The Discovery and Development of LX7101, a Dual LIM-kinase and ROCK Inhibitor for the Treatment of Glaucoma

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Contents:

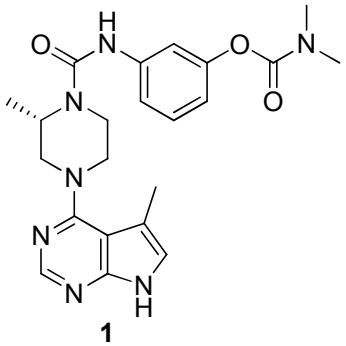
1. PREPARATION OF COMPOUNDS 1-28.

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). ^{1}H 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, 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 F_{254}) 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.

1.2. COMPOUNDS 1, 5-28.

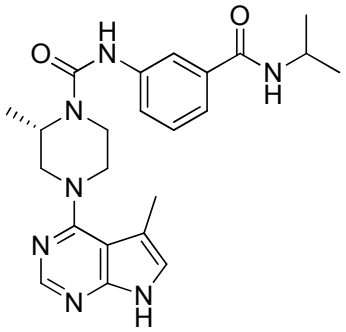


1

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

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 (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. (*S*)-5-methyl-4-(3-methylpiperazin-1-yl)-7H-pyrrolo[2,3-*d*]pyrimidine (231 mg, 1.0 mmol) was added, and the reaction was stirred 1 hour, quenched with MeOH, diluted with EtOAc, washed sequentially with H_2O, sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated under vacuum. The residue was purified by flash chromatography (0-8% MeOH:CH_2Cl_2), suspended in H_2O, and lyophilized to give **1** (365 mg, 0.84 mmol, 84% yield, 100% purity) as a white solid.

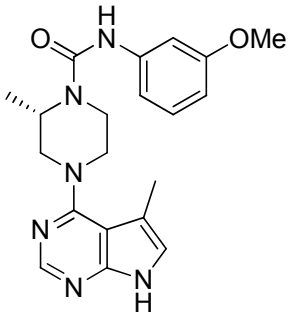
^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.22 (s, 1 H), 7.21 - 7.30 (m, 3 H), 7.03 (d, *J*=1.3 Hz, 1 H), 6.77 (ddd, *J*=7.2, 2.1, 2.0 Hz, 1 H), 4.53 (ddd, *J*=6.3, 3.1, 3.0 Hz, 1 H), 4.16 (dd, *J*=12.6, 2.0 Hz, 1 H), 4.01 (ddd, *J*=13.3, 2.7, 2.5 Hz, 1 H), 3.92 (dt, *J*=12.9, 2.0 Hz, 1 H), 3.52 - 3.62 (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); ^{13}C NMR (100 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.



5

(*S*)-*N*-(3-(Isopropylcarbamoyl)phenyl)-2-methyl-4-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazine-1-carboxamide (5). Prepared from (*S*)-5-methyl-4-(3-methylpiperazin-1-yl)-7H-pyrrolo[2,3-*d*]pyrimidine by an analogous procedure as used to synthesize **1**.

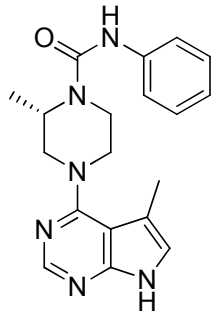
^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.23 (s, 1 H), 7.79 (t, *J*=1.9 Hz, 1 H), 7.51 (ddd, *J*=8.0, 2.2, 1.1 Hz, 1 H), 7.46 (ddd, *J*=8.0, 1.4, 1.3 Hz, 1 H), 7.36 (t, *J*=7.8 Hz, 1 H), 7.04 (d, *J*=1.0 Hz, 1 H), 4.56 (m, *J*=6.4, 3.2, 3.0 Hz, 1 H), 4.13 - 4.24 (m, 2 H), 4.05 (dt, *J*=13.1, 2.5 Hz, 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.



6

(*S*)-N-(3-Methoxyphenyl)-2-methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperazine-1-carboxamide (6). Prepared from (*S*)-5-methyl-4-(3-methylpiperazin-1-yl)-7H-pyrrolo[2,3-*d*]pyrimidine by an analogous procedure as used to synthesize **1**.

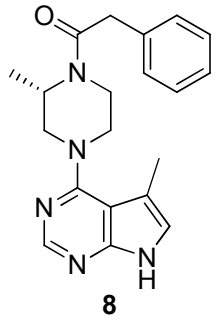
^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.31 (s, 1 H), 7.12 - 7.24 (m, 2 H), 7.01 - 7.09 (m, 1 H), 6.88 - 7.00 (m, 1 H), 6.57 - 6.67 (m, 1 H), 4.53 - 4.66 (m, 1 H), 4.23 - 4.40 (m, 1 H), 4.00 - 4.18 (m, 2 H), 3.77 (s, 3 H), 3.54 - 3.65 (m, 1 H), 3.38 - 3.51 (m, 1 H), 3.06 - 3.23 (m, 1 H), 2.49 (s, 3 H), 1.29 (s, 3 H) MS (ES+) $[M+H]^+$ = 381.



7

(*S*)-2-Methyl-4-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-N-phenylpiperazine-1-carboxamide (7). (*S*)-5-Methyl-4-(3-methylpiperazin-1-yl)-7H-pyrrolo[2,3-*d*]pyrimidine (40 mg) and phenyl isocyanate were combined in MeCN. The reaction was stirred at RT for 1 hour, then diluted with MeOH and filtered. The product was purified by prep HPLC to give compound **7**.

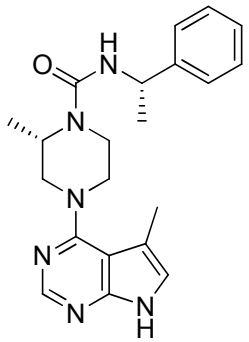
^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.23 (s, 1 H), 7.34 - 7.41 (m, 2 H), 7.23 - 7.32 (m, 2 H), 7.00 - 7.07 (m, 2 H), 4.51 - 4.58 (m, 1 H), 4.13 - 4.20 (m, 1 H), 4.02 (d, *J*=13.4 Hz, 1 H), 3.93 (dt, *J*=12.9, 2.0 Hz, 1 H), 3.51 - 3.66 (m, 1 H), 3.43 (dd, *J*=12.9, 3.8 Hz, 1 H), 3.15 (td, *J*=12.4, 3.5 Hz, 1 H), 2.47 (d, *J*=1.0 Hz, 3 H), 1.30 (d, *J*=6.8 Hz, 3 H); MS (ES+) $[M+H]^+$ = 351.



8

(*S*)-1-(2-Methyl-4-(5-methyl-7H-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazin-1-yl)-2-phenylethanone (8). (*S*)-5-methyl-4-(3-methylpiperazin-1-yl)-7H-pyrrolo[2,3-*d*]pyrimidine (50 mg) was reacted with 2-phenylacetyl chloride (1.1 eq.) and *N*,*N*-diisopropylethylamine (2 eq.) in DCM/DMF. The product was purified by prep HPLC to give **8** (57 mg) as a yellow oil.

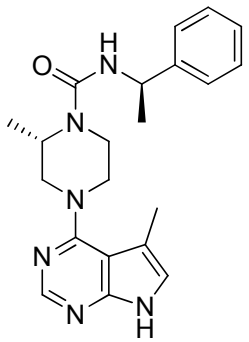
^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm (exhibits rotamers) 8.18 (s, 1 H), 7.19 - 7.35 (m, 5 H), 7.00 - 7.01 (m, 1 H), 4.34 - 4.53 (m, 1 H), 3.14 - 4.21 (m, 7 H), 2.80 - 3.05 (m, 1 H), 2.37 - 2.42 (m, 3 H), 1.13 - 1.23 (m, 3 H); MS (ES+) $[M+H]^+$ = 350.



9

(*S*)-2-Methyl-4-(5-methyl-7H-pyrrolo[2,3-*d*]pyrimidin-4-yl)-*N*-((*S*)-1-phenylethyl)piperazine-1-carboxamide (9). To a solution of (*S*)-α-methylbenzyl isocyanate (53 mg, 0.36 mmol) in THF (3 ml) was added (*S*)-5-methyl-4-(3-methylpiperazin-1-yl)-7H-pyrrolo[2,3-*d*]pyrimidine (83 mg, 0.36 mmol). The reaction was stirred for 1 hour at RT, then concentrated under vacuum. The residue was purified by flash chromatography (12g SiO_2, 0-8% MeOH:DCM) and lyophilized to give **9** (107 mg, 79% yield) as a white powder.

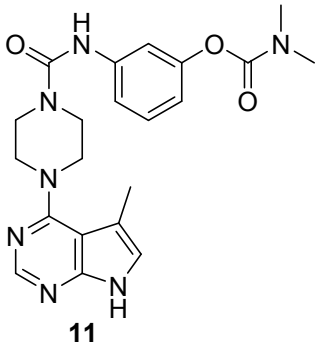
^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.21 (s, 1 H), 7.24 - 7.38 (m, 4 H), 7.15 - 7.24 (m, 1 H), 7.02 (s, 1 H), 6.73 (d, *J*=7.6 Hz, 1 H), 4.92 - 5.01 (m, 1 H), 4.35 - 4.51 (m, 1 H), 4.11 (d, *J*=12.4 Hz, 1 H), 3.92 (d, *J*=13.1 Hz, 1 H), 3.85 (d, *J*=12.9 Hz, 1 H), 3.45 (td, *J*=12.5, 3.3 Hz, 1 H), 3.38 (dd, *J*=12.8, 3.9 Hz, 1 H), 3.08 (td, *J*=12.3, 3.5 Hz, 1 H), 2.44 (s, 3 H), 1.47 (d, *J*=7.1 Hz, 3 H), 1.22 (d, *J*=6.8 Hz, 3 H); MS (ES+) $[M+H]^+$ = 379.



10

(*S*)-2-Methyl-4-(5-methyl-7H-pyrrolo[2,3-*d*]pyrimidin-4-yl)-*N*-((*R*)-1-phenylethyl)piperazine-1-carboxamide (10). Compound **10** (107 mg) was prepared from (*R*)-α-methylbenzyl isocyanate (53 mg, 0.36 mmol) and (*S*)-5-methyl-4-(3-methylpiperazin-1-yl)-7H-pyrrolo[2,3-*d*]pyrimidine (83 mg, 0.36 mmol) by the same procedure as was used to synthesize **9**.

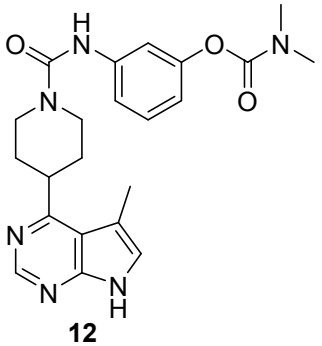
^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.20 (s, 1 H), 7.25 - 7.35 (m, 4 H), 7.14 - 7.24 (m, 1 H), 7.02 (s, 1 H), 6.74 (d, *J*=7.3 Hz, 1 H), 4.92 - 4.99 (m, 1 H), 4.41 (m, 1 H), 4.12 (d, *J*=12.4 Hz, 1 H), 3.81 - 3.94 (m, 2 H), 3.45 (td, *J*=12.4, 3.0 Hz, 1 H), 3.32 - 3.39 (m, 1 H), 3.07 (td, *J*=12.3, 3.2 Hz, 1 H), 2.44 (s, 3 H), 1.47 (d, *J*=7.1 Hz, 3 H), 1.21 (d, *J*=6.6 Hz, 3 H); MS (ES+) $[M+H]^+$ = 379.



11

3-(4-(5-Methyl-7H-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazine-1-carboxamido)phenyl dimethylcarbamate (11). Compound **11** (49 mg) was prepared from 5-methyl-4-(piperazin-1-yl)-7H-pyrrolo[2,3-*d*]pyrimidine by an analogous procedure as used to synthesize **14**.

^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.24 (s, 1 H), 7.23 - 7.31 (m, 3 H), 7.04 (s, *J*=5.9 Hz, 1 H), 6.74 - 6.80 (m, 1 H), 3.72 - 3.79 (m, 4 H), 3.59 - 3.67 (m, 4 H), 3.12 (s, 3 H), 2.99 (s, 3 H), 2.46 (s, 3 H), MS (ES+) $[M+H]^+$ = 424.



12

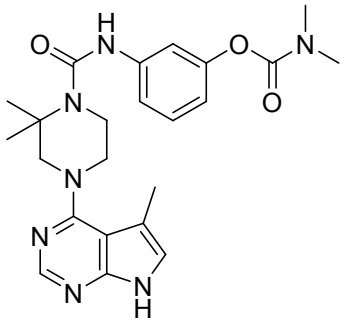
3-(4-(5-Methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidine-1-carboxamido)phenyl dimethylcarbamate (12). *N*-Boc-(1,2,3,6-tetrahydropyridin-4-yl)boronic acid pinacol ester (517 mg, 1.7 mmol), 4-chloro-5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (186 mg, 1.1 mmol), $PdCl_2(PPh_3)_2$ (77 mg, 0.11 mmol), and Na_2CO_3 (175 mg, 1.7 mmol) were combined in 3:1 MeCN:H_2O (15 mL) and heated by microwave to 160 °C for 10 min. The MeCN was removed under vacuum, and the mixture was

extracted with EtOAc. The organic extract was washed with H_2O, dried, and concentrated under vacuum. The residue was purified by flash chromatography (50-100% EtOAc:hexanes) to give *N*-Boc-5-methyl-4-(1,2,3,6-tetrahydropyridin-4-yl)-7H-pyrrolo[2,3-d]pyrimidine (232 mg, 67% yield).

This material was hyrdrogenated over 10% Pd/C, 50% wet (314 mg, 0.15 mmol) under 40 psi H_2 in 9:1 EtOAc:MeOH (10 mL) for 5 hours. The reaction was filtered through celite and concentrated under vacuum to give 187 mg of a white solid. This material was treated with HCl (44 µL, 4 M in dioxane) in MeOH (3 mL) overnight at RT. The reaction was concentrated under vacuum to give 5-methyl-4-(piperidin-4-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (50 mg, 20% yield).

This material (50 mg, 0.23 mmol) was combined with 3-((phenoxycarbonyl)amino)phenyl dimethylcarbamate (69 mg, 0.23 mmol) and triethylamine (0.10 mL, 0.69 mmol) in dioxane (2 mL). The reaction was heated at 55 °C for 3 hours. The reaction was concentrated under vacuum, and the residue was purified by flash chromatography to give **12** (58 mg) as an oil.

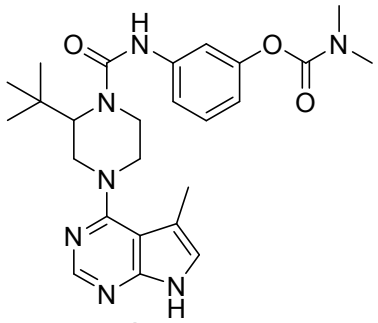
^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.57 (s, 1 H), 7.15 - 7.31 (m, 4 H), 6.76 (dt, *J*=5.2 Hz, 1 H), 4.36 (d, *J*=13.6 Hz, 2 H), 3.67 (tt, *J*=11.7, 3.6 Hz, 1 H), 3.03 - 3.19 (m, 5 H), 2.99 (s, 3 H), 2.52 (s, 3 H), 1.97 - 2.12 (m, 2 H), 1.88 - 1.97 (m, 2 H); MS (ES+) $[M+H]^+$ = 423.



13

3-(2,2-Dimethyl-4-(5-methyl-7H-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazine-1-carboxamido)phenyl dimethylcarbamate (13). Compound **13** (26 mg) was prepared from 4-(3,3-dimethylpiperazin-1-yl)-5-methyl-7H-pyrrolo[2,3-d]pyrimidine (100 mg) by an analogous procedure as used to synthesize **14**.

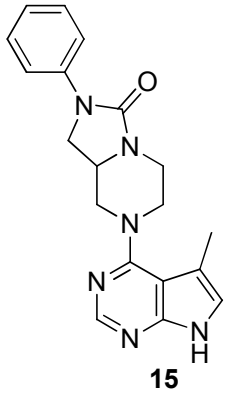
^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.53 (br. s., 1 H), 8.71 (s, 1 H), 8.18 (s, 1 H), 7.34 (s, 1 H), 7.15 - 7.24 (m, 2 H), 7.06 (s, 1 H), 6.62 - 6.69 (m, 1 H), 3.74 (d, *J*=4.5 Hz, 4 H), 3.60 (s, 2 H), 3.03 (s, 3 H), 2.90 (s, 3 H), 2.38 (s, 3 H), 1.43 (s, 6 H); MS (ES+) $[M+H]^+$ = 452.



14

3-(2-(*tert*-Butyl)-4-(5-methyl-7H-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazine-1-carboxamido)phenyl dimethylcarbamate (14). 4-(3-(*tert*-Butyl)piperazin-1-yl)-5-methyl-7H-pyrrolo[2,3-*d*]pyrimidine (150 mg, 0.55 mmol) and 3-((phenoxycarbonyl)amino)phenyl dimethylcarbamate (167 mg, 0.55 mmol) were combined in dioxane and heated at 45 °C for 48 hours and 55 °C for 24 hours. The reaction was concentrated under vacuum, and the residue was purified by flash chromatography (0-10% MeOH:DCM), to give **14** (187 mg, 70% yield) as a white powder.

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 9.96 (br. s., 1 H), 8.33 (s, 1 H), 7.15 - 7.24 (m, 2 H), 7.02 (d, *J*=7.5 Hz, 1 H), 6.89 (s, 1 H), 6.77 (dd, *J*=8.2, 1.4 Hz, 1 H), 6.61 (s, 1 H), 4.23 (d, *J*=6.5 Hz, 2 H), 3.84 - 3.98 (m, 2 H), 3.76 (dd, *J*=13.4, 6.1 Hz, 1 H), 3.41 - 3.60 (m, 2 H), 3.06 (s, 3 H), 2.99 (s, 3 H), 2.39 (s, 3 H), 1.10 (s, 9 H); MS (ES+) $[M+H]^+$ = 480.



15

7-(5-Methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-2-phenylhexahydroimidazo[1,5-a]pyrazin-3(2H)-one (15). 1-*tert*-Butyl 2-methyl piperazine-1,2-dicarboxylate (488 mg, 2.0 mmol), 4-chloro-5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (334 mg, 2.0 mmol), and *N*,*N*-diisopropylethylamine (0.35 mL, 2.0 mmol) were combined in *i*-PrOH (4 mL) in a sealed tube and heated at 110 °C for 24 hours. The reaction was diluted with EtOAc, washed with sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by flash chromatography (10-100%

EtOAc:hexanes) to give 1-*tert*-butyl 2-methyl 4-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazine-1,2-dicarboxylate (345 mg, 46% yield) as a white foam.

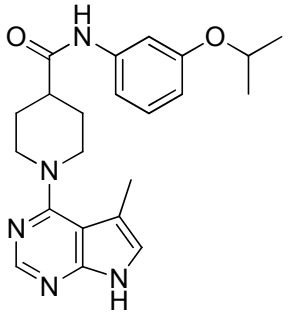
This material was treated with 1 M aq LiOH (2 mL, 2 mmol) in MeOH (1 mL) and THF (2 mL) at 60 °C overnight. The reaction was poured into 1 M aq $NaHSO_4$, saturated with NaCl, and extracted 4x with EtOAc. The organic extracts were dried over Na_2SO_4 and concentrated under vacuum to give 1-(*tert*-butoxycarbonyl)-4-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperazine-2-carboxylic acid (181 mg), which was used in the next step without further purification.

To a solution of the acid from the previous step (181 mg, 0.5 mmol) and HATU (209 mg, 0.55 mmol) in DMF (1 mL) was added aniline (137 µL, 1.5 mmol) followed by *N*,*N*-diisopropylethylamine (0.26 mL,, 1.5 mmol). The reaction was stirred for 1 hour, then diluted with EtOAc, washed with sat. aq. $NaHCO_3$ and brine (with back extraction), dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by flash chromatography (40 g SiO_2, 0-8% MeOH:DCM) to give *tert*-butyl 4-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)-2-(phenylcarbamoyl)piperazine-1-carboxylate (191 mg, 88% yield) as a white solid.

This material was treated with 1:1 TFA:DCM (4.4 mL) for 30 min. The reaction was concentrated under vacuum, diluted with sat. aq. Na_2CO_3, saturated with NaCl, and extracted twice with EtOAc. The combined extracts were dried over $MgSO_4$ and concentrated under vacuum to give 4-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)-N-phenylpiperazine-2-carboxamide as a white solid in quantitative yield.

To a solution of the amide from the previous step (67 mg, 0.2 mmol) in anhydrous THF (1 mL) was added LAH (0.6 mL of a 1.0 M solution in THF, 0.6 mmol). The reaction was heated at 60 °C for 5 hours, then cooled to RT, and carefully quenched with 1 M aq. Rochelle's salt. The mixture was stirred overnight, then diluted with sat. aq. $NaCO_3$, saturated with NaCl, and extracted twice with EtOAc. The combined extracts were dried over $MgSO_4$ and concentrated under vacuum. This crude residue was combined with CDI (32 mg, 0.2 mmol) in THF and stirred at RT for 3 days. The reaction was diluted with EtOAc, washed with sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by flash chromatography (12 g SiO_2, 0-8% MeOH:DCM) to give 13 mg of a white solid, which was triturated with MeOH to give clean compound **15** (8 mg).

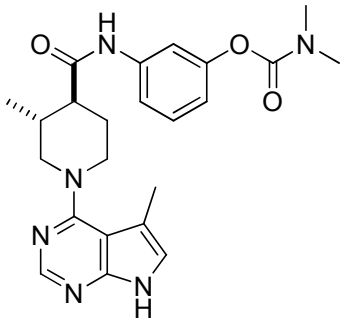
^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.25 (s, 1 H), 7.57 (dd, *J*=8.7, 1.1 Hz, 2 H), 7.34 (dd, *J*=8.7, 7.5 Hz, 2 H), 7.04 - 7.09 (m, 2 H), 4.18 - 4.24 (m, 1 H), 4.02 - 4.16 (m, 3 H), 3.99 (dd, *J*=13.3, 1.9 Hz, 1 H), 3.66 (dd, *J*=8.8, 4.3 Hz, 1 H), 3.06 - 3.15 (m, 1 H), 3.02 (dd, *J*=12.5, 10.5 Hz, 1 H), 2.47 (d, *J*=1.3 Hz, 3 H); MS (ES+) $[M+H]^+$ = 349.



16

***N*-(3-Isopropoxyphenyl)-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamide (16).** To a solution of 1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxylic acid (25 mg, 0.1 mmol) in DMF (1 mL) was added 3-isopropoxyaniline (16 µL, 0.11 mmol), *N*,*N*-diisopropylethylamine (35 µL, 0.2 mmol), and BOP (53 mg, 0.12 mmol). The reaction was stirred overnight and then concentrated under vacuum. The residue was purified by prep HPLC to give **16** (15 mg) as a white solid.

^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.20 (s, 1 H), 7.29 (t, *J*=2.3 Hz, 1 H), 7.18 (t, *J*=7.8 Hz, 1 H), 7.03 - 7.08 (m, 1 H), 7.01 (d, *J*=1.3 Hz, 1 H), 6.64 (ddd, *J*=8.3, 2.5, 0.9 Hz, 1 H), 4.52 - 4.62 (m, 1 H), 4.15 (d, *J*=13.4 Hz, 2 H), 2.99 - 3.13 (m, 2 H), 2.56 - 2.72 (m, 1 H), 2.45 (d, *J*=1.0 Hz, 3 H), 1.96 - 2.04 (m, 4 H), 1.31 (d, *J*=6.1 Hz, 6 H); MS (ES+) $[M+H]^+$ = 394.



17

3-(*trans*-3-Methyl-1-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (17).

To a solution of commercially available cis-N-Boc-3-methylpiperidine-4-carboxylic acid (243 mg, 1 mmol) and DMAP (12 mg, 0.1 mmol) in anhydrous MeOH at 0 °C was added EDC (232 mg, 1.5 mmol). The reaction was allowed to warm to RT and stirred overnight, then concentrated under vacuum, diluted with Et_2O, washed twice with 1 M aq $NaHSO_4$, once with H_2O, sat. aq. $NaHCO_3$, and

brine, and dried over $MgSO_4$. The combined organic layers were concentrated under vacuum to give cis methyl-*N*-Boc-3-methylpiperidine-4-carboxylate (201 mg, 78% yield) as a clear oil.

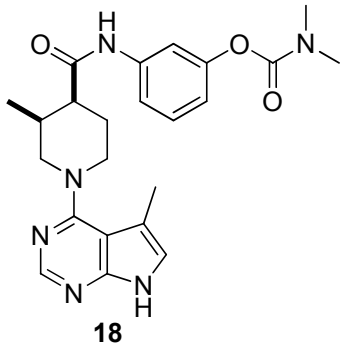
This material (100 mg, 0.39 mmol) was treated with NaOMe (180 µL of 25 wt.% in MeOH, 0.78 mmol) in MeOH (0.4 mL) at 70 °C overnight to epimerize the stereocenter alpha to the ester. Water (50 µL) was added, and heating was continued for 2 hours. The reaction was diluted with H_2O and 1 M aq NaOH and washed with Et_2O. The aqueous layer was acidified to pH=1 with 1 M aq $NaHSO_4$ and extracted twice with Et_2O. The combined organic extracts were washed with brine, dried over Na_2SO_4, and concentrated under vacuum to give *N*-Boc-3-methylpiperidine-4-carboxylic acid (90 mg, 96% yield) as a 2:1 mixture of trans:cis stereoisomers, as determined by NMR.

N-Boc-3-methylpiperidine-4-carboxylic acid (2:1 trans:cis, 169 mg, 0.70 mmol), HATU (291 mg, 0.77 mmol), and *N*,*N*-diisopropylethylamine (0.36 mL, 2.1 mmol) were combined in DMF and stirred 10 minutes. 3-Aminophenyl dimethylcarbamate (150 mg, 0.83 mmol) was added, and the reaction was stirred for 3 hours, then diluted with Et_2O, washed with sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by flash chromatography (40 g SiO_2, 0-50% EtOAc:DCM) to give pure (*trans)*-N-Boc-3-(3-methylpiperidine-4-carboxamido)phenyl dimethylcarbamate (99 mg), plus some cis/trans mixed fractions (114 mg).

The pure trans material (99 mg, 0.24 mmol) was treated with 1:1 TFA:DCM (2.4 mL) for 30 min. The reaction was concentrated, then diluted with sat. aq. $NaHCO_3$ and extracted 5x with EtOAc. The combined extracts were dried over $MgSO_4$ and concentrated under vacuum to give crude 3-(3-methylpiperidine-4-carboxamido)phenyl dimethylcarbamate, which was carried to the next step without further purification.

This crude material was combined with 4-chloro-5-methyl-7*H*-pyrrolo[2,3-*d*] pyrimidine (40 mg, 0.24 mmol) and *N*,*N*-diisopropylethylamine (42 µL, 0.24 mmol) in *i*-PrOH (0.5 mL) and heated at 90 °C overnight. The reaction was diluted with EtOAc, washed with sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by flash chromatography (0-8% MeOH:DCM) and lyophilized to give **17** (61 mg, 67% yield) as a white solid.

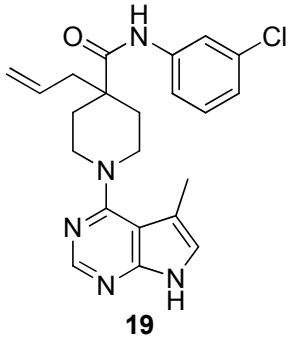
^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.20 (s, 1 H), 7.49 - 7.58 (m, 1 H), 7.36 - 7.42 (m, 1 H), 7.29 - 7.36 (m, 1 H), 7.01 (s, 1 H), 6.85 (d, *J*=7.5 Hz, 1 H), 4.16 (d, *J*=13.6 Hz, 1 H), 4.07 (d, *J*=11.6 Hz, 1 H), 3.12 (s, 3 H), 2.94 - 3.05 (m, 4 H), 2.67 (t, *J*=11.9 Hz, 1 H), 2.43 (s, 3 H), 2.13 - 2.32 (m, 2 H), 1.93 - 2.06 (m, 2 H), 1.00 (d, *J*=6.1 Hz, 3 H); MS (ES+) $[M+H]^+$ = 437.



18

3-(*cis*-3-Methyl-1-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (18). Compound **18** was prepared from commercially available cis-*N*-Boc-3-methylpiperidine-4-carboxylic acid by an analogous procedure as used to synthesize **17**.

^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.18 (s, 1 H), 7.51 (t, *J*=3.5 Hz, 1 H), 7.33 - 7.38 (m, 1 H), 7.27 - 7.33 (m, 1 H), 6.99 (s, 1 H), 6.83 (ddd, *J*=8.0, 2.3, 1.1 Hz, 1 H), 4.06 (dt, *J*=12.9, 4.4 Hz, 1 H), 3.86 (dd, *J*=12.4, 5.1 Hz, 1 H), 3.44 (dd, *J*=12.6, 3.3 Hz, 1 H), 3.26 (ddd, *J*=13.0, 9.7, 3.0 Hz, 1 H), 3.11 (s, 3 H), 2.99 (s, 3 H), 2.81 (dt, *J*=9.2, 4.5 Hz, 1 H), 2.44 (d, *J*=1.3 Hz, 3 H), 2.33 - 2.41 (m, 1 H), 2.19 - 2.33 (m, 1 H), 1.75 - 1.85 (m, 1 H), 1.02 (d, *J*=6.8 Hz, 3 H); MS (ES+) $[M+H]^+$ = 437.



19

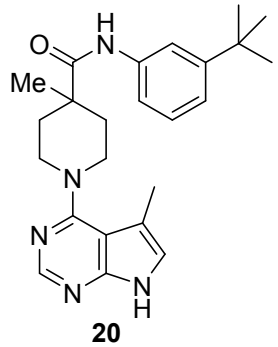
4-Allyl-*N*-(3-chlorophenyl)-1-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidine-4 carboxamide (19). Under an atmosphere of N_2, 4-allyl-1-(*tert*-butoxycarbonyl)piperidine-4-carboxylic acid (0.158 g, 0.59 mmol), pyridine (0.189 mL, 2.34 mmol), and DMF (3 drops) were dissolved in CH_2Cl_2 (10 mL) and cooled in an ice bath. To the cooled solution, oxalyl chloride (0.056 mL, 0.65 mmol) was added dropwise. The reaction was stirred for 40 min, after which time 3-chloroaniline (0.061 mL, 0.59 mmol) was added. The reaction was removed from the ice bath and stirred at room temperature overnight. The crude reaction was washed with 1N aq. HCl and brine, dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by silica gel chromatography EtOAc/hexanes

gradient) to give *tert*-butyl 4-allyl-4-(3-chlorophenylcarbamoyl)piperidine-1-carboxylate in (113 mg, 51% yield) MS (ES+) $[M+H]^+ = 379$.

To a solution of *tert*-butyl 4-allyl-4-(3-chlorophenylcarbamoyl)piperidine-1-carboxylate (0.172 g, 0.453 mmol) in MeOH (10 mL) was added 4M HCl in dioxane (0.340 mL, 1.4 mmol). The reaction was heated at 50 °C for 30 min, and then concentrated under vacuum, redissolved in MeOH and reconcentrated twice to give 4-allyl-*N*-(3-chlorophenyl)piperidine-4-carboxamide as the hydrochloride salt. MS (ES+) $[M+H]^+ = 279$.

This amine (0.142 g, 0.453 mmol), 4-chloro-5-methyl-7*H*-pyrrolo[2,3-*d*] pyrimidine (0.075 g, 0.45 mmol), *N*,*N*-diisopropylethylamine (0.236 mL, 1.35 mmol), and isopropanol (5 mL) were combined and heated at 120 °C overnight in a pressure vessel. The crude reaction was dissolved in CH_2Cl_2, washed with H_2O and brine, dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by silica gel chromatography (MeOH:DCM gradient) to give **19** (113 mg, 61% yield).

^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.19 (s, 1H), 7.73 (s, 1H), 7.48 (d, J=8 Hz, 1H), 7.32 (t, J=8, 16 Hz, 1H), 7.14 (d, J=8 Hz, 1H), 7.00 (s, 1H), 5.78-5.88 (m, 1H), 5.11-5.16 (m, 2H), 3.91 (d, J=13.6 Hz, 2H), 3.24-3.30 (m, 2H), 2.50 (d, J=7.6 Hz, 2H), 2.45 (s, 3H), 2.41 (d, J=13.6 Hz, 2H), 1.80-1.86 (m, 2H); MS (ES+) $[M+H]^+ = 410$.



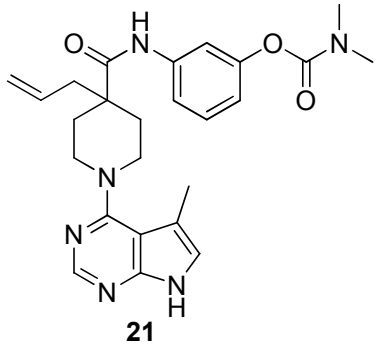
20

***N*-(3-*tert*-Butylphenyl)-4-methyl-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamide (20).** To a solution of 1-(*tert*-butoxycarbonyl)-4-methylpiperidine-4-carboxylic acid (0.30 g, 1.2 mmol), DMF (20 drops), and pyridine (0.38 g, 4.8 mmol) in dichloroethane (10 mL) was added oxalyl chloride (0.15 g, 1.2 mmol). The mixture was stirred for 40 minutes, and 3-*tert*-butylaniline was added. The mixture was stirred for 1 hour, diluted with DCM, washed twice each with sat. aq. $NaHCO_3$ and 1.0 N aq. HCl, dried over $MgSO_4$, and concentrated. The residue was purified by silica gel chromatography (0-2% MeOH:DCM) to give 4-(3-*tert*-butylphenylcarbamoyl)-4-methylpiperidine-1-carboxylate (233 mg, 61 % yield). MS (ES+) $[M+H]^+ = 375$.

4-(3-*tert*-Butylphenylcarbamoyl)-4-methylpiperidine-1-carboxylate (0.40 g, 1.1 mmol) was treated with HCl (4.0 N in dioxane, 1.3 mL, 5.3 mmol) in methanol (5.0 mL) at 50 °C for 30 minutes. The mixture was concentrated to give *N*-(3-*tert*-butylphenyl)-4-methylpiperidine-4-carboxamide as the hydrochloride salt in quantitative yield. MS (ES+) $[M+H]^+ = 275$.

This amine (0.33 g, 1.1 mmol), 4-chloro-5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (0.18 g, 1.07 mmol), *N*,*N*-diisopropylethylamine (0.69 g. 5.34 mmol), and isopropanol (7 mL) were combined in a pressure rated sealed tube and heated at 100 °C for 18 hours. The mixture was concentrated and taken up in dichloromethane from which a precipitate (0.18 g) was collected. The filtrate was chromatographed on silica gel (0-2% MeOH:CH_2Cl_2), and the purified material (0.15 g) was combined with the precipitate (0.18 g) and dissolved in methanol (15 mL) at 60 °C. Water was added (5.0 mL), and the mixture was cooled. The resultant precipitate was filtered, washed with water, and dried to give **20** in (36 mg, 50% yield).

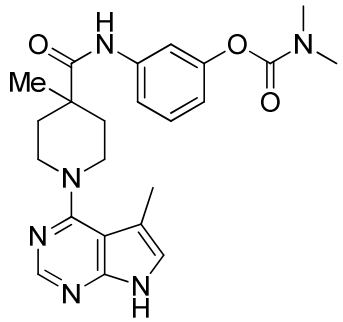
^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.51 (s, 1H), 9.31 (s, 1H), 8.19 (s, 1H), 7.62 (s, 1H), 7.59 (d, J=8.0 Hz, 1H), 7.22 (t, J=8Hz and 16Hz, 1H), 7.08 (d, J= 8.0 Hz, 1H), 3.63-3.68 (m, 2H), 3.28-3.33 (m, 2H), 2.36 (s, 3H), 2.25-2.29 (m, 2H), 1.63-1.68 (m, 2H), 1.31 (s, 3H), 1.27 (S, 9H); MS (ES+) $[M+H]^+ = 406$.



21

3-(4-Allyl-1-(5-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (21). Compound **21** (15 mg) was prepared from 3-aminophenyl dimethylcarbamate by an analogous procedure as used to synthesize **19**.

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 9.37 (br. s., 1 H), 8.38 (br. s., 1 H), 7.43 - 7.49 (m, 1 H), 7.40 (s, 1 H), 7.28 - 7.35 (m, 2 H), 6.88 - 6.93 (m, 2 H), 5.81 (m, *J*=16.8, 10.2 Hz, 1 H), 5.12 - 5.20 (m, 2 H), 3.86 (dt, *J*=13.5, 4.1 Hz, 2 H), 3.45 (ddd, *J*=13.2, 10.3, 2.8 Hz, 2 H), 3.10 (s, 3 H), 3.02 (s, 3 H), 2.44 (d, *J*=7.6 Hz, 2 H), 2.41 (d, *J*=0.8 Hz, 3 H), 2.25 (d, *J*=14.7 Hz, 2 H), 1.83 (ddd, *J*=13.6, 9.9, 3.5 Hz, 2 H); MS (ES+) $[M+H]^+ = 463$.



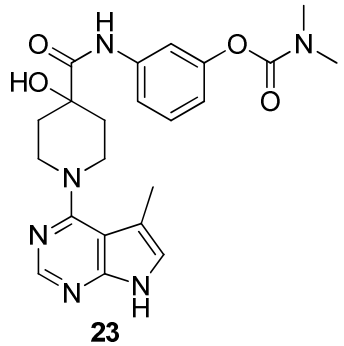
22

3-(4-Methyl-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (22). 1-(*tert*-Butoxycarbonyl)-4-methylpiperidine-4-carboxylic acid (67 mg, 0.28 mmol), EDC (64 mg, 0.33 mmol), HOBT (45 mg, 0.33 mmol) and dichloroethane (2.0 mL) were combined in a pressure rated sealed tube and stirred for 45 min. 3-Aminophenyl dimethylcarbamate (50 mg, 0.28 mmol) was added, and the mixture was heated at 85 °C for 18 hours. The reaction was cooled to room temperature, concentrated, and the crude reaction mixture was purified by silica gel chromatography (0-2% MeOH:CH_2Cl_2) to give *tert*-butyl 4-(3-(dimethylcarbamoyloxy)phenylcarbamoyl)-4-methylpiperidine-1-carboxylate (56 mg, 50% yield). MS (ES+) $[M+H]^+$ = 406.

This material (56 mg, 0.14 mmol) was treated with HCl (4.0 N in dioxane, 0.17 mL, 0.69 mmol) in methanol (2.0 mL) at 40 °C for 3 hours. The mixture was concentrated to give 3-(4-methylpiperidine-4-carboxamido)phenyl dimethylcarbamate as the hydrochloride salt in quantitative yield. MS (ES+) $[M+H]^+$ = 306.

This amine (47 mg, 0.14 mmol), 4-chloro-5-methyl-7H-pyrrolo[2,3-d]pyrimidine (23 mg, 0.14 mmol), *N*,*N*-diisopropylethylamine (54 mg, 0.41 mmol), and isopropanol (1.0 mL) were combined in a pressure rated sealed tube and heated at 100 °C for 18 hours. The reaction mixture was cooled to room temperature, concentrated, and the residue was purified by preparative HPLC to give **22** (32 mg, 53% yield).

^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.18 (s, 1H), 7.50 (s, 1H), 7.42-7.44 (d, J=8 Hz, 1H), 7.34 (t, J=8 and 16 Hz, 1H), 7.0 (s, 1H), 6.87-6.89 (m, 1H), 3.79-3.85 (m, 2H), 3.37-3.44 (m, 2H), 3.14 (s, 1H), 3.01 (s, 3H), 2.44 (s, 3H), 2.36-2.40 (m, 2H), 1.75-1.81 (m, 2H), 1.40 (s, 3H); MS (ES+) $[M+H]^+$ = 437.



23

3-(4-Hydroxy-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (23). To a solution of methyl 4-hydroxypiperidine-4-carboxylate (792 mg, 4.98 mmol) and triethylamine (1.04 mL, 7.47 mmol) in dichloromethane (25 mL) was added di-*tert*-butyl dicarbonate (1.26 mL, 5.48 mmol). Reaction progress was monitored by TLC analysis/ELSD. Upon completion, volatiles were removed under vacuum. Silica gel chromatography (0-10% MeOH:CH_2Cl_2) provided 1-*tert*-butyl 4-methyl 4-hydroxypiperidine-1,4-dicarboxylate as a clear oil (1.25 g, 99% yield). MS (ES+) $[M+H]^+$ = 250.

To a solution of this material (535 mg, 2.1 mmol) in DMF (8.2 mL) was carefully added sodium hydride (60% dispersion in mineral oil, 124 mg, 3.1 mmol). The mixture was stirred for 30 minutes at room temperature. Benzyl bromide (0.29 mL, 2.5 mmol) was added, and the reaction was stirred for another 30 minutes. Upon completion as monitored by TLC analysis, the reaction was quenched with 50 mL of 1:1 saturated aq. NH_4Cl:water. The aqueous layer was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with brine, dried with $MgSO_4$, and concentrated under vacuum. Silica gel chromatography (5-10% EtOAc:hexanes) provided 1-*tert*-butyl 4-methyl 4-(benzyloxy)piperidine-1,4-dicarboxylate as a clear oil (359 mg, 54% yield). MS (ES+) $[M+H]^+$=340.

To this material (350 mg, 1.0 mmol) in 1:2 methanol/water (6 mL) was added lithium hydroxide monohydrate (200 mg). The mixture was heated to 60 °C for 30 min, and then cooled to room temperature and poured onto saturated aqueous $NaHSO_4$ (25 mL). The aqueous layer was extracted EtOAc (3 x 25 mL). The combined organic layers were washed with brine, dried with $MgSO_4$, and concentrated under vacuum to give the 1-(*tert*-butoxycarbonyl)-4-(benzyloxy)-piperidine-4-carboxylic acid as a viscous oil, which was carried on to the next step without further purification.

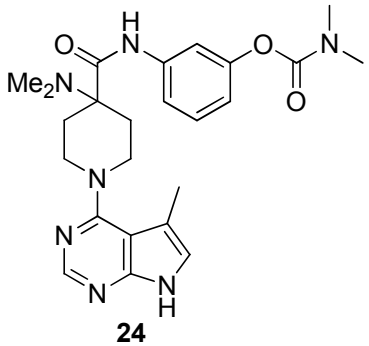
To a solution of this acid (335 mg, 1.0 mmol), pyridine (0.43 mL, 5.4 mmol), and DMF (catalytic, 3 drops) in CH_2Cl_2 (6.8 mL) was added dropwise oxalyl chloride (0.13 mL, 1.5 mmol, caution: exothermic). After gas evolution subsided, the reacton was stirred at ambient temperature for 30 minutes. 3-Aminophenyl dimethylcarbamate (244 mg, 1.4 mmol) was added in one portion to this solution, which was stirred for another 30 minutes. The reaction was quenched with saturated aqueous

NH_4Cl, and the aqueous layer was extracted with eth EtOAc (3 x 50 mL). The combined organic layers were washed with brine, dried with $MgSO_4$, filtered, and concentrated under vacuum. Silica gel chromatography (40% EtOAc:hexanes) afforded *tert*-butyl 4-(benzyloxy)-4-(3-(dimethylcarbamoyloxy)phenyl-carbamoyl)piperidine-1-carboxylate as a white solid (406 mg, 82% yield). MS (ES+) $[M+NH_4]^+ = 515$.

To a solution of this material (400 mg, 0.804 mmol) in methanol (4 mL) was added HCl (4M solution in dioxane, 0.8 mL). The reaction was heated at 50 °C for 1 hour, and then concentrated under vacuum to provide 3-(4-(benzyloxy)piperidine-4-carboxamido)phenyl dimethyl-carbamate as the hydrochloride salt. MS (ES+) $[M+H]^+ = 398$.

To a solution of this amine in isopropanol (1.6 mL) was added 4-chloro-5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (135 mg, 0.80 mmol). The reaction mixture was heated to 110 °C in a sealed high-pressure vial for 17 hours, during which time the mixture became homogenous. The reaction was concentrated under vacuum, and half of the material was taken up in methanol (2 mL), and a catalytic amount of Pearlman's catalyst was added. The system was hydrogenated at 60 psi for 24 hours, after which a small amount of product was observed. Trifluoroacetic acid (0.1 mL) was added, and the hydrogenation was continued at 60 psi H_2 for 3 days, after which the reaction was approximately one third of the way completed. The reaction was filtered, and the product was isolated by prep HPLC to afford **23** (9.6 mg) as a white solid.

^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.20 (s, 1H), 7.55 (t, *J*=2.02 Hz, 1H), 7.44 (ddd, *J*=8.08, 2.02, 1.01 Hz, 1H), 7.33 (t, *J*=8.08 Hz, 1H), 6.99 (app. d., *J*=1.01 Hz, 1H), 6.87 (ddd, *J*=8.08, 2.27, 0.76 Hz, 1H), 3.95-4.03 (m, 2H), 3.73 (s, 3H), 3.41 (dt, *J*=12.63, 2.27 Hz, 2H), 3.13-3.26 (m, 2H), 3.12 (s, 3H), 2.99 (s, 3H), 2.44 (d, *J*=0.76 Hz, 3H), 2.36 (dt, *J*=13.39, 4.29 Hz, 2H), 1.75-1.83 (m, 2H). MS (ES+) $[M+H]^+ = 439$.



24

3-(4-(Dimethylamino)-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (24). *N*-Boc-amino-(4-*N*-Boc-piperidinyl) carboxylic acid (0.114 g, 0.33 mmol), HATU (0.151 g, 0.40 mmol) and *N*,*N*-diisopropylethylamine (128 mg, 1.0 mmol) were combined in isopropyl acetate (2.3 mL) and stirred at room temperature for 15 minutes. 3-aminophenyl dimethylcarbamate (0.072 g, 0.40 mmol) was added, and the mixture heated at 80 °C for 1.5 hours. The reaction mixture was allowed to cool, diluted with EtOAc, washed with 2N Na_2CO_3, 1N HCl, and brine, and then dried over $MgSO_4$ and concentrated. Purification by silica gel chromatography (60% EtOAc/hexanes) gave *tert*-butyl 4-(tert-butoxycarbonylamino)-4-(3-(dimethylcarbamoyloxy)phenylcarbamoyl)piperidine-1-carboxylate (0.075 g, 45% yield) as a white solid. MS (ES+) $[M+NH_4]^+ = 524$.

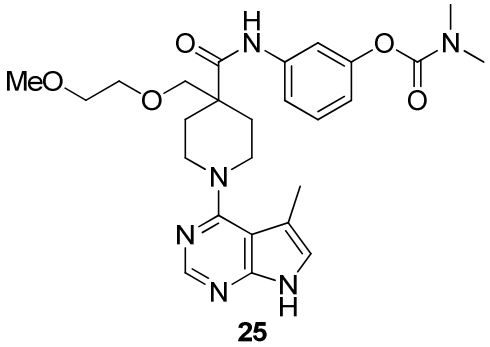
To a solution of this material (0.141 g, 0.28 mmol) in MeOH (2 mL) was added HCl (4N in dioxane, 0.21 mL, 0.84 mmol). The mixture was stirred at room temperature for 14 hours. Further eqivalents of HCl/dioxane were added at this time, and the mixture stirred for a further 24 hours at room temperature. The solvent was removed under vacuum to afford 3-(4-aminopiperidine-4-carboxamido)phenyl dimethylcarbamate (0.084 g, 98% yield), which was carried on crude. MS (ES+) $[M+H]^+ = 307$.

This amine (0.109 g, 0.36mmol), 4-chloro-5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (0.060g, 0.36mmol), and *N*,*N*-diisopropylethylamine (0.277 g, 2.14 mmol) were combined in isopropanol (5 mL) and heated in a sealed tube at 120 °C for 18 hours. The mixture was concentrated, and the residue was purification by silica gel chromatography (0-10% MeOH/CH_2Cl_2) to afford 3-(4-amino-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (0.085g, 55% yield) as a white solid.

^{1}H NMR (400 MHz, CD_3OD) δ ppm 8.25 (s, 1H), 7.61 (m, 1H), 7.47 (m, 1H), 7.38 (m, 1H), 7.05 (d, *J* = 1.0Hz, 1H), 6.91 (m, 1H), 4.00 (m, 2H), 2.48 (m, 2H), 3.18 (s, 3H), 3.05 (s, 3H), 2.51 (s, 3H), 2.45 (m, 2H), 1.72 (m, 2H); MS (ES+) $[M+H]^+ = 438$.

This amine (0.028 g, 0.064 mmol) was dissolved in MeOH (1 mL). Formaldehyde (37 wt% solution in H_2O, 0.15 mmol, 12 µl) was added followed by AcOH (0.26 mmol, 15 µl) and $NaBH_3CN$ (0.008 g, 0.13 mmol). The mixture was stirred at room temperature for 2 hours before concentrating under vacuum. Purification by prep HPLC afforded **24** (0.017 g, 57% yield) as a white solid.

^{1}H NMR (400 MHz, METHANOL-*d*$_4$) δ ppm 8.21 (s, 1H), 7.56 (m, 1H), 7.46 (m, 1H), 7.37 (m, 1H), 7.03 (d, *J* = 1.0Hz, 1H), 6.92 (m, 1H), 4.08 (m, 2H), 3.47 (m, 2H), 3.17 (s, 3H), 3.04 (s, 3H), 2.48 (s, 3H), 2.40 (s, 6H), 2.26 (m, 2H), 2.09 (m, 2H); MS (ES+) $[M+H]^+$ = 466.



25

3-(4-((2-Methoxyethoxy)methyl)-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (25). A. To a solution of 1-*tert*-butyl 4-ethyl piperidine-1,4-dicarboxylate (2.0 g, 7.8 mmol) in THF (70 mL) at -78 °C was added dropwise LDA over 8 minutes, maintaining the temperature below -75 °C. The mixture was stirred at -78 °C for 1 hour. 1-Chloromethoxy-2-methoxy-ethane (0.97 g. 7.8 mmol) was added dropwise while maintaining the temperature below -75 °C. The mixture was stirred at -78 °C for 3 hours, and then allowed to warm slowly to room temperature overnight. The mixture was quenched with sat. aq. NH_4Cl and extracted 3x with EtOAc. The organics were combined, washed with brine, dried over $MgSO_4$, and concentrated. Silica gel chromatography (10-20% EtOAc:hexanes) gave 1-*tert*-butyl 4-ethyl 4-((2-methoxyethoxy)methyl)piperidine-1,4-dicarboxylate in 67% yield. MS (ES+) $[M+H]^+$ = 346.

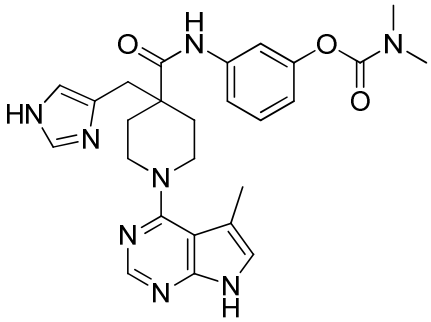
This material (1.78 g, 5.15 mmol) was treated with LiOH (0.62 g. 26 mmol) in ethanol (12 mL) and water (24 mL) at 80 °C for 18 hours. The mixture was cooled, diluted with dichloromethane, and washed with sat. aq. $NaHSO_4$. The aqueous phase was back extracted with dichloromethane (3x), and the organics were combined, washed with brine, dried over $MgSO_4$, and concentrated to give 1-(*tert*-butoxycarbonyl)-4-(2-methoxyethoxy)piperidine-4-carboxylic acid in quantitative yield. MS (ES+) $[M+H]^+$ = 318.

To a solution of this acid (1.6 g, 5.1 mmol) in dichloroethane was added pyridine (1.6 g, 20 mmol) and DMF (0.05 mL), followed by oxalyl chloride (0.65 g, 5.1 mmol). The mixture was stirred for 30 minutes, and then 3-aminophenyl dimethylcarbamate (0.91 g, 5.04 mmol) was added. The mixture was stirred for 18 hours, then diluted with dichloromethane, washed twice with 1N aq. HCl and once with brine, dried over $MgSO_4$, and concentrated. Silica gel chromatography (0-5% MeOH:CH_2Cl_2) gave *tert*-butyl 4-(3-(dimethylcarbamoyloxy)phenylcarbamoyl)-4-(2-methoxyethoxy)piperidine-1-carboxylate in 88% yield. MS (ES+) $[M+H]^+ = 480$.

This material (2.1 g, 4.4 mmol) was treated with HCl (4.0 N in dioxane, 5.5 mL, 22 mmol) in methanol (20 mL) at 50 °C for 30 minutes. The mixture was concentrated to give 3-(4-(2-methoxyethoxy)piperidine-4-carboxamido)phenyl dimethylcarbamate as the hydrochloride salt in quantitative yield. MS (ES+) $[M+H]^+ = 380$.

This amine (1.82 g, 4.38 mmol), 4-chloro-5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (0.73 g, 4.4 mmol), *N*,*N*-diisopropylethylamine (2.8 g. 22 mmol), and isopropanol (50 mL) were combined in a pressure rated sealed tube and heated at 115 °C for 18 hours. The mixture was concentrated and chromatographed (SiO_2, 0-5% MeOH:DCM) to give **25** (1.4 g, 61% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.48 (s, 1H), 9.43 (s, 1H), 8.18 (s, 1H), 7.52 (s, 1H), 7.44 (d, J=8 Hz, 1H), 7.30 (t, J=8 and 16 Hz, 1H), 7.05 (s, 1H), 6.81 (d, J=8 Hz, 1H), 3.68-3.76 (m, 2H), 3.67 (s, 2H), 3.55-3.58 (m, 2H), 3.44-3.47 (m, 2H), 3.24-3.30 (m, 2H), 3.23 (s, 3H), 3.04 (s, 3H), 2.91 (s, 3H), 2.35 (s, 3H), 2.26 (d, J=16 Hz, 2H), 1.69-1.76 (m, 2H); MS (ES+) $[M+H]^+ = 511$.



26

3-(4-((1*H*-Imidazol-4-yl)methyl)-1-(5-methyl-7*H*-pyrrolo [2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (26). To a solution of 1-*tert*-butyl 4-ethyl piperidine-1,4-dicarboxylate (360 mg, 1.39 mmol) in anhydrous THF (10 mL) at -78 °C was added LDA (1.7 M in THF, 1.07 mL, 1.8 mmol) dropwise over 10 min. The reaction mixture was stirred at -78 °C for 1 hour, and 4-(chloromethyl)-1-trityl-1*H*-imidazole (500 mg, 13.9 mmol) in THF (5 mL) was added slowly.

The reaction mixture was allowed to warm to room temperature over 1 hour, quenched with sat. aq. NH_4Cl (10 mL), and extracted with EtOAc (3x20 mL). The combined organic extracts were washed with brine, dried over Na_2SO_4, filtered, and concentrated under vacuum. Purification by silica gel chromatography (10-50% EtOAc:hexanes) afforded 1-*tert*-butyl 4-ethyl 4-((1-trityl-1*H*-imidazol-4-yl) methyl) piperidine-1,4-dicarboxylate (403 mg, 51% yield). MS (ES+) $[M+H]^+ = 580$.

To a solution of this material (330 mg, 0.57 mmol) in 1:1:2 THF:MeOH:H_2O (10 mL) was added LiOH (136 mg, 5.7 mmol). The mixture was refluxed until LCMS showed complete conversion to product, then concentrated under vacuum, dissolved in water (3 mL), neutralized to pH 5 – 6 with aq. $NaHSO_4$, and extracted with EtOAc (3x10 mL). The combined organic phases were washed with brine, dried over Na_2SO_4, and concentrated under vacuum to give 1-(*tert*-butoxycarbonyl)-4-((1-trityl-1*H*-imidazol-4-yl) methyl)piperidine-4-carboxylic acid (273 mg, 86% yield). MS (ES+) $[M+H]^+ = 552$.

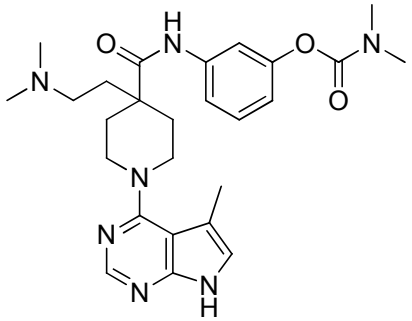
To a solution of this acid (270 mg, 0.6 mmol) in isopropyl acetate (5 mL) were added HATU (273 mg, 0.73 mmol) and *N*,*N*-diisopropylethylamine (205 µL, 1.2 mmol). The mixture was stirred for 5 min, and 3-aminophenyl dimethylcarbamate (108 mg, 0.6 mmol) was added. The resulting mixture was heated at 90 °C for 2 hours. The reaction mixture was diluted with EtOAc (10 mL), washed with aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated under vacuum. The residue was purified by silica gel chromatography (10-70% EtOAc:hexanes) to afford *tert*-butyl 4-(3-(dimethylcarbamoyloxy)phenylcarbamoyl)-4-((1-trityl-1*H*-imidazol-4-yl)methyl)piperidine-1-carboxylate (185 mg, 43% yield). MS (ES+) $[M+H]^+ = 714$.

To a solution of this material (180 mg) in MeOH (5 mL) was added HCl (1N in dioxane, 1 mL). The reaction was heated at 60 °C for 1 hour, and then concentrated under vacuum. The residue was dissolved in EtOAc (5 mL), washed with aq. $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated under vacuum to produce 3-(4-((1*H*-imidazol-4-yl)methyl)piperidine-4-carboxamido) phenyl dimethylcarbamate (84 mg, 93% yield). MS (ES+) $[M+H]^+ = 372$.

This amine (10 mg, 0.027 mmol), 4-chloro-5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (5 mg, 0.03 mmol), and *N*,*N*-diisopropylethylamine (23 µL, 0.135 mmol) were combined in isopropanol (0.5 mL). The resulting mixture was heated at 100 °C in a sealed pressure tube for 24 hours. The reaction mixture was cooled and concentrated under vacuum. The residue was purified by prep HPLC (10-95 % MeOH:H_2O with 0.1% HCOOH), then treated with AcOH in H_2O and lyophilized to give **26** (5.7 mg, 42% yield) as the acetate salt.

^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.17 (s, 1 H), 7.61 (s, 1 H), 7.42 - 7.45 (m, 1 H), 7.31 - 7.39 (m, 2 H), 6.99 - 7.01 (m, 1 H), 6.87 - 6.91 (m, 1 H), 6.79 (s, 1 H), 3.90 - 3.98 (m, 2 H), 3.14

(s, 3 H), 3.02 (br. s., 2 H), 3.01 (s, 3 H), 2.44 (d, *J*=1.01 Hz, 3 H), 2.36 - 2.43 (m, 2 H), 1.94 (s, 3 H), 1.83 - 1.93 (m, 4 H); MS (ES+) $[M+H]^+$ = 503.



27

3-(4-(2-(Dimethylamino)ethyl)-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (27). A. To a solution of 1-*tert*-butyl 4-ethyl piperidine-1,4-dicarboxylate (514 mg, 2.0 mmol) in THF (10 mL) at -78 °C was added dropwise LDA (1.5 M in cyclohexane, 2 mL, 3.0 mmol). After stirring for 30 minutes, 2-bromo-*N*,*N*-dimethylethanamine (370 mg, 2.4 mmol) in THF (3 mL) was added. The reaction was stirred for 30 min at -78 °C, then allowed to warm to room temperature, stirred for 2 hours, then quenched with sat. aq. $NaHCO_3$, diluted with EtOAc, washed with sat. aq. $NaHCO_3$ and brine (with back extraction), dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by flash chromatography (0-15% MeOH:DCM) to give 1-*tert*-butyl 4-ethyl 4-(2-(dimethylamino)ethyl)piperidine-1,4-dicarboxylate (328 mg, 1.0 mmol, 50% yield) as a yellow oil. MS (ES+) $[M+H]^+$ = 329.

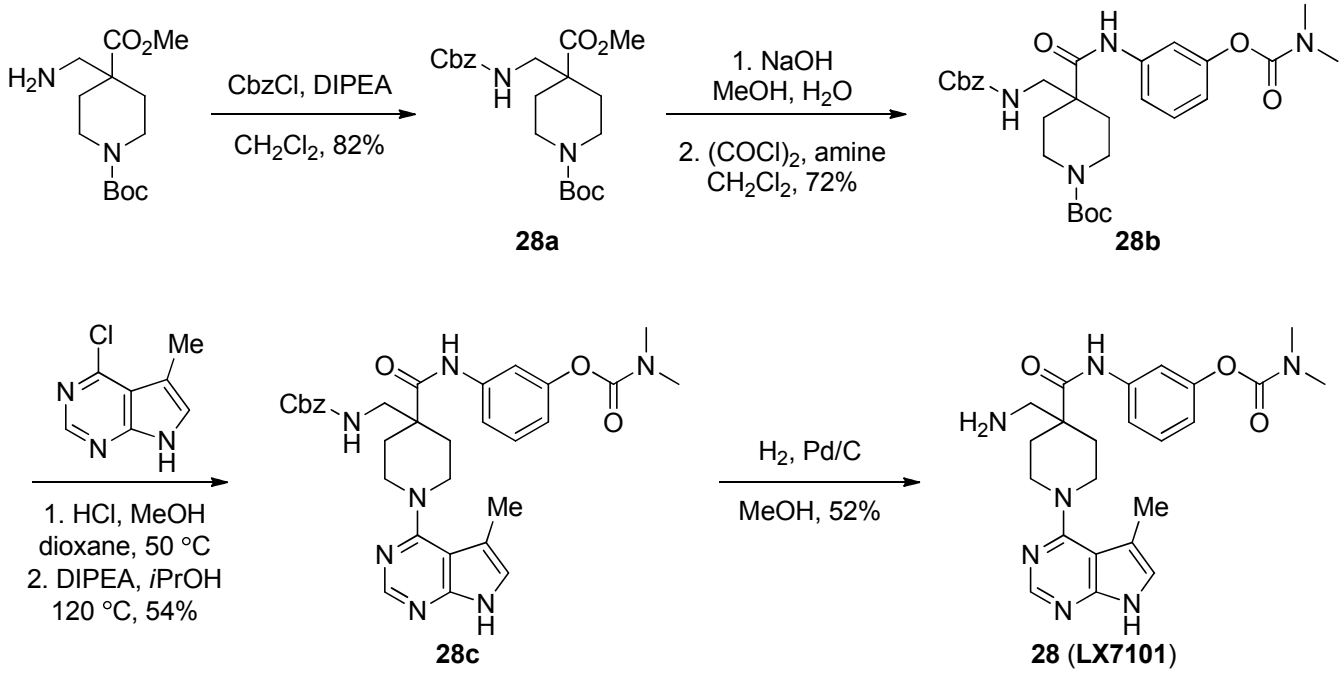
This material (328 mg, 1.0 mmol) was treated overnight with lithium hyrdroxide monohydrate (210 mg, 5 mmol) in 1:1 EtOH:H_2O (5 mL) at 60 °C. The reaction was cooled to room temperature, neutralized to pH 6 with 1N aq. HCl, and concentrated under vacuum. The residue was purified by prep HPLC and lyophilized twice to give 1-(*tert*-butoxycarbonyl)-4-(2-(dimethylamino)ethyl)piperidine-4-carboxylic acid (191 mg, 64% yield) as a white solid. MS (ES+) $[M+H]^+$ = 301.

This acid (100 mg, 0.33 mmol), HATU (175 mg, 0.46 mmol), and *N*,*N*-diisopropylethylamine (0.116 mL, 0.67 mmol) were stirred in isopropyl acetate for 10 minutes. 3-Aminophenyl dimethylcarbamate (90 mg, 0.5 mmol) was added, and the reaction was stirred at 85 °C for 2 hours, cooled to room temperature, stirred overnight, and then diluted with EtOAc, washed with sat. aq. Na_2CO_3 and brine (with back extraction), dried over $MgSO_4$, filtered, and concentrated under vacuum, to give *tert*-butyl 4-(2-(dimethylamino)ethyl)-4-(3-(dimethylcarbamoyloxy)phenylcarbamoyl)piperidine-1-carboxylate as a viscous oil. MS (ES+) $[M+H]^+$ = 463.

This material was treated with 1:1 TFA:CH_2Cl_2 (1.5 mL) for 45 min. The reaction was concentrated under vacuum, and the residue was purified by prep HPLC and lyophilized to give impure 3-(4-(2-(dimethylamino)ethyl)piperidine-4-carboxamido)phenyl dimethylcarbamate (173 mg) as the TFA salt. MS (ES+) $[M+H]^+$ = 363.

This crude amine (173 mg) was combined with 4-chloro-5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (50 mg, 0.30 mmol) and *N*,*N*-diisopropylethylamine (0.174 mL, 5 mmol) in isobutanol (0.4 mL)and heated at 110 °C for 7 hours. The reaction was cooled to room temperature and concentrated under vacuum. The residue was purified by prep HPLC (30x100 mm C18 column, 10-70% MeCN:H_2O (10 mM NH_4OAc), 12 min, 45 mL/min) and lyophilized to give **27** (60 mg) as the di-acetate salt.

^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.19 (s, 1 H), 7.52 (t, *J*=2.15 Hz, 1 H), 7.44 (d, *J*=8.08 Hz, 1 H), 7.35 (t, *J*=8.08 Hz, 1 H), 7.01 (s, 1 H), 6.91 (dd, *J*=7.58, 1.77 Hz, 1 H), 3.91 (d, *J*=13.64 Hz, 2 H), 3.37-3.30 (m, 2 H), 3.14 (s, 3 H), 3.01 (s, 3 H), 2.75 - 2.82 (m, 2 H), 2.58 (s, 6 H), 2.43 - 2.47 (m, 5 H), 2.00 - 2.08 (m, 2 H), 1.79 - 1.89 (m, 2 H); MS (ES+) $[M+H]^+$ = 494.



3-(4-(Aminomethyl)-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (28) To a solution of 1-*tert*-butyl 4-methyl 4-(aminomethyl)piperidine-1,4-dicarboxylate (0.300 g, 1.1 mmol) and *N*,*N*-diisopropylethylamine (0.229 mL, 1.32 mmol) in CH_2Cl_2 (5 mL) at 0 °C was added benzyl chloroformate. The reaction was stirred overnight, and then diluted with CH_2Cl_2 and washed with H_2O and brine, dried over $MgSO_4$, and

concentrated under vacuum. The residue was purified by silica gel chromatography (EtOAc:hexanes gradient) to give 1-*tert*-butyl 4-methyl 4-((benzyloxycarbonylamino)-methyl)piperidine-1,4-dicarboxylate (**28a**) in 82% yield. MS (ES+) $[M+H]^+ = 407$.

To a solution of **28a** in methanol (5 mL) was added 1N aq. NaOH (0.615 mL, 0.615 mmol). The reaction was heated at 50 °C overnight, acidified to pH 5 with 1N aq. HCl, and extracted 3x with CH_2Cl_2. The combined organic extracts were washed with brine, dried over Na_2SO_4, and concentrated under vacuum to give 4-((benzyloxycarbonylamino)methyl)-1-(*tert*-butoxycarbonyl)piperidine-4-carboxylic acid, which was carried on crude. MS (ES+) $[M+H]^+ = 393$.

Under an atmosphere of N_2, this acid (0.240 g, 0.611 mmol), pyridine (0.197 mL, 2.44 mmol) and DMF (3 drops) were dissolved in CH_2Cl_2 (10 mL) and cooled in an ice bath. Oxalyl chloride (0.064 mL, 0.733 mmol) was added drop-wise to the solution. The reaction was then removed from the ice bath and stirred at room temperature for 30 minutes. The reaction was re-cooled in an ice bath, and 3-aminophenyl dimethylcarbamate (0.110 g, 0.611 mmol) was added in one portion. The reaction was stirred overnight, and then washed 1N aq. HCl and brine, dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by silica gel chromatography (EtOAc:hexanes gradient) to give *tert*-butyl 4-((benzyloxycarbonylamino)methyl)-4-(3-(dimethylcarbamoyloxy)phenylcarbamoyl) piperidine-1-carboxylate (**28b**) (245 mg, 72% yield). MS (ES+) $[M+NH_4]^+ = 573$.

To a solution of **28b** (0.245 g, 0.441 mmol) in MeOH (2 mL) was added 4M HCl in dioxane (0.331 mL, 1.32 mmol). The reaction was heated at 50 °C for 1 hour, and then concentrated under vacuum, re-dissolved in MeOH and re-concentrated twice to give 3-(4-((benzyloxycarbonylamino)methyl)piperidine-4-carboxamido)phenyl dimethylcarbamate as the hydrochloride salt. MS (ES+) $[M+H]^+ = 455$.

This amine (0.216 g, 0.441 mmol), 4-chloro-5-methyl-7*H*-pyrrolo[2,3-*d*] pyrimidine (0.073 g, 0.441 mmol), *N*,*N*-diisopropylethylamine (0.230 mL, 1.32 mmol), and isopropanol (5 mL) were combined and heated at 120 °C overnight in a pressure vessel. The crude reaction was diluted with CH_2Cl_2 and washed with H_2O and brine, dried over $MgSO_4$, and concentrated under vacuum. The residue was purified by silica gel chromatography (MeOH:CH_2Cl_2 gradient) to give 3-(4-((benzyloxycarbonylamino)methyl)-1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)piperidine-4-carboxamido)phenyl dimethylcarbamate (**28c**) in 54% yield.

^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.18 (s, 1H), 7.49 (s, 1H), 7.40 (d, J=8 Hz, 1H), 7.26-7.34 (m, 6H), 7.00 (s, 1H), 6.89 (d, J=8 Hz, 1H), 5.06 (s, 2H), 3.92 (d, J=13.2 Hz, 2H), 3.50 (s,

2H), 3.28-3.37 (m, 2H), 3.1 (s, 3H), 2.99 (s, 3H), 2.43 (s, 3H), 2.40 (d, J=12 Hz, 2H), 1.79 (t, J=10.6, 21.2 Hz, 2H); MS (ES+) $[M+H]^+$ = 586.

This material (0.140 g, 0.239 mmol), Pd/C (10% Pd/C, Pearlman's catalyst, 14 mg), and methanol (5 mL) were combined and hydrogenated overnight at atmospheric pressure. The reaction was filtered through celite, concentrated under vacuum, and purified by prep-HPLC (neutral eluent) to give **28** as the acetate salt (56 mg, 52% yield).

^{1}H NMR (400 MHz, D_2O) δ ppm 7.90 (s, 1H), 7.30 (t, J=8, 6 Hz, 1H), 7.18-7.19 (m, 2H), 6.84-6.88 (m, 1H), 3.48 (d, J= 13.6 Hz, 2H), 3.20 (s, 2H), 3.08 (t, J=10.8, 21.6 Hz, 2H), 2.96 (s, 3H), 2.83 (s, 3H), 2.27 (d, J=14.4 Hz, 2H), 2.11 (s, 3H), 1.72 (t, J=10, 20 Hz, 2H); MS (ES+) $[M+H]^+$ = 452.

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 min-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 x 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). 384 well streptavidin FlashPlates from Perkin Elmer (Cat# SMP410A001PK) were used.

Rock1 purified at Lexicon using a similar procedure described above for LIMK2, was used to activate LIMK2. Specifically, a 20 μL mixture of 0.6 nM ROCK1, 5 nM LIMK2, and cold ATP (5 μM for normal assays, 500 μM for high ATP experiments) was preincubated in kinase assay buffer (30 mM

HEPES, pH 8.0; mM $MgCl_2$; 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 ^{33}P-ATP, 0.5 μM biotinylated-cofilin (Vmax is reached at 0.5 μM), and 0.5 μM Rock1 inhibitor Y39983 (Senju/Novartis) in a final reaction volume of 50 μL (Final ATP concentrations of 2 μM for normal assays, 200 μM for high ATP experiments). For kinetic studies, the final compound concentrations varied from 0 to 100 nM. 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.

Calculating IC50 Values

The IC_{50} of a compound with regard to a given target is determined by fitting the relevant data, using the Levenburg Marquardt algorithm, to the equation:

$$y = A + ((B-A)/(1+((C/x)^D)))$$

wherein A is the minimum y value; B is the maximum y value; C is the IC_{50}; and D is the slope. The calculation of the IC_{50} is performed using XLFit4 software (ID Business Solutions Inc., Bridgewater, NJ 08807) for Microsoft Excel (the above equation is model 205 of that software).

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, Rock1 and Rock2 requires 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 2 μM (or 200 μM for high ATP experiments) and 0.5 μM, respectively.

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 μL per hour which would deliver 0.09 mg of dexamethasone per day. The osmotic pumps were implanted into male hybrid mice (C57BL/6J-$Tyr^{c\text{-}Brd}$ 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 ± SEM) in IOP when compared with baseline IOP measurements.

Pharmacological responses were observed between days 21 and 25, following topical administration of LIMK2 inhibitors **25-28**. The compounds were formulated as 1-10 mg/mL aqueous solutions in HPMC/Phosphate buffered vehicle with NaCl as the tonicity agent. 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. Baseline IOP's were taken at time 0, and a 3 μL drop of vehicle or formulated compound was placed on the eye. IOP measurements were taken 2, 4, 6, and 8 hours later.

3.2. *TOPICAL OCULAR PK OF COMPOUND 28*

Topical PK was evaluated in Sprague-Dawley rats (Table 6) following single or multiple doses with dissection of the eye to determine levels in various tissues. Good exposure of **28** was observed in the aqueous humor, with high levels in the cornea and sclera; moderate levels in the iris, vitreous humor, and retina; and low levels in the lens.

Table 6. Topical PK of compound **28**

	Cmax (nM)[a]		AUC 0-24 h (μM*h)	
tissue	Single	Multiple	Single	Multiple
aqueous humor	600	1500	1.7[b]	3.2[b]
cornea	10,400	116,000	45	162
iris	420	620	2.0	2.9
lens	23	49	0.27	0.46
retina	200	540	1.1	1.5
sclera	1,700	3,300	7.3	8.5
vitreous	100	600	0.62	1.8

[a]Maximum concentrations of **28** in optical tissue of Sprague-Daley rats following topical dosing of 10 mg/mL aqueous formulation of compound. [b]AUC 0-6 hours.