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Biological Active Analogues of the Opioid Peptide Biphalin: Mixed *α*/*β*^{3}-Peptides

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Abstract

Natural residues of the dimeric opioid peptide Biphalin were replaced by the corresponding homo-*β*^{3} amino acids. The derivative **1** containing h*β*^{3} Phe in place of Phe showed good μ- and δ-receptor affinities ($K_i^{\delta}=0.72\ \mathrm{nM}$; $K_i^{\mu}=1.1\ \mathrm{nM}$) and antinociceptive activity in vivo together with an increased enzymatic stability in human plasma.

INTRODUCTION

Biphalin is a unique dimeric opioid peptide composed of two enkephalin-based tetrapeptides (Tyr-D-Ala-Gly-Phe) linked by a hydrazide bridge between the two C-terminal phenylalanine moieties. Biphalin displays a strong affinity for both μ and δ receptors, with an EC_{50} in the nanomolar range. When administered intracerebroventricularly (icv) it shows higher potency than morphine and etorphine in animal models of pain.[1] The remarkable activity is due primarily to its dimeric structure which improves the ability to meet the topographical requirements of involved receptors.[2] Furthermore, this opioid octapeptide produced less physical dependence than other opioid agonists.[1a,3]

A major problem concerning the use of peptides, including the opioid peptides, as drugs, is their susceptibility to enzymatic hydrolysis when administered in vivo.[4] Approaches that have been explored in an effort to overcome this problem in opioid peptides include the use of D-amino acids, *β*-amino acids, various types of synthetic residues, and backbone cyclization.[5]

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Supporting Information
Synthetic procedures, characterization of intermediates, biological assays, and stability assay. This material is available free of charge via the Internet at http://pubs.acs.org

A well-known and useful approach is the design and synthesis of α/β hybrid peptides, where one or more native residues of the backbone sequence is replaced by the corresponding β^2- or β^3-amino acid analogues.[6] Hybrid α/β peptides fold the backbone in a manner more similar to the natural α-peptides than full β-peptides and together with high their intrinsic metabolic stability make them good candidates for drug design. β^3-amino acids have been used in the design of several classes of ligands, including platelet aggregation factors, angiotensin II, chemotactic agents, and neuropeptides, including opioid peptides.[7–10]

This work reports the synthesis and in vitro biological evaluation of four biphalin analogues (Figure 1) containing β^3 homoamino acids (hβ^3Xaa). hβ^3Tyr and hβ^3Phe were incorporated in place of the tyrosine$^{1–1'}$ and phenylalanine$^{4–4'}$ residues, respectively, and D-alanine$^{2–2'}$ and glycine$^{3–3'}$ were replaced with a β-alanine residue. The most active product of this series, which contains hβ^3 Phe (**1**), was further tested in in vivo thermal models of nociception by icv and iv administrations in mice, with human plasma stability also evaluated.

BIOLOGICAL EVALUATION

Binding Affinities at μ/δ Opioid Receptors.[11]

To determine the affinity to the μ-opioid (MOR) and the δ-opioid receptors (DOR) of compounds **1**–**4**, tritiated opioid peptides DAMGO ([^{3}H]-[D-Ala(2), *N*-Me-Phe-(4), Gly-ol(5)] enkephalin) and Deltorphin (selective agonists for MOR and DOR, respectively) were used. Binding IC_{50} and K_i values are shown in Table 1.

Biphalin was used as reference.[1b] Analogue **1** has a very good opioid receptor affinity, showing subnanomolar affinity at the DOR and a potent K_i value at the MOR (0.72 and 1.1 nM, respectively). Analogues **2**–**4** display poor affinity for opioid receptors, with a mild μ-selectivity for peptides **3** and **4** ($K_i^{\mu}/K_i^{\delta}=0.13$). Peptide **2** showed little MOR/DOR discrimination, with K_i values higher than 100 nM at both receptors.

GPI and MVD in Vitro Bioassay.[12]

The biological activity was also investigated through isolated tissue-based functional assay using guinea pig ileum/longitudinal muscle myenteric plexus (GPI) and mouse vas deferens (MVD) tissues (Table 1). Biphalin was used as a reference.[13] Compound **1** showed the greatest potency, with no relevant selectivity for μ- and δ-receptor (with K_i values of 33 and 50 nM, respectively, for MVD and GPI). Analogue **4** showed near identical IC_{50} around 700 nM in both MVD and GPI assays, while **2** and **3** had weak activity. These data are in agreement with results of binding assay.

In Vivo “Hot Plate” and “Tail-Flick” Bioassays.[14]

Antinociceptive properties of compound **1** were further investigated using the mouse hot plate and tail-flick assays, following both local (icv, 0.6 nmol) and systemic (iv, 3000 nmol) injections. Morphine and Biphalin were used as reference compounds (Figure 2).

In Vitro Metabolic Stability.[15]

The enzymatic stability of both Biphalin and the Biphalin analogue **1** was tested by incubation in human plasma at 37 °C. Degradation curves (Figure 3) were obtained by plotting the total amount of remaining parent compound (expressed as μg/mL) versus time (expressed as minutes), demonstrating increased stability for the modified compound due to lack of recognition by degrading enzymes present in human plasma.

DISCUSSION AND CONCLUSION

In this work, four new biphalin analogues were synthesized and investigated. The novel compounds were evaluated for their μ/δ receptor activity, and the results are shown in Table 1. Compound **1**, containing hβ^3Phe residues in position 4 and 4′, showed remarkable binding affinity, with K_i values of 0.72 and 1.1 nM, respectively, for DOR and MOR, resulting in a receptor affinity comparable with that of Biphalin. In the GPI and MVD in vitro bioassays (Table 1), compound **1** was still the most potent of the series, but all the peptides showed lower potency than Biphalin, suggesting that the activities of the compounds do not totally reflect the ability to induce a biological response.

The high affinity and the strong in vitro activity of **1**, confirmed by in vivo nociception tests, may be explained by the fact that the distance between the two aromatic rings of Tyr and Phe is the same as the parent peptide, therefore the additional methylene group increases the flexibility of the compound without compromising side chain arrangements. The activity found for compounds **1**–**4** is compatible with previous biological studies on Biphalin analogues with non-hydrazine linkers. Those studies state that an increased distance between the two pharmacophores of Biphalin, obtained by using short diamine bridges (containing one or two methylene groups) or cyclic linkers (e.g., piperazine) is well tolerated or can even improve the in vitro affinity, and our data here confirm those conlcusions.[16] On the other hand, the distance between Phe$^{4,4'}$ and Tyr$^{1,1'}$ side chains, as well as the reciprocal pharmacophores arrangement of the dimeric structure, are crucial for the activity. Thus, the use of β-residues in position 1,1′, 2,2′, and 3,3′ of the backbone led to an evident loss of affinity and activity (see products **2**–**4**), whereas the introduction of the additional methylene group in position 4,4′ is well tolerated (see product **1**).[16b] These data support the importance of D-Ala and Gly as keystructural residues, in addition to the well-known role of tyrosine. The lack of activity and affinity of compounds **2**–**4** is probably due to the β-residues that affect the spacing between the pharmacophoric Tyr and Phe residues. Interestingly, compounds **3** and **4** showed a significant selectivity for MOR (about 8-fold), suggesting a higher sensitivity of the DOR for modifications induced by β-residues. The antinociceptive in vivo profile of compound **1** clearly indicates that **1** is endowed with good activity, several times higher than morphine tested under the same conditions (for icv), but slightly lower than Biphalin, as expected from the MVD/GPI tests. In the hot plate test, after icv administration, the antinociceptive profile of the analogue **1** was very similar to Biphalin, producing 100% of the MPE 30 and 45 min after administration. In both in vivo models, the maximum effect is reached 15–30 min after drug injection, and no significant decrease is observed for the next 30 min.

Following iv administration (hot plate and tail-flick tests), compound **1** displayed a higher (ranging from 40 to 140% 15– 60 min after administration) and more long lasting antinociceptive effect than Biphalin, thus confirming the improved plasma stability, in full accord with in vitro stability data, reported in Figure 3 (for detailed experimental data see Supporting Information).

The improved metabolic stability paired with good antinociceptive activity confirms that Phe moiety modification[16c, d] is a promising strategy in the field of Biphalin analogues development.

EXPERIMENTAL SECTION

Chemistry

Synthesis of all new analogues was performed in solution phase using the N^{α}-Boc strategy. All synthesis began with hydrazine, with repeated steps of coupling/purification/

deprotection of the intermediate products, until the final products were obtained as TFA salts (Scheme 1).

All coupling reactions were performed with the standard method of HOBt/EDC/NMM in DMF.[16b] Deprotection of N^{α}-*tert*-butyloxycarbonyl group was performed using TFA/CH_2Cl_2 1:1 for 1 h, under nitrogen atmosphere. The intermediate TFA salts were used for subsequent reactions without further purification. Boc protected intermediate products were purified by silica gel column chromatography, or in the case of scarcely soluble products, the purification was performed by trituration in EtOAc.[16c] Final products **1**–**4** were purified by RP-HPLC using a Waters XBridge Prep BEH130 C^{18}, 5.0 μm, 250 mm × 10 mm column at a flow rate of 4 mL/min on a Waters Binary pump 1525, using as eluent a linear gradient of H_2O/acetonitrile 0.1% TFA ranging from 5% acetonitrile to 90% acetonitrile in 45 min. The purity of the N^{α}-Boc-protected products was confirmed by NMR analysis on a Varian VXR 300 MHz and mass spectrometry ESI-HRMS. The purity of all final TFA salts was confirmed by NMR analysis, ESI-HRMS, and by analytical RP-HPLC (C_{18}-bonded 4.6 mm × 150 mm) at a flow rate of 1 mL/min, using as eluent a gradient of H_2O/acetonitrile 0.1% TFA ranging from 5% acetonitrile to 95% acetonitrile in 50 min, and was found to be ≥95%. All synthetic procedure and intermediates' characterizations are reported in the Supporting Information.

2 TFA·(Tyr-D-Ala-Gly-hβ^{3}Phe-NH)$_2$ (1)—R_f = 0.61 (*n*-Bu-OH/ AcOH/H_2O 4:1:1). Rt (HPLC) = 20.24 min. ^{1}HNMR (DMSO-d_6) δ: 1.05 (6H, d, D-Ala CH_3), 2.53 (4H, t, hβ^3Phe hβCH_2), 2.71–2.85 (4H, m, Tyr βCH_2), 2.73–2.80 (4H, m, hβ^3Phe βCH_2), 3.53–3.61 (4H, m, Gly αCH_2), 3.85 (2H, t, Tyr αCH), 4.15 (2H, m, hβ^3Phe αCH), 4.34 (2H, t, D-Ala αCH), 6.65–7.01 (8H, m, Tyr Ar), 7.12– 7.27 (10H, m, hβ^3Phe Ar), 7.88 (2H, d, hβ^3Phe NH), 8.05 (6H, d, Tyr NH_3^+), 8.17 (2H, t, Gly NH), 8.52 (2H, d, D-Ala NH), 9.44 (2H, s, OH), 10.03 (2H, s, NH–NH). ESI-HRMS calcd for $C_{52}H_{62}F_6N_{10}O_{14}$ *m*/*z*: 1165.4429 $[M + H]^+$; found 1165.4431.

2 TFA·(Tyr-D-Ala-βAla-Phe-NH)$_2$ (2)—R_f = 0.41 (*n*-Bu-OH/AcOH/ H_2O 4:1:1). Rt (HPLC) = 19.26 min. ^{1}HNMR (DMSO-d_6) δ: 1.21 (6H, d, D-Ala CH_3), 2.16–2.27 (4H, m, βAla CH_2–CO), 2.70–2.80 (4H, m, Tyr βCH_2), 2.77–2.86 (4H, m, Phe βCH_2), 3.54–3.71 (4H, m, βAla CH_2–N), 3.85 (2H, t, Tyr αCH), 4.38–4.49 (2H, m, D-Ala αCH), 4.52 (2H, t, Phe αCH), 6.69–6.96 (8H, m, Tyr Ar), 7.09–7.25 (10H, m, Phe Ar), 7.76 (2H, d, βAla NH), 8.04 (6H, d, Tyr NH_3^+), 8.20 (2H, d, Phe NH), 8.36 (2H, d, D-Ala NH), 9.36 (2H, s, OH), 10.21 (2H, s, NH–NH). ESI-HRMS calcd for $C_{52}H_{62}F_6N_{10}O_{14}$ *m*/*z*: 1165.4429 $[M + H]^+$; found 1165.4431.

2 TFA·(Tyr-βAla-Gly-Phe-NH)^2 (3)—R_f = 0.39 (*n*-Bu-OH/AcOH/H_2O 4:1:1). Rt (HPLC) = 19.55 min. ^{1}HNMR (DMSO-d_6) δ: 2.27– 2.32 (4H, m, βAla CH_2–CO), 2.67–2.81 (4H, m, Tyr βCH_2), 2.87– 2.92 (4H, m, βAla CH_2–N), 2.91–2.99 (4H, m, Phe βCH_2), 3.28–3.39 (4H, m, Gly αCH_2), 3.77 (2H, t, Tyr αCH), 4.24 (2H, t, Phe αCH), 6.65–6.95 (8H, m, Tyr Ar), 7.11–7.28 (10H, m, Phe Ar), 7.71 (2H, d, βAla NH), 8.07 (6H, d, Tyr NH_3^+), 8.16 (2H, t, Gly NH), 8.22 (2H, d, Phe NH), 9.25 (2H, s, OH), 10.19 (2H, s, NH–NH). ESI-HRMS calcd for $C_{50}H_{58}F_6N_{10}O_{14}$ *m*/*z*: 1137.4116 $[M + H]^+$; found 1137.4120.

2 TFA·(hβ^{3}Tyr-D-Ala-Gly-Phe-NH)$_2$ (4)—R_f = 0.53 (*n*-Bu-OH/ AcOH/H_2O 4:1:1). Rt (HPLC) = 29.48 min. ^{1}HNMR (DMSO-d_6) δ: 1.12 (6H, d, D-Ala CH_3), 2.61 (4H, t, hβ^3Tyr βCH_2), 2.78 (4H, t, hβ^3Tyr hβCH_2), 2.96–3.01 (4H, m, Phe βCH_2), 3.37–3.49 (4H, m, Gly αCH_2), 3.66–3.74 (2H, m, hβ^3Tyr αCH), 4.19–4.23 (2H, m, DAla αCH), 4.60 (2H, m, Phe αCH), 6.60–6.93 (8H, m, hβ^3Tyr Ar), 7.07–7.20 (10H, m, Phe Ar), 7.81 (6H, d, hβ^3Tyr NH_3^+), 8.07 (2H, t, Gly NH), 8.21 (2H, d, Phe NH), 8.33 (2H, d, D-Ala NH), 9.38 (2H, s,

OH), 10.15 (2H, s, NH–NH). ESI-HRMS calcd for $C_{52}H_{62}F_6N_{10}O_{14}$ *m*/*z*: 1165.4429 [M + H]$^+$; found 1165.4426.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

DOR	δ-opioid receptor
EDC	1-ethyl-(3-dimethylaminopropyl)-carbodiimide
ESI-HRMS	electrospray ionization–high resolution mass spectrometry
GPI	guinea pig ileum
[^{3}H]-DAMGO	[^{3}H]-[D-Ala(2), *N*-Me-Phe-(4), Gly-ol(5)] enkephalin
HOBt	1-hydroxybenzotriazole
icv	intracerebroventricular
iv	intravenous
MOR	μ-opioid receptor
MVD	mouse vas deferens
NMM	*N*-methylmorpholine
RP-HPLC	reversed phase high performance liquid chromatography

References

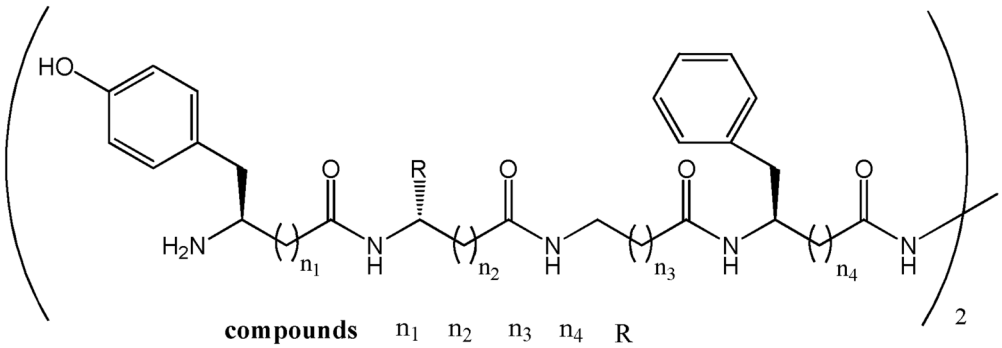
1. (a) Horan PJ, Mattia A, Bilsky EJ, Weber S, Davis TP, Yamamura HI, Malatynska E, Appleyard SM, Slaninova J, Misicka A. Antinociceptive profile of Biphalin, a dimeric enkephalin analog. J Pharmacol Exp Ther. 1993; 265:1446–1454. [PubMed: 8389867] (b) Lipkowski AW, Misicka A, Davis P, Stropova D, Janders J, Lachwa M, Porreca F, Yamamura HI, Hruby V. Biological activity of fragments and analogues of the potent dimeric opioid peptide, Biphalin. Bioorg Med Chem Lett. 1999; 9:2763–2766. [PubMed: 10509931] (c) Silbert BS, Lipkowski AW, Cepeda MS, Szyfelbein SK, Osgood PF, Carr DB. Analgesic activity of a novel bivalent opioid peptide compared to morphine via different routes of administration. Agents Actions. 1991; 3:382–387. [PubMed: 1950824]
2. (a) Flippen-Anderson JL, Deschamps JR, George C, Hruby VJ, Misicka A, Lipkowski AW. Crystal structure of Biphalin sulfate: a multireceptor opioid peptide. J Pept Res. 2002; 59:123–133. [PubMed: 11985706] (b) Abbruscato TJ, Thomas SA, Hruby VJ, Davis TP. Brain and spinal cord distribution of Biphalin: correlation with opioid receptor density and mechanism of CNS entry. J Neurochem. 1997; 69:1236–1245. [PubMed: 9282948]
3. Yamazaki M, Suzuki T, Narita M, Lipkowski AW. The opioid peptide analogue Biphalin induces less physical dependence than morphine. Life Sci. 2001; 69:1023–1028. [PubMed: 11508644]
4. (a) Koda Y, Del Borgo M, Wessling ST, Lazarus LH, Okada Y, Toth I, Blanchfieldm JT. Synthesis and in vitro evaluation of a library of modified endomorphin 1 peptides. Bioorg Med Chem. 2008; 16:6286–6296. [PubMed: 18468445] (b) Tömböly C, Péter A, Tóth G. In vitro quantitative study of the degradation of endomorphins. Peptides. 2002; 23:1573–1580. [PubMed: 12217417]

5. (a) Mollica A, Davis P, Ma SW, Porreca F, Lai J, Hruby VJ. Synthesis and biological activity of the first cyclic Biphalin analogues. Bioorg Med Chem Lett. 2006; 16:367–372. [PubMed: 16275086] (b) Mollica A, Guardiani G, Davis P, Ma SW, Porreca F, Lai J, Mannina L, Sobolev AP, Hruby VJ. Synthesis of stable and potent δ/μ opioid peptides: analogues of H-Tyr-c[D-Cys-Gly-Phe-D-Cys]-OH by ringclosing metathesis. J Med Chem. 2007; 50:3138–3142. [PubMed: 17539621] (c) Zieleniak A, Rodziewicz-Motowidło S, Rusak L, Chung NN, Czaplewski C, Witkowska E, Schiller PW, Ciarkowski J, Izdebski J. Deltorphin analogs restricted via a urea bridge: structure and opioid activity. J Pept Sci. 2008; 14:830–837. [PubMed: 18219707] (d) Weltrowska G, Berezowska I, Lemieux C, Chung NN, Wilkes BC, Schiller PW. N-Methylated cyclic enkephalin analogues retain high opioid receptor binding affinity. Chem Biol Drug Des. 2010; 75:82–88.(e) Hruby VJ. Designing peptide receptor agonists and antagonists. Nature Rev Drug Discovery. 2002; 1:847–858.
6. Seebach D, Beck AK, Bierbaum DJ. The world of *β*- and *γ*-peptides comprised of homologated proteinogenic amino acids and other components. Chem Biodiversity. 2004; 1:1111–1239.
7. (a) Steer DL, Lew RA, Perlmutter P, Smith AI, Aguilar MI. Design and Synthesis of Inhibitors Incorporating *β*-Amino Acids of Metalloendopeptidase EC 3.4.24.15. J Pept Sci. 2000; 6:470–477. [PubMed: 11016884] (b) Seebach D, Abele S, Gademann K, Guichard G, Hintermann T, Jaun B, Matthews JL, Schreiber JV, Oberer L, Hommel U, Widmer H. β^2- and β^3-Peptides with Proteinaceous Side Chains: Synthesis and Solution Structures of Constitutional Isomers, a Novel Helical Secondary Structure and the Influence of Solvation and Hydrophobic Interactions on Folding. Helv Chim Acta. 2003; 86:2098–2103.
8. (a) Seebach D, Matthews JL. *β*-Peptides: a surprise at every turn. Chem Commun. 1997; 21:2015–2126.(b) Seebach D, Abele S, Gademann K, Guichard G, Hintermann T, Jaun B, Matthews JL, Schreiber JV, Oberer L, Hommel U, Widmer H. β^2- And β^3-Peptides with Proteinaceous Side Chains: Synthesis and Solution Structures of Constitutional Isomers, a Novel Helical Secondary Structure and the Influence of Solvation and Hydrophobic Interactions on Folding. Helv Chim Acta. 1998; 81:932–982.
9. (a) Stachowiak K, Khosla MC, Plucinska K, Khairallah PA, Bumpus FM. Synthesis of angiotensin II analogues by incorporating *β*-homotyrosine or *β*-homoisoleucine residues. J Med Chem. 1979; 9:1128–1130. [PubMed: 490561] (b) Zablocki JA, Tjoeng FS, Bovy PR, Miyano M, Garland RB, Williams K, Schretzman L, Zupec ME, Rico JG, Lindmark RJ, Toth MV, McMakins DE, Adams SP, Panzer-Knodle SG, Nicholson NS, Taite BB, Salyers AK, King LW, Campion JG, Feigen LP. A novel series of orally active antiplatelet agents. Bioorg Med Chem. 1995; 5:539–551. [PubMed: 7648203] (c) Giordano C, Lucente G, Mollica A, Nalli M, Pagani Zecchini G, Paglialunga Paradisi M, Gavuzzo E, Mazza F, Spisani S. Hybrid α/β^3-peptides with proteinogenic side chains. Monosubstituted analogues of the chemotactic tripeptide For-Met-Leu-Phe-OMe. J Pept Sci. 2004; 10:510–523. [PubMed: 15347138]
10. (a) Wilczyńska D, Kosson P, Kwasiborska M, Ejchart A, Olma A. Synthesis and receptor binding of opioid peptide analogues containing β^3-homo-amino acids. J Pept Sci. 2009; 15:777–782. [PubMed: 19787815] (b) Bozü B, Fülöp F, Tóth GK, Tóth G, Szücs M. Synthesis and opioid binding activity of dermorphin analogues containing cyclic β-amino acids. Neuropeptides. 1997; 31:367–372. [PubMed: 9308025] (c) Cardillo G, Gentilucci L, Qasem AR, Sgarzi F, Spampinato S. Endomorphin-1 analogues containing β-proline are μ-opioid receptor agonists and display enhanced enzymatic hydrolysis resistance. J Med Chem. 2002; 45:2571–2578. [PubMed: 12036366]
11. Misicka A, Lipkowski AW, Horvath R, Davis P, Kramer TH, Yamamura HI, Hruby VJ. Topographical requirements for delta opioid ligands: common structural features of dermenkephalin and deltorphin. Life Sci. 1992; 51:1025–1032. [PubMed: 1326067]
12. Szekeres PG, Traynor JR. Delta opioid modulation of the binding of guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate to NG108-15 cell membranes: characterization of agonist and inverse agonist effects. J Pharmacol Exp Ther. 1997; 283:1276–1284. [PubMed: 9400003]
13. Yamamoto T, Nair P, Largent-Milnes TM, Jacobsen NE, Davis P, Ma SW, Yamamura HI, Vanderah TW, Porreca F, Lai J, Hruby VJ. Discovery of a potent and efficacious peptide derivative for *δ*/*μ* opioid agonist/neurokinin 1 antagonist activity with a 2′,6′-dimethyl-L-tyrosine: in vitro, in vivo, and NMR-based structural studies. J Med Chem. 2011; 54:2029–2038. [PubMed: 21366266]

14. Pieretti S, Di Giannuario A, De Felice M, Perretti M, Cirino G. Stimulus-dependent specificity for annexin 1 inhibition of the inflammatory nociceptive response: the involvement of the receptor for formylated peptides. Pain. 2004; 109:52–63. [PubMed: 15082126]

15. Al-Fayoumi SI, Brugos B, Arya V, Mulder E, Eppler B, Mauderli AP, Hochhaus G. Identification of stabilized dynorphin derivatives for suppressing tolerance in morphine-dependent rats. Pharm Res. 2004; 21:1450–1456. [PubMed: 15359581]

16. (a) Shimohigashi Y, Costa T, Chen HC, Rodbard D. Dimeric tetrapeptide enkephalins display extraordinary selectivity for the δ opiate receptor. Nature. 1982; 297:333–335. [PubMed: 6281658] (b) Mollica A, Davis P, Ma SW, Lai J, Porreca F, Hruby VJ. Synthesis and biological evaluation of new Biphalin analogues with non-hydrazine linkers. Bioorg Med Chem Lett. 2005; 15:2471–2475. [PubMed: 15863299] (c) Mollica A, Pinnen F, Feliciani F, Stefanucci A, Lucente G, Davis P, Porreca F, Ma SW, Lai J, Hruby VJ. New potent Biphalin analogues containing p-fluoro-L-phenylalanine at the 4,4′ positions and nonhydrazine linkers. Amino Acids. 2011; 40:1503–1511. [PubMed: 20924622] (d) Leone S, Chiavaroli A, Orlando G, Mollica A, Di Nisio C, Brunetti L, Vacca M. The analgesic activity of Biphalin and its analog AM 94 in rats. Eur J Pharmacol. 2012; 685:70–73. [PubMed: 22546229]



compounds	n_1	n_2	n_3	n_4	R
Biphalin	0	0	0	0	$-CH_3$
1	0	0	0	1	$-CH_3$
2	0	0	1	0	$-CH_3$
3	0	1	0	0	-H
4	1	0	0	0	$-CH_3$

Figure 1.
Structure of biphalin and analogues **1–4**.

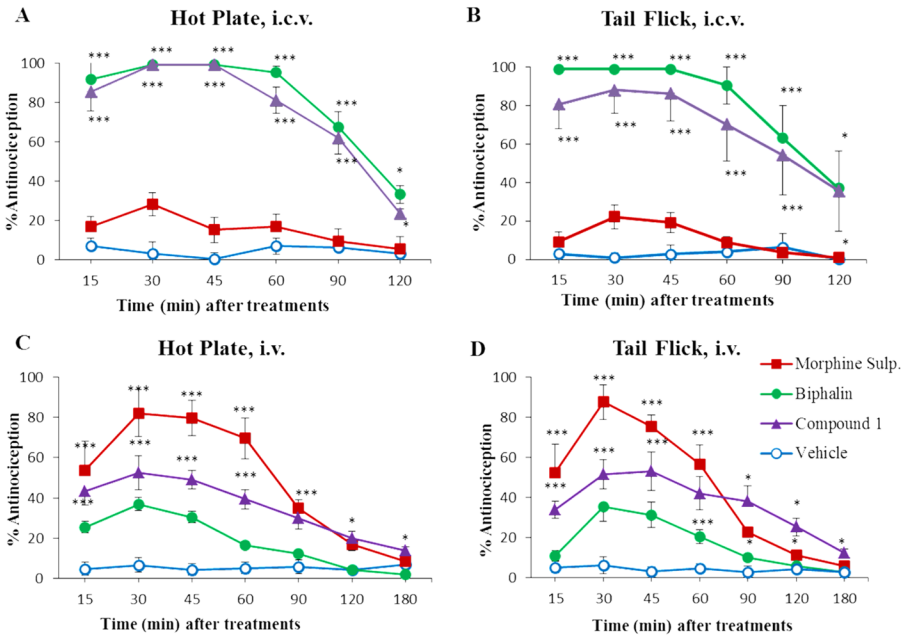


Figure 2.

Antinociceptive results of hot plate and tail-flick in vivo bioassays for compound **1**, Biphalin, and morphine sulfate. Compounds were injected by icv administration (A,B) at a dose of 0.6 nmol, and systemic iv administration (C,D) at a dose of 3000 nmol. The data represent the mean ± SEM. The statistical significance among groups was determined, in comparison with vehicle-treated animals with the analysis of variance (two-way ANOVA test) followed by Bonferroni's posthoc comparisons using the statistical software SPSS. Statistical significance was $P < 0.05$ (*$P < 0.05$; ***$P < 0.001$).

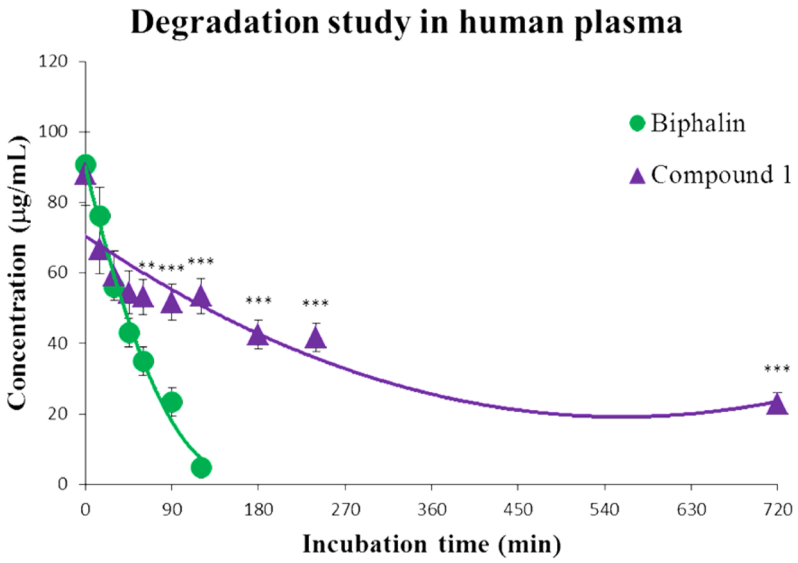
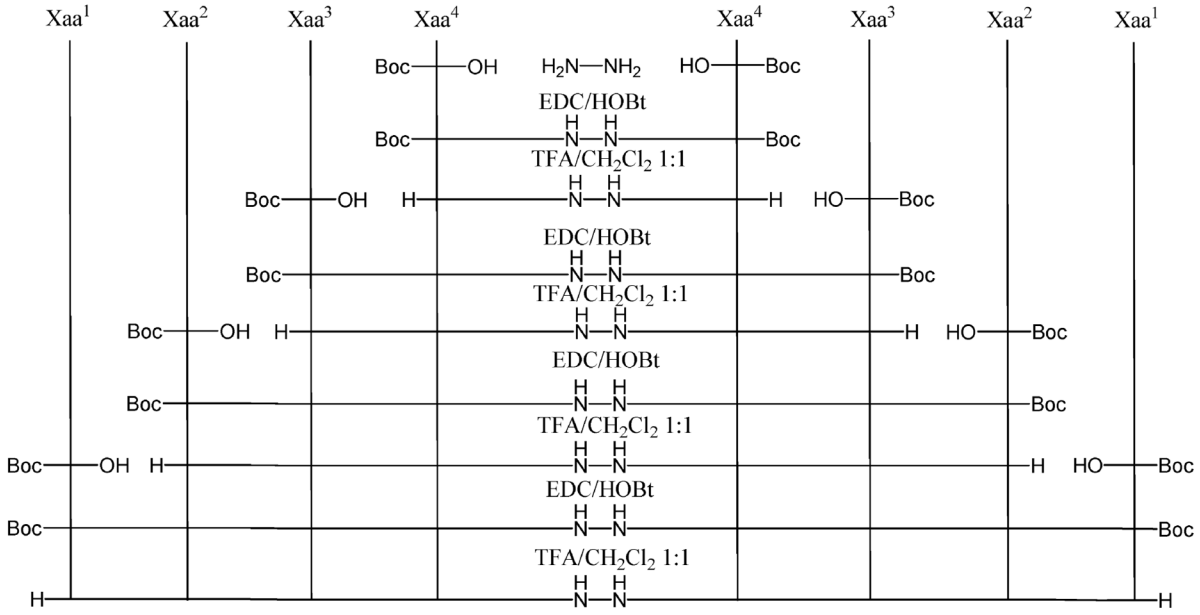


Figure 3.

Stability curves for Biphalin (green line) and the Biphalin derivative **1** (purple line). The samples were tested in three independent experiments ($n = 3$) and represent the mean ± SEM. The significance among groups was evaluated with the analysis of variance (two-way ANOVA test) followed by Bonferroni's posthoc comparisons between compound **1** and Biphalin using the statistical software GraphPad Prism v.4. Statistical significance was $P < 0.05$ (**$P < 0.01$; ***$P < 0.001$); times from t_0 to t_{45} present no statistical significance ($P > 0.05$).



Scheme 1.

Synthesis of Biphalin Analogues 1–4[a]

[a](**1**) Xaa4 = hβ3Phe; Xaa3 = Gly; Xaa2 = D-Ala; Xaa1 = Tyr; (**2**) Xaa4 = Phe; Xaa3 = βAla; Xaa2 = D-Ala; Xaa1 = Tyr; (**3**) Xaa4 = Phe; Xaa3 = Gly; Xaa2 = βAla; Xaa1 = Tyr; (**4**) Xaa4 = Phe; Xaa3 = Gly; Xaa2 = D-Ala; Xaa1 = hβ3Tyr.

Table 1

Binding Affinities and in Vitro Activities for Peptide Derivatives at δ/μ Opioid Receptors

	hDOR[a], [3H]Deltorphin[b]		rMOR[a], [3H]DAMGO[c]			MVD (δ)	GPI (μ)	
compd	**log IC_{50}[d]**	**K_i (nM)**	**log IC_{50}[d]**	**K_i (nM)**	K_i^{μ}/K_i^{δ}	**IC_{50} (nM)[e,f]**	**IC_{50} (nM)[e,f]**	**IC_{50} (GPI)/IC_{50} (MVD)**
1	−8.84 ± 0.11	0.72	−8.64 ± 0.07	1.1	1.53	33 ± 9.4	50 ± 6.5	1.5
2	−4.97 ± 0.16	240	−6.42 ± 0.08	172	0.72	1300 ± 221	9.2% at 1 μM	>0.75
3	−8.75 ± 0.11	450	−6.90 ± 0.08	57	0.13	1800 ± 266	1700 ± 306	0.9
4	−8.27 ± 0.14	480	−6.87 ± 0.11	62	0.13	710 ± 97	670 ± 139	0.9
Bph[g]		2.6		1.4	0.54	2.7 ± 1.5	8.8 ± 0.3	3.2

[a] Displacement of [3H]Deltorphin (μ-selective) and [3H]DAMGO (δ-selective) from rat brain membrane binding site.

[b] K_d = 0.50 ± 0.3 nM.

[c] K_d = 0.50 ± 0.1 nM.

[d] The log IC_{50} ± standard error are expressed as logarithmic values determined from the nonlinear regression analysis of data collected from at least two independent experiments performed in duplicate. The K_i values are calculated using the Cheng and Prusoff equation to correct for the concentration of the radioligand used in the assay.

[e] Concentration at 50% inhibition of muscle contraction in electrically stimulated isolated tissues (n = 4).

[f] ±SEM.

[g] Biphalin (Bhp), refs 1b and 13.