ACS Medicinal Chemistry Letters

Letter

pubs.acs.org/acsmedchemlett

Novel Cyclic Biphalin Analogue with Improved Antinociceptive Properties

Adriano Mollica,*[,†] Alfonso Carotenuto,*[,‡] Ettore Novellino,[‡] Antonio Limatola,[‡] Roberto Costante,[†] Francesco Pinnen,[†] Azzurra Stefanucci,[§] Stefano Pieretti,[∥] Anna Borsodi,[⊥] Reza Samavati,[⊥] Ferenc Zador,[⊥] Sándor Benyhe,[⊥] Peg Davis,[#] Frank Porreca,[#] and Victor J. Hruby[○]

[†]Dipartimento di Farmacia, Università di Chieti-Pescara "G. d'Annunzio", Via dei Vestini, 31, 66100 Chieti, Italy

[‡]Dipartimento di Farmacia, Università di Napoli "Federico II", Via D. Montesano, 49, 80131 Naples, Italy

[§]Dipartimento di Chimica, Sapienza, Università di Roma, P.le A. Moro, 5, 00187 Rome, Italy

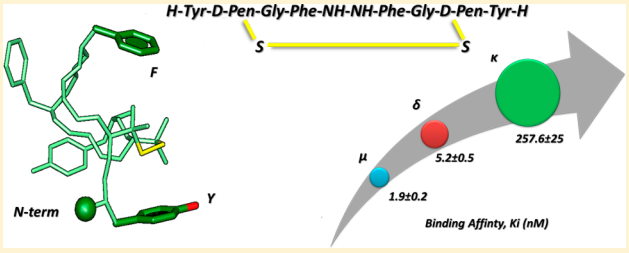
[∥]Department of Therapeutic Research and Medicine Evaluation, Istituto Superiore di Sanità, V.le Regina Elena 299, 00161 Rome, Italy

[⊥]Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, 6726 Szeged, Hungary

[#]Department of Pharmacology and [○]Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721, United States

Supporting Information

ABSTRACT: Two novel opioid analogues have been designed by substituting the native D-Ala residues in position 2,2′ of biphalin with two residues of D-penicillamine or L-penicillamine and by forming a disulfide bond between the thiol groups. The so-obtained compound **9** containing D-penicillamines showed excellent μ/δ mixed receptor affinities (K_i^{δ} = 5.2 nM; K_i^{μ} = 1.9 nM), together with an efficacious capacity to trigger the second messenger and a very good *in vivo* antinociceptive activity, whereas product **10** was scarcely active. An explanation of the two different pharmacological behaviors of products **9** and **10** was found by studying their conformational properties.



KEYWORDS: *Analgesics, biphalin, dimeric opioid peptides, cyclic analogues*

In the field of dimeric opioid peptides, biphalin presents a unique structure based on two enkephalin-like branch (H-Tyr-D-Ala-Gly-Phe, **1**) linked by a hydrazine moiety.[1,2] Its noticeable bioactivity is due to the peculiar structure, which has the ability to match the topographical requirements for both μ and δ opioid receptors.[3−5] Furthermore, this opioid octapeptide induces less physical dependence and toxicities than other opioids.[6−8]

Unfortunately, structural flexibility, scarce metabolic and chemical stability, low bioavailability, and distribution represent some of the major problems concerning the use of native opioid peptides as drugs when administered *in vivo*.[9] Different approaches have been explored in an effort to overcome these limits, including the use of D-amino acids, β-homoamino acids, other types of nonproteinogenic residues, cyclization, and their combinations.[10−13] Particularly appealing is the cyclization of peptides, which has been demonstrated to be a useful approach for developing diagnostic and therapeutic peptidic and peptidomimetic drugs. Cystine or penicillamine containing cyclic peptides are often obtained by substituting nonbonding residues in the linear native peptide sequence with two Cys or Pen residues, followed by oxidation of the thiol groups.[14−16] If compared with the corresponding linear peptides, cyclic derivatives have shown a great improvement of the conformational rigidity, premising meaningful conformational studies to determining the bioactive conformation. Cyclic peptides are blocked to assume the best conformation to interact with their specific receptors, thus the loss of internal rotational entropy compared to the linear analogues upon binding should be smaller.[17,18] Cyclic peptides offer advantages over linear peptides in terms of (i) stability; (ii) conformational rigidity; and (iii) suited templates for orally available small molecule.[5,14−16]

In the last decades we extensively studied several linear and cyclic biphalin analogues,[19−21] and in the present study, we pointed our attention to the design of two novel cyclic biphalin-like structures, as part of our program in search for new antinociceptive agents. This work reports the synthesis, the *in vitro* and *in vivo* biological activity, and the conformational

Received: June 9, 2014
Accepted: July 14, 2014
Published: July 14, 2014

analysis of two novel cyclic biphalin analogues **9** and **10** (Figure 1).

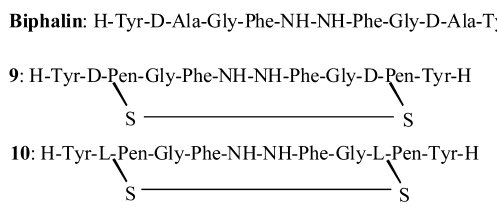
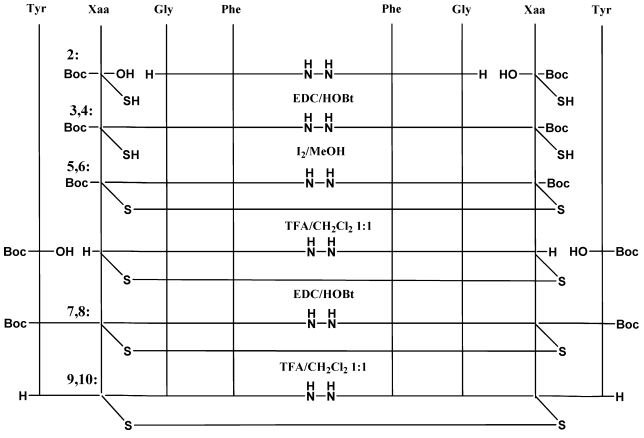


Figure 1. Structures of biphalin and derivatives **9** and **10**.

We initiated this research with the aim to optimize the previous reported first cyclic model of biphalin containing a disulfide bridge.[19−21] Since advantages of using penicillamine residues in place of cysteine were already shown, especially in the field of DPDPE and its derivatives,[14,22−24] the original design of cyclic biphalin analogues was modified accordingly. Thus, two novel cyclic biphalin analogues (**9** and **10**) were developed (Figure 1), and their *in vitro* biological activities were tested. The analgesic activity of the most active model **9** was further investigated by *in vivo* studies. The cyclic final products **9** and **10** were synthesized starting from the previously reported tetrapeptide **2**·TFA-(H-Gly-Phe-NH−)$_2$ by symmetrically coupling the remaining two amino acids (see Scheme 1).[19−21] It is worth noting that no protecting group was adopted for the side chain of the penicillamine residues since the thiol groups were stable in the condition of the reactions.

Scheme 1. Syntheses of Biphalin Analogues 9 and 10 from Tetrapeptide 2[a]



[a]Reference 10. Compounds **3**,**5**,**7**,**9**: Xaa = D-Pen. Compounds **4**,**6**,**8**, **10**: Xaa = L-Pen.

Cyclization was obtained by the oxidation of the thiols group of the D-Pen or L-Pen residues by a treatment of the peptides **3** and **4** with a mixture of MeOH/I_2. The resultant cyclic intermediate products **5** and **6** were deprotected in standard conditions by TFA/DCM and used for the next coupling without further purification to give the final Boc-protected products **7** and **8**. Products **9** and **10** were purified as TFA salts.

To determine the affinity to the μ-opioid receptor (MOR),[25,26] the δ-opioid receptor (DOR), and to the κ-opioid receptor (KOR) of compounds **9** and **10**, tritiated opioid peptides DAMGO ([^{3}H]-[D-Ala^2,N-Me-Phe^4,Gly-ol^5]-enkephalin), Ile^{5,6}deltorphin II, and U69593 (selective agonists for MOR, DOR, and KOR, respectively) were used. K_i values are shown in Table 1 (binding curves are shown in Figure S1, Supporting Information). Analogue **9** has a very good μ and δ opioid receptor affinity, showing comparable K_i values with respect to biphalin for MOR (K_i = 1.9 nM), DOR (K_i = 5.2 nM), and KOR (K_i = 260 nM). Analogue **10** shows very low affinity for all opioid receptors.

Table 1. Binding Affinity and *in Vitro* Bioactivity for Compounds 9 and 10

	binding affinity,[a] K_i (nM)[b]		
compd	δ	μ	κ
Ctrl[c]	1.8 ± 0.5	1.0 ± 0.1	5.7 ± 0.5
Bph	15 ± 2.3	2.6 ± 0.7	283.1 ± 182.3
9	5.2 ± 0.5	1.9 ± 0.2	257.6 ± 25
10	amb.	amb.	amb.

[a]Displacement of [^{3}H]Ile^{5,6}deltorphin II (δ-ligand), [^{3}H]DAMGO (μ-ligand), and [^{3}H]U69593 (κ-ligand) from binding sites on rat brain membrane. [b]±SEM. [c]The control was the appropriate opioid receptor specific ligand. amb.: ambiguous fitting since the compound can inhibit specific receptor binding significantly only in the highest concentration.

Isolated tissue based functional assays were also performed on guinea pig ileum/longitudinal muscle myenteric plexus (GPI) and mouse vas deferens (MVD) (Table 2).[27−29] While compound **9** was potent in inhibiting muscle contraction both in MVD (expressing DOR) and in GPI (expressing MOR) assays, analogue **10** showed activity only in the micromolar range. These data are coherent with those obtained from the binding assays.

The ability of **9** and **10** to stimulate the activation of G-proteins associated with the opioid receptors has been evaluated by [^{35}S]GTPγS binding assay (Table 2 and Figure S2, Supporting Information).[30−33] Analogue **9** has a similar μ and δ opioid receptor activation profile as specific opioid ligands (DAMGO and Ile^{5,6}deltorphin II), unlike the κ opioid receptor. Furthermore, compound **9** has a significantly higher efficacy than biphalin in activating MOR. Interestingly, its efficacy (E_{max}) on MOR is also higher than that of the cyclic Cys derivatives.[19−21]

According to other *in vitro* assays, compound **10** shows a lower activity for all receptors.

Overall *in vitro* results clearly suggest that D-residues in position 2,2′ are crucial for opioid receptor affinity, which is in accordance with our previous SAR.[19−21] Thus, ligand **10**, which possesses a disulfide bridge between L-penicillamines displays a remarkable loss of activity when compared to **9** and biphalin, displaying reduced binding affinities for DOR and MOR, as well as for all the functional activities in the [^{35}S]GTPγS binding and the functional assays.

Product **9** was also tested *in vivo* for its antinociceptive activity. In the "hot plate" and "tail flick" tests, analogue **9** produced about 95% of the MPE 15 min after i.c.v. administration. The maximum effect was obtained 15−30 min after drug injection, and minimal decrease was observed for the next 30 min in both *in vivo* models (Figure 2). Product **9** showed an activity several times higher than morphine after i.c.v. administration. Following i.v. administration ("hot plate" and "tail-flick" tests), compound **9** displayed a greater and longer lasting antinociceptive effect than biphalin, thus suggesting a likely improvement of the pharmacokinetic

dx.doi.org/10.1021/ml500241n | ACS Med. Chem. Lett. 2014, 5, 1032−1036

Table 2. [^{35}S]GTPγS Binding (G-Protein Activation) and Functional Assays

	δ receptor		μ receptor		κ receptor		bioassay, IC_{50}[d] (nM)[b]	
compd	E_{max} (%)[a]	EC_{50} (nM)[b]	E_{max} (%)[a]	EC_{50} (nM)[b]	E_{max} (%)[a]	EC_{50} (nM)[b]	MVD (δ)	GPI (μ)
Ctrl[c]	142.6 ± 1.4	7.7 ± 1.9	465.2 ± 7.7	81 ± 12	202 ± 3.3	7.7 ± 1.8		
Bph	219.6 ± 5.7	90.5 ± 25	178.2 ± 3.6	12 ± 4.6	108.9 ± 4.1	amb.	27 ± 15[e]	8.8 ± 0.3[e]
9	149.5 ± 2.3	7.1 ± 1.7	474.5 ± 4.1	76.2 ± 7.4	126.8 ± 4.4	480 ± 385	7.2 ± 0.8	21 ± 4
10	142.6 ± 2.8	360 ± 121	162.2 ± 3.2	230 ± 82	124.5 ± 2.7	205.1 ± 137	21% at 1 mM	4% at 1 mM

[a]Net total bound/basal binding × 100 ± SEM. [b]±SEM. [c]The control was the corresponding opioid receptor specific ligand (δ, Ile^{5,6}deltorphine II; μ, DAMGO; and κ, U69593). [d]Concentration at 50% inhibition of muscle contraction in electrically stimulated isolated tissues (n = 4). [e]Data according to refs 19−21. amb.: ambiguous fitting since the compound did not stimulate the receptor above basal activity significantly.

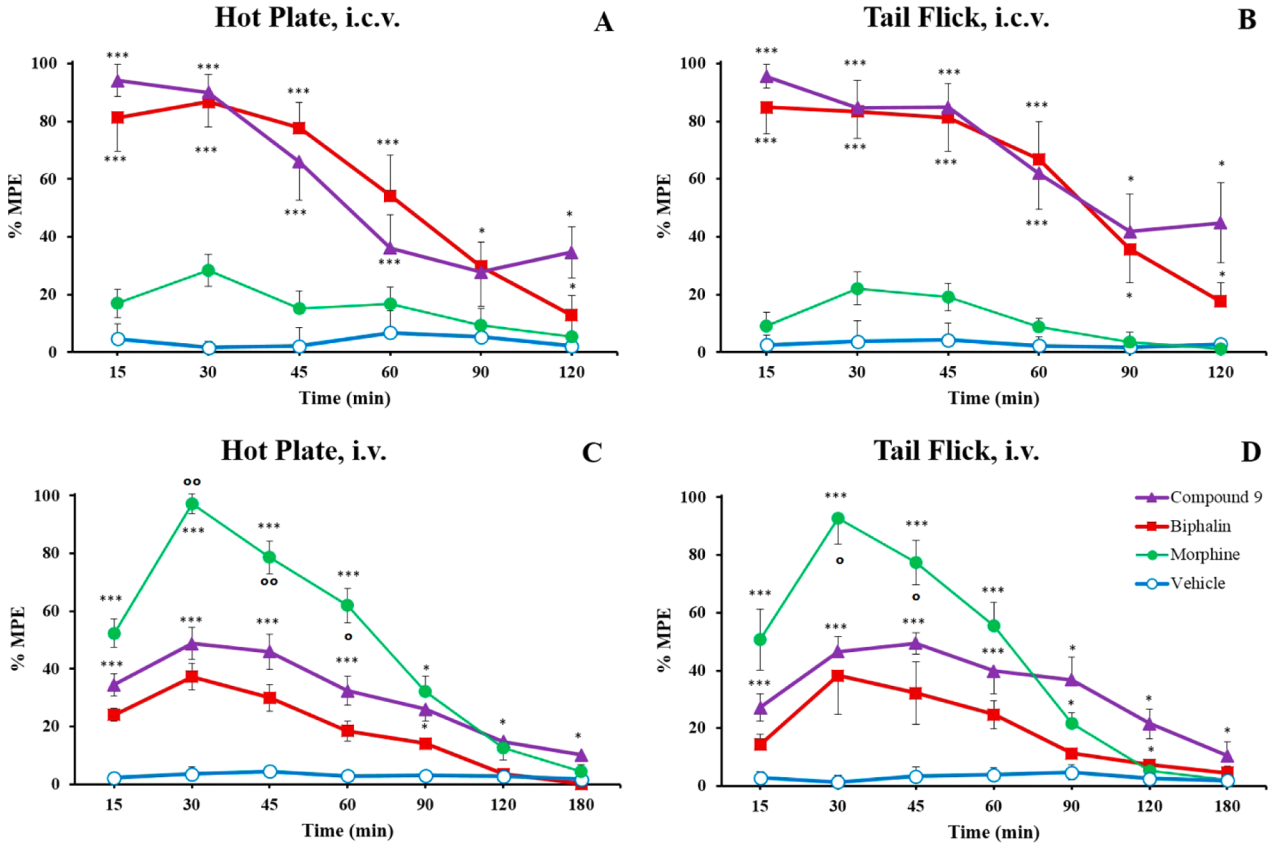


Figure 2. Antinociceptive results, reported as maximum possible effect (MPE), of hot plate and tail flick *in vivo* bioassays for compound **9**, biphalin, and morphine sulfate. Compounds were injected i.c.v. (A,B) at a dose of 0.1 nmol/rat and systemic i.v. administration (C,D) at a dose of 1500 nmol/kg. The data represent the mean ± SEM. Statistical significance was assumed for $P < 0.05$. *$P < 0.05$ and ***$P < 0.001$ vs vehicle-treated animals; °$P < 0.05$ and °°$P < 0.01$ vs biphalin-treated animals. N = 8−10.

parameters if compared to biphalin, in accordance with the cyclization strategy.[34,35] Also, the increased efficacy of **9** at the MOR with respect to biphalin should play a role in this antinociceptive effect. For detailed experimental procedures[36,37] see Supporting Information.

The bioactivity of **9** is still lower than morphine following i.v. administration probably due to a reduced blood−brain barrier penetration of **9** compared to morphine.

To explain the activity differences between **9** and **10**, a conformational analysis of the two analogues was carried out by solution NMR (Supporting Information, Tables S1−S5). Dodecylphosphocoline (DPC) micelle solution was used to mimic a membrane environment considering that opioid peptides interact with membrane receptors.[38,39] Using the NMR data as input, structure calculations by restrained simulated annealing gave the conformers shown in Figure 3. More details are reported in the Supporting Information.

Both peptides **9** and **10** show a well-defined structure encompassing residues 1−4 (backbone root-mean-square deviation values are 0.27 and 0.21 Å, respectively). A γ-turn centered on Gly^3 is seen in peptide **9** (Figure 3A,B; Table S5, Supporting Information). As expected from the NOE cross-peaks between the aromatic rings and the methyl groups of D-Pen^2 (Figure S3, Supporting Information), a sandwich-like π-CH_3-π geometry of the signal sequence of the peptides was observed.

Peptide **9** has similar activity profile of the linear parent biphalin (MOR and DOR agonist) and different from DPDPE, which inspired the D-Pen−D-Pen bridge (selective DOR agonist).[14] To explain peptide **9**'s lack of μ/δ selectivity, we considered the distances between pharmacophoric points obtained by restrained molecular dynamics (Figure S4, Supporting Information). Indeed, these distances are compatible with both μ and δ opioid receptors.[40,41] In fact, considering

dx.doi.org/10.1021/ml500241n | ACS Med. Chem. Lett. 2014, 5, 1032−1036

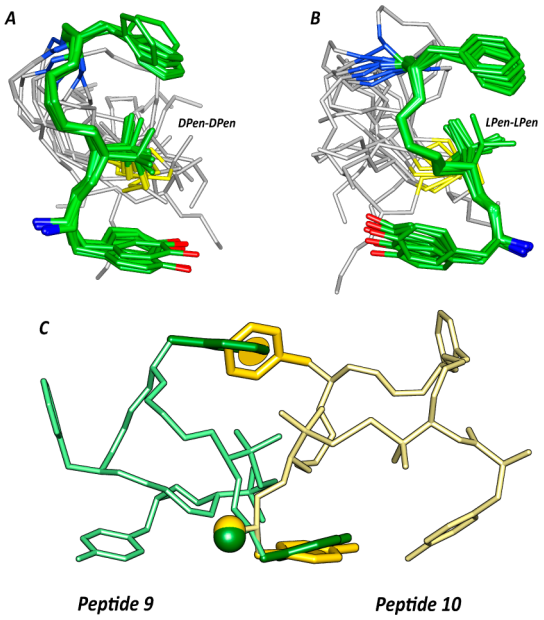


Figure 3. Superposition of the ten lowest energy conformers of **9** (A) and **10** (B). Structure models were superimposed using the backbone heavy atoms of residues 1−4. Heavy atoms have different colors (carbon, green; nitrogen, blue; oxygen, red; sulfur, yellow). Hydrogen atoms are hidden for a better view. (C) Superposition of peptides **9** (green) and **10** (yellow) using the three pharmacophoric points, i.e., terminal amino group (Nterm), center of the Tyr phenol (Y), and center of the Phe phenyl ring (F).

μ-selective peptides, the distances between the aromatic rings of Tyr[1] and Phe[4] should be in the range 10−13 Å,[40] while the range characteristic for peptide and nonpeptide δ-selective compounds is about 7 Å.[41] We found this distance ranging between 6 and 12 Å in peptide **9** (Figure S4c, Supporting Information) thus fitting both the pharmacophores.

In contrast, κ-receptor agonists require a shorter Tyr[1] and Phe[4] distance (about 5 Å) and a g^- orientation of the Tyr[1] side chain.[42] Those criteria are both unsatisfied by peptide **9**. Finally, the inactivity of peptide **10** can be tentatively explained by a comparison of the peptide structures (Figure 3C). As observed, while the three pharmacophoric points (i.e., terminal amino group, center of the Tyr phenol, and center of the Phe phenyl ring) overlap very efficiently, the backbone atoms of residues 2−4 are not overlapping and the palindromic fragments (residues 1′−4′) point in opposite directions.

Those nonfitting regions probably form incompatible interactions with the receptors in the case of peptide **10** thus accounting for its lack of activity.

In conclusion, we have successfully developed two novel cyclic biphalin analogues. Compound **9**, containing a D-Pen residue at position 2,2′, showed improved *in vitro* and *in vivo* activity compared to biphalin. According to previous SARs, compound **10**, containing L-Pen, was virtually inactive. Conformational analysis pointed to a different 3D structure of the two analogues explaining their activity profiles. Further studies on the promising novel compound **9** using additional animal models are currently underway.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures, characterization of intermediates and final products, biological assays, NMR analysis, and structure calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*(A.M.) Phone: +39-0871-3554477. E-mail: a.mollica@unich.it.

*(A.C.) Phone: +39-081678626. E-mail: alfocaro@unina.it.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Lipkowski, A. W.; Konecka, A. M.; Sroczynska, I. Double-enkephalins-synthesis, activity on guinea-pig ileum, and analgesic effect. *Peptides* **1982**, *3*, 697−700.

(2) Feliciani, F.; Pinnen, F.; Stefanucci, A.; Costante, R.; Cacciatore, I.; Lucente, G.; Mollica, A. Structure-activity relationships of biphalin analogs and their biological evaluation on opioid receptors. *Mini-Rev. Med. Chem.* **2013**, *13*, 11−33.

(3) Portoghese, P. S. From models to molecules: opioid receptor dimers, bivalent ligands, and selective opioid receptor probes. *J. Med. Chem.* **2001**, *44*, 2259−2269.

(4) Lipkowski, A. W.; Misicka, A.; Davis, P.; Stropova, D.; Janders, J.; Lachwa, M.; Porreca, F.; Yamamura, H. I.; Hruby, V. Biological activity of fragments and analogues of the potent dimeric opioid peptide, biphalin. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2763−2766.

(5) Silbert, B. S.; Lipkowski, A. W.; Cepeda, M. S.; Szyfelbein, S. K.; Osgood, P. F.; Carr, D. B. Analgesic activity of a novel bivalent opioid peptide compared to morphine via different routes of administration. *Agents Actions* **1991**, *3*, 382−387.1.

(6) Horan, P. J.; Mattia, A.; Bilsky, E. J.; Weber, S.; Davis, T. P.; Yamamura, H. I.; Malatynska, E.; Appleyard, S. M.; Slaninova, J.; Misicka, A. Antinociceptive profile of biphalin, a dimeric enkephalin analog. *J. Pharmacol. Exp. Ther.* **1993**, *265*, 1446−1454.

(7) Yamazaki, M.; Suzuki, T.; Narita, M.; Lipkowski, A. W. The opioid peptide analogue biphalin induces less physical dependence than morphine. *Life Sci.* **2001**, *69*, 1023−1028.

(8) Abbruscato, T. J.; Thomas, S. A.; Hruby, V. J.; Davis, T. P. Brain and spinal cord distribution of biphalin: correlation with opioid receptor density and mechanism of CNS entry. *J. Neurochem.* **1997**, *69*, 1236−1245.

(9) Tömböly, C.; Péter, A.; Tóth, G. In vitro quantitative study of the degradation of endomorphins. *Peptides* **2002**, *23*, 1573−1580.

(10) Mollica, A.; Pinnen, F.; Costante, R.; Locatelli, M.; Stefanucci, A.; Pieretti, S.; Davis, P.; Lai, J.; Rankin, D.; Porreca, F.; Hruby, V. J. Biological active analogues of the opioid peptide biphalin: mixed α/β^3-peptides. *J. Med. Chem.* **2013**, *56*, 3419−3423.

(11) Mollica, A.; Pinnen, F.; Stefanucci, A.; Feliciani, F.; Campestre, C.; Mannina, L.; Sobolev, A. P.; Lucente, G.; Davis, P.; Lai, J.; Ma, S. W.; Porreca, F.; Hruby, V. J. The cis-4-amino-L-proline residue as a scaffold for the synthesis of cyclic and linear endomorphin-2 analogues. *J. Med. Chem.* **2012**, *55*, 3027−3035.

(12) Mollica, A.; Pinnen, F.; Stefanucci, A.; Mannina, L.; Sobolev, A. P.; Lucente, G.; Davis, P.; Lai, J.; Ma, S. W.; Porreca, F.; Hruby, V. J. cis-4-Amino-L-proline residue as a scaffold for the synthesis of cyclic and linear endomorphin-2 analogues: part 2. *J. Med. Chem.* **2012**, *55*, 8477−8482.

(13) Hruby, V. J. Designing peptide receptor agonists and antagonists. *Nat. Rev. Drug Discovery* **2002**, *1*, 847−858.

(14) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. Bis-penicillamine enkephalin possess highly improved specificity toward delta opioid receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871−5874.

(15) Zieleniak, A.; Rodziewicz-Motowidło, S.; Rusak, L.; Chung, N. N.; Czaplewski, C.; Witkowska, E.; Schiller, P. W.; Ciarkowski, J.; Izdebski, J. Deltorphin analogs restricted via a urea bridge: structure and opioid activity. *J. Pept. Sci.* **2008**, *14*, 830−837.

(16) Weltrowska, G.; Berezowska, I.; Lemieux, C.; Chung, N. N.; Wilkes, B. C.; Schiller, P. W. *N*-Methylated cyclic enkephalin analogues retain high opioid receptor binding affinity. *Chem. Biol. Drug Des.* **2010**, *75*, 82−88.
(17) Berezowska, I.; Chung, N. N.; Lemieux, C.; Wilkes, B. C.; Schiller, P. W. Dicarba Analogues of the cyclic enkephalin peptides H-Tyr-c[D-Cys-Gly-Phe-D (or L)-Cys]NH_2 retain high opioid activity. *J. Med. Chem.* **2007**, *50*, 1414−1417.
(18) Mollica, A.; Guardiani, G.; Davis, P.; Ma, S. W.; Porreca, F.; Lai, J.; Mannina, L.; Sobolev, A. P.; Hruby, V. J. Synthesis of stable and potent δ/μ opioid peptides: analogues of H-Tyr-c[D-Cys-Gly-Phe-D-Cys]-OH by ring-closing metathesis. *J. Med. Chem.* **2007**, *50*, 3138−3142.
(19) Mollica, A.; Davis, P.; Ma, S. W.; Porreca, F.; Lai, J.; Hruby, V. J. Synthesis and biological activity of the first cyclic biphalin analogues. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 367−372.
(20) Mollica, A.; Costante, R.; Stefanucci, A.; Pinnen, F.; Lucente, G.; Fidanza, S.; Pieretti, S. Antinociceptive profile of potent opioid peptide AM94, a fluorinated analogue of biphalin with non-hydrazine linker. *J. Pept. Sci.* **2013**, *19*, 233−239.
(21) 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.
(22) Froimowitz, M.; Hruby, V. J. Conformational analysis of enkephalin analogs containing a disulfide bond. Models for δ and μ-receptor opioid agonists. *Int. J. Pept. Protein Res.* **1989**, *34*, 88−96.
(23) Hruby, V. J.; Gehrig, C. A. Recent developments in the design of receptor specific opioid peptides. *Med. Res. Rev.* **1989**, *9*, 343−401.
(24) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Galligan, J. J.; Burks, T. F.; Gee, K.; Yamanura, H. I. [D-Pen^2,L-Cys^5] Enkephalinamide and [D-Pen^2,D-Cys^5]enkephalinamide, conformationally constrained cycle enkephalinamide analogues with delta receptor specificity. *Biochem. Biophys. Res. Commun.* **1982**, *106*, 506−512.
(25) Nevin, S. T.; Kabasakal, L.; Ötvös, F.; Tóth, G.; Borsodi, A. Binding characteristics of the novel highly selective delta agonist, [^{3}H-$Ile^{5,6}$]deltorphin II. *Neuropeptides* **1994**, *26*, 261−265.
(26) Bojnik, E.; Farkas, J.; Magyar, A.; Tömböly, C.; Güçlü, U.; Gündüz, O.; Borsodi, A.; Corbani, M.; Benyhe, S. Selective and high affinity labeling of neuronal and recombinant nociceptin receptors with the hexapeptide radioprobe [(3)H]Ac-RYYRIK-ol. *Neurochem. Int.* **2009**, *55*, 458−466.
(27) Polt, R. L.; Porreca, F.; Szabo, L. Z.; Bilsky, E. J.; Davis, P.; Abbruscato, T. J.; Davis, T. P.; Horvath, R.; Yamamura, H. I.; Hruby, V. J. Glycopeptide enkephalin analogues produce analgesia in mice: evidence for penetration of the blood-brain barrier. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7114−7118.
(28) Colucci, M.; Mastriota, M.; Maione, F.; Di Giannuario, A.; Mascolo, N.; Palmery, M.; Severini, C.; Perretti, M.; Pieretti, S. Guinea pig ileum motility stimulation elicited by *N*-formyl-Met-Leu-Phe (fMLF) involves neurotransmitters and prostanoid. *Peptides* **2011**, *32*, 266−271.
(29) Kramer, T. H.; Davis, P.; Hruby, V. J.; Burks, T. F.; Porreca, F. *In vitro* potency, affinity and agonist efficacy of highly selective delta opioid receptor ligands. *J. Pharmacol. Exp. Ther.* **1993**, *266*, 577−584.
(30) Schmidhammer, H.; Burkard, W. P.; Eggstein-Aeppli, L.; Smith, C. F. Synthesis and biological evaluation of 14-alkoxymorphinans. 2. (−)-*N*-(Cyclopropylmethyl)-4,14-dimethoxymorphinan-6-one, a selective mu opioid receptor antagonist. *J. Med. Chem.* **1989**, *32*, 418−421.
(31) Sim, L. J.; Selley, D. E.; Childers, S. R. In vitro autoradiography of receptor-activated G proteins in rat triphosphate binding. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7242−7246.
(32) Traynor, R.; Nahorski, R.; Traynor, J. R.; Nahorski, S. R. Modulation by mu-opioid agonists of guanosine-5′-O-(3-[35S]thio)-triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol. Pharmacol.* **1995**, *47*, 848−54.
(33) Szekeres, P. G.; Traynor, J. R. δ 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.
(34) Egleton, R. D.; Davis, T. P. The development of neuropeptide drugs that cross the blood-brain barrier. *J. Am. Soc. Exp. NeuroTher.* **2005**, *2*, 44−53.
(35) Gentilucci, L. New trends in the development of opioid peptide analogues as advanced remedies for pain relief. *Curr. Top. Med. Chem.* **2004**, *4*, 19−38.
(36) Tallarida, J.; Murray, R. B. Manual of pharmacologic calculations with computer programs. Second edition. *J. Pharm. Sci.* **1988**, *77*, 284.
(37) 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.
(38) Di Cianni, A.; Carotenuto, A.; Brancaccio, D.; Novellino, E.; Reubi, J. C.; Beetschen, K.; Papini, A. M.; Ginanneschi, M. Novel octreotide dicarba-analogues with high affinity and different selectivity for somatostatin receptors. *J. Med. Chem.* **2010**, *53*, 6188−6197.
(39) Yamamoto, T.; Nair, P.; Jacobsen, N. E.; Davis, P.; Navratilova, E.; Moye, S.; Lai, J.; Yamamura, H. I.; Vanderah, T. W.; Porreca, F.; Hruby, V. J. The importance of micelle-bound states for the bioactivities of bifunctional peptide derivatives for δ/μ opioid receptor agonists and neurokinin 1 receptor antagonists. *J. Med. Chem.* **2008**, *51*, 6334−6347.
(40) Yamazaki, T.; Ro, S.; Goodman, M.; Chung, N. N.; Schiller, P. W. A topochemical approach to explain morphiceptin bioactivity. *J. Med. Chem.* **1993**, *36*, 708−719.
(41) Shenderovich, M. D.; Liao, S.; Qian, X.; Hruby, V. J. A three-dimensional model of the δ-opioid pharmacophore: comparative molecular modeling of peptide and nonpeptide ligands. *Biopolymers* **2000**, *53*, 565−580.
(42) Wu, Y. C.; Lin, J. S.; Hwang, C. C. Structure-activity relationships of αS1-casomorphin using AM1 calculations and molecular dynamics simulations. *J. Phys. Chem. B* **2007**, *111*, 7377−7383.