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A new opioid designed multiple ligand derived from the μ opioid agonist endomorphin-2 and the δ opioid antagonist pharmacophore Dmt-Tic

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Abstract—Opioid compounds with mixed μ agonist/δ antagonist properties could be used as analgesics with low propensity to induce tolerance and dependence. Here we report the synthesis of a new designed multiple ligand deriving from the μ selective agonist endomorphin-2 and the δ selective antagonist pharmacophore Dmt-Tic. As predicted, the resulting bivalent ligand showed a μ agonist/δ antagonist profile deriving from the corresponding activities of each pharmacophore.

1. Introduction

The development of tolerance and physical dependence induced by chronic morphine administration limits its prolonged use in the treatment of pain. Analgesia and tolerance to morphine are abolished in μ-opioid receptor knock-out mice, implicating the μ-opioid receptor as the primary receptor type mediating both these effects.[1–3] However, several lines of evidence suggest the additional involvement of the δ-opioid receptor in morphine tolerance. Initial studies using δ-opioid receptor antagonists[4] and more recent studies using δ-opioid receptor knock-out mice[5] were shown to disrupt the development of tolerance. In pharmacological studies, the selective δ receptor antagonist naltrindole has been shown to interact with alternative receptors because naltrindole binding was still detected in the μ/δ/κ triple knock-out mice.[6]

Furthermore, at high concentrations, naltrindole has been shown to lose its δ selectivity and act as an agonist in some cell types.[7] These observations suggest that the development of opioid ligands possessing mixed μ agonist/δ antagonist activity may provide a novel approach for the development of analgesic agents with low propensity to produce tolerance, physical dependence and other side effects. In this context, interesting compounds were reported. As an example, the pseudotetrapeptide DIPP[Ψ] displayed mixed μ agonist/δ antagonist properties in vitro, and analgesia with reduced physical dependence and tolerance when administered icv in rats.[8] In our previous SAR studies, we demonstrated that the C-terminal elongation of the Dmt-Tic (Dmt, 2′,6′-dimethyl-L-tyrosine; Tic, 1,2,3,4-tetrahydroisoquinoline3-carboxylic acid) δ selective dipeptide antagonist pharmacophore yielded the mixed μ agonist/δ antagonist compound, H-Dmt-Tic-Gly-NH-Bzl [pEC_{50} (Guinea Pig Ileum, GPI) = 8.57 and pA_2 (Mouse Vas Deferens, MVD) = 9.25].[9]

A new interesting strategy in the synthesis of such compounds is actually developed through the designed multiple ligands (DML) obtained by the linkage of two different selective pharmacophores.[10] For example, a mixed μ agonist/δ antagonist pseudopeptide was obtained by linking tail to tail through an ethylene spacer,

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a selective δ antagonist (H-Tyr-TicΨ[CH_2-NH]Cha-Phe-OH) with a selective μ agonist (H-Dmt-D-Arg-Phe-Lys-NH_2).[11] Recently, Neumeyer et al. reported the synthesis and pharmacological evaluation of homo- and heterodimeric designed multiple ligands deriving from morphinans,[12] and from the μ/κ agonist morphinan derivative butorphan, and the δ antagonist dipeptide Dmt-Tic.[13] With the aim to extend our studies in this field, we now report the synthesis of the first DML obtained through the tail to tail condensation of the δ selective Dmt-Tic pharmacophore with the endogenous opioid μ selective agonist endomorphin-2, connected by an ethylenediamine spacer. Although opioid bivalent ligands were developed by tail to tail condensation, such as the μ-opioid selective dermorphin analogues[14] and the opioid mimetics containing the *bis*-[H-Dmt-NH(CH_2)$_n$]-2(1*H*)pyrazinone,[15] or the δ-opioid selective enkephalin[16] and the 1,6-*bis*-(*N*,*N*-dimethyl-Dmt-Tic-NH)hexyl,[17] this is the first report of a C-terminally extended endomorphin-2 with the Dmt-Tic pharmacophore in which both moieties of the new DML retained their inherent opioid receptor preference and biological activities. Bifunctional ligands were also formed between disparate receptor systems, for example combining the weakly μ-opioid selective casomorphine and a substance P antagonist.[18]

2. Chemistry

The DML pseudopeptide was prepared by standard solution peptide synthesis reactions as outlined in Scheme 1. Boc-N-protected endomorphin-2 tetrapeptide (Boc-Tyr-Pro-Phe-Phe-OH)[15e] was obtained by a 2 + 2 condensation via WSC/HOBt (WSC, *N*-(3-dimethyaminopropyl)-*N*′-ethylcarbodiimide; HOBt, 1-hydroxybenzotriazole) starting from the corresponding dipeptides Boc-Tyr-Pro-OH[19] and H-Phe-Phe-OBzl.[20] Z-N-monoprotected ethylenediamine was condensed with Boc-Tic-OH via WSC/HOBt to give Z-NH-CH_2-CH_2-NH ← Tic ← Boc. After Boc deprotection (TFA, trifluoroacetic acid), it was condensed with Boc-Dmt-OH (WSC/HOBt) to obtain Z-NH-CH_2-CH_2-NH ← Tic ← Dmt ← Boc. This compound was Z (benzyloxycarbonyl) deprotected by catalytic hydrogenation and then condensed (WSC/HOBt) with the Boc-N-protected endomorphin-2 tetrapeptide previously deprotected at the C-terminus by catalytic hydrogenation. Removal of Boc (*tert*-butyloxycarbonyl) protecting groups (TFA) gave the final pseudopeptides DML that was purified by preparative reverse phase HPLC. The final compounds (**1**, **4**) were identified by HPLC, elemental analysis, mass spectrometry and NMR spectroscopy.

3. Results and discussion

3.1. Receptor affinity analysis

Receptor binding and functional bioactivities of H-Tyr-Pro-Phe-Phe-NH-CH_2-CH_2-NH ← Tic ← Dmt-H (**1**) and the reference compounds (**2**–**4**) are reported in Table 1. Opioid receptor binding studies were performed using a rat brain synaptosome (P_2) fraction.[9,21] In this assay, (**1**) displayed high μ-receptor binding affinity ($K_i^{\mu} = 1.03$ nM), which is similar to the μ selective reference compound endomorphin-2 (**3**) ($K_i^{\mu} = 0.69$ nM),[22] and high δ receptor binding affinity ($K_i^{\delta} = 1.45$ nM) comparable to the reference δ selective antagonist H-Dmt-Tic-NH_2 ($K_i^{\mu} = 1.22$ nM).[21] Compound (**1**) did not show significant κ receptor binding affinity ($K_i^{\kappa} = 1$ μM).

3.2. Functional bioactivity

The GPI and MVD bioassays were carried out as reported in Section 5.[9,21] The functional bioactivity of compound (**1**) showed a μ agonist activity (IC_{50}, GPI = 25.1 nM) in the same order of magnitude of the reference endomorphin-2 (IC_{50}, GPI = 15 nM).[22] At the same time, its δ antagonist activity (pA_2, MVD = 8.9) was 50 times higher than the reference δ antagonist H-Dmt-Tic-NH_2 (pA_2, MVD = 7.2).[21] This new DML compound endowed of μ agonist/δ antagonist activity showed a functional bioactivity that is in quite good accord with our best μ agonist/δ antagonist compound derived from the Dmt-Tic pharmacophore (H-Dmt-Tic-Gly-NH-Bzl; IC_{50}, GPI = 8.57 nM; pA_2, MVD = 9.25).[9] With the aim to demonstrate that μ activity is independent from the presence of the ethylenediamine spacer we synthesized compound (**4**) which maintained the δ antagonism but is endowed with only poor μ agonism.

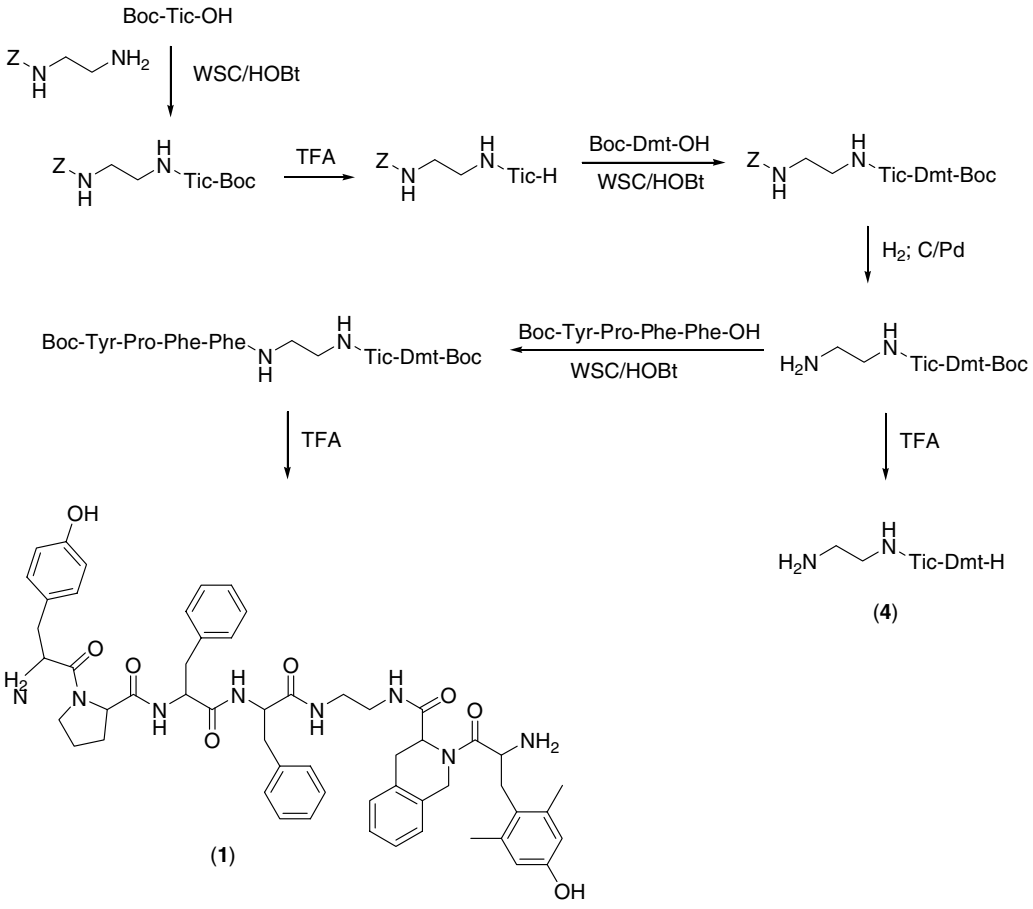
4. Conclusion

The C-terminal joining of endomorphin-2 and the H-Dmt-Tic pharmacophore via an ethylenediamine linker yielded a new DML pseudopeptide, that maintained the high receptor affinity and in vitro biological potency of the parent peptide ligands. In summary, here we confirmed once more the usefulness of the δ selective Dmt-Tic pharmacophore in the synthesis of DML compounds;[13,15e,24] but, more important, here we reported for the first time the possibility to use endomorphin-2 (an endogenous ligand for μ-opioid receptors) in the synthesis of a new DML. As an example, endomorphin-2 could be used in the synthesis of compounds useful for magnetic resonance imaging or PET imaging. H-Tyr-Pro-Phe-Phe-NH-CH_2-CH_2-NH-CO-C_6H_4-pF could represent a potential pharmacological tool for PET imaging of μ-opioid receptors.[25]

5. Experimental

5.1. Chemistry

5.1.1. General methods. Crude peptides were purified by preparative reverse phase HPLC [Waters Delta Prep 4000 system with Waters Prep LC 40 mm Assembly column C18 (30 × 4 cm, 15 μm particle size)] and eluted at a flow rate of 25 mL/min with mobile phase solvent A (10% acetonitrile + 0.1% TFA in H_2O, v/v), and a linear gradient from 25% to 75% B (60%, acetonitrile + 0.1%



Scheme 1. Synthesis of H-Tyr-Pro-Phe-Phe-NH-CH_2-CH_2-NH ← Tic ← Dmt-H.

Table 1. Receptor binding and functional bioactivity

	Compound	Receptor affinity[c] (nM)		Selectivity		Functional bioactivity		
		K_i^{δ}	K_i^{μ}	δ/μ	μ/δ	MVD (IC_{50}) (nM)	MVD pA_2[d]	GPI (IC_{50})[e] (nM)
1	H-Tyr-Pro-Phe-Phe-NH-CH_2-CH_2-NH ← Tic ← Dmt-H	1.45 ± 0.20 (3)	1.03 ± 0.17 (4)	1.41		—	8.9	25.1 ± 3
2	H-Dmt-Tic-NH_2[a]	1.22 ± 0.09	277 ± 26	—	227	—	7.2	>10,000
3	H-Tyr-Pro-Phe-Phe-NH_2[b]	9230 ± 200	0.69 ± 0.16	13,400	—	344 ± 93	—	15 ± 2
4	H-Dmt-Tic-NH-CH_2-CH_2-NH_2	1.81 ± 0.12 (4)	2.72 ± 0.18 (3)		3.36	—	7.6	285.6 ± 21.4

[a] Ref. 21.

[b] Ref. 22.

[c] The K_i values (nM) were determined according to Chang and Prusoff.[23] The mean ± SE with *n* repetitions in parentheses is based on independent duplicate binding assays with five to eight peptide doses using several different synaptosomal preparations.

[d] pA_2 is the negative logarithm to base 10 of the molar concentration of an antagonist that is necessary to double the concentration of agonist needed to elicit the original submaximal response; the antagonist properties of these compounds were tested using deltorphin II as a δ selective opioid agonist.

[e] Agonist activity was expressed as IC_{50} obtained from dose–response curves using guinea-pig ileum (GPI). These values represent means ± SE for at least five fresh tissue samples. Deltorphin II and dermorphin were the internal standards for mouse vas deferens (MVD, δ-opioid receptor bioactivity) and GPI (μ-opioid receptor bioactivity) tissue preparations, respectively.

TFA in H_2O, v/v) in 25 min. Analytical HPLC analyses were performed with a Beckman System Gold (Beckman ultrasphere ODS column, 250 × 4.6 mm, 5 μm particle size). Analytical determinations and capacity factor (K') of the products used HPLC in solvents A and B programmed at flow rate of 1 mL/min with linear gradient from 0% to 100% B in 25 min. Analogues had less than 1% impurities at 220 and 254 nm. TLC was performed on precoated plates of silica gel F254 (Merck, Darmstadt, Germany): (A) 1-butanol/AcOH/H_2O (3:1:1, v/v/v); (B) CH_2Cl_2/toluene/methanol (17:1:2). Ninhydrin (1% ethanol, Merck), fluorescamine (Hoffman-La Roche) and chlorine spray reagents. Melting points were determined on a Kofler apparatus and are uncorrected. Optical rotations were assessed at 10 mg/mL in methanol with a Perkin-Elmer 241 polarimeter in a 10 cm water-jacketed cell. Molecular weights of the compounds were determined by a MALDI-TOF analysis (Hewlett Packard G2025A LD-TOF system mass spectrometer) and α-cyano-4-hydroxycinnamic

acid as a matrix. ^{1}H NMR (δ) spectra were measured, when not specified, in DMSO-d_6 solution using a Bruker AC-200 spectrometer, and peak positions are given in parts per million downfield from tetramethylsilane as internal standard.

5.2. Peptide synthesis

5.2.1. Boc-Tyr-Pro-Phe-Phe-OBzl. To a solution of Boc-Tyr-Pro-OH[19] (0.37 g, 0.97 mmol) and TFA·H-Phe-Phe-OBzl[20] (0.5 g, 0.97 mmol) in DMF (10 mL) at 0 °C, NMM (4-methylmorpholine) (0.11 mL, 0.97 mmol), HOBt (0.16 g, 1.07 mmol) and WSC (0.2 g, 1.07 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF (*N*,*N*-dimethylformamide) was evaporated, the residue was dissolved in EtOAc (ethyl acetate) and washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O) and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/Pe (diethyl ether/petroleum ether) (1:9, v/v): yield 0.66 g (89%); R_f (B) 0.91; HPLC K' 9.14; mp 133–135 °C; $[\alpha]_D^{20}$ −22.6; *m*/*z* 764 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9H), 1.92–2.34 (m, 4H), 2.92–3.29 (m, 6H), 3.41–3.51 (m, 2H), 4.40–4.92 (m, 4H), 5.34 (s, 2H), 6.68–7.21 (m, 19H).

5.2.2. Boc-Tyr-Pro-Phe-Phe-OH[15e]. To a solution of Boc-Tyr-Pro-Phe-Phe-OBzl (0.66 g, 0.86 mmol) in methanol (30 mL) was added Pd/C (10%, 0.1 g), and H_2 was bubbled for 1 h at room temperature. After filtration, the solution was evaporated to dryness. The residue was crystallized from Et_2O/Pe (1:9, v/v): yield 0.56 g (96%); R_f (B) 0.82; HPLC K' 7.39; mp 142–144 °C; $[\alpha]_D^{20}$ −25.1; *m*/*z* 674 $(M+H)^+$.

5.2.3. Boc-Tic-NH-CH_2-CH_2-NH-Z. To a solution of Boc-Tic-OH (0.2 g, 0.72 mmol) and HCl·H_2N-CH_2-CH_2-NH-Z (0.17 g, 0.72 mmol) in DMF (10 mL) at 0 °C, NMM (0.08 mL, 0.72 mmol), HOBt (0.12 g, 0.79 mmol) and WSC (0.15 g, 0.79 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in EtOAc and washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O) and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/Pe (1:9, v/v): yield 0.29 g (89%); R_f (B) 0.81; HPLC K' 5.18; mp 116–118 °C; $[\alpha]_D^{20}$ +7.3; *m*/*z* 455 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9H), 2.92–3.46 (m, 6H), 4.17–4.27 (m, 2H), 4.92–5.34 (m, 3H), 6.96–7.19 (m, 9H).

5.2.4. TFA·H-Tic-NH-CH_2-CH_2-NH-Z. Boc-Tic-NH-CH_2-CH_2-NH-Z (0.25 g, 0.55 mmol) was treated with TFA (2 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) were added to the solution until the product precipitated: yield 0.24 g (92%); R_f (A) 0.45; HPLC K' 3.44; mp 127–129 °C; $[\alpha]_D^{20}$ + 6.9; *m*/*z* 354 $(M+H)^+$.

5.2.5. Boc-Dmt-Tic-NH-CH_2-CH_2-NH-Z. To a solution of Boc-Dmt-OH (0.18 g, 0.59 mmol) and TFA·H-Tic-NH-CH_2-CH_2-NH-Z (0.28 g, 0.59 mmol) in DMF (10 mL) at 0 °C, NMM (0.06 mL, 0.59 mmol), HOBt (0.1 g, 0.65 mmol) and WSC (0.12 g, 0.65 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in EtOAc and washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O) and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/Pe (1:9, v/v): yield 0.33 g (87%); R_f (B) 0.78; HPLC K' 5.14; mp 134–136 °C; $[\alpha]_D^{20}$ +13.7; *m*/*z* 646 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9H), 2.35 (s, 6H), 2.92–3.46 (m, 8H), 4.41–4.51 (m, 2H), 4.92–5.34 (m, 4H), 6.29 (s, 2H), 6.96–7.19 (m, 9H).

5.2.6. Boc-Dmt-Tic-NH-CH_2-CH_2-NH_2. To a solution of Boc-Dmt-Tic-NH-CH_2-CH_2-NH-Z (0.30 g, 0.47 mmol) in methanol (30 mL) was added Pd/C (10%, 0.1 g), and H_2 was bubbled for 1 h at room temperature. After filtration, the solution was evaporated to dryness. The residue was crystallized from Et_2O/Pe (1:9, v/v): yield 0.20 g (86%); R_f (B) 0.61; HPLC K' 4.03; mp 132–134 °C; $[\alpha]_D^{20}$ +15.9; *m*/*z* 512 $(M+H)^+$.

5.2.7. Boc-Tyr-Pro-Phe-Phe-NH-CH_2-CH_2-NH ← Tic ← Dmt ← Boc. To a solution of Boc-Tyr-Pro-Phe-Phe-OH (0.13 g, 0.2 mmol) and Boc-Dmt-Tic-NH-CH_2-CH_2-NH_2 (0.1 g, 0.2 mmol) in DMF (10 mL) at 0 °C, HOBt (0.03 g, 0.22 mmol) and WSC (0.04 g, 0.22 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in EtOAc and washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O) and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/Pe (1:9, v/v): yield 0.2 g (84%); R_f (B) 0.92; HPLC K' 5.30; mp 147–149 °C; $[\alpha]_D^{20}$ −17.2; *m*/*z* 1166 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.40–1.44 (m, 18H), 1.92–2.34 (m, 4H), 2.35 (s, 6H), 2.92–3.51 (m, 16H), 4.41–4.51 (m, 3H), 4.92–4.97 (m, 5H), 6.29 (s, 2H), 6.68–7.21 (m, 18H).

5.2.8. TFA·H-Tyr-Pro-Phe-Phe-NH-CH_2-CH_2-NH ← Tic ← Dmt-H·TFA (1). Boc-Tyr-Pro-Phe-Phe-NH-CH_2-CH_2-NH ← Tic ← Dmt ← Boc (0.17 g, 0.15 mmol) was treated with TFA (1.5 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) were added to the solution until the product precipitated: yield 0.17 g (95%); R_f (A) 0.46; HPLC K' 5.16; mp 153–155 °C; $[\alpha]_D^{20}$ −18.6; *m*/*z* 966 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.92–2.34 (m, 4H), 2.35 (s, 6H), 2.92–3.51 (m, 16H), 3.93–3.97 (m, 2H), 4.40–4.51 (m, 3H), 4.92–4.97 (m, 3H), 6.29 (s, 2H), 6.68–7.21 (m, 18H), Anal. Calcd for $C_{59}H_{66}F_6N_8O_{12}$: C, 59.39; H, 5.58; N, 9.39. Found: C, 58.82; H, 5.91; N, 9.52.

5.2.9. TFA·H-Dmt-Tic-NH-CH_2-CH_2-NH_2·TFA (4). Boc-Dmt-Tic-NH-CH_2-CH_2-NH_2 (0.07 g, 0.14 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) were added to the solution until the product precipitated: yield 0.08 g (93%); R_f (A) 0.36; HPLC K' 3.27; mp 142–146 °C; $[\alpha]_D^{20}$ +14.2; *m*/*z* 412 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.91–3.17 (m, 6H), 3.46–3.95 (m, 3H), 4.41–4.92 (m, 3H), 6.29 (s, 2H), 6.96–7.02 (m, 4H), Anal. Calcd

for $C_{27}H_{32}F_6N_4O_7$: C, 50.78; H, 5.05; N, 8.77. Found: C, 50.62; H, 5.18; N, 8.95.

5.3. Pharmacology

5.3.1. Radioreceptor binding assays. Opioid receptor affinity was determined under equilibrium conditions [2.5 h at room temperature (23 °C)] in a competition assay using brain P_2 synaptosomal membranes prepared from Sprague–Dawley rats.[26,27] Synaptosomes were preincubated to remove endogenous opioid peptides and stored at −80 °C in buffered 20% glycerol.[26,28] Each analogue was analyzed in duplicate assays using 5–8 dosages and 3–5 independent repetitions with different synaptosomal preparations (*n* values are listed in Table 1 in parentheses and results are means ± SE). Unlabelled peptide (2 μM) was used to determine non-specific binding in the presence of 1.9 nM [^{3}H]deltorphin II (45.0 Ci/mmol, Perkin-Elmer, Boston, MA; K_D = 1.4 nM) for δ-opioid receptors and 3.5 nM [^{3}H]DAMGO (50.0 Ci/mmol, Amersham Bioscience, Buckinghamshire, UK; K_D = 1.5 nM) for μ-opioid receptors. Glass fibre filters (Whatman GFC) were soaked in 0.1% polyethylenimine in order to enhance the signal-to-noise ratio of the bound radiolabelled-synaptosome complex, and the filters were washed thrice in ice-cold buffered BSA (bovine serum albumin).[26] The affinity constants (K_i) were calculated according to Cheng and Prusoff.[23]

5.3.2. Biological activity in isolated tissue preparations. The myenteric plexus longitudinal muscle preparations (2–3 cm segments) from the small intestine of male Hartley strain guinea pigs (GPI) measured μ-opioid receptor agonism, and a single mouse vas deferens (MVD) was used to determine δ-opioid receptor agonism as described previously.[6,29] The isolated tissues were suspended in organ baths containing balanced salt solutions in a physiological buffer, pH 7.5. Agonists were tested for the inhibition of electrically evoked contraction and expressed as IC_{50} (nM) obtained from the dose–response curves. The IC_{50} values represent means ± SE of five or six separate assays. δ-Antagonist potencies in the MVD assay were determined against the δ-agonist deltorphin II and are expressed as pA_2 determined using the Schild Plot.[30]

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References and notes

1. Fang, F. G.; Fields, H. L.; Lee, N. M. *J. Pharmacol. Exp. Ther.* **1986**, *238*, 1039.
2. Matthes, H. W.; Maldonado, R.; Simonin, F.; Valverde, O.; Slowe, S.; Kitchen, I.; Befort, K.; Dierich, A.; Le Meur, M.; Dolle, P.; Tzavara, E.; Hanoune, J.; Roques, B. P.; Kieffer, B. L. *Nature* **1996**, *383*, 819.
3. Sora, I.; Funada, M.; Uhl, G. R. *Eur. J. Pharmacol.* **1997**, *324*, R1.
4. Abdelhamid, E. E.; Sultana, M.; Portoghese, P. S.; Takemori, A. E. *J. Pharmacol. Exp. Ther.* **1991**, *258*, 299.
5. Nitsche, F.; Schuller, A. G.; King, M. A.; Zengh, M.; Pasternak, G. W.; Pintar, J. E. *J. Neurosci.* **2002**, *22*, 10906.
6. Gaveriaux-Ruff, C.; Filliol, D.; Simonin, F.; Matthes, H. W.; Kieffer, B. L. *J. Pharmacol. Exp. Ther.* **2001**, *298*, 1193.
7. Chen, Y. L.; Law, P. Y.; Loh, H. H. *Cancer Res.* **2004**, *64*, 8723.
8. Schiller, P. W.; Fundytus, M. E.; Merovitz, L.; Weltrowska, G.; Nguyen, T. M.-D.; Lemieux, C.; Chung, N. N.; Coderre, T. J. *J. Med. Chem.* **1999**, *42*, 3520.
9. Balboni, G.; Guerrini, R.; Salvadori, S.; Bianchi, C.; Rizzi, D.; Bryant, S. D.; Lazarus, L. H. *J. Med. Chem.* **2002**, *45*, 713.
10. Morphy, R.; Rankovic, Z. *J. Med. Chem.* **2005**, *48*, 6523.
11. Weltrowska, G.; Lemieux, C.; Chung, N. N.; Schiller, P. W. *J. Pept. Res.* **2004**, *63*, 63.
12. Peng, X.; Knapp, B. I.; Bidlack, J. M.; Neumeyer, J. L. *J. Med. Chem.* **2006**, *49*, 256.
13. Neumeyer, J. L.; Peng, X.; Knapp, B. I.; Bidlack, J. M.; Lazarus, L. H.; Salvadori, S.; Trapella, C.; Balboni, G. *J. Med. Chem.* **2006**, *49*, 5640.
14. Lazarus, L. H.; Guglietta, A.; Wilson, W. E.; Irons, B. J.; De Castiglione, R. *J. Biol. Chem.* **1989**, *264*, 354.
15. (a) Okada, Y.; Tsuda, Y.; Yokoi, T.; Sasaki, Y.; Ambo, A.; Nagata, M.; Jinsmaa, Y.; Bryant, S. D.; Lazarus, L. H. *J. Med. Chem.* **2003**, *46*, 3201; (b) Jinsmaa, Y.; Miyazaki, A.; Fujita, Y.; Fujisawa, Y.; Shiotani, K.; Li, T.; Tsuda, Y.; Yokoi, T.; Ambo, A.; Sasaki, Y.; Bryant, S. D.; Lazarus, L. H.; Okada, Y. *J. Med. Chem.* **2004**, *47*, 2599; (c) Jinsmaa, Y.; Okada, Y.; Tsuda, Y.; Shiotani, K.; Sasaki, Y.; Ambo, A.; Bryant, S. D.; Lazarus, L. H. *J. Pharmacol. Exp. Ther.* **2004**, *309*, 432; (d) Gao, Y.; Liu, X.; Wei, J.; Zhu, B.; Chen, Q.; Wang, R. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1847; (e) Vázquez, M. E.; Blanco, J. B.; Salvadori, S.; Trapella, C.; Argazzi, R.; Bryant, S. D.; Jinsmaa, Y.; Lazarus, L. H.; Negri, L.; Giannini, E.; Lattanzi, R.; Colucci, M.; Balboni, G. *J. Med. Chem.* **2006**, *49*, 3653.
16. (a) Shimohigashi, Y.; Costa, T.; Chen, H.-C.; Rodbard, D. *Nature* **1982**, *297*, 333; (b) Lipkowski, A. W.; Knonecka, A. M.; Sroczynska, I. *Peptides* **1982**, *3*, 697.
17. Li, T.; Fujita, Y.; Shiotani, K.; Miyazaki, A.; Tsuda, Y.; Ambo, A.; Sasaki, Y.; Jinsmaa, Y.; Marczak, E.; Bryant, S. D.; Salvadori, S.; Lazarus, L. H.; Okada, Y. *J. Med. Chem.* **2005**, *48*, 8035.
18. Lipkowski, A. W.; Misterek, K. *Pol. J. Pharmacol. Pharm.* **1992**, *44*, 25.
19. Salvadori, S.; Marastoni, M.; Balboni, G.; Borea, P. A.; Tomatis, R. *Eur. J. Med. Chem.* **1990**, *25*, 171.
20. Oya, M.; Takahashi, T. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 2075.
21. Salvadori, S.; Attila, M.; Balboni, G.; Bianchi, C.; Bryant, S. D.; Crescenzi, O.; Guerrini, R.; Picone, D.; Tancredi, T.; Temessi, P. A.; Lazarus, L. H. *Mol. Med.* **1995**, *1*, 678.
22. (a) Zadina, J. E.; Hacker, L.; Ge, L. J.; Kastin, A. B. *Nature* **1997**, *386*, 499; (b) Fujita, Y.; Motoyama, T.; Takahashi, M.; Shimizu, Y.; Tsuda, Y.; Yokoi, T.; Li, T.; Sasaki, Y.; Ambo, A.; Kita, A.; Jinsmaa, Y.; Bryant, S. D.; Lazarus, L. H.; Okada, Y. *J. Med. Chem.* **2004**, *47*, 3591.

23. Cheng, Y.-C.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.
24. Balboni, G.; Onnis, V.; Congiu, C.; Zotti, M.; Sasaki, Y.; Ambo, A.; Bryant, S. D.; Jinsmaa, Y.; Lazarus, L. H.; Lazzari, I.; Trapella, C.; Salvadori, S. *Bioorg. Med. Chem.* **2007**, *15*, 3143–3151.
25. (a) Zhang, X.; Cai, W.; Cao, F.; Schreibmann, E.; Wu, Y.; Wu, J. C.; Xing, L.; Chen, X. *J. Nucl. Med.* **2006**, *47*, 492; (b) Tyake, R. J.; Robinson, E. S. J.; Schnabel, R.; Lewis, J. W.; Husbands, S. M.; Nutt, D. J.; Hudson, A. L. *Nucl. Med. Biol.* **2002**, *29*, 455.
26. Lazarus, L. H.; Salvadori, S.; Santagada, V.; Tomatis, R.; Wilson, W. E. *J. Med. Chem.* **1991**, *34*, 1350.
27. Lazarus, L. H.; Salvadori, S.; Attila, M.; Grieco, P.; Bundy, D. M.; Wilson, W. E.; Tomatis, R. *Peptides* **1993**, *14*, 21.
28. Lazarus, L. H.; Wilson, W. E.; De Castiglione, R.; Guglietta, A. *J. Biol. Chem.* **1989**, *264*, 3047.
29. Sasaki, Y.; Sasaki, A.; Niizuma, H.; Goto, H.; Ambo, A. *Bioorg. Med. Chem.* **2003**, *11*, 675.
30. Arunlakshana, Q.; Schild, H. O. *Br. J. Pharmacol.* **1959**, *14*, 48.