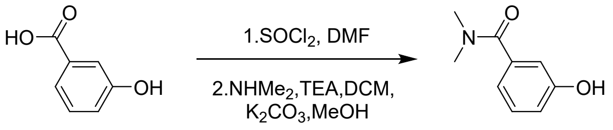
Experimental Procedures

Chemistry

General Methods. Starting materials, reagents, and solvents were purchased from commercial suppliers and were used without further purification unless otherwise noted. All reactions were monitored using a Waters Acquity UPLC/MS system (Waters PDA eλ Detector, QDa Detector, Sample manager-FL, Binary Solvent Manager) using Acquity UPLC® BEH C18 column (2.1 x 50 mm, 1.7 μm particle size): solvent gradient = 85% A at 0 min, 1% A at 1.7 min; solvent A = 0.1% formic acid in Water; solvent B = 0.1% formic acid in Acetonitrile; flow rate : 0.6 mL/min. Reaction products were purified by flash column chromatography using CombiFlash®Rf with Teledyne Isco RediSep® normal-phase silica flash columns (4 g, 12 g, 24 g, 40 g or 80 g) and Waters HPLC system using SunFireTM Prep C18 column (19 x 100 mm, 5 μm particle size): solvent gradient = 80% A at 0 min, 10% A at 25 min; solvent A = 0.035% TFA in Water; solvent B = 0.035% TFA in MeOH; flow rate : 25 mL/min. ^{1}H NMR spectra were recorded on 400 MHz Bruker Avance III spectrometers Chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane (TMS). Coupling constants (J) are reported in Hz. Spin multiplicities are described as br (broad), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet).

3-hydroxy-*N*,*N*-dimethylbenzamide (2)

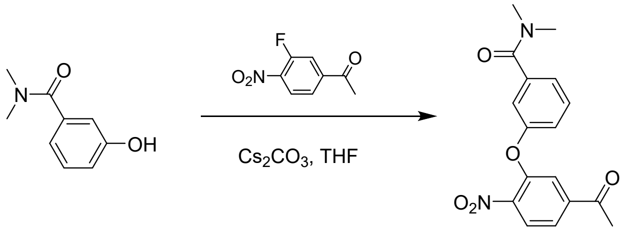


1 **2**

To a solution of 3-hydroxybenzoic acid (5.0 g, 36.20 mmol, 1.0 equiv) in thionyl chloride ($SOCl_2$, 20 mL) was added *N*,*N*-dimethylformamide (DMF, 26.5 mg, 362.0 μmol, 27.9 μL, 0.01 equiv). The reaction mixture was stirred at 80 °C for 2 hours. Upon completion, the residue was diluted with dichloromethane (DCM, 20 mL), concentrated under reduced pressure, re-diluted with DCM (20 mL), and concentrated again to afford crude 3-hydroxybenzoyl chloride (5.7 g) as a yellow oil. To a solution of *N*-methylmethanamine hydrochloride (2.97 g, 36.41 mmol, 3.34 mL, 1.0 equiv) in dichloromethane (DCM, 50 mL) was added triethylamine (TEA, 9.21 g, 91.02 mmol, 12.67 mL, 2.5 equiv). To the resulting mixture, 3-hydroxybenzoyl chloride (5.7 g, 36.41 mmol, 1.0 equiv) was added dropwise. The reaction mixture was stirred at 25 °C for 2 hours. After completion, the mixture was concentrated under reduced pressure to remove the solvent. To the resulting residue, a solution of potassium carbonate (K_2CO_3, 15.09 g, 109.22 mmol, 3.0 equiv) in methanol (30 mL) was added. The reaction was stirred at 25 °C for 12 hours. The mixture was concentrated under reduced pressure, and the residue was diluted with water (20 mL) and extracted with ethyl acetate (EtOAc, 3 × 20 mL). The combined organic layers were dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and concentrated under reduced pressure to afford a crude residue. Purification by flash silica gel chromatography (12 g silica column, 0–50% EtOAc/hexanes gradient, flow rate: 100 mL/min) yielded 3-hydroxy-N,N-dimethylbenzamide (4.0 g, 21.31 mmol, 58.5% yield) as a white solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.64 (br s, 1H), 7.21 (t, J = 7.8 Hz, 1H), 6.83 - 6.74 (m, 2H), 6.74 - 6.71 (m, 1H), 3.00 - 2.83 (m, 6H)

3-(5-acetyl-2-nitrophenoxy)-*N,N*-dimethylbenzamide (3)

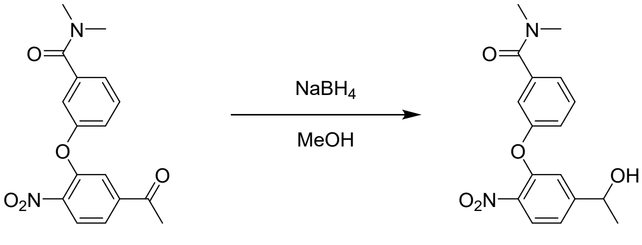


2 3

A mixture of 3-hydroxy-*N,N*-dimethylbenzamide (3.6 g, 21.79 mmol, 1.0 equiv) and 1-(3-fluoro-4-nitrophenyl)ethanone (5.04 g, 27.52 mmol, 1.3 equiv) in tetrahydrofuran (THF, 50 mL) was degassed and purged with nitrogen (×3). The reaction mixture was then stirred at 0 °C for 15 minutes under a nitrogen atmosphere. Cesium carbonate (Cs_2CO_3, 8.64 g, 26.52 mmol, 1.22 equiv) was added, and the mixture was stirred at 0 °C for 1 hour under nitrogen. The reaction mixture was concentrated under reduced pressure to remove the solvent. The crude residue was purified by flash column chromatography (20 g silica gel, 0–55% ethyl acetate/hexanes gradient, flow rate: 120 mL/min) to give the title compound (6.95 g, 19.21 mmol, 88.2% yield) as a yellow oil

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (d, J = 8.4 Hz, 1H), 7.71 (dd, J = 1.8, 8.4 Hz, 1H), 7.55 (d, J = 1.5 Hz, 1H), 7.40 - 7.34 (m, 1H), 7.21 - 7.15 (m, 1H), 7.06 - 6.99 (m, 2H), 3.07 - 2.88 (m, 6H), 2.51 (s, 3H)

3-(5-(1-hydroxyethyl)-2-nitrophenoxy)-*N,N*-dimethylbenzamide (4)

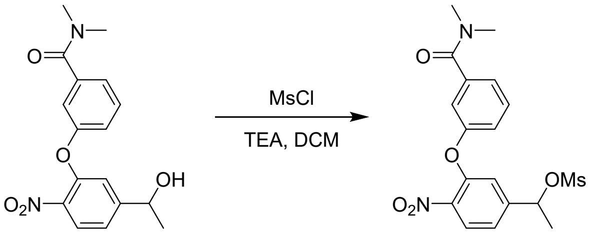


3 4

To a solution of 3-(5-acetyl-2-nitrophenoxy)-*N,N*-dimethylbenzamide (6.95 g, 21.17 mmol, 1 eq) in MeOH (70 mL) was added $NaBH_4$ (808.81 mg, 21.38 mmol, 1.0 eq) at 0°C. The mixture was stirred at 0 °C for 0.5 hr under N_2 atmosphere. The residue was diluted with 50 mL of saturated NH_4Cl aqueous and extracted with EtOAc 150 mL (50 mL × 3). The combined organic phase was dried over by anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a residue. The residue was purified by flash silica gel chromatography (20 g Silica Flash Column, Eluent of 0~70% ethyl acetate/hexanes gradient, flow rate: 120 mL/min) to give the title compound (6.2 g, 17.35 mmol, 81.96% yield) as brown oil.

^1H NMR (400 MHz, $CDCl_3$) δ 7.95 (d, J = 8.5 Hz, 1H), 7.44 - 7.36 (m, 1H), 7.22 (dd, J = 1.6, 8.4 Hz, 1H), 7.18 (td, J = 1.2, 7.6 Hz, 1H), 7.12 - 7.08 (m, 2H), 7.02 (dd, J = 1.6, 2.3 Hz, 1H), 4.85 (d, J = 6.5 Hz, 1H), 3.15 - 2.89 (m, 6H), 1.43 (d, J = 6.5 Hz, 3H)

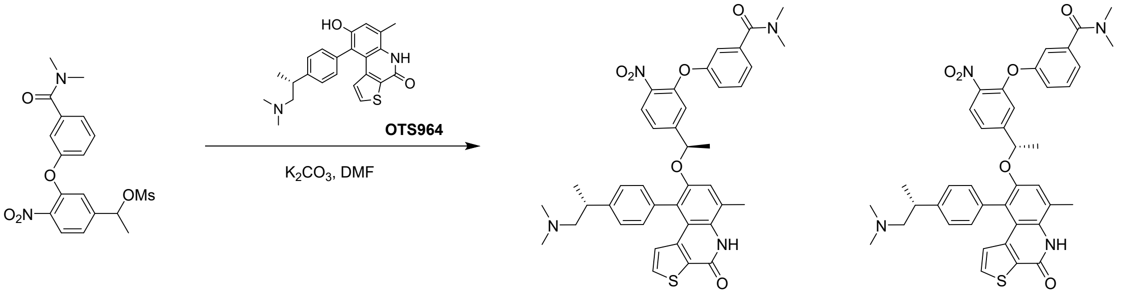
1-(3-(3-(dimethylcarbamoyl)phenoxy)-4-nitrophenyl)ethyl methanesulfonate (5)



4 **5**

To a solution of 3-(5-(1-hydroxyethyl)-2-nitrophenoxy)-*N,N*-dimethylbenzamide (400 mg, 1.21 mmol, 1.0 equiv) in dichloromethane (DCM, 4 mL) was added triethylamine (TEA, 245.1 mg, 2.42 mmol, 337.1 µL, 2.0 equiv). Methanesulfonyl chloride (MsCl, 277.4 mg, 2.42 mmol, 187.4 µL, 2.0 equiv) was then added dropwise at 0 °C. The reaction mixture was stirred at 25 °C for 1 hour. The mixture was quenched with water (5 mL) and extracted with DCM (3 × 5 mL). The combined organic phase was dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and concentrated under reduced pressure to the title compound (500 mg, crude) as a yellow oil.

3-(5-((*R*)-1-((9-(4-((*R*)-1-(dimethylamino)propan-2-yl)phenyl)-6-methyl-4-oxo-4,5-dihydrothieno[2,3-*c*]quinolin-8-*yl*)oxy)ethyl)-2-nitrophenoxy)-*N,N*-dimethylbenzamide (17C-1) and 3-(5-((*S*)-1-((9-(4-((*R*)-1-(dimethylamino)propan-2-yl)phenyl)-6-methyl-4-oxo-4,5-dihydrothieno[2,3-*c*]quinolin-8-yl)oxy)ethyl)-2-nitrophenoxy)-*N,N*-dimethylbenzamide (17C-2)

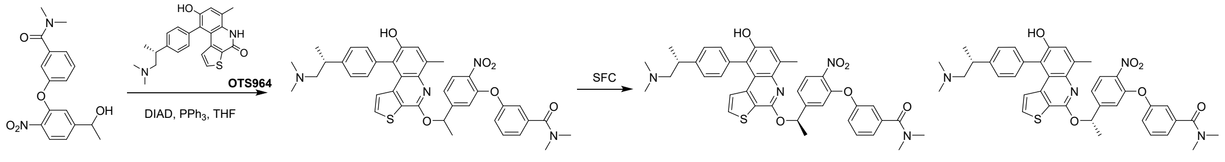


5 **17C-1** **17C-2**

To a solution of 9-[4-[(1*R*)-2-(dimethylamino)-1-methyl-ethyl]phenyl]-8-hydroxy-6-methyl-5*H*-thieno[2,3-*c*]quinolin-4-one (200 mg, 509.54 µmol, 1 eq) in DMF (4 mL) was added K_2CO_3 (211.26 mg, 1.53 mmol, 3 eq). Then 1-(3-(3-(dimethylcarbamoyl)phenoxy)-4-nitrophenyl)ethyl methanesulfonate (312.16 mg, 764.31 µmol, 1.5 eq) was added. The mixture was stirred at 25°C for 12 hr. The reaction mixture was filtered. The residue was purified by prep-HPLC to give the mixed diastereomers (90 mg, yield 25%) as a white solid, which was further separated by SFC (condition: column: DAICEL CHIRALPAK IG (250mm×30mm,10um); mobile phase: [CO_2-EtOH(0.1% NH_3H_2O)];B%:50%, isocratic elution mode) to give **17C-1** (22.53 mg) as a brown solid MS (ESI): *m*/*z* = 705.3 $[M+H]^+$ ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 8.88 (br s, 1H), 7.86 (d, *J* = 8.4J Hz, 1H), 7.40 - 7.33 (m, 3H), z), 7.25 - 7.17 (m, 2H), 7.09 - 7.01 (m, 2H), 6.98 (dd, *J* = 2.3, 8.1 Hz, 1H), 6.94 (d, *J* = 8.5 Hz, 1H), 6.89 (s, 2H), 6.03 (d, *J* = 5.4 Hz, 1H), 5.11 (q, *J* = 6.3 Hz, 1H), 3.14 - 3.00 (m, 4H), 2.94 (br s, 3H), 2.64 - 2.49 (m, 5H), 2.33 (s, 6H), 1.39 (d, *J* = 6.9 Hz, 3H), 1.31 (d, *J* = 6.5 Hz, 3H). **17C-2** (26.55 mg) as a brown solid. MS (ESI): *m*/*z* = 705.3 $[M+H]^+$

^{1}H NMR (400 MHz, $CDCl_3$) *δ* 8.73 (br s, 1H), 7.86 (d, *J* = 8.4 Hz, 1H), 7.39 - 7.32 (m, 3H), 7.29 (dd, *J* = 1.6, 7.9 Hz, 1H), 7.23 (dd, *J* = 1.6, 7.9 Hz, 1H), 7.19 (d, *J* = 7.6 Hz, 1H), 7.07 - 7.01 (m, 2H), 6.98 (td, *J* = 1.2, 8.2 Hz, 1H), 6.93 (dd, *J* = 1.4, 8.5 Hz, 1H), 6.90 (s, 2H), 6.03 (d, *J* = 5.4 Hz, 1H), 5.11 (q, *J* = 6.4 Hz, 1H), 3.14 - 3.01 (m, 4H), 2.94 (s, 3H), 2.62 - 2.46 (m, 5H), 2.33 (s, 6H), 1.39 (d, *J* = 6.9 Hz, 3H), 1.32 (d, *J* = 6.4 Hz, 3H). The stereochemistry for these compounds is arbitrarily assigned.

3-(5-((*R*)-1-((9-(4-((*R*)-1-(dimethylamino)propan-2-yl)phenyl)-8-hydroxy-6-methylthieno[2,3-*c*]quinolin-4-yl)oxy)ethyl)-2-nitrophenoxy)-*N,N*-dimethylbenzamide (17A-1) and 3-(5-((*S*)-1-((9-(4-((*R*)-1-(dimethylamino)propan-2-yl)phenyl)-8-hydroxy-6-methylthieno[2,3-*c*]quinolin-4-yl)oxy)ethyl)-2-nitrophenoxy)-*N,N*-dimethylbenzamide (17A-2)

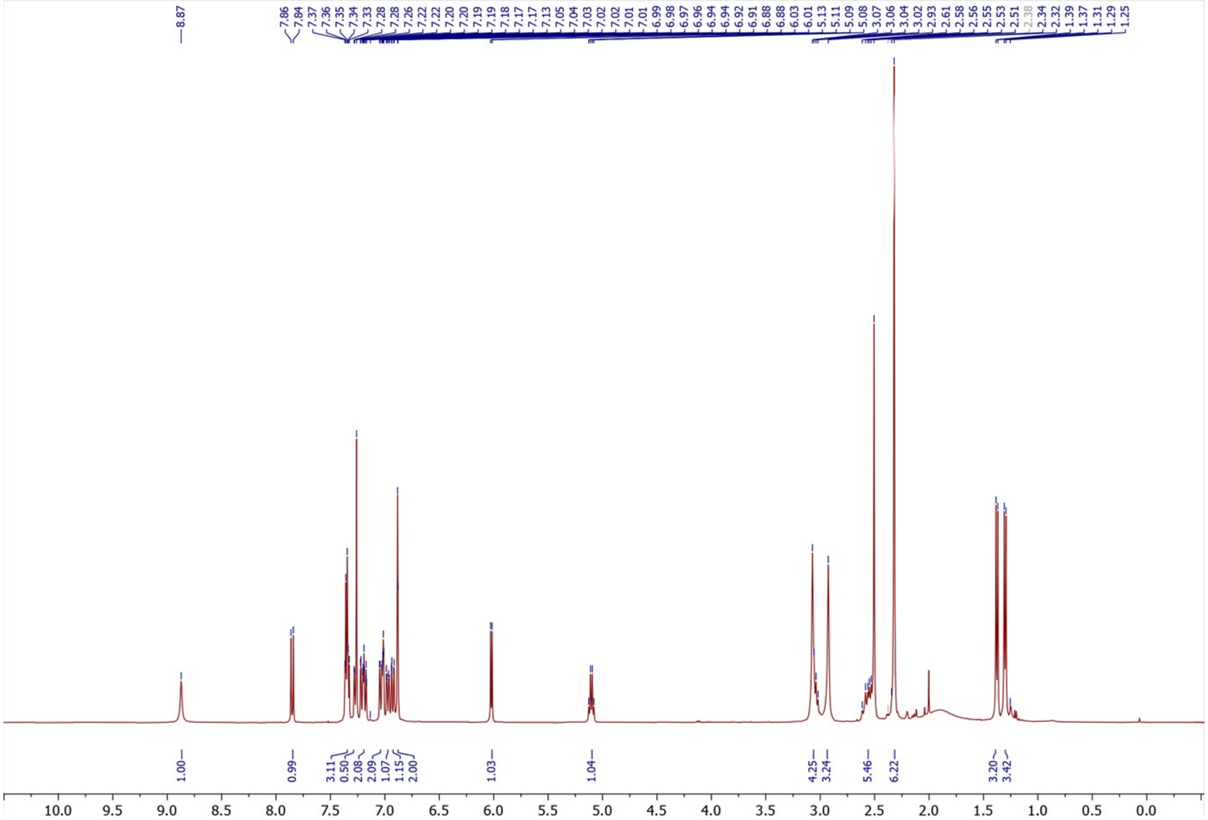


4 17A (-1/-2) 17A-1 17A-2

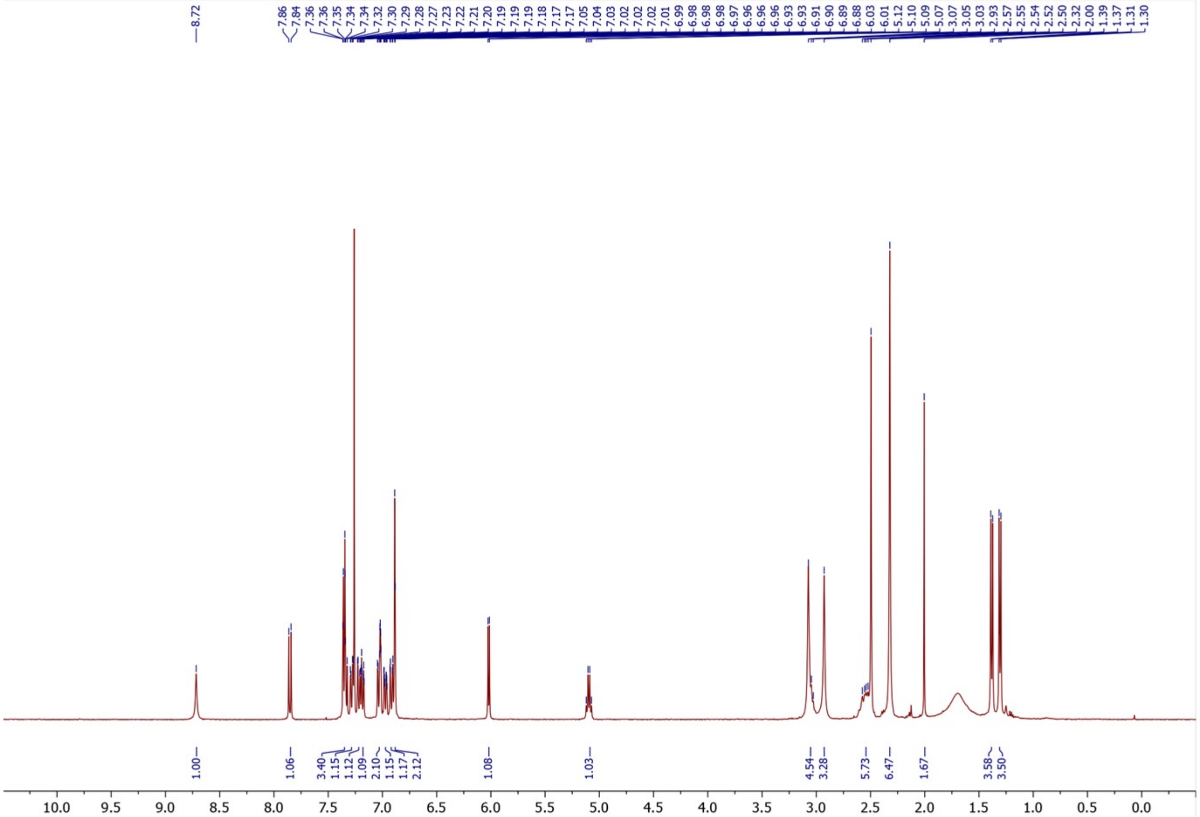
A mixture of 9-[4-[(1*R*)-2-(dimethylamino)-1-methyl-ethyl]phenyl]-8-hydroxy-6-methyl-5*H*-thieno[2,3-c]quinolin-4-one (142.59 mg, 363.27 µmol, 1 eq) , 3-[5-(1-hydroxyethyl)-2-nitro-phenoxy]-*N,N*-dimethyl-benzamide (120 mg, 363.27 µmol, 1 eq) , PPh_3 (142.92 mg, 544.90 µmol, 1.5 eq) , DIAD (110.18 mg, 544.90 µmol, 105.64 µL, 1.5 eq) in THF (3 mL) was degassed and purged with N_2 for 3 times, and then the mixture was stirred at 70 °C for 12 hr under N_2 atmosphere. The reaction mixture was concentrated under reduced pressure to remove solvent. The residue was purified by prep-HPLC to the mixed diastereomers **17A (-1/-2)** (20 mg, 28.38 µmol, 7.81% yield) as a white solid. MS (ESI): *m*/*z* = 705.3 $[M+H]^+$ Followed by SFC sepration (condition: column: DAICEL CHIRALPAK IG (250mm*30mm,10um);mobile phase: [CO_2-MeOH(0.1%NH_3H_2O)];B%:40%, isocratic elution mode) to afford **17A-1** (7.9 mg) as a white solid. MS (ESI): *m*/*z* = 705.4 $[M+H]^+$

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (d, *J* = 8.4 Hz, 1H), 7.48 – 7.37 (m, 3H), 7.29 (dd, *J* = 8.2, 4.9 Hz, 3H), 7.25 – 7.17 (m, 4H), 7.07 (t, *J* = 1.9 Hz, 1H), 6.92 (dd, *J* = 8.1, 2.5 Hz, 1H), 6.44 (q, *J* = 6.6 Hz, 1H), 6.09 (d, *J* = 5.4 Hz, 1H), 3.11 - 3.03 (m,4H), 2.92 (s, 3H), 2.60 – 2.46 (m,5H), 2.30 (s, 6H), 2.01 (s, 6H), 1.74 (d, *J* = 6.6 Hz, 3H), 1.38 (d, *J* = 6.9 Hz, 3H).**17A-2** (7.8 mg) as a white solid. MS (ESI): *m*/*z* = 705.3 $[M+H]^+$ ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (d, *J* = 8.5 Hz, 1H), 7.49 – 7.37 (m, 3H), 7.34 – 7.27 (m, 4H), 7.25 – 7.16 (m, 3H), 7.07 (dd, *J* = 2.6, 1.5 Hz, 1H), 6.93 (ddd, *J* = 8.2, 2.6, 1.1 Hz, 1H), 6.45 (q, *J* = 6.6 Hz, 1H), 6.10 (d, *J* = 5.5 Hz, 1H), 3.11 – 3.00 (m, 4H), 2.92 (s, 3H), 2.59 (d, *J* = 0.9 Hz, 3H), 2.53 (t, *J* = 7.5 Hz, 2H), 2.30 (s, 6H), 1.74 (d, *J* = 6.7 Hz, 3H), 1.38 (d, *J* = 6.9 Hz, 3H). The stereochemistry for these compounds is arbitrarily assigned.

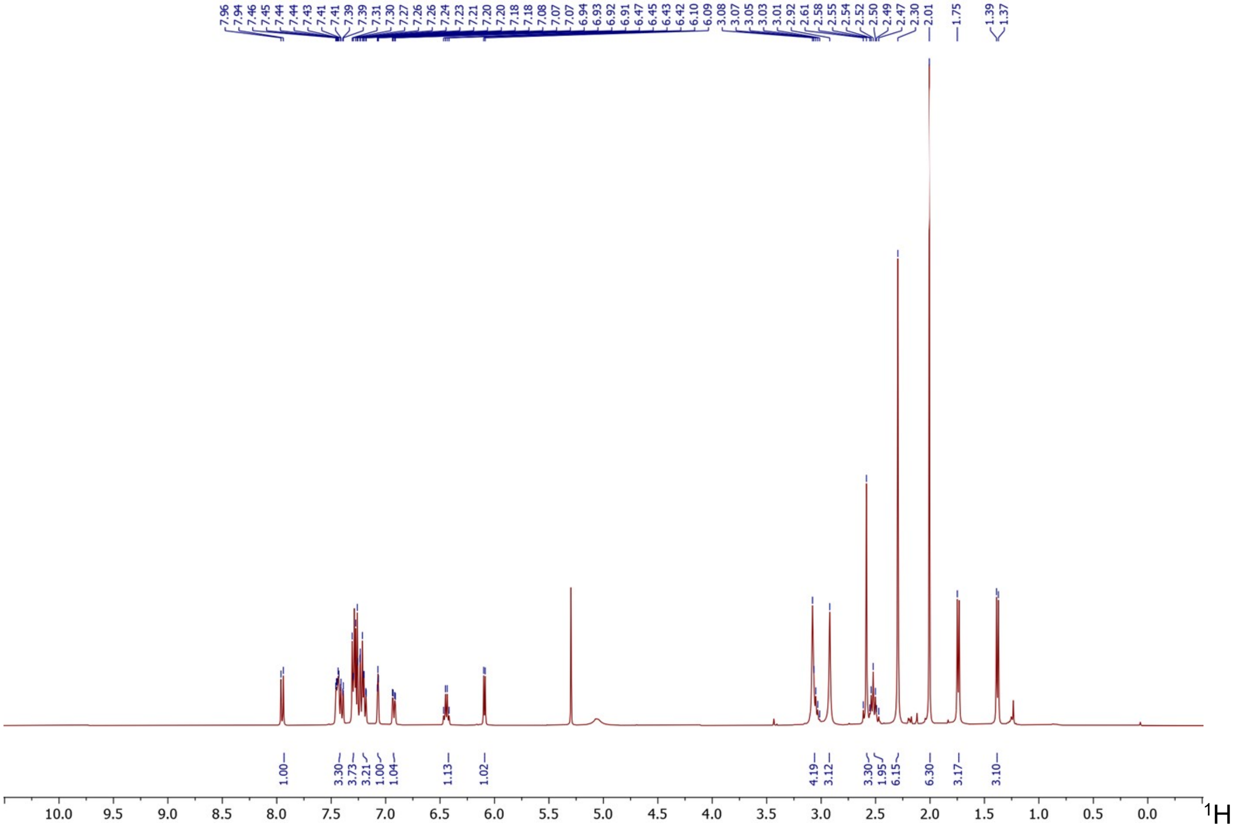
^{1}HNMR spectra of 17C-1



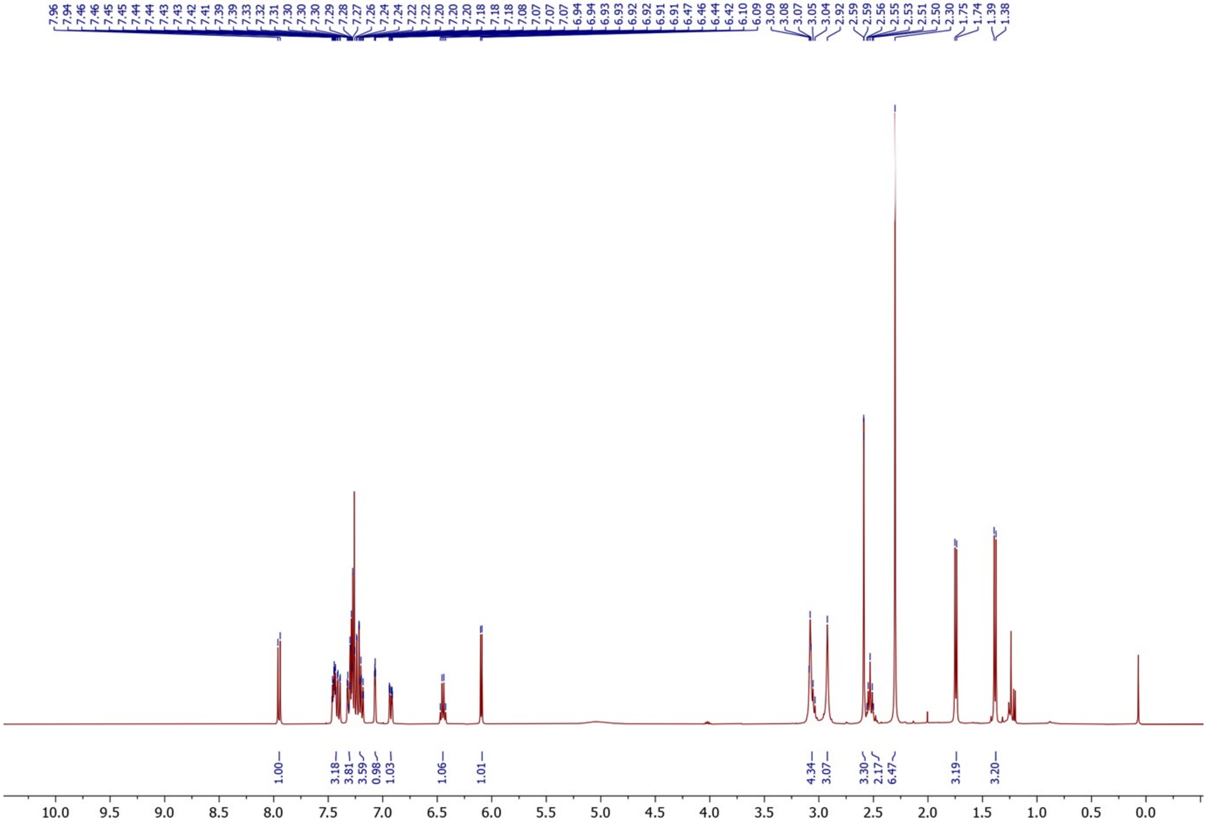
^{1}HNMR spectra of 17C-2



^{1}HNMR spectra of 17A-1



NMR spectra of 17A-2



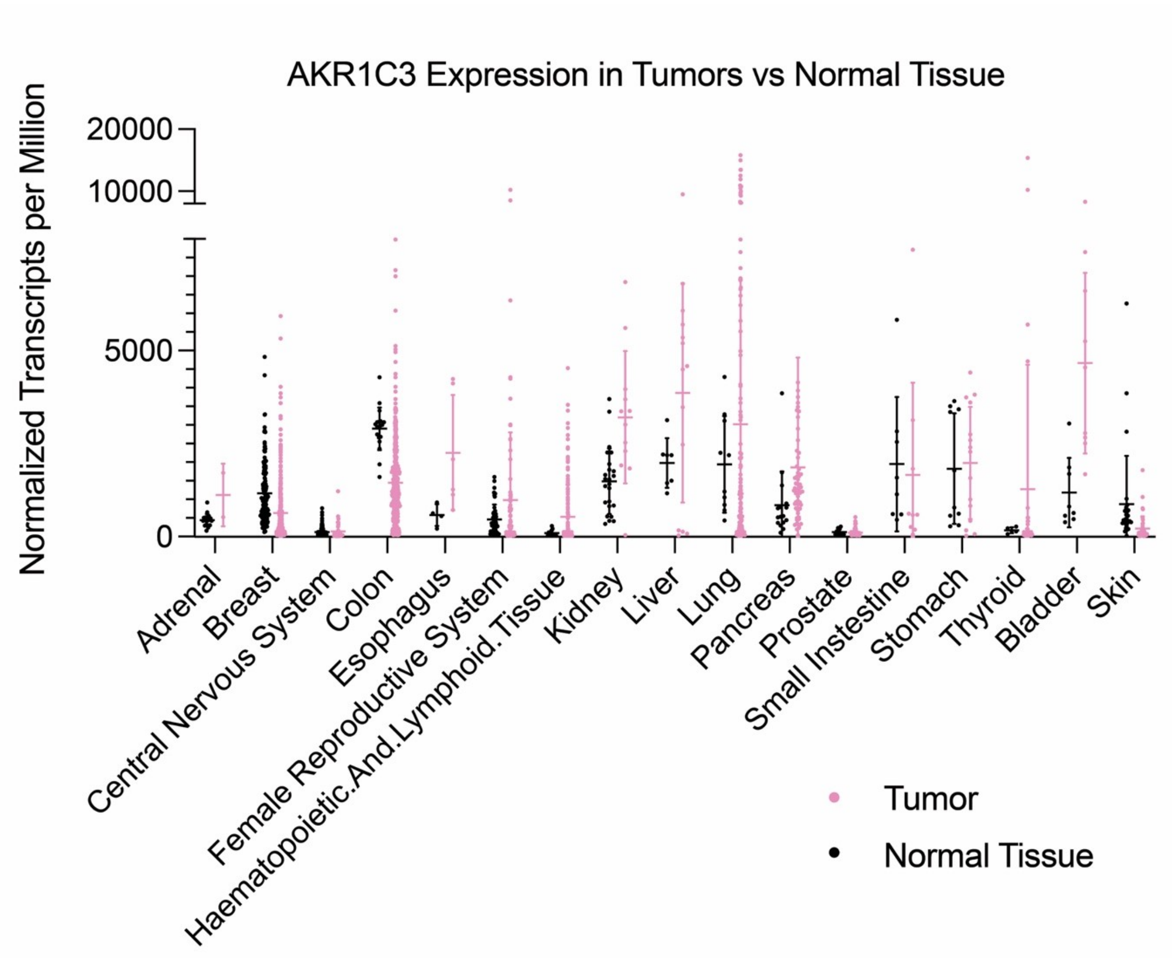


Figure S1 – Expression of AKR1C3 in cancerous and normal tissues
RNAseq expression data was collected from the Metabolic gEne RApid Visualizer (MERAV) online database for primary tumor and normal tissue samples and plotted according to tissue of origin.[4] Kidney and pancreas tumors demonstrate statistically significant enrichment of AKR1C3 RNA in tumor tissue (Kolmogorov-Smirnov tests, unpaired, $p < 0.005$). However, many tumor samples have exceedingly high levels of AKR1C3 mRNA (*e.g.* lung), indicating an opportunity for biomarker-based patient stratification.

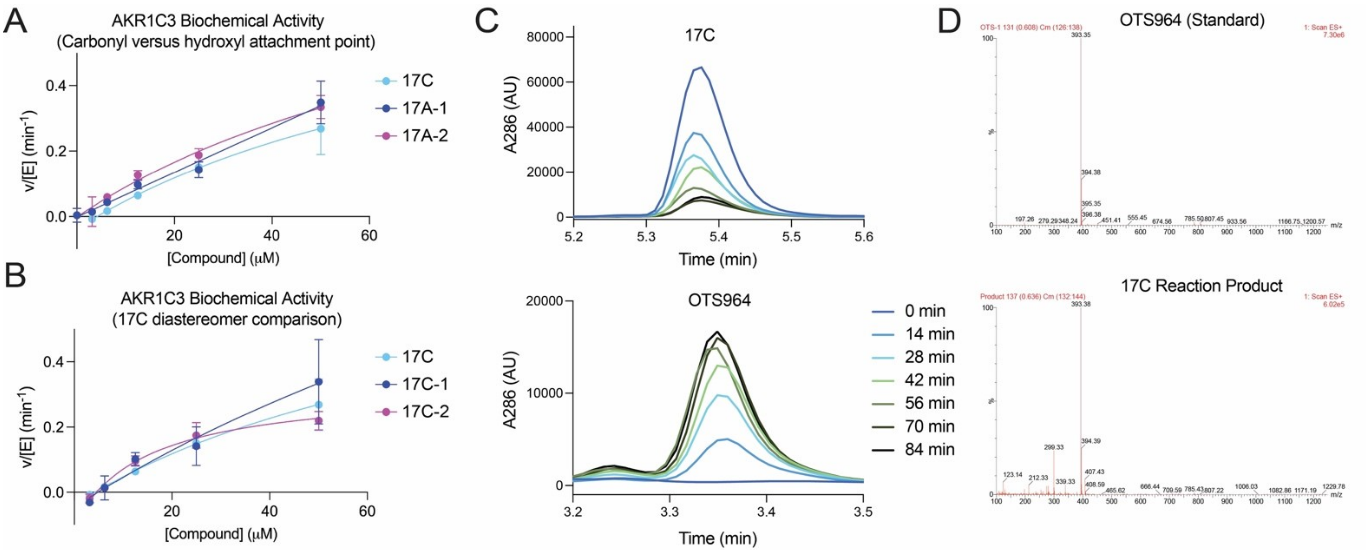


Figure S2 – Detailed assay schematic for AKR1C3 luminescence activity assay
A – Enzymatic assay comparing AKR1C3 activation by 17C with 17A-1 and 17A-2, which feature a protecting group connected to the carbonyl position of OTS964 rather than the hydroxyl position. The 17C data is also shown in Fig. 2C. It is replotted here for comparison.
B – Enzymatic assay as in panel A. 17C-1 and 17C-2 are diastereomers. 17C is racemic. The 17C data is also shown in Fig. 2C. It is replotted here for comparison.
C – Consumption of 17C substrate and production of released OTS kinase inhibitor monitored by reverse phase chromatography. The reaction was initiated by addition of AKR1C3 (2 μM final concentration). Elution profiles of substrate and product peaks were determined by calibration with standards. The experiment was repeated twice with equivalent results.
D – ESI-MS spectrum (positive mode) analysis of OTS964 (top) or the reaction products from panel C (bottom). Samples were resolved by reverse phase chromatography, and spectra corresponding to the reaction product peak are shown (calculated exact mass = 392.16).

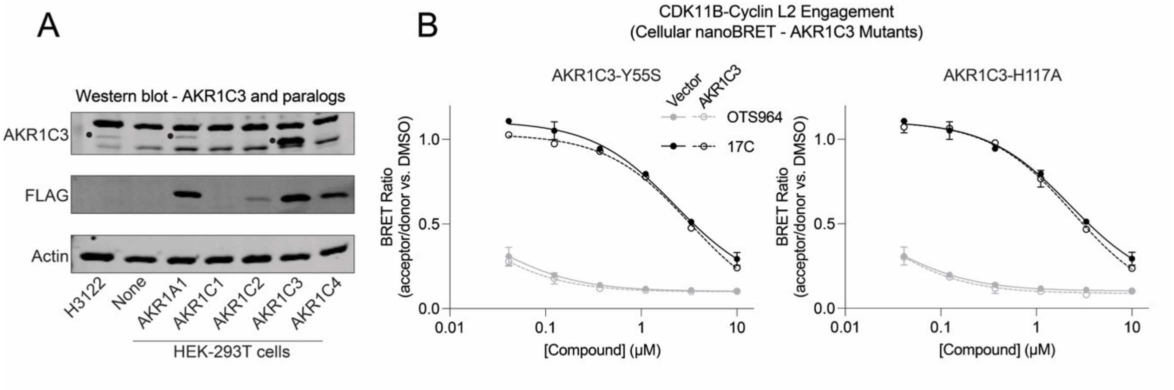


Figure S3 – ARK1C3-dependent CDK11 engagement

A – Western blot showing protein expression levels of AKR1C3 and its paralogs in H3122 cells (left) and transfected 293T cells used for nanoBRET experiments. The black dots (top panel) show reactive non-background bands. Note that AKR1A1 cross-reacts with the AKR1C3 antibody.

B – Example nanoBRET CDK11 engagement assay data showing titration of OTS964 or 17C into cells expressing CDK11B-Nanoluc and Cyclin L2. The dashed curves (empty symbols) show cells co-transfected with AKR1C3-Y55S (left) or -H117A (right). Solid curves show vector-transfected control reporter cells. This data is summarized in Figure 3C.

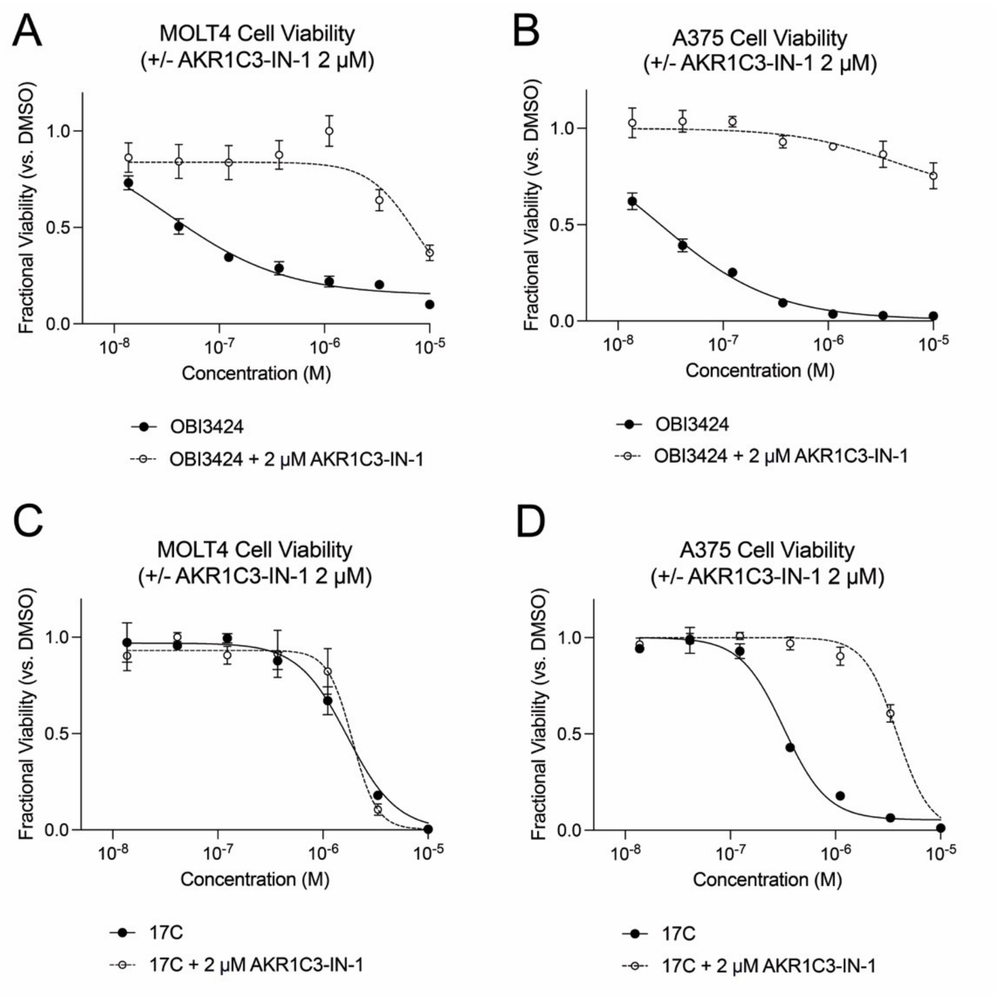


Figure S4 – Example cell proliferation results for diverse cell lines

A-D – 72-hour cell proliferation inhibition curves for AKR1C3-low (Molt4; A,C) and -high (A375; B,D) cell lines. Cell proliferation inhibition curves are shown for OBI-3424 (top) and 17C (bottom).

A

Half life (T1/2 in minutes)

Compound ID	Mouse Hepatocyte
sunitinib	17.65
ZNL-SU2-017C	3.57

Intrinsic clearance (Clint - µl/min/mg)

Compound ID	Mouse Hepatocyte
sunitinib	39.26
ZNL-SU2-017C	194.12

B

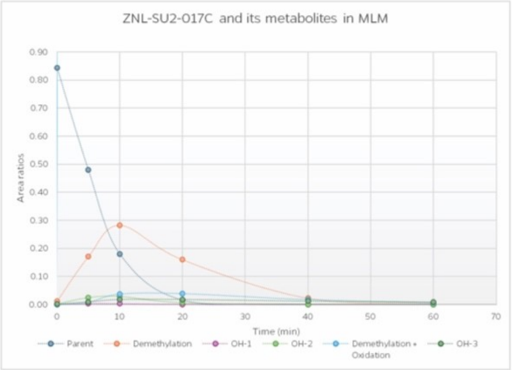


Figure S5 – Pharmacokinetic parameters corresponding to 17C compound stability
A – *In vitro* pharmacokinetic analysis: mouse liver microsome stability assay.
B – Metabolite identification analysis.

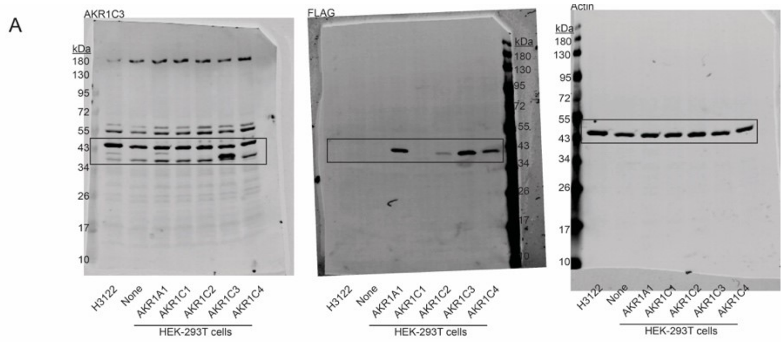


Fig. S2A

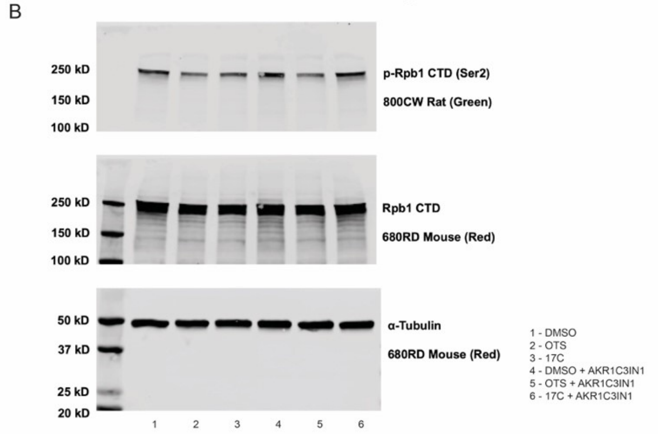


Fig. 5

Figure S6 – Raw images from Western blotting experiments

A – Western blot images from Fig. S3A.

B – Western blot images from Fig. 5.