Supporting Information:

Discovery of Phospholipase D Inhibitors with Improved Drug-like Properties and Central Nervous System Penetrance

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Table of Contents:

1. List of Abbreviations

CO = carbon monoxide

PE = petroleum ether

EtOAc = ethyl acétate

ESI = electrospray ionisation

MeOH = methanol

EtOH = ethanol

TEA = Triethylamine

T3P® = Propanephosphonic acid anhydride

DCM = dichloromethane

DEA = diethylamine

DMF = dimethylformamide

HATU = Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium

HCl = hydrochloric acid

LCMS = liquid chromatography mass spectrometry

HPLC = high pressure liquid chromatography

THF = tetrahydrofuran

MeCN = ACN = acetonitrile

AcOH = acetic acid

TFA = trifluoroacetic acid

DIPEA = diisopropylethyl amine

SFC = Supercritical Fluid Chromatography

N_2 = Nitrogen

MBPR = Manual Back Pressure Regulator

ABPR = Automatic Back Pressure Regulator

NH_4HCO_3 = Ammonium Bicarbonate

CO_2 = Carbon Dioxide

Hunigs Base = N,N-diisopropylethylamine

$MgSO_4$ = magnesium sulfate

NaH = sodium hydride

$LiOH.H_2O$ = lithium hydroxide hydrate

Na_2SO_4 = sodium sulfate

$NaHCO_3$ = sodium bicarbonate

NaOH = Sodium Hydroxide

IPA = isopropanol

DMSO = dimethyl sulfoxide

K_2CO_3 = potassium carbonate

$CDCl_3$ = deuterated chloroform

Na_2SO_3 = sodium sulfite

$KHSO_4$ = potassium bisulfate

2. General experimental:

Unless otherwise stated, all compounds were obtained in purity >95%.

^{1}H nuclear magnetic resonance (NMR) spectra were in all cases consistent with the proposed structures. The 1H NMR spectra were recorded on a Bruker Avance III HD 500 MHz, Bruker Avance III 500 MHz, Bruker Avance III 400 MHz, Varian-400 VNMRS, or Varian-400 MR. Characteristic chemical shifts (δ) are given in parts-per-million downfield from tetramethylsilane (for ^{1}H-NMR) using conventional abbreviations for designation of major peaks: e.g. s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet; dt, double triplet; m, multiplet; br, broad. The following abbreviations have been used for common solvents: $CDCl_3$, deuterochloroform; DMSO-d_6, hexadeuterodimethyl sulfoxide; and MeOH-d_4, deuteron-methanol. Where appropriate, tautomers may be recorded within the NMR data; and some exchangeable protons may not be visible.

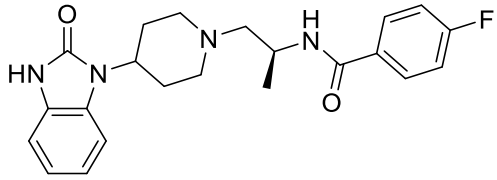
LCMS data: A sample is dissolved in a suitable solvent such as MeCN, dimethyl sulfoxide (DMSO), or MeOH and is injected directly into the column using an automated sample handler. The analysis used one of the following methods: (1) acidic method (1.5, 2, 3.5, 4, or 7 min runs) conducted on a Shimadzu 2010 Series, Shimadzu 2020 Series, or Waters Acquity UPLC BEH. (MS ionization: ESI) instrument equipped with a C18 column (2.1 mm × 30 mm, 3.0 mm or 2.1mm × 50 mm, C18, 1.7 μm), eluting with 1.5 mL/4 L of trifluoroacetic acid (TFA) in water (solvent A) and 0.75 mL/4 L of TFA in MeCN (solvent B) or (2) basic method (3, 3.5, 7 min runs): conducted on a Shimadzu 2020 Series or Waters Acquity UPLC BEH (MS ionization:

ESI) instrument equipped with XBridge Shield RP18, 5um column (2.1 mm × 30 mm, 3.0 mm i.d.) or 2.1 mm × 50 mm, C18, 1.7 μm column, eluting with 2 mL/4 L $NH_3{\cdot}H_2O$ in water (solvent A) and MeCN (solvent B).

Preparation of HRMS samples: The samples were received as solutions in deuterated MeOD (from NMR analysis). The samples were further diluted with 50% aqueous acetonitrile and injected to Sciex Triple TOF5600 for LC/MS analysis. The column used was Waters UPLC Acquity HSS T3 C18 1.8um, 2.1x50mm with mobile phase: A= 0.1% Formic acid in H2O; B= 0.1% Formic acid in ACN.

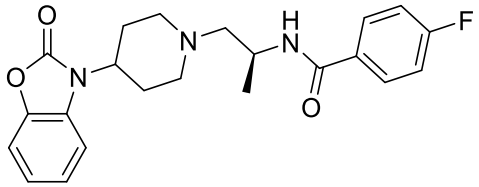
3. Compound Synthesis and Characterization Data for Key Compounds

(S)-4-fluoro-N-(1-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)propan-2-yl)benzamide (4)



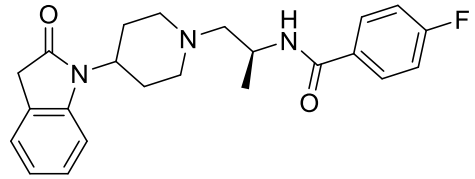
Inhibitor **4** was synthesized in a fashion similar to that described for closely related analog (S)-(S)-N-(1-(4-(1H-indazol-3-yl)piperidin-1-yl)propan-2-yl)-4-fluorobenzamide (**8**) *vide infra* but starting from 1-(piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one, rather than 3-(4-piperidyl)-1H-indazole. HRMS calc. formula: $C_{22}H_{25}FN_4O_2$ [M+H]+ = 397.2035; Found: 397.2045; ^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 7.83 (t, *J*=6.55 Hz, 2 H), 7.06 - 7.18 (m, 3 H), 6.86 - 6.98 (m, 3 H), 4.13 - 4.31 (m, 2 H), 3.10 (br dd, *J*=9.77, 1.53 Hz, 1 H), 3.02 (br dd, *J*=11.52, 1.91 Hz, 1 H), 2.55 (dd, *J*=12.66, 8.70 Hz, 1 H), 2.31 - 2.45 (m, 3 H), 2.22 (td, *J*=11.94, 2.37 Hz, 1 H), 2.11 (td, *J*=11.90, 2.29 Hz, 1 H), 1.63 (ddt, *J*=10.38, 8.13, 2.27, 2.27 Hz, 2 H) 1.17 (d, *J*=6.56 Hz, 3 H). ^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 167.4, 165.74, 163.75, 154.9, 131.09, 131.06, 129.59, 129.52, 128.96, 128.2, 121.04, 120.8, 115.0, 114.8, 109.2, 109.12, 62.9, 53.6, 52.7, 50.7, 43.4, 28.6, 28.5, 18.0.

(S)-4-fluoro-N-(1-(4-(2-oxobenzo[d]oxazol-3(2H)-yl)piperidin-1-yl)propan-2-yl)benzamide (5)



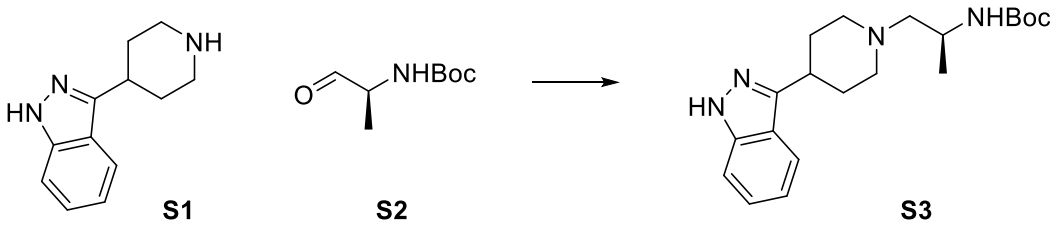
Inhibitor **5** was synthesized in a fashion similar to that described for closely related analog (S)-(S)-N-(1-(4-(1H-indazol-3-yl)piperidin-1-yl)propan-2-yl)-4-fluorobenzamide (**8**) *vide infra* but starting from 3-(piperidin-4-yl)benzo[d]oxazol-2(3H)-one, rather than 3-(4-piperidyl)-1H-indazole. HRMS calc. formula: $C_{22}H_{24}FN_3O_3$ [M+H]+ = 398.1875; Found: 398.1879; ^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 8.00 (t, *J*=6.45 Hz, 2 H), 7.36 (d, *J*=7.93 Hz, 1 H), 7.17 - 7.30 (m, 5 H), 4.64 - 4.74 (m, 1 H), 4.54 (tt, *J*=12.21, 4.04 Hz, 1 H), 4.16 - 4.32 (m, 1 H), 3.74 (br d, *J*=11.29 Hz, 1 H), 3.34 - 3.47 (m, 2 H), 3.17 - 3.30 (m, 1 H), 2.71 - 2.87 (m, 2 H), 2.12 - 2.28 (m, 2 H), 1.41 (d, *J*=6.71 Hz, 3 H), 1.25 - 1.37 (m, 1 H); ^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 168.4, 166.1, 164.1, 153.7, 142.7, 130.0, 129.9, 129.75, 129.73, 123.8, 122.5, 115.1, 114.9, 109.7, 109.1, 62.4, 53.7, 51.5, 48.9, 41.7, 25.63, 25.58, 17.41, 17.38.

(S)-4-fluoro-N-(1-(4-(2-oxoindolin-1-yl)piperidin-1-yl)propan-2-yl)benzamide (6)

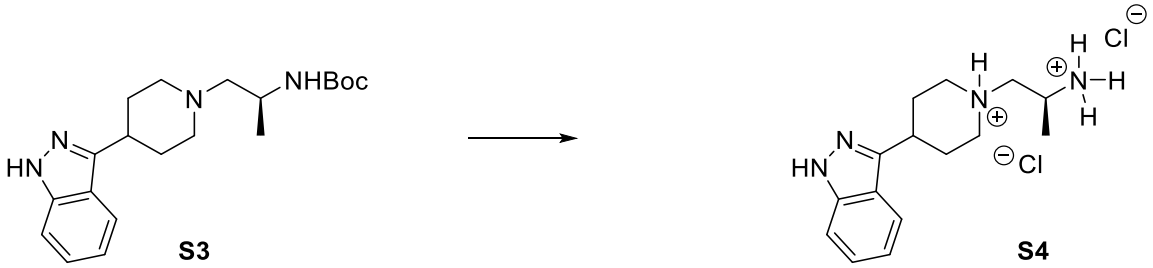


Inhibitor **6** was synthesized in a fashion similar to that described for closely related analog (S)-(S)-N-(1-(4-(1H-indazol-3-yl)piperidin-1-yl)propan-2-yl)-4-fluorobenzamide (**8**) *vide infra* but starting from 1-(piperidin-4-yl)indolin-2-one, rather than 3-(4-piperidyl)-1H-indazole. HRMS calc. formula: $C_{23}H_{26}FN_3O_2$ $[M+H]^+$ = 396.2082; Found: 396.2084; ^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 7.95 - 8.04 (m, 2 H) 7.21 - 7.33 (m, 4 H) 7.06 - 7.19 (m, 2 H) 4.65 - 4.72 (m, 1 H) 4.33 - 4.53 (m, 1 H) 4.21 (br d, *J*=11.90 Hz, 1 H) 4.09 (br s, 1 H) 3.71 (br d, *J*=12.05 Hz, 1 H) 3.57 (s, 1 H) 3.26 - 3.45 (m, 2 H) 3.14 - 3.25 (m, 1 H) 2.77 - 2.99 (m, 2 H) 1.95 - 2.16 (m, 2 H) 1.32 - 1.48 (m, 4 H). ^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 176.4, 166.1, 164.1, 130.0, 129.9, 129.8, 127.5, 124.9, 124.5, 122.3, 115.1, 114.9, 108.8, 92.4, 62.5, 54.1, 51.9, 41.8, 35.5, 25.2, 25.1, 17.4.

(S)-N-(1-(4-(1H-indazol-3-yl)piperidin-1-yl)propan-2-yl)-4-fluorobenzamide (8)

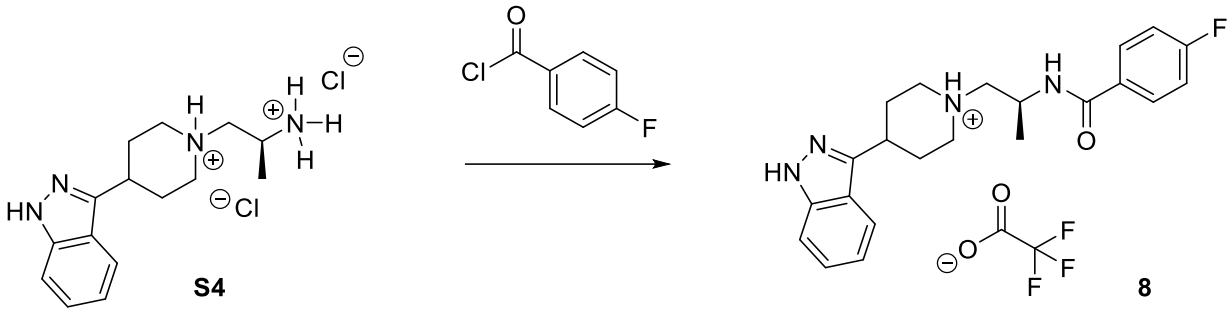


Step 1: A vial was charged with 3-(4-piperidyl)-1H-indazole (**S1**) (308 mg, 1.53 mmol), tert-butyl N-[(1S)-1-methyl-2-oxo-ethyl]carbamate (**S2**) (265 mg, 1.53 mmol), followed by DCM (15.3 mL). To this mixture was added solid sodium triacetoxyborohydride (649 mg, 3.06 mmol). The mixture was stirred at rt for 24 h. The reaction mixture was partitioned between 1 N NaOH (15 mL) and EtOAc (10 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (2x10 mL). The combined organic layers were dried (Na2SO4) and concentrated. The crude residue was taken up in minimal DCM and loaded onto a dry loading column filled with silica gel. The residue was then eluted through a 40 g Reveleris 40 micron silica gel column (Grace X2 automated purification machine) using a gradient of 2% MeOH in EtOAc ramping to 20% MeOH in EtOAc. Clean fractions were collected and concentrated to provide tert-butyl N-[(1S)-2-[4-(1H-indazol-3-yl)-1-piperidyl]-1-methyl-ethyl]carbamate (**S3**) (471 mg, 1.31 mmol, 86% yield). LCMS (ESI) calcd for $C_{20}H_{30}N_4O_2$ [M+H] *m/z* 359.24; found 359.3



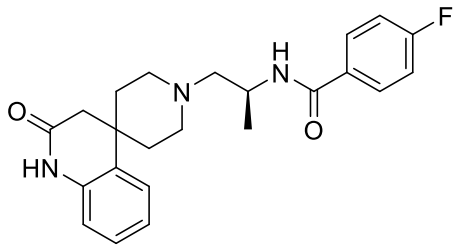
Step 2: A vial was charged with tert-butyl N-[(1S)-2-[4-(1H-indazol-3-yl)-1-piperidyl]-1-methyl-ethyl]carbamate (**S3**) (471 mg, 1.31 mmol) followed by DCM (2.0 mL). A dioxane solution of hydrogen chloride (4 M, 1.0 mL) was added. The vial was sealed, and the mixture was stirred at rt for 18 h to provide a heterogeneous mixture containing a white precipitate. The white solid was collected by vacuum filtration, rinsing with DCM (5 mL) to provide (S)-1-(2-ammoniopropyl)-4-(1H-indazol-3-yl)piperidin-1-ium chloride (**S4**) (430 mg, 1.30 mmol, 99 %

yield). The product was taken forward into the next step without further purification. LCMS (ESI) calcd for $C_{15}H_{22}N_4$ [M+H] *m/z* 259.2; found 259.2.



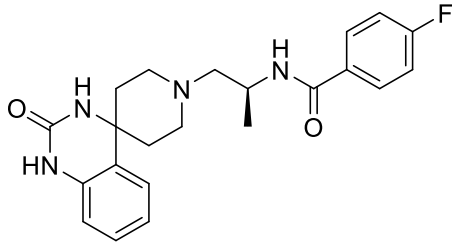
Step 3: A vial was charged with (S)-1-(2-ammoniopropyl)-4-(1H-indazol-3-yl)piperidin-1-ium chloride (68 mg, 206 µmol), DMF (600 µL) and DIPEA (133 mg, 1.03 mmol, 180 µL) followed by 4-fluorobenzoyl chloride (33 mg, 200 µmol, 25 µL) . Some fuming was observed. The solution was maintained at rt for 18 h and then partitioned between 1 N NaOH (15 mL) and EtOAc (10 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (2x10 mL). The combined organic layers were dried (Na2SO4) and concentrated. The crude residue was taken up in minimal MeOH/DMSO, filtered through a 0.45 micron frit, and purified by reverse-phase HPLC (Gilson, column: SunFire Prep C18 OBD, 5 micrometers, 30x50nM column) eluting with an %1 TFA modified gradient of 15% MeCN in H2O to 90% MeCN in H2O to provide (S)-N-(1-(4-(1H-indazol-3-yl)piperidin-1-yl)propan-2-yl)-4-fluorobenzamide (**8**) as the trifluoroacetic acid salt (55 mg, 106 µmol, 51% yield, 95% purity) as a colorless amorphous solid. LCMS (ESI) calcd for $C_{22}H_{25}FN_4O$ [M+H] *m/z* 381.2; found 381.2 ; 1H NMR (400 MHz, METHANOL-d_4) δ ppm 7.94 - 8.05 (m, 2 H), 7.83 (d, J=8.28 Hz, 1 H), 7.50 (d, J=8.53 Hz, 1 H), 7.39 (ddd, J=8.35, 6.96, 1.00 Hz, 1 H), 7.18 - 7.29 (m, 2 H), 7.15 (ddd, J=8.03, 7.03, 0.75 Hz, 1 H), 4.61 - 4.79 (m, 1 H), 4.20 (br d, J=12.30 Hz, 1 H), 3.71 (br d, J=12.05 Hz, 1 H), 3.35 - 3.63 (m, 4 H), 3.13 - 3.30 (m, 1 H), 2.16 - 2.46 (m, 4 H), 1.35 - 1.47 (m, 3 H).

(S)-4-fluoro-N-(1-(2'-oxo-2',3'-dihydro-1'H-spiro[piperidine-4,4'-quinolin]-1-yl)propan-2-yl)benzamide (9)



Inhibitor **9** was synthesized in a fashion similar to that described for closely related analog (S)-(S)-N-(1-(4-(1H-indazol-3-yl)piperidin-1-yl)propan-2-yl)-4-fluorobenzamide (**8**), but starting from 1'H-spiro[piperidine-4,4'-quinolin]-2'(3'H)-one, rather than 3-(4-piperidyl)-1H-indazole. HRMS calc. formula: $C_{23}H_{26}FN_3O_2$ $[M+H]^+$ = 396.2082; Found: 396.2076; ^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 7.79 (t, *J*=6.59 Hz, 2 H), 7.26 (dd, *J*=7.78, 1.22 Hz, 1 H), 7.04 - 7.12 (m, 3 H), 6.94 (td, *J*=7.63, 1.22 Hz, 1 H), 6.78 (dd, *J*=7.86, 1.14 Hz, 1 H), 4.26 (dt, *J*=8.13, 6.31 Hz, 1 H), 2.83 (br d, *J*=11.75 Hz, 1 H), 2.74 (br d, *J*=11.90 Hz, 1 H), 2.50 - 2.59 (m, 3 H), 2.27 - 2.43 (m, 3 H), 1.93 (qd, *J*=12.77, 4.27 Hz, 2 H), 1.49 - 1.62 (m, 2 H), 1.15 (d, *J*=6.56 Hz, 3 H). ^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 171.11, 167.27, 165.71, 163.72, 136.64, 131.97, 131.03, 131.00, 129.55, 129.48, 127.29, 123.66, 123.42, 115.89, 114.96, 114.79, 63.28, 49.24, 48.53, 43.16, 37.62, 34.65, 33.50, 33.48, 18.13.

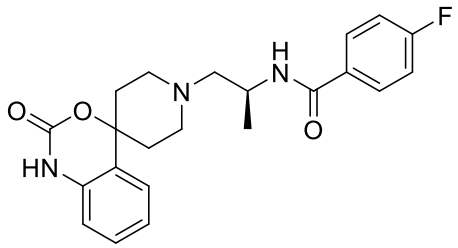
(S)-4-fluoro-N-(1-(2'-oxo-2',3'-dihydro-1'H-spiro[piperidine-4,4'-quinazolin]-1-yl)propan-2-yl)benzamide (10)



Inhibitor **10** was synthesized in a fashion similar to that described for closely related analog (S)-(S)-N-(1-(4-(1H-indazol-3-yl)piperidin-1-yl)propan-2-yl)-4-fluorobenzamide (**8**), but starting from 1'H-spiro[piperidine-4,4'-quinazolin]-2'(3'H)-one, rather than 3-(4-piperidyl)-1H-indazole. HRMS calc. formula: $C_{22}H_{25}FN_4O_2$ $[M+H]^+$ = 397.2035; Found: 397.2023; ^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 7.99 (t, *J*=6.46 Hz, 2 H), 7.18 - 7.32 (m, 4 H), 7.00 - 7.11 (m, 1 H), 6.89 (d, *J*=7.78 Hz, 1 H), 4.64 - 4.77 (m, 1 H), 4.09 (br d, *J*=12.21 Hz, 1 H), 3.55 - 3.65 (m, 2 H), 3.40 - 3.54 (m, 2 H), 3.35 - 3.39 (m, 1 H), 2.68 (s, 1 H), 2.34 - 2.54 (m, 2 H), 2.08 - 2.26 (m, 2 H),

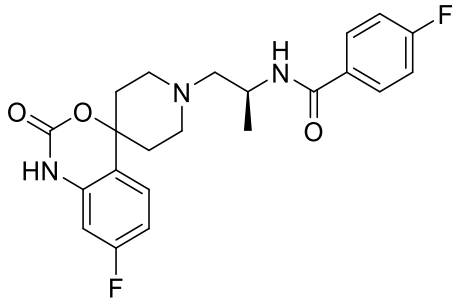
1.43 (d, *J*=6.87 Hz, 3 H). ^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 168.54, 166.09, 164.10, 154.30, 136.18, 130.04, 129.97, 129.73, 128.74, 123.68, 123.35, 122.51, 115.17, 115.10, 114.92, 114.45, 62.48, 52.46, 49.36, 41.53, 39.01, 34.43, 17.29.

(S)-4-fluoro-N-(1-(2-oxo-1,2-dihydrospiro[benzo[d][1,3]oxazine-4,4'-piperidin]-1'-yl)propan-2-yl)benzamide (12)



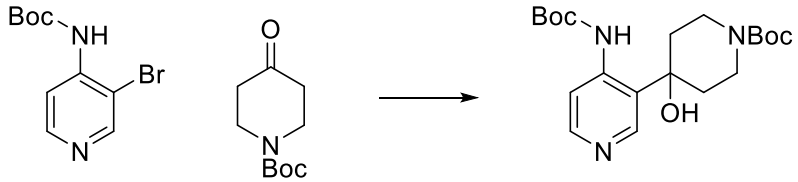
Inhibitor **12** was synthesized in a fashion similar to that described for closely related analog (S)-(S)-N-(1-(4-(1H-indazol-3-yl)piperidin-1-yl)propan-2-yl)-4-fluorobenzamide (**8**), but starting from spiro[benzo[d][1,3]oxazine-4,4'-piperidin]-2(1H)-one, rather than 3-(4-piperidyl)-1H-indazole. HRMS calc. formula: $C_{22}H_{24}FN_3O_3$ $[M+H]^+$ = 398.1875; Found: 398.1872; ^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 7.81 (t, *J*=6.66 Hz, 2 H), 7.05 - 7.18 (m, 4 H), 6.97 (t, *J*=7.71 Hz, 1 H), 6.78 (dd, *J*=7.93, 0.76 Hz, 1 H), 4.17 - 4.37 (m, 1 H), 2.98 - 3.17 (m, 1 H) 2.88 (br s, 1 H) 2.60 - 2.78 (m, 3 H) 2.56 (br s, 1 H) 1.94 - 2.14 (m, 4 H) 1.12 - 1.27 (m, 3 H). ^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 167.55, 165.78, 163.79, 152.05, 134.68, 130.81, 129.72, 129.64, 129.57, 128.88, 124.86, 123.32, 122.95, 115.00, 114.82, 114.18, 80.34, 63.05, 48.84, 34.30, 27.12, 17.95.

(S)-4-fluoro-N-(1-(7-fluoro-2-oxo-1,2-dihydrospiro[benzo[d][1,3]oxazine-4,4'-piperidin]-1'-yl)propan-2-yl)benzamide (13)



Inhibitor **13** was synthesized in a fashion similar to that described for closely related analog (S)-(S)-N-(1-(4-(1H-indazol-3-yl)piperidin-1-yl)propan-2-yl)-4-fluorobenzamide (**8**), but starting from 7-fluorospiro[benzo[d][1,3]oxazine-4,4'-piperidin]-2(1H)-one, rather than 3-(4-piperidyl)-1H-indazole. HRMS calc. formula: $C_{22}H_{23}F_2N_3O_3$ $[M+H]^+$ = 416.1780; Found: 416.1777; ^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 7.99 (t, *J*=6.57 Hz, 2 H), 7.23 (t, *J*=8.16 Hz, 2 H), 7.06 - 7.16 (m, 2 H), 6.96 (dd, *J*=8.77, 4.65 Hz, 1 H), 4.65 - 4.79 (m, 1 H), 4.16 (br d, *J*=10.99 Hz, 1 H), 3.40 - 3.62 (m, 5 H), 2.30 - 2.52 (m, 4 H), 1.42 (d, *J*=6.87 Hz, 3 H). ^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 168.62, 166.09, 164.09, 161.62, 160.06, 158.14, 150.57, 130.99, 130.05, 129.97, 129.75, 129.73, 124.48, 124.42, 116.36, 116.18, 116.13, 115.08, 114.91, 110.31, 110.10, 77.48, 62.62, 49.67, 41.56, 32.23, 32.15, 17.28.

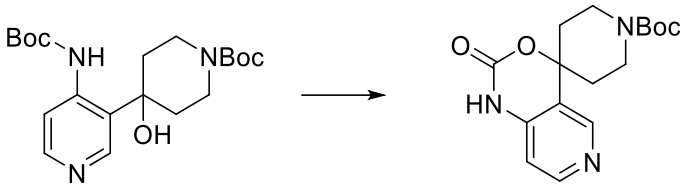
(S)-4-fluoro-N-(1-(2'-oxo-1',2'-dihydrospiro[piperidine-4,4'-pyrido[4,3-d][1,3]oxazin]-1-yl)propan-2-yl)benzamide (**14**)



S5

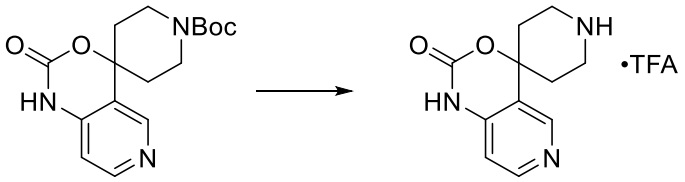
Step 1: A round bottom flask was charged with tert-butyl N-(3-bromo-4-pyridyl)carbamate (509 mg, 1.86 mmol) and sealed with a septum. The flask was flushed with N_2 and THF (6 mL) was added. The solution was cooled to –78 °C, and a solution of methyl lithium (1.6 M in Et_2O, 1.40 mL) was added. The solution was maintained at –78 °C for 15 min, and then tert-butyl lithium (1.7 M in pentane, 2.30 mL) was added dropwise at –78 °C to generate a bright yellow / orange solution. In a separate flask, a THF solution of tert-butyl 4-oxopiperidine-1-carboxylate (667 mg, 3.35 mmol) was prepared. The THF solution of the ketone was added by syringe to the yellow reaction mixture at –78 °C. The orange yellow color slowly dissipated, and the mixture was maintained at –78 °C for 3 h until the reaction became homogeneous (still slightly yellow). A saturated aqueous sodium bicarbonate was added at –78 °C to generate a mixture. The cold bath was removed, and the mixture was allowed to warm to rt. The reaction mixture was partitioned between water (15 mL) and EtOAc (10 mL). The layers were separated, and the aqueous layer

was extracted with EtOAc (1x10 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. The crude residue was taken up in minimal DCM and loaded onto a dry loading column filled with silica gel. The residue was then eluted through a 40 g Reveleris 40 micron silica gel column (Grace X2 automated purification machine) using a gradient of 5% 3:1EtOAc:EtOH in heptanes ramping to 80% 3:1 EtOAc:EtOH in heptanes. Clean fractions were collected and concentrated to provide desired product tert-butyl 4-[4-(tert-butoxycarbonylamino)-3-pyridyl]-4-hydroxy-piperidine-1-carboxylate (**S5**) (610 mg, 1.55 mmol, 83% yield).



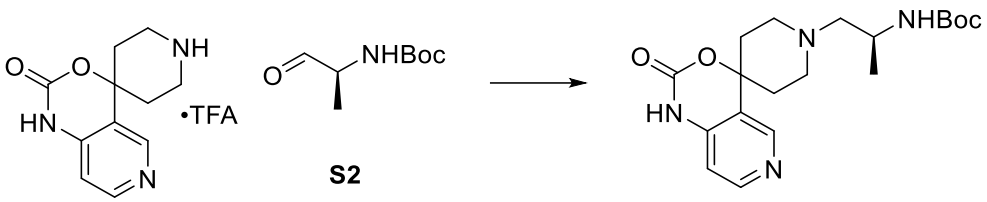
S5 **S6**

Step 2: A vial was charged with tert-butyl 4-[4-(tert-butoxycarbonylamino)-3-pyridyl]-4-hydroxy-piperidine-1-carboxylate (**S5**) (427 mg, 1.09 mmol) and THF (2.2 mL). To this solution was added potassium tert-butoxide (1 M in THF, 2.2 mL) at rt to generate a brown solution. After 5 min, the reaction mixture was partitioned between water (15 mL) and EtOAc (10 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (2x10 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. The crude residue was taken up in minimal DCM and loaded onto a dry loading column filled with silica gel. The residue was then eluted through a 24 g Reveleris 40 micron silica gel column (Grace X2 automated purification machine) using a gradient of 5% 3:1EtOAc:EtOH in heptanes ramping to 80% 3:1 EtOAc:EtOH in heptanes. Clean fractions were collected and concentrated to provide tert-butyl 2-oxospiro[1H-pyrido[4,3-d][1,3]oxazine-4,4'-piperidine]-1'-carboxylate (**S6**) (196 mg, 614 µmol, 56% yield) as a white amorphous solid. LCMS (ESI) calcd for $C_{16}H_{21}N_3O_4$ [M+H] *m/z* 320.2; found 320.2.



S6 **S7**

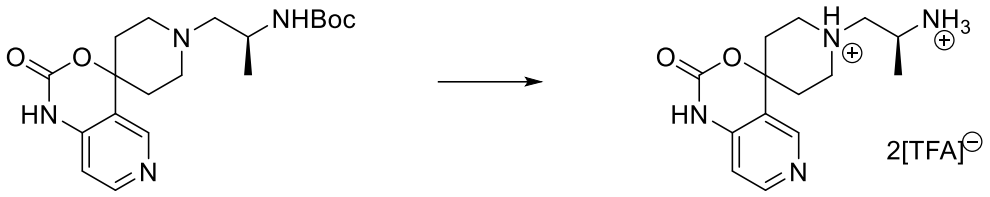
Step 3: A flask was charged with tert-butyl 2-oxospiro[1H-pyrido[4,3-d][1,3]oxazine-4,4'-piperidine]-1'-carboxylate (**S6**) (196 mg, 614 µmol) and DCM (1.23 mL). Trifluoroacetic acid (350 mg, 3.01 mmol, 230 µL) was added to the solution at rt and the solution was maintained at rt for 18 h. The solution was concentrated to provide spiro[1H-pyrido[4,3-d][1,3]oxazine-4,4'-piperidine]-2-one trifluoroacetate (**S7**) (200 mg, 600 µmol, 98% yield) as a brown oil, which was taken forward into the next step without further purification, assuming quantitative yield. LCMS (ESI) calcd for $C_{11}H_{13}N_3O_2$ [M+H] *m/z* 220.1; found 220.0.



S7 **S2** **S8**

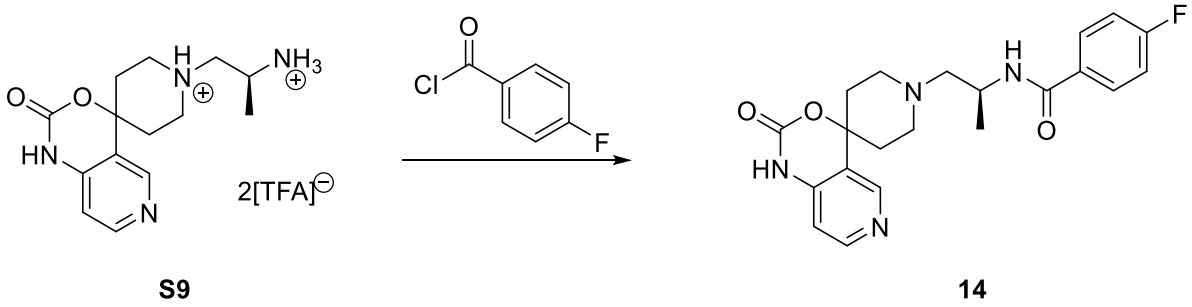
Step 4: A vial was charged with spiro[1H-pyrido[4,3-d][1,3]oxazine-4,4'-piperidine]-2-one trifluoroacetate (**S7**) (200 mg, 600 µmol), tert-butyl N-[(1S)-1-methyl-2-oxo-ethyl]carbamate (**S2**) (114 mg, 660 µmol) and DCM (2.0 mL). The solution was cooled to 0 °C and sodium triacetoxyborohydride (254 mg, 1.20 mmol) was added portion-wise. The mixture was stirred at rt for 48 h. The reaction mixture was partitioned between 1 N NaOH (15 mL) and DCM (10 mL). The layers were separated, and the aqueous layer was extracted with DCM (2x10 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. The crude residue was taken up in minimal DCM and loaded onto a dry loading column filled with silica gel. The residue was then eluted through a 40 g Reveleris 40 micron silica gel column (Grace X2 automated purification machine) using a gradient of 2% MeOH in EtOAC ramping to 20% MeOH in EtOAc. Clean fractions were collected and concentrated to provide desired product tert-butyl N-[(1S)-1-methyl-2-(2-oxospiro[1H-pyrido[4,3-d][1,3]oxazine-4,4'-piperidine]-1'-

yl)ethyl]carbamate (**S8**) (170 mg, 452 µmol, 75 % yield). LCMS (ESI) calcd for $C_{19}H_{28}N_4O_4$ [M+H] *m/z* 377.2; found 377.2 $(M+H)^+$



S8 **S9**

Step 5: A flask was charged with tert-butyl N-[(1S)-1-methyl-2-(2-oxospiro[1H-pyrido[4,3-d][1,3]oxazine-4,4'-piperidine]-1'-yl)ethyl]carbamate (**S8**) (170 mg, 452 µmol) and DCM (1.2 mL) followed by trifluoroacetic acid (260 mg, 2.3 mmol, 170 µL). The solution was maintained at rt for 4 h, and then concentrated to give (S)-1-(2-ammoniopropyl)-2'-oxo-1',2'-dihydrospiro[piperidine-4,4'-pyrido[4,3-d][1,3]oxazin]-1-ium 2,2,2-trifluoroacetate (**S9**) as a brown residue, which was subjected to vacuum for 48 h. The solid darkened a little further but was not purified. The resulting residue was taken directly into the next reaction assuming quantitative yield.

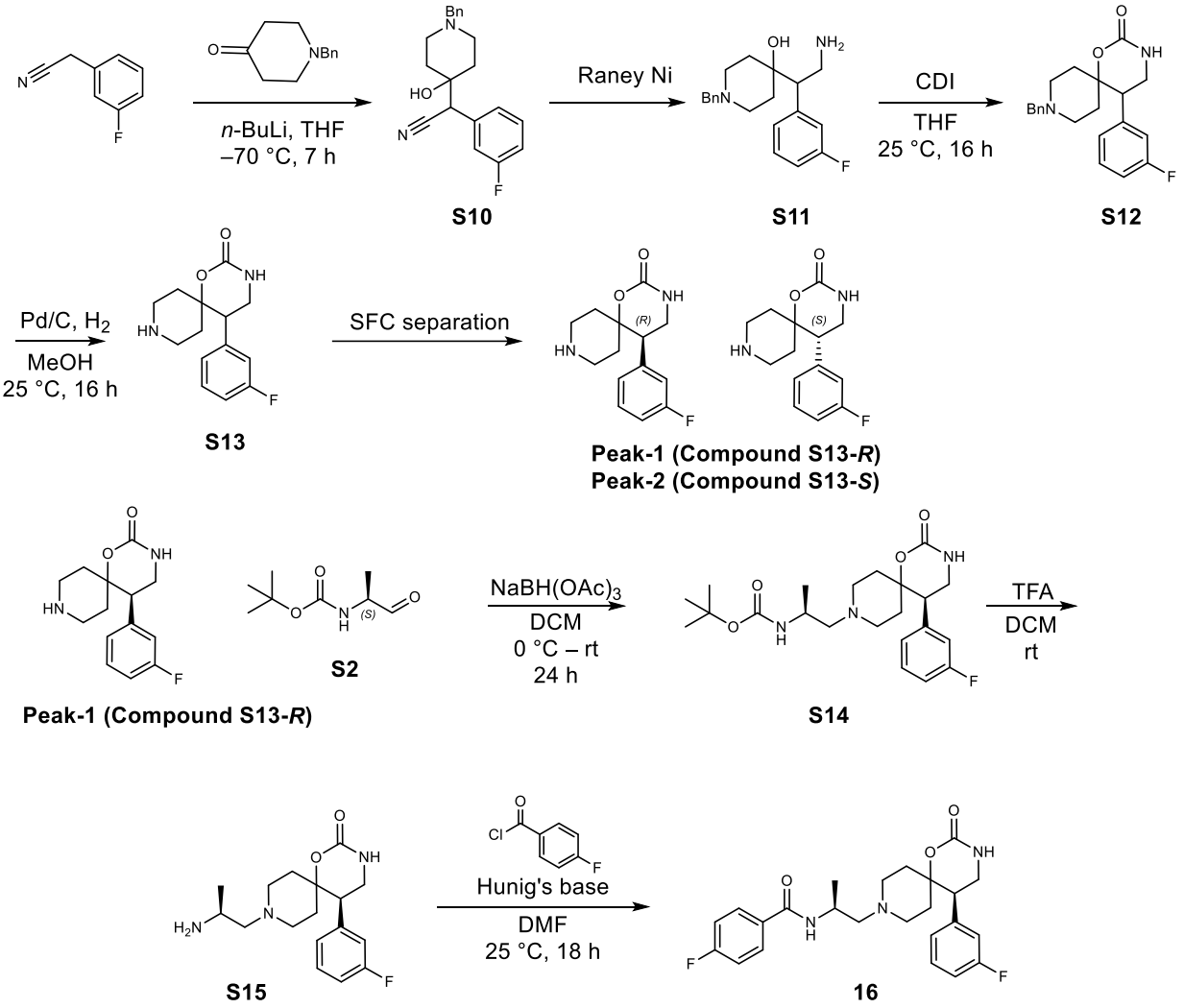


S9 **14**

Step 6: A vial was charged with 1'-[(2S)-2-aminopropyl]spiro[1H-pyrido[4,3-d][1,3]oxazine-4,4'-piperidine]-2-one (**S9**) (279 mg, 1.01 mmol) and DMF was added (650 µL), followed by DIPEA (650 mg, 5.1 mmol, 880 µL) and then 4-fluorobenzoyl chloride (160 mg, 1.0 mmol, 120 µL). The vial was sealed, and the solution was maintained at rt for 18 h. The reaction mixture was partitioned between 1 N NaOH (15 mL) and EtOAc (10 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (2x10 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. The crude residue was taken up in minimal

MeOH/DMSO, filtered through a 0.45 micron frit, and purified by reverse-phase HPLC (Gilson, column: SunFire Prep C18 OBD, 5 micrometers, 30x50nM column) eluting with an %1 TFA modified gradient of 15% MeCN in H2O to 90% MeCN in H2O to provide 4-fluoro-N-[(1S)-1-methyl-2-(2-oxospiro[1H-pyrido[4,3-d][1,3]oxazine-4,4'-piperidine]-1'-yl)ethyl]benzamide 2,2,2-trifluoroacetate (**14**) (100 mg, 128 µmol, 13% yield). HRMS calc. formula C21H23FN4O3 $[M+H]^+$ = 399.1827; Found: 399.1823; ^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 8.22 (d, *J*=4.79 Hz, 2 H), 7.80 (t, *J*=6.70 Hz, 2 H), 7.09 (t, *J*=8.77 Hz, 2 H), 6.79 (d, *J*=5.49 Hz, 1 H), 4.23 - 4.31 (m, 1 H), 2.89 (br d, *J*=11.44 Hz, 1 H), 2.74 - 2.84 (m, 1 H), 2.45 - 2.61 (m, 3 H), 2.39 (dd, *J*=12.66, 5.65 Hz, 1 H), 1.96 - 2.12 (m, 4 H), 1.17 (d, *J*=6.56 Hz, 3 H). ^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 167.34, 165.72, 163.73, 150.70, 149.41, 143.88, 143.05, 131.03, 131.01, 129.60, 129.55, 129.48, 121.43, 114.98, 114.80, 109.08, 80.10, 63.06, 48.44, 43.33, 35.03, 35.01, 17.98.

4-fluoro-N-((*S*)-1-((*R*)-5-(3-fluorophenyl)-2-oxo-1-oxa-3,9-diazaspiro[5.5]undecan-9-yl)propan-2-yl)benzamide (16)



2-(1-benzyl-4-hydroxypiperidin-4-yl)-2-(3-fluorophenyl)acetonitrile (S10). 2-(3-fluorophenyl)acetonitrile (10.0 g, 74.0 mmol) was added to dry THF (100 mL) and the solution was cooled to –70 °C under N_2. *n*-BuLi (2.5 M, 37 mL) was added dropwise and the solution was maintained at –70 °C for 30 min. A THF solution of 1-benzylpiperidin-4-one (17.5 g, 92.5 mmol in 100 mL) was added. The mixture was maintained at –70 °C for 7 h. The mixture was quenched by careful addition of H_2O (200 mL) at –78 °C and extracted with DCM (400 mL). The organic phase was washed with brine (2 x 500 mL), dried (Na_2SO_4), and concentrated to provide a yellow oil, which was re-crystallized from Et_2O (120 mL) to provide 2-(1-benzyl-4-

hydroxypiperidin-4-yl)-2-(3-fluorophenyl)acetonitrile (**S10**) (33.5 g, 70% yield) as a white solid after filtration. LCMS (ESI) cacld for $C_{20}H_{21}FN_2O$ [M+H] *m/z* 325.2; found 325.1. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 7.37-7.30 (m, 6H), 7.15-7.09 (m, 3H), 3.76 (s, 1H), 3.52 (s, 2H), 2.77-2.67 (m, 2H), 2.35-2.24 (m, 2H), 1.90-1.77 (m, 3H), 1.43-1.39 (m, 1H).

4-(2-amino-1-(3-fluorophenyl)ethyl)-1-benzylpiperidin-4-ol (S11). A mixture of 2-(1-benzyl-4-hydroxypiperidin-4-yl)-2-(3-fluorophenyl)acetonitrile (**S8**) (3.5 g, 10.8 mmol) and Raney Nickel (1.5 g) in MeOH (50 mL) was stirred under H_2 (15 psi) for 16 h at 25 °C. The mixture was filtered (Celite) and concentrated to obtain 4-(2-amino-1-(3-fluorophenyl)ethyl)-1-benzylpiperidin-4-ol (**S11**) (14.0 g) as a yellow oil, which was taken forward without further purification.

9-benzyl-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one (S12). A solution of 4-(2-amino-1-(3-fluorophenyl)ethyl)-1-benzylpiperidin-4-ol (**S11**) (14.0 g, 42.6 mmol) and CDI (20.7 g, 128 mmol) in THF (150 mL) was stirred at ambient temperature for 16 h. The mixture was concentrated, and H_2O (200 mL) was added. The mixture was extracted with DCM (3 x 200 mL). The organic phases were washed with brine (200 mL), dried (Na_2SO_4) and concentrated. The residue was purified by silica gel chromatography (eluent: 5% DCM in MeOH) followed by HPLC (Phenomenex Luna C18 250*50 mm*10 um; mobile phase: [water (10 mM NH_4HCO_3)-ACN]; B%: 20%-55%). The eluate was extracted with EtOAc (3 x 300 mL), washed with brine (200 mL), dried (Na_2SO_4), and concentrated to provide 9-benzyl-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one (**S12**) (4.5 g, 30% yield) as a white solid. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 7.30-7.23 (m, 6H), 7.02-6.99 (m, 3H), 6.79-6.69 (d, *J* = 39.2 Hz, 1H), 3.64-3.59 (m, 2H), 3.49 (s, 2H), 3.06-3.04 (m, 1H), 2.71-2.68 (m, 2H), 2.54-2.53 (m, 1H), 2.49-2.43 (m, 1H), 1.85-1.84 (m, 1H), 1.75-1.68 (m, 3H).

(*R*)-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one (S13-*R*). A solution of 9-benzyl-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one (**S12**) (4.5 g, 12.7 mmol) and Pd/C (500 mg) in MeOH (100 mL) was stirred under H_2 (15 psi) at 25 °C for 16 h. The mixture was filtered (Celite) and concentrated to provide 5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one **S13**. Compound **S13** was separated by SFC (AS (250mm*50mm, 10um); mobile phase: [0.1% NH_3H_2O IPA]; B%: 40%-40%) to give (*R*)-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one (**S13-*R***) (peak-1, 2.0 g) as an off-white solid and (*S*)-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one (**S13-*S***) (peak-2, 2.0 g) as an off-white solid. Further purification was performed for Peak 1: (**S13-*R***). To a solution of (*R*)-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one **S13-*R*** (peak-1, 2.0 g, 7.6 mmol) in MeOH (0.5 mL) and DCM (5 mL) was added Et_2O (20 mL) until a precipitate was formed. The solid was collected by filtration and the procedure was repeated twice, and then the material was purified by HPLC (Phenomenex Luna C18 250*50 mm*10 um; mobile phase: [water (10mM NH_4HCO_3)-ACN]; B%: 0%-20%) to obtain (R)-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one **S13-*R*** (195 mg, 10% yield, >99% ee) as a white solid. LCMS (ESI) calcd for $C_{14}H_{17}FN_2O_2$ [M+H] *m/z* 265.1; found 265.1. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 7.40-7.35 (m, 2H), 7.16-7.14 (m, 3H), 3.52-3.49 (m, 2H), 3.18-3.12 (m, 1H), 2.76-2.62 (m, 4H), 1.68-1.60 (m, 1H), 1.55-1.53 (m, 1H), 1.39-1.37 (m, 2H).

tert-butyl ((*S*)-1-((*R*)-5-(3-fluorophenyl)-2-oxo-1-oxa-3,9-diazaspiro[5.5]undecan-9-yl)propan-2-yl)carbamate (S14). To a solution of (*R*)-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one (**S13-*R***) (300 mg, 1.14 mmol) and tert-butyl (*S*)-(1-oxopropan-2-yl)carbamate (**S2**) (198 mg, 0.5 mL, 1.14 mmol) in DCM (11 mL) at 0 °C was added $NaBH(OAc)_3$ (483 mg, 2.28 mmol). The reaction was allowed to warm to ambient temperature

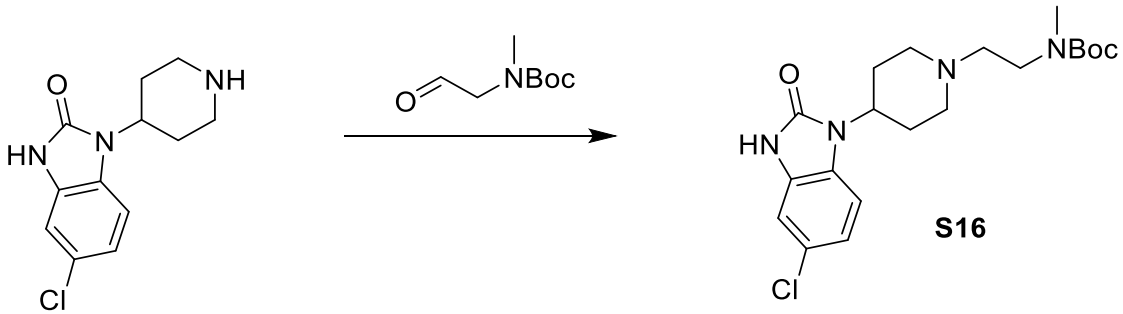
and maintained for 24 h. The reaction mixture was partitioned between 1N NaOH (15 mL) and EtOAc (10 mL). The aqueous layer was extracted with EtOAc (3x10 mL). The organic phases were dried ($MgSO_4$), concentrated, and purified by silica gel chromatography to obtain tert-butyl ((*S*)-1-((*R*)-5-(3-fluorophenyl)-2-oxo-1-oxa-3,9-diazaspiro[5.5]undecan-9-yl)propan-2-yl)carbamate (**S14**) (250 mg, 52% Yield). LCMS (ESI) calcd for $C_{22}H_{32}FN_3O_4$ [M+H] *m/z* 422.2; found 422.2. ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 7.28 - 7.35 (m, 1 H), 6.99 - 7.05 (m, 2 H), 6.93 (dt, *J* = 9.91, 2.07 Hz, 1 H), 5.50 (s, 1 H), 4.55 (br d, *J* = 7.03 Hz, 1 H), 3.56 - 3.72 (m, 3 H), 3.04 (dd, *J* = 8.16, 6.15 Hz, 1 H), 2.72 (br dd, *J* = 10.79, 3.26 Hz, 1 H), 2.42 - 2.63 (m, 3 H), 2.22 - 2.27 (m, 2 H), 1.60 - 1.87 (m, 4 H), 1.38 (s, 9 H), 1.09 (d, *J* = 6.53 Hz, 3 H).

(*R*)-9-((S)-2-aminopropyl)-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one (S15). To a solution of tert-butyl ((*S*)-1-((*R*)-5-(3-fluorophenyl)-2-oxo-1-oxa-3,9-diazaspiro[5.5]undecan-9-yl)propan-2-yl)carbamate (250 mg, 0.59 mmol) in DCM (6 mL, 0.1 M) was added TFA (0.9 mL, 12 mmol). The solution was maintained at 25 °C for 2 h and then quenched by the addition of saturated aqueous $NaHCO_3$, the layers were separated, and the aqueous phase was extracted with DCM (3 x 10 mL). The organic phases were dried ($MgSO_4$) and concentrated to provide (*R*)-9-((S)-2-aminopropyl)-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one (**S15**), which was taken forward without purification. LCMS (ESI) calcd for $C_{17}H_{24}FN_3O_2$ [M+H] *m/z* 322.2; found 322.2.

4-fluoro-N-((*S*)-1-((*R*)-5-(3-fluorophenyl)-2-oxo-1-oxa-3,9-diazaspiro[5.5]undecan-9-yl)propan-2-yl)benzamide (16). A vial was charged with (*R*)-9-((S)-2-aminopropyl)-5-(3-fluorophenyl)-1-oxa-3,9-diazaspiro[5.5]undecan-2-one (**S15**) (190 mg, 0.59 mmol), Hunig's base (382 mg, 2.96 mmol, 0.52 mL) in DMF (6 mL), and then 4-fluorobenzoyl chloride (0.1 mL, 0.6

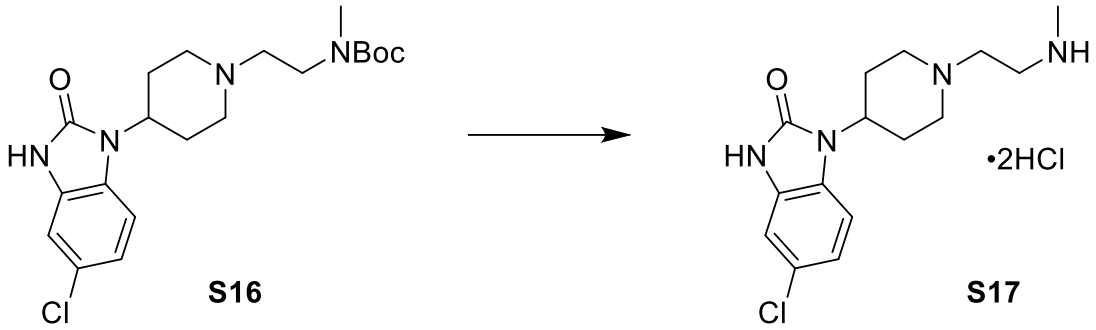
mmol) was added. The solution was maintained at ambient temperature for 18 h and partitioned between 1N NaOH (15 mL) and EtOAc (10 mL). The aqueous layer was extracted with EtOAc (3 x 10 mL) and the combined organic phases were dried ($MgSO_4$), concentrated, and the residue was purified by HPLC to provide 4-fluoro-N-((*S*)-1-((*R*)-5-(3-fluorophenyl)-2-oxo-1-oxa-3,9-diazaspiro[5.5]undecan-9-yl)propan-2-yl)benzamide (**16**) (118 mg, 35% Yield over 2 steps). LCMS (ESI) calcd for $C_{24}H_{27}F_2N_3O_3$ [M+H] *m/z* 444.2; found 444.2. ^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 7.89 (t, *J*=6.30 Hz, 2 H), 7.40 (td, *J*=7.94, 6.10 Hz, 1 H), 7.07 - 7.22 (m, 5 H), 4.60 (ddd, *J*=9.77, 6.71, 3.05 Hz, 1 H), 4.01 (br d, *J*=12.21 Hz, 1 H), 3.74 (dd, *J*=12.51, 9.46 Hz, 1 H), 3.57 (dd, *J*=12.51, 5.80 Hz, 1 H), 3.39 - 3.49 (m, 1 H), 3.32 - 3.38 (m, 3 H), 3.23 - 3.30 (m, 1 H), 2.30 (br dd, *J*=14.96, 2.14 Hz, 1 H), 1.97 - 2.14 (m, 2 H), 1.86 (td, *J*=14.35, 4.27 Hz, 1 H), 1.34 (d, *J*=6.71 Hz, 3 H). ^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 171.05 (s, 1 C), 169.98 (s, 1 C), 165.46 (s, 1 C), 154.94 (s, 1 C), 131.96 (s, 1 C), 131.49 (s, 2 C), 131.41 (s, 2 C), 116.63 (s, 2 C), 116.46 (s, 1 C), 116.40 (s, 1 C), 78.34 (s, 1 C), 64.27 (s, 1 C), 51.37 (s, 1 C), 45.89 (br t, *J*=128.53 Hz, 1 C), 43.10 (s, 1 C), 42.11 (s, 1 C), 33.80 (s, 1 C), 32.86 (s, 1 C), 29.36 (s, 1 C), 18.79 (s, 2 C); HPLC (Purity): 92.84 %; SFC (ee%): 99.34%; HRMS (ESI) Calcd. for $C_{24}H_{27}F_2N_3O_3$ $[M+H]^+$: 444.2093, Found: 444.2093.

N-(2-(4-(5-chloro-2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)ethyl)-N-methylacetamide (17)



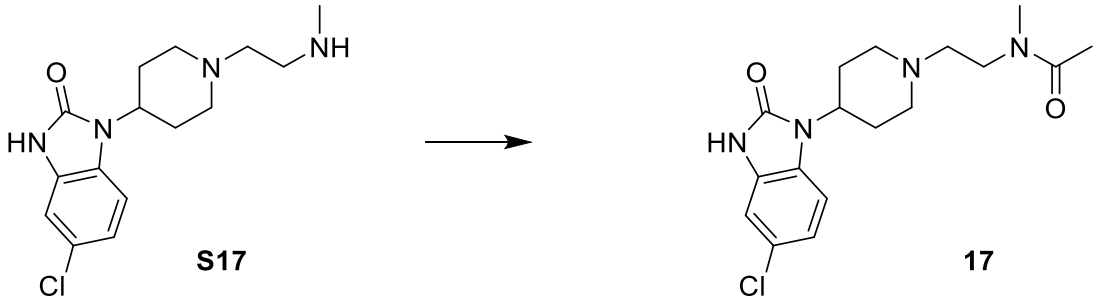
S16

Step 1: A vial was charged with 6-chloro-3-(4-piperidyl)-1H-benzimidazol-2-one (257 mg, 1.02 mmol) and tert-butyl N-methyl-N-(2-oxoethyl)carbamate (177 mg, 1.02 mmol) followed by DCM (3.5 mL). To this mixture was added solid sodium triacetoxyborohydride (432 mg, 2.04 mmol) in a portion-wise manner. The resulting mixture was stirred at rt for 48 h. The reaction mixture was partitioned between 1 N NaOH (15 mL) and EtOAc (10 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (2x10 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. The crude residue was taken up in minimal DCM and loaded onto a dry loading column filled with silica gel. The residue was then eluted through a 40 g Reveleris 40 micron silica gel column (Grace X2 automated purification machine) using a gradient of 2% MeOH in EtOAC ramping to 20% MeOH in EtOAc. Clean fractions were collected and concentrated to provide tert-butyl N-[2-[4-(5-chloro-2-oxo-3H-benzimidazol-1-yl)-1-piperidyl]ethyl]-N-methyl-carbamate (**S16**) (284 mg, 694 µmol, 68% yield). LCMS (ESI) calcd for $C_{20}H_{29}ClN_4O_3$ *m/z* 409.2; found 409.0 $(M+H)^+$.



Step 2: A vial was charged with tert-butyl N-[2-[4-(5-chloro-2-oxo-3H-benzimidazol-1-yl)-1-piperidyl]ethyl]-N-methyl-carbamate (**S16**) (284 mg, 695 µmol) and DCM (2 mL) followed by a dioxane solution of HCl (4 M, 520 µL). The vial was sealed, and the mixture was stirred at rt for 18 h. A white solid precipitated. The solid was collected by vacuum filtration to provide 6-chloro-3-[1-[2-(methylamino)ethyl]-4-piperidyl]-1H-benzimidazol-2-one hydrochloride (**S17**)

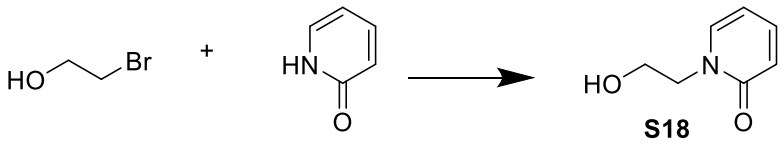
(221 mg, 83% yield) as an amorphous white solid. The solid was taken forward into the next step without further purification.



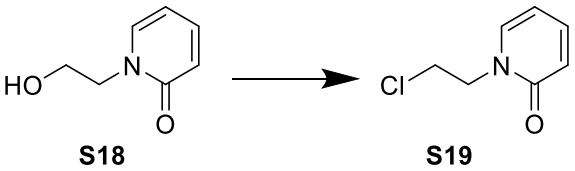
Step 3: To a solution of 6-chloro-3-[1-[2-(methylamino)ethyl]-4-piperidyl]-1H-benzimidazol-2-one dichloride (**S17**) (24 mg, 63 µmol, 2Chloride) and triethylamine (32 mg, 320 µmol, 45 µL) in DMF (0.5 mL) was added acetyl chloride (5 mg, 63 µmol, 5 uL). After 5 minutes, water (1 mL) was added, followed by ethyl acetate (2 mL). the layers were separated, and the aqueous layer was washed with EtOAc (1 mL). The combined organics were dried over $MgSO_4$, filtered and concentrated to provide a mixture of the desired product (N-(2-(4-(5-chloro-2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)ethyl)-N-methylacetamide) and the over-acylated product (N-(2-(4-(3-acetyl-5-chloro-2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)ethyl)-N-methylacetamide) as a yellow oil. The products were separated by reverse phase HPLC (column: Phenomenex Synergi C18 150 x 30 mm x 4 uM; condition: 5% water (0.1% TFA)-ACN; gradiant time: 15 min, flow rate 30 ml/min. N-[2-[4-(5-chloro-2-oxo-3H-benzimidazol-1-yl)-1-piperidyl]ethyl]-N-methyl-acetamide 2,2,2-trifluoroacetate (**17**) (15 mg, 32 µmol, 51% yield) was isolated as a white amorphous solid. LCMS (ESI) calcd for $C_{17}H_{23}ClN_4O_2$ [M+H] *m/z* 351.2; found 351.2 $(M+H)^+$. 1H NMR (400 MHz, DMSO-d_6) δ 11.15 (s, 1H), 7.26 (d, *J*=8.53 Hz, 1H), 7.10 (dd, *J*=2.13, 8.41 Hz, 1H), 7.04 (s, 1H), 4.43-4.60 (m, 1H),

3.64-3.78 (m, 3H), 3.11-3.28 (m, 2H), 3.01 (s, 2H), 2.84 (s, 1H), 2.52-2.65 (m, 3H), 2.45-2.49 (m, 1H), 2.01-2.08 (m, 1H), 1.92-2.00 (m, 1H), 1.85-2.16 (m, 1H).

1-(1-(2-(2-oxopyridin-1(2H)-yl)ethyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one (18)

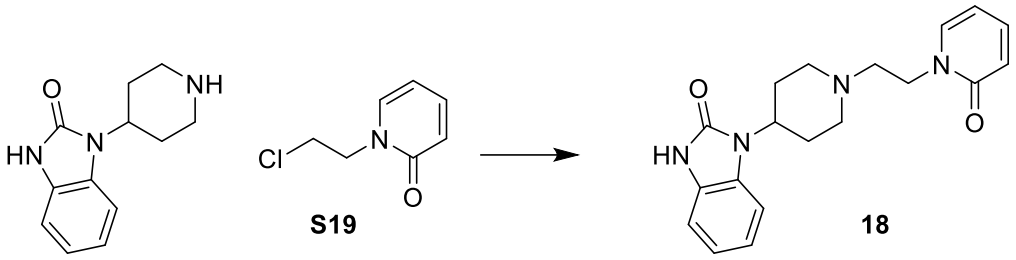


Step 1: To solution of 1H-pyridin-2-one (3.00 g, 31.6 mmol) and 2-bromoethanol (3.94 g, 31.6 mmol, 2.25 mL) in MeCN (20 mL) was added K_2CO_3 (13.1 g, 94.6 mmol) at 50 °C. The mixture was stirred at 50 °C for 16 h. Water (50 mL) was added and the resulting mixture was concentrated under reduced pressure to remove volatiles. The resulting mixture was extracted with EtOAc (3 x 100 mL). The combined organic layers were washed with brine (100 mL), dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica gel chromatography (PE: EA=1:1) to give 1-(2-hydroxyethyl)pyridin-2-one (**S18**) (2.5 g, 18.0 mmol, 57% yield) as a colorless oil.



Step 2: A solution of 1-(2-hydroxyethyl)pyridin-2-one (**S18**) (200 mg, 1.44 mmol) in DCM (6 mL) was cooled to 0 °C and methanesulfonyl chloride (0.39 g, 3.40 mmol, 260 µL) and triethylamine (291 mg, 2.87 mmol, 400 µL) were added under N_2 atmosphere. The solution was allowed to warm to 20 °C and maintained for 1 h. Water was added (10 mL) and the layers were separated. The aqueous layer was extracted with DCM (3 x 50 mL). The combined organic

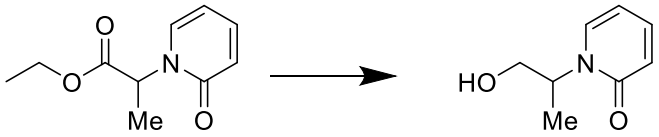
layers were dried over Na2SO4, filtered and concentrated to give 1-(2-chloroethyl)pyridin-2-one (**S19**) (300 mg, crude) as a yellow oil, which was used immediately in the next step without further purification. LCMS (ESI) calcd for C_7H_8ClNO [M+H] *m/z* 158.0; found158.2 $(M+H)^+$.



Step 3: A vial was charged with 3-(4-piperidyl)-1H-benzimidazol-2-one (50.0 mg, 230 µmol), 1-(2-chloroethyl)pyridin-2-one (**S19**)(36.3 mg, 230 µmol) and K_2CO_3 (63.6 mg, 460 µmol). A septum cap was attached and acetonitrile was added. The resulting mixture was stirred at 100 °C for 18 h. The mixture was cooled to rt and filtered through a short plug of celite eluting with MeCN. The filtrate was concentrated *in vacuo* to afford a semi solid, which was dissolved in DMSO and purified by reverse-phase HPLC (column: Phenomenex Synergi C18 150 x 30 mm x 4 uM; condition: 10% MeCN (0.1 wt% TFA) to 90% MeCN in water (0.1 wt% TFA). The clean fractions were concentrated to remove MeCN and then lyophilized to provide 1-(1-(2-(2-oxopyridin-1(2H)-yl)ethyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one (**18**) (57.5 mg, 127 µmol, 55% yield, Trifluoroacetate). ^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 7.73 (dd, *J*=6.64, 1.75 Hz, 1 H), 7.62 (ddd, *J*=9.00, 6.87, 1.98 Hz, 1 H), 7.32 (d, *J*=6.88 Hz, 1 H), 7.07 - 7.15 (m, 3 H), 6.67 (d, *J*=9.16 Hz, 1 H), 6.51 (t, *J*=6.97 Hz, 1 H), 4.61 (ddt, *J*=12.23, 8.14, 4.16, 4.16 Hz, 1 H), 4.48 (t, *J*=6.03 Hz, 2 H), 3.95 (br d, *J*=11.60 Hz, 2 H), 3.61 (br s, 2 H), 3.24 - 3.32 (m, 2 H), 2.81 - 2.93 (m, 2 H), 2.14 (br d, *J*=13.43 Hz, 2 H). ^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 164.35, 154.73, 141.71, 138.56, 128.87, 128.30, 121.52, 121.05,

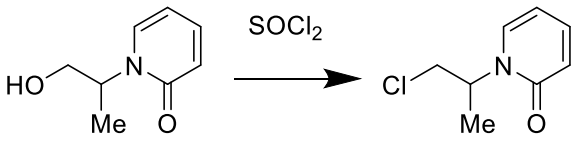
119.66, 109.32, 108.40, 108.26, 56.06, 55.98, 52.62, 52.57, 45.33, 26.40, 26.29. HRMS (ESI) Calcd. for $C_{19}H_{22}N_4O_2$ $[M+H]^+$: 339.1816, Found: 339.1816.

(S)-1-(1-(2-(2-oxopyridin-1(2H)-yl)propyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one (19)



S20

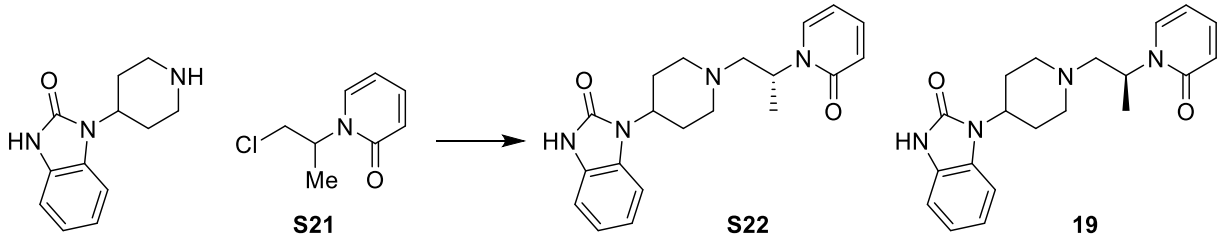
Step 1: A vial was charged with ethyl 2-(2-oxo-1-pyridyl)propanoate (500 mg, 2.56 mmol) and sealed with a septum cap. Under N_2 atmosphere, THF (5.0 mL) was added and the solution was cooled to 0 °C. A THF solution of lithium borohydride (2 M, 1.28 mL) was added via a syringe at 0 °C. The solution was allowed to slowly warm to room temp and stir for overnight. Water (10 mL) was added to the reaction and the resulting suspension was filtered through a funnel and the filtrate was collected and extracted with EtOAc (3 x 10 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated under vacuum to afford 1-(2-hydroxy-1-methyl-ethyl)pyridin-2-one (**S20**) (255 mg, 1.66 mmol, 65% yield), which was used directly for the following chloride formation step.



S20 **S21**

Step 2: A flask was charged with 1-(1-hydroxypropan-2-yl)pyridin-2(1H)-one (**S20**) (250 mg, 1.63 mmol) and DCM (5 mL). Thionyl chloride (388 mg, 3.26 mmol, 240 µL) was added dropwise to avoid violent boiling of the solvent. After the addition, a condenser was attached,

and the solution was heated at 50 °C for 24 h. The reaction was cooled to rt and poured slowly into saturated aqueous $NaHCO_3$ solution; vigorous bubbling occurred. The organic layer was separated, and the aqueous layer extracted with DCM (2x20 mL). The combined layers were dried over $MgSO_4$, filtered and concentrated to give 1-(1-chloropropan-2-yl)pyridin-2(1H)-one (**S21**) (260 mg, 1.52 mmol, 93% yield) as an off-white solid. The material was used directly without any further purification.

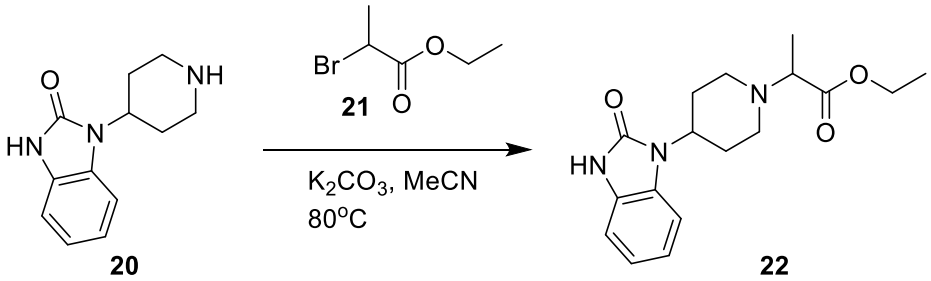


Step 2: A vial was charged with 3-(4-piperidyl)-1H-benzimidazol-2-one (632 mg, 2.91 mmol), 1-(2-chloro-1-methyl-ethyl)pyridin-2-one (**S21**) (500 mg, 2.91 mmol) and K_2CO_3 (1.61 g, 11.64 mmol). Acetonitrile (10 mL) was added and the mixture was stirred and heated at 100 °C for 18 h. The mixture was cooled to rt and filtered through a short plug of celite eluting with MeCN. The filtrate was concentrated *in vacuo* to afford a semi solid, which was dissolved in DMSO and purified by reverse-phase HPLC (column: Phenomenex Synergi C18 150 x 30 mm x 4 uM; condition: 10% MeCN (0.1 wt% TFA) to 90% MeCN in water (0.1 wt% TFA). The clean fractions were concentrated to remove MeCN and then lyophilized to provide racemic 1-(1-(2-(2-oxopyridin-1(2H)-yl)propyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one trifluoroacetate (542 mg, 1.16 mmol, 40% yield). The enantiomers were separated by supercritical fluid chromatography (SFC) using a CHIRALPAK AS-H SFC 5 mic column (Chiral Technologies part # 20475) and using 25% MeOH in 0.1% Et_2NH in CO_2 as the mobile

phase at a flow rate of 100mL/min, (automated backpressure regulator (ABPR) 120 bar, manual backpressure regulator (MBPR) 40 psi.

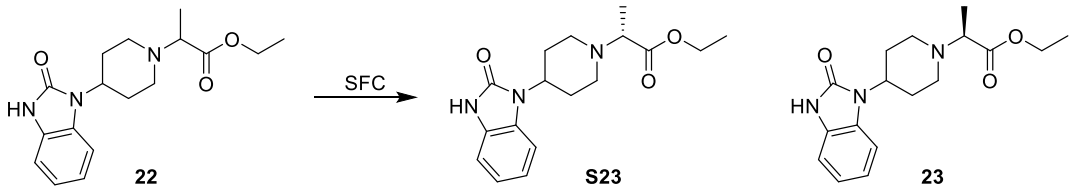
Peak 1: (R)-1-(1-(2-(2-oxopyridin-1(2H)-yl)propyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one (**S22**): (166 mg, 95% purity, 98% ee). Peak 2: (S)-1-(1-(2-(2-oxopyridin-1(2H)-yl)propyl)piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one (**19**) (180 mg, 95% purity, >99% ee). ^{1}H NMR (TFA salt, 500 MHz, METHANOL-d_4) δ ppm 7.75 (dd, *J*=6.94, 1.45 Hz, 1 H), 7.57 (t, *J*=7.82 Hz, 1 H), 7.27 (d, *J*=6.47 Hz, 1 H), 7.06 - 7.11 (m, 3 H), 6.64 (dd, *J*=9.16, 0.76 Hz, 1 H), 6.55 (t, *J*=6.96 Hz, 1 H), 5.59 (ddd, *J*=9.65, 6.90, 2.82 Hz, 1 H), 4.58 (tt, *J*=12.28, 4.20 Hz, 1 H), 4.24 (br s, 1 H), 3.80 (br dd, *J*=13.96, 9.99 Hz, 1 H), 3.62 - 3.74 (m, 1 H), 3.45 - 3.61 (m, 1 H), 3.13 - 3.30 (m, 2 H), 2.74 - 2.91 (m, 2 H), 2.12 (br d, *J*=13.73 Hz, 1 H), 2.00 - 2.09 (m, 1 H), 1.57 (d, *J*=7.02 Hz, 3 H). ^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 163.93, 154.70, 140.63, 134.03, 128.85, 128.28, 121.51, 121.02, 119.92, 109.30, 108.63, 108.37, 60.77, 53.92, 53.78, 51.97, 25.96, 25.88, 25.85, 25.75, 17.40. HRMS (ESI) Calcd. for $C_{20}H_{24}N_4O_2$ $[M+H]^+$: 353.1972, Found: 353.1973

Library synthesis of compounds 25-34



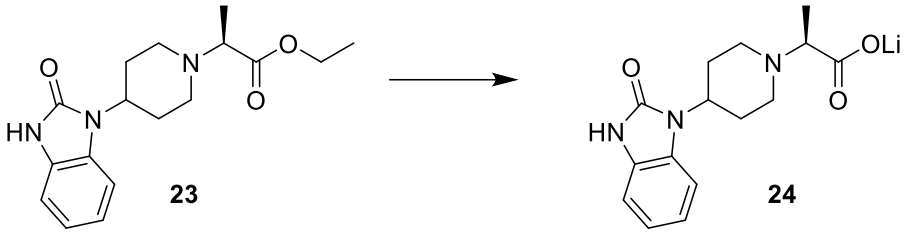
A mixture of 1-(piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one (**20**) (7.3 g, 33.6 mmol, 1.0 eq.), ethyl 2-bromopropanoate (**21**) (6.08 g, 33.6 mmol, 1.0 eq.) and K_2CO_3 (11.6 g, 84 mmol, 2.5 eq.) in MeCN (150 mL) was stirred at 80 °C for 16 h. The reaction was filtered

through a pad of celite and washed with EtOAc (400 mL). The filtrate was concentrated and then purified by column chromatography on silica gel (PE/EA = 10/1 to 1/2, Rf~0.2 in PE/EA 1/1, I_2+H_2O) to give ethyl 2-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)propanoate (**22**) (8.8 g, 82.6% yield) as a white solid.



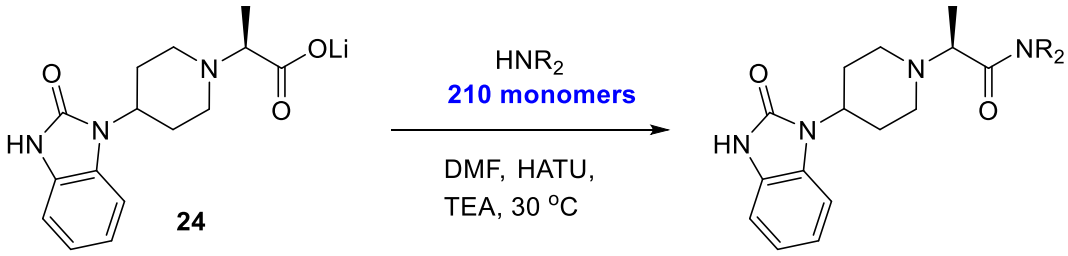
Chiral separation of 22: ethyl 2-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)propanoate (**22**) (10 g, 31.5 mmol, 1.0 eq.) was purified by SFC (Column: AS(250mm x 30mm,10um); Mobile phase: 25% of 0.1%NH_3 in H_2O: EtOH; Flow Rate: 200 mL/min; Detection wavelength: 220 nm) to give ethyl (R)-2-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)propanoate **(S23)** (Peak 1, Rt = 4.134 min, 3.5 g, 35% yield) as a light yellow solid. LCMS (ESI) calcd for $C_{17}H_{23}N_3O_3$ [M+H] *m/z* 318.2; found 318.1; HPLC Purity: 97.5%; Chiral HPLC purity: 99.3%; ^{1}H NMR: (400 MHz, $CDCl_3$) δ: 9.21 (brs, 1H), 7.32-7.29 (m, 1H), 7.11-7.04 (m, 3H), 4.40-4.35 (m, 1H), 4.24-4.18 (m, 2H), 3.42-3.36 (m, 1H), 3.11-3.07 (m, 2H), 2.61-2.41 (m, 4H), 1.86-1.82 (m, 2H), 1.38-1.30 (m, 6H). Peak 2: ethyl (S)-2-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)propanoate (**23**) (Peak 2, Rt = 4.423 min, 4.2 g, 42% yield) as a light yellow solid. HPLC Purity: 98.8%; Chiral HPLC purity: 98.4%; ^{1}H NMR: (400 MHz, $CDCl_3$) δ 9.14 (brs, 1H), 7.33-7.28 (m, 1H), 7.11-7.03 (m, 3H), 4.41-4.34 (m, 1H), 4.25-4.17 (m, 2H), 3.41-3.36 (m, 1H), 3.12-3.07 (m, 2H), 2.61-2.41 (m, 4H), 1.86-1.82 (m, 2H), 1.37-1.30 (m, 6H).

lithium (S)-2-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)propanoate (**24**).



A flask was charged with ethyl (S)-2-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)propanoate (**23**) (8 g, 25.2 mmol, 1.0 eq.), H_2O (10 mL), MeOH (10 mL) and THF (32 mL). To this mixture was added LiOH (2.12 g, 50.4 mmol, 2.0 eq.) at 20 °C. The mixture was stirred at 20 °C for 6 h and then concentrated at 15~18 °C to remove THF and MeOH. The mixture was further diluted with H_2O (20 mL) and then extracted with EtOAc (4 x 40 mL). The aqueous layer was cooled in an ice bath and acidified by the addition of saturated aqueous $KHSO_4$ to pH~4. A white precipitate formed, which was collected by vacuum filtration and washed with H_2O (20 mL) to provide lithium (S)-2-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)propanoate (**24**) (7.2 g, yield: 98.5%) as a white solid. LCMS (ESI) *m/z* 290.2 $(M+H)^+$; HPLC: purity: 99.8%; Chiral HPLC purity: 93.4%; 1H NMR: (400 MHz, MeOD) δ 7.36-7.33 (m, 1H), 7.13-7.07 (m, 3H), 4.62-4.53 (m, 1H), 3.75-3.64 (m, 3H), 3.34-3.32 (m, 1H), 3.28-3.17 (m, 1H), 2.93-2.82 (m, 2H), 2.10-1.95 (m, 2H), 1.57 (d, J = 7.2 Hz, 3H).

Library procedure:

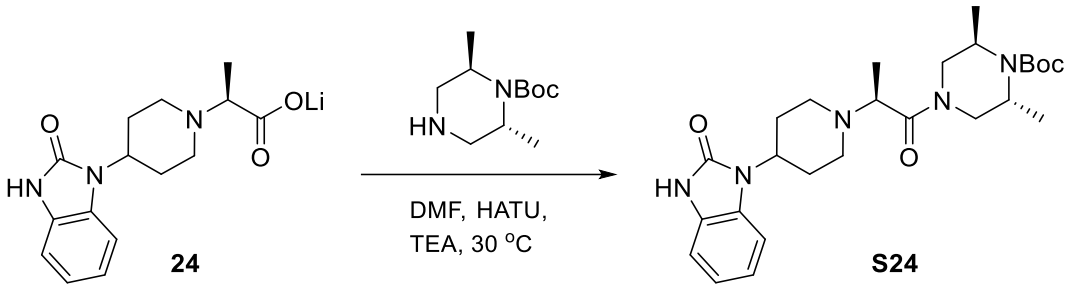


To a mixture of HATU (68.4 mg, 180.0 µmol, 1.0 eq) in 1.0 mL DMF was added the carboxylic acid (50.0 mg, 180.0 µmol), and the reaction mixture was shaken at 30 °C for 2 h. Then the mixture was added into amine (270 µmol, 1.5 eq) and shaken at 30 °C for 16 h. The reaction mixture was purified by preparative HPLC to give the final product.

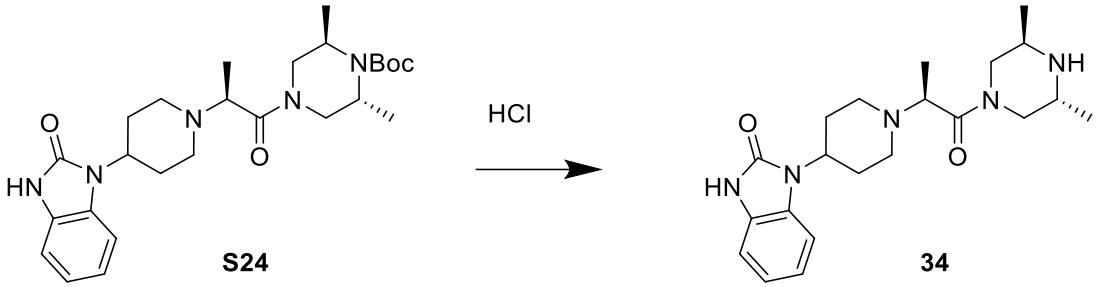
For compounds requiring Boc-deprotection following amine coupling:

The crude reaction mixture from the amide coupling was added to 1 mL H_2O and extracted with DCM (3x2 mL). The combined organic layers were combined and dried over anhydrous Na_2SO_4. The mixture was filtered and the filtrate was concentrated under reduced pressure to give a residue. To this residue was added 2.7 mL mixed solvent DCM/ TFA (V/V=1/ 8) to the residue and the resulting mixture was shaken at 30 °C for 3 h. The mixture was concentrated and the residue purified by preparative HPLC to give the product.

Synthesis of compound 34



Step 1: A flask was charged with lithium (S)-2-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-yl)propanoate (700 mg, 2.42 mmol) and DMF (14 mL) followed by HATU (1.01 g, 2.66 mmol). The solution was maintained at rt for 30 min before the addition of tert-butyl (2S)-2-methylpiperazine-1-carboxylate (727 mg, 3.63 mmol) and DIPEA (375 mg, 2.90 mmol, 510 µL). The solution was maintained at rt for 18h before partitioning between saturated aqueous sodium bicarbonate (1 mL) and ethyl acetate (5 mL). The layers were separated, and the aqueous layer was washed with ethyl acetate (3 x 3 mL). The combined organics were washed with brine (5 mL), dried over MgSO4, filtered and concentrated to afford tert-butyl (2S)-2-methyl-4-[(2S)-2-[4-(2-oxo-3H-benzimidazol-1-yl)-1-piperidyl]propanoyl]piperazine-1-carboxylate (**S24**) as a yellow oil. The material was used in the next experiment without further purification.



Step 2: To a solution of tert-butyl (2S)-2-methyl-4-[(2S)-2-[4-(2-oxo-3H-benzimidazol-1-yl)-1-piperidyl]propanoyl]piperazine-1-carboxylate (1.14 g, 2.42 mmol) in DCM (12 mL) was added hydrochloric acid (4 M in dioxane) (3.0 mL) dropwise at 0 °C. The solution was allowed to warm to rt over 30 min and then was concentrated to a white solid. The material was purified by reverse phase HPLC ((column: Phenomenex Synergi C18 150 x 30 mm x 4 uM; conditions: 90% water (0.1% TFA)/ACN to 50% water/ACN with 0.1% TFA, 15 minutes, flow rate = 30 mL/min) to afford 3-[1-[(1S)-1-methyl-2-[(3S)-3-methylpiperazin-1-yl]-2-oxo-ethyl]-4-piperidyl]-1H-benzimidazol-2-one 2•trifluoroacetate (387 mg, 645 µmol, 27% yield) as a colorless, amorphous solid after lyophilization. ^{1}H NMR (500 MHz, METHANOL-d_4) δ ppm 7.37 - 7.44 (m, 1 H) 7.08 - 7.15 (m, 3 H) 4.90 - 4.97 (m, 1 H) 4.61 - 4.71 (m, 1 H) 4.09 - 4.29 (m, 2 H) 3.96 - 4.08 (m, 1 H) 3.85 (dt, *J*=6.71, 3.36 Hz, 2 H) 3.63 - 3.75 (m, 1 H) 3.43 - 3.61 (m, 3 H) 3.35 - 3.42 (m, 1 H) 2.87 - 3.03 (m, 2 H) 2.08 - 2.22 (m, 2 H) 1.63 (d, *J*=6.87 Hz, 3 H) 1.37 - 1.48 (m, 6 H).

^{13}C NMR (126 MHz, METHANOL-d_4) δ ppm 168.01, 154.74, 128.95, 128.28, 121.51, 121.05, 109.28, 108.46, 61.05, 52.33, 49.13, 46.24, 43.45, 26.17, 25.81, 14.16, 13.06, 12.79. HRMS (ESI) Calcd. for $C_{21}H_{31}N_5O_2$ $[M+H]^+$: 386.2551, Found: 386.2552

4. X-ray Crystallography

Crystallization was performed as before (Metrick, C.M., Peterson, E.A., Santoro, J.C. et al. Human PLD structures enable drug design and characterization of isoenzyme selectivity. Nat Chem Biol 16, 391–399 (2020). https://doi.org/10.1038/s41589-019-0458-4). Briefly, PLD2 protein at 3 mg/ml was set up in sitting drop vapor diffusion plates at 22 °C with 1 mM proprietary compound. Crystals formed in 0.08 M sodium cacodylate pH 6.6, 14% PEG 8000, 20% glycerol, 0.16 M calcium acetate and were soaked in mother liquor supplemented with 25% PEG 3350 and 1 mM cmpd34 for 12 h before freezing. Diffraction data were measured at 100 K

and 1.21 Å in 0.2° rotation frames on a Pilatus detector, using a 10 μm beam at the SLS PXII beamline. The data were integrated with XDS (Kabsch, W. XDS. Acta Crystallogr. D 66, 125–132 (2010).) and scaled using CCP4-Aimless (Winn, M. D. et al. Overview of the CCP4 suite and current developments. Acta Crystallogr. D 67, 235–242 (2011).). Data extending to 3.0 Å were obtained from these crystals. Data collection statistics are listed in Supplementary Table 1.

Molecular replacement was performed in Phenix (Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D 66, 213–221 (2010).) with the human PLD2 structure (PDB: 6OHO Metrick, C.M., Peterson, E.A., Santoro, J.C. et al. Human PLD structures enable drug design and characterization of isoenzyme selectivity. Nat Chem Biol 16, 391–399 (2020). https://doi.org/10.1038/s41589-019-0458-4). The resulting model was improved through iterative rebuilding in Coot (Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. Acta Crystallogr. D 66, 486–501 (2010).) and refinement in Phenix (Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D 66, 213–221 (2010).). The final R_{work} and R_{free} values are provided in Supplementary Table 1 with the collection and refinement statistics, and the model has been uploaded to the RCSB PDB with accession code 7SVP.

4.1 Table S1 Data collection and refinement statistics (molecular replacement)

	PLD2 bound with Cpd **34**
Data collection	
Space group	P 2_1
Cell dimensions	
a, *b*, *c* (Å)	89.7 132.2, 113.8
α, β, γ (°)	90, 100.6, 90
Resolution (Å)	50-3.0
R_{sym} or R_{merge}	0.28 (2.0)
R_{pim}	0.17 (1.3)

$CC_{1/2}$	0.98 (0.44)
I / σI	3.8 (0.5)
Completeness (%)	99.7 (98.1)
Redundancy	3.5 (3.4)
Refinement	
Resolution (Å)	50-3.0
No. reflections	57790 (5748)
R_{work} / R_{free}	0.29/0.32
No. atoms	
Protein	2250
Ligand/ion	112
Water	197
B-factors	
Protein	63.8
Ligand/ion	55.6
Water	47.9
R.m.s. deviations	
Bond lengths (Å)	0.002
Bond angles (°)	0.48

*Values in parentheses are for highest-resolution shell.

[AU: Equations defining various *R*-values are standard and hence are no longer defined in the footnotes.]

[AU: Ramachandran statistics should be in Methods section at the end of Refinement subsection.]

[AU: Wavelength of data collection, temperature and beamline should all be in Methods section.]

5. Development of PD assay

5.1 Summary

PLD1[1] and PLD2[2] hydrolyze a phosphodiester bond of phosphatidylcholine (PC) using water as the nucleophile to generate the secondary messenger signaling lipid, phosphatidic acid (PA). The

enzymes also catalyze a transphosphatidylation reaction of PC by reacting with a short-chained primary alcohol, such as ethanol or 1-butanol, as substrates, to yield a phosphatidyl alcohol, phosphatidylethanol (PtdEtOH) or phosphatidylbutanol (PtdBut).**Error! Reference source not found.** The transphosphatidylation reaction is exclusive to phospholipase D1/D2 and has been employed as a specific assay for assessing the enzyme activity.[3] We have utilized this characteristic transphosphatidylation and developed a LC-MS assay to assess PLD1/PLD2 activity for *in-vivo* mouse model studies.

Two short-chained primary alcohols, ethanol and 1-butanol, have been reported for transphosphatidylation reaction. 1-Butanol has been a favored method to quantify PLD1 and PLD2 cellular activities by measuring PtdBut production but has not been applied to animal study,[4] whereas using ethanol in mouse model has been reported.[5] Our initial experiment was to survey PLD1/PLD2 transphosphatidylation with ethanol and 1-butanol by dosing mice either ethanol or 1-butanol. Figure 1 compared transphosphatidylation products PtdEtOH(34:1) and PtdBut(34:1) detected from ethanol and butanol treated mice brains. PtdBut(34:1) has significantly higher intensity than PtdEtOH(34:1), indicating 1-butanol as a better PLD1/2 substrate than ethanol. Thereafter, we selected 1-butanol for our animal studies and used deuterated 1-butanol-d10 to improve assay specificity. Untargeted high-resolution LC-FT ICR-MS assay profiled the lipid pools and distinguished PtdBut species from the interference of other lipids in tissue. Chromatograms of PtdBut species were optimized to achieve characteristic LC elution profile. The targeted LC-MRM assay was also used to quantify each PtdBut species observed.

To profile the pharmacokinetic character of PLD1/PLD2 transphosphatidylation activity with 1-butanol, a time course study was conducted by sampling the brain and liver tissues at 15, 30 and 60 minutes after the dosing. A panel of phosphatidylbutanol species were identified from the brain and liver, mainly fatty acid with chain lengths of 32, 34, 36, 38 and 40 and various C=C double bonds. The liver PtdBut species distribution was different from brain with much higher abundance of PtdBut (34:2). PtdBut (34:1) was the major species both in the brain and liver. PtdBut (34:1) was quantified using both untargeted high-resolution LC-FTICR-MS and targeted low-resolution LC-MRM assays. Both assays showed consistent results. The study showed time-dependent decrease of PtdBut species. 15-minute sample consisted higher amount of PtdBut in

comparison with 30-minute sample, suggesting that 15-minute was the optimum for *in-vivo* mouse transphosphatidylation study.

The assay sensitivity was evaluated by performing a study with different dosing amounts of 3g/kg, 1.5g/kg and 0.75g/kg 1-butanol. PtdBut (34:1) was quantified using untargeted LC-FTICR-MS and targeted LC-MRM. Both assays showed consistent results of butanol dose-dependent decreasing of transphosphatidylation products. We observed more PtdBut (34:1) in the brain than in the liver. This may be attributed to the fast clearance of PtdBut in the liver. 0.75g/kg butanol dose was on the board-line of the sensitivity limitation for MRM assay and the interference from background ion signals became significant. For high-resolution LC-MS, there were no interferent ions and the assay sensitivity can be increased by targeting specific mass range using CASI scan mode in Solarix FTICR. We also further increased the assay sensitivity by using water/methanol (v/v = 7/3) containing 2mM ammonium format as mobile phase A and 2-propanol/acetone (v/v = 4/1) as mobile phase B.

5.2 PD Assay Materials.

Phosphatidylbutanol (C34:1) was purchased from Enzo Biochem Inc (Farmingdale, New York). Phosphatidylbutanol (C32:0), (C36:2), Phosphatidylethanol (C34:1) and (C32:0) were ordered from Avanti Polar Lipids. Solvents were from Thermo Fisher Scientific (Waltham, MA) unless otherwise stated

5.3 PLD1/2 transphosphatidylation mouse model in vivo experimental

A) Butanol/ethanol mouse study: mice, three per group, were intraperitoneally administrated with deuterated butanol-d10 or ethanol-d6 at the dose of 3g/kg (n=1). The brains were collected 1h after the dosing. B) Time course study**:** 16-20 week aged C57BL/6 mice, four animals per time point, were dosed intraperitoneally with butanol-d10/H2O in the concentration of 0.6g/mL at the amount of 3g/kg. The brain and liver were collected at pre-scheduled time points of 15, 30 and 60 minutes. C) butanol dose study: C57BL/6 mice, three animals per dosing group, were intraperitoneally dosed (n=1) with butanol-d10/H2O in the concentrations of 0.6g/mL, 0.3g/mL or 0.15g/mL at the amount of 3g/kg, 1.5 g/kg or 0.75 g/kg. The brain and liver were collected 15 minutes after the dosing and stored fresh frozen at -80 ^{o}C. D) PLD1/2 inhibitor mouse study: sC57BL/6 mice were subcutaneous pre-treated with Compound **37** at

either a 15 min, 30 min or 1 hour, and followed by intraperitoneal injection of 1.5g/kg butanol-d10 for 15 minutes. The brain and liver were stored fresh frozen for transphosphatidylation assay analysis.

5.4 Lipids extraction from tissue

Fresh frozen tissue was transferred to a pre-chilled Covaris bag and pulverized frozen on a punch using a Covaris CP02 CryoPrep Dry Tissue Pulverizer Impactor. Typically, 10-20 mg the frozen tissue powder per sample was weighted in a pre-chilled glass vial and lipids were extracted using the extraction solution of dichloromethane and methanol (2:1) at the ratio of 40 µL/mg tissue with internal standards of 1 µg/mL phosphatidylbutanol (34:1), (36:2) and (32:0). The samples were vortexed for few seconds and followed by end-to-end rotation for 10-20 minutes at room temperature. 20 µL lipid extract was then pipette into a clean glass vial contained 5 µL methanol and evaporated under N_2 gas. The lipids were reconstructed in 20 µL of methanol/water (v/v = 9:1) and proceeded to LC-MS analysis.

5.5 LC-FTICR-MS analysis

LC-MS were performed on a Waters nano-Acquity chromatographic system in-line with a Bruker Solarix XR FT ICR mass spectrometer. The data were acquired in negative electrospray ionization (ESI) mode at the normal scan range (350-3000 Da) or CASI scan of 738 with the range of 50 Da. The chromatographic method for separation of PtdButs in mouse brain and liver tissues were developed on an Imtakt Cadenza CW-C18, 0.3 x 150 mm column (3µm). The mobile phase A was water/methanol (v/v = 7/3) with 0.1 % formic acid, and the mobile phase B of 10 mM ammonium formate, 95% 2-propanol/5% H2O, 0.1 % formic acid. LC gradient was from 55% to 3% mobile A for 10 minutes at the flow rate of 1.5 µL/min. The column temperature was set to 40 ^{o}C and the sample injection was 2 µL full-loop loading. The LC-MS assay sensitivity was further improved by using mobile phase with A water/methanol (v/v = 7/3) containing 2mM ammonium format and the mobile phase B of 2-propanol/acetone (v/v = 1/4), with isocratic elution condition of 40% mobile A for 10 minutes. The improved LC method was used in BIIB 1618268 study.

5.6 LC-MS/MS analysis

An LC-20 AD system (Shimadzu Corporation, Kyoto, Japan) was used for the chromatography. Samples were separated on an ACE Excel C18AR, 2µm, 2.1 x 50 mm column at 40 ^{o}C. Mobile phase A was 0.1% formic acid in water/methanol (v/v=7:3), mobile phase B was 10 mM ammonium formate in 0.1% formic acid, 95% 2-propanol, 5% water. A binary gradient at a flow rate of 0.4 mL/min was used, in which mobile phase B was maintained at 40% for the first 0.5 min, then ramped up to 95% over 6.5 min and maintained at 95% for 1 min, and then reduced to 40% for 2 min to re-equilibrate the column. The autosampler needle was washed using 0.5% formic acid in 50/50 acetonitrile/water.

MS/MS analysis was performed using an API 5500 from AB Sciex (Framingham, MA). The turboionpray was operated in negative mode at -3000 V, GS1 and GS2 were 60, CUR was 20, CAD was 7, CE was -45, TEM was 600, DP was -60. Q1 and Q3 were both set to unit resolution. The MRM transitions were 738.5/281.3 for 34:1/18:1 butyl ester, 729.5/281.3 for light 34:1/18:1 butyl ester (IS), 738.5/255.2 for 34:1/16:0 butyl ester, 729.5/255.2 for light 34:1/16:0 butyl ester (IS), 680.5/255.2 for 32:0/16:0 ethyl ester, and 675.5/255.2 for light ethyl ester (IS).

5.7 Quantification of phosphatidylbutanol

The ratio of peak area of the deuterated PtdBut (34:1), PtdBut (36:2) and PtdBut (32:0) with their none-deuterated compounds was used to quantify the changes of phosphatidylbutanol.

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