Supporting Information

Brain-Penetrant Picolinamide Derived Small Molecule Inhibitors of Leucine-Rich Repeat Kinase 2 (LRRK2)

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1. General Considerations: Organic Chemistry

Methods:

All reactions were performed in scintillation or microwave vials under a nitrogen atmosphere unless otherwise specified. Reactions were monitored by liquid chromatography/mass spectrometry (LCMS) or by thin layer chromatography (TLC) on Silica Gel 60 F_{254} plates (EMD) and visualized with UV light (254 nm). Flash chromatography was performed using pre-packed RediSep R_f silica gel columns on a Teledyne Isco CombiFlash R_f automated chromatography system. Organic solutions were concentrated under reduced pressure on a Heidolph rotary evaporator. Reversed-phase high-performance liquid chromatography was carried out using an Agilent 1100 HPLC-MSD system consisting of a 6130B single quadrupole mass-selective detector (MSD), G1315B diode array detector, G2258A autosampler, two G1361A preparative pumps, one G1379A quaternary pump with degasser, one G1312A binary pump, and three G1364B fraction collectors from Agilent Technologies. System control and data analysis was performed using Agilent's ChemStation software, revision B.03.01-SR.1. A Waters XBridge C18 OBD Prep Column, 100 Å, 5 μm, 19 mm × 150 mm column was used as the stationary phase (Waters Corporation). Gradient elution was carried out using water and acetonitrile as the mobile phase. An aqueous 10% trifluoroacetic acid or 10% ammonium hydroxide solution was teed into the mobile phase as a modifier using a static mixer prior to the column, pumped at 1% of the total mobile phase flow rate. ESI mass-triggered fraction collected was employed using positive ion polarity scanning to monitor for the target mass. The purity of all compounds screened in the biological assays was examined by LC/MS analysis (100 mm x 3 mm C18 column; 3% to 98% MeCN/water with 0.05% TFA gradient over 5 minute run; UV detection at 215 or 254 nm) and was found to be ≥95%.

Materials and Reagents:

All reagents and solvents were purchased from commercial sources (Sigma-Aldrich, Alfa Aesar, Strem, Acros, Enamine) and used without further purification unless otherwise specified. Deuterated solvents were purchased from Cambridge Isotope Laboratories and used without further purification unless otherwise specified. $CDCl_3$ was treated with and stored over K_2CO_3.

Instrumentation:

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian 500 (500 MHz) NMR spectrometer at ambient temperature. All chemical shifts (δ) are reported in parts per million (ppm). Proton resonances are referenced to residual protium in the NMR solvent. Data are represented as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants (*J*) in Hertz (Hz), integration.

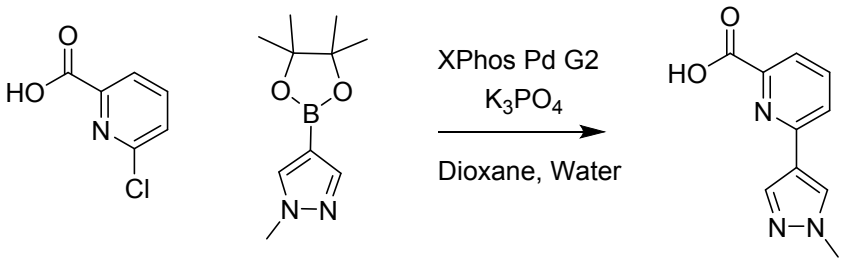
LCMS samples were run on an Agilent 1100 or 1200 system. High-resolution mass spectrometry (HRMS) data were recorded on a Waters Xevo G2 QTof instrument in either ESI^+ or ESI^- (electrospray) ionization mode.

2. Abbreviations

AcOH	Acetic Acid
calc'd	Calculated
COMU	(1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate
DCE	Dichloroethane
DCM	Dichloromethane
DIEA	N,N-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
h	Hours
HATU	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate, *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HCl	Hydrochloric Acid
HRMS	High Resolution Mass Spectra
LRMS	Low Resolution Mass Spectra
MeCN	Acetonitrile
Min	Minutes
NMP	N-Methyl-2-pyrrolidone
NMR	Nuclear Magnetic Resonance
RT	Room Temperature
RuPhos Pd G2	Chloro(2-dicyclohexylphosphino-2′,6′-diisopropoxy-1,1′-biphenyl)[2-(2′-amino-1,1′-biphenyl)]palladium(II) chloride
STAB	Sodium Triacetoxyborohydride
TEA	Triethylamine
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran
XantPhos Pd G2	Chloro[(4,5-bis(diphenylphosphino)-9,9-dimethylxanthene)-2-(2′-amino-1,1′-biphenyl)]palladium(II)
Xphos Pd G2	Chloro(2-dicyclohexylphosphino-2′,4′,6′-triisopropyl-1,1′-biphenyl)[2-(2′-amino-1,1′-biphenyl)]palladium(II), X-Phos aminobiphenyl palladium chloride

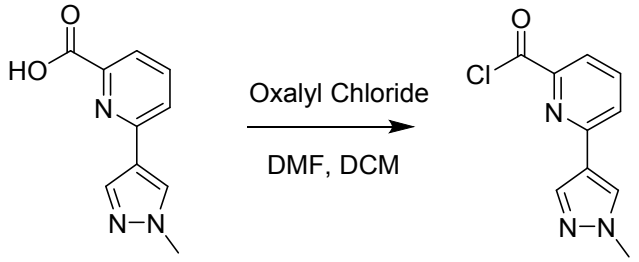
3. Synthesis & Characterization of Intermediates A-K and Compounds 14-16

Scheme 1: Preparation of **A** 6-(1-methyl-1H-pyrazol-4-yl)picolinic acid



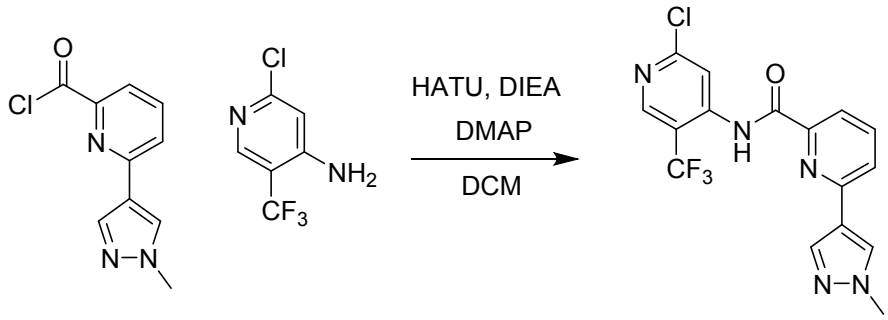
General Procedure: A 100 mL round bottom flask was charged with 6-chloropicolinic acid (2.7 g, 17 mmol), XPhos Pd G2 (0.68 g, 0.86 mmol), 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (3.91 g, 18.8 mmol), dioxane (35 ml), K_3PO_4 (10.9 g, 51.4 mmol), and H_2O (10 mL). The reaction mixture was purged with nitrogen 3 times and stirred at 80 °C for 12 hrs. At 12 hrs, the reaction mixture was cooled to RT, filtered and concentrated under reduced pressure. The crude mixture was dissolved in 3:1 Chloroform/Isopropanol mixture and extracted with saturated NH_4Cl (2 x 50 mL). The combined organic phases were combined, dried over Na_2SO_4, and the solvent removed under reduced pressure to afford 6-(1-methyl-1H-pyrazol-4-yl)picolinic acid (3.48 g, 100%) as a white solid which was taken forward without further purification. LRMS (ESI) m/z 204 [(M+H)+ calc'd for $C_{10}H_9N_3O_2$: 204]

Scheme 2: Preparation of **B** 6-(1-methyl-1H-pyrazol-4-yl)picolinoyl chloride



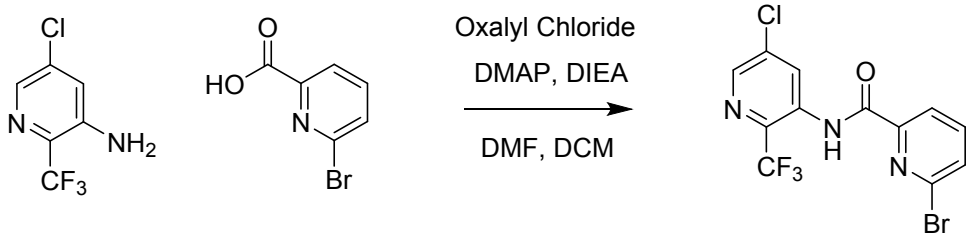
General Procedure: A 30 mL microwave vial was charged with **intermediate A** (801 mg, 3.91 mmol), DCM (8 mL), DMF (0.04 mL, 0.34 mmol), and oxalyl chloride (0.7 mL, 8 mmol). The vial was purged with nitrogen and stirred at RT for 3 hrs. At 3 hrs, the reaction mixture was concentrated and dried over high vacuum overnight to afford 6-(1-methyl-1H-pyrazol-4-yl)picolinoyl chloride (873 mg, 100%) as a white solid which was taken forward without further purification. LRMS (ESI) m/z 222 [(M+H)+ calc'd for $C_{10}H_8ClN_3O$: 222]

Scheme 3: Preparation of **C** *N*-(2-chloro-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide



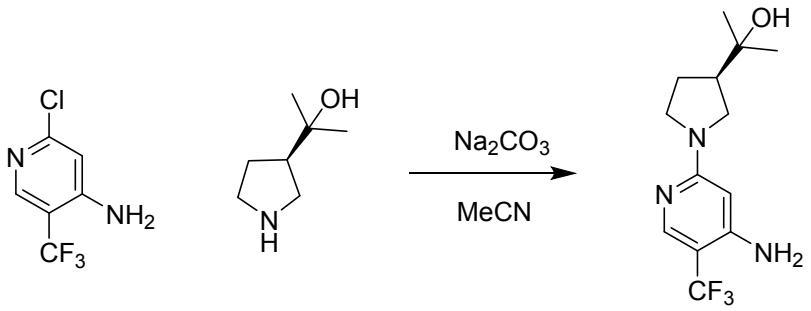
General Procedure: A 30 mL microwave vial was charged with 2-chloro-5-(trifluoromethyl)pyridin-4-amine (2 g, 10.0 mmol), intermediate **B** (4.2 g, 19.0 mmol), DMAP (1.24 g, 10.2 mmol), DIEA (8.9 mL, 51 mmol), DCM (34 mL) and the reaction mixture was stirred at RT for 12 hrs. At 12 hrs, the reaction mixture was poured into a separatory funnel containing deionized water (20 mL). The phases were separated, and the aqueous phase was extracted with DCM (3 x 25 mL). The combined organic phases were combined, dried over Na_2SO_4, and the solvent removed under reduced pressure. The crude mixture was purified by flash chromatography using a pre-packed RediSep Rf silica gel column on a Teledyne Isco CombiFlash Rf automated chromatography system using 0-100% EtOAc/hexanes to afford *N*-(2-chloro-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide (2.3 g, 59%) as a white solid. LRMS (ESI) m/z 382 [(M+H)+ calc'd for $C_{16}H_{11}ClF_3N_5O$: 382]

Scheme 4: Preparation of **D** 6-bromo-*N*-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)picolinamide



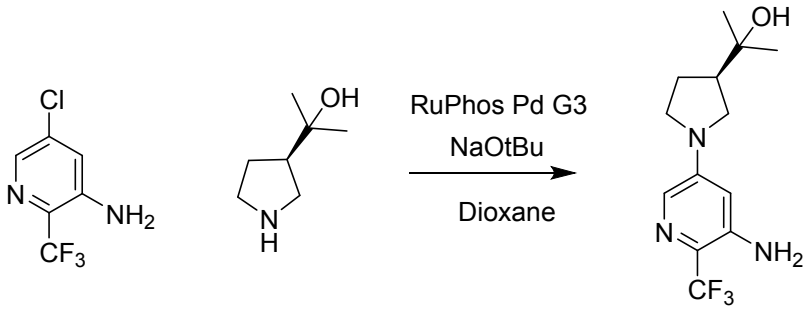
General Procedure: : A 100 mL round bottom flask was charged with 6-bromopicolinic acid (3.3 g, 15 mmol) and DCM (15.3 mL) under inert atmosphere. The reaction mixture was cooled to 0°C and to the stirring mixture, DMF (2 drops) and oxalyl chloride (3.31 mL, 37.6 mmol) were added. The resultant mixture was stirred at 0 °C for 30 min. The reaction mixture was concentrated under reduced pressure and diluted in DCM (15.4 mL). Under inert atmosphere, 5-chloro-2-(trifluoromethyl)pyridin-3-amine, HCl (1.75 g, 7.51 mmol), DIEA (6.56 mL, 37.6 mmol), and DMAP (0.91 g, 7.5 mmol) were added. The reaction mixture was stirred at 0 °C overnight for 12 hrs. At 12 hrs, the reaction mixture was diluted with EtOAc and washed twice with saturated NH_4Cl (2 x 100 mL). The organic fractions were combined, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The crude mixture was purified by flash chromatography using a pre-packed RediSep Rf silica gel column on a Teledyne Isco CombiFlash Rf automated chromatography system using 0-100% EtOAc/hexanes to afford 6-bromo-N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)picolinamide (2.4 g, 84%). LRMS (ESI) m/z 380 [(M+H)+ calc'd for $C_{12}H_6BrClF_3N_3O$: 380]

Scheme 5: Preparation of **E (Compound 14)** (*R*)-2-(1-(4-amino-5-(trifluoromethyl)pyridin-2-yl)pyrrolidin-3-yl)propan-2-ol



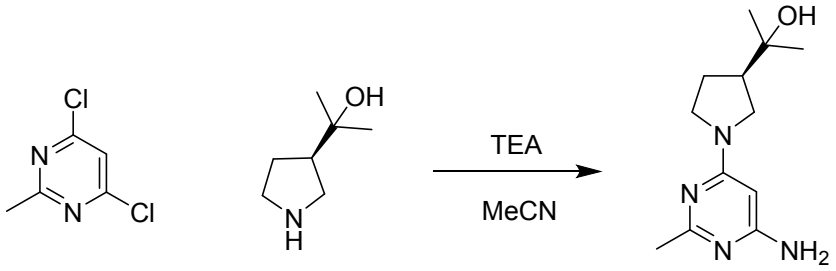
General Procedure: A 30 mL microwave vial was charged with 2-chloro-5-(trifluoromethyl)pyridin-4-amine (170 mg, 0.89 mmol), DMSO (0.89 mL), (*R*)-2-(pyrrolidin-3-yl)propan-2-ol (130 mg, 0.99 mmol), and Na_2CO_3 (190 mg, 1.8 mmol). The reaction mixture was stirred overnight at 90 °C for 12 hrs. At 12 hrs, the reaction mixture was filtered and concentrated. The crude mixture was diluted in DCM and was purified by flash chromatography using a pre-packed RediSep R_f silica gel column on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-100% EtOAc/Hexanes to afford (*R*)-2-(1-(4-amino-5-(trifluoromethyl)pyridin-2-yl)pyrrolidin-3-yl)propan-2-ol (190 mg, 0.66 mmol, 73.8 % yield) as a white solid. LRMS (ESI) m/z 290 [(M+H)+ calc'd for $C_{13}H_{18}F_3N_3O$: 290]

Scheme 6: Preparation of **F (Compound 15)** (*R*)-2-(1-(5-amino-6-(trifluoromethyl)pyridin-3-yl)pyrrolidin-3-yl)propan-2-ol



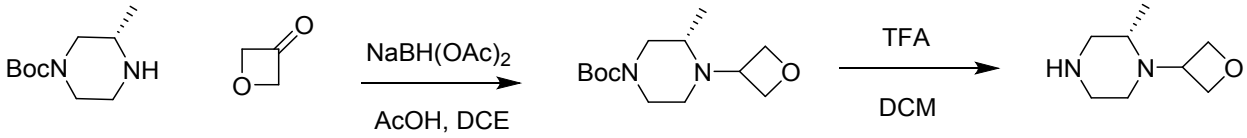
General Procedure: A 30 mL microwave vial was charged with 5-chloro-2-(trifluoromethyl)pyridin-3-amine (400 mg, 2 mmol), (*R*)-2-(pyrrolidin-3-yl)propan-2-ol, HCl (506 mg, 3.05 mmol), RuPhos Pd G3 (171 mg, 0.204 mmol), and sodium tert-butoxide (587 mg, 6.11 mmol). The vial was purged with nitrogen three times and stirred overnight at 80 °C for 12 hrs. At 12 hrs, the crude reaction was filtered, concentrated, diluted in 4 mL of DMSO. The crude product was purified by mass-directed reverse phase chromatography eluting with $MeCN/H_2O$ with base modifier with a linear gradient to afford (*R*)-2-(1-(5-amino-6-(trifluoromethyl)pyridin-3-yl)pyrrolidin-3-yl)propan-2-ol (160 mg, 0.55 mmol, 27.2 % yield) as a white solid. LRMS (ESI) m/z 290 [(M+H)+ calc'd for $C_{13}H_{18}F_3N_3O$: 290]

Scheme 7: Preparation of **G (Compound 16)** (R)-2-(1-(6-amino-2-methylpyrimidin-4-yl)pyrrolidin-3-yl)propan-2-ol



***General Procedure**:* A 30 mL microwave vial was charged with (*R*)-2-(pyrrolidin-3-yl)propan-2-ol hydrochloride (500 mg, 3 mmol), 4,6-dichloro-2-methylpyrimidine (540 mg, 3.3 mmol), MeCN (10 ml), and TEA (1.2 ml, 9.1 mmol). The reaction mixture was stirred overnight at 80 °C for 12 hrs. At 12 hrs, the reaction mixture was concentrated under reduced pressure and was purified by flash chromatography using a pre-packed RediSep R_f silica gel column on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-100% EtOAc/Hexanes to afford (*R*)-2-(1-(6-chloro-2-methylpyrimidin-4-yl)pyrrolidin-3-yl)propan-2-ol (699 mg, 2.74 mmol, 91 % yield) as yellow solid. ^{1}H NMR (499 MHz, DMSO-d_6) δ 5.90 (s, 2H), 5.08 (s, 1H), 4.29 (s, 1H), 3.28 (s, 5H), 3.17 – 3.03 (m, 2H), 2.16 (s, 1H), 1.87 – 1.69 (m, 2H), 1.07 (d, J = 7.8 Hz, 6H). LRMS (ESI+) m/z 237 [(M+H)+ calc'd for $C_{12}H_{20}N_4O$: 237].

Scheme 8: Preparation of **H** (S)-2-methyl-1-(oxetan-3-yl)piperazine, TFA



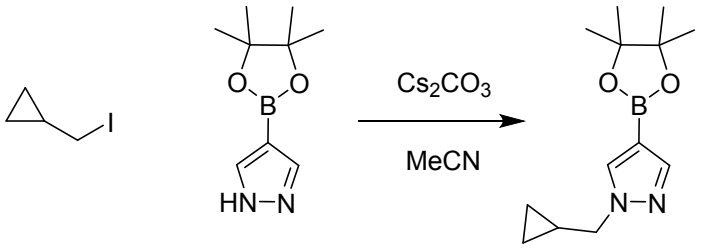
Step 1: Preparation for tert-butyl (S)-3-methyl-4-(oxetan-3-yl)piperazine-1-carboxylate

General Procedure: A 1 L round bottom flask was charged with tert-butyl (3S)-3-methylpiperazine-1-carboxylate (170 g, 851 mmol), DCE (1.7 L), STAB (342 g, 1620 mmol), AcOH (71.4 g, 1190 mmol), and oxetan-3-one (122 g, 1710 mmol). The resulting solution was stirred for 12 h at RT. At 12 hrs, the reaction was quenched by the addition of 500 mL of water. The resulting solution was extracted with DCM (3 x 500 mL) and the organic layers combined. The organic phases were combined, dried over Na2SO4, and the solvent removed under reduced pressure. The crude mixture was purified by flash chromatography using a pre-packed RediSep Rf silica gel column on a Teledyne Isco CombiFlash Rf automated chromatography system using EtOAc/petroleum ether 0-80% to afford tert-butyl (3S)-3-methyl-4-(oxetan-3-yl)piperazine-1-carboxylate (185 g, 84%) as a light yellow solid. LRMS (ESI) m/z 257 [(M+H)+ calc'd for $C_{13}H_{24}N_2O_3$: 257

Step 2: Preparation of (S)-2-methyl-1-(oxetan-3-yl)piperazine, TFA

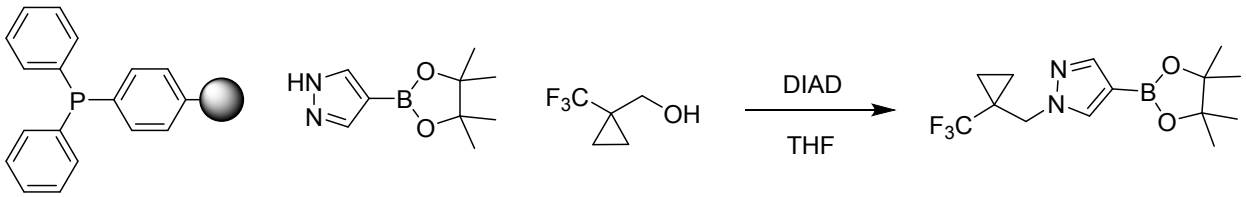
General Procedure: A 1-L round-bottom flask was charged with TFA (554 mL) and the mixture was cooled down at 0 ^{o}C. tert-butyl (3S)-3-methyl-4-(oxetan-3-yl)piperazine-1-carboxylate (185 g) was added slowly to the round bottom flask. The resulting solution was stirred for 4 h at RT. The resulting mixture was concentrated under vacuum to afford (2S)-2-methyl-1-(oxetan-3-yl)piperazinebis(2,2,2-trifluoroacetaldehyde) (261 g, 94 %) as an off-white solid. LRMS (ESI) m/z 157 [(M+H)+ calc'd for $C_8H_{16}N_2O$: 157

Scheme 9: Preparation of **I** 1-(cyclopropylmethyl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole



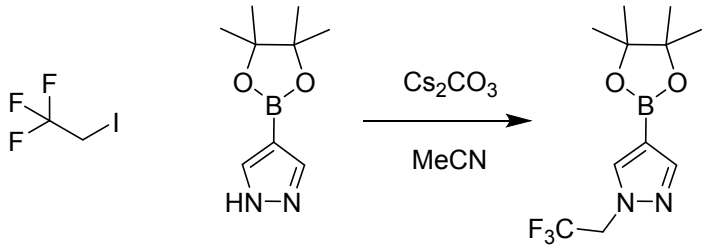
General Procedure: A 30 mL microwave vial was charged with 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (201 mg, 1.03 mmol), (bromomethyl)cyclopropane (167 mg, 1.27 mmol) and Cs_2CO_3 (672 mg, 2.06 mmol) in MeCN (5.0 mL). The reaction was stirred at 80 °C for overnight. The mixture was allowed to cool and was diluted with DCM. The reaction mixture was poured into a separatory funnel containing deionized water (20 mL). The phases were separated and the aqueous phase was extracted with DCM (3 x 25 mL). The combined organic phases were combined dried over Na_2SO_4, and the solvent removed under reduced pressure. The reaction mixture was diluted in DCM and was purified by flash chromatography using pre-packed RediSep R_f silica gel columns on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-100% Hexanes/Ethyl acetate to afford the desired product 1-(cyclopropylmethyl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (250 mg, 1.01 mmol, 98 % yield) as a yellow oil. LRMS (ESI) m/z 249 [(M+H)+ calc'd for $C_{13}H_{21}BN_2O_2$: 249]

Scheme 10: Preparation of **J** 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-((1 (trifluoromethyl)cyclopropyl)methyl)-1H-pyrazole



General Procedure: A 150 mL round-bottom flask was charged with 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (1.71 g, 8.76 mmol), triphenylphosphine-resin (5.87 g, 17.5 mmol), 1-(trifluoromethyl)cyclopropyl)methanol (1.14 g, 8.12 mmol), and THF (22 mL). The flask was cooled to 0 °C and a solution of DIAD (3.44 mL, 17.5 mmol) in THF (22 mL) was added. The reaction mixture was allowed to warm to RT overnight for 12 hrs. At 12 hrs, the mixture was filtered and concentrated under reduced pressure. The crude mixture was purified by flash chromatography using a pre-packed RediSep R_f silica gel column on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-50% EtOAc/hexanes to afford the desired product 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-((1 (trifluoromethyl)cyclopropyl)methyl)-1H-pyrazole (2.11 g, 6.64 mmol, 75 % yield) as an off white solid. MS (ESI) m/z 317 [$(M+H)^+$ calc'd for $C_{14}H_{20}BF_3N_2O_2$: 317]

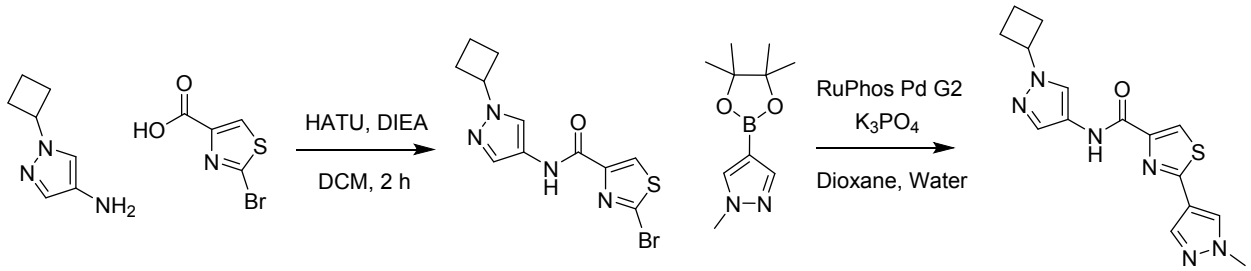
Scheme 11: Preparation of **K** 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-(2,2,2-trifluoroethyl)-1H-pyrazole



General Procedure: A 30 mL microwave vial was charged with 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (5.0 g, 25.8 mmol), Cs_2CO_3 (12.6 g, 38.7 mmol), MeCN (100 mL) and 1,1,1-trifluoro-2-iodoethane (8.11 g, 38.7 mmol). The reaction mixture was stirred at 80 °C overnight. The mixture was allowed to cool, and the reaction mixture was diluted with DCM. The reaction mixture was poured into a separatory funnel containing deionized water (20 mL). The phases were separated, and the aqueous phase was extracted with DCM (3 x 25 mL). The combined organic phases were combined dried over Na_2SO_4, and the solvent removed under reduced pressure. The reaction mixture was diluted in DCM and was purified by flash chromatography using pre-packed RediSep R_f silica gel columns on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-100% Hexanes/Ethyl acetate to afford the desired product 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1-(2,2,2-trifluoroethyl)-1H-pyrazole (2.8 g, 6.29 mmol, 24.4 % yield) as a colorless oil. LRMS (ESI) m/z 277 [(M+H)+ calc'd for $C_{11}H_{16}BF_3N_2O_2$: 277]

4. Synthesis & Characterization of Compounds 1-13 and 17-23

Scheme 12: Preparation of **1** N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-(1-methyl-1H-pyrazol-4-yl)thiazole-4-carboxamide



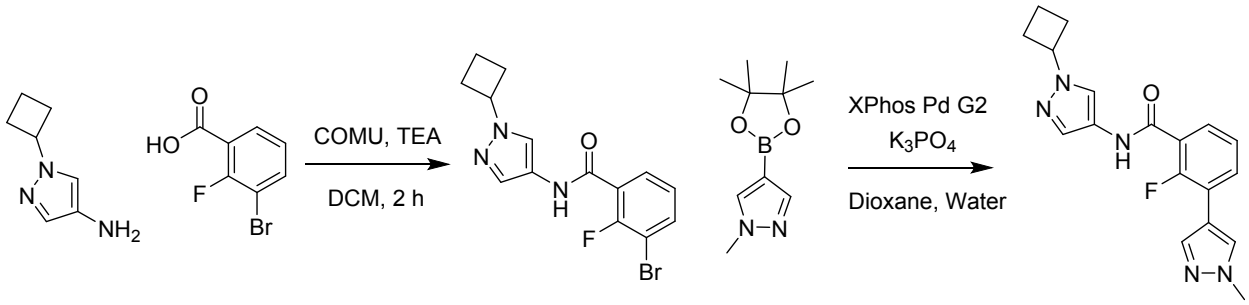
Step 1: Preparation of 2-bromo-N-(1-cyclobutyl-1H-pyrazol-4-yl)thiazole-4-carboxamide

General Procedure: A 30 mL microwave vial was charged with 2-bromothiazole-4-carboxylic acid (651 mg, 3.12 mmol), 1-cyclobutyl-1H-pyrazol-4-amine (435 mg, 3.17 mmol), HATU (1.19 g, 3.12 mmol), DCM (15 ml), DIEA (1.64 ml, 9.37 mmol) and the reaction mixture was stirred at rt for 2 hours. The reaction mixture was poured into a separatory funnel containing deionized water (20 mL). The phases were separated, and the aqueous phase was extracted with DCM (3 x 25 mL). The combined organic phases were combined dried over Na_2SO_4, and the solvent removed under reduced pressure to yield 2-bromo-N-(1-cyclobutyl-1H-pyrazol-4-yl)thiazole-4-carboxamide (1.02 g, 100%) as a pink solid. The crude product was carried forward without any purification. LRMS (ESI+) m/z 327 [(M+H)+ calc'd for $C_{11}H_{11}BrN_4OS$: 327]

Step 2: Preparation of N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-(1-methyl-1H-pyrazol-4-yl)thiazole-4-carboxamide, TFA

General Procedure: A 30 mL microwave vial was charged with 2-bromo-N-(1-cyclobutyl-1H-pyrazol-4-yl)thiazole-4-carboxamide (41 mg, 0.12 mmol), RuPhos Pd G2 (9.6 mg, 0.012 mmol), 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (28 mg, 0.13 mmol), dioxane (610 µL), K_3PO_4 solution (2M in water, 190 µL, 0.37 mmol). The reaction mixture was purged with nitrogen 3 times and stirred at 80 °C overnight. The reaction mixture was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-(1-methyl-1H-pyrazol-4-yl)thiazole-4-carboxamide, TFA (11.9 mg, 0.0271 mmol, 21.8 % yield) as a white solid. ^{1}H NMR (499 MHz, DMSO-d_6) δ 10.35 (s, 1H), 8.41 (s, 1H), 8.20 (s, 1H), 8.11 (s, 1H), 8.03 (s, 1H), 7.72 (s, 1H), 4.87 – 4.75 (m, 1H), 3.91 (s, 3H), 2.47 – 2.41 (m, 2H), 2.37 (s, 2H), 1.77 (d, J = 8.1 Hz, 2H). HRMS (ESI+) m/z 329.11 [(M+H)+ calc'd for $C_{15}H_{16}N_6OS$: 329.11].

Scheme 13: Preparation of **2** N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-fluoro-3-(1-methyl-1H-pyrazol-4-yl)benzamide



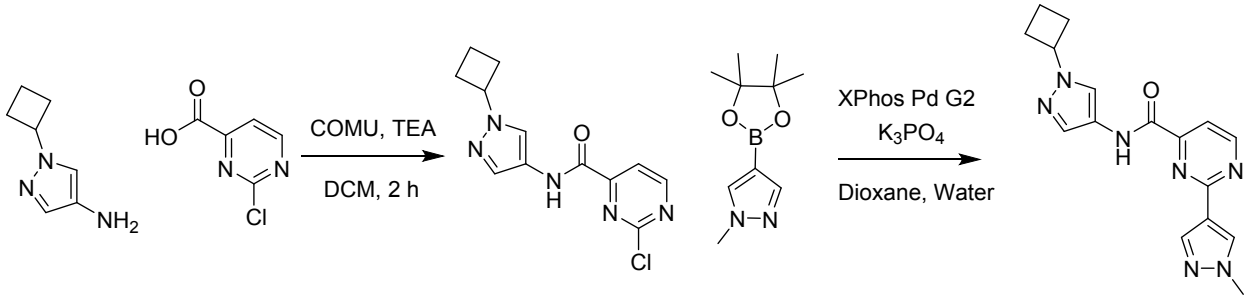
Step 1: Preparation of 3-bromo-N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-fluorobenzamide

General Procedure: A 30 mL microwave vial was charged with 3-bromo-2-fluorobenzoic acid (0.033 g, 0.15 mmol), 1-cyclobutyl-1H-pyrazol-4-amine (0.021 g, 0.150 mmol), COMU (0.064 g, 0.15 mmol), DCM (1 ml), TEA (0.063 ml, 0.45 mmol) and the reaction mixture was stirred at rt for 2 hours. The reaction mixture was poured into a separatory funnel containing deionized water (20 mL). The phases were separated, and the aqueous phase extracted with DCM (3 x 25 mL). The combined organic phases were combined dried over Na_2SO_4, and the solvent removed under reduced pressure to yield 3-bromo-N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-fluorobenzamide (51 mg, 100%) as a pink solid. The crude product was carried forward without any purification. LRMS (ESI+) m/z 338 [(M+H)+ calc'd for $C_{14}H_{13}BrFN_3O$: 338]

Step 2: Preparation of N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-fluoro-3-(1-methyl-1H-pyrazol-4-yl)benzamide, TFA

General Procedure: A 30 mL microwave vial was charged with 3-bromo-N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-fluorobenzamide (51 mg, 0.15 mmol), XPhos Pd G2 (12 mg, 0.015 mmol), 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (35 mg, 0.17 mmol), dioxane (600 µL), K_3PO_4 solution (2M in water, 150 µL, 0.3 mmol). The reaction mixture was purged with nitrogen 3 times and stirred at 80 °C overnight. The reaction mixture was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-fluoro-3-(1-methyl-1H-pyrazol-4-yl)benzamide, TFA (17.4 mg, 0.038 mmol, 25.6 % yield) as a white solid. 1H NMR (499 MHz, DMSO-*d*6) δ 10.53 (s, 1H), 8.20 (s, 1H), 8.08 (s, 1H), 7.95 (s, 1H), 7.84 (s, 1H), 7.55 (s, 1H), 7.43 (s, 1H), 7.30 (s, 1H), 4.83 (s, 1H), 2.47 (d, J = 25.5 Hz, 5H), 2.36 (s, 2H), 1.76 (s, 2H). HRMS (ESI+) m/z 340.15 [(M+H)+ calc'd for $C_{18}H_{18}FN_5O$: 340.15].

Scheme 14: Preparation of **3** N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-(1-methyl-1H-pyrazol-4-yl)pyrimidine-4 carboxamide, TFA



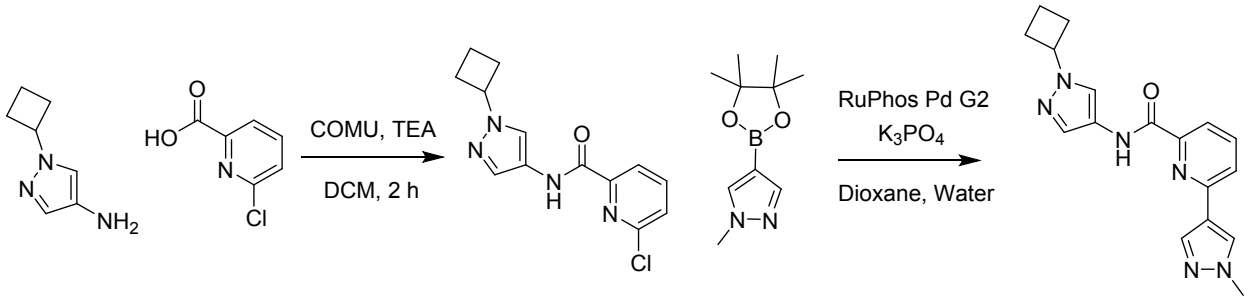
Step 1: Preparation of 2-chloro-N-(1-cyclobutyl-1H-pyrazol-4-yl)pyrimidine-4-carboxamide

General Procedure: A 30 mL microwave vial was charged with 2-chloropyrimidine-4-carboxylic acid (0.024 g, 0.15 mmol), 1-cyclobutyl-1H-pyrazol-4-amine (0.021 g, 0.15 mmol), COMU (0.064 g, 0.15 mmol), DCM (1 ml), TEA (0.08 ml, 0.6 mmol) and the reaction mixture was stirred at rt for 2 hours. The reaction mixture was poured into an separatory funnel containing deionized water (20 mL). The phases were separated, and the aqueous phase was extracted with DCM (3 x 25 mL). The combined organic phases were combined, dried over Na_2SO_4, and the solvent removed under reduced pressure to yield 2-chloro-N-(1-cyclobutyl-1H-pyrazol-4-yl)pyrimidine-4-carboxamide (0.042 g, 100%) as a pink solid. The crude product was carried forward without any further purification. LRMS (ESI+) m/z 278 [(M+H)+ calc'd for $C_{12}H_{12}ClN_5O$: 278]

Step 2: Preparation of N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-(1-methyl-1H-pyrazol-4-yl)pyrimidine-4 carboxamide, TFA

General Procedure: A 30 mL microwave vial was charged with 2-chloro-N-(1-cyclobutyl-1H-pyrazol-4-yl)pyrimidine-4-carboxamide (42 mg, 0.15 mmol), XPhos Pd G2 (12 mg, 0.015 mmol), 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (47 mg, 0.23 mmol), dioxane (750 µL), K_3PO_4 solution (2M in water, 230 µL, 0.46 mmol). The reaction mixture was purged with nitrogen 3 times and stirred at 80 °C overnight. The reaction mixture was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford N-(1-cyclobutyl-1H-pyrazol-4-yl)-2-(1-methyl-1H-pyrazol-4-yl)pyrimidine-4 carboxamide, TFA (12 mg, 0.028 mmol, 18.9 % yield) as a white solid. ^{1}H NMR (499 MHz, DMSO-d_6) δ 10.83 (s, 1H), 9.00 (d, J = 4.6 Hz, 1H), 8.61 (s, 1H), 8.36 (s, 1H), 8.22 (s, 1H), 7.86 – 7.75 (m, 2H), 4.86 (p, J = 8.2 Hz, 1H), 3.95 (s, 4H), 2.45 – 2.35 (m, 3H), 1.78 (dt, J = 18.0, 9.5 Hz, 2H). HRMS (ESI+) m/z 324.15 [(M+H)+ calc'd for $C_{16}H_{17}N_7O$: 324.15].

Scheme 15: Preparation of **4** N-(1-cyclobuty l-1H-pyrazol-4 -yl)-6-(1-meth yl-1H-pyrazol- 4-yl)picolinamide, TFA



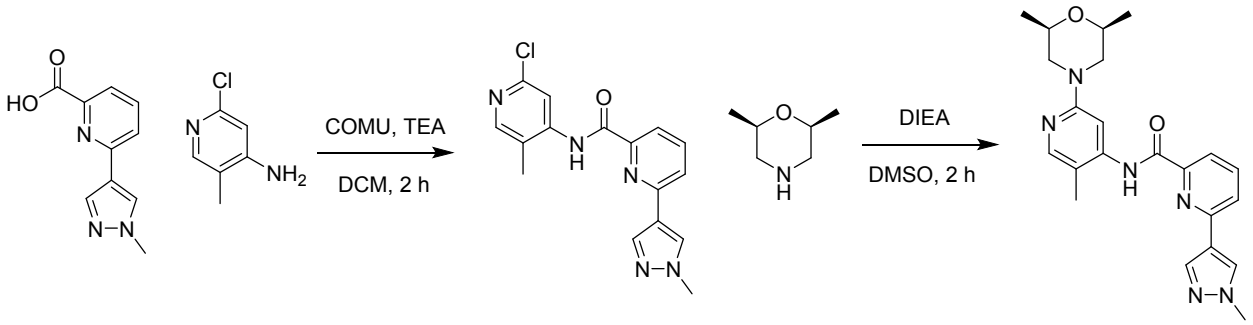
Step 1: Preparation for 6-chloro-N-(1-cyclobutyl-1H-pyrazol-4-yl)picolinamide

General Procedure: A 30 mL microwave vial was charged with 1-cyclobutyl-1H-pyrazol-4-amine (0.021 g, 0.15 mmol), 6-chloropicolinic acid (0.024 g, 0.15 mmol), COMU (0.064 g, 0.15 mmol), DCM (1 ml), TEA (0.084 ml, 0.601 mmol) and the reaction mixture was stirred at rt for 2 hours. The reaction mixture was poured into a separatory funnel containing deionized water (20 mL). The phases were separated, and the aqueous phase was extracted with DCM (3 x 25 mL). The combined organic phases were combined dried over Na_2SO_4, and the solvent removed under reduced pressure to yield 6-chloro-N-(1-cyclobutyl-1H-pyrazol-4-yl)picolinamide (0.042 g, 100%) as a white solid. The crude product was carried forward without any further purification. LRMS (ESI) m/z 277 [(M+H)+ calc'd for $C_{13}H_{13}ClN_4O$: 277]

Step 2: Preparation for N-(1-cyclobuty l-1H-pyrazol-4 -yl)-6-(1-meth yl-1H-pyrazol- 4-yl)picolinamide, TFA

General Procedure: A 30 mL microwave vial was charged with 6-chloro-N-(1-cyclobutyl-1H-pyrazol-4-yl)picolinamide (42 mg, 0.15 mmol), RuPhos Pd G2 (12 mg, 0.015 mmol), 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (47 mg, 0.225 mmol), Dioxane (750 µL), K_3PO_4 solution (2M in water, 0.23 ml, 0.45 mmol). The reaction mixture was purged with nitrogen 3 times and stirred at 80 °C overnight. The reaction mixture was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford N-(1-cyclobuty l-1H-pyrazol-4 -yl)-6-(1-meth yl-1H-pyrazol- 4-yl)picolinamide, TFA (27 mg, 0.027 mmol, 41.2 % yield) as a white solid. 1H NMR (499 MHz, DMSO-*d*6) δ 10.59 (s, 1H), 8.61 (s, 1H), 8.36 (s, 1H), 8.22 (s, 1H), 7.99 (s, 1H), 7.85 (d, *J* = 25.2 Hz, 3H), 4.86 (s, 1H), 2.40 (s, 3H), 1.80 (s, 2H). HRMS (ESI+) m/z 323.15 [(M+H)+ calc'd for $C_{17}H_{18}N_6O$: 323.11].

Scheme 16: Preparation of **5** N-(2-((2S,6 R)-2,6-dimet hylmorpholin o)-5-methyl pyridin-4-yl) -6-(1-methyl -1H-pyrazol -4-yl)picolin amide, TFA



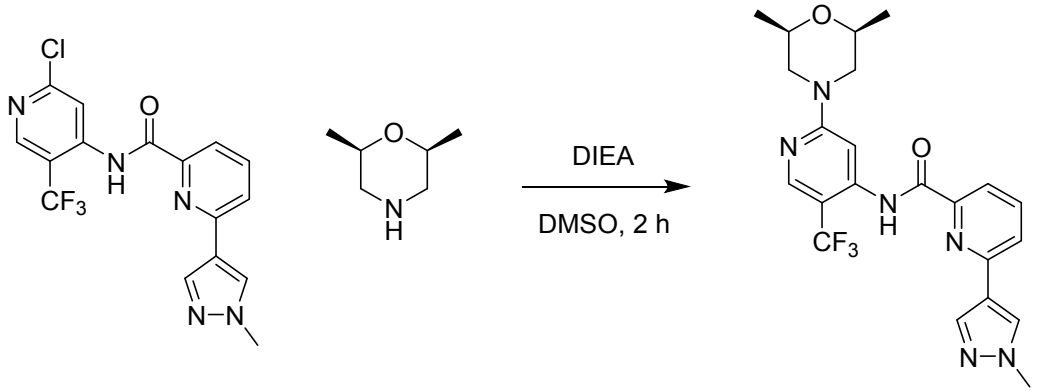
Step 1: Preparation for N-(2-chloro-5-methylpyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide

General Procedure: A 30 mL microwave vial was charged with intermediate **A** (450 mg, 2.2 mmol), COMU (940 mg, 2.2 mmol), DCM (11 ml), TEA (0.9 ml, 6.5 mmol) and the reaction mixture was stirred at rt for 2 hours. The reaction mixture was poured into an separatory funnel containing deionized water (20 mL). The phases were separated, and the aqueous phase was extracted with DCM (3 x 25 mL). The combined organic phases were combined dried over Na_2SO_4, and the solvent removed under reduced pressure to yield N-(2-chloro-5-methylpyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide (717 mg, 100%) as a white solid. The crude product was carried forward without any further purification. LRMS (ESI) m/z 328 [(M+H)+ calc'd for $C_{16}H_{14}ClN_5O$: 328]

Step 2: Preparation for N-(2-((2S,6 R)-2,6-dimet hylmorpholin o)-5-methyl pyridin-4-yl) -6-(1-methyl -1H-pyrazol -4-yl)picolin amide, TFA

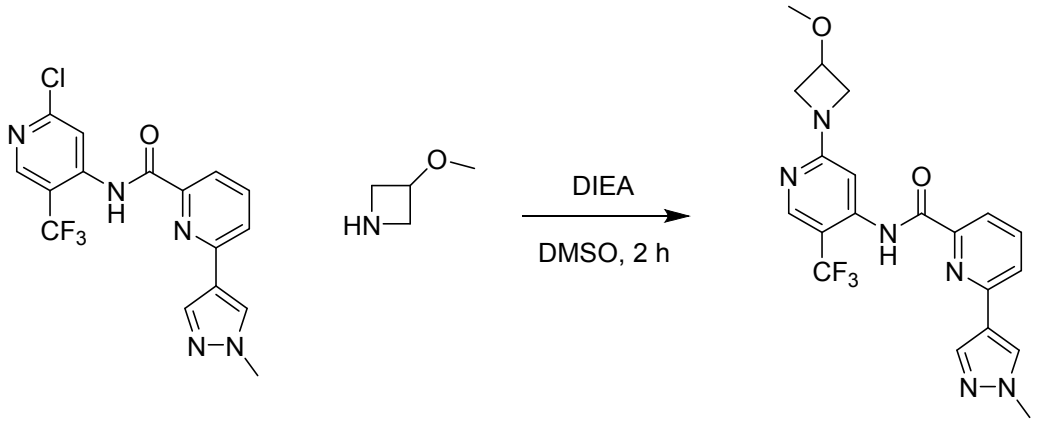
General Procedure: A 30 mL microwave vial was charged with N-(2-chloro-5-methylpyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide (120 mg, 0.11 mmol), DMSO (330 µl), (2R,6S)-2,6-dimethylmorpholine (62 µl, 0.500 mmol), DIEA (96 µl, 0.55 mmol), and the reaction mixture was stirred at 140 °C for 2 hours. The reaction mixture was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford N-(2-((2S,6 R)-2,6-dimet hylmorpholin o)-5-methyl pyridin-4-yl) -6-(1-methyl -1H-pyrazol -4-yl)picolin amide, TFA (3.5 mg, 7.5% yield) as a white solid. ^{1}H NMR (499 MHz, DMSO-d_6) δ 10.73 (s, 1H), 8.47 (s, 1H), 8.20 – 8.07 (m, 3H), 8.00 (s, 3H), 2.76 (s, 3H), 2.43 (s, 3H), 1.20 (s, 12H). HRMS (ESI+) m/z 407.21 [(M+H)+ calc'd for $C_{22}H_{26}N_6O_2$: 407.21].

Scheme 17: Preparation of **6** N-(2-((2S,6R)-2,6-dimethylmorpholino)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide, TFA



General Procedure: A 30 mL microwave vial was charged with intermediate **C** (74 mg, 0.19 mmol) (2R,6S)-2,6-dimethylmorpholine (230 mg, 0.2 mmol), DIEA (340 µl, 0.2 mmol), DMSO (990 µl) and the reaction was heated to 120°C for 2 hours. The reaction was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford N-(2-((2S,6R)-2,6-dimethylmorpholino)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide, TFA (45 mg, 0.2 mmol) as a white solid. ^{1}H NMR (499 MHz, DMSO-d_6) δ 10.76 (s, 1H), 8.37 (s, 1H), 8.33 (s, 1H), 8.04 (d, J = 9.0 Hz, 2H), 7.96 – 7.89 (m, 3H), 4.15 (d, J = 11.5 Hz, 2H), 3.89 (s, 3H), 3.58 (s, 2H), 2.54 (t, J = 11.7 Hz, 2H), 1.14 (d, J = 6.0 Hz, 5H), -0.05 (s, 1H). HRMS (ESI+) m/z 461.18 [(M+H)+ calc'd for $C_{22}H_{23}F_3N_6O_2$: 461.18].

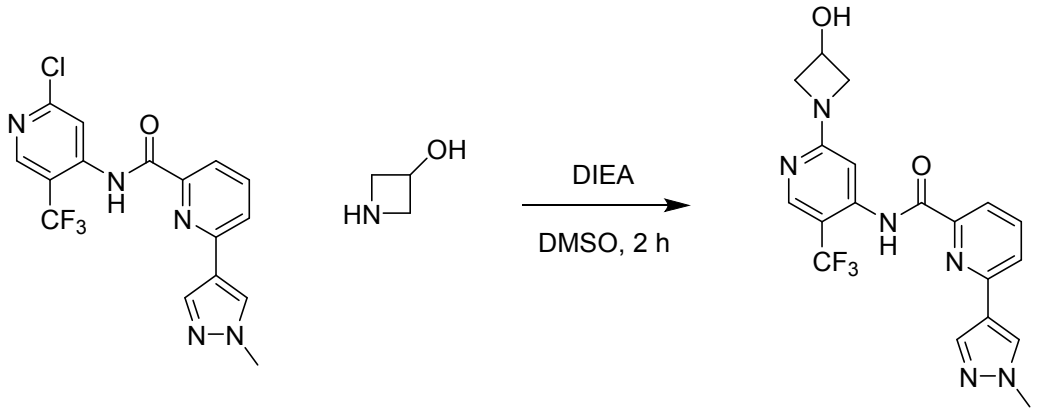
Scheme 18: Preparation of **7** N-(2-(3-methoxyazetidin-1-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide, TFA



General Procedure: A 30 mL microwave vial was charged with intermediate **C** (51 mg, 0.13 mmol), 3-methoxyazetidine, HCl (32 mg, 0.26 mmol), DMSO (0.9 ml) and DIEA (100 µl, 0.6 mmol). The resulting mixture was allowed to stir overnight at 80 °C. The reaction was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford N-(2-(3-methoxyazetidin-1-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-

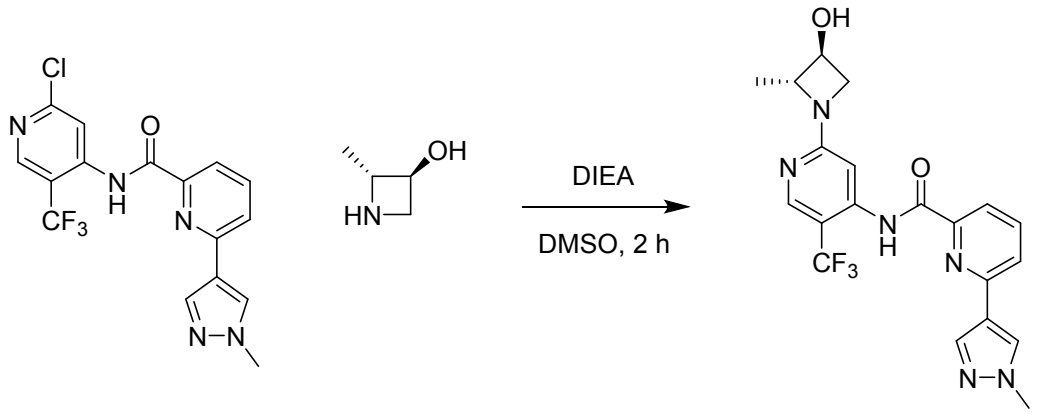
methyl-1H-pyrazol-4-yl)picolinamide, TFA (25.2 mg, 35.2 % yield) as a white solid. ^{1}H NMR (499 MHz, DMSO-d_6) δ 10.84 (s, 1H), 8.39 (d, J = 14.8 Hz, 2H), 8.10 (dd, J = 15.0, 6.8 Hz, 2H), 7.99 (t, J = 6.9 Hz, 2H), 7.59 (s, 1H), 4.42 – 4.24 (m, 3H), 3.93 (d, J = 16.8 Hz, 5H), 3.29 (s, 3H). HRMS (ESI+) m/z 433.15 [(M+H)+ calc'd for $C_{20}H_{19}F_3N_6O_2$: 433.15].

Scheme 19: Preparation of **8** N-(2-(3-hydroxyazetidin-1-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide, TFA



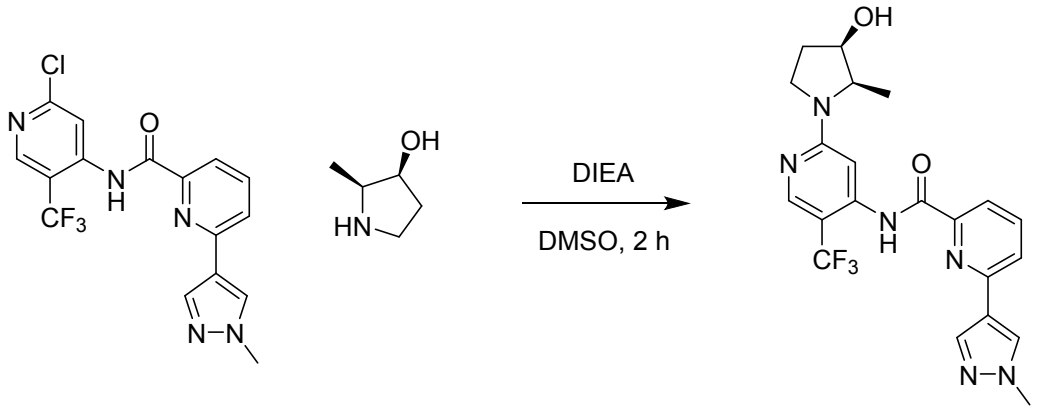
General Procedure: A 30 mL microwave vial was charged with intermediate **C** (20 mg, 0.05 mmol), azetidin-3-ol, HCl (11.5 mg, 0.105 mmol), DIEA (28 µl, 0.16 mmol), and DMSO (260 µl). The resulting mixture was allowed to stir overnight at 80 °C. The reaction was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford N-(2-(3-hydroxyazetidin-1-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide, TFA (14 mg, 0.026 mmol, 49.8 % yield). ^{1}H NMR (499 MHz, DMSO-d_6) δ 10.78 (s, 1H), 8.32 (d, J = 12.3 Hz, 2H), 8.05 (dd, J = 16.6, 9.0 Hz, 2H), 7.93 (t, J = 7.1 Hz, 2H), 7.51 (s, 1H), 6.09 – 5.89 (m, 1H), 4.59 (s, 1H), 4.26 (t, J = 7.3 Hz, 2H), 3.89 (s, 3H), 3.82 – 3.75 (m, 2H). HRMS (ESI+) m/z 419.14 [(M+H)+ calc'd for $C_{19}H_{17}F_3N_6O_2$: 419.14].

Scheme 20: Preparation of **9** N-(2-((2R,3S)-3-hydroxy-2-methylazetidin-1-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide, TFA



General Procedure: A 30 mL microwave vial was charged with intermediate **C** (20 mg, 0.05 mmol), (2R,3S)-2-methylazetidin-3-ol (9.1 mg, 0.105 mmol), DIEA (28 µl, 0.16 mmol), and DMSO (260 µl). The resulting mixture was allowed to stir overnight at 80 °C. The reaction was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford N-(2-((2R,3S)-3-hydroxy-2-methylazetidin-1-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide, TFA (14 mg, 0.026 mmol, 48.9 % yield) as a white solid. ^{1}H NMR (499 MHz, DMSO-d_6) δ 10.79 (s, 1H), 8.33 (d, J = 20.4 Hz, 2H), 8.05 (dd, J = 16.4, 8.8 Hz, 2H), 7.94 (d, J = 7.6 Hz, 2H), 7.59 (s, 1H), 6.08 – 5.93 (m, 1H), 4.22 (d, J = 7.3 Hz, 1H), 4.13 – 4.02 (m, 2H), 3.89 (s, 3H), 3.61 – 3.54 (m, 1H), 1.43 (d, J = 6.0 Hz, 3H). HRMS (ESI+) m/z 433.15 [(M+H)+ calc'd for $C_{20}H_{19}F_3N_6O_2$: 433.15].

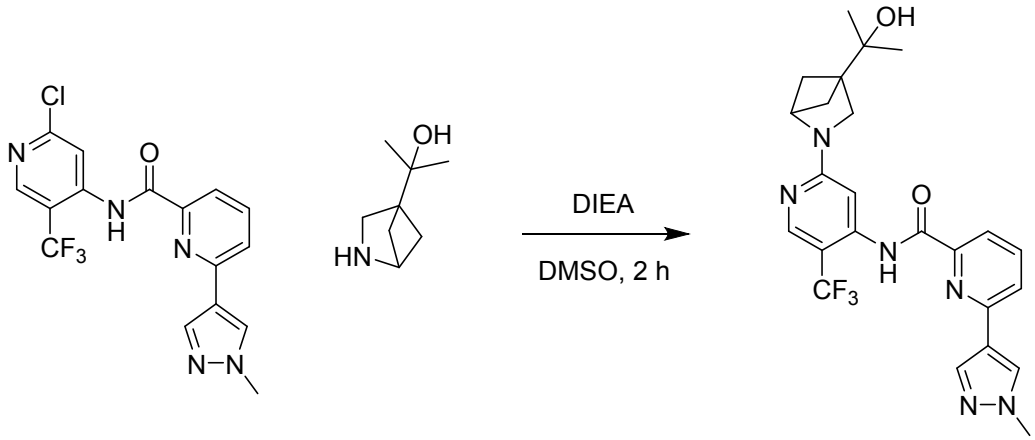
Scheme 21: Preparation of **10** N-(2-((2R,3R)-3-hydroxy-2-methylpyrrolidin-1-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide (Peak 1)



General Procedure: A 30 mL microwave vial was charged with intermediate **C** (50 mg, 0.13 mmol), Rac-(2S,3S)-2-methylpyrrolidin-3-ol, HCl (54 mg, 0.39 mmol), DIEA (110 µl, 0.65 mmol), and DMSO (430 µl). The reaction was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed

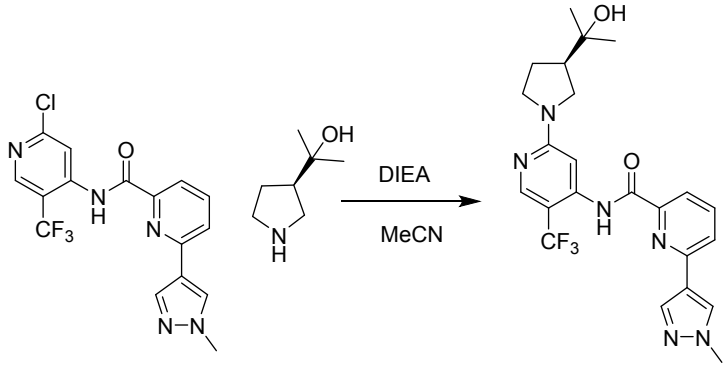
reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford the racemate. The racemic material could be resolved to its component enantiomers by chiral preparative SFC (Column & dimensions: CCA F4, 21 mm x 250 mm; Mobile phase A: CO_2; Mobile phase B: MeOH with 0.1% NH_4OH) to afford N-(2-((2S,3S)-3-hydroxy-2-methylpyrrolidin-1-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide (12 mg, 0.027 mmol, 19.84%). ^{1}H NMR (499 MHz, DMSO-d_6) δ 10.74 (s, 1H), 8.33 (d, *J* = 13.8 Hz, 2H), 8.04 (d, *J* = 10.4 Hz, 2H), 7.93 (d, *J* = 6.6 Hz, 2H), 7.65 (d, *J* = 13.6 Hz, 1H), 5.14 (s, 1H), 4.24 (s, 1H), 4.08 (d, *J* = 6.0 Hz, 1H), 3.50 (d, *J* = 31.8 Hz, 1H), 2.06 (s, 1H), 1.90 (d, *J* = 9.2 Hz, 1H), 1.34 – 1.02 (m, 6H), 0.85 – 0.79 (m, 1H). HRMS (ESI+) m/z 447.17 [(M+H)+ calc'd for $C_{21}H_{21}F_3N_6O_2$: 447.17].

Scheme 22: Preparation of **11** N-(2-(4-(2-hydroxypropan-2-yl)-2-azabicyclo[2.1.1]hexan-2-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide 2,2,2-trifluoroacetate, TFA



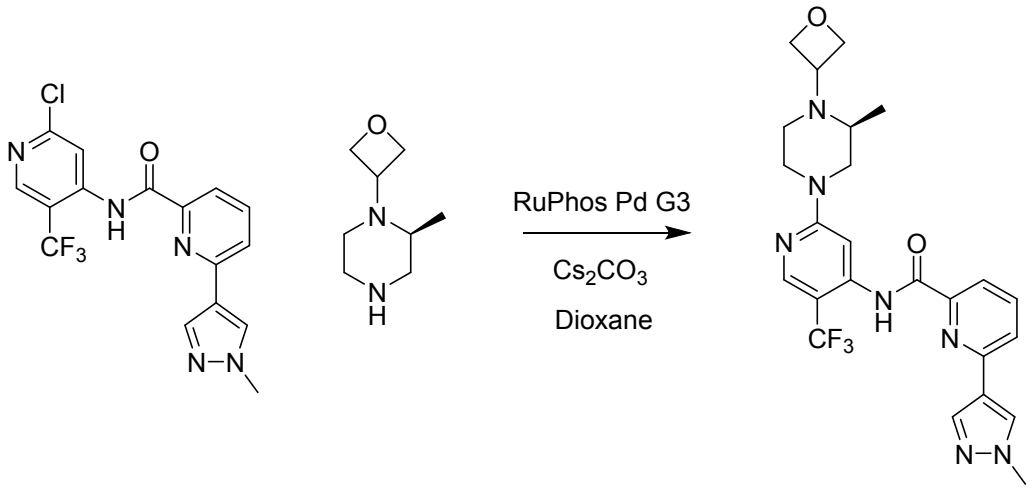
General Procedure: A 30 mL microwave vial was charged with intermediate **C** (70 mg, 0.18 mmol), 2-(2-azabicyclo[2.1.1]hexan-4-yl)propan-2-ol (39 mg, 0.28 mmol), DIEA (160 µl, 0.92 mmol), and DMSO (610 µl). The reaction was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford N-(2-(4-(2-hydroxypropan-2-yl)-2-azabicyclo[2.1.1]hexan-2-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide 2,2,2-trifluoroacetate, TFA (15 mg, 0.025 mmol, 13.6%). ^{1}H NMR (600 MHz, DMSO-d6) δ 10.80 (s, 1H), 8.37 (s, 1H), 8.35 (s, 1H), 8.10 (dd, J = 15.8, 8.0 Hz, 2H), 7.98 (t, J = 6.9 Hz, 2H), 7.76 (s, 1H), 4.79 (s, 1H), 3.94 (s, 3H), 1.93 (s, 2H), 1.43 (s, 2H), 1.17 (s, 6H), 0.00 (s, 3H). HRMS (ESI+) m/z 487.20 [(M+H)+ calc'd for $C_{24}H_{25}F_3N_6O_2$: 487.20].

Scheme 23: Preparation of **12** (R)-N-(2-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide



General Procedure: A 30 mL microwave vial was charged with intermediat **C** (1.3 g, 3.4 mmol), (R)-2-(pyrrolidin-3-yl)propan-2-ol (0.48 g, 3.7 mmol), potassium carbonate (0.71 g, 5.1 mmol), and Acetonitrile (11 ml). The reaction mixture was stirred at 65 °C overnight. The reaction mixture was cooled to rt and was added dropwise to a 100 mL erlenmeyer flask containing 50 mL of deionized water. The precipitate formed was filtered and dried to afford (R)-N-(2-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-5-(trifluoromethyl)pyridin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide (1.5 g, 91%) as a white solid. 1H NMR (600 MHz, DMSO-*d*6) δ 10.74 (s, 1H), 8.33 (d, *J* = 3.3 Hz, 2H), 8.05 (d, *J* = 11.2 Hz, 2H), 7.93 (d, *J* = 7.2 Hz, 2H), 7.64 (s, 1H), 4.39 (s, 1H), 3.89 (s, 3H), 3.31 (s, 1H), 2.32 (d, *J* = 29.0 Hz, 1H), 1.89 (d, *J* = 37.8 Hz, 2H), 1.12 (d, *J* = 12.1 Hz, 6H), -0.05 (s, 3H). HRMS (ESI+) m/z 475.20 [(M+H)+ calc'd for $C_{23}H_{25}F_3N_6O_2$: 475.20].

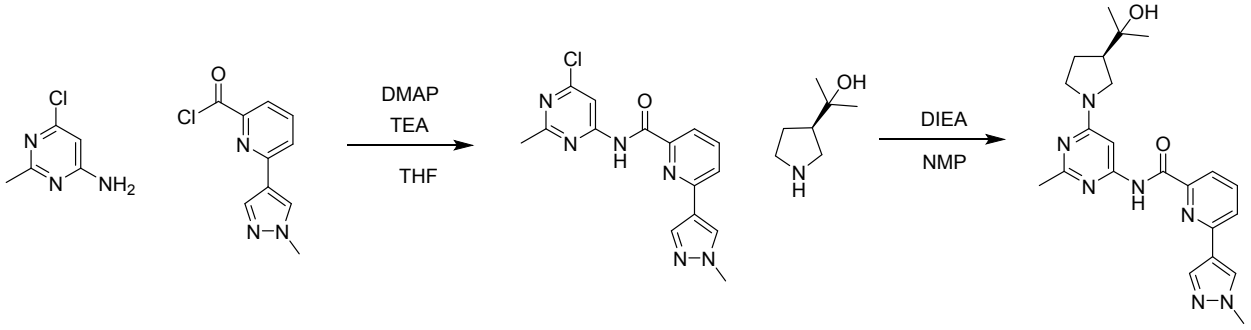
Scheme 24: Preparation of **13** (S)-6-(1-methyl-1H-pyrazol-4-yl)-N-(2-(3-methyl-4-(oxetan-3-yl)piperazin-1-yl) 5-(trifluoromethyl)pyridin-4-yl)picolinamide, TFA



General Procedure: A 30 mL microwave vial was charged with intermediate **C** (75 mg, 0.19 mmol), intermediate **H** (150 mg, 0.39 mmol), RuPhos Pd G3 (30 mg, 0.04 mmol), and cesium carbonate (32 mg, 0.98 mmol). The vial was purged with nitrogen and dioxane (1 mL) was added. The reaction was cooled to rt, concentrated under reduced pressure, diluted in 4 mL of DMSO, filtered and purified by mass-directed reverse phase chromatography eluting with

MeCN/H_2O with 0.1% TFA with a linear gradient to afford (S)-6-(1-methyl-1H-pyrazol-4-yl)-N-(2-(3-methyl-4-(oxetan-3-yl)piperazin-1-yl) 5-(trifluoromethyl)pyridin-4-yl)picolinamide, TFA (38.5 mg, 0.196 mmol, 31.8%). ^{1}H NMR (499 MHz, DMSO-d6) δ 10.85 (s, 1H), 8.48 (s, 1H), 8.39 (s, 1H), 8.09 (t, J = 12.3 Hz, 3H), 7.99 (t, J = 7.4 Hz, 2H), 4.77 (d, J = 29.2 Hz, 3H), 4.66 (s, 1H), 3.94 (s, 3H), 3.52 (s, 7H), 1.26 (d, J = 25.7 Hz, 1H), 1.14 (s, 3H). HRMS (ESI+) m/z 502.21 [(M+H)+ calc'd for $C_{24}H_{26}F_3N_7O_2$: 502.21].

Scheme 25: Preparation of **17** (R)-N-(6-(3-(2- hydroxypropa n-2-yl)pyrrolidi n-1-yl)-2-meth ylpyrimidin-4-yl)-6-(1-methyl-1 H-pyrazol-4-yl) picolinamide, TFA



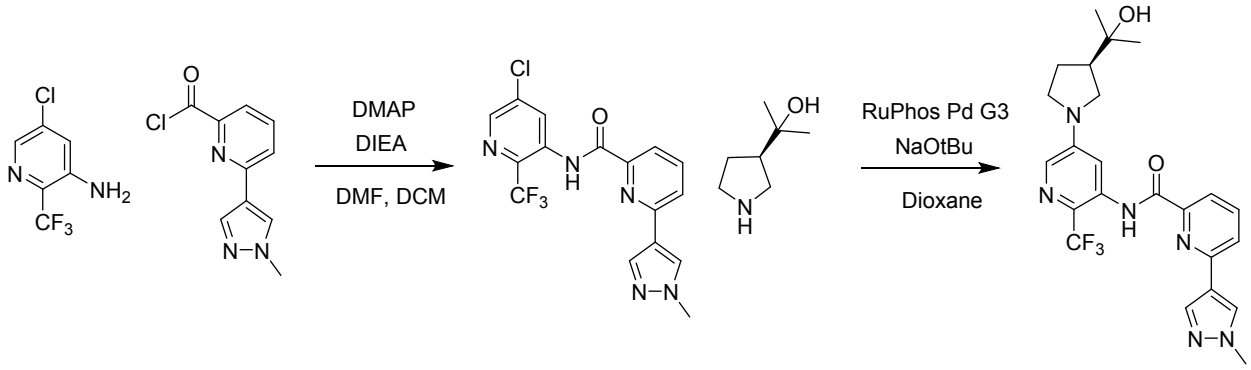
Step 1: Preparation of N-(6-chloro-2-methylpyrimidin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide

General Procedure: A 30 mL microwave vial was charged with 6-chloro-2-methylpyrimidin-4-amine (0.71 g, 4.9 mmol), intermediate **B** (1.09 g, 4.9 mmol), DMAP (1 piece), TEA (2.2 mL, 15 mmol), THF (25 ml) and the reaction mixture was stirred at 80 °C for overnight. The reaction mixture was poured into a separatory funnel containing deionized water (20 mL). The phases were separated, and the aqueous phase was extracted with DCM (3 x 25 mL). The combined organic phases were combined dried over Na_2SO_4, and the solvent removed under reduced pressure to yield N-(6-chloro-2-methylpyrimidin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide (1.6 g, 4.9 mmol, 100%) as a brown solid. The crude product was carried forward without any further purification. LRMS (ESI) m/z 329 [(M+H)+ calc'd for $C_{15}H_{13}ClN_6O$: 329]

Step 2: Preparation of (R)-N-(6-(3-(2- hydroxypropa n-2-yl)pyrrolidi n-1-yl)-2-meth ylpyrimidin-4-yl)-6-(1-methyl-1 H-pyrazol-4-yl) picolinamide, TFA

General Procedure: A 30 mL microwave vial was charged with N-(6-chloro-2-methylpyrimidin-4-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide (130 mg, 0.41 mmol), (R)-2-(pyrrolidin-3-yl)propan-2-ol, HCl (130 mg, 0.81 mmol), NMP (670 µl), DIEA (105 µl, 0.600 mmol), and mixture was heated to 140 °C overnight. The reaction mixture was cooled to rt, filtered, concentrated, and diluted in DMSO (4 mL). The crude product was purified by mass-directed reverse phase chromatography eluting with MeCN/H_2O with 0.1% TFA with a linear gradient to afford (R)-N-(6-(3-(2- hydroxypropa n-2-yl)pyrrolidi n-1-yl)-2-meth ylpyrimidin-4-yl)-6-(1-methyl-1 H-pyrazol-4-yl) picolinamide, TFA (91.5 mg, 42.7%) as a white solid. ^{1}H NMR (499 MHz, DMSO-d6) δ 10.44 (s, 1H), 8.53 (s, 1H), 8.19 (s, 1H), 8.08 (s, 1H), 7.99 (s, 2H), 7.44 (s, 1H), 3.97 (s, 3H), 3.91 – 3.43 (m, 7H), 2.42 (s, 1H), 2.11 (s, 3H), 1.20 (d, J = 13.8 Hz, 6H). HRMS (ESI+) m/z 422.22 [(M+H)+ calc'd for $C_{22}H_{27}N_7O_2$: 422.22].

Scheme 26: Preparation of **18** (R)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide



Step 1: Preparation of 6-bromo-N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)picolinamide

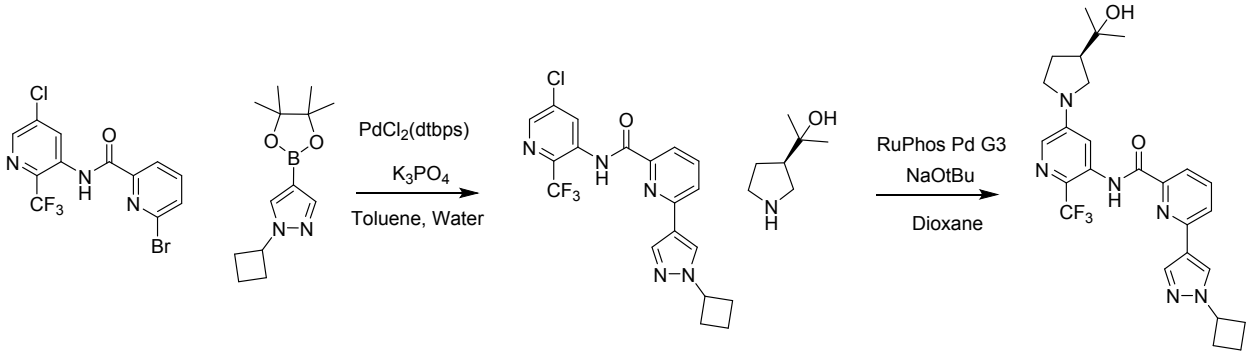
General Procedure: A 30 mL microwave vial was charged with 5-chloro-2-(trifluoromethyl)pyridin-3-amine hydrochloride (230 mg, 0.98 mmol), DIEA (860 µl, 4.9 mmol), **intermediate B** (220 mg g, 0.98 mmol), DMAP (12 mg, 0.098 mmol), DIEA (0.860 mL, 4.9 mmol), DMF (2 drops), DCM (4 mL) and the reaction mixture was stirred at 80 °C for overnight. The reaction mixture was poured into a separatory funnel containing deionized water (20 mL). The phases were separated, and the aqueous phase was extracted with DCM (3 x 25 mL). The combined organic phases were combined dried over Na_2SO_4, and the solvent removed under reduced pressure. The crude mixture was diluted in DCM and was purified by flash chromatography using pre-packed RediSep R_f silica gel columns on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-100% Hexanes/Ethyl acetate to afford the desired product N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide (125 mg, 0.327 mmol, 33.3 % yield) as a brown solid. LRMS (ESI) m/z 382 [(M+H)+ calc'd for $C_{16}H_{11}ClF_3N_5O$: 382]

Step 2: Preparation of (R)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide

General Procedure: A 30 mL microwave vial was charged with 6-bromo-N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)picolinamide (501 mg, 1.31 mmol), (R)-2-(pyrrolidin-3-yl)propan-2-ol (251 mg, 1.94 mmol), RuPhos Pd G3 (200 mg, 0.24 mmol), sodium tert-Butoxide (2M in THF) (2000 µl, 4.00 mmol) and THF (6000 µl). The reaction mixture was purged with nitrogen three times and the reaction mixture was allowed to stir overnight at 80 °C. The reaction mixture was diluted with Ethyl Acetate and washed twice with saturated ammonium chloride and once with brine. The combined organic fractions were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The crude mixture was diluted in DCM and was purified by flash chromatography using pre-packed RediSep R_f silica gel columns on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-100% Hexanes/Ethyl acetate to afford the desired product (R)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)-6-(1-methyl-1H-pyrazol-4-yl)picolinamide (120 mg, 19.31 % yield) as a white solid. 1H NMR (499 MHz, DMSO-*d*6) δ 10.71 (s, 1H), 8.43 (s, 1H), 8.14 (s, 1H), 8.07 (t, *J* = 7.7 Hz, 1H), 7.95 (dd, *J* = 7.6, 3.2 Hz, 2H), 7.84 (d, *J* = 6.2 Hz, 2H), 4.46 (s, 1H), 3.93 (s, 3H), 3.52 (d, *J* = 8.6 Hz, 1H), 3.40 (t, *J* = 8.9 Hz, 1H),

2.36 (p, *J* = 8.8 Hz, 1H), 1.96 (dd, *J* = 38.9, 8.7 Hz, 3H), 1.17 (d, *J* = 10.3 Hz, 7H). HRMS (ESI+) m/z 475.20 [(M+H)+ calc'd for $C_{23}H_{25}F_3N_6O_2$: 475.20].

Scheme 27: Preparation of **19** (R)-6-(1-cyclobutyl-1H-pyrazol-4-yl)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)picolinamide, TFA



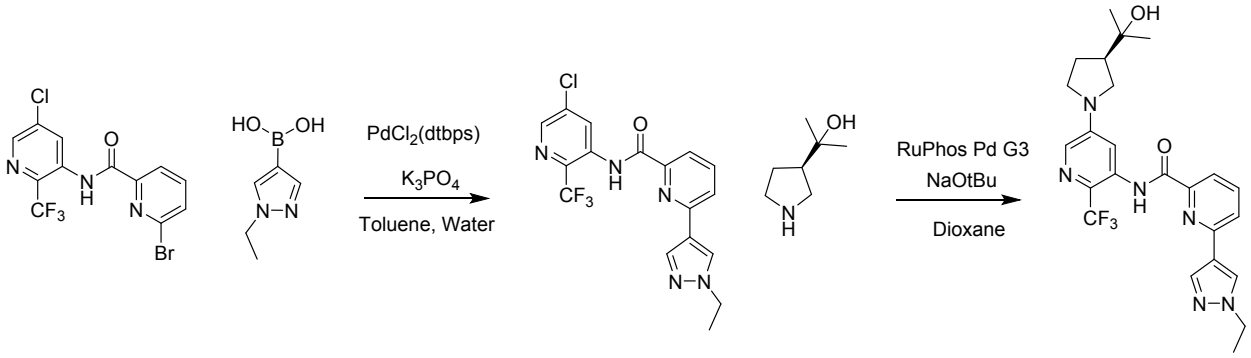
Step 1: Preparation of N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-cyclobutyl-1H-pyrazol-4-yl)picolinamide

General Procedure: A 30 mL microwave vial was charged with intermediate **D** (101 mg, 0.26 mmol), 1-cyclobutyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (82 mg, 0.33 mmol), potassium phosphate (110 mg, 0.52 mmol), and 1,1'-bis(di-tert-butylphosphino)ferrocene palladium dichloride (8.6 mg, 0.013 mmol) under inert atmosphere. Toluene (1.1 mL) and water (0.2 mL) were added and the resulting mixture was allowed to stir at room temperature for 7 hours. At 7 hrs, the reaction mixture was concentrated under reduced pressure. The crude mixture was diluted in DCM and was purified by flash chromatography using pre-packed RediSep R_f silica gel columns on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-100% Hexanes in 3:1 Ethyl acetate/Ethanol to afford N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-cyclobutyl-1H-pyrazol-4-yl)picolinamide (98 mg, 88 % yield). LRMS (ESI) m/z 422 [(M+H)+ calc'd for $C_{19}H_{15}ClF_3N_5O$: 422].

Step 2: Preparation of (R)-6-(1-cyclobutyl-1H-pyrazol-4-yl)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)picolinamide, TFA

General Procedure: A 30 mL microwave vial was charged N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-cyclobutyl-1H-pyrazol-4-yl)picolinamide (101 mg, 0.24 mmol), (R)-2-(pyrrolidin-3-yl)propan-2-ol, HCl (59 mg, 0.35 mmol), RuPhos Pd G3 (20 mg, 0.025 mmol), sodium tert-butoxide (68 mg, 0.71 mmol), and dioxane (0.8 mL) under inert atmosphere. The reaction mixture was purged with nitrogen three times and the reaction was allowed to stir overnight at 80 °C. The crude product was purified by mass-directed reverse phase chromatography eluting with MeCN/H2O with 0.1% TFA with a linear gradient to afford (R)-6-(1-cyclobutyl-1H-pyrazol-4-yl)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)picolinamide, TFA (50 mg, 0.080 mmol, 33.6 % yield) as a white solid. ^{1}H NMR (600 MHz, DMSO-*d*6) δ 10.69 (s, 1H), 8.53 (s, 1H), 8.14 (s, 1H), 8.03 (t, *J* = 7.6 Hz, 1H), 7.92 (dd, *J* = 20.9, 7.5 Hz, 2H), 7.81 (s, 2H), 4.91 – 4.81 (m, 1H), 4.41 (s, 1H), 3.48 (s, 1H), 3.35 (s, 3H), 2.57 (s, 1H), 2.33 (d, *J* = 9.9 Hz, 2H), 2.04 – 1.75 (m, 5H), 1.12 (d, *J* = 12.6 Hz, 7H). HRMS (ESI) m/z 515.23 [(M+H)+ calc'd for $C_{26}H_{29}F_3N_6O_2$: 515.23].

Scheme 28: Preparation of **20** (R)-6-(1-ethyl-1H-pyrazol-4-yl)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)picolinamide, TFA



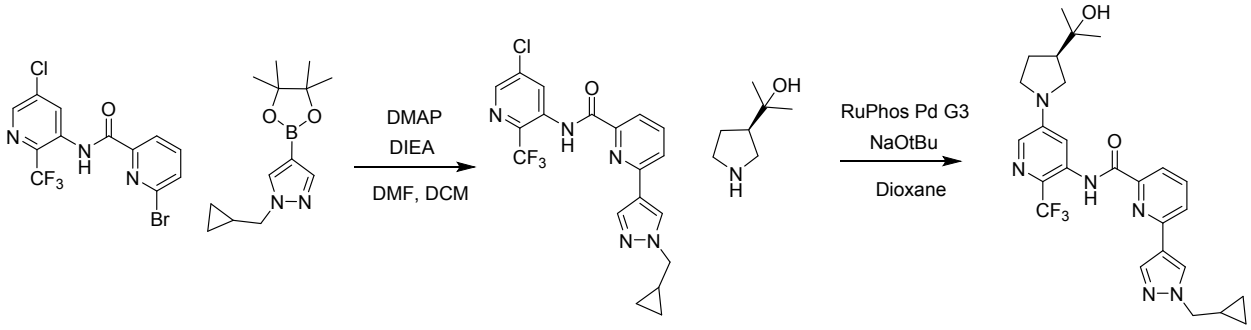
Step 1: Preparation of N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-ethyl-1H-pyrazol-4-yl)picolinamide

General Procedure: A 30 mL microwave vial was charged with intermediate **D** (101 mg, 0.26 mmol), (1-ethyl-1H-pyrazol-4-yl)boronic acid (110 mg, 0.82 mmol), potassium phosphate (280 mg, 1.3 mmol), and 1,1'-bis(di-tert-butylphosphino)ferrocene palladium dichloride (22 mg, 0.033 mmol) under inert atmosphere. Toluene (2.8 mL) and water (0.55 mL) were added and the resulting mixture was allowed to stir at room temperature for 7 hours. At 7 hrs, the reaction mixture was concentrated under reduced pressure. The crude mixture was diluted in DCM and was purified by flash chromatography using pre-packed RediSep R_f silica gel columns on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-100% Hexanes in 3:1 Ethyl acetate/Ethanol to afford N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-ethyl-1H-pyrazol-4-yl)picolinamide (240 mg, 0.61 mmol, 92 % yield). LRMS (ESI) m/z 396 [(M+H)+ calc'd for $C_{17}H_{13}ClF_3N_5O$: 396].

Step 2: Preparation of (R)-6-(1-ethyl-1H-pyrazol-4-yl)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)picolinamide, TFA

General Procedure: A 30 mL microwave vial was charged N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-ethyl-1H-pyrazol-4-yl)picolinamide (25 mg, 0.063 mmol), (R)-2-(pyrrolidin-3-yl)propan-2-ol, HCl (16 mg, 0.095 mmol), RuPhos Pd G3 (5.3 mg, 0.006 mmol), sodium tert-butoxide (18 mg, 0.190 mmol), and dioxane (0.2 mL) under inert atmosphere. The reaction mixture was purged with nitrogen three times and the reaction was allowed to stir overnight at 80 °C. The crude product was purified by mass-directed reverse phase chromatography eluting with MeCN/H2O with 0.1% TFA with a linear gradient to (R)-6-(1-ethyl-1H-pyrazol-4-yl)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)picolinamide, TFA (15 mg, 0.025 mmol, 39.4 % yield) as a white solid. ^{1}H NMR (600 MHz, DMSO-*d*6) δ 10.68 (s, 1H), 8.43 (s, 1H), 8.10 (s, 1H), 8.02 (t, *J* = 7.6 Hz, 1H), 7.95 – 7.87 (m, 2H), 7.80 (d, *J* = 6.5 Hz, 2H), 4.21 – 4.13 (m, 2H), 3.37 – 3.31 (m, 2H), 3.25 (dt, *J* = 18.6, 8.4 Hz, 2H), 2.35 – 2.27 (m, 1H), 2.00 – 1.84 (m, 2H), 1.40 (t, *J* = 7.1 Hz, 3H), 1.12 (d, *J* = 12.6 Hz, 7H). HRMS (ESI) m/z 489.21 [(M+H)+ calc'd for $C_{24}H_{27}F_3N_6O_2$: 489.21].

Scheme 29: Preparation of **21** *(R)*-6-(1-(cyclopropylmethyl)-1H-pyrazol-4-yl)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)picolinamide, TFA



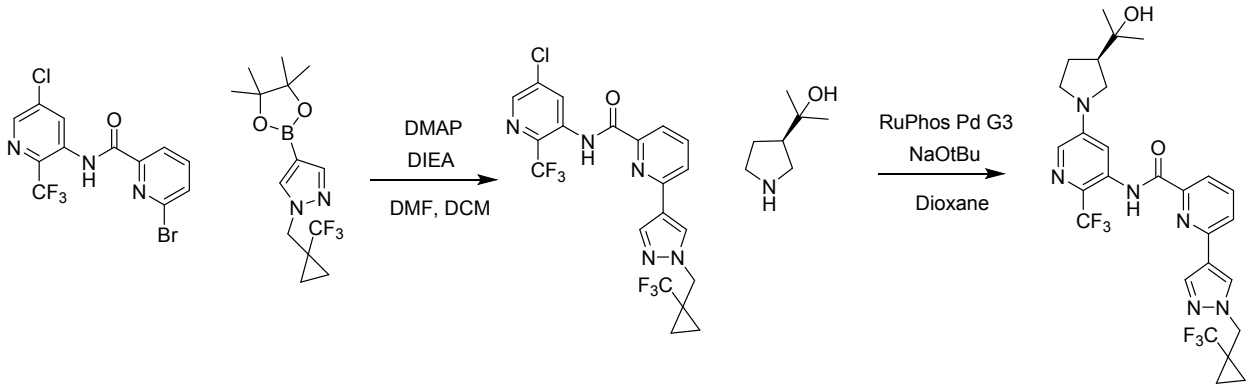
Step 1: Preparation of N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-(cyclopropylmethyl)-1H-pyrazol-4-yl)picolinamide

General Procedure: A 30 mL microwave vial was charged with intermediate **D** (701 mg, 1.84 mmol), 1,1'-bis(di-tert-butylphosphino)ferrocene palladium dichloride (240 mg, 0.37 mmol), potassium phosphate (780 mg, 3.7 mmol), and intermediate **I** (550 mg, 2.2 mmol) under inert atmosphere. Toluene (5.3 ml) was added and the resulting mixture was allowed to stir at room temperature for 7 hours. At 7 hrs, the reaction mixture was concentrated under reduced pressure. The crude mixture was diluted in DCM and was purified by flash chromatography using pre-packed RediSep R_f silica gel columns on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-100% Hexanes in 3:1 Ethyl acetate/Ethanol to afford the desired product N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-(cyclopropylmethyl)-1H-pyrazol-4-yl)picolinamide (540 mg, 1.28 mmol, 69.6 % yield). LRMS (ESI) m/z 422 [(M+H)+ calc'd for $C_{19}H_{15}ClF_3N_5O$: 422].

Step 2: Preparation for *(R)*-6-(1-(cyclopropylmethyl)-1H-pyrazol-4-yl)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)picolinamide, TFA

General Procedure: A 30 mL microwave vial was charged with N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-(cyclopropylmethyl)-1H-pyrazol-4-yl)picolinamide (65 mg, 0.15 mmol), (R)-2-(pyrrolidin-3-yl)propan-2-ol, HCl (38 mg, 0.23 mmol), RuPhos Pd G3 (13 mg, 0.015 mmol), Dioxane (770 µl) and sodium tert-butoxide (44 mg, 0.46 mmol) under inert atmosphere. The reaction mixture was purged with nitrogen three times and the reaction was allowed to stir overnight at 80 °C. The crude product was purified by mass-directed reverse phase chromatography eluting with MeCN/H2O with 0.1% TFA with a linear gradient to afford (R)-6-(1-(cyclopropylmethyl)-1H-pyrazol-4-yl)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)picolinamide, TFA (24 mg, 0.038 mmol, 24.8 % yield). as a white solid. ^{1}H NMR (600 MHz, DMSO-*d*6) δ 10.69 (s, 1H), 8.44 (s, 1H), 8.11 (s, 1H), 8.03 (t, *J* = 7.5 Hz, 1H), 7.93 (d, *J* = 7.6 Hz, 1H), 7.90 (d, *J* = 7.3 Hz, 1H), 7.87 (s, 1H), 7.78 (s, 1H), 4.01 (d, *J* = 6.8 Hz, 2H), 3.47 (s, 1H), 3.33 (d, *J* = 8.1 Hz, 1H), 3.25 (dt, *J* = 18.2, 8.5 Hz, 2H), 2.30 (d, *J* = 7.9 Hz, 1H), 1.98 – 1.84 (m, 2H), 1.25 (s, 1H), 1.12 (d, *J* = 12.9 Hz, 7H), 0.56 (d, *J* = 6.6 Hz, 2H), 0.42 – 0.35 (m, 2H). HRMS (ESI) m/z 515.23 [(M+H)+ calc'd for $C_{26}H_{29}F_3N_6O_2$: 515.23].

Scheme 30: Preparation of **22** *(R)-N*-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)-6-(1-((1-(trifluoromethyl)cyclopropyl)methyl)-1H-pyrazol-4-yl)picolinamide, TFA



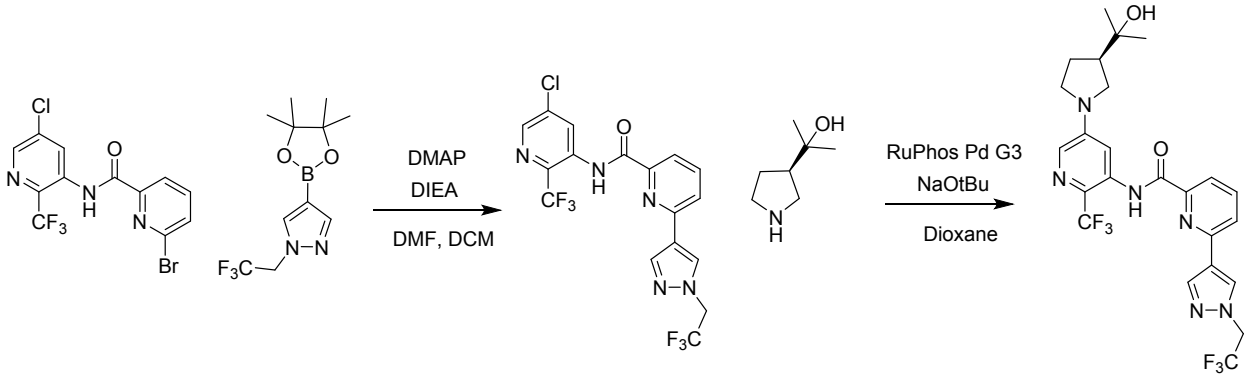
Step 1: Preparation of N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-((1-(trifluoromethyl)cyclopropyl)methyl)-1H-pyrazol-4-yl)picolinamide

General Procedure: A 30 mL microwave vial was charged with intermediate **D** (101 mg, 0.32 mmol), 1,1'-bis(di-tert-butylphosphino)ferrocene palladium dichloride (9.1 mg, 0.013 mmol), potassium phosphate (110 mg, 0.52 mmol), and intermediate **J** (101 mg, 0.32 mmol) under inert atmosphere. Toluene (1.1 mL) and water (0.2 mL) was added and the resulting mixture was allowed to stir at room temperature for 7 hours. At 7 hrs, the reaction mixture was concentrated under reduced pressure. The crude mixture was diluted in DCM and was purified by flash chromatography using pre-packed RediSep R_f silica gel columns on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-100% Hexanes in 3:1 Ethyl acetate/Ethanol to afford the desired product N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-((1-(trifluoromethyl)cyclopropyl)methyl)-1H-pyrazol-4 yl)picolinamide (130 mg, 0.212 mmol, 81 % yield). LRMS (ESI) m/z 490 [(M+H)+ calc'd for $C_{20}H_{14}ClF_6N_5O$: 490].

Step 2: Preparation for *(R)-N*-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)-6-(1-((1-(trifluoromethyl)cyclopropyl)methyl)-1H-pyrazol-4-yl)picolinamide, TFA

General Procedure: A 30 mL microwave vial was charged with N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-((1-(trifluoromethyl)cyclopropyl)methyl)-1H-pyrazol-4 yl)picolinamide (65 mg, 0.13 mmol), (R)-2-(pyrrolidin-3-yl)propan-2-ol, HCl (33 mg, 0.21 mmol), RuPhos Pd G3 (11 mg, 0.013 mmol), Dioxane (440 µl) and sodium tert-butoxide (38 mg, 0.41 mmol) under inert atmosphere. The reaction mixture was purged with nitrogen three times and the reaction was allowed to stir overnight at 80 °C. The crude product was purified by mass-directed reverse phase chromatography eluting with MeCN/H2O with 0.1% TFA with a linear gradient to (R)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)-6-(1-((1(trifluoromethyl)cyclopropyl)methyl)-1H-pyrazol-4-yl)picolinamide, TFA (13 mg, 0.020 mmol, 14.71 % yield). as a white solid. ^{1}H NMR (600 MHz, DMSO-d6) δ 10.69 (s, 1H), 8.46 (s, 1H), 8.14 (s, 1H), 8.04 (t, J = 7.6 Hz, 1H), 7.96 (d, J = 7.6 Hz, 1H), 7.92 (d, J = 7.2 Hz, 1H), 7.86 (s, 1H), 7.79 (s, 1H), 4.44 (s, 2H), 3.52 – 3.43 (m, 1H), 3.34 (d, J = 8.2 Hz, 1H), 3.26 (dt, J = 18.4, 8.5 Hz, 2H), 2.36 – 2.27 (m, 1H), 2.00 – 1.92 (m, 1H), 1.92 – 1.83 (m, 1H), 1.12 (d, J = 12.7 Hz, 9H), 1.06 (s, 2H). HRMS (ESI) m/z 583.22 [(M+H)+ calc'd for $C_{27}H_{28}F_6N_6O_2$: 583.22].

Scheme 31: Preparation of **23** *(R)*-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)-6-(1-(2,2,2-trifluoroethyl)-1H-pyrazol-4-yl)picolinamide, TFA



Step 1: Preparation of N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-(2,2,2-trifluoroethyl)-1H-pyrazol-4-yl)picolinamide

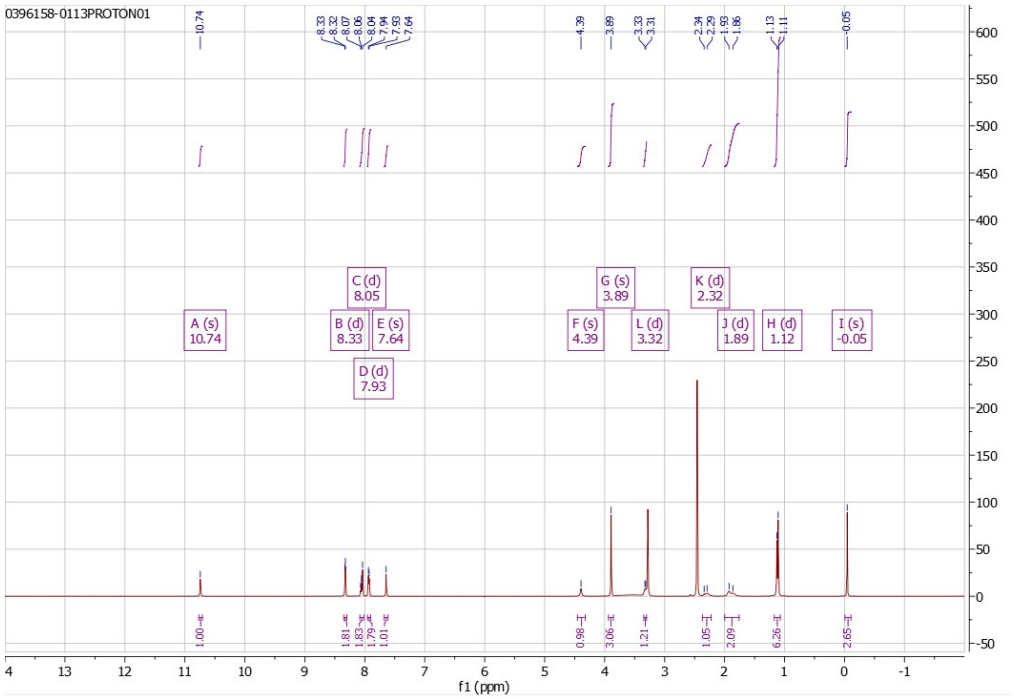
General Procedure: A 30 mL microwave vial was charged with intermediate **D** (501 mg, 1.32 mmol), intermediate **K** (440 mg, 1.6 mmol), aqueous potassium phosphate (1.2 ml, 2.6 mmol), and 1,1'-bis(di-tert-butylphosphino)ferrocene palladium dichloride (43 mg, 0.066 mmol) under inert atmosphere. Toluene (5.3 ml) was added and the resulting mixture was allowed to stir at room temperature for 7 hours. At 7 hrs, the reaction mixture was concentrated under reduced pressure. The crude mixture was diluted in DCM and was purified by flash chromatography using pre-packed RediSep R_f silica gel columns on a Teledyne Isco CombiFlash R_f automated chromatography system using 0-100% Hexanes in 3:1 Ethyl acetate/Ethanol to afford the desired product N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-(2,2,2-trifluoroethyl)-1H-pyrazol-4-yl)picolinamide (260 mg, 44.0 % yield). LRMS (ESI) m/z 450 [(M+H)+ calc'd for $C_{17}H_{10}ClF_6N_5O$: 450].

Step 2: Preparation of *(R)*-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)-6-(1-(2,2,2-trifluoroethyl)-1H-pyrazol-4-yl)picolinamide, TFA

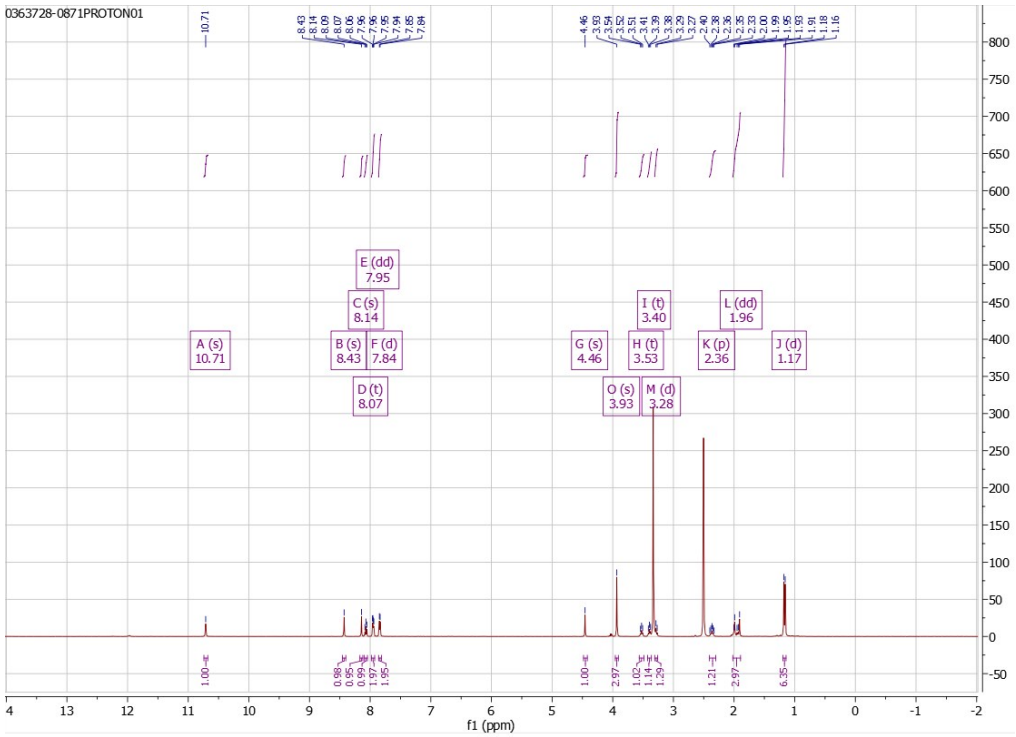
General Procedure: A 30 mL microwave vial was charged N-(5-chloro-2-(trifluoromethyl)pyridin-3-yl)-6-(1-(2,2,2-trifluoroethyl)-1H-pyrazol-4-yl)picolinamide (45 mg, 0.100 mmol), (R)-2-(pyrrolidin-3-yl)propan-2-ol, HCl (25 mg, 0.15 mmol), RuPhos Pd G3 (17 mg, 0.021 mmol), Dioxane (334 µl) and sodium tert-butoxide (29 mg, 0.31 mmol) under inert atmosphere. The reaction mixture was purged with nitrogen three times and the reaction was allowed to stir overnight at 80 °C. The crude product was purified by mass-directed reverse phase chromatography eluting with MeCN/H2O with 0.1% TFA with a linear gradient to afford (R)-N-(5-(3-(2-hydroxypropan-2-yl)pyrrolidin-1-yl)-2-(trifluoromethyl)pyridin-3-yl)-6-(1-(2,2,2-trifluoroethyl)-1H-pyrazol-4-yl)picolinamide, TFA (29 mg, 0.044 mmol, 44 % yield) as a white solid. ^{1}H NMR (600 MHz, DMSO-d6) δ 10.69 (s, 1H), 8.56 (s, 1H), 8.31 (s, 1H), 8.09 (d, J = 7.5 Hz, 1H), 8.03 (d, J = 7.6 Hz, 1H), 7.98 (d, J = 7.3 Hz, 1H), 7.85 – 7.77 (m, 2H), 5.34 – 5.20 (m, 2H), 3.55 – 3.44 (m, 3H), 3.32 – 3.24 (m, 2H), 2.40 – 2.29 (m, 1H), 1.94 (dd, J = 27.6, 17.8 Hz, 2H), 1.16 (d, J = 12.1 Hz, 6H). HRMS (ESI) m/z 543.19 [(M+H)+ calc'd for $C_{24}H_{24}F_6N_6O_2$: 543.19].

5. Copies of ^{1}H NMR spectra for key compounds

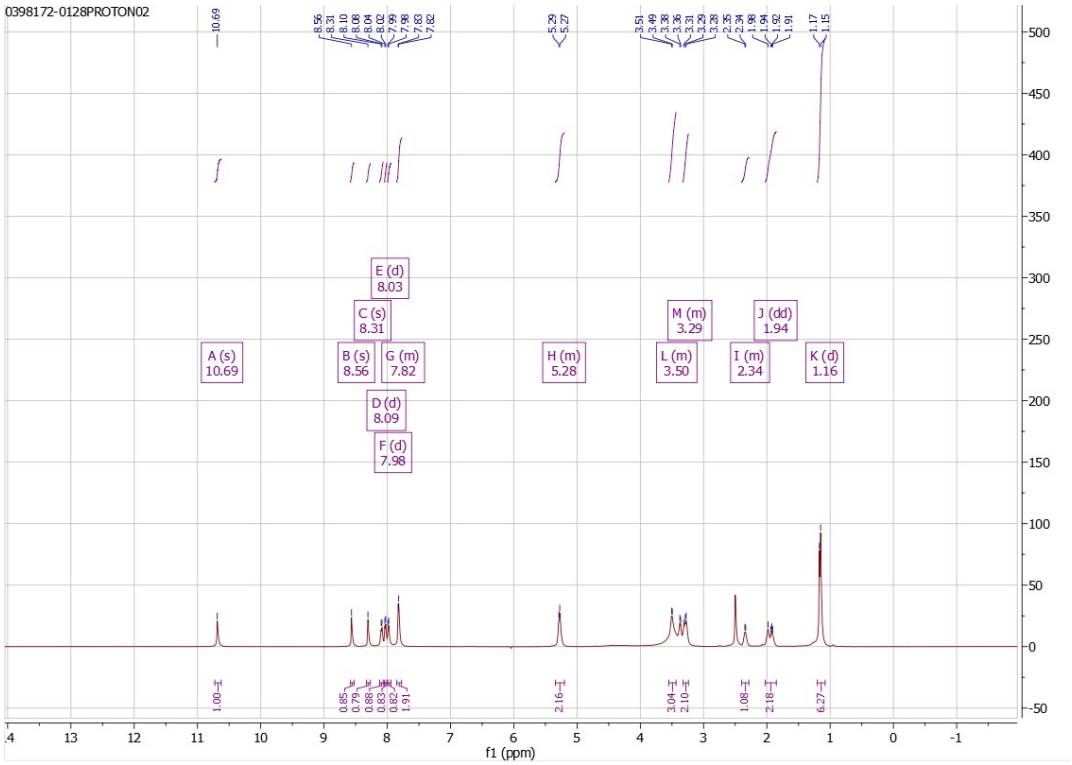
Compound 12



Compound 18



Compound 23


0398172-0128PROTON02
A (s) 10.69
B (s) 8.56
C (s) 8.31
D (d) 8.09
E (d) 8.03
F (d) 7.98
G (m) 7.82
H (m) 5.28
I (m) 2.34
J (dd) 1.94
K (d) 1.16
L (m) 3.50
M (m) 3.29
f1 (ppm)

6. LRRK2 Enzyme Assay: LRRK2 Km ATP LanthaScreen™ Assay

Compound potency against LRRK2 kinase activity was determined using LanthaScreen™ technology from Life Technologies Corporation (Carlsbad, CA) using a GST20 tagged truncated human mutant G2019S LRRK2 in the presence of the fluorescein-labeled peptide substrate LRRKtide® (LRRK2 phosphorylated ezrin/radixin/moesin (ERM)), also from Life Technologies. The data presented for the K_m ATP LanthaScreen™ Assay represents mean IC_{50} values based on several test results and may have reasonable deviations depending on the specific conditions and reagents used. K_m is the Michaelis constant of an enzyme and is defined as the concentration of native substrate (ATP for a kinase) which permits the enzyme to achieve half V_{max} (V_{max} = rate of reaction when the enzyme is saturated with substrate). IC_{50} (half-maximal inhibitory concentration) represents the concentration of inhibitor required to inhibit LRRK2 kinase activity by 50%. Assays were performed in the presence of 134 µM ATP (K_m ATP). Upon completion, the assay was stopped, and phosphorylated substrate detected with a terbium (Tb)-labeled anti-pERM antibody (cat. no. PV4898). The compound dose response was prepared by diluting a 10 mM stock of compound to a maximum concentration of 9.99 µM in 100% DMSO, followed by custom fold serial dilution in DMSO nine times. 20 nL of each dilution was spotted via a Labcyte Echo onto a 384-well black-sided plate (Corning 3575) followed by 15 µl of a 1.25 nM enzyme solution in 1× assay buffer (50 mM Tris pH 8.5, 10 mM $MgCl_2$, 0.01% Brij-35, 1 mM EGTA, 2 mM dithiothreitol, 0.05 mM sodium orthovanadate). Following a 15-minute incubation period at RT, the kinase reaction was started with the addition of 5 µl of 400 nM fluorescein-labeled LRRKtide® (LRRK2 phosphorylated ezrin/radixin/moesin (ERM)) peptide substrate and 134 µM ATP solution in 1× assay buffer. The reaction was allowed to progress at ambient temperature for 90 minutes. The reaction was then stopped by the addition of 20 µl of TR-FRET Dilution Buffer (Life Technologies, Carlsbad, CA) containing 2 nM Tb-labeled anti-phospho LRRKtide® (LRRK2 phosphorylated ezrin/radixin/moesin (ERM)) antibody and 10 mM EDTA (Life Technologies, Carlsbad, CA). After an incubation period of 1 h at RT, the plate was read on an EnVision® multimode plate reader (Perkin Elmer, Waltham, MA) with an excitation wavelength of 337 nm (Laser) and a reading emission at both 520 and 495 nm. Compound IC_{50} values were interpolated from nonlinear regression best-fits of the log of the final compound concentration, plotted as a function of the 520/495-nm emission ratio using activity base "Abase"). Abase uses a 4 parameter (4P) logistic fit based on the Levenberg-Marquardt algorithm.

SI Table A: Number of Experimental Determinations and associated Standard Deviation for LRRK2 Km ATP LanthaScreen™ Assay

Compound Number	Arithmetic Mean LRRK2 G2019S IC_{50}	Standard Deviation (+/-)
1	14 (n=4)	3
2	70 (n=2)	11
3	34 (n=2)	2
4	53 (n=2)	23
5	9 (n=2)	3
6	4 (n=1)	--
7	28 (n=1)	--
8	4.8 (n=1)	--
9	2 (n=1)	--
10	5 (n=1)	--
11	9 (n=1)	--
12	1 (n=3)	0.1
13	1.5 (n=1)	--
17	4 (n=3)	1.1
18	17 (n=2)	13
19	19 (n=1)	--
20	40 (n=1)	--
21	8.6 (n=1)	--
22	8 (n=1)	--
23	10 (n=1)	--

7. LRRK2 Cell assay

LRRK2 G2019S SH-SY5Y human neuroblastoma cell line was used for the LRRK2 pSer935 phosphorylation measurements. The cells were cultured in Dulbecco's modified Eagle's medium/F-12 supplemented with GlutaMax (Life Technologies), 10% tetracycline-free (Tetfree) fetal bovine serum (Clontech), nonessential amino acids (HyClone), pen-strep (100 µg/ml; HyClone) at 37 °C and 5% carbon dioxide. LRRK2 G2019S expression was induced with Tet (2 µg/ml) for 48-72 hours prior to treatment. After 90 minutes of compound incubation, cells were mechanically lifted, pelleted, and lysed with lysis buffer (MSD Lysis Buffer, cat. no. R60TX-3) supplemented with protease (Roche cOmplete Mini, cat. no. 11836170001), and phosphatase inhibitors (Halt Phosphatase Inhibitor, cat. no. 78420; Life Technologies). The lysates were stored at -20 °C until ready for MSD Meso detection. Briefly, two 384-well MSD plates were coated with mouse anti-LRRK2 antibody (Covance) and blocked with 3% BSA. The lysates were dispensed into the MSD plates and incubated with detection antibody rabbit anti LRRK2 pSer935 or rabbit anti-LRRK2 (Abcam) followed by addition of Sulfo-Tag labeled goat antirabbit IgG (MSD) for measuring pSer935 or total LRRK2 levels. The plates were read on S38 SECTOR imager 6000. An IC50 is calculated by fitting the % inhibition data as a function of inhibitor concentration using a standard 4P fit.

SI Table B: Number of Experimental Determinations and associated Standard Deviation for LRRK2 LRRK2 Cell assay

Compound Number	Arithmetic Mean Cell SHSY5Y G2019S IC_{50}	Standard Deviation (+/-)
1	271 (n=1)	--
2	1647 (n=2)	154
3	953 (n=3)	351
4	905 (n=4)	351
5	24 (n=5)	3.7
6	1.5 (n=2)	0.4
7	2.8 (n=2)	1.3
8	7.5 (n=2)	0.9
9	2 (n=2)	1.3
10	3 (n=2)	0.9
11	2 (n=2)	0.7
12	2.5 (n=4)	0.8
13	--	--
17	42 (n=5)	12
18	21 (n=3)	12
19	1.3	--
20	7 (n=2)	4.4
21	0.4 (n=2)	0.3
22	0.3	--
23	0.7 (n=2)	0.6

8. CLK2 Kinase Assay: CLK2 Z'-LYTE Kinase Assay

The CLK2 Z'-LYTE Kinase Assay Kit was purchased from Life Technologies (Carlsbad CA, catalog PV3179), and manufacturer's protocol was followed with some adaptations. The Z´-LYTE biochemical assay employs a fluorescence-based, coupled-enzyme format and is based on the differential sensitivity of phosphorylated and non-phosphorylated peptides to proteolytic cleavage. Briefly, compounds were prepared in 100% DMSO, and diluted serially to produce a 10-pt concentration titration. Using an ECHO dispenser (LabCyte, Sunnyvale CA), compounds were dispensed to empty 384-well black plates (Corning catalog 4514, Corning, NY) using 100nL volumes. Total reaction volumes of CLK2 enzyme and substrate with compound equaled 10uL, and was carried out at ambient temperature for 1 hour. After the addition of development solution and incubation, detection was done by using the Envision (Perkin Elmer, Waltham, MA), using excitation wavelength of 400nM, and measuring emission wavelengths of 445nm and 520nm. Activity of CLK2 enzyme is calculated by using an emission ratio of coumarin at 445nm divided by emission of fluorescein at 520nm. EC50 potency values were determined using a 4-parameter curve fitting algorithm.

9. Kinase Selectivity Screen

Compounds were profiled for in vitro activity against protein kinases using the Invitrogen SelectScreen protein kinase profiling service:

https://www.thermofisher.com/us/en/home/products-and-services/services/custom-services/screening-and-profiling-services/selectscreen-profiling-service/selectscreen-kinase-profiling-service.html

10. P-gp Assay: Determination of bi-directional permeability in the LLC PK-1 cell line

Bi-directional transport was measured across LLCPK1 cell monolayers and monolayers stably expressing human MDR1 (LLC-MDR1, used under a licensing agreement) or rat Mdr1a (LLC-Mdr1a, cell line internally produced) P-gp. Compounds were dosed at 1 μM or 0.1 μM to the apical compartment to determine transport from apical (A) to basolateral (B) and to the basolateral compartment to determine transport from B to A. Samples from both sides of monolayer were taken at 3 h and quantitated by LCMSMS. The Papp B to A/A to B ratio gives an indication whether compounds are P-gp transporter substrates. The following equations were used to calculate the Papp and B to A/A to B ratios:

$P_{app} = [(Volume\ of\ receptor\ chamber\ (ml))/(area\ of\ membrane\ (cm^2)\ x\ initial\ concentration\ (\mu M)]\ x\ [concentration\ in\ receiver\ (\mu M))/(incubation\ time\ (s)]$

$B\ to\ A/A\ to\ B\ ratio\ =\ P_{app}\ (B\ to\ A)/P_{app}\ (A\ to\ B)$

11. Ion channel assay

Cell Dissociation Procedures

Frozen cells for the hERG and I_{Na} assays were prepared before study as follows: cells were dissociated with 0.05% trypsin EDTA, re-suspended in FBS (Fetal Bovine Serum) with 10% DMSO, and cryo-stored in a liquid nitrogen tank. For the experiment, the frozen cells were quickly thawed in a water bath at approximately 37°C, resuspended in external solution and used immediately.

Measurement of I_{Kr} activity: PatchXpress automated patch clamp

Experimental procedures for measurement of I_{Kr} with PatchXpress were previously described in detail elsewhere (1). Briefly, whole-cell hERG currents were measured from cells stably expressing hERG channels using the automated patch clamp system, PatchXpress® 7000A (Molecular Devices), at controlled room temperature (~20-23°C) via the use of an air cooling unit connected to the PatchXpress. Resistance of the planar patch plate ($SealChip_{16}$™ [AVIVA]) chambers (holes) were between 1 and 3 MΩ. For measurement of hERG, currents were elicited with their respective voltage-step protocol at 20-s inter-pulse intervals. hERG current was measured from a holding potential (V_h) of − 80 mV. A 20-ms depolarizing pre-pulse to − 50 mV was applied for measurement of baseline current, followed by a return to V_h for 80 ms, an activating 4-s depolarizing step to a test potential (V_t) of + 20 mV, and a 4-s repolarizing step (V_{tail}) to − 50 mV. hERG current was quantified as peak deactivating tail current amplitude during V_{tail}. Currents were monitored for stability for 5 min before addition of the test agents diluted from stocks in DMSO. Test agents were applied (60 µL) at sequentially increasing concentrations at a rate of 25 µL/s. For each condition or drug concentration, duplicate or triplicate 60 µL additions were made to each test well at 11-s intervals in order to achieve equilibrium and current was monitored for at least 5 min at each condition or test agent concentration.

I_{Na} channel activity measurements: PatchXpress automated patch clamp

Methods for the determination of activity on the cardiac Na^+ channel were described previously, in detail (2). A HEK-293 cell line stably expressing the $hNa_V1.5$ (human cardiac Na + channel α-subunit) channel clone was grown and passaged in a 37°C incubator in presence of 5% CO_2 in flasks containing MEM 'Glutamax' (Invitrogen Corp.) tissue culture media supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.5 mg/ml G418 (Geneticin) antibiotics (Invitrogen Life Technologies). Cells were grown to near confluence in T75 flasks and passaged/plated twice a week. The intracellular PatchXpress Na^+ current recording solution (pipette) contained (in mM): 120 CsF, 30 CsCl, 2 $MgCl_2$, 5 NaF, 10 EGTA, and 5 HEPES adjusted to pH 7.3 with CsOH. The Extracellular PatchXpress Na^+ current recording solution (bath) contained (in mM): 40 NaCl, 1 KCl, 2.7 CaCl2, 0.5 MgCl2, 120 N-Methyl-D-Glucamine Cl, and 5 HEPES, adjusted to pH 7.4 with HCl. PatchXpress experiments were performed at controlled room temperature (~20-23°C) via the use of an air cooling unit connected to the PatchXpress.

PatchXpress hNa$_v$1.5

Whole cell hNa$_v$1.5 currents (I_{Na}) were recorded from isolated HEK-293 cells using 16-chamber planar glass electrodes (Sealchip™) and the whole-cell variant of the patch-clamp technique with the PatchXpress® 7000A automated patch clamp (Molecular Devices, Sunnyvale, CA). SealChip 'hole' resistances were between 1 MΩ and 3 MΩ in the presence of the indicated recording solutions. Whole-cell hNav1.5 currents were low-pass filtered at a cut-off frequency of 3 kHz and digitally sampled at 15 kHz. Na^+ (hNav1.5) current was elicited using 30 ms pulses to − 20 mV from a V_h of -100 mV and quantified using the amplitude of the negative (inward) peak current. Following test article addition, voltage pulses were applied at a rate of 0.2 Hz for at least 5 min to allow equilibration of the current to a new steady state. Subsequently, a train of 60 pulses was applied at a rate of 3 Hz to determine rate-dependent effects of the test article. Typically, three test article concentrations were tested sequentially in half-log increments on each cell. At a given pulsing rate and test concentration, peak inward Na^+ current amplitudes were quantified as the average of final three pulses in a train.

Data analysis

The effects of test agents on currents were normalized to the vehicle control current level for each cell and were expressed as a percent inhibition using DataXpress (Molecular Devices Corp., Union City, California, U.S.A.) for hERG and I_{Na}. The averaged concentration-response data (means ± SEM, $n \geq 3$ for each data point) for current inhibition were fitted with a Hill equation to determine the half-inhibitory concentration (IC_{50}) and the concentration inhibiting 20% of current (IC_{20}) using Microsoft Excel (Microsoft, Inc., Redmond, Washington, U.S.A.):

$$\% \text{ Inhibition} = \frac{100}{1 + (\text{IC50}/[\text{Drug}])^h}$$

$$\text{IC } 20 = (0.25)^{1/h} \times \text{IC } 50$$

where h is the Hill coefficient.

References

1. Zeng H, Penniman JR, Kinose F, et al. Improved throughput of PatchXpress hERG assay using intracellular potassium fluoride. Assay Drug Dev Technol 2008;6: 235–241.
2. Penniman JR, Kim DC, Salata JJ, Imredy JP. Assessing use-dependent inhibition of the cardiac Na(+/−) current ($I_{(Na)}$) in the PatchXpress automated patch clamp. J Pharmacol Toxicol Methods 2010; 62:107-118.

12. Eurofins Cerep Panlabs

The activity of Compound **12** was profiled in Eurofins Cerep Panlab panel (Total number of assays:108) using the Eurofins Panlab service.

https://www.eurofinsdiscoveryservices.com/catalogmanagement/viewitem/Delta%20SpectrumScreen%20-%20SafetyScreen87%20Panel/PP264#biologicalInfo

Methods employed in this study have been adapted from the scientific literature to maximize reliability and reproducibility. Reference standards were run as an integral part of each assay to ensure the validity of the results obtained. Where presented, IC_{50} values were determined by a non-linear, least squares regression analysis using Math IQTM (ID Business Solutions Ltd.,UK). Where inhibition constants (Ki) are presented, the Ki values were calculated using the equation of Chengand Prusoff (Cheng,Y.,Prusoff,W.H.,Biochem.Pharmacol.22:3099-3108,1973) using the observed IC_{50} of the tested compound, the concentration of radioligand employed in the assay, and the historical values for the KD of the ligand (obtained experimentally at Eurofins Panlabs,Inc.). Where presented, the Hill coefficient (nH), defining the slope of the competitive binding curve, was calculated using Math IQTM. Hill coefficients significantly different than 1.0, may suggest that the binding displacement does not follow the laws of mass action with a single binding site. Where IC50, Ki, and/or nH data are presented without Standard Error of the Mean (SEM), data are insufficient to be quantitative, and the values presented (Ki,IC50,nH) should be interpreted with caution.

Summary of Significant Results

Significant responses (≥ 50% inhibition or stimulation for Biochemical assays) were noted in the primary assays listed below:

Cat #	Assay Name	Species	Conc.	% Inh.	IC_{50}*	K_i	n_H
200510	Adenosine A_1	hum	10 µM	68	3.45 µM	2.01 µM	0.79
200610	Adenosine A_{2A}	hum	10 µM	52	8.60 µM	4.83 µM	0.57
255520	Tachykinin NK_1	hum	10 µM	73	2.37 µM	1.72 µM	0.72
202000	Transporter, Adenosine	gp	1 µM	69	0.45 µM	0.15 µM	0.83

13. MSIC Protocol: Hepatocytes

The in vitro intrinsic clearances were determined in cryopreserved rat, dog, and human hepatocytes (Bioreclamation IVT, Baltimore, MD) using a standard substrate depletion method. Compounds were incubated for 90 min at 0.3 □M in phenol red-free Williams' Medium E buffer containing 4 mM L-glutamine at a cell density of 1 million hepatocytes/ml. Supernatant samples from the incubations were quantified via LC/MS/MS. The initial slope of the substrate disappearance curve (ln peak area ratio versus time) was used to calculate the intrinsic clearance.

14. Pharmacokinetic Properties in Han-Wistar Rats

Pharmacokinetic profiles of selected compounds were evaluated in fasted male Han-Wistar rats, male beagle dogs, or male rhesus monkeys following in a discrete single intravenous dose or oral administration. In the IV study, blood samples were collected at 0.03, 0.13, 0.25, 0.5, 1, 2, 4, 6, 8, and up to 24 hours after dosing. In the PO study, blood samples were collected at 0.25, 0.5, 1, 2, 4, 6, 8, and 24 hours after dosing. Plasma samples were prepared from the blood samples by centrifugation and analyzed by LC-MS/MS.

In vivo Sample Analysis: A reference standard (10 mM DMSO stock solution) was used to prepare standard working solutions at 1 and 0.05 mM by diluting suitable amounts of the stock solution with DMSO for the calibration standards (STD) and quality control (QC) samples preparation. Plasma samples obtained from dosed animals, calibration standards, and quality control samples were prepared for analysis employing a single step protein precipitation technique by adding 200 µL of internal standard (IS) crashing solvent to 50 µL aliquots of individual samples. The internal standard solution is prepared by diluting 1 mL ampoule of Cerilliant IS MIX (Diclofenac 200 µM, Labetalol 200 µM, and Imipramine 200 µM) in 1 L of acetonitrile. Samples were mixed by vortexing for homogeneity for 2 min and centrifuged at 3500 rpm for 5 min. The supernatant (200 µL) was transferred into a 96-well plate and injected into the LC-MS/MS for analysis.

Chromatography was performed on a Waters Acquity HSS T3 (2.1 mm x 50mm, 1.8 µm) column at room temperature with an injection volume of 5 µL. The mobile phase consisted of a solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Detection was carried out using a triple quadrupole tandem mass spectrometer (API 6500, Applied Biosystems) equipped with an electrospray interface (ESI). The Analyst 1.6.2 software (Applied Biosystems) was used to control the MS-MS system and MultiQuant 3.0.1 was used for data analyses.

15. Determination of plasma protein binding

Plasma protein binding was assessed using a 96-well equilibrium dialysis apparatus (HTDialysis, Gales Ferry, CT). The compound of interest was added to plasma to achieve a final concentration of 2.5 □M and 150 □L aliquots were loaded into the 96-well equilibrium dialysis chamber. The plasma samples were dialyzed against an equal volume of buffer. The 96-well equilibrium dialysis apparatus was incubated for four hours at 37°C under 5% CO2. At the completion of the incubation compound concentrations in plasma (Cp) and buffer (Cb) were measured by LC/MS/MS. The fraction unbound is calculated ratio of Cb/Cp. Plasma protein binding was assessed for select compounds by spiking the compound of interest in 10% plasma. For these compounds the unbound fraction in undiluted plasma was calculated using the equation below where D = 10:

$$fu{,}p\ undiluted\ =\ \frac{1/D}{(\frac{1}{fu{,}p\ diluted}-1)\ +\ 1/D}$$

16. In vivo rat PK/PD study

Male CD rats (125 -150g) were purchased from Charles River Laboratories (Raleigh, NC). Rats were housed on a 12 h light/dark cycle (lights on at 7:00AM, lights off at 7:00PM) under constant temperature (22 ± 2 °C) and humidity (>45%) conditions and given free access to food and drinking water. The animal protocols were reviewed and approved by IACUC of Merck & Co., Inc., Boston, MA, USA . All procedures were conducted in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

The pharmacokinetic profile of 12 was evaluated in male rats following a single oral (PO) administration. Blood and brain samples were collected at 120 minutes after dosing and plasma was prepared by centrifugation. Brain samples were divided in half. From one half the striatum was isolated and dissected out for determination of pSer935 LRRK2 levels, the other half was dedicated to PK analysis.

Plasma and brain levels of 12 were determined by liquid chromatography and tandem mass spectrometry analyses (LC/MS/MS) on an API-5000 instrument consistent with PK analysis (Applied Biosystems, Waltham, MA). The plasma and brain binding of 12 was determined by equilibrium dialysis at 1 μM.

To assess the effect of 12 on phosphorylation of LRRK2 Ser935 in-vivo, 12 was suspended in 10% Tween 80 and administered in a volume of 5 mL/kg. Rats received 12 (3-100 mg/kg; p.o.), or vehicle two hours prior to euthanasia by excess CO2. Immediately following euthanasia, rat striatum was dissected and frozen on a steel plate over dry ice for analysis of pSer935 LRRK2 via MSD immunoassay (Meso Scale Diagnostics, Maryland, USA). Plasma and brain samples were collected and frozen as described above for determination of 12 by LC/MS/MS. pSer935 LRRK2 data are expressed as the ratio of pSer935 LRRK2/total LRRK2 and expressed as mean ± S.E.M. The effect of 12 on pSer935 LRRK2 levels in the brain and peripheral tissues were analyzed by one-way ANOVA and then post hoc comparisons were made by Dunnett's test using Prism software (GraphPad Software, Inc.).

17. Exploratory Microbial Mutagenesis 3-strain Ames Assay

LRRK2 inhibitors tested over a concentration range of 30 to 5000 μg/plate in a 3-strain (TA1535, TA98 and TA100)Ames assay with metabolic activation by liver extract (S9) from male Wistar HAN rats pretreated with phenobarbital and beta naphthoflavone. The criteria for a positive result in this assay include a 2-fold increase in revertants over concurrent vehicle controls and evidence of a positive dose relation.

Method:

Based on the ICH S2(R1) guideline for this assay, concentrations routinely tested are 30, 100, 300, 1000, 3000, and 5000 μg/plate.

A plate incorporation protocol was used, with full-sized 100 mm plates. A volume of 500 μL of the activation system was added to the various concentrations of the test article and controls, followed by 2 mL of top agar containing 0.1 mL of the suspension of the test strains. The mixture was gently agitated for even dispersion and poured onto an agar plate. Triplicate plates S32 (minimal agar with 0.4% glucose) containing only a trace of histidine were prepared for each treatment group with metabolic activation. After incubation for approximately 48 hours at 37°C, revertant colonies on minimal agar plates were counted.

18. Crystallographic Information

Cotransfection, P0 generation, and P1-BIIC generation

The gene construct used for expression was cloned at, and procured from, GenScript Biotech. Sf21 host cells were defrosted and passaged in shake flasks (50 mL cells/250 mL flask) in a shaking incubator (1" orbit, 110 rpm, 27 °C). Cells were allowed to grow for at least 2 passages before co-transfecting. Approximately 30-60 minutes prior to co-transfection cells were prepared in antibiotic-free media to 5 x 105 cells/mL and then 2 mL were pipetted into each well of a tissue culture-treated 6-well plate (1 x 106 cells/well) and allowed to adhere. Co-transfection media was prepared by gently mixing 1 mL of Sf900-II serum-free media (no antibiotics) in a 1 mL microfuge tube with 10 µL CellfectinII (Invitrogen #10362100), 1 µL (100 ng) of BestBac 2.0, v-cath/chiA deleted linearized baculovirus DNA (Expression Systems #91-002), and 500 ng of the 10-point CHK1-LRRK2 chimera1. This co-transfection media was allowed to incubate for 30 min at RT to allow DNA recombination. Following incubation, the media on the adhered Sf21 host cells was gently removed and 1 mL of co-transfection media was added. The cells were allowed to incubate at 27 °C without shaking. 1 day after co-transfection an additional of another 1 mL of Sf900-II serum free media was added to the cells and incubated for an additional 4-5 days.

Generation of the P1-BIICs was as follows. 50 mL of Sf900-II serum-free media containing 5 µg/mL gentamycin was added to a 250 mL shake flask. Sf21 cells were prepared the day prior to infection to reach a density of 1 x 106 cells/mL. To these cells, 0.5 mL of P0 virus from the 6-well plate were added to the media. The cells were allowed to incubate at 27 °C and a rotational speed of 110 rpm until there was a verified ≥3 µm increase in cell size vs. uninfected cells prior to harvest. The cells were harvested at 2000 x g for 10 minutes at 4 °C. Cells were resuspended in 1-2 mL cryogenic vials to a density of 2 x 107 cells/mL x 1 or 2 mL aliquots in Sf900-II SFM containing 5 µg/mL gentamicin + 10% FBS + 10% DMSO. Cell aliquots were frozen in a -80 °C freezer using a Mr. Frosty freezing container (ThermoFisher) for up to one week or were stored long-term under liquid nitrogen.

P2-BIIC generation and large-scale purification of protein for crystallography

Uninfected Sf21 cells were prepared by growth in 300 mL Sf900-II SFM (ThermoFisher) with 5 µg/mL gentamycin in a 1 L shake flask at 27 °C and 110 rpm rotational speed to a density of 5 x 105 the day prior to infection and allowed to double to 1 x 106 cells/mL overnight. These cells were then infected at MOI=1 with 15 µL of the P1-BIICs assuming that the titer of a P1-BIIC is 2 x 109. Cells were allowed to grow for 72 hours in the 1 L shake flask at 27 °C and 110 rpm rotational speed to expected cell densities of 1-2 x 106 cells/mL and viability ≥ 90%. Prior to harvesting it was verified that there was ≥ 3 µM increase in infected cell size versus uninfected cells. The P2-BIICs were harvested by centrifugation at 2000 x g for 10 minutes at 4 °C. The BIIC pellet was resuspended to a density of 2 x 107 cells/mL in preservation media consisting of Sf900-II SFM containing 5 µg/mL gentamicin + 10% FBS + 10% DMSO and stored in 2 mL aliquots in cryogenic vials. Cell aliquots were frozen in a -80 °C freezer using a Mr. Frosty freezing container (ThermoFisher) for up to one week or were stored long-term under liquid nitrogen.

Large scale 2.5 L expression was carried out in Tni insect cells. 24 h prior to infection, 5 L Thompson flasks containing 2.5 L expression media was seeded with Tni cells at a density of 0.5 x 106 cells/mL at 27 °C and 110 rpm rotational speed. When the density reached 1.2 x 106 cells/mL the cells were infected at an MOI=5 with 7.5 mL of P2-BIICs.

Protein expression was carried out for 48 hours was monitored by recording cell density, viability and cell diameter every 24 h. After 48 hours cells were harvested by centrifugation at 3,400 x g for 15 min at 4 °C. 3 mL of cells were separately centrifuged and used to confirm protein expression via a small scale pulldown on the cell pellet using a PhyNexus Robot and running the total, soluble and eluted fractions on an SDS-PAGE and a performing a Western blot using anti-His antibody.

All following purification steps were carried out at 4 °C. Cells were lysed by passing two times through a cell disrupter (20,000 psi) in a Lysis Buffer containing 25 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 20 mM imidazole, Roche EDTA-free protease inhibitor cocktail, and 10 U/mL benzonase in a ratio of 5 mL buffer/1 g cell pellet. Lysate was clarified by centrifugation at 150,000 x g for 45 min. Cleared lysate was loaded onto a 5 mL HisTrap crude FF column (GE) at a flow rate of 1 mL/min. Column was washed in 10 column volumes (CV) of Wash Buffer (25 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 20 mM imidazole, and Roche EDTA-free protease inhibitor cocktail) at a flow rate of 2 mL/min. Protein was eluted in Elution Buffer containing 25 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 500 mM imidazole, and Roche EDTA-free protease inhibitor cocktail using a gradient of 0-100% Elution Buffer over 20 CV followed by 100% Elution Buffer for 5 CV. 2 mL fractions of eluate were analyzed by SDS-PAGE and fractions containing protein were pooled.

Protein was dialyzed into Dialysis Buffer containing 25 mM Tris-HCl (pH 9.0), 75 mM NaCl, 10% (v/v) glycerol, and 5 mM DTT. The 8x-Histag was removed by adding protease in a 1:40 ratio with protein. Cleaved protein was loaded onto a 1 mL HiTrap Q HP (GE) column and washed in 20 mL Wash Buffer containing 25 mM Tris-HCl (pH 9.0), 75 mM NaCl, 5% (v/v) glycerol, and 5 mM DTT at a flow rate of 1 mL/min. Protein was then eluted with Elution Buffer containing 25 mM Tris-HCl (pH 9.0), 1 M NaCl, 5% (v/v) glycerol, and 5 mM DTT in a gradient of 0-50% Elution Buffer over 50 CV followed by 50-100% Elution Buffer over 10 CV. 1 mL fractions were collected and those containing protein were pooled. Final purification of protein was undertaken by loading protein onto a GE Superdex 75 26/60 column in Running Buffer containing 25 mM Tris-HCl (pH 9.0), 250 mM NaCl, 5% (v/v) glycerol, and 5 mM DTT. Fractions containing protein via SDS-PAGE analysis were pooled and concentrated to 4.1 mg/mL.

Crystallization and Structural Analysis

X-ray diffraction-quality apo crystals of the CHK1-LRRK2 10-point chimera were obtained by sitting drop vapor diffusion at 20 °C by mixing a 1:1 ratio of the protein solution (4.1 mg/mL apoprotein in 25 mM Tris-HCl (pH 9.0), 250 mM NaCl, 5% (v/v) glycerol, and 5 mM DTT) and a precipitant solution (11% PEG 8000, 15-20% ethylene glycol, 0.1 M MES pH 6.5) with 5% v/v final concentration of 6-aminohexanoic acid as an additive. Crystals were seen after approximately 24-48 hours. The apocrystals were soaked for 16 hours in a 5 µL drop containing 12.5% PEG 8000, 17.5% ethylene glycol, 0.1 M MES (pH 6.5) and 0.5 mM Compound **18** over 500 µL reservoir of the same buffer lacking compound. Crystals were looped directly from the soaking drop plunged into LN2 prior to synchrotron data collection. These crystals diffracted to nominal resolutions of 1.7 Å (using staraniso data) and belonged to the space group P21 with one monomer in the asymmetric unit and the following approximate unit cell dimensions: a = 45.0 Å, b = 65.4 Å, and c = 54.1 Å, β = 102.3°.

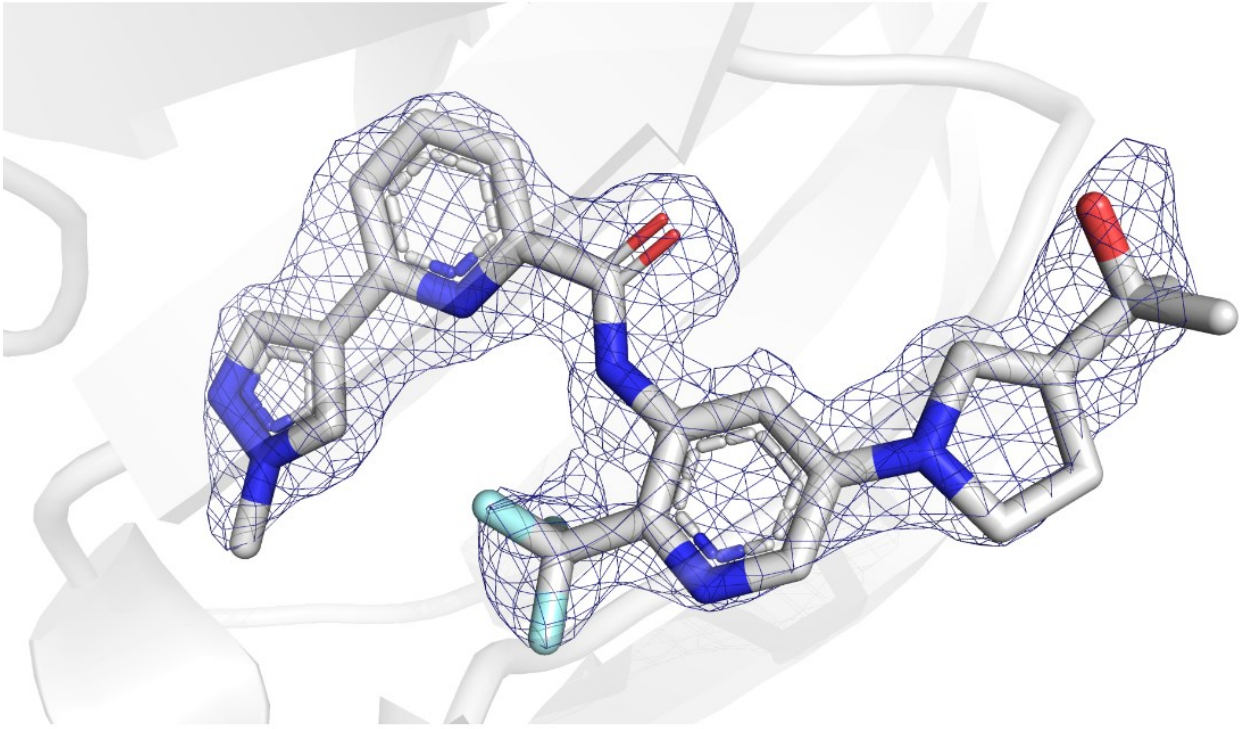
Diffraction data for Compound **18** were collected at the IMCA-CAT at the Advanced Photon Source using a PILATUS 6M detector (Dectris). All data was processed using autoPROC1[1], refined using autoBUSTER2[2], with manual model building using Coot3[3]. Compound geometrical restraints were prepared using grade.[4] Figures were prepared using PyMOL.[5]

Part of the research described in this paper used resources at the Industrial Macromolecular Crystallography Association Collaborative Access Team (IMCA-CAT) beamline 17-ID, supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with HauptmanWoodward Medical Research Institute. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

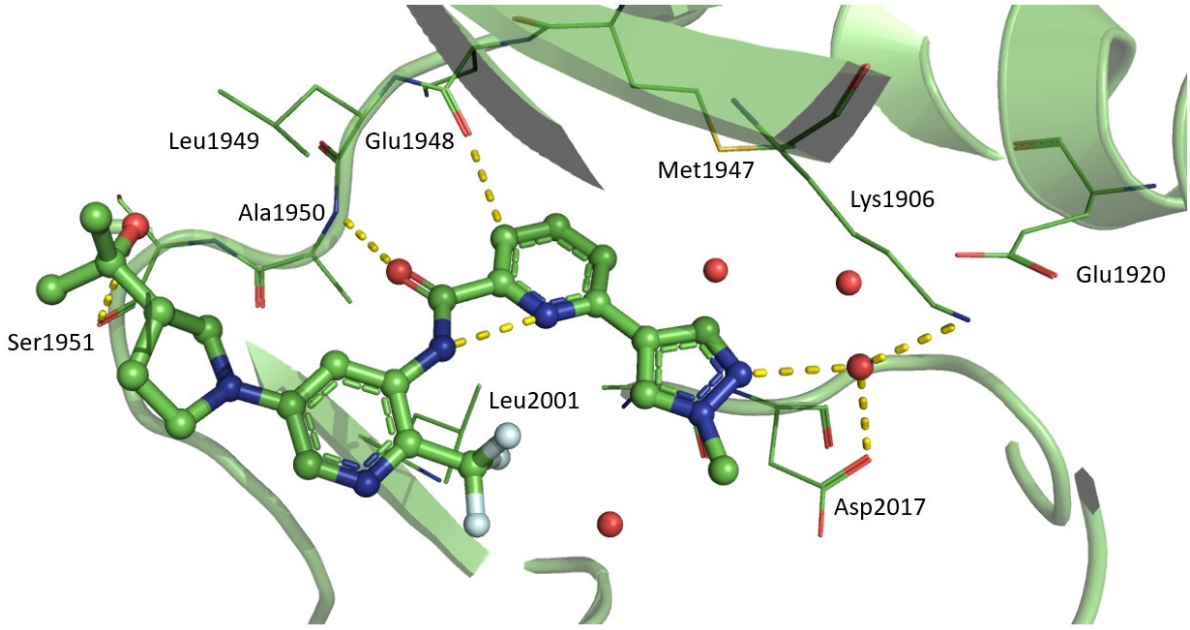
SI Table C: Crystallographic Information

PDB code	**7MCK**
Compound name	**Compound 18**
Data collection	
Space group	$P2_1$
Cell dimensions a, b, c (Å)	45.0, 65.4, 54.1; β=102.3°
Resolution (Å)	43.95-1.66 (1.80-1.66)*
Rmerge	0.07 (0.90)
I/σI	10.0 (1.2)
Completeness (%)	92.6 (52.9)
CC(1/2)	1.0 (0.55)
Redundancy	3.4 (3.3)
Refinement	
Resolution (Å)	43.9-1.65
No. reflections	28354
Rwork / Rfree	0.19/0.23
No. atoms	
Protein	2137
Ligand	34
Solvent	194
B-factors	
Protein (Å2)	33.5
Ligands (Å2)	43.6
Solvent (Å2)	38.7
R.m.s. deviations	
Bond lengths (Å)	0.010
Bond angles (º)	0.96

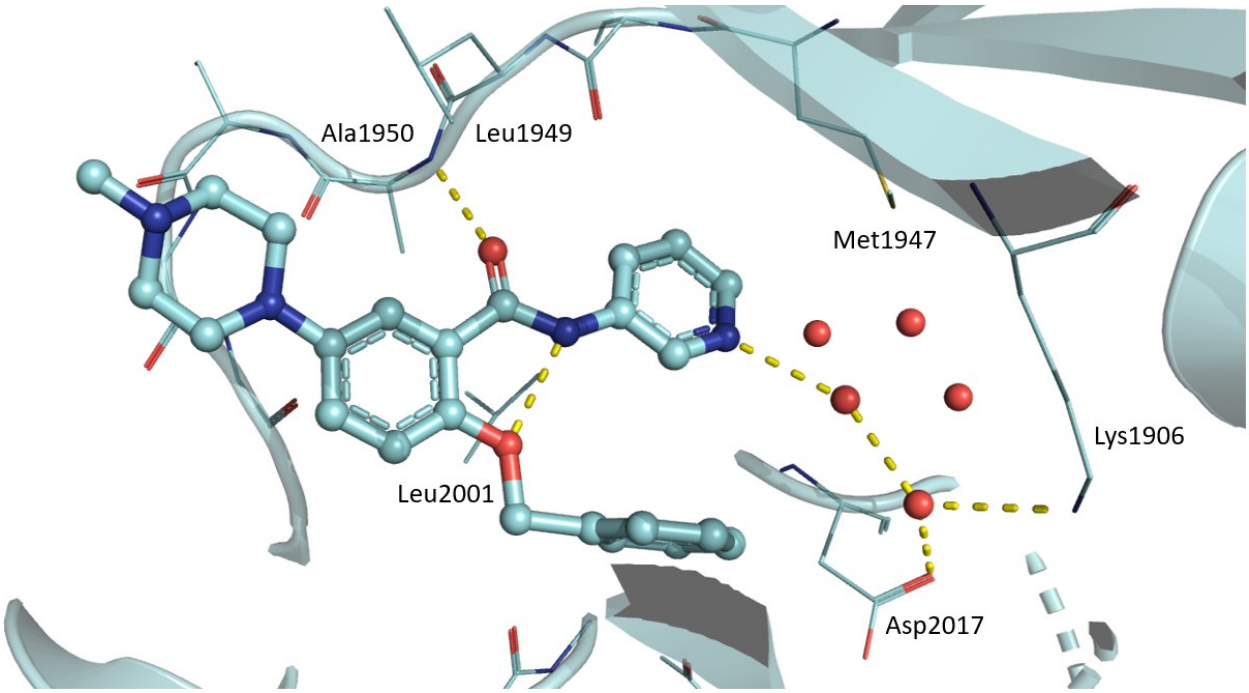
SI Figure A: 2 Fo-Fc density for Compound 18 bound within the active site of this CHK1-LRRK2 chimera. Density is contoured at 1σ



SI Figure B: X-ray crystal structures of compound 18 (PDB Code: 7MCK)



SI Figure C: X-ray crystal structures of BMPPB-32 (PDB Code: 5OP2)



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