

Metal-Ion-Promoted Cleavage, Isomerization, and Desulfurization of the Diastereomeric Phosphoromonothioate Analogues of Uridyl(3',5')uridine

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Metal-ion-promoted hydrolytic reactions of the S_P and R_P diastereomers of the phosphoromonothioate analogues of uridyl(3',5')uridine [3',5'-Up(s)U] and their cleavage products, diastereomeric uridine 2',3'-cyclic phosphates [2',3'-cUMPS], were followed by HPLC as a function of pH (4.7–5.6) and metal ion concentration (1–10 mmol L⁻¹). With 3',5'-Up(s)U, three reactions compete: (i) cleavage to 2',3'-cUMPS, (ii) isomerization to 2',5'-Up(s)U, and (iii) desulfurization to an equilibrium mixture of 2',5'- and 3',5'-UpU. Of these, the cleavage to 2',3'-cUMPS is markedly accelerated by Zn²⁺, Cd²⁺, and Gd³⁺, the rate enhancements observed with the S_P isomer at [M^{z+}] = 5 mmol L⁻¹ and pH 5.6 ($T = 363.2$ K) being 410-, 3600-, and 2000-fold, respectively. The effect of Mn²⁺ and Mg²⁺ on the cleavage rate is, in turn, modest (6- and 1.7-fold acceleration, respectively). The rate-accelerations are almost equal with the S_P and R_P diastereomers. The metal-ion-promoted reaction is first-order in both the hydroxide and metal ion concentration, and it proceeds by inversion of configuration at phosphorus, consistent with an in-line displacement mechanism. The isomerization and desulfurization are much less susceptible to metal ion catalysis: 6.4- and 7.7-fold accelerations were observed with Zn²⁺, respectively. Gd³⁺ does not promote these reactions at all. The isomerization proceeds by retention of configuration at phosphorus, consistent with formation of a pentacoordinated thiophosphorane intermediate having the entering 2'-hydroxy group apical and the leaving 3'-hydroxy equatorial, and subsequent pseudorotation posing the leaving group apical. The hydrolysis of 2',3'-cUMPS is accelerated by metal ions slightly more efficiently than the cleavage of 3',5'-Up(s)U to 2',3'-cUMPS. In striking contrast to the reactions of 3',5'-Up(s)U, the hydrolytic desulfurization to 2',3'-cUMP is accelerated as efficiently as its endocyclic hydrolysis to uridine 2'- and 3'-phosphoromonothioates [2'- and 3'-UMPS]. Somewhat unexpectedly, the latter compounds were observed to undergo metal-ion-promoted cyclization/desulfurization to 2',3'-cUMP. The hydrolysis of 2'- or 3'-UMPS to uridine was, in turn, observed to be retarded by metal ions. The mechanisms of the partial reactions are discussed.

Introduction

Ribonucleoside phosphoromonothioates, containing a chiral S-bonded phosphorus atom, were introduced in RNA chemistry in late 1960s, and since then they have been extensively used as mechanistic probes in elucidation of the action of both protein enzymes^{1,2} and ribozymes.^{3–10} One of the central subjects has been the role of divalent metal ions in ribozyme-catalyzed

reactions.^{11–13} A phosphorothioate linkage, having either an R_P or S_P configuration, has been inserted in a selected position of the sugar–phosphate backbone of either the substrate chain or the ribozyme itself, and the resulting kinetic thio effect has been utilized to pinpoint the binding site of the metal ion^{7–9,14–17} and to elucidate the stereochemistry of the ribozyme-catalyzed cleavage.^{15,16} Phosphorothioate linkages, having either the 5'-bridging^{18,19} or 3'-bridging^{20,21} oxygen replaced with sulfur, have been more recently used for related purposes.

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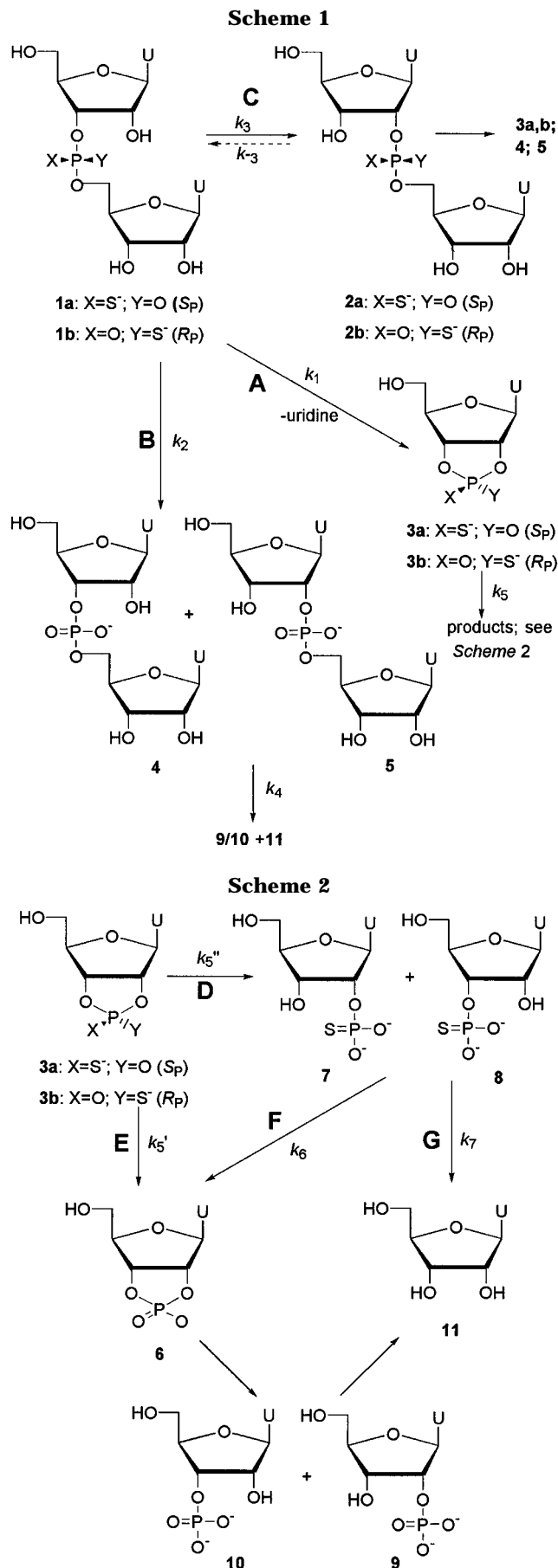
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This kind of use of phosphorothioates as mechanistic probes is largely based on detailed knowledge of the effects of thiosubstitution on the underlying chemical reactivity of phosphoesters. The metal-ion-dependent reactivity of diastereomeric phosphorothioates is, for example, of considerable interest since metal ions are known to exhibit markedly different binding properties to oxygen and sulfur.^{22,23} For this purpose, we now report on the catalytic effects of some metal ions on the nonenzymatic cleavage, isomerization, and desulfurization of diastereomeric dinucleoside 3',5'-phosphoromonothioates (**1a,b**) and the corresponding reactions of diastereomeric nucleoside 2',3'-cyclic phosphoromonothioates (**3a,b**). To the best of our knowledge, no systematic kinetic study on these subjects has been published. The results are compared to those obtained earlier^{24,25} with the same compounds in the absence of metal ions. Moreover, the catalytic effects of the metal ions are compared to those reported^{26,27} for the cleavage of their oxygen counterparts, uridylyl(3',5')uridine (3',5'-UpU; **4**) and uridine 2',3'-cyclic phosphate (2',3'-cUMP; **6**), and correlated with their affinities²³ to a nucleoside 5'-thiophosphate.

Results

Product Distributions. The decomposition of the S_P (**1a**) and R_P (**1b**) diastereomers of uridylyl(3',5')uridine phosphoromonothioate [3',5'-Up(s)U] was followed in the presence of Mg^{2+} , Mn^{2+} , Zn^{2+} , Cd^{2+} , and Gd^{3+} (1–10 mmol L^{-1}) at pH 4.7–5.6 ($T = 363.2$ K). The composition of the aliquots withdrawn at appropriate intervals from the reaction solution was determined by RP HPLC. The signals were assigned by spiking with authentic samples. The same three reactions observed previously²⁴ in the absence of metal ions took place, viz. (i) cleavage to the S_P or R_P diastereomer of uridine 2',3'-cyclic phosphoromonothioate [2',3'-cUMPS; **3a,b**; route A in Scheme 1], (ii) desulfurization to a mixture of 3',5'- and 2',5'-UpU (**4, 5**; route B), and (iii) isomerization to either the S_P or R_P diastereomer of 2',5'-Up(s)U (**2a,b**; route C). 2',3'-cUMPS (**3a,b**) underwent, also consistent with previous²⁵ observations in the absence of metal ions, concurrent hydrolysis to a mixture of uridine 2'- and 3'-phosphoromonothioates [2'- and 3'-UMPS; **7, 8**; route D in Scheme 2] and desulfurization to 2',3'-cUMP (**6**; route E). 2',3'-cUMP was also formed by cyclization/desulfurization of 2'- and 3'-UMPS (route F). This reaction has not been observed to take place in the absence of metal ions at pH > 2.²⁸ The reaction sequence was completed by hydrolysis of 2',3'-cUMP to a mixture of uridine 2'- and 3'-phosphates (2'- and 3'-UMP; **9, 10**) and their dephosphorylation to uridine. 2'- and 3'-UMPS were additionally hydrolyzed to uridine without intermediary accumulation of 2'/3'-UMP (route G), but this reaction was retarded rather than accelerated by metal ions.



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Stereochemistry and Kinetics of the Metal-Ion-Promoted Reactions of 3',5'-Up(s)U (1a,b**). Table 1**

Table 1. First-Order Rate Constants for the Cleavage (k_1), Desulfurization (k_2), and Isomerization (k_3) of (S_P)-3',5'-Up(s)U (1a**), (R_P)-3',5'-Up(s)U (**1b**), and 3',5'-UpU (**4**) at 363.2 K^a**

compd	M ^{z+}	[M ^{z+}]/ mmol L ⁻¹	pH	k_1 / 10 ⁻⁶ s ⁻¹	k_2 / 10 ⁻⁶ s ⁻¹	k_3 / 10 ⁻⁶ s ⁻¹	
1a	Zn ²⁺	1.0	5.60	5.2	1.6	0.22	
		2.0	5.60	15	2.5	0.32	
		5.0	5.60	38	6.3	0.77	
		10.0	5.60	47	20	2.0	
		20.0 ^b	5.60	220	63	4.6	
		40.0 ^c	5.60	260	170	7.4	
		100.0 ^d	5.60	340	38	5.7	
		5.0	5.38	23	3.5	0.70	
		5.0	5.08	9.2	2.8	0.57	
		5.0	4.68	3.6	1.4	0.42	
	Mn ²⁺	1.0	5.60	0.24	0.93	0.12	
		2.0	5.60	0.31	0.90	0.11	
		5.0	5.60	0.55	1.6	0.12	
		10.0	5.60	1.1	1.2	0.12	
		5.0	5.60	0.16	0.72	0.11	
Mg ²⁺	5.0	5.60					
Gd ³⁺	5.0	5.60	190				
1b	none		5.60	0.093	0.82	0.12	
		Zn ²⁺	1.0	5.60	11	2.7	0.31
		10.0	5.60	190	17	2.0	
	Mn ²⁺	1.0	5.60	0.19	2.5	0.18	
		10.0	5.60	0.79	3.2	0.21	
	Mg ²⁺	5.0	5.60	0.071	2.3	0.19	
	Gd ³⁺	5.0	5.60	89			
	Cd ²⁺	1.0	5.60	110			
		5.0	5.60	340			
		10.0	5.60	930			
5.0		5.60	0.12	2.2	0.20		
4^e	none		5.60	0.12	2.2	0.20	
		Zn ²⁺	5.0	5.60	7.22		0.810
	Mn ²⁺	5.0	5.60	0.650		0.760	
		5.0	5.60	0.104		0.650	
	Gd ³⁺	5.0	5.60	>1000			
	Cd ²⁺	5.0	5.60	2.00			
5.0		5.60	0.052		0.650		

^a The pH was adjusted with HEPES or MES buffer and the ionic strength (0.1 mol L⁻¹) with sodium nitrate. ^b The ionic strength was 0.16, ^c 0.24, and ^d 0.3 mol L⁻¹. ^e Data taken from ref 27.

records the rate constants obtained for the cleavage (route A in Scheme 1), desulfurization (B), and isomerization (C) of (S_P)- and (R_P)-3',5'-Up(s)U (**1a,b**) at various pH and metal ion concentrations. As seen, the cleavage to 2',3'-cUMPS (A) is hardly detectably accelerated by Mg²⁺, and the catalytic effect of Mn²⁺ also is modest: a 6-fold acceleration was observed with the S_P diastereomer (**1a**) at [Mn²⁺] = 5 mmol L⁻¹ at pH 5.6. In contrast, Zn²⁺, Cd²⁺, and Gd³⁺ markedly accelerate the cleavage, the rate accelerations obtained with **1a** being 410-, 3600-, and 2000-fold, respectively ([M^{z+}] = 5 mmol L⁻¹, pH 5.6). It is worth noting that the rate-accelerating effects of the soft metal ions, Zn²⁺ and Cd²⁺, are greater than those observed with 3',5'-UpU, whereas Gd³⁺, a typical hard Lewis acid, accelerates the cleavage of 3',5'-Up(s)U considerably less efficiently than that of 3',5'-UpU.²⁷

The metal-ion-promoted cleavage of 3',5'-Up(s)U proceeds by *inversion* at phosphorus, as depicted in Scheme 3. The reaction is first-order in metal ion concentration (Figure 1) and also in hydroxide ion concentration (Figure 2). At high metal ion concentrations, the cleavage rate, however, levels off to a constant value (Table 1), presumably owing to saturation of the starting material with the metal ion. With Zn²⁺ this saturation takes place in the concentration range 10–50 mmol L⁻¹, suggesting the log(K /mol L⁻¹) value of the 1:1 complex to be 1.5. The S_P and R_P diastereomers react at a comparable rate.

Scheme 3

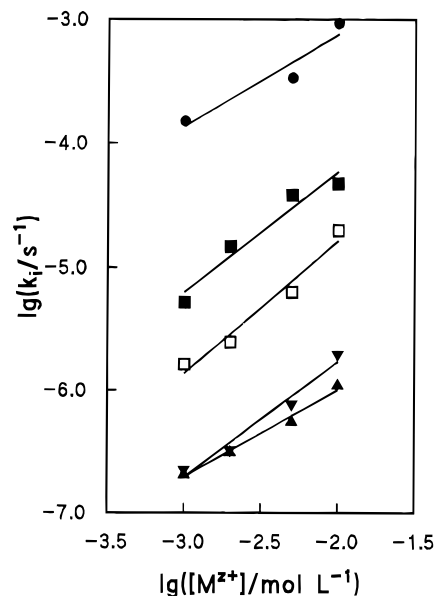
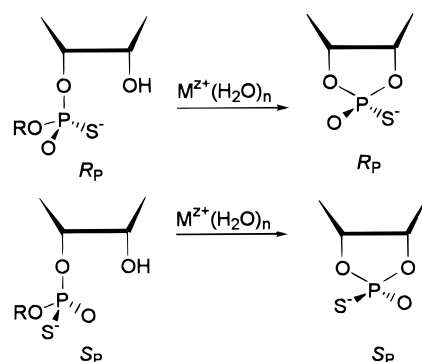


Figure 1. Effect of metal ion concentration on the first-order rate constants of cleavage (k_1), desulfurization (k_2), and isomerization (k_3) of (S_P)-3',5'-Up(s)U (**1a**) at 363.2 K. The pH was adjusted to 5.60 with HEPES buffer and the ionic strength to 0.1 mol L⁻¹ with sodium nitrate. Notation: Cd²⁺-catalyzed cleavage (●); Mn²⁺-catalyzed cleavage (▲), and Zn²⁺-catalyzed cleavage (■), desulfurization (□), and isomerization (▼).

Some stereoselectivity, however, occurs; the Zn²⁺-promoted cleavage of the R_P diastereomer is two to three times as fast as that of the S_P form.

Compared to the cleavage reaction, the desulfurization of 3',5'-Up(s)U is much less susceptible to the metal ion catalysis. Only an 8-fold acceleration was observed in 5 mmol L⁻¹ solution of Zn²⁺. Mg²⁺ and Gd³⁺ exhibited no catalytic effect and Mn²⁺ a barely noticeable rate-accelerating effect (Table 1). Cd²⁺ accelerates the desulfurization very efficiently, but this acceleration appears to be of exceptional origin. The desulfurization of both **1a** and **1b** showed a marked positive deviation from the first-order kinetics, suggesting the reaction to be subject to efficient product catalysis. Consistent with this assumption, desulfurization immediately took place when Na₂S (0.1 mmol L⁻¹) was added to the reaction mixture. With Zn²⁺, no deviation from the first-order kinetics was observed during the first two half-lives. Both diastereomers are desulfurized approximately as fast. The reaction is first-order in the concentration of metal ion (Figure 1) but clearly less than first-order (0.7) in that of hydroxide ion (Figure 2).

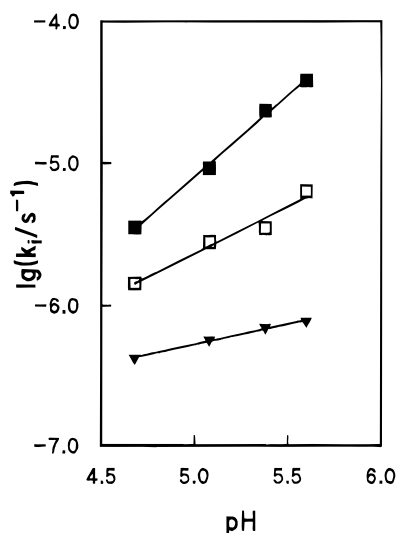
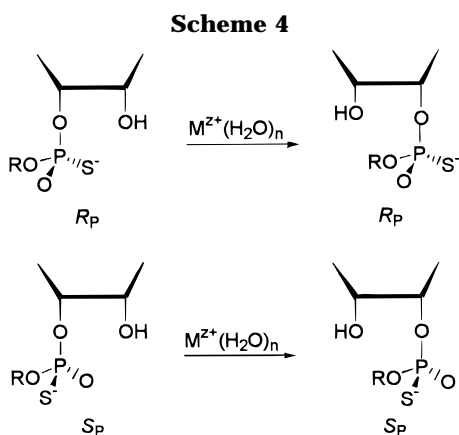


Figure 2. Effect of pH on the cleavage (■), desulfurization (□), and isomerization (▼) of (*S_P*)-3',5'-Up(s)U (**1a**) in the presence of Zn²⁺ (0.005 mol L⁻¹) at 363.2 K. The pH was adjusted with HEPES and MES buffers and the ionic strength (0.1 mol L⁻¹) with sodium nitrate.



Zn²⁺ moderately promotes the isomerization (route C) of 3',5'-Up(s)U, the rate-acceleration at [Zn²⁺] = 5 mmol L⁻¹ at pH 5.6 being 6-fold. In contrast, Mg²⁺, Mn²⁺, and Gd³⁺ showed no such acceleration. With Cd²⁺, the isomerization could not be studied, owing to the rapid desulfurization of the starting material. The Zn²⁺-promoted reaction is nearly pH-independent (Figure 2) and first-order in the metal ion concentration (Figure 1). The effect on the isomerization rate is almost equal with both diastereomers (**1a,b**). For comparison, Zn²⁺ promotes neither the interconversion of 2',5'- and 3',5'-UpU²⁷ nor the isomerization of the phosphodiester bonds of poly(U).²⁹ In striking contrast to the cleavage reaction, the isomerization of **1a** and **1b** proceeds by *retention* of configuration at phosphorus: **1a** gives only **2a** and **1b** gives **2b** (Scheme 4).

Kinetics of the Metal-Ion-Promoted Reactions of 2',3'-cUMPS (3a,b). Metal ions accelerate both the hydrolysis (route D in Scheme 2) and desulfurization (E) of 2',3'-cUMPS (**3a,b**). Determination of the rate constants of these reactions was severely complicated by the fact that the products of the hydrolysis reaction (D), i.e., 2'- and 3'-UMPS (**7, 8**), undergo, in addition to dethio-

Table 2. First-Order Rate Constants for the Hydrolysis (k_5) and Desulfurization (k_5') of (*S_P*)-2',3'-cUMPS (**3a**), (*R_P*)-2',3'-cUMPS (**3b**), and 2',3'-cUMP (**6**) at 363.2 K^a

compd	M ²⁺	[M ²⁺]/mmol L ⁻¹	pH	$k_5'/10^{-6}$ s ⁻¹	$k_5/10^{-6}$ s ⁻¹	
3a	Zn ²⁺	1.0	5.60	110	80	
		2.0	5.60	270	240	
		5.0	5.60	1020	510	
		10.0	5.60	2700	560	
		20.0 ^b	5.60	4700	1900	
		40.0 ^c	5.60	5200	1140	
		100.0 ^d	5.60	6100	1500	
		5.0	5.38	600	240	
		5.0	5.08	260	100	
		5.0	4.68	77	46	
	Mn ²⁺	1.0	5.60	2.0	0.93	
		2.0	5.60	2.6	1.3	
		5.0	5.60	5.9	3.5	
	Mg ²⁺	10.0	5.60	10.4	7.3	
		5.0	5.60	1.1	0.37	
	Gd ³⁺	5.0	5.60	1900	80	
	Cd ²⁺	1.0	5.60	830	350	
2.0		5.60	1600	810		
3b	none	5.0	5.60	3900	1900	
		10.0	5.60	9040	2700	
		5.60	0.64	0.23		
		Zn ²⁺	1.0	5.60	170	130
			2.0	5.60	340	270
	5.0		5.60	1300	520	
	10.0		5.60	2500	1300	
	Mn ²⁺	1.0	5.60	^e		
		10.0	5.60	7.7	7.7	
	Mg ²⁺	5.0	5.60	^f		
Cd ²⁺		1.0	5.60	1100	230	
	2.0	5.60	1990	497		
	5.0	5.60	5200	2100		
6^g	none	5.60	5.60	10300	2600	
		5.60	0.32	1.3		
	Zn ²⁺	5.0	5.60	33.9		
	Mn ²⁺	5.0	5.60	16.4		
	Cd ²⁺	5.0	5.60	1.88		
5.0		5.60	23.8			
none	5.60	1.02				

^a The pH was adjusted with HEPES or MES buffer and the ionic strength (0.1 mol L⁻¹) with sodium nitrate. ^b The ionic strength 0.16, ^c 0.24, and ^d 0.3 mol L⁻¹. ^e $k_5 = 1.9 \times 10^{-6}$ s⁻¹. ^f $k_5 = 1.5 \times 10^{-6}$ s⁻¹. ^g Data taken from ref 26.

phosphorylation to uridine (G), cyclization/desulfurization (F) to 2',3'-cUMP (**6**), and this reaction was observed to exhibit a slight positive deviation from first-order kinetics. Accordingly, accumulation of neither of the intermediates **6** or **7/8** obeys any simple rate law, even though degradation of **3a,b** strictly follows first-order kinetics. That is why the rate constants for the parallel reactions D and E could only be estimated by bisecting the first-order rate constant for disappearance of 2',3'-cUMPS (**3a,b**) with the aid of the concentration ratio of 2',3'-cUMP (**6**) and 2'/3'-UMPS (**7 + 8**) at very early stages of reaction. The results obtained are summarized in Table 2. Both of the parallel reactions (D, E) are strongly promoted by Zn²⁺ and Cd²⁺, the hydrolysis slightly predominating over the desulfurization. Usually, 60–80% of the total disappearance of 2',3'-cUMPS proceeds via 2'- and 3'-UMPS.

The rate-accelerating effects of various metal ions on the hydrolysis of 2',3'-cUMPS are somewhat larger than their effects on the cleavage of 3',5'-Up(s)U (Table 2). The *R_P*-diastereomer (**3b**) reacts slightly faster than the *S_P*-isomer (**3a**), the difference in rate being usually less than 20%. Interestingly, the desulfurization of 2',3'-cUMPS is accelerated by metal ions much more efficiently than the corresponding reaction of 3',5'-Up(s)U.

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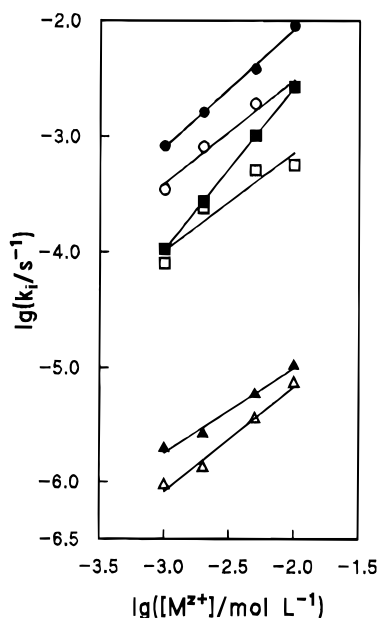


Figure 3. Effect of metal ion concentration on the first-order rate constants of phosphoester hydrolysis (solid notation; k_5^-) and desulfurization (open notation; k_5^-) of (*S_p*)-2',3'-cUMPS (**3a**) at pH 5.60 and 363.2 K. The pH was adjusted with HEPES buffer, and the ionic strength was maintained at 0.1 mol L⁻¹ with sodium nitrate. Notation: Cd²⁺ (●, ○), Zn²⁺ (■, □) and Mn²⁺ (▲, △).

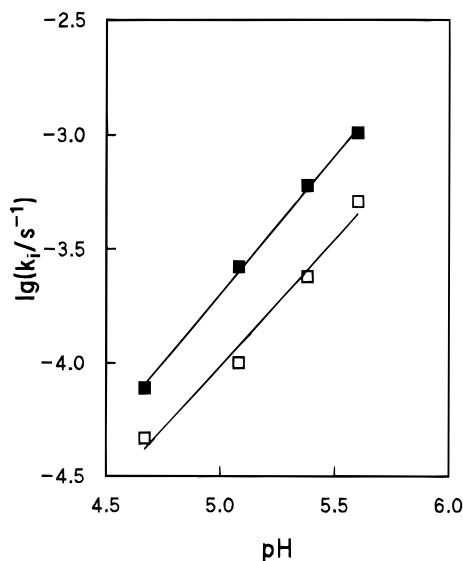


Figure 4. Effect of pH on the hydrolysis (■) and desulfurization (□) of (*S_p*)-2',3'-cUMPS (**3a**) in the presence of Zn²⁺ (0.005 mol L⁻¹) at 363.2 K. The pH was adjusted with HEPES and MES buffers and the ionic strength (0.1 mol L⁻¹) with sodium nitrate.

The Mn²⁺-, Zn²⁺-, and Cd²⁺-promoted hydrolysis (D) and desulfurization (E) of 2',3'-cUMPS are both first-order in the metal ion concentration (Figure 3) and, at least in the case of Zn²⁺, also first-order in the hydroxide ion concentration (Figure 4).

It has been shown previously²⁸ that 2'- and 3'-UMPS undergo under neutral and slightly acidic conditions hydrolytic dethiophosphorylation to uridine (G). The divalent metal ions studied clearly retarded this reaction, though exact kinetic data cannot be obtained due to the concurrent metal-ion-promoted cyclization/desulfuriza-

Table 3. First-Order Rate Constants for the Cyclization (k_6) and Dethiophosphorylation (k_7) of 2'-UMPS (**7**) at 363.2 K^a

compd	M ^{z+}	[M ^{z+}]/mmol L ⁻¹	$k_6/10^{-4} \text{ s}^{-1}$	$k_7/10^{-4} \text{ s}^{-1}$
7	Zn ²⁺	1.0	2.7	3.4
		2.0	2.4	1.5
		5.0	5.9	0.94
		10.0	9.5	0.15
	Cd ²⁺	1.0	5.6	
		2.0	5.9	
		5.0	11.0	
		10.0	7.1	
	Mn ²⁺	1.0	0.94	3.5
		10.0	0.92	1.3
	Mg ²⁺	5.0	0.28	3.0
	none			7.0

^a The pH 5.60 was adjusted with HEPES buffer and the ionic strength (0.1 mol L⁻¹) with sodium nitrate.

tion (F). As mentioned above, the latter reaction does not strictly obey first-order kinetics but is apparently subject to product catalysis. Hence, only approximate values for the rate constants of reactions F and G, referring to early stages of the disappearance of **7** and **8**, may be reported. These data are collected in Table 3.

Discussion

Mechanism of the Metal-Ion-Promoted Reactions of 3',5'-Up(s)U. In the pH range studied (pH 4.7–5.6), all the competing reactions of 3',5'-Up(s)U are nearly pH-independent in the absence of metal ions, the desulfurization (B) predominating over cleavage (A) and isomerization (C).²⁴ It has been suggested^{24,30} that the reactions take place via a common monoanionic thiophosphorane intermediate obtained by the attack of the deprotonated 2'-hydroxy function on the neutral thiophosphate diester, i.e., via a minor but more reactive tautomer. All the metal ions studied accelerate the cleavage reaction (A), but the catalytic effects differ considerably from those observed previously for the cleavage of 3',5'-UpU.²⁷ While Mg²⁺, Mn²⁺, and Gd³⁺ promote the cleavage of 3',5'-Up(s)U less efficiently than that of 3',5'-UpU, the opposite is true with Zn²⁺ and Cd²⁺. Furthermore, Zn²⁺ (and possibly Cd²⁺) also promotes the desulfurization (B) and isomerization (C) of 3',5'-Up(s)U (though less efficiently than the cleavage), in striking contrast to Gd³⁺. These differences in the action most likely result from different binding behavior of the metal ions. The studies of Sigel et al.²³ on adenosine 5'-phosphoromonothioate suggest that soft Lewis acids, such as Zn²⁺ and Cd²⁺, prefer coordination to the sulfur ligand, while hard Lewis acids, such as Mn²⁺ and Mg²⁺ (and in all likelihood also Gd³⁺), are predominantly bound to oxygen. As seen from Figure 5, the catalytic effect of various metal ions on the cleavage of 3',5'-Up(s)U roughly correlates with the stability of their complex with adenosine 5'-phosphoromonothioate.

The mechanism of the action of the catalytically most active metal ions, Zn²⁺ and Cd²⁺, that bind to sulfur instead of oxygen is of special interest of the present study. At low metal ion concentrations, the cleavage (A), desulfurization (B), and isomerization (C) are all first order in the concentration of metal ion (Figure 1), indicating that only one metal ion is involved. The cleavage reaction is also first-order in hydroxide ion

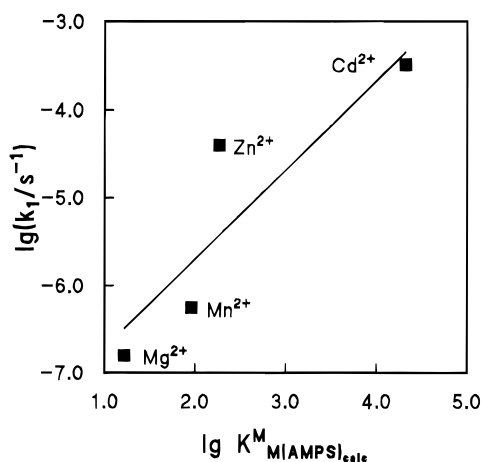


Figure 5. Correlation between the first-order rate constants for the cleavage (k_1) of (*S*_p)-3',5'-Up(s)U (**1a**) in the presence of metal ions ($[M^{2+}] = 5 \text{ mmol L}^{-1}$) and the stability constants $K^M_{M(\text{AMPS})_{\text{calc}}}$ of the complexes the metal ions form with the thiophosphate moiety of adenosine 5'-*O*-thiomonophosphate. Values for the stability constants were taken from ref 23.

concentration. Accordingly, one proton is removed either from the predominant ionic form (i.e., monoanion) of 3',5'-Up(s)U or from the metal aquo ion on going to the transition state, and hence, a dianionic thiophosphorane ($\text{I}^{2-}\text{M}^{z+}$) may be expected to be obtained (Scheme 5). The sulfur-bound metal ion increases the electrophilicity of the phosphorus atom, and most likely it also participates in the deprotonation of the attacking hydroxy group either by direct coordination to the 2'-oxygen, or, as indicated in Scheme 5, by offering an intracomplex base catalyst, the hydroxo ligand. The dianionic thiophosphorane ($\text{I}^{2-}\text{M}^{z+}$) may in principle be regarded either as an intermediate or a transition state. Cleavage of the P–O5' bond then leads to 2',3'-cUMPS. Since the reaction proceeds by *inversion* of configuration at phosphorus (Scheme 3), the attack of the 2'-oxyanion must take place "in-line" to the scissile P–O5' bond: the cleavage products are formed without pseudorotation. Dianionic phosphoranes are usually considered to be so unstable that they are rather regarded as transition states than intermediates,³¹ although opposite opinions have also been presented.³² Quantum chemical calculations³³ suggest that metal ions do not stabilize dianionic oxyphosphoranes in a same manner as a proton does. The fact that isomerization and desulfurization are also promoted by Zn^{2+} , however, suggests that the dianionic thiophosphorane, $\text{I}^{2-}\text{M}^{z+}$, may in this case have a definite lifetime. In other words, it could be regarded as a marginally stable intermediate that predominantly decomposes by an in-line mechanism without pseudorotation but may also be stabilized by protonation to a monoanionic species that pseudorotates. In this respect, the situation differs from that observed with 3',5'-UpU: only cleavage of the latter, not isomerization, is promoted by metal ions.²⁷ Accordingly, the sulfur coordinated metal ion appears to stabilize the dianionic thiophosphorane. We conclude

that the cleavage reaction represents a borderline case between the concerted (dianionic transition state) and stepwise (dianionic intermediate) mechanism.

Evidently, the pseudorotation barrier for $\text{I}^{2-}\text{M}^{z+}$ is high, since both of the negatively charged nonbridging ligands ($-\text{O}^-$ or $-\text{S}^-$) tend to avoid an apical position. It has been shown previously²⁴ that the hydroxide-ion-catalyzed cleavage of 3',5'-Up(s)U, proceeding via a dianionic thiophosphorane, is not accompanied by either desulfurization or isomerization. These reactions take place only via a monoanionic thiophosphorane.²⁴ By analogy, protonation of $\text{I}^{2-}\text{M}^{z+}$ to IH^- might be expected to be required to enable pseudorotation and, hence, isomerization. This protonation may be kinetically visible, since the reaction would be first order in metal ion concentration but pH-independent. As seen from Figure 2, the isomerization rate is only slightly increased with the increasing pH, and the reaction proceeds by complete *retention* of configuration at phosphorus (Scheme 4), consistent with the assumed pseudorotation.

Zn^{2+} also promotes the desulfurization of 3',5'-Up(s)U. The negatively charged sulfur ligand may be expected to prefer an equatorial position upon formation of $\text{I}^{2-}\text{M}^{z+}$. As discussed above, the considerably faster cleavage reaction proceeds by an "in-line" mechanism, and the apical positions may thus be assumed to be predominantly occupied by the attacking 2'-oxygen and the 5'-linked nucleoside. Accordingly, a pseudorotation should precede the cleavage of the PS bond. The reaction could hence be expected to exhibit a pH-dependence similar to that of the isomerization. However, the desulfurization rate is increased with the increasing pH much more markedly than the isomerization rate, the apparent reaction order being 0.7 (Figure 2). This kind of deviation from both zero- and first-order dependence might be accounted for by competing mechanisms, the relative proportions of which are varied with pH (and possibly metal ion concentration). Possibly, the reaction partially proceeds by direct "in-line" displacement of the metal-bound sulfur ligand. In other words, metal ion binding to sulfur would enable the sulfur ligand to adopt initially an apical position in the thiophosphorane, though at a low level compared to the 5'-linked nucleoside. Departure of the sulfur ligand then leads to formation of an unstable cyclic triester that is decomposed to a mixture of 2',5'- and 3',5'-UpU.

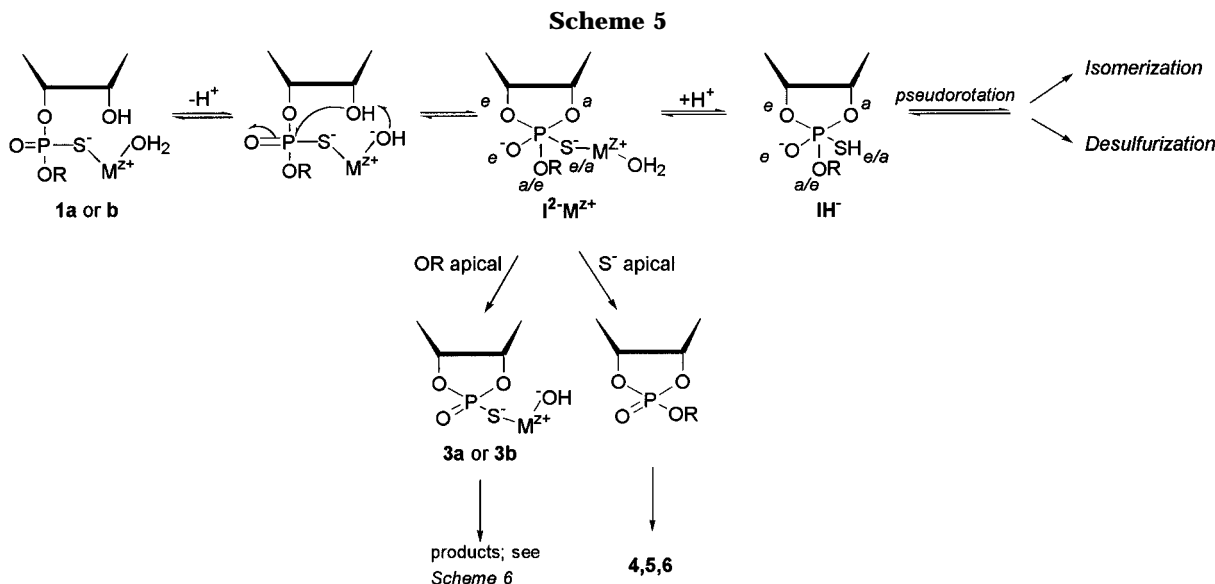
It is worth noting that Gd^{3+} , which accelerates the cleavage of 3',5'-Up(s)U almost as efficiently as Zn^{2+} , does not promote the isomerization or desulfurization. Gd^{3+} as a hard Lewis acid may be expected to prefer coordination to oxygen. Possibly, metal ion binding to oxygen does not stabilize the dianionic thiophosphorane ($\text{I}^{2-}\text{M}^{z+}$) sufficiently to allow pseudorotation, and it does not enable the sulfur ligand to adopt an apical position upon formation of $\text{I}^{2-}\text{M}^{z+}$. In other words, $\text{I}^{2-}\text{Gd}^{3+}$ would rather be a transition state than an intermediate, and it is hence decomposed only to the cleavage products. As mentioned above, metal ions do not accelerate the isomerization of 3',5'-UpU.²⁷ Binding of the metal ion to the sulfur ligand appears to be a prerequisite for the metal ion-promoted isomerization (and desulfurization).

The results of the present study show a completely different dependence of the cleavage rate on the nature of the metal ion than those reported¹⁹ for the metal-ion-promoted cleavage of a 5'-thiolate linkage in a dinucleoside phosphorothiolate, uridylyl-(3',5')-(5'-deoxy-5'-thio-

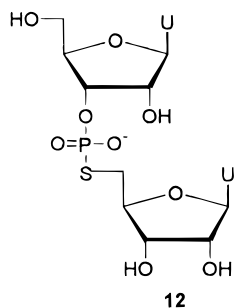
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uridine) (**12**). With the latter compounds, Mg^{2+} , Zn^{2+} , and Cd^{2+} at the concentration of 10 mmol L^{-1} facilitated



the cleavage by factors of 2, 13, and 8, respectively, i.e., even less than the cleavage of 3',5'-UpU.²⁷ With these phosphorothiolates, formation of the pentacoordinated intermediate alone may be expected to be rate-limiting, since an alkylthio group is known to be a several orders of magnitude better leaving group than an alkoxy group. Hence, the observed effects represent the influence on the formation of a pentacoordinated species that is then decomposed in a kinetically invisible manner. The metal ions are coordinated to the nonbridging oxygen and increase the electrophilicity of phosphorus and, in all likelihood, also the nucleophilicity of the attacking 2'-hydroxy group either by direct coordination to the 2'-oxygen or by deprotonating the 2'-hydroxy function with the aid of their hydroxo ligand (intracomplex general base catalysis). Any stabilizing interaction with the leaving group would not be reflected to kinetics. By contrast, with 3',5'-Up(s)U, soft Lewis acids prefer coordination to sulfur. This increases the stability of the metal ion/substrate complex and, hence, the concentration of the reactive dianionic thiophosphorane, $I^{2-}M^{2+}$. Accordingly, a significant thio effect on the metal-ion-promoted cleavage is observed with Zn^{2+} and Cd^{2+} .

In striking contrast to the results obtained with **12**,¹⁹ the catalytic effects of various metal ions on the cleavage of a P-S5' linkage in a chimeric oligonucleotide, d(ApCpGpGpTpCpT)p-rCps-d(ApCpGpApGpC)¹⁸ (for ps linkage, see **12**), closely resemble those observed for the cleavage of 3',5'-Up(s)U in the present study. Mg^{2+} , Mn^{2+} , Zn^{2+} , and Cd^{2+} at a concentration of 5 mmol L^{-1} accelerated

the cleavage by a factor of 10, 71, 370, and 3400, respectively.²⁰ Accordingly, one may ask why a P-S5' bond in a dimeric and polymeric substrate behaves so differently. A tentative explanation may be that with oligomeric substrates, but not with dimers, the bridging sulfur participates in the preequilibrium binding of the metal ion. Additional phosphodiester bonds in a polymeric substrate may result in a multidentate binding that further strengthens the interaction with the bridging sulfur. For comparison, it has been suggested that chimeric ribo/2'-deoxyribo oligonucleotides form macrochelates with metal ions, and this enhanced metal ion binding is reflected as accelerated cleavage. In particular, 3'-terminal monophosphate groups have been shown to influence in this manner.^{34,35}

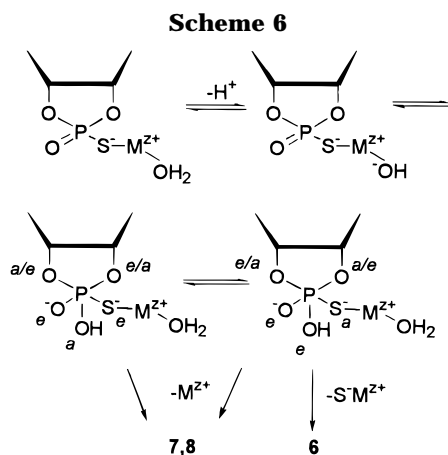
Mechanisms for the Reactions of 2',3'-cUMPS. Zn^{2+} and Cd^{2+} promote the hydrolysis of 2',3'-cUMPS even more markedly than the cleavage of 3',5'-Up(s)U and also much more effectively than the hydrolysis of 2',3'-cUMP (Table 2). Most probably, these metal ions bind to the sulfur ligand in a rapid preequilibrium step. The hydrolysis is first order in both the metal and hydroxide ion concentration, suggesting that the reaction proceeds via a dianionic thiophosphorane, analogously to the cleavage of 3',5'-Up(s)U. As discussed previously for 2',3'-cUMP,²⁶ the hydroxo ligand of the phosphate-bound metal ion may serve either as an intracomplex nucleophile or as an intracomplex general base catalyst that deprotonates the attacking water molecule (Scheme 6).

The striking difference between the metal-ion-promoted reactions of 3',5'-Up(s)U and 2',3'-cUMPS is that with the latter the desulfurization is enhanced as effectively as the diester cleavage. The desulfurization is, similarly to hydrolysis, first-order in both the metal and hydroxide ion concentration. According to the classical guidelines of Westheimer³⁶ on pseudorotating phosphorane intermediates, the apical positions of the thiophosphorane intermediate should initially be occupied by the attacking hydroxide ion and one of the sugar oxygen

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atoms. If it is accepted that the ligands may depart from the intermediate only via an apical position, the dianionic thiophosphorane must pseudorotate to enable desulfurization. This pseudorotation of the dianionic thiophosphorane appears to compete with the “in-line” displacements of the 2'- and 3'-oxyanions much more efficiently than the pseudorotation of the corresponding intermediate derived from 3',5'-Up(s)U competes with the “in-line” displacement of the 5'-linked nucleoside. Possibly, the metal aquo ligand really serves as an intracomplex general acid catalyst in the latter reaction.

Reactions of 2'- and 3'-UMPS (7, 8). The metal-ion-promoted cyclization/desulfurization of 2'- and 3'-UMPS to 2',3'-cUMP (route F in Scheme 2) is also of interest, since this reaction does not take place in the absence of metal ions. One may tentatively assume that the metal ion undergoes a rapid initial binding to the sulfur ligand and, hence, increases the electrophilicity of the phosphorus atom of the monoanionic thiophosphate. Attack of the neighboring hydroxy group then results in an “in-line” displacement of the metal-bound sulfur ligand. Whether the nucleophilicity of the attacking hydroxy group is increased by the metal ion, as suggested in Scheme 5 for the cleavage and desulfurization of 3',5'-Up(s)U, remains unanswered. The dethiophosphorylation (route G in Scheme 2) that competes with the cyclization is not metal-ion-promoted, but the reaction is retarded by increasing concentration of the metal ion. As discussed previously,²⁸ this reaction most likely proceeds by a dissociative mechanism leading to formation of a metathiophosphate ion. Evidently, binding of a metal ion to the sulfur ligand stabilizes more markedly the starting material than the developing metathiophosphate ion.

Experimental Section

Materials. (*S_P*)- and (*R_P*)-3',5'-Up(s)U (**1a,b**),³⁷ (*S_P*)- and (*R_P*)-2',3'-cUMPS (**3a,b**),²⁴ and 2'- and 3'-UMPS (**7, 8**)²⁴ were obtained by the procedures described previously. 3',5'- and 2',5'-UpU (**4, 5**), uridine, and its monophosphates used as reference materials were commercial products of Sigma. The metal salts and buffer acids used were of analytical grade.

Kinetic Measurements. The hydrolytic reactions were carried out in sealed tubes immersed in a thermostated water bath at 363.2 K (±0.1 K). The initial substrate concentration was ca. 10⁻⁴ mol L⁻¹. Aliquots withdrawn from the reaction mixture at appropriate time intervals were cooled in an ice

Table 4. Observed Retention Times for the Hydrolytic Products of 1a and 1b on RP HPLC^a

compd	<i>t_R</i> ^b /min	compd	<i>t_R</i> ^c /min
3a	12.0	1a	53.0
3b	35.5 ^e	2a	12.8
6	7.6	1b	21.0 ^e
7,8^d	13.0	2b	10.3 ^e
9	11.2	3b	7.9 ^e
10	8.9	4	11.5
11	10.6	5	7.6

^a On a Hypersil ODS 5 column (250 × 4 mm, 5 (μm)). Formic acid/sodium formate buffer (0.045/0.015 mol L⁻¹) containing tetramethylammonium chloride 0.1 mol L⁻¹ and an appropriate amount of acetonitrile were used as an eluent. Flow rate was 1 mL min⁻¹. ^b No acetonitrile added. ^c With 3.5% (v/v) acetonitrile in the buffer. ^d **7** and **8** may be separated from each other by using 0.1 mol L⁻¹ ammonium acetate as an eluent. ^e Not detected to accumulate when **1a** was used as the starting material.

bath to quench the reactions. Composition of the samples was analyzed by RP HPLC (UV detection at 260 nm), usually with a combination of two isocratic elutions. The separation methods and observed retention times are reported in Table 4. The signal areas of all monomeric compounds were assumed to be proportional to concentrations, since the base moiety of all the compounds was the same (1-substituted uracil). With the dinucleoside monophosphates and their thioate analogues, the molar absorptivities of the two base moieties were assumed to be additive.

The hydronium ion concentration of the reaction solutions was adjusted with HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] or MES [*N*-morpholinoethanesulfonic acid] buffer. The tendency of these buffer acids to complex with the metal ions studied is known to be low.³⁸ The pH values of the buffers were measured at 298.2 K and extrapolated to 363.2 K by using the known temperature dependence of the p*K_a* values of the buffer acids.³⁸ The pH values remained constant within ±0.2 units during each kinetic run. The metal ions were added as nitrate salts, and the ionic strength was adjusted with NaNO₃.

Calculation of the Rate Constants. The first-order rate constants (*k_{di}*) for the decomposition of 3',5'-Up(s)U (**1a** or **1b**) were calculated by applying the integrated first-order rate equation to the diminution of the peak area of the starting material. Good correlation was obtained in each run for more than 2 half-lives. The concentration of the isomerization products (**2a** or **2b**, respectively) remained low, and their subsequent breakdown was always much faster than isomerization back to the starting material.

Rate constants for the diester hydrolysis (*k₁*) of **1a** or **b** via 2',3'-cUMPS (**3a** or **3b**) were calculated by the rate equation of parallel and consecutive first-order reactions (eq 1). Here,

$$\frac{[2',3'\text{-cUMPS}]_t}{[3',5'\text{-Up(s)U}]_0} = \frac{k}{k_5 - k_{di}} (e^{-k_{di}t} - e^{-k_5t}) \quad (1)$$

[2',3'-cUMPS]_{*t*} stands for the concentration of the appropriate diastereomer of the cyclic 2',3'-thiophosphate at moment *t* and *k₅* for the first-order rate constant of its breakdown (values for *k₅* were determined independently, see below). [3',5'-Up(s)U]₀ is the initial concentration of the starting material.

Rate constants *k₁* for the Cd²⁺-promoted hydrolysis of 3',5'-Up(s)U were calculated by the method of initial rates on the basis of the accumulation of 2',3'-cUMPS during the first 5% of the reaction.

The first-order rate constants for desulfurization (*k₂*) of **1a,b** were calculated by eq 2, where [UpU]_{*t*} stands for the concentration of the equilibrium mixture of 2',5'- and 3',5'-UpU (**4, 5**) at moment *t*, and *k₄* is the first-order rate constant for their hydrolysis.²⁷

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$$\frac{[\text{UpU}]_t}{[3',5'\text{-Up(s)U}]_0} = \frac{k_2}{k_4 - k_{\text{di}}} (e^{-k_{\text{di}}t} - e^{-k_4t}) \quad (2)$$

The first-order rate constants, k_3 , for isomerization of **1a** to **1b** were calculated on the basis of the product distribution at the very early stages of the reaction (eq 3), when the contributions of the slow reverse reaction and the subsequent reactions of 2',5'-Up(s)U may be neglected.

$$k_3 = \frac{[2',5'\text{-Up(s)U}]_t}{[3',5'\text{-Up(s)U}]_0 - [3',5'\text{-Up(s)U}]_t} k_{\text{di}} \quad (3)$$

The first-order rate constants, k_5 , for the disappearance of **3a** or **3b** were calculated by the integrated first-order rate equation on the basis of the diminution of the starting material peak area. The rate constants k_5 were bisected to the first-

order rate constants of desulfurization (k_5) and hydrolysis ($k_{5'}$) on the basis of the product distribution at the very early stages of the reaction: concentration ratio **[6]**:**([7] + [8])** extrapolated to reaction time zero is equal with the ratio of the rate constants of formation of these products. The first-order rate constants for the cyclization/desulfurization (k_6) and dethio-phosphorylation (k_7) of **7** were obtained analogously. Since the total disappearance ($k_6 + k_7$) of the starting material showed a slight positive deviation from the first-order kinetics, the individual rate constants referring to various moments t were extrapolated to time zero. The values obtained were then bisected to the contributions of k_6 and k_7 on the basis of the product distribution during the early stages of the reaction.

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