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Studies on Transfer Ribonucleic Acid and Related Compounds. XI.[1] A Comparative Study on Condensing Reagents in Syntheses of Ribooligonucleotides

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Three types of condensation reaction shown in Chart (Scheme 1–3) were carried out. Scheme (1) shows a condensation of the protected mononucleotide with the protected nucleoside to give UpU. Scheme (2) shows a condensation of the mononucleotides and Scheme (3) involves a reaction of the dinucleotide blocks obtained in reaction (1) and (2). Reagents used for the first reaction were: dicyclohexylcarbodiimide, DCC; triphenylphosphine-2,2′-dipyridyldisulfide, Ph_3P–(PyS–)$_2$; diphenylphosphoryl azide, DPPA; diethylphosphoryl cyanide, DEPC; 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline, iBiBDQ: 2,4,6-triisopropylbenzenesulfonyl chloride, TPS; *p*-toluenesulfonic anhydride, Ts_2O. DCC, TPS and Ph_3P–(PyS–)$_2$ gave almost quantitative yield to form the dinucleoside monophosphate. For the second and the third reactions above three activating reagents were used. These experiments showed that DCC seemed to be suitable for the synthesis of the dinucleotide using aromatic phosphoramidates as protection of the terminal phosphate and TPS for block condensation involving the protected UpU and UpUp to yield the tetranucleotide, UpUpUpU.

One of the important problems in the chemical synthesis of ribooligonucleotides is activation of phosphate esters by dehydrating reagents. Dicyclohexylcarbodiimide (DCC) and 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) are used most frequently in ribooligonucleotide synthesis.[3–6] Systematic studies on the block condensation of ribooligonucleotides have not been performed. We intended to find conditions for quantitative activation of ribodinucleotide blocks. In the present paper we report a block condensation of MMTr-U(OBz)-p-U(OBz)-p[7] (V) with U(OBz)-p-U(OBz)$_2$ (III) together with condensation of the mononucleotide, MMTr-U(OBz)-p (I) with U(OBz)-pNHPh (IV) or U(OBz)$_2$ (II) using various condensing reagents.

Condensation of MMTr-U(OBz)-p (I) and U(OBz)$_2$ (II) using Various Activating Reagents

As shown in Chart, condensation of MMTrU(OBz)-p (I) with U(OBz)$_2$ (II) yields a dinucleoside phosphate U(OBz)-p-U(OBz)$_2$ (III), which is a useful intermediate of ribooligonucleotide synthesis. We have studied this reaction using various condensing reagents shown in Table I. TPS was introduced to ribooligonucleotide synthesis as a condensing reagent which gave less sulfonylation of the primary hydroxyl group of nucleosides.[3b] This reagent and DCC are used most frequently also in the deoxyribooligonucleotide synthesis.[8] Recently Ph_3-(PyS-)$_2$

1) Part X: E. Ohtsuka, K. Fujiyama, M. Ohashi, and M. Ikehara, "in preparation."
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3) *a*) R. Lohrmann and H.G. Khorana, *J. Am. Chem. Soc.*, **86**, 4188 (1964); *b*) *Idem, ibid.*, **88**, 829 (1966); *c*) R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, and H.G. Khorana, *ibid.*, **88**, 819 (1966).
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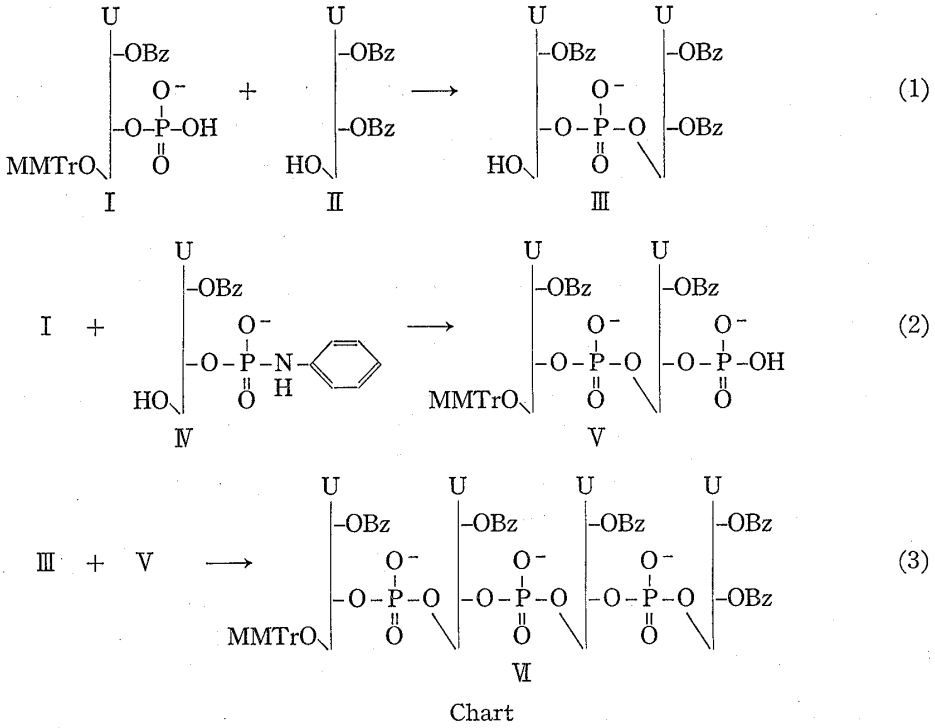
6) E.S. Werstiuk and T. Neilson, *Can. J. Chem.*, **50**, 1283 (1972).
7) MMTr–U(OBz)–p–U(OBz)–p refers to 5′-O-monomethoxytrityl-2′-O-benzoyluridylyl-(3′-5′)-2′-O-benzoyluridine 3′-phosphate. For other abbreviations see ref. 4*a*).
8) H.G. Khorana, *Pure and Appl. Chem.*, **17**, 349 (1968).



Chart

Table I. Reaction Conditions for the Synthesis of U(OBz)-P-U(OBz)$_2$ (III)

Condensing reagent	MMTr-U(OBz)-p (mmoles)	U(OBz)$_2$ (mmoles)	Reagent (mmoles)	Pyridine (ml)	Reaction time (hr)	Yield (%)
DCC	0.42	0.84	5.4	1.5	120	75
Ph_3P-(Pys-)$_2$	0.075	0.13	0.5	1.0	48	65
DPPA	0.07	0.12	0.5	1.0	72	17[a]
DEPC	0.09	0.12	0.5	1.0	72	11[a]
iBiBDQ	0.08	0.18	0.5	1.0	480	2[a]
TPS	0.08	0.12	0.24	0.5	4	80
Ts_2O	0.1	0.13	0.4	0.5	16	0[a]

a) Determined by spectrophotometry.

was shown to be a powerful activating reagent of nucleotides.[9] Diphenylphosphoryl azide (DPPA),[10] diethylphosphoryl cyanide (DEPC)[11] and 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline (iBiBDQ)[12] were reported as peptide reagents. The result of the condensation using these reagents are summarized in Table I. The product was isolated by extraction with organic solvents after detritylation in three cases in which DCC, TPS and triphenylphosphine-2,2′-dipyridyldisulfide (Ph_3P-(PyS-)$_2$) were used. In the other cases the yields indicate the ratio of the product to the starting material. DCC, TPS and Ph_3P-(PyS-)$_2$ gave almost quantitative yields if loss of isolation was considered. In the case of Ph_3P-(PyS-)$_2$, however, the nucleoside recovered after the reaction was modified at the 5′-hydroxyl group. The *Rf* value in paper chromatography and the mobility in paper electrophoresis (Table II) suggested a neutral structure. DPPA and DPPC gave a small amount of the dinucleoside monophosphate (III). With iBiBDQ the favorable reaction occurred to a very small extent and the major product was an isobutyryl ester of the phosphomonoester.

9) T. Mukaiyama and M. Hashimoto, *J. Am. Chem. Soc.*, **94**, 8528 (1972).
10) T. Shioiri, K. Ninomiya, and S. Yamada, *J. Am. Chem. Soc.*, **94**, 6203 (1972).
11) S. Yamada, Y. Kasai, and T. Shioiri, *Tetrahedron Letters*, **1973**, 1595.
12) H. Yajima and Y. Kiso, *Chem. Commun.*, **1972**, 942.

Table II. Paper Chromatography and Paper Electrophoresis

Compound	*Rf* in paper chromatography solvent			*Rm* in paper electrophoresis
	A	B	C	
U	0.40	0.62	0.62	0
Up	0.07	0.20	0.39	1.00
Up cyclic	0.30	0.49	0.60	0.63
UpU	0.11	0.37	0.44	0.48
UpUp	0.03		0.28	1.08
UpUpUpU	0.02		0.21	0.79
MMTr-Up	0.46	0.64	0.84	0.63
MMTr-Up cyclic	0.77	0.78	0.91	0.31
MMTr-UpU	0.58		0.84	0.20
MMTr-UpUp	0.18		0.72	0.80
MMTr-UpUpUpU	0.05		0.59	0.61
U(OBz)-p		0.45		0.89
U(OBz)-p-U(OBz)$_2$		0.73		0.25
U(OBz)-p-U(OBz)-P		0.39		0.71
U(OBz)-p-U(OBz)-p-U(OBz)-p-U(OBz)$_2$				0.60
U(OBz)-p-NHPh		0.76		0.43
MMTr-U(OBz)-p		0.66		
MMTr-U(OBz)-p-U(OBz)-p		0.59		

Table III. Reaction Conditions for the Synthesis of U(OBz)-p-U(OBz)-p

Condensing reagent	MMTr-U(OBz)-p (mmoles)	U(OBz)-pNHPh (mmoles)	Reagent (mmoles)	Pyridine (ml)	Reaction time (hr)	Yield (%)
DCC	0.8	0.48	7	8	96	27
TPS	0.62	0.6	2.9	8	4	16
Ph_3P-(PyS-)$_2$	0.40	0.28	2.5	2	24	22

p-Toluenesulfonic anhydride (Ts_2O) did not give any detectable amount of the product. This was contrary to an experiment of 5′-tritylthymidine and 3′-acetylthymidine 5′-phosphate, in which activation occurred slowly to give 61% of the phosphodiester after 18 hr.[13] Inability of Ts_2O to activate 2′-O-benzoyluridine 3′-phosphate may be due to its bulky structure.

Condensation of MMTr-U(OBz)-p (I) and U(OBz)-pNHPh (IV) using DCC, TPS and Ph_3P-(PyS)$_2$

The reaction scheme (2) is shown in Chart. From the result of the above reaction (Table I), DCC, TPS and Ph_3P-(PyS-)$_2$ were used for the synthesis of the dinucleotide bearing 3′-phosphate (V). The results of the reactions are summarized in Table III. The yiels indicated the amount of the product isolated by chromatography on TEAE-cellulose column. The elution patterns of the chromatography of the product using DCC, TPS and Ph_3P-(PyS-)$_2$ are shown in Fig. 1, 2 and 3. Identification of peaks in Fig. 1, 2 and 3 are shown in Table IV, V and VI, respectively. In all cases the reaction mixtures were rather complex. Among these reactions DCC gave the best yield of the desired product. With TPS further activation of the phosphoramidate could occur during the reaction. As shown in Fig. 3 unchanged mononucleotide (I) is recovered in the reaction using Ph_3P-(PyS-)$_2$. This may be due to comsumption of IV by being modified at the 5′-hydroxyl function. In conclusion complete protection of the phosphomonoester end group may be required to improve the yield of dinucleotides.

13) Unpublished experiments by T. Sugiyama.

Condensation of Dinucleotide Blocks, III and V using DCC, TPS and Ph_3P-$(PyS-)_2$

As shown in scheme (3) the dinucleotides, III and V were condensed with DCC, TPS or Ph_3P-$(PyS-)_2$. The reaction conditions are summarized in Table VII. The elution pattern of chromatography on TEAE-cellulose of the reaction product using TPS is shown in Fig. 4. Peak "d" contained the product (VI). The starting materials III and V were recovered in peak "a" and "c", respectively. Peak "b" contained a pyrophosphate which was tentatively

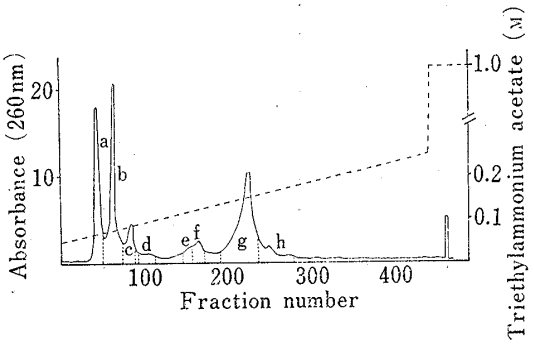


Fig. 1

Chromatography of the products obtained in the synthesis of MMTr-U(OBz)-p-U(OBz)-p (V) using DCC as the condensing reagent on a column (2.8×65 cm) of TEAE-cellulose. Elution was carried out using a linear gradient of triethylammonium acetate in 70% ethanol. The mixing chamber contained 0.05M acetic acid and 0.025M triethylamine (4 liter) and the reservoir contained 0.25M acetate. The identification of peaks is shown in Table IV.

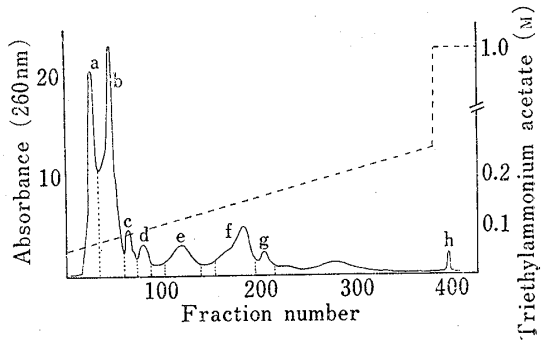


Fig. 2

Chromatography of the dinucleotide (V) synthesized using TPS as the condensing reagent on a column (2.8×54 cm) of TEAE-cellulose. Conditions for elution were as described in Fig. 1. The identification of peaks is shown in Table V.

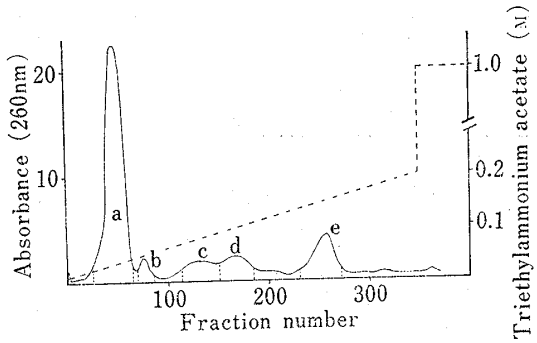


Fig. 3

Chromatography of the dinucleotide (V) synthesized using Ph_3P-$(PyS-)_2$ on a column (2.8×35 cm) of TEAE-cellulose. Elution conditions were as described in Fig. 1 except the total volume of 70% ethanol was 6 liter. The identification of peaks is shown in Table VI.

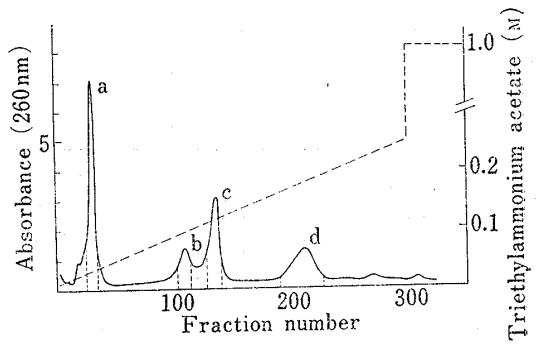


Fig. 4

Chromatography of the tetranucleotide (VI) synthesized using TPS on a column (1.4×43 cm) of TEAE-cellulose. Elution was carried out using a linear gradient of triethylammonium acetate (0.005M—0.25M) in 80% ethanol. The total volume was 6 liter.

TABLE IV. Identification of Peaks in Figure 1

Peak	Fractions pooled	Total A_{260}	Identification
a	40—50	2572	Up cyclic, MMTr-Up cyclic, MMTr-U(OBz)-p
b	57—70	3929	MMTr-U(OBz)-p
c	80—88	613	pyrophosphate of U(OBz)-p
d	94—112	144	pyrophosphate of MMTr-U(OBz)-p
e	147—156	324	U(OBz)-p-U(OBz)-p cyclic. MMTr-U(OBz)-p-
f	157—170	522	U(OBz)-p cyclic, U(OBz)-p-U(OBz)-p
g	190—232	3464	MMTr-U(OBz)-p-U(OBz)-p
h	241—280	576	MMTr-U(OBz)-p-U(OBz)-p+unidentified compounds
	1 M fraction	234	unidentified compounds

Table V. Identification of Peaks in Figure 2

Peak	Fractions pooled	Total A_{260}	Identification
a	23—35	3220	U(OBz)-p, TPS-OH
b	36—58	6650	MMTr-U(OBz)-p
c	61—75	896	pyrophosphate of U(OBz)-p
d	76—89	589	pyrophosphate of MMTr-U(OBz)-p
e	101—135	1425	U(OBz)-p-Up cyclic, MMTr-U(OBz)-p-Up cyclic
f	156—198	2334	MMTr-U(OBz)-p-U(OBz)-p
g	199—218	599	MMTr-U(OBz)-p-U(OBz)-p, unidentified compounds
h		161	unidentified compounds

Table VI. Identification of Peaks in Figure 3

Peak	Fractions pooled	Total A_{260}	Identification
a	27—68	8085	PyS-Up cyclic, U(OBz)-p, unidentified compounds
b	71—88	130	unidentified compounds
c	115—155	615	U(OBz)-p, MMTr-U(OBz)-p
d	156—185	890	MMTr-U(OBz)-p
e	231—260	1520	MMTr-U(OBz)-p-U(OBz)-p

Table VII. Reaction Conditions for the Synthesis of MMTr-U(OBz)-p-U(OBz)-p

Condensing reagent	MMTr-U(OBz)-p-U(OBz)-p (μmoles)	U(OBz)-p-U$(OBz)_2$ (μmoles)	Reagent (μmoles)	Pyridine (ml)	Reaction time (hr)	Yield (%)
DCC	19	37	240	0.2	72	3
TPS	82	39	520	1.0	8	28
Ph_3P-$(PyS\text{-})_2$	17	66	125	0.2	72	9

assigned as a trisubstituted pyrophosphate formed from III and V, since the material from peak "b" gave UpU and MMTr-UpUp cyclic after methanolic ammonia treatment. It is rather unbelievable that this type of compounds survived after prolonged aqueous pyridine treatment. Bulky structures of protected oligonucleotides might affect the rate of hydrolysis of the pyrophosphate. The reaction products in the other two cases were also subjected to chromatography on TEAE-cellulose column. The pyrophosphate, however, was not detected as a distinct peak. The yield of the condensation using DCC was very low compared to the case of thymidine dinucleotide.[14] Oligonucleotides having bulky protecting groups may require powerful activating reagents.

Experimental

Paper chromatography was performed using solvents 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). Paper electrophoresis was performed at 900 V/40 cm using 0.05 M triethylammonium bicarbonate (pH 7.5).

Molar extinction values in water for the nucleotides were as follows: Up, 9,900 (260 nm); MMTr-Up, 12,000 (260 nm); MMTr-U(OBz)-p, 13,000 (260 nm); MMTr-U(OBz)-p-U(OBz)-p, 24,000 (260 nm); U(OBz)-p-(OBz)-p, 23,000 (260 nm). For the protected tetranucleotide, hypochromicity was ignored.

Pyridinium 5′-O-monomethoxytrityl-2′-O-benzoyluridine-3′-phosphate[3c] and 2′,3′-O-dibenzoyluridine[3a] were synthesized with minor modification. Pyridinium 2′-O-benzoyluridine 3′-phosphoranilidate was synthesized as described previously.[4c]

14) H. Kössel, M.W. Moon, and H.G. Khorana, *J. Am. Chem. Soc.*, **89**, 2148 (1967).

Other general methods are as described previously.[4a,c]

General Methods for Condensation of MMTr-U(OBz)-p (I) with U(OBz)$_2$ (II)——The starting materials, I and II were rendered anhydrous by evaporation with pyridine four times. The residue was dissolved in anhydrous pyridine and the condensing reagent was added. After the reaction 50% aqueous pyridine (equal volume to the reaction mixture) was added and the mixture was kept at room temperature for 16 hr. The nucleotides were separated from the activating reagent and its decomposed materials at this stage by extraction with *n*-butanol and precipitation with ether–pentane. The precipitate was treated with 80% acetic acid for 2 hr and the volattile materials were evaporated. The residue was dissolved in *n*-butanol, the mononucleotide, U(OBz)-p, was removed by extraction with 50% aqueous pyridine and the product (III) was freed from the contaminating nucleoside (II) by precipitation with ether.

General Methods for Condensation of MMTr-U(OBz)-p (I) with U(OBz)-pNHPh (IV)——When DCC was used as the condensing reagent the starting materials, I and IV, were dissolved in 50% pyridine separately and passed through columns of pyridinium Dowex 50×2. The nucleotides were precipitated with ether–pentane (3: 2) from its solution in anhydrous pyridine and rendered anhydrous together with pyridinium Dowex 50×2 (equivalent to the anilidate) by evaporation with pyridine. The anion–exchange resin could be omitted in other cases. After the reaction 50% pyridine was added and the mixture was kept at room temperature for 16 hr. The nucleotides were precipitated with ether–pentane from its solution in anhydrous pyridine. Pyridinium arylsulfonate and hydrochloride were removed by extraction with 50% pyridine from butanol solution of the nucleotides. The precipitate was treated with isoamyl nitrite (40—100 fold excess) in pyridine–acetic acid (1: 1) (twice the volume of the nitrite) at room temperature for 4 hr. The volatile materials were evaporated and the residue was added to ether–pentane (3: 2). The sirupy precipitate was dissolved in 70% ethanol (200 ml) and applied to a column of TEAE-cellulose.

General Methods for Condensation of Dinucleotide Blocks——Pyridinium salt of U(OBz)-p-U(OBz)$_2$ (III) and pyridinium salt of MMTr-U(OBz)-p-U(OBz)-p (V) were made anhydrous as described above. The triethylammonium salt of the dinucleotide (V) was used in the case of TBS condensation. After the reaction the mixture was treated with 50% aqueous pyridine and subjected to chromatography on TEAE-cellulose column.

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