Supporting Information to Accompany "**A Mitochondria-Localized Two-Photon Fluorescent Probe for Ratiometric Imaging of Hydrogen peroxide in Live Tissue**"

Goutam Masanta,[†a] Cheol Ho Heo, [†a] Chang Su Lim,[b] Sung Keun Bae,[a] Bong Rae Cho*[b] and Hwan Myung Kim*[a]

[a] *Division of Energy Systems Research and Molecular Science & Technology Research Center, Ajou University, Suwon 443-749, Korea, and* [b]*Department of Chemistry, Korea University, 1-Anamdong, Seoul 136-701, Korea.*

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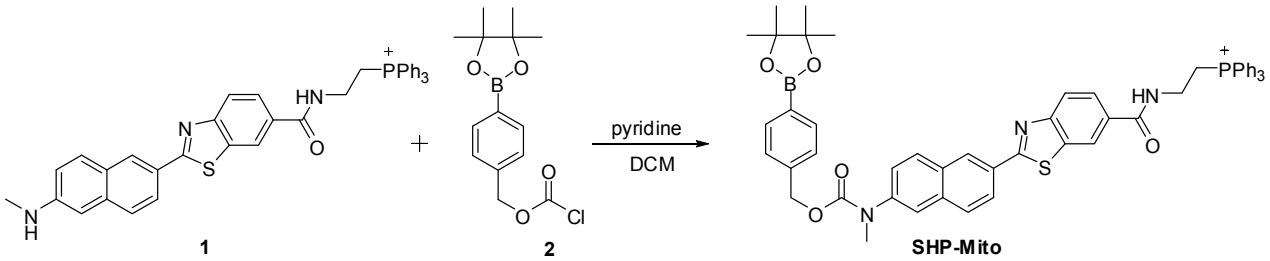
Synthesis of SHP-Mito. 1,[1] and **2**[2] were prepared by the literature methods. Synthesis of SHP-Mito is described below.



Scheme S1. Synthesis of **SHP-Mito**.

Synthesis of **SHP-Mito**. To a stirred solution of **1** (0.30 g, 0.42 mmol) and **2** (0.19 g, 0.64 mmol) in 10 mL dry CH_2Cl_2, was added pyridine (0.35 mL, 4.32 mmol) and the reaction mixture was stirred at room temperature for 2 h under nitrogen atmosphere. The solvent was removed in *vacuo* and the crude product was purified by column chromatography on a silica gel using 8 % methanol in $CHCl_3$ as the eluent to give **SHP-Mito** as a white solid. Yield: 0.11 g (27 %); m.p. 170–172 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.49 (br t, 1H, amide-NH), 8.67 (s, 1H), 8.53 (s, 1H), 8.19-8.16 (m, 2H), 8.06 (d, J = 8.4, 1H), 7.92 (d, J = 8.8 Hz, 1H), 7.88-7.66 (m, 19H), 7.49 (d, J = 8.4 Hz, 1H), 7.32 (d, J = 6.8 Hz, 2H), 5.22 (s, 2H), 4.02-3.99 (m, 4H), 3.44 (s, 3H), 1.33 (s, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.6, 167.0, 156.3, 155.4, 142.4, 139.5, 135.31, 135.1, 135.0, 133.8 (d, J = 9.8 Hz), 131.2, 130.9, 130.7 (d, J = 12.9 Hz), 130.0, 129.7, 128.7, 127.6, 127.1, 126.3, 125.9, 125.1, 122.8, 122.2, 117.6 (d, J = 84.9 Hz), 84.1, 67.7, 38.1, 34.2, 25.2, 23.4 (d, J = 49.3 Hz). ^{31}P NMR (162 MHz, $CDCl_3$): δ 21.9 ppm. HRMS (FAB$^+$): m/z calcd for $[C_{53}H_{50}BN_3O_5PS]^+$: 882.3302, found: 882.3302.

Spectroscopic measurements. Absorption spectra were recorded on a S-3100 UV-Vis spectrophotometer and fluorescence spectra were obtained with FluoroMate FS-2 fluorescence spectrophotometer with a 1 cm standard quartz cell. The fluorescence quantum yield was determined by using coumarin 307 (Φ = 0.95 in MeOH) as the reference by the literature method.[3] Reactive oxygen species (200 μM unless otherwise stated) were administered to SHP-Mito in 30 mM MOPS (pH 7.4, 25 ˚C) as follows. H_2O_2, *tert*-butylhydroperoxide (TBHP), and hypochlorite (NaOCl) were delivered from 30%, 70%, and 10% aqueous solutions respectively. Hydroxyl radical (•OH), and *tert*-butoxy radical (•O^{t}Bu) were generated by reaction of 1 mM Fe^{2+} with 200 μM H_2O_2 or TBHP, respectively. Nitric oxide (NO) was used from stock solution (1.9 mM), prepared by purging phosphate-buffered saline (PBS; 0.01 M, pH 7.4) with N_2 gas for 30 min, followed by NO (99.5 %) for 30 min. Superoxide (O_2^-) was delivered from KO_2. Peroxynitrite was used from stock solution 10 mM in 0.3 M NaOH.

Table S1. Photophysical data for SHP-Mito and **1** in buffer.[a]

Compound	$\lambda_{max}^{(1)}$ (10^{-4} ε) [b]	λ_{max}^{fl} [c]	Φ[d]	R_{max}/R_{min}[e]	$\lambda_{max}^{(2)}$ [f]	$\Phi\delta$[g]
SHP-Mito	342 (1.70)	470	0.13	75 (40)	740	11
1	383 (1.50)	545	0.12		750	55

a) All data were measured in MOPS buffer (30 mM MOPS, 100 mM KCl, pH 7.4) unless otherwise noted. Data for **1** are taken from ref. [1]. b) λ_{max} of the one-photon absorption spectra in nm. The numbers in parentheses are molar extinction coefficients in $M^{-1}cm^{-1}$. c) λ_{max} of the one-photon emission spectra in nm. d) Fluorescence quantum yield, ± 15 %. e) Emission ratio ($F_{530\text{-}600}/F_{400\text{-}470}$) conversion factor, R_{max}/R_{min}, measured by one-photon processes before and 1 h after addition of 1 mM H_2O_2. The number in parentheses is measured by two-photon process. f) The peak two-photon cross section in 10^{-50} cm^4s/photon (GM), ± 15 %. g) Two-photon action cross section.

Water solubility. Small amount of dye was dissolved in DMSO to prepare the stock solutions (1.0×10^{-2} M). The solution was diluted to ($6.0 \times 10^{-3} \sim 6.0 \times 10^{-5}$) M and added to a cuvette containing 3.0 mL of buffer (30 mM MOPS, 100 mM KCl, pH 7.4) by using a micro syringe. In all cases, the concentration of DMSO in H_2O was maintained to be 0.2 %.[4] The plots of fluorescence intensity against the dye concentration were linear at low concentration and showed downward curvature at higher concentration (Figure S1b). The maximum concentration in the linear region was taken as the solubility. The solubility of SHP-Mito in buffer was ~ 3.0 μM.

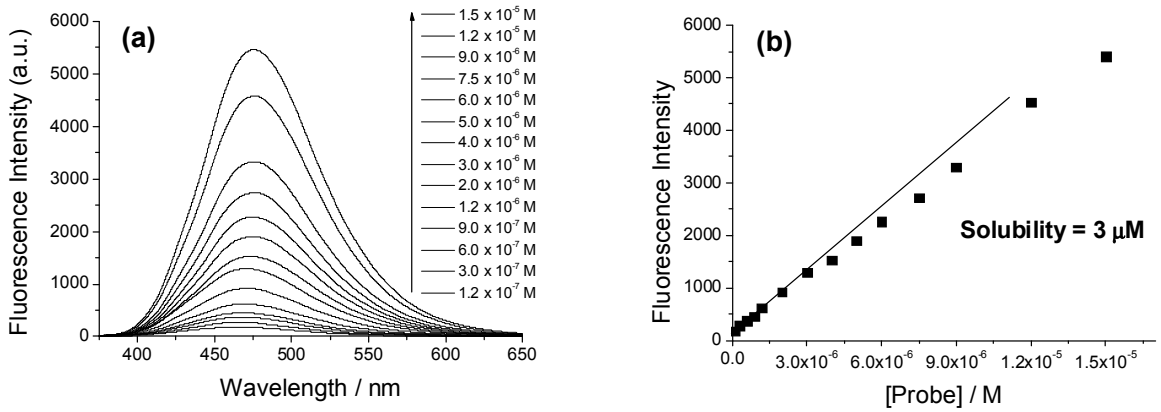


Figure S1. (a) One-photon fluorescence spectra and (b) plot of fluorescence intensity against dye concentration for SHP-Mito in buffer (30 mM MOPS, 100 mM KCl, pH 7.4). The excitation wavelength was 340 nm.

Product analysis. The reaction of SHP-Mito (100 μM) with H_2O_2 (100 mM) was carried out for 30 min at 25 °C in MOPS buffer (pH = 7.4). The LC-MS traces of SHP-Mito, **1**, and the reaction are shown below (Figure S2). The result shows that **1** is the major product.

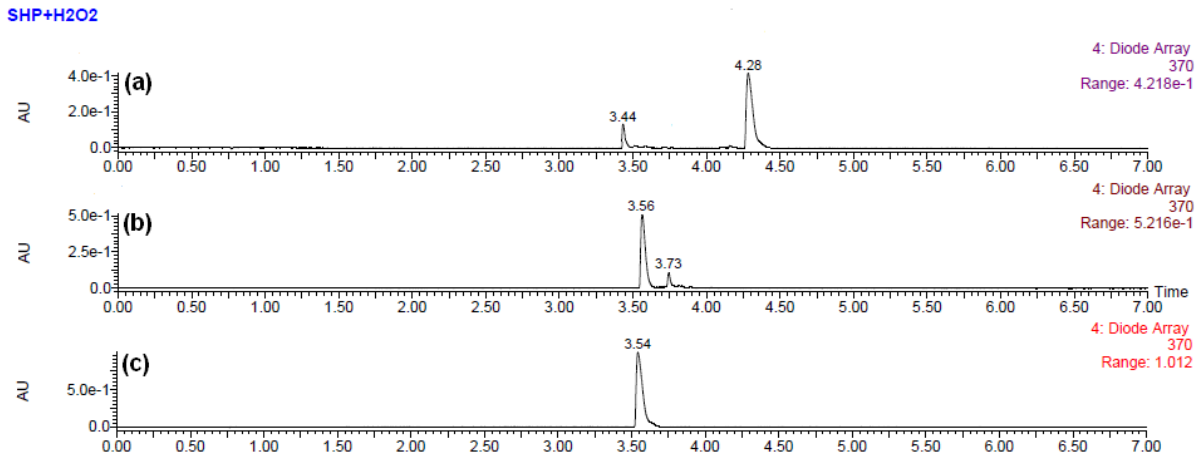


Figure S2. LC-MS traces of (a) SHP-Mito, (b) the reaction product between SHP-Mito and H_2O_2, (c) **1**. Assigned peaks were confirmed by ESI^+. LC-MS conditions: 0.3 mL/min flow rate, 10 % B to 100 % B over 7 min, detected at 370 nm. Solvent A is water with 0.1 % formic acid and solvent B is acetonitrile with 0.1 % formic acid. The peak at 3.44 min in (a) corresponds to the boronic acid form of SHP-Mito ($[M^+]$ = 800.5) produced by the acid-catalyzed hydrolysis of the boronate moiety in the presence of formic acid, and that at 3.73 min in (b) corresponds the side product ($[M^+]$ = 728.3) produced by the reaction between **1** and 4-methylenecyclohexa-2,5-dienone, respectively.

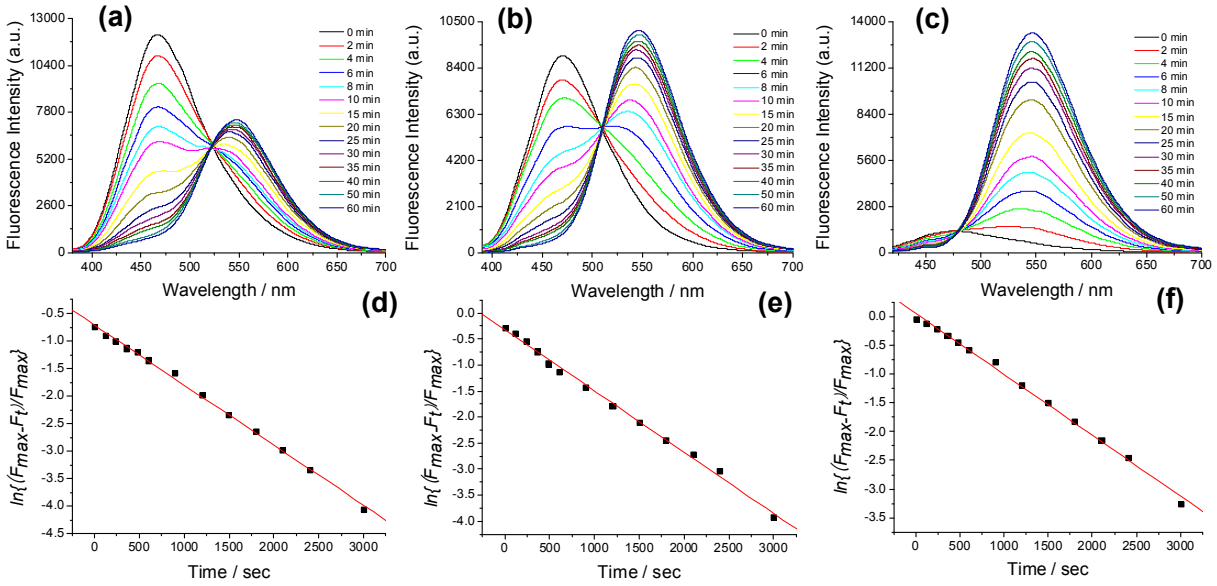


Figure S3. Fluorescence responses of 1 μM SHP-Mito to 1 mM H_2O_2 with excitation at (a) 360, (b) 370, and (c) 390, respectively. The k_{obs} = 1.09 × 10^{-3}, 1.18 × 10^{-3}, and 1.06 × 10^{-3} s^{-1} were obtained from slope of the plot of ln [(F_{max}–F_t)/F_{max}] (measured at 545 nm) vs time using excitation wavelength (d) 360 nm, (e) 370 nm, and (f) 390 nm, respectively.

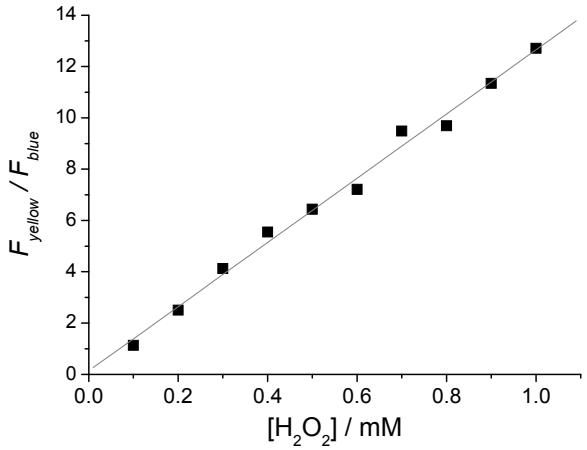


Figure S4. Plot of the F_{yellow}/F_{blue} ratios for SHP-Mito vs [H_2O_2] in MOPS buffer (30 mM MOPS, pH 7.4). Each data was acquired 1 h after H_2O_2 addition at 25 °C. The detection limit (4.6 μM) was calculated with 3σ/k; where σ is the standard deviation of blank measurement, k is the slop in Fig. S4.[5]

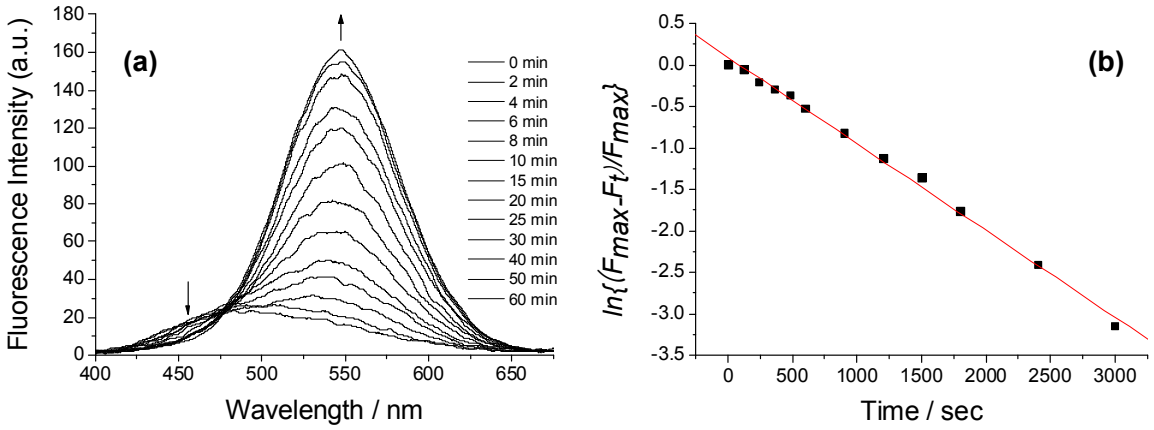


Figure S5. (a) Two-photon fluorescence response of 1 μM SHP-Mito to 1 mM H_2O_2 with excitation at 750 nm. (b) Plot of ln $[(F_{max}–F_t)/F_{max}]$ (measured at 545 nm) vs time. The k_{obs} calculated from the slope is 1.02×10^{-3} s^{-1}.

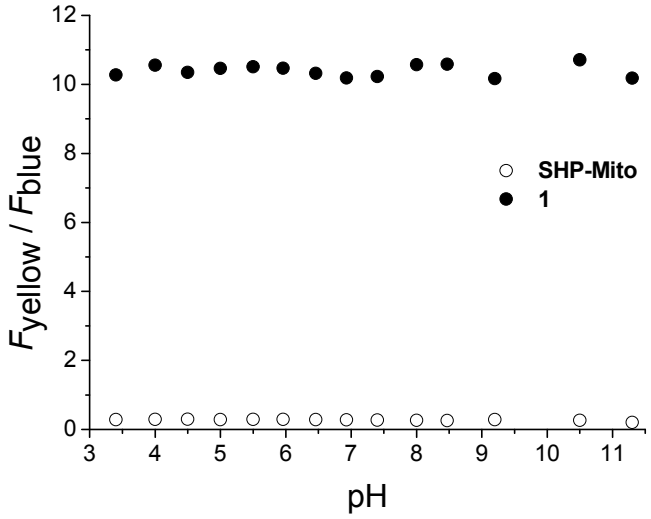


Figure S6. Effect of pH on the F_{yellow}/F_{blue} ratios for SHP-Mito (○) and **1** (●) in MOPS buffer (30 mM MOPS, pH 7.4). The excitation wavelength was 370 nm.

Measurement of Two-Photon Cross Sections. The two-photon cross section (δ) of dyes were determined by using a femto second (fs) fluorescence measurement technique as described.[6] SHP-Mito and **1** were dissolved in 30 mM MOPS buffer (pH 7.4) at concentrations of 3.0×10^{-6} M (SHP-Mito) and 3.0×10^{-6} M (1) and then the two-photon induced fluorescence intensity was measured at 720–940

nm by using Rhodamine 6G in MeOH as the reference, whose two-photon properties have been well characterized in the literature.[7] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$, where the subscripts *s* and *r* stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as *S*. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as *c*. δ_r is the TPA cross section of the reference molecule.

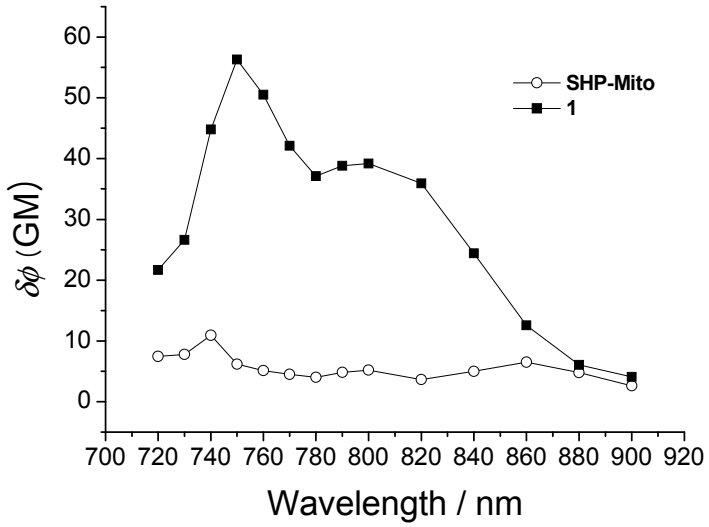


Figure S7. Two-photon action spectra of 3 μM SHP-Mito and **1** in MOPS buffer ([MOPS] = 30 mM, 100 mM KCl, pH 7.4). The estimated uncertainties for the two-photon action cross section values (δΦ) are ± 15%. Data for **1** are taken from ref. 1.

Cell Culture and Imaging. Raw 264.7 cells were cultured in DMEM (Invitrogen) supplemented with 10 % fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). One day before imaging, cells were passaged and plated on 24 well plates with coverslips coated with poly(lysine) at a density of $2x10^5$/well. For labeling, the growth medium was removed and replaced with DPBS containing calcium and magnesium and 3 μM SHP-Mito or **1**. Cells were incubated with the staining solution for 30 min. The coverslips of cells loaded with dye were transferred into a fresh DPBS solution. Hydrogen peroxide was delivered from a 100 mM stock solution in MilliQ water. PMA was added from stock solution of 1 mg/mL in DMSO. For proliferation assays, one day before experiments, Raw 264.7 cells were passaged and plated on 96 well plates at a density of 3×10^4/well. Cells were incubated with WST-1 solution in DMEM containing DMSO, **1**, or SHP-Mito at indicated concentrations for 2 h at 37 °C with 5% CO_2. The formation of the formazan product was measured by absorption at 342 nm using SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). 1 and SHP-Mito in DMEM have no background absorption at 342 nm.

Two-photon fluorescence microscopy. Two-photon fluorescence microscopy images of SHP-Mito- and **1**-labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with ×10, ×40 dry and ×100 oil objectives, numerical aperture (NA) = 0.30, 0.75 and 1.30. The two-photon fluorescence microscopy images were obtained with a DM IRE2 Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 750 nm and output power 1260 mW, which corresponded to approximately 10 mW average power in the focal plane. Data were analyzed using ImageJ software (NIH) and Ratio Plus plugin (programmer: Paulo Magalhes). To obtain images at 400–470 nm and 530–600 nm range, internal PMTs were used to collect the signals in an 8 bit unsigned 512 × 512 and 1024 × 1024 pixels at 800 and 400 Hz scan speed, respectively.

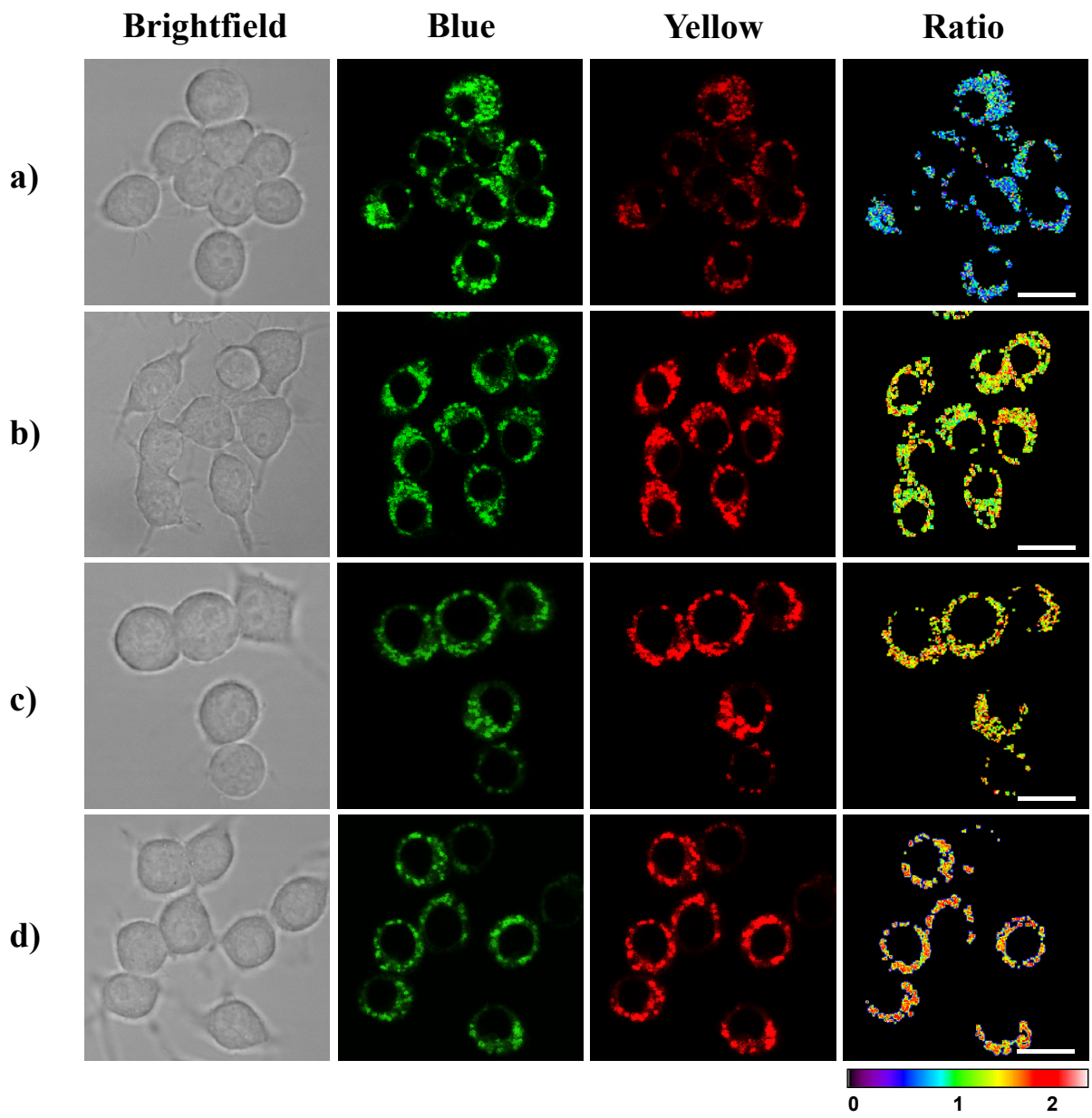


Figure S8. Two-photon microscopy images of Raw 264.7 cells incubated with (a) SHP-Mito and d) **1**. (b,c) Cells were pretreated with (b) 1 μL PMA (1 μg/mL) and (c) 200 μM H_2O_2 for 30 min before labeleing with SHP-Mito. The ratio of yellow to blue (F_{yellow}/F_{blue}) images are shown in pseuedocolor ratiometric image mode with 16 color look-up tables, which represent the ratio from 0 to 2. Images were acquired using 750 nm excitation and fluorescent emission windows: Blue = 400-470 nm, Yellow = 530-600 nm. Scale bar = 15 μm.

Cell viability. To confirm that the probe couldn't affect the viability of Raw 264.7 cells in our incubation condition, we used CCK-8 kit (Cell Counting Kit-8, Dojindo, Japan) according to the manufacture's protocol. The results are shown in Figure S8.

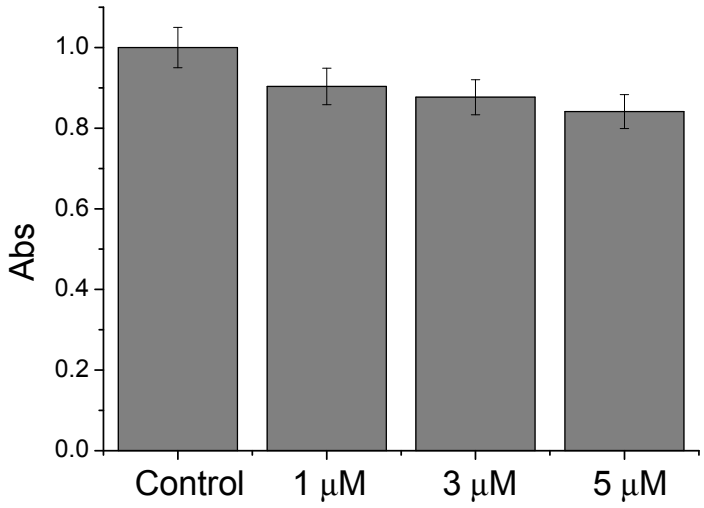


Figure S9. Viability of Raw 264.7 cells in the presence of SHP-Mito as measured by using CCK-8 kit. The cells were incubated with SHP-Mito for 2 h.

Preparation and staining of fresh rat Hippocampal slices. Rat Hippocampal slices were prepared from the hippocampi of 2-days-old rat (SD) according to an approved institutional review board protocol. Coronal slices were cut into 400 μm-thick using a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM D-glucose, 2.4 mM $CaCl_2$, and 1.3 mM $MgSO_4$). Slices were incubated with 20 μM SHP-Mito in ACSF bubbled with 95% O_2 and 5% CO_2 for 2 hr at 37 °C. Slices were then washed three times with ACSF and transferred to glass-bottomed dishes (MatTek) and observed in a spectral confocal multiphoton microscope. The TPM images of fresh rat Hippocampal slice labeled with 20 μM SSH-Mito obtained at 90–180 μm depth are shown in Figure S9, S11, and S12.

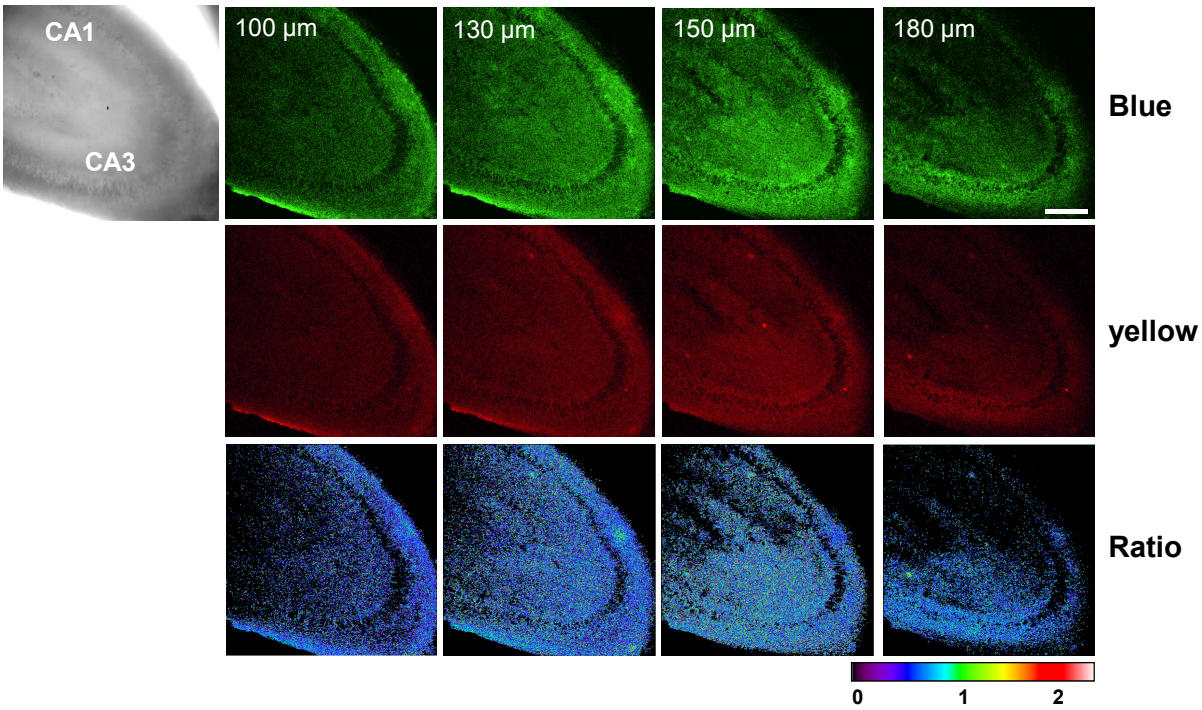


Figure S10. TPM images of a fresh rat hippocampal slice stained with 20 μM SHP-Mito. The TPEF were collected at 400-470 (blue) and 530-600 nm (yellow) upon excitation at 750 nm with fs pulses. The images shown above are representative images out of 10 TPM images taken at depths of 90-180 μm with magnification at 10×. The ratio represents the ratiometric images constructed from the images in the blue and green rows. Scale bar = 300 μm.

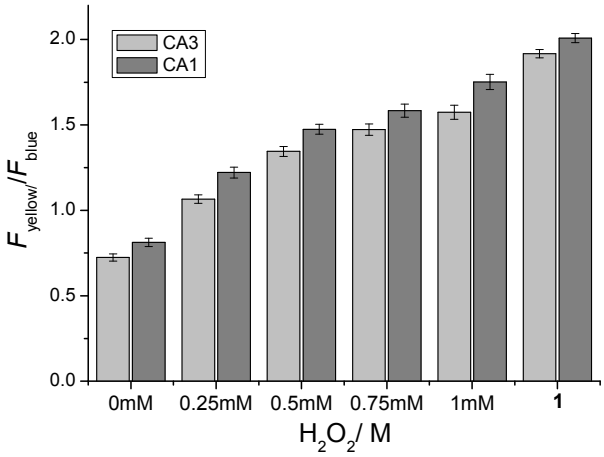


Figure S11. Average F_{yellow}/F_{blue} in the CA3 and CA1 regions of a fresh rat hippocampal slice pretreated with 0, 0.25, 0.50, 0.75, and 1.0 mM H_2O_2 for 30 min before labeling with 20 μM SHP-Mito, and incubated with **1**. The TPEF were collected at two channels (Blue = 400-470 nm, Yellow = 530-600 nm) upon excitation at 750 nm with fs pulse.

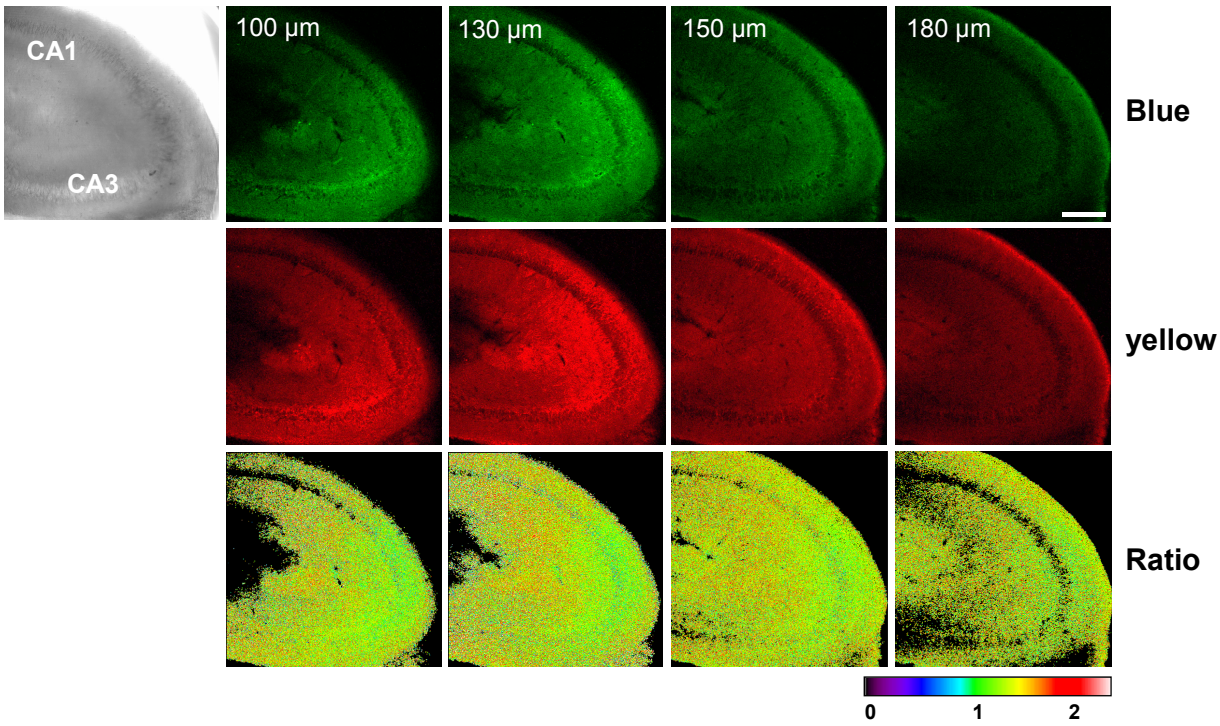


Figure S12. TPM images of a fresh rat hippocampal slice stained with 20 µM SHP-Mito after treatment with 1 mM H_2O_2 for 30 min. The TPEF were collected at 400-470 (blue) and 530-600 nm (yellow) upon excitation at 750 nm with fs pulses. The images shown above are representative images out of 10 TPM images taken at depths of 90-180 µm with magnification at 10×. The ratio represents the ratiometric images constructed from the images in the blue and green rows. Scale bar = 300 µm.

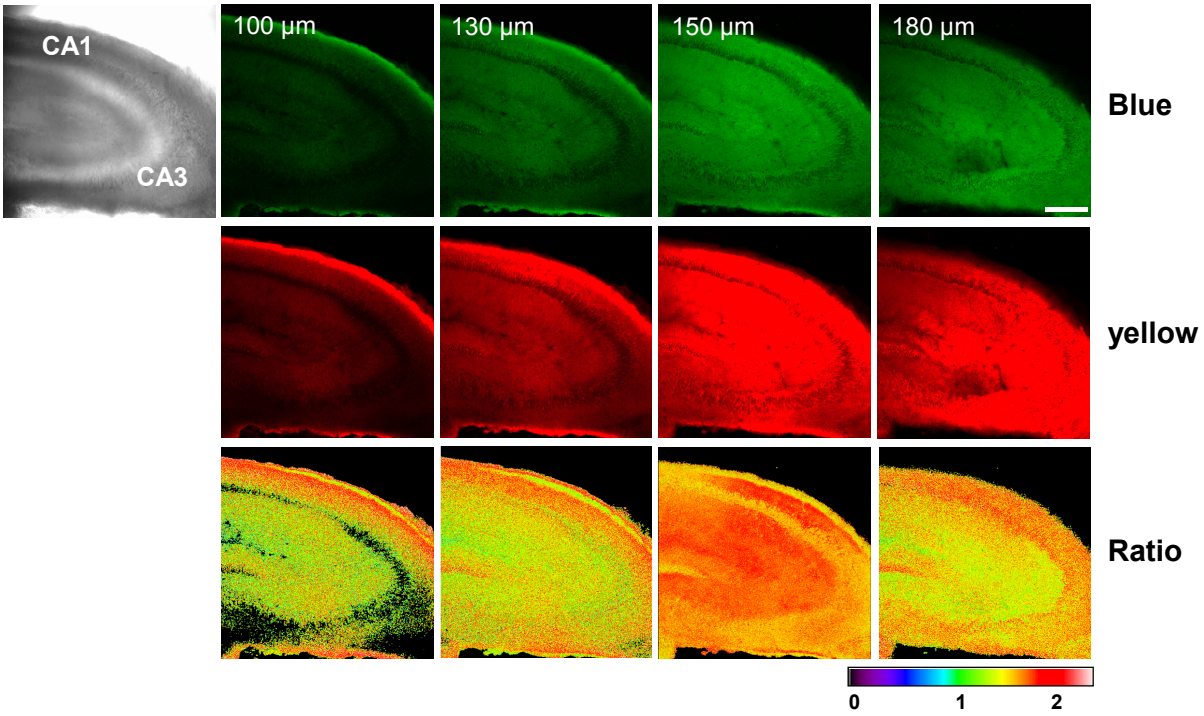


Figure S13. TPM images of a fresh rat hippocampal slice stained with 20 μM **1**. The TPEF were collected at 400-470 (blue) and 530-600 nm (yellow) upon excitation at 750 nm with fs pulses. The images shown above are representative images out of 10 TPM images taken at depths of 90-180 μm with magnification at 10×. The ratio represents the ratiometric images constructed from the images in the blue and green rows. Scale bar = 300 μm.

^{1}H-NMR, ^{13}C-NMR, and HRMS of SHP-Mito

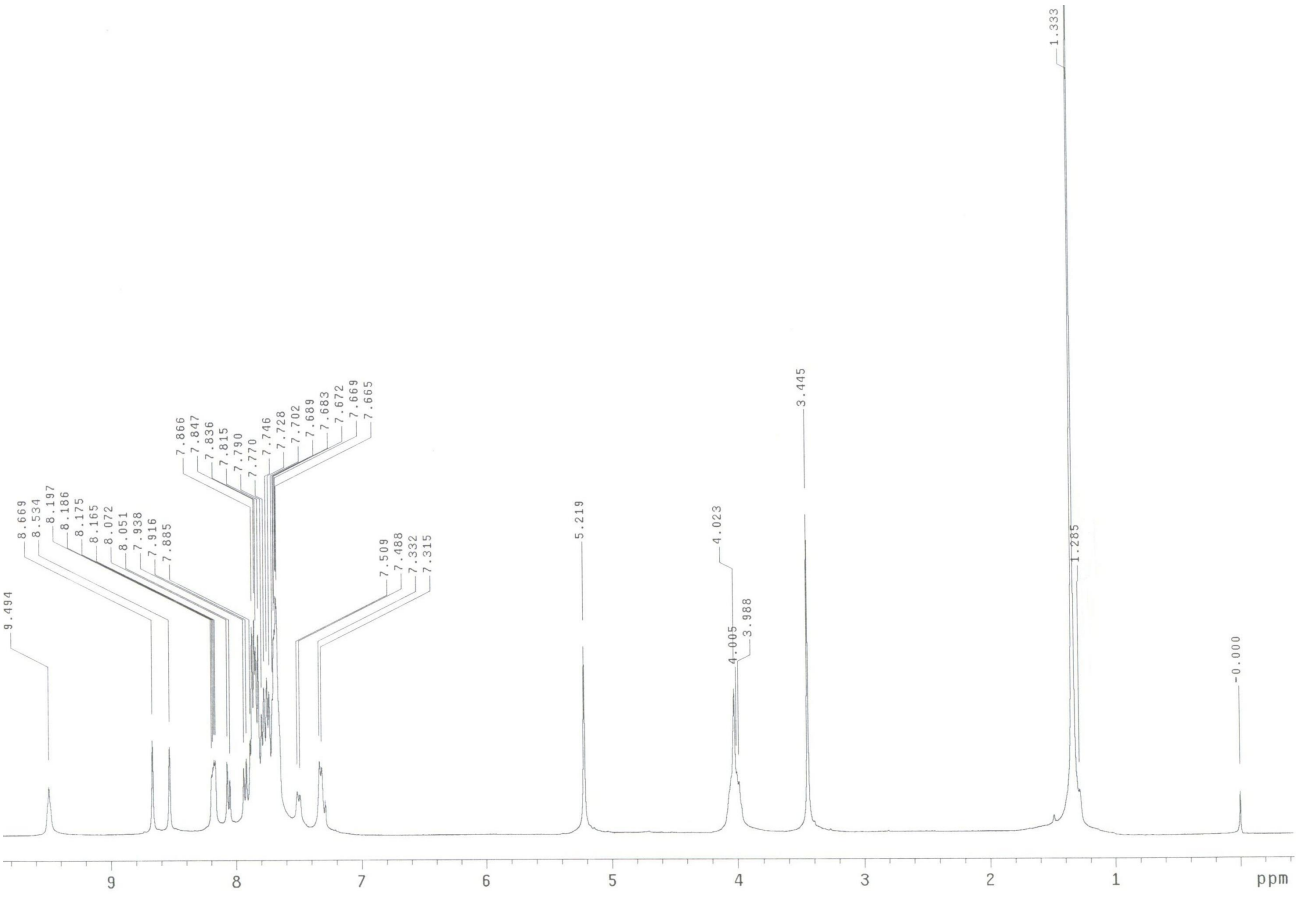


Figure S14. ^{1}H-NMR spectrum (400 MHz) of SHP-Mito in $CDCl_3$.

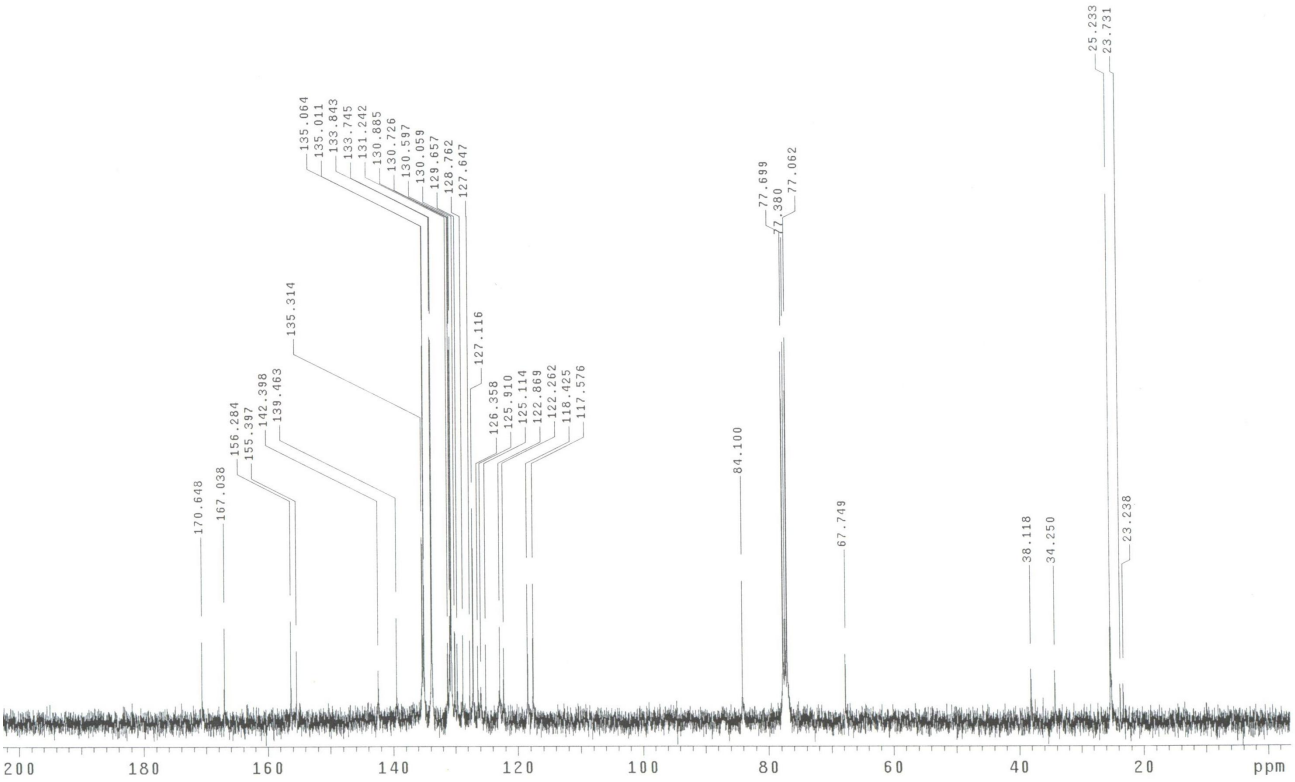


Figure S15. ^{13}C-NMR spectrum (100 MHz) of SHP-Mito in $CDCl_3$.

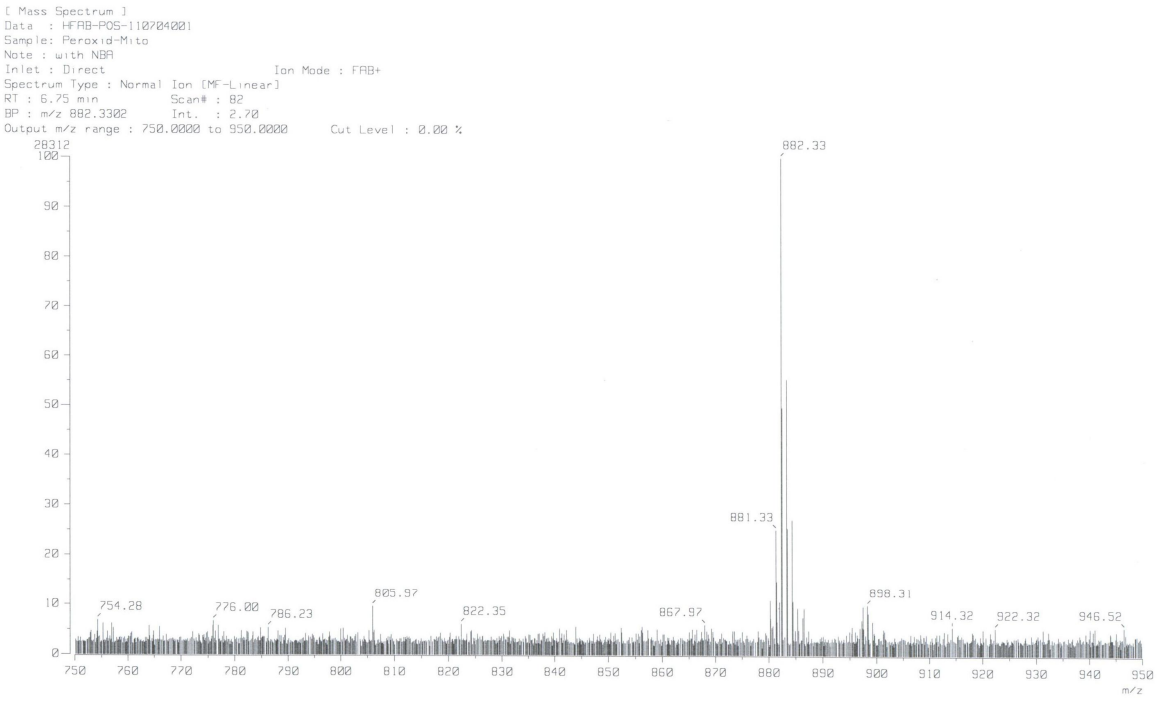


Figure S16. HRMS spectrum of SHP-Mito.

References

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