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Structural effects in aza-peptide bond formation reaction

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Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Estonia

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III. Troska, A.; **Arujõe, M**.; Mastitski, A.; Järv, J.; Ploom, A. Steric Impact of Aza-Amino Acid on Solid-Phase Aza-Peptide Bond Synthesis. *Tetrahedron Lett.* 2021, 152973.

Author's contribution

Paper I: Performed all experimental work, calculations, and data processing. Participation in manuscript writing and submitting.

Paper II: Performed all experiments, calculations and data processing. Participation in manuscript preparation writing and submitting.

Paper III: Performed experiments, calculations, and data processing. Participation in manuscript preparation writing and submitting.

ABBREVIATIONS

6-Cl-HOBt	6-chloro-1-hydroxybenzotriazole
AA	Amino acid
AzAA	Aza-amino acid
Ala	Alanine
BTC	Triphosgene (bis(trichloromethyl)carbonate
COMU	1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate
COVID-19	Coronavirus disease 2019
DCC	*N,N′*-dicyclohexylcarbodiimide
DCM	Dichloromethane
DIC	*N,N′*-diisopropylcarbodiimide
DIPEA	*N,N′*-diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DSC	*N,N′*-disuccinimidyl carbonate
Fmoc	9-fluorenylmethyloxycarbonyl
FDA	U.S. Food and Drug Administration
Gly	Glycine
HATU	*N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-yl-methylene]-*N* methylmethanaminium hexafluorophosphate *N*-oxide
HCTU	2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate
HDMC	*N*-[(5-chloro-3-oxido-1*H*-benzotriazol-1-yl)-4-morpholinylmethylene]-*N*-methylmethanaminium hexafluorophosphate
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
Ile	Isoleucine
LCMS	Liquid chromatography-mass spectrometry
Leu	Leucine
MBHA	4-methylbenzhydrylamine hydrochloride
NMR	Nuclear magnetic resonance
Phe	Phenylalanine
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
PyOxim	[Ethyl cyano(hydroxyimino)acetato-O^2]tri-1-pyrrolidinylphosphonium hexafluorophosphate
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SPPS	Solid-phase peptide synthesis
TBTU	2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate

TCFH	*N* -tetramethylformamidinium hexafluorophosphate
TFA	Trifluoroacetic acid
TIDES	Peptides and oligonucleotides
TIS	Triisopropylsilane
TMS	Tetramethylsilane
Val	Valine

INTRODUCTION

Peptides play a crucial role in regulation of a wide variety of processes in living organisms, targeting selectively peptide receptors and other various binding sites in cells. Therefore, peptides represent fascinating group of compounds for development of therapeutics and drugs. It is significant that this idea has not been harmed by the fact that rapid peptide degradation occurs in living organisms as a rule, and this in most cases hampers the efficiency of peptides as drugs. For this reason, ways to reduce the biodegradation rate of peptides without significantly changing effectiveness of their interaction with their target sites have been sought. One way to achieve this goal is chemical modification of peptides in a way that their stability increases without significant reduction of selectivity of their interaction with target sites. These chemically modified peptide-like compounds are known as peptidomimetics. Drugs based on these compounds constitute a rapidly growing segment of the pharmaceutical industry, as illustrated by the number of peptidomimetic pharmaceuticals approved by the FDA in recent years.[1–3]

Mimics of natural peptides include peptides containing aza-amino acids. These amino acid analogues are obtained by replacing the α-carbon of the natural amino acid, represented by general linear formula NH_2-CαH(R)-COOH, with nitrogen, resulting in the formula NH_2-N(R)-COOH. Compared to a normal amino acid, the spatial structure of an aza-amino acid does not differ much. At the same time, the peptide bonds formed with the participation of aza-amino acids are much more stable than the ordinary peptide bonds that should be good for the wider use of these peptidomimetics in drug design. However, it appears that there has been very little success in research on these drug candidates for several decades.[4] The main reason for this seems to be problematic synthesis of the aza-amino acid containing peptides by using the conventional SPPS (solid-phase peptide synthesis) protocol.[4,5] In this work this question is addressed by analysis of the chemical mechanism of aza-peptide bond synthesis, systematically studying the kinetics of aza-peptide bond formation in model peptides of various structure, and by using different peptide bond synthesis activators. The obtained results and formulated conclusions allow to quantify the role of steric influence of the (aza-)amino acid side groups in the aza-peptide bond synthesis and these findings are important for development of the general protocol for aza-peptide bond synthesis. This, in turn, would probably promote the basic research of aza-peptides and recapitulate their potential for development of novel therapeutics.

LITERATURE OVERVIEW

Peptide-based drugs and peptidomimetics

Bioactive peptides have been an attractive starting point for drug discovery because they are essential regulators in many physiological processes. In recent years, peptides and oligonucleotides (TIDES) are rapidly growing category in drug development.[1–3,6,7] Between 2016 and 2019 FDA (U.S. Food and Drug Administration) approved a total of 175 new drugs for commercialization, where peptides or peptide-containing molecules are accounting for 10% of this total number.[2,7] In 2018 this number was 7% and in 2019 alone, 10%.[2,7] Even in 2020, when the world suffered in global health crises (novel pandemic corona virus disease COVID-19) the pharmaceutical industry continued to develop TIDES which accounted for 10% of new drugs.[1] In 2021 TIDES accounted for approximately 24% of all the FDA-approved drugs, a significant improvement illustrating year-over-year growth.[3]

However, peptides are often unsuitable for therapeutic use because of their cellular membrane barriers, low metabolic stability and biological absorption.[8,9] Due to the intrinsic flexibility of the N-Cα and Cα-CO rotatable bonds in every amino acid, peptides can interact with different targets, sometimes leading to low selectivity and unwanted side reactions.[10]

In the focus of peptide based drugs development is important to overcome the above listed pharmacological drawbacks by using peptidomimetics, which mimic parent peptides.[11,12] Peptidomimetics have been developed using different possibilities for structural changes, taking into account both the peptide backbone and the amino acid side-chains. Some mimetics are designed to mimic the functionality, some are designed to mimic structure.

Over the years, peptidomimetics are traditionally divided into I–III types based on their functional and structural similarity with native substrates:[13]

- Type I, structural mimetics, matching with the native peptide, having only one or some backbone modifications, carrying all functionalities in the same spatial orientation.
- Type II, functional mimetics, which are structurally different, but induce the same biological response as the native peptide receptors or enzymes.
- Type III, structural-functional mimetics, which have a non-peptide scaffold while displaying the interacting elements in the same spatial orientation.

This historical classification of peptidomimetics has evolved over the years as research has progressed. The novel classification, proposed by Grossmann and co-workers,[14] divides peptidomimetics into four classes from A to D, where class A is having the highest and D is having the lowest similarity with parent peptide:[10,14–16]

- Class A, modified peptides. These peptidomimetics are most similar to the amino acid sequence of the parent peptide, like Type I. Peptidomimetics that

are formed primarily from α-amino acids with minor side-chain or backbone changes (a limited number of modifications) to stabilize the bioactive conformation.

- Class B, modified peptides/foldamers. Class B mimetics are modified class A mimetics with different non-natural amino acids, small molecule building blocks and/or major backbone alterations. This class also includes foldamers (β-peptides, α/β-mixed peptides and peptoids) with much more significant backbone and side-chain alterations, but are topologically identical to the parent peptides.
- Class C, structural mimetics. Class C are peptidomimetics which consists of highly modified structures, and their backbone is entirely replaced by a non-peptide unnatural framework (small molecule character). The central scaffold displays orientation of substituents that is comparable to the orientation of the key residues in the bioactive conformation of the native peptide.
- Class D, mechanistic mimetics. These are peptidomimetics with a minor similarity to the parent peptide, molecules that mimic the mode of action of bioactive peptides without direct link to its side-chain functionalities. In general, such molecules can be designed by affinity optimization of a class C mimetics, or they can be identified in screenings of virtual libraries.

Aza-peptides are promising peptidomimetics, usually belonging to type I or class A of these ligands. These compounds have attracted much interest because of their close topological similarity to peptides that seems to be promising for their biological and pharmaceutical applications.[12,17,18]

Aza-peptides

Aza-peptides represent a class of peptidomimetics, which contain at least one aza-amino acid analogue, where Cα in amino acid is substituted with nitrogen in peptide sequence (Figure 1).[19]

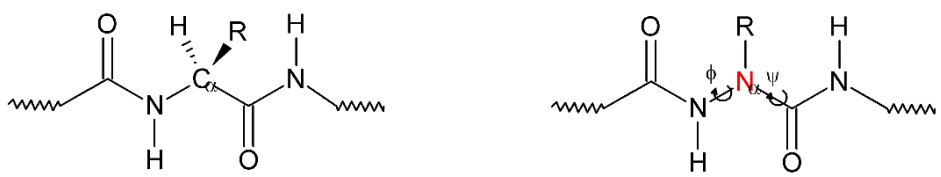


Figure 1. Comparison of amino acid and aza-amino acid constituents in peptide and aza-peptide sequence.

This stereochemical rearrangement of the natural peptide bond, from tetrahedral amide to trigonal-planar urea provides spatially similar peptide analogues, where the second nitrogen atom is still able to bear the side-chain of the natural amino acid.[20] In addition, this structural change causes chemical alterations where carbonyl C atom, located in the formed aza-peptide structural fragment -NH-NR-C(O)-, exhibits lower electrophilicity than that of the amide group in common peptide backbone -NH-CHR-C(O)-. Within the peptide backbone, generated by structural elements of hydrazine and urea, the peptide torsion angle φ and rotatable angle ψ are restricted (Figure 1). Therefore, the chain is able to induce some different types of β-turn secondary conformations as well as helical structures, as proven by NMR spectroscopy, X-ray crystallography and computational analyses.[21–28] The design of such peptide mimics eliminates chirality at the α-position, decreases the electrophilicity of the carbonyl group and causes reduced backbone flexibility.[20–22]

Unnatural conformational compositions that are specific backbone properties of aza-peptides, may cause marked changes in biological and pharmacological properties of these compounds, such as prolonged duration of action, membrane permeability, metabolic stability, target specificity, high receptor affinity and ligand selectivity. All these properties make these peptidomimetics an attractive tool for the structure-active relationship investigations and for the design of drugs.[17,18,29–33] The applications of aza-peptides include design of drug candidates, receptor ligands, hormone analogues, enzyme inhibitors, prodrugs, probes, protease inhibitors active site titrants and imaging agents.[5,12,18,34] Aza-peptide based drugs have already been clinically tested, like Goserelin (Zoladex®), an AzGly peptide analogue for the treatment of prostate and breast cancer, approved by FDA in 1989. The second example is Atazanavir (Reyataz®), orally administrated antiretroviral highly active inhibitor of the HIV protease, approved by FDA in 2003.[35–40] Several researches of Atazanavir show that this drug alone, or in combination with other antiretroviral protease inhibitor might be able to inhibit SARS-CoV-2 enzymes, thus making it a potential drug candidate for treatment of COVID-19.[41–43]

As mentioned above, the development of peptide-based drugs, including aza-peptides, is growing. Recent developments in the field include, for example, the use of thiocarbamate building blocks as aza-amino acid precursors to generate aza-peptides using standard peptide synthesis methods,[44] a novel cyclic (aza-)peptides that promise early diagnosis and treatment development of Alzheimer's disease,[45] and a highly selective method for late-stage *N*-alkylation of aza-peptides, providing rapid access to more than 20 aza-peptoids and N1,N2-dialkylated aza-peptide derivatives of Leu-enkephalin.[46]

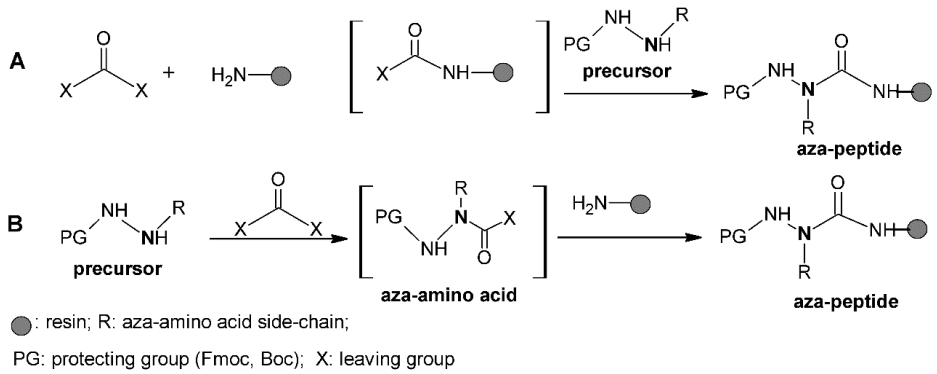
Solid-phase aza-peptide synthesis

The application of aza-peptides in drug discovery is expected to grow following development of effective solid-phase synthetic methods.[23] Differently from classical Merrifield SPPS,[47] solid-phase aza-peptide synthesis involves a combination of hydrazine and peptide chemistry,[4,30,48] because aza-amino acids do not exist as stable compounds and decarboxylate easily if compared with common amino acids.[49] Therefore, aza-peptide synthesis is much more challenging and needs aza-amino acid precursors as building blocks. In addition, the altered steric and electronic properties of *N*-alkylated hydrazine derivatives complicate the aza-amino acid incorporation into peptide sequence and coupling of amino acid to the aza-amino acid residue, if compared with the conventional peptide synthesis.

Incorporation of an aza-amino acid into a peptide sequence

Hydrazine derivatives are mostly used to replace natural amino acid with corresponding aza-analogue in peptide sequence. Preparation of substituted hydrazine that correspond to the side-chains of native amino acids is a challenging task and there are several methods for synthesis of these precursors.[4]

Usually there are two general approaches, used to construct aza-amino acid precursors on solid support (Scheme 1). The first method involves activation of the amino group on resin to an isocyanate or to the active ester, followed by reaction with a substituted monoprotected hydrazine (Scheme 1 A).[20] However, the disadvantage of this approach is the formation of hydantoin as a by-product, caused by the intramolecular nucleophilic attack on the activated *N*-terminal end.[21,50,51] The second method is synthesis through acylation of orthogonally protected mono-substituted hydrazines (Scheme 1 B).[52] This method requires more steps but prevents the formation of hydantoin by-product.[18,21] By this method aza-peptide synthesis starts from acylation of the precursor compound (protected hydrazine derivative), as appropriate acylated hydrazines are very unstable. Some powerful acylating agents, which are carbonyl donors, like phosgene or its derivatives[21,51,53–58] are mainly used for this purpose. The aza-amino acid chloro-anhydride then rapidly binds *in situ* to the amino group on the resin, resulting in the aza-peptide formation.



Scheme 1. Incorporation of an aza-amino acid into solid support: **A** acylation of the amino group on the resin and coupling to hydrazine **B** aza-amino acid precursor acylation and coupling to amino group on the resin.

Several research groups use submonomer method for conventional solid-phase aza-peptide synthesis to simplify the procedure, to avoid problems caused by low reactivity of *N*-alkylated hydrazine derivative and prevent formation of side products.[31,59,60] This is a three-step process: (1) activation of the hydrazone and coupling onto a solid support, (2) alkylation of regioselective semicarbazone for installation of the aza-amino acid side-chain, and (3) deprotection of the chemoselective semicarbazone.[59,60] As mentioned above, this method has several advantages, including the addition of a wide variety of aza-amino acid side-chains that can be installed from easily accessible electrophiles.[60] However, for a more complex side-chains (for example AzVal), alternative methods must be used due to low yields.[53] As the synthesis procedure of the aza-peptide submonomer requires several steps at 0 °C, it has not been automated so far.[60]

Coupling the next amino acid to the aza-amino acid

In addition to the complexity of the synthesis of hydrazine derivatives as precursors for aza-peptides, the solid-phase aza-peptide bond formation is the most challenging step in aza-peptide synthesis. The very low yields for aza-peptide synthesis, observed in the case of the conventional peptide synthesis protocol, can be explained by significantly lower nucleophilicity of the nitrogen atom of the *N*-terminal amino group in the aza-amino acid residue, if compared with the corresponding amino acid nitrogen atom.[61,62] However, this fact has not been recognized, as many attempts have been made to use the conventional peptide chemistry protocol for aza-peptide bond synthesis.[8,21,59,63–65] The slowness of aza-peptide bond formation, if compared with conventional peptide bond formation, can be attributed to the difference in the nucleophilicity of the participating nitrogen atoms. This conclusion can be justified by comparing 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). This reaction is a quite close model of the reaction between aza-peptide and the *N*-terminal group of a common peptide.[61] In these kinetic studies, the potent alkylating reagent benzhydrylium cation, $(dma)_2CH^+$ was used, which allowed explicit monitoring of the kinetics of these reactions.[61]

In addition to the peptide yield, the reaction rate is also an important aspect to be considered in improving peptide synthesis methods. One way to speed up the conventional SPPS is the right choice of coupling reagent for amino acid activation. The ability to assemble peptides faster has many advantages, including increased productivity, identifying difficult couplings in target sequences as well as increasing the production of complex peptides.[66,67] Several scientific groups have been investigated this issue even for decades.[68,69] Today we can conclude that there is no simple answer, because every peptide requires different coupling strategy, and the most reactive compounds could not give the best results. Instead, high reactivity could refer to instability, which could sometimes cause decomposition of reagents before reacting. And in addition, high reactivity can lead to unwanted side reactions.[68] Choosing the right activator also depends on the approach: is it microwave-assisted, automatic SPPS, manual SPPS, cyclization, solution synthesis etc. Moreover, it is important to consider which protecting groups are used.[68] Considering all these aspects, the lower nucleophilicity of the *N*-terminal amino group of the aza-amino acid must also be taken into account, which makes the choice of activators in aza-peptide synthesis particularly important.

Formerly, DCC,[70] DIC[59] and BTC[71] have been used for coupling of amino-acid to semicarbazide moiety for aza-peptide bond formation. But in these cases, the reaction yields were low, especially for branched amino acids. Although, the synthesis of aza-peptides is known to be complex, the effect of activators on the yield of aza-peptide bond formation has not been systematically studied. If the conventional peptide synthesis protocol is applied for aza-peptide synthesis, it is reasonable to test some commonly used triazole based activators, like TBTU,[72] HCTU,[67] HATU,[73] PyBOP,[74] HDMC.[75] And certainly, so-called oxyma-based PyOxim[76] and COMU[77] activators, which have been described as fast coupling reagents, giving good yields in SPPS.[66,67,78] COMU is a new generation uronium-type activator, proposed during the last decade, and has low cost, good solubility, stability and reactivity.[77,79–81] It is widely used in SPPS, but it hadn't been tested for aza-peptide bond synthesis till 2017.[78,80,81]

In addition to COMU,[53] other conventional SPPS activators have been tested for sterically hindered amino acids, recently developed TCFH,[53,82] and the Ghosez's reagent (1-Chloro-*N*,*N*,2-trimethyl-1-propenylamine).[53,83] The results were promising even with the sterically most demanding AzVal, but revealed significant by-product formation and possible epimerization.[53]

As mentioned above, also the nonconventional reaction conditions, such as microwave irradiation, have been used in aza-peptide synthesis, to shorten the reaction time and increase product yield and purity.[8]

However, until now there is no single efficient method proposed, which is good for synthesizing different aza-peptide sequences. To develop the general synthesis protocol for aza-peptides, it is necessary to identify the bottlenecks that affect the course of the synthesis. One way to do this is to study in detail the aza-peptide bond formation step by systematically examining the possible structural effects of the side-chains and the influence of activators.

AIMS OF THE STUDY

The main goal of this dissertation was to study the solid-phase aza-peptide bond formation reaction and to determine the influence of the structure of reactants, including the amino acid activators, on the reaction efficiency. This main goal was divided into the following objectives:

- Investigate the differences between reactivities of *N*-terminal amino groups in amino acids and their aza-derivatives.
- Compare reaction velocities in aza-peptide bond and peptide bond formation reactions, using the conventional SPPS protocol.
- Determine the dependence of the aza-peptide bond formation reaction rate and reaction yield on the activator structure.
- Systematically investigate the kinetics of aza-peptide bond formation reaction in the case of model aza-peptides and characterize the applicability of various amino acid activators in this reaction.
- Characterize the steric effect of bulky amino acids which binds to the semicarbazide group of the preceding aza-amino acid on the rate and yield of aza-peptide formation reaction.
- Elucidate the steric impact of the aza-amino acid side group on the aza-peptide bond formation kinetics.

EXPERIMENTAL

General information

All solvents and reagents were purchased from Merck, Sigma-Aldrich, Iris-Biotech GmbH, or Lach-Ner and were of the highest quality available. Aza-amino acid precursors (AzGly, AzAla, AzLeu, AzVal) were synthesised by Dr. A. Mastitski's research group.[4] NMR spectra were measured using a Bruker Avance 700 MHz instrument using $CDCl_3$ (deuterated chloroform) as the solvent and TMS as the internal reference. Analyses were performed using Shimadzu high-performance liquid chromatography LCMS-2020 instrument with UV-Vis and MS detector. The device was equipped with a Kinetex 5 μm EVO column (C18, 100 Å, 250 × 4.6 mm) and used a gradient where buffer A was 0.1% TFA in water and buffer B was 0.1% TFA in acetonitrile. Samples were injected manually, eluted using a gradient program and analysed at 220 nm.

Solid-phase aza-peptide synthesis

Resin-linked Fmoc-Phe was prepared manually using a stepwise Fmoc solid-phase peptide synthesis strategy. Fmoc-Phe-OH was coupled as a hydroxybenzotriazole (HOBt) ester to the Rink-Amide MBHA resin. Deprotection: 20% piperidine/DMF for 20 min; coupling: Fmoc-Phe-OH (3 eq), TBTU (3 eq), HOBt (3 eq), DIPEA (6 eq) in DMF for 1 h; washes: 3 × DMF/3 × DCM/3 × DMF.

Resin-linked Fmoc-Ala-Phe was synthesized as described above, using Fmoc-Ala-OH × H_2O for the second coupling reaction.

Resin-linked Fmoc-AzAA-Phe (AzAA: AzAla, AzLeu, AzVal) was prepared from resin-linked Fmoc-deprotected Phe via coupling with needed precursor, BTC as the activator, and DIPEA as the base in DMF. In detail, a solution of BTC (1.32 eq, 67 mg, 0.45 mmol) in dry DCM and DIPEA (8 eq, 234 μL, 2.72 mmol) were added to a solution of corresponding hydrazine (4 eq, 1.36 mmol) in dry DCM under argon flow at 0 °C. After 30 min of stirring, the reaction mixture was concentrated *in vacuo*. The resulting Fmoc-AzAA-Cl was suspended in dry DCM and transferred into a vessel containing resin-linked Fmoc-deprotected Phe and DIPEA (8 eq, 234 μL, 2.27 mmol) in DMF. The solution was shaken for 2–3 h, and the residue was washed with 3 × DMF/3 × DCM/3 × DMF.

Resin-linked Fmoc-AzGly-Phe was prepared using a procedure previously described in[53] with slight modifications. In detail, resin-linked Fmoc-Phe (1 eq, 250 mg, 0.17 mmol) was deprotected using 20% piperidine/DMF for 20 min and then was washed with 5 × DMF/1 × DCM. AzGly precursor (3 eq, 0.51 mmol, 130 mg) was dissolved in DMF 1 ml, DSC (3 eq, 0.51 mmol, 131 mg) was added and mixture was stirred at room temperature for 5 min. Pre-activated solution of DSC and 9-*H*-Fluorenyl-9-methyl carbazate was added to resin-bound Phe and was shaken for 24 h. The resin-bound Fmoc-AzGly-Phe was washed then with 3 × DMF/3 × DCM/3 × DMF.

Kinetic measurements

The kinetics of aza-peptide bond formation were measured in DMF at 25 °C. The resin-linked Fmoc-AzAA-Phe (AzAA: AzGly, AzAla, AzLeu, AzVal) (1 eq, 60 mg, 40.8 μmol) was Fmoc-deprotected (20% piperidine/DMF for 20 min) and an excess quantity of 2 min[79] pre-activated mixture of Fmoc-AA-OH (AA: Gly, Ala, Leu, Val, Ile) (10 eq, 0.4 mmol)/Activator (HCTU, PyBOP, COMU, PyOxim, HATU, HDMC, TBTU) (10 eq, 0.4 mmol)/DIPEA (20 eq, 0.8 mmol) (1:1:2) in 10 mL DMF were added to start the coupling reaction. The reactions were carried out in the same volume (10 mL) so that the reagent concentrations would be the same. At appropriate times, 500 μL aliquots with few beads of resin were taken from this reaction mixture; quenching the reaction *via* washing: 3 × DMF/3 × DCM/3 × DMF following Fmoc removal (except for AzGly containing aza-peptides) of resin-bound Fmoc-AA-AzAA-Phe; cleavage: 95% TFA/2.5% TIS/2.5% H_2O for 2 h.

Method for re-protection of the aza-peptide *N*-terminus with the Fmoc group

AzGly-containing aza-peptides were washed 3 × DMF/3 × DCM/3 × DMF/1 × DCM. Then potassium carbonate (36 μmol) and Fmoc-Cl (17 μmol) solution in 2 mL of DCM were added per sample. The heterogeneous reaction mixture was shaken for 24 h and quenched *via* washing: 3 × DMF/2 × H_2O/1 × DMF/3 × DCM. The samples were then cleaved in 95% TFA/2.5% TIS/2.5% H_2O solution for 2 h.

Analysis of kinetic data

The quantities of tripeptide and dipeptide in each sample were determined. The reaction time was dependent on the activator, amino acid or aza-amino acid used. The samples were analysed *via* LC-MS and used a gradient of 5–30% B for Ala, Ile, Val, 5–15% B for Leu and 2% B isocratic flow for Gly, for AzGly, AzAla, AzLeu and AzVal using gradient 5–80%. Buffer A was 0.1% TFA in water and buffer B was 0.1% TFA in acetonitrile. The samples were eluted for 20 min at flow rate of 1 mL/min.

The same conditions were used to investigate the kinetics of conventional peptide bond formation, following synthesis of tripeptide Fmoc-Ala-Ala-Phe from activated Fmoc-Ala-OH and the resin-bound dipeptide H-Ala-Phe. In this case, COMU and PyOxim were used as coupling reagents, and tripeptide formation was complete after 5 min.

Data processing

As the procedure used allowed simultaneous detection of the reaction product (tripeptide) and the remaining starting material (dipeptide) in each sample, it was convenient to characterize the tripeptide formation process using the parameter Y, which was calculated using the dipeptide and tripeptide peak areas from same chromatographic run:

$$Y = \frac{S_{dipeptide}}{S_{dipeptide} + S_{tripeptide}} \quad (1)$$

Using results from the same experiment eliminates errors connected with collection and processing of samples. The parameter Y shows how much of the reactant remains in the reaction mixture, and 1-Y indicates the conventional process yield.

Further, Y vs time plots were used for kinetic analysis. As a 10-fold excess of alanine relative to the number of resin-bound reaction sites was used, the process was described effectively using a first-order rate equation:

$$Y = e^{-k_{obs}t} + Y_{\infty} \quad (2)$$

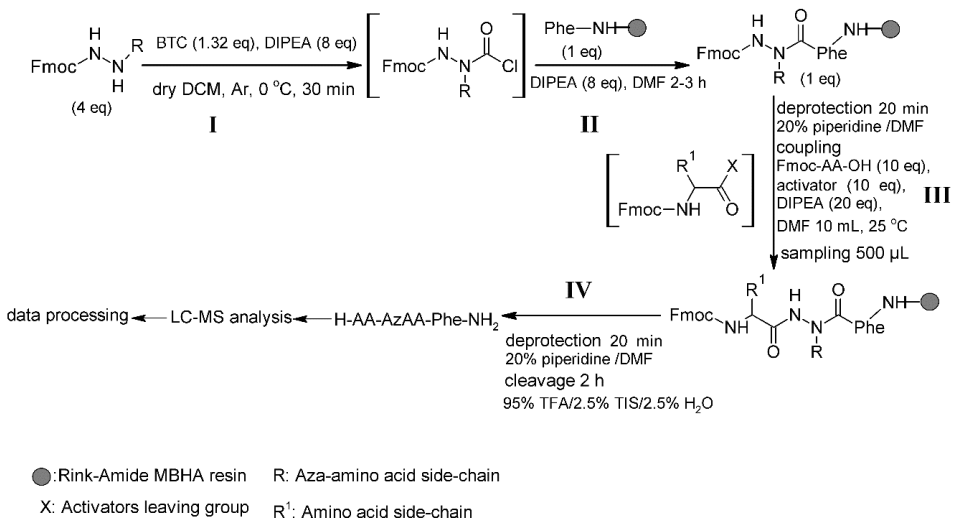
where k_{obs} is the rate constant and Y_{∞} is the plateau value that is reached at the end of the acylation reaction. Importantly, $1-Y_{\infty}$ represents the yield of the reaction.

The kinetic data were analysed using the Graphpad Prism 5 software package. A one-phase exponential decay model was used to calculate the k_{obs} and Y_{∞} values.

RESULTS AND DISCUSSION

Solid-phase aza-peptide synthesis

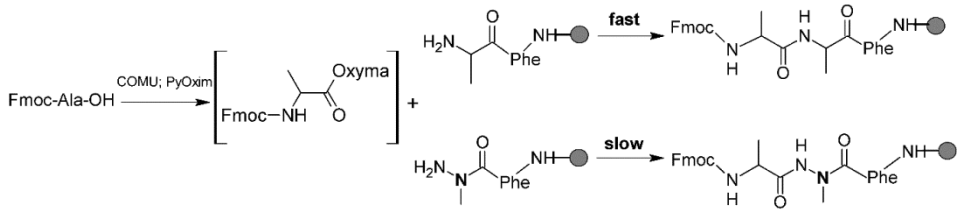
Recently, our group has developed the method to determine quantitatively the peptide bond formation reaction rate and yield,[78] and to the best of our knowledge, no systematic aza-peptide bond formation kinetic study has been published before. Following this method, aza-peptide (H-AA-AzAA-Phe-NH_2) synthesis was started from acylation of the precursor compound using triphosgene as an activator (Scheme 2 step I). Then the activated hydrazine derivative was coupled with the resin-bound Phe (step II) adding aza-amino acid moiety to the peptide sequence. Formed aza-dipeptide was thereafter coupled with the next amino acid using activator (step III). The process is similar to the conventional peptide bond synthesis involving corresponding deprotection and coupling steps. The step III includes sampling from the reaction mixture at appropriate time points for monitoring the time-course of the aza-tripeptide formation reaction and yield of the synthesis. Before LC-MS analysis of these samples, Fmoc deprotection and cleavage from the solid support is needed (step IV).



Scheme 2. General aza-peptide synthesis scheme.

Peptide bond vs aza-peptide bond formation reactions

The abovementioned kinetic investigation method was first used to compare peptide and aza-peptide bond formation rate and yield.[78] Initially, the time evolution of resin-bound aza-tripeptide Fmoc-Ala-AzAla-Phe formation was compared to synthesis of the typical peptide bond in the resin-bound tripeptide Fmoc-Ala-Ala-Phe (Scheme 3). Two oxyma-based coupling reagents, COMU and PyOxim, were selected for Fmoc-protected alanine activation. COMU is a new generation uronium-type activator, and it was not tested for aza-peptide bond synthesis at that time.



Scheme 3. Fmoc-protected alanine activation with activator COMU or PyOxim for resin-bound tripeptide Fmoc-Ala-Ala-Phe and resin-bound aza-peptide Fmoc-Ala-AzAla-Phe formation.

These two oxyma-based activators had similar effects on both reactions; nearly complete acylation of the resin-linked aza-peptide and the conventional peptide were achieved. In the case of peptide synthesis, the reaction half-life[84] ($t_{1/2} = \ln 2/k_{obs}$) is less than 1 min. This is in line with expectations of the conventional Fmoc SPPS protocol, where the amino acid coupling time is 2–5 min at room temperature.[66,85]

Table 1. Kinetic study of tripeptide H-Ala-Ala-Phe-NH_2 and aza-peptide H-Ala-AzAla-Phe-NH_2 formation in DMF at 25 °C using oxyma-based activators COMU and PyOxim for activation. Equation (2) was used to calculate the k_{obs} values using Graphpad 5 software.

(Aza-)peptide	Activator	k_{obs}, min^{-1}
H-Ala-AzAla-Phe-NH_2	COMU	0.022 ± 0.001
H-Ala-AzAla-Phe-NH_2	PyOxim	0.023 ± 0.001
H-Ala-Ala-Phe-NH_2	COMU	1.02 ± 0.29
H-Ala-Ala-Phe-NH_2	PyOxim	1.06 ± 0.24

However, the reaction time of aza-peptide formation is approximately 30 times longer with half-life 17 min. This can be seen from the k_{obs} values listed in Table 1. The difference is also evident from the timescale of the kinetic curve for this reaction in Figure 2. Consequently, the kinetic data demonstrate that synthesis of an aza-peptide bond is much slower than the same reaction in the case of synthesis of the conventional peptide bond.

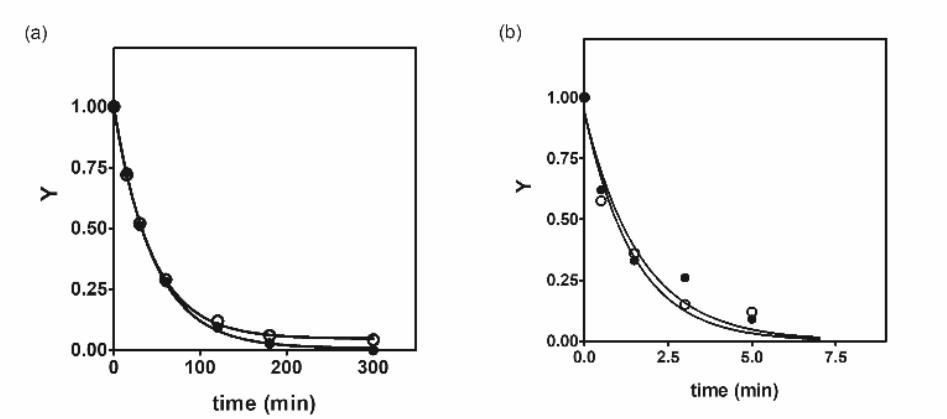


Figure 2. Time course of the reactions between activated Fmoc-alanine and resin-bound H-AzAla-Phe during aza-peptide bond synthesis (a), and with resin-bound H-Ala-Phe during peptide bond synthesis (b) at 25 °C in DMF. Fmoc-alanine was activated with either COMU (●) or PyOxim (○).

The efficiency of coupling reagents in solid-phase aza-peptide bond synthesis

As already shown above (Figure 2, Table 1), the oxyma-based COMU and PyOxim activators led to nearly complete aza-peptide bond formation. Next, applicability of several conventional SPPS amino acid activators, triazole derivatives, which are efficient in peptide bond synthesis,[66] were tested for aza-peptide bond synthesis in model aza-peptide H-Ala-AzAla-Phe-NH_2.[78] These five triazole derivatives HATU, HCTU, HDMC, TBTU and PyBOP all produce similar leaving group X (Table 2). The results obtained using these activating agents are shown in Figure 3. The observed rate constants and calculated acylation yields are listed in Table 3.

Table 2. Activators and their corresponding leaving group structures X of activated amino acid.

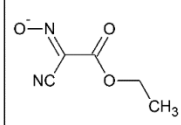
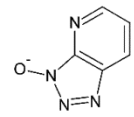
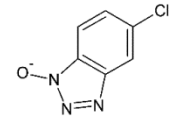
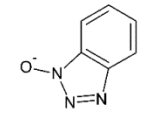
Activator	COMU PyOxim	HATU	HDMC HCTU	TBTU PyBOP
Leaving group X				
	Oxyma	HOAt	6-Cl-HOBt	HOBt

Figure 3 shows that product formation reached an asymptote after 250 min, if HATU, HCTU or HDMC were used. The reaction rates are somewhat lower than those observed in the case of COMU and PyOxim. The half-lives of the first three reactions were ~40 min each. Although HATU results in nearly complete conversion of the dipeptide into the tripeptide, the reactions using HCTU and HDMC were incomplete, and the aza-peptide bond formation yield was only slightly higher than 50% (Figure 3).

Interestingly, aza-peptide bond formation occurs more slowly with TBTU and PyBOP. The half-lives of these reactions were ~150 min. As above, these reactions were incomplete and are characterized by an extrapolated yield of ~0.6.

Table 3. Results of the kinetic study of aza-peptide bond formation *via* the reaction of Fmoc-Ala-OH with the semicarbazide group of the resin-bound H-AzAla-Phe residue at 25 °C using various coupling reagents. Equation (2) was used to calculate the k_{obs} and yield (1- Y_∞) values using Graphpad 5 software.

Activator	Leaving group X	k_{obs}, min^{-1}	Yield	pKa of HX[86]
COMU	Oxyma	0.022 ± 0.001	0.99 ± 0.01	4.24
PyOxim	Oxyma	0.023 ± 0.001	0.95 ± 0.01	4.24
HDMC	6-Cl-HOBt	0.016 ± 0.001	0.55 ± 0.02	4.62
HCTU	6-Cl-HOBt	0.017 ± 0.002	0.68 ± 0.03	4.62
HATU	HOAt	0.017 ± 0.001	0.93 ± 0.01	4.65
TBTU	HOBt	0.004 ± 0.001	0.69 ± 0.05	5.65
PyBOP	HOBt	0.005 ± 0.002	0.65 ± 0.14	5.65

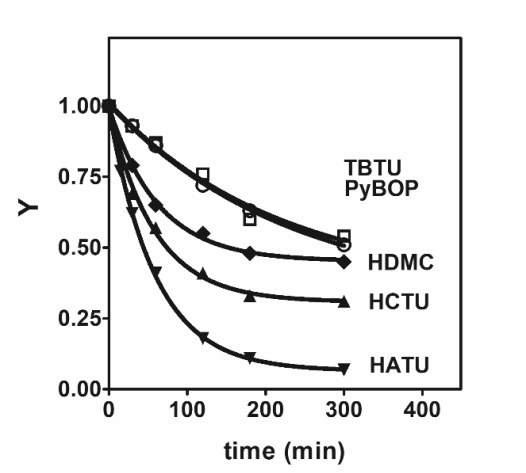


Figure 3. Time evolution of the reaction between activated Fmoc-alanine and resin-bound H-AzAla-Phe during aza-peptide bond synthesis at 25 °C in DMF. Fmoc-alanine was activated with TBTU (□), PyBOP (○), HDMC (♦), HCTU (▲), and HATU (▼).

Thus, it was concluded that more efficient activators are needed to increase the reactivity of the activated amino acid to achieve effective acylation of the semicarbazide moiety of aza-amino acid residue.

All model peptide synthesis reactions were conducted under similar conditions, including reagent concentration and sample processing conditions. Therefore, the kinetic data obtained during experiments with different activators should be comparable. Specifically, the rate constants k_{obs} should characterize the differences in the reactivity of the activated alanine during the acylation reaction. This hypothesis was confirmed by the linear log k_{obs} vs pK_a plot (Figure 4), where the pK_a values (Table 3) measured in a 95% acetonitrile-water mixture at 25 °C[86] quantify the acidities of compounds that correspond to the leaving group HX in the activated Fmoc-alanine with following structures in Table 2. It can be suggested that this plot is useful for design of new activators, as points to the property that should be taken into consideration.

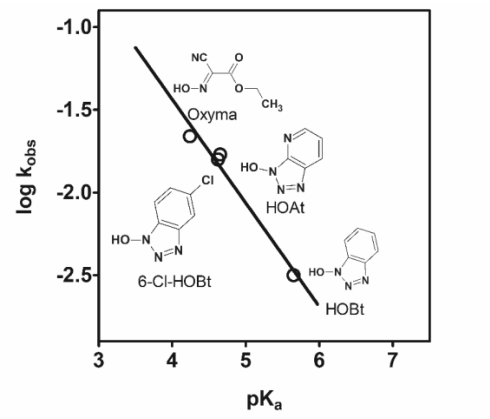
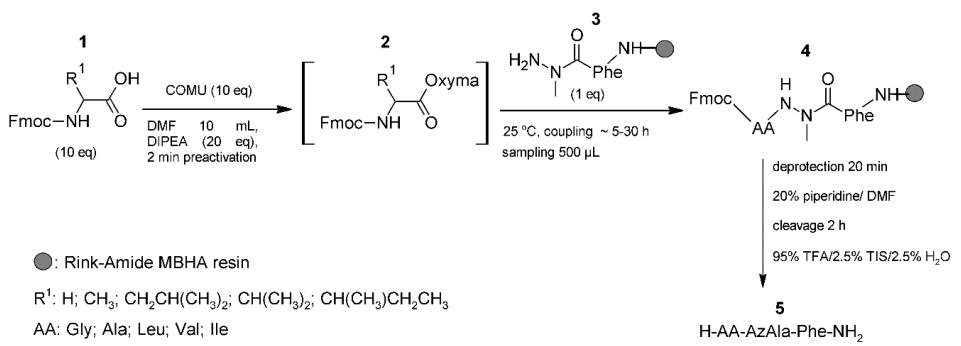


Figure 4. Dependence of log k_{obs} values of the aza-peptide bond synthesis reaction on the pK_a values of acids that correspond to the leaving group HX (Oxyma, 6-Cl-HOBt, HOAt, HOBt) of activated Fmoc-alanine. The log k_{obs} values were taken from Table 3, and the pK_a values were compiled from the literature.[86]

Influence of steric effects of amino acid in solid-phase aza-peptide bond formation

In parallel with the impact of the leaving group acidity that seems to quantify polar effects, it is possible that by analogy with the classical nucleophilic substitution reaction in ester molecules,[87] the rate of the aza-peptide bond formation reaction may also be influenced by the steric effect. The importance of the steric effect in reactions at the carbonyl group has been formulated in the pioneering work of Taft,[88] and since then several experimental procedures for quantification of this influence have been proposed.[87,89,90] In this work, we analysed the influence of the steric effect on the rate of aza-peptide formation in the model compound H-AA-AzAla-Phe-NH_2 **5**, where AA stands for various bulky amino acids linked to the aza-dipeptide *via* an aza-peptide bond (Scheme 4).[91]



Scheme 4. Aza-peptide H-AA-AzAla-Phe-NH_2 synthesis.

For this study, the resin-bound aza-dipeptide H-AzAla-Phe **3** (Scheme 4) was synthesized (see Experimental for details).[91] Thereafter, the kinetics of the reactions of various activated amino acids **2** with the model aza-dipeptide **3** were investigated under conditions defined by the conventional SPPS protocol. The amino acids **1** were chosen so that the substituent (R^1) attached to the α-carbon did not contain a protecting group. COMU was selected as the amino acid activator because oxyma-based activators gave the best yields of aza-peptide bond formation in our previous study.[78]

The kinetic curves obtained under these conditions are shown in Figure 5, and the results of the kinetic experiments are summarized in Table 4.

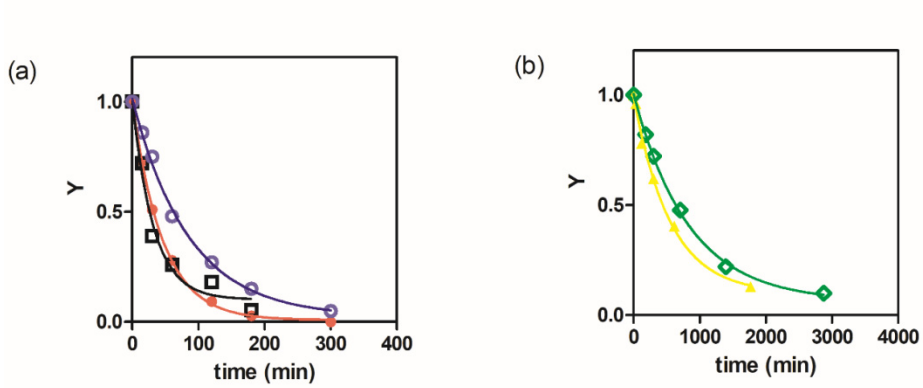


Figure 5. Time evolution of the reactions between resin-bound H-AzAla-Phe and COMU-activated (a) Fmoc-Ala-OH (●), Fmoc-Gly-OH (□), Fmoc-Leu-OH (○); (b) Fmoc-Ile-OH (◇), Fmoc-Val-OH (▲) during aza-peptide bond synthesis at 25 °C in DMF.

Table 4. Kinetic study of aza-peptide bond formation *via* the reaction of Fmoc-AA-OH with the semicarbazide group of the resin-bound H-AzAla-Phe residue in DMF using COMU as an activator at 25 °C. Equation (2) was used to calculate the k_{obs} and yield ($1 - Y_\infty$) values using Graphpad 5 software.

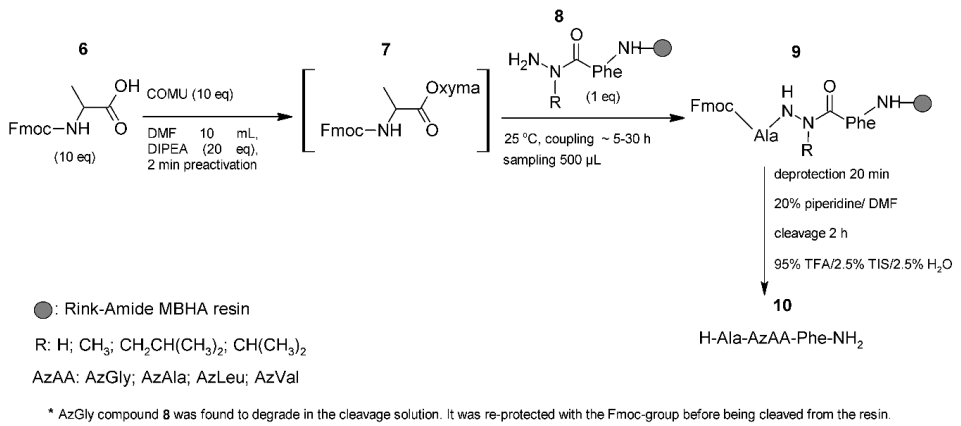
AA	k_{obs}, min^{-1}	Yield
Gly	0.0330 ± 0.006	0.89 ± 0.07
Ala	0.0217 ± 0.001	0.99 ± 0.01
Leu	0.0117 ± 0.001	0.99 ± 0.03
Val	0.0018 ± 0.0002	0.89 ± 0.03
Ile	0.0012 ± 0.00006	0.94 ± 0.02

It can be seen from the reaction yields listed in Table 4 that practically complete conversion of the dipeptide into the tripeptide was achieved using COMU as a coupling reagent. Therefore, COMU may be applicable for the synthesis of various aza-peptide sequences if appropriate reaction times are used.

It can also be seen from Table 4 that the rate of the acylation reaction, characterized by the rate constant k_{obs}, depends on the structure of the side group of the amino acid used. More explicitly, Gly reacts almost 1.5 times faster than Ala and 2.8-fold faster than Leu. Coupling reactions with Val and Ile occur nearly 6.5- to 28-fold slower than in the case of the former amino acids. This difference is clearly seen in Figure 5, where the kinetic curves for these reactions are plotted.

Steric impact of aza-amino acid on solid-phase aza-peptide bond formation

For development of a new aza-peptide synthesis protocol, it is important to elucidate the structural impact of the aza-amino acid side group on the aza-peptide bond formation reaction. For this purpose, a systematic investigation into the reaction kinetics of aza-peptide formation was conducted using the model compound H-Ala-AzAA-Phe-NH_2 **10** (Scheme 5), where AzAA stands for any of the various aza-amino acid residues tested, namely AzGly, AzAla, AzLeu, and AzVal.[4,92] The steric effects of the side group (R) of the aza-amino acid on the rate and yield of aza-peptide bond formation were analysed.



Scheme 5. Synthesis of the model aza-peptide H-Ala-AzAA-Phe-NH_2.

The results of the kinetic experiments are summarized in Table 5 and the obtained kinetic curves are shown in Figure 6. The rate constant (k_{obs}) values, which characterize the acylation reaction velocity, differed significantly when different side groups on the aza-amino acid were used (Table 5). Specifically, activated Ala **7** reacted 5-fold faster with AzAla than with AzLeu. Also, in contrast to the reaction of activated Ala with AzAla, the reaction of activated

Ala with AzLeu did not proceed to completion, showing only a 75% yield of the tripeptide formation (Figure 6). Moreover, no reaction was observed between AzVal and activated Ala using COMU for amino acid activation at 25 °C. Similarly, Proulx and co-workers obtained very low conversion when Fmoc-Tyr(*t*Bu)-OH was coupled to the *N*-terminus of AzVal using COMU as an activator.[53]

Table 5. Results of the kinetic study of aza-peptide bond formation *via* the reaction of Fmoc-Ala-OH with the semicarbazide group of the resin-bound H-AzAA-Phe residue in DMF using COMU as an activator at 25 °C. Equation (2) was used to calculate the k_{obs} and yield ($1 - Y_\infty$) values using Graphpad 5 software.

AzAA	**k_{obs}, min^{-1}**	**Yield**
AzGly	N/A*	N/A
AzAla	0.022 ± 0.001	0.99 ± 0.01
AzLeu	0.004 ± 0.001	0.75 ± 0.05
AzVal	-	Trace

* The previously calculated peptide bond formation rate k_{obs} = 1.02 min^{-1} was used to describe the AzGly *N*-terminus reaction kinetics with activated Ala in calculating inter-relationship $\log k_{obs}$ = -0.17-0,91Ξ, as this reaction was completed within 5 min.

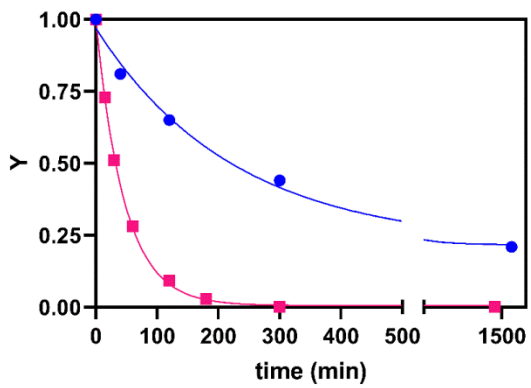


Figure 6. Time evolution of the reactions between COMU-activated Fmoc-Ala-OH and resin-bound H-AzAla-Phe (■) and H-AzLeu-Phe (●) during aza-peptide bond synthesis at 25 °C in DMF.

We also studied the reaction kinetics using AzGly (see Table 5), but LC-MS analysis revealed rapid degradation of dipeptide **8** in the peptide cleavage solution, when Fmoc-group protection of the *N*-terminus was removed. Therefore, we were not able to calculate the kinetic parameters according to equation (2) for this reaction.

As AzGly containing aza-tripeptide is already Fmoc-protected when coupled with activated Ala, the re-protection strategy was needed to prevent AzGly containing aza-dipeptide from decomposition. For this, our group developed an aza-peptide *N*-terminus re-protection method, which was implemented in the experiment before performing cleavage of the aza-peptide mixture from the solid support (see Experimental).

Although we were unable to separate the Fmoc-AzGly-Phe-NH_2 from Fmoc-Ala-AzGly-Phe-NH_2 using LC with a UV-Vis detector, the MS analyses of these two compounds indicated that the reaction with activated Ala was already completed within 5 min when using COMU as the coupling reagent. Unexpectedly, this reaction time was found to be comparable to the durations of typical peptide bond synthesis reactions.[78]

Comparison of the steric effects of the amino acid and aza-amino acid side group in aza-peptide bond formation

Previous results revealed that the rate of aza-peptide bond formation depends on the bulkiness of the aza-amino acid side-chain (**R**) and also of the amino acid side group ($\mathbf{R^1}$) (Figure 7). The influence of these substituents was quantified using the graph shape index values Ξ, which were specifically derived for characterization of the natural amino acid side-chain bulkiness.[93] These parameters are in good correlation with the Taft steric constants E_s,[90] and therefore it was proposed that these parameters characterize the steric influence of the amino acid side group ($\mathbf{R^1}$) and aza-amino acid side-chain (**R**) on the rate of the acylation reaction. Indeed, a linear correlation of the log k_{obs} values of the aza-peptide bond formation reaction and the graph shape index values of amino acids can be observed, as shown in Figure 8.

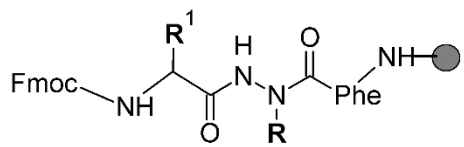


Figure 7. Resin-bound aza-tripeptide Fmoc-AA-AzAA-Phe structure.

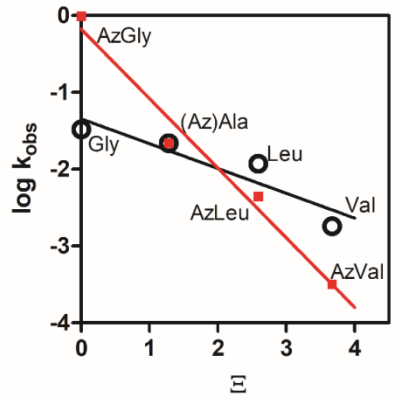


Figure 8. Dependence of $\log k_{obs}$ values of aza-peptide bond synthesis for the model aza-peptides H-AA-AzAA-Phe-NH_2 and graph shape index parameters (Ξ).[93] The k_{obs} values for AzAA (■) and AA (○) were taken from Table 6.

The slope of the obtained relationship $\log k_{obs} = -1.31 - 0.36\Xi$ indicated a clear susceptibility of the aza-peptide bond formation to the steric effects of side group ($\mathbf{R^1}$) in the activated amino acid coupled to the aza-amino acid residue. However, kinetic study demonstrated an even more substantial influence of the aza-amino acid side-chain (**R**) bulkiness on aza-peptide bond formation. Particularly, the aza-peptide bond formation reaction between activated Ala and the least sterically hindered AzGly *N*-terminus was found to be just as fast as the peptide bond formation reaction using COMU for amino acid activation. However, the reaction half-life[84] ($t_{1/2} = \ln 2/k_{obs}$) of activated Ala incorporation with the sterically bulkier AzAla *N*-terminus was 32 min, and that with the AzLeu residue was nearly 2.5 h. Therefore, in the reactions with these aza-amino acids, the aza-peptide bond would form within 4 h and 20 h, respectively. It should be noted that in the case of the reaction involving the more sterically hindered AzLeu, 25% of the starting material remained unreacted. Also, the COMU-activated Ala practically did not react with the *N*-terminus of AzVal, i.e., the sterically most hindered aza-amino acids among of the investigated compounds. Considering that the rate of aza-peptide bond formation between Ala and AzGly was found to be comparable to the rates of typical peptide bond formation, we correlated the kinetic data for AzGly, AzAla and AzLeu with the corresponding graph shape index parameters (Table 6). Analysis of the slope of the obtained relationship $\log k_{obs} = -0.17 - 0.91\Xi$ showed an at least 2.5-fold greater susceptibility of the aza-peptide bond formation to the steric effects of the aza-amino acid substituents than to the bulkiness of the side group of the amino acid coupled to the semicarbazide moiety (Figure 8). As the reaction between COMU-activated Ala and AzVal did not occur, we calculated this rate constant based on the same relationship, and estimated it to be 0.000319 min^{-1} resulting in a reaction half-life of 36 h.

Table 6. Results of the kinetic study of aza-peptide bond synthesis for the model aza-peptides H-AA-AzAA-Phe-NH_2 in DMF using COMU as activator at 25 °C and graph shape index parameters (Ξ). Equation (2) was used to calculate the k_{obs} values using Graphpad 5 software.

(Az)AA	k_{obs}, min^{-1}	Ξ*
Gly	0.0330 ± 0.006	0.00
AzGly	N/A**	
Ala	0.0217 ± 0.001	1.28
AzAla	0.0217 ± 0.001	
Leu	0.0117 ± 0.001	2.59
AzLeu	0.004 ± 0.001	
Val	0.0018 ± 0.0002	3.67
AzVal	-	

* Graph shape index, calculated from the molecular graph structure of the amino acid side-chain.[93]

** The previously calculated peptide bond formation rate k_{obs} = 1.02 min^{-1} was used to describe the AzGly *N*-terminus reaction kinetics with activated Ala in calculating inter-relationship $logk_{obs}$ = -0.17-0,91Ξ, as this reaction was completed within 5 min.

CONCLUSIONS

- Comparison of peptide bond and aza-peptide bond formation. Synthesis of the aza-peptide bond is approximately 30 times slower than formation of a conventional peptide bond if the conventional coupling agents are used to activate the amino acids.
- The efficiency of conventional coupling reagents in solid-phase aza-peptide bond synthesis. Both reaction rates and yields depend on the structure of the amino acid activator. Among the tested activators the oxyma-based activators COMU and PyOxim lead to almost complete aza-peptide bond formation. Triazole-based HATU is equally effective in achieving complete binding but requires a longer reaction time.
- Influence of steric effects of amino acid in solid-phase aza-peptide bond synthesis. The results of the kinetic study revealed that the efficiency of aza-peptide synthesis depends on the reactivity of the amino acid, which binds to the semicarbazide group of the preceding aza-amino acid. Namely, the rate of the acylation reaction, characterized by the rate constant k_{obs}, depends on the structure of the side group of the amino acid used. The sterically least hindered Gly reacts the fastest and the most hindered Ile the slowest.
- Steric impact of aza-amino acid on solid-phase aza-peptide bond formation. It was found that the steric effects of the aza-amino acid side chain governed the aza-peptide bond formation rate. Moreover, no reaction was observed with the most sterically hindered AzVal.
- Comparison of steric influence of the amino acid and aza-amino acid side group in aza-peptide bond formation reaction. The effect of aza-amino acid side chain bulkiness on the reaction rate of aza-peptide bond formation is 2.5-times higher than that of the amino acid side group attached to the semicarbazide fragment, and this difference should be taken into account in aza-peptide synthesis protocol.

SUMMARY

Aza-peptides are promising drug candidates, but wider investigation of their bioactive properties is hindered by the problems encountered in the synthesis of the aza-peptide bond, especially low reaction yields and time-consuming reaction. Determining the kinetic parameters of the reaction is one way to experimentally evaluate the difference in the reactivity of the *N*-terminal amino group of amino and aza-amino acids and to study the suitability of the solid-phase peptide synthesis protocol for aza-peptide bond formation. In the current thesis, these studies were performed by evaluating the effect of amino acid activators and structural effects of amino and aza-amino acid side chains on the efficiency of the acylation reaction.

In the first part of the doctoral thesis, the methodology of kinetic measurements was developed for the systematic study of the aza-peptide bond formation reaction in the aza-peptide. First, the effect of different amino acid activators on the reaction rate and yield depending on the structure of the reactant's leaving group was experimentally evaluated. From the results, it was concluded that the reaction rates and yields depend on the structure of the activator. The oxyma-based activator COMU yielded an almost fully acylated aza-peptide, but the rate of aza-peptide bond formation was ∼30-fold slower than that of a conventional peptide bond.

In the second part of the doctoral thesis, a kinetic method was used to investigate how the spatial structure (steric effect) of the side chain of the amino acid binding to the *N*-terminal of the aza-amino acid affects the rate and yield of aza-peptide bond formation using the activator COMU. For this purpose, a kinetic study was performed for amino acids with different size (steric hindrance) of side group coupled to the *N*-terminal amino group of the aza-amino acid residue. It turned out that the spatial structure of the side group significantly affects the rate of aza-peptide bond synthesis, while it significantly decreases when the steric bulkiness of the amino acid side group increases. For example, for the least sterically hindered glycine, the half-life of the reaction is 21 min, but for the most sterically hindered isoleucine, it is ∼10 h.

In the third part of the doctoral thesis, the influence of the steric hindrance of the aza-amino acid side group on the formation of the aza-peptide bond was investigated. From the results, it was concluded that the increase in the steric hindrance of the aza-amino acid side-chain significantly affects the velocity and the yield of the subsequent amino acid binding reaction. Particularly, the amino acid alanine bounds to AzAla in the model compound 5 times faster than to AzLeu. Moreover, with the given method, the amino acid was practically not attached to the bulkiest of the aza-amino acids, AzVal. Thus, the performed kinetic studies revealed that the aza-amino acid side group affects the rate of aza-peptide bond formation with 2.5 times greater sensitivity than the amino acid side group coupled to the semicarbazide fragment.

In summary, it can be concluded that the performed kinetic studies revealed why the standard solid-phase peptide synthesis protocol cannot be applied directly to aza-peptide synthesis. The reaction of the aza-peptide bond formation is influenced by the effectiveness of the activator used as well as the steric effect of the side chain of the amino acid and the aza-amino acid. Therefore, it is necessary to develop the synthesis methods for aza-peptides in a direction that would enable more effective acylation of the *N*-terminal of the aza-amino acid. This is important for the creation of automated aza-peptide synthesis methods, which in turn would provide an opportunity for more comprehensive studies of the bioactivity of these compounds.

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SUMMARY IN ESTONIAN

Struktuurimõjud asa-peptiidsideme moodustumise reaktsioonis

Asa-peptiidid on perspektiivikad ravimikandidaadid, kuid nende bioaktiivsete omaduste laiemat uurimist takistavad asa-peptiidsideme sünteesil ilmnevad probleemid, eelkõige madalad reaktsioonisaagised ja aeganõudev reaktsioon. Reaktsiooni kineetiliste parameetrite määramine on üheks võimaluseks eksperimentaalselt hinnata amino- ja asa-aminohapete *N*-terminaalse aminorühma reaktsioonivõime erinevust ning uurida tahke faasi peptiidi sünteesi protokolli sobivust asa-peptiidsideme moodustamiseks. Käesolevas töös teostati need uuringud, hinnates aminohappe aktivaatorite ning amino- ja asa-aminohapete külgahelate struktuuriefektide mõju atsüülimisreaktsiooni efektiivsusele.

Doktoritöö esimeses osas töötati välja kineetiliste mõõtmiste metoodika, et uurida süstemaatiliselt asa-peptiidsideme moodustumise reaktsiooni asa-peptiidis. Esimesena hinnati eksperimentaalselt erinevate aminohappe aktivaatorite mõju reaktsiooni kiirusele ja saagisele sõltuvalt reagendi lahkuva rühma struktuurist. Tulemustest järeldati, et reaktsioonikiirused ja saagised sõltuvad aktivaatori struktuurist. Oksüma-põhine aktivaator COMU andis peaaegu täielikult atsüleerunud asa-peptiidi, kuid asa-peptiidsideme moodustumise kiirus oli ~30 korda aeglasem, kui tavapeptiidsideme moodustumine.

Doktoritöö teises osas uuriti kineetilisel meetodil seda, kuidas mõjutab asa-aminohappe *N*-terminaalile seotava aminohappe külgahela ruumiline ehitus (steerika) asa-peptiidsideme moodustumise kiirust ja saagist, kasutades aktivaatorit COMU. Selleks tehti kineetilised mõõtmised aminohapetega, mille külgrühmad olid erineva suurusega (steerikaga) ning mis seoti asa-aminohappejäägi *N*-terminaalsele aminorühmale. Selgus, et külgrühma ruumiline ehitus mõjutab oluliselt asa-peptiidsideme sünteesi kiirust, kusjuures see väheneb oluliselt aminohappe kõrvalrühma steerilise mahukuse suurenemisel. Näiteks steeriliselt kõige vähem takistatud glütsiini korral on reaktsiooni poolestusaeg 21 min, aga steeriliselt enim takistatud isoleutsiini korral on see ~10 tundi.

Doktoritöö kolmandas osas uuriti asa-aminohappe külgrühma steerilise takistuse mõju asa-peptiidsideme moodustumisele. Tulemustest järeldati, et asa-aminohappe külgahela steerilise takistuse suurenemine mõjutab oluliselt sellele järgneva aminohappe sidumise reaktsiooni kiirust ja saagist. Nii seondus aminohape alaniin mudelühendis asuva asaAla külge 5 korda kiiremini, kui asaLeu külge. Veelgi enam, asa-aminohapetest kõige mahukama asaVal-i külge antud meetodil aminohapet ei õnnestunudki praktiliselt siduda. Seega teostatud kineetilised uuringud selgitasid, et asa-peptiidsideme moodustumise kiirust mõjutab asa-aminohappe külgrühm 2,5 korda suurema tundlikkusega, kui semikarbasiidi fragmendi külge kinnitatud aminohappe külgrühm.

Kokkuvõtvalt võib öelda, et teostatud kineetilised uurimused näitasid, miks tavalist tahkefaasi peptiidsünteesi protokolli ei saa rakendada otseselt asa-peptiidsünteesis. Asa-peptiidsideme moodustumise reaktsiooni mõjutavad nii kasu-

tatava aktivaatori efektiivsus kui ka aminohappe ja asa-aminohappe kõrvalahela steeriline efekt. Seega on vajalik asa-peptiidide sünteesimeetodeid arendada suunas, mis võimaldaks asa-aminohappe *N*-terminaali efektiivsemat atsüülimist. See on oluline automatiseeritud asa-peptiidide sünteesimeetodite loomiseks, mis omakorda annaks võimaluse nende ühendite bioaktiivsuse laiaulatuslikumateks uuringuteks.

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PUBLICATIONS

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