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Bifunctional μ/δ Opioid Peptides: Variation of the Type and Length of the Linker Connecting the Two Components

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Abstract

On the basis of evidence that opioid compounds with a mixed μ agonist/ δ antagonist profile may produce an antinociceptive effect with low propensity to induce side effects, bifunctional opioid peptides containing the μ agonist [Dmt¹]DALDA (H-Dmt-D-Arg-Phe-Lys-NH₂; Dmt = 2',6'-dimethyltyrosine) connected tail-to-tail via various α,ω -diaminoalkyl- or diaminocyclohexane linkers to the δ antagonists TICP[Ψ] (H-Tyr-Tic Ψ [CH₂-NH]Cha-Phe-OH; Cha = cyclohexylalanine, Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), H-Dmt-Tic-OH or H-Bcp-Tic-OH (Bcp = 4'-[N-((4'-phenyl)phenethyl)carboxamido]phenylalanine) were synthesized and pharmacologically characterized *in vitro*. Bifunctional [Dmt¹]DALDA $\rightarrow$ NH-(CH₂)_n-NH $\leftarrow$ TICP[Ψ] compounds (n = 0–12) showed decreasing μ and δ receptor binding affinities with increasing linker length. As expected, several of the bifunctional peptides were μ agonist/ δ antagonists with low nanomolar μ and δ receptor binding affinities. However, compounds with unexpected opioid activity profiles, including a μ partial agonist/ δ partial agonist, μ antagonist/ δ antagonists and μ agonist/ δ agonists, were also identified. These results indicate that the binding affinities and intrinsic efficacies of these bifunctional compounds at both receptors depend on the length and type of the linker connecting the μ and δ components. An important recommendation emerging from this study is that the *in vitro* activity profiles of bifunctional compounds containing an agonist and an antagonist component connected via a linker need to be determined prior to their pharmacological evaluation *in vivo*.

Keywords

opioid peptide analogues; peptide synthesis; bifunctional μ/δ opioid compounds; opioid activity profiles; opioid peptide SAR; linkers in bifunctional opioid peptides

Centrally acting μ opioid agonists like morphine are most widely used in severe pain. However, they produce a number of side effects, including analgesic tolerance, dependence, respiratory depression and inhibition of gastrointestinal transit, which often limit their use. It has been shown that selective δ opioid receptor blockade with a δ opioid antagonist reduced the development of morphine tolerance and dependence (1,2). Furthermore, studies indicated that the δ antagonist naltrindole reversed alfentanil (a μ agonist)-induced respiratory depression (3) and enhanced colonic propulsion (4). Taken together, these observations indicate that a mixed μ agonist/ δ antagonist may have a low propensity to

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produce analgesic tolerance, physical dependence, respiratory depression and inhibition of gastrointestinal transit. Compared with a drug combination of a μ agonist and a δ antagonist, the development of a single compound with a mixed μ agonist/ δ antagonist profile is preferable because of the more predictable pharmacokinetic and pharmacodynamic relationship that is the consequence of the administration of a single medicine (5).

The first reported mixed μ agonist/ δ antagonist, DIPP-NH₂[Ψ] (H-Dmt-Tic Ψ [CH₂-NH]-Phe-Phe-NH₂), produced a potent centrally mediated analgesic effect with intracerebroventricular (i.c.v.) administration, no physical dependence and somewhat less tolerance than morphine (6). A number of pyridomorphinans also showed a mixed μ agonist/ δ antagonist profile (7,8) and one of them (SoRI 20411) given i.c.v. showed centrally mediated antinociceptive activity and did not produce analgesic tolerance. Taken together, the results of these studies provided a proof-of-concept for the development of mixed μ agonist/ δ antagonists as analgesic drug candidates with reduced side effects. However, DIPP-NH₂[Ψ] and the pyridomorphinans have limited ability to cross the blood-brain barrier (BBB).

The μ agonist/ δ antagonists described above were discovered by chance and with these compounds no clear distinction can be made between moieties that confer μ agonist properties to the molecule and moieties that are responsible for the δ antagonist behavior. Alternatively, compounds with this bifunctional profile containing distinct μ agonist and δ antagonist components can be designed. An example is the chimeric peptide H-Dmt→D-Arg→Phe→Lys→NH-(CH₂)₂-NH←Phe←Cha[NH-CH₂] Ψ Tic←Tyr-H, in which the μ agonist component H-Dmt-D-Arg-Phe-Lys-NH₂ ([Dmt¹]DALDA) (9) and the δ antagonist component H-Tyr-Tic Ψ [CH₂-NH]Cha-Phe-OH (TICP[Ψ] (10) are connected "tail-to-tail" via a short linker [NH-(CH₂)₂-NH-] (11) (Figure 1, compound 2). [Dmt¹]DALDA is a systemically active μ opioid analgesic and in peptide 2 it plays a dual role as μ agonist component and as vector conferring BBB crossing ability the entire bifunctional construct. Compound 2 showed low nanomolar binding affinity for μ and δ opioid receptors and displayed the expected μ agonist/ δ antagonist profile in vitro (11). Given subcutaneously (s.c.), it produced a centrally mediated antinociceptive effect with a potency comparable to that of morphine and with low propensity to induce analgesic tolerance (12).

In the present paper we describe the syntheses and in vitro opioid activity profiles of analogues of compound 2 in which the length of the α,ω -diaminoalkyl spacer connecting the μ and δ components was varied: -NH-(CH₂)_n-NH- (n = 0, 2, 6, 8, 10, 12) (Figure 1, compounds 1–6). Compounds in which the [Dmt¹]DALDA and TICP[Ψ] components were connected to the more rigid diaminocyclohexane spacers (1*R*,2*R*)-(–)-1,2-diaminocyclohexane (compound 7), (1*S*,2*S*)-(+)-1,2-diaminocyclohexane (compound 8), *cis*-1,2-diaminocyclohexane (compound 9) and *trans*-1,4-diaminocyclohexane (compound 10) were also prepared and characterized. Furthermore, analogues of compound 2 in which the tetrapeptide TICP[Ψ] component was replaced by the dipeptide δ antagonists H-Dmt-Tic-OH (10) (compound 11) or H-Bcp-Tic-OH (Bcp = 4'-*N*-(4'-phenyl)phenylethyl)carboxamido]phenylalanine) (13) (compound 12) were synthesized and pharmacologically characterized.

It should be pointed out that these bifunctional compounds interact in a monovalent fashion either with a μ opioid receptor binding site or a δ receptor binding site, but have not been designed to simultaneously interact in a bivalent binding mode with both receptor binding sites in a μ/δ receptor heterodimer. μ Agonist/ δ antagonists expected to bind in a bivalent mode, such as the MDAN μ agonist/ δ antagonists, would require a much longer spacer length (~20 Å) (14).

Methods and Materials

General Methods

Precoated plates (silica gel 60 F₂₅₄, 250 μ m, Merck, Darmstadt, Germany) were used for ascending TLC in the following systems (all v/v); (I) *n*-BuOH/AcOH/H₂O (4:1:1); (II) *n*-BuOH/pyridine/AcOH/H₂O (15:10:3:12). Preparative reversed-phase HPLC was performed on a Vydac 218-TP1022 column (22 $\times$ 250 mm) with mobile phases of MeOH in 0.1% trifluoroacetic acid (TFA)/H₂O at a flow rate of 12 mL/min. Analytical reversed-phase HPLC was performed on a Vydac 218-TP510 column (10 $\times$ 250 mm) with a linear gradient of 30–70% MeOH in a 0.1% TFA over 40 min at a flow rate of 2.5 mL/min and capacity factors K' defined as $(t_r - t_m)/t_m$ were determined. Molecular masses of the compounds were determined by electrospray mass spectrometry on a hybrid Q-ToF mass spectrometer interfaced to a MassLynx 4.0 data system (Micromass Ltd., Pointe-Claire, QC, Canada).

Peptide synthesis

Peptides were prepared by a combination of solid phase and solution techniques. Crude peptides were purified by preparative reversed-phase HPLC and were found to be at least 97% pure, as assessed by analytical HPLC and TLC.

[Dmt¹]DALDA→NH-NH←TICP[Ψ] (1)—Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH. Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH was prepared on solid phase using a 2-chlorotriyl chloride resin (Bachem, Torrance, Ca, USA), Fmoc α -amino group protection and *O*-benzotriazol-1-yl)-*N,N,N,N*-tetramethyluronium hexafluorophosphate (HBTU) as coupling agent, except for the coupling of Fmoc-Dmt which was performed with 1,3-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) as coupling agents. After cleavage by treatment with CF₃CH₂OH/CH₂Cl₂ (2:8, v/v) the crude product was purified by semipreparative HPLC (linear gradient of 80–92% MeOH in 0.1% TFA over 25 min).

Boc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NHNH₂. Boc-TicΨ[CH₂-NH]Cha-Phe-Merrifield resin was prepared as described (11) and after removal of the Boc group Boc-Tyr(2-Br-Z)-OH was attached using DIC/HOBt as coupling agents. The peptide resin was then shaken in a mixture of hydrazine hydrate and DMF (1:1, v/v) for 24 h at room temperature. The resulting peptide hydrazide was purified by semi-preparative HPLC (linear gradient of 50–80% MeOH in 0.1% TFA over 20 min).

H-Dmt→D-Arg→Phe→Lys→NH-NH←Phe←Cha[NH-CH₂]ΨTic←Tyr-H $\times$ 4TFA (1). Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH (0.08 mmol) and Boc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NHNH₂ (0.12 mmol) were coupled in DMF (1 mL) using HBTU (0.096 mmol) and DIPEA (0.272 mmol) as coupling agents. After letting the reaction mixture stand for 6 h at room temperature, TLC analysis indicated the complete disappearance of Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH. The reaction mixture was diluted with ethyl acetate and washed with saturated NaHCO₃/H₂O and H₂O. The organic layer was evaporated and dried to a white powder. This procedure was used in all the solution phase syntheses described below. After deprotection with 30% diethylamine (DEA) in tetrahydrofuran (THF) and HF/anisole peptide **1** was purified by semi-preparative HPLC (linear gradient of 30–60% MeOH in 0.1% TFA over 30 min). HPLC K' 3.08; TLC R_f 0.492 (I), R_f 0.747 (II); ES-MS $[M+H]^+$ m/z 1263.74.

[Dmt¹]DALDA→NH-(CH₂)₆-NH←TICP[Ψ] (3)—Fmoc-Dmt-D-Arg(Mtr)-Phe-Lys(2-Cl-Z)-NH-(CH₂)₆-NH₂ $\times$ TFA. The peptide segment Fmoc-Dmt-D-Arg(Mtr)-Phe-Lys(2Cl-Z) was assembled on a 1,6-diaminohexane trityl resin (Novabiochem, Lufelfingen, Switzerland) with Fmoc protection of the α -amino function and using DIC/HOBt as

coupling agents. Arg(Mtr) and Lys(2-Cl-Z) protection was used because it is more stable than Arg(Pmc) and Lys(Boc) protection under the cleavage conditions that have to be used with the 1,6-diaminohexane trityl resin. The protected peptide was cleaved from the resin with 10% TFA/CH₂Cl₂ (30 min at room temperature).

H-Dmt→D-Arg→Phe→Lys→NH-(CH₂)₆-NH←Phe←Cha[NH-CH₂]ΨTic←Tyr-H × 4TFA. Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-OH (11) (1 mmol) and Fmoc-Dmt-D-Arg(Mtr)-Phe-Lys(2-Cl-Z)-NH-(CH₂)₆-NH₂ (1.25 mmol) were coupled in a mixture of THF (18 mL) and DMF (2 mL) using HBTU (1 mmol), DIPEA (2 mmol) and *N*-methylmorpholine (1.25 mmol) as coupling agents. The peptide was deprotected by treatment with 30% DEA/THF and HF/anisole. The crude product was purified by two consecutive runs of semi-preparative HPLC using linear gradients of 40–65% MeOH in 0.1% TFA over 20 min and 50–65% MeOH in 0.1% TFA over 20 min. HPLC *K'* 3.68; TLC *R_f* 0.508 (I), *R_f* 0.768 (II); ES-MS [M+H]⁺ *m/z* 1347.83.

[Dmt¹]DALDA→NH-(CH₂)₈-NH ←TICP[Ψ] (4)—Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(CH₂)₈-NH₂ × TFA. Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-OH (11) (0.118 mmol) and Boc-NH-(CH₂)₈-NH₂ (0.177 mmol, prepared by reacting Boc₂O with a 5-fold excess of 1,8-diaminooctane (15)) were coupled in a mixture of DMF (1.4 mL) and CH₂Cl₂ (0.6 mL) using HBTU (0.142 mmol) and DIPEA (0.26 mmol) as coupling agents, followed by removal of the Boc group with TFA. The crude product was purified by semi-preparative HPLC (linear gradient of 50–80% MeOH in 0.1% TFA over 30 min).

H-Dmt→D-Arg→Phe→Lys→NH-(CH₂)₈-NH←Phe←Cha[NH-CH₂]ΨTic←Tyr-H × 4 TFA. Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH (see synthesis of **1**, 0.038 mmol) and Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(CH₂)₈-NH₂ (0.046 mmol) were coupled in 1 mL of DMF using HBTU (0.046 mmol) and DIPEA (0.129 mmol) as coupling agents. After consecutive treatments with 30% DEA/THF and 5% triisopropylsilane (TIS)/TFA the peptide was obtained in deprotected form. The crude peptide was purified by semi-preparative HPLC (linear gradient of 40–65% MeOH in 0.1% TFA over 20 min). HPLC *K'* 4.35; TLC *R_f* 0.525 (I), *R_f* 0.727 (II); ES-MS [M+H]⁺ *m/z* 1375.87.

[Dmt¹]DALDA→NH-(CH₂)₁₀-NH ←TICP[Ψ] (5)—Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(CH₂)₁₀-NH₂ × TFA. Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-OH (11) (0.18 mmol) and Boc-NH-(CH₂)₁₀-NH₂ (0.55 mmol, prepared by reacting Boc₂O with a 5-fold excess of 1,10-diaminododecane (15)) were coupled in a mixture of DMF (8 mL) and CH₂Cl₂ (3.5 mL) using HBTU (0.22 mmol) and DIPEA (0.40 mmol) as coupling agents, followed by removal of the Boc group with TFA. The product was purified by semi-preparative HPLC (linear gradient of 60–80% MeOH in 0.1% TFA over 25 min).

H-Dmt→D-Arg→Phe→Lys→NH-(CH₂)₁₀-NH←Phe←Cha[NH-CH₂]ΨTic←Tyr-H × 4 TFA. Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH (see synthesis of **1**, 0.0175 mmol) and Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(CH₂)₁₀-NH₂ (0.021 mmol) were coupled in 0.7 mL of DMF using HBTU (0.021 mmol) and DIPEA (0.060 mmol) as coupling agents. After consecutive treatments with 30% DEA/THF and 5% TIS/TFA the peptide was obtained in deprotected form. The crude peptide was purified by semi-preparative HPLC (linear gradient of 40–80% MeOH in 0.1% TFA over 30 min). HPLC *K'* 5.10; TLC *R_f* 0.542 (I), *R_f* 0.788 (II); ES-MS [M+H]⁺ *m/z* 1403.55.

[Dmt¹]DALDA→NH-(CH₂)₁₂-NH ←TICP[Ψ] (6)—Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(CH₂)₁₂-NH₂ × TFA. Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-OH (11) (0.118 mmol) and Boc-NH-(CH₂)₁₂-NH₂ (0.177 mmol, prepared by reacting Boc₂O with a 5-fold excess of 1,12-diaminododecane (15)) were coupled in a mixture of DMF (1.4 mL) and CH₂Cl₂ (0.6

mL) using HBTU (0.14 mmol) and DIPEA (0.26 mmol) as coupling agents, followed by removal of the Boc group with TFA. The product was purified by semi-preparative HPLC (linear gradient of 60–80% MeOH in 0.1% TFA over 25 min).

H-Dmt→D-Arg→Phe→Lys→NH-(CH₂)₁₂-NH←Phe←Cha[NH-CH₂]ΨTic←Tyr-H × 4 TFA. Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH (see synthesis of **1**, 0.045 mmol) and Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(CH₂)₁₂-NH₂ (0.054 mmol) were coupled in 1 mL DMF using HBTU (0.054 mmol) and DIPEA (0.153 mmol) as coupling agents. After consecutive treatments with 30% DEA/THF and 5% TIS/TFA the peptide was obtained in deprotected form. The crude peptide was purified by semi-preparative HPLC (linear gradient of 40–80% MeOH in 0.1% TFA over 30 min). HPLC *K'* 5.68; TLC *R_f* 0.551 (I), *R_f* 0.808 (II); ES-MS [M+H]⁺ *m/z* 1431.93.

[Dmt¹]DALDA→NH-(*trans*-1*R*,2*R*-C₆H₁₀)-NH←TICP[Ψ] (7)—Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(*trans*-1*R*,2*R*-C₆H₁₀)-NH₂ × TFA. Fmoc-Tyr-Tic Ψ[CH₂-NH]Cha-Phe-OH (11) (0.118 mmol) and Boc-NH-(*trans*-1*R*,2*R*-C₆H₁₀)-NH₂ (0.177 mmol) prepared by reacting Boc₂O with a 5-fold excess of (1*R*,2*R*)-(-)-1,2-diaminocyclohexane (15)) were coupled in a mixture of DMF (1.4 mL) and CH₂Cl₂ (0.6 mL) using HBTU (0.14 mmol) and DIPEA (0.26 mmol) as coupling agents, followed by removal of the Boc group with TFA. The product was purified by semi-preparative HPLC (linear gradient of 50–80% MeOH in 0.1% TFA over 30 min).

H-Dmt→D-Arg→Phe→Lys→NH-(*trans*-1*R*,2*R*-C₆H₁₀)-NH←Phe←Cha[NH-CH₂]ΨTic←Tyr-H × 4 TFA. Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH (see synthesis of **1**, 0.059 mmol) and Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(*trans*-1*R*,2*R*-C₆H₁₀)-NH₂ (0.071 mmol) were coupled in 1 mL of DMF using HBTU (0.071 mmol) and DIPEA (0.200 mmol) as coupling agents. The peptide was then obtained in deprotected form after consecutive treatments with 30% DEA/THF and 5% TIS/TFA. The crude product was purified by semi-preparative HPLC (linear gradient of 40–60% MeOH in 0.1% TFA over 20 min). HPLC *K'* 3.93; TLC *R_f* 0.542 (I), *R_f* 0.747 (II); ES-MS [M+H]⁺ *m/z* 1345.14.

[Dmt¹]DALDA→NH-(*trans*-1*S*,2*S*-C₆H₁₀)-NH←TICP[Ψ] (8)—Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH(*trans*-1*S*,2*S*-C₆H₁₀)-NH₂ × TFA. Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-OH (11) (0.118 mmol) and Boc-NH-(*trans*-1*S*,2*S*-C₆H₁₀)-NH₂ (0.177 mmol, prepared by reacting Boc₂O with a 5-fold excess of (1*S*,2*S*)-(+)-1,2-diaminocyclohexane (15)) were coupled in a mixture of DMF (1.4 mL) and CH₂Cl₂ (0.6 mL) using HBTU (0.14 mmol) and DIPEA (0.26 mmol) as coupling agents, followed by removal of the Boc group with TFA. The product was purified by semi-preparative HPLC (linear gradient of 50–80% MeOH in 0.1% TFA over 30 min).

H-Dmt→D-Arg→Phe→Lys→NH-(*trans*-1*S*,2*S*-C₆H₁₀)-NH←Phe←Cha[NH-CH₂]ΨTic←Tyr-H × 4 TFA. Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH (see synthesis of **1**, 0.043 mmol) and Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(*cis*-1*S*,2*S*-C₆H₁₀)-NH₂ (0.052 mmol) were coupled in 1 mL DMF using HBTU (0.052 mmol) and DIPEA (0.146 mmol) as coupling agents. After consecutive treatment with 30% DEA/THF and 5% TIS/TFA, the peptide was obtained in deprotected form. The crude product was purified by semi-preparative HPLC (linear gradient of 40–60% MeOH in 0.1% TFA over 20 min). HPLC *K'* 3.92; TLC *R_f* 0.542 (I), *R_f* 0.747 (II); ES-MS [M+H]⁺ *m/z* 1345.27.

[Dmt¹]DALDA→NH-(*cis*-1,2-C₆H₁₀)-NH←TICP[Ψ] (9)—Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(*cis*-1,2-C₆H₁₀)-NH₂ × TFA. Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-OH (11) (0.118 mmol) and Boc-NH-(*cis*-1,2-C₆H₁₀)-NH₂ (0.177 mmol, prepared by reacting Boc₂O with a 5-fold excess of *cis*-1,2-diaminocyclohexane (15)) were coupled in a mixture of DMF

(1.4 mL) and CH₂Cl₂ (0.6 mL) using HBTU (0.14 mmol) and DIPEA (0.26 mmol) as coupling agents. After purification by semi-preparative HPLC (linear gradient of 75–80% MeOH in 0.1% TFA over 30 min) the Boc group was removed with TFA.

H-Dmt→D-Arg→Phe→Lys→NH-(*cis*-1,2-C₆H₁₀)-NH←Phe←Cha[NH-CH₂]ΨTic←Tyr-H × 4 TFA. Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH (see synthesis of **1**, 0.043 mmol) and Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(*cis*-1,2-C₆H₁₀)-NH₂ (0.051 mmol) were coupled in DMF using HBTU (0.052 mmol) and DIPEA (0.146 mmol) as coupling agents. After consecutive treatment with 30% DEA/THF and 5% TIS/TFA, the peptide was obtained in deprotected form. The crude product was purified by semi-preparative HPLC (linear gradient of 40–56% MeOH in 0.1% TFA over 20 min). HPLC *K'* 3.52; TLC *R_f* 0.534 (I), *R_f* 0.808 (II); ES-MS [M+H]⁺ *m/z* 1345.27.

[Dmt¹]DALDA→NH-(*trans*-1,4-C₆H₁₀)-NH←TICP[Ψ] (10)—Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(*trans*-1,4-C₆H₁₀)-NH₂ × TFA. Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-OH (**11**) (0.118 mmol) and Boc-NH-(*trans*-1,4-C₆H₁₀)-NH₂ (0.177 mmol, prepared by reacting Boc₂O with a 5-fold excess of *trans*-1,4-diaminocyclohexane (**15**)) were coupled in a mixture of DMF (1.4 mL) and CH₂Cl₂ (0.6 mL) using HBTU (0.14 mmol) and DIPEA (0.26 mmol) as coupling agents, followed by removal of the Boc group with TFA. The product was purified by semi-preparative HPLC (linear gradient of 50–80% MeOH in 0.1% TFA over 30 min).

H-Dmt→D-Arg→Phe→Lys→NH-(*trans*-1,4-C₆H₁₀)-NH←Phe←Cha[NH-CH₂]ΨTic←Tyr-H × 4 TFA. Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH (see synthesis of **1**, 0.066 mmol) and Fmoc-Tyr-TicΨ[CH₂-NH]Cha-Phe-NH-(*trans*-1,4-C₆H₁₀)-NH₂ (0.079 mmol) were coupled in 1 mL of DMF using HBTU (0.079 mmol) and DIPEA (0.224 mmol) as coupling agents. After consecutive treatment with 30% DEA/THF and 5% TIS/TFA, the peptide was obtained in deprotected form. The crude product was purified by semi-preparative HPLC (linear gradient of 30–60% MeOH in 0.1% TFA over 30 min). HPLC *K'* 3.17; TLC *R_f* 0.525 (I), *R_f* 0.768 (II); ES-MS [M+H]⁺ *m/z* 1345.22.

[Dmt¹]DALDA→NH-(CH₂)₂-NH←Tic←Dmt-H (11)—Fmoc-Dmt-Tic-NH-(CH₂)₂-NH₂ × TFA. The dipeptide segment Fmoc-Dmt-Tic was assembled on a 1,2-diaminoethane trityl resin (Novabiochem) with Fmoc protection of the α-amino function and using HBTU as coupling agent, and was cleaved from the resin with 50% TFA/CH₂Cl₂ (30 min at room temperature). The crude product was purified by semi-preparative HPLC using a linear gradient of 65–90% MeOH in 0.1% TFA over 20 min).

H-Dmt→D-Arg→Phe→Lys→NH-(CH₂)₂-NH←Tic←Dmt-H. Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH (see synthesis of **1**, 0.058 mmol) and Fmoc-Dmt-Tic-NH-(CH₂)₂-NH₂ (0.067 mmol) were coupled in 1 mL of DMF using HBTU (0.067 mmol) and DIPEA (0.190 mmol) as coupling agents. After consecutive treatments with 30% DEA/THF and 5% TIS/TFA, the deprotected peptide was purified by semi-preparative HPLC (linear gradient of 30–60% MeOH in 0.1% TFA over 20 min). HPLC *K'* 1.92; TLC *R_f* 0.364 (I), *R_f* 0.667 (II); ES-MS [M+H]⁺ *m/z* 1033.60.

[Dmt¹]DALDA→NH-(CH₂)₂-NH←Tic-Bcp-H (12)—Boc-Bcp-Tic-(CH₂)₂-NH₂ × TFA. The dipeptide segment Boc-Bcp-Tic was assembled on a 1,2-diaminoethane trityl resin (Novabiochem) using Fmoc-Tic-OH and Boc-Bcp-OH (**13**), and HBTU as coupling agent. After cleavage from the resin with 5% TFA/CH₂Cl₂ (20 min at room temperature) the product was purified by semi-preparative HPLC using a linear gradient of 60–83% MeOH in 0.1% TFA over 20 min.

H-Dmt→D-Arg→Phe→Lys→NH-(CH₂)₂-NH←Tic←Bcp-H. Fmoc-Dmt-D-Arg(Pmc)-Phe-Lys(Boc)-OH (see synthesis of **1**, 0.1 mmol) and Boc-Bcp-Tic-NH-(CH₂)₂-NH₂ (0.12 mmol) were coupled in 1 mL of DMF using HBTU (0.12 mmol) and DIPEA (0.34 mmol) as coupling agents. After consecutive treatment with 30% DEA/THF and 5% TIS/TFA, the deprotected peptide was purified by semi-preparative HPLC (linear gradient of 50–60% MeOH in 0.1% TFA over 20 min). HPLC *K'* 4.62; TLC *R_f* 0.424 (I), *R_f* 0.707 (II); ES-MS [M+H]⁺ *m/z* 1212.67.

***In vitro* bioassays and receptor binding assays**

Binding affinities for μ and δ opioid receptors were determined by displacing, respectively, [³H]DAMGO (Multiple Peptide Systems, San Diego, CA, USA) and [³H]DSLET (Multiple Peptide Systems) from rat brain membrane binding sites, and κ opioid receptor binding affinities were measured by displacement of [³H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2h at 0° C with [³H]DAMGO, [³H]DSLET and [³H]U69,593 at respective concentrations of 0.72, 0.78 and 0.80 nM. IC₅₀ values were determined from log-dose displacement curves, and K_i-values were calculated from the obtained IC₅₀ values by means of the equation of Cheng and Prusoff (16), using values of 1.3, 2.6 and 2.9 nM for the dissociation constants of [³H]DAMGO, [³H]DSLET and [³H]U69,593, respectively. The guinea pig ileum (GPI) (17) and mouse vas deferens (MVD) (18) bioassays were carried out as reported in detail elsewhere (19,20). A dose-response curve was determined with [Leu⁵]enkephalin as standard for each ileum and vas preparation, and IC₅₀ values of the compounds being tested were normalized according to a published procedure (21). K_e values for antagonists were determined from the ratio of IC₅₀ values obtained with an agonist in the presence and absence of a fixed antagonist concentration (22). μ Antagonist K_e values of compounds were determined in the GPI assay against the μ agonist TAPP (H-Tyr-D-Ala-Phe-Phe-NH₂) (23) and δ antagonist K_e values were measured in the MVD assay against the δ agonist DPDPE.

Results and Discussion

Increasing the spacer length in [Dmt¹]DALDA→NH-(CH₂)_n-NH←TICP[Ψ] from n = 0 to n = 12 resulted in a progressive decrease in μ opioid receptor binding affinity from K_i^μ = 11.2 nM (compound **1**) to K_i^μ = 103 nM (compound **6**) (Table 1). Similarly, δ receptor binding affinity progressively decreased upon lengthening the linker from K_i^δ = 2.28 nM (compound **1**) to 41.4 nM (compound **6**), with the exception of compound **3** which displayed slightly higher δ affinity than **2**. These results indicate that the compounds with the shorter spacer lengths (**1–3**) have low nanomolar binding affinity at both receptors, even though these affinities are lower than the extraordinarily high, subnanomolar binding affinities of their components [Dmt¹]DALDA and TICP[Ψ] at the μ and δ receptor, respectively. In general, compounds **1–6** showed fairly balanced μ / δ receptor binding profiles with slight preference for δ receptors over μ receptors (range of K_i^μ/K_i^δ ratios = 2–5 (Table 1)). None of these compounds displayed significant κ receptor binding affinity (K_i^κ > 1 μM).

In the GPI assay, bifunctional peptides (**1–4**) displayed full μ opioid agonist activity with 2–10 times lower potency as compared to morphine (Table 2). These four compounds retained high δ antagonist activity, as indicated by the K_e^δ values (2–15 nM) determined in the MVD assay. Compound **5** turned out to be a weak partial agonist at the μ receptor [IC₃₅(GPI) = 7880 nM] and, unexpectedly, also was a partial δ agonist at the δ receptor [IC₃₅(MVD) = 717 nM]. Surprisingly, the compound with the longest linker length (**6**) was found to be a weak μ opioid antagonist. As expected, this compound showed weak δ antagonist activity at the δ receptor. Thus, lengthening the linker beyond n = 8 produced a progressive decrease in

efficacy at the μ receptor from full agonism ($n = 8$) to partial agonism ($n = 10$) to antagonism ($n = 12$).

Compounds **7–9** contain three different stereoisomers of 1,2-diaminocyclohexane as spacer between the [Dmt¹]DALDA in TICP[Ψ] components. Obviously, the use of these relatively rigid linkers imposes some restrictions with regard to the relative positioning of the two components in these bifunctional compounds. All three of them showed relatively weak μ receptor binding affinities ($K_i^\mu = 70 - 90$ nM) and varying δ receptor binding affinities ($K_i^\delta = 4 - 95$ nM) (Table 1). Surprisingly, compounds **7–9** all showed weak μ antagonist activity in the GPI assay ($K_e^\mu = 680 - 1270$ nM), while maintaining quite good δ antagonist activity as determined in the MVD assay ($K_e^\delta = 5 - 23$ nM) (Table 2). Evidently, the weak μ receptor binding affinities and weak μ antagonist potencies of these compounds is due to some steric interference at the μ receptor as a consequence of the orientational constraints imposed on the two components by the use of the 1,2-diaminocyclohexane linkers. In contrast, the use of the *trans*-1,4-diaminocyclohexane linker resulted in a bifunctional peptide (**10**) showing high μ and δ receptor binding affinity ($K_i^\mu = 7.87$ nM, $K_i^\delta = 5.79$ nM). In the GPI assay this compound was a μ agonist with about half the potency of morphine. Unexpectedly, it was found to be a δ full agonist as determined in the MVD assay.

In bifunctional compounds **11** and **12** the [Dmt¹]DALDA μ agonist component is connected via the -NH-(CH₂)₂-NH- linker to the dipeptide δ opioid antagonists H-Dmt-Tic-OH and H-Bcp-Tic-OH, respectively. Compound **11** showed low nanomolar μ and δ receptor binding affinities and the expected μ agonist/ δ antagonist profile in the functional assays. Bifunctional peptide **12** also displayed high binding affinities at μ and δ receptors and was a μ full agonist in the GPI assay ($IC_{50} = 93.2$ nM). Surprisingly, this compound turned out to be a quite potent δ full agonist in the MVD assay ($IC_{50} = 23.0$ nM).

In general, the μ and δ receptor binding affinities and the activities in the functional assays of the bifunctional peptides described here are lower as compared to those of their non-linked components. This is likely due to some steric interference at both receptors due to the incorporation of the μ and δ components into the bifunctional peptide constructs. However, because of the exceptionally high, subnanomolar receptor binding affinities of the non-connected μ and δ components, several of the bifunctional compounds still display low nanomolar binding affinities at both receptors. While there is qualitative agreement between μ and δ receptor binding data and the data obtained in the functional GPI and MVD assays, quantitative discrepancies between the two sets of data are apparent with some of the compounds. The relatively lower activities (IC_{50} - or K_e -values) observed with most of the compounds as compared to their μ and δ receptor binding affinities are due to the fact that the GPI and MVD assays are relatively insensitive. This is illustrated with morphine which has a K_i^μ of 1 nM in the μ receptor binding assay and an IC_{50}^μ of 29 nM in the GPI assay. Some of the quantitative discrepancies observed could also be due to varying abilities of the bifunctional peptides to access the receptors in the smooth muscle preparations.

Conclusions and Future Directions

A significant result of this study is the observation that the μ and δ receptor binding affinities of bifunctional compounds **1–6** decreased with increasing length of the α,ω -diamino linker connecting the [Dmt¹]DALDA and TICP[Ψ] components. Furthermore, unexpected opioid activity profiles were observed with compounds **5, 6, 7, 8** and **9**, which showed μ partial agonist or μ antagonist activity rather than the expected μ agonist behavior, and δ partial agonist activity rather than δ antagonism in one case. Also unexpected was the δ agonist activity of compounds **10** and **12** which contain the δ antagonist components TICP[Ψ] and H-Bcp-Tic-OH, respectively. Taken together, these

results lead to the conclusion that the type and length of the linker have distinct effects on both the receptor binding affinities and the intrinsic efficacies in this series of bifunctional μ/δ receptor ligands. Therefore, bifunctional compounds constructed by connecting an agonist and an antagonist via a linker need to be fully characterized *in vitro* before they are used in *in vivo* studies. Several of the μ agonist/ δ antagonists described here have low nanomolar μ and δ receptor binding affinities and will be further examined *in vivo* for antinociceptive potency and for analgesic tolerance and physical dependence development. The identified compounds with a mixed μ agonist/ δ agonist profile are also of interest as analgesic drug candidates with reduced propensity to produce side effects (24).

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Abbreviations

BBB	blood-brain barrier
Bcp	4'-[N-((4'-phenyl)phenethyl)carboxamido]phenylalanine
Boc	<i>tert</i> -butyloxycarbonyl
(Boc)₂O	di- <i>tert</i> -butyl-dicarbonate
Cha	cyclohexylalanine
DAMGO	H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol
DEA	diethylamine
DIC	1,3-diisopropylcarbodiimide
DIPEA	<i>N,N</i> -diisopropylethylamine
DIPP-NH₂[Ψ]	H-Dmt-TicΨ[CH ₂ -NH]Phe-Phe-NH ₂
DMF	<i>N,N</i> -dimethylformamide
Dmt	2',6'-dimethyltyrosine
[Dmt¹]DALDA	H-Dmt-D-Arg-Phe-Lys-NH ₂
DPDPE	H-Tyr-c[D-Pen-Gly-Phe-D-Pen]OH
DSLET	H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH
ES-MS	electrospray mass spectrometry
Fmoc	(fluoren-9-ylmethoxy)-carbonyl
GPI	guinea pig ileum
HBTU	<i>O</i> -benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
MVD	mouse vas deferens
Pen	penicillamine
Pmc	2,2,5,7,8-pentamethylchroman-6-sulfonyl

TAPP	H-Tyr-D-Ala-Phe-Phe-NH ₂
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
TICP[Ψ]	H-Tyr-TicΨ[CH ₂ -NH]Cha-Phe-OH
TIS	triisopropylsilane
U69, 593	(5α,7α,8β-(—)- <i>N</i> -methyl- <i>N</i> -[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide
Z	benzyloxycarbonyl

References

1. Abdelhamid EE, Sultana M, Portoghese PS, Takemori AE. Selective blockage of δ opioid receptors prevents the development of morphine tolerance and dependence in mice. *J Pharmacol Exp Ther*. 1991; 258:299–303. [PubMed: 1649297]
2. Fundytus ME, Schiller PW, Shapiro M, Weltrowska G, Coderre TJ. Attenuation of morphine tolerance and dependence with the highly selective delta-opioid receptor antagonist TIPP[psi]. *Eur J Pharmacol*. 1995; 286:105–108. [PubMed: 8566146]
3. Freye E, Latasch L, Portoghese PS. The delta receptor is involved in sufentanil-induced respiratory depression - opioid subreceptors mediate different effects. *Eur J Anaesthesiol*. 1992; 9:457–462. [PubMed: 1330549]
4. Foxx-Orenstein AE, Jin JG, Grider JR. 5-HT₄ receptor agonists and delta-opioid receptor antagonists act synergistically to stimulate colonic propulsion. *Am J Physiol*. 1998; 275:G979–G983. [PubMed: 9815027]
5. Morphy R, Rankovic Z. Designed multiple ligands. An emerging drug discovery paradigm. *J Med Chem*. 2005; 48:6523–6543. [PubMed: 16220969]
6. Schiller PW, Fundytus ME, Merovitz L, Weltrowska G, Nguyen TM-D, Lemieux C, Chung NN, Coderre TJ. The opioid μ agonist/ δ antagonist DIPP-NH₂[Ψ] produces a potent analgesic effect, no physical dependence, and less tolerance than morphine in rats. *J Med Chem*. 1999; 42:3520–3526. [PubMed: 10479285]
7. Ananthan S, Kezar HS III, Carter RL, Saini SK, Rice KC, Wells JL, Davis P, Xu H, Dersch CM, Bilskey EJ, Porreca F, Rothman RB. Synthesis, opioid receptor binding, and biological activities of naltrexone-derived pyrido- and pyrimidomorphinans. *J Med Chem*. 1999; 42:3527–3538. [PubMed: 10479286]
8. Ananthan S, Khare NK, Saini SK, Seitz LE, Bartlett JL, Davis P, Dersch CM, Porreca F, Rothman RB, Bilskey EJ. Identification of opioid ligands possessing μ agonist/ δ antagonist activity among pyridomorphinans derived from naloxone, oxymorphone, and hydromorphone. *J Med Chem*. 2004; 47:1400–1412. [PubMed: 14998329]
9. Schiller PW, Nguyen TM-D, Berezowska I, Dupuis S, Weltrowska G, Chung NN, Lemieux C. Synthesis and in vitro opioid activity profiles of DALDA analogues. *Eur J Med Chem*. 2000; 35:895–901. [PubMed: 11121615]
10. Schiller PW, Weltrowska G, Berezowska I, Nguyen TMD, Wilkes BC, Lemieux C, Chung NN. The TIPP opioid peptide family: Development of δ antagonists, δ agonists, and mixed μ agonist/ δ antagonists. *Biopolymers (Peptide Sci)*. 1999; 51:411–425.
11. Weltrowska G, Lemieux C, Chung NN, Schiller PW. A chimeric opioid peptide with mixed μ agonist/ δ antagonist properties. *J Pept Res*. 2004; 63:63–68. [PubMed: 15009527]
12. Schiller PW. Bi- or multifunctional opioid peptide drugs. *Life Sci*. 2010; 86:598–603. [PubMed: 19285088]

13. Berezowska I, Chung NN, Lemieux C, Wilkes BC, Schiller PW. Agonist vs antagonist behavior of δ opioid peptides containing novel phenylalanine analogues in place of Tyr¹. *J Med Chem*. 2009; 52:6941–6945. [PubMed: 19827750]
14. Daniels DJ, Lenard NR, Etienne CL, Law P-Y, Roerig SC, Portoghese PS. Opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a bivalent ligand series. *Proc Natl Acad Sci USA*. 2005; 102:19208–19213. [PubMed: 16365317]
15. Pons J-F, Fauchère J-L, Lamaty F, Molla A, Lazaro R. A constrained diketopiperazine as a new scaffold for the synthesis of peptidomimetics. *Eur J Org Chem*. 1998:853–859.
16. Cheng YC, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol*. 1973; 22:3099–3108. [PubMed: 4202581]
17. Paton WD. The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br J Pharmacol Chemother*. 1957; 12:119–127. [PubMed: 13413163]
18. Henderson G, Hughes J, Kosterlitz HW. A new example of a morphine-sensitive neuro-effector junction: adrenergic transmission in the mouse vas deferens. *Br J Pharmacol*. 1972; 46:764–766. [PubMed: 4655272]
19. Schiller PW, Lipton A, Horrobin DF, Bodanszky M. Unsulfated C-terminal 7-peptide of cholecystokinin: a new ligand of the opiate receptor. *Biochem Biophys Res Commun*. 1978; 85:1332–1338. [PubMed: 217386]
20. DiMaio J, Nguyen TM-D, Lemieux C, Schiller PW. Synthesis and pharmacological characterization in vitro of cyclic enkephalin analogues: effect of conformational constraints on opiate receptor selectivity. *J Med Chem*. 1982; 25:1432–1438. [PubMed: 6296388]
21. Waterfield AA, Leslie FM, Lord JA, Ling N, Kosterlitz HW. Opioid activities of fragments of β -endorphin and of its leucine⁶⁵-analogue. Comparison of the binding properties of methionine- and leucine-enkephalin. *Eur J Pharmacol*. 1979; 58:11–18. [PubMed: 499333]
22. Kosterlitz HW, Watt AJ. Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (naloxone). *Br J Pharmacol Chemother*. 1968; 33:266–276. [PubMed: 5664147]
23. Schiller PW, Nguyen TM-D, Chung NN, Lemieux C. Dermorphin analogues carrying an increased positive net charge in their "message domain" display extremely high μ opioid receptor selectivity. *J Med Chem*. 1989; 32:698–703. [PubMed: 2537427]
24. Lee YS, Kulkarni V, Cowell SM, Ma SW, Davis P, Hanlon KE, Vanderah TW, Lai J, Porreca F, Vardanyan R, Hruby VJ. Development of potent μ and δ agonists with high lipophilicity. *J Med Chem*. 2011; 54:382–386. [PubMed: 21128594]

No.	Compound
	H-Dmt→D-Arg→Phe→Lys→X←Phe←Cha[NH-CH ₂] ^Ψ Tic←Tyr-H
1	X= —NH—NH—
2	—NH—(CH ₂) ₂ —NH—
3	—NH—(CH ₂) ₆ —NH—
4	—NH—(CH ₂) ₈ —NH—
5	—NH—(CH ₂) ₁₀ —NH—
6	—NH—(CH ₂) ₁₂ —NH—
7
8
9
10
11	H-Dmt→D-Arg→Phe→Lys→NH-(CH ₂) ₂ ←Tic-Dmt-H
12	H-Dmt→D-Arg→Phe→Lys→NH-(CH ₂) ₂ ←Tic-Bcp-H

Figure 1.
Structural formulas of bifunctional μ/δ opioid receptor ligands.

Table 1Opioid receptor binding data of bifunctional μ/δ opioid peptides

Compound	K_i (nM) ^a		K_i ratio
	μ	δ	μ/δ
1	11.2 ± 1.0	2.28 ± 0.09	4.91
2^b	14.0 ± 1.5	4.79 ± 0.50	2.92
3	18.6 ± 2.2	3.50 ± 0.32	5.31
4	28.7 ± 1.7	7.32 ± 1.64	3.92
5	33.4 ± 8.4	15.3 ± 2.2	2.18
6	103 ± 15	41.4 ± 3.7	2.49
7	89.4 ± 15.8	95.2 ± 18.7	0.939
8	80.3 ± 13.4	21.8 ± 6.1	3.68
9	67.8 ± 4.5	4.20 ± 0.85	16.1
10	7.87 ± 1.12	5.79 ± 1.24	1.36
11	4.44 ± 0.97	1.74 ± 0.63	2.55
12	9.19 ± 1.28	19.1 ± 1.0	0.481
[Dmt ¹]DALDA ^b	0.143 ± 0.015	2100 ± 310	0.000068
TICP[Ψ] ^b	1050 ± 10	0.259 ± 0.047	4050
H-Dmt-Tic-OH ^c	1360 ± 160	1.84 ± 0.24	739
H-Bcp-Tic-OH ^c	2270 ± 210	0.646 ± 0.088	3510
Morphine ^d	1.00 ± 0.04	32.6 ± 3.7	0.0306

^aValues represent means of 3–6 determinations ± SEM.^bData taken from Weltrowska et al. (11)^cData taken from Berezowska et al. (13)^dData taken from Schiller et al. (9)

Table 2Guinea pig ileum (GPI) and mouse vas deferens (MVD) assay data of bifunctional μ/δ opioid peptides^a

Compound	GPI		MVD	
	IC ₅₀ (nM)	K _e ^μ (nM)	K _e ^δ (nM)	IC ₅₀ (nM)
1	470 ± 44		11.2 ± 0.3	
2^b	66.5 ± 5.4		2.40 ± 0.58	
3	261 ± 33		5.89 ± 0.71	
4	318 ± 49		15.6 ± 3.8	
5	7880 ± 1490 (IC ₃₅) ^c			717 ± 207 (IC ₃₅) ^c
6		7650 ± 80	1800 ± 90	
7		1270 ± 110	23.2 ± 3.4	
8		883 ± 315	11.4 ± 3.2	
9		681 ± 102	4.97 ± 0.13	
10	56.0 ± 18.2			98.3 ± 2.4
11	284 ± 32		9.11 ± 0.79	
12	93.2 ± 8.5			23.0 ± 2.1
[Dmt ¹]DALDA ^b	1.41 ± 0.29		inactive	-
TICP[Ψ] ^b	inactive		0.175 ± 0.025	
H-Dmt-Tic-OH ^d	inactive		6.55 ± 0.27	
H-Bcp-Tic-OH ^d		5380 ± 380	17.8 ± 0.7	
Morphine ^e	29.3 ± 2.2			155 ± 31

^aMean of 3–6 determination ± SEM.^bData taken from Weltrowska et al. (11)^cPartial agonist (maximal inhibition of contractions = 70%).^dData taken from Berezowska et al. (13).^eData taken from Schiller et al. (9)