**The impact of β-azido(or 1-piperidinyl)methylamino acids in position 2 or 3 on biological activity and conformation of dermorphin analogues**

Maciej Maciejczyka,b#, Anika Lasotac#, Oliwia Frączakc, Piotr Kossond, Aleksandra Misickad,e,Michał Nowakowskif, Andrzej Ejchartg Aleksandra Olmac\*

*a Department of Physics and Biophysics, Faculty of Food Science, University of Warmia and Mazury, Oczapowskiego 4, 10-719 Olsztyn, Poland*

*b Laboratory of Bioinformatics and Protein Engineering, International Institute of Molecular and Cell Biology in Warsaw, ul. Ks. Trojdena 4, 02-109 Warsaw, Poland*

*c Institute of Organic Chemistry, Lodz University of Technology,Żeromskiego116, 90-924 Lodz, Poland*

*dMossakowski Medical Research Centre, Polish Academy of Sciences, Pawińskiego 5, 01-793 Warsaw, Poland*

*e Faculty of Chemistry, University of Warsaw, Pasteura* *1, 02-093 Warsaw, Poland*

*f Centre of New Technologies, University of Warsaw, Banacha 2C, 02-097 Warsaw, Poland*

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

# contributed equally to this work

[\*aleksandra.olma@p.lodz.pl](mailto:*aleksandra.olma@p.lodz.pl),

**Abstract:**

The synthesis of new dermorphin analogues is described. The (*R*)-alanine or phenylalanine residues of natural dermorphin were substituted by the corresponding α-methyl-β-azidoalanine or α-benzyl-β-azido(1- piperidinyl)alanine residues. The potency and selectivity of the new analogues were evaluated by a competitive receptor binding assay in rat brain using [3H]DAMGO (a μ ligand) and [3H]DELT (a δ ligand). The most active analogue in this series, Tyr-(*R*)-Ala-(*R*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 and its epimer were analyzed by 1H and 13C NMR spectroscopy and restrained MD simulations. The dominant conformation of the investigated peptides depended on the absolute configuration around Cα in the α-benzyl-β-azidoAla residue in position 3. The (*R*) configuration led to the formation of a type I β-turn, whilst switching to the (*S*) configuration gave rise to an inverse β-turn of type I’, followed by the formation of a very short β-sheet. The selectivity of Tyr-(*R*)-Ala-(*R*) and (*S*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 wasshown to be very similar; nevertheless, the two analogues exhibited different conformational preferences.

**Keywords** dermorphin analogues, α,α-disubstituted glycines, CSPPS, binding affinity to δ- and μ-opioid receptors, conformational analysis

**INTRODUCTION**

Dermorphin, an amphibian opiate peptide, is a heptapeptide (Tyr-(*R*)-Ala-Phe-Gly-Tyr-Pro-Ser-NH2) that has been isolated from the skin of South American frogs belonging to the subfamily *Phyllomedusa* [1]. Dermorphin shows remarkably high μ selectivity and an extremely potent and long lasting antinociceptive effect with peripheral administration routes, such as *s.c.* and *i.v.* injections [2]; its affinity for μ opioid receptors (MOR) is 100-times higher than that of morphine [3]. Because pain relief is mediated mainly through MOR, it is important to understand the interactions between MOR ligands and the receptor. Therefore hundreds of analogues of the dermorphin and dermorphin-like peptides have been synthesized [4-9].

Conformational, topographical and stereoelectronic structural features of opioid peptides are important for interactions with μ, δ, and κ opioid receptors. Two aromatic amino acids, Tyr1 and Phe3 (deltorphins and dermorphin, endomorphin-2) or Phe4 (enkephalins, endorphins), are crucial structural elements of opioid peptides due to interactions with opioid receptors. The conformational restriction of opioid peptides may increase the potency, selectivity and metabolic stability of the analogues. The incorporation of α,α-disubstituted glycines can offer resistance to enzymatic degradation and increase the conformational stability of the peptides [10].

The present paper describes the synthesis and receptor binding of new analogues with a local constraint. The (*R*)-Ala2 or Phe3 residues in the dermorphin sequence have been substituted by β-azido(or 1-piperidinyl)methylamino acidresidues (Figure 1).



Figure 1. Structures of new dermorphin analogues **I**-**VI**

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 [11]. The incorporation of β-azidomethylamino acidsin positions2 or 3 of deltorphin I revealed that α-methyl- or α-benzyl-β-azidoalanine could be used as successful tool in the modulation of the affinity and selectivity of the peptide [12, 13]. In our previous work, the incorporation of (*R*)-α-benzyl-β-(1-piperidinyl)Ala in the structure of a highly δ-selective peptide deltorphin I, changes receptor binding, consequently leading to a µ-selective compound. Based on NMR and MD studies it was suggested that selectivity inversion might be connected to a hydrophobic interaction of the piperidine ring with Val6 which forces the helical conformation at the *C*-terminal part of the peptide.

Continuing our research on the effects of α-alkyl-β-azido(or 1-piperidinyl)alanine incorporation on the biological properties of opioid peptides, we designed and synthesized dermorphin analogues containing α-methyl-β-azidoalanine in position 2 and α-benzyl-β-azidoalanine or α-benzyl-β-(1-piperidinyl)alanine in position 3 (Figure 1). The phenylalanine in position 3 of δ-selective deltorphins and μ-selective dermorphin plays a key role in binding and discrimination between δ and μ opioid receptors.

The conformational behavior of dermorphin analogues **III** and **IV**, containing (*R*) or (*S*)-α-benzyl-β-azidoalanine in position 3 was analyzed by 1H NMR and restrained molecular dynamics simulation.

**MATERIALS AND METHODS**

*Peptide synthesis*

The protected amino acids were purchased from AK Scientific Inc. (Union City USA), TBTU, HOBt and HOAt were obtained from GLBiochem (Shanghai, China) LTD, HATU from AK Scientific Inc. (Union City USA), whereas Ltd. Fmoc-Rink-Amid AM resin was bought from Iris Biotech Gmbh (Marktredwitz, Germany). All other reagents and solvents were analytical or HPLC grade and were purchased from Sigma-Aldrich (Poland) or Avantor Performance Materials Poland SA.

Optically pure α,α-disubstituted glycines were obtained from available *N*-Boc-(*R* or *S*)-α-alkylserine β-lactone. The treatment of *N*-Boc-(*R* or *S*)-α-methyl(or benzyl)serine β-lactone with sodium azide or piperidine as nucleophile gives suitable, enantiomerically pure *N*-Boc-(*R* or *S*)-α-alkyl-β-azido(1-piperidinyl)alanines [14].

Our previous studies have shown that classic synthesis of deltorphin I analogues (step by step) containing α-methyl-β-azidoalanine on solid phase using Boc strategy was not effective. The dermorphin analogues **I**-**VI** were obtained by convergent peptide synthesis involving the coupling of protected peptide segments on solid support (the fragment approach) (Scheme 1) which we used previously [12,13].



AA1= (*R*) or (*S*)-α-methyl-β-azidoalanine

AA2= (*R*) or (*S*)-α-benzyl-β-azidoalanine, (*R*) or (*S*)-α-benzyl-β-(1-piperidinyl)alanine

Scheme 1. Synthesis of dermorphin analogues **I**-**VI**

The di- and tripeptides (**3**-**5**) were synthesized in solution using HATU/HOAt/NMM coupling method (Scheme 1) The *N*α terminal Boc-protected amino acids were deprotected by 2N HCl in ethyl acetate at room temperature. The resulted hydrochlorides were used in the next reactions without further purification. The *C*-terminal methyl esters in di- and tripeptides were cleavage by mild alkaline hydrolysis in mixtures of water and methanol. Intermidiate fully protected di- and tripeptides **3** and **5** were purified by flash chromatography.

The pentapeptide-resin (Phe-Gly-Tyr(*t*-Bu)-Pro-Ser(*t*-Bu)-resin) and tetrapeptide-resin (Gly-Tyr(*t*-Bu)-Pro-Ser(*t*-Bu)-resin) were synthesized on solid phase (SPPS), following standard Fmoc strategy using TBTU/ HOBt for coupling reactions and piperidine 20% solution in DMF for Fmoc group deprotection. The tetra- and pentapeptide on resin was acylated with hydrolized *N*-protected di or tripeptides (**3** or **5)** using HATU as a coupling reagent in the presence of HOAt and DIPEA in DCM. 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/H2O (95 : 5 by vol) (20 ml/100mg of peptide resin, 3.5 h at room temperature). The final peptides **I**-**VI** were purified by RP-HPLC using semi-preparative column Gemini C18 (Phenomenex, 250 mmx10 mm, 10 μm) (analogues **I**-**IV** HPLC solvent A: 0.1% TFA in water, solvent B: 0.076 TFA in 90% acetonitrile; gradient 10-50 % B over A , in 20 min, flow rate 3 mL/min; analogues **V**-**IV** HPLC solvent A: 0.05% TFA in water, solvent B: 0.038 TFA in 90% acetonitrile; gradient 10-50 % B over A in 20 min , flow rate 3 mL/min).

*Ligand binding assay*

Receptor binding assays were performed as described previously [15, 16]. The radioreceptor binding protocol was based on a protocol decribed by Fichna et al [17] with some modifications. The modification included different incubation time (60min. *vs*. 120min), bacitracin concentration (30 µg/ml vs. 50 µg/ml) and radioligand choice. The modifications were implemented in order to obtain optimal binding conditions. Binding affinities for μ- and *δ*-opioid receptors were determined by displacing [3H]-DAMGO and [3H]-DELT, respectively, from adult male Wistar rat brain membrane binding sites. Binding curves were fitted using nonlinear regression. Compound potency was expressed as IC50 values (Table 2).

*NMR measurements*

NMR samples with a volume of 650 μl contained 5mg of peptide **III** and **IV** dissolved in a (90:10 by vol) H2O/D2O mixture. All spectra were measured on an Agilent 600 MHz DDR2 spectrometer operating at 600 MHz resonance frequency (1H), 60.8 MHz (15N), and 150.9 MHz (13C) at temperature 25°C. Temperature calibration was carefully adjusted using an ethylene glycol reference sample [18]. 2D Homonuclear TOCSY [19] (mixing time 80ms), ROESY [20] (mixing time 300ms), heteronuclear 1H/15N HSQC ( [21], and 1H/13C HSQC (with the offset, spectral widths, and 13C–1H coupling constants tuned to either aliphatic or aromatic carbons) spectra were used to obtain assignments of the 1H, 15N, and 13C resonances. Time domain data were acquired using States-TPPI quadrature detection [22]. Water suppression was achieved with pulsed field gradients echo [23]. All chemical shifts in 1H NMR spectra were reported with respect to external DSS-d4. Chemical shifts of 13C and 15N signals were referenced indirectly using the 0.251449530 and 0.101329118 frequency ratios for 13C/1H and 15N/1H, respectively [24]. Zero filling and a 90°-shifted squared sine-bell filter were performed prior to Fourier transformation. Processed spectra were analyzed with SPARKY software [25]. Intensities of interproton correlations in ROESY spectra, Iij, were used in determining appropriate distances rij from the equation Iij = C⋅rij6 [26]. The constant C was calculated from the intensity of correlations between aromatic hydrogen atoms H and H of fixed distance assumed to be equal to 2.48 Å. This calibration procedure leads to slightly strained final structures and therefore upper limits of distance restraints were scaled by a factor of 1.1.

*Parameterization of modified residues*

For all natural amino-acid residues standard Amber ff10 force-field parameters were applied [27]. The parametrization of α-benzyl-β-azidoAla residue was based on ff10 parameters for phenylalanine residue. Bonded parameters and partial charges were applied to the azido part of the residue from ref. [28] were applied to the azido part of the residue. Four simulated systems, which differ in absolute configuration of α-benzyl-β-azidoAla3 residue and the conformation of Pro6 peptide bond, are shown in Table 3.

*Simulated-annealing procedure*

In all simulations peptide bond of Pro6 residue was restrained either in *cis* or *trans* conformation and ROESY restraints related to selected Pro6 conformation were applied. The peptide chain was built with xleap program of the Amber package. In order to remove close contacts 1000 steps of geometry optimization were applied with steepest-descent energy minimization method. The chain was heated up from 10 to 1200K in 1ps molecular dynamics run, followed by 2ps of high-temperature dynamics and 12ps-long cooling process. NMR distance restraints were slowly switched on during first 3ps of simulated annealing run. Improper dihedral restraints on chiral centers were switched on to prevent chirality flipping during the high-temperature dynamics. Finally the geometry of the peptide was optimized by 1000 steps of steepest-descent and 2000 steps of conjugate-gradient energy minimization procedure. The time-step of the simulation was 1fs and Generalized Born solvation model was applied [29-31] The simulated-annealing cycle was repeated 100 times and the lowest-energy structure was used as the initial structure for time-averaged restrained molecular dynamics simulation.

*Time-averaged restraints* *MD simulations*

The geometry of an initial structure was optimized and the system was equilibrated in 1ns MD run with time-averaged restraints applied. The SHAKE algorithm was used to keep covalent bonds with hydrogens constant and 2fs time-step was applied. The solvation effects were described by Generalized-Born model [29-31]. During 20ns production run proton-proton distance restraints obtained from NMR experiment were time-averaged over 1ps time interval. The average energy of time-averaged distance restraints was smaller than 1kcal/mol for all simulation runs except **IV**-*trans* peptide, for which one distance restraint was slightly stretched (0.1 A), causing increase of average restraint energy to 3.5 kcal/mol. The resulting trajectories were clustered with average-linkage clustering algorithm [32]. The clustering metrics was RMSD of all heavy atoms of the backbone. The number of clusters was chosen to minimize Davies-Bourdin index (DBI) and was equal 5 for Tyr-(*R*)-Ala-(*S*)-α-benzyl-β-azydoAla-Gly-Tyr-Pro-Ser-NH2 (**III**) (for both *cis* and *trans* conformations of Pro-6). For Tyr-(*R*)-Ala-(*R*)-α-benzyl-β-azydoAla-Gly-Tyr-Pro-Ser-NH2 (**IV**) optimal number of clusters was equal 4 and 5 for *cis* and *trans* conformation of Pro6 peptide bond, respectively.

**RESULTS AND DISCUSSION**

The peptides designed **I**–**VI** reported herein were obtained by convergent solid-phase peptide synthesis (CSPPS) involving the coupling of protected peptide segments on solid support (the fragment approach). *N*-Terminal di- and tripeptides containing α,α-disubstituted glycines in *C*-terminal position, were coupled with a tetrapeptide or a pentapeptide on resin (Scheme 1). The final heptapeptides were cleaved from the resin with simultaneous deprotection and purified by RP-HPLC. The purity of the final TFA salts was assessed by analytical RP-HPLC and HR-MS (Table 1)

The affinities of dermorphin analogues for *μ*- and *δ-*receptors were determined by the radioligand binding assay described previously [16, 17] using [3H]-DAMGO and [3H]-DELT as *μ*- and *δ*-receptor-specific ligands, respectively. Table 2 shows the binding affinity of dermorphin analogues to μ- and δ-opioids receptors in comparison with dermorphin.

The substitution of (*R*)-Ala in position 2 with (*R*)- or (*S*)-α-methyl-β-azidoAla led to analogues **I** and **II** with lower μ- and δ-affinity and lower μ-selectivity, irrespective of the Cα chirality on α-methyl-β-azidoAla. The replacement of (*R*)-Ala with amphiphilic conformationally constrained (*R*)- or (*S*)-α-benzyl-β-(1-piperidinyl)Ala resulted in decreased affinity to μ-receptors and a loss of affinity to δ-receptors.

The incorporation of a conformationally restricted α-benzyl-β-azidoalanine in position 3 of dermorphin (**III**, **IV**) led to changes in binding affinities to μ- and δ-opioid receptors, which are strongly affected by the configuration on this residue. Both analogues showed visibly reduced of selectivity. The analogue containing (*R*)-α-benzyl-β-azidoalanine (**IV**) displayed slightly lower µ-receptor affinity than the parent peptide and a significantly higher affinity to δ-receptors.

NMR studies and restrained MD simulations were carried out for analogue **III (**the most active in this series) and its epimer **IV**. The nuclear Overhauser effect (NOE), both in the laboratory and rotating frame, has been the method of choice in studying the conformations of organic and biological molecules [25]. 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.

Most studies of dermorphin and its analogues are complicated by *cis*-*trans* isomerism from the peptide bond Tyr5-Pro6. Based on chemical shift differentiation it was found that both diastereomers exist in equilibrium of *trans* and *cis* conformers for the Tyr5-Pro6 peptide bond. Complete assignment of 1H proton-bearing 13C nuclei was obtained for the *trans* conformer from TOCSY, ROESY and 1H/13C HSQC spectra (Table 3). For the *cis* isomer, however, only approx. 80% of 1H resonances was assigned. Discrimination between the *trans* and *cis* conformers was done basing on the chemical shift differences between Cβ and Cγ carbon atoms (cf. Table 6, Supplementary Material) [34]. The ratio of *trans* to *cis* conformer populations was evaluated as 65 : 35 from signal integration.

For both epimers two sets of ROESY signals, corresponding to the *cis* and *trans* conformers, were acquired and used for the determination of distance constraints. The numbers of distance constraints identified for the *cis* conformers were smaller than those for the *trans* ones owing to the incomplete 1H signal assignments in *cis* conformers. The corresponding constraint numbers for the *trans* and *cis* isomers were 63 and 31 in (*R*)-isomer (peptide **IV**) and 64 and 25 in (*S*)-isomer (peptide **III**) Nevertheless, those constraints sufficed for the determination of well-defined structures.

Four simulated systems with common sequence Tyr-Ala-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2, differs in absolute configuration of modified residue (*R* or *S*) and conformation of the peptide bond of Pro6 residue (*cis* or *trans*). The type of beta turn formed in the most populated cluster is shown in the fourth column and population of the dominant cluster is shown in the last column (Table 4).

The representative structures of two dominant clusters of Tyr-(*R*)-Ala-(*R*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**IV**) are shown in Figure 2.

Populations of dominant clusters are close to 0.6 (see Table 4). The backbone trace of two structures is a very similar with exception of *C*-terminus, which differ in the conformation of Pro6 peptide bond.



Figure 2. The representative structures of two dominant clusters of Tyr-(*R*)-Ala-(*R*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**IV**) with proline peptide bond in *trans* (green) and *cis* (cyan) conformations. Chemically modified residue (*R*)-α-benzyl-β-azidoAla-3 and Gly-4 are involved in type I β-turn stabilized by i->i+3 hydrogen bond (yellow dashed lines). Two structures differ in conformation of *C*-terminal part. The sidechain of Ser7 can form hydrogen bond with one of the oxygens of the backbone.

Both structures form a type I β-turn, stabilized by the hydrogen bond between the (*R*)-Ala2 carbonyl oxygen and the Tyr5 amide nitrogen. The backbone dihedral angles of the β-turn of Tyr-(*R*)-Ala-(*R*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**IV**) and ideal type I β-turn dihedral angles are shown in Table 5. Additionally hydrogen bonds between the side chain of Ser7 and the carbonyl oxygen of (*R*)-Ala2 (**IV***-cis*) or the carbonyl oxygen of (*R*)-α-benzyl-β-azidoAla-3 (**IV***-trans*) can be established.

Tyr-(*R*)-Ala-(*S*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**III**) forms reversed β-turns (type I’) as can be seen in Figure 3. Values of backbone dihedrals of residues of β-turn are shown in Table 5; i->i+3 hydrogen bond between backbone atoms stabilizes β-turn and it is followed another hydrogen bond, which stabilizes a very short β-sheet formed by two antiparallel strands. Two β-strands and β-turn constitute a β-hairpin structural motif. Similarly as in the case of Tyr-(*R*)-Ala-(*R*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**IV**) the difference in *C*-terminal part is caused by different conformation of the Pro6 peptide bond.

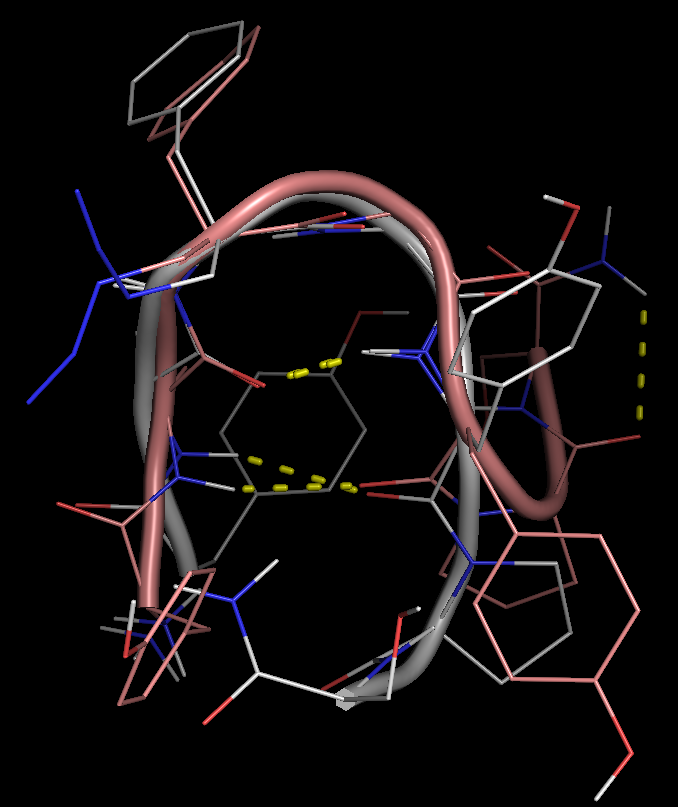


Figure 3. The representative structures of two dominant clusters of Tyr-(*R*)-Ala-(*S*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**III**) with proline peptide bond in *trans* (grey) and *cis* (pink) conformations. Chemically modified residue (*S*)-α-benzyl-β-azidoAla-3 and Gly4 are involved in type I’ β-turn, which is stabilized by two hydrogen bonds (yellow dashed lines), which form a very short β-sheet. Two structures differ mostly in the conformation of *C*-terminal part.

The dominant conformation of the investigated peptides depends on the absolute configuration around Cα in the chemically modified residue in position 3. The (*R*) configuration leads to the formation of a type I β-turn, whilst switching to the (*S*) configuration gives rise to an inversed β-turn of type I’, which is stabilized by two hydrogen bonds, followed by the formation of a very short β-sheet. Therefore Tyr-(*R*)-Ala-(*R*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**IV**) constitutes a β-hairpin protein structural motif. Differences in the dominant conformations of the investigated peptides may be the reason for their different affinities to δ and μ receptors. The selectivities of **III** and **IV** were shown to be very similar; nevertheless, the two analogues exhibited different conformational preferences.

Our results are in agreement with those obtained for dermorphin analogues containing conformationally constrained 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one (α-MeAba) in position 3 [35].

**Acknowledgment**

This research was supported by The National Science Centre (NCN, Poland), grant Preludium 2 (2011/03/N/ST5/04701, A.L.) and Sonata Bis 2 (DEC-2012/07/E/ST4/01386d, M.N.).

NMR measurements were carried out  at the Biological and Chemical Research Centre, University of Warsaw, established within the project co-financed by European Union from the European Regional Development Fund under the Operational Programme Innovative Economy, 2007 – 2013.

All calculations were carried out at the Academic Computer Centre in Gdańsk.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article.

**Conflict of interest** The authors declare that they have no conflict of interests.

**References**

[1] Montecucchi PC, De Castiglione R, Piani S, Gozzin L, Erspamer V. Amino acid composition and sequence of dermorphin, a novel opiate-like peptide from the skin extracts of Phyllomedusa sauvagei. *Int. J*. *Peptide Prot. Res*. 1981; **17:** 275-279; DOI: 10.1111/j.1399-3011.1981.tb01993.x

[2] Erspamer V. The opioid peptides of the amphibian skin.*Int. J. Dev. Neurosci*. 1992;**10**:3-30; DOI: 10.1016/0736-5748(92)90003-I

[3] Melchiorri P, Negri L. The dermorphin peptide family. *Gen. Pharmacol*. 1996; **27:** 1099–1107; [DOI:10.1016/0306-3623(95)02149-3](http://dx.doi.org/10.1016/0306-3623%2895%2902149-3)

[4] Ballet S, Misicka A, Kosson P, Lemieux C, Chung NN, Schiller PW, Lipkowski AW, Tourwé D. Blood-Brain Barrier Penetration by Two Dermorphin Tetrapeptide Analogues: Role of Lipophilicity vs Structural Flexibility. *J. Med. Chem*. 2008; **51**: 2571–2574: **DOI:** 10.1021/jm701404s

[5] Bankowski K, Witkowska E, Michalak OM, Sidoryk K, Szymanek E, Antkowiak B, Paluch M, Filip KE, Cebrat M, Setner B, Szewczuk Z, Stefanowicz P, Cmoch P, Izdebski J. Synthesis, biological activity and resistance to proteolytic digestion of new cyclic dermorphin/deltorphin analogues. *J. Eur. Med. Chem.* 2013; **63**: 457-467; [DOI: 10.1016/j.ejmech.2013.02.019](http://dx.doi.org/10.1016/j.ejmech.2013.02.019)

[6] Biondi L. Filira F, Giannini E, Gobbo M, Lattanzi R, Negri L, Rocchi R. Novel glycosylated [Lys7]-dermorphin analogues: synthesis, biological activity and conformational investigations. *J. Pept. Sci*. 2007; **13:** 179–189; DOI: 10.1002/psc.829

[7] Bozü B, Fülöp F, Tóth GK, Tóth G, Szücs M. Synthesis and opioid binding activity of dermorphin analogues containing cyclic β-amino acids. *Neuropeptides* 1997; **3**: 367-372; [DOI: 10.1016/0306-3623(95)02149-3](http://dx.doi.org/10.1016/0306-3623%2895%2902149-3)

[8] Janecka A, Fichna J, Janecki T. Opioid Receptors and their Ligands. *Curr. Top. Med. Chem.* 2004; **4**: 1-17; DOI: 10.2174/1568026043451618

[9] Vandormael B, De Wachter R, Martins JC, Hendrickx PMS, Keresztes A, Ballet S, Mallareddy JR, Toth F, Toth G, Tourwe D. Asymmetric Synthesis and Conformational Analysis by NMR Spectroscopy and MD of Aba- and α-MeAba-Containing Dermorphin Analogues. *ChemMedChem* 2011; **6**: 2035-2047 DOI: 10.1002/cmdc.201100314

[10] Tran TT, Treutlein H, Burgess AW. Designing amino acid residues with single-conformations. Protein *Eng. Des. Sel*. 2006; **19**:401-408; DOI: 10.1093/protein/gzl024

[11] Oh K-I, Kim W, Joo C, Yoo D-G, Han H, Hwang G-S, Cho M. Azido Gauche Effect on the Backbone Conformation of β-Azidoalanine Peptides. *J. Phys. Chem. B*. 2010; **114**: 13021–13029; **DOI:** 10.1021/jp107359m

[12] Lasota A, Frączak O, Leśniak A, Muchowska A, Lipkowski AW, Nowakowski M, Ejchart A, Olma A. Analogues of deltorphin I containing conformationally restricted amino acids in position 2: Structure and opioid activity. *J. Pept. Sci*. 2015; **21**: 120-125; DOI: 10.1002/psc.2738

[13] Lasota A, Frączak O, Muchowska A,Nowakowski M, Maciejczyk M, Ejchart A, Olma A. Synthesis, biological activity and NMR-based structural studies of deltorphin I analogues modified in message domain with a new α,α-disubstituted glycines, *CBDD*, 2016; DOI: 10.1111/cbdd.12730

[14] A. Kudaj, A. Olma, An efficient synthesis of optically pure α-alkyl-β-azido- and α-alkyl-β-aminoalanines *via* ring opening of 3-amino-3-alkyl-2-oxetanones. *Tetrahedron Lett*. 48 (2007), 6794-6797.

[15] Olma A, Łachwa M, Lipkowski AW. The biological consequences of replacing hydrophobic amino acids in deltorphin I with amphiphilic α-hydroxymethylamino acids. J*. Pept. Res*. 2003: **62:** 45–52; DOI: 10.1034/j.1399-3011.2003.00067.x

[16] Misicka A, [Lipkowski](http://www.ncbi.nlm.nih.gov/pubmed?term=Lipkowski%20AW%5BAuthor%5D&cauthor=true&cauthor_uid=1326067) A.W, Horvath R, P. Davis, T.H. Kramer, H.I. Yamamura, V.J. Hruby, Topographical requirements for delta opioid ligands: common structural features of dermenkephalin and deltorphin. *Life Sci.* 1992; **51**:1025-32; DOI: [10.1016/0024-3205(92)90501-F](http://doi.org/10.1016/0024-3205%2892%2990501-F)

[17] [Fichna](http://www.ncbi.nlm.nih.gov/pubmed?term=Fichna%20J%5BAuthor%5D&cauthor=true&cauthor_uid=15219861)  J, [do-Rego](http://www.ncbi.nlm.nih.gov/pubmed?term=do-Rego%20JC%5BAuthor%5D&cauthor=true&cauthor_uid=15219861) JC, [Costentin](http://www.ncbi.nlm.nih.gov/pubmed?term=Costentin%20J%5BAuthor%5D&cauthor=true&cauthor_uid=15219861) J, [Chung](http://www.ncbi.nlm.nih.gov/pubmed?term=Chung%20NN%5BAuthor%5D&cauthor=true&cauthor_uid=15219861) NN, [Schiller](http://www.ncbi.nlm.nih.gov/pubmed?term=Schiller%20PW%5BAuthor%5D&cauthor=true&cauthor_uid=15219861) PW, [Kosson](http://www.ncbi.nlm.nih.gov/pubmed?term=Kosson%20P%5BAuthor%5D&cauthor=true&cauthor_uid=15219861) P, [Janecka A.](http://www.ncbi.nlm.nih.gov/pubmed?term=Janecka%20A%5BAuthor%5D&cauthor=true&cauthor_uid=15219861) Opioid receptor binding and *in vivo* antinociceptive activity of position 3 substituted morphiceptin analogs. *Biochem. Biophys. Res. Commun*. 2004; **320**: 531-536; [DOI:10.1016/j.bbrc.2004.05.202](http://dx.doi.org/10.1016/j.bbrc.2004.05.202)

[18] Raiford DS, Fisk CL; Becker E.D. Calibration of methanol and ethylene glycol nuclear magnetic resonance thermometers. *Anal. Chem.* 1979; **51**: 2050-2051; DOI:10.1021/ac50048a040

[19] Braunschweiler L, Ernst RR. Coherence transfer by isotropic mixing: application to proton correlation spectroscopy. *J. Magn. Reson.* 1983; **53**: 521-528;

[20] Bax A, Davis DG. Practical aspects of two-dimensional transverse NOE spectroscopy. *J. Magn. Reson*. 1985; **63**: 207-213; [DOI:10.1016/0022-2364(85)90171-4](http://dx.doi.org/10.1016/0022-2364%2885%2990171-4)

[21] Bodenhausen G, Ruben DJ. Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy, *Chem. Phys. Lett*. 1980; **69**: 185-189; [DOI:10.1016/0009-2614(80)80041-8](http://dx.doi.org/10.1016/0009-2614%2880%2980041-8)

[22] Marion D, Ikura M, Tschudin R, Bax A. Rapid recording of 2D NMR spectra without phase cycling. Application to the study of hydrogen exchange in proteins. *J. Magn. Reson*. 1989; **85**: 393-399; DOI: [10.1016/0022-2364(89)90152-2](http://dx.doi.org/10.1016/0022-2364%2889%2990152-2)

[23] Hwang TL, Shaka AJ. Water suppression that works. Excitation sculping using arbitrary waveforms and pulsed field gradients, *J. Magn. Reson*. 1995; **A112**: 275–279; [DOI:10.1006/jmra.1995.1047](http://dx.doi.org/10.1006/jmra.1995.1047)

[24] Wishart DS, Bigam CG, Yao CG, Abildgaad F, Dyson HJ, Oldfield E, Markley JL, Sykes BD. 1H, 13C and 15N chemical shift referencing in biomolecular NMR. *J. Biomol. NMR* 1995; **6**: 135−140; DOI: 10.1007/BF00211777

[25] Goddard TD, Kneller DG. SPARKY3, University of California, San Francisco.

[26] Neuhaus D, Williamson MP. The nuclear Overhauser effect in structural and conformational analysis. VCH Publishers, Inc. Weinheim, 1989.

[27] Case DA, Darden TA, Cheatham III TE. et al, AMBER 12, University of California, San Francisco, 2012.

# [28] Pieffet G., P.A. Petukhov, Parameterization of aromatic azido group: Application for Photoaffinity Probe in Molecular Dynamics Studies, J. Mol. Model. 15 (2009) 1291-1297.

[29] G.D. Hawkins, C.J. Cramer, D.G. Truhlar, Pairwise solute descreening of solute charges from a dielectric medium, Chem. Phys. Lett. 246 (1995) 122–129.

[30] G.D. Hawkins, C.J. Cramer, D.G. Truhlar, Parametrized models of aqueous free energies of solvation based on pairwise descreening of solute atomic charges from a dielectric medium, J Phys. Chem. 100 (1996) 19824–19839.

[31] V. Tsui, D.A. Case, Theory and applications of the generalized Born solvation model in macromolecular simulations. Biopolymers (Nucl. Acid Sci.) 56 (2001) 275–291.

[32] J. Shao, S.W. Tanner, N. Thompson, T.E. Cheatham III, Clustering molecular dynamics trajectories: 1. Characterizing the performance of different clustering algorithms, J. Chem. Theory Comput.3 (2007) 2312-2334.

[33] L.H. Lazarus, A. Guglietta, W.E. Wilson, B.J. Irons, R. de Castiglione, Dimeric dermorphin analogues as μ-receptor probes on rat brain membranes. Correlation between central mu-receptor potency and suppression of gastric acid secretion, J. Biol. Chem. 264 (1989) 354–362.

[34] M. Schubert, D. Labudde, H. Oschkinat, P. Schmieder, A software tool for the prediction of Xaa-Pro peptide bond conformations in proteins based on 13C chemical shift statistics, J. Biomol. NMR 24 (2002) 149-154.

[35] B. Vandormael, R. De Wachter, J.C. Martins, P.M.S. Hendrickx, A. Keresztes, S. Ballet, J.R. Mallareddy, F. Tóth, G. Tóth, D. Tourwé, Asymmetric Synthesis and Conformational Analysis by NMR Spectroscopy and MD of Aba- and α-MeAba-Containing Dermorphin Analogues. ChemMedChem 6 (2011) 2035-2047.

Table 1. Structures and the physicochemical properties of the dermorphin analogues **I-VI**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Peptide | MW [g/mol] | [M +H]+ | HPLC | | TLC | |
| % | tr\* | Rf1 | Rf2 |
| Tyr-(*S*)-α-methyl-β-azidoAla-Phe-Gly-Tyr-Pro-Ser-NH2 (**I**) | 857,9113 | 859,0168 | 99 | 15,01 | 0,77 | 0,45 |
| Tyr-(*R*)-α-methyl-β-azidoAla-Phe-Gly-Tyr-Pro-Ser-NH2 (**II**) | 857,9113 | 858,9068 | 98 | 13,66 | 0,75 | 0,44 |
| Tyr-(*R*)-Ala-(*S*)-α-benzyl-β-azidoAla**-**Gly-Tyr-Pro-Ser-NH2 (**III**) | 857,9113 | 859,0000 | 99 | 13,50 | 0,75 | 0,47 |
| Tyr-(*R*)-Ala-(*R*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**IV**) | 857,9113 | 859,0167 | 95 | 14,34 | 0,77 | 0,48 |
| Tyr-(*R*)-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Gly-Tyr-Pro-Ser-NH2 (**V**) | 900,0308 | 901,0677 | 99 | 11,15 | 0,51 | 0,27 |
| Tyr-(*R*)-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Gly-Tyr-Pro-Ser-NH2 (V**I**) | 900,0308 | 901,0698 | 99 | 10,94 | 0,45 | 0,20 |

HPLC gradient 10-50%B over A, 20 min; TLC: Rf1 (n-butanol:acetic acid:water 4:1:1 *v*/*v*/*v*) Rf2 (acetonitrile:water, 1:1 *v*/*v)*

Table 2. The binding affinity of dermorphin analogues **I-VI** to δ- and μ-opioid receptors

|  |  |  |  |
| --- | --- | --- | --- |
| PEPTIDE | IC50 (nM) | | IC50 ratio δ/μ |
| μa | δb |
| Tyr-(*R*)-Ala-Phe-Gly-Tyr-Pro-Ser-NH2  (DERM) [33] | 1.22 | 178.6c | 146.4 |
| Tyr-*(S)*-α-methyl-β-azidoAla**-**Phe-Gly-Tyr-Pro-Ser-NH2  (**I**) | 52 | 1488 | 28,6 |
| Tyr-*(R)*-α-methyl-β-azidoAla**-**Phe-Gly-Tyr-Pro-Ser-NH2 (**II**) | 34 | 1339 | 39,4 |
| Tyr-(*R*)-Ala-(*S*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**III**) | 151 | 1057 | 7 |
| Tyr-(*R*)-Ala-(*R*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**IV**) | 4.29 | 22.6 | 5,27 |
| Tyr-(*R*)-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Gly-Tyr-Pro-Ser-NH2 (**V**) | 52.48 | >10000 | >191 |
| Tyr-(*R*)-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Gly-Tyr-Pro-Ser-NH2 (**VI**) | 208.9 | >10000 | >48 |

*aversus [3H]DAMGO, bversus [3H]DELT, cversus DADLE*

Table 3. 1H chemical shifts (in ppm) for *trans* conformers of Tyr-(*R*)-Ala-(*S*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**III**) andTyr-(*R*)-Ala-(*R*)-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2 (**IV**) (Aaa = α-benzyl-β-azidoAla)

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| a.a. | HN | Hα | Hβ | Hγ | Hδ | Hε | Hζ |
| Tyr1 (**III**)  (**IV**) | n.a.  n.a. | 4.088  4.088 | 3.062 3.120  3.067 3.120 | ---  --- | 7.110  7.105 | 6.869  6.867 | ---  --- |
| Ala2 (**III**)  (**IV**) | 8.412  8.363 | 4.251  4.276 | 1.161  1.177 | ---  --- | ---  --- | ---  --- | ---  --- |
| Aaa3 (**III**)  (**IV**) | 8.505  8.357 | benzyl  CH2N3  benzyl  CH2N3 | 3.089 3.254  3.608 3.723  3.107 3.197  3.647 3.761 | ---  ---  ---  --- | 7.082  ---  7.080  --- | 7.322  ---  7.313  --- | 7.318  ---  7.313  --- |
| Gly4 (**III**)  (**IV**) | 8.288  8.306 | 3.705 3.891  3.739 3.858 | ---  --- | ---  --- | ---  --- | ---  --- | ---  --- |
| Tyr5 (**III**)  (**IV**) | 7.806  7.885 | 4.827  n.a. | 2.890 3.055  2.852 3.073 | ---  --- | 7.133  7.147 | 6.814  6.810 | ---  --- |
| Pro6 (**III**) | ---  --- | 4.427  4.449 | 1.958 2.257  1.960 2.252 | 1.980 a  1.977 a | 3.543 3.751  3.534 3.737 | ---  --- | ---  --- |
| Ser7 (**III**)  (**IV**) | 8.244  8.252 | 4.338  4.342 | 3.807 3.839  3.817 3.843 | ---  --- | ---  --- | ---  --- | ---  --- |
| *C*-term (**III**)  (**IV**) | 7.136 7.527  7.133 7.518 | ---  --- | ---  --- | ---  --- | ---  --- | ---  --- | ---  --- |

n.a. - not assigned; a - both Hγ protons are isochronous

Table 4. Equilibrium of *trans* and *cis* conformers Tyr-(*R*) or (*S*)-Ala-α-benzyl-β-azidoAla-Gly-Tyr-Pro-Ser-NH2

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | α-Benzyl-β-azidoAla | Proline peptide bond | β-Turn | Population of dominant cluster |
| **IV** *-cis* | (*R*) | *cis* | I | 0.634 |
| **IV***-trans* | (*R*) | *trans* | I | 0.579 |
| **III** *-cis* | (*S*) | *cis* | I' | 0.577 |
| **III***-trans* | (*S*) | *trans* | I' | 0.691 |

Table 5. Comparison of backbone dihedral angles of β-turns of representative conformations of the most populated clusters and their values for ideal β-turns.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Peptide | ϕi+1 | ψi+1 | ϕi+2 | ψi+2 |
| **IV** -cis | -43.1 | -34.4 | -100.1 | 0.3 |
| **IV**-trans | -29.2 | -51.0 | -76.9 | -2.6 |
| ideal β-turn I | -60.0 | -30.0 | -90.0 | 0.0 |
| **III** -cis | 34.5 | 36.9 | 81.5 | 16.4 |
| **III**-trans | 25.5 | 35.5 | 64.7 | 23.0 |
| ideal β-turn I’ | 60.0 | 30.0 | 90.0 | 0.0 |