Hydrolytic Reactions of the Phosphorodithioate Analogue of Uridylyl(3′,5′)uridine: Kinetics and Mechanisms for the Cleavage, Desulfurization, and Isomerization of the Internucleosidic Linkage

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The hydrolytic reactions of the phosphorodithioate analogue of uridylyl(3′,5′)uridine [3′,5′-Up(s)$_2$U] were followed by HPLC over a wide pH range at 363.2 K. Under acidic and neutral conditions, three reactions compete: (i) desulfurization to a mixture of the (R_P)- and (S_P)-diastereomers of the corresponding 3′,5′- and 2′,5′-phosphoromonothioates [3′,5′- and 2′,5′-Up(s)U], which are subsequently desulfurized to a mixture of uridylyl(3′,5′)- and -(2′,5′)uridine [3′,5′- and 2′,5′-UpU], (ii) isomerization to 2′,5′-Up(s)$_2$U, and (iii) cleavage to uridine, in all likelihood via a 2′,3′-cyclic phosphorodithioate (2′,3′-cUMPS$_2$). Under alkaline conditions (pH > 8), only a hydroxide ion catalyzed hydrolysis to uridine via 2′,3′-cUMPS$_2$ takes place. At pH 3−7, all three reactions are pH-independent, the desulfurization being approximately 1 order of magnitude faster than the cleavage and isomerization. At pH < 3, all the reactions are hydronium ion catalyzed. On going to very acidic solutions, the cleavage gradually takes over the desulfurization and isomerization. Accordingly, the cleavage overwhelmingly predominates at pH < 0. The overall hydrolytic stability of 3′,5′-Up(s)$_2$U is comparable to that of (S_P)- and (R_P)-3′,5′-Up(s)U (and to that of 3′,5′-UpU, except at pH < 2). The rate of the hydroxide ion catalyzed hydrolysis of 3′,5′-Up(s)$_2$U is 37% and 53% of that of (S_P)- and (R_P)-3′,5′-Up(s)U, respectively. The reactions, however, differ with the respect of the product accumulation. While the phosphoromonothioates produce a mixture of 2′- and 3′-thiophosphates as stable products, 3′,5′-Up(s)$_2$U is hydrolyzed to uridine without accumulation of the corresponding dithiophosphates. At pH < 3, where the hydrolysis is hydronium ion catalyzed, the kinetic thio-effect of the second thio substitution is small: under very acidic conditions (H_o −0.69), (S_P)-3′,5′-Up(s)U reacts 1.6 times as fast as 3′,5′-Up(s)$_2$U, but the reactivity difference decreases on going to less acidic solutions. In summary, the hydrolytic stability of 3′,5′-Up(s)$_2$U closely resembles that of the corresponding phosphoromonothioate. While replacing one of the nonbridging phosphate oxygens of 3′,5′-UpU with sulfur stabilizes the phosphodiester bond under acidic conditions by more than 1 order of magnitude, the replacement of the remaining nonbridging oxygen has only a minor influence on the overall hydrolytic stability.

Introduction

While nucleoside phosphoromonothioates, i.e., the sulfur analogues of nucleotides having one of the nonbridging phosphate oxygens replaced with sulfur, have been extensively employed as stereochemical probes in mechanistic studies of reactions catalyzed by protein enzymes[1−5] or ribozymes,[6−12] and as substrate analogues with which to distinguish kinetically equivalent mechanisms on the basis of the kinetic thio effects,[13−15] their dithioate analogues have received much less attention from the mechanistic point of view. Oligodeoxyribonucleotides consisting of such internucleosidic linkages have, however, turned out to be resistant toward nucleases and are able to hybridize with complementary oligoribonucleotide sequences.[16] These facts, together with their achirality around phosphorus, have made the phosphorodithioate oligonucleotides interesting as alternatives for the phosphoromonothioate oligonucleotides applied extensively as antisense oligonucleotides. For this reason, the synthetic chemistry of phosphorodithioates has recently received increasing interest,[17−21] and hence a better understanding of the general chemical properties of phosphorodithioates also appears desirable.

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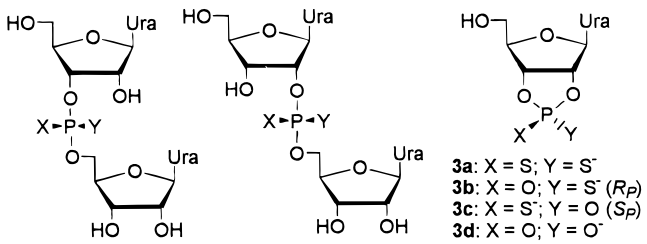
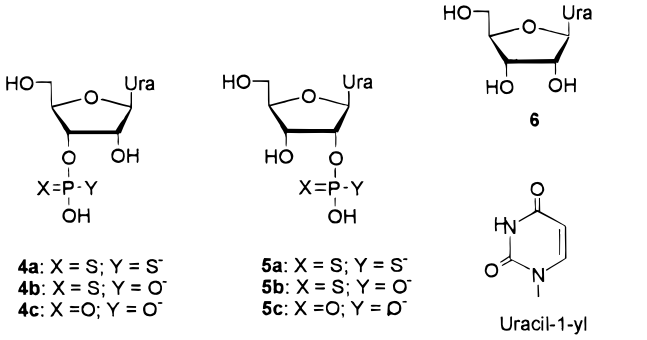
We have previously studied the kinetics and mechanisms of the hydrolytic cleavage, desulfurization, and isomerization of ribonucleoside 3′-phosphoromonothioates in some detail at mono-,[22,23] di-,[23−27] and triester[28] level. Special attention has been paid to the kinetic thio effects, i.e. the effects that replacing a phosphoryl oxygen with sulfur has on the reaction rate. To complete the picture of the effects of the nonbridging sulfur atoms on the course of the reactions of phosphodiesters, we now report on the kinetics and mechanisms of the cleavage, desulfurization, and isomerization of phosphorodithioate analogues of dinucleoside 3′,5′-monophosphates. As far as we know, this is the first quantitative investigation on hydrolytic reactions of this type of compounds. Although the investigation is mechanistic in nature and mainly aimed at furthering the understanding the factors that govern the hydrolytic stability of phosphate esters, we hope that the results also are useful for synthetic chemists attempting to improve the strategies for preparation of phosphorodithioate oligonucleotides.

Results and Discussion

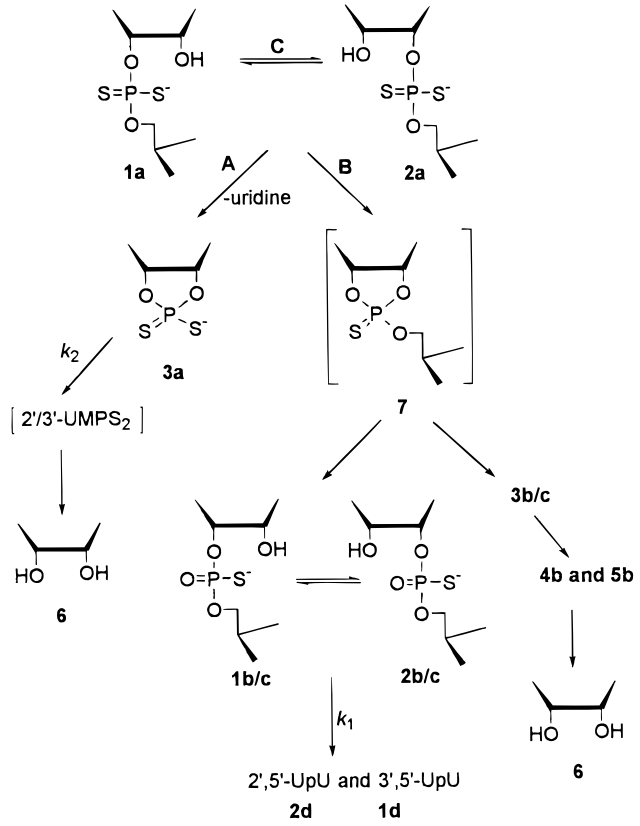
Product Distribution. The hydrolytic reactions of the phosphorodithioate analogue of uridylyl(3′,5′)uridine [3′,5′-Up(s)$_2$U; **1a**] were followed by HPLC over a wide pH





range (from H_o −1.75 to pH 11.4) at 363.2 K. The product distribution turned out to be unexpectedly simple in

Scheme 1



concentrated acidic solutions (pH < 0). The only product accumulated was uridine (**6**). Neither the 2′,5′-isomer of the starting material [2′,5′-Up(s)$_2$U; **2a**] nor the isomeric phosphoromonothioates [(R_P)- and (S_P)-diastereomers of 3′,5′- and 2′,5′-Up(s)U; **1b,c** and **2b**,**c**] nor dinucleoside monophosphates (3′,5′- and 2′,5′-UpU; **1d** and **2d**) were accumulated to such an extent (>2%) that they could have been reliably quantified. 3′,5′-Up(s)$_2$U is decomposed under these conditions only 20% to 60% more slowly than (S_P)- and (R_P)-3′,5′-Up(s)U, and approximately as fast as its 2′,5′-isomer, 2′,5′-Up(s)$_2$U. Accordingly, if these compounds were formed to a significant extent, they would certainly be accumulated. Evidently neither desulfurization to 3′,5′-Up(s)U (route B in Scheme 1) nor isomerization to 2′,5′-Up(s)$_2$U (route C) compete markedly with the cleavage of 3′,5′-Up(s)$_2$U to uridine (route A). The latter reaction in all likelihood proceeds by transesterification of **1a** to uridine 2′,3′-cyclic phosphorodithioate (2′,3′-cUMPS$_2$; **3a**) with concomitant release of the 5′-linked uridine. 2′,3′-cUMPS$_2$ is then rapidly hydrolyzed to a mixture of uridine 2′- and 3′-phosphorodithioates (2′- and 3′-UMPS$_2$; **4a** and **5a**), and these are further dephosphorylated to uridine. It should be, however, noted that the intermediary accumulation of neither 2′,3′-cUMPS$_2$ nor 2′- or 3′-UMPS$_2$ could be detected.

The product distribution at pH 0−8 is much more complicated than that in very acidic solutions. 2′,5′-Up(s)$_2$U (**2a**), 2′,5′- (**2d**) and 3′,5′-UpU (**1d**) and the (R_P)- and (S_P)-diastereomers of 2′,5′- (**2b**/**c**) and 3′,5′-Up(s)U (**1b**/**c**) were observed to accumulate in addition to uridine. In other words, in addition to the cleavage (route A), desulfurization (route B), and isomerization (route C) also take place. In fact, the desulfurization is the fastest reaction between pH 2 and 7. The 3′,5′- and 2′,5′-isomers of Up(s)U are accumulated in a constant 1:1.4 molar ratio

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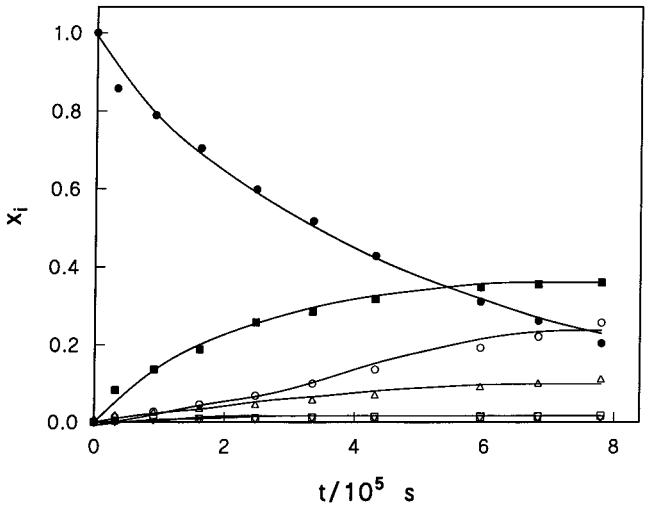


Figure 1. Time-dependent product distribution for the reactions of 3′,5′-Up(s)$_2$U (**1a**) in a MES buffer at pH 5.0 ([MES]/[MESNa] = 0.02/0.01 mol L^{-1}; I = 0.1 mol L^{-1} with NaCl) at 363.2 K. Notation: (●) 3′,5′-Up(s)$_2$U; (□) 2′,5′-Up(s)$_2$U; (▽) 2′,3′-cUMPS$_2$; (○) mixture of 3′,5′- and 2′,5′-UpU, (■) mixture of (R_P)- and (S_P)-2′,5′- and −3′,5′-Up(s)U, (△) uridine.

throughout a kinetic run, the concentration ratio of the diastereomeric forms, [(R_P)]/[(S_P)], being 3 with 2′,5′-Up(s)U and 0.25 with 3′,5′-Up(s)U. Hence, the desulfurization seems to be moderately stereoselective. At pH 5−7, 2′,3′-cUMPS$_2$ was observed to accumulate (>3%) as an intermediate of the phosphodiester hydrolysis. Figure 1 shows as an example the time-dependent product distribution observed at pH 5. In addition to the products indicated in Figure 1, a significant amount of uracil was formed at pH 8 (15% of all products).

At pH > 9, the product distribution is again simple. The only reaction taking place is a hydroxide ion-catalyzed cleavage of 3′,5′-Up(s)$_2$U to uridine and 2′,3′-cUMPS$_2$ (route A). The intermediary appearance of 2′,3′-cUMPS$_2$ can be experimentally observed, whereas the obvious hydrolysis products of this compound, viz. 2′- and 3′-UMPS$_2$, do not accumulate. This is in striking contrast to the results obtained with the phosphoromonothioate diesters. With the latter, the cyclic phosphoromonothioate intermediate (**3b** or **3c**) is rapidly hydrolyzed to 2′- and 3′-phosphoromonothioates (**4b** and **5b)** that quantitatively accumulate.[22,25] Most likely, even in alkaline solutions 2′- and 3′-UMPS$_2$ are dephosphorylated to uridine so rapidly that they do not accumulate.

pH−Rate Profiles. As indicated in Scheme 1, 3′,5′-Up(s)$_2$U may undergo three parallel reactions in aqueous solution: (i) cleavage to 2′,3′-cUMPS$_2$ (route A), (ii) desulfurization to a mixture of phosphoromonothioates (route B), and (iii) isomerization to 2′,5′-Up(s)$_2$U (route C). Table 1 records the pseudo first-order rate constants for the disappearance of 3′,5′-Up(s)$_2$U at different pH, and the contributions of reactions A−C to the overall decomposition. The pH−rate profiles for the latter reactions are depicted in Figures 2−4. The rate profiles reported earlier for (R_P)-3′,5′-Up(s)U and 3′,5′-UpU are included in the same figures for reference purposes. The pH−rate profile for the cleavage reaction closely resembles that of (R_P)-3′,5′-Up(s)U (Figure 2). The cleavage is nearly pH-independent from pH 4 to 6 and becomes hydronium ion-catalyzed at pH < 4 and hydroxide ion-catalyzed at pH > 8. Both the hydronium and hydroxide ion-catalyzed

Table 1. Pseudo First-order Rate Constants for the Disappearance of 3′,5′-Up(s)$_2$U (1a) at 363.2 K,[a] and the Rate Constants for the Concurrent Cleavage (k_{cl}), Desulfurization (k_{ds}), and Isomerization (k_{is})

pH	reaction solution	$k_d/10^{-6}$ s^{-1}	$k_{cl}/10^{-6}$ s^{-1}	$k_{ds}/10^{-6}$ s^{-1}	$k_{is}/10^{-6}$ s^{-1}
−1.75	HCl	13400	−	−	−
−0.75	HCl	1500	−	−	−
0.20	HCl	336	−	−	−
1.00	HCl	85.8	47.4	37.0	6.03
2.00	HCl	27.2	2.18	7.00	1.79
3.01	HCOOH	3.50	0.75	3.49	0.78
4.01	HCOOH	1.49	0.09	1.94	0.26
5.01	MES	1.78	0.17	1.48	0.10
6.01	HEPES	1.49	0.04	1.21	0.15
7.00	HEPES	1.93	0.42	1.06	0.15
7.97	glycine	6.76	4.49	1.29	0.15
8.97	glycine	18.3	183	−	−
10.4	NaOH	324	324	−	−
11.4	NaOH	2860	2860	−	−

[a] The ionic strenght was adjusted at 0.1 mol L^{-1} with sodium chloride. The rate constants indicated refer to buffer concentration zero.

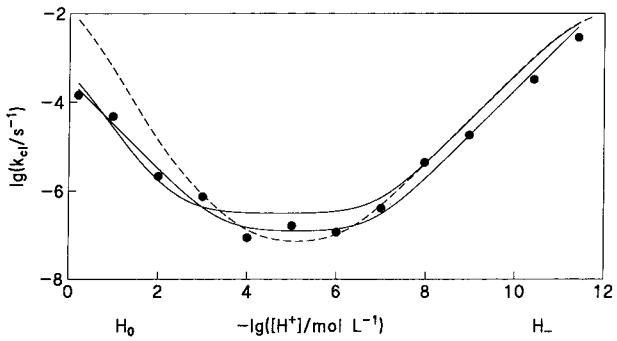


Figure 2. pH−Rate profile for the hydrolytic cleavage of the internucleosidic linkage (k_{cl}) of 3′,5′-Up(s)$_2$U (**1a**, ●) at 363.2 K. The solid and dotted lines show the corresponding curves for the cleavage of (R_P)-3′,5′-Up(s)U (**1c**) and 3′,5′-UpU (**1d**), respectively. The ionic strength was adjusted to 0.1 mol L^{-1} with sodium chloride.

reactions are first-order in the catalyst concentration. The rate of the hydroxide ion catalyzed cleavage of 3′,5′-Up(s)$_2$U is about 60% of that of (R_P)-3′,5′-Up(s)U. Under acidic conditions, the reactivity difference is even smaller.

The pH−rate profile for the desulfurization of 3′,5′-Up(s)$_2$U also closely resembles that of (R_P)-3′,5′-Up(s)U (Figure 3). The reaction is hydronium ion-catalyzed at pH < 3 and pH-independent at higher pH. Almost over the entire pH range, the desulfurization rate of 3′,5′-Up(s)$_2$U falls between those of (R_P)- and (S_P)-3′,5′-Up(s)U. Only at pH < 2, the phosphoromonothioates are desulfurized slightly more readily than 3′,5′-Up(s)$_2$U.

The isomerization responds to changes in pH in the same way as the desulfurization. The reaction is pH-independent at pH > 4 and approximately first-order in hydronium ion concentration under more acidic conditions (Figure 4). The isomerization is slightly faster than that of the diastereomeric 3′,5′-Up(s)U. Owing to much faster cleavage to uridine, the rate constants for desulfurization and isomerization could not be determined at pH < 0.

The shape of the pH-rate profiles shown in Figures 2−4 indicates that the observed rate constant for the cleavage reaction (k_{cl}) may be expressed by eq 1 and those for the desulfurization (k_{ds}) and isomerization (k_{is}) by eq 2. The rate constants k_iH, k_i0 and k_iOH (i = cl, ds, is) in these

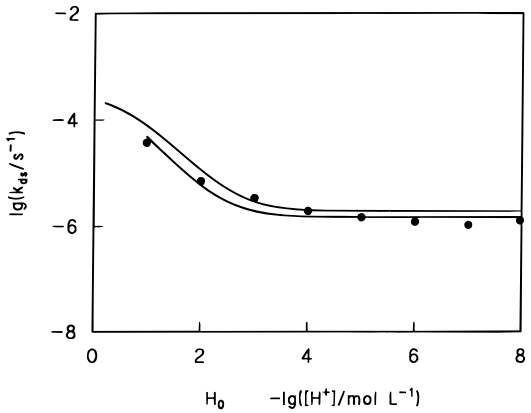


Figure 3. pH−Rate profile for the hydrolytic desulfurization (k_{ds}) of 3′,5′-Up(s)$_2$U (**1a**, ●) at 363.2 K. The solid line shows the corresponding curve for the desulfurization of (R_P)-3′,5′-Up(s)U (**1c**). The ionic strength was adjusted to 0.1 mol L^{-1} with sodium chloride.

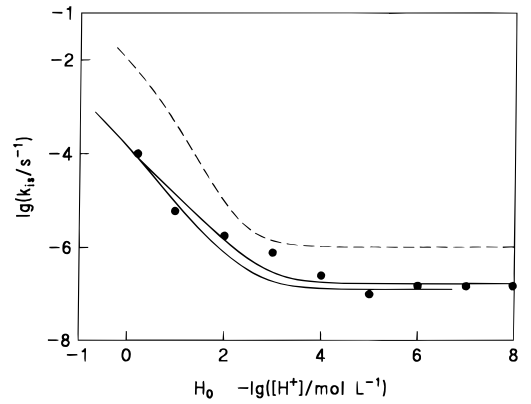


Figure 4. pH−Rate profile for the isomerization (k_{is}) of 3′,5′-Up(s)$_2$U (**1a**, ●) at 363.2 K. The solid and dotted lines show the corresponding curves for the isomerization of (R_P)-3′,5′-Up(s)U (**1c**) and 3′,5′-UpU (**1d**), respectively. The ionic strength was adjusted to 0.1 mol L^{-1} with sodium chloride.

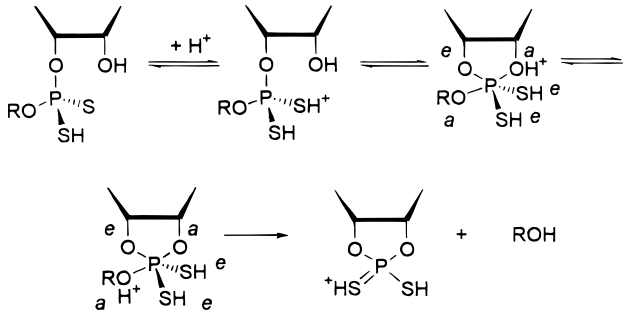
$$k_{cl} = k_{cl}H\,[H^+] + k_{cl}0 + k_{cl}OH(K^w/[H^+]) \qquad (1)$$

$$k_i = k_iH\,[H^+] + k_i0\ (i = ds, is) \qquad (2)$$

equations. refer to the hydronium ion-catalyzed, uncatalyzed, and hydroxide ion-catalyzed partial reactions, respectively. The values obtained by least-squares fitting for these constants are as follows: $k_{cl}H = 3.1 \times 10^{-4}$ M^{-1} s^{-1}, $k_{cl}0 = 1.2 \times 10^{-7}$ s^{-1}, $k_{cl}OH = 5.0 \times 10^{-2}$ M^{-1} s^{-1}, $k_{ds}H = 4.5 \times 10^{-4}$ M^{-1} s^{-1}, $k_{ds}0 = 1.5 \times 10^{-6}$ s^{-1}, $k_{is}H = 1.3 \times 10^{-4}$ M^{-1} s^{-1} and $k_{is}0 = 1.7 \times 10^{-7}$ s^{-1}.

Mechanism of the Cleavage, Desulfurization, and Isomerization under Acidic and Neutral Conditions. As with phosphoromonothioates, the cleavage, desulfurization, and isomerization of 3′,5′-Up(s)$_2$U in all likelihood take place via a common pentacoordinated phosphorane intermediate obtained by the attack of the 2′-hydroxy function on the phosphorus atom. In concentrated acid solutions (H_o < 0), the cleavage to 2′,3′-cUMPS$_2$ (route A) overwhelmingly predominates. Under these conditions, the prevailing ionic form is in all likelihood the neutral phosphorodithioate, and yet the reaction is first-order in hydronium ion activity. Hence,

Scheme 2



the reactive species most likely is the monocation having both sulfur ligands protonated. Evidently the dithiophosphorane intermediate is obtained by the attack of the 2′-hydroxy function on the diprotonated phosphorodithioate moiety (Scheme 2). According to Westheimer's rules,[29] the attacking 2′-oxygen initially occupies an apical and the 3′-oxygen an equatorial position within the intermediate. Since oxygen is a more electronegative element than sulfur, the remaining apical position should be occupied by the oxygen of the 5′-linked nucleoside. Accordingly, the 5′-linked nucleoside may leave without pseudorotation of the intermediate. By contrast, desulfurization, and isomerization are possible only after pseudorotation that converts one of the nonbridging sulfur atoms and the 3′-oxygen to apical positions. The most straightforward explanation for the fact that these reactions are not able to compete with the cleavage reaction under very acidic conditions is that the hydronium ion-assisted cleavage of the P−O5′ bond is a more facile process than the pseudorotation of the dithiophosphorane intermediate.

The cleavage reaction is also first-order in hydronium ion concentration in less acidic solutions (pH 1−3), where the predominant ionic form is the phosphorodithioate monoanion. Accordingly, under these conditions the reaction most likely takes place via the neutral (monoprotonated) species, i.e., by the attack of the 2′-hydroxy function on the monoprotonated phosphorodithioate group (Scheme 3). In this pH range, the desulfurization via a cyclic triester (**7**) and isomerization compete with the cleavage, indicating that the pseudorotation competes with the cleavage of the P−O5′ bond. The departure of the protonated 3′-oxygen from the apical position gives 2′,5′-Up(s)$_2$U, while the cleavage of one of the sulfur ligands as dihydrogen sulfide gives the unstable 2′,3′-cyclic phosphoromonothioate triester (**7**). The latter is not, however, accumulated, but it is rapidly hydrolyzed to a mixture of phosphoromonothioate diesters: 2′,5′- and 3′,5′-Up(s)U, and possibly 2′,3′-cUMPS. The subsequent reactions of these diesters have been described previously.[24,25]

In the pH range 4 to 7, where the cleavage, desulfurization and isomerization all are pH-independent, the reactions proceed via a monoanionic dithiophosphorane intermediate (Scheme 4). As discussed earlier,[24,30] the intermediate is obtained rather by the attack of the 2′-oxyanion on neutral phosphate moiety than by the attack of 2′-hydroxy group on monoanionic phosphate. The monoanionic dithiophosphorane is sufficiently long-lived to undergo pseudorotation, as evidenced by the occur-

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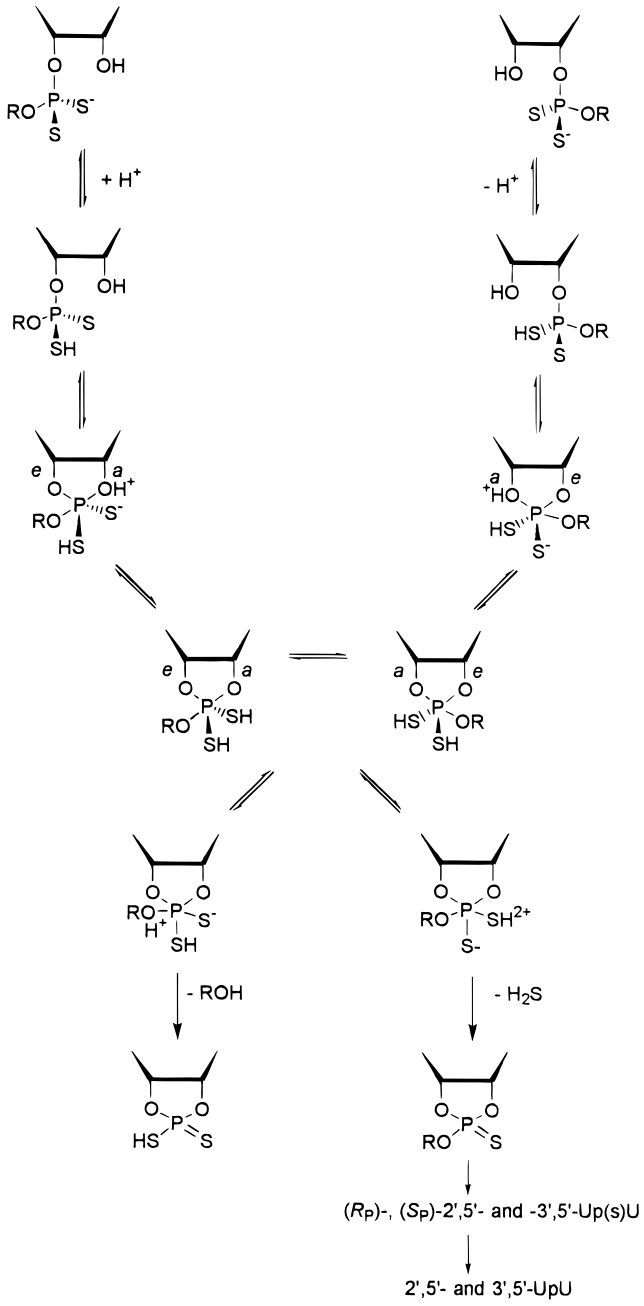
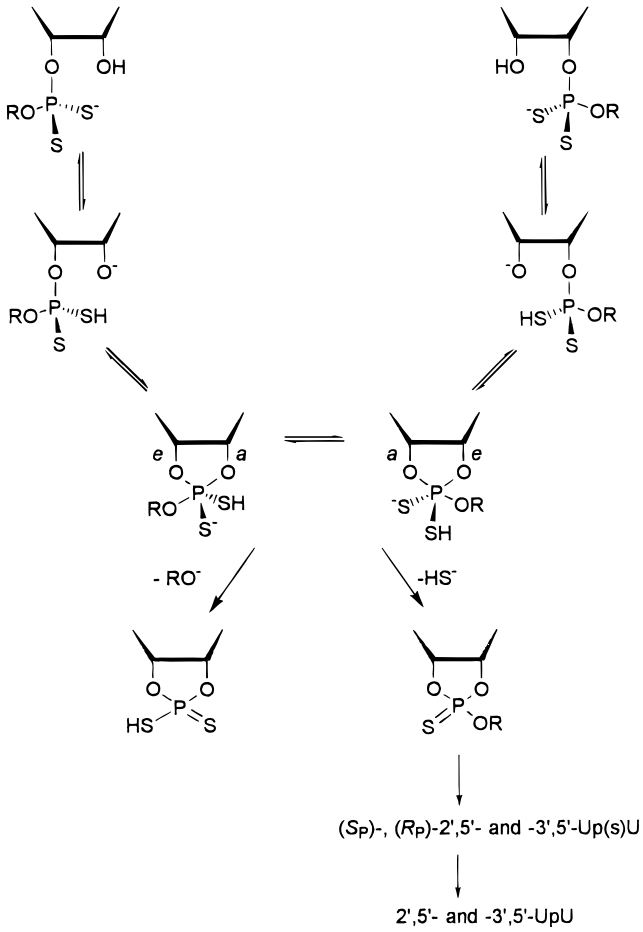
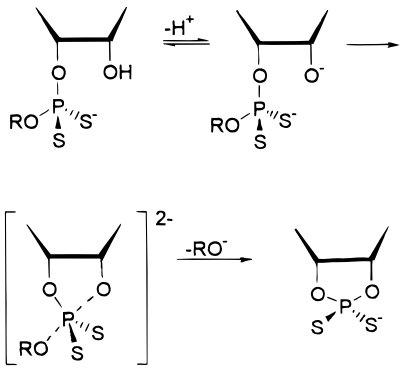
Scheme 3



Scheme 4



Scheme 5



rence of migration of the dithiophosphoryl group. The desulfurization is faster than the cleavage or isomerization, consistent with the fact that hydrogen sulfide ion is less basic than alkoxide ion, and hence a better leaving group.

Mechanism of the Hydroxide Ion-Catalyzed Cleavage. As mentioned above, the intramolecular transesterification of 3′,5′-Up(s)$_2$U to 2′,3′-cUMPS$_2$ with concomitant release of uridine is the only reaction detected in aqueous alkali (pH > 8). In this respect 3′,5′-Up(s)$_2$U behaves analogously to 3′,5′-UpU[31] and 3′,5′-Up(s)U.[24,32] Evidently, the same mechanism[27,33−35] is also utilized: the 2′-oxyanion attacks on the phosphorus atom of the monoanionic phosphorodithiote, resulting in an in-line displacement of the 5′-linked nucleoside (Scheme 5). The lack of hydroxide ion-catalyzed isomerization and desulfurization indicates that the dianionic dithiophosphorane intermediate, if it exists, does not pseudorotate. Evidently the pseudorotation barrier for a dianionic phosphorane is high, since the process would position a negatively charged sulfur in an apical position. In principle, the pseudorotation could, however, proceed by a kinetically invisible intermediary protonation to a dithiophosphorane monoanion known to be able to pseudorotate. In other words, rate-limiting (partially) formation of the dianionic dithiophosphorane would be followed

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Table 2. The Kinetic Thio Effects, $k(PS_2)/k(OPS)$, for the Hydronium Ion Catalyzed, pH-Independent, and Hydroxide Ion Catalyzed Cleavage, Desulfurization, and Isomerization of 3′,5′-Up(s)$_2$U

	cleavage		desulfurization		isomerization	
ionic form	S_P	R_P	S_P	R_P	S_P	R_P
H^+-catalyzed	0.6	1.4	0.7	0.5	2.1	2.1
pH-independent	1.3	0.4	2.0	0.8	1.7	1.3
OH^--catalyzed	0.32	0.46	–	–	–	–

by thermodynamically favored rapid protonation to monoanion, pseudorotation, deprotonation, and rate-limiting (partially) departure of the 3′-oxyanion. The fact that this kind of hydroxide ion-catalyzed isomerization cannot be observed suggests that the dianionic dithiophosphorane is only marginally stable, as also suggested for the corresponding oxyphosphorane dianion.[34,35] 2′- and 3′-UMPS$_2$, the expected hydrolysis products of 2′,3′-cUMPS$_2$, were not observed to accumulate. While ribonucleoside 2′- and 3′-phosphoromonothioates are rather stable in aqueous alkali,[22] this is not the case with the corresponding phosphorodithioates, since the latter appear to be rapidly hydrolyzed to uridine.

Thio Effect. The kinetic thio effect, i.e., the effect that replacing a nonbridging phosphoryl oxygen with sulfur has on the rate of cleavage, desulfurization, and isomerization [$k(PS_2)/k(OPS)$], is given in Table 2. The data clearly shows that replacing the remaining nonbridging oxygen of phosphoromonothioates with sulfur has a surprisingly small effect on the kinetics of their hydrolysis and transesterification. In aqueous alkali, where the cleavage to 2′,3′-cUMPS$_2$ via a dianionic phosphorane (or transition state) is the only reaction detected, the thio effect obtained with 3′,5′-Up(s)$_2$U is more marked than that obtained earlier[24,32] with diastereomeric 3′,5′-Up(s)U. The cleavage rate of 3′,5′-Up(s)$_2$U is 32% and 46% of that of (S_P)- and (R_P)-3′,5′-Up(s)U, respectively. For comparison, the latter compounds are cleaved 1.3 (S_P) and 0.9 (R_P) times as fast as 3′,5′-UpU.

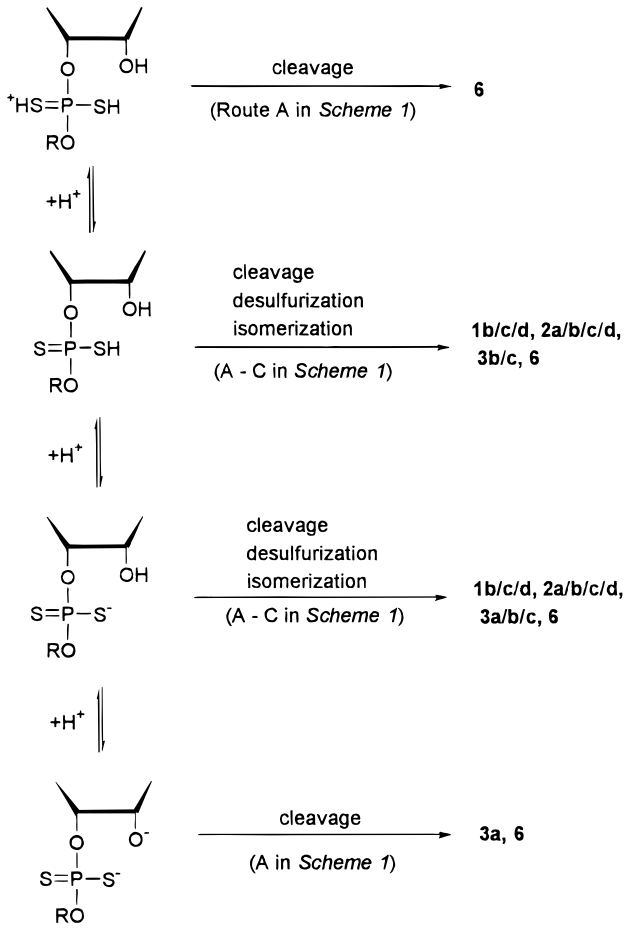
The uncatalyzed cleavage of monoanionic 3′,5′-Up(s)$_2$U is 1.3 and 0.4 times as fast as that of (S_P)- and (R_P)-3′,5′-Up(s)U, respectively. The cleavage via the neutral ionic form, i.e., the reaction first-order in [H^+], is in turn 0.6 and 1.4 times as fast as the corresponding reaction of the (S_P)- and (R_P)-3′,5′-Up(s)U. In other words, while replacing one of the nonbridging phosphoryl oxygens of 3′,5′-UpU with sulfur results in a rate-retardation that is continuously increased with decreasing pH, being up to 100-fold in concentrated acid solutions ($H_o < 0$), the replacement of the remaining oxygen with sulfur has an almost negligible effect on the cleavage rate.

The rate of the pH-independent desulfurization of 3′,5′-Up(s)$_2$U falls between those of the diastereomeric phosphoromonothioates. The acid-catalyzed desulfurization is, in turn, slightly slower than that of the 3′,5′- Up(s)U diastereomers. In fact, the rate of desulfurization shows some indication of leveling off to a constant value at $H_o = 0.2$. The uncatalyzed and acid-catalyzed isomerizations of 3′,5′-Up(s)$_2$U are slightly faster than with the corresponding reactions of phosphoromonothioates.

Conclusions

Scheme 6 summarizes the various ionic forms of 3′,5′-Up(s)$_2$U and the reactions that they undergo. Under very acidic conditions (pH < 0), the reactive species is substrate monocation, which undergoes only cleavage to free nucleoside, probably via sequential formation of 2′,3′-cyclic phosphorodithioate and 2′/3′-diphosphate. At pH 0−4, the reactive species is neutral phosphorodithioate which undergoes in addition to the cleavage reaction desulfurization to diastereomeric 3′,5′-phosphoromonothioates and isomerization to 2′,5′-phosphorodithioate, the desulfurization being the predominant reaction. At pH 4−8, the same three reactions occur, but now the reactive species is phosphorodithioate monoanion. Under alkaline conditions, the only reaction is hydroxide ion-catalyzed cleavage, the reactive ionic form being the substrate dianion. The overall stability of phosphorodithioates closely resembles that of phosphoromonothioates. While replacing one of the nonbridging phosphate oxygens of dinucleoside 3′,5′-monophosphates with sulfur markedly stabilizes the phosphodiester bond under acidic conditions,[24] the replacement of the remaining nonbridging oxygen has only a minor influence on the stability.

Scheme 6



Experimental Section

Materials. Uridylyl(3′,5′)- and -(2′,5′)uridine (**2d** and **1d**), uracil, uridine (**6**), and uridine monophosphates (**5c** and **4c**), all used as reference materials, were commercial products of Sigma. The preparation of the R_P- and S_P- diastereomers of the phosphoromonothioate analogues of uridylyl(3′,5′)- and -(2′,5′)uridine (**1b**,**c** and **2b**,**c**),[36] the diasteromeric uridine 2′,3′-cyclic phosphoromonothioates (**3b**,**c**),[25] and 2′- and 3′-phos-

(36) Almer, H.; Stawinski, J.; Strömberg, R.; Thelin, M. *J. Org. Chem.* **1992**, *57*, 6163.

phoromonothioates (**5b** and **4b**)[22] has been described earlier. Uridylyl(3′,5′)uridine phosphorodithioate (**1a**) was synthesized by a method described in the literature.[37] The crude product was purified by RP HPLC on a Lobar column (37 × 440 mm, 40−63 μm), using a formic acid/sodium formate buffer (0.045/0.015 mol L^{-1}) containing 0.1 mol L^{-1} tetramethylammoniun chloride and 2−10% acetonitrile as an eluent. Finally, the product was desalted and passed through a Dowex 50-W Na^+ (100−200 mesh) column. The product was homogeneous by HPLC on a Hypersil ODS 5 column (see below). **1a**: ^{1}H NMR (D_2O) δ 8.28 (s, 2H), 7.98 (d, 1H, J = 8.1 Hz), 7.77 (d, 1H, J = 8.1 Hz), 5.78 (d, 1H, J = 3.5 Hz), 5.73 (d, 1H, J = 8.1 Hz), 5.70 (d, 1H, J = 3.5 Hz), 5.67 (d, 1H, J = 8.1 Hz), 4.34 (dt, 1H, J = 5.2 Hz), 4.24 (m, 1H), 4.10−4.20 (m, 4H), 3.85 (dd, 2H, J_1 = 12.0 Hz, J_2 = 3.0 Hz), 3.75 (dd, 2H, J_1 = 11.0 Hz, J_2 = 6.0 Hz); ^{31}P NMR (D_2O) δ 118.7; ESI^+-MS m/z 583.

Kinetic Measurements. The hydrolytic reactions were followed by an RP HPLC method described earlier (UV detection at 260 nm).[24] The reactions were carried out in sealed tubes immersed in a thermostated water bath (363.2 K). The hydronium ion concentration of the reaction solutions were adjusted with hydrogen chloride[38] and sodium hydroxide,[39] and formate,[40] 2-(*N*-morpholino)ethanesulfonic acid (MES),[41] (*N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid]) (HEPES),[42] and glycine buffers[43] on the basis of the known pK_a values of the buffer acids under experimental conditions. The composition of the samples withdrawn at appropriate intervals was analyzed 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 4.5% acetonitrile (v/v) as an eluent. The observed retention times (t_R/min) for the hydrolytic products of **1a** on RP HPLC (flow rate was 1 mL min^{-1}) were as follows: 24.3 (**1a**), 19.9 (**1c**), 10.0 (**1b**), 8.9 (**2a**), 7.3 (**3a**), 6.9 (**2c**), 6.0 (**2b**), 5.9 (**1d**), 4.7 (**2d**), and 3.4 (**6**).

The signal areas of the nucleotide analogues were assumed to be proportional to concentrations, since the base moiety of all the compounds was the same. With the dinucleside monophosphates and their thioate analogues, the molar absorptivities of the two base moieties were assumed to be additive. The initial substrate concentration was ca. 2 × 10^{-4} mol L^{-1}.

To identify the reaction products, the mass spectra of the hydrolysis products were recorded (LC/MS). The mixture of acetonitrile and aqueous ammonium acetate (5 mmol L^{-1}) was used as an eluent to separate the products (a linear gradient in 25 min from zero to 20% MeCN). In addition to this, the products formed were identified by spiking with authentic reference compounds.

Two runs at different buffer concentrations were carried out at each pH studied, and the rate constants were extrapolated to zero buffer concentration. The contribution of the buffer catalysis to the observed rate constants was moderate (<70%) even at the higher buffer concentration employed (0.1 mol L^{-1}).

Calculations of the Rate Constants. The pseudo first-order rate constants for the decomposition of 3′,5′-Up(s)$_2$U (k_d) 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_{ds}) for the desulfurization of 2′,5′- and 3′,5′-Up(s)$_2$U were calculated by eq 3, where k_d is the first-order rate constant for the disappearance of 3′,5′- and 2′,5′-Up(s)$_2$U. [3′,5′-Up(s)$_2$U]$_o$ stands for the initial concentration of 3′,5′-Up(s)$_2$U and [Up(s)U]$_t$ denotes the sum concentration of the R_P- and S_P-diastereomers of 3′,5′- and 2′,5′-Up(s)U at moment t. k_1 is the mean of the first-order rate constants of the disappearance of the isomeric Up(s)Us. The mole fraction of the isomerization product (the 2′,5′-isomer) remained low (<10%) during the entire kinetic run. Accordingly, the reverse reaction of the 3′ to 2′ migration could be neglected at the early stages of the reaction.

$$\frac{[\mathrm{Up(s)U}]_t}{[3',5'\text{-Up(s)}_2\mathrm{U}]_o} = \frac{k_{ds}}{k_1 - k_d}[\exp(-k_d t) - \exp(-k_1 t)] \quad (3)$$

The first-order rate constants, k_{cl}, for the phosphodiester hydrolysis of the 3′,5′-Up(s)$_2$U via 2′,3′-cUMPS$_2$ were calculated by eq 4, where [3′,5′-Up(s)$_2$U]$_o$ stands for the initial concentration of the starting material, [2′,3′-cUMPS$_2$]$_t$ denotes the concentration of 2′,3′-cUMPS$_2$ at moment t, k_2 is the first-order rate constant of the hydrolysis of 2′,3′-cUMPS$_2$, and k_d is the first-order rate constant for the disappearance of the starting material and its 2′-isomer.

$$\frac{[2',3'\text{-cUMPS}_2]_t}{[3',5'\text{-Up(s)}_2\mathrm{U}]_o} = \frac{k_{cl}}{k_2 - k_d}[\exp(-k_d t) - \exp(-k_2 t)] \quad (4)$$

Under neutral and acidic conditions, where 2′,3′-cUMPS$_2$ does not accumulate, the first-order rate constants of the phosphodiester hydrolysis, k_{cl}, were calculated by eq 3.

$$k_{cl} = k_d - k_{ds} \quad (5)$$

The first-order rate constants, k_{is}, for the isomerization of 3′,5′-Up(s)$_2$U to 2′,5′-Up(s)$_2$U, were determined by the UFIT 1.0 program of Beckman et al.[44] using the concentrations of 3′,5′- and 2′,5′-Up(s)$_2$U in the least-squares fitting.

Acknowledgment. Financial support from the National Tecnology Agency is gratefully acknowledged.

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