Supporting Information

Structure-Based Design of ASK1 Inhibitors as Potential Agents for Heart Failure

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I/ Synthetic Procedures

General Methods

All reagents and anhydrous solvents were obtained from commercial sources and used without further purification unless noted otherwise. Purchased chiral reagents were assumed to be chirally pure and that no racemisation occurred during the synthesis. Intermediates were analyzed by ^{1}H NMR and/or LCMS. Final compounds were analyzed for integrity and purity by ^{1}H NMR, HPLC and LCMS. Key compounds were analyzed by ^{1}H and ^{13}C NMR, HPLC, LCMS and HR-MS.

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker 400 MHz. ^{1}H NMR chemical shifts are reported in ppm with the solvent as the internal standard (DMSO-d6 2.49 ppm, CDCl3 7.26 ppm, CD3OD 3.31 ppm). ^{13}C NMR chemical shifts are reported in ppm with the solvent as the internal standard (DMSO-d6 39.5 ppm or CDCl3 77.2 ppm).

LC-MS were run on a Waters UPLC/LSMS system. It includes a Waters Acquity binary solvent manager, a Waters Acquity sample manager, a Waters Acquity PDA detector, a Waters Acquity ELS detector, a Waters LCT mass detector, Columns Waters BEH C18, 1.7 µm, ID2.1x50mm; Eluent: 0.05% TFA in water to a gradient of 0.035% TFA in acetonitrile over 1.3 min; Flow Rate: 0.8 mL/min; Column temp. 55°C;

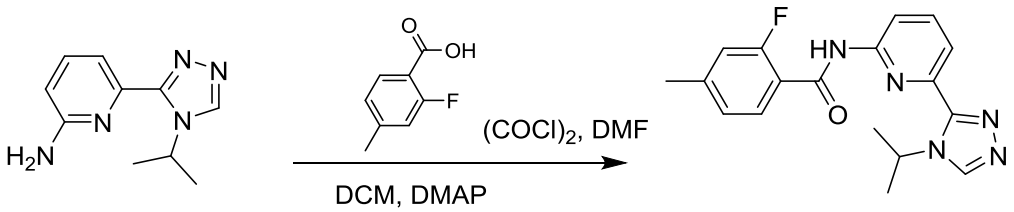
HPLC purification system is a Waters Instrument (Waters 2525 pump, Waters 2767 sample manager, Waters 2487 multi-wavelength UV detector, Alltech 2000 ELS detector, Waters ZQ mass detector). The software is a Waters MassLynx software, the analytical column a Waters XSelect CSH C18, 5 µm, ID4.6x50mm, the Prep column a Waters XSelect CSH Prep C18, 5 µm, ID30x75mm. The mobile phases for Method A were 0.1% FA in water to 0.1% FA in acetonitrile. For Method B, the mobile phase was 10 mMol ammonium hydroxide in water (pH=9.5-10) to 10 mMol ammonium hydroxide in acetonitrile (pH=9.5-10). The flow rate for the analytical channel was 3.5 mL/min; Gradient Time, 4.2 min. For the Prep channel, the flow rate was 50 mL/min; Gradient Time, 6 min, Make Up: 1 mL/min. The mass detector

includes Capillary (kV): 3.50; Source Temperature (°C): 120; Desolvation Temperature (°C): 300; Cone Gas Flow (L/Hr): 40; Desolvation Gas Flow (L/Hr): 500. Purities were assessed by UV at 220 nm and by TIC.
HR-MS system included an autosampler Leap Technologies LC Pal (10uL injection). The LC component is a Shimadzu UFLC LC-20AD and the UV Detector an Agilent 1290 DAD G4212A. The MS Detector is AB Sciex tripleTOF 5600 with a Positive TOF MS (range 100-1000) with IDA (ion range 100-1000), GS1 60, GS2 60, CUR 25, ISVF 5000, TEM 500, DB 80, CES 15, CE=40 (compound **13**), CE=50 (compounds **12, 14, 15**). The LC Column is a Kinetex 2.6µ C18, 100 Å.; mobile Phase: Solvent A= water (0.04 % HCOOH). Solvent B= acetonitrile (0.04 % HCOOH); flow: 0.6mL/min.
Chiral purity was assessed by chiral SFC with methanol and diethylamine (0.1%) using three different columns: AD-H, OD-H and AS-H columns (19x150mm, 5mm) from Chiral Technology.

Preparation of compounds 2-18

Compound **2** was prepared following literature procedures[1]. Analytical data matched the ones described in the literature

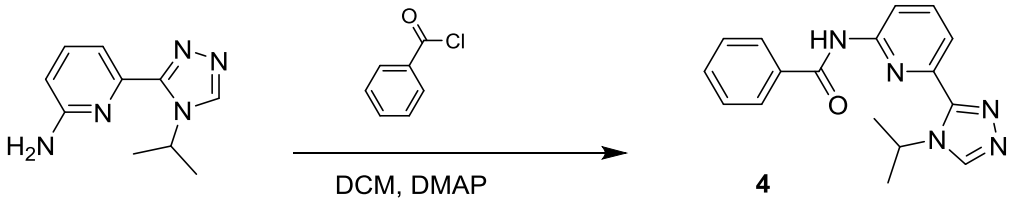
Compound **3**:



3

To a stirred solution of 2-fluoro 4-methylbenzoic acid (77 mg, 0.5 mmol) in DCE (2.5 mL) was added oxalyl chloride (83 mg, 0.65 mmol) and DMF (2 drop) at 16°C under N_2. The mixture was stirred at 16°C for 4 hours, and then the solvent was removed under reduced pressure. The residue was dissolved in DCM (2.5 mL). 6-(4-isopropyl-4H-1,2,4-triazol-3-yl)pyridin-2-amine[1] (100 mg, 0.5 mmol) and DMAP (61 mg, 0.5 mmol) were added with stirring under N_2. The reaction mixture was stirred at 16°C for 2 hours. Aqueous saturated $NaHCO_3$ (5 mL) was added. The mixture was stirred for 10 minutes, and layers were separated. The aqueous layer was washed with DCM (10 mL). The combined organic layers were dried over Na_2SO_4 and concentrated to afford the crude product which was purified by Prep-HPLC (Method B) to give **3** (100 mg, 59% yield) as a white solid.
^{1}H NMR (DMSO-d6, 400 MHz) δ ppm 10.73 (s, 1H), 8.86 (s, 1H), 8.21 (d, *J* = 7.8 Hz, 1H), 8.02 (t, *J* = 8.0 Hz, 1H), 7.89 (dd, *J* = 0.7, 7.6 Hz, 1H), 7.63 (t, *J* = 7.7 Hz, 1H), 7.26 - 7.14 (m, 2H), 5.72 - 5.62 (m, 1H), 2.39 (s, 3H), 1.43 (d, *J* = 6.7 Hz, 6H).
LCMS (ESI$^+$) m/z: 340.4 $(M+H)^+$
Purity TIC 100%, UV 100%

Compound **4**:

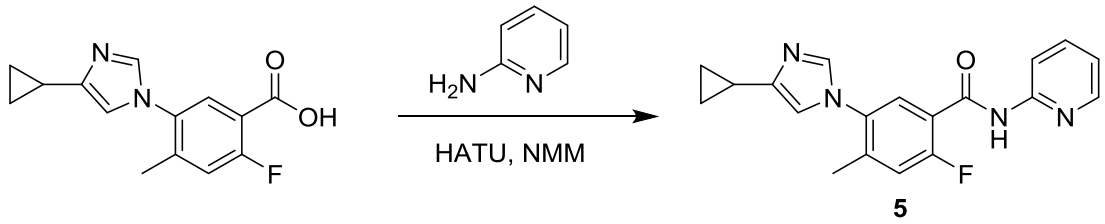


4

Benzoyl chloride (0.073 g, 0.517 mmol) was added to 6-(4-isopropyl-4H-1,2,4-triazol-3-yl)pyridin-2-amine (0.1 g, 0.492 mmol) in pyridine (1 mL) at 0°C. The reaction was warmed up to room temperature and stirred for an additional hour. The reaction was quenched by addition of 10 mL of water. A white precipitate formed and was washed with copious amounts of water followed by hexanes to yield **4** as a white solid (65 mg, 41% yield).

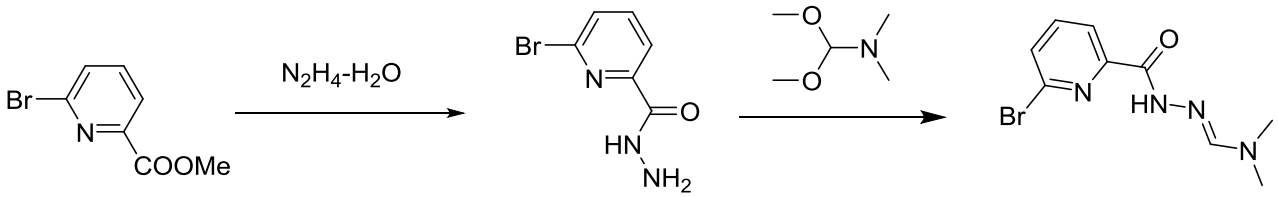
^{1}H NMR (400 MHz, DMSO-d6) δ ppm 10.77 (s, 1H), 8.87 (s, 1H), 8.20 (dd, *J* = 8.34, 0.76 Hz, 1H), 8.02 (t, *J* = 7.96 Hz, 1H), 7.97 (dd, *J* = 8.34, 1.26 Hz, 2H), 7.87 (dd, *J* = 7.58, 0.76 Hz, 1H), 7.62 - 7.66 (m, 1H), 7.54 - 7.59 (m, 2H), 5.70 - 5.78 (m, 1H).
LCMS (ESI$^+$) m/z: 308.4 $(M+H)^+$
Purity TIC 100%, UV >95%

Compound **5**:

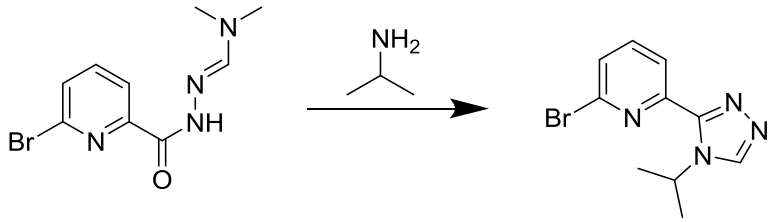


5

A mixture of 2-fluoro-5-(1H-imidazol-1-yl)-4-methylbenzoic acid (100 mg, 0.415 mmol), 2-aminopyridine (58.5 mg, 0.622 mmol), N-methyl morpholine (125.2 mg, 1.24 mmol) and HATU (236.4 mg, 0.622 mmol) in DCM (3 mL) was stirred at room temperature for 3 hours. The solvent was evaporated under reduced pressure. The crude was purified by Prep-HPLC to give **5** (50 mg, 39 % yield) as light brown solid.
^{1}H NMR (400 MHz, CD3OD) δ ppm 9.13 (d, *J* = 1.6 Hz, 1H), 8.44 (d, *J* = 4.5 Hz, 1H), 8.22 - 8.15 (m, 1H), 8.13 - 8.06 (m, 1H), 8.03 (d, *J* = 6.4 Hz, 1H), 7.63 - 7.56 (m, 1H), 7.54 - 7.41 (m, 2H), 2.38 - 2.31 (m, 3H), 2.14 - 2.03 (m, 1H), 1.21 - 1.11 (m, 2H), 0.98 - 0.88 (m, 2H).
LCMS (ESI$^+$) m/z: 337.1 $(M+H)^+$
Purity TIC 95%, UV 95%



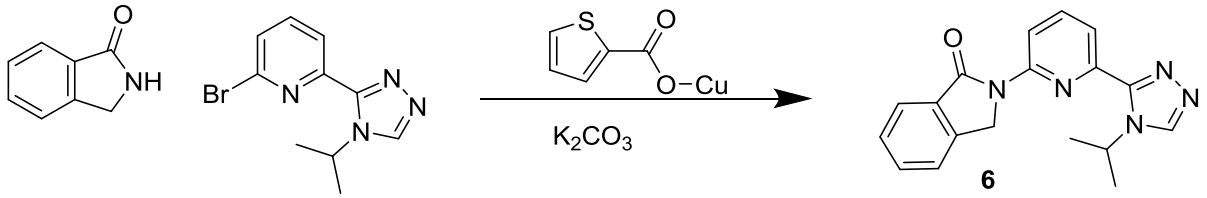
Compound **6**:
Hydrazine hydrate (19.10 mL, 283 mmol) was added to a suspension of methyl 6-bromopicolinate (51 g, 236 mmol) in MeOH (300 mL). The mixture was stirred at room temperature overnight then reduced to 1/3 of its volume under reduced pressure. MTBE was added. The solid was collected by filtration to give 6-bromopicolinohydrazide as an off white solid that was used as is in the next step. DMF-DMA (82 mL, 614 mmol) was added to the solid and the reaction mixture was heated at 80°C overnight. After return to room temperature, diethyl ether (500 mL) was added. A solid formed and was collected by filtration and rinsed with diethyl ether to give 36.3g of N'-(6-bromopicolinoyl)-N,N-dimethylformohydrazonamide. It was used without further purification in the next step.
LCMS (ESI$^+$) m/z: 271.0-273.0 $(M+H)^+$



N'-(6-bromopicolinoyl)-N,N-dimethylformohydrazonamide (5.02 g, 18.52 mmol) and propan-2-amine (7.58 mL, 93 mmol) were dissolved in acetic acid (18.5 mL) and acetonitrile (74.1 mL). The solution was heated at 95°C overnight. The reaction was cooled down and concentrated. The residue was dissolved in water (30 mL), neutralized with 1N NaOH (~20 mL, pH ~5) and extracted with EtOAc (2 x 75 mL). The combined organic extracts were washed with brine (50 mL), dried over magnesium sulfate, filtered and concentrated. The crude product was purified via silica gel column chromatography (0-100% EtOAc-Heptanes). The appropriate fractions were collected, concentrated, and dried in-vacuo to afford 2-bromo-6-(4-isopropyl-4H-1,2,4-triazol-3-yl)pyridine as a pale yellow oil that crystallized upon standing (4.53 g, 92 % yield).

^{1}H NMR (400 MHz, CDCl3) δ ppm 8.38 (br.s., 1H), 8.23 - 8.33 (m, 1H), 7.70 (td, *J* = 7.83, 2.02 Hz, 1H), 7.53 (d, *J* = 7.09 Hz, 1H), 5.41 - 5.72 (m, 1H), 1.57 (dd, *J* = 6.57, 2.53 Hz, 6H).

LCMS (ESI$^+$) m/z: 267.0-269.0 $(M+H)^+$



6

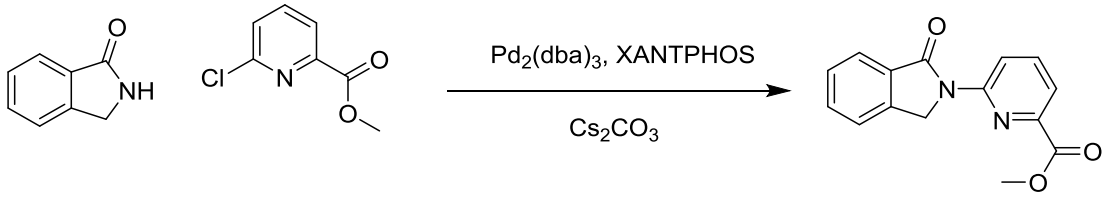
Isoindolin-1-one (50 mg, 0.376 mmol), ((thiophene-2-carbonyl)oxy)copper (7.16 mg, 0.038 mmol), 2-bromo-6-(4-isopropyl-4H-1,2,4-triazol-3-yl)pyridine (150 mg, 0.563 mmol) and K_2CO_3 (104 mg, 0.751 mmol) were mixed in DMSO (3 mL) to give a green suspension. The mixture was heated in a microwave for 60 min at 140°C. The mixture was cooled and diluted with MeOH. The suspension filtered and the filtrate was purified by Prep-HPLC (Method A) to afford **6** as a yellowish green solid (42 mg, 35% yield).

^{1}H NMR (400 MHz, DMSO-d6) δ ppm 9.10 (br. s., 1H), 8.67 (d, *J* = 8.08 Hz, 1H), 8.03 - 8.16 (m, 1H), 7.82 - 7.98 (m, 2H), 7.75 (br. s., 2H), 7.58 (br. s., 1H), 5.54 (br. s., 1H), 5.18 (br. s., 2H), 1.58 (d, *J* = 6.06 Hz, 6H).

LCMS (ESI+) m/z: 320.3 $(M+H)^+$

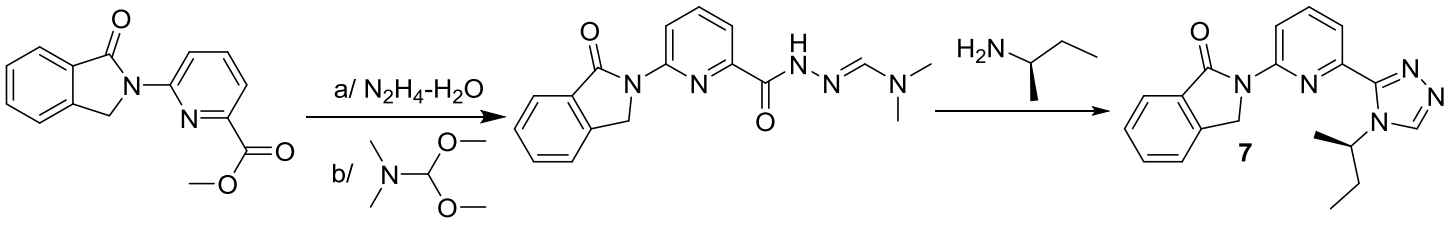
Purity TIC 100%, UV 91%

Compound **7**:



$Pd_2(dba)_3$ (1.03 g, 1.127 mmol), XANTPHOS (1.30 g, 2.253 mmol), cesium carbonate (24.47 g, 75 mmol), isoindolin-1-one (5 g, 37.6 mmol) and methyl 6-bromopicolinate (6.44 g, 37.6 mmol) were combined in dioxane (100 mL). The mixture was degassed with nitrogen, sealed and heated at 80°C for overnight. The reaction mixture was poured into water (100 mL). Some precipitate formed and was filtered off. The filtrate was collected and extracted with EtOAc (3 x 50 mL). The

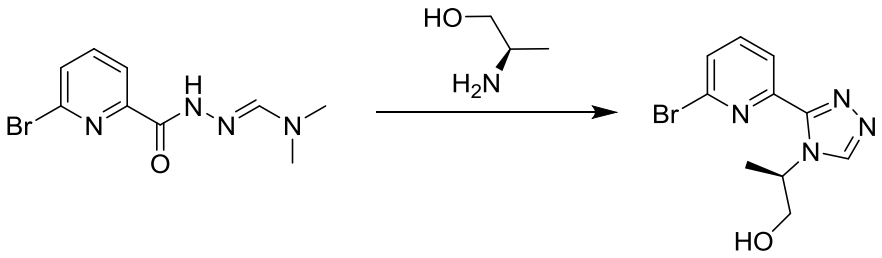
combined organic extracts were combined and concentrated. The resulting solid was triturated in IPA to give methyl 6-(1-oxoisoindolin-2-yl)picolinate as a solid (8.41 g, 83% yield).
LCMS (ESI^+) m/z: 268.3 $(M+H)^+$



To a suspension of 6-(1-oxoisoindolin-2-yl)picolinate (7 g, 26.1 mmol) in MeOH (100 mL) was added hydrazine hydrate (2.6 mL, 52.2 mmol) at 25°C. The reaction mixture was then heated at reflux for 24 hours. The reaction was concentrated in-vacuo and the resulting solid was triturated in IPA to give 6-(1-oxoisoindolin-2-yl)picolinohydrazide (6.56 g). It was used as is in the next step.
LCMS (ESI^+) m/z: 268.3 $(M+H)^+$
6-(1-oxoisoindolin-2-yl)picolinohydrazide (6.56 g, 24.45 mmol) was dissolved in DMF-DMA (65.0 mL, 489 mmol) and heated to 80°C overnight. The reaction mixture was diluted with diethyl ether and the resultant solid, N,N-dimethyl-N'-(6-(1-oxoisoindolin-2-yl)picolinoyl)formohydrazonamide, was collected by filtration, rinsed with diethyl ether then dried in the vacuum oven at 40°C overnight (7.48 g, 95% yield).
LCMS (ESI^+) m/z: 323.3 $(M+H)^+$
N,N-dimethyl-N'-(6-(1-oxoisoindolin-2-yl)picolinoyl)formohydrazonamide (20 mg, 0.062 mmol) and (R)-butan-2-amine (45 mg, 0.619 mmol) were dissolved in acetonitrile/acetic acid (2 mL/0.5 mL). The mixture was then heated to reflux for 3 hours. The reaction mixture was concentrated in-vacuo then purified by Prep-HPLC (Method A) to give a white solid (11.3 mg, 55% yield).
^{1}H NMR (400 MHz, DMSO-d6) δ ppm 9.01 (s, 1H), 8.67 (dd, *J* = 8.59, 0.76 Hz, 1H), 8.11 (dd, *J* = 8.34, 7.58 Hz, 1H), 7.87 - 7.94 (m, 2H), 7.74 - 7.77 (m, 2H), 7.60 (ddd, *J* = 7.83, 4.67, 3.41 Hz, 1H), 5.28 - 5.48 (m, 2H), 5.18 (s, 3H), 1.86 - 2.01 (m, 2H), 1.58 (d, *J* = 6.82 Hz, 3H), 0.82 (t, *J* =7.45 Hz, 3H).

Compound **8**:
Following the same procedure used to prepare **7** and replacing (R)-butan-2-amine by (S)-butan-2-amine, **8** was obtained in similar yields.
^{1}H NMR (400 MHz, DMSO-d6) δ ppm 9.04 (s, 1H), 8.67 (dd, *J* = 8.34, 0.76 Hz, 1H), 8.11 (dd, *J* = 8.46, 7.71 Hz, 1H), 7.84 - 7.96 (m, 2H), 7.71 - 7.81 (m, 2H), 7.55-7.65 (m, 1H), 5.34 - 5.48 (m, 2H), 5.18 (s, 3H), 1.82 - 2.05 (m, 2H), 1.59 (d, *J* = 6.82 Hz, 3H), 0.82 (s, 3H).

Compound **9**:

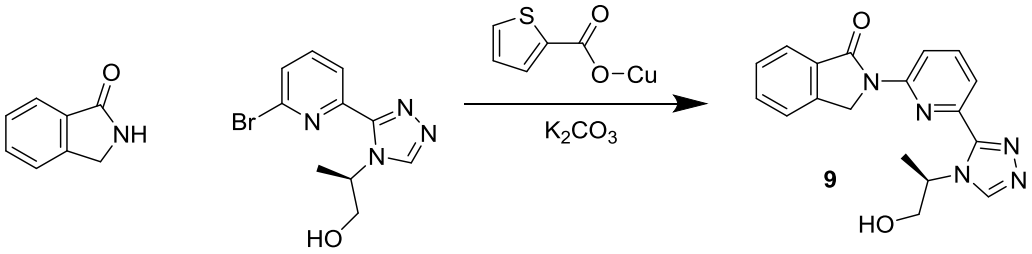


Following the procedure described for 2-bromo-6-(4-isopropyl-4H-1,2,4-triazol-3-yl)pyridine and starting from 7.5 g of N'-(6-bromopicolinoyl)-N,N-dimethylformohydrazonamide (27.7 mmol) and (R)-2-aminopropan-1-ol (6.23 g, 83 mmol),

3.19 g (R)-2-(3-(6-bromopyridin-2-yl)-4H-1,2,4-triazol-4-yl)propan-1-ol was obtained as an off-white solid by recrystallizing the crude in IPA (41% yield).

^{1}H NMR (400 MHz, DMSO-d6) δ ppm 8.85 (s, 1H), 8.09 - 8.18 (m, 1H), 7.89 - 8.01 (m, 1H), 7.74 - 7.83 (m, 1H), 5.17 - 5.30 (m, 1H), 5.00 - 5.09 (m, 1H), 3.70 (s, 2H), 1.47 (d, *J* = 7.07 Hz, 3H).

LCMS (ESI$^+$) m/z: 283.0-285.0 $(M+H)^+$



Following the procedure described for **6** and starting from (R)-2-(3-(6-bromopyridin-2-yl)-4H-1,2,4-triazol-4-yl)propan-1-ol (162 mg, 0.57 mmol) and 99 mg (0.74 mmol) of isoindolin-1-one, 50mg (26% yield) of **9** was obtained.

^{1}H NMR (400 MHz, DMSO-d6) δ ppm 8.94 (s, 1H), 8.66 (d, *J* = 8.6 Hz, 1H), 8.11 (d, *J* = 7.96 Hz, 1H), 7.93 (dd, *J* = 0.76, 7.58 Hz, 1H), 7.88 (d, *J* = 7.58 Hz, 1H), 7.74-7.78 (m, 2H), 7.57-7.63 (m, 1H), 5.47-5.54 (m, 1H), 5.18 (s, 2H), 3.70-3.86 (m, 2H), 1.57 (d, *J* = 6.82 Hz, 3H).

LCMS (ESI$^+$) m/z: 336.3 $(M+H)^+$

Purity TIC 100%, UV 98%

Compound **10**:

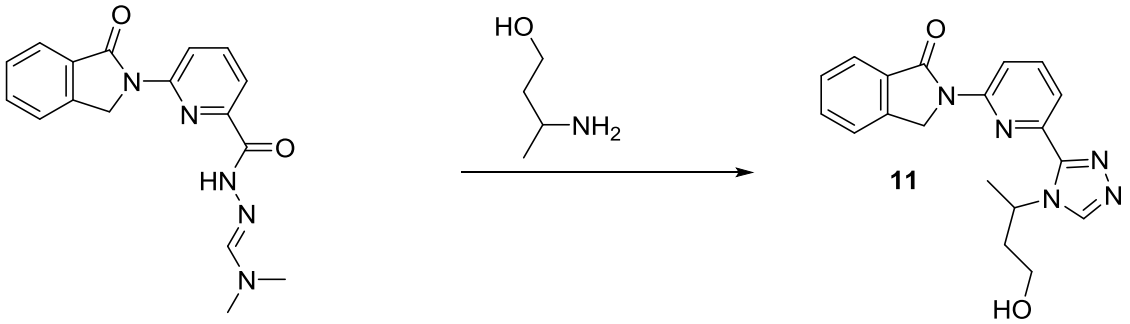
Using the same procedure used to prepare compound **9** replacing (R)-2-aminopropan-1-ol by (S)-2-aminopropan-1-ol, **10** was obtained as an off-white solid (31% yield).

^{1}H NMR (400 MHz, DMSO-d6) δ 8.84 (s, 1H), 8.63 (dd, *J* = 0.76, 8.34 Hz, 1H), 8.08 (dd, *J* = 7.71, 8.46 Hz, 1H), 7.91 (dd, *J* = 0.76, 7.58 Hz, 1H), 7.86 (d, *J* = 7.58 Hz, 1H), 7.71-7.77 (m, 2H), 7.55-7.61 (m, 1H), 5.40-5.53 (m, 1H), 5.16 (s, 2H), 5.08 (t, *J* = 5.31 Hz, 1H), 3.76-3.84 (m, 1H), 3.69-3.76 (m, 1H), 1.55 (d, *J* = 7.07 Hz, 3H).

LCMS (ESI+) m/z: 336.2 $(M+H)^+$

Purity TIC 100%, UV 100%

Compound **11**:



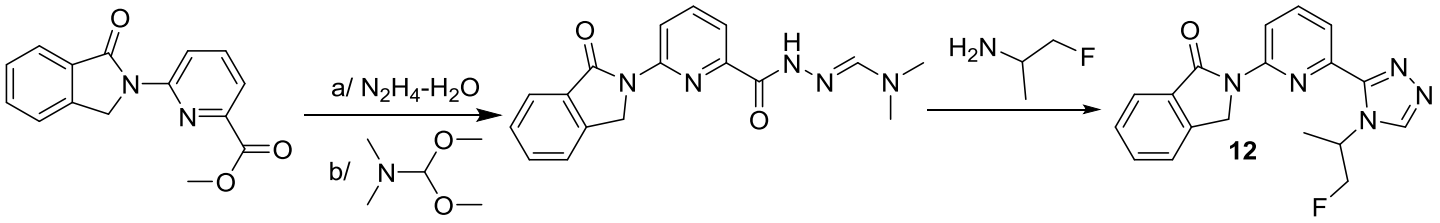
Following the same procedure described for compound **7** and using 200 mg (0.619 mmol) of N,N-dimethyl-N'-(6-(1-oxoisoindolin-2-yl)picolinoyl)formohydrazonamide and 276 mg (3.09 mmol) of 3-aminobutan-1-ol, 5.3 mg (2.4% yield) of **11** were obtained as a colorless liquid.

^{1}H NMR (400 MHz, CD3OD) δ ppm 9.10 - 9.51 (m, 1H), 8.63 - 8.98 (m, 1H), 7.80 - 8.15 (m, 3H), 7.65 - 7.78 (m, 2H), 7.50 - 7.61 (m, 1H), 5.83 - 6.04 (m, 1H), 5.22 (s, 2H), 3.51 - 3.72 (m, 2H), 2.06 - 2.35 (m, 2H), 1.70 (d, *J* = 6.82 Hz, 3H).

LCMS (ESI$^+$) m/z: 350.2 $(M+H)^+$

Purity TIC 94%, UV 100%

Compound **12:**



To a suspension of 6-(1-oxoisoindolin-2-yl)picolinate (7 g, 26.1 mmol) in MeOH (100 mL) was added hydrazine hydrate (2.6 mL, 52.2 mmol) at 25°C. The reaction mixture was then heated at reflux for 24 hours. The reaction was concentrated in-vacuo and the resulting solid was triturated in IPA to give 6-(1-oxoisoindolin-2-yl)picolinohydrazide (6.56 g). It was used as is in the next step.

LCMS (ESI^+) m/z: 268.3 $(M+H)^+$

6-(1-oxoisoindolin-2-yl)picolinohydrazide (6.56 g, 24.45 mmol) was dissolved in DMF-DMA (65.0 mL, 489.0 mmol) and heated to 80°C overnight. The reaction mixture was diluted with diethyl ether and the resultant solid, N,N-dimethyl-N'-(6-(1-oxoisoindolin-2-yl)picolinoyl)formohydrazonamide, was collected by filtration, rinsed with diethyl ether then dried in the vacuum oven at 40°C overnight (7.48 g, 95% yield).

LCMS (ESI^+) m/z: 323.3 $(M+H)^+$

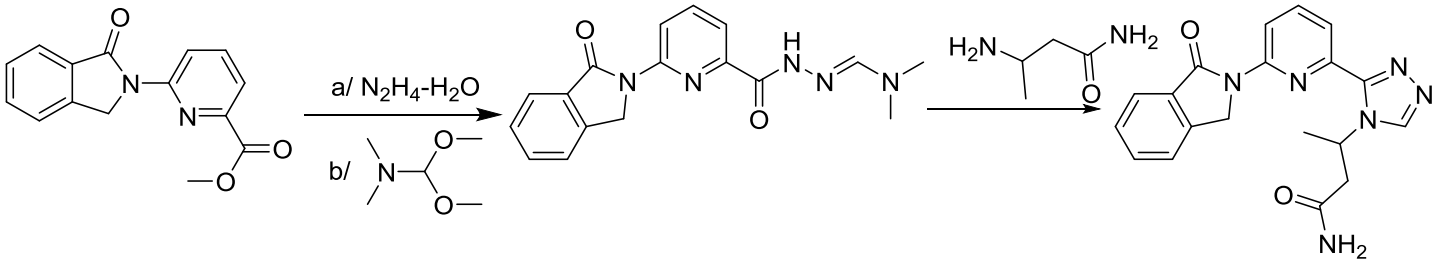
N,N-dimethyl-N'-(6-(1-oxoisoindolin-2-yl)picolinoyl)formohydrazonamide (40 mg, 0.124 mmol) and 1-fluoropropan-2-amine (0.053 mL, 0.113 mmol). The mixture was then heated to reflux for 3 hours. The reaction mixture was concentrated in-vacuo then purified by Prep-HPLC (Method A followed by Method B) to give **12** as a white solid (5.5 mg, 12% yield).

^{1}H NMR (400 MHz, CD3OD) δ ppm 8.79 (bs, 1H), 8.76 (d, J = 12 Hz, 1H), 8.05 (t, J = 8 Hz, 1H), 7.84 - 7.96 (m, 2H), 7.66 - 7.75 (m, 2H), 7.51 - 7.61 (m, 1H), 5.78 - 5.99 (m, 1H), 5.35 (s, 2H), 4.89 - 4.95 (m, 2H), 1.73 (d, J = 3.8 Hz, 3H).

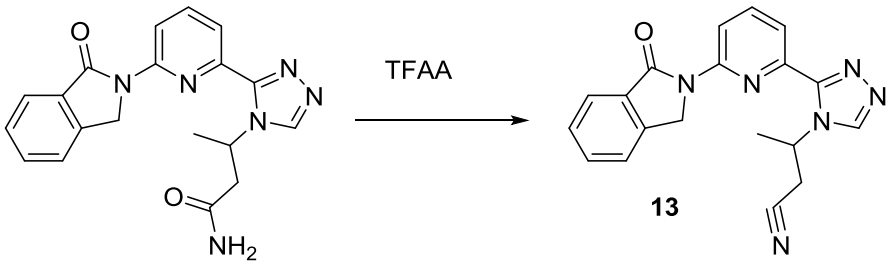
LCMS (ESI^+) m/z: 337.4 $(M+H)^+$

Purity TIC 100%, UV 100%

Compound **13**:



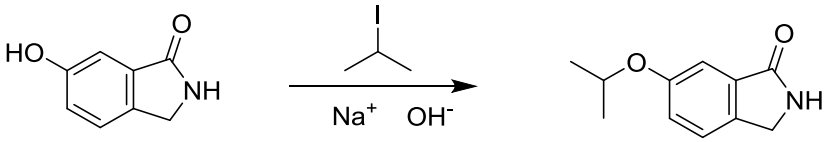
Using the same procedure used to prepare compound **9** replacing (R)-2-aminopropan-1-ol by 3-aminobutanamide 3-(3-(6-(1-oxoisoindolin-2-yl)pyridin-2-yl)-4H-1,2,4-triazol-4-yl)butanamide was obtained as an off-white solid (44% yield).



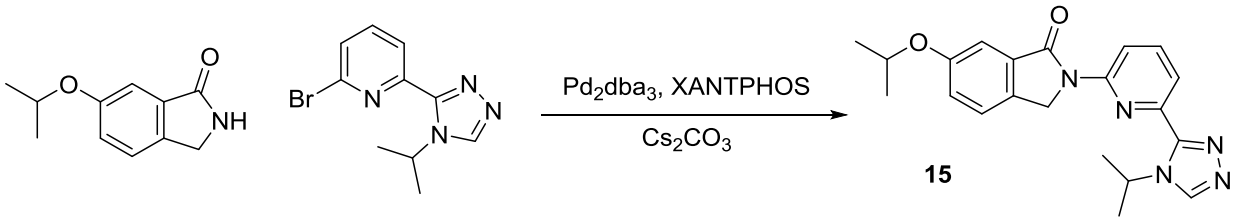
A mixture of 3-(3-(6-(1-oxoisoindolin-2-yl)pyridin-2-yl)-4H-1,2,4-triazol-4-yl)butanamide (49mg, 0.135 mmol) and trifluoroacetic anhydride (0.025 mL) was heated in pyridine (3 mL) at 70°C overnight. Solvents were removed and the crude purified by Prep-HPLC (Method A) to give **13** as a clear film (8% yield).
^{1}H NMR (400 MHz, CD3OD) δ ppm 9.01 - 9.22 (m, 1H), 8.66 - 8.89 (m, 1H), 7.82 - 8.14 (m, 3H), 7.64 - 7.77 (m, 2H), 7.45 - 7.61 (m, 1H), 5.97 - 6.12 (m, 1H), 5.10 - 5.30 (m, 2H), 3.34 - 3.44 (m, 1H), 1.76 - 1.90 (m, 3H).
LCMS (ESI$^+$) m/z: 345.2 $(M+H)^+$
Purity TIC 100%, UV 100%

Compound **14**:
Using the same procedure used to prepare compound **9** replacing (R)-2-aminopropan-1-ol by oxetan-2-ylmethanamine, **14** was obtained as an off-white solid (16% yield).
^{1}H NMR (400 MHz, DMSO-d6) δ ppm 8.60 - 8.73 (m, 12H), 8.05 - 8.15 (m, 6H), 8.00 (dd, *J* = 7.58, 0.76 Hz, 6H), 7.88 (d, *J* = 7.58 Hz, 6H), 7.70 - 7.80 (m, 12H), 7.55 - 7.66 (m, 6H), 5.07 - 5.25 (m, 18H), 5.01 (dd, *J* = 14.27, 6.44 Hz, 6H), 4.90 (dd, *J* = 14.15, 3.79 Hz, 6H), 4.52 (ddd, *J* = 8.59, 7.07, 5.81 Hz, 6H), 4.26 - 4.44 (m, 6H), 2.65 - 2.81 (m, 6H), 2.31 - 2.49 (m, 6H).
LCMS (ESI$^+$) m/z: 348.2 $(M+H)^+$
Purity TIC 100%, UV 100%

Compound **15**:



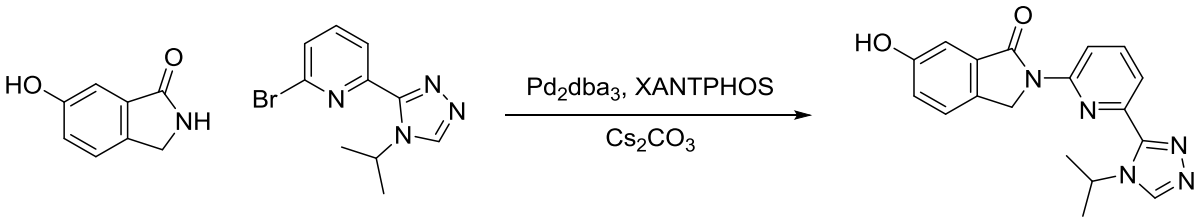
6-hydroxyisoindolin-1-one (60 mg, 0.402 mmol), 2-iodopropane (0.40 mL, 0.402 mmol) and sodium hydroxide (17.7 mg, 0.443 mmol) were dissolved in DMSO (1 mL) and stirred at room temperature for 3 hours. The crude mixture was diluted in MeOH and purified by Prep-HPLC (Method A). 42.5 mg of 6-isopropoxyisoindolin-1-one was obtained (55% yield).
LCMS (ESI$^+$) m/z: 192.1 $(M+H)^+$



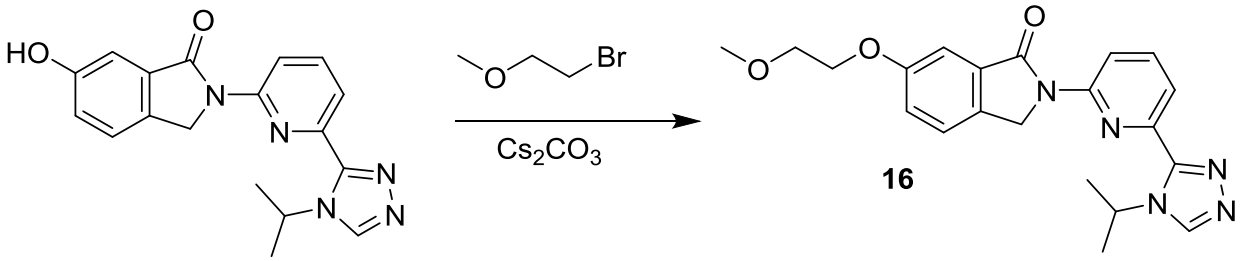
Following the procedure described for methyl 6-(1-oxoisoindolin-2-yl)picolinate and starting from 6-isopropoxyisoindolin-1-one (42.5 mg, 0.222 mmol), and 2-bromo-6-(4-isopropyl-4H-1,2,4-triazol-3-yl)pyridine, 56.5 mg of **15** was obtained (88% yield).
^{1}H NMR (400 MHz, DMSO-d6) δ ppm 9.07 (s, 1H), 8.66 (dd, *J* = 8.59, 0.76 Hz, 1H), 8.10 (dd, *J* = 8.46, 7.70 Hz, 1H), 7.92 (dd, *J* = 7.58, 0.76 Hz, 1H), 7.64 (d, *J* = 8.34 Hz, 1H), 7.27 - 7.33 (m, 2H), 5.51 - 5.59 (m, 1H), 5.09 (s, 2H), 4.78 (quin, *J* = 6.06 Hz, 1H), 1.59 (d, *J* = 6.82 Hz, 6H), 1.32 (d, *J* = 6.06 Hz, 6H).

LCMS (ESI$^+$) m/z: 378.3 $(M+H)^+$
Purity TIC 100%, UV 95%

Compound **16**:



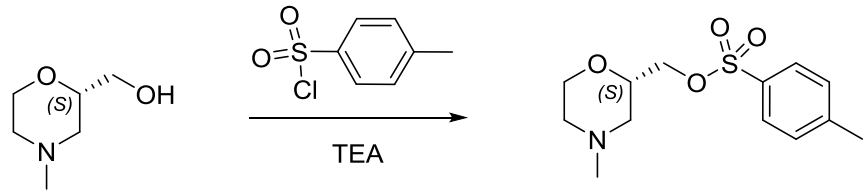
Following the procedure described for methyl 6-(1-oxoisoindolin-2-yl)picolinate and starting from 2-bromo-6-(4-isopropyl-4H-1,2,4-triazol-3-yl)pyridine (4.6 g, 17.22 mmol) and 6-hydroxyisoindolin-1-one (2.57 g, 17.22 mmol), 6-hydroxy-2-(6-(4-isopropyl-4H-1,2,4-triazol-3-yl)pyridin-2-yl)isoindolin-1-one was obtained as an off-white solid (5.15 g, 89 % yield).
^{1}H NMR (400 MHz, DMSO-d6) δ ppm 8.93 (s, 1H), 8.62 (dd, *J* = 8.34, 0.76 Hz, 1H), 8.06 (dd, *J* = 8.34, 7.58 Hz, 1H), 7.89 (dd, *J* = 7.58, 0.76 Hz, 1H), 7.50 (d, *J* = 8.08 Hz, 1H), 7.07 - 7.15 (m, 2 H), 5.45 - 5.54 (m, 1H), 5.03 (s, 2H), 1.56 (d, *J* = 6.82 Hz, 6H).



LCMS (ESI$^+$) m/z: 336.2 $(M+H)^+$

6-hydroxy-2-(6-(4-isopropyl-4H-1,2,4-triazol-3-yl)pyridin-2-yl)isoindolin-1-one (65 mg, 0.194 mmol), 1-bromo-2-methoxyethane (0.02 mL, 0.233 mmol), cesium carbonate (126 mg, 0.388 mmol) were dissolved in DMF (1 mL) and the reaction was purged with nitrogen then capped and heated at 100°C for 7 hours. The crude was filtered, diluted in methanol and purified by Prep-HPLC. 62 mg (81% yield) of a pale yellow solid was obtained.
^{1}H NMR (400 MHz, DMSO-d6) δ ppm 9.03 (s, 1H), 8.66 (dd, *J* = 8.59, 0.76 Hz, 1H), 8.10 (dd, *J* = 8.34, 7.58 Hz, 1H), 7.93 (dd, *J* = 7.58, 1.01 Hz, 1H), 7.66 (d, *J* = 8.08 Hz, 1H), 7.28 - 7.39 (m, 2H), 5.51 - 5.56 (m, 1H), 5.11 (s, 2H), 4.18 - 4.28 (m, 2H), 3.66 - 3.75 (m, 2H), 1.59 (d, *J* = 6.57 Hz, 6H).
^{13}C NMR (101 MHz, DMSO-d6) δ ppm 167.1 (s, 1C), 158.8 (s, 1C), 151.0 (s, 1C), 150.0 (s, 1C), 145.9 (s, 1C), 143.2 (s, 1C), 139.5 (s, 1C), 133.7 (s, 1C), 133.2 (s, 1C), 124.9 (s, 1C), 121.4 (s, 1C), 119.0 (s, 1C), 113.4 (s, 1C), 106.9 (s, 1C), 70.3 (s, 1C), 67.4 (s, 1C), 58.2 (s, 1C), 49.0 (s, 1C), 48.3 (s, 1C), 23.3 (s, 1C).
ESI-HRMS: calcd for 394.1874 C21H24N5O3 $(M+H)^+$, found 394.1865.
LCMS (ESI$^+$) m/z: 394.3 $(M+H)^+$
Purity TIC 100%, UV 100%

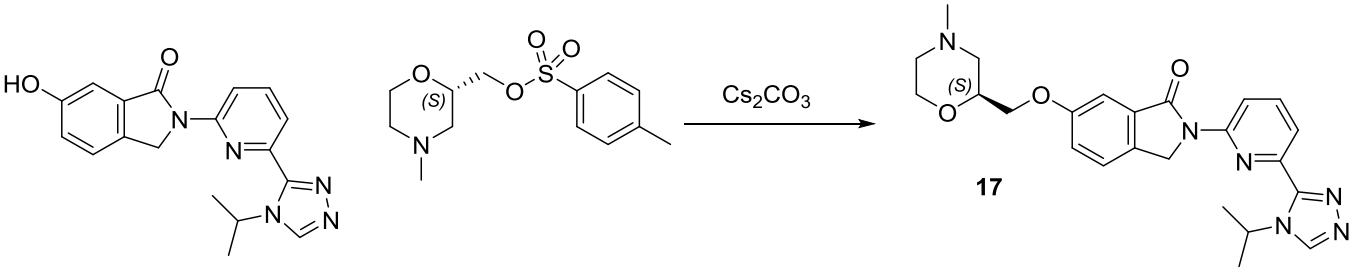
Compound **17:**



To a solution of (S)-(4-methylmorpholin-2-yl)methanol (5 g, 38.1 mmol) in THF (127 mL) at 0°C was added triethyl amine (5.31 mL, 38.1 mmol). The reaction mixture was stirred at 0°C for 10 minutes and then Tosyl-Cl (7.27 g, 38.1 mmol) was added. The reaction mixture was stirred at 0°C for 1.5 hours and then the ice bath was removed and the reaction mixture was slowly warmed to room temperature and stirred for 5.5 hours. The salts were filtered, the filtrate concentrated to yield a reddish-orange semi-solid which was dried under vacuum overnight. Trituration with diethyl ether gave a pale orange solid (9.73g). The orange solid was then dissolved in DCM (150 mL) and washed with a saturated NaHCO3 solution (2 x 50 mL). The organic layer was dried and concentrated to yield (S)-(4-methylmorpholin-2-yl)methyl 4-methylbenzenesulfonate as an amber colored oil (6.51 g, 60% yield).

^{1}H NMR (400 MHz, CD3OD) δ ppm 7.77 - 7.82 (m, 2H), 7.43 - 7.48 (m, 2H), 4.06 - 4.14 (m, 2H), 4.03 (dd, *J* = 13.14, 2.78 Hz, 1H), 3.90 (dtd, *J* = 11.05, 4.20, 4.20, 2.27 Hz, 1H), 3.73 (ddd, *J* = 13.14, 12.13, 2.27 Hz, 1H), 3.35 (d, *J* = 12.13 Hz, 1H), 3.28 (br. s., 1H), 2.90 - 3.02 (m, 1H), 2.78 - 2.88 (m, 4H), 2.46 (s, 3H).

LCMS (ESI$^+$) m/z: 286.1 $(M+H)^+$



Cesium carbonate (11.66 g, 35.8 mmol) was added to 6-hydroxy-2-(6-(4-isopropyl-4H-1,2,4-triazol-3-yl)pyridin-2-yl)isoindolin-1-one (4.00 g, 11.93 mmol) in DMA (220 ml). This mixture was sonicated until all organic solids dissolved. A solution of (S)-(4-methylmorpholin-2-yl)methyl 4-methylbenzenesulfonate (4.08 g, 14.31 mmol) in DMA (20 mL) and the reaction mixture was heated to 65°C for 6 hours. A 10% aq. solution of sodium phosphate, dibasic heptahydrate was added dropwise until a fine milky precipitate formed. The product was dissolved in DMF and the insoluble salts were filtered off. The filtrate was concentrated to near dryness, diluted with EtOAc and washed with a saturated $NaHCO_3$ solution 3 times. Organic layers were combined, dried over magnesium sulfate and concentrated. The isolated orange oil (3.25 g) was purified by liquid chromatography (SiO2, 0-10% MeOH/DCM) to afford **17** as a pale yellow solid (2.27 g, 42% yield).

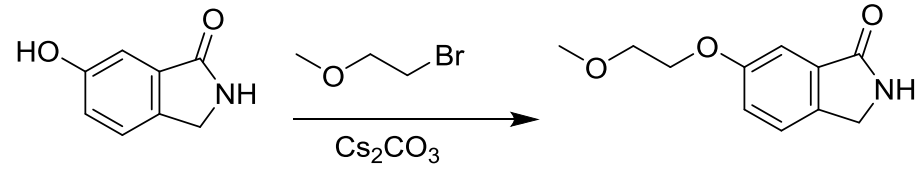
^{1}H NMR (400 MHz, DMSO-d6) δ ppm 8.95 (s, 1H), 8.64 (dd, *J* = 8.34, 0.76 Hz, 1H), 8.09 (dd, *J* = 8.34, 7.58 Hz, 1H), 7.92 (dd, *J* = 7.58, 0.76 Hz, 1H), 7.65 (d, *J* = 8.59 Hz, 1H), 7.30 - 7.36 (m, 2H), 5.47 - 5.58 (m, 1H), 5.10 (s, 2H), 3.79 - 3.86 (m, 2H), 4.10 (d, *J* = 5.05 Hz, 2H), 3.56 (td, *J* = 11.24, 2.27 Hz, 1H), 2.81 (d, *J* = 11.12 Hz, 1H), 2.63 (d, *J* = 11.87 Hz, 1H), 2.22 (s, 3H), 2.03 (td, *J* = 11.37, 3.03 Hz, 1H), 1.92 (t, *J* = 10.61 Hz, 1H), 1.58 (d, *J* = 6.57 Hz, 6H).

^{13}C NMR (101 MHz, DMSO-d6) δ ppm 167.1 (s, 1C), 158.7 (s, 1C), 151.0 (s, 1C), 150.0 (s, 1C), 145.9 (s, 1C), 143.2 (s, 1C), 139.4 (s, 1C), 133.8 (s, 1C), 133.2 (s, 1C), 124.8 (s, 1C), 121.3 (s, 1C), 119.0 (s, 1C), 113.3 (s, 1C), 107.0 (s, 1C), 73.2 (s, 1C), 69.1 (s, 1C), 65.4 (s, 1C), 55.9 (s, 1C), 54.0 (s, 1C), 49.0 (s, 1C), 48.3 (s, 1C), 45.5 (s, 1C), 23.3 (s, 1C).

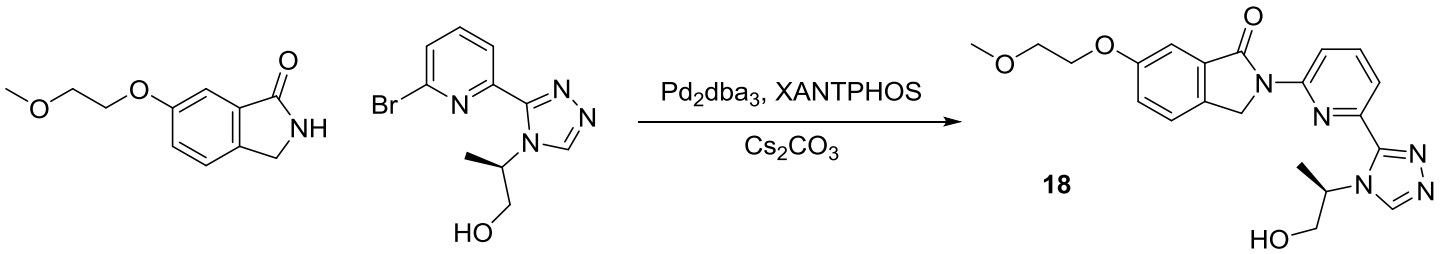
ESI-HRMS: calcd for 449.2296 $C_{24}H_{29}N_6O_3$ $(M+H)^+$, found 449.2301.

LCMS (ESI+) m/z: 449.3 (M+H)+
Purity TIC 100%, UV 100%
Chiral SFC retention times: 20.12 min, 20.20 min, 1.92 min for AD-H, OD-H and AS-H column respectively, 30 minutes run.

Compound **18:**



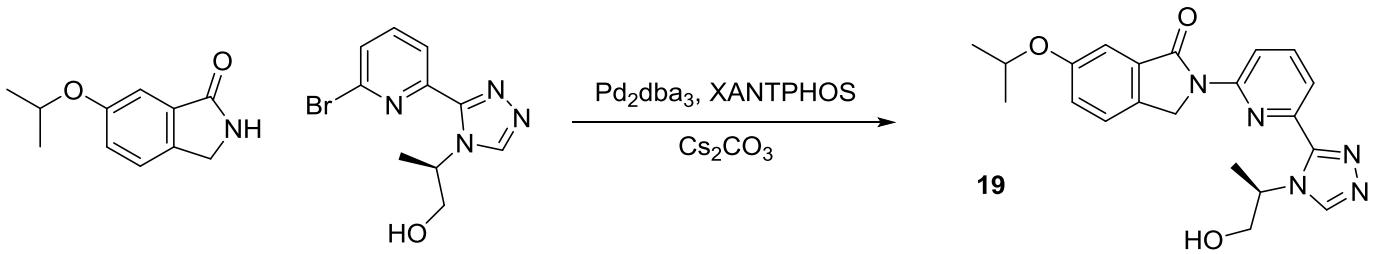
Following the procedure described for **12** and starting from 200 mg (1.34 mmol) of 6-hydroxyisoindolin-1-one and 186 mg (1.34 mmol) of 1-bromo-2-methoxyethane, 6-(2-methoxyethoxy)isoindolin-1-one was obtained with 80% yield.



18

Following the procedure described for methyl 6-(1-oxoisoindolin-2-yl)picolinate and starting from (R)-2-(3-(6-bromopyridin-2-yl)-4H-1,2,4-triazol-4-yl)propan-1-ol (46.1 mg, 0.163 mmol) and 6-(2-methoxyethoxy)isoindolin-1-one (40.5 mg, 0.195 mmol), **18** was obtained (16 mg, 24% yield).
^{1}H NMR (400 MHz, DMSO-d6) δ ppm 8.93 (s, 1H), 8.61 - 8.67 (m, 1H), 8.06 - 8.13 (m, 1H), 7.92 (dd, *J* = 7.58, 0.76 Hz, 1H), 7.64 (d, *J* = 8.08 Hz, 1H), 7.35 (d, *J* = 2.78 Hz, 2H), 5.49 (d, *J* = 5.31 Hz, 1H), 5.09 (s, 2H), 4.23 (br. s., 2H), 3.70 - 3.85 (m, 5H), 3.34 (s, 3H), 1.56 (d, *J* = 7.07 Hz, 3H).
^{13}C NMR (400 MHz, DMSO-d6) δ 167.12 (s, 1C), 158.84 (s, 1C), 150.93 (s, 1C), 150.54 (s, 1C), 145.99 (s, 1C), 143.80 (s, 1C), 139.51 (s, 1C), 133.72 (s, 1C), 133.21 (s, 1C), 124.83 (s, 1C), 121.45 (s, 1C), 119.02 (s, 1C), 113.39 (s, 1C), 106.94 (s, 1C), 70.29 (s, 1C), 67.47 (s, 1C), 64.27 (s, 1C), 58.19 (s, 1C), 53.74 (s, 1C), 49.06 (s, 1C), 17.80 (s, 1C).
LCMS (ESI^+) m/z: 410.4 $(M+H)^+$
Purity TIC 90%, UV 92%
ESI-HRMS: calcd for 410.1823 $C_{21}H_{24}N_5O_4$ $(M+H)^+$, found 410.1810.
Chiral SFC retention times: 4.91min, 8.42 min, 0.88 min for AD-H, OD-H and AS-H column respectively, 10 minutes run.

Compound **19:**



Following the procedure described for methyl 6-(1-oxoisoindolin-2-yl)picolinate and starting from (R)-2-(3-(6-bromopyridin-2-yl)-4H-1,2,4-triazol-4-yl)propan-1-ol (1.39 g, 4.91 mmol) and 6-isopropoxyisoindolin-1-one (938 mg, 4.91 mmol), **19** was obtained as a white solid (1.46 g, 78% yield).

^{1}H NMR (400 MHz, CDCl3) δ ppm 8.62 - 8.68 (m, 1H), 8.51 - 8.55 (m, 1H), 7.77 - 7.88 (m, 2H), 7.41 - 7.47 (m, 1H), 7.37 - 7.41 (m, 1H), 7.16 - 7.22 (m, 1H), 5.55 - 5.65 (m, 1H), 4.88 - 5.04 (m, 2H), 4.61 - 4.70 (m, 1H), 4.12 - 4.21 (m, 1H), 3.94 - 4.02 (m, 1H), 1.61 - 1.68 (m, 3H), 1.34 - 1.44 (m, 6H).

^{13}C NMR (101 MHz, DMSO-d6) δ ppm 167.2 (s, 1 C), 157.8 (s, 1 C), 151.0 (s, 1 C), 150.5 (s, 1 C), 146.0 (s, 1 C), 143.8 (s, 1 C), 139.5 (s, 1 C), 133.4 (s, 1 C), 133.2 (s, 1 C), 124.9 (s, 1 C), 122.4 (s, 1 C) 119.0 (s, 1 C), 113.4 (s, 1 C), 108.2 (s, 1 C), 69.8 (s, 1 C), 64.2 (s, 1 C), 53.7 (s, 1 C), 49.0 (s, 1 C), 21.7 (s, 1 C), 17.8 (s, 1 C)

LCMS (ESI^{+}) m/z: 394.3 $(M+H)^{+}$

Purity TIC 100%, UV 100%

ESI-HRMS: calcd for 394.1874 $C_{21}H_{24}N_5O_3$ $(M+H)^{+}$, found 394.1858.

Chiral SFC retention times: 3.02 min, 5.38 min, 0.74 min for AD-H, OD-H and AS-H column respectively, 10 minutes run.

II/ Protocols for *in vitro* assays (binding assay, functional assay, ADME)

ASK1 Enzyme Inhibition Assay

Material: ASKI recombinant protein was purchased from CarnaBio USA Inc. (Natick, MA, US). HTRF® KinEASE™ STK substrate 3 kit were purchased from CisBio US (Bedford, MA, US). The kinase assay was conducted in 250mM Hepes buffer containing NaN3 0.1%, 0.05% BSA, 0.5mM Orthovanedate, 5mM MgCl2,1mM DTT, 1% DMSO (after compound addition) at pH 7.0 according to the protocol provided by the vendor.

Methods: IC50 determination – the IC50 value for each compound was determined in the presence of compound (various concentrations, from 0 to 10uM) and a fixed amount of ATP (200uM, final concentration), substrate STK3 (1uM, final concentration). The enzymatic reaction was initiated by adding ASK1 (10nM, final concentration). The assay was conducted at room temperature (~ 22°C). After 60 min, the enzymatic reaction was stopped using the stop reagents provided by the CisBio kit. All the reagents were dispensed using a Multidrop Combi reagent dispenser (ThermoFisher Scientific, Waltham, MA US) into white 384 SV Greiner plates. The release of product was detected using a BMG PHERAstar plate reader (BMG LABTECH, Ortenberg, Germany) at 337 nm (excitation wavelength) and measuring the ratio of fluorescence 665/620 nm (emission wavelengths). Experimental data was fitted using equation 1:

$$\frac{V_i}{V_o} = \frac{100}{1+\left(\frac{I}{IC_{50}}\right)^n}$$

Vi and Vo are the rates of the enzyme activity in the presence and in the absence of inhibitor; n is the Hill coefficient; I is the free inhibitor concentration, respectively; IC50 is a measure of potency that is equivalent to a concentration of inhibitor that leads to a 50% inhibition of the enzyme activity.

ASK1-HEK293 P-JNK Cellular Assay. Human ASK1/HEK-293 cells were engineered to express human ASK1 utilizing a tetracycline-inducible gene expression (Tet-on) system, in which the gene of interest is activated by adding tetracycline (Tet) to the culture medium. Cells were maintained in DMEM high glucose (Thermo Fisher Scientific, Cat# 11995-065) medium containing 10% Fetal Bovine Serum (PAA Cat# A15-501), 100units/ml Penicillin G/100ug/mL Streptomycin Sulfate (Thermo Fisher Scientific, Cat# 15140-122), 500ug/ml Geneticin (Thermo Fisher Scientific, Cat# 10131035) and 5ug/ml Blasticidin (Thermo Fisher Scientific, Cat# A1113903). Cells were seeded at 300,000 cell/ml, 145ul/well, in 96 well black clear plates coated with PolyD Lysine (Becton Dickinson #REF356640) and incubated at 37°C under 5% CO2 for 5 hours before ASK1 expression was induced by adding 15ul of tetracycline (11ug/ml) to all wells (except for no Tet/No H2O2 negative controls). Cells were incubated overnight at 37°C under 5% CO2. Five-fold concentrated serial diluted compound plates were prepared the next morning and 0.040 mL from each dilution was transferred to the cell plates. Plates were then incubated for 30 minutes at 37°C under 5% CO2 before activating ASK-1 by adding 20ul of 10mM H2O2 for 30 more minutes to all wells (except for negative controls). At the end of incubation with H2O2, cell supernatants were gently aspirated, and cells were lysed by adding 70ul/well of complete MSD lysis buffer. Phospho- and total JNK levels were determined by MESO Scale ELISA (Cat# K1511D)

Human and Rat Liver Microsomal stability assay: Pooled rat or human microsomes were purchased from BD Biosciences. Liver microsomes were quickly thawed before further processing. The reactions were initiated on a Tecan liquid handler by adding room temperature NADPH solution into pre-warmed wells containing test compound or control compound as well as liver microsomes. The final incubation solutions (20 µL) contained 1 µM test compound or control, liver microsomes (0.5 mg/mL), 2 mM NADPH, and 50 mM potassium phosphate buffer (pH 7.4). Plates were incubated at 37°C for 0, 5, 15 and 30 minutes. The reactions were terminated by the addition of 40 µL of 0.1 M trichloroacetic acid (TCA) quenching solution. The plates were centrifuged at 4500g for 20 min. The peak areas of test compound or controls in the supernatants were measured by LC-MS/MS. A Waters Quattro Premier UPLC-MS/MS System consisting of an Acquity Binary Solvent manager; Waters Acquity sample manager and Waters Acquity Sample Organizer was used for liver microsomal sample analysis. Waters MassLynx software was used for instrument control, data acquisition, data processing and chromatogram/spectrum browsing. Liquid chromatography was performed using a Waters BEH C18, 1.7 µm, ID 2.1 x 50 mm at 50°C column eluting with mobile phase A: 0.04% formic acid in water and mobile phase B: 0.04% formic acid in acetonitrile with a flow rate of 0.35 mL min-1 and a gradient of 1% to 99% B over 1.0 min. The metabolic half life (t1/2) values were calculated using Equation 1, where A is the initial peak area expressed as 100%, k is the first-order rate constant, and t is time in minutes.

Equation 1 Calculation of Metabolic Half Life

$$\%\text{remaining} = Ae^{-kt}$$

The experimental data were fitted to the above equation using IDBS ActivityBase Suite (IDBS, Alameda, CA). The t1/2 was calculated as t when % remaining = 50%. Intrinsic clearance (Cl'int) was calculated from the t1/2 using Equation 2 with microsomal protein yield 45 mg protein g-1 liver, and the scaling factors of 40 and 20g of liver kg-1 of body weight for rat and human respectively.1

Equation 2 Calculation of Hepatic Intrinsic Clearance for Microsomes

Cl'int = (ln2/t1/2)x(ml incubation/mg microsomal protein)x(mg microsomal protein/g of liver)x(g of liver/kg of body weight)

Solubility Measurement: 196 uL of testing medium (In a 1L volumetric flask, dissolve 2.0 g sodium chloride in about 500 ml deionized water. Add 84 ml 1M hydrochloric acid and dilute to volume with deionized water. Mix thoroughly) was

added to compound **19** (5 ul of a 10 mM solution in 100% DMSO dispensed in quadruplets). The plate was then sealed with a cap mat, and equilibrated overnight on a shaker at room temperature (19-23oC). After equilibration, the samples in the 96-well plate were transferred to a 96-well filter plate (1 um, fiber glass filter membrane) and centrifuge-filtered into a new 96-well sample plate (sample filtrate plate). The calibration standards were prepared by adding 195 uL of standard diluent into the standard plate (quadruplets), cap-mat sealed and equilibrated for 2 hours by vigorous shaking on a shaker. The default diluent for standards is 10% DMSO in acetonitrile. The standard plate and sample filtrate plate were then analyzed by HPLC. The calibration response factor was calculated by dividing the average compound peak area from quadruplet standards by 250 (the concentration of standard in uM). The sample solubility in a test medium (in uM) is calculated by dividing the average compound peak area from triplicate samples by the response factor of the corresponding calibration standard. Solubility for **19** was measured at 155uM.

Efflux/permeability assessment: The bottom pieces (basolateral side) of two individual 0.4uM pore size, polycarbonate HTS transwell-96well permeable plates assemblies (Corning Life Sciences, Salt Lake City, UT) were filled with fresh Invitrogen medium 199 fortified with 10% FBS, 1% Geneticin, and 150ng/ml colchicine (cell culture media) to 250uL. The inserts (apical side) of the plate assemblies were filled with 25uL of the cell culture media. The two pieces were put together and preincubated in an atmosphere of 5% CO2/95% air at 37 °C for 30 minutes prior to cell seeding. LLC-PK1 Porcine kidney cells transfected with the human MDR1 gene were then plated into the two inserts (apical side) of the plate assemblies with a seeding density of 112,500 cells per well. Plates were mixed in a manual criss-cross motion and then incubated in an atmosphere of 5% CO2/95% air at 37 °C for 7 days. Culture media was replaced once during the 7 days before the assay.

On the day of the experiment, the cell culture media was replaced with warmed Invitrogen media 199 fortified with 10mM Hepes and 1% BSA (assay media), where the apical side received 75uL of the assay media and the basolateral side received 250uL. Once the media was replaced, the plates were put together and preincubated at an atmosphere of 5% CO2/95% air for 1 hour at 37°C to assure consistent temperature. Prepare 2.5mL of 10uM (0.1% final DMSO concentration) of compound stock in assay media fortified with 10uM atenolol as a cell monolayer intergrity marker. After the preincubation, in one of the two plates, replace the apical side assay media with 75uL of compound stock in assay media and with the other plate replace the basolateral side with 250uL of compound stock in assay media. Once both plates were treated and reassembled, the cell plates were incubated for 2 hours at a temperature of 37 °C in an atmosphere of 5% CO2/95% air without being perturbed. Once the incubation had completed, 50uL samples were taken from both the apical to basolateral and the basolateral to apical test plates. Those samples were combined in separate wells of a 96 well polypropylene plate with 100uL of cold acetonitrile to induce protein precipitation and extraction of the compound. Plates were then shaken for 10 minutes and then centrifuged at 4000xg at 4°C for 10 minutes. 75uL of the sample supernatant was then combined with 75uLof 0.2uM buspirone and taurocholic acid in a solution of 10% acetonitrile and 90% water (as an internal standard) for sample analysis.

Sample were analyzed via LC-MS/MS on a SCIEX API4000 Qtrap, with LC conditions using a kinetix 2.1X 30C18 100A column, mobile phase A of 0.04% formic acid in HPLC grade water, mobile phase B of 0.04% formic acid in acetonitrile. Method run time of 1.6 minutes and a gradient shown in table S1:

TABLE S1: Gradient condition for LC-MS/MS analysis

Time (min)	Flow rate (ml/min)	% A	%B
0.00	0.6	99	1

0.20	0.6	99	1
0.50	0.6	50	50
1.00	0.6	5	95
1.20	0.6	5	95
1.30	0.6	99	1
1.60	0.6	99	1

LC-MS/MS conditions were optimized using 1 µM test compound in 50:50 methanol:water and were quantified using a standard 8 point standard curve prepared in the same fashion as the samples.

Peak area ratio of the compound to the internal standard was then compared to the 8 point standard curve.

The following equations were then utilized to estimate permeability from both the apical to basolateral side and the basolateral side to the apical side.

$$P_{app\ A-B}\ (nm/sec) = \left(Conc_{BL} * 0.25 * 10000\right) \Big/ \left(A * t * 10\right)$$

$$Recovery\%_{A-B} = \frac{\left(Conc_{BL} * 0.25 + Conc_{AP} * 10 * 0.075\right)}{Dosing\ Conc * 0.075}$$

$$P_{app\ B-A}\ (nm/sec) = \left(Conc_{AP} * 0.075 * 10000\right) \Big/ \left(A * t * 10\right)$$

$$Recovery\%_{B-A} = \frac{\left(Conc_{AP} * 0.075 + Conc_{BL} * 10 * 0.25\right)}{Dosing\ Conc * 0.25}$$

$$ER = \frac{P_{app\ B-A}}{P_{app\ A-B}}$$

Where:

Papp A-B = Apparent permeability A-B

Papp B-A = Apparent permeability B-A

Conc $_{BL}$ = Basolateral well concentration

Conc $_{AP}$ = Apical well concentration

Dosing conc = starting concentration

A = well surface area (cm2), A = 0.143 cm2

t= incubation time (seconds), t = 7200 seconds

III/ In vivo assays (PK, Langendorff Perfused Heart Model)

Rat Pharmacokinetics: Cannulated male Sprague-Dawley (SD) rats were fasted overnight then treated with **19** formulated in 20% (2-hydroxypropyl)-β-cyclodextrin in 0.05M methanesulfonic acid at 5 mg/kg by oral gavage. Dose volume was 5 ml/kg and **19** was dosed as a solution at a concentration of 1 mg/ml. Residual dose form was saved and analyzed for exact dose concentration. Following administration of test article, 200 ul of blood was collected through the jugular vein catheter from conscious animals at 0.25, 0.5, 1, 2, 4, 7 and 24 hours. Blood samples were kept on ice until processed for plasma. Plasma was prepared by centrifugation at 5°C, frozen and stored at -70°C until analyzed.

Analysis of **19** plasma concentrations was performed by LC-MS/MS analysis. Briefly, 25 ul of plasma was mixed with 100 ul acetonitrile containing an appropriate internal standard. Samples were vortexed for 1 minute then centrifuged at 300 rpm for 5 min at 2-8°C. Forty ul of supernatant was then diluted 1:2 with water and vortexed for 5 min. Samples were analyzed with a AB Sciex API-4000 triple quadropole mass spectrometer equipped with a Shimadzu LC System and a LEAP autosampler. A reverse-phase gradient method running at a flow rate of 0.500 mL/min on an Phenomenex, Kinetic C18 column (2.1 mm ID × 50 mm; particle size 5.0 μm) was used for the test article separation. The mobile phase used was water (A) and acetonitrile (B), and both were supplemented with formic acid (0.04%, volume-to-volume ratio [v:v]). Samples were ionized and detected in multiple reactions monitoring (MRM) mode by monitoring the transition m/z 394.051→ 336.100. Samples were quantitated by use of analyte standards prepared in pooled rat plasma with internal standard. The lower limit of quantitation in this assay was 1.00 ng/mL and linearity was achieved in the concentration range of 1.00 ng/mL to 2500 ng/mL.

Plasma concentration data was analyzed by non-compartmental analysis in Watson (Thermo Scientific, Version 7.4.2). Pharmacokinetic modeling to support dose selection for pharmacology studies was performed using SAAM II (The Epsilon Group, Version 2.3.1.1) assuming linear pharmacokinetics.

Langendorff Perfused Heart Model: Male Sprague Dawley rats were obtained from Harlan laboratories and were allowed to acclimate at least 48 hours before being used in the study. Rats were housed 2-3 animals/cage and had free access to food and water throughout the study. Rats were dosed with vehicle or compound 1, 4, 6, or 8 hours prior to ex vivo ischemia/reperfusion (I/R). Global no-flow ex vivo I/R was performed on a constant pressure recirculating Langendorff apparatus (Radnoti cat#120102). Rats were treated with 500 U/kg heparin (Sagent cat#25021-400-100) 10 min prior to administration of ketamine (60 mg/kg), xylazine (7.5 mg/kg) via intraperitoneal injection. Analgesics (buprenorphine hydrochloride, 0.05 mg/kg) were administered prior to surgery via subcutaneous injection. Once appropriate depth of anesthesia and analgesia were confirmed, animals were sacrificed and the hearts were quickly removed and placed in ice-cold modified Krebs-Henseleit buffer (Sigma, St. Louis, MO). The aorta was then cannulated and the heart was mounted on a Langendorff apparatus and perfused with oxygenated Krebs-Henseleit buffer at a constant pressure of 80 mmHg. Hearts were submerged in buffer warmed to 37°C at all times. Following a 30 minutes equilibration period, hearts were subjected to 30 minutes of no flow ischemia followed by 90 minutes of reperfusion. At the completion of I/R, hearts were flash frozen on dry ice and sectioned coronally into 3mm pieces (6 in total) using a rat heart matrix and razor blades. Hearts were then stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 10 minutes to visualize viable tissue and then fixed overnight in 10% formalin. Sections were subsequently scanned and the total area and infarcted area of each section of the heart were quantified using Image J (National Institutes of Health).

Relative infarct area (% infarct) was calculated as a sum of infarct areas on each of the 6 heart sections divided by a summative total area of the 6 sections. Statistical analysis was performed on data using non-parametric analysis (Kruskal-Wallis test) given the limited sample size.

V/ Crystal structures information

Table S1: ASK1_ 6 (5UP3)
Table S2: ASK1_17 (5UOR)
Table S3: ASK1_19 (5UOX)

Ask1 Purification and Crystallization

Material The catalytic domain of the human serine/threonine kinase ASK1 (encoding residues 667-952) was modified to contain a C-terminal 6XHis tag and cloned into a modified pET28 vector. Human ASK1 recombinant protein was expressed in E.coli Rosetta2 (DE3) pLysS in 2X YT medium in the presence of 100 ug/ml ampicillin and 34ug/ml chloramphenicol. Expression was induced by 0.5 mM isopropyl β-L-1-thiogalactopyranoside when OD600 reached 0.8, and cell cultures were grown for an additional 3 h at 30 °C.

Protein was purified first with Ni affinity chromatography and then was dephosphorylated by lambda phosphatase. Next anion exchange chromatography was performed to remove phosphatase. Further purification by size-exclusion chromatography was carried out with the final buffer containing 20 mM Tris-HCl (pH8.0), 80 mM NaCl, 1 mM DTT. Purity of purified protein was estimated to be more than 95% by SDS-PAGE. Mass spectrometric analysis confirmed that the protein was prepared as the non-phosphorylated form. For compound co-crystallization experiments an aliquot of the frozen protein was incubated with compound to a final concentration of 1mM in 4^0C overnight and then further concentrated to 20 mg/ml.

Crystals suitable for data collection were obtained by vapor diffusion in sitting drops at 4 °C. Reservoirs contained 5 -9% PEG 2K MME and 50 mM MES pH 5.8 -6.2. Crystals that took 4 -7 days to grow were immersed in mother liquor solution containing 22% ethylene glycol for cryoprotection and flash frozen in liquid nitrogen. Crystals of the Ask1 complexes grew in the hexagonal space group **P 6_5 2 2** and contain two molecules in the asymmetric unit.

Diffraction data from 2.30 to 3.00 A were collected from single cryogenically protected crystals at beam lines 24-ID-C of the Advanced Photon Source at Argonne National Laboratory, 5.0.3 of the Advanced Light Source at Lawrence Berkeley National Laboratory and SLS PXIII at the Swiss Light Source. Data was reduced using the HKL2000 software package.[1] The structure was determined by molecular replacement with either MOLREP[2] or PHASER[3] of the CCP4 program suite and refined with the program REFMAC.[4] several cycles of model building with XtalView[5] or COOT[6] and refinement were performed for improving the quality of the model. The coordinates and structure factors have been deposited in Protein Data Bank with accession code, 5UP3, 5UOR and 5OUX.

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The data and refinement statistics are given in Tables below.

Table S1: ASK1_CMPD 6

Data Collection		*Refinement and Statistics*	
Resolution, (Å)	30-2.9 (3.0-2.95)*	Rwork, (%)	24.2
Space group	P 65 2 2	Rfree, (%)	28.9
a, (Å)	78.8	RMSD from ideal geometry:	
b, (Å)	78.8	Bond lengths, (Å)	0.008
c, (Å)	431.4	Bond angles, (°)	1.30
Molecules per asymmetric unit	2	Numbers of atoms:	
Unique Reflections	17,858 (1,735)*	Protein (non-hydrogen)	4,031
Multiplicity	12.2 (11.8)*	Water oxygen atoms	2
Average I/σ(I)	14.7 (3.2)*	Ligand atoms	48
Rmerge, (%)	17.9 (74.0)*		
Completeness, (%)	99.6 (100.0)*	PDB ID	5UP3
*The values in the parenthesis are those from the highest resolution shell			

Table S2: ASK1_CMPD 17

Data Collection		*Refinement and Statistics*	
Resolution, (Å)	30-2.7 (2.8-2.75)*	Rwork, (%)	20.2
Space group	P 65 2 2	Rfree, (%)	26.7
a, (Å)	78.5	RMSD from ideal geometry:	
b, (Å)	78.5	Bond lengths, (Å)	0.008
c, (Å)	431.7	Bond angles, (°)	1.38
Molecules per asymmetric unit	2	Numbers of atoms:	
Unique Reflections	21,703 (2,113)*	Protein (non-hydrogen)	4229
Multiplicity	5.7 (5.9)*	Water oxygen atoms	75
Average I/σ(I)	15.2 (2.2)*	Ligand atoms	76
Rmerge, (%)	9.42 (77.7)*		
Completeness, (%)	99.7 (99.9)*	PDB ID	5UOR
*The values in the parenthesis are those from the highest resolution shell			

Table S3: ASK1_CMPD 19

Data Collection		*Refinement and Statistics*	
Resolution, (Å)	30-2.5 (2.6-2.5)*	Rwork, (%)	22.8
Space group	P 65 2 2	Rfree, (%)	25.3
a, (Å)	78.5	RMSD from ideal geometry:	
b, (Å)	78.5	Bond lengths, (Å)	0.008
c, (Å)	434.0	Bond angles, (°)	1.15
Molecules per asymmetric unit	2	Numbers of atoms:	
Unique Reflections	28,829 (2,781)*	Protein (non-hydrogen)	4225
Multiplicity	10.4 (9.9)*	Water oxygen atoms	70
Average I/σ(I)	17.6 (3.4)*	Ligand atoms	58
Rmerge, (%)	8.27 (59.5)*		
Completeness, (%)	99.8 (100.0)*	PDB ID	5UOX
*The values in the parenthesis are those from the highest resolution shell			

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