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PEPTIDE CHEMISTRY

Aza-peptides: expectations and reality

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Abstract. The replacement of the α-carbon atom in an α-amino acid structure by a nitrogen atom yields alkylcarbazic acids, also known as α-aza amino acids. Although the topology of α-amino acids and α-aza amino acids is similar, their chemical and stereochemical properties are significantly different. For this reason, the application of the common solid-phase peptide synthesis (SPPS) protocol cannot be used for aza-peptide bond synthesis without changes. On the other hand, the aza-peptide bond is more stable than the common peptide bond, therefore these compounds are very attractive targets for drug design. In this review, we summarize data on aza-peptide bond chemistry, with implications for the improvement of aza-peptide chemical synthesis.

Keywords: aza-amino acid precursors, aza-peptide synthesis, aza-amino acid chemistry, hydrazine derivatives, activators, limits of SPPS protocol for aza-peptide synthesis.

Abbreviations and symbols

Bn	benzyl
Boc	*tert*-butyloxycarbonyl
COMU	(1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate
Ddz	α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl
Fmoc	9-fluorenylmethoxycarbonyl
HATU	(1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HCTU	*O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HDMC	*N*-[(5-chloro-3-oxido-1*H*-benzotriazol-1-yl)-4-morpholinylmethylene]-*N*-methylmethanaminium hexafluorophosphate
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
LFE	linear free energy
Oxyma	ethyl cyano(hydroxyimino)acetate
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
PG	protecting group
PyOxim	[ethyl cyano(hydroxyimino)acetato-O^2]tri-1-pyrrolidinylphosphonium hexafluorophosphate
TBTU	2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate
*t*Bu	*tert*-butyl
Z	benzyloxycarbonyl

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INTRODUCTION

Biologically active peptides are ideal drug candidates due to their biocompatibility and specificity for various pharmacological targets. Therefore, peptide-based pharmaceuticals are a rapidly growing category in drug design, illustrated by an increased number of biologics among FDA-approved drugs in recent years [1,2]. However, peptide druggability is limited by their rapid degradation in living organisms caused by hydrolytic enzymes [3,4]. Hence, the lifetime of peptide-based drugs in organisms could be increased by chemical modification of the natural peptide structure, including exchange of L-amino acids to non-natural D-isomers, *N*-alkylation of natural amino acids or cyclization of peptides [1,2,5]. These compounds are called peptidomimetics, and their elements mimic a natural peptide or protein in 3D space. Thus, it is generally accepted that peptidomimetics retain the ability to interact with biological target sites and produce the same biological effect as natural peptides. In addition to increased stability against proteolysis, certain other properties, such as receptor selectivity or potency, can be even improved in peptidomimetic ligands [6].

One approach to increasing the stability of peptides is chemical modification of their backbone structure via isosteric substitution of the α-carbon atom in α-amino acid (***I***) with a nitrogen atom (Fig. 1.). This replacement yields alkylcarbazic acids (***II***), also known as α-aza amino acids [7,8], and their inclusion in the peptide chain yields aza-peptides.

Although aza-peptides are topological analogs of common peptides, they exhibit no chirality [2,9] and have different hydrogen bonding properties and reduced backbone flexibility [9–20]. Although these structural changes may disrupt the β-sheet secondary structure of the parent sequence [20], several aza-peptides have shown biological effects on a number of therapeutic targets, including the inhibition of various proteases in the treatment of viral infections [21–26]. For example, atazanavir (Reyataz®) is an antiretroviral drug used to treat HIV [27]. Aza-peptide-based agonists and antagonists of several receptors have also been reported as promising lead compounds [10,13,28–30]. Most importantly, the aza-peptide bond

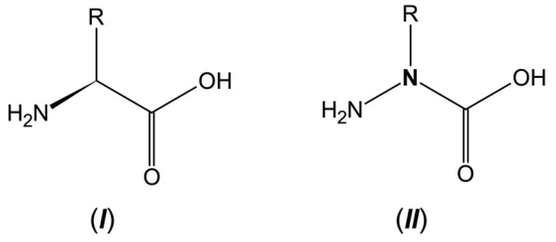


Fig. 1. Structure of α-amino acid (***I***) and α-aza-amino acid (***II***).

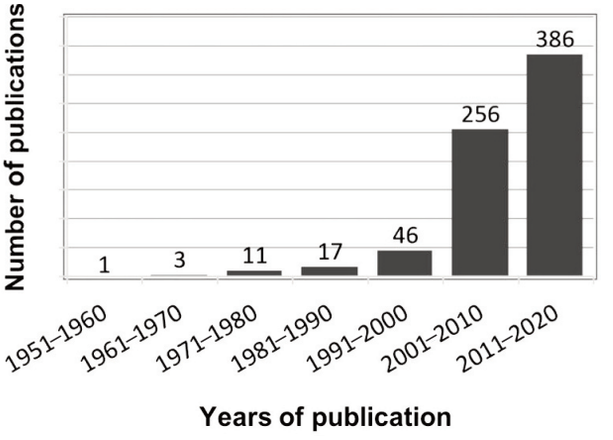


Fig. 2. Number of published papers found in the Google Scholar database under the keyword 'aza-peptide'.

-NH-NR-C(O)- is more resistant to enzymatic hydrolysis than the common peptide bond **-NH-CHR-C(O)-** [2,10,11]. This makes aza-peptides attractive targets for drug design.

Although the aza-peptide structure was first reported in 1951 [31], the aza-peptides were rarely studied for a long time [10,26,30,32–36], and greater interest in these substances has emerged since 2000, as seen in Fig. 2.

This delay in aza-peptide research can be attributed to difficulties in aza-peptide synthesis, as the process is rather complicated compared with the synthesis of conventional peptides. In this review, we focus on these complications, many of which have been studied for several years in our laboratory.

Aza-peptide synthesis needs precursors

In contrast to conventional amino acids (***I***), their aza-analogs (***II***) decarboxylate easily and do not exist as stable compounds [37]. Therefore, aza-peptides are usually synthesized by proceeding from orthogonally protected mono-substituted hydrazines (***III***), which are known as precursors of aza-amino acids (Fig. 3.). In these compounds, the substituent R corresponds to the side chain of the mimicked amino acid, and the selection of the protecting group (PG) is determined by the needs of the appropriate synthesis protocol. Although several of these

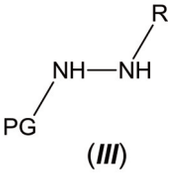


Fig. 3. Structure of α-aza-amino acid precursor.

precursors are commercially available, the preparation of substituted hydrazines that correspond to the sidechains of native amino acids is a rather challenging task. A summary of the structures of these compounds and references to works describing the synthesis of these precursors is given in Table 1.

Table 1. Structures of DNA-encoded amino acids, corresponding α-aza-amino acid precursors, and references to the synthesis of these precursors with different protecting groups (PGs)

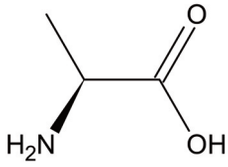
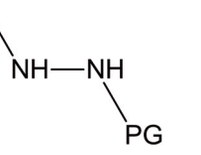
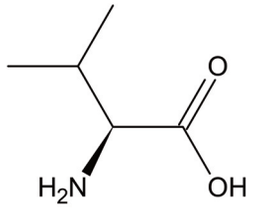
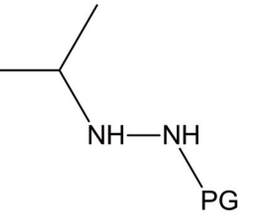
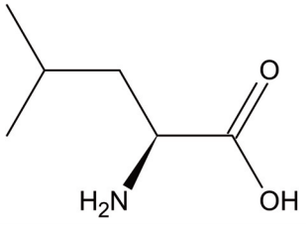
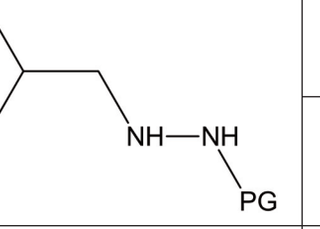
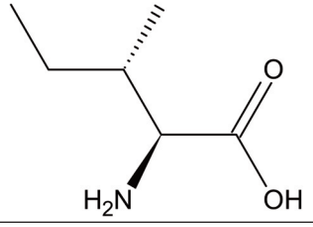
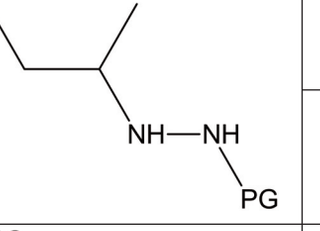
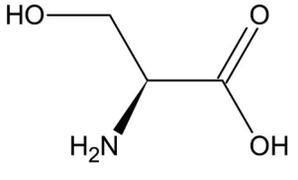
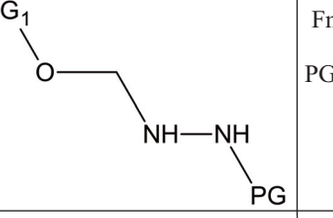
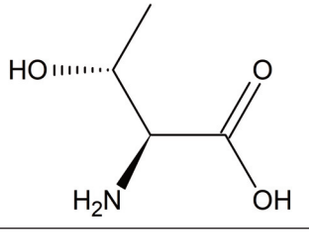
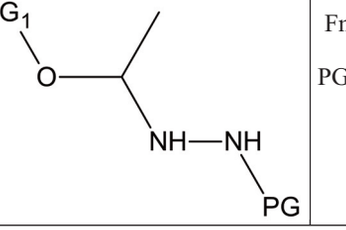
Amino acid	Amino acid structure	Aza-amino acid precursor	PG	References
Alanine			Fmoc	[10,38,39]
			Boc	[13,40,41]
Valine			Fmoc	[10,28,42]
			Boc	[13,32,43]
Leucine			Fmoc	[10]
			Boc	[44,45]
Isoleucine			Boc	[7,43]
			Ddz	[34]
Serine			Fmoc, Boc; PG_1: *t*Bu, Bn	No precursor described
Threonine			Fmoc, Boc; PG_1: *t*Bu, Bn	No precursor described

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Table 1. *Continued.*

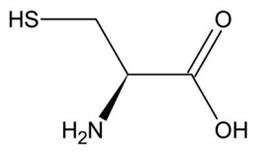
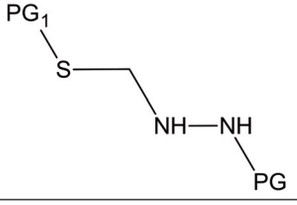
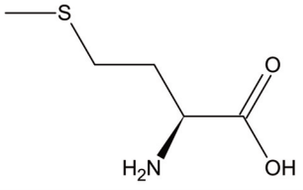
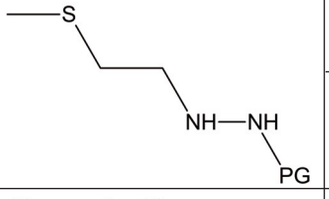
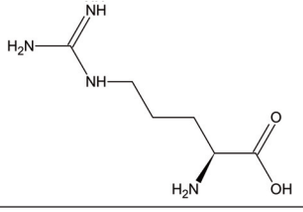
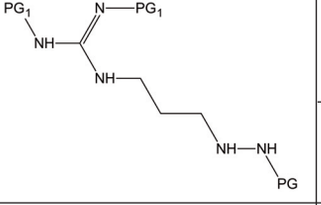
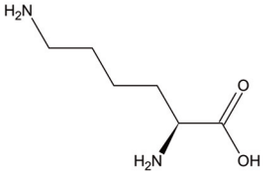
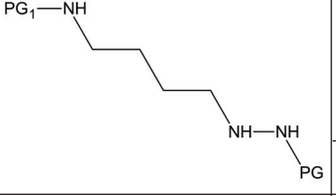
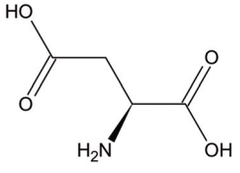
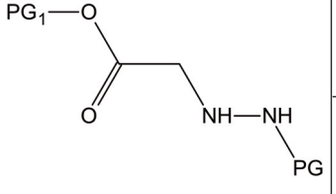
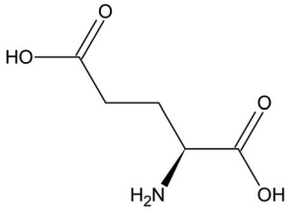
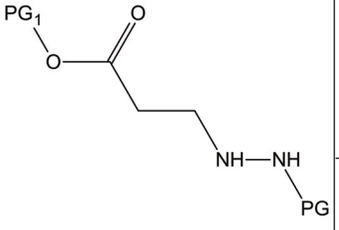
Amino acid	Amino acid structure	Aza-amino acid precursor	PG	References
Cysteine			Fmoc, Boc; PG_1: Trt	No precursor described
Methionine			Fmoc	[46]
			Boc	[46]
Arginine			Fmoc; PG_1: Boc	[26,47]
			On resin	[10,48]
Lysine			Fmoc; PG_1: Boc	[10]
			On resin	[48]
Aspartic acid			Fmoc; PG_1: *t*Bu	[10,28,49,50]
			Boc; PG_1 *t*Bu	[50,51]
Glutamic acid			Fmoc; PG_1: *t*Bu	[39]
			Boc; PG_1: CH_3	[49]

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Table 1. *Continued.*

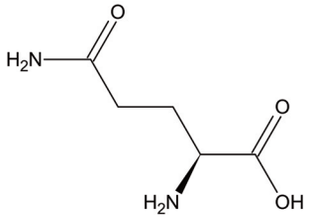
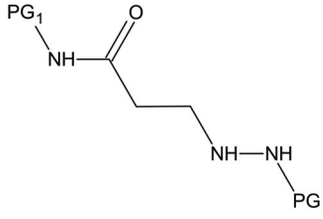
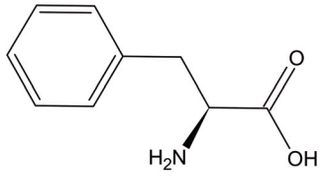
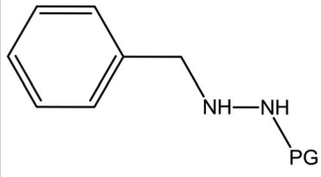
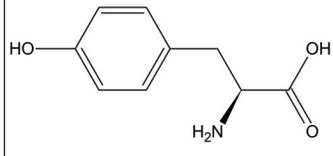
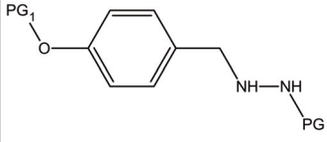
Amino acid	Amino acid structure	Aza-amino acid precursor	PG	References
Glutamine			Boc; PG_1: H	[49,52]
Phenylalanine			Fmoc	[10,28,50,53,54]
			Boc	[13,50,55,56]
Tyrosine			Fmoc; PG_1: $Si(CH_3)_2tBu$	[10]
			Ddz; PG_1: *t*Bu	[34]
			Boc; PG_1: *t*Bu	[7]
			Boc; PG_1: (OH non-protected)	[7]
			Fmoc, Boc; PG_1: Boc, Bn	[56]
			Fmoc, Boc, Z; PG_1: CH_3	[56]

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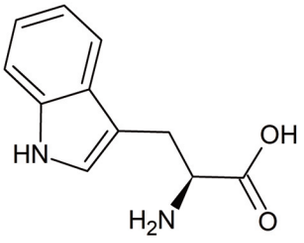
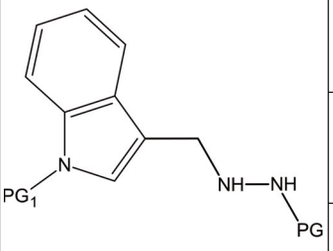
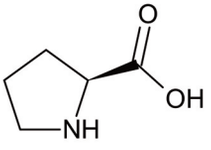
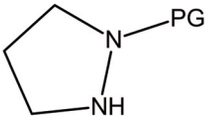
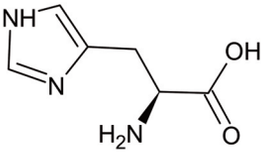
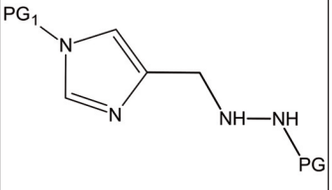
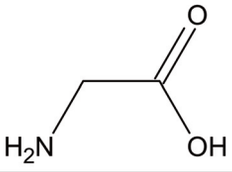
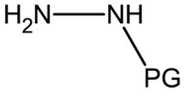
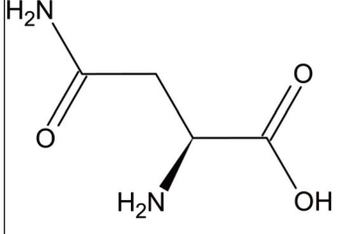
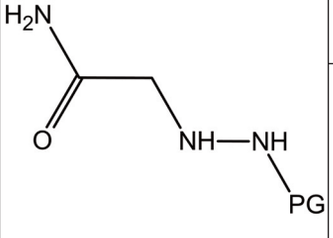
Table 1. *Continued.*

Amino acid	Amino acid structure	Aza-amino acid precursor	PG	References
Tryptophan			Boc; PG_1: H	[7]
			Fmoc; PG_1: Boc	[10,57]
			Ddz; PG_1: Boc	[34]
			Fmoc, Boc; PG_1: Z	[56]
			Z; PG_1: Boc	[57]
Proline			Fmoc	[26]
			Boc	[7]
			Z	[49]
Histidine			Fmoc, Boc; PG_1: Trt, Boc	No precursor described
Glycine			Fmoc	[10,58]
			Boc	[59–61]
			Z	[47,62–64]
Asparagine			Boc	[49]
			Z	[65]

In general, two different approaches can be used to obtain these precursors. First, the direct alkylation of protected hydrazine is schematically the simplest approach that can be used for this synthesis (Scheme 1) [34,38,47,49–51,56].

However, direct alkylation may produce polyalkylated products, as the first alkylation step increases the nucleophilicity of the alkylated nitrogen atom [66]. Although the formation of these side products can be suppressed by applying appropriate reagent concentrations and solvents, it is important to consider this inconvenience in practical synthesis. Secondly, as the alkylation rate depends on the reactivity of halide, iodides are preferred over chlorides. However, as alkyl iodides are relatively unstable compounds, their purification and application for synthesis may cause serious problems. To overcome this complication, we improved the synthetic procedure by using potassium iodide catalysis [50]. This shortens the reaction time and improves the reaction outcome.

Due to the above-mentioned complications, reductive alkylation is more frequently the preferred method of preparing precursors, especially due to commercial availability and the relatively low cost of needed carbonyl compounds. Moreover, this method prevents the formation of polyalkylated compounds.

Another widely used synthetic approach is based on the reduction of hydrazones formed in the reaction of monoprotected hydrazine with a carbonyl compound [7,10,13,26,34,55,57]. These reactions are summarized in Scheme 2.

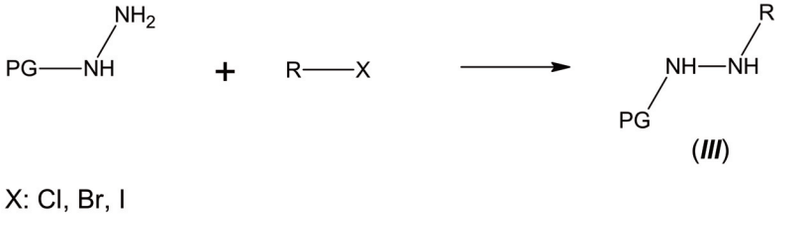
Although the latter reaction path has more steps than the alkylation reaction, there are several reasons why this synthetic protocol is often preferred.

The first step of the reductive hydrazine alkylation procedure occurs selectively under mild conditions, and the formed hydrazones can be reduced by different methods, including catalytic hydrogenation. The selectivity of the process and the possibility of obtaining products with different protecting groups have been used to produce a variety of aza-amino acid precursors (***IV***) (Table 1).

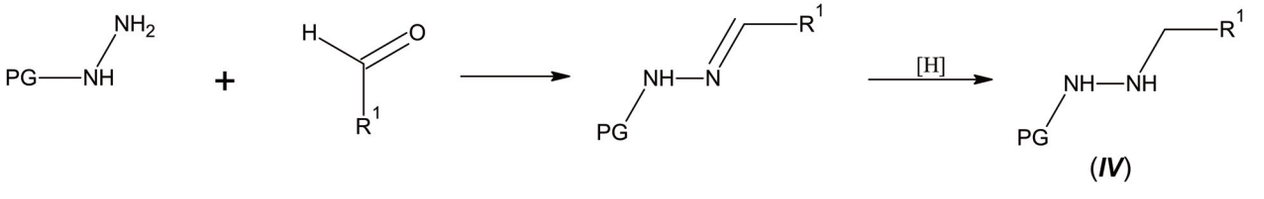
On the other hand, if the aldehyde or ketone contains electronegative groups with heteroatoms or unsaturated substituents, these compounds are prone to self-condensation, and moreover, aldehydes oxidize easily to carboxylic acids. Therefore, these reagents are often used as acetals and ketals, which are more stable but add one more step to the synthesis path.

To overcome aforementioned difficulties, we proposed a one-pot synthesis procedure for the preparation of alkylhydrazines directly from acetals and ketals without converting them to aldehydes or ketones and without the need to isolate the hydrazones as intermediates. This improvement of the synthesis procedure has enabled the preparation of several aza-amino acid precursors. Importantly, the reduction methodology was simplified to avoid expensive catalysts and complicated equipment. Therefore, the developed 'one-pot' synthetic protocol [42,46,54] appears to be a convenient and effective procedure for the preparation of various protected alkylhydrazines from acetals and ketals.

All the improvements discussed above were applied to synthesis of the aza-amino acid precursors, thus enabling to mimic almost the complete set of biologically relevant amino acids. Achieving this has taken us closer to systematic research into the bioactivity of aza-peptides.



Scheme 1. Alkylation of hydrazine.



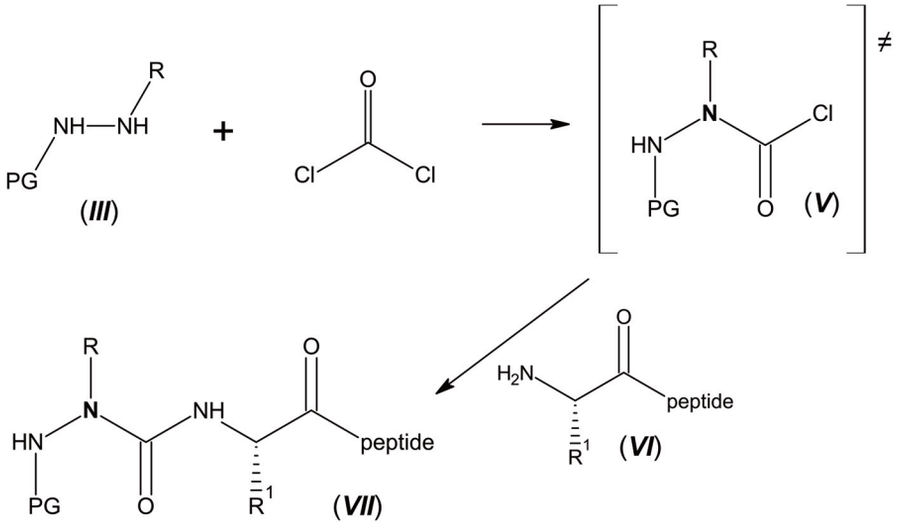
Scheme 2. Reduction of hydrazone.

Aza-peptide bond synthesis

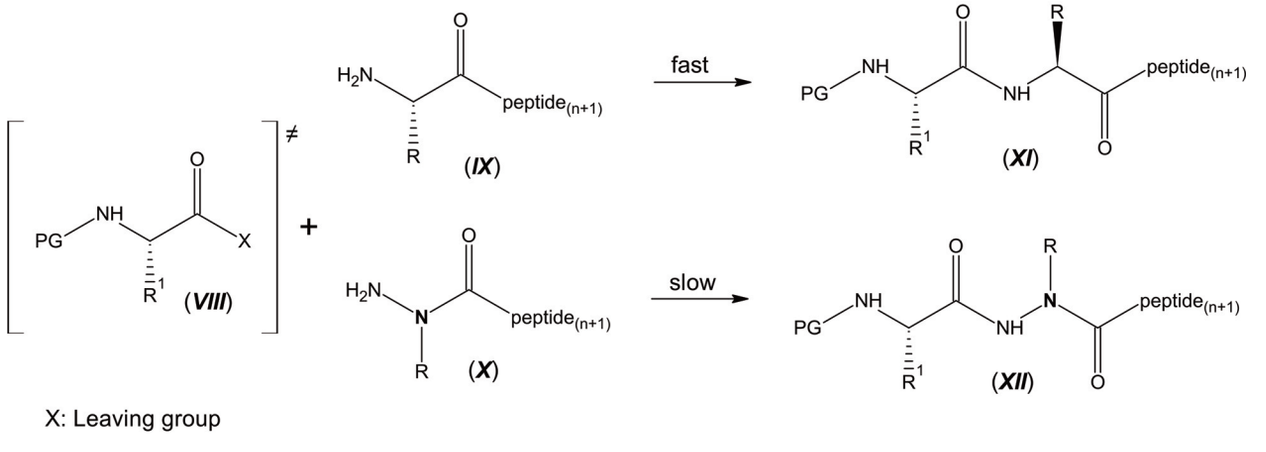
Aza-peptide synthesis starts from acylation of precursor compound (***III***) (hydrazine derivative) (Scheme 3), as appropriate acylated hydrazines are very unstable. Powerful acylating agents, such as phosgene or its derivatives [10,20,67–71], are generally used for this purpose. Thereafter, the chloro-anhydride of the aza-amino acid (***V***) reacts quickly *in situ* with the parent peptide (***VI***), adding the aza-amino acid moiety to the peptide to be synthesized (***VII***).

It is important to mention that performing these reaction steps usually does not cause problems, except for the inconveniences associated with using phosgene (or its derivatives) for acylation of the precursor compound (***III***). However, this conclusion does not apply to the subsequent reaction step, where the next amino acid is added to the alkylcarbazic acid moiety at the end of the synthesizable peptide chain. Although the mechanism of this reaction is similar to conventional peptide bond synthesis and involves nucleophilic attack of the *N*-terminal amino group of the aza-peptide (***X***) or peptide (***IX***) on the activated and *N*-protected amino acid (***VIII***), aza-peptide (***XII***) formation is a slow reaction compared with the synthesis of the conventional peptide (***XI***) (Scheme 4) [72].

This difference explains why conventional peptide synthesis methods cannot be used directly for aza-peptide synthesis. Despite this fact, however, many attempts have been made to use the conventional peptide chemistry protocol for aza-peptide synthesis [7,10,13,26,28,48], and



Scheme 3. Synthesis of aza-peptide (***VII***).



Scheme 4. Comparison of peptide and aza-peptide bond synthesis.

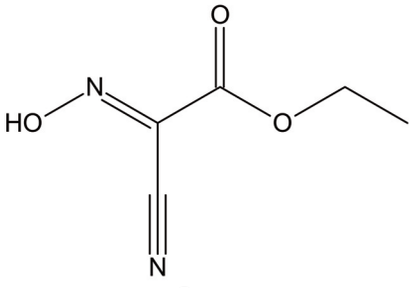
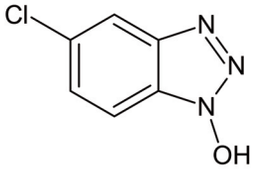
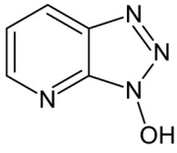
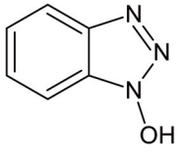
moreover, application of this protocol for aza-peptide synthesis has been promoted in peptide chemistry textbooks [5].

The slowness of aza-peptide bond formation, compared with conventional peptide synthesis, can be attributed to the difference in the nucleophilicity of the participating nitrogen atoms. This conclusion can be justified by comparing the values of the relative second-order rate constants of alkylation of the terminal nitrogen atoms in NH_2-NH-C(O)H (k_{rel} = 0.28) and NH_2-CH_3 (k_{rel} = 260) [73], which quite closely model the aza-peptide ***(II)*** and common peptide ***(I)*** *N*-terminal moieties. In these kinetic studies, the potent alkylating reagent benzhydrylium cation, $(dma)_2CH^+$ was used, which allowed explicit monitoring of the kinetics of these reactions [73].

Additionally, we have demonstrated that the rate of aza-peptide bond formation in compound ***XII*** significantly depends on the bulkiness of the aza-amino acid side chain R and of the amino acid side group R^1 [74,75]. The influence of these substituents was quantified with the graph shape index values Ξ, which were specifically derived for characterization of the amino acid side chain bulkiness [76]. However, as these parameters correlate well with the Taft steric parameter E_s, the influence of the bulkiness of amino acid side groups can be attributed to the well-known steric effect [77].

Using the graph shape index values Ξ, it was revealed that the effect of the bulkiness of group R (***X***) on the reaction rate of aza-peptide synthesis is 2.5 times higher than that of substituent R^1 (***VIII***) [75].

Table 2. Structures of alcohols corresponding to leaving groups X of activated amino acids (structure ***VIII)***

Activator	Alcohol yielding the leaving group in activated amino acid	pK_a of HX [85]
COMU	 Oxyma	4.24
PyOxim		
HDMC	 6-Cl-HOBt	4.62
HCTU		
HATU	 HOAt	4.65
TBTU	 HOBt	5.65
PyBOP		

As steric hindrances govern nucleophilic substitution reactions at carbonyl carbon atoms in general, this effect is also observed in conventional peptide synthesis. However, as this reaction is fast, there was no reason to investigate this phenomenon more thoroughly. As aza-peptide bond synthesis is slow, the steric influence is particularly pronounced in this reaction and may even complicate the synthesis of certain sequences of these peptidomimetics.

Control of aza-peptide bond formation rate

It can be concluded from the information above that successful further development of the aza-peptide synthesis protocol depends on the possibility of increasing the rate of amino acid coupling with the alkylcarbazic acid moiety at the end of the synthesized peptide. As discussed above, the main challenge in achieving this goal seems to be connected with improving the amino acid activators, as the rate of the nucleophilic substitution reaction at the carbonyl C atom in the amino acid derivative (***VIII***) depends obviously on the nature of the leaving group X. Furthermore, it can be suggested that the rate of aza-peptide formation can be influenced by varying the reaction medium, as the nucleophilicity of the amino group may be significantly increased in polar aprotic solvents. However, this aspect of amino group reactivity has not been discussed in depth, as polar aprotic solvents are already in use for aza-peptide synthesis. Therefore, control of the aza-peptide formation reaction by designing efficient activators seems to be the main sound approach.

The influence of the activator reagent on the kinetics of aza-peptide synthesis was studied in the case of the model aza-peptide H-Ala-AzAla-Phe-NH_2 [72] by using various activators proposed for conventional peptide syntheses (COMU, PyOxim, HDMC, HCTU, HATU, TBTU and PyBOP) [78–84]. This study [72] revealed that the rate of aza-peptide bond formation was indeed very sensitive to the structure of the leaving group X in the activated amino acid (***VIII***) (Table 2). Accordingly, among the activators used, the fastest aza-peptide bond formation reaction was observed in the case of the oxyma-based activator COMU. Another oxyma-based activator, PyOxim, was also found to be very efficient, leading to nearly complete aza-peptide bond formation. However, the reaction time of the process was approximately 30 times longer than that of conventional peptide synthesis, which is very inconvenient for the development of aza-peptide synthesis protocols.

Our kinetic studies additionally revealed that the reaction rate (log k_{obs}) correlated well with the pK_a value of the acid HX, corresponding to the leaving group in the activated amino acid, and this interrelationship was described by the LFE relationship. Therefore, the most efficient activator COMU produces leaving group X, whose conjugated acid HX has $pK_a = 4.24$ [85]. This result seems to indicate a promising direction for improving the aza-peptide bond synthesis rate and selecting more reactive activators.

CONCLUSIONS

It is evident that an efficient aza-peptide bond synthesis protocol is still needed, and this can most likely be obtained via the design of new and more efficient activators, which would increase the reactivity of the activated amino acids without inducing massive synthesis of byproducts. Once the activator is available, the synthesis method should be further optimized using the same approach described above to optimize the yield and selectivity of the reaction. We suggest that solving these chemical problems would lead to an explosive rise of interest in the development of aza-peptide-based therapeutic agents.

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Asa-peptiidid: ootused ja tegelikkus

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Asendades looduslike aminohapete struktuuris α-süsiniku aatomi lämmastiku aatomiga, tekivad alküülkarbasiinhapped, mida tuntakse ka α-asa-aminohapetena. Kuigi α-aminohapete ja α-asa-aminohapete topoloogia on sarnane, erinevad nende keemilised ja stereokeemilised omadused oluliselt. Sel põhjusel on tavalise tahkefaasilise peptiidsünteesi protokolli (SPPS) rakendamine asa-peptiidsideme sünteesiks märkimisväärselt keeruline. Teisest küljest suurendavad samad struktuurimuutused asa-peptiidsideme stabiilsust, mis teeb need ühendid väga atraktiivseteks sihtmärkideks peptiidide analoogidel põhinevate ravimite molekulide konstrueerimisel. Selles ülevaates võtame kokku asa-peptiidsideme keemia andmed, millest lähtudes saab edasi arendada asa-peptiidide keemilise sünteesi võimalusi.

Ülevaates esitatud andmetest ilmneb, et eelkõige on vaja tõhusat asa-peptiidsideme sünteesi protokolli, mida saab tõenäoliselt teha uute ja tõhusamate aktivaatorite kavandamise kaudu. Need uued aktivaatorid peavad suurendama aktiveeritud aminohapete reaktsioonivõimet, põhjustamata reaktsiooni kõrvalsaaduste massilist teket. Kui sobiv aktivaator on leitud, on vaja sünteesimeetodit täiendavalt optimeerida. Selleks võib kasutada käesolevas ülevaates kirjeldatud lähenemisviisi. Eeldame, et keemiaga seotud probleemide lahendamise järel kasvab huvi asa-peptiididel põhinevate raviainete loomise vastu plahvatuslikult.