Studies of Nucleosides and Nucleotides. XXIX.[1] Direct Synthesis of Nucleoside-2′,3′ Cyclic Phosphates

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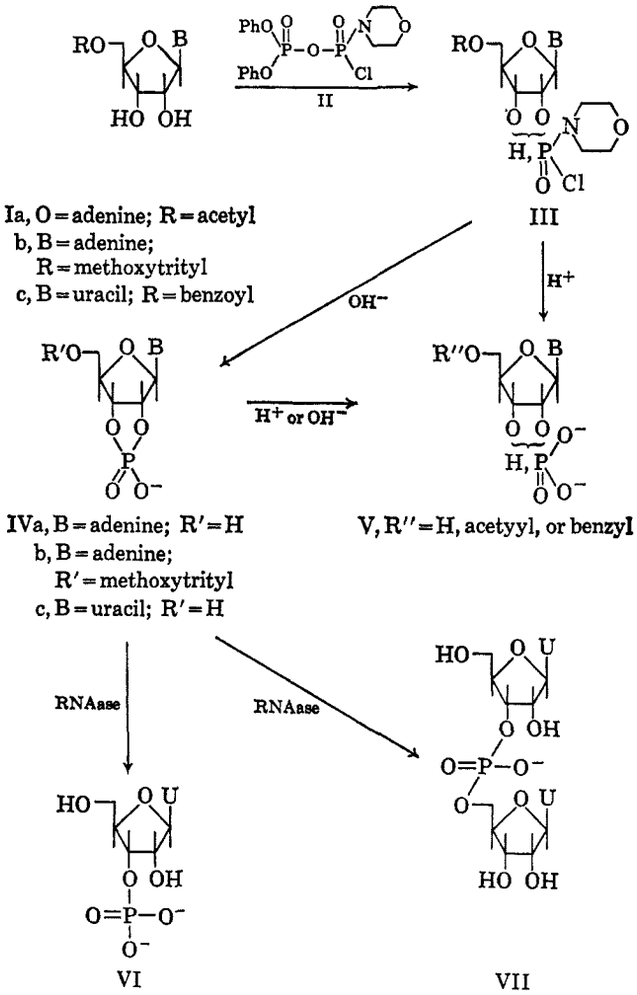
Starting from 5′-O-protected adenosine and uridine, the 2′,3′ cyclic phosphates were synthesized by the simple procedure using P¹-diphenyl P²-morpholinopyrophosphorochloridate as the phosphorylating agent followed by the mild alkaline treatment. Purification of these cyclic phosphates by chromatography on ECTEOLA- and DEAE-cellulose columns gave 2′,3′ cyclic phosphates in 40% yields.

Ribonucleoside-2′,3′ cyclic phosphates have been investigated extensively because of their importance as intermediates in the biological and chemical hydrolysis of ribonucleic acid.[2,3] The use of cyclic phosphates as intermediates for the enzymatic and chemical synthesis of polynucleotides has also been reported.[4-6] The chemical synthesis of the ribonucleoside-2′,3′ cyclic phosphates has been achieved through the action of such diverse condensing agents as dicyclohexylcarbodiimide,[7] ethyl chlorocarbonate,[8] or trifluoroacetic anhydride[9] on mixtures of corresponding 2′- and 3′-phosphates. The synthesis of 3′,5′-cyclic phosphate by the cyclization of monoesterified nucleotide by nucleophile has also appeared in the literature.[10]

In the course of our study of 9-β-D-glucopyranosyl adenine 6′-phosphoromorpholidate[11] and β-D-glucopyranosyl phosphoramidate,[12] the phosphoramidate groups readily reacted with closely situated sugar hydroxyl groups with the formation of cyclic phosphates on alkaline treatment. In this report we describe the conversion of the 2′- and 3′-phosphoromorpholidates of adenosine and uridine to the corresponding 2′,3′ cyclic phosphates.

In order to synthesize the phosphoromorpholidate of ribonucleosides, P¹-diphenyl P²-morpholinopyrophosphorochloridate (DMPC)[13] (II) was employed (Scheme I). The use of this phosphorylating agent in the synthesis of various ATP analogs was reported previously from this laboratory.[11,14-17] When 5′-O-acetyladenosine[18] (Ia) or 5′-O-monomethoxytrityladenosine[19] (Ib) was phosphorylated in dioxane with 2 equiv of DMPC in the presence of 2,6-lutidine as

SCHEME I



the acid acceptor, a mixture of the 2′- and 3′-phosphoromorpholidate was obtained in 60–90% yield. The structure of phosphoromorpholinochloridate (III), which is expected to be formed initially, could not be confirmed either by paper chromatography or electrophoresis, because of the rapid hydrolysis of chloridate. Subsequent treatment of the 2′- or 3′-phosphoromorpholidate (III) with dilute aqueous alkali (0.01–0.1 *N* OH^-) gave adenosine-2′,3′ cyclic phosphate (IVa) and its 5′-methoxytrityl derivative IVb, respectively. The purification of the cyclic phosphates was achieved by chromatography on an ECTEOLA- or a DEAE-cellulose column. A typical pattern of the chromatography is shown in Figure 1. The isolated yield was 52% for adenosine-2′,3′ cyclic phosphate and 36% for the 5′-methoxytrityl derivative.

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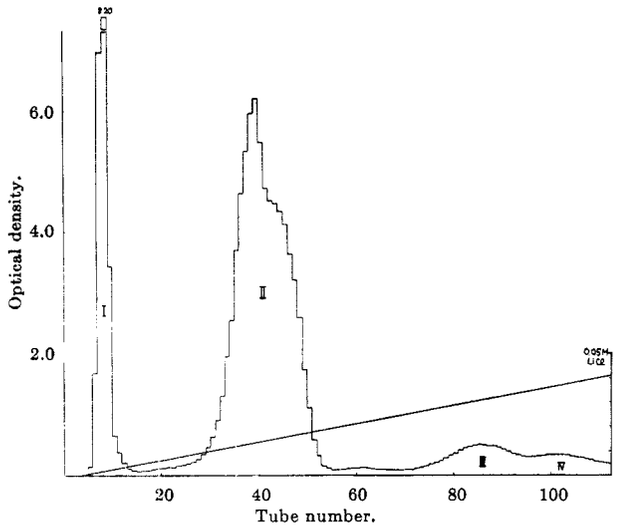

Figure 1.—Ion-exchange chromatography of the synthesis of adenosine-2′,3′ cyclic phosphate: peak I, adenosine; peak II, adenosine-2′,3′ cyclic phosphate; peak III, adenosine 2′-phosphate; peak IV, adenosine 3′-phosphate. Each fraction is 25 ml. Other conditions are given in the text.

Characterization of the 2′,3′ cyclic phosphate was achieved by the comparison by paper chromatography with an authentic sample synthesized according to Khorana.[20] Acid hydrolysis of the cyclic phosphate gave adenosine 2′- and 3′-phosphates (V), which were identical with authentic samples[21] examined by paper chromatography.

In the case of 5′-O-methoxytrityl adenosine-2′,3′ cyclic phosphate, the structure of this sample was confirmed by ultraviolet absorption properties similar to those of 5′-O-methoxytrityl adenosine, R_f values in paper chromatography and paper electrophoresis, and hydrolysis. Treatment of this compound with 0.5 *N* lithium hydroxide at 70° for 1 hr gave a mixture of 5′-O-methoxytrityl adenosine 2′- and 3′-phosphate, which were further hydrolyzed by 0.1 *N* hydrochloric acid at 30° for 2 hr to give adenosine 2′- and 3′-phosphates.

The phosphorylation of 5′-O-benzoyluridine (Ic) with DMPC produced the corresponding phosphoromorpholidates in 73% yield. Treatment with ammonium hydroxide solution gave uridine-2′,3′ cyclic phosphate, which was confirmed by digestion with pancreatic ribonuclease[22] to give uridine-3′ monophosphate (VI) and by comparison with an authentic sample of the cyclic phosphate. When uridine-2′,3′ cyclic phosphate, thus obtained, was incubated with uridine in the presence of pancreatic ribonuclease at 0–1°, uridylyl-(3′→5′)-uridine (VII) was obtained.

Thus, the direct synthesis of ribonucleoside-2′,3′ cyclic phosphate starting from nucleoside was established. This method was particularly useful in the case of various nucleoside analogs[23] whose 2′- and 3′-phosphates are not available.

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(21) Purchased from Schwarz Bioresearch Ltd.

(22) This was the gift from Dr. E. Okuhara, Hokkaido University, to whom the authors give thanks.

(23) N^6-Dimethyladenosine, tubercidin, and 8-hydroxyguanosine were successfuly converted to 2′,3′ cyclic phosphates by the present procedure: M. Ikehara and I. Tazawa, to be published.

Experimental Section

Paper Electrophoresis.—Triethylammonium bicarbonate (0.05 *M*), pH 7.5, was used at 20 v/cm for 1 hr. Toyo filter paper No. 51A was used.

Detection of the Nucleotidic Material.—Ultraviolet absorption was detected under an ultraviolet lamp. Phosphate was visualized by the molybdate spray.[24] Methoxytrityl groups were visualized by spraying with perchloric acid.[25] Vicinal hydroxyl groups were detected using a periodate spray.[26]

Adenosine-2′,3′ Cyclic Phosphate. A. From 5′-O-Acetyladenosine.—Into a dioxane (4 ml) solution containing morpholinophosphorochloridate[13] (102 mg, 0.5 mmole) and diphenyl phosphate (125 mg, 0.5 mmole) was added a dioxane solution (1 ml) of 2,6-lutidine (107 mg, 1.0 mmole). After the precipitation of lutidine hydrochloride appeared, a dioxane (5 ml) solution of 5′-O-acetyladenosine (77 mg, 0.25 mmole) was added with stirring. After stirring for 3 hr at room temperature, the reaction mixture was allowed to stand for 2–3 days with exclusion of moisture. The extent of the phosphorylation estimated after paper electrophoresis was 60–70%. Into the mixture 0.01 *N* lithium hydroxide (or 0.05 *N* ammonium hydroxide) (100 ml) was added, and the solution was set aside at 30° for 1 day. After extraction of the mixture with ether (three 50-ml portions), the water layer was concentrated *in vacuo*. The residual syrup was taken up in 20 ml of water and applied to a column (2.7 × 55 cm) of ECTEOLA-cellulose (Cl^- form).[27] The column was eluted with 20% ethanol solution of 0–0.05 *M* lithium chloride using a linear gradient (1.5 l. of water and 1.5 l. of 0.1 *M* lithium chloride). Adenosine-2′,3′ cyclic phosphate was obtained as a single peak as shown in Figure 1. The fractions containing 2′,3′ cyclic phosphate were pooled and evaporated under reduced pressure below 20°. Lithium chloride was removed by repeated washing with absolute methanol, and 2′,3′ cyclic phosphate lithium salt was obtained. The yield calculated from the optical density units of the peak corresponding to 2′,3′ cyclic phosphate was 52%. This sample is identical with an authentic specimen of adenosine-2′,3′ cyclic phosphate[21] by paper chromatography and paper electrophoresis. The R_f values are summarized in Table I; ultraviolet absorption was at $\lambda_{max}^{H_2O}$ 260 mμ. Hydrolysis of this material in acidic and alkali conditions gave adenosine 2′- and 3′-monophosphate, which were identical with authentic samples in paper chromatography in solvent D.

B. From 5′-O-Monomethoxytrityladenosine.—To a dioxane (15 ml) solution of morpholinophosphorochloridate (408 mg, 2 mmoles) and diphenylphosphate (500 mg, 2 mmoles) was added a dioxane (5 ml) solution of 2,6-lutidine (428 mg, 4 mmoles). After the precipitation of lutidine hydrochloride, a dioxane (35 ml) solution of 5′-O-monomethoxytrityladenosine (539 mg, 1 mmole) was added to the mixture. The stirring was continued for 3 hr and the reaction mixture was stored in a desiccator for 2 days at room temperature. The extent of the phosphorylation estimated after paper electrophoresis was 90%. One-half of the reaction mixture (27.5 ml) was treated with 0.05 *N* ammonium hydroxide (200 ml) and kept overnight at 30°. The TOD_{260}[28] at this step was 16,400. Amines were extracted with ether (three 100-ml portions), and the water layer was evaporated under reduced pressure. The concentrated solution (*ca.* 30 ml) was applied to a column (2.7 × 60 cm) of ECTEOLA-cellulose (Cl^- form) and eluted with 0–0.18 *M* lithium chloride (containing 20% ethanol) by linear gradient elution. Fractions corresponding to 5′-O-monomethoxytrityladenosine-2′,3′ cyclic phosphate were pooled and evaporated under reduced pressure below 20°. The isolated yield was 36% as calculated for methoxytrityladenosine (ϵ_{260} 14,000); ultraviolet absorptions were at $\lambda_{max}^{H_2O}$ 234 and 260 mμ. R_f values are summarized in Table I. Hydrolysis of this sample with 0.01 *N* hydrochloric acid at 30° for 15 hr gave adenosine 2′- and 3′-phosphate which were identical with the authentic samples. Treatment of this sample with 0.5 *N* lithium chloride at 70° for 1 hr gave, presumably, 5′-O-methoxytrityladenosine 2′- and 3′-

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(28) Total optical density units measured at 260 mμ.

TABLE I

R_f VALUES OF NUCLEOSIDES AND NUCLEOTIDES

Solvent	Paper chromatography A[a]	B[b]	C[c]	D[d]	Paper electrophoresis, R_{Up}[e]
5′-Acetyladenosine	0.58	0.55			
Adenosine	0.51	0.46			0.16
Adenosine-2′,3′ cyclic phosphate	0.46	0.38			0.69
Adenosine 2′-phosphate and 3′-phosphate	0.12	0.20		0.31 (2′) 0.18 (3′)	0.91
5′-Methoxytrityladenosine	0.88	0.79			
5′-Methoxytrityladenosine-2′,3′ cyclic phosphate	0.75				0.71
5′-Methoxytrityladenosine 2′-phosphate and 3′-phosphate	0.45				
5′-Benzoyluridine	0.72	0.73	0.72		
Uridine	0.46	0.49	0.57		0.43
Uridine-2′,3′ cyclic phosphate	0.31	0.43	0.40		0.81
Uridine 2′-phosphate and 3′-phosphate	0.09	0.27	0.11		1.00
Uridyl-(3′→5′)-uridine	0.15		0.26		0.66
Diphenyl phosphate	0.89	0.79			
Benzoic acid	0.79	0.66			

[a] Isopropyl alcohol–concentrated ammonia–water, 7:1:12 (descending). [b] Isopropyl alcohol–1% ammonium sulfate, 2:1 (ascending). [c] Ethanol–1 *N* ammonium acetate, 5:2 (ascending). [d] Saturated ammonium sulfate–1 *M* ammonium acetate–isopropyl alcohol, 79:19:2 (descending). [e] R_f values divided by R_f of uridine 5′-phosphate.

phosphates, which were converted to adenosine 2′- and 3′-phosphate by hydrolysis with 0.1 *N* hydrochloric acid at 30° for 2 hr.

Uridine-2′,3′ Cyclic Phosphate.—The reaction was carried out by essentially the same procedure as described in the case of 5′-O-acetyladenosine, using the following reagents: diphenyl phosphate (75 mg, 0.3 mmole) in 2 ml of dioxane; morpholinophosphorochloridate (122 mg, 0.6 mmole) and 2,6-lutidine (128 mg, 1.2 mmoles) in 1 ml of dioxane; and 5′-O-benzoyluridine (105 mg, 0.3 mmole) in 2 ml of dioxane. The extent of the reaction estimated after paper electrophoresis was 73%. After the reaction, 0.1 *N* ammonium hydroxide (40 ml) was added to the reaction mixture, which was kept for 3 hr at room temperature. Extraction with ether (three 30-ml portions) and evaporation of the water layer gave a solution having TOD_{260} 2640. The solution was applied to a column (34 × 2.2 cm) of DEAE-cellulose (bicarbonate form) and the column was eluted with 0–0.25 *M* triethylammonium bicarbonate (pH 7.5) buffer. Evaporation of the fractions corresponding to uridine-2′,3′ cyclic phosphate gave the nucleotide in 38% yield. This material was compared with an authentic sample by paper chromatography and paper electrophoresis. R_f values are shown in Table I. Ultraviolet absorption was at $\lambda_{max}^{H_2O}$ 262 mμ. Hydrolysis with acid or alkaline gave uridine 2′- and 3′-phosphate, which were identical with the authentic samples.

Enzymatic Assay of Uridine-2′,3′ Cyclic Phosphate.—Uridine-2′,3′ cyclic phosphate (triethylammonium salt) obtained as above (15 OD) was dissolved in 0.1 ml of Tris hydrochloride buffer (pH 7, 0.05 *M*), followed by the addition of pancreatic ribonuclease (200 μg) dissolved in 0.2 ml of the buffer. Incubation of this mixture for 24 hr at 37° showed the complete digestion to uridine 3′-phosphate by paper chromatographic tests. A control experiment without addition of RNAase showed no hydrolysis.

Enzymatic Synthesis of Uridylyl-(3′→5′)-uridine.—Uridine-2′,3′ cyclic phosphate (200 OD) was dissolved in 0.3 ml of Tris hydrochloride buffer (pH 7, 0.05 *M*), followed by the addition of uridine (14.6 mg, 60 μmoles) and pancreatic RNAase (3 μg dissolved in 3 μl of buffer). The mixture was incubated for 20 hr at 0–1.° After the reaction, 1.6 ml of chloroform–isoamyl alcohol (1:1 v/v) mixture was added. An aliquot examined by paper chromatography showed a new spot having R_f (A) 0.15 and R_f (C) 0.26 in addition to the spots of uridine, uridine-2′,3′ cyclic phosphate, and uridine 2′- and 3′-phosphate. The newly appeared spot was confirmed as uridylyl-(3′→5′)-uridine by the comparison with an authentic sample.[29]

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Aromatic Polyfluoronitroso Compounds

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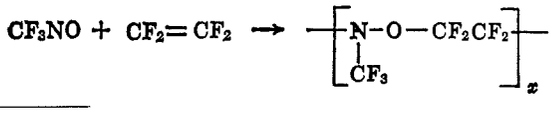
The preparation of 4-nitrosotetrafluorobromobenzene and 4-nitrosotetrafluorobenzoic acid by oxidation of the appropriately substituted anilines with performic acid is reported. The compounds reacted with 1,3-cyclohexadiene to give substituted N-phenyl-2-oxa-3-azabicyclo[2.2.2]oct-5-enes *via* the Diels–Alder reaction.

The reaction of trifluoronitrosomethane and tetrafluoroethylene has been reported to yield a 1:1 copolymer generally referred to as nitroso rubber.[2,3]



This copolymer can be cured only through the use of polyfunctional amines to yield products of low tensile strength. Polyfluoroaromatic nitroso compounds are therefore of interest as termonomers to provide sites for cross-linking the polymer to higher tensile strength products.

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