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4-(Acetylthio)-2,2-dimethyl-3-oxobutyl and 4-(*tert*-Butyldisulfanyl)-2,2-dimethyl-3-oxobutyl as Protecting Groups for Nucleoside 5′-Phosphoramidates Derived from L-Alanine Methyl Ester

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Keywords: Phosphoramidates / Protecting groups / Prodrugs / Kinetics / Nucleosides / Amino acids

Phosphoramidates **1** and **2** were synthesized by *H*-phosphonate methodology and subsequent oxidative amination with L-alanine methyl ester. The removal of the protecting groups at pH = 7.5 and 37 °C in the absence and presence of porcine liver esterase (PLE) or glutathione (GSH) was monitored by HPLC. The stability of phosphoramidate **1** was additionally studied at pH = 9 and 10. The reduction of the disulfide bond with glutathione from **2** triggers the removal of the protecting group by cyclization releasing quantitatively nucleoside 5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]-phosphoramidate} (**7**) as the desired product. With **1**, enzymatic deacetylation or acetyl migration from the sulfur atom to the adjacent hydrated oxo group followed by chemical cyclization produces **7**. The S–S-bond-mediated dimerization (**8**) competes as a side reaction. Prolonged treatment, however, resulted in the conversion of the S–S dimer **8** into **7** that undergoes slow alanine methyl ester hydrolysis to form **10**.

Introduction

The antiviral and anticancer activity of structurally modified nucleosides depends on their uptake into cells and their intracellular conversion into nucleotides. To bypass the initial, usually rate-limiting, phosphorylation,[1] nucleotides protected with biodegradable protecting groups are often used instead of nucleosides as drug candidates.[2] One such class of prodrugs are the 5′-*O*-aryl-*N*-[2-(alkoxycarbonyl)alkyl]phosphoramidates, which have shown higher antiviral potency than their parent nucleoside analogs.[3] 2′-Fluoro-2′-deoxy-2′-*C*-methyluridine 5′-(*O*-phenyl-*N*-isopropoxyalaninyl)phosphoramidate (Sofosbuvir) has even been approved for the treatment of HCV (Hepatitis C). Additionally, 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-[*O*-(4-bromophenyl)-*N*-methoxyalaninyl]phosphoramidate (Stampidine) and 5-(2-bromovinyl)-2′-deoxyuridine 5′-(*O*-phenyl-*N*-methoxyalaninyl)phosphoramidate (Thymectacin), developed as anti-HIV and anticancer drugs, respectively, as well as O^6-methyl-2′-*C*-methylguanosine 5′-(*O*-naphth-1-yl-*N*-neopentoxyalaninyl)phosphoramidate (INX-08189) and O^6-methyl-2′-*C*-methylguanosine 5′-(*O*-phenyl-*N*-isopropoxyalaninyl)phosphoramidate (PSI-353661) for HCV treatment, have advanced to clinical trials.[4] It has been suggested that the carboxylic ester linkages of amino-acid-derived aryloxyphosphoramidates of nucleoside 5′-monophosphates undergo hydrolysis triggered by esterase or cathepsin A,[5] and that this is followed by intramolecular displacement of the phenoxide ion by the carboxylate group. The resulting cyclic mixed anhydride then undergoes hydrolysis to give an *N*-acylated phosphoramidate, and finally intracellular phosphoramidase or HINT-1[6] releases the nucleoside 5′-monophosphate.[7] Another prodrug strategy for nucleoside 5′-phosphoramidates derived from L-amino acid methyl esters is based on protection of the phosphoramidate oxygen atom with an *S*-pivaloyl-2-thioethyl group (SATE).[8] In this case, carboxyesterase-mediated hydrolysis of the pivaloyloxy group is followed by a non-enzymatic departure of the remaining 2-mercaptoethyl linker in a cyclization to give the episulfide.[2] Esterase-labile 2,2-disubstituted 3-(acyloxy)propyl and 3-(acyloxymethoxy)propyl groups have, in turn, been shown to release the amino acid phosphoramidate by an esterase-triggered retroaldol condensation that gives an enone-like by-product.[9] It has also been shown that amino-acid-derived phosphoramidates may themselves become incorporated into DNA by HIV reverse transcriptase.[10]

We now report on the thermally and enzymatically labile 2,2-disubstituted 4-(acylthio)-3-oxobutyl group[11] and the reductively cleavable 4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl group[12] as alternative protecting groups for nucleoside 5′-phosphoramidates. For this purpose, 2′,3′-*O*-isopropylideneuridine 5′-{*O*-[4-(acetylthio)-2,2-dimethyl-3-oxobutyl]-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**1**) and 2′,3′-*O*-isopropylideneuridine 5′-

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{*O*-[4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl]-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**2**) were prepared as model compounds (Figure 1). The protecting groups introduced are released after deacylation or reduction by chemical cyclization to give 4,4-disubstituted dihydrothiophen-3(2*H*)-one, which, as expected, does not show significant alkylation activity,[11] unlike the enone and episulfide by-products mentioned above. The cleavage rate of the 2,2-disubstituted 4-(acylthio)-3-oxobutyl group may be tuned by the size of the 4-(acylthio) group and the size and electronegativity of the 2-substituents.[11] Deprotection of **1** and **2** was monitored by HPLC in the absence and presence of enzyme or glutathione at 37 °C and pH = 7.5.

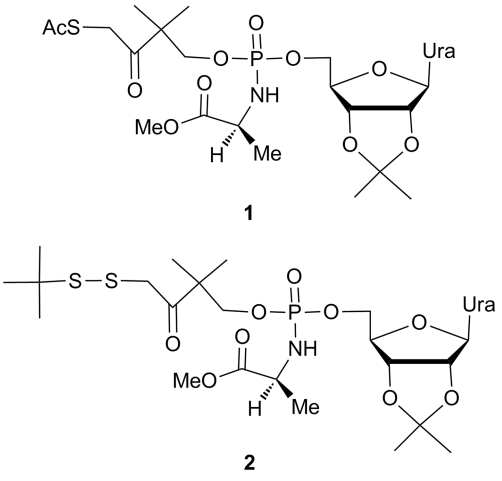


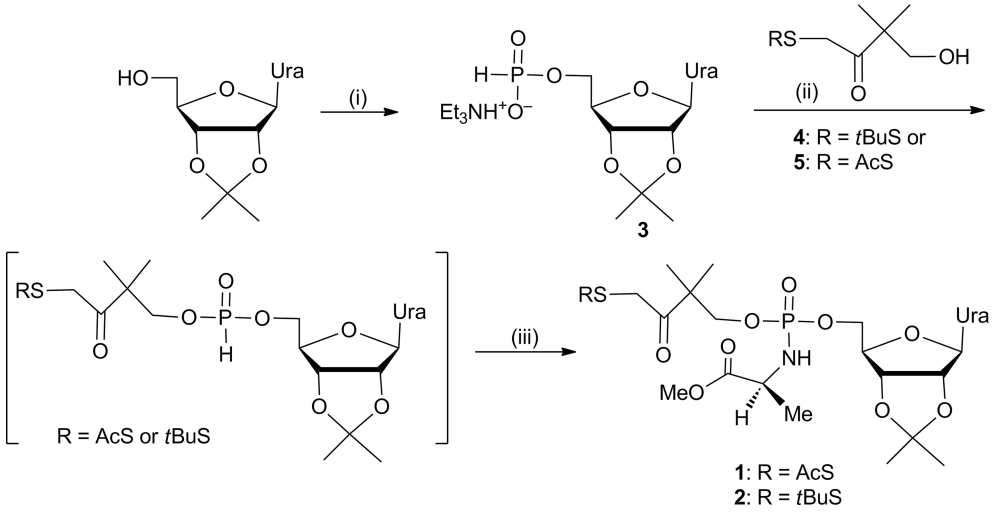
Figure 1. Structures of phosphoramidates **1** and **2**.

Results and Discussion

Synthesis

2′,3′-*O*-Isopropylideneuridine 5′-{*O*-[4-(acetylthio)-2,2-dimethyl-3-oxobutyl]-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**1**) and 2′,3′-*O*-isopropylideneuridine 5′-{*O*-[4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl]-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]-phosphoramidate} (**2**) were prepared by *H*-phosphonate methodology with a subsequent amination with an amino acid methyl ester under Atherton–Todd conditions (Scheme 1).[13] Accordingly, treatment of diphenyl phosphite with commercially available 2′,3′-*O*-isopropylideneuridine in pyridine at room temperature, followed by addition of a mixture of H_2O and Et_3N gave the triethylammonium salt of 2′,3′-*O*-isopropylideneuridine 5′-(*H*-phosphonate) (**3**; Scheme 1). Compound **3** was esterified with 1-(*tert*-butyldisulfanyl)-4-hydroxy-3,3-dimethylbutan-2-one (**4**)[12] or *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl) ethanethioate (**5**)[11] in pyridine using pivaloyl chloride as activator. The product, the 4-(acetylthio)-2,2-dimethyl-3-oxobutyl or 4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl ester of 2′,3′-*O*-isopropylidene 5′-(*H*-phosphonate), was not isolated, but subjected to oxidative amination with L-alanine methyl ester in the presence of CCl_4 and Et_3N in a mixture of MeCN and pyridine. The overall yields of **1** and **2** from **3** were 21 and 23%, respectively.

Phosphorus trichloride was also used as a phosphitylating agent instead of diphenyl phosphite. Accordingly, 2′,3′-*O*-isopropylideneuridine was treated with PCl_3 in CH_2Cl_2 at –20 °C. Reaction of the resulting 5′-dichlorophosphite with *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl) ethanethioate and subsequent hydrolysis in aq. THF gave the 4-(acetylthio)-2,2-dimethyl-3-oxobutyl ester of 2′,3′-*O*-isopropylideneuridine 5′-(*H*-phosphonate). Oxidative amination was then carried out as described above. The yield (10%) was, however, lower than that obtained using diphenyl phosphite as the phosphitylating agent. Attempts to prepare phosphoramidate **1** by oxidative coupling,[12,14] i.e., by iodination of the 5′-[4-(acetylthio)-2,2-dimethyl-3-oxobutyl *H*-phosphonate] and subsequent displacement of iodine from the iodophophate with L-alanine methyl ester, failed.



Scheme 1. Preparation of phosphoramidates **1** and **2**. Reaction conditions: (i) diphenyl phosphite, py; (ii) PivCl; (iii) py/MeCN, CCl_4, Et_3N, H-Ala-OMe·HCl.

Deprotection of 4-(Acetylthio)-2,2-dimethyl-3-oxobutyl- and 4-(*tert*-Butyldisulfanyl)-2,2-dimethyl-3-oxobutyl-Protected Phosphoramidates 1 and 2

The removal of the protecting groups from disastereomeric (R_P/S_P) phosphoramidates **1** and **2** was studied at 37 °C by analysing the composition of aliquots withdrawn as a function of time by reverse-phase HPLC. The deprotection of **1** was monitored in the presence of porcine liver esterase (PLE; 2.6 U mL^{-1}) at pH = 7.5, and in the absence of PLE over a pH range from 7.5 to 10, whereas the deprotection of **2** was carried out in the presence of glutathione (GSH; 5 mM) at pH = 7.5 (see Table 1).

Table 1. First-order rate constants and half-lives for the decomposition of diastereomeric nucleoside 5′-phosphoramidates **1** and **2** at 37 °C (I = 0.1 M with NaCl).

Compound	k [10^{-4} s^{-1}]	$t_{1/2}$ [min]	Reaction solution
1	0.134	862	pH = 7.5
1	6.75	17.1	pH = 9.0
1	36.3	3.2	pH = 10.0
1	ca. 12.0[a]	ca. 10[a]	pH = 7.5 (PLE 2.6 U mL^{-1})
2	2.03	56.9	pH = 7.5 (GSH 5 mM)

[a] 50% of **1** was converted into deacetylated intermediate **6**.

Reductive Deprotection of Phosphoramidate 2

In the presence of glutathione, the disulfide bond of **2** (t_R = 28.6 min) was reductively cleaved. The half-life for the disappearance of the starting material was 56.9 min (k = 2.03×10^{-4} s^{-1}). 2′,3′-*O*-Isopropylideneuridine 5′-{*O*-(4-mercapto-2,2-dimethyl-3-oxobutyl)-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**6**; t_R = 23.5 min; m/z = 602.1 [M + Na]$^+$) accumulated as an intermediate and underwent quantitative conversion into 2′,3′-*O*-isopropylideneuridine-5′-{*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]-phosphoramidate} (**7**; t_R = 16.7 min; m/z = 448.1104 [M – H]$^-$; Figure 2 and Scheme 2), the rate constant being 6.50×10^{-4} s^{-1} ($t_{1/2}$ = 17.8 min). Concurrently, GSH was oxidized to glutathione disulfide (GSSG; t_R = 3.7 min).

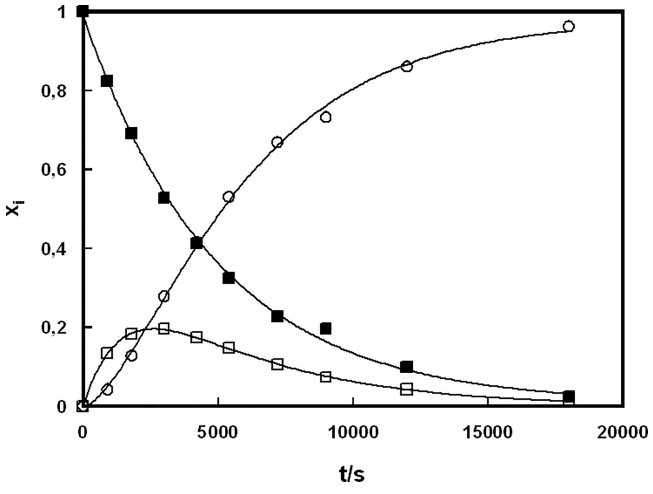
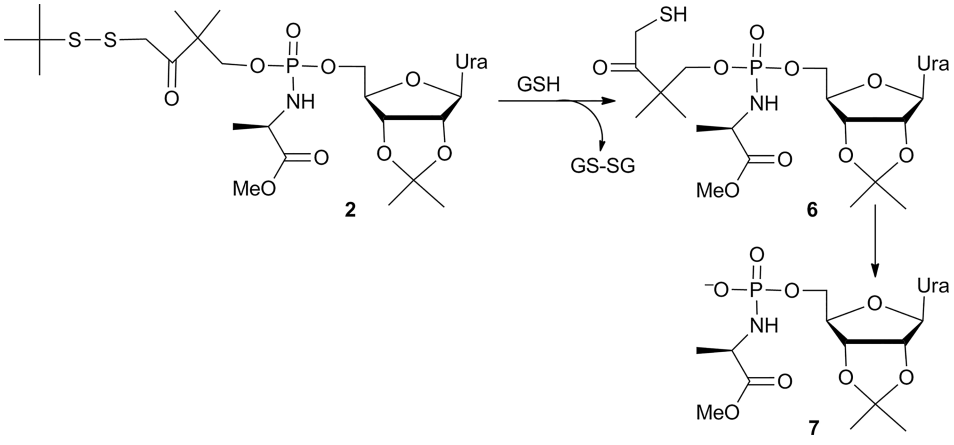


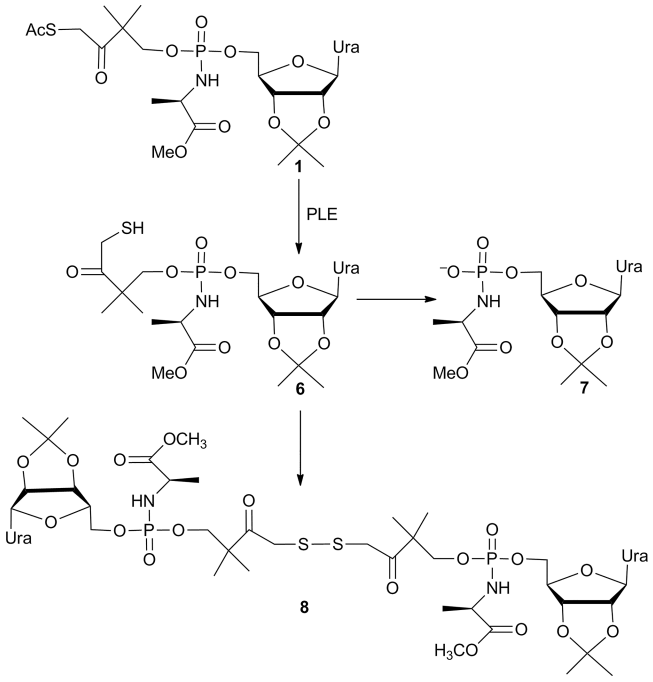
Figure 2. Time-dependent product distribution for the hydrolysis of diastereomeric 2′,3′-*O*-isopropylideneuridine 5′-{*O*-[4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl]-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (**2**) in the presence of GSH at pH = 7.5 and 37 °C. Notation: **2** (filled squares), **7** (circles), and **6** (empty squares).

Enzymatic Deprotection of the Phosphoramidate 1

In the presence of PLE, compound **1** (t_R = 25.7 min) undergoes PLE-triggered deprotection to give **7** (82% of the products; Scheme 3) via deacetylated intermediate **6**, which is also converted into its S–S dimer **8** (18% of the products; t_R = 28.6 min; m/z = 1179.3154 [M + Na]$^+$). This observation is consistent with earlier findings with oligomeric phosphodiesters, which undergo an intrachain S–S-bond-mediated reaction between two deacetylated 4-(acetylthio)-2,2-dimethyl-3-oxobutyl protecting groups.[15] As seen from Figure 3, the enzymatic hydrolysis of **1** is initially rapid, but then becomes slower. For example, 50% of the starting material was converted into deacetylated intermediate **6** within 10 min, but to reach 90% conversion took 125 min. Most probably, one of the two diastereomers (i.e., R_P-**1** or S_P-**1**) reacts more readily than the other.



Scheme 2. Hydrolysis of diastereomeric phosphoramidates **2** in the presence of GSH.



Scheme 3. Enzymatic hydrolysis of diastereomeric phosphoramidates **1**.

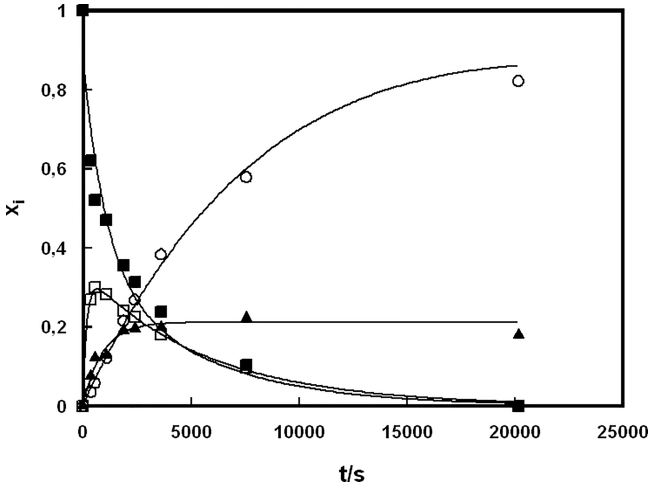
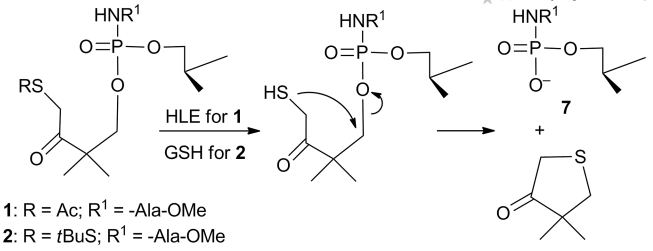


Figure 3. Time-dependent product distribution for hydrolysis of diastereomeric 2′,3′-*O*-isopropylideneuridine 5′-{*O*-[4-(acetylthio)-2,2-dimethyl-3-oxobutyl]-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]-phosphoramidate} (**1**) in the presence of PLE at pH = 7.5 and 37 °C. Notation: **1** (filled squares), **6** (empty squares), **7** (circles), and **8** (triangles).

As discussed previously,[11] the mercapto intermediate (i.e., **6**), obtained by the esterase-catalysed hydrolysis of **1** or thiol/disulfide exchange reaction of **2** with GSH, releases the phosphoramidate monoanion (i.e., **7**) by departure of the remnant of the protecting group in a cyclization reaction to give 4,4-dimethyl-dihydrothiophen-3(2*H*)-one (Scheme 4).



Scheme 4. Mechanism of PLE- and GSH-triggered deprotection.

Non-Enzymatic Deprotection of Phosphoramidate 1

In the absence of PLE at pH = 7.5, **7** was observed as the major product (see Figure 4) and disulfide **8** as an intermediate. In addition, two side-products were formed: 2′,3′-*O*-isopropylideneuridine (**9**; t_R = 18.0 min; 10%; *m*/*z* = 283.0 [M – H]$^-$) and the deprotected phosphoramidate with the alanyl ester function hydrolysed (i.e., **10**; 10%; t_R = 15.5 min; *m*/*z* = 436.1121 [M + H]$^+$). The rate constant for the disappearance of the starting material (i.e., **1**) was 1.34×10^{-5} s^{-1} ($t_{1/2}$ = 14.4 h). Prolonged treatment (4 d) in a HEPES buffer resulted in an accumulation of **7** (70%) and **10** (20%) as the main products.

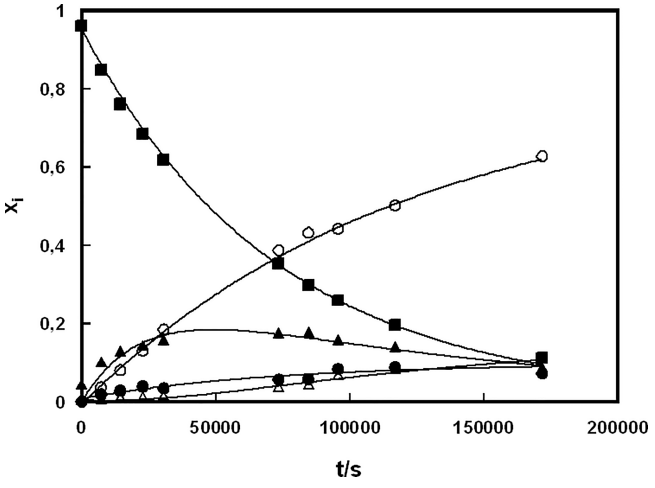
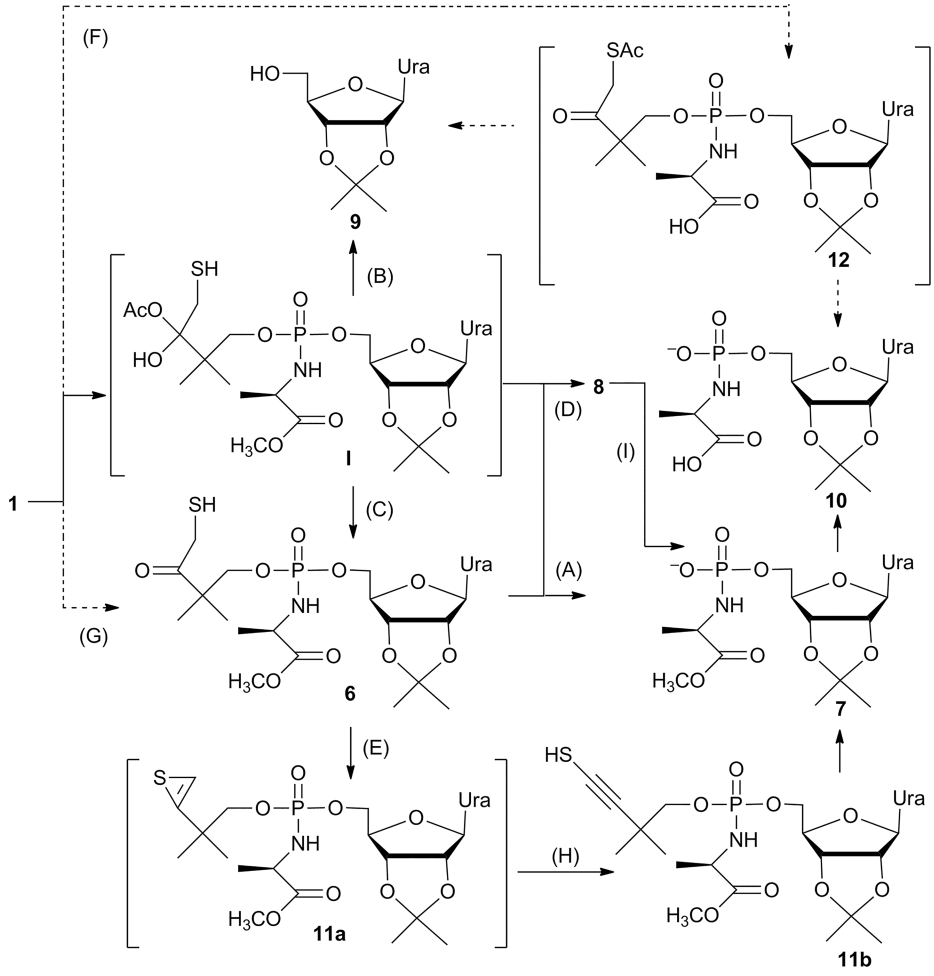


Figure 4. Time-dependent product distribution for hydrolysis of diastereomeric 2′,3′-*O*-isopropylideneuridine 5′-{*O*-[4-(acetylthio)-2,2-dimethyl-3-oxobutyl]-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]-phosphoramidate} (**1**) at pH = 7.5 and 37 °C. Notation: **1** (filled squares), **7** (empty circles),**8** (filled triangles), **9** (filled circles), and **10** (empty triangles).

Under basic conditions (pH = 9–10), the product distribution was more complicated. In addition to the products mentioned above, **6** was observed (at pH = 10), and also the 4-mercapto-2,2-dimethyl-3-oxobutyl group was converted into a 4-mercapto-2,2-dimethylbut-3-yn-1-yl group (to give **11b**) or a 2-methyl-2-(thiiren-2-yl)propyl group (to give **11a**) (Scheme 5). Most probably, a peak observed by HPLC/ESI-MS (t_R = 21.2 min) with *m*/*z* = 562.2 is due to the molecular ion [M + H]$^+$ of **11b**, the thiirene **11a** being unstable. Two unidentified intermediates were also detected. The rate constants for the disappearance of the starting material (i.e., **1**) were 6.75×10^{-4} s^{-1} and 3.63×10^{-3} s^{-1} at pH



Scheme 5. Non-enzymatic hydrolysis of phosphoramidate **1**.

= 9 and 10, respectively. As an example, the product distribution at pH = 10 is shown in Figure 5. Under these conditions, **7** finally underwent hydrolysis to **10**. After 2 d, **7** (15%) and **10** (75%) accumulated as the main products.

In all likelihood, hydration of the oxo group at C-3 initiates the non-enzymatic decomposition of **1**, allowing migration of the acetyl group from the sulfur atom to the adjacent oxygen atom, as described recently (see Schemes 5 and 6).[11] Intermediate **I** does not accumulate, but breaks down as follows: (i) removal of the protecting group [Route (A)] by intramolecular attack of the sulfur atom on C-1, which results in the release of **7**,[11] (ii) cleavage of the P–O-5′ bond [Route (B)] by intramolecular attack of the oxyanion of the hydrated oxo group onto the phosphorus atom[11] to give **9**, and (iii) departure of acetic acid to give **6** [Route (C)], which is further converted into **7**. The S–S-bond-mediated dimerization to give **8**, most probably also takes place via **6** [Route (C)/(D)] after the loss of AcOH, although disulfide bond formation before the elimination of AcOH [Route (D) or Route (G)/(D)] cannot strictly be excluded. No evidence for the appearance of an S–S dimer bearing acetyl group(s) was obtained. Application of the

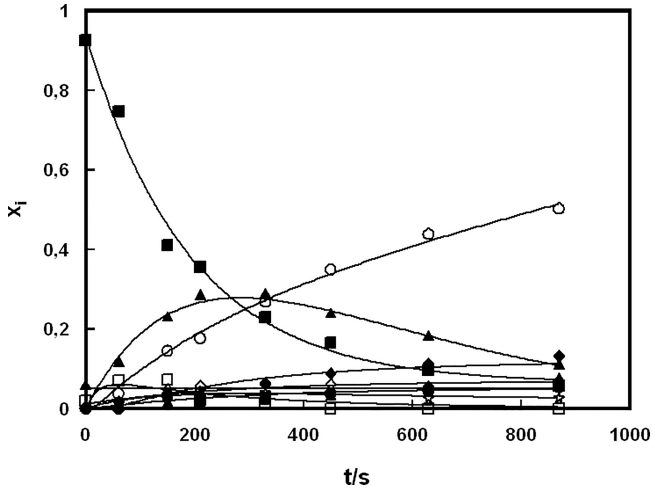
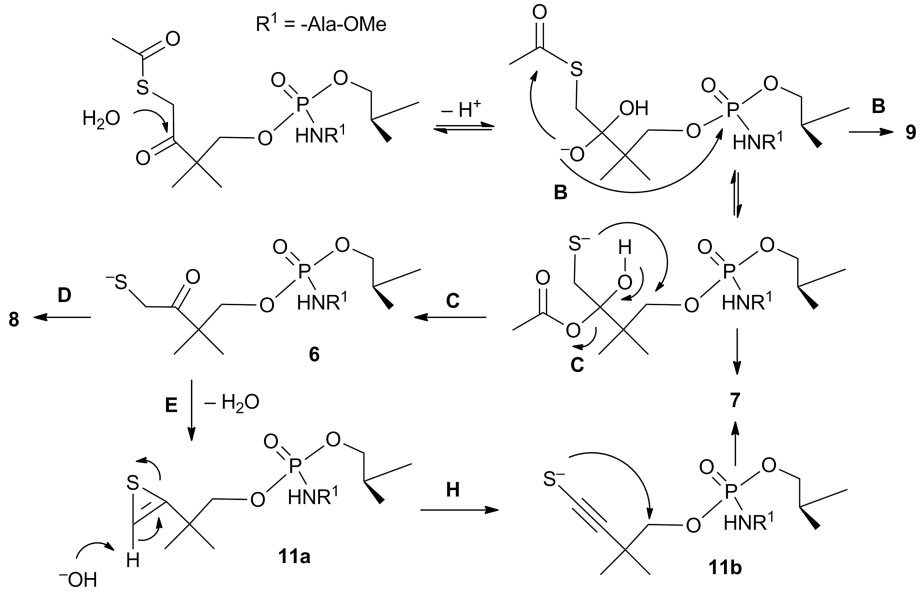


Figure 5. Time-dependent product distribution for hydrolysis of diastereomeric 2′,3′-*O*-isopropylideneuridine 5′-{*O*-[4-(acetylthio)-2,2-dimethyl-3-oxobutyl]-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]-phosphoramidate} (**1**) at pH = 10 and 37 °C. Notation: **1** (filled squares), **7** (empty circles), **8** (filled triangles), **9** (filled circles), **11** (stars), **10** (empty triangles), **6** (empty squares), and two unknown products (filled and empty diamonds).

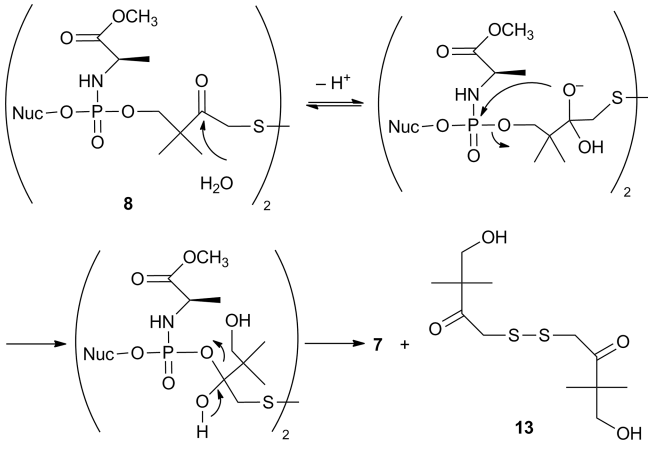


Scheme 6. Mechanism of the non-enzymatic removal of the protecting group, cleavage of the P–O bond, and S–S-bond-mediated dimerization.

rate-law of parallel consecutive first-order reactions for **8** suggests that the S–S-bond-mediated dimerization to give **8** represents 74 and 62% of the overall disappearance of **1** at pH = 7.5 and 9, respectively. At pH = 10, **6** accumulated as an intermediate; so the rate constant for the formation of **8** could not be reliably determined. The intermediate accumulation of **11a** or **11b** [Route (E)] can, in turn, be explained by attack of the sulfur atom of **6** on the adjacent carbonyl group, and subsequent elimination of water (see Schemes 5 and 6). The resulting thiirene (i.e., **11a**) is then converted into **7** via ethynylthiol derivative **11b** [Route (H)] by a ring-opening reaction followed by attack of the sulfur atom on C-1.

The thioester hydrolysis [Route (G); **1** → **6**] and the alaninyl methyl ester hydrolysis [Route (F); **1** → **12**] represent only minor pathways, as evidenced by the following facts. The rate of hydroxide-ion-catalysed hydrolysis of the thioester linkage has been reported to be comparable to that of the ester linkage.[16] Moreover, earlier studies indicate that the acetate ester hydrolysis of 2′-*O*-methylcytidine 5′-{*O*-3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl-*N*-[(1*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} is 11 and 19 times slower than the disappearance of **1** at pH = 9 and 10, respectively.[9] Under these conditions, the hydrolysis of the alaninyl methyl ester group of 2′-*O*-methylcytidine 5′-{*O*-3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl-*N*-[(1*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} is known to take place only 1.5 times faster than the acetate ester hydrolysis.[9] As a conclusion, at most 5–9% and 8–14% of the overall degradation of **1** can be estimated to take place by Routes (G) and (F), respectively.

Dimer **8** breaks down to give mainly **7** [Route (I) in Scheme 5]. Hydration of the oxo group and subsequent attack of the oxyanion of the geminal diol onto the phosphorus atom results in a cleavage of the P–O bond. Finally, hydrolysis of the hemiacetal-like structure takes place to form **7** and 1,1′-disulfanediylbis(4-hydroxy-3,3-dimethylbutan-2-one) (**13**; *m*/*z* = 295.0 [M + H]$^+$; Scheme 7). The conversion of **8** into **9** and **10** is also possible after the alaninyl methyl ester hydrolysis by intramolecular displacement of 2′,3′-isopropylideneuridine (**9**) or the protecting group by the carboxylate ion as described earlier with thymidine 5′-{*O*-phenyl-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate}.[7h] In the latter case, hydrolysis of the resulting cyclic mixed anhydride may then produce **10**. This pathway does not, however, play a significant role. As seen from Figure 5, only a small amount of **9** (<3%) and **10** (<6%), which may also be formed via **12** and/or **I**, is obtained upon decomposition of **8** at pH = 10. Moreover, the decomposition of **8** takes place eight times as fast as the alaninyl methyl ester hydrolysis of 2′-*O*-methylcytidine 5′-



Scheme 7. Mechanism of the conversion of **8** into **7**.

{*O*-3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl-*N*-[(1*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate}, at pH = 9.[9]

Conclusions

Thermally and enzymatically labile (**1**) and reductively labile (**2**) phosphoramidates were prepared by the diphenylphosphite-based *H*-phosphonate strategy and subsequent oxidative amination with L-alanine methyl ester. Glutathione-triggered deprotection of **2** produced exclusively, and enzyme-triggered deprotection of **1** mainly, diester-like phosphoramidate **7** via intermediate **6**, formed by reduction or deacetylation, respectively. In the absence of enzyme, migration of the acetyl group from the sulfur atom to the adjacent hydrated oxo group triggers the deprotection of **1** to give **7**. The S–S-bond-mediated dimerization to give **8** competes with the removal of the protecting group from **1**. In all cases, an attack of the exposed sulfur atom onto C-1 results in the removal of the protecting group by cyclization. Finally, dimer **8** also undergoes degradation to give mainly **7**.

Experimental Section

General Remarks: 1-(*tert*-Butyldisulfanyl)-4-hydroxy-3,3-dimethylbutan-2-oneethanethioate (**5**)[11] were prepared as described previously. Pyridine and dichloromethane were dried with 4 Å molecular sieves. Pivaloyl chloride was distilled before use. ^{1}H, ^{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 with an L-7455 UV detector and an L-7100 pump using an ODS Hypersil C18 column (4 × 250 mm, 5 μm).

Kinetic Measurements: The breakdown of the mixtures of R_P and S_P diastereomers of phosphoramidates **1** and **2** in HEPES (0.036/0.024 M; 3 mL; pH = 7.5) and glycine (0.02/0.04 and 0.05/0.01 M; pH = 9 and 10, respectively) buffers at 37 °C was monitored by reverse-phase HPLC (UV detection at 260 nm, flow rate 0.95 mL min^{-1}) in the absence and presence of porcine liver esterase (PLE; 2.6 U mL^{-1}) or glutathione (GSH; 5 mM). The ionic strength of the solutions was adjusted to 0.1 M with sodium chloride. The initial concentration of the starting material was 0.15 mM. Samples (200 μL) were withdrawn from the reaction solution at appropriate time intervals, and were made acidic (pH = 3) with HCl (1 M, aq.; 10 μL) to deactivate the enzyme and quench the hydrolysis. The solution was filtered with Minisart RC 4 filters (0.2 μm). Products were separated using 7 min isocratic elution with AcOH/AcONa buffer (0.045/0.015 M) containing NH_4Cl (0.1 M) and 0.5% MeCN, followed by a 35 min linear gradient up to 70.0% MeCN. The products detected were identified by HPLC/ESI-MS, or samples were collected and identified by MS analysis.

2′,3′-*O*-Isopropylidene 5′-(*H*-Phosphonate) (3): 2′,3′-*O*-Isopropylideneuridine (1.0 g, 3.5 mmol) was dissolved in dry pyridine (10 mL) while cooling with an ice bath. Diphenyl phosphite (1.16 mL, 5.2 mmol) was added dropwise, and the mixture was stirred for 15 min. The ice bath was removed, and the reaction was allowed to proceed at room temperature for 3 h. A mixture of Et_3N (3 mL) and H_2O (3 mL) was added. The mixture was stirred for 15 min, then the solvent was removed under reduced pressure, and the residue was coevaporated twice with toluene (15 mL). The crude product was purified by column chromatography eluting with dichloromethane containing 2–10% methanol to give **3** (1.43 g, 90%) as a solid. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.83 (d, J = 8.10 Hz, 1 H, 6-H), 6.79 (d, J = 622.31 Hz, 1 H, PH), 5.95 (d, J = 2.90 Hz, 1 H, 1-H), 5.76 (d, J = 8.10 Hz, 1 H, 5-H), 4.95 (dd, J = 6.25 and 2.95 Hz, 1 H, 2′-H), 4.92 (dd, J = 6.30 and 2.85 Hz, 1 H, 3′′-H), 4.36 (m, 1 H, 4′-H), 4.08–4.05 (m, 2 H, 5′-H and 5′′-H), 3.22 (q, J = 7.30 Hz, 6 H, Et_3N), 1.57 (s, 3 H, CH_3), 1.37 (s, 3 H, CH_3), 1.33 (t, J = 7.30 Hz, 9 H, Et_3N) ppm. ^{13}C NMR (126 MHz, CD_3OD): δ = 164.72 (C-4), 150.73 (C-2), 142.10 (C-6), 113.77 (spiro C), 101.64 (C-5), 92.1 (C-1′), 85.16 and 85.10 (C-4′), 84.21 (C-2′), 81.11 (C-3′), 63.38 and 63.35 (C-5′), 46.37 (CH_2 of Et_3N), 26.1 and 24.14 (CH_3), 7.81 (CH_3 of Et_3N) ppm. ^{31}P NMR (202 MHz, CD_3OD): δ = 4.39 ppm. HRMS (ESI$^-$): calcd. for $C_{12}H_{16}N_2O_8P$ [M – H]$^-$ 347.0650; found 347.0650.

2′,3′-*O*-Isopropylideneuridine 5′-{*O*-[4-(Acetylthio)-2,2-dimethyl-3-oxobutyl]-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (1)

Procedure A: 2′,3′-*O*-Isopropylideneuridine 5′-(*H*-phosphonate) (**3**; 150 mg, 0.334 mmol) was dissolved in pyridine (3 mL), and pivaloyl chloride (83 μL, 0.668 mmol) was added dropwise. The mixture was stirred at room temperature for 5 min, then *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl) ethanethioate (**5**; 70 mg, 0.367 mmol) was added dropwise. The reaction mixture was stirred for 30 min. The resulting 2′,3′-*O*-isopropylideneuridine 5′-(*H*-phosphonate) 4-(acetylthio)-2,2-dimethyl-3-oxobutyl ester was not isolated, but CCl_4 (0.25 mL) and Et_3N (0.75 mL) were added. The mixture was stirred for 5 min, then a solution of L-alanine methyl ester hydrochloride (70 mg, 0.501 mmol) in a mixture of pyridine (0.5 mL) and MeCN (1.5 mL) was added dropwise. The mixture was stirred for 2 h, then EtOAc (50 mL) was added, and the organic phase was washed with water (2 × 10 mL). The organic phase was dried with Na_2SO_4, and the solvents were evaporated. The crude product was purified by column chromatography eluting with EtOAc to give **1** (42 mg, 21%) as a white solid foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.50 (br. s, 1 H, NH), 7.42 and 7.38 (d, J = 8.10 and 8.10 Hz, 1 H, 6-H), 5.79 and 5.72 (d, J = 2.50 and 2.40 Hz, 1 H, 1′-H), 5.77 and 5.75 (d, J = 8.05 and 8.05 Hz, 1 H, 5-H), 4.94–4.92 (m, 1 H, 2-H), 4.90 and 4.85 (dd, J = 6.45 and 3.70 Hz, 1 H, 3-H), 4.31–4.29 (m, 1 H, 4-H), 4.24–4.17 (m, 2 H, 5′-H and 5′′-H), 4.06–4.00 (m, 2 H, OCH_2), 3.96–3.87 (3 H, SCH_2 and H^{α}), 3.73 (s, 3 H, OCH_3), 2.37 (s, 3 H, SAc), 1.57, 1.56, 1.36 and 1.35 (s, 6 H, CH_3 of isopropylidene), 1.39 and 1.38 (d, J = 1.80 and 1.95 Hz, 3 H, β CH_3), 1.28, 1.25, 1.23, 1.22 and 1.20 (s, 6 H, 2 CH_3) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 205.56 and 205.51 (C=O), 194.81 and 194.78 (AcS), 174.25, and 174.20 (C=O of amino acid), 170.68 (EtOAc), 163.35 and 135.32 (C-4), 150.16 and 150.10 (C-2), 142.01 and 141.86 (C-6), 114.64, 114.59 and 114.56 (spiro C), 102.82, 102.72 and 102.67 (C-5), 94.02 and 93.35 (C-1), 85.41, 85.34, 84.95, and 84.89 (C-4), 84.38 and 84.20 (C-2), 80.64 and 80.39 (C-3), 71.93, 71.89, 71.84 and 71.79 (OCH_2), 65.98, 65.94, 65.82 and 65.78 (C-5), 52.55 (OCH_3), 49.99 and 49.95 (CH amino acid), 48.73, 48.71, 48.66 and 48.63 (spiro C), 46.14 (spiro C), 36.43 (SCH_2), 30.23 (SAc), 27.29, 27.19, 27.15, 25.32 and 25.30 (CH_3 of isopropylidene), 21.69, 21.64 and 21.60 (CH_3 of the protecting group), 21.00 and 20.98 (β CH_3) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = 7.15, 7.04 ppm. HRMS (ESI$^+$): calcd. for $C_{24}H_{36}N_3NaO_{12}PS$ [M + Na]$^+$ 644.1655; found 644.1650.

Precedure B: 2′,3′-*O*-Isopropylideneuridine (284 mg, 1.0 mmol) was dissolved in CH_2Cl_2 (10 mL), and the resulting solution was added dropwise to a solution of PCl_3 (0.9 mL, 10 mmol) in CH_2Cl_2 (10 mL) at –20 °C over a period of 5 min. The mixture was stirred for 1 h, then the cooling bath was removed, and the reaction mixture was stirred at room temperature for 6 h. The solvent was removed under reduced pressure, and the residue was coevaporated twice with CH_2Cl_2 (2 × 20 mL). The 2′,3′-*O*-isopropylideneuridine 5′-dichlorophosphite obtained was not isolated, but it was dissolved in CH_2Cl_2 (5 mL), and a solution of *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl) ethanethioate (**5**; 190 mg, 1.0 mmol) in a mixture of CH_2Cl_2 (2.5 mL) and pyridine (2.5 mL) was added dropwise. The resulting mixture was stirred for 1 h, then a mixture of THF (2 mL) and water (2 mL) was added, and the reaction was allowed to proceed for 30 min. The solvent was removed under reduced pressure, and the residue was coevaporated twice with anhydrous toluene (2 × 20 mL). The resulting 2′,3′-*O*-isopropylideneuridine 5′-(*H*-phosphonate) was not isolated, but was dissolved in anhydrous MeCN (5 mL), and a mixture of CCl_4 (1.5 mL) and Et_3N (0.5 mL) was added. The mixture was stirred for 5 min, then a solution of L-alanine methyl ester hydrochloride (166 mg, 1.2 mmol) in a mixture of MeCN (4 mL) and pyridine (1 mL) was added dropwise at 0 °C. The mixture was stirred for 2 h, then EtOAc (50 mL) was added, and the organic phase was washed with water (2 × 15 mL). The organic phase was dried with Na_2SO_4, and the solvents were evaporated. The crude product was purified by column chromatography eluting with EtOAc to give **1** (63 mg, 10%) as a white solid foam.

2′,3′-*O*-Isopropylideneuridine 5′-{*O*-[4-(*tert*-Butyldisulfanyl)-2,2-dimethyl-3-oxobutyl]-*N*-[(1*S*)-2-oxo-2-methoxy-1-methylethyl]phosphoramidate} (2): 2′,3′-*O*-Isopropylideneuridine 5′-(*H*-phosphonate) (**3**; 0.50 g, 1.11 mmol) was dissolved in pyridine (5 mL), and pivaloyl chloride (272 μL, 2.22 mmol) was added dropwise. The mixture was stirred at room temperature for 5 min, then 1-(*tert*-butyldisulfanyl)-4-hydroxy-3,3-dimethylbutan-2-one (**4**; 290 mg, 1.22 mmol) was added dropwise. The reaction mixture was stirred for 30 min. The resulting 2′,3′-*O*-isopropylideneuridine 5′-(H-phosphonate) 4-(*tert*-butyldisulfanyl)-2,2-dimethyl-3-oxobutyl ester was not isolated, but CCl_4 (1.5 mL) and Et_3N (0.5 mL) were added. The mixture was stirred for 5 min, then a solution of L-alanine methyl ester hydrochloride (232 mg, 1.67 mmol) in a mixture of pyridine (1 mL) and acetonitrile (3 mL) was added dropwise. After 15 min, EtOAc (50 mL) was added, and the organic phase was washed with water (2 × 10 mL). The organic phase was dried with Na_2SO_4, and the solvents were evaporated. The crude product was purified by column chromatography eluting with EtOAc to give **2** (165 mg, 23%) as a white solid foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.45 (br. s, 1 H, NH), 7.42 and 7.37 (d, J = 8.05 and 8.10 Hz, 1 H, 6-H), 5.80 and 5.72 (d, J = 2.55 and 2.25 Hz, 1 H, 1′-H), 5.78 and 5.75 (d, J = 8.05 and 8.05 Hz, 1 H, 5-H), 4.96–4.93 (m, 1 H, 2-H), 4.89 and 4.86 (dd, J = 6.40 and 3.75 Hz, 1 H, 3-H), 4.31–4.28 (m, 1 H, 4-H), 4.23–4.16 (m, 2 H, 5′-H, 5′′-H), 4.08–3.99 (m, 2 H, OCH_2), 3.93–3.80 (m, 3 H, H^α and SCH_2), 3.74 and 3.73 (s, 3 H, OCH_3), 1.57, 1.56 1.36 and 1.35 (s, 3 H, CH_3 of isopropylidene), 1.40 and 1.38 (d, J = 2.30 and 2.45 Hz, 3 H, β CH_3), 1.34 (s, 9 H, *t*Bu), 1.26 (EtOAc), 1.23, 1.22, 1.21 and 1.19 (s, 6 H, 2 CH_3) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ = 207.16 and 207.12 (C=O), 174.24, 174.19 and 174.14 (C=O of amino acid), 163.32 (C-4), 150.16 and 150.09 (C-2), 142.07 and 141.82 (C-6), 114.67 and 114.61 (spiro C), 102.89 and 102.71 (C-5), 94.05 and 93.31 (C-1), 85.48, 85.42, 84.96 and 84.90 (C-4), 84.37 and 84.18 (C-2), 80.66 and 80.43 (C-3), 71.84, 71.79, 71.73 and 71.69 (OCH_2), 65.99, 65.94, 65.80 and 65.76 (C-5), 52.58 and 52.55 (OCH_3), 49.94 (CH amino acid), 48.38–47.61 (3 spiro C and SCH_2), 29.85 (*t*Bu), 27.18, 27.15, 25.34 and 25.31 (CH_3 of isopropylidene), 21.65, 21.58 and 21.56 (CH_3 of the protecting group), 21.07, 21.02 and 20.98 (β CH_3) ppm. ^{31}P NMR (202 MHz, $CDCl_3$): δ = 7.10, 7.01 ppm. HRMS (ESI$^+$): calcd. for $C_{26}H_{43}N_3O_{11}PS_2$ [M + H]$^+$ 668.2077; found 668.2076.

Supporting Information (see footnote on the first page of this article): Copies of ^{1}H, ^{13}C, and ^{31}P NMR spectra of compounds **1**, **2**, and **3**.

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