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Research Article

Synthesis, Biological Activity, and NMR-Based Structural Studies of Deltorphin I Analogs Modified in Message Domain with a New α,α-Disubstituted Glycines†

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†This article 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.

This article describes new deltorphin I analogs in which phenylalanine residues were replaced by the corresponding (*R*) or (*S*)-α-benzyl-β-azidoalanine, α-benzyl-β-(1-pyrrolidinyl)alanine, α-benzyl-β-(1-piperidinyl)alanine, and α-benzyl-β-(4-morpholinyl)-alanine residues. The potency and selectivity of the new analogs were evaluated by a competitive receptor binding assay in the rat brain using [^{3}H]DAMGO (a μ ligand) and [^{3}H]DELT (a δ ligand). The affinity of analogs 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 displays 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.

Key words: deltorphin I analogues, NMR-based studies, opioid activities, α,α-disubstituted glycines

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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 analogs 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 analogs of the various deltorphins have been synthesized (9–14).

It has been proposed that the δ-receptor selectivity of deltorphins is a result of the formation of special non-equal 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

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(Asp[4])...$CONH_2$ (Gly[7]) is surrounded by dominating lipophilic shells ('hot-dog roll'). Some very potent and selective cyclic analogs, which stabilized this conformation, have supported the proposed model (15). The incorporation amphiphilic amino acid residues (α-hydroxymethylphenylalanine) also support proposed molecular model of the active conformation of deltorphins (16).

The presented paper describes the synthesis and receptor binding of new analogs in which Phe[3] residue in the deltorphin I sequence was substituted by (*R*) or (*S*) α-benzyl-β-azidoalanine, α-benzyl-(1-pyrrolidinyl)alanine, α-benzyl-β-(1-piperidinyl)alanine, and α-benzyl-β-(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.

Optically, pure α,α-disubstituted glycines were obtained from available *N*-Boc-(*R* or *S*)-α-benzylserine β-lactone. 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 (17,18).

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 (19).

Experimental Section

Chemistry

Most chemicals were purchased from Sigma-Aldrich (Poznań, Poland) 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 (17,18). 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. (Union City, USA, CA). All other reagents and solvents were of analytical or HPLC grade and were bought from Sigma-Aldrich 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, δ: 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

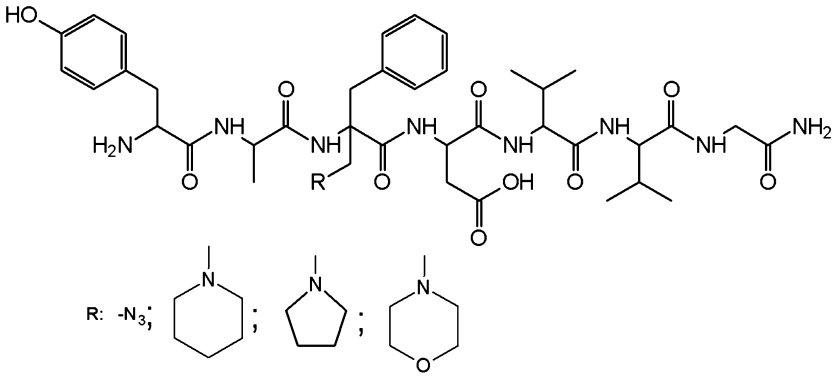


Figure 1: Structure of new deltorphin I analogs.

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, and *N*-Boc-(*R* or *S*)-α-benzyl-β-(*sec*-amino)AlaOMe was 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 h, next portion of 7 mL HCl in AcOEt was added and the stirring was continued for 2–4 h. 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 h, 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) was 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 (3–6 h, 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 (3 × 10 mL) and acidified with 1N $NaHSO_4$ to pH≈2–3 (analogs containing an azido group) or pH≈6–7 (analogs containing a *sec*-amino group). Then, aqueous layer was saturated with NaCl and extracted with ethyl acetate (3 × 15 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 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 threefold excess using TBTU as a coupling reagent in the presence of HOBt and DIPEA in DCM. In the case of a positive Kaiser test (20), 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 twofold 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/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. All heptapeptides were characterized by analytical RP-HPLC and molecular weight determination.

Receptor binding assay

Receptor binding assays were performed as described previously (16). Crude membrane fractions from rat brain were prepared as described by Misicka *et al.* (3) The radioreceptor binding protocol was based on a protocol elaborated by Fichna *et al.* (21) with some modifications. The modification included different incubation time (60 versus 120 min), bacitracin concentration (30 versus 50 μg/mL), and radioligand choice. The modifications were implemented 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

The NMR samples contained 5 mg of Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-NH_2 (**V**) or 4 mg of Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-NH_2 (**VI**) dissolved in 650 μL of 90:10 H_2O/D_2O (v/v).

All spectra were measured on an Agilent DDR2 spectrometer operating at 600 MHz resonance frequency (^1H), 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 (22). 2D Homonuclear TOCSY (23) (mixing time 80 ms), ROESY (24) (mixing time 300 ms), and heteronuclear $^1H/^{15}N$ HSQC (25) and $^1H/^{13}C$ HSQC (with the offset, spectral widths, and ^{13}C–^1H coupling constants tuned to either aliphatic or aromatic carbons) spectra were used to obtain assignments of the ^1H, ^{15}N, and ^{13}C resonances. Time domain data were acquired using States-TPPI quadrature detection (26). Water suppression was achieved with pulsed field gradients echo (27). All chemical shifts in ^1H 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 $^{13}C/^1H$ and $^{15}N/^1H$, respectively (28). Zero filling and a 90°-shifted squared sine-bell filter were performed prior to Fourier transformation. Processed spectra were analyzed with SPARKY software (29).

Calibration

Intensities of interproton correlations in ROESY spectra, I_{ij}, were used in determining appropriate distances r_{ij} from the equation $I_{ij} = C \cdot r_{ij}^{-6}$ (30). 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.

Parametrization of modified residues

For all natural amino acid residues, standard Amber ff10 force-field parameters were applied (31). 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 (32,33). Partial charges were determined by fitting them (RESP) to the electrostatic potential (34) obtained from quantum mechanical computations at the MP2/6–31G(d,p) level with Gaussian 03 package (35).

Simulated-annealing procedure

The peptide chain was built with XLEAP program (University of California, San Francisco, CA, USA) of the Amber package. To remove bad contacts, 1000 steps of geometry optimization were applied with steepest-descent energy minimization method. The chain was heated up from 10 to 1200K in 1 ps molecular dynamics run, followed by 2 ps of high-temperature dynamics and 12 ps cooling process. NMR distance restraints were slowly switched on during first 3 ps 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 1 fs, and generalized Born solvation model was applied (36–38). 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.

MD simulations with time-averaged restraints

The geometry of the initial structure was optimized and equilibrated in 1 ns MD run with time-averaged restraints applied. The SHAKE algorithm was used to keep covalent bonds with hydrogens constant and 2 fs time step

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

Peptide	IC_{50} (nM)		IC_{50} ratio
	μ^a	δ^b	μ/δ
Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 (DTI) (39)	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* [3H]DAMGO, [b]*versus* [3H]DELT, [c]*versus* [3H]DPDPE.

was applied. The solvation effects were described by generalized Born model (36–38). During 20 ns production, run proton–proton distance restraints obtained from NMR experiment were time-averaged over 1 ps 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 was chosen to minimize Davies–Bourdin index (DBI) and was equal to seven for Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-NH_2 (**V**) and five for Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-NH_2 (**VI**).

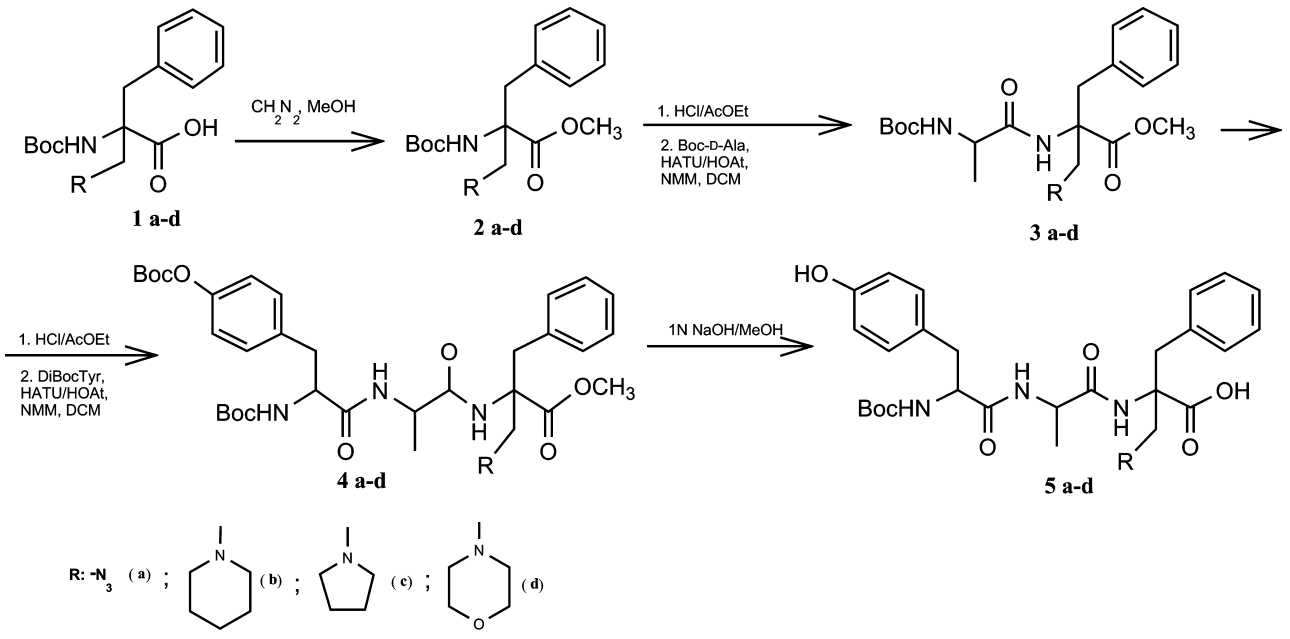
Results and Discussion

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 analogs 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 treating 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.

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 were performed with TFA:water (95:5 by vol). All crude analogs were purified to homogeneity by RP-HPLC, and their structures were verified by mass spectrometry.

The affinities of deltorphin I analogs for μ- and δ-receptors were determined by the radioligand binding assay described previously (15) using [^{3}H]-DAMGO and [^{3}H]-DELT as μ- and δ-receptor-specific ligands, respectively.



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

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

Phenylalanine in position 3 plays a key role in general interaction with receptors of both groups of analogs with common *N*-terminal parts, δ-selective deltorphins, and μ-selective dermorphin. The incorporation of D-Phe^3 in deltorphins resulted in significant decreases in affinity to δ-receptors and partial decrease of receptor selectivity (40). Nevertheless, topographical location of the Phe^3 phenyl ring is probably more critical than the chirality of amino acid in this position. Substitution of Phe^3 with very rigid Aic (tetrahydroisoquinoline-3-carboxylic) or Atc (*R* as well as *S*-2-aminoindan-2-carboxylic acid) increased affinity to both δ- and μ-receptors by almost 300 times, whether replacement Phe^3 with α-MePhe decreased affinity to δ-receptors over 100-fold for both (*R*) and (*S*)-isomers (41). Substitution of Phe^3 with amphiphilic α,α-disubstituted glycine ((*S*)-α-hydroxymethylphenylalanine) gave potent and α-selective ligand (16). Modification of the critical Phe^3 residue to vary steric, electronic, and lipophilic properties may help to determine what features are important for improving δ and μ binding affinities (42).

As reported in Table 1, the affinity of analogs 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 analog **II**, the delocalized charge of azidomethyl group in α-benzyl-β-azidoalanine may partially stabilize the proposed 'hot-dog' conformation (3). The incorporation of more amphiphilic amino acid residues (α-hydroxymethylphenylalanine) gave better stabilization of proposed molecular model of the active conformation of deltorphins (16). 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$^\alpha$ or topographical location of the Phe^3 phenyl ring. 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, analog **V** displays a 192-fold higher selectivity for δ-receptor, while analog **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 analogs **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 (30). 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.

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. These contacts seem to drive helix formation at the *C*-terminus.

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.

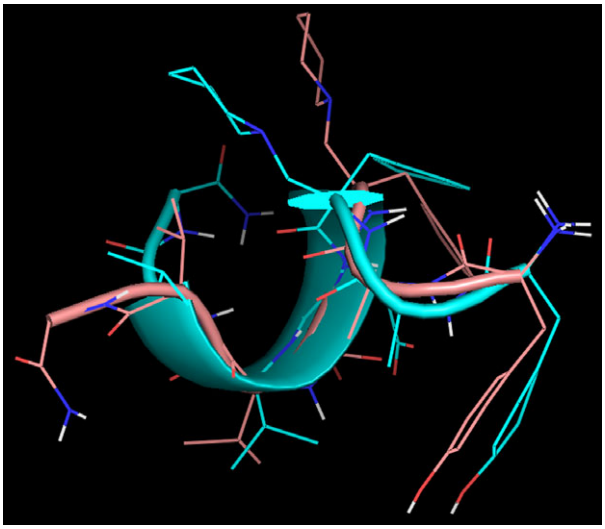


Figure 2: Representative structures of two most populated clusters of the Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-NH_2 (**VI**). Pink and blue structures have populations 0.269 and 0.264, respectively. The *C*-terminus of blue structure forms helix stabilized by interaction of piperidinyl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

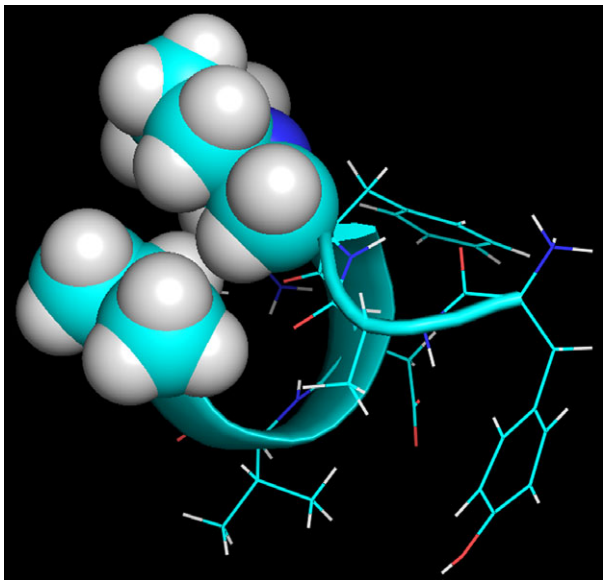


Figure 3: Hydrophobic contact formed by piperidinyl and Val[6] residue in Tyr-D-Ala-(*R*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-NH_2 (**VI**).

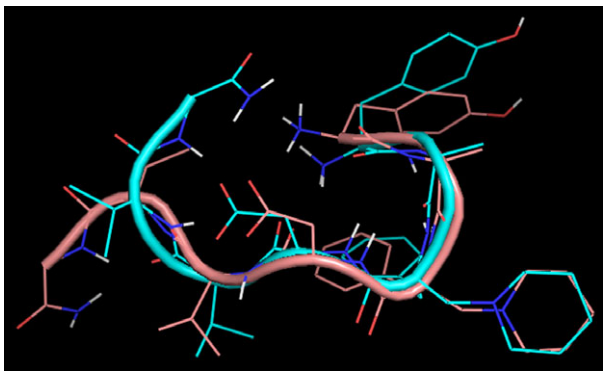


Figure 4: Representative structures of the most populated (blue) and the least populated (pink) clusters of Tyr-D-Ala-(*S*)-α-benzyl-β-(1-piperidinyl)Ala-Asp-Val-Val-Gly-NH_2 (**V**). The peptide lacks helical conformation observed for peptide **VI**.

Analog **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 (43). These two clusters are similar with a major conformational difference at the *C*-terminus. Our studies suggest that μ- or δ-selectivity appears to be forced by conformation adopted by the address domain. These results also support our earlier research on the DTI analogs modified with α-methyl-β-azidoalanine in position 2 (44). In the ROESY spectrum of highly δ-selective analog (Tyr-(*S*)-α-methyl-β-azidoAla-Phe-Asp-Val-Val-Gly-NH_2), only intraresidual and sequential (i/i + 1) correlations were observed, which suggested that the extended conformation was prevalent.

In conclusion, the binding assay showed that the replacement of phenylalanine with α-benzyl-β-azidoalanine, α-benzyl-β-(1-pyrrolidinyl)alanine, α-benzyl-β-(1-piperidinyl) alanine, and α-benzyl-β-(4-morpholinyl)alanine has a strong effect on binding affinity. Our result supports the proposal (45) 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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Conflict of Interest

The authors declare that they have no conflict of interest.

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Supporting Information

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

Appendix S1. Synthesis, biological activity and NMR-based structural studies of deltorphin I analogues modified in message domain with a new ,-disubstituted glycines.