

## Supporting Information

# Design, Synthesis and Biological Evaluation of 3-[4-(2-hydroxyethyl)piperazin-1-yl]-7-(6-methoxypyridin-3-yl)-1-(2-propoxyethyl)pyrido[3,4-b]pyrazin-2(1H)-one, a Potent, Orally Active, Brain Penetrant Inhibitor of PDE5.

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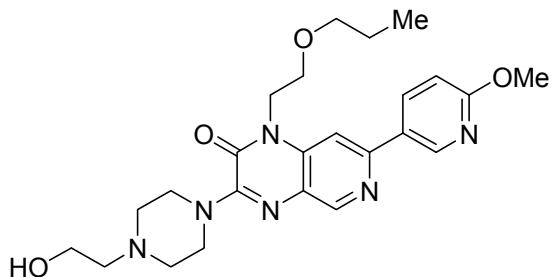
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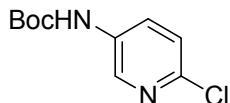
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The purity of all compounds was established to be  $> 95\%$  by HPLC. General HPLC conditions: 5 $\rightarrow$ 95% acetonitrile (0.02% TFA) in 4.5min hold 0.5min(95% acetonitrile), on the 2x30mm 3u Gemini C18 column.  $^1\text{H}$  NMR recorded at 400 MHz unless otherwise specified.

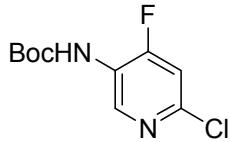


### Preparation of compound 13:



**Step 1:** A solution of 5-amino-2-chloropyridine (30.94 g, 236 mmol) and di-tert-butyldicarbonate (65.36 g, 299 mmol) in 1,4-dioxane (300 mL) was stirred at reflux for 20 hours. Additional di-tert-butyldicarbonate (8.30 g, 38 mmol) was added and the reaction was stirred at reflux for 7 hours. The reaction was cooled to room temperature and poured into water. The layers were separated and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, and solvent was removed at reduced pressure to give a brown oil. The oil was triturated with diethyl ether and filtered to give tert-butyl 6-chloropyridin-3-ylcarbamate as a tan solid. (49.84 g, 92% yield).  $^1\text{H}$  NMR

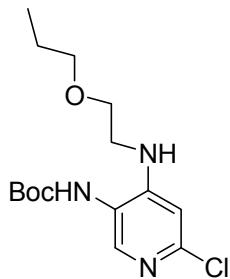
(CDCl<sub>3</sub>) δ 8.24 (m, 1H), 7.96 (m, 1H), 7.27 (m, 1H), 6.65 (br s, 1H), 1.51 (s, 9H).



**Step 2:**

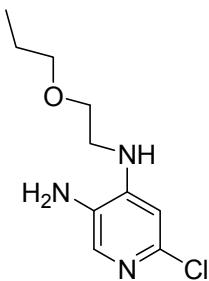
To a -63°C solution of tert-butyl 6-chloropyridin-3-ylcarbamate (24.99 g, 109.3 mmol) and TMEDA (39 mL, 260.0 mmol) in diethyl ether (700 mL) was added a 1.6M n-butyl lithium solution in hexane (193 mL, 308.8 mmol) over a period of 30 minutes while maintaining the temperature of the reaction at -60° to -50°C. The reaction was stirred at -60°C for an additional 10 minutes after the addition was complete then warmed to -10°C and stirred at -25° to -10°C for 2.0 hours. The reaction was cooled to -60°C and a solution of *N*-fluorobenzenesulfonimide (53.49 g, 169.6 mmol) in tetrahydrofuran (155 mL) was added while keeping the temperature below -50°C. It precipitated on addition and stirring became difficult. The reaction was then allowed to slowly warm to 0°C over 1 hour. The reaction was quenched with saturated ammonium chloride solution (400 mL). The layers were separated and the aqueous layer was extracted with ethyl acetate (2 x 250 mL). The combined organic layers were washed with brine, dried over

magnesium sulfate, and solvent was removed at reduced pressure to give an oily brown solid. The material was passed through a column of silica gel with 20% ethyl acetate/ hexane. The 6-chloro-4-fluoropyridin-3-ylcarbamate was obtained as a yellow solid. (15.88 g, 60% yield).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  9.09 (br d, 1H,  $J$  = 9.7 Hz), 7.12 (d, 1H,  $J$  = 9.7 Hz), 6.55 (br s, 1H), 1.54 (s, 9H).



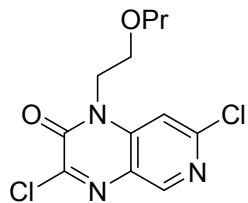
**Step 3:**

A solution of tert-butyl 6-chloro-4-fluoropyridin-3-ylcarbamate (11.96 g, 48.5 mmol) and 2-n-propoxyethylamine (11.8 mL, 97.2 mmol) in ethanol (120 mL) was stirred at reflux for 22 hours. The reaction was cooled to room temperature and solvent was removed at reduced pressure to give a yellow solid which was triturated with diethyl ether and filtered to give 6-chloro-4-[(2-propoxyethyl)amino]pyridin-3-ylcarbamate as a white solid. (13.08 g, 82% yield).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  7.92 (s, 1H), 6.54 (s, 1H), 5.77 (br s, 1H), 5.11 (br s, 1H), 3.65 (t, 2H,  $J$  = 5.2 Hz), 3.44 (t, 2H,  $J$  = 6.6 Hz), 3.34-3.29 (m, 2H), 1.65-1.56 (m, 2H), 1.49 (s, 9H), 0.94 (t, 3H,  $J$  = 7.5 Hz).



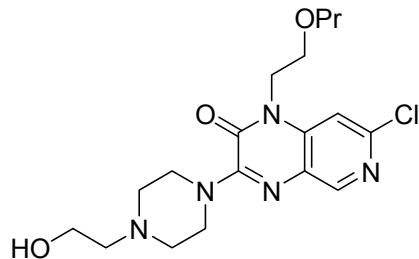
**Step 4:**

A solution of tert-butyl 6-chloro-4-[(2-propoxyethyl)amino]pyridin-3-ylcarbamate (7.08 g, 21.4 mmol) in 1,4-dioxane (20 mL) was treated with 4N HCl in 1,4-dioxane (100 mL) and stirred at room temperature for one hour. The reaction was partitioned between ethyl acetate and saturated sodium bicarbonate solution. The layers were separated and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, and solvent was removed at reduced pressure to give 6-chloro-N<sup>4</sup>-(2-propoxyethyl)pyridine-3,4-diamine as a brown oil. (4.93 g, 100% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.63 (s, 1H), 6.45 (s, 1H), 4.67 (br s, 1H), 3.67 (t, 2H, J = 5.2 Hz), 3.43 (t, 2H, J = 6.6 Hz), 3.32-3.27 (m, 2H), 2.92 (br s, 2H), 1.64-1.55 (m, 2H), 0.93 (t, 3H, J = 7.5 Hz).



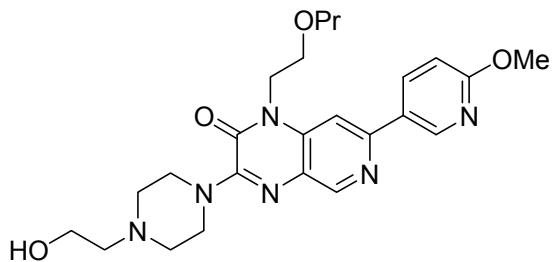
**Step 5:**

A 0°C solution of 6-chloro-N<sup>4</sup>-(2-propoxyethyl)pyridine-3,4-diamine (2.80 g, 12.2 mmol) and diisopropylethylamine (4.6 mL, 25.7 mmol) in dichloromethane (100 mL) was treated with methyl chlorooxoacetate(1.1 mL, 11.7 mmol), allowed to warm to room temperature and stirred for four hours. The reaction was diluted with dichloromethane and washed with saturated sodium bicarbonate solution, dried over magnesium sulfate, and solvent was removed at reduced pressure. The residue was dissolved in toluene (30 mL) and heated at 105°C for four hours. The solvent was removed at reduced pressure and the resulting solid taken up in dichloromethane (100 mL) and treated with oxalyl chloride (2.1 mL, 24.1 mmol) and DMF (3 drops). The reaction was stirred at room temperature for 6 hours. The solvent was removed at reduced pressure to give a brown solid. This was passed through a column of silica gel with 70% ethyl acetate/hexane to give 3,7-dichloro-1-(2-propoxyethyl)pyrido[3,4-b]pyrazin-2(1H)-one as a white solid. (2.44 g, 66% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 8.78 (s, 1H), 7.59 (s, 1H), 4.40 (t, 2H, J = 5.0 Hz), 3.80 (t, 2H, J = 5.0 Hz), 3.35 (t, 2H, J = 6.4 Hz), 1.52-1.46 (m, 2H), 0.82 (t, 3H, J = 7.5 Hz).



**Step 6:**

A solution of 3,7-dichloro-1-(2-propoxyethyl)pyrido[3,4-b]pyrazin-2(1H)-one (200 mg, 0.66 mmol), 1-(2-hydroxyethyl)piperazine (117 mg, 0.90 mmol, Aldrich) and triethylamine (0.27 mL, 1.94 mmol) in THF (3 mL) was stirred at room temperature for one hour. The reaction was partitioned between ethyl acetate and water. The layers were separated and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, and solvent was removed at reduced pressure to give 7-chloro-3-[4-(2-hydroxyethyl)piperazin-1-yl]-1-(2-propoxyethyl)pyrido[3,4-b]pyrazin-2(1H)-one. (218 mg, 83% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 8.48 (1H), 7.34 (1H), 4.33-4.30 (2H), 4.04-4.00 (4H), 3.76-3.72 (2H), 3.69-3.65 (2H), 3.38-3.34 (2H), 2.67-2.64 (4H), 2.62-2.58 (2H), 1.55-1.48 (2H), 0.87-0.82 (3H).

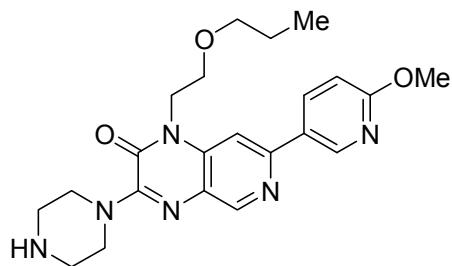


**Step 7:**

A solution of 7-chloro-3-[4-(2-hydroxyethyl)piperazin-1-yl]-1-(2-propoxyethyl)pyrido[3,4-b]pyrazin-2(1H)-one (218 mg, 0.55 mmol) in 1,4-dioxane (3.0 mL) was treated with tetrakis(triphenylphosphine) palladium(0) (19 mg, 0.016 mmol, Strem) and stirred at room temperature for five

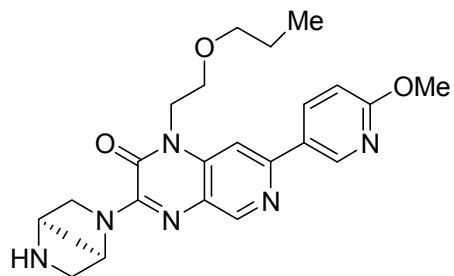
minutes. A warm solution of 2-methoxy-5-pyridineboronic acid (41 mg, 0.27 mmol, Frontier) in ethanol (0.5 mL) and 2.0 M aqueous sodium carbonate (1.5 mL) were added. The mixture was refluxed for 2.0 hours, filtered hot through celite and the filtrate was concentrated under reduced pressure. The residue was partitioned between ethyl acetate and water, and the layers were separated. The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over magnesium sulfate, concentrated under reduced pressure, and passed through a column of silica gel with 2.5% methanol/ dichloromethane. Fractions were concentrated at reduced pressure and triturated with diethyl ether.

The 7-(6-methoxypyridin-3-yl)-3-(piperazin-1-yl)-1-(2-propoxyethyl)pyrido[3,4-b]pyrazin-2(1H)-one was obtained as a pink powder.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  8.75 (s, 1H), 8.73 (d,  $J$  = 2.3 Hz, 1H), 8.22 (dd,  $J$  = 9.0, 2.3 Hz, 1H), 7.62 (s, 1H), 6.81 (d,  $J$  = 9.0 Hz, 1H), 4.40 (t,  $J$  = 5.5 Hz, 2H), 4.01 – 3.98 (m, 7H), 3.76 (t,  $J$  = 5.5 Hz, 2H), 3.65 (t,  $J$  = 5.5 Hz, 2H), 3.34 (t,  $J$  = 6.6 Hz, 2H), 2.74 – 2.86 (br, 1H), 2.65 (m, 4H), 2.59 (t,  $J$  = 5.5 Hz, 2H), 1.50 – 1.44 (m, 2H), 0.76 (t,  $J$  = 7.4 Hz, 3H); HRMS  $m/z$  469.2572 (calcd for  $\text{M}+\text{H}$ , 469.2558); retention time = 1.77min, >95% pure.

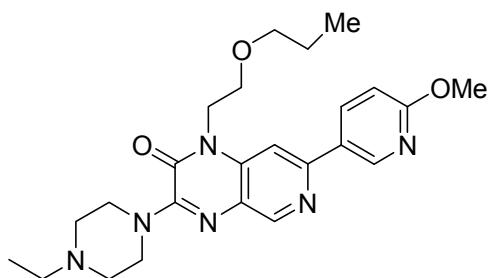


**Compound 10:** Prepared in manner similar to compound **13** replacing 2-(piperazin-1-yl)ethanol with piperazine in step 6.

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 8.78 – 8.77 (m, 2H), 8.28 (m, 1H), 7.66 (s, 1H), 6.87 (d, J = 8 Hz, 1H), 4.45 (t, J = 4.0 Hz, 2H), 4.03 – 4.00 (m, 6H), 3.81 (t, J = 4.0 Hz, 2H), 3.39 (t, J = 4.0 Hz), 3.08 – 3.05 (m, 3H), 1.57- 1.48 (m, 2H), 0.80 (t, J = 4.0 Hz, 3H); LRMS *m/z* 424.9 (calcd for M+H, 425.2); retention time = 1.71 min, >95% pure.

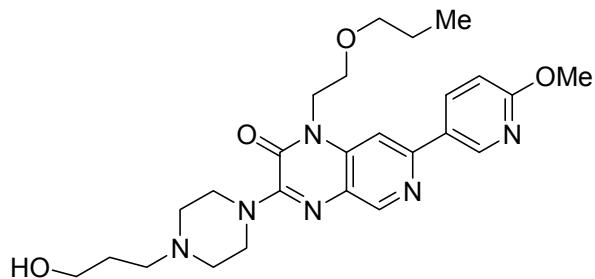


**Compound 11:** Prepared in manner similar to compound **13** replacing 2-(piperazin-1-yl)ethanol with (1R,4R)-2,5-diazabicyclo[2.2.1]heptane in step 6. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 8.74 – 8.70 (2H), 8.41 – 8.22 (1H), 7.59 (1H), 6.84 (1H), 4.39 (2H), 4.00 (3H), 3.85 – 3.73 (5H), 3.37 (2H), 3.17 (2H), 1.87 – 1.76 (2H), 1.54 – 1.47 (3H), 0.87 – 0.76 (4H); HRMS *m/z* 437.2316 (calcd for M+H, 437.2296); retention time = 1.77 min, > 95% pure.



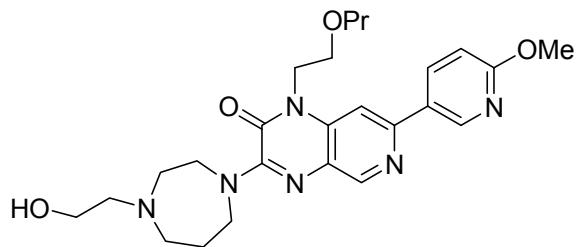
**Compound 12:** Prepared in manner similar to compound **13** replacing 2-(piperazin-1-yl)ethanol with 1-ethylpiperazine in step 6.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ ; 8.73 – 8.72 (m, 2H), 8.21 (dd,  $J$  = 8.6, 2.3 Hz, 1H), 7.60 (s, 1H), 6.82 (d,  $J$  = 8.6 Hz, 1H), 4.39 (t,  $J$  = 5.5 Hz, 2H), 4.02 – 4.00 (m, 2H), 3.97 (s, 3H), 3.75 (t,  $J$  = 5.5 Hz, 2H), 3.34 (t,  $J$  = 6.2 Hz, 2H), 2.58 (t,  $J$  = 5.1 Hz, 2H), 2.46 (q,  $J$  = 7.4 Hz, 2H), 1.49 – 1.44 (m, 2H), 1.11 (t,  $J$  = 7.5 Hz, 3H), 0.75 (t,  $J$  = 7.4 Hz, 3H); LRMS  $m/z$  453.2 (calcd for  $\text{M}+\text{H}$ , 453.3); retention time = 1.75 min, >95% pure.



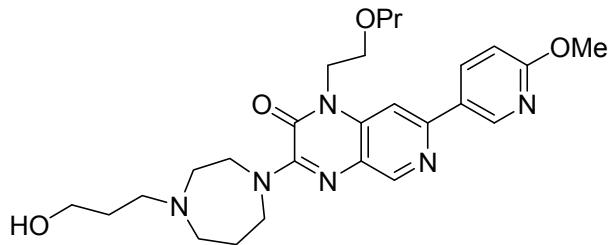
**Compound 14:** Prepared in manner similar to compound **13** replacing 2-(piperazin-1-yl)ethanol with 3-(piperazin-1-yl)propan-1-ol in step 6.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  8.78-8.76 (m, 2H), 8.25 (dd, 1H), 7.65 (s, 1H), 6.86 (d, 1H), 4.42 (t, 2H), 4.13-4.06 (m, 4H), 4.01 (s, 3H), 3.86 (t, 2H), 3.79 (t, 2H), 3.37 (t, 2H), 2.76 (m, 6H), 1.83 (m, 2H), 1.53-1.46 (m, 2H), 0.78 (t, 3H). HRMS  $m/z$  483.2747 (calcd for  $\text{M}+\text{H}$ , 483.2714); retention time = 1.77, 95% pure.



**Compound 15:** Prepared in manner similar to compound **13** replacing 2-(piperazin-1-yl)ethanol with 2-(1,4-diazepan-1-yl)ethanol in step 6.

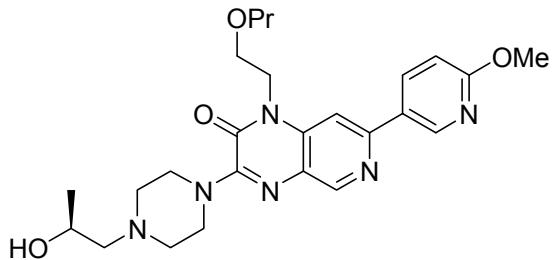
<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 8.75-8.73 (m, 2H), 8.24 (dd, 1H), 7.60 (s, 1H), 6.86 (d, 1H), 4.41 (t, 2H), 4.13-4.11 (m, 2H), 4.05-4.00 (m, 5H), 3.78 (t, 2H), 3.64 (m, 2H), 3.38 (t, 2H), 3.03 (m, 2H). 2.82-2.76 (m, 4H), 2.12 (m, 2H), 1.54-1.47 (m, 2H), 0.80 (t, 3H). HRMS *m/z* 483.2720 (calcd for M+H, 483.2714); retention time = 1.80, 95% pure.



**Compound 16:** Prepared in manner similar to compound **13** replacing 2-(piperazin-1-yl)ethanol with 3-(1,4-diazepan-1-yl)propan-1-ol in step 6.

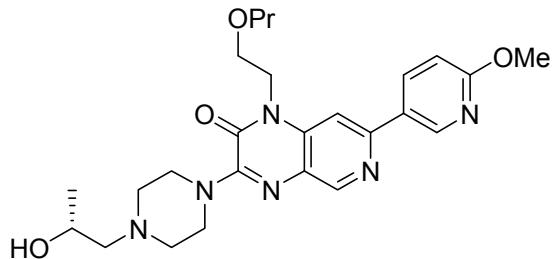
<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 8.69 (d, J = 1.9Hz, 1H), 8.67 (s, 1H), 8.18 (dd, J = 8.6, 2.3 Hz, 1H), 7.54 (s, 1H), 6.78 (d, d = 8.6 Hz, 1H), 4.36 (t, J = 5.5 Hz, 2H),

4.08 – 4.03 (m, 2H), 3.97 – 3.93 (m, 5H), 3.79 – 3.76 (m, 2H), 3.73 (t,  $J$  = 5.5 Hz, 2H), 3.3 (t,  $J$  = 6.6 Hz, 2H), 2.94 – 2.87 (m, 2H), 2.75 – 2.66 (m, 4H), 2.07 – 2.00 (m, 2H), 1.72 – 1.66 (m, 2H), 1.50 – 1.43 (m, 2H), 0.74 (t,  $J$  = 7.4 Hz, 3H); LRMS  $m/z$  497.2 (calcd for M+H, 497.3); retention time = 1.75 min, >95% pure.



**Compound 17:** Prepared in manner similar to compound **13** replacing 2-(piperazin-1-yl)ethanol with (S)-1-(piperazin-1-yl)propan-2-ol in step 6.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  8.78 – 8.75 (m, 2H), 8.27 (dd,  $J$  = 8.0, 2.0 Hz, 1H), 7.65 (s, 1H), 6.86 (d,  $J$  = 8.0 Hz, 1H), 4.44 (m, 2H), 4.02 – 3.90 (m, 8H), 3.80 (m, 2H), 3.38 (m, 2H), 2.83 (m, 2H), 2.57 – 2.52 (m, 2H), 2.38 – 2.33 (m, 2H), 1.52 – 1.48 (m, 2H), 1.17 (m, 3H), 0.79 (m, 3H); LRMS  $m/z$  483.9 (calcd for M+H, 483.2); retention time = 1.82 min, >95% pure.



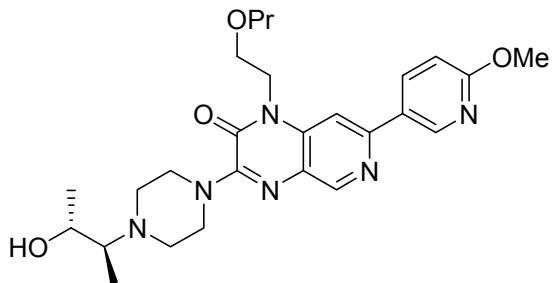
**Compound 18:** Prepared in manner similar to compound **13** replacing 2-(piperazin-1-yl)ethanol with (R)-1-(piperazin-1-yl)propan-2-ol in step 6.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  8.78 – 8.74 (m, 2H), 8.25 (dd,  $J$  = 8.6, 2.3 Hz, 1H), 7.62 (s, 1H), 6.84 (d,  $J$  = 8.6 Hz, 1H), 4.40 (t,  $J$  = 5.5 Hz, 2H), 3.99 – 3.94 (m, 7H), 3.91 – 3.86 (m, 1H), 3.77 (t,  $J$  = 5.5 Hz, 2H), 3.35 (t,  $J$  = 6.6 Hz, 2H), 2.83 (m, 2H), 2.56 – 2.50 (m, 2H), 2.38 – 2.26 (m, 2H), 1.52 – 1.45 (m, 2H), 1.14 (d,  $J$  = 6.3 Hz, 3H), 0.76 (t,  $J$  = 7.4 Hz, 3H); LRMS  $m/z$  483.2 (calcd for  $\text{M}+\text{H}$ , 483.2); retention time = 1.96 min, > 95% pure.

**Compound 19:** Prepared in manner similar to compound **13** replacing 2-(piperazin-1-yl)ethanol with *cis*-3-(piperazin-1-yl)butan-2-ol in step 6.

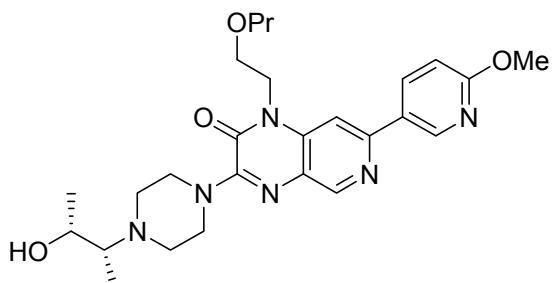
$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  8.78 – 8.74 (m, 2H), 8.25 (dd,  $J$  = 8.6, 2.3 Hz, 1H), 7.62 (s, 1H), 6.84 (d,  $J$  = 8.6 Hz, 1H), 4.40 (t,  $J$  = 5.5 Hz, 2H), 3.99 – 3.94 (m, 7H), 3.91 – 3.86 (m, 1H), 3.77 (t,  $J$  = 5.5 Hz, 2H), 3.35 (t,  $J$  = 6.6 Hz, 2H), 2.83 (m, 2H), 2.56 – 2.50 (m, 2H), 2.38 – 2.26 (m, 2H), 1.52 – 1.45 (m, 2H),

1.14 (d,  $J = 6.3$  Hz, 3H), 0.76 (t,  $J = 7.4$  Hz, 3H); LRMS  $m/z$  497.2 (calcd for M+H, 497.3); retention time = 1.86 min, > 95% pure.



**Compound 20:** Prepared in manner similar to compound **13** replacing 2-(piperazin-1-yl)ethanol with *trans*-3-(piperazin-1-yl)butan-2-ol in step 6.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  8.79 – 8.78 (m, 2H), 8.27 (dd,  $J = 8.5, 2.2$  Hz, 1H), 7.66 (s, 1H), 6.86 (d,  $J = 12.0$  Hz, 1H), 4.44 (t,  $J = 4.0$  Hz, 2H), 4.04 – 3.98 (m, 7H), 3.81 (t,  $J = 4.0$  Hz, 2H), 3.39 (t,  $J = 4.0$  Hz, 2H), 2.85 – 2.65 (m, 3H), 1.54 – 1.47 (m, 2H), 1.27 – 1.20 (m, 10H), 0.80 (t,  $J = 8.0$  Hz, 3H). LRMS  $m/z$  496.9 (calcd for M+H, 497.3); retention time = 1.89 min, > 95% pure.



**Compound 21:** Prepared in manner similar to compound **13** replacing 2-(piperazin-1-yl)ethanol with (2R,3R)-3-(piperazin-1-yl)butan-2-ol in step 6. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 8.69 – 8.67 (m, 2H), 8.28 (dd, J = 8.5, 2.2 Hz, 1H), 7.66 (s, 1H), 6.85 (d, J = 12.0 Hz, 1H), 4.54 (t, J = 4.0 Hz, 2H), 4.03 – 3.95 (m, 7H), 3.82 (t, J = 4.0 Hz, 2H), 3.35 (t, J = 4.0 Hz, 2H), 2.65 – 2.35 (m, 3H), 1.54 – 1.47 (m, 2H), 1.38 – 1.18 (m, 10H), 0.72 (t, J = 8.0 Hz, 3H). LRMS *m/z* 496.9 (calcd for M+H, 497.3); retention time = 1.86 min, >95% pure.

### **PDE5, PDE6 and PDE11 assays:**

PDE5 was isolated from human platelets. PDE6 was isolated from human retinas. PDE11 was recombinant.

*Scintillation Proximity Assay (SPA) for PDE5, 6, and 11 inhibition.* PDE inhibition was determined by monitoring the reduction in the conversion of [<sup>3</sup>H]-cGMP to [<sup>3</sup>H]-GMP in a scintillation proximity assay [5]. The final concentrations of reagents were 20 mM Tris-HCl (pH 7.4), 0.66 mg/mL BSA, 5 mM MgCl<sub>2</sub>, 3.33% DMSO, and 50 nM [<sup>3</sup>H]-cGMP. The final dilutions of enzyme preparations in the assays were: human platelet PDE5 at 1:450, rat platelet PDE5 at 1:3000, human retinal PDE6 at 1:450, and human recombinant PDE11 at 1:9000. Test compound was included at various final concentrations ranging from 2000 nM to 0.00915 nM in either an 8-point or

10-point concentration response curve (in duplicate) depending on the enzyme used for the assay. Enzyme reactions were initiated by adding 30  $\mu$ L substrate to 60  $\mu$ L of a solution containing the enzyme with test compound. Following a 1 hour incubation at room temperature, 30  $\mu$ L/well of yttrium SPA beads were added at a final concentration of 1 mg/mL. The plates were vigorously shaken on a plate shaker for 15 minutes, and the beads were then allowed to settle for 30 minutes. The product, [ $^3$ H]-GMP, selectively binds to the yttrium silicate SPA beads and brings the isotope into close enough proximity to allow radiation from the tritium to excite the scintillant within the bead. The plates were then counted in a Packard TopCount NXT liquid scintillation counter (30 seconds/well) and IC<sub>50</sub>'s determined.

### **Additional selectivity data for 13.**

13 was found to be at minimum 9000-fold selective (PDE5 IC<sub>50</sub>) against the following targets: (data obtained at CEREP).

5-HT Transporter, 5-HT1a, 5-HT2a, 5-HT2b, 5-HT2C, 5-HT3, 5-HT4e, 5HT7, Adenosine 1, Adenosine 2A, ACE, Acetylcholine esterase, Adrenergic alpha 2a, Adrenergic alpha 2b, Angiotensin 1, Adrenergic beta 1, Adrenergic beta 2, Adrenergic beta 3, Cannabinoid 1, Cannabinoid 2, Cholecystokinin 1, Cholecystokinin 2, Choline transporter, COX2, D1, D2,

D3, Dopamine transporter, Endothelan A, Endothelin B, Glucocorticoid, H1, H2, H3, M1, M2, M3, Mu, Nicotinic, NE transporter, NK1, P38 alpha, VGFR1, Kappa, N-type Calcium, L-type Calcium DHP site, L-type Calcium Diltiazem, GABA, Glutamate receptor AMPA site, GABA transporter, Sodium channel site 2, Glutamate receptor NMDA site, Thyroid hormone receptor, Na/K ATPase, MAO-A, Urotensin 2 binding, GABA-B, Vasopressin 1A

The Pfizer Institutional Animal Care and Use Committee reviewed and approved the animal use in these studies. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International

### **Procedure for the in vivo blood pressure experiments.**

Briefly, SHRs (Charles River Laboratories, Wilmington, MA) (14-weeks old, ~300 g) that were housed in a controlled environment (21 °C and 50% humidity) with a 12 hour light-dark cycle and free access to standard rodent chow (Rodent 8640, HARLAN TEKLAD, Madison, WI) and water, were instrumented under anesthesia with bilateral jugular vein and right carotid artery catheters and allowed to recover for approximately 24 hours prior to

connection to the Culex™ system. The Culex ABS system (Bioanalytical System, Inc.) allows for continuous tethered BP monitoring, oral and intravenous administration of compounds and computerized blood sampling at multiple time points. Systemic arterial BP and HR were recorded continuously throughout the course of the study via a pressure transducer (Becton Dickinson, Sandy, Utah) connected to a data acquisition system (ACQ-7700 digital amplifier and PONEMAH P3 Plus software) (Gould Instrument System, Valley View, OH). Compounds were formulated in 0.5% dimethyl sulphoxide (DMSO) (Sigma, St. Louis, MO) in 0.9% saline (Abbott Laboratories, North Chicago, IL). Following an equilibration period, baseline blood pressure (BP) and heart rate (HR) were monitored for 120 minutes prior to compound administration. At time zero each of four animals received three successive, escalating doses of **13** via continuous infusion at 1-hour intervals designed to achieve and maintain steady state plasma concentrations. The change in BP was determined during the 30-50 minute portion of each 1-hour steady state infusion period relative to the mean of the 2-hour pre-dose baseline period. Whole blood (150 ml) was automatically sampled via the right jugular catheter at the 50-minute time point during each infusion period for plasma concentration analysis of compound. Blood was stored in capped, refrigerated vials (4 °C) containing

sodium heparin (Bioanalytical System, Inc.). Plasma was separated by centrifugation at 3000 rpm for 10 minutes at 4 °C and then stored at –80 °C. Plasma sample compound concentration analysis was performed using an LS/MS/MS triple quadropole mass spectrometry system (Siex – API 4000).

### **Procedure for the determination of Brain concentration.**

Male Sprague Dawley rats were obtained from Charles River Laboratories. After an acclimation period, animals were fasted for a period of 12 to 18 hours prior to oral dosing via gavage needle. All dosages were calculated based on body weight just prior to dosing.

Two hours post dose animals were euthanized via CO<sub>2</sub> asphyxiation. Post euthanasia, blood was collected via cardiac veinipuncture and brain was harvested and immediately frozen on dry ice.

Brain tissues were sliced and divided between 4 prep tubes and weighed again to determine amt of brain tissue per tube. 1mL of 70:30 MeOH:H<sub>2</sub>O was added per 0.6g brain tissue directly to the prep tubes followed by manual homogenation using a tissue/plunger apparatus. The contents from each of the 4 tubes were pooled into a single sample. Standards are spiked into control brain homogenate and prepared the same as the samples. 50µL of sample + 150µL of Acetonitrile (w/ IS) was centrifuged and supernatent analyzed via LCMSMS. Calculations of total brain concentration (µM) was

performed using the measured concentration, experimental dilution, and assumed brain density of 1g/mL.