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Synthesis and receptor binding of opioid peptide analogues containing β^3-*homo*-amino acids

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β-Amino acids containing hybrid peptides and β-peptides show great potential as peptidomimetics. In this paper we describe the synthesis and affinity toward the μ- and δ-opioid receptors of β-peptides, analogues of Leu-enkephalin, deltorphin I, dermorphin and α,β-hybrides, analogues of deltorphin I. Substitution of α-amino acid residues with β^3-*homo*-amino acid residues, in general resulted in decrease of affinity to opioid receptors. However, the incorporation β^3*h*-D-Ala in position 2 or β^3*h*Phe in position 3 of deltorphin I resulted in potent and selective ligand for δ-opioid receptor. The NMR studies of β-deltorphin I analogue suggest that conformational motions in the central part of the peptide backbone are partially restricted and some conformational preferences can be expected. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: β^3-*homo*-amino acids; β-peptides; α,β-hybrides of opioid peptides; opioid receptor binding; conformational studies

Introduction

Opioid peptides such as enkephalins [1], endomorphins [2], dermorphin [3] and deltorphins [4,5], have long acted as model compounds for the development of new analgesic drugs. A major problem with opioid peptides as drugs is their susceptibility to enzymatic hydrolysis when administrated *in vivo*. Several chemical approaches, such as the incorporation of D-amino acids, unnatural amino acids, α,α-disubstituted amino acids, cyclic moieties or cyclization of peptides have resulted in obtaining more stable analogues [6–8]. Among the numerous strategies of modification, the substitution of proteinogenic amino acids with β-amino acids represents an interesting possibility. β-Peptides, oligomers of β-amino acids [9,10] are a very actual subject of research. The additional carbon atom in each amino acid residue of β-peptides leads to greater structural diversity. Due to the different dimension, geometries and polarities of the β-peptides, their biological properties differ from those of α-peptides in those cases, where exact fitting is mandatory. β-Peptides do not bind to the active sites of human peptidases and they are entirely stable against proteolytic degradation [11,12]; however, β-peptides can mimic α-peptides. It was demonstrated that small β-peptides with their strong folding preferences have shown pharmacological activity [13]. It shows that β-peptides built of homologated proteinogenic amino acids have great potential in medicinal chemistry [14].

We now report the results of our studies on replacement of α-amino acids with β^3-*homo*-amino acids in the selected opioid peptides deltorphin 1 (DT 1), leu-enkephalin and dermorphin (DRM) (Scheme 1) and its effects on binding to δ- and μ-opioid receptors.

Materials and Methods

Reagents

Protected Boc- and Fmoc-α-amino acid derivatives were purchased from Fluka. AG (Bucks, Switzerland). The following side chain protected amino acids were used: Boc-Asp(OBzl), Boc-Tyr(OBzl) and FmocAsp(OtBu), Fmoc-Tyr(OtBu). *N*-protected β^3-*homo*-amino acids were synthesized using procedures reported in the literature. Optically pure Fmoc- and Boc-β^3-*homo*-amino acids were prepared in two-step *Arndt-Eistert* homologation of commercially available, *N*-protected amino acids (Scheme 2) according to a general procedure [15,16].

Preparation of *N*-protected β^3-*homo*-amino acids 10. General procedure

Synthesis of α-aminodiazo ketones 9. General procedure

The *N*-protected amino acid (10 mmol) was dissolved in anhydrous THF (25 ml) under argon. The solution was cooled to −25 °C, and triethylamine (1.4 ml, 10 mmol) and then ethyl chloroformate (1.3 ml, 10 mmol) were added through a rubber septum. After

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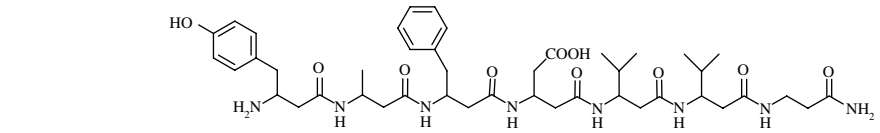
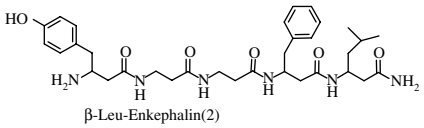
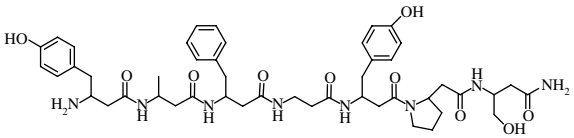


β-Deltorphin I (**1**)

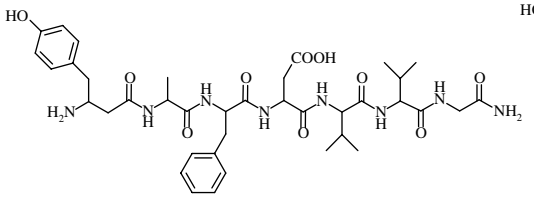


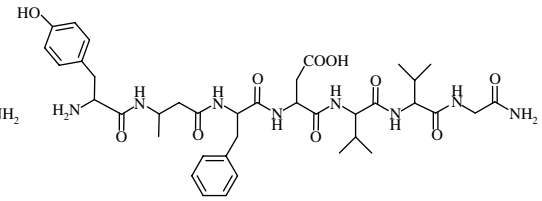
β-Leu-Enkephalin(2)



β-Leu-Enkephalin (**2**)

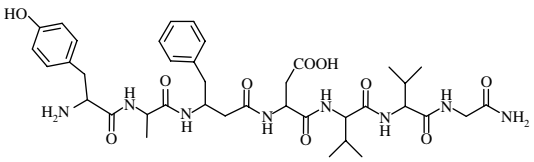
β-Dermorphin (**3**)

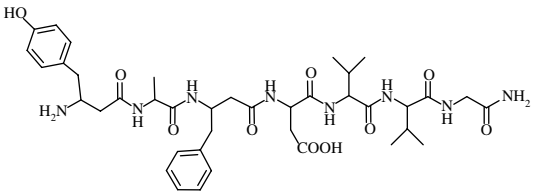




β^3*h*Tyr-D-Ala-Phe-Asp-Val-Val-GlyNH$_2$ (**4**)

Tyr- **β^3*h*-D-Ala**-Phe-Asp-Val-Val-GlyNH$_2$ (**5**)

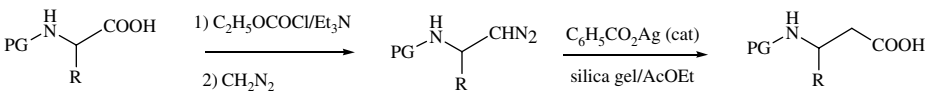




Tyr-D-Ala-**β^3*h*Phe**-Asp-Val-Val-GlyNH$_2$ (**6**)

β^3*h*Tyr-D-Ala-**β^3*h*Phe**-Asp-Val-Val-GlyNH$_2$ (**7**)

Scheme 1. Structure of β-peptides and α,β-hybrides of deltorphin I.



8 **9** **10**

PG=Boc, Fmoc

Scheme 2. Homologation of *N*-protected α – amino acids.

15 min, the suspension was allowed to warm to 0 °C, the septum was replaced by a funnel and an ethereal solution of diazomethane was added until intensive yellow color persisted over a long period (1 h). The mixture was allowed to warm for 1 h without stirring. The excess of diazomethane was destroyed by addition of few drops of acetic acid and saturated aqueous solution sodium bicarbonate (10 ml) was added carefully. The aqueous layer was separated and the organic layer was washed with saturated aqueous sodium chloride (10 ml). The organic layer was dried over $MgSO_4$, filtered off and the solvent was evaporated. The crude product was used directly in the next step.

Homologation of α-aminodiazo ketones. General procedure

N-protected α-aminodiazo ketone was dissolved in ethyl acetate (6 ml per 100 mg of α-aminodiazo ketone). Silver benzoate (4% mol) and silica gel (1g per 100 mg α-amino diazo ketone) were added, and the mixture (under the exclusion of light) was stirred for 30 min – 2 h at 45 °C (the rotary evaporator was used in this step, the reaction was monitored by TLC). The silica gel was filtered off and washed with AcOEt. The ethyl acetate was evaporated to yield *N*-protected β^3-*homo*-amino acids.

The yields and melting points of obtained *N*-protected β^3-*homo*-amino acids are shown in Table 1.

The β-peptides were synthesized by manual SPPS using standard techniques, for *N*-Boc-protected amino acids on 0.2 mM *p*-methylbenzhydrylamine (MBHA) resin × HCl (100 – 200 mesh, 1.05 mM/g, BACHEM, peptides **1**-**2**) or for *N*-Fmoc-protected amino acids on 0.2 mM Rink amide resin (100 – 200 mesh, 0.47 mM/g Novabiochem, peptides **3**-**7**). The *C*-terminal amino acid (3 equiv) was attached to the resin with TBTU (3 equiv) and HOBt (3 equiv) as coupling reagent in the presence of DIPEA (6 equiv) according to the usual protocol. Deprotection of *N*-Boc-protecting group was performed with 50% TFA in CH_2Cl_2. β-Peptides (Leu-ekephalin

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Table 1. Yields and melting points of *N*-protected β^3-*homo*-amino acids

N-protected β^3-*homo*-amino acid	Yield (%)		Mp °C	
	9	10	10	Lit.
Boc-β^3*h*-D-Ala	76	45	Oil	Oil [17]
Boc-β^3*h*Phe	93	99	104–107	96 [15]
Boc-β^3*h*Val	61	92	Oil	71–72 [18]
Boc-β^3*h*Leu	62	99	Oil	Oil [18]
Boc-β^3*h*Asp(OBzl)	96	93	96–99	–
Boc-β^3*h*Tyr(OBzl)	97	97	147–150	148–150 [19]
Fmoc-β^3*h*Ser(OtBu)	97	93	78–80	96–98 [20]
Fmoc-β^3*h*Tyr (OtBu)	96	66	105–110	120–121 [21]
Fmoc-β^3*h*Pro	78	50	175–177	191–192 [22]
Fmoc-β^3*h*-D-Ala	97	31	117–120	115–118 [22]
Fmoc-β^3*h*Phe	96	56	145–150	157–158 [20]

Table 2. Retention times, purity and molecular ions of β–peptides and α,β-hybrides of DT I

No	Peptide	HPLC		MS	
		t_R[min]	purity [%]	MW	$[M+H]^+$
1	β-DT I	15.34[a]	99.9	866	867[d]
2	β-DRM	12.08[b]	98.0	900	901[d]
3	β-Leu-ENK	5.11[a]	98.0	624	625[d]
4	β^3*h*Tyr-D-Ala-Phe-Asp-Val-Val-GlyNH$_2$	7.07[c]	>99%	782.9	783.2[e]
5	Tyr-β^3*h*-D-Ala-Phe-Asp-Val-Val-GlyNH$_2$	7.36[c]	>99%	782.9	783.3[e]
6	Tyr-D-Ala-β^3*h*Phe-Asp-Val-Val-GlyNH$_2$	7.06[c]	>99%	782.9	783.2[e]
7	β^3*h*Tyr-D-Ala-β^3*h*Phe-Asp-Val-Val-GlyNH$_2$	7.15[c]	>99%	796.93	797.6[e]

Linear gradient [a] 30–60%B, 20–80%B; [b] 25–30%B, 25 min flow rate 1 ml/min; [c] 20–80%B, 25 min; [d] FAB-MS; [e] MALDI-MS.

and deltorphin I analogues) were cleaved from the MBHA resin by the treatment with TFMSA in TFA for 2 h at room temperature (5 ml of TFA, 1.9 ml of TFMSA and 1 ml of anisole/g resin). For *N*-Fmoc-protecting group 20% piperidine in DMF was used. Analogues **3**-**7** were cleaved from Rink resin by treatment with TFA/H_2O/triisopropylsilane (TIS) (97.5 : 2.5 : 2.5 v : v : v). The crude peptides were purified by preparative reversed-phase HPLC on a Vydac C_{18} column (25 × 2.2 cm) with linear gradient 30–60% B or 0–90% B at 16 ml/min (A = 0.05% trifluoroacetic acid in water and B = 0.38% trifluoroacetic acid in acetonitrile/H_2O 90 : 10). Each peptide was >98% pure as determined by analytical reversed-phase HPLC on Vydac C_{18} column, 218 TP104) using linear gradient in 25 min at flow rate 1 ml/min, with UV detection at 220 nm (peptides **1**-**3**) and on Supelco C_{18} column 25 cm × 4.5 cm, with detection at 215 nm (peptides **4**-**7**). Molecular weights of all synthetic analogues were confirmed by FAB-MS or MALDI-MS (Table 2).

NMR experiments

About 650 µl NMR samples contained 0.015 mM peptide solutions in dmso-d_6 (Armar Chemicals, Döttingen, Switzerland). All spectra were measured on a VARIAN UNITY PLUS 500 MHz spectrometer at magnetic field of 11.7 T and temperature 298 K. Temperature calibration was carefully performed using ethylene glycol chemical shift thermometer [23]. ^{1}H and ^{13}C chemical shifts were reported with respect to the solvent signals: $\delta(^1H) = 2.54$ and $\delta(^{13}C) = 40.45$ [24]. Chemical shifts of ^{15}N signals were referenced indirectly using the ratio of the zero-point frequencies, $f(^{15}N)/f(^1H) = 0.101329118$ [25]. Homonuclear 2D DQF-COSY [26], TOCSY [27] and ROESY [28] spectra were acquired with 8400 (t_2) × 512 (t_1), 5400 (t_2) × 256 (t_1) and 6000 (t_2) × 512 (t_1) complex data points, respectively, and the sweep widths of 6000 Hz using 32 scans. Mixing times for TOCSY and ROESY were 80 ms and 300 ms, respectively. ^{1}H/^{13}C HSQC [29] spectrum was acquired with 3600 (t_2) × 128 (t_1) complex data points and the sweep widths of 5000 Hz in ^{1}H and 6000 Hz in ^{13}C dimensions. Sixty-four scans were acquired for each increment. Corresponding parameters for ^{1}H/^{15}N HSQC spectrum were: 2006 (t_2) × 96 (t_1) complex data points, sweep widths of 5000 Hz (^{1}H) and 5000 Hz (^{15}N), 96 scans for each increment. The recycle delays in all spectra were equal to 1.4 s. Zero filling was performed prior to the Fourier transformation. Data were processed using the program nmrPipe [30] and analyzed with the program SPARKY [31].

Table 3. Affinity data of β-peptides (**1**–**3**) and α,β-hybrides, analogues of DT I (**4**-**6**)

No	Peptides	IC_{50} [nM]	
		δ[a] ± SEM	μ ± SEM
	DT I [32]	0.6	2140[b]
1	β-DT I	640	>10 000[b]
	DRM [33]	192 ± 51	0.092 ± 0.024[c]
2	β-DRM	>10 000	>10 000[c]
	Leu-ENK [34]	1.43 ± 0.71	2.42 ± 0.93[c]
3	β-Leu-Enk	>10 000	>10 000[c]
4	β^3*h*Tyr-D-Ala-Phe-Asp-Val-Val-GlyNH$_2$	416 ± 5.7	>10 000[b]
5	Tyr-β^3*h*-D-Ala-Phe-Asp-Val-Val-GlyNH$_2$	12.3 ± 2.53	>10 000[b]
6	Tyr-D-Ala-β^3*h*Phe-Asp-Val-Val-GlyNH$_2$	10.47 ± 4.28	>10 000[b]
7	β^3*h*Tyr-D-Ala-β^3*h*Phe-Asp-Val-Val-GlyNH$_2$	>10 000	>10 000[b]

[a] *versus* [^{3}H]-deltorphin II; [b] *versus* [^{3}H]-naloxone; [c] *versus* [^{3}H]-DAMGO.

Results and Discussion

Affinities of the β-peptides, analogues of enkephalin, dermorphin and deltorphin I for μ- and δ–receptors were determined in radioreceptor binding assay method described previously using radioligands [^{3}H]-naloxone or [^{3}H]-DAMGO for μ- and [^{3}H]-deltorphin II for δ-receptors specific ligands, respectively.

Table 3 shows the binding affinity of β-peptides analogues to δ- and μ-opioid receptors in comparison with the respective parent peptide and affinity data of DT I α,β-hybrides containing single or double of β^3-*homo*-amino acid residues. Replacing of each α-amino acid respective with β^3-*homo*-amino acid in DRM and Leu-Enk dramatically reduces the affinity to μ and δ receptors, probably because the Tyr and Phe moieties are not in a favorable position or distance to accomplish the overlapping of the pharmacophore. The additional C^β-atom in the each amino acid caused a higher flexibility and a greater structural variability of peptide chain, which

may result in adopting 'non-active' conformations. Surprisingly, β-DT I showed weak binding affinity at δ-opioid receptors.

Incorporation of β^3h-D-Ala in position 2 of DT I reduced affinity to δ-receptors only 20 times (peptide **5**). The distance between aromatic pharmacophores, which seems to be essential for opioid activity [35], should be the same as in analogue **4**. However, the replacement of Tyr^1 by β^3hTyr (**4**) reduced δ affinity about 700-fold. This may suggest importance of proper location of amide bond between amino acids in position 1 and 2.

Analogue containing β^3hPhe in position 3 (peptide **6**) is about 17-fold less potent in comparison with the DT I. In this case, the distance between two aromatic rings is the same as in the parent peptide. The presence of additional methylene group increased conformational flexibility of analogues, which in case analogues **5** and **6** is well tolerated by the δ-opioid receptor during the peptide ligand–receptor interaction.

The structure of β-DT I, the only β-homopeptide which shows weak binding affinity at δ-opioid receptors, was investigated by NMR spectroscopy.

Structural and conformational analysis of peptides in solution based on NMR techniques consists of several stages. Recognition of ^{1}H signals belonging to closed spin systems, i.e. to individual amino acid residues, is always the first stage of such analysis and can be best achieved with the aid of a number of two-dimensional measurements. Homonuclear techniques, COSY and TOCSY can be supplemented by such heteronuclear methods as ^{1}H/^{13}C HSQC and ^{1}H/^{15}N HSQC, which often allow us to remove some ambiguities in spectral assignments. Identification of structural constraints usually derived from the observation of NOE among ^{1}H nuclei being in close proximity is the subsequent stage of such analysis [36]. Owing to the typical correlation times of rotational diffusion for short peptides (*ca.* 0.5 ns) for which NOE is close to zero, this kind of experiments is performed in the rotating frame [37]. If the sufficient number of structural constraints is available for a rigid molecule, its 3D structure can be determined with a good precision. It is not, however, a usual case in studying peptides which display a high conformational mobility. Nevertheless, even sparse structural constraints can point out to the conformational preferences. It is the case of β-Deltorphin I (**1**), a peptide for which NMR study was carried out.

Almost full assignments of ^{1}H, ^{15}N and proton bearing ^{13}C nuclei in (**1**) was obtained from DQF-COSY, TOCSY, ^{1}H/^{13}C HSQC and ^{1}H/^{15}N HSQC spectra (Table 4).

Two-dimensional NOE in the rotating frame, ROESY spectrum, contained only 15 non-intraresidual cross peaks (Table 5).

Among 12 sequential NOEs, 6 are observed between H_α and subsequent H_N protons, $H_\alpha(i)/H_N(i+1)$, which are of medium or weak intensity and appear independently on the conformation of peptide backbone. The most informative are cross peaks including β^3hAsp^4 H_β and $H_{\beta2}$ protons. Four of them, pointing out to the close vicinity to the amide proton and methyl protons in β^3hVal^5, are the strongest among the non-intraresidual correlations. Together with correlations to H_β of β^3h-D-Ala^2 and H_γ of Val^6 they suggest that conformational motions in the central part of β-DT I backbone are partially restricted and some conformational preferences can be expected. The results of our NMR studies confirm, that only β-peptides constructed from carefully chosen β-amino acids, more conformationally restricted (cyclic β-amino acids, β,β-disubstituted β-amino acids) can adopt different, stable secondary structures [38,39].

Table 4. Nucleus chemical shifts of β-Deltorphin I

Nucleus	Chemical shifts
β^3hTyr^1	
H_N	7.944
N_H	n.a.
H_α	3.579
C_α	50.32
H_β	2.671; 2.866[a]
C_β	37.97[b]
$H_{\beta2}$	2.320[a]
$C_{\beta2}$	36.59[b]
H_δ	7.021
C_δ	131.1
H_ε	6.745
C_ε	116.2
β^3h-D-Ala^2	
H_N	8.081
N_H	126
H_α	4.078
C_α	43.14
H_β	0.979
C_β	20.52
$H_{\beta2}$	2.181; 2.102
$C_{\beta2}$	42.81
β^3hPhe^3	
H_N	7.813
N_H	121.1
H_α	4.249
C_α	48.5
H_β	2.764; 2.649[a]
C_β	39.94[b]
$H_{\beta2}$	2.220[a]
$C_{\beta2}$	35.7[b]
H_δ	7.188
C_δ	129.9
H_ε	7.264
C_ε	128.8
H_z	7.174
C_z	126.7
β^3hAsp^4	
H_N	7.892
N_H	120.8
H_α	4.364
C_α	44.42
H_β	2.352[a]
C_β	40.63[b]
$H_{\beta2}$	2.450[a]
$C_{\beta2}$	38.93[b]
H_δ	na
β^3hVal^5	
H_N	7.636
N_H	118.9
H_α	4.033
C_α	51.59
H_β	1.721
C_β	31.66
$H_{\beta2}$	2.181; 2.289
$C_{\beta2}$	38.94
$C_{\gamma1}$	18.34

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Table 4. (*Continued*)

Nucleus	Chemical shifts
$H_{\gamma 1}$	0.837
$C_{\gamma 2}$	18.34
$H_{\gamma 2}$	0.837
β^3*h*Val^6	
H_N	7.577
N_H	118.6
H_α	3.995
C_α	51.52
H_β	1.708
C_β	31.36
$H_{\beta 2}$	2.123; 2.239
$C_{\beta 2}$	38.76
$H_{\gamma 1}$	0.817
$C_{\gamma 1}$	19.93
$H_{\gamma 2}$	0.817
$C_{\gamma 2}$	19.93
βAla^7	
H_N	7.869
N_H	110.6
H_α	3.244
C_α	35.91
H_β	2.231
C_β	41.1
H_{N2}	6.842; 7.370
N_H	101.7

n.a. – not assigned; [a], [b] – assignments can be interchanged.

Table 5. Type of cross peaks

Type of cross peak	Interacting nuclei	Relative intensity
i/i+1	β^3*h*Tyr^1-H_α/β^3*h*-D-Ala^2-H_N	1.6
i/i+1	β^3*h*-D-Ala^2-H_α/β^3*h*Phe^3-H_N	7.5
i/i+1	β^3*h*-D-Ala^2-H_β/β^3*h*Phe^3-H_N	4.8
i/i+1	β^3*h*Phe^3-H_α/β^3*h*Asp^4-H_N	5.9
i/i+1	β^3*h*Asp^4-H_β/β^3*h*Val^5-H_N	6.0
i/i+1	β^3*h*Asp^4-H_β/β^3*h*Val^5-H_N	33.2
i/i+1	β^3*h*Asp^4-$H_{\beta 2}$/β^3*h*Val^5-H_N	9.5
i/i+1	β^3*h*Asp^4-H_β/β^3*h*Val^5-$H_{\gamma 1\gamma 2}$	17.5
i/i+1	β^3*h*Asp^4-$H_{\beta 2}$/β^3*h*Val^5-$H_{\gamma 1\gamma 2}$	12.0
i/i+1	β^3*h*Val^5-H_α/β^3*h*Val^6-H_N	1.1
i/i+1	β^3*h*Val^6-H_α/βAla^7-H_N	5.9
i/i+1	β^3*h*Val^6-$H_{\gamma 1\gamma 2}$/βAla^7-H_N	4.2
i/i+2	β^3*h*-D-Ala^2-H_β/β^3*h*Asp^4-H_β	5.1
i/i+2	β^3*h*-D-Ala^2-H_β/β^3*h*Asp^4-$H_{\beta 2}$	2.9
i/i+2	β^3*h*Asp^4-H_α/β^3*h*Val^6-$H_{\gamma 1\gamma 2}$	3.5

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