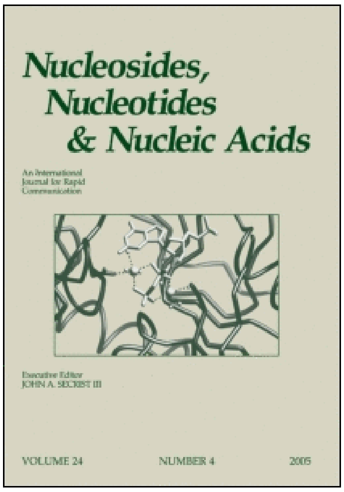

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THE CHEMICAL STABILITY OF *S*-(2-ACYLTHIOETHYL) AND *S*-ACYLOXYMETHYL PROTECTED THYMIDYLYL-3',5'-THYMIDINE PHOSPHOROMONOTHIOLATES AND THEIR DEACYLATION PRODUCTS IN AQUEOUS SOLUTION

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(1&2), 77–91 (2001)

THE CHEMICAL STABILITY OF *S*-(2-ACYLTHIOETHYL) AND *S*-ACYLOXYMETHYL PROTECTED THYMIDYLYL-3′,5′-THYMIDINE PHOSPHOROMONOTHIOLATES AND THEIR DEACYLATION PRODUCTS IN AQUEOUS SOLUTION

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ABSTRACT

The hydrolytic stability of the *S*-(2-acetylthioethyl) (**1a,b**), *S*-(2-pivaloylthioethyl) (**2a,b**), and *S*-acetyloxymethyl (**3a,b**) protected R_P and S_P phosphoromonothiolates of 3′,5′-TpT has been studied. Rather unexpectedly, an intramolecular hydroxide ion catalyzed acetyl migration from the protecting group to the nucleoside 3′- and 5′-hydroxy functions was found to compete with the intermolecular displacement of the $AcSCH_2CH_2S$- or $AcOCH_2S$-ligand from the phosphorus atom of **1a,b** and **3a,b**, respectively. With the *S*-pivaloylthioethyl derivative **2a,b** no such reaction took place. Additionally, the kinetics of the cleavage of the *S*-(2-mercaptoethyl) group from **4a,b**, the products of enzymatic deacylation of **1a,b** and **2a,b**, were studied as a function of pH.

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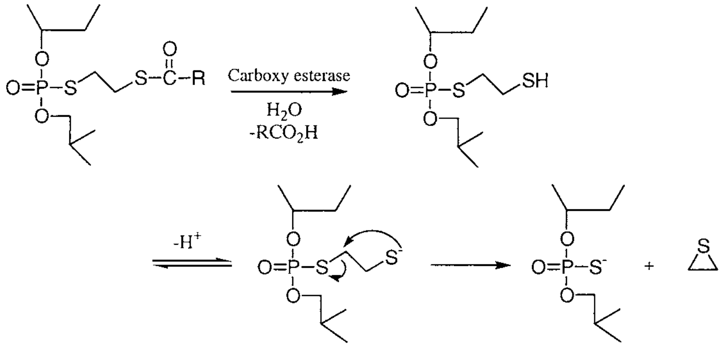
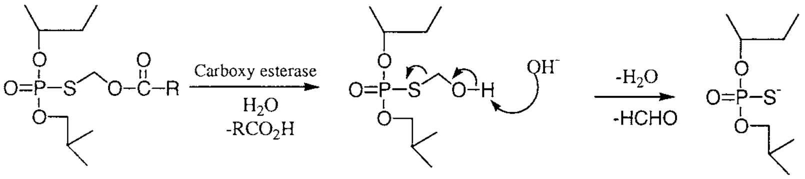
INTRODUCTION

2-Acylthioethyl groups (SATE groups), introduced in nucleic acid chemistry by Imbach et al., show great promise as enzymatically removable protecting groups in the development of prodrug strategies for oligonucleotides (1–5), mononucleotides (6), and phosphopeptides (7). It has been well established that the thioester linkage is readily hydrolyzed by intracellular esterases, and the remaining 2-mercaptoethyl group is subsequently cleaved by episulfide formation (Scheme 1A) (1–3). An alternative approach for biolabile protection of nucleotides is based on acyloxymethyl groups (8–11). Also in this strategy, the intracellular deprotection of the phosphorothioate oligonucleotide is initiated by an esterase catalyzed hydrolysis of the carboxy ester function (Scheme 1B).

To get a better insight into the chemical stability of these nucleotide prodrugs and their deacylation products, we have carried out a kinetic study on the hydrolysis of three diastereomeric pairs of SATE- and acyloxymethyl-protected 3′,5′-dinucleoside phosphoromonothioates (**1a,b; 2a,b; 3a,b**). The data on the chemical stability of the prodrugs may be expected to elucidate the probability and origin of undesired side reactions occurring during various chemical treatments. The acyloxymethyl-protected phosphorothiolates, for example, are known to undergo desulfurization in neutral buffers during the enzyme-catalyzed deblocking (9–11). Additionally, the pH-rate profiles for the breakdown of the (*R*P)- and (*S*P)-*S*-(2-mercaptoethyl)phosphoromonothiolates of 3′,5′-TpT (**4a,b**) were determined to estimate the lifetime and possible accumulation of the products of enzymatic



Scheme 1A.



Scheme 1B.

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deacylation of SATE-Tp(s)T. This fact is of considerable interest, since the deacylation products might be expected to undergo rather readily dimerization by disulfide bond formation.

RESULTS AND DISCUSSION

The thymidylyl-3′,5′-thymidine phosphoromonothiolate triesters bearing the biolabile MeSATE (**1a,b**) (2), *t*BuSATE (**2a,b**) (2), and acetyloxymethyl (**3a,b**) (9) protecting groups at the sulfur were prepared from the appropriate diastereomers of 3′,5′-Tp(s)T (**5a,b**) (12) by methods described earlier.

Hydroxide-ion Catalyzed Hydrolysis of SATE-protected Phosphorothiolates (1a,b; 2a,b)

When the hydrolysis of the R_P- and S_P-*t*BuSATE 3′,5′-Tp(s)T (**2a,b**) in 5 mM aqueous sodium hydroxide (ionic strength 1.0 *M*) was followed by HPLC, 3′,5′-TpT (**6**) was observed to accumulate as the sole nucleotidic product. By contrast, the diastereomeric MeSATE derivatives (**1a,b**), which were observed to decompose in aqueous alkali 3.8 and 2.7 times as fast as their *t*BuSATE counterparts (Tab. 1), gave in addition to 3′,5′-TpT three additional products. As seen from Figure 1, the formation of 3′,5′-TpT represents only for 23 and 36% of the total disappearance of

Table 1. The Hydroxide-Ion Catalyzed Hydrolysis of the Thymidylyl-3′,5′-Thymidine *S*-(2-Acylthioethyl) Phosphoromonothiolates (**1a,b** and **2a,b**): First-Order Rate Constants for Total Degradation (k_{obs}) and for Desulfurization [Accumulation of TpT (k_1)], and the Ratio of the Rate Constants of Desulfurization and Deacylation at 25°C

Compound	pH or H_-	I (mol L^{-1})[a]	$k_{obs}/(10^{-3}\mathrm{s}^{-1})$	k_1 (10^{-3} s^{-1})	k_1/k_2[b]
1a	6.0	0.1	0.000088		
1a	7.5	0.1	0.00255	0.00039	0.18
1a	9.4	0.1	0.117	0.023	0.24
1b	9.4	0.1	0.0475	0.016	0.51
1a	11.7	0.1	6.03	1.3	0.27
1a	13.0	0.1	[c]	[c]	0.29
1a	13.7	0.1	[c]	[c]	0.43
1a	11.5	1.0	9.70	2.2	0.29
1b	11.5	1.0	5.85	2.1	0.56
2a	11.5	1.0	2.55	2.55[d]	
2b	11.5	1.0	2.20	2.20[d]	

[a] The ionic strength of the solutions was adjusted with sodium chloride.

[b] The molar ratio of the products: $k_1/k_2 = x$(TpT)/x(thiophosphate diester products).

[c] Could not be determined by the methods employed.

[d] Accumulation of TpT (k_1) was the only reaction observed.

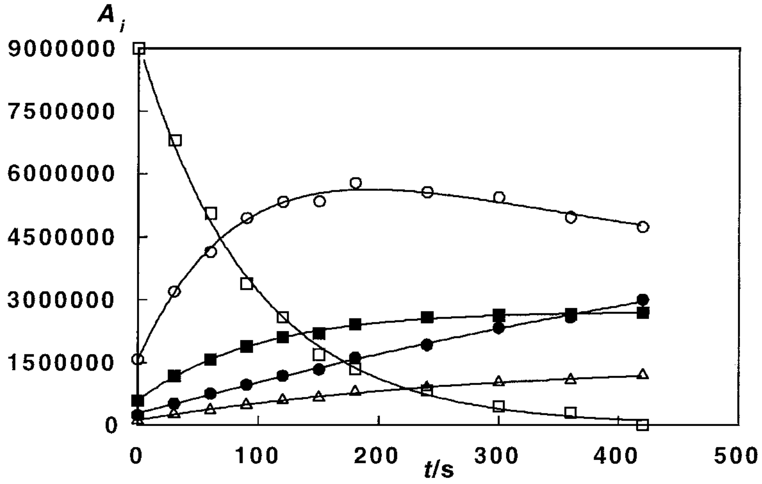
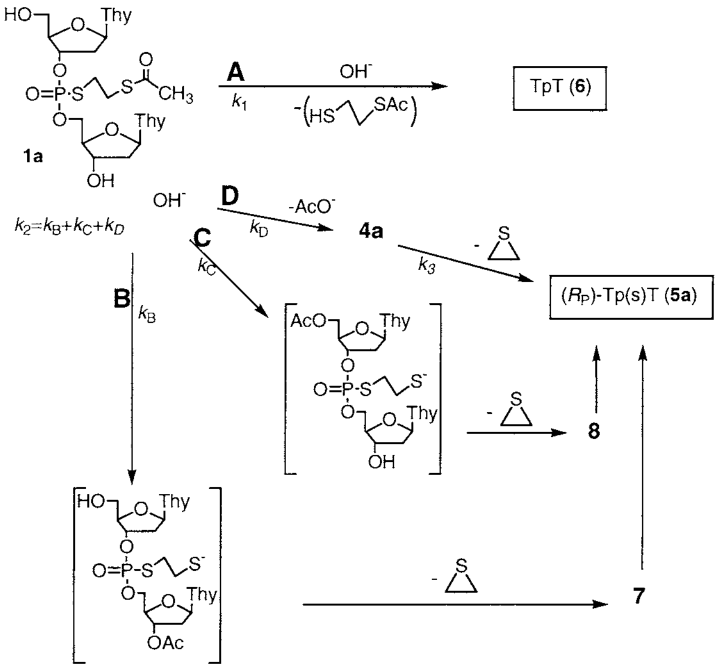


Figure 1. Time-dependent product distribution (A_i = relative peak area in the HPLC chromatogram) of the hydrolysis of (R_P)-*S*-(2-acetylthioethyl) thymidylyl-3′,5′-thymidine phosphoromonothiolate (**1a**) in 5 m*M* sodium hydroxide at 25°C. The ionic strength of the solution was adjusted to 1.0 *M* with sodium chloride [*Notation*: **1a** (□), **5a** [(R_P)-Tp(s)T; ●], **6** (TpT; ■), **7** (○), **8** (△)].

the R_P- (**1a**) and S_P-isomer (**1b**), respectively. This time-dependent product distribution also suggests that two of the additional products are actually intermediates that are slowly converted to 3′,5′-Tp(s)T (**5a** or **5b**), having the same configuration around phosphorus as the starting material.

The two intermediates appear, in turn, to be formed in parallel. The product distribution remains practically unchanged over the pH range 6–13 (Tab. 1), all the partial reactions involved being first-order in hydroxide ion concentration. According to the ESI-MS analysis, both the intermediates have the same molecular mass ($m/z = 605$ for $[M+H]^+$), which is equal to that of an acetyl derivative of 3′,5′-Tp(s)T. By ^{1}H NMR spectroscopy of the intermediates isolated by HPLC, they were assigned as the 3′-*O*- and 5′-*O*-acetyl-3′,5′-Tp(s)T (**7**; **8**), the 3′-*O*-acetylated isomer being somewhat unexpectedly the predominating one ([**7**]:[**8**] = 7:1). The assignment was based on comparison of the proton chemical shifts of the intermediates obtained from **1a** with those of **5a** (data for **5a** not shown). With the major intermediate (**7**), the 3′-H resonance of the 5′-*O*-linked nucleoside occurs 0.7 ppm downfield from the corresponding resonance of 3′,5′-Tp(s)T (5.3 vs. 4.5 ppm). With **8**, in turn, the 5′-H protons of the 3′-linked nucleoside were found to resonate at a lower field compared to those of the unesterified 5′-hydroxymethyl group of (R_P)-3′,5′-Tp(s)T.

Scheme 2 summarizes the reactions occurring with the MeSATE-protected R_P-3′,5′-Tp(s)T (**1a**). Of these only one, viz. displacement of the alkylsulfide ligand by the intermolecular nucleophilic attack of hydroxide ion on the phosphorus (Route A), takes place also with the *t*BuSATE compounds (**2a,b**). It should be noted that no indication of cleavage of the P-O3′ or P-O5′ bonds could be obtained



Scheme 2. Pathways of hydrolysis of MeSATE-Tp(s)T in aqueous alkali.

with either compound, consistent with the fact that an alkylsulfide anion is a much better leaving group than an alkoxy anion. Whether the reaction takes place by an in-line displacement or by an initial formation of a thiophosphorane intermediate, having the less electronegative sulfide ligand in equatorial position according to the rules of Westheimer (13), and subsequent pseudorotation and P–S bond rupture, cannot be deduced on the basis of the data available. Consistent with the suggested mechanism, the rate of desulfurization is insensitive to the structure of the *S*-acyl group (see the values of k_1 in Tab. 1); the acyl group is too far from the reaction center to result in a marked steric hindrance to the attack. Configuration around the phosphorus does not markedly affect the rate of desulfurization either.

The other final product, 3′,5′-Tp(s)T (**5a,b**), obtained only from the MeSATE compounds, may in principle be formed by three alternative routes initiated by a nucleophilic attack on the carbonyl carbon, the nucleophile being either the 3′- or 5′-oxyanion of the starting material, or a hydroxide ion. In all likelihood, the intramolecular reactions (Routes B and C) initially yield 3′-*O*- and 5′-*O*-acetyl-3′,5′-Tp(s)T bearing an *S*-(2-mercaptoethyl) group, while the intermolecular attack of hydroxide ion gives the corresponding deacylated compound, **4a,b**. Evidently, the *t*Bu group is sufficiently bulky to prevent all these attacks. Rapid cyclization of the *S*-(2-mercaptoethyl) group to episulfide (see below) then gives either the *O*-acylated intermediates, **7** and **8**, or **5a,b**. Hydroxide ion catalyzed deacylation of **7** and **8** to **5a,b** finally completes the reaction sequence. Since the formation of **7** and **8** involves an intramolecular attack of a deprotonated hydroxyl function on the carbonyl carbon, the rate of the formation of 3′,5′-Tp(s)T, and hence the competition with desulfurization, is influenced by the stereochemistry around phosphorus. These intramolecular acyl transfer reactions take place more readily with the R_P- than S_P-isomer (see the values k_1/k_2 in Tab. 1).

Stability of the *S*-(2-Mercaptoethyl)-3′,5′-Tp(s)T (4a,b)

Accumulation of the *S*-(2-mercaptoethyl) derivative of R_P-3′,5′-Tp(s)T (**4a**) during a careful enzymic hydrolysis of **2a** with Porcine Liver Esterase (PLE) could be verified by HPLC. Under slightly acidic conditions (pH 4), the maximal accumulation of the intermediate was about 60–70% of the initial amount of **2a**. When analyzed by HPLC/ESI–MS, the intermediate showed an *m/z* value 623, which corresponds to the $[M + 1]^+$ ion of the expected *S*-(2-mercaptoethyl) intermediate **4a**. The same intermediate also accumulated during treatment of **1a** with the PLE enzyme, and its diastereoisomer was obtained by an analogous treatment of **1b** and **2b**.

To follow the decomposition of **4a** and **4b** quantitatively, the pivaloyl protection was removed from the appropriate diastereomer of **1** with the carboxyesterase enzyme at pH 4. When the accumulation of the intermediate was at maximum, the reaction was quenched and the enzyme denaturated by adjusting the pH to 2 with aqueous hydrogen chloride. At this stage, about 10% of **2a** was still intact. The sample could be stored at −18°C without any detectable progress of the reaction. To initiate the kinetic run, the pH of the reaction solution was adjusted by adding an appropriate volume of the acidic stock solution to a known volume of a prebuffered solution. The pH value was in each case measured after the kinetic run. The disappearance of **4a,b** was followed by HPLC, and the rate constants of the reaction were determined over the pH range 2–7 at 25°C. In each run the disappearance of **4a,b** strictly obeyed first-order kinetics for more than 2 half-times. The traces of **2a,b** present in the solution did not show any tendency to react during the runs, indicating that the denaturation of the carboxyesterase was complete.

Over the pH range studied, the rate of decomposition of **4a,b** showed a strictly linear first-order dependence on the hydroxide ion concentration (Fig. 2). The *S*-(2-mercaptoethyl) triester (**4a,b**) was quantitatively converted to 3′,5′-Tp(s)T (**5a,b**) with retention of configuration around phosphorus. The reaction proceeded without detectable accumulation of any intermediates. No reactivity difference between the two diastereomers (**4a,b**) was observed. Assuming that the conversion of **4a,b** to **5a,b** occurs via formation of episulfide, as suggested previously (2), the base catalysis observed may be attributed to deprotonation of the intramolecular nucleophile, viz. the mercapto group. The reaction is under physiological conditions fast, the half-life at pH 7 and 25°C being less than 20 s, and no side reactions, such as oxidation to a disulfide dimer, could be observed. The reaction was also insensitive to the presence of Mg^{2+} and Zn^{2+} ions. Neither of the metal ions at the concentration of 5 or 25 m*M* had any effect on the rate of disappearance of either **4a** or **4b** at pH 5.

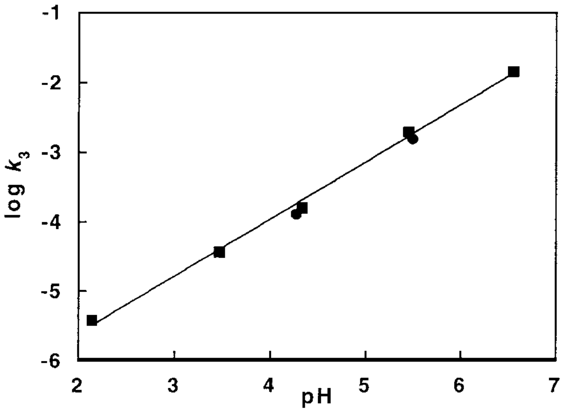


Figure 2. The pH-rate profiles for degradation of (R_{P})- and (S_{P})-*S*-(2-mercaptoethyl) thymidylyl-3′,5′-thymidine phosphoromonothiolates [**4a** (■) and **4b** (●)] at 25°C. The ionic strength of the solutions was adjusted to 0.1 *M* with sodium chloride.

Hydroxide-ion Catalyzed Hydrolysis of *S*-(Acetyloxymethyl)-protected Phosphorothiolates (3a,b)

The *S*-acetyloxymethyl-protected (R_{P})-Tp(s)T (**3a**) when treated with aqueous alkali gave a similar kind of product distribution as **1a,b**. Accordingly, even with **3a** the desulfurization to 3′,5′-TpT was accompanied by formation of R_{P}-3′,5′-Tp(s)T *via* intermediates, which according to HPLC- and MS-analysis were identical to those (**7**; **8**) formed from**1a**. With **3b**, by contrast, the product distribution was slightly more complicated. In addition to **5b** and **6**, four intermediates were observed to accumulate. *Two of these were according to MS- and HPLC-analysis identical to the intermediates formed from **1b** and were assigned as the S_{P}-analogs of **7** and **8**. The other two intermediates were not isolated for full characterization. Nevertheless, it may be noted that the molecular weight determined for them by ESI–MS analysis ($m/z = 529$ for $[\mathrm{M}+\mathrm{H}]^+$ for both of the compounds) is 18 units lower than that of 3′,5′-TpT. A molecule of this weight could be obtained, for example, by an intramolecular attack of either the deprotonated 5′- or 3′-hydroxyl of **3b** on phosphorus, which would lead to departure of the sulfide ligand and formation of a cyclic phosphotriester having the phosphorus as a part of a six-membered ring. This type of compounds are expected to be readily hydrolyzed to 3′,5′-TpT, but be stable enough to accumulate when formed in a sufficient amount under the

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*For the ESI–MS analysis the separations were carried out on a Hypersil ODS 5 column (250–254 mm, 5 μm) eluted with 5 m*M* aqueous ammonium acetate containing 12% acetonitrile. The retention times (flow rate 1 mL/min) for the intermediates of the alkaline hydrolysis of **3b** were 8.7 min ($m/z = 605$ for $[\mathrm{M}+1]^+$), 9.7 min ($m/z = 605$), 15.3 min ($m/z = 529$) and 24 min ($m/z = 529$). The maximum concentration of each of these during the kinetic runs remained below 10% of the initial concentration of **3b**.

Table 2. The Hydroxide-Ion Catalyzed Hydrolysis of the *S*-(Acetyloxymethyl) Thymidylyl(3′,5′)-Thymidine Phosphoromonothiolates (**3a,b**): First-Order Rate Constants of Total Degradation and the Molar Ratio of the Phosphodiester and Thiophosphate Diester Products (k_1/k_2) at 25°C (The ionic strength of the solutions was adjusted at 0.1 *M* with sodium chloride)

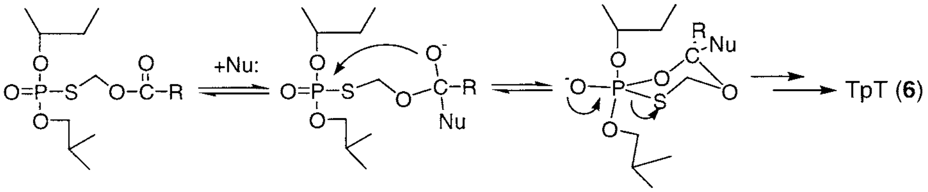
pH or H_-	Compound	k_{obs} (10^{-3} s^{-1})	k_1/k_2
7.5	**3a**	0.00708	0.73
9.4	**3a**	0.330	
11.7	**3a**	23.2	0.97
11.7	**3b**	28.7	0.95
13.0	**3a**	[a]	0.94
13.7	**3a**	[a]	0.94

[a] Could not be determined by the methods employed.

conditions of the kinetic runs. [For kinetics of hydrolysis of phosphotriesters with the phosphate in a six-membered ring, see Ref. (14).] Moreover, the rate of an intramolecular displacement is very likely influenced by the stereochemistry around phosphorus, and this could explain why the corresponding intermediates were not observed to accumulate during hydrolysis of **3a**.

As with the MeSATE-protected compound **1a**, the formation of **7** and **8** from **3a** was hydroxide-ion catalyzed over the pH range studied (pH 6–13), and the 3′-*O*-acetyl derivative predominated over the 5′-*O*-isomer. As seen from the rate constants listed in Tables 1 and 2, the hydrolytic desulfurization to 3′,5′-TpT occurs about eight times, and conversion to *O*-acetylated phosphorothioates (**7**; **8**) about two times more readily with the *S*-(acetyloxymethyl)-protected thiolates than with the MeSATE-protected compounds.

It has been observed earlier that **3a,b** and their analogs undergo desulfurization to phosphodiesters as a minor (typically 3–10%) side reaction also during the enzymic removal of the carboxyester function, and that in chemical hydrolysis in aqueous buffers desulfurization predominates (9–11). In our hands, however, on using a high PLE concentration, **3a,b** yielded only the corresponding phosphorothioate. It has been suggested (9–11) that the desulfurization takes place by an attack of hydroxide ion on the carbonyl carbon and cyclization of the anionic tetrahedral intermediate (Scheme 3) rather than by an intermolecular attack of a hydroxide ion on phosphorus. While this may possibly be the case, the structural effects observed for the hydroxide-ion catalyzed desulfurization in the present study makes a more straightforward explanation, viz. displacement of the alkylsulfide ligand by an intermolecular attack of a hydroxide ion on phosphorus, more attractive. The desulfurization of **3a** is less than one order of magnitude faster than that of **1a**, which proceeds most likely by an intermolecular nucleophilic attack on phosphorus (see above). This reactivity difference is expected



Scheme 3.

on the basis of higher electronegativity of the acetyloxymethyl group compared to that of the acetylthioethyl. It may also be noted that chemical desulfurization may under certain conditions to some extent compete with the enzymic cleavage of **3a**.

Enzymic Cleavage of the *S*-Acetyloxymethyl Group

In contrast to the hydrolysis of **1a,b** and **2a,b**, treatment of the *S*-acyloxymethyl-protected phosphorothiolates **3a,b** with PLE released the appropriate diastereomer of 3′,5′-Tp(s)T without accumulation of any detectable intermediates. Accordingly, formaldehyde is very readily released from the deacetylated compound (Scheme 1B). The enzymatic degradation of **3a,b** in our experiments was slightly slower than that of the *t*BuSATE analogs, but the deacylated derivative released appears to be even more reactive than **4a,b** under neutral conditions.

Conclusions

A significant accumulation of the *S*-(2-mercaptoethyl) derivative **4a,b** of the dithymidine phosphorothioate during the enzymatic deacylation of the SATE-protected pro-drugs (**1a,b**; **2a,b**) was verified. A hydroxide-ion catalyzed conversion to free phosphorothioate diester, however, readily takes place, the half-life of the latter reaction being less than 20 s at pH 7 and 25°C. No side reactions could be observed. With the acetyloxymethyl phosphorothioates **3a,b**, the Tp(s)T diester was released by carboxyesterase treatment without detectable accumulation of any intermediates. In aqueous alkali, the *S*-(2-pivaloylthioethyl)-protected phosphorothiolates (**2a,b**) undergo quantitative hydrolytic desulfurization to 3′,5′-TpT, whereas with both the *S*-(2-acetylthioethyl) and *S*-acetyloxymethyl pro-drugs (**1a,b**; **3a,b**) intramolecular acetyl transfer to the unesterified sugar hydroxy functions compete with the release of 3′,5′-TpT.

EXPERIMENTAL SECTION

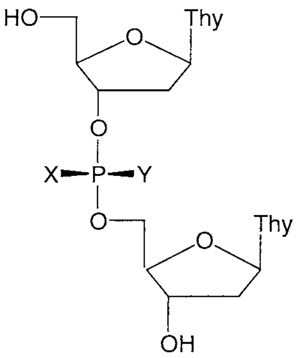
General. The NMR spectra were acquired with a JEOL Alpha 500 spectrometer. The proton chemical shifts were referenced to internal TMS and the ^{31}P shifts to an external orthophosphoric acid (85% in D_2O). The MS analyses were performed on a Perkin-Elmer API 365 Triple Quadrupole LC/MS/MS spectrometer.

Materials. The diastereomeric phosphorothioate analogs of thymidylyl-(3′,5′)-thymidine were prepared by the hydrogen phosphonate procedure described earlier (12). The absolute configuration of the diastereomers was confirmed by an enzymatic digestion with nuclease P1 (from *Penicillium citrinum*; Sigma). The S_P diasteromer is known to be a substrate of this enzyme, while the R_P is not (15). Furthermore, the S_P isomer has a longer retention on an RP column than the R_P analog (15). The two diastereoisomers were separated from each other on a semipreparative RP HPLC column (LiChroSpher 100, RP-18, 10–250 mm, 5 μm) eluted with an 83/17% (v/v) mixture of water and acetonitrile.

The R_P and S_P *S*-(2-acetylthioethyl)phosphoromonothiolates of thymidylyl-3′,5′-thymidine (1a,b). The diastereomeric mixture of R_P and S_P *S*-(2-acetylthioethyl)phosphoromonothiolates of thymidylyl-3′,5′-thymidine was prepared by alkylating the diastereomeric thymidylyl-3′,5′-thymidine phosphoromonothioates with 2-acetylthioethyl iodide (16), as described previously by Barber et al. (2). It is worth noting that the alkylation was accompanied by racemization. Although a pure R_P or S_P phosphoromonothioate diastereomer was employed as a starting material, an approximately equimolar mixture of **1a** and **1b** was always obtained. The diastereomers were separated by semipreparative RP-HPLC on a Lichrospher 100 RP-18 column (250 × 10 mm, 5 μm) using aqueous acetonitrile (MeCN:H_2O 17:83, v/v) as an eluent. Yield 33%. **1a**: ^{1}H NMR (DMSO-d_6, ppm from TMS) δ 11.32 (s, 2H, 2 × N*H*), 7.69 (d, 1H, $J = 1.4$ Hz, *H*6), 7.49 (d, 1H, $J = 1.5$ Hz, *H*6), 6.21 (m, 2H, 2 × *H*1′), 5.48 (d, 1H, $J = 4.3$ Hz, 3′-O*H*), 5.25 (t, 2H, $J = 5.1$ Hz, 5′-O*H*), 5.09 (m, *H*3′ of the 3′-linked T), 4.26 (m, 3H, *H*3′, *H*5′, *H*5″ of the 5′-linked T), 4.11 (dd, 1H, $J = 3.5$ and 3.5 Hz, *H*4′ of the 3′-linked T), 3.95 (m, 1H, *H*4′ of the 5′-linked T), 3.62 (t, 2H, *H*5′, *H*5″ of the 3′-linked T), 3.11 (m, 2H, $-CH_2CH_2SAc$), 2.98 (m, 2H, $-CH_2CH_2SAc$), 2.39 (m, 2H, *H*2′, *H*2″ of the 3′-linked T), 2.34 (s, 3H, Ac), 2.20 (m, 1H, *H*2′ of the 5′-linked T), 2.09 (m, 1H, *H*2″ of the 5′-linked T), 1.79 (dd, 6H, 2 × 5-Me).^{31}P NMR (DMSO, ppm from H_3PO_4, external standard) 27.79. ESI–MS: *m/z* 665 ($[M+H]^+$), 687 ($[M+Na]^+$), 703 ($[M+K]^+$). HPLC: $t_R = 62$ min on a Hypersil ODS 5 column (250–254 mm, 5 μm) eluted with an acetic acid/sodium acetate buffer (0.045/0.015 *M*) containing 0.1 mol/L ammonium chloride and 14 vol.-% of acetonitrile at a flow rate 1.0 mL/min. **1b**: ^{31}P NMR (DMSO, ppm from H_3PO_4, external standard) 27.66. ESI–MS as with **1a**. HPLC: $t_R = 67$ min by the method mentioned

for **1a** (see Fig. 3).

The (R_P)- and (S_P)-*S*-(2-pivaloylthioethyl)phosphoromonothiolates of thymidylyl-3′,5′-thymidine (2a,b). The diastereomeric mixture of **2a** and **2b** was obtained as described above for **1a,b**, but by using 2-pivaloylthioethyl iodide (16) as the alkylating agent. The acetonitrile content of the eluent used in the RP-HPLC separation of the diastereomers was 25 vol.-%. Yield 36%. **2a**: ^{1}H NMR (DMSO-d_6, ppm from TMS) δ 11.32 (s, 2H, 2 × N*H*), 7.66 (d, 1H, $J = 1.5$ Hz, *H*6), 7.47 (d, 1H, $J = 1.2$ Hz, *H*6), 6.20 (m, 2H, 2 × *H*1′), 5.48 (d, 1H, $J = 4.3$ Hz,



1a: X = O; Y = SCH_2CH_2-S-C(O)-CH_3
1b: X=SCH_2CH_2-S-C(O)-CH_3; Y = O
2a: X = O; Y = SCH_2CH_2-S-C(O)-*t*Bu
2b: X = SCH_2CH_2-S-C(O)-*t*Bu; Y = O
3a: X = O; Y = SCH_2-O-C(O)-CH_3
3b: X = SCH_2-O-C(O)-CH_3; Y = O
4a: X = O; Y = SCH_2CH_2SH
4b: X = SCH_2CH_2SH; Y = O
5a: X = O; Y = S^- [(*R*p)-Tp(s)T]
5b: X = S^-; Y = O [(*S*p)-Tp(s)T]
6: X = O; Y = O^- [TpT]

Figure 3.

3′-O*H*), 5.25 (t, 2H, $J = 5.1$ Hz, 5′-O*H*), 5.07 (m, 1H, *H*3′ of the 3′-linked T), 4.25 (m, 3H, *H*3′, *H*5′, *H*5″ of the 5′-linked T), 4.09 (dd, 1H, $J = 3.5$ and 3.5 Hz, *H*4′ of the 3′-linked T), 3.93 (m, 1H, *H*4′ of the 5′-linked T), 3.60 (t, 2H, *H*5′, *H*5″ of the 3′-linked T), 3.08 (m, 2H, $-CH_2CH_2$SPiv), 2.94 (m, 2H, $-CH_2CH_2$SPiv), 2.38 (2H, m, *H*2′, *H*2″ of the 3′-linked T), 2.17 (m, 1H, *H*2′ of the 5′-linked T), 2.07 (m, 1H, *H*2″ of the 5′-linked T), 1.76 (6H, dd, 2 × 5-Me), 1.14 (s, 9H, *t*Bu). ^{31}P NMR (DMSO, ppm from external H_3PO_4) 32.28. ESI–MS: *m/z* 707 ($[M + H]^+$), 729 ($[M + Na]^+$), 751 ($[M + K]^+$). $t_R = 16$ min on a Hypersil ODS 5 column (250–254 mm, 5μm) eluted with a formic acid/sodium formate buffer (0.045/0.015 *M*) containing 0.1 mol/L ammonium chloride and 27 vol.-% acetonitrile at a flow rate 1.0 mL/min. **2b**: ^{31}P NMR (DMSO, ppm from external H_3PO_4) 32.13. ESI–MS as with **2a**. $t_R = 19$ min by the method described for **2a**.

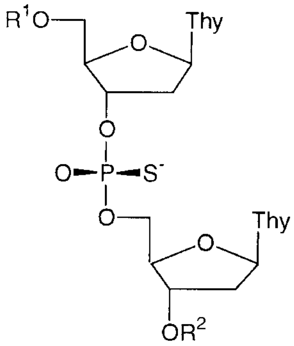
The (*R*$_P$)- and (*S*$_P$)-*S*-(acetyloxymethyl)phosphoromonothiolates of thymidylyl-3′,5′-thymidine 3a,b). **3a** and **3b** were prepared, analogously with a previously described method (9), by reacting the appropriate diastereomer of the monothioate analog of thymidylyl-3′,5′-thymidine (0.17 mmol) with iodomethyl acetate (8,17) (0.68 mmol) in a mixture of Hepes buffer (50 m*M*, pH 7.0, 0.25 mL) and acetonitrile (3 mL) for 4 to 5 h at 37°C. After quenching the reaction with aqueous sodium bisulfite and evaporating the solvents, the product was purified by HPLC on a LiChroSpher RP-18 column (10–250 mm, 10 μm) eluted with a 17:83% (v/v) mixture of acetonitrile and water. The yields were 37 and 18% for **3a** and **3b**, respectively. **3a**: ^{1}H NMR (^2H_2O, ppm from TMS), δ 7.64 (*H*6), 7.56 (*H*6), 6.29 (2H, m, 2 × *H*1′), 5.46 (2H, d, $J = 21$ Hz, C*H*$_2$OAc), 5.23 (m, *H*3′ of 3′linked T), 4.58 (q, $J = 5.3$ Hz, *H*3′ of 5′-linked T), 4.50 (m, *H*5′a of 5′-linked T), 4.45 (m, *H*5′b of 5′-linked T), 4.32 (m, *H*4′), 4.23 (m, *H*4′), 3.82 (2H, m, 2 × *H*5′ of 3′-linked T), 2.64 (dd, $J_1 = 6.0$, $J_2 = 2.5$ Hz, *H*2′), 2.54–2.43 (3H, 3 × *H*2′), 2.13 (3H, s, OC(O)C*H*$_3$), 1.91 (3H, s, C*H*$_3$ of Thy), 1.90 (3H, s, C*H*$_3$ of Thy). ^{31}P NMR (^2H_2O, ppm from external H_3PO_4) δ 28.75. ESI–MS (positive): *m/z* 635 (100%, $[M + H]^+$), 652 (50%, $[M + NH_4]^+$), 657 (20%, $[M + Na]^+$). HPLC: $t_R = 13.8$ min by the method analogous to that described for **2a**, excluding that the acetonitrile content of the eluent was changed to 15 vol.-%. **3b**: ^{1}H NMR (^2H_2O, ppm from TMS), δ 7.63 ($H6$), 7.54 ($H6$), 6.29 (2H, m, 2 × $H1'$), 5.44 (2H, dd, $J_1 = 21$, $J_2 = 5$ Hz, CH_2OAc), 5.23 (m, $H3'$ of 3′-linked T), 4.58 (q, $J = 5$ Hz, $H3'$ of 5′-linked T), 4.47 (m, $H5'$a of 5′-linked T), 4.43 (m, $H5'$b of 5′-linked T), 4.29 (m, $H4'$), 4.19 (m, $H4'$), 3.80 (2H, m, 2 × $H5'$ of 3′-linked T), 2.64–2.42 (4H, 4 × $H2'$), 2.13 (3H, s, $OC(O)CH_3$), 1.89 (3H, s, CH_3 of Thy), 1.88 (3H, s, CH_3 of Thy). ^{31}P NMR (^2H_2O, ppm from external H_3PO_4) δ 29.06. ESI–MS (positive): *m/z* 635 (80%, $[M + H]^+$), 652 (30%, $[M + NH_4]^+$), 657 (100%, $[M + Na]^+$). HPLC: $t_R = 14.6$ min by the method described for **3a**.

Kinetic Measurements

Nonenzymatic hydrolyses. The reactions were carried out in stoppered glass tubes immersed in a thermostatted water bath at (298.2 ± 0.5) K. The hydroxide ion concentrations of the reaction solutions were adjusted with sodium hydroxide or CHES [2-(cyclohexylamino)ethanesulfonic acid] or Hepes [N-(hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid] buffers. The ionic strength of the solutions was adjusted with sodium chloride. The aliquots withdrawn from the reaction solution at appropriate intervals were neutralized with acetic acid and analyzed by HPLC on a Hypersil ODS column (4–250 mm, 5 μm) eluted with an acetic acid/sodium acetate buffer (0.045/0.015 M) containing ammonium chloride (0.1 mol/L) and acetonitrile (7–14%, v/v).

Characterization of the hydrolysis products. The mass spectrometric characterization of the intermediates was performed by HPLC ESI–MS directly from the aliquots of the kinetic runs. Thymidylyl-3′,5′-thymidine and its monothioate derivatives among the reaction products could be identified chromatographically by spiking with authentic samples. The two intermediates formed during hydrolysis of **1a** were isolated and purified by HPLC. According to spectroscopic characterization they were assigned as 3′-*O*- and 5′-*O*-acetyl derivatives of (R_P)-Tp(s)T (**7** and **8**, respectively). By ^{1}H NMR, the change of the chemical shift of the 3′-H of the 5′-linked nucleoside from 4.6 ppm with (R_P)-Tp(s)T (**5a**) to 5.2 ppm with **7** is noticed. With **8**, on the other hand, the 5′-H resonances of the 3′-linked thymidine occur at 4.24–4.30 ppm, while the corresponding protons of (R_P)-Tp(s)T resonate at 3.81 and 3.87 ppm (data for Tp(s)T is not shown).

(R_P)-Thymidylyl-(3′,5′)-(3′-*O*-acetylthymidine)phosphoromonothioate (7). ^{1}H NMR (D_2O, ppm from TMS) δ 7.66 (d, $J = 1.1$ Hz, $H6$), 7.54 (d, $J = 1.2$ Hz, $H6$), 6.24 (dd, $J_1 = 8.5$, $J_2 = 6.1$ Hz, $H1'$), 6.11 (dd, $J_1 = 7.5$, $J_2 = 6.1$ Hz, $H1'$), 5.28 (dt, $J_1 = 6.3$, $J_2 = 2.4$ Hz, $H3'$ of the 5′-linked T), 4.87 (m, $H3'$ of the 3′-linked T), 4.26 (m, $H4'$ of the 5′- linked T), 4.12–4.07 (3H, $H4'$ of the 3′-linked and 2 × $H5'$ of the 5′-linked T), 3.75 (dd, $J_1 = 12.5$, $J_2 = 3.5$ Hz, $H5'$a of the 3′-linked T), 3.69 (dd, $J_1 = 12.5$, $J_2 = 4.5$ Hz, $H5'$b of the 3′-linked T), 2.47–2.22 (4H, 4 × $H2'$), 2.02 (3H, s, CH_3 of OAc), 1.82 (3H, d, $J = 1.0$ Hz, CH_3 of Thy), 1.77 (3H, d, $J = 1.2$ Hz, CH_3 of Thy). ESI–MS: *m/z* 605 ($[M + H]^+$). HPLC: $t_R = 7.5$ and 8.4 min by the methods described for **1a** and **3a**, respectively (see Fig. 4).



7: $R^1 = H; R^2 = Ac$
8: $R^1 = Ac; R^2 = H$

Figure 4.

(R_P)-(5′-*O*-acetylthymidylyl)-(3′,5′)-thymidine phosphoromonothioate (8). ^{1}H NMR (^2H_2O, ppm from TMS) δ 7.60 (d, $J = 1.0$ Hz, $H6$), 7.38 (d, $J = 1.1$ Hz, $H6$), 6.22 (t, $J = 6.8$ Hz, $H1'$), 6.11 (t, $J = 6.8$ Hz, $H1'$), 4.89 (m, $H3'$ of the 3′-linked T), 4.46 (m, $H3'$ of the 5′-linked T), 4.2 (m, 3H, $H4'$ and 2 × $H5'$), 4.03 (3H, m, $H4'$ and 2 × $H5'$), 2.48 (ddd, $H2'$ of the 3′-linked T), 2.30 (m, $H2'$ of the 3′-linked T), 2.25 (m, 2H, 2 × $H2'$ of the 5′-linked T), 2.01 (3H, s, OAc), 1.82 (d, 3H, $J = 0.8$ Hz, CH_3 of Thy), 1.77 (d, 3H, $J = 0.8$ Hz, CH_3 of Thy). ESI–MS: *m/z* 605 ($[M + H]^+$). HPLC: $t_R = 6.3$ and 6.8 min by the methods described for **1a** and **3a**, respectively.

Rate constants. The first-order rate constants for the reactions were calculated by applying the integrated first-order rate equation to the time-dependent diminution of the peak area of the starting compound.

Enzymatic cleavage of the thioacyl function and degradation of the deacylated compounds. The acyl protection groups of **1a,b** and **2a,b** were removed with Porcine Liver Esterase (Sigma) in an acetate buffer at pH 4, since under these conditions the mercaptoethyl intermediate (ESI–MS: $m/z = 623$ for $[M + 1]^+$) accumulated at a higher level than it did at pH 7. Typically, 0.2 mg of the protected nucleotide **2a** was dissolved in 3.0 mL of the acetic acid buffer and 9 mg of the PLE enzyme was added as an aqueous solution (100 μL). After 60 min treatment at 25°C, the pH of the solution was adjusted to 2 by adding 360 μL of 1 M aqueous hydrogen chloride. At this stage, about 60% of the starting compound had been converted to the mercaptoethyl derivative **4a**, and about 10% remained unreacted.

To start the kinetic run with **4a**, the pH of the solution was adjusted by mixing 250 μL of the acidic solution with 750 μL of a prethermostatted buffer solution (298.2 K). Aliquots of 100 μL were withdrawn at appropriate intervals, made acidic (pH 2) with aqueous hydrogen chloride, and cooled in an ice-bath. The samples were immediately analyzed by HPLC on the Hypersil ODS column eluted with a formic acid/sodium formate buffer (pH 3) containing ammonium chloride (0.1 M) and acetonitrile (17 vol.-%).

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