JOC Article

Biodegradable Protections for Nucleoside 5′-Monophosphates: Comparative Study on the Removal of *O*-Acetyl and *O*-Acetyloxymethyl Protected 3-Hydroxy-2,2-bis(ethoxycarbonyl)propyl Groups

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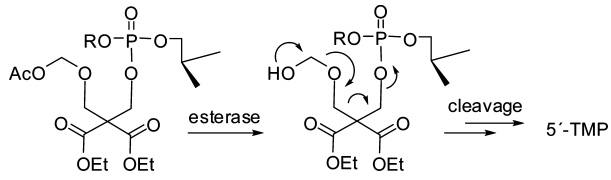

The applicability of 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl and 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl groups as biodegradable phosphate protecting groups for nucleoside 5′-monophosphates has been studied in a HEPES buffer at pH 7.5. Enzymatic deacetylation with porcine carboxyesterase triggers the removal of the resulting 3-hydroxy-2,2-bis(ethoxycarbonyl)propyl and 3-hydroxymethoxy-2,2-bis(ethoxycarbonyl)propyl groups by retro-aldol condensation and consecutive half acetal hydrolysis and retro-aldol condensation, respectively. The kinetics of these multistep deprotection reactions have been followed by HPLC, using appropriately protected thymidine 5′-monophosphates as model compounds. The enzymatic deacetylation of the 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl 5′-triester (**2**) is 25-fold faster than the deacetylation of its 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl-protected counterpart **1**, and the difference in the deacetylation rates of the resulting diesters, **12b** and **12a**, is even greater. With **2**, conversion to thymidine 5′-monophosphate (5′-TMP) is quantitative, while conversion of **1** to 5′-TMP is accompanied by formation of thymidine. Consistent with the preceding observations, quantitative release of 5′-TMP from **2** has been shown to take place in a whole cell extract of human prostate cancer cells.

Introduction

Protected nucleoside monophosphates and their congeners constitute a promising class of therapeutic agents.[1] A viable pronucleotide candidate is reasonably stable in plasma and sufficiently hydrophobic to penetrate into cells, where it releases the parent nucleotide drug by cleavage of the phosphate protecting groups to nontoxic products. The deprotection of the pronucleotide is usually initiated by an enzymatic transformation that triggers a chemical reaction by which the remnants of the protecting groups are removed. The groups employed for this purpose include acyloxymethyl,[2] alkoxycarbonyloxymethyl,[3]

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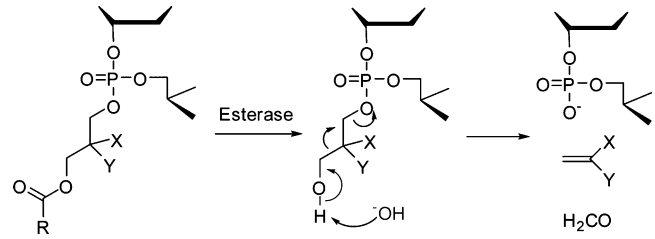
S-acyl-2-thioethyl,[4] 2-(2-hydroxyethyldisulfanyl)ethyl,[5] 4-acyloxybenzyl,[2a,6] 1-acyloxypropan-1,3-diyl,[7] and 1-arylpropan-1,3-diyl[8] functional groups. In addition, cyclosaligenyl nucleotides,[9] phosphoramidates,[8b,10] diamidates,[11] lipid esters,[12] nitrofuranylmethylamidates,[13] and bis-ketol nucleotide triesters[14] have been studied as pro-drugs. Despite the fact that some pronucleotides have been accepted for clinical use and quite many have advanced into clinical phase studies,[1a] many questions have still to be answered to achieve a proper compromise between chemical stability, cellular uptake, rate of biodegradation and byproduct toxicity.

SCHEME 1



Bis(acyloxymethyl) pro-drugs, constituting one of the most commonly used pro-drug categories, have been suggested to undergo an enzyme-triggered conversion to nucleoside monophosphates via a monoanionic diester intermediate.[2g] In spite of the wide interest in this approach, the kinetic data on the consecutive esterase-catalyzed cleavage of the neutral triester pro-drugs to monoanionic diesters and eventually to nucleoside phosphates is surprisingly scarce, being limited to the early studies of Farquhar et al.[15] on the cleavage of bis(acyloxymethyl)esters of benzyl and phenyl phosphates. According to these studies, the rate of the first enzymatic step can be controlled by different acyl groups, but the second step remains slow due to the short distance between the enzyme cleavage site and the negative charge of the phosphodiester intermediate. The studies of Freeman et al. with acyloxybenzyl esters of methylphosphonate,[16a] phosphonoacetate,[16b] and 5′-monophosphate of azidothymidine,[6b] in turn, suggest that the benzene ring as a spacer between the acyl group and the anionic phosphate accelerates the cleavage of both the neutral triester and monoanionic diester by porcine liver carboxyesterase.

We have recently introduced 2,2-bis(substituted)-3-acyloxypropyl groups as biodegradable phosphate protecting groups. Studies with dinucleoside phosphoromonothioates[17] and oligonucleotides[18] have shown that such groups are stable under physiological conditions, but are cleaved by retro-aldol condensation after enzymatic deacylation (Scheme 1).

The hydrolytic stability of the deacylated intermediate may be tuned within wide limits by the polar nature of the substituents at C2.[19,20] The present paper is aimed at clarifying the applicability of one such group, viz., the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group, to protection of nucleoside phosphomonoesters. While the first protecting group obviously is cleaved from the neutral triester at a rate comparable to the cleavage from an internucleosidic phosphodiester linkage, the departure from the resulting nucleoside diester monoanion

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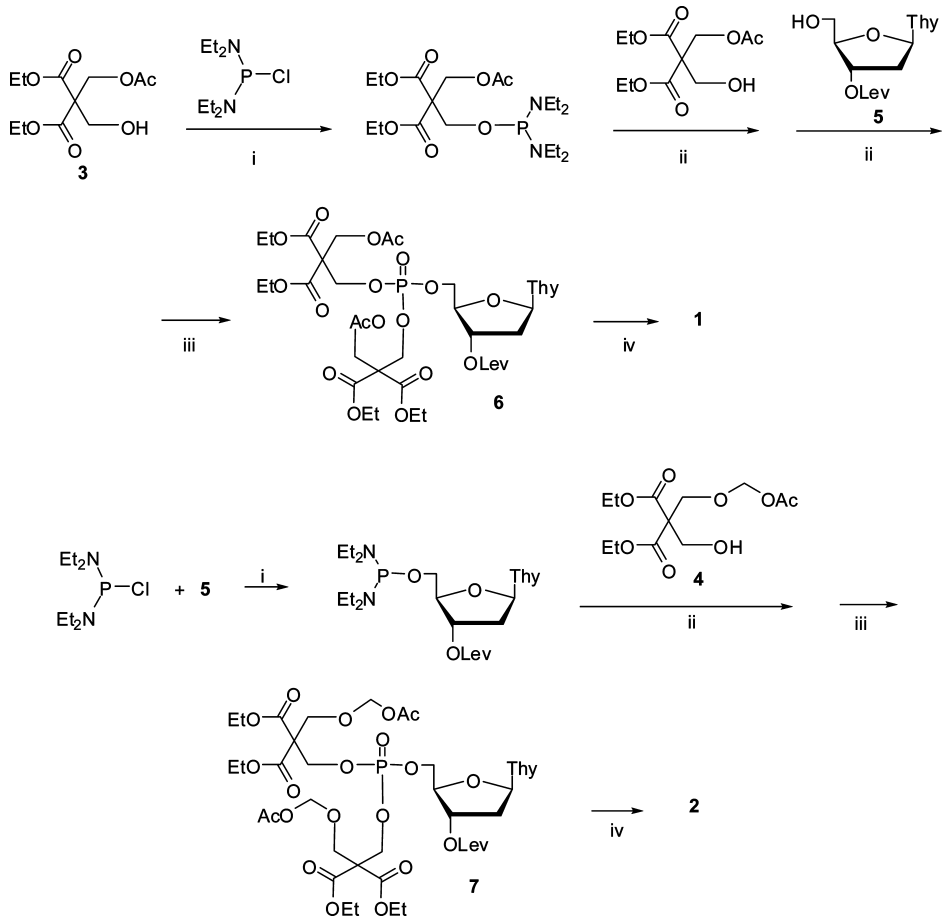
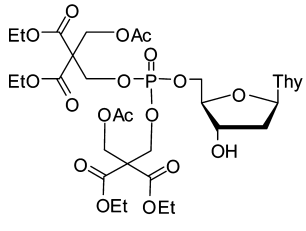
SCHEME 2[a]



[a] Conditions: (i) Et_3N, DCM; (ii) TetH, MeCN; (iii) I_2, THF, H_2O; (iv) NH_2NH_2, AcOH, Py with **6** and NH_2NH_2OAc, DCM, MeOH with **7**.

expectedly is much more difficult. The enzymatic deacetylation may be retarded and dianionic nucleoside monoester is a poor leaving group compared to a monoanionic diester. To facilitate the enzymatic deacetylation of the diester monoanion, the distance between the phosphate moiety and the ester function has been increased by inserting an extra hydroxymethyl fragment in the structure, giving a 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl group. Accordingly, thymidine 5′-bis[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**1**) and 5′-bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**2**) have been prepared and their conversion to thymidine 5′-monophosphate (5′-TMP) by porcine liver carboxyesterase has been studied. In addition, the hydrolysis of **1** in human serum and the hydrolysis of **2** in a whole cell extract of human prostate carcinoma cells have been studied.



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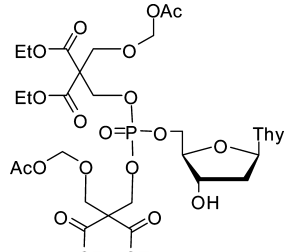

2

Results and Discussion

Preparation of Protected Thymidine 5′-Monophosphates (1, 2). Protected thymidine 5′-monophosphates **1** and **2** were prepared by stepwise alcoholysis of bis(diethylamino)phosphorochloridite with 3′-*O*-levulinoylthymidine (**5**) and the appropriate protecting group reagent, either diethyl 2-acetyloxymethyl-2-hydroxymethylmalonate (**3**) or diethyl 2-acetyloxymethoxymethyl-2-hydroxymethylmalonate (**4**), as outlined in Scheme 2 in more detail. Oxidation to phosphate triester and removal of the levulinoyl protection completed the synthesis. Alcohol **3** was obtained by conversion of commercially available diethyl 2,2-bis(hydroxymethyl)malonate to orthoacetate (**8**) and its subsequent hydrolysis (Scheme 3).[21] Alcohol **4** was, in turn, synthesized by protecting one of the hydroxyl functions of diethyl 2,2-bis(hydroxymethyl)malonate with a *tert*-butyldimethylsilyl group (**9**) and subjecting the other one to methylthiomethylation (**10**) followed by displacement of the methylthio group with acetate ion[22] (**11**) and removal of the *tert*-butyldimethylsilyl group by $Et_3N \cdot 3HF$ treatment.

Hydrolytic Stability of Protected Thymidine 5′-Monophosphates (1, 2). The nonenzymatic hydrolysis of thymidine 5′-phosphotriesters **1** and **2** was first studied by analyzing the composition of the aliquots withdrawn from the reaction mixture

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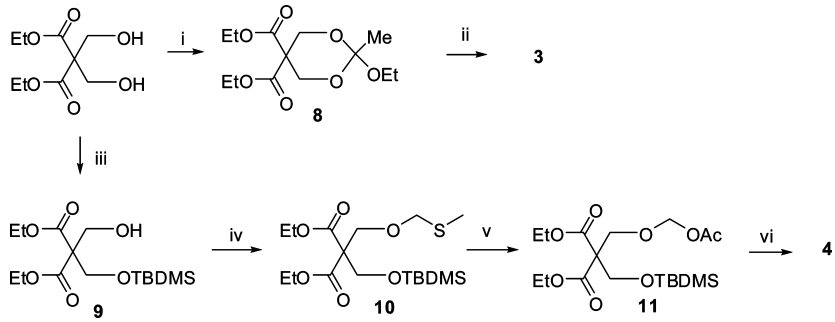
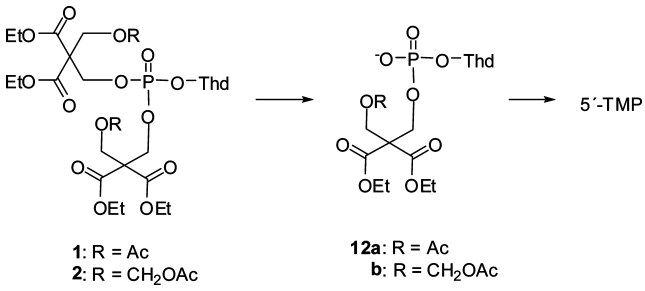
SCHEME 3[a]



[a] Conditions: (i) $(EtO)_3CCH_3$, H_2SO_4, THF; (ii) 80% AcOH; (iii) TBDMSCl, Py; (iv) DMSO, AcOH, Ac_2O; (v) SO_2Cl_2, AcOK, DCM, 18-crown-6; (vi) $Et_3N \cdot 3HF$, THF.

SCHEME 4



at appropriate time intervals by HPLC. The reactions were carried out in HEPES buffers at pH 7.5 and 37 ± 0.1 or 25 ± 0.1 °C. The products were identified by mass spectrometric analysis (HPLC/ESI-MS) and by spiking with authentic samples.

Triester **1** is hydrolytically quite stable. The reaction is first order in hydroxide ion concentration and it in all likelihood proceeds by hydroxide ion-catalyzed deacetylation, followed by hydroxide ion-catalyzed loss of formaldehyde and concomitant elimination of diethyl 2-methylenemalonate (cf. Scheme 1). At pH 7.5 and 25 °C, the half-life for the conversion of **1** to the corresponding diester (**12a** in Scheme 4) is about 20 days ($k = 4.0 \times 10^{-7}$ s^{-1}). The subsequent hydrolysis of the diester to 5′-TMP is almost 1 order of magnitude slower ($k = 5.0 \times 10^{-8}$ s^{-1}). In fact, the nonenxymatic hydroloysis of **12a** is so slow that cleavage of the ethoxycarbonyl groups to carboxylate functions starts to compete.

Triester **2** is hydrolytically almost 1 order of magnitude less stable than **1**. The first-order rate constant for the conversion of **2** to diester **12b** is 6.1×10^{-6} s^{-1} ($\tau_{1/2} = 32$ h) at pH 7.5 and 37 °C, the subsequent hydrolysis of **12b** to 5′-TMP being 5 times slower ($k = 1.3 \times 10^{-6}$ s^{-1}). Evidently, hydroxide ion-catalyzed deacetylation is followed by rapid hydroxide ion-catalyzed loss of formaldehyde. The resulting 3-hydroxy-2,2-bis(ethoxycarbonyl)propyl group is then cleaved by a similar retro-aldol condensation mechanism as with **1**. Besides **12b**, no intermediates appear.

Enzymatic Removal of the 3-Acetyloxy-2,2-bis(ethoxycarbonyl)propyl Groups from Thymidine 5′-Phosphotriester 1. Figure 1 shows the time-dependent product distribution for the treatment of triester **1** with porcine liver esterase (PLE; 26.0 units mL^{-1}) in a HEPES buffer at pH 7.5 ($I = 0.1$ mol L^{-1} with NaCl) and 25 °C. The identification and quantification of the intermediates indicated is based on HPLC-ESI-MS. PLE-catalyzed deacetylation first gives a monodeacetylated triester (**13a**; $[M + H]^+$ 769.5; reaction A in Scheme 5), which then undergoes two subsequent reactions: PLE-catalyzed hydrolysis

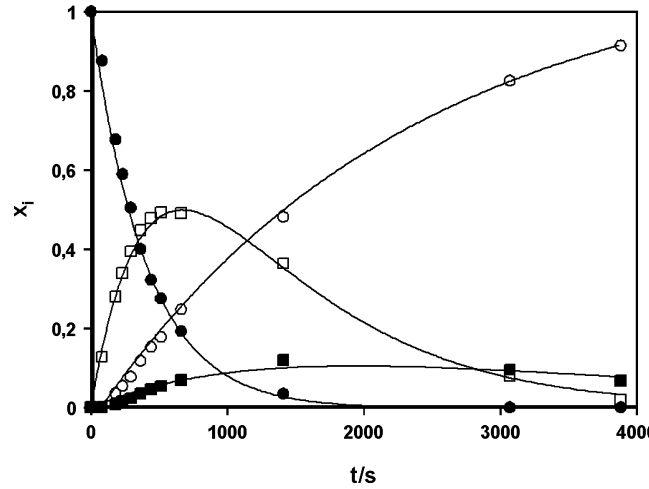
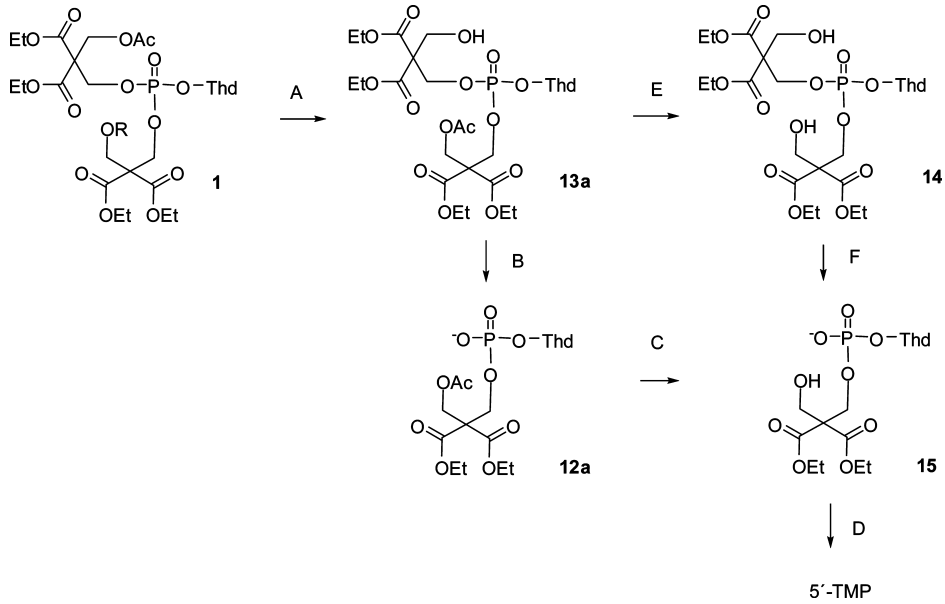


FIGURE 1. Time-dependent product distribution for the PLE-catalyzed hydrolysis of thymidine 5′-bis[3-acetyloxy-2,2-bis(ethoxycarbonyl)]-propylphosphate (**1**) at pH 7.5 and 25.0 °C ($I = 0.1$ mol L^{-1} with NaCl). Notation: (●) **1**; (□) thymidine 5′-[3-acetyloxy-2,2-bis(ethoxycarbonyl)]propyl 3-hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**13a**); (■) thymidine 5′-bis[3-hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**14**); and (○) mixture of thymidine 5′-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**12a**) and thymidine 5′-[3-hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**15**). For the structures, see Scheme 5.

to the fully deacetylated triester (**14**; $[M + H]^+$ 727.7; reaction E) and breakdown to acetylated diester **12a** ($[M + H]^+$ 567.5) by removal of the deacetylated protecting group by retro-aldol condensation (reaction B). The deacetylated triester (**14**) undergoes a similar retro-aldol condensation giving deacetylated diester **15** ($[M + H]^+$ 525.4; reaction F). The subsequent reactions of the diesters **12a** and **15** are slow compared to their formation. At the high PLE concentration employed, the diesters are accumulated in approximately equimolar amounts, but at a lower PLE concentration, the acetylated diester (**12a**) predominates. For example, at [PLE] = 4.3 units mL^{-1}, the ratio [**12a**]/[**15**] is 10:1. Most likely, deacetylation of triester **13a** (E) is not fast enough in the intracellular environment to compete with the nonenzymatic conversion of **13a** to **12a**. In other words, the desired nucleoside 5′-monophosphate should be formed by deacetylation of **12a** to **15**, which then releases the monophosphate by retro-aldol condensation.

The hydrolytic stability of the monodeacetylated triester (**13a**) was determined by applying the rate law of two consecutive first-order reactions to the time-dependent concentration of **13a**. The rate constant obtained on this basis for the disappearance of **13a** (7.5×10^{-4} s^{-1}) was then divided into contributions of

SCHEME 5



retro-aldol condensation (reaction B) and deacetylation (reaction E) with the aid of concentration ratio [**12a**]/([**14**] + [**15**]) at early stages of their appearance. It should be noted that **12a** is considerably more stable than **14**, and hence, at early stages of the appearance of **12a**, **14**, and **15**, the latter compound is formed predominantly via **14**. In this manner, a value of 3.7×10^{-4} s^{-1} was obtained for the first-order rate constant of the retro-aldol condensation of **13a** (reaction B; $\tau_{1/2} = 31$ min). Owing to more complicated kinetics, the rate constant for the conversion of triester **14** to diester **15** (reaction F) could not be determined as accurately, but it appeared to be of the same order of magnitude.

To determine the rate constant for the retro-aldol condensation of the deacetylated diester (**15**, reaction D), triester **1** was first deacetylated with porcine liver esterase (PLE) in a dilute HEPES buffer (pH 7.5, 25 °C) and after 3 h, the enzyme was denaturated by adjusting the pH to 2 with aqueous hydrogen chloride. At this stage, the starting material had been fully converted to a mixture of the acetylated (**12a**) and deacetylated (**15**) diester. The diesters gave separate HPLC signals, but no attempt to isolate them was made. The pH of the filtered solution (1 mL) was then adjusted to 7.5 with a more concentrated HEPES solution (0.1 mol L^{-1}), and the conversion of the deacetylated diester (**15**) to 5′-TMP was followed by HPLC. The rate constant for the process was 8.5×10^{-6} s^{-1} ($\tau_{1/2} = 23$ h). In other words, the 3-hydroxy-2,2-bis(ethoxycarbonyl)propyl group is cleaved from a phosphotriester (**13a**) 45 times as fast as from a phosphodiester (**15**). It should be noted that the nonenzymatic deacetylation of **12a** to **15** (reaction C) is so much slower than the conversion of **15** to 5′-TMP (reaction D) that the concentration of **12a** remains constant, within the limits of experimental errors, during the disappearance of **15**. Table 1 records the rate constants for the partial reactions indicated in Scheme 5.

The problem from the point of view of pro-drug applications is the very slow deacetylation of diester **12a**. While the half-lives for the deacetylation of **1** to **13a** and subsequently to **14** are 4.3 (reaction A) and 31 min (reaction E) at 25 °C, respectively (Table 1), the half-life for the deacetylation of diester **12a** (reaction C) is 190 h. Conversion of the resulting deacetylated diester **15** to thymidine 5′-monophosphate is somewhat faster, although still 45 times slower than the corresponding reaction of **13a** to **12a** (or **14** to **15**), the half-lives for reactions B and D being 0.5 and 23 h, respectively (Table 1). However, as indicated above, **13a** is at a low carboxyesterase activity converted rather to **12a** than to **14**, and hence, deacetylation of **12a** constitutes the rate-limiting step for the release of thymidine 5′-monophosphate. Consistent with this, still after 2 weeks, about 20% of the starting material (**1**) was present as diester **12a**. Thymidine 5′-monophosphate accumulated as an intermediate at maximally 15% level (at $t =$ 5 d) and was largely dephosphorylated to thymidine (Figure 2).

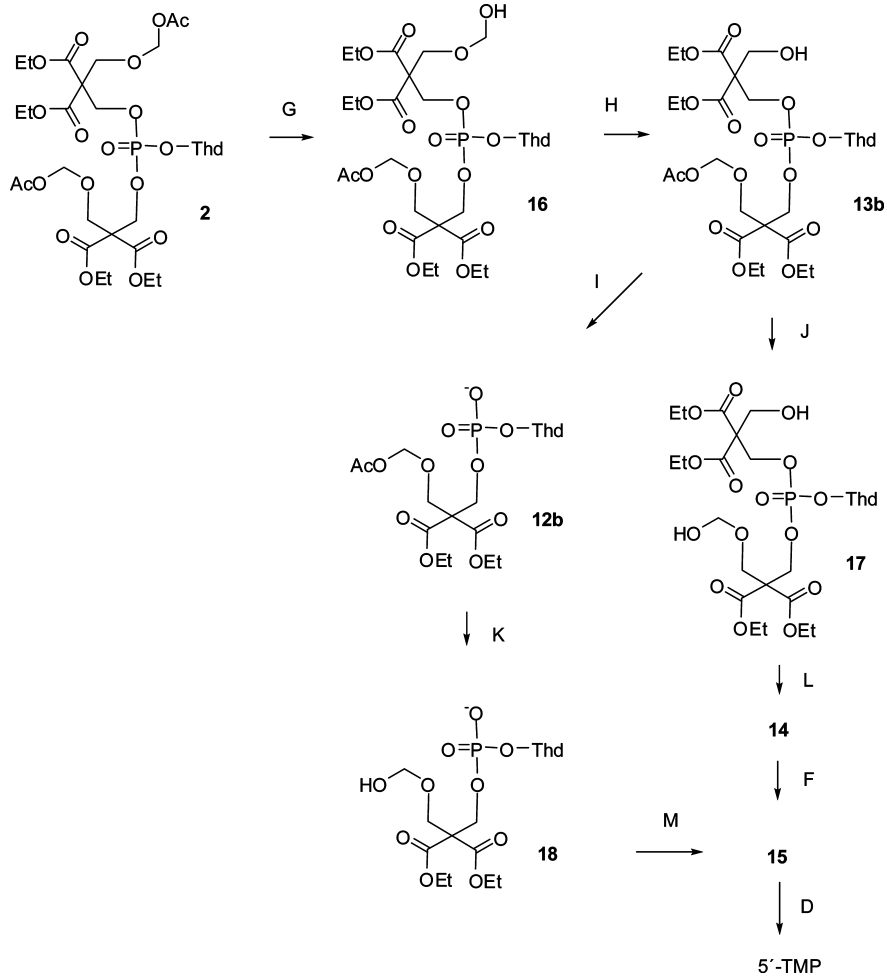
The hydrolytic stability of **1** was additionally determined in human serum at 25 °C. The half-live for the disappearance of **1** was 24 h. In addition to diester **12a**, a considerable amount of thymidine accumulated (25% of the products) during two half-lives of the breakdown of the starting material. In summary, while the 3-acyloxy-2,2-bis(ethoxycarbonyl)propyl group shows

TABLE 1. First-Order Rate Constants for the Partial Reactions Involved in the PLE-Triggered Hydrolysis of Thymidine 5′-Bis[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (1) and Thymidine 5′-Bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)-propyl]phosphate (2) to 5′-TMP at pH 7.5 ($I = 0.1$ mol L^{-1} with NaCl)[a]

reaction	T/°C	$k/10^{-4}$ s^{-1}	$t_{1/2}$/min
1 → **13a** (A)	25.0	27	4.3
	37.0	36	3.2
13a → **12a** (B)	25.0	3.8	31
13a →**14** (E)	25.0	3.8	31
12a → **15** (C)	25.0	0.010	11500
14 → **15** (F)	25.0	≈4	≈30
15 → 5′-TMP (D)	25.0	0.085	1360
2 → **16** (G)	37.0	680	0.17
16 → **13b** (H)	37.0	≈800	≈0.14
13b → **12b** (I)	37.0	11	10.4
13b → **17** (J)	37.0	130	0.90
12b → **18** (K)	37.0	0.54	210
17 → **14** (L)	37.0	≈800	≈0.14
14 → **15** (F)	37.0	≈10	≈10
18 → **15** (M)	37.0	fast	fast
15 → 5′-TMP (D)	37.0	0.31	370

[a] For the reactions, see Schemes 5 and 6. [PLE] = 26 units mL^{-1}.

SCHEME 6



potential as a protecting group for phosphodiester linkages, it does not show promise as a protecting group for phosphomonoesters, such as nucleoside 5′-monophosphates.

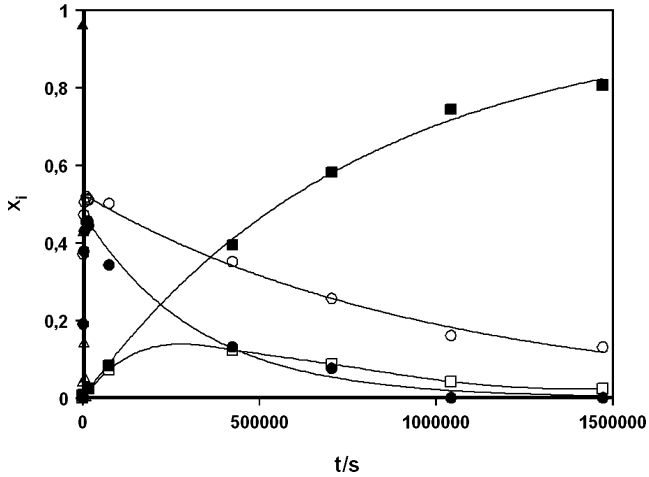


FIGURE 2. Time-dependent product distribution for the prolonged PLE-promoted hydrolysis of **1** at pH 7.5 and 25.0 °C ($I = 0.1$ mol L^{-1} with NaCl). Notation: (▲) (**1**), (△) thymidine 5′-[3-acetyloxy-2,2-bis(ethoxycarbonyl)]propyl 3-[hydroxy-2,2-bis(ethoxycarbonyl)propyl]-phosphate (**13a**) and thymidine 5′-bis[3-hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**14**), (○) thymidine 5′-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**12a**), (●) thymidine 5′-[3-hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**15**), (□) 5′-TMP, and (■) thymidine. For the structures, see Scheme 5.

Enzymatic Removal of the 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl Groups from Thymidine 5′-Phosphotriester 2. As indicated above, the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group is not appropriate for protection of nucleoside 5′-monophosphates for the reason that enzymatic deacetylation of the negatively charged phosphodiester is too slow. To elongate the distance between the scissile ester bond and the phosphate moiety, and to diminish the steric hindrance caused by the bulky ethoxycarbonyl substituents, the 3-acetyloxy group was replaced with a 3-acetyloxymethoxy group. Thymidine 5′-phosphotriester bearing two such groups (**2**) was treated with PLE at 37 °C and the progress of the reaction was followed by HPLC-ESI-MS. Figure 3 gives as illustrative examples the chromatograms obtained at an early stage, in the middle of and at the end of the cleavage of **2**. The time-dependent product distribution after the deacetylation of **2** with PLE is shown in Figure 4.

As seen from Figure 4, the disappearance of the starting material is accompanied by formation of four phosphotriesters formed in the order **16** → **13b** → **17** → **14** (Scheme 6). Among these, **16**, **13b**, and **17** may occur as R_P- and S_P-diastereomers, but the diastereomers could not be separated by the gradient HPLC system applied. The triesters are subsequently hydrolyzed to three nucleoside phosphodiesters (**12b**, **18**, and **15**), and finally, the desired 5′-TMP is released. The time-dependent appearance of the signals reveals that 5′-TMP is formed along the two pathways depicted in Scheme 6. All the intermediates

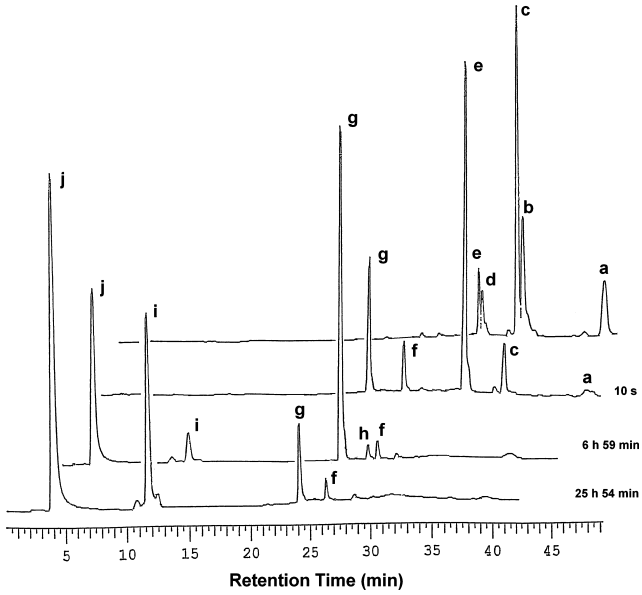


FIGURE 3. RP-HPLC profiles for the PLE-catalyzed hydrolysis of thymidine 5′-bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)]propylphosphate (**2**) at pH 7.5 and 37.0 °C (I = 0.1 mol L^{-1} with NaCl). Notation: (a) **2**, (b) thymidine 5′-[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)]propyl 3-hydroxymethoxy-2,2-bis(ethoxycarbonyl)propyl]-phosphate (**16**), (c) thymidine 5′-[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)]propyl 3-[hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**13b**), (d) thymidine 5′-[3-hydroxymethoxy-2,2-bis(ethoxycarbonyl)]-propyl 3-[hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**17**), (e) thymidine 5′-bis[3-hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**14**), (f) thymidine 5′-[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)]-propylphosphate (**12b**), (g) thymidine 5′-[3-hydroxy-2,2-bis(ethoxycarbonyl)]propylphosphate (**15**), (h) thymidine 5′-[3-hydroxymethoxy-2,2-bis(ethoxycarbonyl)]propylphosphate (**18**), (i) thymidine, and (j) 5′-TMP. For detailed chromatographic conditions, see the Experimental Section. For the structures, see Scheme 6.

were identified on the basis of their *m*/*z* values: $[M + H]^+$ 799.6 (**13b**), $[M + H]^+$ 727.7 (**14**), $[M + Na]^+$ 851,6 (**17**), $[M + Na]^+$ 779.8 (**16**), $[M - H]^-$ 595.5 (**12b**), $[M + H]^-$ 553.2 (**18**), and $[M - H]^-$ 523.4 (**15**). Accordingly, enzymatic removal of one of the acetyl groups from **2** (reaction G) gives triester **16** still bearing the deacetylated hydroxymethoxy group, which is then rather rapidly cleaved (reaction H) giving triester **13b**. This triester then undergoes breakdown by two parallel routes: a retro-aldol condensation produces diester **12b** (reaction I) and enzymatic deacetylation yields triester **17** (reaction J). Both of these products are eventually cleaved to diester **15**, diester **12b** by enzymatic deacetylation (reaction K) and hydroxide ion-catalyzed loss of formaldehyde (reaction M) and triester **17** by loss of formaldehyde (reaction L) followed by retro-aldol condensation (reaction F). In the final step, diester **15** undergoes another retro-aldol condensation to 5′-TMP (reaction D). Prolonged treatment with PLE resulted in partial degradation of 5′-TMP to thymidine.

The initial deacetylation of **2** is much faster than the corresponding reaction of **1**: the half-lives on using 26 units of PLE in 1 mL are 10 s and 3.2 min at 37 °C (Table 1), respectively. Intermediary accumulation of the resulting hydroxymethoxy substituted triester (**16**) is clearly detected, although the loss of formaldehyde giving triester **13b** is fast, approximatively as fast as the deacetylation. The next intermediate, triester **13b**, accumulated as a main product at the early stages of the reaction. At the high PLE concentration employed, this compound undergoes retro-aldol condensation to diester **12b**

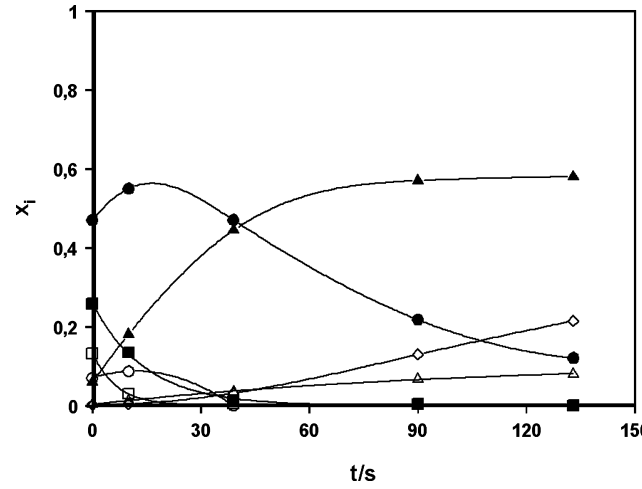


FIGURE 4. Time-dependent product distribution for the PLE-catalyzed hydrolysis of thymidine 5′-bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)]propylphosphate (**2**) at pH 7.5 and 37.0 °C (I = 0.1 mol L^{-1} with NaCl). Notation: (□) **2**; (■) thymidine 5′-[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)]propyl 3-[hydroxymethoxy-2,2-bis(ethoxycarbonyl)-propyl]phosphate (**16**); (●) thymidine 5′-[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)]propyl 3-[hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**13b**); (○) thymidine 5′-[3-hydroxymethoxy-2,2-bis(ethoxycarbonyl)]propyl 3-[hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**17**); (▲) thymidine 5′-bis[3-hydroxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**14**), (△) thymidine 5′-[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)]propylphosphate (**12b**), and (◇) thymidine 5′-[3-hydroxy-2,2-bis(ethoxycarbonyl)]propylphosphate (**15**). For the structures, see Scheme 6.

(reaction I) 12 times slower than the esterase-catalyzed deacetylation (reaction J). At low esterase activity, the route via **12b** may, however, predominate and the enzymatic deacetylation of **12b** (reaction K) most likely becomes rate-limiting. Alternatively, conversion by **15** to 5′-TMP by retroaldol condensation (reaction D) may constitute the rate-limiting step. The half-life for this reaction is 6 h at 37 °C.

The rate-accelerating effect that insertion of a $-CH_2O-$ group between the ester function and C3 of the 2,2-disubstituted propyl group exerts on the esterase-catalyzed deacetylation of both the starting material (**2** compared to **1**) and the monoanionic phosphodiester (**12b** compared to **12a**) is remarkable. At 37 °C, the half-lives for the deacetylation of **1** and **2** are 194 s [$k = (3.6 \pm 0.1) \times 10^{-3}$ s^{-1}] and 10 s [$k = (6.8 \pm 2.0) \times 10^{-2}$ s^{-1}], respectively (see Table 1). The acceleration is thus 20-fold. Deacetylation of the diester accelerated even more markedly. The half-life for the disappearance of **12b** is 2.7 h [$k = (5.4 \pm 0.1) \times 10^{-5}$ s^{-1}], while the half-life for the disappearance of the corresponding 3-acetyloxy derivative (**12a**) is 150 h at this temperature. Since the latter reaction most likely limits the rate of release of nucleoside 5′-monophosphate from its triester prodrug, it appears clear that the 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl group is much more appropriate than its 3-acetyloxy counterpart as a protecting group of nucleoside monophosphates.

Removal of the 3-Acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl Groups from Thymidine 5′-Phosphotriester 2 in a Whole-Cell Extract. The deprotection of thymidine 5′-bis[3-acetyloxymetoxy-2,2-bis(ethoxycarbonyl)propyl]phosphate (**2**) (Figure 5) was additionally studied in a whole cell extract of human prostate carcinoma. The starting material was added into 2 mL of the PC3 cell extract obtained by disrupting 3×10^7 cells in 10 mL of RIPA-buffer (pH 7.5) and diluted in 1:2 ratio with a HEPES buffer. The progress of the deprotection was

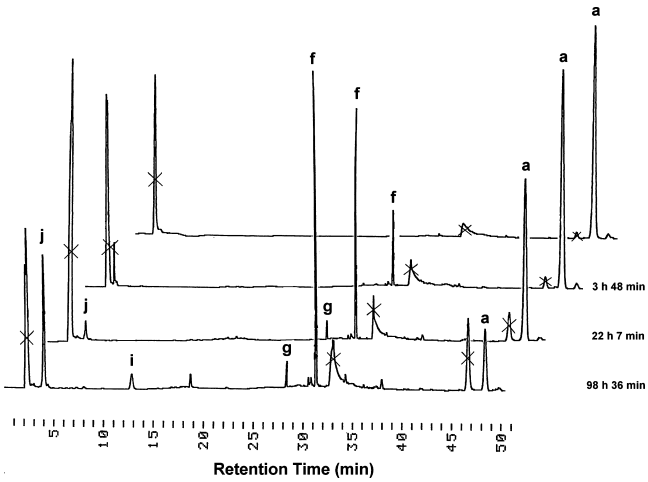


FIGURE 5. RP-HPLC profiles for the PLE-catalyzed hydrolysis of thymidine 5′-bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)]propylphosphate (**2**) in a whole-cell extract of human prostate carcinoma at pH 7.5 and 37.0 °C (I = 0.1 mol L^{-1} with NaCl). Notation: (a) **2**, (f) thymidine 5′-[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)]propylphosphate (**12b**), (g) thymidine 5′-[3-hydroxy-2,2-bis(ethoxycarbonyl)]propylphosphate (**15**), (i) thymidine, and (j) 5′-TMP. For detailed chromatographic conditions, see the Experimental Section. For the structures, see Scheme 6.

followed at 37 ± 0.1 °C. Deacetylation of triester **2** was much slower in the cell extract than at the high concentration of PLE (26 units mL^{-1}), but the expected diester intermediates and 5′-TMP appeared. The acetylated diester **12b** accumulated and was converted to 5′-TMP via the deacetylated intermediate **18**.

In summary, the present study indicates that the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group, shown previously to be a viable biodegradable protecting group for phosphodiesters, does not seem appropriate for the protection of nucleoside 5′-monophosphates as triesters. The diester obtained by departure of the first proteting group undergoes esterase triggered removal very slowly. By contrast, introduction of an additional $-CH_2O-$ group between the enzyme labile ester function and the 2,2-disubstituted propyl group markedly accelerates the enzymatic deacetylation of the triester, but what is more important, the deacetylation of the diester. The latter reaction experiences a 50-fold acceleration by this structural modification. It has additionally been shown that the thymidine 5′-triester released 5′-TMP in a whole cell extract of human prostate carcinoma.

Experimental Section

3′-*O*-Levulinoylthymidine (5). 5′-*O*-(4,4′-dimethoxytrityl)thymidine (16.2 mmol, 8.8 g), prepared by established synthetic procedures, was dissolved in anhydrous 1,4-dioxane (100 mL). A solution of levulinic anhydride, prepared from levulinic acid (49.0 mmol, 5.70 g) in pyridine (60 mL) with 1,3-dicyclohexylcarbodiimide (48.4 mmol, 10.0 g) as a condensing agent, was filtered onto the nucleoside. After being stirred for 4 h at room temperature, the reaction mixture was evaporated to dryness and a conventional aq $NaHCO_3/CH_2Cl_2$ was carried out. The organic phase was evaporated to dryness. The dimethoxytrityl protecting group was removed with 80% aqueous acetic acid (80 mL; 9 h). The reaction mixture was evaporated to dryness and the residue was purified on a silica gel column eluted with a mixture of DCM and MeOH (90:10, v/v). The product was obtained as a white powder in 51% yield (2.8 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.36 (s, 1H), 7.73 (s, 1H), 6.25 (dd, 1H, J = 6.5 and 2.0 Hz), 5.37 (m, 1H), 4.11 (d, 1H, J = 2.0 Hz), 3.90 (m, 2H), 2.80 (m, 2H), 2.59 (t, 2H, J = 6.0 Hz), 2.42 (m, 2H), 2.22 (s, 3H), 1.92 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 206.79, 172.61, 164.11, 150.59, 136.59, 111.32, 86.04, 85.08, 74.96, 62.47, 37.83, 37.15, 29.81, 27.95, 12.55; ESI$^+$-MS m/z obsd 341.6 $[M + H]^+$, calcd 341.1.

Diethyl 2-Ethoxy-2-methyl-1,3-dioxane-5,5-dicarboxylate (8). Concentrated H_2SO_4 (1.3 mmol; 71 μL) was added to a mixture of diethyl 2,2-bis(hydroxymethyl)malonate (43.5 mmol, 9.6 g) and triethyl orthoacetate (65.2 mmol; 11.9 mL) in dry THF (15 mL). The reaction was allowed to proceed overnight and the mixture was then poured into an ice-cold solution of 5% $NaHCO_3$ (50 mL). The product was extracted with diethyl ether (2 × 50 mL), washed with saturated aqueous NaCl (2 × 50 mL), and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified on a silica gel column eluting with a mixture of dichloromethane and methanol (95:5, v/v). The product was obtained as a clear oil in 89% yield (11.3 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.30−4.36 (m, 6H), 4.18 (q, J = 7.1 Hz, 1H), 3.54 (q, J = 7.10 Hz, 2H), 1.46 (s, 3H), 1.32 (t, J = 7.10 Hz, 3H), 1.27 (t, J = 7.1 Hz, 3H), 1.26 (t, J = 7.1 Hz 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.0 and 167.0, 111.1, 62.0 and 61.9, 61.6, 58.7, 52.3, 22.5, 15.1, 14.0, and 13.9; ESI$^+$-MS m/z $[M + Na]^+$ obsd 313.1241, calcd 313.1264.

Diethyl 2-(Acetyloxymethyl)-2-(hydroxymethyl)malonate (3). Compound **8** (17.9 mmol; 5.2 g) was dissolved in 80% aqueous acetic acid (30 mL) and left for 2 h at room tempertature. The solution was evaporated to dryness and the residue was coevaporated three times with water. The product was purified by silica gel colum chromatogaphy eluting with ethyl acetate in dichloromethane (8:92, v/v). The product was obtained as a yellowish oil in 75% yield (3.6 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.76 (s, 2H), 4.26 (q, J = 7.10 Hz, 4H), 4.05 (d, J = 7.10 Hz, 2H), 2.72 (t, J = 7.1 Hz, 1H), 2.08 (s, 3H), 1.27 (t, J = 7.10 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.9, 168.1, 62.3, 62.2, 61.9, 59.6, 20.7, 14.0; ESI$^+$-MS m/z $[M + Na]^+$ obsd 285.0942, calcd 285.0951.

Thymidine 5′-Bis[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]-phosphate (1). Compound **3** was coevaporated once from dry pyridine and three times from dry MeCN after which it was dried over P_2O_5 overnight. To a solution of dried **3** (2.9 mmol; 0.76 g) in dry DCM (2 mL) were added anhydrous triethylamine (14.4 mmol, 2 mL) and bis(diethylamino)chlorophosphine (4.0 mmol; 850 μL), and the reaction mixture was stirred for 1 h under nitrogen. The product was filtered through a short silica gel column eluting with a mixture of anhydrous ethyl acetate and triethylamine in hexane (60:0.5:39.5, v/v/v). The solvent was removed under reduced pressure and the residue was coevaporated three times from dry MeCN to remove the traces of triethylamine. The residue was dissolved in dry MeCN (2.0 mL) and **3** (2.9 mmol; 0.77 g) in dry MeCN (2.0 mL) and tetrazole (7.2 mmol; 16.0 mL of 0.45 mol L^{-1} solution in MeCN) were added under nitrogen. The reaction mixture was stirred for 2.5 h at room temperature. 3′-*O*-Levulinoylthymidine (**5**; 2.9 mmol; 1.0 g), dried over phosphorus pentoxide, and 1-*H*-tetrazole (2.9 mmol; 6.4 mL of 0.45 mol L^{-1} solution in MeCN) were added and the stirring was continued for 1.5 h. The phosphite ester formed was oxidized with I_2 (0.1 mol L^{-1}) in a mixture of THF, H_2O, and lutidine (4:2:1, v/v/v; 10 mL). The crude product (**6**) was isolated by DCM/aq $NaHSO_3$ workup, and purified on a silica gel column eluted with a mixture of hexane and ethyl acetate (40:60, v/v). The purification was repeated with ethyl acetate and a mixture of DCM and MeOH (90:10, v/v) as an eluent. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.75 (s, 1H), 7.48 (d, J = 1.0 Hz, 1H), 6.37 (dd, J = 7.0 and 5.5 Hz, 1H), 5.25 (d, J = 6.5 Hz, 1H), 4.65−4.48 (m, 19H), 2.78 (t, 2H), 2.60 (t, 2H), 2.42 (dd, 1H), 2.22 (dd, 1H), 2.21 (s, 3H), 2.063 (s, 3H), 2.056 (s, 3H), 1.97 (d, 3H), 1.27 (m, 12H); ESI$^+$-MS m/z obsd 909.8 $[M + H]^+$, calcd 909.8.

A mixture of $NH_2NH_2 \cdot H_2O$ (0.5 mol L^{-1}), **6** (0.14 mmol, 0.12 g), pyridine (0.9 mL), and acetic acid (0.2 mL) was stirred for 80 min at 0 °C. The ice−water bath was removed and the solution was stirred for an additional 11 h at room temperature. The crude product was isolated by DCM/aq $NaHCO_3$ workup and purified on a silica

gel column eluted with a mixture of DCM and MeOH (90:10%, v/v) and by reversed phase chromatography on a Lobar RP-18 column (37 × 440 mm, 40−63 μm), eluting with a mixture of water and acetonitrile (60:40%, v/v). The product was obtained as a clear oil in 55% yield. ^{31}P NMR (202 MHz, D_2O) δ −1.96 ppm. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.30 (s, 1H), 7.35 (s, 1H), 6.33 (dd, J = 6.5 Hz, 1H), 4.64−4.48 (m, 9H), 4.25 (m, 10H), 4.40 (br. s, 1H), 2.40 (m, 1H), 2.20 (dd, 1H), 2.06 (s, 6H), 1.94 (s, 3H), 1.25 (t, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.3, 166.3, 163.9, 150.5, 135.5, 111.4, 84.7, 84.3, 84.2, 70.5, 67.1, 65.3, 62.4, 58.8, 39.7, 20.6, 13.9, 12.4; ESI$^+$-MS m/z [M + Na]$^+$ obsd 833.7, calcd 833.2; ESI$^+$-MS m/z [M + H]$^+$ obsd 811.2517, calcd 811.2460.

Diethyl 2-(*tert*-Butyldimethylsilyloxymethyl)-2-hydroxymethylmalonate (9). Diethyl 2,2-bis(hydroxymethyl)malonate (28.3 mmol; 6.23 g) was coevaporated twice from dry pyridine and dissolved in the same solvent (20 mL). *tert*-Butyldimethylsilyl chloride (25.5 mmol; 3.85 g) in dry pyridine (10 mL) was added portionwise. The reaction was allowed to proceed for 4 days. The mixture was evaporated to give solid foam, which was then equilibrated between water (200 mL) and DCM (4 × 100 mL). The organic phase was dried on Na_2SO_4. The product was purified by silica gel chromatography eluting with 10% ethyl acetate in DCM. The product was obtained as a clear oil in 78% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.18−4.25 (m, 4H), 4.10 (s, 2H), 4.06 (s, 2H), 2.63 (br s, 1H), 1.26 (t, J = 7.0 Hz, 6H), 0.85 (s, 9H), 0.05 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.2, 63.3, 62.8, 61.6, 61.4, 25.6, 18.0, 14.0, −3.6; MS [M + H]$^+$ obsd 335.7, calcd 335.2; [M + Na] obsd 357.6, calcd 357.2; ESI$^+$-MS m/z [M + Na]$^+$ obsd 357.1699, calcd 357.1710.

Diethyl 2-(*tert*-Butyldimethylsilyloxymethyl)-2-methylthiomethyloxymethylmalonate (10). 9 (19.7 mmol; 6.59 g) was dissolved into a mixture of acetic anhydride (40 mL), acetic acid (12.5 mL), and DMSO (61 mL), and the mixture was stirred overnight. The reaction was stopped by dilution with cold aq Na_2CO_3 (290 mL, 10% aq solution) and the product was extracted in diethyl ether (4 × 120 mL). The combined organic phase was dried on Na_2SO_4. The product was purified by silica gel chromatography with DCM as an eluent. The product was obtained as a clear oil in 91% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.61 (s, 2H), 4.14−4.19 (m, 4H), 4.06 (s, 2H), 4.00 (s, 2H), 2.06 (s, 3H), 1.22 (t, J = 7.0 Hz, 6H), 0.83 (s, 9H), 0.02 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.3, 75.6, 65.7, 61.4, 61.2, 60.9, 25.6, 18.0, 14.0, 13.7, −3.6 (Si−CH_3); MS [M + H]$^+$ obsd 395.4, calcd 395.2, [M + Na]$^+$ obsd 417.6, calcd 417.2; ESI$^+$-MS m/z [M + Na]$^+$ obsd 417.1732, calcd 417.1743.

Diethyl 2-Acetyloxymethyloxymethyl-2-(*tert*-butyldimethylsilyloxymethyl)malonate (11). 10 (17.9 mmol; 7.08 g) was dissolved in dry DCM (96 mL) under nitrogen. Sulfuryl chloride (21.5 mmol; 21.5 mL of 1.0 mol L^{-1} solution in DCM) was added in three portions and the mixture was stirred for 70 min under nitrogen. The solvent was removed under reduced pressure and the residue was dissolved into dry DCM (53 mL). Potassium acetate (30.9 mmol; 3.03 g) and dibenzo-18-crown-6 (13.5 mmol; 4.85 g) in DCM (50 mL) were added and the mixture was stirred for 1.5 h. Ethyl acetate (140 mL) was added, and the organic phase was washed with water (2 × 190 mL) and dried on Na_2SO_4. The product was purified by silica gel chromatography with DCM as an eluent. The product was obtained as an oil in 71% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.24 (s, 2H), 4.15−4.22 (m, 4H), 4.13 (s, 2H), 4.08 (s, 2H), 2.08 (s, 3H), 1.26 (t, J = 8.0 Hz, 6H), 0.85 (s, 9H), 0.04 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.2, 168.0, 89.3, 67.5, 61.4, 61.1, 60.2, 25.6, 21.0, 18.1, 14.0, −5.7; MS [M + Na]$^+$ obsd 429.6, calcd 429.2; ESI$^+$-MS m/z [M + Na]$^+$ obsd 429.2013, calcd 429.1921.

Diethyl 2-Acetyloxymethyloxymethyl-2-hydroxymethylmalonate (4). 11 (7.2 mmol; 2.93 g) was dissolved in dry THF (23 mL) and triethylamine trihydrofluoride (8.64 mmol; 1.42 mL) was added. The mixture was stirred for 1 week. Aqueous triethylammonium acetate (13 mL of 2.0 mol L^{-1} solution) was added. The mixture was evaporated to dryness and the residue was purified by silica gel chromatography with DCM containing 2−5% MeOH as an eluent. The product was obtained as an oil in 74% yield. ^{1}H NMR (500, $CDCl_3$) δ 5.25 (s, 2H), 4.16−4.29 (m, 6H), 4.13 (s, 2H), 2.10 (s, 3H), 1.81 (br s, 1H), 1.26 (t, J = 9.0 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.41, 168.55, 89.10, 68.95, 63.96, 61.83, 60.29, 20.97, 13.99; MS [M + Na]$^+$ obsd 315.3, calcd 315.1; ESI$^+$-MS m/z [M + Na]$^+$ obsd 315.1058, calcd 315.1056.

Thymidine 5′-Bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)-propyl]phosphate (2). 3′-*O*-Levulinoylthymidine (**5**; 0.47 mmol; 0.166 g) was coevaporated once from dry pyridine and three times from dry MeCN and dissolved in dry DCM (1.2 mL) under nitrogen. Triethylamine (2.35 mmol; 0.34 mL) and bis(diethylamino)chlorophosphine (0.68 mmol; 0.145 mL) were added and the mixture was stirred under nitrogen for 2 h. The product was isolated by passing the mixture through a short silica gel column with a 4:1 mixture of ethyl acetate and hexane containing 0.5% triethylamine. The solvent was removed under reduced pressure and the residue was coevaporated three times from dry MeCN to remove the traces of triethylamine. The residue was dissolved in dry MeCN (1.0 mL) and **4** (1.68 mmol; 0.49 g) in dry MeCN (1.0 mL) and tetrazole (2.91 mmol; 6.46 mL of 0.45 mol L^{-1} solution in MeCN) were added under nitrogen. The reaction was allowed to proceed for 6 h and then iodine (0.73 mmol; 0.185 g) in a mixture of THF (4.0 mL), H_2O (2.0 mL), and 2,6-lutidine (1.0 mL) was added. The oxidation was allowed to proceed overnight. The excess of iodine was destroyed with 5% $NaHSO_3$. The mixture was extracted three times with DCM. The organic phase was washed with brine, dried on Na_2SO_4, and evaporated to dryness. The crude product was purified on a silica gel column eluting with DCM containing 5−10% MeOH. The yield of the 3′-*O*-levulinoyl ester derivative (**7**) of **2** was 15% from **5**.

The 3′-*O*-levulinoylthymidine 5′-bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl]phosphate obtained (0.071 mmol; 69 mg) was dissolved in dry DCM (2.0 mL) and hydrazine acetate (0.12 mmol; 11 mg) in dry MeOH (0.20 mL) was added. After 1 h, hydrazinium acetate (0.05 mmol; 4.6 mg) in a mixture of DCM (100 μL) and MeOH (20 μL) was added. The reaction was allowed to proceed for 2 h and the addition of hydrazinium acetate was repeated. The reaction was quenched with acetone and the mixture was evaporated to dryness. The product was purified on a silica gel column eluting first with ethyl acetate and then with DCM containing 15% MeOH. The product was obtained as a solid in quantitative yield (15% from **5**). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.91 (s, 1H), 7.34 (s, 1H), 6.31 (dd, J = 6.0 and 6.0 Hz, 1H), 5.25 (s, 4H), 4.54 (m, 5H), 4.24 (m, 10H), 4.05 (s, 1H), 3.61 (br s, 1H), 2.42 (m, 1H), 2.24 (m, 1H), 2.11 (s, 6H), 1.95 (s, 3H), 1.27 (m, 12H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.6, 166.6, 163.7, 150.3, 135.5, 111.4, 88.8, 84.8, 84.4, 70.7, 67.2, 67.0, 65.3, 62.3, 58.8, 39.6, 20.9, 13.9, 12.4; ^{31}P NMR (202 MHz, $CDCl_3$) δ −2.1 ppm; ESI$^+$-MS m/z [M + Na]$^+$ obsd 893.8, calcd 893.3; ESI$^+$-MS m/z [M + Na]$^+$ obsd 893.2567, calcd 893.2569.

Preparation of the Cell Extract. A sample of 3 × 10^7 human prostate carcinoma cells (PC3) was treated with 10 mL of RIPA-buffer [15 mmol L^{-1} Tris-HCl, pH 7.5, 120 nmol L^{-1} NaCl, 25 mmol L^{-1} KCl, 2 mmol L^{-1} EDTA, 2 mmol L^{-1} EGTA, 0.1% deoxycholic acid, 0.5% Triton X-100, 0.5% PMSF supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics GmBH, Germany)] at 0 °C for 10 min. Most of the cells were disrupted mechanically. The cell extract obtained was centrifuged (900 rpm, 10 min) and the pellet was discarded. The extract was stored at −20 °C.

Kinetic Measurements. The reactions were carried out in sealed tubes immersed in a thermostated water bath (25.0 and/or 37.0 ± 0.1 °C). The oxonium ion concentration of the reaction solutions (2.85 mL) was adjusted with sodium hydroxide and *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid] (HEPES) buffer. The ionic strength of the solutions was adjusted to 0.1 mol L^{-1} with sodium chloride. The hydronium ion concentration of the buffer solutions was calculated with the aid of the known pK_a values of

the buffer acid under the experimental conditions. The initial substrate concentration was ca. 0.3 mmol L^{-1}.

The enzymatic hydrolysis was carried out at pH 7.5. The acetyl group was removed with Porcine Liver Esterase (26 units mL^{-1}) in a HEPES buffer at pH 7.5 (0.01/0.01 mol L^{-1} at 25 °C; 0.040/0.024 mol L^{-1} at 37 °C). The samples (200 μL) withdrawn at appropriate intervals were made acidic (pH 2) with 1 mol L^{-1} aqueous hydrogen chloride to inactivate enzyme, cooled in an ice bath to quench the hydrolysis, and filtered with SPARTAN 13A filters (0.2 μm). The composition of the samples was analyzed on an ODS Hypersil C18 column (4 × 250 mm 5 μm, flow rate 1 mL min^{-1}), using a mixture of acetic acid/sodium acetate buffer (0.045/0.015 mol L^{-1}) and MeCN, containing ammonium chloride (0.1 mol L^{-1}). A good separation of the product mixtures of **2** was obtained on using a 5 min isocratic elution with the buffer containing 2% MeCN, followed by a linear gradient (23 min) up to 40.0% MeCN. Signals were recorded on a UV-detector at a wavelength of 267 nm. The reaction products were identified by the mass spectra (LC/MS), using a mixture of water and MeCN containing a formic acid (0.1%) as an eluent.

Calculation of the Rate Constants. The first-order rate constants for the nonenzymatic hydrolysis of triesters **1** and **2** to diesters **12a** and **12b**, respectively (Scheme 4), were obtained by applying first-order rate law to the diminution of the concentration of the starting material. The first-order rate constants for the hydrolysis of **12a** and **12b** to 5′-TMP were then calculated with the aid of the rate equation of two consecutive first-order reactions.

The enzymatic deacetylations obeyed first-order kinetics at the high PLE concentrations employed. The pseudo-first-order rate constants, k_A and k_G, for the disappearance of **1** and **2** (reaction A in Scheme 4 and reaction G in Scheme 5, respectively) were obtained by applying the integrated first-order rate equation to the time-dependent diminution of the concentration of the starting material. The first-order rate constant, k_{B+E}, for the disappearance of the deacetylation product of **1** (triester **13** in Scheme 5) was then obtained by least-squares fitting of the time-dependent concentration of **13** with the rate law of two consecutive first-order reactions (eq 1). Here, $[\mathbf{1}]_0$ stands for the initial concentration of **1** and $[\mathbf{13a}]_t$ for the concentration of **13a** at moment t. Breakdown of k_{B+E} to contributions of two parallel first-order reactions with the aid of the product distribution ([**12a**]/[**14**]) at early stages of their formation then gave k_B and k_E. The rate constant, k_C, for the disappearance of **12a** was obtained by first-order rate law, since the formation of this species was so much faster than its breakdown that the intermediary accumulation was virtually quantitative.

$$\frac{[\mathbf{13a}]_t}{[\mathbf{1}]_0} = \frac{k_A}{k_{B+E} - k_A}[\exp(-k_A t) - \exp(-k_{B+E} t)] \quad (1)$$

To determine the rate constant, k_D, for the disappearance of the deacetylated diester, **15**, triester **1** was deacetylated with porcine liver esterase (PLE) in a dilute HEPES buffer (pH 7.5, 25 °C), and after 3 h, the enzyme was denaturated by adjusting the pH to 2 with aqueous hydrogen chloride. In this manner, a mixture of the acetylated (**12a**) and deacetylated (**15**) diester was obtained. The pH of the filtered solution was readjusted to 7.5 with a more concentrated HEPES solution (0.1 mol L^{-1}), and the disappearance of **15** was followed. The rate constant for the disappearance was obtained by the first-order rate law. The nonenzymatic deacetylation of **12a** to **15** (reaction C) was so slow that the concentration of **12a** remained constant during the disappearance of **15**.

The PLE-triggered formation of triester **13b** was so much faster than its breakdown that **13b** was still largely accumulated after complete disappearance of **2** and **16** (cf. Scheme 6). Accordingly, the first-order rate constants for the disappearance of **13b** (k_{I+J}), conversion of **13b** to diester **12b** (k_I), and disappearance of **12b** (k_K) could be obtained by three-parameter fitting of the rate law of parallel and consecutive first-order reactions (eq 2) to the time-dependent concentration of **12b**. In eq 2, $[\mathbf{13b}]_0$ stands for the initial concentration of the triester **13b** and $[\mathbf{12b}]_t$ for the concentration of **12b** at moment t. The rate constant, k_J, for the deacetylation of **13b** is then obtained as a difference of k_{I+J} and k_I.

$$\frac{[\mathbf{12b}]_t}{[\mathbf{13b}]_0} = \frac{k_I}{k_K - k_{I+J}}[\exp(-k_{I+J} t) - \exp(-k_K t)] \quad (2)$$

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Supporting Information Available: Copies of ^{1}H NMR and ^{13}C NMR spectra of compounds **1**−**5** and **8**−**11** and copies of MS spectra of compounds **1**−**4** and **8**−**11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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