

Supporting Information

New potent, short-linker BODIPY-630/650TM labelled fluorescent adenosine receptor agonists.

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General Chemistry Methods: Chemicals and solvents (HPLC grade) were purchased from standard suppliers and were used without further purification. Unless otherwise stated, reactions were carried out at ambient temperature. Reactions were monitored by thin layer chromatography on commercially available pre-coated aluminium backed plates (Merck Kieselgel 60 F254). Visualisation was by examination under UV light (254 and 222 nm). General staining employed KMnO₄. A solution of ninhydrin (EtOH) was used for the visualisation of primary amines. Organic solvents were evaporated *in vacuo* at the temperature of <21 °C (water bath). Purification using preparative layer chromatography was carried out using Fluka silica gel (200 mm x 200 mm x 1 mm). Merck Kieselgel 60, 230-400 mesh, for flash chromatography was supplied by Merck KgaA (Darmstadt, Germany) and deuterated solvents were purchased from Goss International Limited (England).

Melting points were recorded on a Reichert 7905 apparatus and were uncorrected. FT-infrared spectra were recorded as thin films or KBr discs in the range of 600-4000 cm⁻¹ using an Avatar 220 Nicolet FT-IR spectrophotometer. Optical rotation was measured on a Bellingham-Stanley ADP220 polarimeter.

Mass spectra (+/- ES) were recorded on a Waters 2795 Separation Module/Micromass LCT platform. Proton nuclear magnetic resonance spectra were recorded on a Bruker-AV 400 (400.13 MHz). Carbon nuclear magnetic resonance spectra were recorded at 101.62 MHz. Chemical shifts (δ) are recorded in ppm with reference to the chemical shift of the deuterated solvent or internal tetramethylsilane standard. Coupling constants *J* are recorded in hertz (Hz) and the signal multiplicities described by: s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, multiplet; dd, doublet of doublets. Spectra were assigned using appropriate COSY, DEPT and HSQC sequences.

Analytical reverse-phase high performance liquid chromatography (RP-HPLC) was performed on a Waters Millenium 995 LC system using a C8 reverse phase column gradient (20% B to 100% B over 25 min) and a flow-rate of (1 mL/min) unless otherwise stated. Mobile phases were solvent A, water + 0.06% TFA; solvent B, acetonitrile + 0.06% TFA which were degassed by helium and sonication respectively unless otherwise stated.

***N*-2-propenyl-2-thiophenecarboxamide (6):** 2-thiophenecarbonyl chloride (**5**) (12.9 mL, 122 mmol) in anhydrous DCM (50 mL) was added dropwise to a mixture of allylamine (9.20 mL, 124 mmol) and pyridine (19.5 mL, 242 mmol) in anhydrous DCM (200 mL). The reaction was stirred at RT, under a N₂ atmosphere, for 4 h. The reaction was diluted with water (125 mL) and the two layers were separated. The organic layer was washed with 5M HCl (2 x 50 mL), brine (2 x 50 mL), and dried over MgSO₄. The product was concentrated to afford *N*-2-propenyl-2-thiophenecarboxamide (**6**) as a pale yellow solid (17.7 g, 77%). The product was used without further purification: mp 57-59 °C (Lit.¹ 65 °C); δ_H (CDCl₃) 4.07 (2H, m, CH₂), 5.20 (1H, dd, *J* 1.4, 10.2, H_b), 5.25- 5.30 (1H, dd, *J* 1.4, 17.1, H_a), 5.89-5.97 (1H, m, H_c), 6.32 (1H, br s, NH), 7.09 (1H, dd, *J* 3.7, 5.0, ArH), 7.49 (1H, dd, *J* 1.1, 5.0, ArH), 7.54 (1H, dd, *J* 1.1, 3.7 Hz, ArH); δ_C (CDCl₃) 42.4 (CH₂), 116.9 (=CH₂), 127.2, 127.6, 128.1, 129.9 (3 x ArCH, CH=), 138.8 (4°), 161.8 (C=O); *m/z* HRMS (ES⁺) C₈H₉NOS (MH)⁺ calcd, 168.0484; found 168.0299; FT-IR (KBr) 3302, 1621, 1502, 1306 and 708 cm⁻¹.

2-(2-thienyl)pyrrole (7): *N*-2-propenyl-2-thiophenecarboxamide (**6**) (17.0 g, 102 mmol) was dissolved in phosgene solution (20% in toluene, 200 mL), and 20 drops of anhydrous DMF were added to the solution. The reaction was stirred at RT under a N₂ atmosphere for 16 h. The toluene was removed in vacuo to give a pale yellow liquid which was then dissolved in anhydrous THF (100 mL). KOtBu (21.6 g, 316 mmol) was dissolved in anhydrous DMF (155 mL) and the solution cooled to 0 °C. The THF solution was then added dropwise to the KOtBu solution. The reaction was stirred at 0 °C for 10 min and then poured into ice water (500 mL). The reaction was extracted with ether (3 x 150 mL), dried (MgSO₄) and concentrated. The residue was purified using silica column chromatography (hexane:DCM; 1:2) and recrystallised from hexane to yield 2-(2-thienyl)pyrrole (**7**) (10.1 g, 67%) as off-white crystals: mp 73-74 °C (Lit.² 78 °C); δ_H (DMSO-*d*₆) 6.06 (1H, m, ArH of pyrrole), 6.22 (1H, m, ArH of pyrrole), 6.81 (1H, m, ArH of pyrrole), 7.01 (1H, dd, *J* 3.6, 5.0, ArH of thienyl), 7.18 (1H, dd, *J* 1.4, 3.6, ArH of thienyl), 7.29 (1H, dd, *J* 1.1, 5.0, ArH of thienyl), 11.61 (1H, br s, NH); δ_C (DMSO-*d*₆) 106.3 (ArCH of pyrrole), 109.4 (ArCH of pyrrole), 119.4 (ArCH of pyrrole), 120.9 (ArCH of thienyl), 122.8 (ArCH of thienyl), 122.4 (4°), 128.2 (ArCH of thienyl), 123.0 (4°); FT-IR (KBr) 3222, 3111, 722 and 686 cm⁻¹.

2-formyl-5-(2-thienyl)pyrrole^{3,4} (8): Phosphorus oxychloride (6.72 mL, 74.0 mmol) was added dropwise to anhydrous DMF (5.72 mL, 74.0 mmol) and cooled to 0 °C. The solution was warmed to RT and stirred under a N₂ atmosphere for 15 mins. The reaction was cooled to 0 °C and anhydrous 1,2-dichloroethane (130 mL) was added. 2-(2-thienyl)pyrrole (**7**) (10.0 g, 67.0 mmol) was dissolved in anhydrous 1,2-dichloroethane (330 mL) and added dropwise to the reaction. The reaction mixture was then refluxed under N₂ for 1 h. The reaction was cooled to RT and a solution of sodium acetate (50.2 g, 122.8 mmol) in water (128 mL) was added rapidly. The reaction was refluxed with vigorous stirring for 1h. The organic layer was collected and the aqueous layer extracted with chloroform (3 x 200 mL). The combined organics were washed with saturated sodium bicarbonate solution (3 x 100mL), water (3 x 100 mL) and dried (MgSO₄). The organics were concentrated and the residue was purified by silica column chromatography (petrol:EtOAc, 3:1). The desired product was then recrystallised from chloroform and hexane to afford 2-formyl-5-(2-thienyl)pyrrole (**8**) (6.92 g, 57%) as orange/gold crystals: mp 166-167 °C (Lit.^{4,5} 167-168 °C); δ_{H} (DMSO-*d*₆) 6.53 (1H, d, *J* 3.9, ArH of pyrrole), 7.04 (1H, d, *J* 3.9, ArH pyrrole), 7.12 (1H, dd, *J* 3.7, 5.1, ArH of thienyl), 7.55 (1H, dd, *J* 1.1, 5.0, ArH of thienyl), 7.68 (1H, dd, *J* 1.1, 3.6, ArH of thienyl), 9.46 (1H, s, CHO), 12.54 (1H, br s, NH); δ_{C} (DMSO-*d*₆) 109.4 (ArCH of pyrrole), 122.0 (ArCH of pyrrole), 125.3, 122.6, 128.75 (ArCH of thienyl), 133.8, 134.3, 134.7 (4 °), 179.2 (CHO); *m/z* HRMS (ES⁺) C₉H₇NOS (MH)⁺ calcd, 178.0327; found 178.0346; FT-IR (KBr) 3170, 1646, 1481 and 1283 cm⁻¹.

4-methoxybenzyl triphenylphosphonium chloride:^{6,7} A solution of 4-methoxybenzylchloride **14** (10.0 g, 63.8 mmol) and triphenylphosphine in anhydrous benzene was refluxed for 22 h. The precipitated phosphonium chloride salt was collected by filtration, washed several times with benzene and dried to afford 4-methoxybenzyl triphenylphosphonium chloride X (21.5 g, 80%) as a white solid. The product was used without further purification: mp 239-242 °C (Lit.⁷ 238-240 °C; 229 °C⁵⁶; 222-242 °C¹⁹); δ_{H} (CDCl₃) 3.68 (3H, s, OMe), 5.27 (2H, 2 x s, CH₂), 6.60 (2H, d, *J* 8.6, 2 x ArH), 6.96 (2H, dd, *J* 2.5, 8.7 Hz, 2 x ArH), 7.60-7.76 (15H, m, PPh₃); δ_{C} (CDCl₃) 29.7 (CH₂), 55.2 (CH₃), 114.21, 114.24 (ArCH), 117.5, 118.3 (4°), 130.1,

130.2, 132.59, 132.64, 132.70, 134.32, 134.41, 134.90, 134.93(ArCH); m/z HRMS (ES⁺) C₂₂H₂₄OP (MH)⁺ calcd, 384.1643; found 384.1611; FT-IR (KBr) 2779, 1605, 1505, 1438, 1246 and 1111 cm⁻¹.

2-[(E)-2-(4-methoxyphenyl)ethen-1-yl]pyrrole^{8,9} (10): 4-methoxybenzyl triphenylphosphonium chloride (1.19 g, 2.85 mmol) was added to sodium hydride (60% dispersion in mineral oil, 114 mg dispersion, 2.86 mmol NaH) in anhydrous benzene (4 mL) and stirred for 30 mins. Pyrrole-2-carboxyaldehyde (**9**) (222 mg, 2.23 mmol) in anhydrous toluene (3 mL) was added to the solution *via* a cannula. The reaction was refluxed for 3 h. The reaction was poured into water (20 mL), the organic layer was collected and the aqueous layer was extracted with ether (3 x 20 mL). The combined organics were dried over MgSO₄ and concentrated *in vacuo*. The residue was purified using silica column chromatography (hexane:EtOAc, 96:4) and recrystallised from hexane and toluene to yield 2-[(E)-2-(4-methoxyphenyl)ethen-1-yl]pyrrole **17** (95 mg, 20%) as white crystals: mp 176-177 °C (Lit.⁸ 169-170 °C); δ_H (CDCl₃) 3.83 (3H, s, OMe), 6.25 (1H, dd, *J* 2.6, 5.9, ArH of pyrrole), 6.34 (1H, m, ArH of pyrrole), 6.59-6.65 (1H, d, *J* 16.5, CH=), 6.78 (1H, dd, *J* 2.6, 4.2, ArH of pyrrole), 6.83-6.92 (3H, 2 x d, *J* 8.7, 16.4, CH=, 2 x phenyl ArH), 7.38 (2H, d, *J* 8.7, phenyl ArH), 8.28 (1H, br s, NH); δ_C (CDCl₃) 55.3 (OMe), 108.3, 109.9 (2 x ArCH of pyrrole), 114.1 (phenyl CH), 117.1, 118.7, 123.1 (2 x alkenyl C, ArCH of pyrrole), 127.0 (phenyl CH), 130.3 (4°), 131.0 (4°), 158.8 (4°); m/z HRMS (ES⁺) C₁₃H₁₃NO (MH)⁺ calcd, 200.1076; found 200.1100; FT-IR (KBr) 3403, 1602, 1510, 1250, 1031 and 816cm⁻¹.

methyl 2-(4-[(E)-2-(1H-pyrrol-2-yl)ethenyl]phenoxy)acetate¹⁰ (11): Sodium ethanethiolate (90 %, 72.7 mg, 0.78 mmol) was dissolved in anhydrous DMF (5 mL) at 0 °C. 2-[(E)-2-(4-methoxyphenyl)ethen-1-yl]pyrrole (**10**) (156 mg, 0.78 mmol) was added to the suspension. The mixture was refluxed under a N₂ atmosphere for 4h. After cooling to RT, methyl bromoacetate (74 μl, 0.78 mmol) was added to the reaction. The reaction was stirred under a N₂ atmosphere for 2 h, during which time a precipitate formed. The reaction was poured into ice water (40 mL) and extracted with CHCl₃ (3 x 20 mL) and the combined organics were dried and evaporated. The residue was purified using silica column chromatography (CHCl₃) to yield the title

compound **18** (79.5 mg, 40%) as a pale grey solid: mp 130-132 °C; δ_{H} (DMSO- d_6) 3.71 (3H, s, OMe), 4.81 (2H, s, CH_2), 6.05 (1H, dd, J 2.4, 5.6, ArH of pyrrole), 6.78 (2H, m, ArH of pyrrole, CH=), 6.90 (3H, 2 x d, J 8.8, 16.5, 2 x phenyl CH, ArH of pyrrole), 7.22 (2H, d, J 8.7, phenyl CH), 11.1 (1H, br s, NH); δ_{C} (DMSO- d_6) 52.3 (OMe), 65.1 (CH_2), 109.0, 109.3 (2 x ArCH of pyrrole), 115.3 (phenyl ArCH), 118.6, 119.8, 122.2 (2 x alkenyl CH, ArCH of pyrrole), 127.1 (phenyl ArCH), 131.1 (4°), 131.6 (4°), 158.7 (4°), 169.7 (C=O); m/z HRMS (ES^+) $\text{C}_{15}\text{H}_{15}\text{NO}_3$ (MH^+) 258.1131; found 258.1115; FT-IR (KBr) 3330, 1748, , 1511, 1222, 1178 and 731 cm^{-1} .

Methyl 2-(4,4-difluoro-4,4a-dihydro-5-(thiophenyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)vinyl)phenoxy)acetate (12): *NB. The reaction was carried out with the exclusion of light.* 2-[(*E*)-2-[(4-methoxycarbonylmethoxy) phenyl]ethen-1-yl]pyrrole **18** (238 mg, 1.21 mmol) and 2-formyl-5-(2-thienyl)pyrrole **13** (210 mg, 1.22 mmol) were dissolved in anhydrous DCM/MeOH (10:1, 50 mL). POCl_3 (123 μL , 1.21 mmol) was then added and the solution was stirred under a N_2 atmosphere for 18h. The solvent was evaporated and the residue was dissolved in anhydrous DCM (270 mL). *N,N*-diisopropylethylamine (2.23 mL, 13.6 mmol) and $\text{BF}_3 \cdot \text{OEt}_2$ (1.73 mL, 13.6 mmol) were added to the solution and the reaction was stirred under a N_2 atmosphere for 2 h. The solution was washed with brine (3 x 50 mL), dried and concentrated to give a deep blue sticky solid. The solid was purified by silica column chromatography (DCM) to afford the title compound (**12**) (506 mg, 81%) as a blue solid; mp 157-158 °C; δ_{H} (CDCl_3) 3.83 (3H, s, OMe), 4.70 (2H, s, CH_2), 6.79 (1H, d, J 4.2, ArH of pyrrole), 6.94-7.05 (6H, m, 3 x ArH of pyrrole, 2 x phenyl ArH, CH between pyrrole), 7.23 (1H, dd, J 3.9, 5.0, ArH of thienyl), 7.32 (1H, d, J 16.3, CH=), 7.48 (1H, dd, J 0.9, 5.0, ArH of thienyl), 7.58-7.64 (3H, m, 2 x phenyl ArH, CH=), 8.21 (1H, dd, J 0.9, 3.9, ArH of thienyl); δ_{C} (CDCl_3) 52.4 (OMe), 65.2 (CH_2), 115.0, 117.3, 117.7, 119.7, 124.0, 128.7, 128.9, 129.0, 129.4, 129.9, 130.1 (11 x ArCH), 130.57 (ArCH of thienyl), 134.5, 122.6, 122.8 (3 x 4°), 123.5 (ArCH), 149.1 (4°), 156.6 (4°), 158.8 (4°), 169.1 (C=O); $^1\delta_{\text{F}}$ (CDCl_3) -139.97 (1F, d, J 22.0 Hz), -140.15 (1F, d, J 22.0 Hz); m/z HRMS (ES^+) $\text{C}_{24}\text{H}_{19}\text{BF}_2\text{N}_2\text{O}_3\text{S}$ (MH^+) calcd, 487.1068; found 487.1046; FT-IR (KBr) 3433, 2924, 1592, 1511, 1466, 1252, 1207, 1174, 1122, 1087 and 1046 cm^{-1} ; RP-HPLC; R_t 13.80 min; 96% purity.

2-(4,4-difluoro-4,4a-dihydro-5-(thiophenyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)vinyl)phenoxy)acetic acid (13): *NB.* The reaction was carried out with the exclusion of light. Methyl 2-(4,4-difluoro-4,4a-dihydro-5-(thiophenyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)vinyl)phenoxy)acetate (**12**) (400 mg, 0.86 mmol) was dissolved in a mixture of THF (20.8 mL), H₂O (10.4 mL) and phosphoric acid (85%, 1.28 mL) and refluxed under a N₂ atmosphere for 3 days. The reaction was cooled to RT, diluted with water (100 mL) and extracted with DCM (3 x 50 mL). The combined organics were dried and evaporated to give a dark blue solid. The residue was purified using silica column chromatography (DCM:MeOH; 1:1) to yield the title compound (**13**) (174 mg, 49%) as a dark blue iridescent solid: mp 246-247 °C; δ_{H} (DMSO- d_6) 4.76 (2H, s, CH₂), 6.95 (1H, d, J 4.0, ArH of pyrrole), 7.04 (2H, d, J 8.8, 2 x phenyl ArH), 7.27-7.30 (3H, m, 2 x ArH of pyrrole, ArH of thienyl), 7.23-7.42 (2 x d, J 17.0, 4.4, CH=, ArH of pyrrole), 7.59 (3H; 2H d, J 7.6, 2 x phenyl ArH, 1H, s, CH between pyrroles), 7.75 (1H, d, J 16.4, CH=), 7.83 (1H, d, J 4.8, ArH of thienyl), 8.05 (1H, d, J 3.6, ArH of thienyl), 12.56 (1H, br s, OH); δ_{C} (DMSO- d_6) 65.0 (CH₂), 115.8, 116.3, 118.7, 119.8, 125.6, 129.4, 129.6, 130.1, 130.69, 130.76, 131.8 (11 x CH), 134.1, 122.8, 122.9 (3 x 4°), 139.4 (CH), 148.1, 156.9, 159.7 (3 x 4°), 170.4 (C=O); δ_{F} (DMSO- d_6) -138.15 (1F, d, J 33.9 Hz), -138.38 (1F, d, J 33.9 Hz); m/z HRMS (ES⁺) C₂₃H₁₇BF₂N₂O₃S (MH)⁺ calcd, 449.0952; found 449.0963; FT-IR (KBr) 3062, 2220, 1747, 1596, 1510, 1468, 1424, 1222 and 1129 cm⁻¹.

Synthesis of BODIPY 630/650 N⁶-(Aminoalkyl)-5'-ethylamino-5'-oxo-5'-deoxyadenosine conjugates 15a-e: 1,3-dicyclohexylcarbodiimide (DCC) (2 equiv.), 1-hydroxybenzotriazole (HOBt) (2 equiv.) and 2-(4,4-difluoro-4,4a-dihydro-5-(thiophenyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)vinyl)phenoxy)acetic acid (**13**) (1 equiv.) were dissolved in anhydrous DCM. N⁶-(Aminoalkyl)-5'-ethylamino-5'-oxo-5'-deoxyadenosines **14a-e** (2 equiv.) were dissolved in anhydrous DMF and were added to the DCM mixture. The reactions were stirred at RT, under an inert atmosphere, with the exclusion of light for 20-24 h. The mixtures were concentrated and the crude products purified using preparative layer chromatography (9:1; DCM:MeOH) to yield the desired products **15a-e** (69-85%) as dark blue iridescent solids.

(2S,3S,4R,5R)-N-Ethyl-3,4-dihydroxy-5-(6-[2-{2-(4-[(E)-2-{4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-s-indacene-3-yl}ethenyl]phenoxy)-acetamido}ethylamino]-9H-purin-9-yl)-tetrahydrofuran-2-carboxamide 15a:
Mass 3.06 mg (83%); δ_{H} (MeOH- d_4) 0.97 (3H, t, J 7.3, CH_2CH_3), 2.91 (2H, m, CH_2CH_3), 3.05 (2H, t, J 6.0, CH_2), 3.39 (2H, br s, CH_2), 4.23 (1H, dd, J 1.3, 4.8, C^3H), 4.50 (1H, d, J 1.3, C^4H), 4.58, (2H, s, CH_2), 4.75 (1H, dd, J 4.8, 7.6, C^2H), 5.97 (1H, d, J 7.6, C^1H), 6.87 (1H, d, J 3.9, ArH of pyrrole), 7.00-7.12 (6H, m, 3 x ArH of pyrrole, 2 x phenyl ArH, CH=), 7.20 (1H, dd, J 3.9, 5.0, ArH of thienyl), 7.21 (1H, s, -C=CH between pyrroles), 7.57-7.65 (4H, m, CH=, ArH of thienyl, 2 x phenyl ArH), 8.01 (1H, br s, NH), 8.10 (1H, dd, J 1.0, 3.9, ArH of thienyl), 8.15 (1H, br s, amide NH), 8.20 (1H, s, adenine CH), 8.25 (1H, s, adenine CH), 8.93 (1H, t, J 5.6, amide NH); m/z HRMS (ES^+) $\text{C}_{23}\text{H}_{22}\text{BF}_2\text{N}_9\text{O}_6\text{S}$ (MH^+) calcd, 784.2249; found 784.2710; RP-HPLC-1; R_t 10.24 min; 97 % purity (20% B to 100% B over 25 mins); RP-HPLC-2; R_t 16.74 min; 96% purity (20% B to 100% B over 45 mins).

(2S,3S,4R,5R)-N-Ethyl-3,4-dihydroxy-5-(6-[3-{2-(4-[(E)-2-{4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-s-indacene-3-yl}ethenyl]phenoxy)-acetamido}propylamino]-9H-purin-9-yl)-tetrahydrofuran-2-carboxamide 15b:
Mass 1.06 mg (79 %); δ_{H} (DMSO- d_6) 1.08 (3H, t, J 7.3, CH_2CH_3), 1.78 (2H, m, CH_2), 3.15-3.20 (4H, m, CH_2CH_3 , CH_2), 3.76 (2H, br s, CH_2), 4.15 (1H, m, C^3H), 4.46 (1H, d, J 1.2, C^4H), 4.50-4.64 (3H; 2H s, CH_2 , 1H, m, C^2H), 5.57 (1H, d, J 6.4, C^2OH), 5.77 (1H, d, J 4.0, C^3OH), 5.97 (1H, d, J 7.6, C^1H), 6.95 (1H, d, J 4.0, ArH of pyrrole), 7.08 (2H, d, J 8.4, 2 x phenyl ArH), 7.27-7.34 (3H, m, 2 x ArH of pyrrole, ArH of thienyl), 7.23-7.42 (2 x d, J 16.8, 4.4, CH=, ArH of pyrrole), 7.59 (3H; 2H, d, J 7.6, 2 x phenyl ArH, 1H, s, -C=CH between pyrroles), 7.75 (1H, d, J 16.0, CH=), 7.84 (1H, d, J 4.8, ArH of thienyl), 8.05 (2H; 1H, d, J 3.6, ArH of thienyl, 1H, s, NH), 8.23-8.33 (2H, m, adenine CH, amide NH), 8.40 (1H, s, adenine CH), 8.97 (1H, t, J 5.6, amide NH); m/z HRMS (ES^+) $\text{C}_{38}\text{H}_{38}\text{BF}_2\text{N}_9\text{O}_6\text{S}$ (MH^+) calcd, 798.2806; found 798.2803; RP-HPLC-1; R_t 10.17 min; 99% purity (20% B to 100% B over 25 mins); RP-HPLC-2; R_t 17.79 min; 99% purity (20% B to 100% B over 45 mins).

(2*S*,3*S*,4*R*,5*R*)-*N*-Ethyl-3,4-dihydroxy-5-(6-[4-{2-(4-[(*E*)-2-{4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-s-indacene-3-yl}ethenyl]phenoxy)-acetamido}butylamino]-9H-purin-9-yl)-tetrahydrofuran-2-carboxamide 15c:

Mass 5.15 mg (85%); δ_{H} (DMSO- d_6) 1.08 (3H, t, J 7.3, CH_2CH_3), 1.43-1.66 (4H, m, 2 x CH_2), 3.03-3.25 (4H, m, CH_2CH_3 , CH_2), 3.51 (2H, br s, CH_2), 4.12 (1H, m, C^3H), 4.46 (1H, s, C^4H), 4.52, (2H, s, CH_2), 4.63 (1H, m, C^2H), 5.56 (1H, d, J 6.4, C^2OH), 5.77 (1H, d, J 4.0, C^3OH), 5.97 (1H, d, J 7.6, C^1H), 6.95 (1H, d, J 4.0, ArH of pyrrole), 7.08 (2H, d, J 8.4, 2 x phenyl ArH), 7.27-7.34 (3H, m, 2 x ArH of pyrrole, ArH of thienyl), 7.23-7.42 (2 x d, J 16.8, 4.4, CH= , ArH of pyrrole), 7.59 (3H; 2H, d, J 7.6, 2 x phenyl ArH, 1H, s, $-\text{C=CH}$ between pyrroles), 7.75 (1H, d, J 16.0, CH=), 7.84 (1H, d, J 4.8, ArH of thienyl), 8.05 (2H; 1H, d, J 3.6, ArH of thienyl, 1H, s, NH), 8.19 (1H, t, J 5.6, amide NH), 8.28 (1H, s, adenine CH), 8.40 (1H, s, adenine CH), 8.94 (1H, t, J 5.6, amide NH); m/z HRMS (ES^+) $\text{C}_{39}\text{H}_{40}\text{BF}_2\text{N}_9\text{O}_6\text{S}$ (MH^+) 812.2962; found 812.2999; RP-HPLC-1; R_t 11.06 min; 100% purity (20% B to 100% B over 25 mins); RP-HPLC-2; R_t 19.06 min; 100% purity (20% B to 100% B over 45 mins).

(2*S*,3*S*,4*R*,5*R*)-*N*-Ethyl-3,4-dihydroxy-5-(6-[8-{2-(4-[(*E*)-2-{4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-s-indacene-3-yl}ethenyl]phenoxy)-acetamido}octylamino]-9H-purin-9-yl)-tetrahydrofuran-2-carboxamide 15d:

Mass 3.21 mg; Yield (69%); δ_{H} (MeOH- d_4) 1.18 (3H, t, J 7.3, CH_2CH_3), 1.23-1.40 (8H, br s, 4 x CH_2), 1.49 (2H, m, CH_2), 1.63 (2H, m, CH_2), 3.23-3.42 (6 H, 2 x CH_2 , CH_2CH_3), 4.12 (1H, dd, J 1.3, 4.8, C^3H), 4.31 (1H, d, J 1.3, C^4H), 4.52, (2H, s, CH_2), 4.63 (1H, dd, J 4.8, 7.6, C^2H), 5.97 (1H, d, J 7.6, C^1H), 6.84 (1H, d, J 3.9, ArH of pyrrole), 7.05-7.12 (6H, m, 3 x ArH of pyrrole, 2 x phenyl ArH, CH=), 7.22 (1H, dd, J 3.9, 5.0, ArH of thienyl), 7.32 (1H, s, $-\text{C=CH}$ between pyrroles), 7.54-7.62 (4H, m, CH= , ArH of thienyl, 2 x phenyl ArH), 8.00 (1H, br s, NH), 8.12 (1H, dd, J 1.0, 3.9, ArH of thienyl), 8.15 (1H, br s, amide NH), 8.19 (1H, s, adenine CH), 8.20 (1H, s, adenine CH), 8.93 (1H, t, J 5.6, amide NH); m/z HRMS (ES^+) $\text{C}_{43}\text{H}_{48}\text{BF}_2\text{N}_9\text{O}_6\text{S}$ (MH^+) 868.2188; found 868.2162; RP-HPLC-1; R_t 11.19 min; 98% purity (20% B to 100% B over 25 mins); RP-HPLC-2; R_t 21.21 min; 95 % purity (20% B to 100% B over 45 mins).

(2*S*,3*S*,4*R*,5*R*)-*N*-Ethyl-3,4-dihydroxy-5-(6-[11-{2-(4-[(*E*)-2-{4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl}ethenyl]phenoxy)-acetamido}undecanyl-amino]-9*H*-purin-9-yl)-tetrahydrofuran-2-carboxamide
15e: Mass 3.51 mg (81%); δ_{H} (DMSO- d_6) 1.08 (3H, t, J 7.3, CH_2CH_3), 1.34-1.56 (16H, m, 8 x CH_2), 1.78 (2H, m, CH_2), 3.20-3.22 (4H, m, CH_2CH_3 , CH_2), 3.52 (2H, br s, CH_2), 4.15 (1H, m, C^3H), 4.46 (1H, d, J 1.2, C^4H), 4.50-4.64 (3H; 2H s, CH_2 , 1H, m, C^2H), 5.55 (1H, d, J 6.4, C^2OH), 5.75 (1H, d, J 4.0, C^3OH), 5.99 (1H, d, J 7.6, C^1H), 6.97 (1H, d, J 4.0, ArH of pyrrole), 7.01 (2H, d, J 8.4, 2 x phenyl ArH), 7.27-7.22 (3H, m, 2 x ArH of pyrrole, ArH of thienyl), 7.21-7.42 (2 x d, J 16.8, 4.4, $\text{CH}=\text{CH}$, ArH of pyrrole), 7.59 (3H; 2H, d, J 7.6, 2 x phenyl ArH, 1H, s, $-\text{C}=\text{CH}$ between pyrroles), 7.75 (1H, d, J 16.0, $\text{CH}=\text{CH}$), 7.84 (1H, d, J 4.8, ArH of thienyl), 8.03 (2H; 1H, d, J 3.6, ArH of thienyl, 1H, s, NH), 8.20-8.28 (2H, m, adenine CH, amide NH), 8.40 (1H, s, adenine CH), 8.98 (1H, t, J 5.6, amide NH); m/z HRMS (ES⁺) $\text{C}_{46}\text{H}_{54}\text{BF}_2\text{N}_9\text{O}_6\text{S}$ (MH)⁺ calcd, 908.3899; found 908.3939; RP-HPLC-1; R_t 13.15 min; 94% purity (20% B to 100% B over 25 mins); RP-HPLC-2; R_t 23.57 min; 95% purity (20% B to 100% B over 45 mins).

(2*S*,3*S*,4*R*,5*R*)-*N*-Ethyl-3,4-dihydroxy-5-(6-[8-{2-(4-[(*E*)-2-{4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl}ethenyl]phenoxy)-acetamido}-3,6-dioxaoctyl-amino]-9*H*-purin-9-yl)-tetrahydrofuran-2-carboxamide **17a**: DCC (1.8 mg, 8.8 μmol), HOBt (1.2 mg, 8.8 μmol) and 2-(4,4-difluoro-4,4a-dihydro-5-(thiophenyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl)vinyl)phenoxy)acetic acid (**13**) (2.0 mg, 4.4 μmol) were dissolved in anhydrous DCM (1 mL). **16a** (4.0 mg, 9.1 μmol) was dissolved in anhydrous DMF (1 mL) and added to the DCM mixture. The reaction was stirred at RT, under an inert atmosphere, with the exclusion of light for 24 h. The mixture was concentrated and crude product purified using preparative layer chromatography (9:1, DCM:MeOH) to yield the desired product (**17a**) (3.78 mg, 73%) as dark blue iridescent solid: δ_{H} (DMSO- d_6) 1.04 (3H, t, J 7.3, CH_2CH_3), 3.18 (2H, m, CH_2CH_3), 3.42-3.68 (10H, m, 5 x CH_2), 3.70 (2H, br s, CH_2), 4.52 (2H, s, CH_2), 4.95 (1H, m, C^3H), 5.14 (1H, br s, C^4H), 5.25 (1H, m, C^2H), 5.53 (1H, d, J 6.4, C^2OH), 5.72 (1H, d, J 4.0, C^3OH), 5.97 (1H, d, J 7.6, C^1H), 6.95 (1H, d, J 4.0, ArH of pyrrole), 7.03 (2H, d, J 8.4, 2 x phenyl ArH), 7.22-7.30 (3H, m, 2 x ArH of pyrrole, ArH of thienyl), 7.23-7.42 (2 x d, J 16.8, 4.4,

CH=, ArH of pyrrole), 7.59 (3H; 2H, d, *J* 7.6, 2 x phenyl ArH, 1H, s, -C=CH between pyrroles), 7.75 (1H, d, *J* 16.0, CH=), 7.84 (1H, d, *J* 4.8, ArH of thienyl), 8.05 (2H; 1H, d, *J* 3.6, ArH of thienyl, 1H, s, NH), 8.19 (1H, t, *J* 5.6, amide NH), 8.28 (1H, s, adenine CH), 8.40 (1H, s, adenine CH), 8.96 (1H, t, *J* 5.6, amide NH); *m/z* HRMS (ES⁺) C₄₁H₄₄BF₂N₉O₈S (MH)⁺ calcd, 872.3174; found 872.3086; RP-HPLC-1; R_t 9.91 min; 99% purity (20% B to 100% B over 25 mins); RP-HPLC-2; R_t 16.55 min; 97% purity (20% B to 100% B over 45 mins).

(2*S*,3*S*,4*R*,5*R*)-*N*-Ethyl-3,4-dihydroxy-5-(6-[11-{2-(4-[(*E*)-2-{4,4-difluoro-4,4a-dihydro-5-(thiophen-2-yl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl}ethenyl]phenoxy)-acetamido}-3,6,9-trioxaundecylamino]-9*H*-purin-9-yl)-tetrahydrofuran-2-carboxamide **17b:** DCC (1.8 mg, 8.8 μmol), HOBt (1.2 mg, 8.8 μmol) and 2-(4,4-difluoro-4,4a-dihydro-5-(thiophenyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl)vinyl)phenoxy)acetic acid (**13**) (2.0 mg, 4.4 μmol) were dissolved in anhydrous DCM (1 mL). **16b** (4.3 mg, 8.8 μmol) was dissolved in anhydrous DMF (1 mL) and added to the DCM mixture. The reaction was stirred at RT, under an inert atmosphere, with the exclusion of light for 22 h. The mixture was concentrated and crude product purified using preparative layer chromatography (9:1, DCM:MeOH) to yield the desired product **17b** (4.06 mg, 59%) as a dark blue iridescent solid: δ_H (DMSO-*d*₆) 1.09 (3H, t, *J* 7.3, CH₂CH₃), 3.18 (2H, m, CH₂CH₃), 3.42-3.68 (14H, m, 7 x CH₂), 3.70 (2H, br s, CH₂), 4.12 (1H, m, C^{3'}H), 4.33 (1H, d, *J* 1.4, C^{4'}H), 4.52 (2H, s, CH₂), 4.61 (1H, m, C^{2'}H), 5.53 (1H, d, *J* 6.4, C^{2'}OH), 5.72 (1H, d, *J* 4.0, C^{3'}OH), 5.97 (1H, d, *J* 7.6, C^{1'}H), 6.95 (1H, d, *J* 4.0, ArH of pyrrole), 7.03 (2H, d, *J* 8.4, 2 x phenyl ArH), 7.22-7.29 (3H, m, 2 x ArH of pyrrole, ArH of thienyl), 7.23-7.43 (2 x d, *J* 16.8, 4.4, CH=, ArH of pyrrole), 7.57 (3H; 2H, d, *J* 7.6, 2 x phenyl ArH, 1H, s, -C=CH between pyrroles), 7.77 (1H, d, *J* 16.0, CH=), 7.83 (1H, d, *J* 4.8, ArH of thienyl), 8.03 (2H; 1H, d, *J* 3.6, ArH of thienyl, 1H, s, NH), 8.19 (1H, t, *J* 5.6, NH), 8.28 (1H, s, adenine CH), 8.40 (1H, s, adenine CH), 8.97 (1H, t, *J* 5.6, amide NH); *m/z* HRMS (ES⁺) C₄₃H₄₈BF₂N₉O₉S (MH)⁺ calcd, 916.3422; found 916.2100; RP-HPLC-1; R_t 9.99 min; 94% purity (20% B to 100% B over 25 mins); RP-HPLC-2; R_t 16.52 min; 94% purity (20% B to 100% B over 45 mins).

General pharmacology methods: All reagents used were obtained from Sigma chemicals (Poole, Dorset, UK), unless otherwise stated. . CHO cells (CHO-A₁-SPAP) expressing the human adenosine A₁-receptor and a cyclic AMP response element reporter gene (secreted placental alkaline phosphatase; SPAP) (Baker & Hill, 2007) were provided by Dr Jillian Baker. CHO-A₁-SPAP cells were cultured in Dulbecco's modified Eagle's medium/Nutrient mix F12 (DMEM/F12) supplemented with 2mM L-glutamine and 10 % heat-inactivated fetal calf serum (FCS) at 37 °C, 5% CO₂. (PAA Laboratories, Teddington, Middlesex, UK).

CRE-mediated gene transcription-SPAP Assay:

CHO-A1-SPAP cells were grown to confluence in 24-well plates in 1ml/well DMEM/F12 containing 10% fetal calf serum and 2mM L-glutamine. Once confluent, the media was removed, replaced with serum-free media and the cells incubated for an additional 24h. On the day of experimentation, the serum-free medium was removed and replaced with 0.9ml of serum-free medium or 0.9ml of serum free media containing an antagonist drug at the final required concentration and the cells were incubated for 30 min (37°C /5% CO₂). Agonists in 10 µl medium (diluted in serum-free media) were then added to each well and the cells incubated for a further 10 min at 37°C. Forskolin (3 µM, 100 µL/well) was then added to each well and the incubation continued for 5 hours at 37 °C in 5% CO₂. After this 5-hour incubation, media was removed and replaced with serum-free media (300 µL/well) and the cells incubated for a further hour. Samples of the supernatant (20 µL) were then added to a 96-well plate and the plates were heated at 65 °C for 25 min to inactivate endogenous alkaline phosphatases. 200 µl of 5mM 4-nitrophenyl phosphate in diethanolamine buffer was added to each well and the plates incubated until the yellow colour developed (after approximately 45 min - 1 hour). The plates were then read at 405 nm using a Dynatech Laboratories MRX plate reader.

Data analysis

Data are presented as mean ± s.e. of triplicate determinations unless otherwise stated. The n in the text refers to the number of separate experiments. Optical density

measurements were converted to SPAP concentration mU/mL using the following equation:

$$[SPAP](mU / mL) = \frac{A}{18.5tV}$$

Where A is the optical density at 405 nm, t is the incubation time with substrate (min) and V is the volume of sample (mL). Concentration-response data were fitted to the following equations using the computer assisted non-linear equation in the programme Prism (GraphPAD, California, USA):

One-site concentration response curves

$$\text{Response} = \frac{E_{\max} \cdot [A]}{IC_{50} + [A]}$$

Where $E_{\max}$ is the maximal response, $[A]$ is the agonist concentration and IC_{50} is the concentration of agonist that produces 50 % of the maximal inhibitory response.

Two site agonist curves

As many concentration responses clearly contained two components two-site analysis was required for most ligands. This was performed using the following equation

$$\text{Response} = \text{Basal} + (FK - \text{Basal}) \left[1 - \frac{[A]}{[A] + IC_{50}} \right] + S_{\max} \left[\frac{[A]}{[A] + EC_{50}} \right]$$

Where basal is the response in the absence of agonist. FK is the response to a fixed concentration of forskolin, $[A]$ is the concentration of agonist, IC_{50} is the concentration of agonist that inhibits 50 % of the response to forskolin. $S_{\max}$ is the maximum stimulation of the G_s component of the response to agonist and the EC_{50} is the concentration of agonist that stimulated a half maximal G_s response.

Antagonist K_D values were calculated for XAC from the shift of agonist concentration responses in the presence of a fixed concentration of antagonist using the following equation:

$$DR = 1 + \frac{[B]}{K_D}$$

Where DR (dose-ratio) is the ratio of the concentrations of agonist required to produce an identical response in the presence and absence of antagonist. [B] is the concentration of antagonist and K_D is the antagonist dissociation constant.¹²

Radioligand binding assay: Cells were seeded into 96-well white sided plates 24 hours prior to the assay. On the day of experimentation, all media was aspirated and different concentrations of the ligand (in serum-free media) were added (100 μ L/well) in triplicate. Total and non-specific binding was also defined by adding serum free media in triplicate (total binding) and 1 μ M DPCPX in triplicate (non-specific binding). 3 H-DPCPX was made up at the concentration of 2nM in serum free media and was added to all wells (100 μ L/well). The cells were incubated for 2 hours at 37 $^{\circ}$ C, 5 % CO_2 . After 2 hour incubation, the cells were washed twice by addition and removal of ice cold PBS (200 μ L/well). White backing was added to each plate and 100 μ L of MicroScint 20 was added to each well. Finally, the plates were sealed and counted on a TopCount (Packard, Boston, MA).

Data Analysis

All data are represented as mean $\pm$ s.e. of triplicate determinations unless otherwise stated. The n in the text refers to the number of separate experiments. K_D values were determined from the IC_{50} values and concentration of 3 H-DPCPX according to the expression³²

$$KD = \frac{IC_{50}}{(1 + [A] / K_{DDPCPX})}$$

Where [A] is the concentration of 3 H-DPCPX and $K_{D(DPCPX)}$ is the dissociation constant (K_D) of 3 H-DPCPX.

Confocal microscopy

Confocal microscopy was performed using a Zeiss LSM510 laser-scanning microscope with a 40 x 1.3 NA oil immersion lens. Cells were seeded into 6-well

plates containing glass coverslips 48 h prior to the experiment. On the day of the experiment, all media was aspirated and the cells were washed twice with warmed 1 mL HEPES-buffered saline solution (HBS; 147 mM NaCl, 24mM KCl, 1.3 mM CaCl_2 , 1 mM MgSO_4 , 1.5 mM NaHCO_3 , 10 mM HEPES, 1 mM sodium pyruvate, pH 7.4). The coverslip was carefully removed using tweezers and placed cell side up into a specially designed holder. The coverslip formed the base of the holder creating a sealed well capable of holding 1 mL of solution. HBS (450 μL) was added to each well, which provided a medium into which agonists could be added. The holder was transferred to a heated stage on the microscope, which allowed the cells to be maintained at 37 °C throughout the experiment. Cells were incubated for 15 minutes before each agonist (50 μL) was added. When an antagonist was used, cells were incubated with the antagonist for 30 min before the addition of an agonist. All images were taken at 1024 x 1024 pixels.

Images of the BODIPY ligands were taken using 633 nm excitation, with emission collected through a LP650 filter. Images were taken using identical laser power (15 %) and pinhole size (84 μm), however, the gain and offset were adjusted to suit the apparent brightness of the ligands. A gain of 800 was used for the ‘brighter’ ligands (those with shorter alkyl linkers) and a gain of 1000 was used for the ligands with a longer alkyl linker (C11) and polar linkers.

Supplementary References

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