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Supporting Information

A Two-Photon Ratiometric Fluorescent Probe for Imaging of Hydrogen Peroxide Levels in Rat Organ Tissues

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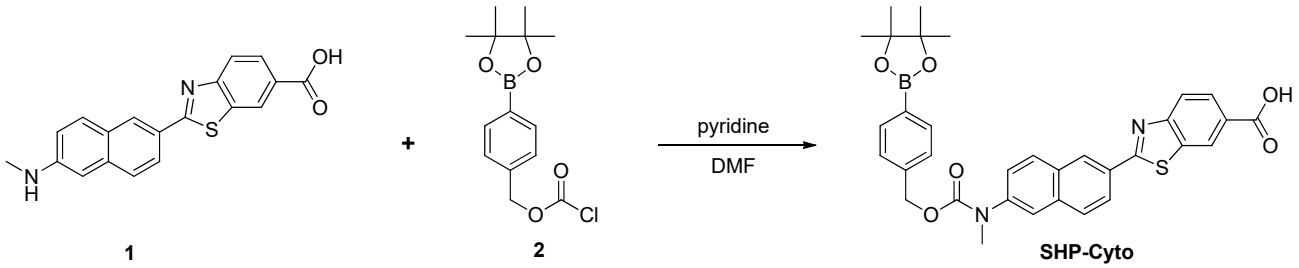
Synthesis of SHP-Cyto. 1,[1] and **2**[2] were prepared by the literature methods. Synthesis of SHP-Cyto is described below.



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

Synthesis of **SHP-Cyto**. To a stirred solution of **1** (0.11 g, 0.32 mmol) and **2** (0.19 g, 0.64 mmol) in 10 mL dry DMF, was added pyridine (0.10 mL, 1.24 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 using 15 % methanol in $CHCl_3$ as the eluent to give **SHP-Cyto** as a white solid. Yield: 0.11 g (27 %); m.p. 170–172 °C; ^{1}H NMR (400 MHz, DMSO): δ 13.13 (br, 1H), 8.78 (s, 1H), 8.70 (s, 1H), 8.21 (d, *J* = 8.4 Hz, 1H), 8.14-8.02 (m, 4H), 7.93 (s, 1H), 7.74 (d, *J* = 7.2 Hz, 1H), 7.63 (d, *J* = 7.2 Hz, 2H), 7.33 (d, *J* = 7.6 Hz, 1H), 7.28 (d, *J* = 7.2 Hz, 1H), 5.15 (s, 2H), 3.38 (s, 3H), 1.26 (s, 12H). ^{13}C NMR (100 MHz, DMSO): δ 171.4, 167.4, 156.9, 143.0, 140.4, 135.3, 135.2, 135.1, 134.9, 134.6, 131.2, 130.4, 130.1, 129.5, 128.3, 128.2, 127.5, 126.3, 126.0, 125.1, 123.2, 84.4, 64.4, 38.2, 25.5. HRMS (FAB$^+$): m/z calcd for $[C_{33}H_{32}BN_2O_6S]^+$: 595.2069, found: 595.2072.

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-Cyto 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.

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-Cyto in buffer was ~ 3.0 μM.

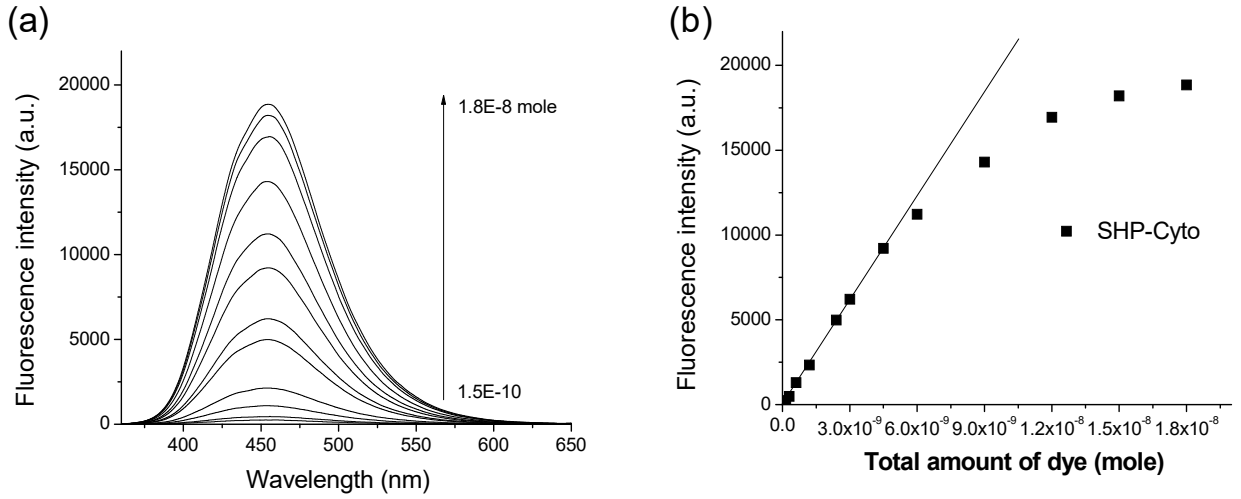


Figure S1. (a) One-photon fluorescence spectra and (b) plot of fluorescence intensity against dye concentration for SHP-Cyto in buffer (30 mM MOPS, 100 mM KCl, pH 7.4). The excitation wavelength was 335 nm.

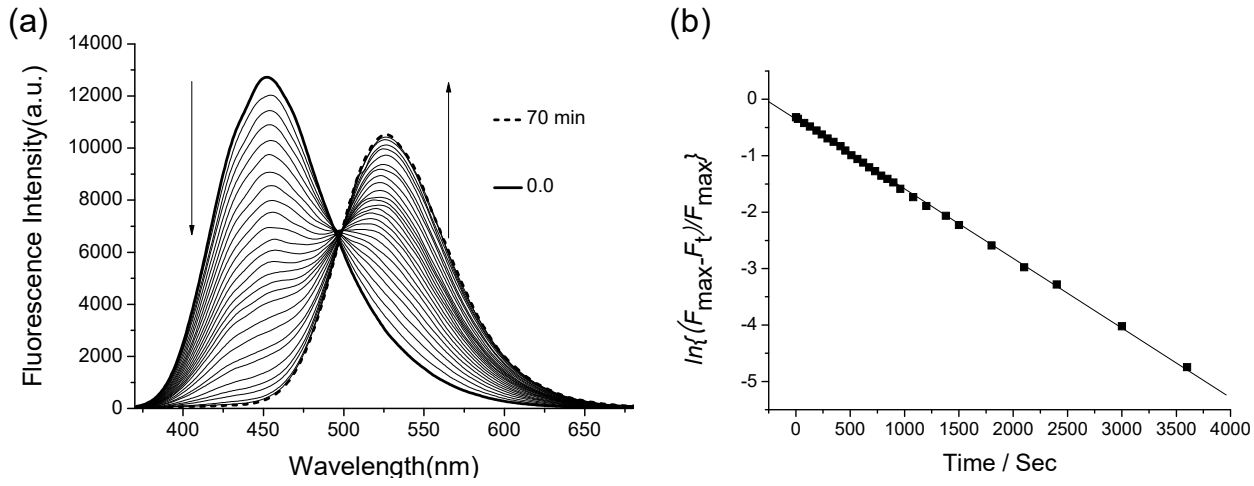


Figure S2 (a) Fluorescence responses of 1 μM SHP-Cyto to 1.15 mM H_2O_2 with excitation at 350. (b) Plot of ln $[(F_{max}–F_t)/F_{max}]$ (measured at 525 nm) vs time. The k_{obs} is 1.23×10^{-3} s^{-1} were obtained from the slope.

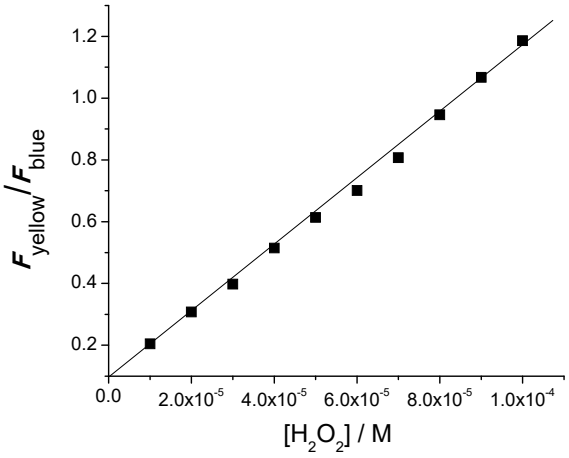


Figure S3. Plot of the F_{yellow}/F_{blue} ratios for SHP-Cyto vs [H_2O_2] in MOPS buffer (30 mM MOPS, 100 mM KCl, pH 7.2). Each data was acquired 2 h after H_2O_2 addition at 25 °C. The detection limit (4.0 μM) was calculated with 3σ/k; where σ is the standard deviation of blank measurement, k is the slop in Fig. S3.

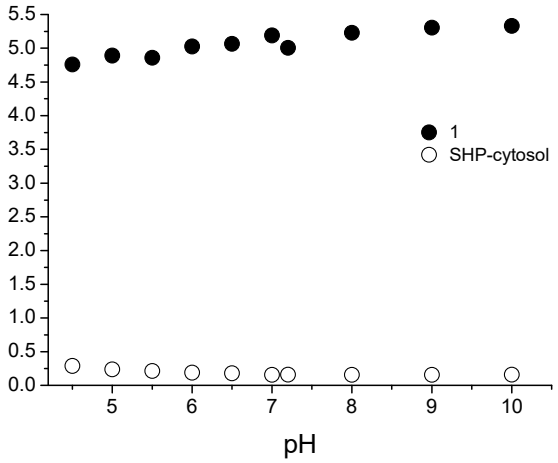


Figure S4. Effect of pH on the F_{yellow}/F_{blue} ratios for SHP-Cyto (○) and **1** (●) in Universal buffer (0.1M KH_2PO_4, 0.1M citric acid, 0.1M Sodium tetraborate, 0.1M Trizma, 0.1M KCl). The excitation wavelength was 350 nm.

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.[5] SHP-Cyto and **1** were dissolved in 30 mM MOPS buffer (pH 7.4) at concentrations of 3.0×10^{-6} M (SHP-Cyto) 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.[5] 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.

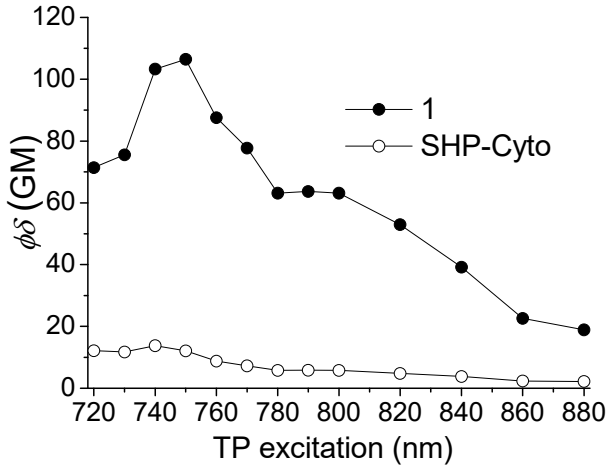


Figure S5. Two-photon action spectra of 1 μM SHP-Cyto and **1** in MOPS buffer (30 mM MOPS, 100 mM KCl, pH 7.4). The estimated uncertainties for the two-photon action cross section values (δΦ) are ± 15%.

Cell viability. To confirm that the probe couldn't affect the viability of HeLa cells in our incubation condition, MTS (Cell Titer 96H; Promega, Maidson, WI, USA) assays were used according to the manufacture's protocol

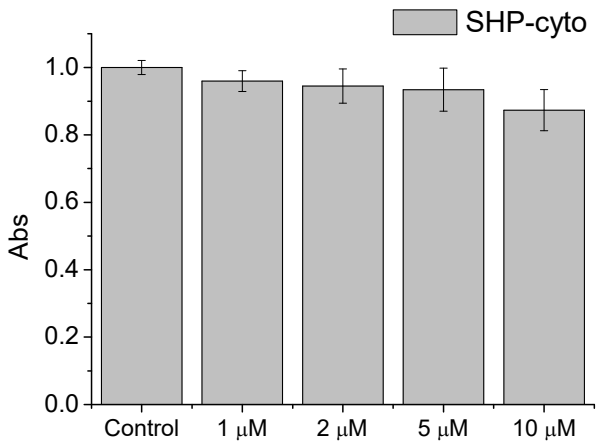


Figure S6. Viability of HeLa cells in the presence of SHP-Cyto as measured by using MTS assay. The cells were incubated with various concentrations of SHP-Cyto for 2 h. Six independent experiments are performed.

Cell Culture and Imaging. All the cells were passed and plated on glass-bottomed dishes (NEST) for two days before imaging. They were maintained in a humidified atmosphere of 5/95 (v/v) of CO_2/air at 37 °C. The cells were treated and incubated with 1 μM SHP-Cyto at 37 °C under 5 % CO_2 for 30 min, washed three times with phosphate buffered saline (PBS; Gibco), and then imaged after further incubation in colorless serum-free media for 30 min. The culture mediums for each cell are as below. HeLa human cervical carcinoma cells (KCLB, Seoul, Korea): MEM (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units per mL), and streptomycin (100 μg/mL).

Two-photon fluorescence microscopy. Two-photon fluorescence microscopy images of SHP-Cyto cells were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP8 MP) with ×10 dry, ×40 oil and ×100 oil objectives, numerical aperture (NA) = 0.30, 1.30, and 1.30. The two-photon fluorescence microscopy images were obtained with a DMI6000B Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Mai Tai HP; Spectra Physics, 80 MHz, 100 fs) set at wavelength 750 nm and output power 2456 mW, which corresponded to approximately 1.52 × 10^8 mW/cm^2 average power in the focal plane. To obtain images at 400−470 nm (F_{blue}) and 530−600 nm (F_{yellow}) range, internal PMTs were used to collect the signals in 8 bits unsigned 512 × 512 and 1024 × 1024 pixels at 400 and 200 Hz scan speed, respectively. Ratiometric image processing and analysis was carried out using MetaMorph software.

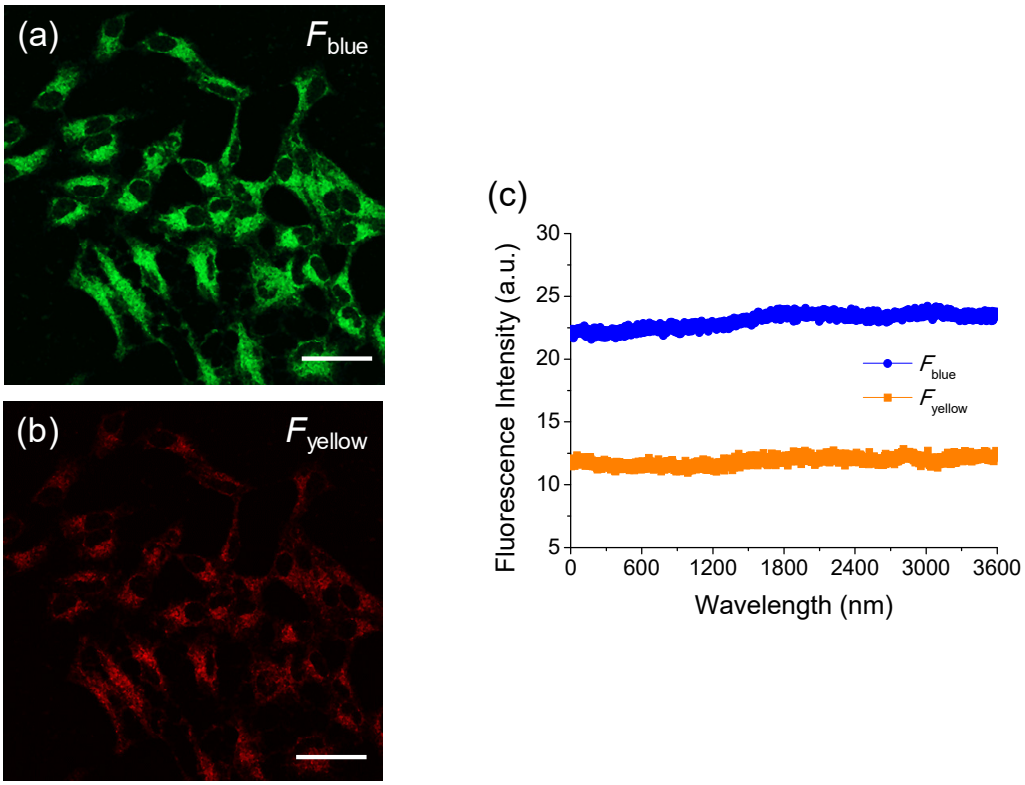


Figure S7. (a, b) TPM images of HeLa cells after 30 min incubation with 1 μM SHP-Cyto. (c) The relative TPEF intensity from (a, b) TPM images as a function of time. The digitized intensity was recorded with 2.00 sec intervals for one hour using *xyt* mode. The TPEF intensities were collected at 400–470 nm (F_{blue}) and 530–600 (F_{yellow}) upon excitation at 750 nm with femto-second pulses. Scale bars = 48 μm. Cells shown are representative images from replicate experiments (n = 3).

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-Cyto 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.

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

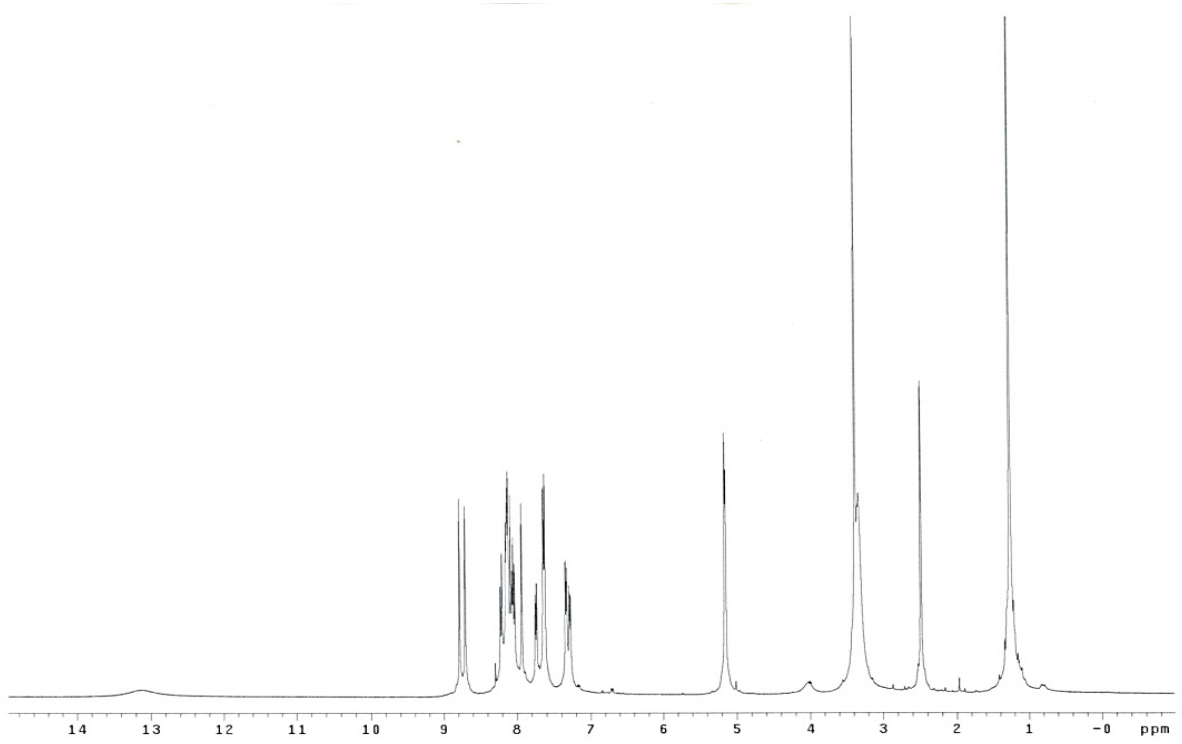


Figure S8. ^{1}H-NMR spectrum (400 MHz) of SHP-Cyto in DMSO.

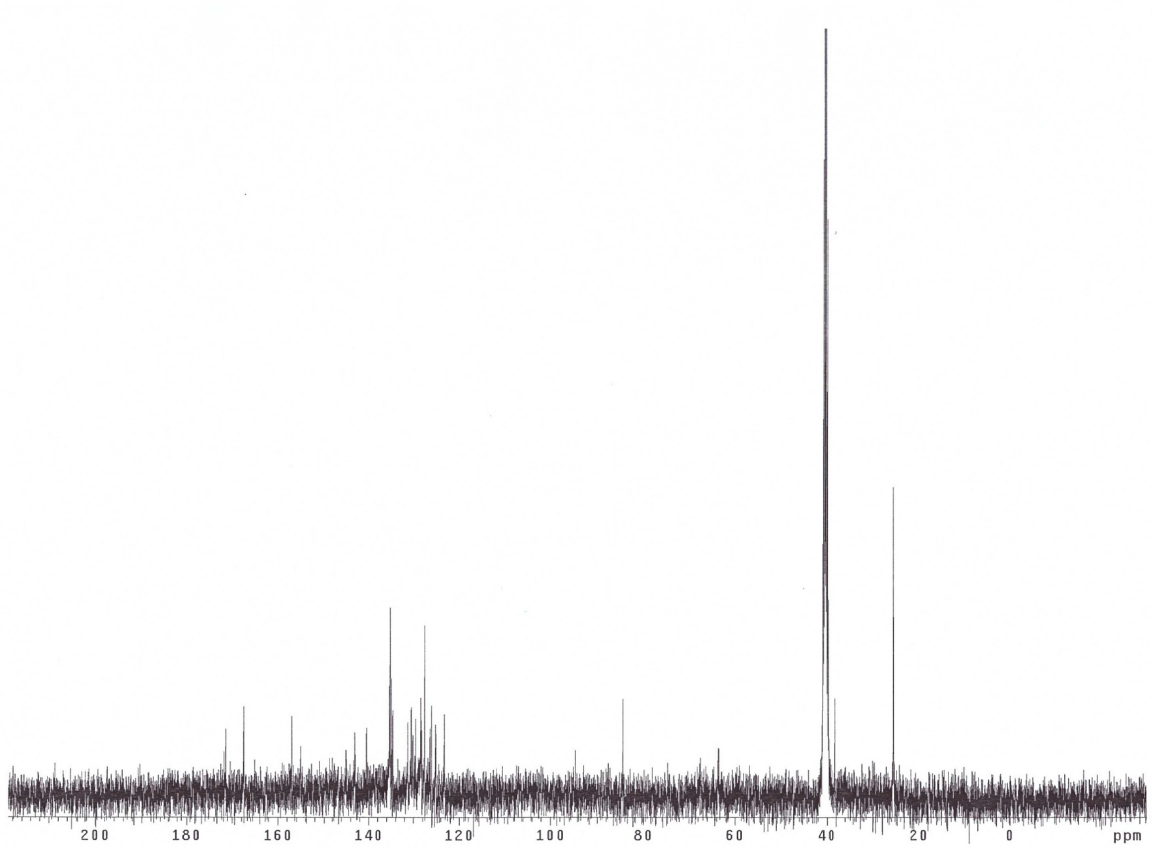


Figure S9. ^{13}C-NMR spectrum (100 MHz) of SHP-Cyto in DMSO.

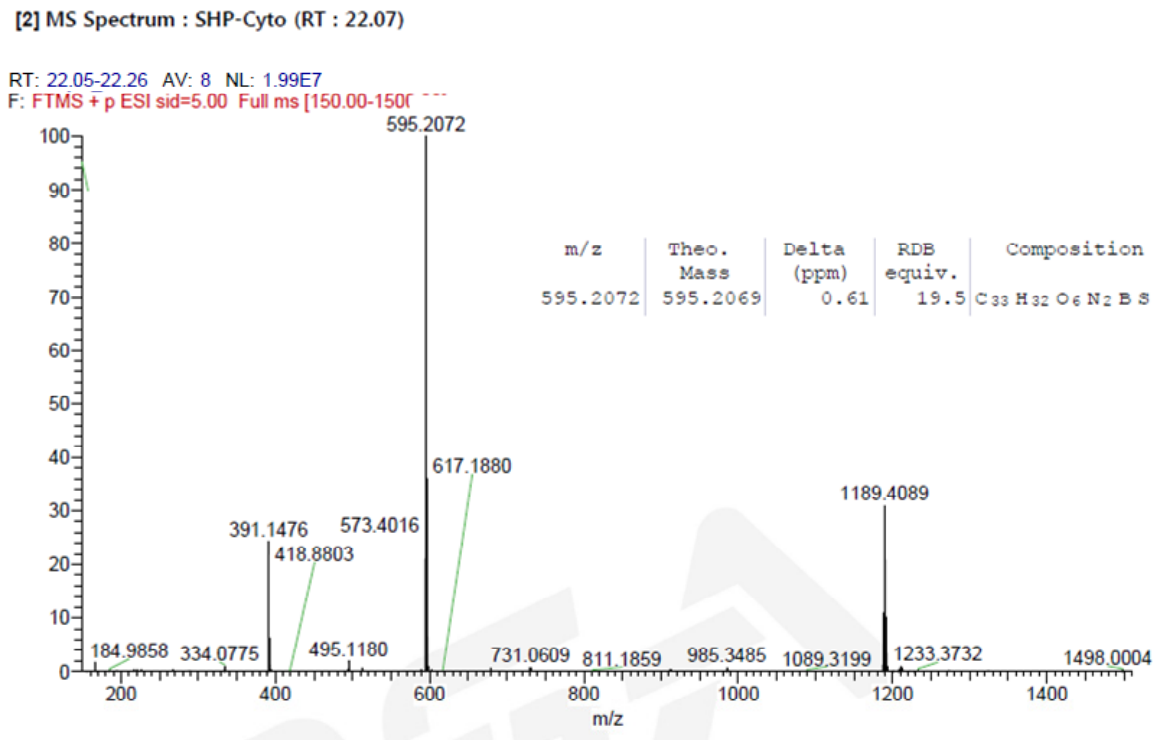


Figure S10. HRMS spectrum of SHP-Cyto

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