Supplemental information

# Microfluidic-enabled intracellular delivery of membrane impermeable inhibitors to study target engagement in human primary cells

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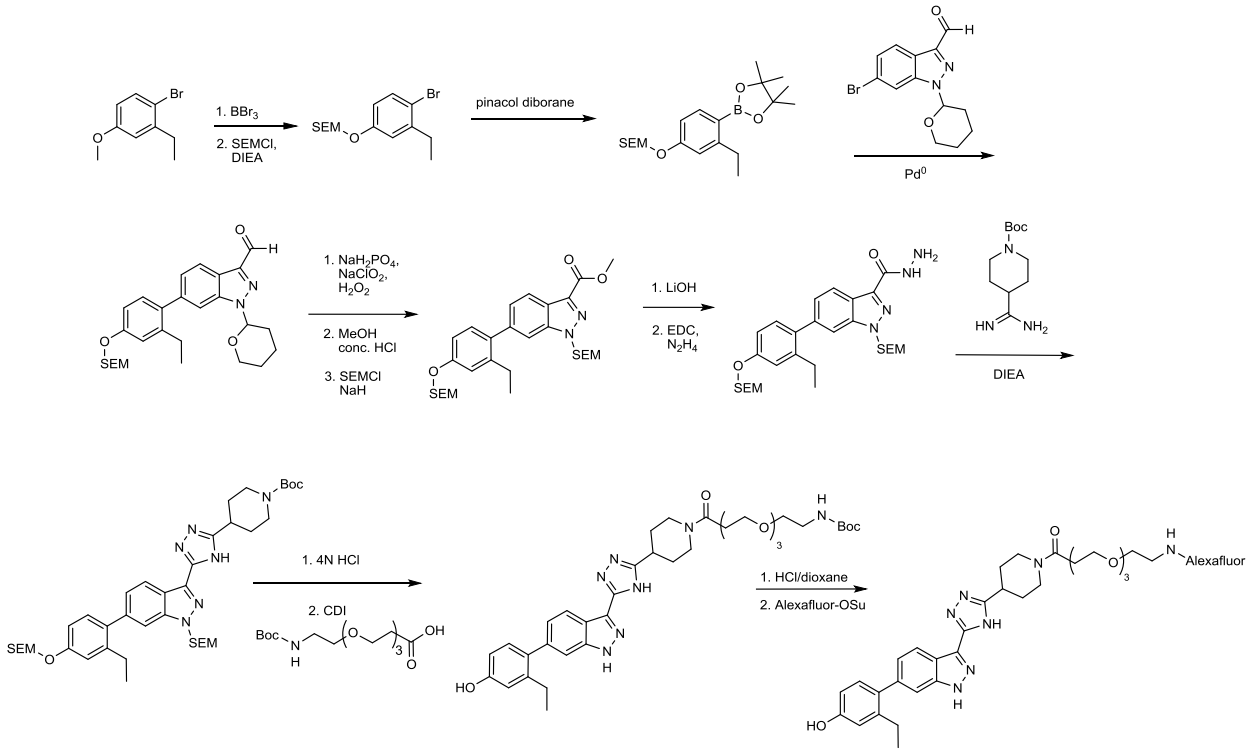
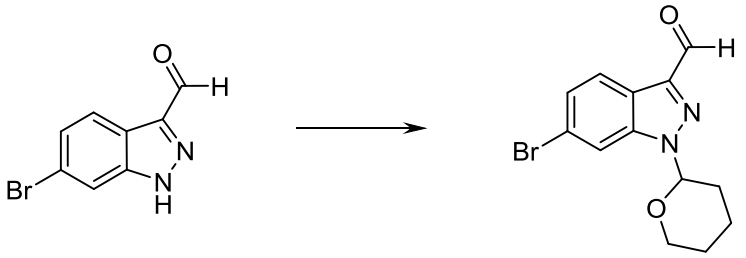
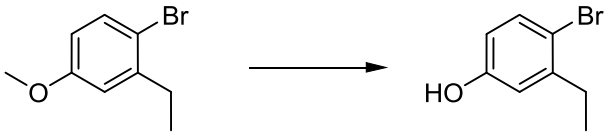
Scheme 1


1. BBr3
2. SEMCl, DIEA
pinacol diborane
Pd0
1. NaH2PO4, NaClO2, H2O2
2. MeOH conc. HCl
3. SEMCl NaH
1. LiOH
2. EDC, N2H4
DIEA
1. 4N HCl
2. CDI
1. HCl/dioxane
2. Alexafluor-OSu
Alexafluor



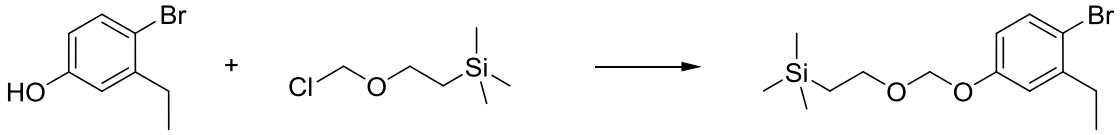

**6-Bromo-1-(tetrahydro-2H-pyran-2-yl)-1H-indazole-3-carbaldehyde**

6-Bromo-1H-indazole-3-carbaldehyde (50 g, 222.18 mmol) and p-toluenesulfonic acid (8.45 g, 44.44 mmol) were dissolved in DCM (517 mL) and cooled to 0°C. DHP (29.7 mL, 333.27 mmol) was added drop-wise and the mixture allowed to warm to ambient temperature overnight. The mixture was diluted with DCM (200 mL) and washed with aqueous sodium bicarbonate. The organics were dried ($MgSO_4$), filtered and concentrated under vacuum. The residue was purified via flash chromatography using 0-20% ethyl acetate in heptane to give (49.55 g, 72%) of the desired product. $^1$H NMR (400 MHz, $CDCl_3$) δ 10.20 (s, 1H), 8.15 (d, 1H), 7.90 (s, 1H), 7.45 (d, 1H), 5.80 (d, 1H), 4.00 (d, 1H), 3.80 (m, 1H), 2.60 (m, 1H), 2.20 (m, 2H), 1.80 (m, 3H). LRMS *m/z* 227 $[M\text{-}THP+H]^+$.



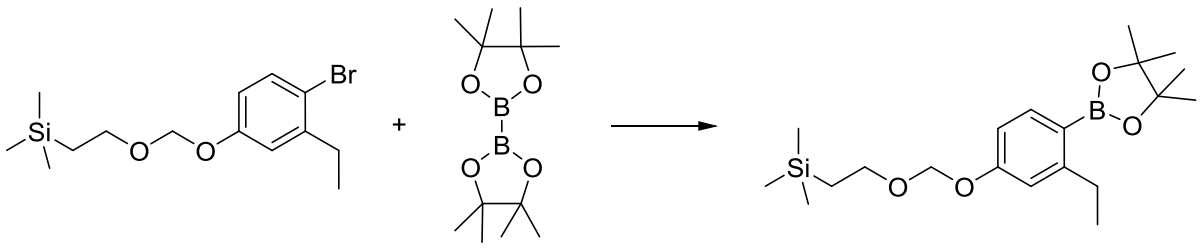

**4-Bromo-3-ethylphenol**

To a chilled 0°C mixture of 1-bromo-2-ethyl-4-methoxybenzene (10 g, 46 mmol) in DCM (100 mL) was slowly added $BBr_3$ (10 mL, 110 mmol). The mixture was stirred in the ice bath, which was allowed to melt, overnight under nitrogen. The mixture was concentrated under reduced pressure. The residue was chilled in an ice water bath and saturated sodium bicarbonate was slowly added to neutralize the residue. The mixture was extracted twice with ethyl acetate. The ethyl acetate extracts were dried over anhydrous sodium sulfate and passed through a silica gel plug and finally concentrated under reduced pressure to give (9 g, 96%) of the desired product. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.34-7.45 (m, 1H), 6.76 (d, *J*=2.73 Hz, 1H), 6.59 (dd, *J*=3.12, 8.59 Hz, 1H), 4.81 (br s, 1H), 2.72 (q, *J*=7.54 Hz, 2H), 1.24 (t, *J*=7.41 Hz, 3H). LRMS *m/z* 200 $[M\text{-}H]^-$.



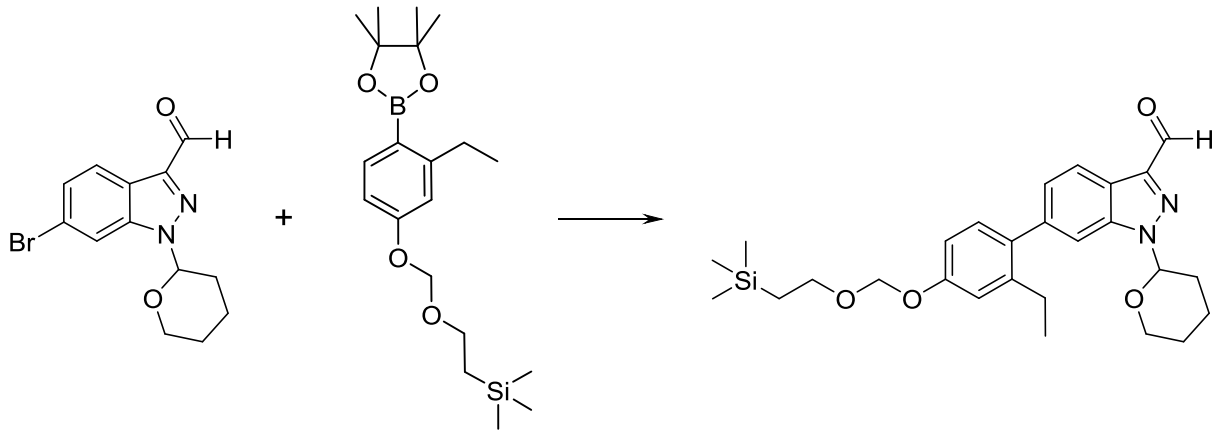

**{2-[(4-Bromo-3-ethylphenoxy)methoxy]ethyl}(trimethyl)silane**

To a mixture of 4-bromo-3-ethylphenol (9 g, 44.76 mmol) in DCM (75 mL) was added DIEA (8.6 mL, 49.2 mmol). The mixture was stirred under nitrogen and SEM chloride (8.32 mL, 47 mmol) was added slowly. The mixture was stirred at ambient overnight under nitrogen. The mixture was partitioned between brine and DCM. The layers were separated and the aqueous phase extracted with DCM. The combined DCM extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was passed through a silica gel plug using 10% ethyl acetate in heptane and concentrated under reduced pressure to give (12.53 g, 85%) of the desired product. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.34-7.45 (m, 1H), 6.92 (d, *J*=2.73 Hz, 1H), 6.76 (dd, *J*=2.93, 8.78 Hz, 1H), 5.18 (s, 2H), 3.69-3.81 (m, 2H), 2.65-2.79 (m, 2H), 1.22 (t, *J*=7.61 Hz, 3H), 0.90-1.02 (m, 2H), 0.00 (s, 9H).



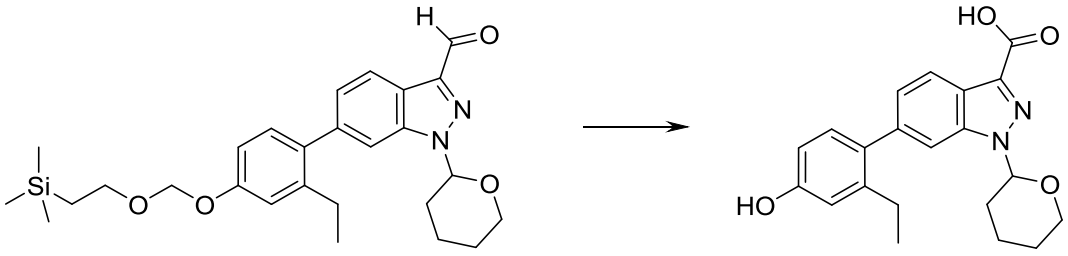

**(2-{[3-Ethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy]methoxy}ethyl)(trimethyl)silane**

To a mixture of {2-[(4-Bromo-3-ethylphenoxy)methoxy]ethyl}(trimethyl)silane (12.5 g, 37.7 mmol) in 1,4-dioxane (100 mL) was added pinacol diborane (12.5 g, 49 mmol) and potassium acetate (11.2 g, 113 mmol). The mixture was degassed by bubbling nitrogen through the mixture for 10 minutes at which time dichloro[1,1'-bis(diphenylphosphino)ferrocene]palladium (II) dichloromethane complex (1.56 g, 1.89 mmol) was added and the mixture heated to 90°C under nitrogen overnight. The mixture was removed from heat and concentrated under reduced pressure. The residue was taken up in ethyl acetate and filtered to remove salts. The filtrates were concentrated under reduced pressure. The residue was purified via flash chromatography using 0-10% ethyl acetate in heptane to give (9 g, 63%) of the desired product. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.73 (d, *J*=7.80 Hz, 1H), 6.84-6.89 (m, 2H), 5.24 (s, 2H), 3.72-3.81 (m, 2H), 2.91 (q, *J*=7.67 Hz, 2H), 1.33 (s, 12H), 1.20 (t, *J*=7.41 Hz, 3H), 0.93-1.00 (m, 2H), 0.89 (t, *J*=6.63 Hz, 2H), -0.01-0.05 (m, 9H).



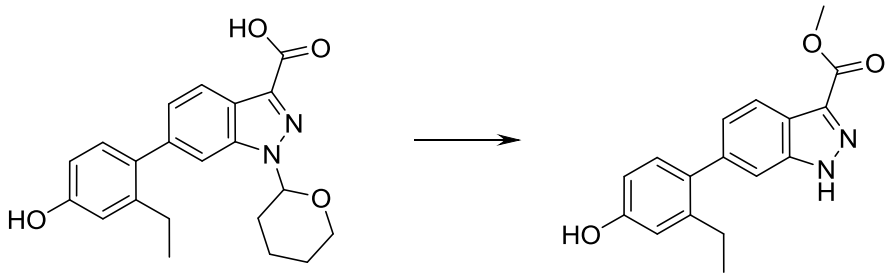
**6-(2-Ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-1-(tetrahydro-2H-pyran-2-yl)-1H-indazole-3-carbaldehyde**

To a solution of 6-bromo-1-(tetrahydro-2H-pyran-2-yl)-1H-indazole-3-carbaldehyde (49.55 g, 160.27 mmol) and (2-{[3-ethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenoxy]methoxy}ethyl)(trimethyl)silane (60.65 g, 160.27 mmol) in 1,4-dioxane (680 mL) was added potassium phosphate tribasic (102.06 g, 480.82 mmol) in water (1080 mL). The mixture was degassed (vacuum-nitrogen x 3). The suspension was treated with tetrakis triphenylphosphine palladium (0) (37.04 g, 32.05 mmol) and heated to reflux overnight. The reaction was cooled and allowed to stand 48 h then filtered through Arbocel® washed with ethyl acetate. The filtrates were reduced to minimal volume under reduced pressure and re-dissolved in ethyl acetate (400 mL) and washed with water (400 mL). The aqueous was re-extracted with ethyl acetate (400 mL) and the combined organic layers were dried ($MgSO_4$), filtered and concentrated. The crude material was filtered through a pad of silica using ethyl acetate. The filtrates were concentrated and the residue purified via flash chromatography using 2-20% ethyl acetate in heptane to give (56.23 g, 73%) of the desired product as a thick brown oil. $^1$H NMR (400 MHz, $CDCl_3$) δ 10.2 (s, 1H), 8.20 (d, 1H), 7.55 (s, 1H), 7.30 (d, 1H), 7.15 (d, 1H), 7.00 (s, 1H), 6.95 (d, 1H), 5.80 (d, 1H), 5.25 (s, 2H), 4.00 (m, 1H), 3.8 (t, 2H), 3.75 (m, 1H), 2.60 (m, 3H), 2.15 (m, 2H), 1.70 (m, 3H), 1.10 (t, 3H), 0.95 (t, 2H), 0,00 (s, 9H). LRMS *m/z* 481 $[M+H]^+$.



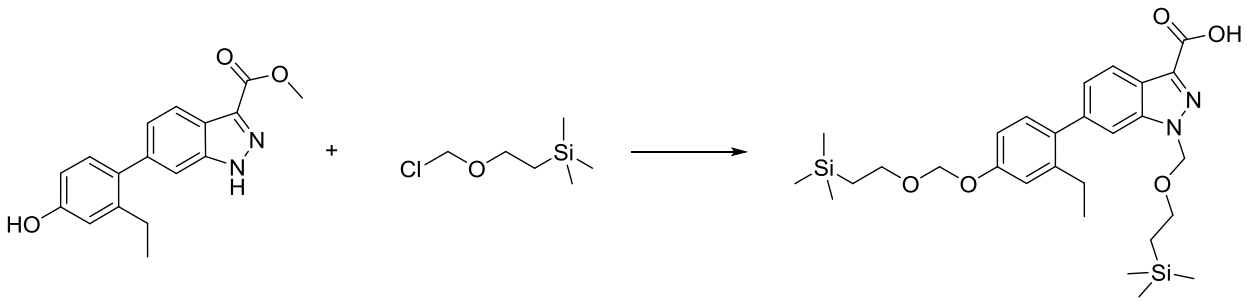
**6-(2-Ethyl-4-hydroxyphenyl)-1-(tetrahydro-2H-pyran-2-yl)-1H-indazole-3-carboxylic acid**
$NaH_2PO_4$ (3.32 g, 24.06 mmol) and $NaClO_2$ (3.17 g, 35 mmol) in water (20 mL) was added drop-wise to a solution of 6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-1-(tetrahydro-2H-pyran-2-yl)-1H-indazole-3-carbaldehyde (8.86 g, 18.45 mmol) in acetonitrile (85

mL). 30% Hydrogen peroxide (6.23 g, 55 mmol) was added drop-wise at 0°C and the mixture stirred at ambient temperature for 2 h. Water (70 mL) was added and the mixture acidified to pH 2 with concentrated hydrochloric acid. The mixture was extracted with ethyl acetate (3 x 100 mL). The combined organic extracts were dried, filtered and evaporated to give (9.82 g, the desired an orange oil/gum which was used as is in the next step without further purification.




**Methyl 6-(2-ethyl-4-hydroxyphenyl)-1H-indazole-3-carboxylate**

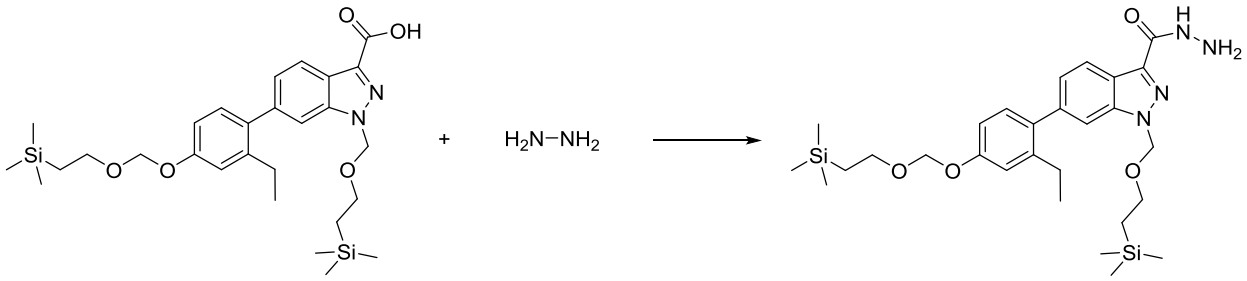
To a mixture of 6-(2-ethyl-4-hydroxyphenyl)-1-(tetrahydro-2H-pyran-2-yl)-1H-indazole-3-carboxylic acid (6.28 g, 17.16 mmol) in methanol (40 mL) was added concentrated hydrochloric acid (10 mL). The mixture was stirred at ambient temperature overnight. The mixture was concentrated under reduced pressure and the residue partitioned between ethyl acetate (40 mL) and saturated aqueous sodium bicarbonate. The layers were separated, the organic phase dried over magnesium sulfate, filtered and concentrated under reduced pressure to give (3.57 g, 70%) of the desired product. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.11 (dd, 1H), 7.44 (m, 1H), 7.22 (dd, 1H), 7.04 (d, 1H), 6.76 (d, 1H), 6.67 (dd, 1H), 4.02 (s, 3H), 2.53 (q, 2H), 1.05 (t, 3H). LRMS *m/z* 297 $[M+H]^+$.




**6-(2-Ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1H-indazole-3-carboxylic acid**

To a mixture of methyl 6-(2-ethyl-4-hydroxyphenyl)-1H-indazole-3-carboxylate (0.515 g, 1.74 mmol) in dry DMF (2 mL) in an ice water bath, under nitrogen, was added 60% sodium hydride* dispersion (0.150 g, 3.75 mmol). The mixture turns dark and opaque. The mixture was stirred for 5 minutes at which time SEM chloride (0.745 mL, 4.2 mmol) was added. Upon the addition of the SEM chloride the mixture lightens and becomes a clear pale yellow solution. The mixture

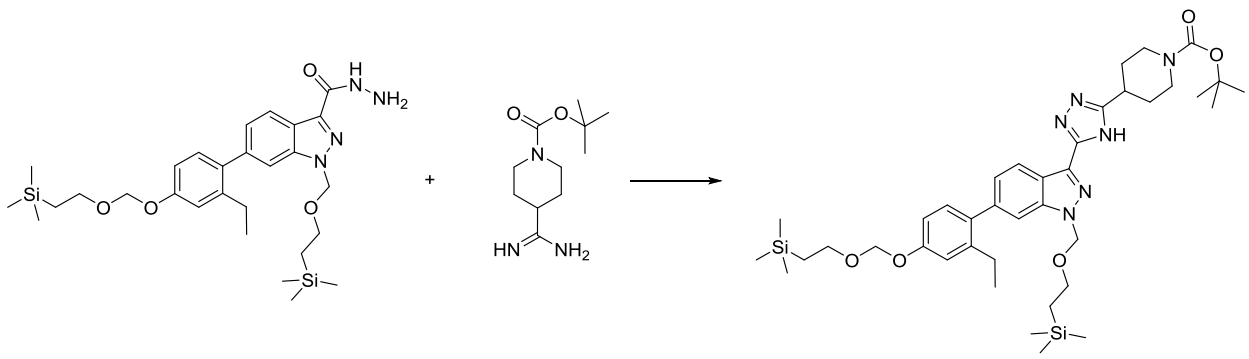
was stirred for 1 h then quenched with saturated sodium bicarbonate. The reaction was partitioned between brine and ethyl acetate. The layers were separated and the aqueous phase extracted with ethyl acetate. The combined ethyl acetate extracts were washed four times with brine, dried over anhydrous sodium sulfate and concentrated under reduced pressure. The reaction was repeated twice on a 3.46 mmol scale. The crude products for the three runs were combined and purified via flash chromatography using methanol:dichloromethane, 0:1 to 1:9 as eluent to give a mixture of regioisomers. The purified material (3.44 g) was dissolved in THF (20 mL) and lithium hydroxide (0.1 g, 4.1 mmol) in 2 mL water was added. The resulting mixture was stirred at rt overnight. The mixture was partitioned between saturated ammonium chloride and ethyl acetate. The layers were separated and the organic phase washed with brine, dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified via flash chromatography using 0-30% ethyl acetate in heptane to give (2.137 g 45%) of the desired product as a mixture of regioisomers 6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1H-indazole-3-carboxylic acid and 6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-2-{[2-(trimethylsilyl)ethoxy]methyl}-2H-indazole-3-carboxylic acid. $^{1}$H NMR (400 MHz, DMSO-*d6*) δ 8.11 (d, *J*=8.19 Hz, 1H), 8.04 (d, *J*=8.59 Hz, 1H), 7.73 (s, 1H), 7.62 (s, 1H), 7.27 (d, *J*=8.20 Hz, 1H), 7.22-7.25 (m, 1H), 7.16 (t, *J*=8.59 Hz, 1H), 6.99-7.03 (m, 1H), 6.91-6.96 (m, 1H), 6.13 (s, 1H), 5.87 (s, 1H), 5.27 (d, *J*=1.56 Hz, 2H), 3.74 (t, *J*=8.00 Hz, 2H), 3.65 (t, *J*=8.00 Hz, 1H), 3.56 (t, *J*=7.81 Hz, 1H), 2.52-2.59 (m, 2H), 1.02 (q, *J*=7.41 Hz, 2H), 0.89-0.95 (m, 2H), 0.81-0.86 (m, 2H), -0.02-0.02 (m, 9H), -0.08 (s, 6H), -0.13 (s, 3H). MS *m/z* 541 $[M-H]^-$.




**6-(2-Ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1H-indazole-3-carbohydrazide**

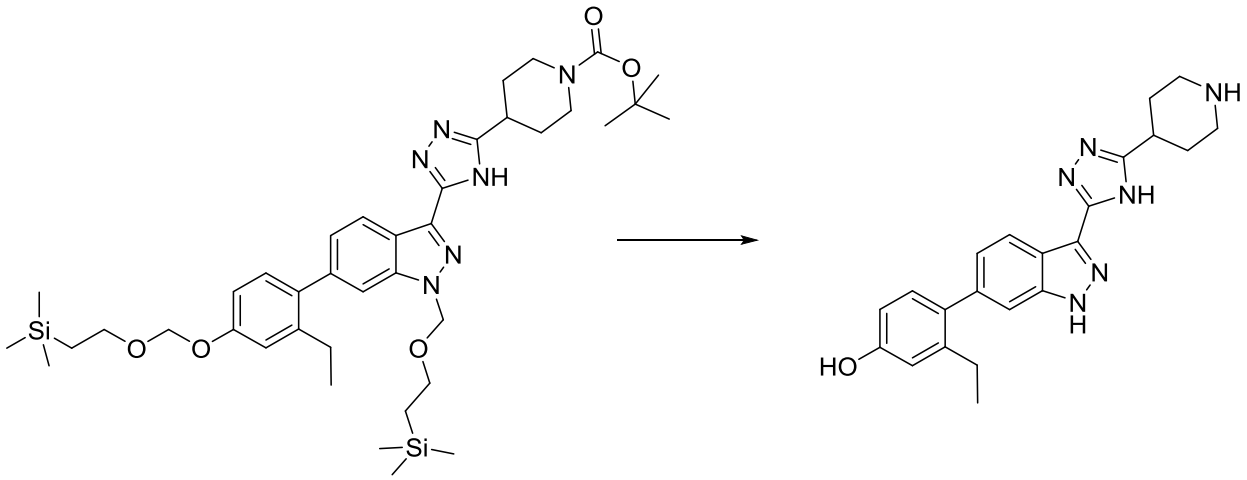
To a mixture of regioisomers of 6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1H-indazole-3-carboxylic acid and 6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-2-{[2-(trimethylsilyl)ethoxy]methyl}-2H-indazole-3-carboxylic acid (1.937 g, 3.57 mmol) in DCM (30 mL) was added hydrazine (0.114 mL, 3.57 mmol), DIEA (0.653 mL, 3.75 mmol), EDC hydrochloride (0.73 g, 3.75 mmol) and HOBT (0.153 g, 1.08 mmol). The mixture was heated to 30°C overnight. The mixture was concentrated under reduced pressure. The residue was partitioned between brine and ethyl acetate. The layers were separated and the organic phase washed with brine, dried over anhydrous sodium sulfate and concentrated under reduced pressure to give (0.95 g, 48%) of the desired product as a mixture of regioisomers 6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1H-indazole-3-carbohydrazide and 6-(2-ethyl-4-{[2-

(trimethylsilyl)ethoxy]methoxy}phenyl)-2-{[2-(trimethylsilyl)ethoxy]methyl}-2H-indazole-3-carbohydrazide. LRMS *m/z* 555 $[M-H]^-$.



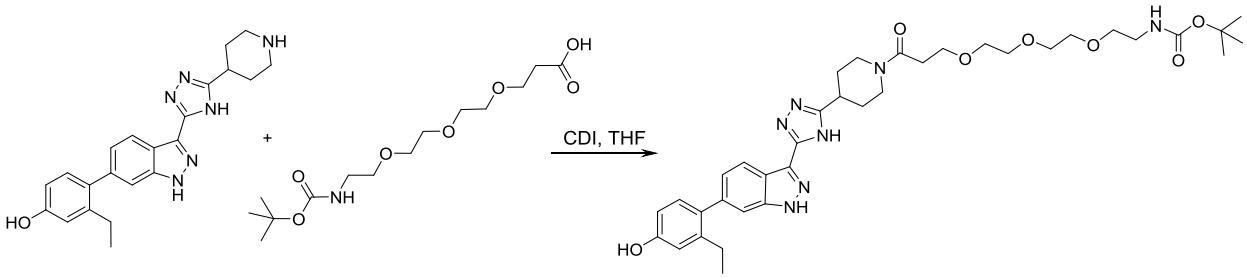
**Tert-butyl 4-{5-[6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1H-indazol-3-yl]-4H-1,2,4-triazol-3-yl}piperidine-1-carboxylate**

To a mixture of 6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1H-indazole-3-carbohydrazide and 6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-2-{[2-(trimethylsilyl)ethoxy]methyl}-2H-indazole-3-carbohydrazide (0.95 g, 1.71 mmol) in n-butanol (80 mL) was added DIEA (0.3 mL, 1.71 mmol) and tert-butyl 4-carbamimidoylpiperidine-1-carboxylate (0.49 g 1.71 mmol). The mixture was heated to 95°C overnight. The temperature was increased to 100°C for 4 h. The mixture was removed from heat and concentrated under reduced pressure. The residue was purified via flash chromatography 0-50% ethyl acetate in heptane as eluent to give (0.595 g 47%) of the desired product as a mixture of regiosomers tert-butyl 4-{5-[6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1H-indazol-3-yl]-4H-1,2,4-triazol-3-yl}piperidine-1-carboxylate and tert-butyl 4-{5-[6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-2-{[2-(trimethylsilyl)ethoxy]methyl}-2H-indazol-3-yl]-4H-1,2,4-triazol-3-yl}piperidine-1-carboxylate. $^1$H NMR (400 MHz, DMSO-*d6*) δ 8.31 (d, *J*=8.59 Hz, 1H), 8.24 (d, *J*=8.59 Hz, 1H), 7.65 (s, 1H), 7.50 (s, 1H), 7.15-7.22 (m, 1H), 7.10 (d, *J*=8.59 Hz, 1H), 6.98-7.04 (m, 1H), 6.91-6.97 (m, 1H), 6.30 (s, 1H), 5.83 (s, 1H), 5.27 (d, *J*=2.34 Hz, 2H), 4.01 (d, *J*=12.10 Hz, 2H), 3.74 (t, *J*=8.00 Hz, 2H), 3.63 (t, *J*=8.00 Hz, 1H), 3.57 (t, *J*=7.81 Hz, 1H), 3.14 (d, *J*=11.32 Hz, 1H), 2.97 (br s, 2H), 2.57 (dd, *J*=3.51, 7.41 Hz, 2H), 1.96-2.10 (m, 2H), 1.68 (q, *J*=11.71 Hz, 2H), 1.33-1.50 (m, 9H), 0.99-1.08 (m, 2H), 0.86-0.97 (m, 2H), 0.81 (dt, *J*=4.10, 7.90 Hz, 2H), 0.00 (d, *J*=0.78 Hz, 9H), -0.19--0.06 (m, 9H). LRMS *m/z* 747 $[M-H]^-$.



**3-Ethyl-4-{3-[5-(piperidin-4-yl)-4H-1,2,4-triazol-3-yl]-1H-indazol-6-yl}phenol**

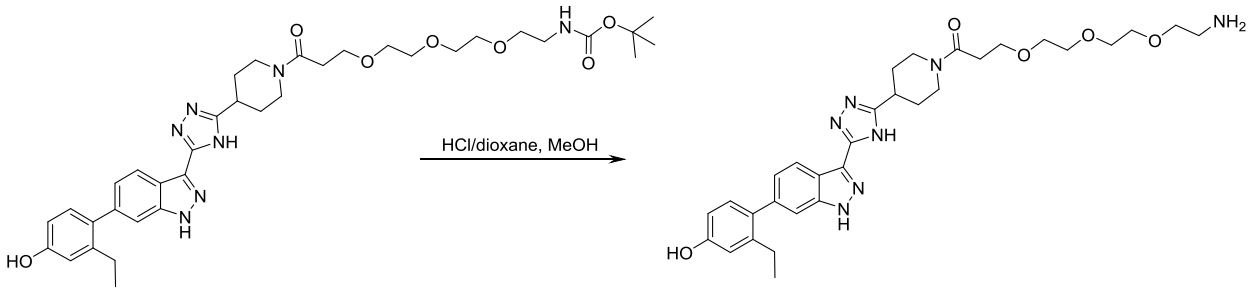
To a mixture of regiosomers tert-butyl 4-{5-[6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-1-{[2-(trimethylsilyl)ethoxy]methyl}-1H-indazol-3-yl]-4H-1,2,4-triazol-3-yl}piperidine-1-carboxylate and tert-butyl 4-{5-[6-(2-ethyl-4-{[2-(trimethylsilyl)ethoxy]methoxy}phenyl)-2-{[2-(trimethylsilyl)ethoxy]methyl}-2H-indazol-3-yl]-4H-1,2,4-triazol-3-yl}piperidine-1-carboxylate (0.595 g, 0.794 mmol) in DCM (20 mL) and methanol (3 mL) was added 4 N HCl in dioxane (1.6 mL, 6.4 mmol). The mixture was stirred at ambient for 4 h then heated to 40°C overnight. The dichloromethane was allowed to evaporate. Water (2 mL) was added and the mixture heated to 50°C for 1 h. The mixture was concentrated under reduced pressure to give (333 mg, 99%) of the desired product which was used as is without further purification. LRMS *m/z* 389 $[M+H]^+$.




**Tert-butyl [2-(2-{2-[3-(4-{5-[6-(2-ethyl-4-hydroxyphenyl)-1H-indazol-3-yl]-4H-1,2,4-triazol-3-yl}piperidin-1-yl)-3-oxopropoxy]ethoxy}ethoxy)ethyl]carbamate**

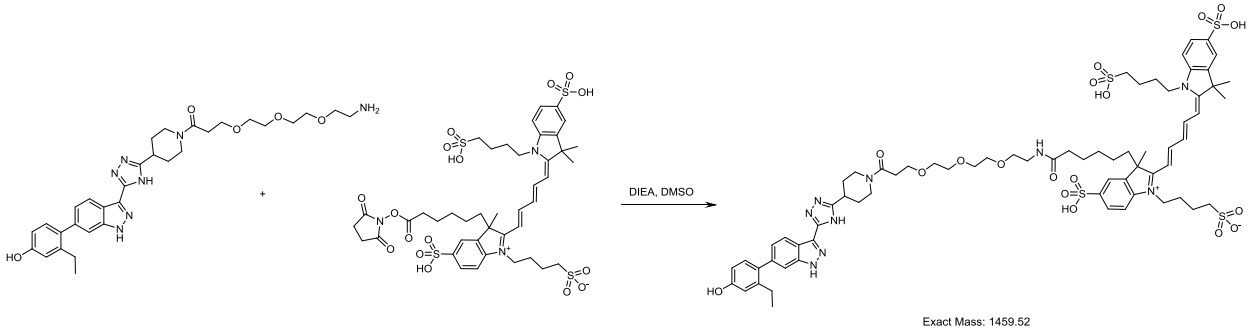
To 2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azaheptadecan-17-oic acid (0.040 g, 0.12 mmol) was added 2 mL of DMSO in a vial at ambient temperature, under nitrogen, and treated with CDI (0.020 g, 0.12 mmol). The resultant reaction solution stirred at ambient temperature for ~3 h then 3-ethyl-4-(3-(5-(piperidin-4-yl)-4H-1,2,4-triazol-3-yl)-1H-indazol-6-yl)phenol (0.050 g, 0.12 mmol) was added and the reaction continued overnight. The reaction solution was then diluted with ~5 mL each of water and ethyl acetate and the phases separated. The aqueous

phase was extracted 2 x 5 mL each of EtOAc and DCM. The combined organics were passed through an SPE cartridge to remove water, adsorbed onto silica gel, then purified by normal phase silica gel chromatography eluting with a methanol\DCM gradient to give the target compound (0.020 g, 24%) as a thick, colorless oil. $^{1}$H NMR (400 MHz, MeOH-*d4*) δ 8.29 (d, *J*=8.20 Hz, 1H), 7.83 (s, 1H), 7.44 (s, 1H), 7.18-7.22 (m, 1H), 7.14 (s, 1H), 7.10 (d, *J*=8.20 Hz, 1H), 6.80 (d, *J*=2.34 Hz, 1H), 6.71 (dd, *J*=2.54, 8.39 Hz, 1H), 4.60-4.66 (m, 1H), 4.17-4.23 (m, 1H), 3.82 (t, *J*=6.24 Hz, 2H), 3.77 (t, *J*=6.44 Hz, 1H), 3.60-3.64 (m, 2H), 3.45-3.60 (m, 4H), 3.38-3.40 (m, 2H), 3.17-3.29 (m, 3H), 2.93-3.00 (m, 1H), 2.78-2.88 (m, 1H), 2.65-2.75 (m, 2H), 2.54-2.64 (m, 2H), 2.17-2.24 (m, 3H), 1.81-2.06 (m, 3H), 1.41-1.50 (m, 9H), 1.10 (t, *J*=7.61 Hz, 3H). LRMS *m/z* 690.2 $[M-H]^-$.




**3-{2-[2-(2-Aminoethoxy)ethoxy]ethoxy}-1-(4-{5-[6-(2-ethyl-4-hydroxyphenyl)-1H-indazol-3-yl]-4H-1,2,4-triazol-3-yl}piperidin-1-yl)propan-1-one**

To tert-butyl (2-(2-(2-(3-(4-(5-(6-(2-ethyl-4-hydroxyphenyl)-1H-indazol-3-yl)-4H-1,2,4-triazol-3-yl)piperidin-1-yl)-3-oxopropoxy)ethoxy)ethoxy)ethyl)carbamate (0.020 g, 0.029 mmol) in ~2 mL of methanol at ambient temperature, was added a 4 M HCl/dioxane solution (0.1 mL, 0.4 mmol), and stirred at ambient temperature for ~4 h. The reaction solution was concentrated under reduced pressure and azeotroped with ether to give the crude HCl salt of 3-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-1-(4-(5-(6-(2-ethyl-4-hydroxyphenyl)-1H-indazol-3-yl)-4H-1,2,4-triazol-3-yl)piperidin-1-yl)propan-1-one (0.020 g, ~100%) as a thick, colorless oil. LRMS *m/z* 592.2 $[M+H]^+$.

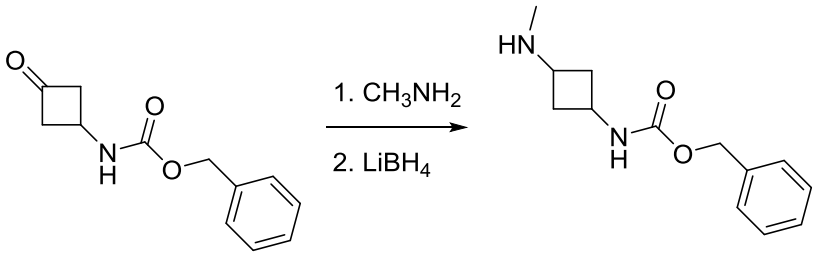


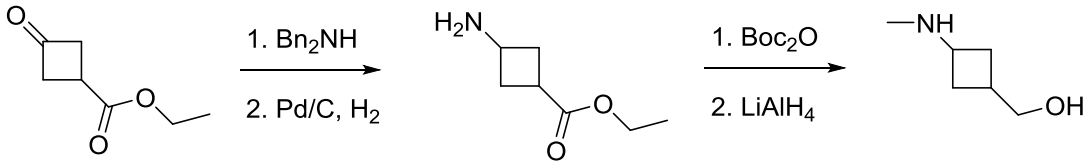

**4-(2-{(1E,3E,5Z)-5-[3,3-Dimethyl-5-sulfo-1-(4-sulfobutyl)-1,3-dihydro-2H-indol-2-ylidene]penta-1,3-dien-1-yl}-3-[19-(4-{5-[6-(2-ethyl-4-hydroxyphenyl)-1H-indazol-3-yl]-4H-**

**1,2,4-triazol-3-yl}piperidin-1-yl)-6,19-dioxo-10,13,16-trioxa-7-azanonadec-1-yl]-3-methyl-5-sulfo-3H-indolium-1-yl)butane-1-sulfonate**

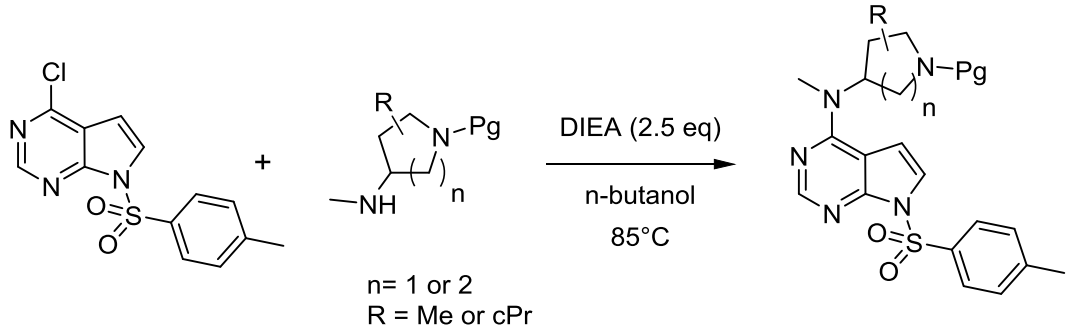
Alexafluor 647 SE® (0.005 g, 0.005 mmol) was combined with 3-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-1-(4-(5-(6-(2-ethyl-4-hydroxyphenyl)-1H-indazol-3-yl)-4H-1,2,4-triazol-3-yl)piperidin-1-yl)propan-1-one (0.0035 g, 0.005 mmol) in a vial at ambient temperature, wrapped in foil, under a stream of nitrogen, then diluted with 1 mL of DMSO-*d6* and treated with DIEA (0.005 mL, 0.03 mmol). The reaction was complete within 1 h. The reaction solution was purified on a Luna (2) C18 150 mm x 21.2 mm 5um column eluting with an acetonitrile/water gradient, 27.0 mL/min to give the target compound (0.0045 g, 64%) as a blue solid after lyophilization. LRMS *m/z* 1459.7 $[M-H]^-$.

Scheme 2 & 3

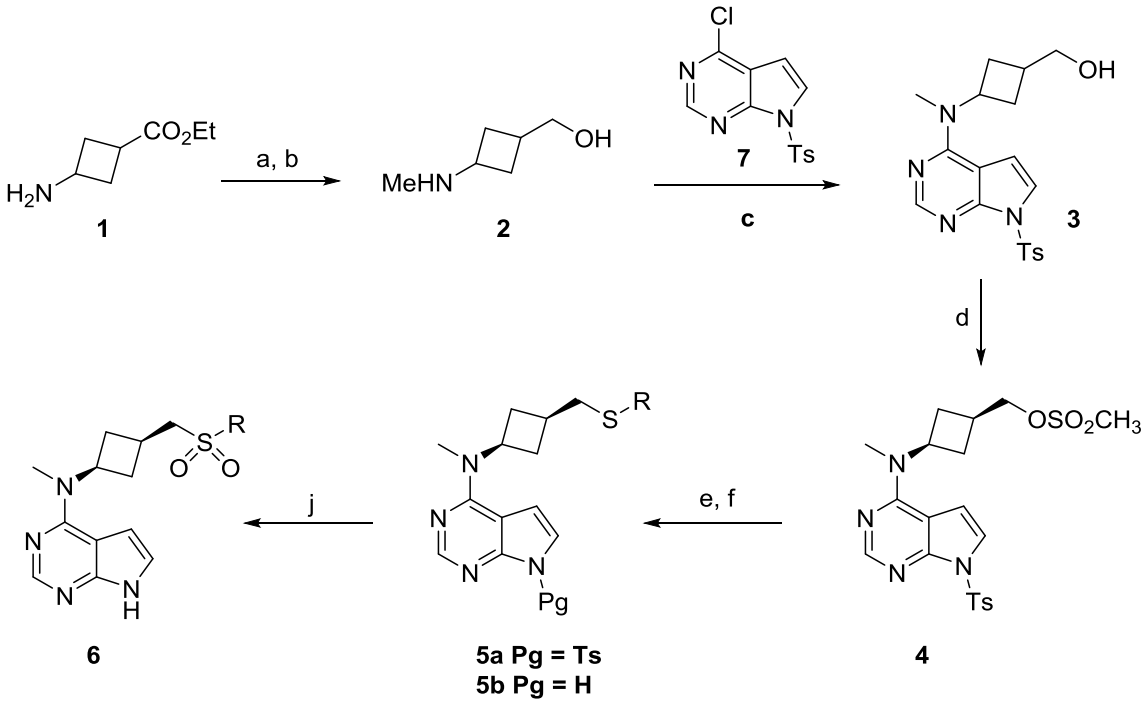


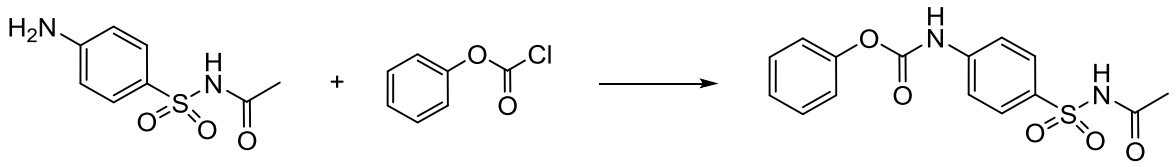

Scheme 4



Scheme 5

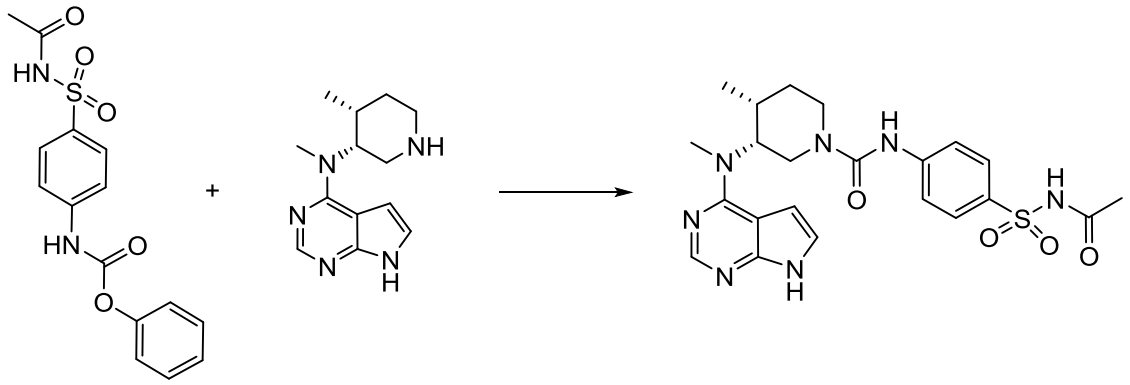



a) **1**·HCl (1 eq.), $Boc_2O$ (1.3 eq.), TEA (2.5 eq.), DCM, 0°C to rt; b) LiAlH4 (5 eq.), THF, 0°C to 66°C; c) **7** (1 eq.), **2** (1.4 eq.), TEA (2.9 eq.), KI (0.002 eq.), acetone, 60°C; d) MsCl (1.5 eq.), $Et_3N$ (3 eq.), DCM, rt; e) 4 (1 eq), RSH (1.1 eq.), NMP, DBU (1.5 eq), 60°C; f) **5a** (1 eq), 1,4-dioxane, 1 N $KOH_{aq}$ (7 eq), 100°C; Various R group modifications, see experimentals; j) 5b (1 eq) potassium peroxymonosulfate (7.7 eq.), THF, EtOH, $H_2O$.



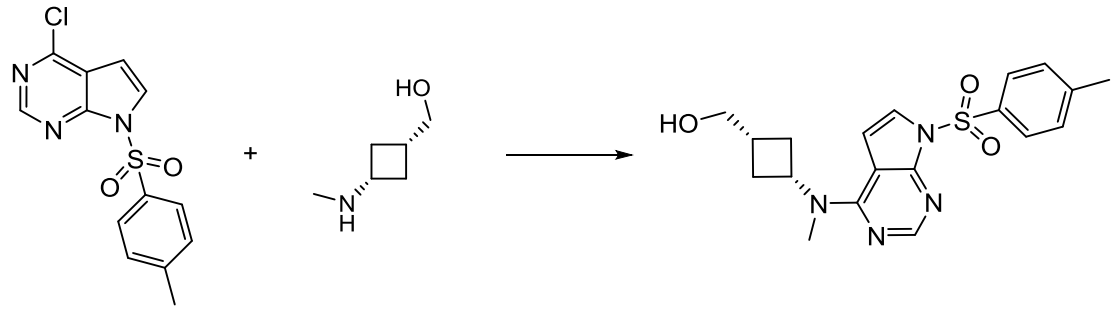
**Phenyl [4-(N-acetylsulfamoyl)phenyl]carbamate**

To a solution of N-[(4-aminophenyl)sulfonyl]acetamide (150 mg, 0.700 mmol) in pyridine (3.5 mL) and anhydrous DCM (3.5 mL) was added phenyl carbonochloridate (109 mg, 0.700 mmol) at 0°C under nitrogen. After the addition, the resulting mixture was stirred at ambient temperature for 1~2 h. The reaction mixture was partitioned between DCM and water. The organic layer was separated and the aqueous layer was extracted with DCM. The combined organic layers were dried over sodium sulfate and concentrated to give crude product. The crude product was purified via flash column (EtOAc: Petroleum 1%-10%) to give (100 mg, 43%) of the desired product.



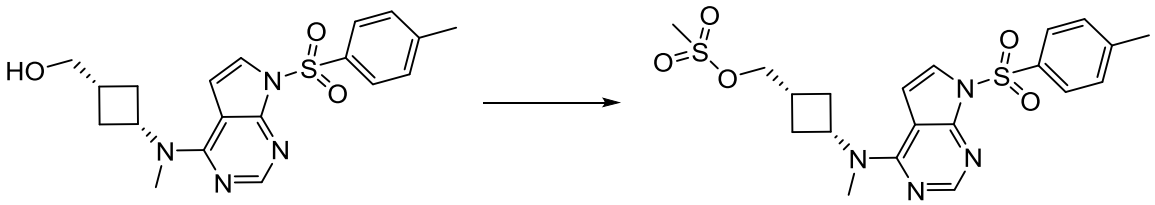
**(3R,4R)-N-[4-(N-acetylsulfamoyl)phenyl]-4-methyl-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]piperidine-1-carboxamide**

To a solution of N-methyl-N-[(3R,4R)-4-methylpiperidin-3-yl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine (100 mg, 0.298 mmol) in anhydrous DCM (3 mL) was added phenyl [4-(N-acetylsulfamoyl)phenyl]carbamate (100 mg, 0.300 mmol) at 0°C, followed by $Et_3N$ (301 mg, 3.0 mmol). The mixture was then warmed to room temperature and stirred overnight. TLC (DCM: MeOH = 10/1) showed the reaction was complete. The solvent was evaporated and the residue was purified via preparative HPLC (mobile phase: 8% acetonitrile in water (ammonia to pH 10) to 17% acetonitrile in water (ammonia to pH 10)) using a Phenomenex® Gemini C18 250x21.2mm 8um column to give (35.1 mg, 24%) of the desired product. $^1$H NMR (400 MHz, DMSO-*d6*) δ 11.63 (s, 1H), 8.93 (s, 1H), 8.09 (s, 1H), 7.67 (d, 2H), 7.55 (d, 2H), 7.12 (m, 1H), 6.55 (m, 1H), 4.90 (m, 1H), 3.84 (m, 2H), 3.67 (m, 1H), 3.46 (m, 1H), 3.23 (s, 3H), 2.40 (m, 1H), 1.76 (m, 4H), 1.60 (m, 1H), 1.02 (d, 3H). LRMS *m/z* 486 $[M+H]^+$.



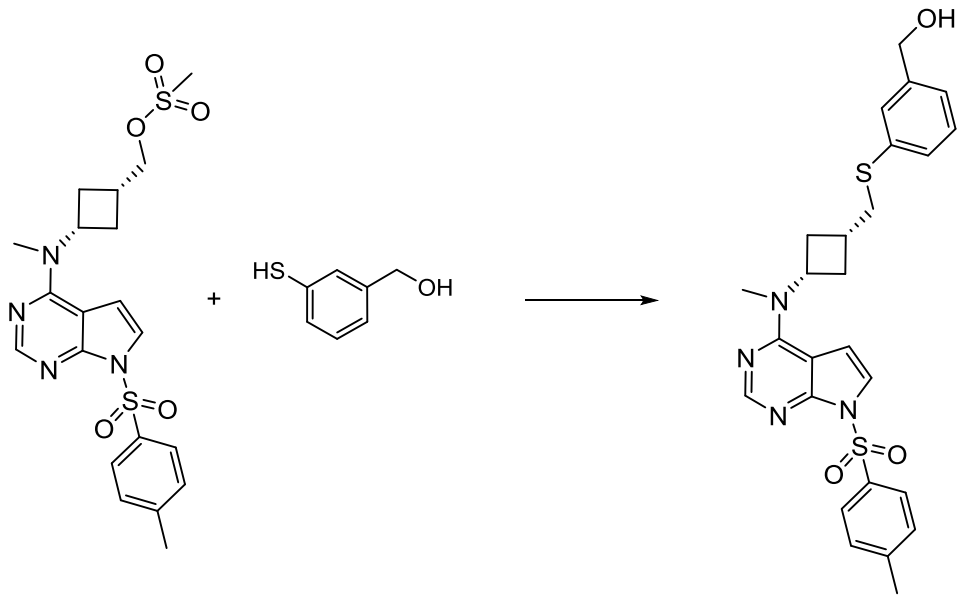
**[Cis-3-(methyl{7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl}amino)cyclobutyl]methanol**

To a solution of 4-chloro-7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidine (2.81 g, 9.0 mmol) and [cis-3-(methylamino)cyclobutyl]methanol (1.50 g, 13.0 mmol) in acetone (15 mL) was added triethylamine (3.64 mL, 26.0 mmol), followed by KI (44 mg, 0.02 mmol). After the addition, the reaction mixture was heated at 60°C for 4 h. TLC (EtOAc: Petroleum ether = 1:1) showed that the reaction was complete. The reaction mixture was diluted in DCM. The organic layer was washed with brine, dried over sodium sulfate and concentrated. The residue was purified via column chromatography (EtOAc: Petroleum ether = 1:10~1:5) to give (3.1 g, 89%) of the desired product.



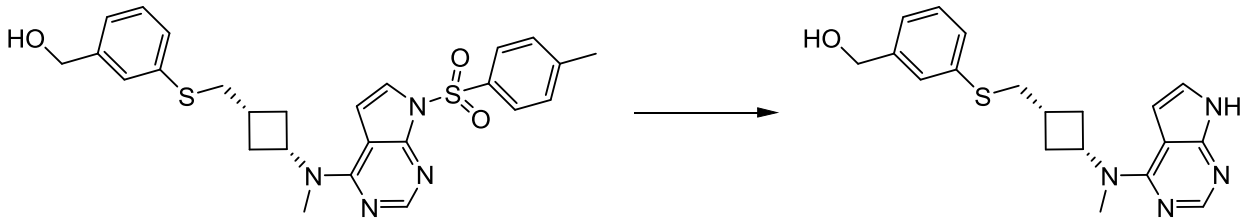
**[Cis-3-(methyl{7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl}amino)cyclobutyl]methyl methanesulfonate**

To a solution of [cis-3-(methyl{7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl}amino)cyclobutyl]methanol (3.3 g, 8.54 mmol) in anhydrous DCM (85 mL) was added methanesulfonyl chloride (1.46 g, 12.8 mmol) at 0°C followed by triethylamine (3.57 mL, 25.62 mmol). After the addition, the reaction mixture was stirred at room temperature overnight. TLC (EtOAc: Petroleum ether = 1:1) showed that the reaction was complete. The reaction mixture was partitioned between DCM and water. The aqueous layer was separated and extracted with DCM. The combined organic layers were washed with brine, dried over sodium sulfate and concentrated to give crude product. The crude product was purified via column chromatography (EtOAc: DCM = 1:20~1:10) to give (2.8 g, 71%) of the desired product.



**[3-({[Cis-3-(methyl{7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl}amino)cyclobutyl]methyl}sulfanyl)phenyl]methanol**

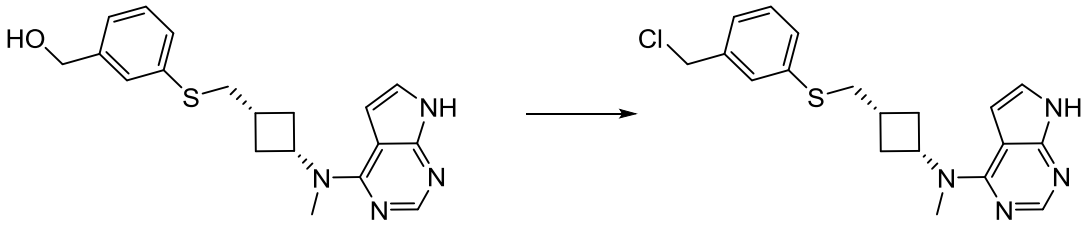
A mixture of [cis-3-(methyl{7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl}amino)cyclobutyl]methyl methanesulfonate (2.8 g, 6.03 mmol) and (3-sulfanylphenyl)methanol (0.92 g, 6.57 mmol) in anhydrous NMP (28 mL) was bubbled with nitrogen for 5 min. To the above solution was added DBU (1.4 g, 9.2 mmol). The resulting mixture was heated at 60°C for 6 h under nitrogen. TLC (EtOAc: Petroleum ether = 1:1) showed that the reaction was completed. The reaction mixture was partitioned between DCM and water. The aqueous layer was separated and extracted with DCM. The combined organic layers were washed with brine, dried over sodium sulfate and concentrated to give crude product. The residue was purified via column chromatography (EtOAc: Petroleum ether = 1:50-1:2) to give the desired product (2.4 g, 78%).



**{3-[({Cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}methyl)sulfanyl]phenyl}methanol**

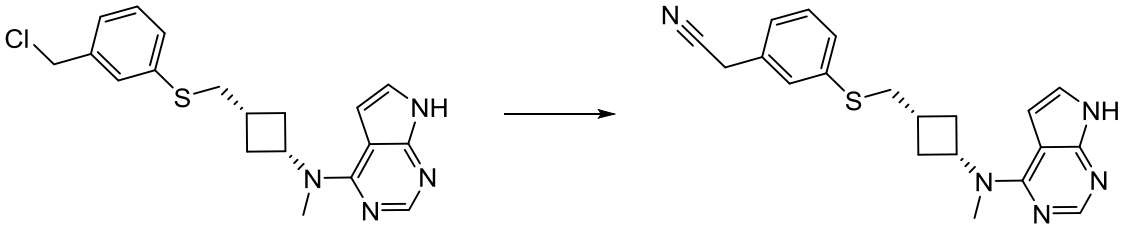
To a mixture of [3-({[cis-3-(methyl{7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl}amino)cyclobutyl]methyl}sulfanyl)phenyl]methanol (2.4 g, 4.72 mmol) in 1,4-dioxane (34 mL) was added aq. KOH (1 N, 34 mL, 34 mmol) at ambient temperature drop wise. After the

addition, the reaction mixture was heated at 100°C for 3 h. TLC (MeOH: DCM = 1:10) showed that the reaction was complete. The reaction mixture was partitioned between DCM and water. The aqueous layer was separated and extracted with DCM. The combined organic layers were washed with brine, dried over sodium sulfate and concentrated to give (1.58 g, 94%) of the desired product.




**N-[cis-3-({[3-(chloromethyl)phenyl]sulfanyl}methyl)cyclobutyl]-N-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine**

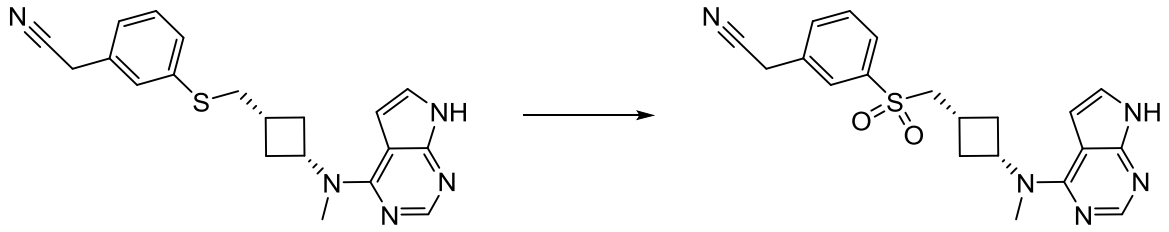
A mixture of {3-[({cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}methyl)sulfanyl]phenyl}methanol (1.58 g, 4.46 mmol) in $SOCl_2$ (15.8 mL) was stirred at ambient temperature for 3 h. TLC (MeOH: DCM = 1:10) showed that the reaction was complete. The reaction mixture was poured into ice-water. The mixture was extracted with DCM. The combined organic layers were washed with brine, dried over $Na_2SO_4$ and concentrated to give crude product. The crude product was purified via flash column (MeOH: DCM = 1%~9%) to give (1.0 g, 60%) of the desired product.




**{3-[({Cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}methyl)sulfanyl]phenyl}acetonitrile**

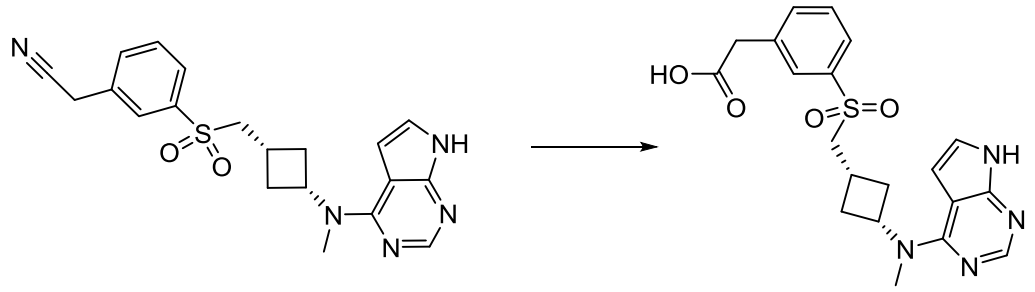
To a solution of N-[cis-3-({[3-(chloromethyl)phenyl]sulfanyl}methyl)cyclobutyl]-N-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine (1.0 g, 2.68 mmol) in DMF (27 mL) and water (14 mL) was added KCN (348 mg, 5.36 mmol). After the addition, the reaction mixture was heated at 80°C for 3 h. TLC (MeOH: DCM = 1:10) showed that the reaction was completed. The reaction mixture was partitioned between DCM and water. The aqueous layer was separated and extracted with DCM. The combined organic layers were washed with brine, dried over sodium sulfate and concentrated to give crude product. The residue was purified via preparative HPLC: using a Phenomenex® Gemini C18 250x21.2mm 8um column and a mobile phase of 40% MeCN in water (ammonia to pH 10) to 60% MeCN in water (ammonia to pH 10) to give (900 mg, 92%)

of the desired product. $^1$H NMR (400Hz, DMSO-*d6*) δ 11.61 (s, 1H), 8.10 (s, 1H), 7.37 (m, 3H), 7.17 (m, 2H), 6.60 (s, 1H), 5.08 (m, 1H), 4.06 (d, 2H), 3.29 (s, 3H), 3.18 (d, 2H), 2.24 (m, 2H), 2.10 (m, 1H), 2.03 (m, 2H). LRMS *m/z* 364 $[M+H]^+$.



**{3-[({Cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}methyl)sulfonyl]phenyl}acetonitrile**

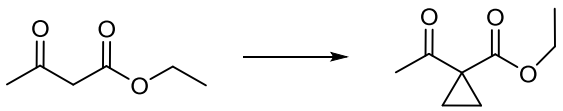
To a solution of {3-[({cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}methyl)sulfanyl]phenyl}acetonitrile (480 mg, 1.32 mmol) in THF (6 mL), EtOH (6 mL) and water (3 mL) was added oxone (6.25 g, 10.16 mmol) in one portion. The resulting mixture was stirred at room temperature for 2 hours. The mixture was filtered and the filter cake was washed with THF/EtOH/water (2/2/1, 50 mL). The filtrate was quenched with 10% $Na_2SO_3$ and neutralized with sat. $NaHCO_3$. The resulting solution was partitioned between DCM and water. The aqueous layer was separated and extracted with DCM. The combined organic layers were washed with brine, dried over sodium sulfate and concentrated to give (300 mg, 57%) of the desired product, which was used directly in next step.



**{3-[({Cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}methyl)sulfonyl]phenyl}acetic acid**

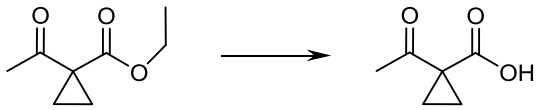
To a solution of {3-[({Cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}methyl)sulfonyl]phenyl}acetonitrile (120 mg, 0.30 mmol) in EtOH (1 mL) was added aq. 2 N KOH (1 mL, 1 mmol) at ambient temperature. The resulting mixture was heated at 80°C overnight. TLC (MeOH: DCM = 1:10) showed that the reaction was complete. The reaction mixture was acidified by aq. 1N HCl to pH 3~4. The aqueous layer was extracted with i-PrOH/$CHCl_3$ (1/3, 10 mL×3). The combined organic layers were dried over $Na_2SO_4$ and concentrated to give crude product. The crude product was purified via preparative HPLC on a

Phenomenex® Gemini 250x21.2mm 8um column using a mobile phase of 40% MeCN in water (ammonia to pH 10) to 60% MeCN in water (ammonia to pH 10) to give (26.3 mg, 21%) of the desired product. $^1$H NMR (400Hz, DMSO-*d6*): δ 11.65 (s, 1H), 8.08 (s, 1H), 7.79 (s, 1H), 7.73 (m, 1H), 7.61(m, 2H), 7.13 (d, 1H), 6.58 (d, 1H), 5.08 (m, 1H), 3.64 (s, 2H), 3.55 (m, 2H), 3.22 (s, 3H), 2.24 (m, 3H), 2.03 (m, 2H). LRMS *m/z* 415 $[M+H]^+$.



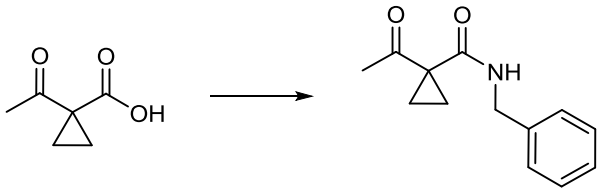
**Ethyl 1-acetylcyclopropanecarboxylate**

To a stirred solution of ethyl 3-oxobutanoate (500 g, 3.8 mol) in DMF (7.5 L) was added potassium carbonate (1063 g, 7.7 mol) and 1, 2-Dibromoethane (356.38 ml, 3.8 mol). The resulting mixture was stirred at ambient temperature for 48 h and monitored by TLC (20% EA/hexane, rf 0.8). After completion, the reaction mixture was filtered, water was added and the mixture extracted with hexane. The organic phase was washed with brine, dried over sodium sulfate and concentrated under reduced pressure to obtain a crude mass, which was vacuum distilled. The fraction boiling at 70°C, (12 mm Hg) was collected to give the desired product (300 g, 50%) as colorless liquid. $^1$H NMR (400 MHz, $CDCl_3$) δ 4.21-4.12 (2H, q), 2.44 (3H, s), 1.44 (4H, s), 1.25 (3H, t). GCMS *m/z* 156 [M].



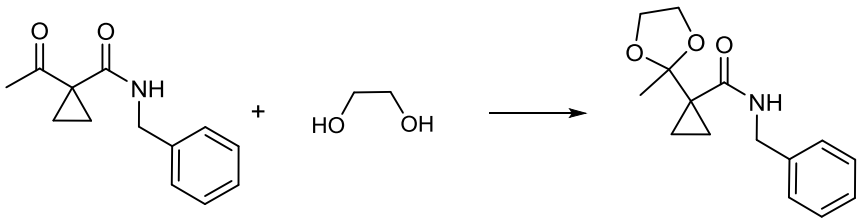
**1-Acetylcyclopropanecarboxylic acid**

To a stirred solution of 25% KOH solution (1.8 L) and TEBA (43.8 g, 0.19 mol) at 50°C was added ethyl 1-acetylcyclopropanecarboxylate (300 g, 1.92 mol) and the mixture was stirred for 5 h and the reaction was monitored by TLC (30% EA/hexane, rf 0.3). Upon completion, the reaction was cooled to ambient temperature, diluted with water (1 L) and washed with ether. The aqueous phase was acidified (pH ~2) with conc. HCl (500 mL) in an ice bath and extracted with ethyl acetate. The organic phase was washed with brine, dried over $Na_2SO_4$ and concentrated under reduced pressure to give the desired product (180 g, 73%) as a colorless liquid. $^1$H NMR (400 MHz, DMSO-*d6*) δ 12.84 (1H, s), 2.35 (3H, s), 1.32 (4H, d). LRMS: *m/z* 129 [M+H]+.



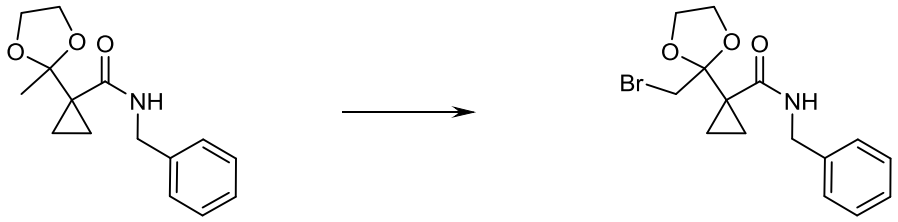
**1-Acetyl-N-benzylcyclopropanecarboxamide**

Ethyl chloroformate (169 mL, 1.76 mol) was added drop wise to a stirred solution of 1-acetylcyclopropanecarboxylic acid (205 g, 1.6 mol) in DCM (2 L) at –40°C. After 15 minutes of stirring triethylamine (267 mL, 1.9 mol) was added drop-wise at –30°C and the mixture stirred for another 40 minutes. Benzylamine (174.68 ml, 1.6 mol) was added drop wise at -20°C and the mixture stirred for 2 h. The reaction was monitored by TLC (30% EA/hexane, rf 0.5). Upon completion, the temperature was allowed to rise to 0°C and 1N HCl (400 mL) was added. The organic phase was separated, washed with sodium bicarbonate, brine, and dried over $Na_2SO_4$. Concentration under reduced pressure afforded a crude mass, which was purified by column chromatography, followed by crystallization with ethyl acetate-hexane to give (300 g, 86%) of the desired product as white crystals. $^1$H NMR (400 MHz, $CDCl_3$) δ 9.21 (1H, br s), 7.33-7.26 (5H, m), 4.49 (2H, d), 1.95 (3H, s), 1.90-1.87 (2H, m), 1.53-1.50 (2H, m). LRMS *m/z* 218 [M+H]+.



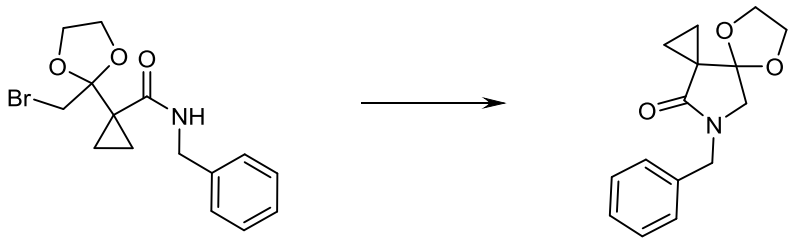

**N-benzyl-1-(2-methyl-1,3-dioxolan-2-yl)cyclopropanecarboxamide**

To a stirred solution of 1-acetyl-N-benzylcyclopropanecarboxamide (300 g, 1.38 mol) in toluene (2.8 L) was added p-toluenesulfonic acid (13.1 g, 0.07 mol) and 1, 2-ethanediol (156 ml, 2.76 mol). The mixture was refluxed using a Dean-Stark apparatus for 16 h and monitored by TLC (30% EA/hexane, rf = 0.6). The reaction mixture was cooled to ambient temperature, and water was added. The organic phase was separated, and the aqueous layer was extracted with ethyl acetate. The combined organic phase was washed with sodium bicarbonate solution, followed by brine and dried over sodium sulfate. Evaporation of solvent under reduced pressure followed by purification on silica gel (100-200 mesh, ethyl acetate:hexane, 1:3) gave the desired product (250 g, 57%) as a pale yellow liquid. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.75 (1H, br s), 7.34-7.26 (5H, m), 4.47 (2H, d), 3.93 (4H, s), 1.48 (3H, s), 1.13-1.10 (2H, m), 0.87-0.84 (2H, m). LRMS *m/z* 262 [M+H]+.



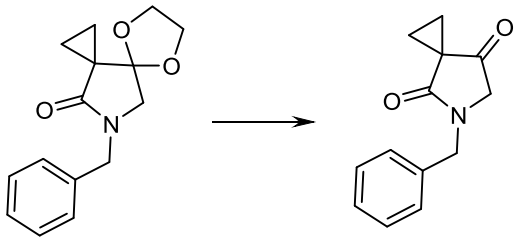

**N-benzyl-1-[2-(bromomethyl)-1,3-dioxolan-2-yl]cyclopropanecarboxamide**

A mixture of bromine (54 ml, 1.03 mol) in DCM (4L) was added drop wise to a solution of N-benzyl-1-(2-methyl-1,3-dioxolan-2-yl)cyclopropanecarboxamide (300 g, 1.14 mol) in DCM (6L) at 0°C, and the mixture was stirred for 4h at ambient temperature and monitored by LCMS. After completion, 10% aq. sodium thiosulfate solution (750 ml) was added to the reaction mixture, and the organic phase was separated, washed with brine, dried over sodium sulfate and concentrated under reduced pressure to give the desired product (390 g, 100%) as pale yellow liquid, which was used to next step without purification. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.68 (1H, s), 7.38-7.24 (5H, m), 4.45 (2H, d), 4.2 (2H, m), 4.03 (2H, m), 3.73 (2H, s), 1.20 (2H, m), 0.93 (2H, m). LRMS *m/z* 342 [M+H]+.



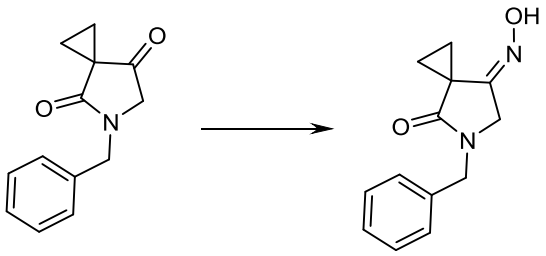

**10-Benzyl-5,8-dioxa-10-azadispiro[2.0.4.3]undecan-11-one**

A solution of N-benzyl-1-[2-(bromomethyl)-1,3-dioxolan-2-yl]cyclopropanecarboxamide (390 g, 1.15 mol) in NMP (2 L) was added drop wise to a suspension of 60% NaH* (60 g, 1.5 mol) in NMP (2 L) at 0°C and the mixture was stirred for 20h at ambient temperature. Completion of the reaction was monitored by TLC (30% EA / hexane, rf 0.4). The reaction mixture was poured in ice-cold water and extracted with ethyl acetate. The organic phase was washed with water and brine, then dried over sodium sulfate and concentrated under reduced pressure to give the desired product (320 g, 107%) as dark brown liquid, which was used in the next step without purification. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.21-7.34 (5H, m), 4.53 (2H, s), 3.84 (4H, s), 3.35 (2H, s), 1.22 (4H, m). LRMS *m/z* 259.9 [M+H]+.



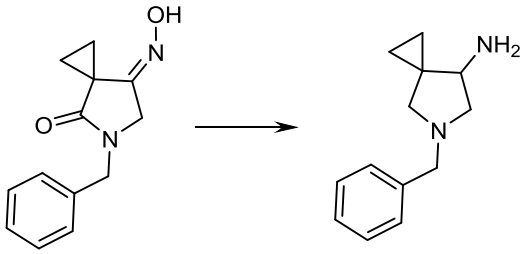
**5-Benzyl-5-azaspiro[2.4]heptane-4,7-dione**

A mixture of 10-benzyl-5,8-dioxa-10-azadispiro[2.0.4.3]undecan-11-one (320 g, 1.08 mol), acetone (5 L) and 1N-HCl (600 mL) was refluxed for 2 h. The reaction mixture was concentrated and the residue was dissolved in ethyl acetate, washed with water, brine and dried over sodium sulfate. The organic phase was concentrated under reduced pressure to obtain (270 g, 116%) of the desired product as light yellow solid, which was used in the next step without purification. $^{1}$H NMR (400 MHz, DMSO-*d6*) δ 7.38-7.16 (5H, m), 3.68-3.67 (2H, m), 2.69 (2H, s), 1.93-1.89 (2H, m), 1.54-1.46 (2H, m). LRMS *m/z* 216 [M+H]+.



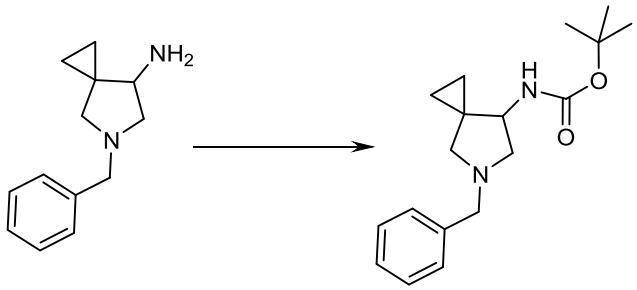
**(E)-5-benzyl-7-(hydroxyimino)-5-azaspiro[2.4]heptan-4-one**

Hydroxylamine hydrochloride (197 g, 1.94 mol) was added portion wise to a solution of 5-benzyl-5-azaspiro[2.4]heptane-4,7-dione (270 g, 1.25 mol), TEA (192 mL, 2.01 mol) and ethanol (4.5 L) in an ice bath and the mixture allowed to warm and stir for 2h at ambient temperature. The reaction mixture was concentrated under reduced pressure to obtain a crude mass, which was washed with water and filtered. The solid mass was further washed with ethyl acetate and dried under reduced pressure to give (140 g, 48%) of the desired product as white solid. $^{1}$H NMR (400 MHz, DMSO-*d6*) δ 10.85 (1H, s), 7.39-7.24 (5H, m), 4.51 (2H, s), 4.01 (2H, s), 1.36-1.34 (2H, t), 1.22-1.21 (2H, t). LRMS *m/z* 231 [M+H]+.



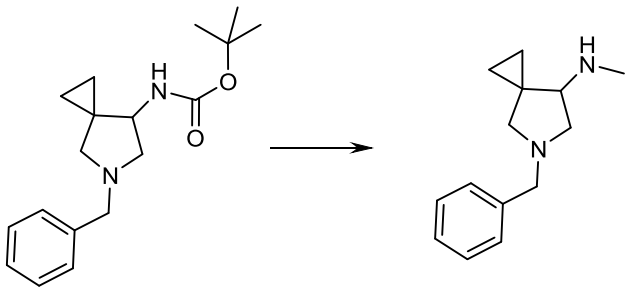

**5-Benzyl-5-azaspiro[2.4]heptan-7-amine**

A solution of (E)-5-benzyl-7-(hydroxyimino)-5-azaspiro[2.4]heptan-4-one (140 g, 0.6 mol) in THF (2.5 L) was added slowly to a suspension of LAH (142 g, 3.76 mol) in THF (4.5 L) at 0°C. The mixture was allowed to warm to room temperature and then refluxed overnight. The reaction was cooled in an ice bath and carefully quenched with a solution of water/THF (142 ml: 1.4 L), 15% aq. NaOH solution (142 ml) and water (300 ml). The mixture was stirred overnight at ambient temperature and the precipitate was filtered through diatomaceous earth. The filtrate was concentrated under reduced pressure to obtain a colorless liquid. The liquid was dissolved in hexane and filtered. The hexane solution was concentrated under reduced pressure to give the desired crude product (110 g) as colorless liquid. LRMS *m/z* 203 [M+H]+.



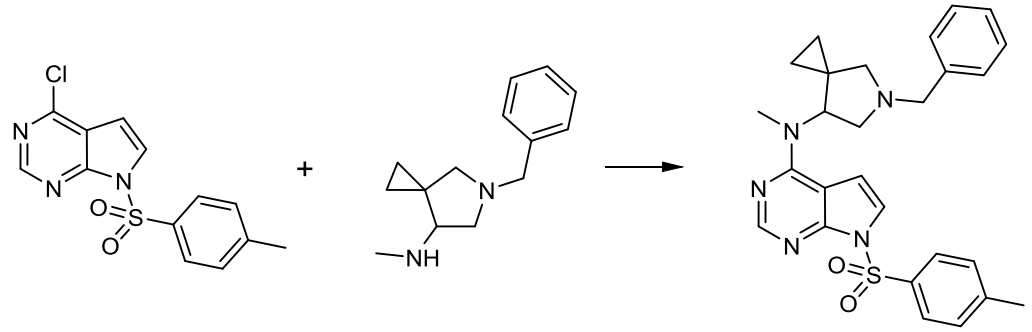

**Tert-butyl (5-benzyl-5-azaspiro[2.4]heptan-7-yl)carbamate**

Di- tert- butyl dicarbonate (140 ml, 0.65 mol) was added portion wise to a mixture of 5-benzyl-5-azaspiro[2.4]heptan-7-amine (110 g, 0.54 mol) and TEA (152 ml, 1.09 mol) in DCM (3.4 L) with cooling in an ice bath. The mixture was allowed to warm and stirred at ambient temperature overnight. Water (2 L) was added to the reaction mixture and the organic phase was separated, washed with sodium bicarbonate solution, dried over sodium sulfate and concentrated under reduced pressure to obtain crude material. The crude product was purified by column chromatography using alumina (ethyl acetate: hexane, 1:4) to give the desired product (125 g, 76%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.30 (5H, m), 4.91-4.93 (1H, d), 3.80 (1H, s), 3.56 (2H, q), 2.85 (1H, m) 2.65 (2H, d), 2.33 (1H, d), 1.40 (9H, s), 0.41- 0.78 (4H, m). LRMS *m/z* 303 [M+H]+.



**5-benzyl-N-methyl-5-azaspiro[2.4]heptan-7-amine**

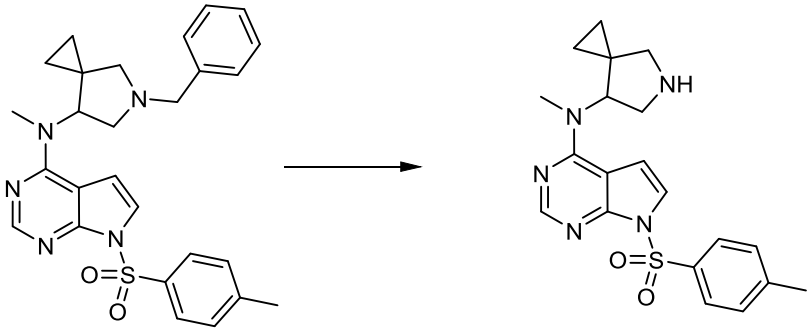
A mixture of tert-butyl (5-benzyl-5-azaspiro[2.4]heptan-7-yl)carbamate (125 g, 0.41 mol) in THF (2.2 L) was added drop wise to a suspension of LAH (79 g, 2.07 mol) in THF (2.2 L) at 0°C. After the addition was complete, the mixture was refluxed for 5 h. The reaction mixture was carefully quenched with a mixture of water and THF (1:10, 880 ml), 15% aq. NaOH solution (80 ml) and water (160 ml) in an ice bath. The mixture was stirred overnight at room temperature and the precipitate was filtered through diatomaceous earth. The filtrate was concentrated under reduced pressure to obtain a crude mass which was purified by column chromatography using silica-gel (100-200 mesh) and 5% methanol in DCM as an eluent to give the desired product (79 g, 88%) as an oil. $^1$H NMR (400 MHz, DMSO-*d6*) δ 7.19-7.27 (5H, m), 3.55 (2H, m), 2.92 (1H, m), 2.82 (1H, t), 2.37-2.34 (3H, m), 2.17 (3H, s), 0.81-0.78 (1H, m), 0.49-0.28 (3H, m). LRMS *m/z* 217 [M+H]+.



**N-(5-benzyl-5-azaspiro[2.4]heptan-7-yl)-N-methyl-7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine**

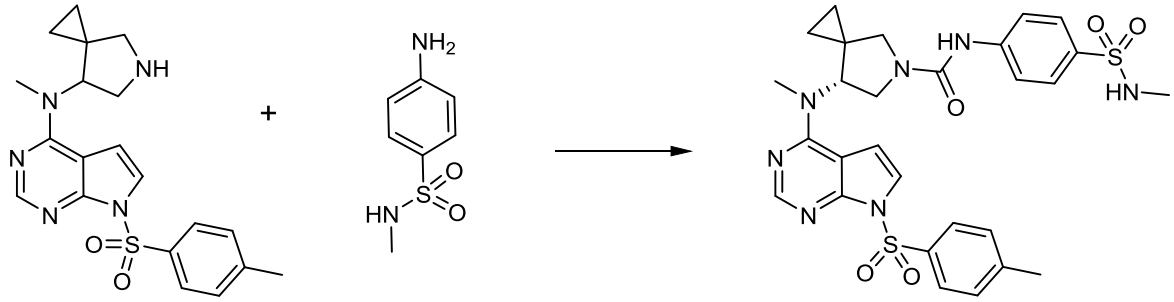
4-Chloro-7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidine (10.26 g, 33.34 mmol), 5-benzyl-N-methyl-5-azaspiro[2.4]heptan-7-amine (7.21 g, 33.3 mmol), and DIEA (15 mL, 86 mmol) were combined in 1-butanol (30 mL) and heated to 85°C for 6 h, then stirred at ambient temperature for 48 h. The reaction mixture was filtered through a plug of silica and the silica washed with 50/50 ethyl acetate/heptane. Evaporation of the solvent afforded a dark yellow oil that was purified by flash column chromatography to give the desired product (10.9 g, 67%) as a light yellow solid. $^1$H NMR (400 MHz, DMSO-*d6*) δ 0.17 - 0.32 (m, 1 H) 0.50 - 0.65 (m, 2 H) 0.76 (dd, *J*=13.66, 4.10 Hz, 1 H) 2.35 (s, 3 H) 2.41 (d, *J*=8.88 Hz, 1 H) 2.68 (d, *J*=8.88 Hz, 1 H) 2.73 - 2.95 (m, 2 H) 3.38 (s, 3 H) 3.58 (s, 2 H) 5.40 (br s, 1 H) 6.92 (d, *J*=4.10 Hz, 1 H) 7.18 -

7.28 (m, 1 H) 7.32 (d, *J*=4.44 Hz, 4 H) 7.42 (d, *J*=8.53 Hz, 2 H) 7.59 (d, *J*=4.10 Hz, 1 H) 7.97 (d, *J*=8.53 Hz, 2 H) 8.13 (s, 1 H).



**N-(5-azaspiro[2.4]heptan-7-yl)-N-methyl-7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine**

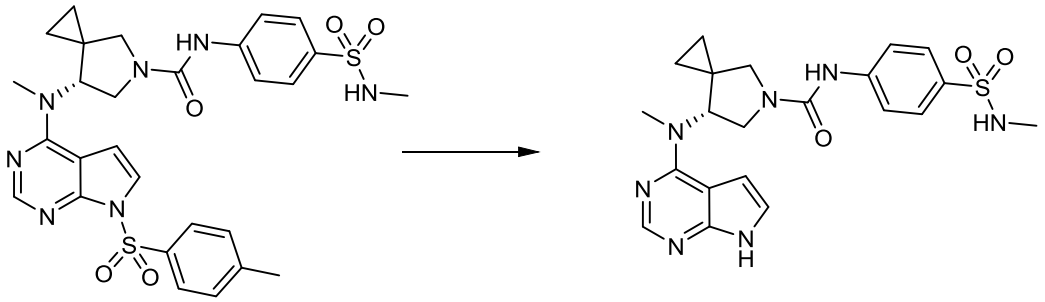
N-(5-benzyl-5-azaspiro[2.4]heptan-7-yl)-N-methyl-7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine (550 mg, 1.13 mmol) was dissolved in methanol (25 mL). Palladium hydroxide (500 mg, 3.5 mmol) and 1,4-cyclohexadiene (922 mg, 11.3 mmol) were added. The reaction was stirred at 60°C for 2 h. The reaction mixture was cooled to ambient temperature and filtered through diatomaceous earth. Evaporation of the methanol afforded the desired product (300 mg, 67%) as a white foam.



**(7R)-7-(methyl{7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl}amino)-N-[4-(methylsulfamoyl)phenyl]-5-azaspiro[2.4]heptane-5-carboxamide**

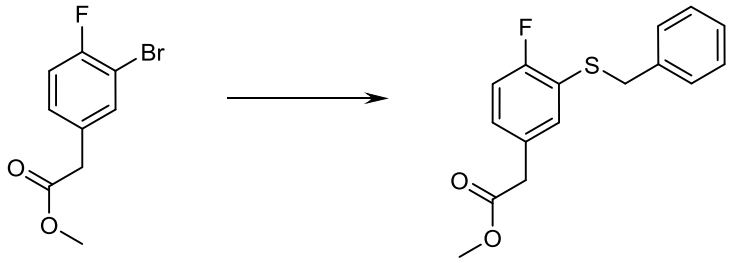
4-Amino-N-methylbenzenesulfonamide (250 mg, l.3 mmol) was combined with phosgene (85.4 mg, 0.855 mmol (20% wt. solution in toluene)). The reaction mixture was placed in a screw cap vial and heated at 70°C for 6 h. The toluene was evaporated and the remaining white solid was dissolved in dichloromethane. N-(5-azaspiro[2.4]hept-7-yl)-N-methyl-7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine (182 mg, 0.855 mmol) and triethylamine (260 mg, 2.56 mmol) were added. The reaction was stirred overnight at ambient temperature. The DCM was evaporated, and the resulting solid was dissolved in DMSO.

Purification by preparative reverse phase HPLC afforded the desired product as a white solid. Chiral separation using AS-H 30 x 250 mm column, eluent 50% methanol at 70 mL/min to give (100 mg, 70%) of the desired product. LRMS *m/z* 610 $[M+H]^+$.



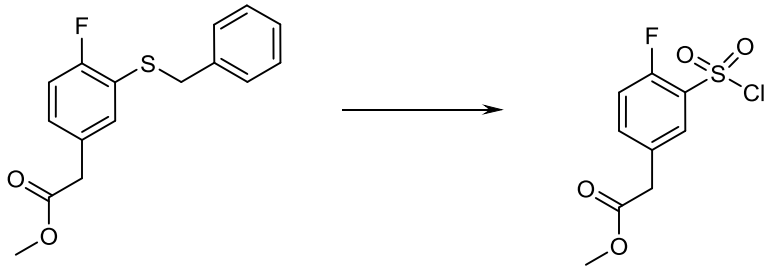
**(R)-7-[Methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]-N-[4-(N-methylsulfamoyl)phenyl]-5-azaspiro[2.4]heptane-5-carboxamide**

(R)-7-(Methyl{7-[(4-methylphenyl)sulfonyl]-7H-pyrrolo[2,3-d]pyrimidin-4-yl}amino)-N-[4-(N-methylsulfamoyl)phenyl]-5-azaspiro[2.4]heptane-5-carboxamide (400 mg, 0.656 mmol) was dissolved in methanol 5 mL). Lithium hydroxide (32.1 mg, 1.31 mmol) in water (1 mL) was added. The reaction was stirred for 3 h at 60°C, cooled to room temperature and the solvent evaporated. Addition of water resulted in the formation of a white solid which was filtered and washed with water. LCMS of the resulting solid showed the desired product and an impurity (M+14). The compound was purified by flash column chromatography on amino silica. The fractions were combined and evaporated to give the desired product (100 mg, 33%). $^1$H NMR (400 MHz, DMSO-*d6*) δ 11.70 (br s, 1H), 8.69 (s, 1H), 8.09 (s, 1H), 7.72-7.78 (m, 2H), 7.64 (d, *J*=8.88 Hz, 2H), 7.23 (q, *J*=5.01 Hz, 1H), 7.14-7.19 (m, 1H), 6.63 (dd, *J*=1.54, 3.24 Hz, 1H), 5.28 (d, *J*=6.14 Hz, 1H), 4.04 (dd, *J*=7.34, 11.78 Hz, 1H), 3.75-3.86 (m, 2H), 3.39-3.46 (m, 1H), 3.34 (s, 6H), 2.38 (d, *J*=5.12 Hz, 3H), 0.89-0.96 (m, 1H), 0.76-0.84 (m, 2H), 0.62-0.69 (m, 1H); peak obscured by water.



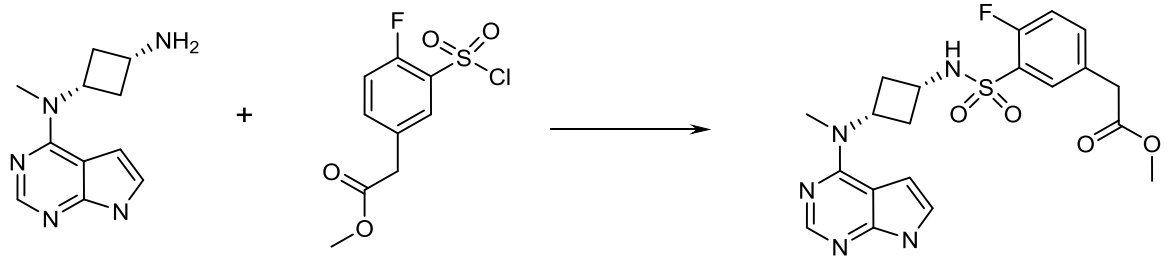
**Methyl [3-(benzylsulfanyl)-4-fluorophenyl]acetate**

Benzylthiol (600 mg, 4.83 mmol), DIPEA (2.0 mL, 10.07 mmol) and Xantphos (230 mg, 0.40 mmol) were added to a solution of methyl (3-bromo-4-fluorophenyl)acetate in dioxane (10 mL). The resulting solution was degassed for 10 min with nitrogen and then $Pd_2(dba)_3$ was added and the reaction mixture again degassed for 10 min. The solution was heated and stirred at 100°C for 16 h. Reaction mixture was filtered through diatomaceous earth and concentrated. The residue was purified by column chromatography on silica gel, by gradient elution with 5-10% EtOAc in petroleum ether to give (700 mg, 63%) of the desired product. $^1$HNMR (300 MHz, DMSO-*d6*) δ 7.18-7.42 (overlapping, 6H), 7.18 (d, 2H), 4.11 (s, 2H), 3.63 (s, 2H), 3.61 (s, 3H).



**Methyl [3-(chlorosulfonyl)-4-fluorophenyl]acetate**

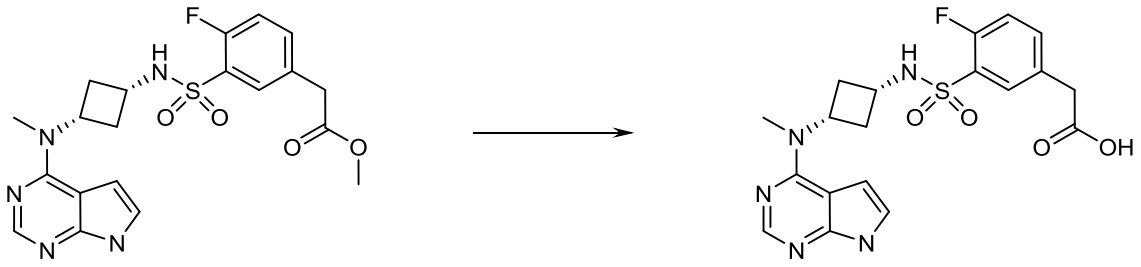
1,3-dichloro-5,5-dimethylhydantion (1.0 g, 5.06 mmol) was added to a solution of methyl [3-(benzylsulfanyl)-4-fluorophenyl]acetate (700 mg, 2.4 mmol) in acetonitrile (40 mL), acetic acid (2 mL) and water (1 mL).The resulting mixture of was stirred at 0°C for 2 h. The solvents were evaporated, and the residue diluted with water and extracted with ethyl acetate, the organic layer was washed with sat $NaHCO_3$ solution and brine. The organic layer was dried over sodium sulfate, filtered and concentrated to give (300 mg, 47%) of the desired product which was used without further purification.



**Methyl 2-[4-fluoro-3-(N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}sulfamoyl)phenyl]acetate**

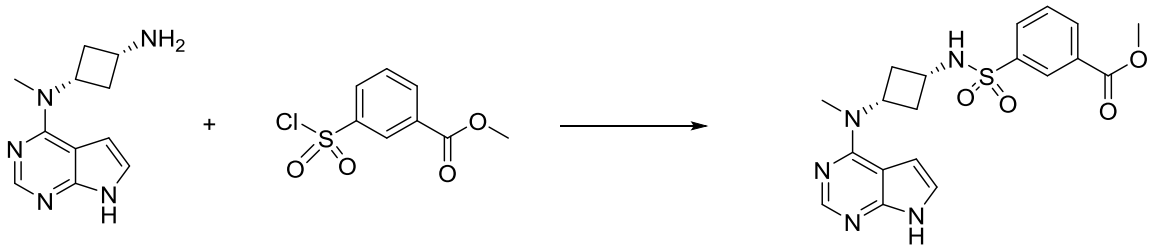
DIPEA (0.4 mL, 2.24 mmol) and methyl 2-(3-(chlorosulfonyl)-4-fluorophenyl)acetate (300 mg, 1.12 mmol) were added to a solution of cis-N-methyl-N-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)cyclobutane-1,3-diamine (243 mg, 1.12 mmol) in DMF (10 mL) and the reaction mixture stirred at ambient temperature for 16 h. The volatiles were concentrated to a volume of 10 mL and ice cold water (25 mL) was added and the mixture stirred for 30 min, then extracted with ethyl acetate. The organic layer was washed with water followed by brine, dried over sodium

sulfate and concentrated. The residue was purified by column chromatography on silica (100-200 mesh), eluting with 80-90 % ethyl acetate in petroleum ether to give (300 mg, 60%) of the desired product**.** LRMS *m/z* 448 $[M+H]^+$.



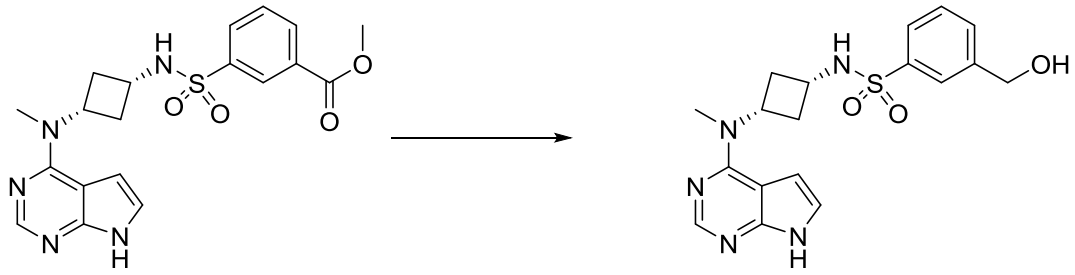
**2-[4-Fluoro-3-(N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}sulfamoyl)phenyl]acetic acid**

Lithium hydroxide (56 mg, 1.34 mmol) was added to a solution of methyl 2-[4-fluoro-3-(N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}sulfamoyl)phenyl]acetate (300 mg, 0.671 mmol) in THF/methanol/water (8mL/4mL/2mL) at ambient temperature. The resulting mixture was stirred at 50°C for 4 h, concentrated, diluted with water and neutralized with 1N HCl. Extracted with dichloromethane and the organic layer washed with water and brine solution, dried over sodium sulfate and concentrated. The residue was purified by preparative HPLC to give (45 mg, 15%) of the desired product. $^1$H NMR (400 MHz, DMSO-*d6*) δ 11.59 (br s, 1H), 8.30 (br s, 1H), 8.06 (br s, 1H), 7.71 (br s, 1H), 7.54 (br s, 1H), 7.35 (br s, 1H), 7.11 (br s, 1H), 6.58 (br s, 1H), 4.84 (br s, 1H), 3.62 (br s, 3H), 3.19 (br s, 3H), 2.31 (br s, 2H), 2.15 (br s, 2H). LRMS *m/z* 434 $[M+H]^+$.



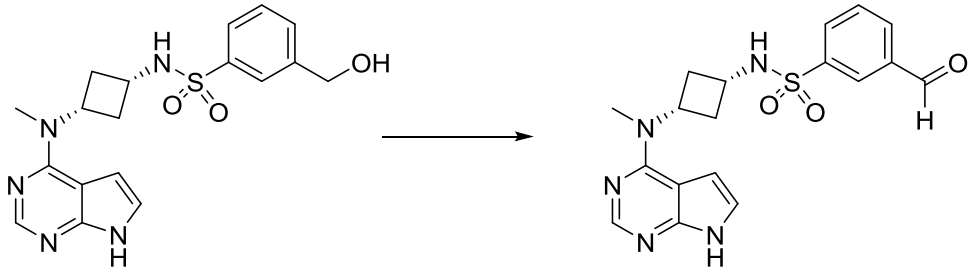
**Methyl 3-(N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}sulfamoyl)benzoate**

To cis-N-methyl-N-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)cyclobutane-1,3-diamine (0.9 g, 4.1 mmol) in dimethylformamide (8 mL) was added diisopropylethylamine (1.1 mL, 6.31 mmol) and methyl 3-(chlorosulfonyl)benzoate (1.1 g, 4.68 mmol). The mixture was stirred at ambient temperature overnight then diluted with ethyl acetate and washed with brine. The aqueous phase was back extracted with ethyl acetate. The combined organic phase was dried over magnesium sulfate and concentrated to dryness to give the desired product (1.997 g, 116%) which was used without further purification.



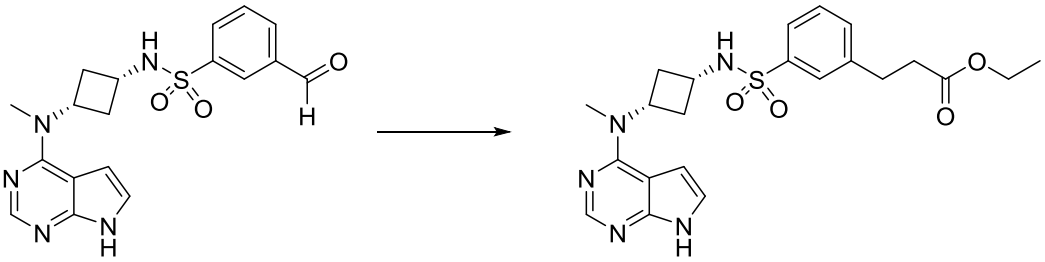
**3-(Hydroxymethyl)-N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}benzenesulfonamide**

To methyl 3-(N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}sulfamoyl)benzoate (1.99 g, 7.79 mmol) in dry tetrahydrofuran (25 mL) was added lithium aluminum hydride (0.4 g, 10.54 mmol). The mixture was stirred overnight at ambient temperature, then quenched with a minimum amount of water until gas evolution ceased. The mixture was diluted with ethyl acetate and stirred for 4 h, then filtered through diatomaceous earth. The solids were washed twice with ethyl acetate. The filtrates were concentrated to give the desired product (1.418 g, 77%). LRMS *m/z* 388 $[M+H]^+$.



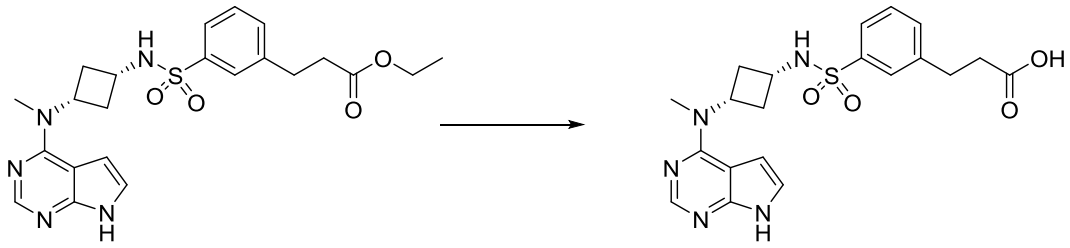
**3-Formyl-N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}benzenesulfonamide**

To a mixture of 3-(hydroxymethyl)-N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}benzenesulfonamide (0.525 g, 1.35 mmol) in dichloromethane (10 mL) was added Dess-Martin periodinane (0.915 g, 2.15 mmol). The mixture was stirred at ambient temperature for 3 h and quenched with the addition of saturated sodium bicarbonate. The mixture was stirred rapidly for 30 minutes. The phases were separated and the aqueous phase back extracted with dichloromethane. The combined organic extracts were washed with saturated sodium bicarbonate, sodium persulfate solution, brine, and dried over magnesium sulfate. The filtrates were concentrated to give the desired product (500 mg, 96%).



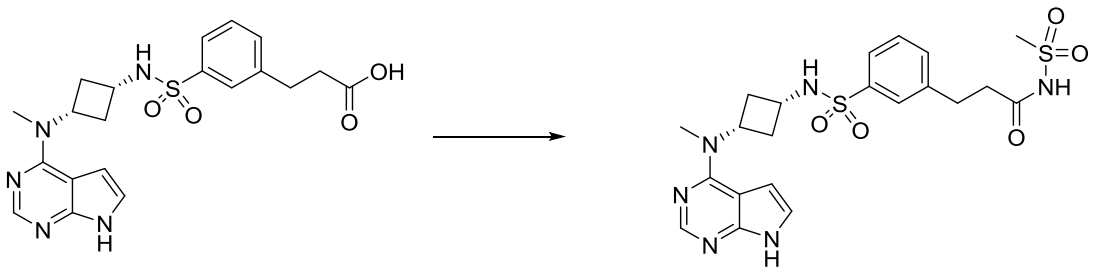
**Ethyl 3-[3-(N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}sulfamoyl)phenyl]propanoate**

To a mixture of 3-formyl-N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}benzenesulfonamide (500 mg, 1.35 mmol) in ethanol (15 mL) was added 6 M potassium carbonate (0.45 mL) and ethyl (diethoxyphosphoryl)acetate (0.535 ml, 2.11 mmol). The mixture was stirred at ambient temperature overnight, concentrated and the residue purified via flash chromatography using 1-4% methanol in chloroform. The intermediate was dissolved in ethanol (50 mL) and platinum oxide (5 mg) was added. The reaction mixture was hydrogenated under 50 psi hydrogen for 18 h. The mixture was filtered through diatomaceous earth and concentrated to give (270 mg, 44%) of the desired product.



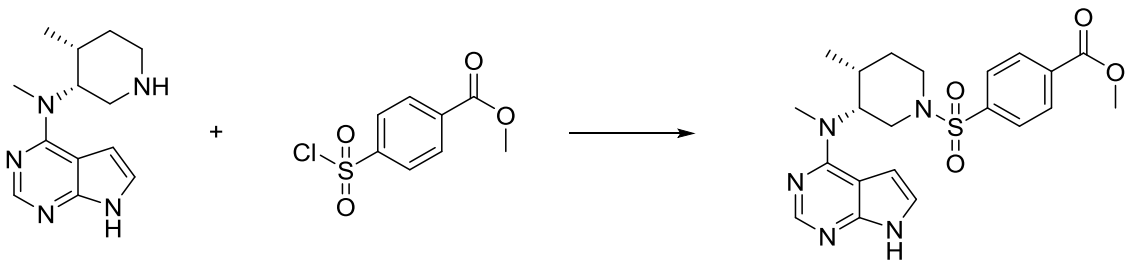
**3-[3-(N-{Cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}sulfamoyl)phenyl]propanoic acid**

To ethyl 3-[3-(N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}sulfamoyl)phenyl]propanoate (40 mg, 0.09 mmol) in THF:MeOH:water (2:2:1) was added lithium hydroxide monohydrate (50 mg, 11.9 mmol). The mixture was stirred at ambient temperature for 4 h, and then acidified to pH 4 with 1 M HCl. The mixture was extracted with ethyl acetate and the aqueous phase was back extracted with ethyl acetate. The combined ethyl acetate extracts were dried over magnesium sulfate and concentrated under reduced pressure to give (23 mg, 61%) of the desired product.



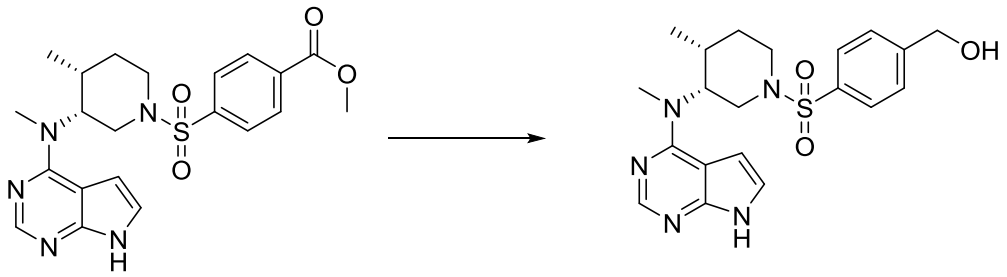
**3-[3-(N-{Cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}sulfamoyl)phenyl]-N-(methylsulfonyl)propanamide**

To 3-[3-(N-{cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}sulfamoyl)phenyl]propanoic acid (50 mg, 0.12 mmol) in dichloromethane (1 mL) was added methane sulfonamide (16 mg, 0.17 mmol) EDC (30 mg, 0.16 mmol), DMAP (21 mg, 0.17 mmol) and triethylamine (0.04 mL, 0.29 mmol). The mixture was stirred at ambient temperature for 48 h. The mixture was purified via flash chromatography using 2-7% methanol in chloroform to give the desired product (48.6 mg, 80%) which appeared to contain some of the triethylammonium salt of the desired product and some methane sulfonamide. $^1$H NMR (400 MHz, DMSO-*d6* with 1 drop $D_2O$) δ 8.28 (s, 1H), 8.19 (d, *J*=6.63 Hz, 1H), 7.64-7.72 (m, 2H), 7.49-7.58 (m, 2H), 7.41 (d, *J*=3.12 Hz, 1H), 6.98 (d, *J*=7.81 Hz, 1H), 6.91 (d, *J*=3.12 Hz, 1H), 4.64 (br s, 1H), 3.59 (br s, 1H), 3.28 (s, 3H), 3.18 (d, *J*=3.51 Hz, 5H), 3.09 (q, *J*=7.28 Hz, 3H), 2.91-2.99 (m, 2H), 2.65 (t, *J*=7.41 Hz, 2H), 2.41 (d, *J*=7.80 Hz, 2H), 2.12 (d, *J*=10.93 Hz, 2H), 1.20 (t, *J*=7.22 Hz, 4H). LRMS *m/z* 507 $[M+H]^+$.



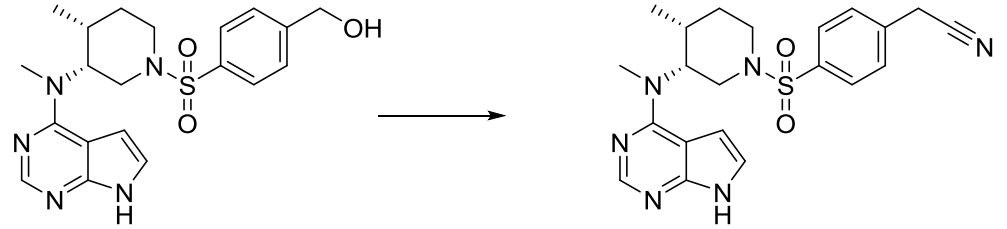
**Methyl 4-({(3R,4R)-4-methyl-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]piperidin-1-yl}sulfonyl)benzoate**

To N-methyl-N-[(3R,4R)-4-methylpiperidin-3-yl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine (500 mg, 2.06 mmol) in DMF (15 mL) was added diisopropylethylamine (0.71 mL, 4.08 mmol), DMAP (25 mg, 0.203 mmol) and methyl 4-(chlorosulfonyl)benzoate (502 mg, 2.14 mmol). The mixture was stirred at ambient temperature overnight, diluted with ethyl acetate and washed 3 times with water. The organic phase was dried over magnesium sulfate and concentrate. The residue was purified via flash chromatography using 5% methanol in DCM to give the desired product (758.6 mg, 84%). LRMS *m/z* 444 $[M+H]^+$.



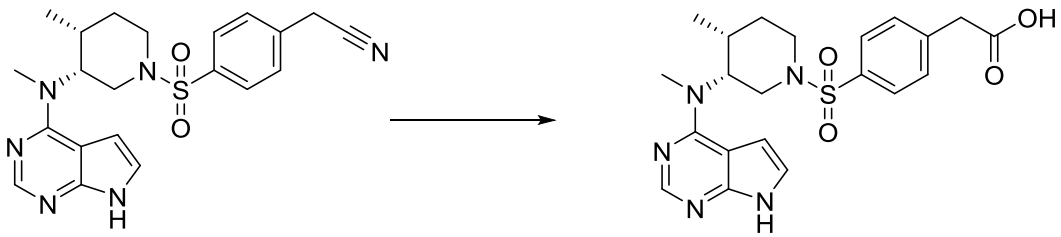
**[4-({(3R,4R)-4-Methyl-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]piperidin-1-yl}sulfonyl)phenyl]methanol**

To methyl 4-({(3R,4R)-4-methyl-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]piperidin-1-yl}sulfonyl)benzoate (700 mg, 1.578 mmol) in tetrahydrofuran (16 mL) was added lithium aluminum hydride (0.12 g, 3.16 mmol). The mixture was stirred at ambient temperature overnight, then carefully quenched with water and saturated sodium bicarbonate. The mixture was extracted three times with ethyl acetate. The ethyl acetate extracts were dried over magnesium sulfate and concentrated to give the desired product (476.6 mg, 73%). LRMS *m/z* 416 $[M+H]^+$.



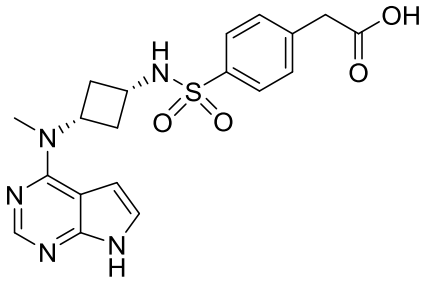
**2-[4-({(3R,4R)-4-Methyl-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]piperidin-1-yl}sulfonyl)phenyl]acetonitrile**

A mixture of [4-({(3R,4R)-4-methyl-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]piperidin-1-yl}sulfonyl)phenyl]methanol (450 mg, 1.08 mmol) and thionyl chloride (11 mL) at 0°C was stirred for 2.5 h. The mixture was concentrated under reduced pressure and the residue was diluted with ethyl acetate and washed with saturated sodium bicarbonate. The aqueous phase was extracted with ethyl acetate and the combined ethyl acetate extracts were dried over magnesium sulfate and concentrated. The residue was dissolved in DMF (3 mL) and water (0.6 mL). To this was added potassium cyanide (147 mg, 2.26 mmol). The mixture was heated to 80°C overnight, cooled to room temperature and diluted with ethyl acetate then washed three times with brine and twice with water. The organic phase was dried over magnesium sulfate and concentrated under reduced pressure. The residue was purified via flash chromatography using 2% methanol in DCM to give the desired product (193.3 mg, 42%). LRMS *m/z* 425 $[M+H]^+$.



**2-[4-({(3R,4R)-4-Methyl-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]piperidin-1-yl}sulfonyl)phenyl]acetic acid**

To 2-[4-({(3R,4R)-4-methyl-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]piperidin-1-yl}sulfonyl)phenyl]acetonitrile (215 mg, 0.506 mmol) in ethanol (4.5 mL) was added water (0.5 mL) and potassium hydroxide (0.144 g, 2.53 mmol). The mixture was heated to 85°C overnight then acidified with 1N HCl to pH 4 and extracted twice with ethyl acetate. The organic extracts were dried over magnesium sulfate and concentrated under reduced pressure to give (130 mg, 53%) of the desired product. $^1$H NMR (400 MHz, DMSO-*d6*) δ 12.52 (br s, 1H), 11.69 (br s, 1H), 8.11 (s, 1H), 7.74 (d, *J*=8.20 Hz, 2H), 7.54 (d, *J*=7.80 Hz, 2H), 7.15 (br s, 1H), 6.58 (br s, 1H), 5.14 (br s, 1H), 3.74 (s, 2H), 3.50 (dd, *J*=5.46, 12.10 Hz, 2H), 3.42-3.46 (m, 3H), 3.06-3.14 (m, 1H), 2.81 (br s, 1H), 2.13 (br s, 1H), 1.74 (br s, 2H), 0.85 (d, *J*=7.02 Hz, 3H). LRMS *m/z* 444 $[M+H]^+$.



**[4-({Cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclobutyl}sulfamoyl)phenyl]acetic acid**

The desired compound was prepared in the same manner as the preparation of the 4-methyl-3-aminopiperidine analog above. $^1$H NMR (400 MHz, DMSO-*d6*) δ 12.44 (br s, 1H), 11.62 (br s, 1H), 8.06 (s, 1H), 7.98 (d, *J*=8.98 Hz, 1H), 7.76 (d, *J*=8.20 Hz, 2H), 7.47 (d, *J*=8.20 Hz, 2H), 7.09-7.15 (m, 1H), 6.59 (d, *J*=1.56 Hz, 1H), 4.76-4.88 (m, 1H), 3.69 (s, 2H), 3.46-3.56 (m, 1H), 3.16 (s, 3H), 2.21-2.36 (m, 2H), 1.96-2.09 (m, 2H). LRMS *m/z* 416 $[M+H]^+$.

NOTE: * These are two examples where NaH is used with DMF or NMP. This is a hazardous reaction mixture which should be avoided.

**Preparation of PBMC**

Frozen PBMCs were purchased from STEMCELL (Cat. No 70025.3, Vancouver, BC, Canada). PBMC were thawed at 37$^{o}$C and transferred to 10ml warm D-PBS/10% FBS in a 50-mL conical tube, and centrifuged at 1,200 RPM at room temperature for 5 min. Then the supernatant was aspirated. Then cells were resuspended in 3ml warm FBS and incubated 37 $^{o}$C in a tissue culture incubator for 1.5 to 2 h.47ml D-PBS was added to PBMC suspension and cells were centrifuged at 1,200 RPM at room temperature for 5 min, and the supernatant was aspirated. Cells were resuspended in 21ml warm RPMI medium and passed through a 40μM cell strainer.

**Dextran and small molecule delivery by CellSqueeze®**

Delivery of 3kDa dextran (ThermoFisher) and small molecules to human primary PBMCs was performed using CellSqueeze®, a microfluidics device and pressure system (SQZ Biotechnologies); chip designs used included 30-4 and 10-4 where X-Y denotes channels with a single constriction site of dimensions Xμm long and Yμm diameter. Human primary PBMCs were suspended at $20 \times 10^6$ cells/mL to $25 \times 10^6$ cells/mL in delivery buffer with 0.2% DMSO (ThermoFisher) 150 μg/mL of Pacific Blue-labelled 3 kDa dextran, 1μM of AF647-labelled small molecule, or 1μM of unlabeled small molecules. The delivery buffer is either PBS (ThermoFisher) or OptiMEM (ThermoFisher). The delivery was done either at room temperature or on ice. When delivering on ice, the cell-material mixture, microfluidics chips and holder set were placed in an ice bath until cold (approximately 5 minutes). The suspension was sent through the device in 100 μL aliquots at 60 psi unless noted otherwise with previously described method.[1–3] Endocytosis control PBMCs were prepared identically in delivery buffer with the materials and 0.2% DMSO (carrier for small molecules), but did not go through the microfluidics device. After delivery, cells were allowed to rest at room temperature for 2 minutes and quenched with 100 μL OptiMEM before further analysis. To assess delivery efficiency and cell viability, uptake of fluorescently-labelled dextran or fluorescently-labelled small molecules and fluorescence from propidium iodide were measured by flow cytometry respectively. To prepare cells for flow cytometry, wash cells twice in PBS with centrifugation at 400rcf, 4°C, for 4 minutes and resuspend cells in PBS buffer with 10 μg/mL propidium iodide. Flow cytometer A with filter settings A1, A2 and flow cytometer B with filter settings B1, B2 were used. ICS cells were quenched, but not washed.

**Small molecule delivery by Electroporation Neon$^{Tm}$**

Human primary PBMCs were suspended at $20 \times 10^6$ cells/mL to $25 \times 10^6$ cells/mL in RPMI with 0.2% DMSO (ThermoFisher) or 1μM of AF647-labelled small molecule, or 1μM of unlabeled small molecules. The cells were electroporated according to the manufacturer's protocol: pulse voltage (2150v); pulse width (20ms); Pulse number (1).

**p-STAT5 inhibition assay**

90 uL of cell suspension was pipetted into each well in a 96-well, deep-well, V-bottom plate (VWR# 82007-292) and the plate was incubated at 37$^{o}$C for 30 min. 5ml compound was added to each well (final 0.2% DMSO. The plate was vortexed gently and incubated at 37$^{o}$C for 75 min. (add 5 uL 4%DMSO/PBS to the control wells). 5 uL 2,000 ng/mL human IL-15 (final 100 ng/mL) was added to each well and the plate was vortexed gently and incubated at 37$^{o}$C for 15 min(add 5 uL PBS to the control wells). 0.3 mL 1% paraformaldehyde buffer (37$^{o}$C) was added to each well, and the plate was incubated at room temperature for 15 min and then centrifuged at 1,200 RPM (Beckman GS-6R or Sorvall Legend) at room temperature for 5 min. The supernatant was aspirated using a 8-channel or 12-channel manifold. 0.8 ml staining buffer (0.5% heat inactivated fetal bovine serum, 0.001% sodium azide in PBS-CMF, which was filtered with a 0.2um filter unit) was added to each well. The plate was centrifuged at 1,200 RPM (Beckman GS-6R or Sorvall Legend) at room temperature for 5 min. The supernatant was aspirated. The plate was then vortexed and 0.35 mL 90% methanol/10% $H_2O$ (-20$^{o}$C) was added to each well. The plate was incubated on ice for 20 min and centrifuged at 1,200 RPM (Beckman GS-6R or Sorvall Legend) at room temperature for 5 min and the supernatant was aspirated. 0.8 ml staining buffer was added to each well and the plate was centrifuged at 1,200 RPM (Beckman GS-6R or Sorvall Legend) at room temperature for 5 min. The supernatant was aspirated again. The plate was then vortexed. 210 ml Alexa Fluor 647 conjugated anti-STAT5 antibody was added to each well (1 to 250 dilution; 1 ml antibody per 250 ml staining buffer). The plate was incubated at 25$^{o}$C overnight in dark (or over weekend at 4 $^{o}$C overnight protected from light). 210 ml/well of the samples were transferred to 96-well U-bottom plate (Falcon #353077) through a 60um nylon filter membrane and perform the FACS analysis.

**FACS analysis**

The samples were analyzed using a BD Calibur or BD FACSCanto flow cytometer equipped with the BD High Throughput Sampler. 10,000 total events were collected with 90 ml sample volume and sample speed of 3ml/sec.

**Data analysis**

Lymphocyte population was gated for the pSTAT5 histogram analysis. A Maker was placed at the foot of the peak from the negative control (~1-2% gated population). Batch analysis was performed using CellQuest or Diva software. The relative the Relative Fluorescence Units (RFU) was created, which indicates the level of pSTAT5, by multiplying the % of M1 within the gated lymphocyte population and the M1 mean fluorescence.

**JAK Biochemical assay**

Caliper Jak Enzyme Endpoint $IC_{50}$ Assays at 1mM ATP: The human Janus Kinase (JAK) activity of was determined by using a microfluidic assay to monitor phosphorylation of a synthetic peptide by the recombinant human kinase domain of JAK1. The condition was optimized for JAK1 concentration and room temperature incubation time to obtain a conversion rate of 20% -

30% phosphorylated peptide product. 250 nL of test compounds and controls solubilized in 100% DMSO were added to a 384 well polypropylene plate (MatricalMP101 or Corning Costar 3676) using an non-contact acoustic dispenser. Kinase assays were carried out at room temperature in a 15 μL reaction buffer containing 20 mM HEPES, pH 7.4, 1mM ATP, 10 mM magnesium chloride, 0.01% bovine serum albumin (BSA), 0.0005% Tween 20 and 1mM DTT. Reaction mixtures contained 1 μM of a fluorescently labeled synthetic peptide, a concentration less than the apparent $K_m$. The JAK1 assay contained 1 μM of the peptide 5FAM-KKSRGDYMTMQID. The assays were initiated by the addition of enzyme. The assays were stopped with 15 μl of a buffer containing 180 mM HEPES, pH=7.4, 20 mM EDTA, 0.2% Coating Reagent, resulting in a final concentration of 10 mM EDTA, 0.1% Coating Reagent and 100 mM HEPES, pH=7.4. Utilizing the LabChip 3000 mobility shift technology (Caliper Life Science), each assay reaction was sampled to determine the level of phosphorylation. This technology is separation-based, allowing direct detection of fluorescently labeled substrates and products. Separations are controlled by a combination of vacuum pressure and electric field strength optimized for each peptide substrate.

**Western Blot**

Cell lysate was mixed with 1X NuPage® LDS Sample Buffer and 1X NuPage® Reducing Agent and loaded into a NuPage™ Novex™ 4-12% Bis-Tris Protein Gel. Gel electrophoresis was performed using NuPAGE® MOPS SDS Running Buffer (1X) (200V, 1.5A, 50min) and proteins transferred to iBlot2® transfer stacks PDVF membrane (P0 protocol). The membrane was blocked for 1 hr at room temperature using SuperBlock™ T20 (TBS) Blocking Buffer. Primary antibodies (pSTAT5 Tyr 694 purchased from Cell signaling #9351) were diluted into blocking buffer (1:1,000) and incubated at 4°C overnight with agitation. After incubation, the primary antibody was removed and membranes washed with TBST (3 x 5 min). The membranes were incubated with secondary antibody (1:5,000) in TBST + 10% blocking buffer for 1 hr at room temperature with agitation. After incubation, membrane was washed with TBST (3 x 5 min) and developed using SuperSignal™ West Femto Maximum Sensitivity Substrate. Membranes were visualized using Kodak Image Station.

**Cell permeability assay**

Specific details are available upon request. RRCK permeability guideline: >10 high; $2.5<x\leq10$ medium; $\leq2.5$ low


1. Sharei, A. *et al.* A vector-free microfluidic platform for intracellular delivery. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 2082–7 (2013).

2. Sharei, A. *et al.* Plasma membrane recovery kinetics of a microfluidic intracellular delivery platform. *Integr. Biol. (Camb).* **6,** 470–5 (2014).

3. Sharei, A. *et al.* Ex vivo cytosolic delivery of functional macromolecules to immune cells. *PLoS One* **10,** (2015).