

Brief Articles

Blood–Brain Barrier Penetration by Two Dermorphin Tetrapeptide Analogues: Role of Lipophilicity vs Structural Flexibility

Steven Ballet,[†] Aleksandra Misicka,^{‡,§} Piotr Kosson,[§] Carole Lemieux,[#] Nga N. Chung,[#] Peter W. Schiller,[#] Andrzej W. Lipkowski,[§] and Dirk Tourwé^{*,†}

Organic Chemistry Department, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium, Department of Chemistry, Warsaw University, Pasteura 1, 02-093 Warsaw, Poland, Medical Research Centre, Polish Academy of Sciences, Pawinskiego 5, 02-106 Warsaw, Poland, and Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute of Montreal, Montreal, PQ H2W 1R7, Canada

Received November 8, 2007

Two dermorphin analogues having an almost identical structure but different structural flexibility were compared for opioid activity. In **1** the aromatic side chains were incorporated into a lactam structure, while in **2** *N*-amide alkylation was retained but the side chains were flexible. Both compounds produced comparable antinociceptive effects in the mouse tail flick test after peripheral administration. This indicates that lipophilicity, rather than side chain flexibility, is the key determinant for blood–CNS barrier penetration.

Introduction

Endogenous opioid peptides have properties that secure their local and short duration actions. Structure–activity studies of opioid peptides have resulted in the development of a large series of analogues that are very potent as analgesics only after direct application to their site of action.¹ The major sites of pain signal modulation are located in the central nervous system (CNS^a). Therefore, systemically administered drugs have to penetrate into the CNS. The permeation through biological barriers, especially the blood–brain barrier (BBB), is still one of the major challenges in the development of peptide analogues as drugs. The BBB is located at the level of endothelial cells in brain capillaries. It utilizes selective molecular mechanisms to prevent certain substances in the blood stream from penetrating into the cerebrospinal fluid. High proteolytic activity at the BBB and the selectivity of active and passive transporters limit the permeation of endogenous and exogenous active compounds from the periphery into the CNS. For the development of active opioid peptide analogues as potential analgesics, high resistance to enzymatic degradation and ability to cross the BBB are essential requirements.

Aromatic amino acids (Tyr, Phe, Trp) are key structural elements of opioid peptides. Conformational restriction of such residues has been a successful strategy to increase the potency, selectivity, and metabolic stability of peptides. Therefore, many constrained analogues of these natural amino acids have been

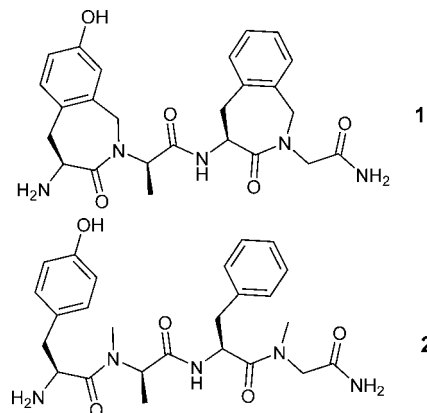


Figure 1. Structures of **1** and **2**.

designed and synthesized.² *N*-Methylation of amide bonds is another well-known structural modification to increase the enzymatic stability of peptides and to improve their oral bioavailability.^{3,4} Recently, we synthesized opioid tetrapeptide analogues structurally derived from dermorphin,⁵ in which the peptide segments around Tyr¹ and Phe³ were conformationally constrained and stabilized by cyclization into 4-aminotetrahydro-2-benzazepin-3-one rings.⁶ The analogue H-Hba-D-Ala-Abagly-NH₂ **1** (SB0304, Figure 1) displayed the highest potency in vitro in the guinea pig ileum (GPI) assay and was selected for in vivo evaluation. After intrathecal injection in rats, analogue **1** produced an antinociceptive effect in the tail-flick assay that was 40–50 times more potent than that of morphine and was also of longer duration.⁶

We now describe the analgesic effect of **1** after intravenous administration. Moreover, we examined the effect of the increased structural rigidity of **1** due to incorporation of the Tyr¹ and Phe³ residues into a lactam ring by comparing its antinociceptive properties to those of the structurally related, more flexible analogue **2** (SB0306, Figure 1). The importance of the rigidity of a compound on the activity is an intriguing question. The opioid peptide sequence H-Tyr-D-Ala-Phe-Gly-NH₂ **3** has

* To whom correspondence should be addressed. Phone: 32 2 629 3295. Fax: 32 2 629 3304. E-mail: datourwe@vub.ac.be.

[†] Vrije Universiteit Brussel.

[‡] Warsaw University.

[§] Polish Academy of Sciences.

[#] Clinical Research Institute of Montreal.

^a Abbreviations: Aba, 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one; AUC, area under the curve; BBB, blood–brain barrier; CNS, central nervous system; DIPEA, diisopropylamine; GPI, guinea pig ileum; Hba, 8-hydroxy-4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one; MBHA, 4-methylbenzhydrylamine; MPE, maximum possible effect; MVD, mouse vas deferens; PSA, polar surface area; RP, reversed phase; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.



Figure 2. Superimposition of [Hba-D-Ala]amide (in green) with oxymorphone (in purple), showing spatial overlap of the *N*-amino groups and phenyl rings.

been constrained by linking the aromatic rings of the Tyr and Phe residues to the amide nitrogen of the succeeding amino acid through a methylene bridge, resulting in **1**.⁶ This compound was a highly potent analgesic after intrathecal administration. To distinguish the effect of cyclization from that of nitrogen alkylation, we synthesized the “ring-opened” analogue **2**, having *N*-methyl-D-Ala and *N*-methyl-Gly (Sar) residues in the sequence (Figure 1). The *in vitro* and *in vivo* activities of both compounds were compared.

Results and Discussion

Evolution has selected flexible linear neuropeptides, including endogenous opioid peptides, to act locally on cell membrane receptors for a very limited time. These peptides are very quickly metabolized and have very limited ability to permeate biological barriers. Therefore, increase of the stability against enzymatic degradation and improvement of membrane permeability are major goals in the development of peptide-derived therapeutic agents. In the case of the opioids, morphine is the gold standard of an exogenous ligand that is able to penetrate the gastrointestinal–blood barrier and the BBB to effectively interact with central opioid receptors. In contrast to the structurally flexible endogenous opioid peptides, morphine is very rigid and has a well defined three-dimensional structure. This led to studies on the development of structurally more rigid opioid peptide analogues. During the interaction with its receptor, the *N*-terminal fragment of an opioid peptide adopts a proper conformation necessary for the stabilization of the active ligand–receptor complex. Therefore, the main challenge of such an approach is to create rigid tyramine structures that correspond to the “active conformation”. Our previous studies identified the 8-hydroxy-4-aminotetrahydro-2-benzazepin-3-one (Hba) structure as a conformationally constrained analogue of tyrosine that when incorporated into an opioid peptide sequence, is well recognized by μ - and δ -opioid receptors.⁶ The results of molecular modeling studies indicated that the conformation of the tyramine moiety in Hba has a high similarity to that contained in the benzomorphan skeleton (Figure 2).

Additionally, we were able to show that constraining the Phe³ residue by formation of the 4-aminotetrahydro-2-benzazepin-3-one (Aba) ring resulted in opioid peptides with high affinity for the δ -opioid receptor.⁷ The synthetic peptidomimetic **1**, in which the conformations of the tyrosine and the phenylalanine residues of the original peptide H-Tyr-D-Ala-Phe-Gly-NH₂ **3** are constrained (Figure 1), showed high binding affinity for opioid receptors and also high antinociceptive activity after intrathecal application.⁶ We now show that this compound also produces a potent analgesic effect when administered intravenously (Figure 3). This indicates that **1** is able to penetrate the BBB to a similar extent as morphine. The incorporation of the

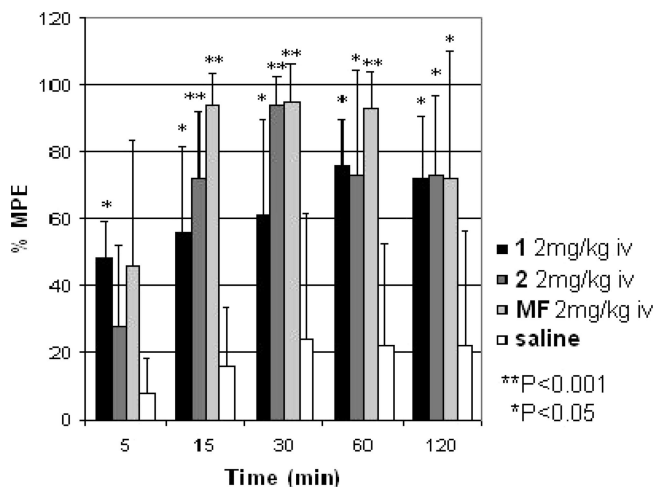


Figure 3. Analgesic effect as % MPE \pm SEM of **1** and **2** in the mouse tail-flick assay at different times after intravenous administration.

Table 1. HPLC Retention Time, ClogP, and tPSA Values of Compounds

| compd | t_R^a (min) | ClogP ^b | tPSA ^b |
|----------|---------------|--------------------|-------------------|
| 1 | 8.87 | 0.6808 | 159.06 |
| 2 | 8.50 | 0.6912 | 159.06 |
| 3 | 6.25 | -0.4752 | 176.64 |

^a RP-HPLC was performed using a RP C-18 column: Supelco Discovery BIO Wide Pore, 25 cm \times 4.6 mm, 5 μ m. System 1: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; gradient, 3–97% B in A over 20 min; flow rate, 1.2 mL/min. System 2: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in MeOH; gradient, 3–97% B in A over 20 min; flow rate, 1.2 mL/min. ^b Calculated using the BioByte estimator and the tPSA calculation algorithm, as implemented in the ChemDraw Ultra software, version 10.0. tPSA = topological polar surface area.

Tyr and Phe side chains into the 2-benzazepinone ring involved the alkylation of the amide nitrogens of the D-Ala and Gly residues of the parent opioid peptide.

N-Alkylation increases the lipophilicity of the peptide analogue, which raises the question of whether the increase of BBB permeation seen with **1** is the result of a conformational stabilization or of an increased lipophilicity due to *N*-alkylation. To answer this question, a “ring opened” peptide analogue **2** with *N*-methylated amino acids in positions 2 and 4 was synthesized (Figure 1). This restores the flexibility of the Tyr and Phe side chains while maintaining the *N*-alkyl (methyl) substitutions. It is mentioned in this context that *N*-alkylation of a peptide bond restricts the conformational space of the amino acid residue to extended conformations, as was also observed for the benzazepine residue,⁸ and permits the *cis*-peptide bond conformation.⁹ The calculated lipophilicities (ClogP) of **1** and **2** are very similar (ClogP = 0.6809 and 0.6912, respectively) and higher than that of the parent peptide **3** (ClogP = -0.4752) (Table 1).

This is confirmed by the similarity of the HPLC retention times (Table 1), indicating that **1** is only slightly more lipophilic than **2** but considerably more lipophilic than **3**. The opioid receptor binding affinities of both compounds are high and quite similar (Table 2). Interestingly, the compound with flexible side chains (**2**) showed moderate preference for δ - over μ -receptors ($IC_{50}^{\mu}/IC_{50}^{\delta}$ = 3.96), whereas the rigid **1** was nonselective ($IC_{50}^{\mu}/IC_{50}^{\delta}$ = 1.17). Neither compound displayed κ -receptor binding affinity at concentrations up to 10 μ M. Like **1**, the peptide **2** displayed agonist activity in the GPI and mouse vas deferens (MVD) assays, albeit with lower potency (Table 2). While in general there is good agreement between the receptor

Table 2. Opioid Receptor Binding Affinities, in Vitro Activities of Compounds, and in Vivo AUC Values for the Analgesic Effect

| compd | IC ₅₀ ± SEM (nM) | | | | AUC ± SEM ^a |
|-------------|-----------------------------|-------------|------------|------------|------------------------|
| | μ | δ | GPI | MVD | |
| 1 | 20.8 ± 3.6 | 17.8 ± 5.0 | 3.64 ± 0.2 | 30.3 ± 4.4 | 7820 ± 551 |
| 2 | 28.1 ± 6.1 | 7.1 ± 5.9 | 22.0 ± 1.6 | 79.7 ± 6.1 | 8732 ± 750 |
| MF/morphine | 9.77 ± 3.45 | 112.2 ± 5.7 | 29.3 ± 2.2 | 155 ± 31 | 9921 ± 589 |

^a AUC values for the analgesic effect after intravenous administration of a dose of 2 mg/kg for each compound.

Table 3. Physicochemical Properties of Opioid Tetrapeptides **1** and **2**

| compd | sequence | <i>m/z</i> (M+H) ⁺ ^a | | TLC Rf ^b | HPLC <i>t</i> _R (min) (% purity) ^c | |
|----------|---|--|------------|---------------------|--|-------------|
| | | calcd M + H | obsd M + H | | system 1 | system 2 |
| 1 | H-Hba-D-Ala-Aba-Gly-NH ₂ | 480.22 | 480.3 | 0.59 | 8.87 (97%) | 12.37 (97%) |
| 2 | H-Tyr-NMe-D-Ala-Phe-Sar-NH ₂ | 484.25 | 484.3 | 0.62 | 8.50 (98%) | 11.85 (98%) |

^a Observed by ESI MS⁺ ionization. ^b TLC system: EtOAc/*n*-BuOH/AcOH/H₂O, 1:1:1:1. ^c HPLC: RP C-18 column, Supelco Discovery BIO Wide Pore, 25 cm × 4.6 mm, 5 μm. System 1: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; gradient, 3–97% B in A over 20 min; flow rate, 1.2 mL/min. System 2: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in MeOH; gradient, 3–97% B in A over 20 min; flow rate, 1.2 mL/min.

binding data and the functional assay data of the compounds, some quantitative discrepancies were observed. Such quantitative discrepancies have often been observed and could be due to possible differences in the structural requirements or receptor access between central and peripheral receptors.

After intravenous application, both compounds produced a similarly high antinociceptive effect in the tail-flick test (Figure 3). This result indicates that both compounds have similar ability to cross the BBB. Whereas **1** produces a significant analgesic response after 5 min, it takes **2** about 15 min. This might reflect a difference in the speed of crossing the BBB between the two analogues. A comparison of the area under the curve (AUC) at the same dose reveals that **1** and **2** have a very similar antinociceptive potency as morphine (Table 2).

A number of physicochemical properties have been identified that influence the ability of drugs to penetrate the BBB.¹⁰ Analysis of CNS and non-CNS drugs revealed that the CNS drugs had fewer hydrogen bond donors, lower polar surface areas (PSA), higher calculated log *P* (ClogP) values, and fewer rotatable bonds.¹¹ Comparison of **1** and **2** reveals that both compounds have the same number of hydrogen bond donors, very similar ClogP values, and identical calculated PSA values (Table 1). However, the main difference between the two compounds is the number of rotatable bonds. It is obvious that in this case the latter is not a determining factor influencing BBB permeability.

It is widely accepted that the structural rigidity of compounds affects receptor binding affinity and selectivity. The results presented here support the hypothesis that the Hba structure in **1** has a conformation that closely resembles the conformation of the tyrosine residue in the receptor-bound conformation of structurally flexible opioid peptide analogues. In this particular comparison of a rigid and a structurally flexible molecule, both compounds showed similar agonist activities. Thus, these findings also support the hypothesis that the 4-aminotetrahydro-2-benzazepinon-3-one core fulfills the conformational requirements for complex formation with the receptor in the "active" conformation. Finally, it is mentioned that opioids with a mixed μ-agonist/δ-agonist profile, such as the peptide analogues described here, are actually of therapeutic interest as potential analgesics with low propensity to produce tolerance and physical dependence.¹²

Experimental Section

General. **1** was prepared as described before.⁶ 4-Methylbenzhydramine (MBHA) resin and Boc-Sar-OH were purchased from Neosystem (Strasbourg, France). *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU) and Boc-Tyr(2,6-

Cl₂Bn)-OH were from Senn Chemicals (Dielsdorf, Switzerland). Boc-NMe-D-Ala-OH was purchased from Bachem (Bubendorf, Switzerland). Trifluoroacetic acid (TFA), *N*-methylmorpholine, and *N,N*-dimethylformamide were obtained from Fluka (Bornem, Belgium).

Solid-phase peptide synthesis was performed on a manual synthesizer. Analytical RP-HPLC was carried out using an Agilent 1100 series system with a RP C-18 column: Supelco Discovery BIO Wide Pore, 25 cm × 4.6 mm, 5 μm. Purification of the peptides was carried out on a semipreparative (HPLC) system (Gilson) using a RP C-18 column: Supelco Discovery BIO Wide Pore, 25 cm × 2.1 cm, 5 μm with a linear gradient (3–80% CH₃CN, containing 0.1% TFA, in 30 min), a flow rate of 13 mL/min, and UV detection at 215 nm. The peptides were analyzed by HPLC using the conditions indicated in Table 3. Mass spectra were recorded on a VG Quatro II spectrometer (electrospray ionization, ESI MS) using MassLynx 2.22 software for data analysis. For thin-layer chromatography (TLC) plastic silica coated plates with F₂₅₄ indicator were used (Merck, Darmstadt, Germany).

The molecular modeling calculations were carried out as described previously using MacroModel 5.0 with Maestro 8.0 as a graphic interface.⁸

Synthesis of 2. Analogue **2** was prepared on a 0.4 mmol scale by manual solid phase synthesis using MBHA resin (loading 1.1 mmol/g) as a solid support and following standard Boc amino protection procedures. The Boc deprotection was performed in a mixture of TFA/CH₂Cl₂/2% anisole (5 min + 20 min). After filtration of the TFA mixture and neutralization with 20% DIPEA/CH₂Cl₂, the couplings were performed by using a 3-fold excess of the amino acids and activating agent (TBTU) and a 9-fold excess of *N*-methylmorpholine. The completeness of the couplings was checked with the ninhydrin or NF 31 color tests.^{13,14} Cleavage of the peptide from the resin and side chain deprotection was accomplished by treatment with HF_{liq} for 1 h at 0 °C. The crude peptide was purified by preparative HPLC. Analytical data of the compound are presented in Table 3.

Receptor Binding Assay. Opioid receptor binding assays were performed as described in detail elsewhere.^{15,16} Binding affinities for μ- and δ-receptors were determined by displacing, respectively, [³H]naloxone and [³H]deltorphin II from rat brain membrane binding sites, and κ-opioid receptor binding affinities were measured by displacement of [³H]U69,593 from guinea pig brain membrane binding sites.

Functional Bioassays (GPI and MVD). The GPI and MVD bioassays were carried out as reported in detail elsewhere.¹⁷ A dose–response curve was determined with [Leu⁵]enkephalin as standard for each ileum and vas preparation, and IC₅₀ values of the compounds were normalized according to a published procedure.¹⁸

In Vivo Antinociception Test. Male C57 mice (20–25 g, from Medical Research Centre Animal House) were housed in groups of five, maintained on a 12 h light–dark cycle, and allowed free

access to food and water. Ten mice were used for each dose–effect test. All procedures were approved by the Local Animal Rights Committee. Peptides were dissolved in saline, injected into the tail vein, and flushed with saline solution. The total volumes injected were 10 mL/kg. The respective amounts of saline were injected in the control group. Analgesia was measured by the warm water (55 °C) tail-flick latency test.¹⁹ Three trials with a maximum exposure to heat (cutoff) of 15 s were performed. The degree of analgesia was expressed as a percentage of maximum possible effect (MPE) calculated as

$$\text{MPE (\%)} = \frac{\text{posttreatment latency} - \text{control}}{\text{cut-off latency} - \text{control}} \times 100$$

The MPE was measured at baseline, 5, 15, 30, 60, and 120 min after administration of the drug or saline. Differences between groups were analyzed using one-way ANOVA for comparison at each time point. The *t* test was used to compare % MPE between treatment groups. *P* < 0.05 was considered statistically significant.²⁰

Acknowledgment. This work has been supported by European Grant “Normolife” (Grant LSHC-CT-2006-037733), by the Fund for Scientific Research Flanders (FWO), and by the U.S. National Institutes of Health (Grants DA-004443 and DA-008924). The authors thank the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT) for financial support, and Rien De Wachter for help with Figure 2.

Supporting Information Available: HPLC tracings of **1and2**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Polt, R.; Porreca, F.; Szabo, L. Z.; Bilsky, E. J. K.; 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.
- (2) Gibson, S. E.; Guilo, N.; Tozer, M. J. Towards control of chi-space: conformationally constrained analogues of Phe, Tyr, Trp and His. *Tetrahedron* **1999**, *55*, 585–615.
- (3) Sagan, S.; Karoyan, P.; Lequin, O.; Chassaing, G.; Lavielle, S. N- and C alpha-methylation in biologically active peptides: synthesis, structural and functional aspects. *Curr. Med. Chem.* **2004**, *11*, 2799–2822.
- (4) Kessler, K.; Chatterjee, J.; Doedens, L.; Opperer, F.; Gilon, C.; Hruby, V. J.; Mierke, D. New perspectives in peptide chemistry by N-alkylation. *Biopolym., Pept. Sci.* **2007**, *88*, 519.
- (5) Montecucchi, P. C.; deCastiglione, R.; Piani, S.; Gozzini, L.; Erspamer, V. Amino acid composition and sequence of dermorphin, a novel opiate-like peptide from the skin of *Phyllomedusa sawagei*. *Int. J. Pept. Protein Res.* **1981**, *17*, 275–283.
- (6) Ballet, S.; Frycia, A.; Piron, J.; Chung, N. N.; Schiller, P. W.; Kosson, P.; Lipkowski, A. W.; Tourwé, D. Synthesis and biological evaluation of constrained analogues of the opioid peptide H-Tyr-D-Ala-Phe-Gly-NH₂ using the 4-amino-2-benzazepin-3-one scaffold. *J. Pept. Res.* **2005**, *66*, 222–230.
- (7) Tourwé, D.; Verschuere, K.; Frycia, A.; Davis, P.; Porreca, F.; Hruby, V. J.; Toth, G.; Jaspers, H.; Verheyden, P.; Van Binst, G. Conformational restriction of Tyr and Phe side chains in opioid peptides: information about preferred and bioactive side-chain topology. *Biopolymers* **1996**, *38*, 1–12.
- (8) Van Rompaey, K.; Ballet, S.; Tomboly, C.; De Wachter, R.; Vanommeslaeghe, K.; Biesemans, M.; Willem, R.; Tourwé, D. Synthesis and evaluation of the beta-turn properties of 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-ones and of their spirocyclic derivative. *Eur. J. Org. Chem.* **2006**, *13*, 2899–2911.
- (9) Misicka, A.; Verheyden, P. M. F.; Van Binst, G. Equilibrium of the cis–trans isomerisation of the peptide bond with *N*-alkyl amino acids measured by 2D-NMR. *Lett. Pept. Sci.* **1998**, *5*, 375–377.
- (10) Hitchcock, S. A.; Pennington, L. D. Structure–brain exposure relationships. *J. Med. Chem.* **2006**, *49*, 7559–7583.
- (11) Mahar Doan, K. M.; Humphreys, J. E.; Webster, L. O.; Wring, S. A.; Shampine, L. J.; Serabjit-Singh, C. J.; Adkison, K. K.; Polli, J. W. Passive permeability and P-glycoprotein-mediated efflux differentiate central nervous system (CNS) and non-CNS marketed drugs. *J. Pharmacol. Exp. Ther.* **2002**, *303*, 1029–1037.
- (12) 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.
- (13) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595–598.
- (14) Madder, A.; Farcy, N.; Hosten, N. G. C.; De Muyenck, H.; De Clerq, P. J.; Barry, J.; Davis, A. P. A novel sensitive assay for visual detection of solid-phase bound amines. *Eur. J. Org. Chem.* **1999**, 2787–2791.
- (15) Olma, A.; Lachwa, M.; Lipkowski, A. W. The biological consequences of replacing hydrophobic amino acids in deltorphin I with amphiphilic alpha-hydroxymethyl-amino acids. *J. Pept. Res.* **2003**, *62*, 45–52.
- (16) Berezowska, I.; Chung, N. N.; Lemieux, C.; Wilkes, B. C.; Schiller, P. W. Dicarba analogues of the cyclic enkephalin peptides H-Tyr-[D-Cys-Gly-Phe-D (or L)-Cys]NH₂ retain high opioid activity. *J. Med. Chem.* **2007**, *50*, 1414–1417.
- (17) DiMaio, J.; Nguyen, T.M.-D.; Lemieux, C.; Schiller, P. W. Synthesis and pharmacological characterization in vitro of cyclic enkephalin analogues: effect of conformational constraints on opiate selectivity. *J. Med. Chem.* **1982**, *25*, 1432–1438.
- (18) Waterfield, A. A.; Leslie, F. M.; Lord, J. A. H.; Ling, N.; Kosterlitz, H. W. Opioid activities of fragments of beta-endorphin and of its leucine 65-analogue. Comparison of the binding properties of methionine- and leucine-enkephalin. *Eur. J. Pharmacol.* **1979**, *5*, 8–11.
- (19) Horan, P. J.; Mattia, A.; Bilsky, E. J.; Weber, S.; Davis, T. P.; Yamamura, H. Y.; Malatynska, E.; Appleyard, S. M.; Slaninova, J.; Misicka, A.; Lipkowski, A. W.; Hruby, V. J.; Porreca, F. Antinociceptive profile of biphalin, a dimeric enkephalin analog. *J. Pharmacol. Exp. Ther.* **1993**, *265*, 1446–1454.
- (20) Kosson, D.; Maszczyńska, B. I.; Carr, D. B.; Mayzner-Zawadzka, E.; Lipkowski, A. W. Antinociceptive properties of biphalin after intrathecal application in rats: a reevaluation. *Pharmacol. Rep.* **2005**, *57*, 545–549.

JM701404S