Synthetic Chemistry Enabling the Discovery and Development of a Series of Pyrazoles as HPK1 Inhibitors

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Supporting Information

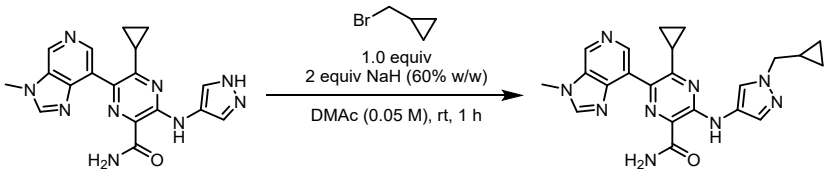
Table of Contents

General Information

All chemicals and solvents used were reagent grade, unless otherwise noted. Anhydrous solvents, DMF, THF, 2-MeTHF, MeOH, PhMe, and DCM were purchased from Sigma-Aldrich. Flash chromatography was carried out using pre-packed normal phase silica or reverse phase C18 cartridges and eluted using either ISCO Combiflash Rf or Biotage Selekt systems. NMR spectra were recorded on either a Bruker NEO 600 mHz with a cryoprobe, Bruker NEO 500 MHz, or a Bruker nano-AV3HD 400 MHz spectrometer. ^{1}H Chemical shifts are reported in parts per million (ppm) relative to solvent peaks as the internal reference. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad peak. Products were characterized using a Waters UPLC-MS system fitted with both DAD and ELSD detectors, as well as a Waters SQD mass spectrometer. Ultra-performance liquid chromatography was performed using a reverse phase C18 Waters Acquity HSS T3,1.8 µm, 2.1 × 30 mm^2 column with flow rate = 1 mL/min and a solvent gradient of 2 to 98% B over 1.5 min, where A = 0.1% formic acid or 0.1% TFA or 0.2% NH_4OH in water and B = 0.1% formic acid or 0.1% TFA or 0.2% NH_4OH in MeCN. Mass spectrometry detection was *via* ESI with positive/negative switching and a cone voltage = 10 V.

Chemical Synthesis.

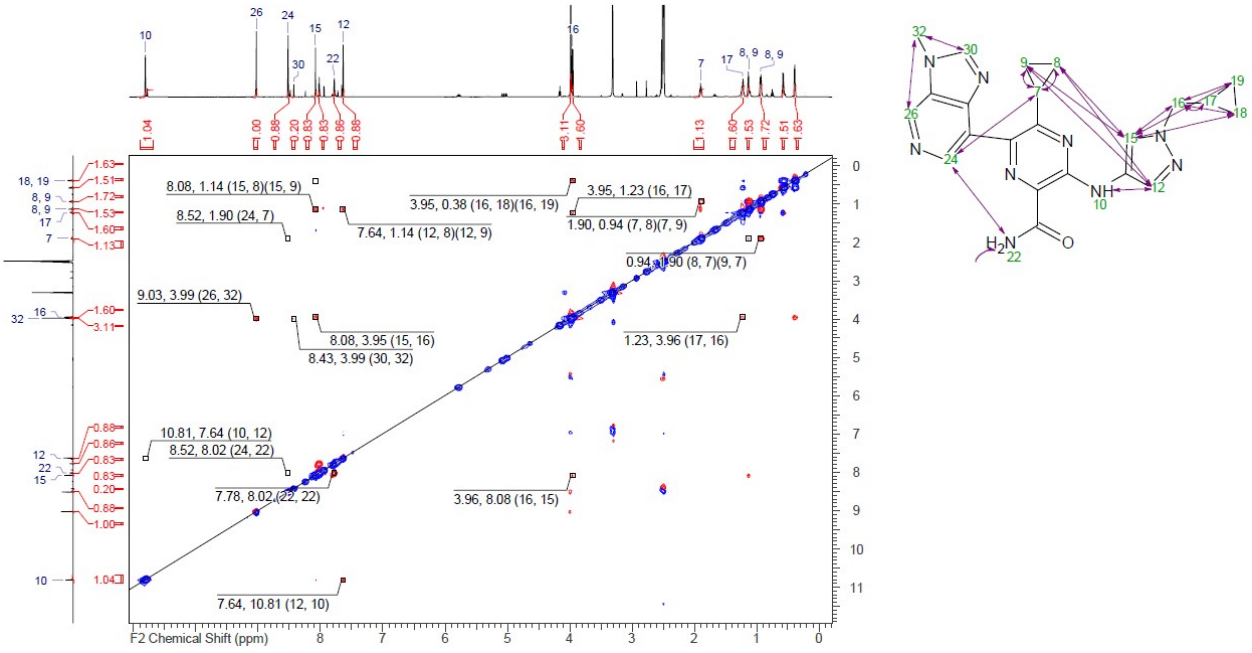
Proof-of Concept for Late-Stage Pyrazole Functionalization: Alkylation of 8 (Scheme 1b)



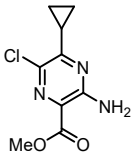
8 **9**

5-Cyclopropyl-3-((1-(cyclopropylmethyl)-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (9). Sodium hydride (60 wt.% mineral oil dispersion, 2.1 mg, 0.05 mmol, 2.0 equiv) was added to a solution of (bromomethyl)cyclopropane (3.6 mg, 0.03 mmol, 1.0 equiv) and 3-((1*H*-pyrazol-4-yl)amino)-5-cyclopropyl-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**8**, 10.0 mg, 0.03 mmol, 1.0 equiv) in DMAc (0.53 mL). The mixture was stirred at rt for 1 h. It was then quenched with 2 drops of methanol and concentrated. The resulting residue was purified by flash silica chromatography with an elution gradient 0 to 20% MeOH in DCM. Product fractions were concentrated under reduced pressure to afford 5-cyclopropyl-3-((1-(cyclopropylmethyl)-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3H-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**9**, 9.0 mg, 70% yield, 80% purity) as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm 0.37 - 0.41 (m, 2H), 0.55 - 0.60 (m, 2H), 0.91 - 0.96 (m, 2H), 1.11 - 1.15 (m, 2H), 1.19 - 1.26 (m, 2H), 1.84 - 2.01 (m, 1H), 3.93 - 3.97 (m, 2H), 3.99 (br s, 1H), 7.61 - 7.66 (m, 1H), 7.78 (br s, 1H), 8.02 (br s, 1H), 8.08 (s, 1H), 8.41 - 8.45 (m, 1H), 8.52 (s, 1H), 9.01 - 9.07 (m, 1H), 10.69 - 10.90 (m, 1H). *m*/*z*: (ES+), $[M + H]^+$ = 431.2.

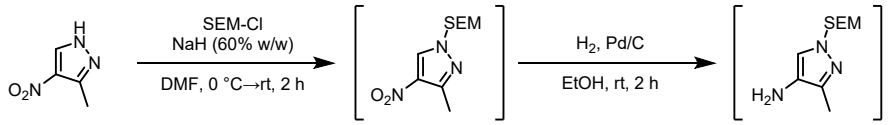
^{1}H-^{1}H ROESY (600 MHz, DMSO-d_6)



Synthesis of C3-Methyl-Pyrazole-Containing Intermediate 7•HCl (Scheme 2)



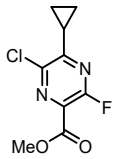
Methyl 3-amino-6-chloro-5-cyclopropylpyrazine-2-carboxylate (11). A 1 L round bottom flask equipped with a magnetic stir bar was charged with methyl 3-amino-5,6-dichloropyrazine-2-carboxylate (**10**, 30.0 g, 135.1 mmol, 1.0 equiv), cyclopropylboronic acid (12.2 g, 141.9 mmol, 1.05 equiv), $Pd_2(dba)_3$ (6.19 g, 6.76 mmol, 5 mol%), cataCXium A (4.84 g, 13.5 mmol, 10 mol%), and potassium carbonate (56.0 g, 405.4 mmol, 3.0 equiv). Next, PhMe (400 mL) and water (40.0 mL) were added in a 10:1 ratio by volume (0.32 M w.r.t. **10**). The resulting slurry was allowed to stir vigorously at rt as the flask was again evacuated/backfilled with nitrogen five times. The reaction mixture was allowed to stir at 90 °C for 4 h. The reaction mixture was cooled to rt, and 2-MeTHF (250 mL) and water (150 mL) were added. The reaction mixture was then filtered through Celite rinsing with 2-MeTHF. The filtrate was transferred to a separatory funnel, and the layers were separated. The aqueous layer was extracted with 2-MeTHF (250 mL), and the combined organics were washed with twice with water (2 x 300 mL) and once with brine (300 mL). The organics were dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo* to provide an orange-brown solid, which was subsequently triturated with 3:1 hexanes/EtOAc (250 mL). The resulting slurry was stirred at 60 °C for 1 h, cooled to rt, and sonicated. The solid was collected by vacuum filtration rinsing with hexanes, and the filtrate was concentrated and re-triturated with 25% EtOAc/hexanes (200 mL). The solid was collected by vacuum filtration rinsing with hexanes. The filtrate was again concentrated *in vacuo*, and the resulting orange residue was triturated with 25% EtOAc/hexanes (200 mL). The solid was collected by vacuum filtration, and the combined solids (23 g) were transferred to a 500 mL round bottom flask and triturated with 5:1 hexanes/diethyl ether. The slurry was allowed to stir at rt for 90 min, then the solids were collected by vacuum filtration and allowed to air dry overnight, providing methyl 3-amino-6-chloro-5-cyclopropylpyrazine-2-carboxylate (21.90 g, 71%) as a beige solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 1.01 (quin, J = 3.7 Hz, 2H), 1.07 - 1.15 (m, 2H), 2.32 - 2.41 (m, 1H), 3.81 (s, 3H), 7.34 (br s, 2H). *m*/*z*: (ES+), $[M + H]^+$ = 228.0, 230.0 (2:1).



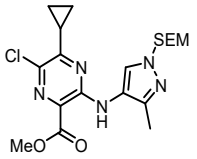
3-Methyl-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrazol-4-amine (12). Sodium hydride (60 wt.% mineral oil dispersion, 3.15 g, 78.7 mmol, 2.0 equiv) was added to a solution of 3-methyl-4-nitro-1*H*-pyrazole (**S1**, 5.00 g, 39.3 mmol, 1.0 equiv) in THF (31.0 mL). The mixture was stirred at 0 °C for 20 minutes, at which point (2-(chloromethoxy)ethyl)trimethylsilane (8.35 ml, 47.2 mmol, 1.2 equiv) was added. The mixture was allowed to warm to rt and was stirred for 2 h. It was then quenched with sat. aq. $NaHCO_3$ (50 mL) and diluted with EtOAc (40 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (3 x 50 mL). The organic layers were combined, dried over Na_2SO_4, and concentrated *in vacuo*. The resulting residue was purified by flash silica chromatography, elution gradient 0 to 30% EtOAc in hexanes. Product fractions were concentrated *in vacuo* to afford 3-methyl-4-nitro-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrazole (**S2**, 10.1 g, 2.4:1 rr by LC) as a yellow oil which was used without further purification in the next step.

Palladium on carbon (4.13 g, 3.89 mmol) was added to a solution of 3-methyl-4-nitro-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrazole (10.0 g, 38.9 mmol) in ethanol (97 mL). The reaction mixture was sparged with hydrogen gas (1 atm) from a balloon for 20 minutes then stirred under an atmosphere of hydrogen for 2 h. The reaction mixture was then sparged with nitrogen and filtered through a pad of Celite rinsing with EtOAc (300 mL).The volatiles were then removed under reduced pressure, and the resulting

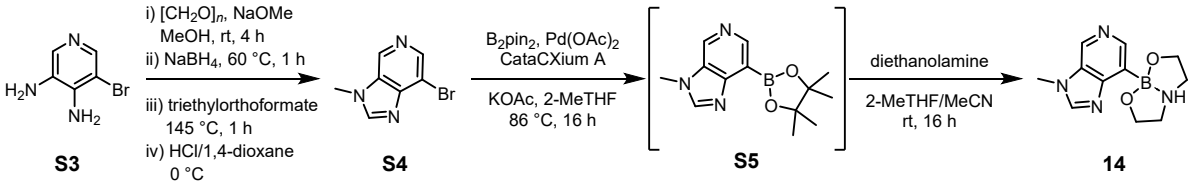
yellow oil 3-methyl-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrazol-4-amine (**12**, 8.95 g, >99%) was used without further purification in the next step. *m*/*z*: (ES+), $[M + H]^+$ = 227.9.



Methyl 6-chloro-5-cyclopropyl-3-fluoropyrazine-2-carboxylate (23). A plastic reaction vessel equipped with a magnetic stir bar was charged with methyl 3-amino-6-chloro-5-cyclopropylpyrazine-2-carboxylate (**11**, 10.0 g, 43.9 mmol). The beige-orange solid was carefully suspended in HF·pyridine (25 mL, 720 mmol), and the suspension was cooled to −5 °C in an ice/brine bath. Sodium nitrite (3.64 g, 52.7 mmol, 1.2 equiv) was added carefully in three portions. The ice bath was removed, and the reaction mixture was stirred for 3 h. The reaction mixture was then diluted with DCM (100 mL) and quenched by the careful addition of water (100 mL). The layers were separated, and the aqueous layer was extracted twice with DCM (20 mL each). The combined organics were washed with brine (200 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated. The resulting residue was treated with 200 mL of 3:1 hexanes/diethyl ether with a small amount of EtOAc and sonicated until all of the solid was suspended. The resulting orange suspension was stirred at rt for 16 h, then filtered and rinsed with copious hexanes. The filtrate was concentrated, and the resulting residue was purified by flash silica gel chromatography, elution gradient 0−30% EtOAc/hexanes, to afford methyl 6-chloro-5-cyclopropyl-3-fluoropyrazine-2-carboxylate (**23**, 6.80 g, 67%) as a waxy, yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.09 (dd, *J* = 4.3, 3.3 Hz, 2H), 1.24,1.37 (m, 2H), 2.51, 2.57 (m,1H), 3.89 (s, 3H). *m*/*z*: (ES+), $[M + H]^+$ = 230.9.



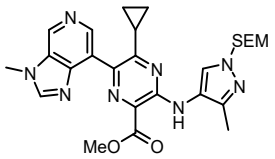
Methyl 6-chloro-5-cyclopropyl-3-((3-methyl-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrazol-4-yl)amino)pyrazine-2-carboxylate (13). A solution of *N*-ethyl-*N*-isopropylpropan-2-amine (DIPEA, 2.24 mL, 13.0 mmol 1.0 equiv), methyl 6-chloro-5-cyclopropyl-3-fluoropyrazine-2-carboxylate (3.00 g, 13.0 mmol), 3-methyl-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrazol-4-amine (**12**, 2.96 g, 13.01 mmol, 1.0 equiv) in DMF (62.8 mL) was stirred at 100 °C for 15 min. The reaction mixture was then concentrated *in vacuo* and telescoped into the next step without further purification. *m*/*z*: (ES–), $[M - H]^-$ = 436.3.



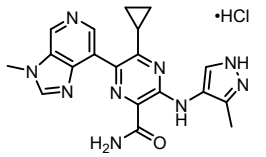
2-(3-methyl-3*H*-imidazo[4,5-*c*]pyridin-7-yl)-1,3,6,2-dioxazaborocane (14). Sodium methoxide in methanol (0.5 M, 425 mL, 213 mmol, 4.0 equiv) was added to a mixture of 5-bromopyridine-3,4-diamine (**S3**, 10.0 g, 53.2 mmol, 1.0 equiv) and paraformaldehyde (1.63 g, 54.3 mmol, 1.02 equiv). The resulting mixture was stirred at 25 °C for 4 h. Sodium borohydride (2.01 g, 53.2 mmol, 1.0 equiv) was added, and the reaction mixture was stirred at 60 °C for 1 h. The reaction mixture was then concentrated. The resulting residue was treated with water and extracted with EtOAc. The extract was dried over anhydrous Na_2SO_4, filtered, and concentrated. The resulting residue was suspended in triethylorthoformate (200 mL) and stirred at 145 °C for 1 h. The reaction mixture was then cooled to 0 °C and acidified with 4 M HCl in dioxane (16.0 mL, 64.0 mmol). The resulting precipitate was filtered to afford a yellow solid, which was partitioned between

saturated aqueous potassium carbonate and ethyl acetate and extracted twice with ethyl acetate. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated to yield 7-bromo-3-methyl-3*H*-imidazo[4,5-c]pyridine (**S4**, 9.00 g, 80%) as a yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.96 (3H, s), 8.48 (1H, s), 8.51 (1H, s), 8.97 (1H, s). *m/z*: (ES+), $[M + H]^+$ = 212.03.

A mixture of 7-bromo-3-methyl-3*H*-imidazo[4,5-*c*]pyridine (**S4**, 29.7 g, 140 mmol, 1.0 equiv), B_2pin_2 (42.7 g, 168 mmol, 1.2 equiv), $Pd(OAc)_2$ (3.14 g, 14.0 mmol, 10 mol%), cataCXium A (10.0 g, 28.0 mmol, 20 mol%), and KOAc (41.2 g, 420 mmol, 3.0 equiv) were suspended in 2-methyl tetrahydrofuran (879 mL, 0.16 M w.r.t. **S4**), and argon was bubbled through the mixture for 20 min. The resulting mixture was then stirred at 86 °C under argon overnight. After 16 h, the reaction mixture was allowed to cool to rt, diluted with DCM (879 mL), filtered through a pad of Celite, and washed twice with DCM (100 mL each). The resulting filtrate was concentrated to afford crude **S5**. 2-Methyl tetrahydrofuran (281 mL) and MeCN (167 mL) were added to the resulting residue. Diethanolamine (16.9 mL, 175 mmol, 1.25 equiv) was added, and the reaction mixture was stirred at rt for 16 h. Additional 2-methyltetrahydrofuran (50 mL) and diethanolamine (0.5 equiv.) were added and the resulting mixture was stirred for 5 h. The reaction mixture was then filtered, rinsing with MeCN (200 mL) to afford 8-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)tetrahydro-8*H*-8l4-[1,3,2]oxazaborolo[2,3-b][1,3,2]oxazaborole (**14**, 31.0 g, 90%) as a light yellow solid. ^{1}H NMR (500 MHz, D_2O) δ 2.82 - 3.10 (m, 4H), 3.67 - 3.87 (m, 4H), 3.94 (s, 3H), 8.32 (s, 1H), 8.34 (s, 1H), 8.77 (s, 1H).



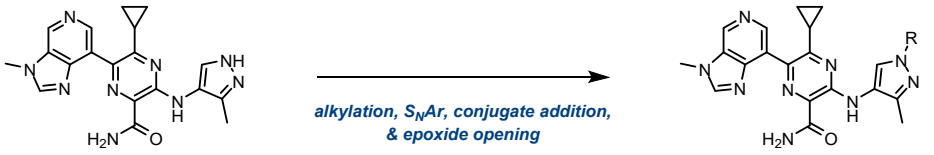
Methyl 5-cyclopropyl-3-((3-methyl-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxylate (15). A 100 mL round bottom flask containing crude methyl 6-chloro-5-cyclopropyl-3-((3-methyl-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrazol-4-yl)amino)pyrazine-2-carboxylate (**13**, 6.54 g, 14.9 mmol, 1.0 equiv) was charged with 8-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)tetrahydro-8*H*-8l4-[1,3,2]oxazaborolo[2,3-b][1,3,2]oxazaborole (4.59 g, 18.7 mmol, 1.25 equiv), $Pd(dppf)Cl_2$•CH_2Cl_2 (1.22 g, 1.49 mmol, 10 mol%), cesium fluoride (6.80 g, 44.8 mmol, 3.0 equiv), and a magnetic stir bar. The vial was sealed and evacuated/backfilled with nitrogen three times. Next, 1,4-dioxane (90 mL) and water (9.05 mL) were added *via* syringe in a 10:1 ratio (0.15 M w.r.t. **13**). The resulting slurry was allowed to stir at rt as the vial was evacuated/backfilled with nitrogen three times more. The reaction mixture was then allowed to stir at 80 °C for 4 h. The crude reaction mixture was cooled to rt and concentrated. The crude product was purified by flash silica chromatography, elution gradient 0–5% MeOH/DCM with 0–0.1% NH_3 modifier over 12 CVs. Product-containing fractions were pooled and concentrated *in vacuo* to provide methyl 5-cyclopropyl-3-((3-methyl-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxylate (4.30 g, 53.9%, 2:1 rr) as yellow foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.00 (s, 10H), 0.88 - 1.04 (m, 4H), 1.18 - 1.33 (m, 1H), 1.24 - 1.27 (m, 1H), 1.88 – 2.04 (m, 1H), 2.34 - 2.41 (m, 3H), 3.59 (dt, *J* = 10.6, 8.3 Hz, 2H), 3.95 - 3.99 (m, 3H), 4.01 (d, *J* = 2.6 Hz, 3H), 5.34 - 5.50 (m, 2H), 7.83 - 8.09 (m, 2H), 8.68 (br d, *J* = 5.2 Hz, 1H), 8.81 - 9.03 (m, 1H), 9.52 - 10.04 (m, 1H). *m/z*: (ES+), $[M + H]^+$ = 535.3.



5-Cyclopropyl-3-((3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide•HCl (7•HCl). A 135 mL pressure vessel was charged with methyl 5-cyclopropyl-3-((3-methyl-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxylate (**15**, 4.30 g, 8.04 mmol, 1.0 equiv) as a solution in MeOH. The carrier solvent was removed by concentration, and 7 M ammonia in methanol (57.4 ml, 402.10 mmol, 50.0

equiv) was added. The flask was sealed securely, and the reaction mixture was allowed to stir at 100 °C for 1 h. The reaction mixture was cooled to rt, transferred to 250 mL round bottom flask and concentrated *in vacuo*. The resulting solid was treated with EtOH (80 mL) and 4 M HCl in 1,4-dioxane (20.0 mL, 80.0 mmol) and allowed to stir at reflux for 2 h. A yellow solid precipiated after ~1 h at 90 °C. The reaction mixture was allowed to cool to rt, and diethyl ether was added portionwise to promote precipitation. The solid was collected by vacuum filtration rinsing with copious diethyl ether. The collected solid was allowed to air dry for multiple hours, providing 5-cyclopropyl-3-((3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide•HCl (**7•HCl**, 3.29 g, 96%) as a yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.89 - 0.99 (m, 2H), 1.06 - 1.14 (m, 2H), 1.82 - 1.95 (m, 1H), 2.26 (s, 3H), 4.12 (s, 3H), 7.88 (br s, 1H), 8.02 (s, 1H), 8.30 (br s, 1H), 8.90 (s, 1H), 9.04 (s, 1H), 9.64 (s, 1H), 10.86 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 390.1.

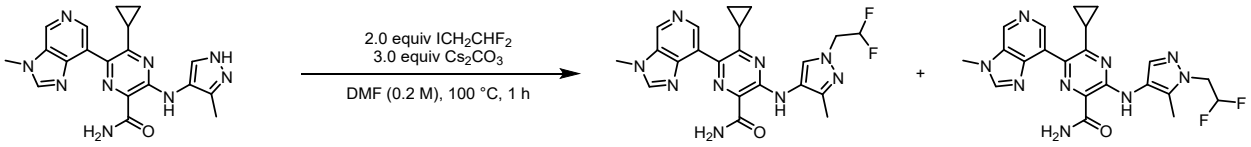
Late-Stage Functionalization of Pyrazole Intermediate 7 (Figure 2)



7

16: R = 1° or 2° alkyl
17: R = 2-pyridyl
18: R = CH_2CH_2EWG
19: R = β-hydroxy alkyl

General Procedure A. A vial equipped with a magnetic stir bar was charged with 5-cyclopropyl-3-((3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**7**, 85 mg, 0.22 mmol, 1.0 equiv) and Cs_2CO_3 (1–3 equiv) or DBU (conjugate addition, 0.75 equiv). DMSO or DMF (typically 0.1–0.2 M w.r.t. **7**) was added, followed by electrophile (1–3 equiv), and the vial was sealed tightly. The reaction mixture was allowed to stir at 100 °C with monitoring by UPLC-MS. The crude reaction mixture was purified directly by reverse phase flash chromatography, typical elution gradient 0–100% MeCN/H_2O with 0.2% NH_3 modifier over 12 CVs. Product-containing fractions were pooled and concentrated *in vacuo* to provide pyrazole functionalized products **16–19**, typically as 1:1 to 3:1 mixture of N1- vs. N2-regioisomers, confirmed by ^{1}H-NMR. The products were then purified by preparative SFC on a chiral stationary phase to separate the regiosomers. Product-containing fractions were pooled and concentrated *in vacuo* to deliver both regioisomeric products for evaluation in our biological assays.

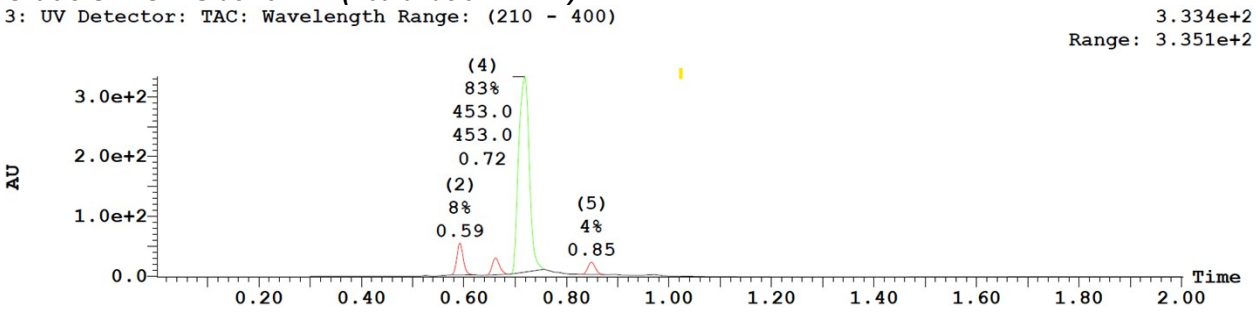


7 **16a** **16a'**

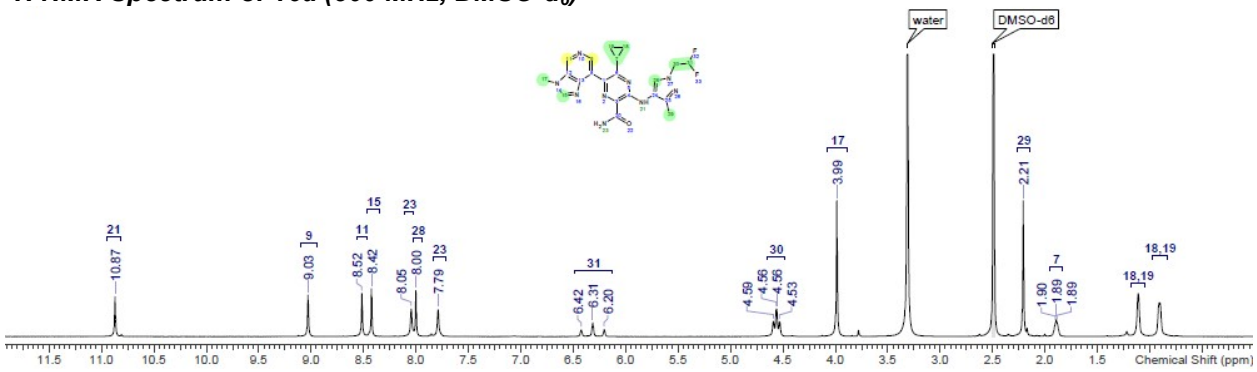
Representative Example: Synthesis of 16a by Late-Stage Alkylation. To a 5 mL microwave vial equipped with a magnetic stir bar was added 5-cyclopropyl-3-((3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (85 mg, 0.22 mmol) as a solution in methanol. The carrier solvent was removed under a stream of nitrogen, and Cs_2CO_3 (213 mg, 0.65 mmol) was added to the vial. DMSO (1.0 mL) was added, followed by 1,1-difluoro-2-iodoethane (38.4 µl, 0.44 mmol), and the vial was sealed. The reaction mixture was allowed to stir at 100 °C for 1 h. The reaction mixture was cooled to rt, and the crude material was purified directly by reverse phase flash chromatography, elution gradient 0–100% MeCN/H_2O with 0.2% NH_4OH modifier over 12 CVs. Product-containing fractions were pooled and concentrated *in vacuo* to provide 88.1 mg (89% yield) of the product as a ~2:1 mixture of **16a** and **16a′** (confirmed by NMR), plus some minor impurities. The product was purified by preparative SFC on a chiral stationary phase (IB column 250 x 21 mm/5 µm, 100–65% $scCO_2$/MeOH w/ 0.2% NH_4OH modifier, 40 °C, 254 nm) to separate the two regioisomers. Product containing fractions were pooled and concentrated *in vacuo* to deliver 5-cyclopropyl-3-((1-(2,2-difluoroethyl)-3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**16a**, 0.048 g, 48.5%) and 5-cyclopropyl-3-((1-(2,2-difluoroethyl)-5-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**16a′**, 0.023 g, 23.2%), both as yellow solids. **16a:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.83 - 0.98 (m, 2H), 1.05 - 1.18 (m, 2H), 1.84 - 1.95 (m, 1H), 2.21 (s, 3H), 3.99 (s, 3H), 4.48 - 4.64 (m, 2H), 6.13 - 6.48 (m, 1H), 7.79 (br s, 1H), 8.00 (s, 1H), 8.05 (br s, 1H), 8.42 (s, 1H), 8.52 (s, 1H), 9.03 (s, 1H), 10.87 (s, 1H). ^{19}F NMR (471 MHz, DMSO-d_6) δ –122.59 (s, 2F). *m/z*: (ES+), $[M + H]^+$ = 454.2. **16b:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.76 - 0.94 (m, 2H), 0.94 - 1.12 (m, 2H), 1.78 - 1.96 (m, 1H), 2.18 - 2.36 (m, 3H), 3.98 (br s, 3H), 4.57 (br t, *J* = 14.0 Hz, 2H),

6.14 - 6.54 (m, 1H), 7.76 (br s, 1H), 7.83 (br s, 1H), 8.02 (br s, 1H), 8.42 (br s, 1H), 8.51 (br s, 1H), 9.03 (br s, 1H), 10.60 (br s, 1H). ^{19}F NMR (471 MHz, DMSO-d_6) δ –122.12 (s, 2F). *m/z*: (ES+), $[M + H]^+$ = 454.2.

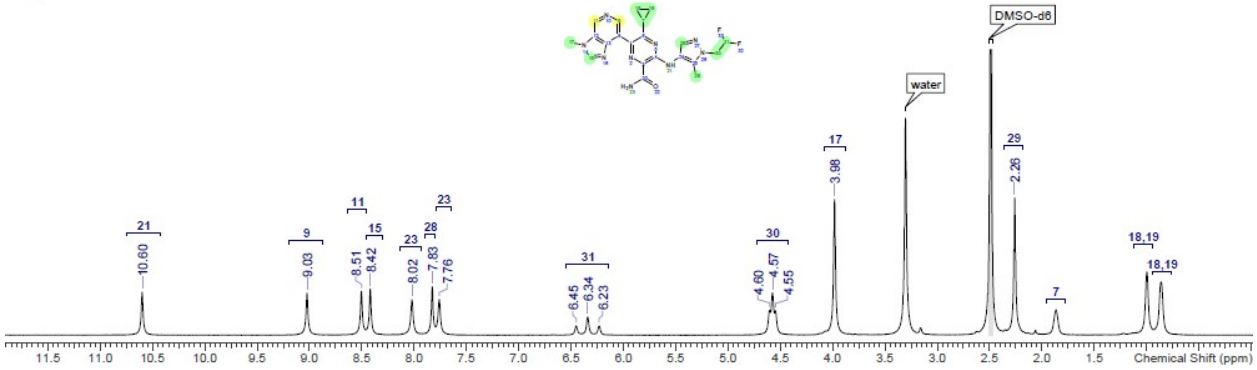
Crude UPLC-MS at 15 min (16a/a' at 0.72 min)



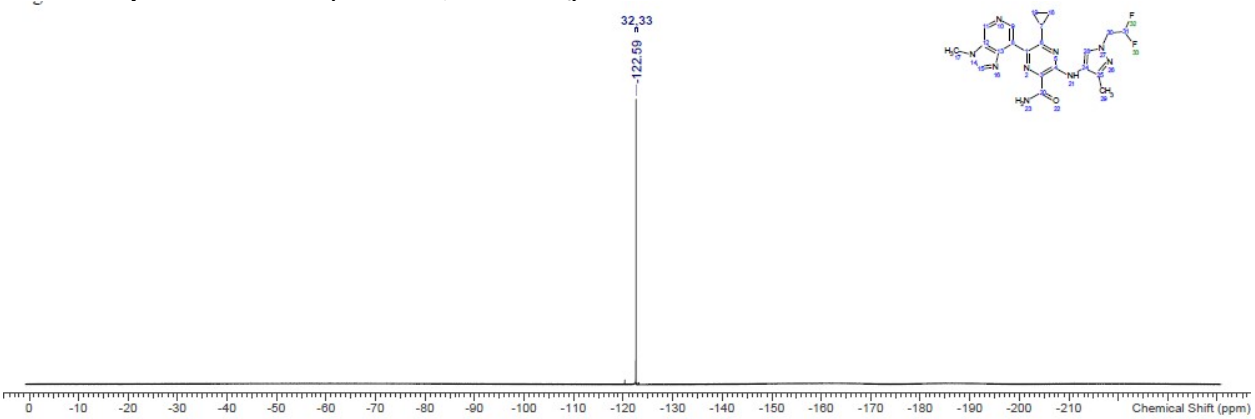
^{1}H NMR Spectrum of 16a (500 MHz, DMSO-d_6)



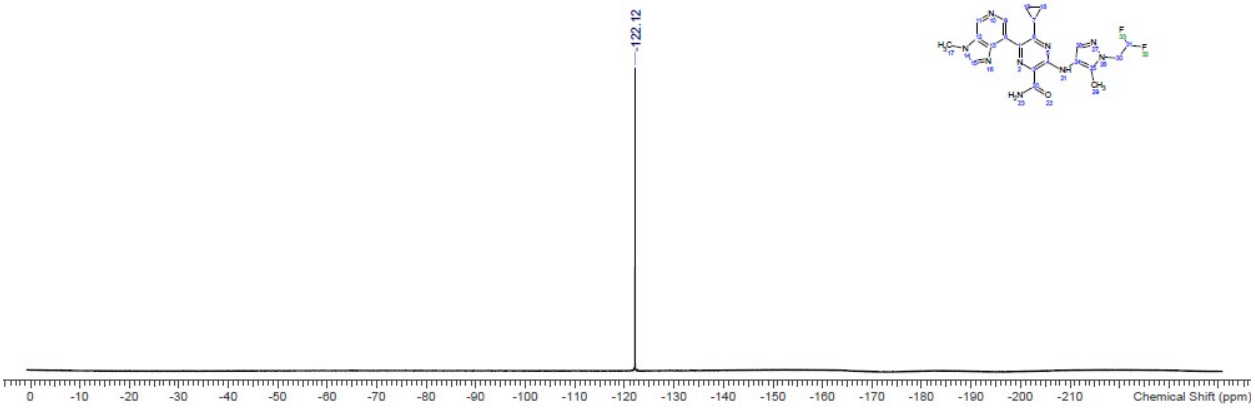
^{1}H NMR Spectrum of 16a' (500 MHz, DMSO-d_6)



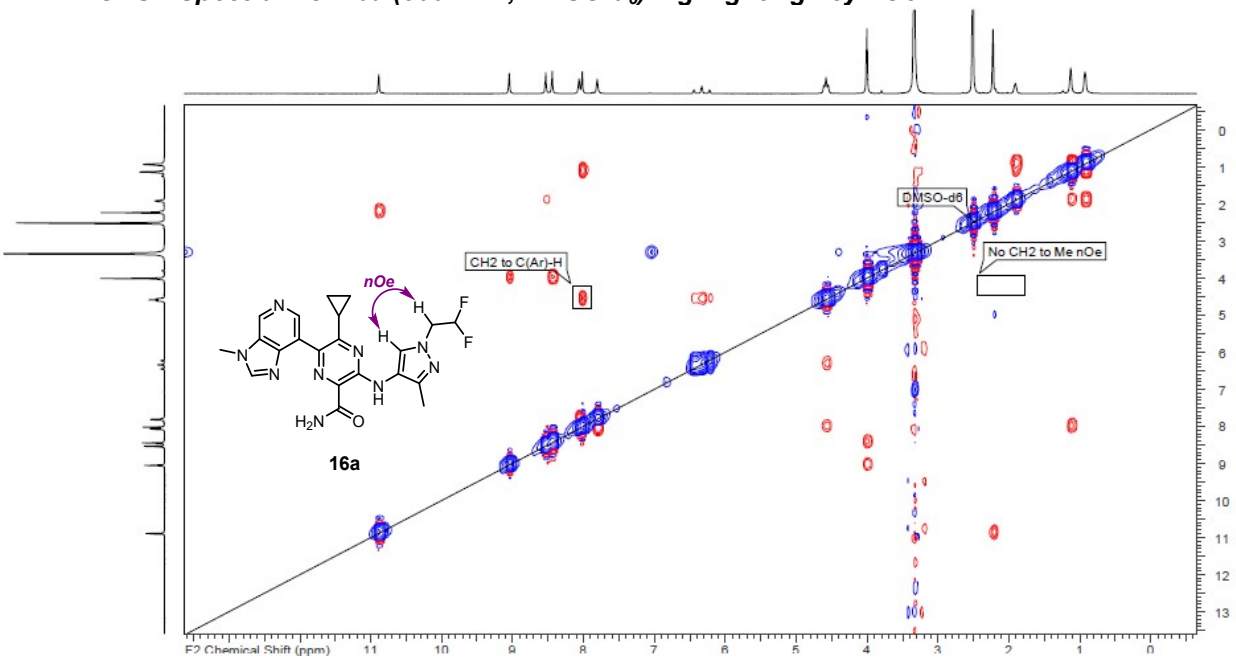
^{19}F NMR Spectrum of 16a (471 MHz, DMSO-d_6)



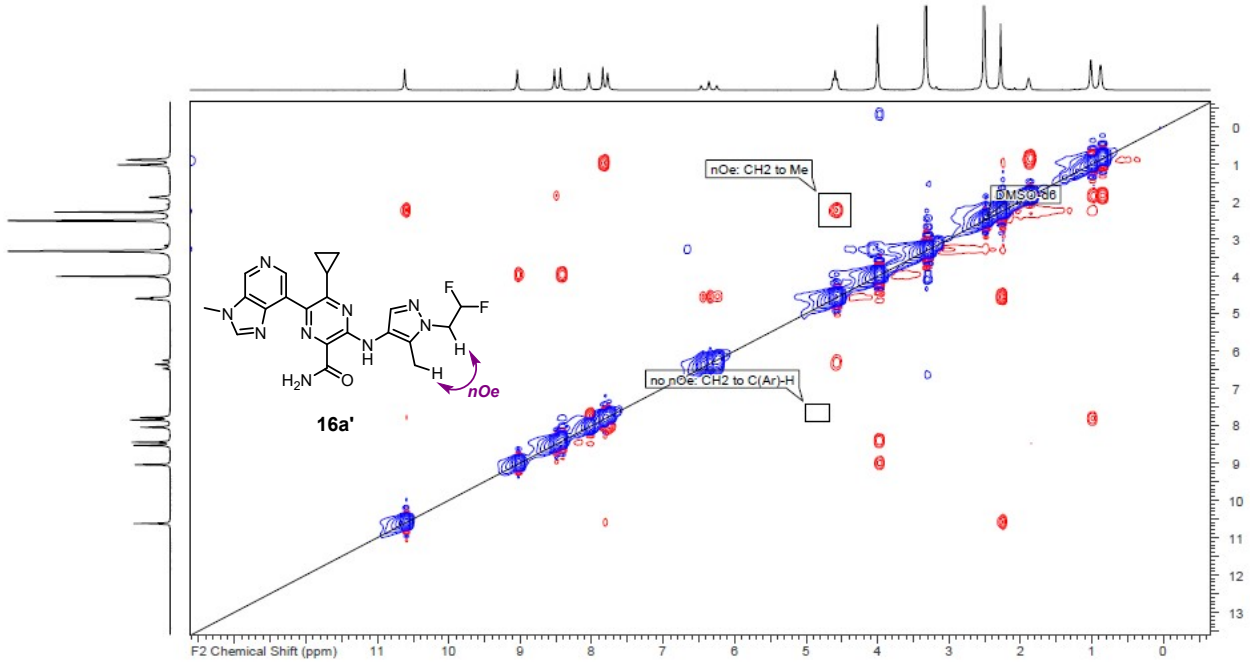
^{19}F NMR Spectrum of 16a' (471 MHz, DMSO-d_6)

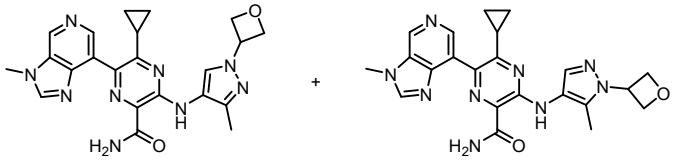


^{1}H-^{1}H ROESY Spectrum of 16a (500 MHz, DMSO-d_6) highlighting key nOe.



^{1}H-^{1}H ROESY Spectrum of 16a' (500 MHz, DMSO-d_6) highlighting key nOe.

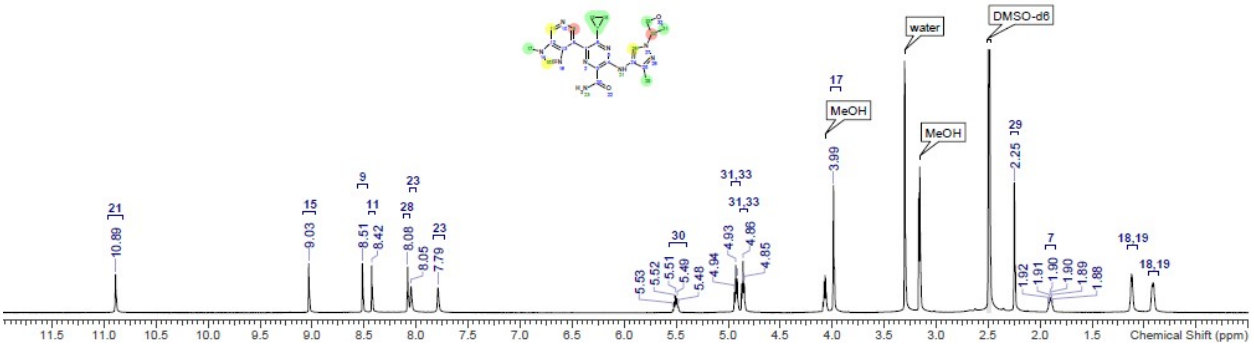




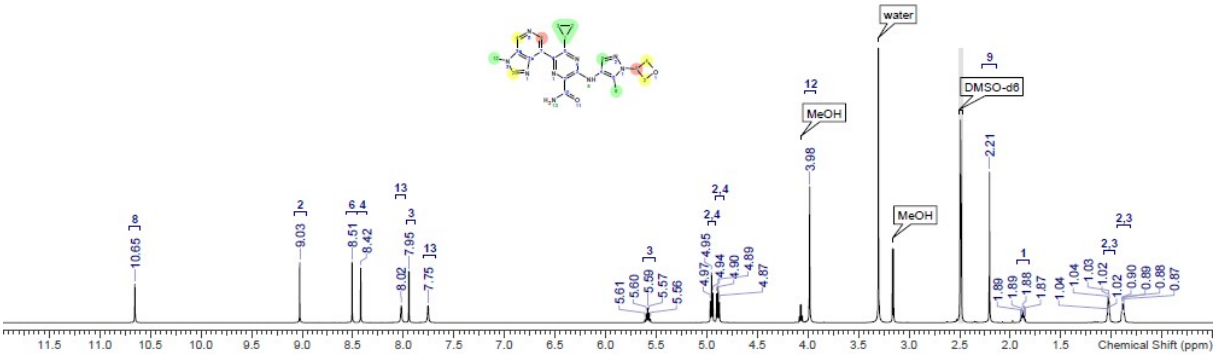
16b **16b'**

Synthesis of 16b by Late-Stage Alkylation. General Procedure A was followed using **7** (75 mg, 0.19 mmol, 1.0 equiv), 3.0 equiv of 3-iodooxetane, 3.0 equiv of Cs_2CO_3, 1.0 mL of DMSO (0.18 M w.r.t. **7**) to deliver the product in ~2:1 rr favoring **16b**. Following preparative SFC on a chiral stationary phase (OJ-H column 250 x 21 mm/5 μ, 100–75% $scCO_2$/MeOH w/ 0.2% NH_4OH modifier, 40 °C, 220 nm), 5-cyclopropyl-3-((3-methyl-1-(oxetan-3-yl)-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**16b**, 0.055 g, 64.1%) and 5-cyclopropyl-3-((5-methyl-1-(oxetan-3-yl)-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo-[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**16b′**, 0.027 g, 31.5%) were obtained, both as yellow solids. **16b:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.86 - 0.96 (m, 2H), 1.06 - 1.16 (m, 2H), 1.86 - 1.94 (m, 1H), 2.25 (s, 3H), 3.99 (s, 3H), 4.86 (br t, *J* = 5.9 Hz, 2H), 4.89 - 4.97 (m, 2H), 5.40 - 5.56 (m, 1H), 7.79 (br s, 1H), 8.05 (br s, 1H), 8.08 (s, 1H), 8.43 (br d, *J* = 0.9 Hz, 1H), 8.52 (d, *J* = 1.4 Hz, 1H), 9.03 (d, *J* = 1.2 Hz, 1H), 10.89 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 446.2. **16b′:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.82 - 0.94 (m, 2H), 1.03 (dt, *J* = 7.0, 3.6 Hz, 2H), 1.81 - 1.94 (m, 1H), 2.21 (s, 3H), 3.98 (s, 3H), 4.83 - 4.92 (m, 2H), 4.92 - 4.99 (m, 2H), 5.58 (quin, *J* = 7.0 Hz, 1H), 7.75 (br s, 1H), 7.95 (s, 1H), 8.02 (br s, 1H), 8.42 (s, 1H), 8.51 (s, 1H), 9.03 (s, 1H), 10.65 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 446.2.

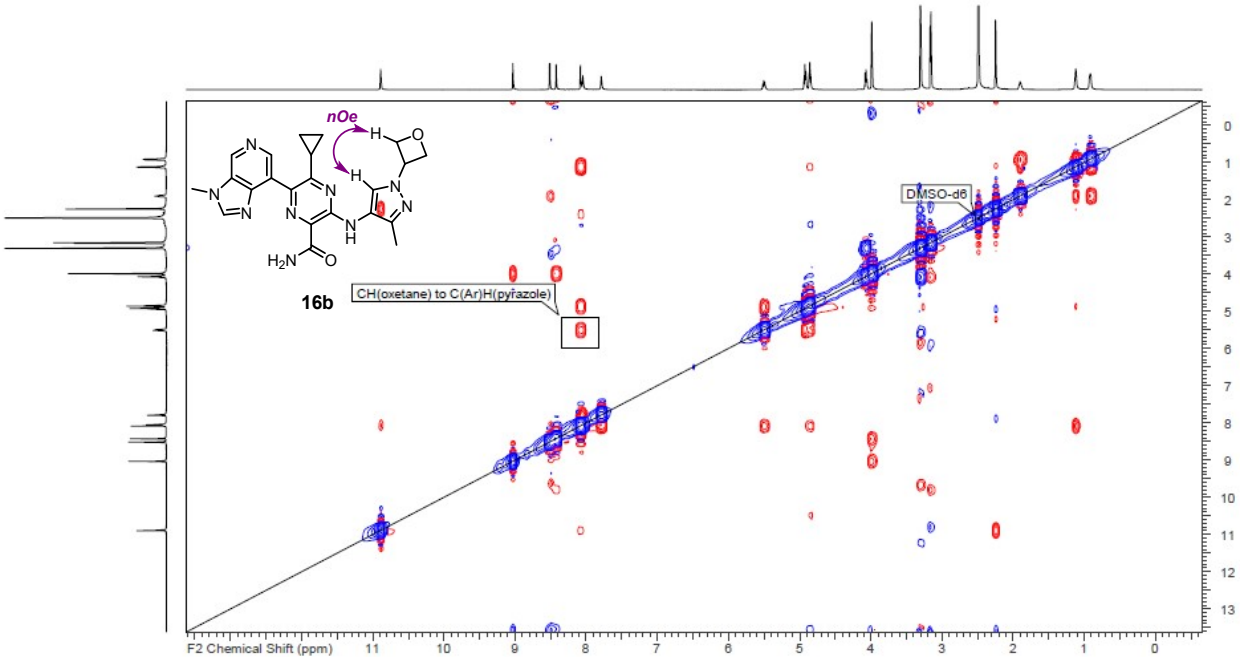
^{1}H NMR Spectrum of 16b (500 MHz, DMSO-d_6)



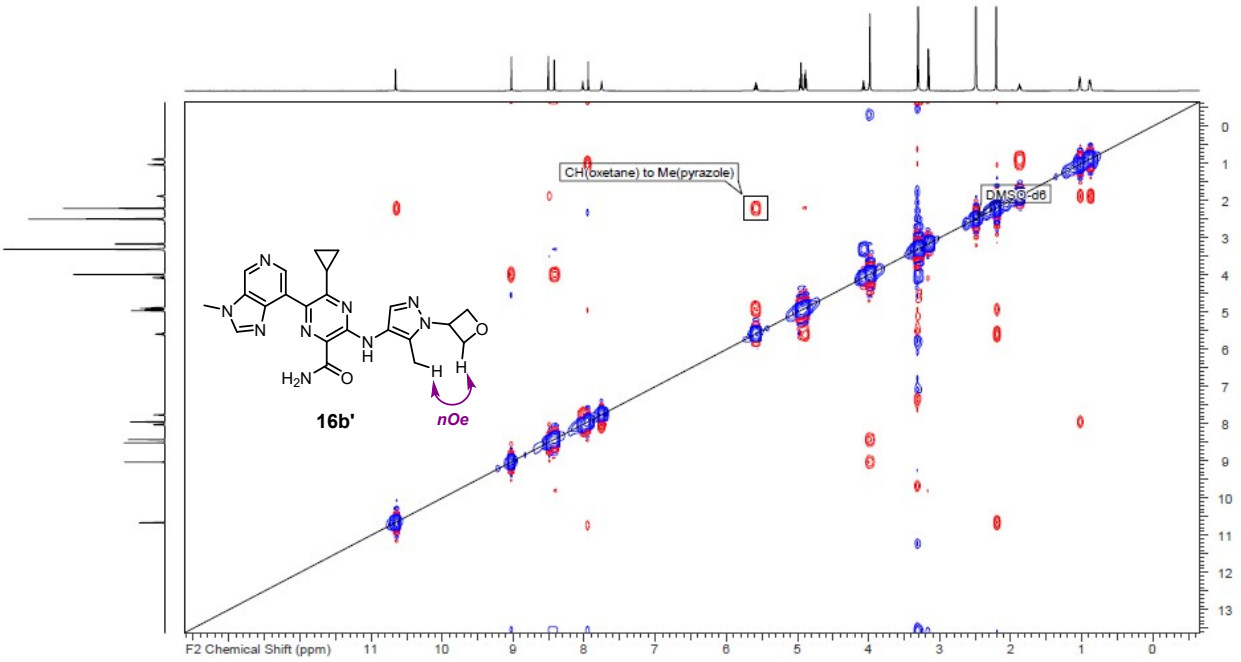
^{1}H NMR Spectrum of 16b' (500 MHz, DMSO-d_6)

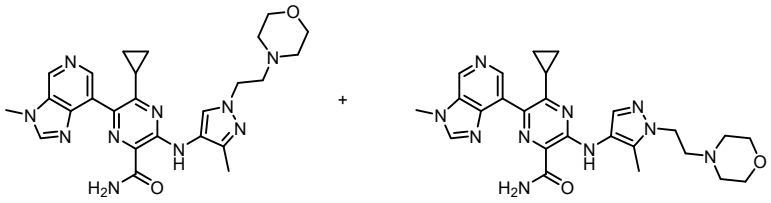


^{1}H-^{1}H ROESY Spectrum of 16a (500 MHz, DMSO-d_6) highlighting key nOe.



^{1}H-^{1}H ROESY Spectrum of 16b' (500 MHz, DMSO-d_6) highlighting key nOe.

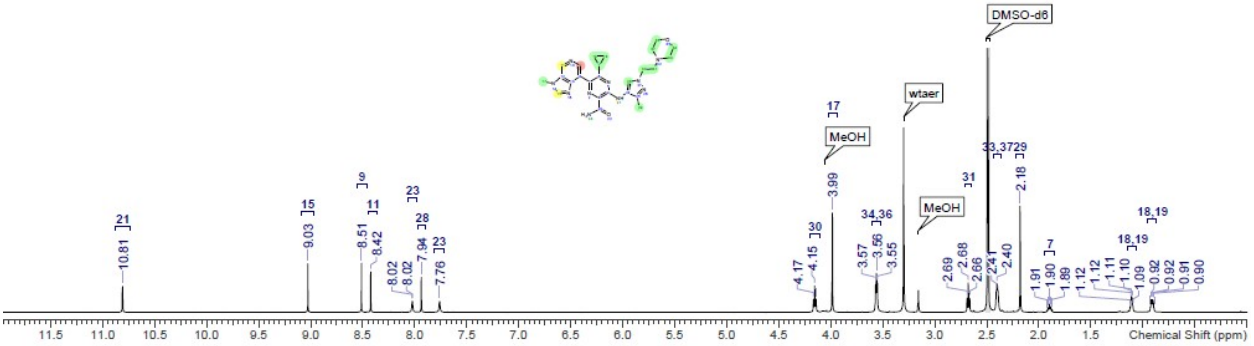




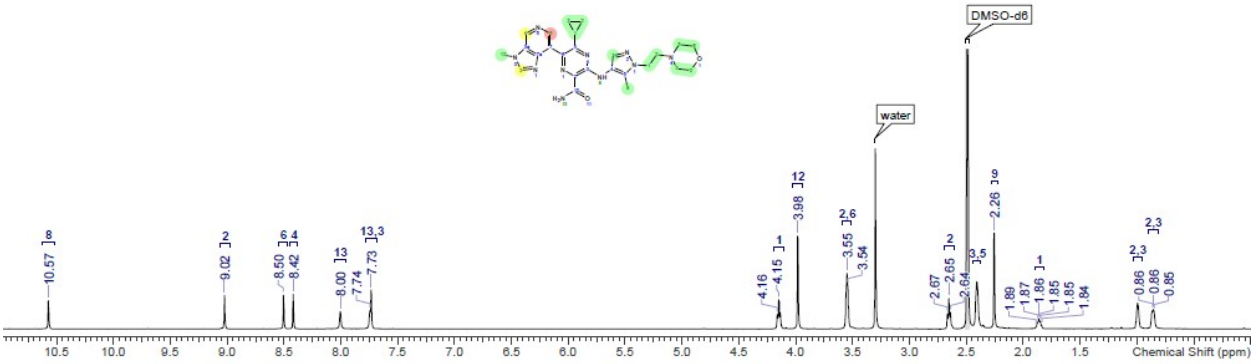
16c **16c'**

Synthesis of 16c by Late-Stage Alkylation. General Procedure A was followed using **7** (70 mg, 0.18 mmol, 1.0 equiv), 3.0 equiv of 4-(2-bromoethyl)morpholine•HCl, 5.0 equiv of Cs_2CO_3, 1.8 mL of DMSO (0.10 M w.r.t. **7**) to deliver the product in ~2:1 rr favoring **16c**. Following preparative SFC on a chiral stationary phase (OJ-H column 250 x 21 mm/5 μ, 100–80% $scCO_2$/MeOH w/ 0.2% NH_4OH modifier, 40 °C, 220 nm), 5-cyclopropyl-3-((3-methyl-1-(2-morpholinoethyl)-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**16c**, 9.00 mg, 10.0%) and 5-cyclopropyl-3-((5-methyl-1-(2-morpholinoethyl)-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**16c′**, 7.00 mg, 7.8%) were obtained, both as yellow solids. **16c:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.87 - 0.95 (m, 2H), 1.07 - 1.14 (m, 2H), 1.85 - 1.94 (m, 1H), 2.18 (s, 3H), 2.40 (br t, *J* = 3.8 Hz, 4H), 2.68 (t, *J* = 6.3 Hz, 2H), 3.52 - 3.61 (m, 4H), 3.99 (s, 3H), 4.15 (t, *J* = 6.3 Hz, 2H), 7.76 (br d, *J* = 1.4 Hz, 1H), 7.94 (s, 1H), 8.02 (br d, *J* = 1.2 Hz, 1H), 8.42 (s, 1H), 8.51 (s, 1H), 9.03 (s, 1H), 10.81 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 503.3. **16c′:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.82 - 0.90 (m, 2H), 0.96 - 1.03 (m, 2H), 1.82 - 1.90 (m, 1H), 2.26 (s, 3H), 2.37 - 2.44 (m, 4H), 2.65 (br t, *J* = 6.5 Hz, 2H), 3.55 (br t, *J* = 3.5 Hz, 4H), 3.98 (s, 3H), 4.15 (br t, *J* = 6.4 Hz, 2H), 7.68 - 7.78 (m, 2H), 8.00 (br s, 1H), 8.42 (s, 1H), 8.50 (s, 1H), 9.02 (s, 1H), 10.57 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 503.3.

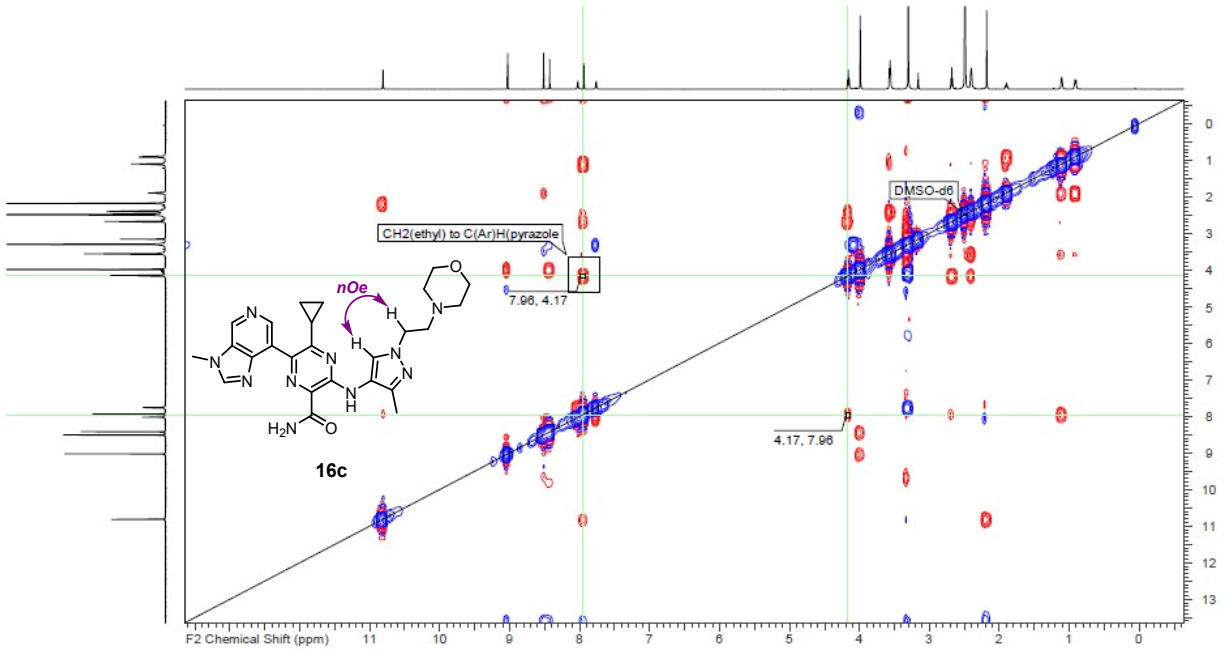
^{1}H NMR Spectrum of 16c (500 MHz, DMSO-d_6)



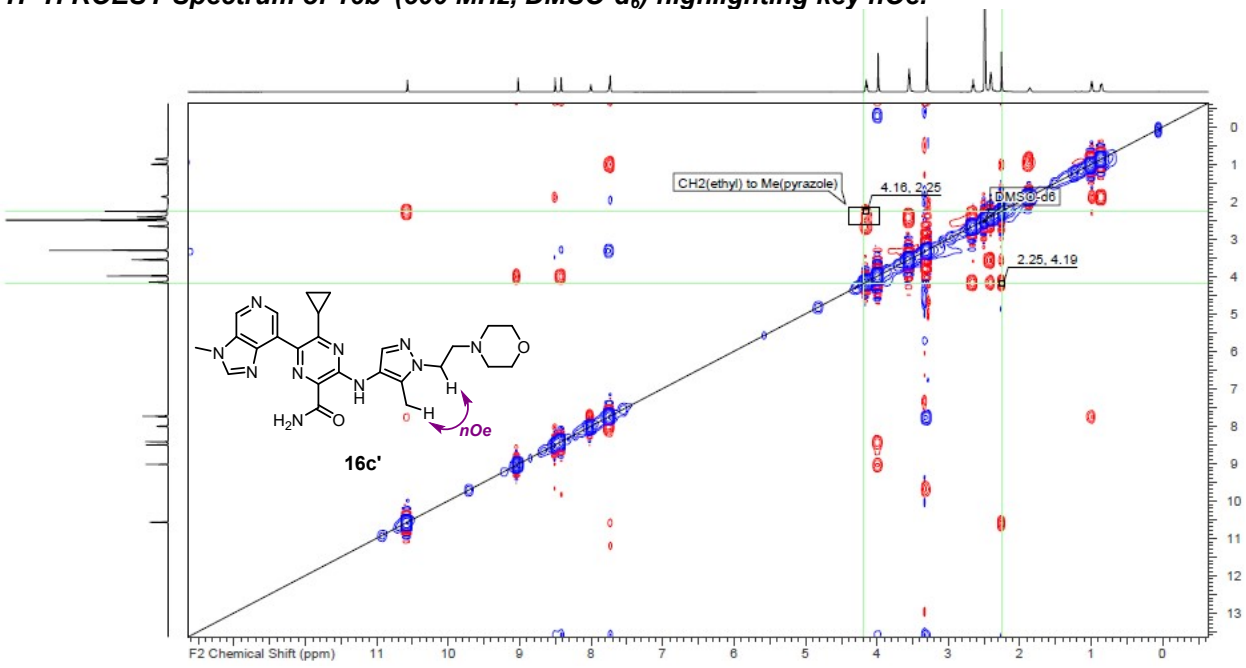
^{1}H NMR Spectrum of 16c' (500 MHz, DMSO-d_6)

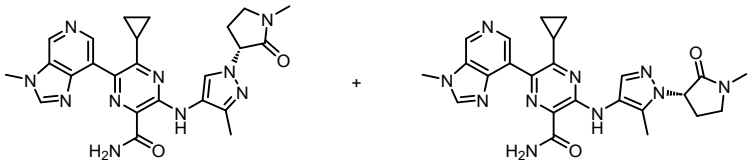


^{1}H-^{1}H ROESY Spectrum of 16c (500 MHz, DMSO-d_6) highlighting key nOe.



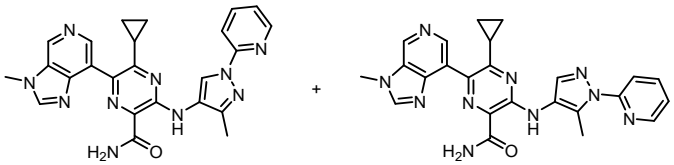
^{1}H-^{1}H ROESY Spectrum of 16b' (500 MHz, DMSO-d_6) highlighting key nOe.





(±)-16d (±)-16d'

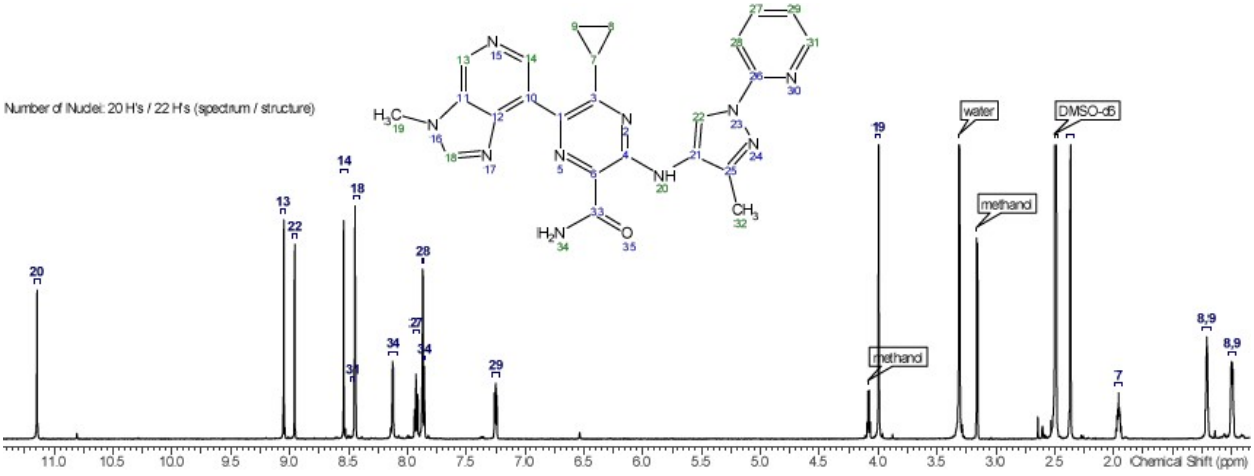
Synthesis of 16d by Late-Stage Alkylation. General Procedure A was followed using **7** (200 mg, 0.51 mmol, 1.0 equiv), 2.5 equiv of 3-bromo-1-methylpyrrolidin-2-one, 3.0 equiv of Cs_2CO_3, 2.5 mL of DMSO (0.20 M w.r.t. **7**) to deliver the product in ~2:1 rr favoring **16d**. Following preparative SFC on a chiral stationary phase (IH column 250 x 21 mm/5 μ, 100–65% $scCO_2$/EtOH, 40 °C, 220 nm), four isomers were obtained: 5-cyclopropyl-3-((3-methyl-1-(1-methyl-2-oxopyrrolidin-3-yl)-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carbox-amide (***ent*-16d**, 33 mg, 13.2%); *ent*-5-cyclopropyl-3-((3-methyl-1-(1-methyl-2-oxopyrrolidin-3-yl)-1H-pyrazol-4-yl)amino)-6-(3-methyl-3H-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**16d**, 36 mg, 14.4%); 5-cyclopropyl-3-((5-methyl-1-(1-methyl-2-oxopyrrolidin-3-yl)-1H-pyrazol-4-yl)amino)-6-(3-methyl-3H-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (***ent*-16d′**, 12 mg, 4.8%); and *ent*-5-cyclopropyl-3-((5-methyl-1-(1-methyl-2-oxopyrrolidin-3-yl)-1H-pyrazol-4-yl)amino)-6-(3-methyl-3H-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**16d′**, 14 mg, 5.6%), all of which were yellow solids. **16d:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.88 - 0.95 (m, 2H), 1.10 - 1.12 (m, 2H), 1.33 - 1.49 (m, 1H), 1.85 - 1.93 (m, 1H), 2.19 (s, 3H), 2.33 - 2.45 (m, 1H), 2.80 (s, 3H), 3.35 - 3.43 (m, 1H), 3.48 (td, *J* = 9.3, 3.4 Hz, 1H), 3.99 (s, 3H), 5.01 (t, *J* = 8.4 Hz, 1H), 7.76 (br s, 1H), 7.99 (s, 1H), 8.04 (br s, 1H), 8.42 (s, 1H), 8.52 (s, 1H), 9.03 (s, 1H), 10.87 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 487.4. ***ent*-16d:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.84 - 0.91 (m, 2H), 1.00 - 1.05 (m, 2H), 1.82 - 1.91 (m, 1H), 2.27 (s, 3H), 2.51 - 2.55 (m, 1H), 2.79 (s, 3H), 3.36 - 3.47 (m, 1H), 3.51 (td, *J* = 9.3, 3.8 Hz, 1H), 3.98 (s, 3H), 5.13 (t, *J* = 8.9 Hz, 1H), 7.76 (br s, 1H), 7.82 (s, 1H), 8.02 (br s, 1H), 8.42 (s, 1H), 8.50 (s, 1H), 9.02 (s, 1H), 10.68 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 487.3. **16d′:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.84 - 0.90 (m, 2H), 1.00 - 1.03 (m, 2H), 1.35 - 1.49 (m, 2H), 1.83 - 1.90 (m, 1H), 2.27 (s, 3H), 2.79 (s, 3H), 3.37 - 3.46 (m, 2H), 3.51 (td, *J* = 9.3, 3.8 Hz, 1H), 3.99 (s, 3H), 5.13 (dt, *J* = 8.5, 1.0 Hz, 1H), 7.83 (s, 1H), 8.01 (br s, 1H), 8.42 (s, 1H), 8.51 (s, 1H), 9.02 (s, 1H), 10.68 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 486.2. ***ent*-16d′:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.84 - 0.97 (m, 2H), 1.05 - 1.14 (m, 2H), 1.84 - 1.97 (m, 1H), 2.19 (s, 3H), 2.34 - 2.45 (m, 1H), 2.51 - 2.56 (m, 1H), 2.81 (s, 3H), 3.35 - 3.43 (m, 1H), 3.48 (td, *J* = 9.5, 3.1 Hz, 1H), 3.98 (s, 3H), 5.01 (t, *J* = 8.9 Hz, 1H), 7.77 (br s, 1H), 7.99 (s, 1H), 8.05 (br s, 1H), 8.43 (s, 1H), 8.52 (s, 1H), 9.03 (s, 1H), 10.87 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 487.3.

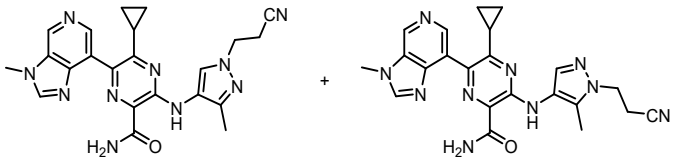


17 **17'**

Synthesis of 17 by Late-Stage S_NAr. General Procedure A was followed using **7** (25 mg, 0.06 mmol, 1.0 equiv), 3.0 equiv of 2-fluoropyridine, 3.0 equiv of Cs_2CO_3, 0.99 mL of DMSO (0.06 M w.r.t. **7**) at 120 °C for 2 h to deliver the product in ~2:1 rr favoring **17**. SFC on chiral stationary phase was not required to separate **17** and **17'**, only a slow gradient on reverse phase flash chromatography. 5-Cyclopropyl-3-((3-methyl-1-(pyridin-2-yl)-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**17**, 0.012 g, 40.1%) was obtained as a yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.96 - 1.02 (m, 2H), 1.17 - 1.25 (m, 2H), 1.92 - 2.00 (m, 1H), 2.36 (s, 3H), 4.00 (s, 3H), 7.23 (quin, *J* = 1.0 Hz, 1H), 7.83 - 7.88 (m, 2H), 7.91 (q, *J* = 1.0 Hz, 1H), 8.13 (br s, 1H), 8.40 - 8.48 (m, 2H), 8.53 (s, 1H), 8.95 (s, 1H), 9.05 (s, 1H), 11.12 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 467.0.

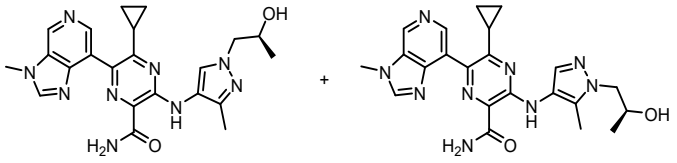
^{1}H NMR Spectrum of 17 (600 MHz, DMSO-d_6)





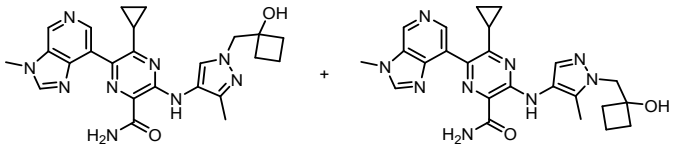
18a **18a′**

Synthesis of 18a by Late-Stage Conjugate Addition. General Procedure A was followed using **7** (100 mg, 0.26 mmol, 1.0 equiv), 1.5 equiv of acrylonitrile, 0.75 equiv of DBU, 5 mL of DMSO (0.05 M w.r.t. **7**) at rt overnight to deliver the product in ~3:1 rr favoring **18a**. Following SFC on a chiral stationary phase (IJ column 250 x 21 mm/5 μ, 80% $scCO_2$/MeOH w/ 0.2% NH_4OH modifier, 40 °C, 254 nm), 3-((1-(2-cyanoethyl)-3-methyl-1*H*-pyrazol-4-yl)amino)-5-cyclopropyl-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**18a**, 0.061 g, 53.9%) and 3-((1-(2-cyanoethyl)-5-methyl-1*H*-pyrazol-4-yl)amino)-5-cyclopropyl-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**18a′**, 0.017 g, 14.9%) were obtained, both as yellow solids. **18a**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.84 - 0.96 (m, 2 H) 1.18 (br s, 2 H) 1.84 - 1.94 (m, 1 H) 2.23 (s, 3 H) 3.04 (t, J=6.26 Hz, 2 H) 4.00 (s, 3 H) 4.36 (t, J=6.33 Hz, 2 H) 7.80 (br s, 1 H) 7.95 - 8.13 (overlap, 2 H) 8.44 (s, 1 H) 8.53 (s, 1 H) 9.04 (s, 1 H) 10.89 (s, 1 H). *m/z*: (ES+), $[M + H]^+$ = 442.9. **18a′**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.84 - 0.96 (m, 2 H) 1.04 (br s, 2 H) 1.84 - 1.94 (m, 1 H) 2.31 (s, 3 H) 3.04 (t, *J*=6.26 Hz, 2 H) 4.00 (s, 3 H) 4.36 (t, *J*=6.33 Hz, 2 H) 7.78 (br s, 1 H) 7.86 (s, 1 H) 8.05 (br s, 1 H) 8.44 (s, 1 H) 8.53 (s, 1 H) 9.04 (s, 1 H) 10.67 (s, 1 H). *m/z*: (ES+), $[M + H]^+$ = 442.7.



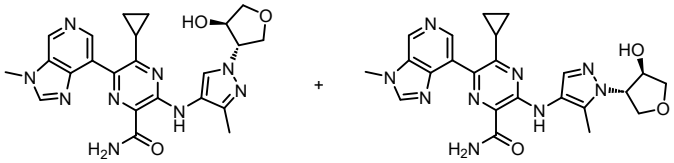
19a **19a′**

Synthesis of 19a by Late-Stage Epoxide Opening. General Procedure A was followed using **7** (100 mg, 0.26 mmol, 1.0 equiv), 3.0 equiv of (*S*)-propylene oxide, 3.0 equiv of Cs_2CO_3, 2 mL of DMF (0.13 M w.r.t. **7**) at 80 °C for 5 h to deliver the product in ~5:1 rr favoring **19a**. SFC on a chiral stationary phase was not required to separate **19a** and **19a′**, only reverse phase flash chromatography, elution gradient 0–20% MeCN/H_2O with 0.1% formic acid modifier, providing (*S*)-5-cyclopropyl-3-((1-(2-hydroxypropyl)-3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**19a**, 62 mg, 36%) and (*S*)-5-cyclopropyl-3-((1-(2-hydroxypropyl)-5-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**19a′**, 43 mg, 25% yield). **19a:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.90 (br dd, *J* = 7.6, 2.8 Hz, 2H), 1.00 - 1.06 (m, *J* = 3.4 Hz, 3H), 1.07 - 1.14 (m, 2H), 1.85 - 1.92 (m, 1H), 2.18 (s, 3H), 3.89 - 3.95 (m, 3H), 3.98 (s, 3H), 4.89 (br s, 1H), 7.76 (br s, 1H), 7.95 (s, 1H), 8.02 (br s, 1H), 8.42 (s, 1H), 8.51 (s, 1H), 9.03 (s, 1H), 10.80 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 448.2. **19a′:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.86 (br dd, *J* = 7.6, 3.1 Hz, 2H), 0.98 - 1.03 (m, 2H), 1.05 (br d, *J* = 5.6 Hz, 3H), 1.83 - 1.90 (m, 1H), 2.25 (s, 3H), 3.87 - 3.93 (m, 1H), 3.94 - 3.97 (m, 2H), 3.98 (s, 3H), 4.87 (br s, 1H), 7.75 (br s, 2H), 8.00 (br s, 1H), 8.42 (s, 1H), 8.50 (s, 1H), 9.02 (s, 1H), 10.59 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 448.2.



19b **19b′**

Synthesis of 19b by Late-Stage Epoxide Opening. General Procedure A was followed using **7** (150 mg, 0.39 mmol, 1.0 equiv), 1.2 equiv of 1-oxaspiro[2.3]hexane, 2.0 equiv of Cs_2CO_3, 3 mL of DMF (0.13 M w.r.t. **7**) at 80 °C for 1 h to deliver the product in ~5:1 rr favoring **19b**. Following SFC on a chiral stationary phase (IG column 250 x 21 mm/5 μ, 90–40% $scCO_2$/EtOH, 40 °C, 220 nm), 5-cyclopropyl-3-((1-((1-hydroxycyclobutyl)methyl)-3-methyl-1H-pyrazol-4-yl)amino)-6-(3-methyl-3H-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**19b**, 0.064 g, 35.1%) and 5-cyclopropyl-3-((1-((1-hydroxycyclobutyl)-methyl)-5-methyl-1H-pyrazol-4-yl)amino)-6-(3-methyl-3H-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**19b′**, 0.029 g, 15.9%) were obtained, both as yellow solids. **19b:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.83 - 0.93 (m, 2H), 1.06 - 1.16 (m, 2H), 1.38 - 1.49 (m, 1H), 1.62 (q, *J* = 10.0 Hz, 1H), 1.82 - 1.98 (m, 3H), 2.02 - 2.10 (m, 2H), 2.19 (s, 3H), 3.98 (s, 3H), 4.08 (s, 2H), 5.34 (br s, 1H), 7.75 (br s, 1H), 8.01 (br s, 1H), 8.05 (s, 1H), 8.42 (s, 1H), 8.51 (s, 1H), 9.03 (s, 1H), 10.82 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 474.2. **19b′:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.82 - 0.89 (m, 2H), 1.02 (br s, 2H), 1.43 - 1.54 (m, 1H), 1.59 - 1.69 (m, 1H), 1.82 - 1.95 (m, 3H), 2.20 (br t, *J* = 9.3 Hz, 2H), 2.27 (s, 3H), 3.98 (s, 3H), 4.09 (s, 2H), 5.27 (br s, 1H), 7.73 (br s, 1H), 7.76 (s, 1H), 8.00 (br s, 1H), 8.42 (s, 1H), 8.50 (s, 1H), 9.02 (s, 1H), 10.61 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 474.2.

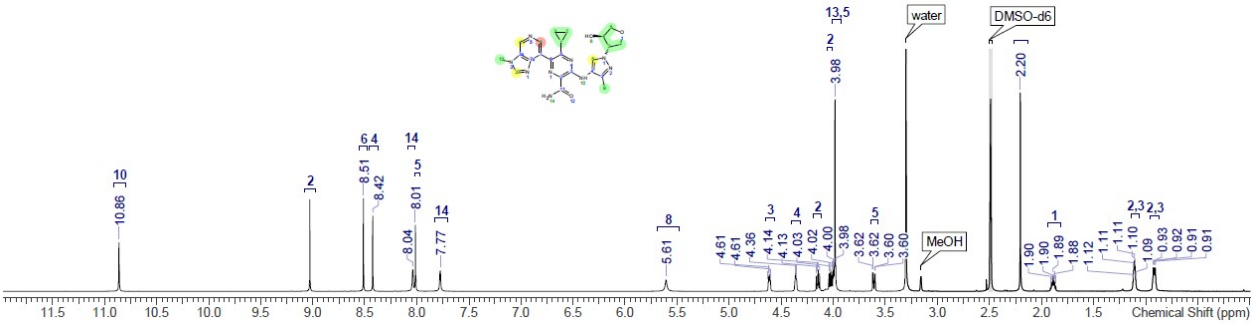


(±)-19c **(±)-19c′**

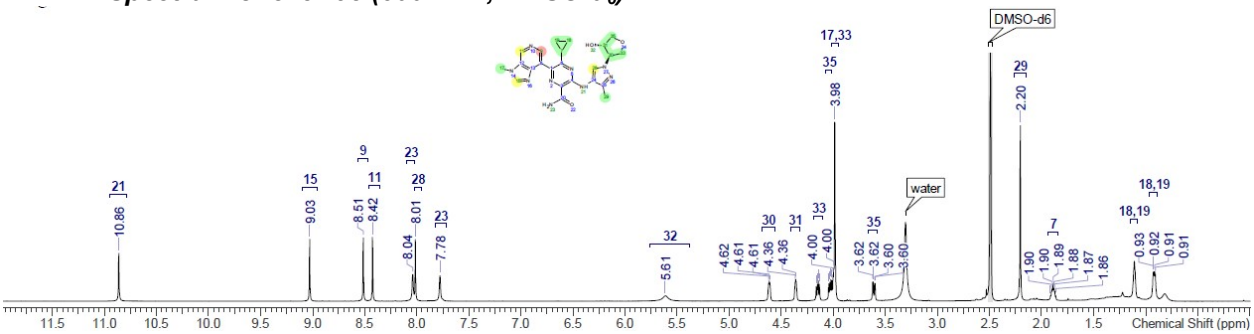
Synthesis of 19c by Late-Stage Epoxide Opening. General Procedure A was followed using **7** (200 mg, 0.51 mmol, 1.0 equiv), 3.0 equiv of 3,6-dioxabicyclo[3.1.0]hexane, 3.0 equiv of Cs_2CO_3, 2 mL of DMSO (0.24 M w.r.t. **7**) at 100 °C for 2 h to deliver the product in ~2:1 rr favoring **(±)-19c**. Following SFC on a chiral stationary phase (IG column 250 x 21 mm/5 μm, 80% $scCO_2$/MeOH with 0.2% NH_4OH modifier, 40 °C, 254 nm), four isomers were obtained: *ent*-5-cyclopropyl-3-((1-((*trans*)-4-hydroxytetrahydrofuran-3-yl)-3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3H-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (***ent*-19c**, 0.035 g, 14.3%), 5-cyclopropyl-3-((1-((*trans*)-4-hydroxytetrahydrofuran-3-yl)-3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**19c**, 0.035 g, 14.3%), *ent*-5-cyclopropyl-3-((1-((trans)-4-hydroxytetrahydrofuran-3-yl)-5-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (***ent*-19c′**, 0.015 g, 6.1%), and 5-cyclopropyl-3-((1-((*trans*)-4-hydroxytetrahydrofuran-3-yl)-5-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (**19c′**, 0.015 g, 6.1%), all of which were yellow solids. **19c:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.92 (br dd, *J* = 7.9, 3.1 Hz, 2H), 1.06 - 1.14 (m, 2H), 1.82 - 1.94 (m, 1H), 2.20 (s, 3H), 3.61 (dd, *J* = 9.4, 2.8 Hz, 1H), 3.93 - 4.01 (m, 4H), 4.03 (dd, *J* = 9.4, 5.1 Hz, 1H), 4.15 (dd, *J* = 9.7, 6.0 Hz, 1H), 4.36 (dt, *J* = 4.9, 2.4 Hz, 1H), 4.61 (dt, *J* = 5.6, 2.7 Hz, 1H), 5.61 (br s, 1H), 7.78 (br d, *J* = 1.4 Hz, 1H), 8.01 (s, 1H), 8.04 (s, 1H), 8.42 (s, 1H), 8.51 (s, 1H), 9.03 (s, 1H), 10.86 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 476.2. ***ent*-19c:** ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.92 (br dd, *J* = 7.9, 3.0 Hz, 2H), 1.08 - 1.15 (m, 2H), 1.84 - 1.94 (m, 1H), 2.20 (s, 3H), 3.61 (dd, *J* = 9.5, 2.6 Hz, 1H), 3.94 - 4.01 (m, 4H), 4.03 (dd, *J* = 9.5, 5.0 Hz, 1H), 4.15 (dd, *J* = 9.5, 6.0 Hz, 1H), 4.32 - 4.41 (m, 1H), 4.56 - 4.68 (m, 1H), 5.61 (br s, 1H), 7.78 (br s, 1H), 8.01 (s, 1H), 8.04 (br s, 1H), 8.42 (s, 1H), 8.51 (s, 1H), 9.03 (s, 1H), 10.86 (s, 1H).). *m/z*: (ES+), $[M + H]^+$ = 476.2. **19c′**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.87 (br dd, *J* = 7.7, 3.1 Hz, 2H), 0.95 - 1.07 (m, 2H), 1.81 - 1.94 (m, 1H), 2.30 (s, 3H), 3.64 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.92 - 4.03 (m, 5H), 4.21 (t, *J* = 8.1 Hz, 1H), 4.38 - 4.48 (m, 1H), 4.69 (br t, *J* = 6.9 Hz, 1H), 5.51 (br s, 1H), 7.76 (br s, 1H), 7.84 (s, 1H), 8.02 (br s, 1H), 8.42 (s, 1H), 8.51 (s, 1H), 9.02 (s, 1H), 10.67 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 476.2. ***ent*-19c′:** ^{1}H NMR (500

MHz, DMSO-d_6) δ 0.84 - 0.91 (m, 2H), 0.98 - 1.05 (m, 2H), 1.82 - 1.92 (m, 1H), 2.30 (s, 3H), 3.64 (dd, *J* = 9.1, 3.1 Hz, 1H), 3.92 - 4.03 (m, 5H), 4.21 (dd, *J* = 9.1, 7.1 Hz, 1H), 4.42 (dt, *J* = 4.8, 2.5 Hz, 1H), 4.69 (ddd, *J* = 7.1, 4.5, 2.7 Hz, 1H), 5.51 (br s, 1H), 7.76 (br d, *J* = 1.5 Hz, 1H), 7.84 (s, 1H), 8.02 (br s, 1H), 8.42 (s, 1H), 8.51 (s, 1H), 9.02 (s, 1H), 10.67 (s, 1H). *m/z*: (ES+), $[M + H]^+$ = 476.2.

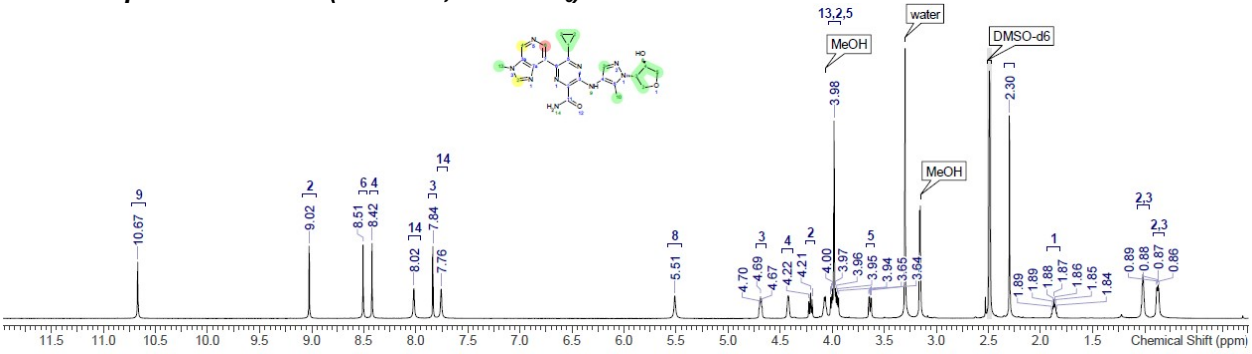
^{1}H NMR Spectrum of 19c (500 MHz, DMSO-d_6)



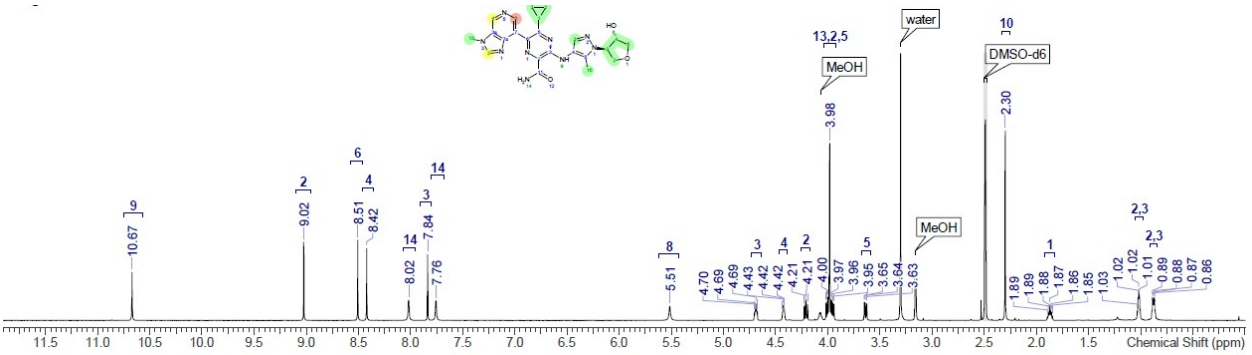
^{1}H NMR Spectrum of ent-19c (500 MHz, DMSO-d_6)



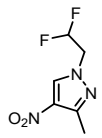
^{1}H NMR Spectrum of 19c' (500 MHz, DMSO-d_6)



^{1}H NMR Spectrum of 19c' (500 MHz, DMSO-d_6)

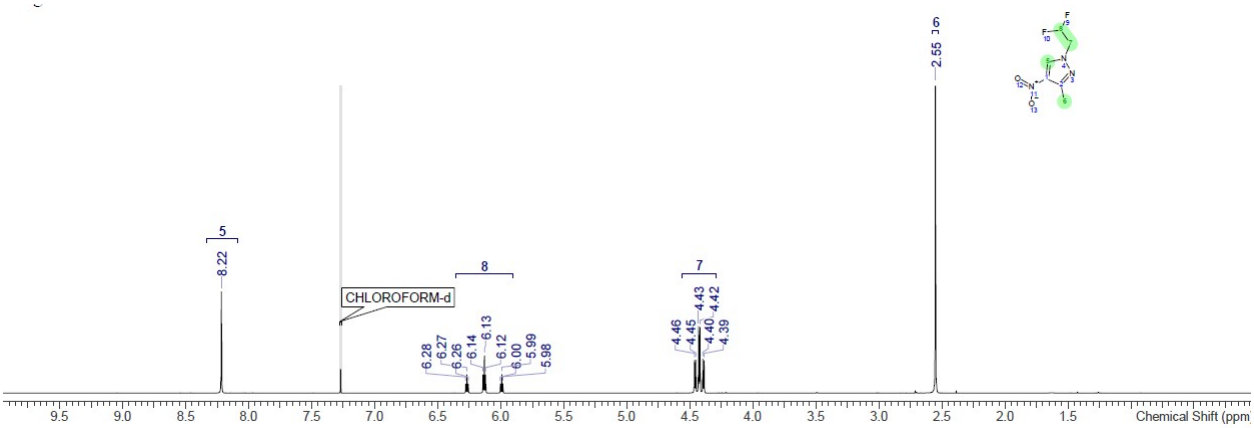


Gram-Scale Synthesis of 16a (Scheme 3).

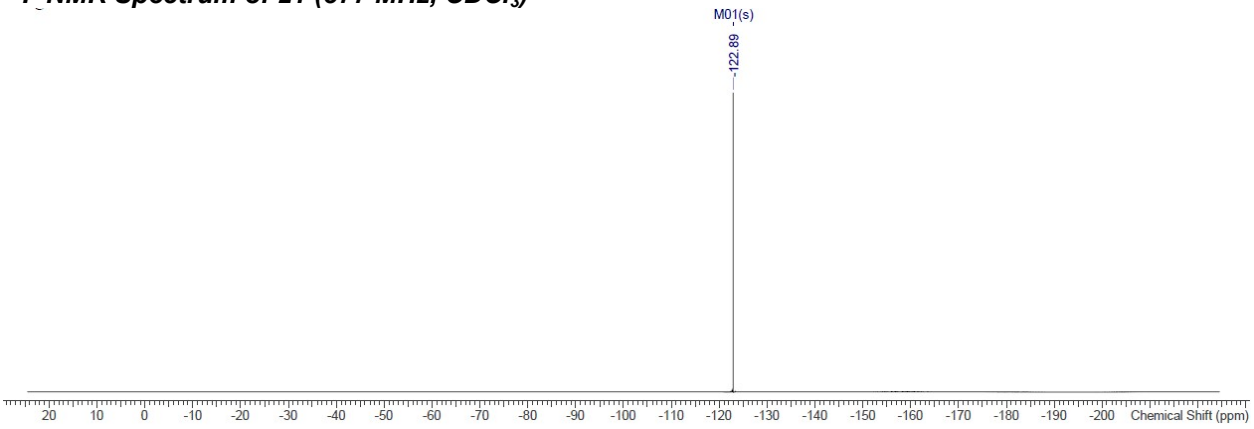


1-(2,2-difluoroethyl)-3-methyl-4-nitro-1*H*-pyrazole (21). A 100 mL round bottom flask was charged with 3-methyl-4-nitro-1*H*-pyrazole (**20**, 5.00 g, 39.3 mmol, 1.0 equiv), Cs_2CO_3 (15.38 g, 47.2 mmol, 1.2 equiv), and a magnetic stir bar. The mixture was suspended in DMF (39.3 mL, 1.0 M w.r.t. **20**), and the flask was sealed with a septum. A nitrogen inlet needle was added through the septum, and 1,1-difluoro-2-iodoethane (9.82 g, 51.1 mmol, 1.3 equiv) was added slowly *via* syringe. The reaction mixture was then allowed to stir at 80 °C for 2 h. The reaction mixture was cooled to rt, quenched with water, and transferred to a separatory funnel rinsing with EtOAc. The aqueous layer was extracted three times with EtOAc. The organics were washed three times with 5% aq. LiCl and once with brine. The combined organics were dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo* to provide a viscous, orange oil. ^{1}H NMR (400 MHz, $CDCl_3$) and ^{19}F NMR (370 MHz, $CDCl_3$) was consistent with the expected product as a 2:1 mixture of pyrazole regiosiomers. The isolated crude product was purified by preparative SFC on a chiral stationary phase (IG column, 250x21 mm, 5μ, 95% $scCO_2$/MeOH with 0.2% NH_4OH modifier). Both regioisomeric products were collected and concentrated. The major peak was dried thoroughly on high vacuum to provide 1-(2,2-difluoroethyl)-3-methyl-4-nitro-1*H*-pyrazole (**21**, 4.17 g, 55.4%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.55 (s, 3H), 4.43 (td, *J* = 13.4, 4.2 Hz, 2H), 5.90 - 6.36 (m, 1H), 8.22 (s, 1H). ^{19}F NMR (377 MHz, $CDCl_3$) δ –122.89 (s, 2F). *m/z*: (ES+), $[M + H]^+$ = 192.0.

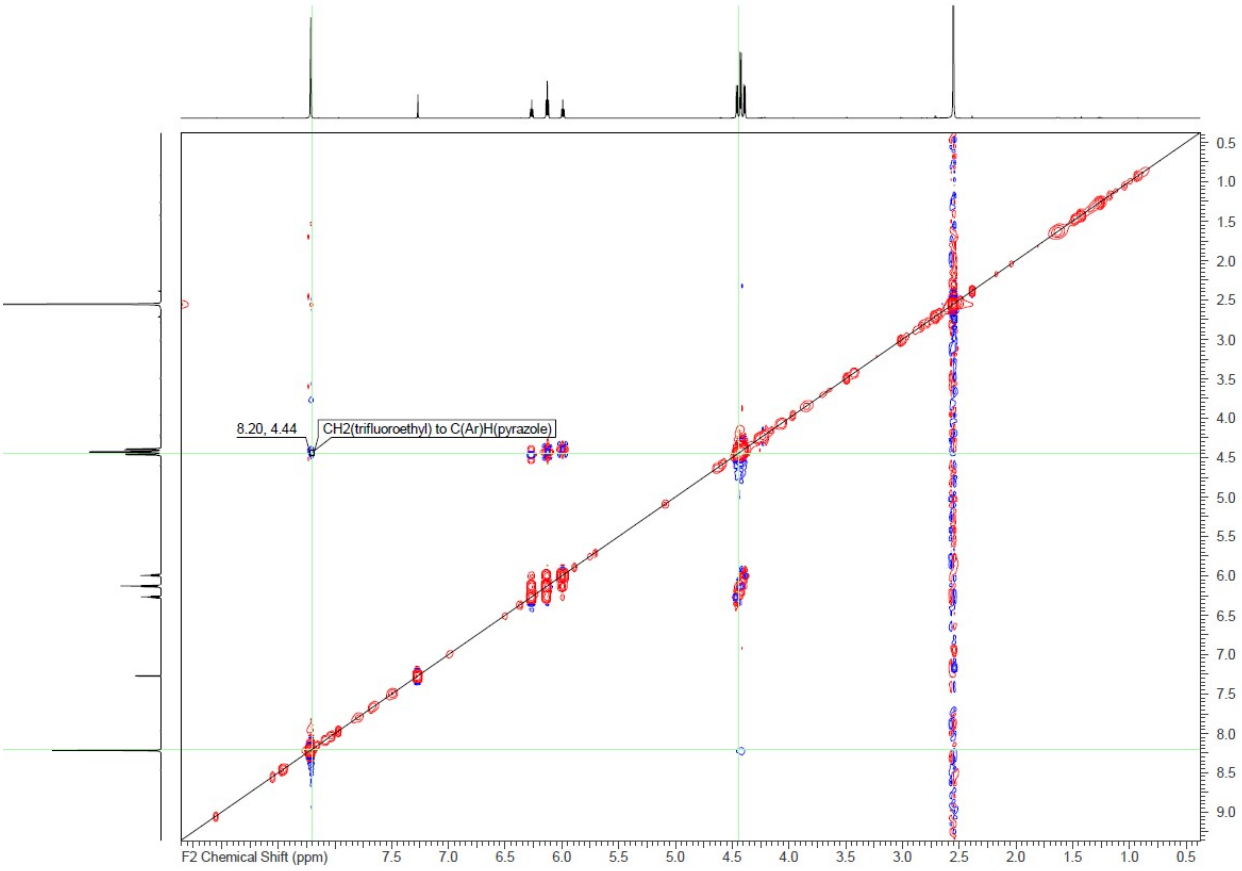
^{1}H NMR Spectrum of 21 (400 MHz, $CDCl_3$)

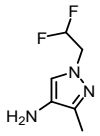


^{19}F NMR Spectrum of 21 (377 MHz, $CDCl_3$)



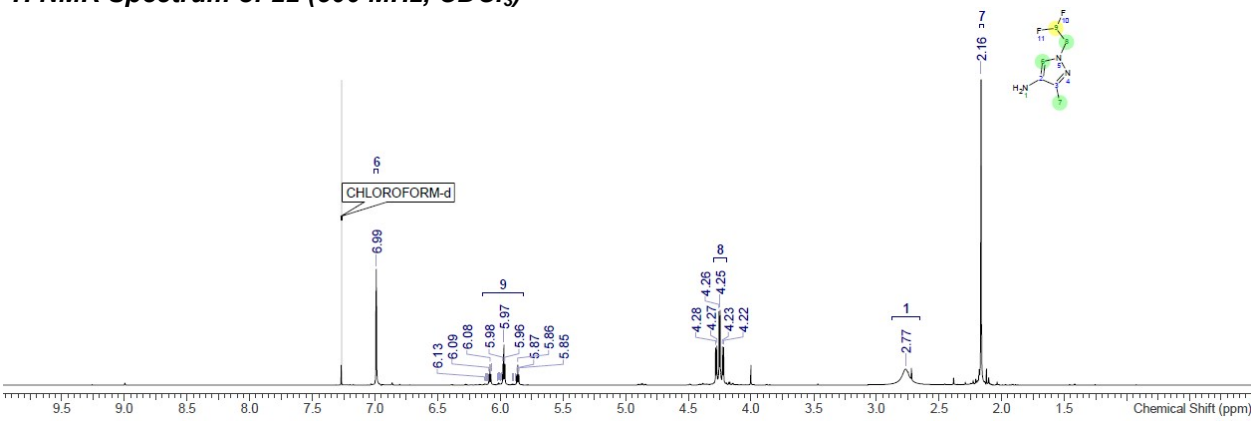
^{1}H-^{1}H ROESY Spectrum of 21 (400 MHz, $CDCl_3$) highlighting key nOe.



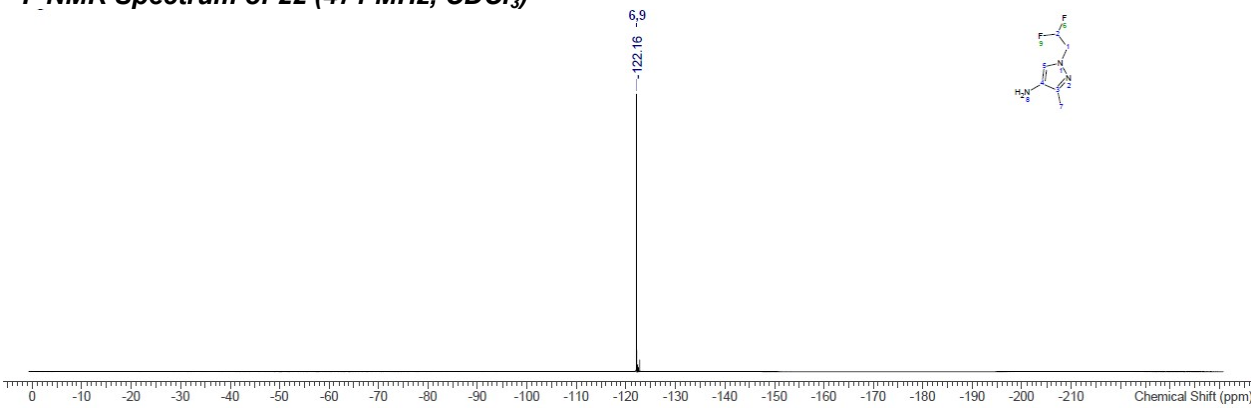


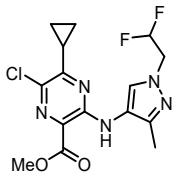
1-(2,2-difluoroethyl)-3-methyl-1*H*-pyrazol-4-amine (22). A 100 mL round bottom flask was charged with 1-(2,2-difluoroethyl)-3-methyl-4-nitro-1*H*-pyrazole (**21**, 4.17 g, 21.8 mmol, 1.0 equiv), palladium on carbon (0.835 g, 7.84 mmol, 20 mass%), and a magnetic stir bar. The flask was sealed and evacuated/backfilled with nitrogen three times. The vial was then charged with MeOH (43.6 mL, 0.5 M w.r.t. **21**) slowly under an atmosphere of nitrogen to avoid exothermic wetting. The resulting black suspension was allowed to stir vigorously at rt as the vial was evacuated/backfilled with hydrogen five times. The reaction mixture was allowed to stir at rt overnight, after which the flask was opened to atmosphere and the Pd was removed by vacuum filtration through a pad of Celite rinsing with copious DCM. The filtrate was concentrated *in vacuo* to provide 1-(2,2-difluoroethyl)-3-methyl-1H-pyrazol-4-amine (**22**, 3.40 g, 97%) as a dark orange oily solid. The product was carried forward to the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.16 (3H, s), 2.74 (2H, br s), 4.25 (2H, td), 5.97 (1H, tt), 6.99 (1H, s). ^{19}F NMR (471 MHz, $CDCl_3$): δ –122.16. *m/z*: (ES+), $[M+H]^+$ = 162.1.

^{1}H NMR Spectrum of 22 (500 MHz, $CDCl_3$)



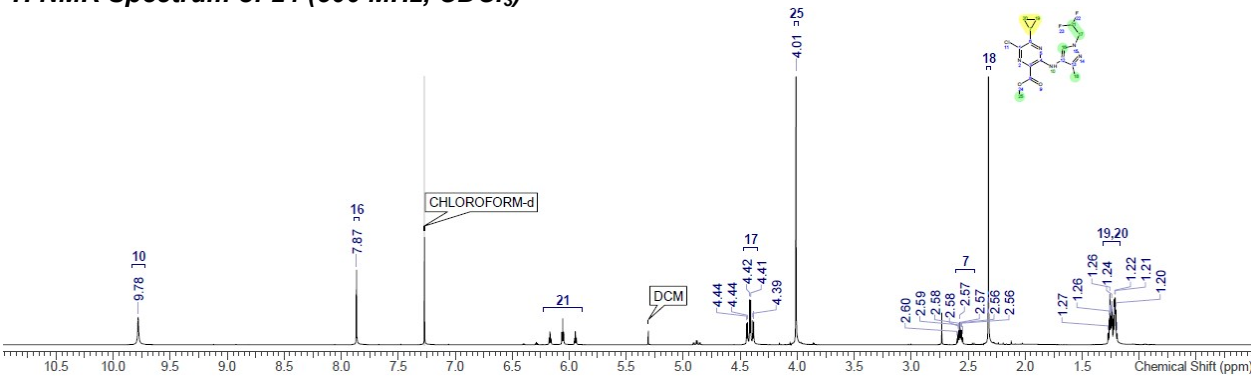
^{19}F NMR Spectrum of 22 (471 MHz, $CDCl_3$)



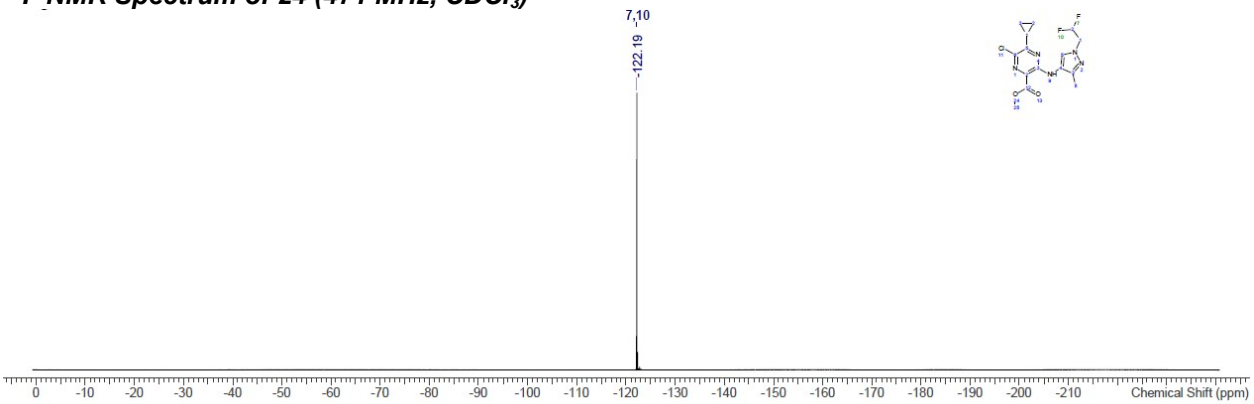


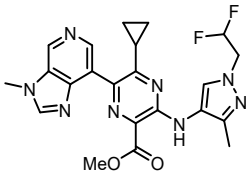
Methyl 6-chloro-5-cyclopropyl-3-((1-(2,2-difluoroethyl)-3-methyl-1H-pyrazol-4-yl)amino)pyrazine-2-carboxylate (24). A 250 mL flask containing 1-(2,2-difluoroethyl)-3-methyl-1*H*-pyrazol-4-amine (**22**, 2.62 g, 16.2 mmol, 1.5 equiv) was charged with methyl 6-chloro-5-cyclopropyl-3-fluoropyrazine-2-carboxylate (**23**, 2.50 g, 10.8 mmol, 1.0 equiv) and a magnetic stir bar. The mixture was dissolved in DMF (48.5 mL, 0.2 M w.r.t. **23**), and DIPEA (5.66 ml, 32.5 mmol, 3.0 equiv) was added *via* syringe. The flask was sealed with a septum, and the reaction mixture was allowed to stir at 100 °C for 1 h. The reaction mixture was cooled to rt, and the solvent was removed *in vacuo*. The crude material was purified directly by flash silica gel chromatography, eluton gradient 0–5% MeOH/DCM with 0 to 0.1% NH_3 modifier over 12 CVs. Product-containing fractions were pooled and concentrated *in vacuo* to provide methyl 6-chloro-5-cyclopropyl-3-((1-(2,2-difluoroethyl)-3-methyl-1*H*-pyrazol-4-yl)amino)pyrazine-2-carboxylate (**24**, 3.67 g, 91%) as a yellow orange solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.17 - 1.32 (m, 4H), 2.32 (s, 3H), 2.45 - 2.61 (m, 1H), 4.01 (s, 3H), 4.41 (td, *J* = 13.6, 4.3 Hz, 2H), 5.89 - 6.22 (m, 1H), 7.87 (s, 1H), 9.78 (s, 1H). ^{19}F NMR (471 MHz, $CDCl_3$): δ –122.19 (s, 2F). *m/z*: (ES+), $[M+H]^+$ = 372.3.

^{1}H NMR Spectrum of 24 (500 MHz, $CDCl_3$)



^{19}F NMR Spectrum of 24 (471 MHz, $CDCl_3$)

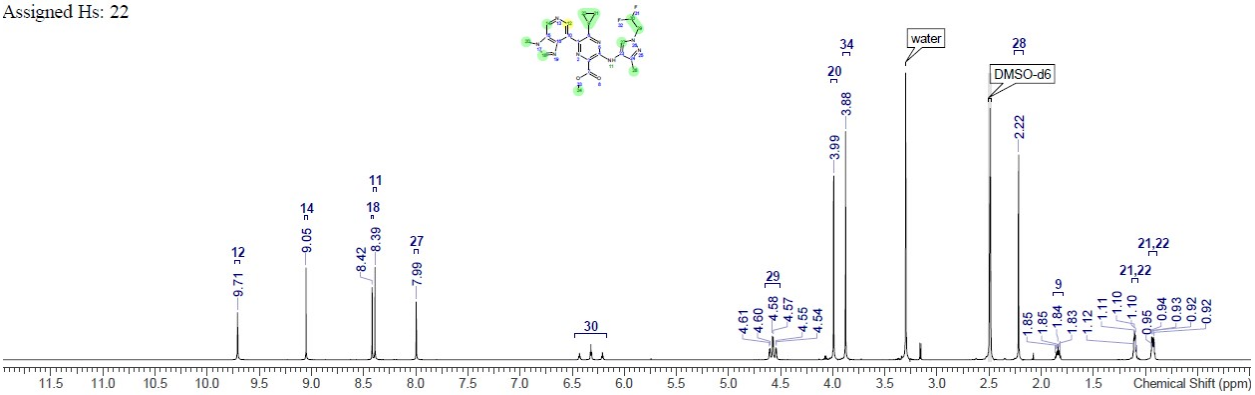




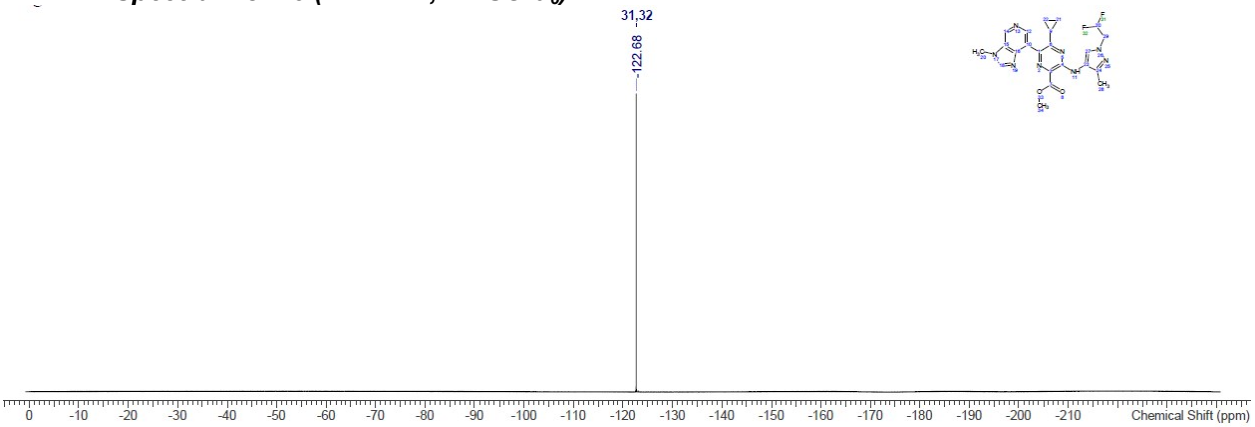
Methyl 5-cyclopropyl-3-((1-(2,2-difluoroethyl)-3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]-pyridin-7-yl)pyrazine-2-carboxylate (25). A 250 mL round bottom flask was charged with methyl 6-chloro-5-cyclopropyl-3-((1-(2,2-difluoroethyl)-3-methyl-1*H*-pyrazol-4-yl)amino)pyrazine-2-carboxylate (**23**, 3.00 g, 8.07 mmol, 1.0 equiv), 8-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)tetrahydro-8*H*-8l4-[1,3,2]oxazaborolo[2,3-b][1,3,2]oxazaborole (**14**, 2.48 g, 10.1 mmol, 1.25 equiv), Pd(dppf)Cl_2•CH_2Cl_2 (0.659 g, 0.81 mmol, 10 mol%), and a magnetic stir bar. Next, 1,4-dioxane (73.4 mL) and water (7.34 mL) were added *via* syringe in a 10:1 ratio (0.1 M w.r.t. **23**). The resulting slurry was allowed to stir at rt as the flask was evacuated/backfilled with nitrogen three times. The reaction mixture was then allowed to stir at 80 °C for 1 h. The reaction mixture was cooled to rt and the solvent was removed *in vacuo*. The crude material was purified by flash silica gel chromatography, elution gradient 0–20% MeOH/DCM with 0–0.4% NH_3 modifier over 12 CVs. Product fractions were pooled and concentrated *in vacuo* to provide a dark yellow solid, which was slurried in ~5:1 MeOH/diethyl ether and allowed to stir at 40 °C for 1 h. The yellow suspension was then allowed to cool to rt, and the solid was collected by vacuum filtration rinsing with diethyl ether. The filtrate was collected and concentrated *in vacuo*, resulting in a dark brownish foam which was suspended in ~5:1 MeOH/diethyl ether and allowed to stir at 40 °C for 30 min. This slurry was allowed to cool to rt, and the solid was collected by vacuum filtration rinsing with diethyl ether. This process was repeated a third time with filtrate, and the solid was again collected rinsing with diethyl ether and allowed to air dry. The combined solids were allowed to dry overnight on high vacuum to provide methyl 5-cyclopropyl-3-((1-(2,2-difluoroethyl)-3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]-

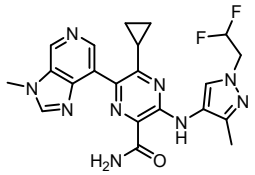
pyridin-7-yl)pyrazine-2-carboxylate (**25**, 2.64 g, 69.9%) as a bright yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.89 - 0.97 (m, 2H), 1.07 - 1.13 (m, 2H), 1.79 - 1.88 (m, 1H), 2.22 (s, 3H), 3.88 (s, 3H), 3.99 (s, 3H), 4.57 (td, *J* = 15.3, 3.6 Hz, 2H), 6.17 - 6.47 (m, 1H), 7.99 (s, 1H), 8.38 - 8.41 (m, 1H), 8.42 (s, 1H), 9.05 (s, 1H), 9.71 (s, 1H). ^{19}F NMR (471 MHz, DMSO-d_6): δ –122.68 (s, 2F). *m/z*: (ES+), $[M+H]^+$ = 469.2.

^{1}H NMR Spectrum of 25 (500 MHz, DMSO-d_6)



^{19}F NMR Spectrum of 25 (471 MHz, DMSO-d_6)

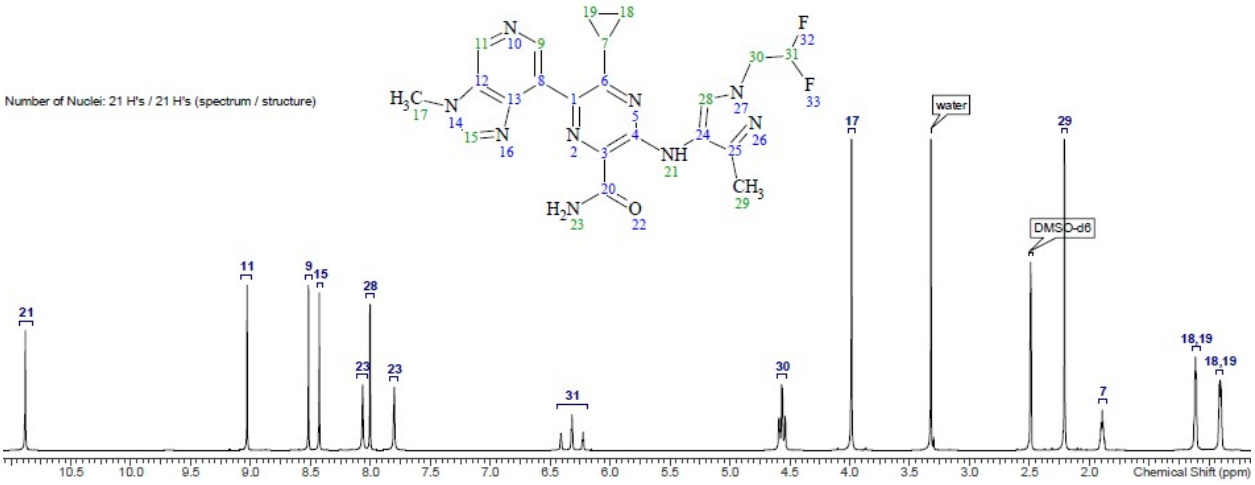




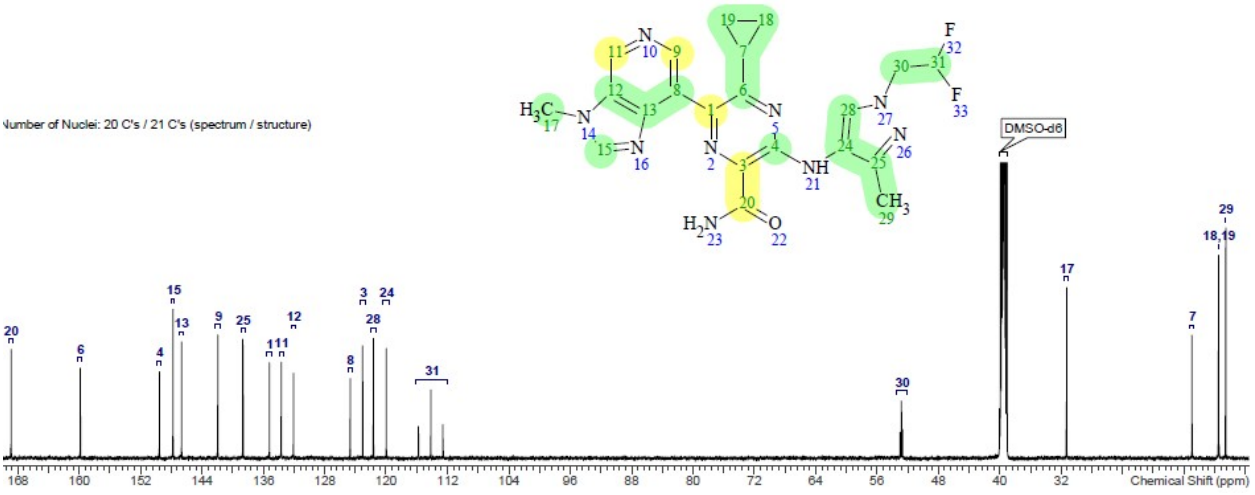
5-cyclopropyl-3-((1-(2,2-difluoroethyl)-3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine-2-carboxamide (16a). 7 N Methanolic ammonia (40 mL, 280 mmol, 50 equiv) was added to methyl 5-cyclopropyl-3-[[1-(2,2-difluoroethyl)-3-methyl-pyrazol-4-yl]amino]-6-(3-Methyl-3*H*-imidazo[4,5-*c*]pyridin-7-yl)pyrazine-2-carboxylate in a pressure flask. The resulting mixture was stirred at 80 °C for 1 hour. The reaction was then allowed to cool to rt, resulting in the formation of a yellow precipitate. The pressure flask was carefully unsealed, and the precipitate was collected by vacuum filtration rinsing with diethyl ether. The isolated material was dried thoroughly on high vacuum to deliver 5-cyclopropyl-3-((1-(2,2-difluoroethyl)-3-methyl-1*H*-pyrazol-4-yl)amino)-6-(3-methyl-3*H*-imidazo[4,5-c]pyridin-7-yl)pyrazine 2-carboxamide (**16a**, 2.38 g, 94% yield) as a yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.89 - 0.94 (m, 2H), 1.08 - 1.15 (m, 2H), 1.86 - 1.92 (m, 1H), 2.21 (s, 3H), 3.99 (s, 3H), 4.56 (td, *J* = 15.0, 3.2 Hz, 2H), 6.32 (br tt, *J* = 55.0, 3.5 Hz, 1H), 7.80 (br s, 1H), 8.00 (s, 1H), 8.07 (br s, 1H), 8.43 (s, 1H), 8.52 (s, 1H), 9.03 (s, 1H), 10.88 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 10.58, 11.50, 14.96, 31.31, 52.84 (br t, *J* = 25.3 Hz, 1C), 114.18 (t, *J* = 241.0 Hz, 1C), 119.96, 121.65, 123.05, 124.67, 132.07, 133.66, 135.21, 138.65, 141.94,

146.66, 147.77, 149.55, 159.86, 168.87.^{19}F NMR (471 MHz, DMSO-d_6): δ –122.59 (s, 2F). *m/z*: (ES+), $[M+H]^+$ = 454.2.

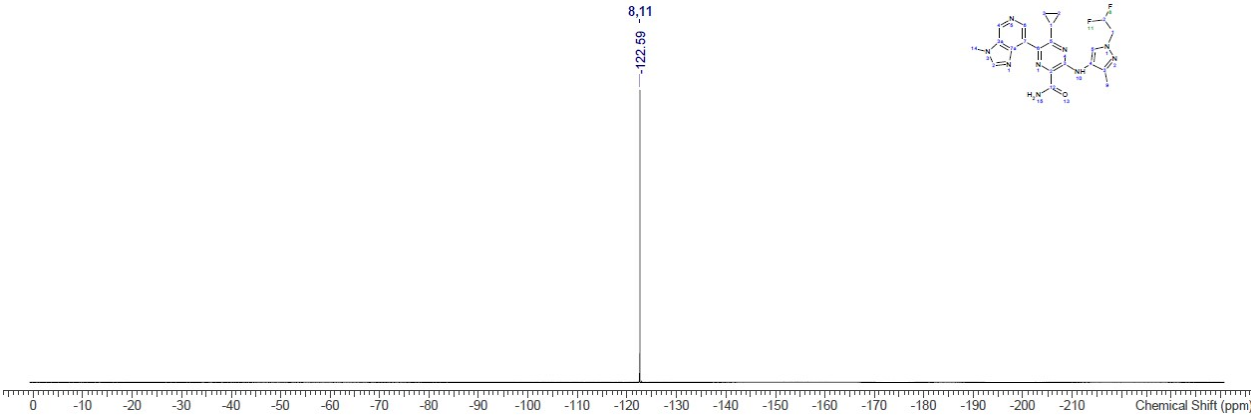
^{1}H NMR Spectrum of 16a (600 MHz, DMSO-d_6)



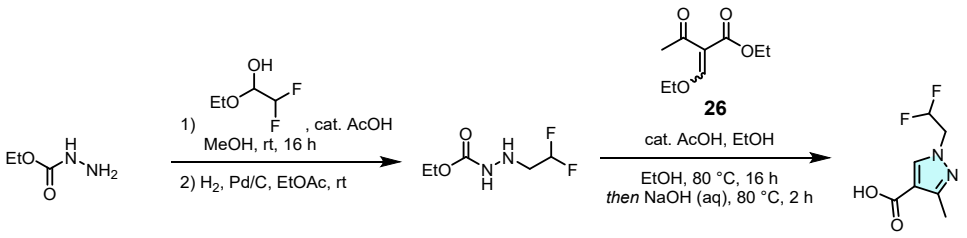
^{13}C NMR Spectrum of 16a (600 MHz, DMSO-d_6)



^{19}F NMR Spectrum of 16a (471 MHz, DMSO-d_6)



Bespoke Difluoroethyl Pyrazole Synthesis (Scheme 4)



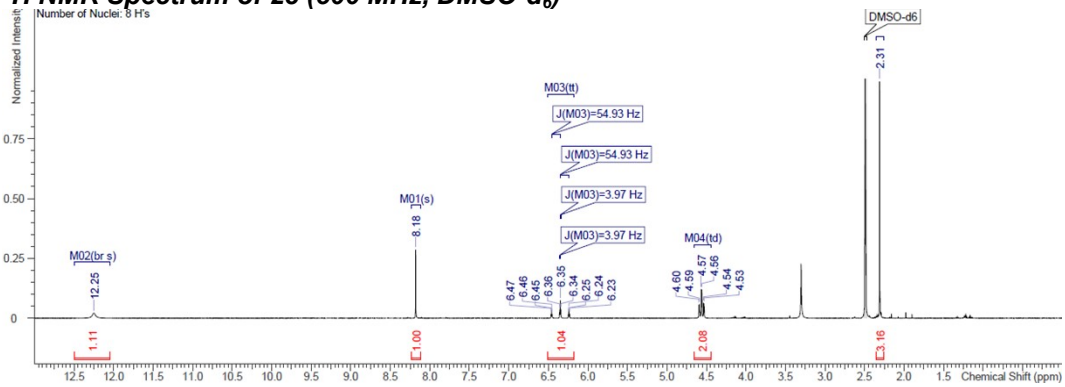
S6 **27** **28**

Ethyl (*E*)-2-(2,2-difluoroethylidene)hydrazine-1-carboxylate. Difluoroacetaldehyde ethyl hemiacetal (0.66 mL, 6.39 mmol, 1.0 equiv) was added to a solution of ethyl carbazate (**S6**, 662 mg, 6.36 mmol, 1.0 equiv) and acetic acid (0.005 mL, 0.09 mmol) in MeOH (8 mL, 0.73 M w.r.t. **S6**). The resulting mixture was stirred at rt for 16 h. The reaction was then concentrated *in vacuo* to afford crude ethyl (*E*)-2-(2,2-difluoroethylidene)hydrazine-1-carboxylate (1.056 g, >99% assumed) as a white solid, which was carried forward without purification. Crude ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.10 - 1.21 (m, 4H), 3.94 - 4.05 (m, 3H), 4.23 - 4.37 (m, 1H), 5.04 (dd, *J* = 5.8, 3.2 Hz, 1H), 5.58 - 5.86 (m, 1H), 6.15 (d, *J* = 6.0 Hz, 1H), 8.39 (br s, 1H).

Ethyl 2-(2,2-difluoroethyl)hydrazine-1-carboxylate (27). A mixture of crude ethyl (*E*)-2-(2,2-difluoroethylidene)hydrazine-1-carboxylate (750 mg, 4.51 mmol, 1.0 equiv) and palladium on carbon (508 mg, 0.48 mmol, 10 mass%) in EtOAc (10 mL, 0.45 M) was stirred at 25 °C for 16 h under a hydrogen atmosphere. The reaction was then filtered through silica, rinsing with EtOAc. The filtrate was concentrated *in vacuo* to afford ethyl 2-(2,2-difluoroethyl)hydrazine-1-carboxylate (**27**, 707 mg, 93%) as a white solid, which was used directly in the next step without purification. Crude ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.15 (t, *J* = 7.1 Hz, 3H), 3.04 (tt, *J* = 15.5, 4.4 Hz, 2H), 4.00 (q, *J* = 7.1 Hz, 2H), 4.98 (q, *J* = 4.2 Hz, 1H), 5.95 (tt, *J* = 56.0, 4.7 Hz, 1H), 8.57 (br s, 1H).

1-(2,2-Difluoroethyl)-3-methyl-1*H*-pyrazole-4-carboxylic acid (28). Ethyl 2-(ethoxymethylene)-3-oxobutanoate (**26**, 770 µL, 4.42 mmol, 1.05 equiv) was added to a solution of crude ethyl 2-(2,2-difluoroethyl)hydrazine-1-carboxylate (**27**, 707 mg, 4.20 mmol, 1.0 equiv) and acetic acid (30 µL, 0.52 mmol) in EtOH (3.4 mL, 0.55 M w.r.t. **27**). The resulting mixture was stirred at 80 °C for 16 h. A solution of sodium hydroxide (444 mg, 11.10 mmol 2.64 equiv) in water (6 mL) was added. The resulting mixture was stirred at 80 °C for 2 h. The reaction mixture was then concentrated *in vacuo*, diluted with water (20 mL), and washed three times with EtOAc (10 mL each). The aqueous layer was then acidified with 2 M aqueous HCl and extracted three times with EtOAc (10 mL each). This second set of combined organics was dried over $MgSO_4$, filtered, and concentrated to afford 1-(2,2-difluoroethyl)-3-methyl-1*H*-pyrazole-4-carboxylic acid (**28**, 496 mg, 62.0%) as a pale yellow solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.31 (s, 3H), 4.56 (td, *J* = 15.0, 3.8 Hz, 2H), 6.35 (tt, *J* = 54.9, 4.0 Hz, 1H), 8.18 (s, 1H), 12.25 (br s, 1H). *m/z*: (ES+), $[M+H]^+$ = 191.0.

^{1}H NMR Spectrum of 28 (500 MHz, DMSO-d_6)



Protein Expression and Purification

The kinase domain of HPK1 bearing phospho-mimetic mutations in the activation loop (6His-ZZ-TEV-Hpk1 (D2-N293) [S171E, T165E]) was recombinantly expressed in *S. frugiperda* SF21 cells. The protein proved poorly tolerated and cells were harvested 24 h post infection with baculovirus. Cell pellets were resuspended in 50 mM Tris pH 7.5, 500 mM NaCl, 10% glycerol, 1 mM TCEP, 20 mM imidazole and lysed by homogenisation. The clarified lysate was subjected to nickel affinity chromatography with Ni^{2+}-NTA resin (Qiagen), and the eluate was de-tagged with TEV protease, before 10-fold dilution and cation exchange chromatography on SP Sepharose (Cytiva). Fractions containing HPK1 were pooled and subjected to size exclusion chromatography on a Superdex 75 column in 20 mM Hepes pH 7.2, 300 mM NaCl, 1 mM TECP. Pure protein was concentrated to 5.5 mg/ml and stored frozen in aliquots.

Protein Crystallography

HPK1 (D2-N293) [S171E, T165E]) was co-crystallized with inhibitors by incubating the enzyme with 1 mM inhibitor (from 100 mM stock in DMSO) for 15 min prior to dispensing sitting-drop vapor diffusion crystallization experiments with precipitant (30 % PEG400, 0.2 M tri-sodium citrate, 0.01 M propionate-cacodylate-bis-tris (PCTP) buffer pH 8.5) in 1: 1 ratio. Crystals belonging to space group C2 formed after 2-3 days and were cryo-protected with 20 % ethylene glycol added to reservoir solution. Diffraction data were collected at Soleil Synchrotron beamline Proxima I, and processed with XDS, autoproc and STARANISO. Structures were solved by molecular replacement with Phaser and refined with Buster. Coot was used for model building, and initial ligand restraints were generated with Grade. Coordinates and structure factors, as well as data collection and refinement statistics are available from the PDB with accession codes (9QT6).

Assay Descriptions

HPK1, GLK, and LCK IC_{50} assays: Activity of purified N-terminal GST-tagged, recombinant, human HPK1, GLK, and LCK enzymes expressed in insect cells (HPK1: amino acids 1-346, ThermoFisher Scientific, #PV6356, Carlsbad, CA; GLK: amino acids 1-380, ThermoFisher Scientific, #PV6351, Carlsbad, CA; LCK: full length, Abeam, #ab79626, Cambridge, MA) was determined *in-vitro* using ADP-Glo Max Assay (Promega, #V7002, Madison, WI), a luminescent ADP detection assay, by quantifying the amount of ADP produced in a kinase reaction.

The luminescent signal generated is proportional to the ADP concentration produced in a kinase assay in the presence and absence of the compound(s) and is correlated with the kinase activity. Two microliters (µL) of enzyme mix consisting of 10 nM HPK1, 30 nM GLK, or 2 nM LCK in lx reaction buffer (50 mM HEPES (pH 7.2), 1 mM *DL*-Dithiothreitol (DTT), 0.005% (vol/vol) Brij35, 20 mM $MgC1_2$) was spotted into Greiner 384-well low volume plate with 0.1 µL of compound, which was dosed at 100, 31.25, 12.5, 3.19, 1, 0.32, 0.1, 0.032, 0.01 and 0.003 µM of final test concentrations and preincubated for 30 minutes at rt. Enzymatic reactions were initiated with 2 µL of peptide substrate/ATP mix (for HPK1: 10 µM LRRKtide (RLGRDKYKTLRQIRQ-amide; Cambridge Research Biochemicals, Billingham, UK), 30 µM ATP; for GLK: 14 µM LRRKtide, 60 µM ATP; for LCK: 100 µM LCKtide (EQEDEDEPEGIYGVLE-amide; Intonation, Boston, MA), 50 µM ATP) in lx reaction buffer and incubated at rt for 60 minutes. 4 µL ADP-Glo Reagent was added to terminate the reactions and deplete the remaining ATP and incubated at rt for 60 minutes. Finally, 8 µL ADP-Glo Max Detection Reagent was added to simultaneously convert ADP to ATP and incubated at rt for 60 minutes. The newly synthesized ATP is converted to a light signal using a luciferase/luciferin reaction. Luminescence was read by PHERAstar FSX plate reader (BMG LABTECH, Cary, NC) and the data was captured by PHERAstar FSX MARS data analysis software. IC_{50} values were processed using GeneData Screener (GeneData AG, Basel, Switzerland).

T cell assay: Two different T cell assays were used over the course of our HPK1 program. They are described below.

Assay #1

Materials

RPMI 1640 (Sigma R5886)
Heat inactivated FBS (Gibco 10270-10
Glutamax 100X (Thermo Fisher 35050061)
HEPES IM (Thermo Fisher 15630080)
Dulbecco's PBS (SigmaD8537)
MultiCyt® QBeads® Human PlexScreen (2) Plex for 1 x 384 plate (Sartorius 90602) Ultra-LEAF™ Purified anti-human CD28 Antibody (Biolegend 302933)
CD3 Monoclonal Antibody (OKT3), Functional Grade, eBioscience™ (Thermo Fisher 16- 0037-85)
β-mercaptoethanol (Sigma M3148)
Propidium Iodide (Abeam ab 14083)
Pen/Strep (Sigma P0781)
Non-essential amino acids (Sigma M7145)
Sodium pyruvate (Sigma S8636)

T cell media preparation

RPMI 1640 + Heat inactivated FBS 10% + Glutamax (100X) 1% + Pen/Strep 1% +
Non-essential amino acids 1% + IM HEPES to make original media 100 mM final concentration
Sodium pyruvate 1% + 1.75ul of 14.3 M original solution β-mercaptoethanol

Method

Cryo-preserved human CD3+ T cells are recovered in warm T cell media overnight. Recovered T cells are seeded at 70000 cells per well in a 384-well Black/Clear Round Bottom Ultra-Low Attachment Spheroid Microplate (Corning 3830). Compounds in an assay-ready 384-well plate (Greiner 781280) are added to the seeded T cells using a Bravo liquid handler. T cells are then left in a humidified incubator at 37°C for 1

hour. At the end of incubation, the cells are transferred to a 384-well flat bottom plate (Greiner 781090) coated with anti-CD3 antibody (5 µg/mL anti-CD3 in PBS, overnight incubation at 4 °C). Anti-CD28 antibody in T cell media is also added to the cells by Bravo liquid handler at 5 µg/mL or l µg/mL in a donor dependent manner. T cells are then incubated in a humidified incubator at 37°C for 4 hours. Cell culture supernatant is collected using a Bravo liquid handler in a v-bottom 384 well plate (Greiner 781280) after the 4-hour incubation. IL2 is detected using a IL2 MultiCyt® QBeads® kit on an iQue Screener flow cytometer (Sartorius). Briefly, capture beads are diluted by 50x in the supplied capture bead diluent. 10 µL per well diluted capture beads is added to each well of a v-bottom 384 well plate (Greiner 781280) using a ThermoFisher multichannel pipette. Using a Bravo liquid handler, 10 µL cell culture supernatant is transferred to the v-bottom plate with diluted capture beads. The plate is then sealed in foil and incubate at rt on a plate shaker set to 900 rpm for 1 hour. At the end of incubation, 10 pL per well of the supplied 5 detection reagent is added to the wells using a ThermoFisher multichannel pipette. The plate is then sealed in foil and incubated again at rt on a plate shaker set to 900 rpm for 2 hours before detection by iQue Screener. iQue Screener flow cytometer uses pre-configured analysis template supplied with the IL2 MultiCyt® QBeads® kit to detect IL2 in each sample well.

Assay #2

Materials

Human CD3+ T cells

CD3 antibody: anti-human CD3 antibody (clone OKC-3)—eBioscience #16-0037-85

CD28 antibody: Anti-Human CD28: Clone CD28.2 (RUO)---BD Bioscience #555725

U-bottom 96 well TC culture plate: Corning #3799

Human IL2 ELISA kit: Invitrogen #88-7025-88

T cell media

To 500 mL media RPMI (Invitrogen #22400089), add the following:

50 mL FBS

5 mL Pen/Strep

3.73 mL 1M HEPES to make 100mM final concentration

5 mL non-essential amino acids (100X)

5 mL sodium pyruvate

5 mL glutamax

1.75 µl of 14.5 M 2-Mercaptoethanol solution (Sigma #M6250)

Method

Thaw desired number of cryo-preserved human CD3+ T cells in T cell medium without stimulation, ~2 x 10^6 cells/mL. Coat plate with CD3 antibody: dilute CD3 antibody using cold PBS into 1-10 µg/mL depending on the donors; add 100 µl of diluted CD3 antibody into each well of 96 well U bottom plate, gently shake at 4 °C overnight. Coat IL2 antibody for ELISA—follow vendor's instruction. Spin down T cells at 1,400 rpm for 5 min, remove the supernatant, and resuspend the cells with fresh T cell medium. Dilute the cells to 1.0 x 10^6 cells/mL. Add 200 µL into each well of assay-ready plate. Incubate at 37 °C for 1 h. Immediately before cell stimulation, wash the CD3-coated plate using 200 µL cold PBS 3 times; invert and dry. Transfer 190 µL of compound pre-treated cells into CD3-coated plate. Prepare a 20 µg/mL CD28 antibody solution (20x) using T cell medium, and add 10 µL/well into cells in the CD3 coated plate. Incubate the cell plate at 37 °C for an additional 4 h. Wash and block the IL2 ELISA plate. Remove 100 µL of cell solution and add to a v-bottom 96-well plate. Spin down the cell plate in the v-bottomed plate at 1,500 rpm for 5 min. Remove 90 µL of supernatant and add it into the IL2 ELISA assay plate. Incubate at 4 °C overnight with gentle shaking. Perform the ELISA assay per the vendor protocol.