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| Complete List of Authors: | Olma, Aleksandra; Lodz University of Techology, Faculty of Chemistry, Institute of Organic Chemistry<br>Lasota, Anika; Lodz University of Technology, Institute of Organic Chemistry<br>Frączak, Oliwia; Lodz University of Technology, Institute of Organic Chemistry<br>Muchowska, Adriana; Polish Academy of Sciences, Mossakowski Medical Research Centre<br>Nowakowski, Michał; University of Warsaw, Centre of New Technologies<br>Maciejczyk, Maciej; University of Warmia and Mazury, Department of Physics and Biophysics, Faculty of Food Science<br>Ejchart, Andrzej; Polish Academy of Sciences, Institute of Biochemistry and Biophysics |





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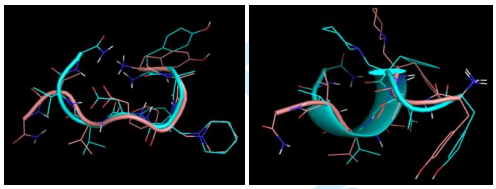
# Synthesis, biological activity and NMR-based structural studies of deltorphin I analogues modified in message domain with a new α,α-disubstituted glycines†

Anika Lasota[a], Oliwia Frączak[a], Adriana Muchowska[b], Michał Nowakowski[c], Maciej Maciejczyk[d], Andrzej Ejchart[e], Aleksandra Olma[a*]



Eight new analogues of DTI were designed, synthesized and tested for receptor affinity and selectivity to μ- and δ-opioid receptors. NMR solution structure of μ-selective Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ and δ-selective Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ were determined and diussed.

# Synthesis, biological activity and NMR-based structural studies of deltorphin I analogues modified in message domain with a new α,α-disubstituted glycines†

Anika Lasota[a], Oliwia Frączak[a], Adriana Muchowska[b], Michał Nowakowski[c], Maciej Maciejczyk[d], Andrzej Ejchart[e], Aleksandra Olma[a*]

[a] *Institute of Organic Chemistry, Lodz University of Technology, Żeromskiego 116, 90-924 Lodz, Poland*

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

[c] *Centre of New Technologies, University of Warsaw, Banacha 2C, 02-097 Warsaw, Poland*

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

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

† This paper is dedicated to the memory of Andrzej Lipkowski (deceased November 27, 2014). The peptide community has lost an excellent scientist and a dear friend, and he will be missed by all of us who were fortunate enough to know him and work with him.

Abstract:

This paper describes new deltorphin I analogues in which phenylalanine residues were replaced by the corresponding (*R*) or (*S*)-α-benzyl-β-azido(1-pyrrolidinyl, 1-piperidinyl, 4-morpholinyl)alanine residues. All analogues were tested for receptor affinity and selectivity to μ- and δ-opioid receptors. The affinity of analogues containing (*R*) or (*S*)-α-benzyl-β-azidoalanine in position 3 to δ-receptors strongly depended on the chirality of the α,α-disubstituted residue. The conformational behavior of peptides modified with (*R*) or (*S*)-α-benzyl-β-(1-piperidinyl)Ala, which display the opposite selectivity, was analyzed by $^{1}$H and

$^{13}$C NMR. The μ-selective Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ lacks the helical conformation observed in the δ-selective Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$. Our results support the proposal that differences between δ- and μ-selective opioid peptides are attributable to the presence or absence of a spatial overlap between the *N*-terminal message domain and the *C*-terminal address domain.

## INTRODUCTION

Opioid peptides include a large group of physiologically active bioregulators exhibiting a broad spectrum of biological activity and interacting with opioid receptors (μ, δ, κ). Deltorphins are heptapeptides that have been isolated from the South American frog belonging to the genus Phyllomedusa (1). Deltorphins show a higher affinity and selectivity for δ opioid receptors than any other endogenous mammalian compound (2).

Deltorphin I and deltorphin II consist of two parts, a biologically important *N*-terminal tripeptide fragment (Tyr-D-Ala-Phe, the message domain), a binding pharmacophore (3), and a *C*-terminal fragment (Asp/Glu-Val-Val-Gly-$NH_2$, the address domain). Anionic and hydrophobic *C*-terminal tetrapeptides decrease μ affinity while at the same time increasing δ affinity (4). The conformational, topographical and stereoelectronic structural features of the opioid peptides are important for interaction with μ, δ, and κ opioid receptors. Two aromatic amino acids, Tyr$^1$ and Phe$^3$ or Phe$^4$, are important structural elements because they interact with opioid receptors. The search for new analogues of deltorphins is an important direction of study because they are likely to be effective analgesic agents for the treatment of cancer pain (5) and neuropathic pain (6) with a low potential for abuse (7,8). Since the discovery of endogenous amphibian peptides hundreds of analogues of the various deltorphins have been synthesized (9).

It has been proposed that the δ-receptor selectivity of deltorphins is a result of the formation of special nonequal amphiphilic topography ('hot-dog' shape) (3). In such a conformation, the hydrophilic strain ('hot-dog') formed by ionic and hydrogen bonds between $NH_2$ ($Tyr^1$)...-COOH($Asp^4$)...$CONH_2$ ($Gly^7$) is surrounded by dominating lipophilic shells ('hot-dog roll'). Some very potent and selective cyclic analogues, which stabilized this conformation, have supported the proposed model (10). The incorporation amphiphilic amino acid residues (α-hydroxymethylphenylalanine) also support proposed molecular models of the active conformation of deltorphins (11).

The presented paper describes the synthesis and receptor binding of new analogues in which $Phe^3$ residue in the deltorphin I sequence was substituted by (*R*) or (*S*)-α-benzyl-β-azido(1-piperidinyl, 1-pyrrolidinyl, 4-morpholinyl)alanine (Figure 1). 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.

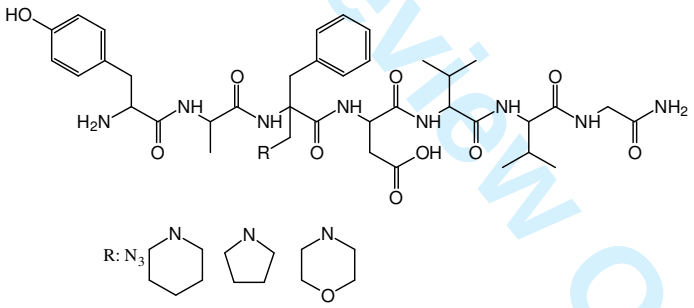



Figure 1. Structure of new deltorphin I analogues

Optically pure α,α-disubstituted glycines were obtained from available *N*-Boc-(*R* or *S*)-α-benzylserine β-lactone (12). The treatment of *N*-Boc-(*R* or *S*)-α-benzylserine β-lactone with sodium azide or free heterocyclic amines (pyrrolidine, piperidine, morpholine) as nucleophile gives suitable, enantiomerically pure *N*-Boc-(*R* or *S*)-α-benzyl-β-azido(*sec*-amino)alanines (13).

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 (14).

## Experimental Section

### Chemistry

Most 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*-Boc-α-benzyl-β-azido(*sec*-amino) alanines were obtained according to a procedure described in the literature (12,13). 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.

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.

$^1$H NMR spectra of protected amino acids, di- and tripeptides were recorded on a Bruker DPX 250 spectrometer (Bruker Biospin GMBH, Rheinstetten, Germany). Proton chemical shifts are reported in ppm (d) relative to internal tetramethylsilane (TMS, d: 0.00). Data are reported as follows: chemical shift {multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m)], coupling constants [Hz], integration}. ESI-LC-MS was recorded on a Bruker amaZon speed ETD trap, with an ESI ion source, positive ion polarity, a maximum resolution mass range, and a 50–2000 m/z range.

**General procedure for synthesis of 2a-2c**

To *N*-Boc-(*R* or *S*)-α-benzyl-β-azido(*sec*-amino)alanine (1,2 mM) in 3 ml of MeOH, a freshly prepared ethereal solution of diazomethane was added until the yellow color of diazomethane persisted. Reaction without stirring was left overnight. The progress of the reactions was monitored with TLC (chloroform:methanol 9:1 *v/v*). Then 99.5 % acetic acid was added carefully to destroy unreacted diazomethane and the solvents are removed under vacuum on a rotary evaporator. The crude methyl ester was diluted with ethyl acetate (25 ml) and washed with three portions of water, 5% $NaHCO_3$, and brine, dried with magnesium sulfate, and concentrated under vacuum. *N*-Boc-(*R* or *S*)-α-benzyl-β-azidoalanines without further purification were used in the next step, *N*-Boc-(*R* or *S*)-α-benzyl-β-(*sec*-amino)AlaOMe were purified by flash chromatography (chloroform: methanol 95:5 *v/v*).

**General Procedure for the deprotection of the *Boc*-group**

To a solution of *N*-Boc-(*R* or *S*)-α-benzyl-β-azido(*sec*-amino)AlaOMe in 1 ml of ethyl acetate 7 ml of 2 N HCl in AcOEt was added. After 2 hours next portion of 7 ml HCl in AcOEt was added and the stirring was continued for 2-4 hours. The reaction was monitored by TLC (chloroform: methanol 9:1 *v/v*). After conversion of all starting material the product was precipitated with diethyl ether. Precipitated amorphous solids were filtered off and washed with ethyl ether and used for the next step without further purification.

**General procedure for synthesis 3a-3d and 4a-4d**

To a stirred solution of Boc-D-alanine or *N,O*-DiBoc-tyrosine (1eq.) in dry DCM, HATU (1eq), HOAt (1 eq) and *N*-methylmorpholine (4 eq for monohydrochlorides, or 5 eq for dihydrochlorides) were added. After 20 min. methyl ester hydrochloride of amino acid **2a**-**2d** or hydrochloride of unprotected dipeptide **3a**-**3d** (1 eq) dissolved in 2 ml of dry dimethylformamide was added. The reaction progress was controlled by TLC in chloroform: methanol 9:1 *v/v*. After 20 hours, if a significant amount of unreacted substrates were present, additional amount of HATU(0.5 eq) and HOAt (0.5 eq) and amine (1 eq) were added. Then, the reaction mixture was stirred at room temperature for 16 h. The solvent was evaporated under reduced pressure; the residue was diluted with ethyl acetate and washed with three portions of water, 1 N $NaHSO_4$ (for dipeptides containing secondary amines this step was omitted due to formation of quaternary ammonium salts), 5% $NaHCO_3$, and brine, dried with magnesium sulfate, and concentrated under vacuum. Purification by flash chromatography (chloroform:methanol 95:5*v/v*) afforded the desired di- or tripeptides.

**General procedure for synthesis of 5a-5d**

To the solution of **4a**-**4d** (1 mM) in 5 ml of methanol cooled in an ice bath 3 ml of 1 N NaOH was added. Stirring was continued at room temperature until no starting material remained (3 to 6 hours, TLC chloroform: methanol 9:1 *v/v*). Then the methanol was evaporated under reduced pressure at room temperature. The residue was diluted with 20 ml of water and the aqueous layer was washed with diethyl ether (3x10 ml) and acidified with 1N $NaHSO_4$ to pH≈2-3 (analogs containing an azido group) or pH≈6-7 (analogues containing a *sec*-amino group). Then aqueous layer was saturated with NaCl and extracted with ethyl acetate (3x15 ml). The combined ethyl acetate layer was dried over $MgSO_4$, and evaporated *in vacuo*. The crude *N*-protected tripeptides (**5a**-**5d**) were used for the next step.

The structures of all isolated compounds were established by nuclear magnetic resonance (NMR). Full characterization as well as detailed experimental procedures for all intermediates is is available in the online supporting information.

**General procedure for synthesis of I-VIII**

Tetrapeptide resin was prepared by the manual solid-phase technique on Rink-amide AM resin (capacity 0.1 mmol/g), according to standard methods for peptides synthesized by the Fmoc/tBu strategy. The protected amino acids were coupled with a 3-fold excess using TBTU as a coupling reagent in the presence of HOBt and DIPEA in DCM. In the case of a positive Kaiser test (15), the coupling was repeated with a 1.5 fold excess of reagents. The Fmoc groups were removed by treatment with 20% piperidine in DMF. The tetrapeptide on resin was acylated with a 2-fold excess of *N*,*O*-protected tripeptides containing (*R*) or (*S*)-α-benzyl-β-azido(*sec*-amino)alanine **5a**-**5d** using HATU as a coupling reagent in the presence of HOAt and DIPEA in DCM. In the case of a positive Kaiser test, the coupling was repeated with a 1.2 fold excess of reagents. The heptapeptides were cleaved from the resin and protecting groups were removed in one step using a mixture of TFA/$H_2O$ (95:5 by vol) (20 ml/100mg 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. All heptapeptides were characterized by analytical RP-HPLC and molecular weight determination.

***Ligand binding assay***

Receptor binding assays were performed as described previously (11). Rat membrane preparation followed the procedure described by Misicka et al. (3) The radioligand receptor binding protocol was based on a study performed by and Fichna et al. (16) 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 [$^3$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 1).

***NMR experiments and computation of peptide structures***

NMR samples with a volume of 650 μL contained 5mg of Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (V) or 4 mg Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (VI) dissolved in a $H_2O/D_2O$ mixture (90:10 by vol). All spectra were measured on an Agilent DDR2 spectrometer operating at 600 MHz resonance frequency ($^1$H), 60.8 MHz ($^{15}$N), and 150.9 MHz ($^{13}$C) at temperature 25°C. Temperature calibration was carefully adjusted using an ethylene glycol reference sample (17). 2D Homonuclear TOCSY (18) (mixing time 80 ms), ROESY (19) (mixing time 300 ms) and heteronuclear $^1$H/$^{15}$N HSQC (20) and $^1$H/$^{13}$C HSQC (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, $^{15}$N and $^{13}$C resonances. Time domain data were acquired using States-TPPI quadrature detection (21). Water suppression was achieved with pulsed field gradients echo (22). All chemical shifts in $^1$H NMR spectra were reported with respect to external DSS-$d_4$. Chemical shifts of $^{13}$C and $^{15}$N signals were referenced indirectly using the 0.251449530 and 0.101329118 frequency ratios $^{13}$C/$^1$H and $^{15}$N/$^1$H respectively (23). Zero filling and a 90º-shifted squared sine-bell filter were performed prior to Fourier transformation. Processed spectra were analyzed with SPARKY software (24).

Intensities of interproton correlations in ROESY spectra, $I_{ij}$, were used in determining appropriate distances $r_{ij}$ from the equation $I_{ij} = C \cdot r_{ij}^{-6}$ (25). The constant C was calculated from the intensity of correlation between tyrosine protons HD and HE of fixed distance assumed to be equal to 2.48 Å in case of **V.** In case of **VI** correlation intensities between tyrosine protons

HA and both HB were used assuming r = 2.5 Å for stronger correlation and r = 3 Å for a weaker one.

*Calibration*

*Parametrization of modified residues*

For all natural amino-acid residues standard Amber ff10 force-field parameters were applied (26). The parametrization of α-benzylo-β-(1-piperydynyl)Ala residue was based on ff10 parameters for phenylalanine residue. The piperydynyl part of amino-acid residue was parameterized in the following manner. Bonded part of the potential was automatically assigned by Antechamber and GAFF force-field (27, 28). Partial charges were determined by fitting them (RESP) to the electrostatic potential (29) obtained from quantum mechanical computations at the MP2/6-31G(d,p) level with Gaussian 03 package (30).

*Simulated annealing procedure*

The peptide chain was built with xleap program of the Amber package. In order to remove bad contacts 1000 steps of geometry optimization was 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 cooling process. NMR distance restraints were slowly switched on during first 3ps of simulated annealing run. Improper dihedral restraints on chiral centers were switch 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 (31-33). 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 restrains MD simulation*

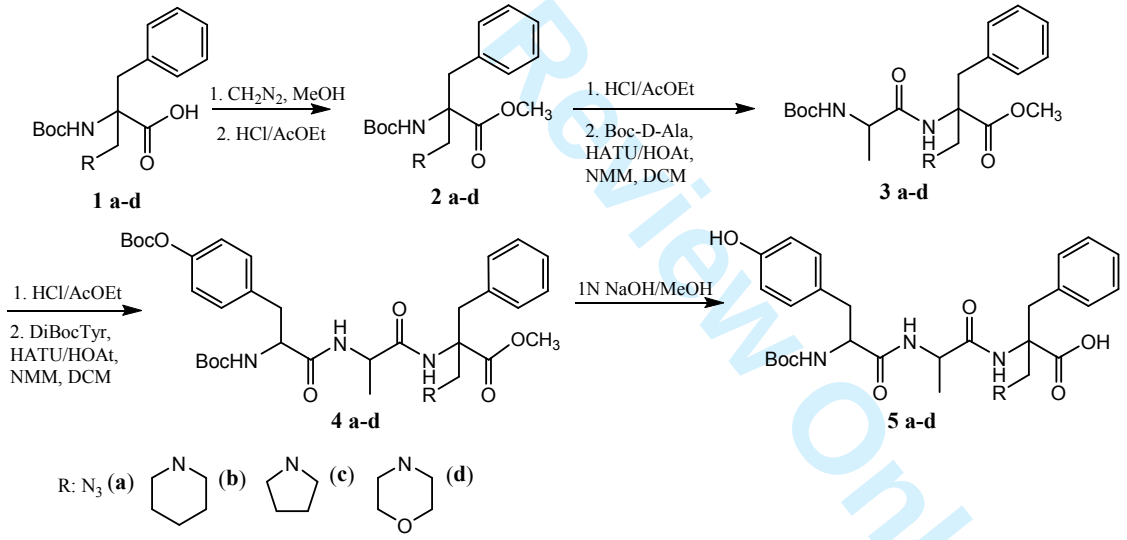
The geometry of the initial structure was optimized and equilibrated in 1ns MD run with time-averaged restraints applied. The SHAKE algorithm was used to keep covalent bonds with hydrogens constant and 2fs time-step was applied. The solvation effects were described by Generalized-Born model (31-33). During 20 ns production run proton-proton distance restraints obtained from NMR experiment were time-averaged over 1ps time interval. The average energies of time-averaged distance restraints were below 1 kcal/mol for both peptides. The resulting trajectories were clustered with average-linkage clustering algorithm. The clustering metrics was RMSD of all heavy atoms of the backbone. The number of clusters were chosen to minimize Davies-Bourdin index (DBI) and was equal to 7 for Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (**V**) and 5 for Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (**VI**).

## RESULTS AND DISCUSSION

*N*-Protected (*R*) and (*S*) α-benzyl-β-azido(*sec*-amino)alanines were synthesized from conveniently available β-lactones of *N*-Boc-(*R*) and (*S*)-α-benzylserine by ring opening with a sodium azide, pyrrolidine, piperidine or morpholine as nucleophile. Incorporation of α,α-disubstituted glycines into peptides in stepwise solid-phase peptide synthesis (SPPS) is difficult due to their steric hindrance and lower reactivity. Our attempts to prepare DT I analogues by solid-phase synthesis using Boc strategy were unsuccessful. The resulting products, despite the use of reagents for difficult coupling and prolonged time of reaction are contaminated with truncated peptides (penta- and hexapeptides) due to inefficient coupling of α,α-disubstituted amino acids.

The designed peptides **I**–**VII** reported here were obtained by convergent solid-phase peptide synthesis (CSPPS) involving the coupling of protected peptide segments on solid support (the fragment approach). *N*-Terminal tripeptides containing α,α-disubstituted glycines in position 3

were obtained in solution using HATU as a coupling reagent and then, after deprotection of the carboxyl function, were coupled with tetrapeptides on resin. *N*,*O*-Protected tripeptides were obtained by the stepwise peptide chain elongation in solution. (Scheme 1). The tetrapeptide Asp-Val-Val-Gly-$NH_2$ was 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 final heptapeptide resins were obtained by segment condensation (fragments 3+4). Cleavage from the resin and removal of the protecting groups were carried out in one step 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.




Scheme 1. Synthesis of *N*,*O*-protected tripeptide units

This strategy allows for a full control and monitoring of the peptide synthesis. The cleavage of heptapeptides from the resin and the removal of the protecting groups was performed with TFA:water (95:5 by vol). All crude analogues were purified to homogeneity by RP-HPLC and their structures were verified by mass spectrometry.

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

Table 1 shows the binding affinity of deltorphin I analogues to δ- and μ-opioid receptors in comparison with deltorphin I.

**Table 1**. Binding affinities of deltorphin analogues **I**-**VIII** to δ and μ opioid receptors

| Peptide | $IC_{50}$ (nM) | | |
|---|---|---|---|
| | μ[a] | δ[b] | select. |
| Tyr-D-Ala-Phe-Asp-Val-Val-Gly-$NH_2$ (DTI) (34) | 976±148 | 3.05±0.10[c] | 320 |
| Tyr-D-Ala-(*S*)-α-benzyl-β-azidoAla-Asp-Val-Val-Gly-$NH_2$ **I** | 2473±113 | 655±108 | 3.77 |
| Tyr-D-Ala-(*R*)-α-benzyl-β-azidoAla-Asp-Val-Val-Gly-$NH_2$ **II** | 1272±55.5 | 8.8±1.0 | 144 |
| Tyr-D-Ala-(*S*)-α-benzyl-(1-pyrrolidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **III** | 1793±54.7 | 3178±430 | 0.56 |
| Tyr-D-Ala-(*R*)-α-benzyl-β-(1-pyrrolidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **IV** | 419±24.31 | 378.7±25.1 | 1.11 |
| Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **V** | 2876±99.5 | 15.0± 1.2 | 192 |
| Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **VI** | 88±3.1 | 669±53.5 | 0.13 |
| Tyr-D-Ala-*(S)*-α-benzyl-β-(4-morpholinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **VII** | 3907± 231 | 2205±166 | 1.77 |
| Tyr-D-Ala-*(R)*-α-benzyl-β-(4-morpholinyl)Ala-Asp-Val-Val-Gly-$NH_2$[b] **VIII** | 2624±116 | 1373±137 | 1.91 |

[a]*versus* [$^3$H]DAMGO, [b]*veresus* [$^3$H]DELT, [c]*versus* [$^3$H]DPDPE

As reported in Table 1, the affinity analogues containing (*R*) or (*S*)-α-benzyl-β-azidoalanine in position 3 depends on the $C^{\alpha}$ chirality of α-benzyl-β-azidoalanine. The replacement of phenylalanine with (*R*)-α-benzyl-β-azidoalanine (peptide **II**) slightly decreases δ- and μ-receptor affinity in comparison with parent peptide, whereas the incorporation of (*S*) isomer gives analog **I**, considerably less potent and δ-selective. In analogue **II**, the delocalized charge of azidomethyl group in α-benzyl-β-azidoalanine may stabilize the proposed 'hot-dog' conformation (3). The substitution of Phe$^3$ with (*R*) or (*S*)-α-benzyl-(1-pyrrolidinyl)alanine

and *(R)* or (*S*)-α-benzyl-β-(4-morpholinyl) results in a loss activity and selectivity (**III**, **IV** and **VII**, **VIII**).

The introduction of the conformationally restricted α-benzyl-β-(1-piperidinyl)alanine (**V**, **VI**) in position 3 of deltorphin I leads to changes in binding affinities to μ and δ opioid receptors, which are strongly affected by the configuration at $C^{\alpha}$. The (*S*) isomer slightly decreases affinity to δ receptors and significantly to μ receptors, yielding δ-selective ligand. Changing the configuration of α-benzyl-β-(1-piperidinyl)alanine reverses selectivity as compared to deltorphin I, giving Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (**VI**), the μ-selective ligand. In a binding assay analogue **V** displays a 192-fold higher selectivity for δ receptor, while analogue **VI** shows a 7.6-fold higher selectivity for μ-receptors (over δ receptors). An NMR study was carried out to explain the opposite selectivities of analogues **V** and **VI**. 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 (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. Complete assignment of $^{1}H$, proton-bearing $^{13}C$ nuclei was obtained from TOCSY, ROESY and $^{1}H/^{13}C$ HSQC spectra.

The representative structures of two dominant clusters of Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (**VI**), with total population over 0.5, are shown in Figure 2.

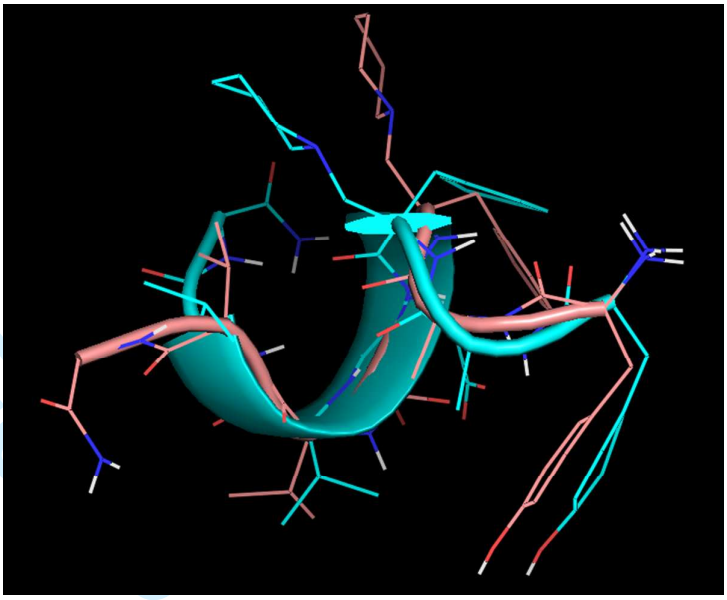


Figure 2. Representative structures of two most populated clusters of the Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (**VI**). Pink and blue structures have populations 0.269 and 0.264, respectively. The *C*-terminus of blue structure forms helix stabilized by interaction of piperidynyl with Val-6.

The populations of two dominating clusters are nearly identical. The backbone trace of two structures is very similar with exception of *C*-terminus. The *C*-terminal part of the peptide can form helix as can be seen in Figure 2 (blue structure). The helical conformation is stabilized partially by hydrogen-bonds, but probably more important is hydrophobic contact between piperydynyl and $Val^6$, as can be seen in Figure 3. This contacts seems to drive helix formation at the *C*-terminus.

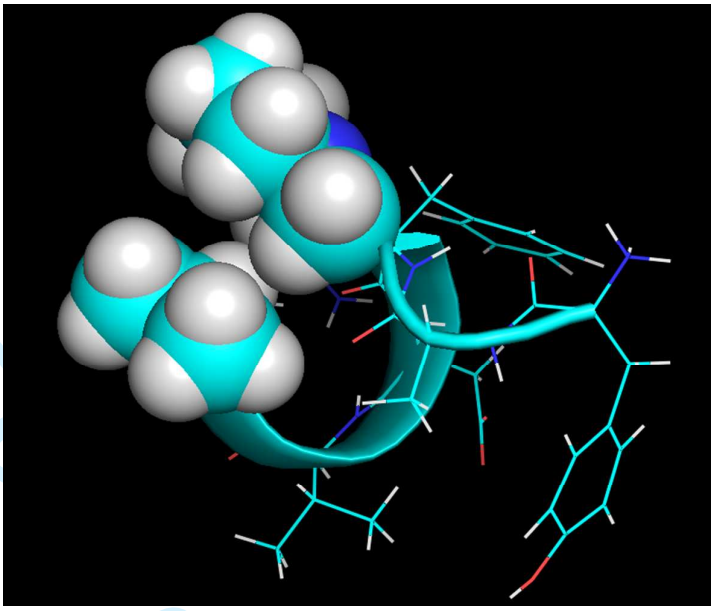


Figure 3. Hydrophobic contact formed by piperidynyl and Val[6] residue in Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (**VI**)

The Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (**V**) lacks contact which seems to drive helix formation and the representative structures of the most populated (0.3) and least populated (0.05) clusters are shown in Figure 4. These two clusters are similar with a major conformational difference at the *C*-terminus.

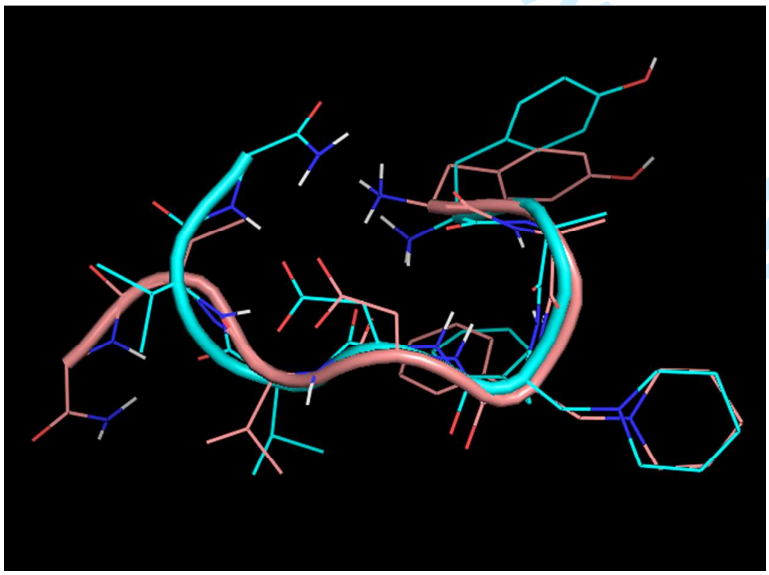


Figure 4. Representative structures of the most populated (blue) and the least populated (pink)

clusters of Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (**V**). The peptide lacks helical conformation observed for peptide **VI**.

Analogue **V** (Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$, Figure 4) lacks helical conformation observed in Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (Figure 2), which can be responsible for its μ-selectivity. This confirms that *C*-terminal tail of this δ-selective deltorphin assumes an extended, rather than helix-like, conformation (35). These two clusters are similar with a major conformational difference at the *C*-terminus. Our studies suggest, that μ or δ selectivity appear to be forced by conformation adopted by the address domain.

In conclusion, the binding assay showed, that the replacement of phenylalanine with α-benzyl-β-azido(1-pyrrolidinyl, 1-piperidinyl, 4-morpholinyl)alanine has a strong effect on binding affinity. Our result supports the proposal (36) that differences between δ- and μ-selective opioid peptides are attributable to the presence or absence of a spatial overlap between the *N*-terminal message domain and *C*-terminal address domain.

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Quantum chemistry and molecular dynamics computations were performed at CI TASK in Gdańsk.

Conflict of interest

The authors declare that they have no conflict of interest

**Supporting Information**

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

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**'Supporting Information'**

**Boc-*(R)*-α-benzyl-β-(azido)AlaOMe ((*R*)-2a)**

Yield: quantitative, colorless oil, $R_f$= 0.85 (chloroform: methanol 95:5 *v/v*), $[\alpha]_D^{20}$ = 133.7 [*c* = 1, $CHCl_3$]

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.49 (s, 9H, Boc); 2.98 and 3.52 (AB system, *J* = 13.25 Hz, 2H, C$\underline{H}_2$Ph); 3.51 and 4.34 (system AB, *J* = 12.33 Hz, 2H, $CH_2N$); 3.78 (s, 3H, $OCH_3$); 5.49 (broad s, 1H, NH); 7.01-7.05 (m, 2H, Ar) and 7.26-7.30 (m, 3H, Ar).

**Boc-*(S)*-α-benzyl-β-(azido)AlaOMe ((*S*)-2a)**

Yield: 98%, colorless oil, $R_f$ = 0.85 (chloroform: methanol 95:5 *v/v*), $[\alpha]_D^{25}$ = -132.0 [*c* = 1, $CHCl_3$]

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.49 (s, 9H, Boc); 2.97 and 3.52 (system AB, *J* = 13.14 Hz, 2H, C$\underline{H}_2$Ph); 3.61 and 4.30 (system AB, *J* = 12.20 Hz, 2H, $CH_2N$); 3.76 (s, 3H, $OCH_3$); 5.49 (s, 1H, NH); 7.01-7.07(m, 2H, Ar) and 7.25-7.30 (m, 3H, Ar).

**Boc-*(R)*-α-benzyl-β-(1-pyrolidinyl)AlaOMe ((*R*)-2b)**

Yield: quantitative, orange oil, $R_f$ = 0.78 (chloroform: methanol 9:1 *v/v*), $[\alpha]_D^{25}$ = 62.1 [*c* = 1, $CHCl_3$]

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.47 (s, 9H, Boc); 1.70-1.79 (m, 4H, $N(CH_2\underline{CH_2})_2$); 2.69-2.79 (m, 4H, $N(\underline{CH_2}CH_2)_2$); 3.13 and 3.14 (system AB, *J* = 13.71 Hz, 2H, $\underline{CH_2}$Ph); 3.46 and 3.49 (system AB, *J* = 13.25 Hz, 2H, $\underline{CH_2}$N); 3.75(s, 3H, $OCH_3$); 7.05-7.09 (m, 2H, Ar); 7.20-7.26 (m, 3H, Ar).

**Boc-*(S)*-α-benzyl-β-(1-pyrolidinyl)AlaOMe ((*S*)-2b)**

Yield: 83%, orange oil, $R_f$ = 0.78 (chloroform: methanol 9:1 *v/v*), $[\alpha]_D^{25}$ = -61.8 [*c* = 1, $CHCl_3$]

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.47 (s, 9H, Boc); 1.65-1.70 (m, 4H, $N(CH_2\underline{CH_2})_2$); 2.48-2.65 (m, 4H, $N(\underline{CH_2}CH_2)_2$); 2.97 and 3.14 (system AB, *J* = 13.25 Hz, 2H, $\underline{CH_2}$Ph); 3.38 and 3.49 (system AB, 2H, *J* = 13.25 Hz, $\underline{CH_2}$N); 3.76 (s, 3H, $OCH_3$); 7.06-7.09 (m, 2H, Ar); 7.20-7.25(m, 3H, Ar).

***N*-Boc-*(R)*-α-benzylo-β-(1-piperidinyl)AlaOMe ((*R*)-2c)**

Yield: quantitative, orange oil, $R_f$ = 0.50 (chloroform: methanol 9:1 *v/v*), $[\alpha]_D^{25}$ = 56.2 [*c* = 1, $CHCl_3$]

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.26-1.52 (m, 6H, $N(CH_2\underline{CH_2})_2\underline{CH_2}$); 1.47 (s, 9H, Boc); 2.36-2.50 (m, 4H, $N(\underline{CH_2}CH_2)_2CH_2$); 2.79 and 3.32 (system AB, *J* = 13.71 Hz, 2H, $\underline{CH_2}$Ph); 3.08 and 3.49 (system AB, *J* = 13.48 Hz, 2H, $\underline{CH_2}$N); 3.75(s, 3H, $OCH_3$); 5.67 (s, 1H, NH); 7.04-7.08 (m, 2H, Ar); 7.20-7.26 (m, 3H, Ar).

**Boc-*(S)*-α-benzyl-β-(1-piperidinyl)AlaOMe ((*S*)-2c)**

Yield:95%, orange oil, $R_f$ = 0.50 (chloroform: methanol 9:1 *v/v*), $[\alpha]_D^{25}$ = -55.8 [*c* = 1, $CHCl_3$]

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.26-1.54 (m, 6H, $N(CH_2\underline{CH_2})_2\underline{CH_2}$); 1.47 (s, 9H, Boc); 2.36-2.50 (m, 4H, $N(\underline{CH_2}CH_2)_2CH_2$); 2.71 and 3.37 (system AB, 2H, *J* = 13.71 Hz, $\underline{CH_2}$Ph); 3.08 and 3.49 (system AB, 2H, *J* = 13.25 Hz, $\underline{CH_2}$N); 3.75(s, 3H, $OCH_3$); 5.59 (s, 1H, NH); 7.04-7.08 (m, 2H, Ar); 7.19-7.24 (m, 3H, Ar).

***N*-Boc-*(R)*-α-benzyl-β-(4-morpholinyl)AlaOMe ((*R*)-2d)**

Yield: 78%, orange oil, $R_f$ = 0.60 (ethyl acetate: hexane 1:1 *v/v*), $[\alpha]_D^{25}$ = 48.5 [*c* = 1, $CHCl_3$]

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.46 (s, 9H, Boc); 2.41-2.49 and 2.54-2.62 (2m, 4H, $N(C\underline{H}_2CH_2)_2$°); 2.76 and 3.42 (system AB, *J* = 13.71 Hz, 2H, $C\underline{H}_2Ph$); 3.02 and 3.53 (system AB, *J* = 13.48Hz, 2H, $CH_2N$); 3.59-3.63 (m, 4H, $N(CH_2C\underline{H}_2)_2$°); 3.76 (s, 3H, $OCH_3$); 5.56 (s, 1H, NH); 7.02-7.06 (m, 2H, Ar); 7.20-7.25 (m, 3H, Ar).

***N*-Boc-*(S)*-α-benzylo-β-(4-morpholinyl)AlaOMe ((*S*)-2d)**

Yield: 86%, orange oil, $R_f$ = 0.60 (ethyl acetate: hexane 1:1 *v/v*), $[\alpha]_D^{25}$ = -48.9 [*c* = 1, $CHCl_3$]

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.46 (s, 9H, Boc); 2.41-2.49 and 2.54-2.62 (2m, 4H, $N(C\underline{H}_2CH_2)_2$); 2.76 and 3.42 (system AB, *J* = 13.71 Hz, 2H, $C\underline{H}_2Ph$); 3.02 and 3.53 (system AB, *J* = 13.25Hz, 2H, $CH_2N$); 3.58-3.62 (m, 4H, $N(CH_2C\underline{H}_2)_2$°); 3.76 (s, 3H, $OCH_3$); 5.56 (s, 1H, NH); 7.02-7.06 (m, 2H, Ar); 7.20-7.25 (m, 3H, Ar).

***N*-Boc-D-Ala-*(R)*-α-benzyl-β-(azido)AlaOMe ((*R*)-3a)**

Yield: 99%, colorless oil, $R_f$ = 0.60 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.33 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.41 (s, 9H, Boc); 3.00 and 3.58 (system AB, *J* = 13.25 Hz, 2H, $C\underline{H}_2Ph$); 3.68 and 4.44 (system AB, *J* = 12.33 Hz, 2H, $CH_2N$); 3.78 (s, 3H, $OCH_3$); 4.13-4.27 (m, 1H, CH); 4.96 (broad s, 1H, NH); 6.98-7.02 (m, 2H, Ar) and 7.22-7.27 (m, 3H, Ar).

***N*-Boc-D-Ala-*(S)*-α-benzyl-β-(azido)AlaOMe ((*S*)-3a)**

Yield: 95%, colorless oil, $R_f$ = 0.58 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.36 (d, 3H, *J* = 7.08 Hz, $CH_3$); 1.44 (s, 9H, Boc); 3.00 and 3.59 (system AB, *J* = 13.48 Hz, 2H, $C\underline{H}_2Ph$); 3.73 and 4.40 (system AB, *J* = 12.33 Hz, 2H, $CH_2N$); 3.78 (s, 3H, $OCH_3$); 4.19 (qw, *J* = 7.08 Hz, 1H, CH); 4.92 (broad s, 1H, NH); 6.98-7.02 (m, 2H, Ar) and 7.22-7.28(m, 3H, Ar).

***N*-Boc-D-Ala-*(R)*-α-benzyl-β-(1-pyrrolidinyl)Ala Ome ((*R*)-3b)**

Yield: 70%, orange oil, $R_f$ = 0.67(chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.28 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.43 (s, 9H, Boc); 1.62-1.71 (m, 4H, $N(CH_2\underline{CH_2})_2$); 2.46-2.62 (m, 4H, $N(\underline{CH_2}CH_2)_2$); 3.00 and 3.51 (system AB, 2H, *J* = 13.14 Hz, $\underline{CH_2}Ph$); 3.20 and 3.60 (system AB, 2H *J* = 13.47, $\underline{CH_2}N$); 3.81 (s, 3H, $OCH_3$); 4.13 (qw, *J* = 7.08 Hz, 1H, CH); 5.00 (d, *J* = 7.08 Hz, 1H, NH); 7.00-7.04 (m, 2H, Ar); 7.19-7.26 (m, 3H, Ar).

***N*-Boc-D-Ala-*(S)*-α-benzylo-β-(1-pyrrolidinyl)AlaOMe ((*S*)-3b)**

Yield: 99%, orange oil, $R_f$ = 0.67(chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.33 (d, *J* = 7.07 Hz, 3H, $CH_3$); 1.43 (s, 9H, Boc); 1.69 (qw, *J* = 3.71 Hz, 4H, $N(CH_2\underline{CH_2})_2$); 2.56-2.69 (m, 4H, $N(\underline{CH_2}CH_2)_2$); 3.07 and 3.50 (system AB, *J* = 13.14 Hz, 2H, $\underline{CH_2}Ph$); 3.18 and 3, 60 (system AB, 2H, *J* = 13.47, $\underline{CH_2}N$); 3.78 (s, 3H, $OCH_3$); 4.15 (qw, *J* = 7.07 Hz,1H, CH); 5.01 (d, *J* = 7.07 Hz, 1H, NH); 7.00-7.04 (m, 2H, Ar); 7.18-7.23 (m, 3H, Ar).

***N*-Boc-D-Ala-*(R)*-α-benzylo-β-(1-piperidinyl)AlaOMe ((*R*)-3c)**

Yield: 72%, orange oil, $R_f$ = 0.55(chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.27 (d, *J* = 7.07 Hz, 3H, $CH_3$); 1.32-1.52 (m, 6H, $N(CH_2\underline{CH_2})_2\underline{CH_2}$); 1.43 (s, 9H, Boc); 2.34-2.44 (m, 4H, $N(\underline{CH_2}CH_2)_2CH_2$); 2.75 and 3.35 (system AB, 2H, *J* = 13.71 Hz, $\underline{CH_2}Ph$); 3.18 and 3.55 (system AB, 2H, *J* = 13.48 Hz, $\underline{CH_2}N$); 3.79 (s, 3H, $OCH_3$); 4.13 (qw, *J* = 7.07 Hz, 1H, CH); 5.01 (d, *J* = 7.07 Hz, 1H, NH); 7.00-7.04 (m, 2H, Ar); 7.18-7.23 (m, 3H, Ar).

***N*-Boc-D-Ala-*(S)*-α-benzylo-β-(1-piperidinyl)AlaOMe ((*S*)-3c)**

Yield: 80%, orange oil, $R_f$ = 0.50 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.33 (d, *J* = 7.07 Hz, 3H, $CH_3$); 1.37-1.50 (m, 6H, $N(CH_2\underline{CH_2})_2\underline{CH_2}$); 1.43 (s, 9H, Boc); 2.29-2.51 (m, 4H, $N(\underline{CH_2}CH_2)_2CH_2$); 2.75 and 3.42 (system AB, 2H, *J* = 13.81 Hz, $\underline{CH_2}Ph$); 3.11 and 3.59 (system AB, 2H, *J* = 13.47 Hz, $\underline{CH_2}N$); 3.78 (s, 3H, $OCH_3$); 4.10-4.16 (m, 1H, CH); 4.93-4.96(m, 1H, NH) 6.98-7.02 (m, 2H, Ar); 7.19-7.23 (m, 3H, Ar).

***N*-Boc-D-Ala-*(R)*-α-benzylo-β-(4-morpholinyl)AlaOMe ((*R*)-3d)**

Yield: 77%, orange oil, $R_f$ = 0.49 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.23 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.41 (s, 9H, Boc); 2.37-2.46 and 2.50-2.58 (2m, 4H, $N(C\underline{H_2}CH_2)_2$º); 2.79 (d, *J* = 13.93 Hz, 1H, HC<u>H</u>Ph); 3.08 (d, *J* = 13.71, Hz 1H, HC<u>H</u>N); 3.30-3.64 (m, 6H, $N(CH_2C\underline{H_2})_2$º, <u>H</u>CHN, <u>H</u>CHPh); 3.80 (s, 3H, $OCH_3$); 4.09 (qw, *J* = 7.08 Hz, 1H, CH); 4.80 (d, *J*=7.08 Hz, 1H, NH); 6.95-7.00(m, 2H, Ar); 7.20-7.24 (m, 3H, Ar).

***N*-Boc-D-Ala-*(S)*-α-benzylo-β-(4-morpholinyl)AlaOMe ((*S*)-3d)**

Yield: 98%, orange oil, $R_f$ = 0.48 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.32 (d, *J* = 7.07 Hz, 3H, $CH_3$); 1.42 (s, 9H, Boc); 2.38-2.46 and 2.51-2.61 (2m, 4H, $N(C\underline{H_2}CH_2)_2O$); 2.76 (d, *J* = 12.46 Hz, 1H) HC<u>H</u>Ph; 3.04 and 3.65 (system AB, *J* = 13.47 Hz, 2H, $CH_2N$); 3.56-3.60 (m, 5H, $N(CH_2C\underline{H_2})_2O$, <u>H</u>CHPh); 3.80 (s,

3H, $OCH_3$); 4.09 (qw, *J* = 7.07 Hz, 1H, CH); 4.80(d, *J* = 7.07 Hz, 1H, NH); 6.95-7.00 (m, 2H, Ar); 7.20-7.24 (m, 3H, Ar).

***N, O*-DiBocTyr-D-Ala-*(R)*-α-benzyl-β-(azido)AlaOMe ((*R*)-4a)**

Yield: 77%, colorless oil, $R_f$ = 0.65 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.22 (d, *J* = 6.85 Hz, 3H, $CH_3$); 1.44 (s, 9H, Boc); 1.59 (s, 9H, OBoc); 3.00-3.08 (m, 2H, $CH_2$ (Tyr) ); 3.08 and 3.54 (system AB, *J* = 13.48 Hz, 2H, C$H_2$Ph); 3.72 and 4.35 (system AB, *J* = 12.33 Hz, 2H, $CH_2N$); 3.82 (s, 3H, $OCH_3$); 4.26-4.46 (m, 2H, 2xCH (D-Ala, Tyr)), 4.99 (broad s, 1H, NH); 6.40 (d, *J* = 7.77 Hz, 1H, NH); 6.90-7.02 (m, 2H, Ar); 7.11-7.28 (m, 7H, Ar).

***N, O*-DiBocTyr-D-Ala-*(S)*-α-benzyl-β-(azido)AlaOMe ((*S*)-4a)**

Yield: 98%, colorless oil, $R_f$ = 0.62 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.20 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.40 (s, 9H, Boc); 1.55(s, 9H, OBoc); 2.88-3.03 (m, 2H, $CH_2$ (Tyr)); 3.05 and 3.51 (system AB, *J* = 13.48 Hz, 2H, C$H_2$Ph); 3.67 and 4.32 (system AB, *J* = 12.33 Hz, 2H, $CH_2N$); 3.79 (s, 3H, $OCH_3$); 4.22-4.48 (m, 2H, 2xCH (D-Ala, Tyr); 4.97 (broad s, 1H, NH); 6.37 (d, *J* = 7.77 Hz, 1H, NH); 6.95-6.99 (m, 2H, Ar); 7.07-7.26 (m, 7H, Ar).

***N, O*-DiBoc-Tyr-D-Ala-*(R)*-α-benzyl-β-(1-pyrrolidinyl)AlaOMe ((*R*)-4b)**

Yield: 76%, yellow oil, $R_f$ = 0.55 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.23 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.41 (s, 9H, Boc); 1.55 (s, 9H, OBoc); 1.62-1.71 (m, 4H, $N(CH_2CH_2)_2$); 2.41-2.57 (m, 4H, $N(CH_2CH_2)_2$); 2.88-3.19 (m, 4H, $CH_2$ (Phe, Tyr) ), 3.50-3.55 (m, 2H, $CH_2N$); 3.78 (s, 3H, $OCH_3$); 4.23-4.43 (m, 2H, 2xCH (D-Ala, Tyr) ); 6, 96-7.00 (m, 2H, Ar); 7.08-7.14 (m, 2H, Ar); 7.16-7.25 (m, 5H, Ar).

***N, O*-DiBoc-Tyr-D-Ala-*(S)*-α-benzyl-β-(1-pyrrolidinyl)AlaOMe ((*S*)-4b)**

Yield: 76%, yellow oil, $R_f$ = 0.50 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.23 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.40 (s, 9H, Boc); 1.55 (s, 9H, OBoc); 1.58-1.71 (m, 4H, $N(CH_2CH_2)_2$); 2.43-2.55 (m, 4H, $N(CH_2CH_2)_2$); 2.87-3.20 (m, 4H, $CH_2$ (Phe, Tyr) ); 3.50-3.55 and 3.68-3.70 (2m, 2H, $CH_2N$); 3.78 (s, 3H, $OCH_3$); 4.28-4.40 (m, 2H, 2xCH (D-Ala, Tyr)); 6.96-7.00 (m, 2H, Ar); 7.08-7.12 (m, 2H, Ar); 7.19-7.23 (m, 5H, Ar).

***N, O*-DiBoc-Tyr-D-Ala-*(R)*-α-benzyl-β-(1-piperidinyl)AlaOMe ((*R*)-4c)**

Yield: 78%, orange oil, $R_f$ = 0.54 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.21 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.25-1.46 (m, 6H, $N(CH_2CH_2)_2CH_2$); 1.41 (s, 9H, Boc); 1.55 (s, 9H, OBoc); 2.31-2.40 (m, 4H, $N(CH_2CH_2)_2CH_2$); 2.74 and 3.36 (system AB, 2H, *J* = 13.71 Hz, $CH_2$ (Phe)); 2.99-3.08 (m,

2H, $CH_2$ (Tyr)); 3.09 and 3.52 (system AB, 2H, *J* = 13.48 Hz, $\underline{CH_2}N$); 3.79 (s, 3H, $OCH_3$); 4.23-4.43 (m, 2H, 2xCH (D-Ala, Tyr)); 6,92-6.99 (m, 2H, Ar); 7.08-7.14 (m, 2H, Ar); 7.17-7.25 (m, 5H, Ar).

***N, O*-DiBoc-Tyr-D-Ala-*(S)*-α-benzyl-β-(1-piperidinyl)AlaOMe ((*S*)-4c)**

Yield: 77%, orange oil, $R_f$ = 0.52 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHZ, $CDCl_3$) δ: 1.20 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.23-1.39 (m, 6H, $N(CH_2\underline{CH_2})_2\underline{CH_2}$); 1.44 (s, 9H, Boc); 1.58 (s, 9H, OBoc); 2.33-2.49 (m, 4H, $N(\underline{CH_2}CH_2)_2CH_2$); 2.87-3.15 (m, 4H, 2x $\underline{CH_2}$ (Phe, Tyr)); 3.39 and 3.56 (system AB, 2H, *J* = 13.71 Hz, $\underline{CH_2}N$); 3.80 (s, 3H, $OCH)_3$; 4.29-4.46 (m, 2H, 2xCH (D-Ala, Tyr) ); 6, 97-7.03 (m, 2H, Ar), 7.11-7.16 (m, 2H, Ar); 7.21-7.26 (m, 5H, Ar )

***N, O*-DiBocTyr-D-Ala-*(R)*-α-benzyl-β-(4-morpholinyl)AlaOMe((*R*)-4d)**

Yield: 76%, orange oil, $R_f$ = 0.58 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.20 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.40 (s, 9H Boc); 1.55 (s, 9H, OBoc); 2.35-2.51 (m, 4H, $N(C\underline{H_2}CH_2)_2O$); 2.76 (d, *J* = 13.71 Hz, 1H, HC<u>H</u>Ph (Phe) ); 2.96-3.04(m, 2H, $CH_2$ (Tyr)), 3.10 (d, *J* = 13.48 Hz, 1H, HC<u>H</u>N); 3.43-3.58(m, 6H, $N(CH_2C\underline{H_2})_2O$, <u>H</u>CHN, <u>H</u>CHPh); 3.80 (s, 3H, $OCH_3$); 4.21-4.38(m, 2H, 2xCH(D-Ala, Tyr)); 5.05 (broad s, 1H, NH); 6.34 (d, *J* = 7.77 Hz, 1H, NH); 6.93-6.98(m, 2H, Ar); 7.08-7.12 (m, 2H, Ar); 7.17-7.24 (m, 5H, Ar)

***N, O*-DiBocTyr-D-Ala-*(S)*-α-benzyl-β-(4-morpholinyl)AlaOMe((*S*)-4d)**

Yield: 85%, orange oil, $R_f$ = 0.60 (chloroform: methanol 9:1 *v/v*)

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.20 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.41 (s, 9H) Boc; 1.55 (s, 9H) OBoc; 2.37-2.55 (m, 4H) $N(C\underline{H_2}CH_2)_2O$; 2.78 (d, *J* = 13.71 Hz, 1H) HC<u>H</u>Ph (Phe); 2.96-3.01(m, 2H) $CH_2$ (Tyr), 3.05 (d, *J* = 13.48 Hz, 1H, HC<u>H</u>N); 3.49-3.59(m, 6H, $N(CH_2C\underline{H_2})_2O$, <u>H</u>CHN, <u>H</u>CHPh); 3.78 (s, 3H) $OCH_3$; 4.24-4.41(m, 2H, 2*CH, D-Ala, Tyr); 4.84-5.05 (m, 1H, NH); 6.21 (d, 1H, NH); 6.92-6.96(m, 2H, Ar); 7.08-7.12 (m, 2H, Ar); 7.19-7.23 (m, 5H, Ar)

***N*-BocTyr-D-Ala-(*R*)-α-benzyl-β-(azido)AlaOH ((*R*)-5a)**

Yield: quantitative, amorphous white solid, $R_f$ = 0.42 (chloroform: methanol 8:2 *v/v*)

**$^1$H NMR** (250 MHz, DMSO) δ: 1.14 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.29 (s, 9H, Boc); 2.57-2.70 (m, 2H, $CH_2$ (Tyr)); 2.75-2.80 and 2.95-3.02 (2m, 2H, $C\underline{H_2}$Ph); 3.21-3.26 and 3.72-3.80 (2m,

2H, $CH_2N$); 4.08-4.10 and 4.36-4.45 (2m, 2H, 2xCH (D-Ala, Tyr)); 6.61-6.66 (m, 2H, Ar) 6.69-7.17 (m, 4H, Ar); 7.19-7.28 (m, 3H, Ar); 9.14 (s, 1H, COOH).

**_N_-BocTyr-D-Ala-_(S)_-α-benzylo-β-(azido)AlaOH ((_S_)-5a)**

Yield: quantitative, amorphous white solid, $R_f$ = 0.40 (chloroform: methanol 8:2 *v/v*)

**$^1$H NMR** (250 MHz, DMSO) δ: 1.14 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.29 (s, 9H, Boc); 2.57-2.70 (m, 2H, $CH_2$ (Tyr)); 2.75-2.80 and 2.95-3.02 (2m, 2H, $C\underline{H_2}Ph$); 3.21-3.26 and 3.72-3.80 (2m, 2H, $CH_2N$); 4.08-4.10 and 4.36-4.45 (2m, 2H, 2xCH (D-Ala, Tyr)); 6.61-6.66 (m, 2H, Ar) 6.69-7.17 (m, 4H, Ar); 7.19-7.28 (m, 3H, Ar); 9.14 (s, 1H) COOH.

**_N_-BocTyr-D-Ala-_(R)_-α-benzylo-β-(1-pirolidynyl)AlaOH ((_R_)-5b)**

Yield: 79%, amorphous white solid, $R_f$ = 0.44 (chloroform: methanol 8:2 *v/v*)

**$^1$H NMR** (250 MHZ, DMSO) δ: 1.10 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.28 (s, 9H, Boc); 1.84-1.96 (m, 4H, $N(CH_2\underline{CH_2})_2$); 2.39-2.74 (m, 4H, $N(\underline{CH_2}CH_2)_2$); 3.02-3.20 (m, 4H, 2x$\underline{CH_2}$ (Phe, Tyr)); 3.63 and 3.85 (2d, *J*=13.48 Hz, 2H, $\underline{CH_2}N$), 4.04-4.20 (m, 2H, 2xCH(D-Ala, Tyr)); 6, 60-6.63 (m, 2H, Ar); 6.98-7.04 (m, 4H, Ar); 7.18-7.24(m, 3H, Ar); 9.16 (s, 1H, COOH).

**_N_-BocTyr-D-Ala-_(S)_-α-benzyl-β-(1-pyrrolidinyl)AlaOH ((_S_)-5b)**

Yield: 81%, amorphous white solid, $R_f$ = 0.45 (chloroform: methanol 8:2 *v/v*)

**$^1$H NMR** (250 MHZ, DMSO) δ: 1.09 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.27 (s, 9H, Boc); 1.84-1.96 (m, 4H, $N(CH_2\underline{CH_2})_2$); 2.40-2.74 (m, 4H, $N(\underline{CH_2}CH_2)_2$); 3.05-3.20 (m, 4H, 2x$\underline{CH_2}$ (Phe, Tyr); 3.63 and 3.85 (2d, *J*=13.48 Hz, 2H, $\underline{CH_2}N$); 4.05-4.20 (m, 2H, 2xCH(D-Ala, Tyr ); 6, 60-6.63 (m, 2H, Ar); 6.98-7.04 (m, 4H, Ar); 7.18-7.24(m, 3H, Ar); 9.16 (s, 1H, COOH).

**_N_-Boc-Tyr-D-Ala-_(R)_-α-benzyl-β-(1-piperidinyl)AlaOH ((_R_)-5c)**

Yield: 84%, amorphous white solid, $R_f$ = 0.57 (chloroform: methanol 8:2 *v/v*)

**$^1$H NMR** (250 MHZ, DMSO) δ: 1.03 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.27 (s, 9H, Boc); 1.34-1.44 (m, 2H, $N(CH_2CH_2)_2\underline{CH_2}$); 1.51-1.64 (m, 4H, $N(CH_2\underline{CH_2})_2CH_2$); 2.66-2.99 (m, 8H, $N(\underline{CH_2}CH_2)_2CH_2$; 2x$\underline{CH_2}$ (Phe, Tyr)); 3.11-3.14 (m, 2H, $\underline{CH_2}N$); 4.11-4.03 (m, 2H, 2xCH (D-Ala, Tyr)); 6.59-6.62 (m, 2H, Ar), 6.96-7.03 (m, 4H, Ar); 7.11-7.17 (m, 3H, Ar), 9.13 (s, 1H, COOH).

**_N_-Boc-Tyr-D-Ala-_(S)_-α-benzyl-β-(1-piperidinyl)AlaOH ((_S_)-5c)**

Yield: 77%, amorphous white solid, $R_f$ = 0.59 (chloroform: methanol 8:2 *v/v*)

**$^1$H NMR** (250 MHZ, DMSO) δ: 1.16 (d *J* = 7.08 Hz, 3H, $CH_3$); 1.28 (s, 9H, Boc); 1.36-1.48 (m, 2H, $N(CH_2CH_2)_2\underline{CH_2}$); 1.52-1.68 (m, 4H, $N(CH_2\underline{CH_2})_2CH_2$); 2.56-3.09 (m, 8H, $N(\underline{CH_2}CH_2)_2CH_2$), 2x$\underline{CH_2}$ (Phe, Tyr)); 3.12-3.17 (m, 2H, $\underline{CH_2}N$); 4.04-4.19 (m, 2H, 2xCH (D-

Ala, Tyr)); 6, 91-6.97 (m, 2H, Ar), 7.01-7.04 (m, 2H, Ar); 7.09-7.29 (m, 5H, Ar), 9.17 (s, 1H, COOH).

**BocTyr-D-Ala-*(R)*-α-benzyl-β-(4-morpholinyl)AlaOH (*(R)*-5d)**

Yield: 90%, amorphous white solid, $R_f$ = 0.44 (chloroform: methanol 8:2 *v/v*)

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.17 (d, *J* = 7.08 Hz, 3H, $CH_3$); 1.30 (s, 9H, Boc); 2.35-2.51 (m, 4H, $N(C\underline{H}_2CH_2)_2O$); 2.96-3.04 (m, 2H, $CH_2$(Tyr)); 3.09-3.70 (m, 8 H, $\underline{CH}_2$Ph, $N(CH_2C\underline{H}_2)_2O$, $C\underline{H}_2N$); 4.08-4.29(m, 2H, 2xCH(D-Ala, Tyr)); 6.62-6.66(m, 2H, Ar); 7.00-7.06 (m, 4H, Ar); 7.23-7.31 (m, 3H, Ar), 9.21 (s, 1H, COOH).

***N*-BocTyr-D-Ala-*(S)*-α-benzylo-β-(4-morpholinyl)AlaOH (*(S)*-5d)**

Yield: 94%, amorphous white solid, $R_f$ = 0.45 (chloroform: methanol 8:2 *v/v*)

**$^1$H NMR** (250 MHz, $CDCl_3$) δ: 1.12 (d, *J* = 7.08 Hz, 3H) $CH_3$; 1.30 (s, 9H, Boc); 2.57-2.79 (m, 4H, $N(C\underline{H}_2CH_2)_2O$); 2.96-3.02 (m, 2H, $CH_2$(Tyr)), 3.09-3.63 (m, 8H, $\underline{CH}_2$Ph, $N(CH_2C\underline{H}_2)_2O$, $C\underline{H}_2N$;)); 4.08-4.29(m, 2H, 2xCH (D-Ala, Tyr)); 6.98-7.07 (m, 4H, Ar); 7.15-7.29 (m, 5H, Ar), 9.20 (s, 1H, COOH).

[a] gradient 10- 50%B over A in 20 min.

Table 2. Structures and the physicochemical properties of the deltorphin I analogues **I-VIII**

| Peptide | MW [g/mol] | $[M+H]^+$ | HPLC[a] Purity% | $t_R$ |
|---|---|---|---|---|
| Tyr-D-Ala-(*S*)-α-benzyl-β-azidoAla-Asp-Val-Val-Gly-$NH_2$ **I** | 823.9 | 823.9 | 99 | 16.4 |
| Tyr-D-Ala-(*R*)-α-benzyl-β-azidoAla-Asp-Val-Val-Gly-$NH_2$ **II** | 823.9 | 824.5 | 99 | 15.0 |
| Tyr-D-Ala-(*S*)-α-benzyl-β-(1-pyrrolidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **III** | 852.0 | 853.0 | 99 | 11.3 |
| Tyr-D-Ala-(*R*)-α-benzyl-β-(1-pyrrolidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **IV** | 852.0 | 852.5 | 99 | 9.9 |
| Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **V** | 866.0 | 867.1 | 97 | 10.4 |
| Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **VI** | 866.0 | 867.0 | 97 | 10.5 |
| Tyr-D-Ala-*(S)*-α-benzyl-β-(4-morpholinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **VII** | 868.0 | 869.0 | 97 | 9.2 |
| Tyr-D-Ala-*(R)*-α-benzyl-β-(4-morpholinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **VIII** | 868.0 | 869.0 | 99 | 9.9 |

Table 3. $^1$H chemical shifts (in ppm) of **V** and **VI** (AA=(*S*) or (*R*)-α-benzyl-β-(1-piperidinyl)alanine)

| Amino acid | | HN | Hα | Hβ | Hγ | Hδ | Hε | Hζ |
|---|---|---|---|---|---|---|---|---|
| Tyr1 | (*S*) | n.a. | 4.095 | 3.031 3.168 | --- | 7.112 | 6.881 | --- |
| | (*R*) | n.a. | 4.111 | 3.080 3.152 | --- | 7.146 | 6.882 | --- |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ala2 | (*S*) | 8.475 | 4.172 | 1.212 | --- | --- | --- | --- |
| | (*R*) | 8.455 | 4.322 | 1.189 | | | | |
| AA3 | (*S*) | 8.825 | benzyl | 3.165 3.398 | --- | 7.108 | 7.341 | 7.343 |
| | | | piperidinyl | 3.565 3.660 | --- | δ 2.998 3.034 | ε 1.531 1.675 | 1.405 1.691 |
| | | | benzyl | | | δ' 3.394 3.562 | ε' 1.834 | |
| | | 9.022 | piperidinyl | 3.156 3.342 | --- | 7.139 | 7.361 | n.a. |
| | (*R*) | | | 3.635 | --- | δ 3.030 3.544 | ε 1.611 | 1.692 |
| | | | | | | δ' 3.107 3.319 | ε' 1.865 | |
| As4 | (*S*) | 8.606 | 4.775 | 2.788 2.869 | --- | --- | --- | --- |
| | (*R*) | 8.305 | 4.598 | 2.729 | --- | --- | --- | --- |
| Val5 | (*S*) | 8.430 | 4.137 | 2.056 | 0.916 | --- | --- | --- |
| | (*R*) | 8.128 | 4.083 | 2.070 | 0.858 0.899 | --- | --- | --- |
| Val6 | (*S*) | 8.292 | 4.063 | 2.037 | 0.929 | --- | --- | --- |
| | (*R*) | 8.171 | 4.048 | 2.024 | 0.906 | --- | --- | --- |
| Gly | (*S*) | 8.490 | 3.864 3.916 | --- | --- | --- | --- | --- |
| 7 | | 8.448 | 3.856 3.905 | | | | | |
| | (*R*) | | | | | | | |
| C- | (*S*) | 7.038 | --- | --- | --- | --- | --- | --- |
| term | (*R*) | 7.424 | | | | | | |
| | | 7.029 | | | | | | |
| | | 7.426 | | | | | | |

n.a. - not assigned, AA=α-benzyl-β-(1-piperidinyl)Ala

Table 4. $^{13}$C chemical shifts (in ppm) of **V** and **VI** (AA=(*S*) or (*R*)-α-benzyl-β-(1-piperidinyl)alanine)

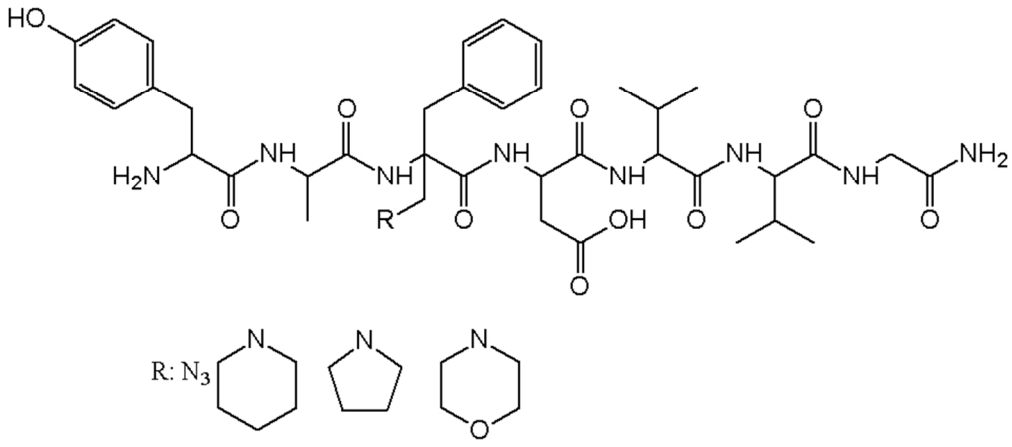
| Amino acid | | Cα | Cβ | Cγ | Cδ | Cε | Cζ |
|---|---|---|---|---|---|---|---|
| Tyr1 | (*S*) | 57.28 | 39.00 | n.a. | 133.37 | 118.67 | n.a. |
| | (*R*) | 57.42 | 39.02 | n.a. | 133.49 | 118.71 | n.a. |
| Ala2 | (*S*) | 52.57 | 18.52 | --- | --- | --- | --- |
| | (*R*) | 52.43 | 18.49 | --- | --- | --- | --- |
| AA3 | (*S*) | benzyl | 44.01 | n.a. | 132.83 | 131.61 | 131.19 |
| | | piperydynyl | 64.98 | --- | δ 59.71 δ' 59.74 | ε 26.17 ε' 26.17 | 23.30 |
| | (*R*) | benzyl | 44.37 | n.a. | 132.88 | 131.51 | n.a. |
| | | piperydynyl | 65.51 | --- | δ 59.33 δ' 60.34 | ε 26.15 ε' 26.19 | 23.20 |
| Asp4 | (*S*) | n.a. | 39.02 | --- | --- | --- | --- |
| | (*R*) | n.a. | 38.70 | --- | --- | --- | --- |
| Val5 | (*S*) | 62.70 | 32.73 | 21.03 | --- | --- | --- |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | (*R*) | 62.49 | 32.66 | 20.88 21.08 | --- | --- | --- |
| Val6 | (*S*) | 62.70 | 32.70 | 20.70 | --- | --- | --- |
| | (*R*) | 62.65 | 32.70 | 20.68 | --- | --- | --- |
| Gly7 | (*S*) | 44.86 | --- | --- | --- | --- | --- |
| | (*R*) | 44.82 | --- | --- | --- | --- | --- |

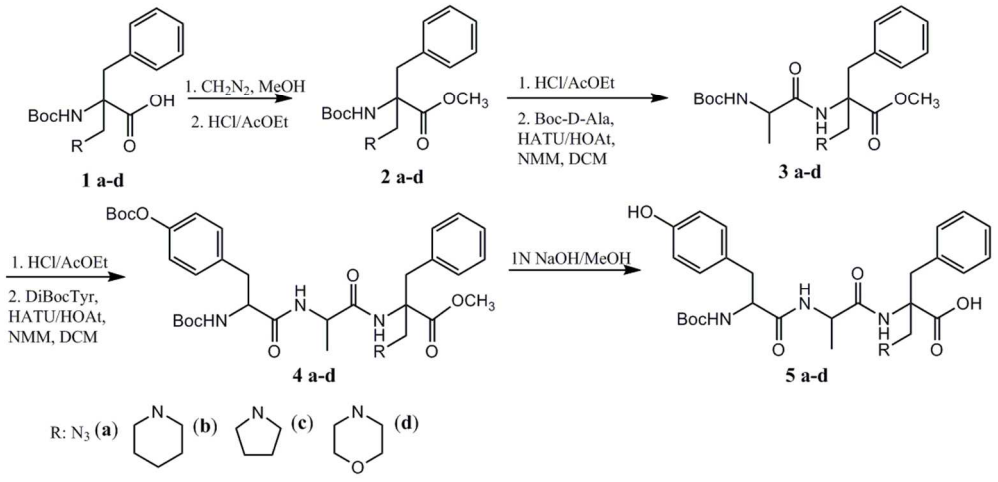
n.a. - not assigned, AA=α-benzyl-β-(1-piperidinyl)Ala

Table 5. $^{15}$N chemical shifts (in ppm) of **V** and **VI** (AA=(*S*) or (*R*)-α-benzyl-β-(1-piperidinyl)alanine)

| Amino acid | | N |
|---|---|---|
| Ala2 | (*S*) | 127.77 |
| | (*R*) | 127.91 |
| AA3 | (*S*) | 142.54 |
| | R | 126.47 |
| Asp4 | (*S*) | 116.20 |
| | (*R*) | 116.85 |
| Val5 | (*S*) | 122.31 |
| | (*R*) | 123.18 |
| Val6 | (*S*) | 125.00 |
| | (*R*) | 124.38 |
| Gly7 | (*S*) | 114.21 |
| | (*R*) | 113.98 |
| C-term | (*S*) | 107.18 |
| | (*R*) | 107.18 |




80x35mm (300 x 300 DPI)




104x50mm (300 x 300 DPI)

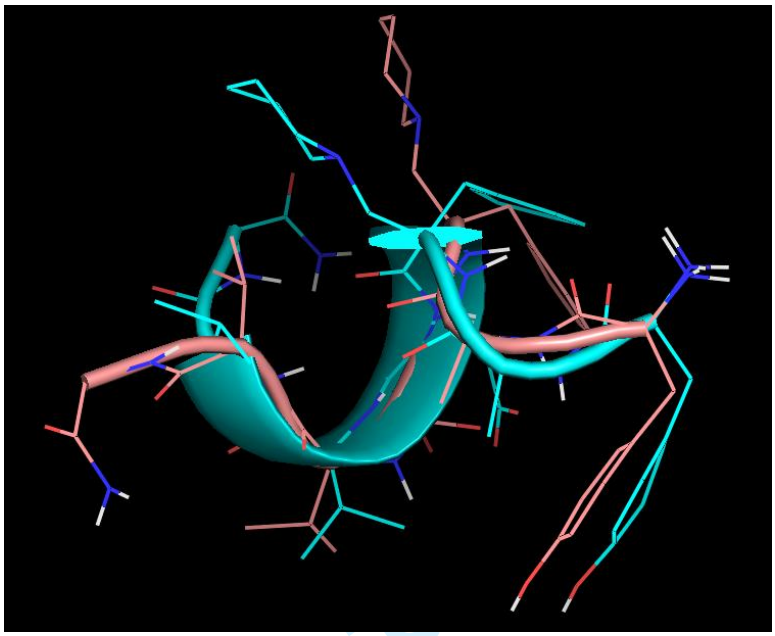


Figure 2. Representative structures of two most populated clusters of the Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (**VI**). Pink and blue structures have populations 0.269 and 0.264, respectively. The *C*-terminus of blue structure forms helix stabilized by interaction of piperidynyl with Val-6.

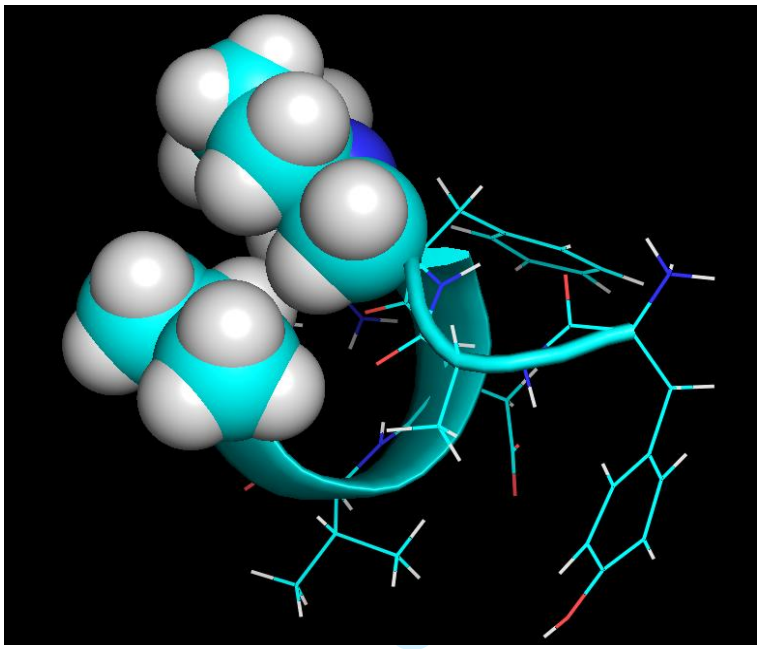


Figure 3. Hydrophobic contact formed by piperidynyl and Val$^{6}$ residue in Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (**VI**)

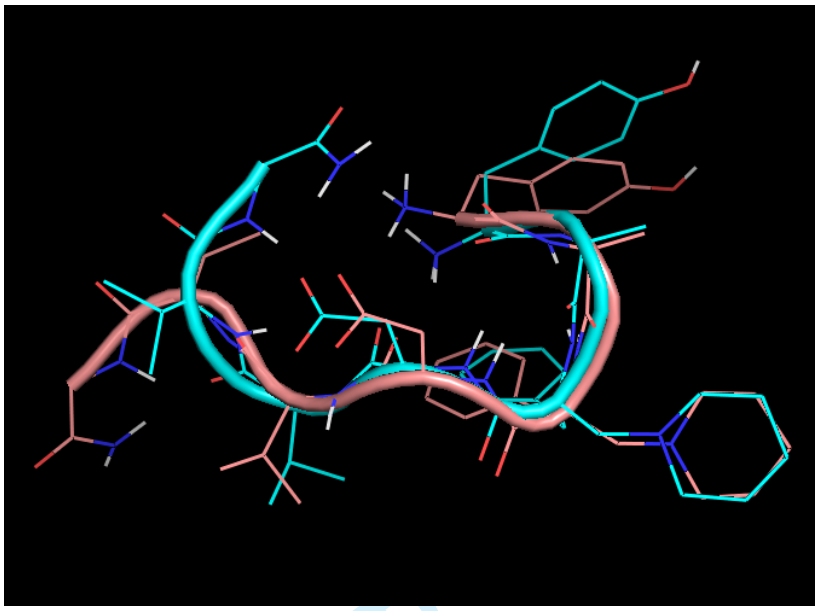


Figure 4. Representative structures of the most populated (blue) and the least populated (pink) clusters of Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ (**V**). The peptide lacks helical conformation observed for peptide **VI**.

# Synthesis, biological activity and NMR-based structural studies of deltorphin I analogues modified in message domain with a new α,α-disubstituted glycines†

Anika Lasota[a], Oliwia Frączak[a], Adriana Muchowska[b], Michał Nowakowski[c], Maciej Maciejczyk[d], Andrzej Ejchart[e], Aleksandra Olma[a*]

**Table 1**. Binding affinities of deltorphin analogues **I-VIII** to δ and μ opioid receptors

| Peptide | $IC_{50}$ (nM) | | |
|---|---|---|---|
| | μ[a] | δ[b] | select. |
| Tyr-D-Ala-Phe-Asp-Val-Val-Gly-$NH_2$ (DTI) (34) | 976±148 | 3.05±0.10[c] | 320 |
| Tyr-D-Ala-(*S*)-α-benzyl-β-azidoAla-Asp-Val-Val-Gly-$NH_2$ **I** | 2473±113 | 655±108 | 3.77 |
| Tyr-D-Ala-(*R*)-α-benzyl-β-azidoAla-Asp-Val-Val-Gly-$NH_2$ **II** | 1272±55.5 | 8.8±1.0 | 144 |
| Tyr-D-Ala-(*S*)-α-benzyl-(1-pyrrolidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **III** | 1793±54.7 | 3178±430 | 0.56 |
| Tyr-D-Ala-(*R*)-α-benzyl-β-(1-pyrrolidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **IV** | 419±24.31 | 378.7±25.1 | 1.11 |
| Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **V** | 2876±99.5 | 15.0± 1.2 | 192 |
| Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **VI** | 88±3.1 | 669±53.5 | 0.13 |
| Tyr-D-Ala-*(S)*-α-benzyl-β-(4-morpholinyl)Ala-Asp-Val-Val-Gly-$NH_2$ **VII** | 3907± 231 | 2205±166 | 1.77 |
| Tyr-D-Ala-*(R)*-α-benzyl-β-(4-morpholinyl)Ala-Asp-Val-Val-Gly-$NH_2$[b] **VIII** | 2624±116 | 1373±137 | 1.91 |

[a]*versus* [$^3$H]DAMGO, [b]*veresus* [$^3$H]DELT, [c]*versus* [$^3$H]DPDPE