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Role of benzimidazole (Bid) in the δ-opioid agonist pseudopeptide H-Dmt-Tic-NH-CH_2-Bid (UFP-502)☆

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Abstract—H-Dmt-Tic-NH-CH_2-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 antidepression. To evaluate the importance of 1*H*-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 1*H*-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] We first reported potent and selective δ-opioid agonist derived from the δ-antagonist Dmt-Tic pharmacophore; it is characterized by the presence of Bid (1*H*-benzimidazole-2-yl) at its C-terminus (H-Dmt-Tic-NH-CH_2-Bid).[4] It is endowed with antidepressant-like effects in rodents, with a propensity

Keywords: Dmt-Tic pharmacophore; δ-Opioid receptors; Opioid peptides; UFP-502.

☆ 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*H*-benzimidazol-2-yl; Boa, benzoxazol-2-yl; Boc, *tert*-butyloxycarbonyl; Bta, benzothiazol-2-yl; DAMGO, [D-Ala^2,*N*-Me-Phe,^{4}Gly-ol^5]enkephalin; DEL C, deltorphin II (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2); DMF, *N,N*-dimethylformamide; DMSO-d_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*H*-imidazol-2-yl; ImidPh, 4-phenyl-1*H*-imidazol-2-yl; Indl, 1*H*-indol-2-yl; Indn, 2,3-dihydro-1*H*-inden-2-yl; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; MVD, mouse vas deferens; NMM, 4-methylmorpholine; pA_2, 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)amino-propyl]-carbodiimide hydrochloride; Z, benzyloxycarbonyl.

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to induce convulsions to a lower degree than non-peptide δ-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**, 1*H*-indol-2-yl (Indl);[15] **4**, 2,3-dihydro-1*H*-inden-2-yl (Indn);[16] **5**, 4-phenyl-1*H*-imidazol-2-yl (ImidPh);[17] and **6**, 1*H*-imidazol-2-yl (Imid).[18] Structures of new compounds and their bioactivities are reported in Figure 1 and Table 1, respectively.

2. Chemistry

Pseudopeptides (**1**–**6**) were prepared in solution by peptide synthetic methods. Simply, Boc-Dmt-Tic-OH[19] was coupled with H_2N-CH_2-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 (H_2N-CH_2-Boa) and (benzo[*d*]thiazol-2-yl)methanamine (H_2N-CH_2-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-CH_2-Boa) by cyclization and dehydration in refluxing propionic acid (~140 °C). N-terminal Z deprotection was accomplished by catalytic hydrogenation (H_2; Pd/C). *Z*-NH-CH_2-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-CH_2-Bta upon treatment with TEA in dioxane. N-terminal Z-deprotection was accomplished by HBr/AcOH treatment. H_2N-CH_2-Indl and H_2N-CH_2-Indn were prepared according to procedures described by Wright et al.[15] and Shinozaki et al.,[16] respectively. H_2N-CH_2-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-CH_2-ImidPh which was finally Boc-deprotected upon TFA treatment. Finally, H_2N-CH_2-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^{δ} = 0.066–0.443 nM); nonetheless, their averaged affinity was 6-fold less than the reference compound (H-Dmt-Tic-NH-CH_2-Bid). As expected, the lack of a free carboxylic function induces an increase in the μ-receptor affinity (K_i^{μ} = 0.16–6.74nM).[5] None of these new compounds exhibited high receptor selectivity; (K_i^{μ}/K_i^{δ} = 5–15) in comparison with the reference; only compound (**4**) does not follow this trend showing an almost complete lack of selectivity (K_i^{δ}/K_i^{μ} = 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 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,

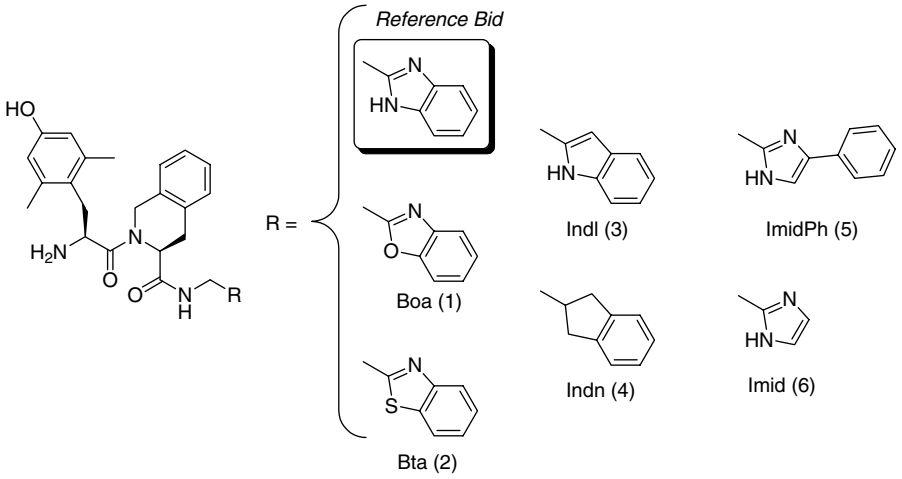
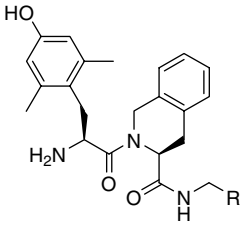
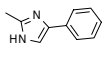


Figure 1. Structures of new compounds **1**–**6**.

Table 1. Receptor binding affinities and functional bioactivities of compounds **1–6**



Compound	Structure	Receptor affinity (nM)[a]			Selectivity	Functional bioactivity (nM)		
		R	K_i^δ	K_i^μ	K_i^μ/K_i^δ	MVD (IC_{50})[b]	MVD (pA_2)[c]	GPI (IC_{50})[b]
Ref.	H-Dmt-Tic-NH-CH_2-Bid		0.035[d]	0.50[d]	14[d]	0.94 ± 0.21		35.5 ± 7.3
1	H-Dmt-Tic-NH-CH_2-Boa		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-Bta		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-Indl		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-Indn		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-ImidPh		0.443 ± 0.13 (3)	6.74 ± 0.98 (5)	15		9.40	153.5 ± 10.4
6	H-Dmt-Tic-NH-CH_2-Imid		0.114 ± 0.017 (4)	1.18 ± 0.12 (6)	10	3.63 ± 0.52		111.3 ± 37.8

[a] The K_i values (nM) were determined according to Cheng and Prusoff.[20] Means ± 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 IC_{50} obtained from dose–response curves. These values represents means ± 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 pA_2 values of opioid antagonists against the agonists (deltorphin II and endomorphin-2) were determined by the method of Kosterlitz and Watt.[21]

[d] Data taken from Balboni et al.[4]

[#] μ Selectivity K_i^δ/K_i^μ.

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- to 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 behavior 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 behavior; 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-CH_2-Bid (UFP-502). The new δ-agonist H-Dmt-Tic-NH-CH_2-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 × 4 cm, 15 μm particle size)] and eluted at a flow rate of 20 mL/min with mobile phase solvent A (10% acetonitrile + 0.1% TFA in H_2O, v/v), and a linear gradient from 10 to 60% solvent B (60%, acetonitrile + 0.1% TFA in H_2O, v/v) in 30 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 a 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/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. Benzyl (benzo[*d*]oxazol-2-yl)methylcarbamate (*Z*-NH-CH_2-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% $NaHCO_3$ and AcOEt. The organic layer was washed with 5% $NaHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and brine. The AcOEt layer was dried (Na_2SO_4) and filtered. The filtrate was concentrated to yield the intermediate amide as a yellow oil which 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 CH_2Cl_2 to CH_2Cl_2/Et_2O (3:1): yield 2.37 g (42%); R_f(B) 0.73; HPLC K' 6.31; oil; *m*/z 283 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 4.18–4.23 (d, 2H), 5.34 (s, 2H), 7.19–7.26 (m, 9H).

5.2.2. H_2N-CH_2-Boa. A mixture of *Z*-NH-CH_2-Boa (1.13 g, 4 mmol) and 400 mg of 10% Pd/C in acetic acid (20 mL) was treated with H_2 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% $NaHCO_3$ and AcOEt. The organic layer was washed with 5% $NaHCO_3$, H_2O, and brine. The AcOEt layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The residual oil was subjected to silica gel chromatography with a linear gradient from CH_2Cl_2 to CH_2Cl_2/Et_2O (3:1): yield 0.53 g (89%); R_f(B) 0.34; HPLC K' 3.41; mp 120–122 °C; *m*/z 149 $(M+H)^+$; ^{1}H NMR (DMSO-d_6)δ 3.87–3.93 (d, 2H), 7.24–7.28 (m, 4H).

5.2.3. Boc-Dmt-Tic-NH-CH_2-Boa. To a solution of Boc-Dmt-Tic-OH[19] (0.14 g, 0.3 mmol) and H_2N-CH_2-Boa (0.04 g, 0.3 mmol) in DMF (10 mL) at 0 °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 °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 0.15 g (82%); R_f(B) 0.75; HPLC K' 6.02; mp 140–142 °C; $[\alpha]_D^{20}$ − 20.8; *m*/z 600 $(M+H)^+$; ^{1}H 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-CH_2-Boa (1). Boc-Dmt-Tic-NH-CH_2-Boa (0.12 g, 0.2 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 0.12 g (97%); R_f(A) 0.36; HPLC K' 4.36; mp 150–152 °C; $[\alpha]_D^{20}$ − 21.5; *m*/*z* 500 $(M+H)^+$; ^{1}H 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 $C_{31}H_{31}F_3N_4O_6$: 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 $(M+H)^+$; ^{1}H 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-CH_2-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 N_2 at room temperature overnight. After solvent evaporation, the residue was purified by column chromatography as reported above for *Z*-NH-CH_2-Boa: yield 1 g (71%); R_f(B) 0.71; HPLC K' 6.12; mp 93–95 °C; *m*/z 299 $(M+H)^+$; ^{1}H NMR (DMSO-d_6)δ 4.18–4.23 (d, 2H), 5.34 (s, 2H), 7.19–8.23 (m, 9H).

5.2.7. H_2N-CH_2-Bta. *Z*-NH-CH_2-Bta (0.8 g, 2.68 mmol) was treated with 4 N 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 H_2N-CH_2-Boa. The purified intermediate was deprotonated with 5% NaHCO3 as reported for H_2N-CH_2-Boa: yield 0.39 g (88%); R_f(B) 0.32; HPLC K' 3.19; mp 125–127 °C; *m*/z 165 $(M+H)^+$; ^{1}H NMR (DMSO-d_6)δ 3.87–3.93 (d, 2 H), 7.55–8.23 (m, 4 H).

5.2.8. Boc-Dmt-Tic-NH-CH_2-Bta. This compound was obtained by condensation of Boc-Dmt-Tic-OH with H_2N-CH_2-Bta via WSC/HOBt as reported for Boc-Dmt-Tic-NH-CH_2-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 $(M+H)^+$; ^{1}H 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-CH_2-Bta (2). Boc-Dmt-Tic-NH-CH_2-Bta was treated with TFA as reported for TFA · H-Dmt-Tic-NH-CH_2-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 $(M+H)^+$; ^{1}H 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 $C_{31}H_{31}F_3N_4O_5S$: 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-CH_2-Indl. This compound was obtained by condensation of Boc-Dmt-Tic-OH with H_2N-CH_2-Indl[15] via WSC/HOBt as reported for Boc-Dmt-Tic-NH-CH_2-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 $(M+H)^+$; ^{1}H NMR (DMSO-d_6)δ 1.40 (s, 9 H), 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-CH_2-Indl (3). Boc-Dmt-Tic-NH-CH_2-Indl was treated with TFA as reported for TFA · H-Dmt-Tic-NH-CH_2-Boa: yield 0.09 g (91%); *Rf*(A) 0.30; HPLC K' 4.16; mp 149–151 °C; $[\alpha]_D^{20}$ − 18.1; *m*/*z* 498 $(M+H)^+$; ^{1}H NMR (DMSO-d_6)δ 2.35 (s, 6 H), 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 $C_{32}H_{33}F_3N_4O_5$: C, 62.94; H, 5.45; N, 9.18. Found: C, 63.15; H, 5.59; N, 9.29.

5.2.12. Boc-Dmt-Tic-NH-CH_2-Indn. This compound was obtained by condensation of Boc-Dmt-Tic-OH with H_2N-CH_2-Indn[16] via WSC/HOBt as reported for Boc-Dmt-Tic-NH-CH_2-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 $(M+H)^+$; ^{1}H 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).

5.2.13. TFA · H-Dmt-Tic-NH-CH_2-Indn (4). Boc-Dmt-Tic-NH-CH_2-Indn was treated with TFA as reported for TFA · H-Dmt-Tic-NH-CH_2-Boa: yield 0.10 g (90%); *Rf*(A) 0.35; HPLC K' 4.44; mp 140–142 °C; $[\alpha]_D^{20}$ − 19.8; *m*/*z* 499 $(M+H)^+$; ^{1}H 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 $C_{33}H_{36}F_3N_3O_5$: C, 64.80; H, 5.93; N, 6.87. Found: C, 64.67; H, 5.76; N, 6.73.

5.2.14. *tert*-butyl (4-phenyl-1*H*-imidazol-2-yl)methylcarbamate (Boc-NH-CH_2-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 NH_4OAc and H_2O were removed using a Dean–Stark trap. The mixture was then cooled to room temperature, diluted with AcOEt (100 mL), and washed with H_2O, 5% $NaHCO_3$, and brine. The organic phase was dried (Na_2SO_4) and concentrated to dryness. The residue was precipitated from Et_2O/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).

5.2.15. H_2N-CH_2-ImidPh. Boc-NH-CH_2-ImidPh (2 g, 7.33 mmol) was treated with TFA (4 mL) for 0.5 h at room temperature. Et_2O/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% $NaHCO_3$ as reported for H_2N-CH_2-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, 2 H), 6.9–7.48 (m, 4H).

5.2.16. Boc-Dmt-Tic-NH-CH_2-ImidPh. This compound was obtained by condensation of Boc-Dmt-Tic-OH with H_2N-CH_2-ImidPh via WSC/HOBt (except washing with aqueous citric acid solution) as reported for Boc-Dmt-Tic-NH-CH_2-Boa: yield 0.11 g (80%); R_f(B) 0.58; HPLC K' 5.65; mp 148–150 °C; $[\alpha]_D^{20}$ − 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).

5.2.17. 2TFA · H-Dmt-Tic-NH-CH_2-ImidPh (5). Boc-Dmt-Tic-NH-CH_2-ImidPh was treated with TFA as reported for TFA · H-Dmt-Tic-NH-CH_2-Boa: yield 0.08 g (93%); *Rf*(A) 0.29; HPLC K' 4.12; mp 152–154 °C; $[\alpha]_D^{20}$ − 23.7; *m*/*z* 525 $(M+H)^+$; ^1H NMR (DMSO-d_6)δ 2.35 (s, 6 H), 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 $C_{35}H_{35}F_6N_5O_7$: C, 55.93; H, 4.69; N, 9.32. Found: C, 55.77; H, 4.62; N, 9.18.

5.2.18. Boc-Dmt-Tic-NH-CH_2-Imid. This compound was obtained by condensation of Boc-Dmt-Tic-OH with H_2N-CH_2-Imid[18] via WSC/HOBt (except washing with aqueous citric acid solution) as reported for Boc-Dmt-Tic-NH-CH_2-Boa: yield 0.09 g (83%); R_f(B) 0.43; HPLC K' 5.01; mp 153–155 °C; $[\alpha]_D^{20}$ − 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).

5.2.19. 2TFA · H-Dmt-Tic-NH-CH_2-Imid (6). Boc-Dmt-Tic-NH-CH_2-Imid was treated with TFA as reported for TFA · H-Dmt-Tic-NH-CH_2-Boa: yield 0.07 g (94%); *Rf*(A) 0.25; HPLC K' 3.97; mp 156–158 °C; $[\alpha]_D^{20}$ − 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 $C_{29}H_{31}F_6N_5O_7$: 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 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.[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 parentheses and results are means ± SE). Unlabeled 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, U. K.; K_D = 1.5 nM) for μ-opioid receptors. Glass fiber 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 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 DPDPE; μ-antagonism in the GPI assay used the μ-agonist PL-017 and both are expressed as pA_2 determined using the Schild Plot.[26]

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