Supporting information for

Visualizing Endogenous Sulfur Dioxide Derivatives in Febrile Seizure-Induced Hippocampal Damage by a Two-Photon Energy Transfer Cassette

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1.Materials and Instruments

All chemicals were obtained from commercial suppliers and used without further purification. Water used in all experiments was doubly distilled and purified via a Milli-Q water system (Millipore, USA). Stock solution (500 μM) of the probe was prepared by dissolving TP-Ratio-SO_2 in ethanol. Na_2SO_3, used as a sulfur dioxide derivates source in all experiments, was dissolved in water at a concentration of 5 mM. Stock solutions of metal ions and anions were prepared from the corresponding inorganic salts. Nitric oxide (NO) was generated from DEA/NONOate (1mM stock solution in 0.01 M NaOH).

UV-Vis absorption spectra were recorded using a Hitachi U-4100 UV/Vis spectrophotometer (Kyoto, Japan) in 1 cm path length quartz cuvettes. The one-photon steady-state fluorescence emission spectra were obtained from a PTI QM4 fluorescence system (Photo Technology International, USA). Two-photon fluorescence spectra were obtained with a mode-locked Ti: sapphire pulsed laser (Chameleon Ultra II, Coherent Inc.) and then recording with a DCS200PC single photon counting (Beijing Zolix Instruments Co., Ltd.). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 spectrometer (Bruker) with TMS employed as an internal standard. All chemical shifts are given in the standard δ notation of parts per million. Mass spectra were measured on LCQ Advantage ion trap mass spectrometer (Thermo Finnigan). The pH was measured with a Mettler-Toledo Delta 320 pH meter. The flow cytometry diagrams were measured by a cell flow meter (Gallios, USA). The fluorescence images were acquired from the green channel (490-520 nm) and red

channel (610-660 nm) upon two-photon excitation at 760 nm with a pulse laser by using an Olympus FV1000 laser confocal microscope (Olympus, Japan) and Nikon Ultra-high resolution spectroscopic confocal microscope (TI-E+AI SI, Japan).

2. Synthesis

Synthesis of Compound 2. 6-amino-1,2,3,4-tetrahydro-1-naphthalenone (161.5 mg, 1.0 mmol), tert-Butyl bromoacetate (210mg, 1.1 mmol), K_2CO_3 (175mg, 1.1 mmol), NaI (18mg, 0.28 mmol) in MeCN (30 mL) was refluxed under nitrogen atmosphere for 18 hours. The product was extracted with ethyl acetate, washed with brine. The solution was concentrated under reduced pressure and then subjected to flash chromatography on silica (CH_2Cl_2: C_2H_5OH = 50:1), yielding compound **2** as a yellow solid (221.1mg, 80.1%).1H NMR(400MHz, DMSO):δ 1.42(s, 9H), 1.97-2.00 (m, 2H), 2.46-2.49 (m, 2H), 2.34-2.37 (m, 2H), 3.77 (s, 2H), 6.22 (s, 1H), 6.40 (d, *J* = 8.0 Hz, 1H), 7.82 (d, *J* = 8.0 Hz, 1H).

Synthesis of Compound 3. Compound **2** (138.2mg, 0.5 mmol) and 4-(diethyl amino) salicylaldehyde (96.8mg, 0.5 mmol) were dissolved in $MeSO_3H$ (2.5 mL) and stirred at 90 ℃ under N2 atmosphere for 6 hours. After being cooled, the mixture was poured into ice-water (30g), and then per-chloric acid (1.0 mL) was added. The resulting mixture was extracted with CH_2Cl_2/CH_3OH solution (60 ml, V/V = 59: 1) for 3-4 times, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure and then subjected to flash chromatography on silica (CH_2Cl_2:C_2H_5OH = 50:1), yielding compound **3** as a red solid (132.1mg, 75%). ^{1}H NMR (400MHz, DMSO): δ 1.20-1.23 (m,6H), 2.95 (s, 4H), 3.61(d, *J* = 12.0 Hz, 4H), 4.10 (s,

2H),6.64(s,1H), 6.82(s,1H), 7.14(s, 1H), 7.23(d, *J* = 8.0 Hz,1H),7.78(d, *J* = 8.0 Hz, 1H),7.89(s, 1H),8.05(d, *J* =12.0 Hz, 1H),8.37(s, 1H).

Synthesis of Compound 4. TP was prepared by one literature method.[1] Compound **TP** (150mg, 0.52 mmol) was treated with 4-(N-Boc-amino)piperidine (95mg, 0.5mmol), EDC (150mg, 0.6 mmol), and HOBt (110mg, 1.5 mmol) in CH_2Cl_2 (5 mL). The reaction mixture was stirred at room temperature under N_2 atmosphere for 12 hours, and the solvent was removed under reduced pressure. The resulting residue was purified by column chromatography (CH_2Cl_2/MeOH, 20:1) on silica gel to give the product **4** as a yellow solid (160.5 mg, yield: 71.1%). ^{1}H NMR (400MHz, $CDCl_3$): δ 1.30 (s, 1H), 1.42 (s, 9H), 1.52 (d, *J* = 12.8 Hz, 1H), 2.58 (s, 3H), 3.51-3.55 (m, 6H), 3.70(s, 1H),4.62(d, *J* = 8.0 Hz, 1H),6.59 (s, 1H),6.79 (d, *J* = 8.0 Hz, 1H),7.51 (d, *J* = 8.2 Hz, 1H),7.7 (d, *J* = 8.0 Hz, 1H),7.83(d, *J* = 8.0 Hz, 1H),8.22 (s, 1H).

Synthesis of Compound 5. Compound **4** (160.5 mg, 0.3554 mmol) was dissolved in CH_2Cl_2/CF_3COOH solution (4 ml, V/V = 1: 1), and the solution was stirred at room temperature for 30 min under a N_2 atmosphere. The solution was concentrated under reduced pressure and then subjected to flash chromatography on silica (CH_2Cl_2:C_2H_5OH = 50:1),yielding compound **5** as a yellow solid (100.3mg, 80.3%).^{1}H NMR (400MHz, DMSO): δ 1.24 (s,3H), 2.01 (d, *J* = 8.0 Hz,3H), 2.30-2.34 (m, 1H), 2.60 (s, 3H), 3.13 (s, 2H),3.20(s, 1H), 3.30(s, 1H), 3.73(s, 1H), 3.83(s, 1H),4.01(d, *J* = 8.0 Hz, 1H),4.97(d, *J* = 12.0 Hz, 1H),6.70(s, 1H),6.97 (d, *J* = 8.0 Hz, 1H),7.66(d, *J* = 8.0 Hz, 1H),7.81(d, *J* = 8.0 Hz, 1H),7.87(d, *J* = 8.0 Hz, 1H),8.43(s, 1H),9.34(s, 1H).

3. DFT Calculations[2]

The ground state structures of **TP-Ratio-SO_2** and **TP-Ratio-SO_2** + Na_2SO_3 were optimized using DFT with B3LYP functional and 6-31+G (d, p) basis set. The initial geometries of the compounds were generated by the Gaussian View software. All of these calculations were performed with Gaussian 09 (Revision A.02).

4. Measurements of Quantum Yield and Two-Photon Absorbance Cross Section

The one-photon quantum yields of samples were estimated using Rhodamine B or quinine sulfate as a reference standard, which was freshly prepared to reduce the measurement error.[3] The quantum yield $\boldsymbol{\Phi}$ as a function solvent polarity is calculated using the following equation (1):

$$\Phi_F = \Phi_{F,cal} \cdot \frac{S}{S_{cal}} \cdot \frac{A_{cal}}{A} \cdot \frac{n^2}{n_{cal}^2} \qquad (1)$$

Where $\boldsymbol{\Phi}_F$ is the quantum yield, $\boldsymbol{S}$ is the areas' integral values of the corrected fluorescence spectra, $\boldsymbol{A}$ stands for the absorbance and $\boldsymbol{n}$ is refractive index. The subscript cal and no denote the standard and sample, respectively.

The two-photon absorption (TPA) cross sections ($\boldsymbol{\delta}$) of samples (in the wavelength range of 740-840 nm) in neutral conditions were determined using TPE method with femtosecond Ti-sapphire laser pulses described in previous literature.[4] the TPE fluorescence emission intensity of **TP-Ratio-SO_2** and **TP-Ratio-SO_2** + Na_2SO_3 in PBS buffer (pH 7.4) was measured in the emission range of 450-700 nm under excitation at 740-840 nm using Rhodamine B as the reference, whose TP properties

have been well-characterized in the previous literature.[5] Intensities of TPE fluorescence emission of the reference and the samples emitted at the same excitation wavelength were determined. The TPA cross section was calculated as the following equation (2): [6]

$$\delta_S = \frac{S_S}{S_R} \cdot \left[\frac{\Phi_R \cdot C_R \cdot n_S}{\Phi_S \cdot C_S \cdot n_R} \right] \cdot \delta_R \qquad (2)$$

Subscript ***S*** and ***R*** denote the sample and the reference, respectively. ***S*** represents the intensity of TPE fluorescence emission, ***Φ*** is the fluorescence quantum yield, ***C*** denotes the concentration, and ***n*** represents the refractive index of the solvents.

5. Cell Incubation and Cytotoxicity Assay

U251 cells were cultured using high-glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 1% penicillin-streptomycin (10,000 U/mL, 10,000 μg mL^{-1}, Invitrogen) and 10% fatal bovine serum (GIBCO) in an atmosphere of 5% CO_2 and 95% air at 37°C.

The cellular cytotoxicity of **TP-Ratio-SO_2** towards U251 cells as the model was evaluated using the standard cell viability assay - the MTT assay.[7] U251 cells were seeded into a 96-well plate at a concentration of 5×10^3 cells $well^{-1}$ in 100 μL of DMEM medium with 10% FBS. Plates were maintained at 37 °C in a 5% CO_2 95% air incubator for 48 h. After the original medium was removed, the U251 cells were incubated with different concentrations of **TP-Ratio-SO_2** (1-10 μM). The cells incubated with the culture medium only were served as the controls. The cells were washed with PBS for three times and then 100 μL MTT solution (0.5 mg mL^{-1} in PBS)

was added to each well. After addition of DMSO (150 μL well^{-1}), the assay plate was allowed to shake at room temperature for 10 min. The spectrophotometrical absorbance of the samples was measured by using a Tecan microplate (ELISA) reader. The cell viability was calculated based on measuring the UV-vis absorption at 570 nm using the following equation (3), where OD_{570} represents the optical density.[8]

$$\text{Cell viability} = [OD_{570(\text{sample})} - OD_{570(\text{blank})}] / [OD_{570(\text{control})} - OD_{570(\text{blank})}] \quad (3)$$

6. Spectroscopic and Imaging Data

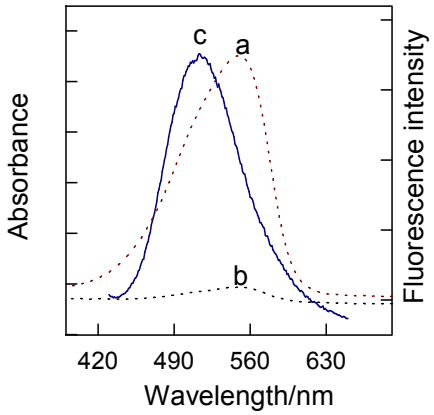


Figure S1. UV-vis absorption spectra (dashed lines) of **TP** before (a) and after addition of 2000 μM Na_2SO_3 (b) and two-photon emission spectra (solid lines) of 10 μM **4** (c) in PBS (pH = 7.4, containing 30% DMSO). (λ_{ex} =760 nm).

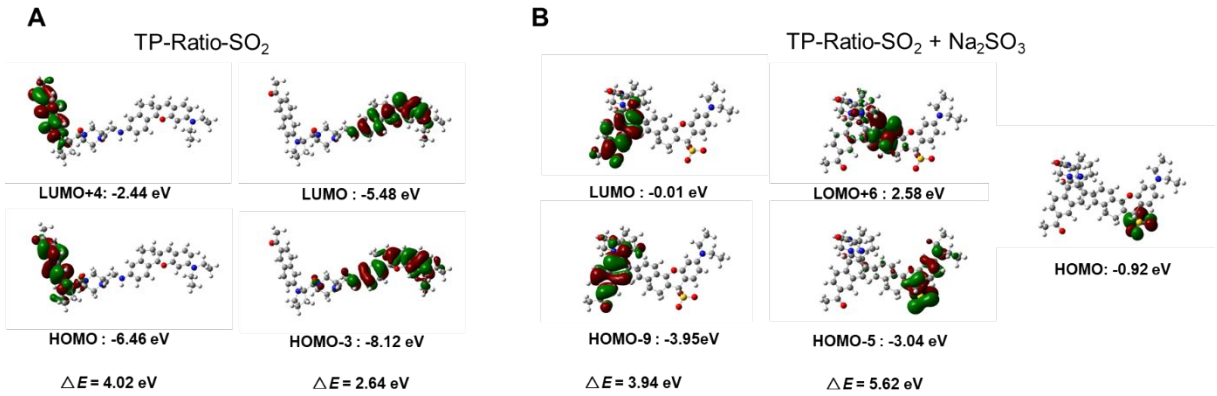


Figure S2. Frontier molecular orbital plots of **TP-Ratio-SO$_2$** (A) and **TP-Ratio-SO$_2$**

+ Na_2SO_3 (B).

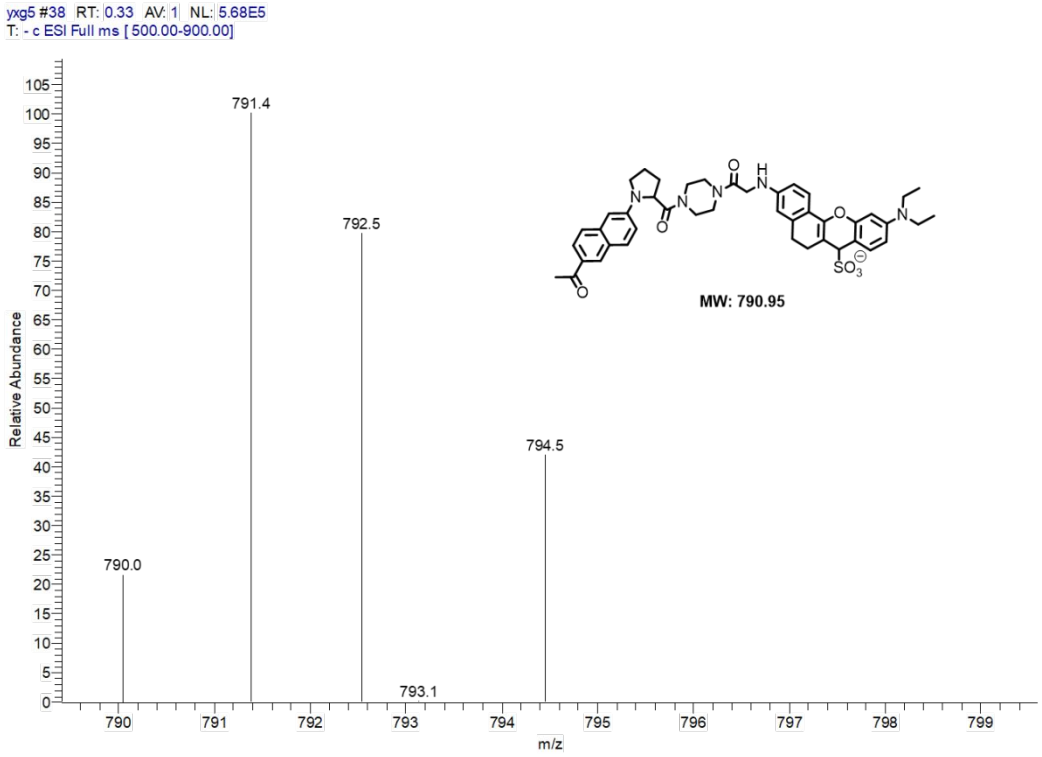


Figure S3. ESI-MS of Michael addition reaction product of **TP-Ratio-SO_2** with Na_2SO_3.

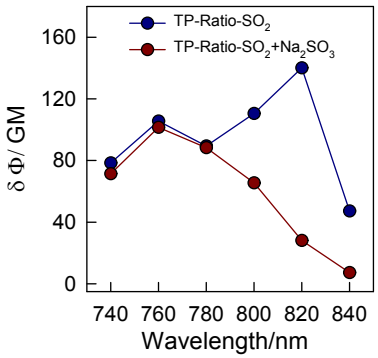


Figure S4. Two-photon absorption action cross sections of **TP-Ratio-SO_2** and **TP-Ratio-SO_2** + Na_2SO_3 in PBS buffer solution (containing 30% DMSO).

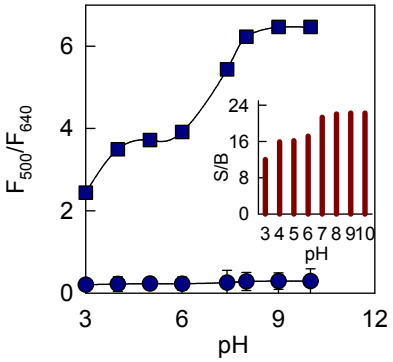


Figure S5. Effect of pH on the fluorescence of **TP-Ratio-SO_2** (1.0 μM) in the absence（●）and presence（■）of 200 μM Na_2SO_3 in 10 mM PBS buffered aqueous DMSO solution (7/3 v/v). λ_{ex} = 390 nm.

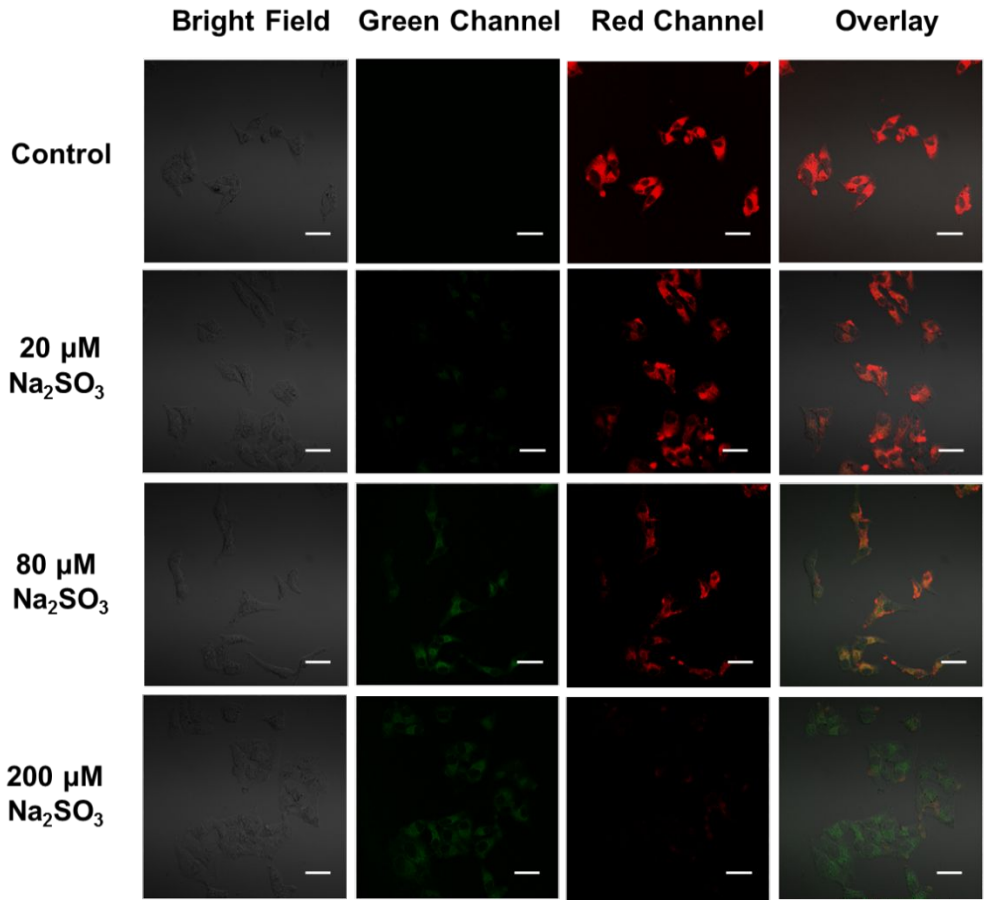


Figure S6. Fluorescence images of exogenous SO_2 derivatives in U251 cells using **TP-Ratio-SO_2.** (up to down) loading with 1.0 μM **TP-Ratio-SO_2** for 30min after

treated with different concentration Na_2SO_3 solution (0, 20.0, 80.0, 200.0 μM) for 5 min. The images were collected at 490-520 nm (green channel) and 610-660 nm (red channel) upon excitation at 760 nm. Scale bar: 20 μm.

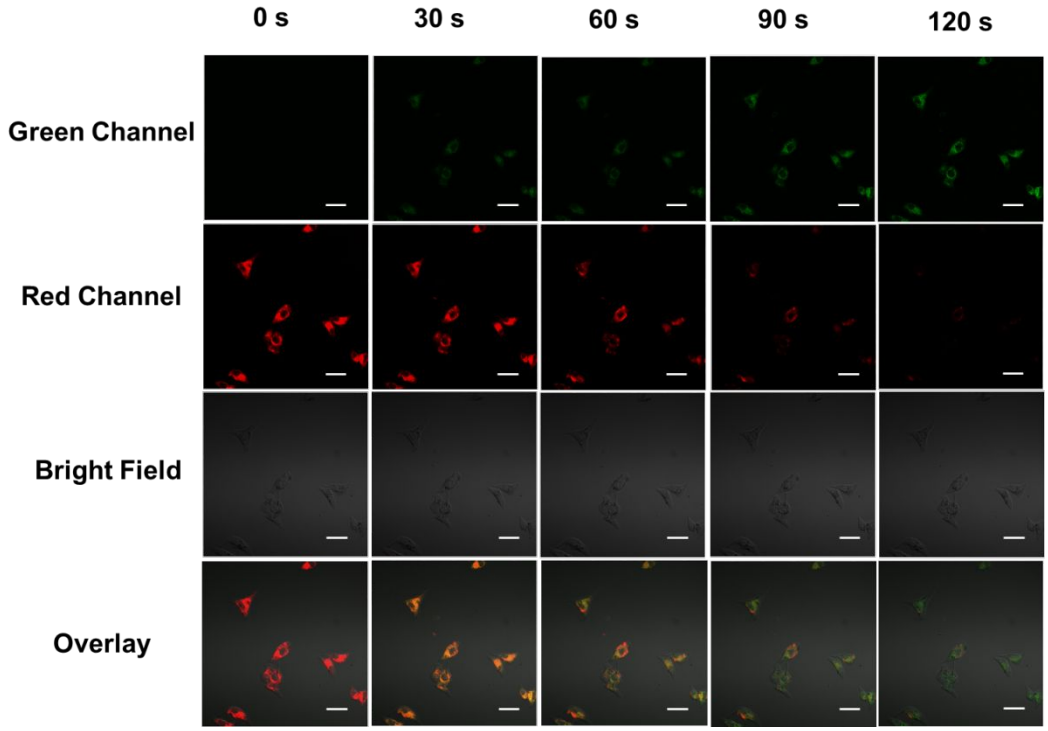


Figure S7. Time-dependent fluorescence images of exogenous SO_2 derivatives in U251 cells using 1.0 μM **TP-Ratio-SO_2.** The images were collected at 490-520 nm (green channel) and 610-660 nm (red channel) upon excitation at 760 nm. Scale bar: 20 μm.

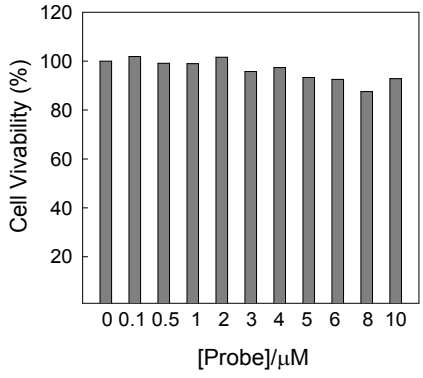


Figure S8. Cell viability of U251 cells treated with different concentrations of **TP-**

Ratio-SO_2 (0-30 μM) for 24 h in fresh medium.

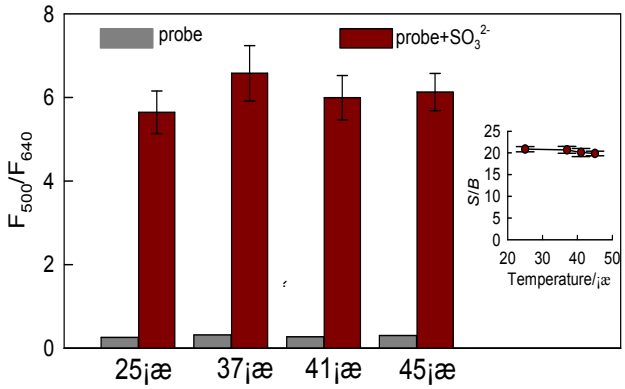


Figure S9. Effect of temperature on the fluorescence of **TP-Ratio-SO_2** (1.0 μM) in the absence and presence of 400 μM Na_2SO_3 in PBS buffer solution (containing 30% DMSO) λ_{ex} = 378 nm.

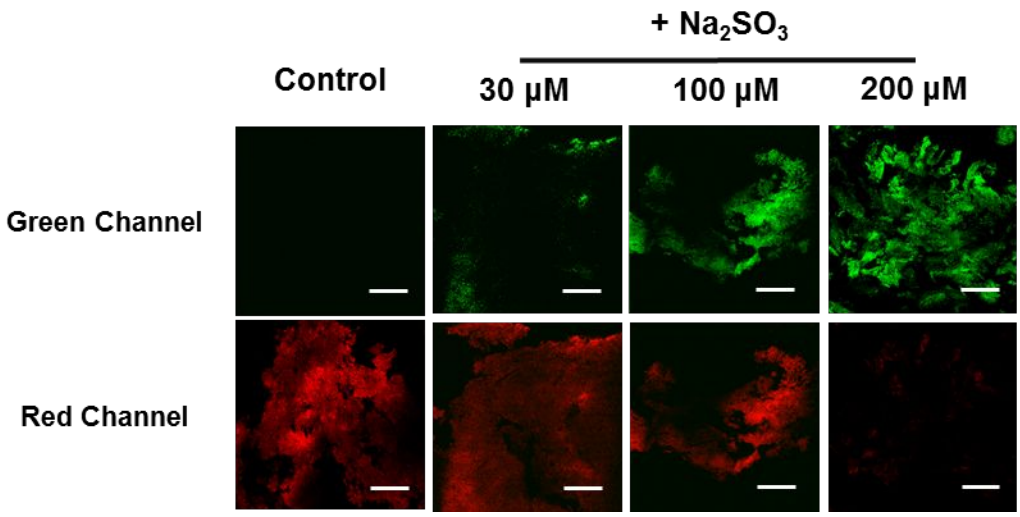


Figure S10. Fluorescence images of exogenous SO_2 derivatives in liver tissue slices using **TP-Ratio-SO_2.** (left to right) loading with 1.0 μM **TP-Ratio-SO_2** for 30min after treated with different concentration Na_2SO_3 solution (0, 30.0, 100.0, 200.0 μM) for 5 min. The images were collected at 490-520 nm (green channel) and 610-660 nm (red channel) upon excitation at 760 nm. Scale bar: 50 μm.

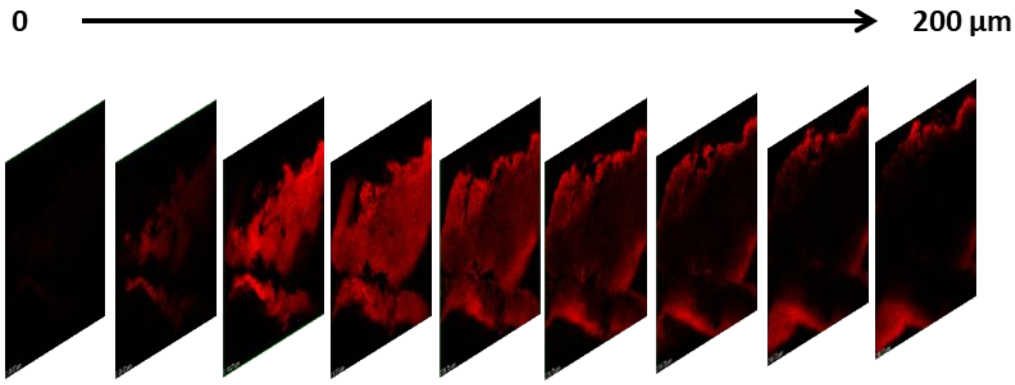
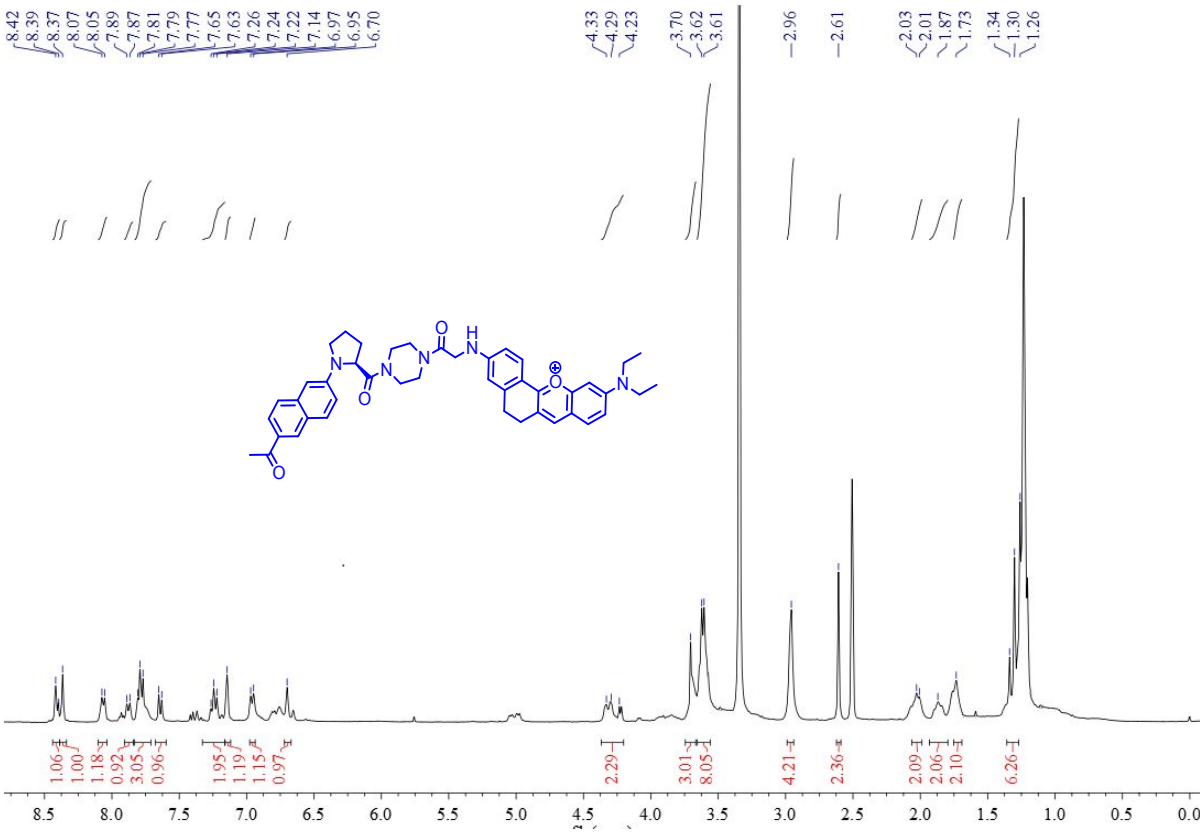


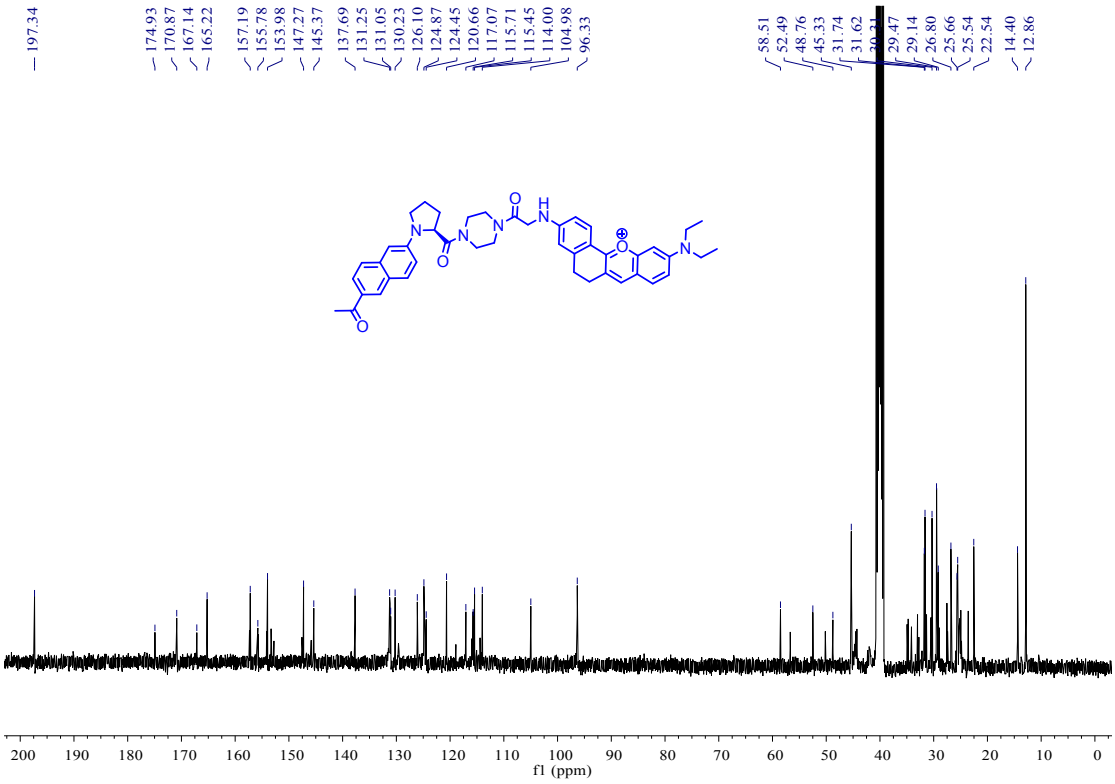
Figure S11. Confocal microscopy fluorescence depth images of exogenous SO_2 derivatives of liver tissue slices using **TP-Ratio-SO_2.** The images were collected at 610-660 nm (red channel) upon excitation at 760 nm with femtosecond pulses.

7. NMR and Mass Spectra of TP-Ratio-SO_2.

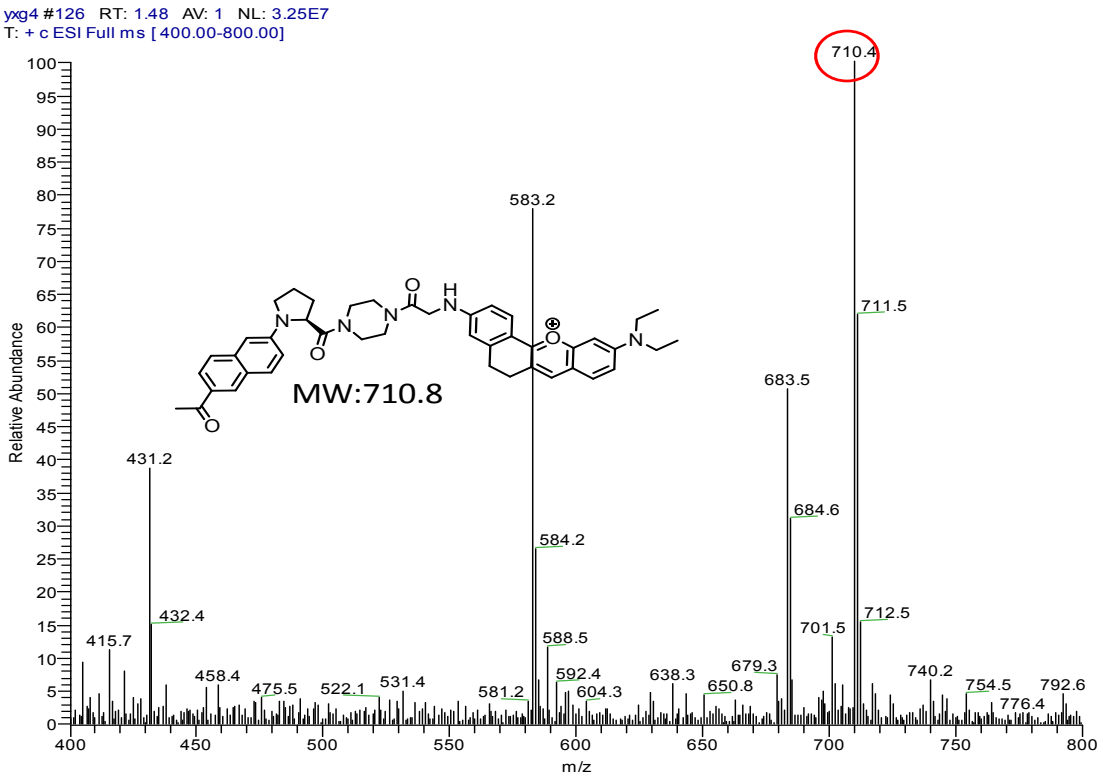
^{1}H NMR of **TP-Ratio-SO_2** in DMSO-d_6



^{13}C NMR of **TP-Ratio-SO$_2$** in DMSO-d$_6$



ESI-MS of **TP-Ratio-SO$_2$**



8. References

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