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Synthesis and Deprotection of Biodegradably and Thermally Protected Dinucleoside-2´,5´-Monophosphate Prodrug Model of 2-5A

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Protected dinucleoside-2´,5´-monophosphate has been prepared to develop a prodrug strategy for 2-5A. The removal of enzymatically and thermally labile 4-acetylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl phosphate protecting group and enzymatically labile 3´-*O*-pivaloyloxymethyl group was followed at pH 7.5 and 37°C by HPLC from the fully protected dimeric adenosine-2´,5´-monophosphate **1** used as a model compound for 2-5A. The desired unprotected 2´,3´-*O*-isopropylideneadenosine-2´,5´-monophosphate (**9**) was observed to accumulate as a major product. Neither the competative isomerization of 2´,5´- to a 3´,5´-linkage nor the P-O5´ bond cleavage was detected. The phosphate protecting group removed faster than the 3´-*O*-protection and, hence, the attack of the neighbouring 3´-OH on phosphotriester moiety did not take place.

Keywords: Prodrugs • Nucleotides • 2-5A • Protecting group • Deprotection

Introduction

The 2-5A/RNase L system is one of the most important immunity pathway that acts against viral infections. As a result of infectious agents cells secrete interferons which activates enzyme, called 2´,5´-adenylate synthetase, to produce 2´,5´-oligoadenylates (2-5A; [$p_x5´A(2´p5´A)_n$; x = 1-3; n ≥ 2]) from adenosine triphosphate. The 2-5A molecules, in turn, activates 2-5A-dependent RNase (RNase L) that cleaves viral RNA resulting in apoptosis.[1-4] Accordingly, the synthetic structurally modified 2´,5´-oligoadenylates which activate RNase L constitute a potential class of therapeutic agents to compact against viral diseases and cancer.[5] To be applicable as a drug, the 2-5A must exhibit a good cellular uptake and resistance against biodegradation. Numerous modified 2-5A pro-nucleotides, such as phosphorothioate,[6-9] alanyltyrosine,[10] 2´-*O*-phosphoglyceryl,[11] 5´-capped,[12] aminofuctionalized[13] and phosphoramidate derivatives[14] as well as 3´-deoxyadenosine,[15] 3´-*O*,4-C-bridged adenosine,[16] 3´-fluoro-3-deoxyadenosine,[17] 3´-*O*-methyladenosine[18] and 3´-amino-3´-deoxyadenosine[19] analogues have been synthesized to increase the stability in serum and cytoplasm. Several techniques have also been tested to enhance the cell delivery of 2-5A and their modified analogues, as an example, encapsulation in liposomes [20] and conjugation

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to a lipophilic groups,[21-23] but no satisfactory prodrug strategy for the enhancement of cellular uptake has been developed. Generally, most of the prodrug approaches of nucleoside 5´-phosphomonoesters and 3´,5´-phosphodiesters are based on enzymatic transformation that triggers a chemical removal of the remnants of phosphate protecting group.[24,25] In case of 2-5A, both the negatively charged phosphate functions and 3´-OH groups must be protected, otherwise 3´-OH attacks readily on adjacent phosphotriester moiety.[26,27]

We have previously studied on removal of esterase labile protecting groups from dimeric adenylyl-2´,5´-adenosines[28] (2´,5´-ApA) (**2a** and **b**) and from 2-5A trimers[29] (pA2´p5´A2´p5´A) (**3a** and **b**) using 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl protection[30,31] for the phosphate moieties and pivaloyloxymethyl ($PivOCH_2$) or acetyloxymethyl ($AcOCH_2$) groups for the 3´-OH. In all cases, the exposed 3´-OH function attacks on the still protected phosphodiester linkage resulting in a complex product distribution. With trimers, a dramatic retardation of the enzymatic removal of 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl protecting group was also observed upon accumulation of the negative charge on the phosphodiester linkages. To develop an improved prodrug strategy, we now report on an approach based on the enzymatically labile 4-acetylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl group[32,33] of the internucleosidic phosphodiester linkage that undergoes also thermolytic removal. 3´-Hydroxyl function was protected with the enzymatically removable pivaloyloxymethyl group. For this reason, the dimeric model compound **1** was synthesized and the removal of the protecting groups was followed by HPLC at 37 ^{o}C and pH = 7.5 in the presence and absence of carboxyesterase.

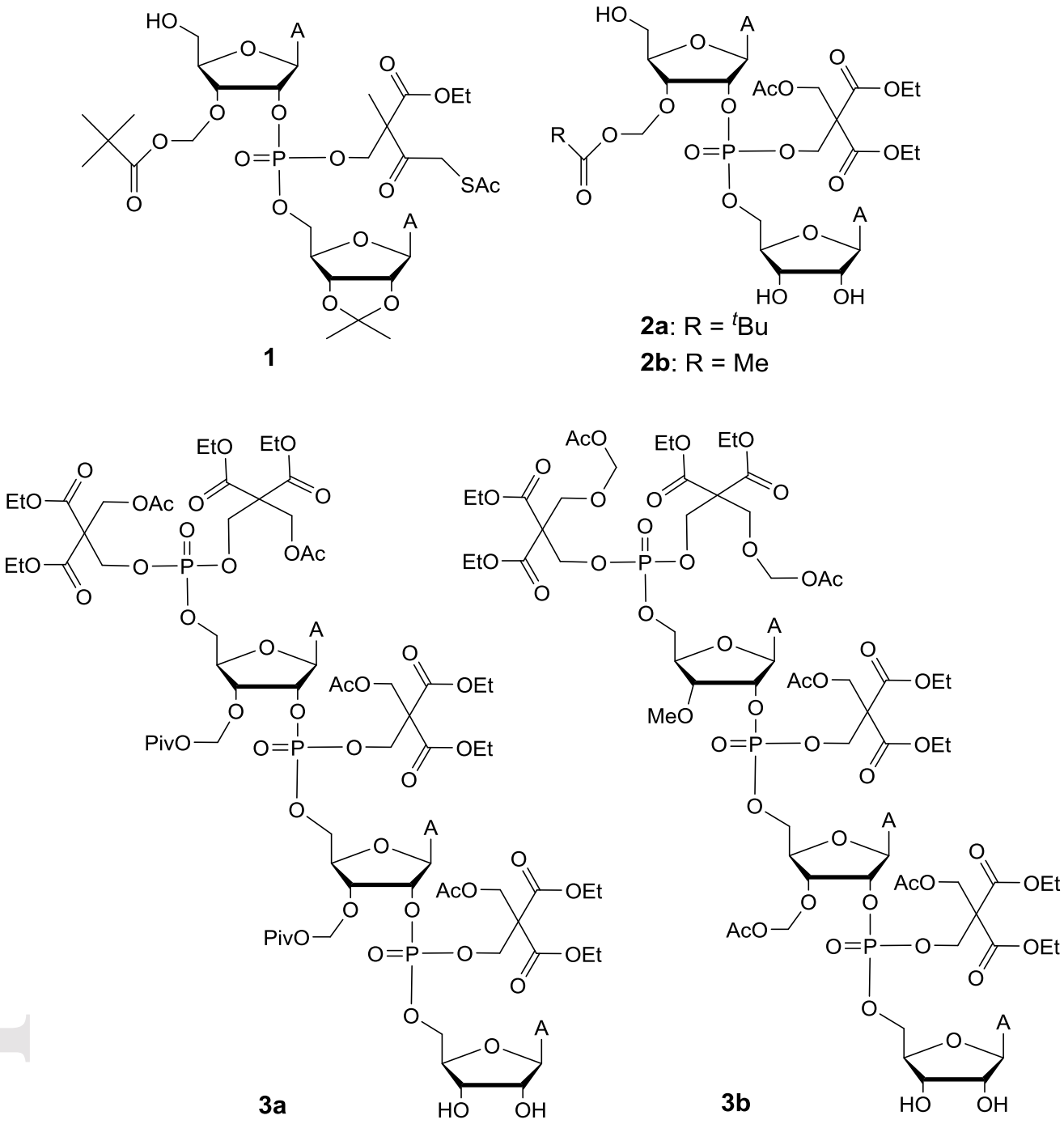
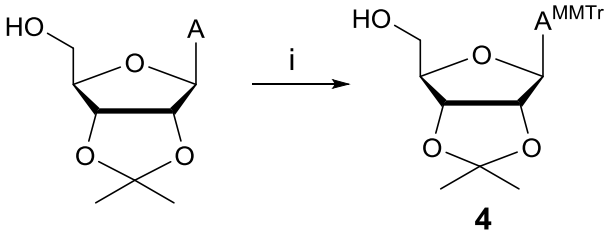


Figure 1. Structures of dimeric adenylyl-2´,5´-adenosines and trimeric 2-5A.

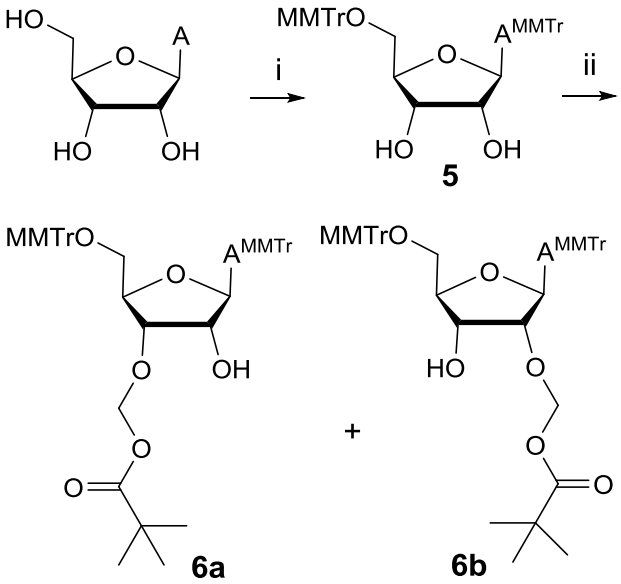
Results and Discussion

Preparation of Fully Protected Adenosine-2´,5´-monophosphate (**1**). 2´,3´-*O*-Isopropylidene-N^6-(4-methoxytrityl)adenosine (**4**) was prepared as describer previously.[34,35] 5´-Hydroxyl group of commercially available 2´,3´-*O*-isopropylideneadenosine was temporarily trimethylsilylated followed by the tritylation of N^6 and the removal of silyl group by treating with ammonia (Scheme 1).



Scheme 1. Conditions: i) 1.TMSCl, Py, 2. MMTrCl, Py, 3. NH_4OH.

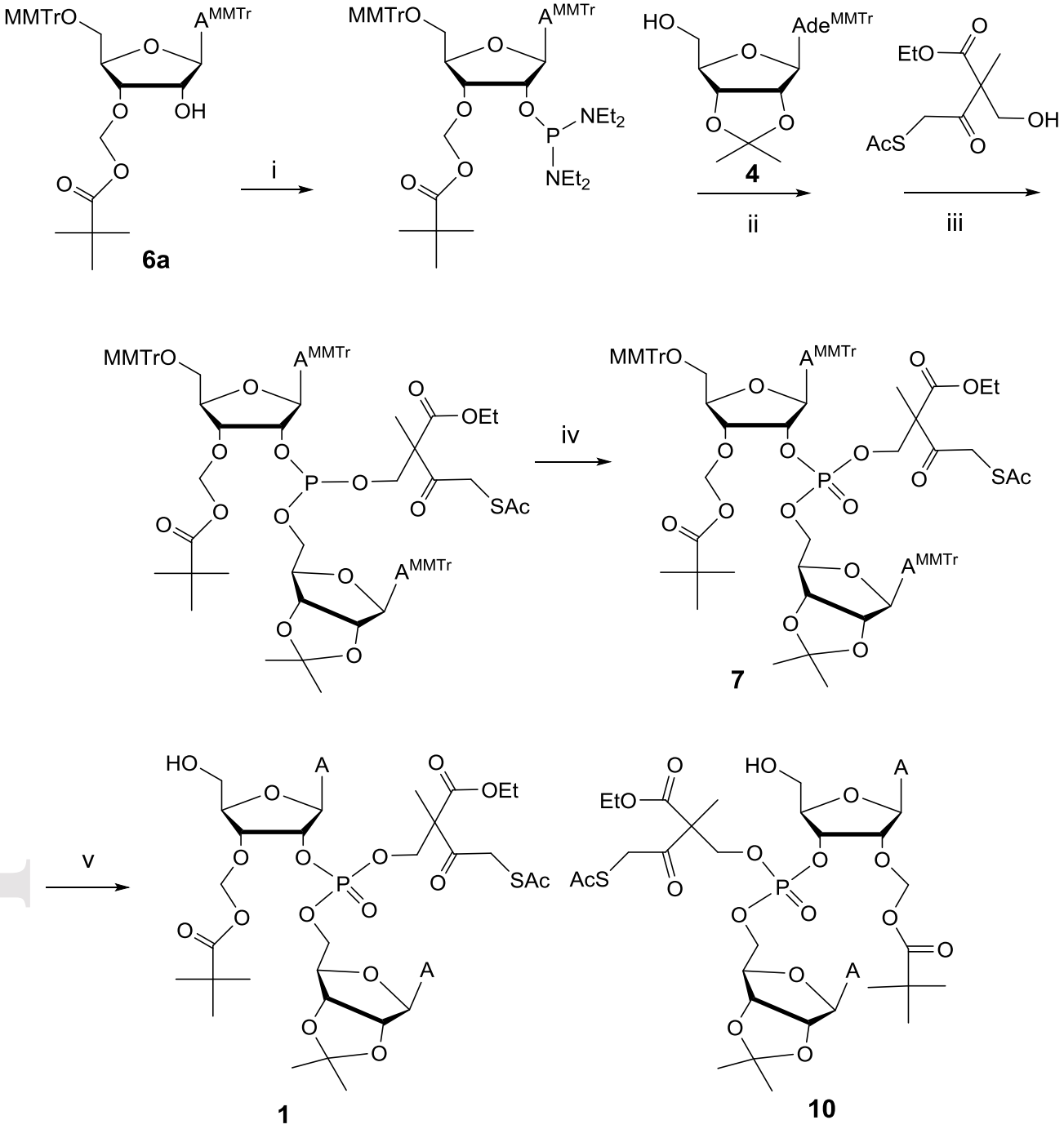
The adenosine building block **6a** was synthesized analogously as reported earlier.[28] Accordingly, 5´-OH and N^6-amino functions of adenosine were first 4-methoxytritylated to afford **5** (Scheme 2). Alkylation of **5** with pivaloyloxymethyl chloride in the presence of NaH and sodium iodine as a catalyst gave a mixture of 3´-*O*- (**6a**) and 2´-*O*-(pivaloyloxymethyl)-5´-*O*,N^6-bis(4-methoxytrityl)adenosine (**6b**). The separation of the isomers proved difficult and, hence, a mixture of **6a** and **6b** was used in a next step. Attempts with 2´-*O*-*tert*-butyldimethylsilylated adenosine failed, since TBDMS group migrated between 2´-and 3´-OH groups upon pivaloyloxymethylation.



Scheme 2. Conditions: i) MMTrCl, pyridine; ii) $PivOCH_2Cl$, NaH, NaI, THF.

Compound **1** was prepared by phosphitylation method using the appropriately protected nucleosides (**4** and **6a**) and ethyl 4-(acetylthio)-2-(hydroxymethyl)-2-methyl-3-oxobutanoate. A mixture of 3´-*O*- (**6a**; see Scheme 3) and 2´-*O*-(pivaloyloxymethyl)-5´-*O*,N^6-bis(4-methoxytrityl)-3´-*O*-(pivaloyloxymethyl)adenosine (**6b**) was first phosphitylated with bis(diethylamino)phosphorochloridite in DCM in the presence of triethylamine. The subsequent tetrazole-promoted displacement of the diethylamino ligands of the diethylaminophosphoramidite by 2´,3´-*O*-isopropylidene-N^6-(4-methoxytrityl)adenosine and ethyl 4-(acetylthio)-2-(hydroxymethyl)-2-methyl-3-oxo-butanoate in acetonitrile gave the phosphite ester that was oxidized with I_2 in a mixture of THF, H_2O and 2,6-lutidine. The phosphate ester **7** obtained was passed through a short silica gel column and used without further purification. Finally, the 4-methoxytrityl groups were removed with 80% aqueous acetic acid to give the

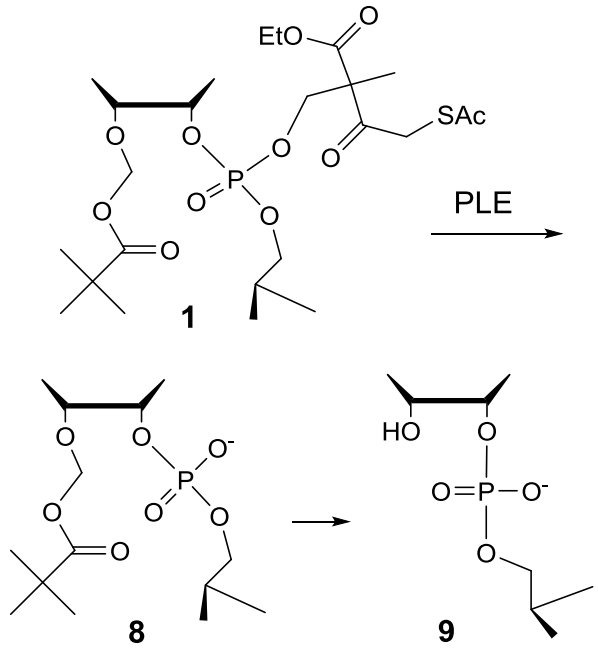
fully protected diadenosine monophosphate as a mixture of 2´,5´- (**1**) and 3´,5´-isomers (**10**) separated by a reverse phase HPLC. Assignment of the isomers **1** and **10** as 2´,5´- and 3´,5´-phosphotriesters is based on the couplings between C2´ or C3´ and CH_2 of POM obtained from HMBC spectra.



Scheme 3. Conditions: i) $(Et_2N)_2PCl$, Et_3N, DCM; ii) TetH, MeCN; iii) TetH, MeCN; iv) I_2, THF, H_2O, 2,6-lutidine; v) 80% AcOH.

Deprotection of Fully Protected Adenosine-2´,5´-monophosphate (**1**). The decomposition of **1** (t_R = 29.4 min) was followed in the presence of porcine liver carboxyesterase (PLE; 2.6 units/ml) in a HEPES buffer at pH 7.5 and at 37 °C. The composition of the aliquot withdrawn at appropriate intervals from the reaction solution was determined by RP HPLC. According to the MS-HPLC

analyses, the 4-acetylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl protecting group was completely removed before the pivaloyloxymethyl group started to release. Accordingly, 3´-*O*-pivaloyloxymethyladenosin-2´-yl 2´,3´-*O*-isopropylideneadenosin-5´-yl phosphate (**8**; t_R = 20.5 min; [M-H$^-$] at m/z 749.2) was observed to accumulate as an intermediate and was further decomposed nearly quantitatively to the desired unprotected adenosin-2´-yl 2´,3´-*O*-isopropylideneadenosin-5´-yl phosphate (**9**; t_R = 15.2 min; [M-H$^-$] at *m/z* 635.1) as shown in Scheme 4 and Figure 2. The half-lives for the disappearance of **1** and **8** were 6.3 min (k = 1.84 x 10^{-3} s^{-1}) and 612 min (k = 1.89 x 10^{-5} s^{-1}), respectively. The small intermediate detected at the retention time 24.9 min accumulated to such an extent (< 2%) that it could have not been reliably quantified but, most likely, it represents the deacetylated starting material. No sign of isomerization of 2´,5´-to 3´,5´-isomer or P-O5´-bond cleavage was detected. Only traces of unknown side products could be observed. We have recently described that the esterase- and thermolabile 2,2-disubstituted 4-acylthio-3-oxobutyl protecting groups are removable also in a cell extract. For example, treatment of 4-acetylthio-2,2-dimethyl-3-oxobutyl protected bis(2´-*C*-methyluridin-5´-yl)phosphate in a whole cell extract of human prostate carcinoma released the desired 5´,5´-phosphodiester as a main product.[32]



Scheme 4. PLE-triggered deprotection of **1**.

The data clearly shows that the now reported protection group strategy is superior compared to the earlier reported one for the dimers **2a** and **b**.[28] During 1 day, approximately 80% of **1** was converted to the desired unprotected phosphodiester **9** the amount of which was finally about 90% (Figure 2). With **2a** and **b**, 2´,5´ApA, in turn, accumulated only as a minor product during 1 day (20% and 5%, respectively), since the isomerization to 3´,5´-isomer and cleavage of P-O5´ bond markedly competed with the deprotection (Figure 3 and 4).[28] The removal of 4-acetylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl from **1** proceeds more than one order of magnitude faster ($t_{1/2}$ = 6.3 min; k = 1.84 x 10^{-3} s^{-1}) than that of 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl from **2a** ($t_{1/2}$ = 395 min; k = 2.9×10^{-5} s^{-1}).

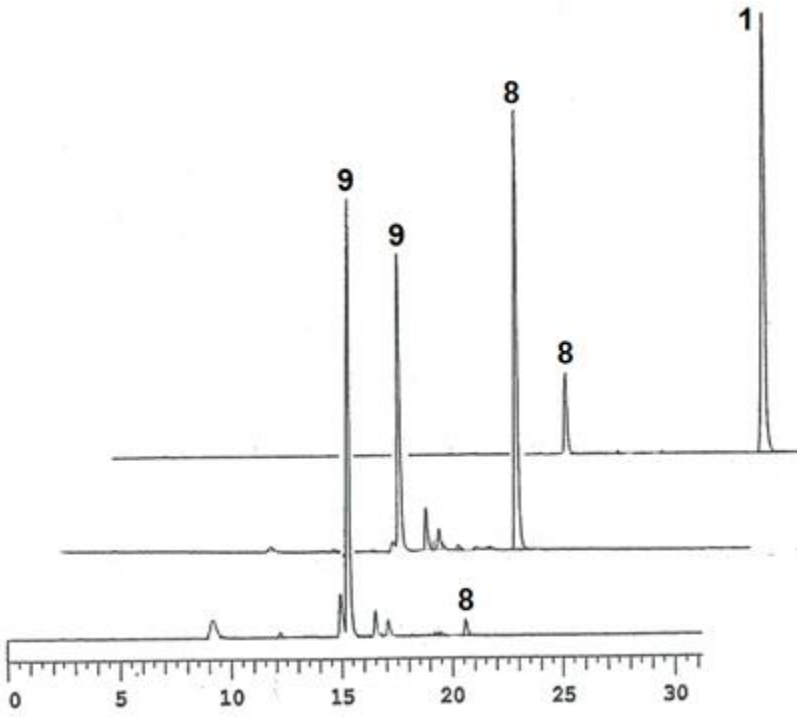


Figure 2. RP-HPLC traces for the carboxyesterase-catalyzed deprotection of **1** at pH 7.5 and 37.0 °C (*I* = 0.1 M with NaCl). The traces from top to bottom refer to aliquots withdrawn at 15 s, 7 h and 48 h. For the chromatographic conditions, see the experimental section.

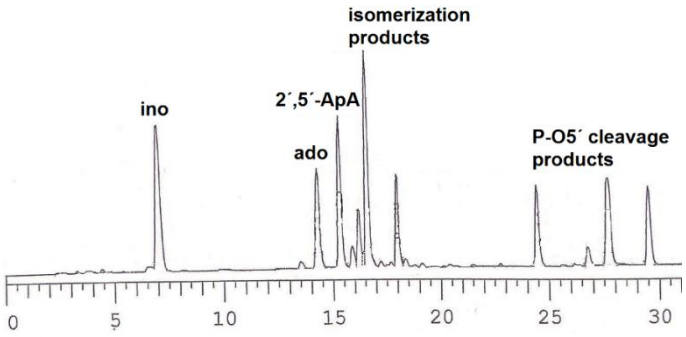


Figure 3. RP-HPLC traces for the HLE-catalyzed hydrolysis of **2b** at pH 7.5 and 37.0 °C (*I* = 0.1 mol L^{-1} with NaCl).[28]

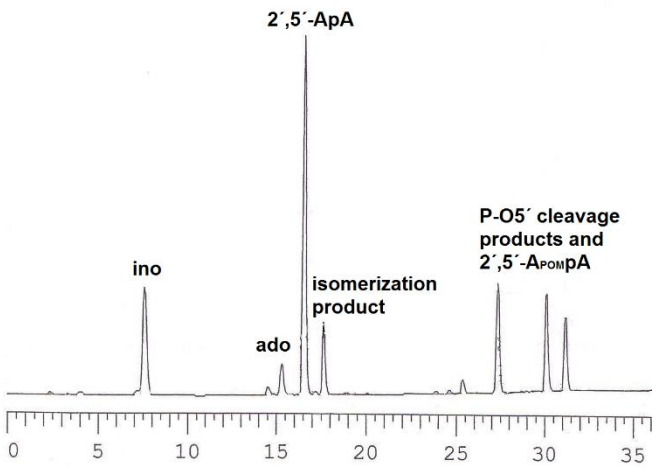
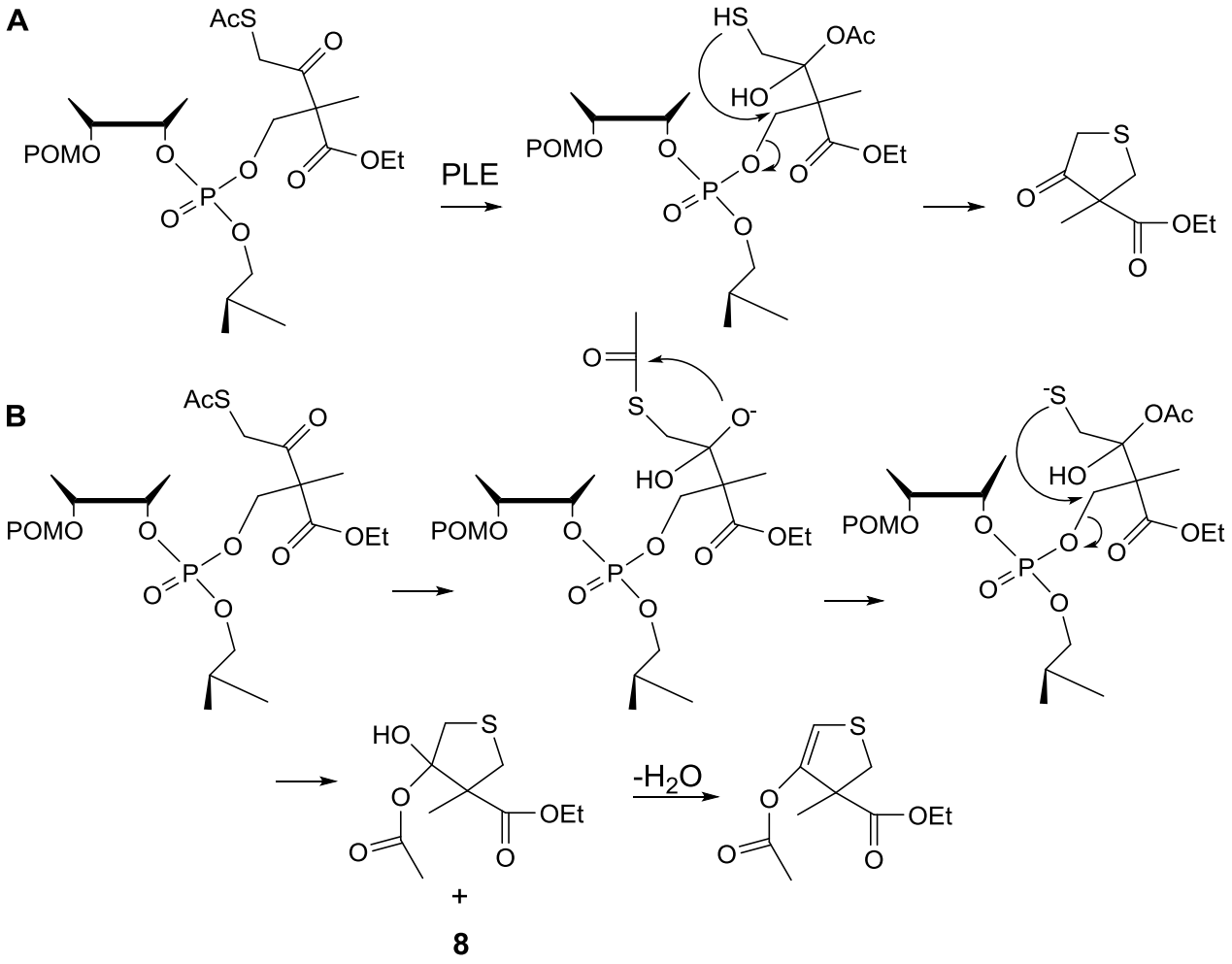


Figure 4. RP-HPLC traces for the HLE-catalyzed hydrolysis of **2a** at pH 7.5 and 37.0 °C (I = 0.1 M with NaCl).[29]

As mentioned above, it is known that enzyme triggered deprotection is markedly retarded upon accumulation of a negative charge on the neighboring phosphodiester linkages as a result of reduced affinity of the enzyme to charges substrates.[29] Our previous studies with thermolabile 4-acetylthio-2,2-dimethyl-3-oxobutyl protected oligomeric 3´,5´-phosphodiesters have been shown that the non-enzymatic removal of the second protecting group from internucleosidic phosphodiester linkages was only slightly retarded (by a factor of 1.15-1.50) compared to the first one.[33] Moreover, now presented 4-acetylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl protecting group is known to be even 19.6 and 1.6 times more labile than 4-acetylthio-2,2-dimethyl-3-oxobutyl group thermolytically and enzymatically, respectively.[32] For these reason it can be assumed that our prodrug strategy, in all likelihood, offers a potential method also for trimeric oligoadenylates (2-5A). To measure the non-enzymatic departure rate of the 4-acetylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl from phosphotriester linkage, the decomposition of **1** was also followed in the absence of enzyme at pH 7.5 and 37.0 °C. Starting material **1** was observed to undergo quantitative conversion into **8** five times slower than in the presence of enzyme, the half-live being 34.4 min (k = 3.36 x 10^{-4} s^{-1}). Accordingly, in the presence of enzyme, intermediate **8** is most likely formed by two competative routes, *i.e.*, a minor non-enzymatic one and a major enzymatic pathway.

Deprotection of Fully Protected Adenosine-3´,5´-monophosphate (**10**). For comparison, removal of the 4-acetylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl was also followed from **10**. The observed rate constant for the PLE-catalyzed deacetylation of the phosphotriester **10** was 3.36 x 10^{-4} s^{-1} ($t_{1/2}$ = 34.4 min). Only product observed to accumulate was 2´-*O*-pivaloyloxymethyladenosin-2´-yl 2´,3´-*O*-isopropylideneadenosin-5´-yl phosphate (**8**). Unexpectedly, the pivaloyloxymethyl group was released much slower. Even after 3 d, we could not reliable verify the release of POM.

Mechanisms. As discussed previously, the mercapto intermediate formed by the esterase-catalyzed deacetylation undergoes an intramolecular cyclization with a concomitant release of the remnants of the protecting group (Scheme 5A).[32] Also the non-enzymatic departure of the protecting group takes place by cyclization after the hydration of the keto group at C3 and by the subsequent migration of the acetyl group from sulfur into the oxyanion of the C3 of geminal diol (Scheme 5B).[32] Earlier studies has also been shown that the 4,4-disubstituted dihydrothiophen-3(2H)-one released is not markedly alkylating and was not observed to form glutathione adduct.[32] The removal of the pivaloyloxymethyl group takes place by an esterase-catalyzed deacylation and a consequtive half-acetal hydrolysis with a loss of formaldehyde.



Scheme 5. A) Mechanism of the enzymatic removal of the protecting group. B) Mechanism of the non-enzymatic removal of the protecting groups.

Conclusions

In summary, the data suggest that decreasing the stability of the phosphate protecting group by replacement of the earlier used 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl group with the esterase and thermolabile 4-acetylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl group allows a feasible prodrug strategy for 2-5A. The fully protected dimeric adenylyl-2´,5´-adenosine prodrug model **1** was observed to undergo the deprotection nearly quantitatively into the desired unprotected product (**9**). We also showed that the deprotection of **1** produced exclusively **8** in the absence of enzyme. An additional advantage of the now used phosphate protecting group is that it is removable even if the enzymatic deprotection is retarded.

Experimental Section

General

4-(Acetylthio)-2-(hydroxymethyl)-2-methyl-3-oxo-butanoate was synthesized as described previously.[32] THF, DCM, MeCN and Py were dried over 4Å molecular sieves. TEA was dried by refluxing over CaH_2 and distilled. The assignment of the NMR signals is based on 2D COSY and HSQC spectra. ^{1}H, ^{13}C, ^{31}P and 2D-NMR spectra were recorded on a *Bruker Avance 400 or 500* NMR spectrometer. High-resolution mass spectra were acquired using a *Bruker Daltonics micrOTOF-Q* and electrospray ionization technique. LC-ESI-MS: Agilent 6120 quadrupole LC-ESI-MS. The kinetic samples were analyzed on a *Merck Hitachi LaChrom D7000* HPLC using Hypersil C18 column (4 x 250 mm x 5 µm).

2´,3´-*O*-Isopropylidene-*N*^{6}-(4-methoxytrityl)adenosine (4). 2´,3´-*O*-Isopropylideneadenosine (13.0 mmol, 4.00 g) was dissolved into dry pyridine (117 ml) and trimethylsilylchloride (26.0 mmol, 3.31 ml) was added. After 1 h 45 min, 4-methoxytritylchloride (13.0 mmol, 4.00 g) was added and the reaction was allowed to proceed for overnight. Aqueous 28% NH_4OH solution (29.0 ml) was added and the reaction mixture was stirred for 45 min. The volatiles were evaporated to dryness and the residue was dissolved in DCM and washed with 5% aq. $NaHCO_3$. The organic phase was dried on Na_2SO_4, filtered and evaporated to dryness. Crude product was purified by silica gel chromatography using a 1:1 (*v/v*) mixture of EtOAc and petroleum ether as an eluent to yield **4** (1.68 g, 22%). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$): 8.43 (*s*, H-C(8)); 7.93 (*s*, H-C(2)); 7.39-7.19 (*m*, 12H of MMTr); 6.84 (*d*, *J* = 8.79, 2H of MMTr); 6.12 (*d*, *J* = 3.20, H-C(1´)); 5.34 (*dd*, *J* = 6.40 and 3.2, H-C(2´)); 4.94 (*dd*, *J* = 6.40 and 2.80, H-C(3´)); 4.20 (*m*, H-C(4´)); 3.71 (*s*, MeO of MMTr); 3.54 (*dd*, *J* = 11.59 and 4.80, H-C(5´)); 3.49 (*dd*, *J* = 11.59 and 5.20, H-C(5´´)); 1.50 (*s*, CH_3); 1.30 (*s*, CH_3). ^{13}C-NMR (101 MHz, $(CD_3)_2SO$): 158.21 (MMTr); 154.12 (C(6)); 151.80 (C(2)); 148.48 (C4); 145.54 (MMTr); 140.85 (C(8)); 137.47, 130.32, 128.94, 128.17, 127.02 (MMTr); 121.30 and 121.29 (spiro C of isopropylidene and C(5)); 113.49 (MMTr); 90.18 (C(1´)); 87.03 (C(4´)); 83.73 (C(2´)); 81.76 (C(3´)); 70.47 (MMTr); 61.99 (C(5´)); 55.47 (OMe); 27.50 (Me); 25.62 (Me). HR-MS: 602.2351 ([*M*+Na]$^{+}$, $C_{56}H_{55}N_5NaO_8^{+}$; calc. 602.2374).

5´-*O*,*N*^{6}-Bis(4-methoxytrityl)adenosine (5). Adenosine (18.7 mmol, 5.00 g) was coevaporated from dry pyridine and dissolved in the same solvent (70 ml). A solution of 4-methoxytritylchloride (39.3 mmol, 12.1 g) in dry pyridine (70 ml) was added. The reaction mixture was stirred at 40 ^{o}C overnight. The reaction was quenched by adding MeOH (18 ml) and the volatiles were removed under reduced pressure. The residue was dissolved in DCM and washed with sat. aq. $NaHCO_3$ and brine. The organic phase was dried on Na_2SO_4, filtered and evaporated to dryness. Crude product was purified by silica gel chromatography using DCM containing 1% MeOH and 0.1% pyridine as an eluent to yield **5** (9.4 g, 62%). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$): 8.34 (*s*, H-C(2)); 7.84 (*s*, H-C(8)); 7.39-7.17 (24H of MMTr); 6.86-6.82 (4H of MMTr); 5.91 (*d*, *J* = 4.80, H-C(1´)); 5.52 (*s*, 2´-OH); 5.21 (*s*, 3'-OH); 4.72 (*t*, H-C(2')); 4.28 (*t*, H-C(3')); 4.05 (*m*, H-C(4')); 3.71 (MeO); 3.20-3.19 (*m,* H-C(5´ and 5´´)). ^{13}C-NMR (101 MHz, $(CD_3)_2SO$): 158.64, 158.21 (MMTr); 154.08 (C(6)); 151.72 (C(2)); 149.0, 145.54, 144.83, 144.57 (C(4) and MMTr); 141.00 (C(8)); 137.53, 135.38, 130.51, 130.32, 128.96, 128.44, 128.35, 128.26, 128.16, 127.27, 127.01, 124.37 (MMTr); 121.43 (C(5)); 113.65 and 113.48 (MMTr); 88.65 (C(1´)); 86.21 (spiro C of MMTr); 83.60 (C(4´)); 73.24 (C(2´)); 70.79 (C(3´)); 70.47 (spiro C of MMTr); 64.22 (C(5´)); 55.47 (OMe). HR-MS: 812.3420 ([*M*+H]$^{+}$, $C_{50}H_{46}N_5O_6^{+}$; calc. 812.3443).

3´-*O*-(Pivaloyloxymethyl)-5´-*O*,N^6-bis(4-methoxytrityl)adenosine (**6a**). Compound **5** (3.08 mmol, 2.50 g) was dissolved in dry THF (26 ml) and 1 equivalent of 60% dispersion NaH (123 mg, 3.08 mmol) was added. After stirring for 1 h at room temperature, the mixture was added into a mixture of pivaloyloxymethyl chloride (3.39 mmol, 0.53 ml) and NaI (0.31 mmol, 46 mg). The reaction was allowed to proceed for 5 h and quenched by adding water (70 ml). The mixture was extracted three times with Et_2O, the ether layer was dried over Na_2SO_4 and evaporated to dryness. The crude products was purfied by silica gel chromatography, eluting with a 1:1 (*v/v*) mixture of EtOAc and petroleum ether to give a mixture of **6a** and **6b** (0.32 g, 11% yield). The isomers were not separated from each other. ^{1}H-NMR of **6a** (500 MHz, ($CDCl_3$): 8.00 (*s*, H-C(2)); 7.99 (*s*, H-C(8));. 7.45-7.19 (24H of MMTr); 7.00 (NH); 6.83-6.80 (4H of MMTr); 5.91 (*d*, *J* = 7.55, H-C(1´)); 5.47-5.30 (CH_2); 4,90 (*t*, *J* = 6.85, H-C(2')); 4.65 (*s*, 2´-OH); 4,50 (*t*, H-C(3')); 4.22 (*m*, H-C(4')); 3.79 (MeO); 3.50 (*dd*, *J* = 13.25 and 4.70, H-C(5´)); 3.38 (*dd*, *J* = 13.20 and 5.50, H-C(5´´)); 1.16 (Me of POM). ^{1}H-NMR of **6b** (500 MHz, ($CDCl_3$): 7.97 (*s*, H-C(2)); 7,94 (*s*, H-C(8));. 7.45-7.19 (24H of MMTr); 6.94 (NH); 6.83-6.80 (4H of MMTr); 6.10 (*d*, *J* = 6.15 Hz, H-C(1´)); 5.47-5.30 (CH_2); 5.11 (*t*, *J* = 6.20 Hz, H-C(2')); 4,50 (*t*, H-C(3')); 4,35 (*m*, H-C(4')); 3.79 (MeO); 3.47 (*dd*, *J* = 13.10 and 4.95 Hz, H-C(5´)); 3.27 (*dd*, *J* = 13.05 and 4.70 Hz, H-C(5´´)); 2.67 (*s*, 3´-OH); 1.14 (Me of POM). ^{13}C-NMR (126 MHz, ($CDCl_3$): 177.74 (C=O); 158.70, 158.35 (MMTr); 154.25 (C(6)); 154.25 and 154.10 (C(2)); 148.59 and 148.30 (C4); 145.20, 145.12, 144.07, 144.00, 143.96, 143.88 (MMTr); 138.86 and 138.61 (C(8)); 137.24, 137.14, 135.19, 134.94, 130.38, 130.33, 130.21, 129.24, 128.90, 128.43, 128.37, 128.27, 128.23, 127.88, 127.06, 126.89 (MMTr); 121.46 and 121.28 (C(5)); 113.23 and 113.19 (MMTr); 89.67 (C(1´)); 89.05 and 88.69 (CH_2); 87.10 (C(1´)); 86.86 and 86.81 (spiro C of MMTr); 83.87 (C(4´)); 83.58 (C(4´)); 81.38 (C(2´)); 79.24 (C(3´)); 74.67 (C(2´)); 71.08 (C(3´)); 70.99 and 70.63 (spiro C of MMTr); 63.18 (C(5´)); 55.21 (OMe); 38.77 and 38.74 (spiro C of POM); 26.96 and 26.87 (Me of POM). HR-MS: 948.3902 ([*M*+Na]$^+$, $C_{56}H_{55}N_5NaO_8^+$; calc. 948,3943).

3´-*O*-Pivaloyloxymethyladenosin-2´-yl 2´,3´-*O*-isopropylideneadenosin-5´-yl 4-acetylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl phosphate (**1**). Compound **6a** (0.34 mmol, 0.314 g) was dried over P_2O_5 overnight and dissolved in dry DCM (1.9 ml) under nitrogen atmosphere. TEA (1.7 mmol, 0.24 ml) and bis(diethylamino)phosphorochloridite (0.44 mmol, 0.093 ml) were added and the mixture was stirred under nitrogen for 2 hours. The product was isolated by passing the mixture through a short silica gel column with a 7:3 mixture of EtOAc and hexane containing 0.5% TEA. The solvent was removed under reduced pressure and the residue was coevaporated from dry MeCN to remove the traces of triethylamine. The phosphitylated nucleoside was dissolved in dry MeCN (0.36 ml) under nitrogen and tetrazole (5.09 mmol, 0.754 ml of 0.45 M solution in MeCN) and 2´,3´-*O*-isopropylidene-N^6-(4-methoxytrityl)adenosine (**5**, 0.238 mmol; 0.14 g) in dry MeCN (0.36 ml) were added. The reaction mixture was stirred for 30 min and then tetrazole (7.64 mmol, 1.13 ml of 0.45 M solution in MeCN) and ethyl 4-(acetylthio)-2-(hydroxymethyl)-2-methyl-3-oxo-butanoate (0.52 mmol, 0.129 g) in dry MeCN were added. The phosphite ester (**6b**) formed was oxidized with iodine (0.2 g) in a mixture of THF (4.0 ml), H_2O (2.0 ml) and 2,6-lutidine (1.0 ml) overnight. A solution of 5% $NaHSO_3$ (100 ml) was added and the mixture was extracted 3 times with DCM. The organic phase was dried on Na_2SO_4 and evaporated to dryness. The crude product was passed through a short silica column eluting first with a 1:1 (*v/v*) mixture of EtOAc and DCM and then with DCM containing 3-5 % MeOH. The product **7** obtained was used without further purification and 80% (*v/v*) aq AcOH (7.0 ml) was added to carry out the detritylation. After stirring overnight at room temperature, the reaction mixture was evaporated to dryness. The residue was coevaporated twice with water. The product was purified first by silica gel chromatography eluting with DCM containing 2-20 % MeOH, and then by HPLC on a SunFire C18 column (250 x 10 mm, 5 µm, flow rate 3.0 ml min^{-1}), using isogratic elution with water and MeCN (45%) to yield **1** (14 mg, 3.9%). ^{1}H-NMR (500

MHz, CD_3CN): 8.23, 8.22, 8.20 and 8.19 (*s*, 2H of H-C(2)); 8.10, 8.09 and 8.01 (*s*, 2H of H-C(8)); 6.23 (br. *s*, NH_2); 6.13 (*d*, *J* = 2.00, H-C(1')); 6.11 (br. *s*, NH_2); 6.043 and 6.041 (*d*, *J* = 7.00, H-C(1')); 5.48 (CH of POM); 5.45 (*dd*, *J* = 6.50 and 2.50, H-C(2´)); 5,45 (*dd*, *J* = 6.50 and 2.50, H-C(2´)); 5,22 (*d*, *J* = 6.50, CH of POM); 5.00 (*m,* H-C(3´)); 4.32 (*m*, H-C(4´)); 4.24 (*m*, H-C(4´)); 4.15-3.98 (*m*, H-C(5´), H-C(5´´), CH_2 of Et and $POCH_2$); 3.87-3.77 (*m*, 1H of CH_2(5´) and CH_2S); 3.65 (br. *d*, *J* = 12.5, 1H of CH_2(5´´)); 2.32 and 2.31 (*s*, SAc); 1.58 and 1.36 (*s*, Me of isopropylidene); 1.20-1.10 (*m*, Me, 3 x Me of POM and CH_3 of Et). ^{13}C-NMR (126 MHz, CD_3CN): 198.73 and 198.70 (C=O); 194.07 (C=O of SAc); 177.58 (C=O of POM); 169.01 (C=OOEt); 156.44 and 156.00 (C(6)); 152.94 and 152.52 (C(2)); 149.28 and 148.61 (C(4)); 141.00, 140.98, 139.86 and 139.83 (C(8)); 120.61 and 119.82 (C(5)); 114.09 (spiro C of isopropylidene); 90.42 (C(1´)); 88.74 (CH_2 of POM); 88.09 and 88.02 (C(1´)); 85.96, 85.94, 85.09 and 85.03 (C(4´)); 84.25 (C(2´)); 81.15 (C(3´)); 76.46 and 76.42 (C(2´)); 69.27, 69.23 and 69.17 ($POCH_2$); 67.91 and 67.87 (C(5´)); 62.17 (CH_2 of Et); 61.85 (C(5´)); 59.72 (spiro C); 38,48 (spiro C of POM); 36.43 and 36.40 (CH_2S); 29.30 (SAc); 26.37 (Me of isopropylidene); 26.37 and 26.24 (Me of POM); 24.54 (Me of isopropylidene); 16.41 (Me); 13.18 (CH_3 of Et). ^{31}P-NMR (202 MHz, CD_3CN): -2.71 and -2.71. (Multiplicity of some signals is due to the presence of R_p and S_p diastereomers.) HR-MS: 981.3171 ($[M+H]^+$, $C_{39}H_{54}N_{10}O_{16}PS^+$; calc. 980.3172).

2´-*O*-Pivaloyloxymethyladenosin-3´-yl 2´,3´-*O*-isopropylideneadenosin-5´-yl 4-acetylthio-2-ethoxycarbonyl-3-oxo-2-methylbutyl phosphate (**10**). The **10** was obtained in 4.7% yield (17 mg). ^{1}H-NMR (500 MHz, CD_3CN): 8.27, 8.24 and 8.22 (*s*, 2H of H-C(2)); 8.10, 8.06, 8,05 and 8.01 (*s*, 2H of H-C(8)); 6.35-6.23 (*m*, NH_2 and 5´-OH); 6.21 (*d*, *J* = 9.50, H-(C1´)); 6.20 (*d*, *J* = 9.00, H-(C1´)); 6.13 (br. *s*, NH_2); 6.11 (br. *s*, NH_2); 5.96 (*d*, *J* = 8.00, H-C(1')); 5.89 (*d*, *J* = 8.00, H-C(1')); 5,47-5.41 (*m*, H-C(2´)); 5.25-5.07 (CH_2 of POM, H-C(2´) and H-C(3´)); 4.53-4.33 (*m*, H-C(4´), H-C(5´) and H-C(5´´); 4.32-4.25 (*m*, $POCH_2$); 4.25-4.19 (*m*, CH_2 of Et); 4.07-3.97 (*m*, CH_2S); 3.81-3.61 (*m*, H-C(5´) and H-C(5´´)); 2.37 and 2.36 (*s*, SAc); 1.61 (*s*, Me of isopropylidene); 1.51 and 1.50 (*s*, Me); 1.36 (*s*, Me of isopropylidene); 1.26 (*dt*, *J* = 7.00 and 2.00, CH_3 of Et); 0.80 and 0.79 (*s*, 3 x Me of POM). ^{13}C-NMR (126 MHz, CD_3CN): 198.73 and 198.70 (C=O); 194.10 (C=O of SAc); 176.97 (C=O of POM); 169.40 (C=OOEt); 156.58 and 156.03 (C6); 152.99, 152.97 and 152.31 (C2); 148.44 and 148.61 (C4); 141.12, 141.04 and 139.96 (C8); 120.81 and 119.82 (C5); 114.10 and 114.05 (spiro C of isopropylidene); 90.40 and 90.26 (C1´); 87.86 and 87.84 (C1´); 87.38 and 87.35 (CH_2 of POM); 85.25 and 85.24 (C4´); 84.22 and 84.18 (C2´); 81.40 and 81.22 (C3´); 77.90 and 77.80 (C2´); 77.00 and 76.90 (C3´); 69.39, 69.33, 69.18 and 69.12 (C5´ and C5´´); 67.64, 67.62 and 67.58 ($POCH_2$); 62.26, 62.25, 62.18 and 62.12 (CH_2 of Et, C5´ and C5´´); 60.00 and 59.72 (spiro C); 38.00 (spiro C of POM); 36.58 (CH_2S); 29.34 (SAc); 26.38 (Me of isopropylidene); 25.70 and 26.24 (Me of POM); 24.56 (Me of isopropylidene); 16.79 (Me); 13.23 (CH_3 of Et). ^{31}P-NMR (202 MHz, CD_3CN): -2.35, -2.37 and -2.51. (Multiplicity of some signals is due to the presence of R_p and S_p diastereomers.) HR-MS: 981.3171 ($[M+H]^+$, $C_{39}H_{54}N_{10}O_{16}PS^+$; calc. 980.3172).

Kinetic Measurements

The reactions were carried out in sealed tubes immersed in a thermostated water bath at 37 ^{o}C. The hydronium ion concentration of the reaction solution was adjusted with (*N*-[2-hydroxyethyl]piperazine-*N*,-[2-ethanesulfonic acid]) (HEPES) buffer (0.036/0.024 M; 3 ml; pH 7.5) and the ionic strength to 0.1 M with sodium chloride. The initial starting material concentration was *ca*. 0.2 mM. The deprotection **1** was followed by reversed-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 units/ml). The samples (200 µl) were withdrawn

from the reaction solution at appropriate time intervals and were made acidic (pH 3) with 1 M aqueous HCl (15 µl) to inactivate the enzyme and to quench the hydrolysis. The solution was filtered with Minisart RC 4 filters (0.2 µm). Products were separated using 5 min isocratic elution with AcOH/AcONa buffer (0.045/0.015 M) containing NH_4Cl (0.1 M) and 2% MeCN, followed by 30 min linear gradient up to 70.0% MeCN. The products were identified by HPLC/ESI-MS.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

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Author Contribution Statement

Dr. Mikko Ora has acted as a supervisor of undergraduate student Ms. Suvi Malmikare who prepared compounds **1**, **4**-**6** and **10**. Mikko Ora has written the article.
Dr. Emilia Kiuru has purified and characterized the compounds **1** and **10**.
Dr. Emilia Kiuru and Mikko Ora have acted as supervisors of undergraduate student Ms. Tuuli-Maaria Tuominen mentioned in acknowledgement who carried out kinetic measurements.

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