Kinetics and Mechanism of Facile and Selective Dephosphorylation of 2′-Phosphorylated and 2′-Thiophosphorylated Dinucleotides: Neighboring 3′−5′ Phosphodiester Promotes 2′-Dephosphorylation

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Received January 2, 1997[⊗]

2′-Phosphorylated and 2′-thiophosphorylated dinucleotides U(2′-p)pU (**1**) and U(2′-ps)pU (**2**) were found to undergo facile 2′-specific dephosphorylation at 90 °C in neutral aqueous solution to give UpU, and the first-order rate constants of these reactions were determined by HPLC. Particularly, U(2′-ps)pU (**2**, $k = 1.38 \pm 0.4 \times 10^{-3}$ s^{-1}, $t_{comp} = 1$ h) was cleanly dephosphorylated *ca.* 100 times more rapidly than U(2′-p)pU (**1**, $k = 1.41 \pm 0.05 \times 10^{-5}$ s^{-1}, $t_{comp} = 72$ h). Dephosphorylations of **1** and **2** were faster than those of thymidine 3′-phosphate (**8**) and thymidine 3′-thiophosphate (**9**), respectively. The kinetic data observed were independent of the 2′- or 3′-position of the phosphate group and the kind of base moiety. The neighboring 3′−5′ phosphodiester function most probably promotes the 2′-dephosphorylation efficiently. A branched trimer, U(2′-pU)pU (**3**), and related compounds having a substituent on the 2′-phosphoryl group, such as U(2′-pp-biotin)pU (**4**) and U(2′-ps-bimane)pU (**5**), were rather resistant to hydrolysis. The addition of divalent metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, and Cd^{2+}) remarkably decreased the rate of 2′-de(thio)phosphorylation of **1** or **2**. Among these metal ions, Zn^{2+} most significantly inhibited the dephosphorylation. On the contrary, trivalent metal ions considerably accelerated the 2′-de(thio)phosphorylation of **1** or **2**. The mechanism of 2′-dephosphorylation in the presence and absence of various metal ions is also discussed.

Introduction

In eukaryotic cells, RNAs transcribed from DNAs are modified at various sites, such as base residues,[1] 5′-terminal phosphate,[2] and hydroxyl groups,[3] to give mature functional molecules. Among these modifications, an unprecedented product that was phosphorylated at the 2′-hydroxyl function was first discovered by Konarska[4,5] in ligation products derived from circular RNA fragments of tobacco mosaic virus RNAΩ73. This 2′-phosphorylated RNA has a unique structure bearing two different neighboring phosphoryl groups on the 2′,3′-*cis*-diol of the nucleoside. Later, Abelson[6] also reported that a 2′-phosphorylated RNA was found in pre-tRNA^{Leu} during tRNA splicing, which is one of several nuclear RNA-processing events. Similar structures have been reviewed by Culbertson and Winey.[7] With respect to another biochemical property of the 2′-phosphate, a 2′-phosphotransferase playing an important role in tRNA splicing was found to transfer the 2′-phosphoryl function from 2′-phosphorylated pre-tRNA to NAD with elimination of nicotinamide to give an ADP-ribose 1″,2″-cyclic phosphate.[8]

Szostak and co-workers reported that, when the selection of the ribozymes bearing a 5′-thiophosphorylation activity was performed by the *in vitro* selection method, some of the products obtained were unexpectedly phosphorylated at the 2′-position.[9] Interestingly, they also disclosed that reverse transcription using a RNA oligomer having a 2′-thiophosphoryl group as the template caused a pause of reverse transcriptase, which ultimately read through the point.[10]

Quite recently, we have found on the basis of NMR spectroscopy and computational molecular modeling that diribonucleotides X(2′-p)pY (X, Y = U or A) having an inner 2′-phosphoryl group exist in a C2′-endo conformation like that seen in the DNA duplex.[11] Since the presence of the 2′-phosphoryl group in the spliced pre-tRNA is expected to inhibit molecular recognition of ARS at the amino acylation step, the chemical property of such

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1997.

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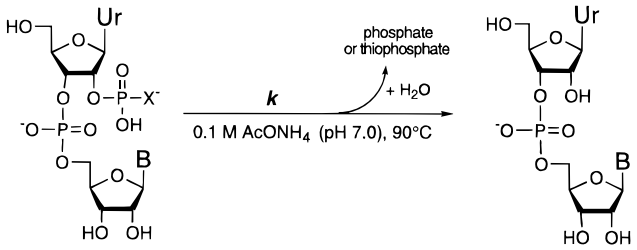
S0022-3263(97)00021-2 CCC: $14.00

Scheme 1



a 2′-phosphoryl group as well as the 3D-structure around the 2′-phosphorylated ligation site are of great importance. In our recent studies, an effective method for the polymer-supported synthesis of 2′-phosphorylated RNAs has already been established.[12−15] To our surprise, these studies disclosed that, when the T_m value of a RNA duplex having a 2′-phosphoryl group was measured, the 2′-phosphate group was hydrolyzed more rapidly than expected at neutral pH, 90 °C. (Scheme 1).

In the field of nucleic acid chemistry, several research groups have reported the acid or base hydrolysis of DNA/RNA as well as nucleotide monomers. More recently, Lönnberg and his co-workers[16−20] have reported detailed studies on the understanding of the behavior of internal and terminal phosphate esters in hydrolysis or migration. On the other hand, much interest has recently been paid to the effect of metal ions on hydrolysis or transesterification of phosphoester bonds of nucleic acids since it is well known that hydrolysis catalyzed by enzymes requires metal ions as cofactors.[21] Binding of metal ions to biomolecules also induces higher order structures. For example, the mature tRNA functions only in the presence of Mg^{2+} ion.[22] Hitherto, it has been known that the addition of metal ions, especially trivalent lanthanide ions, results in promoting the hydrolysis of phosphomono- or phosphodiester bonds in both DNA and RNA[20,23−26] as well as cyclic nucleotides.[26] A number of studies on site-specific cleavage of DNA/RNA using metal binding ligands have been reported.[27]

However, in spite of the importance of the 2′-phosphoryl group in molecular biology, no papers dealing with the fundamental chemistry of 2′-phosphorylated RNAs have been published. Moreover, a few are known about the basic hydrolytic property of alkyl thiophosphates or nucleoside thiophosphate derivatives. Breslow *et al*. first described that 4-nitrophenyl phosphorothioate underwent hydrolysis 63 times as rapidly as 4-nitrophenyl phosphate.[42] Eckstein observed that thymidine 5′-phosphorothioate was hydrolyzed to thymidine to a degree of 67% on heating in 80% acetic acid at 100 °C for 40 min.[40a] Quite recently, Lönnberg *et al*.[19b] revealed the dethiophosphorylation rate of nucleoside phosphorothioate mono- and diesters within an acidic pH range at 90 °C for the first time in nucleic acid chemistry, as far as the kinetic study was concerned.

In this paper, we wish to report the detailed site-specific dephosphorylation or dethiophosphorylation of 2′-phosphorylated or 2′-thiophosphorylated uridylyl(3′-5′)-uridine [U(2′-p)pU (**1**) or U(2′-ps)pU (**2**)] and related compounds under thermal but neutral conditions. The present paper also describes the kinetics and mechanism of these reactions in the presence or absence of metal ions.

Results and Discussion

Hydrolytic 2′-Dephosphorylation of U(2′-p)pU (1). In our continuing studies on chemical synthesis and properties of 2′-phosphorylated RNAs, we found that the 2′-phosphorylated uridylate dimer U(2′-p)pU (**1**) underwent facile site-specific dephosphorylation upon simple heating in aqueous solution to give the corresponding UpU dimer (**12a**) (Scheme 2). The detailed study showed that this reaction proceeded in 0.1 M ammonium acetate (pH 7.0) at 90 °C to give **12a** in *ca*. 80% yield along with small amounts of hydrolyzed and isomerized products [**14a**,**b** (<5%) and **12b** (*ca*. 10%)] derived from UpU after 49 h, as shown in Figure $1A_2$.

In this reaction, the 2′-phosphomonoester bond was first cleaved, since products such as U(2′-p)p (**16a**) directly hydrolyzed at the internal phosphodiester bond could not be detected (see Supporting Information for more detail). As the disappearance of U(2′-p)pU (**1**) strictly obeyed first-order kinetics, the rate constant (*k*)

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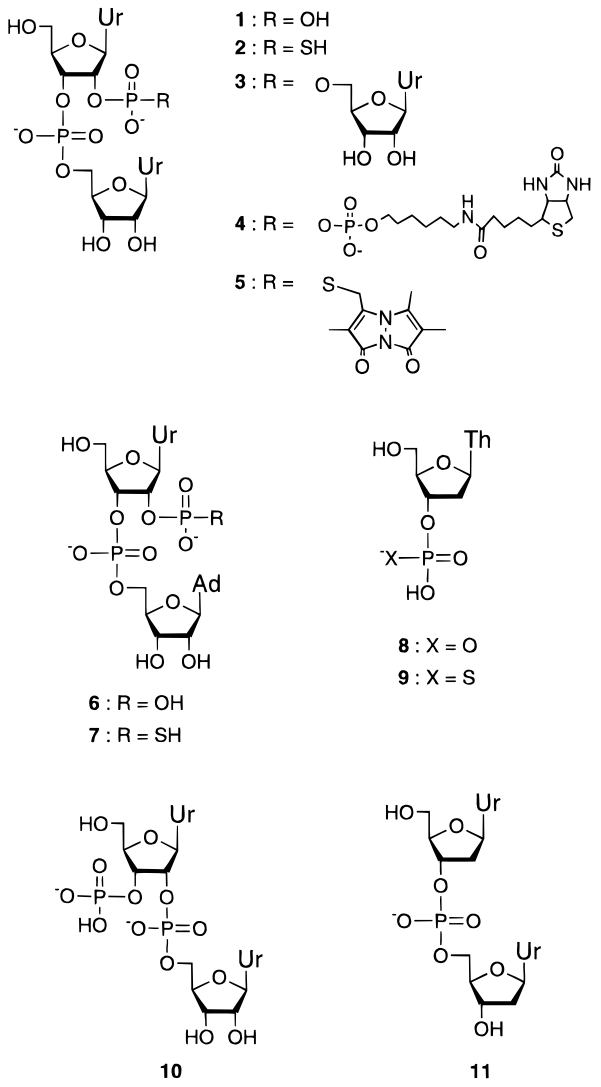
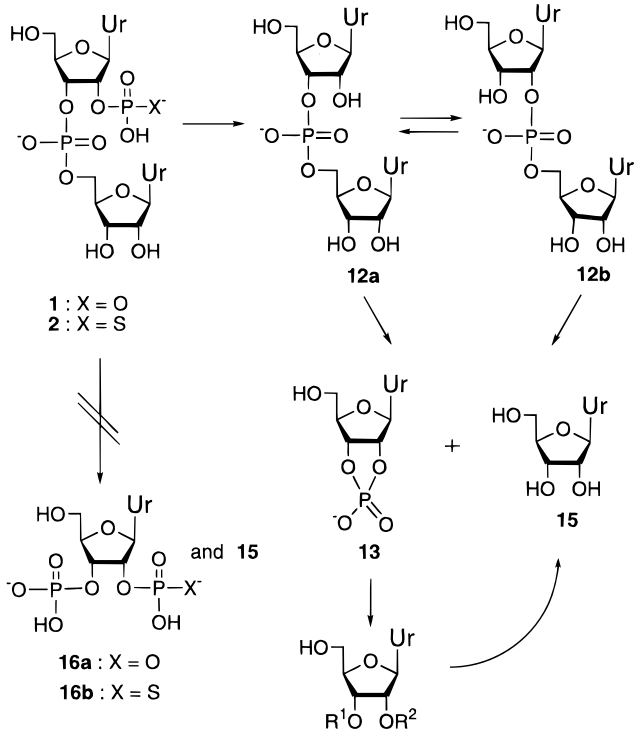
Chart 1



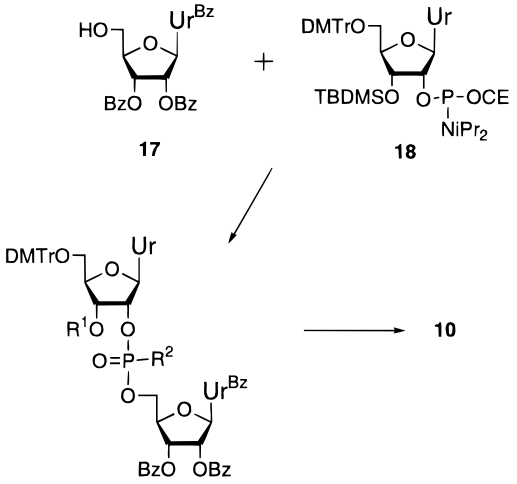
of this 2′-dephosphorylation was calculated to be $1.41 \pm 0.05 \times 10^{-5}$ s^{-1} (t_{comp} = 72 h) by HPLC analysis. To study the mutual effect between the proximal 2′- and 3′-bisphosphoryl groups of **1**, the rate constant of thymidine 3′-phosphate (**8**, Tp) as a reference compound was also determined under the same conditions to be $7.5 \pm 0.2 \times 10^{-5}$ s^{-1}. Consequently, it turned out that 2′-dephosphorylation of U(2′-p)pU (**1**) occurred about 2 times faster than that of Tp (**8**) and considerably faster than that of any other simple monoalkyl phosphates and nucleoside monophosphates reported previously.[16,20b,28]

Next, 3′-dephosphorylation of U(3′-p)pU (**10**), in which the 2′- and 3′-substituents of **1** were interchanged, was examined to study the effect of the regioisomer on the hydrolysis of the 3′-phosphate group. U(3′-p)pU (**10**) was prepared by the use of 3′-phosphorylation of appropriately protected U(2′−5′)pU derivative (**19b**) having a 2′-5′ phosphodiester linkage followed by deprotection. This kind of phosphorylation was first reported by Caruthers and Kierzek.[29] In connection with the 2′-phosphorylation,

Scheme 2



Scheme 3



Chattopadhyaya and his co-workers reported the bis-(cyanoethyl) phosphorylation as an intermediate for the synthesis of branched RNA (Scheme 3).[30] Quite recently, Kierzek also reported the synthesis of U(2′-p)pU (**1**) by this reaction.[31]

Table 3 shows that there was little regiospecificity in hydrolysis between **1** and **10** since the rate constant of

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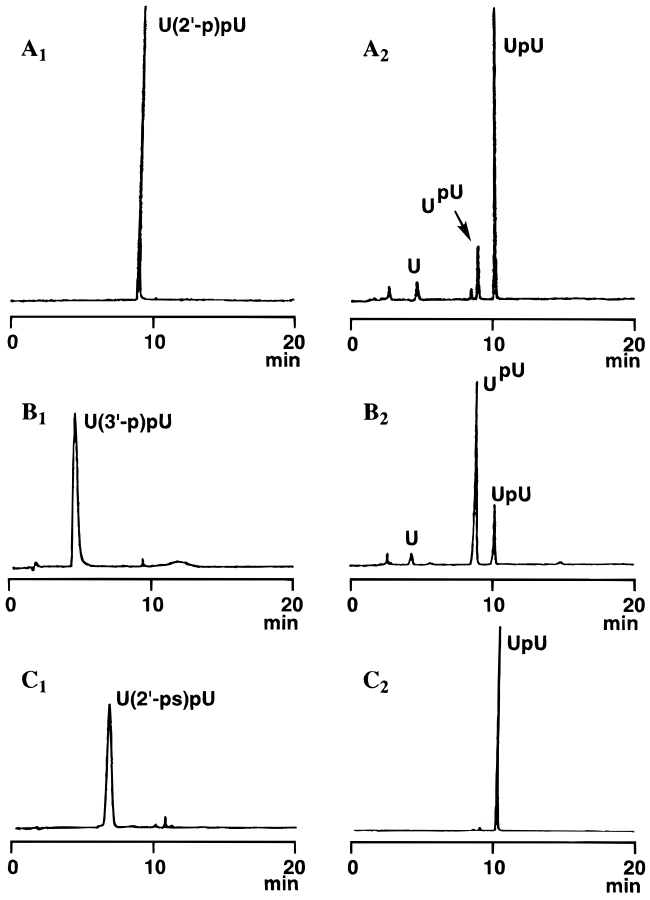

Figure 1. Reversed phase HPLC profiles of the mixtures obtained by the thermolysis of U(2′-p)pU (**1**) (A, after 71 h), U(3′-p)pU (**10**) (B, after 1 h), and U(2′-ps)pU (**2**) (C, after 72 h) in 0.1 M $AcONH_4$ (pH 7.0) at 90 °C. The UV detection was performed at 254 nm.

the dephosphorylation of **10** was similar to that of U(2′-p)pU (**1**) (Figure 1B). The reason why the *k* value of U(3′-p)pU (**10**) was slightly greater than that of **1** might be ascribed to the difference in basicity between the 2′ and 3′-oxygens of **1** and **10**.

Interaction between the uracil residue and the 2′-phosphoryl group should also be taken into account. The 2′-phosphoryl group of U(2′-p)pU (**1**) may be sterically associated with the 3′-downstream base moiety. However, the kinetic data of U(2′-p)pA (**6**), which was prepared by our method reported previously[15] (Scheme 4), did not support this possibility, as shown in Table 4.

Accordingly, the promoted dephosphorylation was only due to the effect of a neighboring 3′−5′ phosphodiester. The rate enhancement promoted by a proximal phosphodiester was most probably attributed to protonation on the 2′-esterified oxygen atom through route B depicted in Scheme 5. A similar mechanism has been quite recently proposed by our laboratory, suggesting that the 3′−5′ phosphodiester promotes the hydrolytic deprotection of the 2′-*tert*-butyldimethylsilyl group in synthetic RNA oligomers under acidic conditions due to the enhancement of protonation on the oxygen of the 2′-silyl ether bond.[32]

In order to examine the relative effect of the 3′,5′-phosphodiester function on the 2′-hydrolysis in more

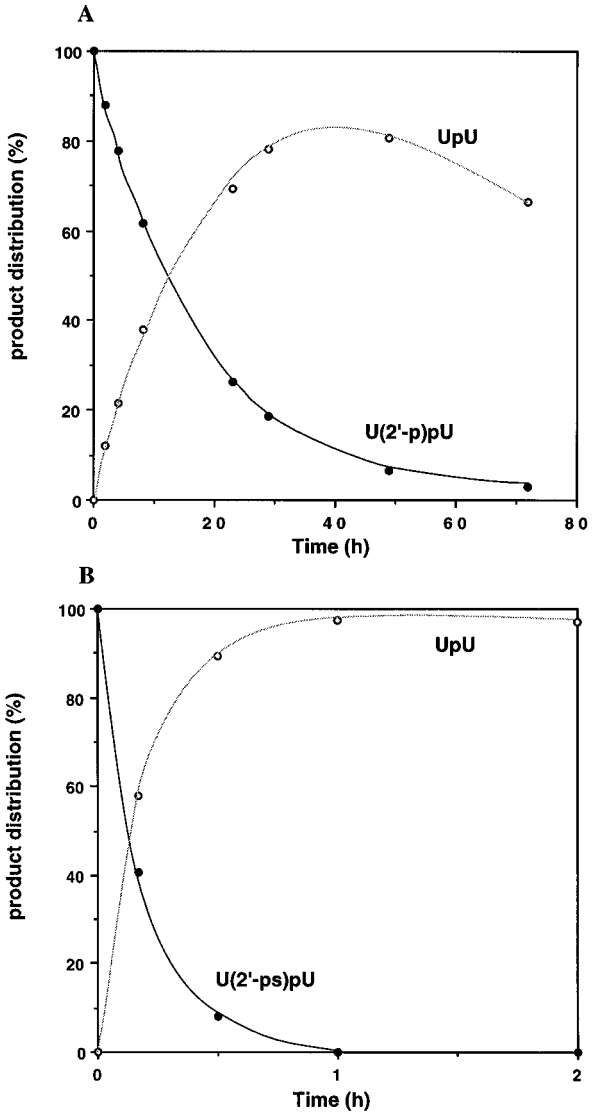

Figure 2. Time-dependent product distribution for de(thio)-phosphorylation of U(2′-p)pU (**1**) (A) and U(2′-ps)pU (**2**) (B) in 0.1 M $AcONH_4$ (pH 7.0) at 90 °C. The concentration of **1** or **2** was 1.5×10^{-4} mol dm^{-1}; (●) **1** or **2**, (○) UpU (**13a**).

detail, the related compounds bearing substituents on the 2′-phosphoryl group, such as 2′-phosphodiester (**3**), pyrophosphate (**4**), and thiophosphodiester (**5**) derivatives, were investigated in the same way (Chart 1).

First, to study the thermal stability of the 2′-phosphodiester derivative U(2′-pU)pU (**3**), it was synthesized by the use of a bifunctional 2′,3′-bisphosphoramidite derivative reported by Damha and Ogilvie.[33] In fact, U(2′-pU)pU (**3**) having two vicinal phosphodiester linkages was hardly hydrolyzed even after 1 week, similarly to the case of d(UpU) (**11**) with an internucleotidic bond which is very stable in neutral conditions (Figure 3A). Furthermore, a 2′-dialkyl pyrophosphate derivative, U(2′-pp-biotin)pU (**4**), was also unexpectedly fairly stable as can be seen in Figure 3B. The 2′-thiophosphodiester derivative **5** was not as stable as U(2′-pp-biotin)pU (**4**), and its decomposition profile was complicated. (Figure 3C). However, it was more stable than U(2′-p)pU (**1**). The mechanism of hydrolysis of these compounds was as-

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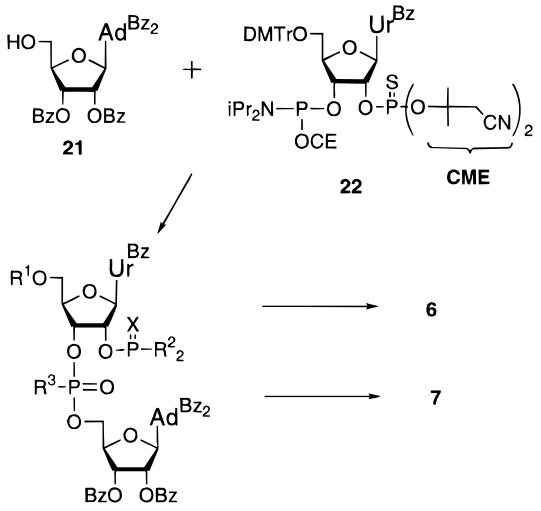
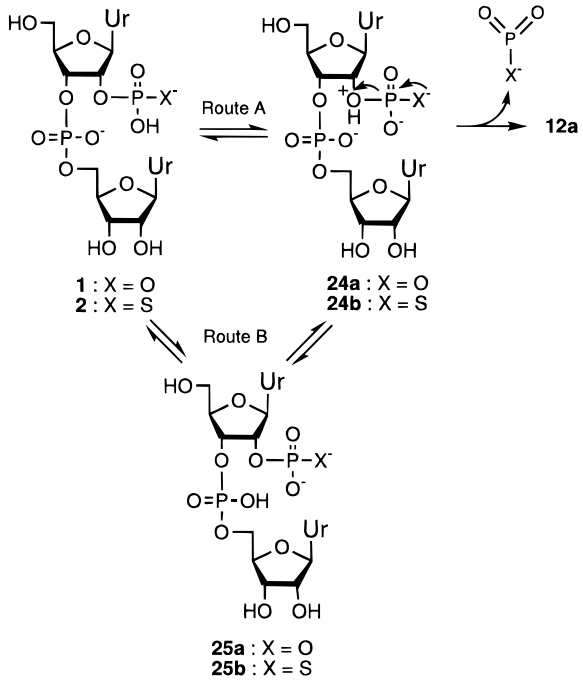
Scheme 4



23a : R^1 = DMTr, R^2 = OCME, R^3 = OCE, X = S
23b : R^1 = DMTr, R^2 = $OSiMe_3$, R^3 = $OSiMe_3$, X = S
23c : R^1 = DMTr, R^2 = O^-DBUH^+, R^3 = O^-DBUH^+, X = S
23d : R^1 = DMTr, R^2 = O^-DBUH^+, R^3 = O^-DBUH^+, X = O
23e : R^1 = H, R^2 = OCME, R^3 = OCE, X = S
23f : R^1 = H, R^2 = $OSiMe_3$, R^3 = $OSiMe_3$, X = S
23g : R^1 = H, R^2 = O^-DBUH^+, R^3 = O^-DBUH^+, X = S

Scheme 5



sumed to be a nucleophilic substitution on phosphorus to give the usual pentacoordinated phosphorus intermediates.[34] Accordingly, the rate of hydrolysis was considerably slow. The substituted 2′-phosphoryl group of this kind was different from that of the 2′-phosphomonoester types as far as their stability and mechanism are concerned.

Hydrolytic 2′-Dephosphorylation of U(2′-ps)pU. As a substrate for the present study, 2′-thiophosphorylated uridylate dimer U(2′-ps)pU (**2**) was also examined. This compound was prepared according to the procedure reported previously.[15] Dephosphorylation of U(2′-ps)pU

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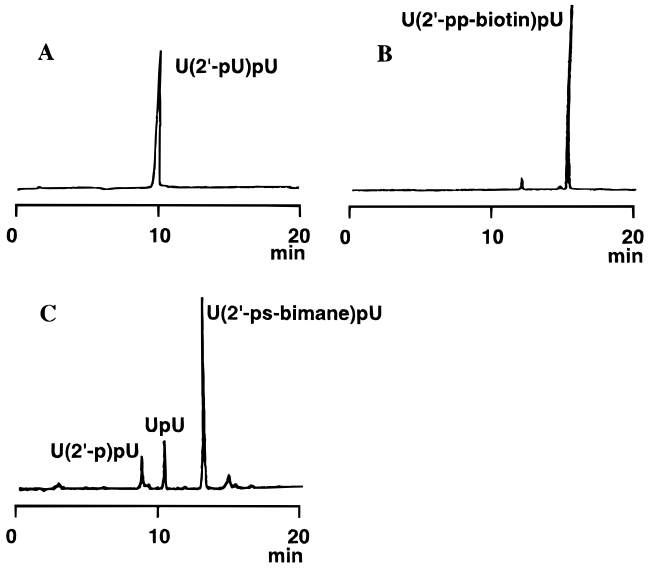

Figure 3. Reversed phase HPLC profiles of the mixtures obtained by the thermolysis of 2′,3′-bisphosphorylated derivatives U(2′-pU)pU (**3**) (A, after 168 h), U(2′-pp-biotin)pU (**4**) (B, after 24 h), and U(2′-ps-bimane)pU (5) (C, after 24 h). The UV detection was performed at 254 nm.

Table 1. First-Order Rate Constants for Dephosphorylation of 1 and 8 in the Presence and Absence of Metal Ions at pH 7.0, 90 °C

	U(2′-p)pU (**1**)		Tp (**8**)	
additives	k (10^{-6} s^{-1})	$t_{1/2}$ (h)	k (10^{-6} s^{-1})	$t_{1/2}$ (h)
none[a]	14.1 ± 0.5[c]	13.6	7.5 ± 0.2	25.7
Ca^{2+}	13.3 ± 0.1	14.5		
Mg^{2+}	11.8 ± 1.0	16.4	6.1 ± 0.2	31.6
Co^{2+}	10.3 ± 0.5	18.6		
Cd^{2+}	10.1 ± 0.4	19.0		
Mn^{2+}	2.9 ± 0.2	66.9	4.3 ± 0.2	44.4
Zn^{2+}	2.3 ± 0.2	84.0	5.2 ± 0.8	36.9
La^{3+}	25.9 ± 0.4	7.4	5.4 ± 0.5	35.9
Y^{3+}	16.9 ± 0.1	11.4	5.3 ± 0.5	36.8
phosphate buffer[b]	11.2 ± 0.2	17.2		

[a] 0.1 M $AcONH_4$ (pH 7.0) was used. [b] 10 mM sodium phosphate buffer (pH 7.0) was used as the solvent. [c] Standard deviation.

Table 2. First-Order Rate Constants for Dethiophosphorylation of 2 and 9 in the Presence and Absence of Metal Ions at pH 7.0, 90 °C

	U(2′-ps)pU (**2**)		Tps (**9**)	
additives	k (10^{-6} s^{-1})	$t_{1/2}$ (h)	k (10^{-6} s^{-1})	$t_{1/2}$ (h)
none[a]	1380 ± 40[c]	0.14	230 ± 10	0.82
Mg^{2+}	710 ± 60	0.27	210 ± 30	0.90
Mn^{2+}	250 ± 10	0.76	190 ± 10	0.99
Zn^{2+}	56 ± 8	3.5	60 ± 3	3.19
phosphate buffer[b]	820 ± 40	0.24		

[a] 0.1M $AcONH_4$ (pH 7.0) was used. [b] 10 mM sodium phosphate buffer (pH 7.0) was used as the solvent. [c] Standard deviation.

(**2**) proceeded site-specifically like that of U(2′-p)pU (**1**) and was completed *in only 1 h* at 90 °C.

Interestingly, we found that the dephosphorylation of U(2′-ps)pU (**2**) was much more rapid (k = 1.38 ± 0.4 × 10^{-3} s^{-1}) than that of U(2′-p)pU (**1**). In general, monoalkyl thiophosphates are known to be more unstable than the corresponding phosphates.[41,42] Lönnberg *et al.*[19c] also reported that uridine 2′- and 3′-thiophosphate (2′/3′-UMPS) underwent dethiophosphorylation to give uridine 200−300-fold more rapidly than dephosphorylation of the corresponding phosphate. Moreover, they disclosed that dethiophosphorylation of 2′/3′-UMPS did not accompany

Table 3. First-Order Rate Constants for Dephosphorylation of 10 in the Presence and Absence of Metal Ions at pH 7.0, 90 °C

	U(3′-p)pU (**10**)	
additives	k (10^{-6} s^{-1})	$t_{1/2}$ (h)
none[a]	15.2 ± 0.4[b]	12.7
Mg^{2+}	10.4 ± 0.3	18.4
Mn^{2+}	4.2 ± 0.3	45.5
Zn^{2+}	3.8 ± 0.1	50.4

[a] 0.1 M $AcONH_4$ (pH 7.0) was used. [b] Standard deviation.

desulfurization at all over an acidic pH range (pH 2−7),[19c] although UpsU was desulfurized over the hydrolytic cleavage of the internucleotidic linkage in the thermal hydrolysis within a similar pH range. No desulfurization of (**2**) giving rise to **1** similarly occurred under these conditions. It was also observed that U(2′-ps)pU (**2**) was hydrolyzed *ca.* 6 times faster than thymidine 3′-thiophosphate (**9**, Tps) at pH 7.0 as depicted in Table 2 and *ca.* 22 times faster than uridine 2′-thiophosphate ($k = ca.\ 6 \times 10^{-5}$) at pH 6.5 as reported by Lönnberg *et al.*[19c] Noteworthy is the very simple reaction shown in Figure 1C, which illustrates the HPLC profile of the reaction. In fact, the present facile dephosphorylation of **2** produced UpU (**12a**) in 98% yield after 1 h. This result suggests a new possibility that the thiophosphoryl group might serve as a tentative 2′-hydroxyl-protecting group as well as a unique thermally removable group.

On the other hand, U(2′-ps)pA (**7**) in which the 3′-downstream nucleoside of **2** was replaced with adenosine was synthesized and subjected to hydrolysis. As a result, there is only a slight difference in the rates of dethiophosphorylation between **2** and **7** as mentioned in the dephosphorylation of **1** and **6**.

pH Dependency of 2′-Dephosphorylation of U(2′-p)pU and U(2′-ps)pU. The kinetic rates of 2′-dephosphorylation of **1** and **2** in the range of pH 2−8 are depicted in Figure 4. As shown in Figure 4A, the dephosphorylation rate of **1** at 90 °C was highest at near pH 4 and almost in proportion to the concentration of the monoanion species of **1**. This result strongly suggested that the rate of 2′-dephosphorylation should depend on the population of this species as described in the hydrolysis of phosphomonoesters.[28a,34] A detailed mechanism of 2′-dephosphorylation is described below. Dethiophosphorylation of U(2′-ps)pU (**2**) at 90 °C was too rapid to measure the kinetic rate (t_{comp} < 10 min at pH 5.8, 90 °C), so that the reaction was carried out at 70 °C. As shown in Figure 4B, the pH dependency of dethiophosphorylation of U(2′-ps)pU (**2**) also gave a similar result, showing the highest rate at pH 4.0. However, the rate constant varied considerably compared with that of **1**. For instance, the k value of dephosphorylation of **2** at pH 4 ($k = 7.4 \pm 0.4 \times 10^{-4}$ s^{-1}) was *ca.* 23 times greater than that at pH 8 ($k = 3.2 \pm 0.1 \times 10^{-5}$ s^{-1}), while **1** showed a lesser difference of 1.6 times.

Mechanism of 2′-Dephosphorylation of U(2′-p)pU and U(2′-ps)pU. The dissociative mechanism proposed for the typical hydrolysis of general phosphomonoesters has been reported as follows:[16a,b,28] Transfer of a proton from the hydroxyl ligand of a phosphomonoester to the esteric oxygen atom results in hydrolysis to an alcohol and metaphosphate through the P−O bond fission. The reactive species is a monoanionic phosphoester that is in equilibrium with the esteric *O*-protonated form (**24a**) via route A depicted in Scheme 3. The pH range most effective for the intramolecular protonation is known to

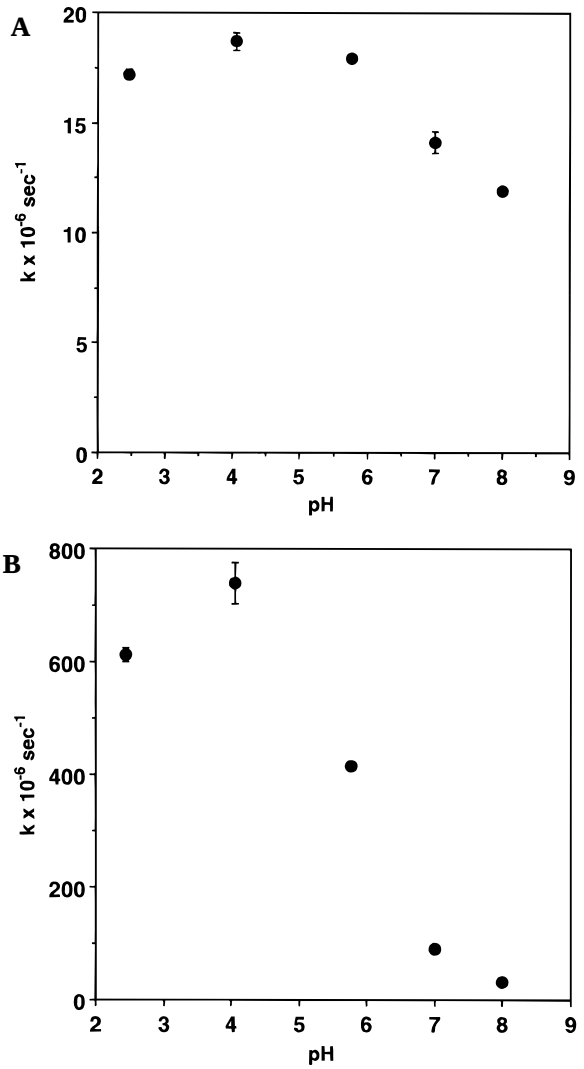

Figure 4. pH dependency of the de(thio)phosphorylation rate of U(2′-p)pU (**1**) at 90 °C (A) and U(2′-ps)pU (**2**) at 70 °C (B). Conditions: 10 mM NaH_2PO_4 (pH 2.5 and 5.8), 0.1 M citric acid−0.2 M Na_2HPO_4 (pH 4.0), 0.1 M NH_4OAc (pH 7.0), 0.1 M Tris-HCl (pH 8.0).

be near pH 4.[28a] However, it was noteworthy that the present dephosphorylation of U(2′-p)pU (**1**) proceeded smoothly even at pH 7.0. Lönnberg and co-workers reported that the rate constant of uridine 2′-phosphate in its hydrolysis at pH 5.6 was $0.87 \pm 0.02 \times 10^{-5}$ s^{-1}.[20b] This finding and our present results suggested that the facile dephosphorylation of **1** should involve a somewhat different mechanism due to the neighboring group participation of the 3′−5′ phosphodiester or the base moiety and the substituent effect around the 2′-phosphate group. Particularly, the proton transfer of the 2′-phosphate via route B involving the help of the 3′−5′ phosphodiester oxygen should be considered as a plausible explanation of the dephosphorylation of **1** based on the above experiment.

It is known that the sulfur atom in thiophosphate esters stabilizes a transition state in the dissociative mechanism and destabilizes that of the associative mechanism.[43] Accordingly, the P−O split of the protonated intermediate **24a** to give the hydrolyzed product requires more activation energy than that of **24b**. Since this knowledge is in good agreement with the present result that **2** undergoes the 2′-dephosphorylation more rapidly than **1**, both the reactions were similarly explained in

terms of the mechanism via metaphosphate[35] or metathiophosphate.[36,19c]

Effect of Metal Ions on Hydrolytic 2′-Dephosphorylation. As the 2′-phosphorylated and 2′-thiophosphorylated ribonucleotides **1** and **2** have two different proximal phosphoryl groups bearing several binding sites of various metal ions, the effect of these compounds on the 2′-dephosphorylation rate in the presence of metal ions was expected. We have chosen several metal ions which can be completely dissolved in 0.1 M ammonium acetate to avoid inherent heterogeneous effects. Table 2 summarizes the kinetic data of the 2′-dephosphorylation of these compounds in the presence of various metal ions as additives. Similar hydrolytic dephosphorylations using Tp (**8**) and Tps (**9**) were studied as control experiments. Kuusela and Lönnberg reported that some di- and trivalent metal ions promoted the hydrolysis of ribonucleoside phosphoesters under slightly acidic conditions without enhancement of intramolecular phosphate migration.[20b] In marked contrast to these observations, it was interestingly found that, at the neutral pH we used in this study, the rate constants of Tp (**8**) were slightly decreased by addition of various divalent metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, and Cd^{2+}), as can be seen in Table 1. Compared with these results, the rate constants of the 2′-dephosphorylation of U(2′-p)pU (**1**) in the presence of metals considerably decreased under the same conditions. Among the metal ions tested, Mn^{2+} and Zn^{2+} ions inhibited the hydrolysis most effectively. It might be concluded that apparently the proximal 3′−5′ phosphodiester anion strongly interferes with this hydrolysis.

On the other hand, the 2′-phosphomonoester hydrolysis of U(2′-p)pU (**1**) in the presence of trivalent metal ions[20a,b,24,25] (La^{3+} and Y^{3+}) was markedly accelerated, but the 3′-phosphomonoester hydrolysis of Tp (**8**) was not affected significantly. In any case, the produced UpU (**12a**) was rapidly hydrolyzed to uridine **15** and Up **14a**,**b** followed by further dephosphorylation of **14a**,**b** to uridine **15**. Consequently, the HPLC profiles of dephosphorylation of U(2′-p)pU (**1**) observed showed a large amount of uridine **15**.

Furthermore, the 2′-dethiophosphorylation of U(2′-ps)pU (**2**) was more greatly suppressed by divalent metal ions than the 2′-dephosphorylation of U(2′-p)pU (**1**). Particularly, addition of Zn^{2+} ion to U(2′-ps)pU (**2**) led to a severe repression with a $^1/_{25}$ rate constant compared with the hydrolysis of U(2′-ps)pU (**2**) in the absence of metal ions, while there is a difference of only *ca*. 4 times in the rate constant between the hydrolysis reactions of Tps measured in the presence and absence of Zn^{2+} ion. It is known that Zn^{2+} ion exists in the active site of alkaline phosphatases as a cofactor.[37] Kimura *et al.* have recently reported enzyme models having Zn^{2+} chelates for dephosphorylation of alkyl monophosphates.[38]

Divalent metal ions such as Mn^{2+} and Zn^{2+} could each bind to two proximate phosphoryl groups. Therefore, the formation of a relatively stable nine-membered ring containing the two phosphoryl groups and a divalent metal ion could probably interrupt the decomposition of the intermediate protonated on the 2′-esteric oxygen atom. According to Schwing-Weil's recent finding, a phenol derivative, in which two phosphonate groups are bound to the 2- and 5-positions, forms a rigid complex with Zn^{2+} and Ni^{2+} ions.[39]

Table 4. First-Order Rate Constants of Hydrolysis in the Presence and Absence of Metal Ions at pH 7.0, 90 °C (k, 10^{-6} s^{-1})

additives	2	3	4	6	7	11
none[a]	nd[d]	nd	*b*	15.1 ± 0.1[c]	1450 ± 30	nd
Mg^{2+}	nd					nd
Mn^{2+}	nd					nd
Zn^{2+}	nd					nd

[a] 0.1 M $AcONH_4$ (pH 7.0) was used. [b] The reaction was complicated. [c] Standard deviation. [d] The term "nd" indicates that the hydrolyzed product could not be detected.

Conclusions

The results of dephosphorylation of Tp (**8**) and Tps (**9**) as reference compounds under neutral conditions showed that phospho- or thiophosphomonoester derivatives were not essentially more stable than phosphodiesters. Especially, hydrolytic dephosphorylation of thiophosphomonoester Tps (**9**) occurred rapidly. In the present study, U(2′-p)pU (**1**) and U(3′-p)pU (**10**), having both the vicinal phosphodiester and phosphomonoester bonds, were found to undergo more facile dephosphorylation only at the monoester site than the other usual phosphomonoesters due to acid catalysis by the neighboring effect of the phosphodiester function. The most rapid hydrolysis was observed in the case of U(2′-ps)pU. One of the reasons for the promoted de(thio)phosphorylation might be explained by the fact that the 2′,3′-bisphosphoryl groups are fixed rigidly by the five-membered ring sugar in the case of RNAs.

Although a lot of nucleoside phosphorothioates of the monoester type have been synthesized[40] and utilized in biological applications such as inhibitors and enzyme-resistant substrates,[41] the chemical properties of these materials have not been elucidated until quite recently, especially as far as the basic kinetics of their hydrolytic behavior are concerned. Since Breslow and Katz[42] first noted that thiophosphate monoesters undergo nucleophilic displacement reactions more rapidly than their oxy counterparts, few studies on their displacement reactions involving hydrolysis have appeared to date.[34] Cullis and co-workers reported the stereochemical thiophosphoryl-transfer reaction of ^{18}O-labeled 4-nitrophenyl phosphorothioate in ethanol[36a] and also noted the hydrolysis of 2,4-dinitrophenyl phosphorothioate at pH 12.7 giving the rate constant of 6.9×10^{-4} s^{-1}. [36b] Lönnberg and his co-workers[19b] described that, in the thermal hydrolysis of UpsU at pH 6−8 at 363.2 K, uridine 2′- and 3′-phosphorothioates accumulated in addition to the products obtained under more acidic conditions, but dephosphorylation of these compounds was not described probably because of the very complicated distribution of products as suggested in their paper.[19b] Later, they disclosed the

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detailed and basic kinetic data of hydrolytic dethiophosphorylation of uridine 2′- and 3′-thiophosphates under similar conditions in their subsequent paper.[19c]

It is anticipated that new methodologies using this characteristic of the thiophosphate group will be developed especially in molecular biology, since thiophosphoryl-transfer reactions have been established by using some enzymatic systems.[41c,44] In consideration of these facts, possibly in extreme thermophilic bacteria, such as *Thermus aquaticus* and *Thermus thermophilus*, 2′-phosphorylated RNA species might not be found in cells, since spliced tRNA containing a 2′-phosphate should be dephosphorylated by the above-mentioned thermal dephosphorylation as well as a tRNA-specific phosphatase. In fact, the reason why there are only a few discoveries of 2′-phosphorylated RNA species to date may be supported by the dephosphorylation described above.

Experimental Section

General Methods. ^{1}H NMR spectra were measured on JEOL-EX 270 and Varian Unity 400 spectrometers at 270 and 400 MHz with TMS (for $CDCl_3$) or DDS (for D_2O) as internal standard. ^{13}C NMR spectra were obtained on JEOL-EX 270 and Varian Unity 400 spectrometers at 67.8 and 100.6 MHz, respectively, with $CHCl_3$ (for $CDCl_3$) as internal standard or dioxane (for D_2O) as external standard. ^{31}P NMR spectra were recorded on a JEOL-EX 270 spectrometer at 109 MHz with the external reference of 85% H_3PO_4. UV spectra were taken on a HITACHI U-2000 spectrophotometer. Reversed phase HPLC was performed on a Waters LC module 1 with a μBondasphere 5 μm C18 100 Å (3.9 × 150 mm) column using a linear gradient of acetonitrile (0−30%) in 0.1 M ammonium acetate buffer (pH 7.0) for 30 min at flow rate of 1 mL/min at 50 °C. Ion exchange HPLC was carried out at a flow rate of 1 mL/min for 30 min at 50 °C on a Whatman Partisil 10 SAX WCS analytical column (4.6 × 250 mm) using a linear gradient of 0−100% solution B (20% CH_3CN in 0.005 M KH_2PO_4) in solution A (20% CH_3CN in 0.5 M KH_2PO_4). Paper chromatography was carried out by use of a descending technique with Whatman 3MM Chr papers using iPrOH−concd NH_3−H_2O (6:1:3, v/v/v). Thin-layer chromatography was performed using Merck Kieselgel 60F-254 (0.25 mm) with developing solvent of CH_2Cl_2−CH_3OH (9:1, v/v) or iPrOH−concd NH_3−H_2O (7:1:2, v/v/v). Column chromatography was performed with silica gel C-200 purchased from Wako Co. Ltd., and a minipump for a goldfish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation. Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology, Nagatsuta, Japan.

Synthesis of Fully Protected 2′-Phosphorylated Dinucleotide U(2′-p)pA (23a). 2′,3′-*O*,4-*N*,4-*N*-tetrabenzoyladenosine **22** (32 mg, 0.05 mmol) and 1*H*-tetrazole (7 mg, 0.1 mmol) were rendered anhydrous by repeated coevaporation with dry pyridine (1 mL × 3) and toluene (1 mL × 1) and finally dissolved in CH_3CN (0.5 mL). To the mixture was added **23** (61 mg, 0.055 mmol). After being stirred for 3 h, the mixture was concentrated under reduced pressure and treated with iodine (127 mg, 0.5 mmol) in pyridine−H_2O (9:1, v/v, 1 mL) for 1 h. The mixed solution was evaporated in vacuo, and the residue was dissolved in CH_2Cl_2 (10 mL) and washed with saturated $Na_2S_2O_3$ (10 mL) and water (10 mL). After the organic layer was dried over Na_2SO_4, the solution was filtered and concentrated under reduced pressure. Silica gel column chromatography was performed with hexane−ethyl acetate (2:3, v/v) containing 0.5% pyridine to give the fully protected dimer **23a** (73 mg, 85%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.50−1.72 (m, 12H), 2.62−2.85 (m, 6H), 3.44−3.59 (m, 2H), 3.76−3.81 (m, 6H), 4.16−4.37 (m, 2H), 4.49−4.76 (m, 4H), 5.29−5.40 (m, 2H), 5.47−5.52 (m, 1H), 6.03−6.26 (m, 2H), 6.33, 6.35 (2d, J = 6.3, 5.3 Hz, 1H), 6.50, 6.52 (2d, J = 5.3, 4.6 Hz, 1H), 6.84−6.89 (m, 4H), 7.16−7.69 (m, 24H), 7.83−8.00 (m, 11H), 8.36, 8.41 (2s, 1H), 8.68, 8.69 (2s, 1H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 19.48, 19.57, 20.40, 26.67, 26.72, 26.76, 26.92, 26.96, 27.17, 27.23, 31.45, 31.50, 31.56, 31.74, 31.77, 55.22, 55.26, 62.79, 62.84, 62.88, 62.91, 63.00, 70.78, 73.87, 74.05, 81.24, 83.02, 83.06, 83.18, 83.29, 83.79, 83.85, 83.97, 86.61, 86.92, 88.00, 102.79, 113.50, 116.48, 116.60, 116.64, 116.91, 125.25, 127.33, 127.71, 128.10, 128.18, 128.23, 128.43, 128.52, 128.64, 128.77, 128.90, 128.99, 129.08, 129.42, 129.76, 129.85, 130.14, 130.23, 130.64, 131.32, 133.05, 133.80, 133.87, 133.94, 134.45, 134.48, 134.74, 135.04, 137.83, 140.14, 140.20, 143.49, 143.76, 149.29, 152.13, 152.51, 152.72, 158.83, 161.87, 165.00, 165.14, 165.21, 165.26, 168.43, 172.18, 172.25; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ −2.20, −1.92 (2s, 1P), 51.2 (s, 1P). Anal. Calcd for $C_{88}H_{80}N_{10}O_{21}P_2S$: C, 61.90; H, 4.72; N, 8.20; S, 1.88. Found: C, 62.03; H, 5.02; N, 7.77; S, 1.61.

Deprotection of Fully Protected 2′-Phosphorylated Dinucleotide U(2′-p)pA (6). The fully protected dinucleotide U(2′-p)pA (**23a**) (29 mg, 0.017 mmol) was dissolved in pyridine (0.5 mL) after repeated coevaporation with dry pyridine (2 mL × 3). To the solution were added BSA (0.1 mL, 0.4 mmol) and DBU (0.05 mL, 0.05 mmol), and the mixture was stirred for 10 min at room temperature. To the mixture was added water (0.05 mL). After being stirred for a few minutes, the solution was treated with iodine (44 mg, 0.17 mmol). After being stirred for an additional 48 h, the mixture was diluted with water. Then, extraction was performed by use of H_2O (2 mL)−Et_2O (2 mL), and the aqueous layer was concentrated under reduced pressure. The residue was dissolved in concd NH_3−pyridine (9:1, v/v, 5 mL). After stirring for 20 h, the mixture was coevaporated with water, and the resulting residue was treated with 80% acetic acid (5 mL) for 15 min. The solution was concentrated under reduced pressure, and then the residue was dissolved in water (2 mL) and washed with Et_2O (2 mL). The aqueous layer was collected and chromatographed on Whatman 3MM Chr papers with iPrOH−concd NH_3−H_2O (6:1:3, v/v/v) to give pure material **6** (248 A_{260}, 58%): ^{1}H NMR (400 MHz, D_2O, DSS) δ 3.69 (dd, J = 3.4, 12.8 Hz, 1H), 3.73 (dd, J = 12.8 Hz, 1H), 4.17 (ddd, J = 4.1, 5.9, 11.8 Hz, 1H), 4.21 (ddd, J = 2.9, 5.2, 11.7 Hz, 1H), 4.25 (ddd, J = 2.6, 3.4, 4.0 Hz, 1H), 4.37 (ddd, J = 2.9, 4.1, 4.3 Hz, 1H), 4.56 (dd, J = 4.3, 5.2 Hz, 1H), 4.71 (ddd, J = 2.6, 5.2, 7.3 Hz, 1H), 4.77 (m, 1H), 4.79 (dd, J = 5.2, 5.6 Hz, 1H), 5.85 (d, J = 8.0 Hz, 1H), 5.94 (d, 1H, J = 6.7 Hz), 6.10 (d, 1H, J = 5.6 Hz), 7.75 (d, 1H, J = 8.0 Hz), 8.24 (s, 1H), 8.45 (s, 1H); ^{13}C NMR (100.6 MHz, D_2O, DSS) δ 63.80, 66.08, 72.93, 76.48, 76.56, 76.96, 86.60, 86.63, 89.80, 90.57, 105.17, 142.60, 145.38, 151.90, 154.40, 155.59, 158.32, 168.92; ^{31}P NMR (109 MHz, D_2O, 85% H_3PO_4) δ 0.14, (brs, 1P), 0.34 (brs, 1P); MS (FAB−) calcd for $C_{19}H_{23}O_{15}N_7P_2Na$ (M^+ − H) 674.0625, found 674.0634; R_f 0.27 (solvent system: iPrOH−concd NH_3−H_2O, 6:1:3, v/v/v).

Synthesis of 2′-Thiophosphorylated Dinucleotide U(2′-ps)pA (7). The fully protected dinucleotide U(2′-p)pA (**23a**) (29 mg, 0.017 mmol) was dissolved in 1% trifluoroacetic acid (5 mL), and the solution was stirred for 15 min. The reaction was quenched with CH_3OH−pyridine (1:1, v/v, 2 mL). After being concentrated under reduced pressure, the mixture was extracted by use of CH_2Cl_2 (20 mL)−5% $NaHCO_3$ (20 mL). The organic layer was collected, washed with H_2O (20 mL), and dried over Na_2SO_4. The residue was rendered anhydrous by repeated coevaporation with pyridine and finally dissolved in pyridine (0.5 mL). To the solution were added successively BSA (0.1 mL, 0.4 mmol) and DBU (0.05 mL, 0.05 mmol). After the mixture stirred for 10 min at room temperature, the reaction was quenched by addition of water (0.05 mL). Stirring was continued for a few minutes, and then the mixture was extracted with H_2O (2 mL)−Et_2O (2 mL). The aqueous layer was concentrated under reduced pressure, and the residue was dissolved in concd NH_3−pyridine (9:1, v/v, 5 mL). After stirring for 24 h, the mixture was coevaporated twice with water, and the resulting residue was treated with 80% acetic acid (5 mL) for 15 min. The solution was concentrated under

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reduced pressure, and then the residue was dissolved in water (2 mL). The solution was washed with Et_2O (2 mL), concentrated under reduced pressure, and chromatographed on Whatman 3MM Chr papers with iPrOH−concd NH_3−H_2O (6:1:3, v/v/v) to give **7** (298 A_{260}, 70%): ^{1}H NMR (400 MHz, D_2O, DSS) δ 3.70 (dd, J = 3.2, 12.7 Hz, 1H), 3.76 (dd, J = 4.1, 12.7 Hz, 1H), 4.20−4.23 (m, 2H), 4.25 (ddd, J = 2.3, 3.2, 4.1 Hz, 1H), 4.38 (m, 1H), 4.62 (m, 1H), 4.76 (ddd, J = 2.3, 5.0, 7.3 Hz, 1H), 4.81 (dd, J = 5.5, 5.8 Hz, 1H), 4.93 (ddd, J = 5.0, 7.2, 10.6 Hz, 1H), 5.83 (d, J = 7.9 Hz, 1H), 6.02 (d, J = 7.2 Hz, 1H), 6.10 (d, J = 5.8 Hz, 1H), 7.77 (d, J = 7.9 Hz, 1H), 8.23 (s, 1H), 8.46 (s, 1H); ^{13}C NMR (100.6 MHz, D_2O, dioxane) δ 64.04, 68.22, 72.99, 76.53, 76.56, 77.53, 86.82, 87.00, 89.71, 90.39, 105.01, 121.50, 142.69, 145.91, 151.95, 154.49, 155.54, 158.29, 169.89; ^{31}P NMR (109 MHz, D_2O, 85% H_3PO_4) δ 0.15 (brs, 1P), 46.07 (brs, 1P); MS (FAB−) calcd for $C_{19}H_{23}O_{14}N_7P_2SNa$ (M^+ − H) 690.0397, found 690.0389; R_f 0.26 (solvent system: iPrOH−concd NH_3−H_2O, 6:1:3, v/v/v).

Synthesis of Fully Protected Dinucleotide U(2′-5′)pU (19a). 2′, 3′-*O*,N^3-Tribenzoyluridine **18** (223 mg, 0.4 mmol) and 1*H*-tetrazole (70 mg, 1.0 mmol) were rendered anhydrous by coevaporation with pyridine (1 mL × 3) and toluene (1 mL × 2) followed by dissolution in CH_3CN (4 mL). To the solution was added 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)uridine-2′-*O*-(2-cyanoethoxy)-*N*,*N*-diisopropylphosphoramidite (**18**) (467 mg, 0.52 mmol). After being stirred for 3.5 h, the reaction mixture was treated with *tert*-butyl hydroperoxide (500 mL, 4.0 mmol) and stirred for an additional 1 h. The solution was diluted with CH_2Cl_2 (15 mL), washed with 5% $NaHCO_3$ (15 mL) and H_2O (15 mL), dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with CH_2Cl_2−CH_3OH to give **19a** (202 mg, 74%): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ −0.02 (3H, s, $SiCH_3$ of TBDMS), 0.09−0.11 (m, 3H), 0.78 (s, 9H), 2.77, 2.90 (2t, J = 5.8, 6.3 Hz, 2H), 3.37−3.40 (m, 1H), 3.81 (s, 3H), 4.15−4.18 (m, 2H), 4.33−4.68 (m, 5H), 4.96−5.05 (m, 1H), 5.30−5.34 (m, 1H), 5.63−5.83 (m, 2H), 5.93−6.02 (m, 2H), 6.15 (d, J = 2.3 Hz, 1H), 6.42 (d, J = 6.3 Hz, 1H), 6.87 (m, 4H), 7.24−7.63 (m, 20H), 7.76−7.97 (m, 7H), 8.10, 8.14 (2d, J = 7.9, 8.3 Hz, 1H), 9.69, 9.82 (2s, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ −5.35, −4.60, 17.88, 19.43, 19.54, 25.47, 55.17, 60.54, 60.74, 62.64, 62.72, 63.22, 66.94, 66.97, 67.14, 69.24, 70.60, 70.84, 73.28, 73.53, 80.06, 80.94, 81.06, 87.26, 88.09, 102.55, 103.31, 113.172, 113.21, 116.30, 117.23, 127.26, 127.91, 128.14, 128.18, 128.27, 128.36, 128.43, 128.55, 129.99, 129.65, 129.775, 130.21, 130.42, 131.20, 133.62, 134.63, 134.72, 134.93, 139.19, 139.50, 139.96, 143.72, 149.42, 150.73, 150.80, 158.74, 161.51, 161.67, 163.13, 165.10, 165.21, 165.43, 168.25, 168.34; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ −1.28 (s, 1P), −0.73 (s, 1P). Anal. Calcd for $C_{69}H_{70}N_5O_{19}PSi\cdot H_2O$: C, 61.37; H, 5.37; N, 5.19. Found: C, 61.22; H, 5.30; N, 5.09.

Synthesis of Protected 2′−5′ Linked 3′-Phosphorylated Diuridylate U(3′-p)pU (20). The fully protected 2′−5′-linked diuridylate U(2′-5′)pU (**19a**) (247 mg, 0.18 mmol) was treated with Et_3N−pyridine (1:3, v/v, 8 mL) for 20 h and then evaporated under reduced pressure. The residue was dissolved in pyridine (3.6 mL) and to the solution was added $Et_3N\cdot 3HF$ (202 μL, 1.2 mmol). After being further stirred for 72 h, the mixture was diluted with CH_2Cl_2 (15 mL), and the usual extraction was performed with 1 M triethylammonium hydrogen carbonate buffer (15 mL). The organic layer was concentrated in vacuo. After addition of 1*H*-tetrazole (378 mg, 5.4 mmol) to the residue, the mixture was rendered anhydrous by repeated coevaporation with pyridine under reduced pressure and finally dissolved in CH_3CN (2 mL). To the solution was added bis(2-cyanoethoxy)-*N*,*N*-diisopropylphosphoramidite (497 mg, 1.8 mmol). After being stirred for 1 h, the mixture was treated with *tert*-butyl hydroperoxide (406 μL, 3.6 mmol) for 1 h. Then, the solution was diluted with CH_2Cl_2 (15 mL), and extraction was performed with CH_2Cl_2−1 M triethylammonium hydrogen carbonate buffer. The organic layer was concentrated, dried over Na_2SO_4, and chromatographed on silica gel with CH_2Cl_2−CH_3OH containing 0.5% pyridine. Finally, extraction was performed with CH_2Cl_2−1 M triethylammonium hydrogen carbonate buffer, and the organic layer was collected to give **20** (35%, 95 mg): ^{1}H NMR (270 MHz, $CDCl_3$, TMS) δ 1.26−1.33 (m, 9H), 2.65−2.93 (m, 4H), 3.08 (q, J = 7.3 Hz, 6H), 3.48−3.64 (m, 2H), 3.76 (s, 6H), 4.18−4.55 (m, 9H), 5.10−5.23 (m, 1H), 5.15−5.31 (d, J = 8.2 Hz, 1H), 5.72−6.08 (m, 3H), 6.22 (d, J = 5.0 Hz, 1H), 6.50 (d, J = 7.3 Hz, 1H), 6.81−6.86 (m, 4H), 7.06−7.57 (m, 20H), 7.75−7.96 (m, 7H), 8.15 (d, J = 8.2 Hz, 1H), 11.45 (brs, 1H); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 8.47, 21.31, 29.54, 45.64, 55.37, 55.10, 55.13, 62.41, 62.48, 63.02, 63.11, 72.15, 73.04, 81.19, 86.99, 87.26, 102.50, 103.22, 112.47, 113.24, 125.16, 126.88, 127.13, 127.67, 127.96, 128.09, 128.25, 128.32, 128.42, 128.68, 128.79, 128.90, 129.02, 129.63, 129.69, 130.15, 130.35, 131.23, 133.51, 134.86, 135.04, 137.72, 139.46, 143.95, 147.33, 149.63, 150.63, 158.44, 158.51, 158.62, 161.80, 163.27, 165.23, 165.32, 168.54; ^{31}P NMR (109 MHz, $CDCl_3$, 85% H_3PO_4) δ −0.32 (s, 1P), 3.66 (s, 1P). Anal. Calcd for $C_{72}H_{75}N_7O_{22}P\cdot 2H_2O$: C, 58.10; H, 5.35; N, 6.59. Found: C, 58.13; H, 5.84; N, 6.79.

Synthesis of 2′−5′-Linked 3′-Phosphorylated Diuridylate U(3′-p)pU (10). The protected 2′-5′-linked 3′-phosphorylated dinucleotide U(3′-p)pU (**20**) (53 mg, 0.028 mmol) was dissolved in 1% trifluoroacetic acid in CH_2Cl_2 (2 mL). The mixture was stirred for 15 min, and then the reaction was quenched with CH_3OH−pyridine (1:1, v/v, 1 mL). After being concentrated in vacuo, the solution was extracted with CH_2Cl_2 (10 mL)−5% $NaHCO_3$ (10 mL). The organic layer was collected and dried over Na_2SO_4. The residue was rendered anhydrous by repeated coevaporation with pyridine and finally dissolved in pyridine (0.3 mL). To the solution were added BSA (159 μL, 0.64 mmol) and DBU (4 μL, 0.084 mmol), and the mixture was stirred for 30 min at room temperature. To the reaction mixture was added water (0.2 mL). After being stirred for a few minutes, the solution was extracted with H_2O (3 mL)−Et_2O (3 mL). The aqueous layer was concentrated under reduced pressure. The residue was dissolved in concd NH_3−H_2O (9:1, v/v, 5 mL). After stirring for 20 h at room temperature, the mixture was coevaporated twice with water under reduced pressure, and then the residue was dissolved in water (2 mL). The solution was washed with Et_2O (2 mL), evaporated under reduced pressure, and chromatographed on Whatman 3MM Chr papers with iPrOH−concd NH_3−H_2O (6:1:3, v/v/v) to give **10** (42%, 228 A_{260}): ^{1}H NMR (400 MHz, D_2O, DSS) δ 3.86 (d, J = 3.4 Hz, 2H), 4.12 (m, 2H), 4.17 (dd, J = 2.4, 4.4 Hz, 1H), 4.27 (t, J = 4.4 Hz, 1H), 4.31−4.35 (m, 2H), 4.67 (ddd, J = 4.9, 4.9, 8.9 Hz, 1H), 4.80 (ddd, J = 4.9, 4.9, 8.7 Hz, 1H), 5.78 (d, J = 8.1 Hz, 1H), 5.89 (d, J = 4.6 Hz, 1H), 5.90 (d, J = 8.1 Hz, 1H), 6.08 (d, J = 4.9 Hz, 1H), 7.83 (d, J = 8.1 Hz, 1H), 7.88 (d, J = 8.1 Hz, 1H); ^{13}C NMR (100.6 MHz, D_2O, dioxane) δ 61.70, 63.35, 65.03, 69.83, 72.88, 74.80, 77.06, 83.42, 84.76, 89.19, 89.49, 103.29, 103.48, 142.28, 143.16, 152.16, 152.47, 166.89, 166.99; ^{31}P NMR (109 MHz, D_2O, 85% H_3PO_4) δ −0.32 (brs, 1P), 3.7 (brs, 1P); R_f 0.10 (solvent system: iPrOH−concd NH_3−H_2O, 6:1:3, v/v/v).

Materials. The used nucleosides were purchased from Yamasa. Preparation of U(2′-p)pU (**1**), U(2′-ps)pU (**2**), U(2′-pp-biotin)pU (**4**), U(2′-ps-bimane)pU (**5**), thymidine 3′-phosphate (**8**), and thymidine 3′-thiophosphate (**9**) was carried out according to our previous paper.[11,12] All compounds were used after checking the purity by HPLC.

Kinetic Measurements. A substrate (1.0 A_{260}) was dissolved in a 0.1 M $AcONH_4$ solution (0.3 mL, pH 7.0) either containing 10 mM metal chloride or not. The initial concentration of the substrate was 1.5 × 10^{-4} mol dm^{-3}, and the reaction temperature was maintained at 90 °C. The reaction was followed by analyzing the compositions of samples withdrawn at appropriate intervals by HPLC. First-order rate constants (k) for the dephosphorylation of U(2′-p)pU (**1**), U(2′-ps)pU (**2**), thymidine 3′-thiophosphate (**9**), and U(3′-p)pU (**10**) in Table 2 were obtained by estimating the time-dependent product distributions. The method of least-squares was applied to fit experimental data using eq 1. The c_0 value is the initial concentration of starting materials, and c is the concentration of the starting material which remained at time t:

$$\ln(c_0/c) = kt \quad (1)$$

In the case of the dephosphorylation (k_1) of thymidine 3′-

phosphate (**8**), since the appreciable hydrolysis of the glycosidic bond occurred concurrently, the first-order rate constants were obtained by eq 2:

$$c = k_1[c_0 \exp(-k_2 t) - \exp\{\ln c_0 - (k_1 + k_3)t\}]/(k_1 + k_3 - k_2) \quad (2)$$

In eq 2, c_0 refers to initial concentration of Tp (**8**), and c is concentration of thymidine at time t. Rate constants k_2 and k_3 are for glycosidic hydrolysis of thymidine and thymidine 3′-phosphate, respectively. The value of k_2 was determined from other experiments in which 0.1 M $AcONH_4$ (pH 7.0) buffer in the presence and absence of metal chlorides as additives was used at 90 °C (none, 0.678×10^{-6} s^{-1}; $MgCl_2$, 0.725×10^{-6} s^{-1}; $MnCl_2$, 0.747×10^{-6} s^{-1}; $ZnCl_2$, 0.681×10^{-6} s^{-1}; $LaCl_3$, 0.758×10^{-6} s^{-1}; YCl_3, 0.753×10^{-6} s^{-1}).

Abbreviations: U(2′-p)pU, 2′-phosphoryluridylyl(3′−5′)uridine; U(2′-ps)pU, 2′-thiophosphoryluridylyl(3′−5′)uridine; UpU, uridylyl(3′−5′)uridine; U(2′-p)p, uridine 2′,3′-bisphosphate; U(3′-p)pU, 2′-phosphoryluridylyl(3′−5′)uridine; U(2′-p)pA, 2′-phosphoryluridylyl(3′−5′)adenosine; U(2′-ps)pU, 2′-thiophosphoryluridylyl(3′−5′)adenosine; Tp, thymidine 3′-phosphate; Tps, thymidine 3′-thiophosphate; U(2′-pU)pU, uridylyl(2′−5′)-(3′−5′)diuridine; U(2′-pp-biotin)pU, 2′-biotinyldiphosphoryluridylyl(3′−5′)uridine; U(2′-ps-bimane)pU, 2′-(*S*-bimanyl)-thiophosphoryluridylyl(3′−5′)uridine; d(UpU), 2′-deoxyuridylyl(3′−5′)-2′-deoxyuridine.

Acknowledgment. This work was supported by the Toray Scientific Foundation, Japan Society for the promotion of Science for Young Scientists (H. Tsuruoka), and a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture, Japan. We express hearty thanks to Prof. H. Lönnberg for his kindness in sending many reprints including galley proofs.

Supporting Information Available: Copies of ^1H, ^{13}C, and ^{31}P NMR data for **6**, **7**, **10**, **19a**, **20**, and **23a**; $^1H-^1H$ COSY NMR data for **6**, **10**, **21a, 20**, and **23a**; $^1H-^{13}C$ COSY NMR data for **6**; FAB mass data for **6** and **7**; detailed HPLC profiles of hydrolysis of **1**, **2**, and **10** (27 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO970021K