DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS

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Synthesis of aza-β^3-amino acid containing peptides and kinetic study of their phosphorylation by protein kinase A

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II Nicolas, I., Kisseljova, K., Bauchat, P., Baudy-Floc'h, M. N^β-Fmoc-N^β-methyl-aza-β^3-amino acids. *SYNLETT,* **2011**, 3, 327–330.

III Kisseljova, K., Kuznetsov, A., Baudy-Floc'h, M., Järv, J. Effect of two simultaneous aza-β^3-amino acid substitutions on recognition of peptide substrates by cAMP dependent protein kinase catalytic subunit. *Bioorg. Chem.*, **2011**, 39, 4, 133–137.

IV Kisseljova, K., Kuznetsov, A., Baudy-Floc'h, M., Järv, J. N^β-methylation changes the recognition pattern of aza-β^3-amino acid containing peptidomimetic substrates by protein kinase A. *Org. Med. Chem. Lett.*, **2011**, 1, 16.

Author's contribution:

I: Synthesis of precursors and peptidomimetics, kinetic studies, data processing and manuscript preparation.

II: Synthesis of N-methylated peptidomimetics, participation in manuscript preparation.

III: Synthesis of precursors and peptidomimetics, kinetic studies, data processing and manuscript preparation.

IV: Synthesis of precursors and peptidomimetics, kinetic studies, data processing, manuscript preparation and submission.

ABBREVIATIONS

A	alanine
Alk	alkyl
ATP	adenosine-5’-triphosphate
Bn	benzyl
Boc	*tert*-butyloxy carbonyl
BOP	benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate
BSA	bovine serum albumine
cAMP	cyclic adenosine monophosphate
DCC	N,N’-Dicyclohexylcarbodiimide
DCM	dichloromethane
DIC	N,N’-diisopropylcarbodiimide
DiPEA	N,N-diisopropylethylamine
DMAP	4-dimethylaminopyridine
EDCI	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
Fmoc	9-fluorenylmethoxycarbonyl
G	glycine
HATU	2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate
HOAT	1-hydroxy-7-azabenzotriazole
HOBT	hydroxybenzotriazole
HPLC	high pressure liquid chromatography
*i*Pr	isopropyl
IR	infrared
k_{cat}	catalytic rate constant
k_{II}	second-order rate constant of the enzymatic reaction
K_m	Michaelis constant
L	leucine
Me	methyl
MS	mass spectrum
NMR	nuclear magnetic resonance
PE	petroleum ether
PG	protecting group
PKA	protein kinase A, catalytic subunit (E.C.2.7.11.11)
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
R	arginine
S	serine
SPPS	solid phase peptide synthesis
Su	succinimide
TBTU	O-(1H-benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium tetrafluoroborate

*t*Bu	*tert*-butyl
Tf	trifyl (trifluoromethanesulfonyl)
TFA	trifluoroacetic acid
TRIS	tris(hydroxymethyl)aminomethane hydrochloride
V	valine
Z	carboxybenzyl

INTRODUCTION

The enormous variety of biological functions, carried out by peptides in living organisms, is based on specific recognition of these compounds by their target sites. This recognition is conventionally associated with the multitude of sequence combinations of side-chains of natural amino acids, while the role of peptide backbone, consisting of repetitive -NH-CHR-CO- motif, is much less discussed. However, the question of backbone role and function rises when the peptide bonds are replaced by analogous structures. This replacement yields peptidomimetic ligands, which may still be recognized by the side group sequence, but may have properties different from those of parent peptides due to the modified backbone structure. These different properties include improved bioavailability and chemical and biological stability of peptidomimetics. For the latter reason peptidomimetics with modified backbone structure may be highly interesting compounds for pharmacology and drug design, as far as these compounds have the biological effects similar to those of their parent peptides.

If various amino acid analogues are considered for peptidomimetics construction, several important aspects should to be taken into account. First, the influence of backbone modification on secondary and tertiary structure of the compounds should be considered. Secondly, feasibility and convenience of synthesis are important. And last but not least, the commercial availability and price of the starting materials are also important factors.

Aza-β^3-amino acids possess several useful properties, and most importantly they lack chirality that significantly simplifies their synthesis. Moreover, the hydrazino bonds in such peptidomimetics are stable towards proteases. The main drawback is that they simultaneously induce two structural alterations: besides the replacement of side chain bearing carbon atom to nitrogen, the additional methylene unit increases both backbone flexibility and length of the monomer residue. In fact, the actual influence of these factors on molecular recognition of peptidomimetics by protein binding sites has never been investigated in detail. In this thesis we have made an attempt to fill this gap by investigating into molecular recognition of aza-β^3-amino acids-containing peptides by protein kinase A.

Protein kinase A is a well-studied enzyme, and its structure, catalytic properties and substrate specificity have been reliably determined. Due to several practical and theoretical aspects, protein kinase A is generally recognized as a model enzyme of the protein kinase superfamily. In the present study this enzyme was used to investigate into recognition of peptidomimetic substrates, which mimic hexapeptide RRASVA sequence, known as the "minimum substrate" of this enzyme. The results obtained revealed several regularities, which seem to govern peptidomimetics recognition by the enzyme active site and may be useful for tailoring of peptidomimetic ligands in general.

1. LITERATURE OVERVIEW

1.1. Peptidomimetics

Many biologically active compounds, e.g. inhibitors, neurotransmitters, immunomodulators, hormones, all of which influence cell-to-cell communications and control metabolism, immune defence, digestion, respiration, sensitivity to pain, reproduction, behaviour and other vital functions, are peptides (Gilon, 2004). Therefore it is not surprising that peptides are potentially ideal drugs, especially those which have undergone natural selection and can be derived from naturally occurring sequences (Gentilucci, 2010).

However, there are also certain complications associated with development of peptide-based drugs (Gentilucci, 2010), among which their rapid degradation and poor permeability across biological barriers should be named first of all. This leads to short half-lives of these compounds *in vivo* (generally <30 min) and thus results in low oral bioavailability (1–2%) (Gentilucci, 2010; Lee, 1990; Bocci, 1990). As a result, many researchers have sought modifications of peptide structures to create molecules less apt for enzymatic cleavage, but still possessing biological activity characteristic of their natural parent peptides. Such modified peptide-like compounds are known as peptidomimetics.

The most common ways to increase stability against enzymatic degradation are peptide backbone modification and N-methylation (Gentilucci, 2010), side chain substitution (Lee, 1990; Bocci, 1990), use of D-amino acids, peptide cyclization and termini modification (Adessi, 2002). Many of these ’tricks’ enhancing peptide stability have been used by evolution (Gentilucci, 2010).

To assess the value of these approaches, the mechanism of molecular recognition of peptides should be understood. It is generally accepted that several appropriately oriented side chains, which interact with the receptor protein, and form the consensus motif for recognition, stipulate biological activity of peptides. Accordingly, the peptide backbone may be viewed as a 3D scaffold, which places the amino acid side chains in right positions and this topology determines binding properties and biological messaging of ligands (Gilon, 2004). Following this concept, the rigid peptide analogues should yield the most efficient pharmacophores (Ripka, 1998; Cowell, 2004). However, this is not so in many cases, where flexibility of peptidomimetics may be important for their effectiveness.

According to Ripka and Rich (Ripka, 1998), peptidomimetics can be divided into three groups:

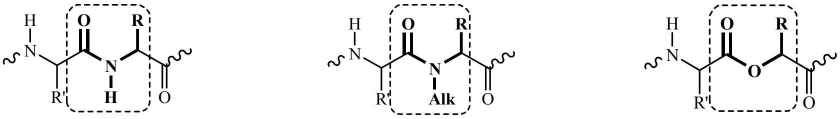
- Type-I mimetics include structures that mimic peptide topography around amide bond (amide bond isosteres, pyrrolinones) or stabilize β-turns (bicycles (Wang, 2002; Eguchi, 2002), aromatic (Jean, 1998; Kaul, 2002) and cyclic compounds (Han, 2002)). These mimetics usually match the peptide backbone atom by atom, but there may be some deviations from this rule. The list of the backbone modifications often used in peptidomimetics is given in **Table 1**.

- Type-II mimetics are known as functional mimetics, which induce the same biological response as the native peptide ligands, but are structurally different from the parent peptide. Normally these compounds are small non-peptide molecules. In many cases these mimetics don't bind to the same sites as the original peptide ligand (Sautel, 1996).
- Type-III mimetics belong to the group of "topographic mimetics", which have a non-peptide scaffold, carrying main functionalities for receptor interactions in similar spatial disposition as that of the native peptide (Moradi, 2009 (and references therein)).

Following this classification, we considered that the "true" peptidomimetic compounds should belong to the first group, and possess well-defined "backbone" and "side groups" by analogy with the natural peptides. Therefore the present work was focussed on these compounds, and more particularly on synthesis and testing of aza-β^3-amino acid containing peptides.

Table 1. The most common structures used for replacement of peptide bond in peptidomimetics (Goodman (Ed.), 2004)

1. **α**-peptide 2. N-alkylated peptide 3. Depsipeptide



4. C$^{\alpha}$-dialkylated peptide 5. Azapeptide

6. Peptides with carbaamide bond replacements: (1) E-... and (2) Z-...

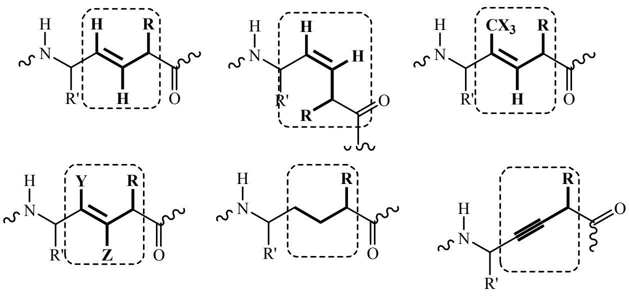
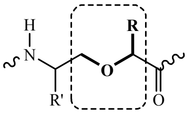
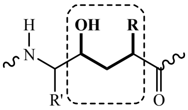
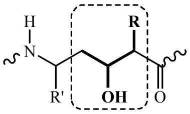
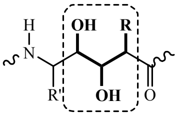
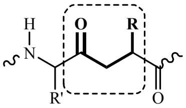
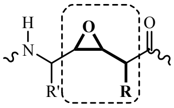
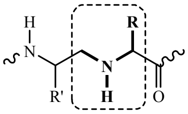
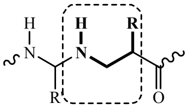
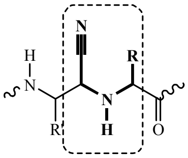
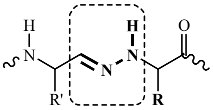
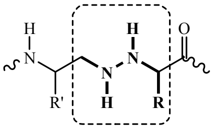


Table 1 (continued)

7. Peptides with C/O bond replacements:

8. C/N amide replacements

9. C/N/O amide bond replacements:

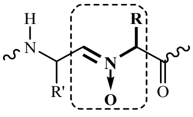
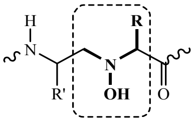
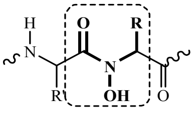
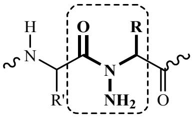
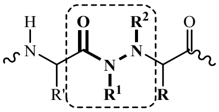
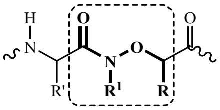
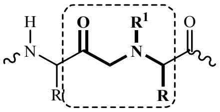
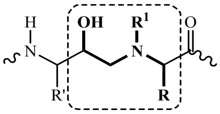
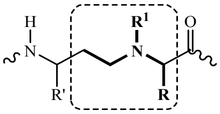
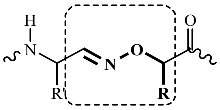
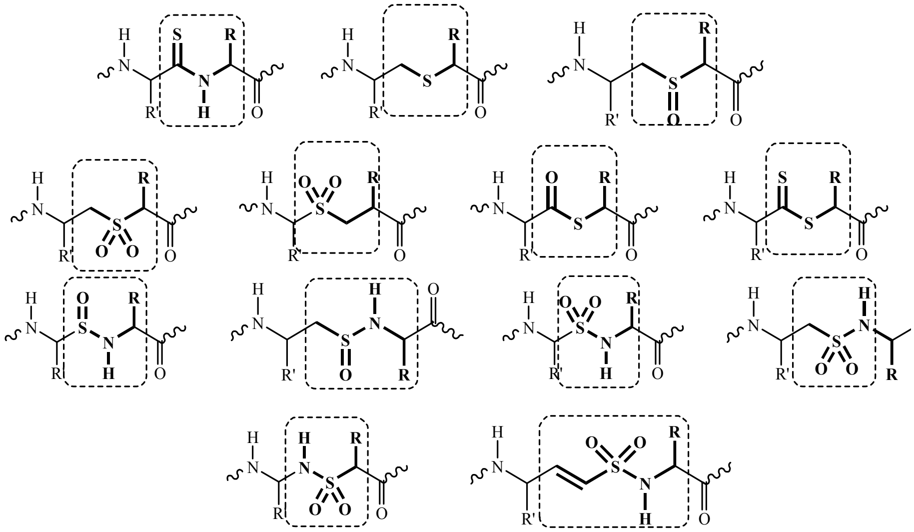
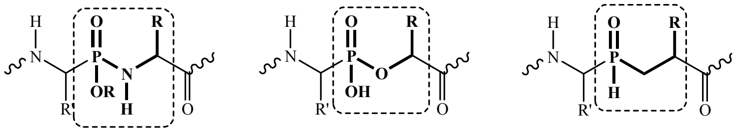











Table 1 (continuous)

10. Sulphur-containing amide bond replacements



11. Phosphorous-containing amide bond replacements



12. Retro-inverso peptides:

L-AA^3-L-AA^2-L-AA^1 D-AA^1-D-AA^2-D-AA^3 D-AA^3-D-AA^2-D-AA^1

13. β-amino acids (2-, 3-, 2,3-)

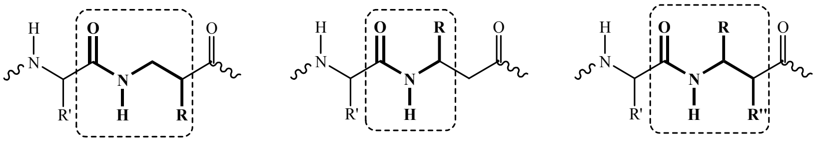
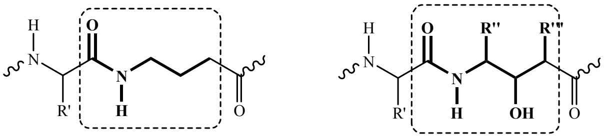
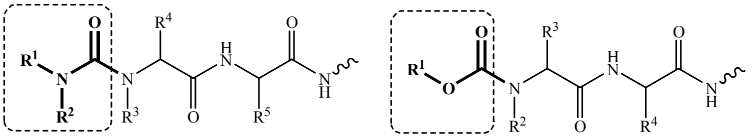


Table 1 (continuous)

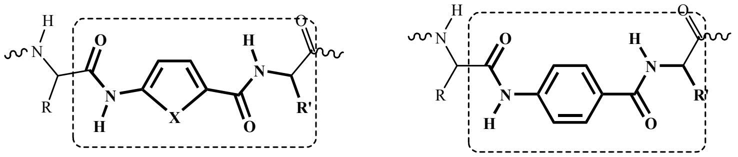
14. γ-amino acids (most are β-OH-substituted)



15. Peptides containing ureine and urethane linkages

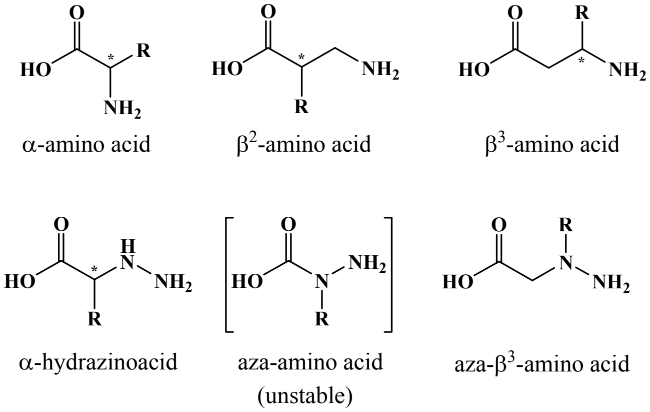


16. Peptides containing an aromatic spacer



1.2. Backbone-modified peptide analogs

Amino acid homologs and hydrazine moiety containing peptidomimetics, which are the closest structural analogs of α-amino acids, are shown in **Scheme 1**. These compounds have both amino and carboxyl groups and may replace the common amino acid in peptides. This replacement can be made in one or in multiple positions. In many cases this replacement is straightforward and amino acid analogs may be introduced into the peptide chain by common methods of peptide synthesis, considering, however, some variation in their reactivity. Exceptions concern the aza-amino acids, as their actual precursors are N'-protected N-alkyl hydrazine derivatives.



Scheme 1. Closest structural and functional analogues of α-amino acids

1.2.1. N-Methylated peptides

N-methylated and N-benzylated peptides can be found in nature (Gilon, 2004), mostly in marine and microbial organisms. Among these peptidomimetics there are many well-known antibiotics (monamycins, echinomycin), insecticides, antitumor agents (buovardin) or anti-inflammatory compounds (Gentilucci, 2010; Sagan, 2004; Wipf, 1995). Some N-alkyl amino acids are commercially available (mainly with non-functionalized side-chains) while the rest can be prepared by methods reported in literature (Gilon, 2004; Sagan, 2004; Aurelio, 2004).

Syntheses of N-methyl derivatives of all natural amino acids have been described and the following approaches may be listed:

- Nucleophilic substitution of optically active α-bromoacetic acids by methylamine or its derivatives, a method first employed by Fischer (Fischer, 1916).
- Methylation with NaH/MeI, a method used on amino acids bearing N-carbamate or N-sulfonyl protecting group (McDermott, 1973; Coggins, 1971; Cheung, 1977).
- Mitsunobu protocol (Mitsunobu, 1981).
- Reductive amination, where various N-alkyl amino acid derivatives can be obtained by reduction of the corresponding Schiff bases by different methods (transition metal catalysis, Raney nickel and activated zinc (Suyama, 1965), borohydride and sodium cyanoborohydride (Jentoft, 1983; Keller-Schierlein, 1988).
- Leuckart reaction (Quitt, 1963).
- Reduction of corresponding 5-oxazolidinones, as proposed by Ben-Ishai et al (Ben-Ishai, 1957). 5-Oxazolidinones can be prepared by reacting N-protected amino acids with paraformaldehyde to give a cyclic product, which is then reduced by triethylsilane/trifluoroacetic acid tandem to give the N-protected-N-methyl-amino acids.

All these procedures generally include the chiral center generation step, as summarized in reviews (Sagan, 2004) and (Aurelio, 2004).

Incorporation of N-methyl-amino acids into the peptide chain is more sophisticated than simple peptide synthesis due to steric hindrance of the methyl group, and therefore this step requires stronger activation or/and longer coupling times. Therefore various groups (Doedens, 2010; Albericio, 1998; Carpino, 1994; Chatterjee, 2010) have used HATU or PyBOP and HOAT for coupling to the N-methylated peptide terminus instead of TBTU and HOBT used for "normal" coupling reactions (Teixido, 2005; Carpino, 1993). Triphosgene-chloroacylation couplings were reported to provide N-methylated peptides in acceptable yields (Wormser, 1986; Laufer, 1986). Somewhat lower yields were common in the case of N-methylated peptides due to the coupling complications and instability N-methyl amino acid residues in N-terminal position in the presence of TFA (Teixido, 2005). Another reason of lower yields could be diketopiperazine formation during peptide cleavage from the resin (Chatterjee, 2010).

N-methylation of an amide moiety can be directly performed on solid support. For instance, N-sulfonyl protected N-terminus of the resin-bound peptide can be methylated with methyl p-nitrobenzenesulfonate in the presence of a strong base (Miller, 1997), or by Matteson rearrangement (Laplante, 2001).

Influence of N-alkylation on peptide structure and properties has been examined on model peptides and the following effects were found:

- N-Methylation supports *cis*-conformation: if amide bonds in open-chain non-methylated polypeptides adopt exclusively *trans* conformation (Goodman, 1967), N-methylated peptides exhibit both *cis*- and *trans*- imide bond geometry due to decreased energy difference between *cis*- and *trans*-configurations (Goodman, 1967; Bovey, 1968; Deber, 1970).
- N-Methylation was found to restrict the side chain conformations of the amino acid (Bach, 1994).
- N-Methylation diminishes inter- and intramolecular hydrogen bonding in peptide due to the absence of proton on the tertiary amide group.
- N-Methylation increases basicity and decreases polarity of the attached carbonyl group (Gilon, 2004).
- N-Methylation significantly increases stability of peptide bond against proteolysis. In some cases this has dramatically increased lifetime of these compounds *in vivo* (Sandberg, 1981; Bruehlmeier, 2002).
- In several cases N-methylated peptides were reported to retain high receptor affinity, comparable to their non-methylated parent compounds (Sandberg, 1981; Bruehlmeier, 2002; Cody, 1997).

N-Methylation can also be used to probe the effect of subtle conformational constraints on the pharmacological profiles (Ron, 1992), and in some cases this may even change subtype-specificity of peptide ligands (Chatterjee, 1998). N-

Methylation is also known to improve the ADMET profile of the corresponding drugs (Bose, 2010).

Although in pioneering studies with poly-N-methylated peptides it was reported that polymeric N-Me-Ala adopts a helical structure (Goodman, 1967; Mark, 1967; Goodman, 1973), it was later agreed that N-Me-amino acid polymers adopt an extended conformations (Zhang, 2006), which are essentially similar to β-strand structure. However, as N-methylated peptides are unable to form hydrogen bonds needed for β-sheet formation, these ligands interfere with peptide aggregation. Therefore these compounds were studied as possible inhibitors of formation of amyloid plaques in AD (Laufer, 1986).

1.2.2. β-Peptides

β-Peptides may include β^2-, β^3- or $\beta^{2,3}$-amino acid monomers. In comparison to α-peptides, β-peptides have an additional backbone methylene unit and therefore were expected to be more flexible, and even unable to adopt definite conformations (Seebach, 1996). However, this belief was proved to be wrong by later studies, showing that β-peptides can still form stable helical structures (Fernandez-Santin, 1984; Fernandez-Santin, 1987; Bella, 1992; Lopez-Carrasquero, 1995), and just like α-peptides, these helical structures are more stable in organic solvents or upon peptide introduction into lipid membranes. Moreover, β-peptides consisting of conformationally restricted β-amino acids (Appella, 1996; Barchi, 2000; Seebach, 1996; 1996; 1998) maintain helical structure in water. It was also observed that helices can be formed by β-peptides consisting of 6 to 12 amino acid residues, while for α-peptides at least 15 residues are required (Godballe, 2011). For this reason β-peptides make good foldamers (Appella, 1996).

In some cases conformational freedom of β-peptides was found to improve bioactivity if compared with the parent peptide (Seebach, 1996 (p913) and references therein). So, English reported bioactive β^2-amino acid oligomers, which were more efficient than their α-amino acid counterparts (English 2006). These β^3-amino acid containing oligomers formed a helical structures based on 12-membered ring, well mimicking α-helix of normal peptides. Liu and co-workers (Liu, 2001) prepared helical magainin analogs, which showed sufficient antimicrobial activity, but had high hemolytic activity as a side-effect. However, if hydrophobic β-amino acids were replaced with less hydrophobic β-alanine, hemolytic activity of these compounds was acceptably low. Gellman and co-workers studied biologically active oligomers of conformationally constrained β-amino acid (2-aminocyclohexanecarboxylic acid, ACHC), which revealed comparable activity to that of magainin and possessed helical structure composed of 12-membered rings (Porter, 2000).

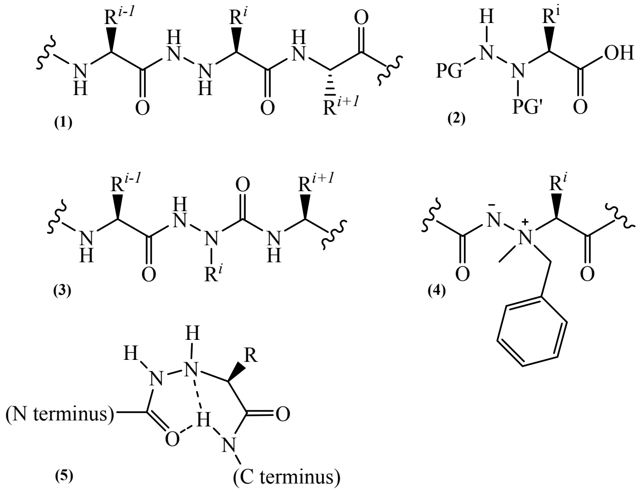
Moreover, β-peptide bond is recognized and degraded by very few proteases that prolongs lifetime of these compounds *in vivo*. It was also reported, that peptides comprised of alternating α- and β-amino acids were extremely stable towards trypsin, chymotrypsin and pronase degrading activity (Schmitt, 2004; Seebach, 1998).

For preparation of β-amino acids several methodologies were proposed (Lelais, 2004). β^2-Amino acids were prepared by diastereoselective alkylation of α-alanine (Juaristi, 1991, 1996; Seebach, 1999; Gutierez-Garcia, 2001, 2002; Nagula 2000), Curtius rearrangement of chiral succinate (Sibi, 2000; Seebach, 2003), conjugate addition of nitrogen (Lee, 2003; Bedow, 2004) or carbon nucleophile (Rimkus, 2003; Eilitz, 2003; Dursma, 2003) to the corresponding Michael acceptor, aminomethylation of a chiral precursor, which bears the side-chain of the amino acid (Evans, 2009; Oppolzer, 1989; Hintermann, 1997, 1998; Arvanatis, 1998; Sebesta, 2003; Gademann, 2001; Seebach, 2004), modifying α-amino acids in Reformatsky reaction with Mannich-type imminium electrophile (Moumne, 2006), by enantioselective aminomethylation of aldehydes via organocatalytic Mannich reaction (Chi, 2006, 2007; Ibrahem, 2006, 2007). β^3-Analogs of the most proteinogenic α-amino acids can be easily prepared by Arndt-Eistert homologation of α-amino acids (Ellmerer-Mueller, 1998), Mannich reactions (Song, 2006), Kowalsky rearrangements (Gray, 2004), organocatalytic (Chi, 2006; Guerin, 2002) and radical reactions (Sibi, 2006), via isoxazoline intermediates (Fuller, 2005), and β-lactams (Li, 2006). β-oligomers can be prepared by standard SPPS methods, using HBTU, HOBT and DiPEA as activators (English, 2006).

1.2.3. Hydrazinopeptides

The use of α-hydrazino acids to increase peptides stability against proteolysis was first proposed by Niedrich (Laure, 1998, and references therein; Lelais, 2003). But such peptide mutants received limited attention over the following decades due to difficulties in preparation of necessary monomers and lack of efficient methodologies for their introduction into peptide chains. Later short hydrazinopeptides were obtained and their structure was studied by X-ray diffraction, NMR and IR spectroscopy and by computational studies. It was observed that all hydrazinopeptides have a common structural feature: 8-membered bifurcated pseudocycle termed „hydrazinoturn" (Aubry, 1991) (**Scheme 2**).

There are four main synthetic methodologies for preparation of α-hydrazino-acids, a task which is generally considered tedious: modified Schmitz electrophilic amination of L- or D-amino acids using oxaziridine derivatives (Vidal, 1997; Bonnet, 1999; Guy, 1998; Niederer, 1993); Mitsunobu protocol (including a solid-phase methodology) (Acherar, 2009; Bouillon, 2004, 2007; Brosse, 2001); hydrogenation of hydrazones (Burk, 1994; Gennari, 1986); Shestakov rearrangement (Amour, 1994; Viret, 1987).



Scheme 2. Hydrazinoturn (5) and examples of molecules possessing this structural feature: hydrazinopeptide (1), azapeptide (3), aminimide (4), and N^{α}-, N^{β}-diprotected L-hydrazinoacid.

In solid-phase peptide synthesis, N^{β}-protected hydrazinoacids can be coupled to the free N-terminus using standard SPPS coupling procedures (Vidal, 1997; Hannachi, 2004). For activation of this reaction DCC, phosgene, PyBop (Guy, 1998), HATU (Lelais, 1998), TBTU/HOBT/DiPEA (Huisgen, 1999) are commonly used. For the coupling to the free hydrazino N-terminus $SOCl_2$, isobutyl chloroformate, cyanuric fluoride ((Werle, 2006)) (Bouillon, 2007), active esters (OSu, (Guy, 1998)) have been employed. It has been stressed that in the case when C^{α} bears a non-bulky side chain, there may be a regioselectivity problem between N^{β} and N^{α} positions, along with the unwanted polymerization of the monomers upon their activation. To avoid this, N^{α} should be protected, e.g. with benzyl group. In general, low preparative yields are common for synthesis of hydrazinopeptides.

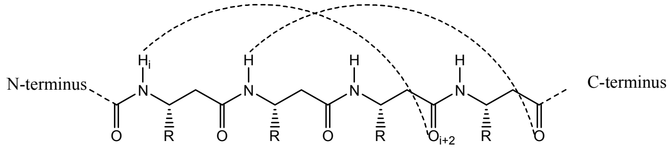
Due to complicated synthesis of hydrazinopeptides only a few studies of hydrazino analogs of biologically active peptides have been published and structural effects of hydrazino acid substitution have been practically not described. Guy (Guy, 1998) has reported a slightly increased binding of hydrazino analogs of human leukocyte elastase substrates to the enzyme, but no detailed analysis of this phenomenon was provided. Günther and Seebach (Günther, 2001) have carried out a computational study in order to establish which conformations hydrazinopeptides are prone to adopt. A number of folding types were found to be favored for these compounds, including the H14 helix, which was also characteristic for β-peptides.

1.2.4. Aza-β^3-peptides

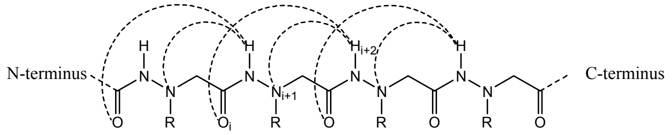
Aza-β^3-amino acids are not chiral and their preparation does not require chiral starting materials or catalysts. This makes their synthesis less sophisticated than preparation of hydrazinoacids. N^β-Fmoc-Aza-β^3-amino acids are essentially prepared by alkylation of Fmoc-, Boc- or Z-protected hydrazine derivatives by convenient and simple techniques, as described in the Experimental section. General procedure of preparation of aza-β^3-amino acids precursors was shown in **Scheme 7** (Experimental section, **2.1**). Aza-β^3-amino acids can be obtained by the carboxymethylation of N-Fmoc-N'-alkyl-hydrazine derivatives.

There are few reports on structure studies of peptides containing aza-β^3-amino acid residues. Thus, it cannot be excluded that structural features (such as 8-membered pseudocycles) can be exclusively attributed to regular aza-β^3-structures and may not be present in mixed α- and aza-β^3-peptides (Salaün, 2005).

Like in all hydrazine-moiety containing peptides reported, hydrazine link in aza-β^3-peptides is known to induce a hydrazinoturn. Unlike their L-β^3-amino acid-containing counterparts, which adopt a left-handed helical structures based on 14-membered hydrogen-bonded pseudocycles, aza-β^3-oligomers preferably form consecutive right-handed (Salaün, 2006) 8-membered hydrogen-bonded pseudocycles (Salaün, 2005) (**Scheme 3**). The replacement of C^2 for N^2 leads to rearrangement of the hydrogen bond network due to N^β participating in it. LeGrel and co-workers have carried out structural studies of cyclic aza-β^3-hexapeptides with both aliphatic and acyl substituents on N^2 atoms. N^β carrying an aliphatic substituent is sp^3-hybridized, while an acyl-substituted N^2 is sp^2-hybridized. Different hybridization states result in different hydrogen bond acceptor ability of these nitrogen atoms. But it was shown, that the influence of such change does not significantly affect the speed of the pyramidal inversion, which, as indicated by NMR (temperature of coalescence, 378 K for N^β-alkyl- and 388 K for N^β-acyl-derivative) remained tremendously low (ca 1 inversion per second).



(L)-β^3-peptides

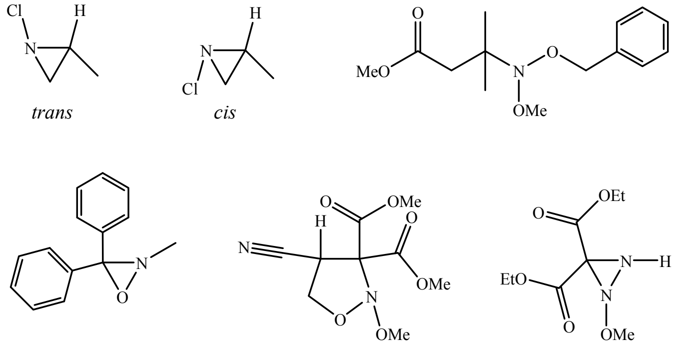


Aza-β^3-peptides

Scheme 3. Comparison of β^3- and aza-β^3-peptide folding patterns (Le Grel, 2006), H-bonds are shown by dotted lines.

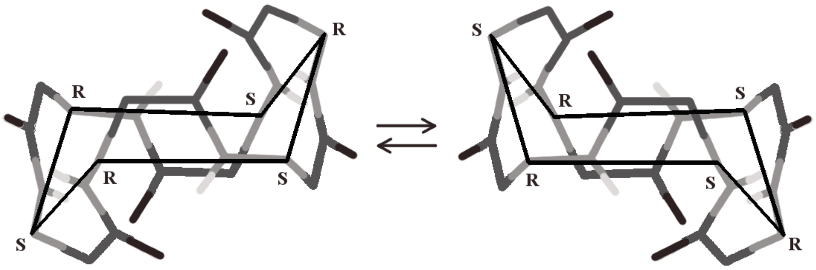
LeGrel and co-workers went on with preparing small cyclic peptides consisting of aza-β^3- and β^3-amino acids and α-hydrazinoacids. They showed that replacing aza-β^3-amino acid residues with their hydrazinoacid or β^3-counterparts, containing chiral C-atoms, resulted in stabilized cyclic peptides, which showed no inversion in NMR studies. In these compounds the chiral carbon center acted as a conformational lock, inhibiting isomerisation of these aza-β^3-cyclopeptide hybrids.

Atoms bearing three different substituents and an electron pair may be expected to exhibit chirality. However, in the case of nitrogen, chirality cannot be generally observed due to the nitrogen inversion. In certain cases, however, the inversion may be slowed down so that the different enantiomers can actually be separated. This applies to three-membered nitrogen cycles, in which the inversion is impeded by the additional tension imposed on the cycle by the sp^3 to sp^2 transition. Another factor to slow down the inversion is an unshared electron pair-bearing substituent at the nitrogen atom, as additional energy is required to overcome the lone-pair repulsion during the inversion process. Several examples of such compounds are shown in **Scheme 4**.



Scheme 4. Compounds with chiral nitrogen atom.

LeGrel and co-workers have observed the nitrogen chirality in aza-β^3-hexacyclopeptides due to slow pyramidal inversion (**Scheme 5**). They have reported that for cyclic aza-β^3-peptide shown in **Scheme 5** the inversion energy was around 75 kJ/mol, that was comparable to that observed for some N-alkyl azaridines (Le Grel, 2006, and references therein).



Scheme 5. 24-membered aza-β^3-cyclohexapeptide undergoing slow inversion to its enantiomeric antipode (invertomer), RRSSRR → SSRRSS. (Le Grel, 2006)

Aza-β^3-amino acid oligomers were prepared in solution from N-Boc protected aza-b^3-amino acids using DCC/DMAP in dry DCM (Cheguillaume, 2001; Salaün, 2005; Salaün, 2008); cyclizations to produce aza-β^3-cyclohexapeptides were performed at 1mM peptide concentration using EDCI/HOBT as activators in DCM (Le Grel, 2006). In the reference (Hayet, 2007) DIC and HOBT were used for aza-β^3-amino acid coupling on solid support.

1.3. Peptide recognition by protein kinase A

1.3.1. Protein kinases

Regulatory phosphorylation, catalyzed by protein kinases, which transfer the γ-phosphate group from ATP onto protein substrates, is the most important mechanism to control activity of enzymes and receptors in living cells (Krebs, 1998). Precision of this regulation depends on the accuracy of recognition of substrate phosphorylation sites by protein kinases. Therefore these enzymes are excellent objects to study details of protein-protein and protein-peptide recognition mechanism and specificity on.

All protein kinases share homologous “kinase domain”, which is also named “catalytic domain” (Hanks, 1988), and contains 12 conserved sub-domains. The catalytic domains of various kinases consist of 250–300 amino acid residues and fold into a similar three-dimensional core structure, properties of which were reviewed in (Taylor, 1992; Hanks, 1995). Alignment of amino acid sequences of the kinase domain was used for classification of protein kinases (Hanks, 1988), and four main enzyme families were thus revealed. These four groups are listed below:

- The AGC group with mainly basic amino acid specificity determinants.
- The CaMK group covers the family of kinases regulated by Ca^{2+}/calmodulin.
- CMGC group includes family of cyclin-dependent kinases (Cdks), the MAP kinase family and casein kinase II.
- The protein –tyrosine kinase group (PTK).

1.3.2. cAMP dependent protein kinase

cAMP dependent protein kinase belongs to the AGC group, and is a tetrameric protein C_2R_2 consisting of two catalytic subunits (C) and two regulatory subunits (R). If cAMP binds to the regulatory subunits, active catalytic subunits C are released (Beebe, Corbin, 1986; Doskeland, 1993). These subunits, further denoted as PKA, are water soluble proteins, have catalytic activity, interact specifically with ATP and peptide/protein substrates and can be isolated and purified from natural sources, or more recently as a product of over-expression of the protein in different expression systems. The catalytic subunit consists of 350 amino acids and 245 of them belong to the conserved catalytic core structure, which is characteristic to all protein kinases (Hanks, 1991). The three-dimensional structure of the catalytic subunit of cAMP-dependent protein kinase (PKA) was elucidated in 1991 (Knighton, 1991), and later structures for different PKA-ligand complexes were resolved (Zheng, 1993 (Biochemistry); Zheng, 1993 (Acta Crystallographica); Engh, 1996; Prade, 1997; Narayana, 1997). Therefore PKA has become the most thoroughly studied enzyme among protein kinases and is so far recognized as a model enzyme of this superfamily. For the same reason the enzyme was selected for peptidomimetics recognition study in this work.

1.3.3. Peptide recognition by protein kinase A

Substrate specificity of PKA may be controlled on three structural levels. Firstly, the enzyme exhibits site-specificity, which means that primary structure motifs in the vicinity of the phophoacceptor amino acid are recognized (Pinna, 1979). Secondly, the recognition of a substrate protein may be mediated by interactions between other site of the kinase apart from the active site and a short sequence of the substrate protein called “docking site” (Biondi, 2003). Thirdly, the targeting of a kinase to its substrate may be supported by specific anchoring proteins, which direct the kinase to the vicinity of its substrate inside the cell (Klussmann, 2001).

Although the enzyme may use all these three levels of substrate recognition, it is known that phosphorylation site specificity is needed for the correct docking and positioning of the phosphoacceptor residue in the active site, while other two levels are not absolutely necessary for recognition. This conclusion is proved by the fact that selective phosphorylation of short peptide substrates by PKA may occur (Kemp, 1977). Therefore thorough analysis of the specificity pattern of PKA was made by using short peptide substrates and this experience was extended to peptidomimetic substrates in this study.

1.3.4. Phosphorylation site specificity of PKA

The first studies of synthetic peptide phosphorylation were made with PKA by using sequences resembling phosphorylation sites in protein substrates (Zetterqvist, 1976; Zetterqvist, 1982; Kemp, 1977; Feramisco, 1980; Meggio, 1981). It was found that peptides based on the phosphorylation site sequence of L-type pyruvate kinase were just as good substrates as the pyruvate kinase itself. Further, these studies revealed that peptide RRASV was efficiently phosphorylated, but further truncation of this sequence leads to dramatic decrease of reactivity. Therefore this sequence was denoted as a "minimal substrate" of PKA, *i.e.* the shortest peptide containing the whole set of necessary specificity determinants (Zetterqvist, 1976).

The presence of two arginines was found to be exclusive substrate specificity determinant for PKA, since they could not be replaced by other amino acids, e.g. basic amino acid lysine, without serious detriment of phosphorylating activity. Further, moving these arginines either closer to or further from the serine residue increased the K_m value (Ragnarsson, unpublished results**)**. These studies resulted in definition of a minimal consensus sequence Arg-Arg-X-Ser-Y, for PKA, where X denotes any amino acid and Y is a hydrophobic amino acid. More recently, the determination of three-dimensional structures of PKA-pseudosubstrate inhibitor peptide complexes (Taylor, 1993; Tsigelny, 1996) confirmed the importance of the arginine pair and shed light onto the peptide recognition mechanism of PKA in atomic detail and the system has become a model of substrate recognition for the whole protein kinase superfamily.

Further attempt to rationalize and systematize the collected peptide phosphorylation data for PKA was performed by Järv and Ragnarsson (Järv, 1991). In this study the amino acid positions from –3 to +2 around serine residue, which position was denoted as 0 in synthetic peptide substrates, were analyzed by linear free energy relationships. Different structural parameters for each amino acid side chain were correlated with kinetic data for peptides and specificity requirements of each position were characterized by the intensity factors obtained from these correlations. Similar approach was used and further developed in two studies where the specificity of PKA and PKC was studied by using peptide substrates derived from the phosphorylation site of myelin basic protein, where peptide structure was varied in position +1 (Järv, 1996; Loog, 1999). It was found that specificity of PKA was quantitatively described by hydrophobicity and bulkiness of amino acid side chains in position +1. By these studies it was understood that it is not possible to express the site specificity of protein kinases by some fixed consensus sequence, since the recognition process may have far more complex nature, involving different structural factors, which may have positive or negative influence on substrate reactivity. On the other hand, the same results also confirmed that peptide recognition by PKA can be presented by a rather wide recognition pattern and this tolerance provides good preconditions for study with peptidomimetic substrates.

Differently from peptidases, influence of chemical modification of backbone structure on catalytic reaction may be excluded from this analysis, as intrinsic reactivity of serine side-chain in the phosphorylation reaction cannot be directly affected by these changes. On the other hand, the presence of the phosphorylation reaction should assure similar positioning of peptidomimetics in their binding site. Therefore the protein kinase catalyzed phosphorylation reaction seemed to us as an appropriate choice for study of peptidomimetics recognition by a protein binding site.

2. AIMS OF THE STUDY

The general objective of this work was to investigate into recognition of aza-β^3-amino acid containing peptides by a protein binding site, using the catalytic subunit of cAMP-dependent protein kinase as a model enzyme. For this study series of peptidomimetic substrates, which were analogs of the minimal peptide substrate of this enzyme, RRASVA, were designed, synthesized and kinetics of their phosphorylation was studied.

More specifically, this objective was achieved through:

- preparation of aza-β^3-amino acids as precursors of peptidomimetic substrates,
- synthesis of aza-β^3-, di-aza-β^3- and N^β-methyl-aza-β^3-amino acid containing peptidomimetics as PKA substrates,
- kinetic study of phosphorylation of peptidomimetic substrates,
- comparison of the kinetic data with results for peptide phosphorylation by PKA, and
- analysis of computer modelling data of peptidomimetics and their comparison with results of kinetic experiments.

3. METHODS

3.1. Synthesis of N$^{\beta}$-Fmoc-aza-β^{3}-alanine, -valine, -arginine and N$^{\beta}$-Fmoc-N$^{\beta}$-Me-aza-β^{3}-arginine

Aza-β^{3}-amino acids cannot be prepared from natural amino acids or by chemical modification of peptides. Fmoc-, Boc- or Z-protected hydrazines serve as convenient precursors for the synthesis, while choice of the protecting group is depended upon the details of the preferred synthetic route.

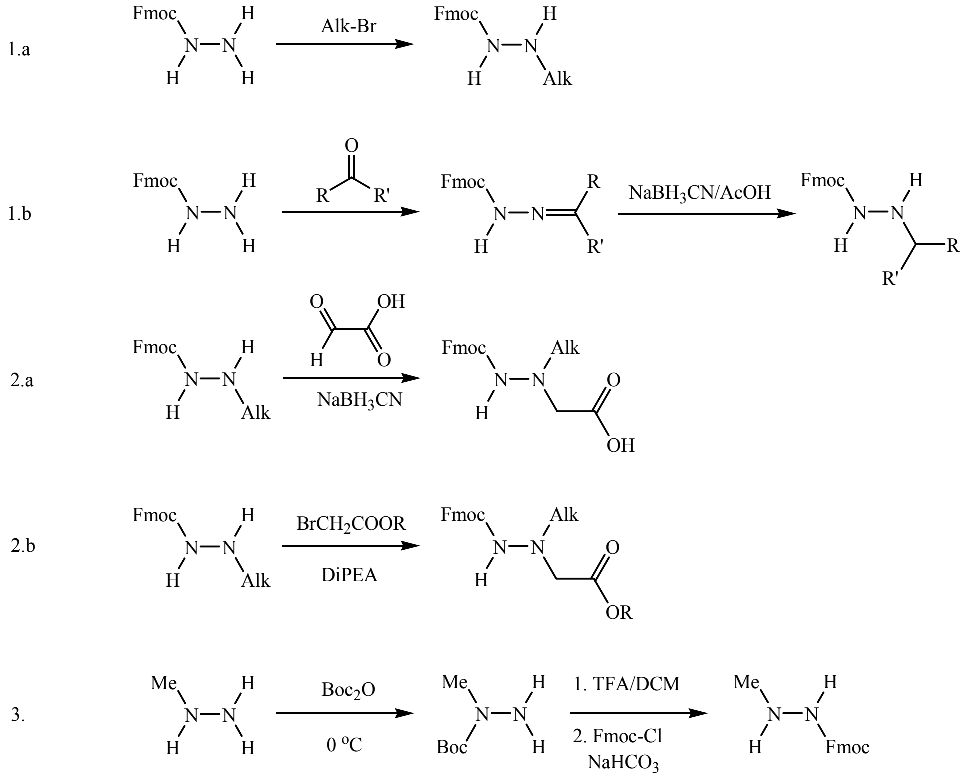
N$^{\beta}$-Fmoc-aza-β^{3}-amino acids were prepared in two steps: alkylation of Fmoc-hydrazine to give FmocNHNHAlk compounds; carboxymethylation of N-alkyl-N'-Fmoc hydrazines yielding FmocNHN(Alk)CH_2COOH-type derivatives (**Scheme 6**).

Fmoc-hydrazine was alkylated by reacting it with corresponding halogenoalkane (**Scheme 6**, route 1a), analogs of Asp, Asn, Glu, Gln (Busnel, 2007; Boeglin, 2005) were prepared in this manner. The second route was reduction of corresponding hydrazones (**Scheme 6**). All aza-β^{3}-amino acids with non-functionalized side chains and some with functionalized chains, adequately protected, were conveniently prepared by using Fmoc-hydrazine condensation with a carbonyl reagent followed by reduction. This procedure did not require intermediate purification and the yields of the alkylation products were high (70 - 90%) (Boeglin, 2005).

For introduction of the carboxymethylene moiety two approaches can be used (**Scheme 6**, routes 2.a and 2.b). Non-functionalized (or adequately protected) side-chain-bearing precursors were reacted with glyoxalic acid in the presence of sodium cyanoboronhydride, giving Fmoc-aza-β^{3}-amino acids as final products. This reaction proceeded with high yields. In certain cases (e.g. Fmoc-aza-β^{3}-arginine) it was strategically necessary to introduce the carboxymethyl group first, protected in form of ester, which was accomplished using a corresponding bromoacetate ester (**Scheme 6**, 2.b). Fmoc-aza-β^{3}-alanine was another exception: it was synthesized from methyl hydrazine, which is commercially available (**Scheme 6**, route 3) (Busnel, 2005).

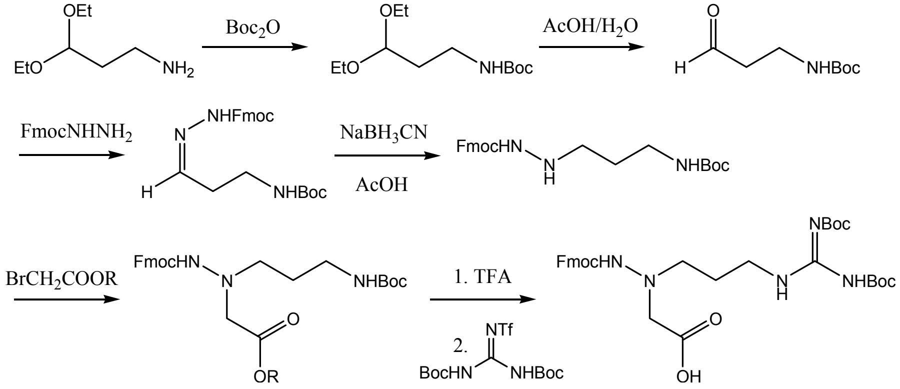
N$^{\beta}$-Fmoc-aza-β^{3}-alanine was prepared from methyl hydrazine by protecting the alkyl bearing nitrogen with *tert*-butoxycarbonyl group and subsequent introduction of the Fmoc protecting group, followed by *tert*-butoxycarbonyl group removal. The FmocNHNHMe obtained is then carboxymethylated with glyoxalic acid and sodium cyanoborohydride.

N$^{\beta}$-Fmoc-aza-β^{3}-valine was prepared by Fmoc-hydrazine condensation with acetone, reduction of the hydrazone formed and carboxymethylation of FmocNHNH*i*Pr with glyoxalic acid hydrate and cyanoborohydride.



Scheme 6. Preparation of N$^{\beta}$-Fmoc-aza-β^3-amino acids. (1a, b) Alkylation and (2a, b) carboxymethylation of Fmoc hydrazine derivatives; (3) preparation of N'-Fmoc-aza-β^3-alanine precursor from commercially available methyl hydrazine.

N$^{\beta}$-Fmoc-aza-β^3-Arg(Boc)$_2$-OH was prepared as shown in **Scheme 7**. 3-aminopropanal is commercially available in diethyl acetal form. Amino group was acylated, acetal protection removed by acid hydrolysis, the aldehyde formed was coupled with Fmoc-hydrazine and intermediate hydrazone was reduced. The FmocNHNH(CH$_2$)$_3$NHBoc obtained was further reacted with BrCH$_2$COOR (R = Me or Bn), the side chain and carboxyl-protected N$^{\beta}$-Fmoc-aza-β^3-ornithine was then N-deprotected and guanylated with N,N'-di-Boc-N''-trifylguanidine (Feichtinger, 1998). The final deprotection of ester group yielded N$^{\beta}$-Fmoc-aza-β^3-(Boc)$_2$-arginine. However, there is an approach which would allow the preparation of this compound in fewer synthetic steps (**Scheme 7**), as was reported by Gilon and co-workers (Freeman, 2011).



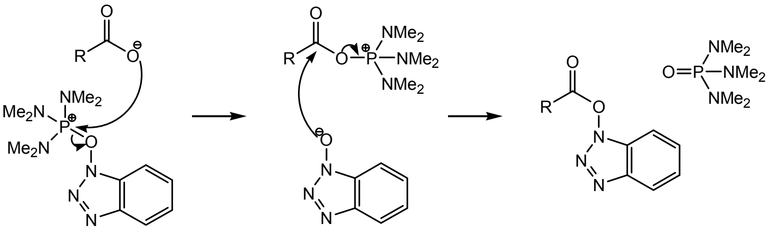
Scheme 7. Preparation of aza-β^3-arginine. R = methyl or benzyl.

Not all aza-β^3-analogs of natural amino acids can be prepared. For example, aza-β^3-serine is not known, since the hemiacetal-type compounds with a -NH-CH$_2$-OH-moiety are sensitive to acids, although their stability can be increased by protecting hydroxyl group and introducing more substituents to the nitrogen atom. The same applies to aza-β^3-threonine. No data are available on aza-β^3-cysteine preparation or potential stability.

3.2. Solid phase peptide synthesis

The solid-phase peptide synthesis (SPPS) is a widely used method for preparation of small batches of relatively short (approximately 20 amino acid residues, the top limit is considered to be fifty amino acid residues) peptides. The advantages of this method are simplicity of removal of excessive reagents and soluble side-products, which allows using the reagents in excess, accelerating the reactions, and possibility of using the automated synthesizers due to the ease of handling the resin-bound substrate. The general drawback of the solid phase method is the possibility of sluggishness of reactions in comparison to reactions in solution (Carpino, 1991; Albericio, 1989). There are debates whether with increasing the length of peptide chain substrate overcrowding and chain aggregation (intra- and intermolecular) occurs, impeding the further amino acid couplings. Merrifield and co-workers have showed that peptide coupling can be effectively carried out in cases where peptide-resin contains up to 81% of petide and 19% polymer resin and excellent peptide-resin bead swelling was observed (Sarin, 1980). It was concluded, that even at high polymerization degree (6000 Dalton) peptide chains are perfectly accessible for solvent molecules, and therefore for reagent molecules, and even more so with increasing the distance of reactive N-terminus from the resin bead as the chain growth progresses. It should be noted, though, that this study was carried out with (Leu-Ala-Gly-Val)-polymer, which does not contain charged side-chain groups, which may have the general responsibility for polypeptide collapsing into an unwanted folded structure (most commonly, β-sheets). For the complicated couplings (most commonly, peptides containing sterically hindered N^{α}- and C^{α}-alkylated amino acids and already mentioned β-sheet-forming peptides) special procedure modifications are employed: intermediate acid treatment, use of more polar solvents and reversible modifications of amino acid monomers (alkylation, forming of pseudoproline) (Coin, 2007, and references therein).

The wide variety of resins and activating reagents has been developed since the SPPS was first introduced by Merrifield in 1959 (Joullie, 2010; Han, 2004). The two commonly used methodologies of peptide synthesis, both in solution and on the solid support, are Fmoc/tBu and Boc/Bn. To elongate the peptide chain various amino acid activation techniques are available. These methods include synthesis of acyl chlorides or symmetrical anhydrides with phosgene, activation with dialkylcarbodiimides and formation of active esters (e.g. 2,4-dinitrophenyl esters, perfluorophenyl esters, benzotriazolyl-type esters). Undoubtely, the most popular are various tandems of activators, like phosphonium and uronium salt-based reagents (e.g. TBTU, HATU, BOP) with corresponding free triazoles (HOBT, HOAT) (Albericio, 1998). The mechanism of their action is illustrated in **Scheme 8**. In this technique, N-protected amino acids are converted into their benzotriazolyl esters, which readily couple to the free N-terminus of the growing peptide. The Fmoc protecting group is removed with solution of piperidine. After the peptide synthesis is completed, the crude material is cleaved off the resin using TFA with certain additives, which are used to scavenge reactive alkyl cations formed from the cleaved alkyl protecting groups. These cations can attack the peptide chain, causing unwanted alkylations. Scavengers also protect the thiol groups from alkylation and oxidation.



Scheme 8. Activation of amino acid by phosphonium-type reagent (BOP) and HOBT.

3.3. Substrate phosphorylation by protein kinase A

Phosphorylation of peptidomimetics and peptides by PKA was monitored by measuring incorporation of radioactivity of ^{32}P-containing phosphate group into substrates. As all studied substrates contained arginine residues, the conventional assay method, using product absorption onto phosphocellulose paper, was used for determination of the amount of phosphorylated product (Järv, 1991). Values of the initial velocity of the reaction were calculated from the linear plot of substrate-bound radioactivity vs time (**Figure 1**).

Kinetic experiments were carried out under steady state conditions at 0.1 mM ATP concentration exceeding significantly the K_m value for this substrate and various concentrations of peptidomimetic substrates below and above their own K_m values were used. Experimental conditions of the phosphorylation reaction were described in attached publications and briefly in legend to **Figure 1.** In parallel with peptidomimetic substrates phosphorylation of two specific peptide substrates, Kemptide (LRRASLG) and RRASVA were measured. These data were used for calculation of specific activity of different enzyme preparations.

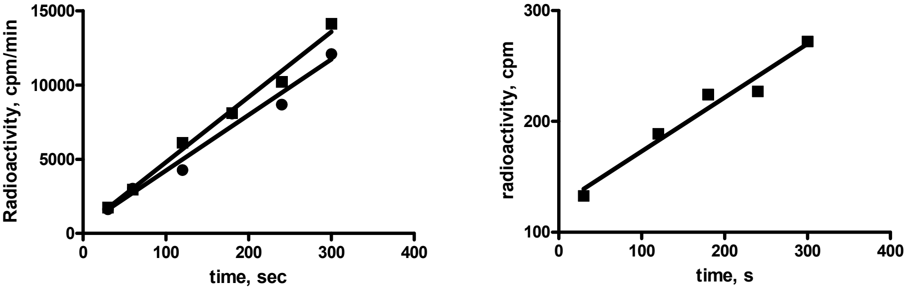


Figure 1. Kinetics of phosphorylation of RRASVA (■) and Kemptide (●) (left panel), and peptidomimetic substrate ψR1-ψV5 (right panel) by protein kinase A. Reaction conditions: 30°C, 50 mM TRIS/HCl, pH 7.5. The reaction mixture (final volume 100 μL, 50 mM TRIS/HCl, pH 7.5) contained: 100 μM γ-(^{32}P)ATP, 100 μM peptide, 10 mM $MgCl_2$, and 0.3 μg/mL of the enzyme, 1 mg/mL BSA. At shown time points 10 μL aliquots were taken from the reaction mixture and spotted onto pieces of phosphocellulose paper, which were subsequently immersed into ice-cold 75 mM phosphoric acid to stop the reaction. These samples were then washed four times with cold 75 mM H_3PO_4 (10 min each time) to remove the excess of γ-(^{32}P)ATP and were dried at 120°C for 15 min. The radioactivity bound onto the paper was measured as Cherenkov radiation using a Beckman LS 7500 scintillation counter.

As the phosphorylation reaction was assayed at excess of ATP over its K_m value, the initial velocities of phosphorylation were analyzed by means of the common Michaelis–Menten equation, where S stands for the variable peptide or peptidomimetic substrate concentration (S):

$$v = \frac{k_{cat}[S][E]_0}{K_m + [S]}$$

This plot is illustrated in **Fig. 2** for peptidomimetic substrate ψR2-ψV5 (see **Table 2**). From these hyperbolic plots the kinetic parameters k_{cat} and K_m were calculated, as listed in **Table 2** of the next part of this dissertation.

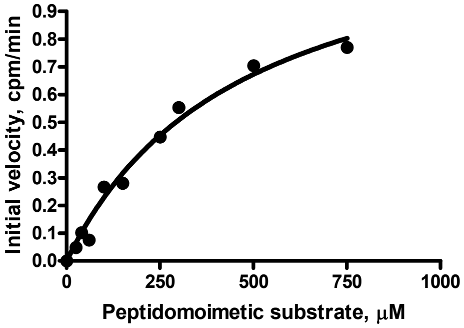


Figure 2. Phosphorylation of peptidomimetic substrate ψR2-ψV5 by protein kinase A. Reaction conditions were specified in caption to **Figure 1**.

Additionally, we used the initial linear part of the Michaelis– Menten plots, characterizing the phosphorylation reaction under the second-order rate conditions where (S) < Km, and calculated the second-order rate constants k_{II}:

$$v = k_{II}[S][E]_0$$

The kinetic parameters k_{II} have the same meaning as the ratio k_{cat}/K_m, as $k_{cat} = V/(E_0)$. However, since the k_{II} values ware determined from different set of experimental data, this approach can be used as an internal test of applicability of the conventional Michaelis-Menten equation.

3.4. Data processing and results of computer modelling of peptidomimetics

For statistical data processing the GraphPad Prism software was used (ver. 4.0, GraphPad Software Inc, USA). Linear and hyperbolic plots were analyzed by using regression analysis and kinetic parameters together with their standard errors were calculated.

All *in silico* modelling experiments with peptidomimetics were performed by Dr. A.Kuznetsov and results of these calculations were kindly provided as personal communication for their further analysis and comparison with results of kinetic analysis. More specifically the results of the following calculations were used.

Peptidomimetics structure modeling was made by using a Spartan 4.0 software suite (Wavefunction, Inc., USA) and the minimum-energy conformations of the compounds were obtained. Conformational searches were made by using molecular mechanics with additional conditions of the aqueous medium for finding stable geometry. All peptides were represented as zwitterions for these calculations.

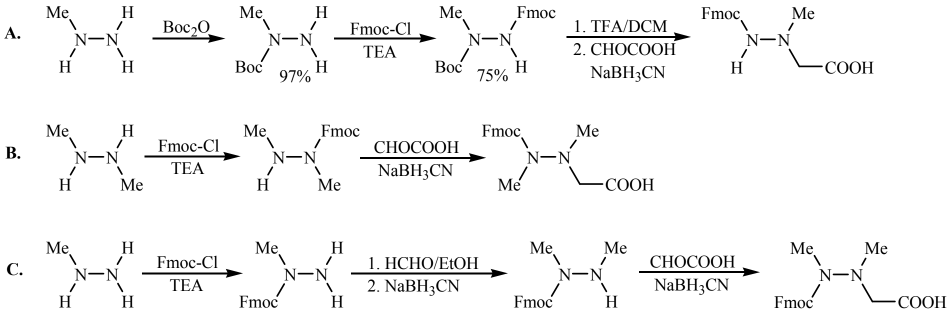
The peptidomimetics docking modeling was carried out by using the AutoDock Vina software (ver. 1.0.3) (Trott, 2010). The initial structure of peptides and their analogs were calculated by Spartan and the docking compatible structure formats of the protein were prepared by AutoDockTools (ver. 1.5.4) (Sanner, 1999). The fitting box with 0.3 Å of grid spacing was defined once and used for all docking calculations. The fitting area covered the peptide binding site of PKA and the best docking pose with the lowest energy for each peptidomimetics within this area was obtained.

4. RESULTS AND DISCUSSION

As mentioned above, the objective of this work was syntyhesis of aza-β^3-amino acid containing peptides and testing the effect of peptide backbone modification on their molecular recognition by protein kinase A binding site. Therefore the main directions of this study were synthesis of precursors of necessary aza-β^3-amino acids, preparation of peptidomimetic substrates for protein kinase A and investigation into interaction of these compounds with the enzyme.

4.1. Fmoc-protected aza-β^3- and N^β-Me- aza-β^3-amino acids and their precursors

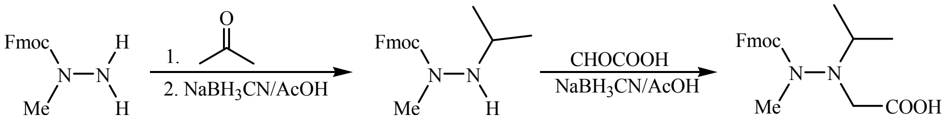
Although syntheses of Fmoc-protected aza-β^3-analogs of Ala, Arg and Val was reported previously (Busnel, 2005), it was necessary to advance several aspects of the synthetic procedures. In this study, N^β-Fmoc-N^β-Me-aza-β^3-amino acids were prepared via the same general route as their non-methylated counterparts, starting from FmocMeNNH$_2$ instead of FmocNHNH$_2$ (**Scheme 9**).



Scheme 9. Preparation of Fmoc-aza-β^3-alanine (**A**) and the two routes for Fmoc-N^β-Me-aza-β^3-alanine preparation (**B**, **C**).

For preparation of N^β-Fmoc-aza-β^3-alanine MeNHNH$_2$ was acylated with di-*tert*-butyl dicarbonate at 0ºC, the yield of the required N,N-isomer was nearly qualitative (97%). The N-Boc-N-Me hydrazine was subsequently acylated with Fmoc-chloride in the presence of triethylamine (yield 75%), Boc protecting group was removed with trifluoroacetic acid in DCM (yield 89%), and the obtained N-Fmoc-N'-methyl hydrazine was coupled with glyoxalic acid in the presence of sodium cyanoborohydride and acetic acid, giving the final product, N^β-Fmoc-aza-β^3-alanine in 65% yield. The overall yield of this procedure was 42% (**Scheme 9**, route **A**). This compound was also obtained by reacting Fmoc-hydrazine with methanal, reducing the hydrazone intermediate and carboxymethylation of FmocNHNHMe (obtained in *ca* 75% overall yield) with glyoxalic acid and cyanoborohydride. Correspondingly, N$^{\beta}$-Fmoc-N$^{\beta}$-Me-aza-β^3-alanine was prepared over two steps starting from commercial *sym*-dimethyl hydrazine (**Scheme 9**, route **B**). The fisrt step, acylation with Fmoc-Cl and TEA at 0ºC or -72ºC afforded a mixture of mono- and diacylated products, despite the fact that 2 equivalents of *sym*-dimethyl hydrazine was used. The yield of the product was relatively low (39%) and tedious purification was required. The usual yield of reductive amination step after chromatographic product purification is *ca* 65%. To avoid the inefficient step of *sym*-dimethyl hydrazine acylation, a different route to N-Fmoc-N-Me-N'-Me was elaborated (**Scheme 9**, C). Methyl-hydrazine was acylated with Fmoc-Cl and TEA at 0ºC or at -78 ºC (Nicolas, 2011). In this procedure Fmoc-Cl solution must be added to the reaction mixture very carefully over 2 hours. The reaction was successfully performed also at 0ºC, and the crude FmocMeNNH$_2$ (ca 80% yield) was usable in subsequent step without purification. On the other hand, it was found that performing the acylation reaction at lower temperature was safer, as in this case less careful addition of Fmoc-Cl solution did not have a disasterous impact on the product quality. N-Fmoc-N-methyl hydrazine was a precursor of all prepared N$^{\beta}$-Fmoc-N$^{\beta}$-Me-aza-β^3-amino acids (N$^{\beta}$-Me-aza-β^3-proline can only be used as an N-terminal amino acid and its synthesis has not been reported). N-Fmoc-N-methyl hydrazine was further methylated by refluxing with formaldehyde (1 equivalent, ~37% aqueous solution) in ethanol (96% hydrazone formation) and subsequent reduction of the formed hydrazone with 100% yield. The final step of this synthesis, the reductive amination, afforded N$^{\beta}$-Fmoc-N$^{\beta}$-Me-aza-β^3-alanine in good yield (72%). Thus the overall yield of the procedure was 55%.

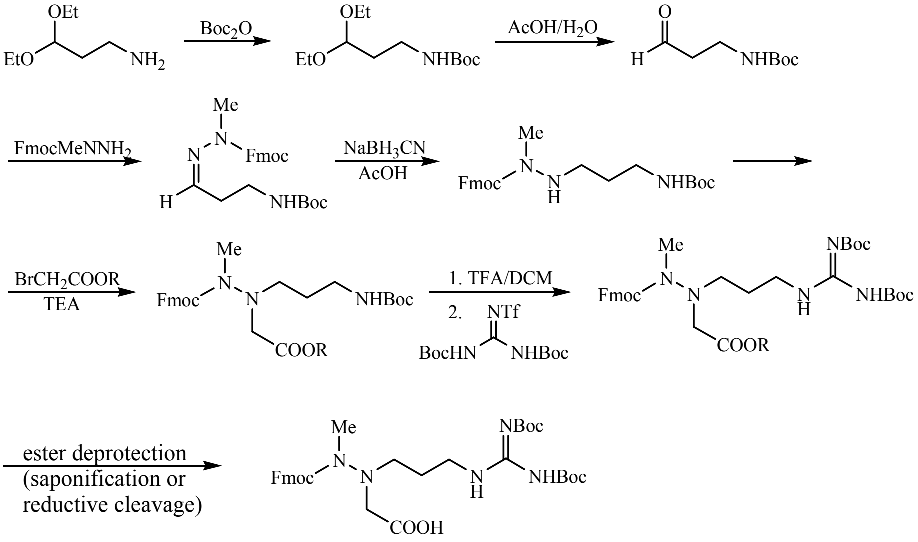
N$^{\beta}$-Fmoc-N$^{\beta}$-Me-aza-β^3-valine was prepared starting from N-Fmoc-N-methyl hydrazine via acetone methyl hydrazone (**Scheme 10**). The reaction with acetone is catalyzed by adding a few drops of acetic acid (crude product yield 99%). Hydrazone reduction with sodium cyanoborohydride and acetic acid yielded the N-Fmoc-N-Me-N'-*i*Pr hydrazine (100%, crude), which was carboxymethylated with glyoxalic acid hydrate and sodium cyanoborohydride to yield the ready N$^{\beta}$-Fmoc-N$^{\beta}$-Me-aza-β^3-valine (58% after chromatographic purification). The overall yield of this reaction path was 57%.



Scheme 10. Synthesis of N$^{\beta}$-Fmoc-N$^{\beta}$-Me-aza-β^3-valine.

Two sets of signals were observed in 1H NMR spectrum of N$^{\beta}$-Fmoc-N$^{\beta}$-Me-aza-β^3-Val-OH for *N*-CH_3 and *i*Pr protons of two stereomers, which indicates that the nitrogen pyramidal inversion was slow due to the sterical hindrance in the tetrasubstituted hydrazine derivative.

The precursor N$^{\beta}$-Fmoc-N$^{\beta}$-Me-aza-β^3-Arg(Boc)$_2$-OH was prepared using N-Fmoc-N-Me hydrazine as starting material (**Scheme 11**). 3-aminopropanal required for ornithine side chain introduction was obtained by protecting the amino group of 3-aminopropionaldehyde diethyl acetal with Boc group and subsequent acidolytic removal of acetal protection in aqueous media. Coupling of 3-(N-Boc)-aminopropionanaldehyde with N-Fmoc-N-Me hydrazine and the following reduction of the resulting hydrazone yielded the N-Fmoc-N-Me-N'-(3-(N-Boc)-aminopropyl) hydrazine. This compound was carboxymethylated with bromoacetate methyl or benzyl ester.



Scheme 11. Preparation of N$^{\beta}$-Fmoc-N$^{\beta}$-Me-aza-β^3-Arg(Boc)$_2$-OH (R = Me or Bn).

Alkylation of Fmoc-protected hydrazine derivative with alkyl bromide was carried out in the presence of base. Althoug the Fmoc group is base-sensitive, stability of such protected hydrazine derivatives increases with increasing degree of N,N'-substitution. N-Fmoc-N'-alkyl hydrazine was refluxed in the presence of 1 equivalent of base for several days, while Fmoc-hydrazine is decomposed after several minutes under such reaction conditions. Both FmocNHNH$(CH_2)_3$NHBoc and FmocNMeNH$(CH_2)_3$NHBoc were sufficiently stable for this procedure and yields of N$^{\beta}$-Fmoc-N$^{\beta}$-Me-aza-β^3-arginine and N$^{\beta}$-Fmoc-aza-β^3-arginine were similar (ca 20% overall yield after 6 steps). As was shown in **Scheme 11**, the carboxyl group was protected with methyl or benzyl ester during the intermediate synthetic steps. Although the benzyl group can be conveniently removed by hydrogenation on palladized carbon, it is known that such conditions can be destructive for Fmoc group. In this work, however, the N^{β}-Fmoc-aza-β^3-arginine was obtained in high yield by reductive elimination of ester protection over 1.5 h. Final products were obtained in 97% (N^{β}-Fmoc-aza-β^3-Arg$(Boc)_2$-OH) and 56% (N^{β}-Fmoc-N^{β}-Me-aza-β^3-Arg$(Boc)_2$-OH) yields.

Deprotection of methyl ester has also certain practical limitations. In the course of this reaction viscous and poorly soluble product was formed that was inconvenient for performing the reaction. The yields for N^{β}-Fmoc-aza-β^3-Arg$(Boc)_2$-OH and N^{β}-Fmoc-N^{β}-Me-aza-β^3-Arg$(Boc)_2$-OH were 67% and 36%, respectively.

The ester protection was necessary for manipulation of amino group of 3-aminopropyl residue, which was after Boc cleavage with TFA guanylated with Goodman reagent $(BocNH)_2C{=}NTf$ to give N^{β}-Fmoc-N^{β}-Me-aza-β^3-Arg$(Boc)_2$-OR (R = Bn or Me). This functional group was finally converted to free acid after removal of the ester protection.

It is important to note that all stages of peptide synthesis (amino acid activation, coupling and deprotection) are commonly carried out at room temperature. In this work, however, microwave activation was used for reaction acceleration. This treatment was possible because of all reagents used in these syntheses were safe to heat up to 85ºC, and there was no danger of amino acid racemization. This modification allowed shortening of the reaction time: for example 5 min deprotection time was used instead of commonly used 20 min at room temperature.

4.2. Peptidomimetics with aza-β^3-amino acids

The use of aza-β^3-amino acids allowed preparation of polypeptide analogs by the convenient methods of peptide chemistry, although nucleophilicity of N-termini of hydrazino compounds were understandably poorer than those of amino acids. Moreover, the methylated N-termini of N^{β}-methyl-aza-β^3-amino acid residues were also sterically hindered. Therefore stronger activation of reagents with HATU (Doedens, 2010) was necessary and some excess of reagents was also used. Although application of 3 or 4 equivalents could be recommended under normal reaction conditions, as little as 1.2 equivalents might be enough if high reagent concentration is maintained. This is possible in the case of small volume of the reaction cell. Moreover, for coupling to the non-methylated aza-termini longer coupling times were also used (10 minutes instead of 5). Like in common synthetic procedure, coupling of arginine analog imposed some problems due to poor reactivity. Therefore repetitive couplings were used by analogy with the common SPPS procedure. But still the yelds of these syntheses reflected low reactivity of arginine analog.

Summary of synthesis results was shown in **Table 2**, where the overall yields of synthesis of the three series of peptidomimetics were listed. These data show that the average yield of synthesis of peptidomimetics containing one aza-β^3 analog was approximately 40%. Certainly exceptions were in the case of arginine analogs, as explained above. However, lower yields were also characteristic for peptidomoimetics with two aza-β^3 analogs (entries VI-XV), and with N$^\beta$-methylated analogs.

Table 2. List of synthesized peptidomoimetics and their overall yield of synthesis.

No.	Abbreviation	Structure	Yield	MW determined
I	ψR1	azaβ^3RRASVA	21%	674.4053
II	ψR2	Razaβ^3RASVA	22%	674.4052
III	ψA3	RRazaβ^3ASVA	69%	674.4054
IV	ψV5	RRASazaβ^3VA	39%	674.4051
V	ψA6	RRASVazaβ^3A	49%	674.4053
VI	ψR1-ψR2	azaβ^3Razaβ^3RASVA	24%	689.4165
VII	ψR1-ψA3	azaβ^3RRazaβ^3ASVA	39%	689.4165
VIII	ψR1-ψV5	azaβ^3RRASazaβ^3VA	19%	689.4165
IX	ψR1-ψA6	azaβ^3RRASVazaβ^3A	4%	689.4165
X	ψR2-ψA3	Razaβ^3Razaβ^3ASVA	16%	689.4165
XI	ψR2-ψV5	Razaβ^3RASazaβ^3VA	25%	689.4165
XII	ψR2-ψA6	Razaβ^3RASVazaβ^3A	22%	689.4165
XIII	ψA3-ψV5	RRazaβ^3ASazaβ^3VA	30%	689.4165
XIV	ψA3-ψA6	RRazaβ^3ASVazaβ^3A	25%	689.4165
XV	ψV5-ψA6	RRASazaβ^3Vazaβ^3A	28%	689.4165
XVI	ψ(Me)R1	(N$^\beta$Me)azaβ^3RRASVA	10%	688.33
XVII	ψ(Me)R2	R(N$^\beta$Me)azaβ^3RASVA	5%	688.19
XVIII	ψ(Me)A3	RR(N$^\beta$Me)azaβ^3ASVA	62%	687.86
XIX	ψ(Me)V5	RRAS(N$^\beta$Me)azaβ^3VA	25%	688.34
XX	ψ(Me)A6	RRASV(N$^\beta$Me)azaβ^3A	20%	688.45

ψ = aza-β^3, ψ(Me) = N$^\beta$-Me-aza-β^3

Knowing that the yield of RRASVA, if prepared under the same conditions, was above 80%, it can be calculated that the average yield of coupling of one normal amino acid was ca 96%. At the same time the average coupling yield of aza-β^3 amino acid was ca 50% (48% and 53% calculated for mono- and di-aza-β^3 series, respectively), and ca 28% for N$^\beta$-methyl-aza-β^3 amino acid. It fact, the lower yield of peptidomometics can be caused by two circumstances: (1) insufficient activation of the incoming aza-β^3-amino acid and (2) low reactivity of hydrazinic H_2N-NR-terminus. Thus, the yields of substrates do not depend entirely on the number of analog amino acids in the peptide, but also on the type of analogs and the position of analog in the chain. If the analog is in the terminal position, no amino acid is to be coupled to its N-terminus, and the less-than-usual reactivity of such N-terminus will not influence the product yield.

Taking together, it can be concluded that there seems still to be room for improvement of the of aza-β^3 amino acid containing peptidomimetics synthesis methodology, taking into consideration the dependence of the reaction yield on the type of the amino acid analog and its positioning in the sequence of peptidomimetic.

4.3. Phosphorylation of peptidomimetic substrates by PKA

All syhthesized aza-β^3-peptidomimetics were phosphorylated by PKA and the results of these kinetic experiments are summarized in **Table 3**. As it was mentioned above (Experimental part), kinetic parameters K_m, k_{cat} and k_{II} were determined for each substrate. This was possible regardless of great variance in substrate reactivity, as all compounds were soluble and enabled experiments at concentration up to 10 mM.

Secondly, it can be seen from these results that the k_{II} values were close to the k_{cat}/K_m ratio. As these parameters were calculated from different parts of the Michaelis-Menten plot, this agreement confirms applicability of the simple Michaelis-Menten rate equation for this phosphorylation reaction and the absence of potential complicating phenomena like substrate inhibition or non-productive binding in the case of these peptidomoimetic substrates (Fersht, 1974). Taking together, it was concluded that all peptidomimetic compounds were effectively recognized by the binding site of protein kinase A and were favourably positioned for the catalytic step of the phosphorylation reaction. Therefore the kinetic parameters should correspond to similar reaction mechanism and should be comparable to each other.

Thirdly, it can be seen that changes in reactivity of peptidomimetic substrates were rather different within the series of substrates and were clearly depending on position of backbone structure modification. This is a clear illustration of the viewpoint that recognition of a biologically active peptide by its target binding site depends not only upon the side group sequence of the peptide, but can also be modulated by altering the backbone structure of this polymer.

If the kinetic parameters K_m characterize interaction of substrate molecule with the enzyme binding site and could be related with the enzyme-substrate complex dissociation constant, their larger values refer to weaker substrate interaction with the enzyme. It can be seen in **Table 3** that for all peptidomimetics this kinetic parameter was larger than the K_m value for the minimum sunstrate RRASVA. Thus introduction of one or two aza-β^3-fragments into the sequence of six amino acids always hindered peptidomimetic binding.

Table 3. Kinetic data for phosphorylation of three series of peptidomimetic substrates and their parent peptide subatrte RRASVA by protein kinase A. Conditions of the reaction were specified in the caption to **Figure 1**.

No.	Abbreviation	Structure	K_m μM	$10^2 \cdot k_{cat}$ μmol·mg^{-1}·s^{-1}	$10^{-4} \cdot k_{II}$ L·mg^{-1}·s^{-1}
I	ψR1	azaβ^3RRASVA	273.5±43.8	28±5	0.1±0.02
II	ψR2	Razaβ^3RASVA	19.1±2.9	32±2	1.7±0.2
III	ψA3	RRazaβ^3ASVA	59.8±9.4	27±1	0.5±0.1
IV	ψV5	RRASazaβ^3VA	122.1±21.6	25±4	0.3±0.04
V	ψA6	RRASVazaβ^3A	43.7±8.8	26±5	0.6±0.2
VI	ψR1-ψR2	azaβ^3Razaβ^3RASVA	920 ± 127	11.1 ±1.0	0.0114 ± 0.00079
VII	ψR1-ψA3	azaβ^3RRazaβ^3ASVA	1572 ± 333	2.87 ± 0.30	0.00194 ± 0.00014
VIII	ψR1-ψV5	azaβ^3RRASazaβ^3VA	254 ± 68	1.08 ± 0.11	0.00441 ± 0.00051
IX	ψR1-ψA6	azaβ^3RRASVazaβ^3A	131 ± 38	19.7 ± 2.0	0.121 ± 0.0064
X	ψR2-ψA3	Razaβ^3Razaβ^3ASVA	69 ± 15	2.35 ± 0.15	0.0359 ± 0.0093
XI	ψR2-ψV5	Razaβ^3RASazaβ^3VA	544 ± 122	1.19 ± 0.13	0.00185 ± 0.00049
XII	ψR2-ψA6	Razaβ^3RASVazaβ^3A	47.4 ± 8.1	4.39 ± 0.27	0.0976 ± 0.0065
XIII	ψA3-ψV5	RRazaβ^3ASazaβ^3VA	217 ± 46	4.58 ± 0.52	0.0230 ± 0.0012
XIV	ψA3-ψA6	RRazaβ^3ASVazaβ^3A	118 ± 35	14.2 ± 1.7	0.121 ± 0.0094
XV	ψV5-ψA6	RRASazaβ^3Vazaβ^3A	73 ± 17	8.80 ± 0.55	0.126 ± 0.013
XVI	ψ(Me)R1	(N$^{\beta}$Me)azaβ^3RRASVA	209 ± 24	24.4 ±1.5	0.098 ± 0.010
XVII	ψ(Me)R2	R(N$^{\beta}$Me)azaβ^3RASVA	79 ± 18	11.9 ± 1.0	0.11 ± 0.07
XVIII	ψ(Me)A3	RR(N$^{\beta}$Me)azaβ^3ASVA	91 ± 15	5.5 ± 0.3	0.034 ± 0.005
XIX	ψ(Me)V5	RRAS(N$^{\beta}$Me)azaβ^3VA	118 ± 36	16 ± 2.0	0.12 ± 0.02
XX	ψ(Me)A6	RRASV(N$^{\beta}$Me)azaβ^3A	57 ± 17	5.0 ± 0.4	0.090 ± 0.015
-	RRASVA	RRASVA	11.1 ± 3.5	36 ± 3	3.2 ± 0.1

Similarly, as can be seen from kinetic data shown in **Table 3**, backbone modification also reduced rate of the catalytic step, characterized by the k_{cat} values. This comparison also revealed that the location of backbone modification was palying crucial role in determining the magnitude of the observed effect. Therefore the following analysis was focussed on two aspects of backbone modification. Firstly, we were discussing manifestation of this effect in the case of kinetic parameters K_m and k_{cat}, characterizing the substrate binding and reaction steps, respectively. And secondly, the dependence of the modification effect upon location of the aza-β^3-fragment in peptidomimetic substrate backbone. As the second order rate constants k_{II} should present summary of structural effects in the binding step and catalysis, these parameters seemed to be more suitable for the latter analysis.

4.4. Effect of backbone modification on binding and catalysis

It can be seen in **Table 3** that introduction of aza-β^3-fragments into substrate backbone affected both kinetic parameters, increasing the K_m up to 150 times and reducing the k_{cat} value up to 30 times. Thus the effect of modification was not equal in these parameters, and in many cases the backbone recognition was more important for the binding step.

The most straightforward way to compare manifestation of structural effects in the binding and catalytic steps is a direct plot of the free-energy related pK_m and log k_{cat} values for the same substrates. This plot was shown in **Figure 3**, where data for all studied compounds (**Table 2**) were included. In addition, kinetic data for some common peptide substrates from literature were also added to this plot. The results of this analysis clearly demonstrated that alteration of peptide and peptidomimetic substrates could be rather different in the binding and reaction steps, and most importantly, depended on backbone structure of these compounds.

Firstly, it can be seen that the phosphorylation step of aza-β^3-amino acid containing peptide analogs was almost indepent on structure variation, while binding effectiveness was significantly affected by the presence of the aza-group. This was illustrated by very small slope of the log k_{cat} vs pK_m plot for these compounds (●). Interestingly, the same regularity was observed in the case of common peptides, as was shown by Järv and Ragnarsson (1991). This phenomenon was illustrated in **Figure 3** by including data for phosphorylation reaction of common peptide (×). It can be seen that kinetic behaviour of peptides and peptidomimetics with single aza-β^3-amino acid substitution is very similar, and the final outcome of backbone modification in peptidomometic substrates and alteration of sequence of common amino acids in peptide substrates resulted in identical log k_{cat} vs pK_m plot. Formally the presence of this common plot for peptide substrates and peptidomimetics with single aza-β^3-substitution should refer to common mechanism of interaction of these compounds with the enzyme binding site.

This conclusion was also in agreement with the results of computer modelling of substrate docking with protein kinase A, which revealed rather similar positioning of peptide RRASVA and peptidomimetic substrates with single aza-β^3-substitution in the enzyme binding site (see **Figure 4**).

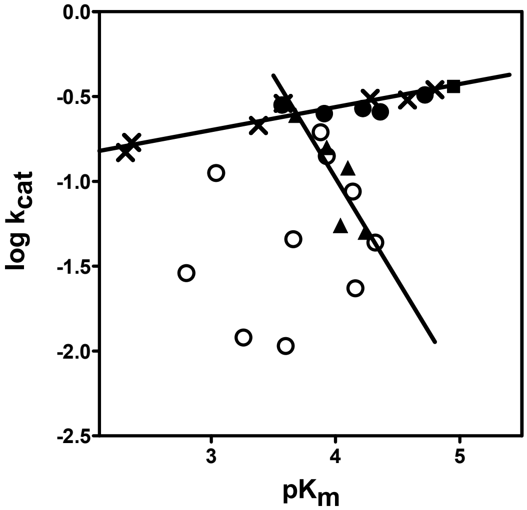


Figure 3. Comparison of influence of structural changes of peptidomimetic and peptide substrates on their binding effectiveness (pK_m) and reactivity ($\log k_{cat}$). Data for peptidomimetic substrates were taken from **Table 3** and were denoted as follows: aza-β^3–derivatives (●), di-aza-β^3-derivatives (○), N^β-Me-aza-β^3-derivatives (▲) and RRASVA (■). Data for peptide phosphorylation (except for RRASVA) were compiled from literature (Järv, 1991) and the following compounds were used in this plot: LRRASLG, RRASLG, LRKASLG, LARASLG, RASLG, LHRASLG (×).

Differently from compounds I–V (**Table 3**), for which the interrelationship "better binding – slightly better reaction" was observed, substrates with the methylated aza-β^3-amino acids revealed completely different interrelationship between $\log k_{cat}$ and pK_m values (filled triangles in **Fig 3**). It can be seen that for these substrates clearly negative slope of the $\log k_{cat}$ vs pK_m plot was observed and more effective binding resulted in lower reaction rate. Formally this change in slope value of the $\log k_{cat}$ vs pK_m plot should indicate change of the mechanism of their interaction with the enzyme binding site. On the other hand, as these compounds were still substrates and transfer of the phosphate group from ATP to serine moiety was observed, the productive enzyme-substrate complexes should exist in this case. Indeed, computer modelling of substrate docking revealed the presence of these complexes, where orientation of reacting groups was favourable for phosphate transfer step. On the other hand, however, in the case of methylated compounds significant population of other binding poses was observed that might be understood as "non-productive" enzyme-substrate complexes. This means thet the negative slope of the $\log k_{cat}$ vs pK_m plot can be explained by this pehenomenon. Indeed, following the concept of non-productive substrate binding both parameters, k_{cat} and K_m, should be divided by similar factor $(1 + 1/K_{np})$, where K_{np} stands for dissociation constant of the non-productive enzyme-substrate complex (Fersht 1974). Some structural arguments for possibility of this phenomenon in the case of substrates with N^{β}-methylated aza-β^3-amino acids were given below, although the topic of multiple positioning of peptidomimetic substrates on surface of enzyme molecule remains outside of the present topic and will be discussed elsewhere.

Finally, the series of peptidomimetic substrates with two aza-β^3-amino acids revealed more complicated specificity pattern, as only some datapoints were described by the linear log k_{cat} vs pK_m plot for N^{β}-methylated derivatives and there was no overlap with this linear plot for substrates with no or single aza-β^3-residue. On the other hand, all these compounds were still phosphorylated by protein kinase A, indicating the possibility of their correct positioning relatively the second substrate ATP. This conclusion was also confirmed by analysis of docking positions of these compounds in the active center. On the other hand, however, this computer modelling study revealed also efficient formation of non-productive complexes, demonstrating possibility of fairy difficult kinetic mechanism of the overall process.

It can be seen in **Figure 3** that datapoints for 4 double-substituted compounds fitted the linear log k_{cat} vs pK_m plot, while other 6 points were randomly scattered. Interestingly, all the former substrates have aza-β^3-alanine at the last position of the parent hexapeptide RRASVA structure. This fact again pointed to the importance of location of the aza-β^3-group in the sequence of the peptidomimetic substrates.

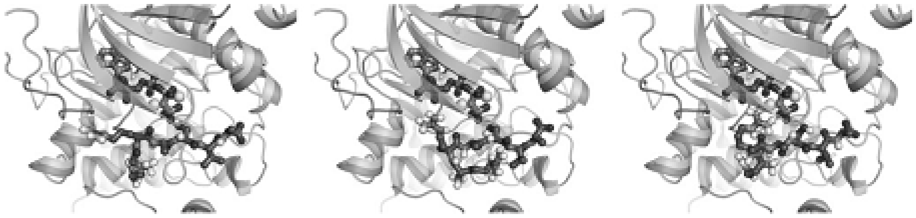


Figure 4. Computer modelling of productively bound peptidomimetic substrates in the protein kinase A active center. ψR1 (left), RRASVA (middle), ψR2 (right).

4.5. Role of the backbone modification position

It can be seen from the previous discussion that influence of modifications made along the backbone of the parent substrate RRASVA on kinetics of the enzyme reaction was significantly dependednt upon location of the modification site, but also on chemical structure of this modification. As both parameters K_m and k_{cat} were affected, these effects cumulated in the second-order rate constants k_{II}. The latter parameter corresponds to the ratio k_{cat}/K_m, which has been often thought of as a measure of efficiency of enzyme catalysis. Therefore the following discussion was based mainly on analysis of these parameters.

To illustrate the importance of location of the modification site the log k_{II} values for studied peptidomimetics were charted in three groups (**Figure 5**), representing the three series of peptidomimetic substrates made. The kinetic data for peptidomimetic substrate phosphorylation were compared with the log k_{II} value of the parent peptide substrate RRASVA, measured under the same experimental conditions.

It can be seen from these data that decreased efficiency of enzymatic phosphorylation reaction was observed in all cases of substrate backbone modification, even if this change was made at the end of the backbone chain. This can be understood, as the minimum peptide substrate was chosen as parent compound for these modification experiments, and therefore all parts of these compounds should indeed participate in the recognition process.

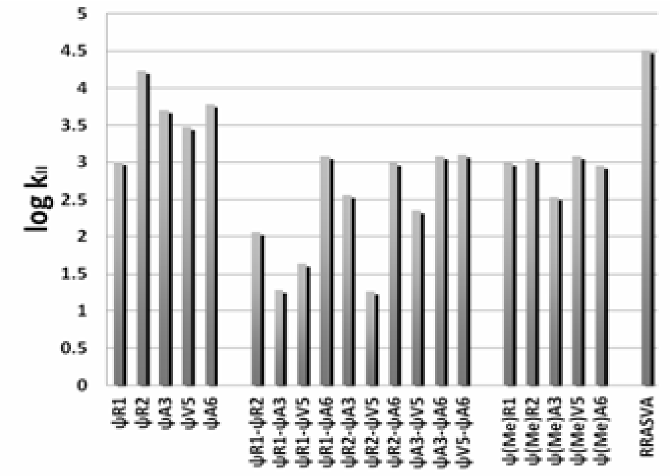


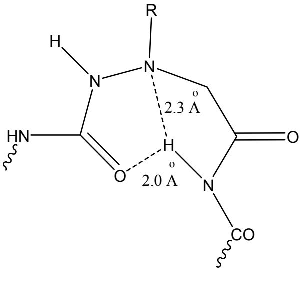
Figure 5. Comparison of the log k_{II} values for the three subseries of peptidomimetic subatrates and for the parent peptide RRASVA.

The smallest decrease of catalysis efficiency was observed in the case of peptidomimetic substrate Razaβ^{3}RASVA, where the modification was made at the second arginine residue. This is surprising, as the presence of consecutive two arginines of the minimal substrate RRASVA has been generally recognized as the main substrate specificity factor for protein kinase A. However, it is clear from the backbone structure of this peptidomimetic substrate that the mutulal positioning of these two arginine side groups was not disturbed in this compound. On the other hand, this mutual positioning should be altered in the case of azaβ^{3}RRASVA, which was indeed the worst substrate of this series, and the decrease of the rate constant exceeded one order of magnitude. Most interestingly, the same conclusions could be drawn also from analysis of the pK_m values listed in **Table 3**, while the k_{cat} was almost not affected by these changes.

These results indicate that the structure elements, which govern specificity of substrate recognition, seem to form clusters, structure of which should not be disturbed to design efficiently interacting peptidomimetic ligands. On the contrary, if efficient ligand binding is not the desired result, these clusters should be identified and selected as location of structural changes. This algorithm of orthogonal structure-activity alterations seems to be important if two distinct binding sites are targeted by ligand design. For example, if minimal alteration of affinity for a receptor site is needed with simultaneous decrease of ligand degradation rate by a proteolytic enzyme.

It can be seen in **Figure 5** that substrates with two aza-β^3-amino acids exhibited the lowest reactivity within the whole series of the peptidomimetic substrates. In this subgroup of compounds the worst substrates azaβ^3RRazaβ^3ASVA and Razaβ^3RASazaβ^3VA were phosphorylated at more that 1000 folds lower rate than RRASVA. Noteworthy, among the poorest substrates almost all compounds had modified arginine residue in the first position. This agrees with the conclusion about the presence of distinct specificity clusters, made above for peptidomimetics containing single aza-β^3-amino acid. At the same time modifications made at the C-terminal amino acid had considerably lower impact in both subseries.

It has been mentioned in literature that the presence of aza-β^3-amino acid in peptidomimetic molecule may cause formation of hydrazinoturns, as shown in **Scheme 12**. LeGrel (Dabbagh, 2009) has calculated the hydrogen bonds lengths, which should be characteristic for formation of these structures.



Scheme 12. Hydrazinoturn.

These distances were 2.0 Å for CO···HN bond and 2.3 Å for N···HN bond.

For all peptidomimetics which were synthesized in this study, the spatial models were calculated by Dr. A. Kuznetsov (private communication). By using this information it was possible to check the possibility of formation of hydrazinoturn-like structures in these models. This analysis revealed that hydrazinoturn formation was obvious only for one calculated structure: azaβ^3RRazaβ^3ASVA. Notoriously this compound was the least efficient substrate in the present reaction series, and its low reactivity could be observed by comparing the log k_{II} values of other substrates, as shown in **Figure 5**, but also by comparing the k_{cat} and K_m valuses for this substrate with the appropriate kinetic data for other compounds listed in **Table 3**. Therefore the possibility that recognition of the short peptide-like structures by enzyme binding sites may be governed by the second-order structure elements seemed rather intriguing, but its more detailed elaboration would need more straightforward experimental approaches.

4.6. Effect of N-methylation of aza-β^3-amino acid residue on peptidomimetics recognition by protein kinase A active center

As it was mentioned above, the log k_{cat} versus pK_m plot (**Figure 3**) revealed that these compounds have rather different specificity patterns. This conclusion was supported by straightforward analysis of the effect of N^β-methylation of aza-β^3-residues in peptidomimetic substrates that was made by comparing the same type kinetic data for congenetic substrates with and without the N^β-methyl group, as shown in **Figure 6**.

Firstly, comparison of the pK_m values for these two subseries of substrates demonstrated that rather good linear interrelationship can be observed in the case of these data (**Figure 6**, left panel), pointing to similar mechanism of interaction of these substrates with the enzyme binding site. Moreover, this linear plot was characterized by slope 1.0±0.3 and intercept 0.08±1.26. This was in agreement with the fact that N^β-methylation had practically no effect on interaction of these peptidomimetic substrates with their binding site, and similar specificity factors should govern the dependence of the K_m values upon position of the backbone modification. Notoriously, this linear plot included also the data-point for the parent peptide RRASVA.

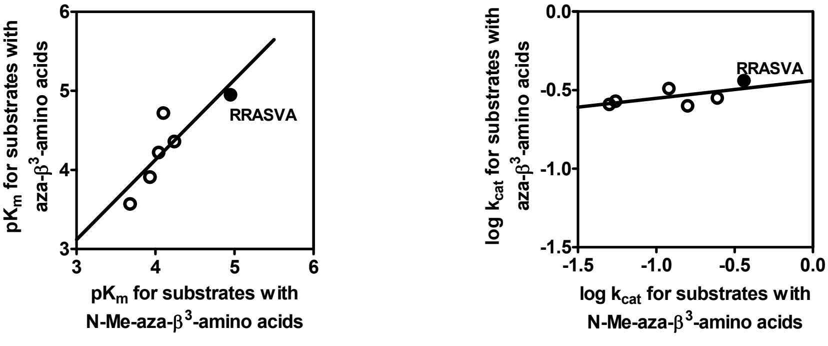


Figure 6. Effect of N^β-methylation on pK_m and log k_{cat} values for the protein kinase A catalyzed phosphorylation of peptidomimetic substrates, derived from RRASVA sequence and containing one aza-β^3-amino acid or N^β-Me-aza-β^3-amino acid subsequently in all positions (except serine).

Secondly, comparison of the log k_{cat} values for these two subseries of substrates was made and the resulting plot was shown in **Figure 6** (right panel). Again, linear plot between kinetic data for N-methylatesd and non-methylated compounds was obtained; however in this case the slope of this plot was close to zero (0.11±0.07), as the rate constant of the phosphorylation step of substrates with aza-β^3-amino acids was practically independent on structure modification. If we accept that the rate constant k_{cat} characterizes the phosphsate transfer step of the catalytic reaction, there should exist some specific mechanism how N^{β}-methylation of substrate backbone could affect specifically this step of the catalytic process.

Rather surprisingly, N^{β}-methylation of aza-β^3-residues reduced the $\log k_{II}$ values almost equally (**Figure 5**), although these modifications had tremendeous effect on the pK_m and log k_{cat} level. This difference pointed most probably to strong influence of these modifications on the kinetic mechanism of this reaction. Firsthand, the possibility of non-productive binding should be considered. This possibility was supported by ligand docking study, however more thorough discussion of these details remains outside of the scope of this work. On the other hand, as mentioned above, the log k_{II} values should describe the efficiency of catalysis and thus should be free from effects caused by drift of the reaction mechanism from simple Michaelis-Menten reaction scheme.

The influence of N-methylation of peptide backbone on their recognition by the enzyme active site could be explained by different stereochemical properties of the tertiary amide bond if compared to the -NH-C(O)- fragment in common peptides. It is known from multiple studies with N-methylated peptides that tertiary amide bonds are prone to adopt *cis* configuration, differently from the strongly preferred *trans* configuration of normal peptide bonds (Gilon, 2004; Goodman, 1967). To obtaine some preliminary information about the possibility of this difference in the case of N-methylated aza-β^3-substrates and non-methylated aza-β^3-substrates, the computer models of substrates were addressed.

Indeed, analysis of the *in silico* models of peptidomimetics revealed that all peptide bonds in non-methylated aza-β^3-amino acids had *trans* configuration, while in the case of N^{β}-methylated aza-β^3-amino acids *cis* configurations of peptide bonds were observed in substrates R(N^{β}Me)azaβ^3RASVA, RR(N^{β}Me)azaβ^3ASVA and RRASV(N^{β}Me)azaβ^3A. Interestingly, these tree substrates were desctribed by the lowest k_{II} values in this subseries of substrates (**Table 3**), while other peptidomimetics, which had only *trans* peptide bonds in their backbone, were phosphorylated more efficiently. However, the latter discourse may be accepted as a starting point for further study of influence of ligand conformation on their molecular recognition by protein binding sites.

CONCLUSIONS

1. Although the common solid-phase peptide synthesis methodology can be used for preparation of peptidomimetic compounds containing aza-β^3-amino acid residues, due to different reactivity of functional groups of amino acids and their aza-β^3-amino-derivatives, stronger activation of the carboxyl group is needed. Although the applied measures were efficient, and the efficiency of synthesis was increased, the overall yields were still lower than the yield of the well-optimized peptide synthesis. The latter fact sets certain limits to the number of replacements of common amino acids by aza-β^3-amino acids in a peptidomimetic compound.

2. Peptidomimetic substrates with aza-β^3-amino acids and their N^β-methylated derivatives were recognized by their target site in the protein kinase A active center. Therfore kinetic analysis of phosphorylation reaction of all synthesized peptidomimetic substrates was possible and the kinetic parameters K_m, k_{cat} and k_{II} were determined for all peptidomimetics. This allowed analysis of substrate backbone modifications on peptidomimetic recognition by protein kinase A.

3. Backbone modification may have significant effect on recognition of peptidomimetic compounds by their binding site, and may also affect the mechanism of their interaction with the enzyme. This influence is the strongest, if structure changes were made within certain ligand regions formulated as “specificity clusters”. If different specificity clusters for different recognition sites could be identified, peptidomimetic ligands with orthogonal effects against these sites can be designed.

4. N-methylation of peptidomimetic substrates affected their binding with target site, as was observed in the case of protein kinase A interaction with ligands containing aza-β^3-amino acid or N^β-Me-aza-β^3-amino acid.

SUMMARY

In this work we have analyzed molecular recognition of peptidomimetic compounds with modified backbone structure by cAMP-dependent protein kinase catalytic subunit (protein kinase A) as target site. For planning of this project literature survey was done on known peptide backbone modifications. Proceeding from this information the further analysis was focussed on analogs of short peptide substrates, derived from the minimum substrate of this enzyme RRASVA, and containing one or two aza-β^3-amino acids and N^β-Me-aza-β^3-amino acids subsequently in all positions except serine. Altogether, three series of peptidomimetic substrates were prepared and studied.

The necessary N^β-Fmoc-aza-β^3- and N^β-Fmoc-N^β-Me-aza-β^3 amino acids were prepared from simple precursors. Synthesis procedure of some compounds was updated to diminish the number of synthesis steps. The solid-phase peptide chemistry approach was used for introduction of amino acid derivatives into peptidomimetics, and if appropriate, the reaction conditions as well as reagents were modified to increase the yield of synthesis. The peptide analogs were assembled by using microwave–assisted coupling technique. But still lower yields of synthesis were observed if compared to normal peptides. For introduction of aza-β^3 monomers into the peptide with reasonable yield slight modification in coupling methodology (prolonged reaction times) was used. But still the reaction was less efficient if compared to normal peptide synthesis. For N^β-methyl-aza-β^3 peptides even lower yields than those for non-methylated analogs were observed, although more reactive activating agent was used.

It was found that all peptidomimetic substrates were phosphorylated by protein kinase A and kinetic parameters K_m, k_{cat} and k_{II} were determined. Using the results of this kinetic study the effect of peptide backbone modification with aza-β^3groups on recognition of these compounds by the protein binding site was analyzed in order to get insight into binding specificity and reaction mechanism of such substrates. It was observed, that binding efficiency of peptidomimetic substrates was dependent upon substitution position. In aza-β^3-analogs, disruption of the two-arginine cluster lead to a considerable decrease in binding activity, while the values of catalytic constants were not seriously altered within the aza-β^3 series, and were comparable to that of parent RRASVA. Binding efficiency of compounds of the N^β-Me-aza-β^3 series was much like that in case of aza-β^3-series, however in this case the catalytic rate constants were also affected. Peptidomimetic substrates with two aza-β^3-analogs revealed even more complex specificity.

The results of this study were used to draw several conclusions, which may be important for understanding of the principles of peptide substrate recognition and may have some practical value for design of peptidomimetic ligands in general.

SUMMARY IN ESTONIAN

Aza-β^3-aminohappeid sisaldavate peptidomimeetikute süntees ja nende fosforüleerimise kineetika uurimine proteiinkinaas A abil

Antud töös analüüsiti modifitseeritud põhiahelaga peptidomimeetikute molekulaarset äratundmist cAMP-sõltuva proteiinkinaasi katalüütilise alaühiku (PKA) aktiivtsentris. Selle dissertatsiooni planeerimise faasis koostati kokkuvõte teadaolevatest peptiidi põhiahela modifitseerimiseks kasutatud struktuuridest. Lähtudes sellest informatsioonist valiti uurimistöö objektiks ühte või kahte aza-β^3–aminohappe jääki või ühte N$^\beta$-Me-aza-β^3-aminohappe jääki sisaldavad lühikesed PKA peptiidsubstraadid, mis olid konstrueeritud lähtudes selle ensüümi miinimum-substraadist RRASVA. Selles peptiidis modifitseeriti aminohappeid kõikides asendites, välja arvatud fosfaatrühma aktseptoriks olev seriin. Kokku sünteesiti ja uuriti kolme petidomimeetiliste substraatide seeriat.

Vajalikud N$^\beta$-Fmoc-aza-β^3 and N$^\beta$-Fmoc-N$^\beta$-Me-aza-β^3 aminohapped sünteesiti lähtudes lihtsatest lähteainetest. Mõnede ainete valmistamismeetodit muudeti, et väheneks sünteesietappide arv. Muudeti ka modifitseeritud aminohapete peptiidiga liitmise metoodikat, mis võimaldas kasutada klassikalist tahkel kandjal teostatavat peptiidsünteesi metoodikat ning suurendada seejuures sünteesietappide saagist. Reaktsioonide kiirendamiseks kasutati ka mikrolainekiirgust. Vaatamata aga kõikidele täiendustele jäi modifitseeritud aminohapete lülitumise efektiivsus ikkagi mõnevõrra madalamaks võrreldes tavaliste peptiidide sünteesiga. On iseloomulik, et N$^\beta$-metüül-aza-β^3-peptiidide korral olid saagised madalamad kui mitte-metüleeritud analoogide korral ja seda vaatamata ka tugevamate aktiveerivate reagentide kasutamisele.

Leiti, et kõiki sünteesitud peptidomimeetilisi substraate fosforüleeritakse PKA poolt. See võimaldas määrata nende reaktsioonide jaoks kineetilised parameetrid K_m, k_{cat} ja k_{II}. Kasutades saadud kineetilise uuringu tulemusi analüüsiti peptiidsubstraadi põhiahela aza-β^3-gruppidega modifitseerimise efekti antud substraatide molekulaarsele äratundmisele nii sidumise staadiumis kui ka fosforüleerimise staadiumis. Ilmnes, et peptidomimeetiliste substraatide sidumise tõhusus sõltus aminohappe asenduse positsioonist. Kahe arginiini poolt moodustatud klastri lõhkumine aza-β^3 peptiidanaloogides viis sidumisaktiivsuse märkimisväärse languseni, samal ajal kui katalüütiliste konstantide väärtused ei olnud tugevasti mõjutatud kogu aza-β^3-seerias ja olid võrreldavad vastava RRASVA konstandi väärtusega. N$^\beta$-metüül-aza-β^3-seeria ensüümiga seostumise efektiivsused olid sarnased aza-β^3-seeria omadega, kuigi antud juhul olid mõjutatud ka katalüütilised kiiruskonstandid. Kahe aza-β^3-analoogiga substraadid näitasid aga keerukamat reaktsioonivõime sõltuvust asenduste positsioonidest.

Antud uurimistöö tulemused võimaldasid teha mitmeid järeldusi, mis võivad olla olulised peptiidsubstraatide äratundmise põhimõtetest arusaamisel ja omada praktilist väärtust peptidomimeetiliste ligandide loomisel üldse.

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PUBLICATIONS

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