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Thio Effects on the Departure of the 3′-Linked Ribonucleoside from Diribonucleoside 3′,3′-Phosphorodithioate Diesters and Triribonucleoside 3′,3′,5′-Phosphoromonothioate Triesters: Implications for Ribozyme Catalysis

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Abstract: To provide a solid chemical basis for the mechanistic interpretations of the thio effects observed for large ribozymes, the cleavage of triribonucleoside 3′,3′,5′-phosphoromonothioate triesters and diribonucleoside 3′,3′-phosphorodithioate diesters has been studied. To elucidate the role of the neighboring hydroxy group of the departing 3′-linked nucleoside, hydrolysis of 2′,3′-*O*-methyleneadenosin-5′-yl bis[5′-*O*-methyluridin-3′-yl] phosphoromonothioate (**1a**) has been compared to the hydrolysis of 2′,3′-*O*-methyleneadenosin-5′-yl 5′-*O*-methyluridin-3′-yl 2′,5′-di-*O*-methyluridin-3′-yl phosphoromonothioate (**1b**) and the hydrolysis of bis[uridin-3′-yl] phosphorodithioate (**2a**) to the hydrolysis of uridin-3′-yl 2′,5′-di-*O*-methyluridin-3′-yl phosphorodithioate (**2b**). The reactions have been followed by RP HPLC over a wide pH range. The phosphoromonothioate triesters **1a**,**b** undergo two competing reactions: the starting material is cleaved to a mixture of 3′,3′- and 3′,5′-diesters, and isomerized to 2′,3′,5′- and 2′,2′,5′-triesters. With phosphorodithioate diesters **2a**,**b**, hydroxide-ion-catalyzed cleavage of the P–O3′ bond is the only reaction detected at pH >6, but under more acidic conditions desulfurization starts to compete with the cleavage. The 3′,3′-diesters do not undergo isomerization. The hydroxide-ion-catalyzed cleavage reaction with both **1a** and **2a** is 27 times as fast as that compared with their 2′-*O*-methylated counterparts **1b** and **2b**. The hydroxide-ion-catalyzed isomerization of the 3′,3′,5′-triester to 2′,3′,5′- and 2′,2′,5′-triesters with **1a** is 11 times as fast as that compared with **1b**. These accelerations have been accounted for by stabilization of the anionic phosphorane intermediate by hydrogen bonding with the 2′-hydroxy function. Thio substitution of the nonbridging oxygens has an almost negligible influence on the cleavage of 3′,3′-diesters **2a**,**b**, but the hydrolysis of phosphoromonothioate triesters **1a**,**b** exhibits a sizable thio effect, $k_{PO}/k_{PS}=19$. The effects of metal ions on the rate of the cleavage of diesters and triesters have been studied and discussed in terms of the suggested hydrogen-bond stabilization of the thiophosphorane intermediates derived from **1a** and **2a**.

Keywords: hydrolysis · kinetics · phosphoesters · phosphorothioates · ribozymes · thio effect

Introduction

Oligonucleotides containing a chiral phosphoromonothioate linkage have been extensively used as substrates in mechanistic studies of large ribozymes.[1] An oxygen atom assumed to be catalytically important is substituted with a sulfur atom and the accompanying change in the reaction rate, known as a "thio effect", is quantified. Usually this approach has been applied to the identification of the nonbridging phosphoryl oxygen atom, either pro-*R* or pro-*S* oxygen, as a coordination site of metal ions playing a catalytic role.[2–4] The catalytic activity of large ribozymes depends on the Mg^{2+} ion, which prefers oxygen over sulfur as a donor atom. Accordingly, replacement of an oxygen atom that participates in binding of the Mg^{2+} ion with sulfur expectedly results in rate-deceleration, which may then be at least partially restored by using a more thiophilic Mn^{2+} or Cd^{2+} ion instead of Mg^{2+} as a cofactor. In addition, replacement of the bridging 3′-oxygen atom with sulfur has been exploited in attempting to elucidate the importance of this atom as a donor of an electron pair for hydrogen bonding or metal-ion binding.[4,5] Thio-substitution may, however, simultaneously affect several structural parameters, such as chain folding, hydrogen bonding, metal-ion binding, solvation, and van der Waals interactions, and, hence, interpretation of the

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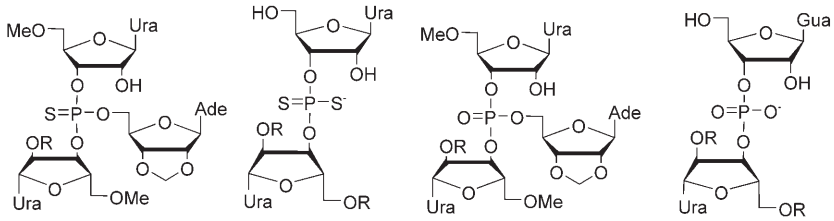
observed changes in reaction rate is not always unambiguous. Comparative studies with small molecule models usually facilitate evaluation of the inherent influence of a given structural modification and help to distinguish between alternative mechanistic interpretations.

The thio effects of phosphoesters have previously been studied in some detail both experimentally[6–13] and by theoretical calculations.[14–16] Generally speaking, the thio effect (k_{PO}/k_{PS}) resulting from replacement of a nonbridging phosphoryl oxygen atom of a phosphodiester with sulfur usually falls in the range 1–10, while phosphotriesters exhibit considerably higher and monoesters lower, that is, inverse thio effects.[6] In other words, this kind of thio-substitution appears to favor a dissociative mechanism via a monoanionic metaphosphate-like intermediate (monoester cleavage) and disfavor an associative mechanism via a pentacoordinated monoanionic phosphorane intermediate (triester cleavage). The magnitude of the thio-effect, hence, allows mechanistic conclusions even in cases for which metal-ion binding does not play a role. A complicating factor, however, is that the electronic and solvation effects of the thio-substitution are opposite. While the soft sulfur atoms stabilize anionic phosphorane-like transition states by more facile delocalization of the negative charge, this rate-accelerating effect is largely compensated by less efficient solvation of the thiophosphorane intermediates compared to their oxygen counterparts.[15] For this reason, the cleavage of phosphodiester via a dianionic transition state usually exhibits a rather small thio effect.[7,10,12] Bearing the importance of solvation in mind, one might expect the thio effects of associative phosphoester cleavage reactions via a phosphorane-like transition state to be sensitive to hydrogen-bonding interactions. In other words, stabilization of a phosphorane intermediate or leaving group by intramolecular hydrogen bonding could well be reflected in the magnitude of the thio effect.

With large ribozymes, the cleavage of 3′,5′-phosphodiester bonds proceeds by an attack of a 3′-hydroxy group of an external nucleoside on the phosphorus atom with concomitant departure of the 3′-linked nucleoside.[1] The neighboring 2′-hydroxy group of the departing nucleoside is rate accelerating, in particular with group I introns,[2,6,17] and may, in principle, donate a hydrogen bond either to an oxyanion of the phosphorane intermediate or to the 3′-oxyanion of the departing nucleoside. We have previously preferred the former alternative for the reason that the 2′-hydroxy group appears to accelerate the departure of the 3′- and 5′-linked nucleosides as efficiently,[18–20] but the opposite view has also received support from model studies, namely from the observation that methoxide-ion-catalyzed methanolysis of ribonucleoside 3′(2′)-dimethylphosphate proceeds by cleavage of the P–O3′ bond.[21] To quantify the influence of this kind of intramolecular hydrogen bonding on the thio effect and to provide a solid chemical basis for mechanistic inferences concerning the action of large ribozymes, we now report on thio effects determined for the cleavage and isomerization of simple chemical models of their cleavage site. For this purpose, cleavage and isomerization of a 3′,3′,5′-phosphoromonothioate triester, 2′,3′-*O*-methyleneadenosin-5′-yl bis[5′-*O*-methyluridin-3′-yl] phosphoromonothioate (**1a**), which



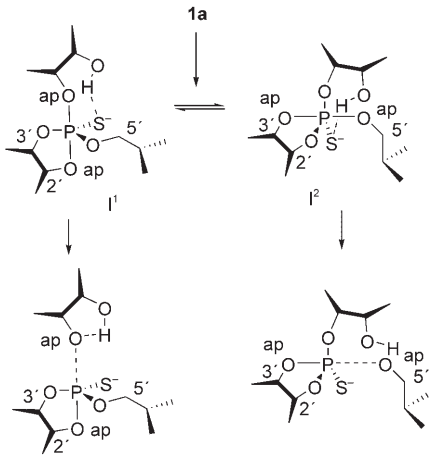
1a: R = H
b: R = Me

2a: R = H
b: R = Me

3a: R = H
b: R = Me

4a: R = H
b: R = Me

gives a tri-*O*-alkyl monoanionic phosphorane upon the attack of hydroxide ion, and a 3′,3′-phosphorodithioate diester, bis[uridin-3′-yl] phosphorodithioate (**2a**), giving a di-*O*-alkyl dianionic phosphorane, have been studied over a wide pH range and the results are compared to those reported previously for their oxygen analogues **3a**,**b**[19,22] and **4a**,**b**.[20] The reasoning behind the choice of **1a** as a model compound is that the 2′-hydroxy group of the leaving 3′-linked nucleoside may donate a hydrogen bond either to the departing 3′-oxyanion or to the oxyanion ligand of the monoanionic phosphorane intermediate obtained by an intramolecular attack of the other 3′-linked nucleoside (Scheme 1). The relative importance of these two processes is estimated by comparing the rate and product distribution (departure of 3′-linked versus 5′-linked nucleoside) to the corresponding quantities obtained with **1b**, containing the



Scheme 1. Thiophosphorane intermediates for the hydrolysis of trinucleoside 3′,3′,5′-phosphorothioate **1a**; ap = apical.

methylated 2′-hydroxy group of the leaving nucleoside. Acceleration of the P–O5′ cleavage by intramolecular hydrogen bonding to the 5′-oxyanion is less probable, as the O2′–H–O5′ hydrogen bond, if ever formed, must be cleaved upon release of the 5′-oxyanion as an immediate product. The influence of the hydrogen-bonding on the thio effect is evaluated by comparing the cleavage rates of **1a**,**b** to the cleavage rates of their oxygen analogues **3a**,**b**. Studies with diesters **2a**,**b** and **4a**,**b** elucidate, in turn, the thio effect related to the cleavage reaction via a dianionic phosphorane.

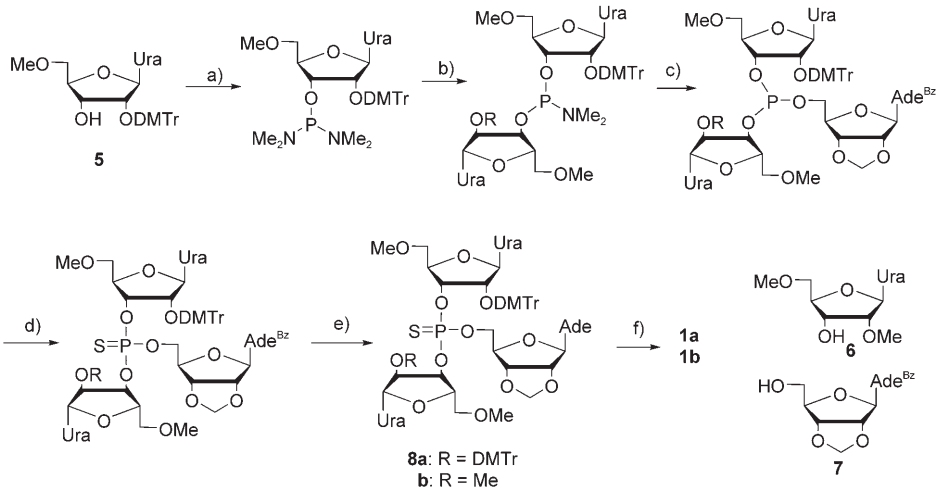
Results

Preparation of the trinucleoside 3′,3′,5′-phosphoromonothioates 1a,b and dinucleoside 3′,3′-phosphorodithioates 2a,b: Trinucleoside 3′,3′,5′-phosphoromonothioates were obtained in a 4,4′-dimethoxytrityl (DMTr)-protected form, **8a**,**b**, essentially as previously[19] described for the corresponding trinucleoside monophosphates, namely by tetrazole-promoted stepwise displacement of the dimethylamino groups from tris(dimethylamino)phosphine with the appropriately protected nucleosides, followed by oxidation with elemental sulfur (Scheme 2). Preparation of the protected nucleosides, 5′-*O*-methyl-2′-*O*-(4,4′-dimethoxytrityl)uridine (**5**),[22] 2′,5′-di-*O*-methyluridine (**6**),[23] and N^6-benzoyl-2,3′-*O*-methyleneadenosine (**7**)[23] has been described earlier. The dimethoxytrityl protections were removed only prior to each kinetic run with hydrogen chloride in 1,4-dioxane.

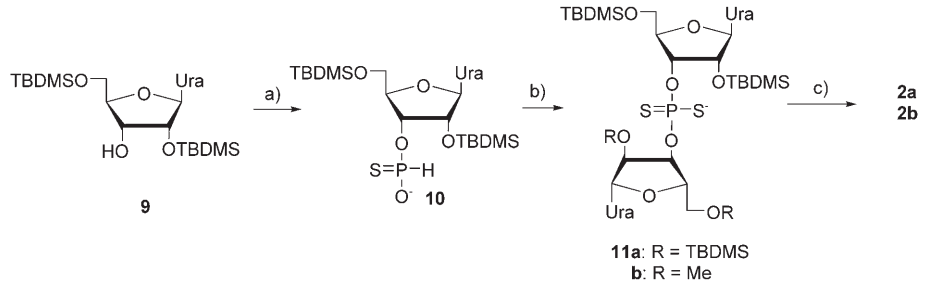
Bis(uridin-3′-yl) phosphorodithioate (**2a**) and 2′,5′-di-*O*-methyluridin-3′-yl uridin-3′-yl phosphorodithioate (**2b**) were synthesized by the hydrogen phosphonate methodology described by Stawinski et al.[24] (Scheme 3). Accordingly, 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)uridine 3′-hydrogenphosphonothioate (**10**) prepared from 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)uridine (**9**) was allowed to react with this nucleoside or 2′,5′-di-*O*-methyluridine (**6**) in the presence of diphenylphosphorochloridate and the product was oxidized with elemental sulfur to obtain **11a**,**b**. Desilylation of the latter products gave the desired phosphorodithioate diesters **2a**,**b**.

Product distributions and pH rate profiles for the reactions of phosphorothioate triesters 1a,b: The hydrolysis of **1a** and **1b** was followed over a wide pH range (from $H_0 = -0.1$ to pH 9) at 25 °C by analyzing the composition of the aliquots withdrawn from the reaction mixture at appropriate time intervals by RP HPLC. The nucleosidic products (**6**, **14**, **20** in Scheme 4) were identified by spiking with authentic samples. The diester products were identified either as di(uridin-3′-yl)esters (**15a**, **16a**, **17** from **1a**; **15b**, **16b** from **1b**) or uridin-3′-yl adenosine-5′-yl esters **18** and **19** by ESI-HPLC/MS. The triester isomers of the starting materials were also identified by ESI-HPLC/MS. Assignment of **12a** and **13** as 2′,3′,5′- and 2′,2′,5′-isomers, respectively, was based on the fact that **12a** appeared as an intermediate in the formation of **13**.

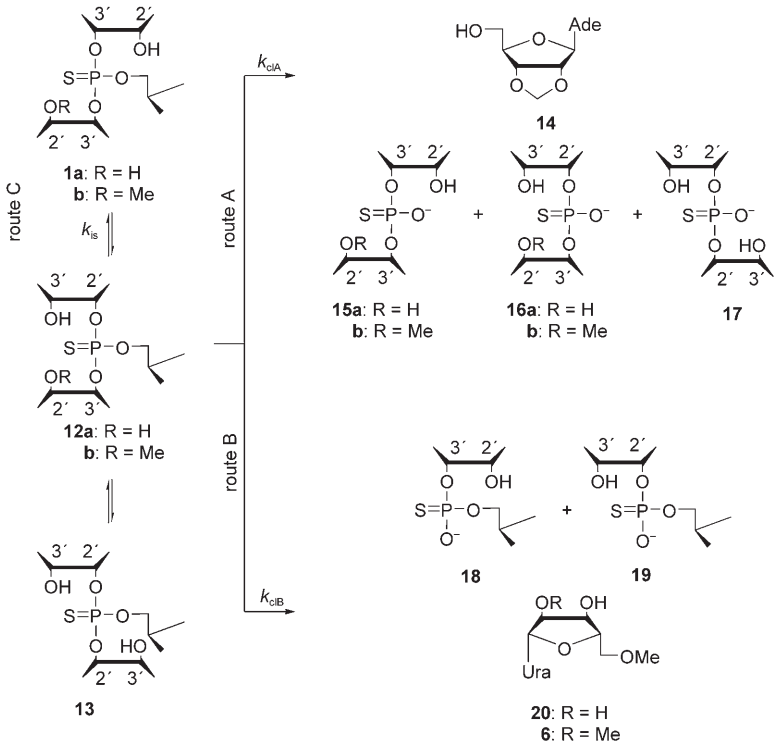
Over the pH range studied, two reactions expectedly took place: isomerization of the 3′,3′,5′-triesters **1a**,**b** to 2′,3′,5′- and 2′,2′,5′-triesters **12a**,**b** and **13**, respectively, and cleavage of the triesters to a mixture of diesters. Two sets of cleavage products were obtained (Scheme 4). Release of 2′,3′-*O*-methyleneadenosine (**14**) by the P–O5′ bond fission (route A) resulted in formation of bis(5′-*O*-methyluridin-3′-yl) phosphorothioate (**15a**) and its 2′,3′- and 2′,2′-isomers **16a**



Scheme 2. a) $P(NMe_2)_3$, tetrazole, MeCN; b) **5** (to obtain **8a**) or **6** (to obtain **8b**), tetrazole, MeCN; c) **7**, tetrazole, MeCN; d) S_8, CH_2Cl_2; e) NH_3, MeOH; f) HCl/1,4-dioxane.



Scheme 3. a) $H_2PO_2^-$ HN^+Et_3, PivCl, Py; b) 1) **9** (to obtain **11a**) or **6** (to obtain **11b**), diphenylphosphorochloridate, Py/MeCN, 2) S_8; c) Bu_4NF, THF.

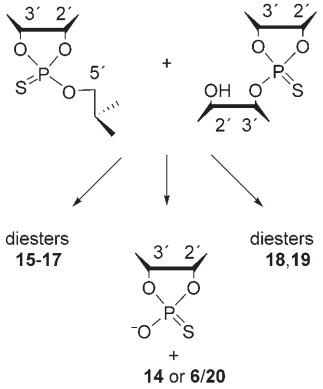


Scheme 4. Products for the cleavage and isomerization of trinucleoside 3′,3′,5′-phosphorothioate triesters **1a** and **1b**.

The pH rate profiles for the cleavage (routes A and B in Scheme 4) and isomerization (route C) of **1a** and **1b** are depicted in Figure 1. The rate constants indicated are those extrapolated to buffer concentration zero. With both compounds, the cleavage is pH independent under acidic conditions. The cleavage of **1a** becomes hydroxide-ion-catalyzed at pH 4–5, while with **1b**, this change takes place at pH 6–7. Between pH 4 and 6, the cleavage of **1b** is still pH independent, although slightly faster than under more acidic conditions. Compound **1b** actually is a mixture of two diastereomers, which can be separated by RP HPLC. Both diastereomers are, however, hydrolyzed as rapidly. With both **1a** and **1b**, the P–O3′ and P–O5′ bonds are cleaved almost equally rapidly under acidic conditions (pH <6), but on going to more alkaline conditions, cleavage of the P–O3′ bond gradually becomes the predominant reaction.

and **17**, respectively, from **1a**, and 5′-*O*-methyluridin-3′-yl 2′,5′-di-*O*-methyluridin-3′-yl phosphorothioate (**15b**) and its 2′,3′-isomer **16b** from **1b**. Release of 5′-*O*-methyluridine (**20**) from **1a** or 2′,5′-di-*O*-methyluridine (**6**) from **1b** by P–O3′ bond fission (route B), in turn, resulted in formation of 2′,3′-*O*-methyleneadenosin-5′-yl 5′-*O*-methyluridin-3′-yl phosphoromonothioate (**18**) and its 2′,5′-isomer **19**. No desulfurization was observed to take place.

The isomerization and cleavage reactions most likely proceed via a common thiophosphorane intermediate indicated in Scheme 1. While endocyclic bond cleavage of this intermediate leads to isomerization, departure of either the 5′- or 3′-linked nucleoside leads to the cleavage products by routes A and B, respectively. The cleavage reactions, in fact, give a 2′,3′-cyclic phosphorothioate triester as the immediate product (Scheme 5). Attack of water on this highly unstable triester gives another thiophosphorane intermediate that by endocyclic cleavage gives the diester products **15**–**19**. In principle, the cyclic thiophosphorane may also undergo exocyclic cleavage to a nucleoside 2′,3′-cyclic phosphate. Previous studies[9] have, however, shown that the cyclic triester undergoes only endocyclic cleavage, except at low pH (pH <3), for which exocyclic cleavage starts to compete as a side reaction (see the discussion on diester cleavage below). Consistent with this, the monomeric nucleosides **14** or **6**/**20** and the respective isomeric phosphorothioate diesters **15**–**17** or **18**,**19** were observed to be formed in a 1:1 ratio at pH >2.



Scheme 5. Breakdown of 2′,3′-cyclic phosphorothioate triester intermediates.

The isomerization exhibits a similar dependence of rate on acidity as the cleavage, except that the pH-independent region is not as wide: the reaction becomes first order in hydroxide-ion concentration already around pH 2 with **1a** and around pH 3 with the diastereomers of **1b**. Over the whole pH range studied, isomerization proceeds at essentially equal rates in both directions. In contrast to the cleavage reaction, the diastereomers of **1b** are isomerized at a different rate, the faster eluting diastereomer somewhat less rapidly than the more slowly eluting one.

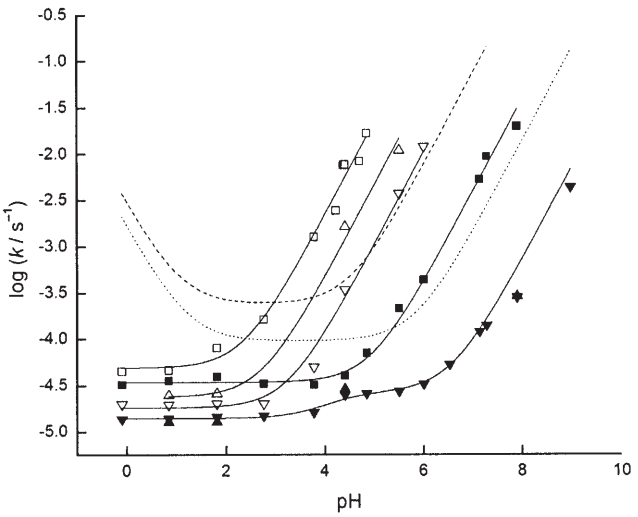


Figure 1. pH rate profiles for the cleavage and isomerization of phosphotriesters **1a** and **1b** at 25 °C, $I(NaNO_3)=1.0\,mol\,L^{-1}$. All the rate constants refer to buffer concentration zero. The curves are obtained by least-squares fitting by Equations (1)–(3). The dashed and dotted lines refer to the cleavage of triesters **3a**[19] and **3b**,[22] respectively. Notation: (■) cleavage of **1a** ($k_{clA}+k_{clB}$), (▲ and ▼) cleavage of the slowly and fast-eluting diastereomer of **1b**, respectively ($k_{clA}+k_{clB}$), (□) isomerization of **1a** (k_{is}), (▽ and △) isomerization of the fast and slowly eluting diastereomer of **1b** (k_{is}). For the rate constants, see Scheme 4. k_{is} stands for the sum of the first-order rate constants for the forward and reverse reaction of **1a** or **1b** to a mixture of other isomers.

As mentioned above, buffer catalysis was observed in carboxylic acid, MES and HEPES buffers for the cleavage, and to a lesser extent, for the isomerization reactions. Measurements at various concentration ratios of the buffer constituents revealed both reactions to be susceptible to general base catalysis. The rate constants obtained at different buffer concentrations (k_{cl}^{obs} and k_{is}^{obs}) and the rate constants for the general base catalysis (k_{cl}^{buf} and k_{is}^{buf}) and the buffer-independent reactions (k_{cl}^{W} and k_{cl}^{W}) are given in Tables 1 and 2.

The buffer-independent rate constant, k_{is}^{W}, for the isomerization of **1a** and **1b** may be expressed by Equation (1), in which k_{is}^{0} and k_{is}^{OH} are the rate constants for the pH-independent and hydroxide-ion-catalyzed reactions. A similar equation, Equation (2), applies to the buffer-independent rate constant, k_{cl}^{W}, of the cleavage of **1a**. K_w is the ionic product of water under the experimental conditions ($1.87\times10^{-14}\,mol^2\,L^{-1}$).[25] The observed first-order rate constant for the cleavage of **1b** may, in turn, be expressed by Equation (3), in which k_{cl}^{X} is the rate constant for the additional pH-independent reaction observed between pH 4 and 6, and K_a is the equilibrium constant referring to the change in the ionic form of the reactants which results in the observed increment in the rate of the otherwise pH-independent cleavage on passing pH 4. The pK_a value, 4.2, coincides with the pK_a value of N1 in the adenine moiety.[26]

$$k_{is}^{W}=k_{is}^{0}+k_{is}^{OH}\,(K_w/[H^+]) \quad (1)$$

$$k_{cl}^{W}=k_{cl}^{0}+k_{cl}^{OH}(K_w/[H^+]) \quad (2)$$

$$k_{cl}^{W}=\{k_{cl}^{0}[H^+]+k_{cl}^{X}K_a+k_{cl}^{OH}K_a(K_w/[H^+])\}/([H^+]+K_a) \quad (3)$$

The rate constants for the overall cleavage, $k_{cl}^{W}=k_{clA}+k_{clB}$ (see Scheme 4), and isomerization, k_{is}^{W}, have been obtained by nonlinear least-squares fitting of the experimental data to Equations (1)–(3). To take into account the fact that in **1a** there are two potential 2′-OH nucleophiles to initiate the reaction, the rate constants corresponding to the cleavage and isomerization of **1a** have been divided by two. The overall rate constants have then be broken down to contributions of k_{clA} and k_{clB} on the basis of observed product distribution. The rate constants obtained in this manner are listed in Table 3.

Table 1. Rate constants for the buffer-catalyzed and -independent cleavage (k_{cl}^{buf} and k_{cl}^{W}) and isomerization (k_{is}^{buf} and k_{is}^{W}) of **1a** at $T=25$ °C and $I(NaNO_3)=1.0\,mol\,L^{-1}$.

Buffer acid	[HA]/[A$^-$]	[HA] [mol L^{-1}]	[A$^-$] [mol L^{-1}]	$k_{cl}^{obs}\times10^5$ [s^{-1}]	$k_{cl}^{buf}\times10^4$ [L mol^{-1} s^{-1}]	$k_{cl}^{W}\times10^5$ [s^{-1}]	$k_{is}^{obs}\times10^4$ [s^{-1}]	$k_{is}^{buf}\times10^4$ [L mol^{-1} s^{-1}]	$k_{is}^{W}\times10^4$ [s^{-1}]
formic	3:1	0.033	0.011	5.30			1.90		
		0.069	0.023	7.58			2.49		
		0.139	0.046	11.7			3.07		
					4.53 ± 0.07[a]	3.32 ± 0.09[b]		8 ± 1[a]	1.6 ± 0.2[b]
	1:3	0.011	0.033	9.71					
		0.023	0.069	13.4			15.7		
		0.046	0.139	23.6			18.7		
					10.0 ± 0.8	5 ± 1		32[c]	13[c]
acetic	1:1	0.024	0.024	15.5			32.5		
		0.048	0.048	20.2			75.2		
		0.095	0.095	43.2			76.9		
					20 ± 3	4 ± 4		18	74
	1:3	0.012	0.036	17.5					
		0.024	0.071	27.3					
		0.048	0.143	48.3					
					21.6 ± 0.3	7.0 ± 0.4			

[a] Statistical error of the slope of the linear regression of the rate constants obtained at various buffer concentrations. [b] Statistical error of the rate constant extrapolated to buffer concentration zero. [c] The values based on rate constants obtained at two buffer concentrations only.

Table 2. Rate constants for the buffer-catalyzed and -independent cleavage (k_{cl}^{buf} and k_{cl}^{W}) and isomerization (k_{is}^{buf} and k_{is}^{W}) of the fast-eluting diastereomer of **1b** at $T=25\,°C$ and $I(NaNO_3)=1.0\,mol\,L^{-1}$.

Buffer acid	[HA]/[A$^-$]	[HA] [mol L^{-1}]	[A$^-$] [mol L^{-1}]	$k_{cl}^{obs}\times10$ [s^{-1}]	$k_{cl}^{buf}\times10^4$ [L mol^{-1} s^{-1}]	$k_{cl}^{W}\times10^5$ [s^{-1}]	$k_{is}^{obs}\times10^4$ [s^{-1}]	$k_{is}^{buf}\times10^4$ [L mol^{-1} s^{-1}]	$k_{is}^{W}\times10^4$ [s^{-1}]
formic	3:1	0.033	0.011	1.87			3.65		
		0.069	0.023	2.33			5.49		
		0.139	0.046	3.14			8.91		
					0.90 ± 0.02[a]	1.48 ± 0.03[b]		3.73 ± 0.03[a]	0.20 ± 0.01[b]
	1:3	0.011	0.033	3.06			1.13		
		0.023	0.069	4.63			1.87		
		0.046	0.139	6.64			3.19		
					2.5 ± 0.3	2.1 ± 0.3		14.6 ± 0.3	0.50 ± 0.03
acetic	1:1	0.024	0.024	3.56			2.76		
		0.048	0.048	4.84			6.37		
		0.095	0.095	7.00			9.33		
					2.4 ± 0.1	2.5 ± 0.1		40 ± 10	1 ± 1
	1:3	0.012	0.036	4.25					
		0.024	0.071	6.00			11.6		
		0.048	0.143	9.35			15.1		
					3.56 ± 0.04	2.58 ± 0.05		37[c]	8.1[c]
MES	3:1	0.057	0.019	3.09					
		0.086	0.029	3.51					
		0.143	0.048	3.80					
					0.6 ± 0.2	2.7 ± 0.2			
	1:1	0.024	0.024	3.60					
		0.048	0.048	3.78					
		0.095	0.095	4.48					
					0.63 ± 0.09	3.3 ± 0.1			
HEPES	3:1	0.071	0.024	6.76					
		0.143	0.048	8.24					
					1.56[c]	5.28[c]			

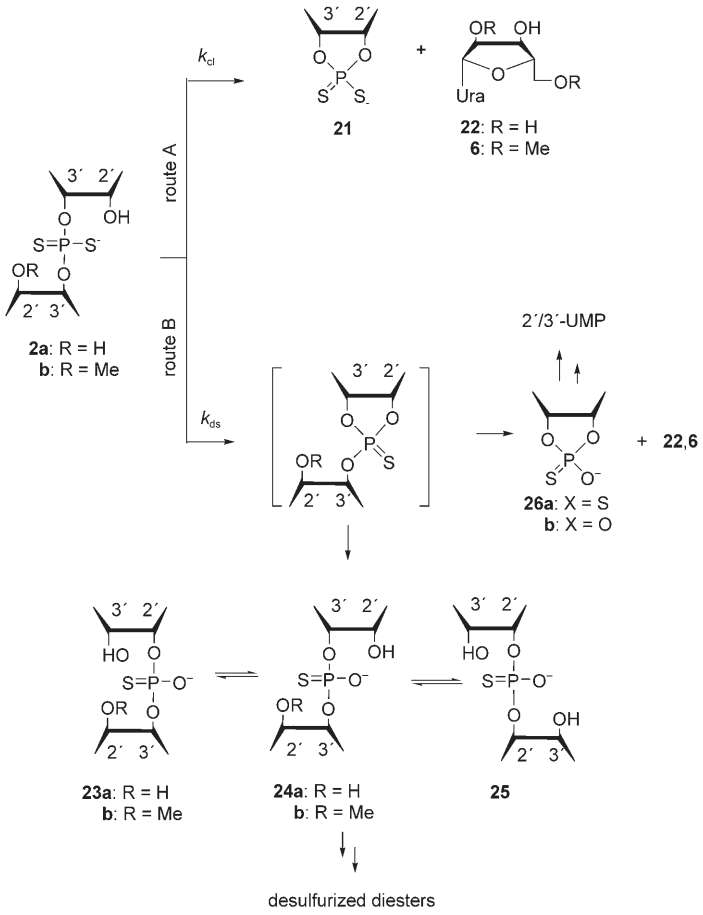
[a] Statistical error of the slope of the linear regression of the rate constants obtained at various buffer concentrations. [b] Statistical error of the rate constant extrapolated to buffer concentration zero. [c] The values based on rate constants obtained at two buffer concentrations only.

Table 3. Rate constants for the pH-independent isomerization (k_{is}^{0}), hydroxide-ion-catalyzed isomerization (k_{is}^{OH}), pH-independent cleavage (k_{cl}^{0}), and hydroxide-ion-catalyzed cleavage (k_{cl}^{OH}) of phosphorothioate triesters **1a** and **1b** at $T=25\,°C$ and $I(NaNO_3)=1.0\,mol\,L^{-1}$. k_{clA} and k_{clB} refer to cleavage by routes A and B (see Scheme 4) and k_{cl}^{X} to the pH-independent cleavage of **1b** at pH 4–6. For comparison, the corresponding data for the oxygen–phosphate counterparts **3a**[19] and **3b**[22] are given.

	1[a]	**1b**[b]	**1b**[c]	**3a**[a]	**3b**
$k_{cl}^{0}\times10^5$ [s^{-1}]	1.7 ± 0.1	1.4 ± 0.1		14 ± 2	9.0 ± 0.9
$k_{clA}^{0}\times10^5$ [s^{-1}]	1.05 ± 0.06	0.83 ± 0.03		9 ± 1	6.8 ± 0.7
$k_{clB}^{0}\times10^5$ [s^{-1}]	0.65 ± 0.04	0.56 ± 0.02		5 ± 1	2.3 ± 0.2
$k_{cl}^{X}\times10^5$ [s^{-1}]			2.7 ± 0.2		
$k_{is}^{0}\times10^5$ [s^{-1}]	2.4 ± 0.4	1.8 ± 0.2	2.4 ± 0.6		
$k_{cl}^{OH}\times10^{-3}$ [L mol^{-1} s^{-1}]	10.5 ± 0.9	0.39 ± 0.07		200 ± 10	7.7 ± 0.9
$k_{clA}^{OH}\times10^{-3}$ [L mol^{-1} s^{-1}]	1.3 ± 0.1	0.02 ± 0.004		22 ± 1	0.9 ± 0.1
$k_{clB}^{OH}\times10^{-3}$ [L mol^{-1} s^{-1}]	9.2 ± 0.8	0.37 ± 0.07		178 ± 9	6.6 ± 0.8
$k_{is}^{OH}\times10^{-5}$ [L mol^{-1} s^{-1}]	58 ± 6	5.5 ± 0.7	24 ± 5		500 ± 100

[a] The rate constants referring to compounds **1a** and **3a** have been subjected to a statistical correction (division by a factor of two) for the reason that these molecules contain two attacking 2′-OH groups, while **1b** and **3b** contain only one 2′-OH. [b] Fast-eluting diastereomer. [c] Slowly eluting diastereomer.

Product distributions and pH rate profiles for the reactions of phosphoroditioate diesters 2a,b: The cleavage of phosphorodithioate diesters is much slower than the hydrolysis of triesters. The reactions of **2a** and **2b** were, hence, followed at an elevated temperature, namely 90 °C. Under basic and neutral conditions (pH >6), the only reaction detected was the hydroxide-ion-catalyzed cleavage of the P–O3′ bond of **2a** (route A in Scheme 6). Release of uridine (**22**) from **2a** and 2′,5′-di-*O*-methyluridine (**6**) from **2b** resulted in accumulation of uridine 2′,3′-cyclic phosphorodithioate (**21**). In addition, a small amount of uracil (2–5%) was formed at pH 7–9 parallel to **21**. Under acidic conditions (pH <6), desulfurization (route B) competed with the formation of **21**. At pH <4, **21** was not any more accumulated, but **2a** and **2b** were converted to a mixture of 2′,3′- (**23a,b**), 3′,3′- (**24a,b**), and 2′,2′-phosphorothioate diesters (**25**), probably via a 2′,3′-cyclic phosphoromonothioate triester (not accumulated). Consistent with previous studies,[11] the latter intermediate underwent only endocyclic fission to acyclic phosphoromonothioate diesters (**23–25**) at pH >4. On going to more acidic solutions, exocyclic fission of the 2′,3′-cyclic phosphoromonothioate triester to 2′,3′-cyclic phosphoromonothioate (**26a**), however, started to compete and these reactions became significant side reactions at pH <2. The phosphoromonothioate diesters containing two dissimilar nucleosides **23a,b** and **24b** were expectedly obtained as pairs of two diastereomeric forms. All the phosphoromono-



Scheme 6. Products for the cleavage and desulfurization of dinucleoside 3′,3′-phosphorodithioate diesters **2a** and **2b**.

thioate diesters **23**–**25** were subsequently desulfurized to a mixture of the corresponding oxygen diesters, as previously[11] described in detail. It is worth noting that no isomerization of the starting material **2a**,**b** took place under any conditions. No sign of participation of the 5′-OH as a nucleophile was observed.

The pH rate profiles for the cleavage and desulfurization of **2a** and **2b** are depicted in Figure 2. The rate constants indicated refer to buffer concentration zero. In fact, the rate constants were independent of the buffer concentration in the low concentration region ($<0.1\,mol\,L^{-1}$) used in the kinetic measurements. The cleavage is oxonium-ion-catalyzed at pH <4, hydroxide-ion-catalyzed at pH >6, and nearly pH independent between these two limiting pH values. Both the oxonium- and hydroxide-ion-catalyzed cleavage is first-order in the catalyst concentration, H_3O^+ and OH^-, respectively. The desulfurization is first order in $[H_3O^+]$ at pH <4, and pH independent under less acidic conditions. To illustrate the effects that replacement of both of the nonbridging oxygens of diribonucleoside 3′,3′-monophosphates with sulfur atoms has on the cleavage rate, the pH rate profiles obtained previously[20] with **4a** and **4b** are also included in Figure 2. The curves indicated have been obtained by nonlinear least-squares fitting based on Equations (4) and (5).

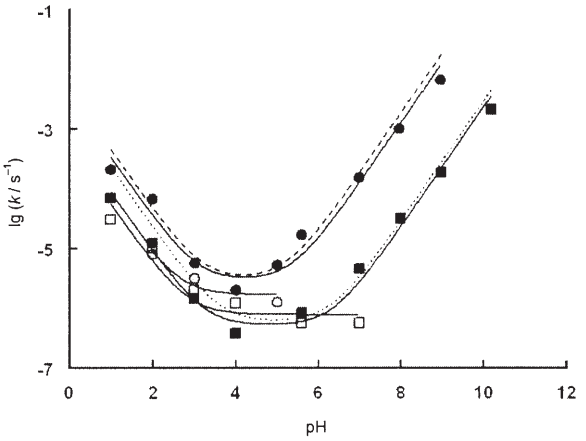


Figure 2. pH rate profiles for the cleavage (k_{cl}) of **2a** (●) and **2b** (■) and desulfurization (k_{ds}) of **2a** (○) and **2b** (□) at 90 °C and I(NaCl) = 0.1 mol L^{-1}. The dashed and dotted lines show the corresponding curves for **4a** and **4b**, respectively.[20]

$$k_{cl} = k_{cl}^{H}[H^+] + k_{cl}^{0} + k_{cl}^{OH}\,(K_w/[H^+]) \quad (4)$$

$$k_{ds} = k_{ds}^{H}[H^+] + k_{ds}^{0} \quad (5)$$

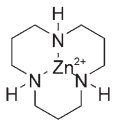
In these equations, the rate constants k_{cl}^{H}, k_{c}^{0}, and k_{cl}^{OH} refer to oxonium-ion-catalyzed, pH-independent and hydroxide-ion-catalyzed cleavage (route A in Scheme 6), respectively, and k_{ds}^{H} and k_{d}^{0} are the rate constants of the oxonium-ion-catalyzed and pH independent desulfurization (route B). A value of $6.17\times10^{-13}\,mol^2\,L^{-2}$ has been used for the ionic product of water.[25] To take into account the fact that in **2a** there are two intramolecular 2′-OH nucleophiles to initiate the reaction, the rate constants corresponding to the cleavage and desulfurization of **2a** have been divided by two. The values of these constants are listed in Table 4.

Table 4. Rate constants for the oxonium-ion-catalyzed cleavage (k_{cl}^{H}) and desulfurization (k_{ds}^{H}), pH-independent cleavage (k_{cl}^{0}) and desulfurization (k_{ds}^{0}), and hydroxide-ion-catalyzed cleavage (k_{cl}^{OH}) of phosphorodithioate diesters **2a** and **2b**. T = 90 °C and I($NaNO_3$) = 0.1 mol L^{-1}. For comparison, the corresponding data for their oxygen–phosphate analogues **4a** and **4b**[20] are given.

	2a[a]	**2b**	**4a**[a]	**4b**
$k_{cl}^{H}\times10^5$ [L mol^{-1} s^{-1}]	170 ± 5	90 ± 30	130 ± 60	230 ± 70
$k_{cl}^{0}\times10^5$ [s^{-1}]	0.15 ± 0.06	0.05 ± 0.02	0.15 ± 0.05	0.06 ± 0.02
k_{cl}^{OH} [L mol^{-1} s^{-1}]	10.1 ± 3.1	0.37 ± 0.08	14.9 ± 3.1	0.46 ± 0.12
$k_{ds}^{H}\times10^5$ [L mol^{-1} s^{-1}]	36 ± 13	55 ± 21		
$k_{ds}^{0}\times10^5$ [s^{-1}]	0.09 ± 0.02	0.08 ± 0.02		

[a] The rate constants referring to compounds **2a** and **4a** have been subjected to a statistical correction (division by a factor of two) for the reason that these molecules contain two attacking 2′-OH groups, while **2b** and **4b** contain only one 2′-OH.

Metal-ion catalysis: The cleavage of phosphorothioate triesters, **1a** and **1b**, was studied in the presence of the Zn^{2+}



27

chelate of 1,5,9,-triazacyclododecane ([12]aneN$_3$; **27**) at pH 7.28 [$T=25\,°C$; $I(NaNO_3)=1.0\,mol\,L^{-1}$]. The Zn^{2+} chelate was used instead of the free metal ion, as the purpose was to study the effect of metal ions on the cleavage rate under conditions in which this reaction is hydroxide-ion-catalyzed, that is, at pH >7. Uncomplexed Zn^{2+} precipitates at such a high pH. Pseudo-first-order rate constants were measured as a function of the concentration of Zn^{2+}[12]aneN$_3$.

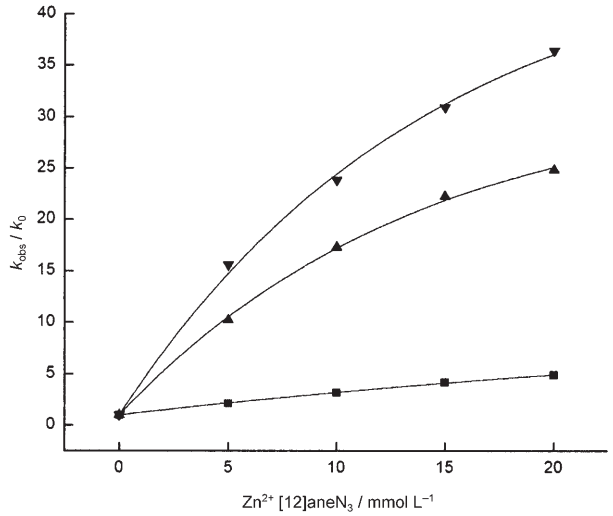


Figure 3. Acceleration of the cleavage of **1a** (■) and the fast (▼) and slowly eluting (▲) diastereomer of **1b** by Zn^{2+}[12]aneN$_3$ at pH 7.28, $T=25\,°C$ and $I(NaNO_3)=1.0$ mol L^{-1}.

Figure 3 shows the observed pseudo-first-order rate constants (k_{obs}) divided by values obtained in the absence of the metal complex (k_o) plotted as a function of the concentration of Zn^{2+}[12]aneN$_3$. As seen, both compounds are subject to catalysis by the complex, but the cleavage of **1b** is catalyzed more efficiently than the cleavage of **1a**. The rate acceleration, k_{obs}/k_o, levels off to a value of 46 with the fast-eluting diastereomer of **1b** and a value of 10 with **1a**.

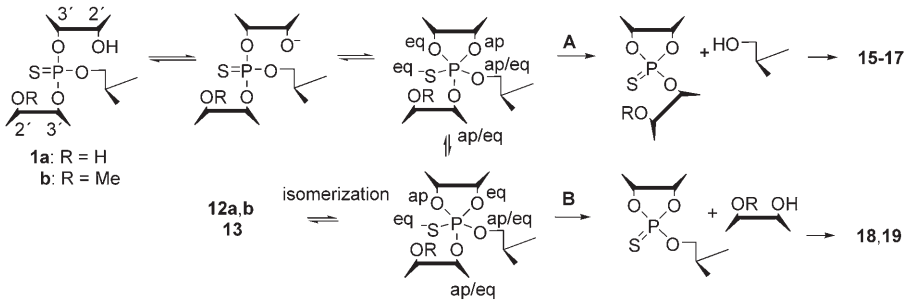
The effect of the Zn^{2+} ion on the cleavage of phosphorodithioate diesters **2a** and **2b** was followed as a function of pH (5.1–5.6) and metal-ion concentration (1–10 mmol L^{-1} at pH 5.6). Cleavage was the only reaction observed. Over the narrow pH and [Zn^{2+}] range studied, the reaction was first-order in both the hydroxide and metal-ion concentration. Cleavage was the only reaction detected at [Zn^{2+}]$=5$ mmol L^{-1} and pH 5.6. In other words, the desulfurization was less susceptible to the Zn^{2+} ion catalysis than the cleavage. The Zn^{2+}-promoted cleavage of **2a** and **2b** was at [Zn^{2+}]$=5$ mmol L^{-1} and pH 5.6 83 and 230 times as fast as in the absence of the metal ion, respectively (Table 5). The catalytic activity of Mg^{2+}, a hard Lewis acid, was negligible, whereas the markedly thiophilic Cd^{2+} ion efficiently accelerated both the cleavage and desulfurization. The cleavage reactions of **2a** and **2b** were accelerated by a factor of 3300 and 21000, respectively, at [Cd^{2+}]$=5$ mmol L^{-1} and pH 5.6. Desulfurization of **2b** was 13000-fold faster than in the absence of the metal ion.

Table 5. First-order rate constants for the cleavage of phosphorodithioate diesters **2a** and **2b** at 90 °C and $I(NaNO_3)=0.1$ mol L^{-1}.

M^{z+}	[M^{z+}] [mmol L^{-1}]	pH	$k_{cl}\times10^3$ [s^{-1}]	
			2a	**2b**
Zn^{2+}	1.0	5.60	0.33	0.0477
	5.0	5.60	1.40	0.328
	10.0	5.60	3.58	1.08
	5.0	5.38	1.27	0.27
	5.0	5.08	0.52	0.134
	5.0	4.68		0.051
Cd^{2+}	5.0	5.60	55.9	24.4
Mg^{2+}	5.0	5.60	0.021	0.0012
none	5.60	0.0169	0.0014	

Discussion

Reactions of phosphorothioate triesters 1a and 1b: The overall cleavage of **1a** and **1b** becomes first-order in hydroxide-ion concentration at pH 4–5 and 6–7, respectively. Under these conditions, the reaction most probably proceeds by rapid initial deprotonation of the 2′-hydroxy group, attack of the resulting oxyanion on the phosphorus atom with concomitant formation of a thiophosphorane intermediate and rate-limiting departure of the 3′- or 5′-linked nucleoside (Scheme 7).[15] The fact that breakdown of the monoanionic thiophosphorane rather than its formation is rate limiting is evidenced by much faster isomerization of the starting material compared to its cleavage. Methylation of the 2′-hydroxy function of one of the 3′-linked nucleosides markedly retards the cleavage: **1a** is cleaved 27 times

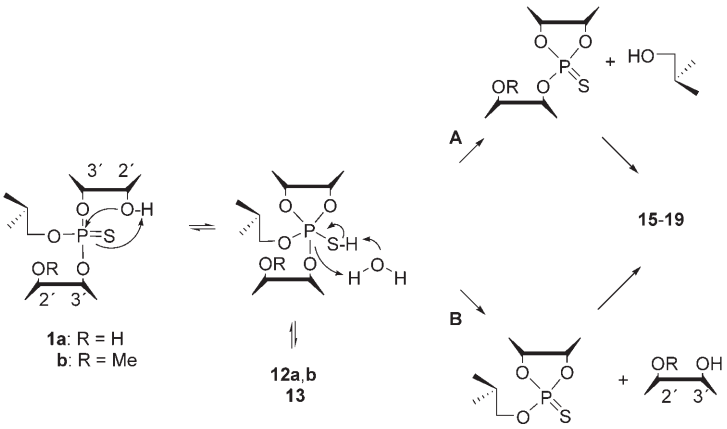


Scheme 7. Mechanism of the hydroxide-ion-catalyzed cleavage of phosphorothioate triesters **1a** and **1b**; eq = equatorial.

as rapidly as **1b**. This value includes the statistical correction arising from the fact that **1a** contains two and **1b** only one hydroxy function serving as an intramolecular nucleophile. In other words, the observed rate acceleration is 54-fold. The cleavage of the P–O3′ bond (route B in Scheme 7) is favored over the cleavage of the P–O5′ bond (route A), reaching a maximum proportion of 88% with **1a** and 95% with **1b** under sufficiently alkaline conditions. As described previously for the oxygen–phosphate counterparts **4a**[19] and **4b**,[22] the fact that 2′-OMe substitution does not specifically retard the P–O3′ bond cleavage may be taken as an evidence for hydrogen-bond stabilization of the phosphorane intermediate (compare I^1 and I^2 in Scheme 1) rather than the leaving group.

Isomerization of the 3′,3′-5′-phophorothioate triester (**1a**) to a mixture of the 2′,3′,5′- and 2′,2′,5′-triesters **12a** and **13**, respectively, is also facilitated by the 2′-OH function of the 3′-linked nucleoside, albeit somewhat less markedly: **1a** is isomerized 11 times as rapidly as the fast-eluting diastereomer of **1b** and 2.4 times as fast as the slowly eluting one. The only viable hydrogen bond acceptors are the nonbridging sulfur and the departing 3′-oxygen atom. Hydrogen bonding of the 2′-OH to the latter is not expected to accelerate isomerization. Accordingly, the observed marked rate acceleration of isomerization by the 2′-OH function may be taken as compelling evidence for hydrogen-bond stabilization of the thiophosphorane intermediate. The fact that the acceleration, however, is less prominent than the acceleration of cleavage, argues against the suggestion that acceleration of the cleavage by leaving group stabilization is of minor importance. Possibly, intramolecular hydrogen bonding, while stabilizing the phosphorane intermediate, simultaneously increases the pseudorotation barrier, argued to limit at least partially the rate of isomerization of monoanionic phosphorane intermediate (though not a thiophosphorane).[27]

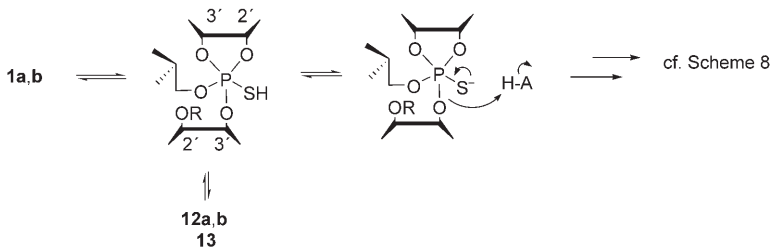
Over a wide range from $H_0 = -0.1$ to pH 4, the overall cleavage of both **1a** and **1b** is pH independent. Evidently nucleophilic attack of the 2′-oxygen on the phosphorus atom concerted with transfer of the 2′-hydroxy proton to the sulfur atom (either direct or water-mediated transfer) gives a thiophosphorane intermediate which undergoes rate-limiting expulsion of the leaving group concerted with water-mediated proton transfer from the mercapto ligand to the departing oxyanion (Scheme 8).[9,18] The rate-retarding effect of the 2′-OMe substitution is again very similar to that observed previously[19,22,28] for the oxygen analogues **3a**,**b**. Compound **1a** is cleaved 2.3 times as rapidly as **1b**, and cleavage of the P–O5′ (route A) and P–O3′ (route B) bonds is equally rapid. With **3a**, the rate acceleration has been reported to be 1.6 compared to **3b**. As the thiophosphorane intermediate is now neutral and the leaving group departs as an alcohol, the neighboring 2′-hydroxy group does not markedly stabilize the phosphorane intermediate or the leaving group by hydrogen bonding. With **1b**, another slightly faster pH independent reaction is observed between pH 4 and 6. The origin of this modest (approximately twofold) rate difference between the two pH-independent reactions remains obscure, but it possibly is related to the protolytic equilibrium at the N1 site of the adenine moiety at around pH 4.[26]



Scheme 8. Mechanism of the pH- and buffer-independent cleavage of phosphorothioate triesters **1a** and **1b**.

Between pH 3 and 5, the overall cleavage of **1a** and **1b** is susceptible to general base catalysis by carboxylate anions. The reaction may be interpreted as a sequential specific base/general acid catalysis, in which the attacking 2′-OH is deprotonated in a pre-equilibrium step and proton transfer from the general acid to the departing oxyanion occurs concerted with the rate-limiting P–O bond fission (Scheme 9).[18] The rate-retarding effect of the 2′-OMe substitution is more marked than with the pH- and buffer-independent reaction, but not as prominent as in the hydroxide-ion-catalyzed reaction: **1a** is cleaved by carboxylic acid buffers 4–6 times as fast as **1b** (the statistical correction made).

The fact that the effects of 2′-OMe substitution on the cleavage rate of **1a** and its oxygen analogue **3a** are almost equal is somewhat unexpected. The sulfur atom is less electronegative than oxygen and, hence, a weaker hydrogen-bond acceptor. Accordingly, one might expect the 2′-OMe substitution to be less rate-retarding with **1a** than with **3a**, assuming that stabilization of the monoanionic phosphorane



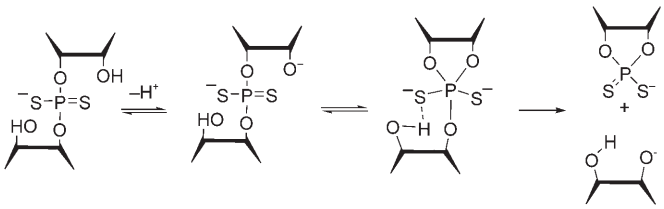
Scheme 9. Mechanism of the general-base-catalyzed cleavage of phosphorothioate trimesters **1a** and **1b**.

intermediate by intramolecular hydrogen bonding is the main source of the reactivity difference between the 2′-OH and 2′-OMe substituted compounds. One should, however, bear in mind that the sulfur atom is considerably larger than the oxygen atom and this may contribute to the strength of intramolecular hydrogen bonding. The cleavage of ribonucleoside 3′-thioesters, which contain a bridging sulfur atom, serves as an example of the importance of geometry upon the formation of a cyclic phosphorane intermediate. The 3′-thioesters are converted to phosphorane intermediates up to 100 times more readily than their oxygen counterparts,[8] although the inductive effect of thio substitution on nucleophilic attack on phosphorus is rather more rate retarding than rate accelerating.

The hydroxide-ion-catalyzed cleavage of **1a** and **1b** exhibits a thio effect of $k_{PO}/k_{PS}=19$. A considerably smaller effect, namely $k_{PO}/k_{PS}=4$, has been reported for the corresponding reaction of uridine 3′-dimethylphosphorothioates,[9] whereas the thio effects for the hydroxide-ion-catalyzed hydrolysis of triethyl phosphate[29] and dialkyl 4-nitrophenyl phosphate[30] are of this magnitude, 12 and 30, respectively. Stabilization of the monoanionic phosphorane intermediate by intramolecular hydrogen bonding, suggested above to be the reason of 27-fold faster cleavage of **1a** compared to **1b**, is not reflected in the thio effect: both compounds exhibit exactly the same effect. The thio effects for the pH-independent cleavage of **1a** and **1b**, proceeding via a neutral phosphorane, are considerably smaller, 4.2 and 6.4, respectively, but still higher than obtained with uridine 3′-dimethylphosphorothioate ($k_{PO}/k_{PS}\approx1$ at pH 4).[9] The fact that the thio effect of the hydroxide-ion-catalyzed reaction is three- to fourfold compared to that of the pH-independent cleavage means that the thioester is less prone to hydroxide-ion catalysis than the oxyester.

The thio effect for the hydroxide-ion-catalyzed isomerization of **1a**,**b** appears to be even larger than that of the cleavage reaction, of the order of 100. Again, ribonucleoside 3′-dimethylphosphorothioates show a much weaker effect, $k_{PO}/k_{PS}=4$. Estimation of the rate constant for the very rapid hydroxide ion-catalyzed isomerization of the oxygen triesters, **3a**,**b**, is, however, susceptible to errors.

Reactions of phosphorodithioate diesters 2a and 2b: In contrast to phosphorothioate triesters, **1a**,**b**, which are hydrolyzed via a monoanionic thiophosphorane intermediate under alkaline conditions, the hydroxide-ion-catalyzed cleavage of phosphorodithioate diesters, **2a**,**b**, proceeds via a dianionic dithiophosphorane (Scheme 10). Methylation of the 2′-OH (and 5′-OH) of the departing nucleoside retards this reaction by a factor of 27 (the statistical correction resulting from the presence of two attacking 2′-OH groups has been taken into account). The corresponding value for the rate-retarding influence of the 2′-*O*-methylation of guanylyl-3′,3′-uridine (**4a** versus **4b**) has been reported to be 33 and attributed, at least predominantly, to stabilization of the phosphorane intermediate by intramolecular hydrogen bonding.[20] As discussed above, sulfide ion expectedly is a



Scheme 10. Mechanism for the hydroxide-ion-catalyzed cleavage of phosphorodithioate diesters **2a** and **2b**.

weaker hydrogen-bond acceptor than oxyanion. Accordingly, the difference between the cleavage rates of the dithioesters **2a** and **2b** could be expected to be smaller than the difference between the cleavage rates of the oxygen esters, **4a**.

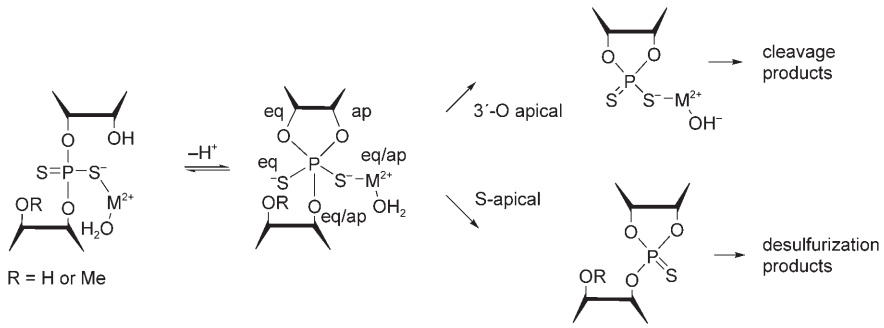
The thio effects are small: the 3′-*O*-linked nucleoside departs 1.5 times more slowly from **2a** than from **4a**,[20] and the corresponding reactivity ratio for **2b** and **4b** is 1.2. In other words, stabilization of the dianionc phosphorane by intramolecular hydrogen bonding is not appreciably reflected to the thio effect. Previously, a small thio-effect has been reported for the cleavage of 3′,5′-phosphoromonothioates[10,11] and their dithioate analogues.[7] Previous theoretical calculations suggest that the small values of the thio effect result from opposite electronic and solvation effects.[14,15] While higher polarizability of sulfur compared to oxygen stabilize the dianionic phosphorane intermediate, poor solvation of the intermediate cancel the resulting rate acceleration.

Desulfurization starts to compete with cleavage only under mildly acidic conditions (pH <5), that is, under conditions in which the reactions turn acid-catalyzed and proceed via a neutral phosphorane intermediate. Under these conditions, methylation of the 2′-OH of the departing alcohol has only a minor effect on the rate of the desufurization and cleavage reactions. In other words, stabilization of the phosphorane intermediate or leaving group by intramolecular hydrogen bonding does not play a role.

Metal-ion-catalyzed cleavage and desulfurization: Zn^{2+}[12]aneN$_3$ promotes the cleavage of phosphorothioate triesters, **1a** and **1b**. The catalytic influence is most likely based on stabilization of the thiophosphorane intermediate by binding of the rather thiophilic Zn^{2+} ion to the sulfide anion. In addition, the aquo ligand of the sulfur-bound Zn^{2+} ion may serve as an intracomplex general acid by donating a proton to the departing 3′-oxyanion.[31] As seen from Figure 3, the catalytic influence of Zn^{2+}[12]aneN$_3$ is more marked with the diastereomeric 2′-OMe-substituted triesters **1b** than with the 2′-OH-substituted **1a**. A possible explanation for this difference is that in case of **1a**, the rate-accelerating effect resulting from the metal-complex binding is partially offset by loss of the stabilizing effect of intramolecular 2′-OH hydrogen bonding with the anionic sulfur ligand. While **1a** is cleaved in the absence of Zn^{2+}[12]aneN$_3$ 27 times as fast as **1b**, the reactivity ratio at saturating con-

centrations of Zn^{2+}[12]aneN$_3$ is five. The situation is qualitatively similar with the phosphorodithioate diesters **2a** and **2b**: the 2′-*O*-methylated **2b** is more susceptible to catalysis by the Zn^{2+} aquo ion. The mechanistic explanation for the difference may well be the same as described for **1a**,**b**. It should be noted, however, that the reaction now proceeds via a dianionic phosphorane and, hence, simultaneous metal-ion binding and 2′-OH hydrogen bonding to the anionic sulfur ligand is, in principle, still possible.

The Cd^{2+} ion accelerates desulfurization in addition to cleavage. In the absence of metal ions, the negatively charged sulfur ligands initially adopt equatorial positions upon formation of the dianionic dithiophosphorane. Evidently, binding of Cd^{2+} to the sulfur atom makes the CdS^+ ligand sufficiently apicophilic to compete with the 3′-linked nucleoside for an apical position. Accordingly, desulfurization by departure of CdS may compete with the cleavage (Scheme 11). As with Zn^{2+}, the cleavage of the 2′-OMe-substituted diester, **2b**, is accelerated by Cd^{2+} more efficiently than its 2′-OH counterpart, **2a**. The cleavage of **2b** is accelerated slightly more markedly than the desulfurization.



Scheme 11. Mechanism of the metal-ion-catalyzed desulfurization of phosphorodithoate diesters **2a** and **2b**.

Conclusion

In summary, comparative kinetic measurements with phosphoromonothioate analogues of trinucleoside 3′,3′,5′-monophosphates **1a** and **1b** and phosphorodithioate analogues of dinucleoside 3′,3′-monophosphates **2a** and **2b** indicate that 2′-OH, as an intramolecular hydrogen-bond donor, accelerates the cleavage of the departing 3′-linked nucleoside. The observed 27-fold rate-acceleration effect is almost as large as that observed previously with the oxygen analogues. In the case of phosphoromonothioate triester **1a**, stabilization of the monoanionic thiophosphorane intermediate by hydrogen bonding of the 2′-OH group of a 3′-linked nucleoside evidently is the major source of the observed rate accelerations, stabilization of the leaving group being only of minor importance: the 2′-OH accelerates as efficiently the departure of both the 3′-and 5′-linked nucleoside. The observed 11-fold rate acceleration of isomerization by the 2′-OH function may be taken as compelling evidence for hydrogen-bond stabilization of the phosphorane intermediate. The thio effect for the cleavage reaction, k_{PO}/k_{PS}, is 19 and independent of the 2′-substitution of the leaving group. In other words, stabilization of the thiophosphorane intermediate by 2′-OH hydrogen bonding is not reflected in the value of the thio effect. The thio effect for the hydroxide-ion-catalyzed isomerization is of the order of 100. Consistent with the proposed stabilization of the thiophosphorane intermediate by hydrogen bonding with the 2′-OH of the departing nucleoside, the difference between the cleavage rates of the 2′-OH (**1a**) and 2′-OMe-substituted (**1b**) triesters is markedly diminished at saturating concentrations of Zn^{2+}[12]aneN$_3$. Evidently binding of Zn^{2+} to the sulfur ligand of the thiophosphorane intermediate does not allow any extra stabilization by hydrogen-bonding and, hence, the cleavage rates of **1a** and **1b** become comparable. Phosphorodithioate diesters **2a**,**b**, which react via a dianionic dithiophosphorane, behave analogously. The rate-retarding effect of 2′-*O*-methylation of the leaving nucleoside is 80% of that observed with the corresponding oxygen diesters, consistent with the fact that sulfur is not as good a hydrogen-bond acceptor as oxygen. The thio effect is small, 1.5 with **2a** and 1.2 with **2b**. As with triesters **1a**,**b**, thiophilic metal ions, Zn^{2+} and Cd^{2+}, accelerate more markedly the cleavage of **2b** than the cleavage of **2a**. Cd^{2+} induces desulfurization, which otherwise occurs only under acidic conditions, in which the reactions proceed via a neutral dithiophosphorane.

Experimental Section

Materials: Nucleosides, uridine 2′,3′-monophosphate and uridine 2′- and 3′-monophosphates, all used as reference materials, were commercial products of Sigma. The preparation of the diasteromeric uridine 2′,3′-cyclic phosphoromonothioates, also used as reference materials, has been described earlier.[12] All the reagents employed were of reagent grade.

2′,3′-*O*-Methyleneadenosin-5′-yl bis[2′-*O*-(4,4′-dimethoxytrityl)-5′-*O*-methyluridin-3′-yl] phosphorothioate (8a): 2′-*O*-(4,4′-Dimethoxytrityl)-5′-*O*-methyluridine[22] (**5**, 0.56 g, 1.00 mmol) was coevaporated three times from anhydrous pyridine. The residue was dissolved in anhydrous MeCN (1.0 mL), and then tris(dimethylamino)phosphine (190 μL, 1.01 mmol) and 1*H*-tetrazole (81.9 mg,1.17 mmol) in anhydrous MeCN (2.6 mL) were added. After being stirred at room temperature for 2 h, the reaction mixture was divided into two portions. 2′-*O*-(4,4′-Dimethoxytrityl)-5′-*O*-methyluridine (**5**, 0.28 g, 0.50 mmol) was coevaporated three times from anhydrous pyridine and the residue was added to one portion of the reaction mixture, followed by 1*H*-tetrazole (69.3 mg, 0.99 mmol) in anhydrous MeCN (2.2 mL). The reaction mixture was stirred at room temperature for 120 h, after which N^6-benzoyl-2′,3′-*O*-methyleneadenosine[23] (**7**, 0.38 g, 0.99 mmol), coevaporated three times from anhydrous pyridine, and 1*H*-tetrazole (63.0 mg, 0.90 mmol) in anhydrous MeCN (2.0 mL) were added. The reaction mixture was stirred at room temperature for 27 h, after which elemental sulfur (0.16 g, 0.62 mmol) and CH_2Cl_2 (1.5 mL) were added. The reaction mixture was stirred at room temperature for 24 h, after which the sulfur was separated by filtration. The filtrate was then neutralized by addition of Et_3N and evaporated to dryness. The residue was dissolved in CH_2Cl_2 and a conventional aq $NaHCO_3$/CH_2Cl_2 workup was carried out. The organic phase was evaporated to

dryness and the residue was dissolved in saturated methanolic ammonia (15 mL). After being stirred at room temperature for 6 h, the reaction mixture was evaporated to dryness and the residue was purified first on a silica-gel column eluting with a mixture of MeOH, Et_3N, and CH_2Cl_2 (4:1:95 v/v) and then by HPLC on a Supelcosil LC-18 column (25 cm × 21.2 mm, 12 μm) eluting with a mixture of water and MeCN (30:70, v/v). The overall yield was 12.7% from **5** (92.8 mg). ^{1}H NMR (500 MHz, [D$_6$]DMSO, 25 °C, TMS): δ = 11.49 (d, 1H, J = 2.1 Hz), 11.36 (d, 1H, J = 2.0 Hz), 8.30 (s, 1H), 8.17 (s, 1H), 7.5–6.7 (m, 28H), 7.16 (d, 1H, J = 8.1 Hz), 7.06 (d, 1H, J = 8.1 Hz), 6.31 (d, 1H, J = 8.0 Hz), 6.19 (d, 1H, J = 8.3 Hz), 6.17 (d, 1H, J = 2.8 Hz), 5.49 (d, 1H, J_1 = 2.1, J_2 = 8.1 Hz), 5.38 (dd, 1H, J_1 = 2.0, J_2 = 8.1 Hz), 5.33 (dd, J_1 = 2.7, J_2 = 6.5 Hz), 5.17 (s, 2H), 4.96 (dd, J_1 = 4.2, J_2 = 6.4 Hz), 4.49 (m, 1H), 4.35 (m, 1H), 4.31 (m, 1H), 4.27 (m, 1H), 4.20 (m, 1H), 4.16 (m, 1H), 4.00 (m, 1H), 3.71 (s, 6H), 3.70 (s, 3H), 3.69 (s, 3H), 3.58–3.48 (m, 2H), 3.28–3.18 (m, 2H), 3.19 (s, 3H), 2.98 (s, 3H), 2.80–2.73 ppm (m, 2H); ^{31}P NMR (202 MHz, [D$_6$]DMSO, 25 °C, H_3PO_4): δ = 66.45 ppm; HRMS (FAB): m/z: calcd for $[M]^-$: 1458.4430; found: 1458.4459.

2′,3′-*O*-Methyleneadenosin-5′-yl 2′-*O*-(4,4′-dimethoxytrityl)-5′-*O*-methyluridin-3′-yl 2′,5′-di-*O*-methyluridin-3′-yl phosphorothioate (8b): 2′,5′-Di-*O*-methyluridine[23] (**6**, 0.136 g, 0.5 mmol), coevaporated three times from anhydrous pyridine, and 1*H*-tetrazole (1.00 mmol, 80.0 mg) in anhydrous MeCN (2.2 mL) were added to the remaining portion of 2′-*O*-(4,4′-dimethoxytrityl)-5′-*O*-methyluridine 3′-(*N*,*N*,*N*′,*N*′-tetramethylphosphoramidite) (0.68 g, 0.5 mmol) in anhydrous MeCN (1.8 mL). The reaction mixture was stirred at room temperature for 120 h, after which N^6-benzoyl-2′,3′-*O*-methyleneadenosine (**7**, 0.38 g, 0.99 mmol), coevaporated three times from anhydrous pyridine, and 1*H*-tetrazole (63.0 mg, 0.90 mmol) in anhydrous MeCN (2.0 mL) were added. The reaction mixture was stirred at room temperature for 27 h, after which elemental sulfur (0.16 g, 0.62 mmol) and CH_2Cl_2 (1.5 mL) were added. The reaction mixture was stirred at room temperature for 24 h. The sulfur was then separated by filtration. The filtrate was neutralized by addition of Et_3N and evaporated to dryness. The residue was dissolved in CH_2Cl_2 and a conventional aq $NaHCO_3$/CH_2Cl_2 workup was carried out. The organic phase was evaporated to dryness and the residue was dissolved in saturated methanolic ammonia (10 mL). After being stirred at room temperature for 6 h, the reaction mixture was evaporated to dryness and the residue was purified first on a silica-gel column, eluting with a mixture of MeOH, Et_3N, and CH_2Cl_2 (4:1:95, v/v) and then by HPLC on a Supelcosil LC-18 column (25 cm × 21.2 mm, 12 μm) eluting with a mixture of water and MeCN (47:53, v/v). Compound **8b** was obtained as two diastereomers in an approximately 2:3 ratio. The overall yield was 9.3% calculated from **6** (54.2 mg). ^{1}H NMR (500 MHz, [D$_6$]DMSO, 25 °C, TMS; for the fast-eluting diastereomer): δ = 11.46 (brs, 2H), 8.31 (s, 1H), 8.18 (s, 1H), 7.69 (d, 1H, J = 8.1 Hz), 7.38 (brs, 2H), 7.33–7.15 (m, 9H), 7.21 (d, 1H, J = 8.1 Hz), 6.77 (m, 4H), 6.27 (d, 1H, J = 8.2 Hz), 6.18 (d, 1H, J = 2.7 Hz), 5.87 (d, 1H, J = 6.1 Hz), 5.74 (d, 1H, J = 8.1 Hz), 5.49 (d, 1H, J = 8.1 Hz), 5.36 (dd, 1H, J_1 = 2.7, J_2 = 6.5 Hz), 5.19 (s, 1H), 5.18 (s, 1H), 5.03 (dd, 1H, J_1 = 4.0, J_2 = 6.4 Hz), 4.95 (m, 1H), 4.29 (m, 3H), 4.25–4.15 (m, 2H), 4.09 (m, 1H), 3.70 (s, 3H), 3.69 (s, 3H), 3.46–3.37 (m, 2H), 3.29 (s, 3H), 3.26 (s, 3H), 3.19 (s, 3H), 3.22–3.10 (m, 2H), 2.75–2.67 ppm (m, 2H); ^{1}H NMR (500 MHz, [D$_6$]DMSO, 25 °C, TMS; for the slowly eluting diastereomer): δ = 11.45 (brs, 2H), 8.31 (s, 1H), 8.15 (s, 1H), 7.68 (d, 1H, J = 8.1 Hz), 7.35 (brs, 2H), 7.42–7.15 (m, 9H), 7.16 (d, 1H, J = 8.1 Hz), 6.82 (m, 4H), 6.23 (d, 1H, J = 2.5 Hz), 6.20 (d, 1H, J = 8.2 Hz), 5.90 (d, 1H, J = 6.1 Hz), 5.76 (d, 1H, J = 8.1 Hz), 5.46 (d, 1H, J = 8.1 Hz), 5.36 (dd, 1H, J_1 = 2.5, J_2 = 6.5 Hz), 5.16 (s, 1H), 5.15 (s, 1H), 5.04 (dd, 1H, J_1 = 3.8, J_2 = 6.4 Hz), 4.95 (m, 1H), 4.34–4.17 (m, 4H), 4.15–4.04 (m, 2H), 3.72 (s, 3H), 3.71 (s, 3H), 3.46–3.37 (m, 2H), 3.25 (s, 3H), 3.19 (s, 3H), 3.18 (s, 3H), 3.22–3.10 (m, 2H), 2.75–2.67 ppm (m, 2H); ^{31}P NMR (202 MHz, [D$_6$]DMSO, 25 °C, H_3PO_4; for the fast-eluting diastereomer): δ = 66.75 ppm; ^{31}P NMR (202 MHz, [D6]DMSO, 25 °C, H_3PO_4; for the slowly eluting diastereomer): δ = 66.13 ppm; HRMS (FAB): m/z: calcd for $[M]^-$: 1170.3280; found: 1170.3283.

Bis(uridin-3′-yl) phosphorodithioate (2a): 2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)uridin3 3′-hydrogenphosphonothioate[24] (**10**, 0.25 g, 0.57 mmol) and 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)uridine[24] (**9**, 0.58 g, 1.21 mmol) were dissolved in 10 mL of a mixture of MeCN and pyridine (1:4, v/v), and then diphenylphosphorochloridate (0.91 g, 3.36 mmol) was added. After stirring for 20 min at ambient temperature, elemental sulfur (0.11 g, 3.36 mmol) was added. After an additional 2 h stirring, the reaction mixture was evaporated to dryness. The crude product was isolated by a conventional aqueous work up and purified on a silica-gel column, eluting with a mixture of CH_2Cl_2 and MeOH (95:5%, v/v). The *tert*-butyldimethylsilyl protecting groups were removed with tetrabutylammonium fluoride (1 mol L^{-1}) in THF (3 mL). After being stirred for 20 h, the solution was evaporated to dryness and the product was purified by reversed-phase chromatography on a Lobar RP-18 column (37 × 440 mm, 40–63 μm), eluting with a mixture of water and MeCN (92:2%, v/v). Finally, the product was desalted and passed through a Dowex 50-W Na^+ (100–200 mesh) column. ^{1}H NMR (500 MHz, D_2O, 25 °C, external TMS): δ = 7.83 (d, 2H, J = 8.0 Hz), 5.90 (d, 2H, J = 5.2 Hz), 5.85 (d, 2H, J = 8.4 Hz), 4.93 (m, 2H), 4.47 (t, 2H, J = 5.2 Hz), 4.38 (m, 1H), 3.91 (dd, 2H, J_1 = 12.8, J_2 = 3.0 Hz), 3.85 ppm (dd, 2H, J_1 = 12.8, J_2 = 4.0 Hz); ^{31}P NMR (202 MHz, D_2O, 25 °C, H_3PO_4): δ = 116.7; ESI-MS: m/z: 581.0.

2′,5′-Di-*O*-methyluridin-3′-yl uridin-3′-yl phosphorodithioate (2b): 2′,5′-Di-*O*-methyluridin-3′-yl uridin-3′-yl phosphorodithioate (**2b**) was prepared in a similar manner as **2a**, but by using 2′,5′-di-*O*-methyluridine[22] (**6**) instead of 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)uridine (**9**). ^{1}H NMR (500 MHz, D_2O, 25 °C, external TMS): δ = 7.82 (d, 1H, J = 8.0 Hz), 7.75 (d, 1H, J = 8.0 Hz), 5.93 (d, 1H, J = 4.5 Hz), 5.86 (d, 1H, J = 4.5 Hz), 5.81 (d, 1H, J = 8.0 Hz), 5.80 (d, 1H, J = 8.0 Hz), 4.98 (m, 1H), 4.89 (m, 1H), 4.24 (m, 1H), 4.43 (m, 2H), 4.34 (m, 1H), 4.18 (t, 1H), 3.87 (dd, 1H J_1 = 13.0, J_2 = 3.0 Hz), 3.81 (dd, 1H, J_1 = 13.0, J_2 = 4.5 Hz), 3.75 (dd, 1H, J_1 = 11.5, J_2 = 2.0 Hz), 3.67 (dd, 1H, J_1 = 11.5, J_2 = 4.5 Hz), 3.44 (s, 3H), 3.35 ppm (s, 3H); ^{31}P NMR (202 MHz, D_2O, 25 °C, H_3PO_4): δ = 116.0 ppm; ESI-MS: m/z: 609.2.

Kinetic measurements: Reactions were carried out in sealed tubes immersed in a thermostated water bath, the temperature of which was adjusted to 25 °C within ±0.1 °C with **1a** and **1b**, and 90 °C with **2a** and **2b**. Prior to the actual kinetic runs, the 2′-*O*-(4,4′-dimethoxytrityl) groups were removed from **8a** and **8b** by adding the starting material in DMSO (3 μL) to 80 μL of 0.1 mol L^{-1} HCl in 1,4-dioxane. After 30 min at 50 °C, 1520 μL of the desired reaction solution (prethermostated to 25 °C) was added. The aliquots were quenched by cooling to 0 °C and adjusting their pH to approximately three with a formic acid buffer when necessary. The oxonium-ion concentration of reaction solutions was adjusted with nitric acid, hydrogen chloride, and sodium hydroxide, and with formate, acetate, 2-(*N*-morpholino)ethanesulfonic acid (MES), (*N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid]) (HEPES) and glycine buffers. The oxonium ion concentrations of the buffer solutions were calculated with the aid of the pK_a values of the buffer acids under the experimental conditions.[32] In addition, the pH values of the solutions were checked with a pH meter. The initial substrate concentration in the kinetic runs was approximately 10^{-4} mol L^{-1}.

The composition of the samples withdrawn at appropriate time intervals was analyzed by HPLC on a Hypersil-Keystone Aquasil C18 column (4 × 150 mm, 5 μm) by using a mixture of 0.06 mol L^{-1} formic acid buffer and MeCN as an eluent. The amount of MeCN was 7% for the first 10 min, after which it was increased linearly to 27% during 20 min. The observed retention times (t_R/min) for the products of **1a** and **1b** (the flow rate was 1.0 mL min^{-1}) were as follows: 35.2 (both diastereomers of **1b**); 32.3 (both diastereomers of **12b**); 32.3 (**1a**); 30.0, 29.4 (diastereomers of **12a**); 27.2 (**13**); 21.1, 18.8, 17.9, 15.8 (**15a**, diastereomers of **16a**, **17**); 23.7, 22.8, 21.4, 20.9 (diastereomers of **15b** and **16b**); 21.7, 19.5, 18.7, 16.8 (diastereomers of **18** and **19**); 7.8 (**6**); 6.8 (**14**); 3.9 (**20**). The nucleosidic products (**6**, **14**, **20**) were identified by spiking with authentic samples. The diester products were identified either as di(uridin-3′-yl)esters (**15a**, **16a**, **17** from **1a**; **15b**, **16b** from **1b**) or uridin-3′-yl adenosine-5′-yl esters (**18**, **19**) by ESI-HPLC/MS. The triester isomers of the starting materials were also identified by ESI-HPLC/MS. Assignment of **12a** and **13** as 2′,3′,5′- and 2′,2′,5′-isomers, respectively, was based on the fact that **12a** appeared as an intermediate of the formation of **13**. In calculations, the total concentrations of di(uridin-3′-yl) esters ([**15a**]+[**16a**]+[**17**] or [**15b**]+[**16b**]), uridin-3′-yl adenosine-5′-yl esters ([**18**]+[**19**]), and isomerized triesters ([**12a**]+[**13**]) were used.

A good separation of the product mixture of **2a** was obtained, when an 8 min isocratic elution with a acetic acid/sodium acetate buffer (0.045/0.015 mol L^{-1}) containing 0.1 mol L^{-1} ammonium chloride and 1% acetonitrile was followed by a linear gradient (32 min) up to 6.0% MeCN. The observed retention times (t_R/min) for the products of **2a** on a Hypersil ODS 5 column (4×250 mm, 5 μm) at flow rate 1 mL min^{-1} were: 27.0, 21.8, 19.8 (**23a**, **24a**, **25**); 22.2, 21.8, 18.6, 17.6 (fully desulfurized diesters); 14.2 (**21**); 11.7 (R_P-**26a**); 7.1 (**22**); 6.1 (S_P-**26a**) and 4.5 (**26b**). The observed retention time for the starting material **2a** was 38.5 min. With **2b**, a sufficient separation of the product mixture was obtained, when an 8 min isocratic elution with buffer was followed by a linear gradient (57 min) up to 14.0% acetonitrile. The observed retention times (t_R/min) for the products of **2b** were as follows: 39.7, 37.8, 31.2 (R_P-**23b**, S_P-**23b**, R_P/S_P-**24b**); 28.3, 27.2 (desulfurized diesters); 18.1 (**21**); 16.0 (R_P-**26**); 9.5 (**22**); 7.2 (S_P-**26**); 7.0 (2′-UMP); 5.3 (**26b**); 5.8 (3′-UMP); and 4.5 (uracil). The observed retention time for starting materials **4** was 49.7. The products were either characterized by spiking with authentic samples monomeric nucleoside/nucleotides or identified as phosphoromonothioate diesters (**23a**, **24a**, **25** from **2a**; **23b**, **24b** from **2b**) or respective fully desulfurized diesters by mass spectrometric analysis (HPLC/ESI-MS) by using a mixture of MeCN and aqueous fomic acid (0.4%) as an eluent. In calculations, the total concentration of phosphoromonothioate diesters ([**23a**]+[**24a**]+[**25**] or [**23b**]+[**24b**]) was used.

The signal areas of the nucleotide analogues, obtained as products from hydrolysis of **2a** and **2b**, were assumed to be proportional to concentrations, as the base moiety of all the compounds was the same. With the dinucleoside monophosphates and their thioate analogues, the molar absorptivities of the two base moieties were assumed to be additive. In the case of **1a** and **1b**, the peak areas were converted to relative concentrations by calibrating the system with uridine and adenosine solutions of known concentrations. The contribution of the buffer catalysis to the observed rate constants of **2a** and **2b** was insignificant 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 starting materials were obtained by applying the integrated first-order rate equation to the time-dependent concentration of the starting material. In cases for which desulfurization competed with the cleavage, the first-order rate constants (k_{ds}) for the desulfurization of **2a** were calculated by applying the kinetics of consecutive parallel first-order reactions [Eq. (6)). In Equation (6), k_d is the first-order rate constant for the disappearance of **2a**, $[\mathbf{2a}]_0$ stands for the initial concentration of **2a** and $[\mathbf{23}–\mathbf{25}]_t$ denotes the sum concentration of all diastereomeric forms of **23a**, **24a**, and **25** at moment *t*. k_1 is the first-order rate constant for the disappearance of the latter compounds.

$$[\mathbf{23}-\mathbf{25}]_t/[\mathbf{2a}]_0 = [k_{ds}/(k_1-k_d)] \times [\exp(-k_d t)-\exp(-k_1 t)] \quad (6)$$

In the case of **2b**, the first-order rate constants (k_{ds}) for the desulfurization were obtained by breakdown of the rate constant of the disappearance of the starting material (k_d) to the rate constants of the two parallel first-order reactions (desulfurization and cleavage) on the basis of the product distribution at the early stages of the reaction. The same method was applied to obtain the rate constants for the parallel reactions of triesters **1a** and **1b**. The resulting diesters did not undergo further decomposition during the time used to follow the disappearance of the triesters. The isomeric triesters, the R_P- and S_P-diasteromers **1a**, **12a** and **13** on one hand, and the R_P- and S_P-diasteromers of **1b** and **12b** on the other hand, were assumed to react at equal rates.

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