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4-Acetylthio-2,2-dimethyl-3-oxobutyl Group as an Esterase- and Thermo-Labile Protecting Group for Oligomeric Phosphodiesters

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Keywords: Synthetic methods / Medicinal chemistry / Protecting groups / Prodrugs / Oligonucleotides / Kinetics

(4-Acetylthio-2,2-dimethyl-3-oxobutyl)-protected oligomeric phosphodiesters **1** and **2** were synthesized and removal of the protecting groups in the presence and absence of hog liver esterase was followed at pH 7.5 and 37 °C. Phosphotriesters **1** and **2** were successfully converted into the desired fully deprotected phosphodiesters **3** and **4**, respectively. Some cleavage of internucleosidic P–O bonds took place, which reduced the yield of **3** and **4**. Non-enzymatic removal of the protecting group was only modestly retarded by accumulation of negative charge on the molecule. With **1**, the half-lives for the departure of the first and second protecting groups were 7.8 and 10.7 h, respectively, and with **2**, 6.2 and 7.2 h, respectively. After 4 d, 70 % of both starting materials **1** and **2** were converted into the unprotected phosphodiester. The presence of hog liver esterase (2.6 units mL^{-1}) resulted in fast removal of the first protecting group ($\tau_{1/2}$ 2.7 min and 36 min with **1** and **2**, respectively), but the appearance of fully deprotected **3** and **4** was accelerated only by a factor of 2, consistent with dramatic retardation of the enzymatic reaction upon accumulation of the negative charge.

Introduction

Structurally modified oligonucleotides have received attention as a means to protect against viral infections, cancer and hereditary diseases.[1–7] To enhance cell penetration, the negatively charged phosphodiester or phosphorothioester backbone has been masked by enzyme-labile acyl-2-thioethyl,[8–11] 4-acyloxybenzyl,[12,13] acyloxyalkyl,[14] and 2,2-bis(substituted)-3-acyloxypropyl[15] groups. In all cases, the protected oligonucleotide is expected to undergo an intracellular carboxyesterase-mediated deacylation followed by a chemical reaction, which eliminates the remnants of the protecting groups and releases the oligonucleotide. Removal of the protecting groups is, however, markedly retarded upon accumulation of a negative charge on the neighboring phosphodiester linkages,[15–17] as a result of reduced affinity of the activating enzyme to charged substrates.[1] In this regard, thermolabile protecting groups appear more attractive. Their removal, as expected, is less sensitive to the charge type of the adjacent phosphoester linkages. Recently, (*N*-formyl-*N*-methyl)-2-aminoethyl,[18] 4-hydroxybutyl[19] and ω-(alkylthio)alkyl groups[20] have been introduced as thermosensitive protecting groups for immunotherapeutic phosphoromonothioate oligomers. Additionally, α-hydroxybenzylphosphonate modified oligonucleotides have been studied as non-enzymatically degradable prooligonucleotide candidates.[21–23]

We have reported that 2,2-disubstituted 4-acylthio-3-oxobutyl groups serve as both thermo- and esterase-labile protecting groups for nucleoside 5′-methylphosphate.[24] The advantage of these protecting groups is that the rate of non-enzymatic deprotection can be controlled by the electronegativity of the 2-substituents and the rate of enzymatic deprotection governed by the bulkiness of the acyl group.

To study the applicability of such a group for protection of oligomeric phosphodiesters, phosphotriesters **1** and **2** that bear (4-acetylthio-2,2-dimethyl-3-oxobutyl)-protected phosphodiester linkages were prepared. Because development of a solid-phase procedure for oligonucleotides that bear phosphate protecting groups, which are not only base labile but also susceptible to amine nucleophiles and leave readily by intramolecular cyclization, is not trivial, we decided to prepare the first model oligomers in solution. The

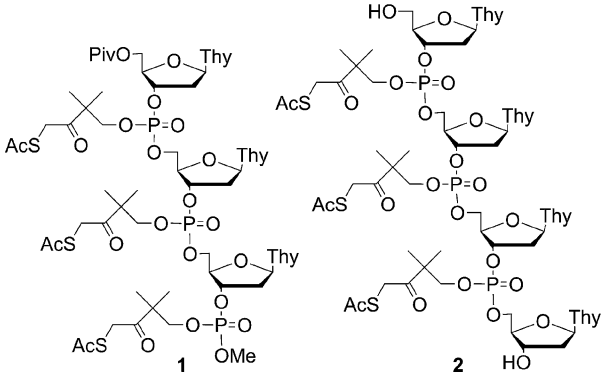

Figure 1. Structures of (4-acetylthio-2,2-dimethyl-3-oxobutyl)-protected phosphotriesters **1** and **2**.

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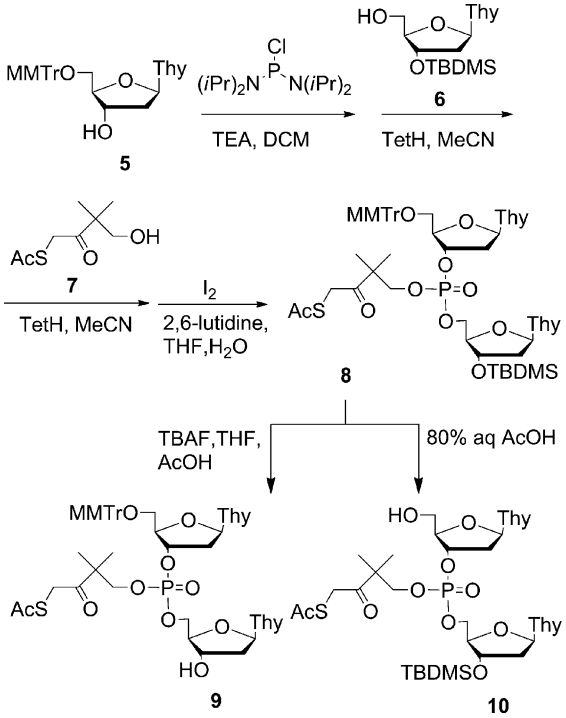
conversion of **1** and **2** to fully deprotected oligonucleotides was investigated in 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer at pH 7.5 and 37 °C in the presence and absence of hog liver esterase (Figure 1).

Results and Discussion

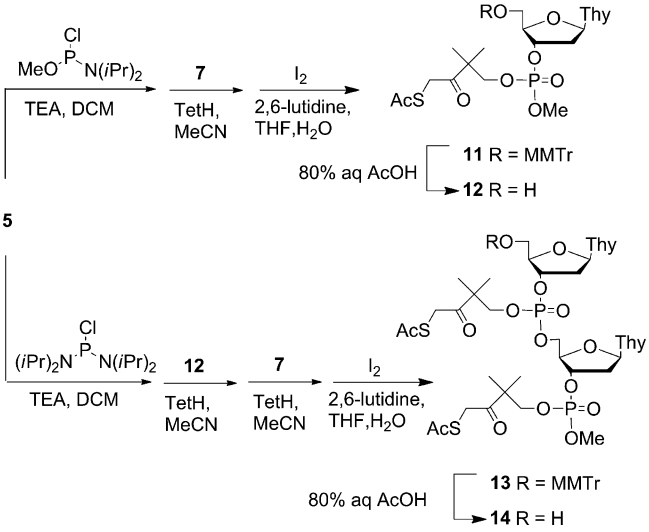
Synthesis

Appropriately protected nucleotide building blocks **9** and **10** were synthesized as described in Scheme 1. 5′-*O*-(4-Methoxytrityl)thymidine (**5**) was phosphitylated with 1-chloro-*N*,*N*,*N*′,*N*′-tetraisopropylphosphanediamine and the diisopropylamino ligands were displaced with 3′-*O*-(*tert*-butyldimethylsilyl)thymidine (**6**) and *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate (**7**). The phosphite triester obtained was oxidized to phosphate triester **8**. The *tert*-butyldimethylsilyl group was then removed by treating with tetrabutylammonium fluoride and the 4-methoxytrityl group with 80% aqueous acetic acid to obtain 5′-*O*-(4-methoxytrityl)thymidin-3′-yl thymidin-5′-yl 4-acetylthio-2,2-dimethyl-3-oxobutyl phosphate (**9**) and thymidine-3′-yl 3′-*O*-(*tert*-butyldimethylsilyl)thymidine-5′-yl 4-acetylthio-2,2-dimethyl-3-oxobutyl phosphate (**10**), respectively.



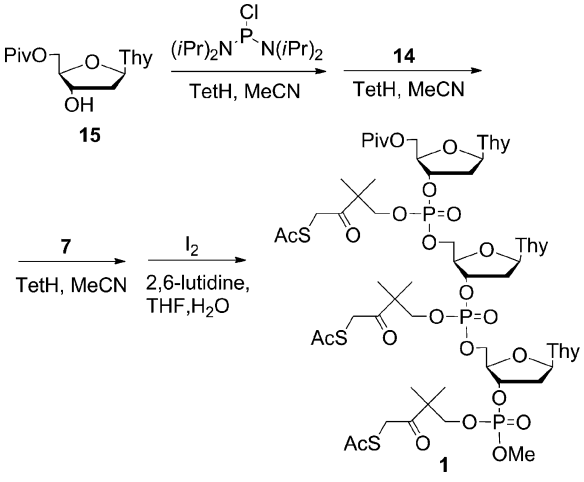
Scheme 1. Preparation of 5′-*O*-(4-methoxytrityl)thymidin-3′-yl thymidin-5′-yl 4-acetylthio-2,2-dimethyl-3-oxobutyl phosphate (**9**) and thymidine-3′-yl 3′-*O*-(*tert*-butyldimethylsilyl)thymidine-5′-yl 4-acetylthio-2,2-dimethyl-3-oxobutyl phosphate (**10**).

4-Acetylthio-2,2-dimethyl-3-oxobutyl thymidin-3′-yl methylphosphate (**12**) was obtained as outlined in Scheme 2. Accordingly, 5′-*O*-(4-methoxytrityl)thymidine (**5**) was first phosphitylated with 1-chloro-*N*,*N*-diisopropyl-1-methoxyphosphinamine, protecting group **7** was coupled and the phosphite ester was oxidized to phosphate ester. Then the monomethoxytrityl group was removed. (4-Acetylthio-2,2-dimethyl-3-oxobutyl)-protected thymidylyl-(3′,5′)thymidine 3′-[4-acetylthio-2,2-dimethyl-3-oxobutyl,methyl] phosphate (**14**) block was, in turn, prepared by phosphitylating the 3′-hydroxy group of **5** with 1-chloro-*N*,*N*,*N*′,*N*′-tetraisopropylphosphanediamine and by displacing the remaining diisopropylamino ligands with **12** and **7**. The phosphite triester formed was oxidized and the resultant phosphate triester was detritylated.



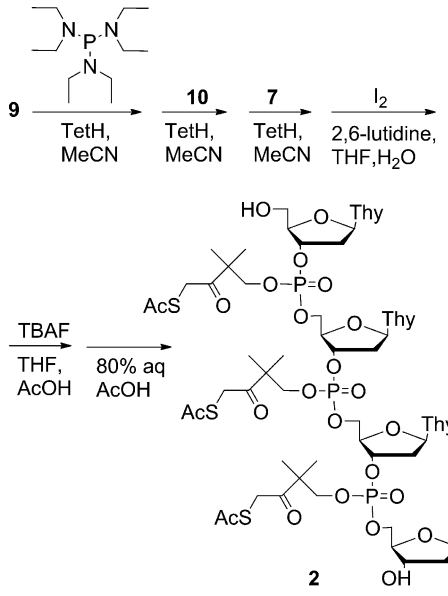
Scheme 2. Preparation of 4-acetylthio-2,2-dimethyl-3-oxobutyl thymidin-3′-yl methylphosphate (**12**) and (4-acetylthio-2,2-dimethyl-3-oxobutyl)-protected thymidylyl-(3′,5′)thymidine 3′-[4-acetylthio-2,2-dimethyl-3-oxobutyl,methyl] phosphate (**14**).

The assembly of (4-acetylthio-2,2-dimethyl-3-oxobutyl)-protected oligomeric phosphotriesters **1** and **2** is outlined in Scheme 3 and Scheme 4, respectively. To obtain **1**, 5′-*O*-pivaloylthymidine (**15**) was phosphitylated with 1-chloro-



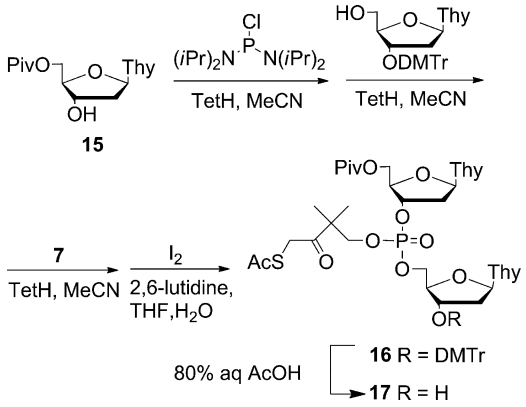
Scheme 3. Preparation of (4-acetylthio-2,2-dimethyl-3-oxobutyl)-protected phosphotriester **1**.

N,*N*,*N*′,*N*′-tetraisopropylphosphanediamine and the diisopropylamino ligands were displaced with **14** and **7**, followed by oxidation. Stepwise displacement of the diethylamino ligands of tris(diethylamino)phosphine with **10** and **7**, followed by oxidation, desilylation and detritylation gave compound **2**.



Scheme 4. Preparation of (4-acetylthio-2,2-dimethyl-3-oxobutyl)-protected phosphotriester **2**.

The synthesis of 4-acetylthio-2,2-dimethyl-3-oxobutyl 5′-*O*-pivaloylthymidin-3′-yl thymidin-5′-yl phosphate (**17**) is described in Scheme 5. 5′-*O*-Pivaloylthymidine (**15**) was phosphitylated with 1-chloro-*N*,*N*,*N*′,*N*′-tetraisopropylphosphanediamine and the diisopropylamino ligands were displaced with 3′-*O*-(4,4′-dimethoxytrityl)thymidine and **7**, followed by oxidation. Detritylation gave compound **17**.

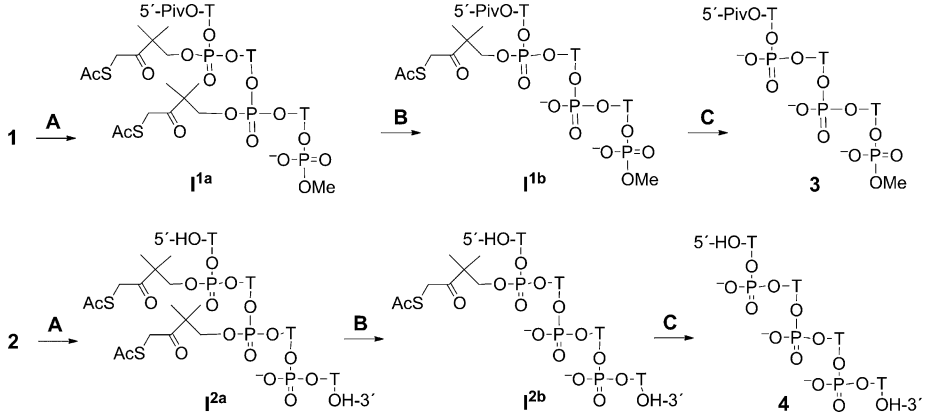


Scheme 5. Preparation of 4-acetylthio-2,2-dimethyl-3-oxobutyl 5′-*O*-pivaloylthymidin-3′-yl thymidin-5′-yl phosphate (**17**).

Non-Enzymatic Deprotection of Oligomeric Phosphotriesters 1 and 2

Removal of the 4-acetylthio-2,2-dimethyl-3-oxobutyl groups from oligomeric phosphotriesters **1** and **2** was followed by HPLC in a HEPES buffer at pH 7.5 and 37.0 ± 0.1 °C. To simplify the product distribution, the mixtures of eight R_P/S_P-diastereomers of the starting materials were fractionated by HPLC and one of the fractions that showed a single chromatographic signal was selected for kinetic measurements. The products were identified by spiking samples for analysis with authentic samples and/or by HPLC/ESI-MS analysis or signals were collected and identified by MS analysis.

The departure of the first 4-acetylthio-2,2-dimethyl-3-oxobutyl group from **1** (Route A in Scheme 6) gave mixture of monodeprotected intermediates $\mathbf{I^{1a}}$ that still have two of the phosphodiester linkages protected (t_R = 25.0–25.5 min; [M – H]$^-$ at *m*/*z* 1371.3; see Figure 2). The half-life for the disappearance of **1** was 7.8 h ($k = 2.47 \times 10^{-5}$ s^{-1}). Monodeprotected products $\mathbf{I^{1a}}$ were subsequently converted into



Scheme 6. Deprotection of oligomeric phosphotriesters **1** and **2** to fully deprotected phosphodiesters **3** and **4** through intermediate mixtures **I**. No attempt was made to clarify which of the three possible diester intermediates were accumulated in case of mixtures $\mathbf{I^{1a}}$, $\mathbf{I^{2a}}$, $\mathbf{I^{1b}}$ and $\mathbf{I^{2b}}$.

mixture of intermediates **I**^{1b}, which still bore one phosphate protecting-group (t_R = 20.0–22.0 min; [M – H]$^-$ at *m*/*z* 1371.3; Route B). The half-life of this conversion was 10.7 h ($k = 1.80 \times 10^{-5}$ s^{-1}). Accordingly, no significant rate-retardation relative to the first step was observed. Removal of the third phosphate protecting-group produced desired fully deprotected phosphodiester **3** ([M – H]$^-$ at *m*/*z* 1027.2) as the main product (Route C). In 4 d, more than 70% of starting material **1** had been converted into **3**. As seen from Figure 2, removal of the last protecting group from the slowest-eluting oligomer among **I**^{1b} (t_R = 21.7 min) is slower than removal from the two faster-eluting compounds (t_R = 20.3 and 20.6). Only small amounts of side products, which included PivO-TpTpTp (*m*/*z* at 1357.3), PivO-TpTpT (*m*/*z* at 933.2), TpT (*m*/*z* at 544.9), PivO-TpT (*m*/*z* at 629.2) and PivO-Tp-$CH_2C(Me_2)C(O)CH_2SAc$ (*m*/*z* at 577.2), could be detected by MS analysis.

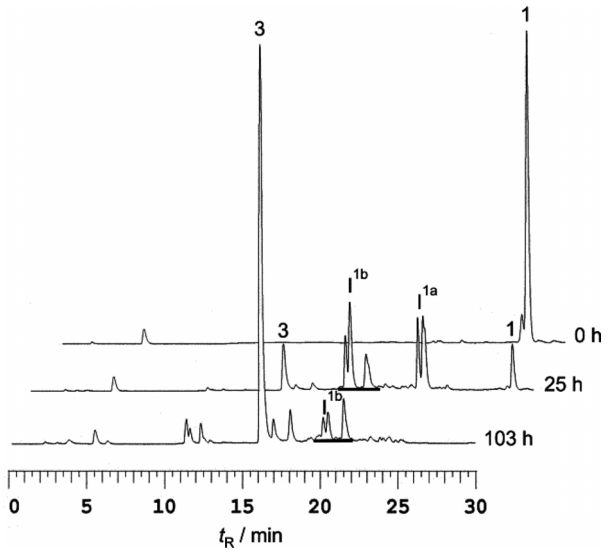


Figure 2. RP-HPLC traces for the non-enzymatic hydrolysis of protected oligomeric phosphotriester **1** to unprotected phosphodiester **3** at pH 7.5 and 37.0 °C (I = 0.1 mol L^{-1} with NaCl). For detailed chromatographic conditions, see the Experimental Section.

Removal of the 4-acetylthio-2,2-dimethyl-3-oxobutyl groups from **2** proceeded analogously (see Scheme 6 and Figure 3). Monodeprotected products **I**^{2a} were observed at t_R = 19.5–20.5 min ([M – H]$^-$ at *m*/*z* 1497.3), and they were subsequently converted into dideprotected intermediates **I**^{2b} (t_R = 15.0–17.0 min; [M – 2H]$^{2-}$ at *m*/*z* 662.2). Then desired fully deprotected phosphodiester **4** ([M – H]$^-$ at *m*/*z* 1153.2; identified by spiking samples for analysis with authentic sample) was accumulated. The half-lives for the disappearance of **2** and **I**^{2a} were 6.2 h ($k = 3.09 \times 10^{-5}$ s^{-1}) and 7.2 h ($k = 2.66 \times 10^{-5}$ s^{-1}), respectively. The amount of side products was somewhat more marked than with **1**, and included TpT (*m*/*z* at 545.1) and Tp-$CH_2C(Me_2)C(O)CH_2SAc$ (*m*/*z* at 493.1), but no attempt was made to characterize all of them.

Because it had been shown earlier[24] that 2′,3′-*O*-isopropylideneuridine 5′-(methyl, 4-benzoylthio-2,2-dimethyl-3-oxobutyl) phosphate and 2′,3′-*O*-isopropylideneuridine

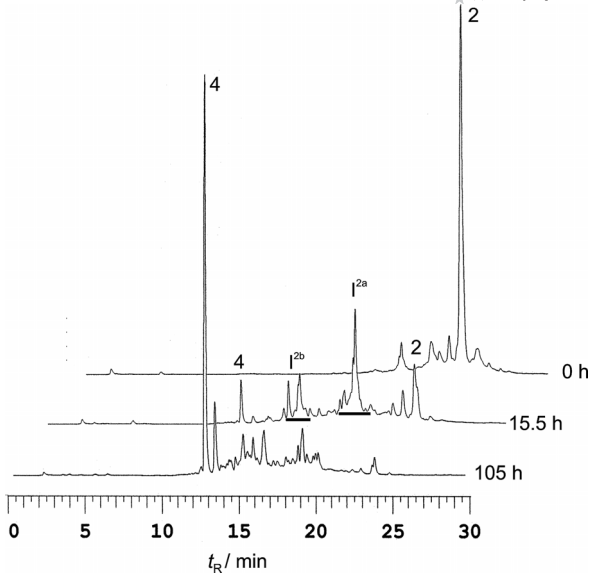


Figure 3. RP-HPLC traces for the non-enzymatic hydrolysis of protected oligomeric phosphotriester **2** to unprotected phosphodiester **4** at pH 7.5 and 37.0 °C (I = 0.1 mol L^{-1} with NaCl). For detailed chromatographic conditions, see the Experimental Section.

5′-(methyl, 2-ethoxycarbonyl-2-methyl-3-oxo-4-pivaloylthiobutyl) phosphate underwent, as a minor side-reaction, hydrolytic cleavage of the P–OMe bond, the possible occurrence of P–O3′ and/or P–O5′ cleavage was studied by using 4-acetylthio-2,2-dimethyl-3-oxobutyl 5′-*O*-pivaloylthymidin-3′-yl thymidin-5′-yl phosphate (**17**) as a model compound. The time-dependent product mixture at pH 7.5 and 37 °C (Figure 4) revealed that although 5′-*O*-pivaloylthymidylyl-3′,5′-thymidine (Piv-TpT) was the predominant product, both thymidine (t_R 11.9 min; 9%) and 5′-*O*-

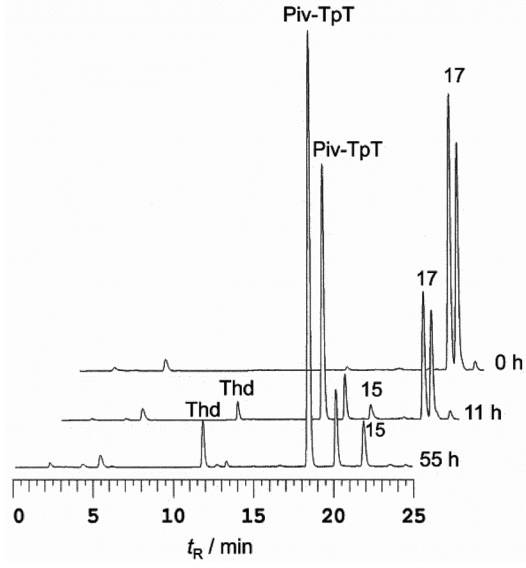


Figure 4. RP-HPLC traces for the nonenzymatic hydrolysis of (4-acetylthio-2,2-dimethyl-3-oxobutyl)-protected dimer **17** to the unprotected phosphodiester (Piv-TpT) at pH 7.5 and 37.0 °C (I = 0.1 mol L^{-1} with NaCl). For detailed chromatographic conditions, see the Experimental Section.

pivaloylthymidine (t_R 21.8 min; 9%) were formed. Accordingly, 5′-(4-acetylthio-2,2-dimethyl-3-oxobutyl)phosphate and 5′-*O*-pivaloylthymidine 3′-(4-acetylthio-2,2-dimethyl-3-oxobutyl)phosphate should also have been formed. The signal at 20.2 min possibly refers to these products, but this could not be verified by MS. The half-life for the disappearance of the faster- and slower-eluting diastereomers of **17** was 9.9 and 10.2 h, respectively.

Although chain cleavage with dimer **17** is rather marked, analysis of the HPLC-traces in Figures 2 and 4 reveals that the extent of this side reaction is not much increased with oligomer **1** that contains three phosphotriester groups. It should be also noted that when the first, and possibly to some extent the second, protecting group is removed enzymatically, as discussed below, deprotection does not suffer from competition by chain cleavage. Only when the enzymatic deprotection becomes so severely retarded that the groups are removed non-enzymatically does competing chain cleavage start to play a role.

Enzymatic Deprotection of Oligomeric Phosphotriesters 1 and 2

Removal of 4-acetylthio-2,2-dimethyl-3-oxobutyl groups from oligomeric phosphotriesters **1** and **2** was also followed in the presence of hog liver carboxyesterase (2.6 units/mL) in HEPES buffer at pH 7.5 and at 37 °C. Under these conditions, starting material **1** underwent deprotection to desired phosphodiester **3** in 2 d. The half-life for the disappearance of **1** was 2.7 min. The enzyme-triggered deprotection to mono-deprotected products $\mathbf{I^{1a}}$ most likely proceeds through deacetylated intermediates,[24] which were not accumulated. Consistent with the initial deacetylation, an interchain disulfide bond formation competed as a side reaction with the chemical removal of the remnants of the protecting group. The S–S bond mediated dimerization reaction was verified by both mass analysis and HPLC (t_R = 28.0–29.0; $[M - H]^-$ signal at *m*/*z* 1457.4). Loss of the second protecting group resulted in the formation of di-deprotected intermediates $\mathbf{I^{1b}}$. Also at this stage, dimerization through disulfide bond formation between two deacetylated protecting groups were detected (t_R = 23.0–24.0, $[M - H]^-$; at *m*/*z* 1285.3). Departure of the third protecting group then gave desired phosphodiester **3** (Figure 5). As minor side products, TpTpMe (t_R = 11.4–11.7 min; $[M - H]^-$ at *m*/*z* 655.1), pTpTpMe (t_R = 11.4–11.7 min; $[M - H]^-$ at *m*/*z* 719.1) and Piv-TpT (t_R = 16.0 min; $[M - H]^-$ at *m*/*z* 629.2) were observed.

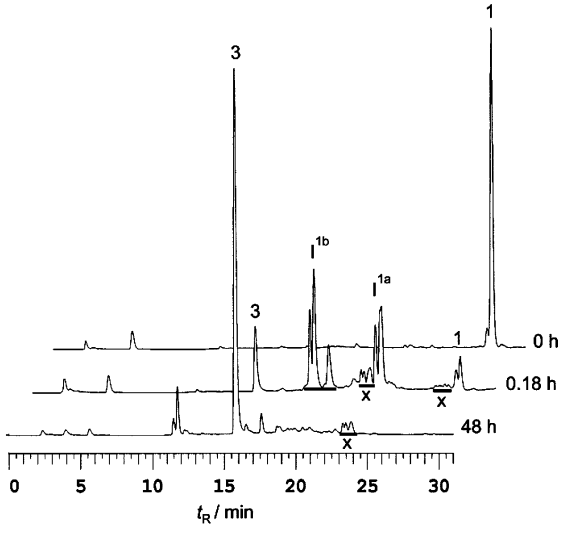


Figure 5. RP-HPLC traces for the enzymatic hydrolysis of protected oligomeric phosphotriester **1** to unprotected phosphodiester **3** at 37.0 °C in HEPES buffer (pH 7.5) that contained HLE 2.6 units/mL (I = 0.1 mol L^{-1} with NaCl). For detailed chromatographic conditions, see the Experimental Section. Signals marked with **x** refer to disulfide products.

The conversion of **2** to **4** via $\mathbf{I^{2a}}$ and $\mathbf{I^{2b}}$ takes place as with **1**, but somewhat more slowly. The half-life for the disappearance of **2** was 36 min, and deprotection to **4** was not complete even after 5 d. In addition to the disulfide products at t_R = 19.0–19.4 and 23.0–23.8 min, a considerable amount of an unknown product at t_R = 12.8 was formed (Figure 6).

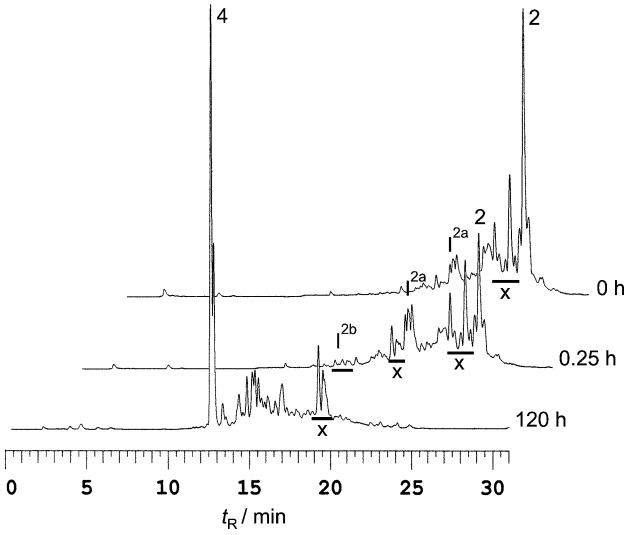
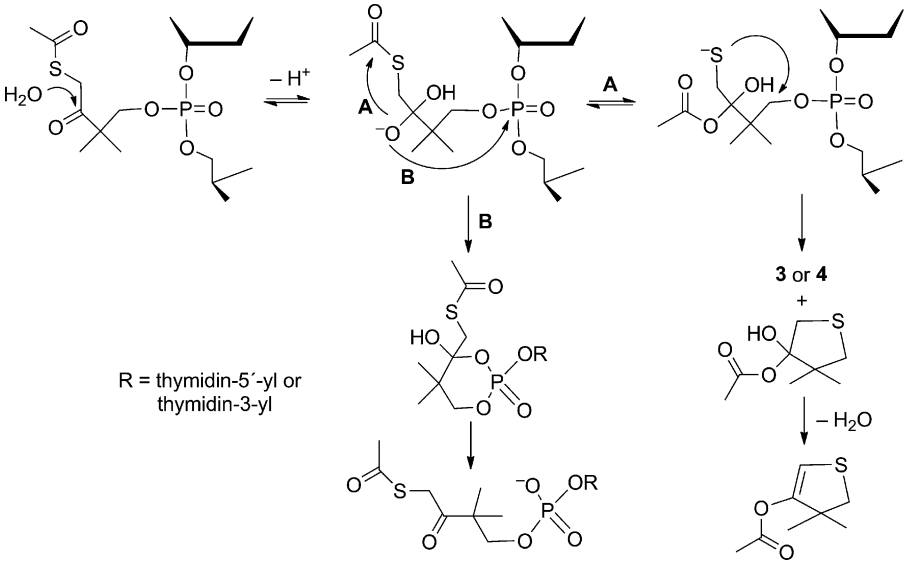


Figure 6. RP-HPLC traces for the enzymatic hydrolysis of protected oligomeric phosphotriester **2** to unprotected phosphodiester **4** at 37.0 °C in HEPES buffer (pH 7.5) that contained HLE 2.6 units/mL (I = 0.1 mol L^{-1} with NaCl). For detailed chromatographic conditions, see the Experimental Section. Signals marked with **x** refer to disulfide products.

In case of dimer **17**, 5′-*O*-pivaloylthymidylyl-3′,5′-thymidine accumulated quantitatively. The rate constants for the enzymatic deacetylation of the slower and faster eluting diastereomers of **17** were 9.52×10^{-3} s^{-1} ($\tau_{1/2}$ = 1.21 min) and 6.71×10^{-3} s^{-1} $\tau_{1/2}$ = 1.72 min, respectively.

Mechanisms

The non-enzymatic departure of the protecting group from the internucleosidic oligomeric phosphodiester linkage in all likelihood takes place as described earlier for the protected nucleoside 5′-methylphosphates.[24] Accordingly, the deprotection involves an acetyl migration from the sulfur



Scheme 7. Mechanism of the non-enzymatic removal of the protecting group (A) and the cleavage of P–O bond (B).

atom to the geminal diol formed by hydration of the 3-oxo group, and subsequent attack of the exposed mercapto group on C1 (Route A in Scheme 7). The harmful cleavage of the internucleosidic P–O3′ and P–O5′ bonds, which occurs as a minor side reaction, proceeds by intramolecular attack of an oxyanion of the hydrated keto group at the phosphorus atom (Route B). The enzymatic removal of the protecting group is, in turn, initiated by deacetylation and followed by attack of the mercapto function on C1 carbon and results in release of the phosphodiester.[24]

To put the results of the present study in perspective, they can be analyzed relative to those obtained previously with a 2–5A trimer, pApApA, which bears four esterase labile phosphate protecting groups, viz. 3-acetyloxy-2,2-bis-(ethoxycarbonyl)propyl groups.[17] Conversion to a tri-anionic diester form (the 5′-terminal phosphate still bears one protecting group) was not complete after two weeks under the conditions of the present study (HLE 2.6 units/mL in HEPES buffer at pH 7.5 and 37 °C). The deprotection of the oligothymidylates used as model compounds in the present study is much faster, but still the thermolability of the 4-acetylthio-2,2-dimethyl-3-oxobutyl group appears to be too low to allow sufficiently fast deprotection of longer oligonucletotides. Fortunately, the lability can be markedly increased by increasing the electronegativity of the 2-substituents.[24] Replacement of one of the 2-methyl substituents with an ethoxycarbonyl group, for example, accelerates the non-enzymatic cleavage reaction by a factor of 20 and reduces the amount of side reactions that result from attack on phosphorus.

Conclusions

The feasibility of 4-acetylthio-2,2-dimethyl-3-oxopropyl group, shown to be both esterase- and thermo-labile,[24] as a phosphate protecting group of short oligonucleotides has been studied. Such a group should be removable by the use of esterases, but when the enzymatic removal becomes retarded by accumulation of negative charge on the oligomeric substrate, the thermolability guarantees removal of the remaining protecting groups. Thymidine-derived trimer **1** and tetramer **2** that bear three such protecting groups have been shown to undergo conversion to fully deprotected phosphodiesters, with some degradation of the phosphate backbone as a side reaction. Accumulation of the negative charge did not markedly retard the non-enzymatic removal of the protecting groups. Unfortunately, the non-enzymatic removal still seems to be too slow to allow efficient deprotection of longer oligonucleotides. Earlier studies with nucleoside 5′-methylphosphates have, however, shown that the thermal stability of 2,2-disubstituted 4-acylthio-3-oxobutyl groups may be considerably decreased by increasing the electronegativity of the C2 substituents.

Experimental Section

General: The NMR spectra (^{1}H, ^{13}C, ^{31}P, DQF-COSY and HSQC) were recorded with a Bruker Avance 500 or 600 spectrometer. The chemical shifts are referenced to tetramethylsilane. 2D NMR spectra were used for peak assignment. The multiplicity of some signals is a result of R_P- and S_P-diastereoisomerism of the compounds. MS analyses were carried out with a Bruker Daltonics microTOF-Q instrument. A Phenomenex Gemini C18 column (2.0 × 150 mm, 5 μm) was used for HPLC/ESI-MS analyses. The HPLC measurements were performed with a Merck Hitachi LaChrom D7000 instrument equipped with a L-7455 UV-detector (λ = 267 nm), L-7100 pump and ODS Hypersil column (4 × 260 mm, 5 μm). Pyridine, MeCN, CH_2Cl_2, tetrahydrofuran (THF) and EtOAc were dried with 4 Å molecular sieves. Triethylamine (TEA) was dried by heating to reflux over CaH_2 and distilled before use. Tetramer T_4 (**4**), which was used as a reference material, was prepared with an Applied Biosystems 392 DNA/RNA synthesizer following a phosphoramidite protocol. 5′-*O*-(4-methoxytrityl)thymidine[25] (**5**), 3′-*O*-(*tert*-butyldimethylsilyl)thymidine (**6**)[26] and *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate[24] (**7**) were synthesized as described previously. The synthesis of **6** was somewhat modified from

the original by using pyridine as solvent and 4-monomethoxytrityl (MMTr) as transient protection in 5′-OH. 5′-*O*-MMTr-protection was removed in 80% aq. AcOH. The ^{1}H and ^{13}C NMR spectra of the compounds were identical with those reported earlier (**5**,[27] **6**,[28,29] and **7**[24]).

4-Acetylthio-2,2-dimethyl-3-oxobutyl 3′-*O*-(*tert*-Butyldimethylsilyl)-thymidin-5′-yl 5′-*O*-(4-Methoxytrityl)thymidin-3′-yl Phosphate (8): Compound **5** (2.29 mmol, 1.18 g) was dried with P_2O_5 overnight and dissolved in CH_2Cl_2 (5 mL) under nitrogen. TEA (11.40 mmol, 1.60 mL) and bis(diisopropylamino)chlorophosphite (2.52 mmol, 0.678 g) were added. The course of the reaction was followed by ^{31}P NMR spectroscopy (202 MHz, CD_3CN) by taking samples from the reaction mixture. The phosphitylation step was completed in 15 min. The product resonated at δ = 115.8 ppm. The reaction mixture was filtered through a small silica gel column by eluting with EtOAc that contained 30% hexane and 0.5% TEA. The fractions that contained product were combined and the solvents evaporated to dryness under reduced pressure and the residue was co-evaporated twice from dry MeCN. The residue was dissolved in MeCN (5 mL) under nitrogen. 1*H*-tetrazole (1.70 mmol, 3.77 mL, 0.45 M solution in MeCN) and compound **6** (1.70 mmol, 0.60 g) were added. After 30 min, ^{31}P NMR signals at δ = 148.4 and 148.6 ppm were observed. Compound **7** (3.34 mmol, 0.635 g) and 1*H*-tetrazole (2.54 mmol, 5.65 mL of 0.45 M solution in MeCN) were added and the mixture was stirred for another 70 min. ^{31}P NMR signals at δ = 139.4 and 139.8 ppm were observed. I_2 (1.70 mmol, 0.42 g) in a mixture of THF, H_2O and 2,6-lutidine (v/v/v, 4:2:1, 10 mL) was added to the reaction mixture and the oxidation was left to proceed overnight. The oxidized products resonated at δ = –2.5 and –2.6 ppm. The reaction mixture was evaporated to dryness and the residue was equilibrated between CH_2Cl_2 and aq. 5% $NaHSO_3$. The organic phase was separated, dried with Na_2SO_4 and the solvents evaporated to dryness. The crude product was purified by silica gel chromatography by eluting with EtOAc that contained 30% hexane to yield **8** (1.39 g, 54%) as a white solid foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.38 (br. s, 2 H, NH); 7.56 and 7.55 (d, *J* = 8.65 Hz, 1 H, H6); 7.40–7.25 (m, 13 H, MMTr and H6); 6.86 (d, *J* = 8.80 Hz, 2 H, MMTr); 6.46–6.43 (m, 1 H, H1′); 6.27–6.22 (m, 1 H, H1′); 5.23–5.20 (m, 1 H, H3′); 4.43–4.38 (m, 1 H, H3′); 4.28 (br. s 1 H, H4′); 4.28–3.88 (m, 7 H, OCH_2, SCH_2, H4′, H5′, H5′′); 3.80 (s, 3 H, OMe); 3.53–3.47 (m, 1 H, H5′); 3.43–3.40 (m, 1 H, H5′′); 2.63–2.61 (m, 1 H, H2′); 2.45–43 (m, 1 H, H2′′); 2.32 and 2.30 (s, 3 H, AcS); 2.28–2.14 (m, 2 H, H2′, H2′′); 1.91, 1.90 and 1.39 (s, 6 H, 2 × CH_3 of Thy); 1.29–1.27 (m, 6 H, 2 × CH_3); 0.90 and 0.89 (s, 9 H, Me_3CSi); 0.10–0.06 (m, 6 H, Me_2Si) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.03 and 204.99 (C=O); 194.32 and 194.25 (C=O of AcS); 163.91, 163.86, 163.80 and 163.79 (C4); 158.91 (MMTr); 150.54, 150.52, 150.37 and 150.36 (C2); 143.61, 143.57 and 143.49 (MMTr), 135.82, 135.67, 135.22 and 135.14 (C6); 134.48, 130.43, 128.40, 128.39, 128.38, 128.06, 127.36 and 113.38 (MMTr); 111.76, 111.71, 111.30 and 111.20 (C5); 87.56 (spiro C of MMTr); 85.58 (C1′); 85.25 (C4′); 84.90, 84.87 and 84.85 and 84.80 (C1′); 84.59 and 84.54 (C4′); 84.26 (C1′); 79.60, 79.52, 79.40 and 79.36 (C3′); 73.14, 73.12, 73.10 and 73.08 (OCH_2); 71.44 (C3′); 67.11, 67.10, 67.24, 66.90 and 66.89 (C5′); 55.27 (OCH_3); 48.62, 48.58 and 48.56 (spiro C); 40.46, 40.39, 39.03, 39.02 and 39.00 (C2′); 36.28 and 36.25 (SCH_2); 30.14 (CH_3 of Ac); 25.71 and 25.69 (Me_3CSi); 23.67, 23.65, 22.63, 22.62, 21.59, 21.57, 21.53 and 21.49 (CH_3); 17.91 and 17.90 (Me_3CSi); 14.20, 12.49, 12.45 and 11.66 (CH_3 of Thy); –4.65 and –4.83 (Me_2Si) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –2.20, and –2.32 ppm. HRMS (ESI^+): *m*/*z* calcd. for $C_{54}H_{68}N_4NaO_{15}PSSi$ [M + Na]$^+$ 1127.3879; found 1127.3836.

5′-*O*-(4-Methoxytrityl)thymidin-3′-yl Thymidin-5′-yl 4-Acetylthio-2,2-dimethyl-3-oxobutyl Phosphate (9): Compound **8** (0.40 mmol, 0.44 g) was dissolved in a mixture of THF (7.6 mL) and acetic acid (0.4 mL) and tetrabutylammoniumfluoride hydrate (TBAF; 0.86 mmol, 0.22 g) was added. The reaction mixture was stirred at room temp. for 4 d. The reaction mixture was evaporated to dryness and the residue was equilibrated between satd. aq. $NaHCO_3$ and CH_2Cl_2. The organic phase was washed with brine, separated, dried with Na_2SO_4 and the solvents evaporated to dryness. The crude product was purified by silica gel chromatography by eluting with CH_2Cl_2 that contained 5% MeOH to yield **9** (0.29 g, 74%) as a white solid foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.13, 9.33, 9.84, 9.79 (s, 2 H, 2 × NH); 7.55 and 7.54 (s, 1 H, H6); 7.39–7.23 (m, 13 H, MMTr and H6); 6.85 (2 × d, *J* = 9.00 and 8.95 Hz, 2 H, MMTr); 6.42–6.38 (m, 1 H, H1′); 6.27 (t, *J* = 6.50 Hz, 1 H, H1′); 5.18 (br. s, 1 H, H3′); 4.53–4.42 (m, 1 H, H3′); 4.31–3.99 (m, 6 H, 2 × H4′, H5′, H5′′, OCH_2); 3.93,3.92 and 3.88 (s, 2 H, SCH_2); 3.80 and 3.79 (s, 3 H, OMe); 3.51–3.40 (m, 2 H, H5′ and H5′′); 2.72–2.62 (m, 1 H, H2′); 2.47–2.34 (m, 2 H, H2′ and H2′′); 2.30 and 2.27 (s, 3 H, AcS); 2.24–2.15 (m, 1 H, H2′′); 1.88 and 1.88 (s, 3 H, CH_3 of Thy); 1.39 (s, 3 H, CH_3 of Thy); 1.27, 1.25, 1.22 and 1.22 (s, 6 H, 2 × CH_3) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.41 and 205.38 (C=O); 194.61 and 194.57 (C=O of AcS); 164.17, 164.12 and 163.98 (C4); 158.91 and 158.89 (MMTr); 151.05, 150.94 and 150.64 (C2); 143.62, 143.56 and 143.54 (MMTr), 135.85, 135.81, 135.28 and 135.00 (C6); 134.50, 134.47, 130.45, 128.11, 127.43 and 113.40 (MMTr); 111.98, 111.85, 111.34 and 111.21 (C5); 87.61 and 87.54 (spiro C of MMTr); 85.33 and 85.25 (C1′); 84.48, 84.47 and 84.45 (C1′and C4′); 79.89, 79.88, 79.59 and 79.58 (C3′); 73.21 and 73.16 (OCH_2); 70.83 and 70.79 (C3′); 67.55, 67.54, 67.24, 67.23, 63.60 and 63.51 (C5′); 55.30 (OCH_3); 48.68, 48.62 and 48.56 (spiro C); 39.81 and 39.74 (C2′); 36.28 (SCH_2); 30.16 (CH_3 of Ac); 21.62, 21.61, 21.60 and 21.50 (CH_3); 12.51, 11.74 and 11.73 (CH_3 of Thy) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –2.43 and –2.63 ppm. HRMS (ESI^+): *m*/*z* calcd. for $C_{48}H_{55}N_4NaO_{15}PS$ [M + Na]$^+$ 1013.3014; found 1013.3024.

4-Acetylthio-2,2-dimethyl-3-oxobutyl 3′-*O*-(*tert*-Butyldimethylsilyl)-thymidin-5′-yl Thymidine-3′-yl Phosphate (10): Compound **8** (1.00 mmol, 1.11 g) was dissolved in aq. 80% AcOH (50 mL) and the reaction mixture was stirred at room temp. overnight. The reaction mixture was evaporated to dryness and the residue was coevaporated from H_2O. The residue was equilibrated between satd. aq. $NaHCO_3$ and CH_2Cl_2. The organic phase was washed with brine, dried with Na_2SO_4 and the solvents evaporated to dryness. The crude product was purified by silica gel chromatography by eluting with CH_2Cl_2 that contained 5% MeOH to yield **10** (0.24 g, 29%) as a white solid foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.97, 9.82, 9.80, 9.78 (s, 2 H, 2 × NH); 7.55 and 7.54 (s, 1 H, H6); 7.36 and 7.34 (s, 1 H, H6); 6.28–6.19 (m, 2 H, 2 × H1′); 5.14–5.12 (m, 1 H, H3′); 4.43–4.39 (m, 1 H, H3′); 4.26–4.07 (m, 5 H, H4′, H5′, H5′′, OCH_2); 4.03–4.00 (m, 1 H, H4′); 3.97 and 3.94 (s, 2 H, SCH_2); 3.88–3.79 (m, 2 H, H5′ and H5′′); 3.73–3.67 (m, 1 H, 5′-OH); 2.51–2.39 (m, 2 H, 2 × H2′′); 2.36 and 2.35 (s, 3 H, AcS); 2.29–2.20 (m, 2 H, 2 × H2′′); 1.92, 1.91, 1.89 and 1.87 (s, 6 H, 2 × CH_3 of Thy); 1.29, 1.28, 1.28 and 1.27 (s, 6 H, 2 × CH_3); 0.88 (s, 9 H, Me_3CSi); 0.09 (s, 6 H, Me_2Si) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.36 and 205.25 (C=O); 194.65 and 194.57 (C=O of AcS); 164.36, 164.23, 164.21 and 164.17 (C4); 150.64, 150.59 and 150.49 (C2); 136.57, 136.51, 136.12 and 136.07 (C6); 111.39, 111.23 and 111.17 (C5); 85.84, 85.76, 85.72, 85.66, 85.61 and 85.41 (C1′ and C4′); 84.91, 84.85, 84.83 and 84.77 (C4′); 78.74, 78.70, 78.54 and 78.50 (C3′); 73.14 and 73.09 (OCH_2); 71.42 and 71.30 (C3′); 67.11 and 67.05 (C5′); 61.96 and 61.89 (C5′); 48.69, 48.66, 48.62 and

48.59 (spiro C); 40.28 and 40.19 (C2′); 38.54, 38.50 and 38.46 (C2′); 36.32 and 36.24 (SCH_2); 30.19 and 30.18 (CH_3 of Ac); 25.69 and 25.65 (Me_3CSi); 21.60, 21.57, 21.55 and 21.53 (CH_3); 17.89 (Me_3CSi); 12.53, 12.52, 12.47 and 12.42 (CH_3 of Thy); –4.67, –4.84 and –4.85 (Me_2Si) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –2.10, and –2.16 ppm. HRMS (ESI^-): *m*/*z* calcd. for $C_{34}H_{53}N_4NaO_{14}PSSi$ $[M - H]^-$ 831.2713; found 831.2746.

4-Acetylthio-2,2-dimethyl-3-oxobutyl 5′-*O*-(4-Methoxytrityl)thymidin-3′-yl Methyl Phosphate (11): Compound **5** (2.66 mmol, 1.37 g) was dried with P_2O_5 overnight. It was dissolved in CH_2Cl_2 (6 mL) and TEA (13.31 mmol, 1.87 mL) and *N*,*N*-diisopropylmethylphosphonamidic chloride (2.69 mmol, 0.52 mL) were added under nitrogen. After 30 min stirring at room temp. the reaction mixture was filtered through a short silica gel column by eluting with EtOAc that contained 40% hexane and 0.5% TEA. The fractions that contained product were combined and the solvents evaporated to dryness under reduced pressure and the residue was coevaporated twice from dry MeCN. The obtained phosphoramidite was dissolved in MeCN (5 mL) under nitrogen and compound **7** (3.78 mmol, 0.72 g) and 1*H*-tetrazole (3.96 mmol, 8.80 mL of 0.45 M solution in MeCN) were added. The mixture was stirred for 2 h. I_2 (2.60 mmol, 0.66 g) in a mixture of THF, H_2O and 2,6-lutidine (v/v/v, 4:2:1, 17.5 mL) was added and the oxidation was left to proceed overnight. Aq. 5% $NaHSO_3$ was added to the reaction mixture and the product was extracted to CH_2Cl_2. The organic fractions were combined, washed with brine, dried with Na_2SO_4 and the solvents evaporated to dryness under reduced pressure. The crude product was purified by silica gel chromatography by eluting first with EtOAc that contained 40% hexane and then with CH_2Cl_2 that contained 5% MeOH to yield **11** (1.38 g, 66%) as a yellowish solid. ^1H NMR (500 MHz, $CDCl_3$): δ = 9.20 and 9.17 (s, 1 H, NH); 7.57–7.55 (m, 1 H, H6); 7.41–7.37 (m, 4 H, MMTr); 7.32–7.23 (m, 8 H, MMTr); 6.86–6.83 (m, 2 H, MMTr); 6.47–6.44 (m, 1 H, H1′); 5.18–5.14 (m, 1 H, H3′); 4.28–4.25 (m, 1 H, H4′); 4.15–4.00 (m, 2 H, OCH_2); 3.96, 3.95 and 3.90 (s, 2 H, SCH_2); 3.80 (s, 3 H, OMe of MMTr); 3.76, 3.74, 3.71 and 3.68 (d, *J* = 11.2, and *J* = 11.3 Hz, 3 H, OMe); 3.53–3.48 (m, 1 H, H5′); 3.42–3.39 (m, 1 H, H5′′); 2.64–2.60 (m, 1 H, H2′); 2.45–2.40 (m, 1 H, H2′′); 2.32 and 2.31 (s, 3 H, AcS); 1.40 (s, 3 H, CH_3 of Thy); 1.29, 1.28, 1.25 and 1.23 (s, 6 H, 2 × CH_3) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.28 and 205.18 (C=O); 194.36 and 194.31 (C=O of AcS); 163.78 and 163.75 (C4); 158.89 (MMTr); 150.47 and 150.45 (C2); 143.64, 143.62, 143.58 and 143.55 (MMTr); 135.28, 134.57 and 134.55 (C6); 130.44, 130.43 and 130.41 (MMTr); 128.42, 128.38, 128.08, 128.02, 127.40 and 113.35 (MMTr); 111.65 and 111.63 (C5); 87.49 and 87.47 (spiro C of MMTr); 84.59, 84.54, 84.49, 84.28 and 84.22 (C1′ and C4′); 78.86, 78.82, 78.60 and 78.56 (C3′); 73.08, 73.03, 72.98 and 72.93 (OCH_2); 63.49 and 63.38 (C5′); 55.28 and 55.27 (OCH_3 of MMTr); 54.73, 54.68 and 54.62 (OCH_3); 48.69, 48.66, 48.63 and 48.60 (spiro C); 39.14, 39.11, 39.08 and 39.05 (C2′); 36.41 and 36.31 (SCH_2); 30.18 and 30.15 (CH_3 of Ac); 21.64, 21.60, 21.58 and 21.56 (CH_3); 11.69 (CH_3 of Thy) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –1.08 and –1.23 ppm. HRMS (ESI^-): *m*/*z* calcd. for $C_{39}H_{44}N_2O_{11}PS$ $[M - H]^-$ 779.2409; found 779.2414.

4-Acetylthio-2,2-dimethyl-3-oxobutyl Thymidin-3′-yl Methyl Phosphate (12): Compound **11** (1.75 mmol, 1.37 g) was dissolved in aq. 80% AcOH (30 mL) and the reaction mixture was stirred at room temp. for 20 h. The reaction mixture was evaporated to dryness and coevaporated from H_2O. The crude product was purified by silica gel chromatography by eluting with CH_2Cl_2 that contained 5% MeOH to yield **12** (0.65 g, 73%) as a white solid. ^1H NMR (500 MHz, $CDCl_3$): δ = 9.66 and 9.65 (s, 1 H, NH); 7.58–7.57 (m, 1 H, H6); 6.27–6.24 (m, 1 H, H1′); 5.14–5.09 (m, 1 H, H3′); 4.21–4.19 (m, 1 H, H4′); 4.16–4.07 (m, 2 H, OCH_2); 3.97 (s, 2 H, SCH_2); 3.81 and 3.79 (d, *J* = 1.05 Hz and *J* = 0.95 s, 3 H, OMe); 3.88–3.87 (m, 2 H, H5′ and H5′′); 2.55–2.48 (m, 1 H, H2′); 2.46–2.40 (m, 1 H, H2′′); 2.38 and 2.37 (s, 3 H, AcS); 1.91 (s, 3 H, CH_3 of Thy); 1.30 (s, 6 H, 2 × CH_3) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.49 and 205.46 (C=O); 194.68 and 194.67 (C=O of AcS); 164.14 (C4); 150.60 (C2); 136.59 and 136.50 (C6); 111.23 (C5); 85.79, 85.75, 85.72, 85.70 and 85.68 (C1′ and C4′); 78.13, 78.08, 78.04 and 78.00 (C3′); 73.06 and 73.02 (OCH_2); 61.93 and 61.89 (C5′); 54.81 and 54.76 (OCH_3); 48.70 and 48.63 (spiro C); 38.62, 38.59, 38.51 and 38.47 (C2′); 36.34 (SCH_2); 30.19 (CH_3 of Ac); 21.61, 21.58, 21.57 and 21.56 (CH_3); 12.55 (CH_3 of Thy) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –1.08 and –1.13 ppm. HRMS (ESI^-): *m*/*z* calcd. for $C_{19}H_{28}N_2O_{10}PS$ $[M - H]^-$ 507.1208; found 507.1175.

(4-Acetylthio-2,2-dimethyl-3-oxobutyl)-Protected 5′-*O*-(4-Methoxytrityl)-thymidylyl-(3′,5′)thymidine 3′-[4-Acetylthio-2,2-dimethyl-3-oxobutyl,methyl] Phosphate (13): Compound **5** (0.91 g, 1.77 mmol) was dried with P_2O_5 overnight and dissolved in CH_2Cl_2 (5 mL). TEA (1.24 mL, 8.82 mmol) and bis(diisopropylamino) chlorophosphite (0.45 g, 1.67 mmol) were added under nitrogen. The phosphitylation step was complete in 30 min, with a product peak that occurred at δ = 115.64 ppm. The reaction mixture was filtered through a short, silica gel column by eluting with EtOAc that contained 60% hexane and 0.5% TEA. The fractions that contained product were combined and the solvents evaporated to dryness under reduced pressure and the residue was coevaporated twice from dry MeCN. The obtained phosphoramidite was dissolved in MeCN (6 mL) under nitrogen and compound **12** (1.23 mmol, 0.63 g) and 1*H*-tetrazole (1.23 mmol, 2.74 mL of 0.45 M solution in MeCN) were added. After 1.5 h, ^{31}P NMR signals (202 MHz, CD_3CN) 149.07, 148.99 and 148.32 ppm were observed. Compound **7** (2.86 mmol, 0.55 g) was dissolved in MeCN (1 mL) and added to the reaction mixture with 1*H*-tetrazole (3.18 mmol, 6.36 mL of 0.45 M solution in MeCN). After 1.5 h, the formation of the phosphitylated product was accompanied by appearance of ^{31}P NMR resonance at 139.72, 139.63, 139.54, 139.47, –1.30, –1.32, –1.38 and –1.41 ppm. I_2 (2.13 mmol, 0.54 g) in a mixture of THF, H_2O and 2,6-lutidine (v/v/v, 4:2:1, 14 mL) was added to the reaction mixture and the oxidation was left to proceed overnight. Aq. 5% $NaHSO_3$ was added to the reaction mixture and the product was extracted to CH_2Cl_2. The organic fractions were combined, washed with brine, dried with Na_2SO_4 and the solvents evaporated to dryness under reduced pressure. The crude product was purified by silica gel chromatography by eluting first with EtOAc that contained 60% hexane and then with CH_2Cl_2 that contained 5% MeOH to yield **13** (0.99 g, 0.64%) as a yellowish solid. ^1H NMR (500 MHz, $CDCl_3$): δ = 9.45, 9.43, 9.35 and 9.27 (s, 2 H, 2 × NH); 7.56 and 7.53 (s, 1 H, H6); 7.40–7.25 (m, 13 H, MMTr and H6); 6.85 (d, *J* = 8.80 Hz, 2 H, MMTr); 6.44–6.41 (m, 1 H, H1′); 6.31–6.28 (m, 1 H, H1′); 5.24–5.18 (m, 1 H, H3′); 5.04–4.98 (m, 1 H, H3′); 4.32–3.89 (m, 12 H, 2 × H4′, H5′, H5′′, 2 × OCH_2 and 2 × SCH_2); 3.80 (s, 3 H, OCH_3 of MMTr); 3.79–3.77 (m, 3 H, OCH_3); 3.50–3.40 (m, 2 H, H5′and H5′′); 2.64–2.33 (m, 4 H, 2 × H2′and 2 × H2′′); 2.36 and 2.35 (s, 3 H, AcS); 2.31 and 2.30 (s, 3 H, AcS); 1.90 (s, 3 H, CH_3 of Thy); 1.38 (s, 3 H, CH_3 of Thy); 1.31–1.22 (m, 12 H, 4 × CH_3) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.37, 205.10, 205.07 and 205.06 (C=O); 194.43, 194.40, 194.39 and 194.30 (C=O of AcS); 163.75 (C4); 158.91 (MMTr), 150.50, 150.42 and 150.40 (C2); 143.57 and 143.51 (MMTr); 134.50 (C6); 130.44, 128.42, 128.38, 128.09, 127.42 and 113.38 (MMTr); 111.70, 111.68 and 111.59 (C5); 87.55 (spiro C of MMTr); 85.19 and 84.24 (C1′); 84.47 and 83.05, 83.03 and 83.00 (C4′); 79.69, 79.66, 76.93 and 76.91 (C3′); 73.17, 73.13, 73.08, 73.06, 63.56,

63.54, 63.53 and 63.52 (OCH_2 and C5′); 55.28 (OCH_3 of MMTr); 54.83, 54.79 and 54.77 (OCH_3); 48.71, 48.69, 48.64, 48.63 and 48.58 (spiro C); 38.98, 38.97, 38.95 and 38.94 (C2′); 38.19, 38.16, 38.10 and 38.08 (C2′); 36.34, 36.32, 36.27 and 36.23 (SCH_2); 30.19 and 30.13 (CH_3 of Ac); 21.59, 21.57, 21.49 and 21.05 (CH_3); 12.47, 12.44, 11.66 and 11.64 (CH_3 of Thy) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –1.02, –1.05, –1.10, –1.12, –2.42, –2.45, –2.51 and –2.55 ppm. HRMS (ESI$^-$): *m*/*z* calcd. for $C_{57}H_{69}N_4O_{20}P_2S_2$ [M – H]$^-$ 1255.3427; found 1255.3457.

(4-Acetylthio-2,2-dimethyl-3-oxobutyl)-Protected Thymidylyl-(3′,5′)-thymidine 3′-[4-Acetylthio-2,2-dimethyl-3-oxobutyl,methyl] Phosphate (14): Compound **13** (0.79 mmol, 0.99 g) was dissolved in aq. 80% AcOH (22 mL) and the reaction mixture was stirred at room temp. overnight. The reaction mixture was evaporated to dryness and the residue was coevaporated from H_2O. The crude product was purified by silica gel chromatography by eluting with CH_2Cl_2 that contained 5% MeOH to yield **14** (0.48 g, 63%) as a white solid foam. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.65, 9.63, 9.49, 9.47 and 9.39 (br. s, 2 H, 2 × NH); 7.56 and 7.54 (s, 1 H, H6); 7.34–7.33 (m, 1 H, H6); 6.32–6.21 (m, 2 H, 2 × H1′); 5.17–5.05 (m, 2 H, 2 × H3′); 4.32–4.04 (m, 8 H, 2 × H4′, H5′, H5′′, 2 × OCH_2); 3.96, 3.96 and 3.95 (s, 4 H, 2 × SCH_2); 3.87–3.77 (m, 5 H, H5′, H5′′, OCH_3); 2.58–2.37 (m, 4 H, 2 × H2′and 2 × H2′′); 2.38, 2.36, 2.36 and 2.36 (s, 6 H, 2 × AcS); 2.31 and 2.30 (s, 3 H, AcS); 1.93, 1.92, 1.90 and 1.89 (s, 6 H, CH_3 of Thy); 1.31–1.29 (m, 12 H, 4 × CH_3) ppm. ^{13}C NMR (151 MHz, $CDCl_3$): δ = 205.43, 205.42, 205.39, 205.37 and 205.25 (C=O); 194.60, 194.55, 194.46 and 194.43 (C=O of AcS); 164.01, 163.97, 163.96 and 163.85 (C4); 150.51, 150.49, 150.46 and 150.44 (C2); 136.32, 136.29, 135.53 and 135.47 (C6); 111.75, 111. 62, 111.22 and 111.16 (C5); 85.84 and 85.80 (C4′); 85.60, 85.56, 85.52, 85.49, 85.47, 85.38, 85.20 and 85.17 (C1′); 83.01 and 82.87 (C4′); 78.78, 78.76, 78.15 and 78.13 (C3′); 73.23, 73.19 and 73.14 (OCH_2), 66.68, 66.58, 61.79 and 61.72 (C5′); 54.97, 54.95, 54.93, 54.62, 54.89 and 54.86 (OCH_3); 48.68, 48.64 and 48.62 (spiro C); 38.59, 38.57, 38.55, 38.53 and 37. 90 (C2′); 36.31, 36.29 and 36.25 (SCH_2); 30.21 and 30.19 (CH_3 of Ac); 21.60, 21.57 and 21.53 (CH_3); 12.58, 12.55, 12.50 and 12.48 (CH_3 of Thy) ppm. ^{31}P NMR (242 MHz, $CDCl_3$): δ = –1.010, –1.16, –2.44, –2.46, –2.51 and –2.57 ppm. HRMS (ESI$^-$): *m*/*z* calcd. for $C_{37}H_{53}N_4O_{19}P_2S_2$ [M – H]$^-$ 983.2226; found 983.2255.

5′-*O*-Pivaloylthymidine (15): Thymidine (2.03 g, 8.38 mmol) was coevaporated from dry pyridine and dissolved in the same solvent (20 mL). The solution was cooled on an ice bath and pivaloyl chloride (1.035 mL, 8.40 mmol) was added in portions. The reaction mixture was kept on an ice bath for 4 h and the stirring was continued at room temp. overnight. The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in EtOAc and washed with satd. aq. $NaHCO_3$, water and brine, and dried with Na_2SO_4. The crude product was purified by silica gel chromatography by eluting with 5% MeOH in CH_2Cl_2 to yield **15** (64%, 1.78 g) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.08 (s, 1 H, NH), 7.31 (d, *J* = 1.15 Hz, 1 H, H6), 6.33 (dd, *J* = 7.85 and 5.90 Hz, 1 H, H1′), 4.42–4.39 (m, 2 H, H3′ and H5′), 4.27–4.22 (m, 2 H, H4′ and H5′′), 3.97 (d, *J* = 4.60 Hz, 1 H, 3′-OH), 2.54–2.49 (m, 1 H, H2′′), 2.10–2.05 (m, 1 H, H2′′), 1.91 (s, 3 H, CH_3 of Thy) 1.23 (s, 9 H, Piv) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.47 (C=O of Piv), 164.21 (C4), 150.77 (C2), 135.10 (C6), 111.31 (C5), 85.14 (C1′), 84.68 (C4′), 71.58 (C3′), 64.08 (C5′), 40.50 (C2′), 38.88 (spiro C), 27.23 (CH_3 of Piv), 12.51 (CH_3 of Thy) ppm. HRMS (ESI$^-$): *m*/*z* calcd. for $C_{15}H_{21}N_2O_6^-$ [M – H]$^-$ 325.1405; found 325.1303.

4-Acetylthio-2,2-dimethyl-3-oxobutyl 3′-*O*-(4,4′-Dimethoxytrityl)-thymidin-5′-yl 5′-*O*-Pivaloylthymidin-3′-yl Phosphate (16): Compound **15** (0.82 mmol, 0.27 g) was dried with P_2O_5 overnight and dissolved in CH_2Cl_2 (2 mL) under nitrogen. Bis(diisopropylamino) chlorophosphine (0.93 mmol, 0.25 g) and TEA (4.13 mmol, 0.58 mL) were added and the reaction mixture was stirred for 40 min. The product exhibited ^{31}P NMR signal (202 MHz, CD_3CN) at δ = 116.08 ppm. The reaction mixture was filtered through a short silica gel column by eluting with EtOAc that contained 40% hexane and 0.5% TEA. The fractions that contained product were combined and the solvents evaporated to dryness under reduced pressure and the residue was co-evaporated three times from dry MeCN. The phosphoramidite was dissolved in dry MeCN (3 mL) and 3′-*O*-(4,4′-dimethoxytrityl)thymidine (0.57 mmol, 0.31 g) and 1*H*-tetrazole (0.64 mmol, 1.42 mL of 0.45 M solution in MeCN) were added under nitrogen. After 30 min, ^{31}P NMR signals at 148.84 and 148.94 ppm were observed. Compound **7** (1.52 mmol, 0.29 g) in MeCN (1 mL) and 1*H*-tetrazole (1.55 mmol, 3.45 mL of 0.45 M solution in MeCN) were added and the mixture was stirred for 60 min. The product resonated at 140.02 and 139.70 ppm. I_2 (0.98 mmol, 0.25 g) in a mixture of THF, H_2O and 2,6-lutidine (v/v/v, 4:2:1, 7 mL) was added to the reaction mixture and the oxidation was left to proceed overnight. Aq. 5% $NaHSO_3$ was added and the product was extracted with CH_2Cl_2 (3 × 20 mL). The organic phase was separated, dried with Na_2SO_4 and the solvents evaporated to dryness. The crude product was purified by silica gel chromatography three times by eluting with CH_2Cl_2 that contained 50% EtOAc, EtOAc that contained 40% hexane and CH_2Cl_2 that contained 5% MeOH to yield **16** (0.43 g, 60%) as a yellowish solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.55, 9.50, 9.42 and 9.32 (s, 2 H, 2 × NH); 7.45–7.42 (m, 2 H, DMTr); 7.35–7.15 (m, 9 H, DMTr and H6); 6.87–6.83 (m, 4 H, DMTr); 6.38–6.33 (m, 1 H, H1′); 6.24–6.16 (m, 1 H, H1′); 4.92–4.90 (m, 1 H, H3′); 4.33–4.22 (m, 4 H, H3′, H4′, H5′ and H5′′); 4.06–3.96 (m, 3 H, H4′ and OCH_2); 3.94–3.90 (m, 3 H, H5′ and SCH_2); 3.80–3.76 (m, 6 H, 2 × OCH_3); 2.75–2.72 (m, 1 H, H5′′); 2.63–2.47 (m, 1 H, H2′); 2.35 and 2.33 (s, 3 H, AcS); 2.15–2.03 (m, 1 H, H2′′); 1.92–1.87 (m, 6 H, 2 × CH_3 of Thy); 1.84–1.82 (m, 1 H, H2′); 1.73–1.65 (m, 1 H, H2′′); 1.30–1.25 (6 H, 2 × CH_3); 1.23–1.21 (9 H, CH_3 of Piv) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.06 and 205.00 (C=O); 194.35 and 194.32 (C=O of AcS); 178.84 and 178.80 (C=O of Piv); 163.90, 163.82 and 163.78 (C4); 158.81 and 158.79 (DMTr); 150.47, 150.28 and 150.19 (C2); 144.82 and 144.80 (DMTr); 135.96, 135.95 and 135.91 (DMTr); 135.61, 135.58, 134.62 and 134.49 (C6); 130.20, 130.17, 130.15 and 130. 13 (DMTr); 128.24, 128.23, 128.10, 127.25 and 127.22 (DMTr); 113.46 and 113.43 (DMTr); 111.46, 111.44, 111.41 and 111.38 (C5); 87.47 and 87.45 (spiro C of DMTr); 85.37, 85.14, 85.00 and 84.77 (C1′); 83.86, 83.82, 82.89, 82.84 and 82.83 (C4′); 77.87, 77.77, 77.73, 77.34 and 73.86 (C3′); 73.07, 73.02, 72.98 and 72.93 (OCH_2); 67.79, 67.78, 67.77, 63.76, 63.59 and 63.43 (C5′); 55.28 (OCH_3 of DMTr); 48.59, 48.55, 48.52 and 48.48 (spiro C); 38.86, 38.85, 38.84, 38.82, 38.81 and 38.78 (spiro C of Piv and C2′); 36.24 and 36.17 (SCH_2); 30.17 and 30.16 (CH_3 of Ac); 27.24, 27.22 and 27.18 (CH_3 of Piv); 21.55, 21.52 and 21.49 (CH_3); 12.54, 12.52 and 12.47 (CH_3 of Thy) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –2.37 and –2.41 ppm. HRMS (ESI$^-$): *m*/*z* calcd. for $C_{54}H_{64}N_4O_{17}PS$ [M – H]$^-$ 1103.3730; found 1103.3760. DMTr = 4,4′-dimethoxytrityl.

4-Acetylthio-2,2-dimethyl-3-oxobutyl 5′-*O*-Pivaloylthymidin-3′-yl Thymidin-5′-yl Phosphate (17): Compound **16** (0.39 mmol, 0.43 g) was dissolved in aq. 80% AcOH (10 mL) and the reaction mixture was stirred at room temp. for 4 h. The reaction mixture was evaporated to dryness. The crude product was purified by silica gel chromatography by eluting with CH_2Cl_2 that contained 10% MeOH to yield **17** (0.20 g, 65%) as a white solid. ^{1}H NMR

(500 MHz, $CDCl_3$): δ = 10.32–10.22 and 10.14–9.92 (br. s, 2 H, 2 × NH); 7.38, 7.36, 7.27 and 7.25 (s, 2 H, H6); 6.31–6.28 (m, 1 H, H1′); 6.25–6.21 (m, 1 H, H1′); 5.06–4.99 (m, 1 H, H3′); 4.50 (br. s, 1 H, H3′): 4.42–4.24 (m, 5 H, H4′, H5′ and H5′′); 4.18–4.10 (m, 3 H, H4′ and OCH_2); 3.95 (s, 2 H, SCH_2); 2.74–2.64 (m, 1 H, H2′); 2.44–2.41 (m, 1 H, H2′′); 2.37 and 2.35 (s, 3 H, AcS); 2.25–2.05 (m, 2 H, H2′ and H2′′); 1.92–1.88 (m, 6 H, CH_3 of Thy); 1.5–1.29 (m, 6 H, 2 × CH_3); 1.22 (s, 9 H, CH_3 of Piv) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.45 and 205.35 (C=O); 194.59 and 194.58 (C=O of AcS); 178.03 and 178.94 (C=O of Piv); 164.28, 164.26 and 164.02 (C4); 150.81, 150.75 and 150.70 (C2); 135.86, 135.85, 134.71 and 134.45 (C6); 111.60, 111.55, 111.33 and 111.21 (C5); 85.30, 85.07 and 85.01 (C1′); 84.54, 84.50, 83.02, 82.96, 82.94 and 82.89 (C4′); 78.51, 78.48, 78.15 and 78.11 (C3′); 73.26, 73.21 and 73.17 (OCH_2); 70.87 and 70.83 (C3′); 67.69, 67.63, 67.50, 63.77, 63.69 and 63.66 (C5′); 48.68, 48.67 and 48.61 (spiro C); 39.78, 39.63, 38.99, 38.96, 38.67 and 38.65 (C2′); 38.83 and 38.81 (spiro C of Piv); 36.30 and 36.21 (SCH_2); 30.20 and 30.18 (CH_3 of Ac); 27.22 (CH_3 of Piv); 21.64, 21.56, 21.52 and 21.50 (CH_3); 12.51 (CH_3 of Thy) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –2.39 and –2.68 ppm. HRMS (ESI^-): *m*/*z* calcd. for $C_{33}H_{46}N_4O_{15}PS$ $[M - H]^-$ 801.2423; found 801.2393.

(4-Acetylthio-2,2-dimethyl-3-oxobutyl)-Protected Phosphotriester 1: Compound **15** (0.49 mmol, 0.16 g) was dried with P_2O_5 overnight and dissolved in CH_2Cl_2 (3 mL). TEA (0.34 mL, 2.38 mmol) and bis(diisopropylamino)chlorophosphite (0.48 mmol, 0.13 g) were added under nitrogen. The phosphitylation step was complete in 30 min, and a ^{31}P NMR signal (202 MHz, CD_3CN) occurred at δ = 116.19 ppm. The reaction mixture was filtered through a short silica gel column by eluting with hexane that contained 40% EtOAc and 0.5% TEA. The fractions that contained product were combined and the solvents evaporated to dryness under reduced pressure and the residue was coevaporated three times from dry MeCN. The obtained phosphoramidite was dissolved in MeCN (3.5 mL) under nitrogen and compound **14** (0.30 mmol, 0.30 g) and 1*H*-tetrazole (0.30 mmol, 0.68 mL of 0.45 M solution in MeCN) were added. After 1.5 h, multiple ^{31}P NMR signals at 149.56–148.36, –1.20 – (–1.40) and 2.40 – (–2.65) ppm were observed. Compound **7** (1.12 mmol, 0.21 g) was dissolved in MeCN (0.5 mL) and added to the reaction mixture with 1*H*-tetrazole (1.12 mmol, 2.49 mL of 0.45 M solution in MeCN). After 1.5 h, multiple ^{31}P NMR signals at 140.29–139.16, –1.25 – (–1.45) and 2.45 – (–2.70) ppm were observed. I_2 (1.02 mmol, 0.26 g) in a mixture of THF, H_2O and 2,6-lutidine (v/v/v, 4:2:1, 7 mL) was added to the reaction mixture and the oxidation was left to proceed overnight. Aq. 5% $NaHSO_3$ was added to the reaction mixture and the product was extracted to CH_2Cl_2. The organic fractions were combined, washed with brine, dried with Na_2SO_4 and the solvents evaporated to dryness under reduced pressure. The residue was eluted through a silica gel column by using CH_2Cl_2 that contained 5% MeOH. From the crude product (0.43 g) 0.29 g was purified with RP-HPLC (SunFire prep C18 10 × 250 mm 5 μm, flow rate 4 mL/min) by using isocratic elution (35% MeCN in H_2O) to yield **1** (overall yield from **14** 23 mg, 4.9%) as a white solid. In pursuance of HPLC purification the mixture of eight R_P/S_P-diastereomers was fractionated. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.55, 7.53 and 7.46 (s, 3 H, H6); 6.29–6.23 (m, 3 H, H1′); 5.15–5.06 (m, 4 H, H3′); 4.45–4.14 (m, 15 H, H4′, H5′, H5′′and OCH_2); 4.10 and 4.08 (s, 6 H, SCH_2); 3.85 and 3.83 (d, *J* = 2.10, and *J* = 2.10 Hz, 3 H, OMe); 2.65–2.40 (m, 8 H, H2′ and H2′′); 2.37–2.35 (m, 9 H, AcS); 1.92, 1.92 and 1.91 (s, 9 H, CH_3 of Thy); 1.32–1.26 (m, 18 H, CH_3); 1.25 (s, 9 H, CH_3 of Piv) ppm. ^{13}C NMR (126 MHz, CD_3OD): δ = 205.96, 205.91, 205.87, 205.79 and 205.75 (C=O); 194.77, 194.75, 194.73 and 194.71 (C=O of AcS); 178.03 (C=O of Piv); 164.85, 164.84 and 164.82 (C4); 150.74, 150.69 and 150.63 (C2); 136.67 136.63 and 135.94 (C6); 110.70 110.68 and 110.45 (C5); 85.60, 85.57, 85.55, 85.52 and 85.25 (C1′); 82.91, 82.90, 82.85, 82.80, 82.74, 82.69 and 82.64 (C4′); 78.36, 78.32, 77.87, 77.82, 77.77, 77.14, 7710, 77.09 and 77.04 (C3′); 73.32, 73.28, 73.24, 73.20, 73.08 and 73.03 (OCH_2); 67.32, 67.31, 67.30, 67.20, 66.70, 66.98 and 66.96 (C5′); 63.30 (spiro C of Piv); 54.40, 54.35 and 54.30 (OCH_3), 38.48 (spiro C); 37.66, 37.63, 37.25, 37.24, 37.22, 37.20 and 37.17 (C2′); 35.90–35.30 (SCH_2); 28.76 and 28.71 (CH_3 of Ac); 26.26 (CH_3 of Piv) 20.42, 20.41, 20.39, 20.37, 20.34 and 20.32 (CH_3); 11.26, 11.25, 11.24 and 11.23 (CH_3 of Thy) ppm. ^{31}P NMR (202 MHz, CD_3OD): δ = –1.45, –1.47, –1.48, –1.51, –2.79, –2.84 and –2.85 ppm. HRMS (ESI^-): *m*/*z* calcd. for $C_{60}H_{86}N_6O_{29}P_3S_3$ $[M - H]^-$ 1543. 3820; found 1543.3777.

(4-Acetylthio-2,2-dimethyl-3-oxobutyl)-Protected Phosphotriester 2: Compound **9** (0.13 mmol, 0.13 g) was dried with P_2O_5 for 2 d and dissolved in MeCN (1.5 mL). 1*H*-tetrazole (0.14 mmol, 325 μL of 0.45 M solution in MeCN) and tris(diethylamino)phosphine (0.13 mmol, 35 μL) were added under nitrogen. After 3 h stirring, compound **10** (0.13 mmol, 0.11 g) in MeCN (0.9 mL) and 1*H*-tetrazole (0.13 mmol, 285 μL of 0.45 M solution in MeCN) were added to the reaction mixture and the stirring was continued for 90 min. Compound **7** (0.27 mmol, 0.05 g) and 1*H*-tetrazole (0.26 mmol, 585 μL of 0.45 M solution in MeCN) were added and the reaction mixture was stirred for another 90 min. I_2 (1.06 mmol, 0.27 g) in a mixture of THF, H_2O and 2,6-lutidine (v/v/v, 4:2:1, 2 mL) was added and the oxidation was left to proceed overnight. The reaction mixture was evaporated to dryness and the residue was equilibrated between CH_2Cl_2 and aq. 5% $NaHSO_3$. The organic phase was separated, dried with Na_2SO_4 and the solvents evaporated to dryness. The crude product was eluted through a silica gel column by eluting first with CH_2Cl_2 that contained 10 to 15% MeOH, then with EtOAc that contained 20 to 30% hexane and finally with CH_2Cl_2 that contained 5% MeOH. The silyl protecting-group was removed by dissolving the crude product (0.12 g) in a mixture of THF (1.25 mL) and AcOH (0.25 mL) and TBAF (0.15 mmol, 0.04 g) and the reaction mixture was stirred at room temp. for 3 d. The mixture was evaporated to dryness and the residue was equilibrated between CH_2Cl_2 and H_2O. The organic phase was separated and the solvents evaporated to dryness under reduced pressure. To remove the 4-methoxytrityl protecting group, the residue was dissolved in aq. 80% AcOH and the reaction mixture was stirred at room temp. for 22 h. The crude product was purified by RP-HPLC (LichroCHART 250 × 10 Hypersil ODS 5 μm, flow rate 4 mL/min) by using gradient elution (first with 30% to 50% MeCN in H_2O in 20 min and then 30% to 40% MeCN in H_2O in 20 min). The mixture of R_P/S_P-diastereomers was fractionated by HPLC (isocratic elution by using 32% MeCN in acetic acid/sodium acetate buffer, 0.045/0.015 mol L^{-1} and finally removing the buffer salts by eluting with 30% MeCN in H_2O) to simplify the product distribution in kinetic measurements. Compound **2** (overall yield from **9** 4 mg, 1.8%) was obtained as a white solid. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.81–7.80 and 7.56–7.51 (m, 4 H, H6); 6.33–6.23 (m, 4 H, H1′); 5.15–5.110 (m, 3 H, H3′); 4.43–4.32 (m, 9 H, H3′, H4′, H5′, H5′′); 4.24–4.18 (m, 7 H, H4′ and OCH_2); 4.08–4.02 (m, 7 H, SCH_2 and H4′); 3.80–3.79 (m, 2 H, H5′ and H5′′): 2.65–2.30 (m, 8 H, H2′ and H2′′); 2.36, 2.35 and 2.34 (s, 9 H, AcS); 1.91–1.987 (m, 12 H, CH_3 of Thy); 1.31–1.29 (m, 18 H, 6 × CH_3) ppm. ^{13}C NMR (126 MHz, CD_3OD): δ = 205.90 and 205.79 (C=O); 194.80 and 194.79 (C=O of AcS); 164.93, 164.91, 164.88, 164.85 and 164.83 (C4); 150.90, 150.89, 150.86, 150.84, 150.71 and 150.70 (C2); 136.60, 136.54, 136.51 and 136.49 (C6);

110.67, 110.58, 110.42 and 111.36 (C5); 85.74, 85.73, 85.60, 85.40 and 85.20 (C1′ and C4′); 84.60, 84.56 and 84.55 (C1′); 83.00, 82.99 and 84.98 (C4′); 79.44 and 79.45, (C3′); 73.21 and 73.20 (OCH_2); 70.35 and 70.20 (C3′); 68.10, 68.09, 61.28 and 61.26 (C5′); 38.97, 38.95, 38.14, 38.13, 37.50 and 37.48 (C2′); 35.84, 35.81 and 35.78 (SCH_2); 28.77, 28.73, 28.72, 28.71 and 28.63 (CH_3 of Ac); 20.47, 20.45, 20.44 and 20.40 (CH_3); 11.33, 11.31, 11.29 and 11.28 (CH_3 of Thy) ppm. ^{31}P NMR (202 MHz, CD_3OD): δ = –2.51, –2.67, –2.69, –2.71, –2.74, –2.77, –2.80 and –2.86 ppm (the impurity that resonated at δ = 20 ppm in ^{31}P NMR spectrum disappeared after HPLC fractionation; see Supporting Information). HRMS (ESI^-): *m*/*z* calcd. for $C_{64}H_{88}N_8O_{32}P_3S_3$ $[M - H]^-$ 1669.3776; found 1669.3770.

Kinetic Measurements: Kinetic measurements were carried out by HPLC as previously reported.[24] Signals were recorded on a UV detector at a wavelength of 267 nm. The samples from the kinetic run were eluted by using first a 5 min isocratic elution with a mixture of acetic acid/sodium acetate buffer (0.045/0.015 mol L^{-1}) and MeCN (2%) at a flow rate of 0.95 mL/min. Then linear gradient was followed from 2% to 70% MeCN in 30 min. The products were identified by HPLC-MS analysis or they were isolated and analyzed by MS. The same gradient system, as in the kinetic runs, was applied for the isolation of the products, but 0.1 mol L^{-1} triethylammonium acetate was used as an eluent instead.

Supporting Information (see footnote on the first page of this article): Copies of the ^{1}H, ^{13}C and ^{31}P NMR spectra of compounds **1**, **2**, **8**–**14**, **16** and **17**.

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