Electronic Supplementary Information

for

Highly Selective Two Photon Imaging of Cysteine in Cancerous Cells and Tissues

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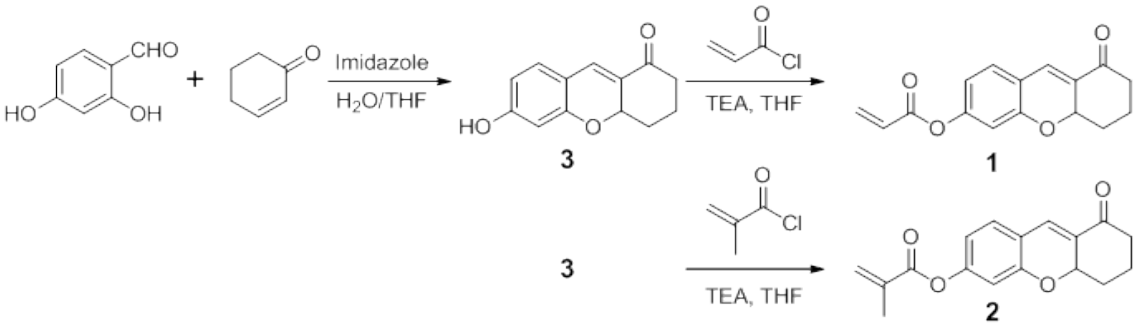
General information and materials.

All fluorescence and UV/Vis absorption spectra were recorded with Shimadzu RF-5301PC and Agilent 8453 spectrophotometers, respectively. All ^{1}H and ^{13}C NMR spectra were collected in $CDCl_3$ on a Varian 300 and 400 MHz spectrometer. All chemical shifts are reported in ppm value using the peak of residual proton signals of TMS as an internal reference. HRMS data received directly from the Korea Basic Science Institute. The fluorescence imaging of cells and tissues was performed with a confocal laser scanning microscope (Carl-Zeiss LSM 5 Exciter, Oberko, Germany). All analytes were purchased from Aldrich and used as received. All solvents were analytical reagents from Duksan Pure Chemical Co., Ltd. All DMSO for spectra detection was HPLC reagent grade, without fluorescent impurity; H_2O was deionized.

Synthesis



Scheme S1. Synthetic pathway for probe **1** and **2**.

Synthesis of 6-hydroxy-2,3,4,4a-tetrahydro-1H-xanthen-1-one (**3**):

To a solution of salicylaldehyde (553 mg, 4 mmol) in THF (3 ml), 1.5 equiv. 2-cyclohexen-1-one (576 mg, 6 mmol) and 1.5 equiv. imidazole (408 mg, 6 mmol) was added. After mixed with deionized water (3 ml), the mixture was stirred at room temperature. After completion of the reaction (as monitored by TLC), the final mixture was treated with 1 M HCl (20 ml) and extracted with ethyl acetate. The organic layer was dried by Na_2SO_4 and concentrated under vacuum. Flash chromatographic purification on silica gel (Hexane/EtOAc = 100% to 30%) afforded pure product 432 mg (50% yield) as a light yellow solid. Spectral data was in agreement with previous reports.[S1]

Synthesis of 1-oxo-2,3,4,4a-tetrahydro-*1H*-xanthen-6-yl acrylate (**1**):

To a solution of 6-hydroxy-2,3,4,4a-tetrahydro-*1H*-xanthen-1-one (200 mg, 0.93 mmol) and triethylamine (151 mg, 1.5 mmol) in THF (20 mL) at 0 °C, acryloyl chloride (84 mg, 0.93 mmol) was added and the mixture was stirred for 2 h. After completion of the reaction, the reaction mixture was concentrated in vacuum and the residue was dissolved in ethyl acetate and then washed with water and brine, dried with Na_2SO_4. The concentrated residue was purified by column chromatography (10% ethyl acetate/methylene chloride) to give an light yellow solid (236 mg, 95%). ^{1}H NMR ($CDCl_3$) δ (ppm): δ 1.65-1.75 (m, 1H), 1.95-2.11 (m, 2H), 2.34-2.61 (m, 3H), 5.00-5.04 (m, 1H), 6.03 (d, *J*=10.4 Hz, 1H), 6.26-6.33 (m, 1H), 6.58-6.75 (m, 2H),7.22 (d, *J*=8.3 Hz, 1H), 7.41 (s, 1H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.16, 29.82, 39.01, 75.06, 110.05, 115.71, 120.24, 127.84, 130.14, 130.65, 131.01, 133.37, 149.64, 153.35, 164.25, 197.62; HRMS: m/z = 270.0892 [M].

Synthesis of 1-oxo-2,3,4,4a-tetrahydro-*1H*-xanthen-6-yl methacrylate (**2**):

To a solution of 6-hydroxy-2,3,4,4a-tetrahydro-*1H*-xanthen-1-one (200 mg, 0.93 mmol) and triethylamine (151 mg, 1.5 mmol) in THF (20 mL) at 0 °C, methacryloyl chloride (97 mg, 0.93 mmol) was added and the mixture was stirred for 2 h. After completion of the reaction, the reaction mixture was concentrated in vacuum and the residue was dissolved in ethyl acetate and then washed with water and brine, dried with Na_2SO_4. The concentrated residue was purified by column chromatography (10% ethyl acetate/methylene chloride) to give an light yellow solid (243 mg, 92%). ^{1}H NMR ($CDCl_3$) δ (ppm): δ 1.65-1.74 (m, 1H), 1.94-2.09 (m, 5H), 2.33-2.60 (m, 3H), 4.99-5.03 (m, 1H), 5.76 (s, 1H), 6.32 (s, 1H), 6.68-6.74 (m, 2H), 7.22 (d, *J*=8.3 Hz, 1H), 7.40 (s, 1H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.16, 18.58, 29.80, 39.00, 75.03, 110.12, 115.80, 120.11, 127.96, 130.06, 130.62, 131.05, 131.46, 135.80, 153.75, 156.94, 165.55, 197.59; HRMS: m/z = 284.1049 [M].

Spectroscopic data. Stock solutions (1.0 mM) of the biologically relevant analytes amino acids (Cys, Hcy, GSH, Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Tau, Thr, Trp, Tyr, Val), metals [K(I), Na(I), Ca(II), Cu(II), Fe(II), Fe(III), Mg(II), Zn(II)] were prepared in twice distilled water. Stock solutions of **1** (1 mM) and **2** (1 mM) were prepared in DMSO (dimethyl sulfoxide). For all measurements of fluorescence spectra, excitation was at 444 nm with all the excitation slit widths at 1.5 nm, and emission at 3.0 nm. All UV/Vis and fluorescence titration experiments were performed using 10 μM of **1** and **2** in PBS solutions (pH=7.4, 10 mM) with varying concentrations of analytes at 37 °C.

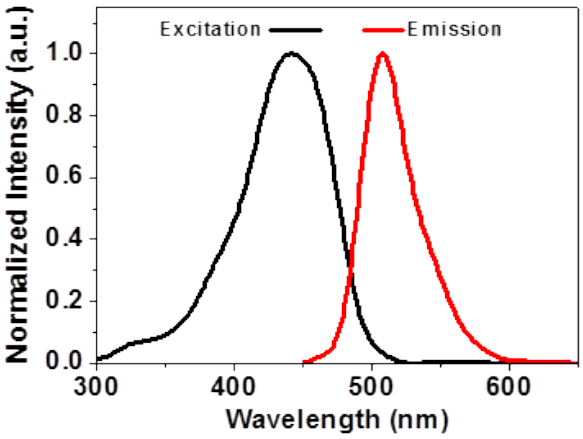


Figure S1. The fluorescence excitation (black) and emission (red) spectra of **1** (10 μM) at 509 nm in the presence of Cys (100 μM) in in ethanol-phosphate buffer (10 mM, pH 7.4, 2:8 *v*/*v*) at 37 °C.

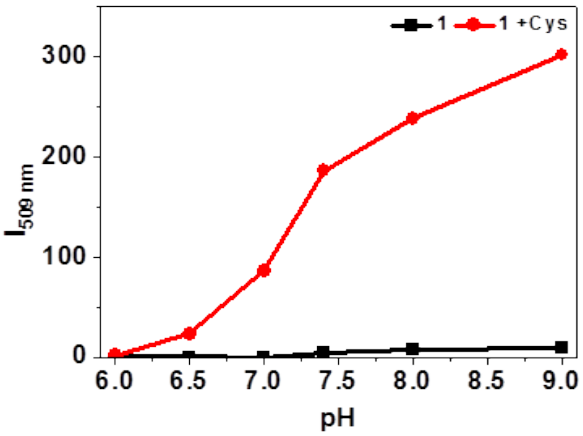


Figure S2. The fluorescence intensity of **1** (10 μM) at 509 nm in the presence (red) and absence (black) of Cys (100 μM) in ethanol-phosphate buffer at different pH value (λ_{ex} = 444 nm; Slit: 1.5 nm/1.5 nm).

Determination of the detection limit:

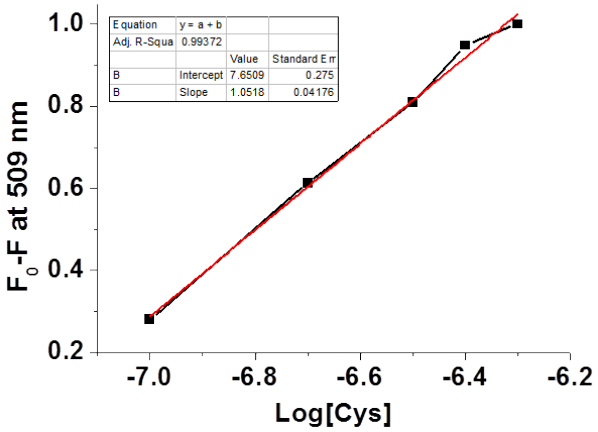


Figure S3. The detection limit of **1** for Cys was estimated from plot of normalized fluorescence changes (509 nm) of **1** verse Log[Cys] using an equation (Log[Cys] = -intercept/slop).[S2]

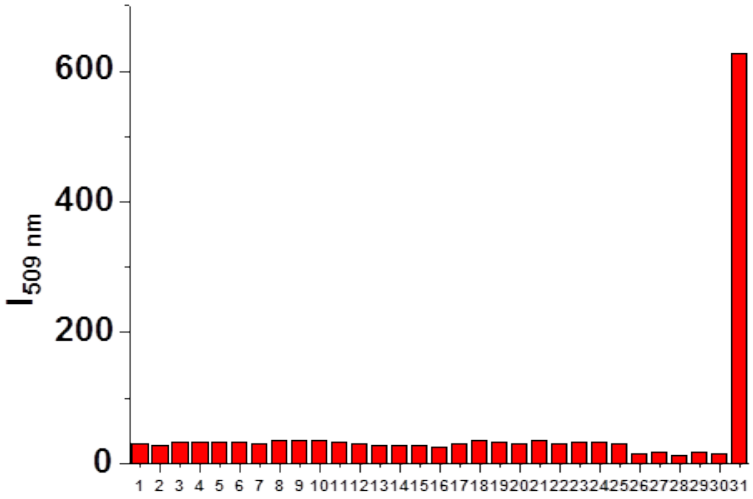


Figure S4. The histogram of the fluorescence intensities (509 nm) of probe **1** (10 μM) upon exposure to 1 mM of different biological analytes [1, only probe **1**; 2, Val; 3, Tyr; 4, Thr; 5, Tau; 6, Ser; 7, Pro; 8, Phe; 9, Met; 10, Lys; 11, Leu; 12, Ile; 13, His; 14, Gly; 15, Gluc; 16, Glu; 17, Gln; 18, Asp; 19, Asn; 20, Arg; 21, Ala; 22, Trp; 23, K(I); 24, Na(I); 25, Ca(II); 26, Cu(II); 27, Fe(II); 28, Fe(III); 29, Mg(II); 30, Zn(II)] except 100 μM of Cys (31). The spectra were acquired in ethanol-phosphate buffer (10 mM, pH 7.4, 2:8 *v*/*v*) after 15 min at 37 °C. Excitation was provided at 444 nm.

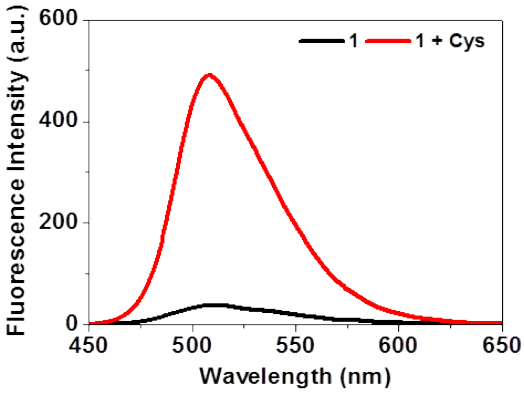


Figure S5. Two-photon fluorescence spectra of **1** (10 μM) in the presence (red) and absence (black) of Cys (100 μM). Excitation was provided at 740 nm. The spectra were recorded 15 min after addition of analyte in ethanol-phosphate buffer (10 mM, pH 7.4, 2:8 *v*/*v*) at 37 °C.

One-Photon Fluorescence Microscopy (OPM).

One-photon fluorescence microscopy images of probe-labeled tissues were obtained using a confocal laser scanning microscope (Carl-Zeiss LSM 710 Exciter, Oberko, Germany). Fluorescence channel was excited at 488 nm with a Si laser and emission was collected by a 500-600 nm band pass filter. One-photon images of various organs and tumor tissue were taken by a Carl-Zeiss Axio Imager M1 microscope (Oberko, Germany) equipped with a CRI Nuance multispectral imaging camera (CRI, Woburn, MA, USA).

Two-Photon Fluorescence Microscopy (TPM).

Two-photon fluorescence microscopy images of probe-labeled tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with ×100 oil and ×20 dry objective, numerical aperture (NA) = 1.30 and 0.50, respectively. The two-photon fluorescence microscopy images were obtained with a DM IRE2Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 740 nm. To obtain images at 450−600 nm range, internal PMTs were used to collect the signals in an 8 bit unsigned 512 × 512 pixels at 400 Hz scan speed.

Cell culture and data analysis

Three human hepatocellular carcinoma cells (HepG2, Hep3B, and Huh7), a human breast cancer cells (MDA-MB-231), and a human cervical cancer cells (HeLa) were purchased from Korean Cell Line Bank (Seoul, Republic of Korea). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO BRL., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO), and 1% penicillin (GIBCO) and streptomycin (GIBCO) at 37˚C in a 5% CO_2 and 95% air environment. When cell density reached 70~80% of confluence, subculture was done. Medium was changed every 3~4 days.

Cells were seeded on 35 mm-glass bottom confocal dish (SPL, Kyong-Gi, Republic of Korea) and stabilized 48 h. The cell density was 2.0 x 10^6 for a 1% gelatin pre-coated 35 mm-confocal dish. Prior to examine total intracellular Cys concentration in all cell lines, we treated probe **1** to HepG2 cells to examine sensitivity of the probe and to compare total intracellular Cys concentration among different cancer cell lines.

HepG2 were incubated with 500 μM *N*-ethylmaleimide (NEM, Sigma), which is a competitive binding chemical against probe **1**, for 30 min then treated probe **1** for 1 h**.** To induce intracellular Cys concentration, HepG2 were also incubated with glucose-free DMEM (GIBCO) for 2 h and then treated with probe **1** for 1 h at 37˚C in a 5% CO_2 air environment. Media was removed and the cells were washed twice with PBS. Fluorescence images were taken using a confocal laser scanning microscope (Carl-Zeiss LSM 710 Exciter, Oberko, Germany) with same condition. Fluorescence channel was excited at 488 nm with a Si laser and emission was collected by a 500-600 nm band pass filter. 3D deconvolution process of MetaMorph Offline software (Version 7.6.1, Molecular Devices, Sunnyvale, CA, USA) was used for analysis of cellular fluorescent images. A randomly selected 5 to 8 field per each cell line was used for quantitative analysis.

Xenograft mouse model and snapshot frozen section of organs/tumor

To examine of probe **1** in the tumor tissue, we used 7- to 8-week-old BALB/c nude mice from RaonBio (Kayonggido, Yonginsi, South Korea; control xenograft: n = 8; treatment and xenograft: n = 8). Before experimentation, animals in the animal facility were accustomed for 48 h and maintained as per the guidelines of the Care and Use of Laboratory Ani-mals published by the National Institutes of Health (Be-thesda, MD, USA). The animals were housed in cages and provided with water ad libitum and sterilized food, and maintained in a 12-h light/dark cycle with 30–40% humidity at room temperature (21 ± 2 °C). The 354234-matrigel (BD, San Jose, California, USA) were mixed with approximately 5.0×10^6 of A549 cells and subcutaneously injected into the right and left flanks of the mice.

Statistical analysis

All statistical analysis was determined using a one-way analysis of variance (ANOVA) of SAS program (Version 8.2, Cary, NC, USA). Paired Student's t-tests were performed to compare the relative means of all tested cell lines with that of HepG2 cells. P-values less than 0.05 were reported as significant difference. Numerical values were expressed as the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicate. Statistical significance was reported when *P* value was <0.05.

Cytotoxicity.

The cytotoxic effect of probe **1** was validated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Life Technologies, Carlsbad, CA, USA) assay as following the manufacture's instruction. Briefly, HeLa, HepG2, Hep3B, Huh7, BJ, and MDA-MB-231 cells (1.7 X 10^4 cells in 150 µl of media) were seeded onto a 96 well plate. On the following day, the culture medium was changed with different concentrations (0, 1, 5, 10, and 50 µM) of probe **1** in 100 µl of fresh medium and incubated them at 37˚C for 24 h. After 24 h, 10 µl of 12 mM MTT solution to each well, including negative controls of 10 µl of distilled water, medium alone, and MTT stock solution added to two wells in absence of probe **1**. After an hour and half incubation, 25 µl of medium was removed from the wells and then added 50 µl of DMSO to each well and mix thoroughly for 20 min. Absorbance was measured at 540 nm using a microplate spectrophotometer (PowerWave XS, Bio-Tek, Winooski, VT, USA).

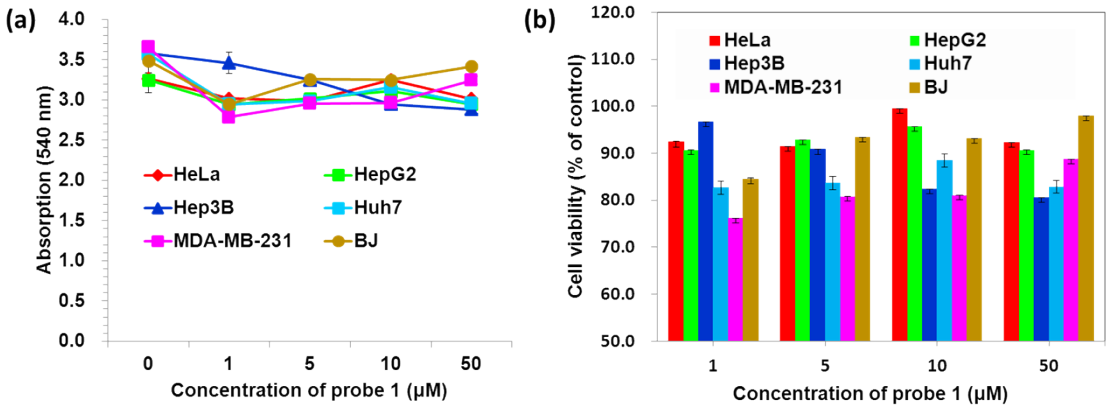


Figure S6. .Cytotoxic effect of probe **1** in HeLa, HepG2, Hep3B, Huh7, MDA-MB-231, and BJ. The cytotoxic effect of MTT was performed followed by manufacture's instruction. Different concentrations (0, 1, 5, 10, and 50 µM) of probe **1** were tested for 24 h incubation. (a) Absorption of 6 different cell lines was not much different, regardless of concentrations. (b) The control cells 100% alive without compound condition. At least 75% of the probe **1** treated cells are alive after 24 h of incubation at 50 µM concentration. This indicates that the probe **1** is nontoxic to various cell lines.

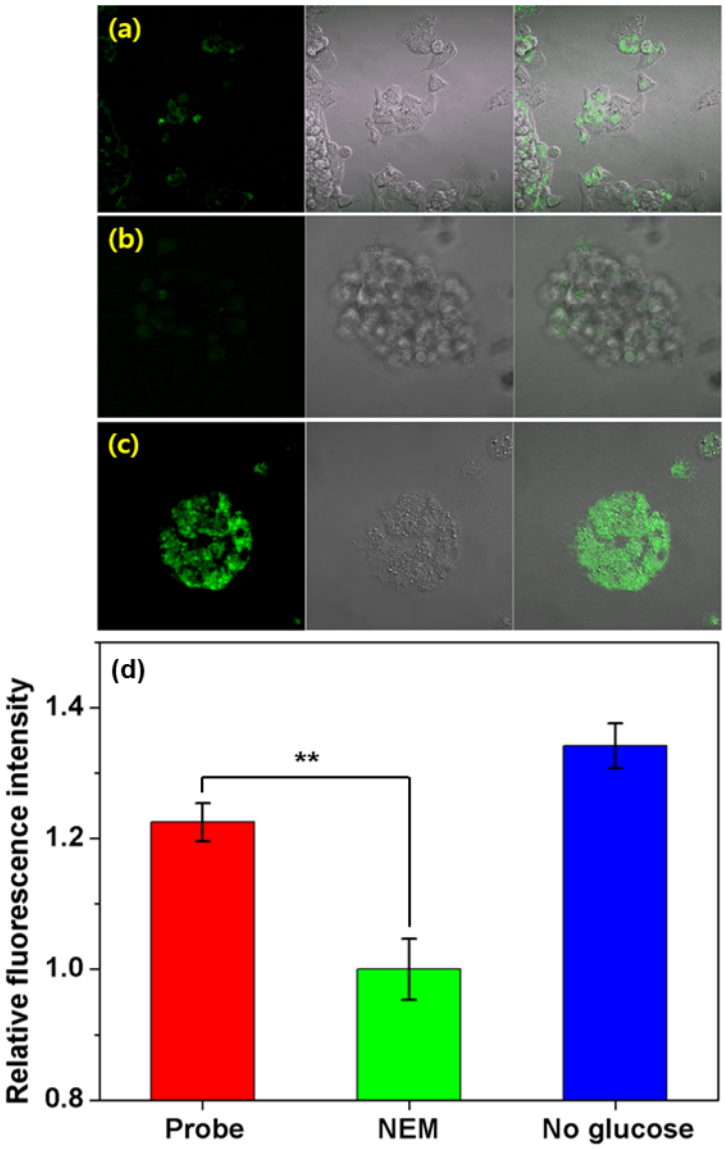


Figure S7. OPM images of **1** in HepG2 cells. (a) Cells were incubated with **1** (10 μM) for 1 h. (b) Cells were pre-incubated with 500 μM NEM for 30 min and washed. Then the cells were treated with **1** (10 μM) for 1 h. (c) Cells were incubated in glucose-free DMEM for 2 h, and then treated with **1** (10 μM) for 1 h. Magnification: x400. (d) Relative fluorescence intensity of (a), (b) and (c). Note that fluorescence intensity was significantly decreased in the presence of NEM (**$p < 0.005$).

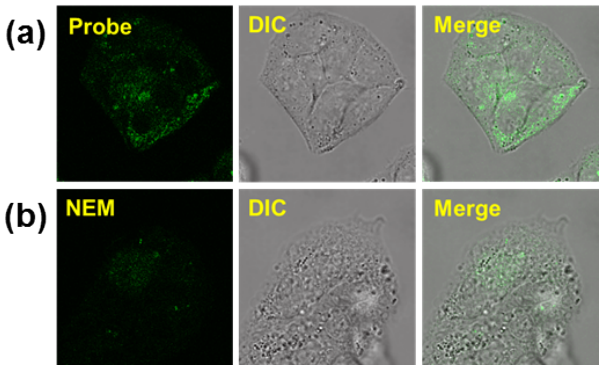


Figure S8. TPM fluorescence images of **1** in HepG2 cells. (a) Cells were incubated with **1** (10 μM) for 1 h. (b) Cells were pre-incubated with 500 μM NEM for 30 min, washed, and then treated with **1** (10 μM) for 1 h. Magnification: x400.

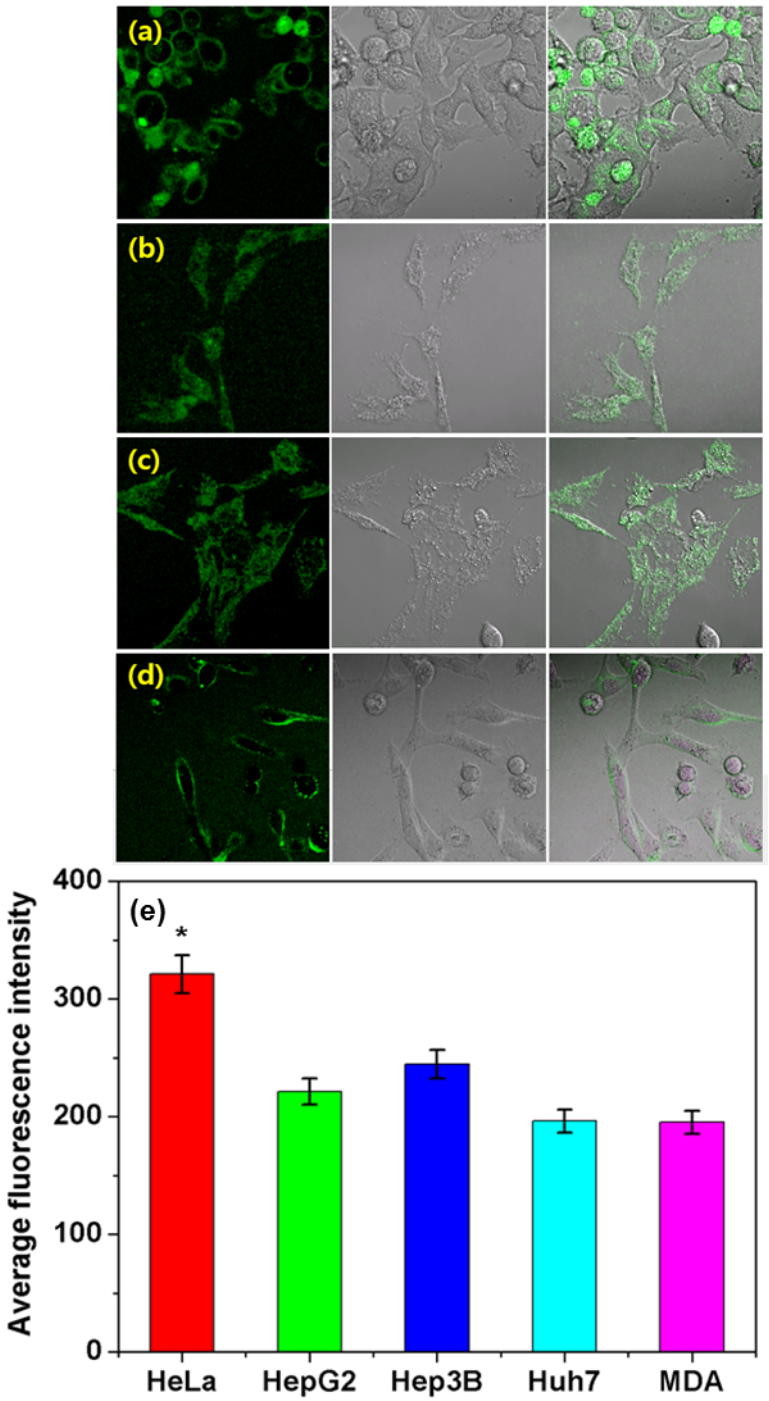


Figure S9. (a-d) OPM images of **1** in four living carcinoma cell lines (HeLa, Hep3B, Huh7, and MDA-MB-231). Magnification: x400. (e) Average fluorescence intensity in different carcinoma cell line. Note that average fluorescence intensity of HeLa was significantly high, compared to the rest of tested cell lines (*$p < 0.05$).

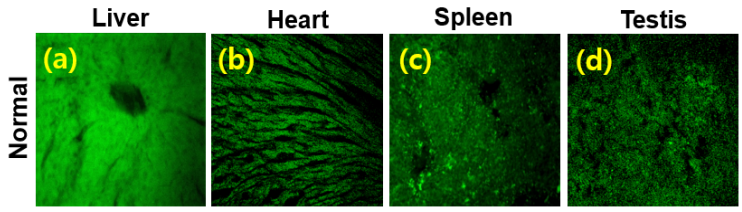


Figure S10. (a-d) TPM images of tissues from different organs (liver, heart, spleen, and testis) stained with probe **1** (10 μM) for 2 h. Note that liver has the highest fluorescent intensity among other major organs. Magnification: x100.

^{1}H-NMR and ^{13}C-NMR spectra

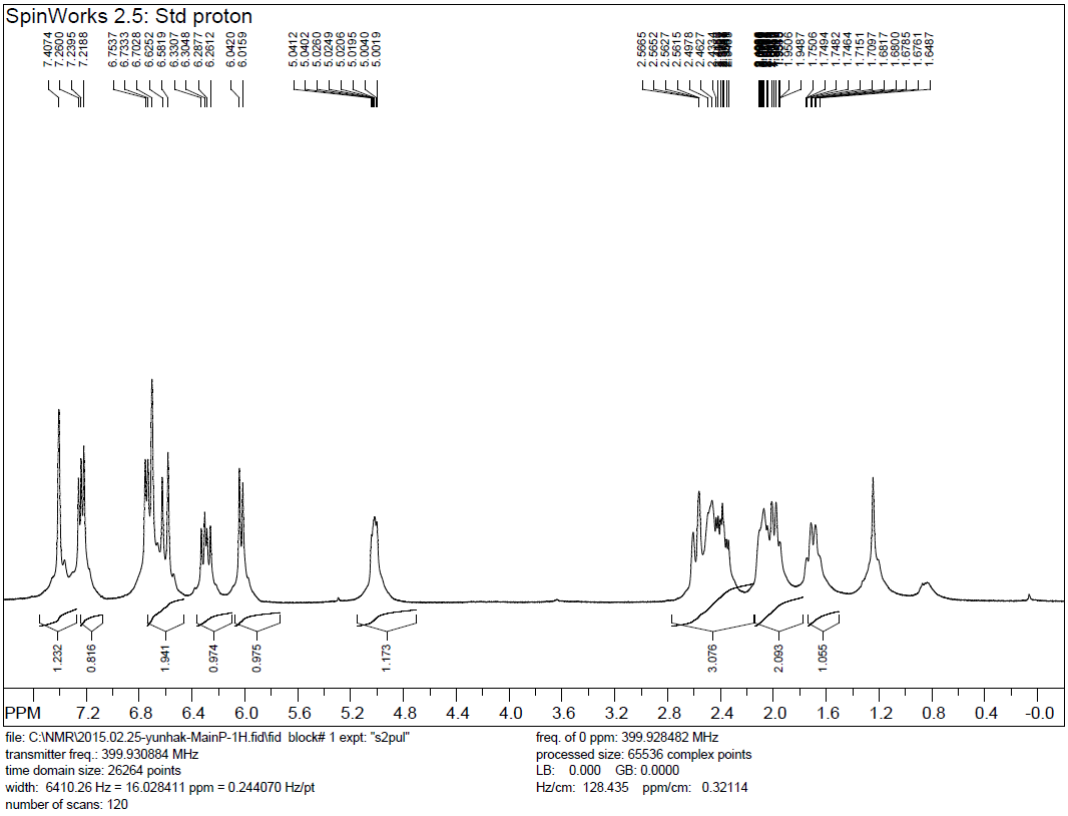


Figure S11. ^{1}H NMR spectra (400 MHz) of **1** in $CDCl_3$.

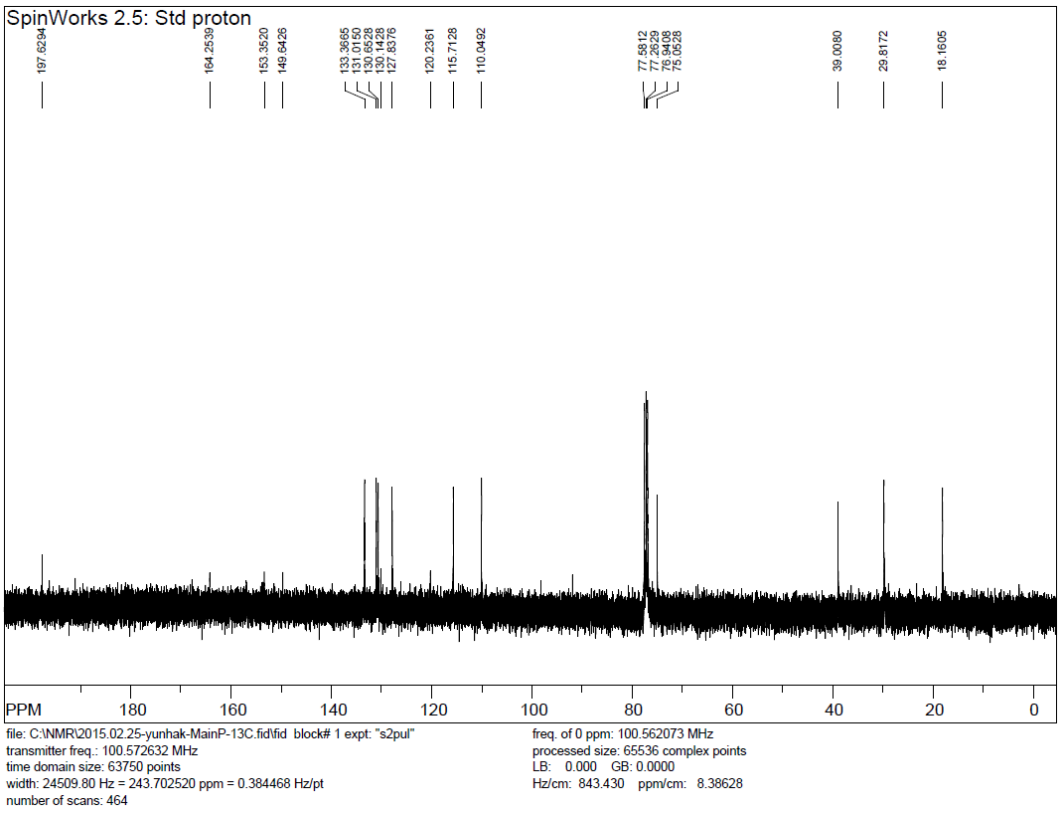


Figure S12. ^{13}C NMR spectra (100 MHz) of **1** in $CDCl_3$.

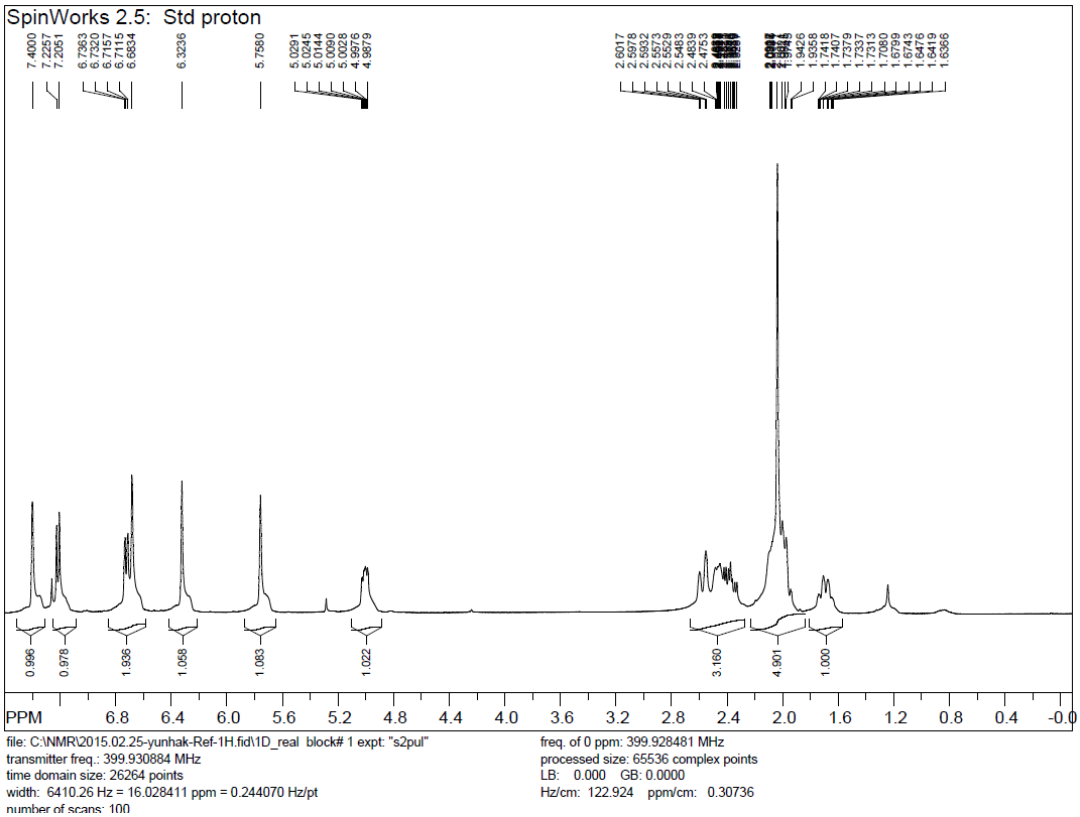


Figure S13. ^{1}H NMR spectra (400 MHz) of **2** in $CDCl_3$.

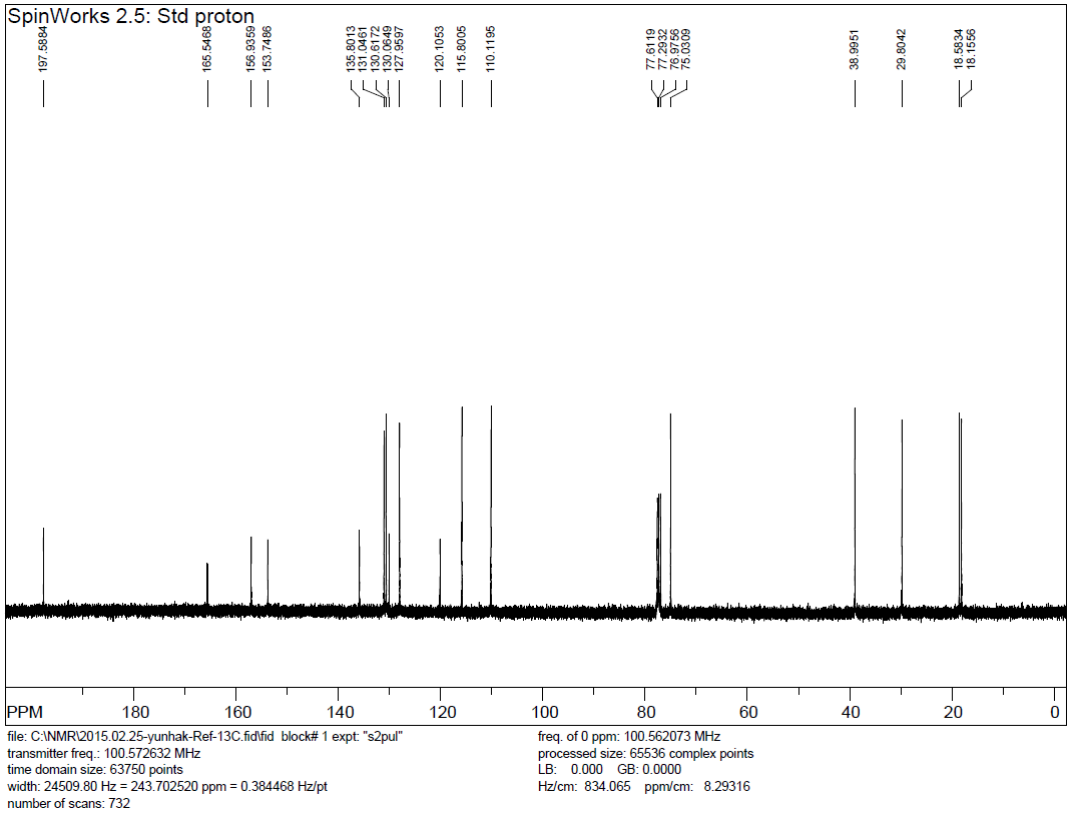


Figure S14. ^{13}C NMR spectra (100 MHz) of **2** in $CDCl_3$.

References

S1. L. Yuan, W. Lin, H. Chen, S. Zhu and L. He, *Angew. Chem. Int. Ed.*, 2013, **52**, 10018.

S2. M. Shortreed, R. Kopelman, M. Kuhn and B. Hoyland, *Anal. Chem.*, 1996, **68**, 1414.