SUPPORTING INFORMATION

Design, Synthesis and Pharmacological Evaluation of Fluorescent and Biotinylated Antagonists of ρ_1 $GABA_C$ Receptors

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Experimental

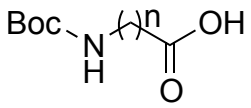
1) Chemistry

1.1 General Details:

All glassware was oven dried prior to use. All chemicals used were purchased from Aldrich Chemical Co. Ltd. (St Louis, MO) and were of highest commercially available purity. All solvents were distilled by standard techniques prior to use. Where stated, reactions were performed under an inert atmosphere of nitrogen. ^{1}H NMR spectra were recorded at 300 MHz and 400 MHz using a Varian (Palo Alto, CA) Gemini 300 and 400 spectrometer respectively. Chemical shifts (δ) are quoted in parts per million (ppm), referenced externally to tetramethyl silane at 0 ppm. ^{13}C NMR spectra were recorded at 100.5 MHz using a Varian (USA) 400 MI spectrometer. Chemical shifts (δ) are quoted in ppm, referenced internally to $CDCl_3$ at 77.0 ppm. Low and high-resolution electrospray ionization (ESI) MS was carried out using a Bruker (USA) Daltronics BioApexII with a 7T superconducting magnet and an analytical ESI source. All synthetic compounds for *in vitro* studies were ≥95% purity as determined by combustion analyses (within ± 0.4% of the calculated values), which were performed at the Research School of Chemistry, Australian National University, Canberra. Thin layer chromatography was performed on Merck aluminium backed plates, precoated with silica (0.2 mm, $60F_{254}$), which were developed using one of the following techniques: UV fluorescence (254 nm), alkaline potassium permanganate solution (0.5% w/v) or ninhydrin (0.2% w/v) and Iodine vapor. Flash chromatography was performed on silica gel (Merck silica gel 60H, particle size 5−40 μm).

1.2 Synthetic Experimental Procedures and Spectral Characterization:

General Procedure for the Synthesis of Boc-protected amino acids:



n = 1, 3, 4, 5, 6, 7, 10

The corresponding amino acid (1 gm, 1.0 equiv) and NaOH (1.0 equiv) was dissolved in a dioxane-H_2O (30 mL, 2:1) solution. This mixture was cooled to 0 °C and then di-*tert*-butyl dicarbonate (Boc_2O; 1.1 equiv) was added in three equal portions. The reaction mixture was stirred at room temperature for 4-16 h. The solution was concentrated under reduced pressure. The basic residue was redissolved in H_2O (50 mL) and washed twice with EtOAc (30 mL). The aqueous phase was acidified to pH 1-2 with aqueous 1 M HCl and then extracted with EtOAc (3 x 50 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford the desired Boc-protected amino acids as colorless oil, which slowly crystallized as colorless solid.

2-*tert*-Butyloxycarbonylamino acetic acid:

Yield 86%.^{1}H NMR (300 MHz, $CDCl_3$): δ 3.15 (br d, 2H), 1.44 (s, 9H). MS (ESI) *m*/*z* = 176.1 [M + 1].

4-*tert*-Butyloxycarbonylamino butanoic acid:

Yield 91%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.14-3.12 (m, 2H), 2.39-2.34 (t, 2H, *J* = 7.5 Hz), 1.84-1.75 (m, 2H), 1.42 (s, 9H). MS (ESI) *m*/*z* = 204.2 [M + 1].

5-*tert*-Butyloxycarbonylamino pentanoic acid:

Yield 94%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.13-3.09 (m, 2H), 2.38-2.33 (t, 2H, *J* = 7.2 Hz), 1.67-1.60 (m, 2H), 1.56-1.49 (m, 2H) 1.43 (s, 9H). MS (ESI) *m*/*z* = 218.2 [M + 1].

6-*tert*-Butyloxycarbonylamino hexanoic acid:

Yield 98%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.11-3.07 (m, 2H), 2.36-2.31 (t, 2H, *J* = 7.4 Hz), 1.67-1.61 (m, 2H), 1.54-1.43 (m, 2H), 1.44 (s, 9H), 1.43-1.32 (m, 2H). MS (ESI) *m*/*z* = 232.2 [M + 1].

7-*tert*-Butyloxycarbonylamino heptanoic acid:

Yield 94%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.12-3.09 (m, 2H), 2.36-2.31 (t, 2H, *J* = 7.5 Hz), 1.65-1.60 (m, 2H), 1.52-1.41 (m, 2H), 1.45 (s, 9H), 1.41-1.26 (m, 4H). MS (ESI) *m*/*z* = 246.2 [M + 1].

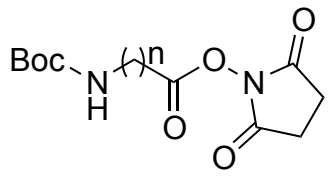
8-*tert*-Butyloxycarbonylamino octanoic acid:

Yield 92%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.11-3.08 (m, 2H), 2.34-2.30 (t, 2H, J = 7.4 Hz), 1.63-1.58 (m, 2H), 1.51-1.44 (m, 2H), 1.45 (s, 9H), 1.40-1.24 (m, 6H). MS (ESI) m/z = 260.2 [M + 1].

11-*tert*-Butyloxycarbonylamino undecanoic acid:

Yield 87%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.12-3.08 (m, 2H), 2.35-2.31 (t, 2H, J = 7.5 Hz), 1.63-1.58 (m, 2H), 1.53-1.42 (m, 2H), 1.44 (s, 9H), 1.42-1.22 (m, 12H). MS (ESI) m/z = 302.3 [M + 1].

General Procedure for the Synthesis of NHS esters (9a-g):



9a, n = 1; 9b, n = 3;
9c, n = 4; 9d, n = 5;
9e, n = 6; 9f, n = 7;
9g, n = 10

The corresponding Boc-protected amino acid (0.75-1 gm, 1 equiv) was dissolved in CH_2Cl_2 (50 mL). *N*-hydroxysuccinimide (1.5 equiv) and catalytic amount of DMAP (0.1 equiv) were added. The reaction mixture was cooled to 0 °C, then dicyclohexylcarbodiimide (1.5 equiv), dissolved in CH_2Cl_2 was added dropwise to the reaction mixture. The reaction mixture was stirred at 0 °C for 45 minutes and then stirred at room temperature for 16 h. The solution was filtered and the filtrate washed with 0.05N HCl (2 x 10 mL) and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was dissolved in a minimum volume of EtOAc and placed at -20 °C to allow urea to precipitate. Filtration and concentration afforded the desired compounds **9a-g** as a colorless solid.

2-*tert*-Butyloxycarbonylamino acetic acid succinimidyl ester (9a):

Yield 74%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.03 (br s, 1H), 4.29 (d, 2H), 2.85 (s, 4H), 1.43 (s, 9H). MS (ESI) m/z = 273.2 [M + 1].

4-*tert*-Butyloxycarbonylamino butanoic acid succinimidyl ester (9b):

Yield 77%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.62 (br s, 1H), 3.19-3.09 (m, 2H), 2.83 (s, 4H), 2.64-2.61 (t, 2H, J = 7.2 Hz), 1.88-1.76 (m, 2H), 1.44 (s, 9H). MS (ESI) m/z = 301.2 [M + 1].

5-*tert*-Butyloxycarbonylamino pentanoic acid succinimidyl ester (9c):

Yield 75%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.64 (br s, 1H), 3.18-3.07 (m, 2H), 2.83 (s, 4H), 2.63-2.60 (t, 2H, J = 7.5 Hz), 1.84-1.76 (m, 2H), 1.64-1.54 (m, 2H) 1.43 (s, 9H). MS (ESI) m/z = 315.2 [M + 1].

6-*tert*-Butyloxycarbonylamino hexanoic acid succinimidyl ester (9d):

Yield 83%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.62 (br s, 1H), 3.18-3.08 (m, 2H), 2.84 (s, 4H), 2.66-2.61 (t, 2H, J = 7.4 Hz), 1.82-1.73 (m, 2H), 1.63-1.53 (m, 2H), 1.43 (s, 9H), 1.41-1.34 (m, 2H). MS (ESI) m/z = 329.2 [M + 1].

7-*tert*-Butyloxycarbonylamino heptanoic acid succinimidyl ester (9e):

Yield 85%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.64 (br s, 1H), 3.21-3.12 (m, 2H), 2.83 (s, 4H), 2.63-2.60 (t, 2H, J = 7.4 Hz), 1.83-1.77 (m, 2H), 1.64-1.55 (m, 2H), 1.43 (s, 9H), 1.43-1.29 (m, 4H). MS (ESI) m/z = 343.3 [M + 1].

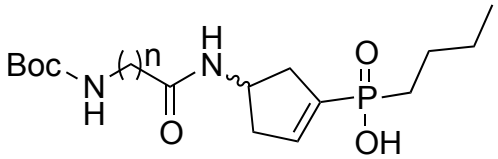
8-*tert*-Butyloxycarbonylamino octanoic acid succinimidyl ester (9f):

Yield 81%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.66 (br s, 1H), 3.22-3.11 (m, 2H), 2.85 (s, 4H), 2.66-2.61 (t, 2H, J = 7.5 Hz), 1.85-1.76 (m, 2H), 1.62-1.51 (m, 2H), 1.44 (s, 9H), 1.39-1.25 (m, 6H). MS (ESI) m/z = 357.3 [M + 1].

11-*tert*-Butyloxycarbonylamino undecanoic acid succinimidyl ester (9g):

Yield 87%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.64 (br s, 1H), 3.19-3.10 (m, 2H), 2.84 (s, 4H), 2.61-2.58 (t, 2H, J = 7.4 Hz), 1.83-1.75 (m, 2H), 1.61-1.50 (m, 2H), 1.43 (s, 9H), 1.44-1.21 (m, 12H). MS (ESI) m/z = 399.4 [M + 1].

General Procedure for Synthesis of Amides from (*S*)-4-ACPBPA (6) or (*R*)-4-ACPBPA (7):



n = 1, 3, 4, 5, 6, 7, 10

A solution of NHS esters (1 equiv) in DME (5 mL) was added to a solution of (*S*)-4-ACPBPA **6** or (*R*)-4-ACPBPA **7** (1 equiv) and $NaHCO_3$ (2 equiv.) in water (5 mL) at room temperature. THF (1.5 mL)

was added to increase solubility. The mixture was stirred at room temperature for 16 h and then 15% citric acid was added. The suspension was extracted with 10% ^{i}PrOH/EtOAc (4 x 20 mL) then organic layer washed with water and brine solution. The combined organic extracts were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford an off-white solid. The product triturated with ether and the resulting white solid was collected by filtration to afford corresponding amide.

((*S*)-4-(2-((*tert*-Butoxycarbonyl)amino)acetamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid:
Yield 82%. ^{1}H NMR (300 MHz, CD_3OD): δ 6.25 (br d, 1H, J = 10.0 Hz), 4.01 (d, 2H), 3.54 (m, 1H), 2.48 (dd, 2H, J = 7.2, 18.2 Hz), 2.16 (dd, 2H, J = 3.2, 18.2 Hz), 1.46 (m, 2H), 1.44 (s, 9H), 1.25-1.15 (m, 4H), 0.91 (t, 3H, J = 7.2 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 172.93, 158.46, 137.56 (d), 133.12 (d), 78.31, 47.83, 45.14 (d), 35.83 (d), 35.51 (d), 29.03, 26.28 (d), 24.92 (d), 23.53 (d), 14.11. MS (ESI) m/z = 361.3 [M + 1].

((*S*)-4-(4-((*tert*-Butoxycarbonyl)amino)butanamido)cyclopent-1-en-1-yl)(butyl)-phosphinic acid:
Yield 85%. ^{1}H NMR (300 MHz, CD_3OD): δ 6.24 (br d, 1H, J = 9.8 Hz), 3.56 (m, 1H), 3.25-3.19 (m, 2H), 2.49 (dd, 2H, J = 7.2, 18.2 Hz), 2.25-2.11 (m, 4H), 1.87-1.76 (m, 2H), 1.46 (m, 2H), 1.44 (s, 9H), 1.25-1.15 (m, 4H), 0.92 (t, 3H, J = 7.2 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 172.21, 157.53, 137.54 (d), 133.12 (d), 77.22, 45.17 (d), 41.53, 35.90 (d), 35.54 (d), 34.02, 28.71, 26.29 (d), 24.92 (d), 24.63, 23.52 (d), 13.95. MS (ESI) m/z = 389.3 [M + 1].

((*S*)-4-(5-((*tert*-Butoxycarbonyl)amino)pentanamido)cyclopent-1-en-1-yl)(butyl)-phosphinic acid:
Yield 84%. ^{1}H NMR (300 MHz, CD_3OD): δ 6.25 (br d, 1H, J = 10.0 Hz), 3.55 (m, 1H), 3.25-3.19 (m, 2H), 2.49 (dd, 2H, J = 7.2, 18.2 Hz), 2.27-2.12 (m, 4H), 1.86-1.75 (m, 2H), 1.62-1.41 (m, 4H), 1.43 (s, 9H), 1.25-1.14 (m, 4H), 0.91 (t, 3H, J = 7.2 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 172.21, 157.53, 137.54 (d), 133.12 (d), 78.22, 45.13 (d), 41.33, 35.83 (d), 36.12, 35.52 (d), 29.92, 26.29 (d), 25.32 (d), 24.98, 24.63, 24.52 (d), 13.95. MS (ESI) m/z = 403.3 [M + 1].

((*S*)-4-(6-((*tert*-Butoxycarbonyl)amino)hexanamido)cyclopent-1-en-1-yl)(butyl)-phosphinic acid:
Yield 85%. ^{1}H NMR (300 MHz, CD_3OD): δ 6.24 (br d, 1H, J = 10.2 Hz), 3.56 (m, 1H), 3.24-3.17 (m, 2H), 2.49 (dd, 2H, J = 7.4, 18.0 Hz), 2.28-2.12 (m, 4H), 1.86-1.75 (m, 2H), 1.65-1.42 (m, 4H), 1.44-1.34 (m, 2H), 1.43 (s, 9H), 1.23-1.14 (m, 4H), 0.92 (t, 3H, J = 7.2 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 172.19, 157.51, 137.52 (d), 133.12 (d), 77.92, 46.01 (d), 42.13, 35.85 (d), 36.13, 35.61 (d), 31.37, 26.29 (d), 26.22, 26.03, 25.93, 24.72 (d), 23.86 (d), 13.93. MS (ESI) m/z = 417.4 [M + 1].

((*R*)-4-(6-((*tert*-Butoxycarbonyl)amino)hexanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid:

Yield 81%, off-white solid. ^{1}H NMR (300 MHz, CD_3OD): δ 6.25 (br d, 1H, J = 10.2 Hz), 3.56 (m, 1H), 3.26-3.18 (m, 2H), 2.49 (dd, 2H, J = 7.1, 18.1 Hz), 2.28-2.13 (m, 4H), 1.87-1.75 (m, 2H), 1.65-1.42 (m, 4H), 1.44-1.34 (m, 2H), 1.44 (s, 9H), 1.23-1.15 (m, 4H), 0.91 (t, 3H, J = 7.2 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 172.20, 157.51, 137.53 (d), 133.12 (d), 77.92, 46.03 (d), 42.13, 35.85 (d), 36.13, 35.61 (d), 31.37, 26.29 (d), 26.22, 26.03, 25.93, 24.72 (d), 23.86 (d), 13.93. MS (ESI) m/z = 417.4 [M + 1].

((*S*)-4-(7-((*tert*-Butoxycarbonyl)amino)heptanamido)cyclopent-1-en-1-yl)(butyl)-phosphinic acid:

Yield 87%. ^{1}H NMR (300 MHz, CD_3OD): δ 6.25 (br d, 1H, J = 10.4 Hz), 3.58 (m, 1H), 3.23-3.16 (m, 2H), 2.44 (dd, 2H, J = 7.0, 18.4 Hz), 2.26-2.14 (m, 4H), 1.89-1.78 (m, 2H), 1.66-1.42 (m, 4H), 1.45-1.34 (m, 2H), 1.43 (s, 9H), 1.34-1.14 (m, 6H), 0.92 (t, 3H, J = 7.0 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 172.17, 157.24, 137.52 (d), 133.14 (d), 78.31, 44.33 (d), 41.98, 35.84 (d), 36.13, 35.65 (d), 31.57, 26.31 (d), 26.10, 26.06, 25.97, 25.73, 24.99 (d), 23.51 (d), 13.91. MS (ESI) m/z = 431.4 [M + 1].

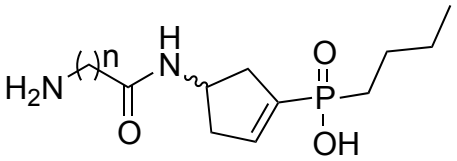
((*S*)-4-(8-((*tert*-Butoxycarbonyl)amino)octanamido)cyclopent-1-en-1-yl)(butyl)-phosphinic acid:

Yield 86%. ^{1}H NMR (300 MHz, CD_3OD): δ 6.25 (br d, 1H, J = 10.0 Hz), 3.60-3.52 (m, 1H), 3.20-3.14 (m, 2H), 2.46 (dd, 2H, J = 7.2, 18.4 Hz), 2.29-2.18 (m, 4H), 1.87-1.77 (m, 2H), 1.65-1.46 (m, 4H), 1.46-1.37 (m, 2H), 1.43 (s, 9H), 1.39-1.14 (m, 8H), 0.92 (t, 3H, J = 7.4 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 172.17, 157.26, 138.84 (d), 133.63 (d), 77.94, 45.56 (d), 42.02, 35.97 (d), 35.89, 35.79 (d), 31.57, 27.12 (d), 26.17, 25.96, 25.72, 25.54, 25.35, 25.01 (d), 23.77 (d), 13.91. MS (ESI) m/z = 445.4 [M + 1].

((*S*)-4-(11-((*tert*-Butoxycarbonyl)amino)undecanamido)cyclopent-1-en-1-yl)(butyl)-phosphinic acid:

Yield 89%.^{1}H NMR (300 MHz, CD_3OD): δ 6.24 (br d, 1H, J = 10.2 Hz), 3.58-3.50 (m, 1H), 3.15-3.09 (m, 2H), 2.49 (dd, 2H, J = 7.2, 18.4 Hz), 2.27-2.19 (m, 4H), 1.86-1.77 (m, 2H), 1.65-1.46 (m, 4H), 1.44 (s, 9H), 1.47-1.09 (m, 16H), 0.94 (t, 3H, J = 7.2 Hz). ^{13}C NMR (75 MHz, CD_3OD): δ 171.94, 157.03, 139.05 (d), 134.12 (d), 78.33, 45.65 (d), 42.81, 36.52 (d), 35.86, 35.83 (d), 32.24, 28.01 (d), 27.35, 27.83, 26.77, 26.53, 26.04, 25.68, 25.57, 25.23, 25.21 (d), 23.73 (d), 13.93. MS (ESI) m/z = 487.5 [M + 1].

Synthesis of Compounds (*S*)-10a-g and (*R*)-10d



(*S*)-10a, n = 1; (*S*)-10b, n = 3;
(*S*)-10c, n = 4; (*S*)-10d, n = 5;
(*R*)-10d, n = 5; (*S*)-10e, n = 6;
(*S*)-10f, n = 7; (*S*)-10g, n = 10

((*S*)-4-(2-Aminoacetamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-10a]:

((*S*)-4-(2-((*tert*-Butoxycarbonyl)amino)acetamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid (150 mg) was dissolved in the mixture of TFA and CH_2Cl_2 (1:1, 5 mL). The reaction mixture was stirred at room temperature for 1-2 h and then concentrated under reduced pressure. The residue was triturated and crystallized with ether to afford compound **(*S*)-10a** (102 mg, 95% yield) as an off-white solid. ^{1}H NMR (300 MHz, CD_3OD): δ 6.27 (br d, 1H, J = 10.0 Hz), 4.08 (d, 2H), 3.57 (m, 1H), 2.51 (dd, 2H, J = 7.4, 18.0 Hz), 2.16 (dd, 2H, J = 3.6, 18.0 Hz), 1.42 (m, 2H), 1.25-1.15 (m, 4H), 0.92 (t, 3H, J = 7.2 Hz). MS (ESI) m/z = 261.1 [M + 1].

Compounds **(*S*)-10b-g and (*R*)-10d** were synthesized using the same procedure described for the preparation of compound **(*S*)-10a** using appropriate starting materials.

((*S*)-4-(4-Aminobutanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-10b]:

Yield 97%. ^{1}H NMR (300 MHz, CD_3OD): δ 6.26 (br d, 1H, J = 10.2 Hz), 3.60 (m, 1H), 3.25-3.19 (m, 2H), 2.49 (dd, 2H, J = 7.2, 18.2 Hz), 2.25-2.11 (m, 4H), 1.87-1.76 (m, 2H), 1.46 (m, 2H), 1.25-1.15 (m, 4H), 0.91 (t, 3H, J = 7.4 Hz). MS (ESI) m/z = 289.2 [M + 1].

((*S*)-4-(5-Aminopentanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-10c]:

Yield 95%. ^{1}H NMR (300 MHz, CD_3OD): δ 6.28 (br d, 1H, J = 10.4 Hz), 3.59 (m, 1H), 3.25-3.17 (m, 2H), 2.50 (dd, 2H, J = 7.0, 18.6 Hz), 2.27-2.12 (m, 4H), 1.86-1.73 (m, 2H), 1.62-1.41 (m, 4H), 1.25-1.14 (m, 4H), 0.91 (t, 3H, J = 7.0 Hz). MS (ESI) m/z = 303.2 [M + 1].

((*S*)-4-(6-Aminohexanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-10d]:

Yield 98%. ^{1}H NMR (300 MHz, CD_3OD): δ 6.29 (br d, 1H, J = 10.2 Hz), 3.62 (m, 1H), 3.24-3.17 (m, 2H), 2.50 (dd, 2H, J = 7.0, 18.4 Hz), 2.28-2.12 (m, 4H), 1.86-1.75 (m, 2H), 1.65-1.42 (m, 4H), 1.44-1.34 (m, 2H), 1.23-1.14 (m, 4H), 0.92 (t, 3H, J = 7.0 Hz). MS (ESI) m/z = 317.3 [M + 1].

((*R*)-4-(6-Aminohexanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*R*)-10d]:

Yield 94%, off-white solid. ^{1}H NMR (300 MHz, CD_3OD): δ 6.30 (br d, 1H, J = 10.2 Hz), 3.63 (m, 1H), 3.25-3.18 (m, 2H), 2.50 (dd, 2H, J = 7.0, 18.2 Hz), 2.30-2.15 (m, 4H), 1.86-1.75 (m, 2H), 1.66-1.44 (m, 4H), 1.44-1.34 (m, 2H), 1.25-1.13 (m, 4H), 0.92 (t, 3H, J = 7.1 Hz). MS (ESI) m/z = 317.3 [M + 1].

((*S*)-4-(7-Aminoheptanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-10e]:

Yield 95%. ^{1}H NMR (300 MHz, CD_3OD): δ 6.27 (br d, 1H, J = 10.4 Hz), 3.62 (m, 1H), 3.23-3.16 (m, 2H), 2.47 (dd, 2H, J = 7.2, 18.4 Hz), 2.26-2.14 (m, 4H), 1.86-1.78 (m, 2H), 1.66-1.42 (m, 4H), 1.45-1.34 (m, 2H), 1.34-1.14 (m, 6H), 0.91 (t, 3H, J = 7.4 Hz). MS (ESI) m/z = 331.3 [M + 1].

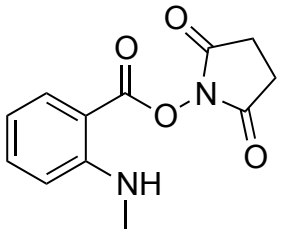
((*S*)-4-(8-Aminooctanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-10f]:

Yield 92%. ^{1}H NMR (300 MHz, CD_3OD): δ 6.28 (br d, 1H, J = 10.2 Hz), 3.67 (m, 1H), 3.20-3.14 (m, 2H), 2.51 (dd, 2H, J = 7.2, 18.2 Hz), 2.29-2.18 (m, 4H), 1.87-1.77 (m, 2H), 1.65-1.46 (m, 4H), 1.46-1.37 (m, 2H), 1.39-1.12 (m, 8H), 0.92 (t, 3H, J = 7.2 Hz). MS (ESI) m/z = 345.3 [M + 1].

((*S*)-4-(11-Aminoundecanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-10g]:

Yield 94%.^{1}H NMR (300 MHz, CD_3OD): δ 6.29 (br d, 1H, J = 10.4 Hz), 3.65 (m, 1H), 3.15-3.06 (m, 2H), 2.47 (dd, 2H, J = 7.0, 18.2 Hz), 2.27-2.19 (m, 4H), 1.86-1.77 (m, 2H), 1.65-1.46 (m, 4H), 1.47-1.07 (m, 16H), 0.92 (t, 3H, J = 7.2 Hz). MS (ESI) m/z = 387.4 [M + 1].

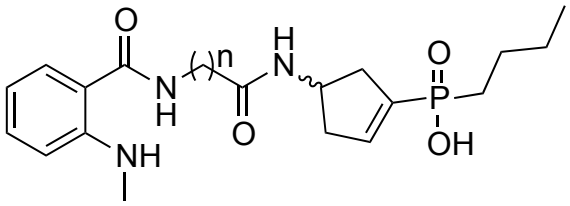
Synthesis of *N*-Methyl Anthranilic acid Succinimidyl Ester:



N-Methyl anthranilic acid succinimidyl ester was synthesized from *N*-methyl anthranilic acid using the same general procedure described for the preparation of compound **9a**.

Yield 81%; yellow color solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.05 (d, 1H, J = 8.1 Hz), 7.50 (t, 1H, J = 8.7 Hz), 6.78 (d, 1H, J = 8.7 Hz), 6.69 (t, 1H, J = 8.4 Hz), 6.28 (br s, 1H), 2.93 (s, 3H), 2.90 (s, 4H). MS (ESI) m/z = 249.1 [M + 1].

Synthesis of Target Compounds [(*S*)-11a-g and [(*R*)-11d



(*S*)-11a, n = 1; (*S*)-11b, n = 3;
(*S*)-11c, n = 4; (*S*)-11d, n = 5;
(*R*)-11d, n = 5; (*S*)-11e, n = 6;
(*S*)-11f, n = 7; (*S*)-11g, n = 10

((*S*)-4-(2-(2-(Methylamino)benzamido)acetamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-11a]:

A solution of *N*-methyl anthranilic acid succinimidyl ester (86 mg, 1 equiv) in DME (5 mL) was added to a suspension of ((*S*)-4-(2-aminoacetamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid **(*S*)-10a** (90 mg, 1 equiv) and $NaHCO_3$ (58 mg, 2 equiv) in water (5 mL) at room temperature. THF (1.5 mL) was added to increase solubility. The mixture was stirred at room temperature for 16 h and then 15% citric acid was added. The suspension was extracted with 10% ^{i}PrOH/EtOAc (4 x 20 mL) and the combined organic extracts washed with water and brine solution, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford a colorless solid. The crude was purified by flash silica column chromatography (5% methanolic ammonia solution:EtOAc) to give desired product. The product was further triturated with ether and the resulting off white solid was collected by filtration to afford compound **(*S*)-11a** (102 mg, 75%).

^{1}H NMR (300 MHz, CD_3OD): δ 8.08 (d, 1H, J = 8.1 Hz), 7.47 (t, 1H, J = 8.8 Hz), 6.84 (d, 1H, J = 8.8 Hz), 6.72 (t, 1H, J = 8.6 Hz), 6.24 (br d, 1H, J = 10.0 Hz), 4.03 (d, 2H), 3.59 (m, 1H), 2.96 (s, 3H), 2.48 (dd, 2H, J = 7.4, 18.2 Hz), 2.21 (dd, 2H, J = 3.8, 18.0 Hz), 1.42 (m, 2H), 1.25-1.16 (m, 4H), 0.92 (t, 3H, J = 7.2 Hz). MS (ESI) m/z = 394.3 [M + 1].

Compounds **(*S*)-11b-g** and **(*R*)-11d** were synthesized using the same procedure described for the preparation of compound **(*S*)-11a** using appropriate starting materials.

((*S*)-4-(4-(2-(Methylamino)benzamido)butanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid

[(*S*)-11b]:

Yield 79%; off white solid. ^{1}H NMR (300 MHz, CD_3OD): δ 8.10 (d, 1H, J = 8.2 Hz), 7.49 (t, 1H, J = 8.8 Hz), 6.82 (d, 1H, J = 8.6 Hz), 6.73 (t, 1H, J = 8.8 Hz), 6.24 (br d, 1H, J = 10.2 Hz), 3.55 (m, 1H), 3.25-3.19 (m, 2H), 2.95 (s, 3H), 2.49 (dd, 2H, J = 7.4, 18.0 Hz), 2.25-2.11 (m, 4H), 1.87-1.76 (m, 2H), 1.46 (m, 2H), 1.25-1.15 (m, 4H), 0.92 (t, 3H, J = 7.4 Hz). MS (ESI) m/z = 422.4 [M + 1].

((*S*)-4-(5-(2-(Methylamino)benzamido)pentanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-11c]:

Yield 83%; light yellow color solid. ^{1}H NMR (300 MHz, CD_3OD): δ 8.08 (d, 1H, J = 8.4 Hz), 7.49 (t, 1H, J = 8.7 Hz), 6.83 (d, 1H, J = 8.8 Hz), 6.70 (t, 1H, J = 8.6 Hz), 6.25 (br d, 1H, J = 10.2 Hz), 3.53 (m, 1H), 3.25-3.17 (m, 2H), 2.97 (s, 3H), 2.50 (dd, 2H, J = 7.2, 18.4 Hz), 2.26-2.10 (m, 4H), 1.86-1.71 (m, 2H), 1.62-1.41 (m, 4H), 1.25-1.14 (m, 4H), 0.91 (t, 3H, J = 7.2 Hz). MS (ESI) m/z = 436.4 [M + 1].

((*S*)-4-(6-(2-(Methylamino)benzamido)hexanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-11d]:

Yield 80%; off-white solid. ^{1}H NMR (300 MHz, CD_3OD): δ 8.10 (d, 1H, J = 8.2 Hz), 7.50 (t, 1H, J = 8.7 Hz), 6.85 (d, 1H, J = 8.7 Hz), 6.71 (t, 1H, J = 8.8 Hz), 6.23 (br d, 1H, J = 10.0 Hz), 3.55 (m, 1H), 3.24-3.15 (m, 2H), 2.96 (s, 3H), 2.49 (dd, 2H, J = 7.2, 18.0 Hz), 2.28-2.12 (m, 4H), 1.86-1.75 (m, 2H), 1.65-1.42 (m, 4H), 1.46-1.34 (m, 2H), 1.25-1.13 (m, 4H), 0.92 (t, 3H, J = 7.1 Hz). MS (ESI) m/z = 450.4 [M + 1].

((*R*)-4-(6-(2-(Methylamino)benzamido)hexanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*R*)-11d]:

Yield 77%, off-white solid. ^{1}H NMR (300 MHz, CD_3OD): δ 8.09 (d, 1H, J = 8.1 Hz), 7.49 (t, 1H, J = 8.8 Hz), 6.85 (d, 1H, J = 8.7 Hz), 6.72 (t, 1H, J = 8.8 Hz), 6.24 (br d, 1H, J = 10.0 Hz), 3.55 (m, 1H), 3.24-3.14 (m, 2H), 2.95 (s, 3H), 2.49 (dd, 2H, J = 7.2, 18.2 Hz), 2.30-2.15 (m, 4H), 1.87-1.75 (m, 2H), 1.65-1.42 (m, 4H), 1.46-1.34 (m, 2H), 1.28-1.15 (m, 4H), 0.91 (t, 3H, J = 7.1 Hz). MS (ESI) m/z = 450.4 [M + 1].

((*S*)-4-(7-(2-(Methylamino)benzamido)heptanamido)cyclopent-1-en-1-yl) (butyl)phosphinic acid [(*S*)-11e]:

Yield 84%; light yellow color solid. ^{1}H NMR (300 MHz, CD_3OD): δ 8.07 (d, 1H, J = 8.6 Hz), 7.52 (t, 1H, J = 8.8 Hz), 6.82 (d, 1H, J = 8.7 Hz), 6.74 (t, 1H, J = 8.8 Hz), 6.24 (br d, 1H, J = 10.4 Hz), 3.54 (m, 1H), 3.23-3.14 (m, 2H), 2.95 (s, 3H), 2.50 (dd, 2H, J = 7.1, 18.5 Hz), 2.26-2.14 (m, 4H), 1.86-1.77

(m, 2H), 1.66-1.42 (m, 4H), 1.46-1.34 (m, 2H), 1.37-1.14 (m, 6H), 0.92 (t, 3H, J = 7.1 Hz). MS (ESI) m/z = 464.5 [M + 1].

((*S*)-4-(8-(2-(Methylamino)benzamido)octanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-11f]:

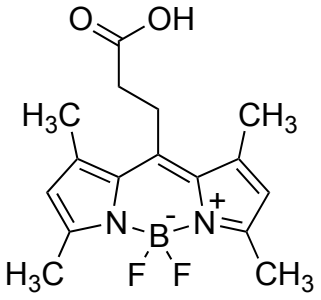
Yield 78%; light yellow color solid. ^{1}H NMR (300 MHz, CD_3OD): δ 8.10 (d, 1H, J = 8.2 Hz), 7.50 (t, 1H, J = 8.7 Hz), 6.83 (d, 1H, J = 8.8 Hz), 6.74 (t, 1H, J = 8.7 Hz), 6.25 (br d, 1H, J = 10.4 Hz), 3.55 (m, 1H), 3.22-3.14 (m, 2H), 2.96 (s, 3H), 2.50 (dd, 2H, J = 7.4, 18.4 Hz), 2.29-2.18 (m, 4H), 1.87-1.77 (m, 2H), 1.65-1.46 (m, 4H), 1.46-1.34 (m, 2H), 1.39-1.11 (m, 8H), 0.91 (t, 3H, J = 7.2 Hz). MS (ESI) m/z = 478.5 [M + 1].

((*S*)-4-(11-(2-(Methylamino)benzamido)undecanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid [(*S*)-11g]:

Yield 81%, light yellow color solid. ^{1}H NMR (300 MHz, CD_3OD): δ 8.09 (d, 1H, J = 8.2 Hz), 7.48 (t, 1H, J = 8.6 Hz), 6.81 (d, 1H, J = 8.4 Hz), 6.70 (t, 1H, J = 8.6 Hz), 6.23 (br d, 1H, J = 10.4 Hz), 3.53 (m, 1H), 3.18-3.06 (m, 2H), 2.95 (s, 3H), 2.49 (dd, 2H, J = 7.2, 18.2 Hz), 2.27-2.16 (m, 4H), 1.86-1.77 (m, 2H), 1.65-1.45 (m, 4H), 1.48-1.07 (m, 16H), 0.92 (t, 3H, J = 7.1 Hz). MS (ESI) m/z = 520.6 [M + 1].

Synthesis of BODIPY *N*-succinimidyl Ester:

3-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8-yl)propionic acid:[1]

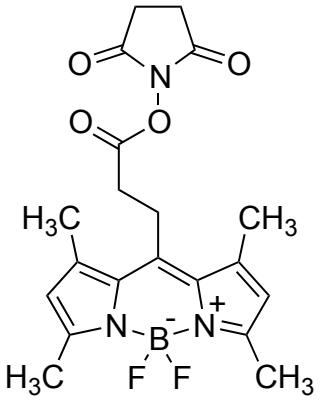


To a solution of succinic anhydride (300 mg, 1 equiv) in a mixture of CH_2Cl_2 (30 mL) and CH_3CN (10 mL) was added 2,4-dimethylpyrrole (713 mg, 2.5 equiv) at room temperature. The resulting mixture was heated to reflux under nitrogen for 8 h. After the solution was cooled to room temperature, 6 equiv of Et_3N (2.50 mL) was added slowly, and after 10 min, 8 equiv of $BF_3.OEt_2$ (2.96 mL) was added slowly. The reaction mixture was stirred under nitrogen at 50-55 °C for another 5 h. The mixture was quenched with water, extracted with CH_2Cl_2 (3 x 100 mL) and then the combined organic extracts

washed with water (3 x 15 mL) and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude was purified by flash silica column chromatography (20 to 50% EtOAc in hexane) to give 3-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8-yl)propionic acid (172 mg, 18% yield) as an orange red solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.08 (s, 2H), 3.37-3-28 (m, 2H), 2.72-2.63 (m, 2H), 2.56 (s, 6H), 2.45 (s, 6H). ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 177.11, 155.48, 142.85, 140.82, 131.47, 122.28, 35.28, 23.59, 16.71, 14.75. MS (ESI) m/z = 321.1 [M + 1].

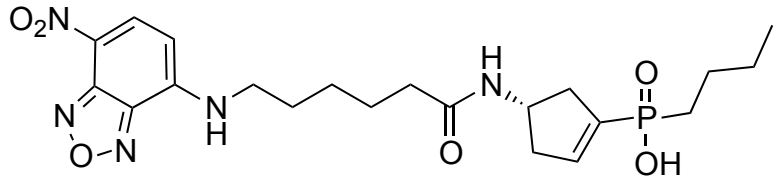
3-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8-yl)propionic acid succinimidyl ester:[1]



To a solution of 3-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8-yl)-propionic acid (150 mg, 1 equiv) in CH_3CN (10 mL) was added *N*-hydrosuccinimide (80 mg, 1.5 equiv) at room temperature. The reaction mixture was cooled to 0 °C, then dicyclohexylcarbodiimide (241 mg, 2.5 equiv) in 1 mL CH_3CN was added dropwise. The reaction mixture was stirred at 0 °C for 45 minutes and then stirred for 24 h at room temperature and then concentrated under reduced pressure at no more than 20 °C. The residue was dissolved and extracted in CH_2Cl_2 (3 x 50 mL), washed with water (2 x 15 mL), and dried over anhydrous Na_2SO_4, and the concentrated solution was purified by flash silica column chromatography (10-20% EtOAc in hexane). The crude compound was recrystallized from CH_2Cl_2 and hexane to afford BODIPY *N*-succinimidyl ester (168 mg, 86% yield) as an orange red solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.10 (s, 2H), 3.46-3.37 (m, 2H), 2.89 (s, 4H), 2.86-2.75 (m, 2H), 2.67 (s, 6H), 2.52 (s, 6H). ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 169.04, 167.47, 155.63, 142.98, 140.71, 131.47, 122.68, 37.53, 26.13, 23.59, 16.75, 14.98. MS (ESI) m/z = 418.2 [M + 1].

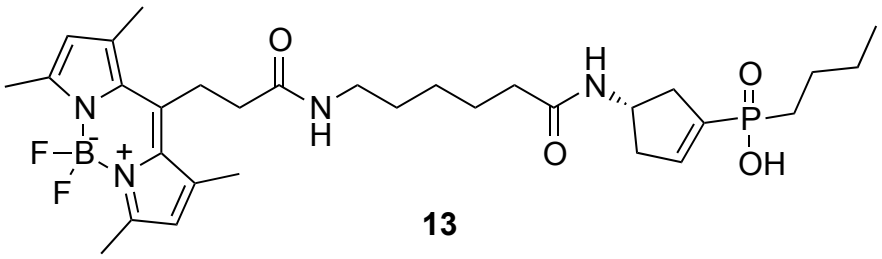
((*S*)-4-(6-((7-Nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino) hexanamido)-cyclopent-1-en-1-yl)(butyl)-phosphinic acid (12):



12

A solution of 7-nitrobenzo-2-oxa-1,3-diazol-4-yl chloride (NBD-chloride) (19 mg, 1.0 equiv) in anhydrous DMF (1.0 mL) was added dropwise to a stirred solution of ((*S*)-4-(6-aminohexanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid **(*S*)-10d** (36 mg, 1.2 equiv) and K_2CO_3 (27 mg, 2.0 equiv) in anhydrous DMF (2.0 mL). The dark solution was stirred with the exclusion of light for 24 h at room temperature. The reaction mixture was concentrated under reduced pressure and crude product purified by flash silica column chromatography, first eluting with $CHCl_3$ and then $CHCl_3$/5% methanolic ammonia (9:1) to give desired compound **12** (35 mg, 76% yield) as yellow solid. ^{1}H NMR (400 MHz, CD_3OD): δ 8.57 (d, 1H, J = 8.8 Hz), 6.48 (d, 1H, J = 8.8 Hz), 6.23 (br d, 1H, J = 10.2 Hz), 3.59 (m, 1H), 3.20-3.09 (m, 2H), 2.81-2.65 (m, 2H), 2.30-2.15 (m, 4H), 1.82-1.75 (m, 2H), 1.65-1.40 (m, 4H), 1.45-1.37 (m, 2H), 1.27-1.16 (m, 4H), 0.92 (t, 3H, J = 7.0 Hz). MS (ESI) m/z = 480.3 [M + 1].

((*S*)-4-(6-(3-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8-yl)propanamido) hexanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid (13):

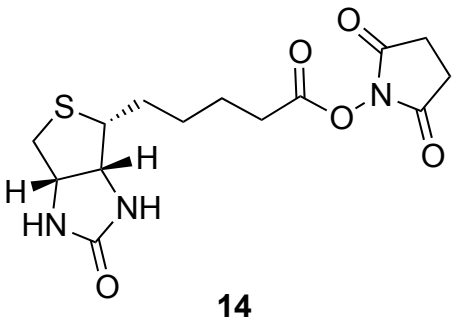


13

A solution of 3-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8-yl)-propionic acid succinimidyl ester (25 mg, 1.0 equiv) in anhydrous DMF (1.0 mL) was added dropwise to a stirred solution of ((*S*)-4-(6-aminohexanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid **(*S*)-10d** (23 mg, 1.2 equiv) and DIPEA (15 mg, 2.0 equiv) in anhydrous DMF (3.0 mL). The dark solution was stirred with the exclusion of light for 24 h at room temperature. The reaction mixture was concentrated under reduced pressure and crude product purified by flash silica column chromatography, first eluting with $CHCl_3$ and then $CHCl_3$/5% methanolic ammonia (8.5:1.5) to give desired compound **13** (26 mg, 72% yield) as brown solid.

^{1}H NMR (400 MHz, CD_3OD): δ 6.22 (br d, 1H, J = 9.8 Hz), 6.11 (s, 2H), 3.57 (m, 1H), 3.39 (t, 2H, J = 8.8 Hz), 3.18-3.08 (m, 2H), 2.79-2.65 (m, 4H), 2.69 (s, 6H), 2.55 (s, 6H), 2.31-2.15 (m, 4H), 1.84-1.78 (m, 2H), 1.63-1.45 (m, 4H), 1.49-1.35 (m, 2H), 1.29-1.13 (m, 4H), 0.91 (t, 3H, J = 7.4 Hz). MS (ESI) m/z = 619.4 [M + 1].

Synthesis of Biotin Succinimidyl Ester (14):



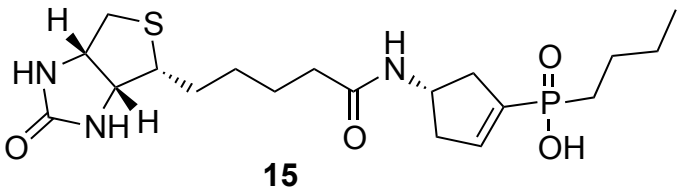
14

Biotin (300 mg, 1.0 equiv) was dissolved in DMF (8 mL) at room temperature and then *N*-hydroxysuccinimide (183 mg, 1.3 equiv), dicyclohexylcarbodiimide (304 mg, 1.2 equiv) and pyridine (0.10 mL, 1.0 equiv) were added to the colourless solution. The reaction mixture was stirred for 24 h at room temperature. The white urea was removed by filtration, the solution was concentrated under reduced pressure and then residue was passed through short silica column chromatography. The product was recrystallized from isopropanol to give biotin succinimidyl ester **14** (389 mg, 93% yield) as a white solid.

^{1}H NMR (400 MHz, DMSO): δ 6.44 (s, 1H), 6.37 (s, 1H), 4.29 (m, 1H), 4.15 (m, 1H), 3.11 (m, 1H), 2.82 (s, 4H), 2.81 (m, 1H), 2.67 (t, 2H, J = 7.5 Hz), 2.58 (d, 1H, J = 12.6 Hz), 1.67-1.41 (m, 6H). ^{13}C NMR (100.5 MHz, DMSO): δ 170.42, 162.85, 61.01, 59.28, 55.33, 30.08, 27.91, 27.68, 25.63. MS (ESI) m/z = 342.3 [M + 1].

Compounds **15** and **16** were synthesized using the same procedure described for the preparation of compound **13** using appropriate starting materials.

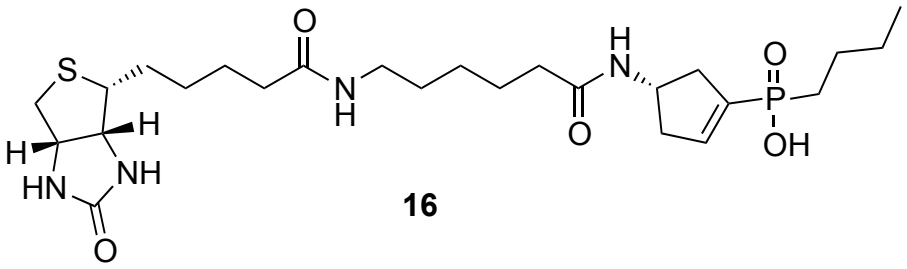
((*S*)-4-(5-((3a*R*, 4*R*, 6a*S*)-2-Oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)-pentanamido)cyclopent-1-en-1-yl)(butyl) phosphinic acid (15):



15

Yield 75%, off-white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 6.23 (br d, 1H, J = 9.8 Hz), 4.27 (m, 1H), 4.14 (m, 1H), 3.57 (m, 1H), 3.13 (m, 1H), 2.86-2.79 (m, 3H), 2.65 (t, 2H, J = 7.4 Hz), 2.60 (d, 1H, J = 12.4 Hz), 2.26 (dd, 2H, J = 3.8, 18.6 Hz), 1.67-1.40 (m, 8H). 1.25 (m, 4H), 0.91 (t, 3H, J = 7.4 Hz). MS (ESI) m/z = 430.4 [M + 1].

((*S*)-4-(6-(5-((3a*R*, 4*R*, 6a*S*)-2-Oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanamido)cyclopent-1-en-1-yl)(butyl)phosphinic acid (16):



16

Yield 81%, off white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 6.25 (br d, 1H, J = 9.4 Hz), 4.28 (m, 1H), 4.15 (m, 1H), 3.56 (m, 1H), 3.22-3.10 (m, 3H), 2.87-2.80 (m, 3H), 2.65 (t, 2H, J = 7.6 Hz), 2.59 (d, 1H, J = 12.6 Hz), 2.32-2.15 (m, 6H), 1.81-1.72 (m, 2H), 1.65-1.1.38 (m, 10H). 1.27 (m, 4H), 0.91 (t, 3H, J = 7.4 Hz). MS (ESI) m/z = 543.5 [M + 1].

1.3 Elemental Analysis:

Compound	Formula	Calculated	Found
(*S*)-11a	$C_{19}H_{28}N_3O_4P.2H_2O$	C, 53.14; H, 7.51; N, 9.78	C, 53.16; H, 7.47; N, 9.77
(*S*)-11b	$C_{21}H_{32}N_3O_4P.H_2O$	C, 57.39; H, 7.80; N, 9.56	C, 57.41; H, 7.77; N, 9.56
(*S*)-11c	$C_{22}H_{34}N_3O_4P.4H_2O$	C, 52.06; H, 8.34; N, 8.28	C, 52.09; H, 8.38; N, 8.27
(*S*)-11d	$C_{23}H_{36}N_3O_4P.H_2O$	C, 59.09; H, 8.19; N, 8.99	C, 59.11; H, 8.23; N, 9.03
(*R*)-11d	$C_{23}H_{36}N_3O_4P.H_2O$	C, 59.09; H, 8.19; N, 8.99	C, 59.10; H, 8.24; N, 9.01
(*S*)-11e	$C_{24}H_{38}N_3O_4P.3H_2O$	C, 55.69; H, 8.57; N, 8.12	C, 55.70; H, 8.61; N, 8.11
(*S*)-11f	$C_{25}H_{40}N_3O_4P.2H_2O$	C, 58.46; H, 8.63; N, 8.18	C, 58.48; H, 8.60; N, 8.20
(*S*)-11g	$C_{28}H_{46}N_3O_4P.2H_2O$	C, 60.52; H, 9.07; N, 7.56	C, 60.56; H, 9.11; N, 7.58
12	$C_{21}H_{30}N_5O_6P.2H_2O$	C, 48.93; H, 6.65; N, 13.59	C, 48.96; H, 6.69; N, 13.62
13	$C_{31}H_{46}BF_2N_4O_4P.H_2O$	C, 58.50; H, 7.60; N, 8.80	C, 58.53; H, 7.64; N, 8.83
15	$C_{19}H_{32}N_3O_4PS.H_2O$	C, 50.99; H, 7.66; N, 9.39	C, 51.03; H, 7.69; N, 9.41
16	$C_{25}H_{43}N_4O_5PS.H_2O$	C, 53.55; H, 8.09; N, 9.99	C, 53.58; H, 8.12; N, 10.01

2) Pharmacology

2.1 General Details and Materials:

Pharmacological experiments were performed as previously described.[2-3]

Human $GABA_C$ ρ_1 DNA encapsulated into the vector pcDNA1.1 (Invitrogen, CA) was donated by Dr. George Uhl (National Institute for Drug Abuse, Baltimore, MD). Human α_1, β_2, and γ_{2L} $GABA_A$ cDNAs encapsulated into pcDM8 were gifts from Dr Paul Whiting (Merck Sharpe and Dohme, Harlow, UK). *Xenopus laevis* were obtained from South African *Xenopus* colony and housed in the Department of Veterinary Science, University of Sydney.

2.2 Expression of Recombinant Human GABA Receptors in *Xenopus* Oocytes:

Female *Xenopus laevis* were anaesthetised with 0.17% ethyl 3-aminobenzoic acid ethyl ester and a lobe of an ovary was removed and rinsed with oocyte releasing buffer, OR2 (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5). It was then treated with Collagenase A (2 mg/ml in OR2 solution, Bohringer Manheim) for 2 h. The released oocytes were rinsed in ND96 storage solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, 2.5 mM pyruvate, 0.5 mM theophylline, 50 ng/mL gentamycin, pH 7.5). Stage V−VI oocytes were collected and stored at 18 °C in ND96 storage solution with constant mixing in an orbital shaker.

Plasmids containing the ρ_1, α_1, and γ_{2L}, were linearized with restriction enzyme Xba1, β_2 was expelled from the vector using a double digest of Sac1 and Sma1. cRNA was synthesized using the "mMessage mMachine" kit from Ambion (Austin, TX).

ρ_1 cRNA (2 ng/nL), and $\alpha_1\beta_2\gamma_{2L}$ cRNAs (8 ng/nL) in a 1:1:2 ratios were injected into the cytoplasm of defolliculated Stage V *Xenopus* oocytes. Oocytes were stored at 18 °C in ND96 solution with constant mixing in an orbital shaker for 1-5 days.

2.3 Electrophysiological Recording:

Two to eight days after injection of the oocyte with mRNA, receptor activity was measured by two-electrode voltage clamp recording using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA), a MacLab 2e recorder (AD Instruments, Sydney, NSW, Australia), and Chart version 5.5.6 program. Oocytes were voltage clamped at -60 mV and continuously superfused with ND96 recording solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5) at a rate of 5 mL/min. For receptor activation measurements, the indicated concentrations of drug were added to the buffer solution. For $GABA_A$ containing oocytes, a washout period of 7 min was allowed to minimize desensitization.

Recombinant GABA receptors where expressed in *Xenopus laevis* oocytes with similar properties to those reported earlier, these were: homomeric ρ_1,[4] and heteromeric $\alpha_1\beta_2\gamma_{2L}$ receptors.[5] The voltage potential was clamped at -60 mV, and the amplitudes of the whole-cell currents varied upon receptor

opening. For all receptors, the currents ranged from 200 to 1000 nA. Compounds were screened for agonist activity by applying increasing concentrations of the compound to the cell bath until the maximal current was attained with and without pre-incubation. Compounds were also screened for antagonist effects by testing the compound in the presence of a submaximal concentration of GABA (1 μM for $GABA_C$ and 30 μM for $GABA_A$) with and without pre-incubation. The effects of antagonists were evaluated for their inhibitory concentration-response actions. For each drug, concentration-response curves were constructed with a minimum of three cells to ensure that an accurate and replicable concentration-response relationship was produced.

2.4 Time Course Experiments (Pre-incubation): The time required for maximal inhibition of the compounds was determined by pre-incubating the compounds with GABA at 0, 1, 3, 5, and 10 min. Since there was no difference in activity between 5 and 10 min, all compounds were pre-incubated for 5 min before the addition of GABA in all the experiments described.

2.5 Data Analysis:

The amplitude of the current (I) recorded in response to each drug concentration [A] was normalized to the maximum amplitude (Imax) of the current response to GABA (ρ_1, 100 μM; $\alpha_1\beta_2\gamma_{2L}$, 300 μM).

IC_{50} values were calculated *via* non-linear regression from concentration-response data by fitting ratios of maximal GABA current as a function of agonist concentration by least squares method to the Hill equation (1).

$$I = (I_{max}[A]^{nH})/IC_{50}{}^{nH} + [A]^{nH})$$

where I is the peak current at a given concentration of agonist, I_{max} is the maximal current generated by the concentration of agonist, [A] is the concentration of GABA, IC_{50} is the antagonist concentration, which inhibits 50% of the maximum GABA response, and n_H is the Hill coefficient.

Data are expressed as means ± standard error of the mean (SEM) or as means (95% confidence intervals (CI)).

3) Homology Modeling and Docking

Homology model of ρ_1 $GABA_C$ was generated by using the 'prime' suite in Maestro. The crystal structure of the *L. stagnalis* acetylcholine binding protein[6] (AChBP) was obtained from the RCSB Protein Data Bank[7] (PDB) (PDB code: 1I9B), and used as a template for generating the model. The amino acid sequence of ρ_1 $GABA_C$ (accession code: P24046) was obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). Sequence alignments were based on the results of Adamian et al[8] and Abdel-Halim et al.[9] and five subunits of the ρ_1 $GABA_C$ were individually made using Prime v2.1.[10] The 'bldstruct' command in Prime was used to merge and build the five alignments, resulting in the ρ_1

$GABA_C$ homopentmaric model. The OPLS_2005 all-atom force field was used for energy scoring of the protein and surface generalized Born (SGB) continuum solvation model for treating solvation energies and effects. The predicted model was then prepared for docking by using protein preparation wizard, wherein hydrogens were added, bond orders assigned and disulphide bonds created. Finally the corrected structure was optimized by restrained minimization using "impref minimization" by selecting hydrogens only so that heavy atoms were left untouched.

Docking studies were conducted by using 'Glide' software as provided in Maestro. A docking model was generated by forming a receptor grid around the active site amino acids of the two adjacent $GABA_C$ monomers. The centroid of Arg104, Ser168 of first chain and Tyr198 of adjacent chain was defined as the active site. (*S*)-4-ACPBPA **6**, (*S*)-4-ACPBPA-C5-NMA **(*S*)-11d**, (*S*)-4-ACPBPA-C5-NBD **12**, (*S*)-4-ACPBPA-C5-BODIPY **13** and (*S*)-4-ACPBPA-C5-Biotin **16** were docked flexibly in to the active site using the extra-precision (XP) mode.

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