SUPPORTING INFORMATION

Synthesis and Biological Evaluation of (-)-Kainic Acid Analogues as Phospholipase D-Coupled Metabotropic Glutamate Receptor Ligands

Chiara Zanato,* Sonia Watson, Guy S. Bewick, William T. A. Harrison and Matteo Zanda*

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General Information

$^1$H (400.13 MHz), $^{13}$C (100.58 MHz) and $^{19}$F (376.45 MHz) NMR spectra were recorded on a Bruker ADVANCE III spectrometer. $^1$H NMR chemical shifts are reported relative to TMS, and the solvent resonance was employed as the internal standard (CDCl$_3$ $\delta$ = 7.26, CD$_3$OD $\delta$ = 3.31, D$_2$O $\delta$ = 4.79). $^{13}$C NMR spectra were recorded with complete proton decoupling, and the chemical shifts are reported relative to TMS with the solvent resonance as the internal standard (CDCl$_3$, $\delta$ = 77.0, CD$_3$OD $\delta$ = 49.00). $^{19}$F NMR spectra were recorded with complete proton decoupling. The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, dd = doublet-doublet, ddd = doublet-doublet-doublet, dt = doublet-triplet, t = triplet, td = triplet-doublet, q = quartet, m = multiplet, bs = broad singlet, bq = broad quartet. All chemical shifts ($\delta$) are expressed in parts per million and coupling constant ($J$) are given in Hertz. LC-MS experiments were performed on an Agilent Technologies 1200 Series HPLC system equipped with a DAD and a 6120 MS detector composed by a ESI ionization source and a Single Quadrupole mass selective detector using an Analytical C18 RP Column (Phenomenex Luna, C18, 250x4.60 mm, 5 $\mu$, 100 Å). HPLC purifications were performed on the Agilent 1200 system using a semi preparative C18 RP Column (Phenomenex Luna, 250x10.00 mm, 5 $\mu$, 100 Å). A CEM Discover® System was used to perform reaction with microwaves. Optical rotation values were measured on an AA-65 Angular Scale automatic polarimeter (Optical Activity Limited) with a 1dm cell at the sodium D line. All reactions were carried out in oven- or flame-dried glassware under nitrogen atmosphere, unless stated otherwise. All commercially available reagents were used as received. Reactions were magnetically stirred and monitored by TLC on silica gel (60 F254 pre-coated glass plates, 0.25 mm thickness). Visualization was accomplished by irradiation with a UV lamp and/or staining with a ceric ammonium molibdate or KMnO$_4$ solution. Flash chromatography was performed on silica gel (60 Å, particle size 0.040–0.062 mm). Yields refer to chromatographically and spectroscopically pure compounds, unless stated otherwise. Abbreviations used: DCM for dichloromethane, EtOAc for ethyl acetate, Et$_2$O for diethyl ether, PfBr for 9-Bromo-9-phenylfluorene, DMSO for dimethyl sulfoxide, NBS for N-bromosuccinimide, DIBAL-H for diisobutylaluminium hydride, DME for dimethoxyethane, DIPEA N,N-diisopropylethylamine, THF for tetrahydrofuran, MeOH for methanol, TEA for triethylamine, EDC for 1-(3-dimethylaminopropyl) -3-ethylcarbodimide, DMF for N,N-dimethylformamide.
**Synthetic Procedures and Compounds Characterizations**

**Methyl (2S, 4R)-4-hydroxypyrrolidine-2-carboxylate hydrochloride**

Thionyl chloride (4.00 mL, 30.5 mmol) was added to methanol (20.0 mL) at 0°C and the reaction was allowed to warm to r.t. 4-trans-L-hydroxyproline (4.00 g, 54.9 mmol) was added, the mixture was heated at 45°C and stirred overnight. The solvent was removed under reduced pressure and the solid was treated with Et₂O (3 x 10 mL) followed by evaporation. Recrystallization of the solid from MeOH-Et₂O gave the desired methyl ester (8.97 g, 90%) as a white solid, used without further purification. ESI MS m/z: [M+H]+ calcd for C₆H₁₂NO₃ 146.07, found (relative intensity) 146.1 (100) [M+H]+. (For the complete characterization refer to D.S. Kemp, P. Curran, M.D. William, J. G. Boyd, C. Muendel, *J. Org. Chem.* 1991, 56, 6672-6682 and J.E. Baldwin, S. J. Bamford, A.M. Fryer, M.P. W. Rudolph, M.E. Wood, *Tetrahedron*, 1997, 53, 5233-5254).

**Methyl (2S, 4R)-1-benzoyl-4-hydroxypyrrolidine-2-carboxylate**

To a solution of methyl (2S, 4R)-4-hydroxypyrrolidine-2-carboxylate hydrochloride (5.45 g, 30.0 mmol) in H₂O/THF (1:1, 20 mL) NaHCO₃ (5.48 g, 63.0 mmol) was slowly added and the mixture was cooled to 0°C. A solution of benzoyl chloride (3.83 mL, 33.0 mmol) in THF (8.8 mL) was added drop wise and the mixture was stirred at r.t for 1.5 h. The solvent was removed under vacuum, CHCl₃ (50 mL) was added, the organic layers were washed with brine dried over Na₂SO₄, filtered and the solvent was evaporated under reduced pressure. The crude product was recrystallized in EtOAc to give the desired benzoyl amide (7.33 mg, 98%) as a white crystalline solid, used without further purification. Rf 0.21 (DCM/EtOAc 1:1); ESI MS m/z: [M+H]+ calcd for C₁₃H₁₆NO₄ 250.10, [2M+Na]+ calcd for C₂₆H₃₀N₂O₈Na 521.19, found (relative intensity) 250.1 (100) [M+H]+, 521.1 (100) [M+H]⁺. (For the complete characterization refer to D.S. Kemp, P. Curran, M.D. William, J. G. Boyd, C. Muendel, *J. Org. Chem.* 1991, 56, 6672-6682).

**Methyl (2S)-1-benzoyl-4-oxopyrrolidine-2-carboxylate (2)**

To a solution of methyl (2S, 4R)-1-benzoyl-4-hydroxypyrrolidine-2-carboxylate (7.30 g, 29.0 mmol) in a mixture of CH₃CN (32 mL), CCl₄ (32 mL) and H₂O (48 mL), NaIO₄ (116.0 mmol, 24.8 g) and ruthenium trichloride hydrate (300 mg, 1.45 mmol) were added. The
mixture was vigorously stirred for 4 h at r.t. then DCM (160 mL) was added and the aqueous phase was further extracted with DCM (3 x 50 mL). The organic layers were dried over Na2SO4, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (DCM/ EtOAc 6:4) to give ketone 2 (5.74 g, 80%) as a light brown oil. Rf 0.50 (DCM/EtOAc 1:1); ESI MS m/z: [M+H]⁺ calcd for C13H14NO4 248.08, found (relative intensity) 248.1 (100) [M+H]⁺. (For the complete characterization refer to D.S. Kemp, P. Curran, M.D. William, J. G. Boyd, C. Muendel, J. Org. Chem. 1991, 56, 6672-6682).

5 Methyl (2S,3R)-1-benzoyl-3-[2-(tert-butoxy)-2-oxoethyl]-4-oxopyrrolidine-2-carboxylate (3)

A solution of nBuLi (1.6 N in hexane, 1.83 mL, 2.92 mmol) was added dropwise to a stirred solution of ketone 2 (687.9 mg, 2.79 mmol) in THF (5.3 mL) and dry HMPA (1.1 mL) at -78°C. The resulting mixture was stirred at -78°C for 30 minutes and then rapidly transferred with a cannula to a solution of NaI (251.0 mg, 1.67 mmol) and tert-butylbromoacetate (1.23 mL, 8.37 mmol) in THF (5.3 mL) at -60°C. The reaction was allowed to warm to -42°C, stirred for an additional hour and treated with a 30% aqueous solution of H3PO4 (5.3 mL), H2O (5.3 mL) was added and the resulting mixture was extracted with EtOAc, the organic layers were washed with brine, dried over Na2SO4, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/EtOAc 7:3) to give compound 3 (276.6 mg, 30%, single diastereoisomer) as a white solid. Rf 0.25 (Hexane/EtOAc 7:3); [α]25_D -36.1 (c: 1.0, CHCl3); ESI MS m/z: [M+H]⁺ calcd for C19H24NO6 361.15, [M+Na]⁺ calcd for C19H23NO6Na 384.14, found (relative intensity) 362.1 (100) [M+H]⁺, 384.1 (55) [M+Na]⁺. (For the complete characterization refer to D.S. Kemp, P. Curran, M.D. William, J. G. Boyd, C. Muendel, J. Org. Chem. 1991, 56, 6672-6682).

5 Methyl (2S)-4-hydroxy-1-(9-phenyl-9H-fluoren-9-yl)pyrrolidine-2-carboxylate

A solution of methyl (2S,4R)-4-hydroxypyrrolidine-2-carboxylate hydrochloride (3.0 g, 16.5 mmol) and chlorotrimethylsilane (5.2 mL, 41.3 mmol) in DCM (40 mL) at 0°C was treated with TEA (8.0 mL, 57.8 mmol) and allowed to reach r.t. The mixture was stirred at reflux for 1 h, cooled to 0°C, treated with MeOH (1.0 mL) in DCM (4.5 mL), allowed to warm to r.t. for 1 h and then treated with PfBr (6.9 g, 21.45 mmol) and Pb(NO3)2 (4.9 g, 14.9 mmol). The mixture was stirred at r.t. for 96 h, filtered and evaporated. The remaining solid was dissolved in MeOH (54 mL) and citric acid (5.5 g) was added. The solution was vigorously stirred for 1
additional hour, solvent was evaporated under reduce pressure. The crude product was purified by flash chromatography (Hexane/ EtOAc 1:1) to give the Pf protected compound (5.7 g, 90%) as a white foam. Rf 0.42 (Hexane/EtOAc 1:1); [α]D 25 -139 (c: 1.2, CHCl3); 1H NMR (400 MHz, CDCl3) δ: 1.79 (ddd, 1H, J = 5.6, 8.9, 13.0 Hz), 1.98 (dt, 1H, J = 5.6, 12.6 Hz), 2.92 (dd, 1H, J = 4.8, 9.9 Hz), 3.24 (s, 3H), 3.29 (dd, 1H, J = 5.3, 8.8 Hz), 3.57 (dd, 1H, J = 5.3, 10.0 Hz), 4.43-4.58 (m, 1H), 7.16 (td, 1H, J = 1.1, 7.5 Hz), 7.21-7.35 (m, 6H), 7.43 (td, 1H, J = 1.1, 7.5 Hz), 7.50-7.59 (m, 3H), 7.65 (dd, 1H, J = 0.7, 6.8 Hz), 7.74 (dd, 1H, J = 0.8, 6.8 Hz); 13C NMR (100MHz, CDCl3) δ: 40.0, 51.3, 56.8, 59.3, 70.4, 76.1, 119.8, 120.1, 126.4, 127.1, 127.2, 127.3 (x 2), 127.4, 127.6, 128.3 (x 2), 128.4, 128.8, 139.9, 141.5, 142.7, 146.1, 147.2, 175.8; ESI MS m/z: [M+H]⁺ calcd for C25H24NO3 385.17, [M+Na]⁺ calcd for C25H23NO3Na 408.16, found (relative intensity) 385.2 (100) [M+H]⁺, 408.2 (70) [M+Na]⁺.

6 Methyl (2S)-4-oxo-1-(9-phenyl-9H-fluoren-9-yl)pyrrolidine-2-carboxylate (4)

DMSO (2.3 mL, 29.5mmol) was added to a solution of oxalyl chloride (2M in DCM, 14.8 mmol, 7.4 mL) at -78°C. After 10 minutes a solution of methyl (2S)-4-hydroxy-1-(9-phenyl-9H-fluoren-9-yl)pyrrolidine-2-carboxylate (2.2 g, 5.68 mmol) in DCM (38 mL) was added. The mixture was stirred at -78°C for 1 h, treated with TEA (18.2 mL) and allowed to reach r.t. A saturated aqueous solution of NaHCO3 (82 mL) was added and the aqueous layer was extracted with DCM (3 x 150 mL). The combined organic layers were extracted, washed with brine, dried over Na2SO4, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/ EtOAc 8:2) to give compound 4 (1.96 g, 90%) as a white solid. Rf 0.32 (Hexane/EtOAc 7:3); [α]D 25 -60 (c: 1.4, CHCl3); 1H NMR (400 MHz, CDCl3) δ: 2.29 (dd, 1H, J = 2.6, 18.4 Hz), 2.44 (ddd, 1H, J = 0.7, 8.6, 18.4 Hz), 3.21 (s, 3H), 3.48 (dt, 1H, J = 1.0, 17.9 Hz), 3.76 (d, 1H, J = 17.9 Hz), 3.76 (dd, 1H, J = 2.9, 8.6 Hz), 7.22-7.32 (m, 6H), 7.36-7.40 (m, 2H), 7.40-7.48 (m, 3H), 7.69-7.74 (m, 2 H); 13C NMR (100MHz, CDCl3) δ: 41.7, 51.5, 55.3, 58.2, 76.0, 120.1, 120.3, 125.5, 126.9 (x 2), 127.0, 127.6, 127.7, 128.1, 128.6 (x 2), 128.9, 129.0, 140.4, 140.9, 141.9, 145.4, 146.5, 173.1, 212.9; ESI MS m/z: [M+H]⁺ calcd for C23H22NO3 383.15, [M+Na]⁺ calcd for C23H21NO3Na 406.14, found (relative intensity) 383.1 (100) [M+H]⁺, 406.1 (60) [M+Na]⁺.
A solution of nBuLi (1.6 N in hexane, 3 mL, 4.8 mmol) was added drop wise to a stirred solution of ketone 4 (1.69 g, 4.4 mmol) in THF (9 mL) and dry HMPA (2 mL) at -78°C. The resulting mixture was stirred at -78°C for 30 minutes and then rapidly transferred with a cannula to a solution of NaI (396 mg, 2.6 mmol) and tert-butylobromoacetate (1.95 mL, 13.2 mmol) in THF (9 mL) at -60°C. The reaction was allowed to warm to -42°C, stirred for an additional hour and treated with a 30% aqueous solution of H₃PO₄ (9 mL) H₂O (9 mL) was added and the resulting mixture was extracted with EtOAc, the organic layers were washed with brine, dried over Na₂SO₄, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/ EtOAc 7:3) to give compound 5a (1.7 g, 80%, dr 16:1) as a white solid. Rf 0.58 (Hexane/EtOAc 6:4); [α]²⁵°D -51.4 (c: 1.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ: 1.35 (s, 9 H), 2.46 (dd, 2H, J = 3.6, 6.3 Hz), 2.87 (dd, 1H, J = 5.8, 11.7 Hz), 3.14 (s, 3H), 3.49 (d, 1H, J = 17.7 Hz), 3.50 (d, 1H, J = 6.1 Hz), 3.82 (d, 1H, J = 17.7 Hz), 7.22-7.33 (m, 5H); 7.40 (t, 2H, J = 7.2 Hz), 7.47 (d, 1H, J = 7.4 Hz), 7.54 (d, 3H, J = 7.0 Hz), 7.73 (d, 2H, J = 7.4 Hz); ¹³C NMR (100MHz, CDCl₃) δ: 27.9 (x 3), 34.3, 49.3, 51.5, 55.7, 63.7, 75.8, 81.4, 120.0, 120.2, 126.0, 127.0 (x 2), 127.3, 127.7, 127.9, 128.0, 128.5 (x 2), 128.9 (x 2), 140.5, 140.1, 141.5, 144.5, 146.1, 169.6, 172.9, 211.8; ESI MS m/z: [M+H]+ calcd for C₃₁H₃₂NO₅ 498.22, [M+Na]+ calcd for C₃₁H₃₁NO₅Na 520.21, found (relative intensity) 498.2 (10) [M+H]+, 520.2 (100) [M+Na]+.

**Methyl-(2S, 3S)-3-[2-(tert-butoxy)-2-oxoethyl]-4-oxo-1-(9-phenyl-9H-fluoren-9-yl)pyrrolidine-2-carboxylate (5b)**

Rf 0.60 (Hexane/EtOAc 6:4); [α]²⁵°D -89.2 (c: 1.4, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ: 1.34 (s, 9 H), 1.83 (dd, 1H, J = 8.9, 17.4 Hz), 2.57 (dd, 1H, J = 5.2, 17.4 Hz), 3.00-3.11 (m, 1H), 3.15 (s, 3H), 3.58 (dd, 1H, J = 1.0, 17.6 Hz), 3.80 (d, 1H, J = 17.6 Hz), 4.02 (d, 1H, J = 8.2 Hz), 7.22-7.28 (m, 6H), 7.30-7.38 (m, 2H), 7.38-7.44 (m, 3H), 7.68 (d, 1H, J = 7.5 Hz), 7.73 (d, 1H, J = 8.1 Hz); ¹³C NMR (100MHz, CDCl₃) δ: 27.9 (x 3), 31.1, 48.0, 51.1, 53.7, 61.9, 75.4, 81.1, 120.2 (x 2), 125.4, 126.7, 126.8 (x 2), 127.6, 127.7, 128.1, 128.6 (x 2), 128.9, 129.0, 140.0, 141.2, 141.8, 145.3, 146.9, 170.2, 171.8, 212.1; ESI MS m/z: [M+H]+ calcd for C₃₁H₃₂NO₅ 498.22, [M+Na]+ calcd for C₃₁H₃₁NO₅Na 520.21, found (relative intensity) 498.2 (20) [M+H]+, 520.2 (100) [M+Na]+. 

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7 Methyl (2S, 3R)-3-[2-(tert-butoxy)-2-oxoethyl]-4-oxo-1-(9-phenyl-9H-fluoren-9-yl)pyrrolidine-2-carboxylate (5a)
Methyl-(2S,3R,4S)-3-[2-(tert-butoxy)-2-oxoethyl]-4-hydroxy-1-(9-phenyl-9H-fluoren-9-yl)-4-(trifluoromethyl)pyrrolidine-2-carboxylate (6)

To a solution of ketone 5a (100 mg, 0.27 mmol), in DMF (4.2 mL), were added trimethylsilyl trifluoromethane (0.2 mL, 1.15 mmol) and Cs₂CO₃ (105 mg, 0.34 mmol). After stirring for 2 h TBAF (1M in THF, 1.6 mL) was added. After 30 minutes the mixture was diluted with H₂O (9 mL) and extracted with EtOAc (3 x 20 mL). Combined organic layer were washed with H₂O, dried over Na₂SO₄, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/EtOAc 1:1) to give compound 6 (56 mg, 48%) as a white solid. R_f 0.55 (Hexane/EtOAc 1:1); [α]²⁵⁺D +142.2 (c: 0.7, CHCl₃);

¹H NMR (400 MHz, CDCl₃) δ: 1.33 (s, 9 H), 1.73 (dd, 1H, J = 10.7, 16.1 Hz), 2.35 (dd, 1H, J = 4.3, 16.1 Hz), 2.55-2.69 (m, 1H), 2.90 (d, 1H, J = 3.4 Hz), 3.27 (s, 3H), 3.36 (d, 1H, J = 10.0 Hz), 3.55 (d, 1H, J = 10.0 Hz), 5.96 (s, OH), 7.19 (td, 1H, J = 1.1, 7.5 Hz), 7.22-7.38 (m, 5H), 7.40-7.46 (m, 1H), 7.51 (dd, 2H, J = 4.3, 10.8 Hz), 7.55-7.61 (m, 2H), 7.67 (d, 1H, J = 7.5 Hz), 7.80 (dd, 1H, J = 1.8, 6.6 Hz); ¹⁹F NMR (376.45 MHz, CDCl₃) δ: -74.6; ¹³C NMR (100MHz, CDCl₃) δ: 27.8 (x 3), 35.7, 48.0, 52.4, 54.4, 64.5, 75.4, 79.8 (q, J_C-F = 28.8 Hz), 81.5, 120.0, 120.3, 124.6 (q J_C-F = 283.0 Hz), 126.3, 127.0, 127.3, 127.4 (x 2), 127.8, 128.0, 128.6 (x 2), 128.8, 129.2, 139.7, 140.8, 141.5, 144.7, 146.7, 170.1, 177.0; ESI MS m/z: [M+H]⁺ calcd for C₃₂H₃₃F₃NO₅ 568.22, [M+Na]⁺ calcd for C₃₂H₃₂F₄NO₅Na 590.21, found (relative intensity) 568.2 (80) [M+H]⁺, 590.2 (100) [M+Na]⁺.

(2S,3R,4S)-3-(carboxymethyl)-4-hydroxy-4-(trifluoromethyl)pyrrolidine-2-carboxylic acid (1a)

To a solution of compound 6 (50.0 mg, 0.089 mmol) in toluene (1 mL) at r.t. was added drop wise aqueous phosphoric acid (85 wt %, 0.02 mL, 0.45 mmol). The mixture was stirred for 2 h and the progress was checked by mass analysis {ESI MS m/z: [M+H]⁺ calcd for C₉H₁₃F₃NO₅ 272.07, [2M+Na]⁺ calcd for C₁₈H₂₄ F₆N₂O₁₀Na 565.13, found (relative intensity) 272.1 (100) [M+H]⁺, 565.1 (30) [2M+Na]⁺}. Once the reaction was completed, water was added (5 mL), and the mixture was washed with hexane (3 x 10 mL). The pH of the aqueous layer was adjusted to 7 (adding a solution of LiOH 0.5 N in water) and the solvent was evaporated under reduce pressure in order to obtain a pale yellow solid. The crude product was diluted with water (2 mL) and an aqueous solution of LiOH is added (0.5 N, 0.35 mL, 0.18 mmol). The mixture was stirred for 6 h at r.t. (progress was checked by mass analysis) then the pH is neutralised by addition of aqueous HCl (1N in water) and the
solvent was evaporated under reduced pressure. The crude product was purified by HPLC (Semi-preparative C18 Luna column, Eluent A: H2O in isocratic condition, 5mL/min, Retention Time: 9.5 min) in order to obtain compound 1a (11 mg, 48% in two steps) as a white solid. $[\alpha]_{25}^D +20.2$ (c: 0.7, H2O); ¹H NMR (400 MHz, D₂O) δ: 2.70 (dd, 1H, $J = 7.2, 17.4$ Hz), 2.80 (ddd, 1H, $J = 1.4, 7.0, 17.4$ Hz), 3.11 (q, 1H, $J = 6.8$ Hz), 3.59 (d, 1H, $J = 13.2$ Hz), 3.79 (d, 1H, $J = 13.2$ Hz), 4.07 (d, 1H, $J = 6.3$ Hz); ¹⁹F NMR (376.45 MHz, CDCl₃) δ: -75.7; ¹³C NMR (100MHz, D₂O) δ: 34.9, 46.7, 50.2, 64.7, 80.1(q, $J_{C-F} = 30.0$ Hz), 124.0 (q, $J_{C-F} = 284.2$ Hz), 171.9, 175.5; ESI MS m/z: [M+H]+ calcd for C₈H₁₁F₃NO₅ 258.05, found (relative intensity) 258.0 (100) [M+H]+; HRMS calcd. for C₈H₁₁F₃NO₅: 258.0511, found: 258.0586.

**Methyl-(2S,3R,4S)-3-[2-(tert-butoxy)-2-oxoethyl]-4-hydroxy-1-(9-phenyl-9H-fluoren-9-yl)pyrrolidine-2-carboxylate (7)**

To a stirred solution of ketone 5a (433 mg, 0.87 mmol) in MeOH (14.0 mL), NaBH₄ (32.4 mg, 0.95 mmol) was added. After 2 h the mixture was poured into a saturated aqueous solution of NH₄Cl (20 mL) and the aqueous layer was extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with aqueous HCl (0.1N in water), brine, dried over Na₂SO₄, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/EtOAc 8:2) to give compound 7 (391 mg, 90%) as a white solid and single diastereoisomer. Rf 0.40 (Hexane/EtOAc 6:4); $[\alpha]_{25}^D +191$ (c: 1.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ: 1.34 (s, 9 H), 1.73 (dd, 1H, $J = 8.4, 15.6$ Hz), 1.90 (dd, 1H, $J = 7.4, 15.6$ Hz), 2.33 (t, 1H, $J = 7.9$ Hz), 2.70 (d, 1H, $J = 2.8$ Hz), 3.20 (dd, 1H, $J = 4.1, 10.2$ Hz), 3.32 (s, 3H), 3.44 (dd, 1H, $J = 1.3, 10.2$ Hz), 3.83 (ddd, 1H, $J = 2.0, 3.9, 8.4$ Hz), 4.46 (d, OH, $J = 10.4$ Hz), 7.15 (td, 1H, $J = 1.1, 7.5$ Hz), 7.22-7.35 (m, 5H), 7.39 (td, 1H, $J = 1.1$ Hz), 7.49 (t, 2H, $J = 7.1$ Hz), 7.58 (d, 2H, $J = 6.9$ Hz) 7.65 (d, 1H, $J = 7.5$ Hz), 7.79 (dd, 1H, $J = 1.6, 6.9$ Hz); ¹³C NMR (100MHz, CDCl₃) δ: 27.9 (x 3), 38.5, 48.3, 51.9, 55.2, 64.1, 75.4, 75.2, 81.1, 120.0, 120.2, 126.2, 126.9, 127.2, 127.4 (x 2), 127.5, 127.8, 128.5 (x 3), 128.9, 139.3, 141.7, 141.8, 145.2, 147.8, 170.5, 177.6; ESI MS m/z: [M+H]+ calcd for C₃₁H₃₄NO₅ 500.24, found (relative intensity) 500.3 (100) [M+H]+.

**Methyl (2S,3R,4S)-3-[2-(tert-butoxy)-2-oxoethyl]-4-methoxy-1-(9-phenyl-9H-fluoren-9-yl)pyrrolidine-2-carboxylate (8)**
To an ice cooled solution of alcohol 7 (50 mg, 0.10 mmol), in DMF (1 mL), NaH (60% in mineral oil, 8.8 mg, 0.22 mmol) was added. The mixture was allowed to stir for 30 minutes at 0 °C, and then MeI (0.007 mL, 0.11 mmol) was added. The resulting solution was stirred for 3h at r.t. then was quenched with a few drops of H₂O and 5 mL of a saturated aqueous solution of NaHCO₃. The aqueous layer was extracted with Et₂O (2 x 10 mL), the combined organic layers were dried over Na₂SO₄, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/EtOAc 8:2) to give compound 8 (36 mg, 70%) as a white solid. Rf/0.45 (Hexane/EtOAc 6:4); [α]²⁵ D +180 (c: 1.1, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ: 1.35 (s, 9 H), 2.03 (d, 2H, J = 7.1 Hz), 2.51-2.61 (m, 1H), 2.74 (d, 1H, J = 6.8 Hz), 3.27 (s, 3H), 3.31 (s, 3H), 3.35-3.49 (m, 3H), 7.15 (td, 1H, J = 1.1, 7.5 Hz), 7.22-7.38 (m, 6H), 7.44 (ddd, 2H, J = 3.2, 7.0, 8.5 Hz), 7.58 (d, 2H, J = 6.9 Hz), 7.64 (d, 1H, J = 7.4 Hz), 7.76 (d, 1H, J = 7.5 Hz); ¹³C NMR (100MHz, CDCl₃) δ: 27.9 (x 3), 31.6, 37.8, 45.8, 51.3, 53.4, 57.5, 65.2, 76.5, 80.6, 83.0, 119.7, 120.2, 125.8, 127.1, 127.2 (x 2), 127.3, 127.5, 128.3, 128.4 (x 2), 128.8, 139.5, 141.8, 143.1, 146.3, 147.1, 170.5, 174.6; ESI MS m/z: [M+H]+ calcd for C₃₂H₃₆NO₅ 513.25, found (relative intensity) 513.2 (100) [M+H]+.

(2S,3R,4S)-3-(carboxymethyl)-4-methoxypyrrolidine-2-carboxylic acid (1b)

To a solution of compound 8 (79.3 mg, 0.15 mmol) in toluene (2 mL) at r.t. was added dropwise aqueous phosphoric acid (85 wt %, 0.03 mL, 0.76 mmol). The mixture was stirred for 2 h and the progress was checked by mass analysis {ESI MS m/z: [M+H]+ calcd for C₉H₁₆NO₅ 218.10, found (relative intensity) 218.1 (100) [M+H]+}. Once the reaction was completed, water was added (8 mL), and the mixture was washed with hexane (3 x 15 mL). The pH of the aqueous layer was adjusted to 7 (adding a solution of LiOH 0.5 N in water) and the solvent was evaporated under reduced pressure in order to obtain a pale yellow solid. The crude product was diluted with water (2 mL) and an aqueous solution of LiOH is added (0.5 N, 0.60 mL, 0.30 mmol). The mixture was stirred for 4 h at r.t. (progress was checked by mass analysis) then the pH is neutralised by addition of aqueous HCl (1N in water) and the solvent was evaporated under reduced pressure. The crude product was purified by HPLC (Semi-preparative C18 Luna column, Eluent A: H₂O in isocratic condition, 5mL/min, Retention Time: 13.2 min) in order to obtain compound 1b (18 mg, 60% in two steps) as a white solid,[α]²⁵ D +16.2 (c: 0.5, H₂O); ¹H NMR (400 MHz, D₂O) δ: 2.29 (dd, 1H, J = 9.9, 15.3 Hz), 2.50 (dd, 1H, J = 6.1, 15.4 Hz), 3.05-3.10 (m, 1H), 3.31 (s, 3H), 3.54 (dd, 1H, J =
4.1, 13.3 Hz), 3.61 (d, 1H, \(J = 13.3\) Hz), 3.94 (d, 1H, \(J = 3.0\) Hz), 3.96 (d, 1H, \(J = 4.0\) Hz); \(^{13}\)C NMR (100MHz, D\(_2\)O) \(\delta\): 39.4, 44.6, 49.2, 56.0, 64.6, 82.8, 173.4, 178.8; ESI MS m/z: [M+H]\(^{+}\) calcd for C\(_{8}\)H\(_{14}\)NO\(_{5}\) 204.08, found (relative intensity) 204.1 (100) [M+H]\(^{+}\); HRMS calcd. for C\(_{8}\)H\(_{12}\)NO\(_{5}\): 202.0794, found: 202.0723.

**Methyl (2S,3R,4R)-3-[2-(tert-butoxy)-2-oxoethyl]-4-ethynyl-4-hydroxy-1-(9-phenyl-9H-fluoren-9-yl)pyrrolidine-2-carboxylate (9)**

To a solution of (trimethylsilyl)acetylene (0.31 mL, 2.2 mmol) in THF (8.0 mL), nBuLi (1.6 N in hexane, 1.4 mL, 2.4 mmol) was added dropwise at -10°C and the resulting mixture was stirred for 1 h. Ketone 5\(\alpha\) (1.0 g, 2.0 mmol) in THF (4.0 mL), was added, after 4 h at -10°C, the temperature was raised to 0°C and NaOH (208 mg, 5.2 mmol) in MeOH (4.0 mL) was added. The mixture was warmed to r.t., the pH was adjusted to 7 adding concentrated acetic acid and the solution was poured into H\(_2\)O (53 mL). The aqueous layer was extracted with EtOAc (3 x 100mL), the combined organic layers were dried over Na\(_2\)SO\(_4\), filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/ EtOAc 7:3) to give compound 9 (1.5 g, 70%) as a white solid. R\(f\) 0.45 (Hexane/EtOAc 7:3); \([\alpha]^{25}_{D} +180.2 \) (c: 1.1, CHCl\(_3\)); \(^{1}\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\): 1.22 (s, 9 H), 1.49 (dd, 1H, \(J = 10.0, 15.5\) Hz), 2.32-2.40 (m, 1H), 2.41 (s, 1H), 2.43 (dd, 1H, \(J = 2.5, 15.5\) Hz), 2.73 (d, 1H, \(J = 2.5\) Hz), 3.14 (d, 1H, \(J = 9.9\) Hz), 3.16 (s, 3H), 3.52 (d, 1H, \(J = 9.7\) Hz), 5.42 (bs, OH), 7.05 (td, 1H, \(J = 0.9, 7.5\) Hz), 7.12-7.24 (m, 5H), 7.32 (t, 1H, \(J = 7.5\) Hz), 7.41 (dd, 2H, \(J = 6.2, 13.6\) Hz), 7.48 (d, 2H, \(J = 6.9\) Hz), 7.55 (d, 1H, \(J = 7.5\) Hz), 7.70 (d, 1H, \(J = 7.5\) Hz); \(^{13}\)C NMR (100MHz, CDCl\(_3\)) \(\delta\): 27.8 (x 3), 38.4, 50.9, 52.2, 59.8, 64.0, 74.6, 75.2, 75.3, 81.0, 81.1, 120.0, 120.2, 126.4, 127.0, 127.2, 127.4 (x 2), 127.6, 127.8, 128.5 (x 2), 128.6, 129.0, 139.4, 141.0, 141.7, 144.5, 147.3, 170.2, 177.4; ESI MS m/z: [M+H]\(^{+}\) calcd for C\(_{33}\)H\(_{34}\)NO\(_{5}\) 524.24, found (relative intensity) 524.2 (100) [M+H]\(^{+}\).

**Methyl (2S,3R,4S)-3-[2-(tert-butoxy)-2-oxoethyl]-4-ethynyl-4-hydroxy-1-(9-phenyl-9H-fluoren-9-yl)pyrrolidine-2-carboxylate (10)**

To solution of alkyne 9 (570 mg, 1.0 mmol) and trimethylsilyl azide (0.17 mL, 1.2 mmol) in DMF/H\(_2\)O (4:1, 8.0 mL), CuSO\(_4\) (8.0 mg, 0.05 mmol) and sodium ascorbate (79.3 mg, 0.4 mmol) were added. The reaction mixture was placed in a microwave reactor and irradiated for 30 minutes at 120 °C. The solution was cooled, ice was added, and it was extracted with EtOAc (3 x 10 mL). The organic layers were washed with H\(_2\)O, brine, dried over Na\(_2\)SO\(_4\),
filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/EtOAc 1:1) to give compound 10 (397 mg, 70%) as a white solid. Rf 0.20 (Hexane/EtOAc 1:1); [α]D25 +101 (c: 1.0, CHCl3); 1H NMR (400 MHz, CDCl3) δ: 1.11 (s, 9 H), 1.42 (dd, 1H, J = 10.4, 15.9 Hz), 1.69 (dd, 1H, J = 5.3, 15.9 Hz), 2.42 (dd, 1H, J = 5.3, 10.4 Hz), 2.75 (d, 1H, J = 1.6 Hz), 3.24 (s, 3H), 3.53 (d, 1H, J = 9.7 Hz), 3.67 (d, 1H, J = 9.7 Hz), 5.89 (s, OH), 7.06 (td, 1H, J = 7.5 Hz), 7.12-7.23 (m, 4 H), 7.26 (d, 1H, J = 7.5 Hz), 7.37 (td, 1H, J = 1.2, 7.5 Hz), 7.44 (td, 1H, J = 1.1, 7.5 Hz), 7.53 (dd, 1H, J = 1.9, 7.1 Hz), 7.57 (d, 1H, J = 7.5 Hz), 7.65 (s, 1H), 7.73 (d, 1H, J = 7.0 Hz); 13C NMR (100MHz, CDCl3) δ: 27.7 (x 3), 38.3, 51.4, 52.3, 57.5, 64.5, 75.4, 78.0, 80.8, 120.0, 120.3, 126.5, 127.0, 127.2, 127.5 (x 2), 127.6, 128.0, 128.5 (x 2), 128.6, 129.0, 130.4, 130.8, 139.3, 141.0, 141.9, 144.4, 147.6, 169.9, 178.3; ESI MS m/z: [M+H]+ calced for C33H35N4O5 566.25, found (relative intensity) 566.2 (100) [M+H]+.

(2S,3R,4S)-3-(carboxymethyl)-4-hydroxy-4-(1H-1,2,3-triazol-4-yl)pyrrolidine-2-carboxylic acid (1c)

To a solution of compound 10 (124.0 mg, 0.22 mmol) in toluene (5 mL) at r.t. was added drop wise aqueous phosphoric acid (85 wt %, 0.05 mL, 1.11 mmol). The mixture was stirred for 2 h and the progress was checked by mass analysis {ESI MS m/z: [M+H]+ calced for C10H14N4O5 271.10, found (relative intensity) 271.1 (100) [M+H]+}. Once the reaction was completed, water was added (10 mL), and the mixture was washed with hexane (3 x 20 mL). The pH of the aqueous layer was adjusted to 7 (adding a solution of LiOH 0.5 N in water) and the solvent was evaporated under reduce pressure in order to obtain a pale yellow solid. The crude product was diluted with water (4 mL) and an aqueous solution of LiOH is added (0.5 N, 0.86 mL, 0.44 mmol). The mixture was stirred for 6 h at r.t. (progress was checked by mass analysis) then the pH is neutralised by addition of aqueous HCl (1N in water) and the solvent was evaporated under reduced pressure. The crude product was purified by HPLC (Semi-preparative C18 Luna column, Eluent A: H2O in isocratic condition, 5mL/min, Retention Time: 3.0 min) in order to obtain compound 1c (37 mg, 65% in two steps) as a white solid,[α]D25 -16.3 (c: 1.1, D2O); 1H NMR (400 MHz, D2O) δ: 2.44 (dd, 1H, J = 7.8, 17.1 Hz), 2.53 (dd, 1H, J = 7.0, 17.1 Hz), 3.38 (td, 1H, J = 4.2, 7.2 Hz), 3.80 (d, 1H, J = 12.6 Hz), 4.12 (d, 1H, J = 12.6 Hz), 4.56 (d, 1H, J = 3.8 Hz), 8.01 (s, 1H); 13C NMR (100MHz, D2O) δ: 39.6, 54.2, 58.7, 68.6, 82.5, 131.0, 149.1, 175.9, 179.0; MS (ESI), calculated m/z

S11
C₉H₁₂N₄O₅ 257.08 [M+H]+, found m/z (relative intensity) 257.0 (100) [M+H]+; HRMS calcd. for C₉H₁₂N₄O₅: 257.0808, found: 257.0884.

5-{(3aS,4S,6aR)-2-oxo-hexahydrothieno[3,4-d]imidazolidin-4-yl}-N-(20-azido 3,6,9,12,15,18-hexaoxaicosan-1-yl)pentanamide (12)

To a solution of D-(+)-biotin (100 mg, 0.40 mmol) and N-hydroxysuccinimide (51 mg, 0.44 mmol) in DMF (5.0 mL) was added EDC.HCl (92 mg, 0.2 mmol). After being stirred for 24 h at room temperature, the reaction solution was concentrated to give white solid. The solid was washed with methanol several times, and excess solvent was evaporated under reduced pressure. The crude product 11 was characterized by mass {ESI MS m/z: [M+H]+ calcd for C₁₄H₁₉N₃O₅S 341.10, found (relative intensity) 341.0 (100) [M+H]+} and submitted to the next step without further purification. To a solution of crude N-hydroxysuccinimido biotin (0.40 mmol) and O-(2-Aminoethyl)-O′-(2-azidoethyl)pentaethylene glycol (70 mg, 0.40 mmol) in DMF (2.0 mL) was added TEA (0.06 mL, 0.40 mmol). After being stirred for 24 h at room temperature, the solvent was evaporated under reduced pressure and the resulting residue was diluted in DCM (30 mL). The organic layer was washed with H₂O, dried over Na₂SO₄, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (MeOH/DCM 5:95) to give compound 12 (76.3 mg, 98%) as a white solid. Rf 0.52 (MeOH/DCM 10:90); ¹H NMR (400 MHz, CD₃OD) δ: 1.40-1.52 (m, 2H), 1.55-1.82 (m, 4H), 2.24 (t, 2H, J = 7.4 Hz), 2.73 (d, 1H, J = 12.7 Hz), 2.95 (dd, 1H, J = 5.0, 12.7 Hz), 3.23 (ddd, 1H, J = 4.6, 5.8, 9.0 Hz), 3.39 (dd, 4H, J = 5.4, 10.7 Hz), 3.56 (t, 2H, J = 5.6 Hz), 3.60-3.74 (m, 22H), 4.33 (dd, 1H, J = 4.5, 7.9 Hz), 4.51 (ddd, 1H, J = 0.7, 4.9, 7.9 Hz); ¹³C NMR (100MHz, CD₃OD) δ: 25.4, 28.1, 28.4, 35.3, 39.0, 39.7, 50.4, 55.6, 60.2, 62.0, 69.2, 69.7, 69.9, 70.1, 70.2 (x 6), 70.3 (x 2), 164.7, 174.7; ESI MS m/z: [M+H]+ calcd for C₂₄H₄₅N₆O₈S 576.29, [M+Na]+ calcd for C₂₄H₄₆N₆O₈S Na 599.28, found (relative intensity) 577.3 (45) [M+H]+, 599.3 (100) [M+Na]+.

Methyl (2S,3R,4S)-4-{1-(20-{5-{(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl}pentanamido}-3,6,9,12,15,18-hexaoxaicosan-1-yl)-1H-1,2,3-triazol-4-yl]-3-[2-(tert-butoxy)-2-oxoethyl]-4-hydroxy-1-(9-phenyl-9H-fluoren-9-yl)pyrrolidine-2-carboxylate (13)
To a solution of biotin azide 12 (54.7 mg, 0.13 mmol) and alkyne 9 (50.0 mg, 0.095 mmol) in tBuOH-H2O (1:1, 0.5 mL) and THF (0.5 mL) at r.t. were added CuSO4 (3.0 mg, 0.012 mmol) and sodium ascorbate (1.0 M in H2O, 3 drops). The reaction mixture was stirred at r.t. for 36 h and then the solvent was evaporated. The residue was diluted with H2O (2 mL) and the mixture was extracted with DCM (3 x 5 mL). The combined organic extract was washed with brine, dried over Na2SO4, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (MeOH/ DCM 5:95) to give compound 13 (87.7 mg, 83%) as a white solid. Rf 0.54 (MeOH/ DCM 10:90); 1H NMR (400 MHz, CD3OD) δ: 1.23 (s, 9 H), 1.39-1.52 (m, 3H), 1.54-1.81 (m, 5H), 2.23 (t, 2H, J = 6.1 Hz), 2.46 (dd, 1H, J = 4.5, 11.2 Hz), 2.71 (d, 1H, J = 12.7 Hz), 2.87 (d, 1H, J = 1.7 Hz), 2.93 (dd, 1H, J = 5.0, 12.7 Hz), 3.17-3.26 (m, 1H), 3.35-3.42 (m, 4 H), 3.49-3.73 (m, 24 H), 3.86 (d, 1H, J = 9.6 Hz), 3.88 (t, 2H, J = 5.0 Hz), 4.30 (dd, 1H, J = 4.6, 7.9 Hz), 4.49 (dd, 1H, J = 4.6, 8.2 Hz) 4.55 (dd, 2H, J = 4.1, 5.6 Hz) 7.18 (dd, 1H, J = 4.6, 7.9 Hz), 7.23-7.38 (m, 5H), 7.50 (td, 1H, J = 1.2, 7.5 Hz), 7.57 (td, 1H, J = 1.2, 7.5 Hz), 7.61 (d, 2H, J = 7.2 Hz), 7.74 (t, 2H, J = 7.1 Hz), 7.92 (d, 1H, J = 7.1 Hz), 8.02 (s, 1H); 13C NMR (100MHz, CD3OD) δ: 25.4, 26.7 (x 3), 28.1, 28.4, 35.3, 37.7, 39.0, 39.6, 50.1, 50.4, 51.4, 51.6, 55.6, 56.9, 60.2, 62.0, 64.6, 68.8, 69.2, 69.7, 69.9, 70.0, 70.2 (x 6), 75.5, 77.7, 80.5, 119.8, 120.1, 124.04, 126.7 (x 2), 126.3, 127.2 (x 2), 127.3, 127.7, 128.2 (x 2), 128.5, 129.0, 139.3, 141.5, 142.1, 144.4, 147.0, 147.7, 164.7, 170.1, 174.7, 178.3; ESI MS m/z: [M+H]+ calcd for C57H78N7O13S 1099.53, [M+Na]+ calcd for C57H77N7O13SNa 1122.52, found (relative intensity) 1100.4 (100) [M+H]+, 1122.4 (80) [M+Na]+.

(2S,3R,4S)-4-[1-(20-{5-[3aS,4S,6aR]-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]pentanamido}-1H-1,2,3-triazol-4-yl]-3-(carboxymethyl)-4-hydroxypyrrolidine-2-carboxylic acid (14)

To a solution of compound 13 (80.0 mg, 0.07 mmol) in DCM (1 mL) at r.t. was added drop wise aqueous phosphoric acid (85 wt %, 0.016 mL, 0.35 mmol). The mixture was stirred for 24 h and the progress was checked by mass analysis {ESI MS m/z: [M+H]+ calced for C34H58N7O13S 803.37, found (relative intensity) 804.3 (100) [M+H]+}. Once the reaction was completed, water was added (2 mL), and the mixture was washed with hexane (3 x 5 mL). The pH of the aqueous layer was adjusted to 7 (adding a solution of LiOH 0.5 N in water) and the solvent was evaporated under reduce pressure in order to obtain a pale yellow solid. The crude product was diluted with water (1 mL) and an aqueous solution of LiOH is added...
(0.5 N, 0.27 mL, 0.14 mmol). The mixture was stirred for 12 h at r.t. (progress was checked by mass analysis) then the pH is neutralised by addition of aqueous HCl (1N in water) and the solvent was evaporated under reduced pressure. The crude product was purified by HPLC (Semi-preparative C18 Luna column, Eluent A: H2O, Eluent B: CH3CN, method, linear gradient from 2% B to 40% B in 15 min, 5mL/min, Retention time: 11.7 min) in order to obtain compound 14 (31.5 mg, 50% in two steps) as a white solid. 1H NMR (400 MHz, D2O) δ: 1.29-1.39 (m, 2H), 1.44-1.72 (m, 4H), 2.20 (t, 2H, J = 7.3Hz), 2.32 (dd, 1H, J = 8.6, 16.9 Hz), 2.43 (dd, 1H, J = 5.9, 16.9 Hz), 2.70 (d, 1H, J = 13.0 Hz), 2.92 (dd, 1H, J = 5.0, 13.1 Hz), 3.11-3.19 (m, 1H), 3.21-3.29 (m, 1H), 3.31 (t, 2H, J = 5.3 Hz), 3.55 (t, 2H, J = 5.3 Hz), 3.51-3.64 (m, 20H), 3.67 (d, 1H, J = 12.6 Hz), 3.90 (t, 2H, J = 5.0 Hz), 3.98 (d, 1H, J = 12.6 Hz), 4.09 (d, 1H, J = 4.6 Hz), 4.35 (dd, 1H, J = 4.5, 7.9 Hz), 4.53 (dd, 1H, J = 4.5, 7.9 Hz), 4.57 (t, 2H, J = 5.0 Hz), 8.03 (s, 1H); 13C NMR (100MHz, D2O): 25.1, 27.7, 27.9, 35.4, 35.8, 38.9, 39.7, 49.8, 50.1, 53.6, 55.3, 60.2, 62.1, 65.1, 68.8, 68.9, 69.4, 69.6 (x 8), 69.7, 77.7, 124.9, 146.2, 165.3, 172.8, 174.8, 176.9; ESI MS m/z: [M+H]+ calcd for C33H56N7O13S 790.36, found (relative intensity) 790.3 (100) [M+H]+; HRMS calcd. for C33H56N7O13S: 790.3657, found: 790.3632.
$^1$H NMR of 5 Methyl (2S)-4-hydroxy-1-(9-phenyl-9H-fluoren-9-yl)pyrrolidine-2-carboxylate
$^1$H NMR of 4
$^1$H NMR of 5a
$^{13}$C NMR of 5a
$^1$H NMR of 5b
$^1$H NMR of 6
$^{19}$F NMR of 6
2D NOESY NMR EXPERIMENT OF COMPOUND 6
$^{19}$F - $^1$H HOESY NMR EXPERIMENT OF COMPOUND 6
$^1$H NMR of 1a
$^{19}$F NMR of 1a
$^1$H NMR of 7
\[ ^{13}\text{C} \text{NMR of 7} \]
2D NOESY NMR EXPERIMENT OF COMPOUND 7
\[ {^1}H \text{ NMR of 8} \]
$^{13}$C NMR of 8
$^1$H NMR of 1b
$^{13}$C NMR of 1b
$^1$H NMR of 9
$^{13}$C NMR of 1b
$^1$H NMR of 10
$^{13}$C NMR of 10
$^1$H NMR of 1c
$^{13}$C NMR of 1c
\(^1\text{H NMR of 12}\)
$^{13}C$ NMR of 12
$^1$H NMR of 13
$^{13}$C NMR of 13
$^1$H NMR of 14
$^{13}\text{C NMR of 14}$
Biological Testing of the Novel Compounds

Tissue harvest, preparation and recording techniques were as previously published (Bewick et al., 2005; Simon et al., 2010) and are only described here briefly. 4th deep lumbrical nerve-muscle preparations were removed from adult Sprague-Dawley rats (male, 278 – 419 g) after killing in accordance with U.K. Animals (Scientific Procedures) Act, 1986 and associated EU guidelines 2013. Experiments were carried out at room temperature (18 – 21°C) in physiological saline (Liley, 1956).

Muscles received 1mm (~10% muscle length) trapezoidal stretches for 5s and muscle spindle (stretch organ) nerve responses were recorded with silver-wire electrodes above the saline surface. Signals were amplified (A103, Isleworth Electronics, Isleworth, UK and 8102, CF Palmer, HighWycombe UK preamplifiers in series), and recorded using PC software (WCP, Strathclyde Electrophysiology Software, University of Strathclyde, UK). Test muscles were incubated 60 min in cumulative concentrations of ligands, before a drug-free wash. Control muscles had time-matched changes in drug-free saline.

The average stretch-evoked firing frequency was calculated from the first 0.5 s at the stretched length over 3 stretch cycles. The significance of difference (significance threshold P = 0.05) between, conditions was determined by two-way ANOVA with Bonferroni’s post-test.