

Supporting Information

Agonist vs Antagonist Behavior Opioid Peptides Containing Novel Phenylalanine Analogues in Place of Tyr¹

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Experimental Section

General Methods. Molecular masses of compounds were determined by electrospray mass spectrometry on a hybrid Q-Tof mass spectrometer interfaced to a Mass Lynx 4.0 data system. ¹H and ¹³C NMR spectra were recorded on a Varian Unity 400 spectrometer or a Varian INOVA 500 MHz spectrometer and referenced with respect to the residual signals of the solvent. The following abbreviations were used in reporting spectra: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Peptides were purified on a Vydac 218-TP1022 column (22 x 250 mm) with a linear gradient of 20-45% acetonitrile in 0.1% TFA/H₂O over 30 min at a flow rate of 12 mL/min. Analytical reversed-phase HPLC was performed on a Vydac 218-TP54 column (5 x 250 mm) with a linear gradient of 35-60% MeOH in 0.1% TFA/H₂O over 30 min at a flow rate of 1 mL/min. All compounds had >95% purity. The same column was used for the determination of the capacity factors K' under the same conditions. Precoated plates (silica gel 60 F₂₅₄, 250 µm, Merck Darmstadt, Germany) were used for ascending TLC in the following solvent systems (all v/v): (I) *n*-BuOH/AcOH/H₂O (4:1:1); (II) *n*-BuOH/pyridine/AcOH/H₂O (15:10:3:12). Specific optical rotations were determined on an AA-5 automatic polarimeter.

Syntheses of Boc-Bcp-OH and Boc-Dbcp-OH

Synthesis of Boc-Bcp-OH (15)

Boc-Phe(4'-COOH)-OMe (13). To 25 g (84.64 mM) of Boc-Tyr-OMe dissolved in dry dichloromethane (150 mL) were added at 0°C under inert atmosphere: 30.84 g (86.34 mM) of *N*-phenyltrifluoromethanesulfonimide and 12.79 mL (91.42 mM) triethylamine. After completion of the reaction as determined by TLC, water (50 mL) was added and the organic layer was washed with water (3 x 20 mL) and brine (3 x 20 mL), and dried over MgSO₄. After filtration and evaporation *in vacuo*, Boc-Tyr(4-OTf)-OMe was obtained as a lightly yellowish oil (35.42 g, 98%) and was then dissolved in DMF (200 mL). To this solution under inert atmosphere were added 56 g (405.6 mM) of K₂CO₃, 1.9 g (8.46 mM) of Pd(OAc)₂ and 10.19 g (17.75 mM) of DPPF, and CO was passed through this mixture for 1.5 h at room temperature. The mixture was then heated to 60°C and stirred under CO atmosphere for 8 h. After cooling to room temperature, the mixture was filtered through Celite and then NaHCO₃ sat. (250 mL) and ethyl acetate (400 mL) were added. The aqueous layer was washed three times with ethyl acetate and was acidified with 12% citric acid at 0°C under a new layer of ethyl acetate. The organic layer was dried over MgSO₄, filtered and evaporated *in vacuo*. The resulting solid was crystallized from ethyl acetate/hexane, yielding **13** as white crystals (10.94 g, 41%); mp 94-96°C. [α]_D²⁰ -11.3 (c 1, DMSO). ¹H NMR (500 MHz, DMSO) δ 12.81 (s, 1H), 7.88-7.83 (d, 2H, *J* = 7.8 Hz), 7.38-7.32 (m, 3H), 4.25-4.18 (m, 1H), 3.64-3.59 (s, 3H), 3.10-3.04 (m, 1H), 2.96-2.88 (m, 1H), 1.35-1.28 (s, 8H), 1.28-1.22 (s, 1H). ¹³C NMR (500 MHz, DMSO) δ 173, 168, 156, 144, 130, 79, 56, 53, 37, 29. HRMS (EI) *m/e* calcd for C₁₆H₂₂NO₆ [M+H]⁺ 324.1447; obsd 324.1438.

N^a-Boc-4'-[N-((4'-phenyl)-phenethyl)carboxamido]phenylalanine methyl ester (Boc-Bcp-OMe, 14). To a solution of 2.548 g (8 mM) of **13** in DMF (10 mL) were added 3.793 g (10 mM) of HBTU dissolved in DMF (12 mL), 1.742 mL (10 mM) of DIEA and 1.97 g (10 mM) of 2-(4-biphenyl)ethylamine. After stirring the mixture for 2 h, the DMF

was evaporated *in vacuo* and the obtained oily crystals were dissolved in a mixture of ethyl acetate (25 mL) and brine (20 mL). The organic layer was washed with brine (3 x 20 mL), dried over MgSO₄, filtered and evaporated *in vacuo*, furnishing white crystals (3.97 g, 99%); mp 196-198° C. [α]_D²⁰ -3.3 (c 1, DMSO). ¹H NMR (DMSO, 500 MHz) δ 8.54-8.50 (m, 1H), 7.76-7.73 (d, 2H, *J* = 7.8 Hz), 7.65-7.62 (d, 2H, *J* = 7.6 Hz), 7.61-7.58 (d, 2H, *J* = 7.8 Hz), 7.47-7.42 (m, 2H), 7.36-7.29 (m, 5H), 4.24-4.18 (m, 1H), 3.64-3.60 (s, 3H), 3.54-3.48 (m, 2H), 3.08-3.01 (m, 1H), 2.93-2.85 (m, 3H), 1.35-1.28 (s, 8H), 1.28-1.22 (s, 1H). ¹³C NMR (DMSO, 500 MHz) δ 173, 166, 156, 141, 140, 139, 138, 133, 129, 127, 79, 55, 52, 41, 36, 35, 28. HRMS (EI) *m/e* calcd for C₃₀H₃₅N₂O₅ [M+H]⁺ 503.2548, obsd 503.2544.

N^a-Boc-4'-[N-((4'-phenyl)-phenethyl)carboxamido]phenylalanine (Boc-Bcp-OH, 15). 3.97 g (7.9 mM) of **14** was dissolved in DMF (50 mL) and 2N NaOH (8 mL) was added dropwise. After completion of the reaction, the DMF was evaporated *in vacuo* and the resulting oily crystals were dissolved in a mixture of water (20 mL) and ethyl acetate (20 mL), followed by acidification with 12% citric acid to pH 5. The organic and aqueous layer were separated, and the organic layer was dried over MgSO₄, filtered and evaporated *in vacuo*, furnishing **15** as white crystals (3.85 g, 100%); mp 252-253°C. [α]_D²⁰ -3.8 (c 1, DMSO). ¹H NMR (500 MHz, DMSO) δ 12.70-12.50 (br s, 1H), 8.75-8.51 (m, 1H), 7.78-7.30 (m, 13H), 7.16-7.11 (d, 1H, *J* = 8.3 Hz) 4.18-4.09 (m, 1H), 3.55-3.48 (m, 2H), 3.10-3.04 (m, 1H), 2.90-2.83 (m, 3H), 1.35-1.28 (s, 8H), 1.28-1.22 (s, 1H). ¹³C NMR (500 MHz, DMSO) δ 174, 167, 156, 141, 140, 139, 134, 130, 128, 79, 56, 42, 37, 36, 29. HRMS (EI) *m/e* calcd for C₂₉H₃₃N₂O₅ [M+H]⁺ 489.2389, obsd 489.2375.

Synthesis of Boc-Dbcp-OH (18)

Boc-L-Phe(2',6'-Me₂,4'-COOH)-OEt (16). To 5.055 g (15 mM) of Boc-L-Dmt-OEt dissolved in dry dichloromethane (40 mL) were added at 0°C under inert atmosphere: 5.46 g (15.3 mM) of *N*-phenyltrifluoromethanesulfonimide and 2.27 mL (16.2 mM) of triethylamine. After completion of the reaction as determined by TLC, water (10 mL) was added and the organic layer was washed with water (2 x 10 mL) and brine (3 x 10 mL), and dried over MgSO₄. After filtration and evaporation *in vacuo*, Boc-Dmt(4-OTf)-OEt was obtained as a transparent, lightly yellowish oil (6.89 g, 98%) and was dissolved in dry DMF (40 mL). To this solution under inert atmosphere were added 9.936 g (72 mM) of K₂CO₃, 0.337 g (1.5 mM) of Pd(OAc)₂ and 1.808 g (3.15 mM) of DPPF, and CO was passed through this mixture for 1.5 h at room temperature. The mixture was then heated to 60°C and stirred under CO atmosphere for 8 h. After cooling to room temperature, the mixture was filtered through Celite, and then NaHCO₃ sat (50 mL) and ethyl acetate (100 mL) were added. The aqueous layer was washed three times with ethyl acetate and was acidified with 12% citric acid to pH 5 at 0° C. The organic layer was dried over MgSO₄, filtered and evaporated *in vacuo*. The resulting light brown crystals were recrystallized from ethyl acetate/hexane, yielding **16** as white crystals (3.002 g, 55%); mp 163-164° C. [α]_D²⁰ -1.2 (c 1, DMSO). ¹H NMR (500 MHZ, DMSO) δ 7.44-7.40 (d, 1H, *J* = 8.3 Hz), 7.12-7.10 (s, 2H), 4.20-4.13 (m, 1H), 4.01-3.93 (m, 2H), 3.12-3.06 (m, 1H), 3.02-2.95 (m, 1H), 2.35-2.30 (s, 6H), 1.35-1.29 (s, 8H), 1.20-1.17 (s, 1H), 1.04-0.98 (t, 3H, *J* = 7.1 Hz). ¹³C NMR (500 MHz, DMSO) δ 172, 156, 147, 140, 136, 120, 79, 61, 53, 31, 28, 20, 14. HRMS (EI) *m/e* calcd for C₁₉H₂₇NO₆Na [M+Na]⁺ 388.1736, obsd 388.1726.

N^a-Boc-2',6'-dimethyl-4'-[N-((4'-phenyl)-phenethyl)carboxamido]phenylalanine ethyl ester (Boc-Dbcp-OEt, 17). To a solution of 3.002 g (8.22 mM) of **16** in DMF (10 mL) were added 3.793 g (10 mM) of HBTU dissolved in DMF (12 mL), 1.742 mL (10 mM) of DIPEA and 1.97 g (10 mM) of 2-(4-biphenyl)ethylamine. After stirring for 2 h the reaction was complete and the DMF was evaporated to yield a brown oil. Ethyl acetate was added and the mixture was briefly refluxed with C_{act} and then was filtered through Celite. It was then washed with brine (3 x 10 mL), dried over MgSO₄, filtered and evaporated *in vacuo*. Spontaneous crystallization occurred, yielding **17** as white crystals (4.246 g, 95%); mp 128-130° C. [α]_D²⁰ -5.3 (c 1, DMSO). ¹H NMR (500 MHz, DMSO) δ 8.44-8.40 (m, 1H), 7.70-7.28 (m, 13H), 4.15-4.09 (m, 1H), 4.00-3.93 (m, 2H), 3.52-3.45 (m, 2H), 3.14-3.08 (m, 1H), 3.03-2.96 (m, 1H), 2.90-2.86 (m, 2H), 2.33-2.28 (s, 6H), 1.35-1.32 (s, 8H), 1.20-1.17 (s, 1H), 1.04-0.98 (t, 3H, J = 7.1 Hz). ¹³C NMR (500 MHz, DMSO) δ 173, 167, 156, 141, 140, 139, 138, 133, 130, 128, 127, 79, 61, 55, 42, 36, 32, 29, 19, 18, 14. HRMS (EI) *m/e* calcd for C₃₃H₄₀N₂O₅Na [M+Na]⁺ 567.2835, obsd 567.2821.

N^a-Boc-2',6'-dimethyl-4'-[N-((4'-phenyl)-phenethyl)carboxamido]phenylalanine. (Boc-Dbcp-OH, 18). To 2.8 g (5.14 mM) of **17** dissolved in methanol (50 mL) 1N NaOH (8 mL) was added dropwise. After completion of the reaction, the methanol was evaporated *in vacuo* and the resulting white crystals were dissolved in a mixture of water (20 mL) and ethyl acetate (20 mL). The solution was acidified with 12% citric acid to pH 5 and then the organic and aqueous layers were separated. The organic layer was dried over MgSO₄, filtered and evaporated, yielding **18** as white crystals (1.09 g, 53%); mp 229-231° C. [α]_D²⁰ -10.8 (c 1, DMSO). ¹H NMR (500 MHz, DMSO) δ 12.70-12.55 (s, 1H), 8.46-8.40 (m, 1H), 7.68-7.30 (m, 1H), 7.24-7.18 (d, 1H, J = 8.5 Hz), 4.14-4.07 (m, 1H), 3.53-3.44 (m, 2H), 3.14-3.08 (m, 1H), 2.98-2.92 (m, 1H), 2.90-2.85 (m, 2H), 2.37-2.29 (s, 6H), 1.35-1.24 (s, 8H), 1.16-1.00 (s, 1H). ¹³C NMR (500 MHz, DMSO) δ 174, 166, 155, 140, 139, 138, 137, 132, 129, 127, 78, 53, 41, 35, 31, 28, 20. HRMS (EI) *m/e* calcd for C₃₁H₃₇N₂O₅ [M+H]⁺ 517.2704, obsd 517.2694.

Table 3. Analytical Parameters of Bcp¹- and Dbcp¹-peptides

No.	Rf (I)	Rf (II)	K'	ES-ML <i>m/e</i>
1	0.475	0.827	1.31	842
2	0.366	0.777	1.40	695
3	0.225	0.913	1.72	548
4	0.439	0.821	1.46	828
5	0.482	0.852	1.37	834
6	0.609	0.839	1.25	870
7	0.329	0.907	1.71	576
12	0.511	0.765	1.22	690

In Vitro Bioassays and Receptor Binding Assays. Opioid receptor binding studies were performed as described in detail elsewhere.¹⁹ Binding affinities for δ and μ receptors were determined by displacing, respectively, [³H]DSLET, (Multiple Peptide Systems, San Diego, CA) and [³H]DAMGO (Multiple Peptide Systems) from rat brain membrane binding sites, and κ opioid receptor affinities were measured by displacement of [³H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 0° C with [³H]DSLET, [³H]DAMGO and [³H]U69,593²⁰ at respective concentrations of 0.78, 0.72 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 Prusoff²¹ using values of 2.6, 1.3 and 2.9 nM for the dissociation constants of [³H]DSLET, [³H]DAMGO and [³H]U69,593, respectively.

The MVD²² and GPI²³ bioassays were carried out as reported in detail elsewhere.^{19,24} For agonists log-dose response curves were determined with [Leu⁵]enkephalin as standard for each vas or ileum preparation, and IC₅₀ values of compounds being tested were normalized according to a published procedure.²⁵ 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 concentration²⁶. δ antagonist K_e values of compounds were determined in the MVD assay against the δ agonist DPDPE. μ and κ antagonist K_e values of compounds were determined in the GPI assay against the μ agonist TAPP²⁷ and against the κ agonist U50,488²⁸, respectively.

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