

Supporting Information:

Discovery of Axelopran (TD-1211): A Peripherally-Restricted μ -Opioid Receptor Antagonist.

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Chemistry Schemes

Reagents (including secondary amines) and solvents were purchased from commercial suppliers (Aldrich, Fluka, Sigma, etc.), and used without further purification. Reactions were run under nitrogen atmosphere, unless noted otherwise. Progress of reaction mixtures was monitored by thin layer chromatography (TLC), analytical high performance liquid chromatography (anal. HPLC), and mass spectrometry, the details of which are given below and separately in specific examples of reactions. Reaction mixtures were worked up as described specifically in each reaction; commonly they were purified by extraction and other purification methods such as temperature-, and solvent-dependent crystallization, and precipitation. In addition, reaction mixtures were routinely purified by preparative HPLC: a general protocol is described below. Characterization of reaction products was routinely carried out by mass and $^1\text{H-NMR}$ spectrometry. For NMR measurement, samples were dissolved in deuterated solvent (CD_3OD , CDCl_3 , or $d_6\text{-DMSO}$), and $^1\text{H-NMR}$ spectra were acquired with a Varian Gemini 2000 instrument (300 MHz) under standard observation conditions. Mass spectrometric identification of compounds was performed by an electrospray ionization method (ESMS) with an Applied Biosystems (Foster City, CA) model API 150 EX instrument or an Agilent (Palo Alto, CA) model 1100 LC/MSD instrument.

Scheme 1:

1. Cyclohexylmethyl-(2-oxo-ethyl)carbamic acid *tert*-butyl ester 12a (and by analogous method 12b-f; 13-15)

a. 2-(Cyclohexylmethylamino)ethanol 9

A mixture of cyclohexylmethylbromide (23.2 g, 131 mmol) and ethanolamine **8** (47.9 g, 786 mmol) in EtOH (131 mL) was heated at 75 °C for 2 hours. NMR analysis of an aliquot showed the reaction was complete. The reaction was then concentrated to remove ethanol and the resulting residue was diluted with DCM. The organic layer was washed successively with water (3 x 100 mL) and brine (100 mL) and then dried over sodium sulfate, filtered, and concentrated to afford **9** as a light yellowish oil (10.57 g). (m/z): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_9\text{H}_{19}\text{NO}$ 158.16; found 158.2. $^1\text{H NMR}$ (CDCl_3 , 300 MHz) δ (ppm): 3.59 (t, $J=5.4$ Hz, 2H), 2.73 (t, $J=5.4$ Hz, 2H), 2.42 (d, $J=6.6$ Hz, 2H), 1.6-1.77 (m, 6H), 1.36-1.6 (m, 1H), 1.10-1.28 (m, 2H), 0.82-0.95 (m, 2H).

b. Cyclohexylmethyl-(2-hydroxyethyl) carbamic acid *tert*-butyl ester 10

To the solution of the product of the previous step (10.57 g, 67.3 mmol) in DCM (200 mL) at 0 °C was added a solution of di-*tert*-butyldicarbonate (13.2 g, 60.57 mmol) in DCM (100 mL) dropwise. The resulting mixture was allowed to slowly warm to room temperature overnight. The mixture was washed successively with 1N aq HCl (3 x 100 mL), saturated sodium bicarbonate (100 mL) and brine (100 mL). After drying over sodium sulfate, the organic layer was filtered and concentrated to afford **10** as a light yellowish oil (16.5 g). $^1\text{H NMR}$ (CDCl_3 , 300MHz) δ (ppm): 3.71-3.73 (m, 2H), 3.37 (brs, 2H), 3.03-3.05 (m, 2H), 1.61-1.72 (m, 6H), 1.3-1.5 (m, 1H), 1.48 (s, 9H), 1.14-1.21(m, 2H), 0.86-0.91 (m, 2H).

c. Cyclohexylmethyl-(2-oxo-ethyl)carbamic acid *tert*-butyl ester 12a

To the solution of the product of the previous step (16.5 g, 64.2 mmol) in DCM (256 mL) at 0 °C was added sequentially dimethylsulfoxide (7.52 g, 96.3 mmol), *N,N*-diisopropylethylamine (20.74 g, 160.5

mmol) and pyridine-sulfur trioxide complex (25.5 g, 160.5 mmol). Thirty minutes later, NMR analysis of an aliquot showed the reaction was complete. The mixture was then washed successively with 1N aq HCl (3 x 100 mL), saturated sodium bicarbonate and brine, filtered through a pad of silica gel and eluted with DCM. After concentration, **12a** was obtained as a light yellowish oil (10.46 g). ¹H NMR (CDCl₃, 300MHz) δ (ppm): 9.55 (s, 1H), 3.88 (s, 1H), 3.79 (s, 1H), 3.07-3.15 (m, 2H), 1.56-1.72 (m, 6H), 1.3-1.5 (m, 1H), 1.4 (s, 9H), 1.1-1.25 (m, 2H), 0.87-0.98 (m, 2H).

Compounds **12b** (R₁= phenethyl-), **12c** (R₁=phenpropyl-), **12e** (R₁=cyclobutylmethyl-), **12f** (R₁=n-hexyl) were prepared by the same general sequence substituting cyclohexylmethylbromide in step 1a for the appropriate alkyl bromide; or in the case of **12d** (R₁=cyclopentylmethyl-) cyclopentylmethylmesylate was used in step 1a. **13-15** were prepared by the same general sequence substituting cyclohexylmethylbromide for 2-ethyl-n-butylbromide and substituting ethanolamine for 3-hydroxypropylamine, 4-hydroxybutylamine and 5-hydroxypentylamine, respectively, in step 1a.

2. Acetic acid [benzyl-(2-oxo-ethyl)carbamoyl]-methyl ester **16a** (and by analogous method **16b**)

a. Acetic acid [benzyl-(2-hydroxyethyl)carbamoyl]methyl ester **11a**

Benzylethanolamine **9** (1.78 g, 11.8 mmol) was weighed into a 25 mL round bottom flask and diluted with dichloromethane. *N,N*-Diisopropylethylamine (2.66 mL, 15.3 mmol) was added quickly via syringe and the reaction was cooled to 0 °C. After stirring at 0 °C for 10 minutes, acetoxyacetyl chloride (1.26 mL, 11.8 mmol) was added dropwise via syringe. The reaction was stirred overnight and allowed to warm to room temperature. The reaction mixture was diluted with ethyl acetate and washed with saturated aqueous sodium bicarbonate and saturated aqueous sodium chloride. The organic layer was separated and dried over anhydrous magnesium sulfate. The drying agent was removed via filtration and solvent was removed in vacuo to give crude title intermediate as a yellow oil. The crude material was chromatographed on silica gel using ethyl acetate as the mobile phase. Fractions were combined and solvent was removed in vacuo to give pure title intermediate as a clear oil (1.75 g, 59% yield). (*m/z*): [M+H]⁺ calcd for C₁₃H₁₇NO₄ 252.13, found 252.3.

Compound **11b** (R₁= 4,4-diF-*c*-hexylmethyl-), was prepared by the same procedure substituting benzylethanolamine for 4,4-diF-*c*-hexylmethylethanolamine (itself prepared from 4,4-diF-*c*-hexylmethyl mesylate according to step 1a above).

b. Acetic acid [benzyl-(2-oxo-ethyl)carbamoyl]-methyl ester **16a** (and by analogous method **16b**)

Compounds **16a-b** were prepared by the same procedure as step 1c substituting **10** for **11a-b**.

Scheme 2:

3-*endo*-(8-{2-[Cyclohexylmethyl-(2-hydroxyacetyl)-amino]-ethyl}-8-azabicyclo[3.2.1]oct-3-yl)benzamide **19h** (and by analogous method **19a-g,i-l**)

a. 3-*endo*-{8-[2-(Cyclohexylmethylamino)ethyl]-8-azabicyclo[3.2.1]oct-3-yl}benzamide **18**

To a stirred solution of 3-*endo*-(8-azabicyclo[3.2.1]oct-3-yl)benzamide **17** (1.18 g, 5.1 mmol), in DCM (20 mL) at 0 °C was added a solution of cyclohexylmethyl-(2-oxo-ethyl)-carbamic acid *tert*-butyl ester

12a (1.57 g, 6.1 mmol) in DCM (5 mL) followed by sodium triacetoxyborohydride (1.29 g, 6.0 mmol). The resulting mixture was warmed to room temperature after addition and stirred at that temperature for 30 minutes until reaction was determined by mass spectrometry to be complete. The mixture was then diluted with DCM, washed twice with saturated sodium bicarbonate, followed by brine, dried over sodium sulfate, filtered and concentrated to give a yellowish oil, which was used in the next step without further purification. (m/z): $[M+H]^+$ calcd for $C_{28}H_{43}N_3O_3$ 470.34; found 470.6. The oily residue of the previous step was dissolved in DCM (12 mL) and treated with TFA (12 mL) at room temperature for about 40 minutes. The reaction was judged complete by mass spectrometry. The mixture was then concentrated, dissolved in 50 % acetic acid in water (15.0 mL) and purified by reverse phase preparative HPLC. Desired fractions were combined and freeze dried to give the bis TFA salt of **18** (1.65 g). 1H NMR (CD_3OD , 300 MHz) δ (ppm): 8.03 (s, 1H), 7.71-7.77 (m, 2H), 7.44-7.50 (m, 1H), 4.11 (brs, 2H), 3.52 (t, $J=6.0$ Hz, 2H), 3.33-3.40 (obscure, 3H, overlap with solvent), 2.96 (d, $J=6.6$ Hz, 2H), 2.67-2.67 (m, 4H), 2.05-2.12 (m, 2H), 1.70-1.84 (m, 8H), 1.20-1.39 (m, 3H), 1.03-1.10 (m, 2H). (m/z): $[M+H]^+$ calcd for $C_{23}H_{35}N_3O$ 370.28; found 370.2.

b. 3-endo-(8-{2-[Cyclohexylmethyl-(2-hydroxyacetyl)-amino]-ethyl}-8-azabicyclo[3.2.1]oct-3-yl)benzamide **19h**

To a solution of the bis TFA salt of 3-endo-{8-[2-(cyclohexylmethylamino)ethyl]-8-aza-bicyclo[3.2.1]oct-3-yl}benzamide **18** (734 mg, 1.23 mmol) in dichloromethane (5 mL) at room temperature was added *N,N*-diisopropylethyl amine (635 mg, 4.9 mmol). The resulting mixture was cooled to $-20^\circ C$ before a solution of acetoxyacetyl chloride (184 mg, 1.35 mmol) in DCM (2 mL) was added. Five minutes later, the reaction went to completion as confirmed by mass spectrometric analysis. The reaction mixture was concentrated, dissolved in EtOH (15 mL) and treated with lithium hydroxide monohydrate (309 mg) in water (5 mL) for about 30 minutes. The reaction mixture was then concentrated, dissolved in 50% acetic acid in water (15 mL), filtered and purified by reversed phase preparative HPLC to give the TFA salt of **19h** (585.9 mg). (m/z): $[M+H]^+$ calcd for $C_{25}H_{37}N_3O_3$, 428.29; found 428.2. 1H NMR (CD_3OD , 300 MHz) δ (ppm): 8.0 (s, 1H), 7.68-7.74 (m, 2H), 7.42-7.47 (t, $J=8.1$ Hz, 1H), 4.27 (s, 2H), 4.09 (brs, 2H), 3.72 (t, $J=5.7$ Hz, 2H), 3.2-3.35 (m, obscure 1H), 3.09-3.14 (m, 4H), 2.59-2.62 (m, 4H), 2.07-2.12 (m, 2H), 1.62-1.83 (m, 8H), 1.15-1.35 (m, 3H), 0.87-1.16 (m, 2H).

Compounds **19a** ($R_2=Ac^-$), **19b** ($R_2=^tBuC(O)^-$), **19c** ($R_2=CF_3C(O)^-$), **19d** ($R_2=MeOC(O)^-$), **19f** ($R_2=MeO_2S^-$), **19g** ($R_2=Me_2NO_2S^-$), **19i** ($R_2=2-furylC(O)^-$) were prepared by the same procedure (without the hydrolysis step) substituting acetoxyacetyl chloride for the appropriate R_2Cl .

c. 3-endo-(8-[2-(1-Cyclohexylmethyl-3-isopropylureido)ethyl]-8-aza-bicyclo[3.2.1]oct-3-yl)benzamide **19e**

To a solution of the bis TFA salt of 3-endo-{8-[2-(cyclohexylmethylamino)ethyl]-8-aza-bicyclo[3.2.1]oct-3-yl}benzamide **18** (30 mg, 0.05 mmol) in DMF (0.4 mL) at room temperature was added *N,N*-diisopropylethylamine (27 mg, 0.20 mmol) followed by isopropylisocyanate (0.075 mmol). The resulting mixture was shaken at room temperature overnight, concentrated, dissolved in 50% acetic acid in water (1.5 mL), filtered, and purified by preparative HPLC to provide the TFA salt of **19e** (24.5 mg) (m/z): $[M+H]^+$ calcd for $C_{27}H_{42}N_4O_2$, 455.33; found 455.4; 1H NMR (CD_3OD , 300 MHz) δ (ppm) 7.97 (s, 1H), 7.71 (d, $J=7.8$ Hz, 1H), 7.66 (d, $J=8.1$ Hz, 1H), 7.42 (dd, $J=7.8$ Hz, 1H), 3.90-3.99 (m, 3H), 3.60 (t, $J=4.8$ Hz,

2H), 3.25-3.29 (m, 1H, overlap with solvent), 3.02-3.09 (m, 4H), 2.55-2.58 (m, 4H), 2.03-2.07 (m, 2H), 1.63-1.78 (m, 8H), 1.07-1.25 (m, 9H), 0.92-1.01 (m, 2H).

d. 3-endo-(8-{2-[Cyclohexylmethyl-((S)-2,3-dihydroxy-propionyl)amino]ethyl}-8-aza-bicyclo[3.2.1]oct-3-yl)benzamide **19i**

To a solution of the bis TFA salt of 3-endo-{8-[2-(cyclohexylmethylamino)ethyl]-8-aza-bicyclo[3.2.1]oct-3-yl}benzamide **18** (400 mg, 0.67 mmol) in DMF (4 mL) at room temperature was added HATU (757 mg, 1.99 mmol). After stirring for 2 h, a solution of (S)-2,2-dimethyl-[1,3]dioxolane-4-carboxylic acid (289 mg, 1.99 mmol) and *N,N*-diisopropylethylamine (0.47 mL, 2.68 mmol) in DMF (4 mL) was added. The resulting mixture was stirred at 45°C overnight and concentrated. The resulting residue was dissolved in 75% acetic acid in water (16 mL) and stirred at 70°C for 1 h, then concentrated and purified by preparative HPLC to provide the TFA salt of **19i** (254 mg). (*m/z*): [M+H]⁺ calcd for C₂₆H₃₉N₃O₂, 458.30; found 458.2. ¹H NMR (CD₃OD, 300M Hz) δ (ppm): 7.98 (s, 1H), 7.67-7.73 (m, 2H), 7.40-7.45 (m, 1H), 4.57 (t, J=5.4 Hz, 1H), 3.94-4.12 (m, 3H), 3.69-3.72 (m, 2H), 3.50-3.58 (m, 1H), 3.35-3.43 (m, 1H), 3.20-3.27 (obscure 2H, partially overlap with solvent), 3.12-3.15 (m, 2H), 2.52 (brs, 4H), 1.98-2.02 (m, 2H), 1.61-1.70 (m, 8H), 1.09-1.22 (m, 3H), 0.88-0.95 (m, 2H).

Compounds **19j** (R₂= MeO₂SCH₂C(O)-), **19k** (R₂=2-furylC(O)-), were prepared by the same procedure (without the acetal removal step) substituting (S)-2,2-dimethyl-[1,3]dioxolane-4-carboxylic acid for the appropriate R₂OH.

Scheme 3:

Compounds **24b-g**, **25**, **26**, **27** were prepared by the same general sequence as Scheme 2, steps 1a and 1b substituting aldehyde **12a** in step 1a for the appropriate aldehyde **12b-f**, **13**, **14**, **15**.

3-endo-(8-{2-[(4,4-Difluorocyclohexylmethyl)-(2-hydroxyacetyl)amino]ethyl}-8-azabicyclo[3.2.1]oct-3-yl)benzamide **24g (and by analogous method **24a**)**

To a solution of 3-endo-(8-azabicyclo[3.2.1]oct-3-yl)-benzamide **17** (2.15 g, 9.34 mmol) in DCM (45.0 mL) at 0 °C was added acetic acid (0.56 g, 9.34 mmol) followed by a solution of acetic acid [(4,4-difluorocyclohexylmethyl)-(2-oxo-ethyl)-carbamoyl]-methyl ester **16b** (2.59 g, 8.9 mmol) in DCM (10.0 mL) and sodium triacetoxyborohydride (2.26 g, 10.7 mmol). The resulting mixture was stirred at 0°C for 30 min and then diluted with DCM (40.0 mL). The organic layer was washed sequentially with saturated sodium bicarbonate (20.0 mL) and brine (20.0 mL), dried over sodium sulfate, filtered and concentrated to give **20g** as a light yellowish foam, which was used in the next step without further purification. The product from the previous step was dissolved in methanol (20.0 mL) at ambient temperature and treated with lithium hydroxide monohydrate (0.56 g, 13.4 mmol) in water (5.0 mL) for 30 min. The reaction mixture was concentrated. The residue was dissolved in 25 % acetic acid in water (48.0 mL), filtered and purified by reverse phase preparative HPLC. Desired fractions were combined and freeze dried to give **24g** as its TFA salt (2.1 g). ¹H NMR (CD₃OD, 300 MHz) δ (ppm) 8.07 (s, 1H), 7.75-7.81 (m, 2H), 7.48 (dd, J=7.8 Hz, 1H), 4.35 (s, 2H), 4.17 (brs, 2H), 3.81 (t, J=6.0 Hz, 2H), 3.35-3.38 (obscure, 1H, overlap with solvent), 3.25 (d, J=6.9 Hz, 2H), 3.20 (t, J=5.4 Hz, 2H), 2.66-2.72 (m, 4H), 2.10-2.19 (m, 4H), 1.77-1.90 (m, 7H), 1.33-1.41 (m, 2H).

Compound **24a** was prepared by the same general procedure substituting aldehyde **16b** for aldehyde **16a**.

Scheme 4:

3-endo-(8-{2-[Tetrahydropyranmethyl-(2-hydroxyacetyl)amino]ethyl}-8-azabicyclo[3.2.1]oct-3-yl)-benzamide 29

a. 3-endo-[8-(2-Oxo-ethyl)-8-azabicyclo[3.2.1]oct-3-yl]benzamide 28

To a solution of the TFA salt of 3-endo-(8-azabicyclo[3.2.1]oct-3-yl)-benzamide **17** (1.03 g, 2.98 mmol) in dichloromethane (15.0 mL) at room temperature was added N,N-diisopropylethylamine (385 mg, 2.98 mmol) followed by 2,2-dimethoxy-acetaldehyde (621 mg, 5.96 mmol) and sodium triacetoxyborohydride (821 mg, 3.87 mmol). The resulting mixture was sonicated to aid dissolution. After about 30 minutes, the reaction was concentrated. The resulting residue was co-evaporated with methanol twice and then dissolved in 50% acetic acid in water (10 mL), filtered and purified by reverse phase preparative HPLC to give the intermediate as its TFA salt (577 mg). (*m/z*): [M+H]⁺ calcd for C₁₈H₂₆N₂O₃ 319.20; found 319.3. The product of the previous step (577 mg) was treated with 6N HCl (20 mL) at room temperature overnight. The reaction mixture was concentrated and the residue was diluted with water and freeze dried to give **28** as its HCl salt (554 mg). (*m/z*): [M+H]⁺ calcd for C₁₈H₂₆N₂O₂ 273.16; found 273.1.

b. 3-endo-(8-{2-[Tetrahydropyranmethyl-(2-hydroxyacetyl)amino]ethyl}-8-azabicyclo[3.2.1]oct-3-yl)-benzamide 29

To a solution of the HCl salt **28** (200 mg, 0.65 mmol) in dichloromethane (7.0 mL) at 0 °C was added sodium triacetoxyborohydride (165 mg, 0.78 mmol) followed by 4-aminomethyl-tetrahydropyran (90 mg, 7.8 mmol). The reaction mixture was concentrated after 5 mins. The residue was redissolved in 50% acetic acid in water (10 mL), filtered, and purified by reverse phase preparative HPLC to give the intermediate compound as its bis TFA salt (75 mg). (*m/z*): [M+H]⁺ calcd for C₂₂H₃₃N₃O₂ 372.27; found 372.5. To a solution of the product of the previous step (75 mg, 0.20 mmol) in DCM (1.0 mL) was added DIPEA (0.07 mL, 0.4 mmol) followed by acetoxyacetyl chloride (0.03 mL, 0.26 mmol) at room temperature. The reaction mixture was stirred for 10 min, and then concentrated. The resulting residue was redissolved in ethanol (1.0 mL) and treated with a solution of lithium hydroxide monohydrate (50.0 mg, 1.2 mmol) in water (0.5 mL) for 1 h and reconcentrated. The residue was dissolved in 50 % acetic acid in water (1.5 mL), filtered and purified by reverse phase preparative HPLC to give **29** as its TFA salt. (*m/z*): [M+H]⁺ calcd for C₂₄H₃₅N₃O₄ 430.27; found 430.2.

Scheme 5:

3-endo-(8-{3-[(2-Ethylbutyl)-(2-hydroxyacetyl)-amino]ethyl}-8-aza-bicyclo[3.2.1]oct-3-yl)-benzamide 32 (and by analogous method 33)

a. {2-[3-endo-(3-Carbamoylphenyl)-8-azabicyclo[3.2.1]oct-8-yl]-ethyl}-carbamic acid benzyl ester 30

A suspension of 3-endo-(8-azabicyclo[3.2.1]oct-3-yl)-benzamide **17** (1.3 g, 5.7 mmol) and (2-oxoethyl)-carbamic acid benzyl ester (0.99 g, 6.2 mmol) in dichloromethane (20 mL) was sonicated for 5 min. To the stirred suspension was added sodium triacetoxyborohydride (1.3 g, 6.1 mmol). After stirring for 30

min, the reaction mixture was concentrated, diluted with ethyl acetate (50 mL) and washed with 1.0 N NaOH (50 mL) and water (50 mL). The organics were separated, dried with anhydrous sodium sulfate, filtered, and concentrated. The crude material was purified by silica gel chromatography, eluting with methanol in dichloromethane (0% to 30% gradient) to provide **30** (1.4 g, 57%). (*m/z*): [M+H]⁺ calcd for C₂₄H₂₉N₃O₃, 408.22; found 408.5.

Compound **31** was prepared by the same general procedure substituting (2-oxoethyl)-carbamic acid benzyl ester for (2-oxopropyl)-carbamic acid benzyl ester.

b. 3-endo-[8-(2-aminoethyl)-8-azabicyclo[3.2.1]oct-3-yl]-benzamide 32

A solution of **30** (1.4 g, 3.4 mmol) in methanol (20 mL) was added to palladium hydroxide on carbon (50 wt % water, 20 % Pd on dry base, 140 mg). The reaction mixture was stirred under an atmosphere of hydrogen overnight. The solution was filtered through Celite and concentrated to give an oil (1.0 g), which was used without further purification. (*m/z*): [M+H]⁺ calcd for C₁₆H₂₃N₃O, 274.19; found 274.5. To a solution of the product of the previous step and 2-ethylbutyraldehyde (37 mg, 0.37 mmol) in dichloromethane (20 mL) was added sodium triacetoxyborohydride (140 mg, 0.66 mmol). After stirring for 2 h, the reaction mixture was diluted with dichloromethane (20 mL) and washed with 0.5 N NaOH (30 mL). The organics were separated, dried with anhydrous sodium sulfate, filtered, and concentrated to give crude solid, which was used without further purification. (*m/z*): [M+H]⁺ calcd for C₂₂H₃₅N₃O, 358.28; found 358.3. The general procedure below was applied to the crude product to give **32** as its TFA salt. (*m/z*): [M+H]⁺ calcd for C₂₄H₃₇N₃O₃, 416.28; found 416.4.

To a solution of 3-endo-[8-[3-(2-ethylbutylamino)propyl]-8-aza-bicyclo[3.2.1]oct-3-yl]-benzamide (400 mg, 1.1 mmol) (derived from **31**) in dichloromethane (10 mL) was added acetoxyacetyl chloride (0.127 mL, 1.18 mmol). After 30 min, the reaction mixture was concentrated and the resulting crude oil was stirred in methanol (10 mL) and 6.0 N NaOH (0.36 mL) for 30 min. The reaction mixture was concentrated and purified by preparative HPLC to give **33** (430 mg) as its TFA salt. (*m/z*): [M+H]⁺ calcd for C₂₅H₃₉N₃O₃, 430.30; found 430.4. ¹H NMR (*d*₆-DMSO, 400MHz) δ (ppm): 9.3-9.2 (br, 1H), 8.1-8.0 (m, 2H), 7.8-7.7 (m, 2H), 7.5-7.4 (m, 2H), 4.2-4.1 (m, 2H), 4.0 (s, 2H), 3.4-3.1 (m, 4H), 3.1 (d, J=7.6 Hz, 1H), 3.0-2.8 (br, 2H), 2.6 (m, 2H), 2.0-1.8 (br, 4H), 1.5 (m, 3H), 1.2 (m, 4H), 0.9-0.8 (m, 6H).

Scheme 6:

3-endo-(8-Azabicyclo[3.2.1]oct-3-yl)benzamide hydrochloride 17

a. Trifluoro-methanesulfonic acid 8-benzyl-8-azabicyclo[3.2.1]oct-2-en-3-yl ester 35

To a 500 mL flask was added 8-benzyl-8-azabicyclo[3.2.1]octan-3-one hydrochloride **34** (50.4 g, 200 mmol), EtOAc (160 mL), and 4 N NaOH (50 mL). The reaction mixture was heated to 30 °C and stirred at that temperature for 1 h. The layers were separated and the aqueous layer was discarded. The volume of the organic layer was reduced to ~40 mL by rotary evaporation and THF (270 mL) was added. The resulting solution was added to a 1 L flask and cooled to -20 °C. A NaHMDS solution (1 M in THF, 230 mL, 230 mmol) was added to the flask over 15 min. The reaction mixture was stirred at -20 ± 5 °C for 1 h. *N*-phenyl-bis(trifluoromethanesulfonimide) (82.2 g, 230 mmol) was added to the reaction mixture in portions over 5 min and the mixture was stirred at -20 °C to -10 °C for 1 h. To the reaction mixture was added 1 N NaOH (200 mL) and the mixture was allowed to warm to 22 °C with stirring. Solvent was

partially removed by rotary evaporation at 30 °C to a volume of 450 mL. To the remaining reaction mixture was added EtOAc (300 mL) and heptane (150 mL). The mixture was stirred at 22 °C for 5 min. The layers were separated and the aqueous layer was discarded. The organic layer was washed with 1N NaOH (3 x 450 mL). The aqueous layers were discarded. The organic layer was concentrated by rotary evaporation to provide **35** (77 g, >96 % purity). ¹H NMR (*d*₆-DMSO, 400 MHz): δ (ppm) 7.25-7.35 (m, 5H), 6.05 (d, *J*=5.2 Hz, 1H), 3.64 (q, *J*=13.2 Hz, 2H), 3.40-3.44 (m, 2H), 2.77 (d, *J*=16.4 Hz, 1H), 1.79-2.09 (m, 5H), 1.52-1.59 (m, 1H).

b. 3-(8-Benzyl-8-azabicyclo[3.2.1]oct-2-en-3-yl)benzamide **36**

To crude **35** (77g) was added THF (420 mL) and the solution was purged with nitrogen for 5 min. To a 2 L flask was added 3-carbamoylphenyl boronic acid (98%, 33.0 g, 200 mmol), palladium (II) acetate (98%, 0.46 g, 2 mmol), 1,1'-bis(diphenylphosphino)ferrocene (97%, 1.1 g, 2 mmol), and potassium fluoride (34.9 g, 600 mmol) followed by the THF solution of trifluoro-methanesulfonic acid 8-benzyl-8-azabicyclo[3.2.1]oct-2-en-3-yl ester. The resulting mixture was purged with nitrogen for 5 min, heated to reflux (67 °C) under nitrogen and stirred for 2 h. The reaction mixture was cooled to 30 °C, then EtOAc (500 mL) and 1 N NaOH (500 mL) were added and the mixture was stirred at 22 °C for 10 min. The layers were separated and the aqueous layer was discarded. The organic layer was washed with a mixture of brine (250 mL) and water (250 mL) and stirred for 5 min. The layers were separated and the aqueous layer was discarded. The organic layer was briefly dried over Na₂SO₄, filtered, and solvent was partially removed. Product precipitated as light yellow solids during solvent removal. The resulting slurry (about 200 mL) was filtered and the solids were washed with cold EtOAc (0 °C, 100 mL) and dried under high vacuum at 25 °C to provide **36** (42.5 g) as a light yellow solid. The mother liquor and the above washes were combined and concentrated and the resulting slurry (about 100 mL) was stirred at 5 °C for 30 min and filtered. The filtered solids were washed with cold EtOAc (0 °C, 30 mL) and dried under high vacuum to provide a second crop of **36** (7 g, combined yield 78 %, > 98.5 % pure). (*m/z*): [M+H]⁺ calcd for C₂₁H₂₂N₂O, 319.18; found 319.4. ¹H NMR (CDCl₃, 400 MHz): δ (ppm) 7.9 (s, 1H), 7.63 (d, *J*=6.4 Hz, 1H), 7.57 (d, *J*=6.4 Hz, 1H), 7.21-7.42 (m, 6H), 6.38 (d, *J*=4.4 Hz, 1H), 6.13 (s, br, 1H), 5.83 (s, br, 1H), 3.68-3.76 (m, 2H), 3.46-3.51 (m, 2H), 2.92 (d, *J*=17.2 Hz, 1H), 2.18-2.26 (m, 1H), 2.04-2.12 (m, 2H), 1.86-1.92 (m, 1H), 1.58-1.65 (m, 1H).

c. 3-endo-(8-Azabicyclo[3.2.1]oct-3-yl)benzamide hydrochloride **17**

To a 1 L hydrogenation vessel was added **36** (40 g, 125 mmol), EtOH (800 mL), 6 M HCl (42 mL) and water (80 mL) and the mixture was stirred at 22 °C until complete dissolution was observed. The reaction mixture was purged with nitrogen for 5 min while being heated to 30 °C over 5 min. To the mixture was added 10 wt % Pd/C (50 % in water, 4 g). The mixture was purged at atmospheric pressure with hydrogen for 5-10 min while being heated. The mixture was stirred at 50 °C under a flow of hydrogen at < 5 psi (<0.34 atmospheres) for 5 h, resulting in >99 % conversion of the reactants, according to HPLC analysis. The solution was cooled to 30 °C and filtered through Celite to provide a solution of crude **17** with an *endo:exo* ratio of ~93:7 by HPLC method 2 *endo* Rt=10.97, *exo* Rt=12.67. Water was removed from the crude product by azeotropic distillation at 30 °C in EtOH (~80 mL) to provide a slurry that was heated to 60 °C until complete dissolution. The solution was cooled to 35 °C and seed crystals of the product (0.05 g) were added. The resulting slurry was stirred at 22 °C for 30 min, MTBE (120 mL) was added slowly, and the slurry was stirred at 22 °C for 4 h and then at 0 °C for 1 h. The resulting solids were filtered, washed with cold EtOH and dried under high vacuum to provide the title compound **17** (24.5 g) as a white powder (75 % yield, >98.5 % purity < 0.4 % *exo* isomer). (*m/z*): [M+H]⁺

calcd for C₁₄H₁₈N₂O 231.15; found 231.2. ¹H NMR (*d*₆-DMSO, 400 MHz): δ (ppm) 9.13 (s, br, 1H), 9.03 (s, br, 1H), 8.05 (s, 1H), 7.93 (s, 1H), 7.73 (d, *J*=7.6 Hz, 1H), 7.58 (d, *J*=7.6 Hz, 1H), 7.40 (t, *J*=7.6 Hz, 2H), 3.97 (s, 2H), 3.17-3.23 (m, 1H), 2.39-2.46 (m, 2H), 2.19-2.24 (m, 2H), 1.86-1.89 (m, 2H), 1.59-1.63 (m, 2H).

Scheme 7:

N*-cyclohexylmethyl-(2-oxoethyl)-carbamic acid benzyl ester bisulfite adduct **39*

a. *N*-Cyclohexylmethyl-(2,2-diethoxyethyl)carbamic acid benzyl ester **38**

To a mixture of 2,2-diethoxyethylamine (209 mL, 1.43 mol) and MeTHF (1050 L) was added cyclohexanecarbaldehyde **37** (107 mL, 0.89 mol). The reaction mixture was stirred for 30 min at room temperature and cooled to 0 °C. Sodium triacetoxyborohydride (378 g, 1.79 mol) was added over 40 min and the reaction mixture was stirred for 2 h and cooled to 0 °C. 1 M NaOH (1 L) was added. The organic layer was washed with brine in water (1:1, 2 x 1 L) and the volume was reduced to ~ 20 %. MeTHF (1 L) was added and the volume reduced to ~ 20 %. To this solution was added MeTHF (2 L) and DIPEA (233 mL, 1.34 mol). The reaction mixture was cooled to 0 °C and benzylchloroformate (140 mL, 0.98 mol) was added dropwise. The reaction mixture was stirred for 30 min at 0 °C, for 2 h at 0 °C to room temperature, and then for 1 h at room temperature. Water (1.6 L) was added and the reaction mixture was stirred for 10 min. The phases were separated and the organic layer was washed with sodium bicarbonate (1.6 L) and water (1.6 L). The layers were separated and the organic layer was reduced to about 20 %. MeTHF (1 L) was added and the volume reduced to ~ 20 %. The solution of the crude intermediate **38** was used directly in the next step.

b. *N*-Cyclohexylmethyl-(2-oxoethyl)-carbamic acid benzyl ester bisulfite adduct **39**

To the solution of the crude intermediate **38** was added acetonitrile (2 L) and 1 M HCl (2 L) and the reaction mixture was stirred at 30 °C for 7 h. Ethyl acetate (2 L) was added and the reaction mixture was stirred for 10 min. The phases were separated, the organic layer was washed with 1 M HCl (1.5 L), the phases were again separated and the organic layer was washed with 0.5 M HCl (1 L). Sodium bisulfite (71.4 g, 0.69 mol) was added and the reaction mixture was stirred overnight, and then filtered. The reactor and filter cake were washed with ethyl acetate (1 L). The resulting solution was dried in air for 2 h and under vacuum overnight to provide the title compound **39** as white solid (199 g, >99 % area purity by HPLC). The filtrate was treated by the same procedure to provide a second lot of the title compound **39** (30 g).

c. *N*-Cyclohexylmethyl-(2-oxoethyl)-carbamic acid benzyl ester **40**

To a 100 mL flask was added *N*-cyclohexylmethyl-(2-oxoethyl)-carbamic acid benzyl ester bisulfite adduct **39** (3.94 g, 1 mmol) and MeTHF (35 mL), followed by water (25 mL). The resulting slurry was stirred at room temperature for 5 min and 1 M NaOH (8 mL) was added. The reaction mixture was stirred at room temperature for 45 min. The layers were separated and the volume of the organic layer was reduced to ~8 mL to provide the crude intermediate **40**.

Scheme 8:

Crystalline 3-endo-(8-{2-[cyclohexylmethyl-((S)-2,3-dihydroxy-propionyl)amino]ethyl}-8-aza-bicyclo[3.2.1]oct-3-yl)benzamide sulfate **19i**

a. 3-endo-{8-[2-(cyclohexylmethylamino)ethyl]-8-aza-bicyclo[3.2.1]oct-3-yl}benzamide **18**

To the solution of *N*-cyclohexylmethyl-(2-oxoethyl)-carbamic acid benzyl ester **40** in MeTHF (~8 mL) from Scheme 7, step c, was added DMF (15 mL) followed by 3-endo-(8-azabicyclo[3.2.1]oct-3-yl)-benzamide hydrochloride **17** (2.67 g, 1 mmol) and then DMF (10 mL). The mixture was stirred at room temperature for 30 min, cooled to 10 °C and then sodium triacetoxyborohydride (4.25 g, 2 mmol) was added. The reaction mixture was stirred at room temperature for 90 min and then cooled to 10 °C. Isopropyl acetate (100 mL) was added, followed by 1 M NaOH (50 mL). The mixture was stirred for 15 min, and the phases were separated. The organic layer was washed with brine in water (1:1, 2 x 50 mL) and the volume of the organic layer was reduced to ~ 10 mL to provide crude intermediate. To the solution of crude intermediate was added EtOH (30 mL) and concentrated HCl (1.5 mL). The solution was purged with nitrogen, 10 % palladium on carbon (470 mg) was added and the mixture was purged with nitrogen for 5 min and then hydrogenated at 30 psi overnight. After purging with nitrogen for 2 min, the solution was filtered through Celite and solvent was removed to ~10 mL. Isopropyl acetate (40 mL) and 1 M NaOH (20 mL) were added. The layers were separated and the organic layer was washed with brine (20 mL), phases were separated and organic solvent removed to 5-10 mL. Isopropyl acetate (20 mL) was added and the volume reduced to ~ 8 mL to which isopropyl acetate (20 mL) was added. The resulting slurry was stirred at room temperature for 2 h. The product was isolated by filtration, the reaction flask and filter cake were washed with isopropyl acetate (10 mL) to yield the **18** (2.4 g, 98 % pure) as an off-white solid. ¹H NMR (600 MHz, *d*₆-DMSO): δ (ppm) 7.81 (s, 1H), 7.68 (d, J=7.7 Hz, 1H), 7.43 (d, J=7.8 Hz, 1H), 7.34 (t, J=8.2 Hz, 1H), 3.82 (s, 6H), 3.32 (s, 2H), 3.06 (t, J=8.3 Hz, 1H), 2.95 (d, J=6.7 Hz, 2H), 2.79 (d, J=6.9 Hz, 2H), 2.67 – 2.46 (m, 2H), 2.31 (dd, J=14.5 Hz, 7.5, 2H), 1.99 – 1.84 (m, 3H), 1.81 (d, J=12.6 Hz, 2H), 1.69 (d, J=13.4 Hz, 4H), 1.59 (q, J=14.0 Hz, 11.2, 3H), 1.44 (d, J=8.3 Hz, 2H), 1.35 – 1.07 (m, 3H), 0.97 (q, J=12.3 Hz, 2H). ¹³C NMR (151 MHz, *d*₆-DMSO) δ (ppm) 168.48, 146.46, 134.43, 130.64, 128.43, 126.63, 125.33, 58.03, 53.04, 48.32, 46.14, 38.41, 34.79, 32.68, 30.60, 28.62, 26.13, 25.54.

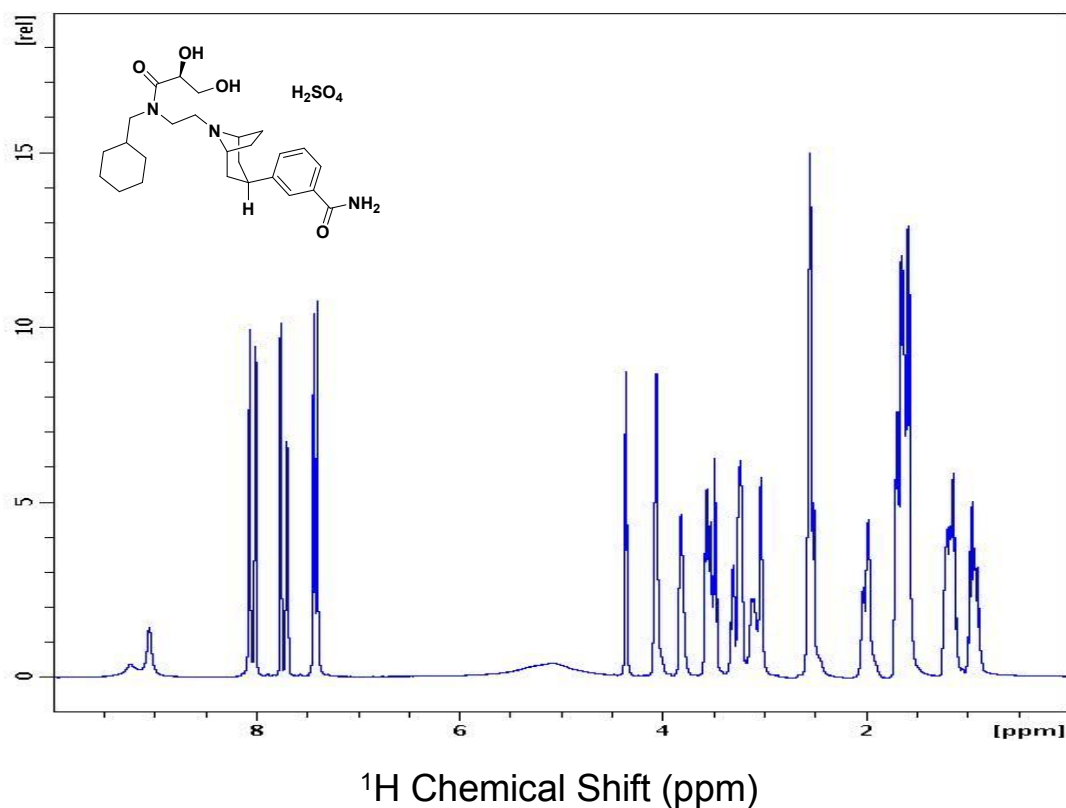
b. 3-endo-(8-{2-[Cyclohexylmethyl-((S)-2,3-dihydroxy-propionyl)amino]ethyl}-8-aza-bicyclo[3.2.1]oct-3-yl)benzamide sulfate (hydrate form) **19i**

To a 500 mL flask was added 3-endo-{8-[2-(cyclohexylmethylamino)ethyl]-8-aza-bicyclo[3.2.1]oct-3-yl}benzamide **18** (31 g, 83.9 mmol) and DMF (150 mL). The mixture was stirred for 10 min and then benzotriazol-1-yloxytris(pyrrolidino)-phosphonium hexafluoro-phosphate (56.8 g, 109 mmol) and lithium (4*S*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate (15.6 g, 92.3 mmol) were added and the mixture was stirred at room temperature for 2 h. Ethyl acetate (600 mL) and 0.5 M NaOH (300 mL) were added and the phases were separated. The organic layer contained crude (*S*)-2,2-dimethyl-[1,3]dioxolane-4-carboxylic acid {2-[3-(3-carbamoyl-phenyl)-8-azabicyclo[3.2.1]oct-8-yl]ethyl}cyclohexylmethyl-amide **41** (~84 mmol) which was not isolated. The organic layer was washed with brine in water (1:1, 2 x 300 mL) and the phases were separated. To the organic layer was added 2 M H₂SO₄ (42 mL) and the reaction mixture was stirred at room temperature overnight. Acetonitrile (300 mL) was added and the resulting slurry was stirred for 2-6 h. The product was isolated by filtration, the filter cake were washed with acetonitrile (200 mL), dried in air for 2 h and then under vacuum at room temperature for 20 h to provide the title compound **19i** (40 g, 97 % pure by HPLC) as a white powder.

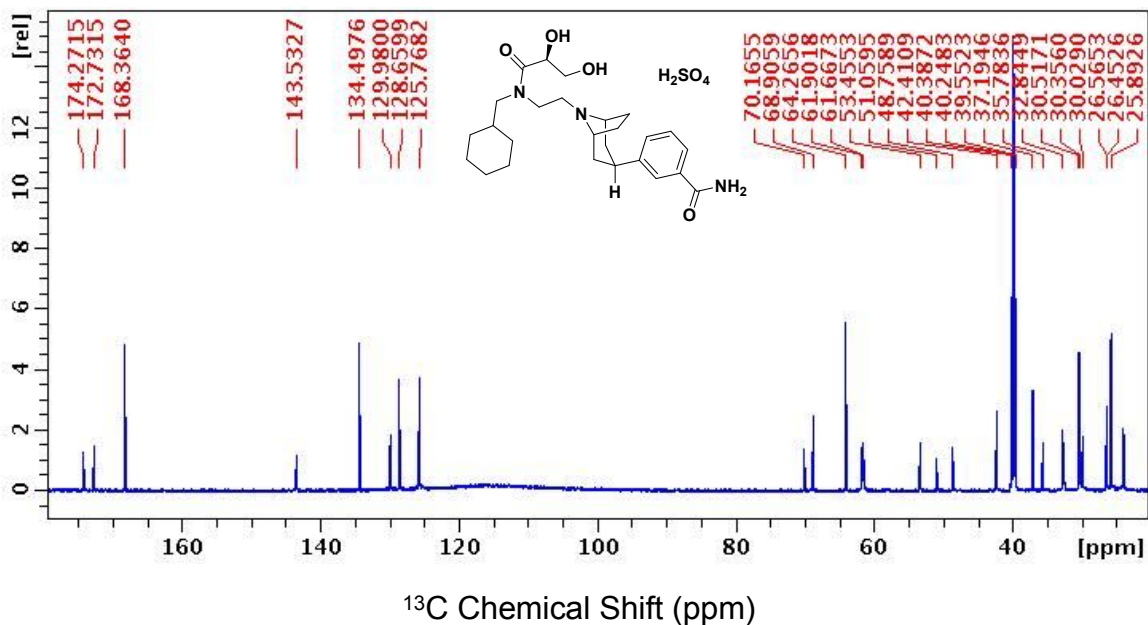
c. Crystalline 3-*endo*-(8-{2-[cyclohexylmethyl]-((*S*)-2,3-dihydroxy-propionyl)amino)ethyl}-8-aza-bicyclo[3.2.1]oct-3-yl)benzamide sulfate **19i**

To a 100 mL flask was added 3-*endo*-(8-{2-[cyclohexylmethyl]-((*S*)-2,3-dihydroxy-propionyl)amino)ethyl}-8-aza-bicyclo[3.2.1]oct-3-yl)benzamide sulfate (hydrate form) **19i** (2 g) and MeOH (40 mL). The resulting slurry was heated to 65 °C under nitrogen for 20 min resulting in complete dissolution. The solution was cooled to room temperature with stirring. About 20 mL of solvent was removed under slightly reduced pressure and the resulting slurry stirred at room temperature overnight. The product was isolated by filtration, and the flask and filter cake were washed with acetonitrile (2 x 5 mL). The filter cake was dried in air for 2 h and then under vacuum at room temperature overnight to provide the title compound **19i** (1.71 g, > 99 % pure by HPLC, ~85 % yield) as a white crystalline powder. (*m/z*): [M+H]⁺ calcd for C₂₆H₃₉N₃O₂, 458.30; found 458.39.

¹H NMR (600 MHz, *d*₆-DMSO) Spectrum of TD-1211 **19i** Sulfate



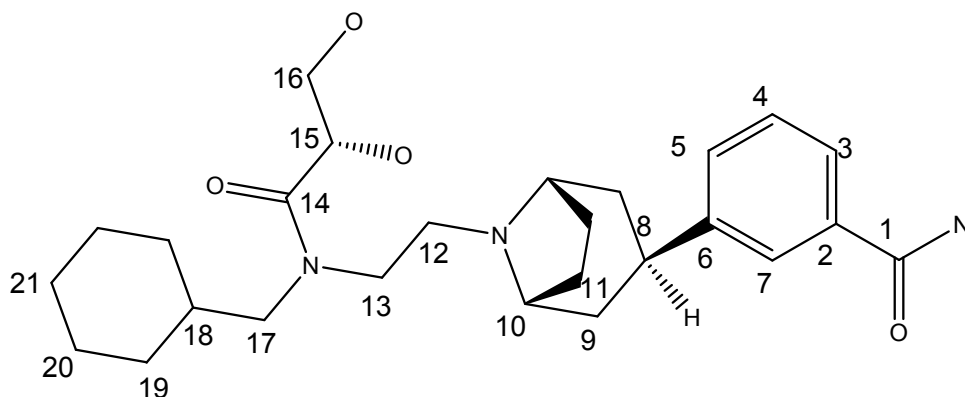
¹³C NMR (150 MHz, *d*₆-DMSO) Spectrum of TD-1211 **19i** Sulfate



Two sets of ¹³C peaks with an intensity ratio ~ 2:1 are observed for most carbons, indicating the presence of 2 conformers. For proton peaks, overlapping prevents the identification of separated peaks for the conformers.

One-dimensional (1D) ¹H, ¹D ¹³C, two-dimensional (2D) COSY, NOESY, TOCSY, HSQC and HMBC data were acquired for the structural elucidation. All chemical shifts were referenced to *d*₆-DMSO multiplet, 2.50 ppm for ¹H and 39.5 ppm for ¹³C.

NMR Data^a for TD-1211 in *d*₆-DMSO



Atom no.	δ_c (type)		δ_H mult, <i>J</i> (Hz)
1	168.4	(C)	
2	143.5	(C)	
3	125.8	(CH)	7.75 d, 7.7
4	128.7	(CH)	7.43 td, 7.7, 2.2
5	130.0	(CH)	7.68 t, 6.8
6	134.5	(C)	
7	125.8	(CH)	8.01 d, 6.8
8	30.0	(CH)	3.24 m
9	32.7,32.8	(CH ₂)	2.54 m
10	61.7,61.9	(CH)	4.06 m
11	24.0,24.1	(CH ₂)	2.00, 1.58 m
12	48.8	(CH ₂)	3.03 t, 6.5; 3.11 m
13	42.4	(CH ₂)	3.80, 3.56 m
14	172.7,174.3	(C)	
15	68.9, 70.2	(CH)	4.36 t, 5.9
16	64.2, 64.3	(CH ₂)	3.57, 3.46 m, 5.9,10.8
17	51.1, 53.5	(CH ₂)	3.31, 3.11 dd, 14.6, 7.4
18	35.8, 37.2	(CH)	1.69,1.65 m
19	30.4,30.5	(CH ₂)	0.93, 1.56 ^b m
20	26.5,26.6	(CH ₂)	1.12,1.63 ^b m
21	25.9	(CH ₂)	1.19, 1.71 ^b m
15-OH			5.1
16-OH			5.1
NH			7.40, 8.06
NH ₂			9.23, 9.05

^a Measured at 600 MHz (¹H) and 151 MHz (¹³C).

^b Multiplicity undetermined due to overlapping signals.

The X-ray single crystal structure of the solid form has been solved, elucidating the molecular structure of the parent, the crystalline nature of the solid, the 1:1 mole ratio of the parent to the sulfate counter ion, and the anhydrous composition of the crystal lattice. The lattice is in the orthorhombic system, with a space group of $P2_12_12_1$ and R factor = 0.0424.

CCDC 1948348 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

In Vitro and In Vivo Pharmacology Methods

Radioligand binding assays

Radioligand binding assays were conducted in 96-well polypropylene assay plates in a total assay volume of 200 μ L containing human μ (4 μ g), human δ (2 μ g), or guinea pig κ (20 - 30 μ g) receptor membrane protein. In all cases, the final assay buffer was 50 mM HEPES pH 7.4, containing 0.025 % BSA.

Displacement assays for determination of pK_i values were conducted at room temperature, using a 90-minute incubation period; the radioligand ($[^3\text{H}]$ -diprenorphine, $[^3\text{H}]$ -DPN) concentrations were 0.5 nM (human μ receptor), 1.3 nM (human δ receptor), or 0.7 nM (guinea pig κ receptor). Eleven different concentrations (10 pM to 100 μ M) of compound were tested. Binding data were analyzed by nonlinear regression analysis with the GraphPad Prism™ Software package (GraphPad Software, Inc., San Diego, CA) using the 3-parameter model for one site competition. K_i values for test compounds were calculated in Prism from the best-fit IC_{50} values using the Cheng Prusoff equation (Cheng and Prusoff 1973), and the negative decadic logarithm of the K_i value, pK_i , was reported.

Guanine nucleotide binding assays

Membrane-based GTP binding assays were performed using either $[^{35}\text{S}]$ -GTP γ S for δ or κ receptors or in DELFIA GTP Binding Assays (Perkin Elmer, Boston MA) for μ receptors. Compound dilution and preparation of opioid receptor membranes were conducted as described above. The DELFIA GTP assays were performed in AcroWell 96-well plates in a total volume of 100 μ L containing μ receptor membrane protein (5 μ g), test compound (10 pM - 20 μ M), GDP (1 μ M), and europium-labeled GTP (Eu-GTP; 10 nM). The GTP γ S binding assays were performed in a total volume of 200 μ L in 96-well polypropylene assay plates containing δ or κ receptor membrane protein (10 μ g), test compound (10 pM - 20 μ M), GDP (10 μ M), and $[^{35}\text{S}]$ -GTP γ S (0.1 nM). In all cases, final buffer composition was HEPES (50 mM, pH 7.4), MgCl_2 (10 mM), NaCl (50 mM for μ and κ , or 100 mM for δ), and 0.0125 % BSA. Reactions were incubated at room temperature for 30 minutes prior to termination by filtration. Concentration-response curves were generated from bound Time Resolved Fluorescence (TRF) or cpm values for both test compounds and receptor-selective agonists (DAMGO, DPDPE, or U69593 for μ , δ , or κ receptors, respectively). pEC_{50} values and the top and bottom of the curve were determined using a 3-parameter sigmoidal dose-response algorithm in GraphPad Prism™. Intrinsic activities (IA) were expressed as a percent of the maximal response evoked by each receptor-selective agonist.

Mouse gastric emptying and intestinal transit

Male mice (19 - 40 g; ICR from Harlan) were acclimated to the holding room (temperature controlled at $70 \pm 2^\circ\text{C}$ and 12:12 hr light-dark cycle commencing at 7:00 am) for at least 3 days following arrival prior to use. Prior to the experiment, animals were fasted overnight with free access to water. Mice received an oral gavage (10 mL/kg) of test article or vehicle (5% dextrose in water) followed 30 minutes later by a subcutaneous injection of morphine (3 mg/kg; 10 mL/kg) or vehicle (0.9% saline). Five minutes later, animals received an oral gavage (0.5 mL) of a non-absorbable test meal (10% charcoal in 1% carboxymethylcellulose with 40 mg/mL corn starch). Sixty minutes after receiving the oral test meal, animals were euthanized by carbon dioxide asphyxiation followed by a thoracotomy. The body cavity was opened and ligatures (4-0 silk) were tied at the pyloric sphincter and distal esophagus prior to excising the stomach and small intestine (from pyloric sphincter to cecum) from the body cavity. The stomach was then clipped from the intestine at the site of the ligature, and its weight recorded. The intestine was stretched out, and the distance traveled by the charcoal meal and the entire intestinal length were measured and recorded.

Data for each treatment group were averaged and expressed as a mean (\pm SEM) gastric weight or intestinal distance. In each study, a Student's t-test was performed, comparing the vehicle/vehicle group to the vehicle/morphine group. A statistically significant ($p < 0.05$) inhibitory effect of morphine defined the study as acceptable for further analysis. Differences between treatments were then determined using one-way analysis of variance (ANOVA) with a Dunnett's post-hoc test ($p < 0.05$ considered to be statistically significant).

Mouse hot plate

Male mice (18 - 48 g; ICR from Harlan) were acclimated to the holding room (temperature controlled at $70 \pm 2^\circ\text{C}$ and 12:12 hr light-dark cycle commencing at 7:00 am) for at least 3 days following arrival. A standard rodent diet (2018, Harlan Teklad) and drinking water were provided ad libitum. Following an overnight fast, mice received an oral gavage (10 mL/kg) of test article or vehicle (5% dextrose in water) followed 30 minutes later by a subcutaneous injection of morphine (10 mg/kg; 10 mL/kg) or its vehicle, saline. Thirty minutes later, animals were placed on a hot plate apparatus (Pan Labs model LE7406). The temperature of the hot plate was kept constant at 53°C . The time taken for the animal to lick its paw was then measured and recorded. A cut-off time of 35 seconds was used to minimize the pain experienced by the animals and to avoid tissue damage.

Data for each treatment group were averaged and expressed as a mean (\pm SEM) paw withdrawal latency. In each study, a Student's t-test was performed, comparing the vehicle/vehicle group to the vehicle/morphine group. A statistically significant ($p < 0.05$) effect of morphine defined the study as acceptable for further analysis. Differences between treatment groups were then determined using one-way analysis of variance (ANOVA) with a Dunnett's post-hoc test ($p < 0.05$ considered to be statistically significant).

Cheng Y, Prusoff WH. 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: 3099-108.