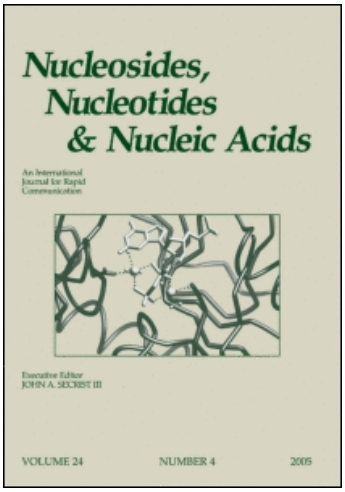

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PREPARATION, HYDROLYSIS AND INTRAMOLECULAR TRANSESTERIFICATION OF 3'-DEOXY-3'-THIOINOSINE 3'-S-DIMETHYLPHOSPHOROTHIOLATE

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ABSTRACT: The hydrolytic reactions of the dimethyl ester of 3'-deoxy-3'-thioinosine 3'-*S*-phosphorothiolate have been followed over a wide aciditiy range by HPLC. At pH > 3, only hydroxide ion catalyzed isomerization to the 2'-dimethylphosphate takes place, whereas under more acidic conditions hydrolysis to the 2'-monomethylphosphate and 3'-*S*-monomethylphosphorothiolate competes. The latter is the only product accumulating in very acidic solutions (1 M hydrochloric acid). Mechanisms of the reactions are discussed.

Among various thiophosphate analogues of nucleotides, the 3'-*S*-phosphorothiolate analogs of ribonucleotides have recently been introduced as tools for mechanistic studies of ribozyme reactions and RNA splicing.[1-4] To elucidate the chemical reactivities of this class of thiophosphate esters in comparison to the corresponding oxyphosphate esters, we have recently carried out a detailed kinetic study on the cleavage and isomerization reactions of 3'-deoxy-3'-thioinosylyl-(3',5')-uridine (**1**; IspU), an RNA dimer containing a 3'-*S*-phosphorothiolate linkage.[5] In the present work, these studies have been extended to the reactivity of a ribonucleoside 3'-phosphorothiolate triester, the dimethyl ester of 3'-deoxy-3'-thioinosine 3'-*S*-phosphorothiolate (**6**), which may be regarded as a mimic of the neutral form of the corresponding phosphorothiolate diester. Besides elucidating the contributions of the various ionic forms to the reactivity of the diester, the present work was aimed at furthering the understanding of the mechanisms behind the destabilizing effects[1,5] of the 3'-thiosubstitution. The kinetics and mechanisms of the reactions are

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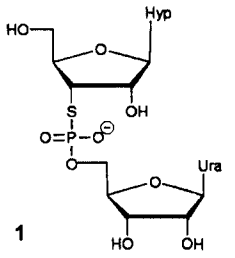
discussed also in comparison with the results obtained earlier with the corresponding nucleoside phosphate[6] and phosphorothioate[7] dialkylesters (**2a,b**).



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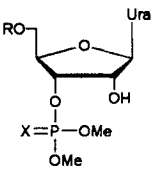
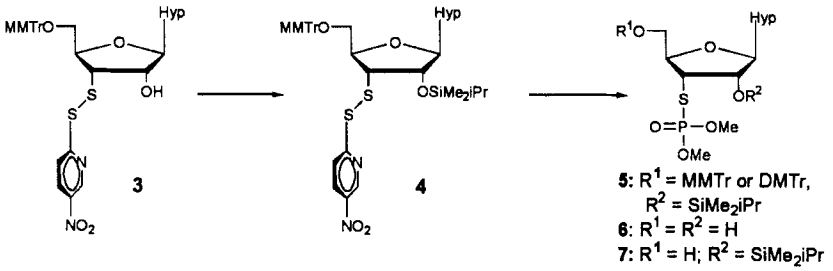


2a : X= O; R = Me or pivaloyl
2b: X= S; R= Me

RESULTS AND DISCUSSION

Synthesis. Compound **5** was synthesized by a previously described procedure,[1,8] with the exception that the *tert*-butyldimethylsilyl (TBDMS) group used earlier for the 2'-*O*-protection was replaced with a more acid labile isopropyldimethylsilyl group. Accordingly, inosine was converted to 3'-deoxy-5'-*O*-(4-methoxytrityl) or (4,4'-dimethoxytrityl)-3'-thioinosine, and the mercapto function was then activated as an aryl disulfide (Scheme 1).[1] The isopropyldimethylsilyl group was introduced by reacting the activated nucleoside (**3**) with the appropriate silyl chloride in pyridine, and the resulting compound (**4**) was phosphitylated with trimethylphosphite in THF to give the 3'-*S*-dimethylphosphorothiolate in a 2',5'-protected form (**5**). The crude product was purified by RP-HPLC and characterized by ^{31}P and ^{1}H NMR spectroscopy and ESI-MS. The detritylated and desilylated product **6** was too unstable for full spectroscopic characterization, but its identity was verified by HPLC/ESI-MS.

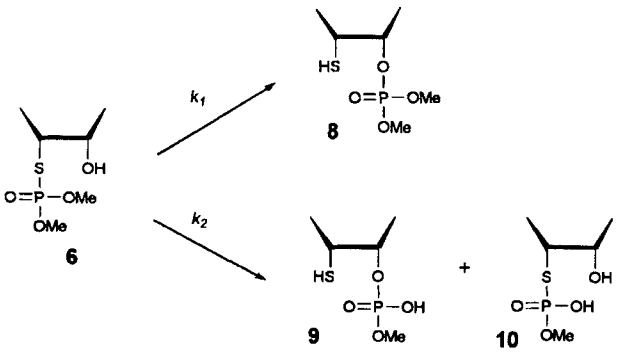


SCHEME 1

Replacing the 2'-*O*-TBDMS protection with a more acid labile isopropyldimethylsilyl group was an indispensable modification to the previously reported route, since it allowed, in contrast to the TBDMS protection, removal of the 2'- and 5'-*O*-protecting groups under conditions that left the phosphorothiolate triester moiety intact. The 5'-*O*-(4-methoxytrityl) group could be removed selectively with 10 % trifluoroacetic acid in a mixture of dichloromethane and methanol in about 70 minutes, and the isopropyl-dimethylsilyl group that withstood this treatment could then be removed with aqueous HCl (0.1-1.0 M) at 0 °C in 10-20 minutes without cleaving the P-S3' bond.

Kinetics of the hydrolysis and 3'S → 2'O isomerization. The reactions of deprotected 3'-deoxy-3'-thioinosine 3'-*S*-dimethylphosphorothiolate (**6**) were followed over an acidity range H_0 -0.2 to pH 5.0 at 273 K by determining the time-dependent product distribution by RP-HPLC. The results obtained indicate that the only buffer-independent reaction that **6** undergoes at pH > 3 is hydroxide ion catalyzed isomerization to the 2'-*O*-dimethyl-phosphate (**8**), whereas under more acidic conditions cleavage to a mixture of the 2'-*O*-(**9**) and 3'-*S*-monomethylphosphates (**10**) also takes place (Scheme 2). The isomerization of **6** to **8** is irreversible; the 2'-dimethylphosphate (**8**) showed no indication of isomerization back to **6**, although it is under both acidic and alkaline conditions hydrolytically much more stable than **6**.

The isomerization is first-order in hydroxide ion concentration at pH > 3 (Table 1), indicating that the reactive nucleophile is the 2'-oxyanion, in spite of the fact that the proportion of this ionic form is very low under acidic conditions (pK_a of the 2'-OH falls in



SCHEME 2

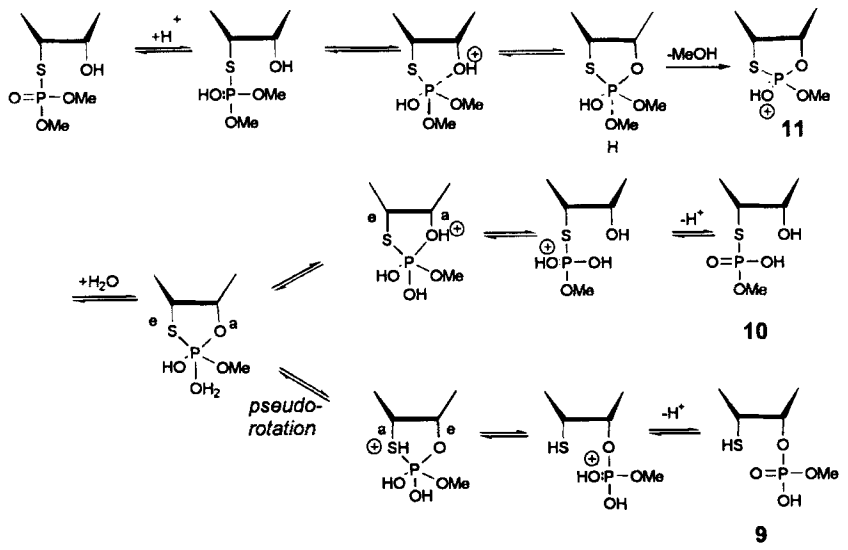
Table 1. The First-order Rate Constants for the Decomposition ($k_1 + k_2$, see Scheme 2) of 3'-Deoxy-3'-thioinosine 3'-*S*-dimethylphosphorothiolate **6** at 273 K.[a]

pH	$(k_1 + k_2)/ 10^{-4}$ s^{-1}	% isomerization
-0.2 (H_0)	25 ± 0.7	0
0.2 (H_0)	4.7 0.2	9
0.6	2.8 0.1	14
1.0	2.4 0.1	17
2.0	1.6 0.1	60
3.0	3.2[b] 0.2	100
	5.5[c] 0.2	~76
4.0	7.9[d] 0.2	100
	13.5[e] 0.3	~91
5.0	92[f] 3	100
	147[g] 6	100

[a]The ionic strength was adjusted to 0.1 M with sodium chloride. [b]Formic acid/sodium formate buffer (0.02 M/0.0033 M). [c]Formic acid/sodium formate (0.08/0.013 M).[d] Acetic acid/sodium acetate (0.02/0.0033 M).[e]Acetic acid/sodium acetate (0.08/0.013 M). [f]Acetic acid/sodium acetate (0.02/0.033 M). [g]Acetic acid/sodium acetate (0.08/0.13 M).

the range 12 - 13).[1] As mentioned above, no buffer-independent hydrolysis to the phosphate monomethyl esters (**9**,**10**) could be observed at pH > 3. Only at high buffer concentrations the isomerization was accompanied by one order of magnitude slower hydrolysis. Evidently the buffer concentration has only a modest effect on the rate of isomerization, whereas the hydrolysis is rather susceptible to buffer catalysis, analogously to the situation with the correponding phosphate triesters.[6,7]

Under more acidic conditions (pH < 3), hydrolysis of **6** to a mixture of the monomethyl esters **9** and **10** competes with the isomerization, and becomes the predominant pathway at pH < 1. In the acidity range from pH 3 to H_0 0.6, the overall rate of the disappearance of **6** is rather independent of acidity, but the hydrolysis gradually takes over the isomerization. Under these conditions, only the 2'-methylphosphate **9** accumulates as a product of hydrolysis. By contrast, under very acidic conditions ([HCl] > 0.5 M), the hydrolysis turns to acid-catalyzed and, interestingly, the product distribution is changed to favor the formation of the 3'-*S*-monomethylphosphate **10**, instead of the 2'-methylphosphate **9**. In 1 M aqueous hydrogen chloride, **10** already was the only product detected. It should be noted that the isomerization product **8** turned to be hydrolytically much more stable than the starting material, and hence **9** must be formed directly form **6**,



SCHEME 3

not *via* intermediary accumulation of **8**. Accordingly, no indication of acid-catalysis for isomerization of **6** to **8** could be observed.

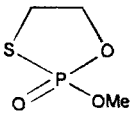
Mechanism of the hydrolysis. The main difference between the hydrolytic behaviour of **6** and its previously studied[6] oxygen analog, uridine 3'-dimethylphosphate **2a**, is that while the latter undergoes concurrent hydronium ion catalyzed hydrolysis to phosphodiesters and isomerization to the 2'-dimethylphosphate, the only acid-catalyzed reaction of **6** seems to be the hydrolysis to the 2'-phosphodiester **9** and 3'-*S*-phosphorothiolate diester **10**. As mentioned above, in 1 M aqueous hydrogen chloride the accumulation of **8** does not exceed the limit of detection. According to separate experiments **8** is, however, so stable in aqueous acid that it should clearly be accumulated if formed even to a small extent during the hydrolysis of **6**. Accordingly, if an acid-catalyzed pathway for the 3'S → 2'O phosphoryl migration exists, this reaction must be at least one order of magnitude – more likely up to two orders of magnitude – slower than the hydrolysis.

In all likelihood, the phosphoester hydrolysis of **6** occurs by an intramolecular attack of the 2'-hydroxy group on the protonated phosphorothiolate triester group (Scheme 3), although the isomerization product that would be a clear indication of the intramolecular nature of the reaction is not formed. The reactions are, however, too fast to be accounted for by intermolecular hydrolysis of phosphorothiolate triesters.[9] The attack of the 2'-

hydroxy function on the protonated 3'-*S*-phosphotriester group leads to formation of a monocationic thiophosphorane intermediate. According to Westheimer's concept of pseudorotating phosphorane intermediates,[10] the attacking sugar-oxygen (2'O) initially adopts an apical position and the ring strain of the resulting five membered ring forces the 3'S to an equatorial position. Since the departure of the leaving group may also take place only from an apical position, isomerization requires pseudorotation of the thiophosphorane intermediate to bring the 3'S atom apical. The lack of isomerization concurrent with the acid-catalyzed hydrolysis strongly suggests that the exocyclic departure of methanol from the thiophosphorane intermediate must be fast compared to the pseudorotation transferring the sulfur atom from equatorial to apical position. If the pseudorotation were fast compared to the breakdown of the thiophosphorane intermediate, the leaving-group ability of various ligands would determine the product distribution, according to the Curtin-Hammett principle.[11] Accordingly, the isomerization proceeding by the departure of a thiol could be expected to compete with the hydrolysis proceeding by the departure of an alcohol. Evidently the 3'-thio substitution markedly increases the pseudorotation barrier, since with uridine 3'-dialkylphosphates the acid-catalyzed isomerization efficiently competes with the hydrolysis.

The breakdown of the thiophosphorane intermediate by the cleavage of methanol gives a cyclic phosphorothiolate triester **11** (Scheme 3), which is rapidly hydrolyzed to either the 2'-phosphodiester **9** or the 3'-*S*-phosphorothiolate diester **10**. Formation of the latter product appears to be favored by the increasing hydronium ion concentration. The third potential product, the 2'-*O*,3'-*S*-cyclic phosphorothiolate, did not accumulate. This product distribution is, in fact, similar to that reported earlier[12] for the hydrolysis of the methyl ester of *O*,*S*-ethylene phosphorothiolate (**12**), an analog of the cyclic phosphorothiolate moiety of **11**. Even in the hydrolysis of **12** the P-S cleavage is predominating at pH > 2, but under more acidic conditions its proportion strongly decreases. At pH 0, already 90 % of the hydrolysis of **12** proceeds by cleavage of the endocyclic P-O bond. The product distribution was suggested[12] even in this case to be consistent with a retarded pseudorotation of the protonated phosphorothiolate. While it can be assumed that the sulfur atom will initially occupy an equatorial position in the thiophosphorane intermediate formed by attack of a water molecule on the phosphorus, the favored P-O cleavage implies that the barrier of pseudorotation of the protonated

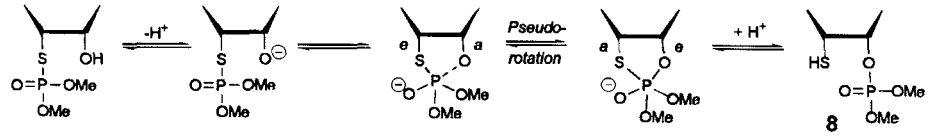
intermediate is higher than the barrier of the ring-opening by P-O bond rupture. One factor possibly favoring the P-O cleavage with the protonated compound is that the oxygen atom may be more easily protonated than the sulfur atom, and thus departure of the alkoxy ligand can be more effectively assisted by protonation.



12

Mechanism of the isomerization. While the pseudorotation appears to be unable to compete with the cleavage of protonated methoxy ligand from the monocationic thiophosphorane intermediate derived from **6**, the situation is entirely different when hydroxide ion catalyzed reactions, proceeding by the attack of 2'-oxyanion and departure of either sulfide or alkoxide ion, are concerned. This kind of isomerization of **6** to **8** is the predominant reaction at pH > 3 (Scheme 4). Pseudorotation of the thiophosphorane is required to bring the 3'S atom to an apical position, from where it may depart as a sulfide ion. Evidently the pseudorotation is fast, and the product distribution is determined by the relative leaving-group abilities of the alkoxy and alkylthio ligands; an alkylsulfide ion is much less basic than an alkoxide ion, and hence the cleavage of the P-S bond predominates. It is worth noting that the hydroxide ion catalyzed isomerization is approximately 10^5 times faster than the hydrolysis even with uridine 3'-dimethylphosphate[6b,7], suggesting that opening of the five-membered ring probably is an extra rate-accelerating factor.

While the hydronium ion catalyzed hydrolysis predominates at pH < 1 and hydroxide ion catalyzed isomerization at pH > 3, between these pH values the rates of both reactions seem to be rather independent of pH. In all likelihood a pH-independent pathway giving both the hydrolysis and isomerization products exists, but it is significant only over a



SCHEME 4

narrow pH range. The reaction may be assumed to proceed by nucleophilic attack of a 2'-hydroxyl group on a neutral triester and departure of the alkoxy/alkylthio ligand as an alcohol/thiol.

Thio effects. The significant acceleration that the 3'-thio substitution exerts on the rate of hydrolysis and isomerization of the 3'-phosphorothiolate triester **6** is in striking contrast to the small or moderately rate retarding effects of a *non*bridging sulfur on the corresponding reactions of **2b**.[7] Evidently the rate-acceleration at least partially results from the fact that the 3'-thio substitution makes the phosphoester group more susceptible to the attack of the neighbouring nucleophile. For example, the hydronium ion catalyzed hydrolysis not involving P-S bond cleavage (*i.e.* hydrolysis of **6** to **10**) is 10 to 20 times as fast as the corresponding reaction of 5'-*O*-methyluridine 3'-dimethylphosphate (**2a**).[6a] By contrast, with uridine 3'-dimethylphosphorothioate (**2b**) containing a nonbridging sulfur, the thio effect in acid-catalyzed hydrolysis is rate-retarding, $k_{s\text{-}p}/k_{o\text{-}p}$ being about 0.02 at 363 K.[7]

The "3'-bridging thio-effect" ($k_{s\text{-}p}/k_{o\text{-}p}$) observed for the base-catalyzed isomerization of the triester **6** falls in the range 20 to 30, being hence comparable to the 50-fold rate-acceleration observed[5] in the pH-independent isomerization of the dinucleoside monophosphates (**1** compared to its oxyanalogs), *i.e.* for a reaction suggested to proceed by an attack of a 2'-oxyanion on the neutral ionic form of the phosphodiester. In the latter cases the superior leaving-group ability of the alkyl sulfide ion over alkoxide ions may also contribute to the thio effects. However, we may note that these thio effects are orders of magnitude lower than the 10^5 fold rate acceleration brought about by thiosubstitution of the 5'-bridging oxygen in the hydroxide-ion-catalyzed hydrolysis of a dinucleoside monophosphate.[13] In contrast to the reactions discussed above, the latter reaction takes place by an in-line (S_N2-type) displacement of the 5'-linked nucleoside by a 2'-alkoxide ion, without formation of a thiophosphorane intermediate able to undergo pseudorotation.[14]

EXPERIMENTAL SECTION

General Methods. ^{31}P, ^{1}H NMR spectra were recorded on JEOL Lambda 400 NMR spectrometer operating at 399.78 MHz for ^{1}H and 161.70 MHz for ^{31}P. The ^{1}H NMR spectra were referenced to tetramethylsilane (TMS), and ^{31}P NMR spectra to an external

85 % phosphoric acid. The MS analyses were performed on Perkin-Elmer API 365 Triple Quadrupole LC/ESI/MS spectrometer.

Materials.

9-[5-O-(monomethoxytrityl)-3-deoxy-3-thio-3-S-(5-nitropyridyl-2-disulfanyl)-2-O-(isopropyldimethylsilyl)-β-D-ribofuranosyl] hypoxanthine (**4**). 100 mg of the aryldisulfide **3**, prepared by a procedure previously described,[8] was dissolved in dry pyridine (5 mL) and 50 μL (2 equi.) of isopropyldimethylsilyl chloride was added. The reaction mixture was stirred overnight and partitioned between dichloromethane and saturated aqueous $NaHCO_3$. The organic layer was washed with water, dried over Na_2SO_4 and evaporated to dryness. The crude product was purified on a silica gel column with a mixture of dichloromethane and methanol (98:2) containing 0.1 % of triethylamine or pyridine. Appropriate fractions were pooled and evaporated *in vacuo* to give **4** in *c.a.* 80 % yield: ^{1}H NMR ($CDCl_3$): δ 9.31 (d, J = 1.9 Hz), 8.21 (dd, J_1 = 2.8, J_2 = 9.0 Hz), 8.04 (s), 7.97 (s), 7.64 (d, J = 8.8 Hz), 7.36-7.21 (12 H, m, trityl H), 6.77 (2 H, d, J = 8.8 Hz, anisyl H), 6.03 (d, J = 2.4 Hz), 4.94 (dd), 4.56 (m), 3.96 (dd), 3.77 (3 H, s, trityl OMe), 3.68 (dd), 3.38 (dd), 1.0 (7 H, m, isopropyl), 0.17 (6 H, d, $SiMe_2$). ESI-MS (positive): *m/z* 811.4 $[M+ H]^+$, 912.4 $[M+ Et_3N]^+$.

9-[5-O-(Monomethoxytrityl)-3-deoxy-3-thio-3-S-(O,O-dimethylphosphorothiolate)-2-O-(isopropyldimethylsilyl)-β-D-ribofuranosyl] hypoxanthine (**5**). To a stirred solution of the disulfide **4** (70 mg) in dichloromethane or THF (5 mL) was added 45 μL (3 equi.) of trimethylphosphite and the resulting solution was stirred overnight. The reaction mixture was partitioned between dichloromethane and saturated aqueous $NaHCO_3$ and the dichloromethane layer was washed with water, dried over Na_2SO_4 and evaporated to dryness. The crude product was purified by HPLC on a LiChrospher RP-18 column eluting with 80 % aqueous acetonitrile. ^{1}H NMR ($CDCl_3$) δ 8.06 (s, hypoxanthine), 8.03 (s, hypoxanthine), 7.52-7.00 (12 H, m, trityl H), 6.84 (2H, d, J = 9.04 Hz, anisyl H), 6.05 (d, J = 1.2 Hz, H1'), 4.69 (d, J = 4.6 Hz, H2'), 4.48 (m, H3'), 3.97 (m, H4'), 3.78 (3H, s, trityl OMe), 3.58 (2H, m, H5'a and H5'b), 3.64 (3H, d, J_{PH} = 12.7 Hz, POMe), 3.05 (3H, d, J_{PH}= 12.76 Hz, POMe), 1.0 (7 H, d, isopropyl), 0.18 (6H, d, $SiMe_2$); ^{31}P NMR ($CDCl_3$) δ 29.32; ESI-MS (positive): *m/z* 765 $[M+H]^+$, 787.2 $[M+Na]^+$, 803.4 $[M+K]^+$; ESI-MS (negative) *m/z* 763.4 [M-1].

Deprotection. The 2',5'-protecting groups of 5 were removed separately for each kinetic run. 5'-MMTr was removed with 10 % trifluoroacetic acid in a 7:2 mixture of dichloromethane and methanol in 70 min at 22 °C, after which the volatile components were evaporated in an ice-bath. The residue was dissolved in a small amount of 0.1 M aqueous hydrochloric acid (at 0 °C), where removal of the 2'-O-silyl protection was completed in about 10 min. The kinetic run was initiated immediately after deprotection by adding an appropriate pre-buffered solution to adjust the reaction conditions (Table 1).

Kinetic measurements. Reactions were carried out in stoppered tubes immersed in an ice-water bath (273 K). 50 μL aliquots were withdrawn at appropriate intervals. Composition of the samples was analyzed by HPLC on a Hypersil ODS 5 column (either 250-4 or 125-4 mm) eluted with a mixture of 0.05 M aqueous formic acid and acetonitrile. With the 250 mm column, using 14 % (*v/v*) acetonitrile in the eluent and flow rate 1 mL min^{-1}, the starting material **6** and its 2'-*O*-phosphotriester isomer **8** were eluted in 4.8 and 9.0 min, respectively. The monomethylester isomers **9** and **10** were eluted with 2 % acetonitrile in 8.8 and 7.6 min, respectively. Assignement of **9** and **10** was based on the finding that the slower migrating of these was the product formed, when the 2'-dimethylphosphate **8** was hydrolyzed in 0.1 M aqueous sodium hydroxide at 363.2 K.[15] Under the same conditions, the faster migrating diester undergoes a fast hydrolysis to a 3'-thioinosine 3'-*S*-phosphorothiolate monoester, as is expected for a 3'-*S*-phosphorothiolate monoalkylester.[5] For the mass spectrometric characterization of the products, see Table 2.

Table 2. Mass Spectrometric Characterization[a] for the Protected and Unprotected Forms of 3'-Deoxy-3'-thioinosine 3'-*S*-dimethylphosphorothiolate (**6**), and for the Hydrolytic Products of **6**.

Compound	*m/z*				
	M^+	$[M+H]^+$	$[M+Na]^+$	$[M+K]^+$	M(calculated)
5 (5'-*O*-DMTr)[b]	-	795.3	817.2	833.4	794
5 (5'-*O*-MMTr)[c]	-	765.3	787.2	803.4	764
6	393.1	-	-	-	393
7	493.3	-	515.3	531	493
8	393.2	-	-	-	393
9	379.1	-	-	-	379
10	379.3	-	-	-	379

[a]By HPLC/ESI-MS directly from the aliquots of the kinetic runs. [b,c]The fully protected compounds were isolated and purified by HPLC.

The first-order rate constants for disappearance of the starting material were calculated by applying the time-dependent diminution of the integrated peak area of 4 to the integrated form of the first-order rate equation. Peak areas of all the nucleoside derivatives occurring in the reactions were assumed to be proportional to concentrations, since the chromophoric base moiety was the same. Kinetic data are given in Table 1.

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