Supporting Information

# A near infrared heptamethine aminocyanine dye with a long-living excited triplet state for photodynamic therapy

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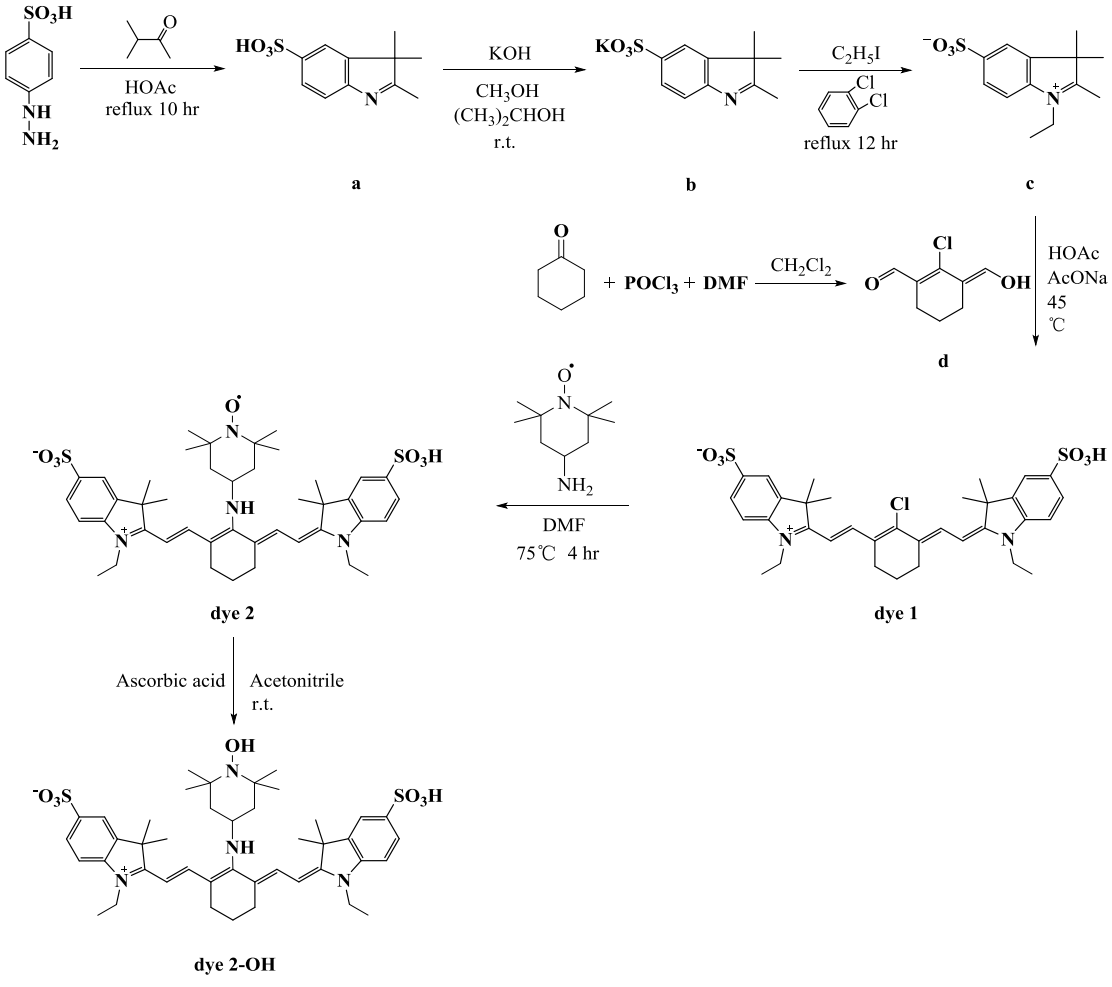
**Contents:**

## 1、Materials and Methods

**Materials.** 4-Hydrazinobenzenesulfonic acid (98%), 3-methyl-2-butanone (98%), 4-bromomethylbenzoic acid (97%), iodoethane (98%), 4-amino-TEMPO (98%), cyclohexanone (99%) and phosphorus oxychloride (99.5%) were purchased from Sigma-Aldrich without further purifications. All reactions have been carried out under a nitrogen atmosphere unless otherwise noted. *N*,*N*-Dimethylformamide (DMF) was dried prior to use with calcium hydride. Ultrapure deionized water from a Milli-Q ultrapure system was used for all synthesis, analysis, separation and purification steps.
**Methods.** $^{1}$HNMR spectra were recorded at room temperature using a VARIAN INOVA-400 spectrometer with chemical shifts reported as ppm (in dimethyl sulfoxide (DMSO), tetramethylsilane (TMS) as internal standard. Mass spectrometric data were obtained on LTQ Orbitrap (LTQ Orbitrap XL) from Thermo Scientific. The element content of dye **2** was measured by Vario EL III CHNS elemental analysis. The crude products of dye **1**,dye **2**and dye **2-OH** were purified by FLEXA Purification System from Agela Technologies using methanol and deionized water as mobile phase.

## 2、Synthesis of Compounds and Preparation of Stock Solutions

**Scheme S1** Synthetic strategy of heptamethine cyanine dye **1**,dye **2** and dye **2-OH**



**Synthesis of dye 1.** Intermediates **a**, **b**, **c** and **d** were synthesized according to our reported work.[1] Intermediates **c** and **d** condensed to form dye **1.**[2] Briefly, Compound **c** (500.00 mg, 1.6 mmol, 2.0 eq.), Compound **d** (140.99 mg, 0.8 mmol, 1.0 eq.), and sodium acetate (160 mg, 2.0 mmol, 2.5 eq.) were dissolved in acetic anhydride (10 mL) and stirred at 45 °C for 1 hour. The crude product was precipitated by adding diethyl ether (100 mL). The resulting solid product was obtained by filtration, and subsequently purified by HPLC using methanol, deionized water as mobile phase. The purified product, dye **1**, was obtained as green amorphous powder. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ (ppm): 8.26 (d, $J$ = 14.0 Hz, 1H), 7.81 (s, 1H), 7.68 (d, $J$ = 7.9 Hz, 1H), 7.39 (d, $J$ = 8.3 Hz, 1H), 6.34 (d, $J$ = 14.1 Hz, 2H), 4.25 (d, $J$ = 6.9 Hz, 2H), 2.72 (s, 2H), 1.86 (s, 1H), 1.68 (s, 6H), 1.30 (t, $J$ = 7.0 Hz, 3H). MS (ESI): m/z calcd. for $C_{34}H_{38}ClN_2O_6S_2^-$ [M-H]$^-$: 669.19, found: 669.49.

**Synthesis of dye 2.** Dye **c** (200 mg, 0.3 mmol, 1.0 eq.) and 4-Amino-Tempo (153.33 mg, 0.9 mmol, 3.0 eq.) were mixed in anhydrous DMF (20 mL) under nitrogen and stirred at 75°C for 4 hours. The crude product was precipitated with anhydrous ether and filtered. Subsequently, the crude product was purified by HPLC using methanol, deionized water as mobile phase. Dye **2** was obtained as purple amorphous powder. MS (ESI): m/z calcd. for $C_{43}H_{56}N_4O_7S_2^-$ [M-H]$^-$: 804.3590, found: 804.3596. Elemental analysis, calcd. for $C_{43}H_{57}N_4O_7S_2^{\cdot}$ (805.3669): C, 64.07; H, 7.13; N, 6.95, S, 7.95%. Found: C, 64.38; H, 7.16; N, 6.90, S, 7.51%. Due to the paramagnetic property, satisfactory NMR spectra of dye **2** can't be recorded. Therefore, dye **2-OH** was synthesized from dye **2** to support the structure characterization

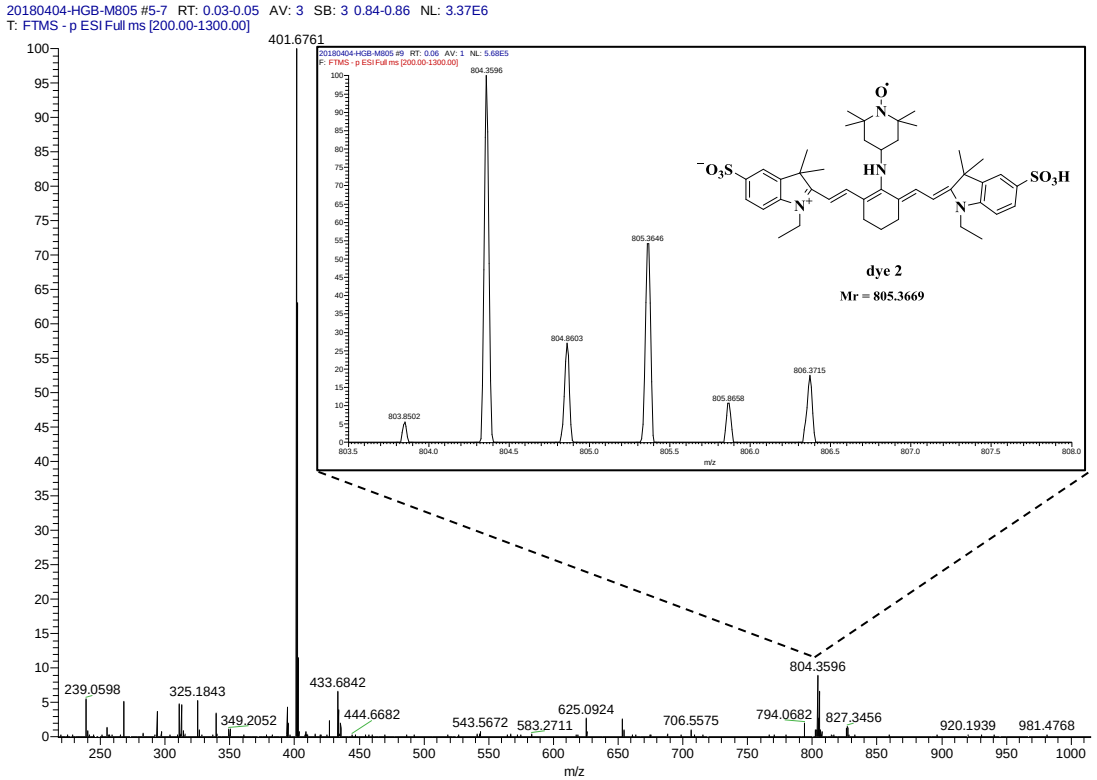



**Figure S1.** HRMS (ESI$^-$) of dye **2**

**Synthesis of dye 2-OH.** Dye **2** (100 mg) was dissolved in 10 mL acetonitrile, and ascorbic acid (109.34 mg, 0.6 mmol, 5.0 eq) was added. The mixture was stirred at room temperature for 30 min under nitrogen. The solvent is evaporated until dry. Subsequently, the crude product was purified by HPLC using methanol, deionized water as mobile phase. Dye **2-OH** was obtained as purple amorphous powder. $^{1}$H NMR (400 MHz, DMSO-$d_6$) $\delta$ (ppm): 7.77 (d, $J$ = 11.0 Hz, 2H), 7.68 (s, 2H), 7.60 (s, 2H), 7.17 (s, 2H), 5.96 (s, 2H), 4.08 (s, 4H), 3.17 (s, 1H), 1.86–1.52 (m, 18H), 1.19 (dd, $J$ = 100.5, 34.0 Hz, 22H). MS (ESI): m/z calcd. for $C_{43}H_{57}N_4O_7S_2^-$ [M-H]$^-$: 805.3669, found: 805.3678.

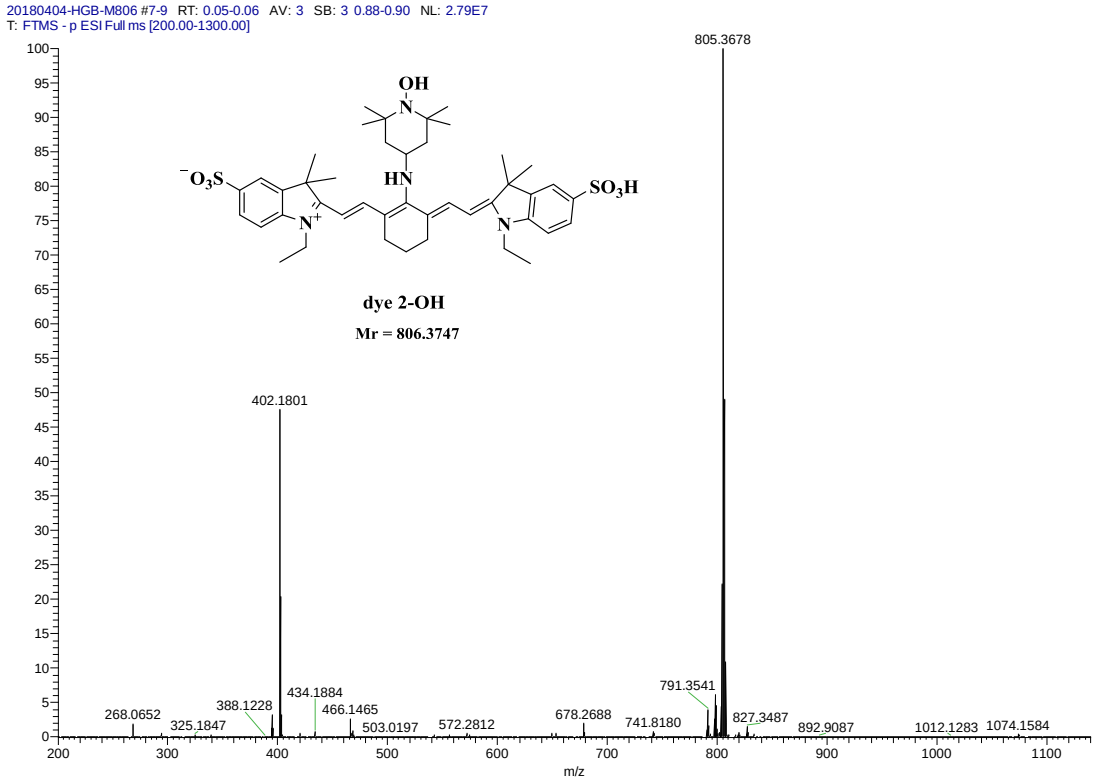



**Figure S2.** HRMS (ESI$^-$) of dye **2-OH**

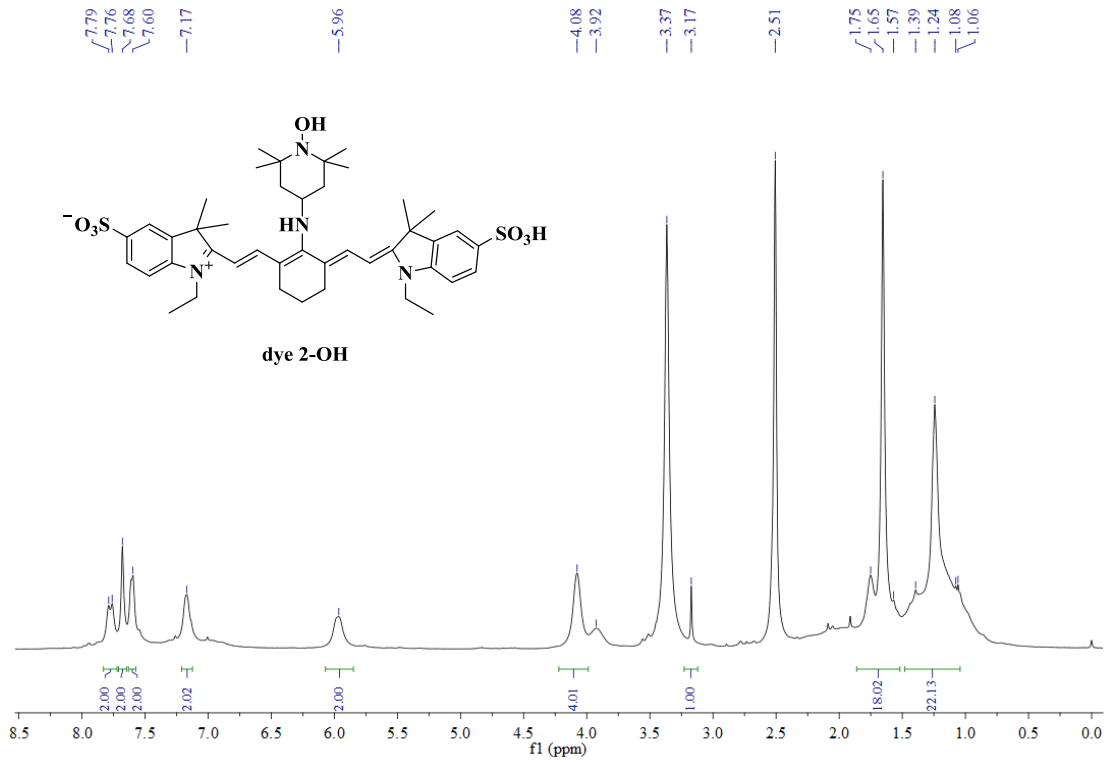



**Figure S3.** $^1$H NMR spectrum of dye **2-OH** (in DMSO-$d_6$)

**Preparation of Stock Solutions**

The 5.0 mM solutions of dye **1** (16.8 mg, 0.025 mmol) and **2** (20.1 mg, 0.025 mmol) were dissolved in DMSO (5 mL) and stored in the dark at 4 °C for measuring absorption, fluorescence spectroscopy, and nanosecond transient difference absorption spectra.

For Hela cells staining, a 1.0 mM solution of dye **2** (4.0 mg, 0.005 mmol) was prepared in complete medium (5 mL) without DMSO. A 5.0 mM (20.1mg, 0.025 mmol) complete medium solution (5 mL) of dye **2** without DMSO was prepared for cytotoxicity assay and further dilution to staining concentration.

## 3. Characterization of Compounds

**Steady-state Absorption and Fluorescence Spectroscopy**

The steady-state absorption and fluorescence spectroscopy were recorded with a UV-Visible spectrometer named Cary 60 and a fluorometer named Cary Eclipse from Agilent Tech at room temperature, respectively. The data were obtained under the control of a Windows-based PC running the manufacturers' supplied software.

**Chemical Stability in Vitro**

The chemical stability evaluation was performed by HRMS. So the time-dependent chemical stability of dye **2** in 100% ICR mice serum at 37 °C for 4 hand 24 h as a function of time were tested. ICR mice serum was provided by the Specific Pathogen Free Animal Laboratory at Dalian Medical University. Under dark condition, the mice serum containing dye **2** (5 μM) was incubated in a constant temperature bath at 37 °C for 4 h and 24 h, respectively. Then the above serum solutions were centrifuged at

6000 rpm. Subsequently, carefully pipette supernatant liquid into the appropriate amount of HPLC methanol for 30 min at room temperature. Finally, the mixtures were centrifuged again, and the appropriate upper liquid was transferred to HPLC methanol for high resolution mass spectrometry (HRMS). The dye **2** methanol solution containing a molar concentration of 10% dye **2-OH** (mole ratio of dye **2-OH**:dye **2** is 1:9) was detected by HRMS as control. All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Dalian Medical University (Dalian, China) and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1996).

**Transient Absorption Measurements**

Nanosecond transient difference absorption spectra was recorded on a LP920 laser flash photolysis spectrometer (Edinburgh Instruments, UK) at room temperature. Dye **1** and dye **2** were dissolved in ethanol at 10 μM, respectively. The oxygen removal was achieved to all samples by argon bubbling for about 30 min. The dye solutions were excited with a Nd:YAG 532 nm laser beam while nanosecond transient difference absorption spectrums were obtained at certain time intervals.

**Singlet Oxygen Generation Measurements**

In singlet-oxygen generation measurements, 1,3-diphenylisobenzofuran (DPBF) was used as capture agent in absolute ethanol. It's a typical procedure for the detection of singlet oxygen generation by using DPBF. Before the measurement, dye **1** and dye **2** were respectively dissolved in absolute ethanol to a final concentration with almost the same optical density (~0.1) at 660 nm and mixed DPBF at 50 μM. Similarly, the concentration of 4-amino-TEMPO (a control group, 3 μM) was adjusted to be the same as dye **2**. A near infrared LED array (power density of 0.8 mW/cm$^2$, $\lambda_{ex}$ = 660 nm) was used as excited light source. The absorbance spectra of DPBF at 410 nm were recorded at different intervals.

**Singlet Oxygen Quantum Yield ($\Phi_\Delta$) Measurements**

The Singlet Oxygen Quantum Yield of dye **1** and dye **2** in ethanol were determined compared to methylene blue (MB). Dye **1** and dye **2** (Optical densities were adjusted to around 0.2-0.3 at 660 nm) under irradiation (power density of 0.8 mW/cm$^2$) in the presence of DPBF (50 μM) reduces their optical absorption, indicating formation of the singlet oxygen. $\Phi_\Delta$ values were calculated by the following equation:

$$\Phi_\Delta = \Phi_{MB} \times \left(\frac{k_{Cy7}}{k_{MB}}\right)\left(\frac{F_{MB}}{F_{Cy7}}\right)$$

where $k$ is the bleach rate of DPBF absorbance (410 nm) with irradiation time, and $F$ is the absorption correction factor, given that $\Phi_{MB}$ = 0.52 in ethanol as reference.[3]

## 4、HeLa Cell Line Culture

HeLa cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM; GIBCO) containing 10% fetal bovine serum (FBS; GIBCO) and 1% antibiotics (80 U mL$^{-1}$ penicillin and 0.08 mg mL$^{-1}$ streptomycin; GIBCO). All the biochemical reagents described in this work were purchased from Nanjing Key GEN Bio TECH of China.

Cultured cells were incubated at 37 °C in an atmosphere of 5% $CO_2$ in air.

## 5、Confocal Fluorescence Microscopy

Confocal Fluorescence images were performed on a confocal laser scanning biological microscope (Fluoview FV1000, Olympus). HeLa cells were seeded in a glass bottom dish (MatTek, 35 mm dish with a 20 mm bottom well) with an amount a density of $1\times10^5$ cells per dish. Dye **2** at 10 μM was then co-cultivated with HeLa cells for 12 h. Prior to imaging, HeLa cells were stained with DAPI for 10 min. After that, the cells were washed three times with PBS (pH = 7.4) to fully remove the free dyes. For dye **2**, a standard 635-nm laser was used and image detection range was 655 nm to 755 nm. For DAPI, a standard 405 nm laser was used with a beam splitter SDM560.

## 6、Intracellular ROS Production after PDT

HeLa cells were seeded in a 35 mm glass bottom dish with an amount a density of $1\times10^5$ cells per dish. Dye **2** at 20 μM was co-cultivated with HeLa cells for 12 h, followed by stained with dihydroethidium (DHE) of 5 μM for 15 min in the dark. The cells were washed three times by PBS and then irradiated for 10 min and 20 min, respectively. After PDT treatment, the cells were imaged under confocal fluorescence microscopy and the non-irradiated cells served as control. Red fluorescent detection channel of DHE was set between 595 to 615 nm by using a standard 515 nm laser. In order to eliminate the effect of temperature on the observation, an ice box was placed under the 35 mm petrie dish for avoiding the temperature increase during PDT.

## 7、Cytotoxicity Assay

The dark cytotoxicity and phototoxicity of dye **1**, dye **2** and PpIX were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HeLa cells harvested in a logarithmic growth phase were seeded in 96-well plates at 100 μL/well (density of $1\times10^4$ cells/well) and incubated for 24 h. Then different concentrations of dye**s** were added into each well and incubated for 12 h. After the removal of samples, fresh medium was added and then irradiated by the LED lamp at a power density of 50 mW/cm$^2$ for 20 min. An ice box was disposed below the 96-well plates to eliminate the influence of temperature. MTT array was immediately tested on HeLa cells in group A, while, the Hela cells in group B were incubated for another 6 hours before being tested. Taking the same cell culture and staining methods under the premise, the dark cytotoxicity was monitored without irradiation at the same time as control. The cells were then subjected to MTT assay with Microplate reader named Multiskan FC from Thermo scientific (Filter: 490 nm).

## 8、AO/EB Staining

HeLa cells were seeded in a 35 mm glass bottom dish with an amount a density of $1\times10^5$ cells per dish. Dye **2** at 20 μM was co-cultivated with HeLa cells for 12 h. In order to eliminate the effect of temperature on the observation, an ice box was placed under the 35 mm petrie dish for avoiding the temperature increase during PDT. After

HeLa cells were irradiated for 20 min, the cells in group A were washed three times with PBS. HeLa cells were stained for 5 minutes with an appropriate amount of acridine orange/ethidium bromide (AO/EB) working solution.[4] Subsequently, cells were washed twice with PBS and imaged under confocal fluorescence microscopy. The cells in group B were incubated for another 6 h. Then the staining method was the same as above. For comparison, HeLa cells were irradiated for 20 min without stained dye **2** as control A, and that were stained with dye **2** for 12 h without irradiation as control B. A standard 488 nm laser was used. Green and red fluorescence were collected using beam splitter SDM560 and SDM640, respectively.

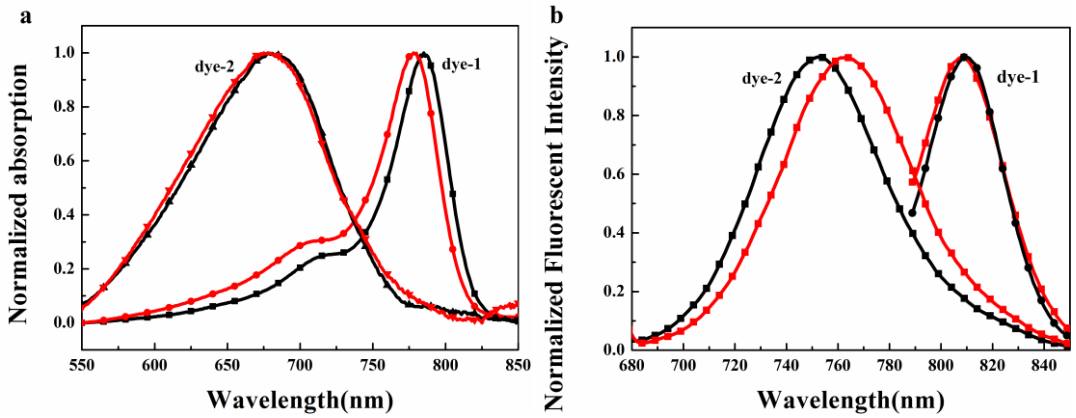



**Figure S4.** Normalized absorption (a) and emission (b) spectra of dye **1** and dye **2** in methanol (black line) and PBS (pH = 7.4, red line).

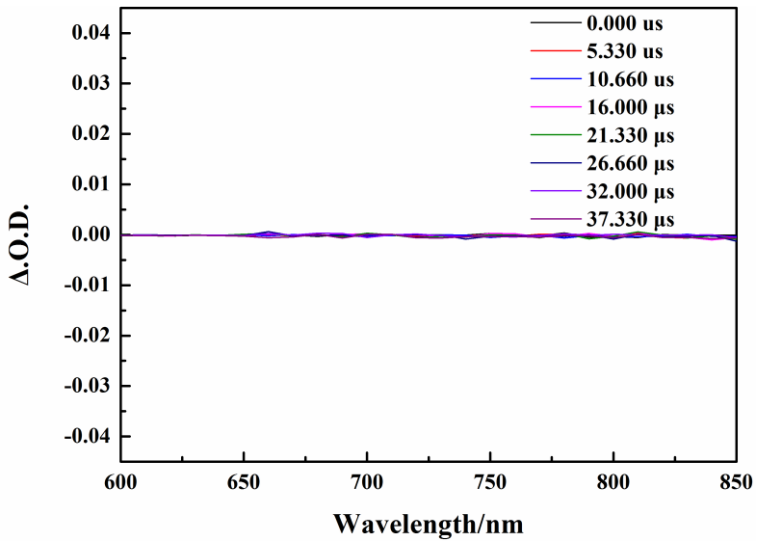



**Figure S5** Nanosecond time-resolved transient difference absorption spectra of dye **1** at 10.0 μM in deaerated ethanol. 532 nm laser pulse, decay times as indicated. All measurements are performed at room temperature.

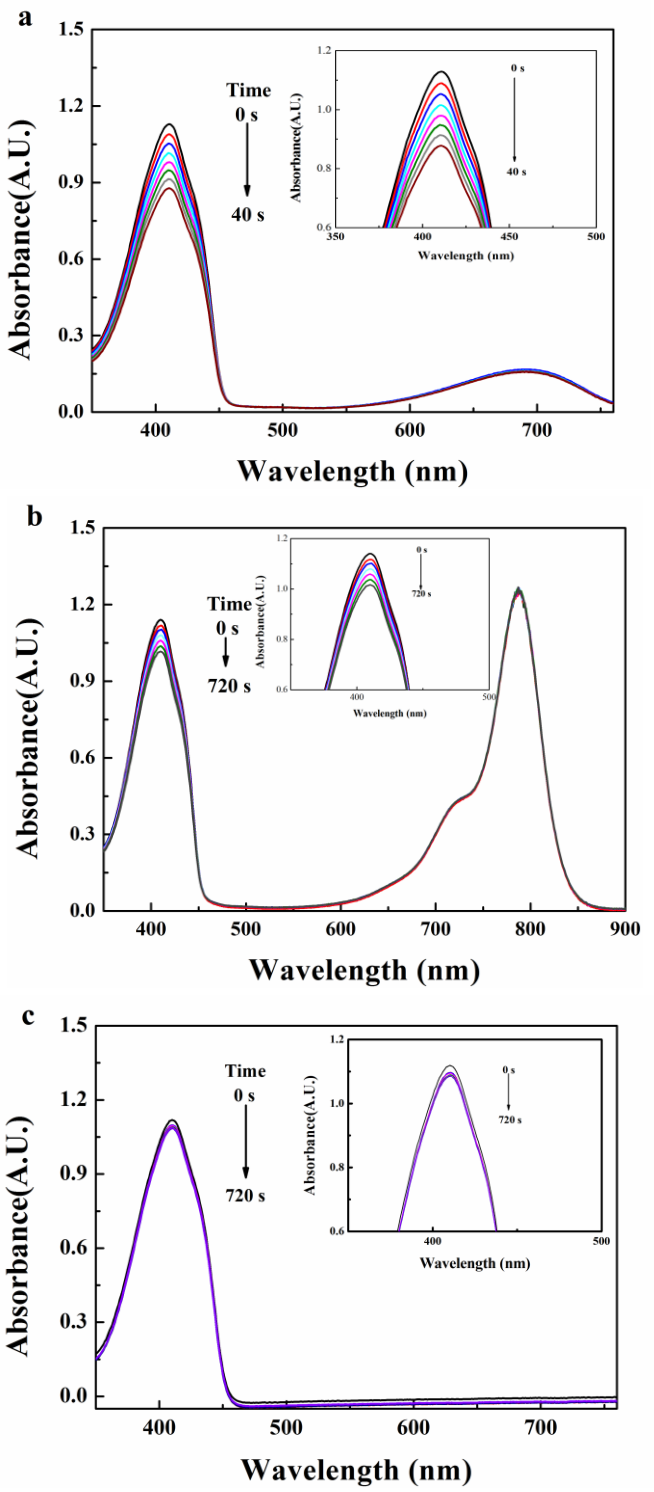
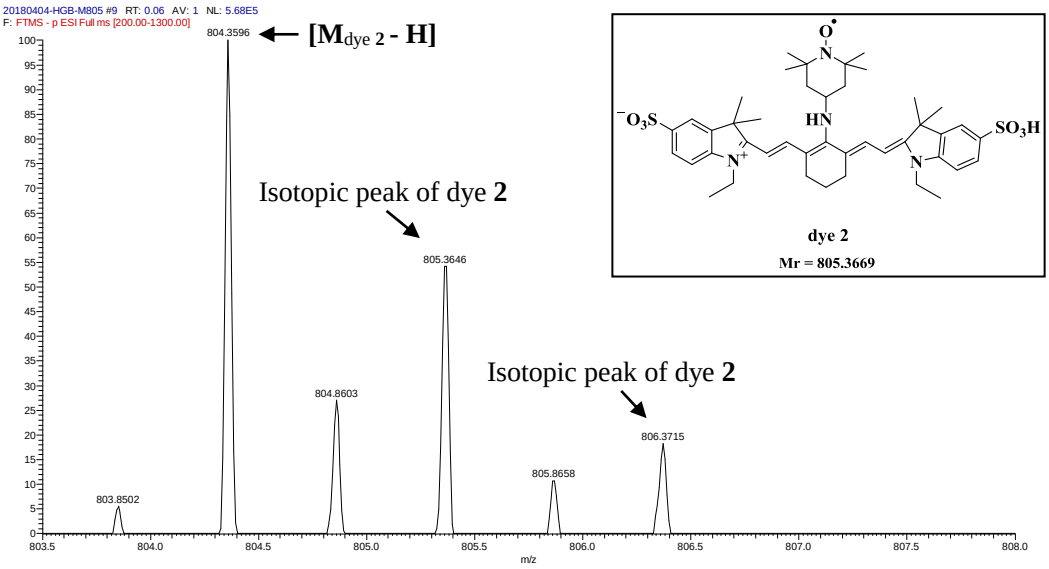
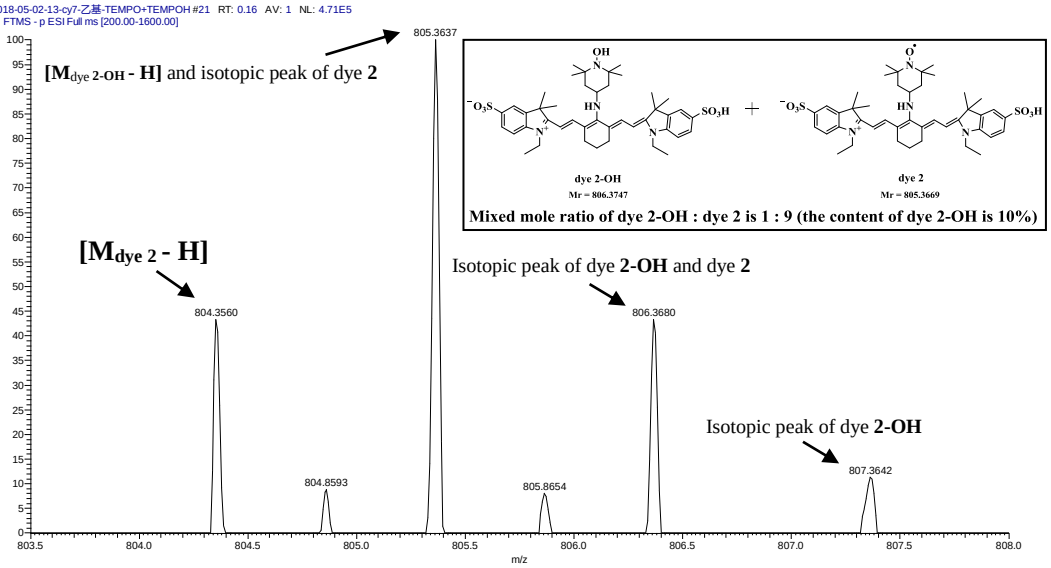



**Figure S6.** Singlet oxygen generation by dye **2** (a), dye **1** (b) and 4-amino-TEMPO (c) in ethanol monitored by disappearance of absorbance of DPBF at 410 nm with an LED array ($\lambda_{ex}$= 660 nm), power density of 0.8 mW/cm$^2$ for different irradiation times. Dye **1** and dye **2** were respectively dissolved in absolute ethanol to a final concentration with almost the same optical density (~0.1) at 660 nm and mixed with DPBF (50 μM). The concentration of 4-amino-TEMPO (3 μM) was adjusted to be the same as dye **2**.

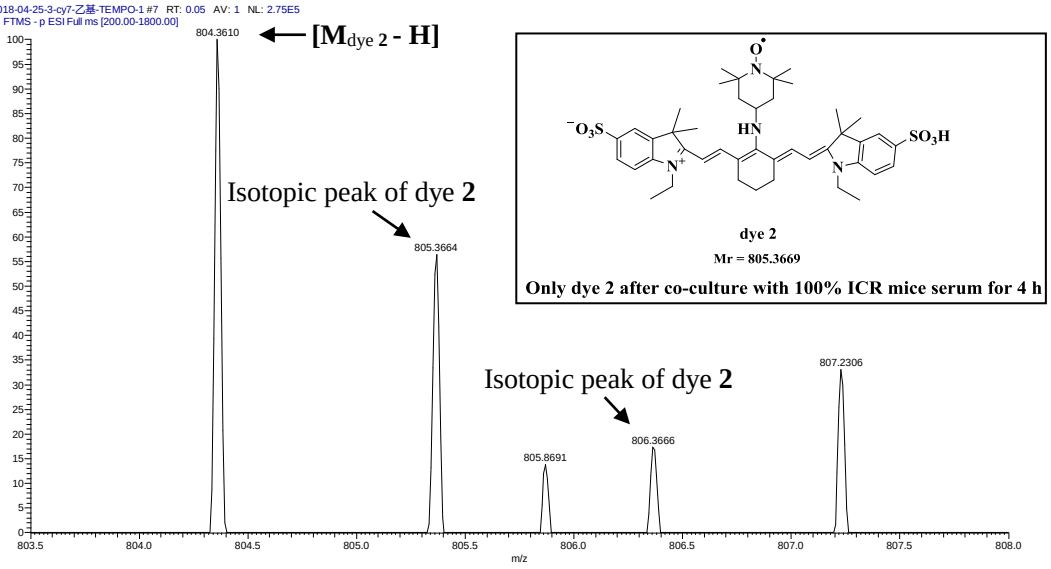
**a**


20180404-HGB-M805 #9 RT: 0.06 AV: 1 NL: 5.68E5
F: FTMS - p ESI Full ms [200.00-1300.00]
804.3596
[M dye 2 - H]
Isotopic peak of dye 2
805.3646
804.8603
806.3715
805.8658
803.8502
m/z
dye 2
Mr = 805.3669

**b**


2018-05-02-13-cy7-乙基-TEMPO+TEMPOH #21 RT: 0.16 AV: 1 NL: 4.71E5
T: FTMS - p ESI Full ms [200.00-1600.00]
805.3637
[M dye 2-OH - H] and isotopic peak of dye 2
dye 2-OH
Mr = 806.3747
dye 2
Mr = 805.3669
Mixed mole ratio of dye 2-OH : dye 2 is 1 : 9 (the content of dye 2-OH is 10%)
[M dye 2 - H]
804.3560
Isotopic peak of dye 2-OH and dye 2
806.3680
Isotopic peak of dye 2-OH
807.3642
804.8593
805.8654
m/z

**c**


2018-04-25-3-cy7-乙基-TEMPO-1 #7 RT: 0.05 AV: 1 NL: 2.75E5
T: FTMS - p ESI Full ms [200.00-1800.00]
804.3610
[M dye 2 - H]
Isotopic peak of dye 2
805.3664
dye 2
Mr = 805.3669
Only dye 2 after co-culture with 100% ICR mice serum for 4 h
807.2306
Isotopic peak of dye 2
806.3666
805.8691
m/z

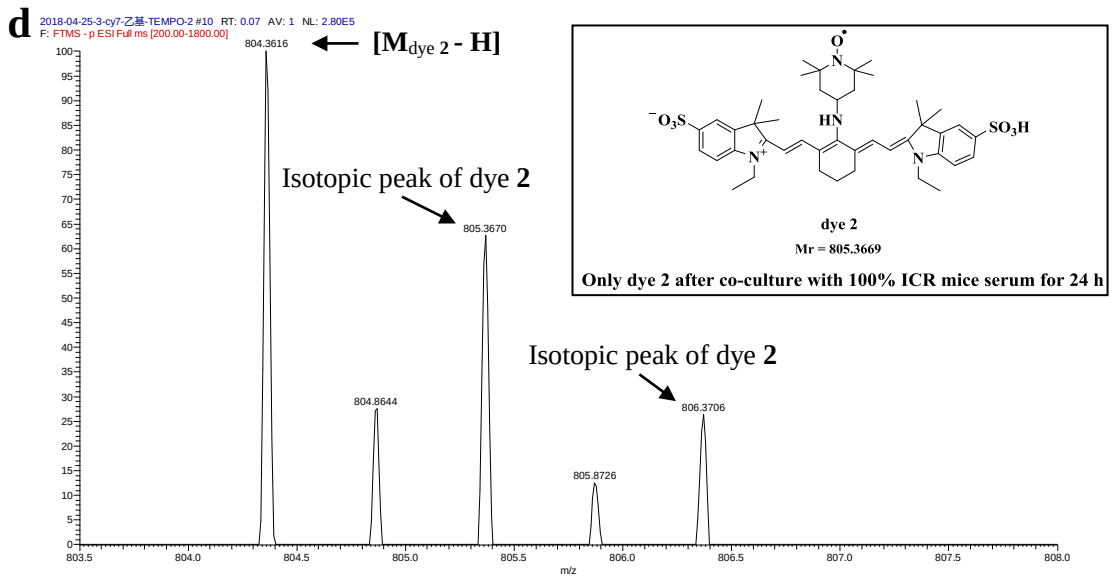



**Figure S7** (a) HRMS (ESI$^-$) of dye **2**; (b) HRMS (ESI$^-$) of dye **2-OH** mixed dye **2** (mole ratio of dye **2**-OH: dye **2** is 1:9)**;** HRMS (ESI$^-$) of dye **2** after co-culture with 100% ICR mice serum for 4 h (c) and 24 h (d).

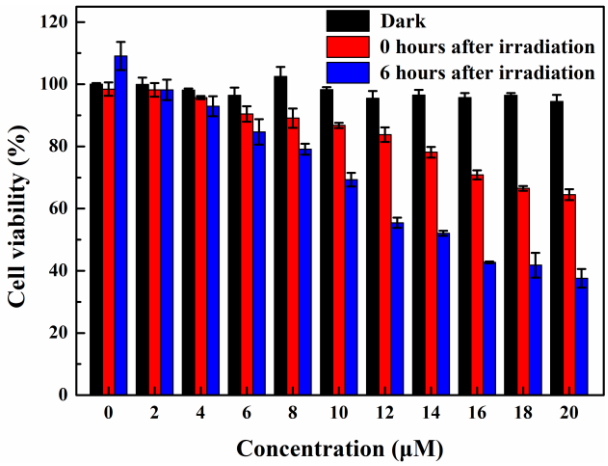



**Figure S8.** Phototoxicity evaluation of dye **2** on Hela cells for 20 minutes irradiation ($\lambda_{ex}$ = 660 nm, 50 mW/cm$^2$). MTT array was measured immediately (red bar), and measured after incubating for 6 h (blue bar). Data are presented as the mean value ± SD (n = 6).

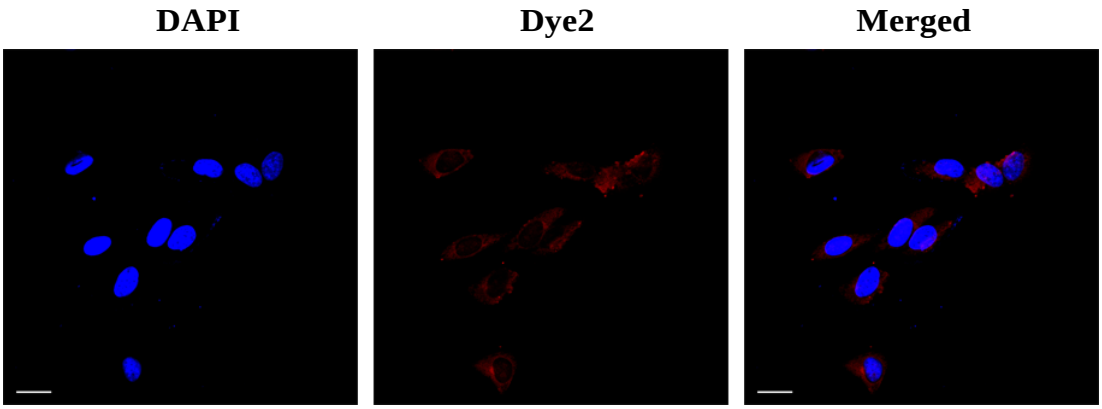



**Figure S9.** HeLa cells imaging stained with dye **2** (10 μM). Scale bar = 20 μm.

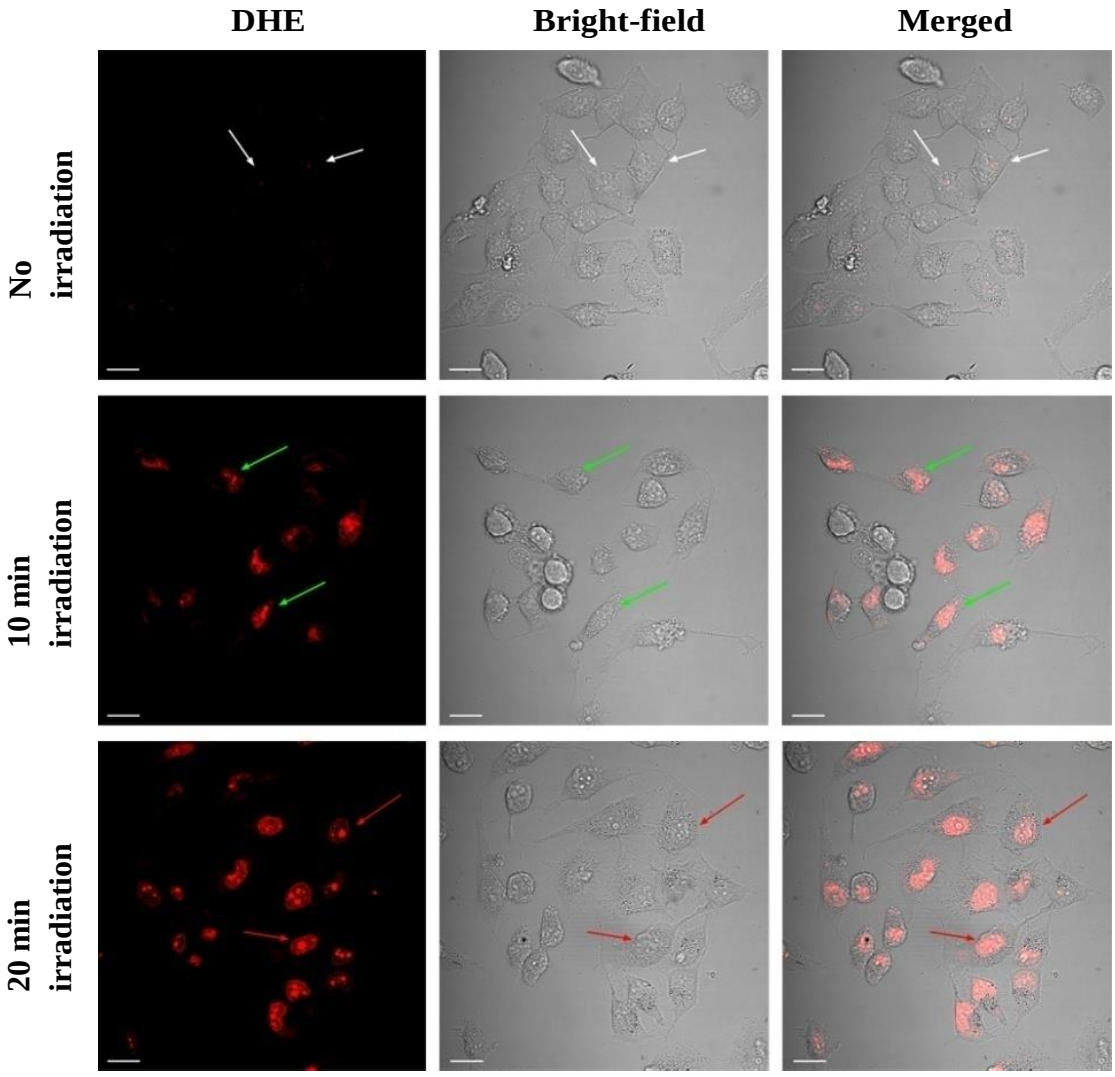



**Figure S10.** Confocal imaging of HeLa cells stained with DHE after different PDT times. LED array irradiation ($\lambda_{ex}$ = 660 nm, power density of 50 mW/cm$^2$). Scale bar = 20 μm.

# References:


1. F. Song, X. Peng, E. Lu, R. Zhang, X. Chen and B. Song, *Journal of Photochemistry and Photobiology A: Chemistry*, 2004, **168**, 53-57.
2. S. G. König and R. Krämer, *DYES PIGMENTS*, 2017, **145**, 80-94.
3. F. M. E. D. Juliano A. Bonacin, *J. Braz. Chem. Soc.*, 2009, **20**, 31-36.
4. M. Leite, M. Quinta-Costa, P. S. Leite and J. E. Guimar Es, 1999, **19**, 139-151.