Electronic Supplementary Information

A novel fluorescent sensor for detection of highly reactive oxygen species, and for imaging such endogenous hROS in the mitochondria of living cells

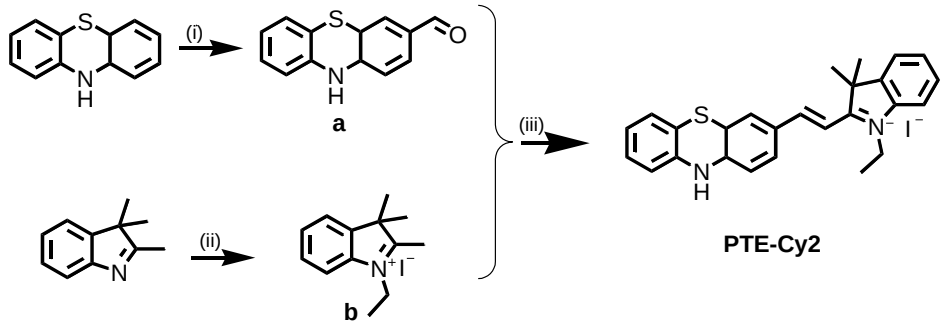
Fei Liu[a], Tong Wu[a], Jianfang Cao[a], Hua Zhang[a], Mingming Hu[a], Shiguo Sun[a], Fengling Song[a], Jiangli Fan[a], Jingyun Wang[b] and Xiaojun Peng*[a]

[a]State Key Laboratory of Fine Chemicals, [b]School of Life Science and Biotechnology, Dalian University of Technology, 2 Linggong Road, Hi-tech Zone, Dalian 116024, P.R. China.

pengxj@dlut.edu.cn

Dye synthesis

The synthetic route for **PTZ-Cy2** is outlined in Scheme S1.



Scheme S1. Synthetic route of dye **PTZ-Cy2**. i) DMF, 1,2-dichloroethane, $POCl_3$, reflux 8 h, yield 63%; ii) toluene, CH_3CH_2I, reflux 10 h, yield 92%; iii) ethanol, piperidine, reflux 12 h, yield 65%.

Intermediate products **a** and **b** were synthesized according to literature procedures. [1, 2]

Synthesis of 3-[2-(1',3',3'-thimethyl-indolium-2'-yl) vinyl]-phenothiazine (PTZ-Cy2)

1-Ethyl-2, 3, 3- trimethyindolenium quaternized salt **b** (1.58 g, 5.0 mmol), and aldehyde **a** (2.4 g, 4.5 mmol) were added to a 100 mL flask with 50 mL ethanol, followed by catalytic piperidine (1.0 mL). The resulting mixture was stirred for 12 h under reflux. The residue was recrystallized from ethanol to give the desired product in 65% yield. 1H NMR (400 MHz, d6-DMSO) δ 9.55 (s, 1H), 8.21 (d, *J* = 16.0 Hz, 1H), 7.84 (m, 4H), 7.57 (m, 2H), 7.36 (d, *J* = 16.0 Hz, 1H), 7.03 (s, *J* = 7.2 Hz,1H), 6.95 (d, *J* = 7.2 Hz, 1H), 6.84 (s, *J* = 7.2 Hz, 1H), 6.72 (m, 2H), 4.61 (d, *J* = 7.2 Hz, 2H), 1.75 (s, 6H), 1.41 (t, *J* = 7.2 Hz, 3H). HRMS-ESI: *m/z* calcd. M^+ for $C_{26}H_{25}N_2S^+$, 397.1733; found, 397.1729.

^{13}C NMR (100 MHz, d6-DMSO) δ 180.55, 153.29, 146.91, 143.90, 140.99, 138.95, 129.86, 129.46, 128.99, 128.68, 128.68, 128.42, 127.98, 123.99, 123.46, 122.29, 114.77, 114.74, 108.27, 105.58, 73.98, 52.03, 30.16, 26.41, 14.00, 10.88.

3-[2-(1',3',3'-thimethyl-indolium -2'-yl)vinyl]-5-oxo-phenothiazine (**OPTZ-Cy2**)

^{1}H NMR (400 MHz, d6-DMSO) δ 11.67 (s, 1H), 8.97 (s, 1H), 8.55 (m, 2H), 8.04 (d, *J* = 7.6 Hz, 1H), 7.90 (d, 2H), 7.72 (d, 1H), 7.63 (m, 3H), 7.52 (m, 2H), 7.35 (t, *J* = 7.2 Hz, 1H), 4.72 (q, *J* = 7.2 Hz, 2H), 1.83 (s, 6H), 1.48 (t, *J* = 7.2 Hz, 3H). HRMS-ESI: *m/z* calcd. M^+ for $C_{26}H_{25}N_2OS^+$, 413.1682; found, 413.1681.

3-carbaldehyde 5-oxo-phenothiazine (**OPTA**)

^{1}H NMR (400 MHz, d6-DMSO) δ 11.56 (s, 1H), 9.96 (s, 1H), 8.55 (s, 1H), 8.05 (m, 2H), 7.70 (m, 1H), 7.51 (m, 2H), 7.33 (t, 1H). HRMS-ESI: *m/z* calcd. M for $C_{13}H_9NO_2S$, 243.0354; found, 243.0358.

Preparation of stock solutions for generation of ROS[3-6]

(a) H_2O_2

H_2O_2 was diluted appropriately in water. The concentration of H_2O_2 was determined based on the molar extinction coefficient at 240 nm (43.6 M^{-1} cm^{-1}). Then, a H_2O_2 stock solution in water was prepared.

(b) •OH

To a solution of H_2O_2 in 100 μM sodium phosphate buffer at pH 7.4 as a cosolvent, the $FeSO_4$ solution (10 μM) was added at room temperature. Then, •OH was generated from Fe^{2+} and H_2O_2 (Fenton reaction).

(c) OCl^-

NaOCl solution was diluted appropriately in 0.1 M NaOH aq. The concentration of OCl^- was determined based on the molar extinction coefficient at 292 nm (350 M^{-1} cm^{-1}). Then, a OCl^- stock solution in 0.1 M NaOH aq. was prepared.

(d) Generation of $•O_2^-$

Superoxide ($•O_2^-$) was added as solid KO_2.

(e) $^1O_2$

A solution of $NaMoO_4$ was added to a solution of H_2O_2 in 0.1 M sodium phosphate buffer at pH 7.4 as a cosolvent at room temperature.

Determination of the detection limit

The detection limit was calculated based on the method used in the previous literature[7]. The fluorescence emission spectrum of **PTZ-Cy2** was measured by three times and the standard deviation of blank measurement was achieved. The fluorescence intensity at 595 nm was plotted as a concentration of NaClO. The detection limit was calculated with the following equation:

$$\text{Detection limit} = 3\sigma/k \qquad (1)$$

Where σ is the standard deviation of blank measurement, k is the slope between the fluorescence intensity versus hROS concentration.

Live cell incubation

HeLa cells were cultured in DEME (Invitrogen) supplemented with 10% FCS (Invitrogen). One day before imaging cells were seeded into 24-well flatbottomed plates. The next day, the cells were incubated with 8.0 μM dye for 40 min at 37 °C under 5% CO_2 and washed with phosphate-buffered saline (PBS) three times.

Hela cells pre-treated with PMA (2 ng mL^{-1}) for 40 min and then incubated with **PTZ-Cy2** (8 μM) plus MitoTracker Deep Red FM (1 μM) for 30 min at 37 ^{0}C. The cells were washed with PBS buffer and the fluorescence images were acquired

Fluorescence imaging

Fluorescence imaging in cells were obtained with spectral confocal multiphoton microscopes (Olympus FV1000 confocal laser scanning microscope).

Photostability

PTZ-Cy2, **OPTZ-Cy2**, **OPTA** were dissolved in DMSO-water (5:5 v) at a concentration of 10.0 μM, respectively. The solutions were irradiated under a 500 W iodine tungsten lamp for 2 h at a distance of 250 mm away. An aqueous solution of sodium nitrite (50.0 g/L) was placed between the samples and the lamp as a heat filter. The photostabilities were expressed in the terms of remaining absorption (%) calculated from the changes of absorbance at the absorption maximum before and after irradiation by iodine tungsten lamp.

Table S1 Spectral data of dyes.

Dyes	Solvents	λ_{abs}	λ_{em}	$\varepsilon \times 10^4$	Φ_f
PTZ-Cy2	DMSO	384/583	485	2.96	0.0008
	DCM	412/ 627	500	3.29	0.0004
	Ethanol	392/ 595	480	3.32	0.0008
	H_2O	375 /550	475	2.62	0.0006
	Methanol	390/ 593	478	3.43	0.0008
	Acetone	383/577	478	2.92	0.0006
OPTZ-Cy2	DMSO	340/ 485	486/622	6.65	0.066
	DCM	352/ 524	498/624	5.87	0.035
	Ethanol	342/ 486	479/605	5.43	0.036
	H_2O	343 /478	479/595	5.23	0.019
	Methanol	339/ 477	480/602	4.65	0.032
	Acetone	346/480	475/603	5.12	0.041
OPTA	DMSO	346	475	2.0	0.81
	DCM	343	454	1.94	0.68
	Ethanol	342	471	1.86	0.58
	H_2O	348	468	2.86	0.24
	Methanol	340	473	1.76	0.48
	Acetone	340	461	1.95	0.36

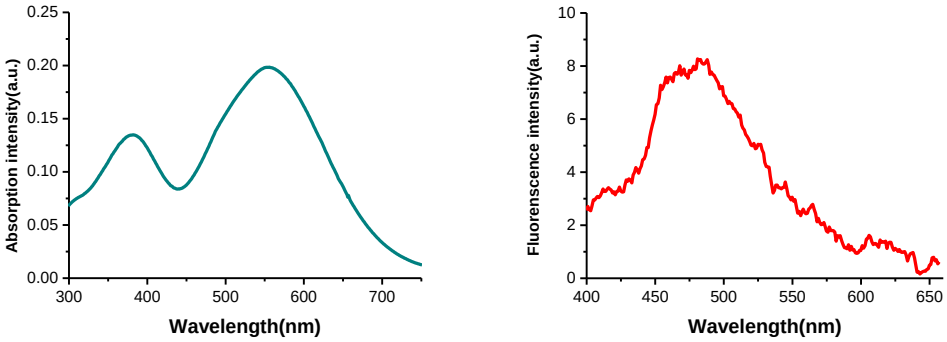


Figure S1. The absorption (left) and emission (right) spectra of dye **PTZ-Cy2** in water. λ_{ex} = 345 nm.

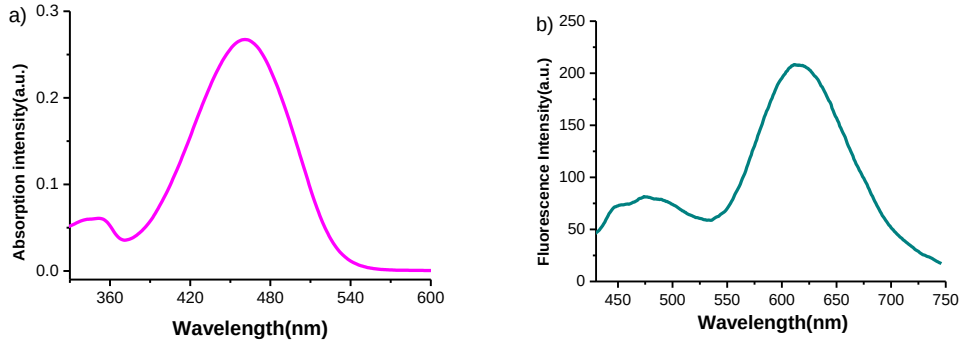


Figure S2. The absorption (left) and emission (right) spectra of dye **OPTZ-Cy2** in water. λ_{ex} = 405 nm.

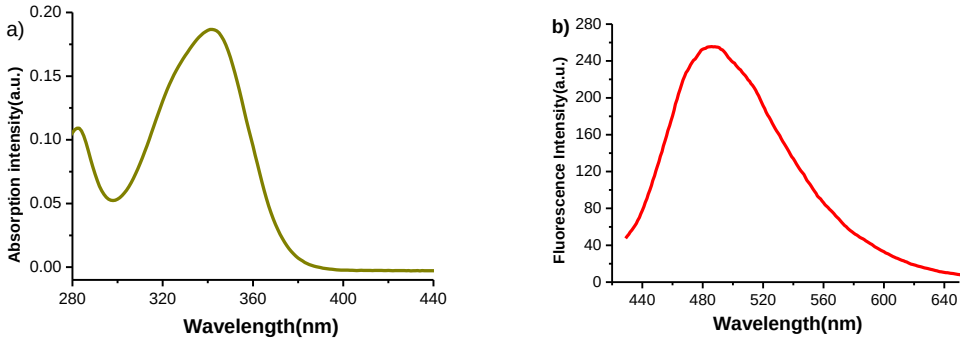


Figure S3. The absorption (left) and emission (right) spectra of dye **OPTA** in water. λ_{ex} = 405 nm.

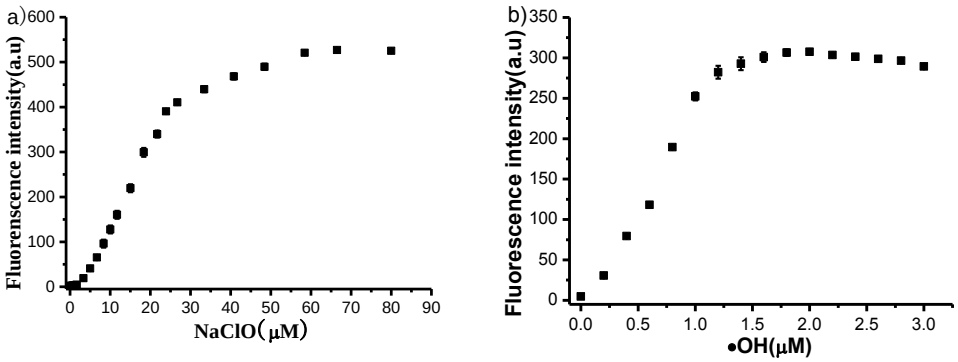


Figure S4 a) Changes in the fluorescence spectrum of **PTZ-Cy2** (10 μM) upon addition of NaClO (0-60 μM). Each spectrum was recorded after 5 min in water. b) Changes in the fluorescence spectrum of **PTZ-Cy2** (10 μM) upon addition of •OH (0-30μM). Each spectrum was recorded after 3 min in water. λ_{ex} = 340 nm.

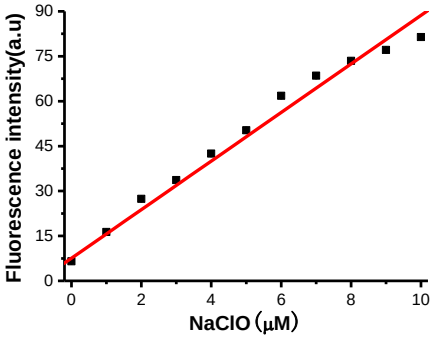


Figure S5 The fluorescence changes at 595 nm, upon addition of lower concentrations of NaClO at 1–10 μM. Excitation: 450 nm.

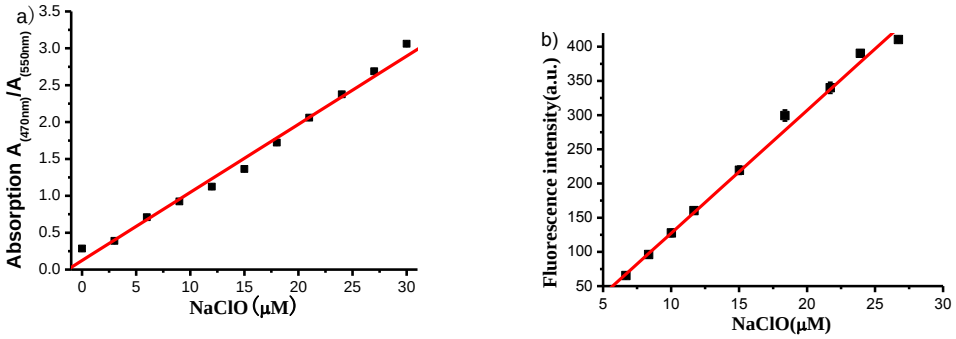


Figure S6 a) Plot of the absorption intensity ratios at 470 nm and 550 nm of **PTZ-Cy2** (10 μM) upon addition of NaClO (0–30 μM). λ_{ex} = 340 nm. b) Fluorescence intensity at 470 nm of **PTZ-Cy2** (10 μM) upon addition of NaClO (0–30 μM). Conditions: each spectrum was recorded after 5 min in water. λ_{ex} = 450 nm.

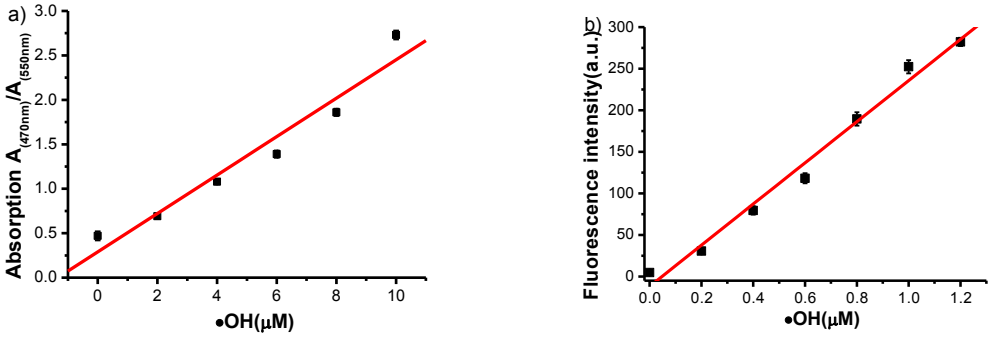


Figure S7 a) Plot of the absorption intensity ratios at 470 nm and 550 nm of **PTZ-Cy2** (10 μM) upon addition of •OH (0–10 μM). b) Fluorescence intensity at 470 nm of **PTZ-Cy2** (10 μM) upon addition of •OH (0–12 μM). λ_{ex} = 340 nm. Conditions: each spectrum was recorded after 3 min in water. λ_{ex} = 450 nm.

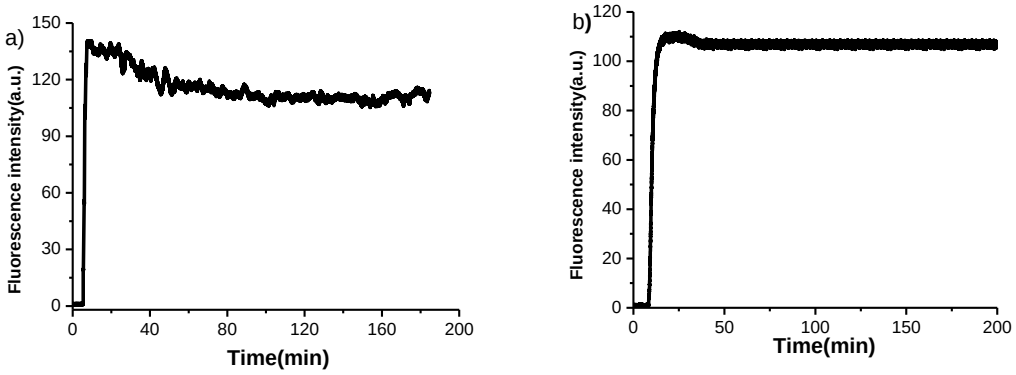


Figure S8. a) Time dependent fluorescence intensity changes of **PTZ-Cy2** (10 μM) at 595 nm in the presence of 50 equiv •OH in water (180min). b) Time dependent fluorescence intensity changes of **PTZ-Cy2** (5 μM) at 595 nm in the presence of 20 equiv NaClO in water (200min), λ_{ex} = 340 nm.

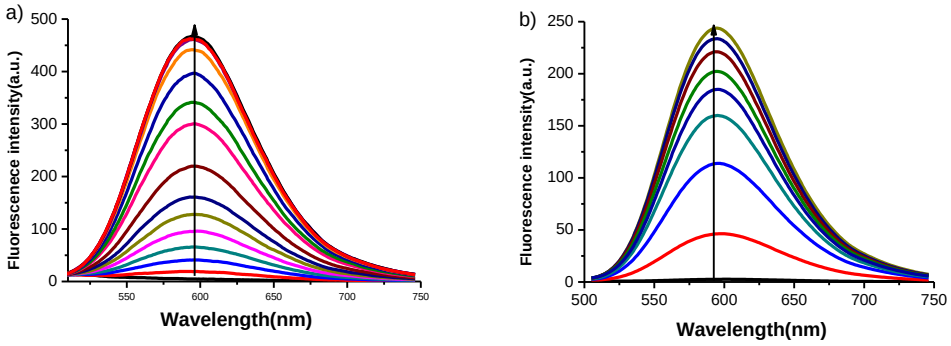


Figure S9. a) Changes in the fluorescence emission spectrum of **PTZ-Cy2** (10 μM) with increases of NaClO concentration (0-30μM) at 595nm in water. b) Changes in the fluorescence emission spectrum of **PTZ-Cy2** (10μM) with increases of the •OH concentration (0-10μM) at 595nm in water, λ_{ex} = 450 nm.

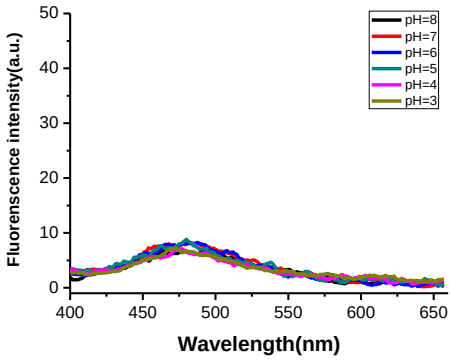


Figure S10. Influence of pH on fluorescence for **PTZ-Cy2** (20 μM) over the range pH 3–8. λ_{ex} = 340 nm.

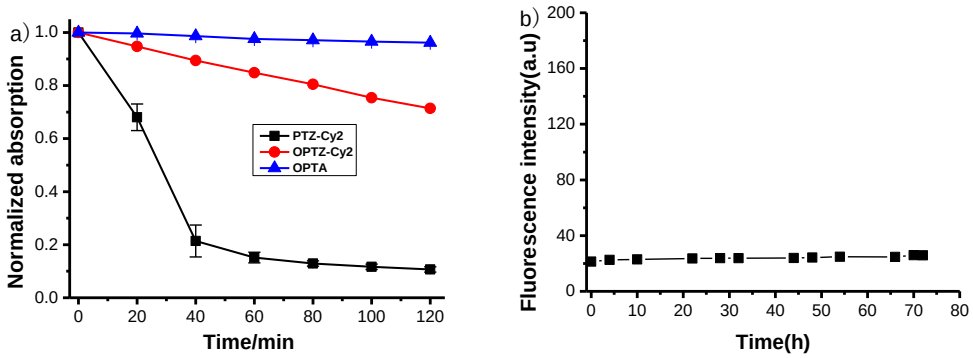


Figure *S11*. a) Photo-fading of dyes (**PTZ-Cy2, OPTZ-Cy2**, **OPTA)** in solvent mixture with the ratio of DMSO-water 5: 5 v/v with radiation by a 500 W iodine-tungsten lamp. **PTZ-Cy2**: λ_{abs} = 550 nm, **OPTZ-Cy2**: λ_{abs} = 470 nm, **OPTA**: λ_{abs} = 350 nm. b) Time-profile of the emission intensities of compound **PTE-Cy** in water at room temperature for 72 h. The fluorescent data were collected at 595 nm.

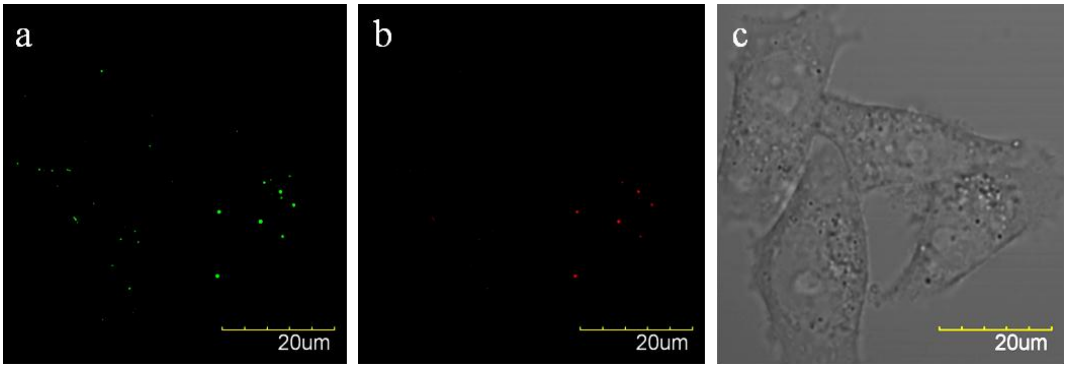


Figure S12. **PTZ-Cy2** (20 μM) was loaded into HeLa cells for 30 min. a) Green emission of the **PTZ-Cy2** (470 ± 20) nm; b) red emission of the **PTZ-Cy2** (590 ± 20) nm, c) the green-red merged image with bright-field image. λ_{ex} = 405 nm.

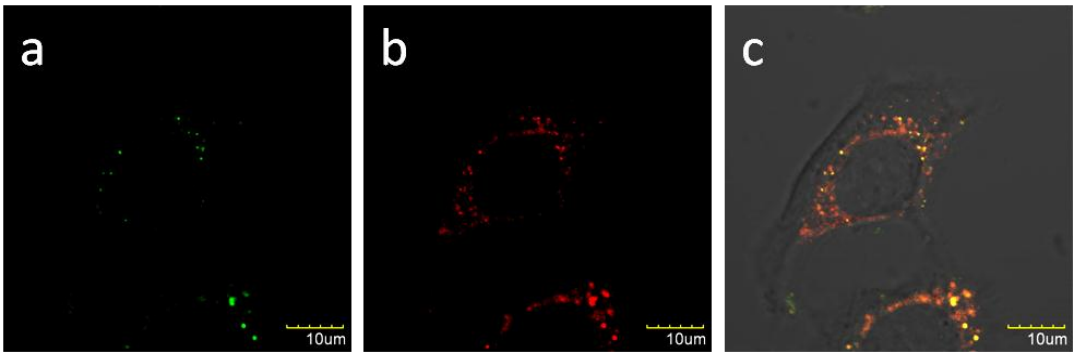


Figure S13. **PTZ-Cy2** (20 μM) was loaded into HeLa cells for 1.5 hours. a) Green emission of the **PTZ-Cy2** (470 ± 20) nm; b) red emission of the **PTZ-Cy2** (590 ± 20) nm, c) the green-red merged image with bright-field image. λ_{ex} = 405 nm.

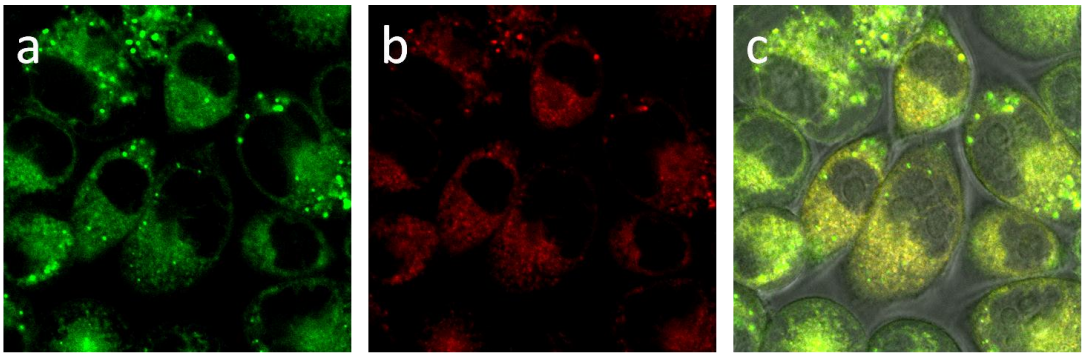


Figure S14. Hela cells pre-treated with NaClO (100 μM) for 40 min and then incubated with **PTZ-Cy2** (8 μM) for 30 min. (a) Green emission of the **PTZ-Cy2** (470 ± 20) nm. (b) Red emission of the **PTZ-Cy2** (590 ± 20) nm. (c) The green-red merged image with bright-field image. λ_{ex} = 405 nm.

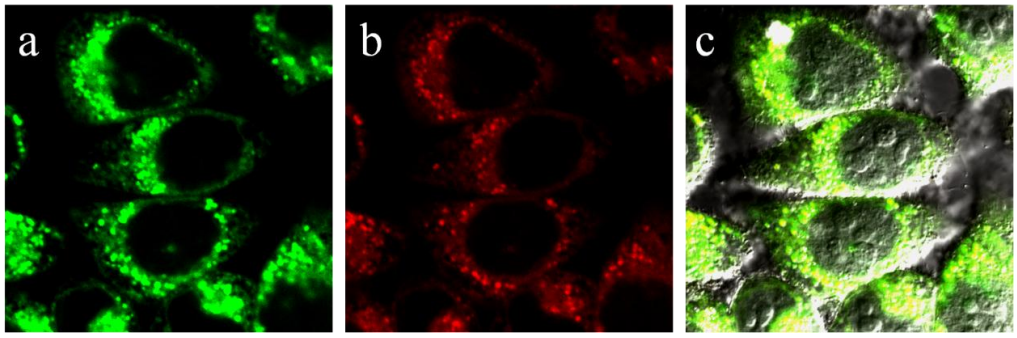


Figure S15. Hela cells pre-treated with **PTZ-Cy2** (8 μM) for 30 min and then incubated with NaClO (100 μM) for 30 min. (a) Green emission of the **PTZ-Cy2** (470 ± 20) nm. (b) Red emission of the **PTZ-Cy2** (590 ± 20) nm. (c) The green-red merged image with bright-field image. λ_{ex} = 405 nm.

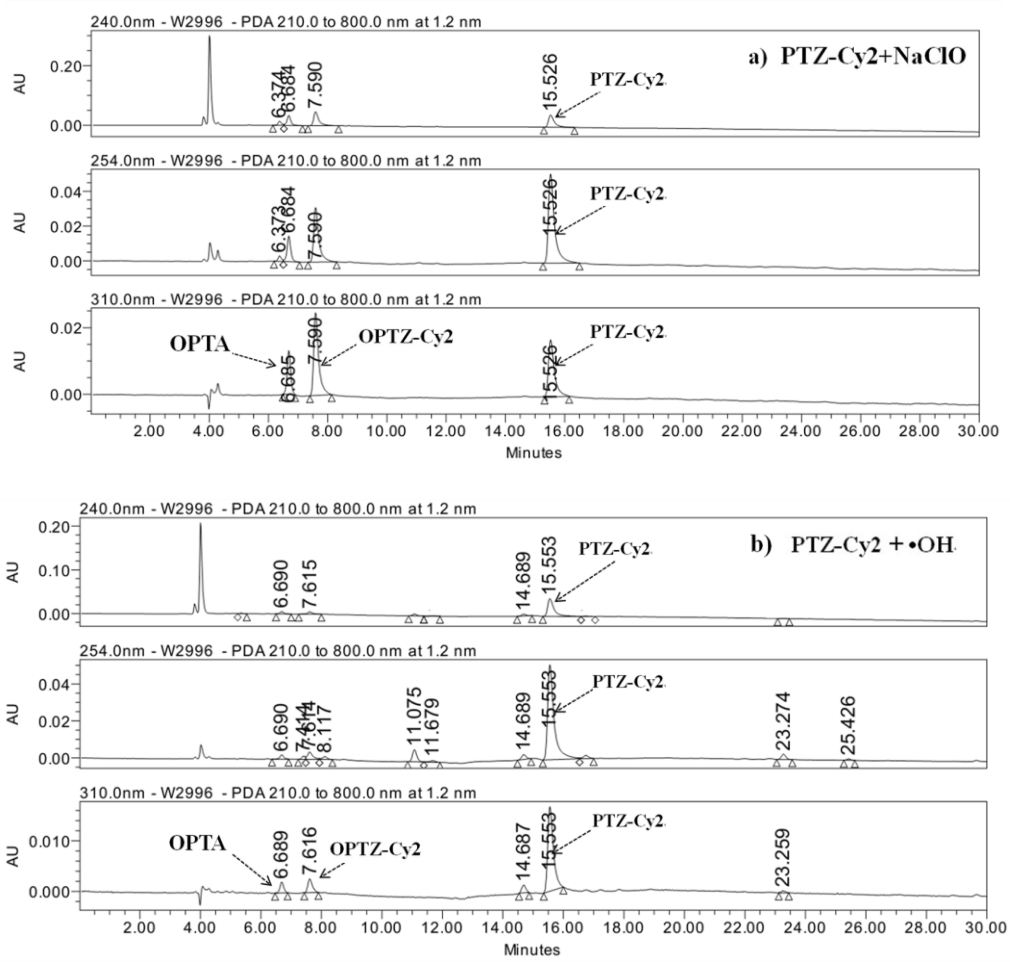


Figure S16. HPLC chromatograms of probe **PTZ-Cy2**, a) after reaction with NaOCl for 10 min, b) after reaction with •OH for 10 min .

In vitro testing: two fluorescent substances, **OPTZ-Cy2** and **OPTA**, were generated when NaClO and were added slowly to the **PTZ-Cy2** solution. To confirm the formation of these substances, the partial MS spectra of the reaction of **PTZ-Cy2** with NaClO and •OH are shown in Fig S11.

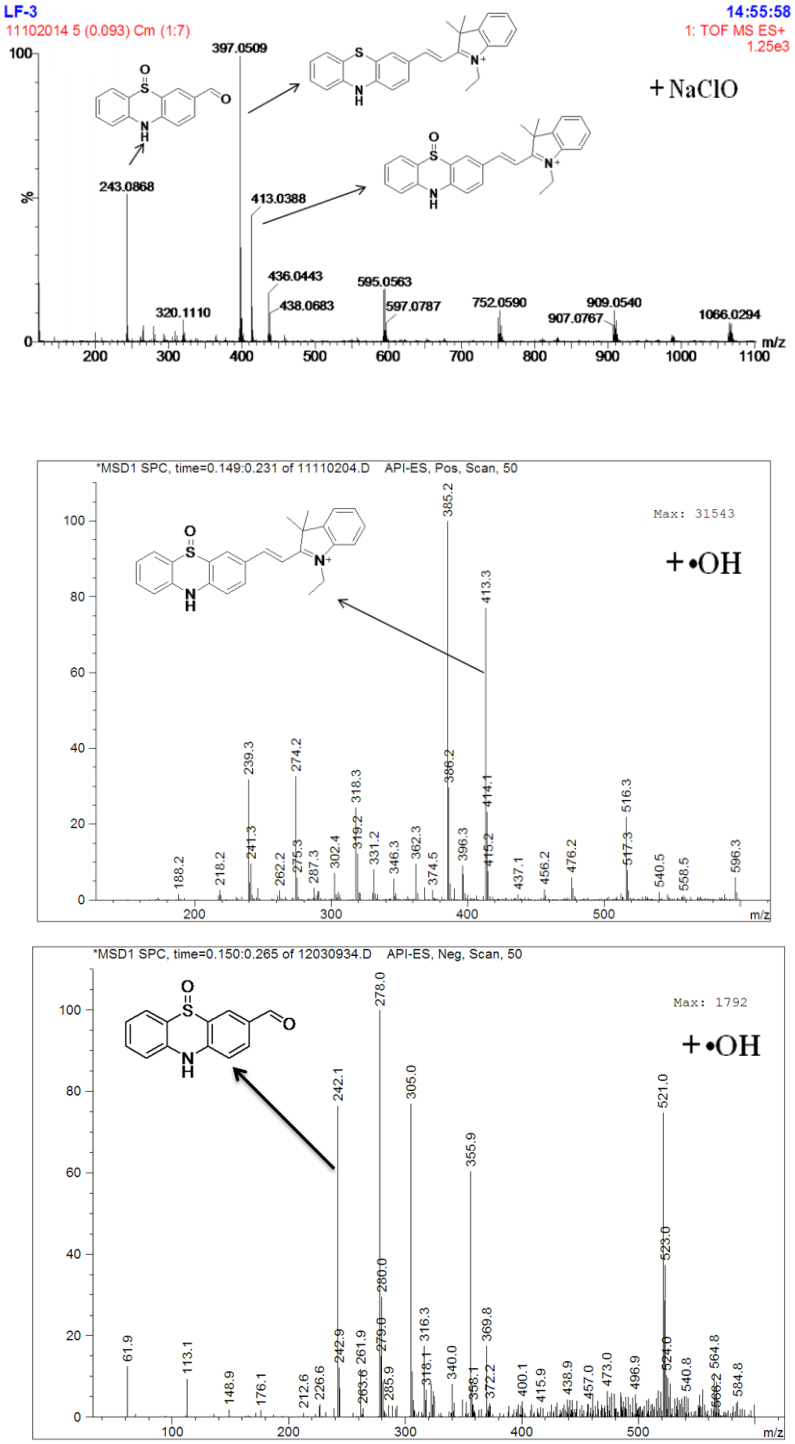


Figure S17. MS monitoring oxidition of the hROS with **PTZ-Cy2** process.

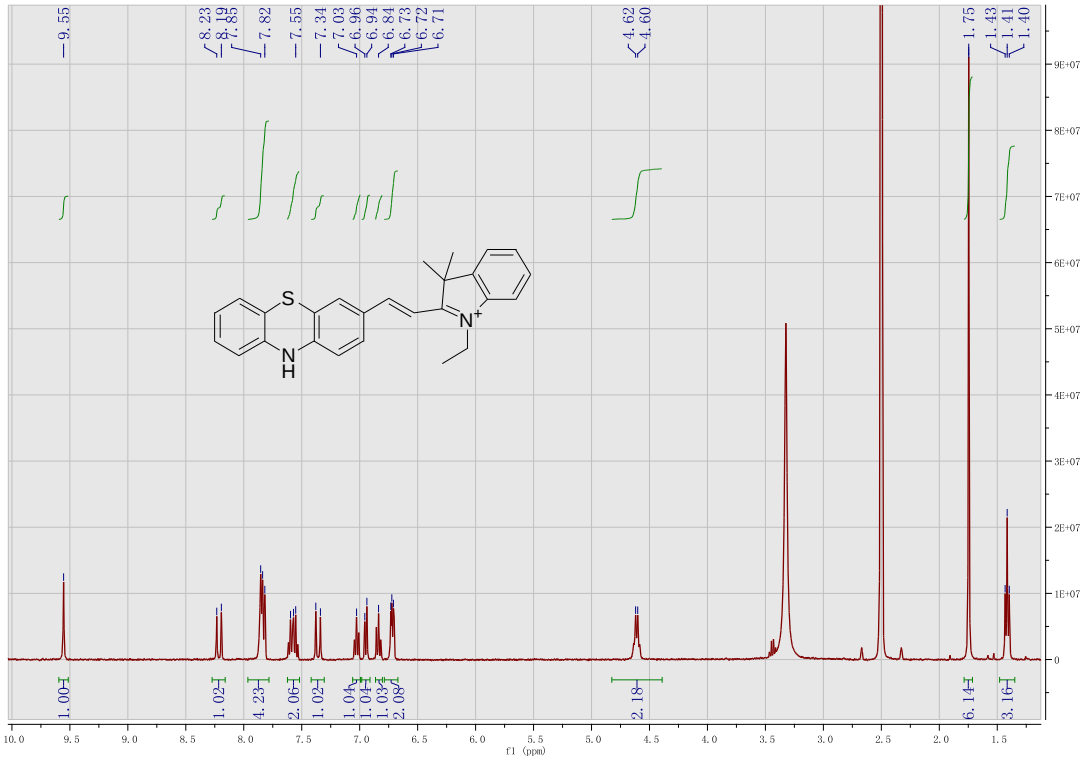
References

1. H. Spreitzer, J. Daub, *Chem. Eur*, **1996**, 9, 1150-1158.
2. B.J. Coe, J.A. Harris, I. Asselberghs, K. Clays, G. Olbrechts, A. Persoons, J.T. Hupp, R.C. Johnson, S.J.

Coles, M.B. Hursthouse, K. Nakatani, *Adv. Funct. Mater*. **2002**, 12, 110-116.

3. D. Oushiki, H. Kojima, T. Terai, M. Arita, K. Hanaoka, Y. Urano, T. Nagano, *J Am Chem Soc* **2010**, *132*, 2795-2801.
4. L. Yuan, W. Lin, J. Song, *Chem. Commun. (Camb)* **2010**, *46*, 7930-7932.
5. S. M. Chen, J. X. Lu, C. D. Sun, H. M. Ma, *Analyst* **2010**, *135*, 577-582.
6 K. Kundu, S. F. Knight, N. Willett, S. Lee, W. R. Taylor, N. Murthy, *Angew. Chem. Int. Edit.* **2009**, *48*, 299-303.
7 B. Zhu, C. Gao, Y. Zhao, C. Liu, Y. Li, Q. Wei, Z. Ma, B. Du and X. Zhang, *Chem. Commun.* **2011**, 47, 8656–8658.

The ^{1}H-NMR and ^{13}C-NMR spectra of the dyes



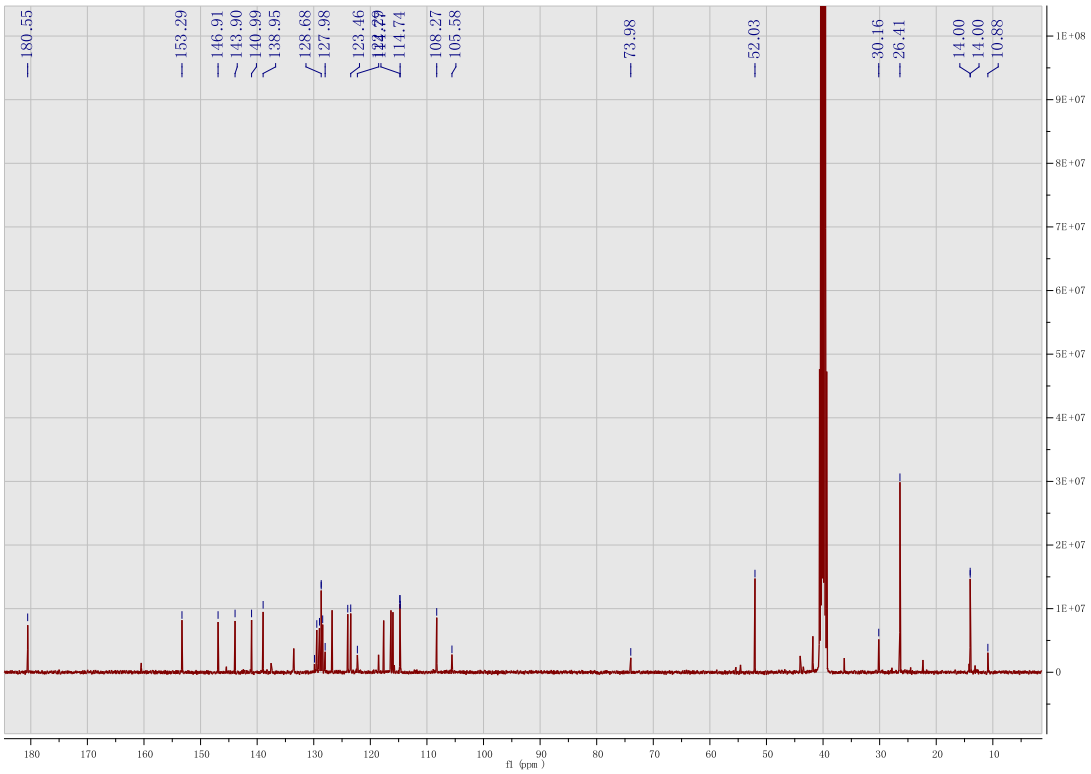


Figure S18. The ^{1}H-NMR and ^{13}C-NMR spectra of **PTZ-Cy2** in DMSO.

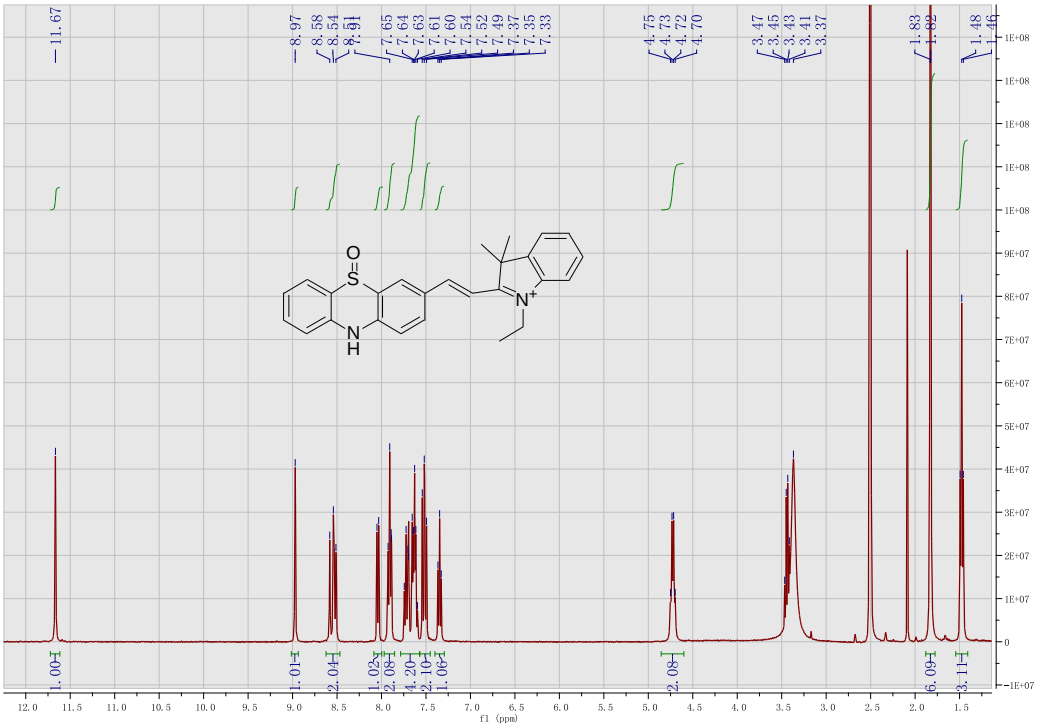


Figure S19. The ^{1}H-NMR spectra of **OPTZ-Cy2** in DMSO.

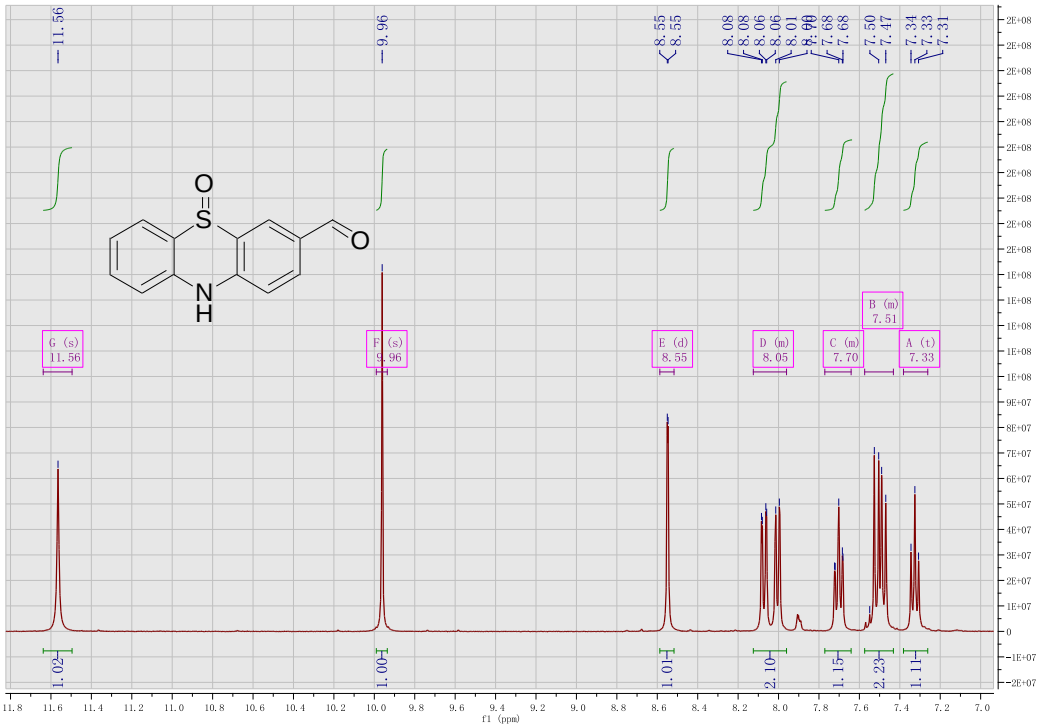


Figure S20. The ^{1}H-NMR spectra of **OPTA** in DMSO.