[Chem. Pharm. Bull. 19(12)2567—2571(1971)] UDC 547.963.32.04.057 : 547.341.04

A Convenient Synthesis of Diribonucleoside Monophosphates by the Use of Unblocked Nucleosides[1]

KAZUNOBU MIURA and TOHRU UEDA

Faculty of Pharmaceutical Sciences Hokkaido University[2]

(Received June 12, 1971)

A convenient modified method for the preparation of ribonucleotidyl-(2′—5′) and (3′—5′)-ribonucleoside by employing unblocked ribonucleoside was developed. Particularly, this method is useful for the synthesis of diribonucleoside monophosphates (XpA or XpG) involving adenosine or guanosine in 2′ or 3′ OH end.

Recently extensive studies of chemical modification of nucleic acids, namely transfer ribonucleic acids, have been done for elucidating the secondary or tertiary structure in relation to their biological activities.

In connection with the above mentioned studies the modification of shorter oligonucleotides such as diribonucleoside monophosphates and triribonucleoside diphosphates will be valuable since even diribonucleoside monophosphates and triribonucleoside diphosphates possess ordered structure in some extent and the analysis of the site of modification and of its effect for physicochemical properties including hydrogen–bonding or base–stacking would be rather easily performed.

This paper describes a convenient modified procedure for the synthesis of ribonucleotidyl-(2′-5′) and (3′-5′)-ribonucleoside by Michelson's method[3] in relatively large quantities serving for the chemical modifications and the survey of their physicochemical properties.

One of the key point of this method is the use of unblocked ribonucleoside in place of suitably protected nucleoside, which is one of the essential advantage in obtaining a larger quantity of oligonucleotides.

Honjo and his co-workers have reported[4] that the treatment of a nucleoside with pyrophosphoryl chloride in *o*-chlorophenol, acetonitrile or ethylacetate at 0—10° afforded the 5′-monophosphate in an excellent yield as the sole nucleotide. Their method is unique in that the reaction is carried out without base (or acid acceptor) such as pyridine or tri-*n*-butylamine. From this finding it would have been expected that ribonucleotidyl-(2′-5′) and (3′-5′)-ribonucleosides could be obtained by the selective phosphorylation of 5′-hydroxyl group of unblocked ribonucleoside with the activated ribonucleoside 2′,3′-cyclic phosphate.

Tri-*n*-butylammonium 5′-0-acetylribonucleoside 2′,3′-cyclic phosphate(I) was treated with triisopropylbenzenesulfonyl chloride (TPS) in N,N-dimethylformamide(DMF) for ten minutes under shaking and the ribonucleoside(II) was added. After 2 hours the product(III) was deacetylated by methanolic ammonia to give the desired diribonucleoside monophosphates(IV). The results of this condensation reaction are summarized in Table I.

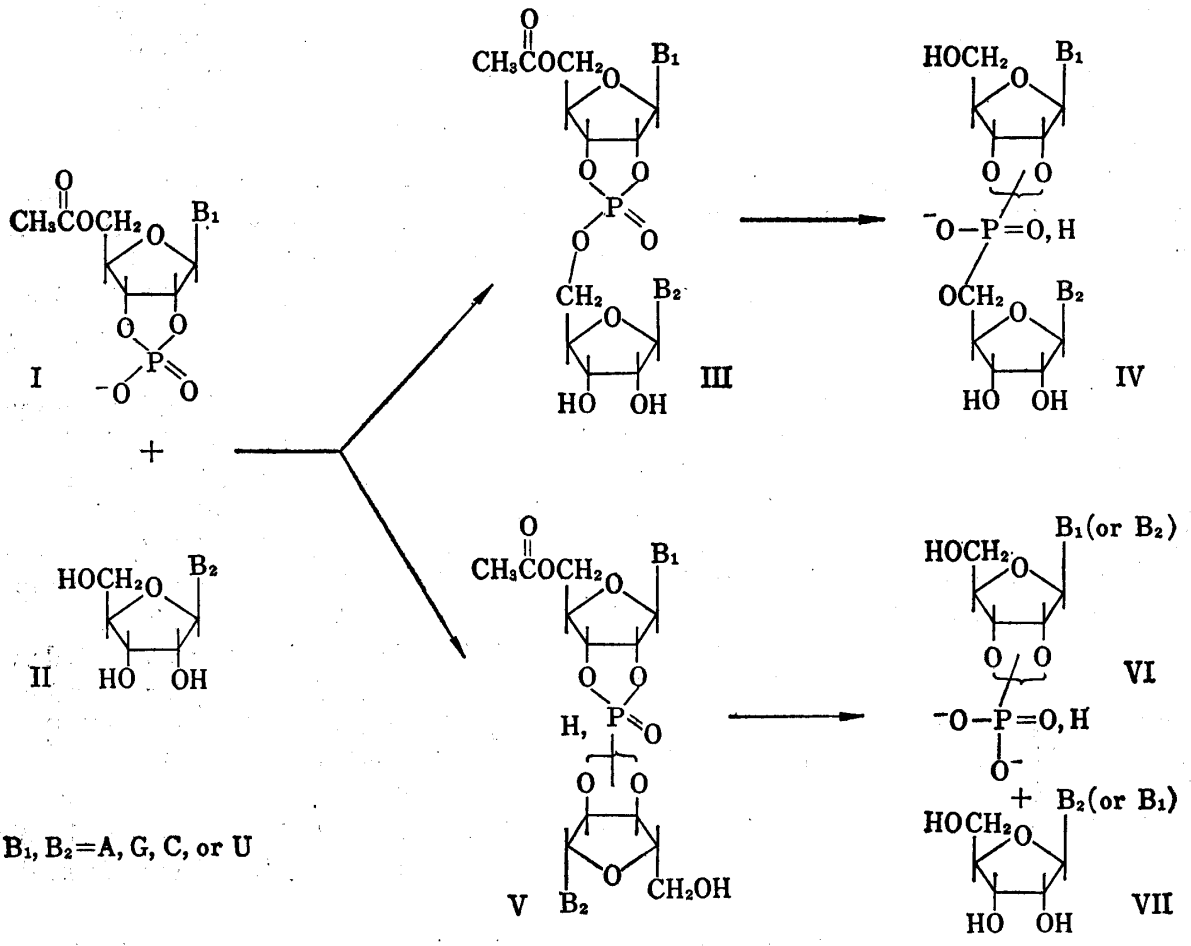
As shown in Table I ApA and ApG[5] were obtained in a good yield, however, the diribonucleoside monosphophates with pyrimidine nucleoside at the 2′(3′) end were formed in

1) Presented at the 89th Annual meeting of Pharmaceutical Society of Japan at Nagoya, April 1969.
2) Location: *Kita-12, Nishi-6, Sapporo.*
3) A.M. Michelson, *J. Chem. Soc.*, **1959**, 3655.
4) K. Imai, S. Fujii, K. Takanohashi, Y. Furukawa, T. Masuda and M. Honjo, *J. Org. Chem.*, **34**, 1547 (1969).
5) Abbreviation are used for ribonucleotidyl-(2′(3′)—5′)-ribonucleoside as XpY; X,Y=A (adenosine), G (guanosine), C (cytidine), and U (uridine).

Table I. Preparation of Diribonucleoside Monophosphates

Acetylated nucleoside 2′,3′-cyclic phosphate	Nucleoside	Solvent	Product XpY[b]	Yield (%)	By-product[a]
Adenosine	adenosine	DMF	ApA	80	—
	guanosine	DMF	ApG	70	—
	cytidine	DMF	ApC	40	adenosine
	N^4-benzoylcytidine	DMF	ApC	50	—
	uridine	DMF	ApU	30	adenosine
	2′,3′-di-O-acetyluridine	pyridine–dioxan	ApU	50	—
Uridine	adenosine	DMF	UpA	80	—
	adenosine	DMF-pyridine	UpA	45	uridine
	adenosine	DMF-$(n\text{-Bu})_3N$[b]	UpA	80	uridine
	guanosine	DMF	UpG	50	—
	cytidine	DMF	UpC	20	uridine
	N^4-benzoylcytidine	DMF	UpC	45	—
	uridine	DMF	UpU	20	—

a) The by-product was identified spectrophotometrically on a paper chromatogram.
b) abbreviation to tri-*n*-butylamine



rather low yield. In the latter case the nucleoside corresponding to the original ribonucleoside 2′,3′-cyclic phosphate was detected in the reaction mixture together with the starting nucleoside. This is explained by the intermediary formation of a ribonucleotidyl-(2′(3′)-2′(3′))-ribonucleoside(V) which would be degraded to the nucleotide(VI) and nucleoside(VII) in

either way. This observation has been already reported by Follmann[6] in the reaction of acetylated nucleoside 3'-phosphate and unblocked ribonucleoside in pyridine. This type of by-product was also detected during the reaction of 5'-0-acetyluridine 2',3'-cyclic phosphate with adenosine in the presence of excess tri-*n*-butylamine or pyridine in DMF. The yield of diribonucleoside monophosphate of type XpC could be increased by the use of N^4-benzoylcytidine in place of cytidine. The by-product was not detected in this case. The use of 2',3'-di-0-acetyluridine in place of uridine improved the yield of ApU.

From these findings it can be concluded that the selectivity of the phosphorylation of unblocked nucleoside decreased if the base (or acid acceptor) such as pyridine or tri-*n*-butylamine is present in the reaction medium. Secondly, the reaction involving pyrimidine nucleoside proceeds with less selectivity than that of purine nucleoside. Furthermore, it is interesting that the protection of amino group of adenosine, guanosine and cytidine is not essential in this modified method. Reese and his co-workers have discussed about the protection of amino group of adenosine.[7]

For the purpose of the simplification of the synthesis of diribonucleoside monophosphates, the acetylated mononucleotide obtained from nucleoside 2'(3')-phosphate with acetic anhydride in pyridine, that is, the mixture of 5'-0-acetylnucleoside 2',3'-cyclic phosphate and 2'(3')5'-di-0-acetylnucleoside 3'(2')-phosphate[8] was used as a starting nucleotide. From acetylated adenosine monophosphate and adenosine, adenylyl-(2'-5') and (3'-5')-adenosine were prepared in a yield of 65% based on adenosine 2'(3')-phosphate.

As shown in Fig. 1 and 2 the mixture of 2'-5' and 3'-5' linked isomers could be separated on a DEAE-cellulose column. The separation of guanosine 2'- and 3'-benzyl phosphate on a DEAE-cellulose column has been reported by Egami and his co-worker.[9]
The structure assignment of dinucleoside monophosphate isomers was performed by alkaline hydrolysis, enzymic hydrolysis (Ribonuclease M from *Aspergillus saitoi*[10]) and ultraviolet (UV) absorption spectral measurement.

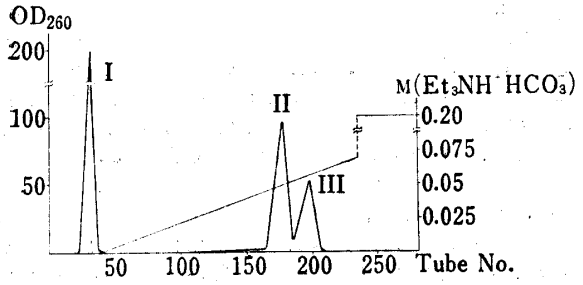


Fig. 1. DEAE-Cellulose Column Chromatography of the Products of the Reaction of Acetylated Uridine 2',3'-Cyclic Phosphate and Adenosine with TPS

column size: 3.3×55 cm, 1 fraction: 20 ml

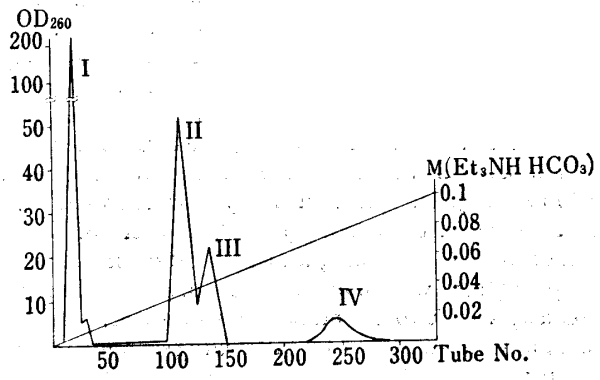


Fig. 2. DEAE-Cellulose Column Chromatography of the Products of the Reaction of Acetylated Adenosine 2',3'-Cyclic Phosphate and 2',3'-Di-O-acetyl Uridine with TPS in Pyridine and Dioxan

coloumn size: 2.7×55 cm, 1 fraction: 20 ml I (No. 10—39): uridine, II (No. 86—123): adenylyl-(2'—5')-uridine, III (No. 124—156): adenylyl-(3'—5')-uridine and a small amount of adenosine 2', 3'-cyclic phosphate, IV (No. 220—280): adenosine 2'(3')-phosphate

6) H. Follmann, *Tetrahedron Letters*, **1967**, 2113.
7) B.E. Griffin, M. Jarman and C.B. Reese, *Tetrahedron*, **24**, 639 (1968).
8) D.H. Rammler and H.G. Khorana, *Biochem. Biophys. Res. Commun.*, **7**, 147 (1962).
9) S. Sato and F. Egami, *Biochem. Z.*, **342**, 437 (1965).
10) M. Irie, *J. Biochem.*, **62**, 509 (1967).

Experimental

The Preparation of 5′-O-Acetylnucleoside 2′,3′-cyclic Phosphate——To a solution of tri-*n*-butylammonium nucleoside 2′,3′-cyclic phosphate, which was prepared from 1 mmole of nucleoside 2′(3′)-phosphate by Khorana's method[11] where tri-*n*-butylamine was used in place of triethylamine, in DMF (3 ml) and dioxan (3 ml) was added acetic anhydride (0.5 ml) and tri-*n*-butylamine (1.45 ml) and allowed to stand at 37° for 2—3 days. The solvent was removed under reduced pressure and the residue was dissolved in water (50 ml), the mixture was subjected to ether extraction to remove excess of the amine. The water layer was concentrated and the residue was coevaporated with DMF (until it had lost odor of amine) and dried at 80° under reduced pressure. The dried gummy residue was used to the reaction without further purification.

In the case of adenosine 2′,3′-cyclicphosphate, acetylation afforded two products, 5′-O-acetyladenosine 2′,3′-cyclic phosphate and N^6-acetyl 5′-O-acetyladenosine 2′,3′-cyclic phosphate, which were identified with an authentic sample by paper chromatography and UV absorption measurements (Table II).

Table II. Paper Chromatography and Ultraviolet Spectra of Acetylated Nucleoside 2′,3′-Cyclic Phosphates

	Rf-value	UV spectra max. (mμ)
Uridine 2′,3′-cyclic phosphate	0.47	260
Acetylated uridine 2′,3′-cyclic phosphate	0.61	260
Adenosine 2′,3′-cyclic phosphate	0.34	260
Acetylated adenosine 2′,3′-cyclic phosphate	0.50 and 0.60	260 and 271

solvent system; EtOH: 1M NH_4OAc (5: 2)

The Synthesis of Uridylyl-(2′—5′) and (3′—5′)-Adenosine, and General Procedure for Diribonucleoside Monophosphate——To the solution of tri-*n*-butylammonium 5′-O-acetyluridine 2′,3′-cyclic phosphate (prepared from 1 mmole of uridine 2′(3′)-phosphate) in 5 ml of DMF was added TPS (3 mmole) and kept for 10 min, and adenosine (2 mmole) was added. The mixture was stirred until the solution became clear (for about 20 min), and allowed to stand at room temperature for 2 hr in anhydrous condition. To the reaction mixture was added water (5 ml) and tri-*n*-butylamine (2 ml) and kept for 30 min at room temperature to decompose TPS and nucleotide triester. More water (45 ml) was added and the mixture was subjected to ether extraction and water layer was concentrated to dryness. The dried residue was treated with methanolic ammonia (100 ml) overnight at room temperature and the solution was evaporated. The residue was dissolved in water and the solution was applied to a column (3.3 × 55 cm) of DEAE-cellulose in a HCO_3^- cycle (see Fig. 1). Elution was performed with the use of a linear gradient concentration of triethylammonium hydrogen carbonate, pH 8.0, (2 liters of water in the mixing chamber and 2 liters of a 0.07M buffer solution in the reservoir), and each 20 ml fraction was collected. The fractions corresponding to dinucleoside phosphate were combined, evaporated under reduced pressure and the residue was coevaporated subsequently with two 100 ml portions of water. Fractions (No. 166—183, II, 10100 OD units at 260 mμ) contained a mixture of two UV abosrbing components with *Rf*'s, 0.47 and 0.28 (solvent system; EtOH: 1M NH_4-OAc (5: 2), ascending). The compound with higher *Rf*-value was probably uridine 2′,3′-cyclic phosphate. The yield of uridylyl-(2′—5′)-adenosine was 40% based on the determination by the OD unit of two compounds. For further purification an aliquot of this fraction was applied to a column of Dowex-1 (formate form).[12] Fractions (No. 185—206, III) contained uridylyl-(3′—5′)-adenosine (8400 OD units, 36%, *Rf*-value, 0.28; EtOH: 1M NH_4OAc (5: 2)).

The yields of other diribonucleoside phosphate listed on Table I were determined spectrophotometrically on paper chromatogram based on the starting nucleotide. The chromatogram of ApU in the reaction of acetylated adenosine 2′,3′-cyclic phosphate (prepared from 2 mmole of adenosine 2′(3′)-phosphate) with 2′,3′-di-O-acetyluridine (3 mmole) and TPS (4 mmole) in pyridine (2 ml) and dioxan (3 ml) is shown in Fig. 2.

Acetylation of Adenosine 2′(3′)-Phosphate and the Synthesis of Adenylyl-(2′—5′) and (3′—5′)-Adenosine——A solution of adenosine 2′(3′)-phosphate (0.1 mmole) and acetic anhydride (0.45 ml) in pyri-

11) H.G. Khorana, "*Methods in Enzymology*," *Vol. VI*, ed. by S.P. Colowick, and N.O. Kaplan, Academic Press, New York, N.Y., 1963, p. 649.

12) T. Ukita, M. Irie, M. Imazawa, Y. Furuichi, H. Nishimura and T. Sekiya, *Seikagaku*, **40**, 363 (1968).

dine (2.5 ml) was allowed to stand at 80° for 3 hr. Methanol (1 ml) was added to the reaction mixture and allowed to stand at room temperature for 2 hr. Solvent was removed to dryness azeotropically with DMF and dried at 60° under reduced pressure. This residue was dissolved in DMF (0.5 ml) and TPS (0.3 mmole) was added to the solution. The solution was stirred at room temperature for 10 min, and adenosine (0.2 mmole) was added. The mixture was treated as described above. Adenylyl (2′—5′) and (3′—5′)-adenosine were obtained in 65% yield.