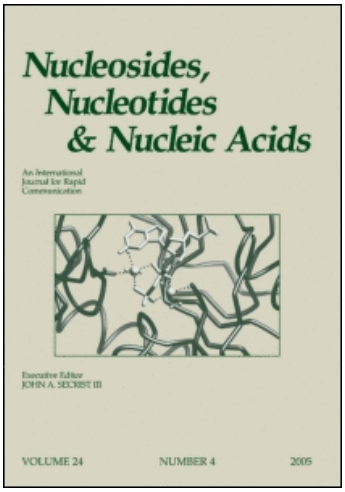

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NEW STRATEGIES FOR THE CHEMICAL SYNTHESIS OF BIOLOGICALLY IMPORTANT NUCLEIC ACID DERIVATIVES

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ABSTRACT: This paper describes general methods for the synthesis of *N*-phosphorylated ribonucleosides and oligonucleotides containing a 2'-*O*-phosphorylated or 2'-*O*-thiophosphorylated ribonucleoside. The NMR-based conformational analysis and computational molecular dynamics simulation of the 2'-*O*-phosphorylated ribonucleoside residue in such modified oligonucleotides suggested that the ribose residue existed preferentially in a C2'-endo conformation. It was also found that simple heating of 2'-*O*-phosphorylated oligonucleotides resulted in rapid dethiophosphorylation.

Ribonucleic acids (RNAs) are modified in a variety of ways in living cells. [1] From the chemical point of view, the nucleobases such as cytosine, adenine, and guanine as well as the 2'-hydroxyl group have sufficient reactivities toward electrophiles such as acylating or phosphorylating reagents. Accordingly, these amino or 2'-hydroxyl functions should be possible sites for biological phosphorylation *via* an enzymatic process. However, no examples of naturally occurring *N*-phosphorylated nucleosides have been reported except for Agrocin 84.[2]

The 2'-hydroxyl group of RNAs is also a potential site for biological phosphorylation. Actually, such 2'-phosphorylated species have been discovered as splicing products of a linear RNA fragment Ω73[3] and pre-tRNAs,[4] in which the 2'-phosphate group is derived from an internucleotidic phosphate group at the junction point. Figure 2 shows the location of the 2'-phosphoryl group in the splicing product of yeast pre-tRNA^{Tyr}.[4a] Although the biological meaning and role of the 2'-phosphate group in the structure-function relationships of tRNAs are of great interest, no basic physicochemical studies have been reported regarding 2'-phosphorylated tRNAs. The chemical synthesis of this kind of RNAs is highly desired for such studies.

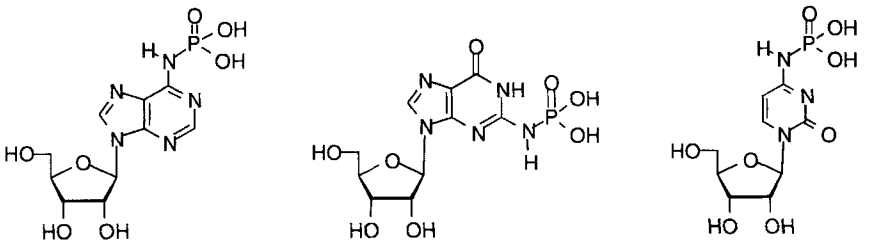


Figure 1. Chemically synthesized *N*-phosphorylated ribonucleosides

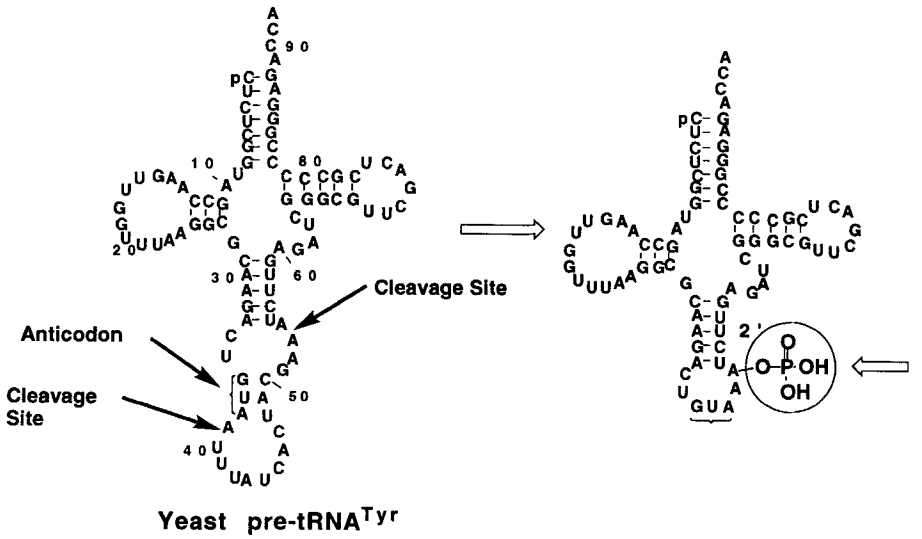


Figure 2. Splicing of yeast pre-tRNA^{Tyr} giving rise to 2'-*O*-phosphorylated tRNA^{Ty}

This paper describes general methods for the chemical synthesis of *N*- and 2'-*O*-phosphorylated nucleic acid derivatives and several chemical properties of these hyperphosphorylated species.

Synthesis of *N*-Phosphorylated Nucleosides

We have recently developed the chemical synthesis of *N*-phosphorylated nucleosides[5] and 2'-phosphorylated RNAs[6] to clarify the chemical and enzymatic properties of such hyperphosphorylated nucleic acid derivatives. Consequently, three *N*-phosphorylated

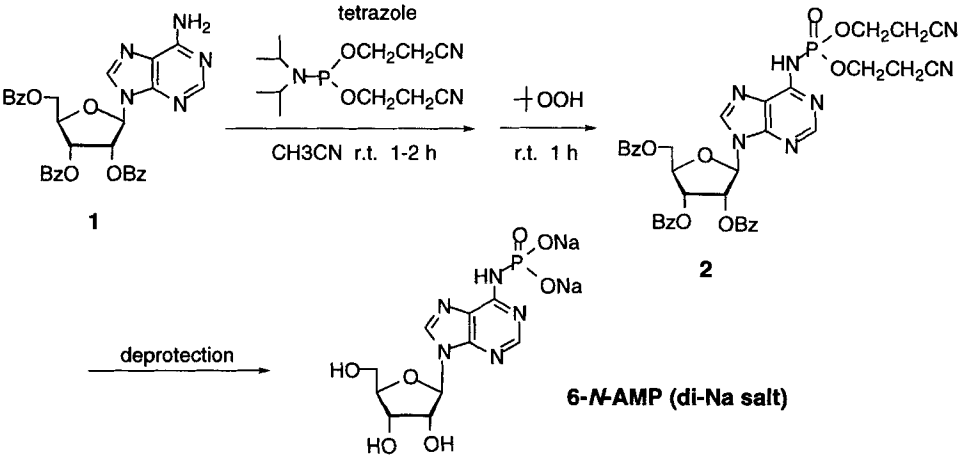
ribonucleosides, *N*-phosphorylated cytidine (4-*N*-CMP), guanosine (2-*N*-GMP), and adenosine (6-*N*-AMP), were successfully synthesized by a series of reactions involving phosphitylation of the amino group and the successive oxidation. A typical synthesis of 6-*N*-AMP *via* a fully protected intermediate **2** starting from 2',3',5'-tri-*O*-benzoyladenosine **1** is shown in Scheme 1. The stabilities of these *N*-phosphorylated ribonucleosides under acidic and basic conditions are summarized in Table 1. Although these substances have not been found, there is a potential possibility of isolation of them in the future because they exhibited relatively sufficient stabilities under neutral conditions. Among these *N*-phosphorylated ribonucleosides, 4-*N*-CMP was the most stable.

Synthesis of Oligonucleotides Incorporating a 2'-Phosphorylated Ribonucleoside

For the synthesis of 2'-*O*-phosphorylated RNAs,[6] 3',5'-*O*-protected 2'-*O*-thiophosphorylated ribonucleoside derivatives **4a-d** were synthesized as the key synthetic intermediates by the successive reactions of 3',5'-*O*-protected ribonucleosides **3a-d** followed by sulfurization. Compounds **4a-d** were converted to 3',5'-*O*-unprotected 2'-*O*-thiophosphorylated products **5a-d** without 2'–3' phosphoryl migration. 5'-*O*-DMTr-3'-phosphoramidite building blocks **7a-d** were obtained by the usual tritylation of **5a-d** followed by the 3'-phosphitylation of the resulting 5'-*O*-protected ribonucleosides **6a-d**, as described in Scheme 2. In compounds **7a-d**, the sterically hindered and electronically controlled 2-cyano-1,1-dimethylethyl (CME) group[7] was introduced into the 2'-thiophosphate residue as the DBU/*N*, *O*-bis(trimethylsilyl)acetamide (BSA)–labile protecting group[8] to avoid the 2'-3' cyclization of the 2'-phosphoryl group due to the neighboring group participation.

Scheme 3 shows the synthesis of 2'-*O*-phosphorylated uridylic acid dimer U(2'-p)pU **10** and 2'-*O*-thiophosphorylated species U(2'-ps)pU **11**. The condensation of **7a** with 5'-hydroxyl component **8** followed by oxidation with tBuOOH gave the fully protected dimer **9** in 90% yield. As evidenced by these syntheses, the CME group could be completely and rapidly removed from **9** by treatment with DBU in the presence of BSA. When the iodine treatment was eliminated, the U(2'-ps)pU **11** was obtained. Since the thiophosphoryl group is known to be easily modified with alkylating reagents to give *S*-alkyl derivatives, such 2'-*O*-thiophosphorylated RNAs would be useful for introduction of a variety of functional groups involving reporter groups into RNAs.

This method was applied to the solid phase synthesis of oligouridylates $[U(2'\text{-}p)p]_nU$ (n = 1, 3, and 5). Pfleiderer[9] and Brown[10] reported that part of the oligodeoxyribonucleotide chain was eliminated from CPG gel because of the intramolecular cyclization of the succinate linker which was catalyzed by DBU as



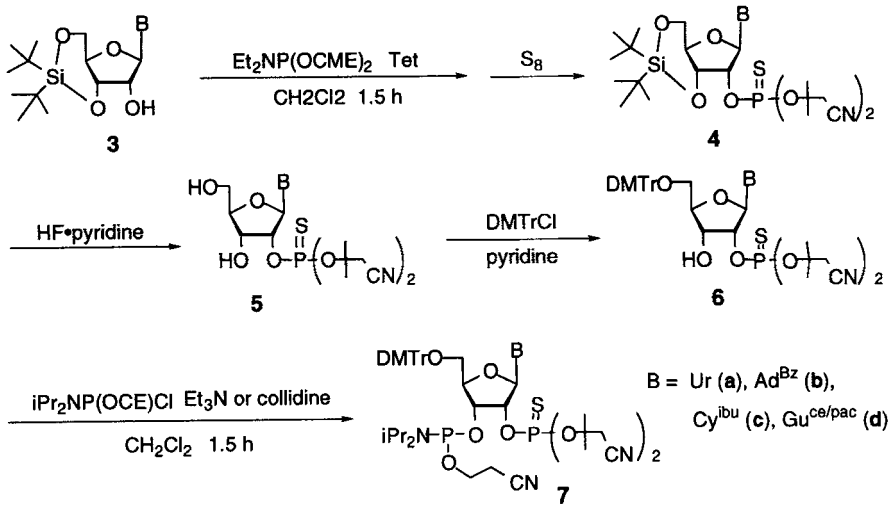
Scheme 1

Table 1. The stability of *N*-phosphorylated ribonucleoside derivatives under various conditions

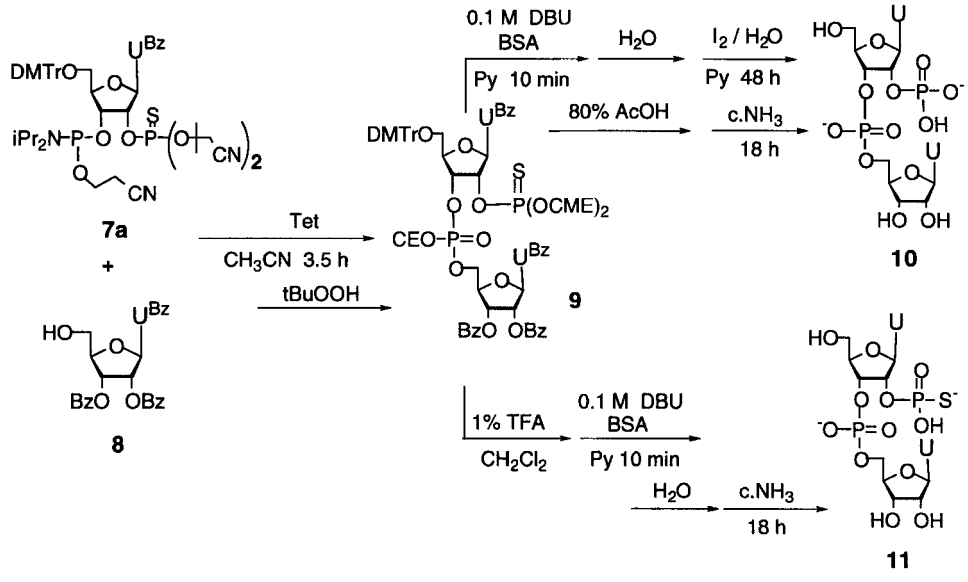
conditions	6-*N*-AMP		4-*N*-CMP		2-*N*-GMP	
	$t_{1/2}$	t_{comp}	$t_{1/2}$	t_{comp}	$t_{1/2}$	t_{comp}
0.1N HCl	4 h	24 h	8 h	3 d	5 h	30 h
80% AcOH	24 h	4 d	4 d	-	7 h	60 h
0.1N NaOH	stable		stable		stable	
concd NH_3	stable		stable		stable	

depicted in Scheme 4. Accordingly, the succinate linker must have been hitherto avoided when DBU was used as reagent for removal of the 4-nitrophenylethyl group or 2-cyanoethyl group.

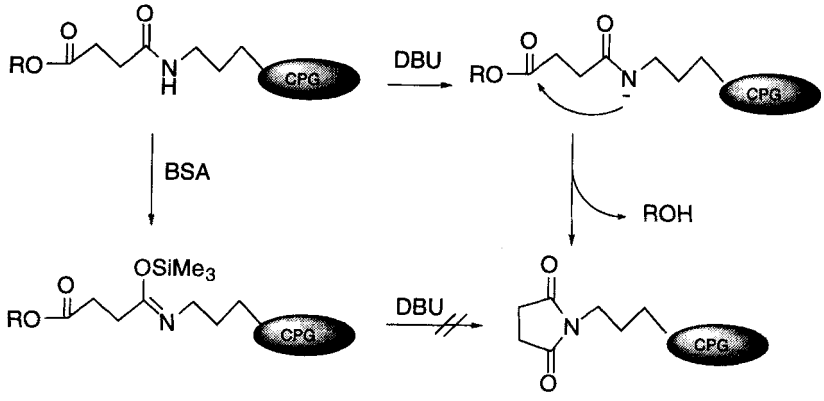
Therefore, it was checked to see if the 2'-phosphorylated oligouridylates were eliminated from CPG gel upon treatment with DBU/BSA as prescribed for removal of the internal CE and 2'-CME groups when the succinate linker was used. Interestingly, we found that DMTrU(2'- or 3'-Bz)-suc-AP-CPG was more stable than DMTrU(2'- or 3'-Bz)-suc-LCAA-CPG, when they were treated with 0.2 M of DBU in pyridine. Furthermore, elimination of DMTrU(2'- or 3'-Bz) from both resins was largely avoided, when BSA was added. It should be, particularly, noted that DMTrU(2'- or 3'-Bz)



Scheme 2



Scheme 3



Scheme 4

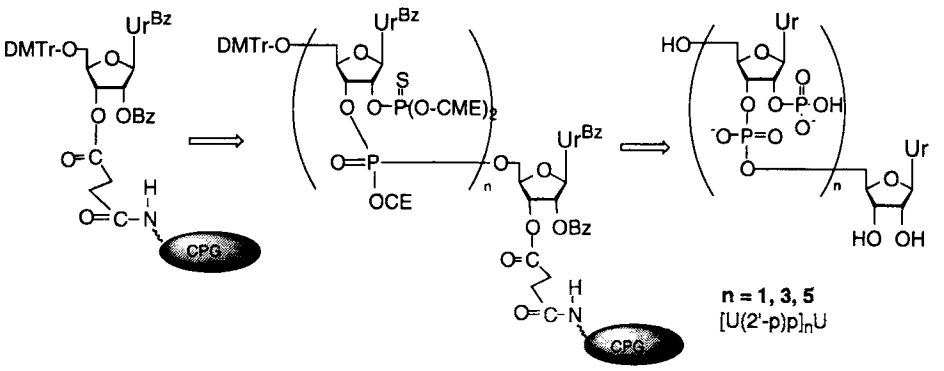
remained almost intact in the case of DMTrU(2'- or 3'-Bz)-suc-AP-CPG even after 100 h. From these results, we finally chose AP-CPG gel for our study. To obtain $[U(2'\text{-p})p]_nU$ on AP-CPG gel, N^3,2'-*O*-dibenzoyl-5'-*O*-(4,4'-dimethoxytrityl)uridine was attached to AP-CPG gel *via* a succinate linker in the usual manner. The chain elongation was performed in the usual manner.

As a result, successive treatments of the gel with 0.2 M of DBU and BSA in pyridine for 40 h and with iodine in aqueous pyridine for 48 h gave satisfactory results. The isolated yield of U(2'-p)pU was 75%.

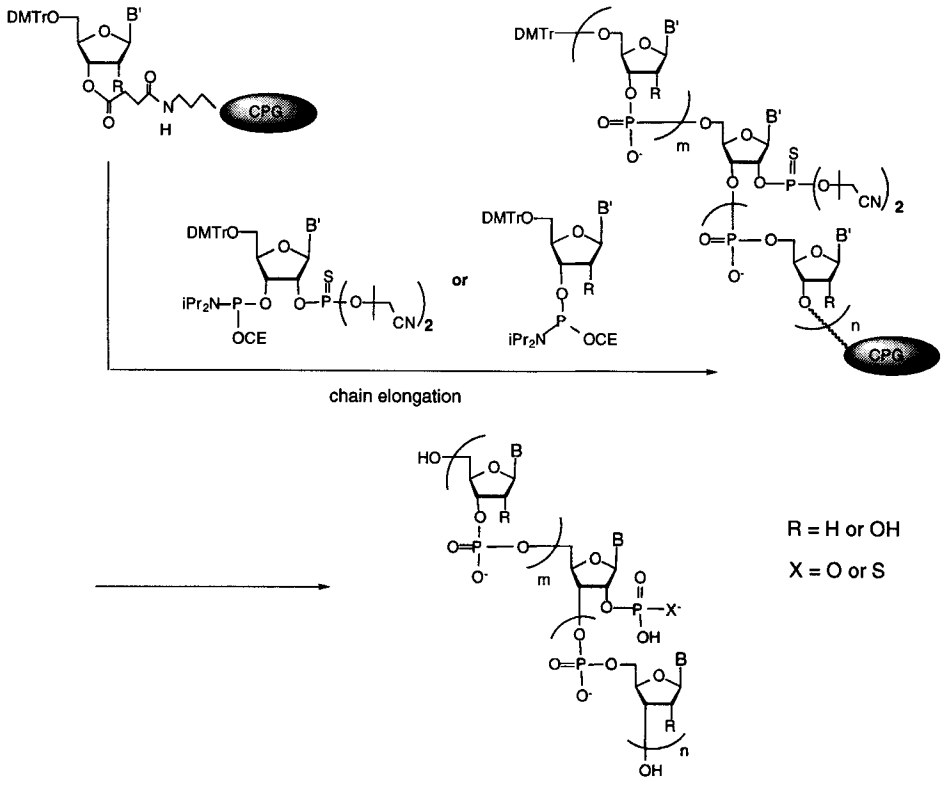
This method was applied to the synthesis of longer oligouridylates $[U(2'\text{-p})p]_nU$ (n= 3 and 5) was examined on CPG gel. The average coupling yield was usually ca. 97% in each synthesis. The 2'-phosphorylated tetrauridylate $[U(2'\text{-p})p]_3U$ was isolated in 19% yield by reverse phase C_{18} HPLC, while $[U(2'\text{-p})p]_5U$ was obtained in 12% yield by anion exchange HPLC using a FAX column which was necessary for its better separation.

Next, DNA and RNA oligomers incorporating a 2'-*O*-phosphorylated and 2'-*O*-thiophosphorylated ribonucleotide were synthesized. Particularly, in the case of oligonucleotides incorporating a 2'-*O*-phosphorylated oligonucleotides, it was found that KI_3 is more effective than I_2[11] for S–O conversion. Thus, several kinds of oligonucleotides containing a 2'-*O*-phosphorylated or 2'-*O*-thiophosphorylated uridine unit were successfully synthesized in good yields.

Finally, a number of oligodeoxynucleotides and oligoribonucleotides containing all four nucleobases and a 2'-*O*-phosphorylated or 2'-*O*-thiophosphorylated ribonucleoside



Scheme 5

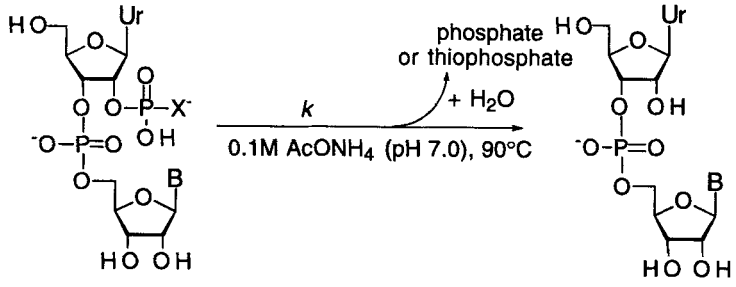


Scheme 6

were also synthesized. The stability of duplexes formed between these modified oligonucleotides and their complementary oligonucleotides was examined. Generally, it has proved that the presence of the 2'-*O*-phosphoryl or thiophosphoryl group resulted in decrease of the Tm values of the duplexes. The Tm value of the duplex formed between tridecamer $d(U_6)U(2'\text{-}p)d(U_6)$ containing a 2'-*O*-phosphorylated uridine and $d(A_{13})$ decreased to an extent of 4.7 °C compared with that of the unmodified duplex, while the Tm value of the duplex formed between the tridecamer $U_6U(2'\text{-}p)U_6$ containing a 2'-*O*-phosphorylated uridine and A_{13} decreased considerably by 11.5 °C compared with that of the unmodified duplex. The detailed conformational analysis of these 2'-*O*-phosphorylated or 2'-thiophosphorylated oligonucleotides by means of H^1 NMR spectroscopy suggested that the ribose puckering of 2'-*O*-phosphoryl or 2'-*O*-thiophosphoryl ribonucleosides is in favor of the C2'-endo conformation, which is typical for B-DNA. This is the reason why the above result was obtained. Molecular dynamics simulation of these modified duplexes also supported this conclusion.

Dephosphorylation of 2'-*O*-Phosphorylated Oligonucleotides by Enzymatic and Non-enzymatic Methods. U(2'-p)pU was digested with snake venom phosphodiesterase to give U(2'-p) and pU while this dimer was resistant to nuclease P1 and spleen phosphodiesterase. Treatment of U(2'-p)pU with calf intestinal alkaline phosphatase (CIAP) gave the usual UpU dimer, which was digestible with nuclease P1 to give U and pU. In the case of $[U(2'\text{-}p)p]_3U$ and $[U(2'\text{-}p)p]_5U$, dephosphorylation also occurred upon treatment with CIAP. The stability of the 2'-phosphoryl group to CIAP increased with an increase in length of the oligomer. Contrary to these results, the 2'-thiophosphorylated uridine dimer U(2'-ps)pU was found to be completely resistant to CIAP. Similarly, oligodeoxynucleotides and oligoribonucleotides incorporating a 2'-*O*-phosphorylated uridine were dephosphorylated by CIAP treatment to give the dephosphorylated products.

During this study, it was observed that, when U(2'-p)pU was heated in 0.1 M NH_4OAc (pH 7.0) at 90 °C, the 2'-phosphoryl group was rapidly removed from the 2'-position.[12] More interestingly, the 2'-thiophosphoryl group of U(2'-ps)pU was more promptly eliminated upon heating to give UpU than the 2'-phosphoryl group of U(2'-p)U. The first-order rate constants of these reactions were determined by HPLC. Particularly, U(2'-ps)pU ($k = 1.38 \pm 0.4 \times 10^{-3}\ sec^{-1}, t_{comp} = 1$ h) was cleanly dephosphorylated ca. 100 times more rapidly than U(2'-p)pU ($k = 1.41 \pm 0.05 \times 10^{-5}\ sec^{-1}, t_{comp} = 72$ h) was dephosphorylated. The kinetic data observed were independent of the 2'- or 3'-position of the phosphate group and the kind of base moiety. These unexpected facile



Scheme 7

Table 2. First order rate constants for dephosphorylation of **10** and **11** in the presence and absence of metal ions at pH 7.0, 90°C

additives	U(2'-p)pU **10** $k/10^{-6}$ sec^{-1}	$t_{1/2}$ (h)	U(2'-ps)pU **11** $k/10^{-6}$ sec^{-1}	$t_{1/2}$ (h)
none[a]	14.1 ± 0.5[c]	13.6	1380 ± 40[c]	0.14
Ca^{2+}	13.3 ± 0.1	14.5		
Mg^{2+}	11.8 ± 1.0	16.4	710 ± 60	0.27
Co^{2+}	10.3 ± 0.5	18.6		
Cd^{2+}	10.1 ± 0.4	19.0		
Mn^{2+}	2.9 ± 0.2	66.9	250 ± 10	0.76
Zn^{2+}	2.3 ± 0.2	84.0	56 ± 8	3.5
La^{3+}	25.9 ± 0.4	7.4		
Y^{3+}	16.9 ± 0.1	11.4		
phosphate buffer[b]	11.2 ± 0.2	17.2	820 ± 40	0.24

[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

dephosphorylations were explained in terms of the neighboring group participation of the proximate internucleotidic phosphate group.

It was found that 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 U(2'-p)pU or U(2'-ps)pU. 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 U(2'-p)pU or U(2'-ps)pU. These results are summarized in Table 2. In connection with this study, it was found that thermal treatment of psT$(pT)_9$ and d[psA$(pA)_9$] resulted in the highly selective dethiophosphorylation at the 5'-terminal position to give T$(pT)_9$ and d[A$(pA)_9$], respectively, without appreciable hydrolysis of 3'-5' phosphodiesters and glycosidic bonds.

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