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Synthesis, binding affinities and metabolic stability of dimeric dermorphin analogs modified with β^3-*homo*-amino acids

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In this study, proteinogenic amino acids residues of dimeric dermorphin pentapeptides were replaced by the corresponding β^3-*homo*-amino acids. The potency and selectivity of hybrid α/β dimeric dermorphin pentapeptides were evaluated by competetive receptor binding assay in the rat brain using [3H]DAMGO (a μ ligand) and [3H]DELT (a δ ligand). Tha analog containing β^3-*homo*-Tyr in place of Tyr (Tyr-D-Ala-Phe-Gly-β^3-*homo*-Tyr-NH-)$_2$ showed good μ receptor affinity and selectivity (IC_{50} = 0.302, IC_{50} ratio μ/δ = 68) and enzymatic stability in human plasma. Copyright © 2016 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: β^3-*homo*-amino acids; hybrid α/β dimeric dermorphin pentapeptides; opioid receptors binding affinity; enzymatic stability in human plasma

Introduction

Opioids have been used for the treatment of moderate-to-severe chronic pain. Unfortunately, they have a large number of side effects [1,2]. Three main types of opioid receptors, μ (MOR), δ (DOR) and κ (KOR), are known [3]. The main target for analgesia is the MOR. Opioid receptors can form homodimers and the following heterodimers: DOR-KOR, DOR-MOR and KOR-MOR [4–7]. Specifically, designed ligands that are able to penetrate the BBB are used to study physiological consequences of opioid receptor homodimerization and heterodimerization, and as new analgesics. Biphalin is one of the most successful applications of the dimeric ligand approach to design enkephalin analogs [8]. It is composed of two tetrapeptide pharmacophores (Tyr-D-Ala-Gly-Phe) connected 'tail to tail' by a hydrazine bridge. Biphalin is 257-fold and 6.7-fold more potent than, respectively, morphine and etorphine (a reference μ agonist), in eliciting antinociception when administrated with intracerebroventricular [9], and leads to lesser physical dependence than morphine [10].

Dermorphin, linear heptapeptide (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), has been isolated from skins of the South American frogs *Phylomedusa sauvagei* [11]. It shows remarkably high μ selectivity and an extremely potent, long-acting antinociceptive effect [12] and has 100-fold higher affinity than morphine for μ opioid receptors [13]. Dermorphin shows potent antinociceptive effects with peripheral injection routes, such as s.c. and i.v. injections [14].

Because pain relief is mediated mainly through MOR, it is important to understand the interactions between MOR ligands and the receptor. Since the discovery of dermorphin, more than 100 dermorphin analogs have been synthesized, and their structure-activity relationships have been investigated [15].

The *N*-terminal tetrapeptide of dermorphin is known to be the minimum sequence required for opioid activity [16]. Synthetic peptides derived from the dermorphin tetrapeptide, which has the sequence Tyr-D-AA^2-Phe-AA^4 (where AA^2 and AA^4 represent a certain amino acid), have been reported to show a potent agonist activity for the μ opioid receptor [17], for example, DALDA (D-AA^2 = D-Arg, AA^4 = Lys-NH_2) [18], TAPA (D-AA^2 = D-Arg, AA^4 = βAla-OH) [19] or amidino-TAPA [20]. Dimeric dermorphin analogs were published by Lazarus *et al.* [21]. Several dimeric dermorphin tripeptides, tetrapeptides and pentapeptides connected 'tail-to-tail' by a different length bridge were synthesized. The most active in this series dimeric dermorphin pentapeptide with a hydrazine bridge (Tyr-D-Ala-Phe-Gly-Tyr-NH-)$_2$ exhibits higher μ affinities than either dermorphin or DAGO [22].

In this paper, we describe the synthesis, binding affinity and enzymatic stability of analogs dimeric dermorphin pentapeptides containing β^3-*homo*-amino acids. The synthesis of α/β hybrid peptides, modified with homologated proteinogenic amino acids, is a useful tool in drug design. The substitution of α-amino acids with their β-isomers in biologically active peptides may result in increased enzyme stability [23] and also in a strong influence on peptide conformation [24]. Hybrid α/β peptides fold the backbone in a manner more similar to the natural α-peptides than full β-peptides, and this, together with their high intrinsic metabolic stability, makes them good candidates for drug design [25]. Various β^3-*homo*-amino acids have been used in the design of several classes of ligands, including

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opioid peptides [25–30]. Incorporation of β^3-*homo*-phenylalanin or its analogs at the 4,4′ positions of biphalin resulted in good opioid receptor affinities and antinociceptive activity [25,26].

Materials and Methods

General

All solvents and reagents were obtained from commercial suppliers and used without further purification. All the amino acid derivatives were purchased from Sigma-Aldrich, except BocTyr(Boc), which was obtained as described by Shevchenko *et al.* [31]. Optically pure isomeric Boc-β^3-*homo*-amino acids were prepared in two-step Arndt–Eistert homologation of *N*-protected amino acids [32–34].

General procedure for synthesis of protected dipeptides, tripeptides and tetrapeptides

To a stirred solution of Boc-amino acids (1 mM) in DCM, TBTU (1 mM), HOBt (1 mM) and DIPEA (3 mM) were added. After stirring the mixture for 10 min, amino component (1 mM) was added. Then the reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure; the residue was diluted with ethyl acetate (20 ml) and washed with 1 N $NaHSO_4$ (three times, 5 ml each), 5% $NaHCO_3$ (three times, 5 ml each) and 5 ml of brine, dried with magnesium sulfate and concentrated under vacuum.

The crude peptides were purified by flash chromatography or crystallization.

General procedure for Boc group removal

Boc peptides were deprotected by 2 N HCl in ethyl acetate or by 90% TFA_{aq} at room temperature for 2 h. Then, ethyl ether was added to the reaction mixture. Precipitated crystals were filtered off, washed with diethyl ether and used for the next step without further purification.

General procedure for methyl ester removal

To a stirred solution of methyl ester (1 mM) in MeOH (1.5 ml), 1 N $NaOH_{aq}$ (1.5 ml) was added. The reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure at room temperature. The residue was diluted with water and washed with diethyl ether (three times, 5 ml each), acidified with 1 N $NaHSO_4$ and extracted with ethyl acetate (three times, 5 ml each). The organic layer was dried with magnesium sulfate, and concentrated under vacuum and used for the next step without further purification.

General procedure for benzyl group removal

To a stirred solution of Bzl-protected peptides (1 mM) in AcOH (5 ml), Pd/C_{act} in MeOH was added. The reaction mixture was stirred under a hydrogen atmosphere at room temperature for 4 h. The catalyst was filtered off through Celite, and the solvent was evaporated under reduced pressure. Purification by crystallization or flash chromatography, as needed, afforded the desired peptides.

Boc-Tyr(Boc)-D-Ala-β^3-homo-Phe-Gly-OH ***1a***

Boc-β^3-homo-Phe-Gly-OBzl The general procedure for synthesis of protected peptides was followed using 1.5 mM of Boc-β^3-*homo*-Phe-OH and HCl × H-Gly-OBzl; yield = 0.57 g (89%); m.p. = 127–130 °C; R_f[$CHCl_3$:CH_3OH 9:1] = 0.59; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.40 (s, 9H, Boc); 2.31–2.53 (m, 2H, CH_2); 2.82–3.01 (m, 2H, CH_2); 4.06–4.16 (m, 3H, CH_2, CH); 5.20 (s, 2H, CH_2); 7.19–7.38 (m, 10H, Ar).

HCl × H-β^3-homo-Phe-Gly-OBzl

The general procedure for Boc group removal was followed using 1.3 mM of Boc-β^3-*homo*-Phe-Gly-OBzl, yield = 0.47 g (100%); colorless oil.

Boc-D-Ala-β^3-homo-Phe-Gly-OBzl

The general procedure for synthesis of protected peptides was followed using 1.3 mM Boc-D-Ala-OH and HCl × H-β^3-*homo*-Phe-Gly-OBzl; yield = 0.57 g (88%); m.p. = 130–132 °C; R_f[$CHCl_3$:CH_3OH 9:1] = 0.69; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.31 (d, 3H, CH_3, J = 6.85 Hz); 1.52 (s, 9H, Boc); 2.40–2.68 (m, 2H,CH_2); 2.95–3.06 (m, 2H, CH_2); 3.83–3.97 (m, 2H, CH_2); 4.47–4.66 (m, 2H, 2 × CH); 5.32 (s, 2H, CH_2); 7.25–7.40 (m, 5H, Ar); 7.47–7.51 (m, 5H, Ar).

HCl × H-D-Ala-β^3-homo-Phe-Gly-OBzl

The general procedure for Boc group removal was followed using 1.1 mM of Boc-D-Ala-β^3-*homo*-Phe-Gly-OBzl; yield = 0.47 g (99%); colorless oil.

Boc-Tyr(Boc)-D-Ala-β^3-homo-Phe-Gly-OBzl

The general procedure for synthesis of protected peptides was followed using 1.05 mM Boc-Tyr(Boc)-OH and HCl × H-D-Ala-β^3-*homo*-Phe-Gly-OBzl; yield = 0.70 g (88%); colorless oil; R_f[$CHCl_3$:CH_3OH 9:1] = 0.64; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.12 (d, 3H, CH_3, J = 7.1 Hz); 1.41 (s, 9H, Boc); 1.53 (s, 9H, Boc); 2.26–2.47 (m, 2H, CH_2); 2.88–3.11 (m, 4H, 2 × CH_2); 3.85–3.94 (m, 2H, CH_2); 4.26–4.45 (m, 3H, 3 × CH); 5.16 (s, 2H, CH_2); 7.06–7.37 (m, 14H, Ar).

Boc-Tyr(Boc)-D-Ala-β^3-homo-Phe-Gly-OH ***1a***

The general procedure for benzyl ester removal was followed using 0.9 mM of Boc-Tyr(Boc)-D-Ala-β^3-*homo*-Phe-Gly-OBzl; yield = 0.53 g (88%); oil; R_f[$CHCl_3$:CH_3OH:CH_3CO_2H 90:10:2] = 0.57; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.10 (bs, 3H, CH_3); 1.38 (s, 9H, Boc); 1.55 (s, 9H, Boc); 2.22–2.43 (m, 2H, CH_2); 2.88–3.17 (m, 4H, 2 × CH_2); 3.91–3.96 (m, 2H, CH_2); 4.37–4.58 (m, 3H, 3 × CH); 7.06–7.28 (m, 9H, Ar).

Boc-Tyr(Bzl)-D-Ala-Phe-βAla-OH ***1b***

Boc-Phe-βAla-OMe The general procedure for synthesis of protected peptides was followed using 2.15 mM Boc-Phe-OH and HCl × H-βAla-OMe; yield = 0.73 g (97%); m.p. = 79–81 °C (m.p. = 84–85 °C [35]); R_f[$CHCl_3$:CH_3OH 9:1] = 0.67; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.41 (s, 9H, Boc); 2.31–2.48 (m, 2H, CH_2); 2.94–3.12 (m, 2H, CH_2); 3.33–3.55 (m, 2H, CH_2); 3.64 (s, 3H,CH_3); 4.22–4.31 (m, 1H, CH); 7.17–7.33 (m, 5H, Ar).

HCl × H-Phe-βAla-OMe

The general procedure for Boc group removal was followed using 2.1 mM of Boc-Phe-βAla-OMe; yield = 0.59 g; (98%); m.p. = 146–147 °C.

Boc-D-Ala-Phe-βAla-OMe

The general procedure for synthesis of protected peptides was followed using 2.0 mM of Boc-D-Ala-OH and HCl × H-Phe-βAla-OMe; yield = 0.80 g (95%); m.p. = 100–102 °C; R_f[$CHCl_3$:CH_3OH 9:1] = 0.70; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.28 (d, 3H, CH_3, J = 7 Hz); 1.44 (s, 9H, Boc); 2.33–2.51 (m, 2H,CH_2); 3.01–3.14 (m, 2H, CH_2); 3.39–3.47 (m, 2H, CH_2); 3.65 (s, 3H, CH_3); 4.01–4.14 (t, 1H, CH, J = 7 Hz); 4.60 (q, 1H, CH, J = 7 Hz); 7.14–7.33 (m, 5H, Ar).

HCl × H-D-Ala-Phe-βAla-OMe

The general procedure for Boc group removal was followed using 1.8 mM of Boc-D-Ala-Phe-βAla-OMe; yield = 0.64 g (99%); colorless oil.

Boc-Tyr(Bzl)-D-Ala-Phe-βAla-OMe

The general procedure for synthesis of protected peptides was followed using 1.2 mM of Boc-Tyr(Bzl)-OH and HCl × H-D-Ala-Phe-β Ala-OMe; yield = 0.70 g (87%); colorless oil; R_f[$CHCl_3$:CH_3OH 9:1] = 0.60; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.11 (d, 3H, CH_3, J = 6.85 Hz); 1.40 (s, 9H, Boc); 2.45 (t, 2H, CH_2, J = 6.15 Hz); 2.92–3.19 (m, 4H, 2 × CH_2); 3.28–3.57 (m, 2H, CH_2); 3.64 (s, 3H, CH_3); 4.23–4.39 (m, 2H, 2 × CH); 4.58 (q, 1H, CH, J = 7.3 Hz); 5.04 (s, 2H, CH_2); 6.90–7.10 (m, 4H, Ar); 7.17–7.45 (m, 10H, Ar).

Boc-Tyr(Bzl)-D-Ala-Phe-βAla-OH ***1b***

The general procedure for methyl ester removal was followed using 1.0 mM of Boc-Tyr(Bzl)-D-Ala-Phe-βAla-OMe; yield = 0.58 g (88%); colorless oil; R_f[$CHCl_3$:CH_3OH:CH_3CO_2H 90:10:2] = 0.62; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.12 (bs, 3H, CH_3); 1.37 (s, 9H, Boc); 2.43–2.54 (m, 2H, CH_2); 2.89–3.18 (m, 4H, 2 × CH_2); 3.34–3.55 (m, 2H, CH_2); 4.44–4.61 (m, 2H, 2 × CH); 4.78–4.89 (m, 1H, CH); 5.01 (s, 2H, CH_2); 6.87–7.45 (m, 14H, Ar).

Boc-Tyr(Boc)-D-Ala-Phe-Gly-OH ***1c***

Boc-Phe-Gly-OBzl The general procedure for synthesis of protected peptides was followed using 2 mM of Boc-Phe-OH and HCl × H-Gly-OBzl; yield = 0.82 g (99%); m.p. = 131–132 °C (m.p. = 132–134 °C [36]); R_f[$CHCl_3$:CH_3O 9:1] = 0.52; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.39 (s, 9H, Boc); 2.98–3.16 (m, 2H, CH_2); 3.92–4.13 (m, 2H, CH_2); 4.37–4.45 (m, 1H, CH); 5.16 (s, 2H, CH_2); 7.18–7.41 (m, 10H, Ar).

HCl × H-Phe-Gly-OBzl

The general procedure for Boc group removal was followed using 1.9 mM of Boc-Phe-Gly-OBzl; yield = 0.64 g (97%); m.p. = 126–128 °C.

Boc-D-Ala-Phe-Gly-OBzl

The general procedure for synthesis of protected peptides was followed using 1.5 mM of Boc-D-Ala-OH and HCl × H-Phe-Gly-OBzl; yield = 0.70 g (97%); m.p. = 158–160 °C; R_f[$CHCl_3$:CH_3O 9:1] = 0.64; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.22 (d, 3H, CH_3, J = 7 Hz); 1.40 (s, 9H, Boc); 3.04–3.21 (m, 2H, CH_2); 3.84–4.16 (m, 3H, CH_2, CH); 4.69 (q, 1H, CH, J = 7 Hz); 5.15 (s, 2H, CH_2); 7.17–7.37 (m, 10H, Ar).

HCl × H-D-Ala-Phe-Gly-OBzl

The general procedure for Boc group removal was followed using 1.45 mM of Boc-D-Ala-Phe-Gly-OBzl; yield = 0.60 g (98%); m.p. = 113–115 °C.

Boc-Tyr(Boc)-D-Ala-Phe-Gly-OBzl

The general procedure for synthesis of protected peptides was followed using 1.4 mM of Boc-Tyr(Boc)-OH and HCl × H-D-Ala-Phe-Gly-OBzl; yield = 0.95 g (91%); oil; R_f[$CHCl_3$:CH_3O 9:1] = 0.62; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.26 (bs, 3H, CH_3); 1.37 (s, 9H, Boc); 1.54 (s, 9H, Boc); 2.87–3.10 (m, 4H, 2 × CH_2); 3.99–4.33 (m, 4H, CH_2, 2 × CH); 4.63–4.75 (m, 1H, CH); 5.15 (s, 2H, CH_2); 7.05–7.36 (m, 14H, Ar).

Boc-Tyr(Boc)-D-Ala-Phe-Gly-OH ***1c***

The general procedure for benzyl ester removal was followed using 1.3 mM of Boc-Tyr(Boc)-D-Ala-Phe-Gly-OBzl; yield = 0.73 g (86%); m.p. = 143–145 °C (m.p. = 149–150 °C [37]); R_f[$CHCl_3$:CH_3OH:CH_3CO_2H 90:10:2] = 0.53; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.09 (bs, 3H, CH_3); 1.36 (s, 9H, Boc); 1.54 (s, 9H, Boc); 2.89–3.23 (m, 4H, 2 × CH_2); 3.98 (bs, 2H, CH_2); 4.56–4.75 (m, 3H, 3 × CH); 7.07–7.23 (m, 9H, Ar).

General procedure for synthesis of diacylated hydrazides

To a stirred solution of Boc-amino acid (2.5 mM) in DCM : DMF (1:1), TBTU (2.5 mM), HOBt (2.5 mM), and DIPEA (7.5 mM) were added. After stirring the mixture for 10 min, hydrazine dihydrochloride (1 mM) was added. Then the reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure; the residue was precipitated out by the addition of ethyl acetate, filtered, washed with ethyl acetate and distilled water and dried.

(Boc-Tyr(Bzl)-NH-)$_2$ ***2a***

The general procedure for synthesis of diacylated hydrazide **2a** was followed using 5 mM of (Boc-Tyr(Bzl)-OH and 2 mM of hydrazine dihydrochloride; yield = 1.31 g (89%); m.p. = 196–198 °C (m.p. = 210–212 °C [38]); R_f[$CHCl_3$:CH_3OH 95:5] = 0.59; R_f[$CHCl_3$:CH_3OH 9:1] = 0.73.

(Boc-β^3-homo-Tyr(Bzl)-NH-)$_2$ ***2b***

The general procedure for synthesis of diacylated hydrazide **2b** was followed using 5 mM of (Boc-β^3-*homo*-Tyr(Bzl)-OH and 2 mM of hydrazine dihydrochloride; yield = 1.03 g (67%); m.p. = 217–219 °C; R_f[$CHCl_3$:CH_3OH 95:5] = 0.44; R_f[$CHCl_3$:CH_3OH 9:1] = 0.54.

(HCl × H-Tyr(Bzl)-NH-)$_2$ ***3a***

The general procedure for Boc group removal was followed using 1.5 mM of (Boc-Tyr(Bzl)-NH-)$_2$; yield = 0.91 g (99%); m.p. = 242–245 °C.

(HCl × H-β^3-homo-Tyr(Bzl)-NH-)$_2$ ***3b***

The general procedure for Boc group removal was followed using 1.5 mM of (Boc- β^3-*homo*-Tyr(Bzl)-NH-)$_2$; yield = 0.95 g (99%); m.p. = 265–268 °C.

General procedure for synthesis of unprotected decapeptides ***I–VI***

To a stirred solution of *N,O*-protected pentapeptides **1a–1c** (2.5 eq.) in DMF, HATU (2.5 eq.) and DIPEA (5 eq.) were added. After stirring the mixture for 10 min, diacylated hydrazides **3a** or **3b** (1 eq.) was added. Then the reaction mixture was stirred at room temperature for 48 h. The solvent was evaporated under reduced pressure; the residue was diluted with ethyl acetate and washed with three portions of 1 N $NaHSO_4$, 5% $NaHCO_3$ and brine, dried with magnesium sulfate and concentrated under vacuum. The crude-protected decapeptides were purified by flash chromatography and then were deprotected using general procedures for Boc and benzyl groups removal. Unprotected decapeptides **I–VI** were purified by RP-high-performance liquid chromatography (HPLC). The purity of the final TFA salts was assessed by analytical HPLC and ESI-MS (Table 1).

Results

Optically pure *N*-Boc-β^3-*homo*-tyrosine(Bzl) and *N*-Boc-β^3-*homo*-phenylalanine were prepared in two-step Arndt–Eistert homologation of protected amino acids [32–34]. Dipeptides, tripeptides and tetrapeptides were synthesized by conventional method in solution according to Scheme 1.

The final step of the synthesis included acylation of the amino groups in the symmetric dihydrazides **3a** or **3b** by *N,O*-protected tetrapeptides **1a–1c** (Scheme 2).

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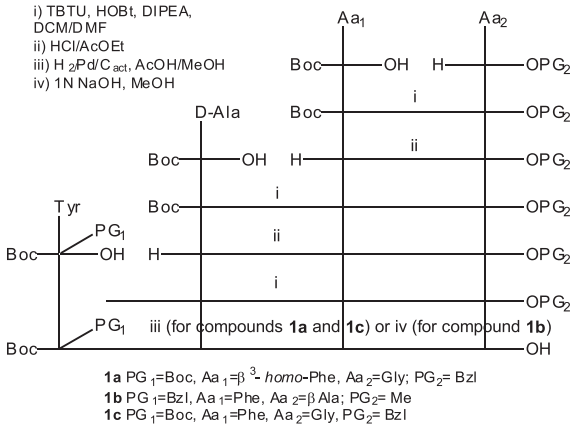
Table 1. Structures and the physicochemical properties of the dimeric pentapeptide dermorphin analogs **I–VI**

Peptides	MW		m/z $[M+H]^+$	RP-HPLC	
	Formula	Calc.	Found	t_R (min)[a]	Purity
(Tyr-D-Ala-Phe-Gly-Tyr-NH-)$_2$ **I**	$C_{64}H_{74}N_{12}O_{14}$	1235.3	1236.5	14.12	98
(Tyr-D-Ala-β^3-*homo*-Phe-Gly-Tyr-NH-)$_2$ **II**	$C_{66}H_{78}N_{12}O_{14}$	1263.4	1264.5	14.27	96
(Tyr-D-Ala-Phe-βAla-Tyr-NH-)$_2$ **III**	$C_{66}H_{78}N_{12}O_{14}$	1263.4	1264.5	13.82	97
(Tyr-D-Ala-Phe-Gly-β^3-*homo*-Tyr-NH-)$_2$ **IV**	$C_{66}H_{78}N_{12}O_{14}$	1263.4	1264.5	13.99	99
(Tyr-D-Ala-β^3-*homo*-Phe-Gly-β^3-*homo*-Tyr-NH-)$_2$ **V**	$C_{68}H_{82}N_{12}O_{14}$	1291.5	1292.5	13.63	98
(Tyr-D-Ala-Phe-βAla-β^3-*homo*-Tyr-NH-)$_2$ **VI**	$C_{68}H_{82}N_{12}O_{14}$	1291.5	1292.5	13.71	97

[a]Linear gradient 10–60% B, 20 min, 20 min flow rate 3 ml/min [solvents (A) 0.05% TFA in water and (B) 0.038% TFA in acetonitrile/H_2O, 90:10].



Scheme 1. Synthesis of protected tetrapeptides **1a–1c**.

The crude peptides were purified by RP-HPLC. The purity of the final TFA salts was assessed by analytical HPLC (purity >96–99%) and ESI-MS (Table 1).

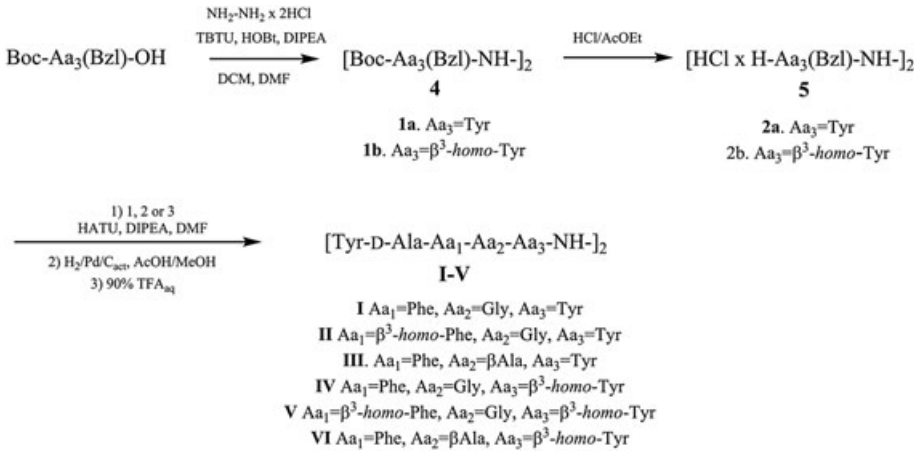
Table 2. Binding affinities of the dimeric pentapeptide dermorphin analogs **I–VI** to δ and μ opioid receptors

Peptides	IC_{50} [nM]		μ/δ
	μ	δ	
Dermorphin	1.22 ± 0.13[a]	178.6 ± 18[a]	146.4[a]
(Tyr-D-Ala-Phe-Gly-Tyr-NH-)$_2$ **I**	0.243 ± 0.005 0.27 ± 0.04[a]	8.754 ± 0.26 7.88 ± 0.07[a]	36.0 29.2[a]
(Tyr-D-Ala-β^3-*homo*-Phe-Gly-Tyr-NH-)$_2$ **II**	18.20 ± 0.43	203.7 ± 9.4	11.2
(Tyr-D-Ala-Phe-βAla-Tyr-NH-)$_2$ **III**	3.543 ± 0.169	85.34 ± 1.83	24.1
(Tyr-D-Ala-Phe-Gly-β^3-*homo*-Tyr-NH-)$_2$ **IV**	0.302 ± 0.006	20.55 ± 1.22	68.0
(Tyr-D-Ala-β^3-*homo*-Phe-Gly-β^3-*homo*-Tyr-NH-)$_2$ **V**	11.2 ± 0.68	>10000	—
(Tyr-D-Ala-Phe-βAla-β^3-*homo*-Tyr-NH-)$_2$ **VI**	40.7 ± 2.9	645 ± 45.8	15.8

[a][20]

Receptor binding assay

Receptor binding assays were performed as described previously [39]. Rat membrane preparation followed the procedure described by Misicka *et al.* [40]. The radioreceptor-binding protocol was based on a study elaborated by Fichna *et al.* [41] with some modifications. The modification included different incubation times (60 min vs 120 min), bacitracin concentrations (30 μg/ml vs 50 μg/ml) and radioligand choices. The modifications were implemented in order to obtain optimal binding conditions. Binding affinities for μ and δ opioid receptors were determined by displacing [3H]DAMGO and [3H]DELT, respectively, from adult-male Wistar rat brain-membrane binding sites. Binding curves were fitted using nonlinear regression. Compound potency was expressed as IC_{50} values (Table 2).



Scheme 2. Synthesis of dimeric dermorphin pentapeptides analogs **I–VI**.

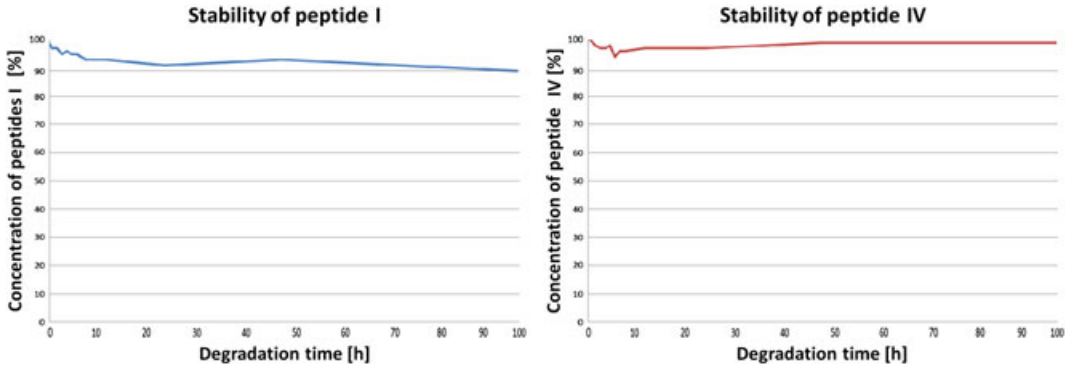


Figure 1. Stability of dimeric pentapeptide dermorphin analogs **I** and **IV** in human plasma *in vitro*.

Plasma stability studies of I and IV

Plasma stability studies were performed as described previously [42], with some modifications. The stability of the peptides **I** and **IV** was tested in human plasma obtained from a healthy donor. A stock solution of the peptide was prepared by dissolving 0.44 μM (**I**) or 0.27 μM (**IV**) in 1 ml of water. In Eppendorf tubes, samples of human plasma (50 μl) were temperature equilibrated at 37 ± 1 °C for 5 min before adding 50 μl of the peptide stock solution. The time was recorded at different intervals (0 , 1, 2, 3, 4, 5, 6, 12, 24, 48 and 96 h). Next, ethanol (200 μl) was added to the samples in order to precipitate the plasma proteins. These cloudy mixtures were shaken 1 min and cooled in a refrigerator (4 °C) for 5 min and subsequently centrifuged for 10 min at 2000 *g* (Eppendorf centrifuge). The reaction supernatant was then analyzed by HPLC/MS using LCMS-2010EV Shimadzu, a Phenomenex Jupiter 4u Proteo 90A; C12 (25 cm × 2 mm × 4 μm) column, a non-linear gradient was used: 0–63% B for 40 min followed by an increase to 97% B from 40 to 45 min, at a solvent flow of 0.3 ml/min. Solvent solutions were solvent A (0.05% TFA in water) and solvent B (0.05% TFA in acetonitrile). As an internal standard, Z-Val-OH was used. Additionally, the activity of the human plasma was tested using Endomorphin-2 (0.47 μM/ml) as a control sample, as it is known that Endomorphin-2 is unstable in human plasma.

Discussion

The general strategy for the synthesis of dimeric dermorphin analogs by (2 × 4) + 2 fragment coupling was similar to the procedure for synthesis of biphalin, originally described by Lipkowski *et al.* [8]. Affinities of α,β-peptides, dimeric dermorphin analogs, for μ receptor and δ receptor were determined by the radioligand-labeled binding assay described previously using [3H]DAMGO and [3H]DELT as μ receptor-specific and δ receptor-specific ligands, respectively. Table 2 shows the binding affinity of α,β-peptide analogs of dermorphin to δ opioid and μ opioid receptors in comparison with dermorphin. Dimeric dermorphin pentapeptide analog **I** exhibits five times better affinity to μ receptor and 20 times to δ receptor than dermorphin, and it is comparable with previously described by Lazarus *et al.* [21].

Our studies have shown that analogs containing of β^3-*homo*-amino acids in positions 3,3′ (**II**), 4,4′ (**III**), 5,5′ (**IV**), 3,3′, 5,5′ (**V**) and 4,4′, 5,5′(**VI**) exhibit various degree of affinity to μ and δ receptors. Analogs **II**, **III**, **V** and **VI** have better or reduced μ affinity compare with dermorphin yet maintain selectivity for μ receptor. The replacement of tyrosine at positions 5 and 5′ with β^3-*homo*-tyrosine (**IV**) exhibits no significant difference relative to **I** in its affinity for the μ receptor, and lower affinity for the δ receptor than **I** giving the most active and μ selective analog containing β^3-*homo*-amino acid residue in this series. Similar results were obtained by the incorporation of β^3-*homo*-amino acids in positions 4 and 4′ of biphalin. The binding affinity and δ selectivity have been improved or remained unchanged. Biphalin analog modified with β^3-*homo*-Phe in position 4,4′ showed remarkable binding affinity, with $K_i\delta = 0.72$ and $K_i\mu = 1.1$ nM and an increased enzymatic stability in human plasma [25]. The incorporation of β^3-*homo*-Phe(NO_2) in position 4,4′ increases μ-affinities and slightly decreases δ-affinities ($IC_{50}\mu = 0.72$ nM; $IC_{50}\delta = 4.66$ nM [43]). It confirms that homologation of residue connected with linker in dimeric opioid analogs is generally well tolerated and improves activity and selectivity.

The enzymatic stability of peptide drugs is important if the drug candidate is to be considered for therapeutic use. The resistance of the **I** and **IV** was determined by HPLC analysis of about 0.5 μM samples incubated with human plasma. Analog **I** exhibited good stability to human plasma, whilst analog **IV**, containing β^3-*homo*-tyrosine in position 5,5′, was found to be extremely stable in human plasma. It was resistant to the blood protease degradation for 96 h (Figure 1).

The metabolic stability of biphalin is lower (the half-life found after incubation in human plasma was 24 h) (Misicka A, Tymecka D. personal communication). Present results suggest that modification of flexibility (an additional CH_2 group in the main chain of the peptide) may facilitate fitting to μ opioid receptor and that these compounds will be not be degraded enzymatically before having an opportunity to produce the desired receptor mediated, analgesic effects.

Preliminary data were presented on the 24th American Peptide Symposium [44].

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