Lysosome targeted photoactivation reveals local sphingosine metabolism signature

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**Supplementary figures**

**Figure S1.** Analysis of uncaging efficiency of caged probes. (A) HPLC traces of Lyso-So after exposing to UV light for indicated time. Spectra indicate absorbance at the wavelength of 360 nm. Lyso-So (500 μM) solution (10 % DMSO in water) was illuminated by UV light (350 - 450 nm) for 30 s, 1 min and 2 min respectively. The peak at ~0.25 min in blank corresponds to the solvent (DMSO) peak. (B) Mass spectrum of Lyso-Chol. Due to the hydrophobicity of Lyso-Chol and cholesterol, the uncaging efficiency was analysed by mass spectrometry. Lyso-Chol was dissolved in a mixture of chloroform and methanol (1:1 v/v) (~ 0.1 mg/mL), and illuminated by UV light for 2 min and 5 min, respectively. The peaks in the red square corresponds to Lyso-Chol.
Figure S2. Effect of pH on fluorescence of the caged probes. Caged probes was first dissolved in water (1% DMSO) with indicated pH. The fluorescence intensity was recorded using a Molecular Devices SpectraMax M5 fluorescence reader. The excitation wavelength was set at 360 nm, cut off at 455 nm, and emission was collected at 465 nm. The fluorescence signals were plotted after normalization to pH 7. Data represents the average of three measurements. Error bars represent standard deviation.

Figure S3. Representative fluorescence images of Lyso-Chol (5 μM) in the presence of LysoTracker Red DND-99 (100 nM) in living HeLa cells. Cells were treated with Lyso-Chol and LysoTracker for 15 min at 37 ºC, washed twice by HBSS (5 min each), and replaced by HBSS prior to fluorescence imaging. Due to the relative weaker fluorescence, Lyso-Chol staining was presented in “fire” mode using the look-up table of ImageJ; and was used as the blue color for image merging. Scale bar: 5 μm.

Figure S4. Analysis of D7-ceramides in cells treated with caged probes. Cells were treated with D7-Lyso-So (2 μM) for 15 minutes, washed twice, replaced by new HBSS solution, with/without illumination under UV light for 2 min on ice. Similarly, cells were treated with D7-Sph-Cou (2 μM) or D7-Mito-So (2 μM) for 15 minutes, replaced by new HBSS solution, with/without illumination under UV light for 2 minutes on ice. After UV illumination, cells were immediately collected for lipid extraction. Data is presented as percentage of total measured lipid signals (% of total). Data represents the average of three independent experiments. Error bars represent SEM. *** p< 0.001, n.s., not significant, students’ t-test.
Figure S5. Analysis of major lipid species in the time course experiments. HeLa cells were treated with D7-Lyso-So (2 μM) for 15 minutes, washed twice, replaced by new HBSS solution, and illuminated under UV light for 2 min on ice. Cells were either immediately collected for lipid extraction or incubated at 37 ºC for indicated time before lipid extraction. Data was normalized by measured total lipid signals and presented as “% of total”. Data represents the average of three independent experiments. Error bars represent SEM. Abbreviations: PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; CL: cardiolipin; SM: sphingo-myelin; CER: ceramide.
Figure S6. Characterization of mutation efficiencies in CERS5/6 double knockout cells. Sanger sequencing was performed to characterize CERS5 and CERS6 loci of CERS5/6 double knockout cells. Sequencing results obtained from wild type cells and CERS5/6 double knockout cells are shown (left). TIDE (tracking of indels by decomposition) analysis was done using these results (right) to characterize indel patterns. The amount of signal corresponding to wild type controls (yellow bar) was used to calculate mutation efficiency, which was 93.2% and 88.7% for CERS5 and CERS6, respectively. PAM: protospacer adjacent motif.

Table S1. Single guide RNA sequences for genome editing.

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<th>Target locus</th>
<th>Guide RNA</th>
<th>Protospacer adjacent Motif</th>
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<tr>
<td>CERS6</td>
<td>TGTATTGGCATCGAGGACAG</td>
<td>TGG</td>
</tr>
<tr>
<td>CERS6</td>
<td>TTTTCAGAATGGCATTGGG</td>
<td>CGG</td>
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<tr>
<td>HPRT</td>
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Table S2. Primers for PCR of genome DNA.

<table>
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<th>Primers</th>
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<td>CERS6</td>
<td>TAATGTACTGCTGGGGGCTA</td>
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<tr>
<td>CERS6</td>
<td>TTAAGACTCTCTGACCTCTCTT</td>
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Figure S7. Endogenous lipid profiles of HeLa wild-type (WT) and CerS 5/6 dKO cells. Cells were treated with D<sub>7</sub>-Lyso-So (2 μM) for 15 minutes, washed twice, replaced by new HBSS solution, and illuminated under UV light for 2 min on ice. The cells were incubated at 37 °C for 5 minutes before lipid extraction. Similarly, cells were treated with D<sub>7</sub>-Sph-Cou (2 μM) or D<sub>7</sub>-Mito-So (2 μM) for 15 minutes, replaced by new HBSS solution, and illuminated under UV light for 2 minutes on ice. The cells were incubated at 37 °C for 5 minutes before lipid extraction. In the control condition, cells were directly collected for lipid extraction. Data was normalized by measured total lipid signals and presented as “% of total”. Data represents the average of three independent experiments. Error bars represent SEM. Abbreviations: PC, phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; CL: cardiolipin; SM: sphingomyelin; CER: ceramide.
Figure S8. Work flow of the uncaging assay. Details are described in the “General procedures” section.
General procedures

Chemicals and reagents

Chemicals and reagents were purchased from commercial sources and were used without further purification unless otherwise mentioned. Sphingosine was purchased from Echelon Bioscience. d7-Sphingosine was purchased from Avanti Polar Lipids. LysoTracker Red DND-99 was a gift from Jean Gruenberg lab (Department of Biochemistry, University of Geneva) originally purchased from Invitrogen, Thermo Fisher Scientific. D7-Sph-Cou and D7-Mito-So were synthesized using previously published protocols [1,2]. Deuterated solvents were obtained from Cambridge Isotope Laboratories, Inc. The plasmid pX330 was deposited to Addgene (plasmid #42230) by Feng Zhang (Broad Institute, Cambridge).

Cell culture and experimental conditions

HeLa MZ cells were cultured at 37 °C and 5 % CO2 in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) with 4.5 g/L glucose, supplemented with 10 % fetal calf serum (FCS, Hyclone) and 1 % Pen/Strep (Gibco). Cell numbers were quantified by Countess II Automated Cell Counter (Invitrogen, ThermoFisher Scientific) following manufacturer’s protocol.

For fluorescence imaging experiments, cells were seeded in 35 mm glass bottom MatTek dishes and grown until ~ 80 % confluency before use. Then cells were treated with caged probes in HBSS buffer (#14025-092, Gibco) for 15 minutes at 37 °C, washed twice (5 min each) by HBSS solution, and finally replaced with new HBSS solution right before fluorescence imaging.

For lipidomic experiments, 0.5 x 10⁶ cells were seeded in 60 mm tissue culture dishes and grown for ~ 48 h to reach 100 % confluency. Cells were then treated with caged probes (2 μM) in HBSS buffer (#14025-092, Gibco) for 15 minutes at 37 °C. As for D7-Sph-Cou (2 μM) and D7-Mito-So (2 μM), cells were replaced by new HBSS solution and subsequently went for uncaging, as previously described [2]. For D7-Lyso-So (2 μM), cells were washed twice (5 min each) by HBSS solution, and finally replaced with new HBSS solution right before uncaging.

The uncaging experiments were performed using a 1000 Watt Arc Lamp Source (#66924, NewPort) equipped with a dichromic mirror (350 - 450 nm, #66226). For live-cell uncaging, cells were placed on the ice under the lamp at a distance of 20 cm, and irradiated 120 seconds at 100 Watt. Cells were either placed back in a 37 °C incubator, and/or immediately processed for lipid extraction. For lipid extract uncaging, the lipid extract was re-suspended in 100 μL water in a glass vial, vortexed and irradiated on ice for 10 min. And then the samples were dried in vacuum for further analysis.

Fluorescence images were captured using a Leica SP8 confocal microscope equipped with a 63 x oil immersion objective. The fluorescence images were analyzed by Fiji software [3] following the same procedures as previously described [2]. In each cell, mean intensity of the lysosomes was measured and compared to mean intensity of the whole cell. The area of a cell was manually selected and the information was kept in “ROI manager”. In order to select the area of lysosomes, the lysosome particles were chosen using the built-in “threshold” function of FIJI, and then the “create selection” function was applied to keep the area information in “ROI manager”. The ratio of lysosome / whole cell was calculated in each cell and the values presented are average.

Lipid extraction and quantification

Lipids were extracted following previously described protocols [2,4]. Briefly, cells were washed by cold PBS and scraped off in 500 μL cold PBS on ice. The suspension was transferred to a 1.5 mL Eppendorf tube in which it was spin down at 2,500 rpm for 5 minutes at 4 °C. After carefully taking off the PBS, samples were stored at -20 °C or directly used for further extraction. For sphingoid base analysis, samples were re-suspended in 150 μL extraction buffer (ethanol, water, diethyl ether, pyridine, and 4.2 N ammonium hydroxide (15:15:5:1:0.018, v/v/v)). A mixture of internal standards (0.04 nmol of C17 sphingosine, 0.04 nmol of C17 sphinganine, 0.4 nmol of C17 sphingosine-1-phosphate, 0.4 nmol of C17 sphinganine-1-phosphate) were added. The samples were vigorously vortexed using a Cell Disruptor Homogenizer (Disruptor Genie, Scientific Industries) for 10 minutes at 4 °C and incubated on ice for 20 minutes. Cell debris was pelleted by centrifugation at 14,000 rpm for 2 min at 4 °C, and the supernatant was collected. The extraction was repeated once more without ice incubation. The supernatants were combined and dried under vacuum in a CentriVap (Labconco, Kansas City, MO). The samples were re-suspended in a mixture of solvents composed of 70 μL of borate buffer (200 mM boric acid pH 8.8, 10 mM tris(2-carboxyethyl)-phosphine, 10 mM ascorbic acid and 33.7μM 15N13C-Valine), and 10μL of formic acid solution (0.1 % aqueous solution), derivatized by reacting with 20 μL 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) solution (2.85 mg/ml in acetonitrile) for 15 minutes at 55 °C. After overnight incubation at 24 °C, samples were analyzed by LC-MS/MS in an Accela HPLC system (ThermoFisher Scientific, Waltham, USA) coupled to a TSQ Vantage (ThermoFisher Scientific, Waltham, USA). MRM-MS was used to identify and quantify sphingoid bases. The amounts of sphingolipids were normalized with respect to the amount of C17 internal standards and cell numbers.
For ceramide and phospholipid analysis, samples were prepared following the MTBE protocol[5]. Briefly, cells were re-suspended in 100 μL of water and transferred into a 2.0 mL Eppendorf tube. 360 μL of MeOH and a mixture of internal standards (0.4 nmol of DLPC, 1 nmol of PE31:1, 1 nmol of PI31:1, 3.3 nmol of PS31:1, 2.5 nmol of C12 sphingomyelin, 0.5 nmol of C17 ceramide and 0.1 nmol of C8 glucosylceramide) was added. Samples were vortexed, following the addition of 1.2 ml of MTBE. The samples were vigorously vortexed at maximum speed for 10 minutes at 4 ºC and incubated for 1 h at room temperature on a shaker. Phase separation was induced by addition of 200 μL MS-grade water and incubation for 10 minutes. Samples were centrifuged at 1,000 g for 10 minutes. The upper phase was transferred into a 13 mm glass tube and the lower phase was re-extracted with 400 μL of a MTBE/MeOH/H2O mixture (10:3:1.5, v/v). The extraction was repeated one more time. The combined upper phase was separated into three equal aliquots before drying under nitrogen flow. One aliquot was treated by alkaline hydrolysis to enrich for sphingolipids, one was used for glycerophospholipid analysis and the third was kept as a backup. To hydrolyze glycerophospholipids, the sample was re-suspended in 1 ml freshly prepared monomethylamine reagent (methylamine/H2O/n-butanol/methanol at 5:3:1:4 (v/v)) and incubated at 53 ºC for 1 h in a water bath before dried by nitrogen flow. The excess salts were removed by extracting samples in 300 μL water-saturated n-butanol solution and 150 μL MS-grade water. The organic phase was collected, and the extraction was repeated with 300 μL water-saturated n-butanol. The combined organic phase was dried by nitrogen flow.

Identification and quantification of phospholipid and sphingolipid molecular species were performed using multiple reaction monitoring (MRM) with a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific) equipped with a robotic nanoflow ion source, Nanomate HD (Advion Biosciences). Each individual ion dissociation pathway was optimized with regard to collision energy. Lipid concentrations were calculated with respect to the corresponding internal standards and were presented as percentage of all lipid signals.

Statistical analysis

Data represent at least the average of three independent experiments. Error bars represent standard error of the mean (SEM) as indicated. Statistical significance was calculated based on two-tailed unpaired student’s t-test using GraphPad Prism 7.

Generation of CerS 5/6 double knockout cells

CERS 5/6 double knockout cells were generated using the CRISPR/Cas9 system, based on the HPRT co-targeting approach[6]. This approach enables an enrichment of mutated cells based on the resistance against 6-thioguanine, which is conferred by the co-disruption of the HPRT1 gene. Cas9 protein and the single guide RNAs (sgRNAs) were expressed from plasmids, which were prepared as previously described[7]. The plasmid backbones were pX330 (deposited by Feng Zhang, Broad Institute, as Addgene plasmid #42230) for CERS5 and CERS6 sgRNAs, and pUC-U6-sg[7] for HPRT1 sgRNA. HPRT1 sgRNA contains mismatches on purpose to improve target knockout efficiency after drug selection (manuscript in preparation). The sequences of sgRNAs are available in Table S1. The three plasmids were co-transfected in HeLa MZ cells (gift from Marino Zerial, Max Planck Institute) using Lipofectamine 3000 (ThermoFisher Scientific). Five days later, cells were selected with 6 µg/mL 6-thioguanine for 1 week, with a medium change at the fourth day of selection. To measure knockout efficiency, each targeted locus was amplified by PCR using genome DNA from control or mutant cells as templates. PCR products were treated with Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (both from Thermo Fisher Scientific), and Sanger sequencing was performed by Fasteris (Plan-les-Ouates, Switzerland). Primer sequences are available in Table S2. Sequencing results were subjected to TIDE (tracking of indels by decomposition) analysis[8], and the degree of reduction in wild type signal (signal corresponding to zero indel) was used as the knockout efficiency.
Chemical Synthesis

General procedures

$^1$H and $^{13}$C-NMR spectra were recorded on a Bruker AMX-400 MHz or 500 MHz spectrometer. Chemical shifts are given in ppm (δ) using the NMR solvent as internal references and J values are reported in Hz. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad. $^{13}$C-NMR spectra were broadband hydrogen decoupled. LC-MS were recorded using a Thermo Electron Corporation HPLC with a Thermo Scientific Finnigan Surveyor MSQ Spectrometer System. High-resolution mass spectra were recorded on a Thermo QExactive mass spectrometer. Flash chromatography purification was carried out using silica gel 40-63 μm (200-400 mesh) from SiliCycle in solvent systems as described. Thin layer chromatography (TLC) was performed on aluminum-backed, pre-coated silica gel plates (Merck TLC silica gel 60 F254). Spots were detected by a UV lamp under 254 nm or 365 nm wavelength. 7-[(carboxymethyl)-methylamino]-4-(hydroxymethyl)coumarin was synthesized according to previously described protocols[2].

Compound 1

To a solution of 7-[(carboxymethyl)-methylamino]-4-(hydroxymethyl)coumarin[2] (52 mg, 0.2 mmol, 1.0 equiv.) and 4-(2-Aminoethyl)morpholine (32 mg, 0.25 mmol, 1.25 equiv.) in 10 mL of dry DCM, DIPEA (87 μL, 0.5 mmol, 2.5 equiv.) and HBTU (100 mg, 0.26 mmol, 1.3 equiv.) were added. The reaction mixture was stirred at room temperature overnight under nitrogen atmosphere. After removing the solvents under reduced pressure, the crude product was purified by flash chromatograph (SiO$_2$, 5 - 10 % MeOH in DCM) to yield the compound 1 as light yellow solid (30 mg, Yield: 40 %). The product was further purified by reverse-phase HPLC. The semi-preparative HPLC runs were carried out with a gradient from 5 % acetonitrile/water system (0.1 % TFA) for 20 min and a flow of 3 mL/min, monitored by a PDA detector at 254 and 360 nm. $^1$H NMR (400 MHz, DMSO-$d_6$) δ = 9.92 (br s, 1H), 8.25 (br s, 1H), 7.46 (d, $J$ = 8.9 Hz, 1H), 6.65 (dd, $J$ = 8.9, 2.5 Hz, 1H), 6.54 (d, $J$ = 2.5 Hz, 1H), 6.13 (s, 1H), 5.54 (br s, 1H), 4.68 (s, 2H), 4.07 (s, 2H), 3.97 - 3.91 (m, 2H), 3.69 - 3.60 (m, 2H), 3.45 - 3.12 (m, 8H, overlaps with H$_2$O peak), 3.08 (s, 3H) ppm. $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ = 169.58, 160.97, 156.83, 155.06, 152.03, 124.80, 109.04, 106.78, 104.83, 97.83, 63.40, 59.05, 55.00, 51.33, 45.72, 33.45, 8.60 ppm. HR-ESI-MS (pos.) C$_{19}$H$_{25}$N$_3$O$_5$, [M+H]$^+$ calculated: 376.1873, found: 376.1865.

Lyso-So

To a solution of compound 1 (15 mg, 0.04 mmol, 1.0 equiv.) in 4 mL of dry DMF, bis-(4-nitrophenyl) carbonate (12 mg, 0.04 mmol, 1.0 equiv.) and DIPEA (20 μL, 0.11 mmol, 2.75 equiv) was added. The reaction mixture was stirred for 3 h at room temperature in dark under nitrogen atmosphere. Sphingosine (12 mg, 0.04 mmol, 1.0 equiv) in 1 mL of DMF was added and the reaction mixture was stirred at 60 ºC for another 3 h in dark under nitrogen atmosphere. After removing the solvents under reduced pressure, the crude product was purified by flash chromatograph (SiO$_2$, 10 % MeOH in DCM) to afford Lyso-So as a yellow solid (20 mg, Yield: 72 %). The product was further purified by reverse-phase HPLC. The semi -preparative HPLC runs were carried out with a gradient from 5 % acetonitrile/water system (0.1 % TFA) for 25 minutes and a flow of 3 mL/min, monitored by a PDA detector at 254 and 360 nm. $^1$H NMR (500 MHz, DMSO-$d_6$) δ = 9.80 (s, 1H, HC), 8.25 (s, 1H, HB), 7.48 (d, $J$ = 9.0 Hz, 1H, H$_6$), 7.12 (d, $J$ = 9.0 Hz, 1H, H$_8$), 6.67 (dd, $J$ = 2.6 Hz, 1H, H$_{20}$), 6.09 (s, 1H, H$_{22}$), 5.86 - 5.51 (m, 1H, H$_5$), 5.48 - 5.35 (m, 1H, H$_4$), 5.27 - 5.13 (m, 2H, H$_{20}$), 4.84 (s, 1H, –OH), 4.51 (s, 1H, –OH), 4.08 (s, 2H, H$_{31}$), 3.97-3.95 (m, 2H, H$_{33}$), 3.90 (m, 1H, H$_3$), 3.65-3.60 (m, 2H, H$_{34}$), 3.56-3.53 (m, 1H, H$_2$), 3.61-3.10 (m, 1H, H$_4$), 3.61-3.10 (m, 1OH, H$_{25}$, H$_{26}$, overlaps with H$_2$O peak), 3.08 (s, 3H, H$_{30}$), 1.94 (d, $J$ = 7.0 Hz, 2H, H$_6$), 1.27-1.16 (m, 22H, H$_7$ - H$_{17}$), 0.85 (t, $J$ = 6.9 Hz, 3H, H$_{18}$) ppm. $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ = 169.49, 160.56, 157.72 (t, $J$ = 31.0 Hz), 155.32, 155.14, 152.25, 151.00, 131.21, 131.12, 124.87, 109.17, 106.26, 105.18, 97.87, 71.38, 63.34, 60.60, 60.52, 57.82, 54.96, 51.29, 48.58, 45.74, 33.39, 31.66, 31.28, 29.05, 29.02, 28.96, 28.75, 28.70, 28.54, 22.08, 13.95, 8.61 ppm. HR-ESI-MS (pos.) C$_{38}$H$_{60}$N$_4$O$_8$, [M+H]$^+$ calculated: 701.4490, found: 701.4484.
Lyso-Chol

To a solution of compound 1 (30mg, 0.08 mmol) in 3 mL dry DCM, cholesteryl chloroformate (40mg, 0.089mmol, 1.1 equiv.) and DMAP (10mg, 0.08 mmol, 1.0 equiv.) were added and the reaction mixture was allowed to stir at room temperature for overnight in dark. The crude product was directly purified by flash chromatograph (SiO₂, 2-5 % MeOH in DCM) to afford Lyso-Chol as a yellow solid (60mg, Yield: 95 %). ¹H NMR (400 MHz, CDCl₃) δ = 7.35 (d, J = 8.8 Hz, 1H), 6.89 (br, 1H), 6.63 (dd, J = 8.9, 2.5 Hz, 1H), 6.59 (d, J = 2.5 Hz, 1H), 6.25 (s, 1H), 5.41 (d, J = 5.0 Hz, 1H), 5.25 (s, 2H), 4.53 (tdd, J = 10.8, 6.5, 4.5 Hz, 1H), 4.02 (s, 2H), 3.55 (s, 4H), 3.40 (s, 2H), 3.17 (s, 3H), 2.63 – 2.36 (m, 8H), 2.08 – 0.96 (m, 30H), 0.91 (d, J = 6.5 Hz, 3H), 0.87 (d, J = 1.8 Hz, 3H), 0.85 (d, J = 1.8 Hz, 3H), 0.68 (s, 3H) ppm. ¹³C NMR (101 MHz, CDCl₃) δ = 167.74, 160.03, 154.75, 152.98, 150.75, 147.69, 138.09, 123.56, 122.23, 108.33, 106.87, 98.32, 77.94, 65.30, 63.13, 56.18, 55.68, 55.13, 52.08, 48.99, 41.31, 38.93, 38.70, 38.51, 36.94, 35.81, 35.53, 35.17, 34.77, 30.90, 30.83, 28.69, 27.21, 27.00, 26.64, 23.27, 22.81, 21.80, 21.55, 20.04, 18.25, 17.70, 10.85 ppm. HR-ESI-MS (pos.) C47H69N3O₇, [M+H]⁺ calculated: 788.5214, found : 788.5206.

References

NL:
8.13E4
nm=253.5-254.5 PDA
cmpd1

cmpd1 #132  RT: 1.22  AV: 1  NL: 1.38E5
T: ITMS + c ESI Full ms [110.00-2000.00]
Lyso-So #254  RT: 2.26  AV: 1  NL: 2.24E5
T: ITMS + c ESI Full ms [110.00-2000.00]
DMSO peak

DCSFD7-2 #262  RT: 2.25  AV: 1  NL: 1.71E5
T: ITMS + c ESI Full ms [110.00-2000.00]
DCSF3_181028121915 #351  RT: 3.11  AV: 1  NL: 4.95E4
T: ITMS + c ESI Full ms [110.00-2000.00]

DMSO peak

Relative Absorbance

Time (min)

Relative Abundance

m/z