

## Supporting Information

### **Agonist vs Antagonist Behavior Opioid Peptides Containing Novel Phenylalanine Analogues in Place of Tyr<sup>1</sup>**

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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.  $^1\text{H}$  and  $^{13}\text{C}$  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/ $\text{H}_2\text{O}$  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/ $\text{H}_2\text{O}$  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 F<sub>254</sub>, 250  $\mu\text{m}$ , Merck Darmstadt, Germany) were used for ascending TLC in the following solvent systems (all v/v): (I) *n*-BuOH/AcOH/ $\text{H}_2\text{O}$  (4:1:1); (II) *n*-BuOH/pyridine/AcOH/ $\text{H}_2\text{O}$  (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  $\text{MgSO}_4$ . 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  $\text{K}_2\text{CO}_3$ , 1.9 g (8.46 mM) of  $\text{Pd}(\text{OAc})_2$  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  $\text{NaHCO}_3$  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  $\text{MgSO}_4$ , 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.  $[\alpha]_{\text{D}}^{20}$  -11.3 (c 1, DMSO).  $^1\text{H}$  NMR (500 MHz, DMSO)  $\delta$  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).  $^{13}\text{C}$  NMR (500 MHz, DMSO)  $\delta$  173, 168, 156, 144, 130, 79, 56, 53, 37, 29. HRMS (EI)  $m/e$  calcd for  $\text{C}_{16}\text{H}_{22}\text{NO}_6$   $[\text{M}+\text{H}]^+$  324.1447; obsd 324.1438.

**$N^\alpha$ -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 MgSO<sub>4</sub>, filtered and evaporated *in vacuo*, furnishing white crystals (3.97 g, 99%); mp 196-198° C. [ $\alpha$ ]<sub>D</sub><sup>20</sup> -3.3 (c 1, DMSO). <sup>1</sup>H NMR (DMSO, 500 MHz)  $\delta$  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). <sup>13</sup>C NMR (DMSO, 500 MHz)  $\delta$  173, 166, 156, 141, 140, 139, 138, 133, 129, 127, 79, 55, 52, 41, 36, 35, 28. HRMS (EI) *m/e* calcd for C<sub>30</sub>H<sub>35</sub>N<sub>2</sub>O<sub>5</sub> [M+H]<sup>+</sup> 503.2548, obsd 503.2544.

**N<sup>α</sup>-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 MgSO<sub>4</sub>, filtered and evaporated *in vacuo*, furnishing **15** as white crystals (3.85 g, 100%); mp 252-253° C. [ $\alpha$ ]<sub>D</sub><sup>20</sup> -3.8 (c 1, DMSO). <sup>1</sup>H NMR (500 MHz, DMSO)  $\delta$  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). <sup>13</sup>C NMR (500 MHz, DMSO)  $\delta$  174, 167, 156, 141, 140, 139, 134, 130, 128, 79, 56, 42, 37, 36, 29. HRMS (EI) *m/e* calcd for C<sub>29</sub>H<sub>33</sub>N<sub>2</sub>O<sub>5</sub> [M+H]<sup>+</sup> 489.2389, obsd 489.2375.

### Synthesis of Boc-Dbcp-OH (18)

**Boc-L-Phe(2',6'-Me<sub>2</sub>,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 MgSO<sub>4</sub>. 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 K<sub>2</sub>CO<sub>3</sub>, 0.337 g (1.5 mM) of Pd(OAc)<sub>2</sub> 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 NaHCO<sub>3</sub> 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 MgSO<sub>4</sub>, 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. [ $\alpha$ ]<sub>D</sub><sup>20</sup> -1.2 (c 1, DMSO). <sup>1</sup>H NMR (500 MHz, DMSO)  $\delta$  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). <sup>13</sup>C NMR (500 MHz, DMSO)  $\delta$  172, 156, 147, 140, 136, 120, 79, 61, 53, 31, 28, 20, 14. HRMS (EI) *m/e* calcd for C<sub>19</sub>H<sub>27</sub>NO<sub>6</sub>Na [M+Na]<sup>+</sup> 388.1736, obsd 388.1726.

***N*<sup>α</sup>-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<sub>act</sub> and then was filtered through Celite. It was then washed with brine (3 x 10 mL), dried over MgSO<sub>4</sub>, filtered and evaporated *in vacuo*. Spontaneous crystallization occurred, yielding **17** as white crystals (4.246 g, 95%); mp 128-130° C. [ $\alpha$ ]<sub>D</sub><sup>20</sup> -5.3 (c 1, DMSO). <sup>1</sup>H NMR (500 MHz, DMSO)  $\delta$  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). <sup>13</sup>C NMR (500 MHz, DMSO)  $\delta$  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 C<sub>33</sub>H<sub>40</sub>N<sub>2</sub>O<sub>5</sub>Na [M+Na]<sup>+</sup> 567.2835, obsd 567.2821.

***N*<sup>α</sup>-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 MgSO<sub>4</sub>, filtered and evaporated, yielding **18** as white crystals (1.09 g, 53%); mp 229-231° C. [ $\alpha$ ]<sub>D</sub><sup>20</sup> -10.8 (c 1, DMSO). <sup>1</sup>H NMR (500 MHz, DMSO)  $\delta$  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). <sup>13</sup>C NMR (500 MHz, DMSO)  $\delta$  174, 166, 155, 140, 139, 138, 137, 132, 129, 127, 78, 53, 41, 35, 31, 28, 20. HRMS (EI) *m/e* calcd for C<sub>31</sub>H<sub>37</sub>N<sub>2</sub>O<sub>5</sub> [M+H]<sup>+</sup> 517.2704, obsd 517.2694.

**Table 3.** Analytical Parameters of Bcp<sup>1</sup>- and Dbcp<sup>1</sup>-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.<sup>19</sup> Binding affinities for  $\delta$  and  $\mu$  receptors were determined by displacing, respectively, [<sup>3</sup>H]DSLET, (Multiple Peptide Systems, San Diego, CA) and [<sup>3</sup>H]DAMGO (Multiple Peptide Systems) from rat brain membrane binding sites, and  $\kappa$  opioid receptor affinities were measured by displacement of [<sup>3</sup>H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 0° C with [<sup>3</sup>H]DSLET, [<sup>3</sup>H]DAMGO and [<sup>3</sup>H]U69,593<sup>20</sup> at respective concentrations of 0.78, 0.72 and 0.80 nM. IC<sub>50</sub> values were determined from log-dose displacement curves and K<sub>i</sub> values were calculated from the obtained IC<sub>50</sub> values by means of the equation of Cheng and Prusoff<sup>21</sup> using values of 2.6, 1.3 and 2.9 nM for the dissociation constants of [<sup>3</sup>H]DSLET, [<sup>3</sup>H]DAMGO and [<sup>3</sup>H]U69,593, respectively.

The MVD<sup>22</sup> and GPI<sup>23</sup> bioassays were carried out as reported in detail elsewhere.<sup>19,24</sup> For agonists log-dose response curves were determined with [Leu<sup>5</sup>]enkephalin as standard for each vas or ileum preparation, and IC<sub>50</sub> values of compounds being tested were normalized according to a published procedure.<sup>25</sup> K<sub>e</sub> values for antagonists were determined from the ratio of IC<sub>50</sub> values obtained with an agonist in the presence and absence of a fixed antagonist concentration<sup>26</sup>.  $\delta$  antagonist K<sub>e</sub> values of compounds were determined in the MVD assay against the  $\delta$  agonist DPDPE.  $\mu$  and  $\kappa$  antagonist K<sub>e</sub> values of compounds were determined in the GPI assay against the  $\mu$  agonist TAPP<sup>27</sup> and against the  $\kappa$  agonist U50,488<sup>28</sup>, respectively.

## References

- (19) Schiller, P. W.; Lipton, A.; Horrobin, D. F.; Bodanszky, M. Unsulfated C-Terminal 7-Peptide of Cholecystokinin: a New Ligand of the Opiate Receptor. *Biochem. Biophys. Res. Commun.* **1978**, *85*, 1332-1338.
- (20) Lahti, R. A.; Mickelson, M. M.; McCall, J. M.; Von Voigtlander, P. F. [3H]U-69593 a Highly Selective Ligand for the Opioid  $\kappa$  Receptor. *Eur. J. Pharmacol.* **1985**, *109*, 281-284.
- (21) Cheng, Y.; Prusoff, W. H. Relationship Between the Inhibition Constant ( $K_i$ ) and the Concentration of Inhibitor Which Causes 50 Per Cent Inhibition ( $I_{50}$ ) of an Enzymatic Reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099-3108.
- (22) Henderson, G.; Hughes, J.; Kosterlitz, H. W. A New Example of a Morphine-Sensitive Neuro-Effector Junction: Adrenergic Transmission in the Mouse Vas Deferens. *Br. J. Pharmacol.* **1972**, *46*, 764-766.
- (23) Paton, W. D. The Action of Morphine and Related Substances on Contraction and on Acetylcholine Output of Coaxially Stimulated Guinea-Pig Ileum. *Br. J. Pharmacol. Chemother.* **1957**, *12*, 119-127.
- (24) DiMaio, J.; Nguyen, T. M.; Lemieux, C.; Schiller, P. W. Synthesis and Pharmacological Characterization in Vitro of Cyclic Enkephalin Analogues: Effect of Conformational Constraints on Opiate Receptor Selectivity. *J. Med. Chem.* **1982**, *25*, 1432-1438.
- (25) Waterfield, A. A.; Leslie, F. M.; Lord, J. A.; Ling, N.; Kosterlitz, H. W. Opioid Activities of Fragments of B-Endorphin and of Its Leucine<sup>65</sup>-Analogue. Comparison of the Binding Properties of Methionine- and Leucine-Enkephalin. *Eur. J. Pharmacol.* **1979**, *58*, 11-18.
- (26) Kosterlitz, H. W.; Watt, A. J. Kinetic Parameters of Narcotic Agonists and Antagonists, With Particular Reference to N-Allylnoroxymorphone (Naloxone). *Br. J. Pharmacol. Chemother.* **1968**, *33*, 266-276.
- (27) Schiller, P. W.; Nguyen, T. M.; Chung, N. N.; Lemieux, C. Dermorphin Analogues Carrying an Increased Positive Net Charge in Their "Message Domain" Display Extremely High  $\mu$  Opioid Receptor Selectivity. *J. Med. Chem.* **1989**, *32*, 698-703.
- (28) Vonvoigtlander, P. F.; Lahti, R. A.; Ludens, J. H. U-50,488: a Selective and Structurally Novel Non-Mu ( $\kappa$ ) Opioid Agonist. *J. Pharmacol. Exp. Ther.* **1983**, *224*, 7-12.