

Supporting Information

A Small Molecule Multifunctional Tool for pH detection, Fluorescence Imaging and Photodynamic Therapy

*Qingbin Zeng,^{†‡} Qianni Guo,^{†‡‡} Yaping Yuan,^{†‡} Xiaoxiao Zhang,[†] Weiping Jiang,^{†‡}
Sa Xiao,^{†‡} Bin Zhang,[†] Xin Lou,[¶] Chaohui Ye,^{†‡} Maili Liu,^{†‡} Louis-S. Bouchard,[§]
Xin Zhou^{†‡*}*

[†]State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics,
National Center for Magnetic Resonance in Wuhan, Wuhan Institute of Physics and
Mathematics, Innovation Academy of Precision Measurement Science and
Technology, Chinese Academy of Sciences-Wuhan National Laboratory for
Optoelectronics, Wuhan, P. R. China.

[‡] University of Chinese Academy of Sciences, Beijing, P. R. China.

[¶] Department of Radiology, Chinese PLA General Hospital, Beijing, P. R. China.

[§] California Nano Systems Institute, Jonsson Comprehensive Cancer Center, The
Molecular Biology Institute, Department of Chemistry and of Bioengineering,
University of California, Los Angeles, USA.

|| These authors contributed equally

* Corresponding Author: xinzhou@wipm.ac.cn

Experimental Section and Procedure

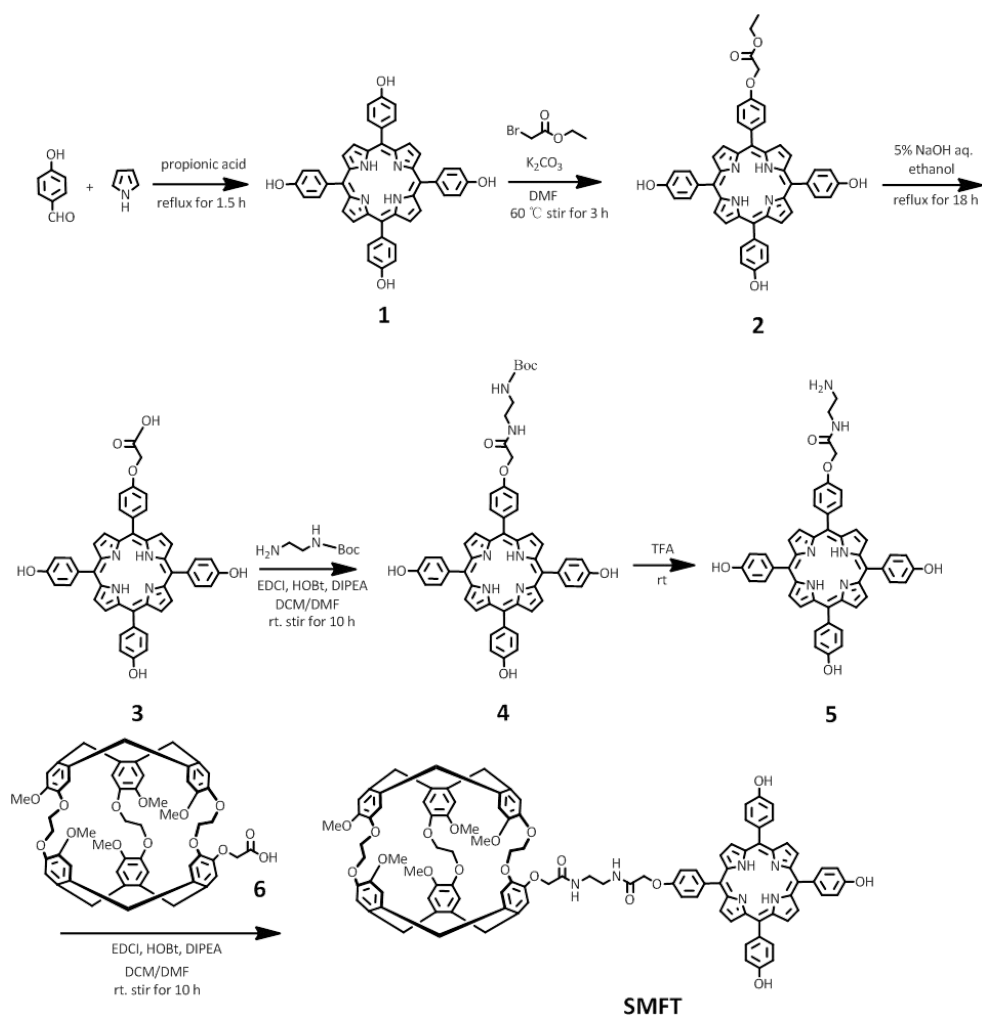
General

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification.

Pyrrole, *p*-Hydroxybenzaldehyde, ethyl bromoacetate, K₂CO₃, NaHCO₃, NaOH, Na₂CO₃, Na₂HPO₄, NaH₂PO₄, HCl, trifluoroacetic acid (TFA), propionic acid, dimethylformamide (DMF), tetrahydrofuran (THF), methanol, ethanol, dichloromethane (DCM), acetone were purchased from Sinopharm Chemical Reagent Co. Ltd., 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) was purchased from Shanghai Medpep Co. Ltd., 1-hydroxybenzotriazole (HOBt) was purchased from GL Biochem Ltd., N,N-diisopropylethylamine (DIPEA) was purchased from Aladdin Chemistry Co. Ltd. (all reagents were of analytical grade). Spectral grade Dimethyl sulfoxide (DMSO) was purchased from Tianjin Kemiou Chemical Reagent Co. Ltd.

Melting points of compounds were obtained using a melting point apparatus. ¹H NMR spectra were recorded on a Bruker AVANCE 500 spectrometer, using TMS as internal standard. ¹²⁹Xe MRS/MRI were recorded on a 400 MHz Bruker AV400 wide bore spectrometer. IR spectra were obtained on a Perkin Elmer PE-983 infrared spectrometer. High resolution mass spectra (HRMS) were taken on Bruker micrOTOF-Q spectrometer or Agilent 6530 Accurate-Mass Q-TOF spectrometer. The pH values were acquired by a Mettler Toledo SevenEasy pH meter. *In vivo* fluorescence images were taken on an IVIS Spectrum. The 650 nm laser source was purchased from Beijing Laserwave Optoelectronics Tech. Co., Ltd.

Synthesis



Scheme S1. Synthetic route of SMFT.

Synthesis of Compound 1. *p*-Hydroxybenzaldehyde (6.11 g, 50 mmol) was dissolved in 100 mL propionic acid and heated to 140 °C. Newly distilled pyrrole (3.35 g, 50 mmol) was dissolved in 20 mL of propionic acid, and then dropped in the solution during a 15 min period. The solution was cooled to room temperature after stirred for 1.5 h at 140 °C, and the precipitate was collected and filtered. The filter cake was washed by propionic acid for 3 times and dispersed in saturated $NaHCO_3$ solution, then stirred for another 3 h. The crude product was obtained by filtering and purified with silica gel column chromatography using DCM/MeOH to yield compound **1** as a

purple solid (1.2 g, 14.2%). The characterization data was identical to that reported in the literature.¹

Synthesis of Compound 2. Compound **1** (1.53 g, 2.25 mmol) was dissolved in 60 mL of dry DMF, followed by addition of K₂CO₃ (0.62 g, 4.5 mmol). After stirred at 60 °C for 1 h under N₂ atmosphere, ethyl 2-bromoacetate (0.75 g, 4.5 mmol) was added in one portion. The mixture was stirred for another 2 h at 60 °C. While the reaction completed, the mixture was cooled to room temperature, and 150 mL of ethyl acetate was added into mixture. The organic phase was washed by water for three times and then dried with anhydrous Na₂SO₄. The solvent was evaporated under vacuum, and the residue was purified via silica gel column chromatography using DCM/MeOH (v/v 100/1-50/1) to give compound **2** as a bluish purple solid (320 mg, 18.6%). The characterization data was identical to that reported in the literature.²

Synthesis of Compound 3. Compound **2** (160 mg, 0.21 mmol) was dispersed in 20 mL of 5% NaOH aqueous ethanol (1/1), and then refluxed for 18 h. After the mixture cooled to room temperature, 10 mL of water was added into the solution. Whereafter, HCl (0.1 M) was dropped in the solution until the solution became neutral. And then, ethyl acetate (100 mL) was added, and the organic layer was washed with water, and dried with anhydrous Na₂SO₄, and the solvent evaporated under vacuum. The residue was purified via silica gel column chromatography using DCM/MeOH (20/1) to give compound **3** as a purple solid (100 mg, 64.9%). The characterization data was identical to that reported in the literature.²

Synthesis of Compound 4. Compound **3** (100 mg, 0.136 mmol) was dissolved in dry DCM (6 mL) and dry DMF (2 mL) mixture, followed by addition of EDCI (31.3 mg, 0.163 mmol). The mixture was stirred at 0 °C under N₂ atmosphere for 30 min, followed by addition of HOBt (22 mg, 0.163 mmol). The mixture was stirred at 0 °C under N₂ atmosphere for another 1 h. After that, N-Boc-ethylenediamine (21.8 mg, 0.136 mmol) and DIPEA (70.3 mg, 0.544 mmol) was added. The mixture was stirred at room temperature for 10 h. After the reaction completed, 40 mL ethyl acetate was added, the organic layer was washed with water for 3 times and dried with anhydrous Na₂SO₄, and the solvent was removed under vacuum. The residue was purified with silica gel column chromatography using DCM/MeOH (v/v 20/1) to give compound **4** as a bluish purple solid (65 mg, 54.5%). M.p.: >300 °C. UV-Vis (MeOH, 20 °C, λ_{max} /nm): 418, 518, 553, 595, 650. IR (KBr): ν_{max} 3317, 1676, 1600, 1510, 1452 cm⁻¹. ¹H-NMR (500 MHz, Acetone-d₆, OH signal not observed): δ 9.12-8.75 (m, 8H, pyrrole), 8.19 (d, J = 8.5 Hz, 2H), 8.07 (dd, J = 8.3, 2.1 Hz, 6H), 7.99 (s, 1H, NH), 7.47 (d, J = 8.4 Hz, 2H), 7.31 (d, J = 8.2 Hz, 6H), 6.23 (s, 1H, NH), 4.81 (s, 2H), 3.51 (m, 2H), 3.35 (m, 2H), 1.44 (s, 9H), -2.70 (s, 2H, pyrrole NH). HRMS (ESI): [M+H]⁺, calc'd for C₅₃H₄₇N₆O₇ 879.3506, found 879.3442.

Synthesis of Compound 5. Compound **4** (80 mg, 0.091 mmol) was disperse in 2 mL dry DCM, and 2 mL TFA was added dropwise carefully. After the mixture was stirred at room temperature for 6 h, 15 mL of water was added into the mixture. And then, saturated NaHCO₃ solution was added dropwise until the solution became neutral. Ethyl acetate (60 mL) was then added, and the organic layer was separated, washed with water, and dried with anhydrous Na₂SO₄. The solvent was evaporated under

vacuum to give compound **5** (60 mg) as a purple solid. The product was taken to the next step without further purification.

Synthesis of Compound 6. Compound **6** was prepared as described in Ref. ³ Cryptophanol-A (100 mg, 0.113 mmol) and ethyl 2-bromoacetate (38.4 mg, 0.23 mmol) were dissolved in 15 mL of acetone, and then K₂CO₃ (77.4 mg, 0.56 mmol) was added. The mixture was cooled to room temperature after refluxed for 6 h, 20 mL of ethyl acetate was then added. The organic phase was washed with water (30 mL×3) and dried with anhydrous Na₂SO₄. After remove the solvent under vacuum, the residue was purified via silica gel column chromatography using DCM/MeOH (40/1) to give a white solid. After the solid was refluxed 10 h in 20 mL of 3 M NaOH aqueous THF (1/1). While the solution was cooled to room temperature, HCl (0.1 M) was dropped into the solution until the solution became neutral. DCM (30 mL) was then added, and the organic layer was washed with water, and dried with anhydrous Na₂SO₄. The solvent was removed under vacuum to yield a white solid as the compound **6** (83 mg, 78.3%). The characterization data was identical to that reported in the literature. ³

Synthesis of SMFT. Compound **6** (30 mg, 0.03 mmol) was dissolved in dry DCM (3 mL) and dry DMF (1 mL) mixture, and then EDCI (7.4 mg, 0.038 mmol) was added. The mixture was stirred at 0 °C under N₂ atmosphere for 30 min, and then HOBt (5.1 mg, 0.038 mmol) was added. After the mixture was stirred at 0 °C under N₂ atmosphere for another hour, compound **5** (23.4 mg, 0.03 mmol) and DIPEA (15.5 mg, 0.12 mmol) were added. 20 mL of ethyl acetate was added into the mixture after react 10 h at room temperature. And then, the organic layer was washed with water for 3

times and dried with anhydrous Na₂SO₄. The solvent was evaporated under vacuum. The residue was purified with silica gel column chromatography using DCM/MeOH (v/v 20/1) to give SMFT as a bronze solid (12 mg, 23.5%). M.p.: >300 °C. UV-Vis (MeOH, 20 °C, λ_{max} /nm): 419, 518, 555, 594, 650. IR (KBr): ν_{max} 3416, 1664, 1607, 1509, 1462 cm⁻¹. ¹H-NMR(500 MHz, acetone-d₆, OH signal not observed): δ 9.10-8.88 (m, 8H, pyrrole), 8.25 (d, J = 8.5 Hz, 2H), 8.16 (d, J = 8.1 Hz, 6H), 7.87 (s, 1H, NH), 7.59 (d, J = 8.6 Hz, 2H), 7.40 (dd, J = 8.2, 3.3 Hz, 6H), 7.03 (s, 1H), 6.97 (s, 1H), 6.92 (d, J = 4.0 Hz, 3H), 6.89 (s, 1H), 6.85-6.77 (m, 5H), 6.75 (s, 1H), 4.97 (d, J = 7.7 Hz, 2H), 4.72-4.50 (m, 6H), 4.45-4.19 (m, 16H), 4.00-3.73 (m, 19H), 3.40-3.33 (m, 4H), 3.21-3.15 (m, 2H), -2.61 (s, 2H, pyrrole NH). ¹³C NMR (151 MHz, Acetone-d₆) δ 169.61, 168.41, 157.95, 157.56, 157.45, 149.47, 149.41, 149.37, 147.12, 147.08, 146.70, 146.29, 146.21, 145.80, 135.58, 135.27, 133.94, 133.91, 133.60, 133.52, 133.29, 133.14, 133.14, 132.19, 131.74, 131.44, 131.38, 131.22, 120.98, 120.25, 120.16, 119.95, 119.60, 119.37, 118.66, 118.61, 116.51, 114.57, 114.34, 114.21, 113.81, 113.73, 113.39, 69.71, 68.83, 68.75, 68.69, 68.64, 68.53, 68.36, 67.63, 56.62, 55.45, 55.39, 39.55, 38.73, 35.28, 35.19, 35.16, 34.93. HRMS (ESI): [M+H]⁺ calc'd for C₁₀₃H₉₁N₆O₁₈ 1700.6423, found 1700.6314.

Hyper-CEST ¹²⁹Xe NMR/MRI Studies

SMFT (25 μ M) was dissolved in different pH values buffer (including 50% DMSO) and then Hyper-CEST spectra and Hyper-CEST MRI were acquired. Hyperpolarized ¹²⁹Xe gas was generated by a home-built ¹²⁹Xe hyperpolarizer. All ¹²⁹Xe NMR/MRI experiments were taken on a 9.4 T Bruker AV400 wide bore NMR spectrometer (Bruker Biospin, Ettlingen, Germany). For all experiments, the sample temperature was controlled by a VT unit installed on the NMR spectrometer and held fixed at 298

K. A gas mixture of 10% N₂, 88% He, and 2% Xe (26.4% ¹²⁹Xe natural abundance) (Spectra Gases) was used. For the Hyper-CEST NMR experiment, the gas was directly bubbled into a 10 mm NMR tube while the gas mixture flowed through the hyperpolarizer. After 20 s bubbled and a 3 s delay, a cw-pulse (B₁=3.0 μT, saturation time is 10 s) was swept across the chemical shift range 55-85 ppm in steps of 1 ppm. This was followed by the acquisition of a spectrum. Each spectrum was acquired in a single scan. All NMR spectra were processed using 1 Hz Lorentz broadening. For the Hyper-CEST MRI experiment, 4 on-resonant (-166 ppm relative to dissolved xenon in solution at 0 ppm) and 4 off-resonant (166 ppm relative to dissolved xenon in solution at 0 ppm) scans were taken and averaged. The image was acquired using a RARE sequence (slice thickness=20 mm, matrix size=32×32, in-plane resolution=1.25×1.25 mm², FOV=40×40 mm², centric k-space encoding, bandwidth=5400 Hz, echo time=10 ms, repetition time=104 ms, no partial Fourier transform acceleration, rare factor=8). For each excitation the xenon gas mixture was bubbled into solution for 20 s followed by a 3 s delay to allow bubbles to collapse. This was followed by a 10 s, 6.5 μT saturation pulse. The MR images were processed on MATLAB (R2014a, MathWorks, Natick, MA), The image matrix 32×32 was interpolated into 128×128 image matrix, and the center area (20×20 mm²) of FOV was selected as new FOV (64×64 image matrix). Hyper-CEST effect for on-resonant saturation was analyzed compared to off-resonant saturation for each pixel by the formula (CEST effect = (off_{Res}-on_{Res})/off_{Res}) point by point. The mask was used in the post-processing which covers the image areas that do not belong to the sample phantom and the normalized signal intensities less than 0.2.

Cellular Hyper-CEST Spectra Studies

The human lung cancer cells A549 were incubated in cell culture medium (RPMI-1640) within a standard 5 % CO₂ incubator at 37 °C. The cells were incubated with SMFT (SMFT (30 μM) was dissolved in culture medium (including 1% DMSO, 1% Cremophor ® EL) for 4 h. The cells were washed three times with PBS buffer, and then trypsinization and resuspended in different pH values buffer (pH 7.4, pH 5.1), the cells concentration kept at 5.06×10⁶ cells/mL. After incubation for 30 min, the cells were transferred to an NMR tube, and the ¹²⁹Xe NMR spectra were acquired by Hyper-CEST. A selective saturation pulse was swept across the chemical shift range 55-85 ppm in steps of 1 ppm (cw-saturation for 10 s with B₁ = 6.0 μT). Temperature was set to 298 K.

Fluorescence Spectra Studies

SMFT (5 μM) was dissolved in different pH values buffer (including 50% DMSO) and then fluorescence spectra were acquired via a fluorescence spectrometer. The pH of the buffer ranged from 3.0 to 10.3. Unless otherwise stated, for all measurements the excitation wavelength was 430 nm, the excitation and emission slit widths were both 4 nm, and the dwell time was 0.1 s.

Cellular Fluorescence Imaging Studies

Human lung cancer cell A549 and human normal lung cell WI-38 were seeded on glass coverslips placed in the 6-well plates. After grown at 37 °C in an atmosphere of 5% CO₂ for 24 h, the regular culture mediums were removed. RPMI-1640 medium (for A549 cell) or MEM medium (for WI-38 cell) containing the SMFT was added (SMFT was dissolved in DMSO, and then stock in RPMI-1640 or MEM medium, including 1% DMSO, 1% Cremophor ® EL. The concentrations of SMFT were 10 μM, 30 μM, 50 μM). After 4 h of incubation at 37 °C, the medium was removed, and the cells were fixed by paraformaldehyde for 10 min, and then the cells were

incubated with DAPI for 5 min. All cells were washed by PBS at least three times. The cells fluorescence images were taken on a Nikon Confocal Laser Scanning Microscope (CLSM, Nikon, Japan).

In Vivo Fluorescence Imaging Studies

A549 tumor-bearing BALB/c male nude mice were injected with SMFT (1 mM, in 100 μ L of PBS solution, including 1% DMSO, 1% Cremophor $\text{\textcircled{R}}$ EL) via intravenous injection. After that, the mice fluorescence imaging was performed with an IVIS Spectrum in epifluorescence mode equipped with 430 and 660 nm filters for excitation and emission. The mice were sacrificed 24 h after intravenous injection, with their major organs including the tumor, liver, heart, lung, spleen, and kidneys collected for ex vivo imaging.

Generation of Singlet Oxygen Studies

The generation of singlet oxygen ($^1\text{O}_2$) was detected by the p-nitrosodimethylaniline and imidazole on a UV-Vis spectrophotometer. The method was followed the Kraljic procedure.⁴ SMFT (0 μ M, 5 μ M, 10 μ M, 15 μ M) or TPPS4 (10 μ M), p-nitrosodimethylaniline (30 μ M) and imidazole (30 μ M) were dissolved in buffer (including 50% DMSO) and equilibrated for 1 h keep in a dark place, and then irradiated by 650 nm laser (100 mW/cm²) for different periods of time. The diminishing of absorbance at 440 nm reflects the production of $^1\text{O}_2$.

Cytotoxicity Studies

The cells were added into a 96-well plate with 1×10^4 cells per well. After incubated at 37 $^{\circ}\text{C}$ in a 5% CO_2 incubator for 24 h, the culture medium were removed. Cells were treated with SMFT in different concentrations (SMFT was dissolved in DMSO, and then stock in RPMI-1640 medium, including 1% DMSO, 1% Cremophor $\text{\textcircled{R}}$ EL. The concentrations of SMFT were 0 μ M, 5 μ M, 10 μ M, 15 μ M, 20 μ M, 25 μ M) for 4 h.

After 4 h of incubation at 37 °C, the medium was replaced by fresh medium. The cells were irradiated with Laser (650 nm) at 100 mW/cm² for 10 min (total light dose 60 J/cm²) or kept under dark conditions, respectively. The cells were further incubated to achieve a total incubation time of 48 h, and A549 cell viability was measured at 490 nm for MTT assay.

In Vivo Anticancer Efficacy Studies

The PDT efficacy of SMFT was investigated using a flank A549 mouse tumor model. Tumor bearing mice were established by subcutaneous inoculation of A549 cell suspension (2×10^6 cells per mouse) into the right flank region of 6-week BALB/c male mice, respectively. Four groups were included for comparison: (1) PBS control, (2) PBS+laser, (3) SMFT, (4) SMFT+laser. For group (1) to (3), every group have 3 mice, for group (4) have 4 mice. Two hours post injection, each mouse in group (2) and (4) was irradiated at the tumor site with laser (100 mW/cm²) for 40 min (240 J/cm²). For (1) to (4) groups, mice are treated every 4 days for total 5 treatments. When tumors reached 50-70 mm³, PBS, SMFT (SMFT was dissolved in DMSO, and then stock in PBS, including 1% DMSO, 1% Cremophor ® EL) (9.75 mg/kg, 1.15 mM*100 uL) were intratumorally injected to animals. At 2 h post-injection, mice were anesthetized with 2% (v/v) isoflurane and tumors were irradiated with a 650 nm laser (100 mW/cm²). To evaluate the therapeutic efficacy, tumor growth and body weight evolution were monitored. The tumor size was measured with a digital caliper every 4 days before treatment. Tumor volumes were calculated as follows: (width²×length)/2. Finally, all mice were sacrificed after 20 days later, and the excised tumors were photographed. The tumors, heart, liver, spleen and kidney were embedded in optimal cutting temperature (OCT) medium, sectioned at 4 μm thickness, and subjected to hematoxylin and eosin (H&E) stain for histopathological analysis.

Additional Data

Additional Hyper-CEST Spectra

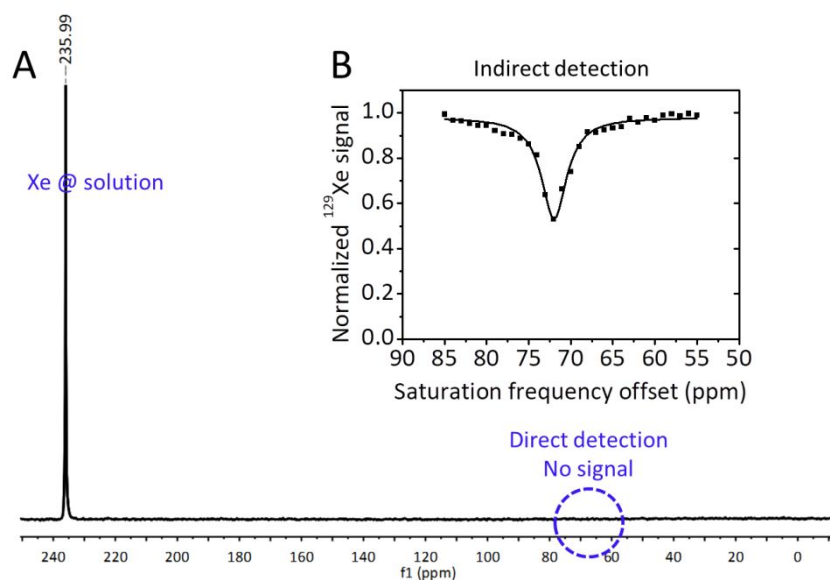


Figure S1. The ^{129}Xe NMR spectra of SMFT obtained by direct and indirect method. The SMFT (25 μM) was dissolved in buffer (pH 3.0, including 50% DMSO). (A) ^{129}Xe NMR spectrum, NS=1, LB=10 Hz; (B) Hyper-CEST ^{129}Xe NMR spectrum. Data was fitted to a Lorentzian line.

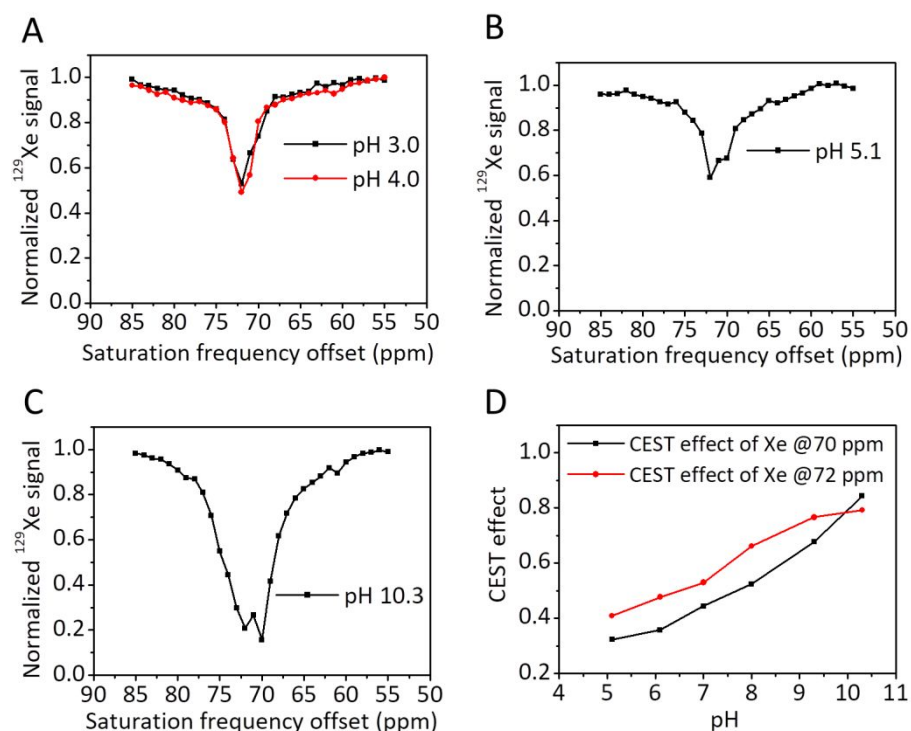


Figure S2. Hyper-CEST spectra of SMFT (25 μM) in different pH buffers (including 50% DMSO). (A) Hyper-CEST spectra of SMFT acquired in pH 3.0 and pH 4.0; (B) Hyper-CEST spectra of SMFT acquired in pH 5.0; (C) Hyper-CEST spectra of SMFT acquired in pH 10.3; (D) Hyper-CEST effect changed trend of SMFT with pH promoted from 5.1 to 10.3.

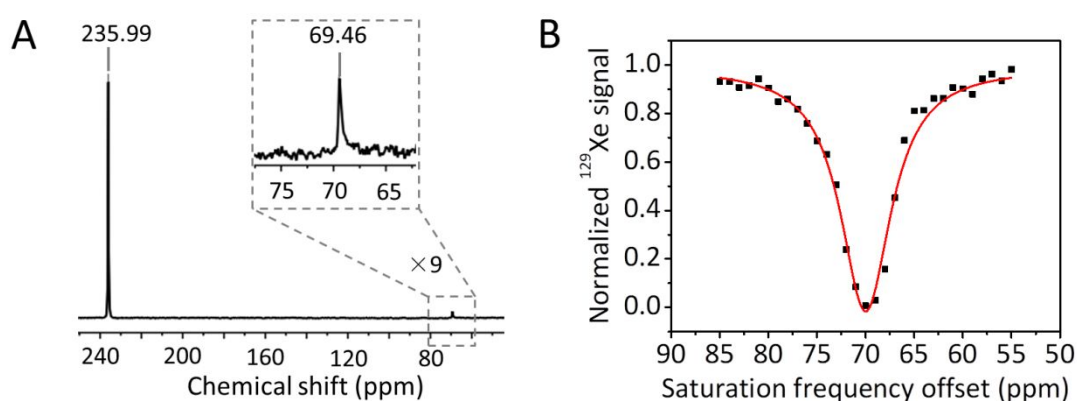


Figure S3. ^{129}Xe NMR spectra of SMFT after treated by NaOH aqueous. The SMFT (25 μM) was dissolved in buffer, including 50% DMSO. (A) The ^{129}Xe NMR

spectrum was obtained by direct method, NS=16, LB=10 Hz. (B) The ^{129}Xe NMR spectrum was obtained by Hyper-CEST.

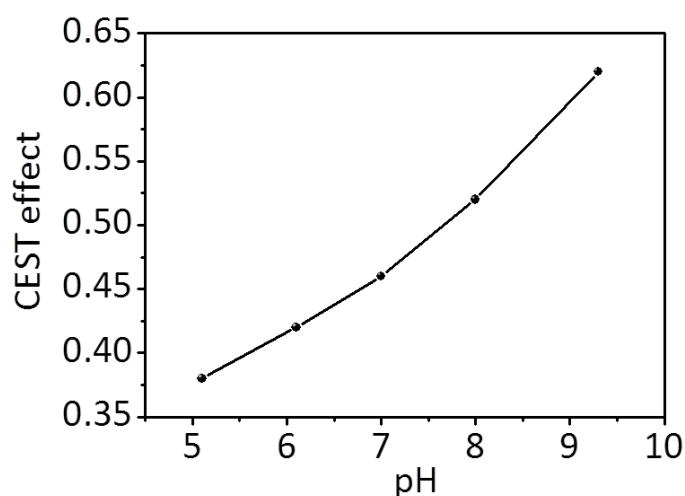


Figure S4. The average Hyper-CEST effect of SMFT calculated from Hyper-CEST images. The CEST effect was calculated based on the average of 4 on-resonant (saturation on Xe @ cage: 70 ppm) and 4 off-resonant (saturation at 402 ppm) images.

Additional Optical Spectra Data

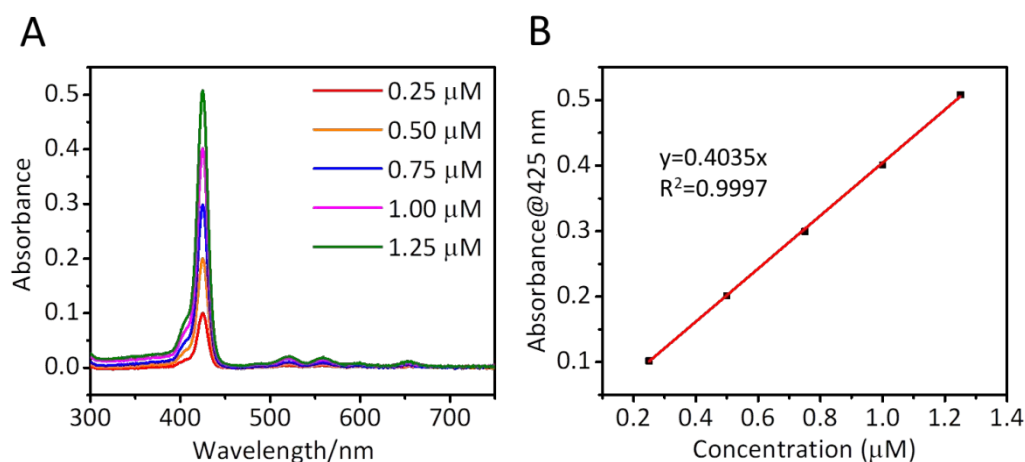


Figure S5. The absorption spectrum of SMFT (0.25 μM ~1.25 μM) in DMSO. (A) The adsorption spectrum for different concentration of SMFT in DMSO. (B) The concentration-dependent of absorbance for SMFT at 425 nm in DMSO. The molar extinction coefficient can be calculated by this equation, $A=\epsilon bc$. Here, ϵ is the molar

extinction coefficient, b is the length of optical path (1 cm), and c is the concentration of sample, respectively.

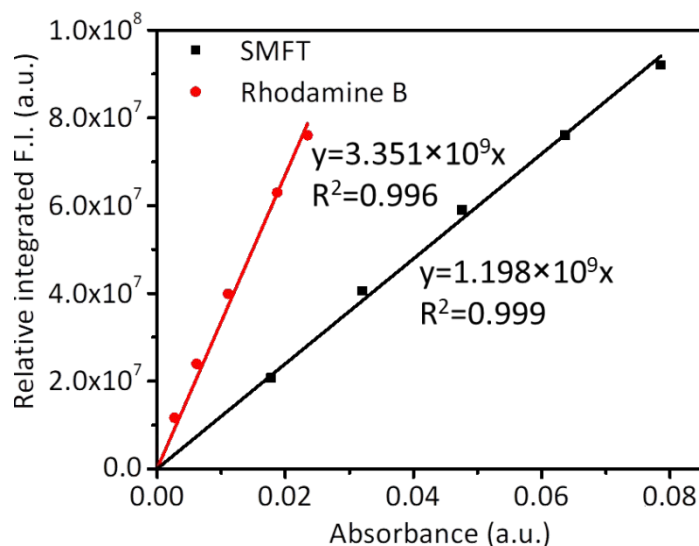


Figure S6. The measurements of fluorescence quantum yield of SMFT, $\lambda_{\text{ex}}=520$ nm, $\lambda_{\text{em}}=530\sim800$ nm. Fluorescence quantum yield determination of SMFT was relative calculated vs. a standard whose quantum efficiency has been accurately determined. Rhodamine B in aqueous solution (literature quantum yield: 0.31) was chosen as the reference. The fluorescence quantum yield can be calculated using the equation: $\Phi_s = \Phi_r(k_s/k_r)(\eta_s/\eta_r)^2$. Here the indices s and r , denote sample and reference, respectively. Where Φ is the fluorescence quantum yield, k is the slope determined by the curves, and η is the refractive index of the solution. In this experiment, the refractive index of DMSO is $\eta_{\text{DMSO}}=1.4795$, and the refractive index of H_2O is $\eta_{\text{H}_2\text{O}}=1.3330$, respectively.

Additional *In Vivo* Fluorescence Images

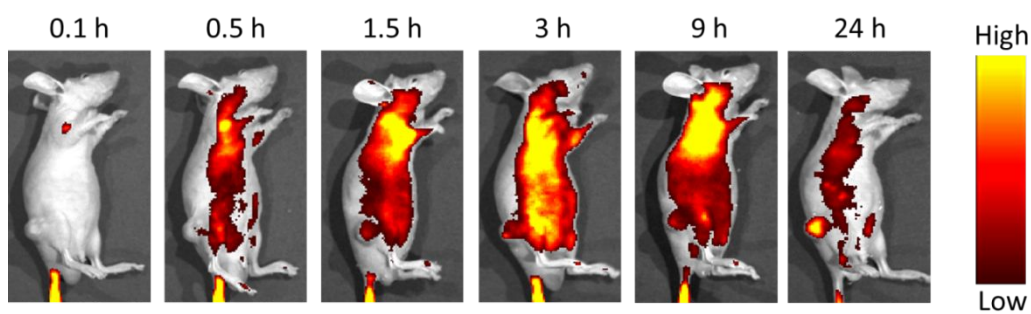


Figure S7. Time based *in vivo* fluorescence imaging of nude mouse before and after injecting SMFT (1 mM, 100 μ L) via intravenous injection.

Additional H&E Data

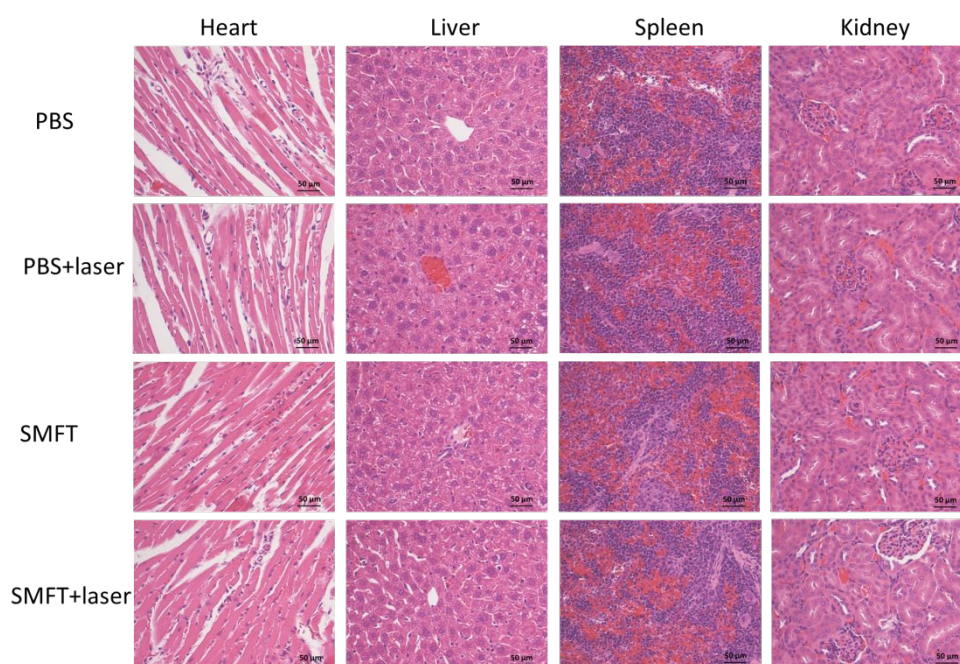


Figure S8. H&E stained of the excised heart, liver, spleen, kidney after different treatments. Scale bar, 50 μ m.

^1H NMR and HRMS Spectra

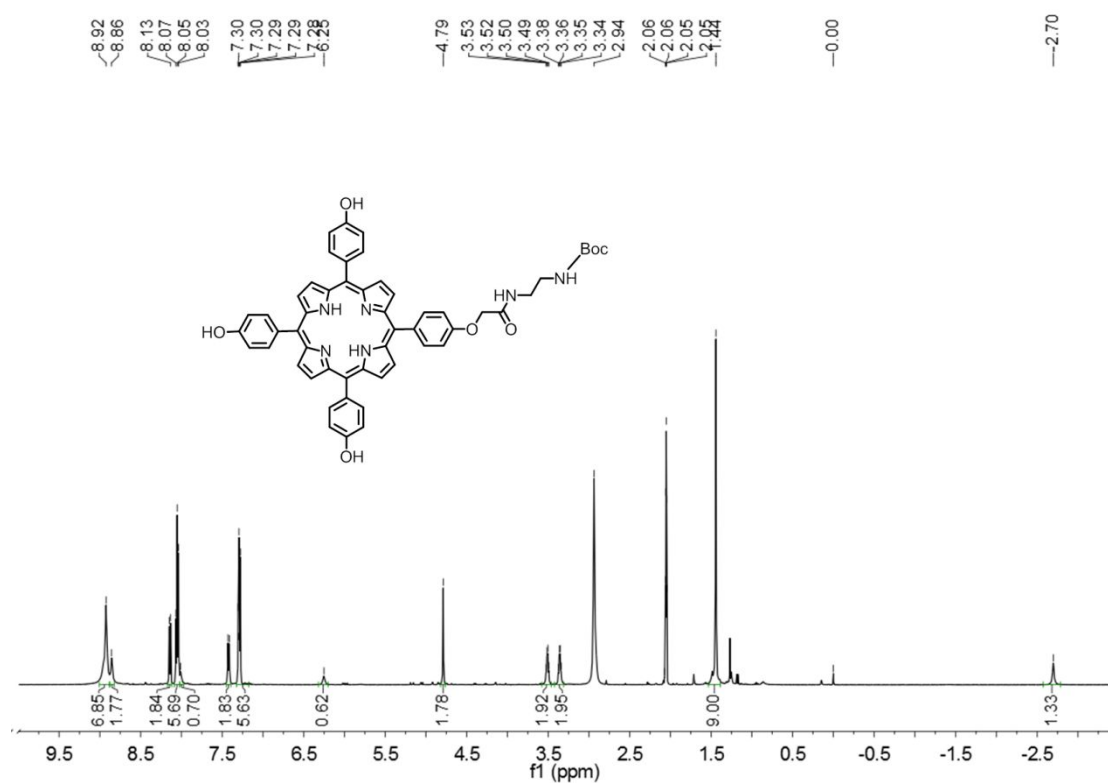


Figure S9. ¹H NMR of compound **4** recorded in acetone-d₆.

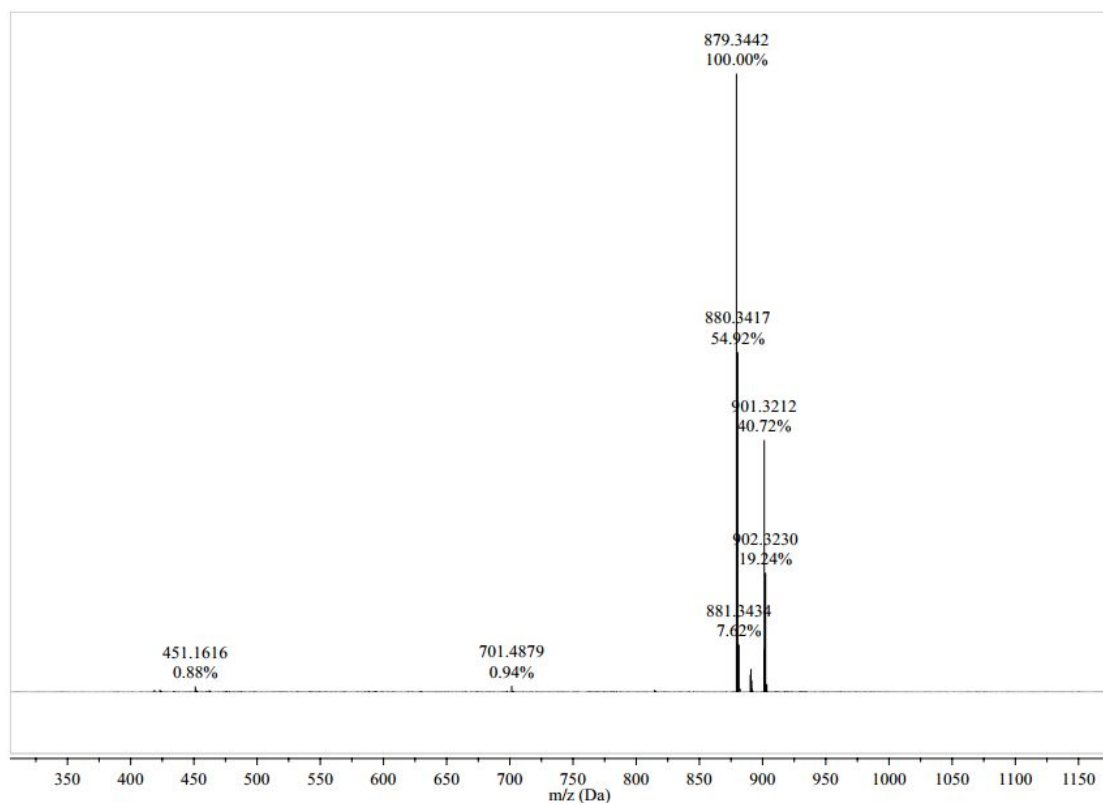


Figure S10. ESI-HRMS spectrum of compound **4**.

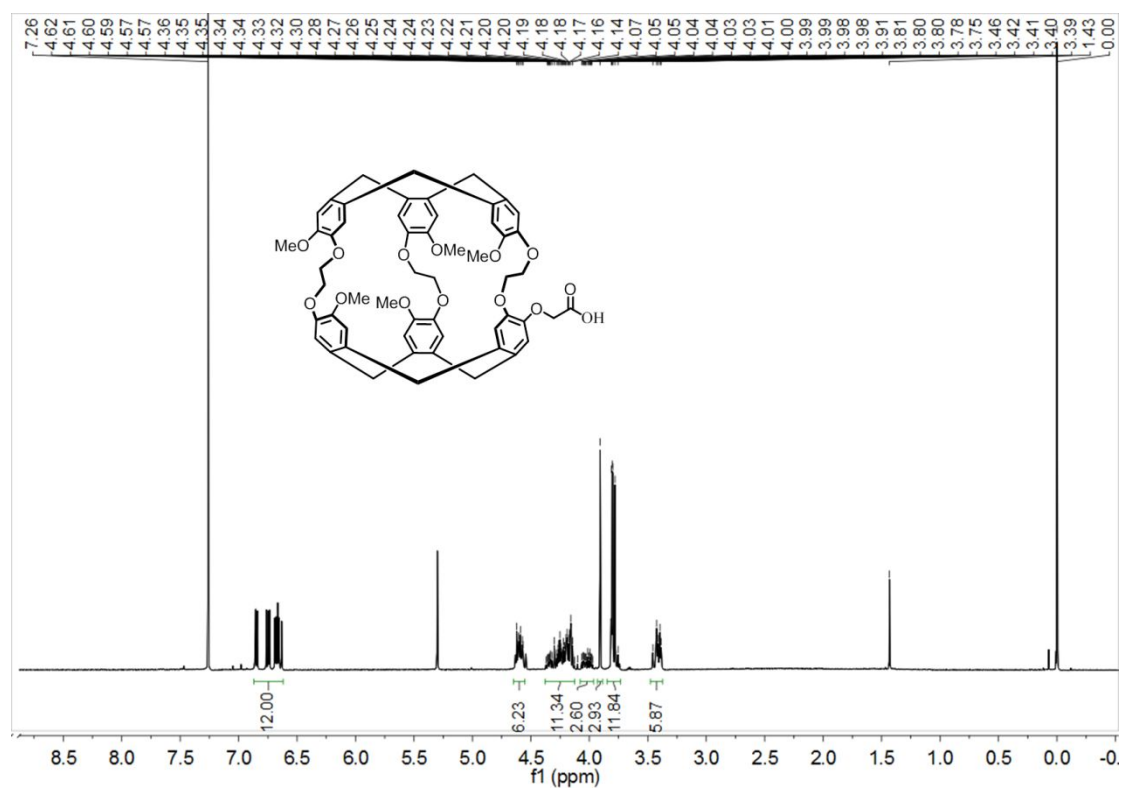


Figure S11. ¹H NMR of compound **6** recorded in CDCl₃.

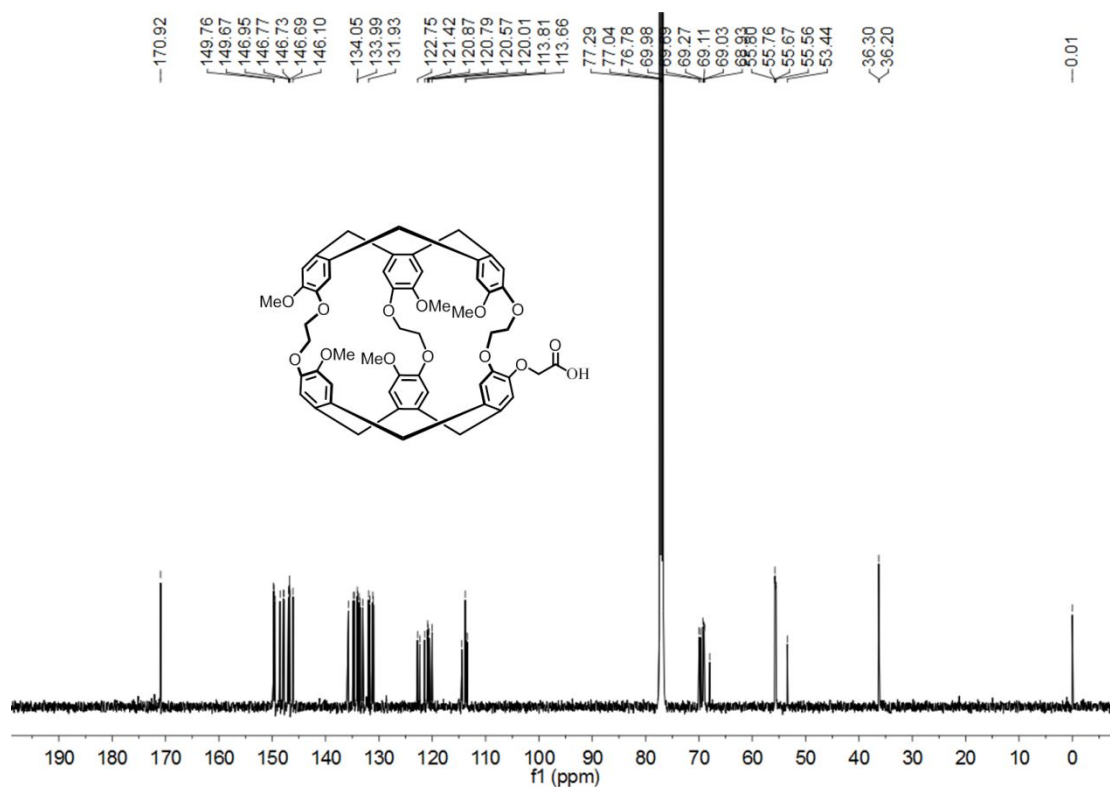


Figure S12. ¹³C NMR of compound **6** recorded in CDCl₃.

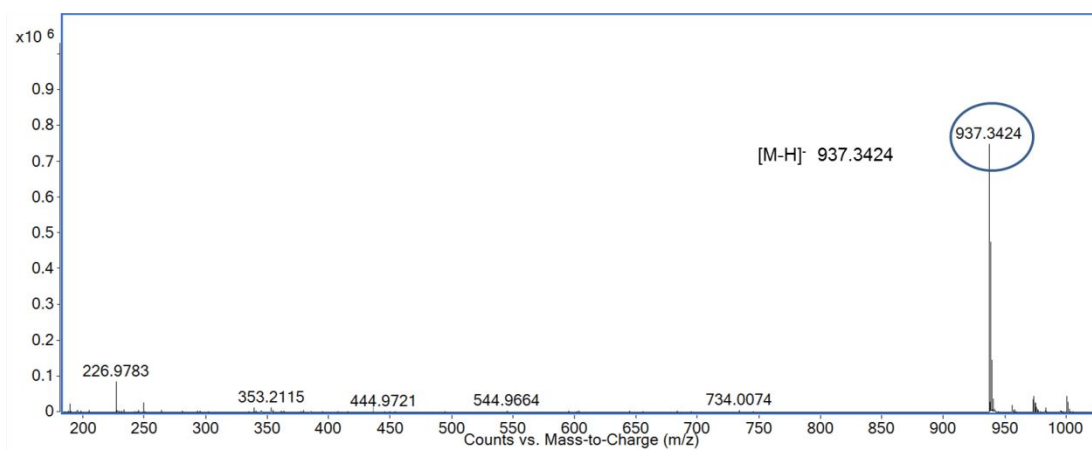


Figure S13. ESI-HRMS spectrum of compound **6**.

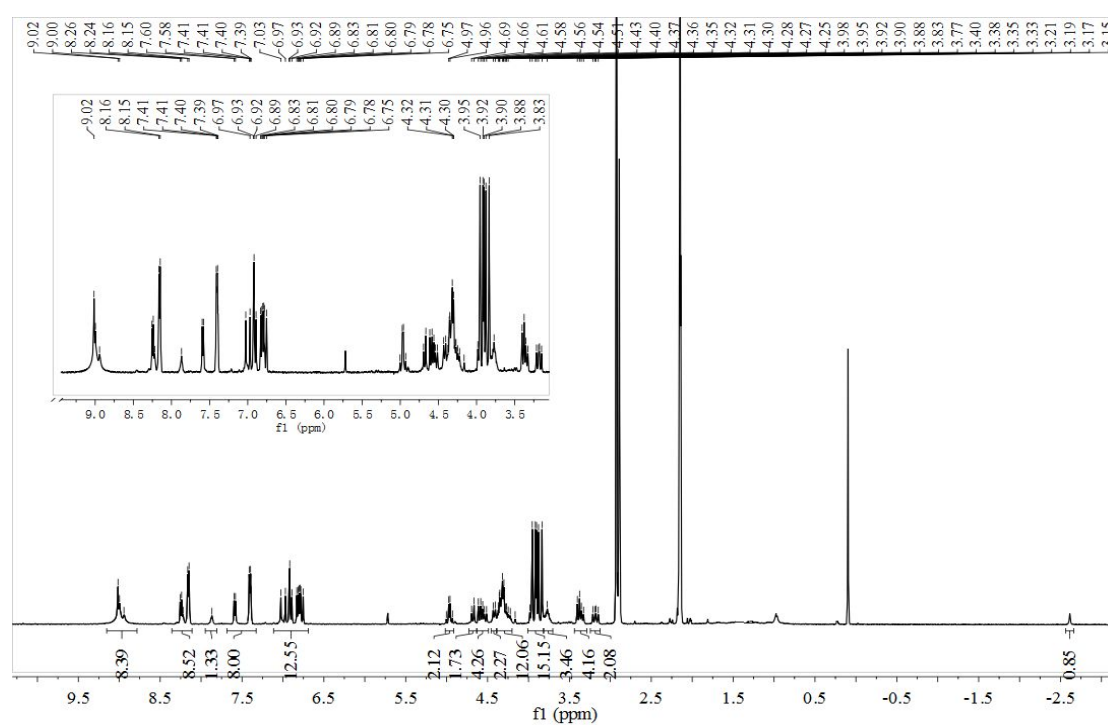


Figure S14. ^1H NMR of SMFT recorded in acetone- d_6 .

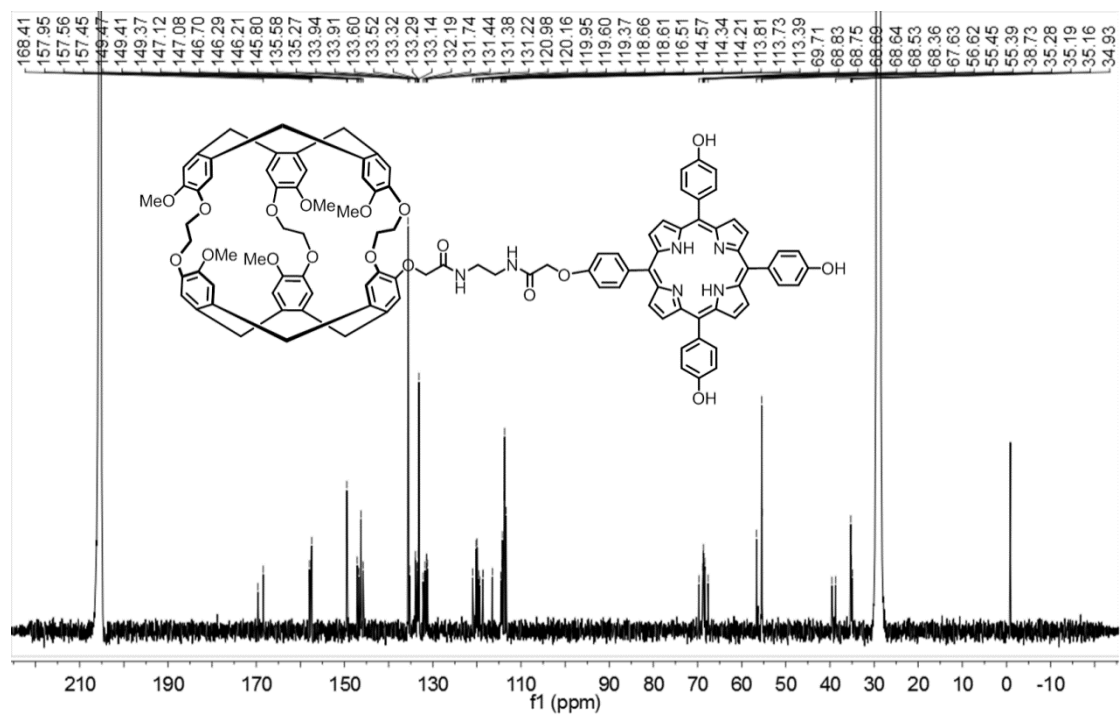


Figure S15. ^{13}C NMR of SMFT recorded in acetone- d_6 .

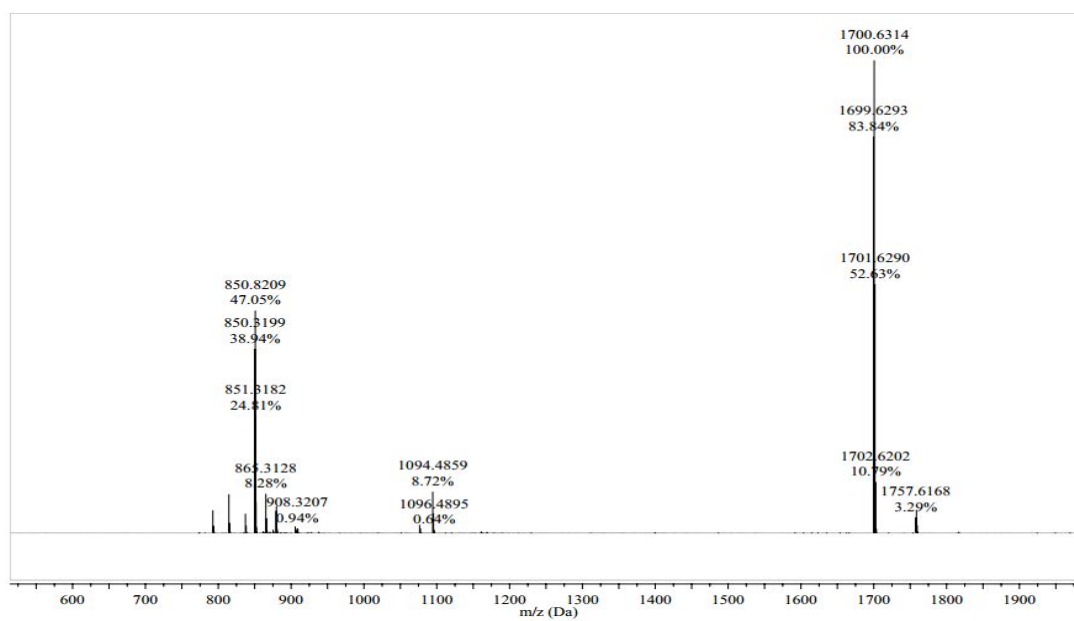


Figure S16. ESI-HRMS spectrum of SMFT.

Reference

1. Guo, X.; An, W.; Shuang, S.; Cheng, F.; Dong, C. Study on spectroscopic characterization of meso-tetrakis (4-hydroxyphenyl) porphyrin (THPP) in β -cyclodextrin and its derivatives. *J. Photochem. Photobiol. A.* **2005**, *173*, 258-263.
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3. Zeng, Q.; Guo, Q.; Yuan, Y.; Yang, Y.; Zhang, B.; Ren, L.; Zhang, X.; Luo, Q.; Liu, M.; Bouchard, L.-S.; Zhou, X. Mitochondria targeted and intracellular biothiol triggered hyperpolarized ^{129}Xe magnetofluorescent biosensor. *Anal. Chem.* **2017**, *89*, 2288-2295.
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