

A Novel Class of LIM Kinase 2 Inhibitors for the Treatment of Ocular Hypertension and Associated Glaucoma

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Contents:

1. Detailed experimental procedures for compounds **1-22r**.
2. Detailed description of LIMK2, LIMK1, ROCK1, ROCK2, and cellular cofilin phosphorylation in vitro assays.
3. Detailed description of in vivo IOP experiments with a dexamethasone induced ocular hypertensive mouse model.
4. Detailed description of pig eye perfusion assay.

1. PREPARATION OF COMPOUNDS 1-22R.

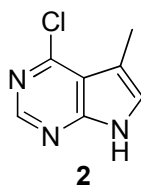
1.1. Chemical Methods.

All reactions were conducted under a static atmosphere of argon or nitrogen and stirred magnetically unless otherwise noted. Reagents, starting materials, and solvents were purchased from commercial suppliers and used as received. Flash column chromatography was carried out using pre-packed silica gel columns from Biotage or ISCO, or by slurry preparation using EMD silica gel 60 (particle size 0.040-0.063 mm). ^1H and ^{13}C NMR spectra were collected on Bruker ARX300, DRX400 or DPX400, or Varian Mercury 400 MHz NMR spectrometers. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane in δ -units, and coupling constants (J -values) are given in

hertz (Hz). Data are reported in the following format: chemical shift, multiplicity, coupling constants, and assignment. Reactions were monitored by TLC using 0.25 mm E. Merck silica gel plates (60 F₂₅₄) and were visualized with UV light. Analytical HPLC spectra were collected on Shimadzu HPLC systems equipped with a UV detector measuring absorbance at 220 and 254 nm. Mass spectra were obtained on Waters ZQ or ZMD LCMS systems equipped with an auto-sampler, and ELSD detector, a UV detector measuring absorbance at 220 and 254 nm, and a mass detector. High resolution mass spectra were obtained on a Waters LCT Premier XE Micromass® MS Technologies instrument equipped with an auto-sampler. Elemental analysis was conducted by Robertson Microlit Laboratory, Madison, NJ.

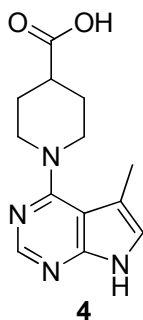
All compounds, except compound **18**, were purified by silica gel flash chromatography or preparatory HPLC to $\geq 95\%$ purity as determined by analytical HPLC. Compound **18** was purified by preparatory HPLC to 94% purity.

1.2. 4-chloro-5-methyl-7H-pyrrolo[2,3-d]pyrimidine



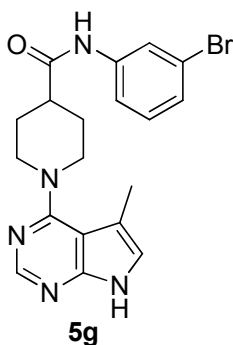
4-chloro-5-methyl-7H-pyrrolo[2,3-d]pyrimidine (2). Compound **2** was prepared according to the procedure from West, R. A. 4-Hydroxypyrrolo[2,3-d]pyrimidine : Mannich Reaction. *J. Org. Chem.* **1961**, *26*, 4959-4961. ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.45 (s, 1 H), 7.26 (d, *J*=1.0 Hz, 1 H), 2.48 (d, *J*=1.0 Hz, 3 H); MS (ES+) [M+H]⁺ = 168.

1.3. Amides 1, 5a-5g



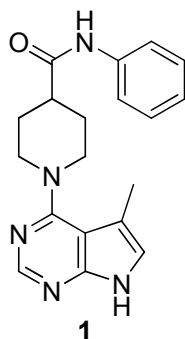
1-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidine-4-carboxylic acid (4). A solution of compound **2** (0.40 g, 2.4 mmol) and ethyl isonipecotatate (**3**) (0.55 mL, 3.6 mmol) in triethylamine (1 mL) and isopropanol (2 mL) was heated at 130 °C in a sealed tube overnight. The reaction was cooled to room temperature and concentrated under vacuum. The residue was treated with LiOH (172 mg, 7.2 mmol.) in THF (5 mL) and H₂O (5 mL) for 4 hours. The reaction was washed 3x with EtOAc, then acidified with 6M aq. HCl, resulting in precipitation of the product. The product was collected by filtration, washed with H₂O and EtOAc, and dried under vacuum. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 11.52 (br. s., 1 H), 8.19 (s, 1 H), 7.01 - 7.08 (m, 1 H), 3.89 (d, *J*=13.3 Hz, 2 H), 2.91 - 3.04 (m, 2 H), 2.44 - 2.48 (m, 1 H), 2.33 (d, *J*=1.0 Hz, 3 H), 1.94 (dd, *J*=13.2, 3.0 Hz, 2 H), 1.63 - 1.76 (m, 2 H); MS (ES+) [M+H]⁺ = 261.

Amides **1** and **5a-g** were prepared from compound **4** according to the procedure for **5g** using BOP, HATU, or PS-carbodiimide/HOBT as the coupling reagents. The compounds were purified by prep HPLC to ≥95% purity (by HPLC).

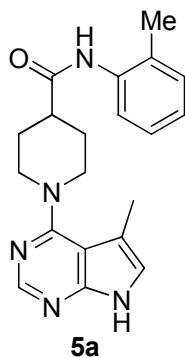


N-(3-bromophenyl)-1-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidine-4-carboxamide (5g). To a solution of acid **4** (50 mg, 0.19 mmol) in DMF (0.5 mL) was added 3-bromoaniline (23 μL, 0.21 mmol), *N,N*-diisopropylethylamine (99 μL, 0.57 mmol), and BOP (127 mg, 0.29 mmol). The reaction was stirred overnight and then concentrated under vacuum. The residue was

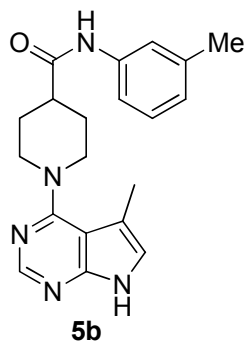
purified by acid phase prep HPLC to give amide **5g** (10 mg) as the TFA salt. ^1H NMR (TFA salt) (400 MHz, METHANOL- d_4) δ ppm 8.30 (br. s., 1 H), 7.92 (d, $J=1.8$ Hz, 1 H), 7.48 (dt, $J=6.8, 2.1$ Hz, 1 H), 7.14 - 7.35 (m, 3 H), 4.34 (d, $J=13.4$ Hz, 2 H), 3.42 - 3.59 (m, 2 H), 2.81 (tt, $J=11.0, 4.1$ Hz, 1 H), 2.48 (d, $J=1.0$ Hz, 3 H), 1.95 - 2.17 (m, 4 H); MS (ES+) $[\text{M}+\text{H}]^+ = 414, 416$.



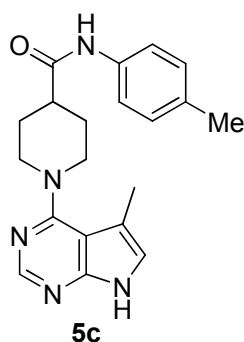
1-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-N-phenylpiperidine-4-carboxamide (1). ^1H NMR (TFA salt) (400 MHz, METHANOL- d_4) δ ppm 8.29 (s, 1 H), 7.51 - 7.60 (m, 2 H), 7.22 - 7.36 (m, 3 H), 7.04 - 7.16 (m, 1 H), 4.35 (d, $J=13.1$ Hz, 2 H), 3.45 - 3.58 (m, 2 H), 2.83 (tt, $J=11.0, 4.2$ Hz, 1 H), 2.48 (d, $J=1.0$ Hz, 3 H), 1.96 - 2.16 (m, 4 H); MS (ES+) $[\text{M}+\text{H}]^+ = 336$.



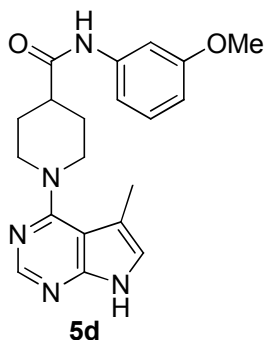
1-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-N-o-tolylpiperidine-4-carboxamide (5a). ^1H NMR (TFA salt) (400 MHz, METHANOL- d_4) δ ppm 8.26 (s, 1 H), 7.21 - 7.27 (m, 1 H), 7.11 - 7.21 (m, 3 H), 7.04 - 7.11 (m, 1 H), 4.35 (d, $J=13.1$ Hz, 2 H), 3.40 - 3.52 (m, 2 H), 3.12 - 3.24 (m, 1 H), 2.48 (d, $J=1.0$ Hz, 3 H), 2.41 (s, 3 H), 1.84 - 2.04 (m, 4 H); MS (ES+) $[\text{M}+\text{H}]^+ = 350$.



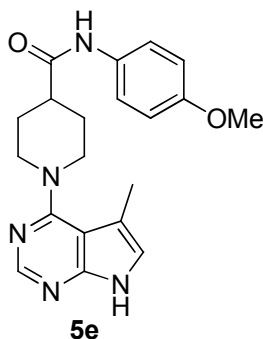
1-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-N-m-tolylpiperidine-4-carboxamide (5b). ^1H NMR (TFA salt) (400 MHz, METHANOL- d_4) δ ppm 8.28 (s, 1 H), 7.40 (s, 1 H), 7.36 (d, $J=8.4$ Hz, 1 H), 7.25 (s, 1 H), 7.19 (t, $J=7.8$ Hz, 1 H), 6.93 (d, $J=7.6$ Hz, 1 H), 4.33 (d, $J=13.3$ Hz, 2 H), 3.42 - 3.54 (m, 2 H), 2.80 (tt, $J=11.0, 4.3$ Hz, 1 H), 2.48 (s, 3 H), 2.32 (s, 3 H), 1.94 - 2.18 (m, 4 H); MS (ES+) $[\text{M}+\text{H}]^+ = 350$.



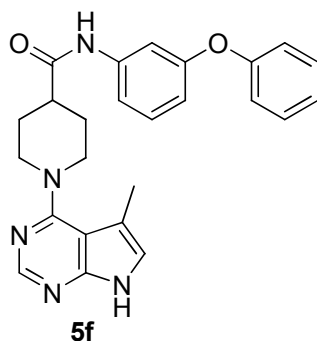
1-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-N-p-tolylpiperidine-4-carboxamide (5c). ^1H NMR (TFA salt) (400 MHz, METHANOL- d_4) δ ppm 8.27 (s, 1 H), 7.40 - 7.46 (m, 2 H), 7.20 - 7.25 (m, 1 H), 7.13 (d, $J=8.2$ Hz, 2 H), 4.31 (d, $J=13.2$ Hz, 2 H), 3.38 - 3.50 (m, 2 H), 2.78 (tt, $J=11.0, 4.4$ Hz, 1 H), 2.48 (d, $J=1.1$ Hz, 3 H), 2.30 (s, 3 H), 1.94 - 2.14 (m, 4 H); MS (ES+) $[\text{M}+\text{H}]^+ = 350$.



***N*-(3-methoxyphenyl)-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamide (5d).** ¹H NMR (TFA salt) (400 MHz, METHANOL-*d*₄) δ ppm 8.28 (s, 1 H), 7.28 - 7.33 (m, 1 H), 7.26 (d, *J*=1.0 Hz, 1 H), 7.21 (t, *J*=8.2 Hz, 1 H), 7.07 (d, *J*=8.0 Hz, 1 H), 6.68 (dd, *J*=7.9, 2.1 Hz, 1 H), 4.34 (d, *J*=13.1 Hz, 2 H), 3.78 (s, 3 H), 3.45 - 3.55 (m, 2 H), 2.74 - 2.88 (m, 1 H), 2.49 (d, *J*=1.2 Hz, 3 H), 1.94 - 2.16 (m, 4 H); MS (ES+) [M+H]⁺ = 366.

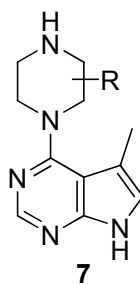


***N*-(4-methoxyphenyl)-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamide (5e).** ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 9.81 (s, 1 H), 8.21 (s, 1 H), 7.52 (m, *J*=9.1 Hz, 2 H), 7.06 (s, 1 H), 6.83 - 6.90 (m, 2 H), 4.03 (d, *J*=12.6 Hz, 2 H), 3.71 (s, 3 H), 2.90 - 3.00 (m, 2 H), 2.52 - 2.58 (m, 1 H), 2.34 - 2.38 (m, 3 H), 1.74 - 1.95 (m, 4 H); MS (ES+) [M+H]⁺ = 366.

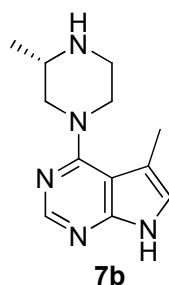


1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)-*N*-(3-phenoxyphenyl)piperidine-4-carboxamide (5f). ¹H NMR (TFA salt) (400 MHz, METHANOL-*d*₄) δ ppm 8.28 (s, 1 H), 7.32 - 7.40 (m, 3 H), 7.22 - 7.31 (m, 3 H), 7.09 - 7.15 (m, 1 H), 7.00 (dd, *J*=8.7, 1.0 Hz, 2 H), 6.70 - 6.77 (m, 1 H), 4.32 (d, *J*=13.2 Hz, 2 H), 3.43 - 3.55 (m, 2 H), 2.78 (tt, *J*=11.0, 4.1 Hz, 1 H), 2.47 (d, *J*=1.1 Hz, 3 H), 1.92 - 2.14 (m, 4 H); MS (ES+) [M+H]⁺ = 428.

1.4. Amines 7



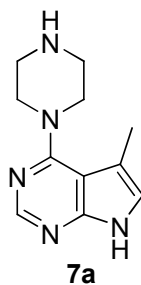
Compounds **7** were prepared from **2** and substituted piperazines according to the procedure for **7b** (Boc-protected piperazines) or **7c** (microwave conditions).



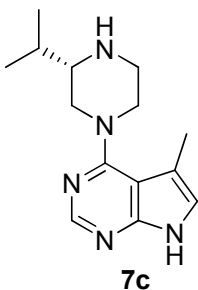
(S)-5-methyl-4-(3-methylpiperazin-1-yl)-7H-pyrrolo[2,3-d]pyrimidine (7b). (*S*)-*tert*-butyl 2-methylpiperazine-1-carboxylate (3 g, 15 mmol), *N,N*-diisopropylethylamine (3 ml), and compound **2** (2 g, 12 mmol) were added to isopropanol (10 ml). The solution was heated at 120 °C in a sealed pressure tube for 12 hours. The reaction was concentrated under vacuum, and the residue was purified by flash chromatography (80 g SiO₂, 0-5% MeOH: CH₂Cl₂) to give intermediate (*S*)-*tert*-butyl 2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboxylate (1.5 g, 4.5 mmol, 38%). ¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 10.42 (br. s., 1 H), 8.39 (s, 1 H), 6.96 (s, 1 H), 4.40 (d, *J*=6.06 Hz, 1 H), 3.83 - 4.01 (m, 2 H), 3.43 (td, *J*=12.57, 3.41 Hz, 1 H), 3.32 (dd, *J*=12.76, 3.92 Hz, 1 H), 3.07 (td, *J*=12.32, 3.41 Hz, 1 H), 2.44 (s, 3 H), 1.50 (s, 9 H), 1.24 (d, *J*=6.82 Hz, 3 H); MS (ES+) [M+H]⁺ = 332.

The Boc-protected intermediate (1.5 g, 4.5 mmol) was treated overnight with 50% trifluoroacetic acid in dichloromethane (10 ml). The reaction was concentrated under vacuum, diluted with dichloromethane, and neutralized with sat. aq. sodium bicarbonate. The layers were separated, and the aqueous layer was back extracted with more dichloromethane. The combined organic fractions were dried over MgSO₄ and concentrated under vacuum to give **7b** (0.80 g, 3.5 mmol, 76%). ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.24 (s, 1 H), 7.05 (d, *J*=1.01 Hz, 1 H), 3.97 - 4.04 (m, 2 H), 3.00 - 3.14

(m, 4 H), 2.74 (dd, $J=12.88, 10.36$ Hz, 1 H), 2.45 (d, $J=1.01$ Hz, 3 H), 1.19 (d, $J=6.32$ Hz, 3 H); MS (ES+) $[M+H]^+ = 232$.

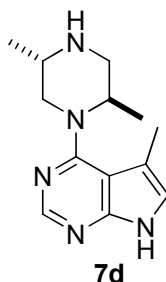


5-methyl-4-(piperazin-1-yl)-7H-pyrrolo[2,3-d]pyrimidine (7a). Prepared from compound **2** and Boc-piperazine according to the procedure for **7b**. ^1H NMR (TFA salt) (400 MHz, DMSO- d_6) δ ppm 11.96 (br. s., 1 H), 8.95 (br. s., 2 H), 8.35 (s, 1 H), 7.21 (d, $J=1.2$ Hz, 1 H), 3.65 - 3.73 (m, 4 H), 3.30 (br. s., 4 H), 2.36 (d, $J=1.0$ Hz, 3 H); MS (ES+) $[M+H]^+ = 218$.



(S)-4-(3-isopropylpiperazin-1-yl)-5-methyl-7H-pyrrolo[2,3-d]pyrimidine (7c). To a solution of (*S*)-3-isopropylpiperazine-2,5-dione (100mg, 0.6mmol) in anhydrous THF was added lithium aluminum hydride 1M in THF (1.2ml, 1.2mmol). The reaction was refluxed for 1 hr, cooled to room temperature, quenched with H₂O, filtered, then concentrated under vacuum to yield (*S*)-2-isopropylpiperazine. This material was combined with compound **2** (85.5 mg, 0.50 mmol) in triethylamine (1 ml) and isopropanol (2 ml). The reaction was heated in a microwave at 180 °C for 30 min, concentrated under vacuum, dissolved in EtOAc, washed with H₂O, and concentrated under vacuum to afford **7c**, carried on without further purification. A small sample was purified by prep HPLC for characterization. ^1H NMR (AcOH salt) (400 MHz, CHLOROFORM- d) δ ppm 10.28 (br. s., 1 H), 8.31 (s, 1 H), 6.91 (s, 1 H), 4.94 (br. s., 2 H), 4.14 (ddd, $J=12.4, 2.3, 2.2$ Hz, 1 H), 4.04 (dd, $J=12.1, 2.0$ Hz, 1 H), 3.20 - 3.27 (m, 1 H), 3.13 (td, $J=11.9, 2.1$ Hz, 1 H), 3.05 (td, $J=11.4, 2.5$ Hz, 1 H), 2.86

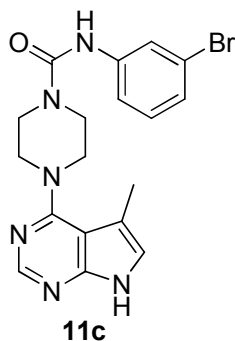
(dd, $J=12.5, 10.5$ Hz, 1 H), 2.70 (ddd, $J=10.2, 7.0, 2.8$ Hz, 1 H), 2.41 (d, $J=1.0$ Hz, 3 H), 1.68 - 1.82 (m, 1 H), 1.03 (t, $J=6.7$ Hz, 6 H); MS (ES+) $[M+H]^+ = 260$.



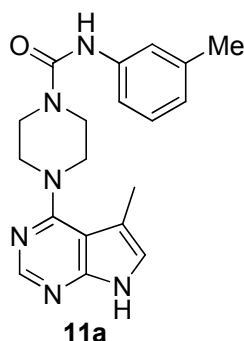
***Trans*-4-(2,5-dimethylpiperazin-1-yl)-5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (7d).** *Trans*-2,5-dimethylpiperazine (1 g, 8.8 mmol), *N,N*-diisopropylethylamine (1 mL) and compound **2** (2 g, 11.9 mmol) were added to isopropanol (10 mL). The solution was heated in a microwave at 150 °C for 6 hours, and then concentrated under vacuum. The material was purified by prep HPLC (Sunfire C18 30x250 mm column, 10-100% MeCN:H₂O (10mM NH₄OAc), 18 min., 45 ml/min.) to give **7d** (0.30 g, 14%) as the acetate salt. ¹H NMR (AcOH salt) (400 MHz, CHLOROFORM-*d*) δ ppm 8.49 (s, 1 H), 6.95 (s, 1 H), 3.61 - 3.71 (m, 1 H), 3.49 (dd, $J=12.5, 2.9$ Hz, 1 H), 3.18 - 3.28 (m, 2 H), 2.81 (dd, $J=12.6, 9.6$ Hz, 1 H), 2.66 - 2.72 (m, 1 H), 2.43 (d, $J=1.3$ Hz, 3 H), 1.12 - 1.18 (m, 6 H); MS (ES+) $[M+H]^+ = 246$.

1.5. Ureas

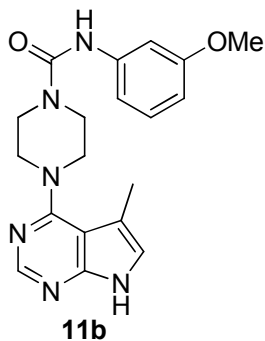
Ureas **11a-c**, **13**, **15**, **16**, **18**, **19**, and **22c** were prepared from amines **7** and isocyanates according to the procedure for **11c**. The products were purified by acid phase prep HPLC to $\geq 95\%$ HPLC purity (except compound **18** which had 94% purity) and isolated as TFA salts.



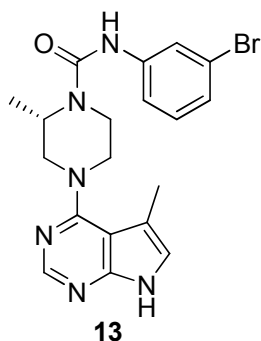
***N*-(3-bromophenyl)-4-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazine-1-carboxamide (11c).** Compound **7a** (22 mg, 0.1 mmol) and 3-bromophenyl isocyanate (20 mg, 0.1 mmol) were combined in DCM (2 mL). The reaction was stirred for 9 hours, and then concentrated under vacuum. The product was purified by prep HPLC to give **11c** (5.7 mg) as the TFA salt. ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.34 (s, 1 H), 7.68 - 7.75 (m, 1 H), 7.32 - 7.39 (m, 1 H), 7.26 (d, *J*=1.0 Hz, 1 H), 7.15 - 7.22 (m, 2 H), 3.91 - 4.01 (m, 4 H), 3.77 - 3.86 (m, 4 H), 2.49 (d, *J*=1.0 Hz, 3 H); MS (ES+) [M+H]⁺ = 415, 417.



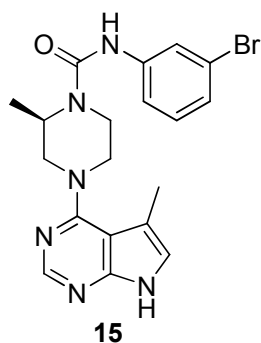
4-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)-*N*-*m*-tolylpiperazine-1-carboxamide (11a). ¹H NMR (300 MHz, METHANOL-*d*₄) δ ppm 8.33 (s, 1 H), 7.10 - 7.34 (m, 4 H), 6.88 (d, *J*=4.0 Hz, 1 H), 3.91 - 4.03 (m, 4 H), 3.76 - 3.90 (m, 4 H), 2.49 (s, 3 H), 2.32 (s, 3 H); MS (ES+) [M+H]⁺ = 351.



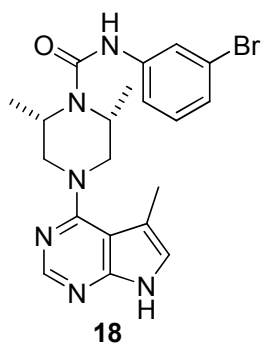
***N*-(3-methoxyphenyl)-4-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazine-1-carboxamide (11b).** ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.34 (s, 1 H), 7.28 (d, *J*=1.0 Hz, 1 H), 7.13 - 7.21 (m, 1 H), 7.07 (t, *J*=2.3 Hz, 1 H), 6.95 (ddd, *J*=8.0, 2.0, 0.9 Hz, 1 H), 6.62 (ddd, *J*=8.2, 2.4, 0.8 Hz, 1 H), 3.94 - 4.01 (m, 4 H), 3.80 - 3.85 (m, 4 H), 3.78 (s, 3 H), 2.49 (d, *J*=1.0 Hz, 3 H); MS (ES+) [M+H]⁺ = 367.



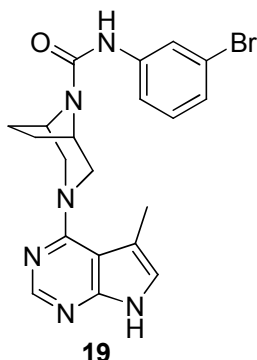
(S)-N-(3-bromophenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboxamide (13). ^1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.36 (s, 1 H), 7.67 - 7.71 (m, 1 H), 7.32 - 7.36 (m, 1 H), 7.25 (d, $J=1.2$ Hz, 1 H), 7.17 - 7.20 (m, 2 H), 4.55 - 4.65 (m, 1 H), 4.34 - 4.42 (m, 1 H), 4.15 - 4.22 (m, 1 H), 4.05 - 4.11 (m, 1 H), 3.86 (dd, $J=13.0, 4.0$ Hz, 1 H), 3.57 - 3.71 (m, 2 H), 2.50 (d, $J=1.2$ Hz, 3 H), 1.29 (d, $J=6.6$ Hz, 3 H); MS (ES+) $[\text{M}+\text{H}]^+ = 429, 431$.



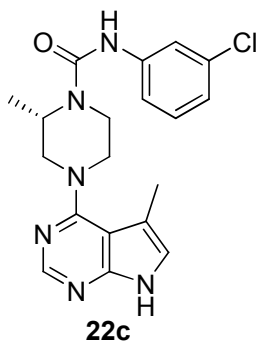
(R)-N-(3-bromophenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboxamide (15). Enantiomer of **13**. ^1H NMR and MS match **13**.



cis-N-(3-bromophenyl)-2,6-dimethyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboxamide (18). ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.38 (s, 1 H), 7.72 (s, 1 H), 7.33 (ddd, *J*=7.3, 2.0, 1.9 Hz, 1 H), 7.11 - 7.21 (m, 3 H), 4.32 - 4.41 (m, 2 H), 3.55 - 3.67 (m, 2 H), 3.18 - 3.28 (m, 2 H), 2.44 (d, *J*=1.0 Hz, 3 H), 1.41 (d, *J*=6.6 Hz, 6 H); MS (ES+) [M+H]⁺ = 443, 445.

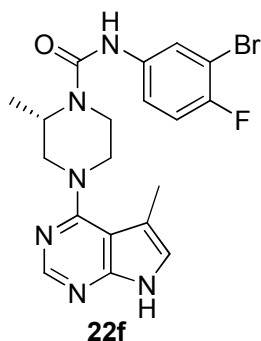


N-(3-bromophenyl)-3-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-3,8-diazabicyclo[3.2.1]octane-8-carboxamide (19). ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.25 (s, 1 H), 7.63 - 7.75 (m, 1 H), 7.31 (ddd, *J*=6.4, 2.5, 2.4 Hz, 1 H), 7.02 - 7.18 (m, 3 H), 4.51 - 4.59 (m, 2 H), 4.39 (d, *J*=12.1 Hz, 2 H), 3.68 (d, *J*=12.1 Hz, 2 H), 2.35 (d, *J*=1.0 Hz, 3 H), 1.94 (m, *J*=10.1 Hz, 2 H), 1.64 - 1.79 (m, 2 H); MS (ES+) [M+H]⁺ = 441, 443.

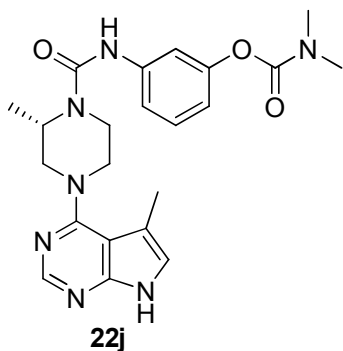


(S)-N-(3-chlorophenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboxamide (22c). ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.35 (s, 1 H), 7.54 (t, *J*=2.0 Hz, 1 H), 7.22 - 7.32 (m, 3 H), 7.01 - 7.05 (m, 1 H), 4.60 (m, *J*=6.7, 6.7, 6.6, 3.5, 3.3 Hz, 1 H), 4.34 - 4.41 (m, 1 H), 4.17 (d, *J*=11.9 Hz, 1 H), 4.05 - 4.11 (m, 1 H), 3.85 (dd, *J*=12.9, 4.1 Hz, 1 H), 3.64 (ddd, *J*=9.9, 6.3, 2.3 Hz, 2 H), 2.50 (d, *J*=1.2 Hz, 3 H), 1.29 (d, *J*=6.8 Hz, 3 H); MS (ES+) [M+H]⁺ = 385.

Ureas **22f,j,l,n,o** were prepared from amine **7b** and anilines according to the procedures for **22f** and **22j**. The products were purified by prep HPLC or silica gel chromatography to $\geq 95\%$ purity (by HPLC).



(S)-N-(3-bromo-4-fluorophenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboxamide (22f). To a solution of triphosgene (104 mg, 0.35 mmol) in anhydrous THF (7.5 ml) at 0 °C was added dropwise 3-bromo-4-fluoroaniline (190 mg, 1.0 mmol) and triethylamine (0.31 mL, 2.2 mmol) in THF (2.5 ml). The reaction was stirred for 10 min at 0 °C then 20 min at room temperature. Amine **7b** (231 mg, 1.0 mmol) was added, and the reaction was stirred 1 hour, quenched with MeOH, diluted with EtOAc, washed with 1 M aq. NaHSO₄, H₂O, sat. aq. NaHCO₃ and brine (with back extraction), dried over MgSO₄, filtered, and concentrated under vacuum. The residue was purified by flash chromatography (40 g SiO₂, 0-6% MeOH:CH₂Cl₂), suspended in H₂O, and lyophilized to give **22f** (261 mg, 0.58 mmol, 58%, 100% purity) as a white solid. ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.22 (s, 1 H), 7.73 (dd, *J*=6.3, 2.5 Hz, 1 H), 7.35 (ddd, *J*=8.8, 4.2, 2.6 Hz, 1 H), 7.12 (t, *J*=8.7 Hz, 1 H), 7.04 (d, *J*=1.0 Hz, 1 H), 4.46 - 4.58 (m, 1 H), 4.12 - 4.21 (m, 1 H), 4.00 (d, *J*=13.2 Hz, 1 H), 3.92 (d, *J*=13.0 Hz, 1 H), 3.52 - 3.63 (m, 1 H), 3.42 (dd, *J*=13.0, 3.7 Hz, 1 H), 3.14 (td, *J*=12.4, 3.4 Hz, 1 H), 2.47 (d, *J*=1.0 Hz, 3 H), 1.29 (d, *J*=6.8 Hz, 3 H); ¹³C NMR (101 MHz, METHANOL-*d*₄) δ ppm 163.0, 157.6, 156.5 (d, *J*=242.2 Hz), 153.6, 151.0, 138.4 (d, *J*=2.9 Hz), 127.0, 123.1, 123.0 (d, *J*=6.6 Hz), 117.1 (d, *J*=22.7 Hz), 110.7, 109.1 (d, *J*=22.0 Hz), 108.2, 53.9, 51.7, 49.2, 40.2, 16.4, 14.2; MS (ES⁺) [M+H]⁺ = 447, 449. HRMS (ES⁺) for C₁₉H₂₀BrFN₆O [M+H]⁺: calcd, 447.0944; found, 447.0933.

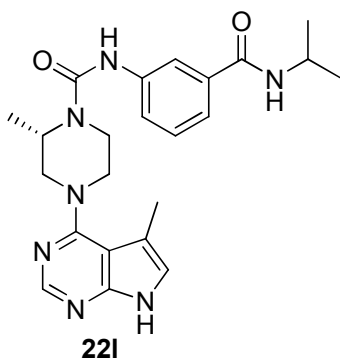


(S)-3-(2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboxamido)phenyl dimethylcarbamate (22j).

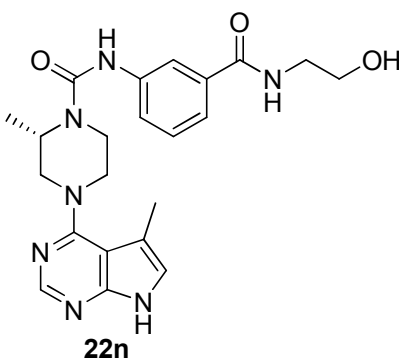
A solution of 3-nitrophenol (1.0 g, 7.2 mmol), *N,N*-dimethylchlorocarbamate (0.79 ml, 8.6 mmol), pyridine (1.7 ml, 21.6 mmol), and triethylamine (1.5 ml, 10.8 mmol) in CH₂Cl₂ (36 mL) was stirred for 3 days. The reaction was quenched with H₂O, stirred for 15 min, diluted with Et₂O, washed with 1 M aq. NaHSO₄, H₂O, sat. aq. NaHCO₃, and brine (with back extraction), dried over MgSO₄, filtered, and concentrated under vacuum. The residue was hydrogenated with balloon pressure H₂ over 10% Pd/C (50% wet, 1.26 g, 0.59 mmol) in THF (36 ml) with AcOH (0.42 ml) for 18 hours. The reaction was filtered through celite with EtOAc and concentrated under vacuum. The residue was purified by flash chromatography (40 g SiO₂, 0-4% MeOH:CH₂Cl₂) to give 3-aminophenyl-*N,N*-dimethylcarbamate (1.15 g, 6.4 mmol, 89%). ¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 7.12 (t, *J*=8.0 Hz, 1 H), 6.52 (t, *J*=2.3 Hz, 1 H), 6.50 (t, *J*=2.3 Hz, 1 H), 6.46 (t, *J*=2.1 Hz, 1 H), 3.71 (br. s., 2 H), 3.08 (s, 3 H), 3.01 (s, 3 H); MS (ES+) [M+H]⁺ = 181.

To a solution of triphosgene (104 mg, 0.35 mmol) in anhydrous THF (7.5 ml) at 0 °C was added slowly 3-aminophenyl-*N,N*-dimethylcarbamate (from the previous step, 180 mg, 1.0 mmol) and triethylamine (0.31 mL, 2.2 mmol) in THF (2.5 ml). The reaction was stirred for 15 min at 0 °C then 15 min at room temperature. Amine **7b** (231 mg, 1.0 mmol) was added, and the reaction was stirred 1 hour, quenched with MeOH, diluted with EtOAc, washed with H₂O, sat. aq. NaHCO₃ and brine, dried over MgSO₄, filtered, and concentrated under vacuum. The residue was purified by flash chromatography (40 g SiO₂, 0-8% MeOH:CH₂Cl₂), suspended in H₂O, and lyophilized to give **22j** (365 mg, 0.84 mmol, 84%, 100% purity) as a white solid. ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.22 (s, 1 H), 7.21 - 7.30 (m, 3 H), 7.03 (d, *J*=1.3 Hz, 1 H), 6.77 (ddd, *J*=7.2, 2.1, 2.0 Hz, 1 H), 4.53 (ddd, *J*=6.3, 3.1, 3.0 Hz, 1 H), 4.16 (dd, *J*=12.6, 2.0 Hz, 1 H), 4.01 (ddd, *J*=13.3, 2.7, 2.5 Hz, 1 H), 3.92 (dt, *J*=12.9, 2.0 Hz, 1 H), 3.52 - 3.62 (m, 1 H), 3.41 (dd, *J*=12.9, 3.8 Hz, 1 H), 3.07 - 3.18 (m, 4 H), 2.99 (s, 3 H), 2.46 (d, *J*=1.0 Hz, 3 H), 1.29 (d, *J*=6.8 Hz, 3 H); ¹³C NMR (101 MHz, METHANOL-*d*₄) δ ppm 163.0, 157.6, 156.9, 153.6, 153.2, 151.0, 142.3, 130.2, 123.1, 118.8, 117.4, 115.5, 110.7, 108.2, 53.9,

51.7, 49.2, 40.3, 37.0, 36.8, 16.4, 14.2; MS (ES+) $[M+H]^+ = 438$; Analysis calculated for $C_{22}H_{27}N_7O_3$: C 60.40, H 6.22, N 22.41, found: C 60.43, H 6.21, N 22.29.

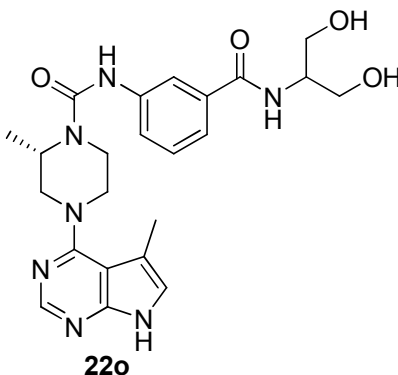


(S)-N-(3-(isopropylcarbamoyl)phenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboxamide (22l). 1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.23 (s, 1 H), 7.79 (t, $J=1.9$ Hz, 1 H), 7.51 (ddd, $J=8.0, 2.2, 1.1$ Hz, 1 H), 7.46 (ddd, $J=8.0, 1.4, 1.3$ Hz, 1 H), 7.36 (t, $J=7.8$ Hz, 1 H), 7.04 (d, $J=1.0$ Hz, 1 H), 4.56 (m, $J=6.4, 3.2, 3.0$ Hz, 1 H), 4.13 - 4.24 (m, 2 H), 4.05 (dt, $J=13.1, 2.5$ Hz, 1 H), 3.94 (dt, $J=12.9, 2.0$ Hz, 1 H), 3.55 - 3.65 (m, 1 H), 3.44 (dd, $J=13.0, 3.9$ Hz, 1 H), 3.16 (td, $J=12.4, 3.5$ Hz, 1 H), 2.48 (d, $J=1.0$ Hz, 3 H), 1.31 (d, $J=6.6$ Hz, 3 H), 1.25 (d, $J=6.6$ Hz, 6 H); MS (ES+) $[M+H]^+ = 436$.



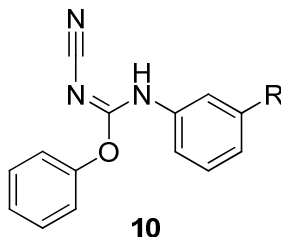
(S)-N-(3-(2-hydroxyethylcarbamoyl)phenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboxamide (22n). Acetate protected **22n** (protection on the hydroxyl group) was prepared according to the procedure for **22j**. The protecting group was removed with K_2CO_3 in MeOH to give **22n**, which was purified by silica gel chromatography. 1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.23 (s, 1 H), 7.84 (t, $J=1.8$ Hz, 1 H), 7.54 (ddd, $J=8.1, 2.3, 1.0$ Hz, 1 H), 7.49 (ddd, $J=8.0, 1.4, 1.0$ Hz, 1 H), 7.38 (t, $J=7.8$ Hz, 1 H), 7.04 (d, $J=1.3$ Hz, 1 H), 4.56 (m, $J=6.7, 3.2$ Hz, 1

H), 4.14 - 4.22 (m, 1 H), 4.01 - 4.09 (m, 1 H), 3.94 (dt, $J=12.9, 1.9$ Hz, 1 H), 3.71 (t, $J=5.8$ Hz, 2 H), 3.55 - 3.65 (m, 1 H), 3.50 (t, $J=1.0$ Hz, 2 H), 3.44 (dd, $J=12.9, 3.8$ Hz, 1 H), 3.16 (td, $J=12.4, 3.5$ Hz, 1 H), 2.48 (d, $J=1.0$ Hz, 3 H), 1.31 (d, $J=6.8$ Hz, 3 H); MS (ES+) $[M+H]^+ = 438$.



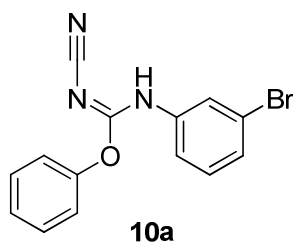
(S)-N-(3-(1,3-dihydroxypropan-2-yl)phenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboxamide (22o). Di-acetate protected **22o** (protection on the hydroxyl groups) was prepared according to the procedure for **22j**. The protecting groups were removed with K_2CO_3 in MeOH to give **22o**, which was purified by silica gel chromatography. 1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.23 (s, 1 H), 7.84 (t, $J=1.8$ Hz, 1 H), 7.49 - 7.55 (m, 2 H), 7.38 (t, $J=7.9$ Hz, 1 H), 7.04 (d, $J=1.0$ Hz, 1 H), 4.52 - 4.61 (m, 1 H), 4.11 - 4.23 (m, 2 H), 4.01 - 4.09 (m, 1 H), 3.94 (dt, $J=12.9, 1.9$ Hz, 1 H), 3.74 (d, $J=5.6$ Hz, 4 H), 3.60 (td, $J=12.5, 3.3$ Hz, 1 H), 3.45 (dd, $J=12.9, 3.8$ Hz, 1 H), 3.16 (td, $J=12.3, 3.5$ Hz, 1 H), 2.48 (d, $J=1.0$ Hz, 3 H), 1.31 (d, $J=6.6$ Hz, 3 H); MS (ES+) $[M+H]^+ = 468$.

1.6. Cyanoguanidines



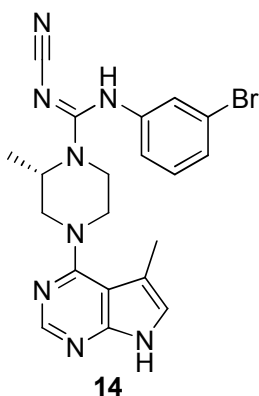
Cyanocarbamimidates **10** were prepared from anilines and diphenyl-*N*-cyanocarbonimidate according to the procedure for compound **10a**. Either products precipitated from the reaction mixture

and were collected by filtration, or the reactions were concentrated under vacuum, and the products were isolated by silica gel chromatography.



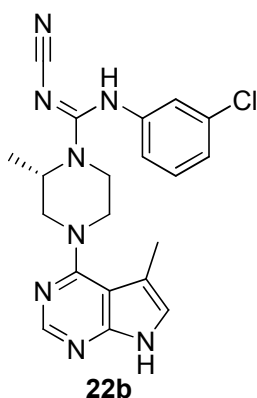
Phenyl *N*-3-bromophenyl-*N'*-cyanocarbamimidate (10a). A solution of 3-bromoaniline (1.44g, 8.4 mmol) and diphenyl-*N*-cyanocarbonimidate (2 g, 8.4 mmol) in acetonitrile (20ml) was heated at 50 °C overnight, and then cooled to room temperature, resulting in precipitation of the product. The white crystalline solid was collected by filtration to give **10a** (2 g, 6.3 mmol, 75%). ¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 7.59 (t, *J*=2.02 Hz, 1 H), 7.42 - 7.47 (m, 2 H), 7.40 (ddd, *J*=8.15, 1.45, 1.26 Hz, 1 H), 7.31 - 7.36 (m, 2 H), 7.24 - 7.29 (m, 2 H), 7.13 - 7.18 (m, 1 H); MS (ES+) [M+H]⁺ = 316, 318.

Cyanoguanidines **12**, **14**, **16**, **17**, **20**, **21a-d**, **22b,d,e,g-i,k,m,p-r** were prepared from amines **7** and cyanocarbamimidates **10** according to the procedures for **14**, **22b**, and **22d**. The compounds were purified by silica gel chromatography or prep HPLC to ≥95% purity.

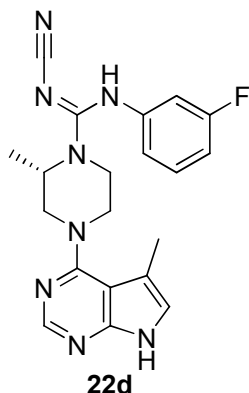


(*S*)-*N*-(3-bromophenyl)-*N'*-cyano-2-methyl-4-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazine-1-carboximidamide (14). A solution of **10a** (6.82 g, 22 mmol), **7b** (5 g, 22 mmol), and triethylamine (3.07 mL, 22 mmol) in MeCN (300 mL) was heated at reflux for 2 hours. The mixture was concentrated and purified by flash chromatography (50% THF/hexanes) to afford **14** (7.95 g, 80%, 98% purity) as a white solid. ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.22 (s, 1 H), 7.30 - 7.34 (m, 1

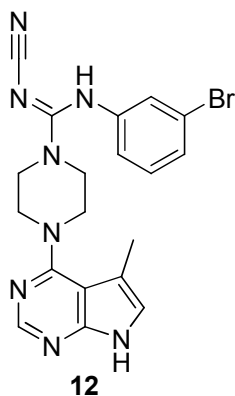
H), 7.23 - 7.30 (m, 2 H), 7.11 (ddd, $J=6.7, 2.3, 2.1$ Hz, 1 H), 7.03 (d, $J=1.0$ Hz, 1 H), 4.56 - 4.70 (m, 1 H), 4.15 (d, $J=12.4$ Hz, 1 H), 4.00 (d, $J=13.6$ Hz, 1 H), 3.92 (dt, $J=13.1, 2.0$ Hz, 1 H), 3.69 (ddd, $J=13.5, 11.9, 3.4$ Hz, 1 H), 3.48 (dd, $J=13.1, 3.8$ Hz, 1 H), 3.19 (ddd, $J=12.9, 12.1, 3.5$ Hz, 1 H), 2.45 (d, $J=1.0$ Hz, 3 H), 1.33 (d, $J=6.8$ Hz, 3 H); ^{13}C NMR (101 MHz, METHANOL- d_4) δ ppm 170.2, 167.3, 162.3, 159.4, 150.4, 140.5, 135.4, 132.3, 131.4, 131.3, 128.8, 125.4, 117.6, 115.6, 61.4, 59.8, 59.1, 51.1, 25.6, 23.2; MS (ES+) $[\text{M}+\text{H}]^+ = 453, 455$; Analysis calculated for $\text{C}_{20}\text{H}_{21}\text{BrN}_8$: C 52.99, H 4.67, N 24.72, found: C 52.66, H 4.67, N 24.48.



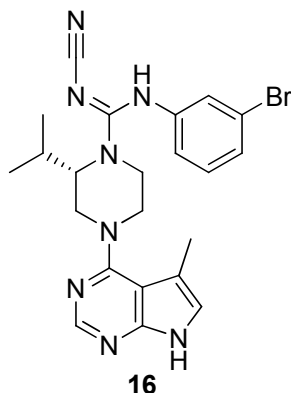
(S)-N-(3-chlorophenyl)-N'-cyano-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamide (22b). A mixture of phenyl *N*-3-chlorophenyl-*N'*-cyanocarbamimidate (0.47 g, 1.7 mmol), **7b** (0.40 g, 1.7 mmol), and *N,N*-diisopropylethylamine (1 mL) in acetonitrile (10 mL) was heated at 85 °C in a sealed pressure tube for 4 hours. The solvent was evaporated, and the residue was purified by flash chromatography (80g SiO_2 , 0-5% MeOH: CH_2Cl_2) to give **22b** (0.32 g, 0.79 mmol, 46%). ^1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.22 (s, 1 H), 7.31 (t, $J=7.96$ Hz, 1 H), 7.01 - 7.16 (m, 4 H), 4.15 (d, $J=13.14$ Hz, 1 H), 4.00 (d, $J=13.39$ Hz, 1 H), 3.91 (d, $J=13.14$ Hz, 1 H), 3.65 (d, $J=3.28$ Hz, 1 H), 3.47 (dd, $J=13.14, 3.79$ Hz, 1 H), 3.34 (s, 1 H), 3.09 - 3.25 (m, 1 H), 2.44 (s, 3 H), 1.32 (d, $J=6.57$ Hz, 3 H); MS (ES+) $[\text{M}+\text{H}]^+ = 409$.



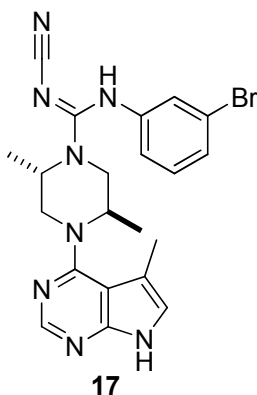
(S)-N'-cyano-N-(3-fluorophenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamide (22d). A mixture of phenyl *N'*-cyano-*N*-(3-fluorophenyl)carbamimidate (0.55 g, 2.2 mmol), **7b** (0.50 g, 2.2 mmol), and *N,N*-diisopropylethylamine (1 mL) in acetonitrile (10 ml) was heated at 85 °C in a sealed pressure tube for 4 hours. The solvent was evaporated, and the residue was purified by prep HPLC (Sunfire C18 30x250 mm column, 10-100% MeCN:H₂O (10mM NH₄OAc), 18 min., 45 ml/min) to give **22d** (0.23 g, 0.58 mmol, 27%). ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.28 (s, 1 H), 7.31 - 7.49 (m, 1 H), 7.08 (d, *J*=1.01 Hz, 1 H), 6.89 - 7.02 (m, 2 H), 4.63 - 4.74 (m, 1 H), 4.20 (d, *J*=13.14 Hz, 1 H), 4.06 (d, *J*=13.39 Hz, 1 H), 3.97 (d, *J*=11.12 Hz, 1 H), 3.67 - 3.86 (m, 1 H), 3.54 (dd, *J*=13.14, 3.79 Hz, 1 H), 3.38 (br. s., 2 H), 3.18 - 3.29 (m, 1 H), 2.50 (d, *J*=1.01 Hz, 2 H), 1.39 (d, *J*=6.57 Hz, 3 H); MS (ES⁺) [M+H]⁺ = 393.



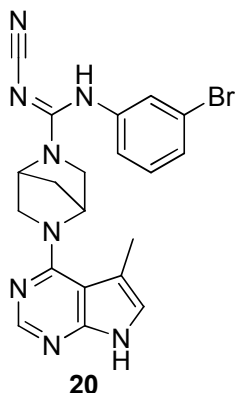
***N*-(3-bromophenyl)-*N'*-cyano-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamide (12).** ¹H NMR (TFA salt) (300 MHz, METHANOL-*d*₄) δ ppm 8.35 (s, 1 H), 7.11 - 7.40 (m, 5 H), 3.94 - 4.04 (m, 4 H), 3.82 - 3.93 (m, 4 H), 2.47 (d, *J*=1.0 Hz, 3 H); MS (ES⁺) [M+H]⁺ = 439, 441.



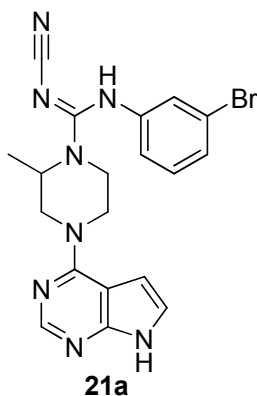
(S)-N-(3-bromophenyl)-N'-cyano-2-isopropyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamide (16). ^1H NMR (TFA salt) (400 MHz, METHANOL- d_4) δ ppm 8.36 (s, 1 H), 7.27 - 7.36 (m, 3 H), 7.19 (d, $J=0.9$ Hz, 1 H), 7.09 - 7.16 (m, 1 H), 5.55 (dt, $J=12.3, 6.2$ Hz, 1 H), 4.62 (d, $J=13.5$ Hz, 1 H), 4.42 (d, $J=9.4$ Hz, 1 H), 3.62 (dd, $J=13.5, 3.5$ Hz, 1 H), 3.52 (d, $J=9.1$ Hz, 2 H), 2.47 (d, $J=1.2$ Hz, 3 H), 2.04 - 2.18 (m, 1 H), 0.94 (d, $J=6.7$ Hz, 6 H); MS (ES+) $[\text{M}+\text{H}]^+ = 482$.



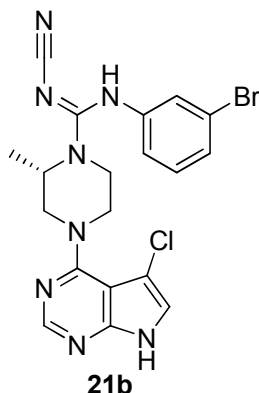
Trans-N-(3-bromophenyl)-N'-cyano-2,5-dimethyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamide (17). ^1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.23 (s, 1 H), 7.30 - 7.35 (m, 1 H), 7.28-7.29 (m, 2 H), 7.09 - 7.17 (m, 1 H), 7.03 (d, $J=1.3$ Hz, 1 H), 4.56 - 4.68 (m, 2 H), 3.83 - 3.95 (m, 3 H), 3.64 (d, $J=13.6$ Hz, 1 H), 2.45 (d, $J=1.0$ Hz, 3 H), 1.28 (d, $J=6.8$ Hz, 3 H), 1.20 (d, $J=6.6$ Hz, 3 H); MS (ES+) $[\text{M}+\text{H}]^+ = 467, 469$.



***N*-(3-bromophenyl)-*N'*-cyano-5-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)-2,5-diazabicyclo[2.2.1]heptane-2-carboximidamide (20).** ^1H NMR (TFA salt) (400 MHz, METHANOL- d_4) δ ppm 8.29 (s, 1 H), 7.38 - 7.43 (m, 1 H), 7.27 - 7.32 (m, 1 H), 7.14 - 7.27 (m, 3 H), 5.37 (s, 1 H), 5.09 (br. s., 1 H), 4.22 (d, $J=9.9$ Hz, 1 H), 4.07 (d, $J=10.1$ Hz, 1 H), 3.89 (d, $J=9.1$ Hz, 1 H), 3.76 (d, $J=10.1$ Hz, 1 H), 2.47 (s, 3 H), 2.28 (s, 2 H); MS (ES+) $[\text{M}+\text{H}]^+ = 451, 453$.

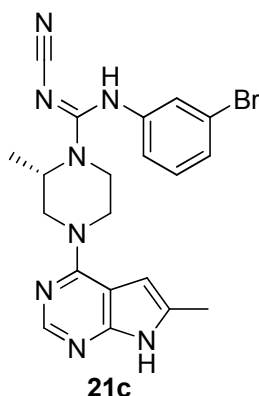


***N*-(3-bromophenyl)-*N'*-cyano-2-methyl-4-(7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazine-1-carboximidamide (21a).** Compound **21a** was prepared from commercially available 4-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine and racemic 2-methylpiperazine according to standard procedures. ^1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.17 (s, 1 H), 7.31 - 7.36 (m, 1 H), 7.23 - 7.31 (m, 2 H), 7.17 (d, $J=3.5$ Hz, 1 H), 7.13 (dt, $J=6.6, 2.3$ Hz, 1 H), 6.67 (d, $J=3.8$ Hz, 1 H), 4.58 - 4.70 (m, 2 H), 4.51 - 4.57 (m, 1 H), 4.02 (ddd, $J=13.5, 3.0, 2.9$ Hz, 1 H), 3.68 (dd, $J=13.5, 3.7$ Hz, 1 H), 3.55 - 3.65 (m, 1 H), 3.43 - 3.53 (m, 1 H), 1.32 (d, $J=6.6$ Hz, 3 H); MS (ES+) $[\text{M}+\text{H}]^+ = 441$.



(S)-N-(3-bromophenyl)-4-(5-chloro-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-N'-cyano-2-methylpiperazine-1-carboximidamide (21b). Commercially available 4-chloro-7H-pyrrolo[2,3-d]pyrimidine (0.50 g, 3.3 mmol) was suspended in anhydrous CH₂Cl₂ (25 ml), and *N*-chlorosuccinimide (0.87 g, 6.5 mmol) was added. The reaction mixture was refluxed for 3 days, then cooled to room temperature. The white solid was collected by filtration to give 4,5-dichloro-7H-pyrrolo[2,3-d]pyrimidine (0.54 g, 2.9 mmol, 88%). ¹H NMR (CD₃OD): δ 8.57 (1 H, s), 7.60 (1H, s); MS (ES+) [M+H]⁺ = 188.

This material was carried on to **21b** according to the standard procedures. ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.27 (s, 1 H), 7.30 - 7.35 (m, 2 H), 7.23 - 7.30 (m, 2 H), 7.09 - 7.15 (m, 1 H), 4.59 - 4.69 (m, 1 H), 4.36 - 4.44 (m, 1 H), 4.16 (ddd, *J*=13.2, 2.1, 2.1 Hz, 1 H), 4.01 (d, *J*=13.1 Hz, 1 H), 3.73 (ddd, *J*=13.5, 11.9, 3.4 Hz, 1 H), 3.48 (dd, *J*=13.3, 3.9 Hz, 1 H), 3.23 (ddd, *J*=13.0, 12.0, 3.5 Hz, 1 H), 1.33 (d, *J*=6.8 Hz, 3 H); MS (ES+) [M+H]⁺ = 475.

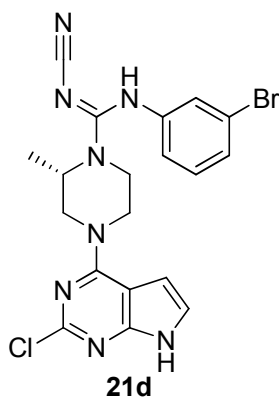


(S)-N-(3-bromophenyl)-N'-cyano-2-methyl-4-(6-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamide (21c). A solution of ethyl 2-amino-5-methyl-1H-pyrrole-3-carboxylate (150 mg, 0.9 mmol, prepared according to literature procedures, *J. Heterocyclic Chem.*, **1985**, *23*, 1555.) in formamide (4.5 ml), formic acid (2.3 ml) and DMF (1.0 ml) was heated to 155 °C

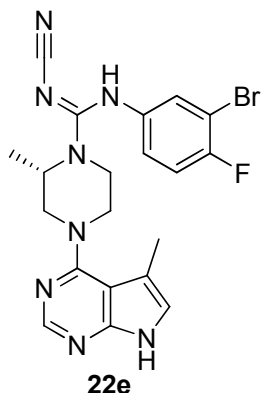
for 12 h. The reaction was concentrated, taken up with NaHCO₃ solution, and extracted with dichloromethane to afford 6-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-ol (70 mg, 0.46 mmol, 51%, MS (ES+) [M+H]⁺ = 150).

This material was dissolved in phosphorous oxychloride (5 ml) and heated at 110 °C for 1 h. The reaction was concentrated, taken up with NaHCO₃, and extracted with DCM to give 4-chloro-6-methyl-7H-pyrrolo[2,3-d]pyrimidine (40 mg, 0.23 mmol, 50%). MS (ES+) [M+H]⁺ = 168.

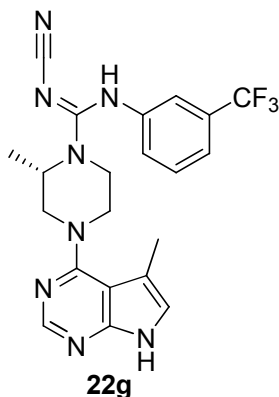
This intermediate was carried on to compound **21c** according to the standard procedures. ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.13 (s, 1 H), 7.32 - 7.33 (m, 1 H), 7.24 - 7.31 (m, 2 H), 7.13 (ddd, *J*=6.7, 2.4, 2.3 Hz, 1 H), 6.36 - 6.41 (m, 1 H), 4.56 - 4.65 (m, 2 H), 4.49 (d, *J*=13.6 Hz, 1 H), 4.02 (d, *J*=13.6 Hz, 1 H), 3.66 (dd, *J*=13.6, 3.8 Hz, 1 H), 3.54 - 3.63 (m, 1 H), 3.41 - 3.50 (m, 1 H), 2.41 (d, *J*=1.0 Hz, 3 H), 1.32 (d, *J*=6.8 Hz, 3 H); MS (ES+) [M+H]⁺ = 455.



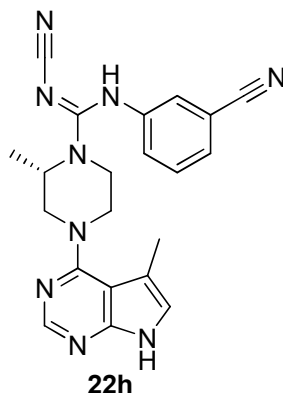
(S)-N-(3-bromophenyl)-4-(2-chloro-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-N'-cyano-2-methylpiperazine-1-carboximidamide (21d). Prepared from 2,4-dichloro-7H-pyrrolo[2,3-*d*]pyrimidine according to the standard procedures. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 7.19 - 7.32 (m, 4 H), 7.04 - 7.11 (m, 1 H), 6.69 (dd, *J*=3.6, 1.9 Hz, 1 H), 4.43 - 4.56 (m, 2 H), 4.37 (dd, *J*=13.5, 2.0 Hz, 1 H), 3.92 (d, *J*=12.5 Hz, 1 H), 3.62 (dd, *J*=13.7, 3.9 Hz, 1 H), 3.37 - 3.52 (m, 2 H), 1.21 (d, *J*=6.6 Hz, 3 H); MS (ES+) [M+H]⁺ = 475.



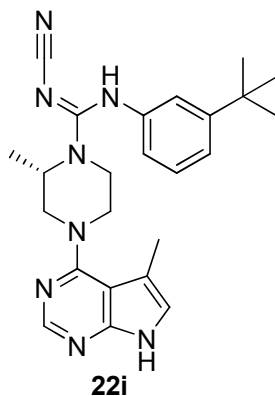
(S)-N-(3-bromo-4-fluorophenyl)-N'-cyano-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamide (22e). ^1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.23 (s, 1 H), 7.42 (dd, $J=5.8, 2.5$ Hz, 1 H), 7.13 - 7.24 (m, 2 H), 7.04 (s, 1 H), 4.62 (br. s., 1 H), 4.16 (d, $J=13.1$ Hz, 1 H), 4.03 (d, $J=13.4$ Hz, 1 H), 3.92 (d, $J=13.1$ Hz, 1 H), 3.63 - 3.73 (m, 1 H), 3.49 (dd, $J=13.0, 3.7$ Hz, 1 H), 3.21 (td, $J=12.4, 3.3$ Hz, 1 H), 2.45 (s, 3 H), 1.33 (d, $J=6.6$ Hz, 3 H); MS (ES+) $[\text{M}+\text{H}]^+ = 471, 473$.



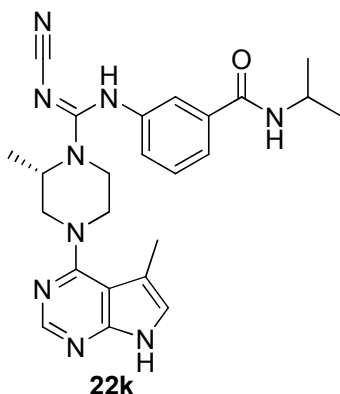
(S)-N'-cyano-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-N-(3-(trifluoromethyl)phenyl)piperazine-1-carboximidamide (22g). ^1H NMR (TFA salt) (400 MHz, METHANOL- d_4) δ ppm 8.37 (s, 1 H), 7.53 - 7.59 (m, 1 H), 7.38 - 7.47 (m, 3 H), 7.24 (d, $J=1.2$ Hz, 1 H), 4.66 - 4.74 (m, 1 H), 4.34 - 4.41 (m, 1 H), 4.21 (d, $J=13.3$ Hz, 1 H), 3.88 (dd, $J=13.2, 4.0$ Hz, 1 H), 3.63 - 3.78 (m, 2 H), 2.48 (d, $J=1.0$ Hz, 3 H), 1.35 (d, $J=6.6$ Hz, 3 H); MS (ES+) $[\text{M}+\text{H}]^+ = 429$.



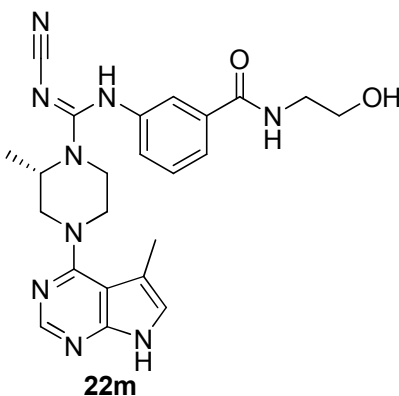
(S)-N'-cyano-N-(3-cyanophenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamide (22h). ^1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.23 (s, 1 H), 7.39 - 7.56 (m, 4 H), 7.04 (s, 1 H), 4.60 - 4.71 (m, 1 H), 4.18 (d, $J=13.4$ Hz, 1 H), 4.05 (d, $J=13.4$ Hz, 1 H), 3.94 (d, $J=13.1$ Hz, 1 H), 3.68 - 3.78 (m, 1 H), 3.51 (dd, $J=13.1, 3.8$ Hz, 1 H), 3.23 (td, $J=12.5, 3.3$ Hz, 1 H), 2.46 (s, 3 H), 1.35 (d, $J=6.8$ Hz, 3 H); MS (ES+) $[\text{M}+\text{H}]^+ = 400$.



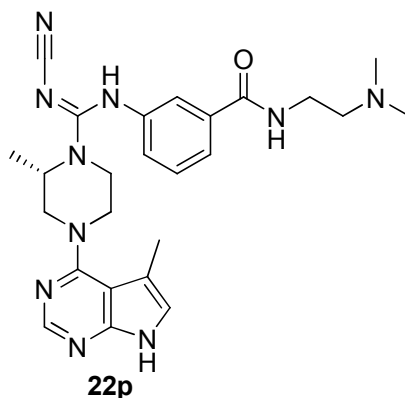
(S)-N-(3-tert-butylphenyl)-N'-cyano-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamide (22i). ^1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.22 (s, 1 H), 7.24 - 7.32 (m, 1 H), 7.16 - 7.21 (m, 2 H), 7.03 (d, $J=1.0$ Hz, 1 H), 6.95 (ddd, $J=7.8, 2.2, 1.1$ Hz, 1 H), 4.57 - 4.68 (m, 1 H), 4.12 (d, $J=12.6$ Hz, 1 H), 3.86 - 3.99 (m, 2 H), 3.59 - 3.70 (m, 1 H), 3.47 (dd, $J=13.1, 3.8$ Hz, 1 H), 3.17 (td, $J=12.4, 3.4$ Hz, 1 H), 2.40 - 2.47 (m, 3 H), 1.27 - 1.37 (m, 12 H); MS (ES+) $[\text{M}+\text{H}]^+ = 431$.



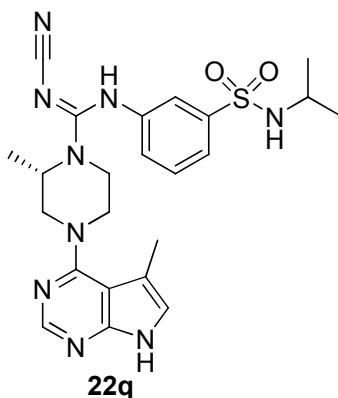
(S)-3-(N'-cyano-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamido)-N-isopropylbenzamide (22k). ¹H NMR (TFA salt) (400 MHz, METHANOL-*d*₄) δ ppm 8.37 (s, 1 H), 7.55 - 7.63 (m, 2 H), 7.42 - 7.47 (m, 1 H), 7.29 - 7.35 (m, 1 H), 7.24 (d, *J*=1.0 Hz, 1 H), 4.63 - 4.73 (m, 1 H), 4.32 - 4.42 (m, 1 H), 4.15 - 4.27 (m, 2 H), 4.04 - 4.11 (m, 1 H), 3.89 (dd, *J*=13.1, 4.0 Hz, 1 H), 3.64 - 3.74 (m, 2 H), 2.47 (d, *J*=1.0 Hz, 3 H), 1.34 (d, *J*=6.8 Hz, 3 H), 1.25 (d, *J*=6.6 Hz, 6 H); MS (ES⁺) [M+H]⁺ = 460.



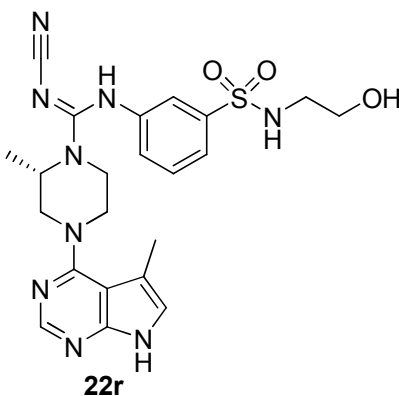
(S)-3-(N'-cyano-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamido)-N-(2-hydroxyethyl)benzamide (22m). ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.23 (s, 1 H), 7.58 - 7.66 (m, 2 H), 7.45 (t, *J*=7.8 Hz, 1 H), 7.31 (ddd, *J*=8.0, 2.2, 0.9 Hz, 1 H), 7.04 (d, *J*=1.0 Hz, 1 H), 4.60 - 4.69 (m, 1 H), 4.16 (d, *J*=12.6 Hz, 1 H), 4.02 (d, *J*=12.9 Hz, 1 H), 3.93 (d, *J*=13.1 Hz, 1 H), 3.65 - 3.75 (m, 3 H), 3.45 - 3.55 (m, 3 H), 3.15 - 3.25 (m, 1 H), 2.45 (d, *J*=1.0 Hz, 3 H), 1.34 (d, *J*=6.8 Hz, 3 H); MS (ES⁺) [M+H]⁺ = 462.



(S)-3-(N'-cyano-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamido)-N-(2-(dimethylamino)ethyl)benzamide (22p). ¹H NMR (AcOH salt) (400 MHz, METHANOL-*d*₄) δ ppm 8.23 (s, 1 H), 7.66 (t, *J*=1.8 Hz, 1 H), 7.62 (dt, *J*=7.8, 1.3 Hz, 1 H), 7.47 (t, *J*=8.0 Hz, 1 H), 7.33 (ddd, *J*=8.1, 2.3, 1.0 Hz, 1 H), 7.04 (d, *J*=1.0 Hz, 1 H), 4.60 - 4.69 (m, 1 H), 4.17 (d, *J*=13.9 Hz, 1 H), 4.04 (d, *J*=13.4 Hz, 1 H), 3.93 (d, *J*=13.1 Hz, 1 H), 3.67 - 3.76 (m, 1 H), 3.64 (t, *J*=6.3 Hz, 2 H), 3.49 (dd, *J*=13.1, 3.8 Hz, 1 H), 3.15 - 3.25 (m, 1 H), 2.97 (t, *J*=6.3 Hz, 2 H), 2.64 (s, 6 H), 2.46 (d, *J*=1.0 Hz, 3 H), 1.35 (d, *J*=6.6 Hz, 3 H); MS (ES⁺) [M+H]⁺ = 489.

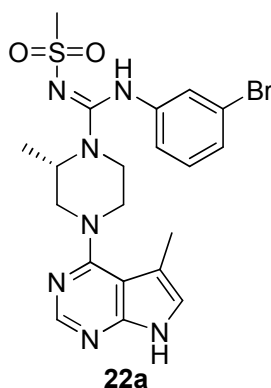


(S)-N'-cyano-N-(3-(N-isopropylsulfamoyl)phenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamide (22q). ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.23 (s, 1 H), 7.58 - 7.66 (m, 2 H), 7.51 - 7.57 (m, 1 H), 7.35 (ddd, *J*=7.9, 2.2, 1.0 Hz, 1 H), 7.04 (d, *J*=1.0 Hz, 1 H), 4.61 - 4.72 (m, 1 H), 4.18 (d, *J*=12.4 Hz, 1 H), 4.03 (d, *J*=13.4 Hz, 1 H), 3.94 (d, *J*=13.1 Hz, 1 H), 3.67 - 3.77 (m, 1 H), 3.50 (dd, *J*=13.3, 3.7 Hz, 1 H), 3.40 (m, *J*=6.6, 6.6, 6.6, 6.6 Hz, 1 H), 3.21 (td, *J*=12.6, 3.4 Hz, 1 H), 2.46 (d, *J*=1.0 Hz, 3 H), 1.35 (d, *J*=6.8 Hz, 3 H), 1.07 (d, *J*=2.0 Hz, 3 H), 1.06 (d, *J*=2.3 Hz, 3 H); MS (ES⁺) [M+H]⁺ = 496.



(S)-N'-cyano-N-(3-(N-(2-hydroxyethyl)sulfamoyl)phenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboximidamide (22r). ^1H NMR (400 MHz, METHANOL- d_4) δ ppm 8.23 (s, 1 H), 7.59 - 7.65 (m, 2 H), 7.52 - 7.58 (m, 1 H), 7.36 (ddd, $J=8.0, 2.1, 1.3$ Hz, 1 H), 7.04 (d, $J=1.0$ Hz, 1 H), 4.63 - 4.70 (m, 1 H), 4.18 (d, $J=13.4$ Hz, 1 H), 4.05 (d, $J=13.1$ Hz, 1 H), 3.94 (d, $J=13.4$ Hz, 1 H), 3.68 - 3.77 (m, 1 H), 3.46 - 3.58 (m, 3 H), 3.18 - 3.27 (m, 1 H), 3.02 (t, $J=5.8$ Hz, 2 H), 2.46 (d, $J=1.0$ Hz, 3 H), 1.35 (d, $J=6.6$ Hz, 3 H); MS (ES+) $[\text{M}+\text{H}]^+ = 498$.

1.7. Sulfonylguanidine 22a



(S)-N-(3-bromophenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-N'-(methylsulfonyl)piperazine-1-carboximidamide (22a). A solution of dichlorodiphenoxymethane (2 g, 7.46 mmol) and methylsulfonamide (1.56 g, 16.41 mmol) in EtOAc (15 mL) was heated at reflux for 12 hours. The reaction was concentrated under vacuum, and purification of the crude mixture by flash chromatography (20% EtOAc/hexanes) afforded diphenyl methylsulfonylcarbonimidate (0.75 g, 2.59 mmol, 35%). ^1H NMR (400 MHz, CHLOROFORM- d) δ ppm 7.38-7.43 (m, 4 H), 7.30 (m, 2H), 7.21 (m, 4H), 3.01 (s, 3H); MS (ES+) $[\text{M}+\text{H}]^+ = 292$.

This material was combined with 3-bromoaniline (0.28 ml, 2.59 mmol) in acetonitrile (5ml) and heated at 70 °C for 12 hours. The reaction was concentrated under vacuum, and the residue was purified by flash chromatography (30% EtOAc/hexanes) to afford phenyl *N*-3-bromophenyl-*N'*-(methylsulfonyl)carbamimidate (0.50 g, 1.35 mmol, 52%). ¹H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 9.25 (s, 1H), 7.54 (m, 1H), 7.40 (m, 3H), 7.26 (m, 2H), 7.13 (m, 2H), 2.96 (s, 3H); MS (ES+) [M+H]⁺ = 369, 371.

A sample of this carbamimidate (100 mg, 0.27 mmol) was combined with **7b** (63mg, 0.27mmol) and triethylamine (77 μL, 0.27 mmol) in MeCN (1.5ml) and heated at reflux for 2 hours. The mixture was concentrated and purified by preparative HPLC to afford **22a**. ¹H NMR (400 MHz, METHANOL-*d*₄) δ ppm 8.19 (s, 1H), 7.40 (s, 1H), 7.30 (m, 2H), 7.16 (m, 1H), 7.01 (s, 1H), 4.46 (m, 1H), 4.01 (m, 1H), 3.78 (m, 2H), 3.52 (m, 1H), 3.40 (m, 1H), 3.08 (m, 1H), 2.98 (s, 3H), 2.39 (d, *J* = 1.0Hz, 3H), 1.26 (d, *J* = 6.8Hz, 3H); MS (ES+) [M+H]⁺ = 506, 508.

2. IN VITRO METHODS

2.1. Expression and Purification of LIMK2

Full length human LIMK2 was expressed using the BAC-to-BAC[®] Baculovirus Expression System (Invitrogen). Recombinant baculovirus was made according to the manufacturer's directions as set forth in the instruction manual. Briefly, the plasmids (pFactBac1 or pFastBacHT) carrying the LIMK2 inserts were transformed into MAX efficiency DH10Bac competent *E. coli* to generate a recombinant bacmid. The DH10Bac *E. coli* host strain contains a baculovirus shuttle vector (bacmid) with a mini-attTn7 target site and a helper plasmid, and allows generation of a recombinant bacmid following transposition between the mini-Tn7 element on the pFastBac vector and the mini-attTn7 target site on the bacmid. The transposition reaction occurs in the presence of transposition proteins supplied by the helper plasmid. Cells were plated and the white colonies picked for bacmid isolation as described in the instruction manual.

The isolated bacmid DNA was transfected into SF9 cells to generate a recombinant baculovirus, and virus was collected five days after transfection. Virus was amplified in T75 flasks at a multiplicity of infection (MOI) of 0.2. The amplified virus was used to infect SF9 cells at a MOI 5 for protein expression.

For small scale purification of the LIMK2 constructs, a 50 ml culture of Sf9 cells infected with the recombinant baculovirus was used. The cells were harvested by centrifugation for 5 minutes at 500 x g. The cells were then resuspended in lysis buffer (5 volumes per gram of cells). A typical lysis buffer contains the following: 50 mM HEPES (pH 8.0), 300 mM KCl, 10% glycerol, 1% NP-40, 15mM imidazole, 1mM benzamidine, and Roche complete protease inhibitors (1 tablet per 50 ml of cell lysate). The cellular suspension was lysed by one passage through a Microfluidics Microfluidizer M-110Y at a liquid pressure of 14,000 to 20,000 psi followed by centrifugation of the lysate at 60,000 x g for 15 minutes at 4 °C.

The supernatant was then loaded directly onto a chromatography matrix containing Cobalt ion covalently attached to nitrilotriacetic acid NTA . The chromatography matrix was equilibrated in the same buffer as the protein loading solution. The ion charged resin typically has a binding capacity equivalent to 5 to 10 mg histidine-tagged protein per ml of packed resin. The amount of extract that can be loaded onto the column depends on the amount of soluble histidine-tagged protein in the extract. The column was then washed in a stepwise fashion, first with: 50 mM HEPES (pH 8.0), 300 mM KCl, 10% glycerol, 1% NP-40, 15mM imidazole, 1mM benzamidine; second, with 20 mM HEPES (pH 8.0), 500mM KCl, 10% glycerol, and 20 mM imidazole; third, with 20 mM HEPES (pH 8.0), 100 mM KCl, 10% glycerol, and 20 mM imidazole; followed by elution with 250 mM imidazole in the same buffer. The LIMK2 protein solution was then analyzed by SDS-PAGE and Western blot using commercial antibodies directed to both the carboxyl terminus and internal catalytic domains of the protein. For storage purposes the protein was dialyzed into 50 mM Tris (pH 7.5), 150mM NaCl, 0.1% BME, 0.03% Brij-35, and 50% glycerol.

Large scale LIMK2 purification was done in a Wave Bioreactor (Wave Biotech) with 10L culture volumes. 10L of cell culture at $2-3 \times 10^6$ viable cells/mL were infected at an MOI=5 pfu/cell and harvested at 48 hours post infection.

2.2. In Vitro LIMK2 Inhibition Assay

An *in vitro* assay used to identify LIMK2 inhibitors was developed. The analytical readout was the incorporation of ^{33}P from ATP into biotinylated-cofilin substrate immobilized on streptavidin coated flash plates (Perkin Elmer Biosciences). Plates were counted on a scintillation counter equipped with a plate reader (TopCount, Packard Bioscience, Meriden, CT). We used 384 well streptavidin FlashPlates from Perkin Elmer (Cat# SMP410A001PK).

Rock1 purified at Lexicon using a similar procedure described above for LIMK2, was used to activate LIMK2. Specifically, a 20 uL mixture of 0.6 nM Rock1, 5 nM LIMK2, and 25 μM ATP was

preincubated in kinase assay buffer (30 mM HEPES, pH 8.0; mM MgCl₂; 5 mM DTT; 0.1% Pluronic F-68) at room temperature for 30 minutes. Compounds were then acoustically dispensed using a Labcyte® Echo™ 550 (Labcyte Inc., Sunnyvale, CA) compound reformatter and 12-point dose responses were performed in four independent dilutions. The LIMK2 assay was then initiated upon addition of ³³P-ATP and 0.5 μM biotinylated-cofilin (overexpressed in *E. coli* and purified at Lexicon) in a final reaction volume of 50 uL. The reaction was incubated at room temperature for 60 minutes, washed 3 times with 75 μl of stop/wash buffer (1X stop/was buffer contains 50 mM EDTA and 20 mM Tris (pH 7.4)), and then the plates were read on the scintillation counter. Each compound was assayed in quadruplicate using four independent dilution series.

2.3. In Vitro LIMK1, Rock1, and Rock2 Inhibition Assays

Human LIMK1 (cat. # 14-656) and Rock2 (cat. # 14-451) were purchased from Millipore (Billerica, MA). Human Rock1 was overexpressed in a baculovirus system and purified at Lexicon using a protocol nearly identical to that described above for LIMK2.

The LIMK1 assay was performed as described above for LIMK2, except no need to preactivate this enzyme. All other reaction conditions were identical to the LIMK2 assay, including the same cofilin substrate, ATP concentration, and kinase buffer. The final LIMK1 concentration in the assay was 1 nM.

Rock1 and Rock2 assays were also performed using a similar streptavidin FlashPlate system described above for LIMK1. However, both Rock1 and Rock2 require the use of a biotinylated myosin light chain (MLC-2) substrate overexpressed in *E. coli* and purified at Lexicon. The final ATP and MLC-2 substrate concentrations in the kinase assays were 10 μM and 0.5 μM, respectively.

Table 6. Profile of Selected Compounds

Cmpopund	IC ₅₀ (nM) ± SDEV			
	LIMK2	LIMK1	ROCK1	ROCK2
14	0.9 ± 0.4	0.50 ± 0.04	260 ± 153	2700 ± 783
22e	1.5 ± 0.7	3.7 ± 1.4	310 ± 82	2200 ± 499
22f	3.2 ± 1.3	6.6 ± 1.4	51 ± 14	280 ± 99
22j	1.2 ± 0.2	3.2 ± 0.4	490 ± 51	1100 ± 848

2.4. Cellular Cofilin Assays

Primary pig trabecular meshwork (PTM) cells were plated on collagen I coated 24-well plates (BD Bio-Coat, Bedford, MA) and allowed to grow to confluence. Confluent cells were treated for 2 hours with LIMK inhibitors in serum free media at doses ranging from 1 nM to 10 μ M. All inhibitors were dissolved in 100% DMSO (Sigma, St. Louis, MO) and diluted 1:1000 to achieve the final working concentration. Serum free media containing 0.1% DMSO was used as the vehicle control in these studies. Two hours post treatment, PTM cells were harvested for protein lysates. The media was aspirated from the cells without disrupting the cell layer. Cells were briefly washed two times with ice cold 1x PBS. After aspiration of the PBS wash, 25 μ l of 3x sample buffer, containing 187.5 mM Tris HCl (pH 6.8), 6% SDS, 30% glycerol, 2% β -mercaptoethanol (β ME), 150 mM DTT, and 0.03% (w/v) bromophenol blue, was dispensed to each well of cells. Cells were then scraped and mixed with a cell scraper and the lysed cells collected into 1.5 ml sample collection tubes. Samples were then heated at 95^o C for 5 minutes, vortexed, and briefly spun down. 5 μ l of each cell lysate was then resolved using precast 10-20% gradient Tris-HCl gels (Bio-Rad, Hercules, CA). Gels ran at 120 constant volts until the running dye front reached the foot of the gel. Proteins were then transferred to PVDF membranes (Bio-Rad, Hercules, CA) using a wet transfer device (Bio-Rad, Hercules, CA). After transfer, membranes were blocked with 5% milk in 1x Tris-buffered saline (Sigma, St. Louis, MO)/0.1%Tween 20 (Acros Organics, Geel, Belgium) (TBS-T) for one hour at room temperature. Membranes were washed at room temperature 3 times for 5 minutes with gentle shaking in 1x TBS-T. Membranes were first incubated overnight with an anti-phospho-cofilin (1:1000)(Cell Signaling #3311, Danvers, MA) primary antibody, and then with a horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody (1:2000)(Amersham Biosciences #NA934V, Pittsburgh, PA) for one hour. Membranes were then developed using ECL plus (Amersham Biosciences, Pittsburgh, PA), and luminescence was captured and measured using a Versa Doc 4000 system with Quantity One analysis software (Bio-Rad, Hercules, CA). The membrane was stripped (following ECL plus protocol), washed in TBS-T, and blocked in 5% milk before incubating it with the second primary antibody against total cofilin (1:1000) (Cell Signaling #3312, Danvers, MA). After overnight incubation with the anti-cofilin antibody, the membrane was incubated with the same HRP conjugated anti-rabbit antibody as mentioned above and developed using ECL Plus. The amount of phospho-cofilin in each lane was normalized by the amount of the total cofilin protein in the same lane. All compound treated phospho-cofilin/cofilin values were compared to the vehicle control values. Compounds were tested in triplicate.

3. IN VIVO METHODS

3.1. In Vivo Dexamethasone Induced Ocular Hypertensive Mouse Model

Alzet micro-osmotic pumps (Model 1004, DURECT Corp., Cupertino, CA) were filled with a PBS solution containing 34.5 mg/mL solution of water soluble dexamethasone (Sigma, Milwaukee, WI). The pump rate for the micro-osmotic pumps was set to 0.11 μ L per hour which would deliver 0.09 mg of dexamethasone per day. The osmotic pumps were implanted into male hybrid mice (C57BL/6J-*Tyr^{c-Brd}* x 129S5/SvEvBrd, F2 generation, 25-35 g). IOP was recorded using a TonoLab tonometer under isoflurane anesthesia before (baseline) and after pump implantation, for a period of 28 days. Continuous administration of dexamethasone resulted in a significant increase of 3.8 ± 0.4 mmHg (mean \pm SEM) in IOP when compared with baseline IOP measurements. Pharmacological responses were observed between days 21 and 25. Three-way crossover studies were designed with a one day washout period between each study. Animal groups were switched during each drug study so each mouse would receive a different compound/vehicle.

For single timepoint experiments, LIMK2 inhibitors **14**, **22f**, and **22j** were formulated as 1 mg/mL suspensions in a xanthan gum based vehicle. A 5 μ L drop of vehicle or formulated compound was placed on the eye of a hypertensive mouse. IOP measurements were taken 1 hour later, and the IOP from compound treated eyes was compared to vehicle treated eyes. In this experiment, for compound **14**, $n = 30$, vehicle $n = 33$; for compound **22f**, $n = 13$, vehicle $n = 14$; compound **22j**, $n = 18$, vehicle $n = 17$.

For the time course/dose response study with **22j**, the compound was formulated at 0.1 mg/mL and 1 mg/mL in a cosolvent/HPMC based vehicle (2% PEG, 1% PG, 0.5% Tween in a 0.54% HPMC based vehicle containing 0.02% Carbopol with 1.25% mannitol). Baseline IOP's were taken at time 0, and a 3 μ L drop of vehicle or formulated compound was placed on the eye of a hypertensive mouse. IOP measurements were taken 1, 2, and 4 hours later. In this experiment, for vehicle, $n = 10$; for 0.3 μ g **22j**, $n = 10$; for 3 μ g **22j**, $n = 9$; for timolol, $n = 10$.

4. EX VIVO METHODS

4.1. Ex Vivo Pig Eye Perfusion Assay

Procedures used in these studies were similar to those previously described by Crosson and colleagues (Craig E. Crosson, Carl F. Sloan, and Philip W. Yates. Modulation of Conventional Outflow Facility by the Adenosine A₁ Agonist *N*⁶-Cyclohexyladenosine. Invest Ophthalmol Vis Sci. 2005;46: S33

3795-3799.). Briefly porcine eyes were obtained from a local slaughter house. After removal, eyes were transported in ice-cold phosphate-buffered saline (pH 7.4). Eyes were bisected at the equator, and the lens was removed. The remaining choroid, iris, and ciliary body were gently teased away. Isolated corneoscleral shells were then attached to a perfusion chamber and perfused with DMEM supplemented with 50 U/mL penicillin and 50 µg/mL streptomycin. The entire perfusion apparatus was placed in an incubator at 37°C and 5% CO₂. Perfusion pressure was maintained at a constant level of 8 mm Hg, and the rate of fluid outflow was monitored continuously by measuring the rate of fluid flow from a reservoir by means of an analytical balance (Model ACCU 124; Fisher, Pittsburgh, PA). The rate of fluid flow was recorded by computer (Dell, Round Rock, TX; with Collect XL software LabTronics, Inc., Guelph, Ontario, Canada). Outflow facility was calculated every 2 minutes as the ratio of flow rate to perfusion pressure (in microliters per minute per mm Hg). Preparations were allowed to stabilize for 5 to 6 hours and baseline facilities were then recorded for 40 to 60 minutes. Only preparations with stable baselines and basal facilities ranging from 0.1 to 0.4 (in microliters per minute x mm Hg) were used in these studies. The test agent was then introduced into the perfusion system by media exchange. For data analysis and presentation individual facility measurements were normalized to baseline values and expressed as the percentage change in facility at the time of media exchange ($t = 0$). All values were presented as means \pm SEM.

Compounds **14** and **22j** were assayed at 100 nM and compared to vehicle and Y-39983 at 10 µM. In this experiment, for vehicle, $n = 6$; for compound **14**, $n = 6$; for compound **22j**, $n = 5$; for Y-39983, $n = 7$.