Hydrolytic dethiophosphorylation and desulfurization of the monothioate analogues of uridine monophosphates under acidic conditions

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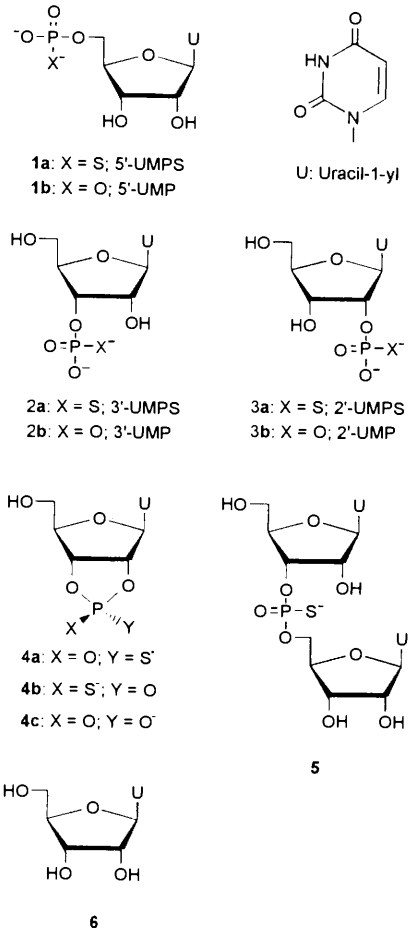
The hydrolytic reactions of uridine 2′-, 3′- and 5′-phosphoromonothioates (2′-, 3′- and 5′-UMPS) under acidic and neutral conditions have been followed by HPLC. Under slightly acidic conditions (pH 2–5), only pH-independent dethiophosphorylation to uridine takes place. This reaction is 200- to 300-fold as fast as dephosphorylation of the corresponding uridine monophosphates (UMP), presumably due to higher stability of the thiometaphosphate monoanion compared to metaphosphate anion. At pH > 5, *i.e.* at pH > pK_{a2} of the thiophosphate moiety, the dethiophosphorylation is retarded with increasing basicity of the solution. At pH < 1, acid-catalysed desulfurization of 2′- and 3′-UMPS to an isomeric mixture of 2′/3′-UMP competes with their dethiophosphorylation. This reaction is suggested to proceed by a nucleophilic attack of the neighbouring hydroxy group on phosphorus. No such reaction occurs with 5′-UMPS. In contrast to 2′- and 3′-UMP, no sign of interconversion of 2′- and 3′-UMPS is detected.

Introduction

The phosphorothioate analogues (**1a**) of nucleoside 5′-monophosphates were introduced in nucleic acid chemistry in the late 1960s when thymidine,[1] uridine[1] and adenosine[2] 5′-phosphoromonothioates were synthesized and shown to be relatively resistant toward alkaline phosphatases. Nucleoside 3′-phosphoromonothioates (**2a**) were, in turn, obtained as products of the RNase-catalysed hydrolysis of the R_p-diastereoisomers of uridine and guanosine 2′,3′-cyclic phosphoromonothioates [**4a**; (R_p)-2′,3′-cUMPS][3–6] and, together with their 2′-isomers (**3a**), as products of the alkaline hydrolysis of **4a**.[3,6] Since then, nucleoside phosphoromonothioates have been widely employed in mechanistic studies of various phosphatases.[7,8] In spite of this, the effect of thiosubstitution on the hydrolytic stability of nucleoside monophosphates, as well as the kinetics of desulfurization of phosphoromonothioates, is not well known.

We have recently described the kinetics of hydrolytic reactions of the diastereoisomeric monothioate analogues of uridylyl(3′,5′)uridine (**5**), having one of the non-bridging phosphate oxygens replaced with sulfur.[9] These kinds of phosphoromonothioate linkages are frequently used as stereochemical probes in mechanistic studies of ribonucleases and ribozymes.[7,10] It was shown that under neutral and acidic conditions the predominant reactions of **5** are hydrolytic cleavage and desulfurization of the phosphoromonothioate bond. The former reaction yields, among other products, 2′- and 3′-phosphoromonothioates, which are subsequently desulfurized to nucleoside monophosphates and/or dethiophosphorylated to nucleoside. This paper is aimed at learning more about these subsequent reactions and at elucidating the hydrolytic behaviour of nucleoside phosphoromonothioates in general. For these purposes we report on the kinetics of the hydrolytic decomposition of uridine 2′-, 3′- and 5′-phosphoromonothioates (**3a**,**2a** and **1a**, respectively). The data obtained are compared to those reported previously[11,12] for the corresponding nucleoside monophosphates (**3b**,**2b**,**1b**). These comparisons enable determination of the so called 'thio effect' that has repeatedly been utilized[13,14] in mechanistic studies of ribozymes and RNases. It has been suggested[13] that since thio substitution considerably accelerates the hydrolysis of phosphate monoesters[15] and retards that of phosphate triesters,[16] the magnitude of the thio effect correlates with the change from a dissociative (phosphomonoester hydrolysis) to an associative (phosphotriester hydrolysis) mechanism.[17] The known values of the thio effect, however, largely refer to hydrolysis of phosphate aryl esters. The results of the present paper, together with those determined previously for the closely related diesters (**5**), provide a broader chemical basis for this kind of reasoning.



Results

The hydrolysis of uridine phosphoromonothioates, 2'-, 3'- and 5'-UMPS (**3a**, **2a**, **1a**) was followed by HPLC, and the products were identified by spiking with authentic samples. Over an acidic pH range (pH 2 to 7), dethiophosphorylation to uridine (**6**) was observed, without the appearance of any intermediates. With 2'- and 3'-UMPS, desulfurization to a mixture of uridine 2'- and 3'-monophosphates (2'-UMP, **3b** and 3'-UMP, **2a**) competed with the dethiophosphorylation at pH < 1, while with 5'-UMPS no such reaction to monophosphate occurred. By contrast, under alkaline conditions (pH > 7), a pH-independent desulfurization of 5'-UMPS to 5'-UMP was found to compete with dethiophosphorylation, whereas the desulfurization of the 2'- and 3'-isomers even then yielded the isomeric mixture of 2'- and 3'-UMP. However, the mechanisms of the alkaline reactions are not discussed here.

No sign of cyclization of 2'- or 3'-UMPS to a cyclic thiophosphate (**4a**,**b**; 2',3'-cUMPS), or of interconversion of 2'- and 3'-UMPS could be obtained under any conditions (detection limit for **4a**,**b** < 1%). Since 2'- and 3'-UMP are known[11,12] to undergo mutual isomerization and dephosphorylation to uridine under acidic conditions, the reactions taking place may be depicted as shown in Scheme 1. Fig. 1 shows the pH–rate profiles obtained for the dethiophosphorylation (k_1) and desulfurization (k_2). Neither of the reactions was markedly susceptible to buffer catalysis. The points presented refer to a buffer concentration of zero.

Discussion

We have shown previously[11,12] that nucleoside 2'- and 3'-monophosphates undergo two competing reactions of the phosphate moiety at pH < 7: (i) mutual isomerization (phosphate migration) and (ii) dephosphorylation (phosphoester hydrolysis) to uridine. The former reaction proceeds by an attack of the neighbouring hydroxy group on phosphorus, and the pentacoordinated intermediate formed is decomposed either to the 2'- or 3'-monophosphate. The latter reaction, in turn, involves unimolecular departure of a metaphosphate monoanion (preassociated with a water molecule) without intramolecular participation of the neighbouring hydroxy function. Protonation of the phosphate moiety facilitates the intramolecular nucleophilic attack on phosphorus, but not the unimolecular departure of the metaphosphate ion, and hence the phosphate migration strongly predominates over phosphoester hydrolysis at pH < 2 (see Fig. 1). At pH 2 to 6, both reactions proceed at comparable pH-independent rates *via* the predominant ionic form, the phosphoester monoanion. At higher pH, the phosphate dianion becomes the prevailing ionic form, and both reactions are retarded with increasing alkalinity. The phosphoester hydrolysis of 5'-UMP is slightly slower than that of 2'- and 3'-UMP, and no phosphate migration occurs.[12]

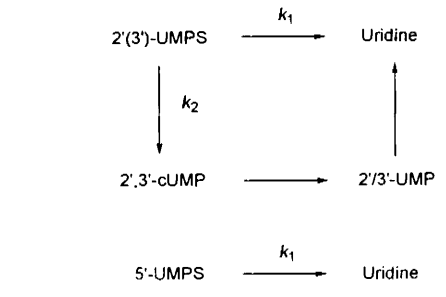
The hydrolytic behaviour of 2'- and 3'-UMPS resembles that of their oxygen counterparts, but is not entirely similar. They undergo, analogously to 2'- and 3'-UMP, a pH-independent dethiophosphorylation to uridine under mildly acidic conditions (Fig. 1), but in striking contrast to nucleoside monophosphates no thiophosphate migration takes place. Instead, desulfurization with intermediary formation of 2',3'-cUMP occurs in very acidic (pH < 1) solutions. 5'-UMPS behaves as 5'-UMP: only phosphomonoester hydrolysis takes place.

The pH–rate profiles of the dethiophosphorylation reaction (Fig. 1) are rather similar to those described for the dephosphorylation of nucleoside 2'-, 3'- and 5'-monophosphates: the rate of the reaction is pH-independent under conditions where the predominant ionic form is the phosphoester monoanion, and a rate-retardation is observed when the monoanionic thiophosphate group becomes deprotonated (pK_{a2} *ca.* 5), and possibly also when it becomes protonated (at Hammett acidity function H_0 *ca.* 0). The rate profiles are, however, shifted about 1 pH unit towards the more acidic region, most likely owing to higher acidity of the thiophosphate group compared to the phosphate group. For comparison, the pK_{a1} and pK_{a2} values of thiophosphoric acid are 0.5 and 1.8 units lower than those of phosphoric acid, respectively.[18,19] Fitting the observed rate constants, k_1, to eqn. (1) gives the following values for the acidity constant, K_{a2},

$$k_1 = k_d/([H^+]K_{a1}^{-1} + 1 + K_{a2}[H^+]^{-1}) \qquad (1)$$

of the substrate monoanion (the reactive ionic form) and the first-order rate constant, k_d, of its dethiophosphorylation. 2'-UMPS: $pK_{a2} = 4.9 \pm 0.2$ and $k_d = (4.2 \pm 0.6) \times 10^{-3}$ s^{-1}. 3'-UMPS: $pK_{a2} = 4.7 \pm 0.2$ and $k_d = (3.4 \pm 0.5) \times 10^{-3}$ s^{-1}. 5'-UMPS: $pK_{a2} = 5.2 \pm 0.2$ and $k_d = (7.6 \pm 0.9) \times 10^{-4}$ s^{-1}. The values of pK_{a1} cannot be obtained with reasonable accuracy, but they appear to be close to zero. The corresponding rate constants for the dephosphorylation of 2'-, 3'- and 5'-UMP are 1.4×10^{-5}, 1.4×10^{-5} and 3×10^{-6} s^{-1}, respectively.[12] Accordingly, the monoanionic 2'-, 3'- and 5'-UMPS are dephosphorylated to uridine 300, 260 and 270 times as fast as 2'-, 3'- and 5'-UMP, respectively. This large difference in the hydrolysis rates strictly excludes all mechanisms involving initial desulfurization of 2'/3'-UMPS to 2',3'-cUMP (and subsequently to 2'/3'-UMP) *via* nucleophilic participation of the neighbouring hydroxy function. No sign of accumulation of 2'/3'-UMP could be detected.

In all likelihood the dethiophosphorylation of isomeric UMPS to uridine proceeds, analogously to the dephosphorylation of isomeric UMP, by a dissociative mechanism generally accepted[20,21] for the hydrolysis of phosphomonoesters: a rapid initial proton transfer from the phosphate hydroxy (or sulfanyl)



Scheme 1

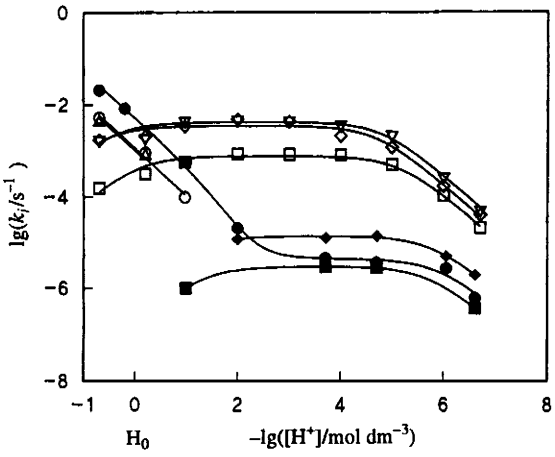
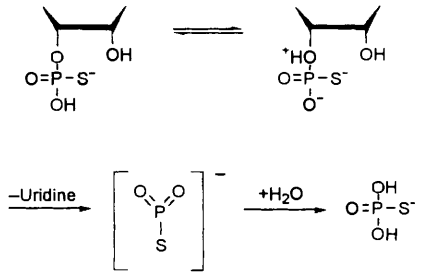


Fig. 1 pH–Rate profiles for hydrolytic reactions of 2'-, 3'- and 5'-UMPS at 363.2 K. The ionic strength of the solutions adjusted to 0.1 mol dm^{-3} with sodium chloride. Notation: (▽) dephosphorylation of 2'-UMPS; (◇) dephosphorylation of 3'-UMPS; (□) dephosphorylation of 5'-UMPS; (△) desulfurization of 2'-UMPS; (○) desulfurization of 3'-UMPS; (◆) dephosphorylation of 2'/3'-UMP;[12] (■) dephosphorylation of 5'-UMP;[12] (●) isomerization of 3'-UMP to 2'-UMP.[12]



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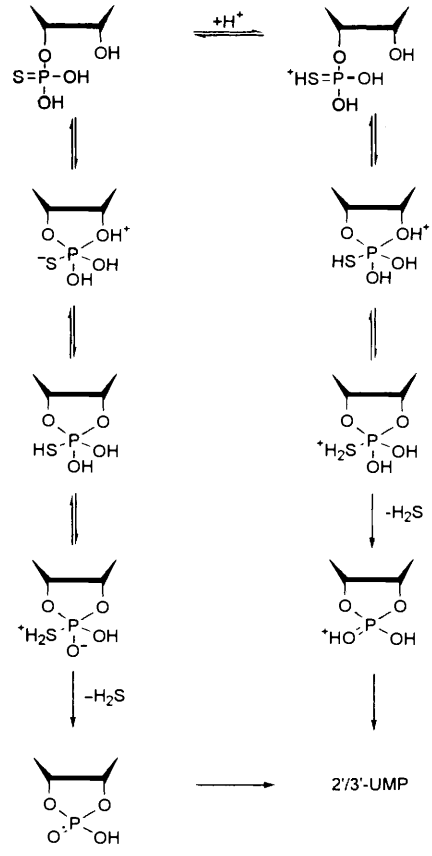
Scheme 2

ligand to the esterified oxygen, results in cleavage of the P–O bond with concomitant release of the thiometaphosphate monoanion, either as a free species or preassociated with a water molecule (Scheme 2). Since the dianionic substrate does not contain a proton that could be transferred to facilitate the departure of the leaving alkoxy group, a rate retardation takes place on passing the pK_{a2} value of the thiophosphate moiety. The neutral thiophosphate ester may also be expected to be less reactive than the monoanionic species, since the transition state leading to the formation of thiometaphosphoric acid is stabilized by resonance less efficiently than the one giving the thiometaphosphate ion. However, no conclusive evidence for rate-retardation under very acidic conditions could be obtained, since the pK_{a1} value is very low and the C–O bond cleavage may start to compete with the P–O bond cleavage.[20a]

The markedly higher rate of phosphoester hydrolysis of UMPS compared to that of UMP reflects the higher stability of the thiometaphosphate ion intermediate (**7** in Scheme 2) compared to its oxygen counterpart. While the metaphosphate anion may exist as a free species only in organic solvents,[22] several lines of evidence suggest[23] that the thiometaphosphate ion has a finite life-time even in protic and aqueous solvents. It has been previously[15] reported that the 4-nitrophenyl monoester of thiophosphoric acid is hydrolysed approximately 50 times as fast as the corresponding phosphoric acid ester. The thio effects observed in the present study for nucleosidic alkyl monoesters, having a much poorer leaving group than 4-nitrophenol, are only moderately larger. Accordingly, the thio effect is not very susceptible to the chemical nature of the esterified alcohol, but replacement of one of the non-bridging oxygens with sulfur invariably results in a 100-fold rate-acceleration as long as a dissociative pathway is followed.

As mentioned above, 2′- and 3′-UMPS do not undergo mutual isomerization, in contrast to 2′- and 3′-UMP. With the latter compounds, the migration of the hydrogen phosphate group is pH-independent at pH 3 to 6 and hydronium ion-catalysed at pH < 3, the observed first-order rate constants being of the order of 10^{-5} s^{-1} at pH = 4 and 10^{-2} s^{-1} at $H_0 = 0$ under the experimental conditions of the present study.[12] Our previous studies[9] with the phosphoromonothioate analogues of uridylyl (3′,5′)uridine [3′,5′-Up(s)U; **5**] suggest that the migration of the monoalkylmonothiophosphate group is one order of magnitude slower than that of the monoalkylphosphate group. Accordingly, the observed first-order rate constants for the migration of the hydrogen thiophosphate group could be expected to be of the order of 10^{-6} at pH = 4 and 10^{-3} s^{-1} at $H_0 = 0$. Obviously the dethiophosphorylation of 2′/3′-UMPS is so fast at pH > 3 ($k_1 > 10^{-3}$ s^{-1}) that thiophosphate migration cannot be expected to compete with it. Under very acidic conditions (H_0 *ca.* 0), nucleophilic attack of the neighbouring hydroxy function on phosphorus takes place, but the pentacoordinated intermediate obtained is decomposed rather by desulfurization to 2′,3′-cUMP than by departure of the originally esterified sugar hydroxy group. These reactions are discussed below in more detail.



Scheme 3

As mentioned, 2′- and 3′-UMPS are partly converted to a mixture of 2′- and 3′-UMP in very acidic solutions (pH < 1). In all likelihood the initial step is desulfurization to 2′,3′-cUMP, which is then hydrolysed to isomeric UMPs. Consistent with this argument, 2′- and 3′-UMP are formed in a 2:3 ratio as in the hydrolysis of 2′,3′-cUMP.[12] Moreover, 5′-UMPS was not observed to undergo desulfurization to 5′-UMP at low pH, which suggests that neighbouring group participation of the 2′/3′-hydroxy function plays a decisive role. The hydrolytic desulfurization is approximately first-order in hydronium ion concentration, the second-order rate constant being (1.1 ± 0.2) × 10^{-3} dm^3 mol^{-1} s^{-1} at 363.2 K. The reaction is most probably initiated by an intramolecular nucleophilic attack of the undissociated neighbouring hydroxy function on a neutral (or possibly monocationic) thiophosphate group, which gives a neutral (or monocationic) pentacoordinated thiophosphorane intermediate (Scheme 3). Since the kinetic measurements could be carried out over a relatively narrow acidity range only, and since the pK_{a1} value of the starting material falls in this region, it is impossible to decide whether the attack on neutral or monocationic thiophosphate predominates. The intermediate obtained is decomposed solely by departure of the sulfanyl ligand, most likely as dihydrogen sulfide, and the cyclic monophosphate obtained is then rapidly hydrolysed to a mixture of 2′- and 3′-UMP.[12] The complete lack of thiophosphate migration is somewhat unexpected. In this respect the behaviour of 2′/3′-UMPS differs from that of 3′,5′-Up(s)U (**5**). The thiophosphorane intermediate formed from the latter compound is decomposed, not only by desulfurization, but also by departure of one of the sugar hydroxy groups. Although the desulfurization is faster than thiophosphate migration, the existence of the latter reaction is a clearcut piece of evidence for a finite life-time and pseudorotation of the thiophosphorane intermediate. With 2′/3′-UMPS no such evidence could be obtained. Anyway, the present results, together with those reported previously for 3′,5′-Up(s)U,

suggest that a non-bridged sulfur departs from thiophosphorane intermediates under acidic conditions more readily than the alkoxy ligands.

As seen from Fig. 1, the rate of desulfurization at pH < 1 of 3′-UMPS to a mixture of 2′- and 3′-UMP is about one quarter of that of the isomerization of 3′-UMP to 2′-UMP. Since no indication of thiophosphate migration was obtained, the thiophosphate group must migrate considerably less readily than the phosphate group. Accordingly, while replacement of one of the non-bridging phosphate oxygens with sulfur accelerates the dissociative phosphomonoester hydrolysis by two orders of magnitude, the effect on reactions proceeding by nucleophilic participation of the neighbouring hydroxy function is rate-retarding. At least in part this rate-retardation may be attributed to the less ready protonation of the thiophosphate group compared to the phosphate group, but still the difference between the thio effect of dissociative and associative processes remains substantial. For comparison, at pH < 1 3′,5′-Up(s)U is hydrolytically more than an order of magnitude more stable than 3′,5′-UpU,[9] while the alkaline hydrolysis shows only a small thio effect, 20% retardation and 30% acceleration for the R_P and S_P diastereoisomers of 3′,5′-Up(s)U, respectively.[24]

Conclusions

The monothioate analogues of uridine 2′-,3′- and 5′-monophosphates are, under neutral and slightly acidic conditions, 200 to 300 times less stable than the corresponding monophosphates due to faster release of the thiometaphosphate monoanion compared to the metaphosphate ion. In contrast to 2′- and 3′-UMP, no mutual isomerization of 2′- and 3′-UMPS is detected under any conditions. However, under acidic conditions (pH < 1) acid-catalysed desulfurization of 2′- and 3′-UMPS to an equilibrium mixture of 2′- and 3′-UMP competes with dethiophosphorylation. The rate of this reaction is one quarter of that of the isomerization of 2′- and 3′-UMP.

Experimental

Materials

The preparation of the monothioates, (R_P)- and (S_P)-2′,3′-cUMPS (**4a**,**b**), and 2′- and 3′-UMPS (**3a**,**2a**), has been described previously.[9] 5′-UMPS (**1a**) was obtained by thiophosphorylation of uridine with thiophosphoryl chloride in trimethylphosphate, as described earlier.[25] The homogeneity of the product was verified by HPLC. The NMR spectra were recorded on a JEOL GX 400 spectrometer. $\delta_H(D_2O)$ 7.87 (1 H, d, *J* 8.1 Hz, H6), 5.79 (1 H, d, *J* 3.7 Hz, H1′), 5.75 (1 H, d, *J* 8.1 Hz, H5), 4.1–4.2 (3 H, m, H2′, 3′,4′), 4.04 (1 H, ddd, *J* 12.2, 4.9 and 1.7 Hz, H5′) and 3.95 (1 H, ddd, *J* 12.2, 6.8 and 2.4 Hz, H5″); $\delta_P(D_2O)$ 52.0 (from external phosphoric acid). Uridine, uridine monophosphates and uracil were products of Sigma and they were used as received after checking the purity by HPLC.

Kinetic measurements

Reactions were followed by the HPLC technique described previously.[9,26] The initial concentration of the starting material was *ca.* 10^{-4} mol dm^{-3}.

The rate constants reported refer to a buffer concentration of zero. The rate constants were determined at each pH at two different (relatively low) buffer concentrations. The effect of buffer concentration on the rate of dethiophosphorylation reactions was almost negligible (less than 10% at [buffer] < 0.05 mol dm^{-3}).

Calculation of the rate constants

The integrated peak areas of the HPLC chromatograms (UV-detection at 260 nm) were assumed to be proportional to the concentrations, since the UV absorbing moiety of all the compounds involved was the same (N1-substituted uracil). First-order rate constants for the hydrolytic decomposition of nucleoside phosphoromonothioates (k_{tot}) were obtained by applying the integrated first-order rate law to the disappearance of the starting material. These rate constants were then bisected to the rate constants of the parallel pseudo-first-order reactions [dethiophosphorylation (k_1) and desulfurization (k_2)] on the basis of product distribution at the early stages of the reaction.

Acknowledgements

The financial support of the Academy of Finland and Turku University Foundation is gratefully acknowledged.

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Paper 6/00090H
Received 4th *January* 1996
Accepted 23rd *January* 1996