SUPPLEMENTAL INFORMATION:

Development of a chemical toolset for studying the paralog-specific function of IRE1

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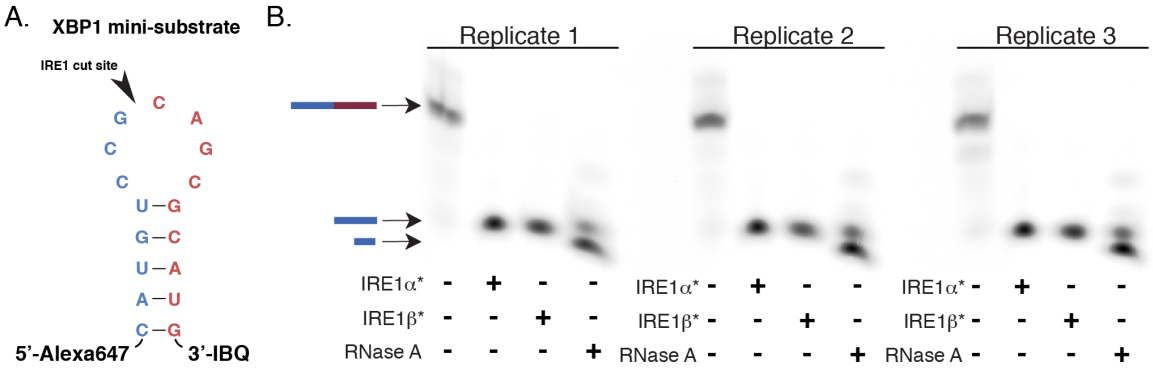
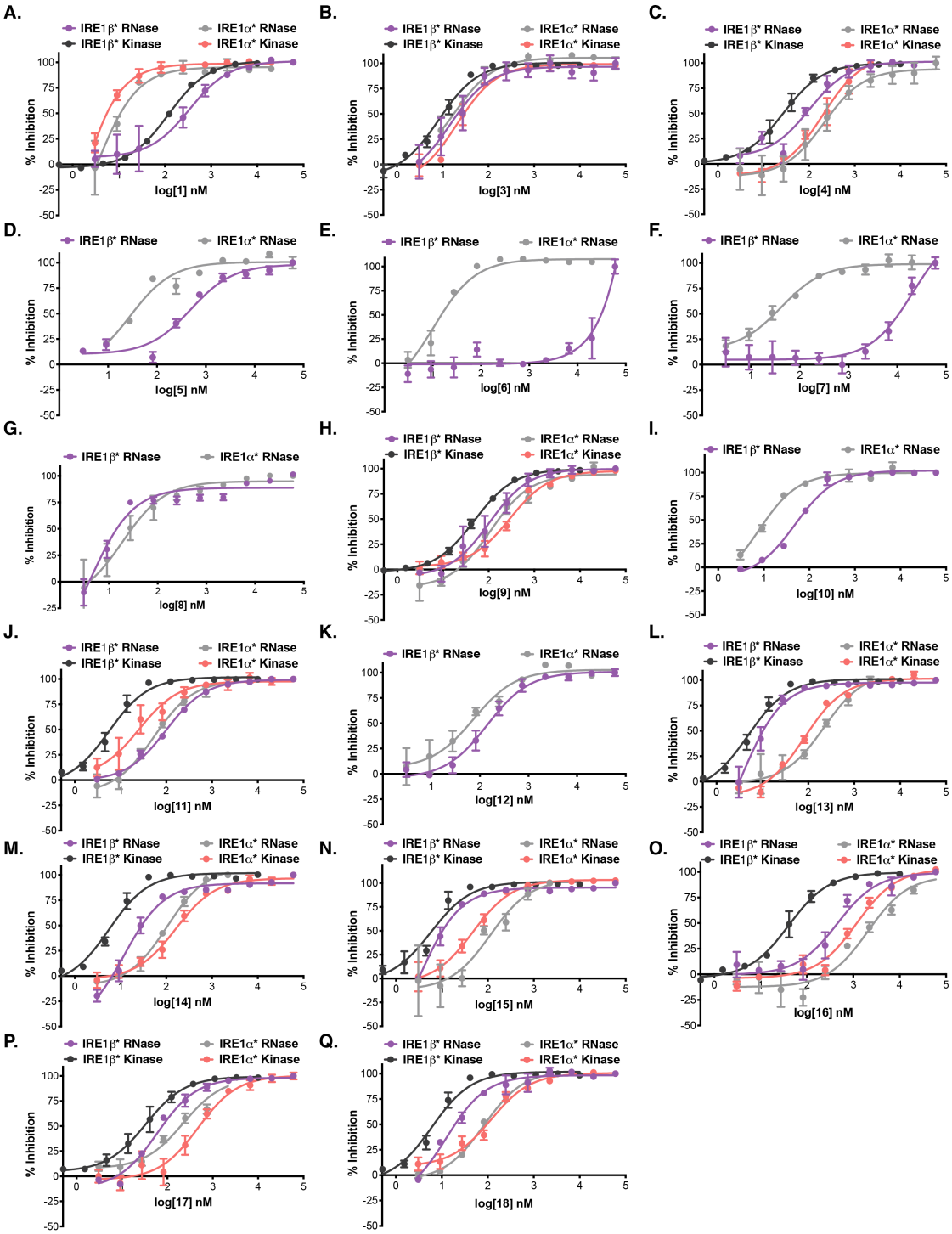
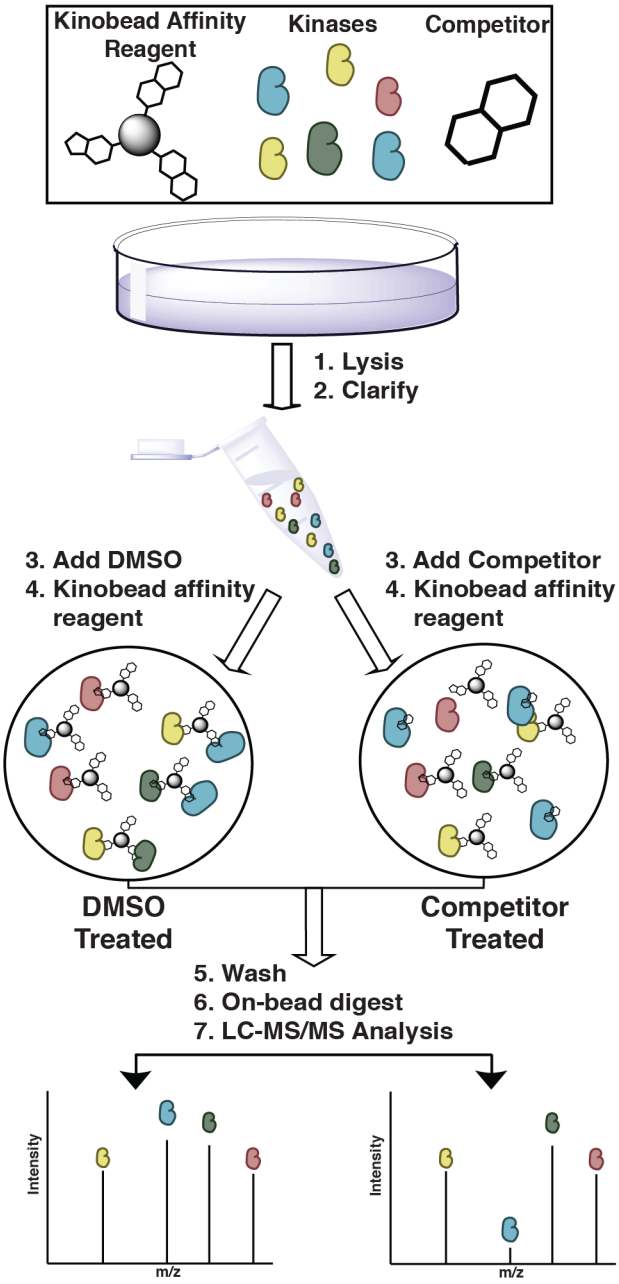
SUPPLEMENTAL FIGURES:



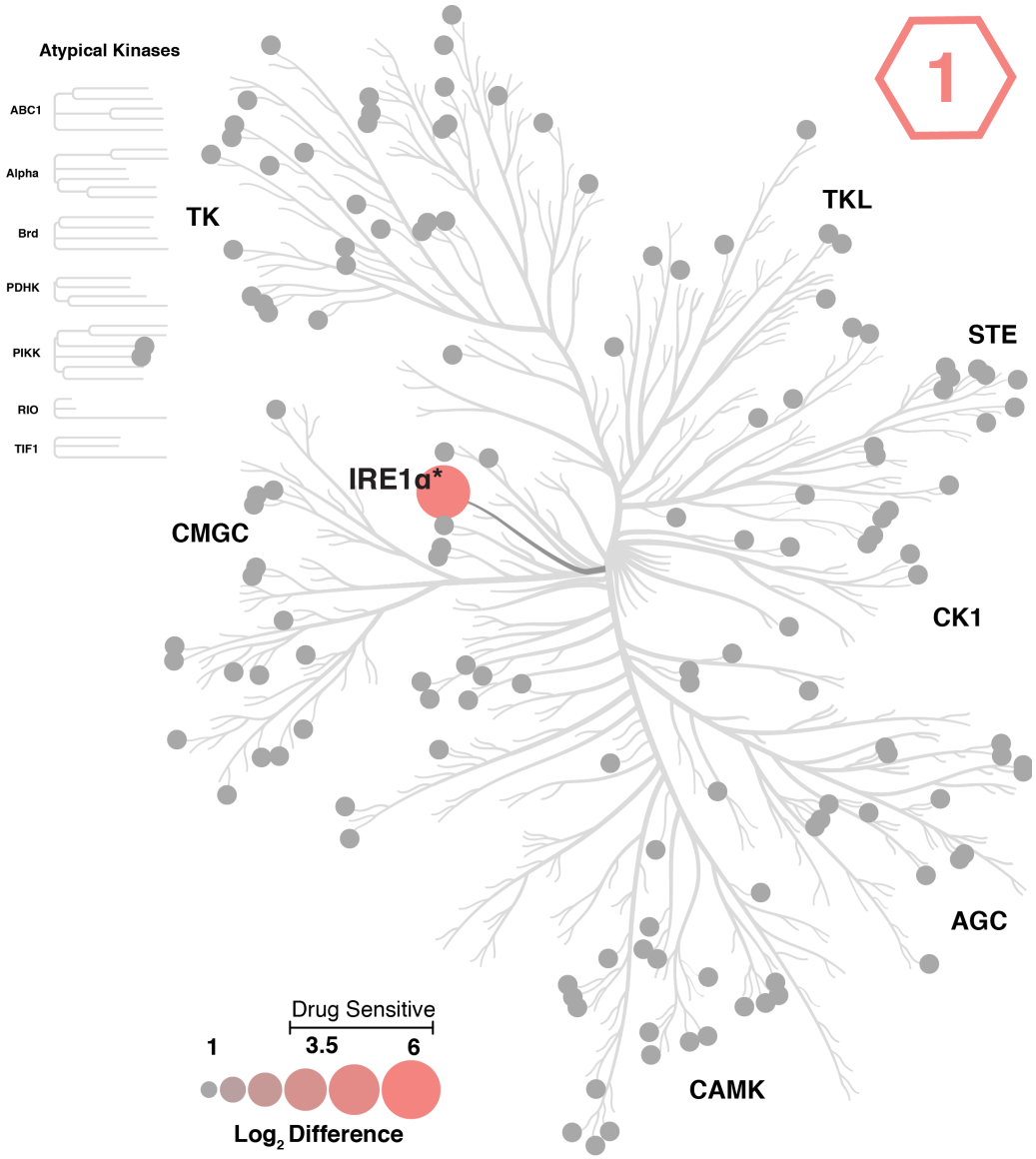
Supplemental Figure 1. IRE1-Mediated Cleavage of the XBP1 mini-substrate
A. Sequence and predicted secondary structure of the XBP1 mini-substrate. The predicted IRE1-mediated cleavage site within the stem loop is denoted with an arrow. B. Urea-PAGE gel of XBP1 mini-substrate cleavage products after treatment with either buffer (Lanes 1), 75 nM IRE1α* (Lanes 2), 75 nM IRE1β* (Lanes 3), or 100 µg/mL RNaseA (Lanes 4). Data shown as three biological replicates run on the same gel.



Supplemental Figure 2. IRE1α*/β* Kinase and RNase IC_{50} Curves for 1-18
Inhibitor titration curves for inhibitors **1-18**. IRE1β* kinase IC_{50} curves are shown in dark gray, IRE1β* RNase IC_{50} curves are shown in lilac, IRE1α* kinase IC_{50} curves are shown in coral, and IRE1α* RNase IC_{50} curves are shown in light gray. Data shown are mean ± SEM, n=3, except for the IRE1β* kinase IC_{50} curves for **4, 14, 16,** and **17** which are shown as a mean of n=2.



Supplemental Figure 3. Kinobead Profiling Experiment Workflow
Cartoon schematic of kinobead profiling assay. In brief, cells are lysed and incubated with either DMSO or an ATP-competitive inhibitor. Kinobead resin is then added to the lysate to enrich kinases with unoccupied ATP-binding sites. Kinobeads are washed and bound kinases are trypsinized follow by LC-MS/MS analysis.



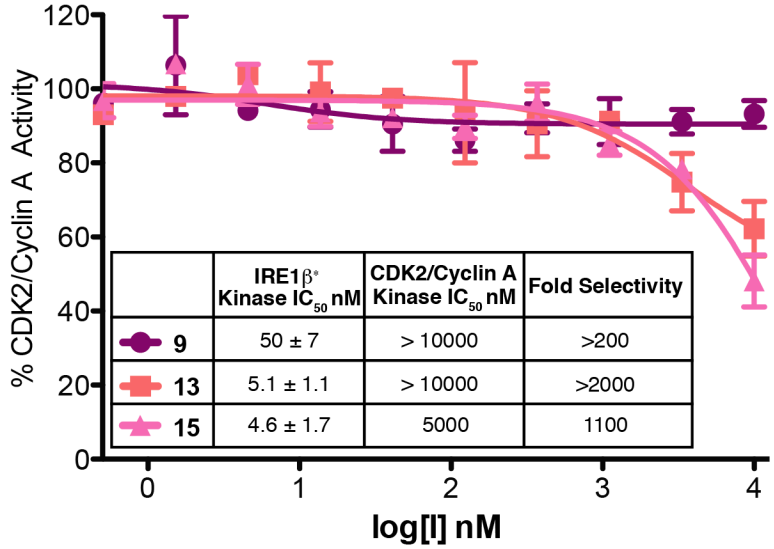
Supplemental Figure 4. Kinobead Profiling and Selectivity of 1

Specificity of **1** as determined by kinobead profiling using lysates spiked with recombinant IRE1α*. Kinases that were ID'd in the experiment are shown as circular nodes, where node size and color has been scaled to the $\log_2$ Difference (difference in LFQ intensity between DMSO treated and inhibitor treated lysates) between DMSO and treatment with 10 µM of **1** (mean of four replicates). Kinases reported as being drug-sensitive (Log_2 Difference > 2) were also required to show significance from a two-sample T-test with FDR of 0.05.

A.

	IRE1β		CDK2	
	Log_2 Ratio	-log(p-value)	Log_2 Ratio	-log(p-value)
9 10 μM	2.9	5.7	2.8	5.3
13 10 μM	5.0	6.2	3.6	5.5
15 10 μM	5.1	5.5	2.9	4.8

B.



Supplemental Figure 5. KIRAs 9, 13, and 15 minimally inhibit the catalytic activity of CDK2/cyclin A

A. Log_2 ratios (difference in LFQ intensities between DMSO treated and inhibitor treated lysates) and –log(p-values) for IRE1β* and CDK2 in the kinobead profiling experiments with inhibitors **9, 13,** or **15**. B. CDK2/cyclin A kinase activity inhibition curves for **9, 13,** and **15**. Kinase IC_{50} values for IRE1β* and CDK2/cyclin A are reported alongside the fold selectivity for IRE1β*. For IRE1β*, IC_{50} values are mean ± SEM, n=3. For CDK2/cyclin A, data shown is the mean of two replicates.

SUPPLEMENTAL TABLES:

Supplemental Table 1. Inhibitor Titration Values for 4, 14, 16, 17[a]

	Percent IRE1β* Kinase Activity							
Inhibitor Conc.(M)	4		14		16		17	
1.00E-05	1.1	0.95	-2.6	2.02	1.4	2.7	1.1	2.2
1.11E-06	0.32	2.7	3.9	2.9	0.81	2.3	1.4	1.7
3.33E-06	1.5	4.7	-0.41	2.8	4.9	4.4	0.2	5.4
3.70E-07	5.6	8.5	1.6	1.4	8.9	12	5.5	10
1.23E-07	11	19	-2.3	2.9	19	29	16	26
4.12E-08	30	48	1.1	3.2	38	64	31	56
1.37E-08	57	80	18	34	79	84	58	77
4.57E-09	84	90	62	70	91	93	78	90
1.52E-09	95	92	92	90	96	96	94	92
5.08E-10	98	96	97	93		106	93	93
IC_{50} Value	4.5	7.0	30	59	18	40	20	54

[a]Percent IRE1β* kinase activity for each inhibitor concentration was determined relative to IRE1β* treated with DMSO. IRE1β* kinase IC_{50} values were then calculated by plotting percent kinase activity versus inhibitor concentration and dose-response curves were fit using “one-site fit logIC50” parameter using GraphPad Prism analysis software. Data reported in Figure 4B is the mean of two replicates.

METHODS:

SYNTHESIS

General Information

Synthetic reagents are commercially available from the vendors (listed below in Reagents Table) were used without further purification. Proton and carbon NMR characterization was obtained using a Bruker AV-300 or 500 MHz instrument at room temperature. Chemical shifts are reported in ppm and coupling constants (J) are reported in Hz. Mass spectrometry on small molecules was preformed using a Bruker Esquire Ion Trap.

Reagent List

Reagent	CAS#	Vendor	Product Number
2-chloro-4-(2-fluoro-3-pyridinyl) pyrimidine	954216-54-7	AstaTech	77359
N-Boc-trans-1,4-cyclohexanediamine	177906-48-8	Sigma	CDS007815
6-Amino-2-aza-spiro[3.3] heptane-2-carboxylic acid tert-butyl ester	1211586-09-2	Sigma	ADV371426272
(S)-tert-Butyl 3-((4-(2-fluoropyridin-3-yl)pyrimidin-2-yl)amino)piperidine-1-carboxylate	1630086-25-7	Sigma	COM497521364
4-Amino-1-naphthol	2834-90-4	Sigma	S57836
5-Amino-1-naphthol	83-55-6	Sigma	376469
4-amino-3-fluorophenol	399-95-1	Sigma	MAT047026697
Cyclobutanesulfonyl chloride	338453-16-0	Sigma	ENA457508270
4-amino-5-chloro-2-fluorophenol	847872-10-0	Enamine Building Blocks	EN300-366591
4-amino-3-chlorophenol	17609-80-2	Combi-Blocks	OR-4616
4-amino-2,3-difluorophenol	163733-99-1	Alfa Aesar	H31555
4-amino-3,5-difluorophenol	135086-76-9	Alfa Aesar	H32493
4-amino-2-fluorophenol	399-96-2	Sigma	MAT047026730
2-chlorobenzylsulfonyl chloride	77421-13-7	Sigma	678643
2-chlorobenzenesulfonyl chloride	2905-23-9	Sigma	546925

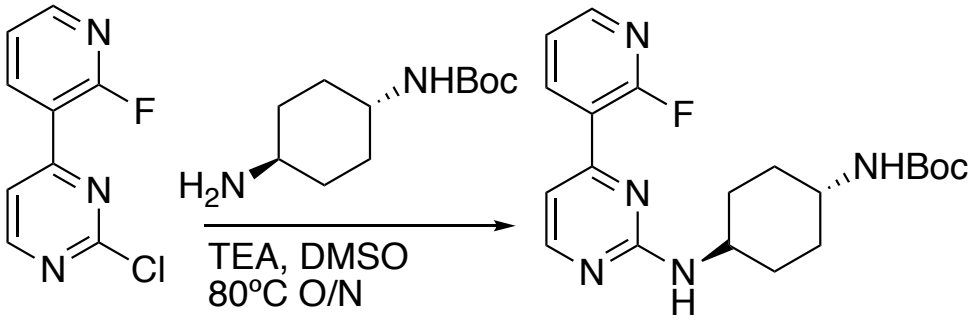
HPLC Preparatory Purification Conditions

Samples were suspended in methanol and injected onto a preparatory reverse-phase C18 column (250 x 21 mm) and separated using a Varian ProStar 210/325 HPLC instrument using a methanol + 0.1% TFA: water + 0.1% TFA solvent system from 1% methanol + 0.1% TFA to 100% methanol + 0.1% TFA over 60 minutes at a flow rate of 8 mL/min. Absorbance was detected via UV absorption at both 220 nm and 254 nm.

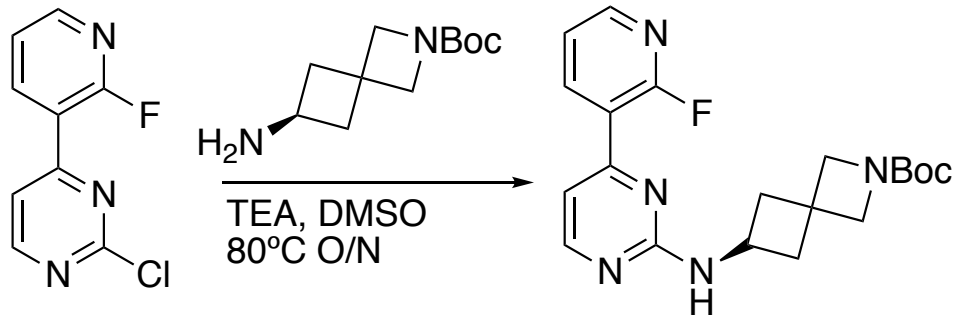
HPLC Analytical Purity Conditions

Post preparatory purification, fractions were injected onto a were injected onto an analytical reverse phase C18 column and separated using a Varian Pro-Star 210 HPLC instrument over 30 minutes at 1 mL/min (acetonitrile/water-0.1% TFA gradient 1%-100% B Phase). Purity of products was determined using UV absorption at 254 nm and 220 nm. Purity was determined by integrating the product peak and calculating its purity as a percentage of background subtracted total area. Percent purity for final compounds was reported for the 220 nm UV absorption channel.

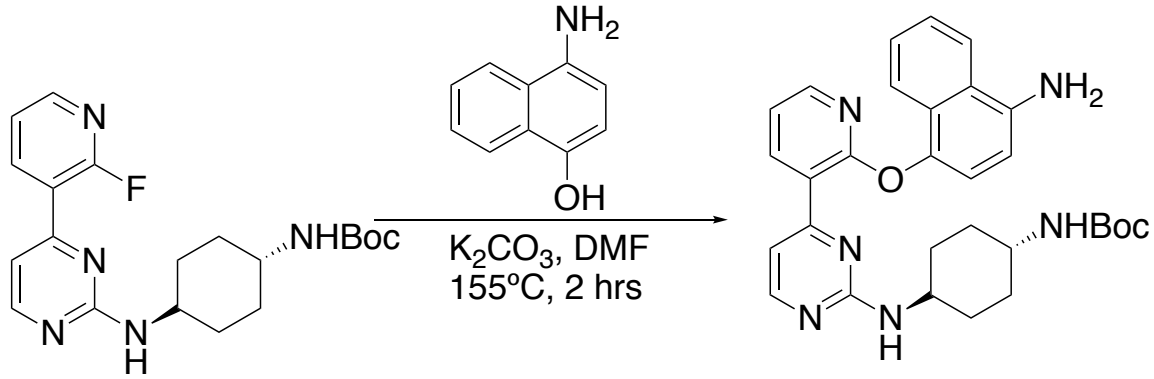
Synthesis and Characterization of Intermediates



tert-butyl (1s,4s)-4-(4-(2-fluoropyridin-3-yl)pyrimidin-2-ylamino) cyclohexylcarbamate: 2-chloro-4-(2-fluoro-3-pyridinyl)pyrimidine (1 mmol, 1 eq., CAS#: 954216-54-7) was added to N-Boc-trans-1,4-cyclohexanediamine (1 mmol, 1 eq., CAS #: 177906-48-8) with triethylamine (1.5 mmol, 1.5 eq.) in 2 mL DMSO. Reaction was heated to 80ºC and stirred for 4 hours. After the 4 hours, the reaction was cooled to room temperature and diluted with 30 mL EtOAc. The diluted reaction mixture was then washed with 25 mL of water, followed by 25 mL of brine. After extraction the organic layer was dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-20% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 50%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.50 (d, J = 7.7 Hz, 1H), 8.30 (d, J = 3.9 Hz, 1H), 8.22 (s, 1H), 7.27 (s, 1H), 7.06 (s, 1H), 5.05 – 4.96 (m, 1H), 4.42 – 4.30 (m, 1H), 3.83 – 3.72 (m, 1H), 2.12 (d, J = 10.1 Hz, 2H), 2.02 (t, J = 12.0 Hz, 2H), 1.38 (s, 9H), 1.32 – 1.18 (m, 5H). Exact Mass: 387.2, $[M+H]^+$ detected: 388.8 m/z.

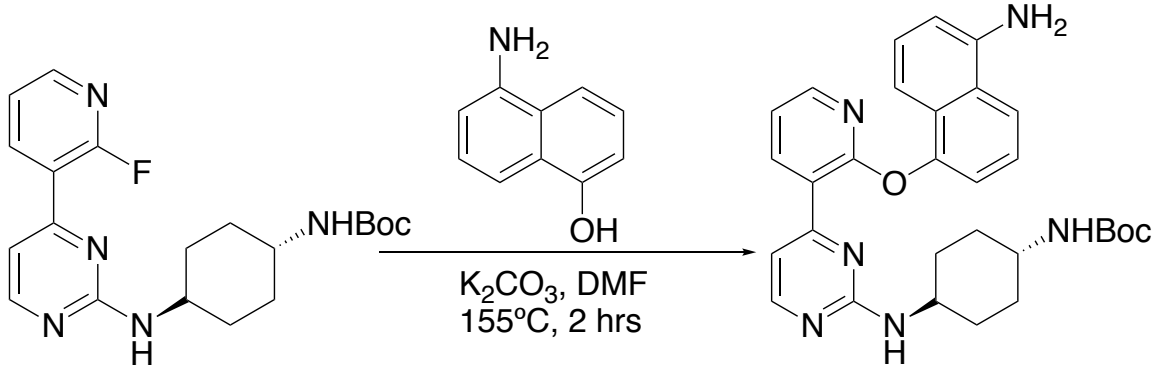


(S)-tert-Butyl 3-((4-(2-fluoropyridin-3-yl)pyrimidin-2-yl)amino)-2-aza-spiro[3.3]heptane-2-carboxylate: 2-chloro-4-(2-fluoro-3-pyridinyl)pyrimidine (0.25 mmol, 1 eq., CAS#: 954216-54-7) was added to 6-Amino-2-aza-spiro[3.3] heptane-2-carboxylic acid tert-butyl ester (0.25 mmol, 1 eq., CAS #: 1211586-09-2) with triethylamine (0.38 mmol, 1.5 eq.) in 1.2 mL DMSO. Reaction was heated to 80ºC and stirred for 4 hours. After the 4 hours, the reaction was cooled to room temperature and diluted with 30 mL EtOAc. The diluted reaction mixture was then washed with 25 mL of water, followed by 25 mL of brine. After extraction the organic layer was dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-20% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 63%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.51 (t, J = 7.9 Hz, 1H), 8.27 (dd, J = 15.5, 4.8 Hz, 2H), 7.35 – 7.25 (m, 1H), 7.12 (d, J = 3.1 Hz, 1H), 4.32 (dd, J = 15.4, 7.7 Hz, 1H), 3.96 (s, 2H), 3.83 (s, 2H), 2.78 – 2.54 (m, 2H), 2.08 (dd, J = 13.5, 7.7 Hz, 2H), 1.37 (s, 9H). Mass: 385.4, $[M+H]^+$ detected: 386.4 m/z.

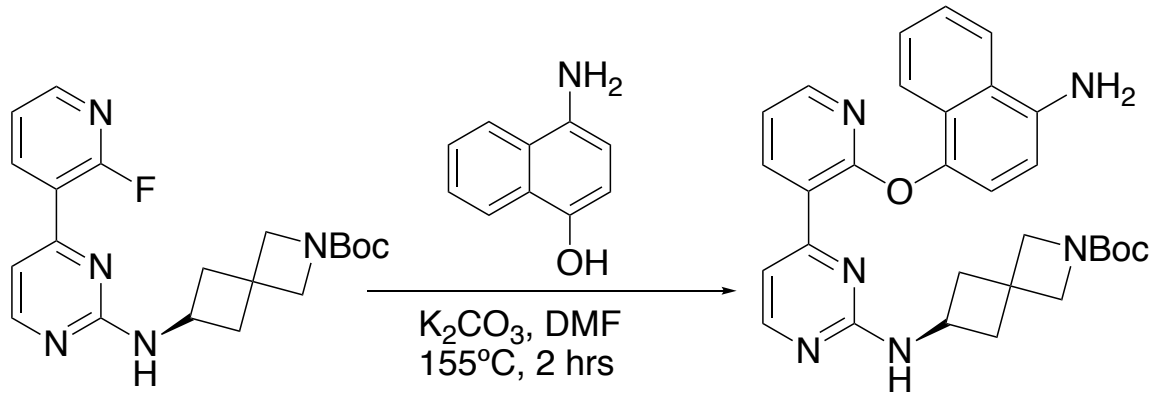


tert-butyl (1s,4s)-4-(4-(2-(4-aminonaphthalen-1-yloxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate: tert-butyl (1s,4s)-4-(4-(2-fluoropyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (1 mmol, 1 eq.) was added to K_2CO_3 (1.5 mmol, 1.5 eq.) in 5 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-naphthalen-1-ol (1 mmol, 1 eq., CAS #: 2834-90-4) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by

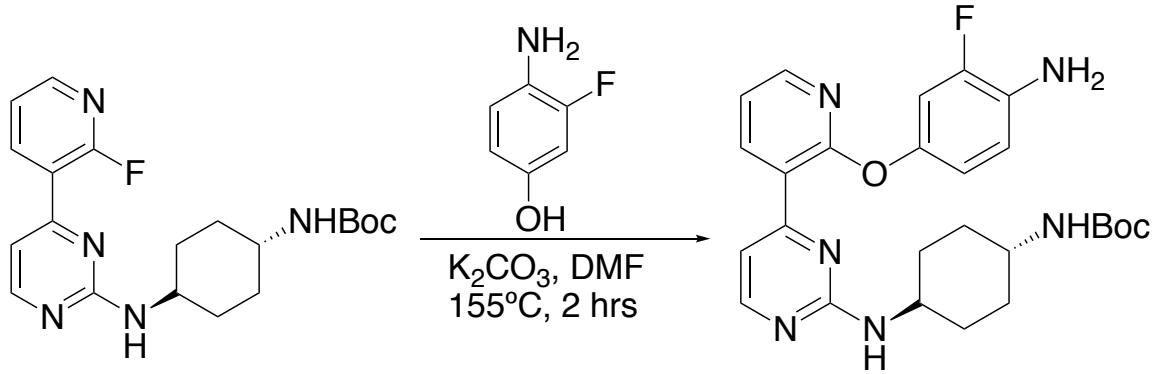
column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 27%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.49 (d, J = 6.9 Hz, 1H), 8.33 (d, J = 4.5 Hz, 1H), 8.11 (d, J = 4.4 Hz, 1H), 7.84 (dd, J = 17.3, 8.4 Hz, 2H), 7.58 (d, J = 5.1 Hz, 1H), 7.47 (t, J = 7.6 Hz, 1H), 7.41 (t, J = 7.5 Hz, 1H), 7.08 (d, J = 7.6 Hz, 2H), 6.79 (d, J = 7.9 Hz, 1H), 4.43 (s, 1H), 3.90 (s, 1H), 3.49 (s, 1H), 2.21 (s, 2H), 2.07 (d, J = 9.0 Hz, 2H), 1.46 (s, 9H), 1.39 – 1.28 (m, 4H). Exact Mass: 526.3, $[M+H]^+$ detected: 527.5 m/z.



tert-butyl (1s,4s)-4-(4-(2-(5-aminonaphthalen-1-yloxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate: tert-butyl (1s,4s)-4-(4-(2-fluoropyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (1 mmol, 1 eq., CAS# 1630086-25-7) was added to K_2CO_3 (1.5 mmol, 1.5 eq.) in 5 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 5-amino-naphthalen-1-ol (1 mmol, 1 eq., CAS #: 83-55-6) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 65%). ^{1}H NMR (500 MHz, MeOD) δ 8.42 (d, J = 4.7 Hz, 1H), 8.20 (d, J = 4.7 Hz, 1H), 8.02 (d, J = 4.4 Hz, 1H), 7.72 (s, 1H), 7.48 – 7.37 (m, 2H), 7.29 (d, J = 8.3 Hz, 1H), 7.17 (t, J = 7.9 Hz, 1H), 7.16 – 7.05 (m, 2H), 6.77 (d, J = 7.1 Hz, 1H), 3.77 (s, 1H), 3.35 (s, 1H), 2.11 (d, J = 11.9 Hz, 2H), 1.97 (d, J = 11.8 Hz, 2H), 1.38 (s, 9H), 1.36 – 1.15 (m, 5H). Exact Mass: 526.3, $[M+H]^+$ detected: 527.6 m/z.

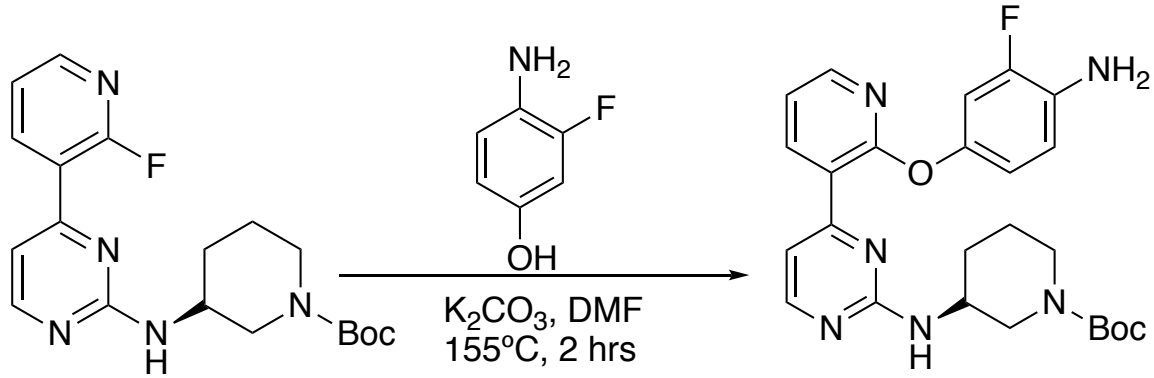


(3R)-tert-butyl 3-(4-(2-(4-aminonaphthalen-1-yloxy)pyridin-3-yl)pyrimidin-2-ylamino)-2-aza-spiro[3.3]heptane-2-carboxylate: (S)-tert-Butyl 3-((4-(2-fluoropyridin-3-yl)pyrimidin-2-yl)amino)-2-aza-spiro[3.3]heptane-2-carboxylate (0.2 mmol, 1 eq.) was added to K_2CO_3 (0.3 mmol, 1.5 eq.) in 1 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-naphthalen-1-ol (0.2 mmol, 1 eq., CAS #: 2834-90-4) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 76%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.48 (d, J = 7.2 Hz, 1H), 8.29 (d, J = 5.2 Hz, 1H), 8.10 – 7.99 (m, 1H), 7.80 (dd, J = 20.4, 8.2 Hz, 2H), 7.60 (d, J = 5.2 Hz, 1H), 7.40 (dt, J = 15.0, 6.9 Hz, 2H), 7.09 – 7.02 (m, 2H), 6.76 (d, J = 8.0 Hz, 1H), 4.40 (dd, J = 15.1, 7.6 Hz, 1H), 3.98 (s, 2H), 3.87 (s, 2H), 3.42 (d, J = 5.8 Hz, 2H), 2.73 – 2.65 (m, 2H), 2.18 – 2.09 (m, 2H), 1.41 (s, 9H).

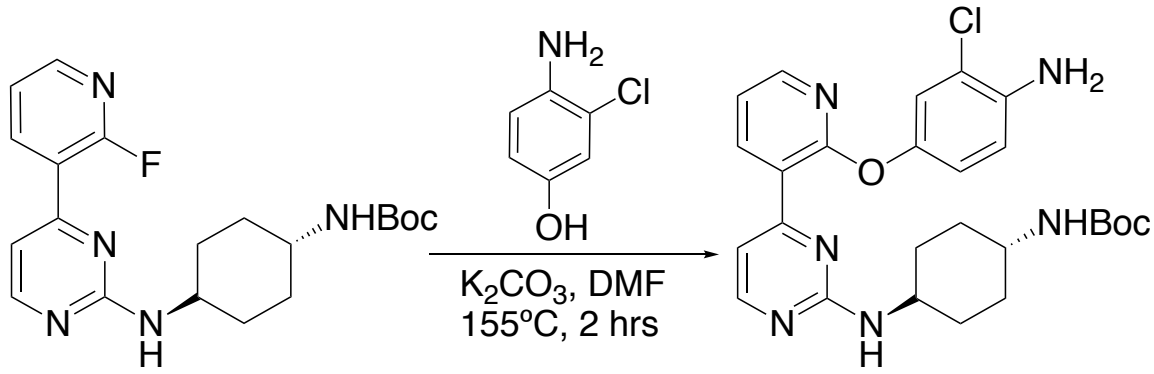


tert-butyl (1s,4s)-4-(4-(2-(4-amino-3-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate: tert-butyl (1s,4s)-4-(4-(2-fluoropyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (1 mmol, 1 eq.) was added to K_2CO_3 (1.5 mmol, 1.5 eq.) in 5 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-3-fluorophenol (1 mmol, 1 eq., CAS #: 399-95-1) was added to the reaction mixture and purged for another 3 minutes with N_2. The

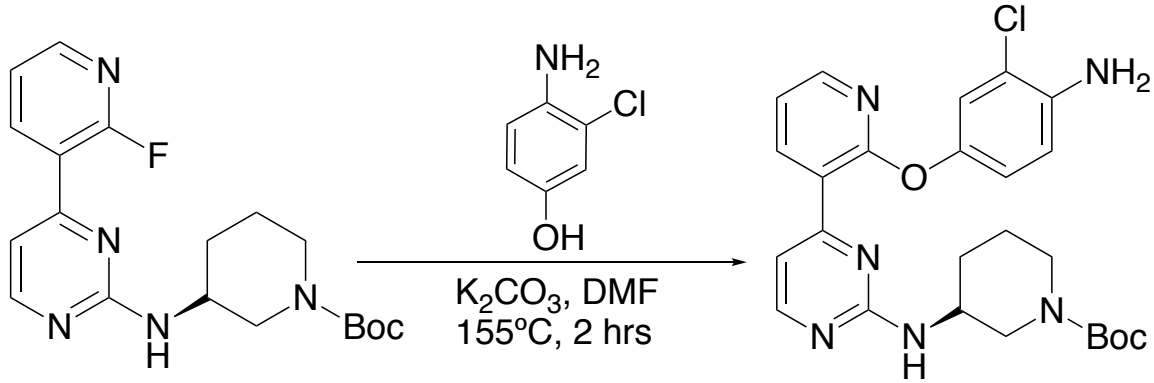
reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 62%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.76 (d, J = 6.9 Hz, 1H), 8.68 (d, J = 4.5 Hz, 1H), 8.56 (s, 1H), 7.70 (d, J = 4.8 Hz, 1H), 7.47 (d, J = 5.4 Hz, 1H), 7.27 – 7.06 (m, 3H), 4.78 (s, 1H), 4.22 (s, 1H), 3.84 (s, 1H), 2.56 (d, J = 11.3 Hz, 2H), 2.43 (d, J = 10.2 Hz, 2H), 1.81 (s, 9H), 1.67 (dt, J = 26.6, 13.3 Hz, 5H). Exact Mass: 494.2, $[M+H]^+$ detected: 495.2 m/z.



(3R)-tert-butyl 3-(4-(2-(4-amino-3-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)piperidine-1-carboxylate: (S)-tert-Butyl 3-((4-(2-fluoropyridin-3-yl) pyrimidin-2-yl)amino)piperidine-1-carboxylate (1 mmol, 1 eq., CAS# 1630086-25-7) was added to K_2CO_3 (1 mmol, 1.5 eq.) in 5 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-3-fluorophenol (1 mmol, 1 eq., CAS #: 399-95-1) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 62%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.42 (s, 1H), 8.25 (s, 1H), 8.12 (s, 1H), 7.43 (s, 1H), 7.05 (s, 1H), 6.92 (t, *J* = 8.0 Hz, 1H), 6.43 (dd, *J* = 26.1, 10.1 Hz, 2H), 4.00 (s, 1H), 3.51 (s, 1H), 3.25 (d, *J* = 30.1 Hz, 2H), 1.96 (d, *J* = 9.9 Hz, 1H), 1.71 (s, 1H), 1.58 (dd, *J* = 23.6, 11.9 Hz, 2H), 1.33 (s, 9H). Exact Mass: 480.5, $[M+H]^+$ detected: 481.4 m/z.

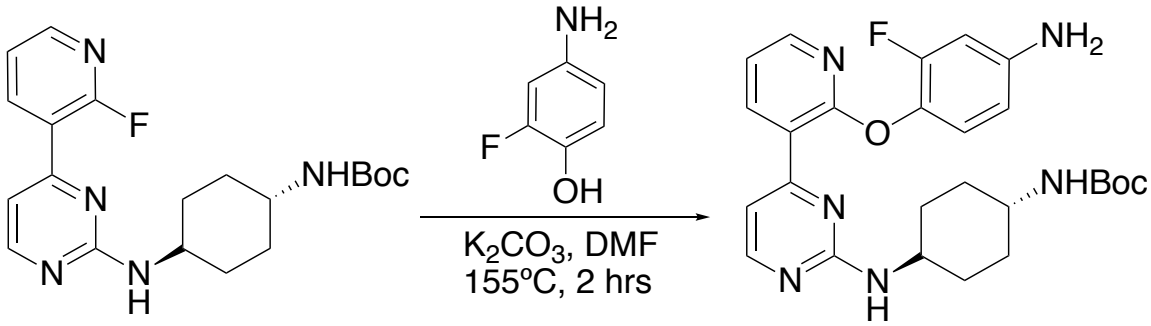


tert-butyl (1s,4s)-4-(4-(2-(4-amino-3-chlorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate: tert-butyl (1s,4s)-4-(4-(2-fluoropyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (0.1 mmol, 1 eq.) was added to K_2CO_3 (0.15 mmol, 1.5 eq.) in 0.5 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-3-chlorophenol (0.1 mmol, 1 eq., CAS #: 17609-80-2) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 29%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (d, J = 7.2 Hz, 1H), 8.25 (d, J = 5.2 Hz, 1H), 8.13 (d, J = 4.7 Hz, 1H), 7.26 (d, J = 5.2 Hz, 1H), 7.04 (dd, J = 6.9, 4.0 Hz, 2H), 6.83 (dd, J = 8.6, 2.4 Hz, 1H), 6.72 (d, J = 8.7 Hz, 1H), 4.97 (d, J = 7.9 Hz, 1H), 4.34 (s, 1H), 3.91 (s, 2H), 3.78 (d, J = 7.3 Hz, 1H), 3.40 (s, 1H), 2.13 (d, J = 9.0 Hz, 2H), 2.05 – 1.95 (m, 2H), 1.38 (s, 9H).

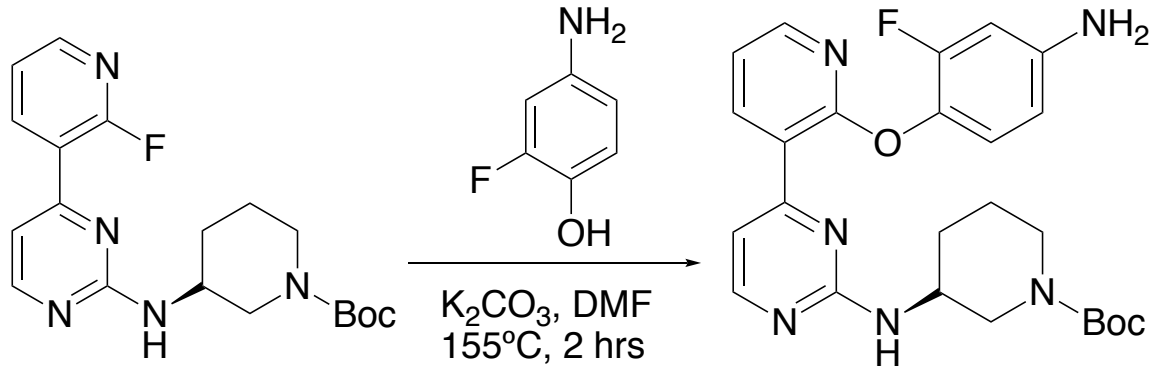


(3R)-tert-butyl 3-(4-(2-(4-amino-3-chlorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)piperidine-1-carboxylate: (S)-tert-Butyl 3-((4-(2-fluoropyridin-3-yl) pyrimidin-2-yl)amino)piperidine-1-carboxylate (0.2 mmol, 1 eq., CAS# 1630086-25-7) was added to K_2CO_3 (0.3 mmol, 1.5 eq.) in 1 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-3-chlorophenol (0.2 mmol, 1 eq., CAS #: 17609-80-2) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was

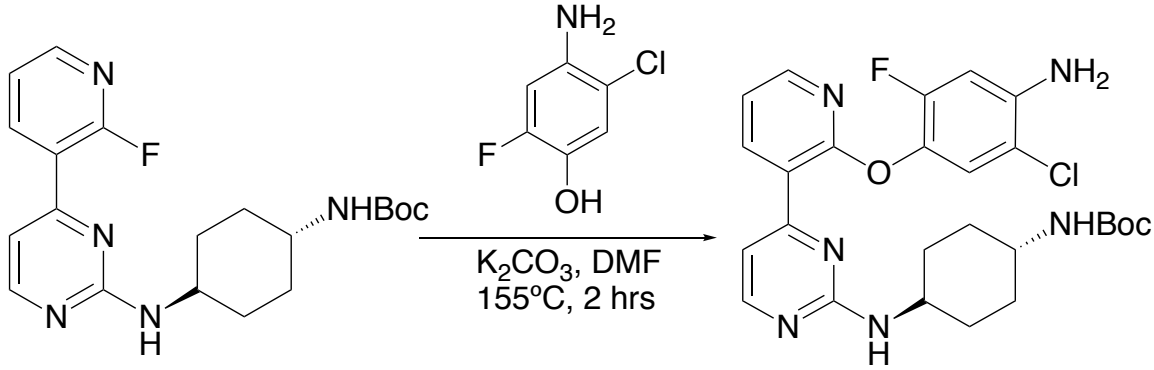
diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na2SO4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 64%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.43 (s, 1H), 8.24 (s, 1H), 8.15 (s, 1H), 7.38 (s, 1H), 7.05 (d, *J* = 15.3 Hz, 2H), 6.83 (d, *J* = 8.1 Hz, 1H), 6.73 (d, *J* = 8.5 Hz, 1H), 3.99 (s, 2H), 3.52 (s, 1H), 3.24 (d, *J* = 37.1 Hz, 2H), 1.95 (s, 1H), 1.71 (s, 1H), 1.58 (dd, *J* = 24.1, 12.3 Hz, 2H), 1.34 (s, 9H). Exact Mass: 497.0, $[M+Na]^+$ detected: 519.2 m/z.



tert-butyl (1s,4s)-4-(4-(2-(4-amino-2-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate: tert-butyl (1s,4s)-4-(4-(2-fluoropyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (0.1 mmol, 1 eq.) was added to K_2CO_3 (0.15 mmol, 1.5 eq.) in 0.5 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-2-fluorophenol (0.1 mmol, 1 eq., CAS #: 399-96-2) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 76%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.32 (d, J = 7.2 Hz, 1H), 8.26 (d, J = 5.2 Hz, 1H), 8.10 (d, J = 4.0 Hz, 1H), 7.33 (d, J = 5.2 Hz, 1H), 7.03 (dd, J = 7.4, 4.9 Hz, 1H), 6.92 (t, J = 8.5 Hz, 1H), 6.50 – 6.36 (m, 2H), 4.98 (d, J = 7.9 Hz, 1H), 4.34 (s, 1H), 3.80 (s, 1H), 3.65 (s, 2H), 3.40 (s, 1H), 2.13 (d, J = 8.0 Hz, 2H), 2.02 (s, 2H), 1.62 (s, 3H), 1.38 (s, 9H). Exact Mass: 494.6, $[M+H]^+$ detected: 495.6 m/z.

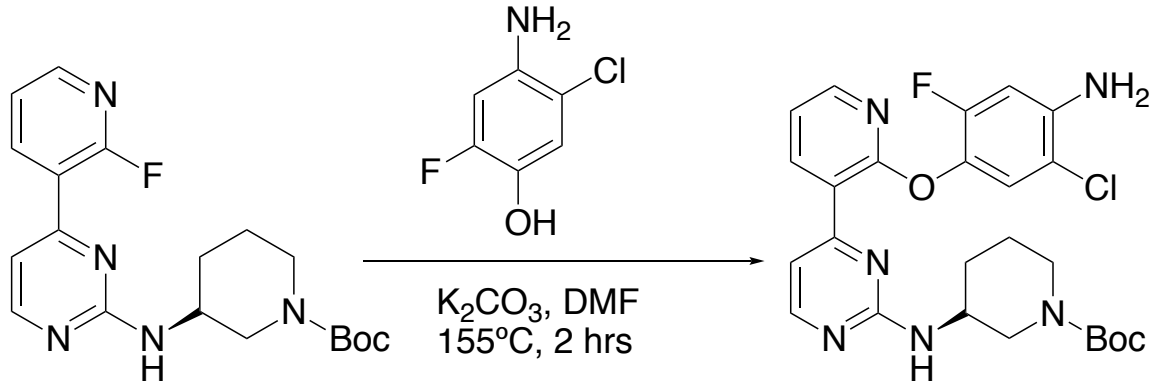


(3R)-tert-butyl 3-(4-(2-(4-amino-2-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)piperidine-1-carboxylate: (S)-tert-Butyl 3-((4-(2-fluoropyridin-3-yl) pyrimidin-2-yl)amino)piperidine-1-carboxylate (0.2 mmol, 1 eq., CAS# 1630086-25-7) was added to K_2CO_3 (0.3 mmol, 1.5 eq.) in 1 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-2-fluorophenol (0.2 mmol, 1 eq., CAS #: 399-96-2) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 34%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.42 (s, 1H), 8.25 (s, 1H), 8.12 (s, 1H), 7.43 (s, 1H), 7.05 (s, 1H), 6.92 (t, *J* = 8.0 Hz, 1H), 6.43 (dd, *J* = 26.1, 10.1 Hz, 2H), 4.00 (s, 1H), 3.51 (s, 1H), 3.25 (d, *J* = 30.1 Hz, 2H), 1.96 (d, *J* = 9.9 Hz, 1H), 1.71 (s, 1H), 1.58 (dd, *J* = 23.6, 11.9 Hz, 2H), 1.33 (s, 9H). Exact Mass: 480.5, $[M+H]^+$ detected: 481.3 m/z.

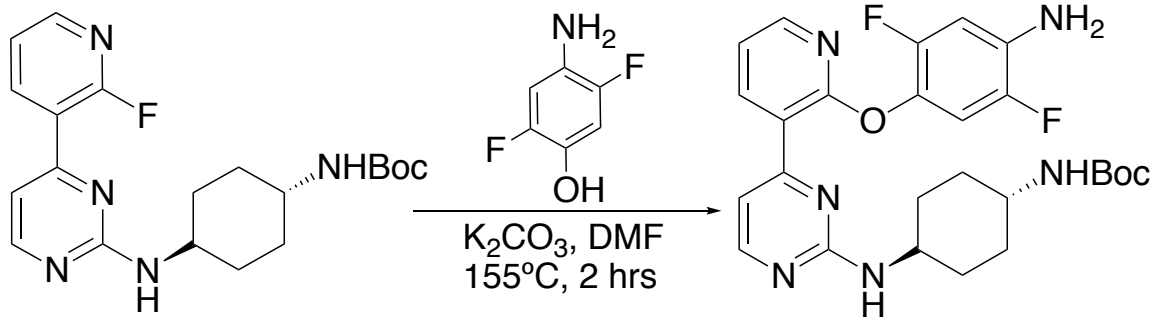


tert-butyl (1s,4s)-4-(4-(2-(4-amino-5-chloro-2-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate: tert-butyl (1s,4s)-4-(4-(2-fluoropyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (0.1 mmol, 1 eq.) was added to K_2CO_3 (0.15 mmol, 1.5 eq.) in 0.5 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-5-chloro-2-fluorophenol (0.1 mmol, 1 eq., CAS #: 847872-10-0) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and

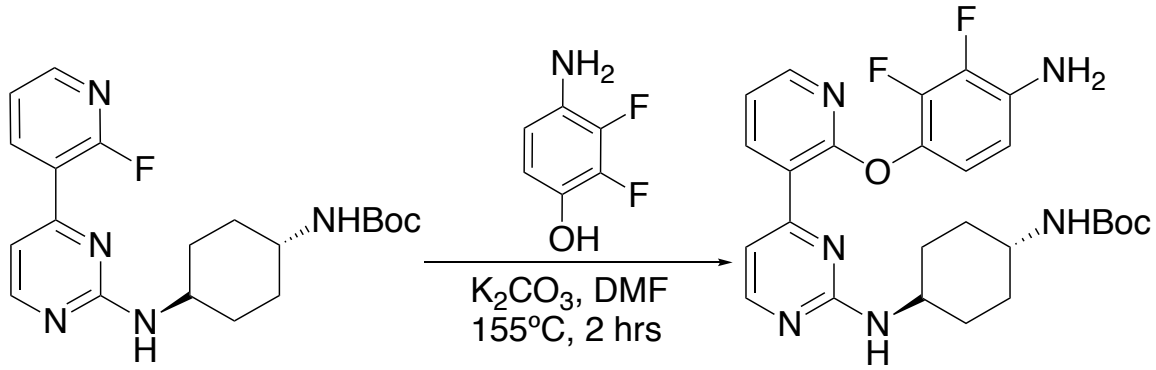
microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 80%). ^{1}H NMR (500 MHz, MeOD) δ 8.45 (d, J = 7.3 Hz, 1H), 8.30 (s, 1H), 8.13 (s, 1H), 7.32 (d, J = 4.3 Hz, 1H), 7.22 (d, J = 4.3 Hz, 1H), 7.17 (d, J = 7.5 Hz, 1H), 6.72 (d, J = 11.9 Hz, 1H), 3.84 (s, 1H), 3.36 (d, J = 21.0 Hz, 1H), 2.14 (d, J = 9.5 Hz, 2H), 1.99 (d, J = 9.9 Hz, 2H), 1.47 (s, 9H), 1.42 – 1.28 (m, 4H). Exact Mass: 529.0, $[M+H]^+$ detected: 530.3 m/z.



(3R)-tert-butyl 3-(4-(2-(4-amino-5-chloro-2-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)piperidine-1-carboxylate: (S)-tert-Butyl 3-((4-(2-fluoropyridin-3-yl)pyrimidin-2-yl)amino)piperidine-1-carboxylate (0.2 mmol, 1 eq., CAS# 1630086-25-7) was added to K_2CO_3 (0.3 mmol, 1.5 eq.) in 1 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-5-chloro-2-fluorophenol (0.2 mmol, 1 eq., CAS #: 847872-10-0) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 77%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.42 (s, 1H), 8.26 (s, 1H), 8.11 (s, 1H), 7.37 (s, 1H), 7.08 (d, *J* = 6.3 Hz, 2H), 6.55 (d, *J* = 11.2 Hz, 1H), 4.00 (s, 2H), 3.50 (s, 1H), 3.23 (s, 2H), 1.96 (d, *J* = 11.4 Hz, 1H), 1.70 (s, 1H), 1.58 (dd, *J* = 22.7, 11.0 Hz, 2H), 1.33 (s, 9H). Exact Mass: 514.99, $[M+Na]^+$ detected: 537.2 m/z.



tert-butyl (1s,4s)-4-(4-(2-(4-amino-2,5-difluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate: tert-butyl (1s,4s)-4-(4-(2-fluoropyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (0.1 mmol, 1 eq.) was added to K_2CO_3 (0.15 mmol, 1.5 eq.) in 0.5 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-3,5-difluorophenol (0.1 mmol, 1 eq., CAS #: 135086-76-9) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 25%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (dd, J = 18.7, 6.2 Hz, 2H), 8.09 (d, J = 4.4 Hz, 1H), 7.27 (d, J = 5.2 Hz, 1H), 7.05 (dd, J = 7.4, 4.9 Hz, 1H), 6.84 (dd, J = 10.7, 7.0 Hz, 1H), 6.54 (dd, J = 10.8, 8.3 Hz, 1H), 4.95 (d, J = 7.7 Hz, 1H), 4.45 – 4.24 (m, 1H), 3.78 (d, J = 14.6 Hz, 2H), 3.40 (dd, J = 15.0, 5.5 Hz, 1H), 2.13 (d, J = 8.5 Hz, 2H), 2.01 (s, 2H), 1.37 (s, 9H).



tert-butyl (1s,4s)-4-(4-(2-(4-amino-2,3-difluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate: tert-butyl (1s,4s)-4-(4-(2-fluoropyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (0.1 mmol, 1 eq.) was added to K_2CO_3 (0.15 mmol, 1.5 eq.) in 0.5 mL DMF in a microwave reaction vial and purged with N_2 for 5 minutes. 4-amino-2,3-difluorophenol (0.1 mmol, 1 eq., CAS #: 163733-99-1) was added to the reaction mixture and purged for another 3 minutes with N_2. The reaction vessel was then capped and microirradiated at 155ºC for 2 hours. After 2 hours, the reaction mixture was diluted with EtOAc and

extracted with water followed by a brine solution. The organic layer was isolated and dried over Na_2SO_4 and concentration via vacuum. The crude material was purified by column chromatography using a 0-75% EtOAc in hexanes over 60 minutes to give the desired compound (yield: 50%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.41 (d, J = 6.7 Hz, 1H), 8.33 (d, J = 5.3 Hz, 1H), 8.17 (dd, J = 4.8, 1.9 Hz, 1H), 7.38 (d, J = 5.2 Hz, 1H), 7.14 (dd, J = 7.5, 4.8 Hz, 1H), 6.85 – 6.77 (m, 1H), 6.56 (td, J = 8.8, 2.2 Hz, 1H), 5.20 – 5.08 (m, 1H), 4.48 – 4.36 (m, 1H), 3.86 (d, J = 14.4 Hz, 1H), 3.77 (s, 2H), 3.48 (dt, J = 23.1, 11.4 Hz, 1H), 2.21 (d, J = 11.3 Hz, 2H), 2.08 (d, J = 12.2 Hz, 2H), 1.45 (s, 9H), 0.94 – 0.65 (m, 1H).

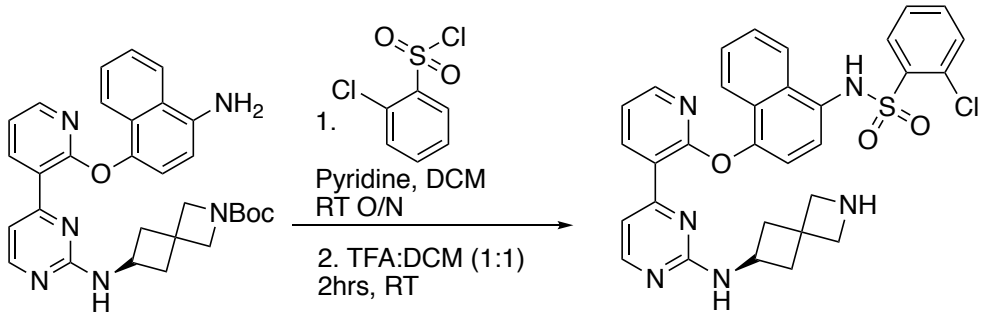
Synthesis and Characterization of Final Compounds

Compound 1: Previously characterized[1]

Compound 2: Previously characterized[1]

Compound 3: Previously characterized[1]

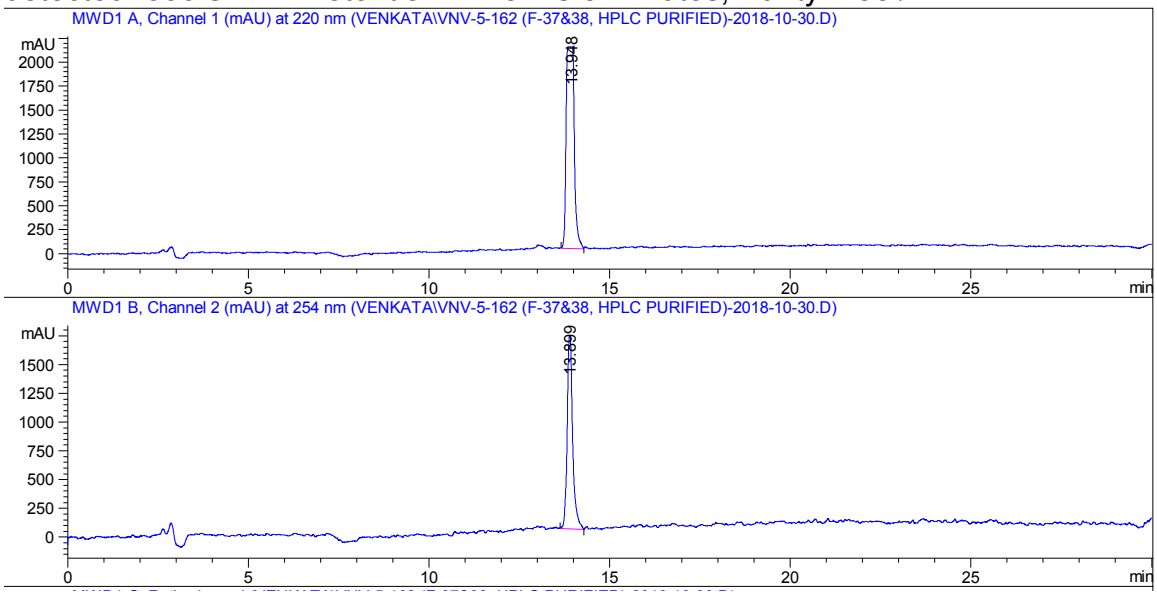
Compound 4:



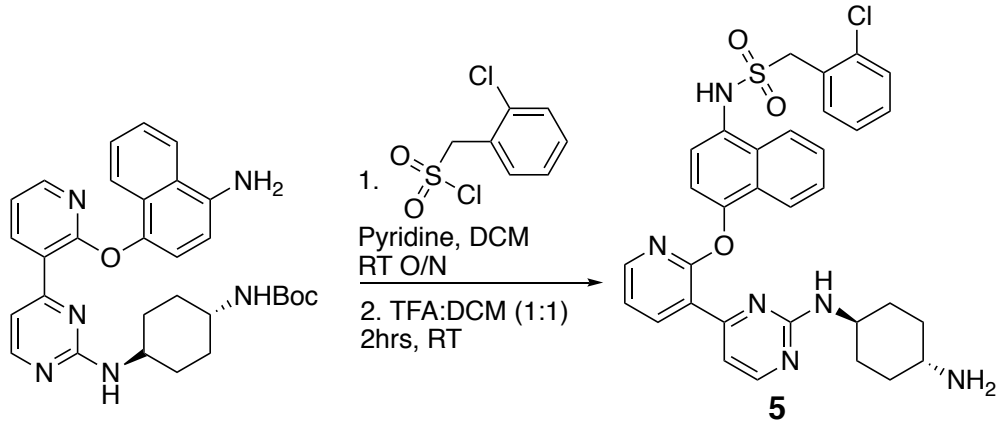
4

(2-chlorophenyl)-N-(4-(3-(2-((S)-2-aza-spiro[3.3]heptane-2-ylamino) pyrimidin-4-yl)pyridin-2-yloxy)naphthalen-1-yl) methane sulfonamide: (3R)-tert-butyl 3-(4-(2-(4-aminonaphthalen-1-yloxy)pyridin-3-yl)pyrimidin-2-ylamino)-2-aza-spiro[3.3]heptane-2-carboxylate (0.025 mmol, 1 eq.) was dissolved in 0.5 mL THF and stirred for 1 minute before addition of pyridine (0.075 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzenesulfonyl chloride (0.038 mmol, 1.5 eq., CAS #: 2905-23-9). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (300 MHz, MeOD) δ 8.49 – 8.39 (m, 1H), 8.18 (dd, J = 16.0, 6.7 Hz, 2H), 7.98 (dd, J = 4.8, 1.9 Hz, 1H), 7.76 (d, J = 7.9 Hz, 2H), 7.52 (d, J = 7.1 Hz, 1H), 7.48 – 7.30 (m, 4H), 7.29 – 7.13 (m, 3H), 6.95 (d, J = 8.1 Hz, 1H), 4.40 – 4.23 (m, 1H), 4.03 (s, 2H), 3.96 (s,

2H), 2.75 – 2.60 (m, 2H), 2.30 – 2.16 (m, 2H). Exact Mass: 599.1, $[M+H]^+$ detected: 600.3 m/z. Retention Time: 13.9 minutes, Purity: >99%



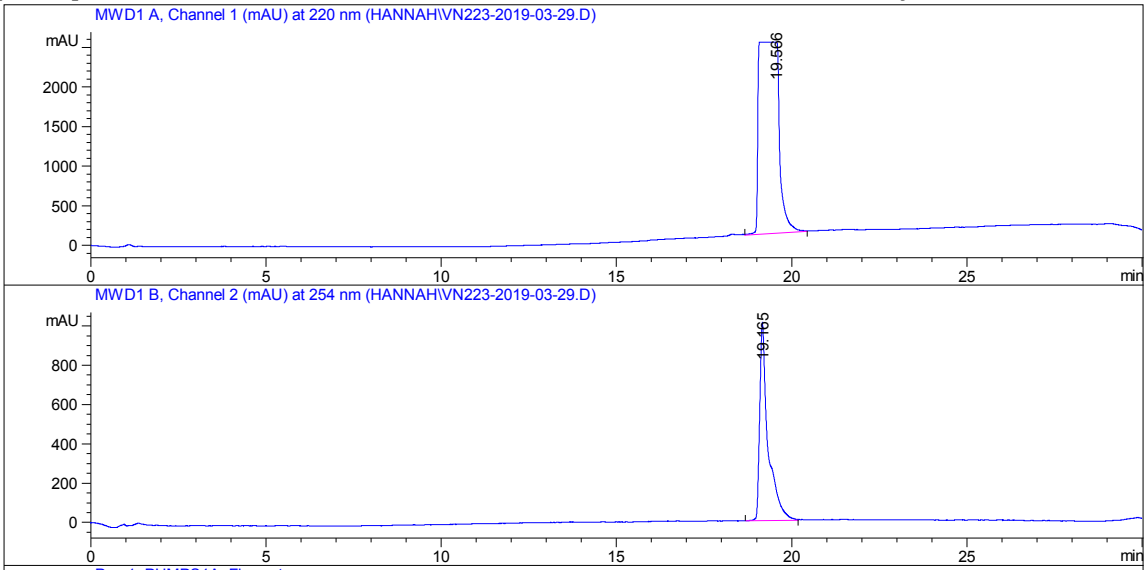
Compound 5:



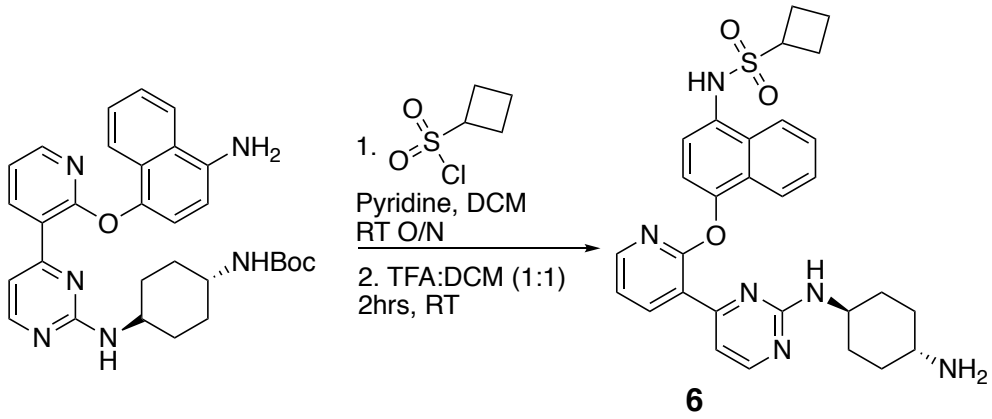
5

N-(4-(3-(2-((1r,4r)-4-aminocyclohexylamino)pyrimidin-4-yl)pyridin-2-yloxy)naphthalen-1-yl)(2-chlorophenyl)methanesulfonamide: tert-butyl (1s,4s)-4-(4-(2-(4-aminonaphthalen-1-yloxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexyl carbamate (0.01 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.03 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzylsulfonyl chloride (0.015 mmol, 1.5 eq., CAS #: 77421-13-7). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (500 MHz, MeOD) δ 8.40 (d, J = 7.6 Hz, 1H), 8.22 (d, J = 5.2 Hz, 1H), 8.06 (d, J = 8.4 Hz, 2H), 7.89

(d, J = 8.4 Hz, 1H), 7.50 (dd, J = 17.2, 8.0 Hz, 2H), 7.46 – 7.39 (m, 2H), 7.37 (d, J = 5.3 Hz, 1H), 7.26 – 7.13 (m, 4H), 7.10 (d, J = 8.2 Hz, 1H), 4.58 (s, 2H), 3.88 – 3.68 (m, 1H), 2.89 (d, J = 11.8 Hz, 1H), 2.15 (d, J = 12.0 Hz, 2H), 1.95 (d, J = 11.3 Hz, 2H), 1.39 – 1.23 (m, 5H), 0.79 (d, J = 7.1 Hz, 1H). Exact Mass: 615.2, $[M+H]^+$ detected: 615.4 m/z. Retention Time: 19.2 minutes, Purity: >99%.



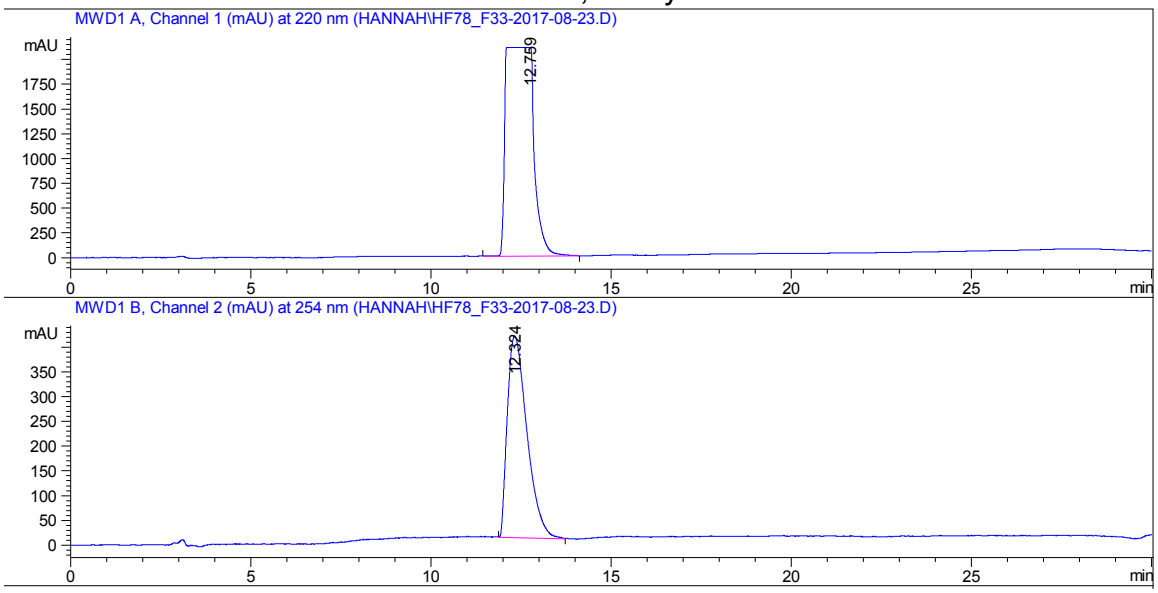
Compound 6:



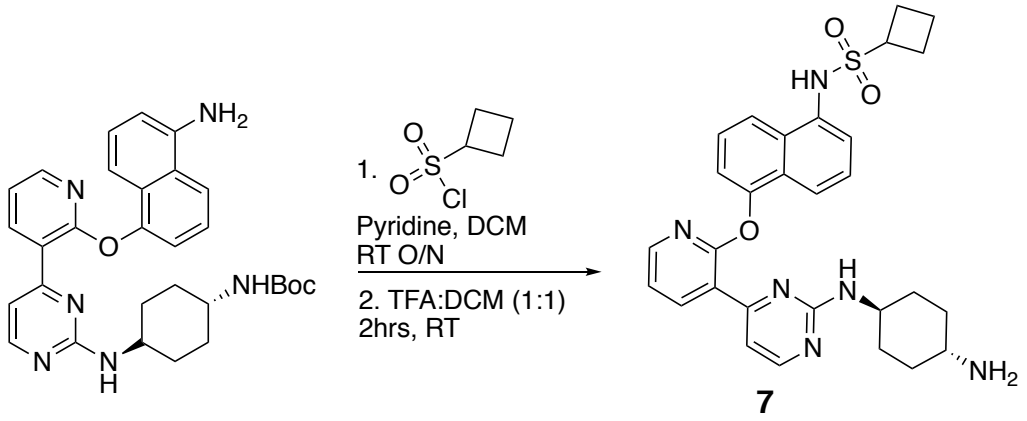
6

N-(4-(3-(2-((1r,4r)-4-aminocyclohexylamino)pyrimidin-4-yl)pyridin-2-yloxy)naphthalen-1-yl)cyclobutanesulfonamide: tert-butyl (1s,4s)-4-(4-(2-(4-aminonaphthalen-1-yloxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (0.02 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.06 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of cyclobutanesulfonyl chloride (0.03 mmol, 1.5 eq., CAS #: 338453-16-0). The reaction was then stirred at room temperature overnight. The reaction mixture was concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1.5 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water

solvent system. ^{1}H NMR (300 MHz, MeOD) δ 8.52 (d, J = 4.9 Hz, 1H), 8.29 (dd, J = 17.9, 6.3 Hz, 2H), 8.10 (s, 1H), 7.91 (d, J = 8.0 Hz, 1H), 7.65 – 7.41 (m, 4H), 7.27 (d, J = 7.0 Hz, 1H), 7.20 (d, J = 8.1 Hz, 1H), 4.02 – 3.79 (m, 2H), 3.12 (td, J = 12.7, 9.6 Hz, 1H), 2.58 – 2.36 (m, 2H), 2.32 – 2.12 (m, 4H), 2.01 (dt, J = 13.0, 8.0 Hz, 4H), 1.47 (tt, J = 9.8, 4.9 Hz, 4H). Exact Mass: 544.23, $[M+H]^+$ detected: 545.4 m/z. Retention Time: 12.3 minutes, Purity: >99%.



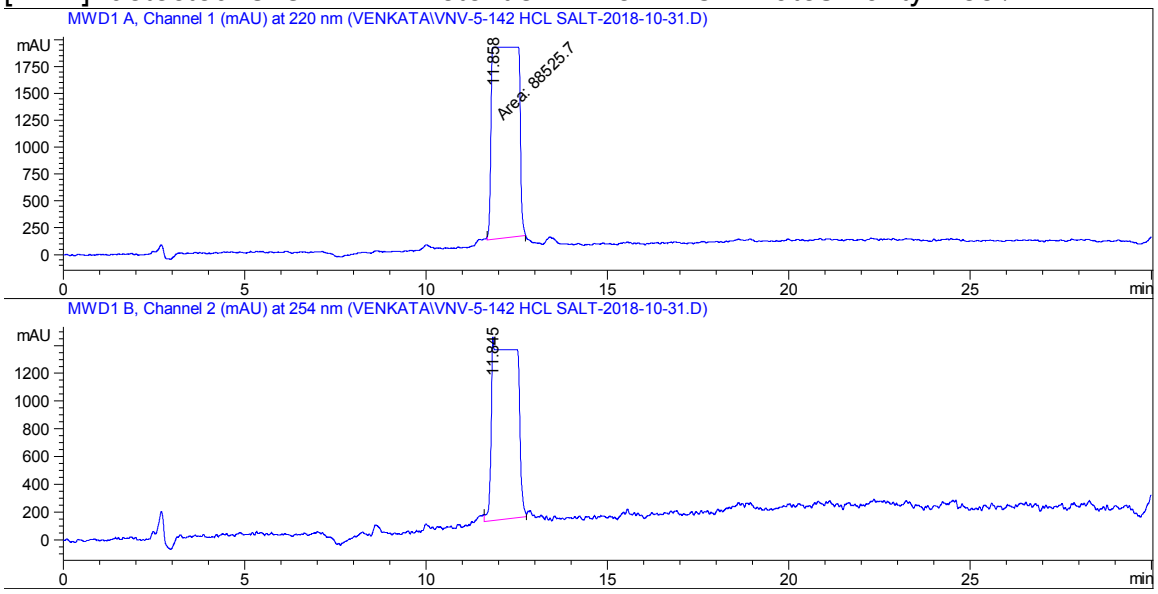
Compound 7:



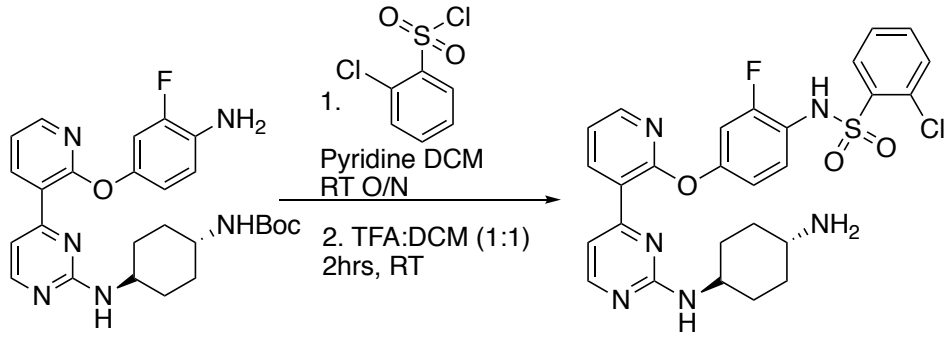
7

N-(5-(3-(2-((1r,4r)-4-aminocyclohexylamino)pyrimidin-4-yl)pyridin-2-yloxy)naphthalen-1-yl)cyclobutanesulfonamide: tert-butyl (1s,4s)-4-(4-(2-(5-aminonaphthalen-1-yloxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (0.09 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.27 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of cyclobutanesulfonyl chloride (0.14 mmol, 1.5 eq., CAS #: 338453-16-0). The reaction was then stirred at room temperature overnight. The reaction mixture was concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1.5 mL TFA:DCM (1:1) and stirred at room temperature for 2

hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (300 MHz, MeOD) δ 8.53 (dd, J = 7.9, 1.5 Hz, 1H), 8.35 (d, J = 5.3 Hz, 1H), 8.18 – 8.09 (m, 2H), 7.86 (d, J = 8.5 Hz, 1H), 7.66 – 7.57 (m, 2H), 7.46 (dd, J = 11.6, 6.5 Hz, 2H), 7.29 (t, J = 7.2 Hz, 2H), 3.95 (dt, J = 14.9, 7.5 Hz, 2H), 3.21 – 3.08 (m, 1H), 2.61 – 2.41 (m, 2H), 2.35 – 2.17 (m, 4H), 2.04 (dt, J = 17.5, 9.9 Hz, 4H), 1.51 (dd, J = 21.2, 10.7 Hz, 4H). Exact Mass: 544.23. $[M+H]^+$ detected: 545.4 m/z. Retention Time: 11.8 minutes Purity: >99%



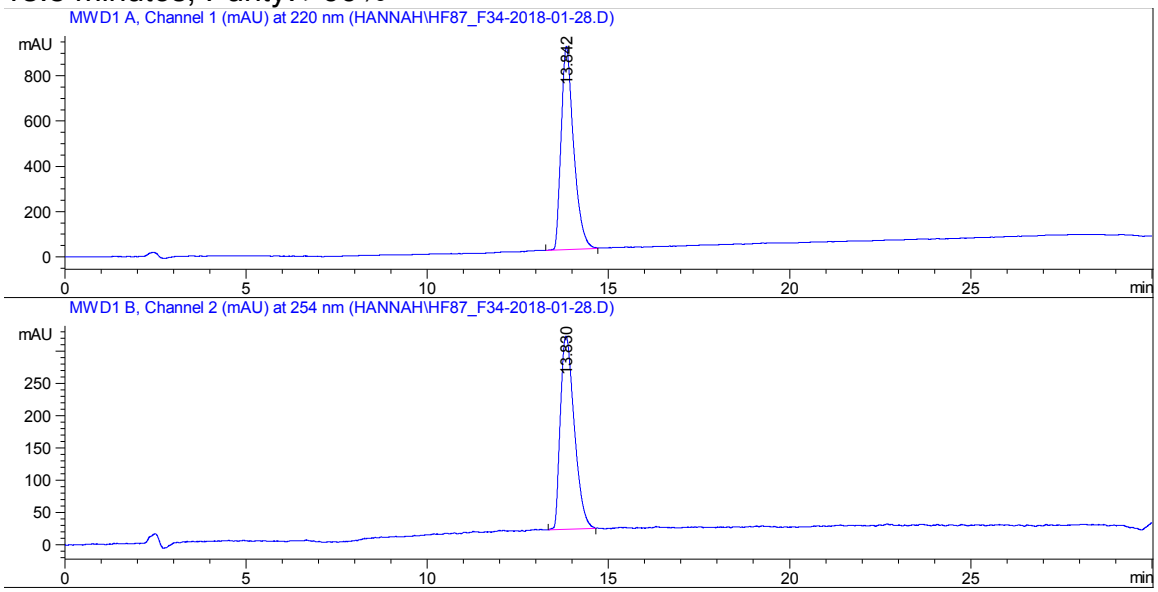
Compound 8:



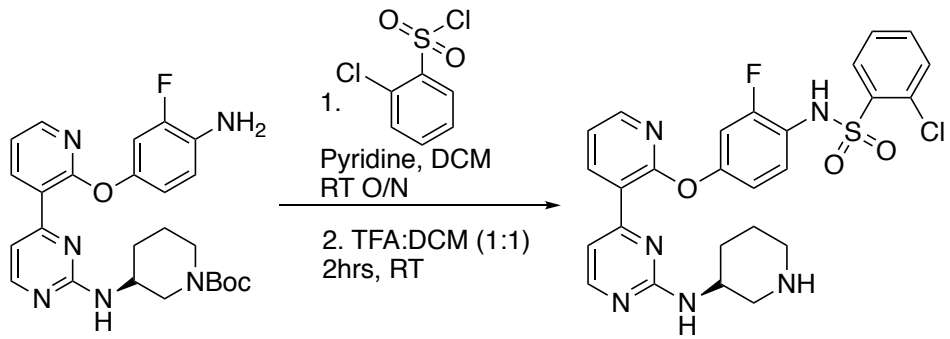
8

N-(4-(3-(2-((1s,4s)-4-aminocyclohexylamino)pyrimidin-4-yl)pyridin-2-yloxy)-2-fluorophenyl)-2-chlorobenzenesulfonamide: tert-butyl (1s,4s)-4-(4-(2-(4-amino-3-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (0.02 mmol, 1 eq.) was dissolved in 0.5 mL THF and stirred for 1 minute before addition of pyridine (0.06 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzenesulfonyl chloride (0.03 mmol, 1.5 eq., CAS #: 2905-23-9). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was

then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (500 MHz, MeOD) δ 8.45 (d, J = 7.4 Hz, 1H), 8.32 (d, J = 4.8 Hz, 1H), 8.21 (s, 1H), 7.99 (d, J = 7.6 Hz, 1H), 7.62 (dd, J = 21.1, 8.0 Hz, 2H), 7.47 – 7.38 (m, 2H), 7.32 – 7.25 (m, 2H), 6.94 (d, J = 11.1 Hz, 1H), 6.88 (d, J = 8.5 Hz, 1H), 3.89 (s, 1H), 3.18 (d, J = 12.8 Hz, 1H), 2.23 (d, J = 13.1 Hz, 2H), 2.12 (d, J = 12.1 Hz, 2H), 1.58 (dd, J = 24.2, 12.2 Hz, 2H), 1.48 (dd, J = 24.6, 12.6 Hz, 2H). Exact Mass: 569.1, $[M+H]^+$ detected: 570.3 m/z. Retention Time: 13.8 minutes, Purity: >99%



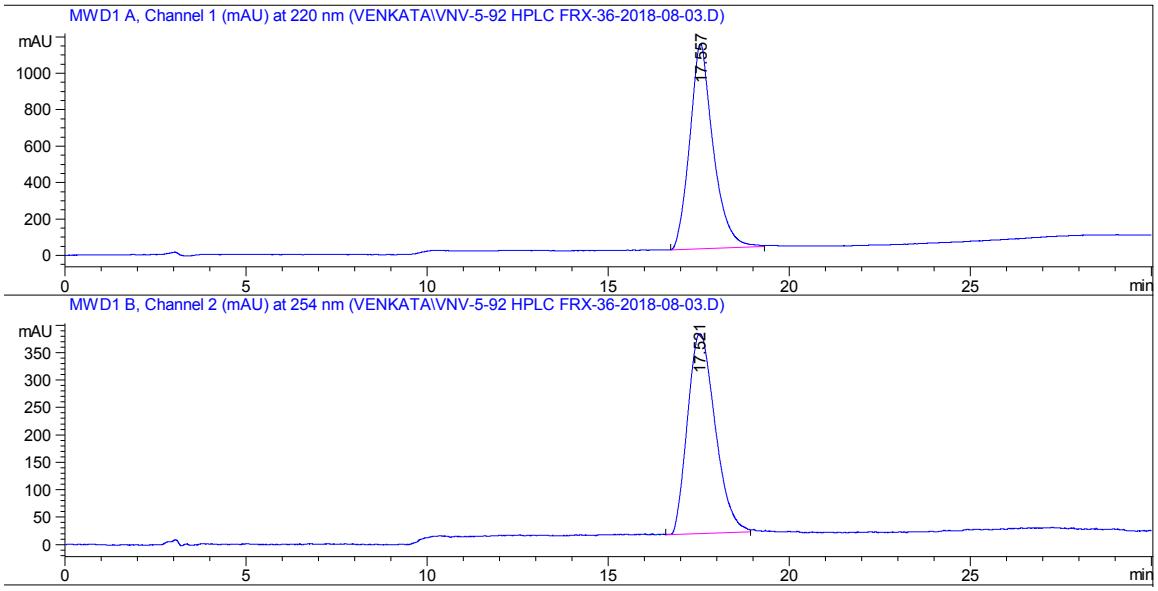
Compound 9:



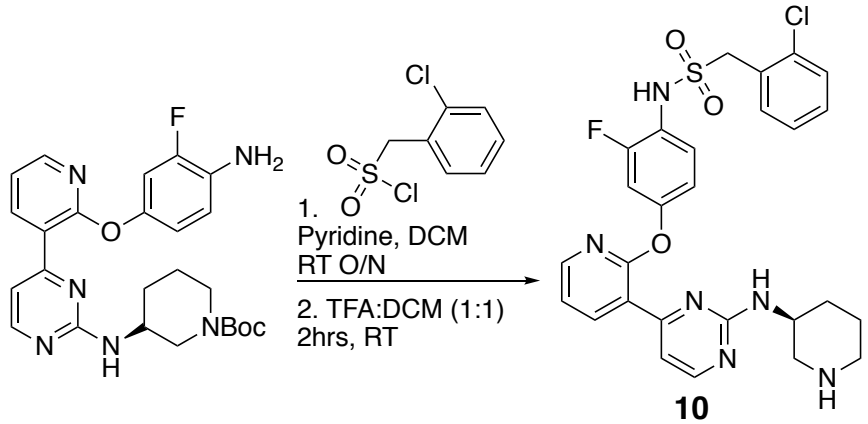
9

2-chloro-N-(2-fluoro-4-(3-(2-((R)-piperidin-3-ylamino)pyrimidin-4-yl)pyridin-2-yloxy)phenyl)benzenesulfonamide: (3R)-tert-butyl 3-(4-(2-(4-amino-3-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)piperidine-1-carboxylate (0.1 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.3 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzenesulfonyl chloride (0.15 mmol, 1.5

eq., CAS #: 2905-23-9). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (300 MHz, MeOD) ^{1}H NMR (300 MHz, MeOD) δ 8.38 (d, J = 7.3 Hz, 1H), 8.25 (d, J = 5.3 Hz, 1H), 8.08 (d, J = 3.1 Hz, 1H), 7.86 (d, J = 7.7 Hz, 1H), 7.49 (q, J = 7.9 Hz, 2H), 7.34 – 7.15 (m, 4H), 6.78 (dd, J = 17.3, 11.4 Hz, 2H), 4.24 – 4.11 (m, 1H), 3.46 (d, J = 11.7 Hz, 1H), 2.92 (dd, J = 20.7, 11.3 Hz, 2H), 2.01 (dd, J = 16.9, 11.9 Hz, 2H), 1.84 – 1.59 (m, 2H), 1.19 (s, 1H). Exact Mass: 555.0, $[M+H]^+$ detected: 556.6 m/z. Retention Time: 17.5 minutes, Purity: 97.4%



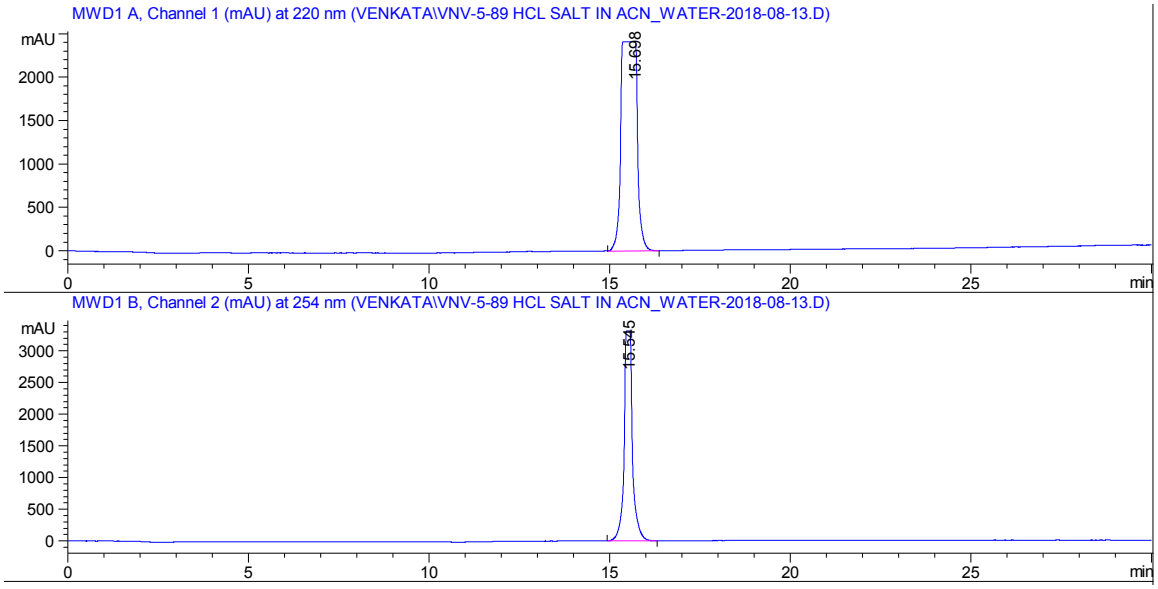
Compound 10:



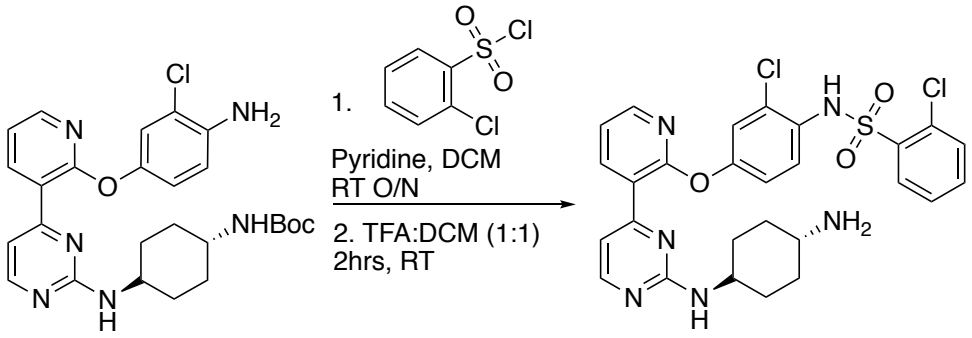
10

(2-chlorophenyl)-N-(2-fluoro-4-(3-(2-((S)-piperidin-3-ylamino)pyrimidin-4-yl)pyridin-2-yloxy)phenyl)methanesulfonamide: (3R)-tert-butyl 3-(4-(2-(4-amino-3-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)piperidine-1-carboxylate

(0.01 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.03 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzylsulfonyl chloride (0.015 mmol, 1.5 eq., CAS #: 77421-13-7). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (300 MHz, MeOD) δ 8.58 (d, J = 7.3 Hz, 1H), 8.36 (s, 1H), 8.23 (d, J = 4.5 Hz, 1H), 7.68 (d, J = 5.4 Hz, 1H), 7.46 (dd, J = 11.9, 5.5 Hz, 1H), 7.37 – 7.19 (m, 5H), 7.02 (d, J = 11.1 Hz, 1H), 6.84 (d, J = 9.0 Hz, 1H), 4.59 (s, 2H), 4.50 – 4.37 (m, 1H), 3.52 (d, J = 9.6 Hz, 1H), 2.97 (t, J = 15.0 Hz, 2H), 2.07 (dd, J = 29.8, 12.4 Hz, 2H), 1.90 – 1.70 (m, 2H), 1.30 – 1.16 (m, 1H). Exact Mass: 569.1, $[M+H]^{+}$ detected: 570.1 m/z. Retention Time: 15.5 minutes, Purity: >99%.

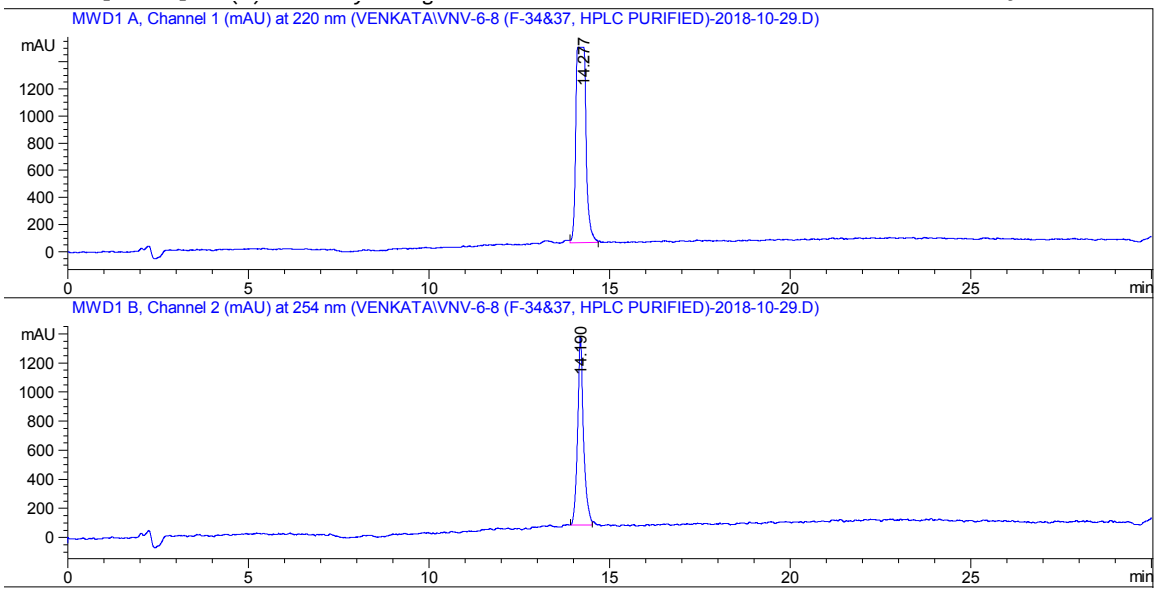


Compound 11:

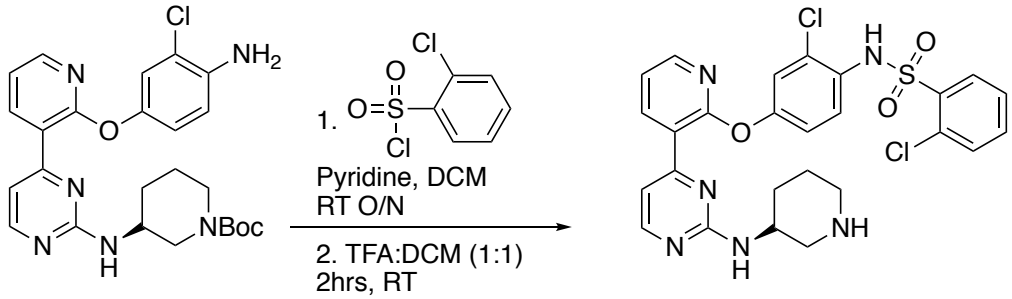


11

N-(4-(3-(2-((1s,4s)-4-aminocyclohexylamino)pyrimidin-4-yl)pyridin-2-yloxy)-2-chlorophenyl)-2-chlorobenzenesulfonamide: tert-butyl (1s,4s)-4-(4-(2-(4-amino-3-chlorophenoxy)pyridin-3-yl)pyrimidin-2 ylamino) cyclohexylcarbamate (0.03 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.09 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzenesulfonyl chloride (0.045 mmol, 1.5 eq., CAS #: 2905-23-9). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (300 MHz, MeOD) δ 8.33 (dd, J = 7.6, 1.9 Hz, 1H), 8.20 (d, J = 5.4 Hz, 1H), 8.10 (dd, J = 4.8, 1.9 Hz, 1H), 7.85 (d, J = 8.6 Hz, 1H), 7.54 – 7.45 (m, 2H), 7.38 – 7.29 (m, 2H), 7.18 (dd, J = 7.3, 5.1 Hz, 2H), 7.08 (d, J = 2.6 Hz, 1H), 6.92 (dd, J = 8.9, 2.7 Hz, 1H), 3.87 – 3.71 (m, 1H), 3.02 (dd, J = 21.3, 9.7 Hz, 1H), 2.06 (dd, J = 32.4, 11.5 Hz, 4H), 1.54 – 1.29 (m, 4H). Exact Mass: 585.5, $[M+H]^+$ detected: 586.9 m/z. Retention Time: 14.2 minutes, Purity: >99%.

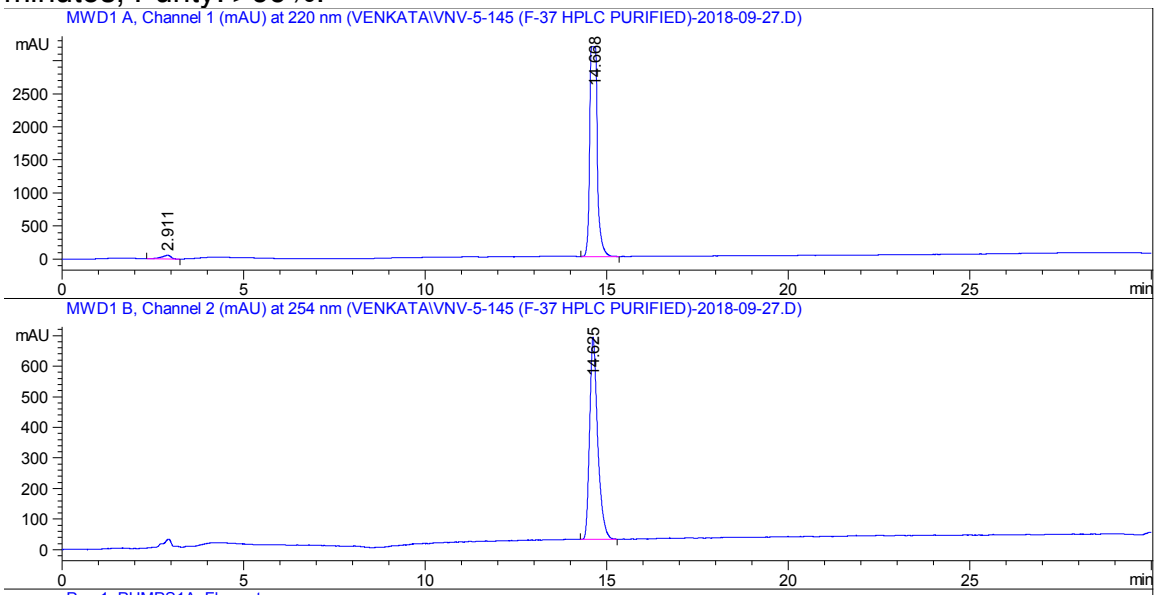


Compound 12:

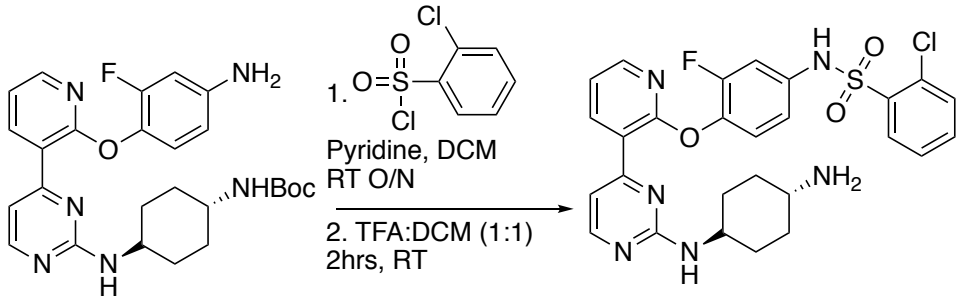


12

2-chloro-N-(2-chloro-4-(3-(2-((R)-piperidin-3-ylamino)pyrimidin-4-yl)pyridin-2-yloxy)phenyl)benzenesulfonamide: (3R)-tert-butyl 3-(4-(2-(4-amino-3-chlorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)piperidine-1-carboxylate (0.04 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.12 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzenesulfonyl chloride (0.06 mmol, 1.5 eq., CAS #: 2905-23-9). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (500 MHz, MeOD) δ 8.48 (d, J = 7.2 Hz, 1H), 8.36 (d, J = 4.6 Hz, 1H), 8.18 (d, J = 2.0 Hz, 1H), 7.94 (d, J = 7.9 Hz, 1H), 7.59 (q, J = 8.1 Hz, 2H), 7.47 – 7.38 (m, 2H), 7.34 (d, J = 4.5 Hz, 1H), 7.32 – 7.25 (m, 1H), 7.16 (s, 1H), 7.00 (d, J = 8.8 Hz, 1H), 4.28 (s, 1H), 3.56 (d, J = 12.0 Hz, 1H), 3.06 – 2.97 (m, 2H), 2.11 (dd, J = 32.5, 13.4 Hz, 3H), 1.91 – 1.81 (m, 1H), 1.80 – 1.69 (m, 1H). Exact Mass: 571.5, $[M+H]^+$ detected: 572.7 m/z. Retention Time: 14.6 minutes, Purity: >99%.

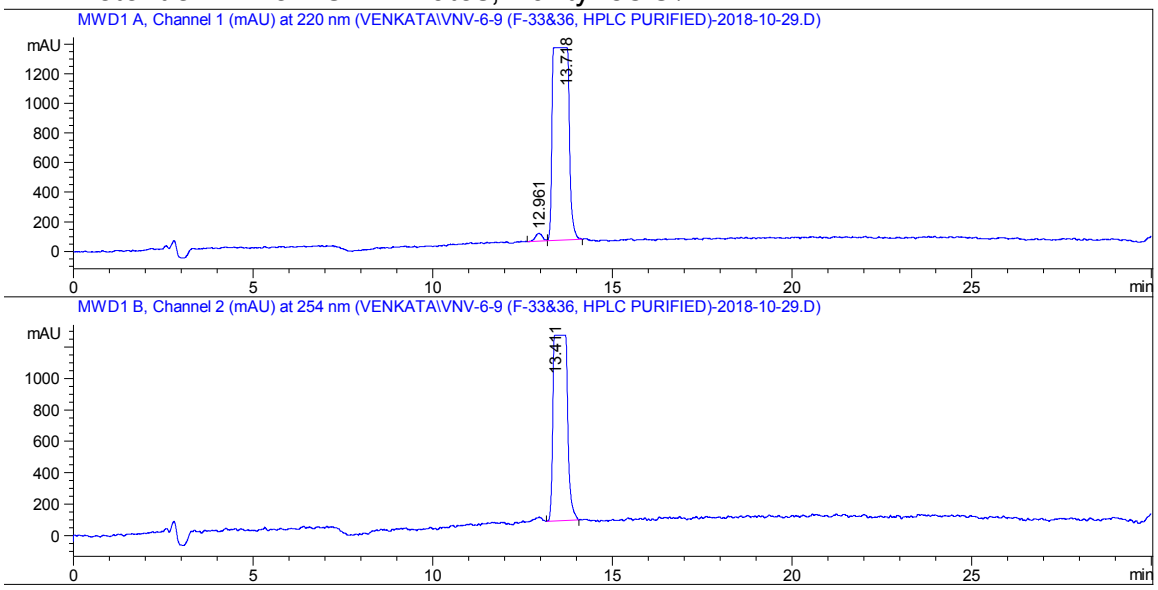


Compound 13:

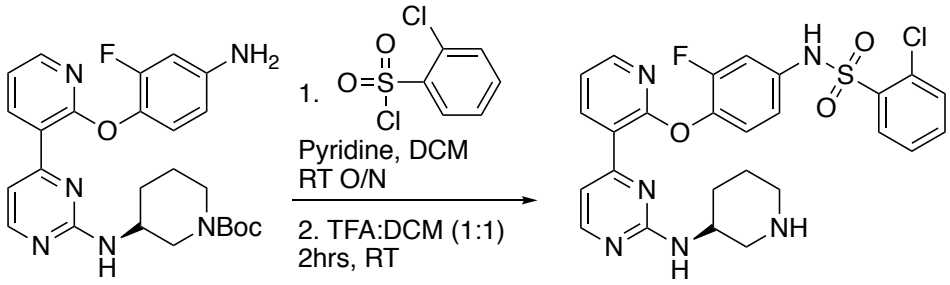


13

N-(4-(3-(2-((1s,4s)-4-aminocyclohexylamino)pyrimidin-4-yl)pyridin-2-yloxy)-3-fluorophenyl)-2-chlorobenzenesulfonamide: tert-butyl (1s,4s)-4-(4-(2-(4-amino-2-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (0.03 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.09 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzenesulfonyl chloride (0.045 mmol, 1.5 eq., CAS #: 2905-23-9). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (300 MHz, MeOD) δ 8.34 (dd, J = 7.6, 1.8 Hz, 1H), 8.20 (d, J = 5.6 Hz, 1H), 8.07 – 7.98 (m, 2H), 7.49 (d, J = 3.4 Hz, 2H), 7.43 – 7.33 (m, 1H), 7.27 (d, J = 5.6 Hz, 1H), 7.13 (dd, J = 7.6, 4.9 Hz, 1H), 6.97 (ddd, J = 22.2, 18.5, 9.0 Hz, 3H), 3.82 (s, 1H), 3.05 (s, 1H), 2.13 (d, J = 12.0 Hz, 2H), 2.01 (d, J = 11.5 Hz, 2H), 1.55 – 1.31 (m, 4H). Exact Mass: 569.1, $[M+H]^+$ detected: 570.2 m/z. Retention Time: 13.7 minutes, Purity: 98.3%.

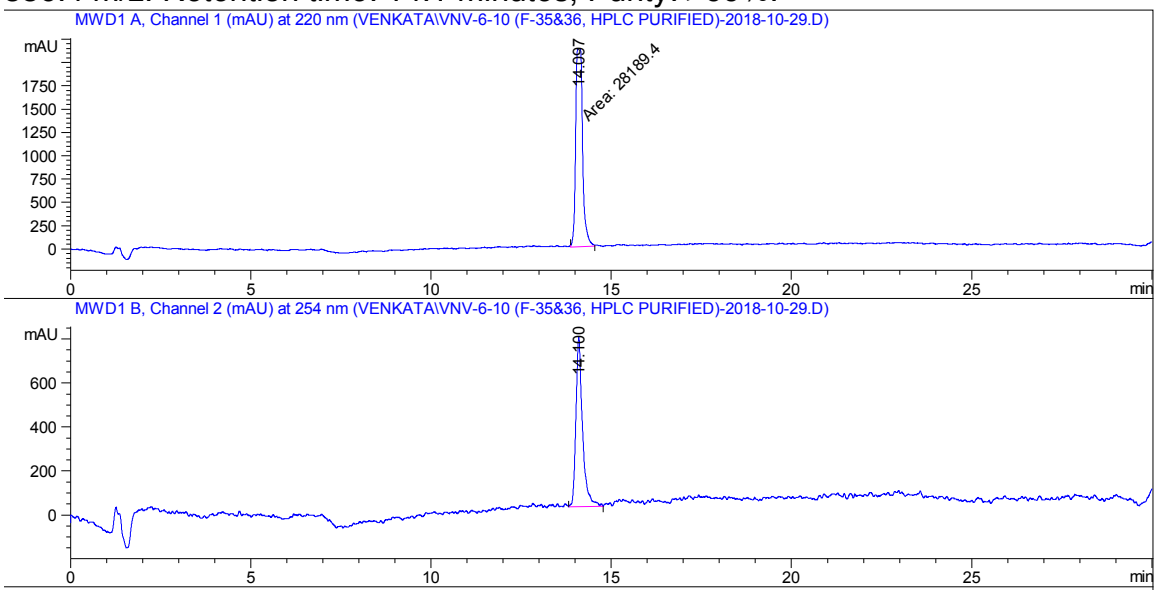


Compound 14:

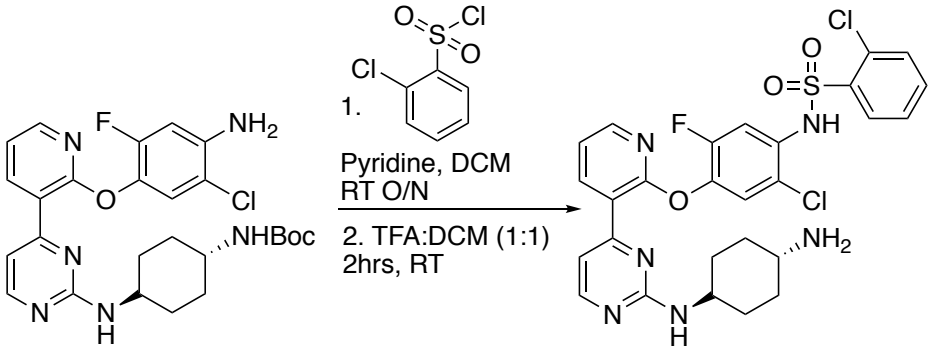


14

2-chloro-N-(3-fluoro-4-(3-(2-((R)-piperidin-3-ylamino)pyrimidin-4-yl)pyridin-2-yloxy)phenyl)benzenesulfonamide: (3R)-tert-butyl 3-(4-(2-(4-amino-2-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)piperidine-1-carboxylate (0.03 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.09 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzenesulfonyl chloride (0.045 mmol, 1.5 eq., CAS #: 2905-23-9). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (300 MHz, MeOD) δ 8.38 (dd, J = 7.6, 1.8 Hz, 1H), 8.26 (d, J = 5.3 Hz, 1H), 7.99 (dd, J = 9.5, 4.7 Hz, 2H), 7.49 (d, J = 3.6 Hz, 2H), 7.43 – 7.34 (m, 1H), 7.30 (d, J = 5.3 Hz, 1H), 7.12 (dd, J = 7.6, 4.9 Hz, 1H), 7.05 – 6.86 (m, 3H), 4.19 (t, J = 9.5 Hz, 1H), 3.49 (dd, J = 12.3, 3.8 Hz, 1H), 2.92 (dd, J = 17.4, 8.2 Hz, 3H), 2.18 – 1.53 (m, 5H). Exact Mass: 555.0, $[M+H]^+$ detected: 556.4 m/z. Retention time: 14.1 minutes, Purity: >99%.

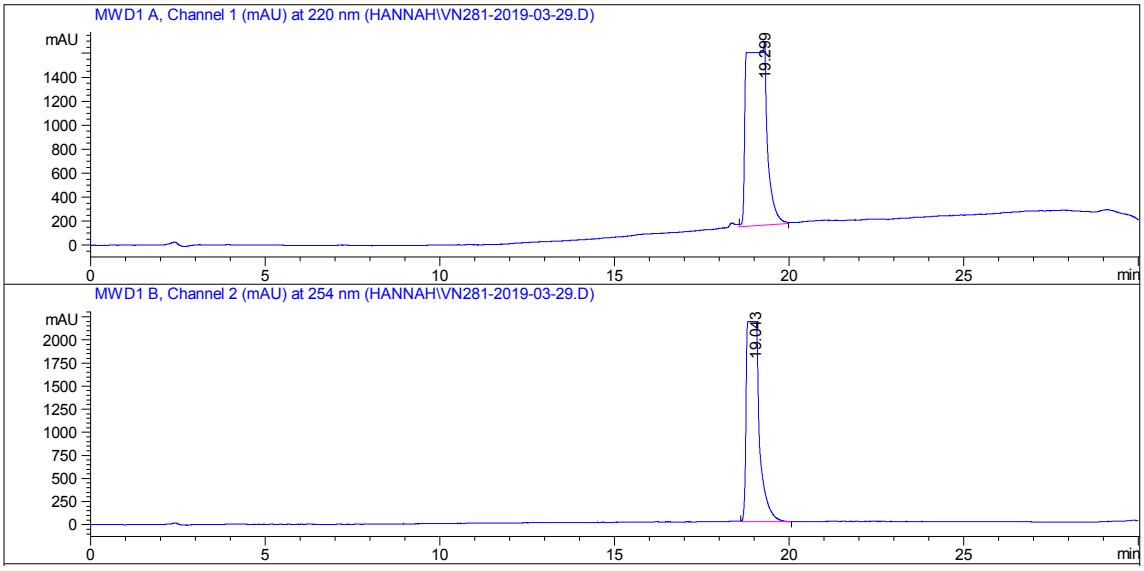


Compound 15:

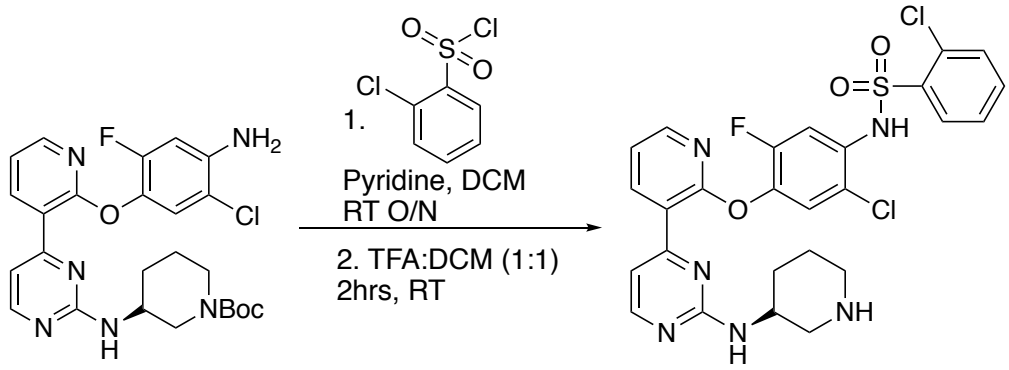


15

N-(4-(3-(2-((1s,4s)-4-aminocyclohexylamino)pyrimidin-4-yl)pyridin-2-yloxy)-2-chloro-5-fluorophenyl)-2-chlorobenzenesulfonamide: tert-butyl (1s,4s)-4-(4-(2-(4-amino-5-chloro-2-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexyl carbamate (0.02 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.06 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzenesulfonyl chloride (0.03 mmol, 1.5 eq., CAS #: 2905-23-9). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (500 MHz, MeOD) δ 8.47 (s, 1H), 8.35 (d, J = 5.2 Hz, 1H), 8.18 (s, 1H), 8.04 (d, J = 7.9 Hz, 1H), 7.63 (d, J = 13.9 Hz, 2H), 7.48 (t, J = 7.1 Hz, 1H), 7.44 (d, J = 11.4 Hz, 1H), 7.40 – 7.27 (m, 3H), 3.92 (s, 1H), 3.18 (d, J = 11.4 Hz, 1H), 2.25 (d, J = 12.7 Hz, 2H), 2.13 (d, J = 11.8 Hz, 2H), 1.66 – 1.42 (m, 4H). Exact Mass: 603.5, [M+H]+ detected: 604.8 m/z. Retention Time: 19.2 minutes, Purity: >99%.

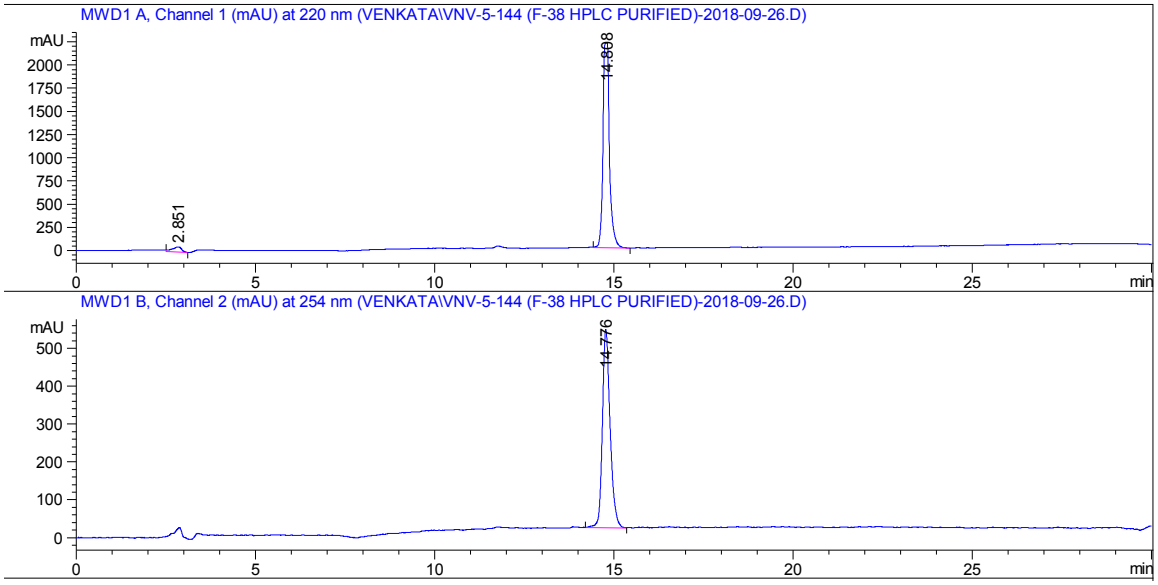


Compound 16:

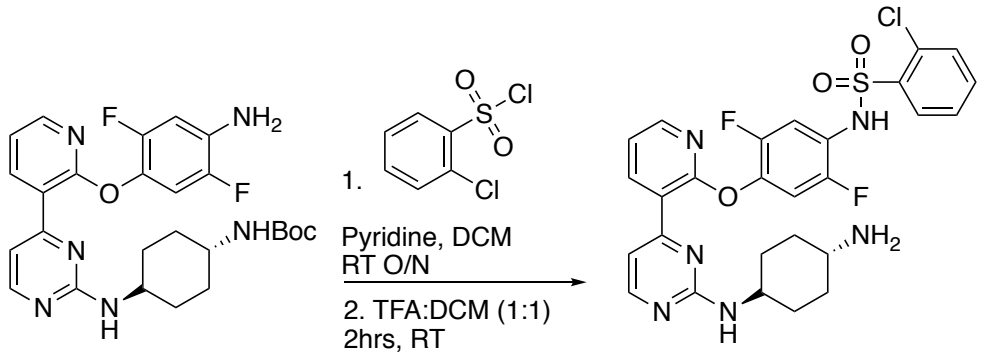


16

2-chloro-N-(2-chloro-5-fluoro-4-(3-(2-((R)-piperidin-3-ylamino)pyrimidin-4-yl)pyridin-2-yloxy)phenyl)benzenesulfonamide: (3R)-tert-butyl 3-(4-(2-(4-amino-5-chloro-2-fluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)piperidine-1-carboxylate (0.04 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.12 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chloro benzenesulfonyl chloride (0.06 mmol, 1.5 eq., CAS #: 2905-23-9). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (300 MHz, MeOD) ^{1}H NMR (500 MHz, MeOD) δ 8.53 (t, J = 8.0 Hz, 1H), 8.41 (d, J = 3.9 Hz, 1H), 8.17 (d, J = 3.2 Hz, 1H), 8.04 (d, J = 7.8 Hz, 1H), 7.65 (s, 2H), 7.54 – 7.34 (m, 4H), 7.30 (t, J = 5.4 Hz, 1H), 4.32 (s, 1H), 3.61 (d, J = 12.7 Hz, 1H), 3.04 (q, J = 10.0 Hz, 2H), 2.16 (dd, J = 34.8, 13.6 Hz, 3H), 1.90 (d, J = 13.5 Hz, 1H), 1.80 (d, J = 20.5 Hz, 1H). Exact Mass: 589.5, $[M+H]^+$ detected: 590.6 m/z. Retention Time: 14.8 minutes, Purity: >99%



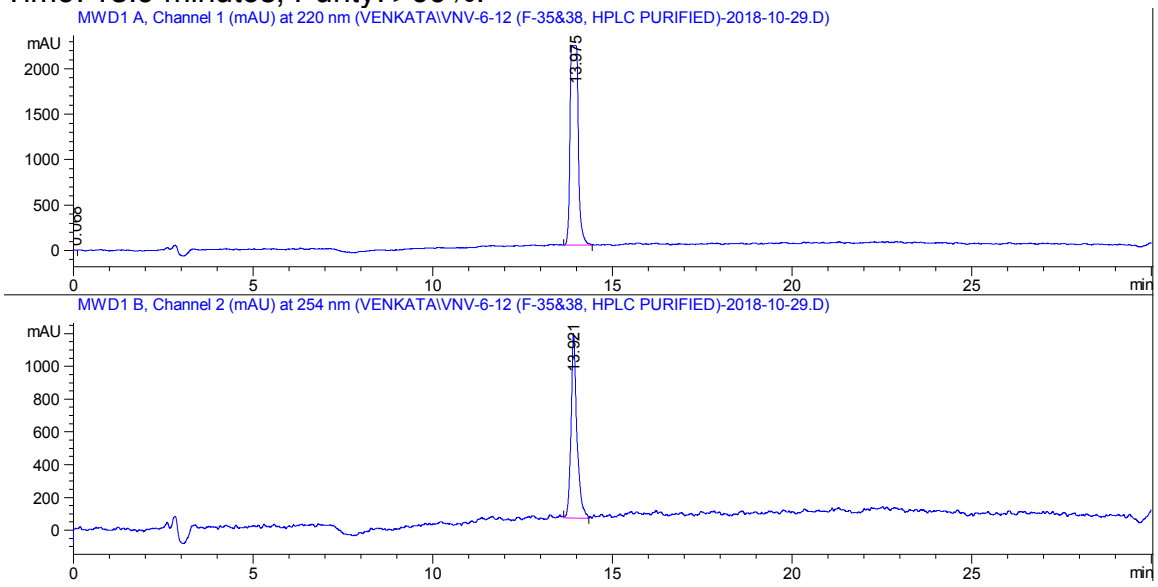
Compound 17:



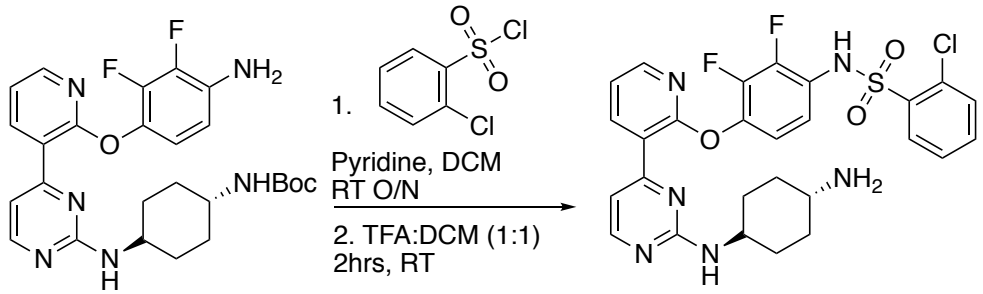
17

N-(4-(3-(2-((1s,4s)-4-aminocyclohexylamino)pyrimidin-4-yl)pyridin-2-yloxy)-2,5-difluorophenyl)-2-chlorobenzenesulfonamide: tert-butyl (1s,4s)-4-(4-(2-(4-amino-2,5-difluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (0.03 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.09 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzenesulfonyl chloride (0.45 mmol, 1.5 eq., CAS #: 2905-23-9). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (300 MHz, MeOD) δ 8.34 (dd, J = 7.6, 1.8 Hz, 1H), 8.21 (d, J = 5.5 Hz, 1H), 8.04 (dd, J = 4.8, 1.8 Hz, 1H), 7.94 (d, J = 7.4 Hz, 1H), 7.60 – 7.46 (m, 2H), 7.36 (ddd, J = 8.5, 6.2, 2.6 Hz, 1H), 7.30 – 7.12 (m, 3H), 7.02 (dd, J = 10.3, 7.0 Hz, 1H), 3.80 (s, 1H), 3.04 (s, 1H), 2.07 (dd, J = 34.2, 11.7 Hz, 4H),

1.56 – 1.30 (m, 4H). Exact Mass: 587.0, $[M+H]^+$ detected: 588.8 m/z. Retention Time: 13.9 minutes, Purity: >99%.



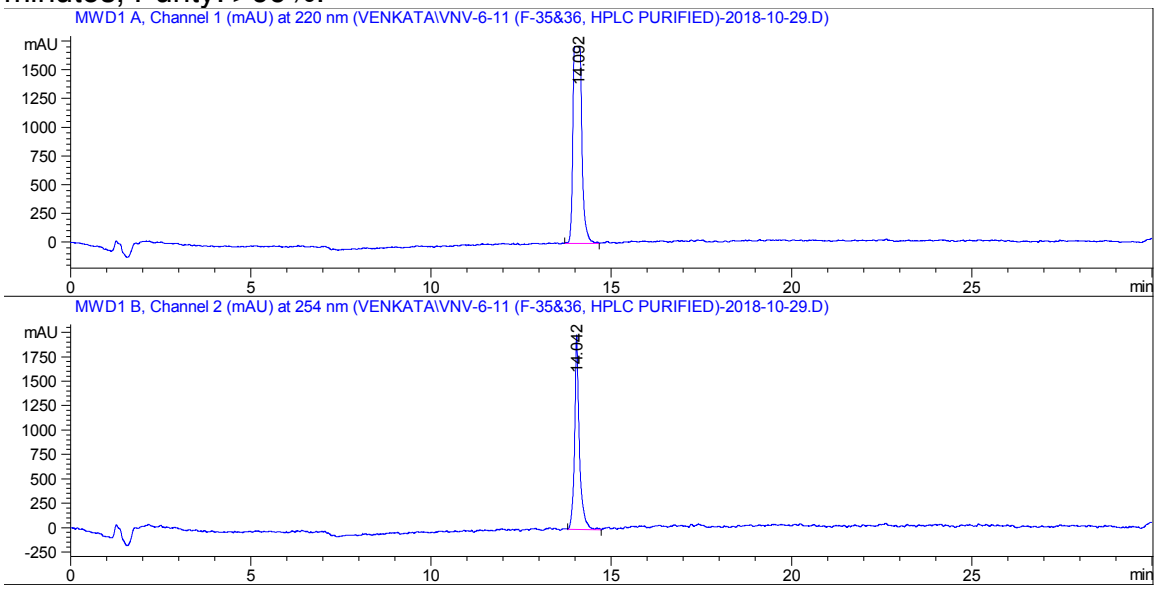
Compound 18:



18

N-(4-(3-(2-((1s,4s)-4-aminocyclohexylamino)pyrimidin-4-yl)pyridin-2-yloxy)-2,3-difluorophenyl)-2-chlorobenzenesulfonamide: tert-butyl (1s,4s)-4-(4-(2-(4-amino-2,3-difluorophenoxy)pyridin-3-yl)pyrimidin-2-ylamino)cyclohexylcarbamate (0.04 mmol, 1 eq.) was dissolved in 1 mL THF and stirred for 1 minute before addition of pyridine (0.12 mmol, 3 eq.). The reaction mixture was stirred another 5 minutes before the dropwise addition of 2-chlorobenzenesulfonyl chloride (0.06 mmol, 1.5 eq., CAS #: 2905-23-9). The reaction was then stirred at room temperature overnight. The reaction mixture was then concentrated in vacuo and purified via flash chromatography (0-50% EtOAc in hexanes). The product was then diluted into 1 mL TFA:DCM (1:1) and stirred at room temperature for 2 hours. The solvent was removed in vacuo and crude reaction mixture was re-suspended in MeOH for further purification using HPLC in a methanol and water solvent system. ^{1}H NMR (300 MHz, MeOD) δ 8.40 (dd, J = 7.6, 1.8 Hz, 1H), 8.27 (d, J = 5.6 Hz, 1H), 8.10 (dd, J = 4.8, 1.8 Hz, 1H), 7.96 (d, J = 7.5 Hz, 1H), 7.63 – 7.49 (m, 2H), 7.46 – 7.36 (m, 1H), 7.35 – 7.11 (m, 3H), 6.99 – 6.89 (m, 1H), 3.86 (s, 1H), 3.08 (d, J = 11.1 Hz, 1H), 2.12 (dd, J = 34.0, 11.6 Hz, 4H), 1.68 – 1.28

(m, 4H). Exact Mass: 587.0, $[M+H]^+$ detected: 588.2 m/z. Retention Time: 14.0 minutes, Purity: >99%.



IRE1α* and IRE1β* Constructs

A construct containing the cytosolic kinase and RNase domains of human IRE1α (residues 547–977, IRE1α*) was expressed in SF9 insect cells by using the Bac-to-Bac baculovirus expression system (Invitrogen) with a His6 tag at the N-terminus and purified with a nickel–nitriloacetic acid (Qiagen) column. IRE1β constructs containing the cytosolic kinase and RNase domains (499-end, IRE1β*) and an N-terminal GST tag were purchased from SignalChem (Cat. No.: E32-11G).

In Vitro Enzymatic Assays

In Vitro Real Time Fluorescence Assay

75 nM IRE1α* and 75 nM IRE1β* (**Figure 1B)** or 1 nM and 5 nM IRE1β* (**Figure 2)** was incubated with inhibitor (50 µM) or DMSO for 30 minutes in assay buffer (20 mM Tris at pH 7.5, 50 mM sodium chloride, 1 mM magnesium chloride, 2 mM DTT, 0.05% Triton X-100 (v/v)). Assays were initiated by adding 10 µL of XBP1-mini substrate (5'-Alex647-CAUGUCCGCAGCGCAUG-IowaBlack-FQ-3'; IDT) to the wells at a final concentration of 250 nM and a final well volume of 30 µL. Fluorescence was detected on a Perkin Elmer Envision Microplate Reader at excitation and emission wavelengths of 650 nm and 665 nm. Reaction process was monitored real time in 10-second intervals for at least 30 minutes. Curves were plotted as fluorescence units as a function of time. Initial rates were determined by taking the slope of linear region from the real time fluorescence curves.

Urea-PAGE Gel of XBP1 Mini-Substrate Cleavage Products

75 nM IRE1α*, 75 nM IRE1β*, or 100 µg/mL RNase A (ThermoFisher, Cat. #: EN0531) was prepared in reaction buffer (20 mM Tris pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 2 mM DTT and 0.05% Triton X-100 (v/v)). Assays were initiated by addition of XBP1-mini substrate (5'-Alex647-CAUGUCCGCAGCGCAUG-IowaBlack-FQ-3'; IDT) to a final concentration of 250 nM and allowed to proceed for 1 hour at room temperature. Reactions were then quenched by the addition of loading buffer (Novex TBE-Urea 2x Sample Buffer, ThermoFisher). Samples were then heated to 70ºC for 1 minute, loaded into a 20% polyacrylamide/8 M urea gel, and ran at 180 V for 60 minutes. Gels were then imaged using AlexaFluor-647 fluorescence on a Typhoon FLA9000 imager.

Determination of Michaelis Constants for IRE1α* and IRE1β*

75 nM IRE1α* and 75 nM IRE1β* was prepared in assay buffer (20 mM Tris at pH 7.5, 50 mM sodium chloride, 1 mM magnesium chloride, 2 mM DTT, 0.05% Triton X-100 (v/v)). Assays were initiated by adding 10 µL of XBP1-mini substrate (5'-Alex647-CAUGUCCGCAGCGCAUG-IowaBlack-FQ-3'; IDT) to the wells at various concentrations (initial concentration of 4 µM, two-fold serial dilultions) and a final well volume of 30 µL. Fluorescence was detected on a Perkin Elmer Envision Microplate Reader at excitation and emission wavelengths of 650 nm and 665 nm. Reaction process was monitored real time in 10-second intervals for at least 30 minutes. Curves were plotted as fluorescence units as a function of time. Initial rates were determined by taking the slope of linear region from the real time fluorescence curves. Initial rates were then plotted as a function of XBP1 mini-substrate concentration and curves were fit using "Enzyme kinetics-Michaelis-Menten" parameter in GraphPad Prism analysis software.

In Vitro IRE1α* Kinase Inhibitor Titrations

Inhibitors (initial concentration of 10 or 60 µM, three-fold serial dilutions) were incubated with IRE1α* in cleavage buffer (20 mM HEPES at pH 7.5, 0.05% Triton X-100 (v/v), 50 mM potassium chloride, 1 mM magnesium chloride, 1 mM DTT, 0.2 mg/mL MBP) for 30 min, followed by incubation with 10 µCi [γ^{32}P] ATP(3,000 Ci $mmol^{-1}$, PerkinElmer) at 23 °C for 3 hours. Samples were then spotted onto glass fiber paper (Easytab-C Glass Fiber Filter Paper, Perkin Elmer) and washed twice with 0.5% phosphoric acid and autoradiographed using a GE Typhoon FLA 9000 Imager. Blots were quantitated using ImageQuant software. The percent inhibition was quantified by setting the background (no kinase well) as 0 and standardizing to IRE1α* without compound treatment (IRE1α* + DMSO). Dose-response curves were fit using "one-site fit logIC50" parameter using GraphPad Prism analysis software.

In Vitro IRE1β* Kinase Inhibitor Titrations[2]

IRE1β* kinase inhibitor titrations were performed by Reaction Biology. In brief, IRE1β* was incubated with inhibitor (initial concentration of 10 µM, three-fold serial dilutions) or DMSO for 20 min in Base Reaction buffer (20 mM HEPES (pH 7.5),

10 mM $MgCl_2$, 1 mM EGTA, 0.02% Brij35, 0.02 mg/ml BSA, 0.1 mM Na_3VO_4, 2 mM DTT, 1% DMSO) supplemented with 20 µM myelin basic protein (MBP). Next, 4 nM [γ^{33}P] ATP and 1 µM ATP were added and reactions were carried out at 25°C for 120 min, followed by spotting of the reactions onto P81 ion exchange filter paper (Whatman). Unbound phosphate was removed by extensive washing of filters in 0.75% phosphoric acid. Kinase activity data were expressed as the percent remaining kinase activity in test samples compared to vehicle (DMSO) reactions. IC_{50} values and curve fits were obtained using Prism (GraphPad Software).

In Vitro IRE1 RNase Inhibition Titrations

IRE1α* (25 nM) or IRE1β* (5 nM) was incubated with inhibitor (initial concentration of 10 or 60 µM, three-fold serial dilutions) or DMSO for 30 min in assay buffer (20 mM Tris at pH 7.5, 50 mM sodium chloride, 1 mM magnesium chloride, 2 mM DTT, 0.05% Triton X-100 (v/v)). Assays were initiated by adding 10 µL of XBP1-mini substrate (5'-Alex647-CAUGUCCGCAGCGCAUG-IowaBlack-FQ-3'; IDT) to the wells at a final concentration of 250 nM and a final well volume of 30 µL. Fluorescence was detected on a Perkin Elmer Envision Microplate Reader at excitation and emission wavelengths of 650 nm and 665 nm. Reaction process was monitored real time in 10-second intervals for at least 30 minutes. Dose-response curves were fit using "one-site fit logIC50" parameter using GraphPad Prism analysis software.

In Vitro CDK2/Cyclin A Inhibitor Titrations[2]

CDK2/Cyclin A kinase inhibitor titrations were performed by Reaction Biology. In brief, CDK2 and Cyclin A were incubated with inhibitor (initial concentration of 10 µM, three-fold serial dilutions) or DMSO for 20 min in Base Reaction buffer (20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 0.02% Brij35, 0.02 mg/ml BSA, 0.1 mM Na_3VO_4, 2 mM DTT, 1% DMSO) supplemented with 20 µM Histone H1. Next, 4 nM [γ^{33}P] ATP and 1 µM ATP were added and reactions were carried out at 25°C for 120 min, followed by spotting of the reactions onto P81 ion exchange filter paper (Whatman). Unbound phosphate was removed by extensive washing of filters in 0.75% phosphoric acid. Kinase activity data were expressed as the percent remaining kinase activity in test samples compared to vehicle (DMSO) reactions. IC_{50} values and curve fits were obtained using Prism (GraphPad Software).

Kinome Profiling and Selectivity

Kinobead Enrichment Protocol[3]

HEK293 and HCT116 cells were plated on 15 cm plates until 90% confluent and lysed into 750 µL modified RIPA buffer (50 mM Tris, 150 mM NaCl, 10 mM NaF, 1% NP40, 0.25% sodium deoxycholate, 5% glycerol, pH=7.8) containing protease inhibitor (Pierce Protease Inhibitor Tablets and 1 mM PMSF) and phosphatase inhibitor (Phosphatase inhibitor cocktail 2 and 3, Sigma Aldrich). Protein content was determined via Bradford assay. HEK293 and HCT116 lysates were combined in a 1:1 ratio and recombinant IRE1α* or IRE1β* was added to the lysate at a final

concentration of 33.3 nM. Lysate (300 µg per sample, 150 µL of 2 mg/mL lysate) was incubated and rotated end over end with 10 µM inhibitor or DMSO (1% v/v) for 1 hour at 4ºC. For kinase enrichment, 10 µL of 50% kinobead slurries (in 20% ethanol) were prepared by washing twice with 500 µL Mod. RIPA buffer. DMSO or inhibitor treated lysates were then added to the washed kinobeads and rotated end over end for 3 hours at 4ºC. After enrichment, the supernatant was aspirated off and the beads were washed twice with 500 µL ice cold Mod. RIPA buffer and three times with 500 µL ice cold TBS (50 mM Tris, 150 mM NaCl, pH = 7.8) to remove detergent. Beads were resuspended in 25 µL of denaturing buffer (6 M guanidinium chloride, 50 mM Tris containing 5 mM TCEP and 10 mM CAM, pH = 8.5). The bead slurries in denaturing buffer were then heated to 95°C for 5 min. After, the bead slurries were diluted 2-fold (add 25 µL) with 100 mM TEAB (triethylammonium bicarbonate buffer, pH 8.5). Then 0.4 µg of LysC (Wako) were added to the beads and. the pH adjusted to 8-9 with 1 N NaOH. The mixture was agitated on a Thermomixer (Eppendorf) at 37°C for 2 hours at 1400 rmp. After the samples were diluted 2-fold (50 µL) with 100 mM TEAB and 0.4 µg of sequencing grade trypsin (Pierce) was added and the samples agitated for another 12 hours at 37°C at ~800 rpm in the Thermomixer. After the overnight trypsinization, samples were diluted 2-fold (100 µL) with Buffer A (5% ACN, 0.1% TFA, H_2O) containing 1% formic acid and the pH adjusted to 2-3 with formic acid if necessary. StageTips[4] were prepared by running 50 µL of Buffer B (80% ACN, 0.1% TFA, H_2O) through them followed by 50 µL of Buffer A (5% ACN, 0.1% TFA, H2O). Beads were spun down and supernatant was added directly to StageTips. Following sample loading, StageTips were washed with 50 µL of Buffer A and eluted with 50 µL Buffer B. Samples were speed vacuumed until dry and re-suspend in Buffer A for injection onto LC-MS.

LC-MS and Data Analysis

Tryptic peptides were separated using a nanoAcquity UPLC instrument with 10 cm fused silica capillary columns made in house and packed with 5 µm 120 Å reverse-phase C18 beads (ReproSil-Pur® (Maisch)). Liquid chromatography was performed over 120 minutes using and initial 20 minute isocratic trapping of 3% Buffer B and a flow rate of 700 nL/minute followed by a 100 minute gradient of 35% Buffer B to 80% Buffer B gradient at 350 nL/minute. LC Buffer A solvent is 0.1% Acetic acid and LC Buffer B is 99.9% ACN, 0.1% Acetic Acid. MS data was analyzed using a Thermo Orbitrap Fusion Tribrid with the following settings:

Thermo Orbitrap Fusion Tribrid Settings	
Ion Source Type	NSI
Spray Voltage	Static
Positive Ion (V)	2100
Negative Ion (V)	600
Sweep Gas (Arb)	0
Ion Transfer Tube Temperature (ºC)	350

Detector Type	Orbitrap
Orbitrap Resolution	120000
Mass Range	Normal
Quadrapole Isolation	ON
Scan Range (m/z)	380-1500
RF Lens (%)	65
AGC Target	40000
Maximum Injection Time (ms)	50
Microscans	1
Data Type	Centroid
Polarity	Positive

Raw files were analyzed using MaxQuant (Andromeda) Version 1.6.0.16 using the following search parameters:

MaxQuant Search Parameters	
Global Parameters: Identification	
Peptide Spectrum Match FDR	0.01
Protein FDR	0.01
Site decoy fraction	0.01
Min. score for modified peptides	40
Min. delta score for un-modified peptides	0
Min. delta score for modified peptides	6
Main search maximum combinations	200
Global Parameters: Sequence	
Database search	UniProt Human Database
Fixed modification	Carbamidomethyl (Cys)
Min. peptide length	7
Max. peptide mass [Da]	4600
Min. peptide length for unspecific search	8
Max. peptide length for unspecific search	25
Global Parameters: Protein Quantification	
Variable modifications	Oxidation (Met), Acetyl (N-terminus)
Label min. ratio count	2
Global Parameters: MS/MS-FTMS	
Match tolerance	20 ppm
Global Parameters: MS/MS-ITMS	
Match tolerance	0.5 Da
Group-specific parameters: Instrument	
First search peptide tolerance	20

Main search peptide tolerance	4.5
Centroid match tolerance	8
Centroid half width	35
Time valley factor	1.4
Group-specific parameters: Label-free quantification	
Label-free quantification	LFQ ON

Files were analyzed further using Perseus (Version 1.6.0.7) by filtering LFQ intensity values only identified by site, reverse, or potential contaminant and log(2x) transformed. To determine kinases that were significantly competed by treatment with 10 µM of inhibitor we applied a two-tailed two-sample t-test in Perseus with an FDR of 0.05. Kinases were reported as being competed by an inhibitor if it had a Log_2 LFQ ratio (Log_2 Difference) >2.0. Kinome tree visualization plots were created using CORAL.[5]

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