Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 367–372

Synthesis and biological activity of the first cyclic biphalin analogues

Adriano Mollica,[a,c] Peg Davis,[b] Shou-Wu Ma,[b] Frank Porreca,[b] Josephine Lai[b] and Victor J. Hruby[a,*]

[a]*Department of Chemistry, University of Arizona, Tucson, AZ 85721, USA*
[b]*Department of Pharmacology, University of Arizona, Tucson, AZ 85721, USA*
[c]*Dipartimento di Studi Farmaceutici, Universita' di Roma "La Sapienza," P.le A. Moro 5, Rome 00185, Italy*

Received 10 August 2005; revised 23 September 2005; accepted 28 September 2005
Available online 3 November 2005

Abstract—Biphalin is a linear octapeptide with strong opioid activity. Its structure is based on two identical sequences derived from enkephalins joined C-terminal to C-terminal by an hydrazide bridge (Tyr-D-Ala-Gly-Phe-NH-NH ← Phe ← Gly ← D-Ala ← Tyr). In this study we present the design, synthesis, and biological evaluation of the first cyclic biphalin analogues. D-Alanine residues in positions 2, 2′ of the parent peptide were replaced by D- and L-cysteine and an intramolecular disulfide bond between the cysteine thiol groups was introduced. We obtained two cyclic analogues with quite different biological profiles.

Determining structure–activity relationships (SAR) for peptides is a stepwise process. The first step may involve many points: (a) replacement of one or more amino acid residues in the primary sequence of native peptide; (b) alanine and D-amino acid scans; (c) truncation, deletion and then evaluation of the biological effects of those modifications, etc.[1] For small linear peptides with flexible conformations such as enkephalins and their potent analogues such as biphalin, the design of conformationally restricted analogues is not obvious. Such a family of compounds forms more compact conformations interacting with the receptor. In this case, the goal is to determine which residues, sequentially separated, may approach each other as a result of the peptide–receptor interaction. Once a hypothesis regarding this has been made, the two involved residues can be replaced by cysteine or penicillamine and then the oxidation of their side chains will form a cyclic peptide with a disulfide bond (Fig. 1).

Disulfide bonds are present in natural proteins and peptides and represent a key structural element in modulating protein tertiary structure. Although many different approaches to cyclization could be adopted, the disulfide based approach was the preferred choice for the present study.

In an effort to develop highly potent and selective ligands based on the endogenous opioid peptide enkephalins (H-Tyr-Gly-Gly-Phe-Leu-OH and H-Tyr-Gly-Gly-Phe-Met-OH),[2a] conformationally and topographically constrained cyclic analogues of enkephalin such as *c*[D-Pen[2], D-Pen[5]] enkephalin (DPDPE) were previously designed.[2] In this case, the Gly[2] and Met[5] (or Leu[5]) residues were replaced by D-penicillamine and a 14-membered ring containing a disulfide bond was formed. DPDPE was found to be highly potent and selective for

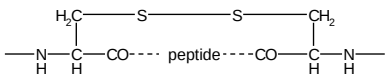


Figure 1. Disulfide cyclization.

Keywords: Biphalin; Cyclic peptides.
Abbreviations: ^{3}H-DAMGO, [D-Ala(2),N-Me-Phe(4),Gly-ol(5)]enkephalin; ^{3}H-DPDPE, [^{3}H]-c[2-D-penicillamine,5-D-penicillamine]enkephalin; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; EDC, 1-ethyl-(3-dimethyl-aminopropyl)carbodiimide; GPI/LMMP, Guinea pig ileum/longitudinal muscle myenteric plexus (μ opioid receptors); GTP, guanosine triphosphate; hMOR, human μ-opioid receptor; HOBt, 1-hydroxybenzotriazole; MVD, mouse vas deferens (δ-opioid receptors); rDOR, rat δ-opioid receptor; TEA, triethylamine; TFA, trifluoroacetic acid.
* Corresponding author. Tel.: +1 520 621 6332; fax: +1 520 621 8407; e-mail: hruby@u.arizona.edu

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.09.080

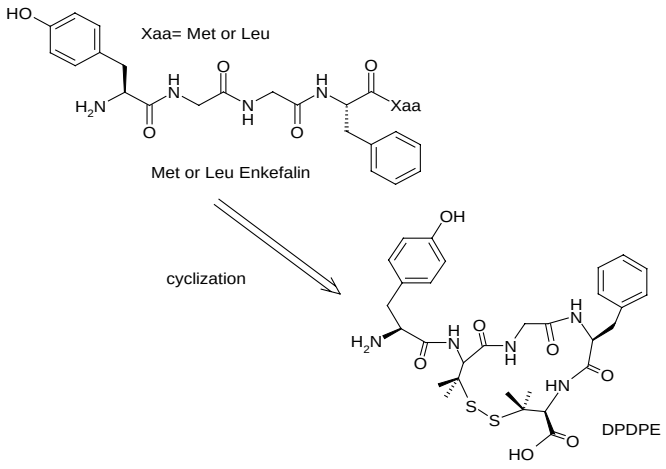


Figure 2. Enkephalin and DPDPE.

the δ-opioid receptor[2d] and to be completely stable to proteolytic breakdown in vitro and in vivo[2] (Fig. 2). However, although a variety of cyclic enkephalin analogues[3] have been described, the cyclization approach has not been applied so far to bivalent analogues such as biphalin. Due to the entropic factors bound to the presence of two pharmacophoric moieties in the molecule, bivalent ligands are expected to exhibit biological potency higher than that of the monomers. Thus, the synthesis of ligands which combine the favourable properties of both cyclic and bivalent ligands appears to be a promising approach.

Biphalin is a flexible, linear octapeptide (Tyr-D-Ala-Gly-Phe-NH-NH ← Phe ← Gly ← D-Ala ← Tyr) which binds to both μ- and δ-opioid receptors with an IC_{50} of about 1–5 nM and exhibits a potent nociceptive effect and low side-effects, in particular to produce no dependency on chronic use.[4c] In the light of the above reported data and considerations and taking into account the interest of obtaining opioid peptides with very high potency, we report here the design and synthesis of two 22-membered cyclic analogues of biphalin. They were obtained by closing a disulfide bridge between two cysteine residues located in positions 2 and 2′ of the backbone of biphalin as shown in Figure 3. Two different structures were obtained by replacing the D-Ala residues of biphalin with two D-Cys or two Cys residues. As a consequence of this design strategy, the D-Cys containing cyclic models **8** and **10** maintain the original biphalin heterochiral structure (mixed L and D amino acids), whereas the Cys containing isomers **7** and **9** present the unusual homochiral L-sequence. However, these new models, at variance with standard cyclopeptides, present an inversion of the direction of the amide bonds caused by the hydrazine bridge joining the two Phe residues which behave as *gem*-diamines in the retro-inverso peptide isomers. As a consequence of this structural feature, the two flanking backbone fragments, departing from the NH–NH moiety of hydrazine, present the same direction of the peptide bonds (Fig. 4).

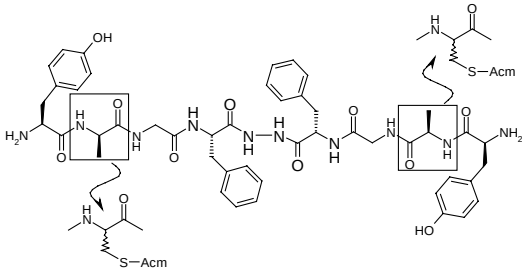


Figure 3. Biphalin. Positions 2, 2′ are marked. D-Alanine residues were substituted with D-cysteine or L-cysteine residues.

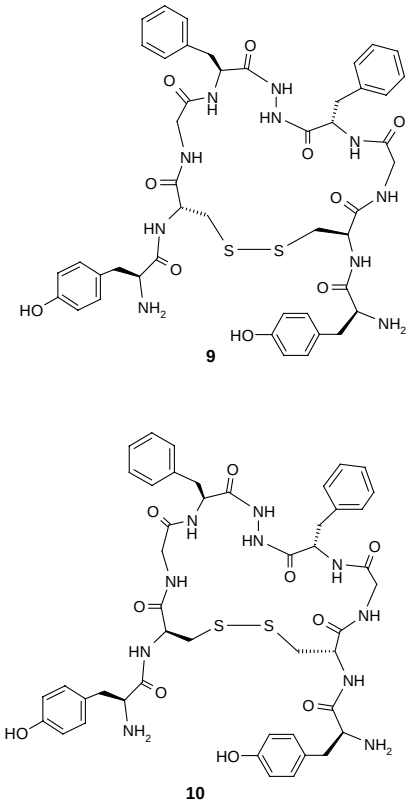


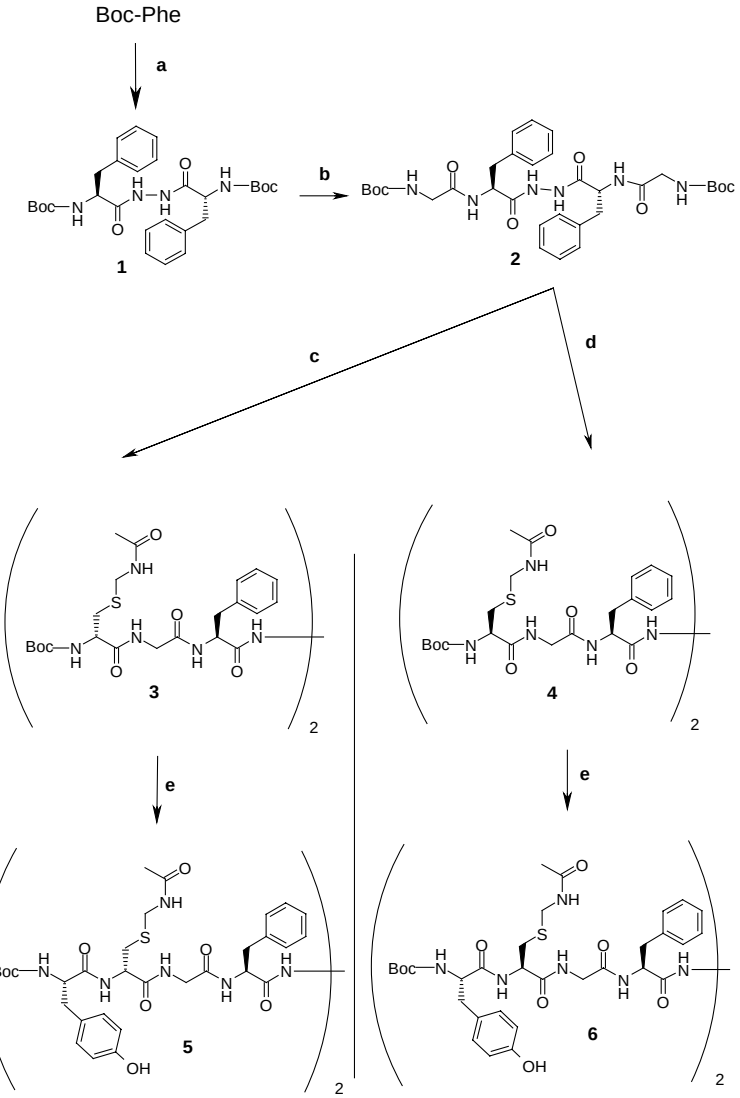
Figure 4. Representation of the molecular structure of the final products **9** and **10**.

The synthesis of the compounds was performed in solution following the N^{α}-Boc strategy. Coupling reactions were performed using the standard method of HOBt·H_2O/EDC/TEA in DMF.[5] The symmetric and linear N^{α}-Boc-protected peptides **5** and **6** were synthesized by elongation of the amino acid sequence simultaneously in both directions starting from the hydrazine group (Scheme 1).

The acetamidomethyl (Acm) group was adopted since it offers an orthogonal protection with the Boc group and allows, at the same time, a one-pot deprotection-oxidation reaction. Cyclization was obtained by dissolving the pure N^{α}-Boc, *S*-Acm-protected linear peptides **5** and **6** (100 mg) in 350 mL of DMSO/water/acetic acid (1:2:3). I_2 (51 mg) was added to the stirred solution at rt. After 3 h, ascorbic acid (60 mg) was added to quench the excess iodine, and then the solvent was evaporated in high vacuum. The orange oily residue was charged into a semi-preparative HPLC column and the pure N^{α}-Boc-protected products were collected and lyophilized (yield 40% ca.).[6]

Deprotection of the *tert*-butyloxycarbonyl groups (Boc) was performed by dissolving the products in a mixture of TFA 50% in DCM for 30 min at rt, under nitrogen atmosphere. The DCM and the TFA were removed under vacuum. The resultant intermediate products were used in the next step without further purification as TFA salts.

All the N^{α}-Boc-protected intermediate products **1**–**8** were purified by reverse-phase semi-preparative high-performance liquid chromatography (HPLC), isolated and characterized by mass spectra and NMR. The final prod-



Scheme 1. Reagents and conditions: (a) Boc-Phe-OH (2.2 equiv), EDC (2.2 equiv), HOBt·H_2O (2.2 equiv), TEA (3.2 equiv), NH_2 NH_2 (1 equiv), DMF (20 mL), 30 min 0 °C, then 12 h rt; (b) TFA 50 % in DCM 30 min rt under N_2 atm, then Boc-Gly-OH (2.2 equiv), EDC (2.2 equiv), HOBt·H_2O (2.2 equiv), TEA (3.2 equiv), DMF (20 mL), 30 min 0 °C, then 12 h rt; (c) TFA 50% in DCM, 30 min rt under N_2 atm, then Boc-D-Cys(Acm)-OH (2.2 equiv), EDC (2.2 equiv), HOBt·H_2O (2.2 equiv), TEA (3.2 equiv), DMF (20 mL), 30 min 0 °C, then 12 h rt; (d) TFA 50% in DCM, 30 min rt under N_2 atm, then Boc-Cys(Acm)-OH (2.2 equiv), EDC (2.2 equiv), HOBt·H_2O (2.2 equiv), TEA (3.2 equiv), DMF (20 mL), 30 min 0 °C, then 12 h rt; (e) TFA 50% in DCM, 30 min rt under N_2 atm, then Boc-Tyr-OH (2.2 equiv), EDC (2.2 equiv), HOBt·H_2O (2.2 equiv), TEA (3.2 equiv), DMF (20 mL), 30 min 0 °C, then 12 h rt (overall yield 50–60%).

ucts **9** and **10** were purified as TFA salts by reverse-phase HPLC using a semi-preparative Vydac (C_{18}-bonded, 300 Å) column and a gradient elution at a flow rate of 10 mL/min. The gradient used was 10–90% acetonitrile in 0.1% aq TFA over 50 min. Approximately 20 mg of crude peptide was injected each time, and the fractions containing the purified peptide were collected and lyophilized to dryness. The purity, determined by NMR analysis and by analytic reverse-phase HPLC, monitored at 220, 254, 280 and 350 nm, was found to be >98%.

In order to confirm the molecular structures, all NMR spectra were acquired on a Bruker DRX-500 spectrometer at 25 °C using a Nalorac triple-resonance single-axis gradient 3-mm probe, processed with the Bruker software XWINNMR. NMR analyses of the products were performed in DMSO-d_6 at a peptide concentration of 2 mM ca. (for analytical data, see Ref. 10).

Receptor binding affinities to the δ- and μ-opioid receptors were performed using cell membrane preparations from transfected cells that stably express the respective receptor type and were evaluated as previously described.[7] The ligands used were [^{3}H]DPDPE and [^{3}H]DAMGO for δ and μ receptors, respectively.

We used [^{35}S]GTP-γ-*S* binding to examine opioid agonist efficacy, for functional characterization of the ligands at the δ- and μ-opioid receptors, which are members of the seven transmembrane G-protein-coupled receptor super family. Agonist efficacy can be determined at the level of receptor G-protein interaction by measuring agonist-stimulated binding with a non-hydrolysable GTP analogue. The ability of μ and δ opioid agonists to activate G-proteins has been demonstrated by studying the binding of the GTP analogue guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTP-γ-*S*). The opioid receptor mediated assay was performed as previously described.[8] Cells expressing hDOR for δ receptor (or rMOR for μ receptor) were incubated with increasing concentrations of the test compounds in the presence of 0.1 nM [^{35}S]GTP-γ-*S* (1000–1500 Ci/mmol, MEN, Boston, MA) in assay buffer (total volume of 1 mL, duplicate samples) as a measure of agonist-mediated G-protein activation. After incubation (90 min, 30 °C), the reaction was terminated by rapid filtration under vacuum through Whatman GF/B glass fibre filters, followed by four washes with ice-cold 15 mM Tris/120 mM NaCl, pH 7.4. Filters were pretreated with assay buffer prior to filtration to reduce non-specific binding. Bound reactivity was measured by liquid scintillation spectrophotometry after an overnight extraction with EcoLite (ICN, Biomedicals, Costa Mesa, CA) scintillation cocktail. The data were analyzed using GraphPad Prism Software (San Diego, CA).

The in vitro tissue bioassays (MVD and GPI/LMMP) were performed as described previously.[9] IC_{50} values represent means of no less than four experiments. IC_{50} values, relative potency estimates and their associated standard errors were determined by fitting the data to the Hill equation by a computerized non-linear least-squares method. All biological data are summarized in Table 1.

In conclusion, we have synthesized the first examples of cyclic analogues of biphalin. Cyclization of peptides is, in fact, a useful approach to develop diagnostic and therapeutic peptidic and peptidomimetic agents. The new cyclic ligands show high in vitro bioactivity (Table 1). As expected, compound **10**, containing D-Cys at the positions 2 and 2′ in place of D-Ala residues of biphalin, reveals binding affinity and bioactivity higher than those of the product **9** built with Cys. Although the binding value of isomer **10** is close to that of biphalin, the GTP binding and E_{max} are surprisingly high. In particular, compound **10** shows a capacity to activate the transduction to the δ receptor (E_{max} = 100%) higher than that to the μ receptor (E_{max} = 47%).

This is in contrast with its binding values which are very similar for both the receptors. The analysis of the data indicates that biphalin and compound **9** are partial agonists at both μ and δ receptors. Compound **10** is a full agonist at the δ receptor and a partial agonist at the μ receptor. Its ability to partially stimulate the μ-opioid receptor is also appreciable by taking into account the fact that the high biphalin analgesic activity seems to be related to its ability to bind both δ- and μ-opioid receptors.[4c] Furthermore, although a certain degree of selectivity is observed for both the isomers **9** and **10**, this is certainly low as already found in the case of biphalin.

In summary, the in vitro studies reported here are certainly promising. However, the evaluation of the therapeutic interest bound to compound **10** high ability to activate the transduction mechanism requires further in vivo studies.

Acknowledgment

This work was supported by a grant from the U.S., Public Health Service, National Institute of Drug Abuse, DA 06284.

Table 1. Binding affinity, GTP binding assay, E_{max} % (net total bound/basal binding × 100), and in vitro activity

Drugs	Binding IC_{50}[a] (nM)		GTP binding IC_{50}[a,b] (nM)				Bioassay IC_{50}[a] (nM)	
	δ	μ	δ	E_{max} (%)	μ	E_{max} (%)	MVD	GPI/LMMP
Biphalin	2.6 ± 0.4[c]	1.4 ± 0.2[c]	2.5 ± 0.5	27 ± 3.5	6 ± 0.2	25 ± 4.7	27 ± 1.5[c]	8.8 ± 0.3[c]
9	53 ± 10.5	130 ± 230	260 ± 100	58 ± 18	120 ± 34	57 ± 2.2	570 ± 130	420 ± 48
10	0.87 ± 0.1	0.60 ± 0.2	0.87 ± 0.3	100 ± 2.3	0.2 ± 0.1	47 ± 5.7	9.9 ± 1.3	25 ± 7.9

[a] ±SEM.
[b] Reference compound: cold [^{35}S]GTP-γ-*S*.
[c] Data according to Ref. 9.

References and notes

1. (a) Hruby, V. J.; Slate, C. A. *Adv. Amino Acid Mimetics Peptidomimetics* **1999**, *2*, 191; (b) Hruby, V. J.; Mosberg, H. I. *Peptides* **1982**, *3*, 329; (c) Hruby, V. J. *Methods Drugs Res.* **1989**, *3*, 43; (d) Hruby, V. J. *J. Med. Chem.* **2003**, *46*, 4215; (e) Cowell, S. M.; Le, Y. S.; Cain, J. O.; Hruby, V. J. *Curr. Med. Chem.* **2004**, *11*, 2785.
2. (a) Hughes, J.; Smith, T. W.; Kosterlitz, H. W.; Fothergill, L. A.; Morgan, B. A.; Morris, H. R. *Nature* **1975**, *258*, 577; (b) DiMaio, J.; Schiller, P. W. *Proc. Nat. Acad. Sci. U.S.A.* **1980**, *77*, 7162; (c) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Galligan, J. J.; Burks, T. F.; Gee, K.; Yamamura, H. I. *Biochem. Biophys. Res. Commun.* **1982**, *106*, 506; (d) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *89*, 5871; (e) Weber, S. J.; Greene, D. L.; Sharma, S. D.; Yamamura, H. I.; Kramer, T. H.; Burks, T. F.; Hruby, V. J.; Hersh, L. B.; Davis, T. P. *J. Pharmacol. Exp. Therap.* **1991**, *259*, 1109; (f) Hruby, V. J.; Bartosz-Bechowski, H.; Davis, P.; Slaninova, J.; Zalewska, T.; Stropova, D.; Porreca, F.; Yamamura, H. I. *J. Med. Chem.* **1997**, *40*, 3957.
3. (a) Shuldiner, A. R.; Newland, R. J.; Rosenblatt, M. *Arch. Biochem. Biophys.* **1985**, *238*, 111; (b) Richman, S. J.; Goodman, M.; Nguyen, T. M.; Schiller, P. W. *Int. J. Pept. Prot. Res.* **1985**, *25*, 648; (c) Bobrova, I. V.; Myshliakova, N. A.; Papsuevich, O. S.; Vosekalna, I. A.; Mekshun, E. I. *Bioorg. Khim* **1995**, *21*, 275; (d) Siemion, I. Z.; Szewczuk, Z.; Herman, Z. S.; Stachura, Z. *Mol. Cell. Biochem.* **1981**, *34*, 23.
4. (a) Lipkowski, A. J.; Koneka, A. M.; Sroczynska, I. *Peptides* **1982**, *3*, 697; (b) Lipkowski, A. W.; Konopka, M.; Osipiak, B.; Gumulka, S. W. In Peptides 1982, *Proceedings of the 17th European Peptide Symposium*; de Gruyter: Berlin, 1983; pp 481–486; (c) Horan, P. J.; Mattia, A.; Bilsky, E. J.; Weber, S. J.; Davis, T. P.; Yamamura, H. I.; Malatynska, E.; Appleyard, S. M.; Slaninova, J.; Misicka, A.; Lipkowski, A. W.; Hruby, V. J.; Porreca, F. *J. Pharmacol. Exp. Ther.* **1993**, *265*, 1446; (d) Lipkowski, A. W.; Misicka, A.; Davis, P.; Stropova, D.; Janders, J.; Lachwa, M.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2763; (e) Misicka, A.; Lipkowski, A. W.; Stropova, D.; Yamamura, H. I.; Davis, P.; Porreca, F.; Hruby, V. J. *Peptide Sci.* **1999**, 726.
5. Han, So.-Y.; Kim, Y.-A. *Tetrahedron* **2004**, *60*, 2447.
6. Cuthbertson, A.; Indrevoll, B. *Org. Lett.* **2003**, *5*, 2955.
7. (a) Polt, R.; Porreca, F.; Szabo, L.; Bilsky, E. J.; Davis, T. P.; Horvath, R.; Abbruscato, T. J.; Yamamura, H. J.; Hruby, V. J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7114; (b) Misicka, A.; Lipkowski, A. W.; Horvath, R.; Davis, P.; Kramer, T. H.; Yamamura, H. I.; Hruby, V. J. *Life Sci.* **1992**, *51*, 1025.
8. (a) Hosohata, K.; Varga, E. V.; Alfaro-Lopez, J.; Tang, X.; Vanderah, T. W.; Porreca, F.; Hruby, V. J.; Roeske, W. R.; Yamamura, H. I. *J. Pharmacol. Exp. Ther.* **2003**, *304*, 683; (b) Wang, A.; Gardell, L. R.; Ossipov, M. H.; Vanderah, T. W.; Brennan, M. B.; Hochgeschwender, U.; Hruby, V. J.; Malan, T. P., Jr.; Lai, J.; Porreca, F. *J. Neurosci.* **2001**, *21*, 1779.
9. Kramer, T. H.; Davis, P.; Hruby, V. J.; Burks, T. F.; Porreca, F. *J. Pharmacol. Exp. Ther.* **1993**, *266*, 577.
10. NMR and MS data: Boc-Phe-NH-NH ← Phe-Boc (1): ^{1}H NMR δ: 1.27 (18H, s, Boc), 2.76–3.05 (4H, m, Phe βCH_2), 4.26 (2H, m, Phe αCH), 6.95 (2H, d, Phe NH), 7.19–7.38 (10H, m, Ar), 10.15 (2H, s, NH–NH). FAB-MS *m/e* 527.1 (M^+). Boc-Gly-Phe-NH-NH ← Phe ← Gly-Boc (2): ^{1}H NMR δ: 1.40 (18H, s, Boc), 2.80–3.05 (4H, m, Phe βCH_2), 3.44–3.59 (4H, m, Gly αCH_2), 4.61 (2H, m, Phe αCH), 6.86 (2H, m, Gly NH), 7.18–7.27 (10H, m, Ar), 8.03 (2H, d, Phe NH), 10.18 (2H, s, NH–NH). FAB-MS *m/e* 641.0 (M^+). Boc-Cys(Acm)-Gly-Phe-NH-NH ← Phe ← -Gly ← Cys(Acm)-Boc (4): ^{1}H NMR δ: 1.40 (18H, s, Boc), 1.85 (6H, s, CH_3-CO), 2.65 and 2.88 (4H, m, Cys βCH_2), 2.75 and 3.08 (4H, m, Phe βCH_2), 3.65–3.78 (4H, m, Gly αCH_2), 4.18–4.28 (6H, m, Acm CH_2 and Cys αCH), 4.60 (2H, m, Phe αCH), 6.96 (2H, d, Cys NH) 7.18–7.31 (10H, m, Ar), 7.95 (2H, m, Gly NH), 8.16 (2H, d, Phe NH), 8.45 (2H, m, Acm NH), 10.18 (2H, s, NH–NH). FAB-MS *m/e* 989.0 (M^+). Boc-Tyr-Cys(Acm)-Gly-Phe-NH-NH ← Phe ← Gly ← Cys(Acm) ← Tyr- Boc (6): ^{1}H NMR δ: 1.30 (18H, s, Boc), 1.82 (6H, s, CH_3CO), 2.63 and 2.89 (4H, m, Tyr βCH_2), 2.74 and 2.90 (4H, m, Cys βCH_2), 2.81–3.05 (4H, m, Phe βCH_2), 3.65–3.80 (4H, m, Gly αCH_2), 4.12 (4H, m, Acm CH_2), 4.15 (2H, m, Tyr αCH), 4.51 (2H, m, Cys αCH), 4.62 (2H, m, Phe αCH), 6.62 and 7.05 (8H, dd, Tyr aromatic), 6.82 (2H, m, Tyr NH), 7.15–7.32 (10H, m, Ar), 8.07–8.12 (4H, m, Gly NH and Cys NH), 8.20 (2H, d, Phe NH), 8.46 (2H, m, Acm NH), 9.12 (2H, br, Tyr OH), 10.22 (2H, s, NH–NH). FAB-MS *m/e* 1315.0 (M^+). Boc-Tyr-Cys(Acm)-Gly-Phe-NH-NH ← Phe ← Gly ← Cys(Acm) ← Tyr- Boc (6): ^{1}H NMR δ: 1.30 (18H, s, Boc), 1.82 (6H, s, CH_3CO), 2.63 and 2.89 (4H, m, Tyr βCH_2), 2.74 and 2.90 (4H, m, Cys βCH_2), 2.81–3.05 (4H, m, Phe βCH_2), 3.65–3.80 (4H, m, Gly αCH_2), 4.12 (4H, m, Acm CH_2), 4.15 (2H, m, Tyr αCH), 4.51 (2H, m, Cys αCH), 4.62 (2H, m, Phe αCH), 6.62 and 7.05 (8H, dd, Tyr aromatic), 6.82 (2H, m, Tyr NH), 7.15–7.32 (10H, m, Ar), 8.07–8.12 (4H, m, Gly NH and Cys NH), 8.20 (2H, d, Phe NH), 8.46 (2H, m, Acm NH), 9.12 (2H, br, Tyr OH), 10.22 (2H, s, NH–NH). FAB-MS *m/e* 1315.0 (M^+). Boc-Tyr-c[Cys-Gly-Phe-NH-NH ← Phe ← -Gly ← Cys] ← Tyr-Boc (7): ^{1}H NMRδ: 1.32 (18H, s, Boc), 2.60 and 2.85 (4H, m, Tyr βCH_2), 2.78 and 3.10 (4H, m, Cys βCH_2), 2.86–3.08 (4H, m, Phe βCH_2), 3.55–3.70 (4H, m, Gly αCH_2), 4.15 (2H, m, Tyr αCH), 4.55 (4H, m, Phe αCH and Cys αCH), 6.62 and 7.05 (8H, dd, Tyr aromatic), 6.76 (2H, m, Tyr NH), 7.18–7.32 (10H, m, Ar), 8.07 (2H, d, Phe NH), 8.15 (2H, d, Cys NH), 8.25 (2H, br, Gly NH), 9.15 (2H, br, Tyr OH), 10.18 (2H, s, NH–NH). FAB-MS *m/e* 1193.3 (M^+ Na). TFA·Tyr-c[Cys-Gly-Phe-NH-NH ← Phe-Gly] ← Cys-Tyr·TFA (9): ^{1}H NMR δ: 1.78 and 3.15 (4H, m, Cys βCH_2), 2.80 and 3.05 (4H, m, Tyr βCH_2), 2.83 and 3.05 (4H, m, Phe βCH_2), 3.50 and 3.81 (4H, m, Gly αCH_2), 3.98 (2H, br, Tyr αCH), 4.55 (4H, m, Phe αCH and Cys αCH), 6.62 and 7.05 (8H, dd, Tyr aromatic), 7.18–7.32 (10H, m, Ar), 8.03 (2H, br, Tyr NH), 8.05 (2H, d, Phe NH), 8.40 (2H, m, Gly NH), 8.83 (2H, d, Cys NH), 9.35 (2H, s, Tyr OH), 10.10 (2H, s, NH–NH). FAB-HRMS *m/e* 971.3472 (M^+). Boc-D-Cys(Acm)-Gly-Phe-NH-NH ← Phe ← Gly ← D-Cys(Acm)-Boc (3): ^{1}H NMR δ: 1.35 (18H, s, Boc), 1.85 (6H, s, CH_3CO), 2.64 and 2.88 (4H, m, Cys βCH_2), 2.80 and 3.08 (4H, m, Phe βCH_2), 3.60–3.88 (4H, m, Gly αCH_2), 4.12–4.28 (6H, m, Acm CH_2 and Cys αCH), 4.62 (2H, m, Phe αCH), 6.98 (2H, d, Cys NH) 7.19–7.30 (10H, m, Ar), 7.93 (2H, m, Gly NH), 8.20 (2H, d, Phe NH), 8.48 (2H, m, Acm NH), 10.22 (2H, s, NH–NH). FAB-MS *m/e* 988.9 (M^+). Boc-Tyr-D-Cys(Acm)-Gly-Phe-NH–NH ← Phe ← Gly ← D-Cys-(Acm) ← Tyr-Boc (5): ^{1}H NMR δ: 0.30 (18H, s, Boc), 1.82 (6H, s, CH_3CO), 2.63 and 2.89 (4H, m, Tyr βCH_2), 2.69 and 2.89 (4H, m, Cys β CH_2), 2.81–3.06 (4H, m, Phe βCH_2), 3.62–3.82 (4H, m, Gly αCH_2), 4.16–4.28 (6H, m, Acm CH_2 and Tyr αCH), 4.55 (2H, m, Cys αCH), 4.62 (2H, m, Phe αCH), 6.63 and 7.05 (8H, dd, Tyr aromatic), 6.80 (2H, m, Tyr NH), 7.15–7.32 (10H, m, Ar), 8.07 (2H,

m, Gly NH), 8.22 (4H, m, Phe NH and Cys NH), 8.48 (2H, m, Acm NH), 9.12 (2H, br, Tyr Tyr OH), 10.22 (2H, s, NH–NH). FAB-MS *m/e* 1314.9 (M^+). Boc-Tyr-c[D-Cys-Gly-Phe-NH-NH ← Phe-Gly ← D-Cys] ← Tyr-Boc (8) ^{1}H NMR δ: 0.28 (18H, s, Boc), 2.65 and 2.89 (4H, m, Tyr βCH_2), 2.81–3.04 (4H, m, Cys βCH_2), 2.87 and 3.14 (4H, m, Phe βCH_2), 3.35 and 3.88 (4H, m, Gly αCH_2), 4.12 (2H, m, Tyr αCH), 4.58 (4H, m, Cys αCH and Phe αCH), 6.62 and7.05 (8H, dd, Tyr aromatic), 6.80 (2H, m, Tyr NH), 7.15–7.30 (10H, m, Ar), 7.81 (2H, d, Phe NH), 8.32 (2H, d, Cys NH), 8.46 (2H, br, Gly NH), 9.15 (2H, br, Tyr OH), 10.05 (2H, s, NH–NH). FAB-MS *m/e* 1170.8 (M^+). TFA·Tyr-c[D-Cys-Gly-Phe-NH–NH ← Phe ← Gly ← D-Cys] ← TyrcdotTFA (10): ^{1}H NMR δ: 1.79 and 3.15 (4H, m, Cys βCH_2), 2.80 and 3.05 (4H, m, Tyr βCH_2), 2.85 and 3.05 (4H, m, Phe βCH_2), 3.55 and 4.05 (4H, m, Gly α CH_2), 3.95 (2H, m, Tyr αCH), 4.60 (2H, m, Phe αCH), 4.68 (2H, m, Cys αCH), 6.65 and 7.08 (8H, dd, Tyr aromatic), 7.18–7.30 (10H, m, Ar), 7.90 (2H, d, Phe NH), 7.97 (2H, br, Tyr NH), 8.68 (2H, br, Gly NH), 8.94 (2H, d, Cys NH), 9.34 (2H, s, Tyr OH), 10.15 (2H, s, NH–NH). FAB-HRMS *m/e* 971.3471 (M^+).