Hydrolytic Reactions of the *cis*-Methyl Ester of 3′-Deoxy-3′-thiothymidine 3′,5′-Cyclic(phosphorothiolate)

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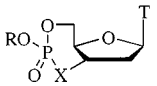
Hydrolysis of the *cis*-methyl ester of 3′-deoxy-3′-thiothymidine 3′-*S*,5′-*O*-cyclic(phosphorothiolate) (**1a**) has been followed by HPLC and MS. At pH < 2 hydrolysis of the thiophosphate triester moiety is acid-catalyzed (first order), while between pH = 2 and 5 the reaction is pH-independent and at pH > 5 first order in hydroxide ion. The uncatalyzed and acid-catalyzed reactions yield two thiophosphate diesters, the 3′-*S*,5′-*O*-cyclic phosphorothiolate **2** and 3′-*S*-phosphorothiolate methyl ester **3**, in a 9:1 and 1:3 molar ratio, respectively. The hydroxide ion catalyzed reaction gives the endocyclic P–O and P–S bond-cleavage products (**3** and **4**, respectively) in a 1:2 molar ratio. The pH-independent reaction is suggested to take place by attack of a water molecule on the carbon atom and concomitant C–O bond rupture, whereas the alkaline and acidic reactions involve attack of the nucleophile on the phosphorus atom and formation of a pseudorotating thiophosphorane intermediate. Under acidic conditions, cleavage of the *N*-glycosidic linkage competes with the phosphoester hydrolysis, corresponding to 20% of the hydrolysis products at pH < 1.

Introduction

In recent years a number of thiophosphate analogs of nucleotides have been introduced either as potential chemotherapeutic agents[1] or as nucleic acid mimics for mechanistic studies.[2] The title compound, the *cis*-methyl ester of 3′-deoxy-3′-thiothymidine 3′,5′-cyclic(phosphorothiolate) (**1a**), belongs to the former category. It was originally prepared[3] as a model compound for elucidation of the applicability of phosphorothiolate triesters as pro-drugs of antiviral nucleotides; intracellular hydrolysis of the P−S bond was aimed at releasing the nucleoside 5′-phosphate. For this reason, and to further the inadequate understanding of the hydrolytic reactions of nucleoside 3′-*S*-phosphorothiolates in general, we undertook a detailed study on the kinetics and mechanisms of the hydrolysis of **1a**. The results are compared with those presented previously[4] for the *cis*-phenyl ester of thymidine 3′,5′-cyclic(monophosphate) (**1b**), an oxyphosphate triester counterpart of **1a**.



1a: R= Me; X= S
1b: R= Ph; X= O

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Results and Discussion

Product Distributions and pH-Rate Profile

The hydrolysis of cyclic(phosphorothiolate) **1a** was followed over a wide acidity range ($H_0 = -0.2 \rightarrow$ pH = 7.6) by determining the time-dependent product distributions by HPLC (Table 1). The products detected (Scheme 1) were assigned by mass-spectrometric analysis (HPLC/ESI-MS; Table 2). As seen from Table 1, the buffer-independent hydrolysis of **1a** is a hydronium ion catalyzed reaction at pH < 2, a pH-independent reaction from pH = 2 to 5, and a hydroxide ion catalyzed reaction at pH > 5. The product distributions of all these reactions are dissimilar. However, at each pH studied the product distribution remained constant during the first half-life of the reaction, showing that only parallel first-order reactions were involved.

Table 1. The first-order rate constants and relative product distributions (see Scheme 1) for the hydrolysis of the *cis*-methyl ester of 3′-deoxy-3′-thiothymidine 3′,5′-cyclic(phosphorothiolate) (**1a**) at 363.2 K

pH	$k_{obs}/10^{-4}$ s^{-1} [a][b]	100 × $c_i/c_{products\ tot.}$ Thy	**2**	**3**	**4**
7.6	151 ± 3	–	–	27	73
6.6	21.5 ± 0.5	–	15	23	62
5.6	4.20 ± 0.05	–	38	21	41
4.4	0.695 ± 0.007	–	90	10	–
3.0	0.560 ± 0.007	–	92	8	–
2.0	0.668 ± 0.009	4	82	14	–
1.0	1.83 ± 0.02	8	43	49	–
0.2 (H_0)	6.48 ± 0.06	14	23	63	–
−0.2 (H_0)	11.7 ± 0.1	19	22	59	–

[a] For the disappearance of **1a**. – [b] The ionic strength of the solutions was adjusted to 0.1 M with sodium chloride.

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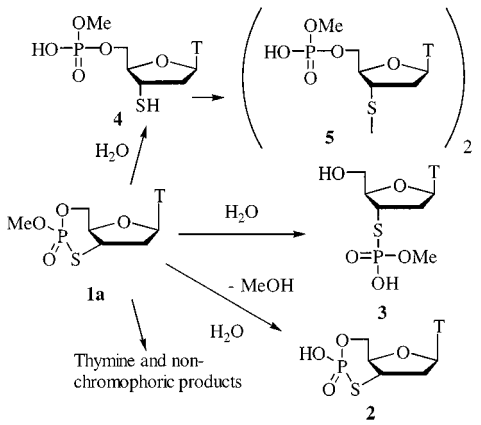


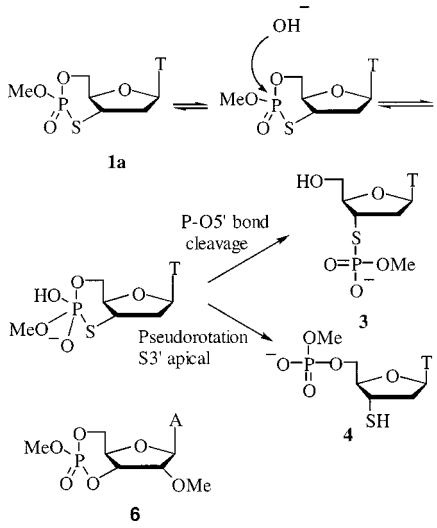
Scheme 1

Table 2. HPLC retention times and mass-spectrometric characterization for the hydrolysis products of the *cis*-methyl ester of 3′-deoxy-3′-thiothymidine 3′-*S*,5′-*O*-cyclic(phosphorothiolate) (**1a**)

Compound	t_R[b]/min	*m*/*z*[a] [M − 1]	[M + 1]$^+$	[M + Na]$^+$	$M_{calcd.}$
1a	29		335.2	357.0	335
2	3.4	319.2	321.2	343.0	320
3[c]	3.8	351.3	353.0	375.0	352
4	5.0		353.0	375.0	352
5	24		703.3	725.3	702
Thymine[c]	2.8		127.2		126

[a] By HPLC/ESI-MS directly from the aliquots of kinetic runs. − [b] On a Hypersil ODS 5 column (4−250 mm, 5μm), eluted with an acetic acid/sodium acetate buffer (0.045/0.015 M) containing 9% (*v*/*v*) acetonitrile at a flow rate 1.0 mL/min. UV detection at wavelength 267 nm. − [c] Assignment could be verified by spiking with an authentic sample (see Experimental Section).

formation of the thiophosphorane intermediate.[9] Accordingly, the P−S bond may be cleaved only after pseudorotation that brings the sulfur atom into an apical position. If the pseudorotation was a rapid pre-equilibrium process, the leaving-group abilities of the 3′-sulfide and 5′-alkoxide would, according to the Curtin−Hammett principle,[10] determine the product distribution. Since the P−S3′ bond is cleaved only twice as rapidly as the P−O5′ bond, this is not the case. In all likelihood, the energy barrier for pseudorotation is only slightly lower than the barrier for the rupture of the apical P−O5′ bond, and hence the competition between the pseudorotation and P−O5′ bond cleavage determines the product distribution.



Scheme 2

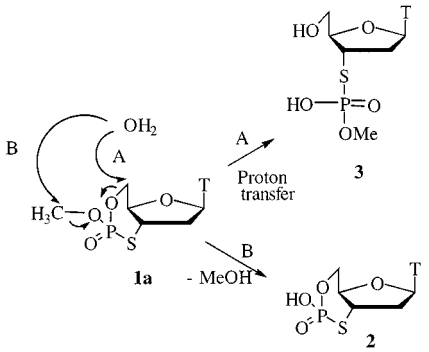
Mechanism of the Hydroxide Ion Catalyzed Hydrolysis

The alkaline hydrolysis of phosphorothiolate triesters has been suggested to proceed by a nucleophilic attack of a hydroxide ion on the phosphorus atom.[5,6] A monoanionic pentacoordinated thiophosphorane intermediate is formed, which may undergo pseudorotation (Scheme 2). The product distribution is thus determined by the relative apicophilicities and leaving-group abilities of the ligands. Since a sulfide ion is a much better leaving group than an alkoxide ion, the observed predominance of the 5′-phosphodiester **4** among the hydrolysis products of **1a** is expected. Thus, while in the hydrolysis of an oxyphosphate triester analog, the *cis*-methyl ester of 2′-*O*-methyladenosine 3′,5′-cyclic(phosphate) (**6**), the 3′-phosphodiester product predominates over the 5′-product in about a 2:1 molar ratio,[7] with **1a** the corresponding ratio is 1:2. However, bearing in mind that thiols are five orders of magnitude more acidic than alcohols,[8] the difference in the product distributions is unexpectedly small. Evidently the leaving-group ability does not alone determine the reaction pathway, but the apicophilicity also plays an important role. Sulfur is a considerably less electronegative element than oxygen, and hence the 3′-sulfur probably adopts an equatorial position upon

Replacment of the 3′-oxygen atom of a nucleoside 3′,5′-cyclic(phosphotriester) with sulfur results in a marked rate acceleration in the hydroxide ion catalyzed hydrolysis. At pH = 8, **1a** is hydrolyzed about 70 times faster than **1b**. Most likely the thio substitution facilitates the nucleophilic attack of a hydroxide ion on the phosphorus atom. As described above, the alkaline hydrolysis of **1a** does not proceed solely by a P−S bond cleavage, but also by rupture of the P−O5′ bond. Accordingly, the cleavage of the latter bond is also accelerated compared to the oxyphosphate analog and the overall rate acceleration thus cannot be attributed to the superior leaving-group ability of the alkyl sulfide ion. Furthermore, it is worth noting that the "3′-bridging thio effect" observed for **1a** is of the same magnitude as those determined for the pH-independent 3′S → 2′O isomerization of a dinucleoside monophosphate analog[11] and the hydroxide ion catalyzed isomerization of 3′-deoxy-3′-thioinosine 3′-*S*-phosphorothiolate dimethyl ester,[12] which both involve an intramolecular attack of the deprotonated 2′-hydroxy group on the phosphorus atom. The rate acceleration may thus be suggested to be of electronic rather than geometric origin.

Mechanism of the pH-Independent Hydrolysis

The dramatic change in the product distribution (Table 1) on going from the hydroxide ion catalyzed hydrolysis to the pH-independent reaction indicates a change in mechanism. Between pH = 2 and 5, more than 90% of the hydrolysis of **1a** proceeds by cleavage of the exocyclic P−OMe bond, releasing the 3′-*S*,5′-*O*-cyclic(phosphorothiolate) diester **2**. We have previously shown that the pH-independent hydrolysis of the *cis*-phenyl ester of thymidine 3′,5′-cyclic(phosphate) (**1b**) proceeds by cleavage of the C5′−O bond, although the hydroxide ion catalyzed reaction takes place by cleavage of one of the P−O bonds. The measurements in ^{18}O-enriched water indicate that this is also the case with **1a**. The site of bond rupture in the pH-independent reaction was determined by hydrolyzing **1a** in a formate buffer (pH = 3.5) prepared in ^{18}O-enriched water (> 90 atom-% ^{18}O) and analyzing the incorporation of the isotopic label into the products by mass spectrometry. The release of Me^{18}OH was detected by GC/EI-MS, while the cyclic(phosphorothiolate) diester **2** in the same sample did not show any sign of incorporation of ^{18}O when analyzed by HPLC/ESI-MS. Accordingly, the solvent molecule attacks the carbon atom of the methyl group, not the phosphorus atom (Scheme 3). The 3′-*S*-phosphorothiolate methyl ester **3** was formed in too small a quantity to allow a reliable HPLC/ESI-MS analysis, but it seems reasonable to assume that even this product is formed by a C−O rather than P−O bond fission. The complete lack of the 5′-phosphodiester **4** among the reaction products is also consistent with the nucleophilic attack on carbon, since the susceptibility of various carbon atoms to the attack of water decreases in the order: CH_3 > C5′ > C3′. Also consistent with the assumed mechanism, the rate of the pH-independent hydrolysis of **1a** is comparable to that of the phenyl ester **1b**, which reacts by the attack of a water molecule on C5′.



Scheme 3

Mechanisms of the Hydronium Ion Catalyzed Reactions

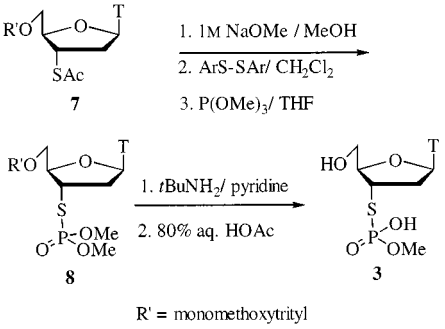
At pH < 2, the hydrolysis of **1a** becomes hydronium ion catalyzed and the product distribution is again changed: Formation of the 3′-*S*-phosphorothiolate methyl ester **3** is favored. Previous studies[13] with trialkyl phosphates have shown that the reaction proceeding by C−O bond cleavage remains pH-independent even under very acidic conditions (3 M perchloric acid). This fact, together with the dissimilar product distribution of the acid-catalyzed and pH-independent cleavage of **1a**, suggests that the acid-catalyzed reaction of the latter proceeds by an attack of water on the phosphorus atom of the monocationic substrate. In analogy with the hydroxide ion catalyzed reaction discussed above, the sulfur atom may be expected to adopt an equatorial position upon formation of the pentacoordinated intermediate.[9] Accordingly, the cleavage of the P−S bond may only take place after pseudorotation. Studies with ribonucleoside 3′-phosphorodithioate diesters,[14] 3′-*S*-phosphorothiolate triesters[12] and a cyclic nonnucleosidic phosphorothiolate triester (methyl *O*,*S*-ethylene phosphorothiolate)[15] have shown that the pseudorotation under very acidic conditions is slow compared to the departure of an apical alkoxy ligand as alcohol. This kind of effect can also be the explanation why **4** is not formed in the acid-catalyzed hydrolysis of **1a**, although we must note that − in contrast to the present study − the studies mentioned[12,14,15] all concern reactions proceeding through a thiophosphorane with the phosphorus atom in a 5-membered ring.

The acid-catalyzed cleavage of the *N*-glycosidic linkage of thymidine has been shown to involve protonation of the 4′-oxygen atom and opening of the sugar ring.[16] An acyclic Schiff base intermediate is formed, which makes the C1′ site susceptible to attack by a solvent molecule. The *N*-glycosidic linkage of thymidine 3′,5′-cyclic(monophosphate) is up to three orders of magnitude more labile than that of free thymidine.[17] This rate acceleration has been attributed to the ring strain that the rigid six-membered cyclic phosphoester moiety causes in the sugar ring; strain is released upon the rate-limiting sugar ring opening. The 3′-*S* substitution can be expected to somewhat diminish the strain of the sugar ring, owing to the fact that the C−S and P−S bonds are longer than their counterparts in the oxyphosphate analog. The 50-fold slower depyrimidination of **1a** compared to that of **1b** may probably be attributed to this geometric effect.

Experimental Section

General Remarks: ^{1}H-, ^{13}C- and ^{31}P-NMR spectra were measured with JEOL Lambda 400 or Alpha 500 NMR spectrometers. ^{1}H and ^{13}C chemical shifts were referenced to an internal tetramethylsilane standard, and ^{31}P shifts to external 85−90% phosphoric acid. − The ESI-MS analyses were performed with a Perkin−Elmer API Triple Quadruple LC/ESI/MS-MS spectrometer. − Pyridine, dichloromethane and dioxane were dried by refluxing over calcium hydride and subsequent distillation. Tetrahydrofuran was dried by distillation from $LiAlH_4$.

Materials: The preparation of **1a** has been described.[3] The 3′-*S*-phosphorothiolate methyl ester **3** was synthesized by applying adopted[18] methods (Scheme 4). Thymine was purchased from Sigma.



Scheme 4

3′-Acetylthio-3′-deoxy-5′-(monomethoxytrityl)thymidine (7): Thiolacetic acid (1.2 mL, 4 equiv.) was added dropwise to a stirred suspension of sodium hydride (60% dispersion in mineral oil; 0.3 g, 3 equiv.) in dry dioxane (10 mL) at 0 °C (ice bath). After stirring for 1 h at room temperature, the mixture was added to a stirred suspension of 1-(2,3′-anhydro-5′-monomethoxytrityl-2′-deoxy-β-D-*threo*-pentofuranosyl)thymine[19] (1.5 g) in dry dioxane (10 mL). The reaction mixture was heated under reflux (100 °C) for 3 h and then for 16 h at 50 °C, after which the mixture was partitioned between dichloromethane and saturated aqueous sodium bicarbonate. The organic layer was separated, washed with water, dried with Na_2SO_4 and concentrated in vacuo. The crude product was purified on a silica gel column eluting with dichloromethane/methanol (99:1). Appropriate fractions were combined and concentrated to dryness to afford **7** (1.2 g, 69%) as a pale yellow syrup. – ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.48 (s, 1 H, N–H), 7.68 (s, 1 H, 6-H), 7.44–7.25 (m, 12 H, trityl-H), 6.85 (d, 2 H, *J* = 9.0 Hz, *O*-anisyl H), 6.28 (t, 1 H, 1′-H), 4.65 (m, 1 H, 4′-H), 4.19 (m, 1 H, 3′-H), 3.79 (s, 3 H, O*CH*$_3$), 3.70 (s, 3 H, SCO*CH*$_3$), 3.50 (m, 2 H, 5′-H$_2$), 2.78 (m, 1 H, 2′-H$_a$), 2.45 (m, 1 H, 2′-H$_b$), 1.49 (s, 3 H, 5-*CH*$_3$). – ^{13}C NMR ($CDCl_3$, 100.40 MHz): δ = 231.0, 163.8, 158.8, 158.8, 150.5, 143.8, 143.7, 143.6, 135.3, 134.8, 130.5, 130.4, 128.4, 128.0, 127.3, 127.2, 113.3, 113.2, 113.2, 111.4, 87.3, 84.8, 84.6, 83.2, 67.0, 64.0, 55.2, 53.4, 48.1, 39.0, 38.1, 11.9. – ESI-MS (positive); *m*/*z*: 595.3 [M + Na]$^+$, 611.2 [M + K]$^+$.

Methyl *S*-(3′-Deoxy-3′-thymidinyl) Hydrogen Thiophosphate (3): The triester **8** (10 mg), prepared from **7** by a previously described[18a] method, was stirred in a mixture of *tert*-butylamine and pyridine (1:9, 10 mL) for 16 h at room temperature. The solvents were evaporated and the residue was dissolved in 80% acetic acid (30 mL) and left standing for 16 h. The mixture was concentrated to dryness and co-evaporated once with ethanol. The residue was partitioned between water and dichloromethane and the water layer was separated and concentrated to dryness. The product was purified by HPLC on a LiChrospher RP-18 column (10–250 mm, 5 μm) eluted with 5% aqueous acetonitrile. The product (2 mg) was obtained after concentration as a colorless solid. – ^{1}H NMR (D_2O, 400 MHz): δ = 7.72 (d, 1 H, *J* = 1.2 Hz, 6-H), 6.19 (t, 1 H, 1′-H), 4.01 (m, 1 H, 4′-H), 3.84 (m, 1 H, 3′-H), 3.62 (d, 3 H, J_{PH} = 12.7 Hz, POC*H*$_3$), 3.54–3.20 (m, 2 H, 5′-H$_2$), 2.57 (m, 2 H, 2′-H$_2$) and 1.37 (s, 3 H, 5- C*H*$_3$). – ^{13}C NMR (D_2O, 125.65 MHz): δ = 188, 170, 154, 140, 114, 88, 87, 65, 62, 49, 29. – ^{31}P NMR (D_2O, 202.35 MHz): δ = 23.38. – ESI-MS (negative); *m*/*z*: 351.3 [M – 1]; (positive); *m*/*z*: 353.0 [M + 1]$^+$, 375 [M + Na]$^+$.

Kinetic Measurements: Reactions were followed by an HPLC method described earlier.[17] For chromatographic separation methods, see Table 2. The hydronium ion concentrations of the reaction solutions were adjusted with hydrogen chloride and formic acid, acetic acid and HEPES buffers.[8,20] Low buffer concentrations (< 0.06 M) were used to minimize the possible catalytic effects of the buffer constituents. The ionic strength of the buffers was adjusted to 0.1 M with sodium chloride. Product distributions were determined from the relative peak areas of the products. Peak areas were assumed to be proportional to concentrations, since the chromophore in all the compounds was the same, i.e. 1-substituted thymine. The first-order rate constants for hydrolysis of **1a** were calculated by applying the integrated first-order rate equation to the time-dependent diminution of the peak area of the compound.

Mass-Spectrometric Analysis of the Products of Hydrolysis in ^{18}O-Enriched Water: A few crystals of **1a** (c.a. 0.5 mg) were dissolved in 100 μL of $H_2{}^{18}O$ (Aldrich; 95 atom-% ^{18}O) and 2 μL of formic acid/sodium formate buffer (1:1 mol L^{-1}) was added. The vial was sealed and immersed in a water bath at 90 °C for 6 h. For reference purposes, an analogous experiment was carried out using a corresponding buffer prepared in normal water.

Acknowledgments

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