellulose column. Since the product was not resolved completely from the pyrophosphate of N,2′-O-dibenzoylcytidine 3′-phosphate, fractions containing the pure dinucleotide (**16b**) were combined and the yield was 21% from **13b**. The condensation of these two dinucleotides **14** and **16b** was carried out using triisopropylbenzenesulfonyl chloride[19] (TPS) as the condensing reagent. The amount of the reagent required to activate the terminal phosphate was estimated on the assumption that each phosphate dissociation, regardless whether primary or secondary, required 1.2 equivalents of sulfonyl chloride. The yield of the tetranucleotide isolated after isoamyl nitrite treatment and chromatography on TEAE-cellulose was 22%. The product was characterized by paper chromatography, paper electrophoresis and enzymatic hydrolysis of the unprotected form (3). The elution pattern is shown in Fig. 5.

Synthesis of the Trinucleotide MMTrC^{Bz}(OBz)-p-G^{iBu}(OiBu)-p-G^{iBu}(OiBu)-p (20)

As shown in Chart 3 the trinucleotide (**20**) corresponding to 5th to 7th nucleotides of the tyrosine tRNA 5′-end was synthesized from the protected dinucleotide MMTrC^{Bz}(OBz)-p-G^{iBu}-(OiBu)-p (**18**) which was described previously.[3] The anisidate of the guanosine 3′-phosphate[3] (**19**) was condensed with the dinucleotide (**18**) using DCC and the reaction mixture was subjected to chromatography on a TEAE-cellulose column after isoamyl nitrite treatment. The trinucleotide (**20**) was obtained in a yield of 17%. Relatively large amounts of unidentified side products were obtained. The unprotected trinucleotide CpGpGp (**2**) was identified by hydrolysis with RNase M. The yield of the trinucleotide was not satisfactory as observed usually in condensations involving guanosine and monomethoxytrityl derivatives. The stability of the phosphoramidate of guanosine derivatives may be different to that of other nucleotides. Isobutyryl protection of the 2′-hydroxyl group of guanosine may not be stable enough for prolonged aqueous pyridine treatment which is requried to cleave tri- or tetra-substituted pyrophosphates.

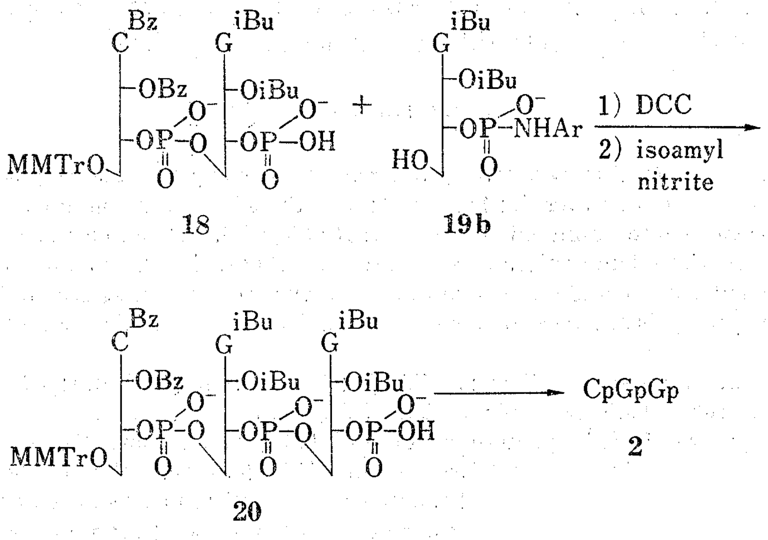

Chart 3

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[17] E. Ohtsuka, S. Morioka, and M. Ikehara, *J. Amer. Chem. Soc.*, **94**, 3229 (1972).

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More stable protecting groups for the 2′-hydroxyl group such as *o*-nitrobenzyl ether,[1] would be desirable especially in the case of guanosine. Methods for introduction of the benzyl group to guanosine and its phosphates will be published in future communications.

General Comments

Ribooligonucleotides of chain length upto five could be synthesized by stepwise condensation of mononucleotides, and isolated by ion-exchange chromatography. A minor nucleoside N^2-methylguanosine was incorporated to the oligonucleotide after phosphorylation and protection of the 2′-hydroxyl group. Although the yield decreased as the chain length increased in spite of using 4 to 5 fold excess of the mononucleotides, this approach did not require protection of phosphomonoesters since the 3′-terminus was not phosphorylated. The recent finding that RNA ligase[20,21] joins short oligonucleotides[22,23] provides various uses of ribooligonucleotides of this size. Ribooligonucleotides having a 3′-phosphate group would be also important substrates in the enzymatic joining reaction, because the 3′-phosphate might become a protecting group of the 3′-hydroxyl group and prevent undesirable reactions such as self-cyclization. The tetranucleotide CpUpCpUp (**3**) and the trinucleotide CpGpGp (**2**) were synthesized by aromatic phosphoramidate method. In the case of the tetranucleotide the anilidate of the dinucleotide (**16b**) was isolated by ion-exchange chromatography on TEAE-cellulose and used for condensation with the dinucleotide (**14**). This is the first block condensation to yield oligonucleotides bearing a terminal phosphate in the ribo-series. The yield was comparable to condensation of mononucleotides but not satisfactory. Studies to improve the yield in block condensations involving protected phosphate end groups are in progress. Chemical and enzymatic joining of these oligonucleotides are under investigation.

Experimental

General Methods——Paper chromatography was performed using the following solvent systems- A, 2-propanol–concentrated ammonia–water (7: 1: 2, v/v); B, ethanol–1 M ammonium acetate (pH 7.5) (7: 3, v/v); C, 1-propanol–concentrated ammonia–water (55: 10: 35, v/v). Paper electrophoresis was performed at 900 V/40 cm in 0.05 M triethylammonium bicarbonate (pH 7.5) and in 0.05 M ammonium formate (pH 2.7). General methods for condensation and removal of the protecting groups were as described previously.[10b] Conditions for enzymatic hydrolysis and base analysis by Varian LCS 1000 Nucleic Acid Analyzer were described in ref.[3]

Molecular extinction values are as follows; MMTrC^{Bz} (OBz)-p, 10000 (305 nm); MMTrm^{2}G(OBz)-p, 15500 (252 nm); MMTrA^{Bz}(OBz)-p, 19000 (280 nm); MMTrU(OBz)-p, 12000 (260 nm).

5′-O-Monomethoxytrityl-N^2-methylguanosine (**4**)——N^2-Methylguanosine (667 mg, 2.2 m moles) was dried over P_2O_5 at 100° for 5 hr *in vacuo* and dissolved in DMSO (4.5 ml). Monomethoxytrityl chloride (2.7 m moles) and pyridine (5.6 ml) were added and the mixture was stirred at room temperature. After 1 day no starting material was detected by thin-layer chromatography (TLC) (chloroform–ethanol, 7: 1) and the mixture was poured into ice-water (80 ml). The precipitate was collected by centrifugation and washed with *n*-pentane. The product was purified by dissolving in dimethyl formamide (DMF) (1 ml) and pouring the concentrated solution into ethyl acetate (160 ml). After cooling the crystallized product was collected by filtration and washed with *n*-pentane. The yield was 845 mg (66%).

5′-O-Monomethoxytrityl-N^2-methylguanosine 2′,3′-cyclic Phosphate (**5**)——(**4**) (569 mg, 1 m mole, dried over P_2O_5 *in vacuo* at 100°) was dissolved in DMF (10 ml) and allowed to react with morpholinophosphorodichloridate (0.41 g, 2 m mole) in dioxane (0.3 ml) and 2,6-lutidine (0.46 ml) for 3 days. The reaction was checked by paper electrophoresis after treatment with 80% acetic acid of aliquots. After 3 days a mixture of 0.05 N ammonium hydroxide (135 ml) and DMF (34 ml) was added. Morpholine was removed by extraction with ether and the aqueous phase was extracted with *n*-butanol. The organic layer was evaporated with added pyridine and the residue was treated with DCC (10 m mole) in pyridine (25 ml) and triethylamine (1.25 ml) for 3 days. Water (67 ml) was added and DCC was removed with ether and the product was extracted

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[20] *a*) R. Silber, V.G. Malathi, and J. Hurwitz, *Proc. Natl. Acad. Sci. U.S.*, **69**, 3009 (1972): *b*) J.W. Cranson, R. Silber, V.G. Malathi, and J. Hurwitz, *J. Biol. Chem.*, **249**, 7447 (1974).

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[23] G.C. Walker, O.C. Uhlenbeck, E. Bedows, and R.I. Gumport, *Proc. Natl. Acad. Sci. U. S.* **72**, 122 (1975).

with methylene chloride. The organic layer was concentrated and the product was precipitated with ether–pentane (1: 1) from its solution in pyridine. The yield was 445 mg (61%).

MMTrm^{2}G(OBz)-p(7)——Triethylammonium salt of **5** (337 mg, 0.46 mmole) was dissolved in DMF (3 ml) and diluted with water (200 ml). Triethylammonium bicarbonate (1 M, pH 7.8, 23 ml) was added to the solution and the nucleotide was hydrolyzed with RNase St (0.8 mg) at 37° for 5 hr. Completion of the reaction was checked by paper electrophoresis (pH 7.5) and the enzyme was removed by centrifugation after shaking with chloroform–isoamyl alcohol (20: 1). The product (**6**) was extracted with 1-butanol from the aqueous solution and 1-butanol was removed in the presence of tetraethylammonium benzoate (pH 7.1, 5 mmoles). The residue was made anhydrous by coevaporation with pyridine 5 times at 55° and a trace of pyridine was further removed with toluene by repeated coevaporation. Benzoic anhydride (10 mmoles) was added and the mixture was warmed at 50°. The homogeneous syrup was kept for 3 days at 25° and treated with aqueous pyridine (50%, 70 ml). The product was extracted with chloroform (30 ml, 3 portions) and the organic layer was washed with water (10 ml). Chloroform was evaporated with added pyridine and the pyridine solution was added to ether–pentane (3: 2). The precipitate was collected by centrifugation and treated with acetic anhydride (5 ml) in pyridine (10 ml) for 24 hr. Volatile materials were removed and the residue was added to 50% pyridine (10 ml) at 0°. The solution was passed through a column (*ca.* 15 ml) of Dowex-50 × 2 (pyridinium form) and kept at 25° for 2 hr. The eluant and washings were collected and evaporated with added pyridine and the product was precipitated with ether–pentane (3: 2) from its solution in pyridine. The precipitate was washed with ether 3 times and kept over phosphorus pentoxide. The yield was 0.18 g (47%). The product was characterized by paper chromatography and paper electrophoresis before and after treatment with 80% acetic acid (1 hr). Spectral properties of m^{2}G(OBz)-p were as follows: $\lambda_{max}^{H_2O}$ (nm) 237, 254 (sh) and 280 (sh); $\lambda_{min}^{H_2O}$ 222; $\lambda_{max}^{H^+}$ 236, 254 (sh) and 280 (sh); $\lambda_{min}^{H^+}$ 224; $\lambda_{max}^{OH^-}$ 255 (sh). The product was completely deprotected by treatment with 80% acetic acid followed by methanolic ammonia treatment.

C^{Bz}(OBz)-p-C^{Bz}(OBz)$_2$ (8)——The dinucleoside monophosphate (**8**) was synthesized by the same method used for the synthesis of C^{Bz}(OBz)-p-A$^{Bz}{}_2$(OBz)$_2$[15] from pyridinium 5′-O-monomethoxytrityl-N^4,2′-O-dibenzoylcytidine 3′-phosphate[10b] (14300 A$_{305}$ units, 1.4 mmoles) and N^4,2′,3′-O-tribenzoylcytidine[24] 1.09 g (1.09 mmoles) in a yield of 75%.

The Trinucleotide m^{2}G(OBz)-p-C^{Bz}(OBz)-p-C^{Bz}(OBz) 2 (9)——The pyridinium salt of the dinucleotide (**8**) (0.59 g, 8000 A$_{305}$, 0.39 mmole) and pyridinium MMTr-m^{2}G(OBz)-p (**1**) (3370 A$_{260}$, 0.37 mmole) were allowed to react with DCC (4.6 mmoles) in anhydrous pyridine (5 ml) at 27° for 5 days. Aqueous pyridine (50%, 15 ml) was added and the solution was kept at room temperature overnight after extraction with *n*-pentane. The filtered solution was evaporated to remove pyridine and the residue was treated with 80% acetic acid for 2 hr at room temperature. Acetic acid was removed, the residue was rendered anhydrous by evaporation with pyridine and precipitated in ether–pentane (3: 2) from its solution in pyridine. The precipitate was collected by centrifugation and dissolved in a small amount of pyridine. The solution was diluted with 90% ethanol (500 ml) and applied to a column (3.0 × 55.5 cm) of TEAE-cellulose (acetate). The column was washed with 90% ethanol and elution was carried out using a linear gradient of trithylammonium acetate in 90% ethanol from 0 to 0.35 M acetic acid containing 0.168 M triethylamine, the total volume being 8 liter. The product (**9**) was eluted with 0.21 to 0.26 M salt and extracted with *n*–butanol after evaporation of ethanol. The organic layer was washed with water and concentrated with added pyridine. The nucleotide was precipitated with ether–pentane from its solution in pyridine, washed with ether and dried over phosphorus pentoxide. The yield was 5360 A$_{260}$ (0.24 g), 38%. The unprotected product m^{2}GpCpC was characterized by enzymatic hydrolysis. Incubation with RNase A (50 mg) in 0.1 M triethylammonium bicarbonate (0.1 ml) at 37° for 6 hr gave cytidine (0.67 A$_{260}$) and m^{2}GpCp (1.65 A$_{260}$). m^{2}GpCp was digested with RNase St (50 μg) at the above condition and one tenth of the mixture was analyzed fy Nucleic Acid Analyzer. The ratio of m^{2}Gp to Cp was 1.0 to 1.0. Spectral properties of m^{2}GpCpC was $\lambda_{max}^{H_2O}$ (nm) 259.5, $\lambda_{min}^{H_2O}$ 230, $\lambda_{max}^{H^+}$ 278, $\lambda_{min}^{H^+}$ 238 and $\lambda_{max}^{OH^-}$ 268.5, $\lambda_{min}^{OH^-}$ 244.5.

The Tetranucleotide A^{Bz}(OBz)-m^{2}G(OBz)-pC^{Bz}(OBz)-p-C^{Bz}(OBz) 2 (10)——The pyridinium salt of the trinucleotide (**9**) (2860 A$_{260}$, 0.079 mmole) and pyridinium MMTr-A^{Bz}(OBz)-p[25] (7200 A$_{280}$, 0.39 mmole) were condensed using DCC (4.3 mmoles) in pyridine (2 ml) at 30°—35° for 5 days with shaking. The reaction mixture was worked up as described in the synthesis of the trimer (**1**) and dissolved in 80% ethanol (200 ml) after acetic acid treatment. The solution was applied to a column of TEAE-cellulose. The elution pattern and identification of peaks are shown in Fig. 2 and Table I. The product (**10**) obtained in peak VI was isolated by *n*-butanol extraction after evaporation of ethanol. The butanol layer was washed with water to remove the salt and the aqueous phase had no absorption at 305 nm. The tetranucleotide (**10**) was precipitated with ether–pentane (3: 2) from its solution in pyridine after removal of butanol by evaporation with pyridine. The yield was 1890 A$_{260}$, 59 mg (31%). The unprotected tetranucleotide Ap^{m}GpCpC was analyzed by Nucleic Acid Analyzer after digestion with RNase M to yield Ap: m^{2}Gp: Cp = 1.00: 0.93: 1.08. The spectral properties of Apm^{2}GpCpC were $\lambda_{max}^{H_2O}$ (nm) 260, $\lambda_{min}^{H_2O}$ 235.5, $\lambda_{max}^{H^+}$ 266.5 $\lambda_{min}^{H^+}$ 237.0 and $\lambda_{max}^{OH^-}$ 262.5 $\lambda_{min}^{OH^-}$ 244.5.

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The Pentanucleotide U(OBz)-p-A^{Bz}(OBz)-p-m^{2}G(OBz)-p-C^{Bz}(OBz)2 (11)——The pyridinium salt of the tetranucleotide (10) (54 mg, 1790 A$_{260}$, 0.023 mmole) and pyridinium MMTrU(OBz)-p (75 mg, 1290 A$_{260}$, 0.09 mmole) were treated with DCC (1 mmole) in pyridine (0.7 ml) at 32° for 5 days with shaking. Aqueous pyridine (50%, 10 ml) was added, extracted with pentane (10 ml) two portions and the mixture was kept overnight. The filtered solution was concentrated and pyridine was removed by coevaporation with water. The monomethoxytrityl group was removed by treatment with 80% acetic acid for 1 hr and acetic acid was removed by evaporation with water-butanol. The residue was applied to a column of TEAE-cellulose (acetate). The elution pattern and conditions are shown in Fig. 3. The product (**11**) was eluted at salt concentration of 0.28—0.32 M and isolated by butanol extraction as described above. The yield was 466 A$_{260}$, 14 mg (21%). The deprotected product UpApm^{2}GpCpC was analyzed by Nucleic Acid Analyzer. RNase M digestion gave Up: Ap: m^{2}Gp: Cp$=$0.75: 1.08: 1.00: 0.94 and the ratio obtained by venom phosphodiesterase hydrolysis was pA: pm^{2}G: pC$=$0.92: 1.01: 2.00. The spectral properties of UpApm^{2}GpCpC are $\lambda_{\max}^{H_2O}$ nm 260.5 $\lambda_{\min}^{H_2O}$ 235.5, $\lambda_{\min}^{H^+}$ 236.0 $\lambda_{\max}^{OH^-}$ 262.5 $\lambda_{\min}^{OH^-}$ 242.0.

BzC^{Bz}(OBz)-p-U(OBz)-p (14)——The pyridinium salt of U(OBz)-pNHPhOCH$_3$ (14000 A$_{260}$ units, *ca.* 1 mmole) and pyridinium N^4,2′,5′-tribenzoylcytidine 3′-phosphate (20800 A$_{305}$, *ca.* 2 mmoles) were passed through a small column of pyridinium Dowex 50×2 in 50% aqueous pyridine separately. The nucleotides were precipitated with ether from their solution in anhydrous pyridine and treated with DCC (4.1 g) in the presence of dry pyridinium Dowex 50×2 resin (1 g) in pyridine (8 ml) at 28° for 5 days. Aqueous pyridine (50%) and pentane were added to the mixture. After 14 hr at room temperature the filtered solution was extracted with pentane (30 ml, 3 portions) to remove DCC and precipitated with ether-pentane (3: 2) from its solution in anhydrous pyridine. The precipitate was treated with isoamyl nitrite (1.5 ml) in pyridine–acetic acid (1: 1) (12 ml) at 28° for 5 hr. The volatile materials were evaporated and the residue was dissolved in pyridine (5 ml) and 70% ethanol (1.4 l). The solution was applied to a column of TEAE-cellulose. The elution pattern and conditions are shown in Fig. 4. Peak II contained the pure dinucleotide (**14**) (19500 A$_{260}$, 0.59 mmole). The yield was 59%. The product was characterized by removing protecting groups and hydrolyzing with RNase A as described previously. The completely hydrolyzed products (Cp and Up) were separated by paper electrophoresis at pH 2.7 and their amounts estimated spectrophotometrically at pH 2. The ratio was 1.0 to 1.0.

C^{Bz}(OBz)-p-U(OBz)-pNHPh (16b)——Pyridinium 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidine 3′-phosphate (6760 A$_{305}$, 0.68 mmole) and pyridinium 2′-O-benzoyluridine 3′-phosphoranilidate (7050 A$_{260}$, 0.54 mmole) were treated with DCC (1.4 g) in pyridine (4 ml) in the presence of pyridinium Dowex 50×2 (500 mg) for 30 hr at 28°. Aqueous pyridine (50%) was added and the mixture was kept at room temperature for 14 hr. N,N′-Dicyclohexylurea was removed, DCC was extracted with *n*-pentane and the nucleotides were precipitated with ether–pentane (3: 2) from their solution in pyridine. The precipitate was washed with ether and treated with acetic acid–chloroform (8: 2) for 8 hr at room temperature to remove the monomethoxytrityl group. The volatile materials were removed and the residue was applied to a column (2.7×50 cm) of TEAE-cellulose (acetate) equilibrated with 75% ethanol. Elution was carried out using a linear gradient of triethylammonium acetate (0—0.25 M) in 75% ethanol. The total volume was 5 liter. The dinucleotide (**16b**) (3500 A$_{260}$, 0.113 mmole, 21%) was eluted with salt concentration of 0.15 M and isolated by *n*-butanol extraction from its concentrated solution in aqueous buffer. The butanol layer was washed with a small amount of water and the product was precipitated in ether–pentane (3: 2) from its solution in pyridine.

C^{Bz}(OBz)-p-U(OBz)-p-C^{Bz}(OBz)-pU(OBz)-p (17)——Ths triethylammonium salt of the dinucleotides (**14**) (960 A$_{305}$, 0.09 mmole) and (**16b**) (920 A$_{305}$, 0.077 mmole) were condensed by using TPS (172 mg, 0.56 mmoles) at 28° for 8 hr. Aqueous pyridine (50%, 8 ml) was added and the solution was kept at room temperature for 24 hr. Tri-*n*-butylamine (0.14 ml) was added and the product was extracted with *n*-butanol. The butanol layer was washed with water, dried by coevaporation with addition of pyridine and the nucleotides were precipitated with ether–pentane (3: 2) from their solution in pyridine. The precipitate was treated with isoamyl nitrite (0.2 ml) in pyridine–acetic acid (1: 1) (3 ml) at 28° for 5 hr. The mixture was applied to a column of TEAE-cellulose after removal of the volatile materials. The elution profile and conditions are shown in Fig. 5. Peak IV contained the protected tetranucleotide as identified by paper chromatography in solvent C after removal of the protecting groups with 15 N methanolic ammonia for 15 hr at 28°. The *Rf* values are shown in Table I. The unprotected tetranucleotide CpUpCpUp (2 A$_{260}$) was completely hydrolyzed with RNase A to give Cp and Up (by paper electrophoresis at pH 2.7). Rnase M hydrolysate (0.1 A$_{260}$) was analyzed by Nucleic Acid Analyzer and the ratio of Cp to Up was 1.0 to 1.3.

MMTrC^{Bz}(OBz)-p-G^{iBu}(OiBu)-p-G^{iBu}(OiBu)-p (20)——The pyridinium salt of the dinucleotide (**18**) (1570 A$_{305}$, 0.126 mmole) and N^2,2′-O-diisobutyrylguanosine 3′-phosphoranisidate (**19a**) (5300 A$_{260}$, 0.28 mmole) were allowed to react with DCC (0.52 g) in pyridine (1 ml) in the presence of pyridinium Dowex 50×2 (250 mg) for 4.5 days at 30°. Aqueous pyridine (50%, 10 ml) and *n*-pentane were added. After 16 hr, the filtered solution was extracted with *n*-pentane (10 ml) 3 portions and the nucleotides were precipitated with ether–pentane (3: 2). The precipitate was treated with isoamyl nitrite (0.6 ml) in pyridine–acetic acid (1: 1) (4 ml) at 30° for 5 hr and added to ether–pentane (3:2). The precipitate was dissolved in a small amount of pyridine and 70% ethanol, and applied to a column (2×48 cm) of TEAE-cellulose (acetate). Elution was carried out using a linear gradient of triethylammonium acetate (0—0.3 M) in 70% ethanol (total 4.4 liter). The trinucleotide (**20**) was eluted at salt concentration 0.19 M. The yield was 1240 A_{260} (17%). The unprotected trinucleotide CpGpGp was characterized by digestion with RNase M. The spectral properties of CpGpGp were $\lambda_{max}^{H_2O}$ (nm) 255, 273 (sh), $\lambda_{max}^{H^+}$ 260, $\lambda_{max}^{OH^-}$ 267 and 280/260=0.71 (H_2O), 0.87 (H^+).