**Evaluation of pyrrolidine and pyrazolone derivatives as inhibitors of trypanosomal phosphodiesterase B1 (TbrPDEB1).**

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**General details**

All starting materials were obtained commercially and were used without further purification. Reaction solvents were puriﬁed by passage through alumina columns on a puriﬁcation system manufactured by Innovative Technology (Newburyport, MA). NMR spectra were obtained on Varian NMR systems, operating at 400 MHz or 500 MHz for 1H acquisitions. LCMS analysis was performed using a Waters Alliance reverse phase HPLC (columns Waters SunFire C18 4.6x50mm, 3.5μm or Waters SunFire C8 4.6x50mm, 3.5μm), with single-wavelength UV–visible detector and LCT Premier time-of-ﬂight mass spectrometer (electrospray ionization). All final compounds were purified by preparative reverse phase HPLC (columns Waters Symmetry RP8 30 × 50 mm, 5 μm column or OBD RP18 30 × 50 mm, 5 μm), with a single-wavelength UV–visible detector and Waters Micromass ZQ (electrospray ionization). All final compounds have purities greater than 95% based upon LC/MS.

**Analytical Chemistry: Preparative Method for Final Compounds Purification Instruments:**

**Instruments:**

Waters 2525 binary pump, Waters 2767 sample manager, Waters 2489 UV/Visible detector, Waters

2x515 pumps, Waters pump control, Waters column fluidics organizer, MicroMass ZQ mass detector

**Detection:**

UV: 254 nm

Mass: ESI + mode, set to the mass of interest compounds

**Collection:**

Mass and UV combination

**Column:**

Waters Symmetry C8 30x50mm, 5μm

Waters Xbridge OBD RP18 30x50mm, 5μm

**Mobile Phase:** phase: A: 0.1% v/v Formic acid in H2O B: Acetonitrile

Typical Preparative HPLC method (start gradient may vary based on compound’s retention time on the analytical system)

|  |  |  |  |
| --- | --- | --- | --- |
| Time (m) | Flow rate | A% | B% |
| 0 | 40 ml/m | 95% | 5% |
| 7.50 | 40 ml/m | 0% | 100% |
| 7.90 | 40 ml/m | 0% | 100% |
| 7.91 | 40 ml/m | 95% | 5% |
| 8.00 | 40 ml/m | 95% | 5% |

Method run time: 8 min

**Analytical Chemistry: Analytical Method for Compounds purity determination Instruments:**

**Instruments:**

Waters e2795 Alliance HPLC separation module, Waters 2489 UV/visible detector, Waters LCT premier micromass

**Detection:**

UV: 254 nm

Mass: ESI + mode, scan mass range 100-1000

**Columns:**

Waters SunFire C18 4.6x50mm, 3.5μm

Waters SunFire C8 4.6x50mm, 3.5μm

**Mobile Phase:** phase: A: 0.1% v/v Formic acid in H2O B: Acetonitrile

|  |  |  |  |
| --- | --- | --- | --- |
| Time (m) | Flow rate | A% | B% |
| 0 | 2 ml/m | 95% | 5% |
| 3.75 | 2 ml/m | 0% | 100% |
| 3.85 | 2 ml/m | 0% | 100% |
| 3.95 | 2 ml/m | 95% | 5% |
| 4.00 | 2 ml/m | 95% | 5% |

Method run time: 4 min



**Scheme S1.** Synthesis of compounds **8** and **14**. Reagents and conditions: (a) Cyclopentyl bromide, K2CO3, DMF, 70 °C, overnight; (b) KMnO4 water solution, MeOH, rt, 1 h



**Scheme S2.** Synthesis of compound **15**. Reagents and conditions: (a) Acetyl chloride, MeOH, rt, overnight; (b) Benzyl bromide, K2CO3, DMF, 70 °C, overnight; (c) LiOH, H2O, MeOH, THF, rt, 2 h;

**General procedure for phenol alkylation (Procedure A)**

In a DMF (40 mL) suspension of 3-(cyclopentyloxy)-4-hydroxybenzaldehyde (32.9 mmol) and potassium carbonate (65.7 mmol), the appropriate alkyl bromide (42.7 mmol) was added and the reaction was stirred for 12 h at 60 °C. The solvent was evaporated to dryness and the residue was dissolved in DCM (100 mL), filtered through Celite and washed with a saturated solution of NaHCO3 (2x30 mL) and brine (1x30 mL). The organic phase was dried with anhydrous Na2SO4, concentrated and the crude product was puriﬁed via silica gel chromatography to give the desired product.



**3-(cyclopentyloxy)-4-methoxybenzaldehyde (8)** (Yield: 80%) (Procedure A). The crude product was puriﬁed via silica gel chromatography, eluting with 0-50% EtOAc in Hexane. 1H NMR (399 MHz, CDCl3) δ 9.83 (s, 1H), 7.36 - 7.45 (m, 2H), 6.96 (d, *J* = 8.1 Hz, 1H), 4.79 - 4.89 (m, 1H), 3.92 (s, 3H), 1.94 - 2.07 (m, 2H), 1.76 - 1.93 (m, 4H), 1.55 - 1.70 (m, 2H). LCMS found 221.1, [M + H]+.



**(E)-ethyl 3-(3-(cyclopentyloxy)-4-methoxyphenyl)acrylate** **(9)** (Yield: 89%). A solution of 3-(cyclopentyloxy)-4-methoxybenzaldehyde (3 g, 13.62 mmol) **(8)** in CH3CN (45 mL) was charged with ethyl 2-(triphenylphosphoranylidene)acetate (4.74 g, 13.62 mmol). The reaction was conducted under microwave irradiation at 150 °C for 30 min. After reaction was complete by LCMS, solvent was removed *in vacuo*. The crude was dissolved in EtOAc (100 mL), washed with a saturated solution of NaHCO3 (2x30 mL) and brine (1x30 mL). The organic layer was dried over Na2SO4, evaporated to dryness and the crude was puriﬁed via silica gel chromatography, eluting with 0-20% EtOAc in Hexane to give the desired product. 1H NMR (400 MHz, CDCl3) δ 7.58 (d, *J* = 16.1 Hz, 1H), 7.00 - 7.05 (m, 2H), 6.80 (d, *J* = 8.1 Hz, 1H), 6.25 (d, *J* = 16.1 Hz, 1H), 4.71 - 4.78 (m, 1H), 4.21 (q, *J* = 7.1 Hz, 2H), 3.81 (s, 3H), 1.73 - 1.96 (m, 6H), 1.52 - 1.64 (m, 2H), 1.29 (t, *J* = 7.1 Hz, 3H). LCMS found 291.1, [M + H]+.



**rac-(trans-3,4)-ethyl 4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methylpyrrolidine-3-carboxylate** **(10a, NEU-493)** (Yield: 70%). A round-bottomed flask with a Dean-Strark trap and a reflux condenser was charged with (E)-ethyl 3-(3-(cyclopentyloxy)-4-methoxyphenyl)acrylate (2.8 g, 9.64 mmol) **(9)**, MgSO4 (0.580 g, 4.82 mmol) and Toluene (40 mL). When the mixture was under reflux, 2-(methylamino)acetic acid (1.031 g, 11.57 mmol) and paraformaldehyde (1.042 g, 34.7 mmol) were added and the oil bath temperature adjusted to 170 °C. This addition was repeated every four hours until the substrate had completely reacted. The solvent was evaporated to dryness and the residue was dissolved in DCM (100 mL), filtered through Celite and washed with NaHCO3 (3x30 mL) and brine (1x30 mL). The organic phase was dried with anhydrous Na2SO4, the solvent concentrated and the crude product was puriﬁed via silica gel chromatography, eluting with 0-5% MeOH in DCM to give the desired product. 1H NMR (500 MHz, CDCl3) δ 6.79 - 6.89 (m, 3H), 4.77 - 4.83 (m, 1H), 4.12 - 4.20 (m, 2H), 3.84 (s, 3H), 3.65 (q, *J* = 7.1 Hz, 1H), 3.03 - 3.09 (m, 2H), 2.96 (br. s., 2H), 2.67 (br. s., 1H), 2.43 (br. s., 3H), 1.80 - 1.99 (m, 6H), 1.57 - 1.67 (m, 2H), 1.25 (t, *J* = 7.1 Hz, 3H). LCMS found 348.2, [M + H]+.

**General procedure for ester hydrolysis (Procedure B)**

A solution of lithium hydroxide (0.144 mmol) in water (0.5 mL) was added to a solution of the appropriate ester (0.072 mmol) in THF (0.5 mL). The mixture was homogenized with methanol (0.5 mL) and stirred at room temperature for 2 h. The solvent was evaporated to dryness and the residue was dissolved in water. The pH was set acidic with 1M HCl, and the desired product was extracted with EtOAc. The combined organic phases were washed with brine, dried with anhydrous Na2SO4 and the solvent removed *in vacuo*. The crudes were purified employing different procedures to give the desired products.



**rac-(trans-3,4)-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methylpyrrolidine-3-carboxylic acid (10b, NEU-615)** (Yield: 34%) (Procedure B). The crude was dissolved in 1 mL DMSO, filtered through 17 mm cellulose syringe filters (0.45 µm) and purified via preparative HPLC to give the desired product. 1H NMR (500 MHz, CDCl3) δ 8.29 (br. s, 1H), 6.89 (s, 1H), 6.84 (s, 2H), 4.78 - 4.84 (m, 1H), 4.13 - 4.21 (m, 2H), 3.91 - 3.98 (m, 1H), 3.85 (s, 3H), 3.10 - 3.22 (m, 2H), 2.88 - 2.92 (m, 1H), 2.86 (s, 3H), 1.79 - 1.99 (m, 6H), 1.55 - 1.66 (m, 2H). LCMS found 320.2, [M + H]+.

**General procedure for pyrrolidine derivatives preparation (Procedure C)**

To a solution of rac-(trans-23,4)-ethyl 4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methylpyrrolidine-3-carboxylate **(10a)** (25 mg, 0.072 mmol) in 1 mL of Toluene at 0 °C was slowly added trimethylaluminum (0.216 mL, 0.108 mmol) 2M solution in Heptane under N2 atmosphere. After 10 min stirring the appropriate amine (0. 216 mmol) was added and the mixture was heated at 80 °C for 5 h. After reaction was complete, a saturated aqueous solution of NH4Cl and few drops of HCl 2N were added. The mixture was washed with a solution of NaOH 2N, and extracted with DCM. The combined organic phases were filtered through pre-packed Na2SO4 column. The filtrate was evaporated with Genevac, solubilized in 1 mL DMSO and filtered through 17 mm cellulose syringe filter (0.45 µm). The crude product was purified via preparative HPLC to give the desired product.



**rac-(trans-3,4)-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methyl-N-(pyridin-4-yl)pyrrolidine-3-carboxamide,** **formate** **salt** **(11a, NEU-505)** (Yield: 21%) (Procedure C). 1H NMR (500 MHz, CDCl3) δ 9.57 (br. s., 1H), 8.47 (d, *J* = 6.3 Hz, 2H), 8.44 (s, 1H), 7.56 (d, *J* = 6.3 Hz, 2H), 6.79 - 6.89 (m, 3H), 4.72 - 4.77 (m, 1H), 3.91 (q, *J* = 9.3 Hz, 1H), 3.83 (s, 3H), 3.60 - 3.72 (m, 2H), 3.47 (d, *J* = 8.8 Hz, 2H), 3.36 - 3.42 (m, 1H), 2.83 (s, 3H), 1.74 - 1.97 (m, 6H), 1.54 - 1.63 (m, 2H). LCMS found 396.2, [M + H]+.



**(rac-(trans-3,4)-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methylpyrrolidin-3-yl)(piperidin-1-yl)methanone, formate salt** **(11b, NEU-506)** (Yield: 6%) (Procedure C). 1H NMR (500 MHz, CDCl3) δ 8.56 (s, 1H), 6.98 (d, *J* = 1.5 Hz, 1H), 6.85 - 6.88 (m, 1H), 6.79 - 6.83 (m, 1H), 4.79 - 4.84 (m, 1H), 3.84 (s, 3H), 3.72 - 3.83 (m, 4H), 3.64 - 3.71 (m, 1H), 3.28 - 3.36 (m, 2H), 3.25 (t, *J* = 10.0 Hz, 1H), 3.12 (t, *J* = 5.4 Hz, 2H), 2.82 (s, 3H), 1.96 - 2.04 (m, 2H), 1.80 - 1.89 (m, 4H), 1.63 (br. s., 2H), 1.45 - 1.53 (m, 3H), 1.25 - 1.41 (m, 2H), 0.79 - 0.87 (m, 1H). LCMS found 387.2, [M + H]+.



**rac-(trans-3,4)-N-benzyl-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methylpyrrolidine-3-carboxamide** **(11c, NEU-524)** (Yield: 3%) (Procedure C). 1H NMR (500 MHz, CDCl3) δ 8.49 (br. s, 1H), 7.25 - 7.28 (m, 2H), 7.07 - 7.11 (m, 2H), 6.90 (d, *J* = 1.5 Hz, 1H), 6.75 - 6.85 (m, 2H), 6.32 (br. s., 1H), 4.71 - 4.79 (m, 1H), 4.46 (dd, *J* = 6.1, 15.0 Hz, 1H), 4.30 (dd, *J* = 5.4, 15.00 Hz, 1H), 3.84 (s, 3H), 3.72 (q, *J* = 9.3 Hz, 1H), 3.59 (br. s., 1H), 3.43 (t, *J* = 8.8 Hz, 1H), 3.23 - 3.35 (m, 3H), 2.74 (s, 3H), 1.90 - 2.00 (m, 2H), 1.77 - 1.88 (m, 4H), 1.53 - 1.68 (m, 2H). LCMS found 409.2, [M + H]+.



**rac-(trans-3,4)-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methyl-N-(p-tolyl)pyrrolidine-3-carboxamide** **(11d, NEU-525)** (Yield: 26%) (Procedure C). 1H NMR (500 MHz, CDCl3) δ 8.23 (br. s., 1H), 7.38 (d, *J* = 8.8 Hz, 2H), 7.13 (d, *J* = 8.3 Hz, 2H), 6.82 - 6.88 (m, 3H), 4.75 - 4.82 (m, 1H), 3.86 (s, 3H), 3.59 (q, *J* = 5.9 Hz, 1H), 3.31 (t, *J* = 8.8 Hz, 1H), 3.20 (dd, *J* = 3.9, 9.8 Hz, 1H), 2.94 - 2.99 (m, 1H), 2.78 - 2.85 (m, 1H), 2.59 (t, *J* = 8.8 Hz, 1H), 2.51 (s, 3H), 2.33 (s, 3H), 1.79 - 1.99 (m, 6H), 1.65 - 1.76 (m, 2H). LCMS found 409.2, [M + H]+.



**rac-(trans-3,4)-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methyl-N-phenylpyrrolidine-3-carboxamide** **(11e, NEU-526)** (Yield: 18%) (Procedure C). 1H NMR (500 MHz, CDCl3) δ 8.42 (br. s, 1H), 7.51 (d, *J* = 8.8 Hz, 2H), 7.33 (t, *J* = 8.1 Hz, 2H), 7.07 - 7.14 (m, 1H), 6.82 - 6.88 (m, 3H), 4.76 - 4.82 (m, 1H), 3.86 (s, 3H), 3.56 - 3.63 (m, 1H), 3.33 (t, *J* = 8.8 Hz, 1H), 3.21 (dd, *J* = 3.9, 9.8 Hz, 1H), 2.94 - 3.00 (m, 1H), 2.75 - 2.83 (m, 1H), 2.56 (t, *J* = 8.6 Hz, 1H), 2.51 (s, 3H), 1.80 - 2.00 (m, 6H), 1.54 - 1.75 (m, 2H). LCMS found 395.2, [M + H]+.



**rac-(trans-3,4)-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-N-isopropyl-1-methylpyrrolidine-3-carboxamide,** **formate salt** **(11f, NEU-527)** (Yield: 6%) (Procedure C). 1H NMR (500 MHz, CDCl3) δ 8.48 (s, 1H), 6.95 (s, 1H), 6.79 - 6.87 (m, 2H), 4.79 - 4.85 (m, 1H), 3.94 - 4.04 (m, 1H), 3.84 (s, 3H), 3.64 - 3.79 (m, 3H), 3.22 - 3.38 (m, 3H), 2.83 (s, 3H), 1.94 - 2.04 (m, 2H), 1.79 - 1.92 (m, 4H), 1.59 - 1.68 (m, 2H), 1.07 (d, *J* = 6.4 Hz, 3H), 0.97 (d, *J* = 6.4 Hz, 3H). LCMS found 361.2, [M + H]+.



**rac-(trans-3,4)-N-cyclohexyl-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methylpyrrolidine-3-carboxamide,** **formate salt** **(11g, NEU-533)** (Yield: 8%) (Procedure C). 1H NMR (500 MHz, CDCl3) δ 8.46 (s, 1H), 6.93 (d, *J* = 1.5 Hz, 1H), 6.79 - 6.87 (m, 2H), 4.77 - 4.85 (m, 1H), 3.85 (s, 3H), 3.79 (s, 1H), 3.62 - 3.75 (m, 3H), 3.26 - 3.38 (m, 3H), 2.84 (s, 3H), 1.93 - 2.03 (m, 2H), 1.76 - 1.92 (m, 5H), 1.53 - 1.73 (m, 6H), 1.23 - 1.36 (m, 2H), 1.00 - 1.16 (m, 2H), 0.87 - 0.97 (m, 1H). LCMS found 401.2, [M + H]+.



**rac-(trans-3,4)-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-N,1-dimethylpyrrolidine-3-carboxamide** **(11h, NEU-534)** (Yield: 10%) (Procedure C). 1H NMR (500 MHz, CDCl3) δ 8.47 (br. s, 1H), 6.90 (d, *J* = 1.5 Hz, 1H), 6.80 - 6.86 (m, 2H), 4.78 - 4.84 (m, 1H), 3.85 (s, 3H), 3.72 - 3.79 (m, 1H), 3.64 - 3.71 (m, 1H), 3.54 (t, *J* = 10.0 Hz, 1H), 3.24 - 3.38 (m, 3H), 2.79 (s, 3H), 2.75 (s, 1H), 2.74 (s, 1H), 2.65 (s, 1H), 1.81 - 2.04 (m, 6H), 1.60 – 1.69 (m, 2H). LCMS found 333.2, [M + H]+.



**rac-(trans-3,4)-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-N-ethyl-1-methylpyrrolidine-3-carboxamide (11i, NEU-535)** (Yield: 11%) (Procedure C). 1H NMR (500 MHz, CDCl3) δ 8.47 (br. s, 1H), 6.91 (d, *J* = 1.9 Hz, 1H), 6.79 - 6.88 (m, 2H), 4.77 - 4.84 (m, 1H), 3.84 (s, 3H), 3.71 - 3.79 (m, 2H), 3.64 (t, *J* = 10.0 Hz, 1H), 3.24 - 3.37 (m, 3H), 3.16 - 3.24 (m, 2H), 2.82 (s, 3H), 1.92 - 2.02 (m, 2H), 1.79 - 1.92 (m, 4H), 1.58 - 1.69 (m, 2H), 1.03 (t, *J* = 7.3 Hz, 3H). LCMS found 347.2, [M + H]+.



**(rac-(trans-3,4)-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methylpyrrolidin-3-yl)(pyrrolidin-1-yl)methanone,** **formate salt** **(11j, NEU-536)** (Yield: 6%) (Procedure C). 1H NMR (500 MHz, CDCl3) δ 8.49 (s, 1H), 6.99 (d, *J* = 1.9 Hz, 1H), 6.87 (dd, *J* = 2.0, 8.3 Hz, 1H), 6.79 - 6.83 (m, 1H), 4.80 - 4.85 (m, 1H), 3.85 - 3.93 (m, 2H), 3.84 (s, 3H), 3.75 - 3.83 (m, 1H), 3.62 - 3.70 (m, 1H), 3.36 - 3.43 (m, 2H), 3.24 - 3.36 (m, 3H), 2.88 (s, 3H), 2.58 - 2.64 (m, 1H), 1.94 - 2.07 (m, 2H), 1.73 - 1.88 (m, 6H), 1.54 - 1.72 (m, 4H). LCMS found 373.2, [M + H]+.



**rac-(trans-3,4)-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methyl-N-(naphthalen-1-yl)pyrrolidine-3-carboxamide,** **formate salt (11k, NEU-544)** (Yield: 8%) (Procedure C). 1H NMR (500 MHz, CDCl3) δ 8.47 (s, 1H), 8.40 (br. s., 1H), 7.81 (d, *J* = 7.8 Hz, 2H), 7.66 (d, *J* = 7.8 Hz, 1H), 7.39 - 7.47 (m, 2H), 7.32 - 7.37 (m, 1H), 7.20 (d, *J* = 8.3 Hz, 1H), 7.06 (d, *J* = 1.9 Hz, 1H), 6.98 (dd, *J* = 2.2, 8.1 Hz, 1H), 6.87 (d, *J* = 8.1 Hz, 1H), 4.76 - 4.81 (m, 1H), 3.82 - 3.90 (m, 5H), 3.75 - 3.82 (m, 1H), 3.63 - 3.73 (m, 1H), 3.41 - 3.53 (m, 2H), 2.87 (s, 3H), 1.69 - 2.00 (m, 6H), 1.49 - 1.62 (m, 2H). LCMS found 445.2, [M + H]+.



**rac-(trans-3,4)-ethyl 4-(3-(cyclopentyloxy)-4-methoxyphenyl)pyrrolidine-3-carboxylate** **(12).** 1-chloroethyl-carbonochloridate (0.466 ml, 4.32 mmol) in ClCH2CH2Cl (5 mL), was added dropwise to a solution of ethyl 4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-methylpyrrolidine-3-carboxylate (500 mg, 1.439 mmol) **(10a)** at 0 °C in ClCH2CH2Cl (10 mL). The mixture was refluxed overnight under N2 atmosphere. The intermediate 1-(1-chloroethyl) 3-ethyl 4-(3-(cyclopentyloxy)-4-methoxyphenyl)pyrrolidine-1,3-dicarboxylate (ACE-pyrrolidine) was deACEylated directly to final by evaporating the reaction mixture *in vacuo* and then heating the residue in MeOH (10 mL) overnight at 60 °C. The solution was cooled, evaporated to dryness, dissolved in DCM (40 mL) and washed with a NaHCO3 saturated solution (2x15 mL) and brine (1x15 mL). The organic phase was dried with anhydrous Na2SO4, the solvent concentrated and the crude intermediate was carried forward without further puriﬁcation. 15 mg of this crude material were dissolved in 1 mL DMSO, filtered through 17 mm cellulose syringe filter (0.45 µm) and purified via preparative HPLC to give the desired product which was used for analytical purposes. 1H NMR (500 MHz, CDCl3) δ 6.75 - 6.91 (m, 3H), 4.75 - 4.84 (m, 1H), 4.14 (q, *J* = 6.3 Hz, 2H), 3.94 - 4.06 (m, 1H), 3.85 (s, 3H), 3.66 - 3.76 (m, 1H), 3.49 - 3.66 (m, 2H), 3.05 - 3.24 (m, 2H), 1.79 - 2.01 (m, 6H), 1.56 - 1.68 (m, 2H), 1.21 (t, *J* = 6.30 Hz, 3H). LCMS found 334.2, [M + H]+.

**Synthetic procedure for compounds 13a and 13b (Procedure D)**

An methanesulfonyl chloride (0.012 ml, 0.150 mmol) or acetyl chloride (0.011 ml, 0.150 mmol) solution in DCM (0.5 mL) was added dropwise to a stirred solution of ethyl 4-(3-(cyclopentyloxy)-4-methoxyphenyl)pyrrolidine-3-carboxylate (25 mg, 0.075 mmol) **(12)** and 4-Dimethylaminopyridine (18.32 mg, 0.150 mmol) in DCM (1 mL) at 0 °C under N2. The mixture was stirred at room temperature for 3 h. After reaction was complete, the solution was washed with NaHCO3 saturated solution (2x1 mL) and brine (1x1 mL). The organic phase was dried over anhydrous Na2SO4 and concentrated *in vacuo*. The crude was dissolved in 1 mL DMSO, filtered through 17 mm cellulose syringe filter (0.45 µm) and purified via preparative HPLC to give the desired product



**rac-(trans-3,4)-ethyl 4-(3-(cyclopentyloxy)-4-methoxyphenyl)-1-(methylsulfonyl)pyrrolidine-3-carboxylate (13a, NEU-538)** (Yield: 12%) (Procedure D). 1H NMR (500 MHz, CDCl3) δ 6.80 - 6.86 (m, 3H), 4.76 - 4.83 (m, 1H), 4.16 (q, *J* = 7.3 Hz, 2H), 3.86 (s, 3H), 3.76 - 3.85 (m, 2H), 3.63 - 3.67 (m, 2H), 3.53 (dd, *J* = 7.6, 10.0 Hz, 1H), 3.18 (q, *J* = 7.8 Hz, 1H), 2.95 (s, 3H), 1.81 - 2.01 (m, 6H), 1.60 - 1.69 (m, 2H), 1.23 (t, *J* = 7.1 Hz, 3H). LCMS found 412.2, [M + H]+.



**rac-(trans-3,4)-ethyl 1-acetyl-4-(3-(cyclopentyloxy)-4-methoxyphenyl)pyrrolidine-3-carboxylate (13b, NEU-539)** (Yield: 10%) (Procedure D). 1H NMR (500 MHz, CDCl3) δ 6.84 (t, *J* = 7.30 Hz, 1H), 6.76 - 6.81 (m, 2H), 4.75 - 4.81 (m, 1H), 4.11 - 4.16 (m, 1H), 4.04 - 4.10 (m, 1H), 3.86 - 3.98 (m, 1H), 3.85 (s, 3H), 3.78 (t, *J* = 10.3 Hz, 1H), 3.65 - 3.72 (m, 1H), 3.49 - 3.60 (m, 2H), 3.11 - 3.24 (m, 1H), 2.11 (d, *J* = 9.3 Hz, 3H), 1.81 - 1.99 (m, 6H), 1.59 - 1.69 (m, 2H), 1.19 (t, *J* = 7.1 Hz, 3H). LCMS found 376.2, [M + H]+.



**methyl 3-hydroxy-4-methoxybenzoate** **(S1)** (Yield: 86%). To cold MeOH (10 mL) was added dropwise acetyl chloride (2.336 ml, 32.9 mmol) and the mixture was stirred for 10 min. 3-hydroxy-4-methoxybenzoic acid (2.5 g, 14.87 mmol), was added in one portion and the reaction was allowed to stir overnight at room temperature. The solvent was removed *in vacuo*, dissolved in EtOAc (100 mL), washed with a saturated solution of NaHCO3 (2x30 mL) and with brine (1x20 mL). The organic layer was dried over Na2SO4, evaporated to dryness and the crude was puriﬁed via silica gel chromatography, eluting with 0-40% EtOAc in Hexane to give the desired product. 1H NMR (500 MHz, CDCl3) δ 7.62 (dd, J = 1.7, 8.3 Hz, 1H), 7.59 (d, J = 1.7 Hz, 1H), 6.87 (d, J = 8.3 Hz, 1H), 5.64 (s, 1H), 3.95 (s, 3H), 3.88 (s, 3H). LCMS found 183.1, [M + H]+.



**methyl 3-(benzyloxy)-4-methoxybenzoate** **(S2)** (Yield: 81%) (Procedure A). The crude product was puriﬁed via silica gel chromatography, eluting with 0-20% EtOAc in Hexane to give the desired product. 1H NMR (500 MHz, CDCl3) δ 7.69 (dd, *J* = 2.0, 8.3 Hz, 1H), 7.61 (d, *J* = 2.0 Hz, 1H), 7.45 - 7.49 (m, 2H), 7.38 (t, *J* = 7.6 Hz, 2H), 7.29 - 7.34 (m, 1H), 6.91 (d, *J* = 8.3 Hz, 1H), 5.18 (s, 2H), 3.93 (s, 3H), 3.87 (s, 3H). LCMS found 273.1, [M + H]+.



**3-(cyclopentyloxy)-4-methoxybenzoic acid** **(14)** (Yield: 71%). In a solution of the 3-(cyclopentyloxy)-4-methoxybenzaldehyde (2 g, 9.08 mmol) **(8)** in MeOH (40 mL), was added potassium permanganate (7.17 g, 45.4 mmol) in Water (20 mL). The reaction was kept at room temperature for 1 h. NaHSO3 10% solution in water was added dropwise until decoloration of the crude. The precipitate was filtered under vacuum and dissolved in NaOH 10N. The aqueous solution was washed with EtOAc (2x30 mL) and then cooled down to 0 °C. Concentrated HCl was slowly added until the pH was set to acidic. The precipitate was filtered under vacuum and dried. 1H NMR (500 MHz, CDCl3) δ 7.73 (dd, J = 1.9, 8.3 Hz, 1H), 7.59 (d, J = 1.9 Hz, 1H), 6.90 (d, J = 8.3 Hz, 1H), 4.83 - 4.87 (m, 1H), 3.92 (s, 3H), 1.96 - 2.04 (m, 2H), 1.80 - 1.94 (m, 4H), 1.59 - 1.67 (m, 2H). LCMS found 237.1, [M + H]+.



**3-(cyclopentyloxy)-4-methoxybenzoic acid** **(15)** (Yield: 98%) (Procedure B). The crude product was puriﬁed via silica gel chromatography, eluting with 0-50% EtOAc in Hexane to give the desired product. 1H NMR (500 MHz, CDCl3) δ 7.76 (dd, *J* = 1.9, 8.8 Hz, 1H), 7.64 (d, *J* = 1.9 Hz, 1H), 7.47 (br. d, *J* = 7.3 Hz, 2H), 7.39 (t, *J* = 7.3 Hz, 2H), 7.30 - 7.34 (m, 1H), 6.94 (d, *J* = 8.8 Hz, 1H), 5.19 (s, 2H), 3.95 (s, 3H). LCMS found 259.1, [M + H]+.

**General procedure for the preparation of β-keto-ester (Procedure E)**

A solution of the appropriate acid (8.47 mmol) in thionyl chloride (8.5 mmol) was stirred for 4 h at reflux. The mixture was cooled and the thionyl chloride removed by distillation. The mixture was washed with toluene (3x20 mL). The acyl chloride was dissolved in dry THF (10 mL), kept under N2 and carried forward for the next step without further puriﬁcation. Diisopropylamine purified by redistillation (26.2 mmol) was dissolved in THF (20 mL) in a flame-dried flask equipped with a septum under N2 atmosphere. The flask was cooled to -78 °C. Butyllithium (25.4 mmol) was slowly added. The temperature was brought to -10 °C by immersion in a water/salt bath for 15 min and then the flask was recooled to -78 C. Methyl acetate (25.4 mmol) was added dropwise. This mixture was stirred at -78 °C for 5 min, and then the acyl chloride (8.5 mmol) was added through a double-tipped needle. The -78 °C bath was removed immediately and the reaction was allowed to warm to room temperature. After 30 min the mixture was diluted with 10% aqueous HCl, extracted with DCM and washed once with brine. The organic phase was dried with anhydrous Na2SO4, the solvent concentrated and the crude products puriﬁed via silica gel chromatography, eluting with 0-20% EtOAc in Hexane to give the desired products.



**methyl 3-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-oxopropanoate** **(16)** (Yield: 67%) (Procedure E). 1H NMR (500 MHz, CDCl3) δ 7.52 - 7.56 (m, 2H), 6.91 (br. d, *J* = 8.8 Hz, 1H), 4.84 - 4.89 (m, 1H), 3.99 (s, 2H), 3.94 (s, 3H), 3.77 (s, 3H), 1.97 - 2.09 (m, 2H), 1.80 - 1.95 (m, 4H), 1.57 - 1.71 (m, 2H). LCMS found 293.1, [M + H]+.



**methyl 3-(3-(benzyloxy)-4-methoxyphenyl)-3-oxopropanoate** **(17)** (Yield: 88%) (Procedure E). 1H NMR (500 MHz, CDCl3) δ 7.54 - 7.58 (m, 2H), 7.43 - 7.48 (m, 2H), 7.34 - 7.40 (m, 2H), 7.29 - 7.34 (m, 1H), 6.92 (d, *J* = 8.3 Hz, 1H), 5.19 (s, 2H), 3.95 (s, 3H), 3.92 (s, 2H), 3.74 (s, 3H). LCMS found 315.1, [M + H]+.



**methyl 1-(3-(cyclopentyloxy)-4-methoxybenzoyl)cyclohexanecarboxylate** **(19, NEU-649)** (Yield: 65%). A stirred suspension of sodium hydride 60% in mineral oil (274 mg, 6.84 mmol) in dry DMF (5 mL) was added dropwise to a solution of methyl 3-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-oxopropanoate **(16)** (500 mg, 1.710 mmol) in dry DMF (5 mL) at 0 °C. After the addition was complete, a solution of 1,5-diiodopentane (0.38 ml, 2.57 mmol) in dry DMF (5 mL) was added dropwise and stirring was continued at room temperature overnight. Water was added, the solvent was removed *in vacuo* and the crude dissolved in DCM (50 mL), washed with a saturated solution of NaHCO3 (2x20 mL) and brine (1x20 mL). The organic phase was dried with anhydrous Na2SO4, concentrated and the crude puriﬁed via silica gel chromatography, eluting with 0-20% EtOAc in Hexane to give the desired product. 1H NMR (500 MHz, CDCl3) δ 7.48 (dd, *J* = 2.2, 8.8 Hz, 1H), 7.45 (d, *J* = 2.2 Hz, 1H), 6.84 (d, *J* = 8.8 Hz, 1H), 4.78 - 4.83 (m, 1H), 3.91 (s, 3H), 3.67 (s, 3H), 2.13 (t, *J* = 5.9 Hz, 4H), 1.98 - 2.06 (m, 2H), 1.80 - 1.93 (m, 4H), 1.55 - 1.70 (m, 6H), 1.41 - 1.55 (m, 2H). LCMS found 361.2, [M + H]+.



**methyl 1-(3-(benzyloxy)-4-methoxybenzoyl)cyclohexanecarboxylate (20, NEU-688)** (Yield: 50%). To a solution of methyl 3-(3-(benzyloxy)-4-methoxyphenyl)-3-oxopropanoate (300 mg, 0.954 mmol) **(17)** in DMF (5 mL) was added potassium carbonate (528 mg, 3.82 mmol) and 1,5-diiodopentane (0.213 ml, 1.432 mmol). The mixture was stirred for 8 h at 90 °C. The solvent was removed *in vacuo* and the crude dissolved in EtOAc (50 mL), washed with a saturated solution of NaHCO3 (2x20 mL) and brine (1x20 mL). The organic phase was dried with anhydrous Na2SO4, the solvent concentrated and the crude puriﬁed via silica gel chromatography, eluting with 0-20% EtOAc in Hexane to give the desired product. 1H NMR (500 MHz, CDCl3) δ 7.49 (dd, *J* = 1.9, 8.8 Hz, 1H), 7.45 - 7.48 (m, 3H), 7.34 - 7.40 (m, 2H), 7.28 - 7.33 (m, 1H), 6.85 (d, *J* = 8.8 Hz, 1H), 5.17 (s, 2H), 3.93 (s, 3H), 3.59 (s, 3H), 1.97 - 2.08 (m, 4H), 1.55 - 1.65 (m, 2H), 1.35 - 1.54 (m, 4H). LCMS found 383.2, [M + H]+.

**General procedure for the preparation of pyrazole derivatives (Procedure F)**

To a neat solution containing the appropriate β-keto-ester (0.684 mmol) and hydrazinium chloride (1.368 mmol) was added Acetic Acid (2 mL). The reaction mixture was heated at 120 °C for 2 h. Acetic Acid was removed *in vacuo* and the crude dissolved in EtOAc (50 mL) washed with a saturated solution of NaHCO3 (2x20 mL) and brine (1x20 mL). The organic phase was dried with anhydrous Na2SO4, concentrated and the crude product puriﬁed via silica gel chromatography, eluting with 0-25% EtOAc in Hexane to give the desired product.



**3-(3-(cyclopentyloxy)-4-methoxyphenyl)-1H-pyrazol-5-ol (18a, NEU-653)** (Yield: 70%) (Procedure F).1H NMR (400 MHz, DMSO-*d*6) δ 7.20 (d, *J* = 1.5 Hz, 1H), 7.13 - 7.18 (m, 1H), 6.94 (d, *J* = 8.8 Hz, 1H), 5.79 (s, 1H), 4.79 - 4.87 (m, 1H), 3.74 (s, 3H), 1.85 - 1.96 (m, 2H), 1.65 - 1.75 (m, 4H), 1.52 - 1.62 (m, 2H). LCMS found 275.1, [M + H]+.



**4-(3-(cyclopentyloxy)-4-methoxyphenyl)-2,3-diazaspiro[4.5]dec-3-en-1-one** **(21a, NEU-654)** (Yield: 52%) (Procedure F). 1H NMR (400 MHz, CDCl3) δ 8.66 (br. s, 1H), 7.41 (d, *J* = 2.0 Hz, 1H), 7.32 (dd, *J* = 2.0, 8.4 Hz, 1H), 6.86 (d, *J* = 8.4 Hz, 1H), 4.78 - 4.86 (m, 1H), 3.89 (s, 3H), 2.23 (tq, *J* = 2.9, 12.5 Hz, 2H), 2.09 (dt, *J* = 4.0, 13.4 Hz, 2H), 1.75 - 2.04 (m, 9H), 1.57 - 1.69 (m, 4H), 1.27 - 1.41 (m, 1H). LCMS found 343.2, [M + H]+.



**4-(3-(benzyloxy)-4-methoxyphenyl)-2,3-diazaspiro[4.5]dec-3-en-1-one** **(22a, NEU-692)** (Yield: 71%). Methyl 1-(3-(benzyloxy)-4-methoxybenzoyl)cyclohexanecarboxylate **(20)** (200 mg, 0.523 mmol), N,N-diisopropylethylamine (0.269 ml, 1.569 mmol) and hydrazinium chloride (71.6 mg, 1.046 mmol) were dissolved in BuOH (10 mL) and the mixture was stirred at 120 °C for 3 h. The solvent was removed *in vacuo* and the crude dissolved in EtOAc (40 mL) washed with a saturated solution of NaHCO3 (2x15 mL) and brine (1x10 mL). The organic phase was dried with anhydrous Na2SO4, concentrated and the crude product puriﬁed via silica gel chromatography, eluting with 0-25% EtOAc in Hexane to give the desired product. 1H NMR (500 MHz, CDCl3) δ 8.25 (br. s, 1H), 7.43 - 7.46 (m, 2H), 7.34 - 7.39 (m, 4H), 7.28 - 7.33 (m, 1H), 6.91 (d, *J* = 8.3 Hz, 1H), 5.20 (s, 2H), 3.93 (s, 3H), 2.18 (tq, *J* = 3.4, 12.7 Hz, 2H), 1.82 - 1.94 (m, 3H), 1.70 (br. d, *J* = 14.2 Hz, 2H), 1.56 (br. s., 2H), 1.25 (tq, *J* = 3.9, 13.2 Hz, 1H). LCMS found 365.2, [M + H]+.



**1-cyclopentyl-3-(3-(cyclopentyloxy)-4-methoxyphenyl)-1H-pyrazol-5-ol (18b, NEU-658)** (Yield: 10%). 3-(3-(cyclopentyloxy)-4-methoxyphenyl)-1H-pyrazol-5(4H)-one (**18a**) (25 mg, 0.091 mmol), bromocyclopentane (0.015 ml, 0.137 mmol) and potassium carbonate (25.2 mg, 0.182 mmol) were suspended in DMF (1 mL) and stirred at 80 °C for 5 h. The mixture was filtered through 17 mm cellulose syringe filters (0.45 µm). and purified via preparative HPLC to give the desired product. 1H NMR (400 MHz, CDCl3) δ 7.04 - 7.10 (m, 2H), 6.89 (d, *J* = 8.1 Hz, 1H), 5.84 (s, 1H), 4.93 - 4.99 (m, 1H), 4.77 - 4.84 (m, 1H), 3.87 (s, 3H), 1.75 - 1.99 (m, 12H), 1.55 - 1.67 (m, 4H). LCMS found 343.2, [M + H]+.

**General procedure for pyrazole alkylation (Procedure G).**

A DMF (0.5 mL) suspension of sodium hydride 60% in mineral oil (11.68 mg, 0.292 mmol) was added dropwise to a cold DMF (0.5 mL) solution of the appropriate pyrazole (0.073 mmol). After addiction was complete neat bromocyclopentane (0.110 mmol) was added all in once. The mixture was stirred for 3 h at 60 °C. Water was added and the reaction stirred for additional 10 min. The solvent was evaporated with Genevac, the crude solubilized in 1 mL DMSO, filtered through 17 mm cellulose syringe filter (0.45 µm) and purified via preparative HPLC to give the desired product.



**2-cyclopentyl-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-2,3-diazaspiro[4.5]dec-3-en-1-one** **(21b, NEU-655**) (Yield: 7%) (Procedure G). 1H NMR (500 MHz, CDCl3) δ 7.45 (d, *J* = 1.9 Hz, 1H), 7.35 (dd, *J* = 1.9, 8.3 Hz, 1H), 6.88 (d, *J* = 8.3 Hz, 1H), 4.81 - 4.86 (m, 1H), 4.64 - 4.71 (m, 1H), 3.90 (s, 3H), 2.28 (tq, *J* = 3.9, 13.2 Hz, 2H), 2.08 (dt, *J* = 4.0, 13.2 Hz, 2H), 1.83 - 2.04 (m, 13H), 1.74 (br. d, *J* = 13.2 Hz, 2H), 1.58 - 1.71 (m, 6H), 1.34 (tq, *J* = 3.4, 13.2 Hz, 1H). LCMS found 411.2, [M + H]+.



**2-cycloheptyl-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-2,3-diazaspiro[4.5]dec-3-en-1-one** **(21c, NEU-656)** (Yield: 11%) (Procedure G). 1H NMR (500 MHz, CDCl3) δ 7.43 (d, *J* = 2.2 Hz, 1H), 7.35 (dd, *J* = 2.2, 8.8 Hz, 1H), 6.88 (d, *J* = 8.8 Hz, 1H), 4.82 - 4.87 (m, 1H), 4.26 - 4.33 (m, 1H), 3.90 (s, 3H), 2.27 (tq, *J*= 3.4, 13.7 Hz, 2H), 2.06 (dt, *J* = 4.2, 13.6 Hz, 2H), 1.78 - 2.02 (m, 13H), 1.51 - 1.75 (m, 12H), 1.34 (tq, *J* = 3.4, 13.2 Hz, 1H). LCMS found 439.2, [M + H]+.



**2-benzyl-4-(3-(cyclopentyloxy)-4-methoxyphenyl)-2,3-diazaspiro[4.5]dec-3-en-1-one** **(21d, NEU-657)** (Yield: 6%) (Procedure G). 1H NMR (500 MHz, CDCl3) δ 7.41 (d, *J* = 1.9 Hz, 1H), 7.32 - 7.39 (m, 5H), 7.27 - 7.32 (m, 1H), 6.87 (d, *J* = 8.3 Hz, 1H), 4.94 (s, 2H), 4.80 - 4.85 (m, 1H), 3.89 (s, 3H), 2.30 (tq, *J* = 3.4, 13.2 Hz, 2H), 2.10 (dt, *J* = 4.4 13.4 Hz, 2H), 1.82 - 2.04 (m, 7H), 1.77 (br. d, *J* = 13.7 Hz, 2H), 1.60 - 1.69 (m, 4H), 1.36 (tq, *J* = 3.4, 13.2 Hz, 1H). LCMS found 433.2, [M + H]+.



**4-(3-(benzyloxy)-4-methoxyphenyl)-2-cyclopentyl-2,3-diazaspiro[4.5]dec-3-en-1-one (22b, NEU-694)** (Yield: 10%) (Procedure G). 1H NMR (500 MHz, CDCl3) δ 7.46 (d, *J* = 7.8 Hz, 2H), 7.34 - 7.39 (m, 4H), 7.28 - 7.32 (m, 1H), 6.89 (d, *J* = 8.3 Hz, 1H), 5.23 (s, 2H), 4.59 - 4.67 (m, 1H), 3.93 (s, 3H), 2.15 - 2.26 (m, 2H), 1.78 - 1.97 (m, 9H), 1.59 - 1.68 (m, 4H), 1.48 - 1.53 (m, 2H), 1.18 - 1.30 (m, 1H). LCMS found 433.2, [M + H]+.



**4-(3-(benzyloxy)-4-methoxyphenyl)-2-cycloheptyl-2,3-diazaspiro[4.5]dec-3-en-1-one** **(22c, NEU-695)** (Yield: 11%) (Procedure G). 1H NMR (500 MHz, CDCl3) δ 7.46 (d, *J* = 7.3 Hz, 2H), 7.33 - 7.40 (m, 4H), 7.28 - 7.33 (m, 1H), 6.89 (d, *J* = 8.3 Hz, 1H), 5.23 (s, 2H), 4.21 - 4.29 (m, 1H), 3.93 (s, 3H), 2.15 - 2.26 (m, 2H), 1.75 - 1.96 (m, 9H), 1.57 - 1.68 (m, 6H), 1.48 - 1.54 (m, 4H), 1.18 - 1.29 (m, 1H). LCMS found 461.2, [M + H]+.



**2-benzyl-4-(3-(benzyloxy)-4-methoxyphenyl)-2,3-diazaspiro[4.5]dec-3-en-1-one** **(22d, NEU-696)** (Yield: 21%) (Procedure G). 1H NMR (500 MHz, CDCl3) δ 7.42 - 7.45 (m, 2H), 7.31 - 7.38 (m, 9H), 7.26 - 7.30 (m, 1H), 6.87 (d, *J* = 8.3 Hz, 1H), 5.19 (s, 2H), 4.89 (s, 2H), 3.91 (s, 3H), 2.22 (tq, *J* = 3.4, 13.2 Hz, 2H), 1.83 - 1.93 (m, 3H), 1.65 (br. d, *J* = 13.7 Hz, 2H), 1.50 - 1.54 (m, 2H), 1.20 - 1.30 (m, 1H). LCMS found 455.2, [M + H]+.

**Data Tables**

**Table S1.** rac-(trans-3,4)-Disubstituted Pyrrolidine analogs tested against TbrPDEB1

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | | | | |
| **Compound** | **NEU Number** | **R** | **R1** | **TbrPDEB1 (% inh)a** |
| **10a** | **NEU-493** | OCH2CH3 | CH3 | 8 ± 11 |
| **10b** | **NEU-615** | OH | CH3 | 17 ± 9 |
| **11a** | **NEU-505** |  | CH3 | 5 ± 5b |
| **11b** | **NEU-506** |  | CH3 | 7 ± 5 |
| **11c** | **NEU-524** |  | CH3 | 8 ± 0 |
| **11d** | **NEU-525** |  | CH3 | 6 ± 6 |
| **11e** | **NEU-526** |  | CH3 | 16 ± 11 |
| **11f** | **NEU-527** |  | CH3 | 10 ± 5 |
| **11g** | **NEU-533** |  | CH3 | 16 ± 6 |
| **11h** | **NEU-534** | NHCH3 | CH3 | 8 ± 0 |
| **11i** | **NEU-535** | NHCH2CH3 | CH3 | 5 ± 4 |
| **11j** | **NEU-536** |  | CH3 | 1 ± 2 |
| **11k** | **NEU-544** |  | CH3 | 10 ± 3 |
| **13a** | **NEU-538** | OCH2CH3 | SO2CH3 | 3 ± 4 |
| **13b** | **NEU-539** | OCH2CH3 | COCH3 | 15 ± 0 |

a Inhibitor concentration 10.0 µM, data shown are average of 2 replicate independent experiments; b Replicate of 3 independent experiments.

**Table S2.** Pyrazolone analogs tested against TbrPDEB1

|  |  |  |  |
| --- | --- | --- | --- |
|  | | | |
| **Compound** | **NEU number** | **R** | **TbrPDEB1 (% inh)a** |
| **18a** | **NEU-653** | H | 0 |
| **18b** | **NEU-658** |  | 5 ± 3 |

aInhibitor concentration 10.0 µM, data shown are average of 2 replicate independent experiments;

**Table S3.** Spiro Pyrazolone analogs tested against TbrPDEB1

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | | | | |
| **Compound** | **NEU number** | **R** | **R1** | **TbrPDEB1 (% inh)a** |
| **21a** | **NEU-654** |  | H | 11 ± 10 |
| **21b** | **NEU-655** |  |  | 18 ± 2 |
| **21c** | **NEU-656** |  |  | 9 ± 4b |
| **21d** | **NEU-657** |  |  | 9 ± 6b |
| **22a** | **NEU-692** |  | H | 14 ± 20 |
| **22b** | **NEU-694** |  |  | 11 ± 10b |
| **22c** | **NEU-695** |  |  | 7 ± 5 |
| **22d** | **NEU-696** |  |  | 10 ± 3 |

aInhibitor concentration 10.0 µM, data shown are average of 2 replicate independent experiments; b Replicate of 3 independent experiments.

**Table S4.** Intermediate compounds tested against TbrPDEB1

|  |  |  |  |
| --- | --- | --- | --- |
|  | | | |
| **Compound** | **NEU number** | **R** | **TbrPDEB1 (% inh)a** |
| **19** | **NEU-649** |  | 9 ± 8 |
| **20** | **NEU-688** |  | 1 ± 2 |

aInhibitor concentration 10.0 µM, data shown are average of 2 replicate independent experiments;

**Biological Assays**

Assays were performed as described previously.[2](#_ENREF_2) In brief: recombinant TbrPDEB1 (Tb09.160.3590, 1 ng/mL) or TbrPDEB2 (Tb09.160.3630 0.4 ng/ml) was assayed at 37°C in 10mM Tris pH 7.4, bovine serum albumin (0.2 mg/ml), 10mM MgCl2, 25µM cAMP (Enzo Lifesciences) and an excess of 5` endonucleotidase (>1000U), as determined by titration (Enzo Lifesciences). Reactions were terminated by the addition of BioMol green (Enzo lifesciences) which was also used to detect changes in the level of phosphate. This was measured by absorbance at 620nm using a Tecan Sunrise plate reader. Inhibitors were dissolved in DMSO and preincubated with the assay mixture at a final DMSO concentration of 2% (v/v) and IC50 values were determined at a final concentration of 10% DMSO. Inhibition was determined by the change in the initial velocity relative to a vehicle only control. Data were analysed using GraphPad Prism 5.0.

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