Synthesis and Enzymatic Deprotection of Fully Protected 2′-5′ Oligoadenylates (2-5A): Towards a Prodrug Strategy for Short 2-5A

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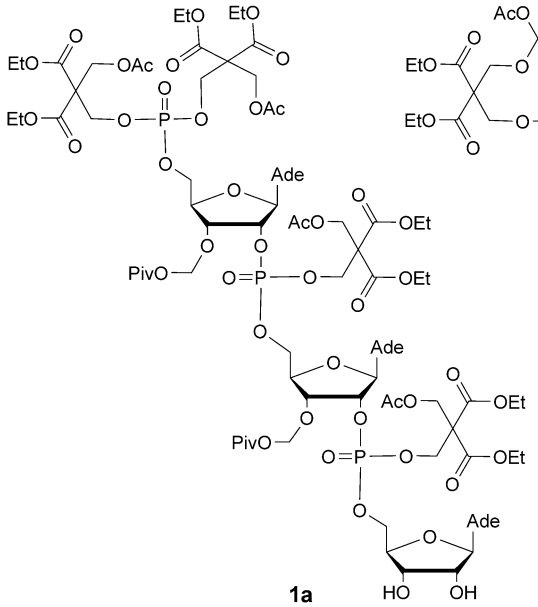
Fully protected pA2′p5′A2′p5′A trimers **1a** and **1b** have been prepared as prodrug candidates for a short 2′-5′ oligoadenylate, 2-5A, and its 3′-*O*-Me analog, respectively. The kinetics of hog liver carboxyesterase (HLE)-triggered deprotection in *HEPES* buffer (pH 7.5) at 37° has been studied. The deprotection of **1a** turned out to be very slow, and 2-5A never appeared in a fully deprotected form. By contrast, a considerable proportion of **1b** was converted to the desired 2-5A trimer, although partial removal of the 3′-*O*-[(acetyloxy)methyl] group prior to exposure of the adjacent phosphodiester linkage resulted in 2′,5′→3′,5′ phosphate migration and release of adenosine as side reactions.

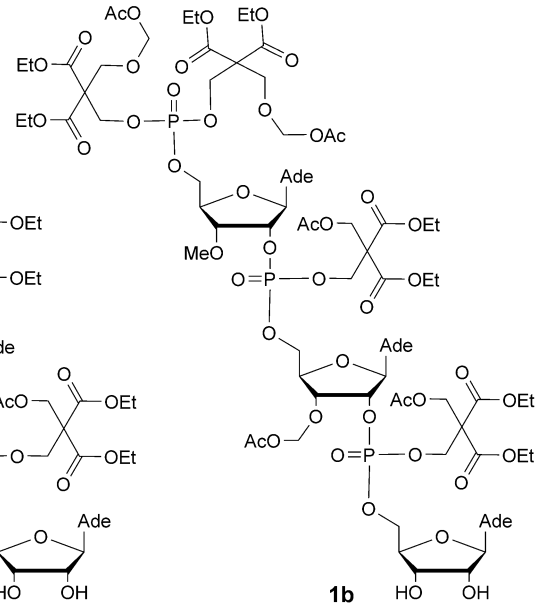
Introduction. – Interferons produced and secreted by cells in response to the presence of infectious agents activate synthetases which produce 2′-5′ oligoadenylates (2-5A) [1]. Intracellular endoribonuclease RNase L activated by 2-5A, in turn, catalyzes the cleavage of viral RNA resulting in apoptosis [2–4]. Recently, several structurally modified 2′-5′-adenylate trimers have been prepared to increase the stability of 2-5A in serum and cytoplasm without loss of activity [5–12]. The results obtained show that the 3′- and 5′-terminal adenosines in a 2-5A trimer may be substituted at 3′-*O*, whereas the 3′-OH of the intervening adenosine is essential for RNase L activation.

Protection of 2-5A with biodegradable groups that facilitate the uptake to cytoplasm and release the parent 2′-5′-nucleotide drug in an intact form offer an alternative approach to therapeutic use of 2-5A. We have previously reported on removal of esterase-labile protecting groups from dimeric adenylyl-2′,5′-adenosines **2a** and **2b** [13]. The present work is aimed at evaluating the feasibility of an enzyme-triggered prodrug strategy for the trimeric 2-5A. The crucial factor is the timing of the exposure of the 3′-OH and the adjacent phosphodiester linkage. The negatively charged phosphate group must be protected to enhance the internalization and the 3′-OH to prevent its attack on the neighboring protected phosphate linkage. The phosphate protecting group must be removed before the 3′-OH protection, since otherwise the extremely fast attack of the free 3′-OH group on the adjacent phosphotriester moiety would lead to isomerization of the 2′,5′ linkage to a 3′,5′ linkage and cleavage of the P–O(5′) bond [14]. In the present work, (acetyloxy)methyl ($AcOCH_2$) and (pivaloyloxy)methyl ($PivOCH_2$) groups have been used for the 3′-*O*-protection. The bulky *t*-Bu group is in all likelihood removed by esterases less readily

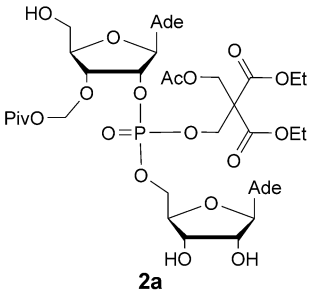
than the Ac group. To find out which one of these is more appropriate for the purpose, trimers bearing 3′-*O*-[(acetyloxy)methyl] and 3′-*O*-[(pivaloyloxy)methyl] protection have been prepared. On using the $AcOCH_2$ protection, the 3′-OH of the 5′-terminal nucleoside has, however, been protected as a methyl ether, since 2-5A has been shown to tolerate this modification without loosing its ability to activate RNase L [12]. The internucleosidic phosphodiester linkages have been protected with 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl groups and the 5′-terminal monophosphate group with 3-[(acetyloxy)methoxy]-2,2-bis(ethoxycarbonyl)propyl group. Our previous studies [15] indicate that the removal of the second 3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl group, *i.e.*, conversion of phosphodiester to monoester, is exceedingly slow, but replacing the Ac group with an $AcOCH_2$ group markedly accelerates the reaction. The hog liver esterase (HLE) triggered multistep release of 2-5A from the protected trimers **1a** and **1b** was followed by HPLC/MS.



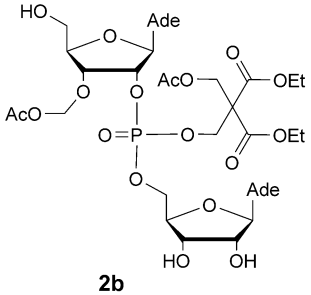
1a



1b

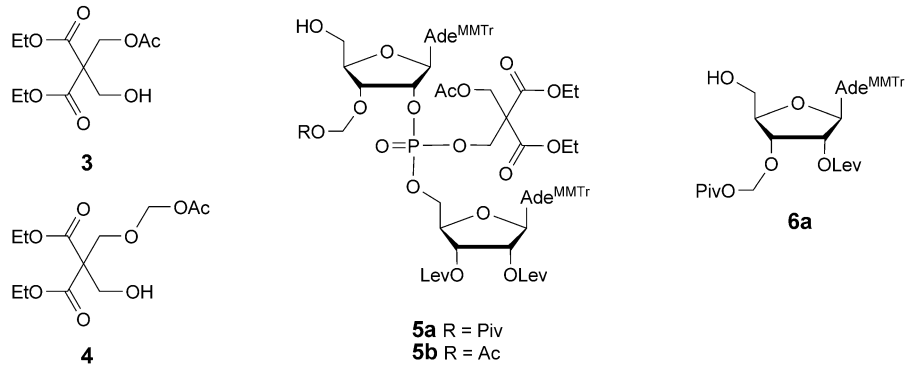


2a

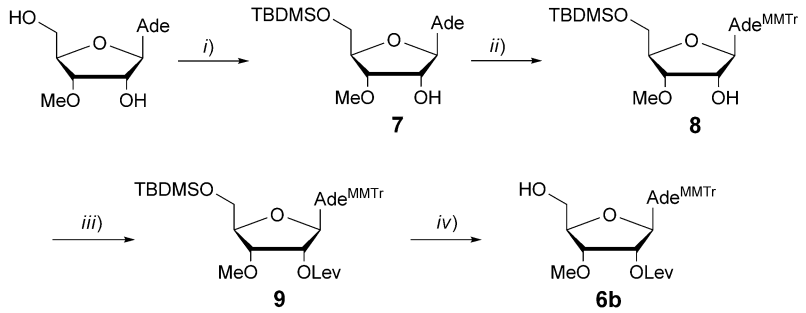


2b

Results and Discussion. – *Preparation of Protected 2-5A Trimers* **1a** *and* **1b**. The preparations of diethyl 2-[(acetyloxy)methyl]-2-(hydroxymethyl)malonate (**3**) [15], diethyl 2-{[(acetyloxy)methoxy]methyl}-2-(hydroxymethyl)malonate (**4**) [15], N^6-(4-methoxytrityl)-3′-*O*-[(pivaloyloxy)methyl]adenosin-2′-yl 2′,3′-di-*O*-levulinoyl-N^6-(4-methoxytrityl)adenosin-5′-yl 3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl phosphate (**5a**) [13], and 3′-*O*-[(acetyloxy)methyl]-N^6-(4-methoxytrityl)adenosin-2′-yl 2′,3′-di-*O*-levulinoyl-N^6-(4-methoxytrityl)adenosin-5′-yl 3-(acetyloxy)-2,2-bis(ethoxycarbonyl)-propyl phosphate (**5b**) [13] have been described earlier. 2′-*O*-Levulinoyl-N^6-(4-methoxytrityl)-3′-*O*-methyladenosine (**6b**) was prepared as depicted in *Scheme 1*. 2′-*O*-Levulinoyl-N^6-(4-methoxytrityl)-3′-*O*-[(pivaloyloxy)methyl]adenosine (**6a**) was obtained by temporary trimethylsilylation of the 5′-OH function of 2′-*O*-levulinoyl-3′-*O*-methyladenosine [13] and subsequent 4-methoxytritylation of the adenine moiety.



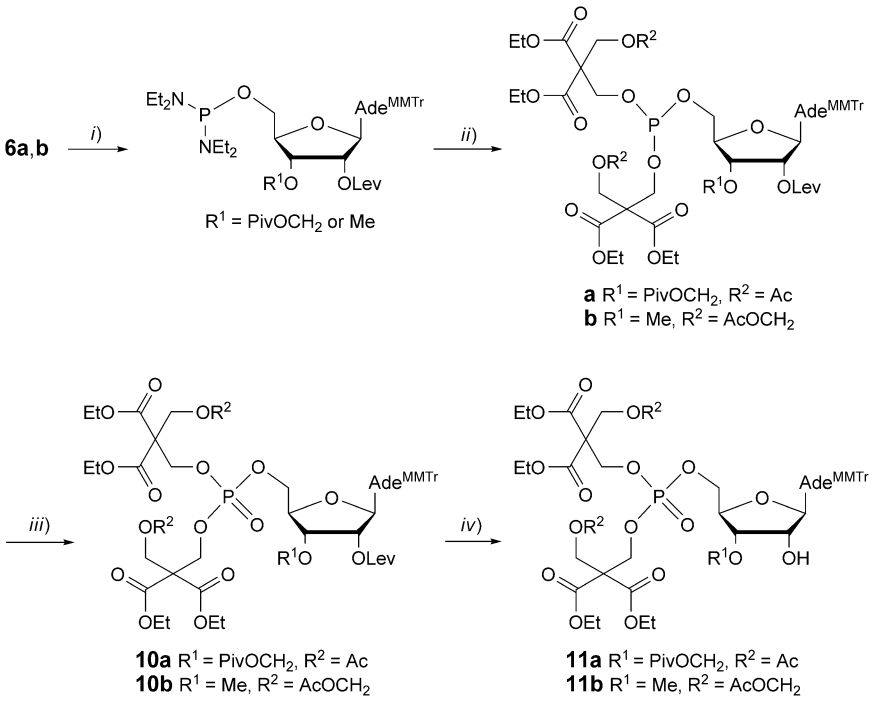
Scheme 1



i) TBDMSCl ((*t*-Bu)Me_2SiCl), Py. *ii*) (4-Methoxyphenyl)(diphenyl)methyl chloride (MMTrCl), Py. *iii*) Lev_2O (Lev = levulinoyl = 4-oxopentanoic acid), 4-(dimethylamino)pyridine (DMAP), dioxane, Py. *iv*) Bu_4NF, THF, AcOH.

Compounds **6a** and **6b** were phosphitylated with chlorobis(diethylamino)phosphine ($(Et_2N)_2PCl$). The Et_2N ligands were then replaced with **3** in the case of **6a** and with **4** in the case of **6b**, using 1*H*-tetrazole as an activator (*Scheme 2*). The resulting phosphite triesters were oxidized to phosphate esters **10a** and **10b** with I_2 in aqueous THF

Scheme 2



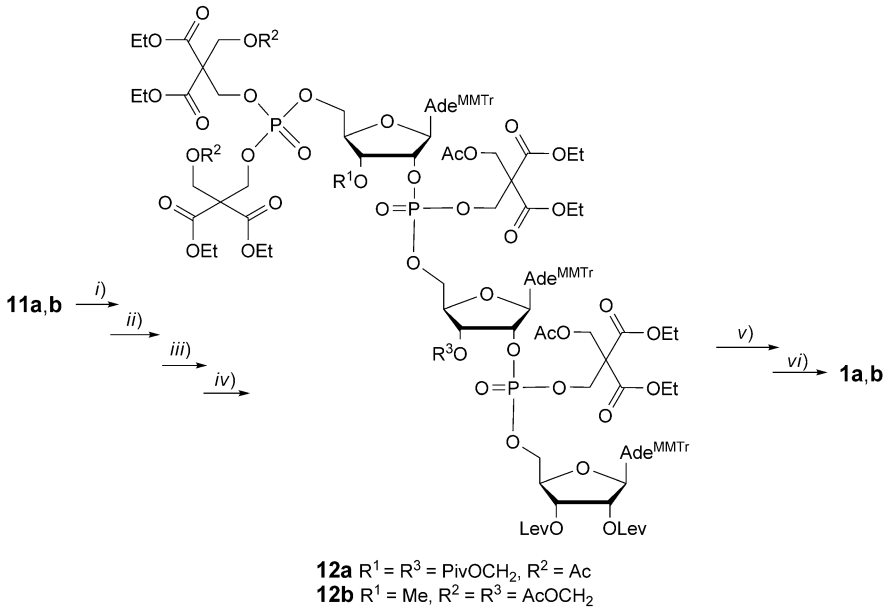
i) $(Et_2N)_2PCl$, Et_3N, CH_2Cl_2. *ii*) **3** with **6a**, **4** with **6b**, 1*H*-tetrazole (TetH), MeCN. *iii*) I_2, THF, H_2O, lutidine. *iv*) NH_2NH_3OAc, Py with **10a**, NH_2NH_3OAc, CH_2Cl_2, THF with **10b**. Piv = pivaloyl = 2,2-dimethylpropanoyl.

containing 2,6-lutidine. The levulinoyl (Lev) group was removed by hydrazinium acetate (NH_2NH_3OAc) treatment to afford building blocks **11a** and **11b**.

Trimers **1a** and **1b** were assembled as described in [13] for the synthesis of **2a** and **2b**. Accordingly, the 2′-OH function of N^6-(4-methoxytrityl)-3′-*O*-[(pivaloyloxy)methyl]-adenosine 5′-{bis[3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl] phosphate} (**11a**) and N^6-(4-methoxytrityl)-3′-*O*-methyladenosine 5′-(bis{[3-(acetyloxy)methoxy]-2,2-bis(ethoxycarbonyl)propyl} phosphate) (**11b**) were phosphitylated with $(Et_2N)_2PCl$ in the presence of Et_3N and the remaining Et_2N ligands were replaced sequentially with **2a** and **3** in the case of **11a**, and with **2b** and **3** in the case of **11b** (*Scheme 3*). In all these replacements, 1*H*-tetrazole was used as an activator. Oxidation of the phosphite triesters to phosphate triesters **12a** and **12b**, and removal of the Lev and (4-methoxyphenyl)(diphenyl)methyl (MMTr) groups completed the synthesis to give **1a** and **1b**, respectively, as a mixture of (R_P)- and (S_P)-diastereoisomers. For kinetic studies, one of the diastereoisomers was separated by reversed-phase (RP) HPLC.

Enzymatic Deprotection of Trimer **1a**. Hydrolysis of the protected 2-5A trimer **1a** in a *HEPES* buffer containing hog liver carboxyesterase (HLE; 2.6 units ml^{-1}) was followed at pH 7.5 and 37° by analyzing the composition of the aliquots withdrawn from

Scheme 3



i) $(Et_2N)_2PCl$, Et_3N, CH_2Cl_2. *ii*) **2a** (1 equiv.) with **11a** and **2b** (1 equiv.) with **11b**, TetH, MeCN. *iii*) **3**, TetH, MeCN. *iv*) I_2, THF, H_2O, lutidine. *v*) NH_2NH_2, AcOH, Py with **12a**, NH_2NH_3OAc, CH_2Cl_2, THF with **12b**. *vi*) 80% AcOH.

the reaction mixture at appropriate time intervals by HPLC. The products were characterized by MS analysis (HPLC/ESI-MS). *Fig. 1* shows the HPLC traces of the reaction mixture at 2 and 8 d. During the first 2 d, only one intermediate accumulated (*cf. Reaction A* in *Scheme 4*). The compound exhibited a $[M+H]^+$ peak at *m*/*z* 1967.3, referring to **1a** that had lost one of the phosphate protecting group.

Evidently, enzymatic deacetylation ($[M+H]^+$ transient occurrence at *m*/*z* 2169.4, referring to a monodeacetylated product, was detected) triggered loss of HCHO and concomitant elimination of the remnant of the protecting group as diethyl 2-methylidenemalonate, as discussed in more detail in [16]. The mere HPLC/ESI-MS data do not allow us to decide which one of the three possible diesters, **13a**, **13b**, or **13c**, was obtained. We are biased to assume that **13a** was first formed, but the other possibilities cannot be strictly excluded.

Loss of the next phosphate protecting group (*Reaction B*, *Scheme 4*) resulted in appearance of three chromatographic signals (t_R 42.0, 47.6, and 34.5 min), which all exhibited the $[M+H]^+$ peak at *m*/*z* 1723.3 (see the lower HPLC traces in *Fig. 1*). Our previous studies with thymidine 5′-(bis{3-[(acetyloxy)methoxy]-2,2-bis(ethoxycarbonyl)propyl} phosphate) [15] have shown that the enzymatic deacetylation of the negatively charged phosphodiester is 10^3 times slower than the deacetylation of the neutral phosphotriester. Accordingly, the two major chromatographic signals (t_R 42.0

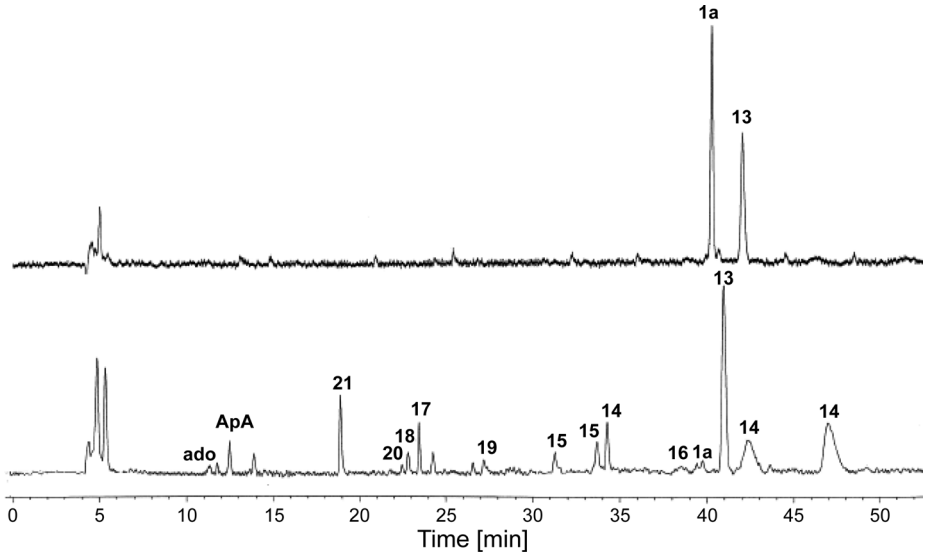
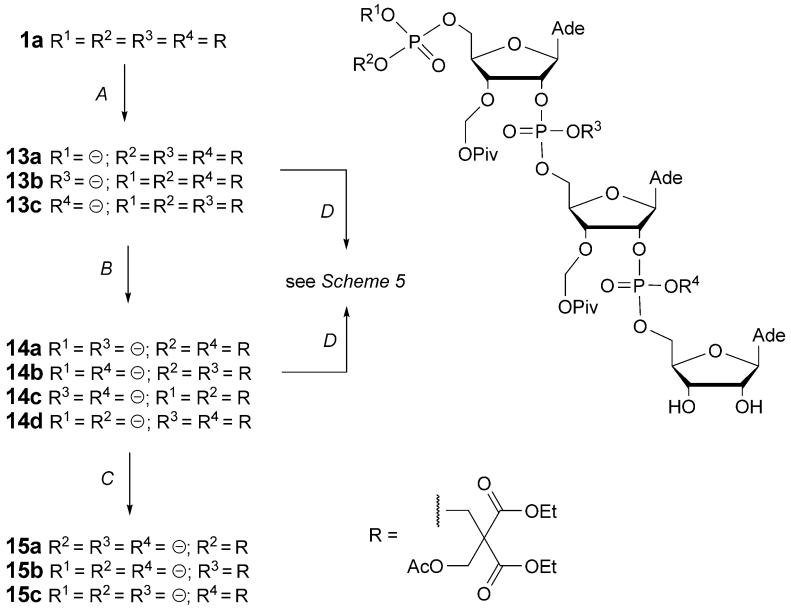


Fig. 1. *RP-HPLC Traces for the HLE-catalyzed hydrolysis of 2-5A trimer* **1a** *at pH 7.5 and 37.0°* ($I = 0.1$M with NaCl). The upper and lower traces refer to aliquots withdrawn at 2 and 8 d, respectively.

Scheme 4. *HLE-Triggered Deprotection of 2-5A Trimer* **1a**

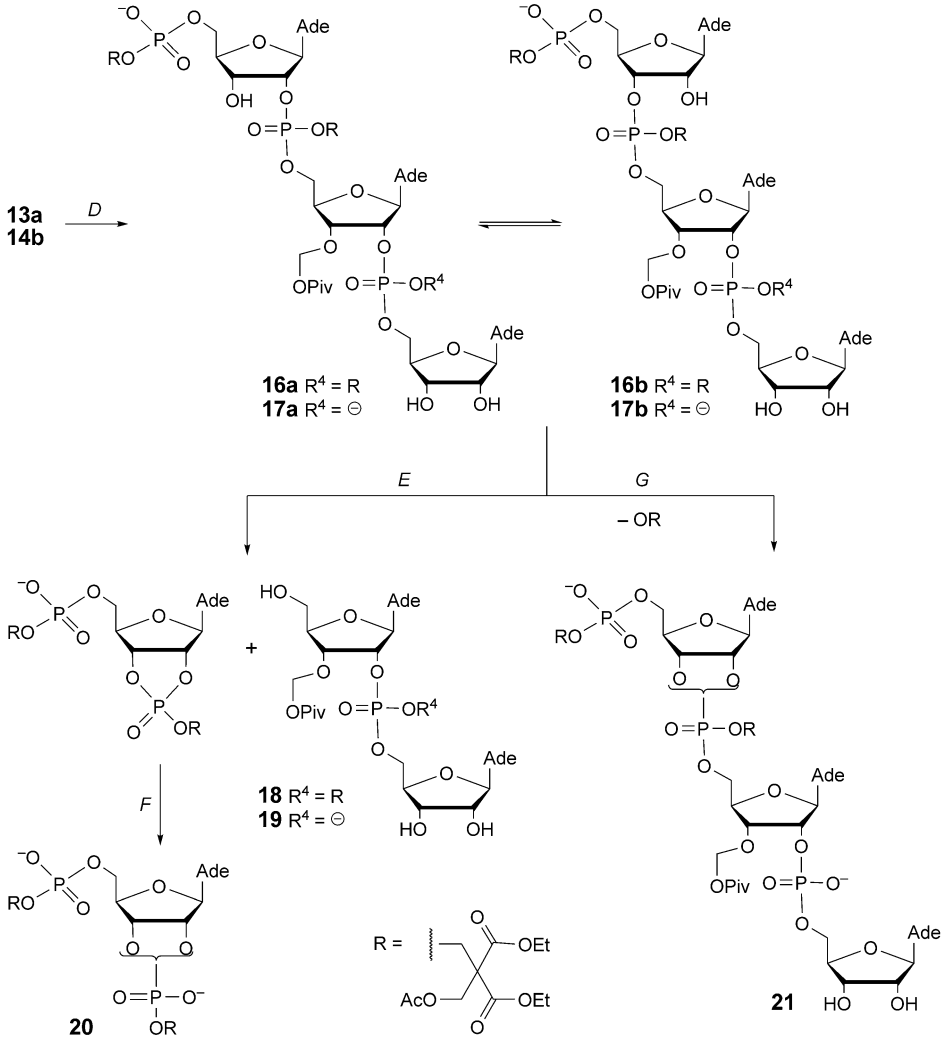


and 47.6 min) most likely refer to **14a** and **14b**, having one of the internucleosidic phosphodiester linkages deprotected in addition to the 5′-phosphate. The minor signal

(t_R 34.5 min) then refers to a trimer bearing either deprotected internucleosidic phosphates (*i.e.*, **14c**) or fully deprotected terminal phosphate (*i.e.*, **14d**).

The next phosphate protecting group was removed only very slowly (*Reaction C*, *Scheme 4*). Two HPLC signals referring to the expected quasi-molecular-ion ($[M + H]^+$) peak at *m/z* 1479.0 appeared. In other words, two of the three possible trianions **15a**–**15c** seemed to be formed. Even a prolonged treatment (2 weeks in HLE; 2.6 units ml^{-1}) resulted in only partial cleavage of the third phosphate protection. The fully phosphate deprotected 2-5A never appeared, but, in parallel to formation of **14** and **15**, compounds **13** and **14** lost a (pivaloyloxy)methyl group, evidently from the 5′-terminal

Scheme 5. *Side Reactions Taking Place during the HLE-Triggered Deprotection of Trimer* **1a**



nucleotide (*Scheme 5*). The latter assumption is based on the fact that, in addition to the $[M+H]^+$ and $[M+2\,H]^{2+}$ peaks at *m/z* 1609.1 and 927.7, referring to **16** and **17**, respectively, *m/z* values corresponding to dimers **18** ($[M+H]^+$ at *m/z* 955.7) and **19** ($[M+H]^+$ at *m/z* 711.5), monomer **20** ($[M+H]^+$ at *m/z* 916.6), and trimers **21** ($[M+2\,H]^{2+}$ at *m/z* 683.5) were detected. All these products were obtained by an attack of the exposed 3′-OH of trimer **16** or **17** on the adjacent protected phosphate group to give a pentacoordinated phosphorane intermediate [14]. Breakdown of this intermediate leads to *i*) isomerization of the 2′,5′ bond to a 3′,5′ bond, *ii*) cleavage of dimer **18** or **19** (*Reaction E*, *Scheme 5*) with concomitant formation of a cyclic phosphotriester that rapidly gives phosphodiesters **20** (*Reaction F*), and *iii*) cleavage of the phosphate protecting group (*Reaction G*), resulting in a cyclic phosphotriester, which is immediately hydrolyzed to isomeric phosphodiesters **21**. Unfortunately, the desired fully deprotedted 2-5A is never detected.

Enzymatic Deprotection of Trimer **1b**. As indicated above, 3′-*O*-[(pivaloyloxy)-methyl] protection did not afford a viable prodrug strategy for 2-5A. This group was removed from the 5′-terminal nucleotide too fast, compared to the removal of the adjacent phosphate protecting group. In addition, the 5′-monophosphate group was not exposed, but the deprotection stopped at the diester level. For these reasons, another prodrug candidate, **1b**, was prepared: *i*) the 3′-OH of the 5′-teminal nucleotide was protected permanently as a methyl ether, *ii*) the 3′-OH of the intevening nucleotide was protected with a more labile 3′-*O*-[(acetyloxy)methyl] group, and *iii*) the 3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl groups at the 5′-terminal phosphate were replaced with more labile 3-[(acetyloxy)methoxy]-2,2-bis(ethoxycarbonyl)propyl groups. Previous studies have shown that replacement of the 3-AcO group with a 3-[(acetyloxy)methoxy] group accelerated the first enzymatic deacetylation of bis-substituted thymidine 5′-monophosphate by a factor of 25, and the deacetylation of the resulting negatively charged diester even more [15].

Fig. 2 shows the HPLC traces of the reaction mixture of trimer **1b** treated with HLE under the same conditions as for **1a**. As seen, **1b** rapidly gave a monodeacetylated triester **22** ($[M+H]^+$ at *m/z* 2057.6; *Reaction H* in *Scheme 6*), which then underwent two parallel reactions: *i*) the HLE-triggered deacetylation and concomitant half-acetal hydrolysis to dideacetylated triester **23** ($[M+H]^+$ at *m/z* 1985.1; *Reaction I*) and *ii*) the *retro*-aldol condensation/elimination to the monoacetylated diester **24** ($[M+H]^+$ at *m/z* 1855.2; *Reaction J*). Both products, **23** and **24**, were subsequently converted to the deacetylated 5′-diester **25** ($[M+H]^+$ at *m/z* 1783.0; *Reactions K* and *L*, resp.) and further to monoester **26** ($[M+H]^+$ at *m/z* 1581.0; *Reaction M*), bearing a fully deprotected 5′-terminal phosphate group.

The final steps on the way to the fully deprotected 2-5A analog differed markedly depending on which one of the remaining internucleosidic phosphodiester protecting group was removed next. In case the 3′-terminal phosphodiester linkage was first exposed (*Reaction N*, *Scheme 7*), to give **27** ($[M-H]^-$ at *m/z* 1334.9; t_R 36.5 min), the desired fully deprotected 2-5A analog was eventually obtained without any side reactions. The last phosphate protection was removed (*Reaction O*), and the fully phosphate deprotected trimer **28** obtained ($[M-H]^-$ at *m/z* 1090.7; t_R 27.0 min) was finally converted to the desired fully deprotected trimer **29** ($[M-H]^-$ at *m/z* 1018.6; t_R 23.5 min) by removal of the 3′-*O*-[(acetyloxy)methyl] group (*Reaction P*).

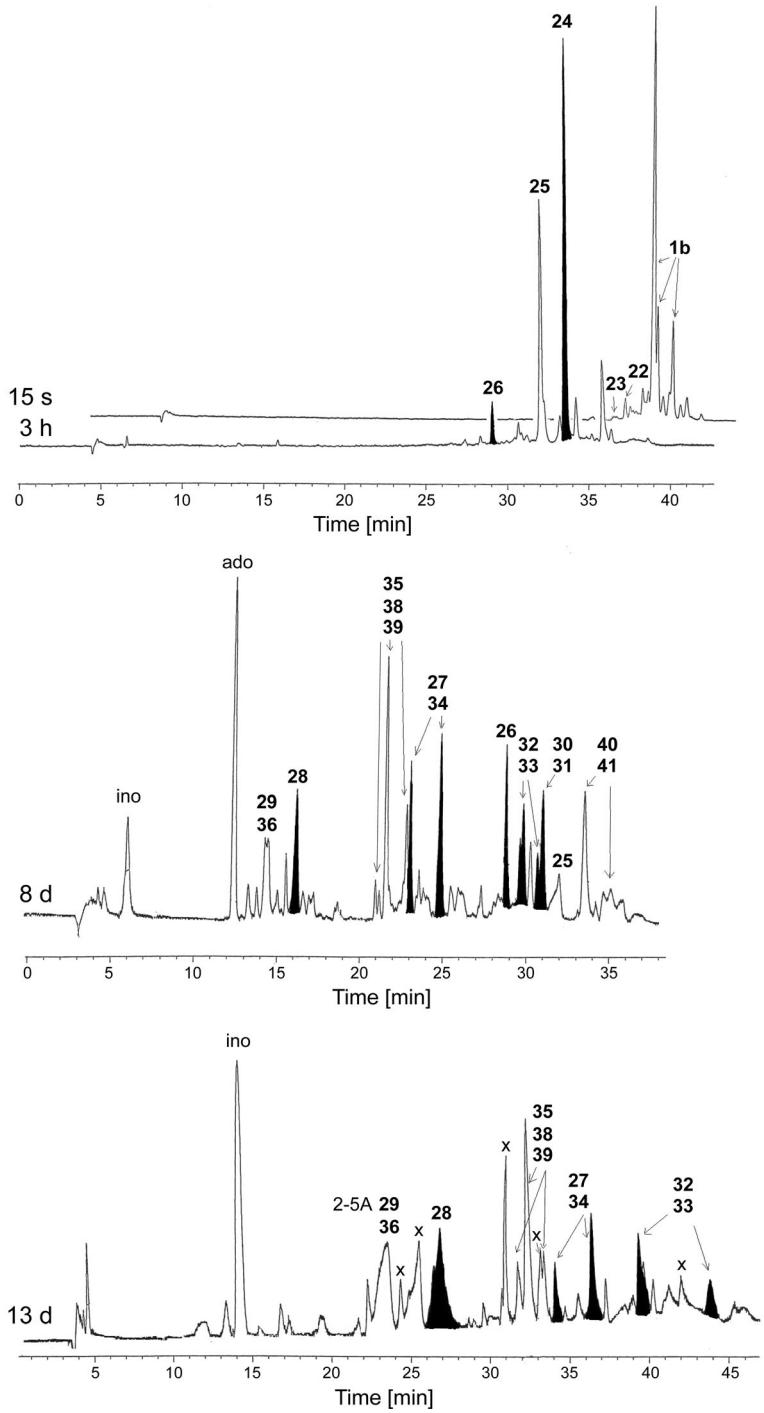
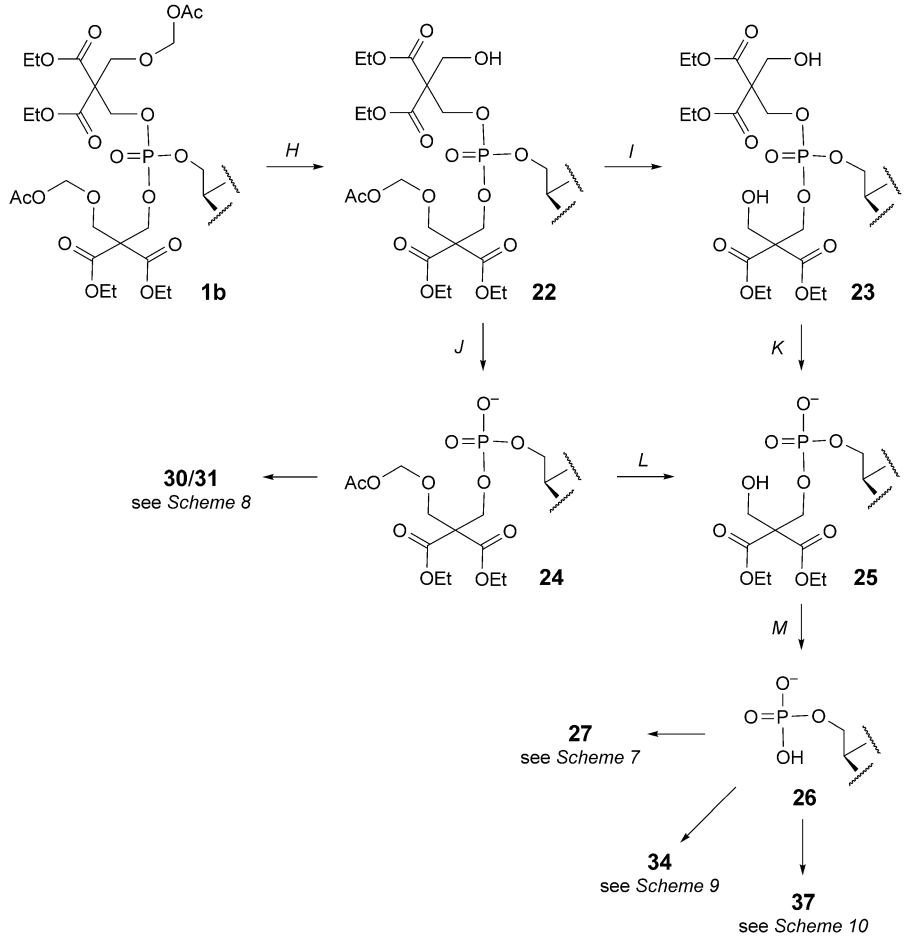


Fig. 2. *HPLC Traces for the HLE-triggered deprotection of 2-5A trimer* **1b** *at pH 7.5 and 37.0°* ($I = 0.1$M with NaCl). The traces from top to bottom refer to aliquots withdrawn at 15 s, 3 h, 8d, and 13 d, respectively. Signals marked with x refer to dephosphorylated products.

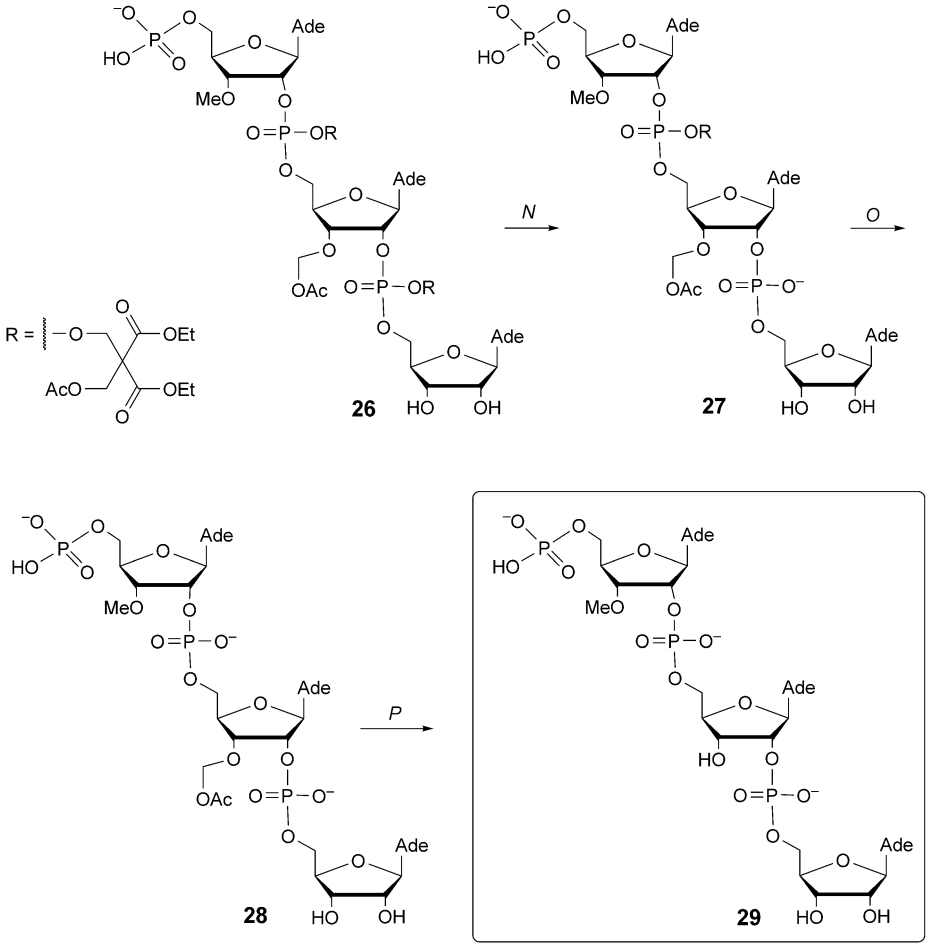
Scheme 6. *First Stages of HLE-Triggered Deprotection of 2-5A Trimer* **1b**



Unfortunately, **1b** was not quantitatively converted to **29**, but several side reactions depicted in *Schemes 8–10* took place. First, deprotection of one of the internucleosidic phosphodiester linkages of **24** (*Reaction Q*) competed with the formation of **26**. Doubly deprotected compounds **30**/**31** ($[M+H]^+$ at *m*/*z* 1610.9; t_R 41 min) were accumulated (*Scheme 8*), and they then lost the remaining 5′-terminal phosphate protecting group. Compound **30**, with a deprotected 3′-terminal diester bond, was converted to **27** *via* the deacetylated 5′-diesters **32** ($[M+H]^+$ at *m*/*z* 1538.7) and it, hence, gave the desired fully deprotected trimer **29** (*Reactions R* and *S*), as indicated in *Scheme 7*. Compound **31**, with a still protected 3′-terminal phosphodiester linkage, was, in turn, converted *via* **33** to **34** (*Reactions R* and *T*), which subsequently yielded several products as indicated in *Scheme 9*.

Second, compound **34**, which, in principle, may also be obtained by deprotection of the 5′-terminal internucleosidic phosphodiester bond of **26** (*Reaction U*, *Scheme 9*),

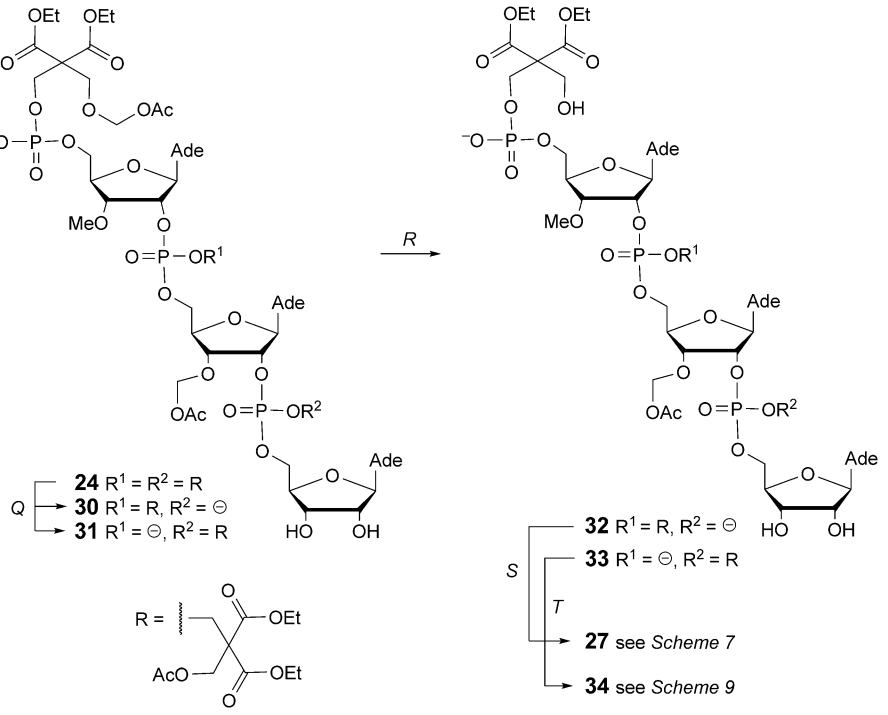
Scheme 7. *Late Stages of HLE-Triggered Deprotection of 2-5A Trimer* **1b**



reacts by two alternative routes (*Scheme 9*). When the remaining 3′-terminal phosphodiester protecting group is removed before the 3′-*O*-[(acetyloxy)methyl] group (*Reaction V*), the desired trimer **29** is obtained, as depicted in *Scheme 7*. By contrast, if the 3′-OH is first exposed (in the case of **35**; *Reaction W*), isomerization (→ **36**) and cleavage of the 2′-terminal phosphoester linkage expectedly take place as a consequence of the facile attack of the 3′-OH on the neighboring phosphotriester. Evidently, the latter reactions really took place, since the *m*/*z* value referring to **35** ($[M-H]^-$ at *m*/*z* 1262.8) was detected among the HPLC signals.

Finally, compound **26** also appeared to loose the 3′-*O*-[(acetyloxy)methyl] group before either of the internucleosidic phosphate protecting groups (→ **37**; *Reaction X*), since HPLC signals referring to trimers **38**/**39** ($[M-H]^-$ at *m*/*z* 1262.8) and dimers **40**/**41** ($[M-H]^-$ at *m*/*z* 1257.9), *i.e.*, the products expected to be obtained by an attack of

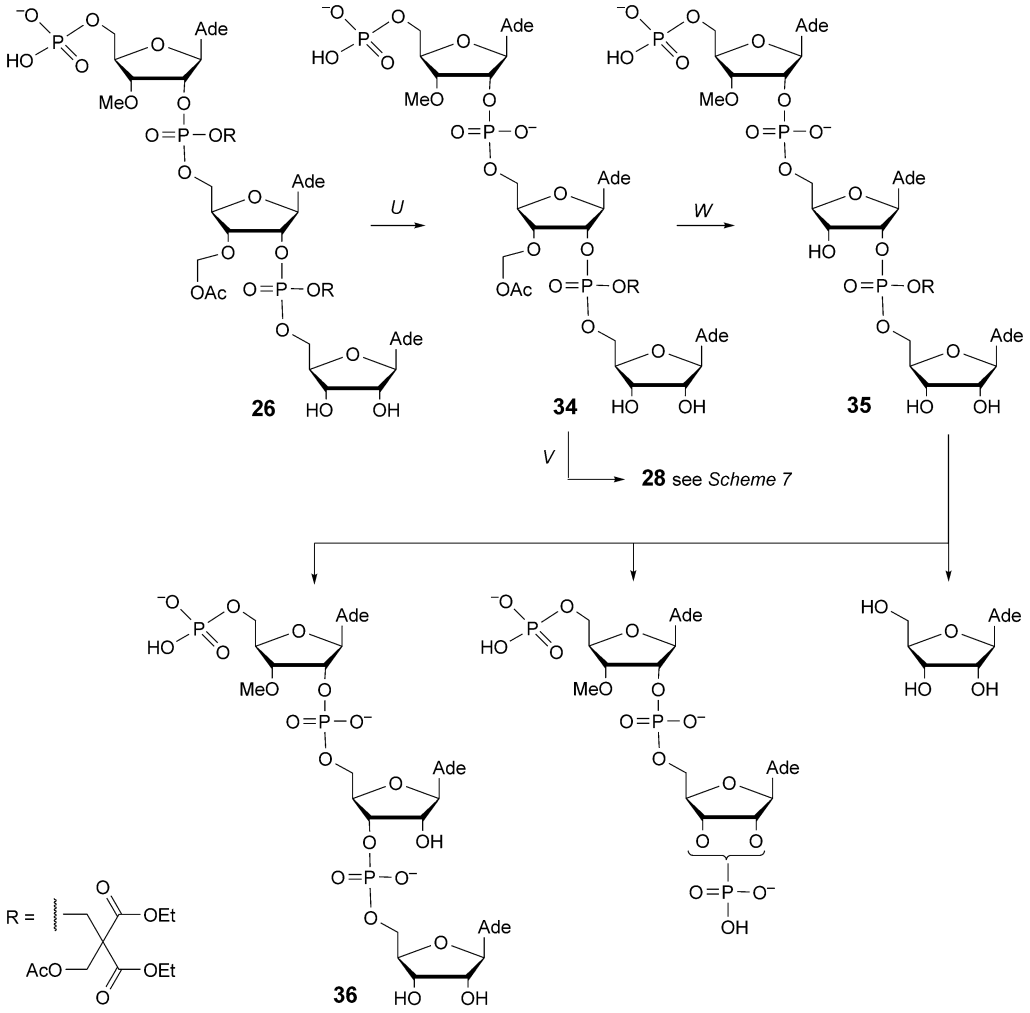
Scheme 8. *Side Reactions* R, S, *and* T *of HLE-Triggered Deprotection of* **1b**



the exposed 3′-OH on the adjacent phosphotriester (*Reactions Y* and *Z*), were observed (*Scheme 10*).

Conclusions. – The results of the present study indicate that an esterase activity-dependent prodrug strategy appears feasible for 2-5A, although the present protecting group scheme, including bis{3-[(acetyloxy)methoxy]-2,2-bis(ethoxycarbonyl)propyl} protection of the 5′-terminal monophosphate group, 3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl protection of the internucleosidic phosphodiester linkages, 3′-*O*-methyl protection of the 5′-terminal nucleotide and 3′-*O*-[(acetyloxy)methyl] protection of the intervening nucleoside, still suffers from formation of several by-products. The main problem is a too slow removal of the protecting group from the 3′-terminal phosphodiester bond compared to the removal of the neighboring 3′-*O*-[(acetyloxy)-methyl] group. Evidently, protection of this phosphodiester linkage with the more labile 3-[(acetyloxy)methoxy]-2,2-bis(ethoxycarbonyl)propyl markedly improves the situation. The advantage of the phosphate protecting groups used in the present study is that the rate of the enzymatic step that triggers the removal and the subsequent chemical step, which eventually results in exposure of the negatively charged phosphate, may be tuned separately. The enzymatic reaction is susceptible to steric properties of the acyl group, while the departure of the remnant of the protecting group may be affected by the polar properties of the 2-substituents.

Scheme 9. *Side Reactions* U, V, *and* W *of HLE-Triggered Deprotection of* **1b**

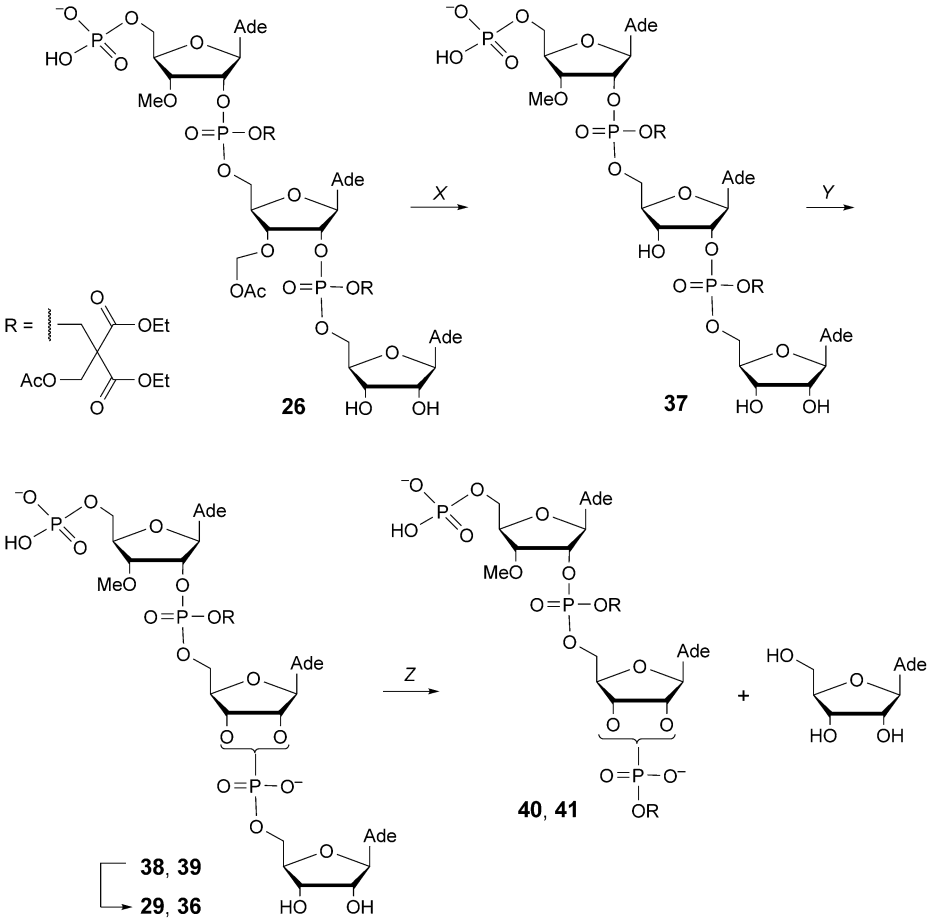


Experimental Part

General. MeCN, CH_2Cl_2, and pyridine were dried over 4-Å and 1,4-dioxane over 3-Å molecular sieves. Et_3N was dried by refluxing over CaH_2 and distilled before use. HPLC: *Merck Hitachi LaChrom D7000* with *L-7455* UV detector and *L-7100* pump. ^{1}H-, ^{13}C-, ^{31}P-, and 2D-NMR spectra: *Bruker Avance 500* NMR spectrometer; δ in ppm, J in Hz. The assignments of the NMR signals are based on 2D-COSY and HSQC spectra. LC/ESI-MS: *Perkin-Elmer Sciex-API-365* triple-quadrupole. HR-ESI-MS: *Bruker Daltonics micrOTOF-Q*.

*2′-*O*-Levulinoyl-*N^6*-[(4-methoxyphenyl)(diphenyl)methyl]-3′-*O*-[(pivaloyloxy)methyl]adenosine* (**6a**). 2′-*O*-Levulinoyl-3′-*O*-[(pivaloyloxy)methyl]adenosine [13] (1.9 mmol, 0.9 g), dried over P_2O_5 overnight, was dissolved in dry pyridine (12 ml). The soln. was cooled on an ice-bath, and Me_3SiCl (9.4. mmol, 1.19 ml) was added. The mixture was stirred for 2.5 h at r.t. (4-Methoxyphenyl)(diphenyl)-

Scheme 10. *Side Reactions* X, Y, *and* Z *of HLE-Triggered Deprotection of* **1b**



methyl chloride (MMTrCl; 2.3 mmol, 0.70 g) was added, and the mixture was stirred over three nights at 37°. The solvent was removed by evaporation under reduced pressure, and the residual oil was partitioned between H_2O and AcOEt. The org. layer was washed with sat. aq. $NaHCO_3$, dried (Na_2SO_4), and evaporated to dryness. The Me_3Si group was removed by treatment with Bu_4NF in THF under acidic conditions. Accordingly, Bu_4NF (2.8 mmol, 0.74 g) was dissolved in dry THF (16 ml), and AcOH (3 ml) was added. The nucleoside was added, and the mixture was stirred at r.t. for 2 h. Sat. aq. $NaHCO_3$ soln. was added, and the mixture was extracted with CH_2Cl_2. The org. phase was dried (Na_2SO_4) and evaporated to dryness. The product was purified by column chromatography (CC; silica gel; CH_2Cl_2 containing 3% MeOH) to give **6a** (1.17 g, 83%). Yellowish foam. ^{1}H-NMR (500 MHz, $CDCl_3$): 8.02 (*s*, H–C(2)); 7.80 (*s*, H–C(8)); 7.24–7.36 (*m*, 12 H of MMTr); 7.05 (*s*, H–N^6); 6.81–6.84 (*m*, 2 H of MMTr); 6.62 (*dd*, *J* = 12, 2, HO–C(5′)); 6.03 (*d*, *J* = 7.5, H–C(1′)); 5.68 (*dd*, *J* = 7.5, 5.3, H–C(2′)); 5.55 (*d*, *J* = 6.5, 1 H of OCH_2O); 5.12 (*d*, *J* = 6.5, 1 H of OCH_2O); 4.82 (*dd*, *J* = 5.3, 1, H–C(3′)); 4.35 (*m*, H–C(4′)); 3.93–3.97 (*m*, H–C(5′)); 3.81 (*s*, MeO of MMTr); 3.72–3.78 (*m*, H–C(5″)); 2.53–2.78 (*m*, 2 CH_2 of Lev); 2.20 (*s*, Me of Lev); 1.25 (*s*, Me_3C of Piv). ^{13}C-NMR (126 MHz, $CDCl_3$): 206.1 (C=O of Lev); 177.7 (C=O of Piv); 171.5 (C=O of Lev); 158.4 (MMTr); 154.6 (C(6)); 151.9 (C(2)); 147.3 (C(4)); 144.9 (MMTr); 139.8

(C(8)); 136.9, 130.2, 128.9, 128.0, 127.0 (MMTr); 122.5 (C(5)); 113.2 (MMTr); 88.9 (OCH_2O); 88.9 (C(1′)); 87.3 (C(4′)); 78.2 (C(3′)); 74.6 (C(2′)); 71.1 (MMTr); 62.7 (C(5′)); 55.2 (MeO of MMTr); 38.8 (Me_3C of Piv); 37.7 (CH_2C=O of Lev); 29.8 (Me of Lev); 27.5 (CH_2COO of Lev); 27.0 (Me of Piv). HR-ESI-MS: 752.3312 (M^+, $C_{41}H_{46}N_5O_9^+$; calc. 752.3290).

*2′-*O*-Levulinoyl-*N^6*-[(4-methoxyphenyl)(diphenyl)methyl]-3′-*O*-[(pivaloyloxy)methyl]adenosine 5′-{Bis[3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl] Phosphate}* (**10a**). Compound **6a** (1.5 mmol, 1.10 g, dried over P_2O_5 overnight) was dissolved in dry CH_2Cl_2 (7 ml) under N_2. Anh. Et_3N (7.3 mmol, 1.02 ml) and chlorobis(diethylamino)phosphine ($(Et_2N)_2PCl$; 2.1 mmol, 0.43 ml) were added, and the mixture was stirred for 2 h. The product was isolated by passing the mixture through a short silica gel column with a 7:3 mixture of AcOEt and hexane containing 0.5% Et_3N. The solvent was removed under reduced pressure, and the residue was co-evaporated from dry MeCN to remove traces of Et_3N. The formation of the phosphitylated product was verified by ^{31}P-NMR spectroscopy. ^{31}P-NMR (202 MHz, CD_3CN): 133.3.

The phosphitylated nucleoside was dissolved in dry MeCN (1 ml) under N_2. *Diethyl 2-[(acetyloxy)methyl]-2-(hydroxymethyl)propanedioate* (**3**; 4.2 mmol, 1.10 g, co-evaporated twice with dry MeCN and dried over P_2O_5 overnight), dissolved in dry MeCN (2 ml), and 1*H*-tetrazole (4.4 mmol, 9.76 ml of 0.45M soln. in MeCN) were added. The course of the reaction was followed by ^{31}P-NMR spectroscopy. The spectrum was recorded after 0.5 h. ^{31}P-NMR (202 MHz, CD_3CN): 138.8. The phosphite ester formed was oxidized with I_2 (0.1M) in a mixture of THF, H_2O, and 2,6-lutidine (4:2:1 (*v*/*v*/*v*), 10 ml) by stirring overnight at r.t. Aq. 5% $NaHCO_3$ soln. was added, and the mixture was extracted twice with CH_2Cl_2. The org. phase was dried (Na_2SO_4) and evaporated to dryness. The product was purified by CC (silica gel; 5% MeOH in CH_2Cl_2). The purification was repeated with CH_2Cl_2/AcOEt 1:1 and then changing to 5% MeOH in CH_2Cl_2 to give **10a** (0.44 g, 22%). Clear oil. ^{1}H-NMR (500 MHz, $CDCl_3$): 8.03 (*s*, H–C(2)); 7.95 (*s*, H–C(8)); 7.23–7.37 (*m*, 12 H of MMTr); 6.94 (*s*, H–N^6); 6.80–6.83 (*m*, 2 H of MMTr); 6.09 (*d*, *J* = 3.5, H–C(1′)); 5.76 (*dd*, *J* = 5.5, 3.5, H–C(2′)); 5.34 (*d*, *J* = 6.5, 1 H of OCH_2O); 5.20 (*d*, *J* = 6.5, 1 H of OCH_2O); 4.97 (*m*, H–C(3′)); 4.51–4.62 (*m*, 2 CH_2OAc, 2 POCH_2C); 4.17–4.33 (*m*, H–C(4′), H–C(5′), H–C(5″), and 4 MeCH_2O); 3.81 (*s*, MeO of MMTr); 2.77–2.80 (*m*, 2 H of CH_2CH_2 of Lev); 2.66–2.70 (*m*, 2 H of CH_2CH_2 of Lev); 2.20 (*s*, Me of Lev); 2.05 (*s*, AcO); 2.01 (*s*, AcO); 1.20–1.32 (*m*, 4 *Me*CH_2, 3 Me of Piv). ^{31}P-NMR (202 MHz, CD_3CN): −2.59. HR-ESI-MS: 1320.4812 (M^+, $C_{63}H_{79}N_5O_{24}P^+$; calc. 1320.4847).

*N^6-[(4-Methoxyphenyl)(diphenyl)methyl]-3′-*O*-[(pivaloyloxy)methyl]adenosine 5′-(Bis{3-[(acetyloxy)methyl]-2,2-bis(ethoxycarbonyl)propyl} Phosphate)* (**11a**). Compound **10a** (0.3 mmol, 0.44 g) was dissolved in a soln. of $NH_2NH_2 \cdot H_2O$ (3.9 mmol, 0.12 ml) in pyridine (4 ml) and AcOH (1 ml) on an ice bath, and the mixture was stirred for 1.5 h. The ice bath was removed, and the reaction was allowed to proceed at r.t. for 2 h. The reaction was quenched with 0.1M NaH_2PO_3 soln., and the mixture was extracted with CH_2Cl_2. The org. phase was washed with H_2O, dried (Na_2SO_4), and evaporated to dryness. The product was purified by CC (silica gel; CH_2Cl_2 containing 3–5% MeOH) to give **11a** (0.35 g, 88%). Clear oil. ^{1}H-NMR (500 MHz, $CDCl_3$): 8.02 (*s*, H–C(2)); 7.98 (*s*, H–C(8)); 7.23–7.37 (*m*, 12 H of MMTr); 6.96 (*s*, H–N^6); 6.80–6.83 (*m*, 2 H of MMTr); 5.93 (*d*, *J* = 5.0, H–C(1′)); 5.51 (*d*, *J* = 6.3, 1 H of OCH_2O); 5.42 (*d*, *J* = 6.3, 1 H of OCH_2O); 4.76 (*dd*, *J* = 5.5, 5.0, H–C(2′)); 4.64 (*m*, H–C(3′)); 4.50–4.63 (*m*, 2 CH_2OAc, 2 POCH_2C); 4.37 (*m*, H–C(4′)); 4.19–4.31 (*m*, 4 MeCH_2O, H–C(5′) and H–C(5″)); 3.88 (*d*, *J* = 5, HO–C(2′)) 3.81 (*s*, MeO of MMTr); 2.05 (*s*, AcO); 2.03 (*s*, AcO); 1.22–1.32 (*m*, 4 *Me*CH_2O, 3 Me of Piv). ^{13}C-NMR (126 MHz, $CDCl_3$): 178.0 (C=O of Piv); 170.1 (C=O of Ac); 166.4 (*C*(=O)OEt) 158.3 (MMTr); 154.3 (C(6)); 152.1 (C(2)); 148.3 (C(4)); 145.2 (MMTr); 138.8 (C(8)); 135.9, 130.2, 128.9, 127.9, 126.9 (MMTr); 123.7 (C(5)); 113.2 (MMTr); 89.4 (C(1′)); 89.0 (OCH_2O); 81.3 (C(4′)); 78.7 (C(3′)); 74.0 (C(2′)); 71.0 (MMTr); 67.2 (C(5′)); 65.4 (POCH_2C); 62.3 (MeCH_2); 61.2 (CH_2OAc); 58.0 (C); 55.2 (MeO of MMTr); 38.8 (Me_3C of Piv); 27.0 (Me of Piv); 20.6 (Ac); 13.9 (*Me*CH_2). HR-ESI-MS: 1222.4485 (M^+, $C_{58}H_{73}N_5O_{22}P^+$; calc. 1222.4479).

*5′-*O*-[(*tert*-Butyl)(dimethyl)silyl]-3′-*O*-methyladenosine* (**7**). Commercially available 3′-*O*-methyladenosine (3.6 mmol, 1.01 g) was co-evaporated twice from anh. pyridine, and the residue was dissolved in the same solvent (7 ml). *t*-Bu(Me_2)SiCl (TBDMSCl; 1.1 equiv.; 4.0 mmol, 0.60 g) was added, and the mixture was stirred overnight at r.t. The reaction was quenched with MeOH, and the mixture evaporated to dryness. The residue was purified by CC (silica gel; CH_2Cl_2 containing 10% MeOH). ^{1}H-NMR (500 MHz, MeOD): 8.41 (*s*, H–C(2)); 8.23 (*s*, H–C(8)); 6.06 (*d*, *J* = 4.2, H–C(1′)); 4.77 (*dd*, *J* = 4.2, 4.6,

H–C(2′)); 4.22 (*m*, H–C(4′)); 4.06 (*dd*, *J* = 4.6, 5.0, H–C(3′)); 4.03 (*dd*, *J* = 11.5, 3.4, H–C(5′)); 3.87 (*dd*, *J* = 11.5, 3.0, H–C(5″)); 3.50 (*s*, 3′-MeO); 0.96 (*s*, ^{t}Bu); 0.14 (*s*, Me_2Si). ^{13}C-NMR (126 MHz, MeOD): 155.9 (C(6)); 152.5 (C(2)); 149.1 (C(4)); 139.3 (C(8)); 119.0 (C(5)); 88.8 (C(1′)); 82.7 (C(4′)); 78.9 (C(3′)); 73.3 (C(2′)); 62.3 (C(5′)); 57.0 (MeO); 25.0 (Me_3C); 17.9 (Me_3C); −6.7 (Me_2Si).

*5′-O-[(*tert*-Butyl)(dimethyl)silyl]-*N^6*-[(4-methoxyphenyl)(diphenyl)methyl]-3′-O-methyladenosine* (**8**). Compound **7** was co-evaporated twice from anh. pyridine, and the residue was dissolved in dry pyridine (6 ml). MMTrCl was added, and the mixture was stirred over three nights at r.t. The reaction was quenched with MeOH, and the mixture was evaporated to dryness. The residue was dissolved in CH_2Cl_2, and washed with sat. aq. $NaHCO_3$ and sat. aq. NaCl. The org. phase was dried (Na_2SO_4) and evaporated to dryness. The product was purified by CC (silica gel; CH_2Cl_2 containing 2–3% MeOH) to afford **8** (180 g, 75% yield from 3′-*O*-methyladenosine). White foam. ^{1}H-NMR (500 MHz, $CDCl_3$): 8.06 (br. *s*, H–C(2), H–C(8)); 7.34–7.37 (*m*, 4 H of MMTr); 7.23–7.30 (*m*, 8 H of MMTr); 6.96 (br. *s*, H–N); 6.81 (*d*, *J* = 8.8, 2 H of MMTr); 5.98 (*d*, *J* = 5.6, H–C(1′)); 4.73 (*m*, H–C(2′)); 4.27 (*m*, H–C(4′)); 4.16 (*d*, *J* = 6.5, HO–C(2′)); 4.05 (*m*, H–C(3′)); 3.91 (*dd*, *J* = 11.2, 4.2, H–C(5′)); 3.78–3.81 (*m*, MeO, H–C(5″)); 3.52 (*s*, 3′-MeO); 0.90 (*s*, ^{t}Bu); 0.09, 0.10 (2*s*, Me_2Si). ^{13}C-NMR (126 MHz, $CDCl_3$): 158.3 (MMTr); 154.1 (C(6)); 152.1 (C(2)); 148.5 (C(4)); 145.2 (MMTr); 138.3 (C(8)); 137.2 (MMTr); 130.2 (MMTr); 128.9 (MMTr); 127.9 (MMTr); 126.9 (MMTr); 121.3 (C(5)); 113.1 (MMTr); 89.3 (C(1′)); 83.1 (C(4′)); 80.0 (C(3′)); 74.3 (C(2′)); 71.0 (MMTr); 63.0 (C(5′)); 58.1 (3′-MeO); 55.2 (MMTr); 25.9 (Me_3C); 18.3 (Me_3C); −5.5 (Me_2Si).

*5′-O-[(*tert*-Butyl)(dimethyl)silyl]-2′-O-levulinoyl-*N^6*-[(4-methoxyphenyl)(diphenyl)methyl]-3′-O-methyladenosine* (**9**). Levulinic anhydride was prepared by dissolving levulinic acid (6.7 mmol, 0.73 g) in dry 1,4-dioxane (10 ml) on an ice bath and adding *N,N′*-dicyclohexylcarbodiimide (DCC; 3.4 mmol, 0.70 g) in small portions within 1 h. The soln. was stirred at r.t. for 2 h. Precipitated dicyclohexylurea was filtered off and washed with 5 ml of dry dioxane. The filtrate was added to a soln. of **8** (2.7 mmol, 1.80 g, dried over P_2O_5 overnight) in dry pyridine (9 ml), and a cat. amount of 4-(dimethylamino)pyridine (DMAP) was added. After stirring overnight at r.t., the mixture was evaporated to dryness. The residue was dissolved in CH_2Cl_2, and washed with sat. aq. $NaHCO_3$ and sat. aq. NaCl. The org. phase was dried (Na_2SO_4) and evaporated to dryness. The product was purified by CC (silica gel; CH_2Cl_2 containing 1–2% MeOH to give **9** (184 g, 89%). White foam. ^{1}H-NMR (500 MHz, $CDCl_3$): 8.10 (*s*, H–C(2)); 8.06 (*s*, H–C(8)); 7.23–7.38 (*m*, 12 H of MMTr); 6.92 (*s*, 2 H–N^6); 6.82 (*m*, 2 H of MMTr); 6.19 (*d*, *J* = 4.0, H–C(1′)); 5.74 (*dd*, *J* = 4.5, 4.0, H–C(2′)); 4.30 (*m*, H–C(3′)); 4.18 (*m*, H–C(4′)); 4.01 (*dd*, *J* = 11.5, 3.0, H–C(5′)); 3.83 (*dd*, *J* = 11.5, 3.0, H–C(5″)); 3.80 (*s*, 3 H of MMTr); 3.42 (*s*, MeO); 2.62–2.81 (*m*, 4 H of Lev); 2.18 (*s*, 3 H of Lev); 0.94 (*s*, ^{t}Bu); 0.12 (*s*, MeSi); 0.11 (*s*, MeSi). ^{13}C-NMR (126 MHz, $CDCl_3$): 206.1 (C=O of Lev); 171.7 (C=O of Lev); 158.3 (MMTr); 154.1 (C(6)); 152.4 (C(2)); 148.5 (C(4)); 145.2 (MMTr); 138.4 (C(8)); 137.2 (MMTr); 130.2 (MMTr); 128.9 (MMTr); 127.9 (MMTr); 126.8 (MMTr); 121.2 (C(5)); 113.1 (MMTr); 86.5 (C(1′)); 82.9 (C(4′)); 77.7 (C(3′)); 74.5 (C(2′)); 71.0 (MMTr); 62.2 (C(5′)); 58.8 (MeO); 55.2 (MMTr); 37.8 (Lev); 29.8 (Lev); 27.8 (Lev); 26.0 (TBDMS); 18.4 (TBDMS); −5.3 (TBDMS); −5.5 (TBDMS).

*2′-O-Levulinoyl-*N^6*-[(4-methoxyphenyl)(diphenyl)methyl]-3′-O-methyladenosine* (**6b**). Compound **9** was desilylated with Bu_4NF in THF under acidic conditions. Accordingly, to a soln. of Bu_4NF (3.6 mmol, 0.94 g) in dry THF (30 ml), AcOH (6 ml) and **9** were added, and the mixture was stirred over two nights at r.t. Sat. aq. $NaHCO_3$ soln. was added, and the mixture was extracted with CH_2Cl_2. The org. phase was washed with sat. aq. $NaHCO_3$ and sat. aq. NaCl, and dried (Na_2SO_4) and evaporated to dryness. The product was purified by CC (silica gel; CH_2Cl_2 containing 1–3% MeOH) to give **6b** (1.25 g, 80%). White foam. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.99 (*s*, H–C(2)); 7.77 (*s*, H–C(8)); 7.21–7.34 (*m*, 12 H of MMTr); 7.00 (*s*, H–N^6); 6.80 (*m*, 2 H of MMTr); 6.59 (*dd*, *J* = 12.0, 2.0, HO–C(5′)); 6.00 (*d*, *J* = 7.5, H–C(1′)); 5.71 (*dd*, *J* = 7.5, 5.0, H–C(2′)); 4.33–4.36 (*m*, H–C(3′), H–C(4′)); 3.98 (*m*, H–C(5′)); 3.78 (*s*, 3 H of MMTr); 3.69 (*m*, H–C(5″)); 3.44 (*s*, MeO); 2.53–2.75 (*m*, 4 H of Lev); 2.17 (*s*, 3 H of Lev). ^{13}C-NMR (126 MHz, $CDCl_3$): 206.1 (C=O of Lev); 171.6 (C=O of Lev); 158.4 (MMTr); 154.6 (C(6)); 151.9 (C(2)); 147.3 (C(4)); 145.0 (MMTr); 139.8 (C(8)); 136.9 (MMTr); 130.2 (MMTr); 128.9 (MMTr); 128.0 (MMTr); 127.0 (MMTr); 122.5 (C(5)); 113.2 (MMTr); 89.1 (C(1′)); 86.1 (C(4′)); 79.4 (C(3′)); 75.0 (C(2′)); 71.1 (MMTr); 63.2 (C(5′)); 58.5 (MeO); 55.2 (MMTr); 37.7 (Lev); 29.8 (Lev); 27.6 (Lev). HR-ESI-MS: 652.2718 (M^+, $C_{31}H_{38}N_7O_9^+$; calc. 652.2726).

2′-O-*Levulinoyl*-N^6-*[(4-methoxyphenyl)(diphenyl)methyl]-3′*-O-*methyladenosine 5′-(Bis{3-[(acetyloxy)methoxy]-2,2-bis(ethoxycarbonyl)propyl} Phosphate)* (**10b**). Compound **6b** (1.5 mmol, 0.99 g, dried over P_2O_5 over three nights) was dissolved in dry CH_2Cl_2 (3 ml) under N_2. Anh. Et_3N (7.60 mmol, 1.056 ml) and $(Et_2N)_2PCl$ (2.13 mmol, 447 µl) were added, and the mixture was stirred for 2 h. The product was isolated by passing the mixture through a short silica-gel column eluting with dry AcOEt containing 1% Et_3N. The solvent was removed under reduced pressure, and the residue was co-evaporated from dry MeCN to remove traces of Et_3N. The identity of the product was checked by ^{31}P-NMR spectroscopy. ^{31}P-NMR (202 MHz, CD_3CN): 133.7. The phosphitylated nucleoside was dissolved in dry MeCN (1 ml) under N_2. *Diethyl 2-{[(acetyloxy)methoxy]methyl}-2-(hydroxymethyl)propanedioate* (**4**; 3.8 mmol, 0.11 g, co-evaporated twice with dry MeCN and dried over P_2O_5 over three nights) and 1*H*-tetrazole (3.80 mmol, 8.440 ml of 0.45M soln. in MeCN) were added. The course of the reaction was followed by ^{31}P-NMR spectroscopy. The spectrum was recorded after 1 h. ^{31}P-NMR (202 MHz, CD_3CN): 139.0. The phosphite ester formed was oxidized with I_2 (0.1M) in a mixture of THF, H_2O, and 2,6-lutidine (4:2:1 (*v*/*v*/*v*); 10 ml) by stirring overnight at r.t. Aq. 5% $NaHCO_3$ soln. was added, and the mixture was extracted twice with CH_2Cl_2. The org. phase was dried (Na_2SO_4) and evaporated to dryness. The product was purified by CC (silica gel; CH_2Cl_2/AcOEt 1:1) to give **10b** (0.85 g, 43%). Clear oil. ^{1}H-NMR (400 MHz, $CDCl_3$): 8.01 (*s*, H–C(2)); 7.92 (*s*, H–C(8)); 7.19–7.35 (*m*, 12 H of MMTr); 6.92 (*s*, H–N^6); 6.77–6.81 (*m*, 2 H of MMTr); 6.06 (*d*, *J* = 3.2, H–C(1′)); 5.76 (*dd*, *J* = 5.2, 3.2, H–C(2′)); 5.20–5.25 (*m*, 2 OCH_2O); 4.50–4.53 (*m*, 2 $POCH_2C$); 4.39 (*m*, H–C(3′)); 4.32 (*m*, H–C(5′)); 4.09–4.27 (*m*, 14 H, H–C(4′), H–C(5″), $MeCH_2O$, CH_2O); 3.78 (*s*, MeO of MMTr); 3.41 (*s*, 3′-MeO); 2.63–2.79 (*m*, CH_2CH_2 of Lev); 2.17 (*s*, Me of Lev); 2.06 (*s*, Ac); 2.04 (*s*, Ac); 1.18–1.28 (*m*, 4 $MeCH_2$). ^{13}C-NMR (101 MHz, $CDCl_3$): 206.0 (C=O Lev); 171.7 (C=O of Lev); 170.2 (Me*C*=*O*); 166.6 (*C*(=O)OEt) 158.3 (MMTr); 154.2 (C(6)); 152.4 (C(2)); 148.3 (C(4)); 145.2 (MMTr); 139.0 (C(8)); 137.2, 130.2, 128.9, 127.9, 126.8 (MMTr); 121.5 (C(5)); 113.2 (MMTr); 89.1 (OCH_2O); 88.8 (OCH_2O); 87.5 (C(1′)); 80.6 (C(4′)); 78.1 (C(3′)); 73.7 (C(2′)); 71.0 (MMTr); 67.1 (C(5′)); 65.2 ($POCH_2C$); 62.1 ($MeCH_2$); 61.8 (CH_2OAc); 59.1 (3′-MeO); 55.2 (MeO of MMTr); 53.4 (C); 37.8 (CH_2 of Lev); 29.7 (Me of Lev); 27.8 (CH_2 of Lev); 20.9 (Ac); 13.9 ($MeCH_2$). ^{31}P-NMR (162 MHz, $CDCl_3$): −2.15. HR-ESI-MS: 1280.4538 (M^+, $C_{60}H_{75}N_5O_{24}P^+$; calc. 1280.4534).

N^6-*[(4-Methoxyphenyl)(diphenyl)methyl]-3′*-O-*methyladenosine 5′-(Bis{3-[(acetyloxy)methoxy]-2,2-bis(ethoxycarbonyl)propyl} Phosphate)* (**11b**). Compound **10b** (0.66 mmol, 0.85 g, dried over P_2O_5 over three nights) was dissolved in dry CH_2Cl_2 (16 ml). NH_2NH_3OAc (1.19 mmol, 0.11 g) in dry MeOH (2 ml) was added. After stirring the mixture for 3 h, another portion of NH_2NH_3OAc (0.60 mmol, 0.055 g) in dry MeOH (1 ml) was added, and the reaction was allowed to proceed for 1 h. The reaction was quenched with acetone, and the mixture was evaporated to dryness. The product was purified by CC (silica gel; CH_2Cl_2/AcOEt 1:4) to give **11b** (0.70 g, 89%). Clear oil. ^{1}H-NMR (500 MHz, $CDCl_3$): 8.01 (*s*, H–C(2)); 7.96 (*s*, H–C(8)); 7.20–7.35 (*m*, 12 H of MMTr); 6.94 (*s*, H–N^6)); 6.77–6.81 (*m*, 2 H of MMTr); 5.91 (*d*, *J* = 5.5, H–C(1′)); 5.20–5.25 (*m*, 2 OCH_2O); 4.80 (*m*, H–C(2′)); 4.49–4.56 (*m*, 2 $POCH_2C$); 4.33 (*m*, H–C(4′)); 4.10–4.30 (*m*, H–C(5′), H–C(5″), H–C(3′), 4 $MeCH_2O$, 2 CH_2O); 3.84 (br. *s*, HO–C(2′)); 3.78 (*s*, MeO of MMTr); 3.54 (*s*, 3′-MeO); 2.06 (*s*, Ac); 2.05 (*s*, Ac); 1.20–1.29 (*m*, 4 $MeCH_2O$). ^{13}C-NMR (126 MHz, $CDCl_3$): 170.3 (Me*C*=O); 166.6 (*C*(=O)OEt); 158.3 (MMTr); 154.2 (C(6)); 152.2 (C(2)); 148.5 (C(4)); 145.2 (MMTr); 138.8 (C(8)); 137.2, 130.2, 128.9, 127.9, 126.9 (MMTr); 121.5 (C(5)); 113.2 (MMTr); 89.5 (C(1′)); 88.7 (OCH_2O); 80.6 (C(4′)); 79.7 (C(3′)); 73.3 (C(2′)); 71.0 (MMTr); 67.1 (C(5′)); 65.3 ($POCH_2C$); 62.2 (CH_2Me); 61.8 (CH_2OAc); 58.9 (3′-MeO); 55.2 (MeO of MMTr); 53.5 (C); 20.9 (Ac); 13.9 ($MeCH_2$). HR-ESI-MS: 1182.4123 (M^+, $C_{55}H_{69}N_5O_{22}P^+$; calc. 1182.4166).

Assembly of Trimer **1a**. Compound **11a** (0.29 mmol, 0.340 g, dried over P_2O_5 overnight) was dissolved in dry CH_2Cl_2 (2 ml) under N_2. Anh. Et_3N (1.42 mmol, 0.198 ml) and $(Et_2N)_2PCl$ (0.34 mmol, 0.072 ml) were added. The mixture was stirred for 2.5 h. The product was isolated by passing the mixture through a short silica-gel column with AcOEt/hexane 7:3, containing 0.5% Et_3N. The solvent was removed under reduced pressure. The product was co-evaporated from dry MeCN to remove traces of Et_3N. The formation of the phosphitylated product was verified by ^{31}P-NMR spectroscopy. ^{31}P-NMR (202 MHz, CD_3CN): 137.4, −2.43.

The phosphitylated nucleoside was dissolved in dry MeCN (400 µl) under N_2. *3′*-O-*[(Pivaloyloxy)methyl]*-N^6-*[(4-methoxyphenyl)(diphenyl)methyl]adenosin-2′-yl 2′,3′-di*-O-*levulinoyl*-N^6-*[(4-methoxy-*

phenyl)(diphenyl)methyl]adenosin-5′-yl 3-(acetyloxy)-2,2-bis(ethoxycarbonyl)propyl phosphate (**2a**; 0.16 mmol, 0.270 g, dried over P_2O_5 overnight) in dry MeCN (600 µl) and 1*H*-tetrazole (0.19 mmol, 0.422 ml of 0.45M soln. in MeCN) were added. The reaction was allowed to proceed for 10 min. Then, **3** (0.29 mmol, 0.080 g; co-evaporated twice with dry MeCN and dried over P_2O_5 overnight) in dry MeCN (200 µl) and 1*H*-tetrazole (0.22 mmol, 0.487 ml of 0.45M soln. in MeCN) were added, and the mixture was stirred for 10 min before the spectrum was recorded. ^{31}P-NMR (202 MHz, CD_3CN): 151.6, 151.3, 150.8, 150.1, 140.5, 140.4, −2.1 – −2.6 (*m*).

The phosphite ester formed was oxidized with I_2 (0.1M) in a mixture of THF, H_2O, and 2,6-lutidine (4 : 2 : 1 (*v*/*v*/*v*); 7 ml) by stirring overnight at r.t. Aq. 5% $NaHCO_3$ soln. was added, and the mixture was extracted twice with CH_2Cl_2. The org. phase was dried (Na_2SO_4) and evaporated to dryness. The product was purified by CC (silica gel; CH_2Cl_2/AcOEt 1 : 1 → AcOEt → CH_2Cl_2 containing 10% MeOH) to give **12a** (diastereoisomer mixture; 0.30, 33%). Yellowish oil. The completion of the oxidation was checked by ^{31}P-NMR spectroscopy. ^{31}P-NMR (202 MHz, CD_3CN): −1.5 – −3.0 (*m*).

The levulinoyl groups were removed by treatment with NH_2NH_3OAc. Accordingly, **12a** (0.100 g) was dissolved in a soln. of $NH_2NH_2 \cdot H_2O$ (2.50 mmol, 0.078 ml) in pyridine (4 ml) and AcOH (1 ml) on an ice bath, and the mixture was stirred for 1 h. The ice bath was removed, and the reaction was allowed to proceed at r.t. for 3 h. The reaction was quenched with 0.1M NaH_2PO_3 soln. (25 ml), and the mixture was extracted with CH_2Cl_2. The org. phase was washed with H_2O, dried (Na_2SO_4), and evaporated to dryness. The product was purified by CC (silica gel; CH_2Cl_2 containing 5% MeOH). The compound was then subjected to detritylation. The product was dissolved in 80% (*v*/*v*) aq. AcOH (10 ml). After stirring overnight at r.t., the mixture was evaporated to dryness. The residue was co-evaporated twice with H_2O. The product was purified first by silica-gel choromatography with CH_2Cl_2 containing 10–20% MeOH, then by HPLC on a *Thermo Hypersil Hypurity™ Elite* C_{18} column (150 × 4.6 mm, 5 µm, flow rate 1.0 ml min^{-1}), using a linear gradient from H_2O to MeCN in 30 min. Overall yield of **1a** starting from **12a** was 39% (27 mg). To study the removal of the enzyme labile protecting groups, 2 mg of the slowest migrating diastereoisomer was separated on a *Sun Fire™ Prep* C_{18} column (250 × 10 mm, 5 µm, flow rate 3.0 ml min^{-1}) eluting with 40–80% MeCN in 30 min. ^{1}H-NMR (500 MHz, CD_3CN): 7.98–8.25 (*m*, 3 H–C(2) and 3 H–C(8)); 6.19–6.53 (*m*, 3 NH_2); 5.90–6.18 (*d*, 3 H–C(1′)); 5.17–5.59 (*m*, H–C(2′), H–C(3′), 2 OCH_2O); 4.85–5.20 (*m*, H–C(3′)); 4.61 (*m*, H–C(2′)); 4.09–4.58 (*m*, H–C(2′), H–C(3′), HO–C(2′), HO–C(3′), 4 OCH_2OAc, 4 POCH_2C, H–C(4′), 3 H–C(5′), 3 H–C(5″), 8 MeCH_2); 1.94–2.04 (*m*, 4 AcO); 1.13–1.27 (*m*, 42 H of Me_3C of Piv, *Me*CH_2). ^{31}P-NMR (202 MHz, $CDCl_3$): −2.7 – −2.0 (*m*). (Multiplicity of some signals is due to the presence of (R_P)- and (S_P)-diastereoisomers.) HR-ESI-MS: 2210.6789 (M^+, $C_{86}H_{123}N_{15}O_{47}P_3^+$; calc. 2210.6903).

Assembly of Trimer **1b**. Compound **11b** (0.42 mmol, 0.500 g, dried over P_2O_5 overnight) was dissolved in dry CH_2Cl_2 (3 ml) under N_2. Anh. Et_3N (2.11 mmol, 294 µl) and $(Et_2N)_2PCl$ (0.59 mmol, 125 µl) were added. The mixture was stirred for 2.5 h. The product was isolated by passing the mixture through a short silica-gel column with AcOEt/hexane 9 : 1, containing 1% Et_3N. The solvent was removed under reduced pressure. The formation of the phosphitylated product was verified by ^{31}P-NMR spectroscopy. ^{31}P-NMR (202 MHz, CD_3CN): 139.2, −2.3. The compound was co-evaporated from dry MeCN to remove traces of Et_3N and dissolved in dry MeCN (0.5 ml) under N_2. Dry CH_2Cl_2 (0.5 ml) was added, because the compound was not completely dissolved. *3′-*O*-[(Acetyloxy)methyl]-*N^6*-[(4-methoxyphenyl)(diphenyl)methyl]adenosine-2′-yl 2′,3′-di-*O*-levulinoyl-*N^6*-[(4-methoxyphenyl)(diphenyl)methyl]-adenosin-5′-yl 3-(acetyloxy)-2,2-bis(ethoxycarbonyl) phosphate* (**2b**; 0.25 mmol, 0.42 g, dried over P_2O_5 overnight) was dissolved in a mixture of dry MeCN (2 ml) and dry CH_2Cl_2 (0.5 ml), and 1*H*-tetrazole (0.51 mmol, 1.128 ml of 0.45M soln. in MeCN) was added. The course of the reaction was followed by ^{31}P-NMR spectroscopy. 1*H*-Tetrazole (0.25 mmol, 0.564 ml of 0.45M soln. in MeCN) was added after 50 min, and the reaction was allowed to proceed for 15 min. Compound **3** (0.42 mmol, 0,110 g; co-evaporated twice with dry MeCN and dried over P_2O_5 overnight) and 1*H*-tetrazole (0.76 mmol; 1.692 ml of 0.45M soln. in MeCN) were added, and the mixture was stirred for 30 min before the spectrum was recorded. ^{31}P-NMR (202 MHz, CD_3CN): 140.2–140.6 (*m*), −2.2 – −2.6 (*m*). The phosphite ester formed was oxidized with I_2 (0.1 mol l^{-1}) in a mixture of THF, H_2O, and 2,6-lutidine (4 : 2 : 1, (*v*/*v*/*v*); 7 ml) by stirring overnight at r.t. Aq. 5% $NaHCO_3$ soln. was added, and the mixture was extracted twice with CH_2Cl_2. The org. phase was dried (Na_2SO_4) and evaporated to dryness. The product was purified by CC (silica gel;

CH_2Cl_2/AcOEt 3:7 → CH_2Cl_2 containing 5% MeOH) to give **12b** (diastereoisomer mixture; 0.58 g, 43%). Yellowish oil. The completion of the oxidation was checked by ^{31}P-NMR spectroscopy. ^{31}P-NMR (202 MHz, CD_3CN): −1.9– −2.9 (*m*).

To remove the levulinoyl groups, **12b** was evaporated once from dry MeCN and dissolved in dry CH_2Cl_2. NH_2NH_3OAc (0.74 mmol, 0.070 g) in dry MeOH (0.9 ml) was added, and the mixture was stirred at r.t. for 2.5 h. The reaction was quenched with acetone, the mixture was stirred for 20 min and evaporated to dryness. The product was purified by silica-gel cromatography with CH_2Cl_2 containing 5% MeOH. The crude product was obtained in 0.510 g yield. A part of the compound (0.280 g) was then subjected to detritylation with 80% (*v*/*v*) aq. AcOH (10 ml). After stirring overnight at r.t., the mixture was evaporated to dryness. The residue was co-evaporated twice with H_2O. The product was purified by HPLC on a *Sun Fire*™ *Prep* C_{18} column (250 × 10 mm, 5 μm, flow rate 3.0 ml min^{-1}), with a linear gradient from 17 to 100% MeOH within 20 min, and by isocratic elution with MeOH for 6 min. Overall yield of **1b** starting from **12b** was 38% (81 mg). To study the removal of the enzyme-labile protecting groups, the diastereoisomers were separated on a *Sun Fire*™ *Prep C18* column (250 × 10 mm, 5 μm, flow rate 3.0 ml min^{-1}) with 55% MeCN for 30 min. ^{1}H-NMR (500 MHz, CD_3OD): 8.07–8.28 (*m*, 3 H–C(2), 3 H–C(8)); 6.19–6.26 (*m*, H–C(1′)); 6.08–6.12 (*m*, H–C(1′)); 5.90–5.95 (*m*, H–C(1′)); 5.19–5.69 (*m*, H–C(2′), 3 OCH_2O); 4.80–5.00 (*m*, H–C(3′)); 4.71–4.75 (*m*, H–C(2′)); 4.09–4.63 (*m*, H–C(2′), 2 H–C(3′), 3 H–C(4′), 3 H–C(5′), 3 H–C(5″), 4 CH_2O, 4 POCH_2C, 8 MeCH_2); 3.49–3.58 (*m*, 3′-MeO); 1.95–2.17 (*m*, 5 AcO); 1.15–1.28 (*m*, 8 *Me*CH_2). ^{31}P-NMR (202 MHz, $CDCl_3$): −2.6– −1.4 (*m*). (Multiplicity of some signals is due to the presence of (R_P)- and (S_P)-diastereoisomers.) HR-ESI-MS: 2129.6054 (M^+, $C_{80}H_{114}N_{15}O_{47}P_3^+$; calc. 2129.6205).

Removal of the Enzyme-Labile Protecting Groups. The removal of the protecting groups was followed by HPLC and HPLC/MS methods. The reactions were carried out in sealed tubes immersed in a thermostated water-bath (37.0 ± 0.1°). The hydronium ion concentration of the reaction solns. (3.0 ml) was adjusted with *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid] (*HEPES*) buffer (0.040/0.024M; pH 7.5). The ionic strength of the solns. was adjusted to 0.1M with NaCl. The hydronium ion concentrations of the buffer solns. were calculated with the aid of the known pK_a values of the buffer acids under the exper. conditions. The initial substrate concentration was 0.15 mM. The Ac group was removed with hog liver carboxyesterase (HLE; 2.6 U ml^{-1}). The samples (200 μl) withdrawn at appropriate intervals were made acidic (pH 2) with 1M aq. HCl to inactivate enzyme and to quench the hydrolysis, cooled in an ice-bath and filtered with minisart *RC 4* filters (0.45 μm). The composition of the samples was analyzed on an *ODS Hypersil* C_{18} column (4 × 250 mm 5 μm, flow rate 1 ml min^{-1}), with a AcOH/AcONa buffer (0.045/0.015M) and MeCN, containing NH_4Cl (0.1M). A good separation of the product mixtures of **1** was obtained by a 5-min isocratic elution with the buffer containing 2% MeCN, followed by a linear gradient (23 min) up to 40.0% MeCN. The reaction products were identified by LC/MS using a mixture of H_2O and MeCN, containing HCOOH (0.1%) as eluent (*Gemini* C_{18} column (2 × 150 mm, 5 μm; flow rate 200 μl min^{-1}). Signals were recorded on a UV detector at a wavelength of 260 nm. The reaction products were identified by means of LC/MS. A mixture of H_2O and MeCN containing HCOOH (0.1%) was used as eluent. The enzymatic deacetylations obeyed first-order kinetics at the HLE concentrations employed.

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