Influence of Nucleobase and Anchimeric Assistance of Carboxyl Acid Groups in the Hydrolysis of Amino Acid Nucleoside Phosphoramidates

Munmun Maiti,^[a] Servaas Michielssens,^[b] Natalia Dyubankova,^[a] Mohitosh Maiti,^[a] Eveline Lescrinier,^[a] Arnout Ceulemans,^[b] Piet Herdewijn*,^[a]

Abstract: Nucleoside phosphoramidates (NPs) are a class of nucleotide analogues that have been developed as potential antiviral/antitumoral prodrugs. Recently we have shown that some amino acid nucleoside phosphoramidates (aaNPs) can act as substrate for viral polymerase like HIV-1 RT. Here we report the synthesis and hydrolysis study of a series of new aaNPs containing either natural or modified nucleobase in order to define the basis for their differential reactivity. Aqueous stability, kinetics and

hydrolysis pathways were studied by NMR in different solution pD (5-7) and temperature. It was observed that kinetics and mechanism (P-N and/or P-O bond cleavage) of hydrolysis largely depend on the nature of nucleobase and amino acid moiety. Aspartyl NPs were found to be more reactive than Gly or β -Ala NPs. For aspartyl NPs, the order of reactivity of nucleobase was 1-deazaadenine > 7-deazaadenine > adenine > thymine $\geq$ 3-deazaadenine. Notably, neutral aqueous solution of Asp-1-deaza-dAMP degraded spontaneously even at 4 °C

via exclusive P-O bond hydrolysis (50-fold reactivity difference of Asp-1-deaza-dAMP vs. Asp-3-deaza-dAMP at pD 5, 70 °C). Conformational studies by NMR and molecular modelling suggest the involvement of protonated N3 atom of adenine and 1/7-deazaadenine in the intramolecular catalysis of the hydrolysis reaction via rare *syn* conformation.

Keywords: synthesis • nucleoside phosphoramidate • prodrugs • physico-chemical properties • NMR spectroscopy

Introduction

Over the past decades, significant progress has been made to improve the therapeutic potency of nucleoside analogues for the treatment of viral infections, which led to the development of nucleotide prodrugs.^[1] The important feature of most nucleotide

prodrugs is that the negatively charged phosphate group remains masked outside the cell, allowing permeation through the lipophilic cell membrane, and the active nucleotide is processed in the cell by chemical and/or by enzymatic degradation.^[2, 3] A suitable nucleotide prodrug also has to be stable in the extracellular medium so that it is not degraded outside the cell.

Nucleoside 5'-phosphoramidates (NPs) constitute one class of such prodrug candidate, pioneered by Jones *et al.* in the early 80's,^[4] and developed by McGuigan *et al.*^[5] Few years later Wagner *et al.* showed that also aromatic amino acid nucleoside phosphoramidates (aaNPs) with a negatively charged phosphate group could exhibit greater potency than the parent nucleoside.^[6, 7] Studies on the activation pathways of aaNP triester prodrug reveal that inside the cell, esterase first catalyses hydrolysis of the carboxylic ester linkage, this results in the cleavage of the P-O-Aryl group by an intramolecular attack of the carboxylate group on the phosphorus atom giving rise to a cyclic structure which is then subsequently hydrolysed to nucleoside 5'-phosphoramidate.^[8] Phosphoramidase enzyme is believed to be involved in the release of the nucleoside 5'-monophosphate (5'-NMP)^[7] by P-N bond cleavage, bypassing the first crucial rate limiting 5'-monophosphorylation step in the kinase pathway. 5'-NMP then undergoes consecutive 5'-di- and 5'-triphosphorylation by cellular kinases which subsequently produce

[a] Munmun Maiti, Dr. Natalia Dyubankova, Mohitosh Maiti, Prof. Dr. Eveline Lescrinier, Prof. Dr. Piet Herdewijn
Laboratory of Medicinal Chemistry
Rega Institute for Medical Research, Katholieke Universiteit Leuven
Minderbroedersstraat 10, 3000 Leuven, Belgium
Fax: (+) 32 16 337340
E-mail: Piet.Herdewijn@rega.kuleuven.be

[b] Servaas Michielssens, Prof. Dr. Arnout Ceulemans
Laboratory of Quantum Chemistry
Department of Chemistry and INPAC Institute for Nanoscale Physics and Chemistry, Katholieke Universiteit Leuven
Celestijnenlaan 200F, 3001 Heverlee, Belgium

Figure 1: Chemical structure and atom numbering of amino acid nucleoside phosphoramidates.

their therapeutic effects as a result of DNA chain termination/polymerase inhibition.^[3, 9]

A considerable challenge, however, is to develop nucleotide analogues that are direct substrates/inhibitors of viral polymerases, thereby bypassing the entire cellular kinase activation cascade. Recently we have shown that some aaNPs, in particular, L-Asp-dAMP (**1**, **Figure 1**) and L-His-dAMP can act as direct substrate for HIV-1 reverse transcriptase (HIV-1 RT).^[10] Notably it was observed that the presence of the free carboxylic acid groups of the L-Aspartic acid leaving group is essential for recognition by HIV-1 RT.^[11, 12] These carboxylic groups, however, are also a source of chemical instability of the NPs. Therefore it is important to study the mechanism of chemical degradation of NPs which will allow designing new modified NPs with improved properties. Recently the kinetics of hydrolysis of L-alanine methyl esters of NP prodrugs was reported and a mechanism of hydrolysis reaction in acidic and basic pH was proposed.^[13-15]

However, the physico-chemical properties and the structural determinants that influence the chemical stability and hydrolysis mechanism of this class of compounds are not known in detail. We have synthesised a series of aaNPs containing either natural or modified nucleobases (**Figure 1**). Aqueous stability, kinetics and reaction pathways of hydrolysis have been studied in pD 5-7 at different temperatures (25, 40, 70 °C) using NMR spectroscopy. The pK_a s, protonation sites and the solution conformation were investigated by NMR for compound **1**, **21**, **32** and **33**. Experimental and theoretical studies showed that the nucleobase as well as the amino acid moiety has a large influence on the chemical stability, kinetics and hydrolysis mechanism. These data contribute to the understanding of the determinants that govern the stability and hydrolysis kinetics of aaNPs and may assist in future design of new therapeutic analogues.

Results and Discussion

Synthesis: The synthesis of methyl ester protected aaNPs (**Figure 1**) was performed according to the method described by Wagner *et al.*^[16] starting from 5'-NMP (**Scheme 1A**). Dimethyl L-aspartic acid is used for the synthesis of aspartyl NPs. Deprotection of methyl esters of the amino acid part was carried out with 0.4 M sodium hydroxide in methanol/water solution at room temperature. An example of such a synthesis is shown in **Scheme 1A** for Asp-dAMP (**1**).

The synthesis of 1-deazaadenine analogues is shown in **Scheme 1B**. The synthesis of 2-deoxy-3,5-di-*O*-*p*-toluoyl- α -D-ribofuranosyl chloride (**10**) was carried out by a reported method^[17] in three steps starting from 2-deoxy- α/β -D-ribose (**Scheme 1B**). Compound **15** was synthesised according to a previously described method starting from commercially available 2,3-diaminopyridine (**11**) with optimizations of some reaction steps for better yield.^[18] Sodium salt glycosylation of **15** with **10** is a stereospecific reaction yielding only N9 β -isomer which is then subsequently converted to 2'-deoxy-1-deazaadenosine (**18**, purine numbering is followed, shown in **Figure S1**) by deprotection and reduction of nitro group. Compound **18** was phosphorylated^[19] at the 5'-hydroxyl group with POCl₃ to obtain **19**. Compound **19** was then converted into phosphoramidates **21-23** in two steps as shown in **Scheme 1A**.

The 7-deazaadenine (**27**) was synthesised according to the method described by Minakawa *et al.*^[20] **Scheme 1C** depicts the synthetic strategy followed to obtain L-Asp-7-deaza-dAMP (**32**). Solid-liquid phase-transfer glycosylation^[21] of **27** with **10** was performed without amino group protection using TDA-1/KOH which afforded **28** with preferred β -selectivity (57 %). Compound **28** was separated from its isomeric mixtures **28a** and **28b** by flash column chromatography. Compound **30** was converted to phosphoramidate **32** as described in **Scheme 1A**.

Synthesis of 2'-deoxy-3-deazaadenosine was followed according to the protocol described by Seela *et al.*^[22] 5'-phosphorylation, DCC assisted coupling and methyl ester deprotection were followed analogously to obtain Asp-3-deaza-dAMP (**33**) as described for

Asp-1/7-deaza analogues. Synthetic strategy is briefly shown in **Scheme 1D**.

Scheme 1: (A) Dicyclohexylcarbodiimide (DCC) assisted coupling and methyl ester deprotection; (B) Synthesis of 1-deazaadenine containing phosphoramidate analogues (**21-23**). For detailed scheme, reagents and conditions see Scheme S1 and S2 in supporting information; (C) Synthesis of Asp-7-deaza-dAMP (**32**). For detailed scheme, reagents and conditions see Scheme S3 in supporting information. (D) Synthesis of Asp-3-deaza-dAMP (**33**).

Hydrolysis kinetics: Generally all aaNPs were found to be stable from neutral to basic solution (pD 7-10, for **21** pD 8-10) in D₂O at room temperature for more than 24 hours, due to the existence of mono anionic phosphoramidate group. Since hydrolysis kinetics of aaNPs (**1**, **21** and **32**) were slow at room temperature in acidic solution (pD 5), the reaction was studied at 70 °C. Knowledge of chemical stability at high temperature is important in view of the potential use of aaNPs as substrate for thermostable polymerases and/or application in PCR.

Hydrolysis was followed by ³¹P and ¹H 1D NMR over pD 5-7 (**Figure 2A**). The reactions are expected to follow pseudo first-order kinetics with respect to phosphoramidate concentration, since D₂O was present in large excess and there was no significant change in solution pD after hydrolysis. The first-order kinetics (as reported earlier for other phosphoramidates)^[13, 23, 24] is also apparent from the exponential decay (**Figure 3: Top left**) and from the corresponding linear transformed data (**Figure 3: Top right**). Temperature and pH dependent kinetics were observed for all aaNPs i.e. faster kinetics at acidic condition (**Figure S2**) and at higher temperature (**Figure S3**).

The acid catalysed hydrolysis demonstrates first-order dependency in hydronium ion concentration [13, 23, 25] as observed by exponential pD-rate profile (**Figure 3**: Bottom left) and from the corresponding linear relationship (**Figure 3**: Bottom right). The overall first-order rate constants for the hydrolysis of aaNPs are presented in **Table 1**. Hydrolysis of **21** was also performed at 40 °C and the rate constants are provided in **Table S1**.

Figure 2: (A) Kinetic profile of the hydrolysis reaction of Asp-1-deaza-dAMP (**21**) at pD 7.0 ($T = 70\text{ }^{\circ}\text{C}$), depicted by the stack plot of ^{31}P spectra at different time intervals, showing the disappearance of the parent ^{31}P peak ($\sim 7\text{ ppm}$) and gradual increase of one ^{31}P peak ($\sim 2\text{ ppm}$). Asterisk (*) indicates the free phosphate peak; (B) ^1H - ^{31}P TOCSY spectrum during hydrolysis of **21** at pD 6.0 ($T = 70\text{ }^{\circ}\text{C}$), depicting the formation of inorganic phosphate by P-O bond cleavage. Inorganic phosphate (*) shows no correlation with any protons, whereas, phosphoramidate ^{31}P showing correlation with Ha and H β of aspartyl group along with H4', H5' and H5'' of sugar moiety.

Influence of amino acid and nucleobase on reactivity: Generally rate constants from **Table 1** indicate that aspartyl NPs containing both α and β -COOH (**1**, **21** and **4**) hydrolysed faster than Gly (with α -COOH) and β -Ala (with β -COOH) NPs (**Figure S4**). For example, hydrolysis of Asp-1-deaza-dAMP (**21**) was 28 and 47-fold faster than Gly-1-deaza-dAMP (**22**) and β -Ala-1-deaza-dAMP (**23**) respectively at pD 6. Glycyl NPs showed moderate reactivity whereas β -Ala NPs were less reactive in the series. The order of reactivity of the amino acids is Asp > Gly > β -Ala.

The aaNPs hydrolysed either by P-N or by P-O bond cleavage or both pathways simultaneously (**Figure S5**). The hydrolysis products as well as reaction pathway were identified by ^1H - ^{31}P TOCSY (**Figure 2B**). The β -Ala phosphoramidates (**3**, **6** and **23**) hydrolysed solely by P-N bond cleavage, which was independent of the nature of nucleobase (**Figure S6**). Hydrolysis of Gly NPs, however, underwent P-N and/or P-O pathways, depending on nucleobase present. In Gly-1-deaza-dAMP (**22**), P-O bond hydrolysis was dominant over P-N bond, while an opposite trend was observed in Gly-dAMP (**2**). However in the presence of thymine, Gly-TMP (**5**) hydrolysed only by P-N bond cleavage despite the presence of α -COOH group. In aspartyl NPs, for Asp-

Figure 3: Kinetic plots for the acid catalysed hydrolysis of amino acid nucleoside phosphoramidates at pD 5, $70\text{ }^{\circ}\text{C}$. Exponential decay of phosphoramidates with time (Top left) and the corresponding linear relationship (Top right, $\ln C$ vs. Time plot) in the hydrolysis reaction, indicating first-order kinetics with respect to phosphoramidate concentration. The exponential (Bottom left, first-order rate constant vs. pD) and linear (Bottom right, $\ln k$ vs. pD) pD-rate profiles for the hydrolysis of nucleoside phosphoramidates at $70\text{ }^{\circ}\text{C}$, showing first-order dependency on the hydronium ion.

TMP (**4**) and Asp-3-deaza-dAMP (**33**) cleavage of the P-N bond was dominant over P-O bond, however, for Asp-dAMP (**1**) and Asp-7-deaza-dAMP (**32**) predominant cleavage of P-O bond was detected over P-N. The most important characteristic observed for Asp-1-deaza-dAMP (**21**) was exclusive P-O bond hydrolysis (**Figure 2**).

During hydrolysis of aspartyl and glycyl NPs (except **5**), corresponding nucleoside was obtained as one of the product and its formation is not due to the hydrolysis of the nucleotide intermediate. The nucleoside was formed directly by the P-O bond cleavage from the parent phosphoramidate. This was verified by studying the stability of the respective nucleotide at the same hydrolytic condition, where no nucleoside product was formed.

A pronounced effect of the nucleobase was observed on (stability) kinetics (**Figure 3, S4**) and mechanism of hydrolysis. For aspartyl NPs, the reactivity of adenine or 1/7-deazaadenine analogues were higher than thymine or 3-deazaadenine analogues. Asp-1-deaza-dAMP (**21**: $t_{1/2} = 18\text{ min}$) hydrolysed 50-fold faster than Asp-3-deaza-dAMP (**33**: $t_{1/2} = 918\text{ min}$) at pD 5, $70\text{ }^{\circ}\text{C}$. The effect of 1-deazaadenine on reactivity was the highest, kinetic acceleration was observed even with Gly and β -Ala analogues. The general order of reactivity of nucleobases is 1-deazaadenine > 7-deazaadenine > adenine > thymine $\geq$ 3-deazaadenine. From rate constants of Asp adenine and deazaadenine conjugates, it is evident that the N3 atom of adenine base has some catalytic role since 1-deaza analogue being the most reactive while 3-deaza being the least.

Accelerated kinetics with preferential P-O bond cleavage in some of the aaNPs containing aspartyl group and adenine/deazaadenine seem to have a dual role in the intramolecular catalysis. Previously the higher reactivity of *N*-diisopropylphosphoryl aspartic acid in comparison with other

Table 1: First-order rate constants ($k \times 10^{-4} \text{ min}^{-1}$) for the hydrolysis of the amino acid nucleoside phosphoramidates at 70 °C (pD 5.0-7.0). “slow” indicates the kinetics of hydrolysis are very slow at 70 °C. P-O > P-N means P-O bond cleavage is observed more than P-N bond cleavage. Half life ($t_{1/2}$) and standard error of the mean (SEM) are provided within parentheses and as $\pm$ SEM respectively.

Compound entry	Mechanism	$k \times 10^{-4}$		
		pD 5.0	pD 6.0	pD 7.0
Asp-dAMP (1)	P-O > P-N	54.19 $\pm$ 6.65 ($t_{1/2}$ = 128 min)	16.65	6.42
Asp-1-deaza-dAMP (21)	P-O	387.76 $\pm$ 56.39 ($t_{1/2}$ = 18 min)	226.46 $\pm$ 5.50	48.40 $\pm$ 0.45
Asp-7-deaza-dAMP (32)	P-O > P-N	79.59 $\pm$ 3.79 ($t_{1/2}$ = 87 min)	35.80 $\pm$ 2.09	7.58
Asp-3-deaza-dAMP (33)	P-O < P-N	7.55 $\pm$ 1.47 ($t_{1/2}$ = 918 min)	slow	slow
Asp-TMP (4)	P-O < P-N	13.43 $\pm$ 0.2 ($t_{1/2}$ = 516 min)	3.33	1.89
Gly-dAMP (2)	P-O < P-N	13.8	slow	slow
Gly-1-deaza-dAMP (22)	P-O > P-N	62.41	8.24	slow
Gly-TMP (5)	P-N	4.93	1.23	slow
β -Ala-dAMP (3)	P-N	13.47	4.85	slow
β -Ala-1-deaza-dAMP (23)	P-N	46.00	4.85	slow
β -Ala-TMP (6)	P-N	7.96	slow	slow

Figure 4: (A) Overlay of ^1H - ^{15}N HSQC (long range) spectra of (^{15}N) Asp-dAMP (1), depicting the chemical shift perturbation (CSP) of phosphoramidate nitrogen in NMR pH-metric titration from pD 9.0 to 2.7 at 25 °C; (B) corresponding sigmoidal response of CSP; (C) Hill plot analysis of the above sigmoidal data is shown as an example; similar analysis was performed for all $\text{p}K_a$ determination; (D) Observed deshielding of the H8 proton of 1 during pH-metric titration, shown by sigmoidal plot; (E) Unusual shielding (wrong way chemical shift) of the H8 proton of Asp-1-deaza-dAMP (21) in the pH-metric titration.

N-phosphoryl amino acids containing only α -COOH or β -COOH group was reported. It was shown that the α -COOH group is involved in the nucleophilic attack on phosphorus [26, 27] as neighbouring group participation reaction (NGP) during the

hydrolysis resulting in the P-O bond cleavage.^[13] The presence of an additional β -COOH group was suggested to have an extra catalytic role via intramolecular hydrogen bonding.^[26, 28] These suggest that in aspartyl and glycyl NPs the α -COOH group is involved in the P-O bond hydrolysis. All aaNPs containing only β -

COOH group (β -Ala) undergo acid-catalysed P–N bond hydrolysis without involvement of any intramolecular catalysis due to the absence of α -carboxyl group. All these observations are consistent with our kinetic data.

pK_a and protonation sites: Determination of pK_a s and protonation sites of aspartyl adenine and 1/3/7-deazaadenine NPs (**1**, **21**, **32**, **33**) has the aim to characterise the reactive protonated tautomeric form(s) under study condition, to explain any conformational preference based on electrostatic interaction and for accurate molecular modelling which may find a rationale for the observed reactivity difference.

Nucleobase pK_a s were determined from the pH dependent chemical shift perturbation (CSP) of nucleobase protons. To obtain the pK_a values of phosphoramidate nitrogen for compounds **1** and **21**, corresponding ^{15}N labelled aspartyl NPs were synthesised. CSP of the ^{15}N atom (**Figure 4A**) showed a typical sigmoidal response (**Figure 4B**). The pK_a value was determined from the Hill plot analysis (**Figure 4C**). **Table 2** represents the pK_a s and protonation sites of aspartyl NPs.

Table 2: Protonation sites and pK_a s of aspartyl nucleoside phosphoramidates (**1**, **21**, **32** and **33**) obtained by NMR pH-metric titration. Protonation site in nucleobase of **21**, **32** and **33** was determined using their corresponding nucleoside (**18**, **29** and 2'-deoxy-3-deazaadenosine respectively). pK_a of the phosphoramidate nitrogen of **1** and **21** was determined using ^{15}N labelled aspartyl nucleoside phosphoramidates. Marker atoms used for pK_a determination are indicated within parentheses. ND = not determined.

Compound entry	Protonation site	pK_a	
		Nucleobase	P–N nitrogen
2'-deoxy-1-deazaadenosine (18)	N3	4.6 (C2)	
2'-deoxy-7-deazaadenosine (29)	N1	4.7 $\pm$ 0.1 (H2/C2)	
2'-deoxy-3-deazaadenosine	N1	7.5 (H2)	
Asp-dAMP (1)	N1	3.6 (H2)	4.3 (^{15}N)
Asp-1-deaza-dAMP (21)	N3	5.5 $\pm$ 0.3 (H1/H2)	4.1 (^{15}N)
Asp-7-deaza-dAMP (32)	N1	4.2 (H2)	ND
Asp-3-deaza-dAMP (33)	N1	7.4 $\pm$ 0.1 (H2)	ND

Determination of the protonation site of nucleobase in deazaadenine analogues (**21**, **32**, **33**), was carried out using the corresponding nucleosides (**18**, **29**). In the titration of **18**, C2 carbon in ^1H - ^{13}C HSQC spectra showed up-field CSP while C8 carbon remains unchanged (**Figure S7**), clearly indicating the protonation site as N3 with pK_a of 4.6. Similarly protonation site for 2'-deoxy-3-deazaadenosine was found to be N1 with pK_a of 7.5. However, in case of **29**, ^1H - ^{13}C HSQC spectra alone cannot provide conclusively the protonation site in the pyrimidine ring, because protonation either at N1 or N3 leads to shielding of C2 carbon (**Figure S8**). For this reason, ^1H - ^{13}C HMBC spectra were recorded to monitor the CSP of C4 and C6 carbon. Although up-field shifts of both C4 and C6 carbon were observed (**Figure S9**), the total change in CSP was 5.6 ppm for C6, while being much smaller ($\Delta\delta$ = 1.8 ppm) for C4. This suggests that the protonation likely occurs at N1 with pK_a of 4.7. N1 atom is the protonation site for 2'-deoxyadenosine (pK_a = 3.8) as reported previously.^[29]

The pK_a of 3-deazaadenine moiety in **33** (ca. pK_a = 7.4) and 1-deazaadenine in **21** (ca. pK_a = 5.5) is much higher than the pK_a of 7-deazaadenine in **32** (pK_a = 4.2) and even higher than the adenine in

1 (pK_a = 3.6). Therefore, it is clear that the population of the protonated species of **33** and **21** is higher than **32** and **1** in acidic solution (pD 5-7). Although the protonated forms of **33** are higher than **21** based on their pK_a s, **33** was much more stable, whereas **21** decomposed rapidly. Therefore it is evident that pK_a data alone cannot explain their reactivity. However, the base protonation site for Asp-1-deaza-dAMP (**21**) is N3 atom whereas N1 for others, this may have a role for the exceptional reactivity of **21**. During pH-metric titration of aspartyl adenine and deazaadenine NPs and their corresponding nucleosides, a significant difference was observed for the CSP of H8 proton of **21**. A shielding effect on H8 proton (wrong way chemical shift, **Figure 4E**, **S10**) was observed during base protonation of **21**, while usual deshielding effect on the H8 proton (**Figure 4D**, **S11**) occurred in all other aspartyl adenine and deazaadenine NPs and nucleosides (even for 2'-deoxy-1-deazaadenosine). This suggests that due to protonation the environment of the purine ring of **21** differs from that of aspartyl adenine and 3/7-deazaadenine NPs, possibly indicating a conformational change of the nucleobase, positioning the H8 of **21** away from the deshielding zone of phosphate group.

Possible mechanism of acid-catalysed P–N and P–O bond hydrolysis: In the acid catalysed hydrolysis of aaNPs, only P–N bond hydrolysis for β -Ala NPs, simultaneous P–N and P–O cleavage for Asp and Gly NPs (except **5**), and for Asp-1-deaza-dAMP only P–O bond hydrolysis was observed. This suggests the involvement of at least two independent reaction mechanisms for P–N and P–O bond hydrolysis which are operating together or independently depending on the molecular nature of aaNPs. In order to explain the hydrolysis pathways we propose reaction mechanism for P–N and P–O bond hydrolysis based on the reported literatures.

The possible protonated forms of aaNP and their tautomeric equilibrium at different solution pH are shown in **Scheme 2A**. The phosphoramidate monoanion (IV) in principle can be protonated either at the amide nitrogen or at the phosphoryl oxygen. Experimental evidence in the favour of *N*-protonation (vs. *O*-protonation) in solution is reported^[25, 30], however, some authors provided theoretical arguments^[23, 31] for partial *O*-protonation and for the existence of an equilibrium between *N* and *O*-protonated forms.

The acid catalysed P–N bond hydrolysis of various non-nucleosidic phosphoramidates has been documented previously^[15, 23, 32] and recently it has been discussed in the perspective of nucleoside phosphoramidates.^[13, 31, 33, 34] P–N bond hydrolysis most probably proceeds through the *N*-protonated zwitterionic form (III), since water attack will be favoured in the zwitterionic phosphate than the monoanionic form, and the leaving group can depart as a neutral amine. The mechanism of P–N bond hydrolysis (**Scheme 2B**) possibly involves a “loose (or hazy)” $\text{S}_{\text{N}}2$ -like transition state (TS) in which the P–N bond cleavage is far more advanced than O–P bond formation i.e. both the entering and the leaving groups are present in the transition state^[25, 32, 35], representing a borderline case between an associative and dissociative mechanism.

The Asp and Gly NPs are also (partly or fully) hydrolysed by P–O bond cleavage, triggered by the intramolecular attack of α -carboxyl group to phosphate centre. A plausible P–O bond hydrolysis mechanism is depicted in **Scheme 2C**. The mechanism possibly involves the *O*-protonated tautomer (I) since attack of neutral phosphate by α -carboxyl oxyanion will be more favourable.

Scheme 2: (A) Possible protonated forms of nucleoside phosphoramidates and their tautomers based on experimental pK_a and solution pH. Possible mechanism for the acid-catalysed hydrolysis of amino acid nucleoside phosphoramidates, (B) Mechanism for P–N bond hydrolysis, (C) Mechanism for P–O bond hydrolysis.

However, recently it has been shown that neighbouring carboxylate anion can also attack monoanionic phosphate group by NMR of ^{18}O -labelled compound.^[36] Initial attack of the α -carboxyl oxyanion on phosphorus takes place along with concomitant transfer of proton from the bulk hydronium ion to the phosphoryl oxyanion. This gives rise to a penta-coordinated five-membered cyclic trigonal bipyramidal (tbp) phosphorane intermediate where the carboxy oxygen as the entering nucleophile takes an apical position,^[13] and hence, the nitrogen atom as a member of the five membered ring is

forced to remain in equatorial position^[37]. The 5'-O linked nucleoside moiety occupies the remaining apical position and hence departs with a proton transfer to the 5'-oxygen. In contrast to a protonated amine leaving group (P–N hydrolysis), a nucleoside is expected to be a rather bad leaving group (P–O hydrolysis) since it is difficult to protonate 5'-oxygen in study condition (pD 5-7). For the removal of the neutral nucleoside leaving group a proton transfer is necessary. Departure of the nucleoside yields a cyclic phosphoramidate which is anticipated to be hydrolytically much

unstable at 70 °C and hydrolysed subsequently to amino acid and inorganic phosphate.

Figure 5: Overview of the mechanisms with the lowest energy barrier for P-O and P-N bond hydrolysis. The red line indicates the role of the 1-deazaadenine base. All energy values are in kcal/mol.

Influence of amino acid and nucleobase from theoretical perspective:

(A) Amino acid: The influence of the amino acid was studied using the model compound, asp-mono-methyl-phosphate shown in **Figure 5**. Here the nucleoside part is replaced by a methyl group and only the effect of the carboxyl groups is investigated. Earlier work^[34] on mono-methyl phosphate pointed out that the barrier for hydrolysis of the P-O bond was about 40 kcal/mol. In asp-mono-methyl-phosphate the α - and β -carboxyl groups offer the possibility for intramolecular catalysis. As shown in **Figure 5**, the barrier is reduced to 25.7 kcal/mol when a 5-membered ring is formed by the attack of α -carboxyl group on phosphorus (**Scheme 2C**). Formation of a 6-membered ring by the attack of β -carboxyl group is 5 kcal/mol higher in energy. Catalytic water molecules are of major importance here (**Figure 5**), the highest barrier is 25.7 kcal/mol for PO_ts2_wat, when the proton transfer doesn't occur via a water molecule the barrier of this step is 35.9 kcal/mol. The most stable reactant is the zwitterionic form with the nitrogen protonated and the

phosphate deprotonated, but since the cyclic intermediate (having pentavalent phosphorus with protonated nitrogen) for P-O bond hydrolysis is not stable with this tautomer the reaction must occur via the tautomer I (**Scheme 2C**) with the proton on the phosphate group. There is an energy penalty of 5.7 kcal/mol for this tautomerisation. The β -carboxyl group might have an enhancing entropic effect via structural role, as can be seen in PO_ts2_base, where a hydrogen bridge between the 3'-hydroxyl group of the sugar moiety and/or N-H group of Asp and β -carboxyl group places the α -carboxyl in the correct position for attack on phosphorus.

The cleavage of the P-N bond occurs via the most stable tautomer III (**Scheme 2B**) and is not influenced by the amino acid since it is geometrically impossible to form a cyclic 5-membered intermediate with nitrogen and the carboxyl group of the amino acid at the apical positions. The P-N bond hydrolysis occurs via a concerted mechanism where P-N bond cleavage and O-P bond formation occur simultaneously. First the *N*-protonation prolongs the P-N bond from 1.6 Å to 1.81 Å before the formation of the O-P

bond starts. In the transition state (PN_{ts_wat}) the P–N bond is further extended to 2.0 Å with a simultaneous partial formation of the O–P bond (1.99 Å). Finally moving from the transition state to the product state the O–P bond is fully formed (1.75 Å) and the P–N bond is cleaved. This is different from the mechanism predicted for *N*-methyl phosphoramidate,^[34] where a more dissociative mechanism is preferred. This can be explained by the presence of an extra methyl group on phosphate here, this disfavours the formation of a metaphosphate intermediate.

(B) Nucleobase: Effect of the nucleobase is most pronounced for the 1-deaza analogues. To catalyse the hydrolysis reaction even further the base must reduce the highest barrier which is 25.7 kcal/mol for PO_{ts2_wat}. Calculations for Asp-1-deaza-dAMP (**21**) show that when the base is protonated at N3 (expected for 1-deazaadenine), the *syn* and *anti* conformations of the base are equal in free energy. This can be favoured by the possibility of the base to form a hydrogen bond between N3-H and the phosphate group. Hydrogen bonding between the base and the catalytic water molecule as shown in PO_{ts2_base} (**Figure 5**) reduces the barrier for P–O cleavage by 6.6 kcal/mol. Although, there is also an alternative possibility with slightly higher in energy where protonated N3 can directly be involved in hydrogen bonding with the 5'-O of nucleoside part thereby effectively delivering a proton to the nucleoside leaving group. These together may explain the increased rate of P–O bond hydrolysis for Asp-1-deaza-dAMP compared to Asp-dAMP, Asp-7-deaza-dAMP and Asp-3-deaza-dAMP.

Conformational study by NMR: To investigate the conformational role in the kinetics and mechanism of P–O bond hydrolysis as suggested by molecular modelling, a comparative solution conformational study for Asp NPs containing adenine and deazaadenines was performed in acidic and basic solution (pD 5 and 8).

The deoxyribose sugar ring of all the conjugates adopts typical S-type (South, 2'-*endo*) puckering in D₂O irrespective of solution pD as indicated by the appearance of a triplet H1' peak (**Figure S12A**, $^3J_{H1'-H2'/H2''} = 7$ Hz). In all Asp NPs, the nucleotide part adopts an orientation (in both pD) as observed in the nucleotide residue of regular DNA duplex. The $^3J_{C4'-P}$ (9.3 Hz) coupling was determined from ¹H decoupled carbon spectrum indicating the β (C4'-C5'-O5'-P) torsion angle within the trans region.^[38] Moreover, a small $^4J_{H4'-P}$ coupling (~1-2 Hz) was detected by comparing ³¹P coupled and decoupled H4' proton peak (**Figure S12B**). This was further confirmed by the appearance of a small cross-peak in the ¹H-³¹P HETCOR spectra (**Figure S12C**). Observation of this long range coupling ($^4J_{H4'-P}$) indicated a planar W-shaped conformation of the molecular fragment H4'-C4'-C5'-O5'-P with β torsion in *anti* region and γ (C3'-C4'-C5'-O5') in *gauche*+ region.^[38] Aspartyl moiety in all these analogues adopts similar conformation in both pD as no significant change in 3J homo and hetero nuclear couplings was observed (**Table S2**). Aspartyl moiety was found to have proximity to sugar residue irrespective of the solution pD since NOE correlation between H3' and Hα/Hβ of Asp was observed in the ROESY spectra (**Figure S13**). This is somewhat in agreement with the proposed molecular model, where H-bonding between β-COOH and 3'-OH was shown.

In order to characterise the existence of any *syn* conformation of nucleobase as suggested by molecular modelling study, ¹H

coupled ¹³C, ¹H-¹H ROESY and ¹H-³¹P HOESY experiments were performed. The glycosyl torsion angle χ was defined by $^3J_{H1'-C4}$ (and $^3J_{H1'-C8}$) coupling and based on ¹H-¹H and ¹H-³¹P NOE interactions in order to discriminate between *syn* and *anti* conformation of nucleobase. The $^3J_{H1'-C4}$ coupling of ca. 2 Hz for Asp-dAMP (**1**) was observed in both pD, indicating predominant *anti* orientation (**Figure S14B**).^[38] The *anti* conformation of nucleobase was further supported by the observed ¹H-¹H NOE cross-peaks (**Figure S14C**).

However, in case of Asp-1-deaza-dAMP (**21**), a significant conformational difference in nucleobase orientation was observed in acidic and basic solution. In basic solution (pD 8), the $^3J_{H1'-C4}$ coupling was ca. 2.0 Hz, indicating predominant *anti* conformation of the nucleobase (**Figure 6A**), further supported by the observed NOE cross-peaks. The H8 proton showed cross-peaks with sugar and aspartyl protons, whereas H2 proton does not show any correlation (**Figure 6B**: Left). Moreover, in the HOESY spectrum, H8 proton shows correlation with phosphorus, while H2 does not (**Figure 6C**: Left). These data clearly indicate that the nucleobase orientation of **21** in the basic solution is in the *anti* domain. However, in acidic condition (pD 5), the $^3J_{H1'-C4}$ coupling was not possible to determine as the C4 carbon peak became too broad (**Figure 6A**), possibly indicating conformational dynamics across glycosyl bond. But from the ROESY and HOESY spectra, the existence of some population of *syn* conformers were identified as H2 proton shows cross-peaks in addition to that of H8 (**Figure 6B**, C: Right). Nucleobase orientation in **32** and **33** was akin to **21**, i.e. some population of *syn* conformers were present in the acidic solution (**Figure S15**, **16**). The change in nucleobase conformation from *anti* to *syn* in acidic solution can be rationalised on the basis of possible electrostatic interaction between positively charged pyrimidine ring and negatively charged phosphate and/or carboxylic acid groups.

In acidic solution for deazaadenine containing analogues the *syn* conformers are detected while not so for adenine analogue. This could be explained on the basis of higher nucleobase pK_a of deazaadenines compared to adenine, where existence of more protonated forms is expected in the acidic solution. As discussed earlier, both the kinetic data and molecular modelling suggest the involvement of protonated N3 atom in catalysing P–O bond hydrolysis. The protonated N3 atom of adenine and 1/7-deazaadenine might be involved in the H-bonding directly or via water molecule with 5'-O of aaNPs, where it can act in stabilising the TS of P–O bond hydrolysis or by effectively delivering a proton to the nucleoside leaving group via rare *syn* conformation. Based on protonation site of nucleobase, the N3 protonated form of Asp-1-deaza-dAMP is expected to be more abundant, while for Asp-7-deaza-dAMP and Asp-dAMP the N3 protonated form is rare as N1 is found to be the predominant protonation site whereas for Asp-3-deaza-dAMP there is no possibility for N3 protonation. This may explain in general the order of reactivity of nucleobase in catalysing P–O bond hydrolysis of Asp and Gly NPs.

Figure 6: (A) ^1H coupled ^{13}C spectra of Asp-1-deaza-dAMP (21), showing the splitting pattern of C4 carbon peak at pD 5 and 8; indicating the change of $^3J_{\text{H}1'-\text{C}4}$ coupling due to conformational change of nucleobase in basic and acidic solution. (B) Expansion of ROESY spectrum of 21 (Left: at pD 8), showing NOE cross-peaks between H8 and sugar protons and $\text{H}\alpha$ of aspartyl group, whereas, H2 shows no NOE cross-peaks with any protons, indicating predominant 'anti-like' conformation of the nucleobase moiety in basic solution, while at pD 5 (Right), H2 proton shows NOE cross-peaks with sugar and aspartyl protons in addition to that of H8 proton, indicating the existence of some population of 'syn' conformers in acidic condition. (C) Expansion of ^1H - ^{31}P HOESY spectrum of 21 (Left: at pD 8), depicting NOE cross-peak between H8 and ^{31}P where no cross-peak appeared between H2 and ^{31}P , while at pD 5 (Right), a small cross-peak appeared between H2 and ^{31}P along with H8 proton, indicating a proximity between H2 proton and phosphorus in acidic condition. All spectra were recorded at 10 $^\circ\text{C}$ in D_2O .

Conclusion

Amino acid nucleoside phosphoramidates can be used in the antiviral prodrug strategy as well as direct substrates for viral polymerases. The 'prodrug' approach relies on the successful delivery of the nucleotide moiety in the cell either by chemical or by enzymatic hydrolysis of the nucleoside phosphoramidate by selective P–N bond cleavage. When functioning as direct substrate for polymerase, the phosphoramidate should be stable enough and survive from the unwanted P–N and P–O bond cleavage before it reaches the enzyme's pocket. In both of these approaches an unnecessary release of nucleoside by means of P–O bond cleavage should be avoided. To establish the aforementioned criteria, it is essential to identify the determinants that govern selectivity for P–N/P–O bond hydrolysis.

In the present study, we attempted to identify the structural factors that modulate the molecule's physico-chemical properties and influence the hydrolysis kinetics and mechanism. The experimental and theoretical studies showed a significant reactivity difference based on the nature of amino acid and nucleobase. The presence of only β -carboxyl group triggered selective P–N bond cleavage whereas the presence of α -carboxyl group made P–O bond more labile and the lability was enhanced in the presence of an additional β -carboxyl group. The rate of hydrolysis reaction was

further accelerated by adenine or 1/7-deazaadenine base for P–O bond hydrolysis. The 1-deazaadenine nucleobase in combination with α - and β -carboxylic acid groups made the molecule exceptionally prone to P–O bond hydrolysis. Kinetic data, molecular modelling and conformational studies suggest the possibility for involvement of protonated N3 atom of adenine and 1/7-deazaadenine in the intramolecular catalysis of P–O bond hydrolysis *via syn* conformation.

Based on the preference (P–N vs. P–O) of hydrolysis it could, for example, be expected that 3-deaza (P–N>P–O) phosphoramidates might favourably deliver nucleoside monophosphates compared to 1/7-deaza's which preferentially deliver nucleosides via chemical degradation. Recently it was shown that 2'-deoxyadenosine-5'-iminodiacetyl phosphoramidate (IDA-dAMP, **Figure S17A**) is an efficient substrate compare to Asp-dAMP (1) for the HIV-1 RT.^[12] Notably its hydrolysis study (**Figure S17B**) revealed that despite the presence of the two α -carboxyl groups it hydrolysed by exclusive P–N bond cleavage (**Figure S17C, D**). This could be due to the increased basicity of the phosphoramidate nitrogen, leading to favourable protonation and release of iminodiacetate by the attack of water molecule. This example and other data from the present study show that property of nucleobase as well as amino acid leaving group have to be considered for explaining P–N/P–O bond hydrolysis of nucleoside phosphoramidates.

Experimental Section

Synthesis and characterization: All detailed synthetic schemes, procedures and molecular characterisation data are provided in the supporting information.

Kinetic study of hydrolytic reaction: Approximately 20 mM sample was prepared in D₂O and pD of the sample was adjusted by addition of small volume (few μ L) of HCl or NaOH solutions in D₂O (0.1M/0.5M). The progress of the reaction was monitored using Bruker Avance II 500 MHz NMR spectrometer and the kinetic cycle was recorded using Bruker Icon NMR automation program. The cycle began with by recording ¹H (NS = 16) and ³¹P (NS = 128) NMR spectra at 25 °C. Each round of kinetic cycle consisted of a heating step at 40 or 70 °C for 25 min inside the NMR instrument, the sample was then cooled down to 25 °C and ¹H and ³¹P spectra were recorded. The peaks appeared in ¹H and ³¹P NMR spectra were integrated and the extent of reaction (%) was calculated from the peak integral. Rate constants were determined by plotting concentration (%) vs. time (min) and fitting the data points to an exponential decay. Reproducibility of the kinetic experiments was verified by repeating the hydrolysis study for compound **1** and **21** twice. No significant discrepancy in the rate constants was observed. Average rate constant and SEM were calculated by considering both disappearances of the ¹H and ³¹P peaks of parent aaNP and the appearances of the products peaks in the ¹H and ³¹P spectra wherever feasible. The products formed after hydrolysis were identified by ¹H-³¹P TOCSY^[39], acquired with 64 scans, 2048 data points in the proton dimension (t_2) and 128 increments in the phosphorus dimension (t_1) over sweep width of 10 and 40 ppm respectively. The residual HOD peak was suppressed by low power on resonance presaturation.

pK_a determination by NMR titration: Samples were dissolved in 0.5 mL of D₂O and the sample's pD was adjusted directly inside the NMR tube. pD was measured at room temperature by using Hamilton SPINRODE electrode. ¹H and ³¹P NMR spectra were recorded at 25 °C in each pH point using 32 and 64 scans respectively. The pK_a of phosphoramidate (¹⁵N labelled **1** and **21**) nitrogen was determined by monitoring ¹⁵N chemical shift using ¹H-¹⁵N HSQC (long range) experiments on Bruker Avance II 600 MHz spectrometer. All ¹H-¹⁵N HSQC spectra in the pH-metric titration were recorded with sensitivity enhancement and gradient coherence selection for 8 scans, 128/2048 complex data points, 100/10 ppm spectral width in t_1 and t_2 respectively. The pH titration studies were done over a pD range of (1.5 < pD < 12). The pH values were obtained using equation: pH = pD – 0.4.^[40] The pK_a determination is based on the linear regression analysis of the Hill plot^[41] using the equation: pH = log [(1- α)/ α] + pK_a, where α represents fraction of the protonated species. The value of α is calculated from the change of chemical shift relative to the neutral state at a given pH divided by the total change in chemical shift between neutral and fully protonated state.

Determination of protonation sites: To determine the protonation site of the nucleobase, pH dependent ¹³C chemical shifts were monitored using ¹H-¹³C HSQC and ¹H-¹³C HMBC NMR. ¹H-¹³C HSQC spectra were recorded with sensitivity enhancement and gradient coherence selection. To measure ¹H-¹³C correlations of only the nucleobase part, J_{CH} was set to 180 Hz and 4 scans, 128/2048 complex data points and 80/10 ppm spectral width in t_1 and t_2 were used respectively. ¹H-¹³C HMBC were measured with 4 scans and 128/ 2048 complex data points and 100/10 ppm spectral widths in t_1 and t_2 respectively. Delays were optimized for transfer in nucleobases using J_{CH} = 145 Hz in the low pass filter and J_{CH} = 8 Hz for long range correlations.

Theoretical calculations: All calculations were performed using the Gaussian 09 package.^[42] Visualization was done with VMD.^[43] The structures were optimized with the B3LYP method^[44] with DGDZVP^[45] basis set. Solvation corrections using continuum solvent were performed during the optimization process using PCM.^[46] Every stationary point was checked by frequency analysis. ZPE corrections using B3LYP were scaled^[47] with a factor of 0.9877.

Conformational studies by NMR: 1D ³¹P coupled/decoupled ¹H spectra were recorded with 32 scans and 66k FIDs at 25 °C. Proton coupled 1D ¹³C spectra with gated ¹H decoupling were acquired using 1024 scans with spectral width of 70 ppm and 100k FIDs. The ¹H-³¹P HETCOR^[48] spectrum was acquired at 25 °C with 128 scans, 2048 data points in the ¹H dimension (t_2) and 512 increments in the ³¹P dimension (t_1) over spectral width of 3500 and 4050 Hz respectively. The ROESY^[49] experiments in D₂O (ROESY-spin lock pulse = 300 ms) were performed at 10 °C with a sweep width of 10 ppm in both dimensions, 64 scans, 2048 data points in t_2 and 512 FIDs in t_1 . All above experiments were repeated in both acidic (pD = 5.0) and basic solution (pD = 8.0). ¹H-³¹P 2D hetero-nuclear NOE (HOESY) experiments^[50] were performed using Bruker pulse program hoesyph. The WALTZ-65 composite pulse sequence for phosphorus decoupling (¹H as observed nuclei) during acquisition was used. Spectra were recorded in the phase-sensitive mode using States-TPPI method, with 512 (t_1) increments and 2K (t_2) data points, 64 scans, 16 dummy scans, and D1 = 1.5 s. The mixing time was set to 0.8 s. Sweep widths were 10 and 40 ppm respectively in the ¹H and ³¹P dimension. The data sets were Fourier transformed using shifted sine-bell window function in both dimensions and processed into a 2K×0.5K matrix without prior zero-filling.

Acknowledgements

This work was financed by KU Leuven grants GOA, IDO. We are indebted to Prof. Jef Rozenski for providing MS and HRMS data and Prof. Roger Busson for helpful discussion. We thank Luc Baudempez for NMR technical assistance, Dr. Elisabetta Groaz and Jolien Claessens for technical support and Chantal Biernaux for editorial help.

- [1] a) C. Meier, *Synlett*. **1998**, 233-242; b) C. R. Wagner, V. V. Iyer, E. J. McIntee, *Med. Res. Rev.* **2000**, *20*, 417-451; c) E. De Clercq, *Nat. Rev. Drug Discov.* **2002**, *1*, 13-25; d) E. De Clercq, *J. Clin. Virol.* **2004**, *30*, 115-133; e) Y. Mehellou, J. Balzarini, C. McGuigan, *ChemMedChem* **2009**, *4*, 1779-1791.
- [2] C. L. Freel Meyers, R. F. Borch, *J. Med. Chem.* **2000**, *43*, 4319-4327.
- [3] C. R. Wagner, S. L. Chang, G. W. Griesgraber, H. Song, E. J. McIntee, C. L. Zimmerman, *Nucleosides & Nucleotides* **1999**, *18*, 913-919.
- [4] a) R. N. Hunston, M. Jehangir, A. S. Jones, R. T. Walker, *Tetrahedron* **1980**, *36*, 2337-2340; b) A. S. Jones, C. McGuigan, R. T. Walker, J. Balzarini, E. de Clercq, *J. Chem. Soc. Perkin Trans. I* **1984**, 1471-1474; c) A. S. Jones, C. McGuigan, R. T. Walker, *J. Chem. Soc. Perkin Trans. I* **1985**, 199-202.
- [5] a) K. G. Devine, C. McGuigan, T. J. O'Connor, S. R. Nicholls, D. Kinchington, *AIDS* **1990**, *4*, 371-373; b) D. Cahard, C. McGuigan, J. Balzarini, *Mini Rev. Med. Chem.* **2004**, *4*, 371-381.
- [6] a) T. W. Abraham, C. R. Wagner, *Nucleosides & Nucleotides* **1994**, *13*, 1891-1903; b) E. J. McIntee, R. P. Remmel, R. F. Schinazi, T. W. Abraham, C. R. Wagner, *J. Med. Chem.* **1997**, *40*, 3323-3331.
- [7] T. W. Abraham, T. I. Kalman, E. J. McIntee, C. R. Wagner, *J. Med. Chem.* **1996**, *39*, 4569-4575.
- [8] a) D. Saboulard, L. Naesens, D. Cahard, A. Salgado, R. Pathirana, S. Velazquez, C. McGuigan, E. De Clercq, J. Balzarini, *Mol. Pharmacol.* **1999**, *56*, 693-704; b) T. F. Chou, J. Baraniak, R. Kaczmarek, X. Zhou, J. Cheng, B. Ghosh, C. R. Wagner, *Mol. Pharm.* **2007**, *4*, 208-217.
- [9] a) S. Chang, G. W. Griesgraber, P. J. Southern, C. R. Wagner, *J. Med. Chem.* **2001**, *44*, 223-231; b) J. Balzarini, *Pharm. World Sci.* **1994**, *16*, 113-126.
- [10] O. Adelfinskaya, P. Herdewijn, *Angew. Chem. Int. Ed.* **2007**, *46*, 4356-4358.
- [11] a) O. Adelfinskaya, M. Terrazas, M. Froeyen, P. Marliere, K. Nauwelaerts, P. Herdewijn, *Nucleic Acids Res.* **2007**, *35*, 5060-5072; b) A. Giraut, N. Dyubankova, X. P. Song, P. Herdewijn, *Chembiochem* **2009**, *10*, 2246-2252.
- [12] A. Giraut, X. P. Song, M. Froeyen, P. Marliere, P. Herdewijn, *Nucleic Acids Res.* **2010**, *38*, 2541-2550.
- [13] M. Ora, J. Ojanpera, H. Lonnberg, *Chem. Eur. J.* **2007**, *13*, 8591-8599.
- [14] A. Leisvuori, Y. Aiba, T. Lonnberg, P. Pojarvi-Virta, L. Blatt, L. Beigelman, H. Lonnberg, *Org. Biomol. Chem.* **2010**, *8*, 2131-2141.
- [15] T. Lonnberg, M. Ora, H. Lonnberg, *Mini-Rev. Org. Chem.* **2010**, *7*, 33-43.
- [16] T. W. Abraham, T. I. Kalman, E. J. McIntee, C. R. Wagner, *J. Med. Chem.* **1996**, *39*, 4569-4575.
- [17] I. Dhimitruka, J. SantaLucia, *Synlett*. **2004**, 335-337.
- [18] D. D. Boehr, A. R. Farley, F. J. LaRonde, T. R. Murdock, G. D. Wright, J. R. Cox, *Biochemistry* **2005**, *44*, 12445-12453.
- [19] Yoshikaw.M, T. Kato, Takenish.T, *Bull. Chem. Soc. Jpn.* **1969**, *42*, 3505-3508.
- [20] N. Minakawa, Y. Kawano, S. Murata, N. Inoue, A. Matsuda, *Chembiochem* **2008**, *9*, 464-470.
- [21] F. Seela, B. Westermann, U. Bindig, *J. Chem. Soc. Perkin Trans. I* **1988**, 697-702.
- [22] F. Seela, H. Rosemeyer, S. Fischer, *Helv. Chim. Acta.* **1990**, *73*, 1602-1611.
- [23] A. W. Garrison, C. E. Boozer, *J. Am. Chem. Soc.* **1968**, *90*, 3486-3494.

- [24] L. M. Qiang, S. X. Cao, X. Y. Zhao, R. Y. Liu, J. H. Liu, J. S. Lu, Y. F. Zhao, *Chin. J. Chem.* **2007**, *25*, 1559-1562.
- [25] J. Rahil, P. Haake, *J. Am. Chem. Soc.* **1981**, *103*, 1723-1734.
- [26] Z. Z. Chen, B. Tan, Y. M. Li, Y. F. Zhao, Y. F. Tong, J. F. Wang, *J. Org. Chem.* **2003**, *68*, 4052-4058.
- [27] B. Tan, M. C. Lee, M. Cui, T. Liu, Z. Z. Chen, Y. M. Li, Y. Ju, Y. F. Zhao, K. X. Chen, H. L. Jiang, *J. Mol. Struct. (Theochem)* **2004**, *672*, 51-60.
- [28] Y.-M. Li, D.-q. Zhang, H.-w. Zhang, G.-J. Ji, Y.-f. Zhao, *Bioorg. Chem.* **1992**, *20*, 285-295.
- [29] Z. Kazimierzczuk, J. A. Vilpo, F. Seela, *Helv. Chim. Acta* **1992**, *75*, 2289-2297.
- [30] a) P. Haake, T. Koizumi, *Tetrahedron Lett.* **1970**, 4849-4850; b) K. E. Debruin, A. G. Padilla, D. M. Johnson, *Tetrahedron Lett.* **1971**, 4279-4280; c) T. Koizumi, P. Haake, *J. Am. Chem. Soc.* **1973**, *95*, 8073-8079; d) M. P. Gamcsik, S. M. Ludeman, E. M. Shulmanroskes, I. J. McLennan, M. E. Colvin, O. M. Colvin, *J. Med. Chem.* **1993**, *36*, 3636-3645.
- [31] M. Ora, K. Mattila, T. Lonnberg, M. Oivanen, H. Lonnberg, *J. Am. Chem. Soc.* **2002**, *124*, 14364-14372.
- [32] S. J. Benkovic, E. J. Sampson, *J. Am. Chem. Soc.* **1971**, *93*, 4009-4016.
- [33] M. Ora, M. Murtola, S. Aho, M. Oivanen, *Org. Biomol. Chem.* **2004**, *2*, 593-600.
- [34] S. Michielsens, N. Tien Trung, M. Froeyen, P. Herdewijn, M. Tho Nguyen, A. Ceulemans, *Phys. Chem. Chem. Phys.* **2009**, *11*, 7274-7285.
- [35] W. P. Jencks, M. Gilchrist, *J. Am. Chem. Soc.* **1964**, *86*, 1410-1417.
- [36] Y. Kajihara, S. Nishigaki, D. Hanzawa, G. Nakanishi, R. Okamoto, N. Yamamoto, *Chem. Eur. J.* **2011**, *17*, 7645-7655.
- [37] Westheim.Fh, *Accounts Chem. Res.* **1968**, *1*, 70-78.
- [38] S. S. Wijmenga, B. N. M. van Buuren, *Prog. Nucl. Magn. Reson. Spectrosc.* **1998**, *32*, 287-387.
- [39] G. W. Kellogg, *J. Magn. Reson.* **1992**, *98*, 176-182.
- [40] P. K. Glasoe, F. A. Long, *J. Phys. Chem.* **1960**, *64*, 188-190.
- [41] S. Chatterjee, W. Pathmasiri, O. Plashkevych, D. Honcharenko, O. P. Varghese, M. Maiti, J. Chattopadhyaya, *Org. Biomol. Chem.* **2006**, *4*, 1675-1686.
- [42] M. J. Frisch, D. J. Fox et al., Gaussian 09, Revision A.1, Gaussian Inc., Wallingford CT, **2009**.
- [43] W. Humphrey, A. Dalke, K. Schulten, *J. Mol. Graph.* **1996**, *14*, 33-38.
- [44] A. D. Becke, *J. Chem. Phys.* **1993**, *98*, 5648-5652.
- [45] N. Godbout, D. R. Salahub, J. Andzelm, E. Wimmer, *Can. J. Chem.* **1992**, *70*, 560-571.
- [46] a) B. Mennucci, J. Tomasi, *J. Chem. Phys.* **1997**, *106*, 5151-5158; b) S. Miertus, E. Scrocco, J. Tomasi, *Chem. Phys.* **1981**, *55*, 117-129.
- [47] M. P. Andersson, P. Uvdal, *J. Phys. Chem. A* **2005**, *109*, 2937-2941.
- [48] V. Sklenar, H. Miyashiro, G. Zon, H. T. Miles, A. Bax, *Febs Lett.* **1986**, *208*, 94-98.
- [49] A. Bax, D. G. Davis, *J. Magn. Reson.* **1985**, *63*, 207-213.
- [50] C. Yu, G. C. Levy, *J. Am. Chem. Soc.* **1984**, *106*, 6533-6537.

Nucleobase in catalysis

Munmun Maiti, Servaas
Michielssens, Natalia Dyubankova,
Mohitosh Maiti, Eveline Lescrinier,
Arnout Ceulemans,
Piet Herdewijn*

Influence of Nucleobase and Anchimeric Assistance of Carboxyl Acid Groups in the Hydrolysis of Amino Acid Nucleoside Phosphoramidates

Nucleobase in catalysis: A dual intramolecular catalytic influence is demonstrated by the nucleobase and carboxyl groups in the chemical hydrolysis of amino acid nucleoside phosphoramidate prodrugs. Replacement of adenine N3 vs. N1/N7 atom is shown to have a conformational role via protonated N3 in crucially regulating kinetics and mechanism of nucleotide (P–N path) vs. nucleoside (P–O path) formation.

Supporting Information

Influence of Nucleobase and Anchimeric Assistance of Carboxyl Acid Groups in the Hydrolysis of Amino Acid Nucleoside Phosphoramidates

Munmun Maiti,^[a] Servaas Michielssens,^[b] Natalia Dyubankova,^[a] Mohitosh Maiti,^[a] Eveline Lescrinier,^[a] Arnout Ceulemans,^[b] Piet Herdewijn*,^[a]

^[a]Laboratory for Medicinal Chemistry, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium; ^[b]Laboratory of Quantum Chemistry, Department of Chemistry and INPAC Institute for Nanoscale Physics and Chemistry, Katholieke Universiteit Leuven, Belgium

* E-mail: Piet.Herdewijn@rega.kuleuven.be, Fax: (+) 32 16 337340

Contents:

	Page No.
Figures S1-S17	S2-S13
Tables S1 & S2	S14
Schemes S1-S3	S15-S16
Synthesis and characterisation data	S17-S34
General synthetic procedure for DCC assisted coupling	S18
General protocol for methyl ester deprotection	S18
General synthesis for direct 5'-phosphorylation of deaza-nucleosides	S19
Characterisation data for compounds 1-6 and their intermediates (1a-6a)	S20-S23
Synthesis of 2-deoxy-3,5-di- <i>O-p</i> -toluoyl- α -D-ribofuranosyl chloride (10)	S23-S24
Synthesis of 1-deazaadenine analogues (21-23)	S25-S30
Synthesis of 7-deazaadenine analogue (32)	S31-S34
Characterisation data for compound (33)	S34

Figure S1: Purine and systematic numbering of the nucleobase atoms mentioned in the text.

Figure S2: The pH dependent kinetics of hydrolytic reaction for β -ala-1-deaza-dAMP (**23**) at 70 °C. Kinetics at pH 5 is faster than at pH 6, and at pH 7 compound **23** is stable within the experimental time.

Figure S3: Temperature dependent kinetic profile of the hydrolytic reaction of Asp-1-deaza-dAMP (**21**) at 40 °C and 70 °C (pD 7).

Figure S4: Influence of the amino acid moiety and the nucleobase on the kinetics of hydrolytic reaction. Comparison of **21** and **22** indicates the influence of amino acids on hydrolysis rate. Comparison of **21** and **32** shows the influence of nucleobase on the rate of hydrolysis. Data are presented for pD 7.0 (70 °C) for these compounds.

Figure S5: (A) Kinetic profile of the hydrolytic reaction of Gly-1-deaza-dAMP (**22**) at pD 5.0 ($T = 70\text{ }^{\circ}\text{C}$), depicted by the stack plot of ^{31}P spectra at different time intervals, showing the disappearance of the parent ^{31}P peak ($\sim 8\text{ ppm}$) and gradual increase of two ^{31}P peaks near 0 ppm. Asterisk (*) indicates the free phosphate peak; (B) ^1H - ^{31}P TOCSY spectrum of Asp-7-deaza-dAMP (B, Compound **32**) and Asp-3-deaza-dAMP (C, Compound **33**) after hydrolytic reaction at pD 5.0 ($T = 70\text{ }^{\circ}\text{C}$), depicting the formation of inorganic phosphate and the corresponding nucleotide by P–O and P–N bond cleavage. Nucleotide phosphorus (peak with arrow) showing correlation with H4', H5' and H5'' of sugar moiety, whereas, inorganic phosphate (#) shows no correlation with any protons. Parent phosphoramidate ^{31}P showing correlation with H α and H β of aspartyl group along with H4' and H5', 5'' of sugar moiety.

Figure S6: ^1H - ^{31}P TOCSY spectrum of β -ala-1-deaza-dAMP (**23**) after the hydrolysis reaction at pH 6.0 (70 °C), depicting the formation of corresponding nucleotide by P-N bond cleavage. Nucleotide phosphorus (peak with arrow) shows correlation with H4' and H5', 5'' of sugar moiety, whereas, the parent phosphoramidate ^{31}P showing correlation with H α and H β of alaninyl group along with H4' and H5', 5'' of the sugar moiety.

Figure S7: Overlay of ^1H - ^{13}C HSQC spectra of 2'-deoxy-1-deazaadenosine (**18**), depicting chemical shift perturbation (CSP) of C2 carbon towards up-field in the NMR pH-metric titration from pD 7.0 to 4.5. The chemical shift of C8 carbon remains unperturbed.

Figure S8: Overlay of ^1H - ^{13}C HSQC of 2'-deoxy-7-deazaadenosine (**29**), depicting chemical shift perturbation of C2 carbon to up-field region in the NMR pH-metric titration from pD 7.4 to 2.7.

Figure S9: Overlay of ^1H - ^{13}C HMBC spectra of 2'-deoxy-7-deazaadenosine (**29**), depicting the chemical shift perturbation (CSP) of C4 and C6 carbon towards up-field in the NMR pH-metric titration from pD 7.4 to 4.4. CSP of C2 carbon towards up-field is also visible by the de-focussed $^1J_{\text{C2-H}}$ cross-peak.

Figure S10: Chemical shift perturbation of the H8 proton of Asp-1-deaza-dAMP (**21**), depicting the up-field chemical shift (shielding effect, the ‘wrong way chemical shift’) during NMR pH-metric titration.

Figure S11: Chemical shift perturbation of the H8 proton of Asp-dAMP (**1**), depicting the down-field chemical shift (deshielding effect) during NMR pH-metric titration.

Figure S12: (A) H1' proton (triplet) peak of Asp-dAMP (**1**) indicating S-type sugar puckering; (B) ^{31}P coupled (blue) and decoupled (red) H4' proton peak of **1**; (C) ^1H - ^{31}P HETCOR spectrum of (^{15}N) Asp-dAMP (**1**), recorded at 25 °C (pD 5), cross-peak for $^4J_{\text{H4}'\text{-P}}$ coupling shown within red box.

Figure S13: (A) Expansion of ROESY spectrum of Asp-dAMP (**1** at pD 5.0, 10 °C), showing NOE correlation between H3' sugar proton and H α proton of aspartyl group; (B) Expansion of ROESY spectrum of **1** (at pD 8.0, 10 °C), showing NOE correlation between H3' proton and H α , H β protons of aspartyl group; (C) Expansion of ROESY spectrum of Asp-1-deaza-dAMP (**21** at pD 8.0, 10 °C), showing NOE correlation between H3' sugar proton and H α proton.

Figure S14: (A) ^1H coupled (red) and decoupled (blue) ^{13}C spectra of Asp-dAMP (**1**, recorded at 25 $^{\circ}\text{C}$), showing the splitting pattern of C8 carbon peak; (B) ^1H coupled (red) and decoupled (blue) C4 carbon peak of **1**, showing the effect of J_{CH} couplings in the ^1H coupled ^{13}C spectrum; (C) Expansion of ROESY spectrum of **1** recorded at 10 $^{\circ}\text{C}$ (at pD 5 and 8), showing NOE correlation of H8 proton with sugar protons and aspartyl protons (H α and H β '/ β ''), whereas, H2 proton shows no NOE correlation.

Figure S15: (A) Expansion of ROESY spectrum of Asp-7-deaza-dAMP (**32**, recorded at pH 8.0, 10 °C), showing NOE correlation of H8 proton with sugar protons and aspartyl proton (Hα), whereas, H2 proton shows no NOE cross-peaks with any other protons, indicating predominant 'anti-like' conformation of the nucleobase moiety in the basic solution. (B) Expansion of ROESY spectrum of **32** (recorded at pH 5.0, 10 °C), showing NOE correlations between H2 proton and the sugar protons and aspartyl proton (Hα) in addition to the NOE cross-peaks from H8 proton, indicating some population of 'syn' conformers in acidic solution.

Figure S16: (A) Expansion of ROESY spectrum of Asp-3-deaza-dAMP (Left: at pD 8), showing NOE cross peaks between H8, H3 protons and sugar protons and H α proton of aspartyl group, whereas, H2 proton shows no NOE cross-peaks with any protons, indicating predominant 'anti-like' conformation of the nucleobase moiety in basic solution, while at pD 5 (Right), H2 proton also shows NOE cross-peaks with sugar and aspartyl protons in addition to that of NOE cross-peaks from H8, H3 protons, indicating some population of 'syn' conformers in acidic condition. (B) Expansion of ^1H - ^{31}P HOESY spectrum of Asp-3-deaza-dAMP (**33**), recorded at pD 5 and 8, depicting NOE cross peak between H8, H3 protons and ^{31}P , where, no cross-peak appeared between H2 proton and ^{31}P , indicating close proximity of H8 and H3 proton to phosphorus under experimental conditions (pD 5 and 8). All spectra were recorded at 25 °C in D $_2$ O.

Figure S17: (a) Chemical structure of 2'-deoxyadenosine-5'-iminodiacetyl phosphoramidate (IDA-dAMP). (b) Progress of the hydrolytic reaction of IDA-dAMP at pD 6.0 ($T = 70\text{ }^{\circ}\text{C}$), depicted by stack plot of ³¹P spectra at different time intervals, showing the disappearance of the parent ³¹P peak (~7.5 ppm) and gradual increase of only one ³¹P peak (~1 ppm) indicated in asterisk (*). (c) ¹H-³¹P TOCSY spectrum of IDA-dAMP after the hydrolysis reaction at pD 6.0 (70 $^{\circ}\text{C}$), depicting the formation of corresponding nucleotide by only P-N bond cleavage. Nucleotide (phosphorus peak in asterisk) showed correlation with H4', H5' and H5'' of sugar moiety, whereas, the parent phosphoramidate showing correlation with H α of iminodiacetyl moiety along with H4' and H5', 5'' of sugar moiety. (d) Concentration decay (%) of the parent phosphoramidate with respect to time is plotted.

Table S1: Rate constants ($k \times 10^{-4} \text{ min}^{-1}$) for the hydrolytic reactions of Asp-1-deaza-dAMP (**21**) at 40 °C (pD 5.0-7.0).

Compound entry	$k \times 10^{-4}$		
	pD 5.0	pD 6.0	pD 7.0
Asp-1-deaza-dAMP (21)	110.26	52.15	11.65

Table S2: Homo-nuclear (^1H - ^1H) and hetero-nuclear (^{31}P - ^1H , ^{31}P - ^{13}C , ^1H - ^{13}C , ^{15}N - ^1H) coupling constants of Asp-dAMP (**1**) and ^{15}N (NH)-Asp-dAMP in D_2O (25 °C, pD 5.0, 8.0). * No hetero-nuclear $^3J/{}^4J_{^{15}\text{N}-^{13}\text{C}}$ couplings were observed.

Couplings	pD 5.0 (Hz)	pD 8.0 (Hz)
$^3J_{\text{H1}'-\text{H2}''/\text{H2}''}$	7	7
$^3J_{\text{H1}'-\text{C8}}$	4.5	4.5
$^3J_{\text{H1}'-\text{C4}}$	-1.8	-1.8
$^3J_{\text{H4}'-\text{H5}''/\text{H5}''}$	3.5	3.8
$^3J_{\text{H5}''/\text{H5}''-\text{P}}$	4.5-5.0	4.5-5.0
$^3J_{\text{C4}'-\text{P}}$	9.3	9.3
$^4J_{\text{H4}'-\text{P}}$	-1-2	-1-2
$^3J_{\text{H}\alpha-\text{HB}''/\text{HB}''}$	6.0	7.7/5.3
$^3J_{\text{H}\alpha-\text{P}}$	8.9	8.3
$^4J_{\text{HB}''/\text{HB}''-\text{P}}$	~0	1.7
$^3J_{\text{P}-\text{CB}}$	4.5-5.0	6.7
$^3J_{^{15}\text{N}-\text{HB}''/\text{B}''}$ *	-1-1.5	2.5/ 3.4

Scheme S1: (i) 1.25M HCl in methanol, MgCO_3 , rt, 10 min, 88%; (ii) *p*-toluoyl chloride (2 eq), Et_3N (2.2 eq), dry acetone, rt, overnight, 67%; (iii) dry HCl, Et_2O , 0 °C, 75%.

Scheme S2: (a) Triethylorthoformate (13 eq), formic acid (57 eq), reflux, 99%; (b) *meta*-chloroperbenzoic acid (1.5 eq), acetic acid, rt, over night, 60%; (c) 90% fuming nitric acid (18 eq), trifluoroacetic acid, 90 °C, 4 h, 63%; (d) phosphorus trichloride (8 eq), dry ACN, 0 °C, 1.5 h, 87%; (e) 2-deoxy-3,5-di-*O-p*-toluoyl- α -D-ribofuranosyl

chloride (**10**) (1.1 eq), NaH (1.1 eq), dry ACN, 0 °C, 6 h, 73%; (f) saturated ammonia in methanol, rt, overnight, 91%; (g) ammonium formate (50 eq), Pd/C, methanol, 75 °C, 3 h, 83%; (h) phosphorus oxychloride (1 eq), dry triethylphosphate, TEAB, 0 °C, 1 h, 95%; (i) L-aspartic acid dimethyl ester hydrochloride (7 eq), Et₃N (14 eq), DCC (10 eq), *tert*-BuOH:H₂O (5:1), 95 °C, 2-3 h, 95% ; (j) 0.4M NaOH in MeOH:H₂O (4:1), TEAA, rt, 2-3h, 91%.

Scheme S3: (a) I₂ (1.1 eq), K₂CO₃ (1.5 eq), H₂O/DMF, 40 °C, 20 h, 81%; (b) trimethylsilylacetylene (2 eq), bis-(triphenylphosphine)-palladium(II)chloride, Et₃N (1.2 eq), CuI, DMF, 60 °C, 2 h, 70%; (c) potassium *tert*-butoxide (1.3 eq), NMP, 90 °C, 5 h, 81%; (d) 2-deoxy-3,5-di-*O*-*p*-toluoyl- α -D-ribofuranosyl chloride (**10**), TDA-1, KOH, dry ACN, rt, 57%; (e) saturated ammonia in methanol, rt, 48 h, 95%; (f) phosphorus oxychloride (1 eq), dry triethylphosphate, TEAB, 0 °C, 1 h, 81%; (g) L-aspartic acid dimethyl ester hydrochloride (7 eq), Et₃N (14 eq), DCC (10 eq), *tert*-BuOH:H₂O (5:1), 95 °C, 2-3 h, 70%; (h) 0.4M NaOH in MeOH:H₂O (4:1), TEAA, rt, 2-3h, 80%.

Note: Intermediates (**12-18** and **27-29**) are assigned according to the systematic numbering (Figure S1) and for the rest of the compounds; purine numbering system (Figure S1) is followed for the atom assignments.

Synthesis:

General: NMR spectra were recorded on Bruker Avance II 300 MHz with 5mm broad band probe, 500 MHz spectrometer equipped with a TXI-HCP Z gradient probe or on Bruker Avance II 600 MHz spectrometer with a 5mm TCI-HCN Z gradient cryo-probe (Biomacs). The spectra were processed with Bruker Topspin 2.1 software. Chemical shifts (δ) were expressed in parts per million (ppm). ^1H and ^{13}C NMR chemical shifts were referenced relative to TMS peak ($\delta = 0.00$ ppm). ^{31}P NMR chemical shifts were referenced to an external 85% H_3PO_4 standard ($\delta = 0.00$ ppm). All signals in Proton and carbon 1D NMR spectra were assigned by 2D COSY, ^1H - ^{13}C (natural abundance) HSQC and ^1H - ^{13}C HMBC NMR spectra. Standard mass spectra were recorded on an ion trap mass spectrometer (LCQ DuO, Thermo Finnigan, San Jose, CA) using electron spray ionization (ESI); data were acquired with the LAC/E³² system (Waters). High resolution mass spectra (HRMS) were obtained with a Q-ToF 2TM (Micromass Ltd.) coupled to a CapLC system (Waters). Chemicals of analytical and synthetic grade were obtained from commercial sources and were used as such. 2,3-diaminopyridine, tris(3,6-dioxahexyl)amine (TDA-1): Alfa Aesar; triethylorthoformate, *meta*-chloroperbenzoic acid, trifluoroacetic acid, dry acetone, *tert*-butanol, 2-deoxy- α/β -D-ribose, *p*-toluoyl chloride, ammonium formate, (trimethylsilyl) acetylene, 4,6-diaminopyrimidine hemisulfate monohydrate: Acros Organics; fuming nitric acid (90%), 1.25 M HCl in methanol, DCC: Fluka; phosphorus trichloride: Merck; Pd/C, phosphorus oxychloride, triethyl phosphate, L-aspartic acid dimethyl ester hydrochloride: Aldrich. Technical solvents were obtained from Brenntag (Deerlijk, Belgium). Flash silica column chromatography was performed on Davisil silica gel 60, 0.040-0.063 mm (Grace Davison). Thin-layer chromatography (TLC) was performed on Alugram silica gel UV254 mesh 60, 0.20 mm (Macherey-Nagel). Celite 545 was purchased from VWR International.

General synthetic procedure for DCC assisted coupling: The example described is for 2'-deoxy-1-deazaadenosine-5'-dimethoxyaspartyl phosphoramidate (**20**): In a two neck flask 2'-deoxy-1-deazaadenosine-5'- monophosphate (**19**) (0.06 g, 0.18 mmol) and L-aspartic acid dimethyl ester hydrochloride (0.25 g, 1.27 mmol) were dissolved in a mixture of *tert*-butanol and water (5:1, 6.0mL). Triethylamine (0.35 mL) was added. 0.37 mL of freshly prepared solution of N,N'-dicyclohexylcarbodiimide (DCC) in *tert*-butanol (0.5 g/mL) was added dropwise to have 5 equivalents of DCC in the reaction mixture. The reaction mixture was heated for 2-3 h at 95 °C while stirring under argon. The progress of the reaction was monitored by TLC (iPrOH: H₂O:NH₃, 6:2:2) every hour. After 1 h, another 5 equivalents of DCC was added to the reaction mixture. Upon completion the reaction mixture was cooled down to room temperature and the solvent was removed by rotary evaporation keeping the temperature of water bath below 30°C. The dry residue was suspended in water and washed 3 times with diethyl ether until the water was clear. The aqueous layer was lyophilized and the residue was purified by silica column chromatography by eluting with gradient of MeOH in CHCl₃ (20% to 40%) to obtain 2'-deoxy-1-deazaadenosine-5'-dimethoxyaspartyl phosphoramidate (**20**) as white solid (95%). TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.73. ¹H NMR (500 MHz, MeOD) δ: 8.41 (s, 1H, H-8), 7.99 (d, 1H, J_{2,1} = 5.9 Hz, H-2), 6.85 (d, 1H, J_{1,2} = 5.9 Hz, H-1), 6.48 (apparent t, 1H, J_{1',2'a} = J_{1',2'b} = 6.4 Hz, H-1'), 4.65–4.58 (m, 1H, H-3'), 4.14–3.96 (m, 4H, H-4', H-5', H5'' & H-α), 3.67 (s, 3H, CH₃), 3.57 (s, 3H, CH₃) 2.77 (d, 2H, J_{H-α,H-β} = 4.75, H-β), 2.70–2.65 (m, 1H, H-2'a), 2.46–2.42 (m, 1H, H-2'b). ¹³C NMR (125 MHz, MeOD) δ: 175.1 & 173.1 (α & β CO), 151.5 (C-6), 143.5 (C-4), 142.0 (C-2), 140.7 (C-8), 124.5 (C-5), 104.2 (C-1), 88.2 (C-4'), 86.7 (C-1'), 73.0 (C-3'), 65.7 (C-5'), 52.9 (C-α), 52.8 (COOCH₃), 52.2 (COOCH₃), 41.6 (C-2'), 39.9 (C-β). ³¹P NMR (121MHz, MeOD) δ: 5.28. ES-MS calculated for C₁₇H₂₄N₅O₉P, 473.13; found (negative mode) 471.7 [M-H]⁻.

General protocol for methyl ester deprotection: The example described is for the synthesis of 2'-Deoxy-1-deazaadenosine-5'-aspartyl phosphoramidate (**21**): A solution (5.9 mL) of 0.4 M NaOH (in MeOH/H₂O, 4:1) to 2'-deoxy-1-deazaadenosine-5'-dimethoxyaspartyl phosphoramidate (**20**) (0.08 g, 0.17 mmol) was added. The reaction mixture was stirred at room temperature under argon for 2-3 h. The course of the reaction was monitored by TLC (iPrOH/H₂O/NH₃, 6:2:2) until the disappearance of the starting material. The reaction mixture was neutralized by addition of TEAA (1 M, pH ~ 7.5). The solvent was removed under reduced

pressure and the resulting residue was dried by lyophilization. The desired product was purified by silica column chromatography eluting with the following gradient [iPrOH, iPrOH:H₂O:NH₃ (20:1:1), iPrOH:H₂O:NH₃ (10:1:1), iPrOH:H₂O:NH₃ (7:1:1)] to obtain 2'-deoxy-1-deazaadenosine-5'-aspartyl phosphoramidate (**21**, 91%). TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.4. ¹H NMR (600 MHz, D₂O) δ: 8.35 (s, 1H, H-8), 7.83 (d, 1H, J_{2,1} = 5.9 Hz, H-2), 6.52 (d, 1H, J_{1,2} = 5.9 Hz, H-1), 6.41 (dd, 1H, J_{1',2'a} = 7.75 Hz, J_{1',2'b} = 6.30 Hz, H-1'), 4.61–4.58 (m, 1H, H-3'), 4.18–4.14 (m, 1H, H-4'), 3.92–3.80 (m, 2H, H-5'), 3.67–3.64 (m, 1H, H-α), 2.73–2.29 (m, 4H, H-2', H2'' & H-β). ¹³C NMR (150 MHz, D₂O) δ: 181.2 & 181.1 (α & β CO), 147.4 (C-6), 144.5 (C-4), 143.6 (C-2), 139.1 (C-8), 122.4 (C-5), 103.8 (C-1), 85.8 (C-4'), 83.4 (C-1'), 71.2 (C-3'), 63.8 (C-5'), 54.7 (C-α), 42.9 (C-β), 38.6 (C-2'). ³¹P NMR (121 MHz, D₂O) δ: 6.50. HRMS (ESI) calculated for C₁₅H₂₀N₅O₉P, 445.0998; found (negative mode) 444.0904 [M-H]⁻.

General synthesis for direct 5'-phosphorylation of deaza-nucleoside: Compounds **19** and **30** were synthesised by selectively phosphorylating at 5'-OH in the unprotected nucleoside. As an example, synthetic procedure for synthesis of 2'-deoxy-1-deazaadenosine-5'-monophosphate (**19**) is described here: To a solution of 7-amino-3-(2'-deoxy-β-D-ribofuranosyl)-imidazo[4,5-*b*]pyridine (**18**) (0.1 g, 0.4 mmol) in dry triethylphosphate (5.5 mL) at 0°C, phosphorus oxychloride (37.0 μl, 0.4 mmol) was added. The mixture was stirred at 0°C for 1 h. Excess of phosphorus oxychloride was hydrolysed by slow addition of aqueous triethylammonium bicarbonate buffer solution (TEAB, 1 M, pH ~7.5). The crude product was purified on silica column eluting with the following gradient to obtain 2'-deoxy-1-deazaadenosine-5'-monophosphate (**19**) as white solid (75%): a) iPrOH, b) iPrOH:H₂O:NH₃ (20:1:1), c) iPrOH:H₂O:NH₃ (10:1:1), d) iPrOH:H₂O:NH₃ (7:1:1). TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.45. ¹H NMR (500 MHz, D₂O) δ: 8.36 (s, 1H, H-8), 7.91 (d, 1H, J_{2,1} = 6.0 Hz, H-2), 6.57 (d, 1H, J_{1,2} = 6.0 Hz, H-1), 6.46 (apparent t, 1H, J_{1',2'a} = J_{1',2'b} = 6.5 Hz, H-1'), 4.71–4.68 (m, 1H, H-3'), 4.28–4.23 (m, 1H, H-4'), 4.05–3.97 (m, 2H, H-5', H5''), 2.77–2.71 (m, 1H, H-2'a), 2.57–2.52 (m, 1H, H-2'b). ¹³C NMR (125MHz, D₂O) δ: 147.6 (C-6), 143.5 (C-4), 142.8 (C-2), 139.1 (C-8), 122.3 (C-5), 103.5 (C-1), 85.7 (C-4'), 83.7 (C-1'), 71.1 (C-3'), 64.1 (C-5'), 38.8 (C-2'). ³¹P NMR (121MHz, D₂O) δ: 1.83. HRMS (ESI) calculated for C₁₁H₁₅N₄O₆P, 330.0729; found (negative mode) 329.0594 [M-H]⁻.

Characterisation data for compounds 1-6 and their intermediates (1a-6a)

2'-Deoxyadenosine-5'-dimethoxyaspartyl phosphoramidate (1a): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.59. ¹H NMR (300 MHz, MeOD) δ : 8.53 (s, 1H, H-8), 8.23 (s, 1H, H-2), 6.51 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 6$ Hz, H-1'), 4.69–4.61 (m, 1H, H-3'), 4.15–4.02 (m, 4H, H-4', H-5', H-5'' & H- α), 3.69 (s, 3H, CH₃), 3.60 (s, 3H, CH₃), 2.89–2.71 (m, 2H, H-2'a & H- β), 2.53–2.34 (m, 1H, H-2'b). ¹³C NMR (75 MHz, MeOD) δ : 175.9 & 173.9 (α & β CO), 158.2 (C-6), 154.7 (C-2), 151.4 (C-4), 141.9 (C-8), 121.1 (C-5), 88.9 (C-4'), 86.2 (C-1'), 74.0 (C-3'), 66.6 (C-5'), 53.7 (C- α), 53.6 (COOCH₃), 53.0 (COOCH₃), 42.3 (C-2'), 40.8 (C- β). ³¹P NMR (121 MHz, MeOD) δ : 5.66. ES-MS calculated for C₁₆H₂₃N₆O₉P, 474.36; found (negative mode) 473.3 [M-H]⁻.

2'-Deoxyadenosine-5'-aspartyl phosphoramidate (1): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.22. ¹H NMR (500 MHz, D₂O) δ : 8.44 (s, 1H, H-8), 8.15 (s, 1H, H-2), 6.41 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 7$ Hz, H-1'), 4.64–4.60 (m, 1H, H-3'), 4.22–4.17 (m, 1H, H-4'), 3.94–3.83 (m, 2H, H-5', H-5''), 3.70–3.61 (m, 1H, H- α), 2.77–2.70 (m, 1H, H-2'a), 2.52–2.32 (m, 3H, H-2'b & H- β). ¹³C NMR (125 MHz, D₂O) δ : 181.9 & 180.1 (α & β CO), 156.1 (C-6), 153.1 (C-2), 149.2 (C-4), 140.4 (C-8), 119.1 (C-5), 86.6 (C-4'), 84.0 (C-1'), 71.8 (C-3'), 64.4 (C-5'), 55.5 (C- α), 43.8 (C- β), 39.5 (C-2'). ³¹P NMR (202 MHz, D₂O) δ : 6.96. ES-MS calculated for C₁₄H₁₉N₆O₉P, 446.31; found (negative mode) 445.2 [M-H]⁻.

2'-Deoxyadenosine-5'-methylglycinyl phosphoramidate (2a): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.49. ¹H NMR (500 MHz, MeOD) δ : 8.53 (s, 1H, H-8), 8.22 (s, 1H, H-2), 6.53–6.50 (m, 1H, H-1'), 4.67–4.64 (m, 1H, H-3'), 4.16–4.14 (m, 1H, H-4'), 4.07–4.02 (m, 2H, H-5', H-5''), 3.64 (s, 3H, CH₃), 3.58 (d, 2H, $J_{H\alpha-P} = 10$ Hz, H- α), 2.85–2.79 (m, 1H, H-2'a), 2.49–2.44 (m, 1H, H-2'b). ¹³C NMR (125 MHz, MeOD) δ : 175.5 (CO), 158.1 (C-6), 154.6 (C-2), 151.3 (C-4), 141.9 (C-8), 121.0 (C-5), 88.9 (C-4'), 86.3 (C-1'), 74.0 (C-3'), 66.4 (C-5'), 53.1 (COOCH₃), 45.2 (C- α), 42.2 (C-2'). ³¹P NMR (202 MHz, MeOD) δ : 6.63. ES-MS calculated for C₁₃H₁₉N₆O₇P, 402.30; found (negative mode) 401.3 [M-H]⁻.

2'-Deoxyadenosine-5'-glycinyll phosphoramidate (2): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.31. ¹H NMR (500 MHz, D₂O) δ : 8.36 (s, 1H, H-8), 8.10 (s, 1H, H-2), 6.39 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 5$ Hz, H-1'), 4.62–4.61 (m, 1H, H-3'), 4.22–4.16 (m, 1H, H-4'), 3.95–3.89 (m, 2H, H-5', H-5''), 3.29 (d, 2H, $J_{H\alpha-P} = 8.7$ Hz, H- α), 2.77–2.72 (m, 1H, H-2'a), 2.55–2.50 (m, 1H, H-2'b). ¹³C NMR (125 MHz, D₂O) δ : 178.3 (CO), 154.5 (C-6), 151.6 (C-2), 148.1 (C-4), 139.6 (C-8), 118.2 (C-5), 85.7 (C-4'), 83.4 (C-1'), 71.0 (C-3'), 63.7 (C-5'), 44.4 (C- α), 38.7 (C-2'). ³¹P NMR (202 MHz, D₂O) δ : 8.13. ES-MS calculated for C₁₂H₁₇N₆O₇P, 388.27; found (negative mode) 387.3 [M-H]⁻.

2'-Deoxyadenosine-5'-methyl- β -alaninyll phosphoramidate (3a): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.55. ¹H NMR (500 MHz, MeOD) δ : 8.54 (s, 1H, H-8), 8.23 (s, 1H, H-2), 6.52 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 7$ Hz, H-1'), 4.68–4.66 (m, 1H, H-3'), 4.18–4.13 (m, 1H, H-4'), 4.02–3.96 (m, 2H, H-5' & H-5''), 3.63 (s, 3H, CH₃), 3.14–3.10 (m, 2H, H- α), 2.84–2.80 (m, 1H, H-2'a), 2.47–2.45 (m, 3H, H-2'b and H- β). ³¹P NMR (202 MHz, MeOD) δ : 7.64. ES-MS calculated for C₁₄H₂₁N₆O₇P, 416.33, found (negative mode) 415.3 [M-H]⁻.

2'-Deoxyadenosine-5'- β -alaninyll phosphoramidate (3): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.33. ¹H NMR (500 MHz, D₂O) δ : 8.41 (s, 1H, H-8), 8.21 (s, 1H, H-2), 6.45 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 6.7$ Hz, H-1'), 4.68–4.66 (m, 1H, H-3'), 4.23–4.19 (m, 1H, H-4'), 3.90–3.88 (m, 2H, H-5' & H-5''), 2.85–2.80 (m, 3H, H- α & H-2'a), 2.58–2.53 (m, 1H, H-2'b), 2.27–2.24 (m, 2H, H- β). ¹³C NMR (125 MHz, D₂O) δ : 177.9 (CO), 154.3 (C-6), 151.0 (C-2), 148.4 (C-4), 139.9 (C-8), 118.5 (C-5), 85.7 (C-4'), 83.5 (C-1'), 70.9 (C-3'), 63.6 (C-5'), 38.5 (C-2'), 37.1 & 36.9 (C- α & C- β). ³¹P NMR (121 MHz, D₂O) δ : 8.60. ES-MS calculated for C₁₃H₁₉N₆O₇P, 402.30; found (negative mode) 401.3 [M-H]⁻.

Thymidine-5'-dimethoxyaspartyl phosphoramidate (4a): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.50. ¹H NMR (500 MHz, MeOD) δ : 7.84 (s, 1H, H-6), 6.36 (dd, 1H, $J_{1',2'a} = 8.01$ Hz, $J_{1',2'b} = 6.05$ Hz, H-1'), 4.50–4.49 (m, 1H, H-3'), 4.17–4.11 (m, 1H, H- α), 4.05–3.99 (m, 3H, H-4' & H-5', H-5''), 3.72 (s, 3H, CH₃), 3.65 (s, 3H, CH₃), 2.90–2.80 (m, 2H, H- β), 2.35–2.19 (m, 2H, H-2', H-2''), 1.95 (s, 3H, CH₃-5). ¹³C NMR (125 MHz, MeOD) δ : 176.0 & 173.9 (α & β CO), 167.4 (C-4), 153.4 (C-2), 139.1 (C-6), 112.8 (C-5), 88.6 (C-4'), 87.0 (C-1'), 73.8 (C-3'), 66.3 (C-5'), 53.7

(C- α), 53.6 (COOCH₃), 53.0 (COOCH₃), 41.7 (C-2'), 40.8 (C- β), 13.4 (CH₃-5). ³¹P NMR (121 MHz, MeOD) δ : 4.81. ES-MS calculated for C₁₆H₂₄N₃O₁₁P, 465.35; found (negative mode) 464.4 [M-H]⁻.

Thymidine-5'-aspartyl phosphoramidate (4): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.30. ¹H NMR (500 MHz, D₂O) δ : 7.73 (s, 1H, H-6), 6.27 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 6.6$ Hz, H-1'), 4.50–4.48 (m, 1H, H-3'), 4.08–4.08 (m, 1H, H-4'), 3.92–3.89 (m, 2H, H-5', H-5''), 3.73–3.69 (m, 1H, H- α), 2.53–2.41 (m, 2H, H- β), 2.33–2.23 (m, 2H, H-2', H-2''), 1.86 (s, 3H, CH₃-5). ¹³C NMR (125 MHz, D₂O) δ : 180.6 & 178.7 (α & β CO), 166.2 (C-4), 151.4 (C-2), 137.1 (C-6), 111.4 (C-5), 85.4 (C-4'), 84.6 (C-1'), 70.9 (C-3'), 63.7 (C-5'), 54.3 (C- α), 42.5 (C- β), 38.2 (C-2'), 11.3 (CH₃-5). ³¹P NMR (202 MHz, D₂O) δ : 6.49. ES-MS calculated for C₁₄H₂₀N₃O₁₁P, 437.30; found (negative mode) 436.2 [M-H]⁻.

Thymidine-5'-methylglycinyl phosphoramidate (5a): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.58. ¹H NMR (300 MHz, MeOD) δ : 7.80 (s, 1H, H-6), 6.35 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 6.5$ Hz, H-1'), 4.48–4.47 (m, 1H, H-3'), 4.04–4.03 (m, 3H, H-4', H-5' & H-5''), 3.70 (s, 3H, CH₃), 3.33 (s, 2H, H- α), 2.36–2.17 (m, 2H, H-2' & H-2''), 1.95 (s, 3H, CH₃-5). ³¹P NMR (121 MHz, MeOD) δ : 5.66. ES-MS calculated for C₁₃H₂₀N₃O₉P, 393.29; found (negative mode) 392.2 [M-H]⁻.

Thymidine-5'-glycinyl phosphoramidate (5): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.33. ¹H NMR (500 MHz, D₂O) δ : 7.69 (s, 1H, H-6), 6.28 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 6.9$ Hz, H-1'), 4.49–4.47 (m, 1H, H-3'), 4.11–4.08 (m, 1H, H-4'), 3.94–3.93 (m, 2H, H-5' and H-5''), 3.37 (d, $J_{H\alpha-P} = 8.6$ Hz, 2H, H- α), 2.31–2.28 (m, 2H, H-2' & H-2''), 1.87 (s, 3H, CH₃-5). ³¹P NMR (202 MHz, D₂O) δ : 7.99. ES-MS calculated for C₁₂H₁₈N₃O₉P, 379.26; found (negative mode) 378.2 [M-H]⁻.

Thymidine-5'-methyl- β -alaninyl phosphoramidate (6a): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.60. ¹H NMR (300 MHz, MeOD) δ : 7.83 (s, 1H, H-6), 6.37 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 6.4$ Hz, H-1'), 4.51–4.49 (m, 1H, H-3'), 4.04–3.99 (m, 3H, H-4', H-5' & H-5''), 3.66 (s, 3H, CH₃), 3.19–3.11 (m, 2H, H- α), 2.54 (t, $J_{H\beta-H\alpha} = 6.7$ Hz, 2H, H- β), 2.38–2.30 (m, 2H, H-2' & H-2''), 1.95

(s, 3H, CH₃-5). ³¹P NMR (121 MHz, MeOD) δ : 6.92. ES-MS calculated for C₁₄H₂₂N₃O₉P, 407.31; found (negative mode) 406.6 [M-H]⁻.

Thymidine-5'- β -alaninyl phosphoramidate (6): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.30. ¹H NMR (500 MHz, D₂O) δ : 7.67 (s, 1H, H-6), 6.25 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 6.9$ Hz, 1H, H-1'), 4.48–4.43 (m, 1H, H-3'), 4.07–4.02 (m, 1H, H-4'), 3.91–3.82 (m, 2H, H-5' & H-5''), 2.93–2.89 (m, 2H, H- α), 2.29–2.24 (m, 4H, H-2', H-2'' & H- β), 1.81 (s, 3H, CH₃-5). ³¹P NMR (202 MHz, D₂O) δ : 8.65. ES-MS calculated for C₁₃H₂₀N₃O₉P, 393.29; found (negative mode) 392.3 [M-H]⁻.

Synthesis of 2-deoxy-3,5-di-*O-p*-toluoyl- α -D-ribofuranosyl chloride, 10 (Scheme S1)

1-*O*-methyl-2-deoxy- α/β -D-ribofuranose (8): To a solution of 2-deoxy- α/β -D-ribose, **7** (10.0 g, 74.6 mmol) in 180.0 mL of anhydrous methanol, 6.4 mL of 1.25 M HCl in MeOH was added to get 8.00 mmol of hydrochloric acid in the reaction mixture. The reaction mixture was kept for 10 minutes at room temperature. After addition of 9.4 g of MgCO₃, the suspension was filtered, and the filtrate was evaporated with silica gel and purified on a silica gel column eluted with CH₂Cl₂/MeOH (98:2 to 96:4) to give 1-*O*-methyl-2-deoxy- α/β -D-ribofuranose (**8**) as a syrup (88%). TLC (DCM/MeOH, 9:1): R_f = 0.35. ¹H NMR (600 MHz, CDCl₃) δ : 5.06–5.02 (m, 2H, H-1 (a & b)), 4.48–4.35 (m, 1H, H-3 (a or b)), 4.13–4.11 (m, 1H, H-3 (a or b)), 4.03–4.01 (m, 1H, H-4 (a or b)), 3.96–3.93 (m, 1H, H-4 (a or b)), 3.70–3.58 (m, 4H, H-5, ((a & b)), 3.34 (s, 3H, OCH₃ (a or b)), 3.32 (s, 3H, OCH₃ (a or b)), 2.20–2.03 (m, 4H, H-2 (a or b)). ¹³C NMR (150 MHz, CDCl₃) δ : 105.4 & 105.3 (C-1 (a & b)), 86.9 & 86.5 (C-4 (a & b)), 72.2 & 71.7 (C-3 (a & b)), 63.6 & 62.5 (C-5 (a & b)), 55.3 & 54.9 (OMe (a & b)), 41.8 & 41.2 (C-2 (a & b)). ES-MS calculated for C₆H₁₂O₄, 148.2; found (positive mode) 148.9 [M+H]⁺.

1-*O*-methyl-2-deoxy-3,5-di-*O-p*-toluoyl- α/β -D-ribofuranose (9): To a solution of 1-*O*-methyl-2-deoxy- α/β -D-ribofuranose (**8**) (9.7 g, 65.5 mmol) in dry acetone (110.0 mL), *p*-toluoyl chloride (17.5 mL, 130.9 mmol) was added. Et₃N (20.0 mL) was added slowly and the solution was stirred overnight. After filtering Et₃N.HCl, the filtrate was evaporated. The crude product was purified by flash chromatography eluting with hexane first and then with hexane-EtOAc

(15:1) to afford 1-*O*-methyl-2-deoxy-3,5-di-*O-p*-toluoyl- α/β -D-ribofuranose (**9**, 67.0%). TLC (Hexane/EtOAc, 15:1): R_f = 0.24. ^1H NMR (500 MHz, $\text{CH}_3\text{COCH}_3\text{-d}_6$) δ : 7.97 (d, 2H, J = 8.1 Hz, aromatic-H), 7.92 (d, 2H, J = 8.1 Hz, aromatic-H), 7.32 (d, 2H, J = 8.1 Hz, aromatic-H), 7.30 (d, 2H, J = 8.1 Hz, aromatic-H), 5.62–5.59 (m, 1H, H-1), 5.25–5.24 (m, 1H, H-3), 4.56–4.42 (m, 3H, H-4 & H-5), 3.33 (s, 3H, -OCH₃), 2.54–2.50 (m, 2H, H-2), 2.41 (s, 3H, aromatic-CH₃), 2.40 (s, 3H, aromatic-CH₃). ^{13}C NMR (125 MHz, $\text{CH}_3\text{COCH}_3\text{-d}_6$) δ : 166.3, 166.1 & 166.0 (-CO), 144.5–129.6 & 127.8–127.5 (aromatic ring-C), 105.8 & 105.4 (C-1), 81.9 & 81.4 (C-4), 75.8 & 75.2 (C-3), 65.4 & 64.8 (C-5), 54.9 & 54.8 (-OCH₃), 39.2 & 39.1 (C-2), 21.3 (aromatic-CH₃). ES-MS calculated for $\text{C}_{22}\text{H}_{24}\text{O}_6$, 384.16; found 407.0 $[\text{M}+\text{Na}]^+$.

2-Deoxy-3,5-di-*O-p*-toluoyl- α -D-ribofuranosyl chloride (10**):** 1-*O*-methyl-2-deoxy-3,5-di-*O-p*-toluoyl- α/β -D-ribofuranose (**9**) (16.9 g, 43.99 mmol) was dissolved in dry ether (154.0 mL) and the solution was cooled to 0°C in an ice bath. To the solution was bubbled HCl gas for *ca.* 20 minutes until the mixture crystallized. The reaction mixture was then kept in a refrigerator (+4°C) overnight. The precipitate was filtered and washed with cold ether. The solid was immediately dried under vacuum over NaOH to give 2-deoxy-3,5-di-*O-p*-toluoyl- α -D-ribofuranosyl chloride (**10**) as a colourless crystalline powder (75%). ^1H NMR (500MHz, CDCl_3) δ : 7.9 (d, 2H, J = 8.2 Hz, aromatic-H), 7.89 (d, 2H, J = 8.2 Hz, aromatic-H), 7.27–7.23 (m, overlapping with CHCl_3 signal, 4H, aromatic-H), 6.48 (d, 1H, $J_{1,2}$ = 5.1 Hz, H-1), 5.56–5.55 (m, 1H, H-3), 4.87–4.85 (m, 1H, H-4), 4.69–4.58 (m, 2H, H-5), 2.89–2.72 (m, 2H, H-2), 2.42 (s, 3H, aromatic-CH₃), 2.41 (s, 3H, aromatic-CH₃). ^{13}C NMR (75 MHz, CDCl_3) δ : 166.4 & 166.3 (-CO), 144.3, 144.0, 130.2–129.2, 126.9 & 126.7 (aromatic ring-C), 95.3 (C-1), 84.7 (C-4), 73.6 (C-3), 63.5 (C-5), 44.5 (C-2), 21.7 (aromatic-CH₃). ES-MS calculated for $\text{C}_{21}\text{H}_{21}\text{ClO}_5$, 388.11; found (positive mode) 389.9 $[\text{M}+\text{H}]^+$.

Synthesis of 1-deazaadenine analogues (Scheme S2)

3*H*-imidazo[4,5-*b*]pyridine (12): A mixture of 2,3-diaminopyridine, **12** (25 g, 229.08 mmol) and triethylorthoformate (500 mL, 3.0 mol) was refluxed for 3 hours at 145°C. The solution was evaporated to dryness in rotary evaporator and then the residue was dissolved in 500 mL of 99% formic acid and refluxed for 2 hours at 110°C. When the reaction was completed, the excess formic acid was removed with the rotary evaporator and the residue dissolved in 500 mL absolute methanol, treated with activated charcoal and stirred overnight at room temperature. Then the charcoal was removed by vacuum filtration through Celite 545 and the filtrate was evaporated to obtain 3*H*-imidazo[4,5-*b*]pyridine (**12**) as a white solid (99%). TLC (CHCl₃/MeOH, 9:1): *R_f* = 0.23. ¹H-NMR (500 MHz, DMSO-*d*₆) δ: 13.01 (br s, 1H, -NH, exchangeable with D₂O), 8.41 (s, 1H, H-2), 8.35 (d, 1H, *J*_{5,6} = 4.7 Hz, H-5), 8.01 (d, 1H, *J*_{7,6} = 8.0 Hz, H-7), 7.23 (dd, 1H, *J*_{6,5} = 4.7 Hz, *J*_{6,7} = 8.0 Hz, H-6). ¹³C (125 MHz, DMSO-*d*₆) δ: 144.0, 143.8, 117.8 (other carbon peaks are overlapped with the mentioned carbon peaks). ES-MS calculated for C₆H₅N₃, 119.05; found (positive mode) 120.1 [M+H]⁺.

3*H*-imidazo[4,5-*b*]pyridine-*N*-oxide (13): To a solution of 3*H*-imidazo[4,5-*b*]pyridine (**12**) (27.12 g, 227.7 mmol) in 99% acetic acid (396 mL), *meta*-chloroperbenzoic acid (84.19 g, 341.5 mmol) was added. The mixture was stirred over the weekend at room temperature. Precipitate was filtered under vacuum. The solid was washed three times with ethyl acetate and dried *in vacuo*. Since NMR results confirmed the presence of *meta*-chlorobenzoic acid, the solid was refluxed in ethyl acetate (300 mL) for 30 minutes and filtered. The crude product was purified by recrystallization from ethanol to provide 3*H*-imidazo[4,5-*b*]pyridine-*N*-oxide (**13**) as white-brown solid (60%). TLC (CHCl₃/MeOH, 9:1): *R_f* = 0.11. ¹H NMR (500 MHz, DMSO-*d*₆) δ: 8.38 (s, 1H, H-2), 8.17 (d, 1H, *J*_{5,6} = 6.4 Hz, H-5), 7.59 (d, 1H, *J*_{7,6} = 8.0 Hz, H-7), 7.19 (dd, 1H, *J*_{6,5} = 6.4 Hz, *J*_{6,7} = 8.0 Hz, H-6). ¹³C (125 MHz, DMSO-*d*₆) δ: 143.4, 132.9, 119.0 (other carbon peaks are overlapped with the mentioned carbon peaks). ES-MS calculated for C₆H₅N₃O, 135.12; found (negative mode) 133.8 [M-H]⁻.

7-Nitroimidazo[4,5-*b*]pyridine-*N*-oxide (14): To a solution of 3*H*-imidazo[4,5-*b*]pyridine-*N*-oxide (**13**) (14.12 g, 104.5 mmol) in trifluoroacetic acid (104.0 mL) at 0°C, 90% fuming nitric acid (68 mL) was added in a drop wise manner. The mixture was heated at 90°C for 4 hours, cooled down, and then poured on to crushed ice. The mixture was neutralized slowly with

concentrated ammonium hydroxide while maintaining the temperature around 30°C. The resulting solid was filtered, washed with ice cold water, and dried to obtain 7-nitroimidazo[4,5-*b*]pyridine-*N*-oxide (**14**) as yellow solid (63%). TLC (iPrOH/NH₃/H₂O, 7:1:1): *R_f* = 0.54. ¹H NMR (500 MHz, DMSO-*d*₆) δ: 13.9 (br s, 1H, -NH exchangeable with D₂O), 8.58 (s, 1H, H-2), 8.31 (d, 1H, *J*_{5,6} = 7.15 Hz, H-5), 8.07 (d, 1H, *J*_{6,5} = 7.15 Hz, H-6). ¹³C NMR (125 MHz, DMSO-*d*₆) δ: 149.3 (C-3a), 145.9 (C-2), 133.5 (C-5), 129.8 (C-7), 123.3 (C-7a), 115.3 (C-6). ES-MS calculated for C₆H₄N₄O₃, 180.1; found (negative mode) 178.9 [M-H]⁻.

7-Nitroimidazo[4,5-*b*]pyridine (15): To a cold solution of 7-nitroimidazo[4,5-*b*]pyridine-*N*-oxide (**14**) (8.93 g, 49.6 mmol) in dry acetonitrile, (180 mL) phosphorus trichloride (39.3 mL) was added dropwise and the mixture was kept at 0°C for 1.5 hours. Then the solution was poured onto crushed ice and then neutralized with solid sodium carbonate with vigorous stirring. The resulting solid was filtered and chromatographed on silica gel eluting with dichloromethane/methanol/ammonia (9:1:0.1) to obtain 7-nitroimidazo[4,5-*b*]pyridine (**15**) as reddish yellow solid (87%). TLC (DCM/MeOH/NH₃, 9:1:0.1): *R_f* = 0.55. ¹H NMR (500MHz, DMSO-*d*₆) δ: 7.96 (s, 1H, H-2), 7.88 (d, 1H, *J*_{5,6} = 7.0 Hz, H-5), 7.68 (d, 1H, *J*_{6,5} = 7.0 Hz, H-6). ¹³C NMR (125 MHz, DMSO-*d*₆) δ: 157.7 (C-2), 153.4 (C-3a), 134.8 (C-7a), 132.3 (C-7), 128.2 (C-5), 110.7 (C-6). ES-MS calculated for C₆H₄N₄O₂, 164.03; found (positive mode) 165.0 [M+H]⁺.

7-Nitro-3-(2'-deoxy-3',5'-di-*O*-*p*-toluoyl-β-D-ribofuranosyl)-imidazo[4,5-*b*]pyridine (16): To a suspension of 7-nitroimidazo[4,5-*b*]pyridine (**15**) (1.0 g, 6.1 mmol) in dry acetonitrile (40.0 mL) under an atmosphere of Ar, NaH (60% in oil, 0.32 g, 6.65 mmol) was added and the mixture was stirred at room temperature for 30 min. To the ice cold mixture, 2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-ribofuranosyl chloride (**10**, 2.7 g, 6.9 mmol) was added and the suspension was stirred at 0°C for 6 h and then kept at room temperature for overnight. The reaction mixture was filtered to remove the insoluble material and the filtrate was concentrated to a black residue which was chromatographed on a silica gel column eluted with hexane/ EtOAc (7:3) to afford 7-nitro-3-(2'-deoxy-3',5'-di-*O*-*p*-toluoyl-β-D-ribofuranosyl)-imidazo[4,5-*b*]pyridine (**16**) as a yellow solid (73.4%). TLC (CHCl₃/CH₃COCH₃, 7:3): *R_f* = 0.3. ¹H NMR (300 MHz, CDCl₃) δ: 8.58 (d, 1H, *J*_{5,6} = 5.3 Hz, H-5), 8.52 (s, 1H, H-2), 8.0 (d, 2H, *J* = 8.2 Hz, aromatic-H), 7.97 (d, 1H, *J*_{6,5} = 5.3 Hz, H-6), 7.89 (d, *J* = 8.2 Hz, aromatic-H), 7.27 (d, 2H, *J* = 8.1 Hz, aromatic-H), 7.23 (d, 2H, *J* =

8.1 Hz, aromatic-H), 6.71 (dd, 1H, $J_{1',2'a} = 8.1$ Hz, $J_{1',2'b} = 5.9$ Hz, H-1'), 5.88–5.85 (m, 1H, H-3'), 4.84–4.66 (m, 3H, H-4', H-5' & H-5''), 3.21–3.14 (m, 1H, H-2'a), 2.97–2.90 (m, 1H, H-2'b), 2.47 (s, 3H, aromatic-CH₃), 2.42 (s, 3H, aromatic-CH₃). ES-MS calculated for C₂₇H₂₄N₄O₇, 516.16; found 538.9 [M+Na]⁺.

7-Nitro-3-(2'-deoxy-β-D-ribofuranosyl)-imidazo[4,5-*b*]pyridine (17): A solution of 7-nitro-3-(2'-deoxy-3',5'-di-*O-p*-toluoyl-β-D-ribofuranosyl)-imidazo[4,5-*b*]pyridine (**16**) (2.0 g, 3.87 mmol) in 100 mL of methanol saturated at 0°C with ammonia was set aside at room temperature for overnight. The reaction mixture was evaporated and the residue was chromatographed on a silica gel column eluted with CHCl₃/MeOH/NH₃ (9.4:0.5:0.08) to obtain 7-nitro-3-(2'-deoxy-β-D-ribofuranosyl)-imidazo[4,5-*b*]pyridine (**17**) as yellow solid (91%). TLC (CHCl₃/MeOH, 9.5:0.5): $R_f = 0.2$. ¹H NMR (300 MHz, MeOD) δ: 8.97 (s, 1H, H-2), 8.65 (d, 1H, $J_{5,6} = 5.3$ Hz, H-5), 8.03 (d, 1H, $J_{6,5} = 5.3$ Hz, H-6), 6.67 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 6.6$ Hz, H-1'), 4.65–4.61 (m, 1H, H-3'), 4.11–4.07 (m, 1H, H-4'), 3.89–3.75 (m, 2H, H-5' & H-5''), 2.90–2.81 (m, 1H, H-2'a), 2.59–2.51 (m, 2H, H-2'b). ES-MS calculated for C₁₁H₁₂N₄O₅, 280.24; found 303.1 [M+Na]⁺.

7-Amino-3-(2'-deoxy-β-D-ribofuranosyl)-imidazo[4,5-*b*]pyridine (mentioned in the article as **2'-deoxy-1-deazaadenosine, 18**): To a solution of 7-nitro-3-(2'-deoxy-β-D-ribofuranosyl)-imidazo[4,5-*b*]pyridine (**17**) (0.94 g, 3.35 mmol) in methanol (180.0 mL) was added 10% Pd/C (0.47 g) and ammonium formate (10.56 g, 167.7 mmol) and the mixture was refluxed at 75°C for 3 h. The catalyst was removed by filtration on celite 545 and the filtrate was evaporated to white solid. The crude product was purified on silica gel column eluting with CHCl₃/MeOH (9:1) to obtain 2'-deoxy-1-deazaadenosine (**18**) as white solid (83%). TLC (CHCl₃/MeOH, 9:1): $R_f = 0.2$. ¹H NMR (500 MHz, MeOD) δ: 8.25 (s, 1H, H-2), 7.85 (d, 1H, $J_{5,6} = 6.0$ Hz, H-5), 6.49 (d, 1H, $J_{6,5} = 6.0$ Hz, H-6), 6.44 (dd, 1H, $J_{1',2'a} = 8.5$ Hz, $J_{1',2'b} = 6.0$ Hz, H-1'), 4.58–4.56 (m, 1H, H-3'), 4.09–4.08 (m, 1H, H-4'), 3.87–3.73 (m, 2H, H-5' & H-5''), 2.89–2.84 (m, 1H, H-2'a), 2.37–2.33 (m, 1H, H-2'b). ¹³C NMR (125 MHz, MeOD) δ: 149.1 (C-7), 147.0 (C-3a), 145.5 (C-5), 141.0 (C-2), 124.8 (C-7a), 104.1 (C-6), 90.1 (C-4'), 87.7 (C-1'), 73.4 (C-3'), 63.9 (C-5'), 41.4 (C-2'). ES-MS calculated for C₁₁H₁₄N₄O₃, 250.25; found (positive mode) 250.9 [M+H]⁺.

2'-Deoxy-1-deazaadenosine-5'-monophosphate (19): To a solution of 7-amino-3-(2'-deoxy-β-D-ribofuranosyl)-imidazo[4,5-*b*]pyridine (**18**) (0.1 g, 0.4 mmol) in dry triethylphosphate (5.5

mL) at 0°C, phosphorus oxychloride (37.0 μ l, 0.4 mmol) was added. The mixture was stirred at 0°C for 1 h. Excess of phosphorus oxychloride was hydrolysed by slow addition of aqueous triethylammonium bicarbonate buffer solution (TEAB, 1 M, pH ~7.5). The crude product was purified on silica column eluting with the following gradient to obtain 2'-deoxy-1-deazaadenosine-5'- monophosphate (**19**) as white solid (75%): a) iPrOH, b) iPrOH:H₂O:NH₃ (20:1:1), c) iPrOH:H₂O:NH₃ (10:1:1), d) iPrOH:H₂O:NH₃ (7:1:1). TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.45. ¹H NMR (500 MHz, D₂O) δ : 8.36 (s, 1H, H-8), 7.91 (d, 1H, $J_{2,1}$ = 6.0 Hz, H-2), 6.57 (d, 1H, $J_{1,2}$ = 6.0 Hz, H-1), 6.46 (apparent t, 1H, $J_{1',2'a}$ = $J_{1',2'b}$ = 6.5 Hz, H-1'), 4.71–4.68 (m, 1H, H-3'), 4.28–4.23 (m, 1H, H-4'), 4.05–3.97 (m, 2H, H-5' & H5''), 2.77–2.71 (m, 1H, H-2'a), 2.57–2.52 (m, 1H, H-2'b). ¹³C NMR (125MHz, D₂O) δ : 147.6 (C-6), 143.5 (C-4), 142.8 (C-2), 139.1 (C-8), 122.3 (C-5), 103.5 (C-1), 85.7 (C-4'), 83.7 (C-1'), 71.1 (C-3'), 64.1 (C-5'), 38.8 (C-2'). ³¹P NMR (121MHz, D₂O) δ : 1.83. HRMS (ESI) calculated for C₁₁H₁₅N₄O₆P, 330.0729; found (negative mode) 329.0594 [M-H]⁻.

2'-Deoxy-1-deazaadenosine-5'-dimethoxyaspartyl phosphoramidate (20): In a two neck flask 2'-deoxy-1-deazaadenosine-5'- monophosphate (**19**) (0.06 g, 0.18 mmol) and L-aspartic acid dimethyl ester hydrochloride (0.25 g, 1.27 mmol) were dissolved in a mixture of *tert*-butanol and water (5:1, 6.0mL). Triethylamine (0.35 mL) was added. 0.37 mL of freshly prepared solution of N,N'-dicyclohexylcarbodiimide (DCC) in *tert*-butanol (0.5 g/mL) was added dropwise to have 5 equivalents of DCC in the reaction mixture. The reaction mixture was heated for 2-3 h at 95 °C while stirring under argon. The progress of the reaction was monitored by TLC (iPrOH: H₂O:NH₃, 6:2:2) every hour. After 1 h, another 5 equivalents of DCC was added to the reaction mixture. Upon completion the reaction mixture was cooled down to room temperature and the solvent was removed by rotary evaporation keeping the temperature of water bath below 30°C. The dry residue was suspended in water and washed 3 times with diethyl ether until the water was clear. The aqueous layer was lyophilized and the residue was purified by silica column chromatography by eluting with gradient of MeOH in CHCl₃ (20% to 40%) to obtain 2'-deoxy-1-deazaadenosine-5'-dimethoxyaspartyl phosphoramidate (**20**) as white solid (95%). TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.73. ¹H NMR (500 MHz, MeOD) δ : 8.41 (s, 1H, H-8), 7.99 (d, 1H, $J_{2,1}$ = 5.9 Hz, H-2), 6.85 (d, 1H, $J_{1,2}$ = 5.9 Hz, H-1), 6.48 (apparent t, 1H, $J_{1',2'a}$ = $J_{1',2'b}$ = 6.4 Hz, H-1'), 4.65–4.58 (m, 1H, H-3'), 4.14–3.96 (m, 4H, H-4', H-5', H5'' & H- α), 3.67 (s, 3H, CH₃), 3.57 (s, 3H, CH₃) 2.77 (d, 2H, $J_{H\alpha,H\beta}$ = 4.75, H- β), 2.70–2.65 (m, 1H, H-2'a), 2.46–2.42 (m,

1H, H-2'b). ^{13}C NMR (125 MHz, MeOD) δ : 175.1 & 173.1 (α & β CO), 151.5 (C-6), 143.5 (C-4), 142.0 (C-2), 140.7 (C-8), 124.5 (C-5), 104.2 (C-1), 88.2 (C-4'), 86.7 (C-1'), 73.0 (C-3'), 65.7 (C-5'), 52.9 (C- α), 52.8 (COOCH₃), 52.2 (COOCH₃), 41.6 (C-2'), 39.9 (C- β). ^{31}P NMR (121 MHz, MeOD) δ : 5.28. ES-MS calculated for C₁₇H₂₄N₅O₉P, 473.13; found (negative mode) 471.7 [M-H]⁻.

2'-Deoxy-1-deazaadenosine-5'-aspartyl phosphoramidate (21): A solution (5.9 mL) of 0.4 M NaOH (in MeOH/H₂O, 4:1) to 2'-deoxy-1-deazaadenosine-5'-dimethoxyaspartyl phosphoramidate (**20**) (0.08 g, 0.17 mmol) was added. The reaction mixture was stirred at room temperature under argon for 2-3 h. The course of the reaction was monitored by TLC (iPrOH/H₂O/NH₃, 6:2:2) until the disappearance of the starting material. The reaction mixture was neutralized by addition of TEAA (1 M, pH ~ 7.5). The solvent was removed under reduced pressure and the resulting residue was dried by lyophilization. The desired product was purified by silica column chromatography eluting with the following gradient [iPrOH, iPrOH:H₂O:NH₃ (20:1:1), iPrOH:H₂O:NH₃ (10:1:1), iPrOH:H₂O:NH₃ (7:1:1)] to obtain 2'-deoxy-1-deazaadenosine-5'-aspartyl phosphoramidate (**21**, 91%). TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.4. ^1H NMR (600 MHz, D₂O) δ : 8.35 (s, 1H, H-8), 7.83 (d, 1H, $J_{2,1}$ = 5.9 Hz, H-2), 6.52 (d, 1H, $J_{1,2}$ = 5.9 Hz, H-1), 6.41 (dd, 1H, $J_{1',2'a}$ = 7.75 Hz, $J_{1',2'b}$ = 6.30 Hz, H-1'), 4.61–4.58 (m, 1H, H-3'), 4.18–4.14 (m, 1H, H-4'), 3.92–3.80 (m, 2H, H-5' & H-5''), 3.67–3.64 (m, 1H, H- α), 2.73–2.29 (m, 4H, H-2', H-2'' & H- β). ^{13}C NMR (150 MHz, D₂O) δ : 181.2 & 181.1 (α & β CO), 147.4 (C-6), 144.5 (C-4), 143.6 (C-2), 139.1 (C-8), 122.4 (C-5), 103.8 (C-1), 85.8 (C-4'), 83.4 (C-1'), 71.2 (C-3'), 63.8 (C-5'), 54.7 (C- α), 42.9 (C- β), 38.6 (C-2'). ^{31}P NMR (121 MHz, D₂O) δ : 6.50. HRMS (ESI) calculated for C₁₅H₂₀N₅O₉P, 445.0998; found (negative mode) 444.0904 [M-H]⁻.

2'-Deoxy-1-deazaadenosine-5'-methylglycyl phosphoramidate (22a): TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.76. ^1H NMR (500 MHz, MeOD, 15 °C) δ : 8.47 (s, 1H, H-8), 7.92 (d, 1H, $J_{2,1}$ = 6.0 Hz, H-2), 6.53 (d, 1H, $J_{1,2}$ = 6.0 Hz, H-1), 6.51 (apparent t, 1H, $J_{1',2'a}$ = $J_{1',2'b}$ = 6.5 Hz, H-1'), 4.61–4.60 (m, 1H, H-3'), 4.12–4.13 (m, 1H, H-4'), 4.04–4.01 (m, 2H, H-5' & H-5''), 3.62 (d, 2H, $J_{\text{H}\alpha\text{-P}}$ = 10.5 Hz, H- α), 3.59 (s, 3H, CH₃), 2.75–2.69 (m, 1H, H-2'a), 2.45–2.40 (m, 1H, H-2'b). ^{13}C NMR (125 MHz, MeOD) δ : 175.5 (CO), 149.9 (C-6), 145.9 (C-4), 144.3 (C-2), 140.3 (C-8), 124.1 (C-5), 104.2 (C-1), 88.1 (C-4'), 85.6 (C-1'), 73.1 (C-3'), 65.6 (C-5'),

52.3 (COOCH₃), 44.3 (C-α), 41.5 (C-2'). ³¹P NMR (121 MHz, MeOD) δ: 6.84. ES-MS calculated for C₁₄H₂₀N₅O₇P, 401.11; found (negative mode) 399.7 [M-H]⁻.

2'-Deoxy-1-deazaadenosine-5'-glycyl phosphoramidate (22): TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.63. ¹H NMR (600 MHz, D₂O, 15 °C) δ: 8.34 (s, 1H, H-8), 7.89 (d, 1H, J_{2,1} = 6.4 Hz, H-2), 6.61 (d, 1H, J_{1,2} = 6.4 Hz, H-1), 6.44 (apparent t, 1H, J_{1',2'a} = J_{1',2'b} = 6.4 Hz, H-1'), 4.63–4.62 (m, 1H, H-3'), 4.24–4.23 (m, 1H, H-4'), 3.98–9.91 (m, 2H, H-5' & H-5''), 3.27 (d, 2H, J_{Hα-P} = 8.6 Hz, H-α), 2.78–2.73 (m, 1H, H-2'a), 2.56–2.52 (m, 1H, H-2'b). ¹³C NMR (150 MHz, D₂O, 15 °C) δ: 178.7 (CO), 149.3 (C-6), 141.8 (C-4), 140.5 (C-2), 139.3 (C-8), 122.6 (C-5), 103.6 (C-1), 85.9 (C-4'), 84.6 (C-1'), 71.2 (C-3'), 63.9 (C-5'), 44.6 (C-α), 38.9 (C-2'). ³¹P NMR (202 MHz, D₂O) δ: 8.13. HRMS (ESI) calculated for C₁₃H₁₈N₅O₇P, 387.0944; found (negative mode) 386.0877 [M-H]⁻.

2'-Deoxy-1-deazaadenosine-5'-methyl-β-alaninyl phosphoramidate (23a): TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.80. ¹H NMR (500 MHz, MeOD) δ: 8.46 (s, 1H, H-8), 7.87 (d, 1H, J_{2,1} = 5.7 Hz, H-2), 6.52 (apparent t, 1H, J_{1',2'a} = J_{1',2'b} = 6.5 Hz, H-1'), 6.45 (d, 1H, J_{1,2} = 5.7 Hz, H-1), 4.61–4.60 (m, 1H, H-3'), 4.14–4.09 (m, 1H, H-4'), 3.98–3.92 (m, 2H, H-5' & H-5''), 3.60 (s, 3H, CH₃), 3.12–3.09 (m, 2H, H-α), 2.79–2.73 (m, 1H, H-2'a), 2.48–2.42 (m, 3H, H-2'b & H-β). ¹³C NMR (125 MHz, MeOD) δ: 175.5 (CO), 149.7 (C-6), 148.5 (C-4), 146.9 (C-2), 140.9 (C-8), 124.8 (C-5), 105.1 (C-1), 88.6 (C-4'), 85.8 (C-1'), 73.9 (C-3'), 66.5 (C-5'), 52.8 (-COOCH₃), 42.2 (C-2'), 39.5 (C-α), 38.2 (C-β). ³¹P NMR (202 MHz, D₂O) δ: 7.71. HRMS (ESI) calculated for C₁₅H₂₂N₅O₇P, 415.1257; found (negative mode) 414.1180 [M-H]⁻.

2'-Deoxy-1-deazaadenosine-5'-β-alaninyl phosphoramidate (23): TLC (iPrOH/H₂O/NH₃, 7:1:2): R_f = 0.60. ¹H NMR (600 MHz, D₂O, 15 °C) δ: 8.32 (s, 1H, H-8), 7.92 (d, 1H, J_{2,1} = 6.1 Hz, H-2), 6.53 (d, 1H, J_{1,2} = 6.1 Hz, H-1), 6.40 (apparent t, 1H, J_{1',2'a} = J_{1',2'b} = 6.8 Hz, H-1'), 4.66–4.65 (m, 1H, H-3'), 4.22–4.21 (m, 1H, H-4'), 3.89–3.88 (m, 2H, H-5' & H-5''), 2.82–2.75 (m, 3H, H-α & H-2'a), 2.55–2.52 (m, 1H, H-2'b), 2.19 (t, 2H, J = 6.9 Hz, H-β). ¹³C NMR (150 MHz, D₂O, 15 °C) δ: 180.77 (CO), 148.1 (C-6), 142.3 (C-4), 142.2 (C-2), 139.0 (C-8), 122.4 (C-5), 103.7 (C-1), 85.8 (C-4'), 83.9 (C-1'), 71.1 (C-3'), 63.8 (C-5'), 39.0 (C-α), 38.7 (C-2'), 38.0 (C-β). ³¹P NMR (121 MHz, D₂O) δ: 8.81. HRMS (ESI) calculated for C₁₄H₂₀N₅O₇P, 401.1100; found (negative mode) 400.1033 [M-H]⁻.

Synthesis of 7-deazaadenine analogue (Scheme S3)

4,6-Diamino-5-iodopyrimidine (25): To a suspension of 4,6-diaminopyrimidine (**24**, available as hemisulfate, monohydrate; 3.9 g, 22 mmol) in H₂O (60 mL) containing K₂CO₃ (4.6 g, 33 mmol), a solution of iodine (6 g, 24 mmol) in DMF (14 mL) was added, and the reaction mixture was heated at 45 °C for 20 h. The reaction was quenched by addition of aqueous solution of sodium thiosulfate (2M, 5 mL). The resulting precipitate was filtered and washed with H₂O to obtain 4,6-diamino-5-iodopyrimidine (**25**) as pale brown solid (81%). TLC (DCM/MeOH, 9.5:0.5): *R_f* = 0.4. ¹H NMR (500 MHz, DMSO-*d*₆) δ: 7.71 (s, 1H, H-2), 6.30 (br s, 4H, -NH₂). ¹³C NMR (125 MHz, DMSO-*d*₆) δ: 162.9 (C-4 & C-6), 157.1 (C-2), 55.3 (C-5). HRMS (ESI) calculated for C₄H₅IN₄, 235.9561; found (positive mode) 236.9624 [M+H]⁺.

4,6-Diamino-5-trimethylsilylethynylpyrimidine (26): A solution of **25** (4 g, 17 mmol) in DMF (200 mL) containing triethylamine (3 mL, 20 mmol), trimethylsilylacetylene (5 mL, 34 mmol), CuI (645 mg, 3.4 mmol), and bis-(triphenylphosphine)-palladium(II)chloride (360 mg, 3.0 mol%) was heated at 60 °C for 2 h. The solvent was removed in vacuo, and the residue was diluted with ethylacetate. The organic layer was washed with 5% EDTA solution, followed by brine. The organic layer was dried (MgSO₄) and concentrated in vacuo. The residue was purified by silica gel column chromatography, eluting with hexane/ethylacetate (1:1 – 1:3) to obtain 4,6-diamino-5-trimethylsilylethynylpyrimidine (**26**) as brown solid (70%). TLC (DCM/MeOH, 9.5:0.5): *R_f* = 0.3. ¹H NMR (300 MHz, CDCl₃) δ: 8.05 (s, 1H, H-2), 5.25 (br s, 4H, -NH₂), 0.27 (s, 9H, Si(CH₃)₃). ¹³C NMR (75 MHz, CDCl₃) δ: 163.3 (C-4 & C-6), 156.9 (C-2), 107.3 (Si(CH₃)₃CC-), 95.7 (Si(CH₃)₃CC-), 81.0 (C-5), 0.1 (Si(CH₃)₃). HRMS (ESI) calculated for C₉H₁₄N₄Si, 206.0988; found (positive mode) 207.1065 [M+H]⁺.

4-Amino-7H-pyrrolo[2,3-*d*]pyrimidine/7-deazaadenine (27): To a solution of **26** (3.3 g, 16 mmol) in *N*-methylpyrrolidinone (30 mL), a solution of potassium *t*-butoxide (2.3 g, 21 mmol) in *N*-methylpyrrolidinone (90 mL) was added slowly at 90 °C. The reaction mixture was then stirred at the same temperature for 5 h. After cooling at room temperature, the reaction mixture was neutralized with 1 N HCl, and then the solvent was evaporated to dryness in vacuo. The residue was purified by silica gel column chromatography, eluting with 5-10% MeOH in CHCl₃ to obtain **27** (1.7 g, 81%). TLC (CHCl₃/MeOH, 9:1) *R_f* = 0.2. ¹H NMR (500 MHz, MeOD) δ: 8.05 (s, 1H, H-2), 7.07 (d, 1H, *J*_{6,5} = 3.5 Hz, H-6), 6.55 (d, 1H, *J*_{5,6} = 3.5 Hz, H-5). ¹³C NMR (125

MHz, MeOD) δ : 158.9 (C-4), 151.8 (C-2), 151.0 (C-7a), 122.8 (C-6), 104.0 (C-4a), 100.2 (C-5). HRMS (ESI) calculated for $C_6H_6N_4$, 134.0592; found (positive mode) 135.0668 $[M+H]^+$.

7-(2'-Deoxy-3',5'-di-O-*p*-toluoyl- β -D-ribofuranosyl)-4-aminopyrrolo-[2,3-*d*]pyrimidine (28):

A suspension of powdered KOH (141 mg, 2.5mmol) in dry acetonitrile (8.0 mL) was stirred at room temperature for 10 minutes. Then TDA-1 (70 μ L, 0.2mmol) was added and again stirred for 30 minutes at room temperature. A suspension of 4-amino-7*H*-pyrrolo[2,3-*d*]pyrimidine (**27**, 0.14 g, 1.0 mmol) in dry acetonitrile (12.0 mL) was added during 10 minutes and stirring was continued for another 30 minutes. A solution of sugar halide (**10**, 0.43 g, 1.1 mmol) was added during 10 minutes and stirring was continued for another 1 hour. Insoluble materials were filtered. The filtrate was evaporated to dryness and the residue was chromatographed on flash silica gel column in 80 % ethyl acetate in hexane to separate α and β isomers (**28a** and **28** respectively). The yield of **28** was found to be 57 %. The more polar N1 isomer (**28b**) was collected by eluting with ethyl acetate (100 %). TLC (DCM/MeOH, 9.5:0.5): R_f = 0.40. 1H NMR (300 MHz, $CDCl_3$) δ : 8.32 (s, 1H, H-2), 7.99–7.94 (m, 4H, aromatic-H), 7.28–7.14 (m, 4H, aromatic-H), 7.15 (d, 1H, $J_{6,5}$ = 3.6 Hz, H-6), 6.82 (dd, 1H, $J_{1',2'a}$ = 8.3 Hz, $J_{1',2'b}$ = 5.7 Hz, H-1'), 6.39 (d, 1H, $J_{5,6}$ = 3.6 Hz, H-5), 5.76–5.74 (m, 1H, H-3'), 5.54 (br s, 2H, NH_2), 4.72–4.59 (m, 3H, H-4', H-5' & H-5''), 2.90–2.69 (m, 2H, H-2'a & H-2'b), 2.43 (s, 3H, aromatic- CH_3), 2.41 (s, 3H, aromatic- CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ : 165.9 & 165.8 (CO), 156.4 (C-4), 151.9 (C-2), 150.5 (C-7a), 144.1 & 143.7 (aromatic ring C), 129.5–126.2 (aromatic ring C), 120.9 (C-6), 103.4 (C-4a), 98.9 (C-5), 83.5 (C-1'), 81.6 (C-4'), 74.9 (C-3'), 64.1 (C-5'), 37.7 (C-2'), 21.4 (aromatic- CH_3). HRMS (ESI) calculated for $C_{27}H_{26}N_4O_5$, 486.1903; found (positive mode) 487.1974 $[M+H]^+$.

7-(2'-Deoxy-3',5'-di-O-*p*-toluoyl- α -D-ribofuranosyl)-4-aminopyrrolo-[2,3-*d*]pyrimidine

(28a): 1H NMR (300 MHz, $CDCl_3$) δ : 8.32 (s, 1H, H-2), 7.99–7.80 (m, 4H, aromatic-H), 7.15 (d, 1H, $J_{6,5}$ = 3.7 Hz, H-6), 7.39–7.23 (m, 4H, aromatic-H), 6.82 (dd, 1H, $J_{1',2'a}$ = 9.2 Hz, $J_{1',2'b}$ = 2.3 Hz, H-1'), 6.43 (d, 1H, $J_{5,6}$ = 3.7 Hz, H-5), 5.69–5.67 (m, 1H, H-3'), 5.50 (br s, 2H, NH_2), 4.87–4.86 (m, 1H, H-4'), 4.61–4.60 (m, 2H, H-5' & H-5''), 3.18–2.80 (m, 2H, H-2'a & H-2'b), 2.44 (s, 6H, aromatic- CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ : 165.8 & 165.7 (CO), 156.4 (C-4), 151.7 (C-2), 150.9 (C-7a), 144.1 & 143.7 (aromatic ring C), 129.5–126.2 (aromatic ring C), 121.5 (C-6),

103.4 (C-4a), 98.0 (C-5), 84.6 (C-1'), 83.3 (C-4'), 75.0 (C-3'), 64.1 (C-5'), 38.2 (C-2'), 21.4 (aromatic-CH₃).

1-(2'-Deoxy-3',5'-di-*O*-*p*-toluoyl- β -D-ribofuranosyl)-4-aminopyrrolo-[2,3-*d*]pyrimidine

(28b): TLC (DCM/MeOH, 9.5:0.5): R_f = 0.1. ¹H NMR (500 MHz, CDCl₃) δ : 8.48 (s, 1H, H-2), 7.99–7.83 (m, 4H, aromatic-H), 7.50 (br s, 1H, H-6), 7.28–7.18 (m, 4H, aromatic-H), 6.98 (apparent t, 1H, $J_{1',2'a} = J_{1',2'b} = 6.7$ Hz, H-1'), 6.51 (br s, 1H, H-5), 5.69–5.67 (m, 1H, H-3'), 5.44 (br s, 2H, NH₂), 4.79–4.73 (m, 3H, H-4', H-5' & H-5''), 3.32–3.23 (m, 1H, H-2'a), 2.64–2.58 (m, 1H, H-2'b), 2.44 (s, 3H, aromatic-CH₃), 2.39 (s, 3H, aromatic-CH₃). ¹³C NMR (125 MHz, CDCl₃) δ : 166.5 & 166.4 (CO), 155.9 (C-4), 146.4 (C-7a), 144.9 & 144.6 (aromatic ring C), 139.4 (C-6), 137.9 (C-2), 130.3–126.7 (aromatic ring C), 105.5 (C-4a), 98.8 (C-5), 89.1 (C-1'), 84.3 (C-4'), 75.2 (C-3'), 64.5 (C-5'), 40.5 (C-2'), 22.0 (aromatic-CH₃).

7-(2'-deoxy- β -D-ribofuranosyl)-4-aminopyrrolo-[2,3-*d*]pyrimidine/2'-deoxy-7-

deazaadenosine (29): Toluoyl group deprotection reaction was performed according to the procedure mentioned for compound **17**, keeping the reaction mixture at room temperature for 48 hours. TLC (DCM/MeOH, 9.5:0.5): R_f = 0.1. ¹H NMR (300 MHz, DMSO-*d*₆) δ : 8.04 (s, 1H, H-2), 7.34 (d, 1H, $J_{6,5} = 3.6$ Hz, H-6), 7.02 (br s, 2H, NH₂), 6.58 (d, 1H, $J_{6,5} = 3.6$ Hz, H-5), 6.47 (dd, 1H, $J_{1',2'a} = 8.01$ Hz, $J_{1',2'b} = 6.0$ Hz, H-1'), 5.26 (d, 1H, 3'-OH), 5.18 (t, 1H, 5'-OH), 4.34–4.33 (m, 1H, H-3'), 3.82–3.41 (m, 3H, H-4', H-5' & H-5''), 2.51–2.12 (m, 2H, H-2'a & H-2'b). ¹³C NMR (75 MHz, DMSO-*d*₆) δ : 158.5 (C-4), 152.6 (C-2), 150.6 (C-7a), 122.6 (C-6), 103.9 (C-4a), 100.7 (C-5), 88.2 (C-4'), 84.3 (C-1'), 72.1 (C-3'), 63.1 (C-5'), 40.4 (C-2'). Yield = 95%. HRMS (ESI) calculated for C₁₁H₁₄N₄O₃, 250.1066; found (positive mode) 251.1132 [M+H]⁺.

2'-Deoxy-7-deazaadenosine-5'-monophosphate (30): TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.3.

¹H NMR (500 MHz, D₂O) δ : 8.04 (s, 1H, H-2), 7.49 (d, 1H, $J_{8,7} = 3.7$ Hz, H-8), 6.57–6.54 (m, 2H, H-7 & H-1'), 4.68–4.67 (m, 1H, H-3'), 4.19–4.18 (m, 1H, H-4'), 4.00–3.98 (m, 2H, H-5' & H-5''), 2.69–2.63 (m, 1H, H-2'a), 2.47–2.45 (m, 1H, H-2'b). ¹³C NMR (125 MHz, D₂O) δ : 154.5 (C-6), 147.7 (C-4 & C-2), 122.5 (C-8), 102.3 (C-5), 100.7 (C-7), 85.1 (C-4'), 82.6 (C-1'), 71.1 (C-3'), 64.1 (C-5'), 38.5 (C-2'). ³¹P NMR (121MHz, D₂O) δ : 1.87. Yield = 81%. HRMS (ESI) calculated for C₁₁H₁₅N₄O₆P, 330.0729; found (negative mode) 329.0653 [M-H]⁻.

2'-Deoxy-7-deazaadenosine-5'-dimethoxyaspartyl phosphoramidate (31): TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.8. ¹H NMR (500 MHz, D₂O): δ : 8.15 (s, 1H, H-2), 7.58 (d, 1H, $J_{8,7}$ = 3.7 Hz, H-8), 6.72 (d, 1H, $J_{7,8}$ = 3.7 Hz, H-7), 6.60 (apparent t, 1H, $J_{1',2'a}$ = $J_{1',2'b}$ = 6.8 Hz, H-1'), 4.69–4.68 (m, 1H, H-3'), 4.20–4.19 (m, 1H, H-4'), 4.00–3.96 (m, 3H, H-5', H-5'' & H- α), 3.69 (s, 3H, CH₃), 3.59 (s, 3H, CH₃), 2.72–2.67 (m, 3H, H-2'a & H- β), 2.52–2.48 (m, 1H, H-2'b). ¹³C NMR (125 MHz, D₂O) δ : 174.8 & 172.9 (α & β CO), 151.6 (C-6), 146.8 (C-4), 143.6 (C-2), 123.9 (C-8), 101.9 (C-5), 101.6 (C-7), 85.2 (C-4'), 83.0 (C-1'), 71.1 (C-3'), 63.9 (C-5'), 52.5 (COOCH₃), 51.9 (COOCH₃), 50.9 (C- α), 38.7 (C-2'), 38.1 (C- β). ³¹P NMR (121MHz, D₂O) δ : 5.77. Yield = 70%. HRMS (ESI) calculated for C₁₇H₂₄N₅O₉P, 473.1312; found (negative mode) 472.1235 [M-H]⁻.

2'-Deoxy-7-deazaadenosine-5'-aspartyl phosphoramidate (32): TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.3. ¹H NMR (500 MHz, D₂O): δ : 8.14 (s, 1H, H-2), 7.56 (d, 1H, $J_{8,7}$ = 3.8 Hz, H-8), 6.69 (d, 1H, $J_{7,8}$ = 3.8 Hz, H-7), 6.65 (apparent t, 1H, $J_{1',2'a}$ = $J_{1',2'b}$ = 6.8 Hz, H-1'), 4.69–4.65 (m, 1H, H-3'), 4.21–4.17 (m, 1H, H-4'), 3.99–3.86 (m, 2H, H-5' & H-5''), 3.79–3.75 (m, 1H, H- α), 2.82–2.67 (m, 4H, H-2'a, H-2'b & H- β). ¹³C NMR (125 MHz, D₂O) δ : 180.9 & 177.1 (α & β CO), 156.1 (C-6), 149.7 (C-2), 148.6 (C-4), 122.2 (C-8), 102.7 (C-5), 100.5 (C-7), 85.2 (C-4'), 82.7 (C-1'), 71.4 (C-3'), 63.8 (C-5'), 54.5 (C- α), 42.8 (C- β), 38.3 (C-2'). ³¹P NMR (121MHz, D₂O) δ : 7.00. Yield = 80%. HRMS (ESI) calculated for C₁₅H₂₀N₅O₉P, 445.0998; found (negative mode) 444.0919 [M-H]⁻.

2'-Deoxy-3-deazaadenosine-5'-aspartyl phosphoramidate (33): TLC (iPrOH/H₂O/NH₃, 6:2:2): R_f = 0.3. ¹H NMR (500 MHz, D₂O): δ : 8.51 (s, 1H, H-8), 7.56 (d, 1H, $J_{2,3}$ = 7.1 Hz, H-2), 7.20 (d, 1H, $J_{3,2}$ = 3.8 Hz, H-3), 6.85 (apparent t, 1H, $J_{1',2'a}$ = $J_{1',2'b}$ = 6.8 Hz, H-1'), 4.70–4.67 (m, 1H, H-3'), 4.28–4.24 (m, 1H, H-4'), 4.06–3.97 (m, 2H, H-5' & H-5''), 3.80–3.75 (m, 1H, H- α), 2.74–2.41 (m, 4H, H-2'a, H-2'b & H- β). ¹³C NMR (125 MHz, D₂O) δ : 180.8 & 178.9 (α & β CO), 147.7 (C-6), 142.8 (C-8), 138.9 (C-4), 129.3 (C-2), 125.4 (C-5), 99.1 (C-3), 86.0 (C-4'), 85.4 (C-1'), 70.6 (C-3'), 63.5 (C-5'), 54.4 (C- α), 42.6 (C- β), 39.3 (C-2'). ³¹P NMR (121MHz, D₂O) δ : 6.95. Yield = 50%. HRMS (ESI) calculated for C₁₅H₂₀N₅O₉P, 445.0998; found (negative mode) 444.0910 [M-H]⁻.