Syntheses of Ribonucleoside 5'-S-Methyl Phosphorothiolates and Ribonucleoside 3': 5'-Cyclic Phosphates from Nucleosides Applying a New Phosphorylating Agent, MTBO[†]

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When adenosine (**Ia**) was allowed to react with 2-methylthio-4H-1,3,2-benzodioxaphosphorin-2-oxide (MTBO) (**II**), a new phosphorylating agent, in the presence of cyclohexylamine, adenosine 5'-S-methyl phosphorothiolate (**IVa**) and adenosine 2': 3'-cyclic phosphate (**Va**) were obtained.

Ribonucleoside 5'-S-methyl phosphorothiolates (**IV**) were selectively synthesized directly from borate complexes of ribonucleosides (**VI**) and MTBO in the presence of cyclohexylamine, followed by removal of metaboric acid by co-distillation with methanol, in 68~86% yields.

Under anhydrous conditions, the phosphorothiolates (**IV**) were cyclized to give ribonucleoside 3': 5'-cyclic phosphates (**X**) by iodine oxidation. Five 3': 5'-cyclic nucleotides including cyclic AMP were synthesized by this procedure in 38~64% yields after purification.

Ribonucleoside 3': 5'-cyclic phosphates (**X**) are biologically important substances because one of these, adenosine 3': 5'-cyclic phosphate, occurs widely in living cells, where it plays essential roles in the hormonal control of many biochemical reactions.^{1,2)} Several methods in which the 5'-phosphates were generally employed as starting materials for the syntheses of ribonucleoside 3': 5'-cyclic phosphates have been reported.^{3~6)} We tried to synthesize ribonucleoside cyclic phosphates directly from ribonucleosides using the new phosphorylating agent, 2-methylthio-4H-1,3,2-benzodioxaphosphorin-2-oxide (MTBO) (**II**), which was readily prepared from its thiono isomer salithion.^{7~9)}

It has been reported in previous communications^{7~9)} that MTBO reacts with aliphatic alcohols in the presence of cyclohexylamine to form O-alkyl-S-methyl phosphorothiolates, which can be converted in good yield to dialkyl hydrogen phosphates or alkyl phosphates by

iodine or isoamyl nitrite oxidation in aliphatic alcohols or water.

Some phosphorylating agents which carry protecting groups may have advantages not only in blocking excess phosphorylation, but also in enhancing the reactivity of the phosphorus atom by removal of these protecting groups. MTBO phosphorylates an alcohol with opening the benzodioxaphosphorin ring by the attack of the hydroxyl group in the presence of an amine catalyst. MTBO is highly reactive in phosphorylation, having two easily removable protective groups, an *o*-hydroxybenzyl group and a methylthio group. The former is formed by ring opening in the course of the initial phosphorylation reaction and can be removed by cyclohexylamine. The latter is removable by the action of oxidizing agents such as iodine^{7,10)} and isoamyl nitrite.⁹⁾ It was expected that ribonucleoside might react with MTBO to form ribonucleoside S-methyl phosphorothiolates which would be converted into ribonucleoside cyclic phosphates by removal of the methylthio group.

This paper describes the general syntheses

[†] A New Phosphorylating Agent, 2-Methylthio-4H-1,3,2-benzodioxaphosphorin-2-oxide. Part II. For Part I see reference 9).

of ribonucleoside 5'-S-methyl phosphorothiolates and ribonucleoside 3': 5'-cyclic phosphates from ribonucleosides and MTBO. MTBO is also useful for the syntheses of ribonucleoside 2': 3'-cyclic phosphates, which will be reported in a following paper.

Reaction of adenosine with MTBO in the presence of cyclohexylamine

When adenosine (**Ia**) dissolved in dimethylformamide was treated with MTBO in the presence of cyclohexylamine, adenosine 2': 3'-cyclic phosphate (**Va**) and adenosine 5'-S-methyl phosphorothiolate (**IVa**) were produced as shown in Scheme 1. The major product **Va** was characterized by its hydrolysis to adenosine 2'-phosphate and 3'-phosphate in 0.4 N barium hydroxide at 100°C, as well as by its chromatographic, electrophoretic, and UV spectroscopic identity with an authentic specimen synthesized according to the method described by Khorana.¹¹⁾ The minor product **IVa** was characterized by a positive test to palladium chloride reagent¹⁷⁾ indicating the existence of a methylthio group, the UV spectrum, and iodine oxidation to give adenosine 5'-phosphate.

These facts indicate that MTBO reacts unselectively with three hydroxyl groups of the ribonucleoside. When MTBO was attacked by the 5'-hydroxyl group of the ribonucleoside, ribonucleoside 5'-S-methyl phosphorothiolate was produced. When the 2'- or 3'-hydroxyl group of the ribonucleoside attacked MTBO,

ribonucleoside 2'- or 3'-S-methyl phosphorothiolate was produced, which then gave spontaneously ribonucleoside 2': 3'-cyclic phosphate by the participation of the neighboring hydroxyl group. In order to obtain a better yield of a specific compound, the hydroxyl group needs to be suitably blocked by some selective protecting group.

Syntheses of ribonucleoside 5'-S-methyl phosphorothiolates (IVa~e)

The syntheses of **IVa~e**, which are possible intermediates for the syntheses of ribonucleoside 3': 5'-cyclic phosphates were attempted. Metaboric acid was found to be the best protecting agent for the syntheses of **IVa~e**.

Adenosine (**Ia**) together with an equimolar amount of metaboric acid was dissolved in dimethylformamide on gentle heating. Benzene was added to the solution and evaporated in order to remove traces of water present in the solvent. When the solution was examined by paper electrophoresis (triethylammonium bicarbonate, pH 7.5), quantitative formation of borate complex (**VIa**) was observed. The solution was then treated with MTBO in the presence of cyclohexylamine. Metaboric acid was removed by co-distillation with methanol without any decomposition of the product **IVa**. Similarly three other 5'-S-methyl phosphorothiolates, **IVb**, **IVc**, and **IVe** were obtained from uridine (**Ib**), guanosine (**Ic**), and inosine (**Ie**) in good yields (68~86%). In the case of cytidine (**Id**), borate complex was not formed

SCHEME 1. Reaction of Adenosine with MTBO in the Presence of Cyclohexylamine.

TABLE I. YIELDS^{a)} OF RIBONUCLEOSIDE 5'-S-METHYL PHOSPHOROTHIOLATES

	Nucleosides (I)	Solvents	MTBO (moles)	Yields (%)	
				5'-S-Methyl phosphorothiolates(IV)	By-products
a,	Adenosine	DMF	4	68 ^{b)}	0
b,	Uridine	DMF	3	78	0
		DMF	4	86	0
c,	Guanosine	DMF	3	71	0
d,	Cytidine	DMSO	5	84 ^{b)}	0
		DMF	5	50	20
e,	Inosine	DMF	4	79	0

^{a)} Estimated spectrophotometrically after elution of the spot from the paper electrophorograms with water.

^{b)} Isolated yields.

FIG. 1. NMR Spectrum of Adenosine 5'-S-Methyl Phosphorothiolate Triethylammonium Salt in D_2O ; Internal Standard, DSS.

sufficiently because of the low solubility of the complex in dimethylformamide. The difficulty was overcome by the use of dimethyl sulfoxide as a solvent. When the borate complex of cytidine (**VId**) was treated with MTBO in the presence of cyclohexylamine, **IVd** was exclusively produced as shown in Table I. After purification by DEAE-cellulose or Dowex 1 column chromatography, **IVa**~**e** were isolated in solid form. The yields, listed in Table I, indicate that nucleotidic by-products were not produced. These results suggest that the borate complex is stable under the present phosphorylation conditions. This was also confirmed by an

observation that only a small amount of deborated product was found on the paper electrophorogram of the reaction mixture. Therefore the phosphorylation can be repeatedly carried out by further supplementation with MTBO and cyclohexylamine to the reaction mixture; for example, the yield of **IVb** was increased from 78% to 86% by the second addition of MTBO and the catalyst (see Table I). The repeated addition of MTBO and cyclohexylamine was particularly useful in the syntheses of 5'-S-methyl phosphorothiolates from precious nucleoside analogs.

Adenosine 5'-S-methyl phosphorothiolate

SCHEME 2. Syntheses of Ribonucleoside 5'-S-Methyl Phosphorothiolate.

a, B=adenine; b, B=uracil; c, B=guanine; d, B=cytosine; e, B=hypoxanthine.

(IVa) was characterized by paper electrophoresis, paper chromatography, elemental analysis, UV spectrum, and NMR spectrum which indicated the existence of the P-S-CH₃ group; δ 2.13 (3H), doublet, $J=14$ Hz,¹²⁾ as shown in Fig. 1. It gave positive tests with palladium chloride and periodate reagents indicating the existence of methylthio and vicinal hydroxyl groups. There was no evidence for the presence of adenosine 2'- or 3'-S-methyl phosphorothiolate, because iodine oxidation of the reaction product gave only adenosine 5'-phosphate, which was identical with an authentic specimen in paper chromatography with

two different solvent systems. The products IVb~e were characterized in the usual manner.

Thus, the general method for the syntheses of ribonucleoside 5'-S-methyl phosphorothiolates (IV) was established as shown in Scheme 2.

It was reported in a previous communication⁹⁾ that the decomposition products of MTBO in the presence of cyclohexylamine were N-cyclohexylsalicylamine (III) and O-[o-(α -cyclohexylamino)tolyl] S-methyl phosphorothiolate (VII). In the reaction of nucleoside with MTBO in the presence of cyclohexylamine, another decomposition product was found. It migrated faster toward the anode

SCHEME 3. Decomposition Products of MTBO in the Presence of Cyclohexylamine.

than **IV** on paper electrophoresis and gave a positive test with palladium chloride indicating the existence of a methylthio group. This product was characterized as saligenin bis (S-methyl phosphorothiolate) (**IX**) by paper chromatography, paper electrophoresis, elemental analysis, UV spectrum, and NMR spectra which indicated the presence of a benzyl group and two S-methyl phosphorothiolate groups. The formation of this product may be explained by the following mechanism: the cyclic structure of MTBO may be hydrolyzed at the P-O-alkyl bond by nucleophilic attack by traces of water present in the solvent to give *o*-hydroxymethylphenyl S-methyl phosphorothiolate (**VIII**), which then attacks another MTBO molecule to give **IX** (see Scheme 3 (3)).

The borate complex was employed as a protecting group in the preparation of ribonucleoside 5'-phosphate.^{13,14} Ikehara *et al.*¹⁴ used boric acid as the protecting agent and obtained adenosine 5'-phosphate in a 27.6% yield. Imai *et al.*¹³ used metaboric acid as the protecting agent, acetone as solvent, and pyrophosphoryl chloride as phosphorylating agent to give the best yield of the 5'-phosphate (60%). However, the latter authors reported that by-products such as the 2'(3'),5'-diphosphates and bases were produced in considerable yield. In the present procedure, metaboric acid had perfect protecting effect; the best yield of the 5'-S-methyl phosphorothiolate was 86% and nucleotidic by-product was not observed.

Syntheses of ribonucleoside 3':5'-cyclic phosphates (**Xa~e**)

In the reaction of MTBO with the 2'- or 3'-hydroxyl group of the ribonucleoside, the proposed intermediate 2'- or 3'-S-methyl phosphorothiolate spontaneously cyclized to give the 2':3'-cyclic phosphate by the participation of the favorably located neighboring hydroxyl group. When MTBO reacts with the 5'-hydroxyl group of a ribonucleoside, the 3'-hydroxyl group of the resulting 5'-S-methyl phosphorothiolate is not situated in such a favorable position to permit the spontaneous

TABLE II. YIELDS^{a)} OF RIBONUCLEOSIDE-3':5'-CYCLIC PHOSPHATES

Nucleosides (I)	Yields (%)	
	3',:5'-Cyclic phosphates (X)	
a, Adenosine	64	
b, Uridine	54	
c, Guanosine	38	
d, Cytidine	52	
e, Inosine	49	

^{a)} Estimated from optical density unit after purification by charcoal treatment followed by DEAE-cellulose column chromatography.

closure of the 3':5'-phosphate ring. In order to synthesize the 3':5'-cyclic phosphate, it is necessary to activate the phosphoryl center of the S-methyl phosphorothiolate. The compounds **IVa~e** were readily converted into the 5'-phosphates by iodine oxidation under aqueous conditions. These results suggested that when the methylthio group of **IVa~e** was removed by iodine oxidation under anhydrous and high dilution conditions, **Xa~e** could be produced.

Pyridine was used as a reaction solvent because it is a good solvent for nucleotides, and acts as an acceptor of iodide ions formed in the course of the reaction. The compounds **IVa** and **IVb** were dissolved in pyridine as the triethylammonium salts or pyridinium salts. However, in the cases of **IVc**, **IVd**, and **IVe**, the 4-morpholine-N,N'-dicyclohexylcarboxamidinium salts were used because of their higher solubility. The use of the carboxamidine increased the solubility of **IVa** as well.

Dry pyridine solution of the appropriate salt of **IV** was added dropwise to a boiling solution of iodine in pyridine under stirring. The paper electrophoresis of the reaction mixture showed that the corresponding **X** was produced as a main product which migrated toward the anode at a speed between nucleosides and 5'-phosphates, and gave negative tests with periodate reagent and palladium chloride reagent. The results indicate the absence of vicinal hydroxyl groups and the methylthio group, respectively. The products **Xa~e** were purified by repeated

SCHEME 4. Syntheses of Ribonucleoside 3': 5'-Cyclic Phosphates.

a, B=adenine; b, B=uracil; c, B=guanine; d, B=cytosine; e, B=hypoxanthine.

FIG. 2. NMR Spectra of Adenosine 3': 5'-Cyclic Phosphate in D_2O ; Internal Standard, DSS.

extractions with water, by charcoal treatment, and then by DEAE-cellulose column chromatography. The products **Xa~e** were isolated in solid form as sodium salts by passing them through a Dowex 50 W (sodium form) column. The isolated yields of the 3': 5'-cyclic phosphate **Xa~e** are summarized in Table II.

Adenosine 3': 5'-cyclic phosphate (**Xa**) was characterized by elemental analysis and by comparison with an authentic specimen synthesized according to the method reported by Khorana⁴⁾ in paper electrophoresis, paper chromatography, UV spectra, and NMR spectra. The NMR spectrum of the 3': 5'-cyclic phosphate prepared by the present procedure was identical with that of the authentic specimen, as shown in Fig. 2. Jardetzky^{16,21)} reported that the peaks for the anomeric protons of adenosine- and uridine 3': 5'-cyclic

phosphate did not split. Our result agreed with that of Jardetzky. The anomeric protons of other ribonucleoside 3': 5'-cyclic phosphates may give singlets, which could be employed as an evidence for the 3': 5'-cyclic phosphate ring. In fact, all the ribonucleoside 3': 5'-cyclic phosphates **Xa~e** prepared by the present procedure gave the signals of the anomeric protons (δ 5.83 ~ 6.19) as almost singlets ($J < 0.5$ Hz). The products **Xb~e** were characterized in the similar way.

Ribonucleoside 5'-phosphates and P^1, P^2 -diribonucleoside 5'-pyrophosphates were isolated as by-products. The 5'-phosphates were presumably produced by the action of traces of water present in the solvent. The P^1, P^2 -diribonucleoside 5'-pyrophosphates might be formed by the reaction of the 5'-phosphates with the 5'-S-methyl phosphorothiolate.

Thus, an alternative general method for the syntheses of ribonucleoside 3':5'-cyclic phosphates (**X**) starting with ribonucleosides (**I**) was established as shown in Schemes 2 and 4. Ribonucleoside 3':5'-cyclic phosphates were prepared by several methods: (A) from adenosine 5'-triphosphate by barium hydroxide hydrolysis,³⁾ (B) from the 5'-phosphates by condensation with dicyclohexylcarbodiimide,⁴⁾ (C) from ribonucleoside *p*-nitrophenyl phosphates and other substituted phenyl phosphates with potassium *t*-butoxide in dimethyl sulfoxide,⁵⁾ and (D) from the 5'-phosphates by triphenylphosphine and 2,2'-dipyridyl disulfide.⁶⁾ Ribonucleoside 5'-phosphates or their derivatives were employed as starting materials in these conventional methods (A)–(D). The use of MTBO as a phosphorylating agent for the synthesis of ribonucleoside 3':5'-cyclic phosphates gives several advantages as follows: 1) Nucleosides can be employed as the starting materials; 2) MTBO reacts with nucleoside borate complexes under mild basic conditions; 3) At the first step of phosphorylation reaction, *o*-hydroxybenzyl group is readily removed by amines; 4) The intermediates, ribonucleoside 5'-S-methyl phosphorothiolates, are activated by iodine oxidation to yield readily cyclic nucleotides. Each step of reactions proceeds smoothly under mild conditions and consequently the formation of by-products is restricted. Since the ease of the formation of **X** from nucleoside is characteristic of the present method, this method seems particularly useful for the 3':5'-cyclic phosphates from nucleoside analogs.

In the reaction of **IVc** with iodine at lower temperature (80°C), P¹,P²-diguanosine 5'-pyrophosphate was produced together with the 5'-phosphate. This result suggests that **IVa~e** would be useful as intermediates for the synthesis of coenzymes containing ribonucleoside 5'-pyrophosphate.

EXPERIMENTAL

General methods

All nucleosides were dried *in vacuo* at 95°C for more than 5 hr. Reagent grade dimethylformamide (DMF)

was distilled and dried over Linde molecular sieve 4A. Dimethyl sulfoxide (DMSO) and pyridine were dried over calcium hydride, distilled and stored over Linde molecular sieve 4A. Cyclohexylamine was dried over potassium hydroxide. MTBO was prepared from 2-methoxy-4H-1, 3, 2-benzodioxaphosphorin-2-sulfide (Salithion®) (supplied by Sumitomo Chemical Co.) by thiono-thiol conversion as described in the previous paper.⁹⁾

Paper electrophoresis was performed with Toyo Roshi No. 51 paper at 700 V (35 cm) using 0.1 M triethylammonium bicarbonate buffer at pH 7.5 for 1 hr. Paper chromatography was performed by ascending technique using Toyo Roshi No. 51 paper. The following systems were used: solvent A, isopropanol-28% ammonia-water (7:1:2); solvent B, isopropanol-28% ammonia-0.1 M boric acid (7:1:2); solvent C, *n*-butanol-acetic acid-water (5:2:3); solvent D, ethanol-M ammonium acetate (5:2); solvent E, isopropanol-1% ammonium sulfate (2:1); solvent F, saturated ammonium sulfate-M sodium acetate-isopropanol (80:18:2). The *R_f*'s and the paper electrophoretic mobility of nucleotides and related compounds are listed in Table III. Thin-layer chromatography was performed with silica gel G according to Stahl (Merck). The solvent system used was chloroform-ether (9:1 v/v). Nucleotides and related compounds were detected by viewing under an ultraviolet lamp, phosphorus containing compounds by molybdate-perchloric acid spray,¹⁶⁾ sulfur containing compounds*¹ and iodide ion*² by palladium chloride spray,¹⁷⁾ phenolic compound by spraying of alcoholic potassium hydroxide followed by diazotized sulfanilic acid,¹⁸⁾ and vicinal hydroxyl groups by periodate spray.¹⁹⁾

NMR spectra were obtained with a Hitachi Perkin Elmer R-20 High Resolution NMR Spectrometer (60 MHz). UV spectra were recorded on a Shimadzu spectrometer MPS-50. Phosphorus analysis was carried out by the method of King.²⁰⁾

All reaction systems were protected from atmospheric moisture. Ion-exchange column chromatography was carried out using Brown DEAE-cellulose or Dowex 1×8 resins in the bicarbonate form. Dowex 1 column had much greater loading capacity than DEAE-cellulose column, but required more concentrated elution buffer, and elution peak was broader than in DEAE-cellulose column. Elution was carried out using a linear gradient with triethylammonium bicarbonate solution. The elution of the products was followed spectrophotometrically. The eluate was

*¹ P-S-CH₃ group was detected as a yellow spot, and P=S group as a dark brown spot by spraying with the reagent.

*² Iodide ion could be detected as a dark brown spot on paper electrophorogram by spraying with the reagent.²²⁾

TABLE III. *Rf*'s and *Rm*'s of NUCLEOTIDES AND RELATED COMPOUNDS

Solvent	<i>Rf</i> 's						<i>Rm</i> 's ^{a)}
	A	B	C	D	E	F	
Adenosine	0.60	0.57	0.63	0.59	0.62		-0.08
Adenosine 2': 3'-cyclic phosphate	0.53	0.54	0.44	0.44	0.54		0.45
Adenosine 5'-phosphate	0.12	0.03	0.36	0.09	0.34	0.28	0.79
Adenosine 2'-phosphate	0.16	0.18	0.41	0.10	0.36	0.25	
Adenosine 3'-phosphate	0.16	0.18	0.41	0.10	0.36	0.16	
Adenosine 5'-S-methyl phosphorothiolate	0.55	0.49	0.50	0.49	0.57		0.39
Adenosine 3': 5'-cyclic phosphate	0.50	0.47	0.45	0.39	0.53		0.44
Uridine	0.45	0.40	0.60	0.82	0.62		0.17
Uridine 5'-phosphate	0.05	0.03	0.37	0.16	0.32		1.0
Uridine 2'(3')-phosphate		0.18					
Uridine 5'-S-methyl phosphorothiolate	0.34	0.30	0.47	0.68	0.46		0.64
Uridine 3': 5'-cyclic phosphate	0.29	0.28	0.40	0.57	0.45		0.76
Guanosine	0.32	0.24	0.45	0.51	0.49		0.44
Guanosine 5'-phosphate	0.04	0.01	0.23	0.05	0.25		0.82
Guanosine 2'(3')-phosphate		0.09					
Guanosine 5'-S-methyl phosphorothiolate	0.28	0.20	0.38	0.44	0.43		0.47
Guanosine 3': 5'-cyclic phosphate	0.26	0.20	0.35	0.38	0.41		0.51
Cytidine	0.48	0.46	0.60	0.62	0.53		0.04
Cytidine 5'-phosphate	0.07	0.04	0.04	0.09	0.28		0.87
Cytidine 2'(3')-phosphate		0.17					
Cytidine 5'-S-methyl phosphorothiolate	0.40	0.38	0.48	0.47	0.40		0.53
Cytidine 3': 5'-cyclic phosphate	0.35	0.33	0.38	0.41	0.37		0.55
Inosine	0.49	0.48	0.53	0.59	0.56		0.34
Inosine 5'-S-methyl phosphorothiolate	0.35	0.33	0.43	0.48	0.46		0.64
Inosine 3': 5'-cyclic phosphate	0.31	0.29	0.39	0.37	0.40		0.81

^{a)} Ratio of electrophoretic mobility of nucleotides and their related compounds to that of uridine 5'-phosphate.

evaporated to dryness, and the residue was redissolved in water. The procedure was repeated till the residue became amine-odorless. Charcoal treatment was carried out using a column of Wako activated charcoal pretreated appropriately.

The yields of the reaction products were estimated by two methods: the yield from paper electrophorogram and the isolated yield. The former was estimated spectrophotometrically from the relative absorbance of water extracts of appropriate spots on the paper electrophorogram of the reaction mixture. The latter was also estimated spectrophotometrically, after isolation by ion-exchange chromatography, based on original nucleoside or nucleotide. The former agreed sufficiently with the latter.

Reaction of adenosine with MTBO

Adenosine (534 mg, 2 mmole) was dissolved in DMF (20 ml) on gentle heating. After cooling to room temperature, MTBO (864 mg, 4 mmole) was dissolved in the solution, and cyclohexylamine (0.8 ml, 10 mmole) was added. Thin-layer chromatography of the reaction mixture showed MTBO to be consumed. The mixture was heated at 100°C for 6 hr. An aliquot of the reaction mixture examined by paper electrophoresis showed five substances were produced. The reaction mixture was treated with a mixture of water (50 ml) and chloroform (50 ml). The aqueous layer was separated and applied to a DEAE-cellulose column (bicarbonate form, 2.5 × 27 cm). After washing with water, elution was carried out using a linear gradient with 0.002 M triethylammonium bicarbonate (pH 7.5,

2 liters) in the mixing vessel and 0.1 M triethylammonium bicarbonate (pH 7.5, 2 liters) in the reservoir. Twenty-ml fractions were collected. The fractions containing a major peak (tubes 28~58) were examined by paper electrophoresis. Two substances (A and B) were found on the paper electrophorogram.

1) Characterization of B

The fractions containing main product B (tubes 48~58) were combined and evaporated to dryness. The residue was dissolved in a small amount of water. To the solution was added acetone (30 ml) followed by an acetone solution of barium iodide (1 mmole in 2.5 ml). The resulting white precipitate was collected by centrifugation, washed with acetone (three 30 ml portions), and dried over phosphorus pentoxide *in vacuo*. Sample B was identical with an authentic specimen of adenosine 2': 3'-cyclic phosphate in paper chromatography and paper electrophoresis. UV absorption was at $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ 260 nm. Hydrolysis of the material by 0.4 N barium hydroxide solution at 100°C for 30 min gave adenosine 2'-phosphate and 3'-phosphate which were identical with authentic specimens in paper chromatography in solvent B and F. From these results the product B was shown to be adenosine 2': 3'-cyclic phosphate (Va).

2) Characterization of A

The fractions containing A (tubes 28~44) were combined and evaporated. The residue was dissolved in water, and the solution applied to a DEAE-cellulose column (1.5 × 20 cm). Elution was carried out using a linear gradient with water (2 liters) in the mixing vessel and 0.025 M triethylammonium bicarbonate (pH 7.5, 2 liters) in the reservoir. Twenty-ml fractions were collected. Two peaks, tubes 35~60 and tubes 61~68, were obtained. The fractions containing the main peak (tubes 35~60) were combined and evaporated. The residue was dissolved in water and examined by paper electrophoresis. Material A was detected by UV absorption under UV lamp, and with palladium chloride reagent, which showed the presence of a methylthio group. To an aliquot (O.D._{260nm} unit=19) of the residue dissolved in water (0.5 ml) was added an acetone solution of iodine (3.2 mg in 0.5 ml). The solution was allowed to stand for 4 hr at room temperature. To the solution was added water (5 ml) and extracted with ether (four 3 ml portions). The solution was adjusted to pH 7 with 1 N ammonium hydroxide, and examined by paper chromatography in solvent B and F. Oxidation of this material by aqueous iodine gave only adenosine 5'-phosphate. UV absorption of material A was at $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ 260nm. From these results the product A was shown to be adenosine 5'-S-methyl phosphorothiolate (IVa).

Adenosine 5'-S-methyl phosphorothiolate (IVa)

A mixture of adenosine (2.67 g, 10 mmole) and metaphoric acid (481.8 mg, 11 mmole) was dissolved in DMF (20 ml) on gentle heating to 120°C. The solution was dried by co-evaporation with benzene (three 10 ml portions). An aliquot of the solution was examined by paper electrophoresis, which showed the borate complex formation in quantitative yield. MTBO (8.64 g, 40 mmole) was added to the solution. The solution was then chilled with liquid nitrogen until just before the solvent would be solidified. Cyclohexylamine (14.3 ml, 133 mmole) was added to the solution. The mixture was heated at 70°C for 30 min, and evaporated to dryness *in vacuo*. The residue was treated with a mixture of chloroform (50 ml) and water (50 ml). The aqueous portion was extracted with chloroform (three 50 ml portions) and evaporated to dryness. The residue was dissolved in methanol (1 liter). Methanol was distilled away at atmospheric pressure to remove the borate as its methyl ester. Paper electrophoresis showed that borate was removed completely. The residue was dissolved in methanol (20 ml), ethyl ether (180 ml) was added, and the resulting precipitate was collected by centrifugation. The procedure was repeated to remove the decomposition product of MTBO IX. The precipitate was dissolved in water and submitted to Dowex 1 column (bicarbonate form, 1.5 × 30 cm). After washing with water (4 liters), elution was carried out using a linear gradient with 0.1 M triethylammonium bicarbonate (pH 7.5 4 liters) in the mixing vessel, and 0.45 M triethylammonium bicarbonate (pH 7.5, 4 liters) in the reservoir, and 20-ml fractions were collected. The fractions containing the product (tubes 50~300) were combined and evaporated to dryness. After removal of residual triethylammonium bicarbonate, the residue was dissolved in water and converted into the sodium salt by passing it through a Dowex 50W column (sodium form). The eluate was evaporated to dryness and ethanol (50 ml) was added to the residue then dried by evaporation of ethanol. A resulting white solid was dissolved in methanol (20 ml), and addition of ethyl ether (180 ml) precipitated a white solid. The material was collected by centrifugation, washed with ethyl ether, and dried *in vacuo* to give 2.92 g (69%) of IVa as the sodium salt.

UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 258 (14,050) at pH 2, 260 (14,500) at pH 7 and pH 12.

Anal. Found: C, 33.07; H, 4.72; N, 16.55; P, 7.32. Calcd. for $\text{C}_{11}\text{H}_{15}\text{N}_5\text{O}_6\text{PSNa} \cdot \text{CH}_3\text{OH}$: C, 33.45; H, 4.45; N, 16.25; P, 7.02%.

NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 1.32 (9H, triplet, $J=7.5$ Hz, $\text{CH}_2\text{-CH}_3$), 2.13 (3H, doublet, $J=14$ Hz, P-S- CH_3), 3.24 (6H, quartet, $J=7.5$ Hz, $\text{CH}_2\text{-CH}_3$) 4.1~4.6 (5H, H2', H3', H4', and H5'), 6.16 (1H, doublet, $J=5.5$ Hz, H1'), 8.19 (1H, singlet, H2), 8.48 (1H, singlet, H8).

The product IVa was also isolated directly in solid

form as its triethylammonium salt by evaporation of the eluent from column chromatography. The NMR spectrum was obtained from the triethylammonium salt (see Fig. 1). The δ 2.13 (3H, doublet, $J=14$ Hz) peak indicated the presence of S-methyl phosphorothiolate group.

Iodine oxidation of **IVa** under aqueous conditions gave only adenosine 5'-phosphate, which was identical with an authentic specimen in paper chromatography in solvent B and F.

Uridine 5'-S-methyl phosphorothiolate (IVb) was prepared in analogy to the preparation of **IVa** from uridine (10 mmole), metaphoric acid (11 mmole), DMF (20 ml), MTBO (30 mmole), and cyclohexylamine (100 mmole). The spectrophotometric yield of **IVb** was 78%. The reaction mixture was chilled again as before and a solution of MTBO (10 mmole) in DMF (5 ml) followed by cyclohexylamine (33 mmole) was added. The spectrophotometric yield of **IVb** was 86%. Ion-exchange chromatography was carried out using a DEAE-cellulose column (4.5 $\times$ 55 cm) and a linear gradient with water (5 liters) in the mixing vessel and 0.05 M triethylammonium bicarbonate (pH 7.5, 5 liters) in the reservoir. The eluate was converted into sodium salt, dried *in vacuo* to give 3.43 g (81.5%) of **IVb**.

UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 263 (9300) at pH 2, 263 (9420) at pH 7, 262.5 (7120) at pH 12.

Anal. Found: C, 33.70; H, 4.50; N, 6.20; P, 7.41. Calcd. for $\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_8\text{PSNa} \cdot \text{C}_2\text{H}_5\text{OH}$: C, 34.10; H, 4.77; N, 6.64; P, 7.33%.

NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 2.20 (3H, doublet, $J=14$ Hz, P-S-CH₃), 4.05~4.45 (5H, H2', H3', H4', and H5'), 5.95 (1H, doublet, $J=8$ Hz, H5), 5.99 (1H, doublet, $J=4$ Hz, H1'), 7.93 (1H doublet, $J=8$ Hz, H4).

Guanosine 5'-S-methyl phosphorothiolate (IVc)

A mixture of guanosine (10 mmole) and metaphoric acid (11 mmole) was dissolved in DMF (50 ml) on heating at 70°C for 4 hr with stirring. A clear solution was obtained by co-evaporation with benzene (three 10 ml portions). The solution was concentrated to about 20 ml* which was treated with MTBO (30 mmole) followed by cyclohexylamine (100 mmole) in the usual manner. The spectrophotometric yield of **IVc** was 71%. The product **IVc** was purified and isolated in solid form as its sodium salt (2.66 g, 64%) by the same method as used in the preparation of **IVb**.

UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 257 (12,410) at pH 2, 254 (13,800) at pH 7, 265 (11,750) at pH 12.

Anal. Found: C, 31.95; H, 3.96; N, 16.78; P, 7.97.

* Guanosine set to a gel and became insoluble in DMF when the solution of the borate complex was completely evaporated to dryness.

Calcd. for $\text{C}_{11}\text{H}_{16}\text{N}_5\text{O}_7\text{PSNa}$: C, 31.81; H, 3.96; N, 16.86; P, 7.46%.

NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 2.11 (3H, doublet, $J=13.5$ Hz, P-S-CH₃), 4.05~4.50 (5H, H2', H3', H4', and H5'), 5.96 (1H, doublet, $J=5.5$ Hz, H1'), 8.12 (1H, singlet with fine structure, H8).

Cytidine 5'-S-methyl phosphorothiolate (IVd)

A mixture of cytidine (10 mmole) and metaphoric acid (11 mmole) was dissolved in DMSO (20 ml) on heating at 80°C for 3 hr, and dried in the usual manner. MTBO (50 mmole) was dissolved in the solution. The solution was chilled with ice until just before the solvent would be solidified. Cyclohexylamine (165 mmole) was added to the solution. The mixture was allowed to stand at room temperature for 2 hr. Ion-exchange chromatography was carried out by the same procedure as used in the preparation of **IVb**. The product **IVd** (3.16 g, 84.3%) was isolated in solid form as its sodium salt in the usual manner. (O.D._{272nm} unit=73,700).

UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 281.5 (12,630) at pH 2, 273 (8730) at pH 7, 273 (8700) at pH 12.

Anal. Found: C, 31.96; H, 4.30; N, 10.79; P, 8.70. Calcd. for $\text{C}_{10}\text{H}_{15}\text{N}_5\text{OPSNa}$: C, 32.35; H, 4.30; N, 11.20; P, 8.25%.

NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 2.14 (3H, doublet, $J=14$ Hz, P-S-CH₃), 4.08~4.45 (5H, H2', H3', H4', and H5'), 6.02 (1H, doublet, $J=4$ Hz, H1'), 6.12 (1H, doublet, $J=7.5$ Hz, H5), 7.94 (1H, doublet, $J=7.5$ Hz, H4).

Inosine 5'-S-methyl phosphorothiolate (IVe) prepared by the same procedure as used in the preparation of **IVa**. The spectrophotometric yield of **IVe** was 79%. The product **IVe** was isolated as its sodium salt (3.2 g, 72.5%).

UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 250 (11,970) at pH 2, 250 (12,300) at pH 7, 254 (13,050) at pH 12.

Anal. Found: C, 32.99; H, 4.36; N, 12.58; P, 7.16. Calcd. for $\text{C}_{11}\text{H}_{14}\text{N}_4\text{O}_7\text{PSNa} \cdot \text{CH}_3\text{OH}$: C, 33.30; H, 4.20; N, 12.95; P, 7.01%.

NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 2.09 (3H, doublet, $J=14$ Hz, P-S-CH₃), 4.08~4.65 (5H, H2', H3', H4', and H5'), 6.15 (1H, doublet, $J=5.3$ Hz, H1'), 8.21 (1H, singlet, H2), 8.43 (1H, singlet, H8).

Adenosine 3': 5'-cyclic phosphate (Xa)

A mixture of pyridinium salt of **IVa** (1 mmole) and 4-morpholine-N, N'-dicyclohexylcarboxamidine (293 mg, 1 mmole) was dissolved in a solution of pyridine (10 ml) and water (10 ml). After removal of traces of water by co-evaporation with dry pyridine (four 10 ml portions). The residue was dissolved in dry pyridine (200 ml). The solution was added dropwise over a period of 1 hr to a boiling solution of iodine

(1.52 g, 6 mmole) in pyridine (200 ml) with stirring. The reaction mixture was further heated under reflux for an additional 10 min and then evaporated to dryness. The black residue was extracted thoroughly with water. The extract was centrifuged and the supernatant was adsorbed on a charcoal (8 g, 40 ml) column. The column was washed with water (2 liters) and eluted with ethanol-28% ammonia-water (50: 5: 45) (400 ml). The eluate was evaporated to dryness below 30°C *in vacuo*. The residue was dissolved in water, and applied to a DEAE-cellulose column (bicarbonate form, 3×28 cm). After washing with water, elution was carried out using a linear gradient with water (2 liters) in the mixing vessel and 0.05 M triethylammonium bicarbonate (pH 7.5, 2 liters) in the reservoir, and 20-ml fraction were collected. The fractions containing the major peak (70~137), **Xa** (O.D._{260nm} unit=9300, 64%) were combined and evaporated to dryness. After removal of residual triethylammonium bicarbonate, the residue was dissolved in water and converted into its sodium salt by passing it through a sodium Dowex 50 W column. The eluate was evaporated to dryness and ethanol (50 ml) was added to the residue, and then dried by evaporation of ethanol. The residue was dissolved in methanol (10 ml) and addition of ethyl ether (90 ml) precipitated a light yellow solid. The material was collected by centrifugation, washed with ethyl ether, and dried *in vacuo* to give 236.5 mg (57.6%) of **Xa** as its sodium salt.

UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 257.5 (13,940) at pH 2, 260 (14,350) at pH 7 and pH 12.

Anal. Found: C, 32.13; H, 3.85; N, 17.08; P, 8.70. Calcd. for $\text{C}_{10}\text{H}_{11}\text{N}_5\text{O}_6\text{PNa}\cdot\text{CH}_3\text{OH}$: C, 32.20; H, 4.43; N, 17.08; P, 8.79%.

NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 4.45 (2H, singlet with fine structure, H5'), 6.14 (1H, singlet, H1'), 8.17 (1H, singlet, H2), 8.21 (1H, singlet, H8).

The product **Xa** was identical with an authentic specimen in paper chromatography and paper electrophoresis. The *Rf*'s are summarized in Table III. Barium hydroxide hydrolysis of **Xa** gave only adenosine 3'-phosphate and 5'-phosphate, which were identical with authentic specimens in paper chromatography in solvent B and F. A peak for the anomeric proton of **Xa** appeared as a singlet in NMR spectrum, which was identical with that of an authentic sample, as shown in Fig. 2. The product **Xa** isolated after ion-exchange chromatography was also crystallized from acidic aqueous ethanol at pH 2.0 as the monohydrate.

Uridine 3': 5'-cyclic phosphate (Xb) was prepared by the same procedure as used in the preparation of **Xa** except that a dry pyridine solution of pyridinium salt of **IVb** was used. The product **Xb** (O.D._{262nm} unit=5048, 53.7%) was isolated as its sodium salt

(190 mg, 52.3%).

UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 261.5 (9980) at pH 2, 261.5 (10,110) at pH 7, 261.5 (7430) at pH 12.

Anal. Found: C, 30.10; H, 3.67; N, 7.58; P, 8.54. Calcd. for $\text{C}_9\text{H}_9\text{N}_2\text{O}_8\text{PNa}\cdot 2\text{H}_2\text{O}$: C, 29.80; H, 3.61; N, 7.75; P, 8.79%.

NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 4.32 (2H, singlet with fine structure, H5'), 5.83 (1H, singlet, H1'), 5.90 (1H, doublet, $J=8.0$ Hz, H5), 7.66 (1H, doublet, $J=8.0$ Hz, H4).

Guanosine 3': 5'-cyclic phosphate (Xc) was prepared by the same procedure as used in the preparation of **Xa**. The product **Xc** (O.D._{264nm} unit=3920, 38.3%) was isolated as its sodium salt (143 mg 37%).

UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 256.5 (11,400) at pH 2, 254 (12,540) at pH 7, 266 (10,750) at pH 12.

Anal. Found: C, 30.18; H, 3.39; N, 17.73; P, 8.01. Calcd. for $\text{C}_{10}\text{H}_{11}\text{N}_5\text{O}_7\text{PNa}\cdot\text{H}_2\text{O}$: C, 31.18; H, 3.68; N, 18.18; P, 8.04%.

NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 4.20~4.45 (5H, H2', H3', H4', and H5'), 5.95 (1H, singlet, H1'), 7.87 (1H, singlet with fine structure, H8).

Cytidine 3': 5'-cyclic phosphate (Xd) was prepared by the same procedure as used in the preparation of **Xa** except that 3 mmole of iodine was used. The product **Xd** (O.D._{272nm} unit=4960, 52%) was isolated as its sodium salt.

UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 280 (13,650) at pH 2, 272 (9550) at pH 7 and 12.

Anal. Found: C, 31.99; H, 4.12; N, 10.99; P, 8.24. Calcd. for $\text{C}_9\text{H}_{11}\text{N}_5\text{O}_7\text{PNa}\cdot\text{H}_2\text{O}\cdot\text{CH}_3\text{OH}$: C, 31.83; H, 4.55; N, 11.14; P, 8.19%.

NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 4.2~4.52 (5H, H2', H3', H4', and H5'), 5.83 (1H, singlet, H1'), 6.04 (1H, doublet, $J=7.5$ Hz, H5), 7.61 (1H, doublet, $J=7.5$ Hz, H4).

Inosine 3': 5'-cyclic phosphate (Xe) was prepared by the same procedure as used in the preparation of **Xa**. The products **Xe** (O.D._{260nm} unit=5600, 49%) was isolated as its sodium salt.

UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 250 (11,300) at pH 2, 250 (11,375) at pH 7, 253.5 (12,100) at pH 12.

Anal. Found: C, 33.30; H, 3.01; N, 15.18; P, 8.60. Calcd. for $\text{C}_{10}\text{H}_{10}\text{N}_4\text{O}_7\text{PNa}\cdot 1/2\cdot\text{H}_2\text{O}$: C, 33.25; H, 3.07; N, 15.52; P, 8.49%.

NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 4.40 (4H, singlet with fine structure, H3', H4', and H5'), 6.19 (1H, singlet, H1'), 8.22 (2H, singlet, H2 and H8).

Saligenin bis (S-methyl phosphorothiolate) (IX)

The chloroform extract of the reaction mixture in the synthesis of **IVd** was extracted with water. The aqueous portion was evaporated to dryness and redis-

solved in water. The solution was applied to a DEAE-cellulose column (3×14 cm). After washing with water, elution was carried out using a linear gradient with water (2 liters) and 0.05 M triethylammonium bicarbonate (pH 7.5, 2 liters). The fractions containing the major peak were examined by paper electrophoresis at every 10th fraction. The product IX was detected as a yellow spot by spraying palladium chloride reagent. The fractions containing only IX (tubes 30~85) were combined and evaporated to dryness. The residue was dissolved in water and converted into sodium salt by passing it through sodium Dowex 50W column. The eluate was evaporated to dryness and dried by co-evaporation with ethanol. The residue was dissolved in methanol (5 ml), and addition of ethyl ether (45 ml) precipitated a white solid. The product IX was collected by centrifugation and dried *in vacuo*.

UV $\lambda_{\text{max}}^{\text{H}_2\text{O}}$ nm (ϵ): 266 (695) at pH 2, 7, and 12.

Anal. Found: C 27.16; H, 3.32; P, 15.65. Calcd. for $\text{C}_9\text{H}_{12}\text{O}_5\text{P}_2\text{S}_2\text{Na}_2\cdot\text{H}_2\text{O}$: C, 27.7; H, 3.62; P, 15.80%.

NMR $\delta_{\text{DSS}}^{\text{D}_2\text{O}}$: 2.15 (3H, doublet, $J=14$ Hz, P-S-CH₃), 2.25 (3H, doublet, $J=14$ Hz, P-S-CH₃), 5.10 (2H, doublet, $J=8$ Hz, Ph-CH₂-O), 7.18~7.68 (4H, Ph-H).

NMR $\delta_{\text{TMS}}^{\text{DMSO}-d_6}$: 2.04 (6H doublet, $J=12.5$ Hz, P-S-CH₃), 4.87 (2H, doublet, $J=6$ Hz, Ph-CH₂-O), 6.85~7.25 (2H, Ph-H), 7.25~7.60 (2H, Ph-H).

The product IX gave a positive test with palladium chloride and was identified as the expected saligenin bis(S-methyl phosphorothiolate) by elemental analysis.

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