2-[(Acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutyl Group: A Thermolabile Protecting Group for Phosphodiesters

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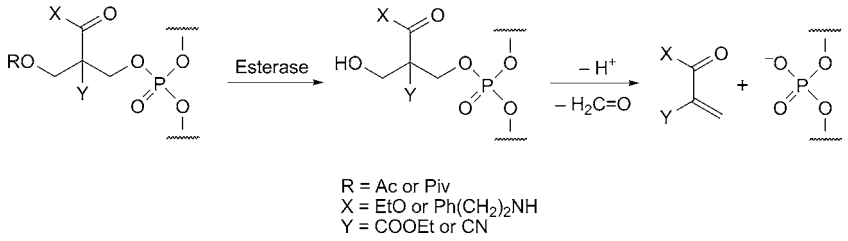
The phosphodiester linkage of 3′-*O*-levulinoylthymidine 5′-methylphosphate (**5**) has been protected with 2-[(acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutyl group (to give **1**) to study the potential of this group as an esterase- and thermolabile protecting group. The group turned out to be unexpectedly thermolabile, being removed as ethyl 3-(acetyloxy)-4-(acetylsulfanyl)-2-methylidenebut-3-enoate (**10**) without accumulation of any intermediates. The half-life of this reaction at pH 7.5 and 37° is 14 min. Hog liver esterase (HLE), in turn, removes the protecting group as ethyl 4-(acetylsulfanyl)-2-methylidene-3-oxobutanoate (**12**). On using 2.6 units of HLE in 1 ml, the rate of the enzymatic deprotection was still only one third of that of the nonenzymatic reaction. The mechanisms of both reactions have been studied and discussed. The crucial step seems to be removal of the O-bound Ac group, either by esterase or by migration to the neighboring 3-oxo group (nonenzymatic removal). This triggers the removal by *retro*-aldol condensation/elimination mechanism. No alkylation of glutathione (GSH) upon the deprotection of **1** could be detected.

Introduction. – We have previously reported on 3-(acyloxy)-2,2-bis(ethoxycarbonyl)propyl [1–4] and 3-(acyloxy)-2-[(alkylamino)carbamoyl]-2-cyanopropyl [5] groups as esterase-labile protecting groups for phosphoesters. Enzymatic deacylation triggers removal of formaldehyde by *retro*-aldol condensation, and the remnants of the group are released as an enone (*Scheme 1*). With the enzymatically more labile 3-[(acetyloxy)methoxy]-2,2-bis(ethoxycarbonyl)propyl group [6][7], deacetylation is followed by hydrolysis of the resulting hemiacetal, the rest of the reaction sequence being identical to that described above. The enone, unfortunately, is an alkylating agent which has been shown to form a covalent adduct with glutathione [5]. For this reason, we have tried to modify the protecting group structure by introducing an intramolecular S-containing nucleophile to trap the enone structure by cyclization. 5′-*O*-[{2-[(Acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutoxy}(methoxy)-phosphoryl]-2′-deoxy-3′-*O*-levulinoylthymidine (**1**; *Fig. 1*) was prepared as a model to study the feasibility of this approach. As indicated below, the original aim was not achieved, probably owing to too-slow enzymatic deacetylation of the sulfanyl function. However, the 2-[(acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutyl group turned out to be useful as a thermolabile protecting group. Although the group still gave an enone structure upon deprotection, no indication of formation of a covalent adduct with glutathione could be detected. Thermolabile protecting groups are of special interest for protection of oligonucleotides and their congeners, since the esterase-dependent removal is dramatically reduced upon accumulation of negative charge on the substrate [4][7].

Scheme 1. *Enone Formation upon Removal of 2,2-Disubstituted 3-(Acyloxy)propyl Groups from a Phosphodiester Linkage*



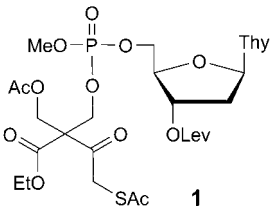
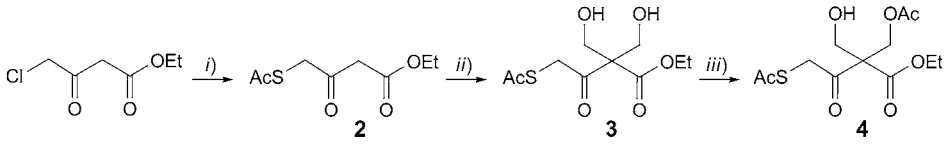


Fig. 1. *5′-O-({2-[(Acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutoxy}(methoxy)phosphoryl)-2′-deoxy-3′-O-levulinoylthymidine* (**1**)

Results and Discussion. – *Syntheses.* For the synthesis of 2-[(acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutyl-protected phosphodiester **1**, the corresponding alcohol **4** was first prepared as depicted in *Scheme 2*. Treatment of ethyl 4-chloroacetoacetate with AcSH acid in the presence of Et_3N gave ethyl 4-(acetylsulfanyl)-3-oxobutanoate (**2**), which was then converted to ethyl 4-(acetylsulfanyl)-2,2-bis(hydroxymethyl)-3-oxobutanoate (**3**) by bis(hydroxymethylation) with HCHO in the presence of a tertiary amine [8]. Diol **3** reacted with $(EtO)_3CMe$ in the presence of acid to afford ethyl 5-[(acetylsulfanyl)acetyl]-2-ethoxy-2-methyl-1,3-dioxane-5-carboxylate, as reported previously [9] for closely related compounds. NMR and MS analyses revealed that the cyclic orthoacetate was hydrolyzed during the silica-gel chromatography, resulting in formation of the desired ethyl 2-[(acetyloxy)methyl]-4-(acetylsulfanyl)-2-(hydroxymethyl)-3-oxobutanoate (**4**).

Phosphotriester **1** was then prepared as outlined in *Scheme 3*. Phosphitylation of 3′-*O*-levulinoylthymidine with methyl-*N*,*N*-diisopropylchlorophosphoramidite and sub-

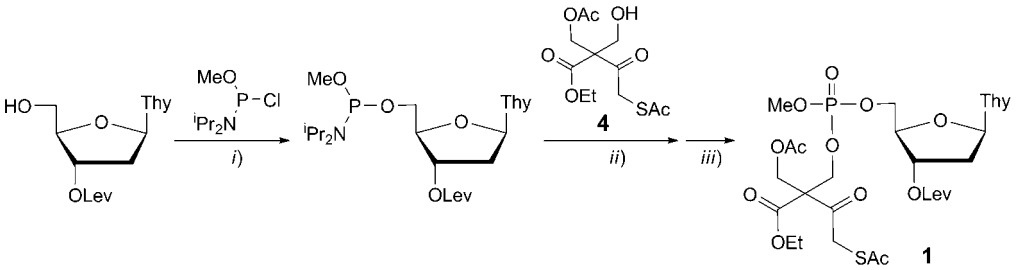
Scheme 2. *Preparation of the 2-[(Acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutyl Protecting Group*



i) AcSH, Et_3N, Et_2O. *ii*) HCHO, Et_3N, 1,4-dioxane. *iii*) 1. $(EtO)_3CMe$, H_2SO_4, THF; 2. CC (SiO_2; CH_2Cl_2/MeOH).

sequent 1*H*-tetrazole-promoted displacement of the $^{i}Pr_2N$ ligand by **4** gave the phosphite triester, which was oxidized to phosphate ester with I_2 in aqueous THF containing 2,6-lutidine. Triester **1** was obtained as a mixture of four diastereoisomers, since the protecting group contained, besides the stereogenic P-atom, an additional stereogenic center. The mixture could be separated to slower- and faster-eluting pairs of diastereoisomers by reversed-phase HPLC, but no attempt was made to assign the absolute configurations of the diastereoisomers.

Scheme 3. *Preparation of* **1**



i) Et_3N, CH_2Cl_2. *ii*) 1*H*-Tetrazole, MeCN. *iii*) I_2, THF, H_2O, 2,6-lutidine.

Hydrolytic Stability of Phosphotriester **1**. The nonenzymatic deprotection of the slower-eluted diastereoisomer pair of phosphotriester **1** was studied in a 2-[4-(2-hydroxyethyl)piperazin-1-yl)]ethanesulfonic acid (*HEPES*) buffer at pH 7.5 at 37.0 ± 0.1°. The composition of the aliquots withdrawn from the reaction mixture at appropriate time intervals was analyzed by RP-HPLC. The products were identified by mass-spectrometric analysis (HPLC/ESI-MS). The removal of the 2-[(acetyloxy)-methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutyl group from **1** gave 3′-*O*-levulinoylthymidine 5′-methylphosphate (**5**; $[M+H]^+$ at *m*/*z* 435.6) without accumulation of any intermediates. The observed *pseudo*-first-order rate constant was $8.44 \times 10^{-4}\,s^{-1}$ ($t_{1/2}$ 14 min). The deprotection was additionally followed in anhydrous MeCN/$BnNH_2$ 50:1 (*v*/*v*), since it is known that amine nucleophiles cleave thioesters more rapidly than oxygen esters [10]. Under these conditions, the rate constant for the conversion of **1** to **5** was $8.20 \times 10^{-4}\,s^{-1}$.

Esterase-Triggered Deprotection of the Phosphotriester. To study the enzymatic stability of **1**, deprotection of the faster-eluted diastereoisomer pair of **1** was followed in the presence of Hog Liver Carboxyesterase (HLE; 2.6 units/ml) in a *HEPES* buffer at pH 7.5 at 37.0°. As shown in *Fig. 2*, upon 95% disappearance of the starting material, the main product was **5** (85%). The *O*-deacetylated phosphotriester (**6**; $[M+H]^+$ at *m*/*z* 682.0) was observed to accumulate as an intermediate (up to 6% of the products). Additionally, delevulinoylated products, *i.e.*, 5′-*O*-[{2-[(acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutoxy}(methoxy)phosphoryl]-2′-deoxy-3,4-dihydrothymidine (**7**; $[M+H]^+$ at *m*/*z* 625.8) and thymidine 5′-methylphosphate (**8**; $[M+H]^+$ at *m*/*z* 337.6), were detected as minor products (*Scheme 4*). Among these, **8** predominated. The observed *pseudo*-first-order rate constant for the disappearance of

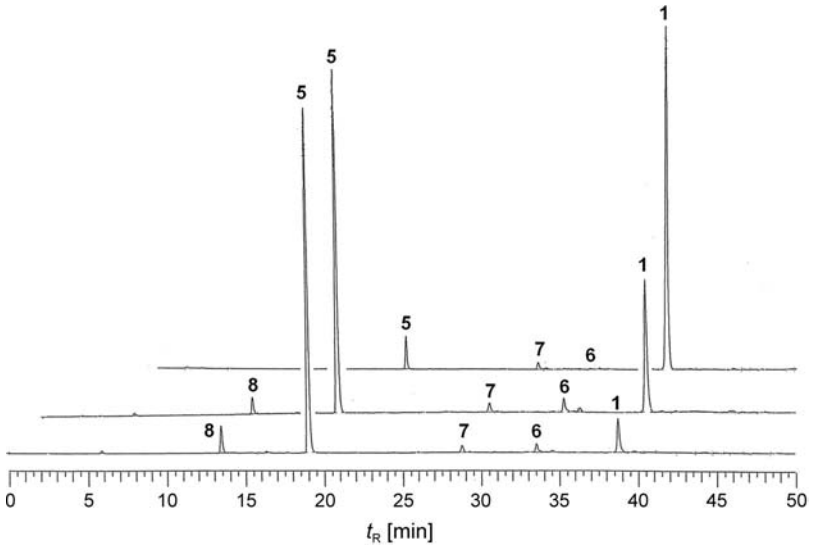
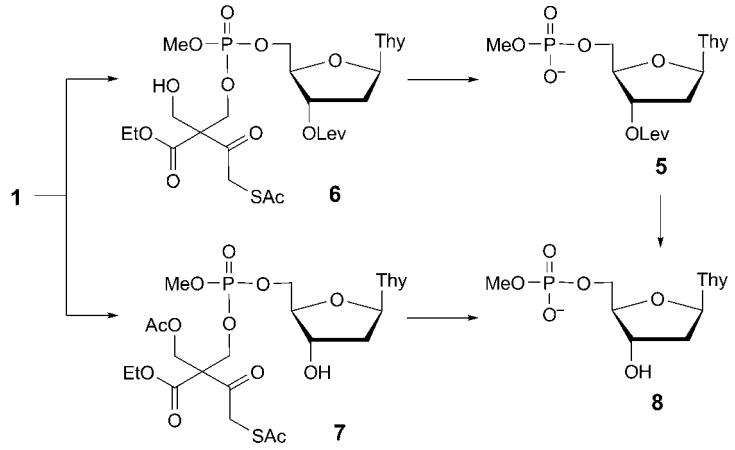


Fig. 2. *RP-HPLC Traces of the hydrolysis of* **1** *at pH 7.5 and 37.0°* ($I = 0.1$M with NaCl) *in the presence of hog liver carboxyesterase* (HLE; 2.6 units/ml). For detailed chromatographic conditions, see the *Exper. Part*.

1 was 1.17×10^{-3} s^{-1} ($t_{1/2}$ 10 min). Accordingly, more than 70% of the removal was still nonenzymatic. By subtracting the contributions of this reaction and delevulinoylation (< 5%), the half-life for the enzymatic deprotection may be estimated to be 40 min.

Mechanism of Nonenzymatic Removal of the Phosphate-Protecting Group. We have reported previously that the half-life for the HO^--catalyzed deacetylation of thymidine 5′-bis[3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl]phosphate is 20 d ($k =$

Scheme 4. *Esterase-Triggered Deprotection of Phosphotriester*



4.0×10^{-7} s^{-1}) at pH 7.5 and 37° [6]. In all likelihood, hydrolysis of the thioester linkage is approximately as fast, since thioesters and their O-counterparts are known to be almost as susceptible to O-nucleopiles [11]. The surprisingly fast nonenzymatic conversion of **1** to **5** ($t_{1/2}$ 14 min) cannot, hence, be initiated by intermolecular deacetylation of the acetylsulfanyl or (acetyloxy)methyl substituent. Phosphodiester **5** is rather released by one of the alternative mechanisms depicted in *Scheme 5*. In other words, migration of the Ac group from the hydroxymethyl to the neighboring 3-oxo group is the key event. As a consequence, the hydroxymethyl group becomes exposed and departs by *retro*-aldol condensation, accompanied by phosphate elimination to give enone, analogously to the enzyme-triggered reaction. This initial Ac group migration may possibly proceed by initial hydration of the C=O group, as indicated in *Scheme 5*,*a*. Dehydration of enone **9** obtained then gives ethyl 3-(acetyloxy)-4-(acetylsulfanyl)-2-methylidenebut-3-enoate (**10**) as the final product. Alternatively, the *O*-Ac migration may take place by an attack of the enolized O-atom of the starting material on the C=O C-atom (*Scheme 5*,*b*). These mechanistic suggestions are based, in addition to the MS data, on the following NMR spectroscopic observations.

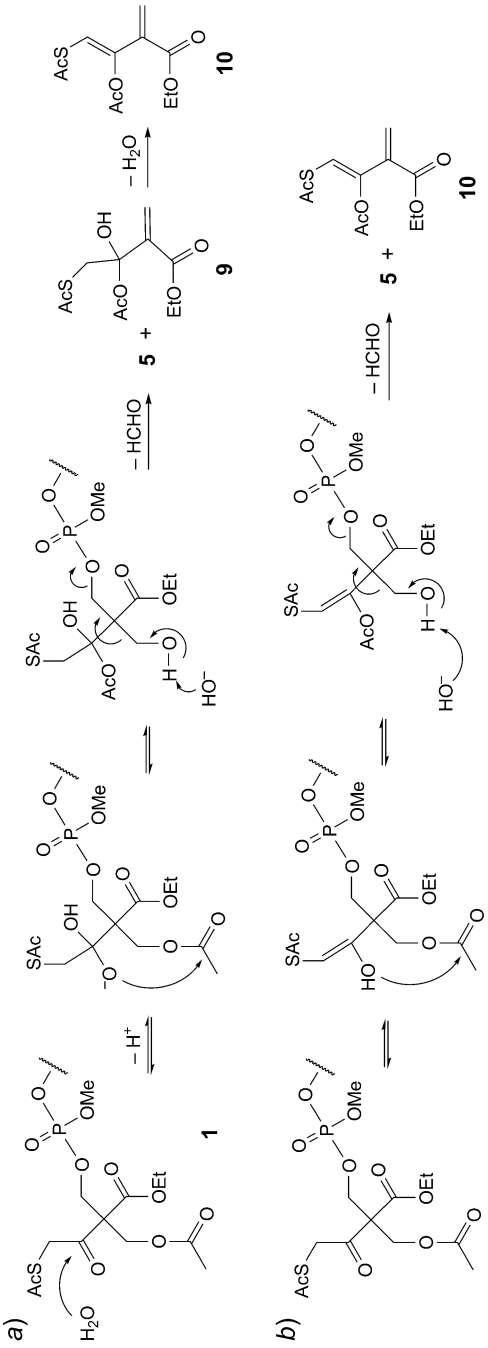
The course of removal of the phosphate-protecting group was followed by NMR spectroscopy in D_2O/CD_3CN 9:1 (*v*/*v*) buffered with potassium phosphate (0.005M; pH 7.0). When 70% of **1** diasappeared, ^{1}H-NMR signals at 6.54 (*s*, 1 H, $=CH_2$), 6.13 (*s*, 1 H, $=CH_2$), 4.38 (*q*, $J = 7.0$, $MeCH_2$), 2.52 (*s*, MeC(O)S), 2,24 (*s*, MeC(O)O), and 1.42 (*t*, $J = 7.0$, $MeCH_2$) appeared. These most likely refer to formation of **10** (*Fig. 3*). From HSQC spectra, the two signals at 130.0 ppm could be assigned to $=CH_2$, while the signals referring to $MeCH_2$ and $MeCH_2$ groups appeared at 62.5 and 13.5 ppm, respectively. The ^{1}H-NMR resonance signal at 4.93 ppm (*s*, 2 H), which was coupled to ^{13}C resonance at 63.0 ppm in HSQC spectrum, could not be firmly assigned. One might speculate that the signal refers to H–C(4) of **10**, but these H-atoms between the acetylsulfanyl and keto groups of **1** undergo deuteration rather readily and, hence, the presence of the signal of the H–C(4) appears doubtful.

To elucidate the potential of **10** as an electrophilic alkylating agent, phosphotriester **1** was hydrolyzed in a 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (0.005M) at pH 6.1. The reaction mixture was treated with glutathione ([GSH] = 5.0 mM) after the the phosphate deprotection was completed. As seen from *Fig. 4*, an *m*/*z* value of 454.4 due to the molecular ion $[M + MES]^+$ of **10** could be observed by HPLC/ESI-MS analysis. No GSH adduct of the product **10** was observed.

When phosphotriester **1** was treated with $BnNH_2$ in MeCN to obtain **5**, the protecting group was, according to HPLC/MS analysis, released as ethyl 3-[(acetyloxy)methyl]-4-oxotetrahydrothiophene-3-carboxylate (**11**; $[M + Na]^+$ at *m*/*z* 268.8) instead of **10**. In all likelihood, the selective *S*-deacetylation by the primary amine triggers an intramolecular nucleophilic attack of SH group at C(1), resulting in cleavage of **5** with concomitant formation of **11**, as depicted in *Scheme 6*.

Mechanism of Enzymatic Removal of the Phosphate-protecting Group. The esterase-catalyzed conversion of **1** to the corresponding diester **5** is most likely initiated by *O*-deacetylation (*Scheme 7*). The exposure of the $HOCH_2$ group results in a HO^--ion catalyzed *retro*-aldol condensation with a loss of HCHO and concomitant release of phosphodiester **5**. The formation of ethyl 4-(acetylsulfanyl)-2-methylidene-3-oxobutanoate (**12**; $[M + H]^+$ at *m*/*z* 217.5) was verified by MS analysis (*Fig. 5*).

Scheme 5. *Mechanism of Nonenzymatic Removal of the Phosphate-Protecting Group*



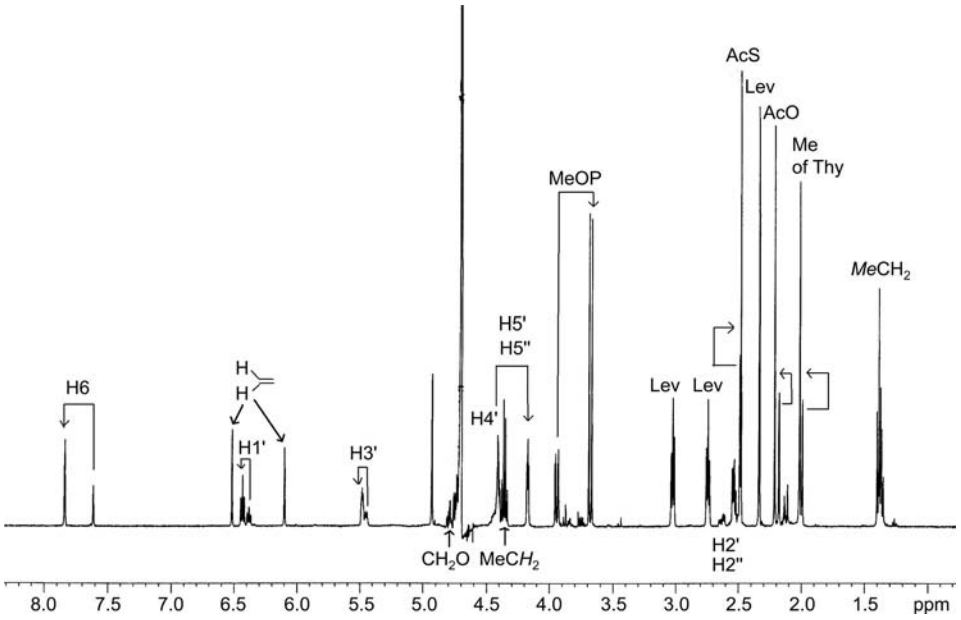


Fig. 3. *^{1}H-NMR Spectrum* (500 MHz, D_2O/CD_3CN 9:1, buffered with potassium phosphate (0.005M; pH 7.0)) *recorded during deprotection of the slower-eluting diastereoisomer pair of* **1**

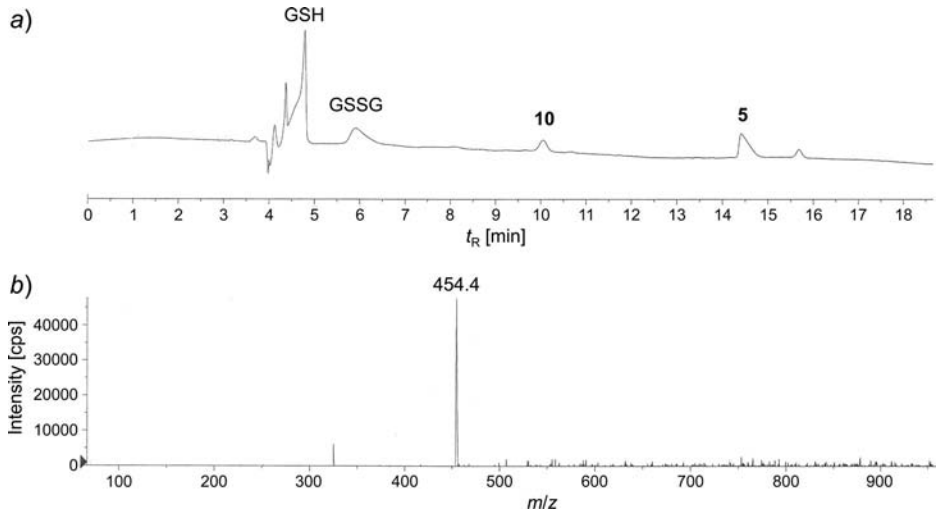
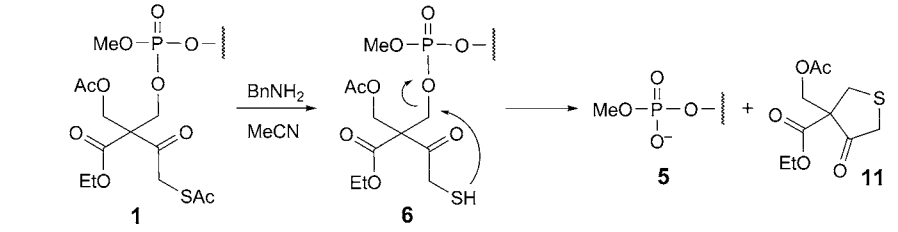


Fig. 4. a) *RP-HPLC Profile for the hydrolysis of the slower-eluting diastereoisomer pair of* **1** *in the presence of GSH at pH 6.1* (*MES* buffer; 0.005M) *and 37.0°*. For detailed chromatographic conditions, see the *Exper. Part* (HPLC signals were recorded with a UV detector at λ 220 nm). b) *Mass spectrum obtained from HPLC signal of the product* **10**.

Scheme 6. *Mechanism of Nonenzymatic Removal of the Protecting Group in the Presence of $BnNH_2$ and MeCN*



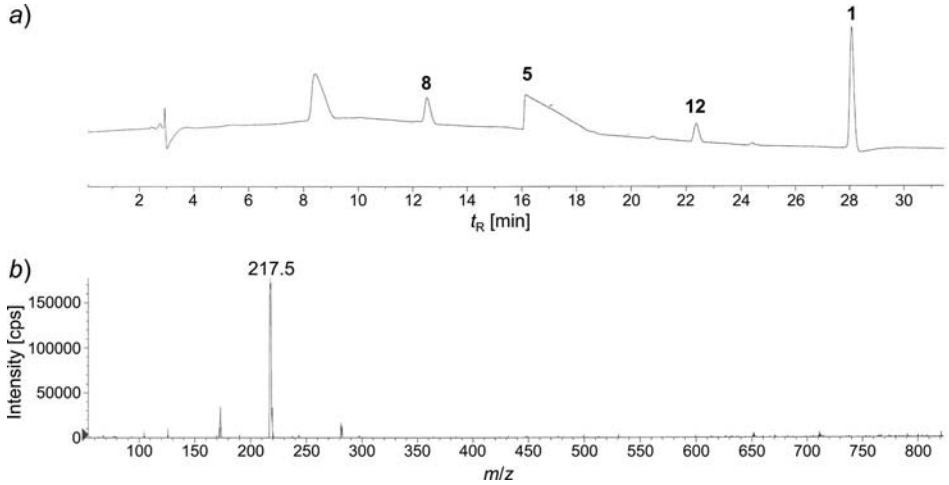
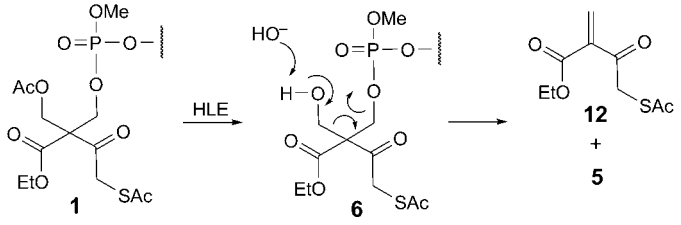


Fig. 5. a) *RP-HPLC Profile of the hydrolysis of the faster-eluting diastereoisomer pair of* **1** *in the presence of HLE at 37.0° in D_2O/CD_3CN 4:1 buffered with potassium phosphate* (0.01M; pH 7.0). For detailed chromatographic conditions, see the *Exper. Part* (HPLC signals were recorded with a UV detector at λ 220 nm). b) *Mass spectrum obtained from HPLC signal of the product* **12**.

As mentioned above, the nonenzymatic hydrolysis of **1** competes with the enzymatic one. The ^{1}H-NMR spectrum recorded in the presence of HLE in a mixture of D_2O and CD_3CN 4:1 (*v*/*v*), buffered with potassium phosphate (0.01M; pH 7.0), exhibited signals of the olefinic H-atoms (=CH_2) at 6.62/6.21 and 6.50/6.12 ppm. The signals most likely resulted from the formation of **12** and **10**, respectively (*Fig. 6*). The ^{13}C-NMR signal of =CH_2 group assigned from HSQC spectra was observed at 127.0 and 130.0 ppm for **12** and **10**, respectively. Additionally, the ^{1}H-NMR signals at 4.50–4.44 (*m*, MeCH_2), 2.59 (*s*, SC(O)Me), 2.33 (*s*, C(O)Me), 1.51 (*t*, J = 7.0, *Me*CH_2), and 1.37 (*t*, J = 7.0, *Me*CH_2) ppm were observed. No attempt was made to assign the ^{1}H-NMR resonances at 6.16, 5.77, 5.04, 4.97, and 2.10 ppm.

The observation that even the enzymatic removal of the protecting group gives enone **12**, and not the cyclic structure **11**, obtained by aminolytic *S*-deacetylation of **1**, is rather unexpected. Consistent with this observation, treatment of compound **10**,

Scheme 7. *Mechanism of Enzymatic Removal of the Protecting Group*



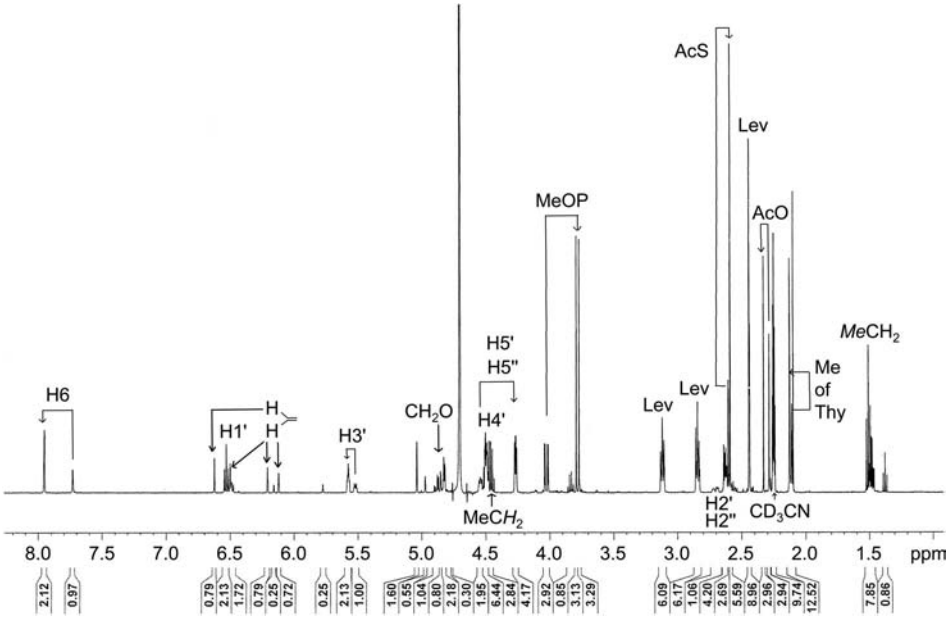


Fig. 6. *^{1}H-NMR Spectrum* (500 MHz, D_2O/CD_3CN 4:1, buffered with potassium phosphate (0.005M; pH 7.0)) *recorded during a deprotection of the faster-eluting diastereoisomer pair of* **1** *in the presence of HLE*

obtained by nonenzymatic removal of the protecting group from **1**, with HLE gave, according to NMR analysis, compound **12** by *O*-deacetylation, with the AcS substituent remaining intact. In striking contrast, phosphate-bound 4-(acetylsulfanyl)-2,2-dimethyl-3-oxobutyl group has been shown to undergo esterase-catalyzed *S*-deacetylation quite rapidly [12].

To evaluate the potential of the released protecting group as an electrophilic alkylating agent, the phosphotriester **1** was treated with glutathione (5 mM) in the presence of HLE (2.6 U/ml) in a MES buffer (0.005M) at pH 6.1. The experiment was repeated, but glutathione was not added until the phosphate deprotection moiety was completed. In neither case, any glutathione adduct was found.

Conclusions. – 2-[(Acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutyl group has been introduced as a phosphodiester-protecting group that may be removed by esterases, but is also thermolabile. The group is removed either as ethyl 4-(acetylsulfanyl)-2-methylidene-3-oxobutanoate (**12**; enzymatic removal) or as ethyl 3-(acetyloxy)-4-(acetylsulfanyl)-2-methylidenebut-3-enoate (**10**; nonenzymatic removal). Neither of these appears to alkylate glutathione. The group is expected to be useful as a thermolabile protecting group of oligonucleotides and their congeners containing several phosphodiester likages, since negatively charged compounds are poor substrates for carboxyesterases.

We thank Mr. *Joonas Mäkelä* (M.Sc.) and Ms. *Anna Leisvuori* (M.Sc.) for the synthesis of **1**.

Experimental Part

General. The preparation of 3′-*O*-levulinoylthymidine has been described in [6]. Reactions requiring anh. conditions were carried out under N_2. The solvents were dried over 4-Å molecular sieves. Et_3N was dried by refluxing over CaH_2 and distilled before use. Column chromatography (CC): *Fluka* silica gel *60* (230–400 mesh was used). Hydrolytic reactions were followed by *Merck Hitachi LaChrom D7000 HPLC*. ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra: *Bruker Avance* 500 or 400-MHz NMR spectrometer; δ in ppm rel. to Me_4Si, *J* in Hz. HR-MS: *Bruker Daltonics micrOTOF-Q* instrument. LC/MS: *Perkin-Elmer Sciex-API-365* triple-quadrupole instrument; in *m*/*z*.

Ethyl 4-(Acetylsulfanyl)-3-oxobutanoate (**2**). Ethyl 4-chloroacetoacetate (8.27 ml, 0.06 mmol) and AcSH (6.51 ml, 0.09 mmol) were dissolved in 150 ml of Et_2O. Et_3N (10.0 ml, 0.07 mmol) in Et_2O (30 ml) was added dropwise with ice cooling. After complete addition, the mixture was stirred for 2 h at r.t. The soln. was washed with H_2O (3 × 100 ml), 5% aq. $NaHCO_3$ (100 ml), and sat. aq. NaCl (100 ml). The org. phase was dried (Na_2SO_4), evaporated, and the crude product was purified by CC (SiO_2; CH_2Cl_2) to give **2** (5.5 g, 45%). Oil. ^{1}H-NMR (400 MHz, $CDCl_3$): 4.11 (*q*, *J* = 7.2, MeCH_2O); 3.80 (*s*, SCH_2); 3.51 (*s*, CH_2); 2.31 (*s*, Ac); 1.20 (*t*, *J* = 7.2, *Me*CH_2O). ^{13}C-NMR (126 MHz, $CDCl_3$): 196.5 (C=O); 194.0 (SC=O); 166.6 (OC=O); 61.5 (OCH_2); 48.0 (CH_2); 39.0 (SCH_2); 30.0 (*Me*C=O), 14.0 (*Me*CH_2O). HR-ESI-MS: 227.0358 ([*M* + Na]$^+$, $C_8H_{12}NaO_4S^+$; calc. 227.0349).

Ethyl 4-(Acetylsulfanyl)-2,2-bis(hydroxymethyl)-3-oxobutanoate (**3**). Compound **2** was bis(hydroxymethylated) as described in [8]. The crude product was purified by CC (SiO_2, CH_2Cl_2/MeOH 96:4). The product including some impurities was obtained as oil in low yield (0.48 g, 7%) and used without further purification. ^{1}H-NMR (500 MHz, $CDCl_3$): 4.32–4.05 (*m*, MeCH_2O, 2 CH_2OH); 4.10–4.03 (*m*, CH_2S); 2.40 (*s*, Ac); 1.32 (*m*, *Me*CH_2O). ^{13}C-NMR (101 MHz, $CDCl_3$): 200.5 (C=O); 195.3 (SC=O); 169.3 (OC=O); 67.0 (spiro-C); 63.6 (Me*C*H_2O); 62.3 (CH_2OH); 38.0, 37.8 (CH_2S); 30.1 (*Me*C=O); 14.0 (*Me*CH_2O). HR-ESI-MS: 265.0729 ([*M* + H]$^+$, $C_{10}H_{17}O_6S^+$; calc. 265.0740).

Ethyl 2-[(Acetyloxy)methyl]-4-(acetylsulfanyl)-2-(hydroxymethyl)-3-oxobutanoate (**4**). The cyclic orthoester, *ethyl 5-[(acetylsulfanyl)acetyl]-2-ethoxy-2-methyl-1,3-dioxane-5-carboxylate* was synthesized as desribed in [9]. Conc. H_2SO_4 (4.5 µl, 0.08 mmol) was added to a mixture of **3** (0.46 g, 1.75 mmol) and triethyl orthoacetate (0.64 ml, 3.50 mmol) in dry THF (0.6 ml). The reaction was allowed to proceed 3 d, and the mixture was then poured into an ice-cold soln. of 5% $NaHCO_3$ (50 ml). The product was extracted with Et_2O (3 × 50 ml), washed with sat. aq. NaCl (3 × 50 ml), and dried (Na_2SO_4). The solvent was evaporated, and the crude product was subjected to CC (SiO_2; CH_2Cl_2/MeOH 98:2 and 96:4). The cyclic orthoester was hydrolyzed, most likely, during CC to give **4** (0.28 g, 47%). Oil. ^{1}H-NMR (500 MHz, $CDCl_3$): 4.67 (*d*, *J* = 11.65, CH_2OAc); 4.62 (*q*, *J* = 11.65, CH_2OAc); 4.29 (*qd*, *J* = 7.20, 1.25, MeCH_2O); 4.21–3.91 (*m*, CH_2OH, SCH_2); 2.38 (*s*, AcS); 2.07 (*s*, AcO); 1.31 (*t*, *J* = 7.20, *Me*CH_2O). ^{13}C-NMR (101 MHz, $CDCl_3$): 198.0 (C=O); 194.7 (SC=O); 170.8 (OC=O); 168.2 (Me*C*=O); 65.2 (spiro C); 62.4, 62.3, 62.1 (MeCH_2O, *C*H_2OC=O, CH_2OH); 37.5 (SCH_2); 30.1 (*Me*C(O)S); 20.70 (*Me*C=O); 13.98 (*Me*CH_2O). HR-ESI-MS: 329.0667 ([*M* + Na]$^+$, $C_{12}H_{18}NaO_7S^+$; calc. 329.0665).

5′-O-*({2-[(Acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutoxy}(methoxy)phosphoryl)-2′-deoxy-3′*-O-*(4-oxopentanoyl)thymidine* (**1**). To a soln. of dried 3′-*O*-levulinoylthymidine (0.17 g, 0.50 mmol) in dry CH_2Cl_2 (2.0 ml), anh. Et_3N (0.35 ml, 2.48 mmol) and methyl-*N*,*N*-diisopropylchlorophosphoramidite (160 µl, 0.55 mmol) were added, and the mixture was stirred for 1 h under N_2. The product was filtered through a short silica-gel column (anh. AcOEt/Et_3N 99.5 : 0.5). The solvent was removed *in vacuo*, and the residue was co-evaporated three times from dry MeCN to remove the traces of Et_3N. The residue was dissolved in dry MeCN (1.0 ml), and **4** (0.17 g, 0.55 mmol) in dry MeCN (1.0 ml) and 1*H*-tetrazole (1.00 mmol; 2.22 ml of 0.45M soln. in MeCN) were added under N_2. The mixture was stirred for 1 h at r.t. The phosphite ester formed was oxidized with I_2 (0.1M) in THF/H_2O/lutidine 4 : 2 : 1 (4 ml) by stirring 2 h at r.t. The crude product **1** was isolated by workup with CH_2Cl_2/aq. 5% $NaHSO_3$, and purified by CC (SiO_2; AcOEt/MeOH 95 : 5, followed by CH_2Cl_2/MeOH 90 : 10) to afford **1** (94 mg, 26%). Solid foam. The diastereoisomer pairs were separated by CC (*SunFire™ C18* column, 250 × 10 mm, 5 µm; H_2O/MeCN 65 : 35). ^{1}H-NMR (500 MHz, CD_3OD): 7.56 – 7.57 (*m*, H–C(6)); 6.28 – 6.33 (*m*, H–C(1′)); 5.33 (br. *s*, H–C(3′)); 4.68 – 4.59 (*m*, C*H*$_2$OAc, POCH_2); 4.37 – 4.29 (*m*, H–C(5′), H–C(5″), MeC*H*$_2$O); 4.23 – 4.24 (*m*, H–C(4′)); 4.07 – 4.05 (*m*, SCH_2); 3.86 – 3.82 (*m*, POMe); 2.86 (*t*, *J* = 6.50, CH_2 of Lev); 2.61 (*t*, *J* = 6.50, CH_2 of Lev); 2.47 – 2.35 (*m*, H–C(2′), H–C(2″)); 2.37 (*s*, AcS); 2.20 (*s*, Me of Lev); 2.06 – 2.07 (*m*, AcO); 1.93 (*m*, Me of Thy); 1.33 – 1.27 (*m*, *Me*CH_2O). ^{13}C-NMR (126 MHz, CD_3OD): 208.0 (Me*C*=O of Lev); 193.9 (SC=O); 172.6 (OC=O of Lev); 170.4 (OC=O); 166.4 (Me*C*=O); 164.9 (C(4)); 150.9 (C(2)); 135.9 (C(6)); 110.8 (C(5)); 85.0 (C(1′)); 82.5 (C(4′)); 73.9 (C(3′)); 67.5 (C(5′)); 65.0 (POCH_2); 63.5 (spiro C); 62.6 (Me*C*H_2O); 60.9 (*C*H_2OAc); 54.3 (POMe); 37.2 (CH_2 of Lev); 36.1 (SCH_2, C(2′)); 28.4 (*Me*C(O)S); 28.2 (Me of Lev); 27.5 (CH_2 of Lev); 19.1 (*Me*C(O)O); 12.8 (*Me*CH_2O); 11.1 (Me of Thy). ^{31}P-NMR (202 MHz, CD_3CN): −0.42; −0.44; −0.53; −0.57. HR-ESI-MS: 723.1655 ([*M* + H]$^+$, $C_{28}H_{40}N_2O_{16}PS^+$; calc. 723.1831). 745.1661 ([*M* + Na]$^+$, $C_{28}H_{39}N_2NaO_{16}PS^+$; calc. 745.1650).

Kinetic Measurements. The reactions were carried out in sealed tubes immersed in a thermostated H_2O bath (37.0 ± 0.1°). The hydronium ion concentration, [H_3O^+], of the reaction solns. (3.0 ml) was adjusted with NaOH and *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid] (*HEPES*) buffer. The ionic strength of the solns. was adjusted to 0.1M with NaCl. The [H_3O^+] of the buffer solns. 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 mM.

The enzymatic and nonenzymatic hydrolyse were carried out in a *HEPES* buffer at pH 7.5 (0.036/0.024M). The acyl group was removed with Hog Liver Esterase (2.6 units/ml). The samples (200 µl) withdrawn at appropriate intervals were made acidic (pH 2) with 1M aq. HCl or 1M AcOH to inactivate enzyme and to quench the hydrolysis, cooled in an ice bath and filtered with *RC4* syringe filters (0.2 µm). The composition of the samples was analyzed on a *C18* column (4 × 250 mm 5 µm, flow rate 0.95 ml min^{-1}), using a mixture of AcOH/AcONa buffer (0.045/0.015M) and MeCN, containing NH_4Cl (0.1M). A good separation of the products was obtained by using a 5-min isocratic elution with the buffer containing 2% MeCN, followed by a 30-min linear gradient up to 50% MeCN. Signals were recorded with a UV detector at of 267 nm. The reaction products were separated and identified by LC/MS on a *Phenomenex Gemini C18* column (2.0 × 150 mm 5 µm; flow rate 0.4 ml min^{-1}) using a 5-min isocratic elution with H_2O containing MeCN (2 %) and HCOOH (0.1%), followed by a 35-min linear gradient up to 60% MeCN.

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