

A Small Molecule Fluorogenic Probe for the Detection of Sphingosine in Living Cells

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General Procedures

Quenching efficiency of Black Hole Quencher-1 (BHQ-1) in probe 1.

10 μM solutions of Bodipy FL or probe 1 were prepared in PBS buffer. Fluorescence emission spectra from 505-550 nm (ex. 480 nm) for each sample were obtained on a Tecan Spark multimode microplate reader.

General procedure for synthesis of large unilamellar vesicles (LUVs)

5 μmol of phospholipid in a 20 mg/mL CHCl_3 solution was added to a glass vial and dried into a film under a stream of nitrogen. 1 mL of buffer (1x PBS) was added to the vial and then the vial tumbled at RT for 1 hr. After tumbling, the formed solution was subjected to 10 freeze/thaw cycles using a dry ice/ethanol bath and a warm water bath with vortexing before every freeze step. The vesicle solution was then extruded 10 times through a 100 nm polycarbonate filter (Whatman, GE Healthcare Bio-Sciences) using an Avanti Mini-Extruder (Avanti Polar Lipids) to produce LUVs.

Fluorescence measurements in DOPC vesicles

Solutions of 5 mM 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) vesicles with or without sphingosine (250 μM), sphinganine (250 μM), sphingosine-1-phosphate (250 μM), and serine (2.5 mM) were treated with Compound 1 (200 μM stock in PBS) at a final concentration of 5 μM . Reactions were incubated on a tube rotator at 37 $^\circ\text{C}$ for 24 h. Fluorescence emission spectra from 500-560 nm (ex. 480 nm) for each sample were obtained on a Jasco FP-8500 spectrofluorometer.

Probe reaction in DOPC vesicles

Solutions of 5 mM DOPC vesicles in PBS with or without sphingosine (1 mM) or sphinganine (1 mM) were treated with Compound 1 (100 μM). Reactions were incubated on a tube rotator at 37 $^\circ\text{C}$ for 24 h. Samples were analyzed using HPLC Method A before and after 24 h incubation.

Probe stability and reactivity toward naturally abundant molecular species

Solutions of 5 mM 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles in PBS with or without 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) (1 mM), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) (1 mM), cholesterol (Chol) (1 mM), cysteine (Cys) (1 mM), ethanolamine (1 mM), cysteamine (1 mM), or cystamine (1 mM) were treated with Compound 1 (100 μM). Reactions were incubated on a tube rotator at 37 $^\circ\text{C}$ for 24 h. Samples were analyzed using HPLC Method A before and after 24 h incubation.

Cell culture

Healthy Human Fibroblasts (GM05399) and Neimann-Pick Type C Human Fibroblasts (GM03123) were obtained from Coriell Institute (Camden, NJ). HeLa S3 (CCL-2.2), T24, BxPC-3, and MIA PaCa-2 cell lines were obtained from ATCC (Manassas, VA). Cells were maintained in DMEM supplemented with Penicillin (50 units/mL), Streptomycin (50 $\mu\text{g}/\text{mL}$) and 10 % FBS (Healthy Fibroblasts, HeLa S3, T24, BxPC-3, and MIA PaCa-2) or 15 % FBS (NPC1 Fibroblasts) at 37 $^\circ\text{C}$, 5 % CO_2 .

Probe dose-response in live cells

HeLa, T24, BxPC-3, or MIA PaCa-2 cells were plated in an 8-well Lab-Tek chamber slide (Sigma-Aldrich) at a density of 40,000 cells/well and allowed to attach overnight. Media was removed and

then 500 μL of media containing 7.5 μM **1** or **2** was added to each well and cells incubated at 37 $^{\circ}\text{C}$, 5 % CO_2 for 2 h. Media in each well was then exchanged for 500 μL of media containing the indicated concentrations of sphingosine or sphinganine and the cells incubated at 37 $^{\circ}\text{C}$, 5 % CO_2 for 20 h before imaging.

Imaging of Bodipy FL carboxylic acid and sphingolipid products in HeLa cells

HeLa cells were plated in an 8-well Lab-Tek chamber slide (Sigma-Aldrich) at a density of 40,000 cells/well and allowed to attach overnight. Media was removed and then 500 μL of media containing 5 μM Bodipy FL carboxylic acid, **3**, or **4** was added to each well and cells incubated at 37 $^{\circ}\text{C}$, 5 % CO_2 for 1 h before imaging.

Detection of endogenous sphingosine/sphinganine in HeLa cells

HeLa cells were plated in an 8-well Lab-Tek chamber slide (Sigma-Aldrich) at a density of 40,000 cells/well and allowed to attach overnight. Media was removed and each well washed once with OptiMEM media (- FBS) before adding 500 μL OptiMEM (- FBS) containing 20 μM **1** or **2**. Cells were incubated at 37 $^{\circ}\text{C}$, 5 % CO_2 for 4 h before exchanging media in each well for growth media. Cells were then maintained at 37 $^{\circ}\text{C}$, 5 % CO_2 for 16 h while imaging.

Detection of sphingosine/sphinganine in Healthy and NPC1 fibroblasts

Healthy and NPC1 fibroblast cells were plated in an 8-well Lab-Tek chamber slide (Sigma-Aldrich) at a density of 20,000 cells/well and allowed to attach overnight. Media was removed and 500 μL DMEM (15 % FBS) containing 7.5 μM **1** was added to each well. Cells were incubated at 37 $^{\circ}\text{C}$, 5 % CO_2 for 24 h before imaging.

Quantification of cellular fluorescence

Using ImageJ,¹ the mean fluorescence of individual cells was measured within 4 images taken across at least 2 biological replicates for each condition. The mean cellular fluorescence intensity was then calculated for each of the 4 images and data reported as the mean of these 4 means \pm SD.

Sphingosine/sphinganine extraction and measurement in Healthy and NPC1 fibroblasts

Cells seeded in 6-well plates were extracted with 250 μL -20 $^{\circ}\text{C}$ methanol, 150 μL of saline, 100 μL of water and spiked with a mixture of deuterated standards (sphingosine-d7, sphinganine-d7 (Avanti Lipids)) of a known concentration as internal control. 500 μL of chloroform was added and the samples were vortexed for 5 min followed by centrifugation at 4 $^{\circ}\text{C}$ for 5 min at 16,000 x g. The organic phase was collected and 1 μL of formic acid was added to the remaining polar phase which was re-extracted with 500 μL of chloroform. Combined organic phases were dried and the pellet was resuspended in 100 μL of buffer containing 100% methanol, 1 mM ammonium formate and 0.2% (v/v) formic acid. Quantification of these species were determined via liquid chromatography mass-spectrometry (Agilent 6460 QQQ). The sphingoid bases were separated on C8 column (Spectra 3 μm C8SR 150 x 3mm ID, Peeke Scientific, CA) as previously described.² Mobile phase A was composed of 100% HPLC grade water containing 2 mM ammonium formate and 0.2% (v/v) formic acid and mobile phase B consisted of 100% methanol containing 0.2% (v/v) formic acid and 1 mM ammonium formate. The gradient elution program consisted of holding at 82% B for 3 min, up to 90% B over 1 min and linearly increasing to 99% B over 14 min maintaining it for 7 min and down to 82% B over 2 min. The 82% mobile phase B was maintained for 3 min followed by a re-equilibration for 10 min. The capillary voltage was set to 3.5 kV, the

drying gas temperature was 350 °C, the drying gas flow rate was 10 L/min, and the nebulizer pressure was 60 psi. Sphingoid base species were analyzed by SRM of the transition from precursor to product ions at associated optimized collision energies and fragmentor voltages (Table below). Abundances of these species were then quantified from spiked internal standards.

Sphingolipid species	Parent	Daughter	Fragmentor	CE
SA d18:0	302.4	284.4	120	9
SA-d7 d18:0	309.4	291.5	120	9
SO d18:1	300.3	282.4	112	9
SO-d7 d18:0	307.3	289.4	112	9

Microscopy

Imaging was performed on an Axio Observer Z1 inverted microscope (Carl Zeiss Microscopy Gmb, Germany) with Yokogawa CSU-X1 spinning disk confocal unit using a 20x, 0.8 NA objective to an ORCA-Flash4.0 V2 Digital CMOS camera (Hamamatsu, Japan). Fluorophores were excited with a diode laser (488 nm; 30 mW). Where indicated, cells were maintained at 37 °C, 5% CO₂ using an Okolab stagetop incubator (Italy). Images were acquired using Zen Blue software (Carl Zeiss) and processed using Image J.

HPLC

Method A

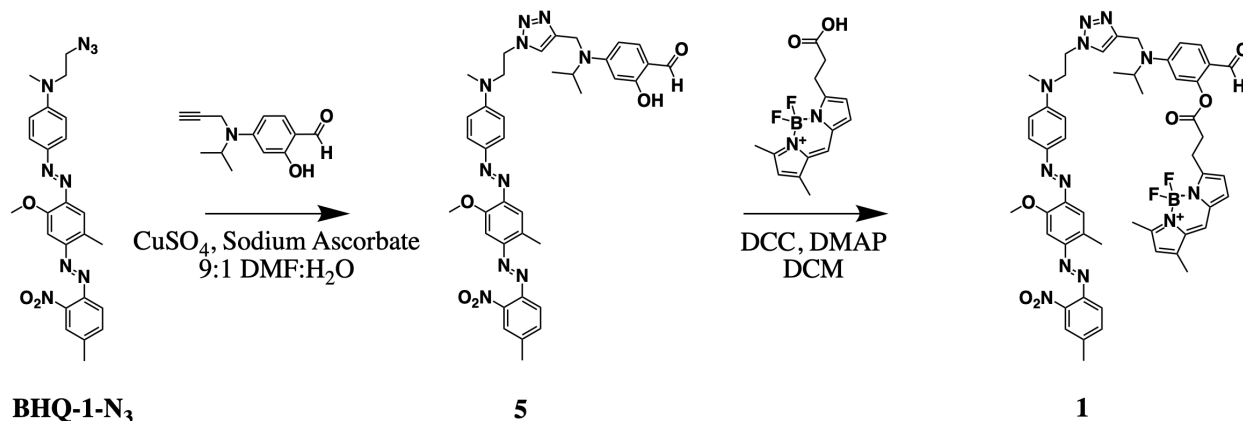
Samples were analyzed or purified on an Agilent 1100 series HPLC equipped with a diode array detector (DAD) using a Zorbax SB-C18 column (Agilent) with HPLC grade H₂O and MeOH each containing 0.1% formic acid. Samples were eluted using a gradient: 75:25 to 95:5 MeOH:H₂O from 0-5 min, 95:5 MeOH:H₂O from 5-17 min.

Statistical Methods

All statistical analysis was performed using Prism 7 software. Where shown, error bars represent standard deviation.

Synthesis

Synthesis of Sph/Spa Fluorescent Probe 1

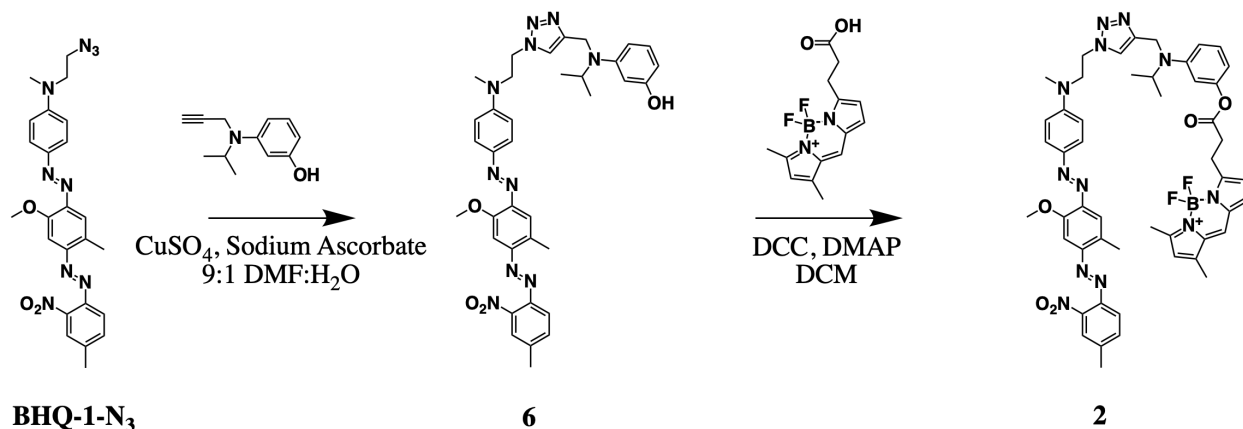


2-hydroxy-4-(isopropyl(BHQ-1-N₃)amino)benzaldehyde (5). BHQ-1-N₃³ (11.3 mg, 0.023 mmol) and 2-hydroxy-4-(isopropyl(prop-2-yn-1-yl)amino)benzaldehyde⁴ (7.6 mg, 0.035 mmol) were dissolved in 1.5 mL 9:1 DMF:H₂O. CuSO₄ pentahydrate (0.6 mg, 0.002 mmol) and sodium ascorbate (0.9 mg, 0.005 mmol) were added and the reaction stirred at 50 °C under inert atmosphere for 12 h. Solvent was removed by rotary evaporation, the residue dissolved in CH₃CN and purified using HPLC method A. Solvent was removed by rotary evaporation to yield **5** as a dark red solid (11.6 mg, 71 % yield). ¹H NMR (500 MHz, Chloroform-d) δ 11.54 (s, 1H), 9.54 (s, 1H), 7.86 (d, J = 8.8 Hz, 2H), 7.72 (s, 1H), 7.67 (d, J = 8.2 Hz, 1H), 7.58 (s, 1H), 7.49 (d, J = 8.1 Hz, 1H), 7.41 (s, 1H), 7.24 (d, J = 8.9 Hz, 1H), 7.11 (s, 1H), 6.60 (d, J = 8.8 Hz, 2H), 6.29 (d, J = 8.8 Hz, 1H), 6.15 (s, 1H), 4.62 – 4.53 (m, 4H), 4.29 – 4.19 (m, 1H), 4.03 (s, 3H), 3.96 (t, 2H), 2.83 (s, 3H), 2.72 (s, 3H), 2.53 (s, 3H), 1.19 (d, J = 6.5 Hz, 6H). ¹³C NMR (125 MHz, Chloroform-d) δ 192.5, 164.1, 154.9, 154.9, 151.3, 150.5, 147.5, 146.6, 145.2, 145.0, 143.4, 141.7, 135.4, 133.5, 132.9, 125.7, 124.3, 122.9, 119.0, 119.0, 112.2, 111.4, 105.3, 99.4, 98.3, 56.3, 52.6, 48.8, 47.7, 40.3, 39.0, 21.3, 20.2, 16.7. MS (ESI) calculated [M+H]⁺: 705.3, found: 705.3.

2-(Bodipy FL)-4-(isopropyl(BHQ-1-N₃)amino)benzaldehyde (1). BDP FL carboxylic acid [Lumiprobe Hunt Valley, MD] (1.6 mg, 5.5 μmol) was dissolved in 200 μL dry DCM. 1 M N,N'-Dicyclohexylcarbodiimide in DCM (5.5 μL, 5.5 μmol) was added and the reaction stirred at rt for 10 min. **5** (3.0 mg, 4.3 μmol) and 4-(Dimethylamino)pyridine (0.8 mg, 6.4 μmol) were dissolved in 300 μL dry DCM and added to the reaction before stirring under inert atmosphere at rt for 4 h. The reaction was filtered through a 0.2 μm filter and dried by rotary evaporation. The residue was dissolved in CH₃CN and purified using HPLC method A. Solvent was removed by rotary evaporation to yield **1** as a dark red solid (2.8 mg, 67 % yield). ¹H NMR (500 MHz, dimethyl sulfoxide-d₆) δ 9.65 (s, 1H), 8.35 (s, 1H), 7.94 (m, 2H), 7.75 (d, J = 8.1 Hz, 1H), 7.70 (m, 2H), 7.60 (d, J = 9.0, 1H), 7.50 (s, 1H), 7.28 (s, 1H), 7.09 (d, J = 4.0, 1H), 6.73 (m, 3H), 6.55 (s, 1H), 6.47 (d, J = 4.0, 1H), 6.31 (s, 1H), 4.57 (t, J = 5.8 Hz, 2H), 4.53 (s, 2H), 4.26 (m, 1H), 3.91 (m, 5H), 3.22 (t, J = 8.0, 2H), 3.06 (t, J = 8.0, 2H), 2.77 (s, 3H), 2.62 (s, 3H), 2.53* (s, 3H), 2.46 (s, 3H), 2.25 (s, 3H), 1.13 (d, J = 6.5, 6H). *peak obscured by residual dimethyl sulfoxide solvent peak but predicted based on equivalent CH₃ peak in precursor (**5**). ¹³C NMR (126 MHz, dimethyl

sulfoxide-d6) δ 186.9, 170.8, 159.9, 156.0, 154.5, 153.8, 153.1, 151.4, 150.3, 146.4, 145.3, 144.6, 143.9, 142.7, 142.3, 134.8, 134.0, 133.2, 133.1, 132.3, 128.8, 128.8, 125.6, 125.6, 125.3, 124.4, 123.6, 120.6, 120.2, 118.6, 116.6, 111.5, 109.8, 105.7, 105.7, 99.1, 79.2, 55.9, 51.6, 48.4, 47.1, 38.1, 32.1, 23.3, 20.8, 19.8, 16.3, 14.6, 11.1. MS (ESI) calculated $[M+H]^+$: 979.4, found: 979.4.

Synthesis of Control Probe 2

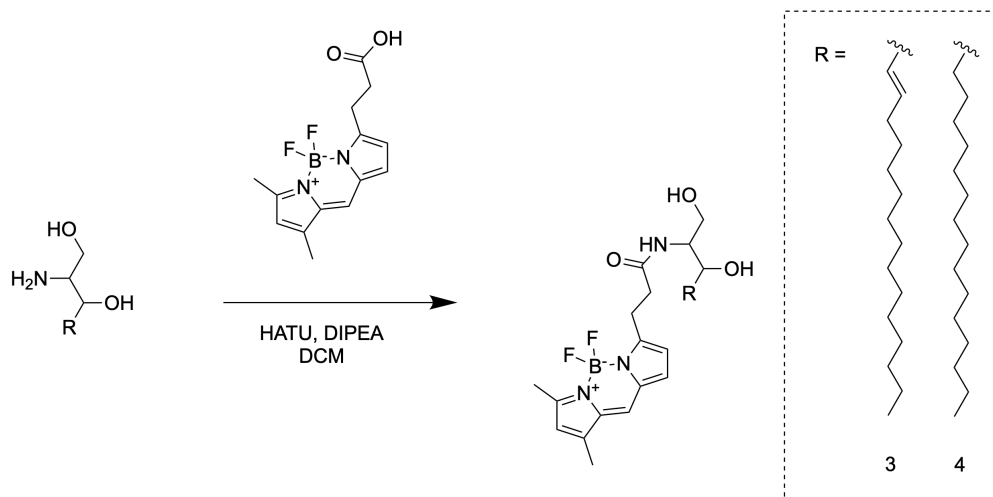


3-(isopropyl(BHQ-1-N₃)amino)phenol (6). BHQ-1-N₃³ (9.3 mg, 0.019 mmol) and 3-(isopropyl(prop-2-yn-1-yl)amino)phenol⁴ (5.4 mg, 0.029 mmol) were dissolved in 1.5 mL 9:1 DMF:H₂O. CuSO₄ pentahydrate (0.5 mg, 0.002 mmol) and sodium ascorbate (0.8 mg, 0.004 mmol) were added and the reaction stirred at 50 °C under inert atmosphere for 12 h. Solvent was removed by rotary evaporation, the residue dissolved in CH₃CN and purified using HPLC method A. Solvent was removed by rotary evaporation to yield **6** as a dark red solid (8.7 mg, 67 % yield). ¹H NMR (500 MHz, Chloroform-d) δ 7.86 (d, J = 8.9 Hz, 2H), 7.73 (s, 1H), 7.67 (d, J = 8.2 Hz, 1H), 7.59 (s, 1H), 7.49 (d, J = 8.2 Hz, 1H), 7.42 (s, 1H), 7.08 (s, 1H), 7.04 – 6.97 (m, 1H), 6.53 (d, J = 9.0 Hz, 2H), 6.30 (d, J = 8.6 Hz, 1H), 6.20 (d, J = 6.0 Hz, 2H), 4.54 (t, J = 5.5 Hz, 2H), 4.46 (s, 2H), 4.10 – 4.05 (m, 1H), 4.05 (s, 3H), 3.93 (t, J = 5.6 Hz, 2H), 2.74 (s, 3H), 2.72 (s, 3H), 2.53 (s, 3H), 1.13 (d, J = 6.6 Hz, 6H). ¹³C NMR (125 MHz, Chloroform-d) δ 157.1, 154.7, 151.2, 150.6, 149.8, 147.5, 145.0, 144.9, 143.4, 141.7, 133.6, 133.5, 133.1, 130.0, 125.9, 124.4, 123.2, 119.0, 119.0, 111.5, 106.0, 104.3, 101.0, 99.3, 56.2, 52.6, 48.9, 47.5, 40.0, 38.9, 21.4, 20.1, 16.8. MS (ESI) calculated $[M+H]^+$: 677.3, found: 677.3.

3-(isopropyl(BHQ-1-N₃)amino)phenyl Bodipy FL (2). BDP FL carboxylic acid [Lumiprobe, Hunt Valley, MD] (1.9 mg, 6.4 μ mol) was dissolved in 200 μ L dry DCM. 1 M N,N'-Dicyclohexylcarbodiimide in DCM (6.4 μ L, 6.4 μ mol) was added and the reaction stirred at rt for 10 min. **6** (4.3 mg, 6.4 μ mol) and 4-(Dimethylamino)pyridine (0.8 mg, 6.4 μ mol) were dissolved in 300 μ L dry DCM and added to the reaction before stirring under inert atmosphere at rt for 12 h. The reaction was filtered through a 0.2 μ m filter and dried by rotary evaporation. The residue was dissolved in 1:1 MeOH:CH₃CN and purified using HPLC method A. Solvent was removed by rotary evaporation to yield **1** as a dark red solid (0.6 mg, 10 % yield). ¹H NMR (500 MHz, dimethyl sulfoxide-d6) δ 7.95 (s, 1H), 7.87 (s, 1H), 7.71 (m, 4H), 7.50 (s, 1H), 7.28 (s, 1H), 7.12 (d, J = 8.2 Hz, 1H), 7.09 (m, 1H), 6.73 (d, J = 9.3 Hz, 2H), 6.56 (d, J = 8.6 Hz, 1H), 6.42 (m, 2H), 6.33 (d, J = 7.9 Hz, 1H), 6.31 (s, 1H), 4.55 (t, J = 5.9 Hz, 2H), 4.36 (s, 2H), 4.07 (m, 1H), 3.91 (m, 5H), 3.19

(t, J = 7.4 Hz, 2H), 2.97 (t, J = 7.4 Hz, 2H), 2.75 (s, 3H), 2.62 (s, 3H), 2.53* (s, 3H), 2.46 (s, 3H), 2.25 (s, 3H), 1.11 (d, J = 6.4 Hz, 6H). *peak obscured by residual dimethyl sulfoxide solvent peak but predicted based on equivalent CH₃ peak in precursor (6). ¹³C NMR (126 MHz, Chloroform-d) δ 171.4, 160.8, 156.5, 155.0, 152.0, 151.3, 150.8, 149.6, 148.0, 147.6, 145.2, 144.2, 143.6, 141.8, 135.4, 133.7, 133.4, 133.1, 130.0, 129.2, 128.4, 128.1, 126.0, 125.4, 124.9, 124.5, 124.1, 123.3, 120.7, 119.2, 119.1, 116.9, 111.5, 110.9, 109.8, 106.9, 99.4, 56.4, 52.7, 51.1, 48.9, 47.6, 40.4, 39.0, 33.7, 31.1, 29.9, 24.1, 21.6, 21.5, 20.1, 16.9, 15.1, 11.5. MS (ESI) calculated [M+H]⁺: 951.4, found: 951.4.

Synthesis of Bodipy FL-Sph 3 and Bodipy FL-Spa 4



Bodipy FL-sphingosine (3). BDP FL carboxylic acid [Lumiprobe, Hunt Valley, MD] (3.5 mg, 12.0 μmol) was dissolved in 200 μL dry DCM and stirred at 0 °C for 10 min. HATU (5.0 mg, 13.2 μmol) and DIPEA (8.4 μL, 47.9 μmol) were then added and the reaction stirred at 0 °C for 10 min. D-erythro-sphingosine (3.6 mg, 12.0 μmol) [Avanti Polar Lipids, Alabaster, AL] was then added and the reaction stirred at rt for 1 h. Solvent was removed by rotary evaporation and the residue dissolved in MeOH and purified using HPLC method A. Solvent was removed by rotary evaporation to yield **3** as a red solid (5.0 mg, 73 % yield). ¹H NMR (500 MHz, Chloroform-d) δ 7.09 (s, 1H), 6.89 (d, J = 3.9 Hz, 1H), 6.63 (bs, 1H), 6.31 (d, J = 3.9 Hz, 1H), 6.13 (s, 1H), 5.73 (m, 1H), 5.45 (m, 1H), 4.25 (m, 1H), 3.84 (m, 2H), 3.63 (m, 1H), 3.30 (t, J = 7.4 Hz, 2H), 2.75 (t, J = 7.4 Hz, 2H), 2.59 (s, 3H), 2.25 (s, 3H), 2.00 (q, J = 7.2 Hz, 2H), 1.38-1.18 (m, 22H), 0.87 (t, J = 7.1 Hz, 3H). ¹³C NMR (125 MHz, Chloroform-d) δ 173.2, 161.1, 156.5, 144.5, 135.5, 134.4, 133.4, 128.5, 128.1, 124.0, 120.9, 117.5, 74.3, 62.3, 55.1, 35.8, 32.4, 32.1, 29.9, 29.8, 29.6, 29.5, 29.4, 29.2, 25.1, 22.8, 15.2, 14.3, 11.5. MS (ESI) calculated [M+Na]⁺: 596.4, found: 596.5.

Bodipy FL-sphinganine (4). BDP FL carboxylic acid [Lumiprobe, Hunt Valley, MD] (6.1 mg, 20.9 μmol) was dissolved in 200 μL dry DCM and stirred at 0 °C for 10 min. HATU (8.7 mg, 23.0 μmol) and DIPEA (14.6 μL, 83.5 μmol) were then added and the reaction stirred at 0 °C for 10 min. D-erythro-sphinganine (6.3 mg, 20.9 μmol) [Avanti Polar Lipids, Alabaster, AL] was then added and the reaction stirred at rt for 1 h. Solvent was removed by rotary evaporation and the residue dissolved in MeOH and purified using HPLC method A. Solvent was removed by rotary evaporation to yield **4** as a red solid (9.7 mg, 81 % yield). ¹H NMR (500 MHz, Chloroform-d) δ

7.09 (s, 1H), 6.89 (d, J = 3.9 Hz, 1H), 6.80 (bs, 1H), 6.32 (d, J = 3.9 Hz, 1H), 6.13 (s, 1H), 3.90 (m, 1H), 3.76 (bs, 1H), 3.68 (m, 2H), 3.29 (t, J = 7.0 Hz, 2H), 2.75 (t, J = 7.0 Hz, 2H), 2.55 (s, 3H), 2.25 (s, 3H), 1.43 (m, 2H), 1.24 (m, 26H), 0.87 (t, 7.1 Hz, 3H). ¹³C NMR (125 MHz, Chloroform-d) δ 172.9, 161.1, 156.5, 144.5, 135.5, 133.4, 128.1, 124.0, 120.9, 117.5, 73.8, 62.3, 54.6, 35.7, 34.3, 32.1, 29.9, 29.8, 29.5, 26.1, 25.1, 22.8, 15.2, 14.3, 11.5. MS (ESI) calculated [M+Na]⁺: 598.4, found: 598.5.

Supporting Figures

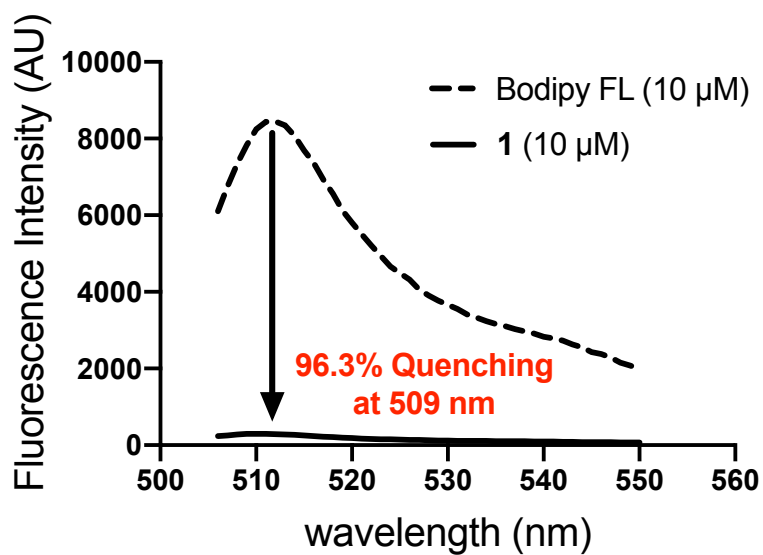


Figure S1. Quenching efficiency of Black Hole Quencher-1 (BHQ-1) in probe **1**. 10 μM solutions of Bodipy FL or probe **1** were prepared in PBS buffer. Fluorescence emission spectra from 505-550 nm (ex. 480 nm) for each sample were obtained on a Tecan Spark multimode plate reader.

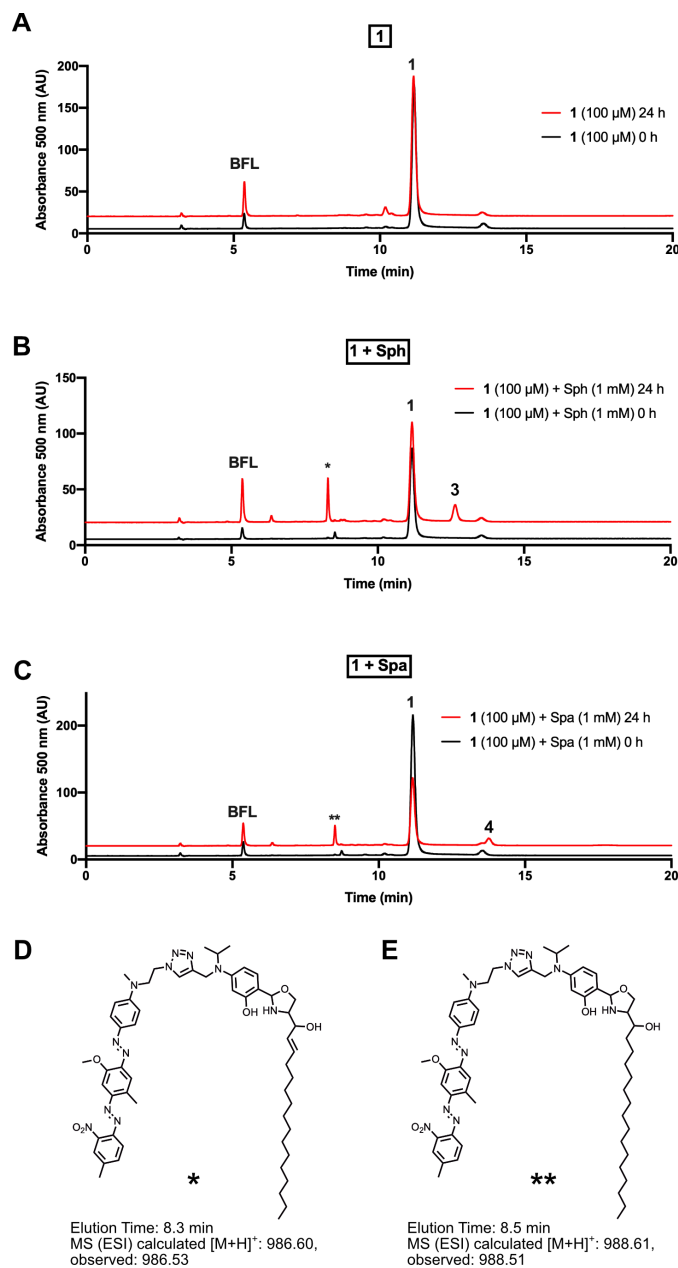


Figure S2. Probe **1** reacts with Sph and Spa to form Bodipy FL-labeled sphingolipid products **3** and **4**. (A-C) HPLC A500 traces before and after incubation of **1** (100 μ M) alone (A), with Sph (1 mM) (B), or with Spa (1 mM) (C). Under reaction conditions, small amounts of Bodipy FL carboxylic acid **BFL** are generated due to background hydrolysis. (D-E) Side products were identified upon reaction of **1** with Sph or Spa. These were identified as adducts between the 2-hydroxy-4-(isopropyl(BHQ-1- N_3)amino)benzaldehyde **5** (which is released during the formation of the fluorescent lipid product) and Sph (D) or Spa (E). Analogous side products were previously reported for the reaction between Sph and other salicylaldehyde esters.⁵ Peaks were identified from known standards of **1**, **3**, **4**, **BFL** (Bodipy FL carboxylic acid) or by MS analysis of purified peaks * (D) and ** (E) (see *Mass Spectra*).

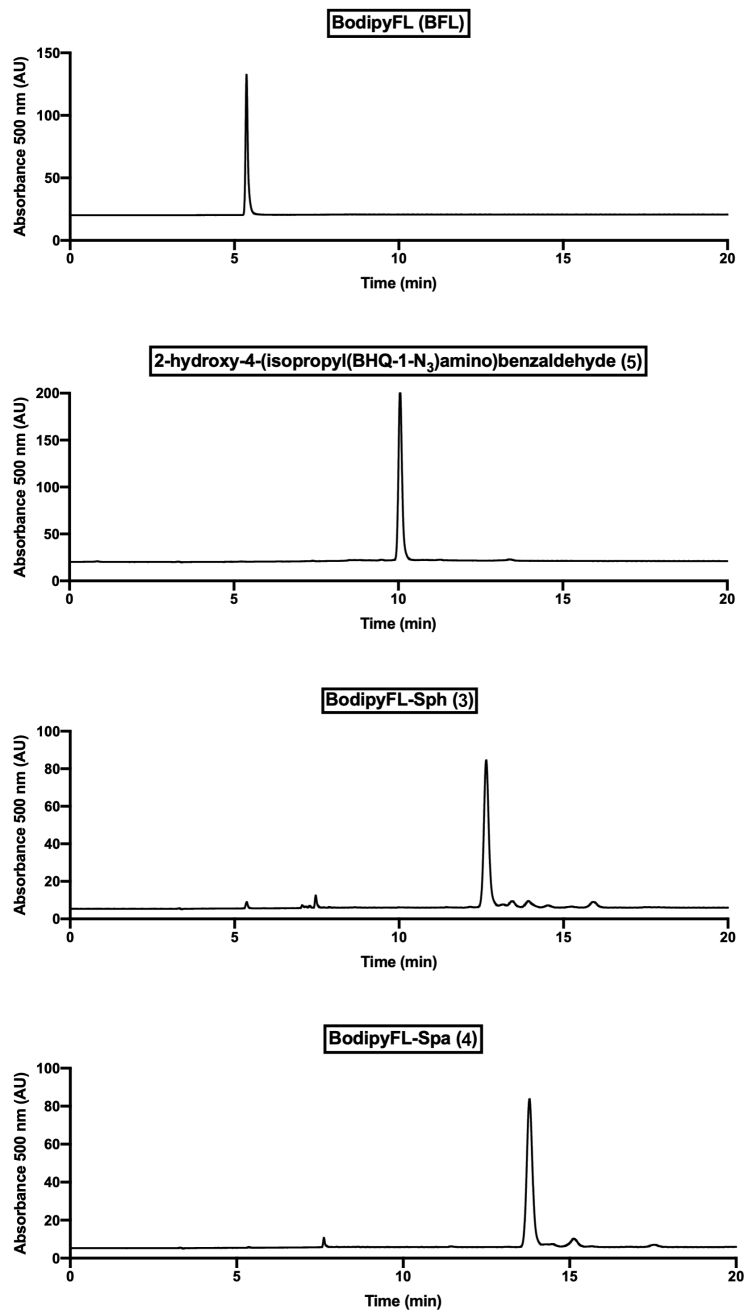


Figure S3. HPLC A500 traces of known standards of Bodipy FL carboxylic acid (A), 2-hydroxy-4-(isopropyl(BHQ-1-N₃)amino)benzaldehyde **5** (B), Bodipy FL-Sph **3** (C), and Bodipy FL-Spa **4** (D).

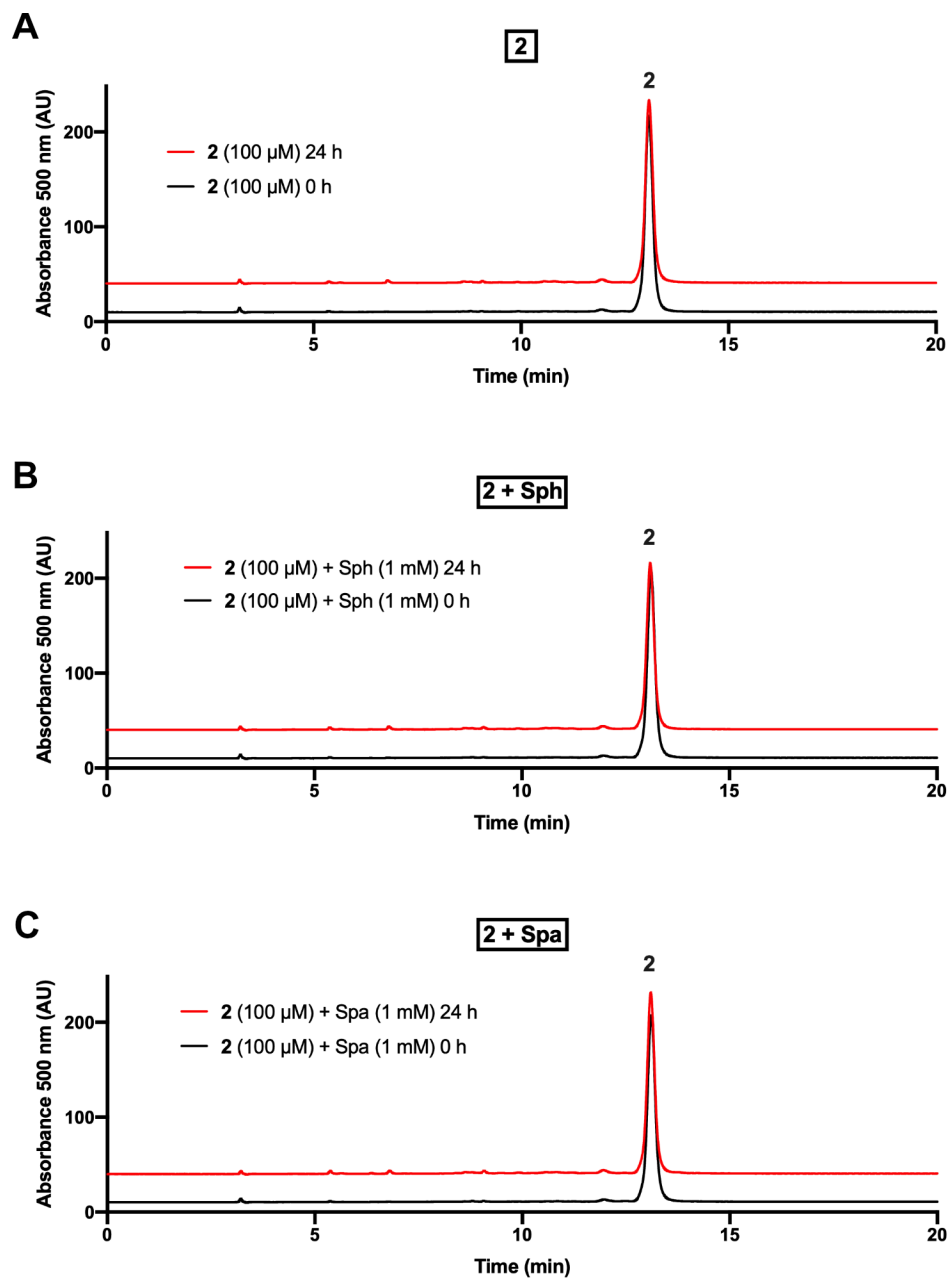


Figure S4. Probe **2** is unreactive toward Sph and Spa. (A-C) HPLC A500 traces before and after incubation of **2** (100 μM) alone (A), with Sph (1 mM) (B), or with Spa (1 mM) (C).

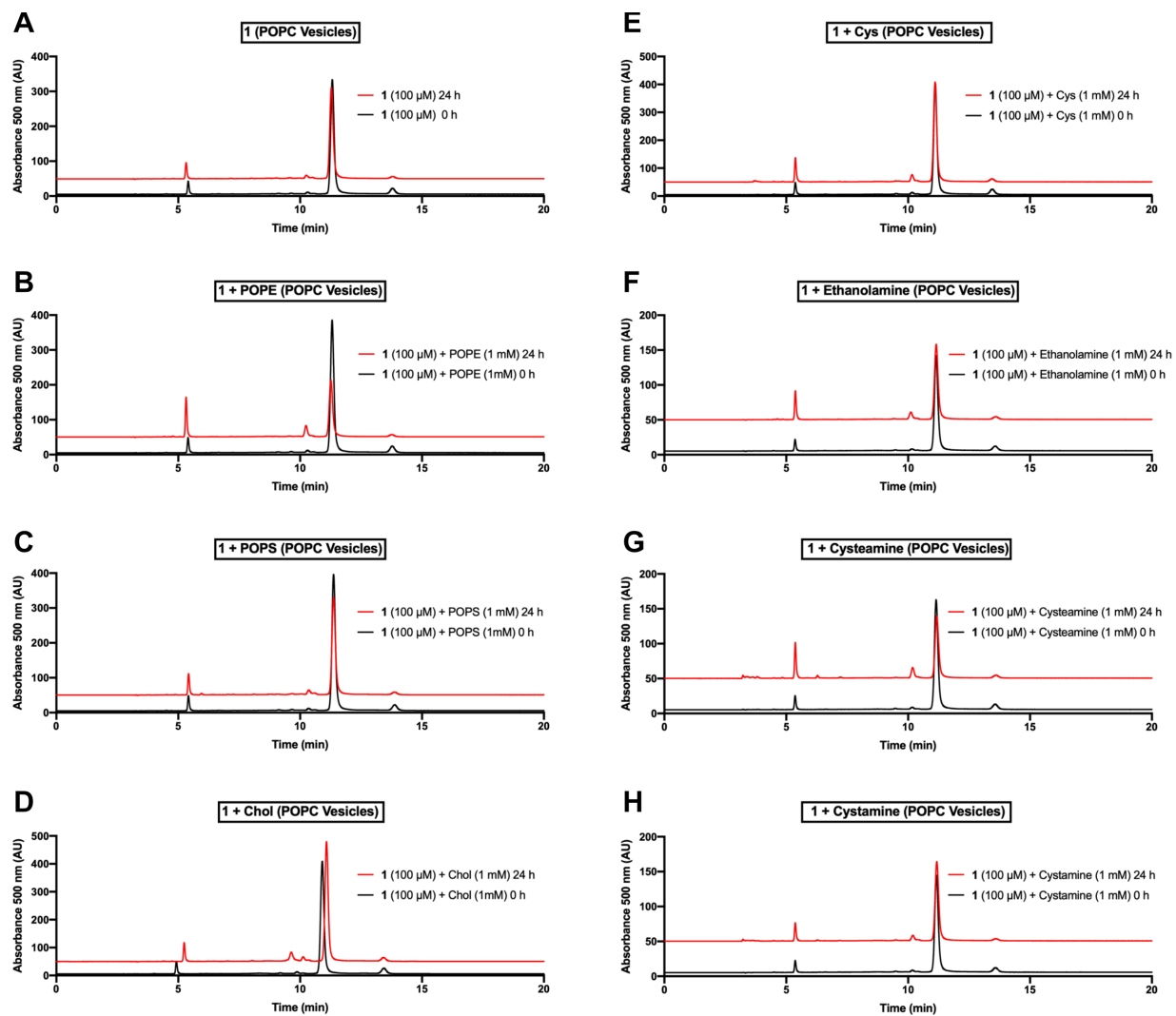


Figure S5. Probe **1** stability and reactivity in the presence of naturally abundant lipid species, and cysteine, ethanolamine, cysteamine, and cystamine. (A-H) HPLC A500 traces before and after incubation of **1** (100 μ M) in POPC Vesicles (5 mM) Alone (A), and with POPE (1 mM) (B), POPS (1 mM) (C), cholesterol (1 mM) (D), cysteine (1 mM) (E), ethanolamine (1 mM) (F), cysteamine (1 mM) (G), or cystamine (1 mM) (H). Elution times normalized to $t = 14$ min peak.

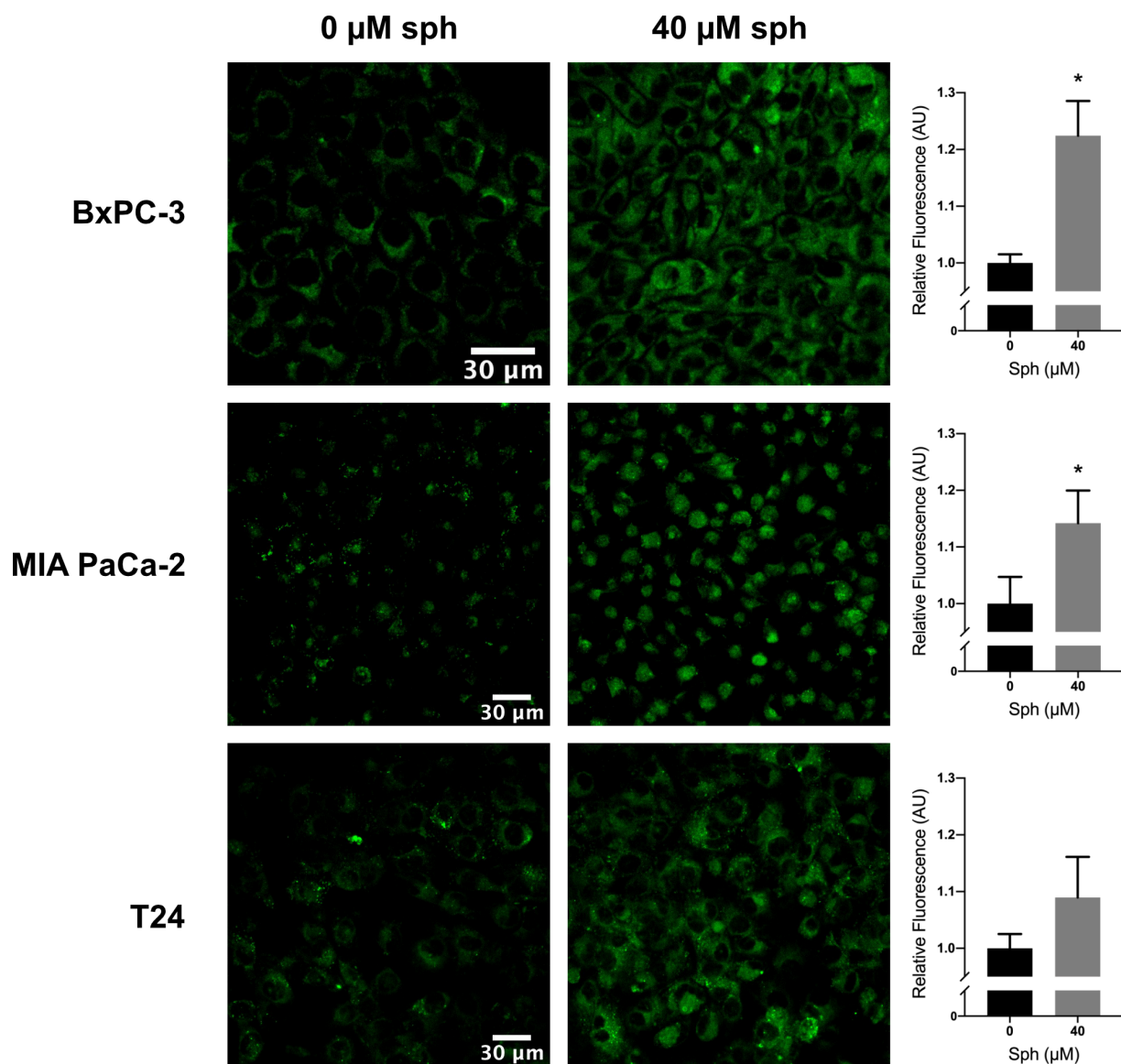


Figure S6. Fluorescence microscopy images of BxPC-3, MIA PaCa-2, and T24 cells treated with probe 1 and exposed to Sph (40 μM) for 20 h. Quantified fluorescence response of 1 within large populations of cells after treatment with Sph (40 μM) is shown to the right of each pair of representative images. Values are reported as means ± SD. Statistically significant changes in fluorescence are indicated as determined by an unpaired t-test: *P<0.05.

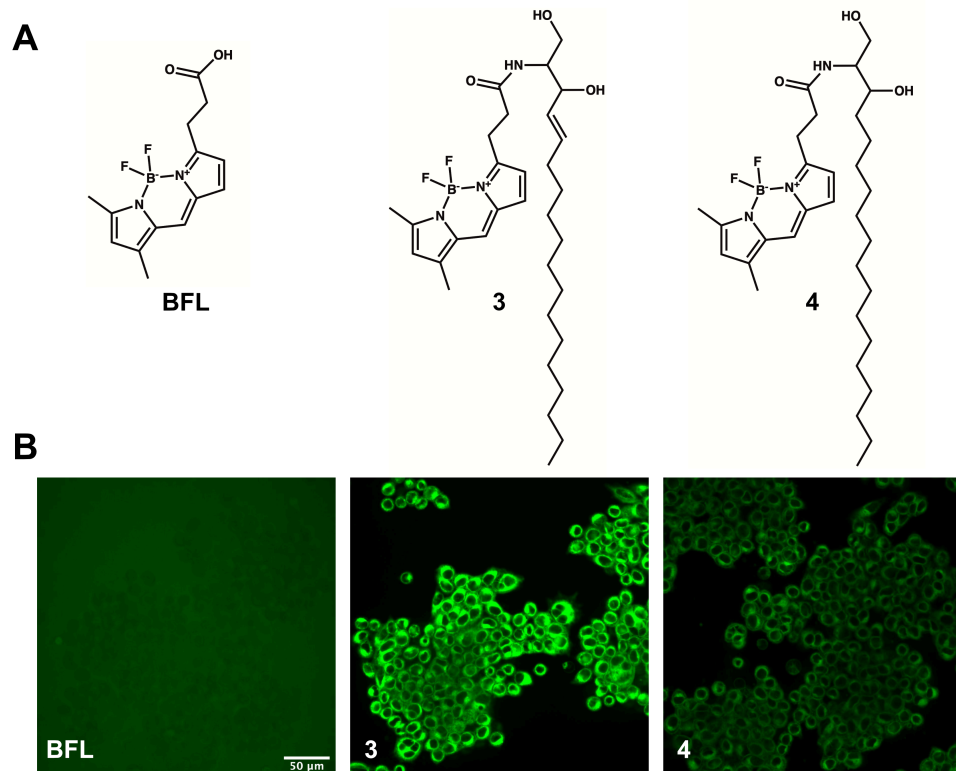


Figure S7. (A) Chemical structures of Bodipy FL carboxylic acid (**BFL**), Bodipy FL-sphingosine (**3**), and Bodipy FL-sphinganine (**4**). (B) Fluorescence microscopy images of HeLa cells after a 1 h incubation with 5 μ M **BFL**, **3**, and **4**. **BFL** does not partition into biological membranes while both Bodipy FL-labeled sphingoid bases **3** and **4** show membrane localization similar to that observed in cells treated with compound **1**.

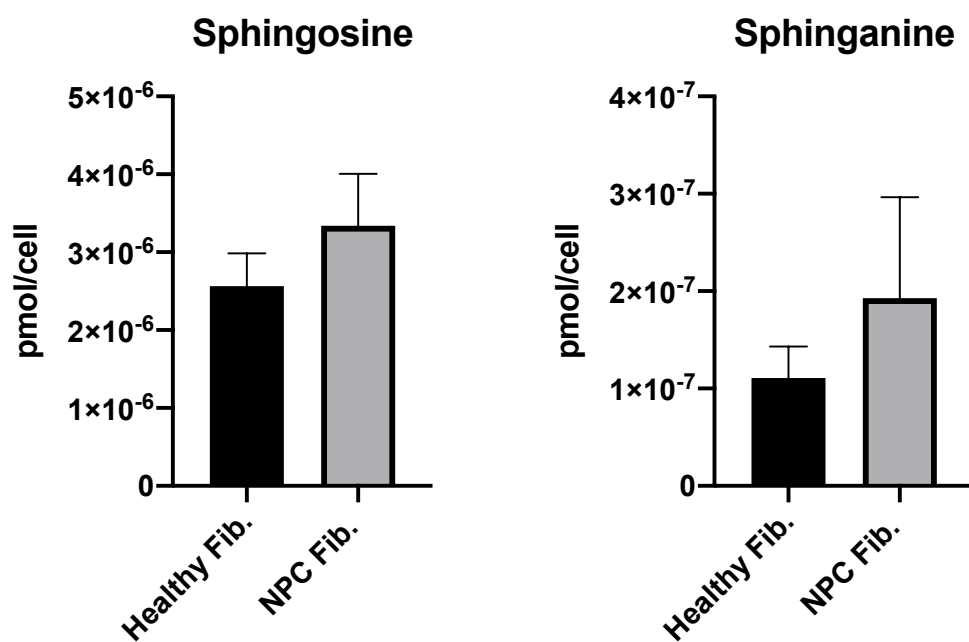
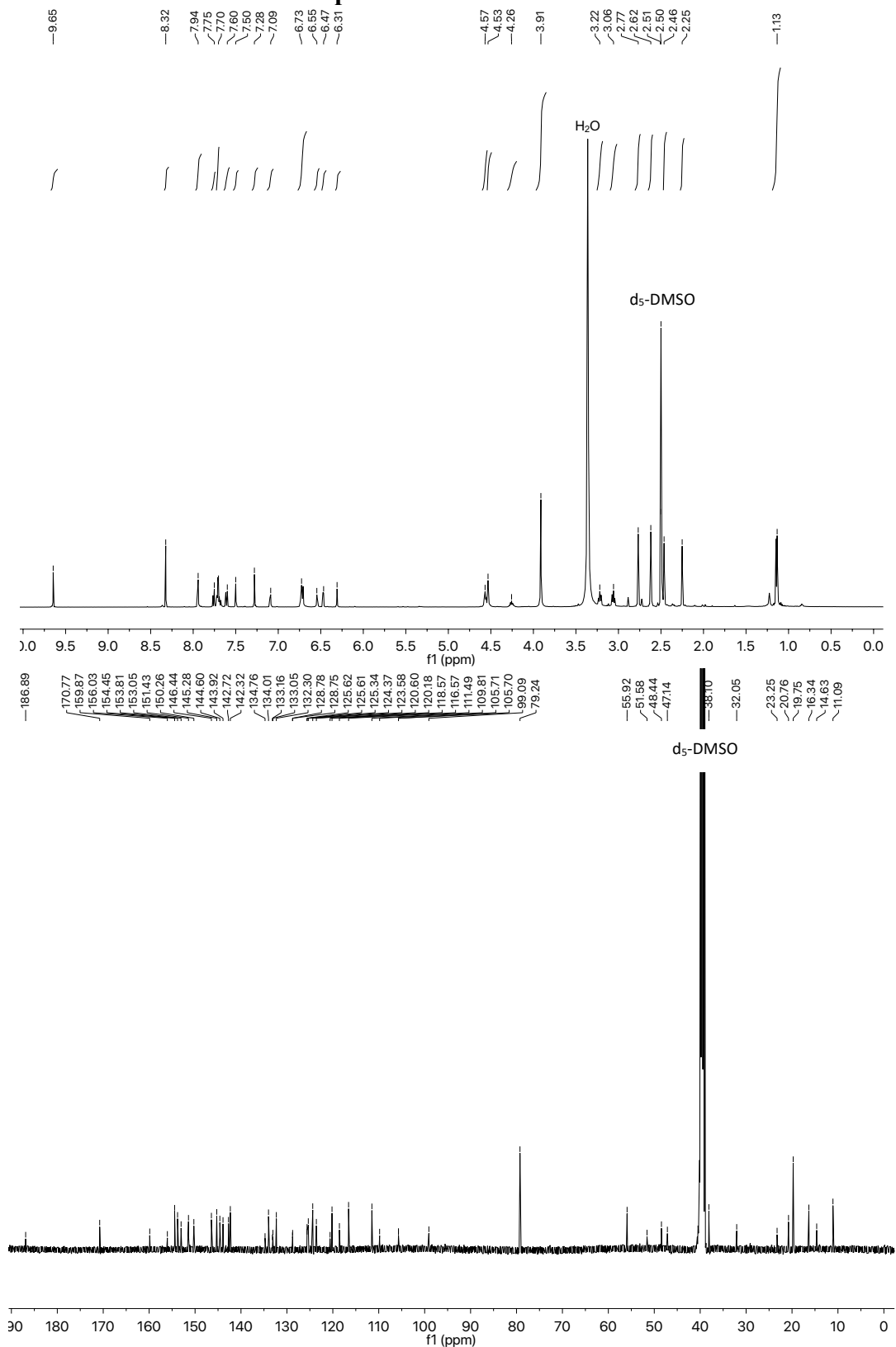


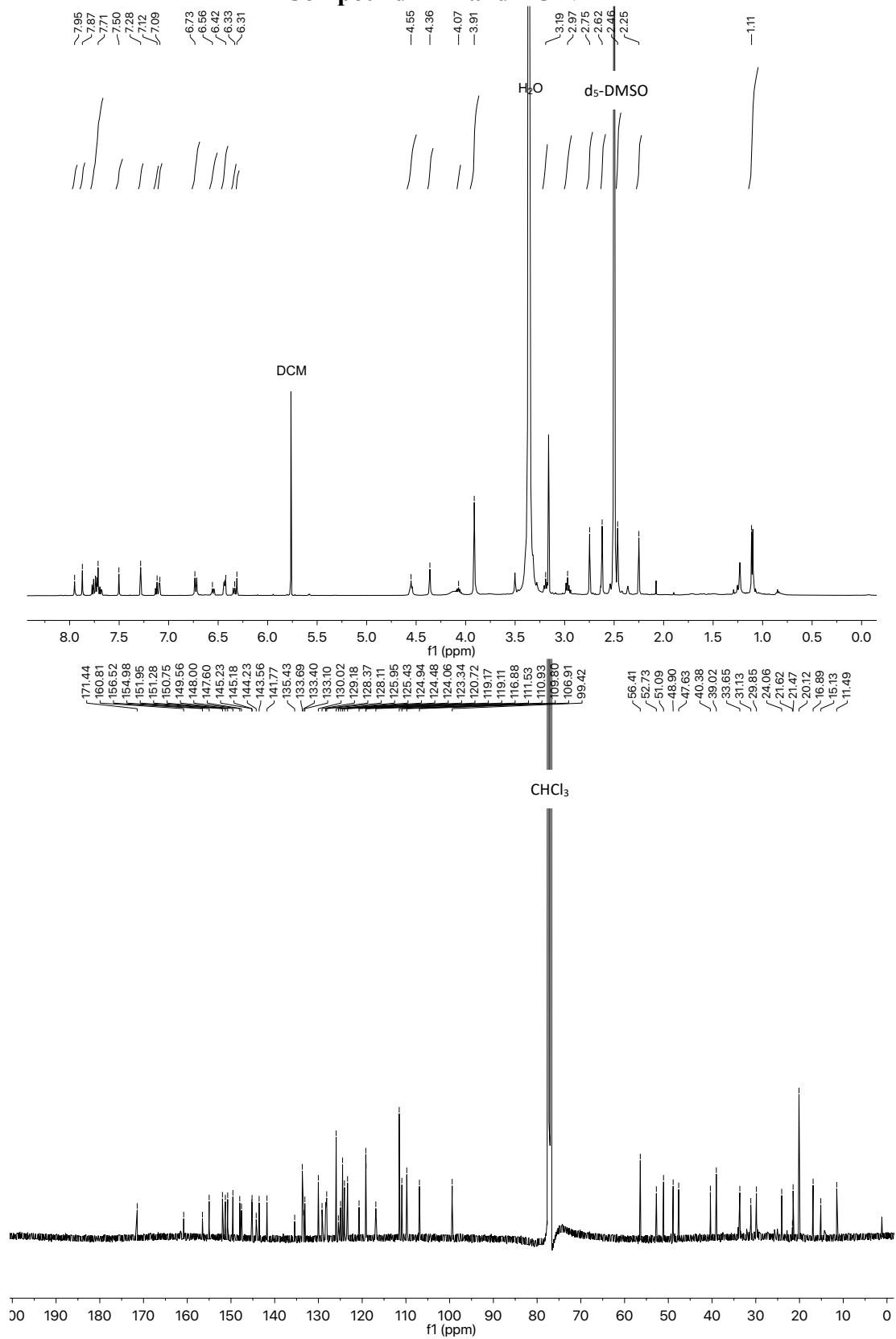
Figure S8. LCMS detection of Sph and Spa in Healthy and NPC1 fibroblasts. Values are reported as means \pm SD.

NMR Spectra

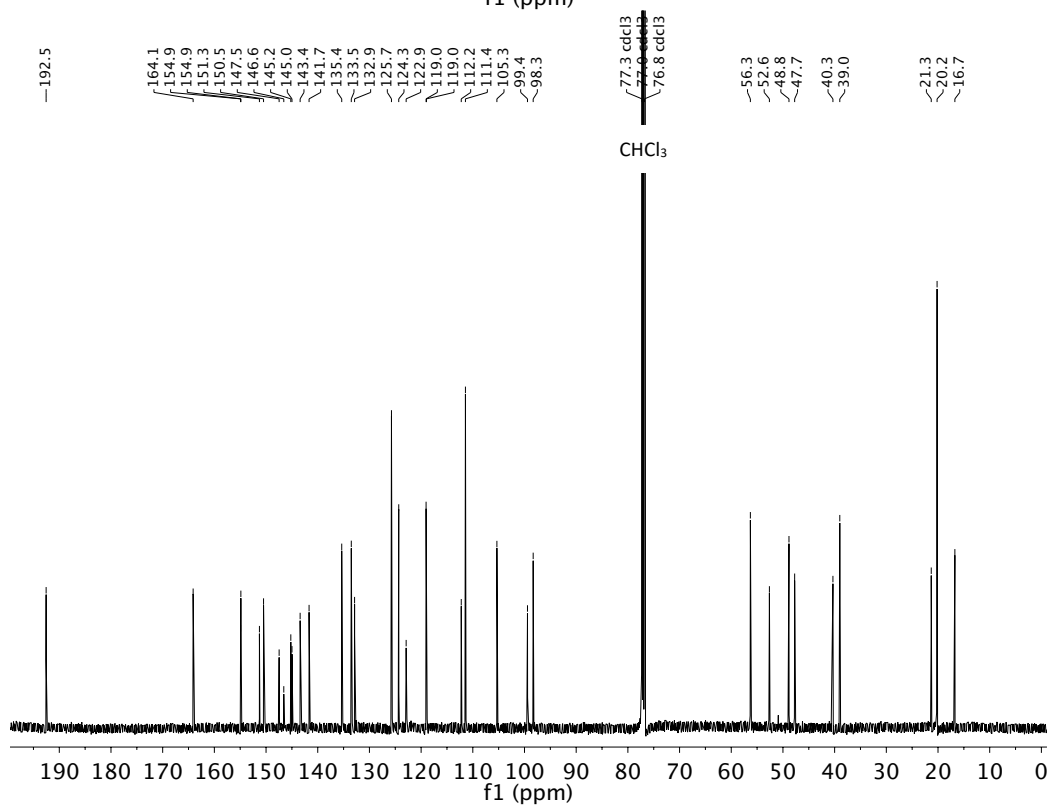
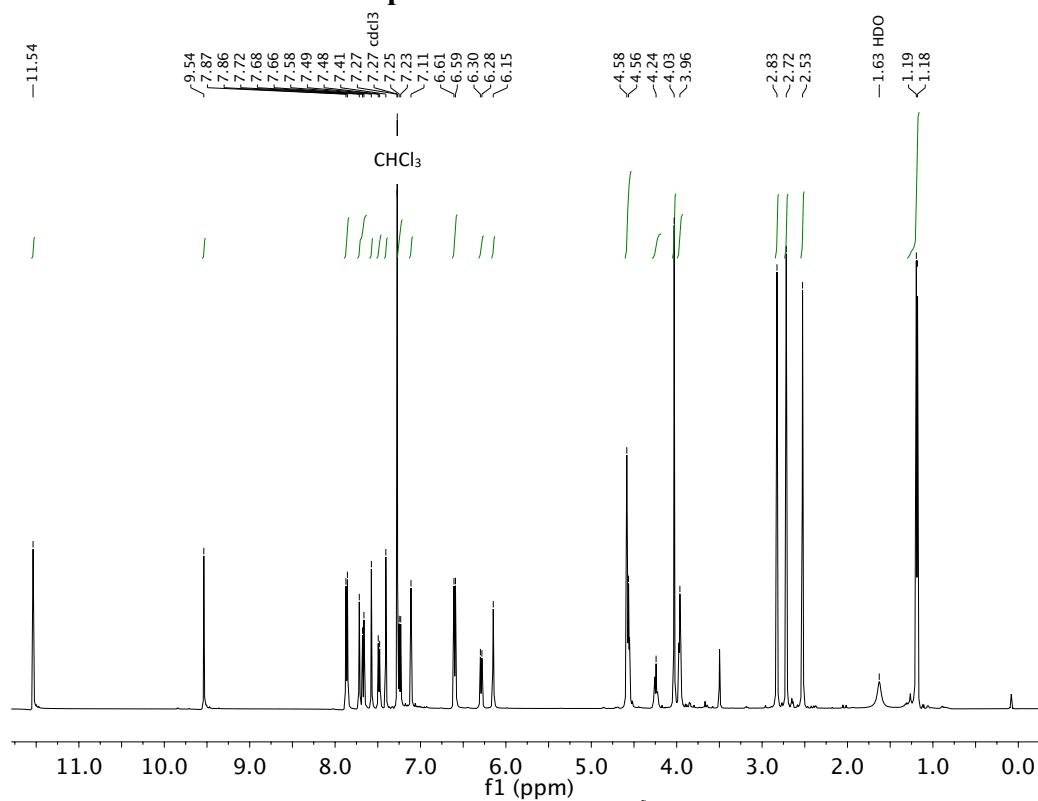
Compound 1 ^1H and ^{13}C NMR



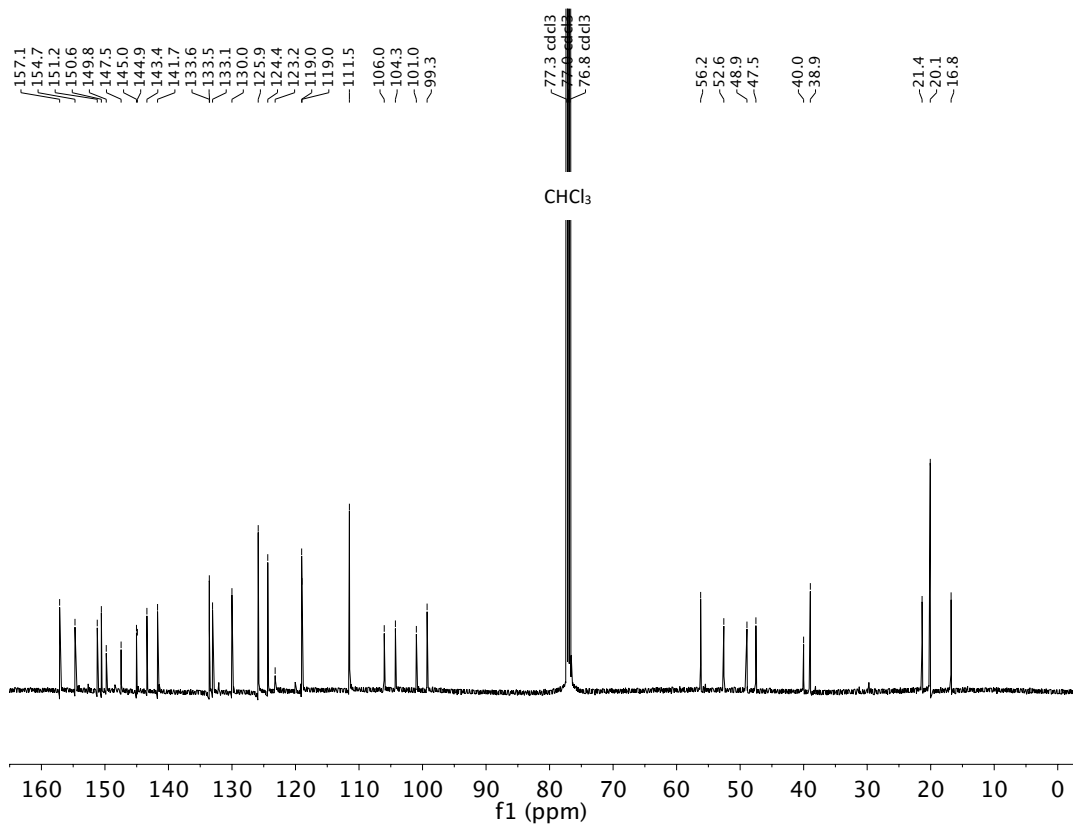
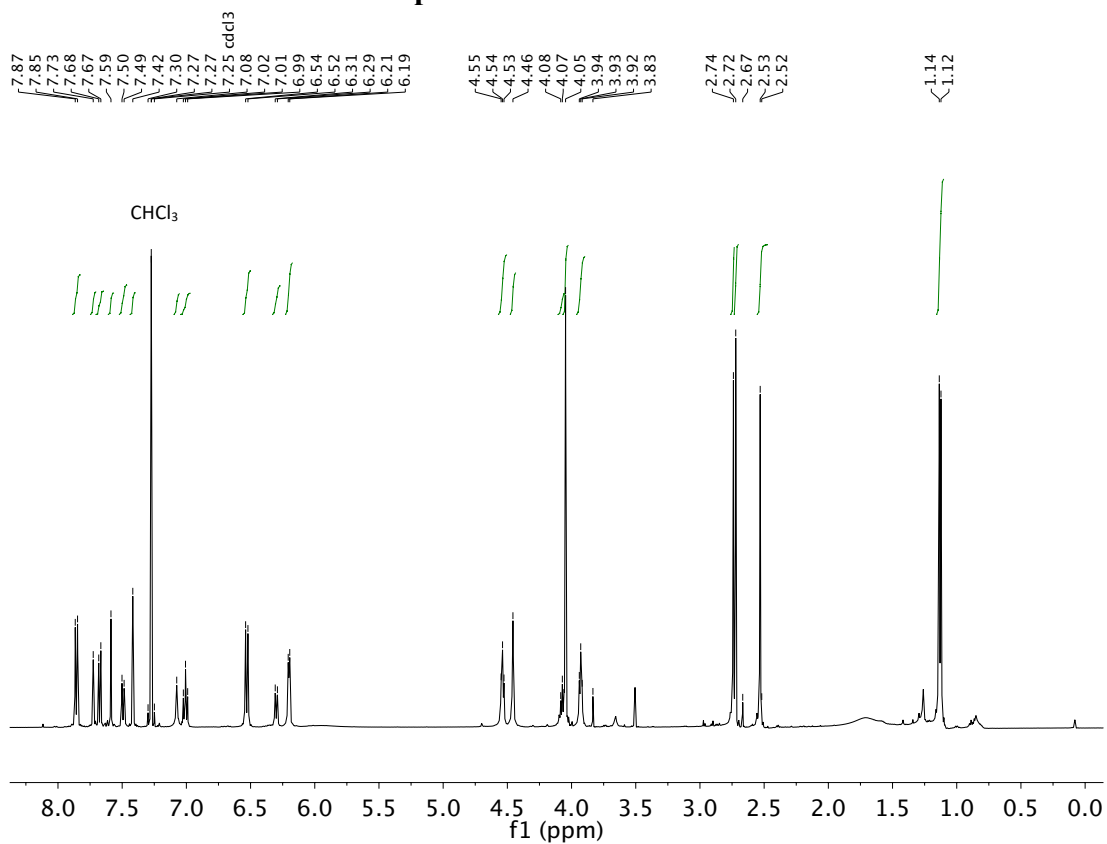
Compound 2 ¹H and ¹³C NMR



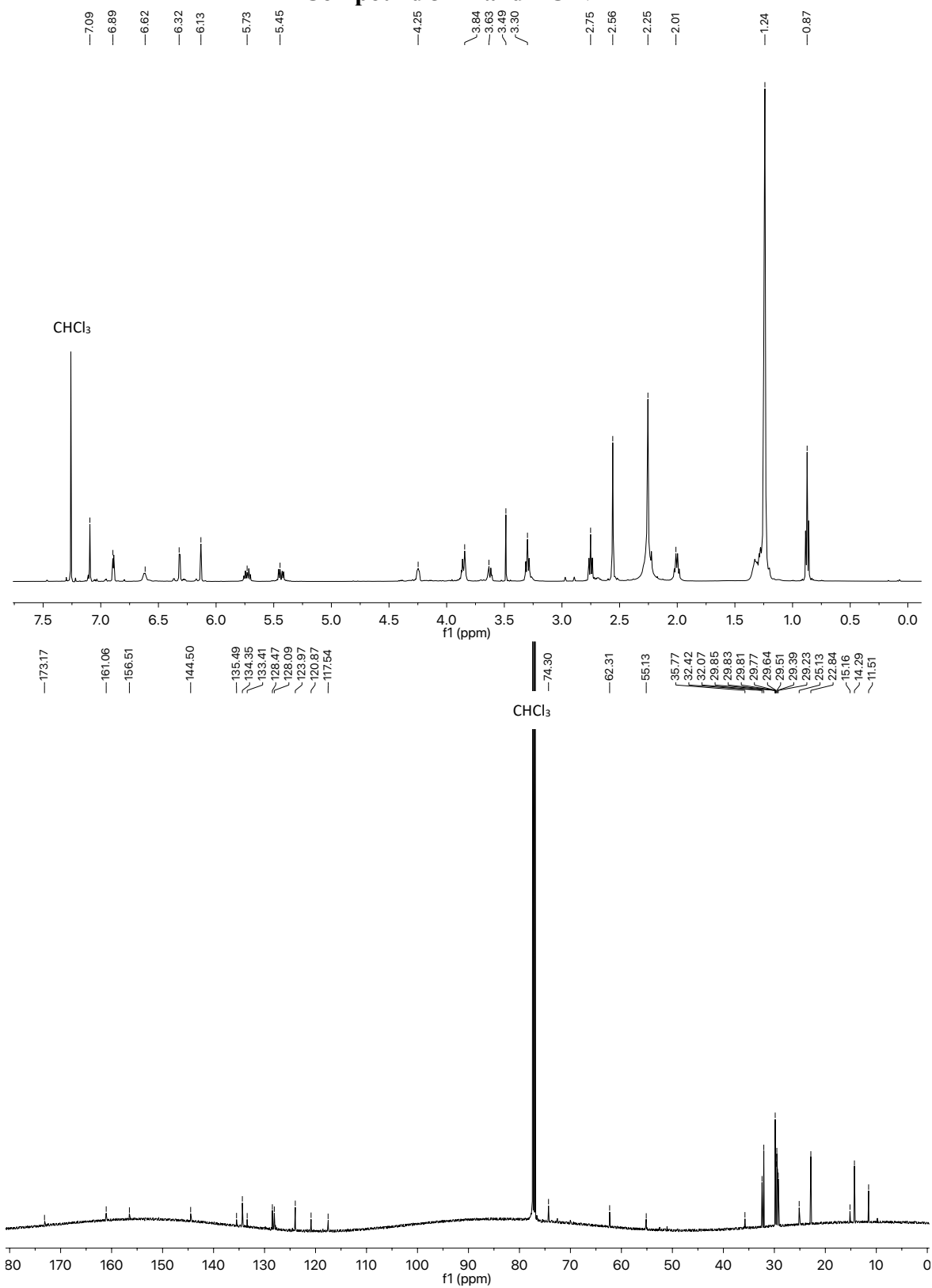
Compound 5 ¹H and ¹³C NMR



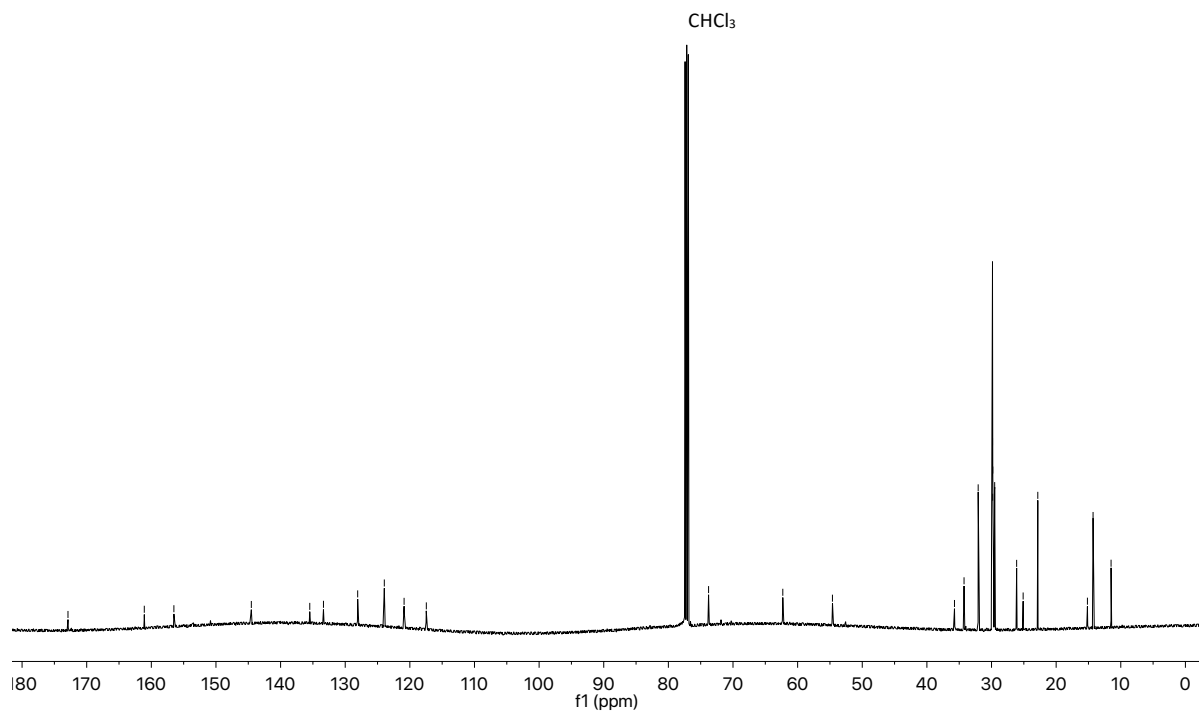
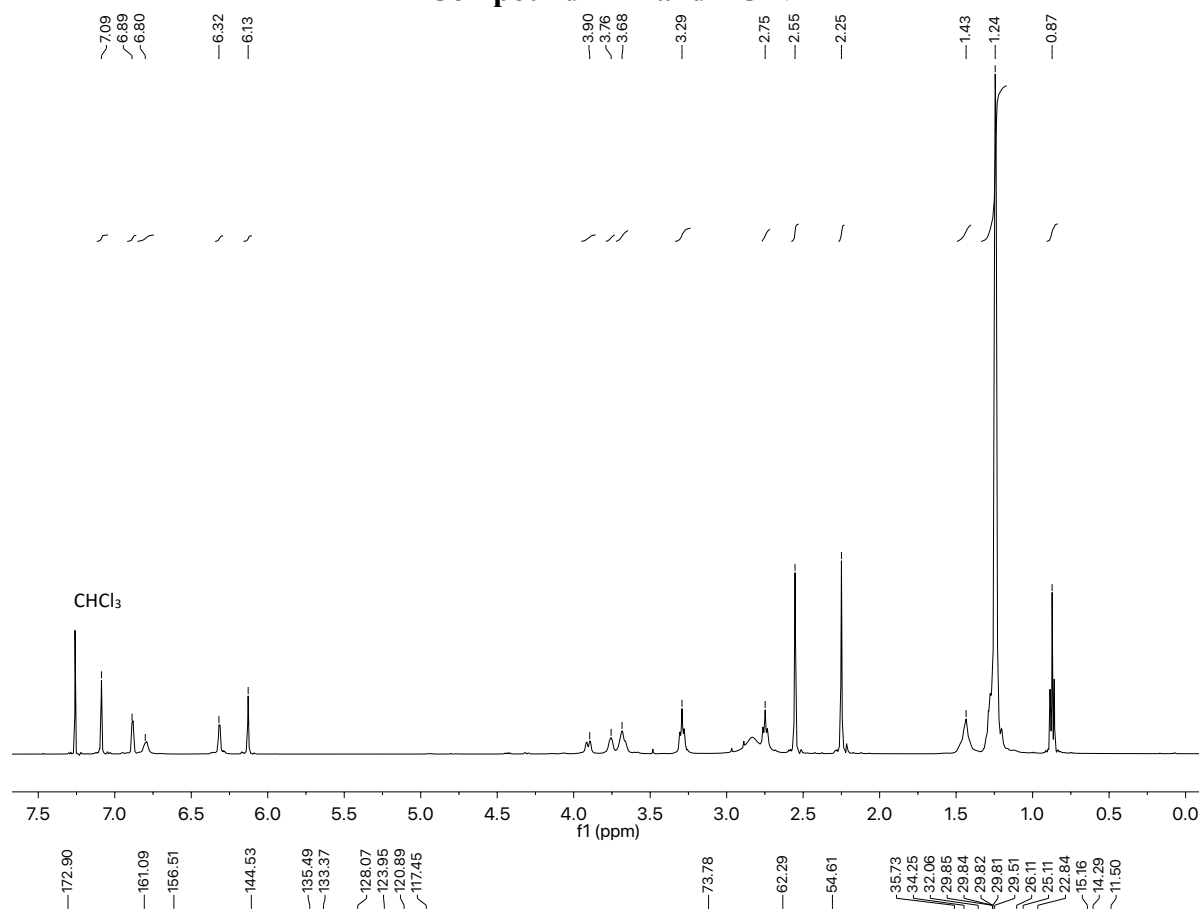
Compound 6 ¹H and ¹³C NMR



Compound 3 ¹H and ¹³C NMR



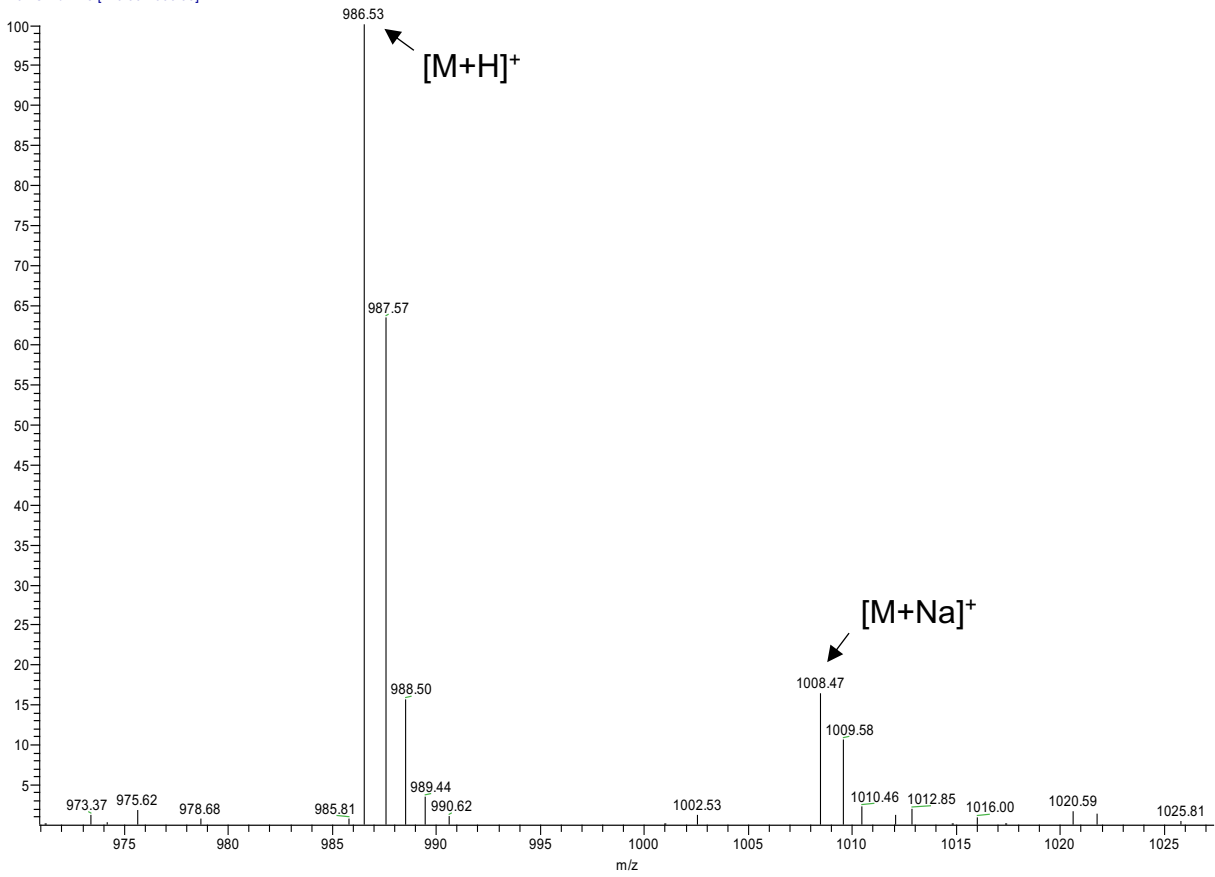
Compound 4 ¹H and ¹³C NMR



Mass Spectra

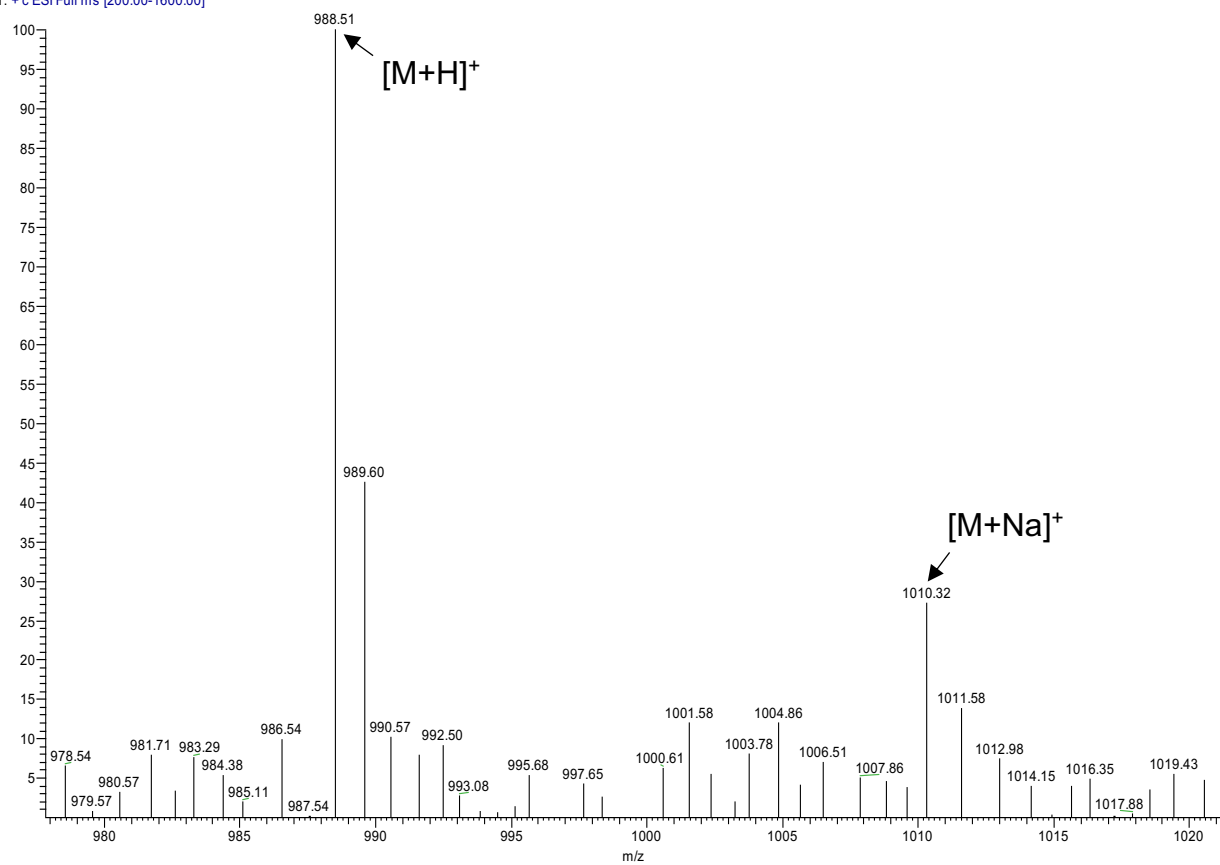
Side Product *

AP-R1-a #86-90 RT: 1.86-1.94 AV: 5 SB: 4 1.24-1.31 NL: 2.86E7
T: + c ESI Full ms [120.00-1600.00]



Side Product **

AR-P2-a #54-58 RT: 1.13-1.21 AV: 5 SB: 5 0.18-0.26 NL: 1.23E7
T: + c ESI Full ms [200.00-1600.00]



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