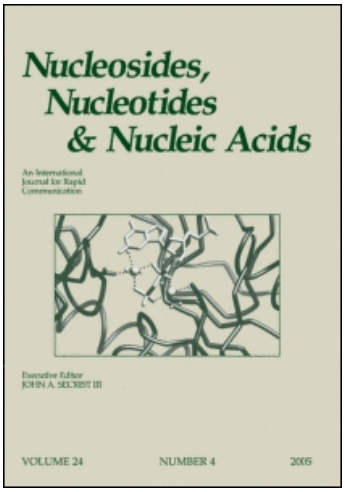

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HYDROLYTIC REACTIONS OF 3'-DEOXY-3'-THIOINOSYLYL-(3'→5')-URIDINE; AN RNA DINUCLEOTIDE CONTAINING A 3'-*S*-PHOSPHOROTHIOLATE LINKAGE

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ABSTRACT: The course of hydrolysis of 3'-deoxy-3'-thioinosylyl-(3'→5')-uridine (IspU) has been followed by HPLC over a wide pH-range. Two reactions of the internucleosidic thiophosphate linkage compete: (i) cleavage yielding thioinosine monophosphates and uridine, and (ii) isomerization to the 2',5'-isomer of IspU. Under very acidic conditions, even acid-catalyzed depurination of the inosine moiety is observed. The stability of the thiophosphate linkage and the mechanisms of its rupture are discussed.

Nucleoside phosphorothioates and -thiolates are well known analogs of nucleoside phosphoesters in nucleic acid chemistry and biochemistry. With ribonucleotides, the thioate analogs bearing a non-bridging sulfur have been widely used over the past 30 years in studying the mechanisms of cleavage of RNA by enzymes and ribozymes.[1] More recently also nucleoside phosphorothiolate diesters with either the 5'-bridging[2] or 3'-bridging[3-5] oxygen replaced by sulfur have received considerble interest for related purposes.

The 3'-thiolate ribonucleotides have proven to be potent tools in studying the metal ion catalysis in RNA splicing[5] and other applications may be expected to emerge after resolving the synthetic problems of introducing the 3'-phosphorothiolate linkage into

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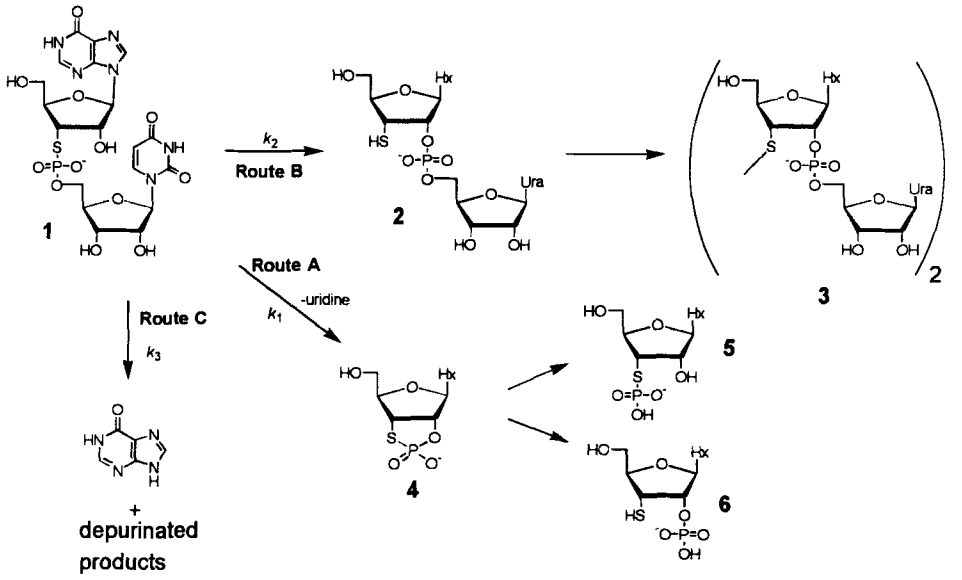
oligonucleotides.[6] Accordingly, it appears important to collect detailed data even on the intrinsic chemical reactivity of this class of compounds. For this purpose we have now studied the kinetics and mechanisms of the non-enzymatic hydrolytic reactions of an RNA dinucleoside analog, 3'-deoxy-3'-thioinosylyl-3',5'-uridine (IspU; **1**).[3] Some preliminary data on the subject have previously been published.[3,4] They show that the 3'-phosphorothiolate linkage in a ribonucleotide dimer is, depending on system, 2 to 3 orders of magnitude more susceptible to alkaline hydrolysis than is the corresponding linkage of a native dinucleoside monophosphate. In aqueous acid, by contrast, the reactivities are more comparable to each other. Furthermore, the pH-rate profile for the hydroxide ion catalyzed degradation of p*IspU (a derivative of **1** having a ^{32}P labeled monophosphate function at the 5'-site of the inosine moiety) in the pH range of 10 - 14 was determined electrophoretically and it was observed to exhibit a similar leveling towards a constant value as was obtained with its oxyphosphate analog p*IpU.[3]

In the present work, we have carried out a quantitative kinetic analysis of the hydrolysis of IspU, focusing attention particularly to the reactions in neutral and acidic solutions, which have not been very thoroughly studied earlier.

RESULTS AND DISCUSSION

Product distributions and pH-rate profiles. The course of hydrolysis of IspU was followed over a wide acidity range by determining the time-dependent product distributions by HPLC. The products were characterized either by chromatographic comparison with authentic samples, by mass spectrometric analysis (HPLC/ESI-MS), or by NMR spectroscopic characterization of an isolated sample. The products detected, under various conditions, and the minimal reaction scheme needed to describe their formation are depicted in Scheme 1. The first-order rate constants for the competing parallel reactions were determined from the time-dependent product distributions.

Table 1 contains rate constants for the cleavage and isomerization of the inter-nucleosidic linkage of IspU under alkaline, neutral and acidic conditions together with the corresponding constants for UpU. In the OH^--catalyzed reaction, IspU (**1**) is degraded about 350 fold faster than the native dinucleoside monophosphate. pH-Independent isomerization of IspU, between pH 3-6, is 50 times faster than the corresponding reaction



SCHEME 1

TABLE 1. The First-order Rate Constants for Phosphoester Hydrolysis (k_2) and Isomerization (k_2) of IspU (**1**) and Uridylyl-(3'→5')-uridine at 363.2 K.[a]

pH	$k_1/10^{-6}$ s^{-1}		$k_2/10^{-6}$ s^{-1}	
	IspU	UpU[b]	IspU	UpU[b]
0.2	11200	6800	3000	6400
1.0	890	680	580	55
2.0	60	10.9	110	11.6
3.0	c	1.07	54	2.9
5.0	c	0.054	40	0.72
7.1	200	0.97	42	c
7.6	680	2.4	c	c
8.3	3100	8.9	c	c

[a] The ionic strength was adjusted to 0.1 M with sodium chloride. [b] Data from Ref. 7. [c] Not observed.

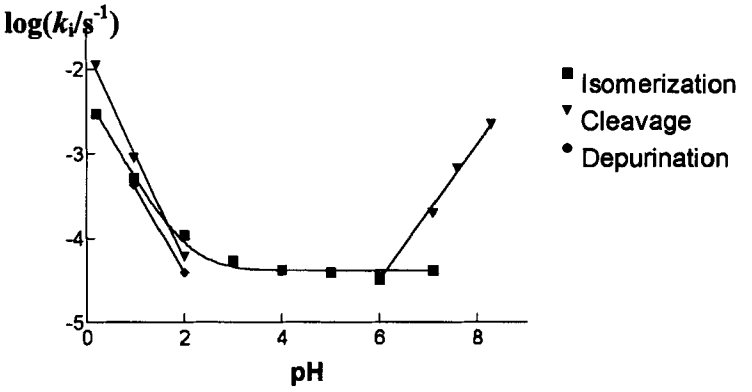


FIG. 1. pH-rate profiles for the competing hydrolytic reactions of IspU (**1**) at 363.2 K (I = 0.1 M).

of UpU (comparison with IpU was less accurate, due to competing depurination of the latter. However, the structure of the base moiety is expected to have only a minor effect on the rate[7]). At pH ≤ 2, both reactions (isomerization and phosphoester hydrolysis) are acid-catalyzed and the total reactivity of IspU is close to that of dinucleoside monophosphates.

Under alkaline conditions (pH > 8), the base-catalyzed cleavage (first-order in [OH^-], see rate-profiles in Figure 1) of the phosphorothiolate linkage is the only reaction detected (Route A in Scheme 1). Accumulation of uridine is accompanied initially by 2'-*O*,3'-*S*-cyclic phosphorothiolate (**4**; *m/z* = 345 by negative scan ESI-MS), which is subsequently hydrolyzed to a single thioinosine monophosphate (*m/z* = 363). As evidenced earlier,[3,4] the latter is most likely the 3'-*S*-phosphorothiolate **5**. No sign of formation of the other thioinosine monophosphate, the 2'-*O*-phosphate **6**, could be detected. The accumulation of the cyclic thiolate is considerable, the concentration reaching its maximum at 40 % of the initial IspU concentration at about one half-life of the hydrolysis of IspU. Accordingly, the rate constant of hydrolysis of **4** is of the same order of magnitude as that of degradation of IspU. This is in contrast to the hydrolysis of native nucleoside monophosphates, in which 2',3'-cNMP is too unstable to accumulate during alkaline hydolysis of a dinucleoside monophosphate.[7] In other words, the intermolecular attack of a hydroxide-ion leading to hydrolysis of the 2',3'-cyclic monophosphate is not as

efficiently facilitated by the 3'-S substitution as is the intramolecular attack of the 2'-oxyanion leading to cleavage of the internucleosidic linkage.

At pH < 7, isomerization of IspU to the 3'-deoxy-3'-thioinosylyl-2',5'-uridine **2** (Route B in Scheme 1) competes with the phosphoester hydrolysis. The isomerization is pH-independent above pH 3 and was the only reaction detected between pH 4 to 6. The 2',5'-isomer (**2**) subsequently oxidizes to the disulfide (a "tetranucleoside") **3**. The assignment of the products **2** and **3** was based on NMR and MS analysis of samples isolated by HPLC from the reaction solutions. The rate of oxidation appears to be pH-dependent. At pH 3, the starting material was quantitatively converted to dimer **2**, without any significant subsequent oxidation. At pH 5, by contrast, the concentration of **2** reached its maximum value (48 % of total peak area) at 60 % reaction. At higher pH (pH > 7), the oxidation could not be quantified due to the predominating cleavage reaction of **1**. In contrast to the behaviour of the native RNA constituents, isomerization is not reversible: IspU (**1**) could not be shown to accumulate when the hydrolysis of **2** was followed. This was the case even under acidic conditions, where the isomerization of **1** to **2** is acid-catalyzed and is considerably faster than the subsequent formation of the disulfide. However, in acidic solutions **2** is also degraded *via* depurination of the inosine moiety, which limits the possibility to detect a slow isomerization. Nevertheless, we may conclude that isomerization of **2** to **1** has to be considerably, certainly more than one order of magnitude, slower than the reverse reaction.

At pH < 3, both isomerization and hydrolysis are acid-catalyzed and their rates are comparable with each other. However, under these conditions even acid-catalyzed depurination of the inosine moiety competes with the phosphoester reactions and thereby severely complicates the reaction system. As an example of the acid-catalyzed reactions, Figure 2 shows the time-dependent product distribution for hydrolysis of IspU at pH 1.

It is noteworthy that the acid-catalyzed phosphoester hydrolysis yields a different thioinosine monophosphate (with a slightly longer HPLC retention, but with equal molecular mass by ESI-MS; m/z = 363, see Table 2) than the hydroxide-ion-catalyzed reaction. Namely, as was shown earlier,[3] in aqueous acid thioinosine 2'-*O*-phosphate (**6**) is formed, without any sign of parallel formation of the 3'-*S*-phosphorothiolate **5**. Most likely, however, both the acid- and base-catalyzed cleavage proceed by intermediate

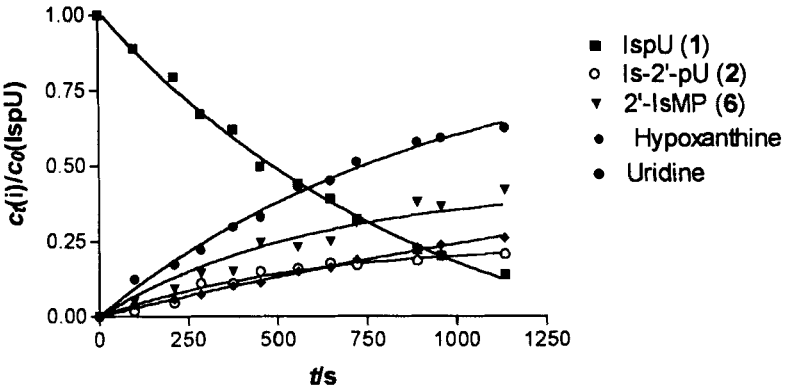


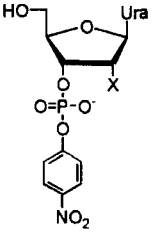
FIG. 2. Time-dependent product distributions of the hydrolysis of IspU (**1**) in 0.1 mol dm^{-3} aqueous hydrogen chloride at 363.2 K.

formation of cyclic 2'-*O*,3'-*S*-phosphorothiolate (**4**), although accumulation of **4** could be verified by HPLC only during alkaline hydrolysis.

As seen from Figure 2, the concentrations of both the 2',5'-isomer **2** and thioinosine 2'-*O*-phosphate (**6**) steadily increase during the first two half-times of the degradation of IspU in aqueous acid. Accordingly, the subsequent hydrolytic reactions of **2** and **6** must be relatively slow compared to their formation. From the product distributions during the first half-life, it may be estimated that 31 % of the degradation of IspU at pH 1 takes place by the phosphoester migration, 44 % by phosphoester hydrolysis and 26 % by depurination. At pH 2, isomerization is already faster than the cleavage (Figure 1). In 0.5 M hydrochloric acid, on the other hand, hypoxanthine did not accumulate. This can be understood on the basis of the known[8] pH-rate profile of depurination of inosine, since it appears reasonable to assume that the depurination of IspU proceeds by a mechanism similar to that of the nucleoside.[9] Namely, whereas the rate profiles for the *N*-glycosidic hydrolysis of purine nucleosides usually show a strictly linear first-order dependence on the acid concentration,[9] the rate profile of hydrolysis of inosine shows some curvature (leveling-off) at pH < 1.[8] Furthermore, the slope of the rate profile of diester hydrolysis of IspU seems to slightly exceed unity between pH 1 and 0.2 (Figure 1), which also partially explains the lack of depurination in the more acidic solution. As was shown earlier,[7] even

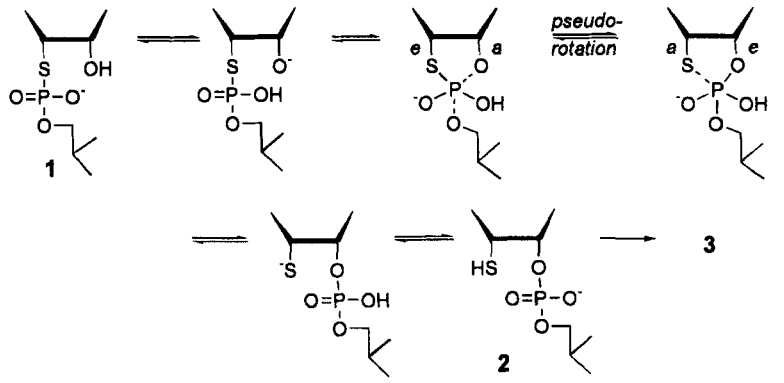
with ApU and UpA the depurination competes most efficiently with the phosphoester transesterification at pH close to 2. The rate of depurination of inosine appears to be moderately affected by the 3'-phosphorothiolate moiety. The first-order rate constants for depurination in 0.1 M aqueous HCl at 363 K were 1.2×10^{-3}, 3.6×10^{-4} and 4.2×10^{-4} s^{-1} for inosine, IpU and IspU, respectively.

Kinetics and Mechanisms. Besides the overall reactivity differences, significant differences between the thiolate **1** and its native phosphate analogs were also noticed in the product distributions of both the isomerization and phosphoester cleavage reactions. Firstly, while isomerization of native dinucleoside monophosphates leads to a nearly equimolar mixture of the 3',5'- and 2',5'-isomers, the isomerization of the thiolate analog is not reversible: no evidence for isomerization of the 2',5'-phosphate **2** back to the 3'-thiolate could be obtained. Accordingly, since the phosphoester migration inevitably involves attack of the neighboring functional group (either 2'-hydroxyl or 3'-thiol) on phosphorus, this means that the 3'-sulfhydryl function is a much weaker nucleophile towards phosphorus than a corresponding hydroxyl function. This finding is consistent with the previous report of Reese *et al.*[10] For comparison, Dantzman and Kiesling[11] were able to follow the hydrolysis involving attack of the 2'-thiol function of 2'-thiouridine on the neighboring 3'-phosphodiester when the 5'-esterified nucleoside was replaced by a much better leaving-group, 4-nitrophenyl (in **7a**). With this compound, the hydrolysis was shown under neutral conditions to be 27 fold slower than that the corresponding 2'-OH analog (**7b**).[11] Furthermore, since the reaction of the 2'-SH analog **7a** is not acid-catalyzed like that of the hydroxyl analog at pH < 3 but remains pH-independent from pH 6 to 1, the reactivity of **7b** under acidic conditions (pH < 1) is several orders of magnitude higher than that of **7a**.[11]



7a: X = SH
7b: X = OH

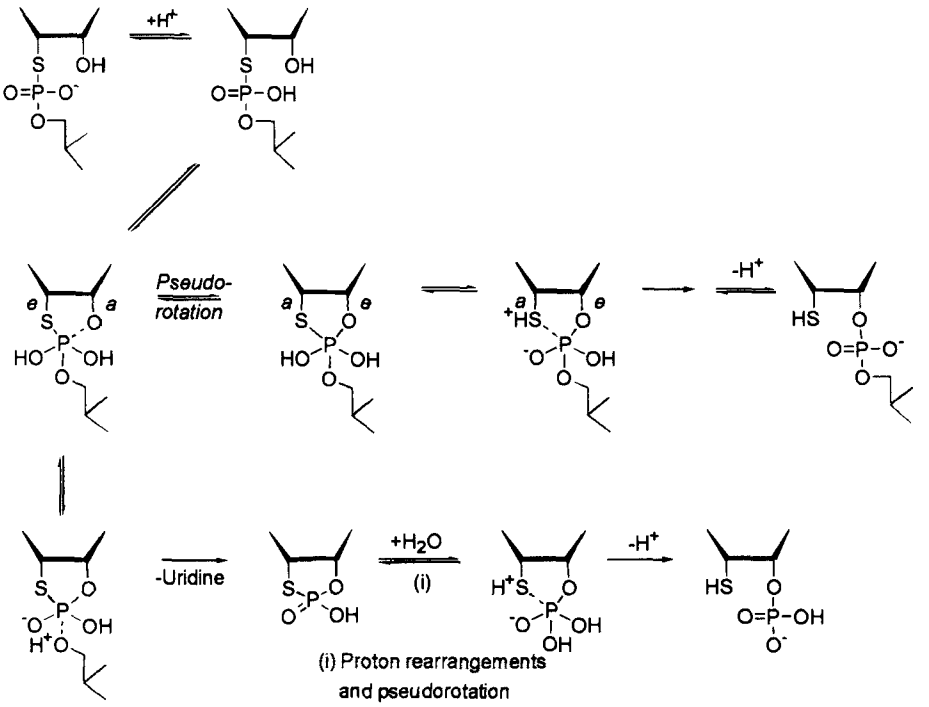
The occurrence of the isomerization of IspU (**1**) to its 2',5'-analog **2** may be taken as a piece of evidence for the formation and pseudorotation of a cyclic thiophosphorane



SCHEME 2

intermediate (Scheme 2). According to the well known Westheimer's guidelines,[12] the attacking nucleophile (2'-OH in the case of IspU) initially attains an apical position in the phosphorane intermediate, but departure of the leaving group can also occur only from an apical position. In the present case, however, the leaving group (3'-S) must initially be equatorial in the thiophosphorane formed, since it belongs to the same five-membered ring formed by the incoming nucleophile; diapical and diequatorial locations within this type of cyclic structure are geometrically forbidden.[12a] Accordingly, pseudorotation of the thiophosphorane is required to bring the thioinosine 3'-sulfhydryl to an apical position, from where it may depart.

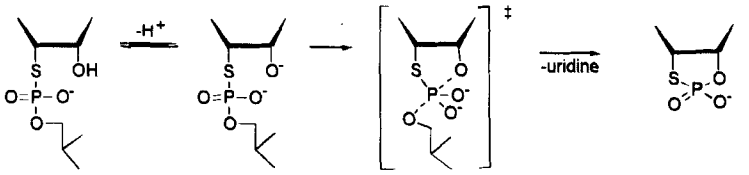
Since the isomerization of IspU is acid-catalyzed at pH < 3, it may be seen that both the monoanionic and neutral (or monocationic) forms of the thiophosphorane are able to pseudorotate. Accordingly, mechanisms shown in Scheme 3 may be suggested for the acid-catalyzed reactions. The present data do not allow a quantitative distinction between the reactions proceeding *via* a neutral or monocationic thiophosphate. According to the rate profile (Figure 1), the apparent reaction order of the diester hydrolysis with respect to $[H^+]$ slightly exceeds unity, suggesting that a reaction *via* a monocation is also involved. For the isomerization, on the other hand, only first-order dependence is detected. The predominating ionic form of IspU between pH 1- 3 is a monoanion, since the pK_a of the neutral molecule is expected to be lower - rather than higher - than that of a



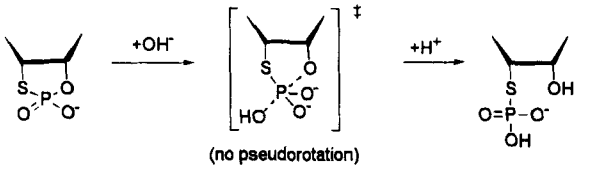
SCHEME 3

corresponding phosphodiester (the kinetically determined first pK_a of UpU is 0.7 under the experimental conditions[7]. The hydrolysis and isomerization of UpU[7] are second order in [H^+] between pH 1 and 2).

The rate of the base-catalyzed hydrolysis of IspU shows a first-order dependence on hydroxide-ion concentration at pH > 6. The rate profile between pH 10 to 14 (at 25 °C) was earlier studied electrophoretically for the hydrolysis of p*IspU (a derivative of **1**, having a ^{32}P labeled monophosphate group at the 5'-site of the inosine moiety) in comparison with its phosphodiester analog (p*IpU).[3] Although p*IspU was 2000 fold more labile towards alkaline hydrolysis than p*IpU, it exhibited an analogous dependence of rate on hydroxide ion concentration.[3] Namely, with both compounds, the rate is first-order in [OH^-] between pH 10 to 13, but then levels off to a constant value under more alkaline conditions. Accordingly, even the hydroxide ion catalyzed hydrolysis of IspU most likely follows the mechanism generally accepted[13] for hydrolysis of the



SCHEME 4a



SCHEME 4b

internucleosidic phosphodiester linkage of RNA constituents: the 2'-hydroxyl is deprotonated in a pre-equilibrium stage and the alkoxide ion thus formed attacks the phosphorus, leading to a concomitant (in-line) departure of the 5'-esterified nucleoside (Scheme 4). The curvature of the pH-rate profiles thus facilitated determination of the pK_a -values of the 2'-hydroxyls, which were shown to be equal (pK_a = 13.0) for p*IspU and p*IpU.[3] Accordingly, the relative lability of the 3'-thiolate linkage does not derive from altered acidity of the 2'-hydroxyl.

For comparison, substitution of the 5'-bridging oxygen with sulfur makes an RNA dinucleotide as much as 10^5 times more reactive towards alkaline hydrolysis.[2] In that case main part of the rate acceleration has been explained[2] to derive from the better leaving-group ability of a thiolate compared to an alcoholate anion. In contrast, the destabilizing effects of the 3'-*S*-substitution are not as clear, and several potential factors have been discussed earlier.[3] The 3'-sulfur may affect both by facilitating the attack of the anionic nucleophile and by stabilizing the developing cyclic transition state and, moreover, at both stages the effects may be either of geometric or electronic origin. The destabilizing effects of both the 3'- and 5'-thiosubstitutions under acidic conditions are smaller, in fact negligible, compared to those observed for alkaline hydrolysis. However, since the thiophosphates are somewhat more acidic than the corresponding oxyphosphates, the

reduced protonation of the thionucleotide may, at least partly, cancel the rate accelerating effects of the thiosubstitution in acid-catalyzed hydrolysis. It must be noted that the reactions of a native phosphodiester linkage in this pH-region proceed mainly *via* a monocationic substrate,[7] whereas the contribution of this ionic form in the reactions of IspU could not be quantified.

Hydrolysis of the cyclic 2'-O,3'-S-phosphorothiolate. As mentioned above, the base-catalyzed hydrolysis of a 2',3'-cyclic nucleotide appears to be less efficiently facilitated by the 3'-S substitution than is the internucleosidic cleavage under same conditions. From the present results, we can estimate that the second-order rate constant for the alkaline hydrolysis of inosine 2'-*O*,3'-*S*-cyclic phosphorothiolate (**4**) is 40 dm^3 mol^{-1} s^{-1}, which can be compared to a value 0.7 dm^3 mol^{-1} s^{-1} determined[14] for adenosine 2',3'-cyclic monophosphate at the same temperature (363.2 K). Accordingly, the "3'-thio effect" means that even in this case there is a rate acceleration of about 60 fold which is, however, somewhat smaller than the 350 fold effect observed with the hydrolysis of IpU. While it may be assumed that for example the polarizability and larger size of the sulfur atom may stabilize the transition states in about equal manner in both cases, the geometries of the initial states are affected differently. With the dinucleoside monophosphate, 3'-thiosubstitution could be concidered to make the phosphate more flexible and perhaps more easily accessible for the attacking nucleophile. In the hydrolysis of the cyclic phosphate, involving an intermolecular nucleophilic attack, this kind of effect on the kinetics may be expected to play a smaller role.

The different product distribution in alkaline and acidic hydrolysis of IspU (3'-*S*-phosphorothiolate is formed under alkaline *cf.* to 2'-monophosphate under acidic conditions) reflects the hydrolytic behaviour of a 2',3'-cyclic phosphorothiolate (**4**). It seems reasonable to assume that even the acidic hydrolysis takes place through this intermediate, even though its accumulation did not exceed the lower limit of detection. Accordingly, it appears that the product distribution in the hydrolysis of **4** differs significantly from that observed with its phosphate analogs. Namely, with the nucleoside 2',3'-cyclic phosphates, both acid- and base-catalyzed hydrolyses yield a mixture of nucleoside 2'- and 3'-monophosphates, the molar composition of which (close to equimolar with all the usual 2',3'-cNMP's) is rather independent of pH.[14,15] The change in

product distribution may well result from the fact that, whereas the thiophosphorane intermediate of the hydrolysis of the cyclic phosphorothiolate **4** may undergo pseudorotation in acidic or neutral solutions, the alkaline hydrolysis proceeds *via* a dianionic thiophosphorane, which is too unstable to pseudorotate. It is expected that the 3'-sulfur, being less electronegative than the 2'-oxygen, initially attains an equatorial position in the thiophosphorane.[12] Thus, when the reaction takes place *via* a dianionic species, only the 3'-*S*-phosphate is formed. Since a thiolate-ion is, anyway, expected to be a much better leaving-group than an alkoxide-ion (R-SH is about 5 orders of magnitude more acidic than R-OH), the complete lack of formation of the 2'-phosphate monoester in alkaline hydrolysis shows that a dianionic thiophosphorane intermediate either is not formed at all, or has a very high barrier for pseudorotation. By contrast, while pseudorotation of the intermediate is possible (or fast enough) in acidic solutions, departure of the 3'-mercapto ligand (a better leaving group) becomes predominating. Furthermore, it was noted that the phosphoryl group of the 3'-*S*-phosphorothiolate monoester **5** under alkaline conditions shows no tendency at all to migrate to the 2'-oxygen. On the other hand, the exclusive formation of the 2'-phosphate (**6**) under acidic conditions is consistent even with the occurrence of the irreversible isomerization of the dinucleoside phosphorothiolate **1** to the 2',5'-phosphodiester.

It may be noted, for comparison, that the change in product distribution of the hydrolysis of **4** due to a change from alkaline to acidic solutions, is analogous to that observed earlier[16] with hydrolysis of ethylene *O,S*-cyclic phosphorothiolate.

EXPERIMENTAL SECTION

Materials. 3'-Deoxy-3'-thioinosylyl-(3',5')-uridine (**1**) was prepared as described earlier.[5b] Inosylyl-(3',5')-uridine, inosine, hypoxanthine and uridine were purchased from Sigma.

Kinetic Measurements. The pH-rate profiles (pH 0.2 to 9 at 363.2 K) for the competing hydrolytic reactions of IspU were determined by an HPLC method described earlier.[7] Composition of the aliquots withdrawn from the reaction solution was analyzed on a Hypersil ODS5 column (4-250 mm, 5μm). Either an acetic acid/sodium acetate buffer (0.045/0.015 M) containing 0.1 M ammonium chloride and 3 - 5 % acetonitrile

TABLE 2. HPLC Retention Times[a] and Mass Spectrometric Characterization[b] for the Hydrolytic Products of 3'-Deoxy-3'-thioinosylyl-(3'→5')-uridine (**1**).

Compound	t_R/min	*m/z* [c] negative	*m/z* [c] positive	*M*(calculated)
IspU (**1**)	23.8	589	591	589.4
Is-2'-pU (**2**)	15.0	589	591	589.4
2',3'-cIsMP (**4**)	4.0	345		345.2
3'-IsMP (**5**)	5.1	363		363.2
2'-IsMP (**6**)	5.9	363		363.2
Uridine	4.3	243		244.2
Hypoxanthine	3.9			

[a] On a Hypersil ODS 5 column (4-250 mm, 5 μm), eluted with an acetic acid/ammonium acetate buffer (0.07 M/0.01 M) containing 2 % (*v/v*) acetonitrile at flow rate 1.0 mL/min. [b] By HPLC ESI-MS directly from the aliquots of the kinetic runs. [c] For the molecular ion. By positive scan, the corresponding sodium adduct-ion was additionally observed in each case.

(eluent A), or an acetic acid/ammonium acetate buffer (0.07/0.01 M) containing 2 % acetonitrile (eluent B) was used as an eluent. The latter eluent was employed in the HPLC/ESI MS analysis, the results of which are given in Table 2. The MS analyses were performed on a Perkin-Elmer API Triple Quadrupole LC/MS/MS spectrometer.

The integrated peak areas (UV detection) of the compounds were converted to relative concentrations by using the ratio of the absorptivities of hypoxanthine and uridine determined by comparison of the peak areas obtained from standard solutions of equal concentrations. For example, with eluent B and at a wavelength 255 nm the ratio of the absorbance of hypoxanthine to uridine was 1.27. Applying this ratio, the peak areas of the hydrolysis products were converted to concentrations relative to the initial concentration of the starting material. This method requires, of course, the assumption that the molar absorption coefficients of the nucleoside bases in these compounds are independent of the molecular environment

Calculation of the rate constants. The integrated first-order rate equation was applied to the diminution of the integrated peak area of the starting material (IspU), to obtain the

combined rate constant (k_{tot}) for the parallel hydrolytic and isomerization reactions. First-order rate constants for the parallel reactions (k_1, k_2 and k_3 for diester hydrolysis, isomerization and depurination, respectively) were obtained by bisecting k_{tot} to the individual rate constants on the basis of the product distribution (relative concentrations of Is-2'-pU (**2**), 2'-IsMP (**5**) and hypoxanthine) at the early stages of the reaction. This appears to be reliable method since the rate of the subsequent hydrolysis was, for each product, relatively slow compared to the rate of its formation.

Characterization of the hydrolysis products. The product accumulating at pH 4 was isolated by HPLC under same conditions as those used for the kinetic runs. The buffer salts were subsequently removed using the same column by eluting with a mixture of water and acetonitrile. ESI-MS (positive): *m/z* 591 ($[M + H]^+$); 613 ($[M + Na]^+$). After removal of the solvents and preparation of an NMR sample, the compound had partially converted to one with a *m/z* 1179 ($[M + H]^+$). According to the identical mass spectrum and HPLC retentions times, the latter compound was the same as that observed in the kinetic run at pH 5. Thus, the NMR data for the first product (assigned as **2**) in a pure isolated form could not be recorded, but its conversion to the subsequent product (**3**) could be followed even by NMR (in ^2H_2O). By ^{31}P NMR (202 MHz, ^2H_2O, 278 K, 85 % H_3PO_4 in ^2H_2O as external standard), conversion of a compound resonating at $\delta = -0.82$ ppm to one resonating at $\delta = -1.15$ ppm was observed. The changes in ^1H NMR (500 MHz, ^2H_2O, 278 K) resonances were small, but those of aromatic and anomeric signals could be followed. Thus, the following chemical shifts were assigned for **2**: $\delta_H = 8.25$ (s, H2 or 8 of Hx), 8.120 (s, H8 or H2 of Hx), 7.613 (d, $J = 8.3$ Hz, H6 of Ura), 6.27 (d, $J = 1.1$ Hz, H1'), 5.73 (d, $J = 3.2$ Hz, H1'), 5.47 (d, $J = 8.1$ Hz, H5 of Ura). The signals of the conversion product were the same as those observed for the isolated sample of the final hydrolysis product at pH 5.

Compound **2** was let to react further at pH 5 and the final product was isolated by HPLC. The spectral data is consistent with the structure for **3**. ^1H NMR (500 MHz, ^2H_2O): $\delta_H = 8.32$ (s, Hypoxanthine), 8.125 (s, Hypoxanthine), 7.610 (d, $J = 8.3$ Hz, H6 of Ura), 6.31 (d, $J = 2.7$ Hz, H1' of Ino), 5.70 (d, $J = 3.1$ Hz, H1' of Urd), 5.54 (d, 8.1 Hz, H5 of Ura), 5.39 (octet, H2' of Ino; by phosphorus decoupling turned to dd, $J_1 = 6.0$, $J_2 = 2.8$ Hz), 4.60 (dtr, $J_1 = 8.1$, J_2, $J_3 = 2.6$ Hz, H4' of Ino), 4.35 (dd, $J_1 = 8.1$, $J_2 = 5.2$ Hz,

H3' of Ino), 4.20 (dd, J_1 = 6.2, J_2 = 5.4 Hz, H4' of Urd), 4.12 - 4.07 (m, H2', 3', 5'a and 5'b of Urd), 4.04 (dd, J_1 = 13.2, J_2 = 2.2 Hz, H5'a of Ino), 3.95 (dd, J_1 = 13.2, J_2 = 3.1 Hz, H5'b of Ino). ^{31}P NMR (202 MHz, ^2H_2O, 278 K): δ = −1.15 ppm.

FAB$^+$ MS: *m/z* 1213 $[M + K]^+$; 1234 $[M + K + Na]^+$; 1255 $[M + 2K]^+$. ESI MS (positive): *m/z* 1179 ($[M + 2]^+$).

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