Synthesis and Enzymatic Deprotection of Fully Protected 2-5A Trimers: Towards a Pro-drug Strategy for Short 2´,5´-Oligoadenylates

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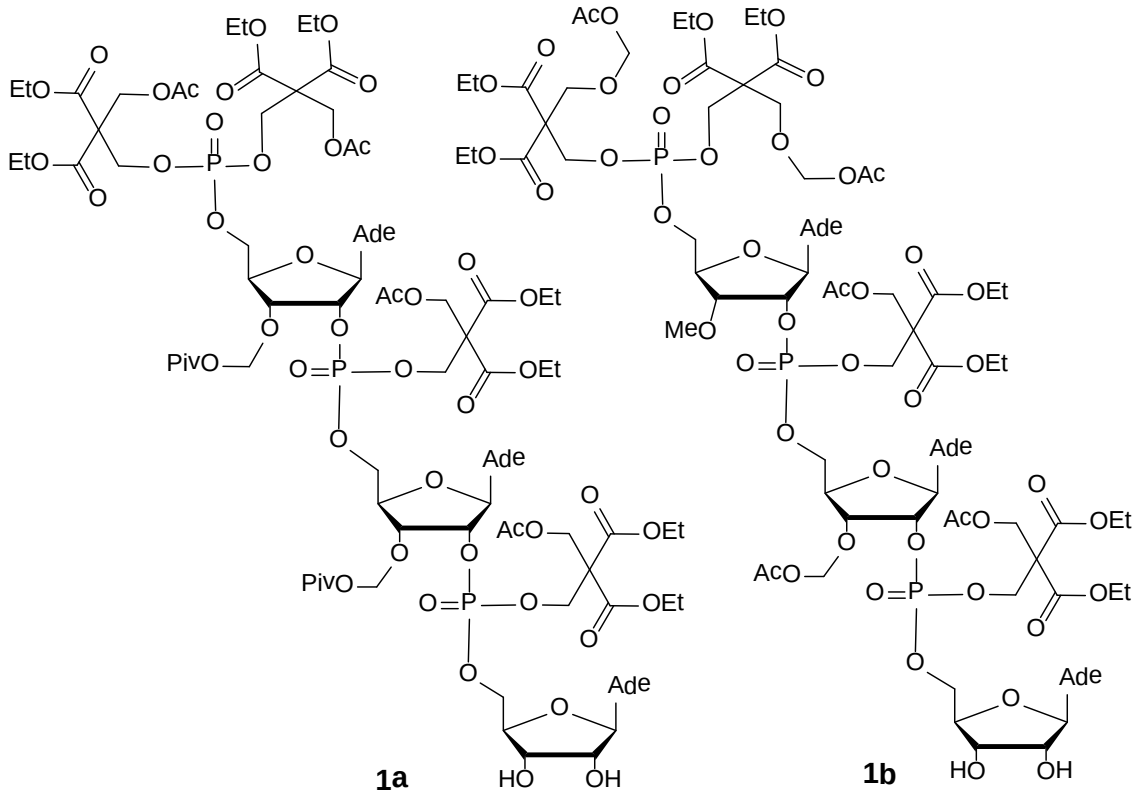
Fully protected pA2′p5′A2′p5′A trimers **1a** and **1b** have been prepared as pro-drug candidates for a short 2′,5′-oligoadenylate, 2-5A, and its 3′-*O*-methyl analog, respectively. The kinetics of hog liver carboxyesterase (HLE) triggered deprotection in HEPES-buffer (pH 7.5) at 37 ^{o}C has been studied. 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 departure of the 3′-*O*-acetyloxymetyl group prior to exposure of the adjacent phosphodiester linkage resulted in 2′,5′- → 3′,5′-phosphate migration and release of adenosine as side reactions.

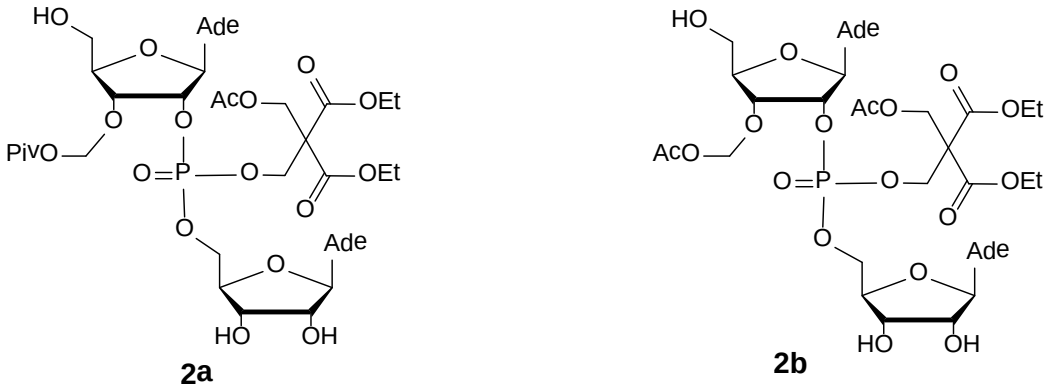
Keywords: Prodrug, 2-5A trimer, protecting group, deprotection

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

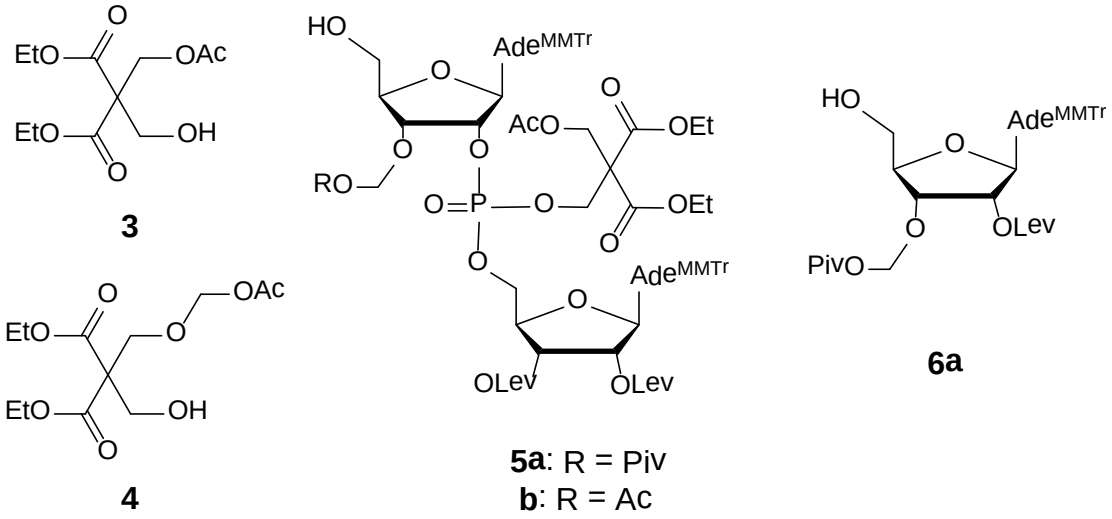
esterase-labile protecting groups from dimeric adenylyl-2′,5′-adenosines **2a** and **2b** [13]. The present work is aimed at evaluating the feasibility of an enzyme-triggered prodrug strategy for the trimeric 2-5A. The crucial thing is the timing of the exposure of the 3′-OH and the adjacent phosphodiester 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 leads to isomerization of the 2′,5′-linkage to a 3′,5′-linkage and cleavage of the P-O5′ bond [14]. In the present work, acetyloxymethyl and pivaloyloxymethyl groups have been used for the 3′-*O*-protection. The bulky *tert*-butyl group is in all likelihood removed by esterases less readily than the acetyl group. To find out which one of these is more appropriate for the purpose, trimers bearing 3′-*O*-acetyloxymethyl and 3′-*O*-pivaloyloxymethyl protection have been prepared. On using the acetyloxymethyl 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 ativate RNase L [12]. The internucleosidic phosphodiester linkages have been protected with 3′-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group and the 5′-terminal monophosphate group with 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl groups. 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 acetyl group with an acetyloxymethyl 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.





Results and discussion. – *Preparation of Protected 2-5A Trimers* (**1a, b**). The preparation of diethyl 2-acetyloxymethyl-2-hydroxy-methylmalonate (**3**) [15], diethyl 2-acetyloxymethoxymethyl-2-hydroxymethylmalonate (**4**) [15], N^6-(4-methoxytrityl)-3´-*O*-pivaloyloxymethyladenosin-2´-yl 2´,3´-di-*O*-levylinoyl-N^6-(4-methoxytrityl)adenosin-5´-yl 3-acetyloxy-2,2-bis(ethoxy-carbonyl)propyl phosphate (**5a**) [13] and 3´-*O*-acetyloxymethyl-N^6-(4-methoxytrityl)adenosin-2´-yl 2´,3´-di-*O*-levylinoyl-N^6-(4-methoxytrityl)-adenosin-5´-yl 3-acetyloxy-2,2-bis(ethoxy-carbonyl)propyl phosphate (**5b**) [13] has 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*-pivaloyloxymethyladenosine (**6a**) was obtained by temporary trimethylsilylation of the 5´-hydroxy function of 2´-*O*-levulinoyl-3´-*O*-methyladenosine [13] and subsequent 4-methoxytritylation of the adenine moiety.



Scheme 1: (i) TBDMSCl, Py; MMTrCl, Py; (iii) Lev_2O, DMAP, dioxane, Py; (iv) Bu_4NF, THF, AcOH.

2´-*O*-Levulinoyl-N^6-(4-methoxytrityl)-3'-*O*-pivaloyloxy-methyladenosine (**6a**) and 2´-*O*-levulinoyl-N^6-(4-methoxytrityl)-3'-*O*-methyladenosine (**6b**) were phosphitylated with chlorobis(diethylamino)phosphine. The diethylamino ligands were then replaced with diethyl 2-acetyloxymethyl-2-hydroxymethylmalonate (**3**) in the case of **6a** and with diethyl 2-acetyloxymethoxymethyl-2-hydroxymethylmalonate (**4**) in the case of **6b**, using tetrazole as an activator (Scheme 2). The resulting phosphite triesters were oxidized to phosphate esters **10a** and **10b** with iodine in aqueous THF containing 2,6-lutidine. The levulinoyl group was removed by hydrazinium acetate treatment to afford building blocks **11a** and **11b**.

Scheme 2: (i) $(Et_2N)_2PCl$, Et_3N, DCM; (ii) **3** with **6a**, **4** with **6b**, TetH, MeCN; (iii) I_2, THF, H_2O, lutidine; (iv) NH_2NH_2, AcOH, pyridine with **10a**, NH_2NH_3OAc, DCM, THF with **10b**.

Trimers **1a** and **b** were assembled as previously [13] desribed for the synthesis of **2a** and **b**. Accordingly, the 2´-hydroxy function of N^6-(4-methoxytrityl)-3´-*O*-pivaloyloxymethyladenosine 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 chlorobis(diethylamino)phosphine in the presence of Et_3N and the remaining diethylamino 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 displacements, tetrazole was used as an activator. Oxidation of the phosphite triesters to phosphate triesters (**12a, 12b**) and removal of the levulinoyl and trityl groups completed the synthesis, giving **1a** and **b** as a mixture of R_P and S_P diastereomers. For kinetic studies, one of the diastereomers was separated by reversed phase HPLC. The RP HPLC profiles of the purified products, **1a** and **1b**, are included in the ESI.

Scheme 3: (i) $(Et_2N)_2PCl$, Et_3N, DCM; (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, pyridine with **12a**, NH_2NH_3OAc, DCM, THF with **12b**; (vi) 80% AcOH.

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 °C by analyzing the composition of the aliquots withdrawn

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

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

Scheme 4. HLE-triggered deprotection of 2-5A trimer **1a**.

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

Loss of next phosphate protecting group (Reaction **B**) resulted in appearance of three chromatographic signals (t_R = 42.0, 47.6 and 34.5 min), which all exhibited the *m/z* $[M+H]^+$ 1723.3 (see the lower HPLC traces in Fig.1). Our previous [15] studies with thymidine 5´-bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)-propyl] phosphate 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 sinals (t_R = 42.0 and 47.6) 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) then refers to a trimer bearing either deprotected internucleosidic phosphates (**14c**) or fully deprotected terminal phosphate (**14d**).

The next phosphate protecting group was removed only very slowly (Reaction **C**). Two HPLC signals referring to the expected molecular ions $[M+H]^+$ = 1479.0 appeared. In other words, two of the three possible trianions (**15a**, **b** or **c**) 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 pivaloyloxymethyl group, evidently from the 5´-terminal nucleotide (Scheme 5). The latter assumption is based on the fact that in addition to the *m/z* values $[M+H]^+$ = 1609.1 and $[M+2H]^{2+}$ = 927.7, referring to **16** and **17**, respectively, *m/z* values referring to values referring to dimers **18** ($[M+H]^+$ = 955.7) and **19** ($[M+H]^+$ = 711.5), monomer **20** ($[M+H]^+$ = 916.6) and trimers **21** ($[M+2H]^{2+}$ = 683.5) were detected. All these products are obtained by an attack of the exposed 3´-OH of trimer **16** or **17** on the adjacent protected phosphate group, giving 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**) 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 never appeared.

Scheme 5. Side reactions taking place during the HLE-triggered deprotection of trimer **1a**.

Enzymatic Deprotection of Trimer **1b.** As indicated above, 3´-*O*-pivaloyloxymethyl 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 pro-drug 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*-acetyloxymethyl group and (iii) the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl groups at the 5´-terminal phosphate were replaced with more labile 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl groups. Previous studies have shown that replacement of the 3-acetyloxy group with a 3-acetyloxymethoxy group accelerates 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].

Figure 2. HPLC traces for the HLE-triggered deprorection of 2-5A trimer **1b** at pH 7.5 and 37.0 ^{o}C (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. Signals marked with x refer to dephosphorylated products.

Scheme 6. First stages of HLE-triggered deprotection of 2-5A trimer **1b**.

Scheme 7. Late stages of HLE-triggered deprotection of 2-5A trimer **1b**.

Fig. 2 shows the HPLC traces of the reaction mixture of trimer **1b** treated with HLE under the same conditions as used for **1a**. As seen, **1b** rapidly gave a monodeacetylated triester **22** ($[M+H]^+$ = 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** ($[M+H]^+$ = 1985.1; Reaction **I**) and (ii) the retro-aldol condensation/elimination to the monoacetylated diester **24** ($[M+H]^+$ = 1855.2; Reaction **J**). Both products (**23**, **24**) were subsequently converted to the deacetylated 5´-diester **25** ($[M+H]^+$ = 1783.0; Reactions **K** and **L**, respectively) and further to monoester **26** ($[M+H]^+$ = 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**), giving **27** ($[M-H]^-$ = 1334.9; t_R = 36.5 min), the desired fully deprotected 2-5A analog was eventually obtained without any side reactions (Scheme 7). The last phosphate protection was removed (Reaction **O**) and the fully phosphate deprotected trimer **28** obtained ($[M-H]^-$ = 1090.7; t_R = 27.0 min) was finally converted to the desired fully deprotected trimer (**29**; $[M-H]^-$ = 1018.6; t_R = 23.5 min) by departure of the 3´-*O*-acetyloxymethyl group (Reaction **P**).

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

Scheme 8. Side reactions of HLE-triggered deprotection of **1b**.

Secondly, compound **34**, which in principle may also be obtained by deprotection of the 5´-terminal internucleosidic phosphodiester bond of **26** (Reaction **U**), reacts by two alternative routes (Scheme 9). When the remaining 3´-terminal phosphodiester protecting group is removed before the 3´-*O*-acetyloxymethyl group (Reaction **V**), the desired trimer **29** is obtained, as depicted in Scheme 7. By contrast, if the 3´-OH is first exposed (**35**; Reaction **W**), isomerization 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** ($[M-H]^-$ = 1262.8) was detected among the HPLC signals.

Finally, compound **26** also appeared to loose the 3´-*O*-acetyloxymethyl protection before either of the internucleosidic phosphate protecting groups (Reaction **X**), since

HPLC signals referring to trimers **38**/**39** ($[M-H]^-$ = 1262.8) and dimers **40**/**41** ($[M-H]^-$ = 1257.9), *i. e.* the products expected to be obtained by an attack of the exposed 3´-OH on the adjacent phosphotriester (Reactions **Y** and **Z**), were observed (Scheme 10).

Scheme 9. Side reactions of HLE-triggered deprotection of **1b**.

Scheme 10. Side reactions of HLE-triggered deprotection of **1b**.

Conclusions. - The results of the present study suggests that an esterase activity dependent pro-drug strategy appears feasible for 2-5A, although the present protecting group scheme, including bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl protection of the 5´-terminal monophosphate group, 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl protection of the intenucleosidic phosphodiester linkages, 3´-*O*-methyl protection of the 5´-terminal nucleotide and 3´-*O*-acetyloxymethyl protection of the intervening nucleoside, still suffers from formation of several by-products. The main problem is too slow removal of the protecting group from the 3´-terminal phosphodiester bond compared to the removal of the neighboring 3´-*O*-acetyloxymethyl protection. Evidently, protection of this phosphodiester linkage with the more labile 3-acetyloxymethoxy-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 with 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.

Experimental Part

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

2´-O-Levulinoyl- N^6-(4-methoxytrityl)-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 solution was cooled on an ice-bath and trimethylsilyl chloride (9.4. mmol, 1.19 ml) was added. The mixture was stirred for 2.5 h at room temperature. 4-Methoxytrityl chloride (2.3 mmol, 0.70 g) was added and the mixture was stirred over three nights at 37 ^{o}C. The solvent was removed by evaporation under reduced pressure and the residual oil was partitioned between water and EtOAc. The organic layer was washed with saturated aq $NaHCO_3$, dried over Na_2SO_4 and evaporated to dryness. The trimethylsilyl 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 room temperature for 2 hours. Saturated aq

$NaHCO_3$ was added and the mixture was extracted with DCM. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The product was purified by Silica gel chromatography, eluting with DCM containing 3% MeOH. Compound **6a** was obtained as yellowish foam in 83% yield (1.17 g). ^{1}H-NMR (500MHz, $CDCl_3$) δ: 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, 2H of MMTr), 6.62 (dd, *J* = 12 and 2 Hz, HO-C(5´) 6.03 (d, *J* = 7.5 Hz, H-C(1´)), 5.68 (dd, *J* = 7.5 and 5.3 Hz, H-C(2´)), 5.55 (d, *J* = 6.5 Hz, 1 H of OCH_2O), 5.12 (d, *J* = 6.5 Hz, 1 H of OCH_2O), 4.82 (dd, *J* = 5.3 and 1 Hz, 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 x CH_2 of Lev), 2.20 (s, CH_3 of Lev), 1.25 (s, $(CH_3)_3C$ of Piv). ^{13}C-NMR (126 MHz, $CDCl_3$) δ: 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 (CH_3 of Lev), 27.5 (-CH_2-COO of Lev) 27.0 (CH_3 of Piv). HRMS (ESI) Calcd for $C_{41}H_{46}N_5O_9$, 752.3290; Found 752.3312.

2´-O-Levulinoyl-N^6-(4-methoxytrityl)-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 over night) was dissolved in dry DCM (7 ml) under nitrogen. Anhydrous Et_3N (7.3 mmol, 1.02 ml) and chlorobis(diethylamino)phosphine (2.1 mmol, 0.43 ml) were added and the mixture was stirred 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% triethylamine. The solvent was removed under reduced pressure and the residue was coevaporated from dry MeCN to remove the traces of

Et_3N. The formation of the phosphitylated product was verified by ^{31}P spectroscopy. ^{31}P-NMR (202 MHz, CD_3CN) δ: 133.3.

The phosphitylated nucleoside was dissolved in dry MeCN (1 ml) under nitrogen. 3-Acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propanol (**4**) (4.2 mmol, 1.10 g, coevaporated twice with dry MeCN and dried over P_2O_5 over night), dissolved in dry MeCN (2 ml), and tetrazole (4.4 mmol, 9.76 ml of 0.45 M solution in MeCN) were added. The course of the reaction was followed by ^{31}P NMR spectroscopy. The spectrum was recorded after half an hour. ^{31}P-NMR (202 MHz, CD_3CN) δ: 138.8. The phosphite ester formed was oxidized with I_2 (0,1 M) in a mixture of THF, H_2O and 2,6-lutidine (4:2:1, v/v/v, 10 ml) by stirring overnight at room temperature. Aqueous 5% $NaHCO_3$ was added and the mixture was extracted twice with DCM. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The product was purified by Silica gel chromatography eluting with 5% MeOH in DCM. The purification was repeated eluting with a mixture of DCM and EtOAc (1:1) and then changing to 5% MeOH in DCM. Compound **10a** was obtained as clear oil in 22% yield (0.44 g). ^1H-NMR (500MHz, $CDCl_3$) δ: 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 Hz, H-C(1´)), 5.76 (dd, *J* = 5.5 and 3.5 Hz, H-C(2´)), 5.34 (d, *J* = 6.5 Hz, 1 H of OCH_2O), 5.20 (d, *J* = 6.5 Hz, 1 H of OCH_2O), 4.97 (m, H-C(3´)), 4.51- 4.62 (m, 2x C*H*$_2$OAc and 2x POC*H*$_2$C), 4.17-4.33 (m, H-(4´), H-C(5´), H-(5´´) and 4 x OC*H*$_2$$CH_3$), 3.81 (s, MeO of MMTr), 2.77-2.80 (m, 2 H of CH_2CH_2 Lev), 2.66-2.70 (m, 2 H of CH_2CH_2 Lev), 2.20 (s, CH_3 of Lev), 2.05 (s, OAc), 2.01 (s, OAc), 1.20-1.32 (m, 4 x CH_2C*H*$_3$ and 3 x CH_3 of Piv). ^{31}P-NMR (202 MHz, CD_3CN) δ: -2.59. HRMS (ESI) Calcd for $C_{63}H_{79}N_5O_{24}P$, 1320.4847; Found 1320.4812.

N^6-(4-Methoxytrityl)-3´-O-pivaloyloxymethyladenosine 5´-bis[3-acetyloxymethyl-

2,2-bis(ethoxycarbonyl)propyl]-phosphate (**11a**). Compound **10a** (0.3 mmol, 0.44 g) was dissolved in a solution of hydrazine hydrate (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 hours. The ice bath was removed and the reaction was allowed to proceed at room temperature for 2 hours. The reaction was quenched with 0.1 M NaH_2PO_3-solution and the mixture was extracted with DCM. The organic phase was washed with water, dried over Na_2SO_4 and evaporated to dryness. The product was purified by Silica gel chromatography using DCM containing 3-5% MeOH as eluent. Compound **11a** was obtained as clear oil in 88% yield (0.35 g). ^{1}H-NMR (500MHz, $CDCl_3$) δ: 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 Hz, H-C(1´)), 5.51 (d, *J* = 6.3 Hz, 1 H of OCH_2O), 5.42 (d, *J* = 6.3 Hz, 1 H of OCH_2O), 4.76 (dd, *J* = 5.5 and 5.0 Hz, H-C(2´)), 4.64 (m, H-C(3´)), 4.50- 4.63 (m, 2x C*H*$_2$OAc and 2 x POC*H*$_2$C), 4.37 (m, H-C(4´)), 4.19-4.31 (m, 4 x OC*H*$_2$Me, H-C(5´) and H-(5´´)), 3.88 (d, *J* = 5 Hz, 1H, HO-C(2')) 3.81 (s, MeO of MMTr), 2.05 (s, OAc), 2.03 (s, OAc), 1.22-1.32 (m, 4 x OCH_2CH_3 and 3 x CH_3 of Piv). ^{13}C-NMR (126 MHz, $CDCl_3$) δ: 178.0 (C=O of Piv), 170.1 (C=O of Ac), 166.4 (*C*=OOEt) 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 (*C*H_2CH_3), 61.2 (*C*H_2OAc), 58.0 (-C-), 55.2 (OCH_3 of MMTr), 38.8 (Me_3C of Piv), 27.0 (CH_3 of Piv), 20.6 (Ac), 13.9 (CH_2CH_3). HRMS (ESI) Calcd for $C_{58}H_{73}N_5O_{22}P$, 1222.4479; Found 1222.4485.

5´-O-tert-Butyldimethylsilyl-3´-O-methyladenosine (**7**). Commercially available 3´-*O*-methyladenosine (3.6 mmol, 1.01 g) was coevaporated twice from anhydrous pyridine and the residue was dissolved in the same solvent (7 ml). 1.1 equiv. of *tert*-

butyldimethylsilyl chloride (4.0 mmol, 0.60 g) was added and the mixture was stirred overnight at room temperature. The reaction was quenched with MeOH and evaporated to dryness. The residue was purified by Silica gel chromatography using DCM containing 10 % MeOH as eluent. ^{1}H-NMR (500 MHz, MeOD) 8.41 (s, H-C(2)), 8.23 (s, H-C(8)), 6.06 (d, *J* = 4.2 Hz, H-C(1´)), 4.77 (dd, *J* = 4.2 and 4.6 Hz, H-C(2´)), 4.22 (m, H-C(4´)), 4.06 (dd, *J* = 4.6 and 5.0 Hz, H-C(3´)), 4.03 (dd, *J* = 11.5 and 3.4 Hz, H-C(5´)), 3.87 (dd, *J* = 11.5 and 3.0 Hz, H-C(5´´)), 3.50 (s, 3´-OMe), 0.96 (s, 9 H,of ^{t}Bu), 0.14 (s, 6 H of ^{t}Bu). ^{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 (OMe), 25.0 (C-*Me*$_3$), 17.9 (*C*Me$_3$), -6.7 (Si*Me*$_2$).

5´-O-tert-Butyldimethylsilyl-N^{6}-(4-methoxytrityl)-3´-O-methyladenosine (**8**). Compound **7** was coevaporated twice from anhydrous pyridine and the residue was dissolved in dry pyridine (6 ml). 4-Methoxytrityl chloride was added and the mixture was stirred over three nights at room temperature. The reaction was quenched with MeOH and the mixture was evaporated to dryness. The residue was dissolved in DCM and washed with saturated aq $NaHCO_3$ and saturated aq NaCl. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The product was purified by Silica gel chromatography using DCM containing 2-3% MeOH as eluent. Compound **8** was obtained as white foam in 75% yield from 3´-*O*-methyladenosine (1.80 g). ^{1}H-NMR (500 MHz, $CDCl_3$) 8.06 (br s, H-C(2) and 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 Hz, 2 H of MMTr), 5.98 (d, *J* = 5.6 Hz, H-C(1´)), 4.73 (m, H-C(2´)), 4.27 (m, H-C(4´)), 4.16 (d, *J* = 6.5 Hz, HO-C(2´)), 4.05 (m, H-C(3´)), 3.91 (dd, *J* = 11.2 and 4.2 Hz, H-C(5´)), 3.78-3.81 (m, OMe and H-C(5´´)), 3.52 (s, 3´-OMe), 0.90 (s, 9 H of ^{t}Bu), 0.09 and 0.10 (2 × s, 6 H of ^{t}Bu). ^{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´-OMe), 55.2 (MMTr), 25.9 (C-*Me*$_3$), 18.3 (*C*Me$_3$), -5.5 (Si*Me*$_2$).

5´-O-tert-Butyldimethylsilyl-2´-O-levulinoyl-N^6-(4-methoxy-trityl)-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 DCC (3.4 mmol, 0.70 g) in small portions within an hour. The solution was stirred at room temperature for two hours. Precipitated dicyclohexylurea was filtered off and washed with 5ml of dry dioxane. The filtrate was added to a solution of compound **8** (2.7 mmol, 1.80 g, dried over P_2O_5 over night) in dry pyridine (9 ml) and a catalytic amount of 4-dimethylaminopyridine was added. After stirring over night at room temperature, the mixture was evaporated to dryness. The residue was dissolved in DCM and washed with saturated aq $NaHCO_3$ and saturated aq NaCl. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The product was purified by Silica gel chromatography using DCM containing 1-2% MeOH as eluent. Compound **8** was obtained as white foam in 89% yield (1.84 g). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 8.10 (s, H-C(2)), 8.06 (s, H-C(8)), 7.23-7.38 (m, 12 H of MMTr), 6.92 (s, H-(N^6)), 6.82 (m, 2 H of MMTr), 6.19 (d, *J* = 4.0 Hz, H-C(1´)), 5.74 (dd, *J* = 4.5 and 4.0 Hz, H-C(2´)), 4.30 (m, H-C(3´)), 4.18 (m, H-C(4´)), 4.01 (dd, *J* = 11.5 and 3.0 Hz, H-C(5´)), 3.83 (dd, *J* = 11.5 and 3.0 Hz, H-C(5´´)), 3.80 (s, 3 H of MMTr), 3.42 (s, 3 H of OMe), 2.62-2.81 (m, 4 H of Lev), 2.18 (s, 3 H of Lev), 0.94 (s, SiCMe_3), 0.12 (s, Si-Me), 0.11 (s, Si-Me). ^{13}C-NMR (126 MHz, $CDCl_3$) δ: 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 (OMe), 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-methoxytrityl)-3´-O-methyladenosine (**6b**). Compound **9** was desilylated with Bu_4NF in THF under acidic conditions. Accordingly, to a solution of Bu_4NF (3.6 mmol, 0.94 g) in dry THF (30 ml), AcOH (6 ml) and compound **9** were added and the mixture was stirred over two nights at room temperature. Saturated aq $NaHCO_3$ was added and the mixture was extracted with DCM. The organic phase was washed with saturated aq $NaHCO_3$ and saturated aq NaCl and dried over Na_2SO_4 and evaporated to dryness. The product was purified by Silica gel chromatography using DCM containing 1-3% MeOH as eluent. Compound **6b** was obtained as white foam in 80% yield (1.25 g). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 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 and 2.0 Hz, HO-C(5´)), 6.00 (d, *J* = 7.5 Hz, H-C(1´)), 5.71 (dd, *J* = 7.5 and 5.0 Hz, H-C(2´)), 4.33-4.36 (m, H-C(3´) and 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, OMe), 2.53-2.75 (m, 4 H of Lev), 2.17 (s, 3 H of Lev). ^{13}C-NMR (126 MHz, $CDCl_3$) δ: 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 (OMe), 55.2 (MMTr), 37.7 (Lev), 29.8 (Lev), 27.6 (Lev). HRMS (ESI) Calcd for $C_{31}H_{38}N_7O_9$, 652.2726; Found 652.2718.

2´-O-Levulinoyl-N^6-(4-methoxytrityl)-3´-O-methyladenosine 5´-bis[3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl]-phosphate (**10b**). Compound **6b** (1.5 mmol, 0.99 g, dried over P_2O_5 over 3 nights) was dissolved in dry DCM (3 ml) under

nitrogen. Anhydrous Et_3N (7.60 mmol, 1.056 ml) and chlorobis(diethylamino)phosphine (2.13 mmol, 447 µl) were added and the mixture was stirred for 2 hours. The product was isolated by passing the mixture through a short silica gel column eluting with dry ethyl acetate containing 1% Et_3N. The solvent was removed under reduced pressure and the residue was coevaporated from dry MeCN to remove the traces of Et_3N. The identity of the product was checked by ^{31}P spectroscopy. ^{31}P-NMR (202 MHz, CD_3CN) δ: 133.7. The phosphitylated nucleoside was dissolved in dry MeCN (1 ml) under nitrogen. 3-Acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propanol (3.8 mmol, 0.11 g, coevaporated twice with dry MeCN and dried over P_2O_5 over 3 nights) and tetrazole (3.80 mmol, 8.440 mL of 0.45 M solution in MeCN) were added. The course of the reaction was followed by ^{31}P-NMR spectroscopy. The spectrum was recorded after 1 hour. ^{31}P-NMR (202 MHz, CD_3CN) δ: 139.0. The phosphite ester formed was oxidized with I_2 (0.1 M) in a mixture of THF, H_2O and 2,6-lutidine (4:2:1, v/v/v, 10 ml) by stirring overnight at room temperature. Aqueous 5% $NaHCO_3$ was added and the mixture was extracted twice with DCM. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The product was purified by Silica gel chromatography eluting with a mixture of DCM and ethyl acetate (1:1). Compound **10b** was obtained as clear oil in 43% yield (0.85 g). ^1H-NMR (400MHz, $CDCl_3$) δ: 8.01 (s, 1H, H2), 7.92 (s, 1H, H8), 7.19-7.35 (m, 12H, MMTr), 6.92 (s, 1H, N^6H), 6.77-6.81 (m, 2H, MMTr), 6.06 (d, *J* = 3.2 Hz, 1H, H1´), 5.76 (dd, *J* = 5.2 and 3.2 Hz, 1H, H2´), 5.20-5.25 (m, 4H, OCH_2O), 4.50-4.53 (m, 4H, $POCH_2C$), 4.39 (m, 1H, H3´), 4.32 (m, 1H, H5´) 4.09- 4.27 (m, 14H, H4´, H5´´, OCH_2CH_3 and CH_2O), 3.78 (s, 3H, MeO MMTr), 3.41 (s, 3H, 3´-OMe), 2.63-2.79 (m, 4H, CH_2CH_2 Lev), 2.17 (s, 3H, CH_3 Lev), 2.06 (s, 3H, Ac), 2.04 (s, 3H, Ac), 1.18-1.28 (m, 12H, CH_2CH_3). ^{13}C-NMR (101 MHz, $CDCl_3$) δ: 206.0 (C=O Lev), 171.7 (C=O Lev), 170.2

(C=O Ac), 166.6 (*C*=OOEt) 158.3 (MMTr), 154.2 (C6), 152.4 (C2), 148.3 (C4), 145.2 (MMTr), 139.0 (C8), 137.2, 130.2, 128.9, 127.9, 126.8 (MMTr), 121.5 (C5), 113.2 (MMTr), 89.1 (OCH_2O), 88.8 (OCH_2O), 87.5 (C1´), 80.6 (C4´), 78.1 (C3´), 73.7 (C2´), 71.0 (MMTr), 67.1 (C5´), 65.2 ($POCH_2C$), 62.1 (CH_2CH_3), 61.8 (CH_2OAc), 59.1 (3´-OMe), 55.2 (OCH_3 MMTr), 53.4 (-C-), 37.8 (CH_2 Lev), 29.7 (CH_3 Lev), 27.8 (CH_2 Lev), 20.9 (Ac), 13.9 (CH_2CH_3). ^{31}P NMR (162 MHz, $CDCl_3$) δ: -2.15. HRMS (ESI) Calcd for $C_{60}H_{75}N_5O_{24}P$, 1280.4534; Found 1280.4538.

N^6-(4-Methoxytrityl)-3´-O-methyladenosine 5´-bis[3-acetyl-oxymethoxy-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 DCM (16 ml). Hydrazinium acetate (1,19 mmol, 0,11 g) in dry methanol (2 ml) was added. After stirring the mixture for 3 hours, another portion of hydrazinium acetate (0.60 mmol, 0.055 g) in dry methanol (1 ml) was added and the reaction was allowed to proceed for 1 hour. The reaction was quenched with acetone and the mixture was evaporated to dryness. The product was purified by Silica gel chromatography, eluting with a mixture of DCM and EtOAc (1:4). Compound **11b** was obtained as clear oil in 89% yield (0.70 g). ^{1}H-NMR (500MHz, $CDCl_3$) δ: 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 Hz, H-C(1´)), 5.20-5.25 (m, 2 x OCH_2O), 4.80 (m, H-C(2´)), 4.49-4.56 (m, 2 x $POCH_2C$), 4.33, (m, H-C(4´)), 4.10- 4.30 (m, H-C(5´), H-C(5´´), H-C(3´), 4 x OCH_2CH_3 and 2 x CH_2O), 3.84 (br. s, HO-C(2´)), 3.78 (s, MeO of MMTr), 3.54 (s, 3´-OMe), 2.06 (s, Ac), 2.05 (s, Ac), 1.20-1.29 (m, 4 x OCH_2CH_3). ^{13}C-NMR (126 MHz, $CDCl_3$) δ: 170.3 (C=O of Ac), 166.6 (*C*=OOEt) 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

(PO*C*H_2C), 62.2 (*C*$H_2$$CH_3$), 61.8 (*C*$H_2$OAc), 58.9 (3´-OMe), 55.2 (OCH_3 MMTr), 53.5 (-C-), 20.9 (Ac), 13.9 (CH_2*C*H_3). HRMS (ESI) Calcd for $C_{55}H_{69}N_5O_{22}P$, 1182.4166; Found 1182.4123.

Assembly of trimer (**1a**). N^6-(4-Methoxytrityl)-3´-*O*-pivaloyloxymethyladenosine 5´-bis[3-acetyloxymethyl-2,2-bis-(ethoxycarbonyl)propyl]phosphate (**11a**) (0.29 mmol, 0.340 g, dried over P_2O_5 over night) was dissolved in dry DCM (2 ml) under nitrogen. Anhydrous Et_3N (1.42 mmol, 0.198 ml) and chlorobis(diethylamino)phosphine (0.34 mmol, 0.072 ml) were added. The mixture was stirred for 2.5 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% triethylamine. The solvent was removed under reduced pressure. The product was coevaporated from dry MeCN to remove the traces of Et_3N. The formation of the phosphitylated product was verified by ^{31}P NMR spectroscopy. ^{31}P-NMR (202 MHz, CD_3CN) δ: 137.4 and -2.43.

The phosphitylated nucleoside was dissolved in dry MeCN (400 µl) under nitrogen. 3´-*O*-Pivaloyloxymethyl-N^6-(4-methoxytrityl)adenosine-2´-yl 2´,3´-di-*O*-levulinoyl-N^6-(4-methoxytrityl)adenosine-5´-yl 3-acetyloxy-2,2-bis(ethoxy-carbonyl)propyl phosphate (**2a**) (0.16 mmol, 0.270 g, dried over P_2O_5 over night) in dry MeCN (600 µl) and tetrazole (0.19 mmol, 0.422 ml of 0.45 M solution in MeCN) were added. The reaction was allowed to proceed for 10 minutes. After this period, 3-acetyloxy-2,2-bis(ethoxycarbonyl)propanol (**3**) (0.29 mmol, 0.080 g, coevaporated twice with dry MeCN and dried over P_2O_5 over night) in dry MeCN (200 µl) and tetrazole (0.22 mmol, 0.487 ml of 0,45 M solution in MeCN) were added and the mixture was stirred for 10 minutes before the spectrum was recorded. ^{31}P-NMR (202 MHz, CD_3CN) δ: 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 (0.1 M) in a mixture of THF, H_2O

and 2,6-lutidine (4:2:1, v/v/v, 7 ml) by stirring overnight at room temperature. Aqueous 5% $NaHCO_3$ was added and the mixture was extracted twice with DCM. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The product was purified by silica gel chromatography, eluting with a mixture of DCM and EtOAc (1:1) and changing the eluent to EtOAc and finally to DCM containing 10% MeOH. Compound **12a** (diastereomeric mixture) was obtained as yellowish oil in 33 % yield (0.30 g). The completion of the oxidation was checked by ^{31}P-NMR spectroscopy. ^{31}P-NMR (202 MHz, CD_3CN) δ: -1.5-(-3.0) (m).

The levulinoyl groups were removed by treatment with hydrazinium acetate. Accordingly, the trimer **12a** (0.100 g) was dissolved in a solution of hydrazine hydrate (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 hour. The ice bath was removed and the reaction was allowed to proceed at room temperature for 3 hours. The reaction was quenched with 0.1 M NaH_2PO_3-solution (25 ml) and the mixture was extracted with DCM. The organic phase was washed with water, dried over Na_2SO_4 and evaporated to dryness. The product was purified by Silica gel chromatography eluting with DCM 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 room temperature, the reaction mixture was evaporated to dryness. The residue was coevaporated twice with water. The product was purified first by Silica gel choromatography eluting with DCM containing 10-20% MeOH, then by HPLC on a Thermo Hypersil Hypurity^{TM} Elite C18 column (150 x 4.6 mm, 5 µm, flow rate 1.0 ml min^{-1}), using a linear gradient elution from water to MeCN in 30 minutes. 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 diastereomer was separated on a Sun Fire^{TM} Prep C18 column (250 x 10 mm, 5 µm,

flow rate 3.0 ml min^{-1}) eluting from 40% to 80% MeCN in 30 minutes. ^{1}H-NMR (500 MHz, CD_3CN) δ: 7.98-8.25 (m, 3 x H-C(2) and 3 x H-C(8)), 6.19-6.53 (m, 3 x NH_2), 5.90-6.18 (d, 3 x H-C(1´)), 5.17-5.59 (m, H-C(2´), H-C(3´) and 2 x O*C*H_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 x O*C*H_2OAc, 4 x POCH_2C, H-C(4´), 3 x H-C(5´), 3 x H-C(5´´), 8 x C$H_2$$CH_3$), 1.94-2.04 (m, 4 x OAc), 1.13-1.27 (m, 42 H of CMe_3 Piv and CH_2CH_3). ^{31}P-NMR (202 MHz, $CDCl_3$) δ: -2.7-(-2.0) (m). (Multiplicity of some signals is due to the presence of R_p and S_p diastereomers. HRMS (ESI) Calcd for $C_{86}H_{123}N_{15}O_{47}P_3$, 2210.6903; Found 2210.6789.

Assembly of trimer (**1b**). N^6-(4-Methoxytrityl)-3´-*O*-methyladenosine 5´-bis[3-acetyloxymethoxy-2,2-bis(ethoxy-carbonyl)propyl]phosphate (**11b**) (0.42 mmol, 0.500 g, dried over P_2O_5 over night) was dissolved in dry DCM (3 ml) under nitrogen. Anhydrous Et_3N (2.11 mmol, 294 µl) and chlorobis(diethylamino)phosphine (0.59 mmol, 125 µl) were added. The mixture was stirred for 2.5 hours. The product was isolated by passing the mixture through a short silica gel column eluting with a 9:1 mixture of dry ethyl EtOAc and hexane 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) δ: 139.2, -2.3. The compound was coevaporated from dry MeCN to remove the traces of Et_3N and dissolved in dry MeCN (0.5 ml) under nitrogen. Dry DCM (0.5 ml) was added, because the compound was not completely dissolved. 3´-*O*-Acetyloxymethyl-N^6-(4-methoxytrityl)adenosine-2´-yl 2´,3´-di-*O*-levulinoyl-N^6-(4-methoxytrityl)adenosine-5´-yl 3-acetyloxy-2,2-bis(ethoxy-carbonyl) phosphate (**2b**) (0.25 mmol, 0.42 g, dried over P_2O_5 over night), was dissolved in a mixture of dry MeCN (2 ml) and dry DCM (0.5 ml), and tetrazole (0.51 mmol, 1.128 ml of 0.45 M solution in MeCN) was added. The course of the reaction

was followed by ^{31}P-NMR spectroscopy. Tetrazole (0.25 mmol, 0.564 ml of 0.45 M solution in MeCN) was added after 50 minutes and the reaction was allowed to proceed yet for 15 minutes. 3-Acetyloxy-2,2-bis(ethoxycarbonyl)propanol (**3**) (0.42 mmol, 0,110 g, coevaporated twice with dry MeCN and dried over P_2O_5 over night) and tetrazole (0.76 mmol, 1.692 ml of 0.45 M solution in MeCN) were added and the mixture was stirred for 30 minutes before the spectrum was recorded. ^{31}P-NMR (202 MHz, CD_3CN) δ: 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 room temperature. Aqueous 5% $NaHCO_3$ was added and the mixture was extracted twice with DCM. The organic phase was dried over Na_2SO_4 and evaporated to dryness. The product was purified by Silica gel chromatography eluting with a mixture of DCM and EtOAc (3:7) and changing eluent to DCM containing 5% MeOH. Compound **12b** (diastereomeric mixture) was obtained as yellowish oil in 43% yield (0.58 g). The completion of the oxidation was checked by ^{31}P-NMR spectroscopy. ^{31}P-NMR (202 MHz, CD_3CN) δ: -1.9-(-2.9) (m).

To remove the levulinoyl protections, trimer **12b** was evaporated once from dry MeCN and dissolved in dry DCM. Hydrazinium acetate (0.74 mmol, 0.070 g) in dry MeOH (0.9 ml) was added and the mixture was stirred at room temperature for 2.5 hours. The reaction was quenched with acetone, stirred for 20 minutes and evaporated to dryness. The product was purified by Silica gel cromatography eluting with DCM 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 room temperature, the reaction mixture was evaporated to dryness. The residue was coevaporated twice with water. The product was purified by HPLC on a Sun Fire™ Prep C18 column (250 x 10 mm, 5 µm, flow rate 3.0 ml min^{-1})

(150 x 4.6 mm, 5 µm, flow rate 1.0 ml min^{-1}), using a linear gradient elution from 17% to 100% MeOH in 20 minutes and isocratic elution with MeOH for 6 minutes. Overall yield of **1b** starting from **12b** was 38% (81 mg). To study the removal of the enzyme labile protecting groups, the diastereomers were separated, on a Sun Fire™ Prep C18 column (250 x 10 mm, 5 µm, flow rate 3.0 ml min^{-1}) eluting with 55% MeCN for 30 minutes. ^{1}H-NMR (500 MHz, CD_3OD) δ: 8.07-8.28 (m, 3 x H-C(2) and 3 x 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´) and 3 x 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 x H-C(3´), 3 x H-C(4´), 3 x H-C(5´), 3 x H-C(5´´), 4 x CH_2O, 4 x POCH_2C, 8 x C$H_2$$CH_3$), 3.49-3.58 (m, 3´-OMe) 1.95-2.17 (m, 5 x OAc), 1.15-1.28 (m, 8 x CH_2CH_3). ^{31}P NMR (202 MHz, $CDCl_3$) δ: -2.6-(-1.4) (m). (Multiplicity of some signals is due to the presence of R_p and S_p diastereomers.) HRMS (ESI) Calcd for $C_{80}H_{114}N_{15}O_{47}P_3$, 2129.6205; Found 2129.6054.

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 °C). The hydronium ion concentration of the reaction solutions (3.0 ml) was adjusted with *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid] (HEPES) buffer (0.040/0.024 M; pH 7.5). The ionic strength of the solutions was adjusted to 0.1 M with sodium chloride. The hydronium ion concentrations of the buffer solutions were calculated with the aid of the known pK_a values of the buffer acids under the experimental conditions. The initial substrate concentration was 0.15 mM. The acetyl group was removed with hog liver carboxyesterase (2.6 U ml^{-1}). The samples (200 µl) withdrawn at appropriate intervals were made acidic (pH 2) with 1 mol L^{-1} aqueous hydrogen chloride 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 C18 column (4 × 250 mm 5 μm, flow rate 1 ml min^{-1}), using a mixture of AcOH/AcONa buffer (0.045/0.015 M) and MeCN, containing NH_4Cl (0.1 M). A good separation of the product mixtures of **1** was obtained on using 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 the mass spectra (LC/MS) using a mixture of water and MeCN, containing a formic acid (0.1%) as an eluent (Gemini C18 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 the mass spectra (LC/MS). A mixture of water and MeCN containing a formic acid (0.1 %) was used as an eluent. The enzymatic deacetylations obeyed first-order kinetics at the HLE concentrations employed.

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Scheme 1: (i) TBDMSCl, Py; MMTrCl, Py; (iii) Lev_2O, DMAP, dioxane, Py; (iv) Bu_4NF, THF, AcOH.

Scheme 2: (i) $(Et_2N)_2PCl$, Et_3N, DCM; (ii) **3** with **6a**, **4** with **6b**, TetH, MeCN; (iii) I_2, THF, H_2O, lutidine; (iv) NH_2NH_2, AcOH, pyridine with **10a**, NH_2NH_3OAc, DCM, THF with **10b**.

Scheme 3: (i) $(Et_2N)_2PCl$, Et_3N, DCM; (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, pyridine with **12a**, NH_2NH_3OAc, DCM, THF with **12b**; (vi) 80% AcOH.

Scheme 4. HLE-triggered deprotection of 2-5A trimer **1a**

Scheme 5. Side reactions taking place during the HLE-triggered deprotection of trimer **1a**

Scheme 6. First stages of HLE-triggered deprotection of 2-5A trimer **1b**

Scheme 7. Late stages of HLE-triggered deprotection of 2-5A trimer **1b**

Scheme 8. Side reactions of HLE-triggered deprotection of **1b**

Scheme 9. Side reactions of HLE-triggered deprotection of **1b**

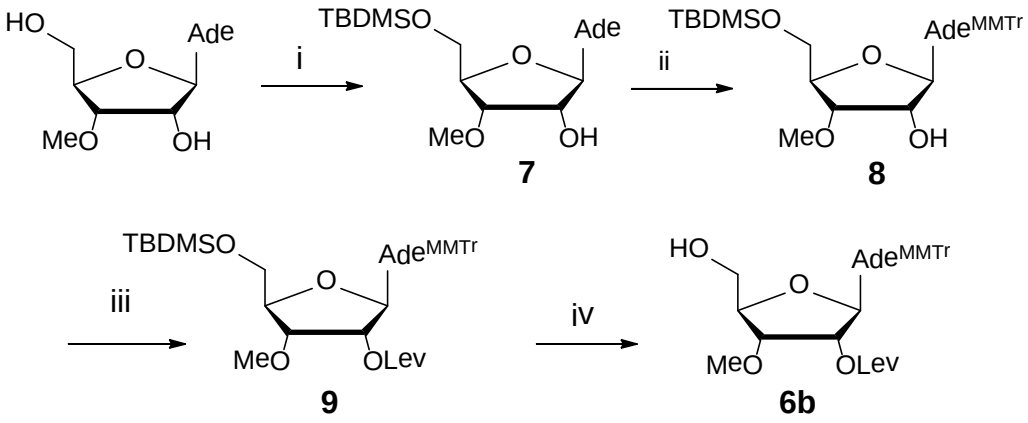
Scheme 10. Side reactions of HLE-triggered deprotection of **1b**

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

Figure 2. HPLC traces for the HLE-triggered deprorection of 2-5A trimer **1b** at pH 7.5 and 37.0 ^oC (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. Signals marked with x refer to dephosphorylated products.

Scheme1


HO
Ade
O
MeO
OH
i
TBDMSO
Ade
O
MeO
OH
7
ii
TBDMSO
Ade^MMTr
O
MeO
OH
8
iii
TBDMSO
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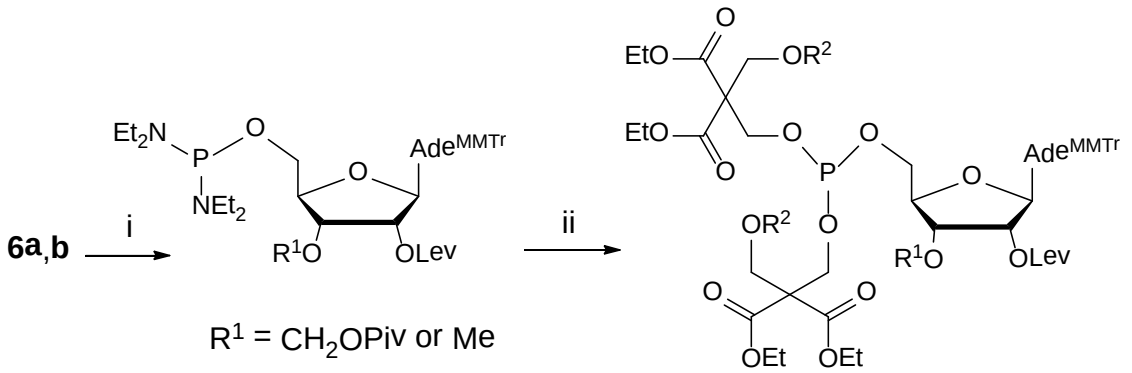
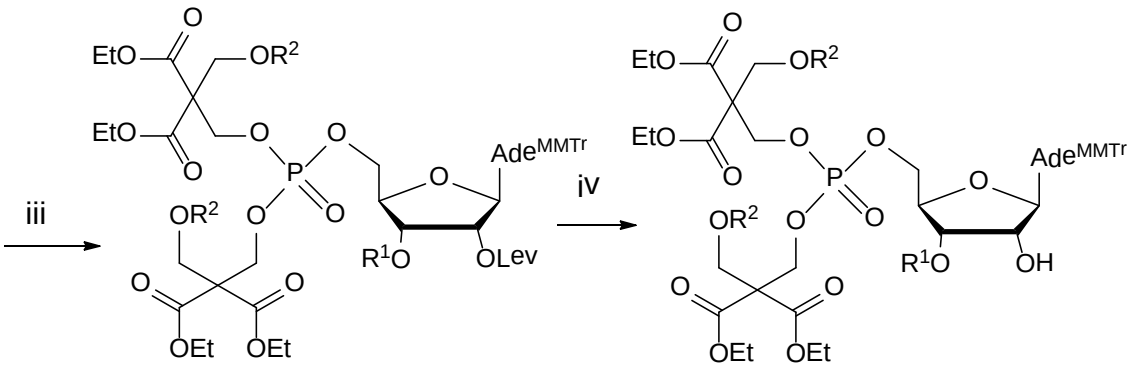
O
MeO
OLev
9
iv
HO
Ade^MMTr
O
MeO
OLev
6b

Scheme 2



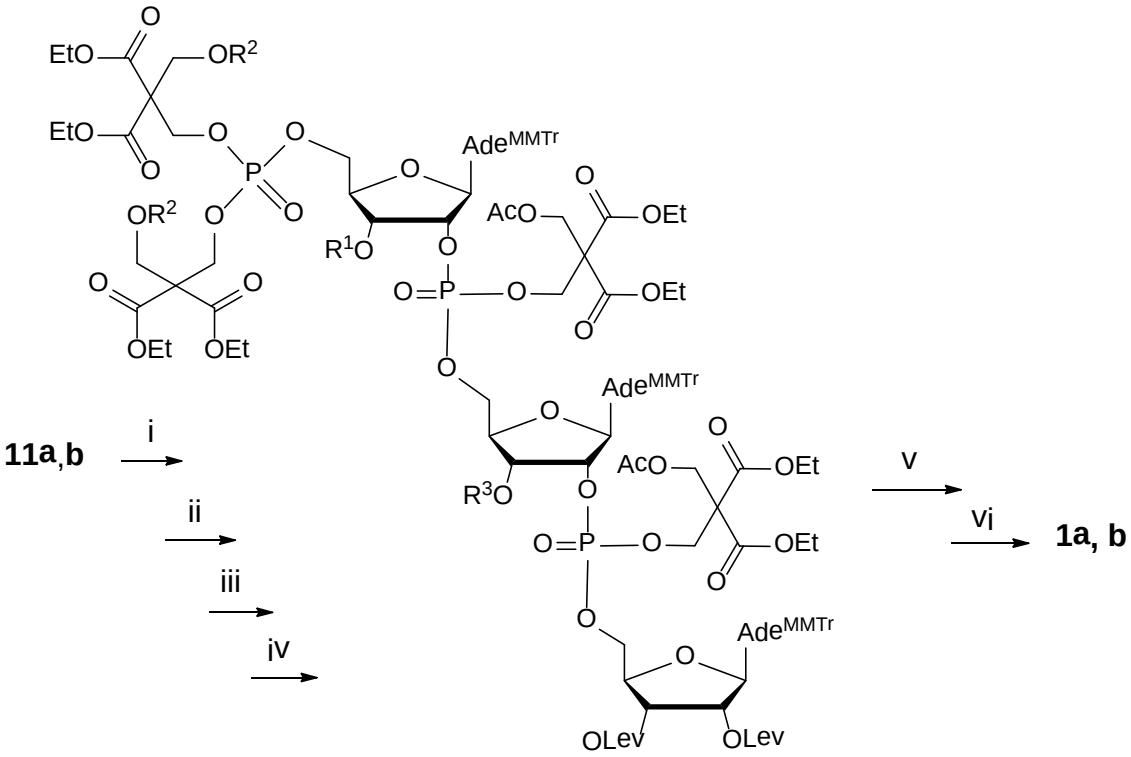
a: $R^1 = CH_2OPiv$, $R^2 = Ac$
b: $R^1 = Me$, $R^2 = CH_2OAc$



10a: $R^1 = CH_2OPiv$, $R^2 = Ac$
b: $R^1 = Me$, $R^2 = CH_2OAc$

11a: $R^1 = CH_2OPiv$, $R^2 = Ac$
b: $R^1 = Me$, $R^2 = CH_2OAc$

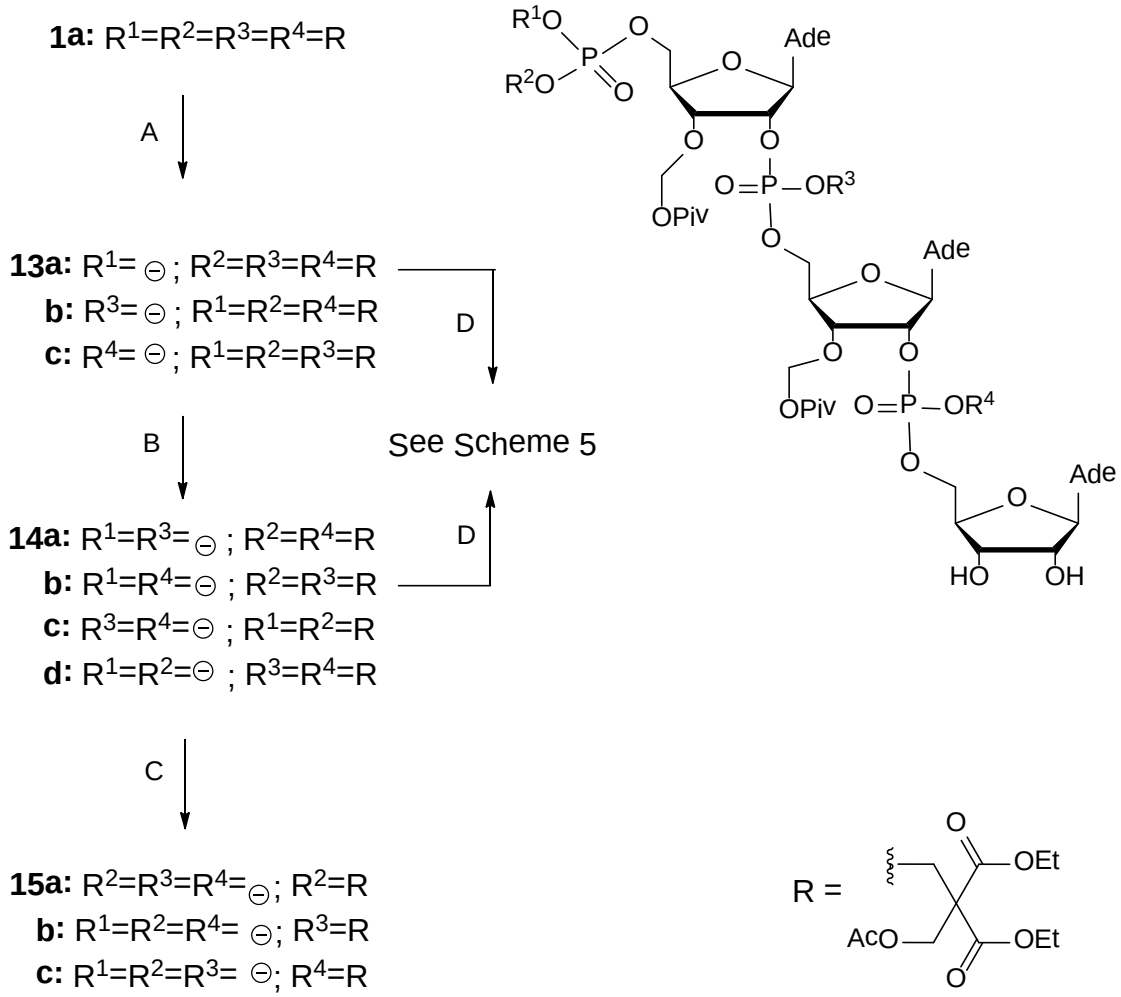
Scheme 3



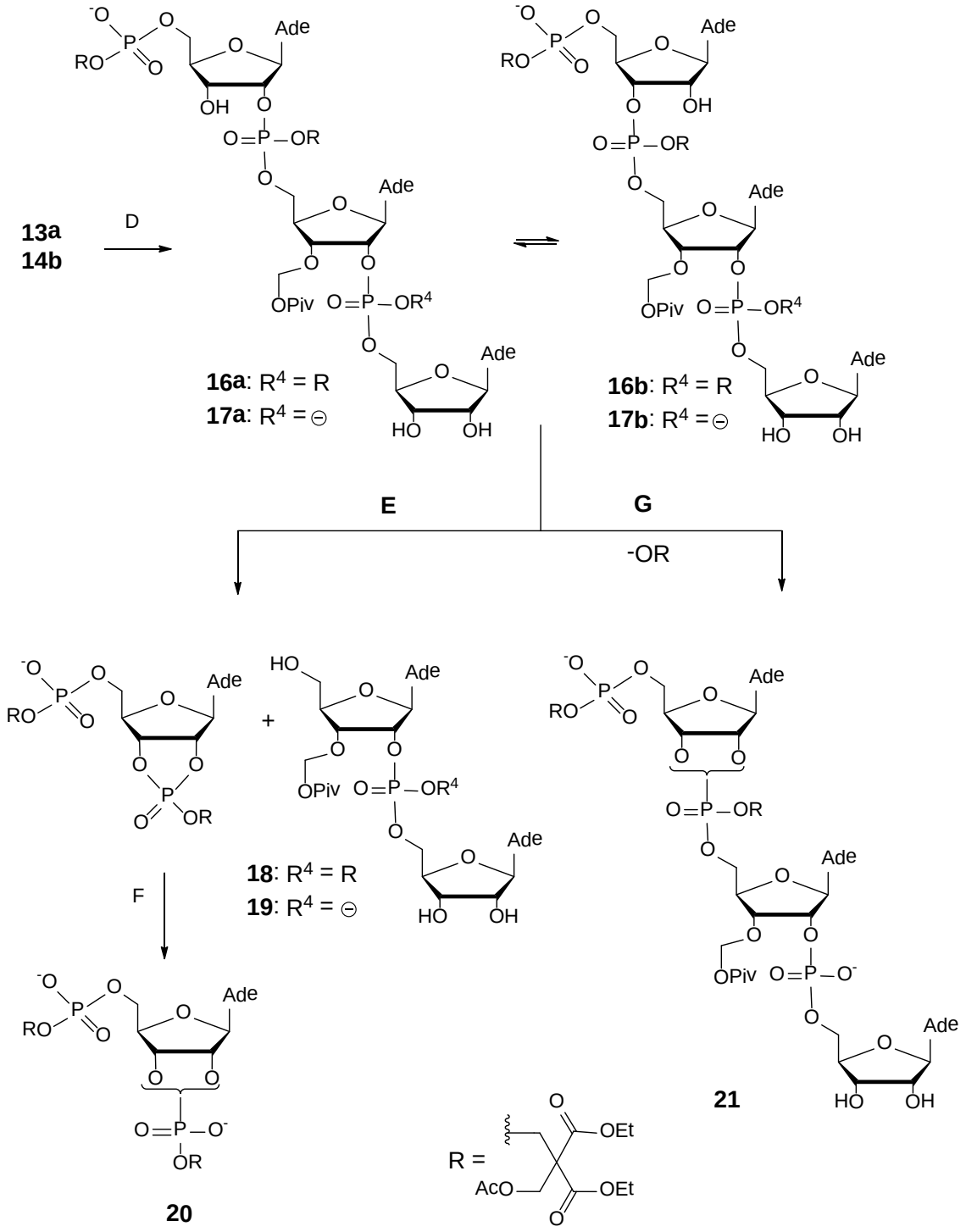
12a: $R^1 = R^3 = CH_2OPiv$, $R^2 = Ac$

b: $R^1 = Me$, $R^2 = R^3 = CH_2OAc$

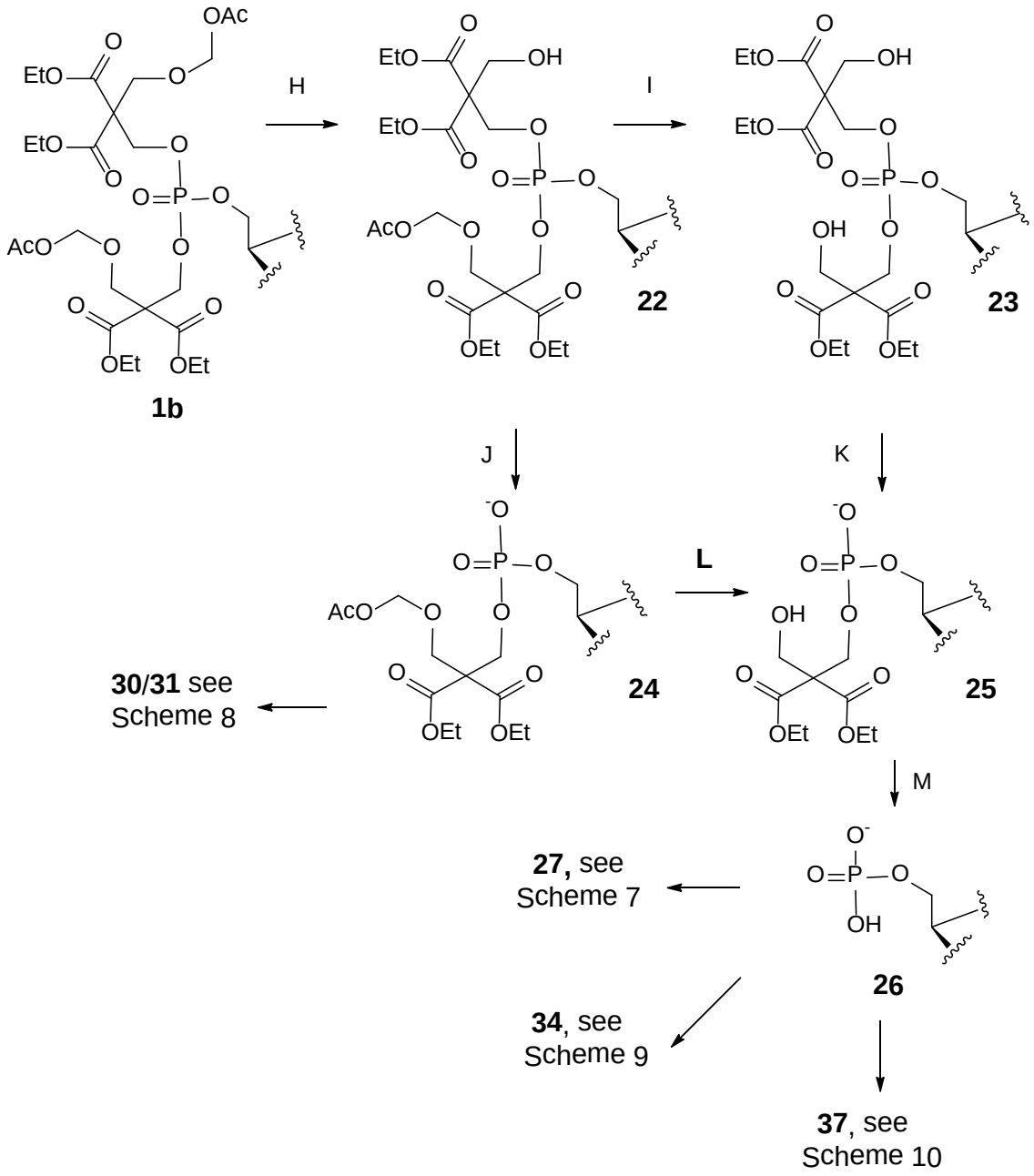
Scheme 4


1a: R1=R2=R3=R4=R
A
13a: R1= ⊖ ; R2=R3=R4=R
b: R3= ⊖ ; R1=R2=R4=R
c: R4= ⊖ ; R1=R2=R3=R
D
B
See Scheme 5
14a: R1=R3= ⊖ ; R2=R4=R
b: R1=R4= ⊖ ; R2=R3=R
c: R3=R4= ⊖ ; R1=R2=R
d: R1=R2= ⊖ ; R3=R4=R
D
C
15a: R2=R3=R4= ⊖ ; R2=R
b: R1=R2=R4= ⊖ ; R3=R
c: R1=R2=R3= ⊖ ; R4=R
R1O
R2O
P
O
Ade
OPiv
O=P–OR3
O=P–OR4
HO
OH
R =
OEt
AcO

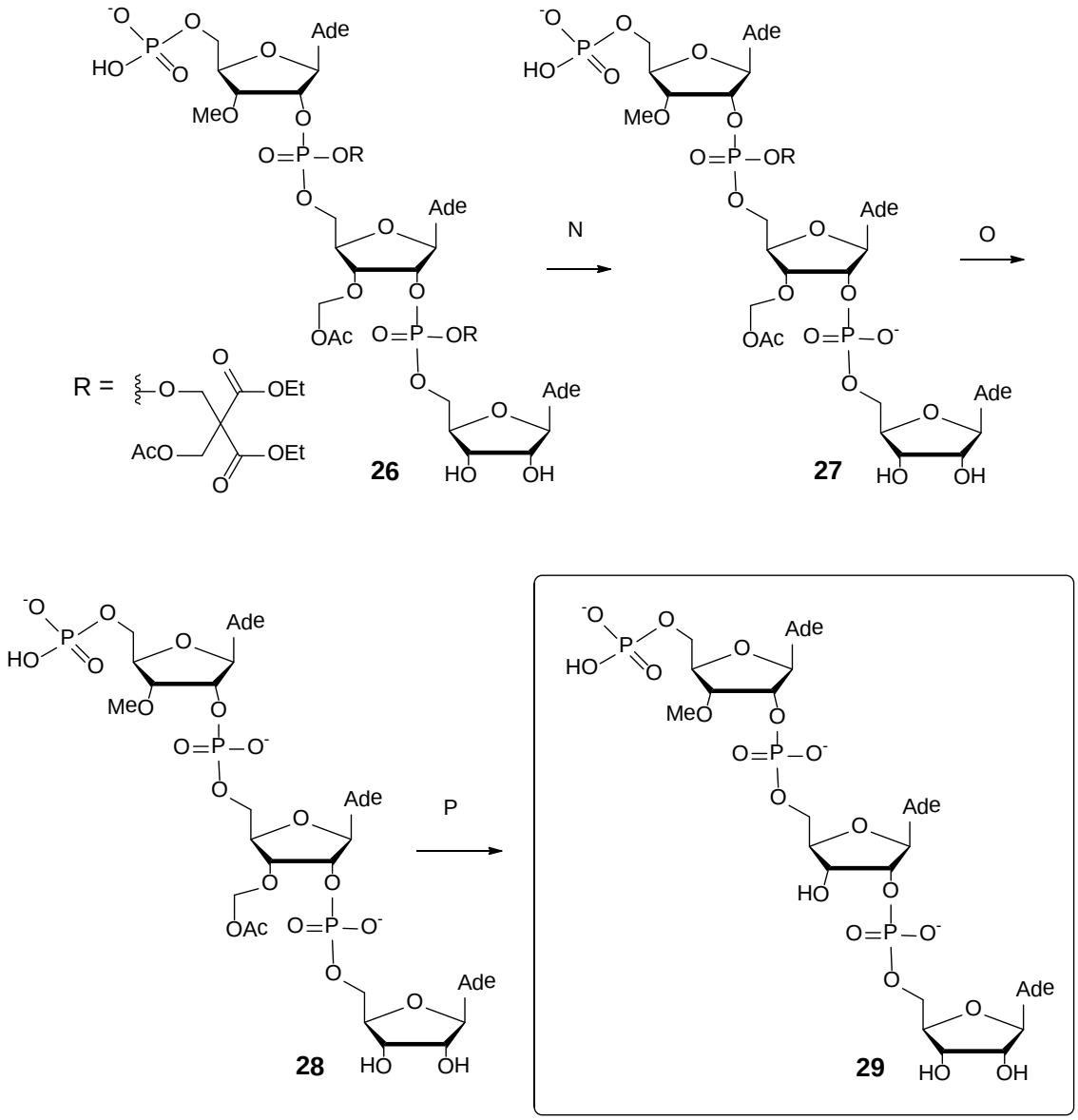
Scheme 5


13a
14b
D
16a: R4 = R
17a: R4 = ⊖
16b: R4 = R
17b: R4 = ⊖
E
G
-OR
18: R4 = R
19: R4 = ⊖
F
20
21
R =
AcO
OEt
OPiv
Ade
RO
HO
OH

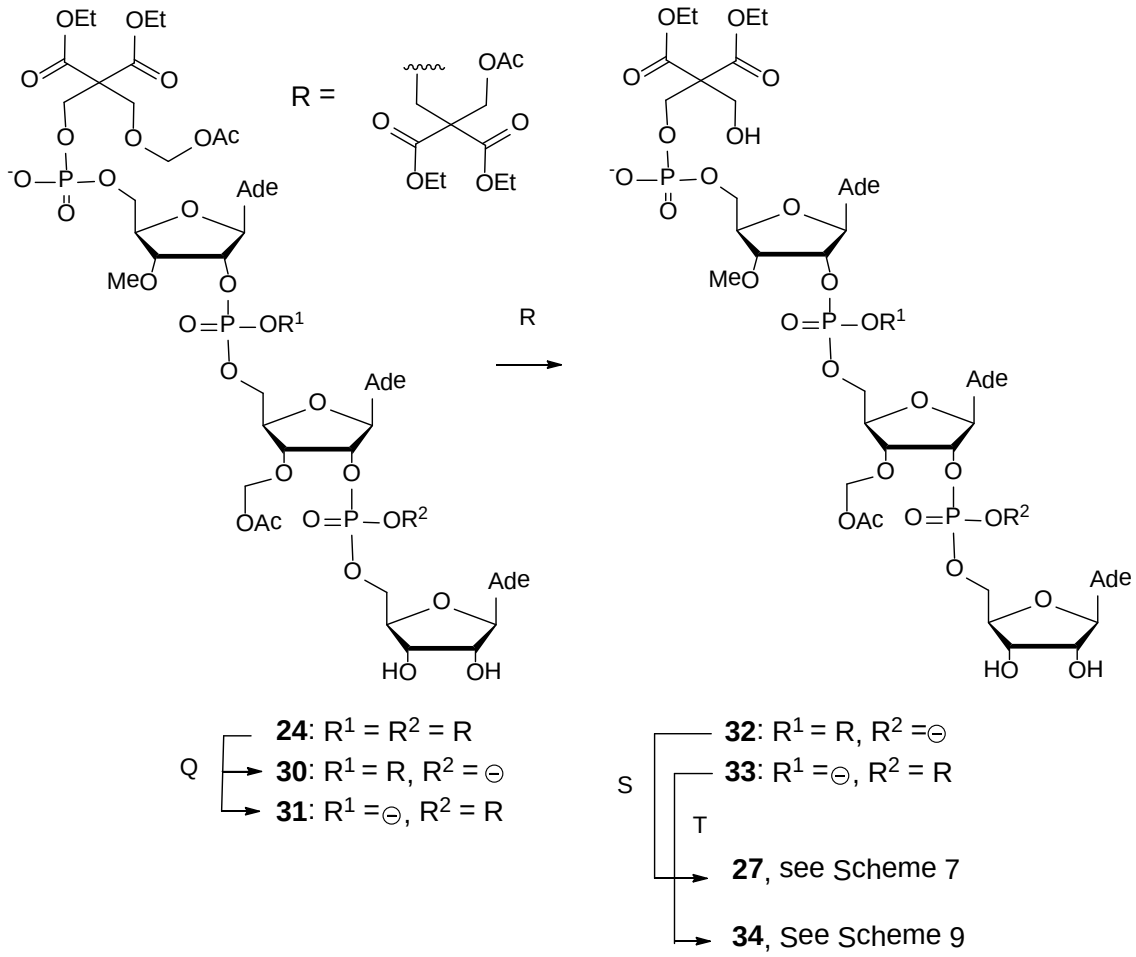
Scheme 6


H
I
J
K
L
M
1b
22
23
24
25
26
30/31 see Scheme 8
27, see Scheme 7
34, see Scheme 9
37, see Scheme 10

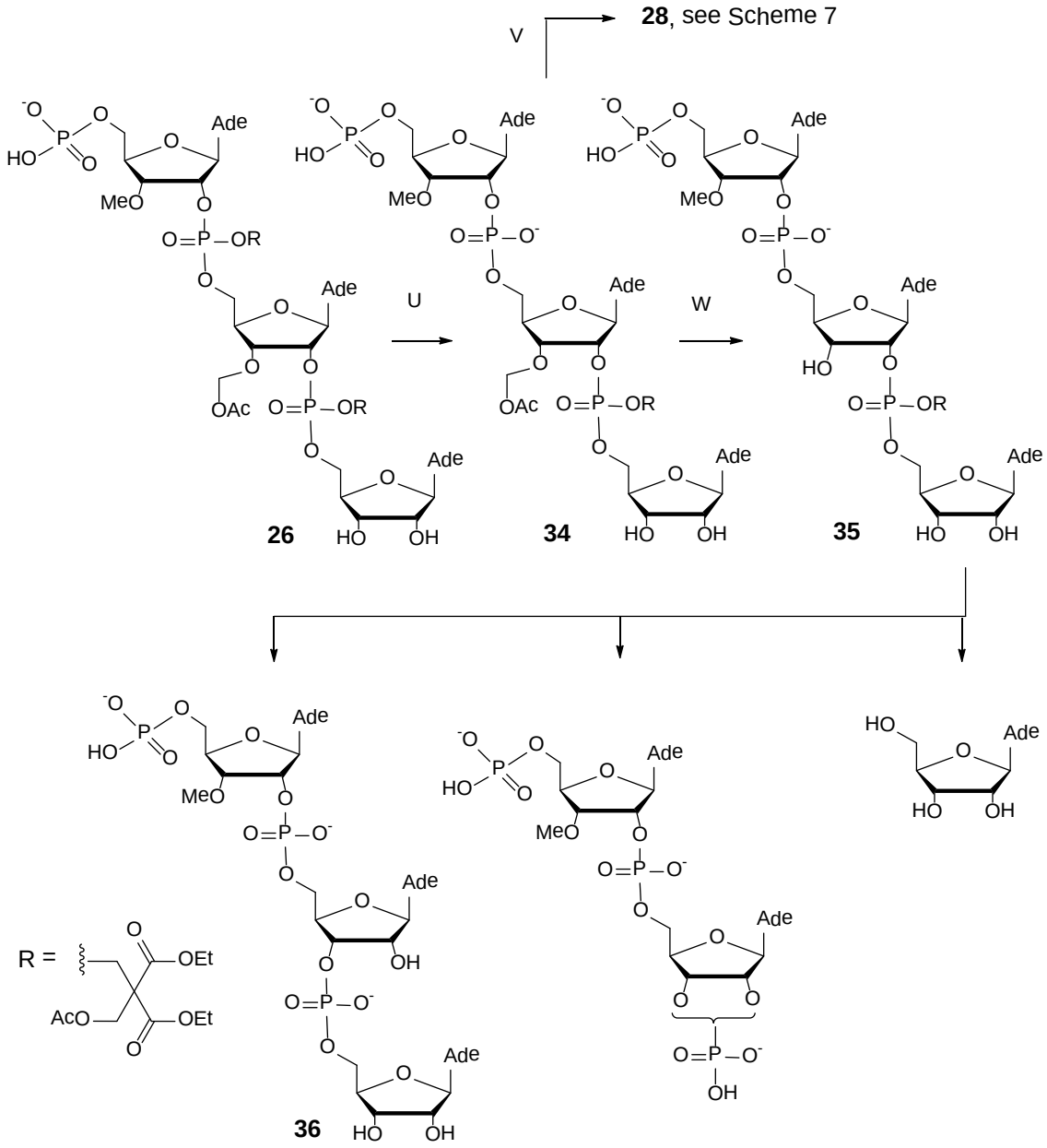
Scheme 7



Scheme 8


R =
R
Q
24: R1 = R2 = R
30: R1 = R, R2 = ⊖
31: R1 = ⊖, R2 = R
S
32: R1 = R, R2 = ⊖
33: R1 = ⊖, R2 = R
T
27, see Scheme 7
34, See Scheme 9

Scheme 9



Scheme 10

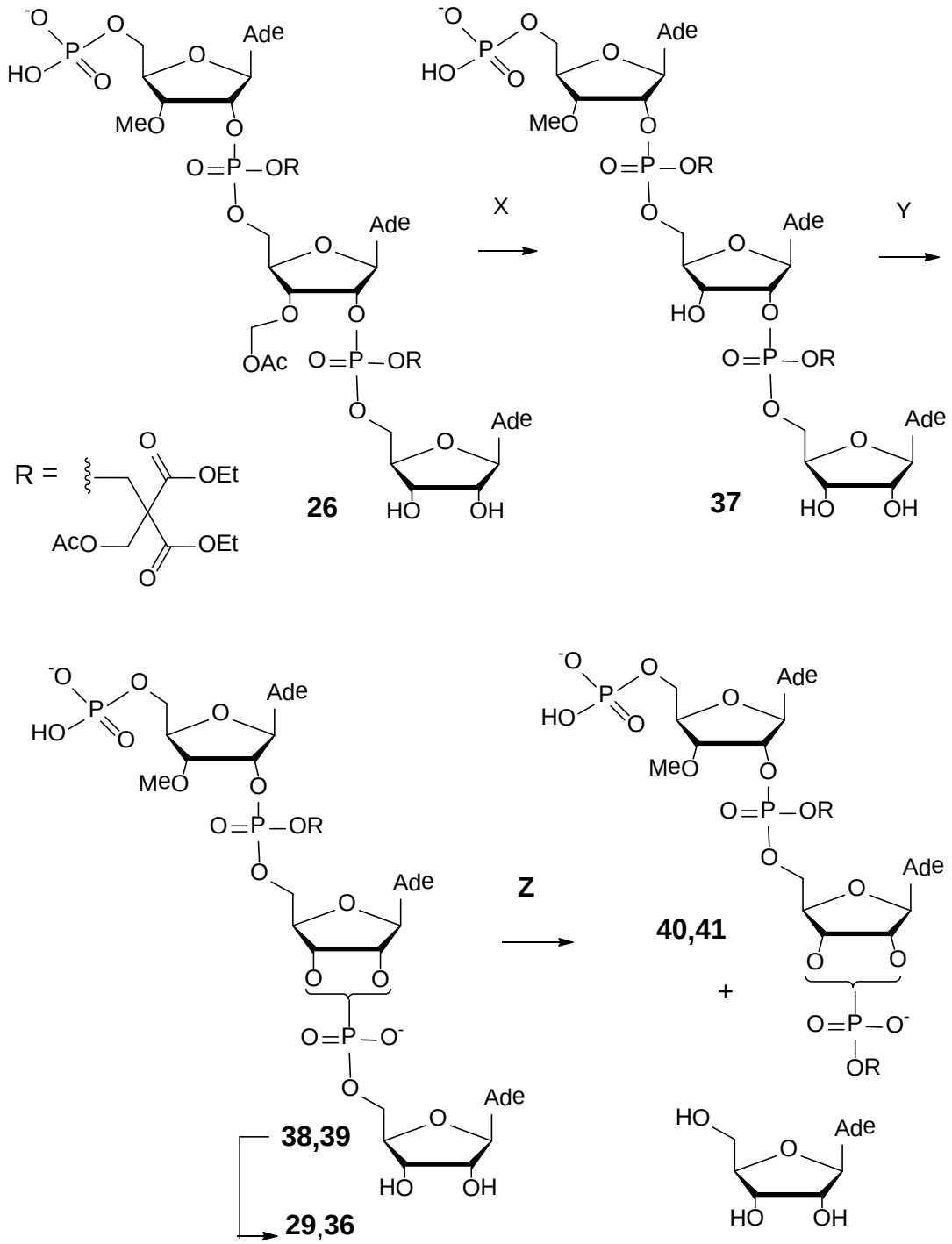
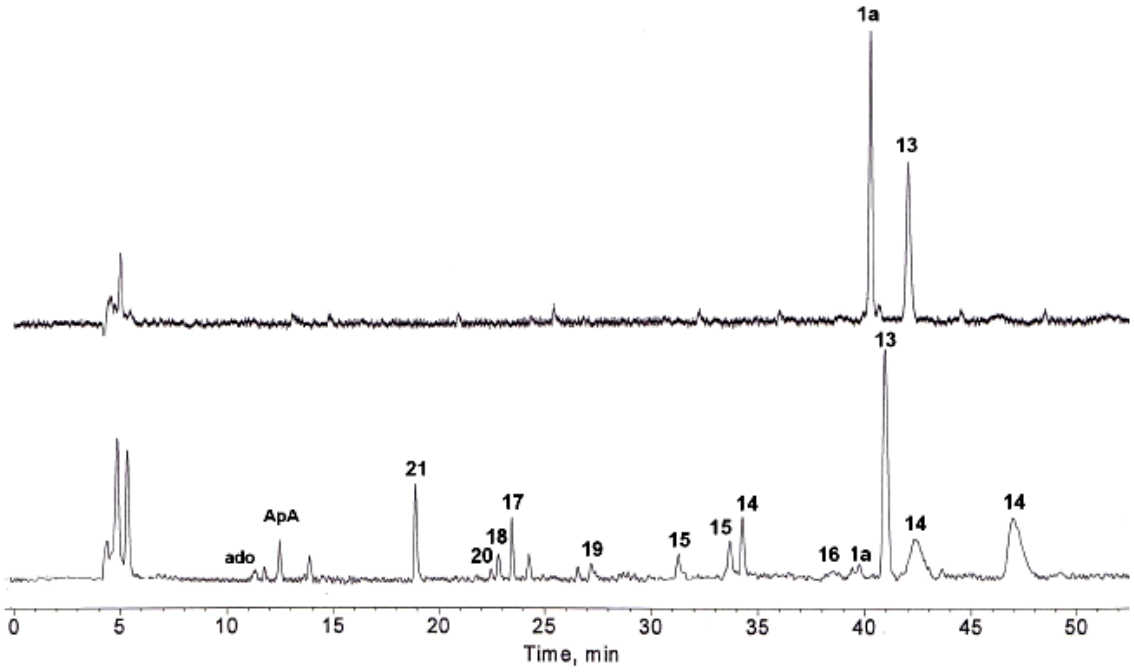


Figure 1


1a
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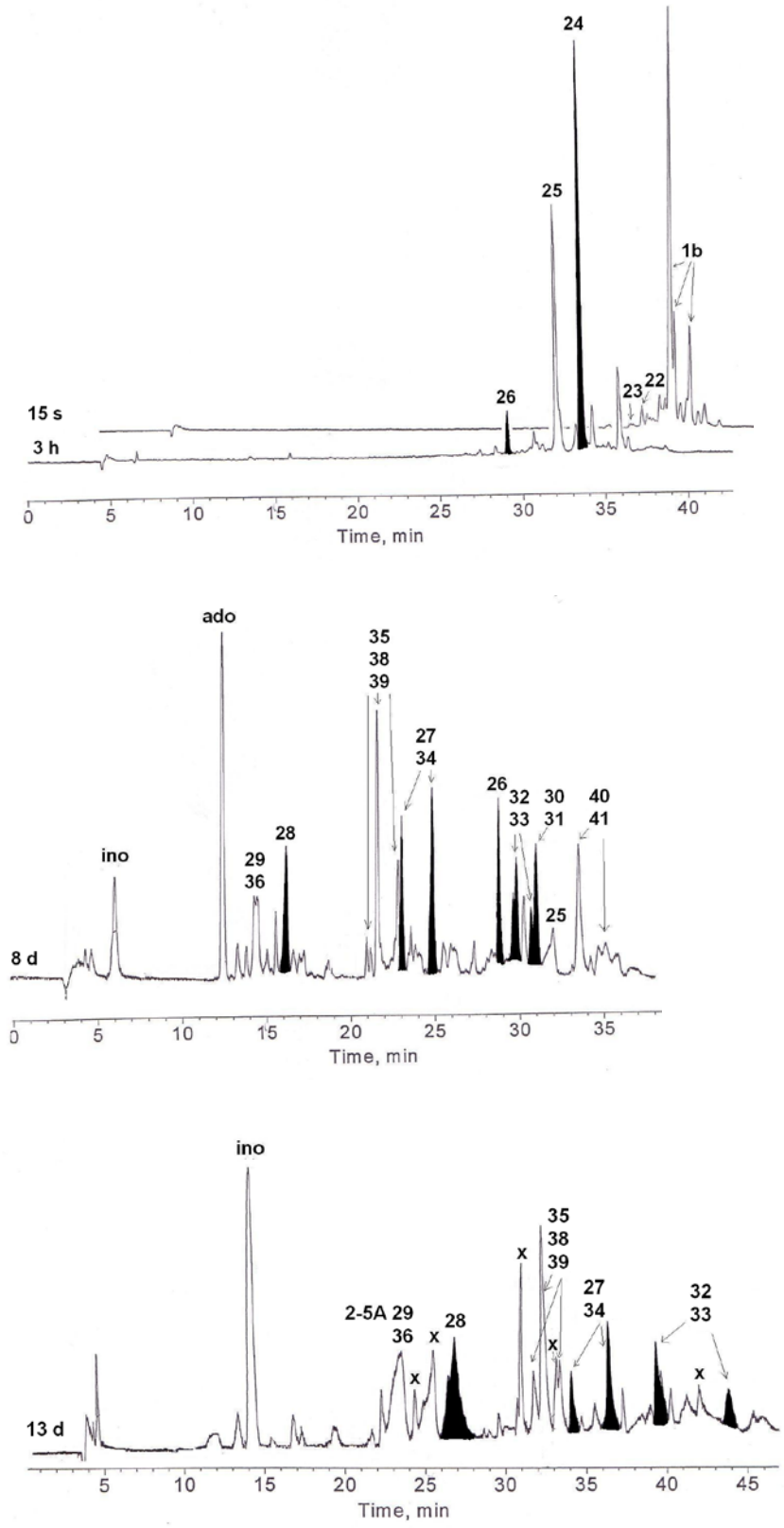
13
21
ApA
ado
17
18
20
19
15
15
14
16
1a
14
14
0
5
10
15
20
25
30
35
40
45
50
Time, min

Figure 2


24
25
1b
23
22
26
15 s
3 h
0 5 10 15 20 25 30 35 40
Time, min
ado
35
38
39
27
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32
33
30
31
40
41
ino
28
29
36
25
8 d
0 5 10 15 20 25 30 35
Time, min
ino
35
38
39
x
27
34
32
33
2-5A 29
36
x
28
x
x
x
13 d
5 10 15 20 25 30 35 40 45
Time, min

Graphical Abstract