Supporting Information

A photosensitizer with conformational restriction for enhanced photodynamic therapy

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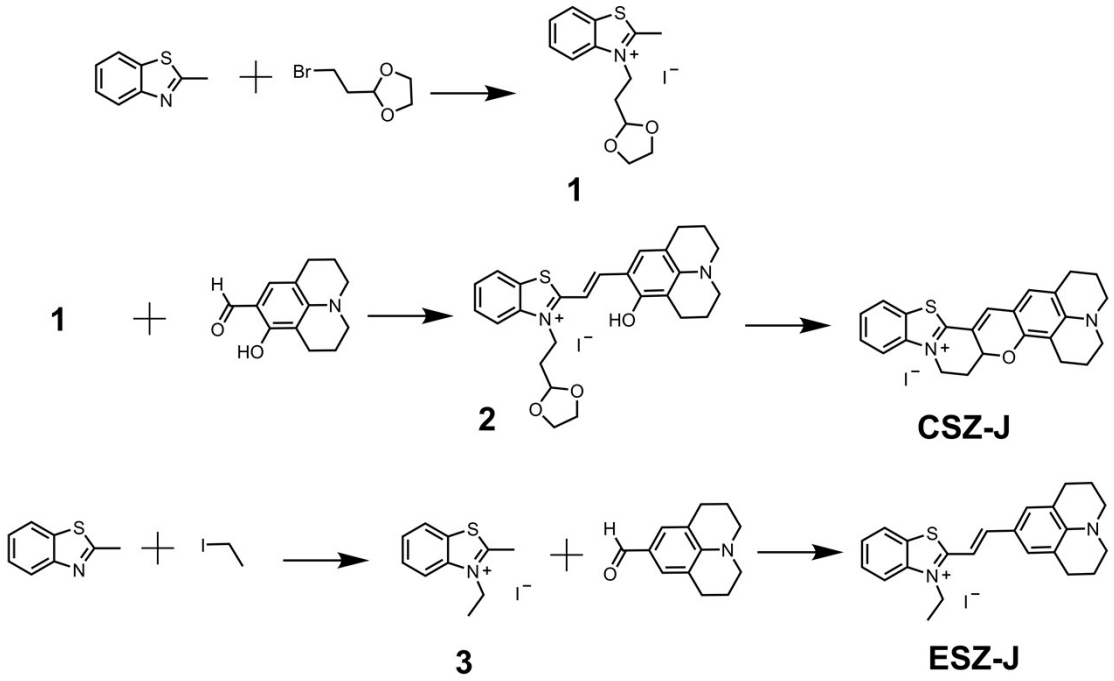
1. Synthesis of CSZ-J and ESZ-J

The synthesis pathway for **CSZ-J and ESZ-J** were shown in Scheme S1.



Scheme S1 Synthetic scheme for preparation of **CSZ-J** and **ESZ-J**

1.1: **Synthesis of compound 1.**

To a suspension of KI (2.988 g, 18.00 mmol) in distilled Acetonitrile (MeCN, 20 mL), under an argon atmosphere, was added bromoethyldioxolane (2.172 g, 12.00 mmol) and the yellow suspension was stirred at 60°C for 1 h. Benzothiazole (0.896 g, 6.00 mmol) was added and the reaction stirred at reflux for 48 h. The reaction was allowed to cool to rt and filtered to remove KBr and KI salt. The solvent was poured into 200 mL of ether to allow precipitation. The resulting solid precipitate was then filtered, washed with ether, and dried so that brown solid powder (0.652 g, 1.73 mmol, yield = 29 %) was obtained. The final product was used directly in the dye synthesis without further purification.

1.2 Synthesis of the compound 2.

A mixture of compound 1 (0.69 mmol, 260 mg) in anhydrous ethanol (10 mL) and 9-formyl-8-hydroxyjulolidin (0.69 mmol, 150 mg) was refluxed for 6 h. The reaction mixture was allowed to cool slowly to room temperature, and evaporated in a vacuum. The residue was purified using chromatography (silica gel), in which 60:1 DCM/methanol (v/v) was used as the eluting solvent. The intermediate **Compound 2** (0.246 g, 0.43 mmol, 62% yield) was obtained.

^{1}H NMR (400 MHz, CD_3OD) δ : 8.25 (t, J = 13.3 Hz, 1H), 7.95 (d, J = 7.9 Hz, 1H), 7.80 (d, J = 8.1 Hz, 1H), 7.67 (t, J = 8.0 Hz, 1H), 7.54 (t, J = 7.8 Hz, 1H), 7.32 (t, J = 7.4 Hz, 2H), 5.45 (d, J = 4.9 Hz, 1H), 5.02 (t, J = 3.9 Hz, 1H), 4.68 (t, J = 6.6 Hz, 2H), 3.92 (m, 3H), 3.40 (m, 5H), 2.75 (t, J = 5.4 Hz, 2H), 2.66 (t, J = 6.2 Hz, 2H), 2.33 (dd, J = 10.0, 6.9 Hz, 1H), 1.99 (dd, J = 13.6, 8.3 Hz, 4H), 1.14 (t, J = 6.9 Hz, 1H).

^{13}C NMR (126 MHz, CD_2Cl_2) δ 169.49, 156.07, 149.93, 144.68, 141.16, 128.42, 126.31, 125.97, 123.51, 116.54, 114.69, 111.59, 106.32, 101.41, 101.11, 64.40, 49.95, 49.22, 42.51, 31.12,

26.76, 21.03, 20.67, 20.03, 15.05.

HRMS (ESI): *m/z* $[M]^+$ = calcd. for $C_{26}H_{29}N_2O_3S^+$ 449.1893; found 449.1894.

1.3 Synthesis of CSZ-J.

To a solution of **compound 2** (132 mg, 0.23 mmol) in $CHCl_3$ (6 mL) was added 50 % aq. H_2SO_4 (130 μL) and the mixture was stirred vigorously for 120 min. The reaction mixture was diluted with $CHCl_3$ (20 mL) and washed with H_2O (3×60 mL). The aqueous phases were combined, washed with $CHCl_3$ (2×60 mL) and the combined organic phase was dried (Na_2SO_4) then the solvent was removed in vacuum. The residue was purified using chromatography (silica gel), in which 70:1 DCM/methanol (v/v) was used as the eluting solvent. The product **CSZ-J** (105 mg, 0.20 mmol, 89% yield) was obtained as a dark pink solid.

^{1}H NMR (400 MHz, CD_3OD) *δ*: 7.98 (d, *J* = 8.0 Hz, 1H), 7.77 (d, *J* = 8.3 Hz, 1H), 7.70 (m, 2H), 7.58 (t, *J* = 7.6 Hz, 1H), 6.86 (s, 1H), 5.31 (dd, *J* = 11.1, 5.3 Hz, 1H), 4.82 (d, *J* = 13.2 Hz, 1H), 4.27 (t, *J* = 12.9 Hz, 1H), 3.41 (m, 4H), 2.92 (d, *J* = 13.2 Hz, 1H), 2.77 (t, *J* = 6.0 Hz, 1H), 2.71 (t, *J* = 5.9 Hz, 2H), 2.61 (m, 2H), 1.96 (dd, *J* = 11.9, 5.9 Hz, 4H).

^{13}C NMR (126 MHz, CD_2Cl_2) *δ*: 141.01, 139.40, 130.74, 130.42, 129.94, 129.45, 129.22, 127.53, 126.98, 125.86, 124.59, 123.72, 117.16, 114.40, 101.54, 71.04, 65.67, 51.33, 50.84, 30.11, 27.43, 27.07, 21.63, 20.56.

ESI-HRMS: *m/z* $[M]^+$ = calcd. for $C_{24}H_{23}N_2OS^+$ 387.1526; found 387.1529.

1.4 Synthesis of the intermediate Compound 3.

To a suspension of benzothiazole (1.490 g, 10.00 mmol) in distilled MeCN (40 mL), under an argon atmosphere, was added iodoethane (7.798 g, 50.00 mmol) and the yellow suspension was stirred at reflux for 24 h. The reaction was allowed to cool to RT. The solvent was poured into 200 ml of ether to allow precipitation. The resulting solid precipitate was then filtered, washed with ether, and dried so that brown solid powder (0.802 g, 2.60 mmol, yield = 26 %) was obtained. The final product was used directly in the dye synthesis without further purification.

1.5 Synthesis of the ESZ-J.

Compound 3 (166 mg, 0.55 mmol) and 9-julolidinecarboxaldehyde (0.100 g, 0.50 mmol) were dissolved in ethanol (6.0 mL) under nitrogen. The mixture was stirred at reflux for 2 h. After accomplished, the reaction solution was then cooled down and evaporated in a vacuum. The residue was purified using chromatography (silica gel), in which 65:1 DCM/methanol (v/v) was used as the eluting solvent. The product **ESZ-J** (137 mg, 0.28 mmol, 56% yield) was obtained as a dark pink solid.

^{1}H NMR (400 MHz, CD_3OD) *δ*: 8.04 (d, *J* = 7.5 Hz, 1H), 7.94 (d, *J* = 8.4 Hz, 1H), 7.89 (d, *J* = 14.8 Hz, 1H), 7.73 (m, 1H), 7.61 (m, 1H), 7.41 (s, 2H), 7.31 (d, *J* = 14.8 Hz, 1H), 4.73 (q, *J* = 7.2 Hz, 2H), 3.41 (dd, *J* = 17.0, 11.2 Hz, 4H), 2.80 (m, 4H), 1.98 (dt, *J* = 12.0, 6.2 Hz, 4H), 1.53 (t, *J* = 7.3 Hz, 3H).

^{13}C NMR (126 MHz, CD_2Cl_2) *δ*: 169.70, 151.91, 149.82, 141.36, 131.74, 129.40, 127.47, 127.08, 123.73, 122.69, 121.28, 114.81, 102.70, 51.05, 44.69, 27.78, 21.43, 14.11.

ESI-HRMS: *m/z* calcd for: $C_{23}H_{25}N_2S^+$ $[M]^+$, 361.1733; found, 361.1728.

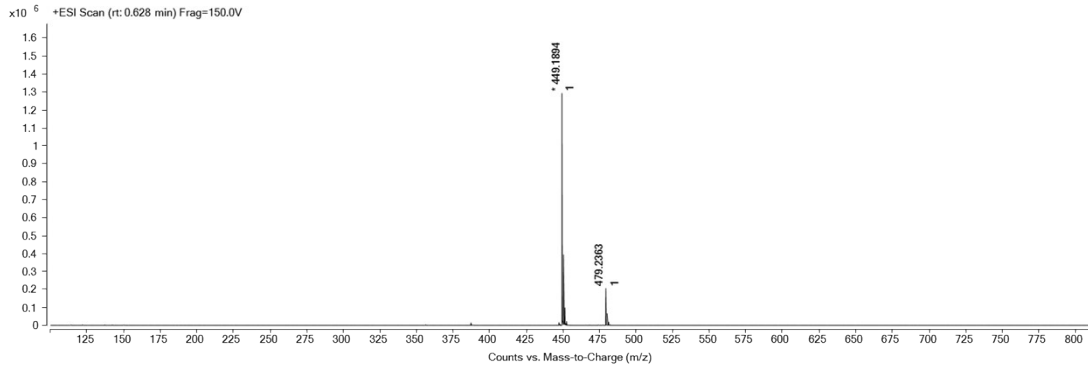


Fig. S1 HRMS spectrum of **Compound 2**

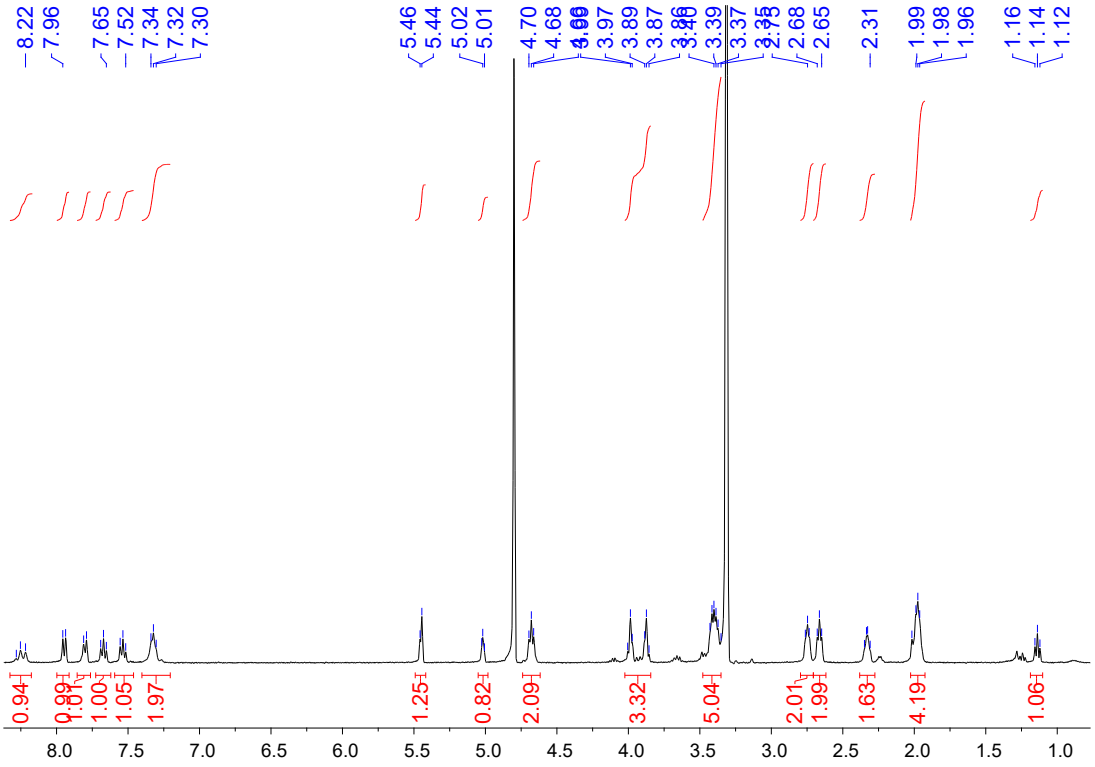


Fig. S2 ^{1}H NMR spectrum of **Compound 2** in CD_3OD

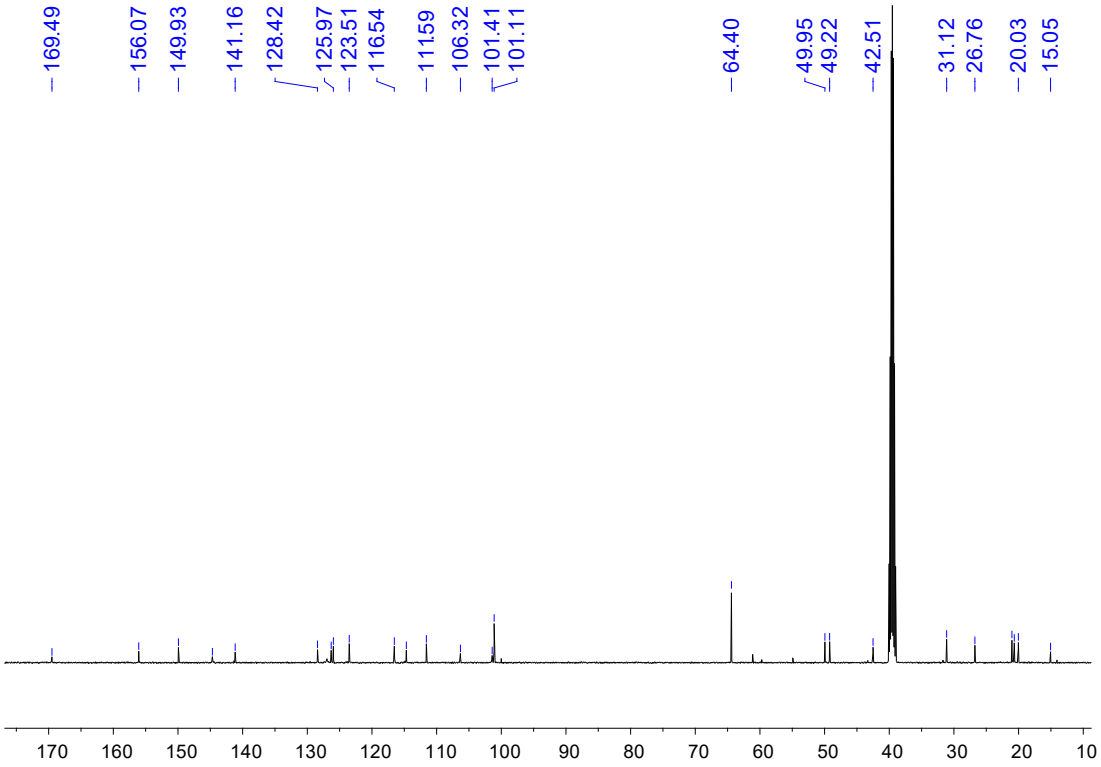


Fig. S3 ^{13}C NMR spectrum of **Compound 2** in CD_2Cl_2

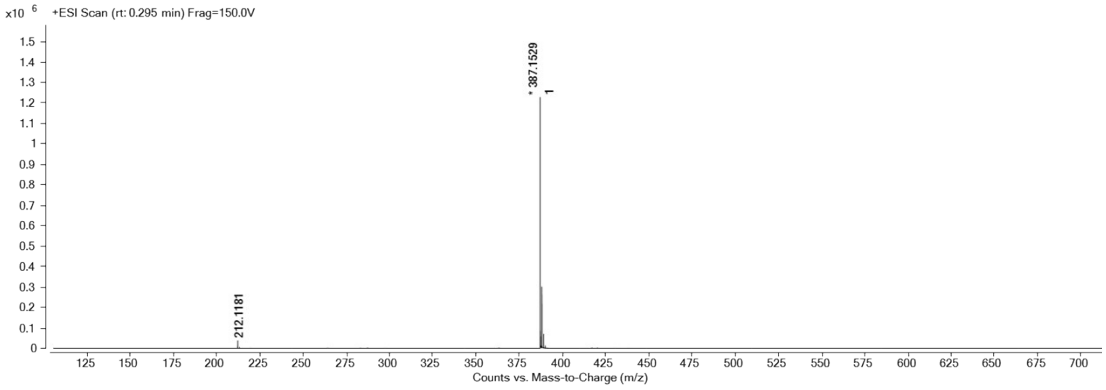


Fig. S4 HRMS spectrum of **CSZ-J**

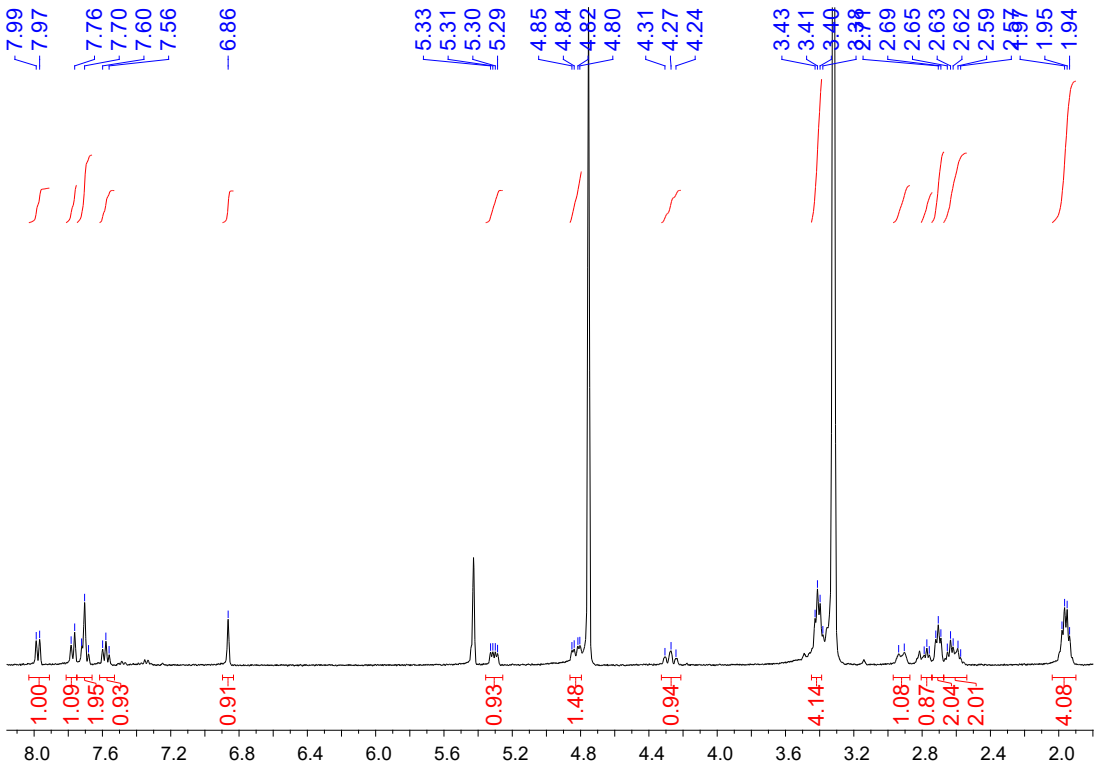


Fig. S5 ^{1}H NMR spectrum of **CSZ-J** in CD_3OD

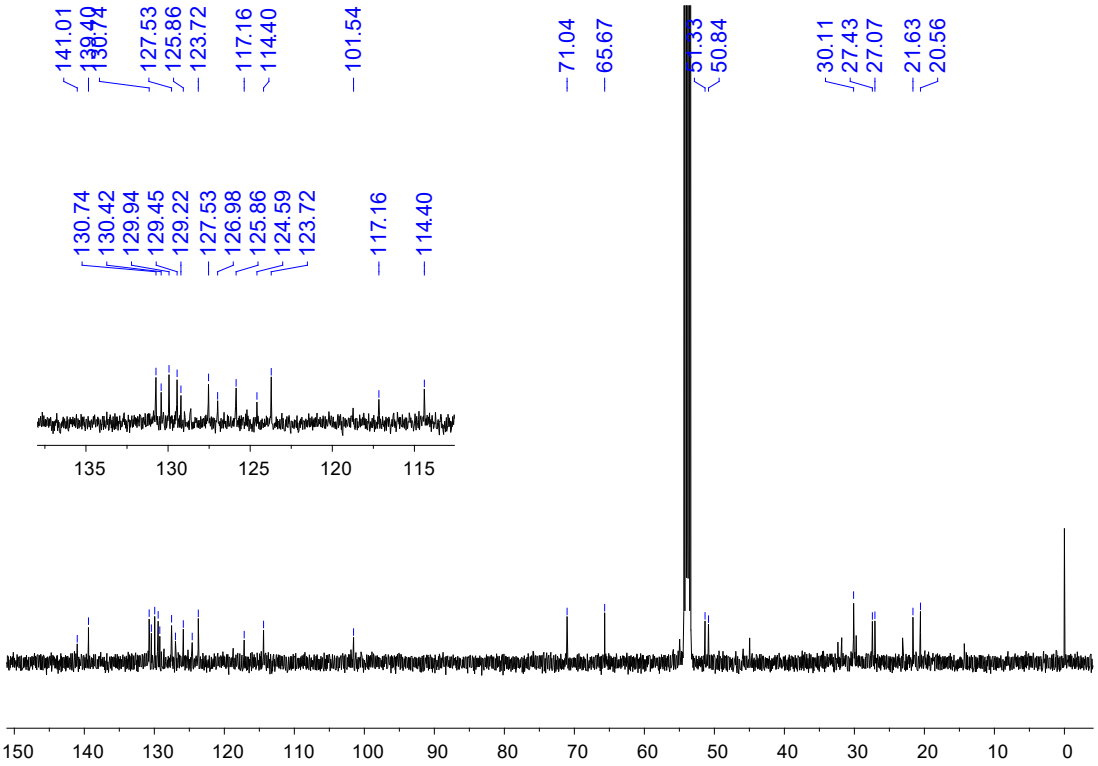


Fig. S6 ^{13}C NMR spectrum of **CSZ-J** in CD_2Cl_2

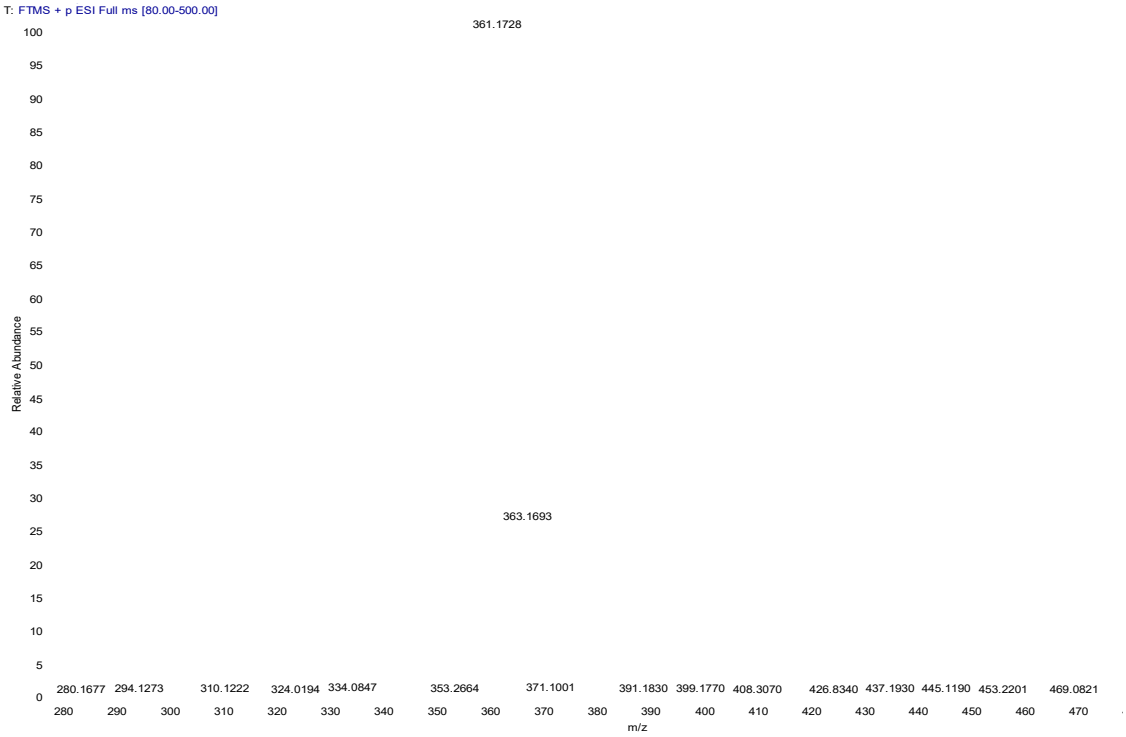


Fig. S7 HRMS spectrum of **ESZ-J**

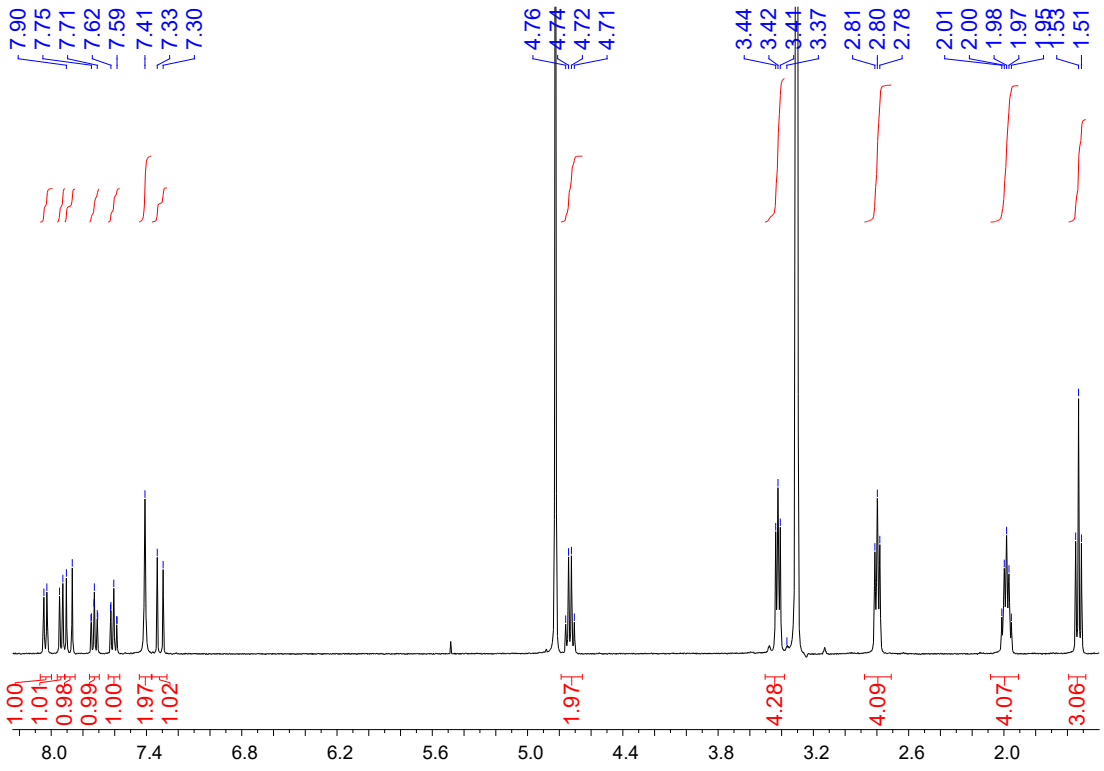


Fig. S8 ^{1}H NMR spectrum of **ESZ-J** in CD_3OD

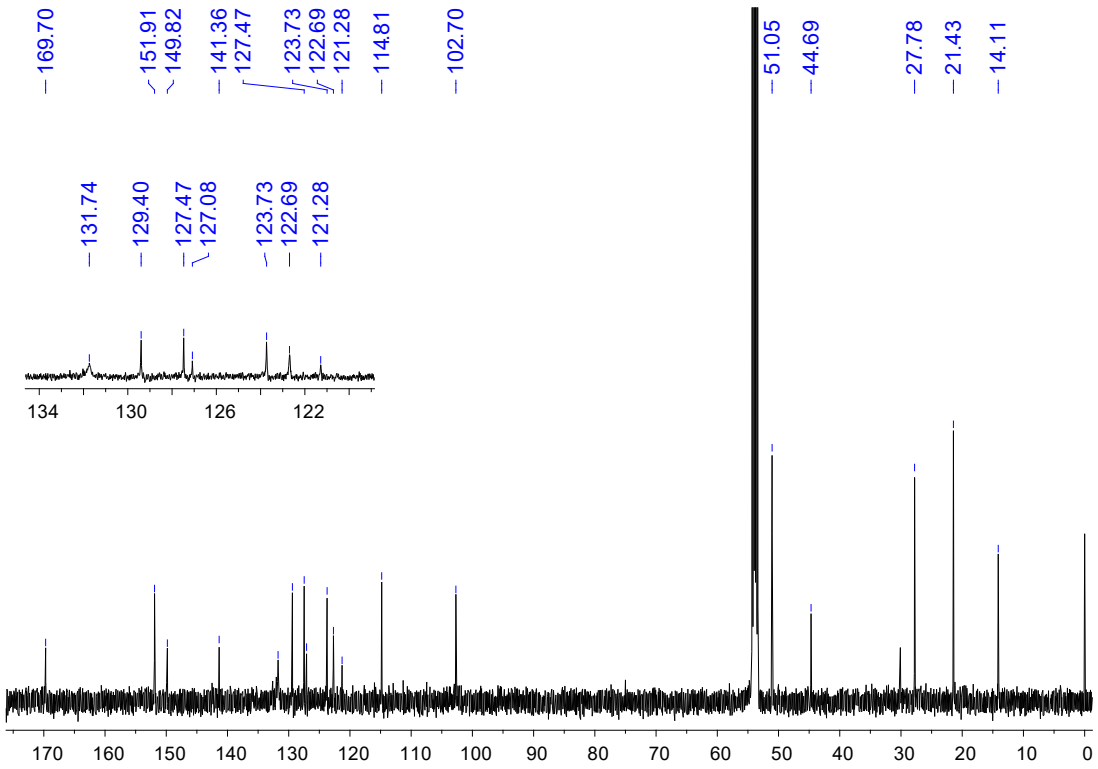


Fig. S9 ^{13}C NMR spectrum of **ESZ-J** in CD_2Cl_2

Materials and Instrumentation

The general chemicals used in the report were purchased from Energy Chemical Co., and J&K Scientific Ltd., and all of the solvents were of analytic grade. 1,3-diphenylisobenzofuran (DPBF) were obtained from Energy Chemical Co.. Annexin V-FITC apoptosis detection kit, Reactive Oxygen Species Assay Kit (DCFH-DA) and mitochondrial membrane potential assay kit (JC-1) was purchased from KeyGEN BioTECH. Hoechst 33324 and MitoTracker Green FM were purchased from Thermo (invitrogen).

^{1}H NMR and ^{13}C NMR spectra were detected by Vaian DLG400 Varian DLG400 spectrometer and Bruker Avance III 500 spectrometer. Mass spectrometric (MS) data were carried out using LTQ Orbit rap XL instruments and TOF LC/MS instruments. Absorption spectra were measured on a PerkinElmer Lambda 35 UV/VIS spectrophotometer (PerkinElmer). Fluorescence spectra were obtained with a VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812:M018). Absolute photoluminescence quantum yields were obtained from Absolute PL Quantum Yields Spectrometer C11347 (Quantaurus-QY). EPR signals were recorded by a Bruker EPR instrument (E500). The triplet excited state lifetime of dyes were obtained from laser flash photolysis spectrometer (LP980, Edinburgh, U.K.). Confocal laser scanning microscope (CLSM) images were performed on Olympus FV3000 confocal laser scanning microscope.

Determination of octanol-water partition coefficient (log P_{oct})

The 1-octanol-water partition coefficient (log P_{oct}) was calculated according to a reported procedure.S15 Firstly, Solution A and Solution B were prepared by pre-mixing 1-octanol and PBS buffer (10 mM, pH 7.4) in a ratio of 1:4 and 4:1, respectively. Then, the solutions were stirred at rt for 24 h. Thereafter, small aliquots (5 μL) of a 5 mM probe solution in DMSO was added to Solution A and Solution B (each 5 ml), respectively. The above solutions were then mixed and stirred for 30 min. The concentration of the probe in each layer was measured by UV-Vis

spectroscopy using the molar coefficients of the probes. The log P_{oct} value was calculated using following equation:

$\log P_{oct} = \log [PS]_{oct} - \log [probe]_{PBS}$

Detection Production of ROS in Solution.

The generation of ROS was detected indirectly using 3-diphenylisobenzofuran (DPBF) as a chemical probe, whose absorbance would be diminished in the presence of ROS. Thus, a solution of DPBF (45 μM) and hemicyanine dyes (optical density was adjusted to around 0.2–0.3 at the irradiation wavelengths) in 3 mL Dichloromethane (DCM) was irradiated monochromatic light (590 nm) intervals. The power density of the laser point is 10 mW cm^{-2}. The solutions were irradiated for 60 second and their absorbance spectra were recorded at 10 s intervals using a spectrometer.

Detection of OH• and $^1O_2$ in Solution.

Hydroxyl radical (OH•) Detection.

The hydroxyl radical generation ability was evaluated by specific probe, 3'-(4-hydroxyphenyl) fluorescein (HPF). Briefly, PS (5 μM) and HPF (5 μM) were prepared as in aqueous solution, then the mixture was exposed to different time irradiation (0, 1, 2, 3, 4, 5min), and corresponding fluorescence spectra were captured (Ex: 480 nm). Light power density: 590 nm laser (10 mW cm^{-2}). The fluorescence of each sample was read, ex/em 488/500-550 nm.

Singlet oxygen Detection.

The singlet oxygen generated by **CSZ-J** and **ESZ-J** was measured using singlet oxygen capture agent, 9, 10-anthracenedipropanoic acid (ABDA). Briefly, the absorbance of ABDA at 380 nm was adjusted to about 1.0 in aqueous solution. Then, 5 μM **CSZ-J** or **ESZ-J** was added to this cuvette. Then, the cuvette was irradiated with 590 nm monochromatic light for various time, and absorption spectra were measured immediately. To confirm the ability of Sodium azide (NaN_3) to scavenge ROS from **CSZ-J**. DPBF was also used to monitor the ROS generation of **CSZ-J** in the presence of NaN_3 at concentrations of 50 μM.

The $^1O_2$ quantum yield was measured using methylene blue (MB) as the reference photosensitizer and calculated using the following eq :[1]

$$\Phi_{ps} = \Phi_{MB}\frac{k_{ps} \times A_{MB}}{k_{MB} \times A_{ps}}$$

where k is the slope of the absorbance versus irradiation time. A represents the light absorbed by PS or MB at 590 nm. Φ_{MB} is the $^1O_2$ quantum yield of reference MB, which is 0.52 in water.

The Electron Paramagnetic Resonance (EPR) technique was employed to monitor ROS signals

O_2•– Detection by EPR:

The generation of $O_2^{\bullet-}$ was qualitatively characterized using EPR spectrometer. In detail, **CSZ-J** or **ESZ-J** (10 μM) and DMPO (10 mg mL^{-1}) was dissolved in aqueous solution. The mixed solution was exposed to Xe lamp (20 mW cm^{-2}) for 3 min. The product DMPO- $O_2^{\bullet-}$ was immediately tracked with EPR spectrometer. DMPO with laser irradiation was tested as controls.

$^1O_2$ Detection by EPR:

To detect $^1O_2$, EPR spectroscopy was employed. In detail, **CSZ-J** or **ESZ-J** (10 μM) and TEMP (2 mg mL^{-1}) was dissolved in aqueous solution. The mixed solution was exposed to Xe lamp (20 mW cm^{-2}) for 3 min. Then TEMP-$^1O_2$ adduct was immediately tracked with EPR spectrometer. **CSZ-J** or **ESZ-J** without lamp irradiation were tested as controls.

Fluorescence lifetime measurements.

Time resolved fluorescence measurements were performed on freshly prepared samples using the time-correlated single photon counting (TCSPC) method by Steady-state/transient fluorescence spectrometer FLS1000 (Edinburgh. UK) at room temperature. The picosecond pulsed diode laser (EPL-375) at 375 nm was selected as excitation source. Emission was monitored at the wavelength of maximum fluorescence. Data analysis was performed with FluoFit software using an exponential decay model.

Cell culturing and cellular uptake assays

MCF-7 cells were incubated on the cell culture plate in DMEM medium (Gibco) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin at 37°C in a humidified, 5% CO_2 atmosphere. Particularly, for real-time cellular uptake assays, MCF-7 cells were stained with 1 μM **CSZ-J or ESZ-J**, immediately after that, the red fluorescence at various time points (5, 10, 20, 30, 45, 90 min) was captured using confocal laser scanning microscopy (CLSM). The excitation wavelength was 561nm, and collection wavelength was from 600 nm to 700 nm.

Intracellular PDT analysis by MTT assay

We further investigated intracellular PDT effect of PS on cancer cells. MCF-7 cells and 4T1 cell were cultured in 96-well plates with 5×10^4 cells/well, and incubated in 100 μL growth medium for 24 h at 37°C under a humidified 5% CO_2 atmosphere. Thereafter, the cells were treated with various concentrations (0–2.5 μM) of **CSZ-J** or **ESZ-J** and further incubated for 1 h prior to irradiation with 590 nm light at 10 mW cm^{-2} for 0 and 10 min. After an overnight incubation, the standard MTT assay was carried out to determine cell viability. Briefly, the cells were labelled with 0.5 mg mL^{-1} of MTT solution in full culture medium for 4 h. Then, the supernatant was removed and replaced with 200 μL DMSO. The absorbance of formazan at 490 nm was measured using a microplate reader. Optical density was determined on a microplate reader. Cell viability was expressed as percentage of control and calculated using equation, as following:
Cell viability (%) = $(OD_{PS} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$

Intracellular PDT analysis by Calcein-AM
4T1 cells were were planted onto 35 mm confocal dishes and incubated for 24 h. After incubated with various concentrations (0 as control, 0.32, 0.64, 1.25 μM) **CSZ-J** or **ESZ-J** for 1 h then irradiation with 590 nm light at 10 mW cm^{-2} for 10 min. After incubation 8 h, Calcein AM was performed. The excitation wavelength of Calcein AM were 488 nm, and emission signals were collected from 505 to 545 nm with green fluorescence.

Subcellular Colocalization Assay

MCF-7 cells were planted onto 35 mm confocal dishes and incubated for 24 h. After incubated with 1 μM **CSZ-J** or **ESZ-J** for 1 h at 37ºC under 5% CO_2, the cells were further stained by MitoTracker Green FM (200 nM), or Hoechst 33342 (2 μg/mL). Cells were then observed with laser confocal microscopy. **CSZ-J** or **ESZ-J** was excited with 561 nm wavelength, while the excitation wavelength for MitoTracker Green FM were 488 nm, and Hoechst 33342 was 405 nm. The emission wavelength was collected from 600 to 700 nm for **CSZ-J** or **ESZ-J**, 500 to 540 nm for Mito Tracker Green, and 440 to 480 nm for Hoechst 33342.

In *Vitro* ROS Generation Tests.

Intracellular reactive oxygen species production was studied by measuring the fluorescence intensity of DCF. The MCF-7 cells were seeded in 35 mm Petri dish with cover slip at a density of 1×10^4 cells and incubated for 24 h. Then 4T1 cells were incubated with 1 μM **CSZ-J** or **ESZ-J** for 60 min for cell uptake followed by incubation with 10 μM H_2DCF-DA for 30 min. Thereafter, the cells were washed with PBS for three times followed by irradiation with 590 nm light (10 mW cm^{-2}) for 0 min (dark) or 10 min (light). Then,

confocal fluorescence imaging was used to observe the intracellular ROS level. The excitation wavelength for DCF was 488 nm and emission wavelength was collected from 500 nm to 530 nm.

Confocal Imaging of Photon-induced Cell Death

Annexin V-FITC Apoptosis Detection Kit (Beyotime, China) used to analysis the **CSZ-J** mediated photon-induced cell death. Briefly, MCF-7 cells were seeded onto 35 mm confocal dishes for 24 h, then cells were treated with following different treatments: group 1, incubated with 1 μM **CSZ-J** for 1 h**;** group 2, incubated with 1 μM **CSZ-J** at 37°C for 1 h and irradiated with 590 nm LED light for 10 min. The power density of light source was 10 mW cm^{-2}. After different treatments, cells were stained with Annexin V-FITC Apoptosis Detection Kit according to the manual. The cell death process was visualized by fluorescence microscopy and flow cytometry. (AnnexinV-FITC, λex: 488 nm, λem: 500–550 nm).

JC-1 Mitochondrial membrane potential assay

To identify the dark toxicity of cyanine dyes for MCF-7 cells, the Mito membrane potential was detected using JC-1 kits. MCF-7 cells were seeded and cultured in 35 mm confocal dishes for 24 h. 1 μM **CSZ-J** was then added into the medium and incubated with cells at 37°C under 5% CO_2 for 4 h. Subsequently, the cells were stained by JC-1 kit according to the instruction manual. Healthy cell with high mitochondrial membrane potential shows red color (JC-1 is J-aggregates) and apoptotic cell with a low mitochondrial membrane potential shows green color (JC-1 is J-monomer). Because the emission of **CSZ-J** overlaps with that of J-aggregates, only j-monomer emission is studied in this experiment. To identify the apoptosis process for MCF-7 cells in PDT. Another set of experiments was carried out. Briefly, MCF-7 cells were seeded in 35-mm CLSM-special cell dishes, and then cells were incubated with different following treatments at 37°C for 1 h: group 1, incubated with 1 μM **CSZ-J**; group 2, incubated with 1 μM **CSZ-J** and irradiated with 590 nm LED light for 10 min. The power density of light source was 20 mW cm^{-2}. Lastly, the same treatments were carried out. Then the fluorescence of J-M (Ex:488 nm, Em: 505–550 nm) was imaged on CLSM.

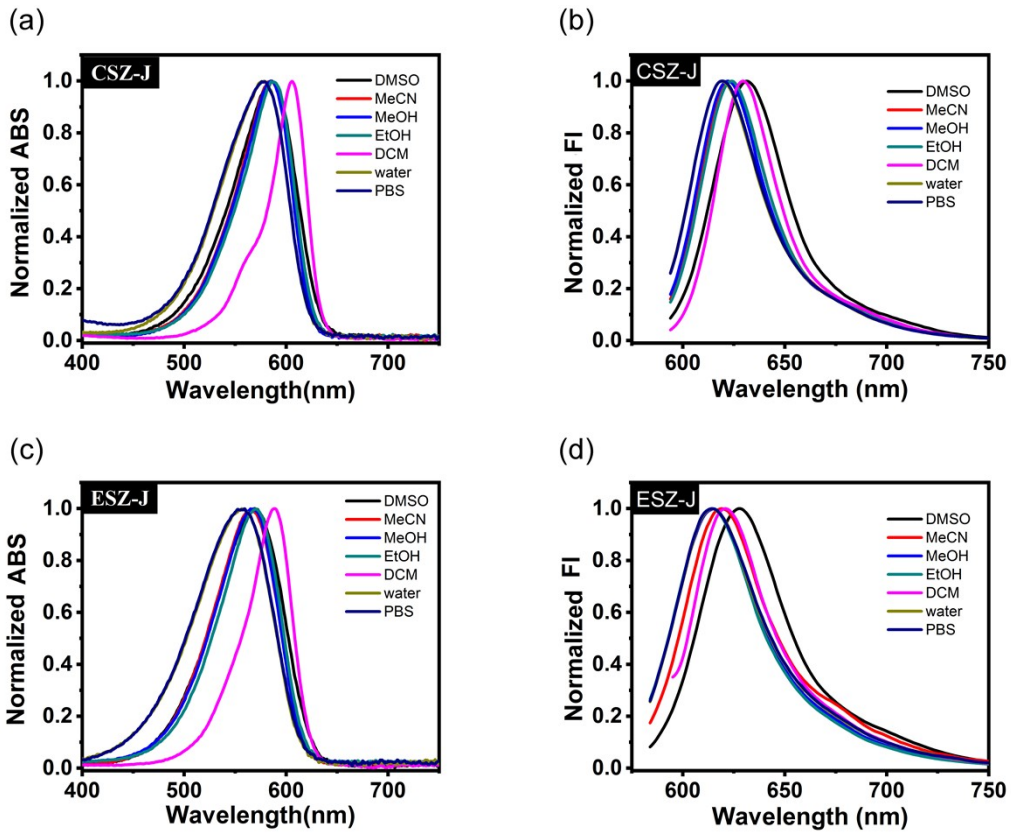


Fig. S10 Normalized UV-Vis spectral absorption spectra and emission spectra for **CSZ-J** and **ESZ-J** in different solvents.

Table S1 Spectral Data of the Dye **CSZ-J** and **ESZ-J** in Different Solvents

Dyes	Solvent	λabs) (nm)[a]	λem) (nm)[b]	ε)[c]	Φ_F)[d]	BT = ε×Φ_f [e]
	DMSO	585	631	8.5	0.88	7.4885
	MeCN	586	624	9.9	0.87	8.5734
	MeOH	585	622	10.4	0.803	8.3512
CSZ-J	EtOH	585	624	10.3	0.835	8.6005
	Water	578	619	7.9	0.634	4.9769
	PBS	577	620	7.6	0.62	4.681
	DCM	606	630	9.8	0.871	12.7166
	DMSO	569	627	8.1	0.017	0.1377
	MeCN	566	622	9.3	0.002	0.0186
	MeOH	565	618	10	0.004	0.0398
ESZ-J	EtOH	571	621	10.3	0.007	0.07175
	Water	557	615	7.3	0.004	0.0292
	PBS	559	616	6.9	0.005	0.03425
	DCM	588	624	9.6	0.005	0.0755

The all data of the solvents were measured at 25°C, the concentration of dyes was 2 μM. *a*: The max absorption peaks of dyes (nm). *b*: The max fluorescence peaks of dyes (nm). *c*: Molar Extinction Coefficient(×10^4 $M^{-1}cm^{-1}$). *d*: The fluorescence quantum yield. *e*: Brightness is defined

as $\boldsymbol{\varepsilon \times \Phi_f}$. ($\times 10^4$).

The fluorescence quantum yield (Φ_f) and the fluorescence lifetime (τ_f) were used to calculate the average rate constant of the fluorescent decay ($\overline{k_f}$) and the nonradiative decay ($\overline{k_{nr}}$), using eqs 1and 2.[2, 3]

$$\overline{k_f} = \Phi_f / \tau_f \qquad (1)$$

$$\overline{k_{nr}} = (1 - \Phi_f) / \tau_f \qquad (2)$$

Table S2 The rate constant of the fluorescent decay and the rate constant of nonradiative decay for two dyes

Dyes	Solvent	$\overline{k_f}$ ($\times 10^8$ s^{-1})	$\overline{k_{nr}}$ ($\times 10^8$ s^{-1})
CSZ-J	DCM	2.17	0.32
	MeOH	2.12	0.52
ESZ-J	DCM	0.03	6.14
	MeOH	0.06	13.83

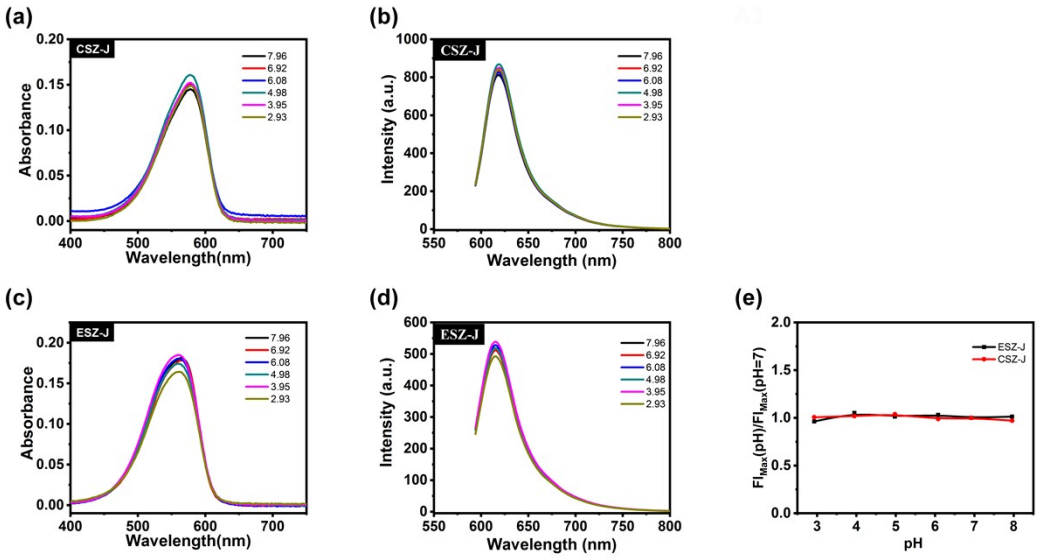


Fig. S11 UV-Vis spectral absorption spectra and emission spectra for **CSZ-J** (a, b) and **ESZ-J** (c, d) in PBS at different pH. (e) Changes in fluorescence ratio of **CSZ-J** and **ESZ-J** (the intensity fluorescence at different pH / the intensity fluorescence at pH=7) in PBS.

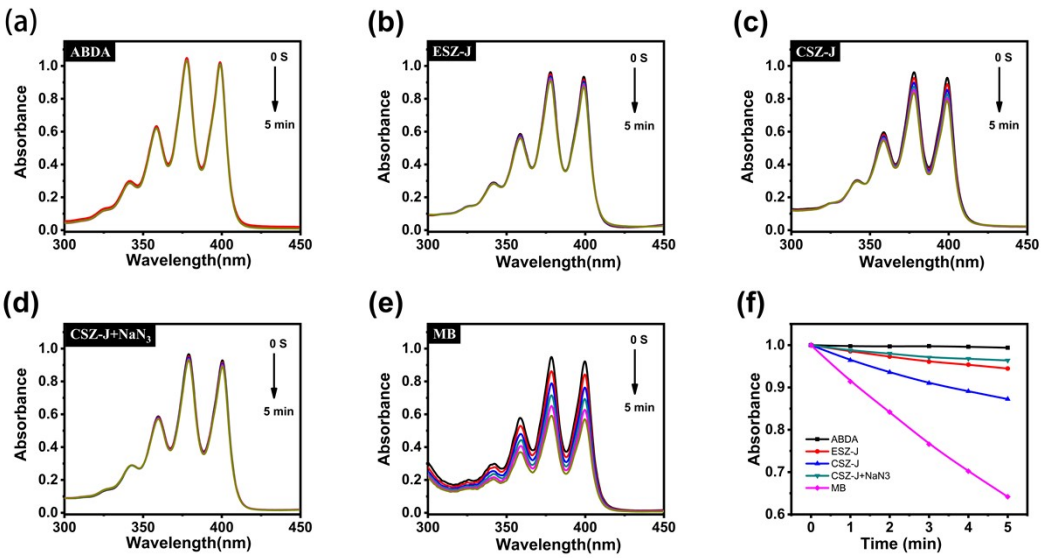


Fig. S12 Photodegradation curves of ABDA with different conditions under 590 nm light irradiation (a-e) and (f) The decomposition rates of ABDA under different conditions at different irradiation times(0-5 min).

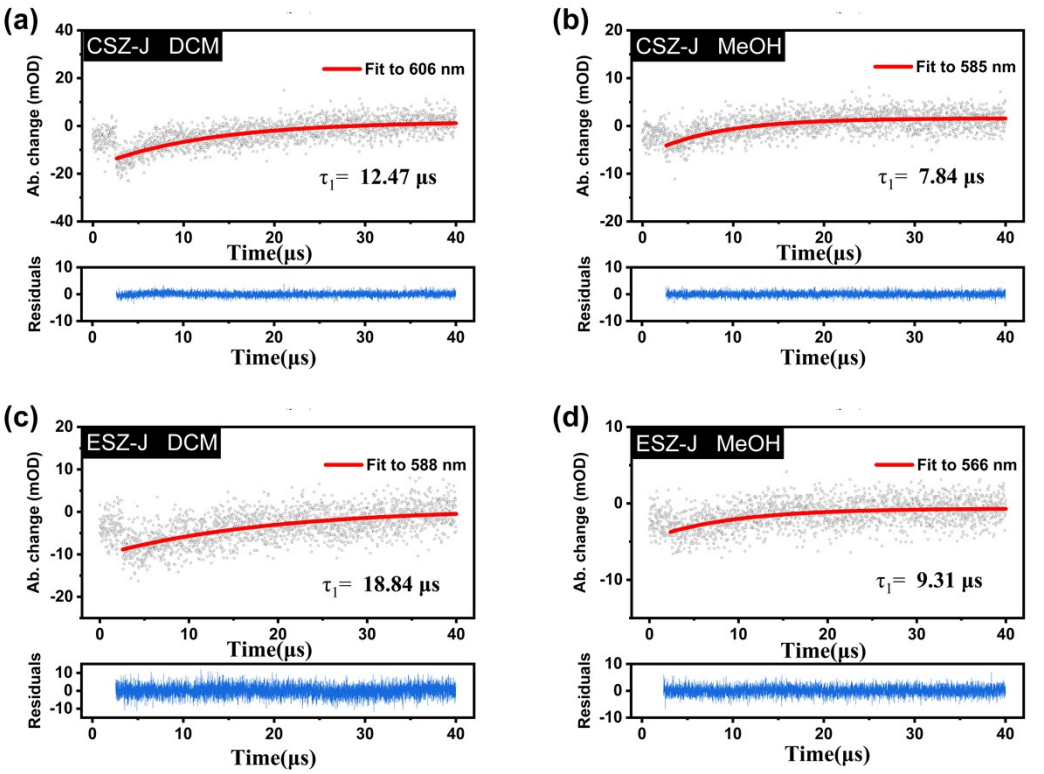


Fig. S13 Nanosecond time-resolved transient dynamics decay traces of **CSZ-J** (a, b) and **ESZ-J** (c, d) at corresponding wavelength in oxygen-free DCM or MeOH.

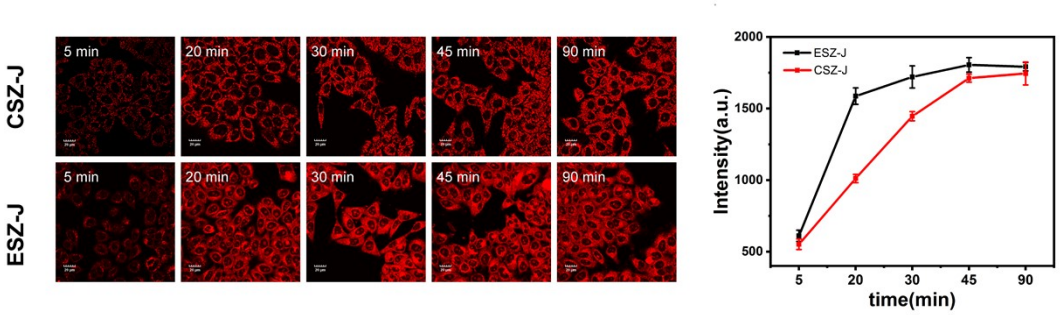


Fig. S14 The cellular uptake of **CSZ-J** and **ESZ-J** in MCF-7 cells (left). Intracellular average fluorescence intensity at different time points after incubation with **CSZ-J** and **ESZ-J** (right).

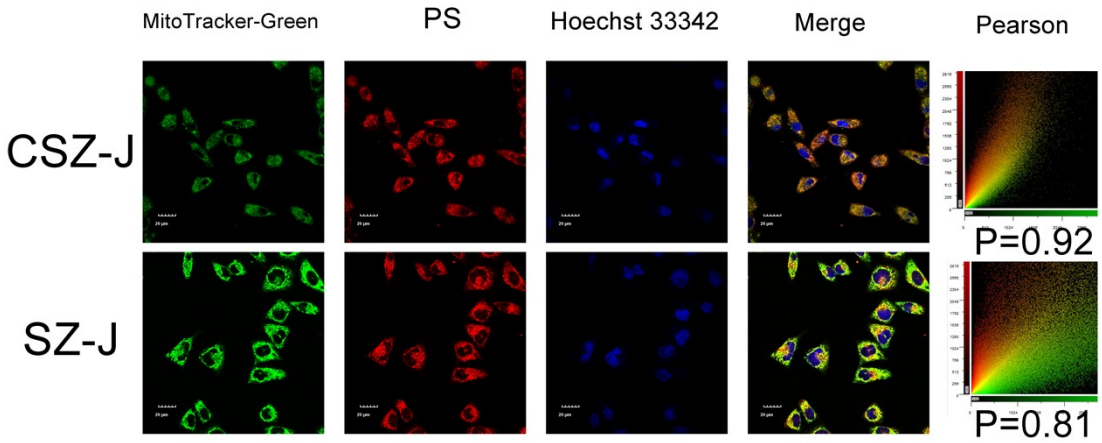


Fig. S15 Co-localization of **CSZ-J** and **ESZ-J** (500 nM) with Mito-Tracker Green FM (150 nM) and Hoechst 33342 (2 μg/mL) in MCF-7 cells.

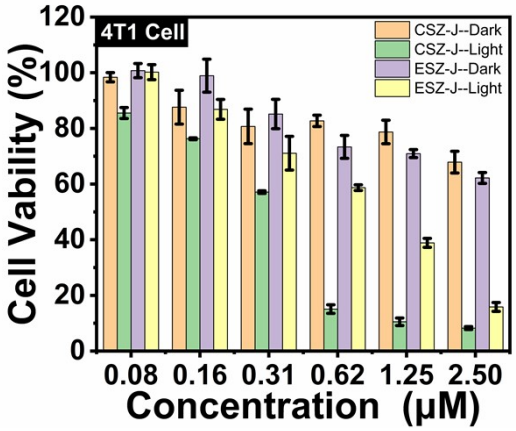


Fig. S16 Cytotoxic effects of CSZ-J and ESZ-J on 4T1 cells in the absence and presence light.

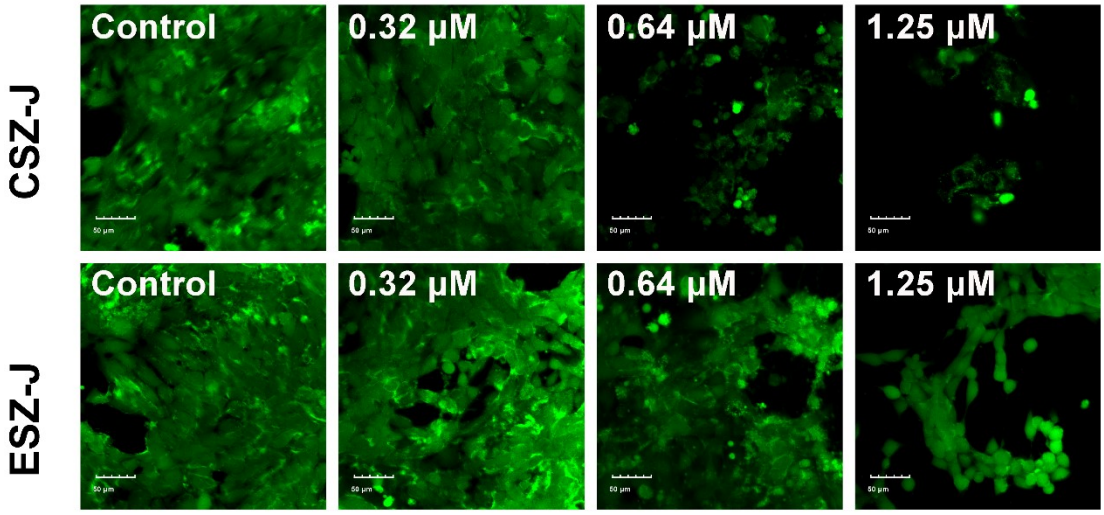


Fig. S17 Fluorescence microscopic images of Calcein AM stained 4T1 Cells treatment with two PS after PDT.

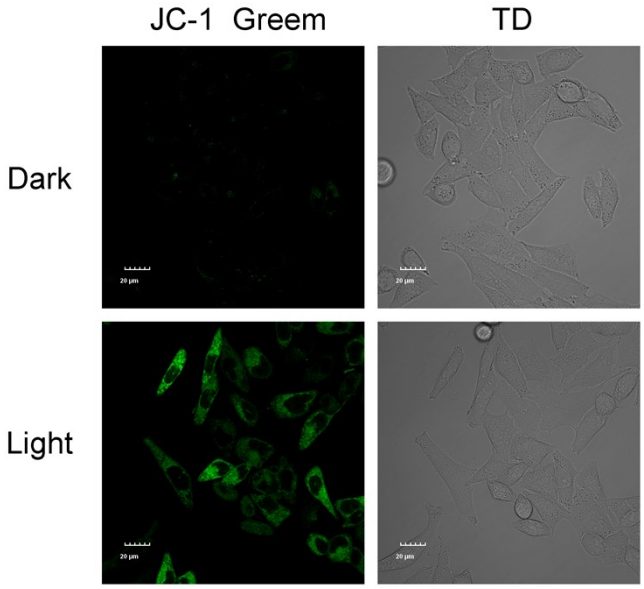


Fig. S18 Mitochondrial membrane potential assay for **CSZ-J** mediated photodamage of mitochondria. Scale bars = 20 μm.

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