# Hydrolysis and Desulfurization of the Diastereomeric Phosphoromonothioate Analogs of Uridine 2′,3′-Cyclic Monophosphate

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Hydrolyses of the two diastereomeric phosphoromonothioate analogs of uridine 2′,3′-cyclic monophosphate [($R_P$)- and ($S_P$)-2′,3′-cUMPS] at 363.2 K have been followed by HPLC over pH-range 0−12. In aqueous alkali (pH > 9) only base-catalyzed endocyclic phosphoester hydrolysis to a nearly equimolar mixture of uridine 2′- and 3′-phosphoromonothioates (2′- and 3′-UMPS) takes place, analogously to the hydrolysis of uridine 2′,3′-cyclic monophosphate (2′,3′-cUMP). The ($R_P$)- and ($S_P$)-2′,3′-cUMPS are hydrolyzed 50 and 30%, respectively, more slowly than 2′,3′-cUMP. Under neutral and acidic conditions, desulfurization of the cyclic thiophosphates to 2′,3′-cUMP competes with the phophoester hydrolysis, both reactions being acid-catalyzed at pH < 5. The desulfurization is most pronounced in strongly acidic solutions ([HCl] > 0.1 mol $L^{-1}$), where more than 90% of the starting material is degraded *via* this route. At pH < 2, the thioates are considerably, *i.e.*, more than 1 order of magnitude, more stable than 2′,3′-cUMP. While the hydrolysis of 2′,3′-cUMP is second-order in hydronium-ion concentration, that of 2′,3′-cUMPS exhibits a first-order dependence. The reactivities of the two diastereomers are comparable with each other over the entire pH-range studied, the most significant difference being that the pH-independent desulfurization at pH > 5 is with the $R_P$-isomer 5-fold faster than with the $S_P$-isomer. In contrast to 2′,3′-cUMP, depyrimidination of the starting material (*i.e.*, release of the uracil base) competes with the hydrolysis of the thiophosphate moiety under neutral conditions (pH 6−8).

## Introduction

The usefulness of chiral nucleoside phosphorothioates as stereochemical probes in mechanistic studies of enzyme-catalyzed phosphate transfer was first demonstrated by Eckstein *et al.*, who synthesized the diastereomeric uridine 2′,3′-cyclic phosphoromonothioates (2′,3′-cUMPS; **1a,b**)[1] and showed that of these only the one having the *endo* ($R_P$) configuration at phosphorus (**1a**) is a substrate for RNase A.[2,3] Subsequently, the diastereomers of 2′,3′-cUMPS and their guanosine analog, 2′,3′-cGMPS, were used to elucidate the stereochemistry of the second step of RNA-hydrolysis catalyzed by RNase A and RNase $T_1$, respectively.[4] Since these pioneering works, chiral thiophosphate esters have become one of the most widely used tools for stereochemical and mechanistic studies of biological reactions of nucleic acid constituents.[5] More recently, the stereochemical course of the ribozyme-catalyzed phosphodiester hydrolysis and the potential metal ion binding sites have been probed by inserting an $R_P$ or $S_P$ phosphorothioate linkage at a given site of the substrate chain.[6,7]

In spite of extensive usage of nucleoside phosphorothioates in biochemical studies, only few kinetically relevant investigations on their chemical hydrolysis have been reported. We have recently described the kinetics for concurrent hydrolysis, desulfurization, and intramolecular transesterification of the diastereomeric phosphoromonothioate analogs (**2a**,**b**) of uridylyl(3′,5′)-uridine over a wide pH range.[8] One of the parallel reactions, *viz.* hydrolysis, proceeds *via* intermediary formation a 2′,3′-cylic phosphorothioate (**1a** or **1b**), although this initial product is usually not markedly accumulated. A similar cyclic intermediate is also formed by the action of RNases and hammerhead ribozymes on internucleosidic phosphoromonothioate linkages.[5,7] Accordingly, quantitative information on the hydrolytic behavior of **1a**,**b** appears relevant. It has been shown previously[1,2] that **1a** is hydrolytically more stable than its phosphate analog, uridine 2′,3′-cyclic monophosphate (2′,3′-cUMP; **1c**), the so-called "thio-effect" being 6 in aqueous alkali and 200 in aqueous acid (0.15 mol $L^{-1}$ $HClO_4$). The base-catalyzed reaction was reported to yield a mixture of uridine 2′- and 3′-thiomonophosphates (**3a**;**4a**), whereas under acidic conditions the major products (>70%) were desulfurized nucleotides, 2′- and 3′-UMP (**3b**;**4b**). The present study was undertaken to learn more about the kinetics of the competing hydrolysis and desulfurization reactions of **1a** and **1b** in comparison with the hydrolysis of the natural nucleotide **1c**. We feel that these results are useful in analyzing the fate of phosphoromonothioate linkages in ribozyme studies.

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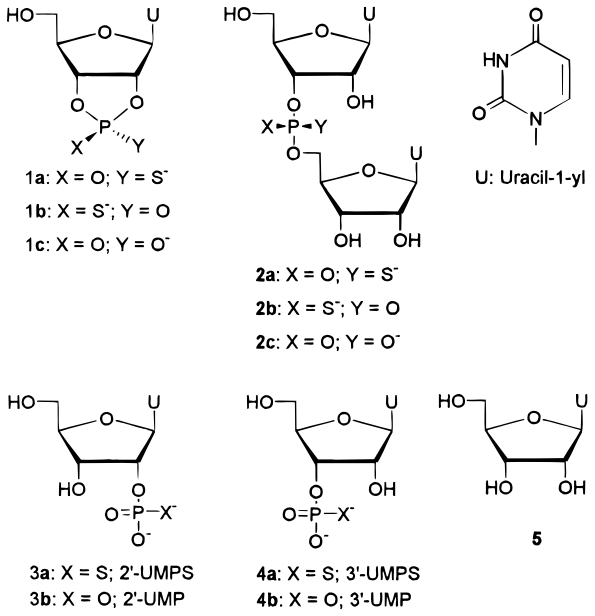



## Results and Discussion

**pH-Dependent Product Distributions.** Decomposition of the diastereomeric monothioate analogs of 2′,3′-cUMP (2′,3′-cUMPS; **1a**,**b**) was followed by HPLC over a wide acidity range [from $H_0 = 0$ to $-\lg([H^+]/\text{mol L}^{-1}) = 12$] at 363.2 K, and the products were identified by spiking with authentic samples. Under strongly acidic conditions ($[H^+] > 0.1$ mol $L^{-1}$), both diastereomers are hydrolyzed to a mixture of 2′- and 3′-UMP ([**3b**]/[**4b**] = 2/3) and their thioate analogs 2′- and 3′-UMPS ([**3a**]/[**4a**] = 2/3). During the early stages of the reaction the ratio [2′/3′-UMP]/[2′/3′-UMPS] is 9:1 (the data for **1b** at pH 1 given as an example in Figure 1). 2′- and 3′-UMPS (**3a**, **4a**) are subsequently dethiophosphorylated to uridine (**5**) and desulfurized to 2′/3′-UMP, as described previously.[9] 2′- and 3′-UMPS are neither interconverted nor cyclized to **1a**,**b** under the experimental conditions.[9] Accordingly, **1a** and **1b** undergo two parallel reactions: irreversible ring opening to 2′/3′-UMPS and desulfurization to 2′,3′-cUMP that is rapidly hydrolyzed[10−12] to 2′/3′-UMP. Under less acidic conditions (pH 2 to 5), appearance of 2′,3′-cUMP as an intermediate is experimentally observed (Figure 2). Owing to relatively rapid dephosphorylation of 2′- and 3′-UMP to uridine at pH > 2,[11,13] these compounds are not accumulated as markedly as in more acidic solutions. In summary, the reactions taking place under acidic conditions may be depicted by routes A and B in Scheme 1.

Under neutral conditions (pH 6−8), hydrolysis of both ($R_P$)- and ($S_P$)-2′,3′-cUMPS yields a considerable amount of uracil (up to 40% of the products) in addition to 2′,3′-cUMP, 2′/3′-UMP and uridine. In striking contrast, neither 2′,3′-cUMP nor 2′/3′-UMP[13] give uracil under the same conditions, and only traces of uracil (<5%) were detected to be formed from 2′- and 3′-UMPS. Accordingly, the base moiety appears to be cleaved directly from 2′,3′-cUMPS. To verify the identity of the product assumed to be uracil, this product was isolated by HPLC and characterized spectroscopically. Both the mass spectrum (EI) and $^1$H NMR spectrum were identical with those of an authentic sample of uracil. It is worth noting that the release of uracil is significant only at pH < p$K_a$ of the uracil base (pH < 9). Evidently, deprotonation of the base moiety of 2′,3′-cUMPS retards the depyrimidination. No attempts were made to clarify the mechanism of this reaction.

At high pH (pH > 9), the endocyclic phosphoester hydrolysis of 2′,3′-cUMPS to a mixture of 2′- and 3′-UMPS (route A, [**3a**]/[**4a**] = 0.45/0.55) is the only reaction detected. Accordingly, in addition to routes A and B,

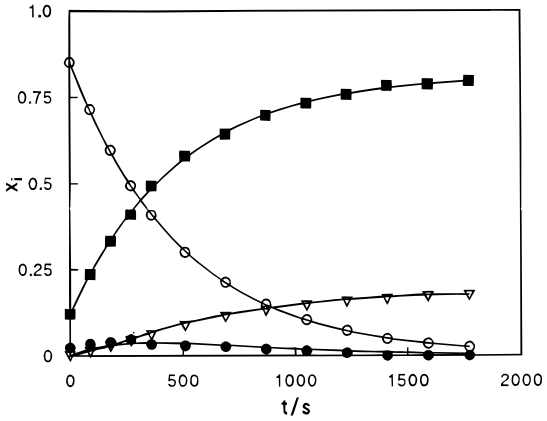



**Figure 1.** Time-dependent product distribution for the hydrolysis of ($S_P$)-2′,3′-cUMPS (**1b**) in 0.1 mol $L^{-1}$ aqueous hydrogen chloride at 363.2 K. Notation: (○) ($S_P$)-2′,3′-cUMPS; (●) mixture of 2′- and 3′-UMPS; (■) mixture of 2′- and 3′-UMP; (▽) uridine.

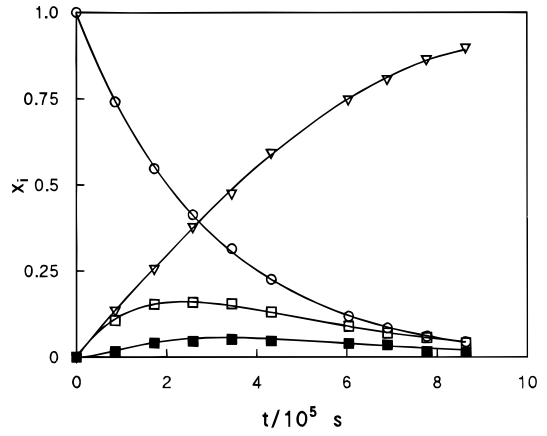
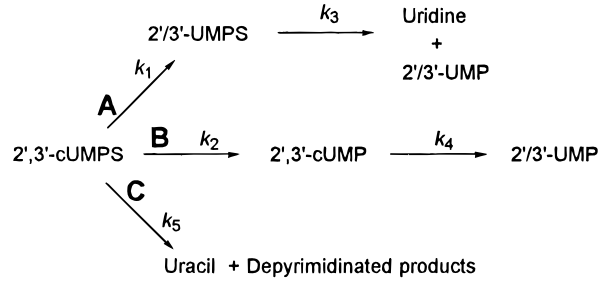



**Figure 2.** Time-dependent product distribution for the hydrolysis of ($S_P$)-2′,3′-cUMPS (**1b**) in a formic acid buffer at pH 4.0 ([HCOOH]/[HCOONa] = 0.01/0.02 mol $L^{-1}$; $I$ = 0.1 mol $L^{-1}$ with NaCl) at 363.2 K. Notation: (○) ($S_P$)-2′,3′-cUMPS; (□) 2′,3′-cUMP; (■) mixture of 2′- and 3′-UMP; (▽) uridine.

**Scheme 1**

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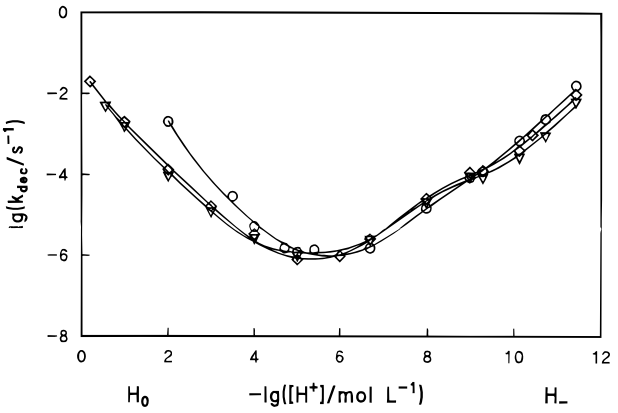



**Figure 3.** pH−rate profiles for the decomposition of ($R_P$)-2′,3′-cUMPS (**1a**, ▽), ($S_P$)-2′,3′-cUMPS (**1b**, ◇), and 2′,3′-cUMP (**1c**, ○) at 363.2 K. The ionic strength of the solutions was adjusted to 0.1 mol $L^{-1}$ with sodium chloride.

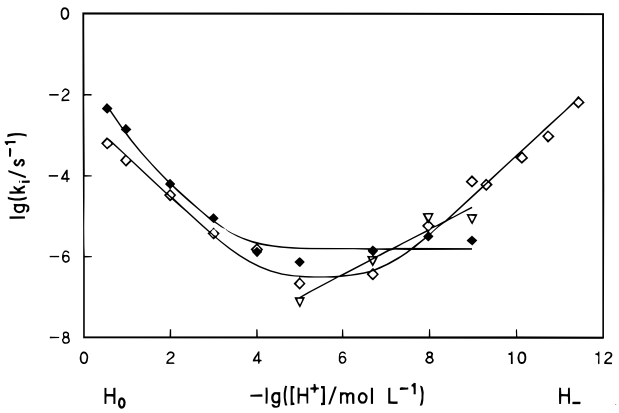



**Figure 4.** pH−rate profiles for the hydrolytic reactions of ($R_P$)-2′,3′-cUMPS (**1a**) at 363.2 K. The ionic strength of the solutiuons adjusted to 0.1 mol $L^{-1}$ with sodium chloride. Notation: (◇) phosphoester hydrolysis; (◆) desulfurization; (▽) depyrimidination.

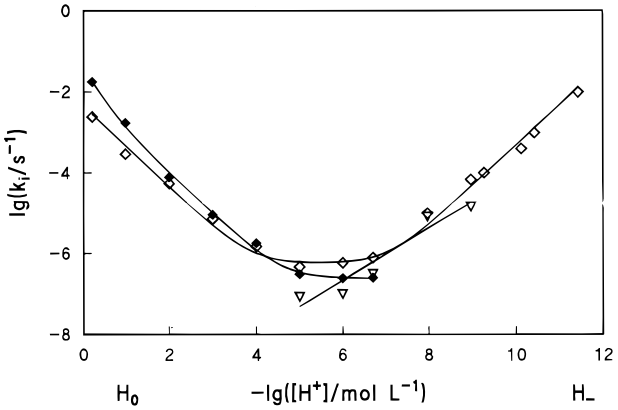



**Figure 5.** pH−rate profiles for the hydrolytic reactions of ($S_P$)-2′,3′-cUMPS (**1b**) at 363.2 K. The ionic strength of the solutiuons adjusted to 0.1 mol $L^{-1}$ with sodium chloride. Notation: (◇) phosphoester hydrolysis; (◆) desulfurization; (▽) depyrimidination.

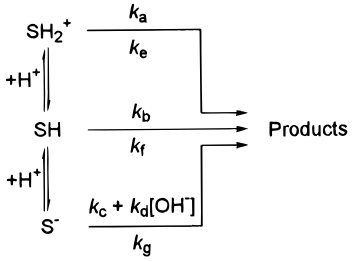
route C in Scheme 1 is also utilized under neutral and slightly alkaline conditions.

To exclude the possibility of an oxidative desulfurization at the pH-independent region (pH > 4), the reaction solutions were, firstly, thoroughly purged with nitrogen before initiating the reactions. Secondly, EDTA (1 mmol $L^{-1}$) was added to the reaction buffers (acetate and triethanolamine buffers), and thirdly, the effect of added dithiotreitol (1 mmol $L^{-1}$) on reaction kinetics was also determined. While purging with nitrogen and addition of EDTA had no effect on the kinetics of desulfurization, addition of dithiotreitol slightly accelerated both the hydrolysis and desulfurization. Accordingly, no indication of possible oxidative nature of the desulfurization could be obtained.

The time-dependent product distributions discussed above were used to calculate the partial rate constants for the phosphoester hydrolysis ($k_1$), desulfurization ($k_2$) and depyrimidination ($k_5$), of ($R_P$)- and ($S_P$)-2′,3′-cUMPS (see Scheme 1), as described in detail in the Experimental Section. The rate constants obtained at different hydronium ion concentrations gave the pH−rate profiles discussed below.

**pH−Rate Profiles and Mechanisms for the Phosphoester Hydrolysis and Desulfurization.** Figure 3 shows the pH−rate profile for the decomposition of ($R_P$)- and ($S_P$)-2′,3′-cUMPS at 363.2 K. As seen, the reactivities of the two diastereomers are comparable, the $S_P$-isomer being 20−50% more reactive than the $R_P$-diastereomer over the entire pH range studied. Under basic and neutral conditions, the diastereomeric 2′,3′-cUMPS react approximately as rapidly as 2′,3′-cUMP, but under acidic conditions they are considerably more stable. On comparing the decomposition rates of 2′,3′-cUMPS and 2′,3′-cUMP, one has to bear in mind that with 2′,3′-cUMP the only reaction over the entire pH range is the endocyclic phosphodiester hydrolysis to 2′/3′-UMP, whereas with the thioate analogs desulfurization to 2′,3′-cUMP competes with the phosphodiester hydrolysis, in particular in acidic solutions where this reaction predominates. Furthermore, depyrimidination markedly contributes to the total decomposition rate at pH 6−8. The pH−rate profiles for the individual partial reactions are depicted in Figures 4 and 5. Generally speaking, the competition between hydrolysis, desulfurization, and depyrimidination is very similar with both ($R_P$)- and ($S_P$)-2′,3′-cUMPS. In fact, the only significant difference is that the desulfurization of

**Scheme 2**




the $R_P$-isomer is around neutral pH slightly more favored over the phosphodiester hydrolysis than that of the $S_P$-isomer.

It has been shown previously[10,11] that the observed pseudo-first-order rate constant, $k_1$, of the hydrolysis of nucleoside 2′,3′-cyclic monophosphates may be expressed by eq 1 over the pH range where the predominant ionic

$$k_1 = k_a[H^+]^2 + k_b[H^+] + k_c + k_d[OH^-] \qquad (1)$$

form is the phosphodiester monoanion (pH > 0.5). The partial rate constants $k_a$, $k_b$, $k_c$, and $k_d$ are those indicated in Scheme 2. Table 1 records the values obtained for the decomposition of ($R_P$)- and ($S_P$)-2′,3′-cUMPS and 2′,3′-

**Table 1. Rate Constants for the Various Partial Reactions of the Hydrolysis and Desulfurization of ($R_P$)- and ($S_P$)-2′,3′-cUMPS (1a,b) and of Hydrolysis of 2′,3′-cUMP (1c) at 363.2 K[a]**

| | ($R_P$)-cUMPS | ($S_P$)-cUMPS | cUMP |
|---|---|---|---|
| $k_a$/L$^2$ mol$^{-2}$ s$^{-1}$ | b | b | 16.1 |
| $k_b$/10$^{-3}$ L mol$^{-1}$ s$^{-1}$ | 3.1 | 4.5 | 51 |
| $k_c$/10$^{-6}$ s$^{-1}$ | 0.29 | 0.57 | 0.98 |
| $k_d$/L mol$^{-1}$ s$^{-1}$ | 0.085 | 0.13 | 0.18 |
| $k_e$/L$^2$ mol$^{-2}$ s$^{-1}$ | 0.045 | 0.030 | |
| $k_f$/10$^{-3}$ L mol$^{-1}$ s$^{-1}$ | 6.0 | 10.1 | |
| $k_g$/10$^{-6}$ s$^{-1}$ | 1.5 | 0.24 | |

[a] The ionic strength adjusted to 0.1 mol L$^{-1}$ with sodium chloride. For the rate constants see the schemes. [b] Could not be determined.

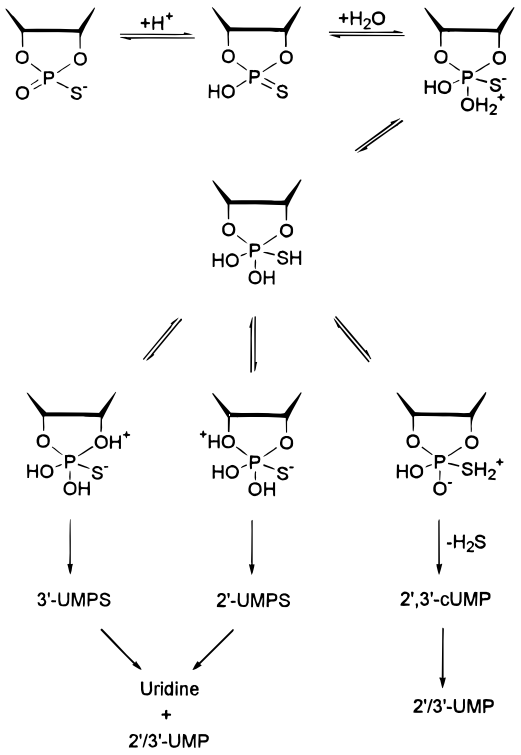
cUMP by fitting the observed rate constants of hydrolysis ($k_1$) to eq 1 and those of desulfurization to eq 2.

$$k_2 = k_e[H^+]^2 + k_f[H^+] + k_g \qquad (2)$$

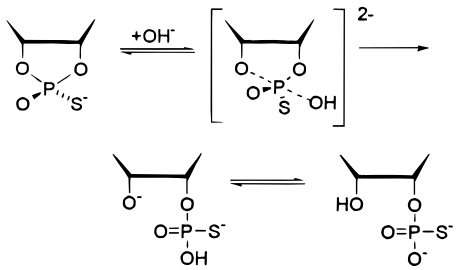
These data, and the shape of the pH−rate profiles (Figures 4 and 5), reveal one basic difference between the hydrolysis of 2′,3′-cUMP and 2′,3′-cUMPS. The dominant kinetic term at pH < 4 is $k_b[H^+]$ with the hydrolysis and desulfurization of 2′,3′-cUMPS and $k_a[H^+]^2$ with the hydrolysis of 2′,3′-cUMP. In other words, 2′,3′-cUMPS exhibits first-order and 2′,3′-cUMP second-order dependence on hydronium ion concentration. Only the desulfurization to 2′,3′-cUMP shows some indication of second-order dependence on hydronium ion concentration at pH < 2. Mechanistically, this means that the hydrolytic reactions of 2′,3′-cUMPS proceed *via* neutral phosphodiester, while 2′,3′-cUMP reacts *via* the phosphodiester monocation. A similar difference has previously been observed in the behavior of 3′,5′-UpU (**2c**) and its phosphoromonothioate analogs (**2a**,**b**).[8] Replacement of one of the nonbridging phosphate oxygens with sulfur appears to retard protonation of neutral phosphodiester to such an extent that hydrolysis and desulfurization *via* the monocationic starting material cannot compete with the reactions occuring *via* the neutral species. Consequently, the monothioates are in very acidic solutions hydrolytically much more stable than their oxygen counterparts, while the hydrolysis rates under mildly acidic conditions are comparable.

In all likelihood, the acid-catalyzed hydrolysis and desulfurization of 2′,3′-cUMPS proceed *via* a common pentacoordinated intermediate[14] obtained by the attack of a water molecule on the phosphorus atom of the neutral (and possibly monocationic) cyclic phosphorothioate (Scheme 3). Cleavage of the mercapto ligand as dihydrogen sulfide gives 2′,3′-cUMP (desulfurization), while departure of one of the sugar hydroxy groups produces either 2′- or 3′-UMPS (hydrolysis). At pH 2−3, breakdown of the intermediate to desulfurization products is moderately favored; desulfurization is twice as fast as hydrolysis. Under more acidic conditions, the product distribution is even more strongly biased in favor of desulfurization. Evidently, this change in product distribution results from the fact that the reaction *via* monocationic phosphorothioate becomes more important, and this partial reaction appears to favor desulfurization products over hydrolysis products more strongly than the reaction *via* neutral phosphorothioate. In this respect the situation is opposite to that observed for 3′,5′-Up(s)U.[8] With the latter compound, desulfurization predominates over phosphodiester hydrolysis and phosphate

**Scheme 3**




**Scheme 4**




migration at pH > 1 but becomes slower than these reactions under more acidic conditions. Why the thiophosphorane intermediates derived from 2′,3′-cUMPS and 3′,5′-Up(s)U do behave so differently remains to be answered.

The base-catalyzed hydrolysis of the diastereomeric 2′,3′-cUMPS to an almost equimolar mixture of 2′- and 3′-UMPS exhibits first-order dependence on hydroxide-ion concentration at pH > 9, analogously to the hydrolysis of 2′,3′-cUMP. The rate constants $k_d$ in Table 1 indicate that ($R_P$)- and ($S_P$)-2′,3′-cUMPS are hydrolyzed 50% and 30% less rapidly than 2′,3′-cUMP, respectively. The hydrolysis under these conditions most likely involves an "in-line" displacement of the leaving 2′- or 3′-hydroxy group by hydroxide ion.[14] In other words, the reaction takes place *via* a pentacoordinated transition state rather than *via* a pentacoordinated intermediate (Scheme 4). According to quantum chemical calculations,[15] the dianionic phosphorane species can hardly be stable enough to undergo ligand reorganization by a pseudorotation process. Consistent with the assumed instability of the

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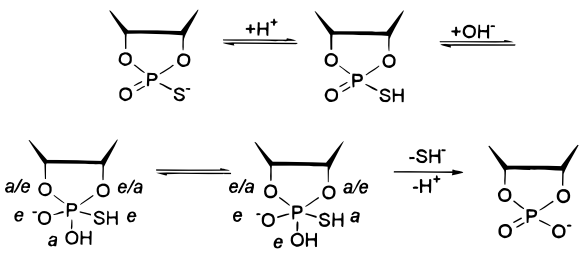
**Scheme 5**



dianionic phosphorane, desulfurization of 2′,3′-cUMPS does not compete with the phosphoester hydrolysis in alkaline solutions. According to Westheimer's concept on pseudorotating phosphorane intermediates,[14] the attacking hydroxide ion adopts an apical position on formation of a pentacoordinated transition state. The remaining apical position is occupied by either the 2′- or 3′-sugar oxygen, since one of these must be apical and the other equatorial. If the pentacoordinated species obtained is too unstable to pseudorotate, the sulfur ligand cannot adopt an apical position, and hence it is unable to leave. The small "thio-effect" of the alkaline hydrolysis is comparable to that reported for the base-catalyzed hydrolysis of 3′,5′-Up(s)U,[8,16] which proceeds by an "in-line" displacement of the 5′-linked nucleoside by the neighboring 2′-oxyanion.

The pH−rate profiles of the phosphoester hydrolysis of neither 2′,3′-cUMP nor the diastereomeric 2′,3′-cUMPS consist of a clearcut pH-independent part. In contrast, the desulfurization of 2′,3′-cUMPS is pH-independent at pH > 5. One may tentatively assume that this reaction proceeds either by attack of a molecule of water on the tetracoordinated phosphorus of a phosphate monoanion or by attack of a hydroxide ion on a neutral phosphate. The mercapto ligand may depart as hydrogen sulfide ion after protolytic rearrangement and pseudorotation of the monoanionic pentacoordinated intermediate obtained (Scheme 5). Similarly, the desulfurization of 3′,5′-Up(s)U has been shown to be pH-independent at pH > 3 and suggested to proceed by departure of hydrogen sulfide ion from the monoanionic pentacoordinated intermediate.[8]

**Conclusions.** The ($R_P$)- and ($S_P$)-diastereomers of 2′,3′-cUMPS are hydrolyzed in aqueous alkali to an almost equimolar mixture of 2′- and 3′-UMPS 2.1 and 1.5 times less readily than 2′,3′-cUMP to 2′- and 3′-UMP. Accordingly, the thiosubstitution only moderately retards the nucleophilic attack on tetracoordinated phosphorus, consistent with the earlier observations on the alkaline hydrolysis of methyl 2,4-dinitrophenyl phosphate[17] and dinucleoside monophosphates.[8,16−18] However, ($R_P$)- and ($S_P$)-2′,3′-cUMPS are in aqueous acid from 1 to 2 orders of magnitude more stable than 2′,3′-cUMP, the hydrolytic desulfurization to 2′,3′-cUMP predominating over the phosphoester hydrolysis. A similar stability difference has previously[8] been observed between dinucleoside monophosphates and their thioate analogs under acidic conditions. These large thio effects largely result from less ready protonation of phosphorothioate diesters to a reactive monocationic form. Unlike with dinucleoside phosphoromonothioates, the pentacoordinated intermediate derived from the monocationic 2′,3′-cUMPS is decomposed to desulfurization products to a larger extent than the one obtained from neutral starting material. In other words, the predominance of desulfurization to 2′,3′-cUMP over hydrolysis to 2′/3′-UMPS is increased with decreasing pH. Near neutral pH, release of uracil base from the starting material (depyrimidination) competes with the reactions of the phosphate moiety.

## Experimental Section

**Materials.** The preparation of the monothioates ($R_P$)- and ($S_P$)-2′,3′-cUMPS (**1a**,**b**) and 2′- and 3′-UMPS (**3a**, **4a**) has been described previously.[8] Uridine, uridine monophosphates, and uracil were products of Sigma. They were used as received after the purity was checked by HPLC.

**Kinetic Measurements.** Reactions were followed by the HPLC method described previously,[8,19] employing analytical methods and reaction solutions similar to those of the earlier study.[8]

The rate constants reported refer to buffer concentration zero. Two runs at different buffer concentrations were carried out at each pH studied. The effect of the buffer concentration ([buffer] < 0.05 mol $L^{-1}$) on the rate of phosphoester hydrolysis and desulfurization was relatively small (<30%).

**Calculation of the Rate Constants.** The pseudo-first-order rate constants for the decomposition of 2′,3′-cUMPS ($k_{dec}$) were obtained by applying the integrated first-order rate equation to the time-dependent concentration of the starting material. The first-order rate constants ($k_1$) for the phosphodiester hydrolysis of the diastereomers of 2′,3′-cUMPS were calculated by eq 3, where $c_0$(cUMPS) stands for the initial

$$\frac{c_t(\text{UMPS})}{c_0(\text{cUMPS})} = \frac{k_1}{k_3 - k_{dec}}[\exp(-k_{dec}t) - \exp(-k_3 t)] \quad (3)$$

concentration of 2′,3′-cUMPS, $c_t$(UMPS) denotes the sum concentration of 2′- and 3′-UMPS at moment $t$, and $k_3$ is the first-order rate constant of the disappearance of 2′/3′-UMPS. The values of $k_3$ have been reported previously.[9]

The first-order rate constants, $k_2$, for the desulfurization of 2′,3′-cUMPS under acidic conditions were calculated by eq 4,

$$\frac{c_t(\text{cUMP})}{c_0(\text{cUMPS})} = \frac{k_2}{k_4 - k_{dec}}[\exp(-k_{dec}t) - \exp(-k_4 t)] \quad (4)$$

where $c_0$(cUMPS) stands for the initial concentration of the starting material, $c_t$(cUMP) denotes the concentration of 2′,3′-cUMP at moment $t$, and $k_4$ is the first-order rate constant of the hydrolysis of 2′,3′-cUMP.

The rate constant for depyrimidination ($k_5$) was calculated by eq 5, where $c_t$(Ura) and $c_t$(cUMPS) stand for the concentrations of uracil and 2′,3′-cUMPS at moment $t$, respectively.

$$k_5 = [c_t(\text{Ura})/[c_0(\text{cUMPS}) - c_t(\text{cUMPS})]]k_{dec} \quad (5)$$

Usually, only either $k_1$ or $k_2$ could be calculated by means of the equation of two consecutive first-order reactions (eqs 3 and 4). Equation 6 was then applied to obtain the other rate constant.

$$k_{dec} = k_1 + k_2 + k_5 \quad (6)$$

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