Dipeptides as Leaving Group in the Enzyme-Catalyzed DNA Synthesis

by **Xiao-Ping Song**, **Camille Bouillon**, **Eveline Lescrinier**, and **Piet Herdewijn***

Laboratory of Medicinal Chemistry, Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10, B-3000 Leuven
(phone: +3216337387; fax: +3216337340; e-mail: Piet.Herdewijn@rega.kuleuven.be)

Conjugates of 2′-deoxyadenosine monophosphate with dipeptides have been synthesized and tested as substrates for several polymerases. Although the incorporation efficiency is not very high, it demonstrates that some of these dipeptides can be accommodated in the active site of polymerases and function as leaving groups in the enzymatic synthesis of DNA.

Introduction. – Nucleic acids are synthesized in a cell making use of nucleoside triphosphates as substrate and polymerases as enzymes. Pyrophosphate functions as leaving group in this process. Diversifying this leaving group could serve the aim to develop a new biochemistry for the enzymatic synthesis of nucleic acids. Besides the potential application in drug design (developing modified nucleoside triphosphate mimics as direct substrates for polymerases), these molecules can also be applied in synthetic biology (developing an XNA information system) [1], and as tools to better understand the chemical origin of nucleic acid replication in life.

The constructions of an XNA system could rely on a systematic diversification of the sugar moiety, the base moiety, and the leaving group, as well as enzyme evolution. Generally, native polymerases have limited ability to recognize modified nucleotide building blocks. However, some engineered polymerase mutants are able to efficiently incorporate unnatural substrates. For example, *Pinheiro et al.* reported that some engineered polymerases are able to generate XNA sequences (HNA, CeNA, ANA, FANA, TNA, and LNA) up to 72 nt, while still retaining canonical base-pairing selectivity [2]. Previously, we have demonstrated that some natural amino acids (*e.g.*, L-aspartic acid) or unnatural amino acids (*e.g.*, L-β-imidazole lactic acid, iminodiacetate (IDA), iminodipropionate (IDP), and 3-phosphono-L-alanine) are able to act as pyrophosphate mimics in enzymatic DNA polymerization [3–7]. We also observed that d4TMP adducts of 3-phosphono-L-alanine (3-phosphono-L-alanine-d4TMP) fails to deliver d4TMP into CEM/TK$^-$ cells, suggesting the metabolic instability of this nucleotide adduct [8].

In the present study, we have evaluated if dipeptides can function as leaving groups in the DNA polymerization reaction. Dipeptides could have an additional advantage, as they might contribute to the cell-penetration ability of the DNA precursors bearing an alternative leaving group. Dipeptides might facilitate the membrane delivery of these nucleotide adducts with the help of membrane transporters [9]. Dipeptides consisting of two amino acid molecules joined by a peptide bond are metabolically

degradable by hydrolase enzymes *in vivo* [10]. Therefore, the dipeptide that would be generated in the cell after nucleotide incorporation can be degraded into single amino acids, making the polymerization reaction irreversible. Dipeptides bearing two additional carboxylic acid residues could retain the chelating properties necessary for binding the nucleotide substrates in the active site of polymerases. Compared to a single amino acid, a dipeptide is a larger molecule, allowing us to probe the geometry of the enzyme-active-site pocket.

Results and Discussion. – *Synthesis of Compounds* **1a**–**1k**. The eleven compounds synthesized are compiled in *Table 1*. The synthetic routes used to obtain compounds **1a**–**1k** are outlined in *Schemes 1–4*.

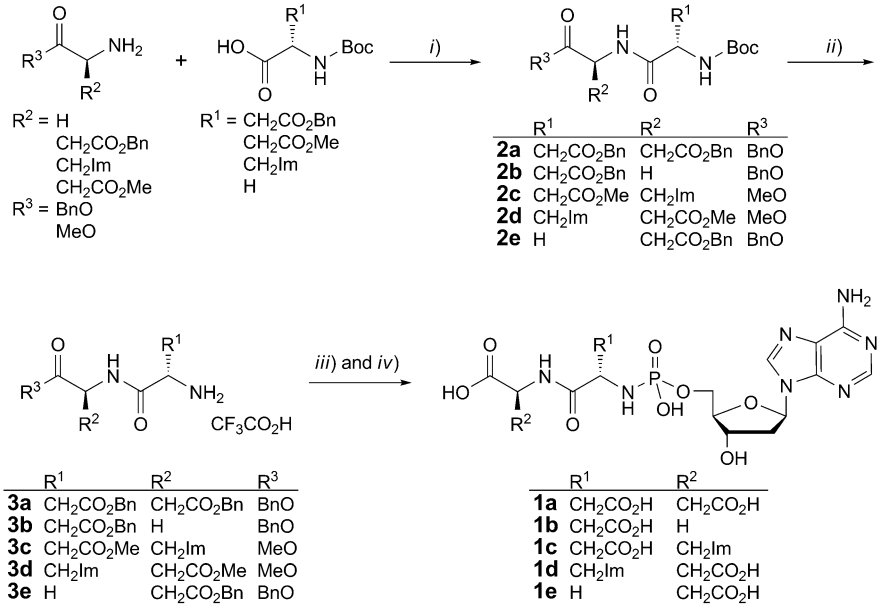
Table 1. *Synthesized Compounds* **1a**–**1k**

Compound No.	Structure	Compound No.	Structure
1a	Asp-Asp-dAMP	**1g**	Gly-IDA-dAMP
1b	Gly-Asp-dAMP	**1h**	Asp-IDA-dAMP
1c	His-Asp-dAMP	**1i**	Gly-IDP-dAMP
1d	Asp-His-dAMP	**1j**	Asp-IDP-dAMP
1e	Asp-Gly-dAMP	**1k**	Gly-Gly-dAMP
1f	His-IDA-dAMP		

Scheme 1 depicts the synthesis of the phosphoramidate nucleoside dipeptide analogs **1a**–**1e**, obtained in four steps. First, the protected dipeptides **2a**–**2e** were obtained in good yield starting from commercially available amino acids, using the conventional coupling reagent *N*,*N*′-dicyclohexylcarbodiimide (DCC)/*N*-hydroxysuccinimide (HOSu) in CH_2Cl_2. The Boc protecting group was removed by treating **2a**–**2e** with CF_3COOH (TFA) to give the corresponding amines **3a**–**3e**, respectively, in excellent yields. The synthesis of compounds **1a**–**1e** was carried out according to the procedure described by *Wagner* and co-workers [11], starting from 2′-deoxyadenosine-5′-monophosphate (dAMP) and amines **3a**–**3e** with DCC as coupling reagent. Finally, deprotection of the carboxylic acid ester function with NaOH in $MeOH/H_2O$ solution (for compounds **1a**, **1b**, and **1e**) or by hydrogenation with 10% Pd/C (for compounds **1c** and **1d**) provided the target compounds **1a**–**1e**.

The synthesis of compound **1f**–**1h** was accomplished as outlined in *Scheme 2*. *N*-Alkylation of *N*-benzylglycine ethyl ester with *tert*-butyl 2-bromoacetate in the presence of EtN^iPr_2 gave compound **4**, which was further treated with TFA to remove selectively the ^{t}Bu group to give the corresponding acid **5** as TFA salt. Then, the obtained compound **5** was coupled to commercially available amino acids, using the coupling reagent DCC/HOSu or *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride (EDCI)/1-hydroxybenzotriazole (HOBt) to afford compounds **6a**–**6c** in good yields. Compounds **7a**–**7c** were obtained after removal of the $PhCH_2$ (Bn) protecting groups, and the resulting secondary amines were coupled with dAMP in the presence of DCC to provide the corresponding protected nucleoside analogs. The deprotection of the carboxylic acid functions with NaOH in $MeOH/H_2O$ solution gave compounds **1f**–**1h**.

Scheme 1. *Synthesis of Compound* **1a**–**1e**



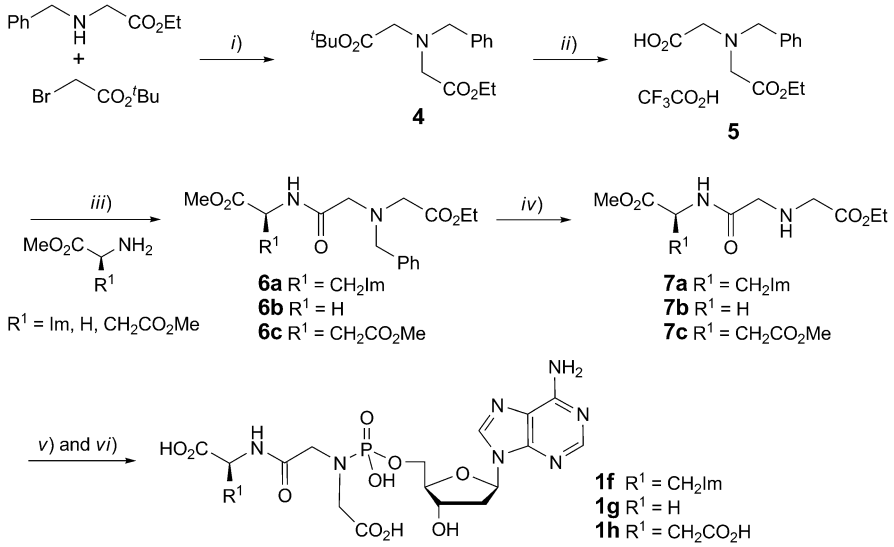
i) *N*,*N*′-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (HOSu), *N*-methylmorpholine, CH_2Cl_2, r.t. *ii*) CF_3COOH (TFA), CH_2Cl_2, r.t. *iii*) 2′-Deoxyadenosine-5′-monophosphate (dAMP), DCC, 1,4-dioxane/H_2O, Et_3N, 80°. *iv*) 10% Pd/C, H_2, r.t. for **1a**, **1b**, and **1e**; 0.4M NaOH (MeOH/H_2O), r.t. for **1c** and **1d**. Im = 1*H*-Imidazol-4-yl.

The synthesis of compound **1i** and **1j** is depicted in *Scheme 3*. Two *Michael* addition reactions of $BnNH_2$ on methyl acrylate and *tert*-butyl acrylate successively gave compounds **8** and **9**, respectively. Treatment of the latter with TFA provided compound **10**. The TFA salt **10** was coupled with glycine methyl ester or L-aspartic acid dimethyl ester in the presence of EDCI/HOBt to furnish **11a** and **11b** respectively. The hydrogenolyses gave the free amines **12a** and **12b**. Finally, as described previously, a coupling reaction of **12a** and **12b** with dAMP, followed by a saponification reaction, gave the final compounds **1i** and **1j**, respectively.

Scheme 4 displays the synthesis of compound **1k** obtained in four steps, starting with a coupling reaction between diethyl aminomalonate and *N*-(benzyloxy)carbonyl (Cbz)-glycine. Then, compound **13** was engaged in a hydrogenation reaction in the presence of HCl to avoid intermolecular and intramolecular side-reactions. The HCl salt **14** was coupled with dAMP, and deprotection of the carboxylate function provided compound **1k**, after a decarboxylation reaction.

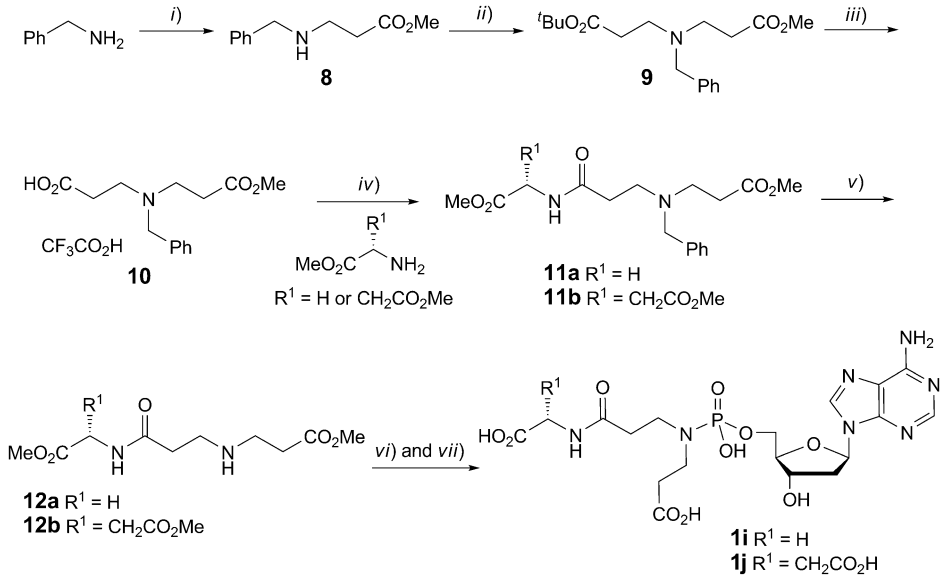
Single-Nucleotide-Incorporation Experiments. Polymerases play a central role during the enzyme-catalyzed DNA synthesis. They promote DNA synthesis by catalyzing the nucleophilic attack of the 3′-terminal OH group of a primer to the α-phosphate of the incoming nucleoside triphosphate, leading to formation of a 5′-3′ phosphodiester bond [12]. To investigate the substrate property of compounds **1a**–**1k**, polymerases from different families were tested (HIV-1 RT, *Taq*, *Vent* (exo–)

Scheme 2. *Synthesis of Compound* **1f**–**1h**



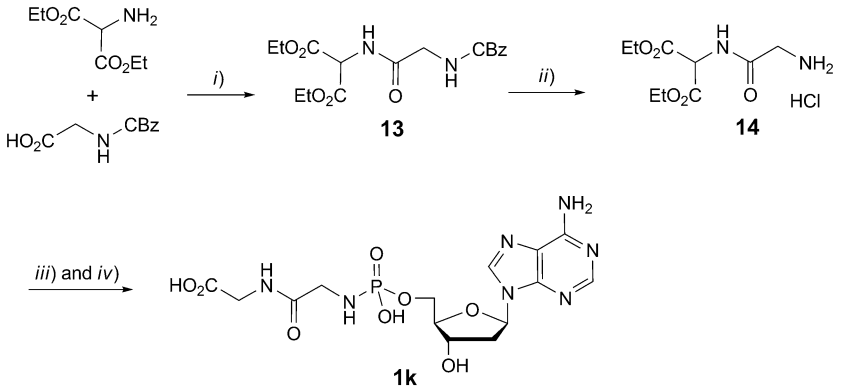
i) EtN^iPr_2, MeCN, r.t. – reflux. *ii*) TFA, CH_2Cl_2, r.t. *iii*) 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDCI), 1-hydroxybenzotriazole (HOBt), CH_2Cl_2, Et_3N, r.t. for **6a**; DCC, HOSu, *N*-methylmorpholine, CH_2Cl_2, r.t. for **6b**–**6c**. *iv*) H_2, 10% Pd/C, MeOH, r.t. *v*) dAMP, DCC, 1,4-dioxane/H_2O, Et_3N, 80° for **1g** and **1h**; dAMP, DCC, ^{t}BuOH/H_2O, Et_3N, 80° for **1f**. *vi*) 0.4M NaOH (MeOH/H_2O), r.t.

Scheme 3. *Synthesis of Phosphoramidate Analogs* **1i** *and* **1j**



i) Methyl acrylate, MeOH, r.t. *ii*) *tert*-Butyl acrylate, MeOH, r.t. *iii*) TFA, CH_2Cl_2, r.t. *iv*) EDCI, HOBt, CH_2Cl_2, Et_3N, r.t. *v*) H_2, 10% Pd/C, MeOH, r.t. *vi*) dAMP, DCC, 1,4-dioxane/H_2O, Et_3N, 80°. *vii*) 0.4M NaOH (MeOH/H_2O), r.t.

Scheme 4. *Synthesis of Phosphoramidate Analog* **1k**



i) EDCI, HOBt, CH_2Cl_2, Et_3N, r.t. *ii*) H_2, 10% Pd/C, MeOH, HCl, r.t. *iii*) dAMP, DCC, ^{t}BuOH/H_2O, Et_3N, 80°. *iv*) 0.4M NaOH (MeOH/H_2O), r.t.

polymerase, and *Therminator* DNA polymerase). Primer P_1 and template T_1 were used as duplex (*Table 2*), 2′-deoxyadenosine triphosphate (dATP) was used as positive control. Incorporation efficiency was evaluated by the polyacrylamide gel-based single-nucleotide-incorporation assay [13][14].

Table 2. *Primer–Template Complex Used in the Incorporation Assay*

Oligonucleotide	Sequence
P_1	5′-CAGGAAACAGCTATGAC-3′
T_1	3′-GTCCTTTGTCGATACTG**TCCC**-5′

Single-Nucleotide Incorporation by HIV-1 RT. HIV-1 RT is an error-prone polymerase and therefore, has high mutation rate [15]. Its flexibility and high tolerance towards chemically modified nucleotide substrates made it the primary choice as enzyme catalyst for our pyrophosphate-mimic screening. The single-nucleotide-incorporation results of compound **1a**–**1k** by HIV-1 RT are compiled in *Table 3*. Unfortunately, all eleven compounds were not good substrates for HIV-1 RT. Most of the compounds only showed 10–35% yield of primer extension. Compound **1c**, **1g**, and

Table 3. *Results of Incorporation of Compounds* **1a**–**1k** *by HIV-1 RT*[a])

Compound No.	Incorporation [%]	Compound No.	Incorporation [%]
1a	33	**1g**	0
1b	0	**1h**	0
1c	20	**1i**	18
1d	15	**1j**	5
1e	14	**1k**	21
1f	20		

[a]) Conditions: primer, P_1; template; T_1, [HIV-1 RT], 0.025 U/μl.

1h were not able to extend the primer after 2 h reaction time. The best single incorporation result was obtained by using compound **1a** as substrate, with 33% yield of P+1 strand. It is interesting to note that compounds **1a**, **1b**, and **1c**, all of which contain an L-aspartic acid moiety in the leaving group, showed quite different incorporation results, with 30, 0, and 20% primer conversion, respectively. This is much less than the 90% conversion obtained when using L-Asp-dAMP as substrate.

Single-Nucleotide Incorporation by Therminator *DNA Polymerase. Therminator* DNA polymerase is a 9°N variant, recognized for its ability to incorporate a broad spectrum of modified nucleotides [16][17]. As shown in *Fig. 1*, compounds **1a**–**1k** exhibited good substrate properties using *Therminator* as catalyst. Except for **1d**, all of the tested analogs were accepted as substrate by *Therminator* DNA polymerase quite efficiently, some moderate-to-high yields of primer conversion were obtained. However, using *Therminator* polymerase as catalyst, a higher ratio of misincorporation was observed. For example, using compound **1g** as substrate in the single-nucleotide-incorporation assay over 90% primer conversion was obtained with *Therminator* polymerase (*Fig. 2*), but only 22% of the primer was converted to the P+1 level, 49% converted to the P+2 level, and 19% primer converted to the P+3 and P+4 level.

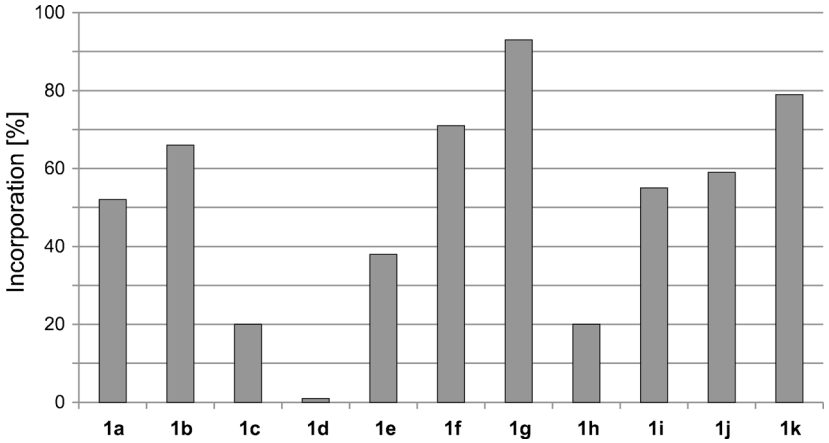


Fig. 1. *Single-nucleotide incorporation of compounds* **1a**–**1k** *into* P_1T_1 *by* Therminator *DNA polymerase.* [*Therminator* pol] = 0.05 U/μl.

Single-Nucleotide Incorporation by Other DNA Polymerases. Furthermore, the substrate properties of compound **1a**–**1k** were investigated by *Taq* and *Vent* (exo–) polymerases (*Fig. 3*). The incorporation efficiency was significantly less appealing than using *Therminator* DNA polymerase as catalyst. When using *Taq* as polymerase, only compounds **1d** (30% primer conversion) and **1g** (27% primer conversion) gave primer extension. Most of the analogs were poor substrates for *Taq* polymerase. In contrast, *Vent* (exo–) polymerase showed a slightly better catalytic ability. Compound **1f** with iminodiacetic acid joint by L-histidine as leaving group demonstrates 75% primer extension at a concentration of 500 μM (*Fig. 4*). Compound **1g** containing an iminodiacetic acid moiety and a glycine in the leaving group gave 60% P+1 product. On the contrary, compound **1h** composed of iminodiacetic acid and L-aspartic acid in

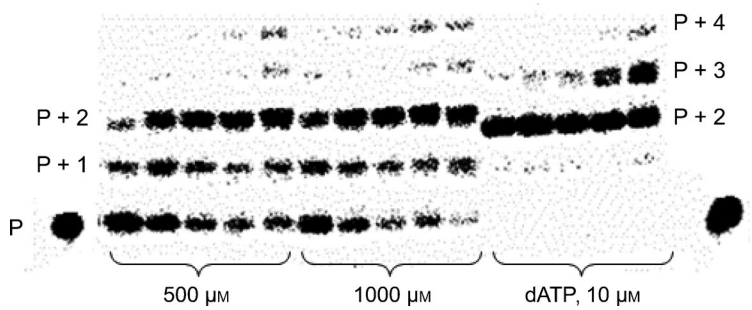


Fig. 2. *Single incorporation of compound* **1c** *into* P_1T_1 *by* Therminator *DNA polymerase.* [*Therminator* pol] = 0.05 U/μl. Time points: 10, 20, 30, 60, and 120 min.

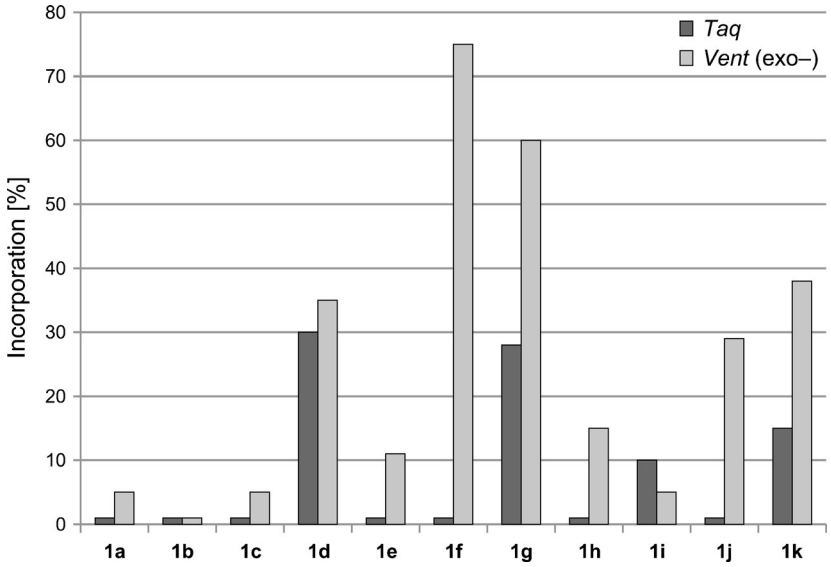


Fig. 3. *Single-nucleotide incorporation of compounds* **1a**–**1k** *by* Taq *and* Vent *(exo–) DNA polymerase.* [*Vent* (exo–) pol] = 0.1 U/μl, [*Taq* pol] = 0.15 U/μl.

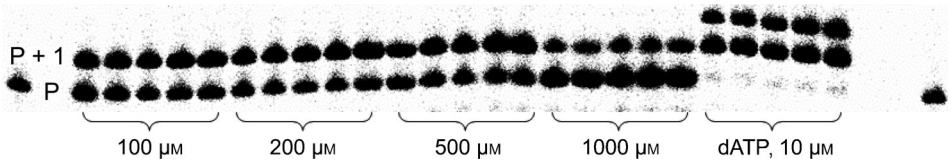


Fig. 4. *Single incorporation of compound* **1f** *into* P_1T_1 *by* Vent *(exo–) DNA polymerase.* [*Vent* (exo–) pol] = 0.1 U/μl. Time points: 10, 20, 30, 60, and 120 min.

the leaving group was a poorer substrate than **1f** and **1g**, and only 15% yield of P + 1 product was obtained. The different incorporation results obtained for compounds **1f**

and **1g** indicated that it is still challenging to balance binding properties and potential substrate properties for polymerases of modified nucleoside triphosphates.

It is worth noting that compound **1k** is the smallest-sized dipeptide among the tested analogs. It contains two glycine molecules and only one carboxylic acid group in the leaving group, and it still can be accepted as substrate by all four enzymes tested. It showed better substrate properties than some of the analogs with two or three carboxylic acid groups, especially when using *Therminator* polymerase as catalyst (*Fig. 1*). Enzyme-catalyzed nucleotide incorporation is a very complicated procedure. Several factors might be involved in this process. Based on the results obtained for compound **1k** and for other analogs that have been studied here, it is clear that the size of the dipeptide leaving group is a very important factor influencing the incorporation reactions. Co-crystallization experiments and molecular modeling to analyze the possible interactions between the substrate and the enzyme active site will be very helpful for the future rational design of new pyrophosphate mimics.

Conclusions. – A series of phosphoramidate analogs bearing dipeptides as leaving groups were synthesized and tested as substrates by HIV-1 RT, *Taq*, *Vent* (exo–), and *Therminator* DNA polymerases. *Therminator* polymerase exhibited the best catalytic efficiency of all the four enzymes used, with moderate-to-high yield of primer conversions. A higher level of misincorporation was also observed with *Therminator* polymerase. Compound **1k** bearing only one carboxylic acid residue in the leaving group is still well-accepted by the four polymerases. This suggests that the spacial size of the dipeptide leaving group might play an important role in the recognition by the enzyme active site. Although these dipeptide leaving groups show inferior incorporation efficiencies when compared with, for example, iminodipropionate, the obtained results are encouraging. We will further explore the potential of using such protected or unprotected dipeptide–dNMP analogs as potential prodrugs for modified nucleotides. Another interesting question is, if such conjugates would be able to freely penetrate the membrane of vesicles and/or function as substrate in a nonenzymatic nucleic acid polymerization reaction.

Experimental Part

General. For all reactions, chemicals of anal. or synthetic grade were obtained from commercial sources and used without purification. Technical-grade solvents were obtained from *Brenntag* (B-Deerlijk). Flash chromatography (FC): *Davisil*® silica gel *60*, 0.040–0.063 mm (*Grace Davison*). Anal. TLC: *Alugram*® silica gel *UV254* mesh 60, 0.20 mm (*Macherey–Nagel*). NMR Spectra: *Bruker Avance II* 300- or 500-MHz NMR spectrometer; chemical shifts, δ, expressed in ppm downfield from TMS (Me_4Si) for ^{1}H- and ^{13}C-NMR; *J* in Hz. ^{31}P-NMR: chemical shifts are referenced to an external 85% H_3PO_4 standard (δ 0.00 ppm). MS: a quadrupole/orthogonal-acceleration time-of-flight tandem mass spectrometer (*Q-Tof 2*, *Micromass*) equipped with a standard electrospray ionization (ESI) source; in *m*/*z*.

*Dibenzyl (2S)-2-({(2S)-4-(Benzyloxy)-2-[(*tert*-butoxycarbonyl)amino]-4-oxobutanoyl}amino)butanedioate* (**2a**). *General Procedure I* (*GP I*). To a soln. of Boc-L-aspartic acid benzyl ester (646 mg, 2.0 mmol) and L-aspartic acid dibenzyl ester (971 mg, 2.0 mmol) in CH_2Cl_2 (15 ml), *N*-methylmorpholine (NMM; 205 µl, 2.0 mmol), DCC (619 mg, 3.0 mmol) and HOSu (232 mg, 2.0 mmol) were added. The mixture was stirred for 14 h under Ar at r.t., white precipitate was removed by filtration, and the resulting crude material was purified by FC (hexane/AcOEt 5:1). Yield: 92%. White solid. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.45–7.30 (*m*, 3 Ph); 5.14–5.07 (*m*, 3 PhCH_2); 4.92–4.86 (*m*, H–C(α)(Asp_a)); 4.60–4.55 (*m*,

H–C(α)(Asp$_b$)); 3.11–3.03 (*m*, CH$_2$(β)(Asp$_a$)); 2.93–2.70 (*m*, CH$_2$(β)(Asp$_b$)); 1.47 (*s*, ^{t}Bu). ^{13}C-NMR (75 MHz, $CDCl_3$): 171.2; 170.3; 170.1; 169.7; 155.2; 135.1; 134.8; 127.9–128.3 (15 C); 81.2; 67.2; 66.5 (2 C); 50.3; 48.6; 36.0; 35.8; 27.9. HR-ESI-MS: 641.2450 ($[M+Na]^+$, $C_{34}H_{38}N_2NaO_9^+$; calc. 641.2470).

*Benzyl (3*S*)-4-{[2-(Benzyloxy)-2-oxoethyl]amino}-3-[(*tert*-butoxycarbonyl)amino]-4-oxobutanoate* (**2b**). *GP I* was applied using glycine benzyl ester (404 mg, 2.0 mmol), Boc-L-aspartic acid benzyl ester (646 mg, 2.0 mmol), NMM (205 µl, 2.0 mmol), DCC (619 mg, 3.0 mmol), and HOSu (232 mg, 2.0 mmol). Purification was carried out according to the method used for **2a**. Yield: 82%. White solid. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.37–7.35 (*m*, 2 Ph); 5.19 (*s*, PhCH_2); 5.15 (*s*, PhCH_2); 4.61–4.60 (*m*, NHC*H*CH$_2$); 4.06 (*d*, *J* = 5.0, NHCH_2CO$_2$Bn); 3.12–3.05 (*m*, 1 H, NHCHCH_2); 2.80–2.72 (*m*, 1 H, NHCHCH_2); 1.47 (*s*, ^{t}Bu). ^{13}C-NMR (75 MHz, $CDCl_3$): 171.7; 171.1; 169.4; 155.6; 135.5; 135.3; 128.4–128.7 (10 C); 80.7; 67.3; 66.9; 50.6; 36.2; 34.0; 28.4. HR-ESI-MS: 471.2112 ($[M+H]^+$, $C_{25}H_{32}N_2O_7^+$; calc. 471.2126).

*Methyl (3*S*)-3-[(*tert*-Butoxycarbonyl)amino]-4-{[(2*S*)-3-(1*H*-imidazol-4-yl)-1-methoxy-1-oxopropan-2-yl]amino}-4-oxobutanoate* (**2c**). *GP I* was applied using L-histidine methyl ester dihydrochloride (484 mg, 2.0 mmol), Boc-L-aspartic acid methyl ester (495 mg, 2.0 mmol), NMM (205 µl, 2.0 mmol), DCC (619 mg, 3.0 mmol), and HOSu (232 mg, 2.0 mmol). Purification was carried out according to the method used for **2a**. Yield: 58%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.72 (*s*, NHC*H*=N); 6.79 (*s*, NHC*H*=C); 4.72–4.70 (*m*, NHC*H*CH$_2$CO$_2$Me); 4.51–4.50 (*m*, NHC*H*CH$_2$Im); 3.65 (*s*, MeO); 3.63 (*s*, MeO); 3.12–3.10 (*m*, ImCH_2); 2.88–2.73 (*m*, NHCHCH_2CO$_2$Me); 1.39 (*s*, ^{t}Bu). ^{13}C-NMR (75 MHz, $CDCl_3$): 171.7; 170.9; 170.6; 155.3; 135.0; 131.1; 118.7; 80.1; 52.6; 52.1; 51.7; 50.6; 36.0; 29.3; 27.9. HR-ESI-MS: 399.1873 ($[M+H]^+$, $C_{17}H_{27}N_4O_7^+$; calc. 399.1880).

Dimethyl N-*(*tert*-Butoxycarbonyl)-L-histidyl-L-aspartate* (**2d**). *GP I* was applied using Boc-L-histidine (510 mg, 2.0 mmol), Boc-L-aspartic acid dimethyl ester hydrochloride (395 mg, 2.0 mmol), NMM (205 µl, 2.0 mmol), DCC (619 mg, 3.0 mmol), and HOSu (232 mg, 2.0 mmol). Purification was carried out according to the method used for **2a**. Yield: 74%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.77 (*s*, NHC*H*=N); 6.79 (*s*, NHC*H*=C); 4.76–4.74 (*m*, NHC*H*CO$_2$Me); 4.37–4.35 (*m*, NHC*H*CH$_2$Im); 3.63 (*s*, MeO); 3.57 (*s*, MeO); 2.99–2.82 (*m*, ImCH_2); 2.81–2.60 (*m*, NHCHCH_2CO$_2$Me); 1.31 (*s*, ^{t}Bu). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.7; 171.8; 170.9; 155.5; 135.0; 132.1; 118.0; 79.9; 54.3; 52.6; 51.9; 48.5; 35.8; 29.2; 28.1. HR-ESI-MS: 399.1871 ($[M+H]^+$, $C_{17}H_{27}N_4O_7^+$; calc. 399.1880).

Dibenzyl N-*(*tert*-Butoxycarbonyl)glycyl-L-aspartate* (**2e**). *GP I* was applied using Boc-L-glycine (350 mg, 2.0 mmol), L-aspartic acid dibenzyl ester (971 mg, 2.0 mmol), NMM (205 µl, 2.0 mmol), DCC (619 mg, 3.0 mmol), and HOSu (232 mg, 2.0 mmol). Purification was carried out according to the method used for **2a**. Yield: 95%. White solid. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.35–7.11 (*m*, 2 Ph); 5.19 (*s*, PhCH_2); 5.06 (*s*, PhCH_2); 4.95–4.92 (*m*, NHC*H*CO$_2$Bn); 3.82 (*s*, NHCH_2CO); 3.13–2.82 (*m*, NHCHCH_2CO$_2$Bn); 1.46 (*s*, ^{t}Bu). ^{13}C-NMR (75 MHz, $CDCl_3$): 170.3; 169.9; 169.1; 155.6; 135.0; 134.8; 127.9–128.3 (10 C); 79.9; 67.3; 66.5; 48.3; 43.8; 36.0; 28.0. HR-ESI-MS: 493.1957 ($[M+Na]^+$, $C_{25}H_{31}N_2NaO_7^+$; calc. 493.1945).

*Dibenzyl (2*S*)-2-{[(2*S*)-2-Amino-4-(benzyloxy)-4-oxobutanoyl]amino}butanedioate Trifluoroacetate* (**3a**). *General Procedure II* (*GP II*). Compound **2a** was dissolved in CH_2Cl_2 (10 ml), and TFA (5 ml) was added to the soln. The soln. was stirred for 24 h at r.t. After removal of solvent and excess of TFA, **3a** was obtained as colorless oil. Yield: 98%. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.30–7.22 (*m*, 3 Ph); 5.06–4.99 (*m*, 3 PhCH_2); 4.56–4.50 (*m*, H–C(α)(Asp$_a$)); 4.09–4.05 (*m*, H–C(α)(Asp$_b$)); 3.01–2.88 (*m*, CH$_2$(β)(Asp$_a$), CH$_2$(β)(Asp$_b$)). ^{13}C-NMR (75 MHz, $CDCl_3$): 170.4; 170.4; 169.4; 135.0; 134.7; 134.6; 127.9–128.2 (15C); 67.2; 66.6; 64.0; 49.3; 48.7; 35.2; 34.6. HR-ESI-MS: 519.2131 ($[M+H]^+$, $C_{29}H_{32}N_2O_7^+$; calc. 519.2126).

*Benzyl (3*S*)-3-Amino-4-{[2-(benzyloxy)-2-oxoethyl]amino}-4-oxobutanoate Trifluoroacetate* (**3b**). *GP II* was applied using **2b** (1.4 g, 3 mmol) and TFA (5 ml) to give **3b** as TFA salt. Yield: 98%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.31–7.30 (*m*, 2 Ph); 5.11–5.09 (*m*, 2 PhCH_2); 4.66–4.65 (*m*, NH$_2$C*H*CH$_2$); 4.00–3.99 (*m*, NHCH_2CO$_2$Bn); 3.04–3.03 (*m*, NH$_2$CHCH_2). ^{13}C-NMR (75 MHz, $CDCl_3$): 170.9; 169.5; 168.6; 134.9; 134.6; 128.5–128.8 (10 C); 68.1; 67.8; 50.2; 34.7; 33.2. HR-ESI-MS: 371.1592 ($[M+H]^+$, $C_{20}H_{24}N_2O_5^+$; calc. 371.1601).

*Methyl (3*S*)-3-Amino-4-{[(2*S*)-3-(1*H*-imidazol-4-yl)-1-methoxy-1-oxopropan-2-yl]amino}-4-oxobutanoate Trifluoroacetate* (**3c**). *GP II* was applied using **2c** (1.2 g, 3 mmol) and TFA (5 ml) to give **3c** as TFA salt. Yield: 95%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.60 (*s*, NHC*H*=N); 6.77 (*s*,

NHC*H*=C); 4.08–4.03 (*m*, NH*CH*CH_2CO_2Me); 3.87–3.83 (*m*, NH*CH*CH_2Im); 3.66 (*s*, MeO); 3.61 (*s*, MeO); 3.53–3.30 (*m*, NHCHCH_2CO_2Me); 2.56–2.43 (*m*, NHCHCH_2Im). ^{13}C-NMR (75 MHz, $CDCl_3$): 172.9; 171.6; 169.9; 134.4; 133.2; 117.6; 55.3; 54.3; 52.2; 51.5; 36.6; 28.9. HR-ESI-MS: 299.1346 ($[M+H]^+$, $C_{12}H_{19}N_4O_5^+$; calc. 299.1355).

Dimethyl L-*Histidyl*-L-*aspartate Trifluoroacetate* (**3d**). *GP II* was applied using **2d** (1.2 g, 3 mmol) and TFA (5 ml) to furnish **3d** as TFA salt. Yield: 90%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.64 (*s*, NHC*H*=N); 6.90 (*s*, NHC*H*=C); 4.79–4.76 (*m*, NH*CH*CH_2CO_2Me); 4.30–4.26 (*m*, NH*CH*CH_2Im); 3.65 (*s*, MeO); 3.60 (*s*, MeO); 3.15–3.06 (*m*, NHCHCH_2CO_2Me); 2.85–2.80 (*m*, NHCHCH_2Im). ^{13}C-NMR (75 MHz, $CDCl_3$): 174.9; 172.4; 169.3; 136.5; 134.2; 119.5; 56.3; 53.2; 52.7; 52.6; 38.7; 32.6. HR-ESI-MS: 299.1350 ($[M+H]^+$, $C_{12}H_{19}N_4O_5^+$; calc. 299.1355).

Dibenzyl Glycyl-L-*aspartate Trifluoroacetate* (**3e**). *GP II* was applied using **2e** (1.5 g, 3 mmol) and TFA (5 ml) to afford **3e** as TFA salt. Yield: 90%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.28–7.21 (*m*, 2 Ph); 5.00–4.97 (*m*, 2 PhCH_2, NHCH_2CO); 3.95–3.86 (*m*, NHCH_2CO); 3.02–2.85 (*m*, NHCHCH_2). ^{13}C-NMR (75 MHz, $CDCl_3$): 170.5; 170.2; 134.9; 134.6; 127.9–128.2 (10 C); 67.5; 66.7; 48.7; 40.7; 35.5. HR-ESI-MS: 371.1609 ($[M+H]^+$, $C_{20}H_{24}N_2O_5^+$; calc. 371.1601).

*5′-O-{[((1*S*)-1-(Carboxymethyl)-2-{[(1*S*)-1,2-dicarboxyethyl]amino}-2-oxoethyl)amino]hydroxyphosphinyl}-2′-deoxyadenosine* (**1a**). *General Procedure III* (*GP III*). 2′-Deoxyadenosine-5′-monophosphate (dAMP; 100 mg, 0.30 mmol) and **3a** (777 mg, 1.5 mmol) were dissolved in 1,4-dioxane (8 ml)/H_2O (2ml) in the presence of one drop of Et_3N. A soln. of DCC (433 mg, 2.10 mmol) in 1,4-dioxane (2 ml) was added, and the mixture was heated at 85° for 3 h under Ar. After completion, the mixture was cooled, and the solvent was removed *in vacuo*. The resulting residue was purified by FC ($CHCl_3$/MeOH/H_2O 5 : 2 : 0.5) to provide the intermediate compound, *5′-O-[({(1*S*)-3-(benzyloxy)-1-[({(1*S*)-3-(benzyloxy)-1-[(benzyloxy)carbonyl]-3-oxopropyl}amino)carbonyl]-3-oxopropyl}amino)hydroxyphosphinyl]-2′-deoxyadenosine*, as a white solid. Yield: 43%. ^{1}H-NMR (300 MHz, D_2O): 8.47 (*s*, H–C(8)); 8.17 (*s*, H–C(2)); 7.29–7.24 (*m*, 3 Ph); 6.47 (*t*, $J=6.5$, H–C(1′)); 5.04–4.99 (*m*, 3 PhCH_2); 4.64–4.60 (*m*, H–C(3′)); 4.12–4.10 (*m*, H–C(4′)); 4.02–3.95 (*m*, CH_2(5′)); 3.47–3.44 (*m*, H–C(α)(Asp_a), H–C(α)(Asp_b)); 2.87–2.75 (*m*, H_a–C(2′), CH_2(β)(Asp_a), CH_2(β)(Asp_b)); 2.50–2.45 (*m*, H_b–C(2′)). ^{13}C-NMR (125 MHz, D_2O): 173.8; 171.2; 170.2; 170.0; 158.1; 155.5; 152.1; 148.7; 139.3; 135.6; 135.5; 135.2; 127.4–127.8 (15C); 118.6; 86.2; 83.6; 71.2; 66.6; 66.0; 65.6; 64.0; 52.0; 48.6; 39.6; 37.2; 35.2. ^{31}P-NMR (121 MHz, D_2O): 5.70. HR-ESI-MS: 830.2591 ($[M-H]^-$, $C_{39}H_{4O}N_7O_{12}P^-$; calc. 830.2556).

This intermediate was dissolved in MeOH (5 ml) and reduced under H_2 for 24 h, in the presence of 10% Pd/C (20 mg). The mixture was filtered over *Celite* and concentrated *in vacuo*, and the resulting crude material was purified by FC ($CHCl_3$/MeOH/H_2O 5 : 2 : 0.5, 5 : 2 : 1, 5 : 3 : 0.5) to provide **1a** (64 mg, 85%). White solid. ^{1}H-NMR (600 MHz, D_2O): 8.45 (*s*, H–C(8)); 8.22 (*s*, H–C(2)); 6.49 (*t*, $J=6.6$, H–C(1′)); 4.68–4.64 (*m*, H–C(3′)); 4.34–4.31 (*m*, H–C(4′)); 4.24–4.22 (*m*, H–C(α)(Asp_a)); 3.99–3.90 (*m*, CH_2(5′)); 3.81–3.77 (*m*, H–C(α)(Asp_b)); 2.83–2.78 (*m*, 1 H–C(2′)); 2.63–2.44 (*m*, 1 H–C(2′), CH_2(β)(Asp_a), CH_2(β)(Asp_b)). ^{13}C-NMR (150 MHz, D_2O): 178.5; 178.3; 178.1; 175.7; 155.5; 152.6; 148.7; 139.9; 118.6; 86.1; 83.6; 71.4; 64.2; 53.1; 52.7; 40.3; 39.4; 38.9. ^{31}P-NMR (121 MHz, D_2O): 6.28. HR-ESI-MS: 560.1154 ($[M-H]^-$, $C_{18}H_{22}N_7O_{12}P^-$; calc. 560.1148).

*5′-O-[({(1*S*)-1-(Carboxymethyl)-2-[(carboxymethyl)amino]-2-oxoethyl}amino)hydroxyphosphinyl]-2′-deoxyadenosine* (**1b**). *GP III* was applied using dAMP (100 mg, 0.30 mmol), **3b** (555 mg, 1.5 mmol), and DCC (433 mg, 2.10 mmol). After purification, the desired intermediate was obtained as white solid. Yield: 76%. ^{1}H-NMR (600 MHz, D_2O): 8.33 (*s*, H–C(8)); 8.09 (*s*, H–C(2)); 7.33–7.12 (*m*, 2 Ph); 6.35 (*t*, $J=7.2$, H–C(1′)); 5.08 (*s*, PhCH_2); 4.88 (*s*, PhCH_2); 4.64–4.63 (*m*, H–C(3′)); 4.18–4.17 (*m*, H–C(4′), NH*CH*CH_2CO_2Bn); 3.95–3.94 (*m*, CH_2(5′)); 3.88 (*d*, $J=2.4$, NHCH_2CO_2Bn); 2.67–2.64 (*m*, 1 H–C(2′), NHCHCH_2CO_2Bn); 2.63–2.61 (*m*, 1 H–C(2′)). ^{13}C-NMR (150 MHz, D_2O): 175.3; 172.2; 170.9; 154.8; 151.8; 148.3; 139.7; 135.1; 135.1; 127.5–128.7 (10C); 118.5; 85.8; 83.7; 70.9; 67.4; 63.9; 52.1; 46.7; 41.4; 39.1; 37.9. ^{31}P-NMR (121 MHz, D_2O): 7.81. HR-ESI-MS: 682.2038 ($[M-H]^-$, $C_{30}H_{29}N_7O_9P^-$; calc. 682.2032).

The intermediate was dissolved in MeOH (5 ml) and reduced under H_2 for 24 h, in the presence of 10% Pd/C (22 mg). The mixture was filtered on *Celite*, and concentrated *in vacuo*, the resulting crude material was purified by FC ($CHCl_3$/MeOH/H_2O 5 : 2 : 0.5, 5 : 2 : 1, 5 : 3 : 0.5) to provide **1b**. Yield: 88%. White solid. ^{1}H-NMR (600 MHz, D_2O): 8.45 (*s*, H–C(8)); 8.24 (*s*, H–C(2)); 6.49 (*t*, $J=8.4$, H–C(1′));

4.70–4.68 (*m*, H–C(3′)); 4.27–4.24 (*m*, H–C(4′)); 4.02–3.91 (*m*, CH_2(5′)); 3.80–3.75 (*m*, NHC*H*CH_2CO_2H); 2.85–2.79 (*m*, 1 H–C(2′)); 2.61–2.45 (*m*, 1 H–C(2′), NHCHCH_2CO_2H). ^{13}C-NMR (150 MHz, D_2O): 178.6; 176.4; 175.9; 155.6; 152.8; 148.8; 139.9; 118.7; 86.1; 83.8; 71.5; 64.2; 53.2; 43.4; 40.6; 39.0. ^{31}P-NMR (121 MHz, D_2O): 6.21. HR-ESI-MS: 502.1074 ($[M-H]^-$, $C_{16}H_{21}N_7O_{10}P^-$; calc. 502.1093).

*5′-O-{[((1*S*)-2-{[(1*S*)-1-Carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-1-(carboxymethyl)-2-oxoethyl)-amino]hydroxyphosphinyl}-2′-deoxyadenosine* (**1c**). *GP III* was applied using dAMP (100 mg, 0.30 mmol), **3c** (447 mg, 1.5 mmol), and DCC (433 mg, 2.10 mmol). After purification, the desired intermediate was obtained as white solid. The resulting crude product was treated with 0.4M NaOH soln. in MeOH/H_2O 1:4 (5 ml) for 3 h, the solvent was removed *in vacuo*, and the crude product was purified by FC ($CHCl_3$/MeOH/H_2O 5:2:0.5, 5:2:1, 5:3:0.5) to provide **1c**. Yield: 22% (over two steps). White solid. ^{1}H-NMR (600 MHz, D_2O): 8.41 (*s*, H–C(8)); 8.14 (*s*, H–C(2)); 7.51 (*s*, NHC*H*=N); 6.73 (*s*, NHC*H*=C); 6.41 (*t*, *J* = 6.7, H–C(1′)); 4.60–4.58 (*m*, H–C(3′)); 4.31–4.27 (*m*, H–C(*α*)(His)); 4.17–4.15 (*m*, H–C(4′)); 3.90–3.82 (*m*, CH_2(5′)); 3.75–3.67 (*m*, H–C(*α*)(Asp)); 2.97–2.86 (*m*, CH_2(*β*)(His)); 2.83–2.68 (*m*, H_a–C(2′)); 2.52–2.42 (*m*, CH_2(*β*)(Asp), H_b–C(2′)). ^{13}C-NMR (150 MHz, D_2O): 179.3; 172.6; 170.7; 155.2; 153.5; 146.8; 139.4; 136.5; 134.9; 119.2; 118.6; 89.4; 84.3; 72.6; 65.2; 56.1; 43.2; 40.9; 39.8; 32.4. ^{31}P-NMR (121 MHz, D_2O): 6.61. HR-ESI-MS: 582.1471 ($[M-H]^-$, $C_{19}H_{25}N_9O_{10}P^-$; calc. 582.1467).

*2′-Deoxy-5′-O-{[((1*S*)-2-{[(1*S*)-1,2-dicarboxyethyl]amino}-1-(1*H*-imidazol-4-ylmethyl)-2-oxoethyl)amino]hydroxyphosphinyl}adenosine* (**1d**). *GP III* was applied using dAMP (100 mg, 0.30 mmol), **3d** (447 mg, 1.5 mmol), and DCC (433 mg, 2.10 mmol). After purification, the desired intermediate was obtained as white solid, and the crude product was then treated with 0.4M NaOH soln. in MeOH/H_2O 1:4 (5 ml) for 3 h. The solvent was removed *in vacuo*, and the resulting crude product was purified by FC ($CHCl_3$/MeOH/H_2O 5:2:0.5, 5:2:1, 5:3:0.5) to provide **1d**. Yield: 30% (over two steps). White solid. ^{1}H-NMR (600 MHz, D_2O): 8.43 (*s*, H–C(8)); 8.13 (*s*, H–C(2)); 7.45 (*s*, NHC*H*=N); 6.70 (*s*, NHC*H*=C); 6.41 (*t*, *J* = 7.1, H–C(1′)); 4.58–4.56 (*m*, H–C(3′)); 4.28–4.26 (*m*, NHC*H*CO_2H); 4.15–4.13 (*m*, H–C(4′)); 3.77–3.71 (*m*, CH_2(5′), NHC*H*CH_2Im); 2.50–2.46 (*m*, 1 H–C(2′), NHCHCH_2CO_2H); 2.61–2.59 (*m*, 1 H–C(2′), NHCHCH_2Im). ^{13}C-NMR (150 MHz, D_2O): 177.4; 176.9; 170.6; 155.3; 152.1; 148.9; 140.3; 139.6; 132.0; 118.8; 116.7; 89.8; 87.4; 70.2; 63.0; 52.2; 51.8; 38.4; 34.9; 32.1. ^{31}P-NMR (121 MHz, D_2O): 6.02. HR-ESI-MS: 582.1475 ($[M-H]^-$, $C_{20}H_{25}N_9O_{10}P^-$; calc. 582.1467).

*2′-Deoxy-5′-O-{[(2-{[(1*S*)-1,2-dicarboxyethyl]amino}-2-oxoethyl)amino]hydroxyphosphinyl}adenosine* (**1e**). *GP III* was applied using dAMP (100 mg, 0.30 mmol), **3e** (555 mg, 1.5 mmol), and DCC (433 mg, 2.10 mmol). After purification, the desired intermediate was dissolved in MeOH (10 ml) and reduced under H_2 for 24 h in the presence of 10% Pd/C (22 mg). The mixture was filtered on *Celite*, and concentrated *in vacuo*, and the resulting crude material was purified by FC ($CHCl_3$/MeOH/H_2O 5:2:0.5, 5:2:1, 5:3:0.5) to provide **1e**. Yield: 49% (over two steps). White solid. ^{1}H-NMR (600 MHz, D_2O): 8.43 (*s*, H–C(8)); 8.22 (*s*, H–C(2)); 6.47 (*t*, *J* = 6.6, H–C(1′)); 4.67–4.66 (*m*, H–C(3′)); 4.33–4.31 (*m*, NHC*H*CO_2H); 4.23–4.22 (*m*, H–C(4′)); 3.97–3.95 (*m*, CH_2(5′)); 3.42–3.39 (*m*, NHCH_2CONH); 2.83–2.78 (*m*, 1 H–C(2′)); 2.64–2.53 (*m*, 1 H–C(2′), NHCHCH_2CO_2H). ^{13}C-NMR (150 MHz, D_2O): 178.8; 178.2; 173.4; 155.6; 152.7; 148.8; 139.8; 118.6; 85.9; 83.6; 71.2; 64.2; 52.5; 44.4; 39.5; 38.9. ^{31}P-NMR (121 MHz, D_2O): 7.81. HR-ESI-MS: 502.1090 ($[M-H]^-$, $C_{16}H_{20}N_7O_{10}P^-$; calc. 502.1093).

N-*Benzyl*-N-*[2-(*tert-*butoxy)-2-oxyethyl]glycine Ethyl Ester* (**4**). To a soln. of *N*-benzylglycine ethyl ester (2.06 g, 10.7 mmol) and EtN^iPr_2 (3.5 ml, 21.3 mmol) in anh. MeCN (10 ml), *tert*-butyl 2-bromoacetate (1.55 ml, 10.7 mmol) was added dropwise. The mixture was stirred for 30 min at r.t. and refluxed for 4 h, the solvent was then removed *in vacuo*. The residue was diluted with AcOEt (10 ml), washed with 10% $NaHCO_3$ soln. (2 × 10 ml), and the org. layer was dried (Na_2SO_4), and concentrated *in vacuo*. The resulting crude material was purified by FC (hexane/AcOEt 5:1) to provide **4**. Yield: 87%. Yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.42–7.27 (*m*, Ph); 4.18 (*q*, *J* = 7.2, 6 H, OCH_2Me); 3.93 (*s*, NCH_2Ph); 3.55 (*s*, NCH_2CO_2Et); 3.45 (*s*, NC$H_2CO_2{}^t$Bu); 1.49 (*s*, ^{t}Bu); 1.29 (*t*, *J* = 7.2, OCH_2*Me*). ^{13}C-NMR (125 MHz, $CDCl_3$): 171.3; 170.5; 138.4; 129.0; 128.3; 127.2; 81.0; 60.3; 57.7; 55.1; 54.3; 28.2; 14.2. HR-ESI-MS: 308.1837 ($[M+H]^+$, $C_{17}H_{26}NO_4^+$; calc. 308.3926).

2-[Benzyl(2-ethoxy-2-oxoethyl)amino]acetic Acid Trifluoroacetate (**5**). Ester **4** (2.84 g, 9.2 mmol) was treated by TFA (6.8 ml, 92.3 mmol) in CH_2Cl_2 (60 ml) for 2 d at r.t. After removal of solvent and

excess of TFA, the oily crude residue was triturated with Et_2O and filtered to afford **5** as a TFA salt. Yield: 78%. White solid. ^{1}H-NMR (300 MHz, MeOD): 7.52–7.44 (*m*, Ph); 4.42 (*s*, NCH_2Ph); 4.23 (*q*, *J* = 7.1, OCH_2Me); 4.07 (*s*, NCH_2CO$_2$Et); 4.02 (*s*, NCH_2CO$_2$H); 1.27 (*t*, *J* = 7.1, OCH$_2$*Me*). ^{13}C-NMR (75 MHz, MeOD): 170.6; 168.5; 139.8; 132.3; 131.0; 130.3; 63.4; 60.3; 54.9; 54.6; 14.3. HR-ESI-MS: 252.1229 ([*M* + H]$^+$, $C_{13}H_{18}NO_4^+$; calc. 252.1236).

Methyl N-*Benzyl*-N-*(2-ethoxy-2-oxoethyl)glycyl*-L-*histidinate* (**6a**). *General Procedure IV* (*GP IV*). A soln. of **5** (TFA salt; 500.0 mg, 1.4 mmol), L-histidine methyl ester dihydrochloride (331.4 mg, 1.4 mmol), and Et_3N (630 µl, 4.5 mmol) in CH_2Cl_2 (5 ml) was stirred at r.t. for 30 min. The mixture was cooled to 0°, and HOBt (278.5 mg, 2.1 mmol) and EDCI (393.6 mg, 2.1 mmol) were added. The soln. was stirred for 24 h at r.t. After removal of the solvent, the residue was diluted with AcOEt (20 ml), washed with sat. $NaHCO_3$ soln. (2 × 10 ml) and brine (10 ml), dried ($MgSO_4$), and concentrated *in vacuo*. The resulting crude material was purified by FC (CH_2Cl_2/MeOH 10 : 1) to provide **6a** (431.0 mg, 75%). White solid. ^{1}H-NMR (300 MHz, $CDCl_3$): 9.77 (br. *s*, NH); 8.39 (*d*, NH); 7.49 (*s*, NHC*H*=N); 7.26–7.18 (*m*, Ph); 6.78 (*s*, NHC*H*=C); 4.85–4.78 (*m*, NHC*H*CO$_2$Me); 4.10 (*q*, *J* = 7.1, OCH_2Me); 3.82–3.69 (*m*, NCH_2CONH); 3.61 (*s*, CO$_2$*Me*); 3.31 (*s*, NCH_2Ph, NCH_2CO$_2$Et); 3.14–3.11 (*m*, ImCH_2); 1.19 (*t*, OCH$_2$*Me*). ^{13}C-NMR (75 MHz, $CDCl_3$): 171.5; 170.9; 170.8; 137.1; 135.1; 132.9; 128.9; 128.2; 127.4; 116.7; 60.4; 58.6; 57.5; 54.1; 52.0; 51.9; 29.2; 13.9. HR-ESI-MS: 403.1981 ([*M* + H]$^+$, $C_{20}H_{19}N_4O_5^+$; calc. 403.1981).

Methyl N-*Benzyl*-N-*(2-ethoxy-2-oxoethyl)glycylglycinate* (**6b**). *GP I* was applied using glycine methyl ester (279 mg, 2.0 mmol), **5** (502 mg, 2.0 mmol), NMM (205 µl, 2.0 mmol), DCC (619 mg, 3.0 mmol), and HOSu (232 mg, 2.0 mmol). Purification was carried out according to the method used for **2a**. Yield: 45%. White solid. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.25–7.40 (*m*, Ph); 4.17–4.12 (*m*, 2 H, OCH_2Me); 3.90 (*d*, *J* = 12.4, CH$_2$(Gly)); 3.72 (*s*, MeO); 3.32–3.49 (*m*, 2 NCH_2CO); 1.29–1.22 (*t*, *J* = 7.4, OCH$_2$*Me*). ^{13}C-NMR (75 MHz, $CDCl_3$): 170.5; 169.0; 166.2; 137.4; 129.0; 128.3; 127.5; 60.4; 58.9; 57.4; 53.8; 51.7; 40.6; 14.0. HR-ESI-MS: 323.1601 ([*M* + H]$^+$, $C_{16}H_{24}N_2O_5^+$; calc. 323.1601).

Dimethyl N-*Benzyl*-N-*(2-ethoxy-2-oxoethyl)glycyl*-L-*aspartate* (**6c**). *GP I* was applied using L-aspartic acid dimethyl ester hydrochloride (395 mg, 2.0 mmol), **5** (502 mg, 2.0 mmol), NMM (205 µl, 2.0 mmol), DCC (619 mg, 3.0 mmol), and HOSu (232 mg, 2 mmol). Purification was carried out according to the method used for **2a**. Yield: 75%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.36–7.27 (*m*, Ph); 4.91–4.87 (*m*, NHC*H*CO$_2$Me); 4.20–4.15 (*m*, 2 H, OCH_2Me); 3.85–3.77 (*m*, PhCH_2); 3.74 (*s*, MeO); 3.69 (*s*, MeO); 3.39–3.35 (*m*, 2 NCH_2CON); 3.06–3.02 (*m*, 1 H, NHCHCH_2CO$_2$Me); 2.84–2.79 (*m*, 1 H, NHCHCH_2CO$_2$Me); 1.28 (*t*, *J* = 8.4, OCH$_2$*Me*). ^{13}C-NMR (75 MHz, $CDCl_3$): 171.2; 171.1; 170.8 (2C); 137.4; 129.1; 128.6; 127.7; 60.7; 58.8; 57.6; 54.6; 52.7; 52.0; 48.0; 36.2; 14.2. HR-ESI-MS: 395.1810 ([*M* + H]$^+$, $C_{19}H_{27}N_2O_7^+$; calc. 395.1818).

Methyl N-*(2-Ethoxy-2-oxoethyl)glycylglycinate* (**7b**). *General Procedure V* (*GP V*). Compound **6b** (322.0 mg, 1.0 mmol) was dissolved in MeOH (5 ml) and reduced under H_2 for 24 h in the presence of 10% Pd/C (37 mg). The mixture was filtered on *Celite* and concentrated *in vacuo* to give **7b**. Yield: 280.0 mg, 100%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 4.23–4.16 (*m*, OCH_2Me); 4.07 (*d*, *J* = 5.7, NHCH_2CO$_2$Me); 3.76 (*s*, MeO); 3.47 (*s*, CH_2CO$_2$Et); 3.42 (*s*, NHCH_2CON); 1.28 (*d*, *J* = 7.1, OCH$_2$*Me*). ^{13}C-NMR (75 MHz, $CDCl_3$): 171.8; 171.6; 170.3; 61.2; 52.4; 51.9; 50.6; 40.8; 14.2. HR-ESI-MS: 233.1138 ([*M* + H]$^+$, $C_9H_{18}N_2O_5^+$; calc. 233.1132).

Dimethyl N-*(2-Ethoxy-2-oxoethyl)glycyl*-L-*aspartate* (**7c**). *GP V* was applied using **6c** (788.0 mg, 2.0 mmol) and 10% Pd/C (37 mg) under H_2 to afford **7c**. Yield: 95%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 4.93–4.87 (*m*, NHC*H*CO$_2$Me); 4.23–4.16 (*m*, PhCH_2); 3.77 (*s*, MeO); 3.70 (*s*, MeO); 3.42 (*s*, CH_2CO$_2$Et); 3.36 (*s*, NHCH_2CON); 3.08–2.82 (*m*, NHCHCH_2CO$_2$Me); 1.28 (*t*, *J* = 7.1, OCH$_2$*Me*). ^{13}C-NMR (75 MHz, $CDCl_3$): 172.1; 171.3; 171.2; 171.2; 61.1; 52.8; 52.2; 52.1; 50.7; 48.1; 36.3; 14.1. HR-ESI-MS: 305.1455 ([*M* + H]$^+$, $C_{12}H_{21}N_2O_7^+$; calc. 305.1349).

*5′-*O*-{[(2-{[(1*S*)-1-Carboxy-2-(1*H*-imidazol-4-yl)ethyl]amino}-2-oxoethyl)(carboxymethyl)amino]-hydroxyphosphinyl}-2′-deoxyadenosine* (**1f**). *GP V* was applied using **6a** (431.0 mg, 1.1 mmol) and 10% Pd/C (37 mg) under H_2 to furnish **7a** (358 mg) as colorless oil. dAMP (50 mg, 0.15 mmol), **7a** (200 mg, 0.75 mmol), and DCC (156 mg, 0.75 mmol) were dissolved in a mixture ^{t}BuOH (3 ml)/H_2O (1 ml). The mixture was heated at 85° for 6 h under Ar. After completion, the mixture was cooled, and solvent was removed *in vacuo*. The resulting crude material was purified by FC (^{i}PrOH/H_2O/NH_3 7 : 0.5 : 0.5, 7 : 1 : 1,

7:1:2), the obtained product was treated with 0.4M NaOH soln. in MeOH/H_2O 1:4 (3 ml) for 4 h, the solvent was removed *in vacuo*, and the resulting crude material was purified by HPLC to give **1f**. Yield: 22% (over two steps). White solid. ^{1}H-NMR (500 MHz, D_2O): 8.40 (*s*, H–C(8)); 8.11 (*s*, H–C(2)); 7.50 (*s*, NHC*H*=C); 6.66 (*s*, NHC*H*=N); 6.41 (*t*, *J*=5.8, H–C(1′)); 4.58–4.56 (*m*, H–C(3′)); 4.28–4.26 (*m*, CONHC*H*); 4.15–4.14 (*m*, H–C(4′)); 4.01–3.94 (*m*, CH_2(5′)); 3.70–3.56 (*m*, NC$H_2$$CO_2$H); 3.41–3.13 (*m*, NC$H_2$CONH); 2.97–2.82 (*m*, CHCH_2Im) 2.55–2.48 (*m*, CH_2(2′)). ^{13}C-NMR (75 MHz, D_2O): 178.2; 171.6; 173.1; 155.2; 152.2; 148.4; 139.5; 135.3; 135.2; 118.2; 118.1; 85.8; 83.2; 71.1; 64.3; 54.8; 51.7; 51.1; 38.3; 29.9. ^{31}P-NMR (121 MHz, D_2O): 7.98. HR-ESI-MS: 582.1469 ([*M* – H]$^-$, $C_{20}H_{25}N_9O_{10}P^-$; calc. 582.1467).

5′-O-[((Carboxymethyl){2-[(carboxymethyl)amino]-2-oxoethyl}amino)hydroxyphosphinyl]-2′-deoxyadenosine (**1g**). *GP III* was applied using dAMP (100 mg, 0.30 mmol), **7b** (348 mg, 1.5 mmol), and DCC (433 mg, 2.10 mmol). Purification gave the desired intermediate as a white solid. Yield: 33%. ^{1}H-NMR (600 MHz, D_2O): 8.40 (*s*, H–C(8)); 8.17 (*s*, H–C(2)); 6.42 (*t*, *J*=7.8, H–C(1′)); 4.63–4.62 (*m*, H–C(3′)); 4.24–4.23 (*m*, H–C(4′)); 4.09–3.99 (*m*, CH_2(5′), OCH_2Me); 3.93 (*s*, NHC$H_2$$CO_2$H); 3.69 (*s*, MeO); 3.68–3.67 (*m*, NC$H_2$$CO_2Et, NCH_2$CON); 2.88–2.82 (*m*, 1 H–C(2′)); 2.64–2.59 (*m*, 1 H–C(2′)); 1.14 (*t*, *J*=9.0, OCH_2*Me*). ^{13}C-NMR (150 MHz, D_2O): 174.3; 173.4; 172.8; 172.2; 155.5; 152.6; 148.7; 139.8; 118.5; 85.9; 83.5; 71.2; 64.4; 62.0; 52.7; 50.2; 48.8; 38.7; 35.2; 13.2. ^{31}P-NMR (121 MHz, D_2O): 6.89. HR-ESI-MS: 544.1545 ([*M* – H]$^-$, $C_{19}H_{26}N_7O_{10}P^-$; calc. 544.1562).

This intermediate was then treated with 0.4M NaOH soln. in MeOH/H_2O 1:4 (5 ml) for 3 h, the solvent was removed *in vacuo*, and the resulting residue was purified by FC (^{i}PrOH/H_2O/NH_3 7:0.5:0.5, 7:1:1, 7:1:2) to provide **1g**. Yield: 44%. White solid. ^{1}H-NMR (600 MHz, D_2O): 8.49 (*s*, H–C(8)); 8.24 (*s*, H–C(2)); 6.49 (*t*, *J*=7.8, H–C(1′)); 4.70–4.67 (*m*, H–C(3′)); 4.24–4.22 (*m*, H–C(4′)); 4.10–4.02 (*m*, CH_2(5′)); 3.75 (*d*, *J*=9.6, NC$H_2$$CO_2$H); 3.73 (*s*, NHC$H_2$$CO_2$H); 3.59 (*d*, *J*=13.2, NCH_2CON); 2.85–2.80 (*m*, 1 H–C(2′)); 2.61–2.59 (*m*, 1 H–C(2′)). ^{13}C-NMR (150 MHz, D_2O): 178.8; 176.5; 174.2; 155.6; 152.7; 148.8; 134.0; 118.7; 86.1; 83.7; 71.3; 64.3; 52.4; 52.2; 43.1; 39.0. ^{31}P-NMR (121 MHz, D_2O): 8.12. HR-ESI-MS: 502.1079 ([*M* – H]$^-$, $C_{16}H_{21}N_7O_{10}P^-$; calc. 502.1088).

5′-O-{[(Carboxymethyl)(2-{[(1S)-1,2-dicarboxyethyl]amino}-2-oxoethyl)amino]hydroxyphosphinyl}-2′-deoxyadenosine (**1h**). *GP III* was applied using dAMP (100 mg, 0.30 mmol), **7c** (456 mg, 1.5 mmol), and DCC (433 mg, 2.10 mmol). After purification, the desired intermediate as a obtained as a white solid. Yield: 59%. ^{1}H-NMR (600 MHz, D_2O): 8.40 (*s*, H–C(8)); 8.19 (*s*, H–C(2)); 6.43 (*t*, *J*=6.6, H–C(1′)); 4.69–4.67 (*m*, H–C(3′), NHC*H*CO_2Me); 4.19–4.18 (*m*, H–C(4′)); 4.06–3.97 (*m*, CH_2(5′), OCH_2Me); 3.68 (*s*, MeO); 3.64 (*s*, MeO); 2.87–2.82 (*m*, NHC$H_2$$CO_2$Me, 1 H–C(2′)); 2.60–2.56 (*m*, 1 H–C(2′)); 1.14 (*t*, *J*=7.2, OCH_2*Me*). ^{13}C-NMR (150 MHz, D_2O): 173.4; 173.4; 172.8; 172.2; 155.5; 152.6; 148.7; 139.8; 118.6; 85.9; 83.5; 71.2; 64.4; 62.0; 53.1; 52.5; 52.0; 50.3; 48.8; 38.7; 35.2; 13.2. ^{31}P-NMR (121 MHz, D_2O): 7.14. HR-ESI-MS: 544.1545 ([*M* – H]$^-$, $C_{19}H_{26}N_7O_{10}P^-$; calc. 544.1562).

This intermediate was then treated with 0.4M NaOH soln. in MeOH/H_2O 1:4 (5 ml) for 3 h, the solvent was removed *in vacuo*, and the resulting residue was purified by FC (^{i}PrOH/H_2O/NH_3 7:0.5:0.5, 7:1:1, 7:1:2) to provide **1h**. Yield: 38%. White solid. ^{1}H-NMR (600 MHz, D_2O): 8.47 (*s*, H–C(8)); 8.17 (*s*, H–C(2)); 6.44 (*t*, *J*=7.8, H–C(1′)); 4.67–4.65 (*m*, H–C(3′)); 4.43–4.40 (*m*, H–C(*α*)(Asp)); 4.23–4.22 (*m*, H–C(4′)); 4.08–3.97 (*m*, CH_2(5′)); 3.82–3.72 (*m*, NC$H_2$$CO_2$H); 3.70–3.47 (*dd*, *J*=11.5, *J*=17.9, NCH_2CONH); 2.81–2.76 (*m*, 1 H–C(2′)); 2.69–2.65 (*m*, 1 H, CH_2(*β*)(Asp_a)); 2.57–2.50 (*m*, 1 H–C(2′), CH_2(*β*)(Asp_b), 1 H of CH_2(*β*)(Asp_a)). ^{13}C-NMR (150 MHz, D_2O): 178.2; 178.1; 178.0; 173.1; 155.1; 152.3; 148.3; 139.6; 118.1; 85.8; 83.2; 71.0; 64.1; 52.9; 51.5; 51.0; 39.4; 38.5. ^{31}P-NMR (121 MHz, D_2O): 8.05. HR-ESI-MS: 560.1153 ([*M* – H]$^-$, $C_{19}H_{24}N_7O_{10}P^-$; calc. 560.1142).

Methyl 3-(benzylamino)propanoate (**8**). A soln. of $BnNH_2$ (1.0 g, 9.33 mmol) in MeOH (1 ml) was stirred at r.t. for 1 d, in the presence of methyl acrylate (756 µl, 8.40 mmol). The mixture was then concentrated *in vacuo*, the resulting crude material was purified by FC (hexane/AcOEt 5:1) to furnish **8**. Yield: 93%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.34–7.26 (*m*, Ph); 3.82 (*s*, NHCH_2Ph); 3.70 (*s*, MeO); 3.92 (*t*, *J*=6.5, NHC$H_2$$CH_2$$CO_2$Me); 2.55 (*t*, *J*=6.5, NHCH_2C$H_2$$CO_2$Me). ^{13}C-NMR (75 MHz, $CDCl_3$): 173.1; 140.0; 128.4; 128.1; 127.0; 53.7; 51.6; 44.4; 34.5. HR-ESI-MS: 194.1190 ([*M*+H]$^+$, $C_{11}H_{16}NO_2^+$; calc. 194.1181).

tert-*Butyl Methyl 3,3′-(Benzylimino)dipropanoate* (**9**). A soln. of **8** (1.67 g, 8.65 mmol) in MeOH (1 ml) was stirred at r.t. for 1 d in the presence of *tert*-butyl acrylate (1.40 ml, 9.52 mmol). The mixture

was then concentrated *in vacuo*, and the resulting crude material was purified by FC (hexane/AcOEt 5:1) to give **9**. Yield: 66%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.27–7.22 (*m*, Ph); 3.61 (*s*, MeO); 3.56 (*s*, NCH_2Ph); 2.79–2.73 (*m*, NCH_2CH$_2$CO$_2$Me, NCH_2CH$_2$CO$_2$^{t}Bu); 2.44 (*t*, *J* = 7.2, NCH$_2$CH_2-CO$_2$Me); 2.36 (*t*, *J* = 7.2, NCH$_2$CH_2CO$_2$^{t}Bu); 1.41 (*s*, ^{t}Bu). ^{13}C-NMR (75 MHz, $CDCl_3$): 172.9; 171.8; 139.1; 128.7; 128.1; 126.9; 80.2; 58.3; 51.4; 49.4; 49.1; 33.8; 32.5; 28.0. HR-ESI-MS: 322.2016 ([*M*+H]$^+$, $C_{18}H_{28}NO_4^+$; calc. 322.4192).

N-*Benzyl*-N-*(3-methoxy-3-oxopropyl)-β-alanine Trifluoroacetate* (**10**). Diester **9** (1.83 g, 5.69 mmol) was treated with TFA (4.23 ml, 56.9 mmol) in CH_2Cl_2 (40ml) for 2 d at r.t. After removal of the solvent and excess of TFA, the crude residue was triturated with Et_2O and filtered to afford **10** as TFA salt. Yield: 97%. White solid. ^{1}H-NMR (300 MHz, (D_6)DMSO): 7.55–7.46 (*m*, Ph); 4.40 (*s*, NCH_2Ph); 3.62 (*s*, MeO); 3.35–3.27 (*m*, NCH_2CH$_2$CO$_2$Me, NCH_2CH$_2$CO$_2$H); 2.89 (*t*, *J* = 7.4, NCH$_2$CH_2CO$_2$Me); 2.79 (*t*, *J* = 7.4, NCH$_2$CH_2CO$_2$H). ^{13}C-NMR (75 MHz, (D_6)DMSO): 171.5; 170.4; 131.2; 129.8; 129.7; 129.0; 56.6; 51.9; 47.7; 47.4; 28.2; 28.0. HR-ESI-MS: 266.1386 ([*M*+H]$^+$, $C_{14}H_{20}NO_4^+$; calc. 266.3129).

Methyl N-*Benzyl*-N-*(3-methoxy-3-oxopropyl)-β-alanylglycinate* (**11a**). *GP IV* was applied using **10** (TFA salt) (500.0 mg, 1.32 mmol), glycine methyl ester hydrochloride (166.0 mg, 1.32 mmol), Et_3N (550 µl, 3.95 mmol), HOBt (267.1 mg, 1.97 mmol), and EDCI (379.0 mg, 1.97 mmol). After purification, **11a** was obtained. Yield: 57%. White solid. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.08 (br. *s*, NH); 7.38–7.30 (*m*, Ph); 3.95 (*d*, *J* = 5.4, NHCH_2CO$_2$Me); 3.70 (*s*, NCH$_2$CH$_2$CO$_2$*Me*); 3.57 (*s*, NHCH$_2$CO$_2$*Me*); 3.55 (*s*, NCH_2Ph); 2.80 (*t*, *J* = 6.9, NCH$_2$CH_2CONH); 2.73 (*t*, *J* = 6.2, NCH$_2$CH_2CO$_2$Me); 2.50 (*t*, *J* = 6.9, NCH_2CH$_2$CONH); 2.40 (*t*, *J* = 6.2, NCH_2CH$_2$CO$_2$Me). ^{13}C-NMR (75 MHz, $CDCl_3$): 172.6; 172.5; 170.2; 137.6; 129.0; 128.2; 127.1; 66.0; 51.8; 51.4; 49.8; 48.9; 40.7; 32.8; 31.8. HR-ESI-MS: 337.1763 ([*M*+H]$^+$, $C_{17}H_{25}N_2O_5^+$; calc. 337.3908).

Dimethyl N-*Benzyl*-N-*(3-methoxy-3-oxopropyl)-β-alanyl*-L-*aspartate* (**11b**). *GP IV* was applied using **10** (TFA salt) (500.0 mg, 1.32 mmol), L-aspartic acid dimethyl ester hydrochloride (260.8 mg, 1.32 mmol), Et_3N (550 µl, 3.95 mmol), HOBt (267.1 mg, 1.97 mmol) and EDCI (379.0 mg, 1.97 mmol). After purification, **11b** was obtained. Yield: 57.5%. White solid. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.20 (br. *s*, NH); 7.27–7.21 (*m*, Ph); 4.89–4.83 (*m*, NHCHCO$_2$Me); 3.70 (*s*, NHCHCO$_2$*Me*); 3.62 (*s*, NHCHCH$_2$CO$_2$*Me*); 3.60 (*s*, NCH$_2$CH$_2$CO$_2$*Me*+NCH_2Ph); 2.97–2.83 (*m*, NHCHCH_2CO$_2$Me); 2.81–2.71 (*m*, NCH$_2$CH_2CONH, NCH$_2$CH_2CO$_2$Me); 2.49 (*t*, *J* = 7.2, NCH_2CH$_2$CO$_2$Me); 2.38 (*t*, *J* = 6.0, NCH_2CH$_2$CONH). ^{13}C-NMR (75 MHz, $CDCl_3$): 172.5; 171.9; 171.0; 17O.5; 137.6; 128.8; 128.1; 127.0; 57.8; 52.3; 51.6; 51.3; 49.8; 48.5; 48.1; 36.0; 33.0; 31.3. HR-ESI-MS: 409.1969 ([*M*+H]$^+$, $C_{20}H_{29}N_2O_7^+$; calc. 409.1975).

Methyl N-*(3-Methoxy-3-oxopropyl)-β-alanylglycinate* (**12a**). *GP V* was applied using **11a** (250.0 mg, 0.74 mmol) and 10% Pd/C (26 mg) under H_2. After filtration and concentration, **12a** was obtained. Yield: 96%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.23 (br. *s*, NH); 3.98 (*s*, NHCH_2CO$_2$Me); 3.70 (*s*, NHCH$_2$CO$_2$*Me*, NHCH$_2$CH$_2$CO$_2$*Me*); 3.35–3.23 (*m*, NHCH_2CH$_2$CO$_2$Me, NHCH_2CH$_2$CONH); 3.01–2.94 (*m*, NHCH$_2$CH_2CO$_2$Me, NHCH$_2$CH_2CONH). ^{13}C-NMR (75 MHz, $CDCl_3$): 171.0; 170.9; 170.5; 52.3; 52.2; 44.5; 43.6; 40.9; 31.1; 30.2. HR-ESI-MS: 247.1285 ([*M*+H]$^+$, $C_{10}H_{19}N_2O_5^+$; calc. 247.2683).

Dimethyl N-*(3-Methoxy-3-oxopropyl)-β-alanyl*-L-*aspartate* (**12b**). *GP V* was applied using **11b** (310.0 mg, 0.76 mmol) and 10% Pd/C (26 mg) under H_2. After filtration and concentration, **12b** was obtained. Yield: 71%. Colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): 8.24 (*d*, NH); 4.82–4.76 (*m*, NHCHCO$_2$Me); 4.66 (br. *s*, NH); 3.67 (*s*, NHCHCO$_2$*Me*); 3.63 (*s*, NHCHCH$_2$CO$_2$*Me*); 3.61 (*s*, NHCH$_2$CH$_2$CO$_2$*Me*); 3.00–2.93 (*m*, NHCH_2CH$_2$CONH, NHCH_2CH$_2$CO$_2$Me); 2.92–2.74 (*m*, NHCH-CH_2CO$_2$Me); 2.64 (*t*, *J* = 6.2, NHCH$_2$CH_2CO$_2$Me); 2.56–2.52 (*m*, NHCH$_2$CH_2NCONH);^{13}C-NMR (75 MHz, $CDCl_3$): 172.2; 171.6; 171.1; 171.0; 52.5; 51.8; 51.7; 48.3; 44.7; 44.0; 35.9; 33.7; 32.6. HR-ESI-MS: 319.1501 ([*M*+H]$^+$, $C_{13}H_{23}N_2O_7^+$; calc. 319.1505).

5′-O-*[((2-Carboxyethyl)[3-[(carboxymethyl)amino]-3-oxopropyl}amino)hydroxyphosphinyl]-2′-deoxyadenosine* (**1i**). *GP III* was applied using dAMP (100 mg, 0.30 mmol), **12a** (369 mg, 1.5 mmol), and DCC (433 mg, 2.10 mmol). After purification, the obtained crude material was treated with 0.4M NaOH soln. in MeOH/H_2O (1:4) (5 ml) for 3 h. Solvent was removed *in vacuo*, and the resulting crude product was purified by FC (^{i}PrOH/H_2O/NH_3 7:0.5:0.5, 7:1:1, 7:1:2) to furnish **1i**. Yield: 28% (over two steps). White solid. ^{1}H-NMR (600 MHz, D_2O): 8.42 (*s*, H–C(8)); 8.18 (*s*, H–C(2)); 6.45 (*t*, *J* = 6.8, H–C(1′)); 4.70–4.69 (*m*, 2 H, H–C(3′)); 4.21–4.20 (*m*, H–C(4′)); 3.89–3.85 (*m*, CH$_2$(5′)); 4.64–3.58 (*m*,

NHCH_2COOH); 3.14–3.12 (*m*, NCH_2CH$_2$CONH); 3.05–3.02 (*m*, NCH_2CH$_2$COOH); 2.88–2.79 (*m*, 2 H, CH$_2$CH_2COOH, 1 H–C(2′)); 2.59–2.52 (*m*, 1 H–C(2′)); 2.34–2.56 (*m*, CH$_2$CH_2COOH); 2.24–2.17 (*m*, 1 H, NCH$_2$CH_2CONH). ^{13}C-NMR (150 MHz, D$_2$O): 178.6; 176.0; 173.7; 156.9; 152.8; 149.7; 141.3; 119.9; 85.8; 85.1; 70.6; 63.4; 42.7 (3C); 38.0; 35.5; 35.3. ^{31}P-NMR (121 MHz, D$_2$O): 8.48. HR-ESI-MS: 530.1409 ($[M-H]^-$, $C_{18}H_{25}N_7O_{10}P^-$; calc. 530.1401).

*5′-*O*-{[(2-Carboxyethyl)(3-{[(1*S*)-1,2-dicarboxyethyl]amino}-3-oxopropyl)amino]hydroxyphosphinyl}-2′-deoxyadenosine* (**1j**). *GP III* was applied using dAMP (100 mg, 0.30 mmol), **12b** (477 mg, 1.5 mmol), and DCC (433 mg, 2.10 mmol). After purification, the resulting crude product was treated with 0.4M NaOH soln. in MeOH/H$_2$O (1:4) (5 ml) for 3 h. Solvent was removed *in vacuo*, and the resulting crude product was purified by FC (^{i}PrOH/H$_2$O/NH$_3$ 7:0.5:0.5, 7:1:1, 7:1:2) to furnish **1j**. White solid. Yield: 32%. ^{1}H-NMR (600 MHz, D$_2$O): 8.43 (*s*, H–C(8)); 8.19 (*s*, H–C(2)); 6.43 (*t*, *J* = 6.6, H–C(1′)); 4.70–4.68 (*m*, H–C(3′)); 4.39–4.37 (*m*, H–C(α)(Asp)); 4.19–4.17 (*m*, H–C(4′)); 3.87–3.83 (*m*, CH$_2$(5′)); 3.06–3.00 (*m*, 4 H, NCH_2CH$_2$CO); 2.86–2.81 (*m*, 1 H–C(2′)); 2.68–2.65 (*m*, 1 H, CH$_2$(β)(Asp$_a$)); 2.58–2.53 (*m*, 2 H, CH$_2$(β)(Asp$_b$), 1 H–C(2′)); 2.31–2.29 (*m*, 4 H, NCH$_2$CH_2CO). ^{13}C-NMR (150 MHz, D$_2$O): 179.7; 178.6; 178.0; 175.0; 155.6; 150.4; 149.7; 139.9; 119.9; 85.8; 85.6; 83.6; 70.6; 63.3; 51.3; 42.8; 42.4; 38.5; 35.6; 34.8. ^{31}P-NMR (121 MHz, D$_2$O): 8.48. HR-ESI-MS: 588.1462 ($[M-H]^-$, $C_{20}H_{27}N_7O_{12}P^-$; calc. 588.1455).

Ethyl N*-[(Benzyloxy)carbonyl]glycyl-*O*-ethyl-3-oxoserinate* (**13**). *GP IV* was applied using diethyl aminomalonate hydrochloride (1.0 g, 4.72 mmol), Et$_3$N (722 µl, 5.20 mmol), *N*-[(benzyloxy)carbonyl]-glycine (988.4 mg, 4.72 mmol), HOBt (957.5 mg, 7.809 mmol), and EDCI (1.36 g, 7.09 mmol). After purification, **13** was obtained. Yield: 87%. White solid. ^{1}H-NMR (300 MHz, CDCl$_3$): 7.38–7.29 (*m*, Ph); 6.91 (br. *s*, NH); 5.36, (*s*, NH); 5.16–5.14 (*m*, PhCH_2, C*H*CO$_2$Et); 4.33–4.23 (*m*, 2 CO$_2$CH_2Me); 4.00–3.98 (*m*, NHCH_2); 1.30 (*t*, *J* = 7.0, 2 CO$_2$CH$_2$*Me*). ^{13}C-NMR (75 MHz, CDCl$_3$): 168.7; 167.3; 166.0; 139.9; 128.5; 128.2; 128.1; 67.3; 62.8; 55.1; 44.3; 14.0. HR-ESI-MS: 367.1508 ($[M+H]^+$, $C_{17}H_{23}N_2O_7^+$; calc. 367.1500).

*Ethyl Glycyl-*O*-ethyl-3-oxoserinate Hydrochloride* (**14**). Compound **13** (300 mg, 0.82 mmol) was dissolved in EtOH (5 ml) and reduced under H$_2$ for 6 h, in the presence of 10% Pd/C (29 mg) and HCl (50 µl). The mixture was then filtered on *Celite* and concentrated *in vacuo*. Compound **14** was obtained as a colorless oil without additional purification. Yield: 88%. ^{1}H-NMR (300 MHz, CDCl$_3$): 9.12 (br. *s*, NH); 5.20, (*s*, C*H*CO$_2$Et); 4.27–4.20 (*m*, 2 CO$_2$CH_2Me); 3.78 (*s*, CH_2NH$_2$); 1.29 (*t*, *J* = 7.0, 2 CO$_2$CH$_2$*Me*). ^{13}C-NMR (75 MHz, CDCl$_3$): 167.4; 167.3; 63.7; 57.9; 41.5; 14.3. HR-ESI-MS: 233.1135 ($[M+H]^+$, $C_9H_{17}N_2O_5^+$; calc. 233.1132).

*5′-*O*-[({2-[(Carboxymethyl)amino]-2-oxoethyl}amino)hydroxyphosphinyl]-2′-deoxyadenosine* (**1k**). dAMP (50 mg, 0.15 mmol), **14** (194 mg, 0.76 mmol), and DCC (156 mg, 0.76 mmol) were dissolved in a mixture ^{t}BuOH (3 ml)/H$_2$O (1 ml). The mixture was heated at 85° for 6 h under Ar. After completion, the mixture was cooled, and the solvent was removed *in vacuo*. The resulting crude material was purified by FC (^{i}PrOH/H$_2$O/NH$_3$ 7:0.5:0.5, 7:1:1, 7:1:2), the obtained product was treated with 0.4M NaOH soln. in MeOH/H$_2$O 1:4 for 4 h, solvent was removed *in vacuo*, the resulting crude material was purified by HPLC to give **1k**. Yield: 32% (over two steps). White solid. ^{1}H-NMR (500 MHz, D$_2$O): 8.39 (*s*, H–C(8)); 8.20 (*s*, H–C(2)); 6.45 (*t*, *J* = 5.8, H–C(1′)); 4.70–4.67 (*m*, H–C(3′)); 4.17–4.14 (*m*, H–C(4′)); 4.03–3.92 (*m*, CH$_2$(5′)); 3.64 (*s*, NHCH_2CO$_2$H); 3.54 (*d*, NHCH_2CONH); 2.84–2.52 (*m*, CH$_2$(2′)). ^{13}C-NMR (75 MHz, D$_2$O): 178.2; 171.6; 155.5; 152.6; 148.6; 139.7; 118.6; 85.6; 83.6; 71.2; 64.1; 44.3; 42.8; 38.6. ^{31}P-NMR (121 MHz, D$_2$O): 7.73. HR-ESI-MS: 444.1042 ($[M-H]^-$, $C_{14}H_{19}N_7O_8P^-$; calc. 444.1033).

Enzymatic Reactions. DNA Duplexes. Oligonucleotides P$_1$ and T$_1$ were purchased from *Sigma Genosys*. The concentrations were determined with a *Varian Cary-300-Bio* UV spectrophotometer. The primer P$_1$ was 5′-^{33}P-labeled with 5′-[γ^{33}P]-ATP (*Perkin-Elmer*) using T4 polynucleotide kinase (*New England Biolabs*) according to the procedure provided by the supplier. The labeled oligonucleotide was further purified using *IllustraTM Microspin TM G-25* columns (*GE Healthcare*).

DNA Polymerase Reactions. End-labeled primer was annealed to its template by combining primer and template in a molar ratio of 1:2 and heating the mixture to 75° for 10 min, followed by slowly cooling to r.t. over a period of 1.5 h. For the incorporation of **1a**–**1k**, a series of 20-µl-batch reactions were performed with the enzyme (HIV-1 RT, *Taq*, *Vent* (exo–), *Therminator* DNA polymerases). The final mixture contained 125 nM primer–template complex, reaction buffer (50 mM *Tris*·HCl, 50 mM KCl,

10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol (DTT); pH 8.3 for HIV-1 RT; 20 mM *Tris*·HCl, 10 mM KCl, 2 mM $MgSO_4$, 0.1% *Triton X-100*, pH 8.8 for thermostable polymerases), appropriate concentration of enzyme, and different concentrations of phosphoramidate building blocks (1 mM, 500 μM, 200 μM, and 100 μM). In the control reaction, a 10-μM dATP was used as reference. The mixture was incubated at 37° or 75°, resp., aliquots were quenched after 10, 20, 30, 60, and 120 min.

Polyacrylamide Gel Electrophoresis. All polymerase-reaction aliquots (2.5 μl) were quenched by addition of 10 μl of loading buffer (90% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, and 50 mM ethylenediaminetetraacetic acid (EDTA)). Samples were heated at 85° for 3 min prior to analysis by electrophoresis for 2.5 h at 2000 V on a 30 cm × 40 cm × 0.4 mm 20% (mono/bis 19 : 1) denaturing gel in the presence of a 100 mM *Tris*-borate, 2.5 mM EDTA buffer; pH 8.3. Products were visualized by phosphorimaging. The amount of radioactivity in the bands corresponding to the products of enzymatic reactions was determined by using the imaging device *Cyclone*® and the Optiquant image analysis software (*Perkin-Elmer*).

The authors thank *FWO* and K.U. Leuven (IDO, GOA) for financial support, *Jef Rozenski* for providing HR-MS, *Luc Baudemprez* for recoding the NMR spectra and *Chantal Biernaux* for editorial help.

REFERENCES

[1] P. Herdewijn, P. Marlière, *Chem. Biodiversity* **2009**, *6*, 791.
[2] V. B. Pinheiro, A. I. Taylor, C. Cozens, M. Abramov, M. Renders, S. Zhang, J. C. Chaput, J. Wengel, S.-Y. Peak-Chew, S. H. McLaughlin, P. Herdewijn, P. Holliger, *Science* **2012**, *336*, 341.
[3] O. Adelfinskaya, P. Herdewijn, *Angew. Chem., Int. Ed.* **2007**, *46*, 4356.
[4] X.-P. Song, C. Bouillon, E. Lescrinier, P. Herdewijn, *ChemBioChem* **2011**, *12*, 1868.
[5] A. Giraut, P. Herdewijn, *ChemBioChem* **2010**, *11*, 1399.
[6] A. Giraut, X.-P. Song, M. Froeyen, P. Marlière, P. Herdewijn, *Nucleic Acids Res.* **2010**, *38*, 2541.
[7] S. Yang, M. Froeyen, E. Lescrinier, P. Marlière, P. Herdewijn, *Org. Biomol. Chem.* **2011**, *9*, 111.
[8] S. Yang, C. Pannecouque, E. Lescrinier, P. Marlière, A. Giraut, P. Herdewijn, *Org. Biomol. Chem.* **2012**, *10*, 146.
[9] J. W. Payne, C. Gilvarg, *J. Biol. Chem.* **1968**, *243*, 335.
[10] N. M. Hooper, in 'Handbook of Proteolytic Enzymes' 2nd edn., Eds. A. J. Barrett, N. D. Rawlings, J. F. Woessner, Elsevier, London, 2004, p. 994.
[11] T. W. Abraham, T. I. Kalman, E. J. McIntee, C. R. Wagner, *J. Med. Chem.* **1996**, *39*, 4569.
[12] C. M. Joyce, T. A. Steitz, *Annu. Rev. Biochem.* **1994**, *63*, 777.
[13] M. S. Boosalis, J. Petruska, M. F. Goodman, *J. Biol. Chem.* **1987**, *262*, 14689.
[14] S. Creighton, L. B. Bloom, M. F. Goodman, *Methods Enzymol.* **1995**, *262*, 232.
[15] H. Huang, R. Chopra, G. L. Verdine, S. C. Harrison, *Science* **1998**, *282*, 1669.
[16] A. F. Gardner, W. E. Jack, *Nucleic Acids Res.* **1999**, *27*, 2545.
[17] A. F. Gardner, W. E. Jack, *Nucleic Acids Res.* **2002**, *30*, 605.

Received July 26, 2012