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Studies on *t*-RNA's and Related Compounds. IV.[1] A Simple Method for the Synthesis of Ribotrinucleotides

Eiko Ohtsuka, Haruko Tagawa and Morio Ikehara

Faculty of Pharmaceutical Sciences, Osaka University[2]

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A trinucleoside diphosphate, GpUpA was synthesized using a dinucleotide 2′,3′-cyclic phosphate as an intermediate. The synthetic approach involved a condensation of N,2′,5′-O-triacetylguanosine 3′-phosphate (I) and uridine 2′(3′)-phosphate (II) to yield the dinucleotide with 2′,3′-cyclic phosphate (III) (Chart 1). Pancreatic RNase treatment of the product was followed by acetylation of the 2′-hydroxyl group of uridine to give the protected dinucleotide (VII). The overall yield from the mononucleotide was 41%.

The unfractionated dinucleotide was used for the condensation with the protected nucleoside bearing the 5′-hydroxyl group (VIII). The isolated yield of the unprotected product was 12% from the dinucleotide.

Previously we reported the synthesis of protected ribotrinucleotides which could be used for further condensation reactions to obtain longer oligonucleotides.[3–5] A dinucleotide such as MMTr-G^{Ac}-OAc-p-Up-cyclic[6] was used as an intermediate for the synthesis of trinucleotide MMTr-C^{Bz}-OBz-p-G^{Ac}-OAc-p-U-OAc-p after selective removal of the trityl group.[5] Söll and Khorana[7] described the synthesis of dinucleotides without specific protection of the 5′-hydroxyl group. A fully acetylated dinucleotide such as Ac-G^{Ac}-OAc-p-U-OAc-p was thought to be an intermediate for the condensation with nucleosides or nucleotides bearing free 5′-hydroxyl groups.

Although a general method for the synthesis of ribotrinucleotides without phosphate end groups was achieved to give all the possible trinucleotides from four major nucleosides,[8] properly protected dinucleoside monophosphate had to be purified by ion exhcange chromatography. In this paper it is reported that a trinucleotide, GpUpA was synthesized without column chromatography. The synthetic approach is shown in Chart 1. The condition of the first condensation was essentially as described previously.[5,7]

N, 2′,5′-O-Triacetylguanosine 3′-phosphate[9] (I) and uridine 2′(3′)-phosphate (II) were condensed using dicyclohexylcarbodiimide (DCC) to give III. It has been reported that five membered cyclic phosphates react further with DCC to yield phosphorylureas, which can be degraded by treatment with acid.[10] The phosphorylurea (IV) was formed as a major side product. To decompose IV to V the mixture was treated with acidic ion exchange resin Dowex 50. Since this treatment cleaved the 2′,3′-cyclic phosphate of III to give also V, the

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6) The system of abbreviation is same as described in ref. 4. MMTr-G^{Ac}-OAc-p-Up-cyclic refers to 5′-O-monomethoxytrityl-N,2′-O-diacetyl-guanylyl-(3′-5′)-uridine 3′-cyclic phosphate.
7) D. Söll and H.G. Khorana, *J. Am. Chem. Soc.*, **87**, 350 (1965).
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10) *a*) C.A. Dekker and H.G. Khorana, *J. Am. Chem. Soc.*, **76**, 3522 (1954); *b*) G.M. Tener and H.G. Khorana, *ibid.*, **77**, 5348 (1955); *c*) H.G. Khorana, G.M. Tener, R.S. Wright and J.G. Moffatt, *ibid.*, **79**, 430 (1957).

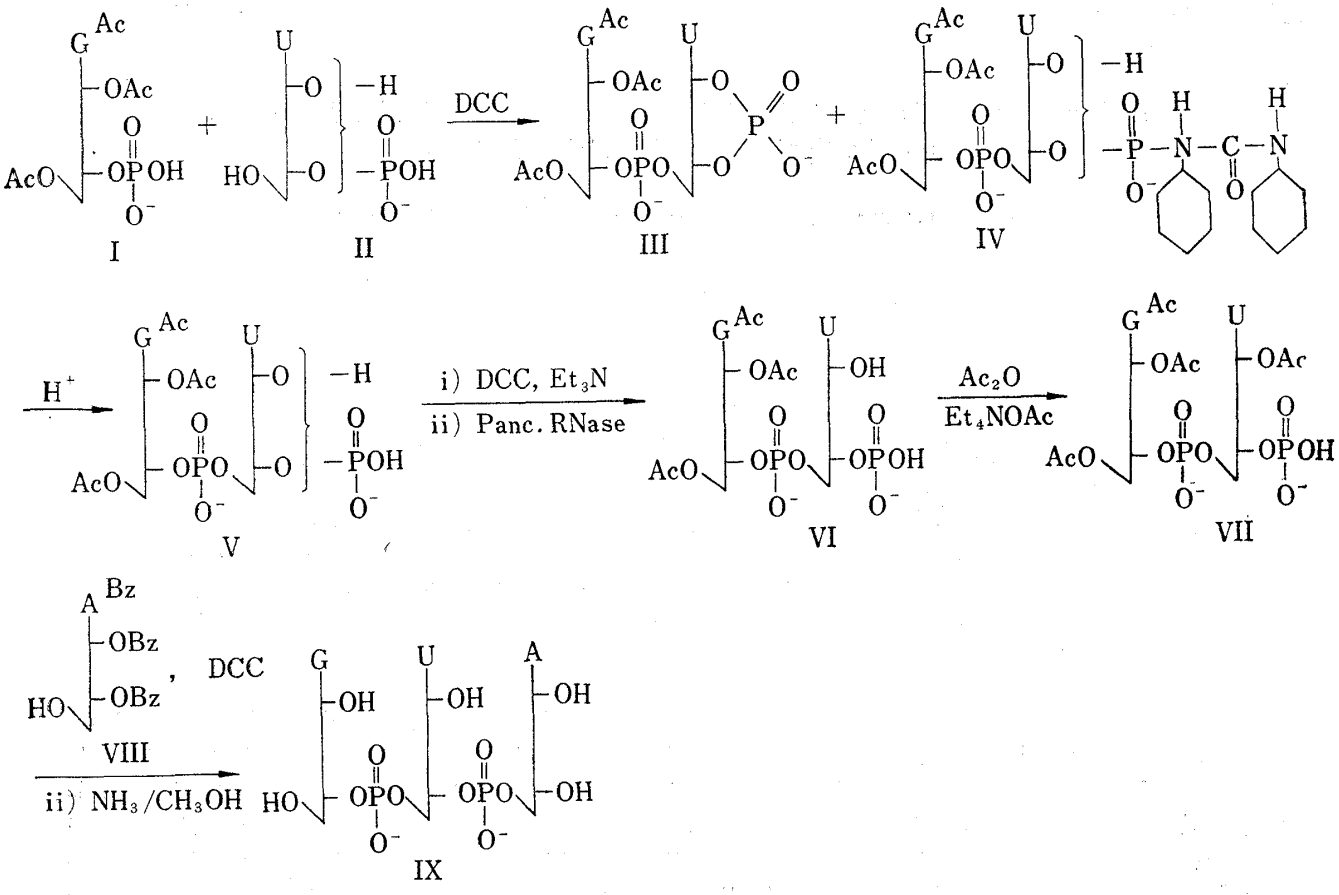

Chart 1

mixture was treated with DCC in the presence of the trialkylamine for cyclization. The unchanged starting material II yielded uridine 2′,3′-cyclic phosphate by this treatment. Cyclic phosphates were hydrolyzed enzymatically to give 3′-phosphates (*e.g.* VI). Paper chromatography of the reaction mixture showed very little starting materials. It was decided to use the mixture in the condensation with tetrabenzoyladenosine (VIII) since I and acylated II react only with the nucleoside to give the side products which could be separated from the final product.

The 2′-hydroxyl group was acylated with acetic anhydride in the presence of tetraethylammonium acetate.[11] The acylation of the mixture yielded mainly VII contaminated with I and 2′,5′-di-O-acetyluridine 3′-phosphate. The overall yield of VII was 41%.

For the synthesis of the trinucleotide the acylated mixture was allowed to react with tetrabenzoyladenosine (VIII) using DCC as condensing reagent. The product was isolated by paper chromatography and paper electrophoresis after removal of the protecting groups. *Rf* values of the unprotected trinucleotide IX are shown in Table I. The yield of the purified product was 12% from VII. The product was characterized using RNase M[12] and found to give Gp, Up and adenosine in the expected ratio.

Although this method does not require the specific protection of mononucleotide at the 5′-hydroxyl group, cautions have to be taken to prevent non-enzymatic hydrolysis of the terminal 2′,3′-cyclic phosphate of the dinucleotide intermediate. The extension of this method to the synthesis of trinucleotides with adenylic acid or cytidylic acid at the middle position is possible if one uses the selective N-debenzoylation with hydrazine.[13] Because

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Table I. Properties of Different Nucleotides in Paper Chromatography and Paper Electrophoresis

Compounds	Paper chromatography			Paper electrophoresis[a] (pH 7.5)
	Solvent A	B	C	
Up	0.21	0.23	0.33	1.00
Gp		0.12	0.18	0.90
Adenosine			0.62	
Ac-G^{Ac}-OAc-p	0.25	0.55		0.91
Ac-G^{Ac}-OAc-p-Up	0.11	0.23		0.95
Gp Up	0.07	0.11	0.11	1.06
Ac-G^{Ac}-OAc-Up>		0.50		0.68
Gp Up>		0.15	0.19	0.68
Ac-G^{Ac}-OAc-p-U-OAc-p		0.46		0.95
Gp Up A			0.16	0.64

a) Relative mobility to Up was shown.

N-protocted nucleoside 2′,3′-cyclic phosphates resist to nucleases,[5] selective deprotection of amino groups or reprotection of 2′-hydroxyl groups of dinucleotide was required for the enzymatic hydrolysis of the terminal phosphate of the intermediate. Condensation of VII with N,2′-O-protected nucleoside phosphoranilidates[3,4] would give another route for the synthesis of trinucleotides with 3′-phosphates.

The activation of the dinucleotide with DCC in the condensation reaction with nucleosides may not be advantageous compared to the reaction between mononucleotides and dinucleotide phosphates. A use of arenesulfonyl chloride would accelerate the rate and the extent of the reaction. Investigations along this line is in progress.

Experimental

General Methods——Paper chromatography was performed by the descending technique in the solvent systems: A, isopropanol–conc. ammonia–water (7:1:2, v/v); B, ethanol–1M ammonium acetate, pH 7.5 (7:3, v/v); C, *n*-propanol–conc. ammonia–water (55:10:35, v/v). Paper electrophoresis was performed at 900v/40 cm using 0.05M triethylammonium bicarbonate, pH 7.5. Other methods are as described previously.[4,5]

Ac-G^{AC} OAc-p-U-2′(3′)-p (V)——Pyridinium N, 2′,5′-O-triacetylguanosine 3′-phosphate (0.41 mmole) and pyridinium uridine 2′(3′)-phosphate (0.44 mmole) were passed through a column (1×10 cm) of ion exchange resin Dowex 50×2 (pyridinium form). The effluent and washings were evaporated with added pyridine and the residue was made anhydrous together with pyridinium Dowex 50×2 (330 mg) by coevaporation with pyridine three times. The mixture was allowed to react with DCC (6.6 mmoles) in pyridine (6.6 ml) for 7 days. Water (15 ml) was added and the mixture was extracted with cyclohexane (22 ml) three times. The solution was filtered to remove the urea. After 16 hr the solution was evaporated to dryness and the residue was dissolved in 50% ethanol (105 ml). The mixture was treated with acidic Dowex 50 (55 g in wet) under shaking. Paper electrophoresis at this stage showed no phosphorylureas. *Rf* values were listed in Table I.

Recyclization of the Terminal 2′(3′)-Phosphates and Hydrolysis of the Cyclicphosphates with Pancreatic RNase to Yield (VI)——N, 2′,5′-O-Triacetylguanylyl-(3′-5′)-uridine 2′,3′-cyclic phosphate was rendered anhydrous by repeated evaporation of added pyridine and the nucleotides were treated with DCC (1 mmole) for 3 days at room temperature. Water (7 ml) was added and the mixture was extracted with *n*-hexane. The aqueous solution was filtered and evaporated with 1M triethylammonium bicarbonate to prevent nonenzymatic hydrolysis of the terminal cyclic phosphate. The residue was dissolved in 20% aqueous DMF containing 0.1M triethylammonium bicarbonate, pH 7.5 (20 ml), and treated with pancreatic RNase (8 mg) for 4 hr. After the hydrolysis being checked by paper chromatography and electrophoresis, the solution was passed through a column (1.5×25 cm) of pyridinium Dowex 50×2 preequilibrated with 20% aqueous DMF containing 0.05M pyridinium acetate to remove enzyme. The effluent and washings were evaporated with pyridine. Properties in paper chromatography and electrophoresis were summarized in Table I.

Acetylation of the 2′-Hydroxyl Group to Yield VII——The residue from above experiment, containing the dinucleotide (0.08 mmole), and tetraethylammonium acetate (1 mmole) was rendered anhydrous by evaporation with pyridine for several times. The residue was coevaporated with toluene four times to

remove pyridine. Acetic anhydride (0.4 ml) was added to the mixture and kept for 4 days at room temperature. A mixture of methanol and pyridine (4:1, v/v, 10 ml) was added to the mixture and after 1 hr cold aqueous pyridine (10%, 20 ml) was added. The solution was passed through a column of pyridinium Dowex 50×2 resin (2×25 cm). The effluent and washings were evaporated after the solution was kept at room temperature for 2 hr. Volatile materials were evaporated with added pyridine and the residue was dissolved in pyridine (7 ml) and DMF (1 ml). The solution was centrifuged to remove insoluble materials. The supernatant was added to ether (200 ml). The precipitate was collected by centrifugation and washed with ether three times. The yield of the protected dinucleotide was 4460 optical density $(OD)_{260}$ units, 41% from the mononucleotide. *Rf* values of the protected and unprotected dinucleotides are shown in Table I. The spectral properties of the protected dinucleotide were $\lambda_{max}^{H_2O}$ 258 nm; $\lambda_{min}^{H_2O}$ 231 nm. $\varepsilon_{280/260}=0.56$.

The Trinucleotide, GpUpA (IX)——Pyridinium salt of VII (1600 OD_{260} units, 0.058 mmole) ane N,N′, 2′,3′-O-tetrabenzoyladenosine (VIII) (62 mg, 0.09 mmole) were made anhydrous by evaporating with added pyridine in the presence of pyridinium Dowex 50×2 (50 mg). The mixture was treated with DCC (0.1 mmole) in pyridine (1 ml) and DMF (0.2 ml) for 5 days in a dark place at room temperature. Water (2.5 ml) was added and the mixture was extracted with *n*-hexane (15 ml, 3 portions). The insoluble materials were removed by filtration. After 18 hr the solution was concentrated to a small volume and treated with methanolic ammonia. The trinucleotide GpUpA was separated from starting materials by paper electrophoresis. The *Rf* values of the product were shown in Table I. The product was contaminated with guanosine 2′(3′)-cyclic phosphate at this stage. The eluant from the corresponding spot from the paper electrophoreogram was applied to preparative paper chromatography in solvent C. The trinucleotide GpUpA was eluted with water by ascending technique; namely the compound was eluted from the filter paper after being collected to the top of the paper. The yield was 202 OD_{260} units, 0.0067 mmole, 12%. The spectral properties of GpUpA were $\lambda_{min}^{H_2O}$ 258 nm; $\lambda_{min}^{H_2O}$ 230 nm and $\varepsilon_{280/260}=0.43$.

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