Published in final edited form as:

Bioorg Med Chem. 2008 March 15; 16(6): 3032–3038.

Role of Benzimidazole (Bid) in the δ Opioid Agonist Pseudopeptide H-Dmt-Tic-NH-CH₂-Bid (UFP-502)

Severo Salvadori[‡], Stella Fiorini[‡], Claudio Trapella[‡], Frank Porreca[§], Peg Davis[§], Yusuke Sasaki[^], Akihiro Ambo[^], D Marczak Ewa[#], Lawrence H. Lazarus[#], and Gianfranco Balboni^{†,‡,*}

[‡] Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, I-44100 Ferrara, Italy

[§] Department of Pharmacology, University of Arizona, Tucson, Arizona 85721, USA

[^] Tohoku Pharmaceutical University, 4-1, Komatsushima 4-chome, Aoba-Ku, Sendai 981-8558, Japan

[#] Medicinal Chemistry Group, Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA

[†] Department of Toxicology, University of Cagliari, I-09124, Cagliari, Italy

Abstract

H-Dmt-Tic-NH-CH₂-Bid (UFP-502) was the first δ opioid agonist prepared from the Dmt-Tic pharmacophore. It showed interesting pharmacological properties, such as stimulation of mRNA BDNF expression, and antidepressant. To evaluate the importance of 1H-benzimidazol-2-yl (Bid) in the induction of δ agonism, it was substituted by similar heterocycles: The substitution of NH(1) by O or S, transforms the reference δ agonist into δ antagonists. Phenyl ring of benzimidazole is not important for δ agonism; in fact 1H-imidazole-2-yl retains δ agonist activity.

1. Introduction

Extensive structure-activity studies on the prototype δ -opioid receptor antagonist, H-Dmt-Tic-OH, ¹ revealed that even minor chemical modifications changed its pharmacological profile, ² including enhanced δ -antagonism, ³ the appearance of mixed μ -agonism/ δ -agonism, ⁴ as well as formation of mixed μ -agonism/ δ -antagonism, ⁴ μ -agonism, ⁵ μ -antagonism, ⁵ and δ agonism. ^{4, 6} δ -Opioid receptor agonists are known to produce many pharmacological effects in rodents, including analgesia, ⁷ antidepressant, ⁸ neuroprotection/neurogenesis, ⁹ and anti-Parkinson activities. ¹⁰ Our first reported potent and selective δ -opioid agonist derived from the δ -antagonist Dmt-Tic pharmacophore; it is characterized by the presence of Bid (1H-benzimidazole-2-yl) at its C-terminus (H-Dmt-Tic-NH-CH₂-Bid). ⁴ It is endowed with antidepressant-like effects in rodents, with a propensity to induce convulsions to a lower degree than non peptide -agonists. ^{11, 12} With the aim of evaluating the importance of Bid in the induction of δ -agonism, it was substituted by related heterocycles, and an homocycle, as follows: **1**, benzoxazol-2-yl (Boa); ¹³ **2**, benzothiazol-2-yl (Bta); ^{13, 14} **3**, 1H-indol-2-yl (Indl); ¹⁵ **4**, 2,3-dihydro-1H-inden-2-yl (Indn); ¹⁶ **5**, 4-phenyl-1H-imidazol-2-yl (ImidPh); ¹⁷ and **6**,

*To whom correspondence should be addressed. Phone: +39-532-455-275. Fax: +39-532-455-953. E-mail: gbalboni@unica.it; bbg@unife.it.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1*H*-imidazol-2-yl (Imid).¹⁸ Structures of new compounds and their bioactivities are reported in Fig 1. and Table 1, respectively.

2. Chemistry

Pseudopeptides (**1–6**) were prepared in solution by peptide synthetic methods. Simply, Boc-Dmt-Tic-OH¹⁹ was coupled with H₂N-CH₂-hetero-/homo-cycle via WSC/HOBt. Final N-terminal Boc deprotection with TFA and purification by preparative HPLC, gave compounds (**1–6**). The intermediates (benzo[*d*]oxazol-2-yl)methanamine (H₂N-CH₂-Boa) and (benzo[*d*]thiazol-2-yl)methanamine (H₂N-CH₂-Bta) were prepared according to the procedure of Nestor et al.¹³ Mixed carbonic anhydride coupling of Z-Gly-OH with *o*-aminophenol gave the crude intermediate amide, which was converted without purification to the desired benzoxazole (Z-NH-CH₂-Boa) by cyclization and dehydration in refluxing propionic acid (~140°C). N-terminal Z deprotection was accomplished by catalytic hydrogenation (H₂; Pd/C). Z-NH-CH₂-Bta was prepared in a similar manner using the disulfide of *o*-aminothiophenol which reacts more effectively than the monomeric *o*-aminothiophenol). The dimeric intermediate amide was reduced with Zn/AcOH to the sulfhydryl compound, which underwent cyclization to Z-NH-CH₂-Bta upon treatment with TEA in dioxane. N-terminal Z-deprotection was accomplished by HBr/AcOH treatment. H₂N-CH₂-Indl and H₂N-CH₂-Indn were prepared according to procedures described by Wright et al.¹⁵ and Shinozaki, et al.,¹⁶ respectively. H₂N-CH₂-IndPh was prepared according to Poitout et al. starting from Boc-Gly-OH instead of Boc-Trp-OH.¹⁷ Boc-Gly-OH was treated with cesium carbonate followed by condensation with phenacyl bromide. Cyclization of the resulting ketoester using ammonium acetate in refluxing xylene yielded the desired Boc-NH-CH₂-ImidPh which was finally Boc-deprotected upon TFA treatment. Finally, H₂N-CH₂-Imid was prepared as reported by Bastiaansen et al.¹⁸

3. Results and Discussion

3.1. Receptor Affinity Analysis

Receptor binding data for μ - and δ -receptors and selectivity (K_i^μ/K_i^δ or K_i^δ/K_i^μ) are reported in Table 1. All new compounds (**1–6**) had subnanomolar affinity for δ -opioid receptors ($K_i^\delta=0.066–0.443$ nM); nonetheless, their averaged affinity was 6 fold less than the reference compound (H-Dmt-Tic-NH-CH₂-Bid). As expected, the lack of a free carboxylic function induces an increase in the μ -receptor affinity ($K_i^\mu = 0.16–6.74$ nM).⁵ None of these new compounds exhibited high receptor selectivity; ($K_i^\mu/K_i^\delta = 5–15$) in comparison with the reference; only compound (**4**) doesn't follow this trend showing an almost complete lack of selectivity ($K_i^\delta/K_i^\mu = 1.2$).

3.2. Functional Bioactivity

Compounds (**1–6**) were tested in the electrically stimulated MVD and GPI pharmacological assays for intrinsic functional bioactivity (Table 1). As often seen, especially in the Dmt-Tic pharmacophore field, a close correlation between binding and functional bioactivity data is often lacking. We and other investigators have previously discussed this discrepancy; unfortunately, until now we have neither definitive nor comprehensive answers for these observations.⁵ The substitution of Bid with benzoxazole (Boa), benzothiazole (Bta), indole (Indl) and 4-phenyl-imidazole heterocycles transforms the potent and moderately selective reference -agonist (MVD; IC₅₀ = 0.94 nM) into potent -antagonists (MVD, pA₂ = 9.37–9.45). Compound **1–6** were tested in the electrically stimulated MVD and GPI pharmacological assays for intrinsic functional bioactivity (Table 1). As often seen, especially in the Dmt-Tic pharmacophore field, a close correlation between binding and functional bioactivity data is often lacking. We and other investigators have previously discussed this discrepancy;

unfortunately, until now we have neither definitive nor comprehensive answers for these observations.⁵ The substitution of Bid with benzoxazole (Boa), benzothiazole (Bta), indole (Indl) and 4-phenyl-imidazole heterocycles transforms the potent and moderately selective reference δ -agonist (MVD; $IC_{50} = 0.94$ nM) into potent δ -antagonists (MVD, $pA_2 = 9.37$ – 9.45). Compound **4**, containing the 2,3-dihydro-1*H*-indene nucleus (Indn) exhibited a δ -agonist activity that was 137-fold lower than the reference, but maintained a similar μ -agonist activity. Finally, compound **6**, characterized by the presence of the only imidazole (Imid) ring instead of the benzimidazole (Bid), substantially maintained the δ - and μ -agonist activities (3.86 and 3.14 fold lower than the reference, respectively). In general, all compounds (except **4**) decreased in μ -agonist activity (3.1–5.9 fold) which could be useful to improve their selectivity as δ -agonists or antagonists.

4. Conclusion

As confirmation that even minor chemical modifications change the pharmacological profile of the opioid Dmt-Tic pharmacophore; this study demonstrates that the transformation of a potent δ -agonist into potent δ -antagonists can be affected by simply changing N^1 -H in the benzimidazole ring by O, S, or by N^3 deletion. Surprisingly, compound **4** shows μ agonist activity comparable to the reference, accomplished by a weak δ agonist activity; its behaviour is reversed in comparison with the reference. Finally, compounds **5** and **6** containing the imidazole heterocycle with (**5**) or without a phenyl ring (**6**), are characterized by a completely different behaviour; in fact the presence of the phenyl ring induces a potent antagonist activity (**5**) while its absence (**6**) yield potent δ agonist activity. The conclusions drawn from this series of compounds are as follows: *i*) Bid is important, but not essential for δ agonist activity; since the substitution of N^1 -H by O or S results in δ antagonism. *ii*) Deletion of N^3 in the benzimidazole ring provides δ -antagonism. *iii*) Finally and more important, the removal of the phenyl ring from the benzimidazole function (Bid) to form an imidazole, maintains the δ -agonism of the reference compound H-Dmt-Tic-NH-CH₂-Bid (UFP-502). The new δ -agonist H-Dmt-Tic-NH-CH₂-Imid could be a useful tool in future pharmacological studies; for example, preliminary data obtained in animal models for Parkinson's disease with δ -opioid agonists containing the Dmt-Tic pharmacophore, provide evidence of an interesting activity profile that differs from that of SNC-80.¹⁰

5. Experimental

5.1. Chemistry

5.1.1. General Methods—Crude compounds were purified by preparative reversed-phase HPLC [Waters Delta Prep 4000 system with Waters Prep LC 40 mm Assembly column C18 (30 x 4 cm, 15 μ m particle size)] and eluted at a flow rate of 20 mL/min with mobile phase solvent A (10% acetonitrile + 0.1% TFA in H₂O, v/v), and a linear gradient from 10 to 60% solvent B (60%, acetonitrile + 0.1% TFA in H₂O, v/v) in 30 min. Analytical HPLC analyses were performed with a Beckman System Gold (Beckman ultrasphere ODS column, 250 x 4.6 mm, 5 μ m 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 when monitored at 220 and 254 nm.

TLC was performed on precoated plates of silica gel F254 (Merck, Darmstadt, Germany): (A) 1-butanol/AcOH/H₂O (3:1:1, v/v/v); (B) CH₂Cl₂/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. ^1H 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. Benzyl (benzo[*d*]oxazol-2-yl)methylcarbamate (Z-NH-CH₂-Boa)—A solution of Z-Gly-OH (4.18 g, 20 mmol) and TEA (2.8 mL, 20 mmol) in dry THF (70 mL) was treated at -20°C with isobutyl chloroformate (2.6 mL, 20 mmol). After 30 min at -20°C , a solution of *o*-hydroxyaniline (2.4 g; 22 mmol) in THF (50 mL) was added. The reaction mixture was stirred while slowly warming to room temperature (1 h). The solution was partitioned between 5% NaHCO₃ and AcOEt. The organic layer was washed with 5% NaHSO₄, H₂O, 5% NaHCO₃, H₂O, and brine. The AcOEt layer was dried (Na₂SO₄) and filtered. The filtrate was concentrated to yield the intermediate amide as a yellow oil; that was directly cyclized without purification by refluxing in propionic acid for 4 h. The solution was concentrated in vacuo, and the residual oil was subjected to silica gel chromatography with a linear gradient from CH₂Cl₂ to CH₂Cl₂/Et₂O (3:1): yield 2.37 g (42%); *R*_f(B) 0.73; HPLC *K'* 6.31; oil; *m/z* 283 (M+H)⁺; ^1H -NMR (DMSO- d_6) δ 4.18–4.23 (d, 2H), 5.34 (s, 2H), 7.19–7.26 (m, 9H).

5.2.2. H₂N-CH₂-Boa—A mixture of Z-NH-CH₂-Boa (1.13 g, 4 mmol) and 400 mg of 10% Pd/C in acetic acid (20 mL) was treated with H₂ for 1 h at atmospheric pressure. The catalyst was filtered through Celite, and the filtrate was concentrated in vacuo to yield a pale yellow oil which was partitioned between 5% NaHCO₃ and AcOEt. The organic layer was washed with 5% NaHCO₃, H₂O, and brine. The AcOEt layer was dried (Na₂SO₄), filtered and evaporated to dryness. The residual oil was subjected to silica gel chromatography with a linear gradient from CH₂Cl₂ to CH₂Cl₂/Et₂O (3:1): yield 0.53 g (89%); *R*_f(B) 0.34; HPLC *K'* 3.41; mp 120–122 $^\circ\text{C}$; *m/z* 149 (M+H)⁺; ^1H -NMR (DMSO- d_6) δ 3.87–3.93 (d, 2H), 7.24–7.28 (m, 4H).

5.2.3. Boc-Dmt-Tic-NH-CH₂-Boa—To a solution of Boc-Dmt-Tic-OH¹⁹ (0.14 g, 0.3 mmol) and H₂N-CH₂-Boa (0.04 g, 0.3 mmol) in DMF (10 mL) at 0 $^\circ\text{C}$, HOBT (0.05 g, 0.33 mmol), and WSC (0.06 g, 0.33 mmol) were added. The reaction mixture was stirred for 3 h at 0 $^\circ\text{C}$ and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in EtOAc and washed with citric acid (10% in H₂O), NaHCO₃ (5% in H₂O), and brine. The organic phase was dried (Na₂SO₄) and evaporated to dryness. The residue was precipitated from Et₂O/Pe (1:9, v/v): yield 0.15 g (82%); *R*_f(B) 0.75; HPLC *K'* 6.02; mp 140–142 $^\circ\text{C}$; $[\alpha]_{\text{D}}^{20}$ -20.8; *m/z* 600 (M+H)⁺; ^1H -NMR (DMSO- d_6) δ 1.40 (s, 9H), 2.35 (s, 6H), 2.92–3.17 (m, 4H), 4.41–4.92 (m, 6H), 6.29 (s, 2H), 6.96–7.26 (m, 8H).

5.2.4. TFA.H-Dmt-Tic-NH-CH₂-Boa (1)—Boc-Dmt-Tic-NH-CH₂-Boa (0.12 g, 0.2 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et₂O/Pe (1:1, v/v) were added to the solution until the product precipitated: yield 0.12 g (97%); *R*_f(A) 0.36; HPLC *K'* 4.36; mp 150–152 $^\circ\text{C}$; $[\alpha]_{\text{D}}^{20}$ -21.5; *m/z* 500 (M+H)⁺; ^1H -NMR (DMSO- d_6) δ 2.35 (s, 6H), 2.92–3.17 (m, 4H), 3.95–4.92 (m, 6H), 6.29 (s, 2H), 6.96–7.26 (m, 8H). Anal. Calcd. for C₃₁H₃₁F₃N₄O₆: C, 60.78; H, 5.10; N, 9.15. Found: C, 60.62; H, 5.03; N, 8.98.

5.2.5. *N*-(benzyloxycarbonyl)-glycine *o*-mercaptoanilide, disulfide derivative—A solution of Z-Gly-OH (1.57 g, 7.5 mmol) and TEA (1.05 mL, 7.5 mmol) in dry THF (25 mL) was treated at -20°C with isobutyl chloroformate (0.98 mL, 7.5 mmol). After 30 min at -20°C , a solution of *o*-aminophenyl disulfide (0.93 g; 3.75 mmol) in THF (25 mL) was added. The reaction mixture was stirred while slowly warming to room temperature (4 h). The solvent was evaporated at reduced pressure, and the residue was slurried in EtOH. The solid was filtered,

washed with EtOH and dried in vacuo: yield 1.77 g (75%); $R_f(B)$ 0.82; HPLC K' 6.54; mp 160–162 °C; m/z 632 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 3.87–3.92 (d, 2H), 5.34 (s, 2H), 7.19–7.48 (m, 9H).

5.2.6. Benzyl (benzo[*d*]thiazol-2-yl)methylcarbamate (Z-NH-CH₂-Bta)—The above disulfide derivative (1.5 g, 2.38 mmol) in glacial acetic acid (200 mL) was warmed to 50 °C. Zn powder (4 g, 60 mmol) was added slowly while most of the disulfide dissolved. The Zn was filtered, the solution was concentrated to dryness. The residue was dissolved in dioxane (200 mL), and the pH was adjusted to 10 with TEA. The solution was stirred under N₂ at room temperature overnight. After solvent evaporation, the residue was purified by column chromatography as reported above. For Z-NH-CH₂-Boa: yield 1 g (71%); $R_f(B)$ 0.71; HPLC K' 6.12; mp 93–95 °C; m/z 299 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 4.18–4.23 (d, 2H), 5.34 (s, 2H), 7.19–8.23 (m, 9H).

5.2.7. H₂N-CH₂-Bta—Z-NH-CH₂-Bta (0.8 g, 2.68 mmol) was treated with 4N HBr/AcOH (30 mL) for 1.5 h at room temperature. The mixture was concentrated in vacuo and purified by column chromatography as reported for H₂N-CH₂-Boa. The purified intermediate was deprotonated with 5% NaHCO₃ as reported for H₂N-CH₂-Boa: yield 0.39 g (88%); $R_f(B)$ 0.32; HPLC K' 3.19; mp 125–127 °C; m/z 165 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 3.87–3.93 (d, 2H), 7.55–8.23 (m, 4H).

5.2.8. Boc-Dmt-Tic-NH-CH₂-Bta—This compound was obtained by condensation of Boc-Dmt-Tic-OH with H₂N-CH₂-Bta via WSC/HOBt as reported for Boc-Dmt-Tic-NH-CH₂-Boa: yield 0.14 g (83%); $R_f(B)$ 0.71; HPLC K' 5.89; mp 143–145 °C; $[\alpha]_D^{20}$ -19.3; m/z 616 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 1.40 (s, 9H), 2.35 (s, 6H), 2.92–3.17 (m, 4H), 4.41–4.92 (m, 6H), 6.29 (s, 2H), 6.96–8.23 (m, 8H).

5.2.9. TFA.H-Dmt-Tic-NH-CH₂-Bta (2)—Boc-Dmt-Tic-NH-CH₂-Bta was treated with TFA as reported for TFA.H-Dmt-Tic-NH-CH₂-Boa: yield 0.1 g (93%); $R_f(A)$ 0.34; HPLC K' 4.22; mp 155–157 °C; $[\alpha]_D^{20}$ -18.7; m/z 516 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 2.35 (s, 6H), 2.92–3.17 (m, 4H), 3.95–4.92 (m, 6H), 6.29 (s, 2H), 6.96–8.23 (m, 8H). Anal. Calcd. for C₃₁H₃₁F₃N₄O₅S: C, 59.23; H, 4.97; N, 8.91. Found: C, 59.52; H, 5.13; N, 8.71.

5.2.10. Boc-Dmt-Tic-NH-CH₂-Indl—This compound was obtained by condensation of Boc-Dmt-Tic-OH with H₂N-CH₂-Indl15 via WSC/HOBt as reported for Boc-Dmt-Tic-NH-CH₂-Boa: yield 0.11 g (84%); $R_f(B)$ 0.67; HPLC K' 5.78; mp 147–149 °C; $[\alpha]_D^{20}$ -20.8; m/z 598 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 1.40 (s, 9H), 2.35 (s, 6H), 2.92–3.17 (m, 4H), 4.41–4.92 (m, 6H), 6.13–6.29 (m, 3H), 6.96–7.08 (m, 8H).

5.2.11. TFA.H-Dmt-Tic-NH-CH₂-Indl (3)—Boc-Dmt-Tic-NH-CH₂-Indl was treated with TFA as reported for TFA.H-Dmt-Tic-NH-CH₂-Boa: yield 0.09 g (91%); $R_f(A)$ 0.30; HPLC K' 4.16; mp 149–151 °C; $[\alpha]_D^{20}$ -18.1; m/z 498 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 2.35 (s, 6H), 2.92–3.17 (m, 4H), 3.95–4.92 (m, 6H), 6.13–6.29 (m, 3H), 6.96–7.08 (m, 8H). Anal. Calcd. for C₃₂H₃₃F₃N₄O₅: C, 62.94; H, 5.45; N, 9.18. Found: C, 63.15; H, 5.59; N, 9.29.

5.2.13. Boc-Dmt-Tic-NH-CH₂-Indn—This compound was obtained by condensation of Boc-Dmt-Tic-OH with H₂N-CH₂-Indn16 via WSC/HOBt as reported for Boc-Dmt-Tic-NH-CH₂-Boa: yield 0.14 g (87%); $R_f(B)$ 0.74; HPLC K' 6.21; mp 139–141 °C; $[\alpha]_D^{20}$ -21.4; m/z 599 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 1.40 (s, 9H), 2.35 (s, 6H), 2.66–3.17 (m, 11H), 4.41–4.92 (m, 4H), 6.29 (s, 2H), 6.96–7.20 (m, 8H).

5.2.14. TFA.H-Dmt-Tic-NH-CH₂-Indn (4)—Boc-Dmt-Tic-NH-CH₂-Indn was treated with TFA as reported for TFA.H-Dmt-Tic-NH-CH₂-Boa: yield 0.10 g (90%); *R*_f(A) 0.35; HPLC *K*' 4.44; mp 140–142 °C; [α]_D²⁰ -19.8; *m/z* 499 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 2.35 (s, 6H), 2.66–3.95 (m, 12H), 4.41–4.92 (m, 3H), 6.29 (s, 2H), 6.96–7.20 (m, 8H). Anal. Calcd. for C₃₃H₃₆F₃N₃O₅: C, 64.80; H, 5.93; N, 6.87. Found: C, 64.67; H, 5.76; N, 6.73.

5.2.15. *tert*-butyl (4-phenyl-1*H*-imidazol-2-yl)methylcarbamate (Boc-NH-CH₂-ImidPh)—A solution of Boc-Gly-OH (2.87 g, 16.4 mmol) and cesium carbonate (2.7 g, 8.3 mmol) in EtOH (50 mL) was shaken for 30 min at room temperature, and then evaporated under reduced pressure. To the resulting salt in DMF (60 mL) was added 2-bromoacetophenone (3.26 g, 16.4 mmol). The mixture was stirred for 1 h at room temperature under argon and then concentrated under reduced pressure. AcOEt (40 mL) was added, the mixture filtered, and the CsBr washed with AcOEt. The filtrate was then concentrated under reduced pressure. A solution of the resulting oil and ammonium acetate (2.5 g, 32 mmol) in xylene (200 mL) was refluxed for 45 min. Excess NH₄OAc and H₂O were removed using a Dean-Stark trap. The mixture was then cooled to room temperature, diluted with AcOEt (100 mL) and washed with H₂O, 5% NaHCO₃ and brine. The organic phase was dried (Na₂SO₄), and concentrated to dryness. The residue was precipitated from Et₂O/Pe (1:1): yield 2.6 g (58%); *R*_f(B) 0.57; HPLC *K*' 5.68; mp 181–183 °C; *m/z* 274 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 1.40 (s, 9H), 4.18–4.23 (d, 2H), 6.9–7.48 (m, 6H).

5.2.16. H₂N-CH₂-ImidPh—Boc-NH-CH₂-ImidPh (2 g, 7.33 mmol) was treated with TFA (4 mL) for 0.5 h at room temperature. Et₂O/Pe (1:1, v/v) were added to the solution until the product precipitated. The solid was filtered and dissolved in AcOEt for deprotonation with 5% NaHCO₃ as reported for H₂N-CH₂-Boa: yield 1.14 g (90%); *R*_f(B) 0.25; HPLC *K*' 2.87; mp 121–123 °C; *m/z* 174 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 3.87–3.93 (d, 2H), 6.9–7.48 (m, 4H).

5.2.17. Boc-Dmt-Tic-NH-CH₂-ImidPh—This compound was obtained by condensation of Boc-Dmt-Tic-OH with H₂N-CH₂-ImidPh via WSC/HOBt (except washing with aqueous citric acid solution) as reported for Boc-Dmt-Tic-NH-CH₂-Boa: yield 0.11 g (80%); *R*_f(B) 0.58; HPLC *K*' 5.65; mp 148–150 °C; [α]_D²⁰ -22.5; *m/z* 625 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 1.40 (s, 9H), 2.35 (s, 6H), 2.92–3.17 (m, 4H), 4.41–4.92 (m, 6H), 6.29 (s, 2H), 6.9–7.48 (m, 10H).

5.2.18. 2TFA.H-Dmt-Tic-NH-CH₂-ImidPh (5)—Boc-Dmt-Tic-NH-CH₂-ImidPh was treated with TFA as reported for TFA.H-Dmt-Tic-NH-CH₂-Boa: yield 0.08 g (93%); *R*_f(A) 0.29; HPLC *K*' 4.12; mp 152–154 °C; [α]_D²⁰ -23.7; *m/z* 525 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 2.35 (s, 6H), 2.92–3.17 (m, 4H), 3.95–4.92 (m, 6H), 6.29 (s, 2H), 6.9–7.48 (m, 10H). Anal. Calcd. for C₃₅H₃₅F₆N₅O₇: C, 55.93; H, 4.69; N, 9.32. Found: C, 55.77; H, 4.62; N, 9.18.

5.2.19. Boc-Dmt-Tic-NH-CH₂-Imid—This compound was obtained by condensation of Boc-Dmt-Tic-OH with H₂N-CH₂-Imid18 via WSC/HOBt (except washing with aqueous citric acid solution) as reported for Boc-Dmt-Tic-NH-CH₂-Boa: yield 0.09 g (83%); *R*_f(B) 0.43; HPLC *K*' 5.01; mp 153–155 °C; [α]_D²⁰ -23.6; *m/z* 549 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 1.40 (s, 9H), 2.35 (s, 6H), 2.92–3.17 (m, 4H), 4.41–4.92 (m, 6H), 6.29 (s, 2H), 6.87–7.02 (m, 6H).

5.2.20. 2TFA.H-Dmt-Tic-NH-CH₂-Imid (6)—Boc-Dmt-Tic-NH-CH₂-Imid was treated with TFA as reported for TFA.H-Dmt-Tic-NH-CH₂-Boa: yield 0.07 g (94%); *R*_f(A) 0.25; HPLC *K*' 3.97; mp 156–158 °C; [α]_D²⁰ -24.1; *m/z* 449 (M+H)⁺; ¹H-NMR (DMSO-*d*₆) δ 2.35 (s, 6H), 2.92–3.17 (m, 4H), 3.95–4.92 (m, 6H), 6.29 (s, 2H), 6.87–7.02 (m, 6H). Anal. Calcd. for C₂₉H₃₁F₆N₅O₇: C, 51.56; H, 4.63; N, 10.37. Found: C, 51.74; H, 4.79; N, 10.18.

5.3. Pharmacology

5.3.1. Radioreceptor binding assays—Opioid receptor affinity were determined under equilibrium conditions [2.5 h at room temperature (23 °C)] in a competition assay using brain P₂ synaptosomal membranes prepared from Sprague-Dawley rats.^{22, 23} Synaptosomes were preincubated to remove endogenous opioid peptides and stored at –80 °C in buffered 20% glycerol.^{22, 24} Each analogue was analyzed in duplicate assays using five to eight dosages and three to five independent repetitions with different synaptosomal preparations (*n* values are listed in Table 1 in parenthesis and results are mean ± SE). Unlabeled peptide (2 μM) was used to determine non-specific binding in the presence of 1.9 nM [³H]deltorphan II (45.0 Ci/mmol, Perkin Elmer, Boston, MA; *K_D* = 1.4 nM) for δ-opioid receptors and 3.5 nM [³H]DAMGO (50.0 Ci/mmol), Amersham Bioscience, Buckinghamshire, U. K.; *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 radiolabeled-synaptosome complex, and the filters were washed thrice in ice-cold buffered BSA.²² The affinity constants (*K_i*) were calculated according to Cheng and Prusoff.²⁰

5.3.2. Biological activity in isolated tissue preparation—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.²⁵ 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 IC₅₀ (nM) obtained from the dose-response curves. The IC₅₀ values represent the mean ± SE of five or six separate assays. δ-antagonist potencies in the MVD assay were determined against the δ-agonist DPDPE; μ-antagonism in the GPI assay used the μ-agonist PL-017 and both are expressed as pA₂ determined using the Schild Plot.²⁶

Acknowledgements

This research was supported in part by the University of Cagliari, University of Ferrara and the Intramural Research Program of NIH and NIEHS. The authors appreciate the professional expertise and assistance of the library staff at NIEHS.

Abbreviations

In addition to the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1985**, *260*, 14-42), this paper uses the following additional symbols and abbreviations

AcOEt	ethyl acetate
AcOH	acetic acid
Bid	1 <i>H</i> -benzimidazol-2-yl
Boa	benzoxazol-2-yl
Boc	<i>tert</i> -butoxycarbonyl
Bta	benzothiazol-2-yl

DAMGO	[D-Ala ² ,N-Me-Phe ⁴ ,Gly-ol ⁵]enkephalin
DEL C	deltorphan II (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH ₂)
DMF	<i>N,N</i> -dimethylformamide
DMSO-<i>d</i>₆	hexadeuteriodimethyl sulfoxide
Dmt	2',6'-dimethyl-L-tyrosine
DPDPE	(D-Pen ² ,D-Pen ⁵)-enkephalin
GPI	guinea-pig ileum
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
Imid	1 <i>H</i> -imidazol-2-yl
ImidPh	4-phenyl-1 <i>H</i> -imidazol-2-yl
Indl	1 <i>H</i> -indol-2-yl
Indn	2,3-dihydro-1 <i>H</i> -inden-2-yl
MALDI-TOF	matrix assisted laser desorption ionization time-of-flight
MVD	mouse vas deferens
NMM	4-methylmorpholine
pA₂	negative log of the molar concentration required to double the agonist concentration to achieve the original response
PL-017	[methyl-Phe ³ ,D-Pro ⁴]morphiceptin
TEA	triethylamine
TFA	

trifluoroacetic acid

Tic

1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid

TLC

thin-layer chromatography

WSC

1-ethyl-3-[3'-dimethyl)aminopropyl]-carbodiimide hydrochloride

Z

benzyloxycarbonyl

References

- Salvadori S, Attila M, Balboni G, Bianchi C, Bryant SD, Crescenzi O, Guerrini R, Picone D, Tancredi T, Temussi PA, Lazarus LH. *Mol Med* 1995;1:678–689. [PubMed: 8529134]
- Bryant SD, Jinsmaa Y, Salvadori S, Okada Y, Lazarus LH. *Biopolymers* 2003;71:86–102. [PubMed: 12767112]
- Salvadori S, Balboni G, Guerrini R, Tomatis R, Bianchi C, Bryant SD, Cooper PS, Lazarus LH. *J Med Chem* 1997;40:3100–3108. [PubMed: 9301674]
- Balboni G, Guerrini R, Salvadori S, Bianchi C, Rizzi D, Bryant SD, Lazarus LH. *J Med Chem* 2002;45:713–720. [PubMed: 11806723]
- Balboni G, Onnis V, Congiu C, Zotti M, Sasaki Y, Ambo A, Bryant SD, Jinsmaa Y, Lazarus LH, Trapella C, Salvadori S. *J Med Chem* 2006;49:5610–5617. [PubMed: 16942034]
- Balboni G, Salvadori S, Guerrini R, Negri L, Giannini E, Jinsmaa Y, Bryant SD, Lazarus LH. *J Med Chem* 2002;45:5556–5563. [PubMed: 12459023]
- Dai X, Cui SG, Li SR, Chen Q, Wang R. *Behav Brain Res* 2007;182:21–27. [PubMed: 17568695]
- Jutkiewicz EM. *Mol Interv* 2006;6:162–169. [PubMed: 16809477]
- Narita M, Kuzumaki N, Miyatake M, Sato F, Wachi H, Seyama Y, Suzuki TR. *J Neurochem* 2006;97:1494–1505. [PubMed: 16696856]
- Hill MP, Hille CJ, Brothie JM. *Drug News Perspect* 2000;13:261–268. [PubMed: 12937640]
- Torregrossa MM, Jutkiewicz EM, Mosberg HI, Balboni G, Watson SJ, Woods JH. *Brain Res* 2006;1069:172–181. [PubMed: 16364263]
- Vergura R, Valenti E, Hebbes CP, Gavioli EC, Spagnolo B, McDonald J, Lambert DG, Balboni G, Salvadori S, Regoli D, Calò G. *Peptides* 2006;27:3322–3330. [PubMed: 16963157]
- Nestor JJ Jr, Horner BL, Ho TL, Jones GH, McRae G, Vickery BH. *J Med Chem* 1984;27:320–325. [PubMed: 6366232]
- Baudet P, Otten C. *Helv Chim Acta* 1970;53:1683–1693.
- Wright WB Jr, Brabander HJ. *J Med Chem* 1968;11:1164–1167. [PubMed: 5680031]
- Shinozaki K, Sato H, Iwakuma T, Sato R, Kurimoto T, Yoshida K. *Bioorg Med Chem Lett* 1999;9:401–406. [PubMed: 10091692]
- Poitout L, Roubert P, Contour-Galcéra M-O, Moinet C, Lannoy J, Pommier J, Plas P, Bigg D, Thurieau C. *J Med Chem* 2001;44:2990–3000. [PubMed: 11520208]
- Bastiaansen LAM, Godefroi EF. *J Org Chem* 1978;43:1603–1604.
- Salvadori S, Guerrini R, Balboni G, Bianchi C, Bryant SD, Cooper PS, Lazarus LH. *J Med Chem* 1999;42:5010–5019. [PubMed: 10585210]
- Cheng YC, Prusoff WH. *Biochem Pharmacol* 1973;22:3099–3108. [PubMed: 4202581]
- Kosterlitz HW, Watt AJ. *Br J Pharmacol* 1968;33:266–276.
- Lazarus LH, Salvadori S, Santagada V, Tomatis R, Wilson WE. *J Med Chem* 1991;34:1350–1355. [PubMed: 1849997]
- Lazarus LH, Salvadori S, Attila M, Grieco P, Bundy DM, Wilson WE, Tomatis R. *Peptides* 1993;14:21–28. [PubMed: 8382810]

24. Lazarus LH, Wilson WE, de Castiglione R, Guglietta A. J Biol Chem 1989;264:3047–3050. [PubMed: 2563375]
25. Sasaki Y, Sasaki A, Niizuma H, Goto H, Ambo A. Bioorg Med Chem 2003;11:675–678. [PubMed: 12537996]
26. Arunlakshana Q, Schild HO. Br J Pharmacol 1959;14:48–58.

Fig. 1.
Structures of new compounds **1-6**.

Table 1
Receptor Binding Affinities and Functional Bioactivities of Compounds **1–6**.

Comp.	Structure	R	Receptor affinity ^a (nM)		Selectivity	Functional bioactivity (nM)		
			K_1^δ	K_1^μ		MVD (IC ₅₀) ^b	MVD (pA ₂) ^c	GPI (IC ₅₀) ^b
Ref.	H-Dmt-Tic-NH-CH ₂ - <i>Bid</i>		0.035 ^d	0.50 ^d	14 ^d	0.94±0.21		35.5±7.3
1	H-Dmt-Tic-NH-CH ₂ - <i>Boa</i>		0.283±0.048 (3)	1.42±0.25 (6)	5.0		9.42	169.9±32.2
2	H-Dmt-Tic-NH-CH ₂ - <i>Bia</i>		0.145±0.018 (3)	1.06±0.11 (4)	7.3		9.37	129.1±21.5
3	H-Dmt-Tic-NH-CH ₂ - <i>Indl</i>		0.066±0.013 (3)	0.7±0.18 (4)	10.6		9.45	208.9±48.1
4	H-Dmt-Tic-NH-CH ₂ - <i>Indn</i>		0.199±0.032 (3)	0.16±0.019 (4)	1.2 [#]	129.0±23.9		28.41±1.74
5	H-Dmt-Tic-NH-CH ₂ - <i>ImidPh</i>		0.443±0.13 (3)	6.74±0.98 (5)	15		9.40	153.5±10.4
6	H-Dmt-Tic-NH-CH ₂ - <i>Imid</i>		0.114±0.017 (4)	1.18±0.12 (6)	10	3.63±0.52		111.3±37.8

[#] μ selectivity K_1^δ / K_1^μ .

^a The K_1 values (nM) were determined according to Cheng and Prusoff.²⁰ The mean ± SE with n repetitions in parenthesis is based on independent duplicate binding assays with five to eight peptide doses using several different synaptosomal preparations.

^b Agonist activity was expressed as IC₅₀ obtained from dose-response curves. These values represents the mean ± SE for at least four tissue samples. DPDPE and PL-017 were the internal standards for MVD (δ-opioid receptor bioactivity) and GPI (μ-opioid receptor bioactivity) tissue preparation, respectively.

^c The pA₂ values of opioid antagonists against the agonists (deltorphin II and endomorphin-2) were determined by the method of Kosterlitz and Watt.²¹

^dData taken from Balboni et al.⁴