

## **Supporting Information 1**

### **Modulating $\beta$ -Arrestin-2 Recruitment at the $\delta$ and $\mu$ Opioid Receptors**

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## General Synthetic Considerations

Unless specified, chemicals were purchased from commercial sources and used without further purification. Organic solvents used for synthesis were of analytical grade and used without further purification.

Proton nuclear magnetic resonance ( $^1\text{H}$  NMR) spectra, fluorine ( $^{19}\text{F}$  NMR), and carbon nuclear magnetic resonance ( $^{13}\text{C}$  NMR) spectra were recorded on a Bruker AVIII 500 AVANCE spectrometer with a BBFO cryoprobe (500, 376 and 126 MHz, respectively), a Bruker DRX 500 spectrometer with HR-mas probe (500, 376 and 126 MHz, respectively), Bruker AVIIIHD 400 MHz NMR with a broadband X-channel detect gradient probe (400, and 101 MHz, respectively), and Bruker AVIII 600 MHz NMR spectrometer equipped with a multi-nuclear broadband probe (600, 471 and 151 MHz, respectively). Chemical shifts ( $\delta$ ) for protons are reported in parts per million (ppm) downfield from tetramethylsilane, and are referenced to proton resonance of solvent residual peak in the NMR solvent (MeOD- $d_4$ :  $\delta = 4.87$  ppm or DMSO- $d_6$ :  $\delta = 2.50$  ppm). Chemical shifts ( $\delta$ ) for carbon atoms are reported in ppm downfield from tetramethylsilane, and are referenced to the carbon resonances of the solvent residual peak (MeOD- $d_4$ :  $\delta = 49.1$  ppm or DMSO- $d_6$ :  $\delta = 39.52$  ppm). Chemical shifts ( $\delta$ ) for fluorine atoms are reported in ppm relative to  $\text{PhCF}_3$  ( $\delta = -63.72$  ppm). NMR data are represented as follows: chemical shift (ppm), multiplicity (s = singlet, d = doublet, dd = doublet of doublets, dt = doublet of triplets, dq = doublet of quartets, t = triplet, q = quartet, m = multiplet), coupling constant in Hertz (Hz), and integration. Exact mass analyses were furnished by using electrospray ionization using a time of flight mass analyzer (Waters LCT Premiere).

Peptidomimetics were synthesized using a Biotage Initiator microwave synthesizer using a solution-phase peptide synthesis protocol using Boc chemistry and 4N HCl in 1,4-dioxane for deprotection. Purification was conducted using a Teledyne ISCO EZ Prep system with a RediSep® C18 Prep column (30x250 mm, 100 Å). Purity analysis of final tested compounds was carried out using a Waters UPLC Aquity system equipped with a PDA  $e\lambda$  detector (200 to 400 nm) and a HSS T3 C18 column, (1.8  $\mu\text{m}$ , 2.1x50 mm column), using one of two methods. Protocol A: gradient elution of 2% MeCN / 98%  $\text{H}_2\text{O}$  (with 0.1% formic acid) to 98% MeCN over 2.5 min, then holding at 98% MeCN for 1 min at a flow rate of 0.7 mL/min at 40 °C. Protocol B: gradient elution of 2% MeCN / 98%  $\text{H}_2\text{O}$  (with 0.1% formic acid) to 98% MeCN over 2.5 min, then holding at 98% MeCN for 3 min at a flow rate of 0.8 mL/min.

## Synthetic Chemistry

**Synthesis of Boc-Tyr- $\psi$ [(Z)CF=CH]-Gly-OH (7).** Synthesis of Boc-Tyr- $\psi$ [(Z)CF=CH]-Gly-OH (7) was carried out by previously developed and reported protocol from our laboratory.<sup>1</sup> All steps of this multistep synthetic sequence were repeated as reported, and all intermediates were characterized and found to be consistent with the reported literature.

**Synthesis of 3b–d.** Synthesis of *N*- $\alpha$ -Boc-Gly-Phe-Leu-OMe was achieved using Boc strategy of solution phase peptide synthesis under microwave irradiation with Leu-OMe.HCl (5 mmol) as starting material.<sup>2</sup> For synthesis of compounds **3b–d**, *N*- $\alpha$ -Boc-Gly-Phe-Leu-OMe (**2a**, 1 equiv., 2 mmol) was transferred to a round bottom flask, and the corresponding amine (NH<sub>4</sub>OH, 5 mL or methylamine 40 wt% in H<sub>2</sub>O, 5 mL or ethylamine solution 66–72% in H<sub>2</sub>O, 5 mL) and methanol (1:1, 5 mL) were added to the flask at 0 °C. The reaction mixture was allowed to warm slowly to room temperature and was then stirred for 14 h. The reaction was monitored using TLC, and after consumption of starting material, the reaction mixture was concentrated under vacuum. The crude mixture was dissolved in a mixture of ethyl acetate and water, and then the layers were separated. The organic layer was concentrated under vacuum to afford desired compounds (**3b–d**). The synthesized peptides **3b–d** were characterized by NMR and mass spectrometry.

**Synthesis of 3e–f.** *N*- $\alpha$ -Boc-Gly-Phe-Leu-OH (**2**, 435 mg, 1 equiv., 1.4 mmol) was dissolved in DMF (1 mL) in a microwave (MW) reaction vessel. Amine (dimethylamine solution 2.0 M in THF, 1.4 mL, or cyclopropylamine, 195  $\mu$ L, 2 equiv., 2.8 mmol), DIC (285  $\mu$ L, 1.3 equiv., 2.8 mmol), and HONB (325 mg, 1.3 equiv., 2.8 mmol) were added to the reaction vessel, and the reaction mixture was heated under MW irradiation at 60 °C for 30 min.<sup>2,3</sup> Then, the crude peptides were dissolved in a mixture of ethyl acetate and water, and the layers were separated. The organic layer was concentrated under vacuum. The crude peptides were purified by RP-HPLC using a combination of acetonitrile and water (0.1% acetic acid) to afford compounds **3e–f**.

**Synthesis of 3g.** Leu-tetrazole (**5**) was synthesized following the previously reported a multistep synthetic protocol.<sup>4</sup> Leu-tetrazole (**5**, 200 mg, 1.0 equiv., 1 mmol) was dissolved in DMF (1 mL) in a microwave (MW) reaction vessel, and *N,N*-diisopropylethylamine (DIEA, 695  $\mu$ L, 4 equiv., 4 mmol) was added. The mixture was stirred for 5 min at room temperature. *N*- $\alpha$ -Boc-Gly-Phe-OH (**2b**) was obtained from *N*- $\alpha$ -Boc-Gly-Phe-OMe (**2a**, 450 mg, 1 mmol) in THF (5 mL), and an aqueous solution of LiOH (96 mg, 4 equiv., 4 mmol dissolved in 5 mL of water) was added. The reaction was allowed to stir at room temperature for 24 h.<sup>1</sup> Next, *N*- $\alpha$ -Boc-Gly-Phe-OH (**2b**, 322 mg, 1 equiv., 1.0 mmol), DIC (205  $\mu$ L, 1.3 equiv., 1.3 mmol), and HONB (232 mg, 1.3 equiv., 1.3 mmol) were added to the reaction vessel, and the vial was sealed. The reaction mixture was heated under MW irradiation at 60 °C for 30 min. Then, the crude *N*- $\alpha$ -Boc-Gly-Phe-Leu-tetrazole (**3g**) was dissolved in a mixture of ethyl acetate and water, and the layers were separated. The organic layer was concentrated under vacuum. The crude peptide was purified by RP-HPLC using a combination of acetonitrile and water (0.1% acetic acid) to afford pure compound **3g**, which were characterized by NMR and the mass spectroscopy.

**Synthesis of 3h.** Compound **6** was purchased from a commercial source and used in the synthesis of compound **3h**. Initially, **6** (300 mg, 1.2 equiv., 1.12 mmol) was dissolved in DMF (1 mL) in a microwave (MW) reaction vessel, and *N,N*-diisopropylethylamine (DIEA, 523  $\mu$ L, 3 equiv., 3.36 mmol) was added, and the solution was stirred to 5 min at room temperature. Next, *N*- $\alpha$ -Boc-Gly-Phe-OH (**2**, 300 mg, 1 equiv., 0.9 mmol), DIC (184  $\mu$ L, 1.3 equiv., 1.17 mmol), and HONB (208 mg, 1.3 equiv., 1.17 mmol) were added to the reaction vessel, and the vial was sealed. The reaction mixture was heated under MW irradiation at 60 °C for 30 min to generate the crude *N*- $\alpha$ -Boc-Gly-Phe-

*N*-phenyl-*N*-(piperidin-4-yl)propionamide (**3h**). Then, the crude peptide was dissolved in a mixture of ethyl acetate and water, and the layers were separated. The organic layer was concentrated under vacuum. The crude peptide was purified by RP-HPLC using a combination of acetonitrile and water (0.1% acetic acid) to afford pure compound **3h**, which was characterized by NMR and the mass spectroscopy.

**General Procedure for Synthesis of Boc-Tyr-ψ[(Z)CF=CH]-Gly-Gly-Phe-Leu-X (1aa–ff).** The synthesized compounds **3a–f** (0.2 mmol) were dissolved in a solution of 4N HCl in 1,4-dioxane (5 mL), and reaction mixtures were stirred at room temperature for 30 min. The removal of 4N HCl in 1,4-dioxane under vacuum afforded the desired the Boc-deprotected peptidomimetics, and then, compounds were transferred to the MW vials for the coupling step. Next, deprotected compounds were neutralized with DIEA (3 equiv.) in DMF (1 mL) for 5 min at room temperature and then Boc-Tyr-ψ[(Z)CF=CH]-Gly-OH (**7**, 88 mg, 1.3 equiv., 0.26 mmol), DIC (40 μL, 1.3 equiv., 0.26 mmol), and HONB (46 mg, 1.3 equiv., 0.26 mmol) were added to the vial. The vial was sealed, and the reaction mixture was subjected to MW irradiated heating at 60 °C for 30 min at constant stirring. After completion of reaction, the crude peptidomimetics (**1aa–ff**) were dissolved in a mixture of ethyl acetate and water, and the layers were separated. The organic layer was concentrated under vacuum. The crude peptidomimetics (**1aa–ff**) were purified by RP-HPLC using a combination of acetonitrile and water (0.1% acetic acid) to afford pure compounds (**1aa–ff**), which was characterized by NMR and the mass spectroscopy.

**General Procedure for Synthesis of H<sub>2</sub>N-Tyr-ψ[(Z)CF=CH]-Gly-Gly-Phe-Leu-X (1a–f).** Boc deprotection reactions were carried out under acidic conditions to afford the final compounds (**1a–f**). Compounds **1a–f** (0.15 mmol) were dissolved in a solution of 4N HCl in 1,4-dioxane (5 mL), and reaction mixtures were stirred at room temperature for 30 min. The removal of 4N HCl in 1,4-dioxane under vacuum afforded desired the Boc-deprotected peptidomimetics. The crude peptidomimetics (**1a–f**) were subjected to purification by RP-HPLC using a combination of acetonitrile and water (0.1% acetic acid) to afford pure compounds (**1a–f**), which were characterized by NMR and the high-resolution mass spectroscopy. The final purity of synthesized compounds was also analyzed using RP-UPLC and found to be ≥95%.

**Synthesis of Boc-Tyr-ψ[(Z)CF=CH]-Gly-Gly-Phe-Leu-tetrazole (1gg).** Tetrazole derivative **3g** (100 mg, 1 equiv., 0.20 mmol) was dissolved in a solution of 4N HCl in 1,4-dioxane (5 mL), and reaction mixture was stirred at room temperature for 30 min. 4N HCl in 1,4-dioxane was removed under vacuum and afforded desired the Boc-deprotected peptidomimetic, which was transferred to a MW vial for the coupling step. Next, the deprotected compound was dissolved in DMF (1 mL) and neutralized with DIEA (105 μL, 3 equiv., 0.6 mmol) for 5 min at room temperature. Then, Boc-Tyr-ψ[(Z)CF=CH]-Gly-OH (**7**, 82 mg, 1.2 equiv., 0.24 mmol), DIC (46 μL, 1.3 equiv., 0.26 mmol), and HONB (46 mg, 1.3 equiv., 0.26 mmol) were added, and the reaction mixture was subjected to MW irradiated heating at 60 °C for 30 min at constant stirring. After completion of reaction, the crude peptidomimetic **1gg** was dissolved in a mixture of ethyl acetate and water, and the layers were separated. The organic layer was concentrated under vacuum. The crude peptidomimetic **1gg** was purified by RP-HPLC using a

combination of acetonitrile and water (0.1% acetic acid) to afford pure tetrazole derivative **1gg**, and characterized by NMR and the high-resolution mass spectroscopy.

**Synthesis of H<sub>2</sub>N-Tyr-ψ[(Z)CF=CH]-Gly-Gly-Phe-Leu-tetrazole (1g).** Deprotection of Boc-Tyr-ψ[(Z)CF=CH]-Gly-Gly-Phe-Leu-tetrazole (**1gg**, 68 mg, 0.1 mmol) was carried out under acidic conditions to afford final peptidomimetic **1g**. The synthesized compounds **1gg** was dissolved in a solution of 4N HCl in 1,4-dioxane (5 mL), and reaction mixture was stirred at room temperature for 30 min. The removal of 4N HCl in 1,4-dioxane under vacuum afforded desired the Boc-deprotected crude peptidomimetic **1g**. The reaction mixture of **1g** was subjected to purification by RP-HPLC using a combination of acetonitrile and water (0.1% acetic acid) to afford pure compound **1g**, which was characterized by NMR and the high-resolution mass spectroscopy. The final purity of compound **1g** was analyzed using RP-UPLC and found to be ≥95%.

**Synthesis of Boc-Tyr-ψ[(Z)CF=CH]-Gly-Gly-Phe-N-phenyl-N-(piperidin-4-yl)propionamide (1hh).** The synthesized tripeptidomimetic **3h** (110 mg, 1 equiv., 0.2 mmol) was dissolved in a solution of 4N HCl in 1,4-dioxane (5 mL), and reaction mixture was stirred at room temperature for 30 min. 4N HCl in 1,4-dioxane was removed under vacuum and afforded desired the Boc-deprotected derivative, which was transferred to the MW vial for the coupling step. Next, the deprotected compound was dissolved in DMF (1 mL) and neutralized using DIEA (108 μL, 3 equiv., 0.6 mmol) for 5 min at room temperature. Then, Boc-Tyr-ψ[(Z)CF=CH]-Gly-OH (**7**, 88 mg, 1.3 equiv., 0.26 mmol), DIC (41 μL, 1.3 equiv., 0.26 mmol), and HONB (46 mg, 1.3 equiv., 0.26 mmol) were added, and the mixture was stirred well before subjecting to the microwave. The reaction mixture was subjected to MW irradiated heating at 60 °C for 30 min. After completion of reaction, the crude peptidomimetic **1hh** was dissolved in a mixture of ethyl acetate and water, and the layers were separated. The organic layer was concentrated under vacuum. The crude peptidomimetic **1hh** was purified by RP-HPLC using acetonitrile and water (0.1% acetic acid) solvent combination to afford pure compound, Boc-Tyr-ψ[(Z)CF=CH]-Gly-Gly-Phe-N-phenyl-N-(piperidin-4-yl)propionamide (**1hh**), which was characterized by NMR and the high-resolution mass spectroscopy.

**Synthesis of H<sub>2</sub>N-Tyr-ψ[(Z)CF=CH]-Gly-Gly-Phe-N-phenyl-N-(piperidin-4-yl)propionamide (1h).** Deprotection of Boc-Tyr-ψ[(Z)CF=CH]-Gly-Gly-Phe-N-phenyl-N-(piperidin-4-yl)propionamide (**13g**) was carried out under acidic conditions to afford final peptidomimetic **1h**. Compound **1h** (33 mg, 0.04 mmol) was dissolved in a solution of 4N HCl in 1,4-dioxane (5 mL), and reaction mixture was stirred at room temperature for 30 min. The removal of 4N HCl in 1,4-dioxane under vacuum afforded the desired the Boc-deprotected crude peptidomimetic **1h**. The crude product was subjected to purification by RP-HPLC using a combination of acetonitrile and water (0.1% acetic acid) to afford pure compound **1h**, which was characterized by NMR and the high-resolution mass spectroscopy. The final purity was analyzed using RP-UPLC and found to be ≥95%.

## Spectroscopic Data

**Boc-Gly-Phe-Leu-NH<sub>2</sub> (3b).** Yield 85%, 1.38 g colorless solid. <sup>1</sup>H NMR (500 MHz, MeOD-*d*<sub>4</sub>) δ 7.33–7.19 (m, 5H), 4.67–4.59 (m, 1H), 4.41–4.34 (m, 1H), 3.74–3.56 (m, 2H), 3.22–3.13 (m, 1H), 3.00 (dd, *J* = 16.1, 8.4 Hz, 1H), 1.68–1.59 (m, 3H), 1.45 (d, *J* = 4.2 Hz, 9H), 0.98–0.89 (m, 6H); <sup>13</sup>C NMR (126 MHz, MeOD-*d*<sub>4</sub>) δ 175.8, 171.9, 171.4, 157.1, 136.7, 128.9, 128.2, 126.5, 79.4, 54.7, 51.6, 43.3, 41.3, 40.2, 36.9, 27.3, 24.3, 22.1; HRMS (ESI<sup>+</sup>) mass calculated for [M+H]<sup>+</sup> (C<sub>22</sub>H<sub>35</sub>N<sub>4</sub>O<sub>5</sub>) *m/z* 435.2607, found *m/z* 435.2617.

**Boc-Gly-Phe-Leu-NHMe (3c).** Yield 84%, 335 mg colorless solid. <sup>1</sup>H NMR (400 MHz, MeOD-*d*<sub>4</sub>) δ 7.34 (s, 1H), 7.23–7.06 (m, 6H), 4.50 (dd, *J* = 8.2, 5.6 Hz, 1H), 4.27–4.19 (m, 1H), 3.63–3.45 (m, 2H), 3.05 (dd, *J* = 13.9, 5.6 Hz, 1H), 2.91–2.81 (m, 1H), 2.58 (s, 3H), 1.58–1.42 (m, 3H), 1.32 (s, 9H), 0.86–0.75 (m, 6H); <sup>13</sup>C NMR (101 MHz, MeOD-*d*<sub>4</sub>) δ 173.5, 171.9, 171.6, 157.1, 136.7, 128.9, 128.1, 126.5, 79.4, 54.8, 51.9, 43.3, 41.3, 40.2, 36.9, 27.2, 25.2, 24.3, 22.1, 22.1, 20.3; HRMS (ESI<sup>+</sup>) mass calculated for [M+H]<sup>+</sup> (C<sub>23</sub>H<sub>36</sub>N<sub>4</sub>O<sub>5</sub>) *m/z* 449.2764, found *m/z* 449.2774.

**Boc-Gly-Phe-Leu-NHEt (3d).** Yield 96%, 407 mg colorless solid. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.29 (s, 1H), 7.21–7.13 (m, 4H), 7.08–7.05 (m, 2H), 7.01 (s, 1H), 5.42 (s, 1H), 4.76–4.67 (m, 1H), 4.50–4.40 (m, 1H), 3.85–3.67 (m, 2H), 3.27–3.10 (m, 3H), 3.02 (dd, *J* = 13.9, 6.0 Hz, 1H), 2.90 (dd, *J* = 13.8, 6.4 Hz, 1H), 1.68–1.57 (m, 1H), 1.45–1.28 (m, 11H), 1.07 (t, *J* = 7.3 Hz, 3H), 0.89–0.77 (m, 6H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 171.5, 170.4, 169.4, 156.4, 136.0, 129.3, 128.6, 127.1, 80.4, 54.4, 51.9, 44.4, 41.1, 38.2, 34.4, 28.2, 24.6, 22.8, 14.6; HRMS (ESI<sup>+</sup>) mass calculated for [M+Na]<sup>+</sup> (C<sub>24</sub>H<sub>38</sub>N<sub>4</sub>NaO<sub>5</sub>) *m/z* 485.2740, found *m/z* 485.2744.

**Boc-Gly-Phe-Leu-NMe<sub>2</sub> (3e).** Yield 60%, 390 mg colorless solid. <sup>1</sup>H NMR (400 MHz, MeOD-*d*<sub>4</sub>) δ 8.00 (s, 1H), 7.33–7.14 (m, 5H), 4.69 (dd, *J* = 8.1, 5.5 Hz, 1H), 3.75–3.60 (m, 2H), 3.18–3.07 (m, 4H), 2.99–2.89 (m, 4H), 1.69–1.54 (m, 3H), 1.46 (s, 9H), 0.99–0.87 (m, 6H); <sup>13</sup>C NMR (101 MHz, MeOD-*d*<sub>4</sub>) δ 173.9, 172.4, 170.7, 163.4, 136.7, 134.1, 129.0, 128.0, 126.3, 79.3, 54.0, 50.8, 44.2, 42.4, 36.1, 35.5, 34.7, 30.2, 27.3, 24.4, 22.1, 20.6; HRMS (ESI<sup>+</sup>) mass calculated for [M+H]<sup>+</sup> (C<sub>24</sub>H<sub>39</sub>N<sub>4</sub>O<sub>5</sub>) *m/z* 463.2920, found *m/z* 463.2938.

**Boc-Gly-Phe-Leu-tetrazole (3g).** Yield 67%, 306 mg colorless solid. <sup>1</sup>H NMR (500 MHz, MeOD-*d*<sub>4</sub>) δ 7.31–7.08 (m, 5H), 5.35 (dd, *J* = 9.6, 6.1 Hz, 1H), 4.69–4.60 (m, 1H), 3.75–3.55 (m, 2H), 3.14 (dd, *J* = 13.8, 5.9 Hz, 1H), 2.93 (dd, *J* = 13.8, 8.2 Hz, 1H), 1.98–1.89 (m, 1H), 1.88–1.78 (m, 1H), 1.67–1.57 (m, 1H), 1.42 (s, 9H), 1.09–0.91 (m, 6H); <sup>13</sup>C NMR (126 MHz, MeOD-*d*<sub>4</sub>) δ 171.7, 171.2, 158.0, 157.1, 136.5, 128.8, 128.0, 126.3, 79.4, 54.4, 43.2, 42.6, 41.4, 37.1, 27.2, 24.2, 21.6, 20.5; HRMS (ESI<sup>+</sup>) mass calculated for [M+H]<sup>+</sup> (C<sub>22</sub>H<sub>34</sub>N<sub>7</sub>O<sub>4</sub>) *m/z* 460.2672, found *m/z* 460.2672.

**Boc-Gly-Phe-*N*-phenyl-*N*-(piperidin-4-yl)propionamide (3h).** Yield 42%, 210 mg colorless solid. <sup>1</sup>H NMR (500 MHz, MeOD-*d*<sub>4</sub>) δ 7.67–7.38 (m, 3H), 7.32–7.11 (m, 5H), 7.11–6.92 (m, 2H), 5.10–5.02 (m, 1H), 4.72–4.58 (m, 1H), 4.54–4.46 (m, 1H), 3.87 (dd, *J* = 30.6, 13.8 Hz, 1H), 3.77–3.52 (m, 2H), 3.10–3.00 (m, 1H), 2.99–2.84 (m, 2H), 2.67–2.52 (m, 1H), 2.01–1.73 (m, 4H), 1.46 (s, 9H), 1.04–0.92 (m, 3H); <sup>13</sup>C NMR (126 MHz, MeOD-*d*<sub>4</sub>) δ 174.4, 170.3, 169.8, 156.9, 138.1, 135.9, 129.9, 129.3, 129.1, 128.9, 128.5, 128.1, 128.0, 126.8, 79.3, 52.2, 49.7,

44.9, 43.0, 41.2, 38.4, 30.5, 29.6, 27.8, 27.2, 8.5. HRMS (ESI<sup>+</sup>) mass calculated for [M+Na]<sup>+</sup> (C<sub>30</sub>H<sub>40</sub>N<sub>4</sub>NaO<sub>5</sub>) m/z 559.2896, found m/z 559.2906.

**Boc-Tyr-ψ[(Z)CF=CH]-Gly-Phe-Leu-NH<sub>2</sub> (1bb).** Yield 59%, 80 mg colorless solid. <sup>1</sup>H NMR (600 MHz, MeOD-*d*<sub>4</sub>) δ 7.39–7.18 (m, 5H), 7.05 (d, *J* = 8.4 Hz, 2H), 6.72 (d, *J* = 8.0 Hz, 2H), 5.00–4.91 (m, 1H), 4.66–4.60 (m, 1H), 4.42–4.36 (m, 1H), 4.35–4.27 (m, 1H), 3.90–3.67 (m, 2H), 3.19 (dd, *J* = 14.0, 5.5 Hz, 1H), 3.10–2.86 (m, 4H), 2.74 (dd, *J* = 13.7, 8.7 Hz, 1H), 1.73–1.56 (m, 3H), 1.39 (s, 9H), 0.96 (d, *J* = 5.9 Hz, 3H), 0.93 (d, *J* = 5.9 Hz, 3H); <sup>13</sup>C NMR (151 MHz, MeOD-*d*<sub>4</sub>) δ 177.4, 173.7, 173.4, 172.1, 161.3 (d, *J* = 260.6 Hz), 157.5, 157.1, 138.2, 131.4, 130.3, 129.6, 128.0, 116.2, 100.5 (d, *J* = 12.7 Hz), 80.4, 56.4, 54.8 (d, *J* = 28.9 Hz), 53.1, 49.9, 41.6, 38.4, 31.4 (d, *J* = 5.1 Hz), 28.7, 25.8, 23.6, 21.8; <sup>19</sup>F NMR (471 MHz, MeOD-*d*<sub>4</sub>) δ -119.46 (dd, *J* = 36.3, 16.5 Hz); HRMS (ESI<sup>+</sup>) mass calculated for [M+H]<sup>+</sup> (C<sub>34</sub>H<sub>47</sub>FN<sub>5</sub>O<sub>7</sub>) m/z 656.3460, found m/z 656.3480.

**Boc-Tyr-ψ[(Z)CF=CH]-Gly-Phe-Leu-NHMe (1cc).** Yield 62%, 265 mg colorless solid. <sup>1</sup>H NMR (500 MHz, MeOD-*d*<sub>4</sub>) δ 7.34–7.20 (m, 5H), 7.05 (d, *J* = 8.5 Hz, 2H), 6.71 (d, *J* = 8.4 Hz, 2H), 4.95 (dt, *J* = 36.2, 7.5 Hz, 1H), 4.66–4.57 (m, 1H), 4.41–4.23 (m, 2H), 3.90–3.69 (m, 2H), 3.17 (dd, *J* = 14.0, 5.6 Hz, 1H), 3.08–2.94 (m, 3H), 2.91 (dd, *J* = 13.7, 6.4 Hz, 1H), 2.79–2.64 (m, 4H), 1.68–1.54 (m, 3H), 1.38 (s, 9H), 0.95 (d, *J* = 5.6 Hz, 3H), 0.90 (s, 3H); <sup>13</sup>C NMR (126 MHz, MeOD-*d*<sub>4</sub>) δ 173.5, 172.3, 171.9, 170.6, 159.9 (d, *J* = 260.2 Hz), 156.1, 155.7, 136.7, 129.9, 128.9, 128.1, 128.1, 126.5, 114.7, 99.0 (d, *J* = 12.6 Hz), 79.0, 54.9, 53.3 (d, *J* = 29.1 Hz), 51.9, 42.4, 40.2, 36.9, 29.9 (d, *J* = 5.0 Hz), 27.3, 25.0, 24.3, 22.1, 20.4; <sup>19</sup>F NMR (471 MHz, MeOD-*d*<sub>4</sub>) δ -119.38 (dd, *J* = 36.2, 16.0 Hz); HRMS (ESI<sup>+</sup>) mass calculated for [M+Na]<sup>+</sup> (C<sub>35</sub>H<sub>48</sub>FN<sub>5</sub>NaO<sub>7</sub>) m/z 692.3435, found m/z 692.3456.

**Boc-Tyr-ψ[(Z)CF=CH]-Gly-Phe-Leu-NHEt (1dd).** Yield 56%, 230 mg colorless solid. <sup>1</sup>H NMR (500 MHz, MeOD-*d*<sub>4</sub>) δ 7.31–7.21 (m, 5H), 7.05 (d, *J* = 8.5 Hz, 2H), 6.71 (d, *J* = 8.5 Hz, 2H), 5.03–4.89 (m, 1H), 4.60 (dd, *J* = 8.5, 5.4 Hz, 1H), 4.39–4.25 (m, 2H), 3.88–3.66 (m, 2H), 3.26–3.12 (m, 3H), 3.05–2.86 (m, 4H), 2.73 (dd, *J* = 13.7, 8.6 Hz, 1H), 1.68–1.55 (m, 3H), 1.38 (s, 9H), 1.12 (t, *J* = 7.3 Hz, 3H), 0.95 (d, *J* = 5.9 Hz, 3H), 0.92 (d, *J* = 5.8 Hz, 3H); <sup>13</sup>C NMR (126 MHz, MeOD-*d*<sub>4</sub>) δ 172.7, 172.3, 171.9, 170.6, 159.9 (d, *J* = 260.7 Hz), 156.1, 155.7, 136.7, 129.9, 128.9, 128.1, 128.1, 126.5, 114.7, 99.0 (d, *J* = 12.7 Hz), 79.0, 54.9, 53.3 (d, *J* = 28.6 Hz), 51.9, 42.5, 40.2, 36.9, 33.9, 30.0 (d, *J* = 5.2 Hz), 27.3, 24.4, 22.1, 20.4, 13.3; <sup>19</sup>F NMR (471 MHz, MeOD-*d*<sub>4</sub>) δ -119.38 (dd, *J* = 36.2, 16.2 Hz); HRMS (ESI<sup>+</sup>) mass calculated for [M+H]<sup>+</sup> (C<sub>36</sub>H<sub>51</sub>FN<sub>5</sub>O<sub>7</sub>) m/z 684.3773, found m/z 684.3793.

**Boc-Tyr-ψ[(Z)CF=CH]-Gly-Phe-Leu-NMe<sub>2</sub> (1ee).** Yield 44%, 98 mg colorless solid. <sup>1</sup>H NMR (500 MHz, MeOD-*d*<sub>4</sub>) δ 7.31–7.18 (m, 5H), 7.05 (d, *J* = 8.5 Hz, 2H), 6.71 (d, *J* = 8.5 Hz, 2H), 5.03–4.88 (m, 1H), 4.69 (dd, *J* = 8.6, 5.4 Hz, 1H), 4.35–4.25 (m, 1H), 3.85–3.74 (m, 2H), 3.19–3.08 (m, 5H), 3.03 (d, *J* = 7.5 Hz, 2H), 2.97–2.87 (m, 5H), 2.73 (dd, *J* = 13.8, 8.6 Hz, 1H), 1.72–1.56 (m, 2H), 1.47 (ddd, *J* = 13.7, 9.1, 4.4 Hz, 1H), 1.38 (s, 9H), 0.98–0.93 (m, 6H); <sup>13</sup>C NMR (126 MHz, MeOD-*d*<sub>4</sub>) δ 172.5, 172.1, 171.5, 169.8, 159.9 (d, *J* = 260.4 Hz), 156.1, 155.7, 136.7, 129.9, 128.9, 128.9, 128.0, 126.4, 114.7, 114.7, 99.0 (d, *J* = 12.7 Hz), 78.9, 54.2, 53.3 (d, *J* = 29.1 Hz), 42.1, 40.4, 37.4, 36.9, 36.1, 34.8, 30.1 (d, *J* = 5.2 Hz), 27.3, 24.4, 22.2, 20.6; <sup>19</sup>F NMR (471 MHz, DMSO-*d*<sub>6</sub>) δ -116.7 (dd, *J* = 38.5, 14.5 Hz), -118.72 (dd, *J* = 30.4, 20.7 Hz); HRMS (ESI<sup>+</sup>) mass calculated for [M+H]<sup>+</sup> (C<sub>36</sub>H<sub>51</sub>FN<sub>5</sub>O<sub>7</sub>) m/z 684.3773, found m/z 684.3772.

**Boc-Tyr-ψ[(Z)CF=CH]-Gly-Phe-Leu-NH<sup>Cr</sup>Pr (1ff).** Yield 56%, 120 mg colorless solid. <sup>1</sup>H NMR (600 MHz, MeOD-*d*<sub>4</sub>) δ 7.34–7.28 (m, 2H), 7.28–7.22 (m, 3H), 7.06 (d, *J* = 8.6 Hz, 2H), 6.72 (d, *J* = 8.5 Hz, 2H), 5.00–4.91 (m, 1H), 4.57 (dd, *J* = 8.6, 5.2 Hz, 1H), 4.35–4.26 (m, 2H), 3.85–3.67 (m, 2H), 3.18 (dd, *J* = 14.1, 5.2 Hz, 1H), 3.03 (d, *J* = 7.4 Hz, 2H), 3.01–2.88 (m, 2H), 2.76–2.64 (m, 2H), 1.73–1.52 (m, 3H), 1.39 (s, 9H), 0.97–0.90 (m, 6H), 0.77–0.68 (m, 2H), 0.59–0.48 (m, 2H); <sup>13</sup>C NMR (151 MHz, MeOD-*d*<sub>4</sub>) δ 175.9, 173.7, 173.3, 172.2, 161.3 (d, *J* = 260.2 Hz), 157.6, 157.1, 138.1, 131.3, 130.3, 129.6, 129.5, 127.9, 116.1, 100.4 (d, *J* = 13.0 Hz), 80.4, 56.5, 54.8 (d, *J* = 28.9 Hz), 53.2, 49.8, 43.9, 41.5, 38.4, 38.2, 31.4 (d, *J* = 5.0 Hz), 28.7, 25.8, 23.5, 23.5, 21.8, 6.5, 6.4; <sup>19</sup>F NMR (471 MHz, MeOD-*d*<sub>4</sub>) δ -119.4 (dd, *J* = 36.4, 16.3 Hz, 1F); HRMS (ESI<sup>+</sup>) mass calculated for [M+H]<sup>+</sup> (C<sub>37</sub>H<sub>51</sub>FN<sub>5</sub>O<sub>7</sub>) m/z 696.3773, found m/z 696.3783.

**Boc-Tyr-ψ[(Z)CF=CH]-Gly-Phe-Leu-tetrazole (1gg).** Yield 52%, 70 mg colorless solid. <sup>1</sup>H NMR (500 MHz, MeOD-*d*<sub>4</sub>) δ 7.23–7.10 (m, 5H), 7.05 (d, *J* = 8.5 Hz, 2H), 6.71 (d, *J* = 8.5 Hz, 2H), 5.37 (dd, *J* = 9.5, 6.2 Hz, 1H), 5.01–4.91 (m, 1H), 4.63 (dd, *J* = 8.4, 5.8 Hz, 1H), 4.34–4.25 (m, 1H), 3.85–3.70 (m, 2H), 3.13 (dd, *J* = 13.9, 5.8 Hz, 1H), 3.08–2.97 (m, 2H), 2.96–2.86 (m, 2H), 2.73 (dd, *J* = 13.8, 8.7 Hz, 1H), 1.98–1.87 (m, 1H), 1.86–1.76 (m, 1H), 1.67–1.55 (m, 1H), 1.38 (s, 9H), 1.00–0.93 (m, 6H); <sup>13</sup>C NMR (126 MHz, MeOD-*d*<sub>4</sub>) δ 173.8, 172.3, 171.7, 170.1, 160.0 (d, *J* = 260.5 Hz), 158.5, 156.1, 155.7, 136.5, 129.9, 128.8, 128.1, 128.0, 126.4, 114.7, 99.0 (d, *J* = 12.7 Hz), 79.0, 54.5, 53.3 (d, *J* = 29.0 Hz), 42.7, 42.3, 41.7, 37.1, 36.9, 30.0 (d, *J* = 5.3 Hz), 27.3, 24.3, 21.6, 20.7; <sup>19</sup>F NMR (471 MHz, MeOD-*d*<sub>4</sub>) δ -119.33 (dd, *J* = 36.4, 16.2 Hz, 1F); HRMS (ESI<sup>+</sup>) mass calculated for [M+Na]<sup>+</sup> (C<sub>34</sub>H<sub>45</sub>FN<sub>8</sub>NaO<sub>6</sub>) m/z 703.3344, found m/z 703.3533.

**Boc-Tyr-ψ[(Z)CF=CH]-Gly-Phe-*N*-phenyl-*N*-(piperidin-4-yl)propionamide (1hh).** Yield 62%, 95 mg colorless solid. <sup>1</sup>H NMR (400 MHz, MeOD-*d*<sub>4</sub>) δ 7.53–7.27 (m, 3H), 7.23–7.10 (m, 1H), 7.10–7.04 (m, 2H), 7.00–6.77 (m, 6H), 6.64–6.56 (m, 2H), 4.93 (dt, *J* = 12.0, 6.7 Hz, 1H), 4.63–4.48 (m, 1H), 4.42 (d, *J* = 13.6 Hz, 1H), 4.31–4.17 (m, 1H), 3.85–3.62 (m, 4H), 3.05–2.88 (m, 3H), 2.89–2.71 (m, 3H), 2.70–2.40 (m, 4H), 1.89–1.62 (m, 4H), 1.29 (s, 9H), 0.88 (td, *J* = 7.5, 5.5 Hz, 3H); <sup>19</sup>F NMR (376 MHz, MeOD-*d*<sub>4</sub>) δ -118.90–119.29 (m, 1F); HRMS (ESI<sup>+</sup>) mass calculated for [M+Na]<sup>+</sup> (C<sub>42</sub>H<sub>52</sub>FN<sub>5</sub>NaO<sub>7</sub>) m/z 780.3748, found m/z 780.3768.

**H<sub>2</sub>N-Tyr-ψ[(Z)CF=CH]-Gly-Phe-Leu-NH<sub>2</sub> (1b).** Yield 81%, 55 mg colorless solid. <sup>1</sup>H NMR (600 MHz, MeOD-*d*<sub>4</sub>) δ 7.32–7.21 (m, 5H), 7.07 (d, *J* = 8.4 Hz, 2H), 6.76 (d, *J* = 8.5 Hz, 2H), 5.12–4.98 (m, 1H), 4.63 (dd, *J* = 8.7, 5.5 Hz, 1H), 4.38 (dd, *J* = 10.8, 3.6 Hz, 1H), 4.00–3.87 (m, 1H), 3.84 (d, *J* = 16.4 Hz, 1H), 3.73 (d, *J* = 16.4 Hz, 1H), 3.19 (dd, *J* = 14.0, 5.5 Hz, 1H), 3.12 (dd, *J* = 16.5, 7.9 Hz, 1H), 3.03–2.89 (m, 4H), 1.70–1.59 (m, 3H), 0.96 (d, *J* = 5.9 Hz, 3H), 0.92 (d, *J* = 5.8 Hz, 3H); <sup>13</sup>C NMR (151 MHz, MeOD-*d*<sub>4</sub>) δ 177.3, 173.4, 173.1, 172.0, 157.8, 157.8 (d, *J* = 257.8 Hz), 138.2, 131.4, 130.3, 129.6, 127.9, 127.6, 116.5, 104.8 (d, *J* = 11.7 Hz), 56.4, 55.3 (d, *J* = 27.2 Hz), 53.0, 49.9, 43.8, 41.6, 38.4, 37.7, 31.2 (d, *J* = 4.5 Hz), 25.8, 23.6, 21.8; <sup>19</sup>F NMR (471 MHz, MeOD-*d*<sub>4</sub>) δ -123.08 (dd, *J* = 36.6, 22.1 Hz, 1F); HRMS (ESI<sup>+</sup>) mass calculated for [M+H]<sup>+</sup> (C<sub>29</sub>H<sub>39</sub>FN<sub>5</sub>O<sub>5</sub>) m/z 556.2935, found m/z 556.3002; purity ≥95%, rt 1.58 min (method-A).

**H<sub>2</sub>N-Tyr-ψ[(Z)CF=CH]-Gly-Phe-Leu-NHMe (1c).** Yield 83%, 50 mg colorless solid. <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 8.14–8.07 (m, 2H), 8.00 (d, *J* = 8.3 Hz, 1H), 7.57 (q, *J* = 4.6 Hz, 1H), 7.29–7.15 (m, 5H), 6.98 (d, *J* =



8.4 Hz, 2H), 6.66 (d,  $J = 8.5$  Hz, 2H), 4.91 (dt,  $J = 38.8, 7.1$  Hz, 1H), 4.53–4.45 (m, 1H), 4.26–4.18 (m, 1H), 3.72 (dd,  $J = 16.4, 5.7$  Hz, 1H), 3.56 (dd,  $J = 16.4, 5.7$  Hz, 1H), 3.51–3.39 (m, 1H), 3.02 (dd,  $J = 13.8, 4.5$  Hz, 1H), 2.95–2.85 (m, 2H), 2.79 (dd,  $J = 13.8, 9.3$  Hz, 1H), 2.72 (dd,  $J = 13.5, 5.9$  Hz, 1H), 2.56 (s, 3H), 1.59–1.41 (m, 3H), 0.88 (d,  $J = 6.4$  Hz, 3H), 0.83 (d,  $J = 6.4$  Hz, 3H);  $^{13}\text{C}$  NMR (126 MHz, DMSO- $d_6$ )  $\delta$  172.5, 171.0, 170.5, 169.5, 162.5 (d,  $J = 257.8$  Hz), 156.1, 138.1, 130.5, 129.6, 128.7, 128.5, 126.7, 115.4, 99.2 (d,  $J = 12.3$  Hz), 54.5, 54.1 (d,  $J = 27.9$  Hz), 51.5, 42.5, 41.2, 37.7, 30.4 (d,  $J = 4.6$  Hz), 26.0, 24.6, 23.4, 22.0;  $^{19}\text{F}$  NMR (470 MHz, DMSO- $d_6$ )  $\delta$  -121.02 (dd,  $J = 39.1, 15.7$  Hz, 1F); HRMS (ESI $^+$ ) mass calculated for  $[\text{M}+\text{H}]^+$  ( $\text{C}_{30}\text{H}_{41}\text{FN}_5\text{O}_5$ )  $m/z$  570.3092, found  $m/z$  570.3098; purity  $\geq 95\%$ , rt 1.60 min (method-A).

**H<sub>2</sub>N-Tyr- $\psi$ [(Z)CF=CH]-Gly-Phe-Leu-NHEt (1d).** Yield 62%, 79 mg colorless solid.  $^1\text{H}$  NMR (500 MHz, MeOD- $d_4$ )  $\delta$  7.32–7.19 (m, 5H), 7.06 (d,  $J = 8.4$  Hz, 2H), 6.75 (d,  $J = 8.4$  Hz, 2H), 4.99 (dt,  $J = 36.5, 7.4$  Hz, 1H), 4.61 (dd,  $J = 8.5, 5.4$  Hz, 1H), 4.37–4.32 (m, 1H), 3.88–3.69 (m, 3H), 3.26–3.03 (m, 4H), 3.03–2.84 (m, 4H), 1.68–1.54 (m, 3H), 1.12 (t,  $J = 7.3$  Hz, 3H), 0.99–0.87 (m, 6H);  $^{13}\text{C}$  NMR (126 MHz, MeOD- $d_4$ )  $\delta$  172.7, 171.8, 171.8, 170.5, 157.9 (d,  $J = 259.5$  Hz), 156.2, 136.7, 130.0, 128.9, 128.1, 126.6, 126.5, 115.1, 102.4 (d,  $J = 12.0$  Hz), 54.9, 53.9 (d,  $J = 27.0$  Hz), 51.8, 48.1, 47.9, 47.7, 47.6, 47.4, 47.2, 47.1, 42.3, 40.3, 37.0, 36.8, 33.9, 29.8 (d,  $J = 4.8$  Hz), 24.4, 22.0, 20.5, 13.3;  $^{19}\text{F}$  NMR (471 MHz, MeOD- $d_4$ )  $\delta$  -123.80 (dd,  $J = 34.8, 22.3$  Hz, 1F); HRMS (ESI $^+$ ) mass calculated for  $[\text{M}+\text{H}]^+$  ( $\text{C}_{31}\text{H}_{43}\text{FN}_5\text{O}_5$ )  $m/z$  584.3248, found  $m/z$  584.3268; purity  $\geq 95\%$ , rt 1.71 min (method-A).

**H<sub>2</sub>N-Tyr- $\psi$ [(Z)CF=CH]-Gly-Phe-Leu-NMe<sub>2</sub> (1e).** Yield 66%, 83 mg colorless solid.  $^1\text{H}$  NMR (500 MHz, MeOD- $d_4$ )  $\delta$  7.30–7.17 (m, 5H), 7.06 (d,  $J = 8.1$  Hz, 2H), 6.75 (d,  $J = 8.0$  Hz, 2H), 5.02 (dt,  $J = 36.5, 7.4$  Hz, 1H), 4.69 (dd,  $J = 8.6, 5.3$  Hz, 1H), 3.90–3.77 (m, 3H), 3.20–3.04 (m, 5H), 3.02–2.82 (m, 7H), 1.70–1.55 (m, 2H), 1.47 (ddd,  $J = 13.7, 9.0, 4.5$  Hz, 1H), 0.96 (d,  $J = 6.5$  Hz, 6H);  $^{13}\text{C}$  NMR (126 MHz, MeOD- $d_4$ )  $\delta$  174.0, 173.1, 173.0, 171.2, 159.4 (d,  $J = 259.1$  Hz), 157.7, 138.2, 131.5, 130.4, 129.5, 128.1, 127.9, 116.6, 104.0 (d,  $J = 11.6$  Hz), 55.7, 55.4 (d,  $J = 27.2$  Hz), 43.5, 41.8, 38.9, 38.3, 37.6, 36.3, 31.4 (d,  $J = 4.9$  Hz), 25.9, 23.6, 22.1;  $^{19}\text{F}$  NMR (471 MHz, MeOD- $d_4$ )  $\delta$  -123.7 (dd,  $J = 33.9, 22.7$  Hz, 1F); HRMS (ESI $^+$ ) mass calculated for  $[\text{M}+\text{H}]^+$  ( $\text{C}_{31}\text{H}_{43}\text{FN}_5\text{O}_5$ )  $m/z$  584.3248, found  $m/z$  584.3278; purity  $\geq 95\%$ , rt 1.62 min (method-A).

**H<sub>2</sub>N-Tyr- $\psi$ [(Z)CF=CH]-Gly-Phe-Leu-NH<sup>Cy</sup>Pr (1f).** Yield 85%, 80 mg colorless solid.  $^1\text{H}$  NMR (600 MHz, MeOD- $d_4$ )  $\delta$  7.38–7.14 (m, 5H), 7.07 (d,  $J = 8.2$  Hz, 2H), 6.76 (d,  $J = 8.4$  Hz, 2H), 5.19–5.00 (m, 1H), 4.59 (dd,  $J = 8.6, 5.2$  Hz, 1H), 4.33 (dd,  $J = 10.0, 4.2$  Hz, 1H), 4.01–3.89 (m, 1H), 3.85 (d,  $J = 16.4$  Hz, 1H), 3.72 (d,  $J = 16.4$  Hz, 1H), 3.23–3.05 (m, 2H), 3.05–2.91 (m, 4H), 2.66 (tt,  $J = 7.4, 3.9$  Hz, 1H), 1.68–1.52 (m, 3H), 0.94 (d,  $J = 6.0$  Hz, 3H), 0.91 (d,  $J = 5.8$  Hz, 3H), 0.73 (dq,  $J = 6.3, 2.4$  Hz, 2H), 0.60–0.49 (m, 2H);  $^{13}\text{C}$  NMR (151 MHz, MeOD- $d_4$ )  $\delta$  175.9, 173.3, 173.1, 172.1, 157.8 (d,  $J = 259.1$  Hz), 157.8, 138.2, 131.5, 130.3, 129.6, 128.0, 127.6, 116.6, 105.0, 56.5, 55.3 (d,  $J = 27.2$  Hz), 53.1, 43.9, 41.6, 38.4, 38.4, 37.6, 31.2 (d,  $J = 4.4$  Hz), 25.9, 23.5, 23.5, 21.9, 6.5, 6.5;  $^{19}\text{F}$  NMR (471 MHz, DMSO- $d_6$ )  $\delta$  -118.3 (dd,  $J = 39.1, 16.8$  Hz, 1F); HRMS (ESI $^+$ ) mass calculated for  $[\text{M}+\text{H}]^+$  ( $\text{C}_{31}\text{H}_{43}\text{FN}_5\text{O}_5$ )  $m/z$  584.3248, found  $m/z$  584.3278; purity  $\geq 95\%$ , rt 2.91 min (method-B).

**H<sub>2</sub>N-Tyr- $\psi$ [(Z)CF=CH]-Gly-Phe-Leu-tetrazole (1g).** Yield 83%, 48 mg colorless solid.  $^1\text{H}$  NMR (500 MHz, MeOD- $d_4$ )  $\delta$  7.11–6.97 (m, 5H), 6.83 (d,  $J = 7.4$  Hz, 2H), 6.78 (d,  $J = 8.2$  Hz, 2H), 5.34 (t,  $J = 7.9$  Hz, 1H), 4.76–

4.48 (m, 2H), 3.91 (d,  $J = 16.7$  Hz, 1H), 3.76–3.58 (m, 2H), 3.16 (dd,  $J = 15.1, 9.4$  Hz, 1H), 3.11–2.93 (m, 3H), 2.88 (dd,  $J = 13.8, 6.5$  Hz, 1H), 2.76 (dd,  $J = 15.4, 5.7$  Hz, 1H), 1.92–1.77 (m, 2H), 1.59–1.47 (m, 1H), 0.96 (d,  $J = 6.6$  Hz, 3H), 0.92 (d,  $J = 6.6$  Hz, 3H);  $^{13}\text{C}$  NMR (126 MHz, MeOD- $d_4$ )  $\delta$  172.6, 171.8, 170.8, 163.6, 158.0, 155.8 (d,  $J = 257.4$  Hz), 137.4, 131.4, 130.6, 129.3, 127.7, 126.7, 116.6, 107.0, 55.2 (d,  $J = 26.8$  Hz), 54.8, 45.4, 44.2, 43.5, 38.8, 36.5, 31.8, 25.9, 22.8, 22.5;  $^{19}\text{F}$  NMR (471 MHz, DMSO- $d_6$ )  $\delta$  -125.87 (dd,  $J = 38.4, 23.4$  Hz, 1F); HRMS (ESI $^+$ ) mass calculated for  $[\text{M}+\text{H}]^+$  ( $\text{C}_{29}\text{H}_{38}\text{FN}_8\text{O}_4$ )  $m/z$  581.2995, found  $m/z$  581.2994; purity  $\geq 95\%$ , rt 1.56 min (method-A).

**H<sub>2</sub>N-Tyr- $\psi$ [(Z)CF=CH]-Gly-Phe-N-phenyl-N-(piperidin-4-yl)propionamide (1h).** Yield 80%, 23 mg colorless solid.  $^1\text{H}$  NMR (500 MHz, DMSO- $d_6$  at room temperature)  $\delta$  8.14 (t,  $J = 7.4$  Hz, 1H), 7.95 (dt,  $J = 41.8, 6.0$  Hz, 1H), 7.46 (dp,  $J = 40.0, 7.3$  Hz, 3H), 7.24–7.08 (m, 4H), 7.06–6.89 (m, 5H), 6.67 (d,  $J = 8.0$  Hz, 2H), 5.01–4.80 (m, 2H), 4.59 (dt,  $J = 24.2, 12.1$  Hz, 1H), 4.36 (d,  $J = 13.2$  Hz, 1H), 3.84 (dd,  $J = 36.5, 14.4$  Hz, 3H), 3.02–2.66 (m, 7H), 2.53 (d,  $J = 15.7$  Hz, 3H), 1.89–1.42 (m, 5H), 1.09–0.82 (m, 4H);  $^1\text{H}$  NMR (500 MHz, DMSO- $d_6$  at 100 °C)  $\delta$  7.73 (s, 1H), 7.62 (s, 1H), 7.51–7.40 (m, 1H), 7.23–6.92 (m, 9H), 6.69 (d,  $J = 8.1$  Hz, 2H), 5.03–4.88 (m, 2H), 4.61–4.51 (m, 1H), 3.73–3.46 (m, 3H), 2.90 (dt,  $J = 20.7, 6.8$  Hz, 4H), 2.77 (dd,  $J = 13.2, 6.8$  Hz, 3H), 2.67–2.43 (m, 3H), 2.05–1.82 (m, 4H), 1.77–1.63 (m, 2H), 0.90 (d,  $J = 7.4$  Hz, 3H);  $^{13}\text{C}$  NMR (126 MHz, DMSO- $d_6$ )  $\delta$  171.9, 169.8, 168.7, 168.5, 168.1, 162.2 (d,  $J = 258.4$  Hz), 155.6, 138.5, 138.4, 137.2, 136.6, 130.2, 130.0, 129.3, 129.2, 128.9, 128.2, 128.0, 127.8, 126.4, 126.3, 114.9, 98.7 (d,  $J = 12.0$  Hz), 53.7, 53.5, 51.5 (d,  $J = 15.5$  Hz), 49.5, 49.0, 44.4, 44.1, 41.78, 41.7, 41.0, 40.8, 38.1, 37.6, 30.1, 29.9 (d,  $J = 15.5$  Hz), 27.6, 9.4;  $^{13}\text{C}$  NMR (126 MHz, DMSO- $d_6$  at 100 °C)  $\delta$  171.7, 169.4, 168.4, 167.6, 162.4 (d,  $J = 259.3$  Hz), 155.3, 138.5, 136.5, 129.7, 129.5, 128.8, 128.6, 128.0, 127.6, 127.5, 125.9, 114.7, 98.3–97.8 (m), 53.2 (d,  $J = 28.0$  Hz), 51.8, 49.0, 41.8, 37.8, 29.6, 27.2, 8.8;  $^{19}\text{F}$  NMR (471 MHz, DMSO- $d_6$  at room temperature)  $\delta$  -118.1 (dd,  $J = 35.5, 18.7$  Hz, 1F);  $^{19}\text{F}$  NMR (471 MHz, DMSO- $d_6$  at 100 °C)  $\delta$  -117.1 (dd,  $J = 37.6, 15.1$  Hz, 1F); HRMS (ESI $^+$ ) mass calculated for  $[\text{M}+\text{H}]^+$  ( $\text{C}_{37}\text{H}_{45}\text{FN}_5\text{O}_5$ )  $m/z$  658.3399, found  $m/z$  658.3366; purity  $\geq 95\%$ , rt 2.98 min (method-B).

## Pharmacological Characterization

**Materials Used in the Cellular Signaling Assays.** Leu-Enk and forskolin, were purchased from Sigma-Aldrich (St. Louis, MO USA). [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-ol] enkephalin (DAMGO) was purchased from Tocris Bioscience (Minneapolis, MN, USA). Radiolabels were from Perkin Elmer (Waltham, MA, USA)

**Cell culture and biased signaling assays.** cAMP inhibition and  $\beta$ -Arr 2 recruitment assays were performed as previously described.<sup>5</sup> In brief, for cAMP inhibition assays HEK 293 (Life Technologies, Grand Island, NY, USA) cells were transiently transfected in a 1:3 ratio with FLAG-mouse  $\delta$ OR, or HA-mouse  $\mu$ OR and pGloSensor22F-cAMP plasmids (Promega, Madison, WI, USA) using Xtremegene9 (Sigma). Two days post-transfection cells (20,000 cells/well, 7.5  $\mu$ l) were seeded in low volume Greiner 384-well plates (#82051-458, VWR, Batavia, IL,

USA) and were incubated with GloSensor reagent (Promega, 7.5  $\mu$ l, 2% final concentration) for 90 min at room temperature. Cells were stimulated with 5  $\mu$ l drug solution for 20 min at room temperature prior to stimulation with 5  $\mu$ l forskolin (final concentration 30  $\mu$ M) for an additional 15 min at room temperature. For  $\beta$ -Arr recruitment assays, CHO-human  $\mu$ OR PathHunter  $\beta$ -Arr 2 cells or CHO-human  $\delta$ OR PathHunter  $\beta$ -Arr 2 cells (DiscoverX, Fremont, CA, USA) were plated (2,500 cells/well, 10  $\mu$ l) one day prior to stimulation with 2.5  $\mu$ l drug solution for 90 min at 37  $^{\circ}$ C/5% CO<sub>2</sub>, after which cells were incubated with 6  $\mu$ l cell PathHunter assay buffer (DiscoverX) for 60 min at room temperature as per the manufacturer's protocol. Luminescence for each of these assays was measured using a FlexStation3 plate reader (Molecular Devices, Sunnyvale, CA, USA).

**Radioligand Binding Assay.** For the binding assay 50  $\mu$ l of a dilution series of peptide was added to 50  $\mu$ l of 3.3 nM [<sup>3</sup>H]DPDPE ( $K_d$  = 3.87 nM) or 2.35 nM of [<sup>3</sup>H]DAMGO ( $K_d$  = 1.07 nM) in a clear 96 well plate. Next, 100  $\mu$ l of membrane suspension containing 7  $\mu$ g protein was added to the agonist wells and incubated for 90 min at room temperature. The reaction mixture was then filtered over a GF-B filter plate (Perkin Elmer) followed by 4 quick washes with ice-cold 50 mM Tris HCl. The plate was dried overnight, after which 50  $\mu$ l scintillation fluid (Ultimagold uLLT) was added and radioactivity was counted on a Packard TopCount NXT scintillation counter. All working solutions were prepared in a radioligand assay buffer containing 50 mM Tris HCl, 10 mM MgCl<sub>2</sub>, and 1 mM ethylenediaminetetraacetic acid at pH 7.4.

**Calculation of Bias Factor.** Bias factors were calculated using the operational model equation in Prism 8 to calculate Log R ( $\tau$ /KA) (Table S2) as previously described.<sup>5</sup> Subsequently, bias factors were calculated using Leu-Enk as reference compound for  $\delta$ OR and  $\mu$ OR. Leu-Enk is more potent in the cAMP (G protein) assay than in the  $\beta$ -Arr 2 recruitment assay, and thus is not unbiased, but rather G protein-biased to begin with. A bias factor >1 meant that the agonist was more G protein-biased than the reference compound; A bias factor <1 meant that the agonist was less G protein-biased than the reference compound.

**Data and Statistical analysis.** All data are presented as means  $\pm$  standard error of the mean, and analysis was performed using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA). For *in vitro* assays, nonlinear regression was conducted to determine pIC<sub>50</sub> (cAMP) or pEC<sub>50</sub> ( $\beta$ -Arr 2 recruitment). Technical replicates were used to ensure the reliability of single values, specifically each data point for binding and  $\beta$ -Arr recruitment was run in duplicate, and for the cAMP assay in triplicate. The averages of each independent run were counted as a single experiment and combined to provide a composite curve in favor of providing a 'representative' curve. In each experimental run, a positive control/reference compound was utilized to allow the data to be normalized and to calculate the log bias value.

## Solubility and Stability Studies

**Assessment of Stability.** Compounds **1e–h** were further characterized using standard physicochemical characterization and stability assays (Table SI-1) according to our previously published procedures.<sup>6</sup> Compounds

**1e–1h** were generally soluble in simulated gastric fluid (SGF) and PBS buffer (0.46–0.8 mM), though Pip-N(Ph)(COPh) analog **1h** displayed poor aqueous solubility (0.047–0.075 mM). This poor solubility of **1h** correlated with an increased logP value (2.09) relative to C-terminal amides **1e–f** (1.48–1.69) and C-terminal tetrazole **1g** (0.24). All analogs showed >1 mM solubility in 20% Captisol, and >0.78 mM in MeCN, MeOH, IPA. Overall, the analogs decomposed moderately in MeCN (ca. 30–50% decomposition at 48 h), though the compounds were generally stable in MeOH and IPA (ca. <5% decomposition at 48 h), which can be important for assay development. C-terminal NMe<sub>2</sub> and tetrazole analogs (**1e,g**) showed excellent stability in simulated gastric fluid, PBS buffer, and SGF, PBS 20% captisol solution, though NHCyPr analog **1f** decomposed slowly in PBS and 20% Captisol (ca. 15–20% decomposition over 24 h). In contrast, Pip-N(Ph)(COPh) analog **1h** readily decomposed in SGF and PBS (ca. 40–90% decomposition over 48 h), indicating potential poor stability under biologically relevant conditions. Finally, stability of all compounds to mouse and human plasmas was assessed to study the influence of C-terminal modifications on in vivo plasma stability, which is the predominant route *in vivo* of metabolism and clearance,<sup>7–9</sup> for the endogenous enkephalins. C-terminal substituted analogs **1e–1f** were completely stable in mouse plasma, while Pip-N(Ph)(COPh) analog **1h** slowly degraded (25% degradation over 2 h). In human plasma.

Compound	Solubility (mM) SGF / PBS	Solution Stability (%T <sup>0</sup> at 48 h) SGF / PBS / 20% Captisol	Plasma Stability (%T <sup>0</sup> at 2 h) Mouse / Human
<b>1a</b> (OH)	0.19 / 0.20	>99% / 91% / 97%	>99% / >99%
<b>1e</b> (NMe <sub>2</sub> )	0.60 / 0.45	95% / 92% / 93%	>99% / 88%
<b>1f</b> (NHCyPr)	0.61 / 0.45	>99% / 81% / 84%	>99% / >99%
<b>1g</b> (Tetrazole)	0.80 / 0.71	>99% / >99% / 93%	>99% / >99%
<b>1h</b> [Pip-N(Ph)(COPr)]	0.075 / 0.047	59% / 11% / 100%	75% / >99%

**Table SI-1.** Experimental Solubility and Stability of Select Analogs.

The stabilities of analogs were monitored in mouse and human plasma (Innovative Research) at 37 °C.

Compounds were spiked into plasma to obtain a concentration of 50 μM. At predetermined time points, samples were taken and quenched with acetonitrile, and the compounds were analyzed by LC-MS/MS. Samples were prepared in duplicate.

## Computational Methods

Agonist-bound structures were downloaded from the PDB: delta opioid receptor (DOR), PDB ID 6PT2,<sup>10</sup> and mu opioid receptor (MOR), PDB ID 5C1M.<sup>11</sup> Structures were prepared using the Protein Preparation Wizard,<sup>12,13</sup> which

added hydrogens, generated preferred heteroatom states, optimized hydrogen bonding (including assigning protein protonation states), and minimizing into the OSPL3e scoring function. Waters not involved with the hydrogen bonding network involving the N-terminal (modified) tyrosine side chain were removed.

Compounds were prepared for docking using LigPrep.<sup>13</sup> Although they generated the cationic as well as the uncharged states, initial docking results strongly disfavored the uncharged state, so those were subsequently left out of the modeling studies.

Small molecules were initially docked into the DOR structures using Glide<sup>13-15</sup> with the following protocol: extended (XP) precision, a core constraint of the tyrosine side chain with the C-terminal nitrogen (which is the tyraminium portion of the agonist), rewarding intramolecular hydrogen bonds, and a maximum of 10 poses per compound. This protocol essentially samples potential interactions while maintaining the well-established, conserved interactions.

Enhanced sampling protocols included the above XP precision protocol with the following settings: extended (XP) precision, a core constraint of the entire pose of interest, rewarding intramolecular hydrogen bonds, enhanced sampling, 50,000 poses per ligand in the first phase of docking, 1,000 scoring window for keeping initial poses, keeping the 1,000 best poses for in-docking energy minimizations, including aromatic H as donors, including 20 poses for post-docking minimization, and a maximum of 10 poses per compound. Additionally, the SP-peptide precision was used (standard precision scoring with enhanced sampling for peptides).

Once a particular pose was selected a biased docking approach was also used, which used the enhanced sampling protocol, where constrained docking was performed with a core constraint of the entire pose of interest.

Refinements used Prime MMGBSA,<sup>13,16,17</sup> allowing for flexibility of any residue within 8 Å of the ligand.

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