Mediating K^+/H^+ Transport on Organelle Membranes to Selectively Eradicate Cancer Stem Cells by a Small Molecule

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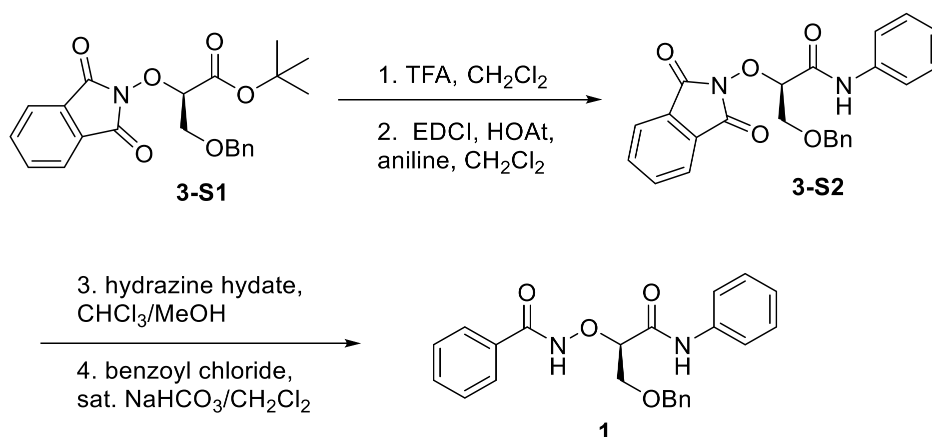
1. Synthesis of aminoxy-acid molecules

1.1 Material and methods

All reagents and solvents were purchased from Sigma Aldrich, TCI or Acros and used without further purification. All reactions requiring anhydrous conditions were carried out under argon atmosphere using oven-dried glassware. AR-grade solvents were used for all reactions. Air and moisture sensitive compounds were introduced via syringes through rubber septa under argon atmosphere. Reaction progress was monitored by TLC on pre-coated silica plates (Merck 60 F254 nm, 0.25 μm) and spots were visualized by UV and/or staining in phosphomolybdic acid (PMA) or $KMnO_4$ solution followed by heating. Flash column chromatography was performed using Merck silica gel (230−400 mesh ASTM).

NMR spectra were acquired on Bruker Avance DPX 300 Fourier Transform Spectrometer operating at 300 MHz for ^{1}H and at 75 MHz for ^{13}C, Bruker Avance DPX 400 Fourier Transform Spectrometer operating at 400 MHz for ^{1}H, 100 MHz for ^{13}C, 376 MHz for ^{19}F and Bruker Avance III HD 500 Spectrometer operating at 500 MHz for ^{1}H, 125 MHz for ^{13}C, 470 MHz for ^{19}F. Chemical shifts were reported in parts per million (ppm) referenced with respect to appropriate internal standards or residual solvent peaks ($CDCl_3$ = 7.26 ppm, DMSO-d_6 = 2.50 ppm, CD_3OD = 3.31 ppm and CD_3CN = 1.94 ppm for ^{1}H; $CDCl_3$ = 77.16 ppm, DMSO-d_6 = 39.52 ppm, CD_3OD = 49.00 ppm and CD_3CN = 1.32, 118.26 ppm for ^{13}C). The following abbreviations were used in reporting spectra, br s (broad singlet), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets) and AB_q (AB quartet). High-resolution mass spectra were obtained on Bruker maXis II High-Resolution QTOF using ESI mode. Fluorescence measurements were carried out on a Hitachi F-2500 fluorescence spectrometer with a slit width of 2.5 nm and photomultiplier at 400 V or 700 V. X-ray crystallographic spectra were collected with APEX3 v2015.5-2 (Bruker-AXS, 2015). Circular dichroism (CD) spectra were recorded on a Jasco-815 spectropolarimeter at 180−300 nm wavelengths at room temperature. The path lengths of CD cells were 1 mm or 0.1 mm. Data were converted to molar ellipticity according to the equation: $[\theta] = \Psi M/(100lc)$, where Ψ is the ellipticity in degrees; M is the molecular weight of test compound; l is the length of the CD cell in dm, and c is the concentration of test compound in g/mL.

1.2 Synthesis of compounds 1–6

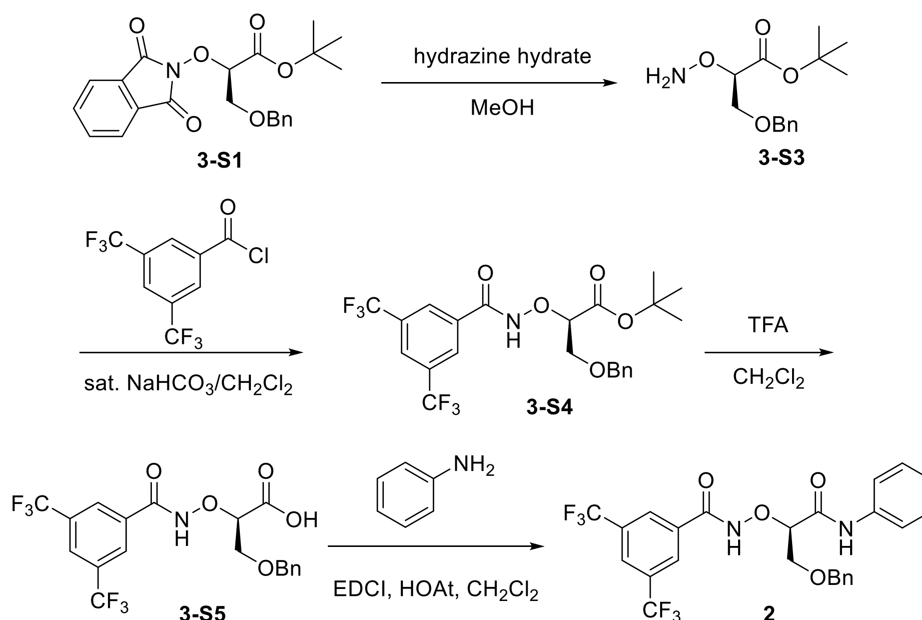


Scheme S1. Synthesis of compound **1**.

Compound **3-S1** was synthesized according to the literature reported procedures[1]. To a solution of compound **3-S1** (198.7 mg, 0.50 mmol) in CH_2Cl_2 (2.5 mL) was added an equal volume of CF_3COOH (2.5 mL) through a syringe at room temperature. After being stirred at room temperature for 3 h, the reaction mixture was concentrated *in vacuo*. The residue was azeotroped with toluene twice to give a free acid, which was used directly in the following steps without further purification. To a solution of the free acid in CH_2Cl_2 (2.5 mL) were sequentially added EDCI (143.8 mg, 0.75 mmol), HOAt (88.5 mg, 0.65 mmol) and aniline (51.2 mg, 0.60 mmol). The reaction mixture was stirred overnight at room temperature, then diluted with CH_2Cl_2, washed with 5% $NaHCO_3$, 0.5 N HCl and brine, and dried over anhydrous Na_2SO_4. The organic layer was concentrated *in vacuo*. The residue was purified by silica gel column chromatography to afford compound **3-S2** (156.2 mg, 75%) as a white solid. Compound **3-S2** was characterized by the following data: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.65 (s, 1H), 7.80−7.69 (m, 6H), 7.36−7.21 (m, 7H), 7.12 (t, J = 7.4 Hz, 1H), 4.92−4.91 (m, 1H), 4.63 (AB_q, J_{AB} = 12.0 Hz, 2H), 4.24−4.21 (m, 1H), 4.16−4.12 (dd, J = 12.0, 3.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.5, 163.9, 137.5, 137.5, 135.1, 134.2, 129.0, 128.5, 128.4, 127.7, 124.7, 124.1, 120.0, 87.8, 73.7, 69.7; HRMS (ESI) for $C_{24}H_{21}N_2O_5$ (M+H$^+$): calcd 417.1445, found 417.1441.

To a solution of compound **3-S2** (124.9 mg, 0.30 mmol) in MeOH/$CHCl_3$ (1:1, 2.5 mL) was added $NH_2NH_2{\cdot}H_2O$ (25.0 mg, 0.50 mmol). After being stirred at room temperature for 2.5 h, the reaction mixture was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 and washed with 5% $NaHCO_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4 and

was concentrated *in vacuo* to give a crude product of the free amine, which was used directly in the following steps without further purification. To a solution of the free amine in MeOH (1.0 mL) was added saturated $NaHCO_3$ solution (1.0 mL). Then, benzoyl chloride (56.2 mg, 0.40 mmol) was added dropwise. After being stirred overnight, the reaction mixture was diluted with CH_2Cl_2. The aqueous layer was extracted with CH_2Cl_2 for three times. The combined organic layer was dried over anhydrous Na_2SO_4 and was concentrated *in vacuo*. The crude oil was purified by flash column chromatography to afford compound **1** (296.7 mg, 76%) as a white solid: ^1H NMR (400 MHz, $CDCl_3$) δ 10.62 (s, 1H), 9.75 (s, 1H), 7.67 (d, J = 7.7 Hz, 2H), 7.62 (d, J = 7.3 Hz, 2H), 7.48−7.44 (m, 1H), 7.35−7.32 (m, 2H), 7.28−7.22 (m, 7H), 7.06−7.02 (m, 1H), 4.60 (dd, J = 8.7, 2.7 Hz, 1H), 4.56, 4.52 (ABq, J_{AB} = 12.0 Hz, 2H), 4.15 (dd, J = 11.2, 2.8 Hz, 1H), 3.89 (dd, J = 11.1, 8.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.1, 166.2, 137.8, 137.3, 132.8, 130.4, 128.9, 128.8, 128.6, 128.2, 128.1, 127.2, 124.4, 119.9, 87.0, 73.8, 70.1; HRMS (ESI) for $C_{23}H_{22}N_2O_4Na$ ($M+Na^+$): calcd 413.1477, found 413.1456.



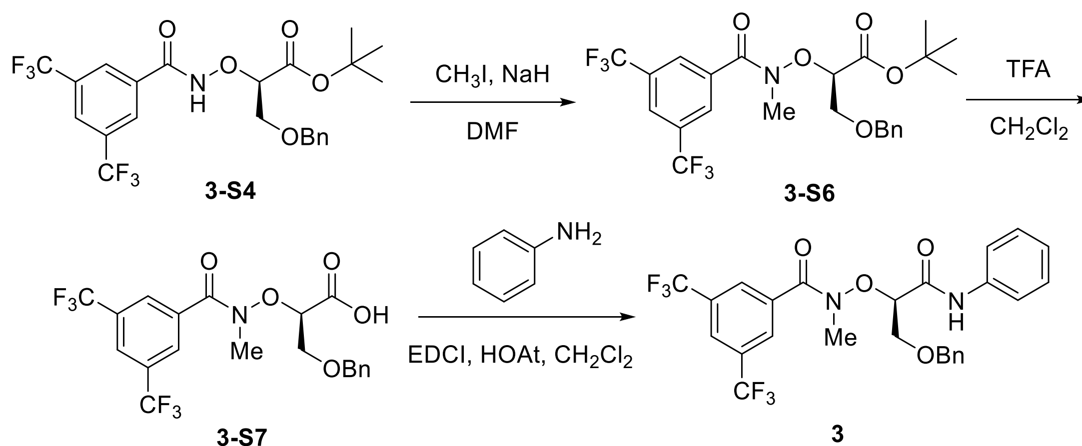
Scheme S2. Synthesis of compound **2**.

To a solution of compound **3-S1** (794.9 mg, 2.0 mmol) in MeOH (20 mL) was added $NH_2NH_2{\cdot}H_2O$ (400.4 mg, 8.0 mmol). After being stirred at room temperature for 2.5 h, the reaction mixture was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 and washed with 5% $NaHCO_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4 and was concentrated *in vacuo* to give a crude product of compound **3-S3**, which was used directly in the following steps without further purification.

To a solution of compound **3-S3** dissolved in CH_2Cl_2 (10 mL) was added saturated aqueous $NaHCO_3$ solution (10 mL). Then, 3,5-bis(trifluoromethyl)benzoyl chloride (553.1 mg, 2.0 mmol) was added dropwise in an ice bath. After being stirred at 0°C for 1 h, the reaction mixture was diluted with CH_2Cl_2. The aqueous layer was extracted with CH_2Cl_2 for three times. The combined organic layer was dried over anhydrous Na_2SO_4 and was concentrated *in vacuo*. The crude oil was purified by flash column chromatography to afford compound **3-S4** (862.6 mg, 85%) as colorless oil. ^{1}H NMR (500 MHz, CD_3CN) δ 10.17 (br s, 1H), 8.24 (s, 2H), 8.16 (s, 1H), 7.36−7.29 (m, 5H), 4.63 (t, J = 3.3 Hz, 1H), 4.54 (AB_q, J_{AB} = 11.6 Hz, 2H), 3.93−3.87 (m, 2H), 1.46 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.0, 163.4, 137.4, 133.8, 132.2 (q, $^2J_{C,F}$ = 34.0 Hz), 128.5, 128.0, 127.9, 127.6, 125.3, 123.0 (q, $^1J_{C,F}$ = 271.3 Hz), 83.4, 77.4, 73.7, 69.2, 28.1; ^{19}F (376 MHz, $CDCl_3$) δ −63.0; HRMS (ESI) for $C_{23}H_{23}F_6NO_5Na$ ($M+Na^+$): calcd 530.1378, found 530.1395.

To a solution of compound **3-S4** (152.2 mg, 0.30 mmol) in CH_2Cl_2 (1.5 mL) was added an equal volume of CF_3COOH (1.5 mL) at room temperature. After being stirred at room temperature for 3 h, the reaction mixture was concentrated *in vacuo*. The residue was azeotroped with toluene twice to give compound **3-S5** as a white solid, which was used directly in the following step without further purification.

To a solution of compound **3-S5** (22.6 mg, 0.05 mmol) in CH_2Cl_2 (1 mL) were added sequentially EDCI (15.3 mg, 0.08 mmol), HOAt (9.5 mg, 0.07 mmol) and aniline (5.6 mg, 0.06 mmol). The reaction mixture was stirred overnight at room temperature, then was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$, 0.5 N HCl, brine and dried over anhydrous Na_2SO_4. The organic layer was concentrated *in vacuo*. The residue was purified by silica gel column chromatography to afford compound **2** (21.6 mg, 82%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.34 (br s, 2H), 8.17 (s, 2H), 8.02 (s, 1H), 7.66 (s, 1H), 7.65 (s, 1H) 7.34−7.25 (m, 7H), 7.12 (tt, J = 7.4, 1.0 Hz, 1H), 4.70−4.68 (m, 1H), 4.62, 4.57 (AB_q, J_{AB} = 11.8 Hz, 2H), 4.13 (dd, J = 11.6, 2.5 Hz, 1H), 3.95 (dd, J = 11.1, 8.2 Hz, 1H); ^{13}C (125 MHz, $CDCl_3$) δ 166.3, 164.9, 137.4, 137.2, 132.8, 132.6 (q, $^2J_{C,F}$ = 33.2 Hz), 129.1, 128.8, 128.4, 128.0, 127.8, 126.2, 125.0, 122.9 (q, $^1J_{C,F}$ = 273.1 Hz), 120.2, 87.2, 74.1, 70.1; ^{19}F (376 MHz, $CDCl_3$) δ −62.9; HRMS (ESI) for $C_{25}H_{21}F_6N_2O_4$ ($M+H^+$): calcd 527.1400, found 527.1413.



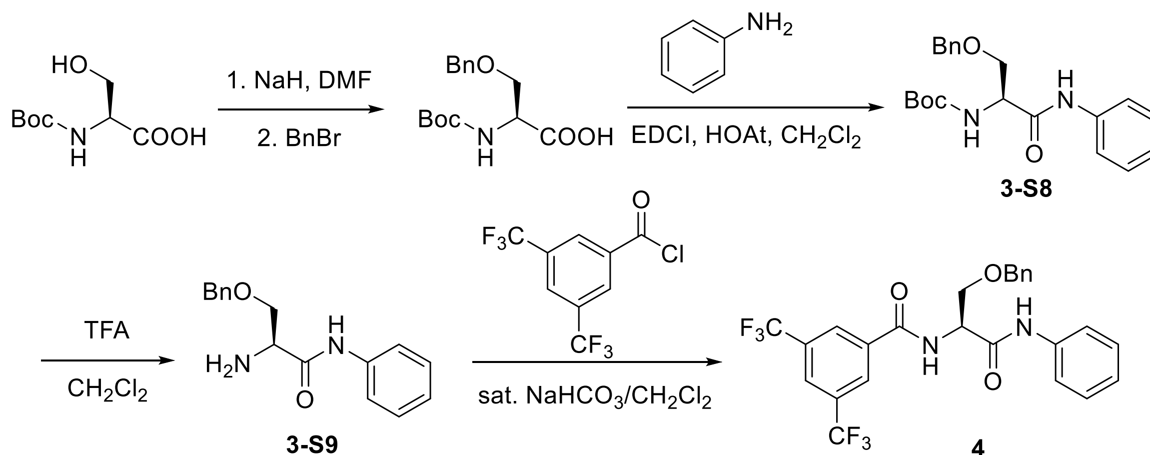
Scheme S3. Synthesis of compound **3**.

To a solution of compound **3-S4** (50.7 mg, 0.10 mmol) in DMF (1 mL) was added 60% NaH (5.2 mg, 0.13 mmol) at 0°C. After being stirred for 0.5 h, CH_3I (18.5 mg, 0.13 mmol) was added dropwise. The reaction mixture was stirred for another 2 h at room temperature, then concentrated *in vacuo*. The residue was purified by silica gel column chromatography to afford compound **3-S6** (37.1 mg, 52% yield) as an oil. ^1H NMR (500 MHz, $CDCl_3$) δ 8.17 (s, 2H), 7.87 (s, 1H), 7.34−7.28 (m, 5H), 4.75 (dd, J = 5.7, 3.3 Hz, 1H), 4.66, 4.59 (AB_q, J_{AB} = 15.0 Hz, 2H), 4.31 (s, 3H), 3.94−3.88 (m, 2H), 1.48 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.8, 153.2, 139.1, 135.3, 133.0 (q, $^2J_{C,F}$ = 33.4 Hz), 129.7, 129.1, 129.0. 128.1, 124.7, 124.3 (q, $^1J_{C,F}$ = 273.2 Hz), 84.1, 83.5, 74.8, 70.4, 62.6, 29.4; ^{19}F (376 MHz, CD_3CN) δ −63.0. HRMS (ESI) for $C_{20}H_{17}F_6NO_5$ (M−*t*-Bu+H^+): calcd 466.1089, found 466.1079.

To a solution of compound **3-S6** (35.7 mg, 0.05 mmol) in CH_2Cl_2 (0.5 mL) was added an equal volume of CF_3COOH (0.5 mL) through a syringe at room temperature. After being stirred at room temperature for 3 h, the reaction mixture was concentrated *in vacuo*. The residue was azeotroped with toluene twice to give free acid compound **3-S7** as a white solid, which was used directly in the next step.

To a solution of compound **3-S7** in CH_2Cl_2 (1 mL) were added EDCI (15.3 mg, 0.08 mmol), HOAt (9.5 mg, 0.07 mmol) and aniline (5.6 mg, 0.06 mmol), sequentially. The reaction was stirred overnight at room temperature, then diluted with CH_2Cl_2, washed with 5% $NaHCO_3$, 0.5 N HCl and brine and dried over anhydrous Na_2SO_4. The organic layer was concentrated *in vacuo*. The residue was purified by silica gel column chromatography to afford compound **3** (22.2 mg, 82% yield) as an oil. ^1H NMR (400 MHz, $CDCl_3$) δ 10.37 (s, 1H), 8.02−8.00 (m, 3H), 7.68 (s, 1H), 7.65 (s, 1H), 7.38−7.28 (m, 7H), 7.13−7.08 (m, 1H), 4.69 (dd, J = 8.3, 2.2

Hz, 1H), 4.63 (s, 2H), 4.18−4.15 (m, 1H), 3.85 (dd, J = 11.2, 8.3 Hz, 1H), 3.42 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.2, 165.6, 137.6, 137.3, 135.5, 132.4 (q, $^{2}J_{C,F}$ = 34.1 Hz), 129.0, 128.6, 128.3, 128.0, 127.8, 125.4, 124.5, 122.7 (q, $^{1}J_{C,F}$ = 277.1 Hz), 119.7, 86.8, 73.8, 69.9, 40.2; ^{19}F (376 MHz, CD_3CN) δ −62.9. HRMS (ESI) for $C_{26}H_{23}F_6N_2O_4$ (M+H$^+$): calcd 541.1557, found 541.1556.



Scheme S4. Synthesis of compound **4**.

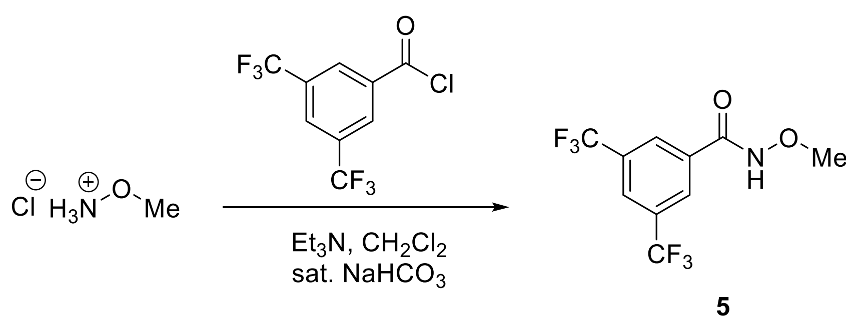
To a solution of *N*-(*tert*-butoxycarbonyl)-L-serine (205.1 mg, 1.0 mmol) in anhydrous DMF (10 mL) was added 60% NaH (96.0 mg, 2.40 mmol) in an ice bath. After being stirred for 1 h, benzyl bromide (188.1 mg, 1.10 mmol) was added. The reaction mixture was stirred at room temperature for 2 h, then diluted with water and extracted with diethyl ether twice. The aqueous layer was adjusted to pH below 3.5 and extracted with ethyl acetate 3 times. The combined organic layer was dried over Na_2SO_4 and was concentrated *in vacuo* to give the free acid, Boc-*O*-benzyl-L-serine, as oil, which was used directly in the next step.

To a solution of Boc-*O*-benzyl-L-serine obtained from the last step in CH_2Cl_2 (10 mL) were added EDCI (287.6 mg, 1.50 mmol), HOAt (176.9 mg, 1.30 mmol) and aniline (102.4 mg, 1.10 mmol) subsequently. The reaction mixture was stirred overnight at room temperature, then diluted with CH_2Cl_2, washed with 5% $NaHCO_3$, 0.5 N HCl and brine. The organic layer was dried over anhydrous Na_2SO_4 and was concentrated *in vacuo*. The residue was purified by silica gel column chromatography to afford compound **3-S8** (262.8 mg, 71%) as an oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.43 (s, 1H), 7.46 (d, J = 7.9, 2H), 7.35−7.25 (m, 7H), 7.09 (t, J = 7.4 Hz, 1H), 5.56 (br, 1H), 4.62, 4.54 (AB$_q$, J_{AB}= 10.0 Hz, 2H), 4.43 (br, 1H), 3.98 (dd, J = 9.1, 3.7 Hz, 1H), 3.65 (dd, J = 9.5, 7.0 Hz, 1H), 1.47 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.6, 155.8,

137.6, 137.4, 129.1, 128.7, 128.2, 128.0, 124.5, 120.0, 80.6, 73.7, 69.9, 54.4, 28.4;

To a solution of compound **3-S8** (262.8 mg, 0.71 mmol) in CH_2Cl_2 (7 mL) was added an equal volume of CF_3COOH (7.0 mL) through a syringe at room temperature. After being stirred at room temperature for 3 h, the reaction mixture was concentrated *in vacuo*. The residue was azeotroped with toluene twice to give free amine **3-S9** as a white solid, which was used directly in the next step without further purification.

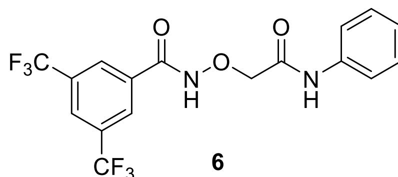
To a solution of compound **3-S9** in CH_2Cl_2 (10 mL) was added saturated $NaHCO_3$ solution (10 mL). Then 3,5-bis(trifluoromethyl)benzoyl chloride (221.2 mg, 0.80 mmol) was added dropwise. After being stirred overnight, the reaction mixture was diluted with CH_2Cl_2. The aqueous layer was extracted with CH_2Cl_2 for three times. The combined organic layer was dried over anhydrous Na_2SO_4 and was concentrated *in vacuo*. The crude oil was purified by flash column chromatography to afford compound **4** (293.6 mg, 81% yield) as a white solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.63 (s, 1H), 8.20 (s, 2H), 7.93 (s, 1H), 7.53 (d, J = 6.5 Hz, 1H), 7.36−7.18 (m, 8H), 7.05 (t, J = 7.3 Hz, 1H), 4.92−4.88 (m, 1H), 4.62, 4.52 (AB_q, J_{AB} = 12.0 Hz, 2H), 3.99 (dd, J = 9.5, 6.1 Hz, 1H), 3.69−3.64 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.9, 164.6, 137.3, 137.0, 135.7, 132.4 (q, $^2J_{C,F}$ = 34.0 Hz), 129.2, 128.9, 128.5, 128.2, 127.7, 125.6, 125.0, 122.8 (q, $^1J_{C,F}$ = 273.0 Hz), 120.1, 74.0, 69.2, 53.4; ^{19}F (376 MHz, $CDCl_3$) δ −62.9; HRMS (ESI) for $C_{25}H_{21}F_6N_2O_3$ (M+H$^+$): calcd 511.1451, found 511.1465.



Scheme S5. Synthesis of compound **5**.

To a solution of *O*-methylhydroxylammonium chloride (16.7 mg, 0.20 mmol) in CH_2Cl_2 (2 mL) was added triethylamine (32.4 mg, 0.32 mmol) at 0°C. The reaction was stirred for 0.5 h, then 3,5-bis(trifluoromethyl)benzoyl chloride (60.8 mg, 0.22 mmol) was added dropwise. After being stirred overnight, the reaction mixture was diluted with CH_2Cl_2. The aqueous layer was extracted with CH_2Cl_2 for three times. The combined organic layer was dried over anhydrous Na_2SO_4 and was concentrated *in vacuo*. The crude oil was purified by flash column

chromatography to afford compound **5** (48.8 mg, 85%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 8.38 (s, 1H), 8.20 (s, 2H), 3.88 (s, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 163.8, 135.9, 133.4, (q, $^{2}J_{C,F}$ = 38.3 Hz), 128.9, 126.5, 124.7 (q, $^{1}J_{C,F}$ = 274.5 Hz), 64.6; ^{19}F (376 MHz, CD_3CN) δ −63.0; HRMS (ESI) for $C_{10}H_7F_6NO_2$ (M+H$^+$): calcd 288.0454, found 288.0460.



According to the same procedures of compound **2**, compound **6** (65%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.57 (s, 1H), 10.55 (s, 1H), 8.28 (s, 2H), 8.00 (s, 1H), 7.57 (d, *J* = 7.1 Hz, 2H), 7.29–7.25 (m, 2H), 7.10 (t, *J* = 7.3 Hz, 1H), 4.60 (s, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 169.1, 164.5, 139.0, 135.4, 133.2 (q, $^{2}J_{C,F}$ = 33.8 Hz), 129.9, 128.9, 126.3, 125.7, 124.5 (q, $^{1}J_{C,F}$ = 271.9 Hz), 121.3, 76.6; ^{19}F NMR (376 Hz, $CDCl_3$) δ −63.0; HRMS (ESI) for $C_{17}H_{13}F_6N_2O_3$ (M+H$^+$): calcd 407.0825, found 407.0842.

2. Ion transport assays and mechanism studies

2.1 General procedures

These procedures refer to the ion transport assays and mechanism studies in the main text. EYPC (egg yolk L-α phosphatidylcholine) was purchased from Avanti and was stored at –20°C in chloroform/methanol solution. Intravesicular and external solutions can vary for the different experiments (see caption of figures). Ion transport activities were monitored by fluorescent probes, and 5% Triton X-100 was used as a detergent.

Preparation of HPTS-loaded vesicles

Egg yolk L-α-phosphatidylcholine (EYPC, 25 mg/mL) solution (2 mL) was concentrated *in vacuo* and then dried under a high vacuum for at least 3 h. The lipid film was rehydrated in extravesicular buffer (0.65 mL), which contained 10 mM HEPES, 100 or 75 mM M_nX (M = Na^+, K^+; X = $Cl^−$, $SO_4^{2−}$), 0.1 mM 8-hydroxy-1,3,6-pyrenetrisulfonate (HPTS) buffered at pH 6.8, for 2 h. During rehydration, the suspension was subjected to 5 freeze-thaw cycles, in which the suspension was frozen in liquid nitrogen, followed by thawing in a water bath at room temperature. The suspension (0.5 mL) was then submitted to 25 high-pressure extrusions at room temperature through a 100 nm polycarbonate membrane. LUV suspension was formed with an average diameter of 100 nm and then passed through size exclusion chromatography (stationary phase: Sephadex G-50; mobile phase: buffer B containing 10 mM HEPES at pH 6.8

and 100 or 75 mM M_nX (M = Na^+, K^+; X = Cl^-, SO_4^{2-})) to remove extravesicular dye. The LUV suspension was diluted with elusion buffer (5 mL) to give a stock solution with a lipid concentration of 10 mM (assuming 100% of lipid was incorporated into liposomes) and stored at 4°C for less than 4 days.

Base-pulsed HPTS assay

HPTS-loaded LUV suspension (100 μL) prepared as described above was added in isotonic HEPES buffer (1.9 mL) and placed into a fluorometric cuvette. HPTS fluorescence was monitored with excitation at 403 and 460 nm, and emission at 510 nm. At t = 100 s, DMSO solution of the test compounds (20 μL) and 0.5 M NaOH/KOH aqueous solution (20 μL) were added through an injection port. Addition of the base caused about 1 pH unit increase in the extravesicular buffer. At t = 500 s, 40 μL of 5% Triton X-100 was added to lyse the liposomes. The fluorescence ratio of F_{460}/F_{403} of initial 100 s was set as 0% ion transport and the final fluorescence ratio of F_{460}/F_{403} induced by Triton X-100 was set as 100% ion transport. DMSO or other solvents as indicated was used as control.

$$F_{460}/F_{403} = I,$$

$$I_{\mathrm{rel}}\% = [(I_t - I_0)/(I_{\max} - I_0)] \times 100\%$$

Preparation of SPQ-loaded EYPC vesicles

Egg yolk L-α-phosphatidylcholine (EYPC, 25 mg/mL) solution (2 mL) was concentrated *in vacuo* and dried under high vacuum for at least 3 h. The lipid film was rehydrated in buffer A (0.65 mL), which contained 10 mM HEPES at pH = 6.8, 200 mM $NaNO_3$ and 0.5 mM SPQ, for 2 h. During rehydration, the suspension was subjected to 5 freeze-thaw cycles, in which the suspension was frozen in liquid nitrogen, followed by thawing in a water bath at room temperature. The suspension (0.5 mL) was submitted to high-pressure extrusion at room temperature for 25 extrusions through a 100 nm polycarbonate membrane. LUV suspension was formed with an average diameter of 100 nm, and then passed through size exclusion chromatography (stationary phase: Sephadex G-50; mobile phase: extravesicular buffer B contained 10 mM HEPES at pH = 6.8 and 200 mM NaCl)) to remove extravesicular dye. The LUV suspension was diluted with elusion buffer (5 mL) to give a stock solution with a lipid concentration of 10 mM (assuming 100% of lipid was incorporated into liposomes) and stored at 4°C for less than 4 days.

SPQ assay

SPQ-loaded vesicles (100 μL) prepared as described above were suspended in extravesicular HEPES buffer B (1.9 mL) contained 200 mM NaCl and placed into a fluorometric cuvette. SPQ fluorescence was monitored with excitation at 360 nm and emission at 430 nm. At 100 s, MeOH solution of ion transporters (20 μL) was added through an injection port. MeOH was used as control. The fluorescence decrease indicates the chloride influx. F_t = fluorescence intensity at time t, F_0 = the average fluorescence intensity before the addition of compounds.

Safranin O membrane potential assay

Liposomes encapsulated with 100 mM KCl and 10 mM HEPES buffered at pH 7.4 were prepared as described above. Liposomes (100 μL) were suspended in a buffer (1.9 mL) contained 100 mM NaCl or 100 mM KCl (for no potassium gradient), 100 nM safranin O and 10 mM HEPES buffered at pH 7.4. Safranin O fluorescence was monitored with excitation at 522 nm and emission at 581 nm. At t = 50 s, DMSO solution (20 μL) of the compound at the concentration of 5 μM was added through an injection port. F_t = fluorescence intensity at time t, F_0 = the average fluorescence intensity before the addition of the compound.

Preparation of carboxyfluorescein-loaded vesicles

Egg yolk L-α-phosphatidylcholine (EYPC, 25 mg/mL) solution (2 mL) was concentrated *in vacuo* and then dried under a high vacuum for at least 3 h. The lipid film was rehydrated in buffer A (0.65 mL) contained 10 mM HEPES at pH = 7.4, 100 mM NaCl, and 50 mM carboxyfluorescein (CF) for 2 h. During rehydration, the suspension was subjected to 5 freeze-thaw cycles, in which the suspension was frozen in liquid nitrogen, followed by thawing in a water bath at room temperature. The suspension (0.5 mL) was submitted to high-pressure extrusion at room temperature for 25 extrusions through a 0.1 μm polycarbonate membrane. LUV suspension was formed with an average diameter of 100 nm and then passed through size exclusion chromatography (stationary phase: Sephadex G-50; mobile phase: extravesicular buffer containing 10 mM HEPES at pH = 7.4 and 100 mM NaCl) to remove extravesicular dye. The LUV suspension was diluted with elusion buffers (5 mL) to give a stock solution with a lipid concentration of 10 mM (assuming 100% of lipid was incorporated into liposomes) and stored at 4°C for less than 4 days.

Carboxyfluorescein release assay

Typically, CF-loaded liposomes (100 μL) prepared as described above were suspended in a buffer (1.9 mL) contained 100 mM NaCl and 10 mM HEPES at pH = 7.4, and placed into a fluorometric cuvette. CF fluorescence was monitored with excitation at 492 nm and emission at 514 nm. At t = 100 s, 20 μL of DMSO solution of the ion transporters was added through an injection port. At the end of the experiment, 5% Triton X-100 (20 uL) was added to lyse the liposomes. The final emission intensities monitored at 514 nm were normalized to 100% of transport. The fractional CF release (I_{rel}) was calculated as follows: I_t = fluorescence intensity at time t, I_0 = the average of fluorescence intensity before the addition of compounds and I_{max} = fluorescence intensity after addition of detergent.

$$I_{rel} = (I_t - I_0)/(I_t - I_{max})$$

Transport behaviors in the presence of FCCP or valinomycin

In HPTS assay, the extravesicular addition of a controlled amount of base will result in pH gradient across the bilayer membrane, which drives the ion transporter to facilitate proton efflux or hydroxide influx to generate membrane potential. If the ion transporter can transport another cation or anion (H^+/M^+ or OH^-/A^- antiport and H^+/A^- or M^+/OH^- symport mechanisms are possible) to balance the membrane potential, the pH may continuously change.

Valinomycin is a natural potassium ionophore, which can form a cationic complex with K^+, and then passes through the lipid membrane, releases K^+ at the other interface (usually with lower K^+ concentration), and eventually, uncomplexed valinomycin travels back. FCCP is a proton transporter, which can only facilitate proton translocation in the lipid bilayers. In the conventional HPTS assay, the detected activity of H^+ transport rate of the test compound decreases with increased H^+ selectivity, because the rate of the disfavored M^+ influx limited the fluorescence change of HPTS. However, with valinomycin, a disfavored M^+ influx can be accelerated. Increasing activity in the presence of valinomycin identifies transporters with $H^+ > M^+$ selectivity. Alternatively, increasing activity in the presence of FCCP identifies transporters with $M^+ > H^+$ selectivity.

HPTS-loaded LUV suspension (100 μL) prepared as described above was added in isotonic HEPES buffer (1.9 mL) and placed into a fluorometric cell. HPTS fluorescence was excited at 403 and 460 nm alternatively, and the emission was monitored at 510 nm. At t = 50 s, DMSO

solution (20 μL) of the transporter, FCCP or valinomycin was added through an injection port, immediately followed by the addition of 0.5 M NaOH or KOH aqueous solution (20 μL). Addition of the base caused about 1 pH unit increase in the extravesicular buffer. At t = 250 s, 5% Triton X-100 (40 μL) was added to lyse the liposomes. The fluorescence of the initial 50 s was set as 0% ion transport and the final fluorescence change induced by Triton X-100 was set as 100% ion transport. DMSO was used as a control.

2.2 pK_a determination

The pK_a values of aminoxy acid NH of compounds **1** and **2** were calculated according to the reported method[2], by monitoring absorbance changes in the UV/Vis spectra as a function of variations in pH of phosphate buffer. Compounds dissolved in MeOH (10 mM) were added into phosphate buffer (0.1 M) with different pH values (from 2 to 11) to give the final concentration of 30 μM. The UV/Vis spectra were recorded in a CARY 50 bio UV/Visible spectrophotometer at 25°C. Standard 10 mm quartz glass cells were used. The pK_a values were determined from a plot of log (ionization ratio) vs pH (Eq.1). For compound **1**, the absorbance at 260 nm was used. AA- was take at pH = 11.01, AAH was take at pH = 2.22. For compound **2**, the absorbance at 230 nm was used. AA- was take at pH = 11.01, AAH was take at pH = 2.22.

A: Absorbance at each pH

AA^-: Absorbance of the deprotonated form

AAH: Absorbance of the protonated form

$$\log [(A - AA^-)/(AAH - A)] = -pH + pKa \quad \text{(Eq. 1)}$$

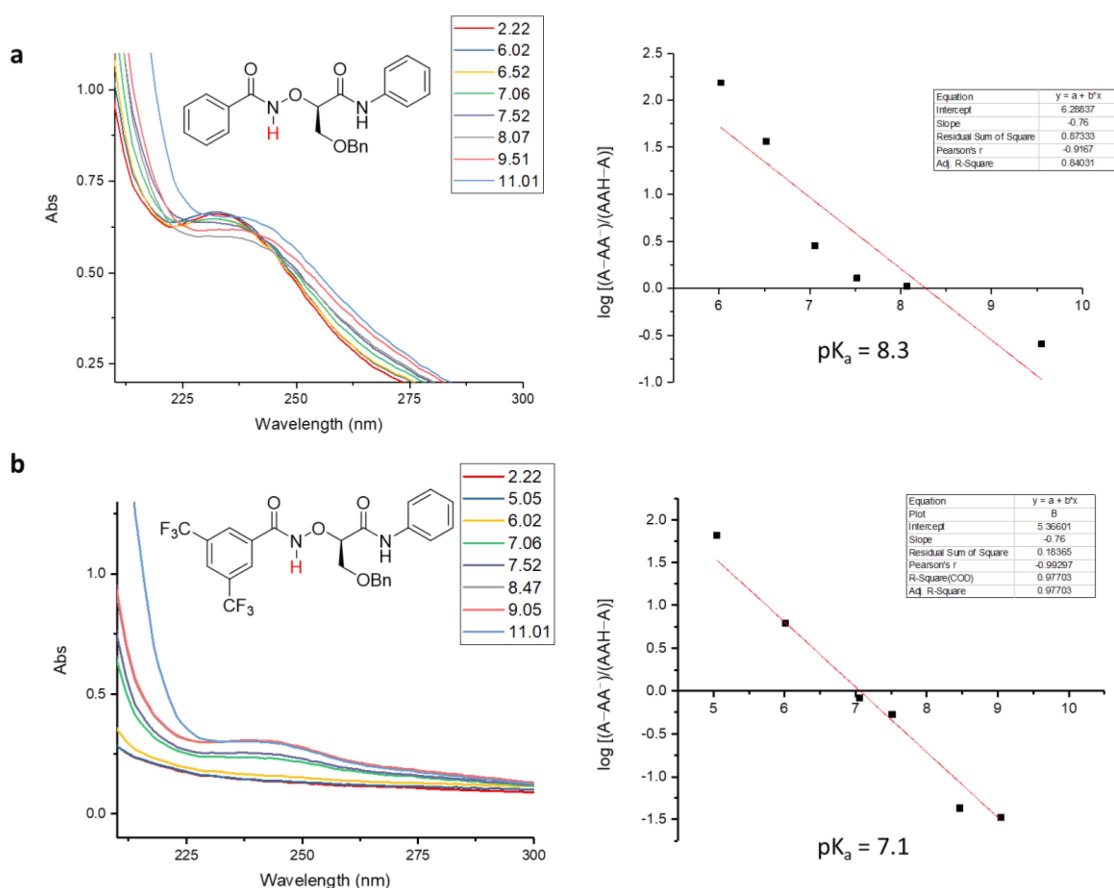


Figure S1. UV/Vis spectra and log(ionization ratio) vs pH for **a**, compound **1** and **b**, compound **2** as a function of pH in 0.1 M phosphate buffer at 25 °C.

2.3 ^{39}K NMR studies

The stoichiometry of ion complex between K^+ and compound **2** was determined according to the literature reported procedures[3]. In brief, LUVs with a diameter of 1 μm were prepared with the same procedure described above. Both intravesicular and extravesicular buffers contained 10 mM HEPES pH = 7.4 and the same concentration of K^+ (75 mM, 100 mM and 125 mM). The intravesicular anion was Cl^- and the external anion was triphosphate ($pppi^{5-}$). NMDG chloride was added in the extravesicular buffer to balance the ionic concentration on both sides of the membrane. $Tb(NO_3)_3$ at the final concentration of 5 mM was added to the extravesicular buffer to create a chemical shift difference between intravesicular and extravesicular K^+. Lifetimes T was calculated from the equation $1/T$ = (line broadening) × л. The line broadening was calculated from the observed linewidth of inner K^+ in the presence of compound **2** minus the original linewidth. As shown in Figure S3, experiments with liposomes loaded different concentrations of KCl (75 mM, 100 mM and 1501 mM) were conducted. For every

concentration of K^+ studied, $1/T$ varies linearly with compound to lipid ratio [L] ([compound **2**] : [EYPC]), which indicated the first-order rate constant for K^+ efflux $1/T = k[L]$. This linear relationship confirmed the 1:1 complex between K^+ and compound **2**.

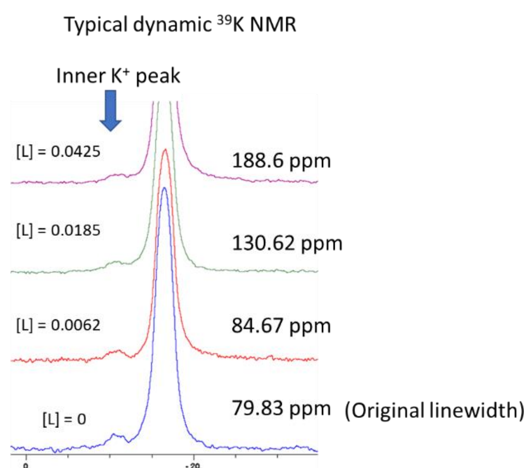


Figure S2. Representative dynamic ^{39}K NMR spectra. Liposomes containing 75 mM KCl was used. [L] = [compound **2**]/[lipid].

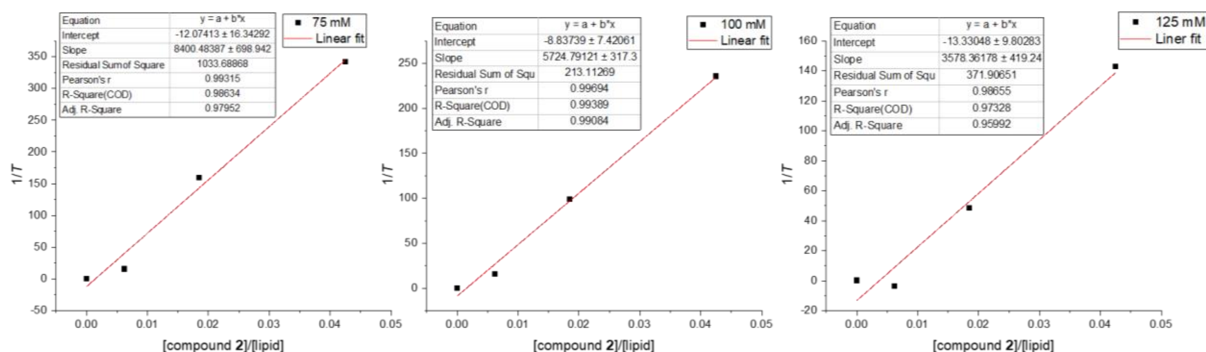


Figure S3. Graph of $1/T$ vs [L], LUVs encapsulated 75, 100 and 125 mM of K^+ were used.

2.4 K^+/Na^+ selectivity studies

EYPC liposomes (100 μL) prepared according to the procedures described above were immersed into extravesicular solution (1.9 mL) containing 75 mM K_2SO_4 (for K^+ transport) or 75 mM Na_2SO_4 (for Na^+ transport). The sensitivity of transporters to extravesicular cation indicates their ability to differentiate potassium and sodium ions. The intravesicular solution contained 0.1 mM HPTS, 10 mM HEPES, and 75 mM K_2SO_4, buffered at pH 6.8; the extravesicular solution contained 10 mM HEPES at pH 6.8, 75 mM K_2SO_4 (blue) or Na_2SO_4 (Red). At t = 100 s, compound **2** or DMSO (as blank) was injected into extravesicular buffer, immediately followed by the addition of KOH or NaOH to trigger a pH gradient. Liposomes

were lysed at 500 s with 5% Triton X-100 to measure the maximum fluorescence change. Hill equation $Y = 1/(1 + (EC_{50}/[C])^n)$ was employed to calculate EC_{50} and Hill coefficient n for the transport of potassium and sodium ions of synthetic ion transporters. Y was calculated for each curve using the normalized value of I_{460}/I_{403}, from 0 (ratio for the blank) to 1 (highest ratio obtained, i.e. a plateau). C is the concentration of transporter used. Experimental data were analyzed by a non-linear least-square fitting method[4].

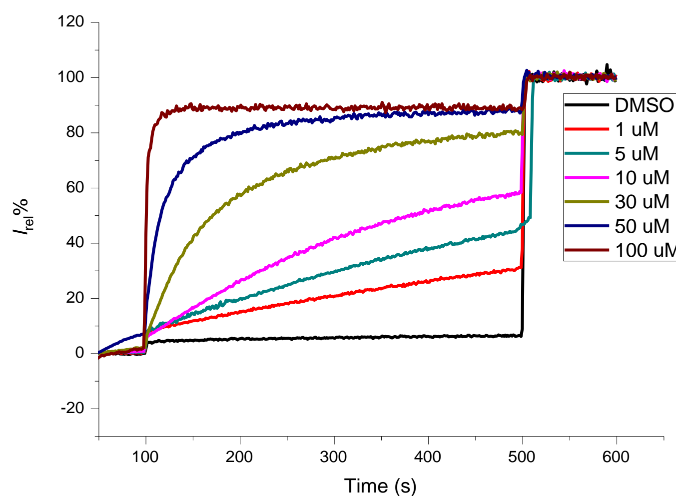


Figure S4. K^+ and H^+/OH^- transport induced by compound **2** at the final concentration of 1, 5, 10, 30, 50, and 100 μM. The intravesicular solutions contained 0.1 mM HPTS, 75 mM K_2SO_4 and 10 mM HEPES, buffered at pH 6.8. And extravesicular solutions contained 75 mM K_2SO_4 and 10 mM HEPES, buffered at pH 6.8. At 100 s, 20 μL of DMSO (as blank) or DMSO solution of compound **2** was injected into extravesicular buffer, immediately followed by the addition of KOH to trigger a pH gradient. Liposomes were lysed at 500 s with 5% Triton X-100 to measure maximum fluorescence change.

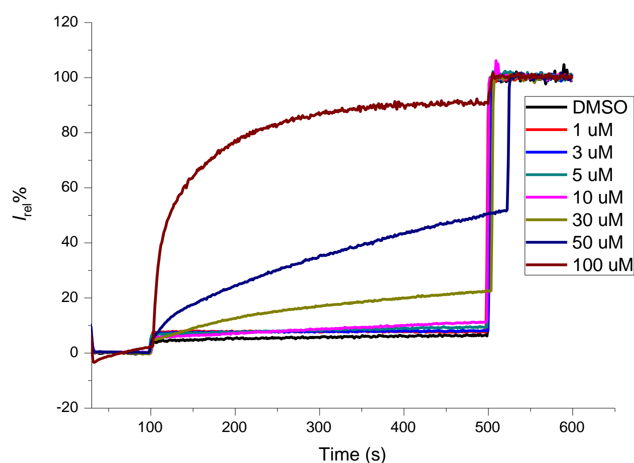


Figure S5. Na^+ and H^+/OH^- transport induced by compound **2** at the final concentration of 1, 3, 5, 10, 30, 50, and 100 μM. The intravesicular solutions contained 0.1 mM HPTS, 75 mM K_2SO_4 and 10 mM HEPES, buffered at pH 6.8. And extravesicular solutions contained 75 mM Na_2SO_4 and 10 mM HEPES, buffered at pH 6.8. At 100 s, 20 uL of DMSO (as blank) or DMSO solution of compound **2** was injected into extravesicular buffer, immediately followed by the addition of NaOH to trigger a pH gradient. Liposomes were lysed at 500 s with 5% Triton X-100 to measure maximum fluorescence change.

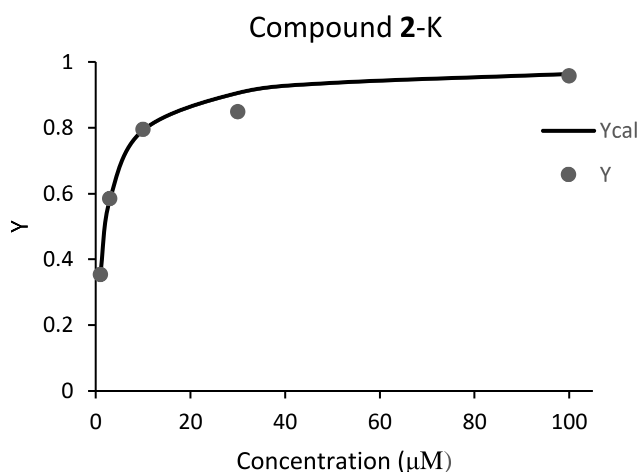


Figure S6. Fractional activity $Y = 1/(1 + (EC_{50}/[C])^n)$ to calculate EC_{50} and Hill coefficient n for the transport of K^+ cations of compound **2**: $EC_{50} = 2.02$ μM, n = 0.84. Solid circles (•) are experimental data, and the curves are the best-calculated fit from the Hill equation.

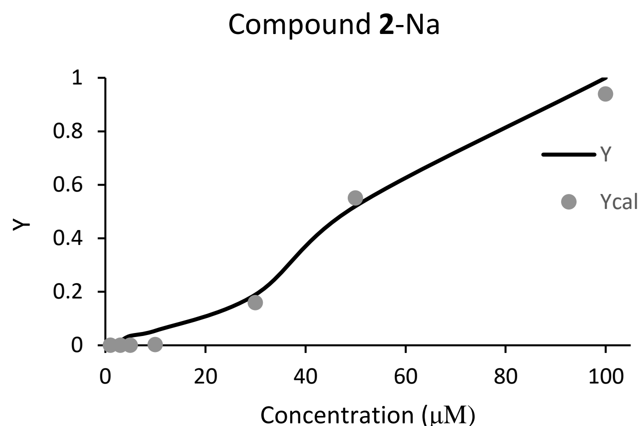


Figure S7. Fractional activity $Y = 1/(1 + (EC_{50}/[C])^n)$ to calculate EC_{50} and Hill coefficient n for the transport of Na^+ cations of compound **2**: EC_{50} = 47.26 µM, n = 3.66. Solid circles (•) are experimental data, and the curves are the best-calculated fit from the Hill equation.

The K^+/Na^+ selectivity of compound **2** was also determined by the ratio of the fractional ion transport activity (R_M^+) at a concentration where the K^+ transport activity reaches its highest capacity[5-6]. In brief, EYPC liposomes (100 µL) prepared according to the procedures described above were immersed into extravesicular solution (1.9 mL) containing 75 mM K_2SO_4 (for K^+ transport) or 75 mM Na_2SO_4 (for Na^+ transport). At t = 100 s, compound **2** at the final concentration of 15 µM or DMSO (as blank) was injected into extravesicular buffer, immediately followed by the addition of KOH or NaOH to trigger a pH gradient. Liposomes were lysed at 1100 s with 5% Triton X-100 to measure the maximum fluorescence change. Y was calculated for each curve using the normalized value of I_{460}/I_{403}. The fractional change R_M^+ was calculated for each curve using Y at 1100 s before the addition of Triton X-100, with the ratio of blank that contains only DMSO normalized to 0.

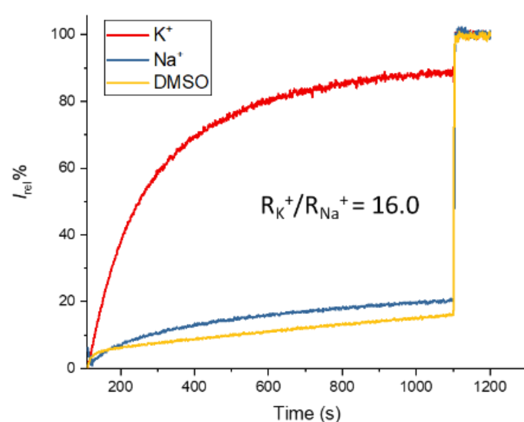


Figure S8. Ratio of fractional transport activities R_K^+ / R_{Na}^+ of compound **2**.

2.5 Selectivity study towards some other alkali metal cations

The selectivity of compound **2** towards some other alkali metal cations was also evaluated using HPTS assay, in which the intravesicular solution contained 100 mM NaCl and the extravesicular solution contained 100 mM MCl (M = Li, Na, K, and Cs). Both ion transport is driven by concentration gradient (before t = 200 s) or by pH gradient (after addition of 20 μL of 0.1 N base at t = 200 s) were investigated. At t = 30 s, 20 μL of DMSO solution of compound **2** was added, and at t = 200 s, 20 μL of 0.1 M MOH (M = Li, Na, K, Cs) was added to generate a pH gradient. Liposomes were lysed by the addition of Triton X-100 at t = 500 s.

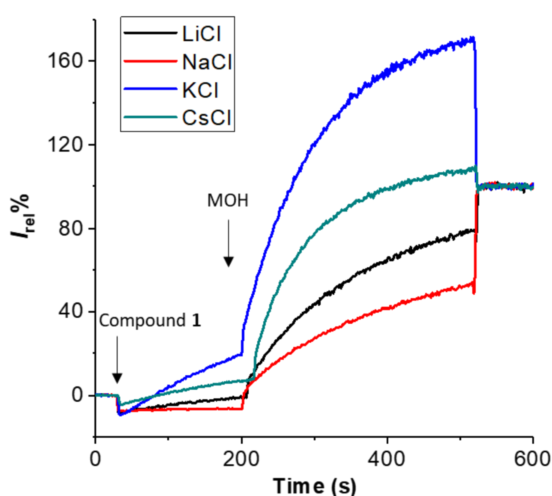


Figure S9. The ion selectivity of compound **2** towards some other alkali metal cations at the concentration of 10 μM. Liposomes loaded with 100 mM NaCl and 0.1 mM HPTS were suspended in 100 mM MCl solutions (M = Li, Na, K and Cs).

2.6 Carboxyfluorescein release study

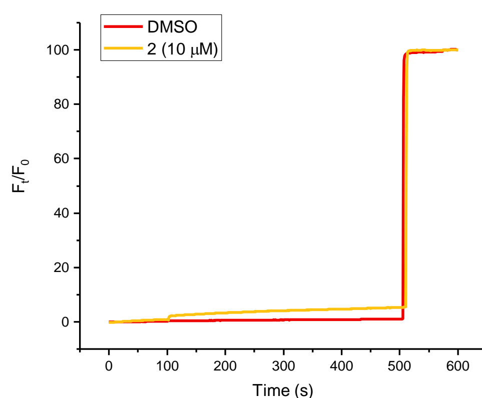


Figure S10. Carboxyfluorescein release assay of compound **2**.

2.7 EC_{50} calculation of proton transport activities

Valinomycin is a highly selective potassium ionophore. Here, in the presence of valinomycin, the proton transport activities of compound **2** at different doses in liposome assays were evaluated. As valinomycin can facilitate K^+ transport for compensation, that compounds can show their maximum proton transport capabilities.

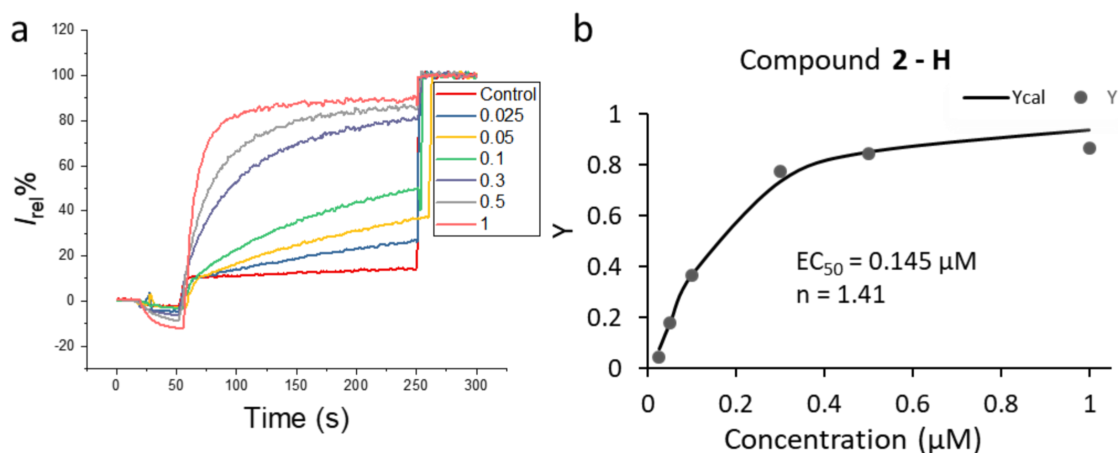


Figure S11. **a**, Proton transport activity induced by compound **2** in the presence of 25 nM Val. The intravesicular solutions contained 0.1 mM HPTS, 75 mM K_2SO_4 and 10 mM HEPES, buffered at pH 6.8; and extravesicular solutions contained 75 mM K_2SO_4 and 10 mM HEPES, buffered at pH 6.8. At t = 30 s, 20 μL of 0.5 M KOH aqueous solution and 20 μL of DMSO (as blank) or DMSO solution of compound **2** were injected into extravesicular buffer. Liposomes were lysed at 500 s with 5% Triton X-100 to measure maximum fluorescence change. **b**, Fractional activity $Y = 1/(1 + (EC_{50}/[C])^n)$ to calculate EC_{50} and Hill coefficient n for the transport of H^+. Solid circles (•) are experimental data, and the curves are the best-calculated fit from the Hill equation. EC_{50} = 0.15 μM, n = 1.41.

3. Biological studies

3.1 Reagents and antibodies

Reagents. CellTiter-Glo® Luminescent Cell Viability Assay (CellTiter reagent, G7570, Promega), APG-2AM (Potassium probe, Asante Potassium Green-2m, TEF labs), DiSBAC2(3) (Bis-(1,3-Diethylthiobarbituric Acid)Trimethine Oxonol, B413, ThermoFisher), SNARF (5-(and-6)-Carboxy SNARF™-1, Acetoxymethyl Ester, Acetate, C1272, ThermoFisher), Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 & Propidium Iodide (PI) (V13245,

ThermoFisher), JC-1 (Mitochondrial membrane potential probe, T3168, ThermoFisher), MitoTracker™ Green FM (MTG, M7514, ThermoFisher), LysoTracker™ Red DND-99 (LTR, L7528, ThermoFisher), Acridine Orange (AO, A6014, Sigma Aldrich), Valinomycin (V0627, Sigma Aldrich), Nigericin (N1495, ThermoFisher), Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, C2920, ThermoFisher), Oligomycin (75351, ThermoFisher), Antimycin A (A0274, ThermoFisher) and Rotenone (R8875, Sigma Aldrich).

Antibodies. PE-conjugated CD133 (Clone: AC133, Miltenyi Biotec), PARP1/2 (sc-7150, Santa Cruz Biotec), Caspase 9 (#9520, Cell Signaling Technology), Caspase 3 (ab4051, Abcam), β-tubulin (#2146, Cell Signaling), P62 (sc-28359, Santa Cruz Biotec), LC3B (#3868, Cell Signaling Technology), GAPDH (#2118, Cell Signaling Technology), COX IV (#4844, Cell Signaling Technology).

3.2 Methods for cell cultures and biological experiments

Cell cultures

Human ovarian carcinoma cell lines HEYA8 and SKOV3 were grown in Dulbecco's Modified Eagle (DMEM) medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Isolation and culture of spheres were performed in serum-free stem-cell-selective conditions as previously described[7]. Briefly, 1–2 weeks after plating, non-adherent spherical clusters of cells could be observed and were separated from single cells by low-speed centrifugation. After 8th to 10th passages, the non-adherent spherical clusters of cells appeared as distinct spheres. Using this selection condition, HEYA8 spheres (HEYA8 CSCs) could be enzymatically dissociated and reformed into spheres within 3 days under the stem-cell-selective condition as described in literature[8]. To allow differentiation, dissociated sphere cells were plated on tissue culture plates in medium (MCDB 105: M199 = 1:1) supplemented with 10% FBS and 1% penicillin-streptomycin.

HeLa cells (cervical cancer cells), NIH3T3 cells (mouse fibroblast cells) and HEK293 cells (human embryonic cells) were grown in DMEM medium with high glucose supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. MDCK cells (Madine Darby Canine kidney cells) were grown in the EMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Statistics

All data were mean ± s.e.m., unless otherwise noted. Statistical analysis represents *p* values obtained from one-way ANOVA or two-sided unpaired Student's t-test where necessary, ns, not significant; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Measurement of cell viability

Cells were plated in triplicate in 0.1 mL full medium in 96-well plates for 24 h. After that, the medium was changed to the freshly prepared medium with various concentrations of test compounds. Cells were incubated for another 48 h. Then the cell viability was measured by CellTiter-Glo® Luminescent Cell Viability reagent according to the manufacture's instruction. The luminescence at 550 nm was measured using a microplate reader (DTX 880 Multimode Detector, Beckman Coulter). Data were analyzed by GraphPad software 7.00.

Measurement of CSCs viability

HEYA8 or SKOV3 CSCs (5×10^4 cells/mL) were seeded in triplicate in 10 mL serum-free MCDB105/M199 = 1/1 medium in 100-mm Petri dish for 7 days to form spheres. Then, test compounds at different concentrations were added. The cells were further incubated at 37°C for 48 h. After that cells were collected by centrifugation and the medium was removed. Then 100 μL of CellTiter-Glo® Luminescent Cell Viability reagent was added into each tube, which was incubated for 10 min with shaking. After that, the reagent was transferred into 96-well plates and cell viability was measured using a microplate reader (DTX 880 Multimode Detector, Beckman Coulter).

Measurement of the population of $CD133^+$ cells by FACS

HEYA8 cells (2.5×10^4 cells/mL) were seeded into 60-mm cell culture dishes in DMEM medium. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h, followed by the addition of 4 mL fresh medium or medium containing Paclitaxel (100 nM) or compound **2** (15 μM). Medium containing 0.1% DMSO was used as a negative control. After 3 days, the medium was removed and cells were washed with cold PBS twice, and then stained with PE-conjugated CD133 antibody according to the manufacturer's instruction. The fluorescence was analyzed by flow cytometry.

Measurement of the sphere-forming ability

Dissociated HEYA8 CSCs at the density of 2.5×10^4 cells/mL were grown in cell culture dishes with full medium and treated with DMSO or compound **2** at the indicated concentrations for 48 h. Then cells were allowed to recover and grow in fresh medium for 3 days. After that, cells at the density of 5×10^4 cells/mL were transferred into low attachment dishes in sphere-forming conditions for 5 days. The number of spheres in each dish was counted.

Measurement of the *in vivo* tumor-forming ability

All animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) at the University of Hong Kong. *In vivo* tumor-forming ability of cells after treatment with compound **2** was evaluated. Paclitaxel was used as a negative control. For this experiment, HEYA8 CSCs were pretreated with **2** (4 μM) or paclitaxel (0.1 μM) for 2 days in a suspension medium, respectively. Then cells were allowed to proliferate in full medium in the absence of drugs for 10 days. After that, 10^6 cells were subcutaneously (s.c.) injected into the flank of athymic nude female mice bilaterally. The length and width of a tumor were measured with a caliper for 25 days after injection and tumor volume was calculated as Tumor volume $V = (\pi \times L \times W^2)/6$, where L represents the largest tumor diameter and W represents the perpendicular tumor diameter.

Measurement of the *in vivo* tumor-size after treatment of compound 2

For this experiment 10^6 HEYA8 cells in PBS buffer were subcutaneously (s.c.) injected into the flank of athymic nude female mice. Compound **2** treatment was initiated with intraperitoneal injection (i.p.) 8 days after tumor seeding. Animals were administered with PBS buffer or compound **2** at the dosage of 5 mg/kg three days a week. The length and width of a tumor were measured with a caliper for 18 days after injection and tumor volume was calculated as Tumor volume $V = (\pi \times L \times W^2)/6$, where L represents the largest tumor diameter and W represents the perpendicular tumor diameter.

Western blotting

Cells were treated as indicated, then washed twice with PBS buffer and lysed with lysis buffer (0.1% Triton X-100, 150 mM NaCl, 0.1 U Benzonase, Roche EDTA-free protease inhibitor cocktail, 20 mM Tris-HCl, pH 8.0). Protein extracts were quantified by Pierce BCA Protein Assay Kit with a Nanodrop 2000 (Thermo Fisher Scientific) according to the manufacturer's instruction. Protein lysates (~ 50 µg/lane) were resolved by hand-cast SDS-PAGE and

transferred onto poly(vinylidene difluoride) (PVDF) membranes. Membranes were incubated with the membrane blocking solution (Thermo Fisher Scientific) for 1 h. The blots were then probed with the relevant primary antibodies in blocking solution at 4°C overnight with gentle agitation. Membranes were washed 5 min with 0.1% Tween 20/TBS for three times and were incubated with Horseradish Peroxidase (HRP) conjugated secondary antibody for 1 h at room temperature. Antigens were detected by SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). The membranes were visualized by chemiluminescent mode of ThermoFischer myECL™ Imager.

Measurement of plasma membrane potential by FACS

HEYA8 cells at the density of 5×10^5 cells/mL in DMEM medium were seeded into 60-mm cell culture dishes. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. After washing with PBS twice, the cells were incubated with 0.5 mL of trypsin-EDTA (0.05% trypsin, 0.02% EDTA, Sigma-Aldrich) for 5−10 min at 37°C and collected. Cells were re-suspended in HBSS buffer with *bis*-oxonol (Bis-(1,3-Diethylthiobarbituric Acid) Trimethine Oxonol) (Thermo Fisher Scientific) at the final concentration of 200 nM and incubated at 37°C for 30 min. Cells were centrifuged and washed with PBS buffer, and re-suspended in 0.5 mL HBSS buffer containing 0.1% DMSO or compound **2** (10 μM) 30 min before analysis by flow cytometry (BD FACSCanto II Analyzer). The fluorescence was measured using an excitation wavelength of 488 nm and an emission filter (530/30 nm). Quantitative data were obtained using FlowJo and GraphPad Prism software.

Measurement of cytosolic potassium concentration

HEYA8 cells at the density of 5×10^5 cells/mL in DMEM medium were seeded into 60-mm cell culture dishes. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. After washing with PBS twice, the cells were incubated with 0.5 mL of trypsin-EDTA (0.05% trypsin, 0.02% EDTA, Sigma-Aldrich) for 5−10 min at 37°C and collected. Cells were re-suspended in HBSS buffer with K^+ probe APG-2AM at the final concentration of 5 μM and incubated at 37°C for 30 min. Cells were centrifuged and washed with PBS buffer, and re-suspended in 0.5 mL HBSS buffer containing 0.1% DMSO or compound **2** (10 μM) 30 min before analyzed by flow cytometry. The fluorescence was measured using an excitation wavelength of 488 nm. Quantitative data were obtained using FlowJo and GraphPad Prism software.

Mito-SypHer transfection

Cells at the density of 10^5 cells/mL in DMEM medium were plated into 30-mm cell culture dishes with a glass-bottom (MatTek). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Cells were transfected with 2 μg plasmid DNA containing Mito-SypHer genes and 6 μL X-tremeGeneHP transfection reagent for 24 h.

Measurement of mitochondrial pH

Cells transfected with Mito-SypHer were washed with PBS buffer twice, and 0.8 mL HBSS buffer was added. The fluorescence of Mito-SypHer was monitored by a confocal microscope (ZEISS LSM 780). During the kinetics studies, Mito-SypHer was alternately excited at 405 nm and 488 nm. At t = 210 s, 0.2 mL HBSS buffer containing 0.1% DMSO or compound 2 (10 μM) was added. Fluorescence ratios (I_{488}/ I_{405}) were calculated in ZEN software and analyzed in Excel (Microsoft) and GraphPad Prism software.

Measurement of matrix potassium concentration

SKOV3 cells at the density of 6×10^4 cells/mL in DMEM medium were seeded into 30-mm cell culture dishes with a glass-bottom (MatTek). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. After that cells were washed with PBS buffer twice. Mitochondrial potassium probe KS6 was diluted with 10% (weight/volume) Pluronic F127 (1:1) and added to HBSS buffer at 1:1000 dilution. Cells were incubated in HBSS containing KS6 at the final concentration of 5 μM for 1 h. Then the loading buffer was aspirated, and cells were washed twice with PBS buffer. Cells were imaged in 0.8 mL HBSS buffer using a confocal microscope (ZEISS LSM 780). Potassium probe KS6 was excited at 561 nm. Synthetic cation transporter **2** dissolved in 0.2 mL HBSS buffer was added during the experiment at given time points and fluorescence images were taken. Quantitative data were obtained using the ZEN and GraphPad Prism software packages.

Measurement of mitochondrial membrane potential by confocal imaging

HEYA8 cells at the density of 5×10^4 cells/mL in DMEM medium were seeded into 30-mm cell culture dishes with a glass-bottom (MatTek). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. The medium was aspirated and cells were washed with PBS buffer twice. Then 1 mL HBSS buffer containing 2.5 μM JC-1 was added. Cells were incubated at 37°C for 20 min before the buffer was aspirated. Cells were washed with PBS buffer twice and treated with 0.1% DMSO or compound 2 at indicated concentrations for 10

min. Fluorescence was monitored by confocal imaging with ZEISS LSM 780 (Red channel: λ_{ex} = 543 nm, Green channel: λ_{ex} = 488 nm). Quantitative data were obtained using the ZEN and GraphPad Prism software packages.

FACS analysis of CSCs

After obtained in serum-free medium from ovarian cancer cell lines, spheres were collected by centrifugation. The medium was aspirated and spheres were washed with PBS buffer twice. After that, spheres were trypsinized to afford single cells and washed with PBS buffer. Then cells were incubated with indicated fluorescent probes in HBSS buffer. After staining, cells were centrifuged again and 0.5 mL cold PBS was added. After pipetting up and down, the single-cell suspension was analyzed by FACS (BD FACSCanto II Analyzer).

Measurement of mitochondrial ROS production

HEYA8 cells at the density of 5×10^4 cells/mL in DMEM medium were seeded into 30-mm cell culture dishes with a glass-bottom (MatTek). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. The medium was aspirated and cells were washed with PBS buffer twice. Then 1 mL HBSS buffer containing HKSOX-2m in DMF (4 μM) was added. Cells were incubated at 37°C for 30 min before the buffer was aspirated and washed twice with HBSS buffer. Cells were imaged in 0.8 mL HBSS buffer. Fluorescence was monitored by a confocal microscope (ZEISS LSM 710; Red channel: λ_{ex} = 543 nm). At t = 180 s, 0.1% DMF or compound **2** in 0.2 mL HBSS buffer was added to give the final concentration as indicated in Figure 4a. Quantitative data were obtained using the ZEN and GraphPad Prism software.

Observation of mitochondrial morphology and fusion of lysosomes and mitochondria

HEYA8 cells at the density of 5×10^4 cells/mL in DMEM medium were seeded into 30-mm cell culture dishes with a glass-bottom (MatTek). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. The medium was aspirated and cells were washed with PBS buffer twice. Then 1 mL HBSS buffer containing 100 nM MitoTracker Green and 50 nM LysoTracker Red was added. Cells were incubated at 37°C for 30 min. After that, the buffer was aspirated, and cells were washed twice with HBSS buffer. Then cells were treated with 0.1% DMSO or compound **2** (10 μM) for 1 h. After washing with HBSS buffer once, fluorescence was monitored by confocal imaging on ZEISS LSM 700 (Green channel λ_{ex} = 488 nm, Red channel λ_{ex} = 543 nm). Images were further processed by Zen software.

Oxygen consumption assay

Cell respiration was measured by using an XF24 Extracellular Flux Analyzer (Seahorse, Bioscience), which measures the oxygen consumption rate (OCR). Adherent HEYA8 cells were seeded at 50000 cells/well in 200 µL of their culture medium and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. The medium was then replaced with 670 µL/well of high-glucose DMEM without serum and supplemented with 1 mM sodium pyruvate and 2 mM L-glutamine. The oxygen consumption rate (OCR) was measured with an extracellular flux analyzer (Seahorse) at preset time intervals upon the preprogrammed additions of the following compounds: Oligomycin to 1 µM, FCCP to 500 nM, Antimycin A and Rotenone to 0.5 µM final concentrations.

Annexin V and PI analysis of apoptotic cells by FACS

HEYA8 cells at the density of 5×10^4 cells/mL in DMEM medium were seeded into 60-mm cell culture dishes. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h followed by the addition of 4 mL fresh medium or medium containing compound **2** at various concentrations. Medium containing 0.1% DMSO was used as a negative control. After 2 days, the medium was aspirated. Cells were washed with cold PBS twice, and then stained with PI and Annexin V according to the manufacturer's instruction (Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 & Propidium Iodide, Thermo Fisher), and analyzed immediately by flow cytometry (BD FACSCanto II Analyzer).

Acridine orange (AO) assay

Cells were treated with DMSO or compound **2** for 1 h prior to treatment with a 2 µg/mL Acridine Orange solution for 20 min (37°C, 5% CO_2), then washed with PBS. Images were obtained using an LSM 700 confocal microscope equipped with an argon laser (ex. 488 nm). AO produces red fluorescence (emission peak at about 650 nm) in the lysosomal compartments and green fluorescence (emission peak between 530 and 550 nm) in the cytosolic and nuclear compartments.

3.3 Transport activities across plasma membranes

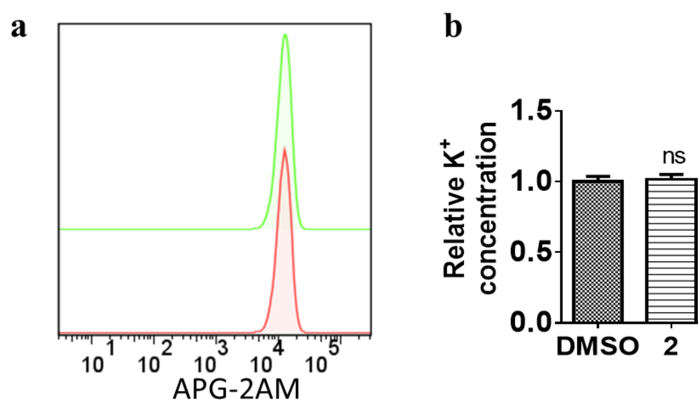


Figure S12. a, Representative result of cytosolic potassium concentration in HEYA8 cells measured by flow cytometry. Cells were treated with compound **2** at the concentration of 10 μM for 10 min. The fluorescence of APG-2AM was analyzed with an excitation wavelength of 488 nm and an emission filter (530/30 nm). Three independent experiments were repeated. **b**, Quantification results of cytosolic potassium concentration in cells treated with DMSO or compound **2** (mean ± s.e.m., n = 3).

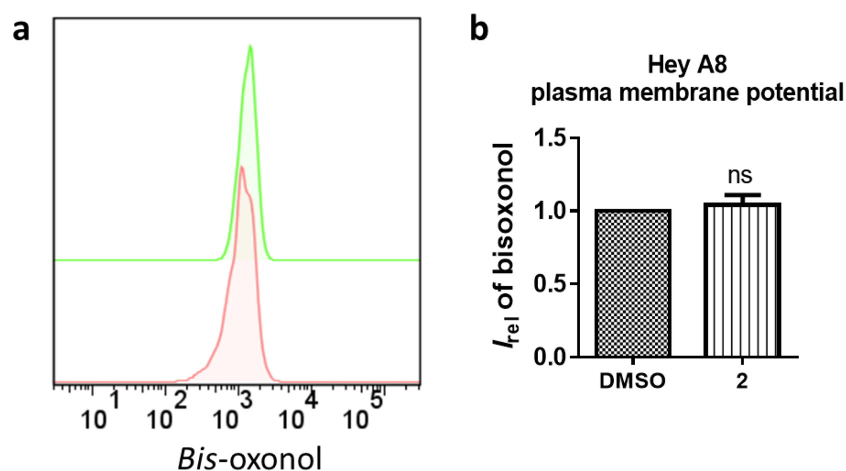


Figure S13. a, Representative result of HEYA8 cells plasma membrane potential measured by flow cytometry. Cells were treated with compound **2** at the concentration of 10 μM for 30 min. The fluorescence of *bis*-oxonol was analyzed with an excitation wavelength of 488 nm and an emission filter (530/30 nm). Three independent experiments were repeated. **b**, Quantification results of plasma membrane potential in HEYA8 cells treated with DMSO or compound **2** (mean ± s.e.m., n = 3).

3.4 Transport activities across the mitochondria inner membranes

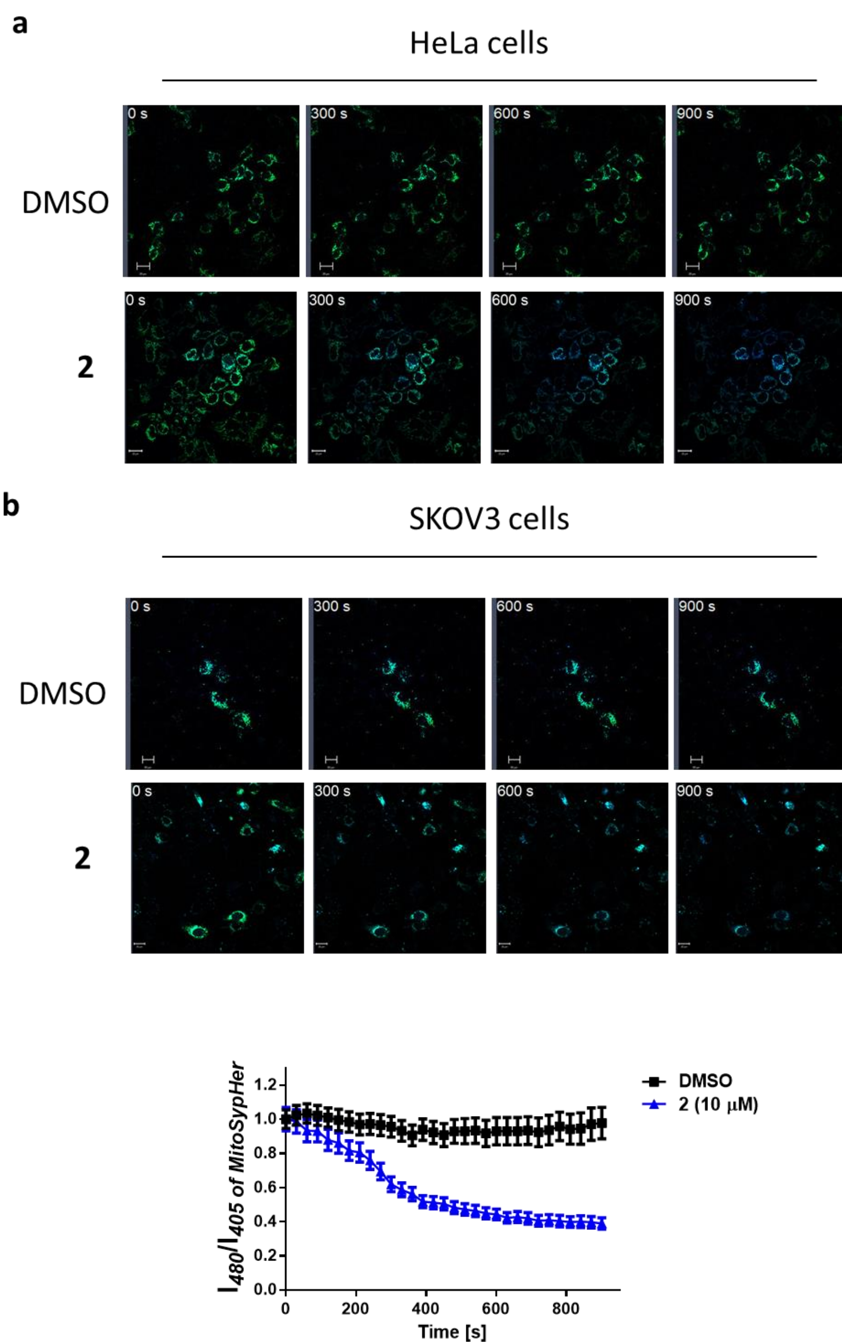


Figure S14. a, Fluorescence confocal images of Mito-SypHer showing the mitochondrial matrix pH levels in HeLa cells cells after treated with compound **2** (10 μM) for 10 min. **b**, Fluorescence confocal images and quantification results of Mito-SypHer showing the mitochondrial matrix pH levels in SKOV3 cells after treated with compound **2** (10 μM) for 10 min. of matrix pH (mean ± s.e.m., n = 20 − 30 cells). Images were obtained from kinetics experiments. Scale bar: 20 μm.

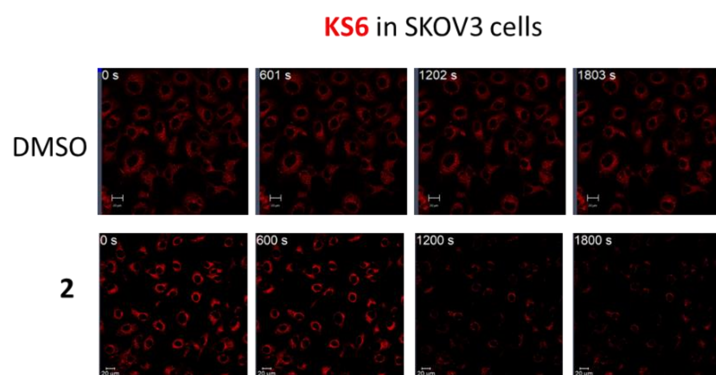


Figure S15. Fluorescence confocal images of KS6 showing the mitochondrial matrix K^+ concentration in SKOV3 cells after treated with compound **2** (10 µM) for 10 min. Images were obtained from kinetics experiments. Scale bar: 20 µm.

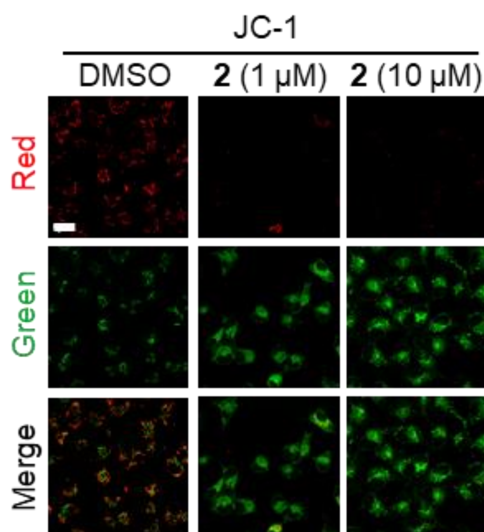


Figure S16. Fluorescence confocal images of JC-1 showing the mitochondrial inner membrane potential in HEYA8 cells after treated with compound **2** (10 µM) for 10 min. Images were obtained from kinetics experiments. Scale bar: 20 µm.

3.5 Mitochondrial superoxide production

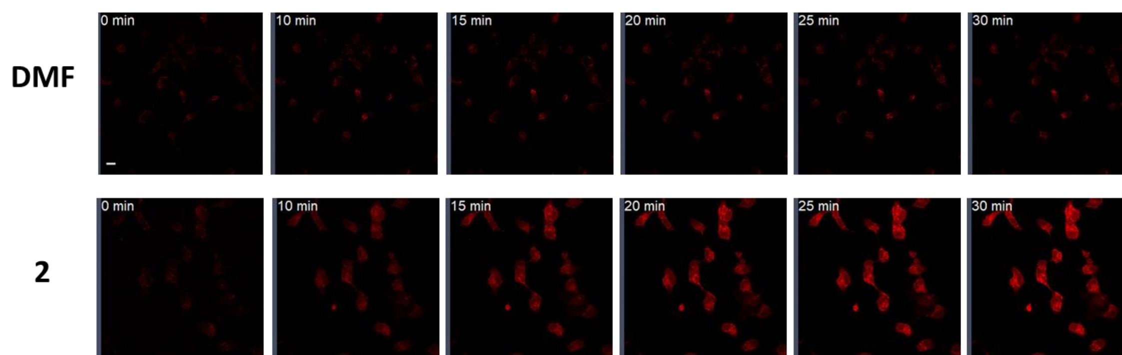


Figure S17. Fluorescence confocal images of HKSOX-2m probe showing the mitochondria superoxide production of HEYA8 cells after treatment with 0.1% DMF or compound **2** (5 µM) for 30 min. Scale bar: 20 µm.

3.6 Characteristics of mitochondria in cancer stem cells

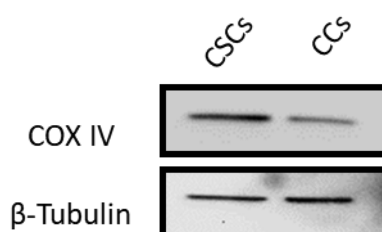


Figure S18. Immunoblotting of COX IV as a protein marker for mitochondria. β-Tubulin was used as the loading control. The upregulated COX IV suggested the higher mitochondrial mass of CSCs than adherent cancer cells (CCs).

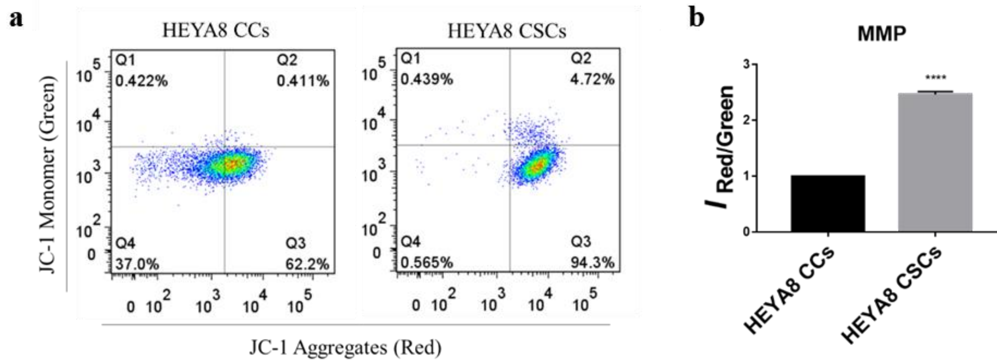


Figure S19. a, CSCs and adherent cancer cells (CCs) were stained with mitochondria membrane potential probe JC-1 and analyzed by FACS. **b**, Quantitative results from **a** (mean ± s.e.m., n = 3, **** $p < 0.0001$. The higher ratio of red/green fluorescence indicated the hyperpolarized mitochondrial membrane potential of CSCs.

3.7 Effects on the mitochondrial membrane potential of CSCs and adherent cancer cells

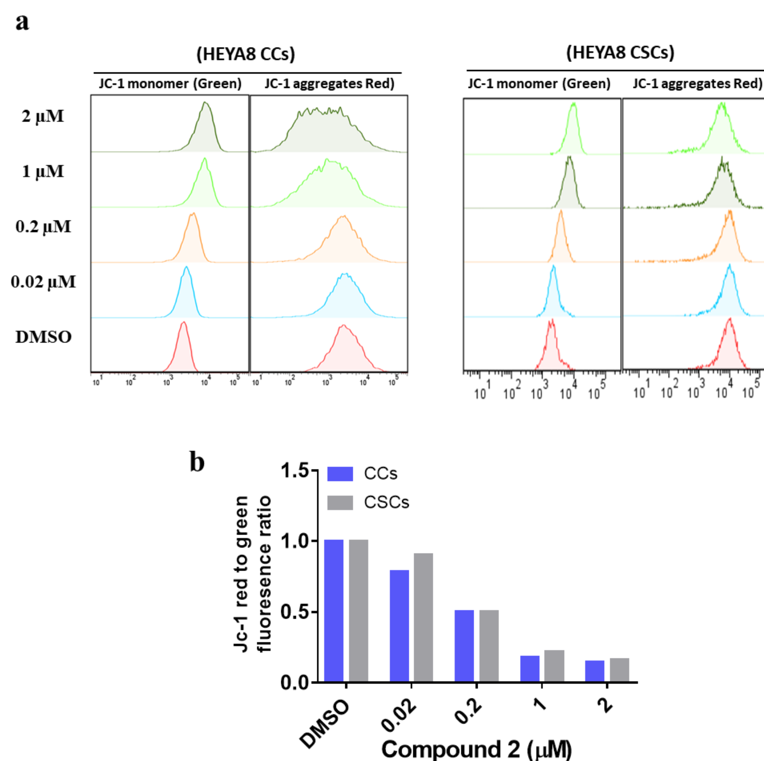


Figure S20. a, FACS analysis of JC-1 probe in HEYA8 adherent cancer cells and CSCs treated with DMSO or compound **2** at the indicated concentration for 10 min. **b**, Quantitative results from **a** to show the effects of compound **2** on the mitochondrial membrane potential of adherent cancer cells and CSCs.

3.8 Cytotoxicity of cation transporters

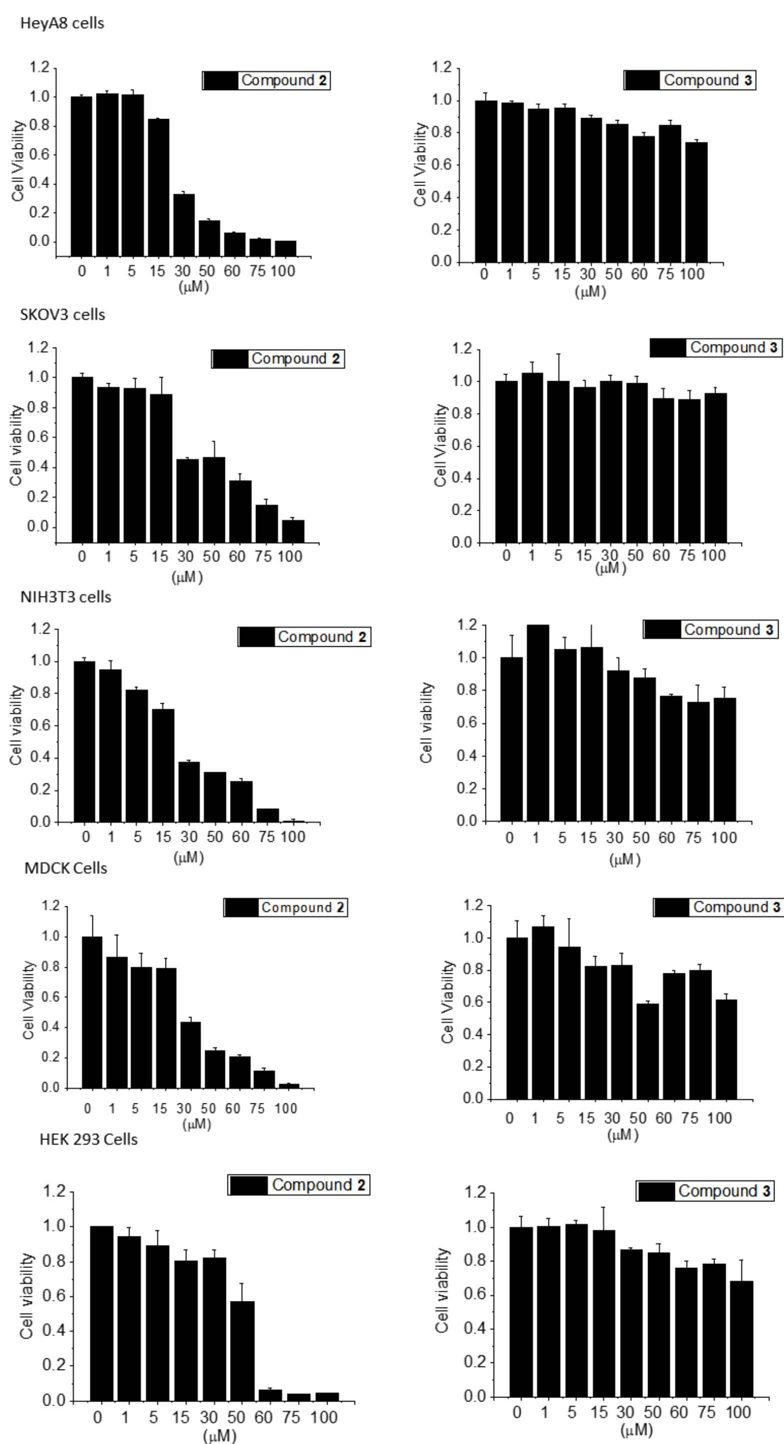


Figure S21. Representative results of cell viability after treatment with compound **2** or **3** for 48 h. Values are reported as means of viable cells normalized with respect to untreated cells (mean ± s.e.m., n = 3).

3.9 ABC transporter expression

The expression levels of ABC drug transporters in ovarian cancer HEYA8 adherent cancer cells and CSCs were investigated by immunoblotting. CSCs have expressed significantly higher amounts of ABCB1 and ABCG2 than that of HEYA8 adherent cancer cells.

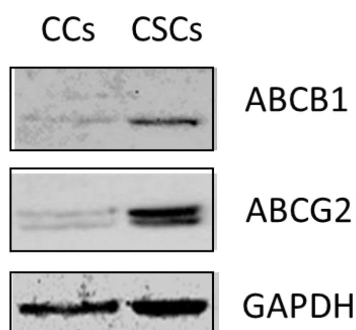


Figure S22. Immunoblots showing the levels ABCB1, ABCG2 and GAPDH (internal loading control) in HEYA8 cancer cells and CSCs.

3.10 *In vivo* anti-tumor effect of compound 2

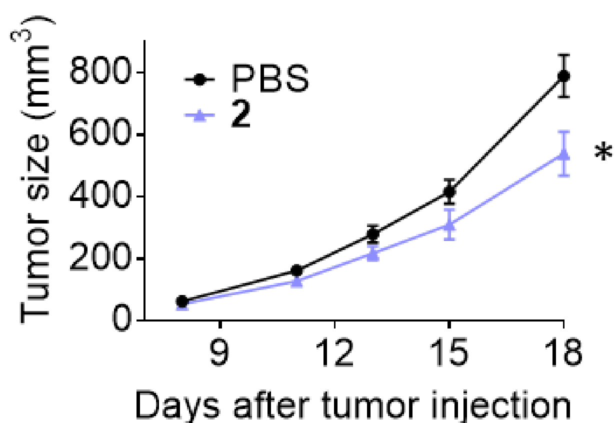


Figure S23. HEYA8 tumor-growth curves of mice treated with compound **2** (5 mg/kg three days a week) or PBS buffer (mean ± s.e.m., $n = 5$ mice per group, $*p < 0.05$).

4. References

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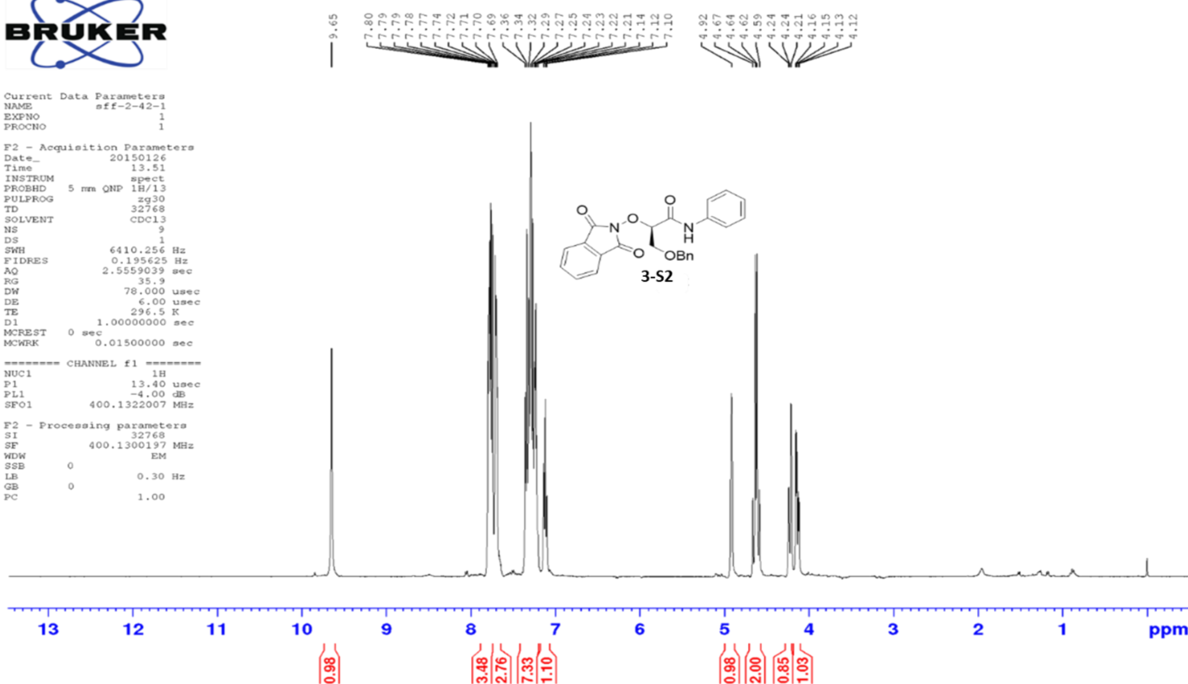
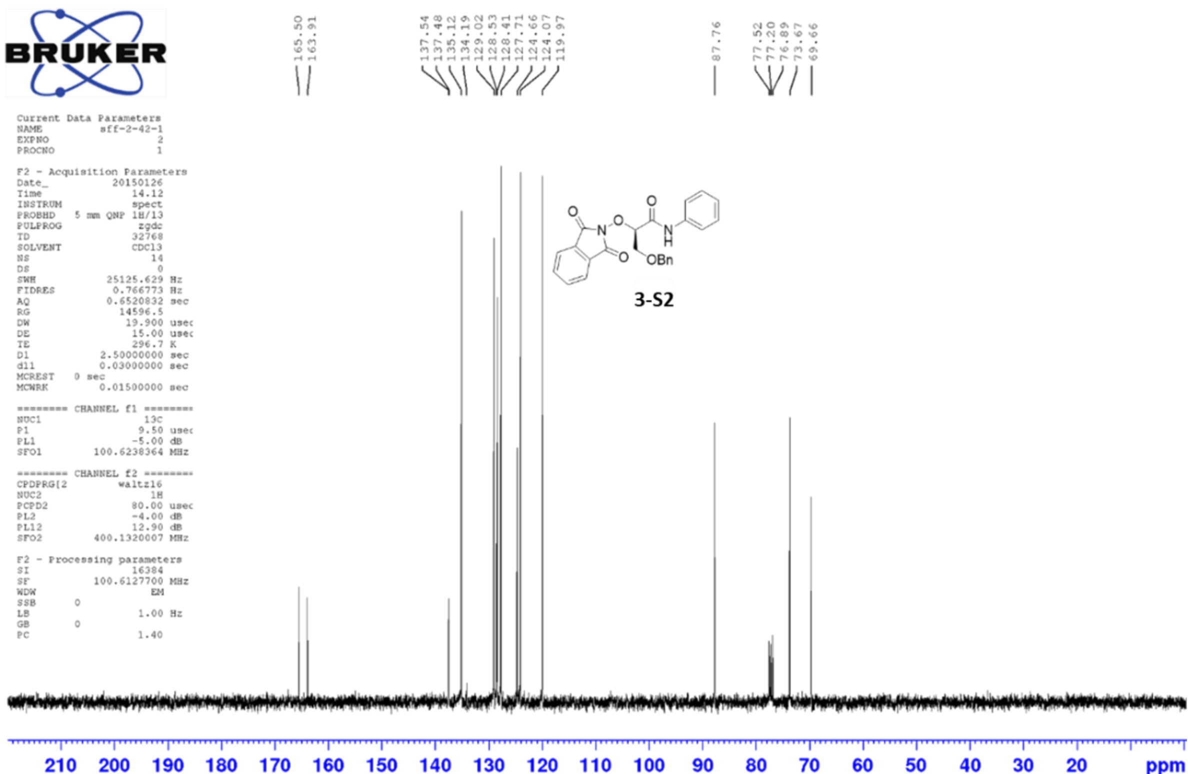
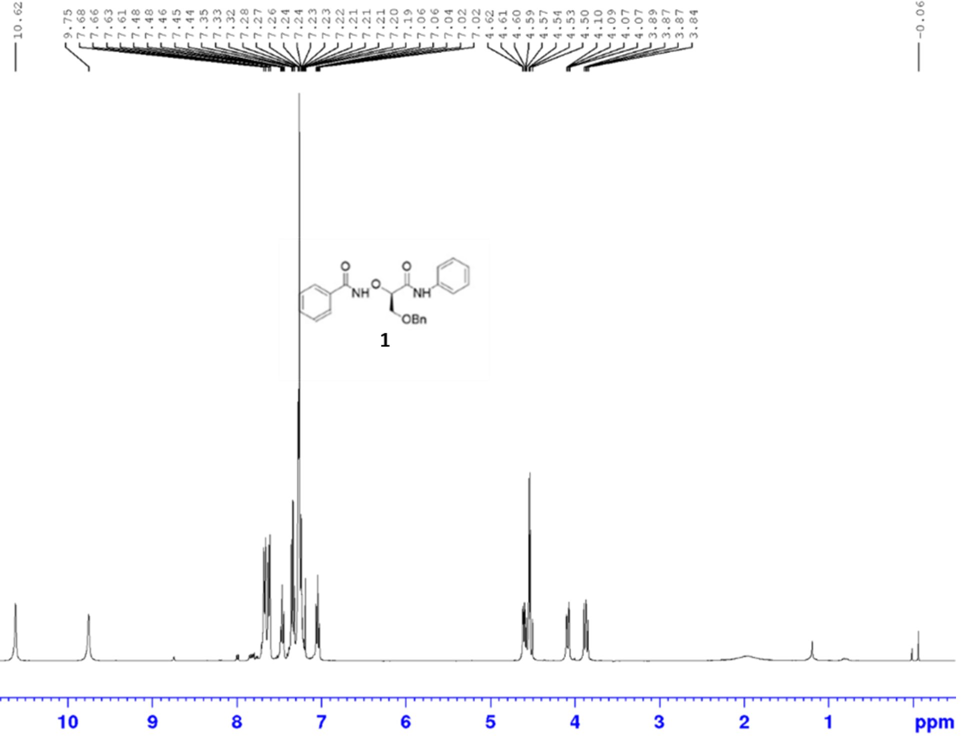
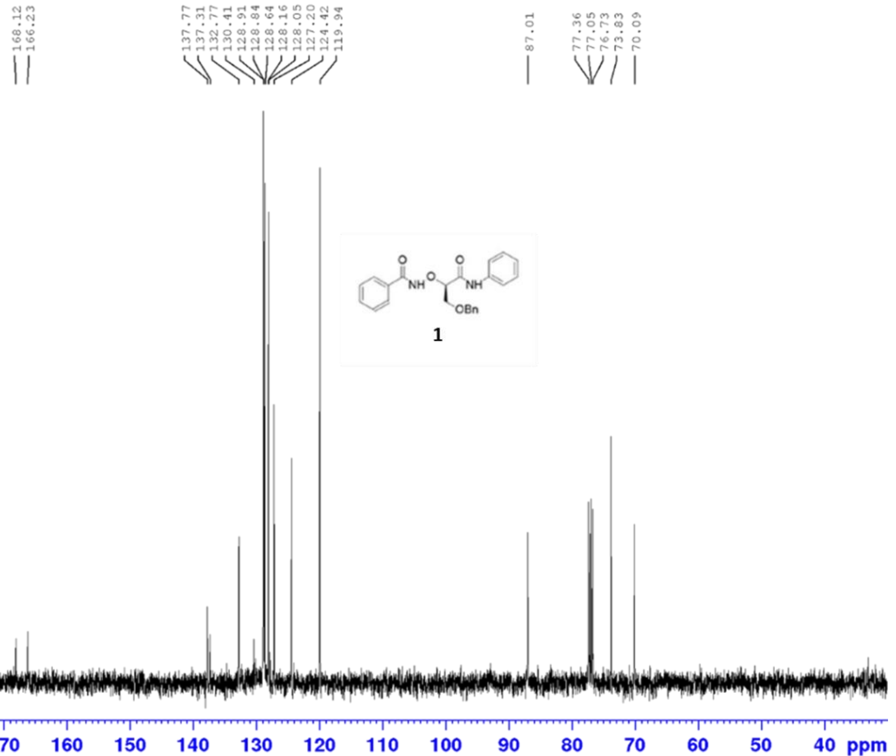
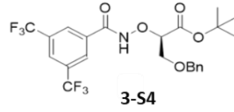
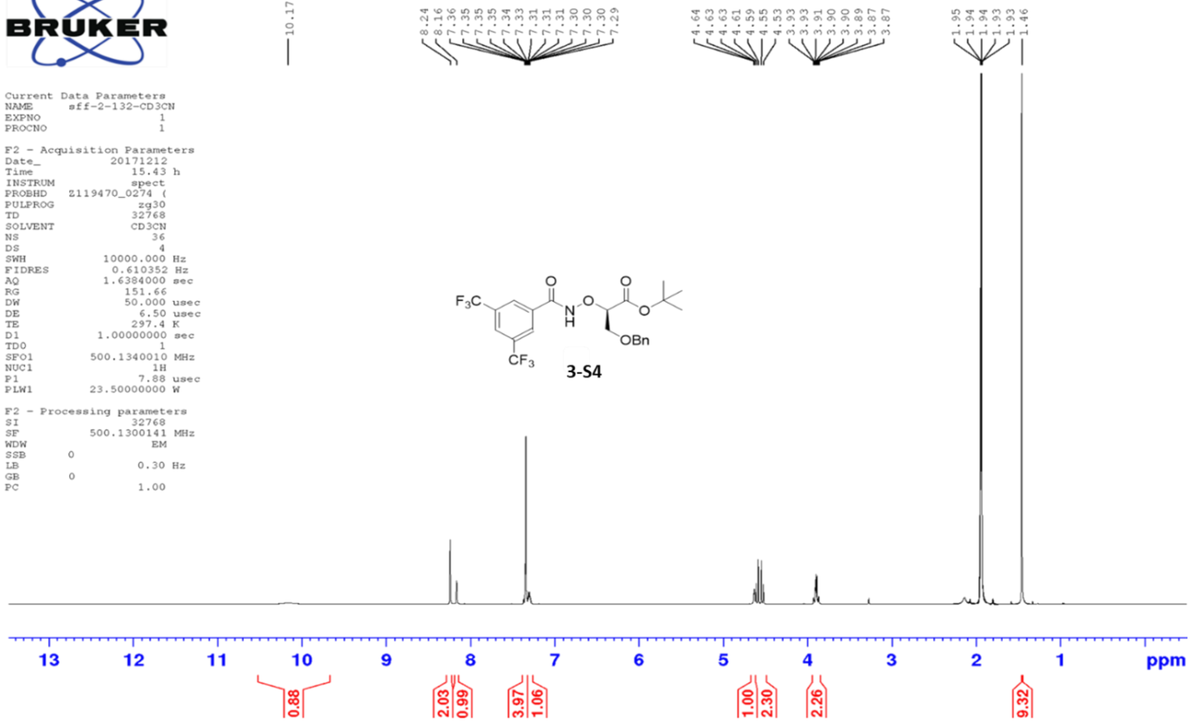
5. Appendix

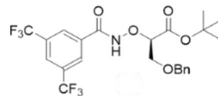
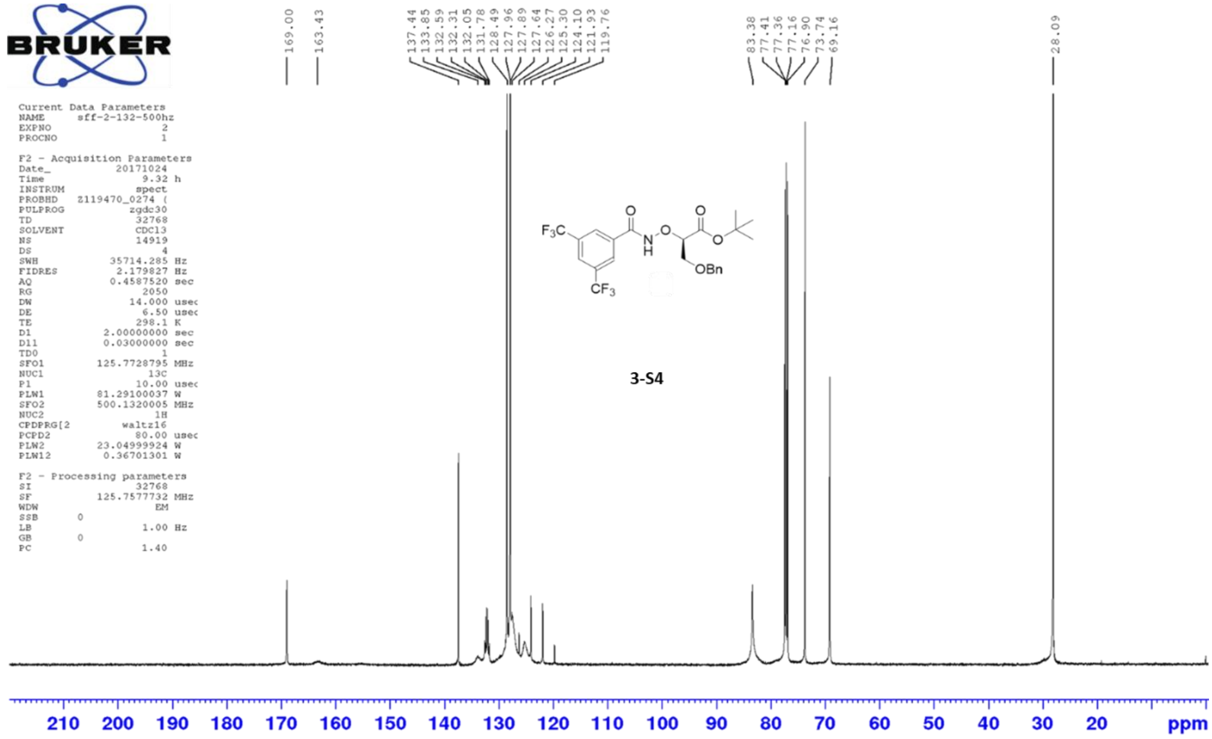


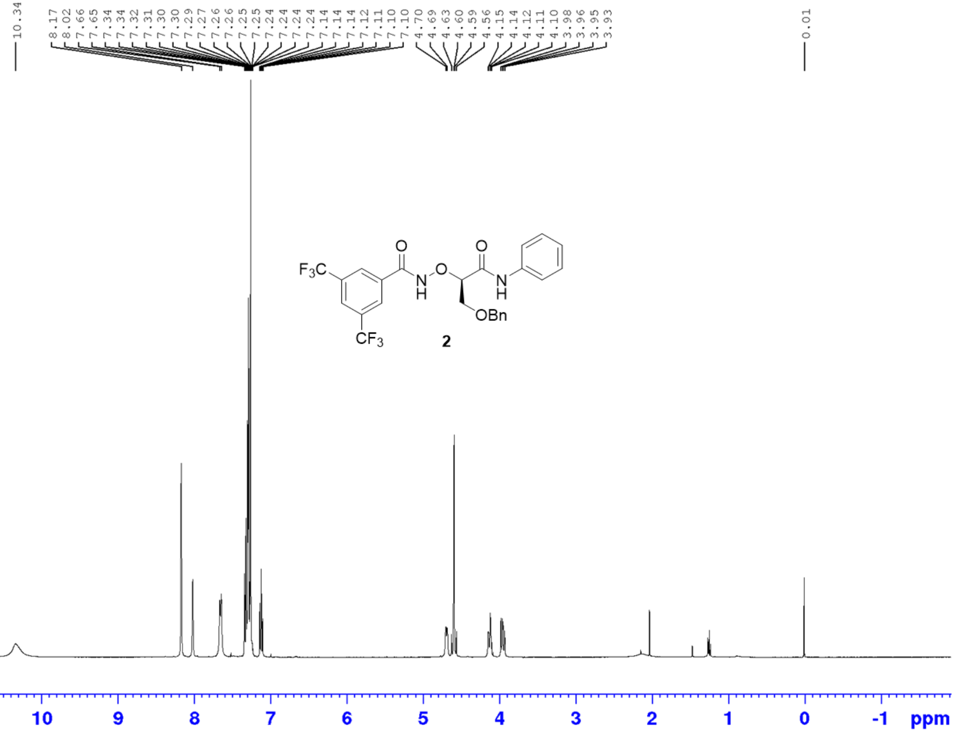


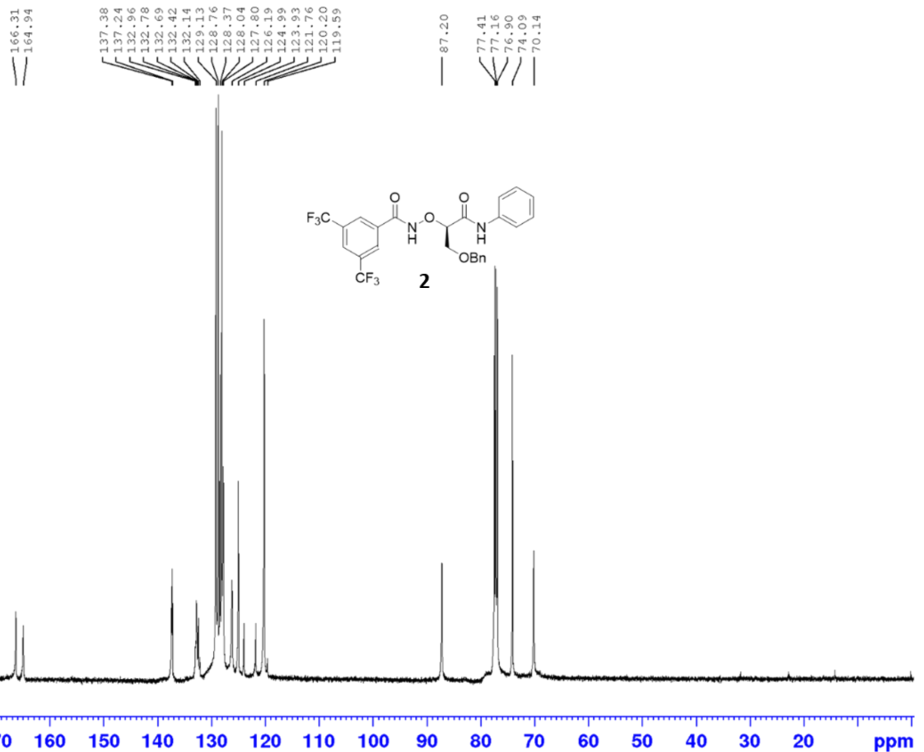








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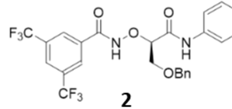

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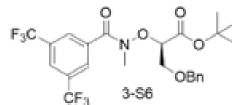

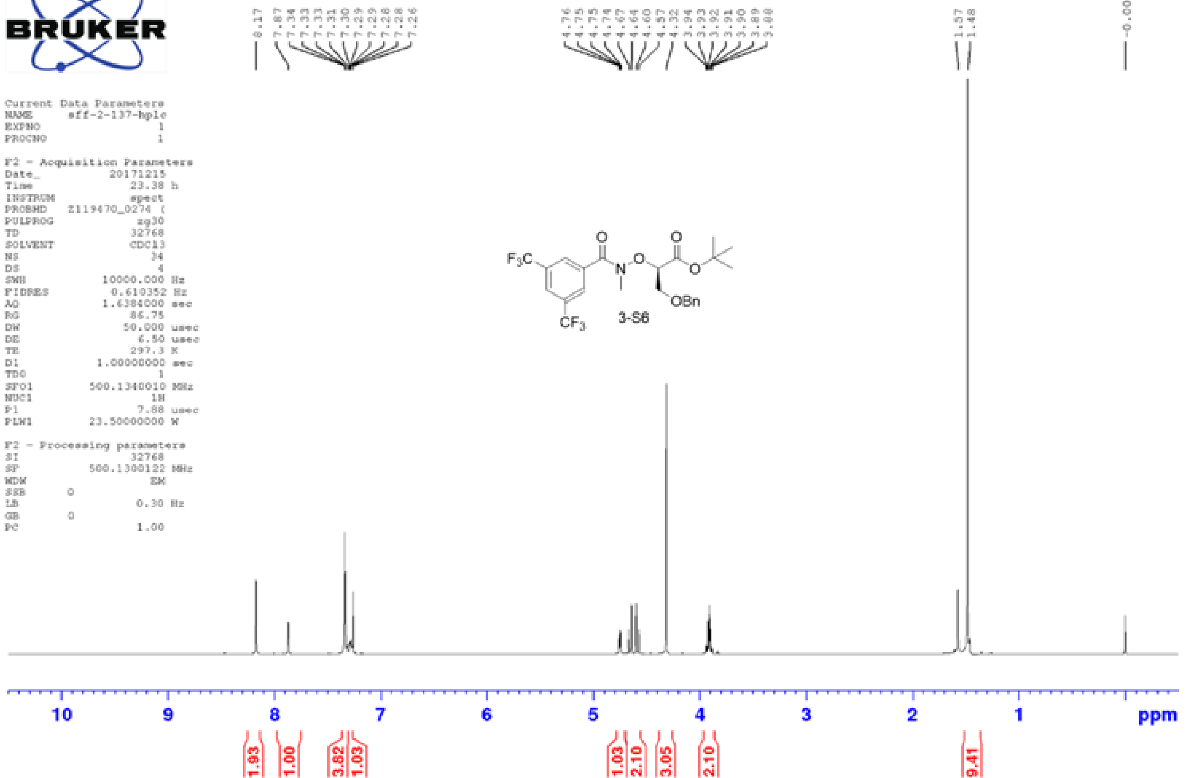

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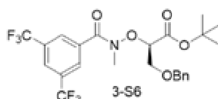

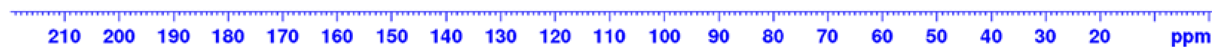

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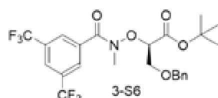
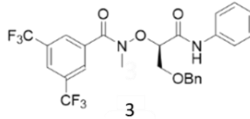
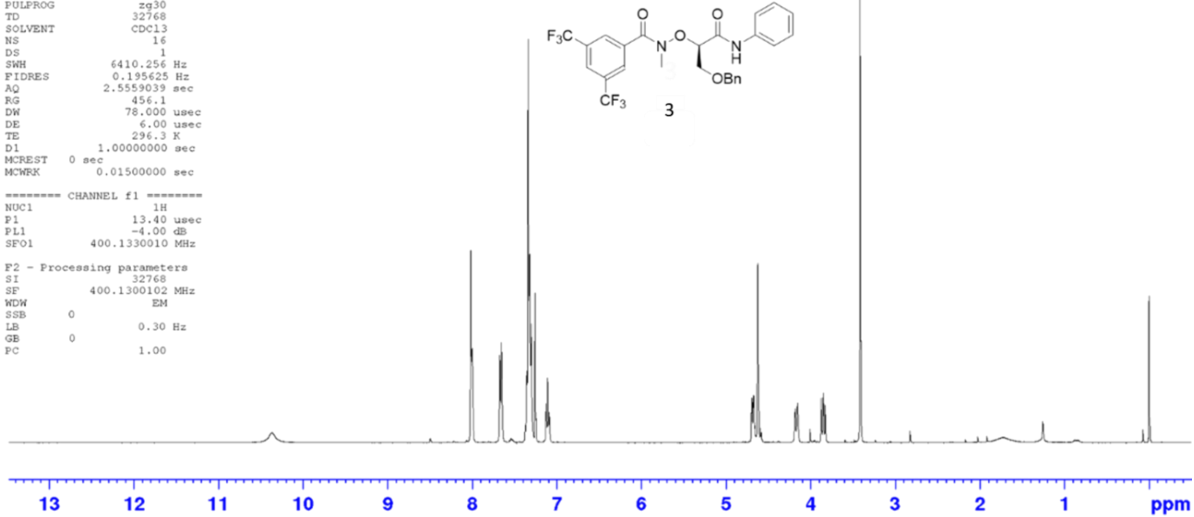
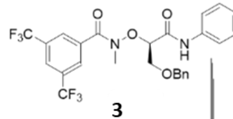
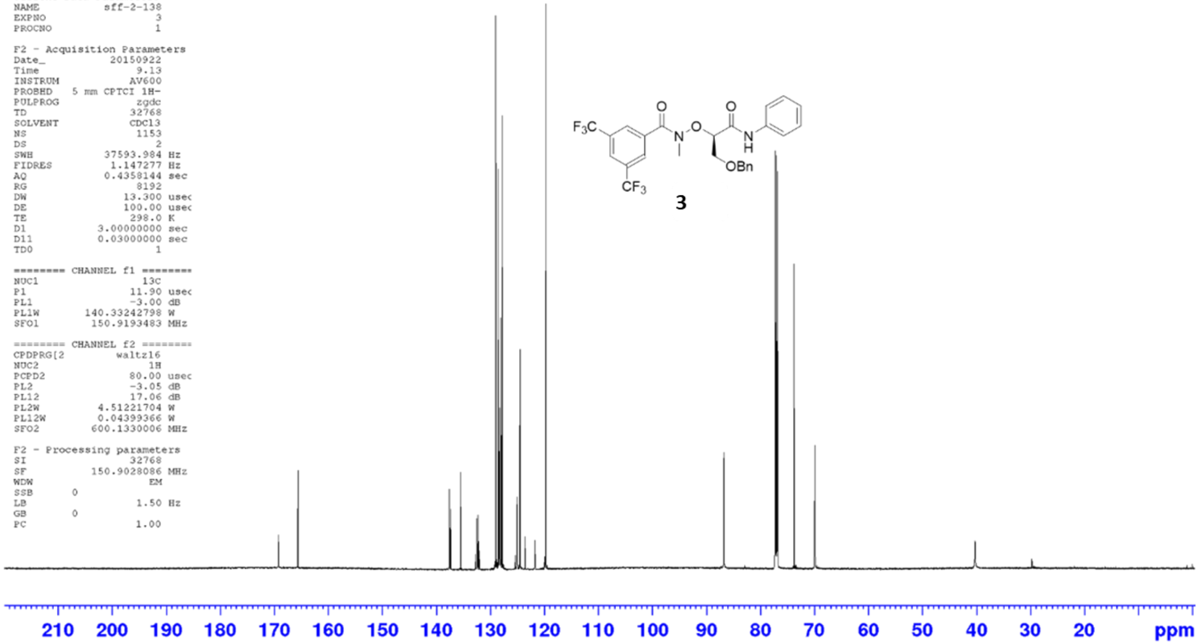
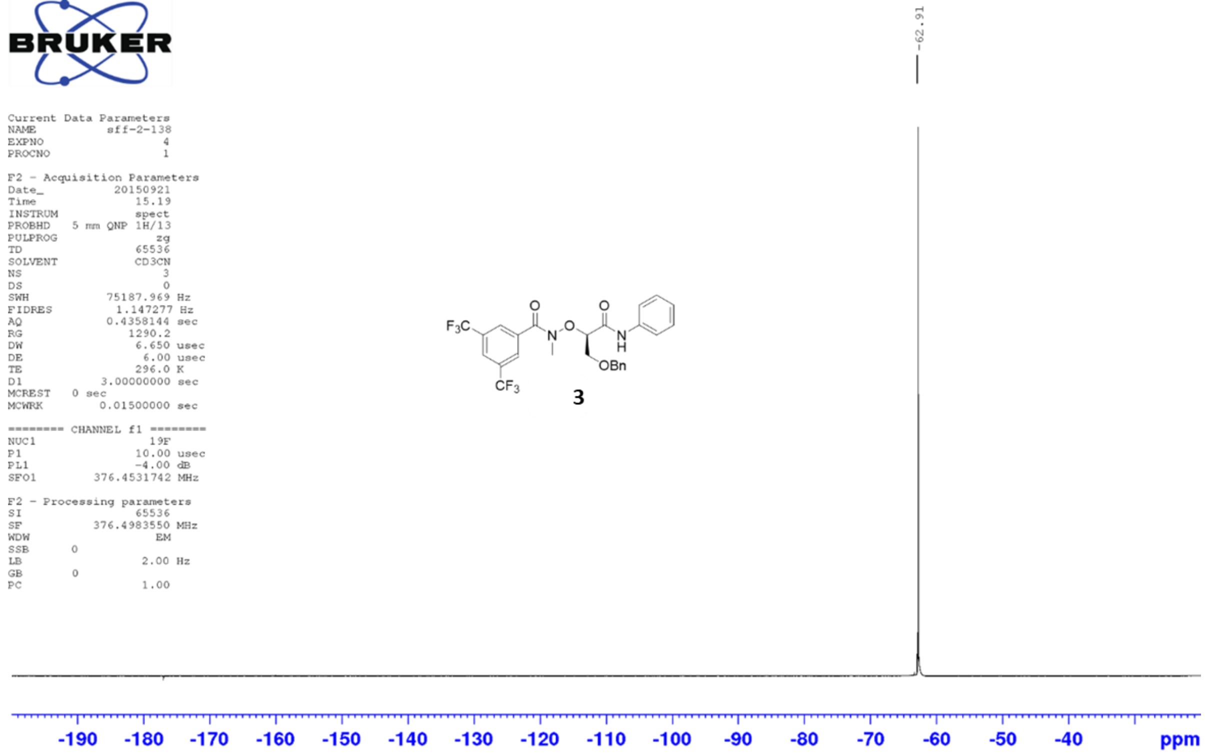




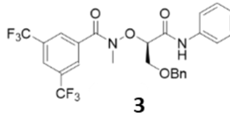




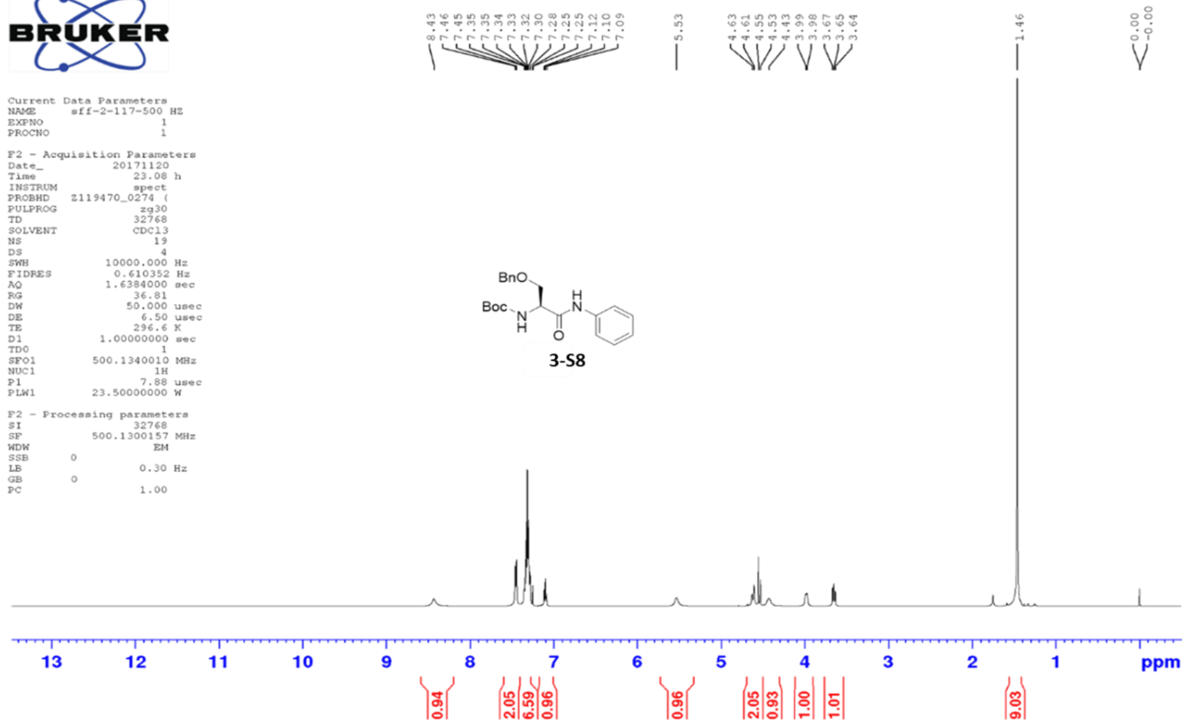




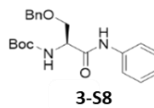
Current Data Parameters
NAME sff-2-138
EXPNO 4
PROCNO 1
F2 - Acquisition Parameters
Date_ 20150921
Time 15.19
INSTRUM spect
PROBHD 5 mm QNP 1H/13
PULPROG zg
TD 65536
SOLVENT CD3CN
NS 3
DS 0
SWH 75187.969 Hz
FIDRES 1.147277 Hz
AQ 0.4358144 sec
RG 1290.2
DW 6.650 usec
DE 6.00 usec
TE 296.0 K
D1 3.00000000 sec
MCREST 0 sec
MCWRK 0.01500000 sec
======== CHANNEL f1 ========
NUC1 19F
P1 10.00 usec
PL1 -4.00 dB
SFO1 376.4531742 MHz
F2 - Processing parameters
SI 65536
SF 376.4983550 MHz
WDW EM
SSB 0
LB 2.00 Hz
GB 0
PC 1.00
-62.91
ppm



3



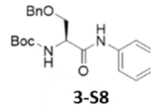
Current Data Parameters
NAME sff-2-117-500 HZ
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20171120
Time 23.08 h
INSTRUM spect
PROBHD Z119470_0274 (
PULPROG zg30
TD 32768
SOLVENT CDCl3
NS 19
DS 4
SWH 10000.000 Hz
FIDRES 0.610352 Hz
AQ 1.6384000 sec
RG 36.81
DW 50.000 usec
DE 6.50 usec
TE 296.6 K
D1 1.00000000 sec
TD0 1
SFO1 500.1340010 MHz
NUC1 1H
P1 7.88 usec
PLW1 23.50000000 W
F2 - Processing parameters
SI 32768
SF 500.1300157 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
ppm



3-S8

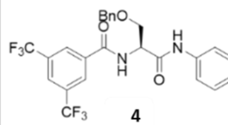
168.60
155.82
137.62
137.38
129.10
128.70
128.20
128.01
124.55
119.99
80.64
77.41
77.15
76.90
73.74
69.88
54.42
28.42

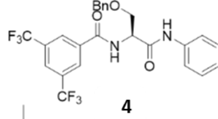
```
Current Data Parameters
NAME      sff-2-117-500 HZ
EXPNO                 2
PROCNO                1

F2 - Acquisition Parameters
Date_          20171121
Time               8.53 h
INSTRUM           spect
PROBHD   Z119470_0274 (
PULPROG          zgdc30
TD                32768
SOLVENT           CDCl3
NS                13846
DS                    4
SWH           35714.285 Hz
FIDRES         2.179827 Hz
AQ            0.4587520 sec
RG                 2050
DW               14.000 usec
DE                 6.50 usec
TE                297.7 K
D1           2.00000000 sec
D11          0.03000000 sec
TD0                   1
SFO1        125.7728795 MHz
NUC1                13C
P1                10.00 usec
PLW1        81.29100037 W
SFO2        500.1320005 MHz
NUC2                 1H
CPDPRG[2        waltz16
PCPD2             80.00 usec
PLW2        23.04999924 W
PLW12        0.36701301 W

F2 - Processing parameters
SI                32768
SF          125.7577763 MHz
WDW                  EM
SSB      0
LB                 1.00 Hz
GB       0
PC                 1.40
```

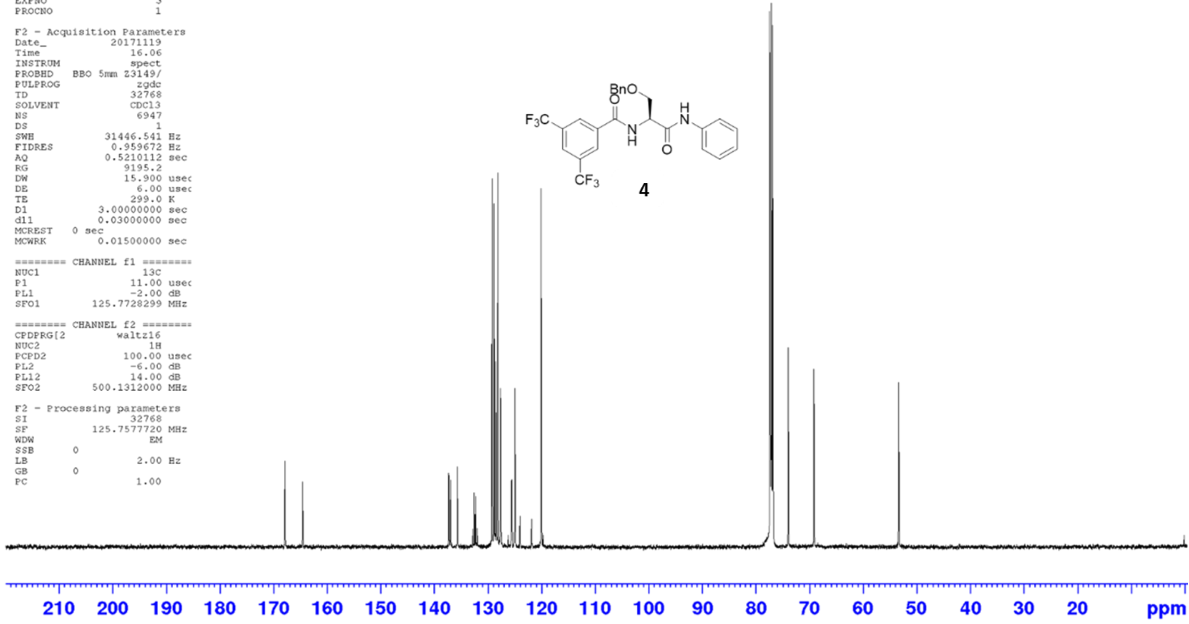


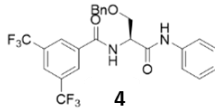
8.63
8.20
7.93
7.54
7.52
7.36
7.34
7.26
7.23
7.21
7.18
7.07
7.05
7.04
4.92
4.91
4.90
4.89
4.88
4.63
4.60
4.54
4.51
4.01
4.00
3.99
3.97
3.69
3.66
3.64
1.64
-0.07

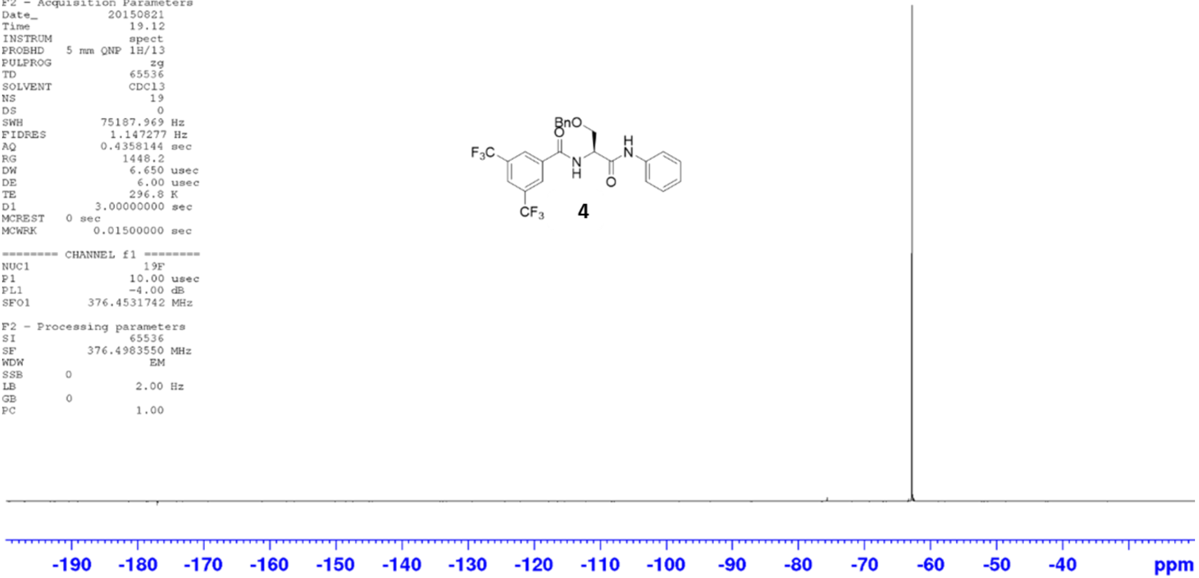
```
Current Data Parameters
NAME         sff-2-118-2
EXPNO                  1
PROCNO                 1

F2 - Acquisition Parameters
Date_           20150821
Time               19.06
INSTRUM            spect
PROBHD   5 mm QNP 1H/13
PULPROG             zg30
TD                 32768
SOLVENT            CDCl3
NS                    16
DS                     1
SWH             6410.256 Hz
FIDRES          0.195625 Hz
AQ             2.5559039 sec
RG                 287.4
DW                78.000 usec
DE                  6.00 usec
TE                 296.4 K
D1            1.00000000 sec
MCREST    0 sec
MCWRK         0.01500000 sec

======== CHANNEL f1 ========
NUC1                  1H
P1                 13.40 usec
PL1                -4.00 dB
SFO1        400.1322007 MHz

F2 - Processing parameters
SI                 32768
SF           400.1300407 MHz
WDW                   EM
SSB       0
LB                  0.30 Hz
GB        0
PC                  1.00
```











BRUKER

8.35
8.17
4.89
3.85
3.31
3.31
3.31

Current Data Parameters
NAME sff-2-140-3
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20151006
Time 14.11
INSTRUM spect
PROBHD 5 mm QNP 1H/13
PULPROG zg30
TD 32768
SOLVENT CDCl3
NS 16
DS 1
SWH 6410.256 Hz
FIDRES 0.195625 Hz
AQ 2.5559039 sec
RG 287.4
DW 78.000 usec
DE 6.00 usec
TE 295.7 K
D1 1.00000000 sec
MCREST 0 sec
MCWRK 0.01500000 sec

======== CHANNEL f1 ========
NUC1 1H
P1 13.40 usec
PL1 -4.00 dB
SFO1 400.1322007 MHz

F2 - Processing parameters
SI 32768
SF 400.1300075 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

F_3C, O, N, O, H, CF_3

5

13 12 11 10 9 8 7 6 5 4 3 2 1 ppm

1.99 1.00 3.13

BRUKER

163.76

135.91
133.69
133.47
133.24
133.02
128.93
127.32
126.47
126.44
126.42
125.52
123.71
121.91

64.56
49.56
49.53
49.42
49.28
49.23
49.22
49.20
49.18
49.14
49.09
49.07
49.06
48.99
48.85
48.71

Current Data Parameters
NAME sff-2-140
EXPNO 3
PROCNO 1

F2 - Acquisition Parameters
Date_ 20151006
Time 16.31
INSTRUM AV600
PROBHD 5 mm CPTCI 1H-
PULPROG zgdc
TD 32768
SOLVENT MeOD
NS 791
DS 2
SWH 37593.984 Hz
FIDRES 1.147277 Hz
AQ 0.4358144 sec
RG 8192
DW 13.300 usec
DE 100.00 usec
TE 298.0 K
D1 3.00000000 sec
D11 0.03000000 sec
TD0 1

======== CHANNEL f1 ========
NUC1 13C
P1 11.90 usec
PL1 -3.00 dB
PL1W 140.33242798 W
SFO1 150.9193483 MHz

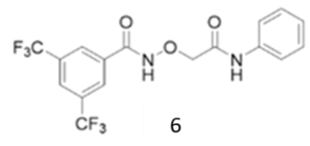
======== CHANNEL f2 ========
CPDPRG[2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -3.05 dB
PL12 17.06 dB
PL2W 4.51221704 W
PL12W 0.04399366 W
SFO2 600.1330006 MHz

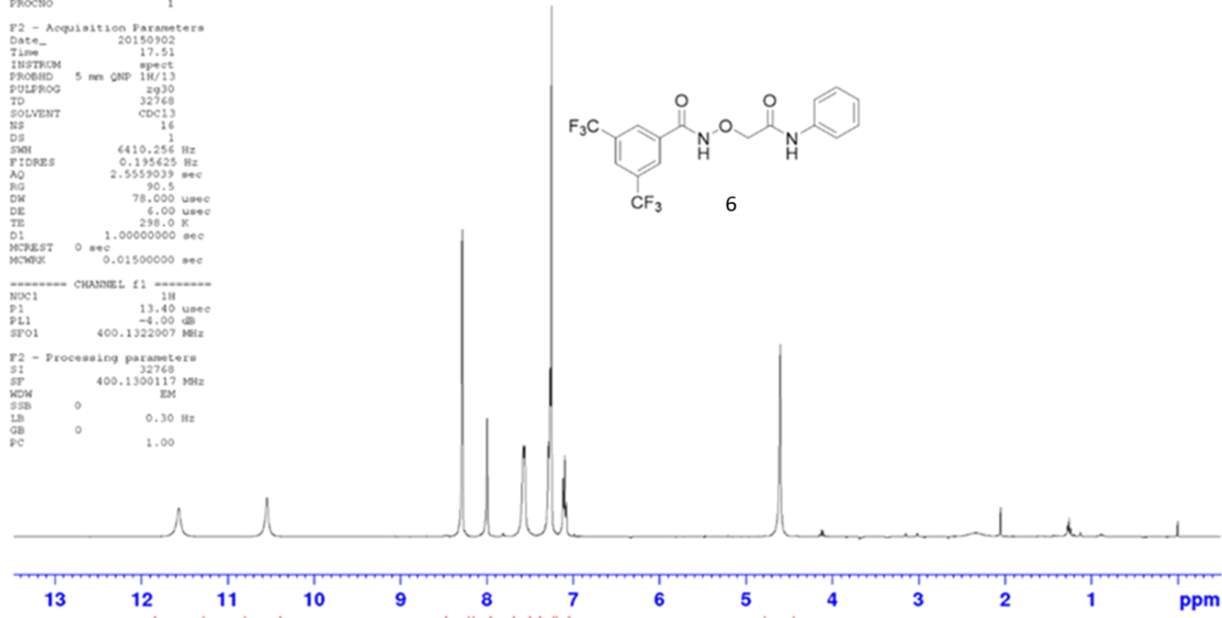
F2 - Processing parameters
SI 32768
SF 150.9025740 MHz
WDW EM
SSB 0
LB 1.50 Hz
GB 0
PC 1.00

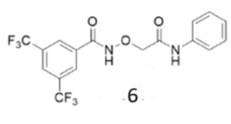
F_3C, O, N, O, H, CF_3

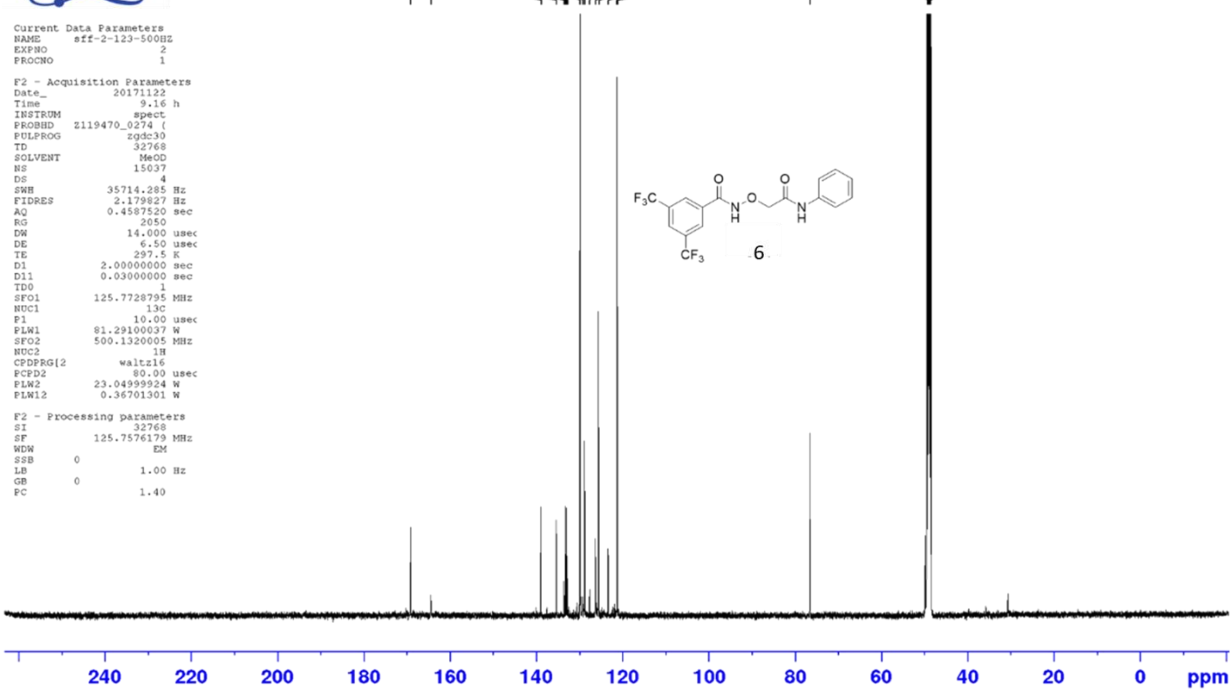
5

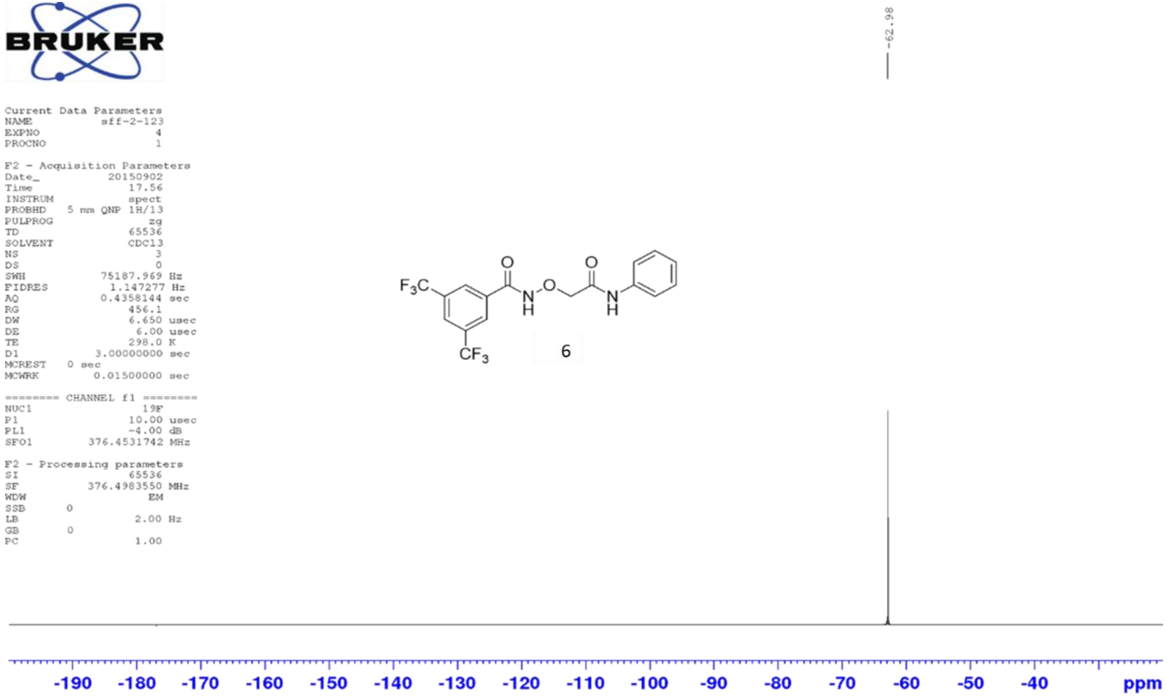
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 ppm

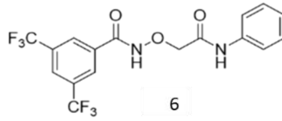










BRUKER
Current Data Parameters
NAME sff-2-123
EXPNO 4
PROCNO 1
F2 - Acquisition Parameters
Date_ 20150902
Time 17.56
INSTRUM spect
PROBHD 5 mm QNP 1H/13
PULPROG zg
TD 65536
SOLVENT CDCl3
NS 3
DS 0
SWH 75187.969 Hz
FIDRES 1.147277 Hz
AQ 0.4358144 sec
RG 456.1
DW 6.650 usec
DE 6.00 usec
TE 298.0 K
D1 3.00000000 sec
MCREST 0 sec
MCWRK 0.01500000 sec
======== CHANNEL f1 ========
NUC1 19F
P1 10.00 usec
PL1 -4.00 dB
SFO1 376.4531742 MHz
F2 - Processing parameters
SI 65536
SF 376.4983550 MHz
WDW EM
SSB 0
LB 2.00 Hz
GB 0
PC 1.00
-62.98
-190 -180 -170 -160 -150 -140 -130 -120 -110 -100 -90 -80 -70 -60 -50 -40 ppm

F3C
O
N
H
O
O
N
H
CF3
6