Discovery of the Highly Potent PI3K/mTOR Dual Inhibitor PF-04691502 through Structure Based Drug Design

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Supporting Information

PI3-Kα Biochemical Assay

Compounds were evaluated for potency against PI3-Kα using an *in vitro* kinase assay. PI3-Kα activity is measured *in vitro* by determining the level of phosphorylation of the substrate $PI(4,5)P_2$. The formation of product $PI(3,4,5)P_3$ is monitored by binding to the Grip1 PH domain in a ligand displacement fluorescence polarization (FP) assay, in which the TAMRA-labeled $PI(3,4,5)P_3$ complexed with Grip1 PH domain is displaced by $PI(3,4,5)P_3$ formed in the PI3-Kα reaction resulting in a decrease in FP signal. Mouse PI3-Kα P110 and P85 subunits were co-expressed in insect cells and co-purified to homogeneity. $PI(4,5)P_2$ were obtained from Cayman. TAMRA-labeled $PI(3,4,5)P_3$ were from Echelon, Grip1 PH domain from Dundee and other reagents were from Sigma.

All assays were performed in a Corning solid black 96-well half area plate using LJL Analyst (Molecular Devices) at room temperature. The assay buffer contained 50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM DTT, and 0.05% CHAPS. Dry powder $PI(4,5)P_2$ was dissolved in 50 mM TRIS, pH 8 to make 1 mM stock solution. The $PI(4,5)P_2$ stock solution was then diluted in the assay buffer to 60 μM, and sonicated for 30 sec before use. To the assay plate, the following reagents were added in sequence: 10 μL of 60 μM $PI(4,5)P_2$, 5 μL of 4 nM PI3-Kα, 2 μL of compound in 25% DMSO, 3 μL of mixture containing 200 μM ATP and 33 mM $MgCl_2$. The final volume for the reaction was 20 μL. The reaction mixture was incubated at room temperature for 35 min. The reaction was then stopped by 20 μL of 20mM EDTA. After the reaction was stopped, 15 μL of the assay mixture was transferred to a 96-well half area plate containing 15 μL detection mixture of 480 nM Grip1 PH domain and 12 nM TAMRA-labeled $PI(3,4,5)P_3$. The FP signal was allowed to develop for 40 min before reading on a LJL analyst at excitation 535 nm and emission 580 nm.

The percentage of inhibition was calculated based on the following equation
$\%\text{inhibition} = [1 - (FP_{compound} - FP_{max})/(FP_{min} - FP_{max})] \times 100$,
where $FP_{compound}$ is the FP reading at a given compound concentration, FP_{min} is the FP signal of the PI3-Kα reaction in the absence of a compound, and FP_{max} is the background

FP signal in the absence of PI3-Kα and a compound. The IC_{50} was determined by fitting the FP signal vs. compound concentration to a sigmoidal dose response equation using GraphPad Prism curve fitting program. The K_i was calculated from IC_{50} based on the equation $K_i = IC_{50} /(1+[ATP]/K_m)$, where [ATP] = 30 uM and K_m = 25 uM

Table 1: mPI3Kα Ki

Compound Number	**1**	**2**	**3**	**4**	**5**	**15**	**16**	**17**	**18**
mPI3Kα Ki (nM)	0.57 ± 0.062	1.4 ± 0.50	1.4 ± 0.3	1.2 ± 0.12	1.1 ± 0.028	0.9 ± 0.017	3.0 ± 0.092	3.6 ± 0.23	6.6 ± 0.43

Data is reported in arithmetic mean ± standard deviation.

mTOR Biochemical Assay

Compounds were evaluated for potency against mTOR using an *in vitro* kinase assay. mTOR activity is measured *in vitro* by determining the level of phosphorylation of the protein substrate 4EBP-1. The phosphorylation of GFP-4E-BP1 at tyrosine residue is recognized by Ab Tb-anti-p4E-BP1, which results in time-resolved fluorescence resonance energy transfer (TR-FRET) between GFP and terbium in the Ab – product complex. Recombinant mTOR kinase domain, GFP-4E-BP1, and Lantha Ab Tb-anti-p4E-BP1, and TR-FRET dilution buffer were from Invitrogen. All other reagents were from Sigma.

All assays were performed in a Corning white 384-well non-binding surface plate using Analyst plate reader (Molecular Devices) at room temperature. The assay buffer contained 50 mM HEPES pH 7.5, 0.01% Tween 20, 1 mM EGTA and 10 mM MnCl2. To the assay plate, substrate GFP-4E-BP-1 and mTOR in the assay buffer were mixed first and the reaction was initiated by addition of ATP. The final concentrations in the reactions were 1 nM mTOR, 400 nM GFP-4E-BP1 and 4 uM ATP. The total volume of the reaction was 10 uL. The reaction mixture was incubated for 20 min followed by addition of 10 uL of 20 mM EDTA, and 4 mM Ab Tb-anti-p4E-BP1 to stop the reaction and detect the product. The FRET signal was allowed to develop for 15 minutes before reading on Analyst plate reader using multi-method with the following filters: Excitation: 330 nm and Emission 490 nm (donor) and 520 nm (acceptor).

The percentage of inhibition was calculated based on the following equation
$\%inhibition = [1 – (FRET_{compound} – FRET_{min})/(FRET_{max} – FRET_{min})] \times 100$,
where $FRET_{compound}$ is the FRET reading at a given compound concentration, $FRET_{max}$ is the FRET signal of the mTOR reaction in the absence of a compound, and $FRET_{min}$ is the background FRET signal in the absence of mTOR and a compound. The IC_{50} was

determined by fitting the FP signal vs. compound concentration to a sigmoidal dose response equation using GraphPad Prism curve fitting program. The K_i was calculated from IC_{50} based on the equation $K_i = IC_{50} /(1+[ATP]/K_m)$, where [ATP] = 4 uM and K_m = 2.6 uM.

Table 3: mTOR Ki

Compound Number	**1**	**2**	**3**	**4**	**5**	**15**	**16**	**17**	**18**
mTOR Ki (nM)	16 ± 4.9	840 ± 86	200 ± 30	82 ± 24	700 ± 139	17 ± 0.56	90 ± 9.0	110 ± 32	150 ± 26

Data is reported in arithmetic mean ± standard deviation.

pAKT ELISA assays

Cells (BT20, SKOV3) were seeded at 25,000 cells/well in volume of 100 ul in 0.5%FBS McCoy's 5A or DMEM depending on the cell line being in a 96-well flat-bottom plate (Costar, cat#3595) and incubated overnight at 37°C, 5% CO_2. Cells were then treated with test compounds (prepared in 0.5%FBS in growth media, and serially diluted for 11 test concentrations in 0.5% DMSO in 0.5% FBS in growth media and incubated for 1 h at 37°C, 5% CO_2. Cell conditioned medium was removed and cells were lysed with 100 ul/well of 1X lysis buffer (Assay Designs Cell Lysate Buffer 22 Concentrate 5X, cat #80-1114 diluted to1X and supplemented with 1 mM PMSF and 2 mM Na_3VO_4) and shaken at room temperature for 15 min. Then, 100 µl of cell lysates were transferred to the ELISA plate (pre-coated with anti-pS473-AKT rabbit monoclonal antibody, Cell Signaling, cat#40**58** or anti-pT308-AKT mouse monoclonal antibody, Cell Signaling, cat#5103) and incubated with gentle shaking at room temperature for 2 h. ELISA plates were washed four times (Wash Buffer Cell Signaling, cat #7175 diluted to 1X)) and incubated with 100 ul of anti-AKT1 mouse monoclonal detection antibody (green, Cell Signaling, cat#2967) per well on a plate shaker for 1 h at room temperature. Subsequently, plates were emptied and washed four times and incubated with 100 ul of anti-mouse IgG HRP-Linked antibody (red, Cell signaling cat#7076) at room temperature on a plate shaker for 1 h. Finally, plate was emptied, washed four times, and incubated with 100 ul of TMB substrate (Sigma, cat#T0440) at room temperature on a plate shaker for 15 to 20 min. Reaction was stopped with 100 ul of stop solution (1N solution of hydrochloric acid in water) and plate was read at 450 nm on Envision 2100 plate reader. PathScan Phospo-AKT (Ser473) Sandwich ELISA Kit (Cell Signaling, cat#7160), PathScan Phospo-AKT (Thr308) Sandwich ELISA Kit (Cell Signaling, cat#7252) and AKT antibody coated microwell, cat#9272 kits were also used as per manufacturer's instructions.

Table 4: BT20 S473 pAKT IC_{50}

Compound Number	**1**	**2**	**3**	**15**	**16**
pAKT IC_{50} (nM)	13 ± 6.3	15 ± 5.0	16 ± 3.6	12 ± 5.8	22 ± 11

Data is reported in arithmetic mean ± standard deviation

Table 5: Cellular Data Summary for PF-04691502

In vitro cellular assay	**IC50 (nM)**
pAKT S473 BT20	13 ± 6.3
pAKT S473 SKOV3	7.4 ± 0.58

Data is reported in arithmetic mean ± standard deviation.

Table 6: Summary of human liver microsomal (HLM) clearance extraction ratio (ER) and kinetic solubility measured at Analiza

Compound Number	**1**	**2**	**3**	**15**	**16**	**17**	**18**
HLM clearance ER	<0.26	0.68	<0.25	<0.25	<0.26	<0.26	<0.26
Analiza Kinetic Solubility (μM)	14 ± 1.9	65 ± 4.0	3.9 ± 0.51	16 ± 1.4	60 ± 19	40 ± 1.3	144 ± 7.1

Data is reported in arithmetic mean ± standard deviation.

Preclinical PK profile of 1

The pharmacokinetics of PF-04691502 (free base) were studied following single intravenous and oral administration to male rats. The IV solution formulation for rats contained 10% (v/v) ethanol, 40% (v/v) PEG-400 in 50 mM citrate buffer. The PO formulation was prepared as suspension in 0.5% methylcellulose. Oral bioavailability was estimated with a non-crossover study design. Plasma samples were assayed for PF-04691502 using protein precipitation with acetonitrile:methanol (75:25, v/v) solution followed by LC-MS/MS analysis employing positive-ion Turbo Ion Spray ionization. The pharmacokinetic parameters showed in Table were determined using non-compartmental analysis with Watson (version 7.2).

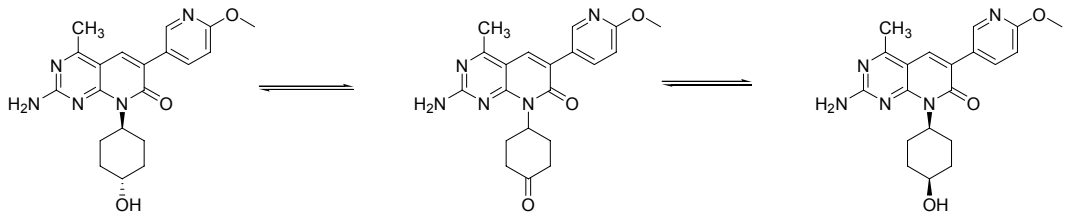
Table 7: PK Parameters for PF-04691502

	Dose (mg/kg)	CL (ml/min/kg)	CL_u (ml/min/kg)	V_{dss} (L/kg)	T1/2 (h)	F (%)
Rat[1]	2 (IV)/5(PO)	5.2 ± 0.33	25.9 ± 1.6	1.39 ± 0.078	3.1 ± 0.53	56 ± 16

Data represents mean ± SD (n=2 or 3)
[1]Rat, IV (n=2) and PO (n=3)

In vivo isomerization of 13 and 14:

The isomerization was determined by assessing the incubation and the plasma samples and comparing the retention times of both the synthetic standards to the peaks in the samples.



13 **ketone** **14**

Table 8: Distribution of 13 and 14 from rat and mouse PK study plasma samples

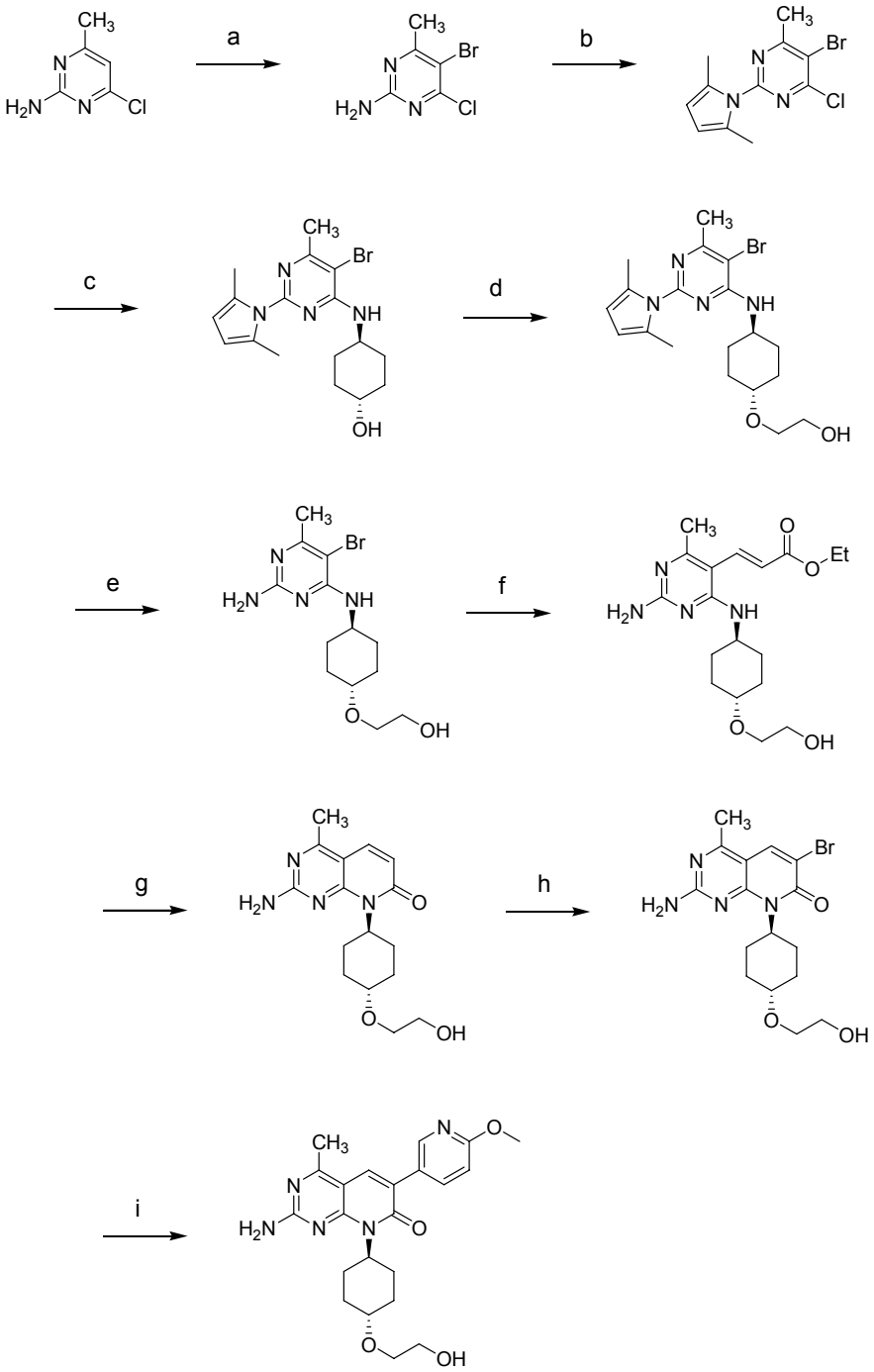
Compound Dosed	Species (compound administration route)	% of **13** in plasma	% ketone in plasma	% of **14** in plasma
13	Rat (iv)	98.4	1.2	0.4
13	Mouse (po)	98	0	2
14	Rat (iv)	7.2	3.8	89
14	Mouse (po)	64.7	7.8	27.5

Table 10: Kinase Selectivity Screen with PF-04691502

Kinase	1uM	10uM
Dundee KSS panel		
rat AMPKa2	8.735	18.675
MNK2	1.52	-1.785
AKT	-10.6	-16.23
PKCa	1.86	18.125
MLCK	-3.685	-2.325
p70S6K	6.355	2.89
p38g	18.46	30.165
CSK	9.19	24.13
rat ROCKII	7.02	21.675
DYRK3	-2.705	7.58
rat RSK1	10.605	2.84
PRK2	-24.485	15.8
JNK2	-1.915	12.11
PBK	4.35	-3.67
NEK6	14.705	9.37
CaMK1a	25.605	18.865
RSK2	7.655	5.97
MST2	-7.245	-3.825
p38	22.44	21.645
MAPKAPK5	14.5	7.855
KINS0239	3.73	26.965
rabbit MEK1	37.545	26.07
SGK	18.385	23.815
mouse ERK2	6.25	-1.89
PIM1	2.22	-1.35
AKT2	-4.37	6.91
chicken CSK	2.655	19.37
NEK2	3.375	45.065
CDK2	-2.805	3.64
ERK7	9.295	13.26
JNK1a	6.9	4.32
DYRK2	9.895	18.89
PIM3	30.735	30.88
PLK1, preactivated	0.905	3.435
CAMKKB	-0.695	2.135
IKKb	6.7	5.1
rat MNB	0.825	7.81
MARK3	5.605	6.89
CHK1	-9.52	4.82
PKD1	20.52	30.965
SRPK1	1.19	7.525
mouse LCK	16.74	20.27

Kinase	1 uM	10 uM
MNK1	1.435	5.145
MSK1	-0.415	-5.32
p38d	3.445	8.135
PLK1	6.235	7.88
Invitrogen KSS panel		
ZC1	-10.5	
PRKCB2	-8	
EGFR	11	
PAK4	-8.5	
GSK3b	0.5	
AKT	-1	
LCK	6.5	
TRKA	13	
CHK2	2.5	
MARK1	-4	
TAO2	-6.5	
HGFR	12	
PIM2	-1.5	
p38	-1	
MST2	9.5	
ROCKI	2	
PDK1	-2	
SGK	-8.5	
ERK2	-7	
NEK2	0	
MST4	0	
KDRVEGF	2.5	
AURA	-2	
JAK3	0	
CKId	14	
IRK	3.5	
IKKb	-3	
PKACa	-1.5	
CHK1	4	
EphA2	-0.5	
CKIIa	4.5	
CDK2, /CyclinA	3.5	
MLCK_sk	-8	
MK2	-3.5	
FGFR1	-1.5	

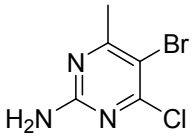
Example for Scheme 1: synthesis of 2-amino-8-(*trans*-4-(2-hydroxyethoxy)cyclohexyl)-6-(6-methoxypyridin-3-yl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one (1, PF-04691502)



Conditions: a) Br_2, CH_2Cl_2, rt; b) 2,5-hexanedione, p-TsOH, toluene, reflux; c) trans-aminocyclohexanol hydrochloride, diisopropylethylamine, DMF; d) 1,3,2-dioxathiolane 2,2-dioxane, NaH, DMA; 1,4-dioxane, H_2O, pTsOH; e) hydroxylamine hydrochloride,

ethanol, H_2O; f) ethyl acrylate, $Pd(PPh_3)_4$, Et_3N; g) thiophenol, benzenethiol sodium salt, 1,8-diazabicyclo(5,4,0)undec-7-ene, diisopropylethyl amine, DMF; h) NBS, DMF; i) 2-methoxy-5-pyridine boronic acid, potassium carbonate, bis(tripehnylphosphine) palladium (II) chloride, 5:1 dimethylformade: water.

5-bromo-4-chloro-6-methylpyrimidin-2-amine

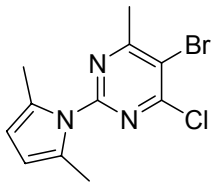


To a mixture of the 2-amino-4-chloro-6-methyl pyrimidine (5.00 g, 34.8 mmol) in dichloromethane (240 mL) was added bromine (1.88 mL, 36.6 mmol). The resulting suspension was stirred at room temperature for 1.5 hours. The mixture was diluted with dichloromethane (1.3 L) and washed with saturated sodium bicarbonate (2 x 200 mL) and brine (200 mL), dried ($MgSO_4$), filtered and concentrated to afford the title compound (7.68 g, 99%).

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.40 (s, 3 H) 7.19 (s, 2 H)

M+H 223, 224, 226

5-bromo-4-chloro-2-(2, 5-dimethyl-1H-pyrrol-1-yl)-6-methylpyrimidine

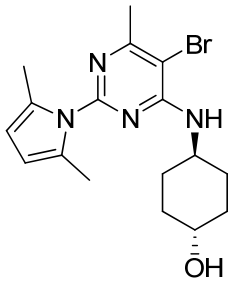


A flask containing a mixture of 5-bromo-4-chloro-6-methylpyrimidin-2-amine (7.68 g, 34.5 mmol), 2, 5-hexanedione (6.1 mL, 52 mmol), and p-toluenesulfonic acid (328 mg, 1.73 mmol) in toluene (150 mL) was fitted with a Dean-stark apparatus and condenser and the mixture was heated to reflux. After refluxing overnight the solution was cooled to room temperature and concentrated. The crude product was purified by flash chromatography eluting with hexanes/dichloromethane (0-20%) to afford the title compound (8.05 g, 77%).

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.30 (s, 6 H) 2.67 (s, 3 H) 5.86 (s, 2 H)

M+H 300, 302, 304

trans-4-(5-bromo-2-(2, 5-dimethyl-1H-pyrrol-1-yl)-6-methylpyrimidin-4-ylamino)cyclohexanol

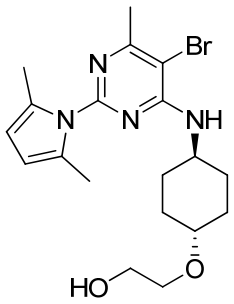


A mixture of 5-bromo-4-chloro-2-(2, 5-dimethyl-1H-pyrrol-1-yl)-6-methylpyrimidine (1.50 g, 5.00 mmol), *trans*-4-aminocyclohexanol hydrochloride (1.17 g, 6.24 mmol), and diisopropylethyl amine (2.61 mL, 15.0 mmol) in dimethylacetamide (25.0 mL) was heated at 130^{o}C in a sealed tube overnight. The reaction mixture was concentrated and the residue was dissolved in methyl *tert*-butyl ether (~400 mL) then washed with saturated ammonium chloride (2 x) and brine. The aqueous layers were combined and extracted with dichloromethane (3 x 150 mL). The combined organics were dried ($MgSO_4$) and concentrated. The crude product was purified by flash chromatography eluting with chloroform/methanol (0-3%) afford the title compound (1.76 g, 93.0%).

M+H 379, 381

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.15 - 1.26 (m, 2 H) 1.46 - 1.57 (m, 2 H) 1.74 - 1.80 (m, 2 H) 1.81 - 1.87 (m, 2 H) 2.26 (s, 6 H) 2.41 (s, 3 H) 3.35 - 3.45 (m, 1 H) 3.86 - 3.96 (m, 1 H) 4.57 (d, *J*=4.29 Hz, 1 H) 5.76 (s, 2 H) 6.82 (d, *J*=8.34 Hz, 1 H)

2-_trans_-4-(5-bromo-2-(2, 5-dimethyl-1H-pyrrol-1-yl)-6-methylpyrimidin-4-ylamino)cyclohexyloxy)ethanol



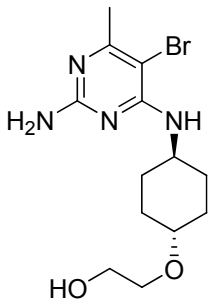
To a cooled (0^{o}C) solution of *trans*-4-(5-bromo-2-(2,5-dimethyl-1H-pyrrol-1-yl)-6-methylpyrimidin-4-ylamino)cyclohexanol (25.0 g, 65.9 mmol) in dimethylacetamide (330 mL) was added sodium hydride (60% dispersion in oil, 13.2 g, 330 mmol). After 2.5 hr, 1,3,2-dioxathiolane 2,2 dioxane (12.3 g, 98.9 mmol) was added in 0.25 eq portions every 15 minutes. The reaction was quenched with methanol and concentrated.

The residue was then diluted with 1, 4 dioxane (2580 mL) and water (70 ml). P-toluenesulfonic acid (18.8 g, 98.9 mmol) was added and the mixture was heated to 40^{o}C for 3 hours. Additional p-toluenesulfonic acid (18.8 g, 98.9 mmol) was added and the mixture was heated to 40^{o}C for another 3 hours. More additional p-toluenesulfonic acid (25.1 g, 132 mmol) was added and the mixture was heated to 40^{o}C for another 1 hour. The reaction was cooled and quenched with a mixture of sodium bicarbonate (100 g) in water (500 mL) then dichloromethane (1 L) was added forming an emulsion. The mixture was concentrated and the residue was taken up in water (3 L) and extracted with dichloromethane (3 x 500 mL). The remaining emulsion was diluted with water until a total volume of 1 L and extracted with dichloromethane (2 x 1 L). The combined organics were dried (Na_2SO_4), filtered and concentrated. The crude product was purified by flash chromatography eluting with hexanes/ethyl acetate (10-100%) to afford 10.3 g, (37%) of the title compound. Impure fractions containing product were combined and repurified by flash chromatography eluting with hexanes/ethyl acetate (10-70%) to yield an additional 4.0 g (14%) of the title compound.

M+H 424

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ ppm 1.13 - 1.26 (m, 2 H) 1.46 - 1.59 (m, 2 H) 1.78 - 1.86 (m, 2 H) 1.98 - 2.06 (m, 2 H) 2.26 (s, 6 H) 2.41 (s, 3 H) 3.18 - 3.28 (m, 1 H) 3.39 - 3.51 (m, 4 H) 3.89 - 4.01 (m, 1 H) 4.53 (m, 1 H) 5.76 (s, 2 H) 6.84 (d, *J*=8.34 Hz, 1 H)

2-(*trans*-4-(2-amino-5-bromo-6-methylpyrimidin-4-ylamino)cyclo hexyloxy)ethanol

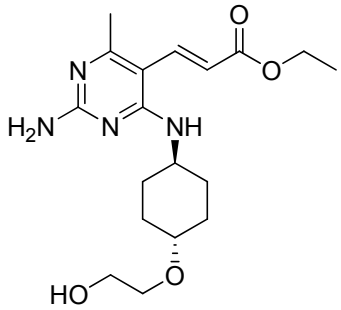


A solution of 2-(*trans*-4-(5-bromo-2-(2,5-dimethyl-1H-pyrrol-1-yl)-6-methylpyrimidin-4-ylamino)cyclohexyloxy)ethanol (17.3 g, 40.7 mmol) and hydroxylamine hydrochloride (14.2 g, 204 mmol) in 10:1 ethanol:water (33 mL) was heated to reflux overnight. The reaction mixture was concentrated and the residue was basified with 50% saturated sodium bicarbonate. The aqueous mixture was extracted with dichloromethane (3x) and the combined organics with dried ($MgSO_4$), filtered, and concentrated. The crude product was purified by flash chromatography eluting with chloroform/methanol (0-10%) to afford the title compound (10.5 g, 74.6%).

M+H 345, 347

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.11 - 1.23 (m, 2 H) 1.33 - 1.47 (m, 2 H) 1.77 - 1.87 (m, 2 H) 1.94 - 2.02 (m, 2 H) 2.17 (s, 3 H) 3.17 - 3.25 (m, 1 H) 3.39 - 3.48 (m, 4 H) 3.81 - 3.92 (m, 1 H) 4.54 (t, *J*=5.43 Hz, 1 H) 5.86 (d, *J*=8.34 Hz, 1 H) 6.10 (s, 2 H)

(E)-ethyl 3-(2-amino-4-(*trans*-4-(2-hydroxyethoxy)cyclohexylamino)-6-methylpyrimidin-5-yl)acrylate

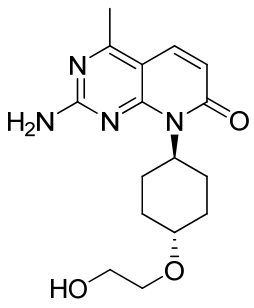


In 2, 150 mL sealed tubes was added half the total amount of 2-(*trans*-4-(2-amino-5-bromo-6-methylpyrimidin-4-ylamino)cyclohexyloxy)ethanol (10.5 g, 30.4 mmol), ethyl acrylate (6.61 mL, 608 mmol), and triethylamine (150 mL). The mixture were bubbled with argon for ~10 minutes then tetrakis(triphenylphosphin)-palladium (0) (3.51 g, 3.04 mmol total) was added. The vials were sealed and the reactions were heated to 130^{o}C overnight. The reactions were cooled to room temperature, combined, and concentrated. The crude product was purified by flash chromatography eluting with chloroform/methanol (0-10%) to afford the title compound (9.53 g, 86.0%).

M+H 365

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.11 - 1.20 (m, 2 H) 1.24 (t, *J*=7.07 Hz, 3 H) 1.34 - 1.47 (m, 2 H) 1.78 - 1.88 (m, 2 H) 1.93 - 2.05 (m, 2 H) 2.21 (s, 3 H) 3.15 - 3.25 (m, 1 H) 3.40 - 3.49 (m, 4 H) 3.91 - 4.03 (m, 1 H) 4.15 (q, *J*=7.24 Hz, 2 H) 4.54 (t, *J*=5.31 Hz, 1 H) 5.95 (d, *J*=15.92 Hz, 1 H) 6.31 (d, *J*=8.08 Hz, 1 H) 6.37 (s, 2 H) 7.59 (d, *J*=15.92 Hz, 1 H)

2-amino-8-(*trans*-4-(2-hydroxyethoxy)cyclohexyl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one

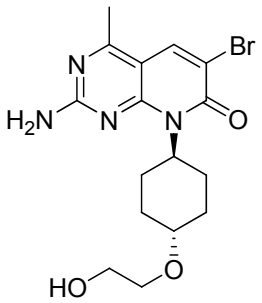


A solution of (E)-ethyl 3-(2-amino-4-(*trans*-4-(2-hydroxyethoxy)cyclohexylamino)-6-methylpyrimidin-5-yl)acrylate (9.50 g, 26.1 mmol), thiophenol (2.87 g, 26.1 mmol), benzenethiol, sodium salt (3.83 g, 26.1 mmol), 1,8-diazabicyclo(5,4,0)undec-7-ene (15.6 mL, 104 mmol), and diisopropylethyl amine (27.5 mL, 156 mmol) in dimethylformamide (200 mL) was heated to 100^{o}C overnight. The reaction mixture was concentrated and the residue was purified by flash chromatography eluting with 3:1 chloroform:ethyl acetate/methanol (0-10%). The fractions containing the desired product were concentrated and the solids were triturated in a minimum amount of chloroform to afford the title compound (5.00 g, 60.2%).

M+H 319

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.14 - 1.32 (m, 2 H) 1.43 - 1.55 (m, 2 H) 2.03 - 2.13 (m, 2 H) 2.46 (s, 3 H) 2.59 - 2.83 (m, 2 H) 3.34 - 3.42 (m, 1 H) 3.43 - 3.52 (m, 4 H) 4.57 (t, *J*=5.31 Hz, 1 H) 5.12 - 5.60 (m, 1 H) 6.14 (d, *J*=9.35 Hz, 1 H) 7.10 (br. s., 2 H) 7.82 (d, *J*=9.60 Hz, 1 H)

2-amino-6-bromo-8-(*trans*-4-(2-hydroxyethoxy)cyclohexyl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one

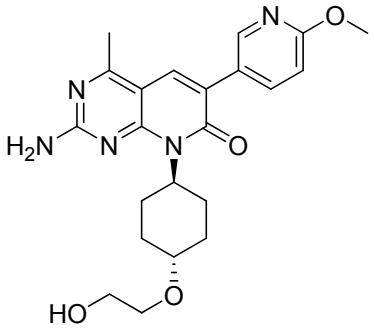


To a solution of 2-amino-8-(*trans*-4-(2-hydroxyethoxy)cyclohexyl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one (6.50 g, 20.4 mmol) in dimethylformamide (136 mL) was added N-bromosuccinimide (3.92 g, 22.2 mmol). After stirring for 30 min at room temperature the solution was concentrated. The residue was slurried in water and filtered. The filter cake was then triturated first with ethyl acetate then a second time with chloroform to afford the title compound (6.14 g, 75.7 %). The combined filtrates were concentrated and purified by flash chromatography eluting with chloroform/7N ammonia in methanol (0-10%) to give an additional 303 mg, 3.74 % of the title compound.

M+H 397, 399

^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.17 - 1.31 (m, 2 H) 1.50 - 1.60 (m, 2 H) 2.06 - 2.16 (m, 2 H) 2.49 (s, 3 H) 2.58 - 2.79 (m, 2 H) 3.34 - 3.42 (m, 1 H) 3.44 - 3.52 (m, 4 H) 4.54 (t, *J*=5.31 Hz, 1 H) 5.27 - 5.51 (m, 1 H) 7.25 (br. s., 2 H) 8.34 (s, 1 H)

2-amino-8-(*trans*-4-(2-hydroxyethoxy)cyclohexyl)-6-(6-methoxypyridin-3-yl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one

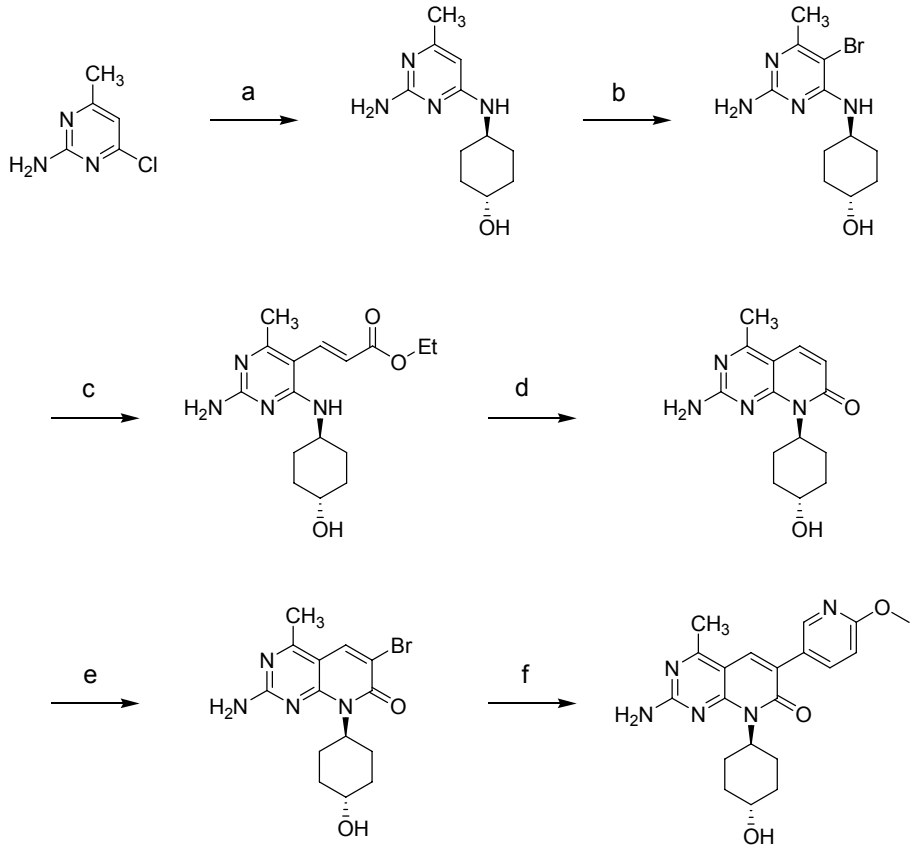


In 4, 20 mL microwave vials was weighted a quarter of the total amount of 2-amino-6-bromo-8-(*trans*-4-(2-hydroxyethoxy)cyclohexyl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one (4.00 g, 10.1 mmol), 2-methoxy-5-pyridine boronic acid (2.31 g, 15.1 mmol), and potassium carbonate (4.17 g, 30.2 mmol). A solution of 5:1 dimethylformade: water (68 mL total) was added to each vial and the mixtures were bubbled with argon for ~10 minutes. To each vial was added bis(tripehnylphosphine) palladium (II) chloride (707 mg, 1.01 mmol total). The vials were sealed and the mixtures were again bubbled with argon (~2 min) before they were heated in the microwave for 20 min at 100°C. The contents of all 4 vials were combined and concentrated in vacuo. To the residue was added a ~1:1 solution of chloroform/methanol and the inorganics were removed by filtration. The crude product was purified by flash chromatography eluting with chloroform:ethyl acetate (3:1)/7 N ammonia in methanol (0-10%). The fractions containing the desired product were combined and concentrated. The solids were dissolved in chloroform (~200 mL) and stirred with activated carbon (5 g) at room temperature for 30 min. The mixture was filtered through a pad of celite and the filtrate was concentrated. The solids were slurried in 3:1 chloroform:methanol (100 mL) and stirred at room temperature for 2 days. The solids were collected by filtration and dried in a vacuum oven at 60°C to afford the title compound (3.37 g, 78.7%). Purity: 99.34% determined by HPLC. CHN calculated for M+2H_2O: C: 57.26%; H: 6.77%; N: 15.17%. Observed: C: 57.22%; H: 6.79%; N: 15.06%.

M+H 426

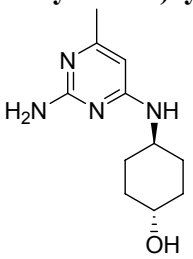
^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.10 - 1.36 (m, 2 H) 1.47 - 1.63 (m, 2 H) 2.03 - 2.18 (m, 2 H) 2.55 (s, 3 H) 2.62 - 2.96 (m, 2 H) 3.34 - 3.44 (m, 1 H) 3.44 - 3.52 (m, 4 H) 3.88 (s, 3 H) 4.52 - 4.62 (m, 1 H) 5.26 - 5.61 (m, 1 H) 6.84 (d, *J*=8.59 Hz, 1 H) 7.16 (br. s., 2 H) 7.97 (s, 1 H) 8.00 (dd, *J*=9.47, 2.40 Hz, 1 H) 8.42 (d, *J*=2.02 Hz, 1 H)

Example for Scheme 1: synthesis of 2-Amino-8-(*trans*-4-hydroxycyclohexyl)-6-(6-methoxypyridin-3-yl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one (15)



Conditions: a) trans-4-aminocyclohexanol, DIEA, K_2CO_3, DMAC, 160 ^oC; b) NBS, $CHCl_3$; c) ethyl acrylate, tri-o-tolylphosphine, palladium (II) acetate, Et_3N, 130 ^oC; d)1,8-diazbicyclo[5,4,0]undec-7-ene, potassium tert-butoxide, DMA, 150 ^oC; e) NBS, DMF; f) 2-methoxy-5-pyridine boronic acid, K_2CO_3, $Pd(PPh_3)_2Cl_2$, DMF/water, 100 ^oC.

***trans*-4-(2-amino-6-methylpyrimidin-4-ylamino)cyclohexanol:**



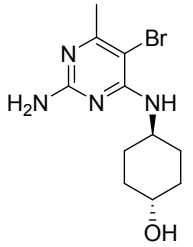
A mixture of 2-amino-4-chloro-6-methyl pyrimidine (1.18 g, 8.24 mmol), *trans*-4-aminocyclohexanol (1.00 g, 6.60 mmol), potassium carbonate (1.82 g, 13.2 mmol), and diisopropylethyl amine (1.44 mL, 8.24 mmol) in dimethylacetamide (20.0 mL) was heated at 160 ^oC in a sealed tube overnight. The reaction mixture was diluted with ethyl acetate, filtered, and the filtrate was concentrated. The residue was purified by flash

chromatography eluting with chloroform/7 N ammonia in methanol (0.5-5%) to afford the title compound as a foamy solid (1.47 g, 99%).

M+H 223

^{1}H NMR (400 MHz, DMSO-*d*6) δ ppm 1.14 - 1.24 (m, 4 H) 1.77 - 1.86 (m, 4 H) 1.97 (s, 3 H) 3.35 - 3.40 (m, 1 H) 3.57 - 3.69 (m, 1 H) 4.52 (d, *J*=4.55 Hz, 1 H) 5.53 (s, 1 H) 5.73 (s, 2 H) 6.43 (d, *J*=4.29 Hz, 1 H)

trans-4-(2-amino-5-bromo-6-methylpyrimidin-4-ylamino)cyclohexanol:

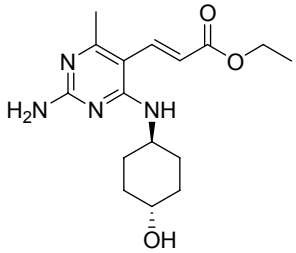


To a solution of *trans*-4-(2-amino-6-methylpyrimidin-4-ylamino)cyclohexanol (1.33 g, 5.98 mmol) in chloroform (15 mL) was added N-bromosuccinamide (1.08 g, 6.04 mmol). After stirring at room temperature for 1.5 hr, the solution was concentrated. The residue was purified by flash chromatography eluting with chloroform/7 N ammonia in methanol (0.5-5%) to afford the title compound (1.14 g, 63%).

M+H 301, 303

^{1}H NMR (400 MHz, DMSO-*d*6) δ ppm 1.14 - 1.25 (m, 2 H) 1.34 - 1.45 (m, 2 H) 1.74 - 1.85 (m, 4 H) 2.17 (s, 3 H) 3.34 - 3.43 (m, 1 H) 3.79 - 3.89 (m, 1 H) 4.55 (d, *J*=4.55 Hz, 1 H) 5.83 (d, *J*=8.34 Hz, 1 H) 6.11 (s, 2 H)

(E)-ethyl 3-(2-amino-4-(_trans_-4-hydroxycyclohexylamino)-6-methylpyrimidin-5-yl)acrylate:



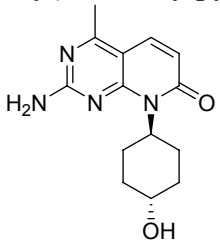
A sealed tube containing *trans*-4-(2-amino-5-bromo-6-methylpyrimidin-4-ylamino)cyclohexanol (655 mg, 2.17 mmol), tri-o-tolylphosphine (298 mg, 0.979 mmol), ethyl acrylate (355 uL, 3.26 mmol) and palladium (II) acetate (73 mg, 0.33 mmol) in triethylamine (20 mL) was evacuated and back-filled with nitrogen (3 x). The reaction mixture was heated overnight at 130oC, filtered and concentrated. The residue was

purified by flash chromatography eluting with chloroform/7 N ammonia in methanol (0.5-5%) to afford the title compound (364 mg, 52%).

M+H 321

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ ppm 1.13 - 1.22 (m, 2 H) 1.24 (t, *J*=7.07 Hz, 3 H) 1.34 - 1.45 (m, 2 H) 1.80 (m, 4 H) 2.21 (s, 3 H) 3.34 - 3.41 (m, 1 H) 3.90 - 4.01 (m, 1 H) 4.15 (q, *J*=7.07 Hz, 2 H) 4.52 (d, *J*=4.55 Hz, 1 H) 5.95 (d, *J*=15.92 Hz, 1 H) 6.27 (d, *J*=8.08 Hz, 1 H) 6.37 (s, 2 H) 7.58 (d, *J*=15.92 Hz, 1 H)

2-amino-8-(*trans*-4-hydroxycyclohexyl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one:

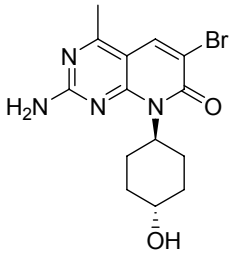


To a solution of (E)-ethyl 3-(2-amino-4-((*trans*-4-hydroxycyclohexylamino)-6-methylpyrimidin-5-yl)acrylate (233 mg, 0.727 mmol) in dimethylacetamide was added 1,8-diazabicyclo[5,4,0]undec-7-ene (544 uL, 3.64 mmol) followed by potassium tert-butoxide (1 M in THF, 364 uL, 364 mmol). The resulting solution was heated at 150oC overnight then concentrated. The residue was purified by flash chromatography eluting with chloroform/7 N ammonia in methanol (0.5-5%). The product was then triturated with 1:1 chloroform:hexanes to afford the title compound (119 mg, 60%).

M+H 275

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ ppm 1.18 - 1.30 (m, 2 H) 1.37 - 1.48 (m, 2 H) 1.87 - 1.94 (m, 2 H) 2.45 (s, 3 H) 2.70 (m, 2 H) 3.46 - 3.57 (m, 1 H) 4.59 (d, *J*=4.29 Hz, 1 H) 5.08 - 5.61 (m, 1 H) 6.13 (d, *J*=9.60 Hz, 1 H) 7.09 (s, 2 H) 7.81 (d, *J*=9.35 Hz, 1 H)

2-amino-6-bromo-8-(trans-4-hydroxycyclohexyl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one:



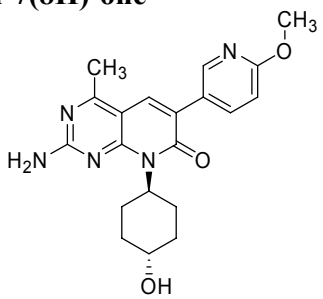
To a solution of 2-amino-8-(trans-4-hydroxycyclohexyl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one (115 mg, 0.419 mmol) in dimethylformamide (2.0 mL) was

added N-bromosuccinimide (75 mg, 0.42 mmol). After stirring for 1.5 hr at room temperature the solution was concentrated. The residue was slurried in methanol, filtered solids, and the filtrated was concentrated and purified by flash chromatography eluting with chloroform/7 N ammonia in methanol (0.5-3%). Combined solids to afford the title compound (120 mg, 81%).

M+H 353/355

^{1}H NMR (400 MHz, DMSO-*d6*) δ ppm 1.21 - 1.32 (m, 2 H) 1.43 - 1.53 (m, 2 H) 1.86 - 1.96 (m, 2 H) 2.48 (s, 3 H) 2.59 - 2.71 (m, 2 H) 3.46 - 3.57 (m, 1 H) 4.62 (d, *J*=3.03 Hz, 1 H) 5.08 - 5.76 (m, 1 H) 7.26 (s, 2 H) 8.34 (s, 1 H)

2-Amino-8-(*trans*-4-hydroxycyclohexyl)-6-(6-methoxypyridin-3-yl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one



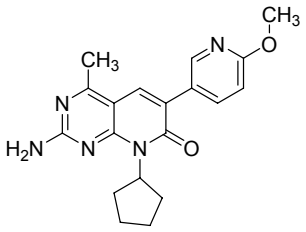
A flask containing 2-amino-6-bromo-8-(trans-4-hydroxycyclohexyl)-4-methylpyrido[2,3-d]pyrimidin-7(8H)-one (105 mg, 0.297 mmol), potassium carbonate (123 mg, 0.892 mmol), and 2-methoxy-5-pyridine boronic acid (52 mg, 0.34 mmol) was evacuated and back-filled with nitrogen (2 x). A solution of 5:1 dimethylformade: water (1.8 mL) was bubbled with argon for 15 min then added to the flask followed by bis(tripehnylphosphine) palladium (II) chloride (10 mg, 0.015 mmol). The flask was fitted with a cold finger, evacuated and back filled with nitrogen (2 x) then heated to 100^{o}C for 4 hr. The mixture was cooled overnight, diluted with methanol and chloroform then filtered through a glass fiber filter to filter out palladium. The filtrate was concentrated and the residue was purified by flash chromatography eluting with chloroform/7 N ammonia in methanol (0.5-6%) to afford the title compound (80 mg, 71%).

LRMS $(M + H)^+$: 382

^{1}H NMR (400 MHz, DMSO-d6) δ ppm 1.23 - 1.34 (m, 2 H) 1.45 - 1.55 (m, 2 H) 1.89 - 1.98 (m, 2 H) 2.55 (s, 3 H) 2.70 - 2.82 (m, 2 H) 3.48 - 3.60 (m, 1 H) 3.82 - 3.91 (m, 3 H) 4.61 (d, J=4.29 Hz, 1 H) 5.16 - 5.62 (m, 1 H) 6.84 (d, J=8.59 Hz, 1 H) 7.16 (s, 2 H) 7.97 (s, 1 H) 8.00 (dd, J=8.72, 2.40 Hz, 1 H) 8.42 (d, J=2.53 Hz, 1 H)

MS and NMR Characterization of compounds **3, 16, 17, 18.** Determined by LCMS, the purity is 98.95% for compound **4**, and >95% for compound **5**.

Compound 3:

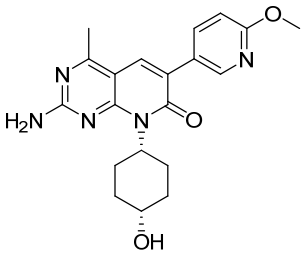


Purity: >95% determined by LCMS.

MS: 352 $(M+H)^+$

^{1}H NMR (DMSO-d_6, 400 MHz): δ ppm 8.43 (1H, s), 8.02 (1H, m), 7.99 (1H, s), 7.17 (2H, bs), 6.86-6.84 (1H, m), 6.02-5.98 (1H, m), 3.88 (3H, s), 2.56 (3H, s), 2.24-2.23 (2H, bm), 2.02 (2H, bm), 1.76 (2H, bm), 1.59 (2H, bm).

Compound 16

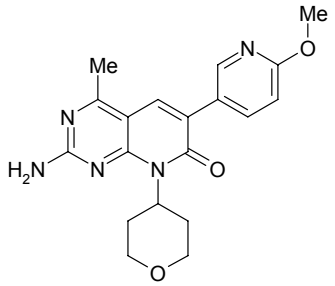


Purity: >99% determined by LCMS.

M+H 382

^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.47 - 1.59 (m, 2 H) 1.63 - 1.78 (m, 2 H) 1.85 - 2.02 (m, 3 H) 2.61 (s, 3 H) 2.96 - 3.20 (m, 2 H) 3.98 (s, 3 H) 4.11 (br. s., 1 H) 5.21 (br. s., 2 H) 5.53 (t, *J*=12.76 Hz, 1 H) 6.81 (d, *J*=8.59 Hz, 1 H) 7.75 (s, 1 H) 7.99 (dd, *J*=8.59, 2.53 Hz, 1 H) 8.32 (d, *J*=2.27 Hz, 1 H)

Compound 17

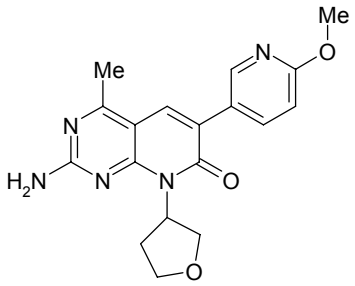


Purity: 96.7% determined by HPLC.

M+H: 368.

^{1}H NMR (400 MHz, DMSO):): δ ppm 8.43-8.42 (d, 1H), 8.01-8.00 (d, 1H), 7.99 (s, 1 H), 7.18 (bs, 2 H), 6.86-6.84 (d, 1 H), 5.25-5.12 (m, 1 H), 4.01-3.97 (m, 2 H), 3.88 (s, 3H), 3.42-3.37 (m, 2H), 3.05-2.85 (m, 2H), 2.55 (s, 3H), 1.49-1.46 (m, 2H)

Compound 18



Purity: 96.3% determined by HPLC.

M+H: 354

^{1}H NMR (400 MHz, DMSO): δ ppm 8.34-8.33 (d, 1 H), 7.93-7.91 (m, 2 H), 7.14 (bs, 2 H), 6.77-6.75 (d, 1 H), 6.13-6.12 (m, 1 H), 4.15-4.13 (m, 1 H), 3.88-3.75 (m, 6 H), 2.47 (s, 3H), 2.33-2.31 (m, 1H), 2.05-1.95 (m, 1H)