Amino Acids

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Amino Acid Phosphoramidate Nucleotides as Alternative Substrates for HIV-1 Reverse Transcriptase**

*Olga Adelfinskaya and Piet Herdewijn**

There has been significant progress in the design and synthesis of numerous nucleotide analogues bearing a modified nucleobase moiety or unnatural sugar and that are substrates for polymerases. Modifications at the phosphate moiety are introduced to increase the stability of a nucleotide toward enzymatic degradation or to mask the phosphate negative charge and facilitate its penetration into a cell. A common strategy in nucleotide prodrug design is protecting a phosphate moiety with a labile masking group. Removal of a masking group liberates a nucleoside monophosphate entity to be transformed to a nucleoside triphosphate, a substrate for intracellular enzymes.[1–4] However, even after removal of the masking group, phosphorylation and activation of nucleoside monophosphates remain a problem owing to substrate specificity of cellular kinases.[3,5,6] Therefore, design of a nucleotide analogue that would allow bypassing of the kinase activation pathway while behaving as a direct polymerase substrate/inhibitor would be a considerable challenge.

The present study focuses on the ability of amino acid phosphoramidates of 2′-deoxyadenosine-5′-monophosphate (AA-dAMP) to serve as substrates for HIV reverse transcriptase (RT). It was rationalized that a successful triphosphate mimetic should possess structural and electronic features that enable it to provide a proper alignment of the α-phosphorus atom in the polymerase active site and coordination of catalytic metal ions. Furthermore, reactivity and susceptibility of the α-phosphate to a nucleophilic attack can be enhanced by attachment of a good leaving group through the P–O or P–N bond. A well-known example of a similar approach is phosphoimidazolide nucleotides in which the α-phosphorus atom is activated by imidazolide or methyl imidazolide moieties. Such deoxy- and ribonucleotides phosphoimidazolides were successfully demonstrated as efficient substrates for nonenzymatic templated and nontemplated oligomerization.[7,8]

Therefore, we explored the possibility of activating a nucleoside monophosphate by coupling it to the natural amino acid moiety through the P–N bond. A rationale behind this approach was that amino acid residues could provide a negative charge (aspartic and glutamic acids) and/or complex with metals (histidine, serine)[9] while serving as a leaving group. Additionally, steric and hydrophobic effects were probed by coupling deoxyadenosine monophosphate to glycine, proline, alanine, and tyrosine. As the leaving groups are natural amino acids and nontoxic, in principle, such an approach could be used in vivo.

A series of L-amino acid phosphoramidate nucleotide analogues and their respective methyl ester derivatives were synthesized according to a previously reported method (Figure 1).[10] Deprotection with 0.04 M NaOH afforded amino acid phosphoramidate nucleotides in moderate yields. Furthermore, our work demonstrates efficient incorporation of **1** and **6**, suggesting that Asp and His amino acid side chains can mimic the pyrophosphate moiety possibly by providing structural and electrostatic features essential for metal coordination and arrangement of the catalytic residues in the polymerase active site.[11,12]

The ability of HIV RT to incorporate amino acid phosphoramidates **1**–**8** and **1a**–**8a** into a growing DNA chain was explored by using a gel-based single-nucleotide-incorporation assay[13,14] with primer (P1) and template (T1; Figure 2).

Among all the analogues, the most remarkable results were observed with Asp-dAMP (**1**). Steady-state kinetic analysis (Table 1) of Asp-dAMP incorporation[13,14] indicates that V_{max} for incorporation of **1** is only theefold lower than that of an HIV RT natural substrate (dATP). As a result, the specificity, or a V_{max}/K_m value, for insertion of amino acid phosphoramidate against a natural nucleobase is decreased 1300-fold.

The kinetic analysis for the other amino acids phosphoramidates (**2**–**8**) was not attempted owing to an apparently slow reaction rate. However, compared with Asp-dAMP (**1**), His-dAMP(**6**) was the only other analogue that showed considerable incorporation. No incorporation occurred when respective methyl ester derivatives **1a**–**8a** were used as substrates in the polymerase reaction. Another unexpected result was obtained with the Glu-dAMP analogue (**2**), which behaved very poorly as an HIV RT substrate. These observations suggest that the process of recognition and incorporation of amino acid dAMPs is very specific.

Further investigation also revealed that HIV RT is able to use phosphoramidate nucleotides for a continuous template-dependent DNA synthesis. Among a series of phosphoramidate nucleotides (**1**–**8**), the most encouraging results were observed with **1** and **6**, which resulted in primer extension by up to three deoxyadenine nucleobases (T3 template; Figures 1 and 2). However, stalling occurs after the addition of two nucleobase residues with the $(n+2)$ product dominant to

[*] Dr. O. Adelfinskaya, Prof. P. Herdewijn
Rega Institute for Medical Research, K.U.Leuven
Laboratory of Medicinal Chemistry
Minderbroedersstraat 10, 3000 Leuven (Belgium)
Fax: (+32) 16-337-340
E-mail: piet.herdewijn@rega.kuleuven.be

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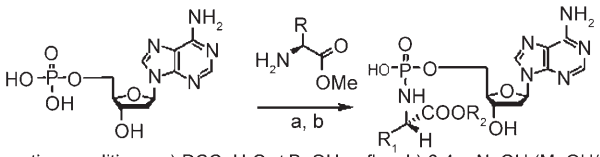
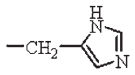
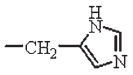
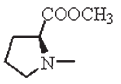


Reaction conditions: a) DCC, H_2O, *t*-BuOH, reflux. b) 0.4 M NaOH (MeOH/H_2O)

	R^2	R^1		R^2	R^1
1	H	-CH_2-COOH	**1a**	CH_3	-CH_2-$COOCH_3$
2	H	-CH_2-CH_2-COOH	**2a**	CH_3	CH_2-CH_2-$COOCH_3$
3	H	-H	**3a**	CH_3	-H
4	H	-CH_3	**4a**	CH_3	-CH_3
5	H	-CH_2-Ph-OH	**5a**	CH_3	-CH_2-Ph-OH
6	H		**6a**	CH_3	
7	H	-CH_2OH	**7a**	CH_3	-CH_2OH
8	H		**8a**	CH_3	

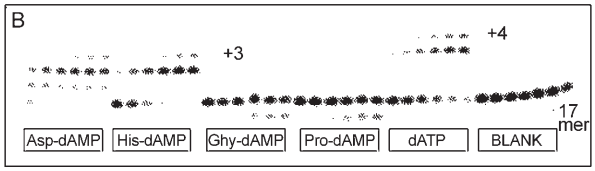


Figure 1. A) General scheme for the synthesis of amino acid phosphoramidates. B) Primer extension with amino acid phosphoramidates by HIV RT. Conditions: Primer (P1) was 5′-labeled with ^{33}P followed by annealing to a template T3. Reactions were carried out with 125 nM primer/template (P1/T3), [AA-dAMP] = 500 μM, [dATP] = 50 μM, [HIV RT] = 0.03 U μL^{-1}, time points: 5, 15, 30, 60, 90, 120 min. Blank: 125 nM primer/template (P1/T3), [HIV RT] = 0.03 U μL^{-1}, no nucleotide substrate. In the case of compounds **8** and **8a**, the structure of the whole amino acid (L-proline and L-proline methyl ester, respectively) is depicted in the table and not only of the amino acid side chain. DCC = 1,3-dicyclohexylcarbodiimide.

P1 5'-p- CAG GAA ACA GCT ATG AC
T1 GTC CTT TGT CGA TAC TGT CCC C-5'

P1 5'-p- CAG GAA ACA GCT ATG AC
T3 GCT CTT TGT CGA TAC TGT TTC C-5'

Figure 2. Primer and templates used to study the chain-elongation reaction with amino acids derivatives of dAMP.

Table 1: Steady-state kinetics: incorporation of Asp-dAMP by HIV-1 RT.

Substrate	V_{max} (pmol min^{-1} U)	K_M (μM)	V_{max}/K_M ($\times 10^6$)
dATP	8.39 ± 0.82	0.46 ± 0.15	18.1
1	2.63 ± 0.13	185.3 ± 24.55	0.014

the ($n+3$) product (56.3% versus 5.2% for **1** and 67.1% versus 13.5% for **6**). It is not yet clear what causes the stalling of the HIV RT after incorporation of two adenine nucleotides. The fact that the same result was observed with **6** could suggest that a common mechanism is involved. We do not have direct evidence, at this moment, for competitive or noncompetitive substrate or product inhibition, and additional experiments are required to determine the cause of the observed stalling.

Based on the presented results, it can be implied that successful recognition of an incoming nucleotide is controlled by certain structural and electrostatic features. It was suggested previously that initial recognition of an incoming dNTP occurs through the binding of the triphosphate moiety.[15] Structural and genetic analysis of several DNA polymerases and reverse transcriptase indicates that amino acid residues involved in the triphosphate binding are highly conserved.[11,12,15] In the case of HIV RT, binding of the incoming dNTP is coordinated by Arg72 and Lys65 which interact with the α- and γ-phosphates, respectively.[12]

Importantly, studies have shown that the binding of the incoming dNTP and catalytic metal ions is responsible for further rearrangements of the catalytic amino acid residues as well as the relocation of the 3′-primer terminus to a position that is effective for nucleotidyl transfer.[12] Incorporation of a nucleotide residue takes place through an "in-line" attack of the 3′-OH group, activated by the catalytic metal ion at the α-phosphorus atom of the triphosphate moiety.

Therefore, a proper geometric and spatial arrangement of all reacting residues and atoms is essential for the formation of the productive tertiary complex.[11,16,17] Efficient incorporation of **1** might imply that an aspartyl amino acid effectively replaces the β- and γ-phosphate groups, likely, by providing electrostatic interactions for metal coordination and contacts to Lys65. In line with this observation, anionic citrate was previously suggested as being a good mimetic of a triphosphate moiety.[18] It can also be suggested that the aspartate moiety acts as a good leaving group. The study presented herein clearly demonstrates the requirement for the presence of the negative charge and electrostatic interactions for the efficient triphosphate binding. This is evident from the study with the aspartyl phosphoramidate (**1**) and bismethyl aspartyl phosphoramidate (**1a**) in which the protection of carboxylate groups brings drastic changes in the ability of HIV RT to recognize and incorporate these two modified substrates.

Therefore, Asp-dAMP (**1**) and His-dAMP (**6**) demonstrate unique substrate properties and could serve as alternative substrates for reverse transcriptase. Coupling of modified nucleoside monophosphates (such as analogues of dideoxynucleosides) with alternative leaving groups (instead of pyrophosphate) could, theoretically, convert a modified nucleoside into an active antiviral by allowing a bypass of the nucleoside/nucleotide kinase activation cascade. With the exception of the bypass of the kinase pathway for intracellular activation of modified nucleosides (antiviral and antitumoral application), this finding can be important in two other research fields: nucleoside triphosphate mimetics, which are substrates for polymerases, can be used in a polymerase chain reaction and in synthetic biology, for which further study must

be expanded to other (thermostable) polymerases. The study of the potential of amino acids to function as a leaving group in prebiotic chemistry should also not be overlooked.

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