Supporting Information

A TP-FRET-Based Two-Photon Fluorescent Probe for Ratiometric Visualization of Endogenous Sulfur Dioxide Derivatives in Mitochondria of Living Cells and Tissues

Xiaoguang Yang,[a§] Yibo Zhou,[b§] Xiufang Zhang,[b] Sheng Yang, *[a] Yun Chen,[b] Jingru Guo,[a] Xiaoxuan Li,[a] Zhihe Qing,[a] and Ronghua Yang*[ab]

[a]School of Chemistry and Biological Engineering, Changsha University of Science and Technology, Changsha, 410114, P. R. China

[b]State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, P. R. China

[§]These authors contributed equally.

*To whom correspondence should be addressed:

E-mail: yangrh@pku.edu.cn; ysh1127@hnu.edu.cn

Fax: +86-731-8882 2523

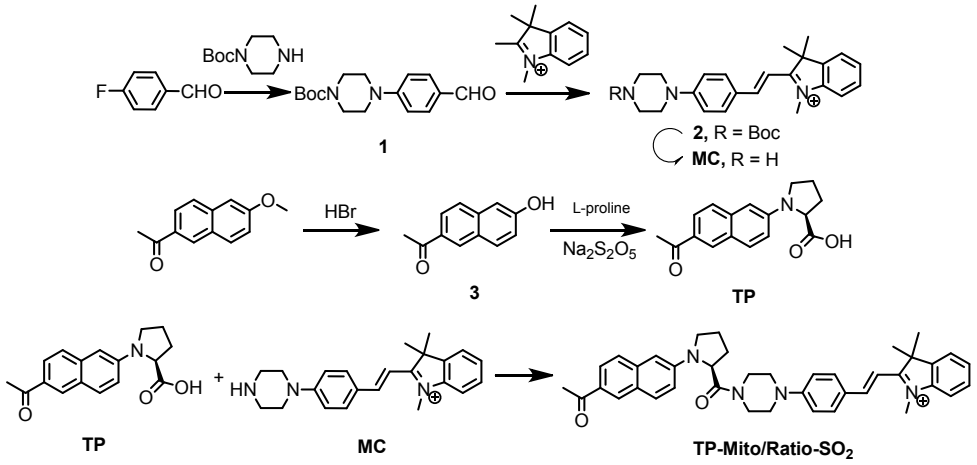
1.Materials and Instruments

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-Mito/Ratio-SO_2 in ethanol. $NaHSO_3$, 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 InstrumentsCo., 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 ä 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 fluorescence images were acquired from the green channel (490-520 nm) and red channel (580-630 nm) upon two-photon excitation at 760 nm with a pulse laser by

using an Olympus FV1000 laser confocal microscope (Olympus, Japan).

2. Synthesis



Scheme S1. Synthetic Procedure of **TP-Mito/Ratio-SO_2**.

Synthesis of Compound 1.[1] To a solution of 4-Fluorobenzaldehyde (250 mg, 2.0 mmol) and 1-Boc-piperidine (558.75 mg, 3.0 mmol) in DMF (3.5 mL) was added K_2CO_3 (414.6 mg, 3.0 mmol). The solution was heated at 90 ℃ for 30 h under N_2 atmosphere in the dark, then was added water and the resulting solution was extracted with ethyl acetate. The organic layer was separated, washed with water(20 mL), brine (20 mL). Then the organic phase was dried with Na_2SO_4, and the solvent was evaporated under reduced pressure. The residue was purified by the silica gel chromatography using Petroleum ether\ethyl acetate (4:1, v/v) as eluent to afford compound **1** as a faint yellow soild (357.1 mg, 61.5%). ^{1}H NMR (400MHz, $CDCl_3$): δ 1.40 (s, 9H), 3.29-3.31 (m, 4H), 3.49-3.52 (m, 4H), 6.82 (d, *J* = 8.0 Hz, 2H), 7.67 (d, *J* = 8.0 Hz, 2H), 7.90 (s, 1H).

Synthesis of Compound 2. Compound **1** (150mg, 0.52 mmol) was treated with 1,2,3,3-tetramethyl-3H-indoliumiodide (186.7 mg, 0.62 mmol) and the proper

piperidine (0.1 mL) in anhydrous ethanol (5 mL). The reaction mixture was stirred at 80℃ under N_2 atmosphere for 3 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 **2** as a purple solid (188.9 mg, yield: 63.7%).^{1}H NMR (400MHz, $CDCl_3$): δ 1.40 (s, 9H), 1.73 (s, 6H), 3.46-3.49 (m, 8H), 4.14 (s, 3H), 6.89 (d, 2H), 7.34-7.46(m, 5H), 8.07-8.10 (m, 3H).

Synthesis of Compound MC. Compound **2** (188.9 mg, 0.3294 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 2 h. The solution was concentrated under reduced pressure and then subjected to flash chromatography on silica (CH_2Cl_2:C_2H_5OH = 15:1), yielding compound **MC** as a purple solid (116.9 mg, 75%).^{1}H NMR (400MHz, $CDCl_3$): δ 1.77(s, 6H), 3.27(brs, 4H), 3.75(brs, 4H), 4.06 (s, 3H), 6.89 (d, 2H), 7.17 (d, *J* = 8.0 Hz, 1H), 7.44 (d, *J* = 16.0 Hz, 1H), 7.54-7.59 (t, 3H), 7.79-7.83 (m, 2H), 8.15(d, *J* = 8.0 Hz, 2H), 8.35(d, *J* = 16.0 Hz, 1H), 9.45(s, 1H). EI-MS (m/z): calcd for $C_{23}H_{28}N_3^+$: 346.49; found: 346.3.

Synthesis of TP-Mito/Ratio-SO_2. compound **TP** (75 mg, 0.277mmol), EDC (90 mg, 0.436 mmol) and catalytic amount DMAP were added into a solution containing compound **MC** (115 mg, 0.243 mmol) in CH_2Cl_2 (4.0mL). The mixture was stirred under N_2 atmosphere for 12 h at the room temperature, and after the solvent was removed under reduced pressure, the resulting residue was purified by column chromatography (CH_2Cl_2/MeOH, 15:1) on silica gel to give the final product **TP-Mito/Ratio-SO_2** as a purple solid (140.3 mg, yield: 77.8%).^{1}H NMR (400MHz,

DMSO): δ 1.77 (s, 6H), 2.61 (s, 3H), 3.17-3.27 (m, 1H), 3.48-3.58 (m, 14H), 4.05 (s, 3H), 6.71 (s, 1H), 6.97 (d, *J* = 8.8 Hz, 1H), 7.10-7.16 (m, 1H), 7.41 (d, *J* = 15.6 Hz, 1H), 7.50-7.60 (m, 2H), 7.64 (d, *J* = 8.8 Hz, 1H), 7.77-7.82 (m, 3H), 7.88 (d, *J* = 8.8 Hz, 1H), 8.17 (d, *J* = 8.0 Hz, 2H), 8.36 (dd, *J* = 15.6, 5.6 Hz, 1H), 8.43 (s, 1H). ^{13}C NMR (100MHz, DMSO): δ 220.01, 185.36, 175.44, 163.10,162.79, 159.11, 158.86, 151.90, 147.93,147.11, 142.34, 138.87, 135.79, 133.85, 133.09, 129.48, 129.22, 128.98, 127.76, 123.88, 120.89, 118.70, 112.36, 112.00, 109.57, 76.36, 72.37, 67.73, 63.11, 60.04, 56.29, 53.39, 50.95, 48.95, 38.49, 35.12, 31.46, 31.12, 28.20, 25.08. EI-MS (m/z): calcd for $C_{40}H_{43}N_4O_2^+$: 611.79; found: 611.5.

3.Spectrophotometric Experiments. Test solutions were prepared by mixturing **TP-Mito/Ratio-SO_2** and appropriate analyte stock into a tube and then diluting the solution to 500 μL with 10mM phosphate buffer (PB) (containing 20 % EtOH, pH 7.4). After incubation at room temperature for 10 min, the absorption or fluorescence spectra measurements were then performed. The one-photon fluorescence spectra were recorded at emission wavelength range from 450 to 650 nm with excitation wavelength of 380 nm. The two-photon fluorescence emission spectra were acquired by recording with a DCS200PC single photon counting using a mode-locked Ti:sapphire pulsed laser(exciting the samples at 760 nm).

4. Kinetic Studies

In kinetic studies, the apparent rate constant *k* for the reaction of **TP-Mito/Ratio-SO_2** with different concentrations of $NaHSO_3$ were determined by fitting the fluoresence intensity ratios to the pseudo first-order:

$$\ln[([F_{500}/F_{590}]_{max} - [F_{500}/F_{590}]_t)/ [F_{500}/F_{590}]_{max}] = -kt^2$$

Where $(F_{500}/F_{590})_t$ and $(F_{500}/F_{590})_{max}$ are the fluoresence intensity ratios of **TP-Mito/Ratio-SO_2** at a time t and the maximum value obtained after the reaction was complete.

The observed rate constant *k*', contains the concentration of $NaHSO_3$ as a constant and is related to the second-order rate constant, *k* ($M^{-1}s^{-1}$), by equation:

$$k' = k[NaHSO_3]$$

5. DFT Calculations[3]

The ground state structures of **TP-Mito/Ratio-SO_2** and **TP-Mito/Ratio-SO_2** + $NaHSO_3$ 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 GaussView software. All of these calculations were performed with Gaussian 09 (Revision A.02).

6. Measurements of One-photon Quantum Yields and Two-Photon Absorbance Cross Section

The one-photon quantum yields (QY) of samples were estimated using Rhodamine B or quinine sulfate as a reference standard, which was freshly prepared to reduce the measurement error.[4] The quantum yield $\boldsymbol{\Phi}$ as a function solvent polarity is calculated using the following equation:

$$\Phi_F = \Phi_{F,\mathrm{cal}} \cdot \frac{S}{S_{cal}} \cdot \frac{A_{cal}}{A} \cdot \frac{n^2}{n^2_{cal}}$$

Where $\boldsymbol{\Phi}_F$ is the quantum yield, ***S*** is the areas' integral values of the corrected fluorescence spectra, ***A*** stands for the absorbance and ***n*** is refractive index. The

subscript cal and no denote the standard and sample, respectively.

The two-photon absorption (TPA) cross sections (***δ***) of samples (in the wavelength range of 690-860 nm) in neutral conditions were determined using TPE method with femtosecond Ti-sapphire laser pulses described in previous literature.[5] the TPE fluorescence emission intensity of **TP-Mito/Ratio-SO_2** and **TP-Mito/Ratio-SO_2** + HSO_3^- in PBS buffer (pH 7.4) was measured in the emission range of 450-620 nm under excitation at 690-840 nm using Rhodamine B as the reference, whose TP properties have been well-characterized in the previous literature.[6] 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: [7]

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

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.

7. Cell Incubation and Cytotoxicity Assay

HepG2 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-Mito/Ratio-SO_2** towards HepG2 cells as the model was evaluated using the standard cell viability assay - the MTT assay.[8] HepG2 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 24 h. After the original medium was removed, the HepG2 cells were incubated with different concentrations of **TP-Mito/Ratio-SO_2** (0-30 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, where OD_{570} represents the optical density.[9]

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

8. Preparation and Staining of Rat Liver Tissue Slices

Rat liver slices were prepared from the liver of 2-weeks-old rat (SD) according to the protocol No. SYXK (Xiang) 2008-0001, approved by Laboratory Animal Center of Hunan. The tissue slices were cut into 400 μm in size using a vibrating-blade microtome. The slices were cultured under different conditions, followed by incubating with **TP-Mito/Ratio-SO_2** for 30min in the culture medium. The residual probe was removed by washing three times using PBS before the imaging by two photon confocal microscope.

9.Ratiometric TPM Imaging of SO_2 Derivatives in Living Cells and Tissues. For the colocalization experiment, **TP-Mito/Ratio-SO_2** (5.0 μM) and

mitochondrial tracker MitoTracker Green FM (1.0 μM) simultaneously stained the HepG2 cells in the incubator at 37 °C with 5 % CO_2 for 30 min and then rinsed for three times with PBS. For imaging of exogenous SO_2, the cells were pretreated with **TP-Mito/Ratio-SO_2** (5.0 μM) for 30 min before washed three times with PBS, then incubated with different concentrations of $NaHSO_3$ solution for another 30 min. In order to monitor the generation of endogenous SO_2 in mitochondria, the HepG2 cells were incubated with the probe in the absence or presence of TST substrates thiosulfate /GSH (250 μM/500 μM) for 1h. As a negative control, another group's HepG2 cells were dealt with TST inhibitor SNAP (100 nM) for 1h before incubation of thiosulfate (250 μM) and GSH (500 μM), and then loaded with the probe for 30 min. After rinsing with PBS buffer, these cells were exposed to imaging experiment on the two photon confocal microscope. For TPM tissue imaging, the rat liver tissue slices were pre-incubated with α-lipoic acid (5 mM) for 1 day or N-ethylmaleimide (10 mM) for 1h to regulate GSH production of tissue, and then treated with TST substrates thiosulfate (2.5 mM) for 1h, followed by loading with 50 μM probe for 30min. As a negative control, another tissue slice was dealt with TST inhibitor SNAP (2.0 μM) for 1h before incubation of α-lipoic acid (5 mM) and thiosulfate (2.5 mM), and then loaded with the probe for 30 min. Procedures of imaging operations for tissue slices were similar as cellular ones. Two-photon imaging patterns were obtained from the green channel (490-520 nm) and red channel (580-630 nm) with two-photon excitation wavelength of 760 nm. The ratiometric imaging patterns were analyzed by Image J software.

10. References

(1) A.Papagni, P.Del Buttero, C.Bertarelli, L.Miozzo, M. Moret, M. T. Pryce and S.Rizzato, *New. J. Chem.*,2010, **34**, 2612.

(2) T. J.Dale,J. Rebek, *J. Am. Chem. Soc*.,2006, **128**, 4500.

(3) M. J.Frisch, G. W.Trucks, H. B.Schlegel, G. E.Scuseria, M. A.Robb, J. R.Cheeseman, G.Scalmani, V.Barone, B.Mennucci, G. A.Petersson, H.Nakatsuji, M.Caricato, X.Li, H. P.Hratchian, A. F.Izmaylov, J.Bloino, G.Zheng, J. L.Sonnenberg, M.Hada, M.Ehara, K.Toyota, R.Fukuda, J.Hasegawa, M.Ishida, T.Nakajima, Y.Honda, O.Kitao, H.Nakai, T.Vreven, J. A.Montgomery, J. E.Peralta, F.Ogliaro, M.Bearpark, J. J.Heyd, E.Brothers, K. N.Kudin, V. N.Staroverov, R.Kobayashi, J.Normand, K.Raghavachari, A.Rendell, J. C.Burant, S. S.Iyengar, J.Tomasi, M.Cossi, N.Rega, J. M.Millam, M.Klene, J. E.Knox, J. B.Cross, V.Bakken, C.Adamo, J.Jaramillo,R.Gomperts, R. E.Stratmann, O.Yazyev, A. J.Austin, R.Cammi, C.Pomelli, J. W.Ochterski, R. L.Martin, K.Morokuma, V. G.Zakrzewski, G. A.Voth, P.Salvador, J. J.Dannenberg, S.Dapprich, A. D.Daniels, O.Farkas, J. B.Foresman, J. V.Ortiz, J.Cioslowski, Fox, D. J. Gaussian, Inc., Wallingford CT, GAUSSIAN 09 (Revision A.02), Gaussian, Inc., Pittsburgh, PA, 2009.

(4) J.Demasa, G. A. Crosby, *J. Phys. Chem*.,1971, **75**, 991.

(5)S. Lee, W.Yang, J.Choi, C.Kim, S. Jeon andB.Cho, *Org. Lett.*, 2005, **7**, 323.

(6)N.Makarov, M. Drobizhev andA.Rebane, *Opt. Express.*,2008, **16**, 4029.

(7) C. Xu and W.Webb, *J. Opt. Soc. Am. B*.,1996, **13**, 481.

(8) T. J.Mosmann, *Immunol. Methods.*, 1983, **65**, 55.

(9) J.Tang, B.Kong, H.Wu, M.Xu, Y.Wang, Y.Wang, D. Zhao andG.Zheng, *Adv. Mater.*, 2013, **25**, 6569.

11. Spectroscopic Data

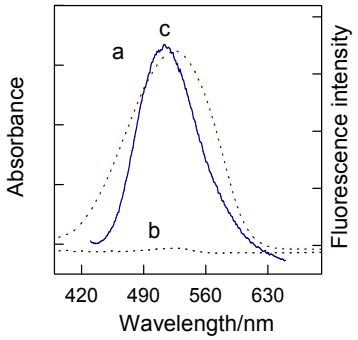


Fig. S1. UV-vis absorption spectra (dashed lines) of **MC** before (a) and after addition of 200 μM $NaHSO_3$ (b) and two-photon emission spectra (solid lines) of 10 μM **TP** (c) in PB (pH = 7.4, containing 20% EtOH). (λ_{ex} =760 nm).

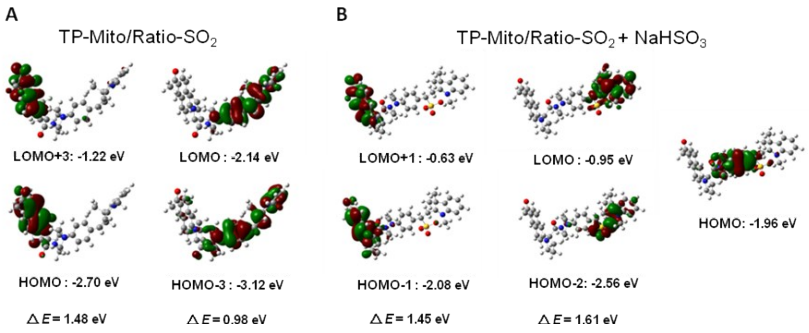


Fig. S2. Frontier molecular orbital plots of **TP-Mito/Ratio-SO_2** (A) and **TP-Mito/Ratio-SO_2** + $NaHSO_3$ (B).

For **TP-Mito/Ratio-SO_2**, the HOMO-LUMO energy gap of the merocyanine moiety (ΔE = 0.98 eV) is less than that of the acedan moiety (ΔE = 1.48 eV), facilitating the intramolecular energy transfer from acedan to merocyanine. However, after treatment with $NaHSO_3$, both of the HOMO and LUMO show that the π electrons are not located around the whole acceptor moiety, indicating that the π-conjunction structure of merocyanine is destroyed, moreover, its energy gap (ΔE = 1.61 eV) is greater than that of the energy donor (ΔE =1.45 eV), thus the FRET process would be suppressed

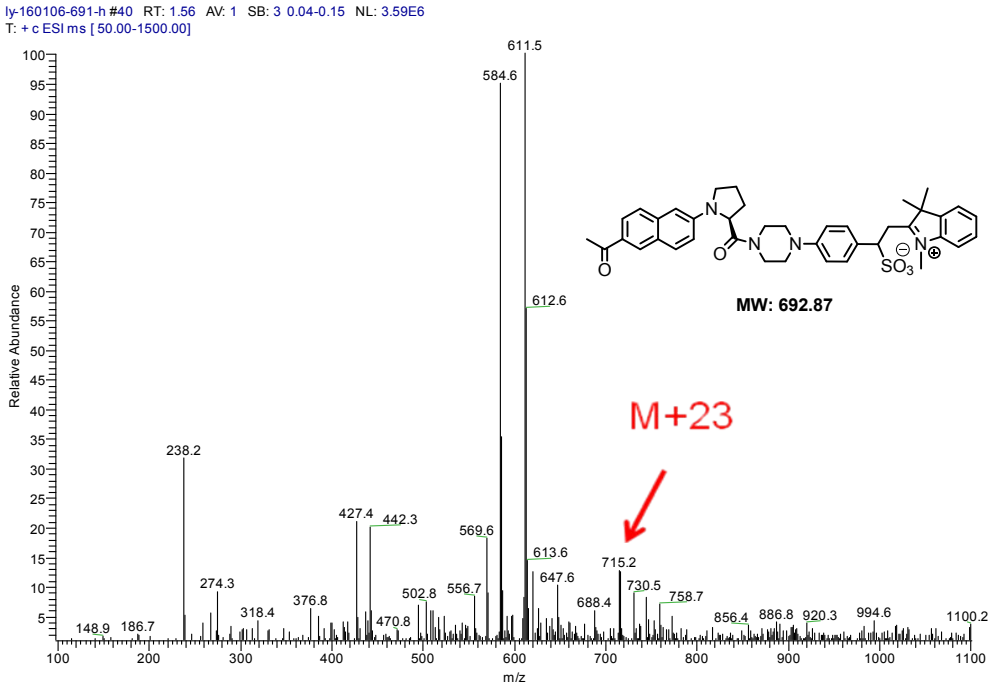


Fig. S3. ESI-MS of Michael addition reaction product of TP-Mito/Ratio-SO_2 with $NaHSO_3$.

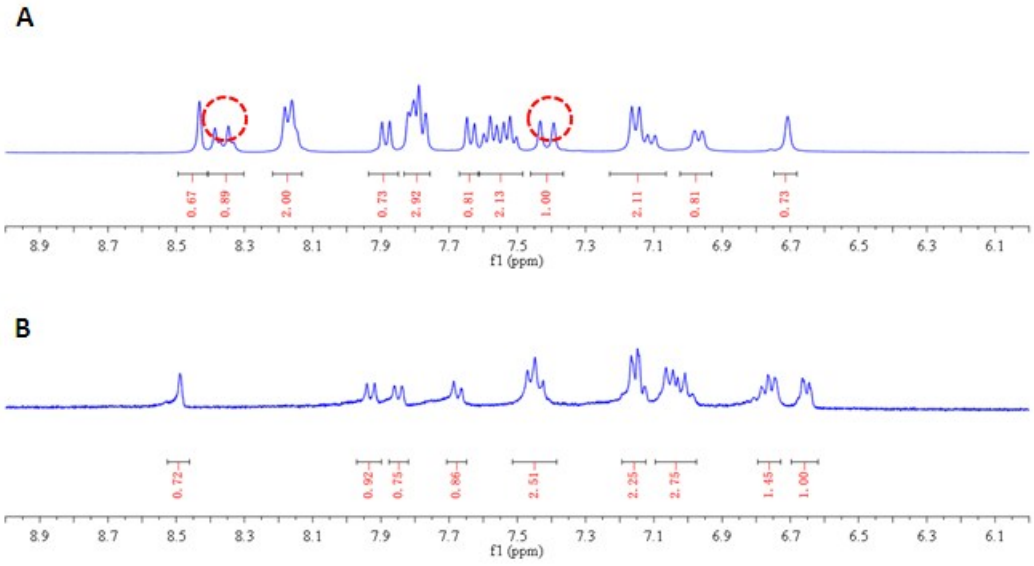


Fig. S4. ^1H NMR of Michael addition reaction product of TP-Mito/Ratio-SO_2 with $NaHSO_3$. (A) Partial ^1H NMR spectra of TP-Mito/Ratio-SO_2 in DMSO-d_6; (B) Partial ^1H NMR spectra of TP-Mito/Ratio-SO_2 in the presence of $NaHSO_3$ in DMSO-d_6:D_2O = 4:1

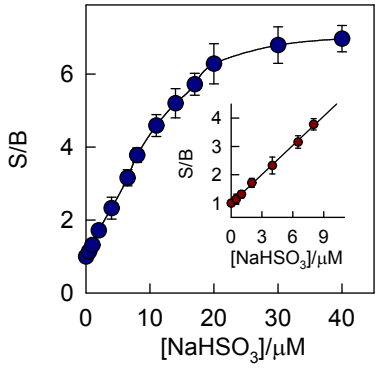


Fig. S5. Signal-to-background ratios (S/B) of **TP-Mito/Ratio-SO$_2$** as a function of $NaHSO_3$ concentrations (0-40 μM) in PB solution (pH = 7.4, containing 20% EtOH). Inset: linear responses of S/B of **TP-Mito/Ratio-SO$_2$** to changing $NaHSO_3$ concentrations (0−10 μM).

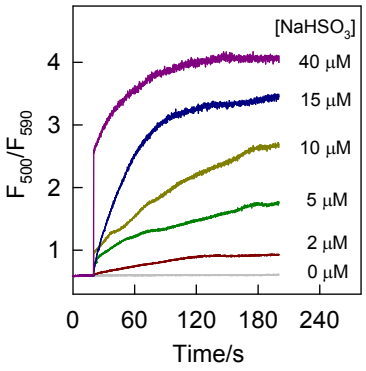


Fig. S6. Real-time records for fluorescence ratio (F_{500}/F_{590}) changes of **TP-Mito/Ratio-SO$_2$** in the presence of different concentrations of $NaHSO_3$ (0.0, 2.0, 5.0, 10.0, 15.0, 40.0 μM). λ_{ex} = 380 nm.

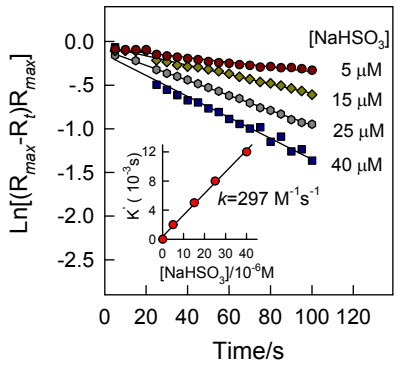


Fig. S7. Plots of $Ln[(R_{max}-R_t)/R_{max}]$ *vs* time for the reaction of TP-Mito/Ratio-SO_2

with different concentrations $NaHSO_3$ in PB solution (20 mM, 20% EtOH, pH 7.4), R = F_{500}/F_{590}. Inset: Plot of k_1 *vs* concentration of $NaHSO_3$.

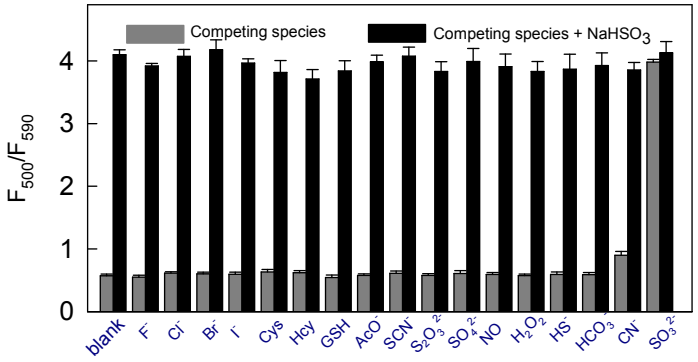


Fig. S8. Fluorescence ratio (F_{500}/F_{590}) changes of TP-Mito/Ratio-SO_2 for $NaHSO_3$ in the presence of other common species in PB (pH = 7.4, containing 20% EtOH). The concentrations of $NaHSO_3$ and other species were 50 μM and 100 μM, respectively. The magnitudes of the error bars were calculated by three independent measurements.

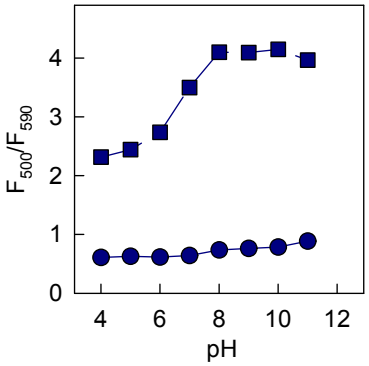


Fig. S9. Effect of pH on the fluorescence of TP-Mito/Ratio-SO_2 (5.0 μM) in the absence（●）and presence（■）of $NaHSO_3$ in PB solution (containing 20% EtOH), λ_{ex} = 380 nm.

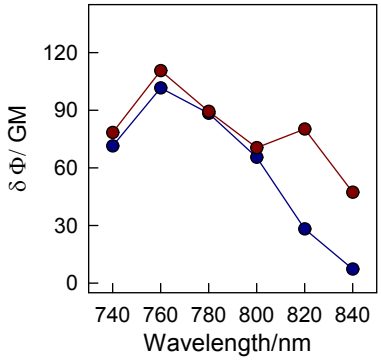


Fig. S10. Two-photon absorption action cross sections of TP-Mito/Ratio-SO_2 (●)

and TP-Mito/Ratio-SO_2 + $NaHSO_3$ (●) in PB (pH = 7.4, containing 20% EtOH).

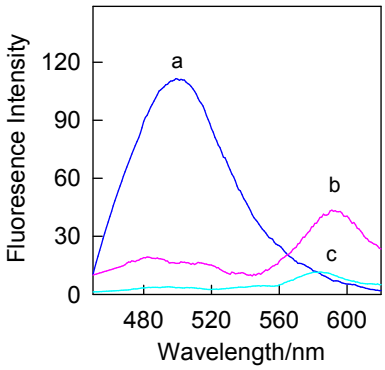


Fig. S11. Two-photon emission spectra of 5 μM TP-Mito/Ratio-SO_2 (a), 5 μM TP-Mito/Ratio-SO_2 + 40 μM $NaHSO_3$ (b), and 5 μM MC (c) in PB (pH = 7.4, containing 20% EtOH). (λ_{ex} =760 nm).

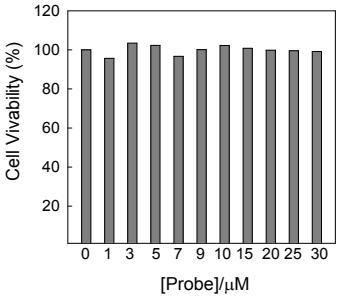


Fig. S12. Cell viability of HepG2 cells treated with different concentrations of TP-Mito/Ratio-SO_2 (0-30 μM) for 24 h in fresh medium.

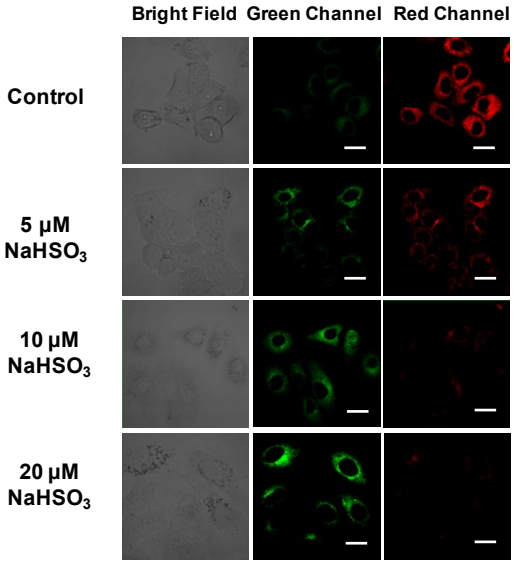


Fig. S13. Fluorescence images of exogenous SO_2 derivatives in mitochondria of HepG2 cells using **TP-Mito/Ratio-SO_2.** (a-d) loading with 5.0 μM **TP-Mito/Ratio-**

SO_2 for 30min after treated with different concentration $NaHSO_3$ solution (0, 5.0, 10.0, 20.0 μM) for 30min. The greed channel λ_{ex} = 760 nm; λ_{em} = 500-520 nm. The red channel λ_{ex} = 760 nm; λ_{em} = 575-630 nm. Scale bar: 20 μm.

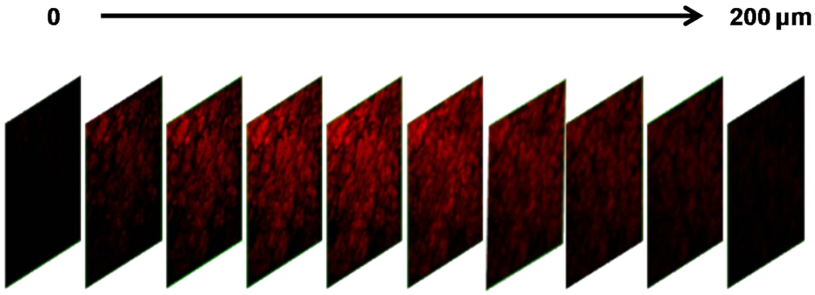
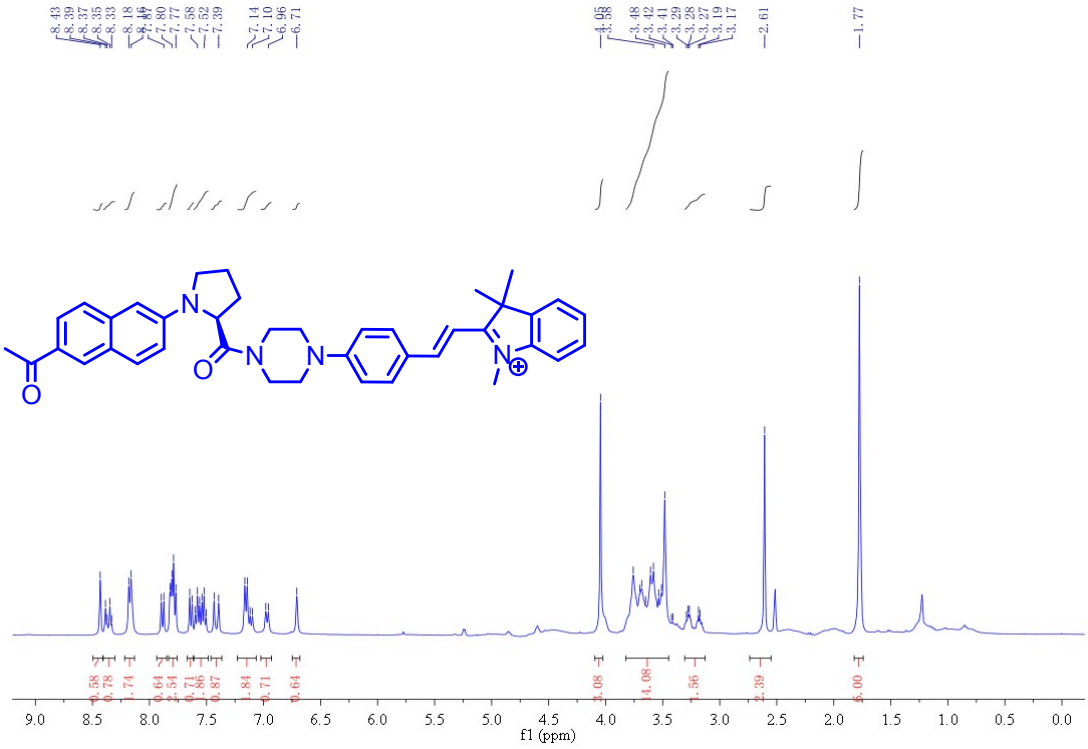


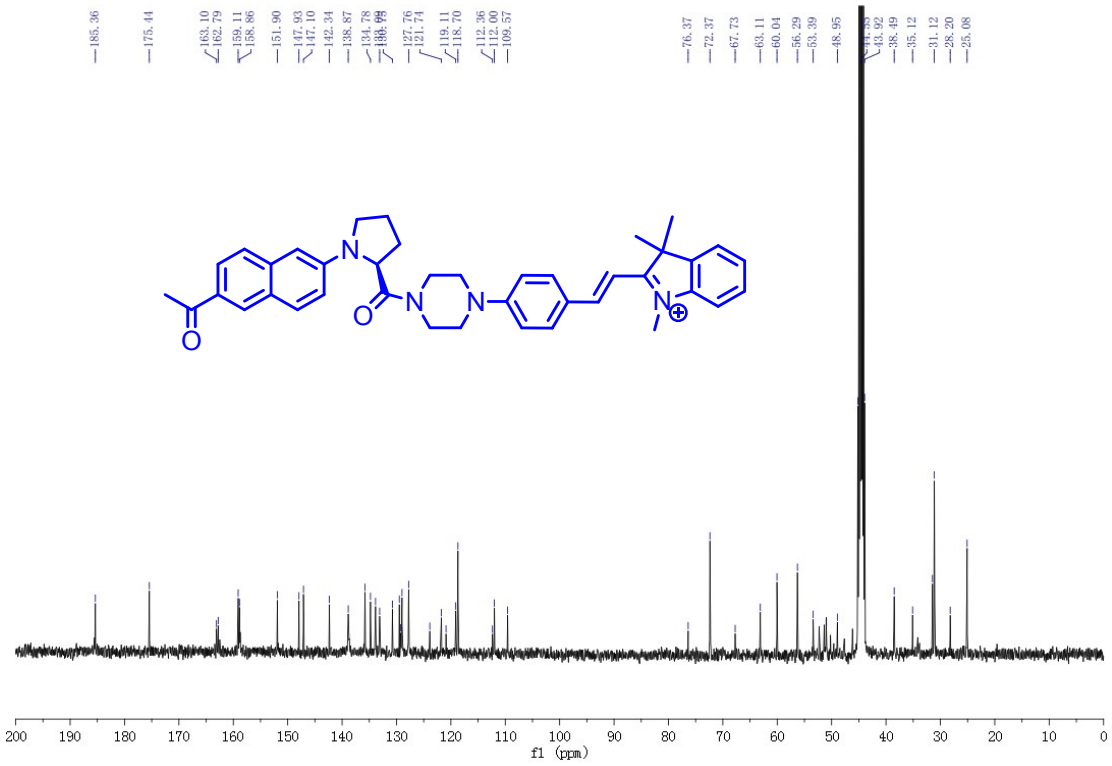
Figure S14. Confocal microscopy fluorescence depth images of exogenous SO_2 derivatives in mitochondria of liver tissue slices using **TP-Mito/Ratio-SO_2.** The images were collected at 580-630 nm (red channel) upon excitation at 760 nm with femtosecond pulses.

12. NMR and Mass Spectra of TP-Mito/Ratio-SO_2 .

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



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



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