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2,2-Disubstituted 4-Acylthio-3-oxobutyl Groups as Esterase- and Thermolabile Protecting Groups of Phosphodiesters

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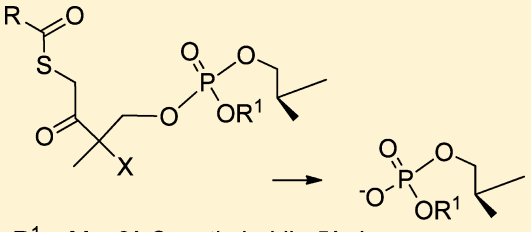
Supporting Information

ABSTRACT: Five different 2,2-disubstituted 4-acylthio-3-oxobutyl groups have been introduced as esterase-labile phosphodiester protecting groups that additionally are thermolabile. The phosphotriesters **1**−**3** were prepared to determine the rate of the enzymatic and nonenzymatic removal of such groups at 37 °C and pH 7.5 by HPLC-ESI-MS. Additionally, ^{1}H NMR spectroscopic monitoring was used for structural characterization of the intermediates and products. When treated with hog liver esterase, these groups were removed by enzymatic deacylation followed by rapid chemical cyclization to 4,4-disubstituted dihydrothiophen-3(2*H*)-one. The rate of the enzymatic deprotection could be tuned by the nature of the 4-acylthio substituent, the benzoyl group and acetyl groups being removed 50 and 5 times as fast as the pivaloyl group. No alkylation of glutathione could be observed upon the enzymatic deprotection. The half-life for the nonenzymatic deprotection varied from 0.57 to 35 h depending on the electronegativity of the 2-substituents and the size of the acylthio group. The acyl group evidently migrates from the sulfur atom to C3-*gem*-diol obtained by hydration of the keto group and the exposed mercapto group attacks on C1 resulting in departure of the protecting group as 4,4-disubstituted 3-acyloxy-4,5-dihydrothiophene with concomitant release of the desired phosphodiester.



R^1 = Me, 2′-*C*-methyluridin-5′-yl
R = Me, Ph, *t*-Bu
X = Me, CO_2Et

INTRODUCTION

Most of the prodrug approaches are based on a strategy consisting of an initial enzymatic activation, usually deacylation by esterases, followed by nonenzymatic removal of the remaining linker attached to phosphate function.[1,2] The protecting groups falling in this esterase-labile category include acyloxymethyl,[3] alkoxycarbonyloxymethyl,[4] *S*-acyl-2-thioethyl,[5] 4-acyloxybenzyl,[6] 2,2-bis(substituted)-3-acyloxypropyl,[7,8] 2,2-bis(substituted)-3-acyloxymethoxypropyl,[9] and 1-acyloxypropan-1,3-diyl[10] groups. In addition, the conversion of extensively studied *O*-aryl-*N*-[2-(alkoxycarbonyl)alkyl]phosphoramidates to phosphate monoesters is trigged enzymatically by esterases, but hydrolysis of the deprotected phosphoramidate to phosphate monoester depends on another enzyme, phosphoramidase.[11,12] Esterases accept a wide range of neutral molecules as substrates, and hence, the first phosphate protecting group is usually removed without difficulty. The cleaving activity of esterases is, however, dramatically reduced upon accumulation of negative charge on the substrate.[9,10,13,14] For this reason, removal of esterase-labile protecting groups from molecules containing more than one phosphodiester linkages still is problematic. Another shortcoming is that the nonenzymatic removal of the remnant of the protecting group often produces potentially toxic products, such as episulfide, formaldehyde, enones, phenols or quinoid structures. In fact, thermolabile protecting groups appear to offer an attractive alternative for esterase-labile groups as long as several phosphodiester linkages have to be protected in a single molecule. The group of Beaucage has introduced several purely thermolytic groups, including (*N*-formyl-*N*-methyl)-2-aminoethyl,[15] 4-hydroxybutyl,[16] and several from *ω*-(alkylthio)alkyl groups,[17] for the protection of immunostimulatory phosphoromonothioate oligomers. All these groups are removed by cyclization, the most appropriate being 5-(methylthio)pentyl, 5-(isopropylthio)pentyl, 4-(methoxymethylthio)butyl, and 2-(methylthiomethoxy)ethyl groups, which exhibit half-lives of removal ranging from 6 to 35 h at 37 °C. Cyclosaligenyl group has, in turn, shown some promise as a thermolabile protection of phosphate monoesters.[18−21]

We now introduce a set of 2,2-disubstituted 4-acylthio-3-oxobutyl groups as novel protecting groups for phosphodiester linkages. These groups are esterase-labile, but they undergo thermolytic removal in case the enzymatic reaction becomes severely retarded. The groups are released by cyclization to substituted tetrahydrothiophenones which do not form adducts with glutathione and, hence, most likely are not markedly alkylating. The rate of the esterase-catalyzed thioester hydrolysis may be varied by the size of the *S*-acyl group, and the thermolytic stability may be tuned by the polar nature of 2-substituents. The following compounds were prepared as model compounds to determine the rate of the enzymatic and

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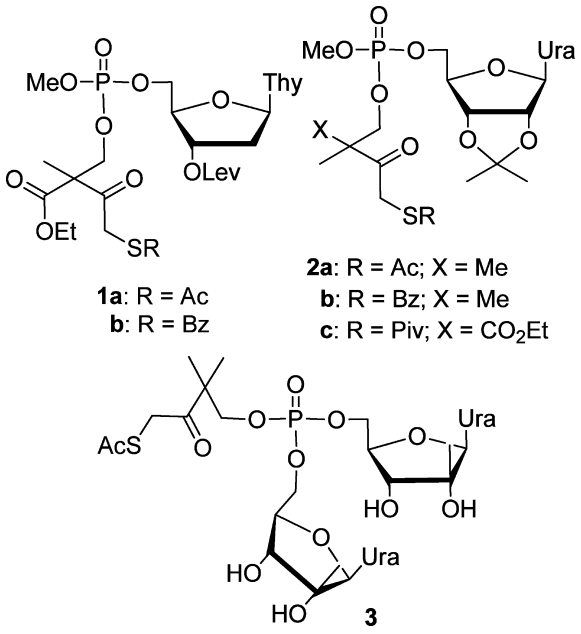
nonenzymatic removal of such groups at physiological pH: 3′-*O*-levulinoylthymidine 5′-methylphosphate protected with 4-acetylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl (**1a**), 4-benzoylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl (**1b**) groups, 2′,3′-*O*-isopropylideneuridine 5′-methylphosphate protected with 4-acetylthio-2,2-dimethyl-3-oxobutyl (**2a**), 4-benzoylthio-2,2-dimethyl-3-oxobutyl (**2b**), and 2-ethoxycarbonyl-2-methyl-3-oxo-4-pivaloylthiobutyl (**2c**) groups, and bis(2′-*C*-methyluridin-5′-yl) phosphate protected with 4-acetylthio-2,2-dimethyl-3-oxobutyl group (**3**). The progress of the deprotection of **3**, containing potentially antiviral 2′-*C*-methyl nucleosides, was additionally followed in a whole cell extract of human prostate carcinoma.



RESULTS AND DISCUSSION

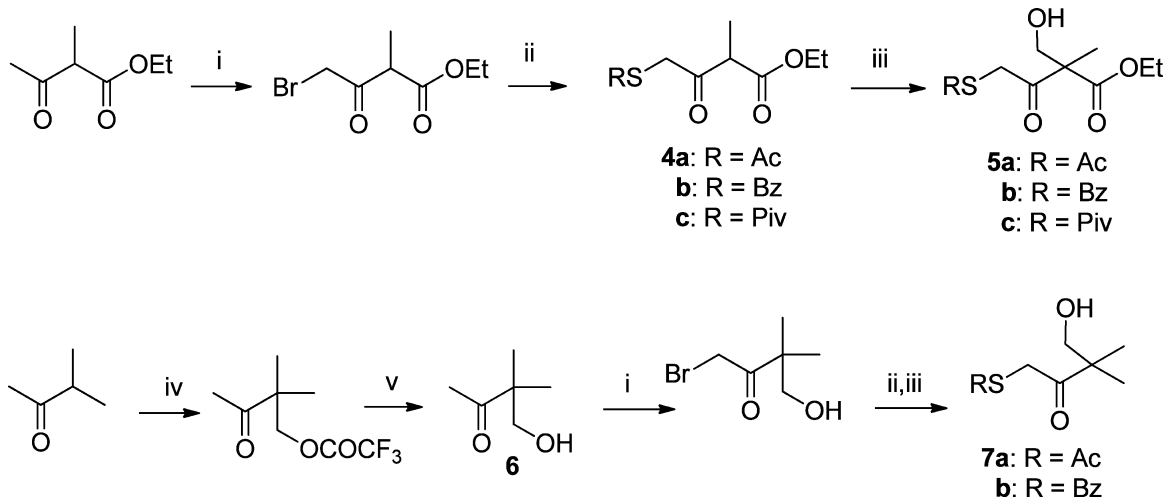
Syntheses. The preparation of various substituted butanols required for preparation of compounds **1**–**3** is outlined in Scheme 1. 4-Acylthio-2-hydroxymethyl-2-methyl-3-oxobutanoates (**5a**–**c**) were obtained by bromination of commercial ethyl 2-methyl-3-oxobutanoate,[22] followed by treatment with thioacetic, thiobenzoic, or thiopivalic acid in the presence of triethylamine to give thioesters **4a**–**c**, and these were finally subjected to triethylamine-promoted hydroxymethylation[23] to **5a**–**c**. *S*-(4-Hydroxy-3,3-dimethyl-2-oxobutyl)thioacetate (**7a**) and thiobenzoate (**7b**) were, in turn, prepared by trifluoroacetoxymethylation of 3-methylbutan-2-one and subsequent alkaline hydrolysis to 4-hydroxy-3,3-dimethylbutan-2-one[24] (**6**), which then was converted to **7a** and **7b** by bromination and subsequent displacement of the bromo substituent with thioacetic or thiobenzoic acid.

Phosphitylation of 3′-*O*-levulinoylthymidine[25] with 1-chloro-*N*,*N*-diisopropyl-1-methoxyphosphinamine and subsequent tetrazole-promoted displacement of the diisopropylamino ligand by alcohols **5a** and **b** gave after oxidation triesters **1a**,**b** (Scheme 2). Similarly, 2′,3′-*O*-isopropylideneuridine was converted to triesters **2a**–**c** with the aid of alcohols **7a**,**b** and **5c**. All of the triesters were mixtures of R_P and S_P diastereomers. In addition, the protecting group of triesters **1a**,**b** and **2c** contained a stereogenic center which increased the number of stereoisomers from two to four. No attempt was made to assign the absolute configuration of the diastereomers.

Phosphotriester **3** was prepared by displacing first the two chloro ligands of 1,1-dichloro-*N*,*N*-diisopropylphosphinamine with 2′,3′-*O*-isopropylidene-2′-*C*-methyluridine[26] (**8**) and the diisopropylamino ligand subsequently with *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl) thioacetate (**7a**), using tetrazole as an activator (Scheme 3). The phosphite triester formed was oxidized to phosphate ester (**9**) with iodine in aqueous THF containing 2,6-lutidine. The isopropylidene groups were then removed by treatment with aq 80% AcOH, giving 4-acetylthio-2,2-dimethyl-3-oxobutyl bis[2′-*C*-methyluridin-5′-yl] phosphate (**3**).

Hydrolytic Stability of Phosphotriesters 1–3. Nonenzymatic removal of the substituted butyl protecting groups from phosphotriesters derived from 3′-*O*-levulinoylthymidine (**1a**,**b**) or 2′,3′-*O*-isopropylideneuridine (**2a**–**c** and **3**) was studied in a 2-[4-(2-hydroxyethyl)piperazin-1-yl)]-ethanesulfonic acid (HEPES) buffer at pH 7.5 and at 37.0 ± 0.1 °C. The composition of the aliquots withdrawn from the

Scheme 1[a]



[a]Conditions: (i) Br_2, DCM; (ii) RSH, TEA, Et_2O; (iii) H_2CO, TEA, dioxane; (iv) H_2CO, TFA, reflux; (v) 15% aq $NaHCO_3$.

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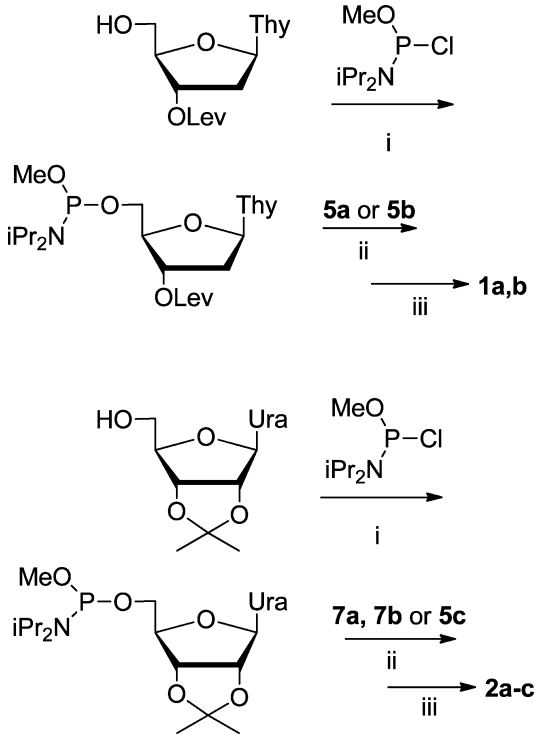
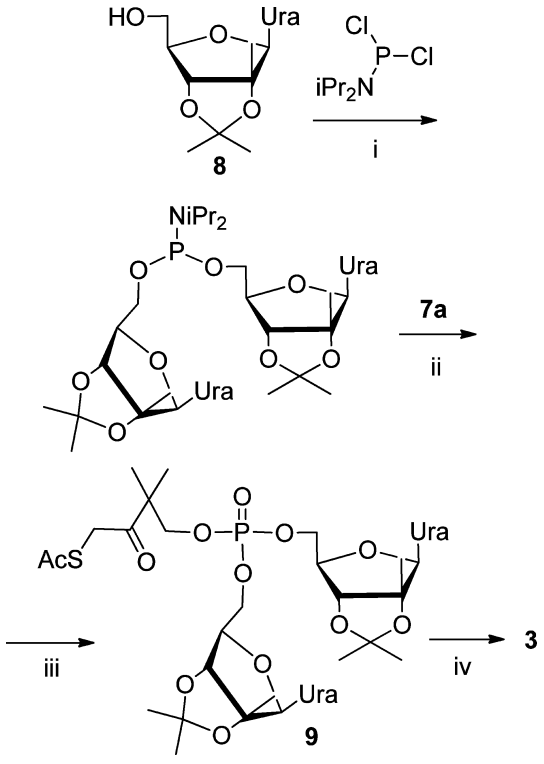
Scheme 2[a]



[a]Conditions: (i) TEA, DCM; (ii) TetH, MeCN; (iii) I_2, THF, H_2O, 2,6-lutidine.

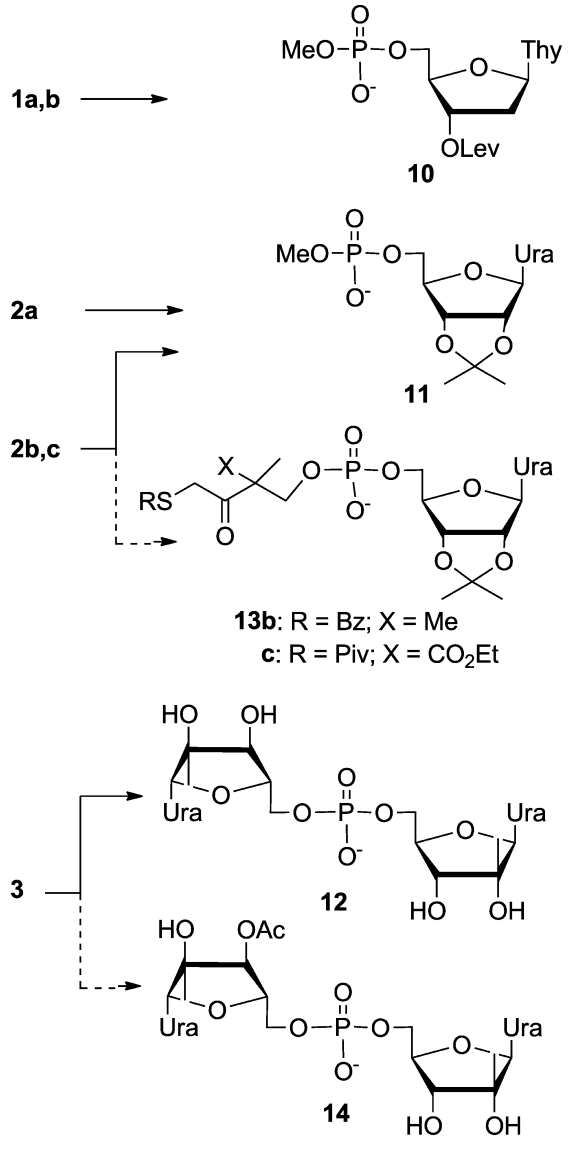
Scheme 3[a]



[a]Conditions: (i) TEA, DCM; (ii) TetH, MeCN; (iii) I_2, THF, H_2O, 2,6-lutidine; (iv) 80% AcOH.

reaction mixture at appropriate time intervals was analyzed by RP-HPLC and the products were identified by mass spectrometric analysis (HPLC/ESI-MS). Diastereomeric mixtures were used as starting materials, but the rate constants were calculated separately for the disappearance of individual diastereomers that exhibited different HPLC signals. With all the phosphotriesters studied, the desired phosphodiester was the main product: 3′-*O*-levulinoylthymidine 5′-methylphosphate (**10**, $[M + H]^+$ at *m*/*z* 435.6) from **1a**,**b**, 2′,3′-*O*-isopropylideneuridine 5′-methylphosphate (**11**, $[M + H]^+$ at *m*/*z* 379.6) from **2a**–**c**, and bis[2′-*C*-methyluridin-5′-yl] phosphate (**12**, $[M + H]^+$ at *m*/*z* 621.2) from **3**. In fact, the only marked side products were formed from **2b** and **2c** that underwent demethylation in 15% and 5% yield, respectively, giving phosphodiesters **13b**,**c** still bearing the original protecting group (Scheme 4). Compound **2b** additionally yielded an unidentified byproduct, the amount of which was less than 5%. Otherwise, more than 95% of the triester was converted to the diester. The R_P and S_P diastereomers reacted at very similar rates. For example, the more slowly eluting diastereomers of **2a** and **2b** reacted 2% and 4% faster,

Scheme 4[a]



[a]Conditions: HEPES buffer, pH 7.5.

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respectively, than the faster eluting ones. Table 1 records the rate constants and half-lives for the departure of the protecting groups at pH 7.5 and 37 °C. At pH 6.5 in a MES buffer, the reaction was 1 order of magnitude slower.

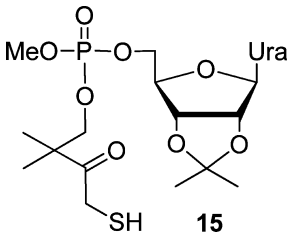
Table 1. First-Order Rate Constants and Half-Lives for the Departure of Various 2,2-Disubstituted 4-Acylthio-3-oxobutyl Groups from Nucleoside 5′-Phosphtriesters in HEPES Buffer at pH 7.5 and 37 °C (I = 0.1 mol L^{-1} with NaCl)

group RS	2-substituents	triester	k (10^{-6} s^{-1})[a]	$\tau_{1/2}$ (h)
AcS	Me, COOEt	**1a**	338 ± 2	0.57
BzS	Me, COOEt	**1b**	121 ± 3	1.59
PivS	Me, COOEt	**2c**	14.9 ± 0.1[b]	12.9
BzS	Me, Me	**2b**	5.45 ± 0.05[c]	35.3
AcS	Me, Me	**2a**	17.2 ± 0.3	11.2
AcS	Me, Me	**2a**	2.42 ± 0.03[d]	79.6
AcS	Me, Me	**3**	28 ± 0.5[e]	6.9

[a]Mean of the rate constants of various diastereomers, which differed by less than 5%. [b]Contains a 5% contribution of side reactions not giving the desired diester. [c]Contains a 20% contribution of side reactions not giving the desired diester. [d]At 20 °C. [e]32% of the diester obtained had one of the sugar hydroxyl groups acetylated.

The removal of the 2,2-dimethyl-4-acetylthio-3-oxobutyl group from the internucleosidic phosphodiester bond of bis(2′-*C*-methyluridin-5′-yl) phosphate (**3**) was also studied. The phosphodiester linkage was exposed 60% faster than with the similarly protected 2′,3′-*O*-isopropylideneuridine 5′-methylphosphate (**2a**), but unexpectedly, 32% of the phosphodiester formed was still acetylated. Prolonged treatment with HEPES buffer resulted in slow deacetylation of the side product ([M + H]$^+$ at m/z 621.2) to the desired phosphodiester (**12**, [M + H]$^+$ at m/z 578.9). Most likely, the acetyl group of the phosphate protecting group had migrated to the 3′-OH function of the sugar moiety. To ensure this assumption, the kinetic analysis was also carried out with the 2′,3′-*O*-isopropylidene-protected counterpart (**9**). The only product observed to be accumulated was bis[2′,3′-*O*-isopropylidene-2′-*C*-methyluridin-5′-yl] phosphate, the half-life for the deprotection being 11.4 h (k = 1.69 × 10^5 s^{-1}).

Esterase-Triggered Deprotection of Triesters 1−3. To elucidate the susceptibility of triesters **1**−**3** to esterase-triggered deprotection, they were treated with hog liver carboxyesterase (HLE; 2.6 units/mL) in a HEPES buffer at pH 7.5 and 37 °C. Under these conditions, the conversion of triesters **1**−**3** to the corresponding phosphodiesters (**10**−**12**) is nearly quantitative. The half-life for the disappearance of **1a**,**b** and **2a**−**c** ranged from 0.17 to 14 min, the deacylation of the 4-benzoylthio-2,2-dimethyl-3-oxobutyl and 2-ethoxycarbonyl-2-methyl-3-oxo-4-(pivaloylthio)butyl group being the fastest and slowest reactions, respectively. Triesters bearing the deacylated 4-mercapto group were accumulated as intermediates. With the 4-benzoylthio-2,2-dimethyl-3-oxobutyl-protected triester, **2b**, and its 4-acetylthio analogue, **2a**, the maximal accumulation level of the intermediate (**15**, [M + H]$^+$ at m/z 509.7) was 80% and 25%, respectively (Figure 1). Accordingly, the rate constants for the chemical removal of deacylated protecting group were 7.2 × 10^{-3} s^{-1} ($\tau_{1/2}$ 1.6 min) and 9.6 × 10^{-3} s^{-1} ($\tau_{1/2}$ 1.2 min), respectively. With their 4-pivaloylthio analogue, **2c**, the accumulation of the mercapto intermediate remained, owing to slow deacylation, at a low level. The removal of 2-ethoxycarbonyl-4-mercapto-2-methyl-3-oxobutyl group appeared, in turn, to be so fast that the mole fraction of the mercapto intermediate ([M + H]$^+$ at m/z 623.4) remained below 0.02 with **1a**,**b** and no accumulation was detected with **2c**. This means that the lower limit for the rate constants of the removal of the deacylated group is 1 s^{-1} ($\tau_{1/2}$ < 1s). Table 2 records the rate constants and half-lives for the esterase-catalyzed deacylation.



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2,2-Dimethyl-2-(4-acetylthio-3-oxo)butyl bis[2′-*C*-methyluridin-5′-yl] phosphate (**3**) was converted by HLE to diester (**12**) more slowly than **2a**, having one of the nucleoside moieties replaced with a methyl group. No intermediate accumulated,

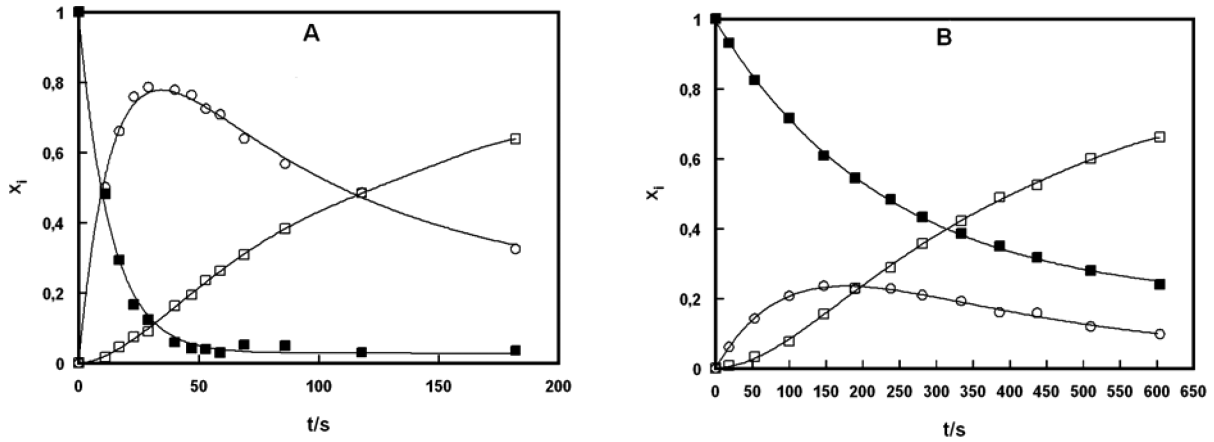

Figure 1. Time-dependent product distribution for the HLE triggered deprotection of diastereomeric (R_P/S_P) 5′-phosphotriesters of 2′,3′-*O*-isopropylideneuridine at pH 7.5 and 37 °C (I = 0.1 mol L^{-1} with NaCl). (A) Removal of the (4-benzoylthio-2,2-dimethyl-3-oxo)butyl group: (■) **2b**, (○) **15**, (□) **11**. (B) Removal of the (4-acetylthio-2,2-dimethyl-3-oxo)butyl group: (■) **2a**, (○) **15**, (□) **11**.

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Table 2. First-Order Rate Constants and Half-Lives for the Esterase-Catalyzed Deacylation of Phosphotriesters 1−3 in a HEPES Buffer (pH 7.5, 37 °C, I = 0.1 mol L^{-1}) Containing HLE 2.6 Units/mL

group RS	2-substituents	triester	deacylation k (10^{-3} s^{-1})	$\tau_{1/2}$ (min)
AcS	Me, COOEt	**1a**	4.05 ± 0.06	2.9
BzS	Me, COOEt	**1b**	39.1 ± 1.6	0.30
PivS	Me, COOEt	**2c**	0.81 ± 0.02	14
BzS	Me, Me	**2b**	67.4 ± 3.4	0.17
AcS	Me, Me	**2a**	2.45 ± 0.10	4.7
AcS	Me, Me	**3**	0.14 ± 0.01[a]	83

[a]Contains a 10% contribution of formation of a diester having one of the sugar hydroxyl groups acetylated.

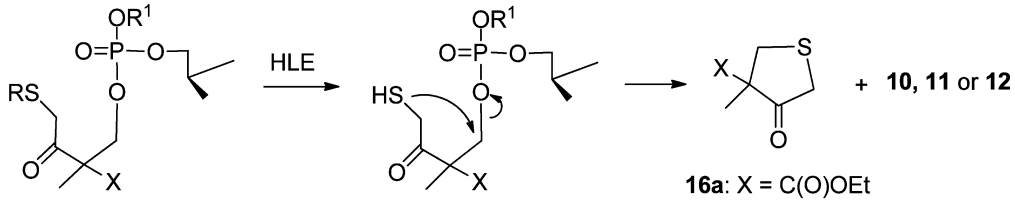
but sugar-acetylated diester **14** appeared as a side product, representing about 10% of the disappearance of **3**. Deprotection of **3** was additionally studied in a whole cell extract of human prostate carcinoma PC3 cells, obtained by disrupting 3 × 10^6 cells in 1 mL of RIPA buffer (pH 7.5) and diluting to triple volume with a HEPES buffer. The 5′,5′-phosphodiester (**12**) was released, the half-life being 8.3 h. The sugar acetylated diester (**14**) was again observed as a side product, now representing 25% of the overall disappearance of **3**. No indication of the desired subsequent hydrolysis to 2′-*C*-methyluridine and 2′-*C*-methyluridine-5′-monophosphate was obtained.

Mechanism of Esterase-Triggered Departure of the 2,2-Disubstituted 4-Acylthio-3-oxobutyl Groups. As indicated above, the carboxyesterase-mediated conversion of phosphotriesters **1**−**3** to phosphodiesters by departure of the 2,2-disubstituted 4-acylthio-3-oxobutyl group is initiated by deacylation that exposes the mercapto group. The 4-mercapto intermediate clearly accumulates when the protecting group is 2,2-dimethylated and the removable acyl group is benzoyl or acetyl. The exposed mercapto group then attacks the phosphate-bound carbon atom resulting in departure of the group as 4,4-disubstituted dihydrothiophen-3(2*H*)-one (**16** in Scheme 5). When one of the methyl groups at C2 is replaced with a more electronegative ethoxycarbonyl group, the electron density at the neighboring methylene group is reduced and the intramolecular attack of the mercapto function is facilitated. Accordingly, accumulation of the deacylated intermediate can hardly be detected with the 2-ethoxycarbonyl substituted compounds **1a**,**b** and **2c**.

Consistent with the suggested removal of the protecting group by intramolecular cyclization, the ESI^--MS signal of **16a** ($[M - H]^-$ at m/z 187.0 see the Supporting Information, Figure S46) was detected upon enzymatic deprotection of a single (HPLC-separated) diastereomer of **1a**. To obtain additional evidence for the structure of this species, the course of the reaction was followed by ^1H NMR spectroscopy in a 10 mmol L^{-1} phosphate buffer (pH 7.0) in a mixture of D_2O and CD_3CN (10:1 *v*/*v*) (see the Supporting Information, Figure S47). Appearance of ^1H resonaces at 3.77 (q, 2H, J = 7.0 Hz, 4-OCH_2CH_3), 3.33 (d, 1H, J = 11.5 Hz, *H*5) and 2.89 (d, 1H, J = 11.5 Hz, *H*5), 1.28 (q, 3H, J = 7.0 Hz, 4-OCH_2CH_3), and 1.25 (s, 3H, 4-CH_3) was consistent with the assumed structure, but no resonances referring to the protons at C2 could be identified because of rapid deuteration as a consequence of keto−enol tautomerism. When the experiment was repeated with **2b** (D_2O/CD_3CN 4:1), containing a methyl group instead of the ethoxycarbonyl group, all four ring proton resonances of **16b** were observed. On the basis of HSQC and HMBC correlations, C2 protons correlated at 3.39 and 2.98 ppm and C4 protons at 2.49 and 2.34 ppm.

Mechanism of Nonenzymatic Departure of the Phosphate Protecting Groups. The nonenzymatic conversion of triesters **1**−**3** to the corresponding diesters **10**−**12** is too fast to be initiated by hydroxide ion catalyzed hydrolysis of the thioester linkage. The rate of hydroxide ion catalyzed hydrolysis of thioesters is comparable to that of oxoesters,[27] and the half-life for the hydroxide ion catalyzed deacetylation of thymidine 5′-bis-[3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl]-phosphate, for example, is 20 days (k = 4.0 × 10^{-7} s^{-1}) at pH 7.5 and 37 °C.[7] The keto group at C3 seems to play a decisive role by allowing migration of the acyl group from sulfur atom to the neighboring oxygen atom. Two alternative mechanisms may be proposed: either a hydroxyl group of the *gem*-diol formed by hydration of the keto group attacks as oxyanion on the carbonyl carbon of the sulfur-bound acyl group, as depicted in Scheme 6, or the attacking neighboring oxyanion is obtained by enolization of the starting material. The exposed mercapto group may then attack on C1 resulting in removal of the protecting group by cyclization. The reaction through hydration of the keto group appears more attractive for the reason that an ESI^+-MS signal at 313.3, possibly referring to the Na^+ adduct of **17**, was observed during the course of the deprotection of **2c**. This intermediate then undergoes dehydration to a 4,4-disubstituted 3-acyloxy-4,5-dihydrothiophen (**18b**). Formation of ethyl 4-acetyloxy-3-methyl-2,3-

Scheme 5



1a: R = Ac; X = CO_2Et; R^1 = Me
b: R = Bz; X = CO_2Et; R^1 = Me
2a: R = Ac; X = Me; R^1 = Me
b: R = Bz; X = Me; R^1 = Me
c: R = Piv; X = CO_2Et; R^1 = Me
3 : R = Ac; X = Me; R^1 = 2′-*C*-methyluridin-5′-yl

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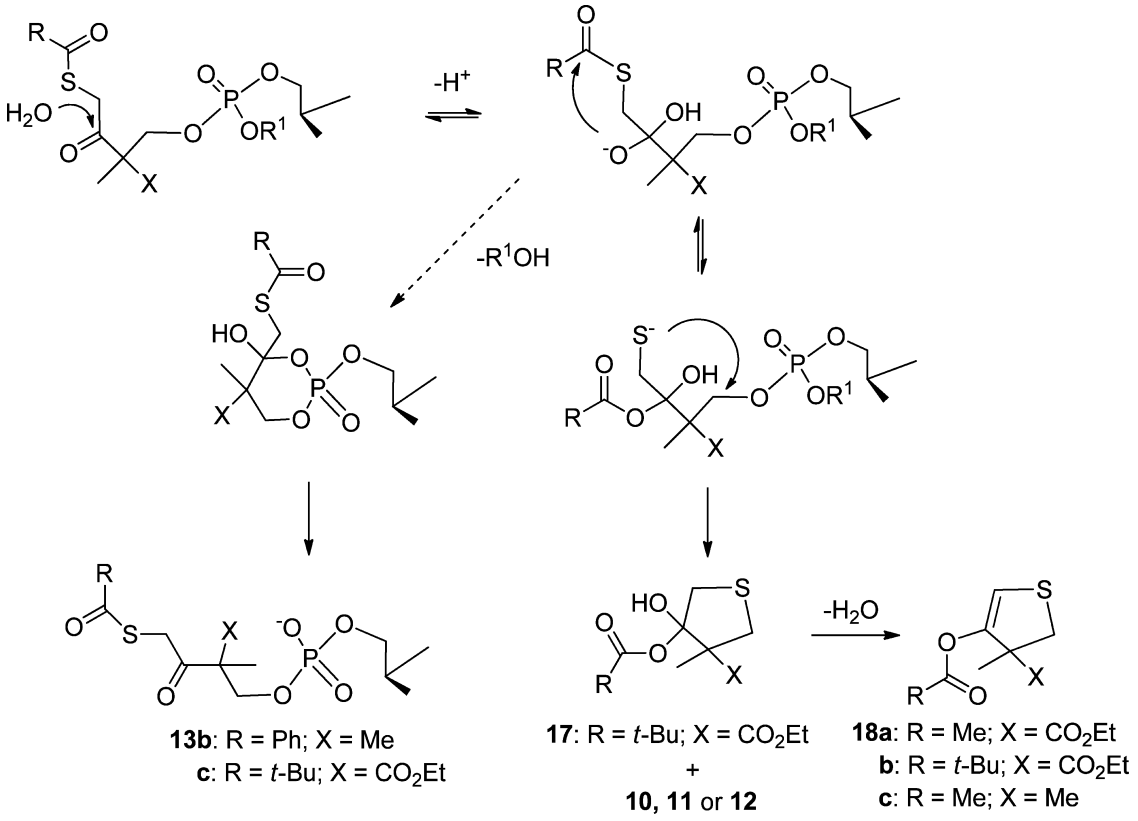
Scheme 6



dihydrothiophene-3-carboxylate (**18a**, $[M + H]^+$ at m/z 231.3 and $[M - H]^-$ at m/z 229.1) from **1a** was detected by ESI-MS (see the Supporting Information, Figure S46). When **2a** was used as a starting material, a HEPES adduct of 4,4-dimethyl-4,5-dihydrothiophen-3-yl acetate (**18c**) was observed.

As mentioned in the foregoing, triesters **2b** and **2c** unexpectedly underwent demethylation as a minor side reaction concurrent with the removal of the protecting group. Conversion to diesters bearing the original protecting group instead of methyl group may also be explained by attack of the C3-oxyanion (formed from the C3-*gem*-diol or -enol) on phosphorus with concomitant displacement of methoxide ion. The cyclic phosphotriester intermediate then collapses to phosphodiester by hydrolysis of the CO bond involved in the half acetal of vinyl ether structure obtained. It is worth noting that a related mechanism, viz. nucleophilic attack of hydrated 2-oxopropyl group on phosphorus, has been proposed for the hydrolysis of bis(ketol) pro-drugs of nucleoside 5′-phosphates.[28]

The removal of the phosphate protecting group was also followed by ^{1}H NMR spectroscopy. Experiments with **1a** in D_2O/CD_3CN (10:1, v/v) buffered with potassium phosphate (0.010/0.010 mol L^{-1}; pH 7.0) revealed the release of a product assigned as ethyl 4-acetyloxy-3-methyl-2,3-dihydrothiophene-3-carboxylate (**18a**). ^{1}H resonances at 4.35 (q, 2H, J = 7.0 Hz, 4-$CO_2CH_2CH_3$), 3.65 (d, 1H, J = 12.0 Hz, *H*2), 3.13 (d, 1H, J = 12.0 Hz, *H*2), 2.06 (s, 3H, AcO), 1.54 (s, 3H, CH_3) 1.36 (t, 3H, J = 7.0 Hz, 4-$CO_2CH_2CH_3$) (see the Supporting Information, Figure S47). It is worth noting that the methylene protons adjacent to keto group of **1a** are subject to rapid deuteration under the experimental conditions, as evidenced by the fact that the aliquots withdrawn from the reaction mixture exhibited the $[M + H]^+$ signal at m/z = 667.6, i.e., two units higher than the authentic **1a**. For this reason, the ^{1}H resonance of *H*4 on **18a** was not detected.

Deprotection in the Presence of Glutathione. To evaluate the potential of the released protecting group as an alkylating agent, triester **2b** was treated with HLE in the presence of glutathione. The only indication of glutathione adduct formation was the appearance of a weak HPLC signal exhibiting a m/z value of 814.4 on positive mode. This signal, representing a few percent of the starting material, most likely refers to debenzoylated **2b** that has formed a disulfide linkage with the mercapto group of glutathione. No alkylation products were detected. The results were similar when the glutathione was added after **2b** had already debenzoylated.

■ CONCLUSIONS

2,2-Disubstituted 4-acylthio-3-oxobutyl groups have been introduced as applicable protection groups for phosphodiesters. These groups resemble other esterase-labile protecting groups in the sense that deacylation by carboxyestarases triggers rapid removal of the remnants of the group. The rate of the enzymatic deprotection may, hence, be tuned by the nature of the 4-acylthio substituent. The benzoyl group, for example, is removed 50 times as fast as the pivaloyl group. Exposure of the mercapto function results in removal of the group by cyclization to 4,4-disubstituted dihydrothiophen-3(2*H*)-one. It is worth noting that this product is not markedly alkylating and was not observed to form glutathione adduct. The novel feature of these esterase-labile groups, however, is that they additionally are thermolabile. The nonenzymatic stability may be tuned within wide limits be electronegativity of the 2-substituents. The acyl group evidently migrates from the sulfur atom to C3-

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gem-diol obtained by hydration of the keto group and the exposed mercapto group attacks on C1 resulting in departure of the protecting group as 4,4-disubstituted 3-acyloxy-4,5-dihydrothiophene. The advantage of thermolability is that these groups will be removed even when the enzymatic reaction becomes severely retarded. This seems to be the case with molecules containing several phosphodiester linkages; accumulation of negative charge in the molecule makes it an increasingly poor substrate for esterases.

EXPERIMENTAL SECTION

General Methods. 2′,3′-*O*-Isopropylideneuridine was a product of Carbosynth. The preparation of 3′-*O*-levulinoylthymidine[25] and 2′,3′-*O*-isopropylidene-2′-*C*-methyluridine (**8**)[26,29] has been described previously. Reactions requiring anhydrous conditions were carried out under argon or nitrogen gas. The solvents were dried over 4 Å molecular sieves. Triethylamine was dried by refluxing over CaH_2 and distilled before use. For column chromatography, silica gel 60 (230−400 mesh) was used. 2D spectra (COSY and HSQC) have been used in assigning ^{1}H and ^{13}C signals.

*Ethyl 4-(Acetylthio)-2-methyl-3-oxobutanoate (**4a**).* Ethyl 2-methyl-3-oxobutanoate (4.00 mL, 28.3 mmol) was dissolved in 25 mL of dry DCM. The solution was cooled on an ice bath, and bromine (28.3 mmol, 1.45 mL) in DCM (3 mL) was added. The ice bath was removed, and the reaction mixture was stirred for 12 h at room temperature. The solvent was removed under reduced pressure, and the residue was coevaporated from dry DCM. Ethyl 4-bromo-2-methyl-3-oxobutanoate (28.27 mmol, 6.3 g, dried over P_2O_5 overnight), used without further purification, was dissolved in dry diethyl ether (60 mL). The solution was cooled on an ice bath, and thioacetic acid (42.4 mmol, 3.03 mL) was added. Triethylamine (4.60 mL, 33.1 mmol) in diethyl ether (14 mL) was added dropwise. The ice bath was removed, and the reaction mixture was stirred for 3.5 h at room temperature. Diethyl ether (40 mL) was added, and the organic phase was washed twice with water, 5% aq $NaHCO_3$, and saturated aq NaCl, dried over Na_2SO_4, and evaporated to dryness. The product was purified by silica gel chromatography using DCM as eluent. Compound **6a** was obtained as oil in 33% yield from ethyl 2-methyl-3-oxobutanoate (2.08 g). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 4.22 (q, 2H, J = 7.50 Hz, CH_3CH_2O); 3.92 and 3.89 (d, 2H, J = 17.00 and 16.50 Hz, CH_2S); 3.75 (q, 2H, J = 7.50 Hz, $CHCH_3$); 2.39 (s, 3H, $CH_3C{=}O$); 1.39 (d, 3H, J = 7.50 Hz, $CHCH_3$); 1.30 (t, 3H, J = 7.50 Hz, CH_3CH_2O). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 199.46 (C=O); 194.18 (SC=O); 169.94 (OC=O); 61.67 (OCH_2CH_3); 51.85 ($CHCH_3$); 38.44 (CH_2S); 30.08 ($CH_3C{=}O$); 14.05 (CH_3CH_2); 12.81 (CH_3CH). ESI$^+$-HRMS: m/z [M + Na]$^+$ calcd for $C_9H_{14}NaO_4S$ 241.0505, found 241.0509.

*Ethyl 4-(Benzoylthio)-2-methyl-3-oxobutanoate (**4b**).* Compound **4b** was prepared in a similar manner as **4a**, but by using thiobenzoic acid instead of thioacetic acid. The product was purified by silica gel chromatography using DCM as eluent. The yield of **4b**, obtained as a solid, was 21% (3.2 g) from ethyl 2-methyl-3-oxobutanoate. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.97 (dd, 2H, J = 7.21 and 1.00 Hz, Bz); 7.79 (t, 1H, J = 7.61 Hz, Bz), 7.46 (t, 2H, J = 7.61 Hz, Bz), 4.23 (q, 2H, J = 7.20 Hz, CH_3CH_2O); 4.14 (d, 1H, J = 16.39 Hz, CH_2S); 4.05 (d, 1H, J = 16.79 Hz, CH_2S); 3.84 (q, 1H, J = 7.20, CH); 1.43 (d, 3H, J = 8.00 Hz, CH_3CH); 1.29 (t, 3H, J = 7.20 Hz, CH_3CH_2O). ^{13}C NMR (100.5 MHz, $CDCl_3$): δ = 199.63 (C=O); 190.31 (SC=O); 170.04 (OC=O); 136.04 (Bz); 133.89 (Bz) ; 128.75 (Bz); 127.47 (Bz); 61.66 (CH_3CH_2O); 51.85 ($CHCH_3$); 38.26 (SCH_2); 14.07 (CH_3CH_2O), and 12.88 ($CHCH_3$). ESI$^+$-HRMS: m/z [M + Na]$^+$ calcd for $C_{14}H_{16}NaO_4S$ 303.0662, found 303.0662.

*Ethyl 2-Methyl-3-oxo-4-(pivaloylthio)butanoate (**4c**).* Compound **4c** was prepared in a similar manner as **4a**, but by using trimethylthioacetic *S*-acid instead of thioacetic acid. The product was purified by silica gel chromatography using DCM as eluent. Compound **4c** was obtained as a solid in 62% yield (6.92 g) from ethyl 2-methyl-3-oxobutanoate. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.21 (q, 2H, J = 7.21 Hz, CH_3CH_2O); 3.90 (d, 1H, J = 16.43 Hz, CH_2S); 3.82 (d, 1H, J = 16.43 Hz, CH_2S); 3.74 (q, 1H, J = 7.21, CH); 1.39 (d, 3H, J = 7.21 Hz, CH_3CH); 1.36 (s, 3H, CH_3 of Piv); 1.29 (t, 3H, J = 7.21 Hz, CH_3CH_2O); 1.26 (s,6H, CH_3 of Piv). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 205.25 (C=O); 199.89 (SC=O); 170.07 (OC=O); 61.58 (CH_3CH_2O); 51.76 ($CHCH_3$); 46.39 (spiro C); 37.99 (SCH_2); 27.25 (CH_3 of Piv); 14.05 (CH_3CH_2O), and 12.86 ($CHCH_3$). ESI$^+$-HRMS: m/z [M + Na]$^+$ calcd for $C_{12}H_{20}NaO_4S$ 283.0975, found 283.0968.

*Ethyl 4-(Acetylthio)-2-(hydroxymethyl)-2-methyl-3-oxobutanoate (**5a**).* Compound **4a** (2.02 g, 8.62 mmol) was dissolved in dioxane (9.0 mL). The solution was cooled on an ice bath, and 20% aq formaldehyde (9.50 mmol) and TEA (1 M in THF, 0.17 mmol, 0.17 mL) were added. The mixture was stirred for 20 min. The water bath was removed, and TEA (0.17 mmol, 0.17 mL) was added. The acidity of the mixture was kept at pH 7.5−8.5 by addition of triethylamine twice in small portions (0.17 mL, 0.17 mmol) within next 4 h. The solvent was removed by evaporation under reduced pressure, and the residue was coevaporated from toluene. The product was purified by silica gel chromatography using DCM containing 2% MeOH as eluent. Compound **5a** was obtained as an oil in 72% yield (1.56 g). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.27 (qd, 2H, J = 7.20 and 0.4 Hz, CH_3CH_2O); 4.05 (d, 2H, J = 17.19 Hz, CH_2S); 4.00 (dd, 1H, J = 6.40 and 5.60 Hz, CH_2OH); 3.96 (d, 1H, J = 17.19 Hz, CH_2S); 3.85 (dd, 1H, J = 8.00 and 7.60 Hz, CH_2OH); 2.84 (dd, 1H, J = 8.00 and 7.60 Hz, OH); 2.39 (s, 3H, $CH_3C{=}O$); 1.47 (s, 3H, CH_3) 1.31 (t, 3H, J = 7.20 Hz, CH_3CH_2O). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 201.62 (C=O); 194.74 (SC=O); 171.85 (OC=O); 66.56; (CH_2OH); 61.97 (OCH_2CH_3); 61.37 (spiro C); 37.35 (CH_2S); 30.09 ($CH_3C{=}O$); 17.49 (CH_3); 14.01 (CH_3CH_2O). ESI$^+$-HRMS: m/z [M + Na]$^+$ calcd for $C_{10}H_{16}NaO_5S$ 271.0611, found 271.0627.

*Ethyl 4-(Benzoylthio)-2-(hydroxymethyl)-2-methyl-3-oxobutanoate (**5b**).* Compound **5b** was prepared as described for **5a.** The product was not purified by silica gel chromatography owing to its lability. The reaction mixture was evaporated to dryness. After addition of water (60 mL), the mixture was extracted with DCM (3 × 40 mL). The organic phase was dried over Na_2SO_4 and evaporated to dryness. Compound **5b** was obtained as solid in 90% yield (0.17 g). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.99 (dd, 2H, J = 8.50 and 1.00 Hz, Bz); 7.62 (t, 1H, J = 7.50 Hz, Bz), 7.48 (t, 2H, J = 7.50 Hz, Bz), 4.32 (q, 2H, J = 7.00 Hz, CH_3CH_2O); 4.26 (d, 1H, J = 17.00 Hz, CH_2S); 4.18 (d, 1H, J = 17.50 Hz, CH_2S); 4.09 (d, 1H, J = 11.50, CH_2O); 3.92 (d, 1H, J = 11.50, CH_2O); 1.55 (s, 3H, CH_3CH); 1.35 (t, 3H, J = 7.00 Hz, CH_3CH_2O). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 201.67 (C=O); 190.89 (SC=O); 171.95 (OC=O); 136.10, 134.00, 129.37, 128.83, 127.55, and 127.44 (Bz); 67.10 and 66.66 (CH_3CH_2O); 62.00 and 61.50 (CH_2OH); 37.29 and 36.74 (SCH_2); 17.52 (CH_3CH_2O) and 14.04 ($CHCH_3$). ESI$^+$-MS: m/z [M + H]$^+$ calcd for $C_{15}H_{19}O_5S$ 311.4, found 311.5. ESI$^+$-HRMS: m/z [M + Na]$^+$ calcd for $C_{15}H_{18}NaO_5S$ 333.0767, found 333.0771.

*Ethyl 2-(Hydroxymethyl)-2-methyl-3-oxo-4-(pivaloylthio)-butanoate (**5c**).* Compound **5c** was prepared as described for **5a.** Compound **5c** was obtained as a solid in quantitative yield (4.13 g). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.27 (q, 2H, J = 7.20 Hz, CH_3CH_2O); 4.01 (d, 1H, J = 11.60 Hz, CH_2OH); 4.00 (d, 1H, J = 17.19 Hz, CH_2S); 3.92 (d, 1H, J = 17.19 Hz, CH_2S); 3.86 (d, 1H, J = 12.00 Hz, CH_2OH); 1.48 (s, 3H, CH_3); 1.40 (s, 3H, CH_3 of Piv); 1.33 (t, 3H, J = 7.20 Hz, CH_3CH_2O); 1.26 (s, 6H, CH_3 of Piv). ^{13}C NMR (101 MHz, $CDCl_3$): δ = 205.89 (C=O); 201.85 (SC=O); 171.92 (OC=O); 66.62 (CH_2OH); 61.90 (CH_3CH_2O); 61.40 (spiro C); 46.43 (spiro C of Piv); 36.93 (SCH_2); 27.29 (CH_3 of Piv); 17.52 ($CHCH_3$) and 14.02(CH_3CH_2O). ESI$^+$-HRMS: m/z [M + Na]$^+$ calcd for $C_{13}H_{12}NaO_5S$ 313.1080, found 313.1081.

*4-Hydroxy-3,3-dimethylbutan-2-one (**6**).* Compound **6** was prepared as described previously.[24] A mixture of 3-methylbutan-2-one (5.00 g, 58.0 mmol), paraformaldehyde (1.74 g, 58.0 mmol), and TFA (8.90 mL, 116.1 mmol) was heated at reflux for 7 h. Saturated aq $NaHCO_3$ (100 mL) was added, and the resulting suspension was stirred at room temperature for 1 d. The mixture was extracted with DCM (8 × 50 mL), after which the organic phase was dried over

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Na_2SO_4 and evaporated to dryness. The product was purified by silica gel chromatography using a 6:4 mixture of hexane and EtOAc as eluent. Compound **6** was obtained as a yellow oil in 73% yield (4.91 g). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.49 (s, 2H, CH_2OH); 2.09 (s, 3H, CH_3C═O); 1.09 (s, 6H, C(CH_3)$_2$). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 215.10 (C═O); 69.31 (CH_2OH); 49.28 C(CH$_3$)$_2$); 25.56 (CH$_3$C═O); 21.53 (C(CH_3)$_2$). ESI$^+$-MS: m/z [M + Na]$^+$ calcd for $C_6H_{12}NaO_2$ 139.15, found 139.13.

*S-(4-Hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate (**7a**).* The synthesis of **7a** was performed in a similar manner as described below for **7b**, but by using thioacetic acid instead of thiobenzoic acid. The product was purified by silica gel chromatography using a 6:4 mixture of hexane and EtOAc as eluent. Compound **7a** was obtained as oil in 53% yield (1.33 g) from 4-hydroxy-3,3-dimethylbutan-2-one (**6**). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.91 (s, 2H, CH$_2$S); 3.59 (s, 2H, CH_2OH); 2.70 (s, 1H, OH); 2.34 (s, 3H, CH$_3$C═O); 1.20 (s, 6H, 2 × CH$_3$). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 208.49 (C═O); 195.39 (SC═O); 69.83 (CH$_2$OH); 49.97 (spiro C); 36.65 (CH$_2$S); 30.15 (CH$_3$C═O) and 21.56 (2 × CH$_3$). ESI$^+$-HRMS: m/z [M + Na]$^+$ calcd for $C_8H_{14}NaO_3S$ 213.0556, found 213.0562.

*S-(4-Hydroxy-3,3-dimethyl-2-oxobutyl)benzothioate (**7b**).* Compound **7b** was prepared from **6** essentially as described for **4b** and **5b**. 4-Hydroxy-3,3-dimethylbutan-2-one (**6**) (1.08 g, 9.3 mmol) was dissolved in 13 mL of dry DCM. The solution was cooled on an ice bath, and bromine (0.476 mL, 9.3 mmol) in DCM (3 mL) was added. The reaction mixture was stirred for 1 h at 0 C. The solvent was removed under reduced pressure, and the residue was coevaporated from dry DCM. 1-Bromo-4-hydroxy-3,3-dimethylbutan-2-one (1.80 g, 9.23 mmol), used without further purification, was dissolved in dry diethyl ether (23 mL). The solution was cooled on an ice bath, and thiobenzoic acid (1.62 mL, 13.84 mmol) was added. Triethylamine (1.5 mL, 10.79 mmol) in diethyl ether (5 mL) was added dropwise. The ice bath was removed, and the reaction mixture was stirred for 3.5 h at room temperature. Diethyl ether (15 mL) was added, and the organic phase was washed twice with water, 5% aq $NaHCO_3$ and saturated aq NaCl and dried over Na_2SO_4 and evaporated to dryness. The product was purified by silica gel chromatography using a 6:4 mixture of hexane and EtOAc as eluent. Compound **7b** was obtained as a yellow solid in 85% yield (2.08 g) from 4-hydroxy-3,3-dimethylbutan-2-one (**6**): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.97 (d, 2H, J = 8.00 Hz, Bz); 7.58 (t, 1H, J = 7.50 Hz, Bz); 7.45 (t, 2H, J = 7.50 Hz, Bz); 4.11 (s, 2H, CH$_2$S); 3.68 (s, 2H, CH_2OH); 2.62 (s, 1H, OH); 1.29 (s, 6H, 2 × CH$_3$). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 208.54 (C═O); 191.47 (SC═O); 136.19 (Bz), 133.87 (Bz); 128.72 (Bz); 127.47 (Bz); 70.08 (CH$_2$OH); 50.16 (spiro C); 36.64 (CH$_2$S) and 21.67 (2 × CH$_3$). ESI$^+$-HRMS: m/z [M + H]$^+$ calcd for $C_{13}H_{17}O_3S$ 253.0893, found 253.0897.

*3′-O-Levulinoylthymidine 5′-(Methyl 4-acetylthio-2-ethoxycarbonyl-2-methyl-3-oxobutyl) Phosphate (**1a**).* To a solution of dried 3′-*O*-levulinoylthymidine[25] (0.39 g, 1.15 mmol) in dry DCM (4.0 mL) were added anhydrous triethylamine (0.80 mL, 5.73 mmol) and methyl-*N*,*N*-diisopropylchlorophosphoramidite (0.244 mL, 1.26 mmol), and the reaction mixture was stirred for 1 h 15 min under nitrogen. The product was filtered through a short silica gel column eluting with a mixture of anhydrous ethyl acetate and triethylamine (99.5:0.5 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 (1.5 mL), and compound **5a** (0.31 g, 1.26 mmol) in dry MeCN (1.5 mL) and 0.45 mol L^{-1} tetrazole solution in MeCN (1.83 mmol, 4.0 mL) were added under nitrogen. The reaction mixture was stirred for 1 h at room temperature. The phosphite ester formed was oxidized with I_2 (0.3 g) in a mixture of THF, H_2O, and lutidine (4:2:1, v/v/v; 10.5 mL) by stirring overnight at room temperature. The solvent was removed under reduced pressure, and the residue was dissolved in DCM and washed twice with saturated 5% aq $NaHSO_3$. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The product was purified by silica gel chromatography, eluting with a mixture of EtOAc containing 5% MeOH. Compound **1a** (diastereomeric mixture) was obtained as as solid foam in 71% yield (0.54 g). The diastereomer pairs were separated on a SunFire C18 column (250 × 10 mm, 5 μm) eluting with a mixture of water and MeCN (65:35, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.18 (s, 1H, NH); 7.48 (m, 1H, H6); 6.37 (dd, 1H, J = 9.00 and 5.50 Hz, H1′); 5.29 (1H, H3′), 4.47 (m, 1H, H5″); 4.39 (m, 1H, H5′); 4.31 (m, 2H, POCH$_2$); 4.26 (m, 2H, OCH_2CH$_3$); 4.17 (m, 1H, H4′); 3.95 (m, 2H, CH$_2$S); 3.81 and 3.79 (s, 3H, POCH$_3$); 2.78 (m, 2H CH$_2$ Lev); 2.59 (m, 2H, CH$_2$ Lev); 2.42 (dd, 1H J = 14.00 and 5.50 Hz, H2′); 2.36 (d, 3H, J = 1.50 Hz, CH$_3$C═O); 2.22−2.18 (m, 4H, H2″ and CH$_3$ Lev); 1.95 (s, 3H, CH$_3$ Thy); 1.58, 1.57, 1.56, and 1.56 (s, 3H, CCH$_3$); 1.29 (m, 3H, CH_3CH$_2$O). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 206.33 (C═O); 198.56 (C═O Lev); 193.75 (SC═O); 172.38 (OC═O Lev), 169.52 (OC═O), 163.52 (C4); 150.40 (C2); 135.05 (C6); 111.84 and 111.79 (C5); 84.51 (C1′); 82.67 and 82.61 (C4′); 74.50 (H3′); 69.46 and 69.33 (C5′); 67.50 and 67.46 (POCH$_2$); 62.46 (OCH$_2$CH$_3$); 60.07 and 60.00 (spiro C); 54.82 and 54.77 (CH$_3$OP); 37.79 (CH$_2$ Lev); 37.02 (C2′); 36.73 and 36.66 (CH$_2$S); 30.07 (CH$_3$C═O); 29.76 (CH$_3$ Lev); 27.86 (CH$_2$ Lev); 17.85 (CH$_3$); 13.96 (CH$_3$CH$_2$O); 12.37 (CH$_3$ Thy). ^{31}P NMR (202 MHz, CD_3CN): δ = −0.30, −0.35, −0.52, and −0.58 ppm. ESI$^+$-HRMS: m/z [M + Na]$^+$ calcd for $C_{26}H_{37}N_2NaO_{14}PS$ 687.1595, found 687.1585.

*3′-O-Levulinoylthymidine 5′-(Methyl 4-benzoylthio-2-ethoxycarbonyl-2-methyl-3-oxobutyl) Phosphate (**1b**).* The phosphotriester **1b** was prepared in a similar manner as **1a**, but by using ethyl 4-(benzoylthio)-2-(hydroxymethyl)-2-methyl-3-oxobutanoate (**5b**) instead of ethyl-4-(acetylthio)-2-(hydroxymethyl)-2-methyl-3-oxobutanoate (**5a**). The crude product **1b** was purified on a silica gel column eluting with a mixture of ethyl acetate and methanol (95:5, v/v). The product (**1b**) was obtained as solid foam in 42% yield (0.36 g). The diastereomers were separated on a SunFire C18 column (250 × 10 mm, 5 μm) eluting with a mixture of water and MeCN (57:43, v/v). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.17 (s, 1H, H6); 7.91 (d, 2H, J = 7.00 Hz, Bz); 7.60 (t, 1H, J = 7.50 Hz, Bz); 7.46 (m, 2H, Bz); 6.37 (dd, 1H, J = 9.00 and 5.50 Hz, H1′); 5.29 (d, 1H, J = 6.50 Hz, H3′), 4.53 (dd, 1H, J = 10.00 and 4.50 Hz, POCH$_2$); 4.44 (dd, 1H, J = 10.00 and 4.50 Hz, POCH$_2$); 4.34−4.26 (m, 4H, H5″, H5′ and OCH_2CH$_3$); 4.20−4.10 (m, 3H, H4′ and CH$_2$S); 3.82 and 3.82 and 3.80 (s, 3H, POCH$_3$); 2.77 (m, 2H CH$_2$ Lev); 2.58 (m, 2H, CH$_2$ Lev); 2.41 (dd, 1H J = 13.50 and 5.50 Hz, H2′); 2.22−2.17 (m, 4H, H2″ and CH$_3$ Lev); 1.94 (d, 3H, J = 0.50 Hz, CH$_3$ Thy); 1.63−1.61 (m, 3H, CCH$_3$); 1.31 (t, 3H, J = 7.00 Hz, CH_3CH$_2$O). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 206.30 (C═O); 198.56 (C═O Lev); 189.89 (SC═O); 172.38 (OC═O Lev), 169.52 (OC═O), 163.20 (C4); 150.17 (C2); 136.00 (H6); 135.07 (Bz); 133.93 (Bz); 128.78 (Bz); 127.41 (Bz); 111.76 (C5); 84.33 (H1′); 82.72 and 82.71 (C4′); 74.61 (C3′); 69.46 and 69.42 (POCH$_2$); 67.50 and 67.46 (C5′); 62.49 (OCH$_2$CH$_3$); 60.13 and 60.05 (spiro C); 54.84 and 54.79 (CH$_3$OP); 37.79 (CH$_2$ Lev); 37.07 (C2′); 36.65 (CH$_2$S); 29.78 (CH$_3$ Lev); 27.85 (CH$_2$ Lev); 17.71 (CH$_3$); 14.01 (CH$_3$CH$_2$O); 12.38 (CH$_3$ Thy). ^{31}P NMR (202 MHz, CD_3CN): δ = −0.26, −0.32, −0.50, and −0.56 ppm. ESI$^+$-HRMS: m/z [M + H]$^+$ calcd for $C_{31}H_{39}N_2NaO_{14}PS$ 749.1752, found 749.1785.

*2′,3′-O-Isopropylideneuridine 5′-(Methyl 4-benzoylthio-2,2-dimethyl-3-oxobutyl) Phosphate (**2b**).* To a solution of dried 2′,3′-*O*-isopropylidineuridine (0.333 g, 1.17 mmol) in dry DCM (5.0 mL) were added anhydrous triethylamine (0.81 mL, 5.85 mmol) and methyl-*N*,*N*-diisopropylchlorophosphoramidite (0.249 mL, 1.29 mmol), and the reaction mixture was stirred for 1 h 15 min under argon. The product was filtered through a short silica gel column eluting with a mixture of anhydrous EtOAc and triethylamine (99.5:0.5 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 (1.5 mL) and *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl) benzothioate (**7b**) (0.325 g, 1.29 mmol) in dry MeCN (1.5 mL) and 0.45 mol L^{-1} of tetrazole solution in MeCN (7.8 mL, 3.51 mmol) were added under argon. The reaction mixture was stirred for 1 h at room temperature. The phosphite ester formed was oxidized with I_2 (0.3 g) in a mixture of THF, H_2O, and lutidine (4:2:1, v/v/v; 10.5 mL) by stirring overnight at room temperature. The solvent was removed under

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reduced pressure, and the residue was dissolved in DCM and washed twice with saturated 5% aq $NaHSO_3$. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The product was purified by silica gel chromatography, eluting with a mixture of EtOAc containing 5% MeOH. Compound **2b** was obtained as solid foam in 73% yield (0.52 g). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.15 (br, 1H, NH); 7.96 (dd, 2H, J = 10.00 Hz, J = 2.00 Hz, Bz); 7.58 (t, 1H, J = 10.00 Hz, Bz); 7.45 (t, 2H, J = 10.00 Hz, Bz); 7.35 (d, 1H, J = 10.00 Hz, H6); 5.73 (m, 2H, H5 and H1′); 4.93 (m, 1H, H2′); 4.86 (m, 1H, H3′); 4.32 (m, 1H, H4′), 4.29−4.24 (m, 2H, H5″ and H5′); 4.16−4.12 (m, 4H, CH_2O and CH_2S); 3.79 and 3.76 (s, 3H, $POCH_3$); 1.55 (s, 3H, CH_3); 1.34 (s, 3H, CH_3); 1.33 (s, 6H, 2 × CH_3). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.40 (C═O); 190.61 (SC═O); 163.23 (4′C═O); 149.98 (C2′C═O); 141.97 and 141.91 (H6); 136.23 (Bz); 133.81 (Bz); 128.74 (Bz); 127.42 (Bz); 114.70 (spiro C of isopropylidene); 102.76 and 102.73 (C5); 93.98 and 93.94 (C1′); 85.34 and 85.28 (C4′); 84.33 (C2′); 80.61 and 80.55 (C3′); 73.07 and 73.02 (CH_2OH); 67.13 and 67.08 (C5′); 54.77 and 54.73 (CH_3OP); 48.84 and 48.82 (spiro C); 36.42 (CH_2S); 27.11; 25.27 and 25.25 (CH_3) ; 21.71 and 21.67 (CH_3); 21.65 (CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ = −0.28 and −0.40 ppm. ESI$^+$-HRMS: m/z [M + H]$^+$ obsd 613.1617, calcd for $C_{26}H_{34}N_2O_{11}PS$ 613.1615. ESI$^+$-HRMS: m/z [M + Na]$^+$ calcd for $C_{26}H_{33}N_2NaO_{11}PS$ 635.1435, found 635.1424.

*2′,3′-O-Isopropylideneuridine 5′-(Methyl 4-acetylthio-2,2-dimethyl-3-oxobutyl) Phosphate (**2a**).* Phosphotriester **2a** was prepared in a similar manner as **2b**, but by using *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl) ethanethioate (**7a**) instead of *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl) benzothioate (**7b**). The crude product **2a** was purified on a silica gel column eluting with a mixture of ethyl acetate and methanol (95:5, *v*/*v*). The product **2a** was obtained as solid foam in 29% yield (325 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.04 (br, 1H, NH); 7.33 (d, 1H, J = 8.00 Hz, H6); 5.68 (m, 2H, H5 and H1′); 4.90 (m, 1H, H2′); 4.79 (m, 1H, H3′); 4.25 (m, 1H, H4′), 4.21−4.15 (m, 2H, H5″ and H5′); 4.04 (m, 2H, CH_2O); 3.89 (d, 2H, J = 3.00 Hz, CH_2S); 3.70 and 3.68 (d, 3H, J = 1.00 Hz, $POCH_3$); 2.29 (2*s*, CH_3C═O); 1.49 (s, 3H, CH_3); 1.28 (s, 3H, CH_3); 1.21 (s, 3H, CH_3) 1.20 (s, 3H, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.34 (C═O); 194.44 and 194.43 (SC═O); 163.68 (C4); 150.22 (C2); 142.17 (C6); 114.55 (spiro C of isopropylidene); 102.66 and 102.64 (C5); 93.97 and 93.90 (C1′); 85.36, 85.34, 85.31, and 85.28 (C4′); 84.28 (C2′); 80.63 and 80.58 (C3′); 72.91 and 72.89 (CH_2OH); 67.18 and 67.14 (C5′); 54.73, 54.68, and 54.64 (CH_3OP); 48.67, 48,65, 48,60 and 48.58 (spiro); 36. 33 (CH_2S); 30.15 (CH_3); 27.06 (CH_3); 25.23 and 25.21 (CH_3); 21.52, 21.50, 21.49 (CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ = −0.40 and −0.50 ppm. ESI$^+$-MS: m/z [M + H]$^+$ obsd 551.1454, calcd for $C_{21}H_{32}N_2O_{11}PS$ 551.1459. ESI$^+$-MS: m/z [M + Na]$^+$ calcd for $C_{21}H_{31}N_2NaO_{11}PS$ 573.1278 found 573.1282.

*2′,3′-O-Isopropylideneuridine 5′-(Methyl 2-ethoxycarbonyl-2-methyl-3-oxo-4-pivaloylthiobutyl) Phosphate (**2c**).* Phosphotriester **2c** was prepared in a similar manner as **1a**, but by using ethyl 2-(hydroxymethyl)-2-methyl-3-oxo-4-(pivaloylthio)butanoate (**5c**) instead of ethyl-4-(acetylthio)-2-(hydroxymethyl)-2-methyl-3-oxobutanoate (**5a**). The crude product was purified on a silica gel column eluting with a mixture of EtOAc and MeOH (95:5, v/v). The product **2c** was obtained as solid foam in 13% yield (0.10 g). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.75 (s br. 1H, NH); 7.34 (d, 1H, J = 8.00 Hz, H6); 5.73−5.71 (m, 2H, H1′ and H5); 4.93−4.91 (m, 1H, H2′), 4.84−4.82 (m, 1H, H3′); 4.43−4.35 (m, 1H,); 4.32−4.27 (dd, 1H, H4′); 4.26−4.19 (m, 4H, H5″, H5′ and OCH_2CH_3); 3.93−3.83 (m, 3H, H4′ and CH_2S); 3.74−3.72 (4*s*, 3H, $POCH_3$); 1.53 (s, 3H, CCH_3); 1.53 (s, 3H, CH_3 of isopropylidene); 1.32 (s, 3H, CH_3 of Piv); 1.26 (t, 3H, J = 7.00 Hz, CH_3CH_2O); 1.21 (s, 3H, CH_3 of Piv); 1.85 (s, 3H, CH_3 of isopropylidene). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 204.79 and 204.77 (C═O); 198.91,198.86, 198.85, and 198.83 (SC═O); 169.52 and 169.49 (OC═O), 163.59 (C4); 150.16 and 150.14 (C2); 142.08 and 142.04 (H6); 114.68 and 114.65 (C of isopropylidene); 102.74 and 102.72 (C5); 93.92 and 93.89 (C1′); 85.30, 85.24, and 85.18 (C4′); 84.29 and 84.27 (C2′); 80.55 (C3′); 69.46 and 69.43 ($POCH_2$); 67.22 and 67.19 (C5′); 62.34 (OCH_2CH_3); 60.15−60.03 (spiro C of protecting group); 54.80, 5476, 54.72, and 54.71 (CH_3OP); 46.37 (spiro C of Piv); 38.40, 36.31, 36.28, and 36.26 (CH_2S); 27.22; 25.25 and 25.23 (CH_3 of Piv); 27.10 (CH_3 of isopropylidene); 17.55, 17.53, and 17.51 ($CHCH_3$); 13.95 (CH_3CH_2O). ^{31}P NMR (202 MHz, CD_3CN): δ = −0.41, −0.44, −0.57, and −0.60 ppm. ESI$^+$-HRMS: m/z [M + Na]$^+$ calcd for $C_{26}H_{39}N_2NaO_{13}PS$ 673.1803, found 673.1832.

*4-Acetylthio-2,2-dimethyl-3-oxobutyl Bis(2′,3′-isopropylidene-2′-C-methyluridin-5′-yl) Phosphate (**9**).* 2′,3′-*O*-Isopropylidene-2′-*C*-methyluridine[26,29] (0.60 mmol; 0.18 g) was dried over P_2O_5 for 2 days and dissolved in dry DCM (4 mL) under nitrogen. Dry Et_3N (5.0 mmol; 0.70 mL) and 1,1-dichloro-*N*,*N*-diisopropylphosphinamine (0.50 mmol; 93 μL) were added. After the reaction mixture was stirred at rt for 1 h, the mixture was passed through a short silica gel column eluting with EtOAc that contained 0.5% Et_3N. The solvents were removed under reduced pressure. The residue was coevaporated three times from dry MeCN and dissolved in dry MeCN (1.5 mL), (4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate (**7a**) (1.50 mmol, 0.29 g) in dry MeCN (1.5 mL) and 1-*H*-tetrazole (1.50 mmol, 3.33 mL of 0.45 mol L^{-1} solution in MeCN) were added under nitrogen, and the reaction mixture was stirred at rt for 2.5 h. The phosphite ester formed was oxidized with I_2 in a 4:2:1 mixture of THF, H_2O, and 1,2-lutidine by stirring overnight at room temperature. The product was isolated by conventional 5% aq $NaHSO_3$/DCM workup. The crude product was purified on a silica gel column eluting with 2 to 6% MeOH in DCM. Compound **9** was obtained as a white solid in 10% yield (52 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.56 (s, 2H, NH); 7.53 (d, 1H, J = 5.00 Hz, H6); 7.51 (d, 1H, J = 5.00 Hz, H6); 6.02 (s, 1H, H1′); 6.01 (s, 1H, H1′); 5.74 (d, 2H, J = 5.00 Hz, H5); 4.43−4.41 (m, 2H, H3′), 4.38−4.32 and 4.18−4.12 (m, 8H, H4′, H5′, H5″ and CH_2O); 3.94 (s, 2H, CH_2S); 2.35 (s, 3H, CH_3C═O); 1.31 (s, 3H, CH_3); 1.30 (s, 3H, CH_3); 1.28 (s, 3H, CH_3); 1.26 (s, 3H, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.13 (C═O); 194.46 (SC═O); 163.28 (C4); 150.11 (C2); 140.67 (C6); 114.92 (spiro C of isopropylidene); 102.22 (C5); 93.29 (C1′); 89.80 and 89.77 (C2′); 85.37 and 85.34 (C3′); and 81.68 (C4′); 73.30 and 73.26 (CH_2O); 67.38 and 67.33 (C5′); 48.66 and 48.60 (spiro C); 36.26 (CH_2S); 30.23 (CH_3 of Ac); 28.46, 28.44, 27,57 and 27.55 (CH_3 of isopropylidene); 21.64 and 21.55 (CH_3); 19.38 and 19.36 (2′-CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ = −1.21 ppm. ESI$^+$-HRMS: m/z [M + H]$^+$ calcd for $C_{34}H_{48}N_4O_{16}PS$ 831.2518, found 831.2487.

*4-Acetylthio-2,2-dimethyl-3-oxobutyl Bis(2′-C-methyluridin-5′-yl) Phosphate (**3**).* Compound **9** was dissolved in 80% aqueous AcOH (5 mL) and stirred at 90 °C for 36 h. The solvent was removed under reduced pressure and the residue was coevaporated two times from water. The crude product was purified on a silica gel column with gradient elution from 5% to 10% MeOH in DCM. Compound **3** was obtained as solid in 42% yield (20 mg). ^{1}H NMR (500 MHz, CD_3OD): δ = 7.65 (d, 1H, J = 5.00 Hz, H6); 7.63 (d, 1H, J = 5.00 Hz, H6); 5.97 (s, 2H, H1′); 4.53−4.47 (m, 2H, H3′); 4.43−4.37 (m, 2H, CH_2O); 4.30−4.20 (m, 2H, H4′); 4.13−4.11 (m, 2H, H5″), 4.07 (s, 2H, CH_2S); 3.81 (t, 2H, J = 10.00 Hz, H5′); 2.34 (s, 3H, CH_3 of Ac); 1.30 (s, 6H, 2 × 2′-CH_3); 1.18 (s, 6H, 2 × CH_3). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.84 (C═O); 194.86 (SC═O); 164.44 (4′C═O); 150.87 (C2′C═O); 140.62 (C6); 101.69 (C5); 92.5 (C1′); 80.04 and 80.05, 78.19 (C4′, C3′ and C2′); 73.17, 72.89, and 72.84 (CH_2O); 66.66 (C5′); 48.13 (spiro C); 35.89 (CH_2S); 28.65 (CH_3 of Ac); 20.42 (CH_3); 18.83 (2′-CH_3). ^{31}P NMR (202 MHz, $CDCl_3$): δ = −1.88. ESI$^+$-HRMS: m/z [M + H]$^+$ calcd for $C_{28}H_{40}N_4O_{16}PS$ 751.1892, found 751.1880.

Kinetic Measurements. The reactions were carried out in sealed tubes immersed in a thermostated water bath (37.0 ± 0.1 °C). The hydronium ion concentration of the reaction solutions (3.0 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. 0.2 mmol L^{-1}.

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The enzymatic and nonenzymatic hydrolysis was carried out in a HEPES buffer at pH 7.5 (0.036/0.024 mol L^{-1}) at 37 °C. The acyl group was removed with hog liver esterase (2.6 units mL^{-1}). The samples (200 μL) withdrawn at appropriate intervals were made acidic (pH 2) with 1 mol L^{-1} aqueous hydrogen chloride or 1 mol L^{-1} AcOH to inactivate enzyme and to quench the hydrolysis, cooled in an ice bath, and filtered with RC4 syringe (0.2 μm). The composition of the samples was analyzed on an C18 column (4 × 250 mm 5 μm, flow rate 0.95 mL min^{-1}), using a mixture of MeCN and 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 mixture of **1a**, **2a**, **2b**, **2c**, and **3** was obtained on using a 5 min isocratic elution with the buffer containing 2% MeCN, followed by a 30 min linear gradient (40 min with **2c**) up to 50% MeCN (75% with **2c**). Signals were recorded on a UV-detector at a wavelength of 267 or 260 nm. In case of **1b**, an isocratic eluation with the buffer containing 35% MeCN was used. 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.

Esterase-Triggered Deprotection in the Presence of Glutathione. The hydrolytic reactions of **2b** were carried out in the presence of glutathione (GSH; 5.0 mmol L^{-1}) and carboxyesterase (HLE; 2.6 units mL^{-1}) in a 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (0.005/0.005 mol L^{-1}) at pH 6.1 and 37 °C. The initial substrate concentration was ca. 0.5 mmol L^{-1}. Glutathione was present in the reaction mixture from the beginning or it was added 20 min after addition of **2b**.The samples (250 μL) withdrawn at appropriate intervals were made acidic (pH 2) with 1 mol L^{-1} aq. HCl, cooled in an ice-bath and filtered with minisart RC4 filters (0.2 μm). The reaction products were identified by mass spectra (LC/MS) on a Phenomenex Gemini C18 column (2.0 × 150 mm 5 μm; flow rate 0.4 mL min^{-1}). A good separation of the products was obtained using 5 min isocratic elution with 0.1% formic acid containing 4% MeCN, followed by a linear gradient to 100% MeCN in 40 min. The signals were recorded on a UV detector at a wavelength of 220 and 260 nm.

In 4 days, the starting material was converted to phosphodiester **11** ($[M + H]^+$ = 379.3 and t_R = 15.6 min). The mixture additionally contained debenzoylated **2b** (**15**; $[M + H]^+$ = 509.4 and t_R = 24.3 min) and a disulfide adduct of **15** with glutathione ($[M + H]^+$ = 814.4 and t_R = 19.3 min). In addition, glutathione was partially converted to its oxidized form, glutathione disulfide (GSSG; $[M + H]^+$ = 613.2 and t_R = 3.8 min). A minor signal of an unidentified byproduct was detected at *m*/*z* value of 316.1 (t_R = 17.8).

■ ASSOCIATED CONTENT

Supporting Information

HPLC chromatograms of compounds **1**−**3** and NMR spectral data for compounds **1**−**7** and **9**. ESI-MS and ^1H NMR spectra of compounds **16a** and **18a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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