Hydrolytic Reactions of the Diastereomeric Phosphoromonothioate Analogs of Uridylyl(3′,5′)uridine: Kinetics and Mechanisms for Desulfurization, Phosphoester Hydrolysis, and Transesterification to the 2′,5′-Isomers

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Hydrolytic reactions of the R_P and S_P diastereomers of the phosphoromonothioate analog of uridylyl-(3′,5′)uridine (3′,5′-UpU), having a nonbridging oxygen replaced with sulfur, have been followed by HPLC over a wide pH range at 363.2 K. Under neutral and acidic conditions three reactions compete: (i) desulfurization to an equilibrium mixture of 3′,5′- and 2′,5′-UpU, (ii) hydrolysis to uridine 2′- and 3′-monophosphates with release of uridine (either *via* a 2′,3′-cyclic phosphoromonothioate, or a desulfurized cyclic triester), and (iii) isomerization to the 2′,5′-dinucleoside phosphoromonothioate. With both diastereomers, desulfurization predominates over hydrolysis and migration at pH 1–8. Migration proceeds by retention of configuration at phosphorus and is most pronounced in very acidic solutions (H_0 < 0.2, *i.e.*, [HCl] > 0.5 mol L^{-1}), representing 20–30% of the total disappearance of the starting material. At pH 3–6, the proportion of this reaction is less than 10%. In the latter pH range, all the reactions are pH-independent. At lower pH, first-order dependence on acidity is observed, but at H_0 < 0.2 desulfurization becomes slower than the competing reactions. The R_P diastereomer is at pH < 7 up to three times as reactive as the S_P isomer. Under alkaline conditions (pH > 9), only base-catalyzed hydrolysis to uridine 2′- and 3′-thiophosphates with release of uridine takes place. At pH < 1, the thioate analogs are more than 1 order of magnitude more stable than UpU, while at higher pH the reactivities are comparable.

Introduction

Nucleoside phosphoromonothioates containing a chiral *S*-bonded phosphorus atom in either an internucleosidic phosphodiester linkage, a cyclic phosphodiester group, or a 5′-triphosphate group have repeatedly been employed as substrates in mechanistic studies of various enzymatic reactions.[1–3] For example, the diastereomeric phosphorothioate analogs of RNA fragments having a nonbridging oxygen atom replaced with sulfur, as well as their 2′,5′-isomers,[4,5] have proved to be powerful tools for elucidation of the stereochemistry of the reactions catalyzed by RNases[1–3,6] and ribozymes.[7,8] These applications are largely based on pioneering works of Eckstein *et al.*, who developed chemical methods for synthesis of both the R_P- and S_P-diastereomers and showed that the action of snake venom phosphodiesterase and RNases A and T_2 is stereospecific.[6] Besides stereochemistry, comparisions of kinetic "thio effect" in enzymatic and nonenzymatic reactions have been utilized in probing the rate-limiting step of enzymic hydrolysis[7,8] and distinguishing between the various mechanisms proposed for the action of RNase A.[9] Apart from these mechanistic studies, phosphoromonothioate analogs of oligonucleotides have received increasing attention as antisense oligonucleotides,[10] and consequently, stereocontrolled synthesis of thioate analogs of oligodeoxynucleotides has been extensively investigated.[11] Very recently, synthesis of stereochemically homogenous oligoribonucleotide analog having all-R_P-phosphorothioate bonds was also reported.[12]

In spite of the extensive use of ribonucleoside phosphoromonothioates as research tools for molecular biology, quantitative information on their chemical reactions in aqueous solutions is scarce. It is known[13] that replacement of one of the nonbridging oxygens of 3′,5′-UpU (**1c**) with sulfur does not significantly affect the rate of base-catalyzed hydrolysis. Both R_P and S_P diastereomers, (R_P)-3′,5′-Up(s)U (**1a**) and (S_P)-3′,5′-Up(s)U (**1b**), exhibit first-order dependence of the hydrolysis rate on hydroxide ion concentration at pH 9–12, similarly to 3′,5′-UpU, **1a** reacting 30% more slowly and **1b** 30% faster than 3′,5′-UpU. Absence of a significant "thio-effect" in alkaline cleavage of 3′,5′-UpA[6] and an RNA oligomer[8] has also been reported. Under neutral conditions, **1a** and **1b** were observed to undergo marked desulfurization.[13] 3′,5′-Dinucleoside phosphoromonothioates have also been suggested to be less susceptible to acid-catalyzed hydrolysis than unmodified ribo dimers,[6] but no quantitative data are available. In contrast, diribonucleoside 3′,5′-phosphoromonothioates having the 5′-bridging oxygen replaced with sulfur have been shown

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(13) (a) Almer, H.; Strömberg, R. *Tetrahedron Lett.* **1991**, *32*, 3723. (b) Almer, H.; Stawinski, J.; Strömberg, R.; Thelin, M. *J. Org. Chem.* **1992**, *57*, 6163.

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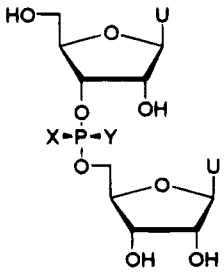
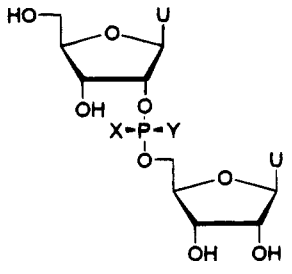
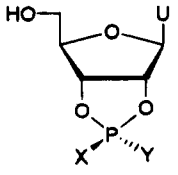
to be considerably (up to 4 orders of magnitude) more susceptible than the corresponding natural phosphates to hydrolysis under neutral and slightly basic conditions.[14a] In addition, these compounds appear to be relatively unstable upon removal of protecting groups by acids or fluoride ion.[14b]



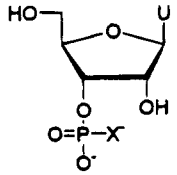
1a: X = O; Y = S⁻
1b: X = S⁻; Y = O
1c: X = O; Y = O⁻



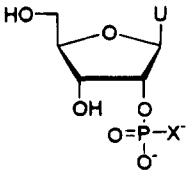
2a: X = O; Y = S⁻
2b: X = S⁻ ; Y = O
2c: X = O; Y = O⁻



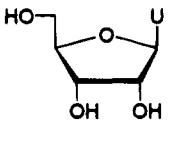
3a: X = O; Y = S⁻
3b: X = S⁻; Y = O
3c: X = O; Y = O⁻
3d: X/Y = RO; Y/X = O



4a: X = S
4b: X = O



5a: X = S
5b: X = O



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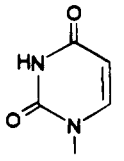


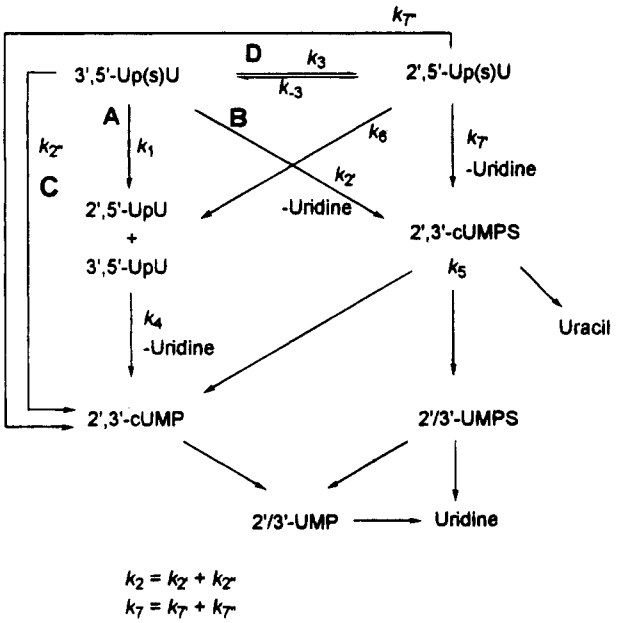
U: Uracil-1-yl

The present work is aimed at providing quantitative information on the kinetics of hydrolysis, intramolecular transesterification, and desulfurization of the internucleosidic phosphoromonothioate bonds of RNA monothioate analogs. For this purpose the reactions of diastereomeric 3′,5′-phosphoromonothioates, **1a** and **1b**, have been studied over a wide pH range. The results are compared with those obtained previously with 3′,5′-UpU[15] to elucidate the effects that replacement of a nonbridging sulfur has on the competition between the hydrolysis and intramolecular transesterification reactions, both of which proceed *via* a common pentacoordinated intermediate, and may hence be influenced by the apicophilicity of the ligands of phosphorus. The usage of pure diastereomers, **1a** and **1b**, as starting materials also allows one to follow the stereochemical course of the reactions, which cannot be done with unlabeled UpU.

Results and Discussion

Product Distributions. Diribonucleoside 3′,5′-monophosphates undergo two competing reactions in aqueous

Scheme 1



$k_2 = k_{2'} + k_{2''}$
$k_7 = k_{7'} + k_{7''}$

acid:[15] The starting material is partly converted to its 2′,5′-isomer (phosphate migration) and the isomeric mixture formed is hydrolyzed to 5′-linked nucleoside and a mixture of 2′- and 3′-monophosphates of the 3′-linked nucleoside (phosphodiester hydrolysis). The latter reaction takes place *via* intermediary formation of a cyclic 2′,3′-monophosphate, but this is hydrolytically too unstable to accumulate under acidic conditions. With the thioate analogs of 3′,5′-UpU, **1a** and **1b**, the product distribution is more complicated, since desulfurization of the starting material to UpU competes with migration and hydrolysis of the thiophosphate diester. The parallel and consecutive reactions observed are depicted in Scheme 1.

When the decomposition of the diastereomers **1a** and **1b** under acidic conditions was followed by RP HPLC, the release of uridine and the mixture of 2′- and 3′-UMP was found to be accompanied by accumulation of three intermediates, two of which were common in the reaction mixtures of both diastereomers. By comparison with authentic samples these two were shown to be 3′,5′- and 2′,5′-UpU (**1c** and **2c**, respectively), and they appeared in a constant 1:1 molar ratio throughout the kinetic run. The rate constant (k_4) of hydrolysis of the equilibrium mixture of **1c** and **2c** is known,[15] and thus the first-order rate constant (k_1) for the desulfurization of Up(s)U to UpU could be calculated by applying the equation of two consecutive first-order reactions to the time-dependent total concentration of UpU's. At pH 1, for example, this pathway was observed to represent 65 and 52% of the total decomposition of the (R_P)- and (S_P)-Up(s)U (**1a** and **1b**), respectively. The final products of route A in Scheme 1, *viz.* 2′/3′-UMP (**5b**/**4b**) and uridine (**6**), are, however, accumulated too rapidly to be entirely accounted for by this pathway, but under acidic conditions (pH < 3) 30–50% of the starting material is hydrolyzed to these products without any detectable intermediates. This phosphodiester hydrolysis may proceed *via* two different routes (routes B and C). Firstly (route B), the thiophosphate diester is cleaved to yield uridine and cyclic uridine 2′,3′-thiophosphate (2′,3′-cUMPS, **3a** or **3b**), the latter of which is rapidly hydrolyzed to 2′- and 3′-UMP. Secondly (route C), the starting material is desulfurized to the

(14) (a) Liu, X.; Reese, C. B. *Tetrahedron Lett.* **1995**, *36*, 3413. (b) Sund, C.; Chattopadhyaya, J. *Tetrahedron* **1989**, *45*, 7523.
(15) Järvinen, P.; Oivanen, M.; Lönnberg, H. *J. Org. Chem.* **1991**, *56*, 5396.

unstable cyclic triester (**3d**), which is hydrolyzed, besides 2′,5′- and 3′,5′-UpU, also directly to the monomeric products (2′/3′-UMP and uridine).

The third intermediate was assigned as a dinucleoside 2′,5′-phosphoromonothioate (**2a,b**), the product of intramolecular transesterification of the starting material (route D). The following observations are consistent with this assignment. (i) With both diastereomers (**1a,b**), the intermediate was UV-spectroscopically identical with the starting material and UpU. (ii) The chromatographic behavior of the intermediates was expected: they were eluted on the RP column faster than **1a** and **1b**, and the intermediate formed from the R_P-diastereomer had a shorter retention time than the one formed from the S_P-isomer.[16] (iii) Only the intermediate derived from the R_P-diasteromer was hydrolyzed by snake venom phosphodiesterase, though slightly slower than the starting material. With both regioisomers, only the R_P-diastereomers are known to be substrates for this enzyme.[1-3,5,6] (iv) RNase A, which cleaves only the 3′,5′-phosphodiester bonds, did not hydrolyze these intermediates or 2′,5′-UpU, although 3′,5′-UpU and its thioate analogs (**1a,b**) were hydrolyzed (the S_P-isomer much more slowly than R_P). (iv) The intermediate formed during hydrolysis of (R_P)-3′,5′-Up(s)U (**1a**) in 0.5 M aqueous hydrogen chloride was isolated by HPLC. The isolated compound, when hydrolyzed in aqueous acid, gave the expected product distribution: intermediary accumulation of 2′,5′- and 3′,5′-UpU was accompanied by appearance of **1a**, the final products being 2′/3′-UMP and uridine.

At pH 3–5, the rates of all the reactions discussed above (routes A–D in Scheme 1) are pH independent. The same reactions as in more acidic solutions take place, although the product distribution is slightly different. Desulfurization (A) strongly predominates in this pH range, and since it is much faster than the hydrolysis of UpU, the equilibrium mixture of 2′,5′- and 3′,5′-UpU constitutes the main product. 2′,3′-cUMP (**3c**) accumulates (<5%) as an intermediate of the phosphodiester hydrolysis, whereas 2′- and 3′-UMP (**5c**, **4c**) are so rapidly dephosphorylated to uridine[15,17] that they are not detected. No monothiophosphates were observed to accumulate at pH < 5.

The product distribution at pH 6–8 is very complicated. All the compounds shown in Scheme 1 were observed in a reaction mixture. In addition to the products obtained under more acidic conditions, 2′,3′-cUMPS (**3a** or **3b**, one diastereomer in each run) and 2′- and 3′-UMPS (**5a** and **4a**, respectively) were accumulated as products of the phosphodiester hydrolysis of 3′,5′-Up(s)U. More surprisingly, a significant amount of uracil (>20% of the monomeric products at pH 8) was obtained. Mechanism of the release of uracil remains obscure, but it seems to be released from 2′,3′-cUMPS.

At pH > 9, only base-catalyzed hydrolysis of Up(s)U to uridine and a mixture of 2′- and 3′-UMPS (route B in Scheme 1) takes place, as described previously.[13] The mechanism of this reaction may now be discussed in more detail, since accumulation of 2′,3′-cUMPS as an intermediate could be detected.

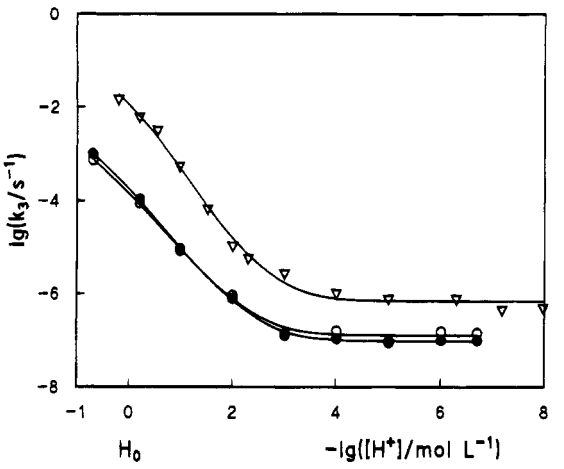


Figure 1. pH–rate profiles for isomerization (3′,5′ → 2′,5′) of (R_P)- and (S_P)-Up(s)U (○ and ●, respectively) and 3′,5′-UpU[15] (▽) at 363.2 K (I = 0.1 mol L^{-1} with sodium chloride).[18]

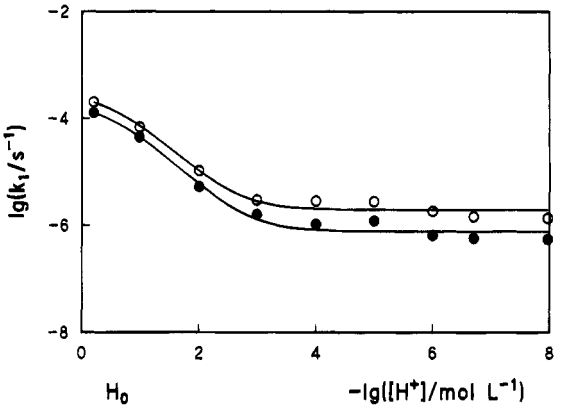


Figure 2. pH–rate profiles for desulfurization (k_1) of R_P (○) and S_P (●) diastereomers of 3′,5′-Up(s)U at 363.2 K (The ionic strength adjusted to 0.1 mol L^{-1} with sodium chloride).[18]

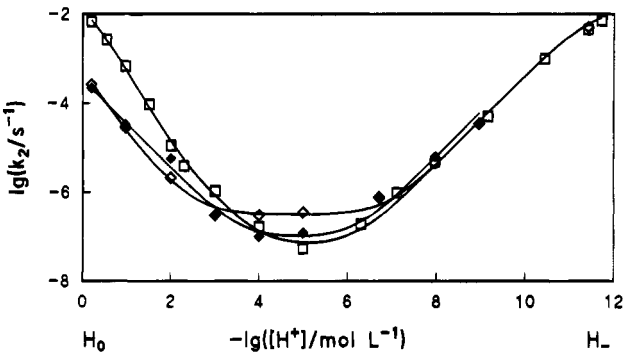


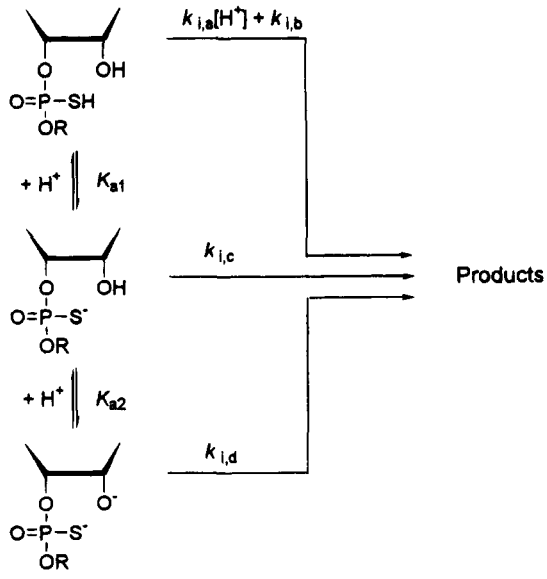
Figure 3. pH–rate profiles for phosphodiester hydrolysis of (R_P)- and (S_P)-3′,5′-Up(s)U ($k_{2'} + k_{2''}$; **1a**, ◇ and **1b**, ◆), and 3′,5′-UpU[15] (k_4, □) at 363.2 K (I = 0.1 mol L^{-1} with sodium chloride).[18,25]

pH–Rate Profiles. Comparison of the pH–rate profiles (Figures 1–3) of the hydrolysis and isomerization of 3′,5′-UpU (**1c**) and its thioate analogs (**1a,b**) reveals

(16) (a) 2′,5′-Dinucleoside monophosphates are eluted faster than the corresponding 3′,5′-isomers.[15] (b) Migration of the phosphodiester group involves pseudorotation of the pentacoordinated intermediate formed by nucleophilic attack of the 2′-hydroxyl group at phosphorus,[15] and the interconversion thus proceeds by *retention* of configuration. Accordingly, the migration product of the R_P-diastereomer should also show R_P configuration and the isomerization product of the S_P-diastereomer should be S_P. Both with dinucleoside 3′,5′-thiophosphates[2] and with their 2′,5′-isomers[5] the R_P-diastereomers have been shown to have shorter retention times on RP HPLC.

(17) (a) Oivanen, M.; Lönnberg, H. *Acta Chem. Scand.* **1990**, *44*, 239. (b) Oivanen, M.; Lönnberg, H. *J. Org. Chem.* **1989**, *54*, 2556.

Scheme 2



one basic difference. The reactions of 3′,5′-UpU exhibit a second-order dependence on acidity at pH 1 to 2 and first-order dependence under more acidic conditions, while the second-order dependence is barely noticeable with Up(s)U's. In other words, while the reactive ionic form of UpU is the phosphodiester monocation (see route $k_{i,a}[H^-] + k_{i,b}$ in Scheme 2), its thioate analogs react mainly *via* the neutral form ($k_{i,b}$ more predominating). Only under very acidic conditions ($H_0 < 0.2$)[18] does the reaction *via* monocationic phosphorothioate appear to be important, since the hydrolysis and migration remain hydronium-ion-catalyzed on passing the pK_{a1} value of the internucleosidic phosphorothioate group.[19] At pH > 3, the reactions of 3′,5′-UpU and 3′,5′-Up(s)U show a similar dependence on pH: the hydrolysis becomes base-catalyzed at pH > 6, and the migration remains pH-independent under these conditions. The pH–rate profile of the desulfurization resembles that of phosphate migration. However, the desulfurization rate tends to level off to a constant value at $H_0 = 0.2$. Due to very rapid hydrolysis of 3′,5′-UpU under more acidic conditions, a conclusive evidence for this change in the reaction order cannot be obtained. At any rate, the desulfurization is slower than the phosphate migration at pH < 1, whereas under less acidic conditions it is the prevailing reaction.

The curves shown in Figure 1–3 were obtained by least-squares fitting of the observed rate constants to eq 1 ($k_i = k_1, k_3$) or 2 (phosphodiester hydrolysis; $k_2 = k_{2'} + k_{2''}$). The rate constants thus obtained for the partial reactions ($k_{i,a}$ to $k_{i,d}$ in Scheme 2) are listed in Table 1 together with the corresponding values reported previously[15] for 3′,5′-UpU. To reduce the number of adjustable parameters, the pK_{a1} value was constrained to 0.5, but even when this was not done very similar results were obtained for the migration and desulfurization. The kinetic data on hydrolysis fitted to eq 2, however, turned out to be too inaccurate to be treated reliably without this constraint. These data (Table 1) allow the following conclusions: (i) The monocationic forms of the diastereomers **1a** and **1b** are hydrolyzed at a comparable rate, which is 3% of that of the corresponding monocationic form of 3′,5′-UpU ($k_{2,a}$ in Table 1). (ii) The neutral forms of **1a** and **1b** are, in turn, hydrolyzed approximately as rapidly as the corresponding form of 3′,5′-UpU ($k_{2,b}$). (iii) The uncatalyzed hydrolysis of the monoanionic form ($k_{2,c}$) of (R_P)-3′,5′-Up(s)U (**1a**) is 3 and 10 times as fast as that of the S_P diastereomer (**1b**) and 3′,5′-UpU, respectively. (iv) The "thio-effect" plays a minor role in alkaline hydrolysis; **1a** and **1b** are hydrolyzed to 2′,3′-cUMPS 0.8 and 1.3 times as fast, respectively, as 3′,5′-UpU to 2′,3′-cUMP ($k_{2,d}$). (v) Both diastereomers of 3′,5′-Up(s)U undergo intramolecular transesterification to 2′,5′-Up(s)U approximately as readily, and considerably more slowly than 3′,5′-UpU to 2′,5′-UpU: the reaction rates of the neutral and monocationic forms, $k_{3,b}$ and $k_{3,a}$, respectively, are 10 and 1.3% of the corresponding values of 3′,5′-UpU. (vi) The uncatalyzed migration of the monoanionic phosphorothioate group is as rapid with **1a** and **1b** and nearly 1 order of magnitude slower than with 3′,5′-UpU ($k_{3,c}$). (vii) **1a** is desulfurized from 1.5 to 3 times as fast as **1b**. The monoanionic forms ($k_{1,c}$) exhibit a greater reactivity difference than the neutral forms ($k_{1,b}$). No values for $k_{1,a}$ were obtained from the data of desulfurization.

Table 1. Partial Rate Constants for the Desulfurization (k_1), Phosphodiester Hydrolysis (k_2), and Intramolecular Transesterification (k_3) of (R_P)- and (S_P)-3′,5′-Up(s)U (1a and 1b, Respectively), and for Hydrolysis and Transesterification of 3′,5′-UpU (1c) at 363.2 K[a]

	1a	1b	1c[b]
pK_{a1}	0.5[c]	0.5[c]	0.7
$k_{1,b}/10^{-3}$ s^{-1} [d]	0.31	0.21	
$k_{1,c}/10^{-6}$ s^{-1}	1.93	0.764	
$k_{2,a}/10^{-3}$ L mol^{-1} s^{-1}	0.69	0.34	15.4[e]
$k_{2,b}/10^{-3}$ s^{-1}	0.072	0.17	0.066[e]
$k_{2,c}/10^{-6}$ s^{-1}	0.31	0.094	0.029[e]
$k_{2,d}$/s^{-1}	0.013	0.019	0.017[e]
pK_{a2}	11.5[c]	11.5[c]	11.5
$k_{3,a}/10^{-3}$ L mol^{-1} s^{-1}	0.16	0.22	13.3
$k_{3,b}/10^{-3}$ s^{-1}	0.020	0.022	0.19
$k_{3,c}/10^{-6}$ s^{-1}	0.13	0.096	0.66

[a] The ionic strength adjusted to 0.1 mol L^{-1} with sodium chloride. For rate and equilibrium constants see Schemes 1 and 2. [b] From ref 15. [c] A fixed value used for the least-squares fitting. [d] Rate constant ($k_{1,a}$) for desulfurization *via* a monocationic species was not obtained. [e] Corresponds to k_4 in Scheme 1.

$$k_i = \frac{\frac{k_{i,a}}{K_{a1}}[H^+]^2 + \frac{k_{i,b}}{K_{a2}}[H^+] + k_{i,c}}{1 + \frac{[H^+]}{K_{a1}}} \quad (1)$$

$$k_2 = \frac{\frac{k_{2,a}}{K_{a1}}[H^+]^2 + \frac{k_{2,b}}{K_{a1}}[H^+] + k_{2,c} + \frac{k_{2,d}K_{a2}}{[H^+]}}{\frac{K_{a2}}{[H^+]} + 1 + \frac{[H^+]}{K_{a1}}} \quad (2)$$

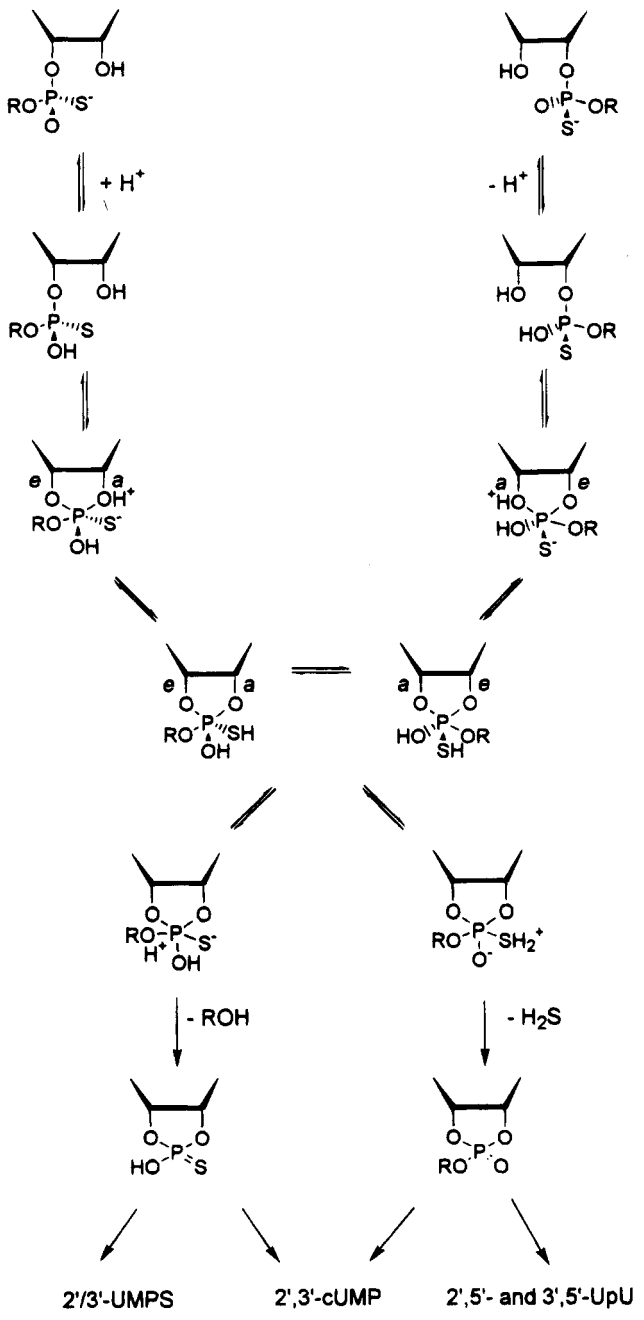
Mechanism of the Desulfurization, Phosphodiester Hydrolysis, and Thiophosphate Migration under Acidic and Neutral Conditions. 3′,5′-UpU (**1c**) undergoes under acidic and neutral conditions concurrent

(18) Acidity function H_0 was used instead of pH, when [HCl] > 0.1 mol L^{-1}. Values for H_0 were taken from: Paul, M. A.; Long, F. A. *Chem. Rev.* **1957**, *57*, 1.

(19) Thiophosphoric acid is known to be 0.4 pK_a units more acidic than orthophosphoric acid,[20] and the pK_a of UpU has been shown to be 0.7 under the experimental conditions.[15] Accordingly, the pK_a value of Up(s)U is expected to be slightly below 0.5.

(20) Perrin, D. D., Ed. *Ionisation Constants of Inorganic Acids and Bases in Aqueous Solution. IUPAC Chemical Data Series, No. 29*, 2nd ed.; Pergamon: Oxford, 1982.

Scheme 3



interconversion to 2′,5′-UpU (**2c**) and phosphodiester hydrolysis to monomeric products (uridine and 2′/3′-UMP).[15] Both reactions in all likelihood take place *via* a common intermadiate, *viz.* a pseudorotating pentacoordinated phosphorane formed by an intramolecular nucleophilic attack of the neighboring 2′-hydroxyl group on phosphorus. The mechanism of isomerization and decomposition of the diastereomeric 3′,5′-Up(s)U's may be discussed on the basis of the same concept, taking the altered apicophilicity of the ligands of phosphorus into account.

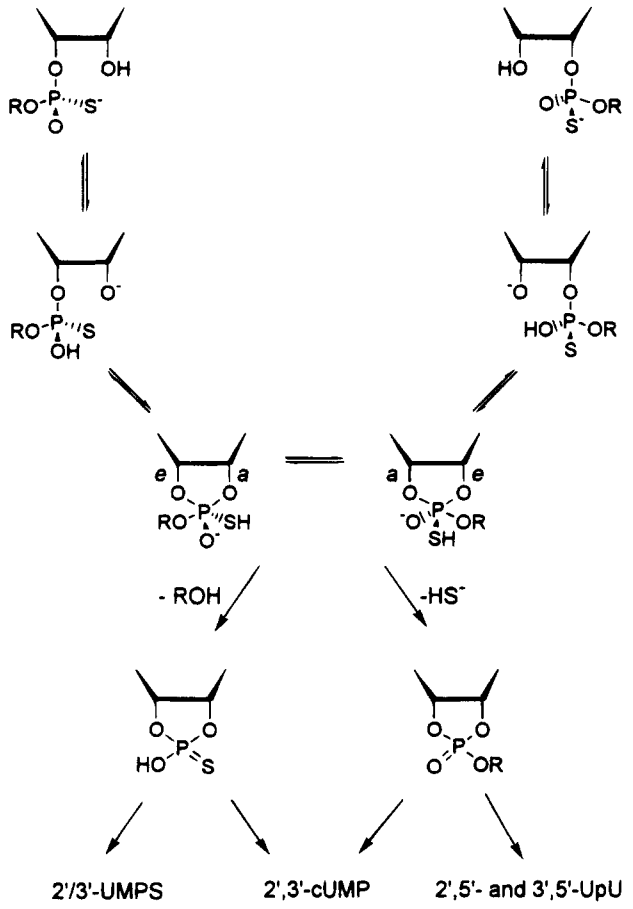
The shape of the pH–rate profiles suggests, as discussed above, that the acid-catalyzed reactions of **1a** and **1b** proceed by intramolecular nucleophilic attack of the 2′-hydroxy group on either the neutral or the monocationic phosphorothioate group (route $k_{i,a}[H^+] + k_{i,b}$ in Scheme 2; see Scheme 3), resulting in formation of the thiophosphorane intermediate. According to Westheimer's rules,[21] the attacking 2′-OH initially occupies an apical position in the trigonal bipyramid thiophosphorane, and hence the 3′-oxyligand, as a member of the same five-membered ring, adopts an equatorial position. Since the departure of the leaving group also takes place from an apical position, migration of the phosphodiester group to the 2′-position necessarily requires pseudorotation[21] of the intermediate, which accompanied by proton rearrangement brings the 3′-hydroxy group to an apical position, and hence makes it able to depart. The stereochemical outcome of the process is retention of configuration at phosphorus. Our results are consistent with this: as described above, the migration product isolated from the reaction mixture of (R_P)-3′,5′-Up(s)U (**1a**) behaved in enzymatic digestion as expected for (R_P)-2′,5′-Up(s)U (**2a**), and it was also isomerized back to **1a** concurrent with acidic hydrolysis. Migration is clearly stereospecific, since a different isomerization product was obtained from the R_P- and S_P-diastereomers. The fact that 3′,5′-Up(s)U is isomerized to 2′,5′-Up(s)U in both neutral and very acidic solutions indicates that the thiophosphorane intermediate is able to pseudorotate as a monoanionic, neutral, and monocationic species.

The interconversion of the 3′,5′- and 2′,5′-Up(s)U was never observed to reach the equilibrium position, due to fast competing reactions. This intramolecular transesterification is most pronounced under strongly acidic conditions. In 2 mol L^{-1} aqueous hydrogen chloride nearly 30% of the starting material initially reacts *via* this route, whereas at pH 1 its proportion is about 10% and in less acidic solutions even smaller. When **2a** was employed as a starting material, no migration product could be detected at pH > 3, although at $H_0 = 0.2$ interconversion was as fast as with **1a** (kinetic data given in the supporting information). The reason is that under slightly acidic conditions (pH > 3) **1a** gives desulfurization products three to six times as fast as **2a**, and hence its accumulatation is difficult to detect. Under more acidic conditions ($H_0 = 0.2$), the rates of decomposition of **1a** and **2a** differ less, the 3′,5′-isomer (**1a**) being hydrolyzed 1.7 times as fast as the 2′,5′-isomer (**2a**).

Accordingly, the competition between hydrolysis and migration appears to be different with Up(s)U's and UpU's. The monocationic and neutral forms of the phosphorane intermediate derived from 3′,5′-UpU undergo nearly symmetrical partition to hydrolysis and migration products, while the monoanionic form gives predominantly migration products.[15] With Up(s)U's, in turn, the rate of hydrolysis is at least comparable to the rate of migration over the entire pH range. It should be noted, however, that the hydrolysis products may now be obtained by two different mechanisms, as depicted above in Scheme 1. In other words, either dihydrogen sulfide or the 5′-linked nucleoside may be released from the apical position (see Schemes 3 and 4). Neither of the initial products obtained, *viz.* the 2′,3′-cyclic phosphotriester (**3d**) or 2′,3′-cUMPS (**3a,b**), accumulated under acidic conditions, and hence, the proportional rates of these two routes cannot be estimated. However, near neutral pH (6–9) **3a** and **3b** accumulated as intermediates, and thus the rate constant for hydrolysis *via* route B ($k_{2'}$ in Scheme 1) could be determined (see Experimental Section). At pH 6–8, the values obtained for $k_{2'}$ were 50–70% of the total rate constants for diester hydrolysis ($k_{2'} + k_{2''}$) obtained by subtracting k_1 and k_3 from the rate constant of disappearance of the 3′,5′-Up(s)U. The

(21) Westheimer, F. *Acc. Chem. Res.* **1968**, *1*, 70.

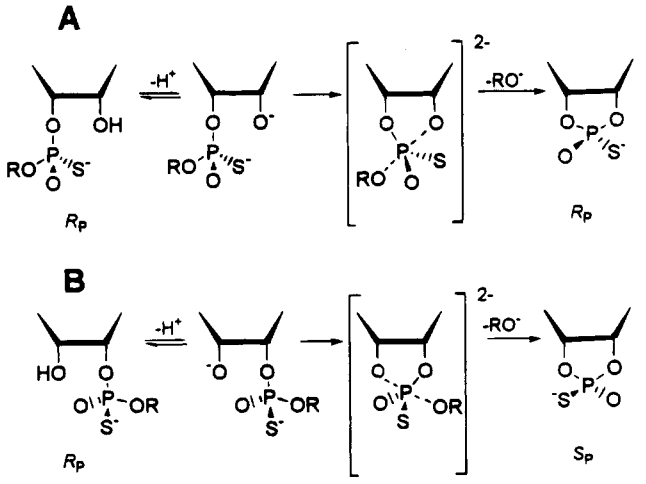
Scheme 4



observed predominance of the hydrolysis over migration may, thus, well result from facile desulfurization of the thiophosphorane intermediate (formation of **3d**) and not from enhanced departure of the 5′-linked nucleoside (formation of **3a**,**b**). **3d** is decomposed, besides the desulfurization products (isomeric UpU's), to 2′,3′-cUMP, which rapidly gives the mixture of 2′- and 3′-UMP. This assumption receives some additional support from the fact that the stereochemistry of the starting material affects in a similar manner the rate of desulfurization (k_1) and hydrolysis ($k_{2'} + k_{2''}$); the R_P-diastereomer is 2.5–3.5 times more reactive than the S_P-isomer. In contrast, the rates of thiophosphate migration (k_3) are nearly equal with both diastereomers.

Desulfurization of Up(s)U's efficiently competes with their mutual isomerization and hydrolysis at pH > 1. Only at pH < pK_{a1} (pH < 0.5) do the latter reactions predominate over the desulfurization. Under such conditions the reactions proceed *via* a monocationic thiophosphorane intermediate. The alkoxy ligands appear to depart from this species as alcohols more readily than the SH-ligand as dihydrogen sulfide, consistent with the expected site of protonation (O favored over S). The situation is completely different when the reactions take place *via* a monoanionic thiophosphorane (pH > 3; see Scheme 4): up to 90% of **1a** or **1b** and 50% of **2a** is desulfurized to 2′,5′/3′,5′-UpU. Furthermore, a major part of hydrolysis to 2′/3′-UMP may in this pH region proceed, as discussed above, *via* initial desulfurization to a 2′,3′-cyclic phosphotriester (**3d**). Evidently this results from the hydrogen sulfide ion being a much better leaving group than the alkoxide ions (pK_a of H_2S and the sugar hydroxy groups are 6.5 and 12,[15,20] respectively, under the experimental conditions).[22] The pH-independent desulfurization most likely proceeds *via* a pentacoordinated intermediate, and not *via* a pentacoordinated transition state, as evidenced by the concurrent occurence of pH-independent thiophosphate migration.

Scheme 5



Mechanism and Stereochemistry of the Base-Catalyzed Thiophoshate Diester Hydrolysis. Replacement of a nonbridging phosphoryl oxygen with sulfur does not markedly affect the kinetics of the base-catalyzed hydrolysis of dinucleoside monophosphates[13] or oligoribonucleotides.[8] The mechanism suggested for the hydrolysis of the phosphodiester bonds of RNA in the 1950's[24] most probably also applies to the reactions of the thioate analogs. Accordingly, the 2′-hydroxy group is deprotonated, and the alkoxide ion formed intramolecularly attacks the phosphorus (Scheme 5). The 5′-linked nucleoside departs "in-line" with the nucleophilic attack, and no pentacoordinated intermediate, only a transition state, is formed. 2′,3′-cUMPS is formed by inversion of configuration at phosphorus. Our results are consistent with this mechanism: the complete inversion of configuration upon the formation of the 2′,3′-cUMPS could be verified by HPLC (pH 6.7–9) and ^{31}P NMR spectroscopy (pH < 13). The concentration of this compound remained low (<10% of the products), but only one diastereomer was observed in each run. Accordingly, (R_P)-3′,5′-Up(s)U gave only (R_P)-2′,3′-cUMPS (**3a**) (Scheme 5A, note the assignment of configurations), while only (S_P)-2′,3′-cUMPS (**3b**) accumulated during the hydrolysis of (S_P)-3′,5′-Up(s)U. On the other hand, the hydrolysis of (R_P)-2′,5′-Up(s)U (**2b**) gave at pH 9 only (S_P)-2′,3′-cUMPS (**3b**, Scheme 5B). At pH 9, the rate constant obtained for route B (Scheme 1) on the basis of accumulation of 2′,3′-cUMPS had the same value (within ±10%) as the rate constant of disappearance of the starting material. In contrast, at pH 6–8, as discussed above, route B accounts for only 50–70% of the diester hydrolysis. The rate constants obtained in the latter pH region for route B exhibited the same pH-dependence as hydrolysis of 3′,5′-UpU. As reported previously,[13] **1a** and **1b** reacted 0.8

(22) The pH-independent hydrolysis and interconversion of 2′,5′- and 3′,5′-UpU have been suggested[23] to proceed by the attack of hydroxy oxyanion on neutral phosphodiester, rather than by the attack of the neutral hydroxyl group on a monoanionic phosphate (see Scheme 4). Since the attacking nucleophile is an oxyanion, the leaving nucleophile may also be assumed to be anionic.

(23) Kosonen, M; Oivanen, M; Lönnberg, H. *J. Org. Chem.* **1994**, 3704.

(24) Brown, D. M.; Magrath, D. I.; Neilson, A. H.; Todd, A. R. *Nature* **1956**, *177*, 1124.

and 1.3 times as fast, respectively, as 3′,5′-UpU (**1c**). **2a** was hydrolyzed 1.3 and 1.5 times as fast as **1a** at pH 9 and at H_- = 11.4,[25] respectively (see the supporting information). For comparison, Herschlag *et al.*[8] have studied the kinetic thio effects in reactions of methyl 2,4-dinitrophenyl phosphate with external nucleophiles and reported 4- to 11-fold rate retardations.

Conclusions. The diastereomeric thioate analogs (**1a,b**) of 3′,5′-UpU are hydrolyzed at a comparable rate with each other both under acidic and basic conditions. Under very acidic conditions (pH < 1), where the thioates are 30 times more stable than UpU, three competing reactions proceed at comparable rates: desulfurization to 2′,5′- and 3′,5′-UpU, cleavage of the thiophosphate diester bond, and isomerization of 3′,5′-Up(s)U's to their 2′,5′-isomers. At pH 3−6 the pH-independent desulfurization is the predominating reaction. This reaction is fast enough to make the thioates slightly less stable than UpU. By contrast, in the base-catalyzed hydrolysis (pH > 9) the kinetic "thio effect" is negligible; the S_P isomer (**1b**) is hydrolyzed to 2′- and 3′-thiomonophosphates and uridine slightly faster, and the R_P isomer (**1a**) slightly slower than 3′,5′-UpU to uridine and 2′/3′-UMP.

Experimental Section

Materials. Preparation of the thioate analogs (**1a,b**) of uridylyl(3′,5′)uridine has been described previously.[13] Uridylyl(3′,5′)- and -(2′,5′)uridine, as well as uridine, its monophosphates, and uracil, employed as reference materials, were commercial products of Sigma. Snake venom phosphodiesterase (*Crotalus Adamanteus* venom) was purchased from USB and ribonuclease A (Bovine Pancreas) from Sigma.

The R_P- and S_P-diastereomers of uridine 2′,3′-cyclic phosphorothioates (**3a** and **3b**) were prepared by modification of the thiophosphorylation method described earlier.[28] Accordingly, 5′-*O*-(dimethoxytrityl)uridine[29] was thiophosphorylated with 2 equiv of thiophosphoryl tris(triazole) in dry acetonitrile containing 10% dry pyridine (v/v), analogously to the oxyphosphorylation[30] (see also ref 23). After 20 h treatment at room temperature, the reaction was quenched with water and conventionally worked up. The dimethoxytrityl group was cleaved (1 h) in a 1:1 (v/v) mixture of acetonitrile and aqueous hydrogen chloride (pH 3). The solution was neutralized with aqueous sodium hydroxide, and the aqueous solution was extracted with dichloromethane and concentrated. The diastereomers **3a** and **3b** were separated by low pressure LC on a Lobar LiChroprep RP-18 column (37 × 440 mm, 40−63 μm). An acetic acid/sodium acetate buffer (pH 4.2) containing 0.1 mol L^{-1} ammonium chloride was employed as eluant. At a flow rate of 2 mL min^{-1}, one of the diastereomers was eluted in 10−12 h and the other in 21−28 h. The buffer salts were removed by eluting the products through the same column with water containing 5% acetonitrile. Both products were UV spectroscopically identical with 2′,3′-cUMP. Also, the ^1H NMR spectra were very similar to that of 2′,3′-cUMP, whereas the ^{31}P chemical shifts in ^2H_2O were typical for phosphorothioates:[31,32] 75.9 and 72.0 ppm for the faster and slower eluted isomers, respectively (compared to external orthophosphoric acid). The slower running isomer was hydrolyzed faster by RNase A, and it was assigned as the R_P- or *endo*-diastereomer (**3a**).[31,33] The overall yield of the synthesis (15%) was comparable to that of the original method.[28]

Uridine 2′- and 3′-Monothiophosphates (5a and 4a, Respectively). **3b** was treated overnight in 0.1 mol L^{-1} aqueous sodium hydroxide at room temperature to yield a mixture of uridine 2′- and 3′-monothiophosphates.[28,31] The solution was neutralized with acetic acid and concentrated *in vacuo*. The two products formed were separated by HPLC on a LiChrospher RP 18 column (10 × 250 mm, 5 μm) with 0.2 mol L^{-1} aqueous ammonium acetate. Retention times at flow rate 3 mL min^{-1} were 9.5 and 11 min. Separation was not complete, and several subsequent runs were needed. The buffer salts were removed by eluting the separated products through the same column with water. The faster migrating isomer was chromatographically identical with the product of enzymatic hydrolysis of **3a** and was thus assigned as the 3′-monothiophosphate (**4a**). Both products were UV-spectroscopically identical with uridine monophosphates. Also the ^1H NMR spectra were very similar to those of 2′- and 3′-UMP. The ^{31}P NMR chemical shifts in ^2H_2O were 44.3 and 46.4 ppm for 3′- and 2′-isomers, respectively (compared to external orthophosphoric acid).

Kinetic Measurements. Reactions were followed by an HPLC technique described earlier.[15] The separations were carried out on a Hypersil ODS 5 column (4 × 250 mm, 5 μm) using formic acid/sodium formate buffer (0.045/0.015 mol L^{-1}) containing 0.1 mol L^{-1} tetramethylammonium chloride and 0−5% (v/v) acetonitrile as an eluant. Under these conditions, 2′- and 3′-UMPS eluted as an isomeric mixture, but they could be separated on the same column employing 0.1 mol L^{-1} ammonium acetate as eluant. Signal areas were assumed to be proportional to concentrations, since the base moiety of all the compounds was the same. With the dinucleoside monophosphates and their thioate analogs the molar absorptivities of the two base moieties were assumed additive.

The hydronium ion concentrations of the reaction solutions were adjusted with hydrogen chloride[18] and sodium hydroxide,[25] and formate, acetate, triethanolamine, and glycine buffers on the basis of the known pK_a values of the buffer acids under experimental conditions.[34−37]

Two runs with different buffer concentrations were carried out at each pH studied, and the rate constants were then extrapolated to zero buffer concentration. The effect of the buffer concentration on the rate was less than 30% (buffer concentration <0.05 mol L^{-1}).

Calculation of the Rate Constants. The integrated first-order rate equation was applied to the disappearance of 3′,5′-Up(s)U (or its 2′,5′-isomer). As the concentration of the migration product (2′,5′-isomer) remained low, disappearance of the starting material (rate constant k_{di}) obeyed first-order kinetics for 2 half-lifes. The first-order rate constants for the isomerization of 3′,5′-Up(s)U to 2′,5′-Up(s)U (k_3; k_{-3} analogously) were calculated by bisecting k_{di} to rate constants of parallel first-order reactions on the basis of the product distribution at the early stages of the reaction, *i.e.*, under conditions where the reverse reaction of the 3′ to 2′ migration may be neglected.

First-order rate constants for the desulfurization (route A in Scheme 1, k_1; k_6 analogously) were calculated by means of eq 3, where c_0(UpsU) stands for the initial concentration of

$$\frac{c_t(\mathrm{UpU})}{c_0(\mathrm{UpsU})} = \frac{k_1}{k_4 - k_{di}}[\exp(-k_{di}t) - \exp(-k_4 t)] \quad (3)$$

the starting material, c_t(UpU) for the concentration of the equilibrium mixture of 2′,5′- and 3′,5′-UpU at moment t, k_4 for the first order rate constant of the hydrolysis of UpU,[15] and k_{di} for the first-order rate constant of the disappearance

(25) The H_- values determined for sodium hydroxide solutions at 298.2 K[26] were corrected with respect to the change of the ionic product of water on going to 363.2 K.[27]

(26) Yagil, G. J. *J. Phys. Chem.* **1967**, *71*, 1034.
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(37) King, E. J. *J. Am. Chem. Soc.* **1951**, *73*, 155.

of the starting material. Rate constants for phosphodiester hydrolysis under acidic conditions (routes B and C, $k_2 = k_{2'} + k_{2''}$; for the 2′,5′-isomer $k_7 = k_{7'} + k_{7''}$) were calculated by eqs 4 and 5.

$$k_2 = k_{di} - k_1 - k_3 \quad (4)$$

$$k_7 = k_{di} - k_6 - k_{-3} \quad (5)$$

Rate constants for the diester hydrolysis of Up(s)U *via* 2′,3′-cUMPS (route B, $k_{2'}$) were calculated by eq 6, where c_t(cUMPS)

$$\frac{c_t(\text{cUMPS})}{c_0(\text{UpsU})} = \frac{k_{2'}}{k_5 - k_{di}}[\exp(-k_{di}t) - \exp(-k_5 t)] \quad (6)$$

stands for the concentration of the appropriate diastereomer of cyclic 2′,3′-thiophosphate at moment t and k_5 for the first-order rate constant of its hydrolysis. Values for k_5 were determined separately.

Acknowledgment. Financial support from the Swedish Natural Science Research Council and Turku University Foundation is gratefully acknowledged.

Supporting Information Available: Tables of first-order rate constants k_i for **1a**, **1b**, and **2a** (2 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.

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