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Synthesis and Stability of Nucleoside 3′,5′-Cyclic Phosphate Triesters Masked with Enzymatically and Thermally Labile Phosphate Protecting Groups

Vyankat A. Sontakke,[a,b] Vaishali S. Shinde,[b] Harri Lönnberg,[a] and Mikko Ora*[a]

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Appropriately protected structurally modified nucleoside 3′,5′-cyclic monophosphates are known to show antiviral activity. For this reason, a straightforward synthesis of nucleoside 3′,5′-cyclic phosphates protected with three different enzymatically removable groups, viz. 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl (in **1** and **4**), 4-acetylthio-2,2-dimethyl-3-oxobutyl (in **2**), and 4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl (in **3**) groups, is described. Removal of these protecting groups at pH 7.5 and 37 °C was monitored by reverse-phase HPLC.

Introduction

To be biologically active, antiviral nucleosides must be phosphorylated by intracellular kinases to their 5′-triphosphates via 5′-mono- and 5′-diphosphates. The first phosphorylation is the most discriminating.[1,2] For this reason, several nucleoside monophosphate prodrugs showing enhanced cellular uptake have been studied as potential antiviral agents.[3,4] Appropriately protected nucleoside 3′,5′-cyclic phosphates represent one class of pronucleotides that has recently received increasing interest.[5–7] These molecules are expected to undergo, after removal of the protecting group, phosphodiesterase-catalysed ring opening to give the desired nucleoside 5′-monophosphate.[6] Pivaloyloxymethyl, 2-(acylthio)ethyl and isopropoxycarbonyloxymethyl esters of base-modified 2′-*C*-methylribonucleoside 3′,5′-cyclic phosphates have shown enhanced inhibition of hepatitis C virus (HCV) replication compared to the parent nucleoside.[8] Even alkyl and aryl esters of base-modified 2′-deoxy-2′-fluoro-2′-*C*-methylribonucleoside 3′,5′-cyclic phosphates have been reported to inhibit the RNA-dependent RNA polymerase of HCV.[9]

Synthetic methods used for the preparation of nucleoside 3′,5′-cyclic phosphotriester prodrugs include the alkylation of cyclic phosphodiesters with an appropriate alkylating agent, and the condensation of cyclic phosphodiesters with suitable alcohols in the presence of a condensation agent.[8] The phosphoramidite strategies reported involve phosphitylation of nucleosides with bis(dialkylamino)alkoxyphosphines followed by intramolecular cyclization and oxidation of the phosphite intermediate formed.[7,9]

[a] University of Turku, Department of Chemistry, 20014 Turku, Finland
E-mail: mikora@utu.fi
http://www.utu.fi/en/units/sci/units/chemistry
[b] University of Pune, Department of Chemistry, Garware Research Centre, Pune 411007, India

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We have previously prepared enzyme-labile *S*-pivaloyloxymethyl-protected 2′-*C*,O^6-dimethylguanosine 3′,5′-cyclic phosphorothioate, a potential prodrug of 2′-*C*-methylguanosine, by alkylation of the cyclic phosphorothioate diester with pivaloyloxymethyl chloride.[10] Although the cyclic phophorothioate is undoubtedly more nucleophilic than its oxygen counterpart, the yield of the alkylation reaction was low (14%). Attempts to prepare the 2,2-disubstituted 4-acylthio-3-oxobutyl-protected nucleoside 3′,5′-cyclic phosphates by the phosphoramidite approach failed. These thermolabile and enzyme-labile protecting groups are not only base labile, but they are also susceptible to cleavage by amine nucleophiles, and they also leave rather easily as a result of intramolecular cyclization. Accordingly, an alternative method is needed for the efficient protection of 3′,5′-cyclic phosphodiesters with enzymatically removable groups. We now show that the oxidative coupling recently described by the Kraszewski group[11] for the synthesis of simple *O*-alkyl and aryl esters of nucleoside 3′,5′-cyclic phosphates offers a workable method for the introduction of structurally more complex enzymatically removable phosphate protecting groups, such as 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl (in **1** and **4**), 4-acetylthio-2,2-dimethyl-3-oxobutyl (in **2**), and 4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl (in **3**) groups (Figure 1). In addition, data on the stability of the cyclic phosphotriesters (i.e., **1**–**4**) and their conversion into cyclic diesters is given.

Regarding the removal of the protecting groups, 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl has been shown to undergo deacetylation by carboxyesterase, and the resulting 3-hydroxy-2,2-bis(ethoxycarbonyl)propyl group is then cleaved in a retro-aldol reaction.[12] The 4-acetylthio-2,2-dimethyl-3-oxobutyl group is also removed by esterases, but it is also thermolabile, and can thus be removed even if the enzymatic reaction is sluggish.[13,14] The enzymatic and nonenzymatic deprotection processes both proceed by intramolecular cyclization to give a substituted tetrahydro-

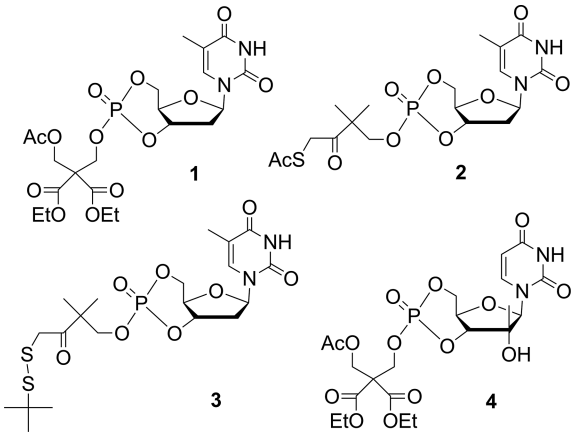


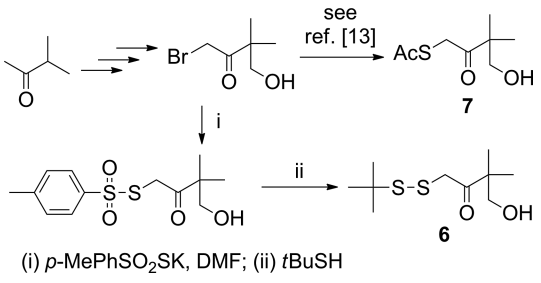
Figure 1. Structures of protected nucleoside 3′,5′-cyclic phosphotriesters **1**–**4**.

thiophenone. Tetrahydrothiophenones do not form adducts with glutathione, and thus they probably do not show significant alkylating activity. The removal of the 4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl protecting group should proceed by enzymatic or glutathione-induced reductive cleavage of the disulfide linkage,[15] followed by removal through cyclization, as in the case of the 4-acetylthio-2,2-dimethyl-3-oxobutyl group. The kinetics of these processes have been studied with triesters **1**–**4**.

Results and Discussion

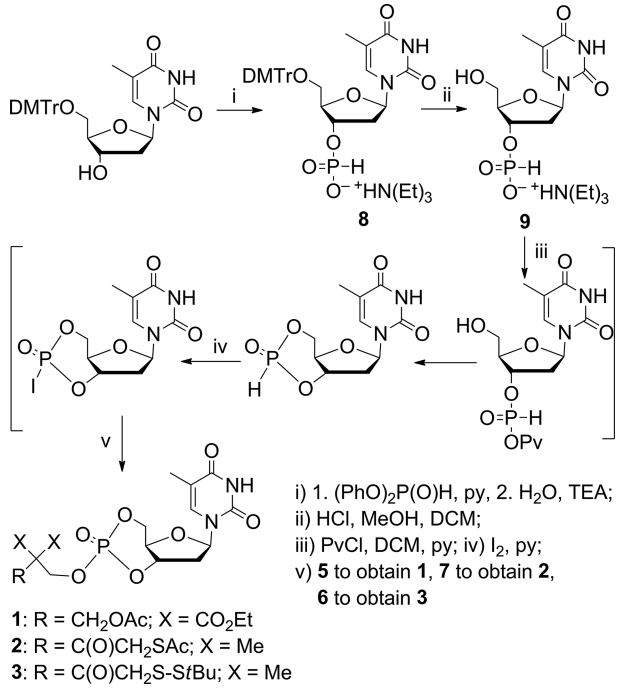
Synthesis

Of the alcohols used for introduction of the enzymatically removable protecting groups, diethyl 2-(acetyloxymethyl)-2-(hydroxymethyl)malonate (**5**)[12] and *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate (**7**)[13] were prepared as described previously. 1-(*tert*-Butyldisulfanyl)-4-hydroxy-3,3-dimethylbutan-2-one (**6**) was obtained by treatment of 1-bromo-4-hydroxy-3,3-dimethylbutan-2-one[13] with potassium *p*-toluenethiosulfonate in DMF, and a subsequent thiol–disulfide interchange reaction with 2-methylpropane-2-thiol (Scheme 1). A similar strategy has previously been used in the synthesis of 2′-*O*-(*tert*-butyldisulfanylmethyl)ribonucleosides.[16]



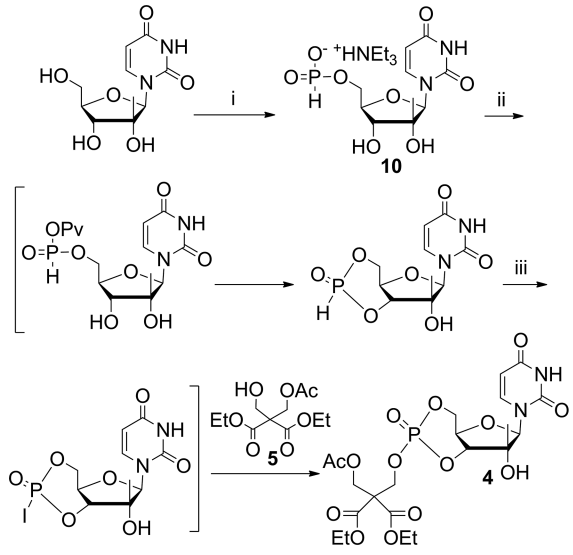
Scheme 1. Preparation of 1-(*tert*-butyldisulfanyl)-4-hydroxy-3,3-dimethylbutan-2-one (**6**).

Thymidine 3′,5′-cyclic phophotriesters **1**–**4** were then synthesized by using an oxidative coupling concept recently described for the synthesis of *O*-alkyl and aryl esters of nucleoside 3′,5′-cyclic phosphates.[11] Accordingly, thymidine 3′-*H*-phosphonate (**9**) was prepared by the acid-catalysed detritylation of 5′-*O*-(4,4′-dimethoxytrityl)thymidine-3′-*H*-phosphonate (**8**).[17] It was subjected to pivaloyl-chloride-promoted intramolecular cyclization in a mixture of CH_2Cl_2 and pyridine (95:5) at 0 °C (Scheme 2). The resulting 3′,5′-cyclic *H*-phosphonate diester was not isolated, but oxidized immediately to the iodophosphate, and the iodine ligand was displaced by alcohols **5**–**7** to give cyclic triesters **1**–**3**. Triester **4** was prepared similarly from 2′-*C*-methyluridine 5′-*H*-phosphonate (**10**), which was obtained by phosphitylation of 2′-*C*-methyluridine with diphenyl phosphite in pyridine at –5 °C and subsequent treatment with aq. Et_3N (Scheme 3). The products were obtained as mixtures of R_P and S_P diastereomers, the ratio of the major and minor diastereomers being 7:3 for **1**–**3**, and 19:1 for **4**. No attempt was made to assign the relative configuration of the diastereomers.



Scheme 2. Preparation of thymidine 3′,5′-cyclic *O*-3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl phosphate (**1**), thymidine 3′,5′-cyclic *O*-4-acetylthio-2,2-dimethyl-3-oxobutyl phosphate (**2**), and thymidine 3′,5′-cyclic *O*-4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl phosphate (**3**). DMTr = 4,4′-dimethoxytrityl.

As mentioned above, attempts to prepare the nucleoside 3′,5′-cyclic phosphates using phosphoramidite methodology failed. N^6-Dimethoxytrityl-2′-*O*-methyladenosine and 2′-*O*-tetrahydropyranyl-2′-*C*-methyluridine were phosphitylated with *N*,*N*-diisopropylchlorophosphoramidite and *N*,*N*-diethylchlorophosphoramidite, respectively, in CH_2Cl_2 containing Et_3N. In the case of unprotected 2′-*C*-methylur-



i) 1. $(PhO)_2P(O)H$, py, 2. H_2O, TEA; ii) PvCl, DCM, py; iii) I_2, py

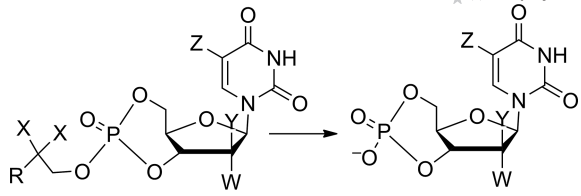
Scheme 3. Preparation of 2′-*C*-methyluridine 3′,5′-cyclic *O*-3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl phosphate (**4**).

idine, tris(dimethylamino)phosphine was used as the phosphitylating agent, and tetrazole as the activator in MeCN. The formation of the phosphitylated products was verified by ^{31}P NMR spectroscopy. The products resonated around δ = 148–150 ppm, depending on the nucleoside and phosphitylating reagent. Displacement of the dialkylamino ligand by cyclization with the 3′-OH group, however, gave complex reaction mixtures. None of the desired products were observed after coupling of the 4-acetylthio-2,2-dimethyl-3-oxobutyl or the 4-pivaloylthio-2,2-dimethyl-3-oxobutyl protecting groups in the presence of tetrazole followed by oxidation with iodine in aqueous THF containing 2,6-lutidine.

Stability

We also investigated the stability of cyclic phosphotriesters **1**–**4**. The mixtures of R_P and S_P diastereomers of the phosphotriesters (i.e., **1**–**4**) were deprotected in the presence of porcine liver esterase (PLE; 2.6 units mL^{-1}) or glutathione in a HEPES buffer (0.036/0.024 M; 3 mL) at pH 7.5 and 37 °C to give nucleoside 3′,5′-cyclic monophosphates (Scheme 4). The deprotection was monitored by HPLC. Aliquots taken from the reaction mixture at appropriate time intervals were acidified (pH 2) to deactivate the enzyme and quench the hydrolysis. Thymidine 3′,5′-cyclic phosphate (**11a**) was identified by spiking with an authentic sample, while 2′-*C*-methyluridine 3′,5′-cyclic phosphate (**11b**) was collected and identified by ESI-MS analysis.

Carboxyesterase-triggered deprotection of phosphotriester **2** (t_R = 22.1 min) produced, as expected, 3′,5′-cyclic diester **11a** (t_R = 14.5 min), the half-life at 37 °C being 6.0 min using 2.6 units mL^{-1}. A minor intermediate accumulated, which was probably the deacetylated starting material. The disappearance of this intermediate was seven



3′,5′-cTMP (**11a**):
W = Y = H; Z = Me
3′,5′-cU$_{2'\text{-CMe}}$MP (**11b**):
W = OH; Z = H; Y = Me

1: W = Y = H; Z = Me; R = CH_2OAc; X = CO_2Et
2: W = Y = H; Z = Me; R = $C(O)CH_2SAc$; X = Me
3: W = Y = H; Z = Me; R = $C(O)CH_2S$-StBu; X = Me
4: W = OH; Y = Me; Z = H; R = CH_2OAc; X = CO_2Et

Scheme 4. Deprotection of 3′,5′-cyclic phosphotriesters **1**–**4** to give cyclic phosphodiesters **11**.

times as fast ($\tau_{1/2}$ = 0.84 min) as the disappearance of **2**. The second step evidently involves the attack of the exposed mercapto group onto the phosphate-bound carbon atom with concomitant release of 3′,5′-cTMP. The product distribution is shown in Figure 2.

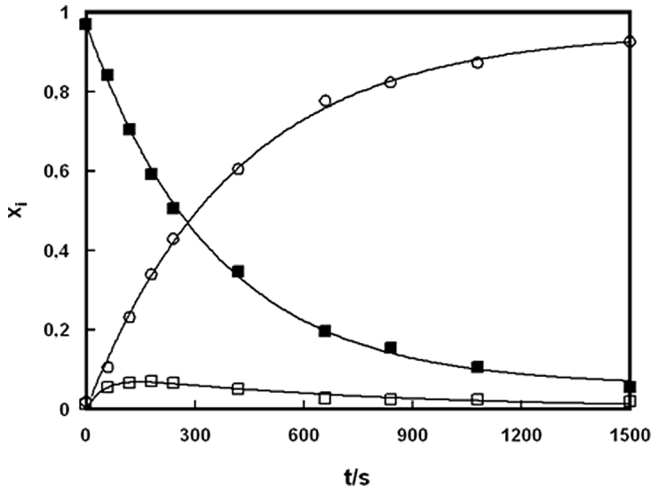


Figure 2. Time-dependent product distribution for the esterase-triggered deprotection of diastereomeric (R_P/S_P) thymidine 3′,5′-cyclic *O*-4-acetylthio-2,2-dimethyl-3-oxobutyl phosphate (**2**) at pH 7.5 (I = 0.1 M with NaCl) and 37 °C. Notation: (■) **2**, (○) 3′,5′-cTMP, (□) deacetylated **2**.

The 4-acetylthio-2,2-dimethyl-3-oxobutyl protecting group may also undergo nonenzymatic cyclization to 4,4-dimethyl-4,*S*-dihydrothiophen-3-yl acetate.[13] In the absence of enzyme, compound **2** underwent quantitative conversion to 3′,5′-cTMP (**11a**), the half-life being 9.3 h.

It is known that glutathione acts as a reducing agent to cleave disulfide bridges. Accordingly, the removal of the 4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl protecting group from **3** was carried out in the presence of glutathione (5 mM) in a HEPES buffer. According to the product distribution, the starting material (i.e., **3**; t_R = 27.9 and 28.4 min for the R_P and S_P diastereomers) was converted into 3′,5′-cTMP, the half-life for the overall disappearance of **3** being

74 min. In addition, glutathione disulfide (GS–SG; t_R = 3.6 min; m/z = 631.4 $[M + H]^+$) was accumulated.

As with **2** and **3**, 3′,5′-cTMP (**11a**) was also obtained as the product of the esterase catalysed deprotection of **1** (t_R = 24.3). The enzymatic deacetylation of the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group was, however, more than one order of magnitude slower than the corresponding reaction of the 4-acetylthio-2,2-dimethyl-3-oxobutyl group on **2** (half-lives 93 and 6.0 min, respectively, at 37 °C using 2.6 units mL^{-1} of carboxyesterase). The deacetylated intermediate was not accumulated, but a minor amount of an unknown intermediate was formed besides **11a**.

The deprotection of phosphotriester **1** was also studied in a whole-cell extract of human prostate cancer (PC3) cells. To obtain a PC3 cell extract, 3×10^6 cells were disrupted in RIPA buffer (pH 7.5; 1 mL), and then this mixture was diluted in a 1:2 ratio with HEPES buffer. Compound **1** was added to 3 mL of this extract. Under these conditions, more than 60% of **1** was converted into 3′,5′-cTMP (**11a**) in 6 d, the half-life being 47.5 h for the disappearance of **1**.

Unexpectedly, the 3-acetyloxy-2,2-bis(ethoxycarbonyl)-propyl-protected phosphotriester (i.e., **4**; t_R = 23.4 min) released the 3′,5′-cyclic phosphodiester (i.e., **11b**; t_R = 14.0; m/z = 343.0 $[M + Na]^+$) even more slowly ($\tau_{1/2}$ = 35 h) than **1** reacted to form **11a**. Repeated purification of **4** by HPLC did not have any appreciable effect on the rate. As **1** and **4** are deacetylated more slowly than **2**, the 4-acetylthio-2,2-dimethyl-3-oxobutyl group appears to be a more attractive protecting group than the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group for 3′,5′-cyclic phosphates of nucleosides.

To study whether **11a** could be further converted into nucleoside 5′-monophosphate (5′-TMP; **12a**) by phosphodiesterases, phosphotriester **2** was first nonenzymatically converted into **11a**, and human phosphodiesterase 5A1 [10 μg enzyme in 40 mM Tris-HCl containing glycerol (20%), NaCl (110 mM), KCl (2.2 mM), and DTT (dithiothreitol; 3 mM); pH 8.0; 30 μL] was added into the reaction solution (200 μL). 5′-TMP was not, however, observed to be formed under these conditions, although human recombinant phosphodiesterases (PDE) have been reported to hydrolyse not only 3′,5′-cAMP and 3′,5′-cGMP, but also 3′,5′-cTMP and 3′,5′-cUMP to their 5′-phosphates.[18] Instead, more than 70% of the 3′,5′-cTMP (**11a**) was converted into thymine (t_R = 9.2 min; m/z = 125.1 $[M - H]^-$) after 3 d. The same conversion was also attempted using phosphodiesterase 3A (PDE 3A). Phosphotriester **1** was first deprotected by PLE, and then the phosphodiesterase [10 μg in 25 mM Tris-HCl containing NaCl (100 mM), TWEEN®-20 (0.05%), glycerol (50%), glutathione (20 mM), and DTT (3 mM); pH 8.0; 20 μL] was added into the reaction solution, but no trace of thymidine 5′-phosphate was observed.

Conclusions

The synthesis using oxidative coupling of nucleoside 3′,5′-cyclic phosphates **1**–**4** bearing enzymatically removable protecting groups has been described. All the cyclic phosphotriesters were converted into the desired cyclic phosphodiesters in the presence and/or absence of enzyme at pH 7.5 and 37 °C. 3-Acetyloxy-2,2-bis(ethoxycarbonyl)-propyl (in **1** and **4**) and 4-acetylthio-2,2-dimethyl-3-oxobutyl (in **2**) groups were removed by carboxyesterase (half-lives 93 min, 35 h, and 6 min, respectively). The 4-acetylthio-2,2-dimethyl-3-oxobutyl protecting group (**2**) was also thermolabile (half-life 9.3 h). The 4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl group (in **3**) was removable by treatment with glutathione (half-life 74 min).

Experimental Section

General Remarks: Pyridine and CH_2Cl_2 were dried with molecular sieves (4 Å). Pivaloyl chloride was distilled before use. ^1H, ^{13}C, ^{31}P, and 2D NMR spectra were recorded with a Bruker Avance 500 spectrometer. High-resolution mass spectra were recorded with a Bruker Daltonics microTOF-Q instrument using electrospray ionization. The composition of the samples was analysed with a Merck Hitachi LaChrom D7000 HPLC instrument with an L-7455 UV-detector and an L-7100 pump, using an ODS Hypersil C18 column (4 × 250 mm, 5 μm, flow rate 0.95 mL min^{-1}).

Thymidine 3′-*H*-Phosphonate (9): 5′-*O*-(4,4′-Dimethoxytrityl)thymidine 3′-*H*-phosphonate[17] (**8**; 2.00 g, 2.81 mmol) was dissolved in a mixture of CH_2Cl_2 (2.5 mL) and MeOH (2.5 mL), and conc. HCl (25 μL) was added dropwise. The mixture was stirred at room temperature for 3 h, then it was neutralized with Et_3N. The solvent was removed under reduced pressure, and the residue was coevaporated twice from toluene. The product was purified by silica gel chromatography using CH_2Cl_2 containing 5–15% MeOH as eluent. Compound **9** (0.90 g, 79%) was obtained as a solid. ^1H NMR (500 MHz, CD_3OD): δ = 7.87 (d, J = 1.15 Hz, 1 H, 6-H), 6.83 (d, J = 626.46 Hz, 1 H, PH), 6.33 (dd, J = 7.50, 6.15 Hz, 1 H, 1′-H), 4.91–4.89 (m, 1 H, 3-H), 4.13–4.11 (m, 1 H, 4-H), 3.82 (d, J = 3.10 Hz, 1 H, 5′-H), 3.23 (q, J = 7.30 Hz, 2 H, Et_3N), 2.45 (ddd, J = 14.28, 6.05, 3.15 Hz, 1 H, 2′-H), 2.36–2.30 (m, 1 H, 2′′-H), 1.90 (d, J = 1.10 Hz, 3 H, CH_3), 1.34 (t, J = 7.30 Hz, 3 H, Et_3N) ppm. ^{13}C NMR (125 MHz, CD_3OD): δ = 164.99 (C-4), 150.93 (C-2), 136.74 (C-6), 110.20 (C-5), 86.50 (C-1′), 84.72 (C-4′), 73.31 (C-3′), 61.14 (C-5′), 46.38 (Et_3N), 38.81 (C-2′), 11.05 (CH_3 of Thy), 7.80 (Et_3N) ppm. ^{31}P NMR (202 MHz, CD_3OD): δ = 3.20 ppm. MS (ESI): calcd. for $C_{10}H_{16}N_2O_7P$ $[M - H]^-$ 305.2; found 305.4.

2′-*C*-Methyluridine 5′-*H*-Phosphonate (10): A solution of 2′-*C*-methyluridine (1.00 g, 3.87 mmol) in anhydrous pyridine (10.0 mL) was added dropwise to diphenyl phosphite (112 μL, 5.80 mmol) in anhydrous pyridine (5.0 mL) at –5 °C. The mixture was stirred for 30 min at room temperature, then a mixture of triethylamine (2.5 mL) and water (2.5 mL) was added. The mixture was stirred for a further 20 min, then it was subjected to a conventional H_2O/CH_2Cl_2 work up. The aqueous layer was evaporated to dryness. The residue was purified by silica gel column chromatography with gradient elution (5–10% MeOH in CH_2Cl_2) to give compound **10** (1.3 g, 79%) as a solid. ^1H NMR (500 MHz, CD_3OD): δ = 8.01 (d, J = 8.15 Hz, 1 H, 6-H), 6.80 (d, J = 623.31 Hz, 1 H, PH), 5.97 (s, 1 H, 1′-H), 5.81 (d, J = 8.15 Hz, 1 H, 5-H), 4.25 (ddd, J = 12.08, 6.20, 1.95 Hz, 1 H, 5′-H), 4.10 (ddd, J = 12.03, 6.85, 2.25 Hz, 1 H, 5′′-H), 4.02–4.00 (m, 1 H, 4′-H), 3.88 (d, J = 9.15 Hz, 1 H, 3′-H), 3.20 (q, J = 7.35 Hz, 2 H, Et_3N), 1.31 (t, J = 7.35 Hz, 3 H, Et_3N), 1.16 (s, 3 H, CH_3) ppm. ^{13}C NMR (125 MHz, CD_3OD): δ = 164.53 (C-4), 151.06 (C-2), 140.93 (C-6), 101.47 (C-5), 91.59 (C-1′), 80.93

and 80.86 (C-4′), 78.58 (C-2′), 71.9 (C-3′), 60.93 and 60.89 (C-5′), 46.43 (Et_3N), 18.79 (CH_3), 7.80 (Et_3N) ppm. ^{31}P NMR (202 MHz, CD_3OD): δ = 4.91 ppm. HRMS (ESI): calcd. for $C_{10}H_{15}N_2NaO_8P$ $[M + H]^+$ 345.0458; found 345.0454.

1-(*tert*-Butyldisulfanyl)-4-hydroxy-3,3-dimethylbutan-2-one (6): 1-Bromo-4-hydroxy-3,3-dimethylbutan-2-one (0.020 mol, 4.00 g), prepared as described earlier,[13] was dissolved in dry DMF (30 mL). Potassium *p*-toluenethiosulfonate (0.022 mol, 5.10 g) was added, and the mixture was stirred for 1 h. 2-Methyl-2-propanethiol (0.030 mol, 4.70 mL) was added dropwise, and the mixture was stirred for 1 h. CH_2Cl_2 (150 mL) was added, and the organic phase was washed with water (3 × 25 mL), and dried with Na_2SO_4, and the solvents were evaporated to dryness. The crude product was purified on a silica gel column eluting with 20% EtOAc in hexane. Compound **6** (4.20 g, 87%) was obtained as a colourless oil. ^1H NMR (500 MHz, $CDCl_3$): δ = 3.80 (s, 2 H, SCH_2), 3.59 (s, 2 H, OCH_2), 2.13 (s, 1 H, OH), 1.34 (s, 9 H, 3 CH_3 of *t*Bu), 1.20 (s, 6 H, 2 CH_3) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 208.95 (C=O), 67.78 (OCH_2), 47.53, 46.59, and 45.96 (2 spiro C and SCH_2), 28.10 (CH_3 of *t*Bu), 19.89 (CH_3) ppm. HRMS (ESI): calcd. for $C_{10}H_{21}$ NaO_2S_2 $[M + Na]^+$ 259.0802; found 259.0802.

Thymidine 3′,5′-Cyclic *O*-3-Acetyloxy-2,2-bis(ethoxycarbonyl)-propyl Phosphate (1): Thymidine 3′-*H*-phosphonate (**9**; 1.22 mmol, 0.500 g) was dissolved in a mixture of CH_2Cl_2 and pyridine (95:5; 10 mL), and the solution was cooled to 0 °C. Pivaloyl chloride (2.45 mmol, 0.302 mL) was added dropwise, and the reaction mixture was stirred for 10 min. A solution of iodine (2.45 mmol, 0.623 g) in pyridine (1.6 mL) was added dropwise, and the mixture was stirred for 15 min at 0 °C. A solution of diethyl 2-(acetyloxymethyl)-2-(hydroxymethyl)malonate (**5**; 2.45 mmol, 0.643 g) in CH_2Cl_2 (5 mL) was added dropwise, and the reaction mixture was stirred at room temperature for 8 h. After the reaction was complete, the excess iodine was quenched with sodium thiosulfate solution (10%). The product was extracted with CH_2Cl_2 (4 × 20 mL). The organic phase was dried with Na_2SO_4, and the solvents were evaporated to dryness. The crude product was purified by silica gel column chromatography eluting with EtOAc to give compound **1** (186 mg, 28%) as a white solid. ^1H NMR (500 MHz, $CDCl_3$): δ = 9.23 and 9.20 (s, 1 H, NH), 7.10 and 6.98 (s, 1 H, 6-H), 6.27 and 6.23 (dd, *J* = 8.93, 2.48 Hz, 1 H, 1′-H), 4.83 (q, *J* = 9.10 Hz, 3′-H), 4.70–4.39 (m, 6 H, 3′-H, 5′-H, $POCH_2$, CH_2OAc), 4.26–4.15 and 4.05 (m, 5 H, 5′′-H, 2 OCH_2CH_3), 3.84 and 3.81 (m, 1 H, 4′-H), 2.58–2.49 (m, 1 H, 2′-H), 2.44–2.38 (m, 1 H, 2′′-H), 2.02 and 1.98 (s, 3 H, AcO), 1.99 and 1.89 (s, 3 H, CH_3 of Thy), 1.25–1.16 (m, 6 H, 2 OCH_2CH_3) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.45 and 170.09 (Me*C*=O), 166.50, 166.39, 166.30, and 166.27 (OC=O), 163.46 and 163.38 (C-4), 150.02 and 149.92 (C-2), 135.61 and 135.32 (C-6), 112.41 and 112.37 (C-5), 85.37 and 84.84 (C-1′), 77.90, 77.84, 77.24, and 77.20 (C-3′), 73.59 and 73.53 (C-4′), 69.88, 69.79, 69.41, and 69.34 (C-5′), 66.41, 66.36, 64.87, and 64.83 ($POCH_2$), 62.52 and 62.40 (OCH_2CH_3), 61.40 and 61.29 (CH_2OAc), 58.26, 58.17, 58.03, and 57.95 (spiro C), 35.35, 35.19, 35.09, and 35.07 (C-2′), 20.74 and 20.63 [*Me*C(O)O], 14.18 and 13.95 (OCH_2CH_3), 12.52 and 12.32 (CH_3 of Thy) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –4.52 and –7.84 ppm. HRMS (ESI): calcd. for $C_{21}H_{30}N_2NaO_{13}P$ $[M + Na]^+$ 571.1299; found 571.1306.

Thymidine 3′,5′-Cyclic *O*-4-Acetylthio-2,2-dimethyl-3-oxobutyl Phosphate (2): Compound **2** was prepared as described for **1**, using *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate (**7**) as the source of the protecting group. The reaction mixture was stirred at room temperature for overnight. Compound **2** (60 mg, 15%) was obtained as a solid. ^1H NMR (500 MHz, $CDCl_3$): δ = 9.66 (s, 1 H, NH), 7.18 and 7.08 (d, *J* = 1.02 Hz, 1 H, 6-H), 6.36 (d, *J* = 8.90 Hz, 1 H, 1′-H), 4.91 and 4.73 (q, *J* = 9.10 Hz, 1 H, 3′-H), 4.65–4.48 and 4.39–4.34 (m, 2 H, 5′-H, 5′′-H), 4.25–4.10 (m, 2 H, $POCH_2$), 4.00–3.85 (m, 3 H, SCH_2, 4′-H), 2.65–2.58 (m, 1 H, 2′-H), 2.52–2.47 (m, 1 H, 2′′-H), 2.40 and 2.35 (s, 3 H, AcS), 2.04 (s, EtOAc), 1.99 and 1.95 (d, *J* = 0.75 Hz, 3 H, CH_3 of Thy), 1.38, 1.33, 1.32, and 1.30 (s, 3 H, CH_3) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 205.88 and 205.33 (C=O), 194.56 and 194.53 (C=O of AcS), 163.67 and 163.64 (C-4), 150.29 and 150.20 (C-2), 135.74 and 135.35 (C-6), 112.38 and 113.35 (C-5), 84.90 and 84.67 (C-1′), 78.00, 77.98, 76.96, and 76.94 (C-3′), 74.03, 73.98, 73.71, and 73.66 (C-4′), 73.44, 73.38, 72.52, 72.48, 69.71, 69.64, 69.35, and 69.29 ($POCH_2$ and C-5′), 48.72, 48.66, and 48.60 (spiro C), 36.43 and 36.41 (SCH_2), 35.27, 35.21, 35.10, and 35.03 (C-2′), 30.23, 30.18, and 29.67 (Me*C*=O), 21.81, 21.66, 21.56, and 21.50 (CH_3), 12.62 and 12.53 (CH_3 of Thy) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –4.48 and –7.27 ppm. HRMS (ESI): calcd. for $C_{18}H_{26}N_2NaO_9PS$ $[M + Na]^+$ 499.0911; found 499.0915.

Thymidine 3′,5′-Cyclic *O*-4-(*tert*-Butyldisulfanyl)-2,2-dimethyl-3-oxobutyl Phosphate (3): Compound **3** was prepared as described for **1** and **2**. A solution of 1-(*tert*-butyldisulfanyl)-4-hydroxy-3,3-dimethylbutan-2-one (**6**), used as the source of the protecting group, in CH_2Cl_2 (5 mL) was added dropwise to the reaction mixture at 0 °C. Compound **3** (80 mg, 13%) was obtained as a solid. ^1H NMR (500 MHz, $CDCl_3$): δ = 9.07 (s, 1 H, NH), 7.22 and 7.03 (s, 1 H, 6-H), 6.39 and 6.34 (dd, *J* = 8.95, 2.50 Hz, 1 H, 1′-H), 4.90 and 4.70 (q, *J* = 9.30 Hz, 1 H, 3′-H), 4.66–4.37 (m, 2 H, 5′-H, 5′′-H), 4.26–3.87 (m, 3 H, $POCH_2$, 4′-H), 3.88, 3.87, and 3.84 (s, 2 H, SCH_2), 2.68–2.59 (m, 1 H, 2′-H), 2.54–2.47 (m, 1 H, 2′′-H), 2.05 and 1.97 (s, 3 H, CH_3 of Thy), 1.36 and 1.35 (s, 9 H, CH_3 of *t*Bu), 1.37 and 1.26 (s, 6 H, CH_3) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 207.97 and 206.73 (C=O), 163.43 (C-4), 150.14 and 149.96 (C-2), 135.42 and 135.21 (C-6), 112.64 and 112.41 (C-5), 85.08 and 84.41 (C-1′), 77.65, 77.61, 77.14, and 77.10 (C-3′), 74.15, 74.10, 73.70, 73.66, 73.61 72.33, and 72.28 (C-4′ and $POCH_2$), 69.84, 69.80, 69.27, 69.65, and 69.21 (C-5′), 48.65, 48.48, 48.41, 48.34, 48.25, and 48.19 (SCH_2), 47.70 and 47.59 (spiro C), 35.21 and 35.15 (C-2′), 29.86 and 29.82 (CH_3 of *t*Bu), 21.86, 21.56, 21.52, and 21.47 (CH_3), 12.69 and 12.58 (CH_3 of Thy) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –4.30 and –7.50 ppm. HRMS (ESI): calcd. for $C_{20}H_{31}N_2NaO_8PS_2$ $[M + Na]^+$ 545.1152; found 545.1156.

2′-*C*-Methyluridine 3′,5′-Cyclic *O*-3-Acetyloxy-2,2-bis(ethoxycarbonyl)propyl Phosphate (4): Compound **4** was prepared as described for **1**, **2**, and **3**. Compound **4** (80 mg, 20%) was obtained as a solid. ^1H NMR (500 MHz, $CDCl_3$): δ = 9.54 (s, 1 H, NH), 7.38 (d, *J* = 8.10 Hz, 1 H, 6-H), 6.22 (s, 1 H, 1′-H), 5.80 (d, *J* = 8.05 Hz, 1 H, 5-H), 5.30 (CH_2Cl_2), 4.77–4.29 (m, 7 H, OCH_2, $POCH_2$, 5′-H, 5′′-H, 4′-H), 4.27 (q, *J* = 7.10 Hz, 4 H, OCH_2CH_3), 4.07 (d, *J* = 9.15 Hz, 1 H, 3′-H), 2.18 (acetone), 2.07 and 2.05 (s, 3 H, AcO), 1.34–1.23 (m, 9 H, 2′-Me and 2 CH_3 of Et) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.58 (Me*C*=O), 166.37 (OC=O), 162.72 (C-4), 150.40 (C-2), 139.16 (C-6), 103.12 (C-5), 93.80 (C-1′), 82.68 and 82.63 (C-3′), 74.80 (C-2′), 69.87, 69.79, and 69.76 (C-4′ and C-5′), 64.81 ($POCH_2$), 62.60 (OCH_2CH_3), 61.28 (CH_2OAc), 58.27 (spiro C), 29.70 (acetone), 20.77 [*Me*C(O)O], 19.77 (2′-*Me*), 13.96 (OCH_2CH_3) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = –6.35 and –7.55 ppm. HRMS (ESI): calcd. for $C_{21}H_{30}N_2O_{14}P$ $[M + H]^+$ 565.1435; found 565.1435.

Kinetics Experiments: Mixtures of R_P and S_P diastereomers of phosphotriesters **1**–**4** were transformed into nucleoside 3′,5′-cyclic monophosphates in the presence of porcine liver esterase (PLE; 2.6 units mL^{-1}) or glutathione in a HEPES buffer (0.036/0.024 M;

3 mL) at pH 7.5 and 37 °C. The deprotection of phoshotriester **2** was also followed in the absence of PLE at pH 7.5 and 37 °C. The initial concentration of the starting materials was 0.15 mM. The conversion was monitored by HPLC. Samples (200 μL) were taken from the reaction solution at appropriate time intervals, and were made acidic (pH 2) with aqueous HCl (1 M; 10 μL) to inactivate the enzyme and to quench the hydrolysis. A 7 min isocratic elution with AcOH/NaOAc buffer (0.045/0.015 M) containing NH_4Cl (0.1 M) and 0.5% MeCN, followed by a 30 min linear gradient up to 70.0% MeCN was used to separate the products. The ionic strength of the solutions used in the HPLC analysis was adjusted to 0.1 M with sodium chloride.

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**–**4**, **6**, **9**, and **10**.

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