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**ARTICLE** *in* JOURNAL OF PEPTIDE SCIENCE · DECEMBER 2014

Impact Factor: 1.55 · DOI: 10.1002/psc.2738



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Received: 7 November 2014 Revised: 9 December 2014 Accepted: 12 December 2014 Published online in Wiley Online Library

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# Analogues of deltorphin I containing conformationally restricted amino acids in position 2: structure and opioid activity

Anika Lasota,[a] Oliwia Frączak,[a] Anna Leśniak,[b] Adriana Muchowska,[b] Andrzej W. Lipkowski,[b†] Michał Nowakowski,[c] Andrzej Ejchart[d] and Aleksandra Olma[a]*

**New analogues of deltorphin I (DT I, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-$NH_2$), with the D-Ala residue in position 2 replaced by α-methyl-β-azido(amino, 1-pyrrolidinyl, 1-piperidinyl or 4-morpholinyl)alanine, were synthesized by a combination of solid-phase and solution methods. All ten new analogues were tested for receptor affinity and selectivity to μ- and δ-opioid receptors. The affinity of analogues containing (*R*) or (*S*)-α-methyl-β-azidoalanine in position 2 to δ-receptors strongly depended on the chirality of the α,α-disubstituted residue. Peptide II, containing (*S*)-α-methyl-β-azidoalanine in position 2, displayed excellent δ-receptor selectivity with its δ-receptor affinity being only three times lower than that of DT I. Copyright © 2014 European Peptide Society and John Wiley & Sons, Ltd.**

**Keywords:** α,α-disubstituted glycines; deltorphin I analogues; opioid peptides; opioid activities

## Introduction

Deltorphin I (DT I, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-$NH_2$), along with deltorphin II (DT II, Tyr-D-Ala-Phe-Glu-Val-Val-Gly-$NH_2$) and dermenkephalin (Tyr-D-Met-Phe-His-Leu-Met-Asp-$NH_2$), belongs to the class of deltorphins, linear heptapeptides isolated from amphibian skin [1]. Deltorphins display extremely high affinity and selectivity to δ-opioid receptors (DOR). Just as all naturally occurring opioid peptides, DT I and DT II consist of two parts, a biologically important *N*-terminal tripeptide fragment (Tyr-D-Ala-Phe, the 'message' domain), which is responsible for the interaction with opioid receptors [2], and a *C*-terminal fragment (Asp/Glu-Val-Val-Gly-$NH_2$, the 'address' domain). Anionic and hydrophobic *C*-terminal tetrapeptides simultaneously decrease μ-affinity and increase δ-affinity [3]. DOR agonists are interesting compounds because they have been found to produce a broad spectrum of antinociceptive effects in preclinical rodent models. When injected into the brain of mice or rats, the highly selective δ-opioid ligands DT I and DT II produce analgesia [4,5], locomotor stimulation [6] and motivational rewarding [7] without the development of physical dependence [8] or respiratory depression [9]. The search for new analogues of deltorphins is an important direction of studies because they have the potential to be used as effective analgesic agents for the treatment of cancer pain [10] and neuropathic pain [11] with potentially low abuse liability[12,13]. Agonists of the δ-opioid receptor induce few or no adverse gastrointestinal effects and no respiratory depression [14]. However, some data suggest that δ-opioid receptors are also critically involved in the mediation of tolerance and dependence [15]. Deltorphins and their analogues are useful tools for elucidation of the structure–activity relationships of DOR agonists [16].

Deltorphins are structurally flexible molecules and need to be conformationally restricted to obtain their bioactive conformation or to resolve structure–activity relationships. Several chemical approaches, such as the incorporation of D-amino acids and cyclic moieties or peptide cyclization [17,18], have resulted in conformationally restricted analogues. The opioid activity of deltorphin analogues is determined either by local conformational constrains introduced in residues in positions 2 and 3 or by more global conformational restrictions resulting from various peptide cyclizations [17]. An appropriate orientation and hydrophobicity at position 5 seem to be prerequisites for binding δ-receptors [19], but the amphiphilic α-hydroxymethylvaline residue is well tolerated at this position [20]. The high δ-affinity and selectivity of DT I analogues containing (*S*)-α-hydroxymethylphenylalanine in position 3 and (*R*)-α-hydroxymethylvaline in position 5 support the previously proposed molecular models of the active conformation of deltorphins, which results from the formation of a special amphiphilic topography with unequal hydrophilic and lipophilic parts (hot-dog shape) [2].

* *Correspondence to: Aleksandra Olma, Institute of Organic Chemistry, Lodz University of Technology, Zeromskiego 116, 90-924 Lodz, Poland. E-mail: aleksandra.olma@p.lodz.pl*

† *Deceased November 27, 2014.*

a *Institute of Organic Chemistry, Lodz University of Technology, Zeromskiego 116, 90-924, Lodz, Poland*

b *Mossakowski Medical Research Centre, Polish Academy of Sciences, Pawinskiego 5, 01-793, Warsaw, Poland*

c *Centre of New Technologies, University of Warsaw, Zwirki i Wigury 93, 02-089, Warsaw, Poland*

d *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5A, 02-106, Warsaw, Poland*

In the present paper, we report on the synthesis and biological properties of DT I analogues in which D-Ala were replaced with α-methyl-β-azido(*sec*-amino or amino)alanines (Figure 1).

Optically active α-methyl-β-azidoalanines can be obtained from available *N*-Boc-α-methylserine β-lactone [21]. *N*-Boc-α-alkylserine β-lactones are useful starting materials for further derivatization yielding potentially interesting building blocks for medicinal chemistry. The treatment of *N*-Boc-α-methylserine β-lactone with free heterocyclic amines (pyrrolidine, piperidine, morpholine or thiomorpholine) gives suitable, enantiomerically pure *N*-protected α-methyl-β-(*sec*-amino)alanines [22].

*N*-Protected α-alkyl-β-azidoalanines are excellent surrogates of α-alkyl-β-aminoalanines in peptide synthesis, suitable for direct incorporation into peptide chains using coupling methods for hindered residues. The azido group can be reduced to an amino group in the last step of peptide synthesis. The incorporation of α-substituted α,β-diaminopropionic acid into a peptide chain requires masking of the side-chain amino function. Efficient orthogonal protection of two amino groups in α-substituted α,β-diaminopropionic acids can be challenging. The incorporation of azidoalanine in the peptide chain is associated with specific conformational consequences. The β-azido group is an effective C7-conformation-directing element, which may be useful for tuning the structures of other amino acids and polypeptides. However, it has not been clarified yet whether the azido group can induce any conformational change via stereoelectronic effects when introduced into the β-carbon of alanine [23].

## Materials and Methods

### Chemistry

All chemicals were purchased from Sigma-Aldrich and used as received without further purification. All untreated solvents used were of HPLC grade.

Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids and Fmoc-Rink amide AM resin were purchased from IrisBiotech (Marktredwitz, Germany). (*R*) and (*S*) *N*-protected α-methyl-β-azido (*sec*-amino)alanines were obtained according to a procedure described in the literature [21,22].

All solvents and reagents used for solid-phase synthesis were of analytical quality and used without further purification. Thin layer chromatography (TLC) was performed on UV plates (Fluka Analytical, Silica on TLC Alufoils, with a 254 nm fluorescent indicator). The coupling reagents HATU and HOAt were purchased from AK Scientific, Inc. (CA, USA). All other reagents and solvents were of analytical or HPLC grade and were bought from Sigma-Aldrich (Poland) or Avantor Performance Materials Poland S.A.

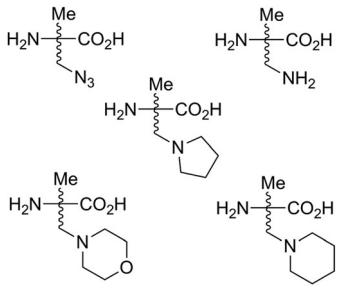



**Figure 1.** Structures of α-methyl-β-azido(amino/sec-amino)alanines.

Analytical reverse-phase HPLC was performed on a GraceSmart C18 column (Grace, 4.6 mm × 250 mm, 5 μm), flow rate 1.0 ml/min, detection at 220 nm, solvents (A) 0.05% trifluoroacetic acid (TFA) in water and (B) 0.038% TFA in acetonitrile/water 90 : 10 in linear gradient elution. The final peptides were purified by RP-HPLC on a Thermoseparation Products P400 Spectra System (detection at 220 nm) using a Gemini C18 column (Phenomenex, 250 mm × 10 mm, 10 μm), flow rate 3.0 ml/min.

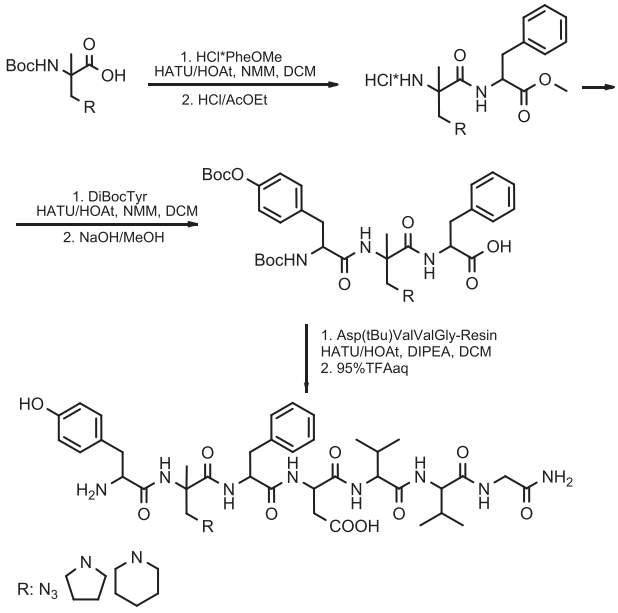
*General procedure for peptide synthesis*

Stepwise solid-phase peptide synthesis (SPPS) is difficult in the case of peptides containing an α,α-disubstituted amino acid because of their steric hindrance and low reactivity. Initial attempts to prepare DT I analogues by solid-phase synthesis were unsuccessful. The mixture of products contained mainly truncated peptides (penta- and hexapeptides) because of inefficient coupling of α,α-disubstituted amino acids.

The peptides reported here were obtained by convergent solid-phase peptide synthesis (CSPPS) involving the coupling of protected peptide segments on solid support according to Schemes 1 and 2. For the synthesis of peptides **I**, **II**, and **V–VIII**, *N*-terminal tripeptides containing an α,α-disubstituted amino acid in position 2 were synthesized in solution (Scheme 1).

After deprotection of the carboxyl group and purification, *N*, *O*-protected tripeptide segments were coupled with tetrapeptides on resin. The synthesis of peptides **IX** and **X** in the same manner gave two epimers (4 : 1) because of racemization on the phenylalanine residue. To avoid that racemization, we prepared *N*-terminal dipeptides that were coupled with pentapeptides on resin. Dipeptides containing (*R*) or (*S*)-α-methyl-β-(4-morpholinyl)alanine in position 2 are not prone to racemization. The change of strategy required the synthesis of (*R*) and (*S*)-α-methyl-β-(4-morpholinyl) alanine methyl ester hydrochlorides. The Boc derivatives were esterified with a freshly prepared solution of diazomethane, and suitable building blocks were obtained after deprotection of the amine group with HCl/AcOH (Scheme 2).

Tetra- and pentapeptides were prepared by the manual solid-phase technique on Rink amide AM resin (capacity 0.1 mmol/g),




**Scheme 1.** Synthesis of analogues **I**, **II** and **V–VIII**.

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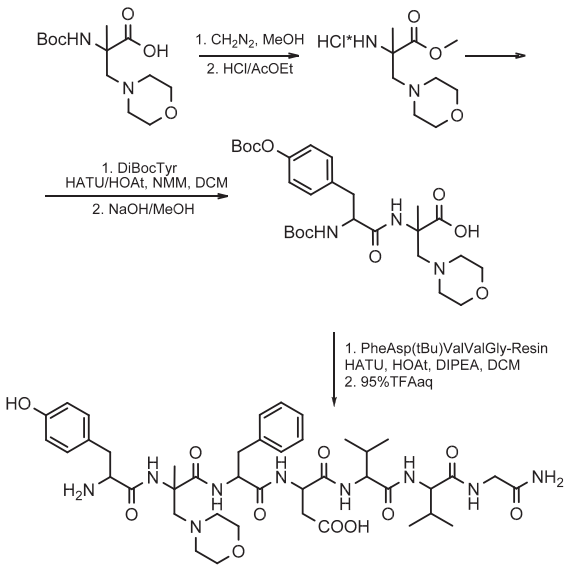


**Scheme 2.** Synthesis of analogues **IX** and **X**.

according to standard methods for peptides synthesized by the Fmoc/tBu strategy. The protected amino acids were coupled with a threefold excess using HATU as a coupling reagent in the presence of HOAt and DIPEA in DCM. The Fmoc groups were removed by treatment with 20% piperidine in DMF. After the coupling of fragments on resin, the final heptapeptides were obtained. Cleavage from the resin and removal of the protecting groups were simultaneously achieved by treatment with a mixture of TFA/$H_2O$ (95 : 5 by vol) (20 ml/100 mg of peptide resin, 3.5 h at room temperature). The acid solution was concentrated *in vacuo*, and the crude peptides were dissolved in water/*t*-butanol (1 : 1 by vol), lyophilized and then purified by RP-HPLC. Catalytic hydrogenation in dry methanol of the azide group of analogues **I** and **II** resulted in analogues **III** and **IV**, respectively. Reduction required anhydrous conditions; otherwise, a mixture of the product and a side product would be obtained. Exact analysis of the MS spectrum showed that the side product contained an α-methylserine residue in position 2. All products were characterized by analytical RP-HPLC and molecular weight determination (Table 1).

### *Molecular modeling*

To investigate structure–activity relationships, the previously proposed computer-assisted intuitional analysis [24] was employed. SYBYL (Tripos Associates, St. Louis, MO, USA) software was used for molecular modeling. Full geometry optimization was performed with an AMBER 12 [25] force field using the SYBYL program module MAXI-MIN. The initial conformations containing α,α-disubstituted amino acid residues were constructed by replacing the respective amino acid of DT I with an α-aminoisobutyric acid residue, followed by energy minimization. Next, respective side-chain substitutions were introduced to form the final analogues with appropriate stereochemistry. The representative structure of each analogue that has been used in structure–activity relationship analysis has been intuitively and arbitrarily selected from low-energy populations of **A–C** (Figure 2).

### *NMR experiments*

NMR sample with a volume of 650 μl contained 5 mg of Tyr-(*S*)-α-methyl-β-azidoAla-Phe-Asp-Val-Val-Gly-$NH_2$ solution in a (90 : 10 by vol) $H_2O/D_2O$ mixture. All spectra were measured on Varian INOVA spectrometer operating at 400 MHz resonance frequency

**Table 1.** Structures and the physicochemical properties of the deltorphin I analogues **I–X**

| Peptide | MW (g/mol) | $[M+H]^+$ | HPLC purity % | $t_r$* |
|---|---|---|---|---|
| Tyr-(***R***)-α-**methyl-β-azidoAla**-Phe-Asp-Val-Val-Gly-$NH_2$ (**I**) | 823.8951 | 824.7583 | 99 | 15.19 |
| Tyr-(***S***)-α-**methyl-β-azidoAla**-Phe-Asp-Val-Val-Gly-$NH_2$ (**II**) | 823.8951 | 824.8073 | 99 | 15.18 |
| Tyr-(***R***)-α-**methyl-β-aminoAla**-Phe-Asp-Val-Val-Gly-$NH_2$ (**III**) | 797.8976 | 798.8128 | 99 | 10.86 |
| Tyr-(***S***)-α-**methyl-β-aminoAla**-Phe-Asp-Val-Val-Gly-$NH_2$ (**IV**) | 797.8976 | 798.7928 | 96 | 8.71 |
| Tyr-(***R***)-α-**methyl-β-(1-pyrrolidinyl)Ala**-Phe-Asp-Val-Val-Gly-$NH_2$ (**V**) | 851.9880 | 853.9397 | 99 | 10.84 |
| Tyr-(***S***)-α-**methyl-β-(1-pyrrolidinyl)Ala**-Phe-Asp-Val-Val-Gly-$NH_2$ (**VI**) | 851.9880 | 852.9391 | 97 | 10.06 |
| Tyr-(***R***)-α-**methyl-β-(1-piperidinyl)Ala**-Phe-Asp-Val-Val-Gly-$NH_2$ (**VII**) | 866.0146 | 866.8564 | 98 | 10.52 |
| Tyr-(***S***)-α-**methyl-β-(1-piperidinyl)**-Phe-Asp-Val-Val-Gly-$NH_2$ (**VIII**) | 866.0146 | 866.9663 | 99 | 10.32 |
| Tyr-(***R***)-α-**methyl-β-(4-morpholinyl)Ala**-Phe-Asp-Val-Val-Gly-$NH_2$ (**IX**) | 867.9874 | 868.3738 | 99 | 10.36 |
| Tyr-(***S***)-α-**methyl-β-(4-morpholinyl)Ala**-Phe-Asp-Val-Val-Gly-$NH_2$ (**X**) | 867.9874 | 868.9801 | 99 | 12.92 |

* gradient 10- 50%B over A in 20 min.

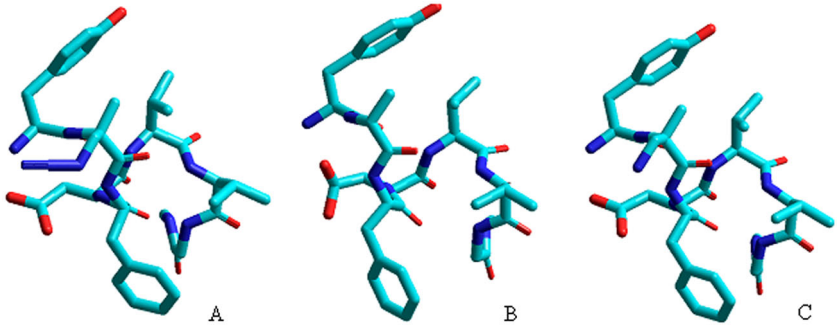


**Figure 2.** Structures of Tyr-(*S*)-α-methyl-β-azidoAla-Phe-Asp-Val-Val-Gly-NH2 (**II**, A), DT I (B) and Tyr-(*S*)-α-methyl-β-aminoAla-Phe-Asp-Val-Val-Gly-NH2 (**IV**, C).

($^1$H) and 100.6 MHz ($^{13}$C) at temperature 25 °C. Temperature calibration was carefully performed using an ethylene glycol reference sample [26]. 2D homonuclear TOCSY [27] (mixing time 80 ms), ROESY [28] (mixing time 300 ms) and heteronuclear $^1$H/$^{13}$C HSQC [29] (with the offset, spectral widths, and $^{13}$C–$^1$H coupling constants tuned to either aliphatic or aromatic carbons) spectra were used to obtain assignments of the $^1$H and $^{13}$C resonances. The ROESY spectrum was checked for inter-residual $^1$H–$^1$H interactions. Time domain data were acquired using States-TPPI quadrature detection [30]. All chemical shifts in $^1$H NMR spectra were reported with respect to external DSS-$d_4$. Chemical shifts of $^{13}$C signals were referenced indirectly using the 0.251449530 frequency $^{13}$C/$^1$H ratio [31]. Zero filling and a 90°-shifted squared sine-bell filter were performed prior to Fourier transformation. Processed spectra were analyzed with SPARKY software [32].

*Ligand binding assay*

Receptor binding assays were performed as described previously [20]. Rat membrane preparation followed the procedure described by Misicka et al. [2]. The radioligand receptor binding protocol was based on a study performed by Fichna et al. [33] with some modifications. The modification included different incubation times (60 min vs 120 min), bacitracin concentrations (30 μg/ml vs 50 μg/ml) and radioligand choices. The modifications were implemented in order to obtain optimal binding conditions. Binding affinities for μ- and δ-opioid receptors were determined by displacing [$^3$H]-DAMGO and [$^3$H]-DELT, respectively, from adult male Wistar rat brain membrane binding sites. Binding curves were fitted using nonlinear regression. Compound potency was expressed as $IC_{50}$ values (Table 2).

## Results and Discussion

*N*-Protected (*R*) and (*S*)-α-methyl-β-azido(*sec*-amino) alanines were synthesized from easily available β-lactones of *N*-protected α-alkylserines by ring opening with a suitable nucleophile. The designed peptides **I–X** were generated by CSPPS involving the coupling of protected peptide segments on solid support. An initial attempt to synthesize the desired peptides on resin gave a mixture of desired products and truncated peptides. *N*-terminal di- or tripeptides containing α,α-disubstituted glycines were obtained in solution using HATU as a coupling reagent and then, after deprotection of the carboxyl function, were coupled with penta- or tetrapeptides on resin. The cleavage of peptides from the resin and the removal of the protecting groups were performed with TFA/water (95 : 5 by vol). All crude peptides were purified to homogeneity by RP-HPLC, and their structures were verified by mass spectrometry (Table 1).

The affinities of DT I analogues for μ- and δ-receptors were determined by the radioreceptor binding assay described previously using [$^3$H]-DAMGO and [$^3$H]-DELT as μ- and δ-receptor-specific ligands, respectively.

Table 2 shows the binding affinity of DT I analogues to δ- and μ-opioid receptors in comparison with DT I.

As reported in Table 2, the affinity of analogues containing (*R*) or (*S*)-α-methyl-β-azidoalanine in position 2 to δ-receptors strongly depends on the chirality of the α,α-disubstituted residue. Peptide **II**, containing (*S*)-α-methyl-β-azidoalanine, displays slightly lower δ-receptor affinity than the parent peptide [34] but higher δ-receptor selectivity, whereas the (*R*) isomer is less potent and δ-selective. It should be noted that the topographical location of the methyl group in (*S*)-α-methyl-β-azidoalanine is related to the methyl group of D-Ala, whose position is crucial for chemical activity. The reduction of the azide group (*R*)-α-methyl-β-azidoalanine in analogue **I** into amphiphilic (*R*)-α-methyl-β-aminoalanine resulted in compound **III**, which has 8 and 100 times the δ- and μ-receptor affinity of peptide **I**, respectively. The reduction of (*S*)-α-methyl-β-azidoalanine (analogue **IV**) resulted in a 120-fold decrease in affinity for δ-receptors and in only about a twofold decrease in affinity to μ-receptors as compared with peptide **II**. Both analogues containing α-methyl-β-aminoalanine in position 2 (**III** and **IV**) lost δ-selectivity. In the proposed model of DT I and DT II, [2] the corresponding topographic locations of the *N*-amino group of tyrosine are stabilized by ionic interaction with the amino acid residue in position 4 (Figure 2). The delocalized charge of the azide moiety in position 2 of the amino acid residue in the (*S*) configuration can further stabilize the ion interactions 1–4 (Figure 2A). In contrast, an amino group in the same position can create misleading (Figure 2C) interactions resulting in the destabilization of the tyramine residue in *N*-terminal tyrosine. The amino group in the (*R*) conformer may promote dermorphin-like conformations [35].

**Table 2.** Binding affinities of deltorphin I analogues **I–X** to δ- and μ-opioid receptors

| Peptide | $IC_{50}$ (nM) | | $IC_{50}$ ratio |
|---|---|---|---|
| | μ[a] | δ[b] | μ/δ |
| Tyr-D-Ala-Phe-Asp-Val-Val-Gly-$NH_2$ (deltorphin I) [34] | 976 ± 148 | 3.05 ± 0.1* | 320 |
| Tyr-**(*R*)-α-methyl-β-azidoAla**-Phe-Asp-Val-Val-Gly-$NH_2$ (**I**) | 3805 | 116.5 | 32.8 |
| Tyr-**(*S*)-α-methyl-β-azidoAla**-Phe-Asp-Val-Val-Gly-$NH_2$ (**II**) | 4762 | 9.74 | 488.9 |
| Tyr-**(*R*)-α-methyl-β-aminoAla**-Phe-Asp-Val-Val-Gly-$NH_2$ (**III**) | 37.32 ± 2 | 13.92 ± 1.05 | 2.68 |
| Tyr-**(*S*)-α-methyl-β-aminoAla**-Phe-Asp-Val-Val-Gly-$NH_2$ (**IV**) | 2797 ± 214 | 1201 ± 79.8 | 2.3 |
| Tyr-**(*R*)-α-methyl-β-(1-pyrrolidinyl)Ala**-Phe-Asp-Val-Val-Gly-$NH_2$ (**V**) | 2641 ± 126.8 | 3742 ± 275.0 | 0.71 |
| Tyr-**(*S*)-α-methyl-β-(1-pyrrolidinyl)Ala**-Phe-Asp-Val-Val-Gly-$NH_2$ (**VI**) | 1896 ± 106.9 | 2768 ± 247.4 | 0.68 |
| Tyr-**(*R*)-α-methyl-β-(1-piperidinyl)Ala**-Phe-Asp-Val-Val-Gly-$NH_2$ (**VII**) | 2904 ± 126.3 | 2336 ± 101.1 | 2.37 |
| Tyr-**(*S*)-α-methyl-β-(1-piperidinyl)Ala**-Phe-Asp-Val-Val-Gly-$NH_2$ (**VIII**) | 3752 ± 157.6 | 1581 ± 187.2 | 1.25 |
| Tyr-**(*R*)-α-methyl-β-(4-morpholinyl)Ala**-Phe-Asp-Val-Val-Gly-$NH_2$ (**IX**) | 1726 ± 74 | 4269 ± 456.8 | 2.37 |
| Tyr-**(*S*)-α-methyl-β-(4-morpholinyl)Ala**-Phe-Asp-Val-Val-Gly-$NH_2$ (**X**) | 583.1 ± 21 | 2380 ± 385.7 | 0.24 |

[a] *versus* [$^3$H]DAMGO.
[b] *versus* [$^3$H]DELT.
* *versus* [$^3$H]DPDPE.

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**Table 3.** $^{1}H$ and $^{13}C$ chemical shifts (in ppm) of Tyr-(*S*)-α-methyl-β-azidoAla-Phe-Asp-Val-Val-Gly-$NH_2$ (**II**) (AA = α-methyl-β-azidoalanine)

| a.a. | HN | Hα | Hβ | Hγ | Hδ | Hε | Hζ |
|---|---|---|---|---|---|---|---|
| Tyr1 | n.a. | 4.143 | 2.910 3.046 | – | 7.161 | 6.882 | – |
| AA2 | 8.583 | – | 1.125[a] 3.578[b] 3.659[b] | – | – | – | – |
| Phe3 | 7.605 | 4.550 | 3.052 3.188 | – | 7.218 | 7.343 | 7.253 |
| Asp4 | 8.139 | 4.694 | 2.785 2.887 | – | – | – | – |
| Val5 | 8.087 | 4.101 | 2.054 | 0.921 0.895 | – | – | – |
| Val6 | 8.251 | 4.069 | 2.033 | 0.929 0.921 | – | – | – |
| Gly7 | 8.473 | 3.859 3.909 | – | – | – | – | – |
| C-term | 7.039; 7.423 | – | – | – | – | – | – |

| a.a. | Cα | Cβ | Cγ | Cδ | Cε | Cζ |
|---|---|---|---|---|---|---|
| Tyr1 | 57.26 | 38.68 | n.a. | 133.58 | 118.67 | n.a. |
| AA2 | n.a. | 23.24[a] 57.01[b] | – | – | – | – |
| Phe3 | 57.73 | 38.66 | n.a. | 131.85 | 131.53 | 130.00 |
| Asp4 | n.a. | 38.376 | – | – | – | – |
| Val5 | 62.43 | 32.65 | 20.78 21.18 | – | – | – |
| Val6 | 62.69 | 32.65 | 20.53 21.00 | – | – | – |
| Gly7 | 44.84 | – | – | – | – | – |

[a] $CH_3$.
[b] $CH_2$.
n.a. not assigned.

A possible alternative explanation of the significant change in the binding affinities of analogues **III** and **IV** as compared with **I** and **II** can be the influence of the β-azido group and its interactions with the two neighboring peptide bonds, which are facilitated by the azido gauche effect [36]. Analogues containing (*R*) or (*S*)-α-methyl-β-aminoalanine with a positively charged side chain could also be involved in electrostatic interactions with the negatively charged μ-receptor site or with the negatively charged membrane compartment that contains μ-receptors [37].

The substitution of D-Ala in position 2 with (*R*) or (*S*)-α-methyl-β-(sec-amino)alanine leads to a significant reduction of affinity to μ- and δ-receptors and decreased selectivity (peptides **V–X**). The incorporation of α-methyl-β-(1-pyrrolidinyl, 1-piperidinyl or 4-morpholinyl)alanine in position 2 may reduce conformational freedom, which suggests that these analogues are not able to adopt the bioactive conformation.

For more than 50 years now, the nuclear Overhauser effect (NOE), both in the laboratory and rotating frame, has been the method of choice in studying conformations of organic and biological molecules [38]. Short linear peptides are usually characterized by high structural flexibility. Therefore, long-range correlations have been seldom observed in their NOESY/ROESY spectra. Nevertheless, one could expect peptides containing α,α-disubstituted amino acid residue(s) to exhibit increased conformational rigidity. In order to resolve this issue, an NMR study was carried out for the most active analogue in the investigated series (analogue **II**). Nearly complete assignment of $^{1}H$ and proton-bearing $^{13}C$ nuclei in **II** was obtained from TOCSY, ROESY and $^{1}H/^{13}C$ HSQC spectra (Table 3).

In the ROESY spectrum, only intra-residual and sequential ($i/i+1$) correlations were observed, clearly demonstrating that the extended conformation of **II** was prevalent. There is no evidence of close interactions between aromatic rings because no Ar(Tyr1)/Ar(Phe3) correlations were detected. The latter can be explained by steric crowding at the modified alanine residue.

In summary, the reported results indicate that α-methyl-β-azido(amino)alanine could be used as a successful tool for modulating the affinity and selectivity of peptide ligands, with the induced effect being strongly dependent on the chirality of the introduced amino acid. It should be noted that the azide group can be treated as an orthogonal protection of the amine group in peptide synthesis. Reduction of the conformational freedom of DT I by the incorporation of α,α-disubstituted glycines with the *sec*-amino group (α-methyl-β-(1-pyrrolidinyl, 1-piperidinyl or 4-morpholinyl)alanine) probably prevents the adoption of the bioactive conformation.

### Acknowledgements

This research was supported by the National Science Centre (NCN, Poland), grant Preludium 2 (2011/03/N/ST5/04701).

## Conflict of Interest

The authors declare that they have no conflict of interest.

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