Phosphoester Hydrolysis and Intramolecular Transesterification of Ribonucleoside 2′- and 3′-Phosphoromonothioate Triesters: Kinetics and Mechanisms for the Reactions of 5′-*O*-Methyluridine 2′- and 3′-Dimethylphosphoromonothioates

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The hydrolytic reactions of the monothioate analogs of 5′-*O*-methyluridine 2′- and 3′-dimethylphosphates have been followed over a wide acidity range, $H_0 = -1.7$ ([HCl] = 5 mol L^{-1}) to pH 9. Two reactions were found to compete: mutual interconversion of the 2′- and 3′-isomers and phosphoester hydrolysis to a mixture of phosphorothioate diesters, *viz.*, the R_P and S_P diastereomers of 2′,3′-cyclic thiophosphate and 2′/3′-monomethylthiophosphates (*i.e.*, three pairs of diastereomers). No marked desulfurization could be observed. The interconversion and hydrolysis both show first-order dependence of rate on acidity at pH < 0, the isomerization being 3−4 times as fast as the phosphoester hydrolysis. Under less acidic conditions, the hydrolysis remains pH-independent up to pH 7, while the isomerization becomes hydroxide-ion-catalyzed (first-order in [OH^-]) already at pH 2. The hydrolysis is susceptible to general base catalysis in carboxylic acid buffers, the Brönsted β value being 0.8. In contrast, no conclusive evidence for buffer-catalyzed isomerization could be obtained. All these reactions are suggested to proceed *via* a pentacoordinated thiophosphorane intermediate, obtained at pH < 1 by an attack of the neighboring hydroxy function on a protonated (monocationic) thiophosphate group and at pH > 2 by an attack of a deprotonated hydroxy function (oxyanion) on a neutral thiophosphate. The monocationic intermediate (pH < 1) may collapse to hydrolysis and isomerization products without further catalysis (departure of alcohol). The monoanionic thiophosphorane (pH > 2) also gives isomerization products without catalysis (departure of 2′/3′-oxyanion), whereas breakdown to the hydrolysis products needs either a specific or a general acid catalysis process (departure of methanol). Accordingly, the observed general-base-catalyzed hydrolysis most likely consists of consecutive specific base/general acid catalysis. The phosphorothioate triesters studied are, under very acidic conditions, more than 2 orders of magnitude more stable than their oxyphosphate counterparts, whereas the rate-retarding "thio effect" ($k_{P=O}/k_{P=S}$) is much smaller with the hydroxide ion-catalyzed reactions (*ca.* 4) and almost negligible with the pH-independent hydrolysis.

Introduction

Replacing one of the nonbridging oxygen atoms of the RNA phosphodiester bond with sulfur gives a phosphoromonothioate linkage that contains a chiral phosphorus center. This chirality has recently been exploited in mechanistic studies of ribozymes by inserting a phosphoromonothioate linkage in a selected position of the sugar−phosphate backbone of either the substrate chain or ribozyme itself.[1−12] The aim has been to study the stereochemical requirements of the ribozyme-catalyzed reactions,[1−5] to probe the metal ion-binding sites in ribozymes,[6−10] and to elucidate the nature of the rate-limiting step.[11] Phosphoromonothioates have also been used as stereochemical probes in mechanistic studies of RNases.[13,14] The so called "thio effect", *i.e.*, the ratio of the reaction rates of phosphate and thiophosphate esters, has been utilized in determining the rate-limiting step and distinguishing between kinetically equivalent pathways of RNase catalysis.[15]

Interpretation of the results of all the studies indicated above is eventually based on comparisons with the chemical behavior of an isolated phosphoromonothioate linkage, such as the one in dinucleoside 3′,5′-thiophosphates. To obtain a solid chemical basis for these comparisons, we have previously studied the kinetics and mechanisms of the hydrolytic reactions of the diastereomeric (R_P and S_P) phosphoromonothioate analogs of 3′,5′-uridylyluridine [3′,5′-Up(s)U][16] and their reaction products, *viz.*, uridine 2′,3′-cyclic phosphoromonothioate [2′,3′-cUMPS][17] and uridine 2′- and 3′-phosphoromonothioates.[18] Kinetics for the concurrent desulfurization and transesterification to 2′,5′-Up(s)U and 2′,3′-cUMPS have been determined over a wide acidity range. Under alkaline conditions (pH > 8) the situation is clearcut: transesterification to 2′,3′-

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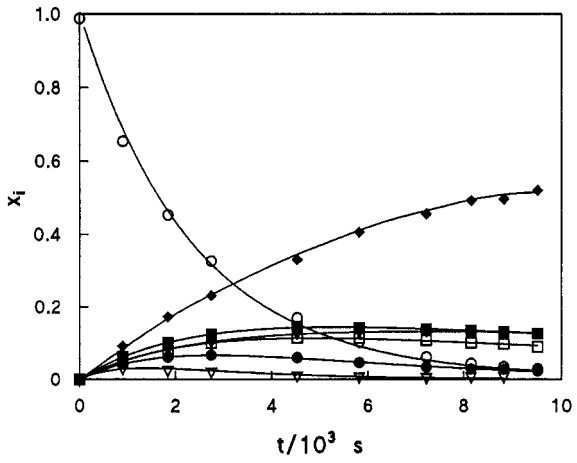

Figure 1. Time-dependent product distribution for the reactions of 5′-*O*-methyluridine 3′-dimethylphosphoromonothioate (**2b**) in 0.1 mol L^{-1} aqueous hydrogen chloride at 363.2 K (ionic strength adjusted to 1.0 mol L^{-1} with sodium chloride): **2b** (○), 2′-phosphorothioate triester **1b** (●), 2′,3′-cyclic phosphorothioates **3a**,**b** (△), 2′- and 3′-monomethylphosphoromonothioates **4a**,**b** (▼) and **5a**,**b** (□, ■), and equilibrium mixture of 2′/3′-monophosphates **6** and **7** (◆).

cUMPS is the only reaction detected.[16,19] The reaction leads to complete inversion and exhibits an almost negligible thio effect. At lower pH, the reactions most likely proceed *via* a thiophosphorane intermediate, as evidenced by the fact that a phosphate migration from 3′O to 2′O with retention of configuration is observed.[16] Under such conditions, desulfurization efficiently competes with the transesterifications. This to a large extent prevents firm mechanistic conclusions, since the competing pathways may lead to accumulation of a single product. In other words, transesterification to 2′,3′-cUMPS (which does not accumulate) cannot be strictly distinguished from desulfurization, and hence mechanistically relevant thio effects are not obtained.

To learn more about the mechanistic details and thio effects under neutral and acidic conditions, we now report a kinetic study on the hydrolytic reactions of 5′-*O*-methyluridine 2′- and 3′-dimethylphosphoromonothioates (**1b**, **2b**). These may be regarded as mimetics for a fixed ionic form of the corresponding diesters [2′,5′- and 3′,5′-Up(s)U], *viz.*, the one having the phosphorothioate OH ligand un-ionized. As far as we know, this is the first attempt to quantify the hydrolytic reactions of ribonucleoside 3′-phosphoromonothioate triesters.

Results

The hydrolysis and interconversion of 5′-*O*-methyluridine 2′- and 3′-dimethylphosphoromonothioates (**1b**, **2b**), obtained by deblocking the corresponding 3′/2′-*O*-tetrahydropyranyl derivatives **1a** and **2a** under acidic conditions, were followed over a wide pH range ($H_0 = -1.7$ to pH 9) by determining by RP HPLC the composition of the aliquots withdrawn at appropriate intervals from the reaction solution. The products formed were identified by spiking with authentic reference compounds. Figure 1 shows, as an illustrative example, the time-dependent product distribution observed at pH 1, when the 3′-isomer **2b** was used as the starting material. These data clearly show that two competing reactions take place as follows: (i) isomerization to the 2′-dimethylphosphoromonothioate **1b** and (ii) hydrolysis to a mixture of phosphorothioate diesters, *viz.*, the R_P and S_P diastereomers of 2′,3′-cyclic phosphoromonothioates **3a**,**b** and 2′- and 3′-monomethylphosphoromonothioates **4a**,**b** and **5a**,**b**. These reactions are depicted in Scheme 1. Additionally, 5′-*O*-methyluridine 2′- and 3′-monophosphates (**6**, **7**) were observed to accumulate. As shown previously[16,17] in detail, these compounds are formed by consecutive desulfurization and hydrolysis of the phosphoromonothioate diesters **3**−**5**. However, the question remains whether they are formed solely *via* **3**−**5**. One might also speculate that **6** and **7** are produced *via* desulfurization of **1b** and **2b** to 5′-*O*-methyluridine 2′- and 3′-dimethylphosphates (**8**, **9**) and their subsequent hydrolysis. Although the occurrence of this pathway under acidic conditions cannot be strictly excluded, in all likelihood it is of minor importance, representing less than 20% of the total disappearance of **1b** and **2b**, if any. This conclusion is based on the following facts. Firstly, no sign of intermediary accumulation of **8** or **9** was detected. As discussed in more detail below, the rate of hydrolysis of **8** and **9** at pH 1 is only 1 order of magnitude faster than that of **1b** and **2b**. Accordingly, if desulfurization of **1b** and **2b** would appreciably compete with their hydrolysis to **3**−**5**, intermediary accumulation of **8** and **9** should be detectable. Secondly, the rate constants for the decomposition of the phosphoromonothioate diesters **3**−**5** may be estimated from the data measured for the closely related diesters.[16,17] These data enable one to estimate, by the rate equation of parallel consecutive first-order reactions,[20] to what extent **3**−**5** should accumulate, assuming that no marked side reaction occurs. The values obtained agreed with the experimental observations within the limits of experimental errors. Accordingly, the routes *via* **3**−**5** must strongly predominate.

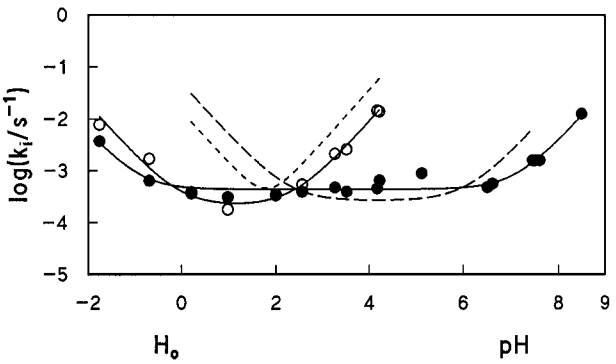

Figure 2. pH−rate profiles for the interconversion (○) and hydrolysis (●) of 5′-*O*-methyluridine 2′- and 3′-dimethylphosphoromonothioates (**1b**, **2b**) at 363.2 K (I = 1.0 mol L^{-1} with sodium chloride). The dotted lines show the corresponding curves for the reactions of 5′-*O*-methyluridine 2′/3′-dimethyl phosphates.[21]

Under less acidic conditions (pH > 2), the product distribution is much simpler than that depicted in Figure 1 and discussed above; in addition to the isomerized starting material, only the R_P and S_P diastereomers of 2′- and 3′-monomethylphosphoromonothioates **4a**,**b** and **5a**,**b** were observed to accumulate. No desulfurized products were observed. It may also be noted that the 2′,3′-cyclic thiophosphates **3a**,**b** did not accumulate as products of the pH-independent or alkaline hydrolysis.

Figure 2 shows the pH−rate profile for the interconversion and hydrolysis of **1b** and **2b**. All the experimen-

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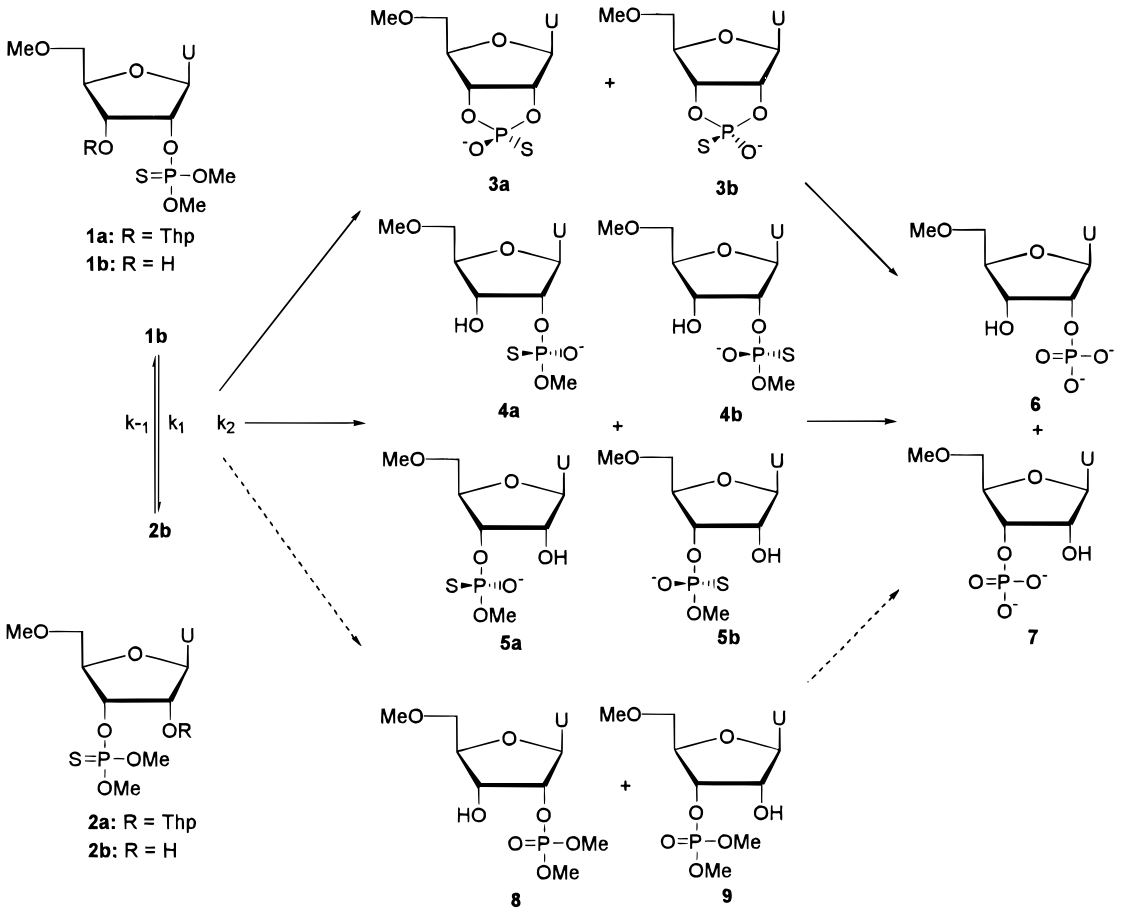
Scheme 1



tal points indicated refer to buffer concentration zero. As seen, the hydrolysis catalyzed by the solvent species (H_2O, H_3O^+, OH^-) is nearly pH-independent over a wide pH range from pH 0 to 7, becoming hydronium-ion-catalyzed at pH < 0 and hydroxide-ion-catalyzed at pH > 7. Both the hydronium- and hydroxide-ion-catalyzed reactions are first-order in the catalyst concentration. The interconversion of **1b** and **2b** is, in turn, first-order in acidity at pH < 0.5, and under very acidic conditions it is about 3.6 times as fast as the hydrolysis. At pH 1, the isomerization rate passes through a broad minimum and already becomes first-order in hydroxide ion concentration at pH > 2, in striking contrast to the hydrolysis reaction. Accordingly, the pH−rate profile for the interconversion may be expressed by eq 1, and that for the hydrolysis by eq 2. In these equations, k_1, k_{-1}, and k_2

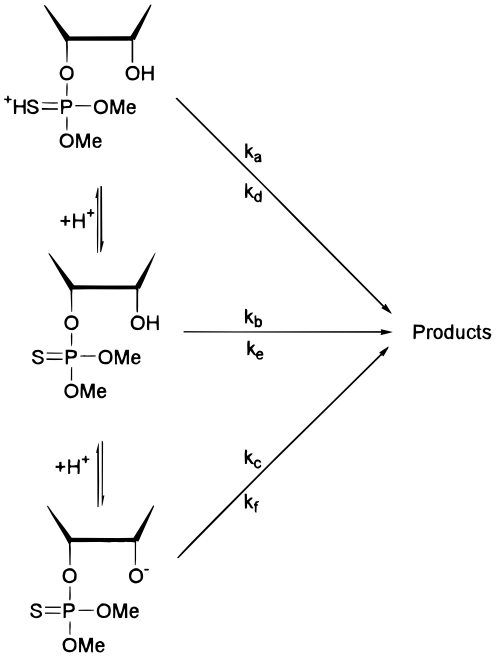
$$k_1 + k_{-1} = k_a[H^+] + k_b + k_c(K_w/[H^+]) \qquad (1)$$

$$k_2 = k_d[H^+] + k_e + k_f(K_w/[H^+]) \qquad (2)$$

are the observed pseudo-first-order rate constants for the interconversion (k_1, k_{-1}) and hydrolysis (k_2). K_w is the ionic product of water under the experimental conditions, and k_a−k_f are the adjustable parameters, *i.e.*, the rate constants referring to various ionic forms (see Scheme 2). The lines indicated in Figure 2 were obtained by least-squares fitting to eqs 1 and 2. The values obtained for the partial rate constants of the interconversion were $k_a = 1.9 \times 10^{-4}$ M^{-1} s^{-1}, $k_b = 2.1 \times 10^{-4}$ s^{-1}, and $k_c = 1.2 \times 10^6$ M^{-1} s^{-1}. The corresponding values for the hydrolysis were $k_d = 0.53 \times 10^{-4}$ M^{-1} s^{-1}, $k_e = 4.4 \times 10^{-4}$ s^{-1}, and $k_f = 51$ M^{-1} s^{-1}.

Table 1 contains the pseudo-first-order rate constants for the interconversion and hydrolysis of **1b** and **2b** in

Scheme 2



buffer solutions. As can be seen, the hydrolysis (k_2) is clearly a buffer-catalyzed reaction. With each of the buffers studied, the catalytic rate constant, k_{cat} (for definition, see eq 3), obtained at [HA]/[A^-] = 1/3 (or

$$k_2 = k_{cat}([HA]+[A^-]) = k_{HA}[HA] + k_{A-}[A^-] \qquad (3)$$

[BH^+]/[B] = 1/3) is approximately 3-fold greater compared to that obtained at [HA]/[A^-] = 3/1 (or [BH^+]/[B] = 3/1).

Table 1. First-Order Rate Constants for the Hydrolysis (k_2) and Interconversion ($k_1 + k_{-1}$) of 5′-*O*-Methyluridine 2′- and 3′-Dimethylphosphoromonothioates (1b, 2b) in Aqueous Buffer Solutions at 363.2 K[a]

buffer acid	[HA] (mol L^{-1})	[A^-] (mol L^{-1})	k_2 (10^{-3} s^{-1})	$k_1 + k_{-1}$ (10^{-1} s^{-1})
chloroacetic acid	0.0375	0.0125	0.408 (12)[b]	0.558 (9)[b]
	0.075	0.025	0.409 (9)	0.558 (9)
	0.225	0.075	0.495 (20)	0.498 (15)
	0.375	0.125	0.501 (5)	0.520 (12)
	0.0125	0.0375	0.472 (6)	2.80 (7)
	0.025	0.075	0.589 (5)	2.47 (6)
	0.075	0.225	0.992 (18)	3.12 (7)
	0.125	0.375	1.33 (2)	3.21 (10)
formic acid	0.0375	0.0125	0.581 (9)	2.22 (2)
	0.075	0.025	0.78 (10)	2.35 (5)
	0.225	0.075	1.45 (3)	2.72 (7)
	0.375	0.125	1.92 (2)	3.18 (12)
	0.0125	0.0375	0.941 (20)[d]	14.9 (9)
	0.025	0.075	1.75 (2)	15.6 (9)
	0.075	0.225	4.03 (4)[e]	19.2 (2)
	0.125	0.375	5.54 (7)[f]	21.8 (5)
acetic acid	0.0375	0.0125	0.764 (14)	14.8 (2)
	0.075	0.025	1.35 (2)	16.1 (2)
	0.225	0.075	2.62 (4)	18.7 (8)
	0.375	0.125	4.30 (3)	20.6 (6)
	0.0125	0.0375	1.97 (2)	
	0.025	0.075	3.60 (3)	
	0.075	0.225	9.05 (6)	
	0.125	0.375	13.8 (3)	
triethanolammonium ion	0.0375	0.0125	0.601 (13)	
	0.075	0.025	0.731 (11)	
	0.225	0.075	1.19 (2)	
	0.375	0.125	1.69 (2)	
	0.0125	0.0375	1.87 (2)	
	0.025	0.075	2.36 (2)[g]	
	0.075	0.225	3.72 (2)[h]	
	0.125	0.375	5.13 (5)	
triethanolammonium ion[c]	0.0375	0.0125	0.007	
	0.075	0.025	0.008	
	0.225	0.075	0.014	
	0.375	0.125	0.018	
	0.0125	0.0375	0.019	
	0.025	0.075	0.023	
	0.075	0.225	0.042	
	0.125	0.375	0.044	
(*N*-methylamino)acetic acid[c]	0.0375	0.0125	0.408 (22)	
	0.075	0.025	0.72 (9)	
	0.225	0.075	1.78 (5)	
	0.375	0.125	3.22 (6)	
	0.0125	0.0375	1.58 (4)	
	0.025	0.075	2.05 (12)	
	0.075	0.225	6.48 (10)	
	0.125	0.375	10.0 (2)	

[a] The ionic strength was adjusted at 1.0 mol L^{-1} with sodium chloride. [b] In parentheses is given the standard deviation of the mean (for 10−12 samples) affecting the last one or two figures. [c] At 298.2 K. [d] For the phosphate triester analog **9**: $k_2 = 0.983 \times 10^{-3}$ s^{-1}. [e] For **9**: $k_2 = 3.81 \times 10^{-3}$ s^{-1}. [f] For **9**: $k_2 = 6.47 \times 10^{-3}$ s^{-1}. [g] For **9**: $k_2 = 9.79 \times 10^{-3}$ s^{-1}. [h] For **9**: $k_2 = 17.9 \times 10^{-3}$ s^{-1}.

Moreover, the catalytic efficiency of the buffer increases with the increasing basicity of the buffer base (A^- or B). Accordingly, the hydrolysis reaction is undoubtedly susceptible to general base catalysis. The catalytic rate constants, k_A-, obtained with various buffer bases are listed in Table 2. Application of the Brönsted catalysis law to the data referring to carboxylate ions gave a β value of 0.8. The points referring to amine catalysis fall markedly below this correlation line.

While the hydrolysis undoubtedly is a general-base-catalyzed reaction, the situation with the interconversion is much more obscure. In most buffers, a modest rate enhancement as a function of buffer concentration is observed. However, this rate enhancement does not seem to depend on either the buffer ratio or the acidity of the buffer constituents. A 20−25% rate enhancement is always observed on going from buffer concentration zero to 0.5 mol L^{-1}. This kind of a rate enhancement may result from medium effects and cannot be taken as a conclusive evidence for buffer catalysis.

Table 2. Catalytic Rate Constants for the Buffer-Catalyzed Hydrolysis of 5′-*O*-Methyluridine 2′/3′-dimethylphosphoromonothioates at 363.2 K[a]

buffer acid	$k_{A-}/10^{-4}$ M^{-1} s^{-1} [b]
chloroacetic acid	27.3
formic acid	137
acetic acid	354
triethanolammonium ion	94.8
	12.8[c]
(*N*-methylamino)acetic acid	260[c]

[a] The ionic strength of the solutions was adjusted to 1.0 mol L^{-1} with sodium chloride. [b] Equation 3 always gave for k_{HA} a small negative value, which within the limits of experimental errors does not deviate from zero. [c] At 298.2 K.

Discussion

Mechanisms of Hydrolysis and Migration of Phosphoromonothioate Triesters. Figure 2 shows the pH−rate profiles of the phosphoester hydrolysis and interconversion of the phosphoromonothioate triesters **1b** and **2b** at 363.2 K. For comparison, the corresponding curves for the 2′/3′-dimethylphosphate analogs[21] **8** and **9** are shown in the same figure. As seen, the characteristic features of the pH dependence are similar with phosphotriesters **8** and **9** and their monothioate analogs **1b** and **2b**. Most likely, the thio substitution does not change the basic mechanisms of the reactions. However, some differences in the kinetics occur. The pH-independent phosphoester hydrolysis (at pH 0−6 with **1b** and **2b** and at pH 3−6 with **8** and **9**) is about as fast with both compounds, but the hydroxide-ion-catalyzed reactions, both the hydrolysis and migration, are slower with the thioates than with their phosphate analogs ($k_{PO}/k_{PS} \sim 4$). Under very acidic conditions ($H_0 < 0$), the rate-retarding thio effect is considerably greater, more than 2 orders of magnitude for the rate of triester hydrolysis and about 50 for the rate of phosphate migration. Accordingly, the reactions of thioates first become acid-catalyzed at 2 units lower pH than those of phosphotriesters. Another noticeable difference is that with **8** and **9** the phosphoester hydrolysis predominates in very acidic solutions, while with the thioate analogs the acid-catalyzed thiophosphate migration is 3−4-fold faster than the hydrolysis.

Both reactions, *i.e.*, hydrolysis and interconversion, of nucleoside 2′- and 3′-dimethylphosphates **8** and **9** have been suggested[22] to proceed *via* a common pentacoordinated phosphorane intermediate, which is formed either by an attack of the neighboring hydroxy group on the protonated phosphotriester moiety (acid-catalyzed reactions) or by an attack of the deprotonated hydroxy function on the neutral phosphotriester (base-catalyzed and pH-independent reactions). The marked similarity of the pH−rate profiles suggests that the same mechanisms also operate with the dimethylphosphoromonothioates **1b** and **2b** (Schemes 3 and 4). The most

(21) The pH−rate profiles reported[22a] for the phosphate triesters, 5′-*O*-pivaloyluridine 2′/3′-dimethylphosphates, at 298.2 K were extrapolated to 363.2 K on the basis of comparative measurements carried out with **9** at pH 0.2, 4.2, and 7.4 at 363.2 K.

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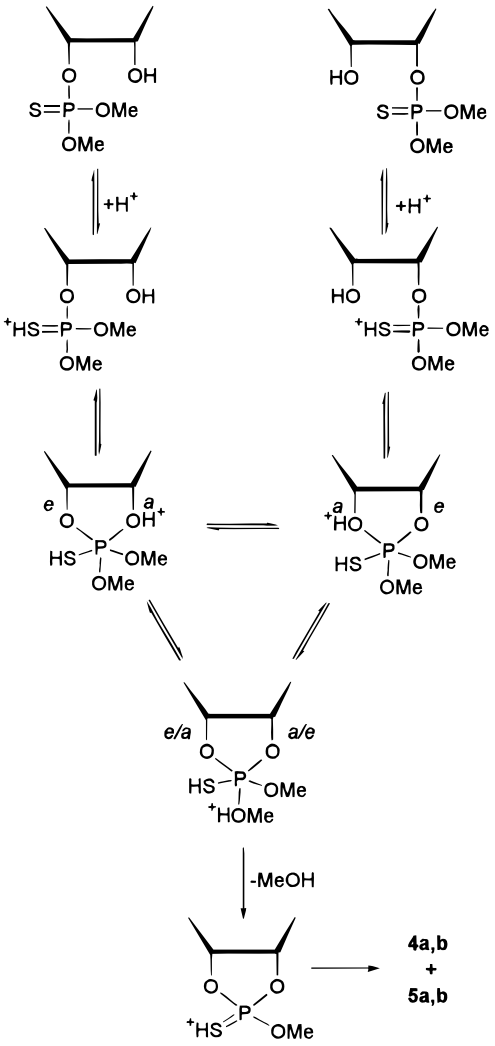
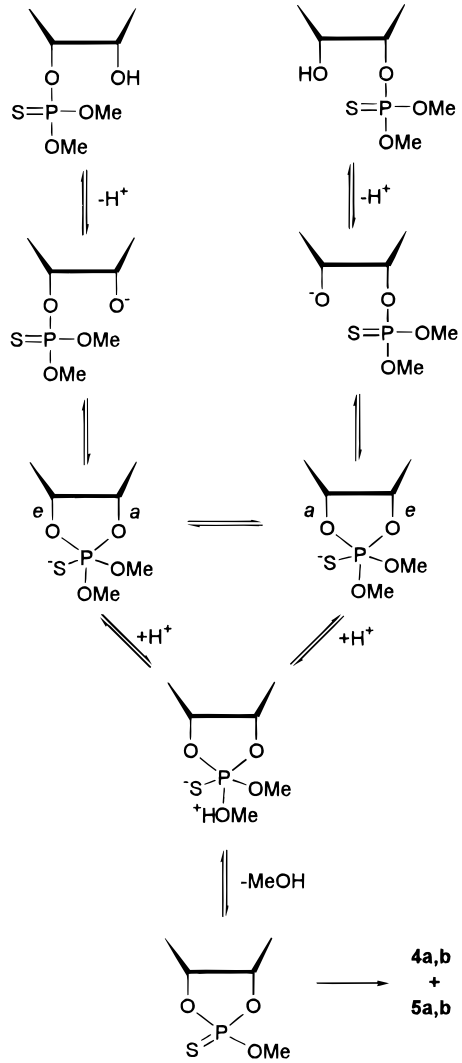
Scheme 3



Scheme 4



unexpected feature of both rate profiles is that the hydrolysis remains pH-independent under conditions where the migration becomes hydroxide-ion-catalyzed, *i.e.*, at pH 0−6 with **1b** and **2b**. Moreover, the hydrolysis, but not the phosphate migration, is susceptible to general base catalysis (Table 1). The striking difference in the reaction order with respect to hydronium ion concentration may be explained as suggested previously[21] for **8** and **9**: the thiophosphorane intermediate is formed even under acidic conditions by an attack of deprotonated neighboring hydroxy group (*i.e.*, an oxyanion) on phosphorus, and either the 2′- or 3′-oxygen ligand departs as an alkoxide ion. By contrast, the departure of a methoxy ligand requires protonation, either by hydronium ion or by an acidic buffer constituent. In other words, the migration is a specific-base-catalyzed reaction that is not susceptible to buffer catalysis. The hydrolysis, in turn, involves specific-base-catalyzed formation of the phosphorane followed by general- or specific-acid-catalyzed breakdown to the hydrolysis products (Scheme 4). Alternatively, one might speculate that the thiophosphorane is formed by an attack of un-ionized hydroxy function on neutral phosphate, and the base catalysis observed with the migration results from deprotonation of thiophosphorane, which would accelerate its pseudorotation, as suggested by *ab initio* calculations on PF_4OH.[23] In other words, the rate-limiting step of migration would be specific-base-catalyzed pseudorotation, while the hydrolysis would proceed by specific-base-catalyzed deprotonation of the thiophosphorane followed by the specific- and/or general-acid-catalyzed rate-limiting departure of methanol. Although this alternative cannot be strictly excluded, it appears less attractive for the following reason: The predominant ionic form of the thiophosphorane under acidic conditions is the neutral species (the first pK_a value of the oxyphosphorane is estimated[24] to be 9). Accordingly, first-order dependence of migration rate on hydroxide ion concentration could be observed only if the pseudorotation of neutral thiophosphorane would be several orders of magnitude slower than that of its monoanion. This is unlikely. Thiophosphate migration also takes place under very acidic conditions (pH < 0), as rapidly as at pH 2−5, where the specific base catalysis is observed. The acid-catalyzed reaction is first-order in hydronium ion concentration, reflecting in all likelihood rapid initial protonation of the thiophosphate (Scheme 3). Evidently the pseudorotation step does not require base catalysis but may proceed *via* a neutral thiophosphorane. This means that the pseudorotation of the neutral thiophosphorane cannot be exceedingly slow compared to that of the monoanion, and hence the mechanism depicted in Scheme 4 appears more likely.

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Assuming the mechanism suggested above (Scheme 4) is followed, sugar 2′- and 3′-oxyanions leave the thiophosphorane intermediate 5 orders of magnitude more rapidly than does methoxide ion. Sugar hydroxyl groups are more acidic than methanol, but the acidity difference is only 3 pK_a units[25] and hence probably too small to completely explain the observed reactivity difference. The initial product of hydrolysis is a cyclic phosphotriester that is undoubtedly rather strained. However, as discussed earlier,[21] the ring strain of this intermediate is hardly of crucial importance, since under acidic conditions the hydrolysis effectively competes with the migration. It may be more relevant to consider the stabilization of the two alternative transition states by the groups that remain bonded to the phosphorus. When one of the thiophosphate alkoxy ligands departs as an oxyanion, the phosphorus atom becomes more electron deficient, and this deficiency is compensated for by electron donation from the remaining ligands. Since a methyl group is less electron-withdrawing than a 2′/3′-*O*-bonded nucleoside, one might speculate that formation of an acyclic triester bearing two methyl groups is favored compared to formation of a cyclic triester. This effect is parallel to the leaving group effect and may still strengthen it. One may also ask why the breakdown of the thiophosphorane to hydrolysis products is susceptible to general acid catalysis, whereas the depature of 2′/3′-oxyanions appears to be uncatalyzed. Tentatively one may argue that since methoxide ion is more basic than the sugar oxyanion, general acid catalysis occurs when it is needed most: to stabilize the most basic of the leaving groups by protonation. It is also worth noting that while the departure of methanol is clearly the rate-limiting step of hydrolysis, the situation is different with migration. The free energy profile for the migration is rather symmetrical. In other words, the transition states for the formation and breakdown of thiophosphorane to the isomeric starting material are approximately at the same energy level, and hence the breakdown of thiophosphorane to the migration product cannot alone be rate-limiting.

Desulfurization. The phosphoromonothioate triesters **1b** and **2b** do not undergo desulfurization to the corresponding phosphate triesters **8** and **9**. This is in striking contrast to the results obtained[16] with the phosphoromonothioate diesters. With the latter, desulfurization is the main reaction at pH 3−7, and it efficiently competes with phosphoester hydrolysis and migration under more acidic conditions. This difference in behavior is consistent with the previous[16] conclusion, according to which desulfurization is a hydrolytic reaction proceeding *via* the thiophosphorane intermediate, not a redox process. The thiophosphorane derived from a phosphoromonothioate diester may decompose to a cyclic phosphotriester by desulfurization, whereas the same reaction with phosphoromonothioate triesters would give a tetraalkoxy-substituted phosphorus compound as the initial product. Evidently the formation of this kind of an intermediate is unfavorable.

Thio Effect. The hydroxide-ion-catalyzed hydrolysis and migration of the phosphoromonothioate triesters **1b** and **2b** show a thio effect of $k_{PO}/k_{PS} \sim 4$. The thio effect is thus considerably smaller than that reported for the alkaline hydrolysis of simple phosphotriesters, proceeding by an intermolecular attack of hydroxide ion, *viz.*, 12 and 30 for the hydrolysis of triethyl phosphate[26] and alkyl,*p*-nitrophenyl phosphotriesters,[27] respectively. Herschlag *et al.*[11] have suggested that the magnitude of the rate-retarding thio effect correlates with the associative nature of the reaction. Accordingly, the hydrolysis and interconversion of ribonucleoside 2′/3′-phosphotriesters would proceed *via* a somewhat less stable pentacoordinated intermediate than the alkaline hydrolysis of simple phosphotriesters. With phosphodiesters being hydrolyzed *via* a dianionic pentacoordinated transition state, rather than phosphorane intermediates, the thio effects are usually still smaller: 4 with methyl 2,4-dinitrophenyl phosphate,[11] 1.7−2.5 with various aryl esters of uridine 3′-thiophosphates,[28] 1.4 and 2.1 with uridine S_P- and R_P-2′,3′-cyclic phosphoromonothioates,[17] and close to unity with dinucleoside phosphoromonothioates[16,19] and corresponding oligomers.[11] In summary, the results of the present paper and those reported previously[16,19] for phosphoromonothioate diesters reveal that the thio effects referring to the attack of a neighboring oxyanion on either a monoanionic phosphodiester center or a neutral phosphotriester center differ by only a factor of 3−5 (depending on stereochemistry at phosphorus). Hence, as also noted by Herschlag,[15] care should be exercised on interpreting enzyme mechanisms on this basis.

Under acidic conditions (pH < 1), the phosphorothioate triesters **1b** and **2b** are hydrolyzed and isomerized considerably less readily than their phosphate analogs. We have shown previously[16,17] that thio substitution markedly stabilizes phosphodiesters toward acid-catalyzed hydrolysis and transesterification. The thio effect was observed to be pH-dependent: close to unity at pH 4 and up to 100 in 1 mol L^{-1} aqueous hydrogen chloride. Accurate values for the thio effects could not be obtained, owing to competing desulfurization. Moreover, it was impossible to assign the observed thio effect to a reaction of any particular ionic form, since the phosphorothioates react predominantly *via* a neutral ionic form under conditions where the phosphodiesters already react *via* a monocationic form. The results of the present study help, at least partially, to overcome these problems. Hydrolysis and interconversion of both **1b** and **2b** and their oxygen analogs **8** and **9** show first-order dependence on hydronium ion concentration, and hence the second-order rate constants may be directly compared. These comparisons give thio effects of 300 and 50 for the acid-catalyzed hydrolysis and isomerization, respectively. To a very large extent these thio effects undoubtedly result from more difficult protonation of phosphorothioate triesters compared to phosphotriesters, since the reactions of **1b** and **2b** become acid-catalyzed at 2 units lower pH than those of **8** and **9**. To what extent the thio effects originate from reduced (or increased) electrophilicity of the protonated thiophosphate triester compared to the protonated oxyphosphate cannot be accurately estimated. However, in all likelihood, this factor is of minor importance. The markedly higher acidity of the conjugate acids of **1b** and **2b** over **8** and **9** is expected, since the protonation probably takes place at sulfur rather than at oxygen; the higher acidity of sulfhydryl groups compared to hydroxy groups is a generally known fact. The

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values indicated above may presumably be regarded as the limiting values for the thio effect of phosphodiester reactions, in other words, the values that the thio effect would reach under conditions where the reactive ionic form of both phosphoromonothioates and their oxygen analogs is the monocationic phosphodiester. Evidently, this kind of a situation is only reached in solutions of strong acids at concentrations of several molar. Under less acidic conditions, the thio effect gradually diminishes toward unity.

Experimental Section

Methods. The NMR spectra were recorded on a Jeol JNMA 500 spectrometer. The ^{1}H NMR chemical shifts (500 MHz, 300 K) were referred to internal TMS and ^{31}P NMR shifts (202 MHz, 300 K) to external orthophosphoric acid.

5′-*O*-Methyl-3′-*O*-(2-tetrahydropyranyl)uridine 2′-dimethylphosphoromonothioate (1a) and 5′-*O*-methyl-2′-*O*-(2-tetrahydropyranyl)uridine 3′-dimethylphosphoromonothioate (2a). 5′-*O*-Methyl-2′-*O*-(2-tetrahydropyranyl)-uridine and 5′-*O*-methyl-3′-*O*-(2-tetrahydropyranyl)uridine were obtained as an isomeric mixture by treating 5′-*O*-methyluridine[29] with 1 equiv of 2,3-dihydro-4*H*-pyran in acidic acetonitrile, as described previously in detail.[22b] The mixture was separated into pure diastereomers of both compounds by RP chromatography on a Lobar RP-18 column (37 × 440 mm, 40−63 μm), using aqueous acetonitrile (15%, v/v) as an eluent. The faster eluted diastereomers of both the 2′- and 3′-*O*-tetrahydropyranyl derivatives were thiophosphorylated with thiophosphoryltris(triazole) in dry acetonitrile, and the remaining triazole ligands were displaced by treatment with excess of methanol, analogously to the method described earlier[22b] for the phosphotriester analogs **8** and **9**.

1,2,4-Triazole (330 mg, 4.8 mmol) and triethylamine (670 μL, 4.8 mmol) were dissolved in dry acetonitile (10 mL), and thiophosphoryl chloride (160 μL, 1.6 mmol) was added. After 45 min of stirring, the mixture was filtered onto the nucleoside (280 mg, 0.8 mmol), predried by coevaporations with dry pyridine. The reaction mixture was left to stand overnight, after which the completeness of the reaction was confirmed by TLC analysis on silica gel 60 ($CHCl_3$/MeOH, 9/1, v/v). The remaining triazole ligands were then replaced by treatment with an excess of methanol (30 mL). After 12 h of stirring at room temperature, the mixture was evaporated to dryness. The residue was dissolved in dichloromethane, washed with phosphate buffer (0.1 mol L^{-1}, pH 7), and dried with $MgSO_4$. Silica gel chromatography ($CHCl_3$/MeOH, 50/1, v/v) gave **1a** and **2a** in 28% and 29% yields, respectively. The homogeneity of the products was verified by HPLC on a Hypersil ODS 5 column (see Kinetic Measurements). The site of the dimethylthiophosphate group was assigned by heteronuclear ^{1}H,^{31}P NMR couplings, after verification of the identity of H2′ and H3′ signals of **1a** and **2a** by irradiation at the frequency of the resonance of the anomeric proton.

1a: ^{1}H NMR (C^2HCl_3) δ 7.86 (s, 1H), 7.77 (d, 1H, J = 8.1 Hz), 6.13 (d, 1H, J = 4.7 Hz), 5.74 (dd, 1H, J_1 = 8.2 Hz, J_2 = 2.4 Hz), 4.99 (ddd, 1H, J_1 = 12.2 Hz, J_2 = 5.0 Hz, J_3 = 5.0 Hz), 4.82 (m, 1H), 4.40 (t, 1H, J = 4.9 Hz), 4.27 (m, 1H), 3.79 (m, 1H), 3.75 (d, 3H, J = 13.6 Hz), 3.74 (dd, 1H, shielded), 3.73 (d, 3H, J = 13.7 Hz), 3.58 (dd, 1H, J_1 = 10.8 Hz, J_2 = 2.3 Hz), 3.54 (m, 1H), 3.45 (s, 3H), 1.9−1.4 (m, 8H); ^{31}P NMR (C^2HCl_3) δ 69.2; FAB$^+$-MS: 467.

2a: ^{1}H NMR (C^2HCl_3) δ 7.98 (s, 1H), 7.68 (d, 1H, J = 8.2 Hz), 6.21 (d, 1H, J = 7.3 Hz), 5.75 (dd, 1H, J_1 = 8.2 Hz, J_2 = 2.4 Hz), 5.03 (ddd, 1H, J_1 = 11.6 Hz, J_2 = 5.2 Hz, J_3 = 1.4 Hz), 4.77 (m, 1H), 4.32 (m, 1H), 3.79 (d, 3H, J = 13.4 Hz), 3.77 (d, 3H, J = 13.7 Hz), 3.65 (dd, 1H, J_1 = 10.7 Hz, J_2 = 2.4 Hz), 3.58 (dd, 1H, J_1 = 10.7 Hz, J_2 = 2.4 Hz, partly shielded), 3.56 (m, 1H, shielded), 3.44 (s, 3H), 3.42 (m, 1H), 1.8−1.4 (m, 6H); ^{31}P NMR (C^2HCl_3) δ 68.3; FAB$^+$-MS 467.

To identify the hydrolysis products of 5′-*O*-methyluridine 2′- and 3′-dimethylphosphoromonothioates (**1b**, **2b**), the following reference compounds were prepared. The R_P and S_P diasteromers of 5′-*O*-methyluridine 2′,3′-cyclic phosphoromonothioate (**3a**,**b**) were obtained by thiophosphorylation of 5′-*O*-methyluridine by the method described previously for 5′-*O*-dimethoxytrityluridine.[16] With the exception of the 5′-*O*-methyl signal at 3.2 ppm (in ^2H_2O), the ^{1}H NMR spectra of **3a**,**b** were practically identical with those reported for their nonmethylated analogs[1] and commercial uridine 2′,3′-cyclic monophosphate. Alkaline hydrolysis of **3a** or **3b** gives an equimolar mixture of 5′-*O*-methyluridine 2′- and 3′-monothiomonophosphates.[16,17] The preparation of 5′-*O*-methyluridine 2′- and 3′-dimethylmonophosphates (**8**, **9**) and their hydrolysis products (2′/3′-monomethylphosphates, 2′,3′-cyclic monophosphate, and 2′- and 3′-monophosphates) has been described.[22b]

The products of alkaline hydrolysis of **1b** and **2b**, tentatively assigned as the R_P and S_P diastereomers of 5′-*O*-methyluridine 2′- and 3′-monomethylphosphoromonothioates (**4a**,**b** and **5a**,**b**), were isolated by RP HPLC on a semipreparative LiChrospher RP 18 column (10 × 250 mm, 5 μm) and characterized by ^{1}H and ^{31}P NMR spectrometry. No attempts were made to assign the absolute configuration of the diastereomers. The indicative resonance in the ^{1}H NMR of each isomer was the three-proton doublet of the phosphate methyl protons (J = 12.8 −13.0 Hz) at 3.3−3.4 ppm (in DMSO-d_6). The spectra were nearly identical with those earlier recorded[22b] for 5′-*O*-methyluridine 2′- and 3′-monomethylphosphates (**8**, **9**). According to this comparison, the two isomers migrating fastest on the RP column were assigned as the diastereomeric 2′-monomethyl phosphoromonothioates (**4a**,**b**) and the two slower migrating ones as their 3′-esterified isomers **5a**,**b**. The ^{31}P NMR chemical shifts for the compounds, in the order of decreasing mobility on the RP column, were 60.3, 56.4, 56.8, and 57.5 ppm.

Kinetic Measurements. The reactions were carried out in sealed tubes immersed in a thermostated water bath, the temperature of which was adjusted to the desired within ±0.1 K. Before initiating the reaction, the protected starting compound (**1a** or **2a**) was first treated at room temperature for 80 min with 50 μL of 0.1 M aqueous hydrogen chloride, to remove the tetrahydropyranyl protecting group. The acidic solution was evaporated to dryness and the residue dissolved in methanol (25 μL). To start a kinetic run, this methanol solution was added to the prethermostated reaction buffer. The initial substrate concentration was *ca.* 2 × 10^{-4} mol L^{-1}. The hydronium ion concentrations of the buffer solutions were calculated from the literature data of the pK_a values of the buffer acids under the experimental conditions.[30]

The hydrolytic reactions were followed by RP HPLC (UV detection at 260 nm), by analyzing the composition of the aliquots withdrawn from the reaction solution at appropriate time intervals. The separations were carried out on a Hypersil ODS 5 column (4 × 250 mm, 5 μm). Mixtures of acetonitrile and a formic acid/sodium formate buffer (0.045/0.015 mol L^{-1}), containing 0.1 mol L^{-1} tetramethylammonium chloride, were employed as eluents. A good separation of the product mixture (Scheme 1) was obtained, when a 15 min isocratic elution with a 2.5% content of MeCN was followed by a linear gradient (10 min) up to 30% MeCN (flow rate 1 mL min^{-1}). The integrated peak areas were assumed to be proportional to the relative concentrations, while the chromophoric base moiety of all the compounds was the same (*N*1-substituted uracil).

Calculation of the Rate Constants. The first-order rate constants (k_1 + k_{-1}) for the mutual isomerization of **1b** and **2b** were calculated by eq 4, where x_t and x_e represent the mole fraction of the starting isomer in the isomeric mixture at moment t and at equilibrium, respectively. A two-parameter

$$x_t = (1 - x_e)\exp[-(k_1 + k_{-1})t] + x_e \qquad (4)$$

least-squares fitting of the observed values of x_t and t to eq 4

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was used to obtain both $k_1 + k_{-1}$ and x_e. The first-order rate constants (k_2) for the phosphoester hydrolysis of **1b** and **2b** were obtained by applying the integrated first-order rate equation to the diminution of the total peak area of the isomeric mixture.

Application of these rate equations requires the assumption that both components of the isomeric mixture (**1b** and **2b**) are hydrolyzed to diesters at an equal rate. This assumption appears justified, since the diminution of the isomeric mixture strictly obeyed first-order kinetics from the very beginning of the reaction, *i.e.*, even when the isomerization had not yet settled to the equilibrium. The rate constant obtained was also independent of the choice of the isomer used as starting material. These criteria could be applied, of course, only for reactions carried out at pH < 4, where the rates of both the reactions are comparable. Under more alkaline conditions, the isomerization becomes so much faster than hydrolysis that the rate constants could be determined only for hydrolysis of the equilibrium mixture.

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