Studies on Transfer Ribonucleic Acids and Related Compounds. I. Synthesis of Ribooligonucleotides Using Aromatic Phosphoramidates as a Protecting Group

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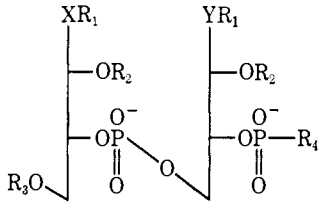
Abstract: Aromatic phosphoramidates of protected nucleosides were found to be converted to the corresponding phosphate with isoamyl nitrite without damaging other protecting groups. By use of such phosphoramidates a trinucleotide, 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidyldyl-(3′–5′)-N,2′-O-dibenzoylcytidylyl-(3′–5′)-N,2′-O-dibenzoyladenosine 3′-phosphate (Chart II, VIII), was prepared as follows: condensation of the aromatic phosphoramidate of N,2′-O-dibenzoylcytidine (II) with 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidine 3′-phosphate (IV) gave the protected dinucleotide intermediate (V). After selective decomposition of the amidate group with isoamyl nitrite, the protected dinucleotide (VI) was condensed with the aromatic phosphoramidate of N,2′-O-dibenzoyladenosine (VII). Repetition of isoamyl nitrite treatment gave the protected trinucleotide (VIII) which was isolated by an anion-exchange chromatography. Dicyclohexylcarbodiimide was used as a condensing reagent and the yields in different steps were *ca.* 30%.

The stepwise condensation of preformed oligonucleotides has been shown to be an attractive method for the synthesis of medium length in the deoxyribopolynucleotide series.[1,2] A dinucleotide such as I could be a versatile intermediate for the synthesis of ribopolynucleotides, providing R_3 and/or R_4 could be removed selectively without damaging other protecting groups. If R_4 were removed to give a phosphomonoester end group, the dinucleotide could be condensed with a nucleotide bearing a 5′-hydroxyl group. If R_3 and R_4 were removed the dinucleotide could be



I

self-polymerized. The condensation of this type of compound would involve reaction between the 5′-hydroxyl group and the 3′-phosphate. Another approach to 3′–5′ internucleotide linkages, which utilizes 5′-phosphate and the 3′-hydroxyl groups, requires specific protection of the 2′-hydroxyl group. The selective acylation of nucleosides is possible in some cases,[3b,4] but further studies are necessary if this technique is to be useful generally. We preferred to use 3′-phosphates in this study.

Trityl derivatives for protection of primary hydroxyl groups and acyl protections for heterocyclic amino groups and 2′-hydroxyl groups have been used successfully in the synthesis of ribotrinucleotides[5] and other ribooligonucleotides.[3] Acid-labile groups for the protection of 2′-hydroxyl groups have been used by several investigators.[3a,6] Acid removal of protecting groups of the 2′-hydroxyl groups, however, possesses a possible danger of migration of the phosphodiester linkage. For the protection of 3′-phosphomonoester groups, acid-[7] and alkaline-[8]labile groups were used in the synthesis of ribodinucleotides. 2′,3′-Cyclic phosphates of pyrimidine nucleosides served as protecting groups of phosphomonoesters and 2′-hydroxyl groups during the condensation using dicyclohexylcarbodiimide (DCC) to yield dinucleotides.[9,10] The accompanying paper describes a synthesis of a protected ribonucleotide using this technique.[11] The trichloroethyl group[12] was introduced recently for the protection of 5′-phosphomonoesters of deoxyribonucleotides.[13] This group can be removed by zinc in acid or organic solvents. The specific cleavage, under neutral conditions of phosphomonoester protecting group, is required for the synthesis of ribooligonucleotides with trityl and acyl derivatives.

In this report it is shown that aromatic amidates of protected nucleotides can be converted to the phosphates with isoamyl nitrite selectively and that the amidate is useful as a protecting group of phosphomonoesters in the synthesis of protected ribooligonucleotide blocks.[14] Since it is known that thymidine

(1) H. G. Khorana, *Pure Appl. Chem.*, 71, 349 (1968).
(2) (a) H. Kössel, H. Büchi, and H. G. Khorana, *J. Amer. Chem. Soc.*, **89**, 2185 (1967); (b) E. Ohtsuka and H. G. Khorana, *ibid.*, **89**, 2195 (1967); (c) J. Hachmann and H. G. Khorana, *ibid.*, **91**, 2749 (1969).
(3) (a) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *ibid.*, **84**, 430 (1962); (b) D. H. Rammler and H. G. Khorana, *ibid.*, **84**, 3112 (1962); (c) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3852 (1963); (d) R. Lohrmann and H. G. Khorana, *ibid.*, **86**, 4188 (1964).
(4) H. P. Fromageot, C. B. Reese, and J. H. Sulton, *Tetrahedron*, **24**, 3533 (1968).
(5) R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, and H. G. Khorana, *J. Amer. Chem. Soc.*, **88**, 819 (1966).
(6) (a) S. Chladek and J. Smrt, *Chem. Ind.*, 1719 (1964); (b) J. Zemlicka and S. Chladek, *Tetrahedron Lett.*, 3057 (1965); (c) F. Eckstein and F. Cramer, *Ber.*, **98**, 995 (1965); (d) C. B. Reese, R. Satthill, and J. E. Sulton, *J. Amer. Chem. Soc.*, **89**, 3366 (1967).
(7) (a) F. Cramer and K. H. Scheit, *Ann.*, **679**, 150 (1964); (b) F. Cramer, H. Neunhoeffer, K. H. Scheit, G. Schneider, and J. Tennegheit, *Angew. Chem.*, **74**, 387 (1962).
(8) D. Söll and H. G. Khorana, *J. Amer. Chem. Soc.*, **87**, 360 (1965).
(9) D. Söll and H. G. Khorana, *ibid.*, **87**, 350 (1965).
(10) H. J. Rhaese, W. Siehr, and F. Cramer, *Ann.*, **703**, 215 (1967).
(11) E. Ohtsuka, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, **92**, 3445 (1970).
(12) R. B. Woodward, K. Heusler, J. Gosteli, P. Naegeli, W. Oppolzer, R. Ramage, S. Ranganathan, and H. Vorbruggen, *ibid.*, **88**, 852 (1966).
(13) (a) F. Eckstein, *Ber.*, **100**, 2228 (1967); (b) F. Eckstein, *ibid.*, **100**, 2236 (1967); (c) A. Franke, F. Eckstein, K. H. Scheit, and F. Cramer, *ibid.*, **101**, 944 (1968).
(14) A preliminary communication was reported; E. Ohtsuka, K.

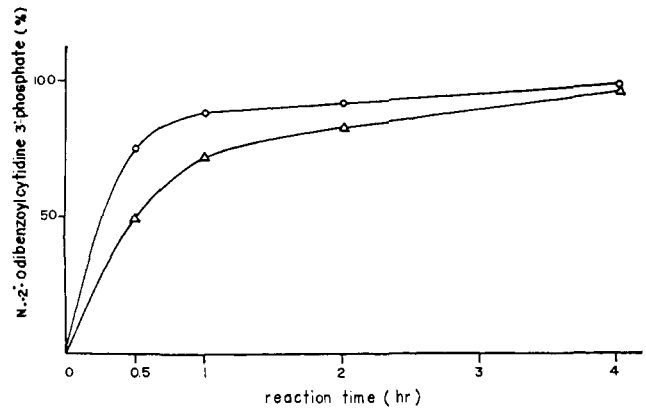
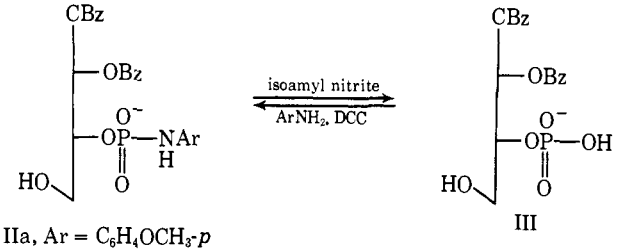


Figure 1. Kinetics of the decomposition of N,2'-O-dibenzoylcytidine 3'-phosphoro-*p*-anisidate. The reaction mixture 1, indicated by circles, contained the amidate (1.5 μmol), DMF (20 μl), pyridine (10 μl), acetic acid (10 μl), and isoamyl nitrite (10 μl); mixture 2, indicated by triangles, contained triethylamine (5 μl) instead of pyridine in the above mixture. Aliquots of 10 μl were taken as shown and applied to paper chromatography in solvent B. The ratio of the compound was estimated spectrophotometrically after eluting the spots in water.

5'-phosphoramidate was decomposed to thymidine 5'-phosphate with isoamyl nitrite,[15] we have examined this reaction with 3'-phosphoro-*p*-anisidate of protected nucleosides. N,2'-O-Dibenzoylcytidine 3'-phosphoro-*p*-anisidate (IIa) (Chart I) was prepared

Chart I. Formation and Decomposition of Aromatic Phosphoramidates



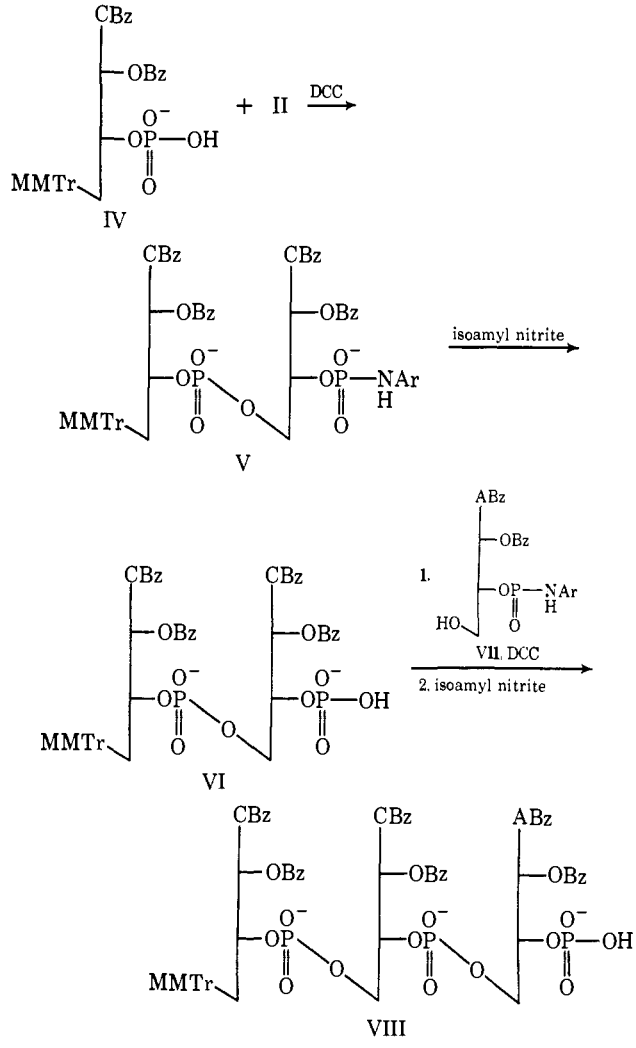
IIa, Ar = $C_6H_4OCH_3$-*p*

b, Ar = C_6H_5

from N,2'O-dibenzoylcytidine 3'-phosphate (III) by condensing the latter and *p*-anisidine with DCC.[16] Treatment of IIa with isoamyl nitrite in DMF gave mainly III but a small amount of deacylated and deaminated products was also formed. Probably these side reactions were caused by the acidity of the medium. In buffered solution containing pyridine–acetic acid or triethylamine–acetic acid, IIa gave III exclusively upon treatment with isoamyl nitrite. The results shown in Figure 1 indicate IIa was converted to the corresponding phosphate in the buffered solution at 20° after 4 hr.

As the reactivity of phosphoramidates parallels the basicity of the amine[17] it is considered that the use of phosphoramidates of weaker bases such as aniline is more suitable for the protection of phosphomonoesters. N,2'-O-Dibenzoylcytidine 3'-phosphoranilidate (IIb) was also converted to the phosphate (III) within 4 hr under conditions similar to that described in Figure 1 except using no DMF.[18] It was also ascertained that other protecting groups were not removed under the conditions used. Thus, both of the aromatic phosphoramidates could be used as protecting groups of phosphomonoesters in the condensation reaction. For the synthesis of the protected di- and trinucleotides, these aromatic phosphoramidates were used as shown in Chart II.

Chart II. The Synthesis of the Trinucleotide



5'-O-Monomethoxytrityl-N,2'-O-dibenzoylcytidine 3'-phosphate (IV) and the anisidate (IIa) were condensed with DCC in pyridine. The selective removal of the amidate group of the intermediate V with isoamyl nitrite in buffered solution gave the protected dinucleotide, MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*[19] (VI), which was isolated by TEAE-cellulose column chromatography.[20] It was found that the monomethoxytrityl group was stable during chromatography at room

(15) M. Ikehara, S. Uesugi, and T. Fukui, *Chem. Pharm. Bull.* (Tokyo), **15**, 440 (1967).

(16) J. G. Moffatt and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 649 (1961).

(17) V. M. Clark, G. W. Kirby, and A. R. Todd, *J. Chem. Soc.*, 1497 (1957).

(18) Detailed studies on the reactivity of different phosphoramidates are under investigation.

(19) The system of abbreviation is principally as has been used in *J. Biol. Chem.*, **241**, 531 (1966). For the protected ribonucleotide essentially the same system is used as described in ref 6. MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p* refers to 5'-O-monomethoxytrityl-N,2'-O-dibenzoylcytidylyl-(3'–5')-N,2'-O-dibenzoylcytidine 3'-phosphate. N,2'-O-Dibenzoylcytidine 3'-phosphoro-*p*-anisidate is abbreviated to C^{Bz}-OBz-*p*$NHPhOCH_3$.

(20) An example of the elution pattern of the dinucleotide is shown in ref 14.

temperature in triethylammonium acetate buffer pH 6.5, in 95% ethyl alcohol. The main peak, which was eluted at 0.18–0.2 *M* salt concentration, contained essentially pure dinucleotide (VI). The yield was 28%. The yield of the dinucleotide in another run using the anilidate (IIb) was 35%. The purity of the product was checked by paper chromatography and paper electrophoresis after partial removal of the protecting groups. R_f values are shown in Table II.

For the synthesis of the trinucleotide, MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*-A^{Bz}-OBz-*p* (VIII), the dinucleotide VI was condensed with N,2′-O-dibenzoyladenosine 3′-phosphoroanilidate (VII) using DCC as the condensing reagent. Using a similar procedure as above, the phosphoramidate was converted to the phosphate VIII which was isolated by a column chromatography on TEAE-cellulose (acetate). The elution pattern and the conditions are shown in Figure 2. The identification of some of the peaks is given in Table I. Peak

Table I. Chromatography of the Products Obtained in the Synthesis of the Trinucleotide MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*-A^{Bz}-OBz-*p* (Figure 2)

Peak no.	Fractions pooled	OD_{260} units	Identification
I	36–45	924	A^{Bz}-cyclic *p*
II	58–64	192	A^{Bz}-OBz-*p*
III	93–105	1080	MMTr-C^{Bz}-OBz-*p*C^{Bz}-OBz-*p* (almost pure)
IV	186–215	2110	MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*-A^{Bz}-OBz-*p* (almost pure)
V	265–278	1350	MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*-A^{Bz}-OBz-*p*-A^{Bz}-OBz-*p* (mainly)

IV contained almost pure product (VIII), the yield being 28%. R_f values of the trinucleotide before and after removing protecting groups are shown in Table II. The completely deprotected trinucleotide, CpCpAp, was degraded with pancreatic RNase to give cytidine phosphate and adenosine 3′-phosphate in the ratio of 2:1 (see Experimental Section).

General Comments

The properly protected ribotrinucleotide with 3′-phosphate which can be used for condensation and polymerization has been synthesized and isolated by column chromatography. The yield could be improved by minimizing side reactions during the condensation. The main side reactions in the condensation of phosphates and the hydroxyl group of the nucleoside with phosphoramidates using DCC in pyridine could be (1) the formation of the unsymmetrical pyrophosphate and activation of the pyrophosphate by DCC and (2) the further activation of the phosphoramidate to give the higher oligonucleotide with the amidate of phosphodiester. The RNase M digestion of the main part of the 1 *M* fraction of Figure 2, after removal of the protecting groups, showed complete degradation to give cytidine phosphate and adenosine 3′-phosphate in a ratio of 1:1, which suggested the formation of the linear tetranucleotide. The formation of the pyrophosphate might be difficult to be proved directly, because pyrophosphates adjacent to hydroxyl groups are cleaved by ammonia treatment.[21]

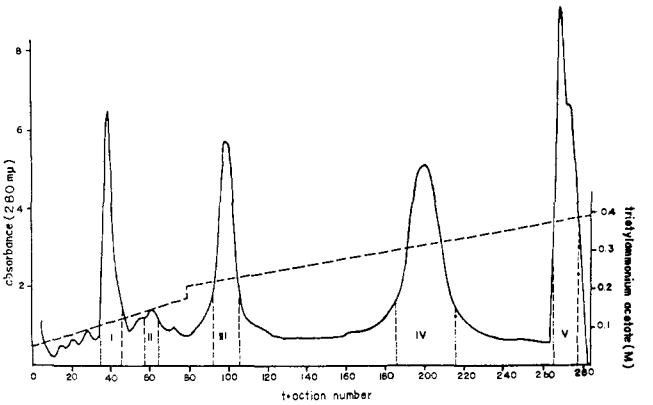


Figure 2. Chromatography of the products obtained in the synthesis of the trinucleotide, MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*-A^{Bz}-OBz-*p*, on a column (2.7 × 50 cm) of TEAE-cellulose (acetate) preequilibrated with 0.02 *M* triethyammonium acetate in 90% ethyl alcohol. Elution was carried out using a linear salt gradient of triethylammonium acetate in 90% ethyl alcohol (1 l. of 0.05 *M* salt in the mixing chamber and the equal volume of 0.2 *M* salt in the reservoir, followed by elution using 2 l. of 0.2 *M* salt and 2 l. of 0.4 *M* salt). Fractions of 20 ml were collected about every 15 min. For identification of peaks, see Table I.

Although the isoamyl nitrite treatment of the phosphoramidate gave the corresponding phosphate exclusively, the possible presence of impurities which do not absorb uv cannot be excluded. The isolated products were characterized enzymatically and found to contain the expected nucleotides. The nature of the reaction with isoamyl nitrite, however, has to be investigated further. Another serious problem is the cleavage of phosphomono- and diester linkages during the condensation reaction. Conditions to improve the yield must be found by preventing this kind of side reaction. It is possible to reduce the loss during isolation if the scale of the reaction is increased.

Further condensation of the trinucleotide with the oligonucleotide having the 5′-hydroxyl group and the application of this method to the synthesis of deoxyribooligonucleotides bearing 5′-phosphate are reported elsewhere. The extension of this method to the synthesis of oligonucleotides containing various kinds of base sequences and the use of phosphoramidate resin[22] of protected ribonucleotide 3′-phosphates are under investigation.

Experimental Section

General Methods. Paper chromatography was performed by the descending technique using Toyoroshi No. 51A paper. The solvent systems used were: solvent A, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); solvent B, ethyl alcohol–1 *M* ammonium acetate, pH 7.5 (7:3, v/v); solvent C, saturated ammonium sulfate–isopropyl alcohol–water (79:2:19, v/v); solvent D, *n*-propyl alcohol–concentrated ammonia–water (55:10:35, v/v). The R_f readings of different compounds are given in Table II. Paper electrophoresis was performed at 900 V/40 cm. Triethylammonium bicarbonate buffer (0.05 *M*, pH 7.5) was used.

Monomethoxytrityl group was detected by spraying the chromatogram with 10% aqueous perchloric acid and drying in warm air.

Pyridine was treated with *p*-toluenesulfonyl chloride before fractional distillation. It was redistilled after refluxing over potassium hydroxide and decanting and stored over molecular sieve

(21) Studies on pyrophosphate formation by phosphoramidates in the presence of DCC and decomposition of phosphoramidates in this medium are under investigation.

(22) G. M. Blackburn, M. J. Brown, and M. R. Harris, *J. Chem. Soc.*, 2438 (1967).

Table II. Paper Chromatography and Electrophoresis

Compounds	Paper chromatography, R_f Solvent A	B	C	D	Paper electrophoresis, relative mobilities to Cp pH 7.5
	N-protected Nucleotides				
A^{Bz}-OBz-*p*		0.71			0.80
A^{Bz}-OBz-*p*NHPhOCH$_3$		0.84			0.48
A^{Bz}-OBz-*p*NHPh		0.86			0.49
C^{Bz}*p*		0.43			0.86
C^{Bz} cyclic *p*		0.70			0.53
C^{Bz}-OBz-*p*		0.68			0.80
C^{Bz}-OBz-*p*NHPhOCH$_3$		0.86			0.42
C^{Bz}-OBz-*p*NHPh		0.86			0.42
C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*		0.71			0.81
C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*-A^{Bz}-OBz-*p*		0.79			0.81
MMTr-A^{Bz}-OBz-*p*		0.86			
MMTr-C^{Bz}-OBz-*p*		0.82			
MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*		0.81			
MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*-A^{Bz}-OBz-*p*		0.87			
	Unprotected Nucleotides				
Ap	0.16		0.11		0.96
Cp	0.14		0.66	0.33	1.0
A-cyclic *p*	0.50		0.06		0.71
C-cyclic *p*	0.42		0.41	0.53	0.73
CpCp	0.43 (R_{Cp})		0.49	0.24	1.03
CpCpAp	0.14 (R_{Cp})		0.14	0.15	1.03
	5′-Monomethoxytrityl Compounds				
MMTr-Cp	0.61		0	0.82	0.79
MMTr-C-cyclic *p*	0.77				0.45
MMTr-CpCp	0.27		0	0.74	0.86
MMTr-CpCpAp	0.14		0	0.45	0.93

(4A) obtained from Linde Co. All reaction mixtures at the start of condensation reactions were made anhydrous by repeated evaporation of added dry pyridine; the last three times the flask was opened in a drybox in which an anhydrous atmosphere was maintained by phosphorous pentoxide. The molar extinction values in water for the nucleotides are as follows: Cp, 13,000 (280 nm, at pH 2); Ap, 15,100 (260 nm, at pH 2); C^{Bz}-OBz-*p*, 11,000 (304 nm); MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*, 17,600 (304 nm); MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*-A^{Bz}-OBz-*p*, 21,200 (304 nm); A^{Bz}-OBz-*p*, 18,300 (280 nm). The abbreviation OD refers to the extinction of a nucleotidic solution in a 1-ml volume using a 1-cm light-path quartz cell. The wavelength used for this measurement is indicated as a subscript after this abbreviation.

For removal of the N- and O-acyl protecting groups the compounds were treated with a large excess of 15 *N* methanolic ammonia for appropriate hours according to base compositions.[3d] Monomethoxytrityl groups were removed using 80% aqueous acetic acid (1 hr) at 20°.

Pancreatic RNase was purchased from Worthington Biochemical Corp. The stock solution contained 10 mg of enzyme/ml. The unprotected oligonucleotide, 5 OD_{260} units, was digested with 5 μl of the enzyme solution in 0.05 *M* Tris-HCl, pH 7.5 (0.1 ml), for 4 hr at 37°. RNase M was a generous gift from Dr. Irie of the University of Kyoto. The stock solution contained 2 mg/ml of the enzyme and was kept at 4°. For the nonbase-specific cleavage *ca.* 5 OD_{260} units of the oligonucleotide in 0.1 *M* ammonium acetate, pH 6.7 (0.1 ml), was incubated at 37° for 4 hr with 20 μl of the stock solution.

Pyridinium 5′-O-Monomethoxytrityl-N,2′-O-dibenzoylcytidine 3′-Phosphate. This compound was prepared by a procedure similar to that used for the 5′-O-dimethoxytrityl derivative.[5] The reaction temperature was kept at 50° until benzoylation of 2′-hydroxyl group was complete. An aliquot was treated with acetic anhydride and 80% acetic acid for the analysis by paper chromatography to check for the completion of benzoylation on the 2′-hydroxyl group. The yield was 85%, as estimated spectrophotometrically by the use of the value of 12,100 for ϵ_{304}.

Pyridinium N,2′-O-Dibenzoylcytidine 3′-Phosphate. Pyridinium 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidine 3′-phosphate (1 mmol) was treated with 80% acetic acid (20 ml) for 1 hr at room temperature. Acetic acid was removed by coevaporation with water and the residue was made anhydrous by repeated evaporation with added pyridine. The pyridine solution (7 ml) was added to ether (300 ml) under vigorous agitation. The resultant precipitate was collected by centrifugation and washed with ether three times. The yield was almost quantitative. Spectral properties were λ_{max} 236, 262, and 304 nm, λ_{min} 247 and 290 nm, and $\epsilon_{304}/\epsilon_{280}$ = 0.80 in water.

Pyridinium N,2′-O-Dibenzoyladenosine 3′-Phosphate. The compound was prepared by the same method as described above from pyridinium 5′-O-monomethoxytrityl-N,2′-O-dibenzoyladenosine 3′-phosphate.[5] Spectral properties were λ_{max} 233 and 282 nm, λ_{min} 259 nm, and $\epsilon_{304}/\epsilon_{308}$ = 0.23.

N,2′-O-Dibenzoylnucleoside 3′-Aromatic Phosphoramidates. A General Method. Pyridinium N,2′-*O*-dibenzoylnucleoside 3′-phosphate (1 mmol) was allowed to react with the aromatic amine (7 mmol) and DCC (5 mmol) in a mixture of *t*-butyl alcohol (10 ml) and water (2 ml) at 90–100° for 2 hr. After checking the extent to which the reaction had gone to completion by paper electrophoresis, the reaction mixture was concentrated to a gum. The gum was dissolved in 50% aqueous pyridine and extracted with *n*-hexane three times. Cyclohexylurea was removed by filtration. The aqueous pyridine solution was passed through a column (1 × 15 cm) of pyridinium Dowex 50−X2 to remove the carboxyamidine. The solution was rendered anhydrous and the residue was dissolved in pyridine (3 ml). The pyridine solution was immediately added to a mixture of ether (200 ml) and *n*-hexane (100 ml) under vigorous stirring. The precipitate was collected by centrifugation and washed with ether three times. The yield was nearly quantitative. Spectral properties in water of different phosphoramidates were as follows: A^{Bz}-OBz-*p*NHPhOCH$_3$, λ_{max} 235 and 282 nm, λ_{min} 224 and 263 nm; A^{Bz}-OBz-*p*NHPh, λ_{max} 232 and 278 nm, λ_{min} 220 and 264 nm; C^{Bz}-OBz-*p*NHPhOCH$_3$, λ_{max} 235, 259, and 300 nm, λ_{min} 252 and 292 nm; C^{Bz}-OBz-*p*NHPh, λ_{max} 233, 261, and 303 nm, λ_{min} 248 and 293 nm.

5′-O-Monomethoxytrityl-N,2′-*O*-dibenzoylcytidylyl-(3′–5′)-N,2′-O-dibenzoylcytidine 3′-Phosphate (MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*) (VI). Pyridinium N,2′-O-dibenzoylcytidine 3′-phosphoro-*p*-anisidate (1 mmol) and pyridinium 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidine 3′-phosphate (0.72 mmol) were dissolved in 50% aqueous pyridine and passed through a column (1 × 7 cm) of pyridinium Dowex 50−X2. The effluent and washings were made anhydrous by coevaporation with pyridine. The pyridine solution (10 ml) was added to a mixture of ether (200 ml) and *n*-hexane (100

ml). The precipitate was collected by centrifugation and washed with ether three times. The dried precipitate was coevaporated with anhydrous pyridine three times in the presence of pyridinium Dowex 50−X2 (1 g). The residue was allowed to react with DCC (7 mmol) in pyridine (6 ml) under the exclusion of moisture for 4 days at 20°. Aqueous pyridine (50%, 6 ml) was added and the solution was extracted with *n*-hexane. After overnight treatment at room temperature, the aqueous pyridine solution was filtered to remove the urea. The filtrate and washings were evaporated with pyridine and the residue was dissolved in a mixture of pyridine (5 ml) and acetic acid (5 ml). Isoamyl nitrite (1.33 ml) was added and the mixture was kept at 20° for 4 hr. Volatile materials were evaporated and the residue was dissolved in pyridine (5 ml). The nucleotides were precipitated in a mixture of ether (300 ml) and *n*-hexane (100 ml) and collected by centrifugation. The precipitate was dissolved in pyridine and 95% ethyl alcohol (100 ml) and applied to a column (2.7 × 50 cm) of TEAE-cellulose preequilibrated with 95% ethyl alcohol. Elution was carried out using a linear salt gradient of triethylammonium acetate (pH 6.5) in 95% ethyl alcohol (2 l. of 0.05 *M* salt in the mixing vessel and an equal volume of 0.25 *M* salt in the reservoir). The dinucleotide was eluted at salt concentration of 0.18–0.2 *M*. The center of the peak contained 3500 OD_{304} units, 0.2 mmol (28% yield). Treatment with methanolic ammonia gave MMTr-CpCp and the removal of monomethoxytrityl group yielded C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*. The R_f values of these compounds are shown in Table II. Pooled fractions were evaporated and triethylammonium acetate was removed either by precipitation of the anhydrous pyridine solution of the protected nucleotide with ether or extraction of the product with *n*-butyl alcohol from the aqueous buffer solution. Spectral properties of the protected dinucleotide (VI) were λ_{max} 230 (sh), 262, and 304 nm, λ_{min} 249 and 290 nm, and $\epsilon_{304}/\epsilon_{280}$ = 0.81 in ethyl alcohol. Those of CpCp were $\lambda_{max}^{H_2O}$ 265 nm, $\lambda_{min}^{H_2O}$ 235 nm, $\epsilon_{250}/\epsilon_{260}$ = 0.62 in water, and $\lambda_{max}^{pH\ 1}$ 273 nm, $\lambda_{min}^{pH\ 1}$ 237 nm, $\epsilon_{280}/\epsilon_{260}$ = 1.1 at pH 1.

Trinucleotide MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*-A^{Bz}-OBz-*p* (VIII). The pyridinium salt of the dinucleotide VI (3250 OD_{304} units, 0.185 mmol) was allowed to react with pyridinium N,2′-O-dibenzoyladenosine 3′-phosphoranilidate (3110 OD_{280} units) and DCC (2 mmol) in the presence of pyridinium Dowex 50−X2 (0.2 g) in pyridine (1.5 ml) for 6 days at 20°. The aqueous pyridine treatment was as above. The anhydrous mixture was treated with isoamyl nitrite (0.87 ml) in a mixture of pyridine (4 ml) and acetic acid (4 ml) at 20° for 4 hr. After evaporation of the volatile materials the residue was dissolved in pyridine (1.5 ml) and precipitated in ether (100 ml) and *n*-hexane (30 ml). The trinucleotide VIII was isolated by column chromatography using TEAE-cellulose (acetate). The elution conditions and the pattern are shown in Figure 2. The identification of peaks is given in Table I. Peak IV contained almost pure trinucleotide VIII (1090 OD_{304} units, 0.051 mmol). The yield was 28%. The purity of the compound was checked by paper chromatography and electrophoresis with and without protecting groups. The R_f values are given in Table II. Debenzoylation of the trinucleotide yielded MMTr-CpCpAp and a trace of trityl-negative side product. Detritylation of the product (VIII) gave a single spot in paper chromatography and electrophoresis. The completely deprotected trinucleotide CpCpAp (3 OD_{260} units) was hydrolyzed with pancreatic RNase. The ratio of cytidine phosphate and adenosine 3′-phosphate was found to be 2.07:1.00 as estimated spectrophotometrically after paper chromatography in solvent C. Spectral properties of the protected trinucleotide (VIII) were λ_{max} 264, 280 (sh), and 300 (sh) nm, λ_{min} 250 nm, and $\epsilon_{304}/\epsilon_{280}$ = 0.50 in ethyl alcohol. CpCpAp had $\lambda_{max}^{H_2O}$ 264 nm, $\lambda_{min}^{H_2O}$ 234 nm, $\epsilon_{280}/\epsilon_{260}$ = 0.58 in water, and $\lambda_{max}^{pH\ 1}$ 269 nm, $\lambda_{min}^{pH\ 1}$ 236 nm, $\epsilon_{280}/\epsilon_{260}$ = 0.93 at pH 1.

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Studies on Transfer Ribonucleic Acids and Related Compounds. II. A Method for Synthesis of Protected Ribooligonucleotides Using a Ribonuclease

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Abstract: A protected trinucleotide 3′-phosphate, 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidylyl-(3′–5′)-N,2′-O-diacetylguanylyl-(3′–5′)-2′-O-acetyluridine 3′-phosphate, was synthesized using the 2′,3′-cyclic phosphate as protection for the terminal phosphomonoester and the 2′-hydroxyl group. The synthetic steps involved (1) the condensation of uridine 2′(3′)-phosphate with 5′-O-monomethoxytrityl-N,2′-diacetylguanosine 3′-phosphate using dicyclohexylcarbodiimide, (2) acidic removal of the monomethoxytrityl group, and (3) condensation with 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidine 3′-phosphate. The terminal uridine cyclic phosphate was hydrolyzed with pancreatic RNase and the 2′-hydroxyl group was acetylated. The overall yield of the protected trinucleotide from the protected mononucleotide was 10%. Methods for the synthesis of protected guanosine 3′-phosphate using an extraction procedure are described.

Suitably protected ribooligonucleotides are required as key intermediates for chemical synthesis of ribonucleotides. The preceding paper reports the synthesis of a protected trinucleotide with 3′-phosphate using aromatic phosphoramidates as a protecting group for phosphomonoesters.[1] In this paper, another approach, one using a 2′,3′-cyclic phosphate as protection for the hydroxyl and phosphomonoester groups, is used to synthesize the protected trinucleotide 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidylyl-(3′–5′)-N,2′-O-diacetylguanylyl-(3′–5′)-2′-O-acetyluridine 3′-phosphate (MMTr-C^{Bz}-OBz-*p*-G^{Ac}-OAc-*p*-U-OAc-*p*).[2] The trinucleotide, CpGpUp, is the third triplet from the 3′ end of yeast alanine $tRNA_1$.[3] For

(1) E. Ohtsuka, K. Murao, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, 92, 3441 (1970).

(2) The system of abbreviation is the same as described in the preceding paper.[1]