Phosphorylation of Nucleosides and Nucleotides with Inorganic Monoimido-*cyclo*-Triphosphate

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The phosphorylation of nucleosides (adenosine, guanosine, cytidine, and uridine) and nucleotides (adenosine 5′-monophosphate, guanosine 5′-monophosphate, cytidine 5′-monophosphate and uridine 5′-monophosphate) has been achieved using inorganic monoimido-*cyclo*-triphosphate (MCTP, $Na_3P_3O_8NH$) in aqueous solution. In this reaction, the 2′-OH or 3′-OH group of the β-D-ribofuranose unit was phosphorylated and the total yield was more than 30% and 14%, respectively. The main products were 2′-diphosphoramidophosphononucleoside and 2′-diphosphoramidophosphononucleoside 5′-monophosphate.

Key words phosphorylation; monoimido-*cyclo*-triphosphate; multinuclear NMR; HPLC

Nucleosides and related organic compounds are usually phosphorylated by phosphoryl chloride,[1] polyphosphoric acid[2—4] and various organic phosphorus compounds.[5—8] Because phosphorylation with these agents is accompanied by various side reactions, protection of other functional groups is necessary and complicated procedures are typically required.

Sodium *cyclo*-triphosphate, $Na_3P_3O_9$ (P_{3m}), is a simple and efficient inorganic phosphorylating agent. One of the authors reported the phosphorylation of nucleosides[9] and nucleotides[10] by P_{3m}. The 2′- and 3′-OH groups of the β-D-ribofuranosyl unit on nucleosides and nucleotides were selectively phosphorylated in high yield without the need for protection of the other hydroxyl groups. The main phosphorylated products were 2′- and 3′-monophosphate esters of the nucleosides and nucleotides. We also reported that alkylamines,[11] aminoalcohols,[12] and carbohydrates[13—18] are readily phosphorylated with P_{3m} to give the corresponding triphosphate derivatives. Unfortunately, phosphorylated carbohydrates are easily decomposed to monophosphate derivatives.[9,10,13—18]

We have developed new inorganic phosphorylating reagents, imido-*cyclo*-triphosphates, $Na_3P_3O_{9-n}(NH)_n$. Compared with the P–O–P linkage, the P–NH–P linkage is stable and difficult to hydrolyze.[19] We therefore explored the use of imido-*cyclo*-triphosphates for the phosphorylation of biologically important compounds. Monoimido-*cyclo*-triphosphate (MCTP), diimido-*cyclo*-triphosphate (DCTP), and triimido-*cyclo*-triphosphate (TCTP), shown in Fig. 1, were synthesized. Our current interest is to disclose the phosphorylation mechanism by MCTP and DCTP. TCTP did not react even under strict conditions such as pH 13 and 70 °C.

MCTP is a six-membered ring composed of one P–NH–P and two P–O–P linkages. We recently demonstrated that the phosphorylation of methylamine[20] and amino acids[21] proceeded with MCTP. More recently, we reported that D-glucose, D-glucuronic acid and 2-deoxy-D-glucose reacted with MCTP to form 1-*O*-diphosphoramidophosphono-β-D-aldoses stereoselectively.[22] Organic compounds containing amino or hydroxyl group were easily phosphorylated by MCTP. We also reported that D-glucose and gluco-oligosaccharides reacted with DCTP.[23]

In the present work, we chose MCTP and first studied the reaction of nucleosides with MCTP in aqueous solution, followed by phosphorylation of nucleotides, in order to synthesize triphosphate derivatives of nucleosides and nucleotides.

Results and Discussion

Phosphorylation of Adenosine (1), Guanosine (2), Cytidine (3) and Uridine (4) with MCTP Nucleosides used in the present study are shown in Fig. 2. Phosphorylation was carried out essentially according to the previously described

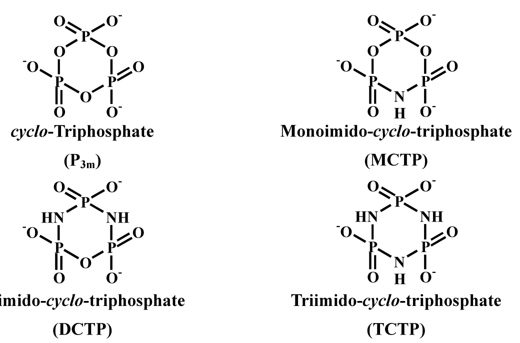
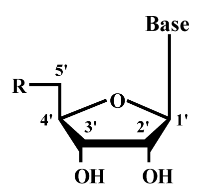


Fig. 1. Structure of *cyclo*-Triphosphate (P_{3m}), Monoimido-*cyclo*-Triphosphate (MCTP), Diimido-*cyclo*-Triphosphate (DCTP), and Triimido-*cyclo*-Triphosphate (TCTP)



1	Base = adenine	R = OH
2	Base = guanine	R = OH
3	Base = cytosine	R = OH
4	Base = uracil	R = OH
13	Base = adenine	R = PO_4^{2-}
14	Base = guanine	R = PO_4^{2-}
15	Base = cytosine	R = PO_4^{2-}
16	Base = uracil	R = PO_4^{2-}

Fig. 2. Structure of Nucleosides and Nucleotides Studied in This Work

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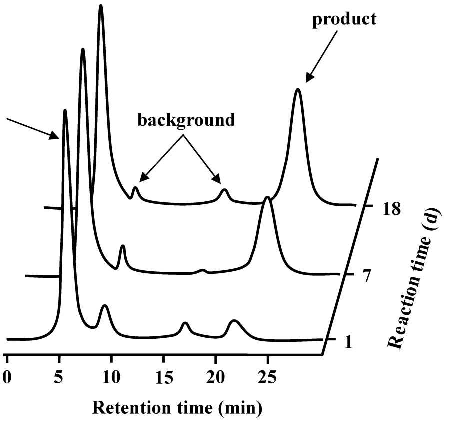


Fig. 3. HPLC Profiles for the Reaction Mixture of **1** and MCTP

MCTP : adenosine (**1**)=0.4 M : 0.1 M, pH 12, and 40 °C.

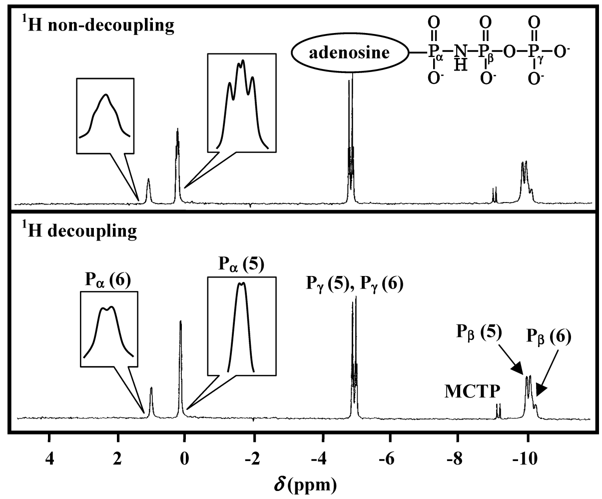


Fig. 4. ^{31}P-NMR Spectra of **5** and **6**

MCTP : adenosine (**1**)=0.4 M : 0.1 M, pH 12, and 40 °C, after 22 d.

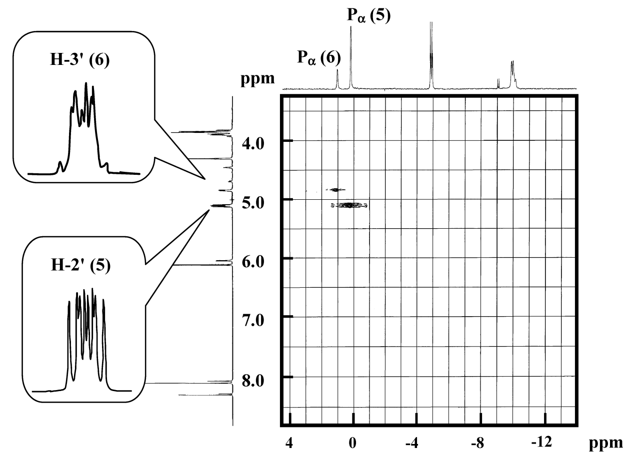


Fig. 5. ^{1}H–^{31}P 2D HMBC NMR Spectrum of **5** and **6**

MCTP : adenosine (**1**)=0.4 M : 0.1 M, pH 12, and 40 °C, after 22 d.

method.[20—22] HPLC analysis served as a tool for evaluating the yields of products from their peak area. Figure 3 shows HPLC profiles for the reaction mixture of adenosine (**1**) (0.1 M) and MCTP (0.4 M) incubated at pH 12 and 40 °C. A peak attributed to the phosphorylated product appeared at a retention time of about 20 min. The other chromatographic peaks were assigned to adenosine and background peaks, respectively. Although, the HPLC profile of the reaction of **1** with MCTP showed a single peak attributable to the reaction product, ^{31}P-NMR spectra (Fig. 4) showed two imidotriphosphate esters, **5** and **6**, which could not be separated by HPLC. The total yield of **5** and **6** was 57% after 18 d and the compounds remained stable for 50 d without hydrolysis of the imidotriphosphate esters.

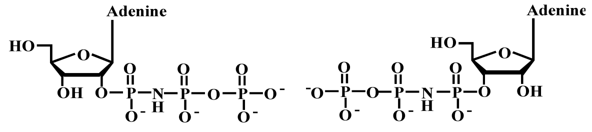
To identify **5** and **6**, ^{31}P- and ^{1}H-NMR spectra were measured. In the ^{31}P-NMR spectra, the peak at 0.3 ppm was assigned to P_α of **5**, and the peak at 1.1 ppm to P_α of **6**. A previous study indicated that the phosphorylation products of D-glucose derivatives[22] with MCTP are diphosphoramidophosphono-D-aldoses with an –O–P_α–NH–P_β– bond. These products show a characteristic P_α signal at around 0 ppm in their ^{31}P-NMR spectra. Therefore, the two doublets of doublets at 0.3 and 1.1 ppm in the ^{1}H non-decoupled ^{31}P-NMR spectrum, which collapsed to two doublets in the ^{1}H decoupled spectrum, exhibited the characteristic peak pattern of P_α similar to those of monoimidotriphosphate derivatives.[20—22] The other doublets at −4.8 and −4.9 ppm and the doublets of doublets at −10.0 and −10.1 ppm in the ^{1}H decoupled spectrum did not change when the decoupler was turned off. The chemical shifts of the middle phosphorus atom (P_β) and the end phosphorus atom (P_γ) of monoimidotriphosphate derivatives usually appear at −10.0 and −6.0 ppm, respectively.[20—22] Therefore, the doublets at −4.8 and −4.9 ppm and the doublets of doublets at −10.0 and −10.1 ppm were assigned to P_γ and P_β, respectively. Compared with the triphosphate ester of D-glucose, the chemical shifts of P_α and P_β of **5** and **6** were shifted downfield, whereas there was no shift for P_γ. Also, the values of J_{P_α,P_β} of **5** and **6** were one-third of J_{P_α,P_β} for the triphosphate ester of D-glucose,[13] and the values of J_{P_β,P_γ} of **5** and **6** were the same as that of the triphosphate ester of D-glucose.[13] These results suggest the existence of an –O–P_α–NH–P_β– bond in the phosphorylated products **5** and **6**. Therefore, **5** and **6** were confirmed to be diphosphoramidophosphonoadenosines.

Figure 5 shows the ^{1}H–^{31}P heteronuclear multiple bond correlation (HMBC) NMR spectrum of **5** and **6**. The ^{1}H–^{31}P 2D HMBC NMR experiment showed a correlation between P_α at 0.3 ppm and the ^{1}H signal at 5.10 ppm. The signal at 5.10 ppm was assigned to H-2′ of **5** based on the ^{1}H–^{1}H COSY spectrum. The $^{3}J_{P_\alpha,H\text{-}2'}$ value (8.8 Hz) from the ^{1}H-NMR spectrum is consistent with that deduced from ^{31}P-NMR data. From these results, **5** was confirmed to be 2′-diphosphoramidophosphonoadenosine.

Figure 5 also shows the correlation between P_α at 1.1 ppm (due to **6**) and the ^{1}H signal at 4.78 ppm. The signal at 4.78 ppm was assigned to H-3′ of **6** by the ^{1}H–^{1}H COSY experiment.[13] Product **6** was determined to be 3′-diphosphoramidophosphonoadenosine (**6**). This shows that **1** reacts with MCTP to form both 2′-diphosphoramidophosphonoadenosine (**5**) and 3′-diphosphoramidophosphonoadenosine (**6**). The main product was found to be 2′-diphosphoramidophosphonoadenosine (**5**) from the comparison of the intensities of the P_α signals (0.3, 1.1 ppm) in the ^{1}H non-decoupled ^{31}P-NMR spectrum.

Table 1 summarizes the total yield of **5** and **6** obtained from the reaction of **1** with MCTP under various conditions.

Table 1. Total Yields of Phosphoylated Products, **5** and **6** at Various Reaction Condition



5 6

Conc. (mol/l)		Temp. (°C)	pH	Time (d)	Yield (%)
MCTP					
0.4	0.2	40	12	15	42
0.4	0.1	25	12	77	50
		40	12	18	57
		70	12	4	36
		40	11	67	56
		40	13	9	33
0.4	0.04	40	12	15	60

Considering the yield and reaction time, the appropriate condition for phosphorylation of **1** with MCTP are pH 12, 40 °C, and a molar ratio of MCTP : **1**=10 (0.4 M) : 1 (0.04 M). The total yield of **5** and **6** remained constant after 50 d without hydrolysis of the imidotriphosphate ester. This is in contrast to the reaction of **1** with P_{3m}. The products of the reaction of **1** with P_{3m} are 2′-monophosphate, 3′-monophosphate, and 2′,3′-cyclicmonophosphate. The triphosphate derivatives of **1** are also produced as intermediates and decomposed to monophosphate derivatives immediately. It was concluded that the stability of the phosphorylated nucleoside was improved by use of MCTP.

The reactions of guanosine (**2**), cytidine (**3**) and uridine (**4**) with MCTP were also carried out under the same reaction conditions as **1**. In HPLC profile of the reaction solution of **2**—**4** with MCTP, a single peak due to each phosphorylated product was obtained. However, each ^{31}P-NMR spectrum showed two imidotriphosphate esters, **7** and **8** for **2**, **9** and **10** for **3**, and **11** and **12** for **4**, respectively. The yields of the products at pH 12, 40 °C, and a molar ratio of MCTP : **2**—**4**=10 (0.4 M) : 1 (0.04 M) increased with reaction time and the total yield of **7** and **8** reached 45% (after 22 d), that of **9** and **10** reached 34% (after 21 d), and that of **11** and **12** reached 30% (after 21 d). The yields of **5**—**8** are higher than that of **9**—**12** due to the effect of the base. Therefore, **2**—**4** react with MCTP to form 2′-diphosphoramidophosphononucleoside (**7**, **9**, **11**) and 3′-diphosphoramidophosphononucleoside (**8**, **10**, **12**) by ^{1}H–^{1}H COSY and ^{1}H–^{1}H total correlation spectroscopy (TOCSY) NMR experiments.

Phosphorylation of Adenosine 5′-Monophosphate (13), Guanosine 5′-Monophosphate (14), Cytidine 5′-Monophosphate (15) and Uridine 5′-Monophosphate (16) with MCTP Figure 6 shows the changes of the amounts of reaction products in the reaction of adenosine 5′-monophosphate (**13**) (0.1 M) with MCTP (0.4 M) at pH 12 and 40 °C. The HPLC profile of the reaction of **13** with MCTP showed two peaks attributable to the reaction products **17** and **18** although they could not be separated completely. The total amounts of **17** and **18** increased with reaction time to reach 22% after 21 d and constant after 50 d without hydrolysis of the imidotriphosphate ester.

In the reactions of **14**, **15**, and **16** with MCTP, two phosphorylated products were also observed in the ^{31}P-NMR spectra. The maximum yield of **19** and **20** for **14**, that of **21** and **22** for **15**, and that of **23** and **24** for **16**, were 21, 19 and 14%, respectively. Compared with the yields of **5**—**12**, the yields of **17**—**24** were lower. This is due to electrostatic repulsion between the MCTP and the monophosphate group at the 5′ position.

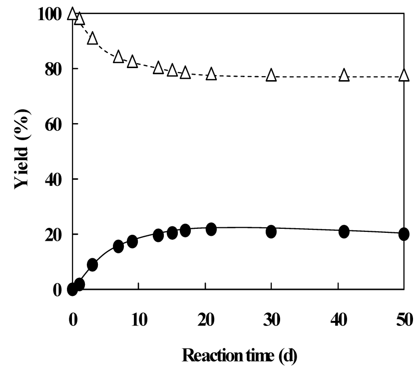


Fig. 6. Changes of the Amounts of Reaction Products in the Reaction of Adenosine 5′-Monophosphate (0.1 M) with MCTP (0.4 M) at pH 12 and 40 °C

●: **17** and **18**, △: adenosine 5′-monophosphate.

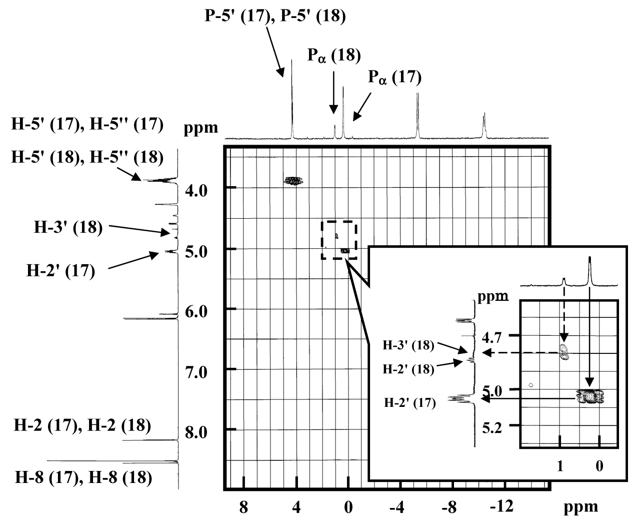


Fig. 7. ^{1}H–^{31}P 2D HMBC NMR Spectrum of **17** and **18**

MCTP : adenosine 5′-monophosphate (**13**)=0.4 M : 0.1 M, pH 12, and 40 °C, after 18 d.

To identify **17**—**24**, ^{31}P- and ^{1}H-NMR spectra were measured. Figure 7 shows a representative ^{1}H–^{31}P 2D HMBC correlation spectrum of **17** and **18**. In the ^{31}P-NMR spectrum, the peak at 0.3 ppm was assigned to P_α of **17**, and the peak at 0.9 ppm to P_α of **18**. The doublet at −5.3 ppm and the doublet of doublets at −10.5 ppm were assigned to P_γ and P_β, respectively. The spectrum showed a correlation between P_α at 0.3 ppm and the ^{1}H signal at 5.04 ppm. The signal at 5.04 ppm was assigned to H-2′ of **17** based on the ^{1}H–^{1}H COSY spectrum. The downfield shift from 4.62 ppm (H-2′ of **17**) to 5.04 ppm is the result of phosphorylation. The other ^{1}H-NMR signals of **17** were assigned by ^{1}H–^{1}H COSY and ^{1}H–^{1}H TOCSY NMR experiments. In this way, the main product **17** was confirmed to be 2′-diphosphoramidophosphonoadenosine 5′-monophosphate (**17**).

Figure 7 also shows a correlation between P_α at 0.9 ppm (due to **18**) and the H-3′ at 4.78 ppm. The signal at 4.78 ppm was assigned to H-3′ of **18** by a ^{1}H–^{1}H COSY experiments. Therefore, product **18** was determined to be 3′-diphosphoramidophosphonoadenosine 5′-monophosphate. In the reaction of **14**—**16** with MCTP, the phosphorylated products **19**—**24** were verified to be 2′-diphosphoramidophosphonoguanosine 5′-monophosphate (**19**), 3′-diphosphoramidophosphonoguanosine 5′-monophosphate (**20**), 2′-diphosphoramidophosphonocytidine 5′-monophosphate (**21**), 3′-diphosphoramidophosphonocytidine 5′-monophosphate (**22**), 2′-diphosphoramidophosphonouridine 5′-monophosphate (**23**), and 3′-diphosphoramidophosphonouridine 5′-monophosphate (**24**), respectively, from the results of ^{31}P-, ^{1}H-, ^{1}H–^{31}P 2D HMBC, ^{1}H–^{1}H COSY and ^{1}H–^{1}H TOCSY NMR experiments. As mentioned above, **13**—**16** were phosphorylated by MCTP at the 2′-OH and 3′-OH positions of the β-D-ribofuranosyl unit similar to **1**—**4**.

Reaction Mechanism of Nucleosides and Nucleotides with MCTP The reaction of nucleosides (**1**—**4**) or nucleotides (**13**—**16**) with MCTP may be explained by the following mechanism (Chart 1). At pH 12, MCTP is easily attacked by nucleophilic reagents such as amines,[20] amino acids[21] and D-glucose derivatives.[22] In the present study, the lone electron pair on the hydroxyl group of the β-D-ribofuranose unit nucleophilicly attacks a phosphorus atom of MCTP, cleaving its six-membered ring. It is noteworthy that the existence of hydrogen bonding between the 2′-OH or 3′-OH of β-D-ribofuranose and the oxygen atom of MCTP would make attack of MCTP easier. Therefore, nucleosides (**1**—**4**) or nucleotides (**13**—**16**) react with MCTP to form 2′-diphosphoramidophosphononucleosides (**7**, **9**, **11**) and 3′-diphosphoramidophosphononucleosides (**8**, **10**, **12**).

The reaction of nucleosides (**1**—**4**) or nucleotides (**13**—**16**) with P_{3m} gave triphosphate derivatives of nucleosides and nucleotides.[10] The triphosphate derivatives is immediately hydrolyzed to give nucleoside 2′-monophosphate and nucleoside 3′-monophosphate or nucleoside 2′,5′-diphosphate and nucleoside 3′,5′-diphosphate *via* a 2′,3′-cyclicmonophosphate derivative. Although the P–O–P linkages of the triphosphate derivatives of nucleosides and nucleotides were hydrolyzed to give monophosphate derivatives, the P–N–P linkages of monoimidotriphosphate derivatives are stable and difficult to hydrolyze.

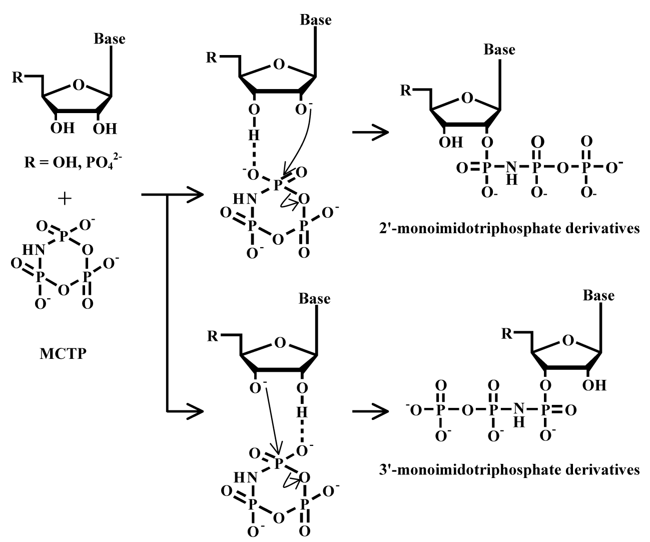


Chart 1. Phosphorylation Mechanism of Nucleosides and Nucleotides with MCTP

Conclusion In the reactions of adenosine (**1**), guanosine (**2**), cytidine (**3**), and uridine (**4**) with MCTP, 2′-diphosphoramidophosphononucleosides (**5**, **7**, **9**, **11**) and 3′-diphosphoramidophosphononucleosides (**6**, **8**, **10**, **12**) were synthesized in yields of more than 30%. The reactions of nucleotides (**13**—**16**) with MCTP produced 2′-diphosphoramidophosphononucleoside 5′-monophosphates similar to **1**—**4**. In the reactions of nucleosides and nucleotides with MCTP, the 2′-OH or 3′-OH of the β-D-ribofuranose unit was phosphorylated. These results suggest that the synthesis of nucleoside triphosphate derivatives by MCTP is a promising area for development.

Experimental

Materials and Methods Monoimido-*cyclo*-triphosphate, $Na_3P_3O_8NH$ (MCTP), was prepared according to a previous paper.[24] Adenosine (**1**), guanosine (**2**), cytidine (**3**) uridine (**4**), adenosine 5′-monophosphate (**13**), guanosine 5′-monophosphate (**14**), cytidine 5′-monophosphate (**15**), uridine 5′-monophosphate (**16**), and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) were purchased from Yamasa Shoyu Co., Ltd. (Tokyo, Japan) and Sigma (St. Louis, U.S.A.). Unless otherwise stated, guaranteed grade reagents from Wako Chemical Industries, Ltd. (Osaka, Japan), were used.

^{1}H-NMR spectra were measured with Varian Gemini 300 spectrometer. Samples were dissolved in D_2O (99.9%) with DSS as an external standard. ^{31}P-NMR spectra with and without broad band ^{1}H-decoupling and ^{1}H–^{31}P 2D HMBC spectra were obtained with a Varian INOVA-500 spectrometer. As an external standard, 85% H_3PO_4 was used.

HPLC analysis was carried out with a JASCO HPLC-800 system (Tokyo, Japan). A column (150×6.0 mm i.d.) packed with a polystyrene-based anion-exchanger (TSK gel, SAX, 5 μm, TOSOH, Tokyo, Japan), was used for the analysis of phosphate. The column temperature was maintained at 45 °C. An isocratic elution technique using 0.27—0.35 M potassium chloride solutions was employed. The flow rate of the eluent was 1.0 ml·min^{-1}. The UV absorbance of the effluent was monitored continuously at 260 nm for the adenosine and 5′-AMP systems, 258 nm for the guanosine and 5′-GMP systems, 271 nm for the cytidine and 5′-CMP systems, and 262 nm for the uridine and 5′-UMP systems.

Synthetic Procedure The reaction of adenosine (**1**) (0.0214 g, 0.04 M) with MCTP (0.2583 g, 0.4 M) were dissolved in H_2O (4 ml), and then the solution was adjusted to pH 12 and 40 °C. The pH of the mixed solution was adjusted by adding 6 M sodium hydroxide solution. The separation of **5** and **6** from the reaction mixture was accomplished by anion-exchange chromatography with a 2×80 cm column filled with Dowex 1-X2 resin (100—200 mesh, chloride form). Elution was carried out with 0.3 M potassium chloride aqueous solution, and each 50 ml fraction was measured by HPLC. The solution fractionated was concentrated at −113 °C *in vacuo* (freeze-drying). An aliquot of the obtained product was dissolved in D_2O for HPLC, ^{1}H- and ^{31}P-NMR measurements. Similar procedures were used for the syntheses of **7**—**12** and **17**—**24**.

2′-Diphosphoramidophosphonoadenosine (**5**): ^{1}H-NMR (D_2O) δ: 6.12 (1H, d, $J_{1',2'}$=6.6 Hz, H-1′), 5.10 (1H, ddd, $J_{1',2'}$=6.6 Hz, $J_{2',3'}$=5.0 Hz, $J_{P_\alpha,H\text{-}2'}$=8.8 Hz, H-2′), 4.68 (1H, dd, $J_{2',3'}$=5.0 Hz, $J_{3',4'}$=2.5 Hz, H-3′), 4.30 (1H, ddd, $J_{3',4'}$=2.5 Hz, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.5 Hz, H-4′), 3.86 (1H, ddd, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.5 Hz, $J_{5',5''}$=13.0 Hz, H-5′), 3.82 (1H, ddd, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.5 Hz, $J_{5',5''}$=13.0 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.3 (1P, dd, J_{P_α,P_β}=6.1 Hz, $J_{P_\alpha,H\text{-}2'}$=8.8 Hz, P_α), −10.0 (1P, dd, J_{P_α,P_β}=6.1 Hz, J_{P_β,P_γ}=20.2 Hz, P_β), −4.8 (1P, d, J_{P_β,P_γ}=20.2 Hz, P_γ).

3′-Diphosphoramidophosphonoadenosine (**6**): ^{1}H-NMR (D_2O) δ: 6.07 (1H, d, $J_{1',2'}$=5.0 Hz, H-1′), 4.78 (1H, dd, $J_{1',2'}$=5.0 Hz, $J_{2',3'}$=5.0 Hz, H-2′), 4.78 (1H, ddd, $J_{2',3'}$=5.0 Hz, $J_{3',4'}$=3.0 Hz, $J_{P_\alpha,H\text{-}3'}$=8.5 Hz, H-3′), 4.45 (1H, ddd, $J_{3',4'}$=3.0 Hz, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.0 Hz, H-4′), 3.92 (1H, ddd, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.0 Hz, $J_{5',5''}$=13.0 Hz, H-5′), 3.88 (1H, ddd, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.0 Hz, $J_{5',5''}$=13.0 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 1.1 (1P, d, J_{P_α,P_β}=6.1 Hz, $J_{P_\alpha,H\text{-}3'}$=8.5 Hz, P_α), −10.1 (1P, dd, J_{P_α,P_β}=6.1 Hz, J_{P_β,P_γ}=20.0 Hz, P_β), −4.9 (1P, d, J_{P_β,P_γ}=20.0 Hz, P_γ).

2′-Diphosphoramidophosphonoguanosine (**7**): ^{1}H-NMR (D_2O) δ: 5.85

(1H, d, $J_{1',2'}$=6.5 Hz, H-1′), 4.99 (1H, ddd, $J_{1',2'}$=6.5 Hz, $J_{2',3'}$=5.5 Hz, $J_{P_\alpha,H-2'}$=9.1 Hz, H-2′), 4.56 (1H, dd, $J_{2',3'}$=5.5 Hz, $J_{3',4'}$=4.5 Hz, H-3′), 4.14 (1H, ddd, $J_{3',4'}$=4.5 Hz, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, H-4′), 3.70 (1H, ddd, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, $J_{5',5''}$=13.0 Hz, H-5′), 3.65 (1H, ddd, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, $J_{5',5''}$=13.0 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.3 (1P, dd, J_{P_α,P_β}=6.7 Hz, $J_{P_\alpha,H-2'}$=9.1 Hz, P_α), −10.0 (1P, dd, J_{P_α,P_β}=6.7 Hz, J_{P_β,P_γ}=20.6 Hz, P_β), −4.7 (1P, d, J_{P_β,P_γ}=20.6 Hz, P_γ).

3′-Diphosphoramidophosphonoguanosine (**8**): ^{1}H-NMR (D_2O) δ: 5.79 (1H, d, $J_{1',2'}$=6.0 Hz, H-1′), 4.75 (1H, dd, $J_{1',2'}$=6.0 Hz, $J_{2',3'}$=5.5 Hz, H-2′), 4.82 (1H, ddd, $J_{2',3'}$=5.5 Hz, $J_{3',4'}$=3.5 Hz, $J_{P_\alpha,H-3'}$=9.7 Hz, H-3′), 4.32 (1H, ddd, $J_{3',4'}$=3.5 Hz, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, H-4′), 3.75 (1H, ddd, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, $J_{5',5''}$=13.0 Hz, H-5′), 3.68 (1H, ddd, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, $J_{5',5''}$=13.0 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.9 (1P, dd, J_{P_α,P_β}=6.7 Hz, $J_{P_\alpha,H-3'}$=9.7 Hz, P_α), −10.1 (1P, dd, J_{P_α,P_β}=6.7 Hz, J_{P_β,P_γ}=20.2 Hz, P_β), −4.8 (1P, d, J_{P_β,P_γ}=20.2 Hz, P_γ).

2′-Diphosphoramidophosphonocytidine (**9**): ^{1}H-NMR (D_2O) δ: 5.97 (1H, d, $J_{1',2'}$=6.5 Hz, H-1′), 4.60 (1H, ddd, $J_{1',2'}$=6.5 Hz, $J_{2',3'}$=5.5 Hz, $J_{P_\alpha,H-2'}$=9.5 Hz, H-2′), 4.41 (1H, dd, $J_{2',3'}$=5.5 Hz, $J_{3',4'}$=3.5 Hz, H-3′), 4.01 (1H, ddd, $J_{3',4'}$=3.5 Hz, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, H-4′), 3.67 (1H, ddd, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, $J_{5',5''}$=13.0 Hz, H-5′), 3.67 (1H, ddd, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, $J_{5',5''}$=12.5 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.3 (1P, dd, J_{P_α,P_β}=6.2 Hz, $J_{P_\alpha,H-2'}$=9.5 Hz, P_α), −10.3 (1P, dd, J_{P_α,P_β}=6.2 Hz, J_{P_β,P_γ}=20.8 Hz, P_β), −5.0 (1P, J_{P_β,P_γ}=20.8 Hz, P_γ).

3′-Diphosphoramidophosphonocytidine (**10**): ^{1}H-NMR (D_2O) δ: 5.81 (1H, d, $J_{1',2'}$=5.5 Hz, H-1′), 4.33 (1H, dd, $J_{1',2'}$=5.5 Hz, $J_{2',3'}$=5.5 Hz, H-2′), 4.51 (1H, ddd, $J_{2',3'}$=5.5 Hz, $J_{3',4'}$=2.5 Hz, $J_{P_\alpha,H-2'}$=9.3 Hz, H-3′), 4.13 (1H, ddd, $J_{3',4'}$=2.5 Hz, $J_{4',5'}$=2.5 Hz, $J_{4',5''}$=4.5 Hz, H-4′), 3.79 (1H, ddd, $J_{4',5'}$=2.5 Hz, $J_{4',5''}$=4.5 Hz, $J_{5',5''}$=12.5 Hz, H-5′), 3.74 (1H, ddd, $J_{4',5'}$=2.5 Hz, $J_{4',5''}$=4.5 Hz, $J_{5',5''}$=12.5 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.8 (1P, dd, J_{P_α,P_β}=6.5 Hz, $J_{P_\alpha,H-3'}$=9.3 Hz, P_α), −10.5 (1P, dd, J_{P_α,P_β}=6.5 Hz, J_{P_β,P_γ}=21.0 Hz, P_β), −5.2 (1P, d, J_{P_β,P_γ}=21.0 Hz, P_γ).

2′-Diphosphoramidophosphonouridine (**11**): ^{1}H-NMR (D_2O) δ: 5.98 (1H, d, $J_{1',2'}$=6.5 Hz, H-1′), 4.65 (1H, ddd, $J_{1',2'}$=6.5 Hz, $J_{2',3'}$=5.0 Hz, $J_{P_\alpha,H-2'}$=8.5 Hz, H-2′), 4.45 (1H, dd, $J_{2',3'}$=5.0 Hz, $J_{3',4'}$=3.0 Hz, H-3′), 4.04 (1H, ddd, $J_{3',4'}$=3.0 Hz, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, H-4′), 3.72 (1H, ddd, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, $J_{5',5''}$=13.0 Hz, H-5′), 3.69 (1H, ddd, $J_{4',5'}$=3.5 Hz, $J_{4',5''}$=3.5 Hz, $J_{5',5''}$=13.0 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.1 (1P, dd, J_{P_α,P_β}=6.1 Hz, $J_{P_\alpha,H-2'}$=8.5 Hz, P_α), −10.8 (1P, dd, J_{P_α,P_β}=6.1 Hz, J_{P_β,P_γ}=20.8 Hz, P_β), −5.4 (1P, d, J_{P_β,P_γ}=20.8 Hz, P_γ).

3′-Diphosphoramidophosphonouridine (**12**): ^{1}H-NMR (D_2O) δ: 5.84 (1H, d, $J_{1',2'}$=5.0 Hz, H-1′), 4.38 (1H, dd, $J_{1',2'}$=5.0 Hz, $J_{2',3'}$=5.0 Hz, H-2′), 4.58 (1H, ddd, $J_{2',3'}$=5.0 Hz, $J_{3',4'}$=3.5 Hz, $J_{P_\alpha,H-3'}$=10.0 Hz, H-3′), 4.16 (1H, ddd, $J_{3',4'}$=3.5 Hz, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.0 Hz, H-4′), 3.77 (1H, ddd, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.0 Hz, $J_{5',5''}$=13.0 Hz, H-5′), 3.70 (1H, ddd, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.0 Hz, $J_{5',5''}$=13.0 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.6 (1P, dd, J_{P_α,P_β}=6.3 Hz, $J_{P_\alpha,H-3'}$=10.0 Hz, P_α), −10.8 (1P, dd, J_{P_α,P_β}=6.3 Hz, J_{P_β,P_γ}=20.8 Hz, P_β), −5.4 (1P, d, J_{P_β,P_γ}=20.8 Hz, P_γ).

2′-Diphosphoramidophosphonoadenosine 5′-Monophosphate (**17**): ^{1}H-NMR (D_2O) δ: 6.14 (1H, d, $J_{1',2'}$=6.0 Hz, H-1′), 5.04 (1H, ddd, $J_{1',2'}$=6.0 Hz, $J_{2',3'}$=5.5 Hz, $J_{P_\alpha,H-2'}$=9.0 Hz, H-2′), 4.58 (1H, dd, $J_{2',3'}$=5.5 Hz, $J_{3',4'}$=3.0 Hz, H-3′), 4.27 (1H, ddd, $J_{3',4'}$=3.0 Hz, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=4.5 Hz, H-4′), 3.89 (1H, m, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=4.5 Hz, $J_{5',5''}$=12.0 Hz, $J_{P-5',H-5'}$=3.6 Hz, H-5′), 3.85 (1H, m, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=4.5 Hz, $J_{5',5''}$=12.0 Hz, $J_{P-5',H-5''}$=3.6 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.3 (1P, dd, J_{P_α,P_β}=5.5 Hz, $J_{P_\alpha,H-2'}$=9.0 Hz, P_α), −10.6 (1P, dd, J_{P_α,P_β}=5.5 Hz, J_{P_β,P_γ}=20.6 Hz, P_β), −5.5 (1P, d, J_{P_β,P_γ}=20.6 Hz, P_γ), 4.3 (1P, dd, $J_{P-5',H-5'}$=3.6 Hz, $J_{P-5',H-5''}$=3.6 Hz, P-5′).

3′-Diphosphoramidophosphonoadenosine 5′-Monophosphate (**18**): ^{1}H-NMR (D_2O) δ: 6.07 (1H, d, $J_{1',2'}$=6.0 Hz, H-1′), 4.81 (1H, dd, $J_{1',2'}$=6.5 Hz, $J_{2',3'}$=4.5 Hz, H-2′), 4.78 (1H, ddd, $J_{2',3'}$=4.5 Hz, $J_{3',4'}$=4.5 Hz, $J_{P_\alpha,H-3'}$=8.7 Hz, H-3′), 4.45 (1H, ddd, $J_{3',4'}$=4.5 Hz, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=4.0 Hz, H-4′), 3.92 (1H, m, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=4.0 Hz, $J_{5',5''}$=13.0 Hz, $J_{P-5',H-5'}$=3.4 Hz, H-5′), 3.88 (1H, m, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=4.0 Hz, $J_{5',5''}$=13.0 Hz, $J_{P-5',H-5''}$=3.4 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 1.3 (1P, dd, J_{P_α,P_β}=6.1 Hz, $J_{P_\alpha,H-3'}$=8.7 Hz, P_α), −10.0 (1P, dd, J_{P_α,P_β}=6.1 Hz, J_{P_β,P_γ}=19.4 Hz, P_β), −5.3 (1P, d, J_{P_β,P_γ}=19.4 Hz, P_γ), 4.2 (1P, dd, $J_{P-5',H-5'}$=3.4 Hz, $J_{P-5',H-5''}$=3.4 Hz, P-5′).

2′-Diphosphoramidophosphonoguanosine 5′-Monophosphate (**19**): ^{1}H-NMR (D_2O) δ: 5.96 (1H, d, $J_{1',2'}$=5.5 Hz, H-1′), 4.98 (1H, ddd, $J_{1',2'}$=5.5 Hz, $J_{2',3'}$=5.5 Hz, $J_{P_\alpha,H-2'}$=9.7 Hz, H-2′), 4.53 (1H, dd, $J_{2',3'}$=5.5 Hz, $J_{3',4'}$=5.0 Hz, H-3′), 4.21 (1H, ddd, $J_{3',4'}$=5.0 Hz, $J_{4',5'}$=4.0 Hz, $J_{4',5''}$=4.0 Hz, H-4′), 3.91 (1H, m, $J_{4',5'}$=4.0 Hz, $J_{4',5''}$=4.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5'}$=3.6 Hz, H-5′), 3.84 (1H, m, $J_{4',5'}$=4.0 Hz, $J_{4',5''}$=4.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5''}$=3.6 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.5 (1P, dd, J_{P_α,P_β}=6.5 Hz, $J_{P_\alpha,H-2'}$=9.7 Hz, P_α), −9.8 (1P, dd, J_{P_α,P_β}=6.5 Hz, J_{P_β,P_γ}=19.6 Hz, P_β), −5.3 (1P, ddd, J_{P_β,P_γ}=19.6 Hz, P_γ), 4.3 (1P, dd, $J_{P-5',H-5'}$=3.6 Hz, $J_{P-5',H-5''}$=3.6 Hz, P-5′).

3′-Diphosphoramidophosphonoguanosine 5′-Monophosphate (**20**): ^{1}H-NMR (D_2O) δ: 5.85 (1H, d, $J_{1',2'}$=6.0 Hz, H-1′), 4.77 (1H, dd, $J_{1',2'}$=6.0 Hz, $J_{2',3'}$=5.0 Hz, H-2′), 4.75 (1H, ddd, $J_{2',3'}$=5.0 Hz, $J_{3',4'}$=3.0 Hz, $J_{P_\alpha,H-3'}$=8.7 Hz, H-3′), 4.39 (1H, ddd, $J_{3',4'}$=3.0 Hz, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.0 Hz, H-4′), 3.91 (1H, m, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5'}$=3.8 Hz, H-5′), 3.88 (1H, m, $J_{4',5'}$=3.0 Hz, $J_{4',5''}$=3.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5''}$=3.8 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 1.3 (1P, dd, J_{P_α,P_β}=6.1 Hz, $J_{P_\alpha,H-3'}$=8.7 Hz, P_α), −10.0 (1P, dd, J_{P_α,P_β}=6.1 Hz, J_{P_β,P_γ}=19.4 Hz, P_β), −5.3 (1P, d, J_{P_β,P_γ}=19.4 Hz, P_γ), 4.2 (1P, dd, $J_{P-5',H-5'}$=3.8 Hz, $J_{P-5',H-5''}$=3.8 Hz, P-5′).

2′-Diphosphoramidophosphonocytidine 5′-Monophosphate (**21**): ^{1}H-NMR (D_2O) δ: 6.32 (1H, d, $J_{1',2'}$=5.0 Hz, H-1′), 4.87 (1H, ddd, $J_{1',2'}$=5.0 Hz, $J_{2',3'}$=5.5 Hz, $J_{P_\alpha,H-2'}$=9.1 Hz, H-2′), 4.67 (1H, dd, $J_{2',3'}$=5.5 Hz, $J_{3',4'}$=5.0 Hz, H-3′), 4.46 (1H, ddd, $J_{3',4'}$=5.0 Hz, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=5.0 Hz, H-4′), 4.17 (1H, m, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=5.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5'}$=3.6 Hz, H-5′), 4.09 (1H, m, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=5.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5''}$=3.6 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.7 (1P, dd, J_{P_α,P_β}=6.7 Hz, $J_{P_\alpha,H-2'}$=9.1 Hz, P_α), −9.4 (1P, dd, J_{P_α,P_β}=6.7 Hz, J_{P_β,P_γ}=19.6 Hz, P_β), −4.2 (1P, d, J_{P_β,P_γ}=19.6 Hz, P_γ), 4.8 (1P, dd, $J_{P-5',H-5'}$=3.6 Hz, $J_{P-5',H-5''}$=3.6 Hz, P-5′).

3′-Diphosphoramidophosphonocytidine 5′-Monophosphate (**22**): ^{1}H-NMR (D_2O) δ: 6.17 (1H, d, $J_{1',2'}$=5.0 Hz, H-1′), 4.69 (1H, dd, $J_{1',2'}$=5.0 Hz, $J_{2',3'}$=4.5 Hz, H-2′), 4.78 (1H, ddd, $J_{2',3'}$=4.5 Hz, $J_{3',4'}$=5.0 Hz, $J_{P_\alpha,H-3'}$=8.5 Hz, H-3′), 4.59 (1H, ddd, $J_{3',4'}$=5.0 Hz, $J_{4',5'}$=2.5 Hz, $J_{4',5''}$=5.0 Hz, H-4′), 4.23 (1H, m, $J_{4',5'}$=2.5 Hz, $J_{4',5''}$=5.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5'}$=3.4 Hz, H-5′), 4.10 (1H, m, $J_{4',5'}$=2.5 Hz, $J_{4',5''}$=5.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5''}$=3.4 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 1.0 (1P, dd, J_{P_α,P_β}=6.1 Hz, $J_{P_\alpha,H-3'}$=8.5 Hz, P_α), −9.5 (1P, dd, J_{P_α,P_β}=6.1 Hz, J_{P_β,P_γ}=20.2 Hz, P_β), −4.3 (1P, d, J_{P_β,P_γ}=20.2 Hz, P_γ), 4.8 (1P, dd, $J_{P-5',H-5'}$=3.4 Hz, $J_{P-5',H-5''}$=3.4 Hz, P-5′).

2′-Diphosphoramidophosphonouridine 5′-Monophosphate (**23**): ^{1}H-NMR (D_2O) δ: 6.02 (1H, d, $J_{1',2'}$=6.0 Hz, H-1′), 4.67 (1H, ddd, $J_{1',2'}$=6.0 Hz, $J_{2',3'}$=5.5 Hz, $J_{P_\alpha,H-2'}$=8.5 Hz, H-2′), 4.43 (1H, dd, $J_{2',3'}$=5.5 Hz, $J_{3',4'}$=3.5 Hz, H-3′), 4.17 (1H, ddd, $J_{3',4'}$=3.5 Hz, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=4.0 Hz, H-4′), 3.90 (1H, m, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=4.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5'}$=3.3 Hz, H-5′), 3.83 (1H, m, $J_{4',5'}$=1.5 Hz, $J_{4',5''}$=4.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5''}$=3.3 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.4 (1P, dd, J_{P_α,P_β}=6.7 Hz, $J_{P_\alpha,H-2'}$=8.5 Hz, P_α), −9.7 (1P, dd, J_{P_α,P_β}=6.7 Hz, J_{P_β,P_γ}=20.8 Hz, P_β), −5.3 (1P, d, J_{P_β,P_γ}=20.8 Hz, P_γ), 4.2 (1P, dd, $J_{P-5',H-5'}$=3.3 Hz, $J_{P-5',H-5''}$=3.3 Hz, P-5′).

3′-Diphosphoramidophosphonouridine 5′-Monophosphate (**24**): ^{1}H-NMR (D_2O) δ: 5.94 (1H, d, $J_{1',2'}$=6.0 Hz, H-1′), 4.62 (1H, dd, $J_{1',2'}$=6.0 Hz, $J_{2',3'}$=5.0 Hz, H-2′), 4.78 (1H, ddd, $J_{2',3'}$=5.0 Hz, $J_{3',4'}$=5.0 Hz, $J_{P_\alpha,H-3'}$=8.7 Hz, H-3′), 4.34 (1H, ddd, $J_{3',4'}$=5.0 Hz, $J_{4',5'}$=2.0 Hz, $J_{4',5''}$=4.0 Hz, H-4′), 3.93 (1H, m, $J_{4',5'}$=2.0 Hz, $J_{4',5''}$=4.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5'}$=3.6 Hz, H-5′), 3.86 (1H, m, $J_{4',5'}$=2.0 Hz, $J_{4',5''}$=4.0 Hz, $J_{5',5''}$=11.5 Hz, $J_{P-5',H-5'}$=3.6 Hz, H-5″). ^{31}P-NMR (D_2O) δ: 0.8 (1P, dd, J_{P_α,P_β}=6.3 Hz, $J_{P_\alpha,H-3'}$=8.7 Hz, P_α), −10.0 (1P, dd, J_{P_α,P_β}=6.3 Hz, J_{P_β,P_γ}=20.8 Hz, P_β), −5.2 (1P, d, J_{P_β,P_γ}=20.8 Hz, P_γ), 4.2 (1P, dd, $J_{P-5',H-5'}$=3.6 Hz, $J_{P-5',H-5''}$=3.6 Hz, P-5′).

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