Supplementary Information

A highly selective near-infrared fluorescent probe for imaging peroxynitrite in the brains of epileptic mice

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Table of Content

1. Additional Experiments

Table S1. Photophysical properties of **CCy7-DP** and **CCy7**.

Compound	λ_{abs_max} (nm)	λ_{em_max} (nm)	Φ_f
CCy7-DP	775	800	0.058[a]
CCy7	545	633	0.156[b]

[a]ICG (Φ = 0.106 in DMSO) was used as the reference standard and the excitation wavelength was 740 nm.

[b]Sulforhodamine 101 (Φ = 0.95 in EtOH) was used as the reference standard and the excitation wavelength was 540 nm.

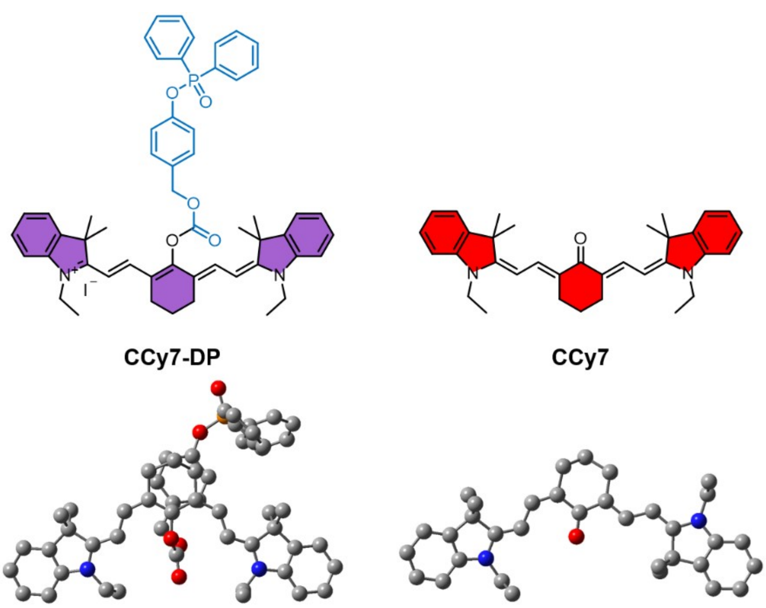


Figure S1. The optimized ground state geometries of **CCy7-DP** and **CCy7** obtained from ωB97XD/6-31G(d,p) method.

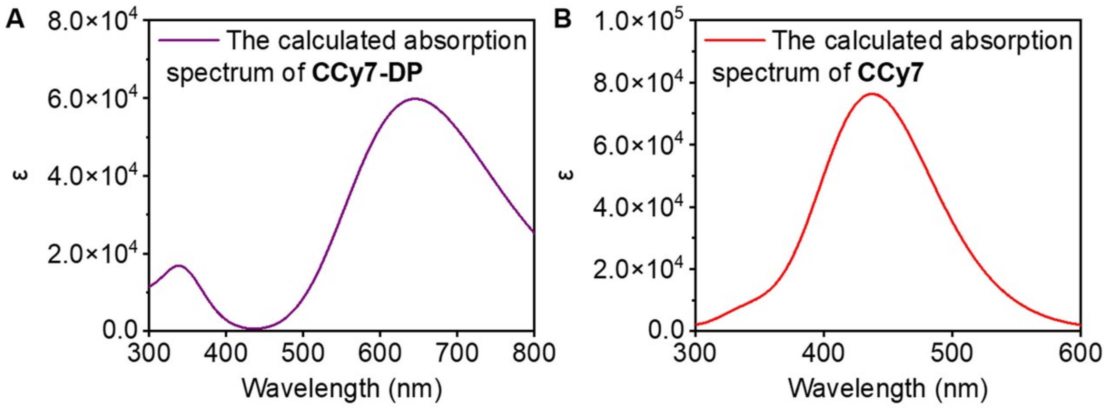


Figure S2. The calculated absorption spectra of **CCy7-DP** and **CCy7** obtained from wB97XD/6-31G(d,p) method.

Table S2. Calculated TD-DFT singlet excitation energies (nm), oscillator strengths (*f*), composition and contributions in terms of molecular orbital contributions.

Compound	Method	Transition	E (nm)	*f*	Composition and contribution	
CCy7-DP	wB97XD/6-31G(d,p)	$S_0 \rightarrow S_1$	645	1.4766	HOMO → LUMO	95%
					HOMO-1 → LUMO+1	3%
CCy7	wB97XD/6-31G(d,p)	$S_0 \rightarrow S_1$	438	1.8793	HOMO → LUMO	90%
					HOMO-1 → LUMO+1	6%

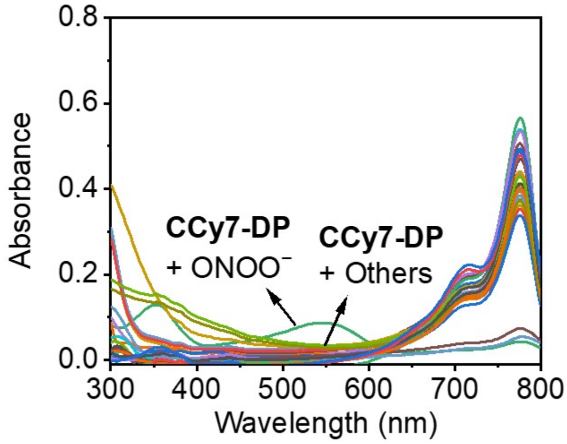


Figure S3. Absorption spectra of **CCy7-DP** (2 μM) in the presence of $ONOO^-$ (100 μM) or other competing species (100 μM or 10 U/mL; 1: Blank; 2: $ONOO^-$; 3: H_2O_2; 4: NaClO; 5: $O_2^{\bullet-}$; 6: $^1O_2$; 7: •OH; 8: Cys; 9: HCy; 10: GSH; 11: Trp; 12: Tyr; 13: Glu; 14: Lys; 15: ATP; 16: NAC; 17: Cellulase; 18: Lysozyme; 19: Fe^{2+}; 20: Mg^{2+}; 21: NO_2^-; 22: NO_3^-) after incubation for 45 min. All experiments were conducted in PBS buffer (10 mM, pH 7.4, V/V, PBS/CH_3CN = 3/1).

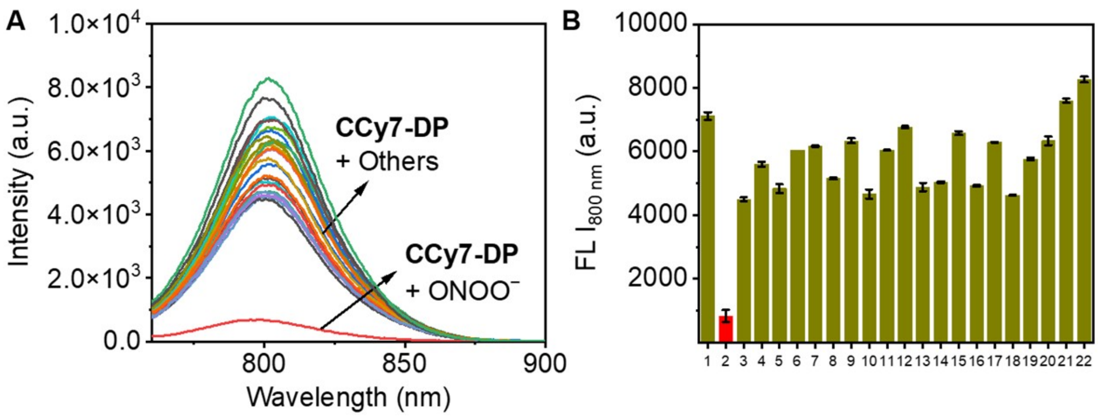


Figure S4. Fluorescence spectra (A) and fluorescence intensity at 800 nm (B) of **CCy7-DP** (2 μM) in the presence of $ONOO^-$ (100 μM) or other competing species (100 μM or 10 U/mL; 1: Blank; 2: $ONOO^-$; 3: H_2O_2; 4: NaClO; 5: $O_2^{\bullet -}$; 6: $^1O_2$; 7: •OH; 8: Cys; 9: HCy; 10: GSH; 11: Trp; 12: Tyr; 13: Glu; 14: Lys; 15: ATP; 16: NAC; 17: Cellulase; 18: Lysozyme; 19: Fe^{2+}; 20: Mg^{2+}; 21: NO_2^-; 22: NO_3^-) after incubation for 45 min. All experiments were conducted in PBS buffer (10 mM, pH 7.4, V/V, PBS/CH_3CN = 3/1) with an excitation wavelength of 740 nm.

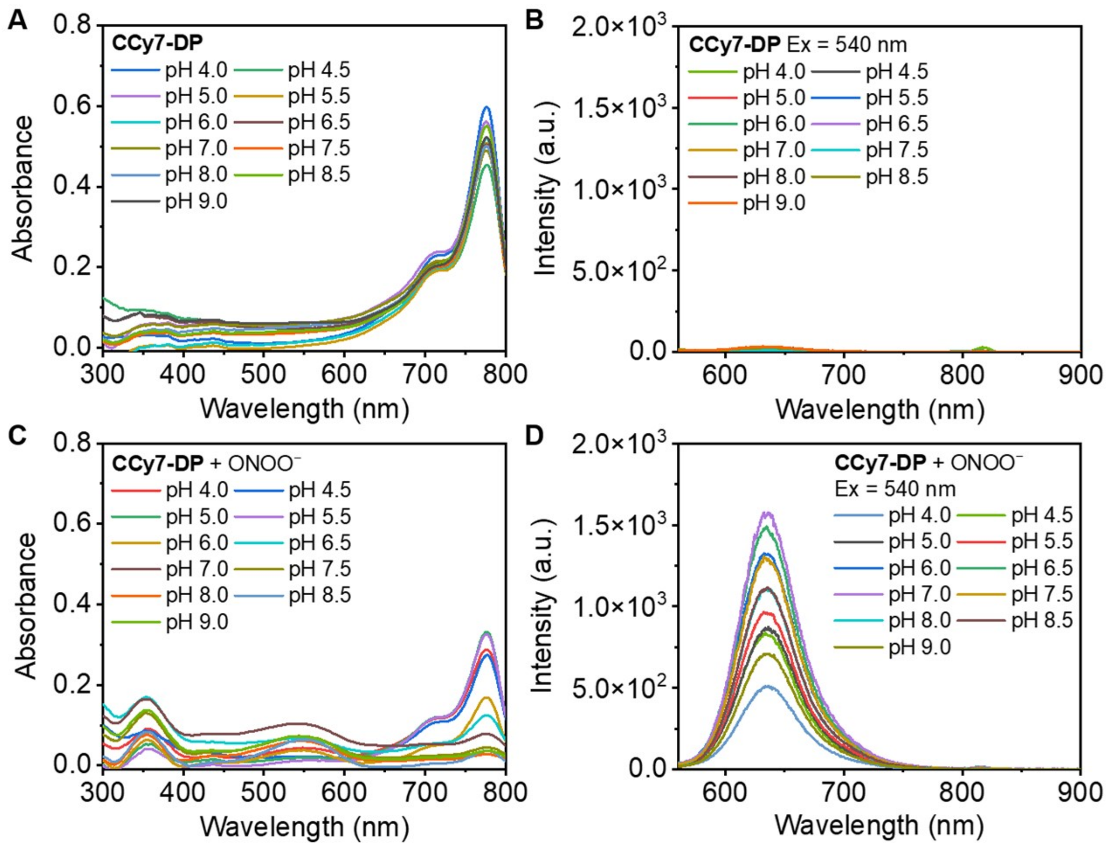


Figure S5. pH-dependent absorption (A) and fluorescence (B) spectra of **CCy7-DP** (2 μM) in PBS buffer (10 mM, V/V, PBS/CH_3CN = 3/1) from pH 4.0 to 9.0 for 45 min. pH-dependent absorption (C) and fluorescence (D) spectra of **CCy7-DP** (2 μM) in the presence of $ONOO^-$ (100 μM) in PBS buffer (10 mM, V/V, PBS/CH_3CN = 3/1) from pH 4.0 to 9.0 for 45 min. All fluorescence experiments were conducted with an excitation wavelength of 540 nm.

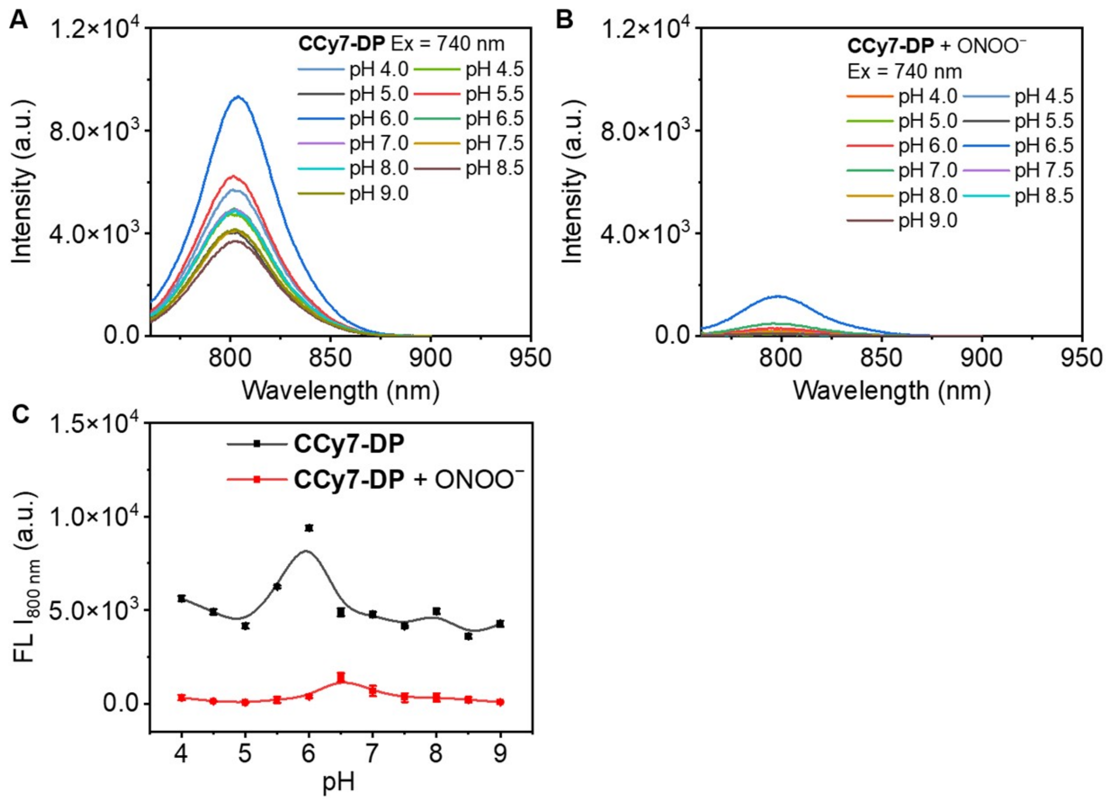


Figure S6. pH-dependent fluorescence spectra of **CCy7-DP** (2 μM) in the absence (A) or presence (B) of $ONOO^-$ (100 μM) in PBS buffer (10 mM, V/V, PBS/CH_3CN = 3/1) from pH 4.0 to 9.0 for 45 min. (C) pH-dependent fluorescence intensity at 800 nm of **CCy7-DP** (2 μM) in the presence or absence of $ONOO^-$ (100 μM) for 45 min. All fluorescence experiments were conducted with an excitation wavelength of 740 nm.

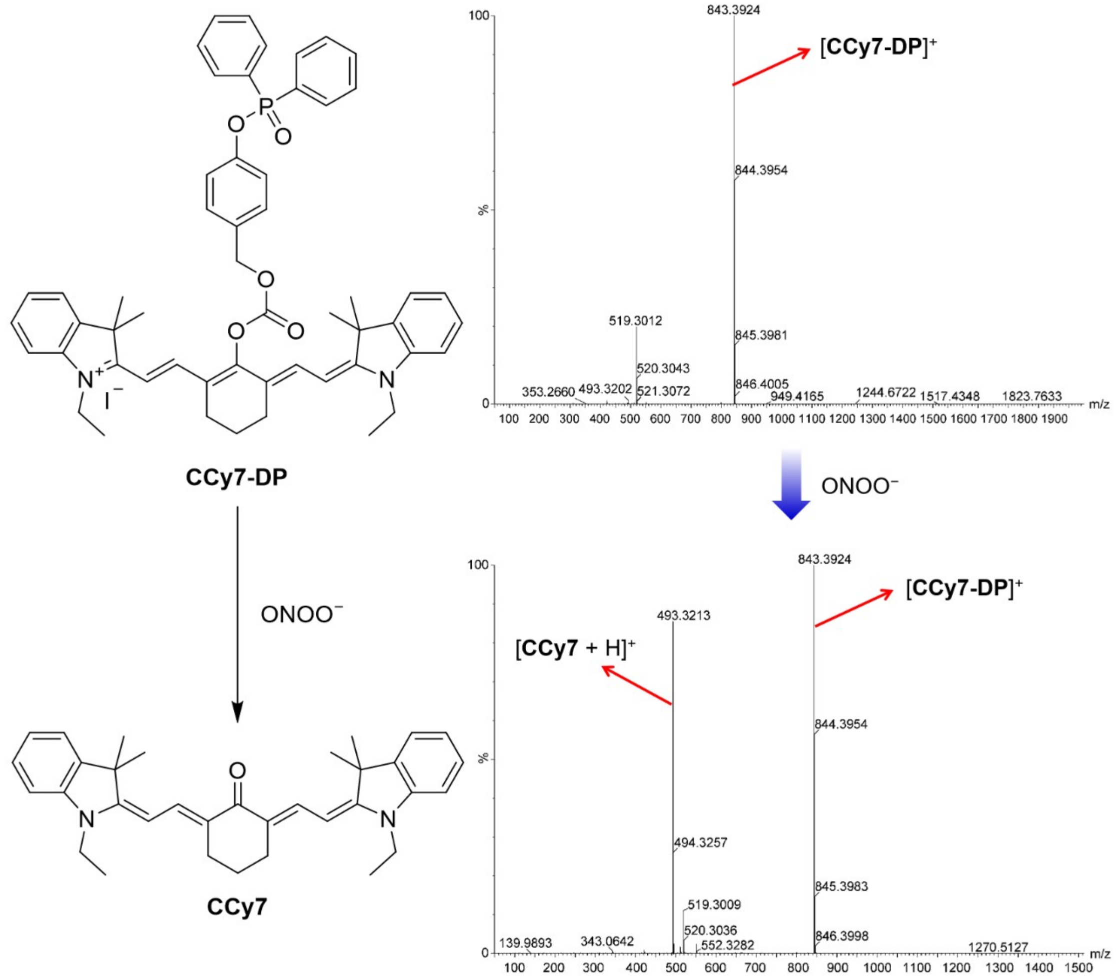


Figure S7. Mass spectra of **CCy7-DP** treated with or without ONOO⁻.

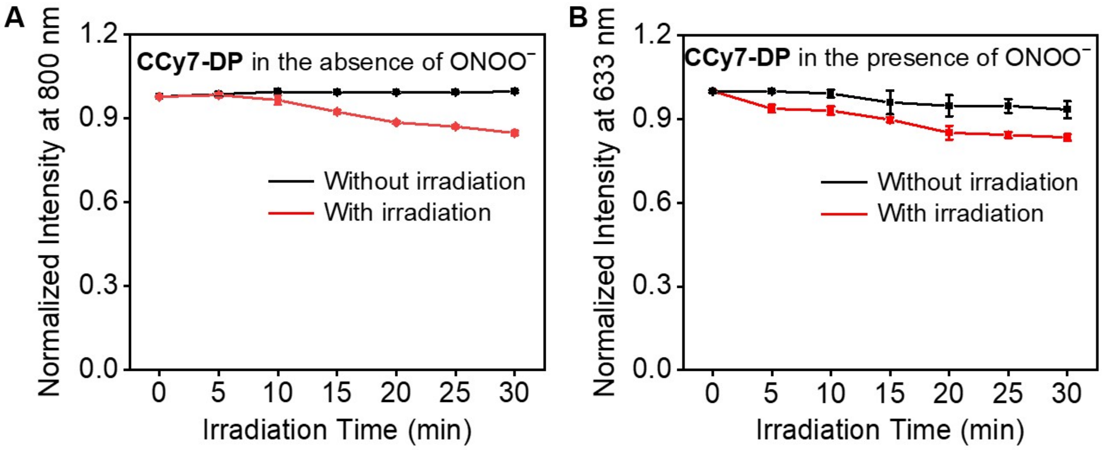


Figure S8. (A) Fluorescence intensity changes of **CCy7-DP** at 800 nm in the absence of $ONOO^-$, without and with white light irradiation (0.3 mW/cm^2) for 30 min. The excitation wavelength was 740 nm. (B) Fluorescence intensity changes of **CCy7-DP** at 633 nm in the presence of $ONOO^-$, without and with white light irradiation (0.3 mW/cm^2) for 30 min. The excitation wavelength was 540 nm. All experiments were conducted in PBS buffer (10 mM, pH 7.4, V/V, PBS/CH_3CN = 3/1).

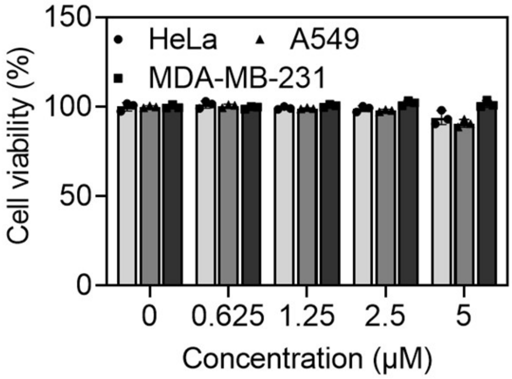


Figure S9. Cytotoxicity evaluation of **CCy7-DP** (0, 0.625, 1.25, 2.5, 5 μM, 24 h) on HeLa, A549, and MDA-MB-231 cells. Data are represented as mean ± SD (n = 3).

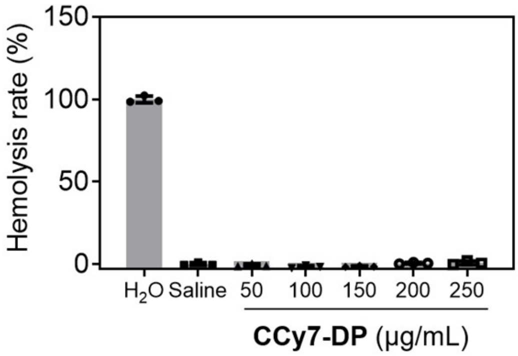
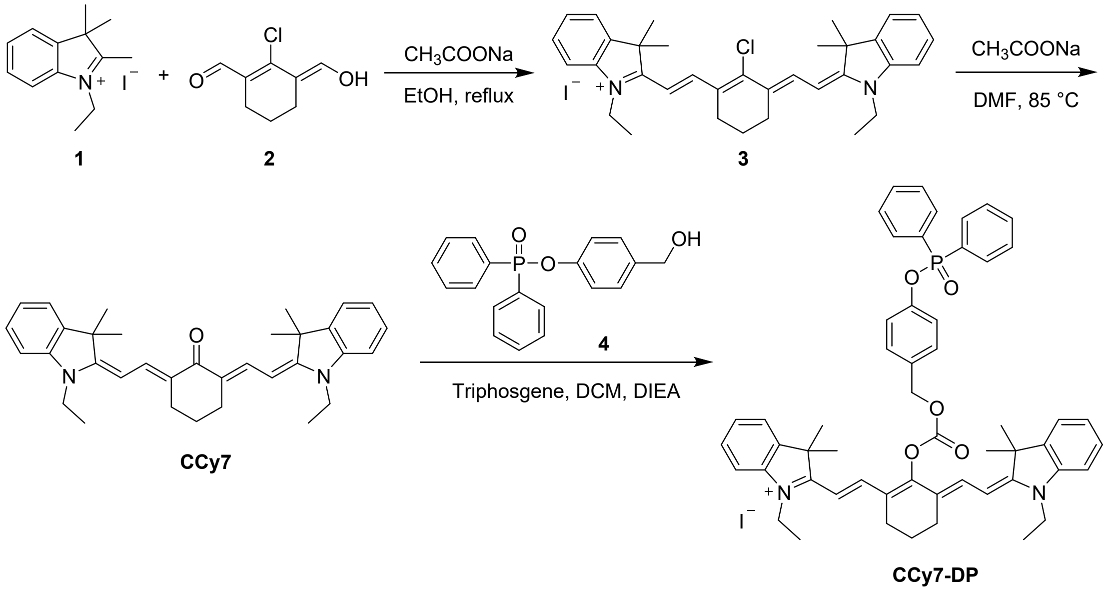


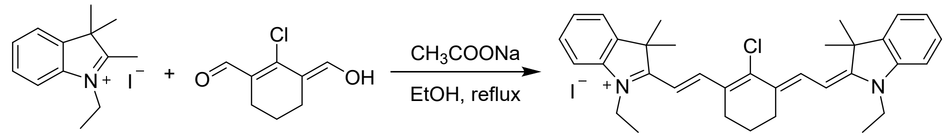
Figure S10. Hemolysis rates of **CCy7-DP** at 50–250 μg/mL and control groups (saline, negative control; H_2O, positive control). Data are presented as mean ± SD (n = 3).

2. Synthesis and characterization



Scheme S1. Synthesis of probe **CCy7-DP**.

Synthesis of compound 3:

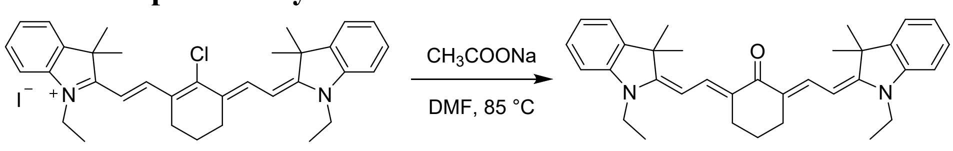


1 2 3

In a 50 mL Schlenk flask, compound **1** (228.26 mg, 724 mmol) and compound **2** (50 mg, 290 mmol) were combined with sodium acetate (68.7 mg, 837 mmol). The flask was purged with nitrogen, degassed, and dried. Anhydrous ethanol (2 mL) was added, and the mixture was heated under reflux at 85 °C for 3 hours. The crude mixture was poured into diethyl ether to precipitate the solid, which was collected by filtration to afford the compound **3** as a green powder (150 mg, 81.0% yield).

^{1}H NMR (400 MHz, DMSO-D_6) δ 8.23 (d, J = 14.1 Hz, 2H), 7.60 (d, J = 1.1 Hz, 2H), 7.47 – 7.35 (m, 4H), 7.29 – 7.22 (m, 2H), 6.29 (d, J = 14.2 Hz, 2H), 4.22 (q, J = 7.1 Hz, 4H), 2.69 (t, J = 6.1 Hz, 4H), 1.63 (s, 12H), 1.52 – 1.48 (m, 2H), 1.27 (t, J = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-D_6) δ 172.32, 148.52, 143.62, 142.19, 141.74, 129.19, 126.64, 125.72, 123.13, 111.87, 101.85, 49.55, 27.92, 26.42, 20.90, 12.78. HRMS-ESI (m/z): $[M]^+$ Calcd for $C_{34}H_{40}ClN_2^+$ 511.2875, found 511.2882.

Synthesis of compound CCy7:

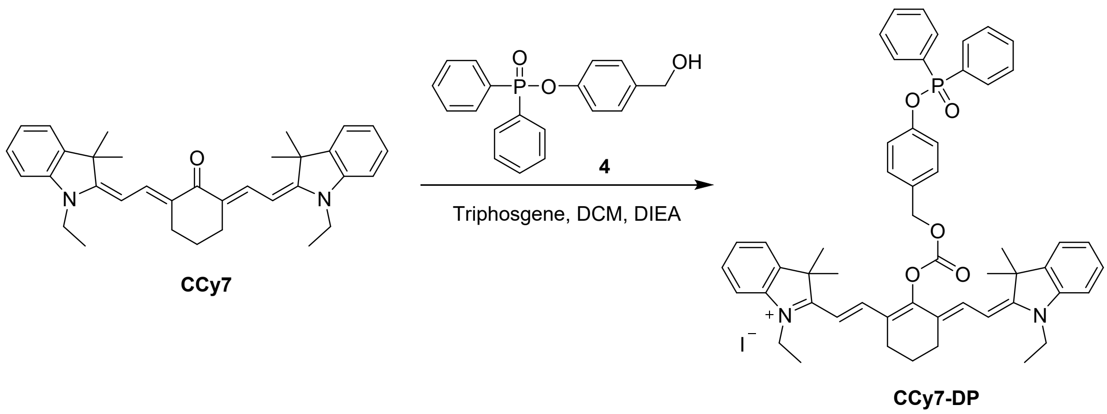


3 CCy7

To a 50 mL Schlenk flask were added compound **3** (100 mg, 156 mmol) and sodium acetate (68.7 mg, 837 mmol). The flask was purged with nitrogen, degassed, and dried. Dry DMF (4 mL) was then added, and the mixture was refluxed at 90 °C for 4 hours. After cooling to room temperature, the mixture was concentrated and purified by silica gel column using PE/EA (3:1, v/v) as the eluent to yield compound **CCy7** (23 mg, 30.1% yield).

^{1}H NMR (400 MHz, CHLOROFORM-*D*) δ 8.20 (d, *J* = 13.0 Hz, 2H), 7.25 – 7.15 (m, 4H), 6.92 (t, *J* = 7.4 Hz, 2H), 6.69 (d, *J* = 7.9 Hz, 2H), 5.48 (d, *J* = 13.3 Hz, 2H), 3.75 (d, *J* = 7.6 Hz, 4H), 2.61 (t, *J* = 6.2 Hz, 4H), 1.87 (p, *J* = 6.5 Hz, 2H), 1.67 (s, 12H), 1.29 (t, *J* = 6.9 Hz, 6H). ^{13}C NMR (101 MHz, CHLOROFORM-*D*) δ 161.93, 143.82, 139.92, 132.94, 127.74, 126.57, 121.92, 120.53, 106.50, 92.21, 46.66, 37.13, 33.93, 32.02, 29.79, 29.46, 29.26, 29.05, 28.78, 25.93, 22.79, 22.64, 14.23, 11.24. HRMS-ESI (m/z): $[M+H]^+$ Calcd for $C_{34}H_{40}N_2O$ 493.3213, found 493.3219.

Synthesis of compound CCy7-DP:



Compound **4** was synthesized according to a previously reported procedure.[1] Compound **4** (33 mg, 1.0 mmol) was added to a Schlenk tube under a nitrogen atmosphere. The tube was purged with nitrogen to remove oxygen and moisture. Anhydrous DCM (4 mL) was added, followed by DIEA (40 mg, 3.0 mmol). After stirring in an ice bath for 10 min, a solution of triphosgene (30 mg, 1.0 mmol) in anhydrous DCM (1 mL) was added dropwise. The reaction was then allowed to proceed at room temperature for 2 hours. Subsequently, a solution of compound **CCy7** (50 mg, 1.01 mmol) in anhydrous DCM (1 mL) was added, and the mixture was stirred at room temperature in the dark for 24 hours, turning deep green. The reaction mixture was concentrated under reduced pressure, and the crude product was purified by silica gel column chromatography (eluent: DCM/MeOH, 10:1, v/v). Concentration of the purified fractions afforded **CCy7-DP** as a green product (14.3 mg, 43.3% yield).

^{1}H NMR (400 MHz, DMSO-D_6) δ 7.93 – 7.85 (m, 4H), 7.62 – 7.54 (m, 6H), 7.51 – 7.42 (m, 10H), 7.41 – 7.36 (m, 2H), 7.30 – 7.24 (m, 2H), 6.22 (d, *J* = 14.2 Hz, 2H), 5.28 (s, 2H), 4.22 (q, *J* = 7.2 Hz, 4H), 2.64 (t, *J* = 6.2 Hz, 4H), 1.88 – 1.77 (m, 2H), 1.41 (s, 12H), 1.28 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-D_6) δ 171.76, 157.86, 152.76, 151.75, 151.67, 142.19, 141.62, 139.21, 133.35, 132.03, 131.92, 131.54, 131.42, 130.51, 129.55, 129.41, 129.23, 125.62, 123.08, 121.40, 121.34, 111.82, 101.20, 70.52, 55.47, 49.26, 27.75, 24.38, 24.24, 20.89, 12.74, 0.64. HRMS-ESI (m/z): $[M]^+$ Calcd for $C_{54}H_{56}N_2O_5P^+$ 843.3912, found 843.3924.

3. Experimental procedures

Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers (Adamas, Energy Chemical, etc.) and used without further purification. All oxygen- or water-sensitive reactions were performed under an N_2 atmosphere using the standard Schlenk method. The ^1H NMR and ^{13}C NMR spectra of compounds were recorded ona JNM-ECZ400 S/L1 spectrometer, using tetramethylsilane (TMS) as an internal reference and $CDCl_3$, MeOD, or DMSO-d_6 as solvents. Mass spectra of compounds were measured on a Waters Xevo G2-XS QTof. The absorption spectra were recorded by a Hitachi U-3900. The emission spectra were recorded on an Edinburgh FS5 Spectrofluorometer or Hitachi F-4700.

General Procedure for Spectra Measurements

For absorption and fluorescence titration, $ONOO^-$ with different concentrations from 0 to 100 μM was added into 2.0 mL of phosphate buffered saline (PBS) (10 mM, pH 7.4, V/V, PBS/CH_3CN = 3/1) containing 2 μM probe **CCy7-DP**. After the mixture was incubated for 45 min, the absorption and fluorescence spectra were measured. To demonstrate its selectivity, **CCy7-DP** (2 μM) was incubated with competing species (100 μM or 10 U/mL) including H_2O_2, NaClO, $O_2^{\bullet -}$, $^1O_2$, •OH, Cys, HCy, GSH, Trp, Tyr, Glu, Lys, ATP, NAC, Cellulase, Lysozyme, Fe^{2+}, Mg^{2+} for 45 min, and the UV−vis absorption and fluorescence of the resulting mixtures were measured.

Determination of the Fluorescence Quantum Yields

The relative fluorescence quantum yields of **CCy7-DP** and **CCy7** were determined using indocyanine green (ICG, Φ = 0.106 in DMSO) and sulforhodamine 101 (Φ = 0.95 in EtOH) as reference standards, respectively. The following equation was used to determine the relative quantum yield of unknown compounds:

$$\frac{\Phi_\Delta^a}{\Phi_\Delta^{ref}} = \frac{1 - 10^{-A_{ref}}}{1 - 10^{-A_a}} \cdot \frac{S_a}{S_{ref}} \cdot \left(\frac{n_a}{n_{ref}}\right)^2 \quad equation\ (1)$$

Where Φ_Δ^a and Φ_Δ^{ref} are the fluorescence quantum yields of the sample and reference standard, respectively; S_a and S_{ref} are the integrated intensities (areas) of the sample and standard spectra, respectively; A_a and A_{ref} are the absorbances of the sample and standard, respectively; and n_a and n_{ref} are the refractive indices of the sample and standard solutions, respectively.

Theoretical Calculations

All theoretical calculations were performed using the Gaussian 16 software package. The ωB97XD functional combined with the 6-31G(d,p) basis set was employed for all geometry optimizations and excited-state calculations. Solvent effects of water were modeled using the SMD (Solvation Model based on Density) continuum solvation model in all calculations. The absorption spectra were calculated using time-dependent density functional theory (TD-DFT) at the same ωB97XD/6-31G(d,p) level. The first 20 excited states were computed to obtain the absorption maxima and oscillator strengths. Molecular orbitals (HOMO and LUMO) were visualized using GaussView, and the orbital energy gaps were derived from the optimized ground-state structures.

Cell Culture

HeLa and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified incubator at 37°C and 5% CO_2. A549 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator at 37°C and 5% CO_2. The cells were split when they reached 90% confluence.

Cytotoxicity Assays

HeLa, MDA-MB-231 and A549 cells were separately seeded in 96-well plates and cultured overnight at 37 °C under 5% CO_2. After that, cells were treated with serially diluted **CCy7-DP** (0, 0.625, 1.25, 2.5, 5 μM) for 24 h. Then, 10 μL of CCK-8 reagent was added, and the plates were incubated for 1 h before absorbance measurement at 450 nm on an EnVision multilabel plate reader.

Hemolysis Assay

For hemolysis experiments, varying concentrations (50, 100, 150, 200, 250 μg/mL) of **CCy7-DP** were incubated with fresh mouse blood. Control groups were established, including a positive control with deionized water and a negative control with saline, both maintained at 37 °C for 1 h. Following incubation, the absorbance of the supernatant at 540 nm was measured using an EnVision multilabel plate reader. The hemolysis ratio was calculated as: [$(A_e - A_n)/(A_p - A_n)$] × 100%. In the formula, A_e denotes the absorbance value of the experimental group, A_p denotes the absorbance value of the positive control group, and A_n denotes the absorbance value of the negative control group.

In Vivo Fluorescence Imaging

Mice from the control group and epileptic model group were selected for the experiment, with the scalp hair of mice was shaved. The mice then received tail vein injection of **CCy7-DP** at a dose of 1 mg/kg. Fluorescence imaging was conducted at different time points post-injection to assess and compare the fluorescence intensity of the probe in the brains of mice between the two groups.

Establishment of Epileptic Mouse Model

All animal experimental protocols and related in vivo studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Yantai Institute of Materia Medica (Approval No. 20260303-02), and were conducted in compliance with the Regulations on the Administration of Laboratory Animals of the People's Republic of China, the national Guidelines for Welfare and Ethical Review of Laboratory Animals (GB/T 35892-2018), as well as institutional animal care guidelines. Male C57BL/6 mice were selected to establish a pentylenetetrazol (PTZ)-induced chronic kindled epilepsy model. The experimental mice were randomly allocated into two groups: the normal control group (wild-type, WT) and the epileptic model group (PTZ group). The body weights of all mice were measured and recorded prior to the experiment. Starting from the first day of the experiment, mice in the PTZ group were intraperitoneally (i.p.) injected every other day with PTZ (50 mg/kg), with a total of 14 administrations; mice in the WT group received an equal volume of saline via the same injection route as the control. After each PTZ administration, each mouse was continuously observed for 30 min, and the seizure latency was documented, including mild seizure latency (corresponding to Racine Grade I-III) and severe seizure latency (corresponding to Racine Grade IV-V).

Seizure severity was graded in accordance with the Racine scale, with the grading criteria as follows: Grade 0, no convulsive responses and normal behavioral status in mice; Grade I, facial muscle spasms characterized by rhythmic chewing, eye blinking and whisker twitching; Grade II, cervical muscle spasms manifested as head nodding, with or without tail erection; Grade III, forelimb clonus or rhythmic twitching; Grade

IV, hindlimb rigidity or tonic standing; Grade V, generalized clonus accompanied by tonic standing, falling and loss of body balance. The chronic kindled epilepsy model was deemed successfully established (i.e., mice achieved complete epileptic kindling) when the animals exhibited three consecutive episodes of convulsions at Grade IV or above.

4. References

(1) Chai X, Li B, Chen C, et al. A highly sensitive and selective near-infrared fluorescent probe for imaging peroxynitrite in living cells and drug-induced liver injury mice[J]. Analytical Chemistry, 2023, 95(13): 5747-5753.

5. Original spectra of synthesized compounds

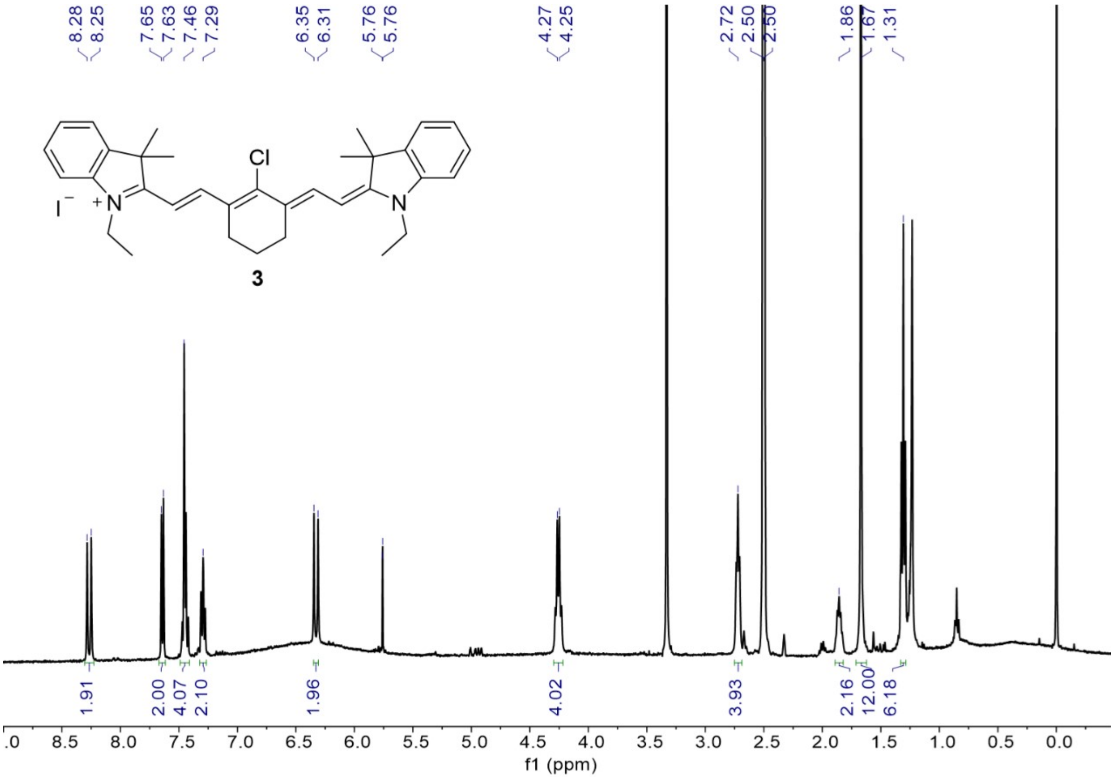


Figure S11. ^{1}H NMR spectrum of compound **3**.

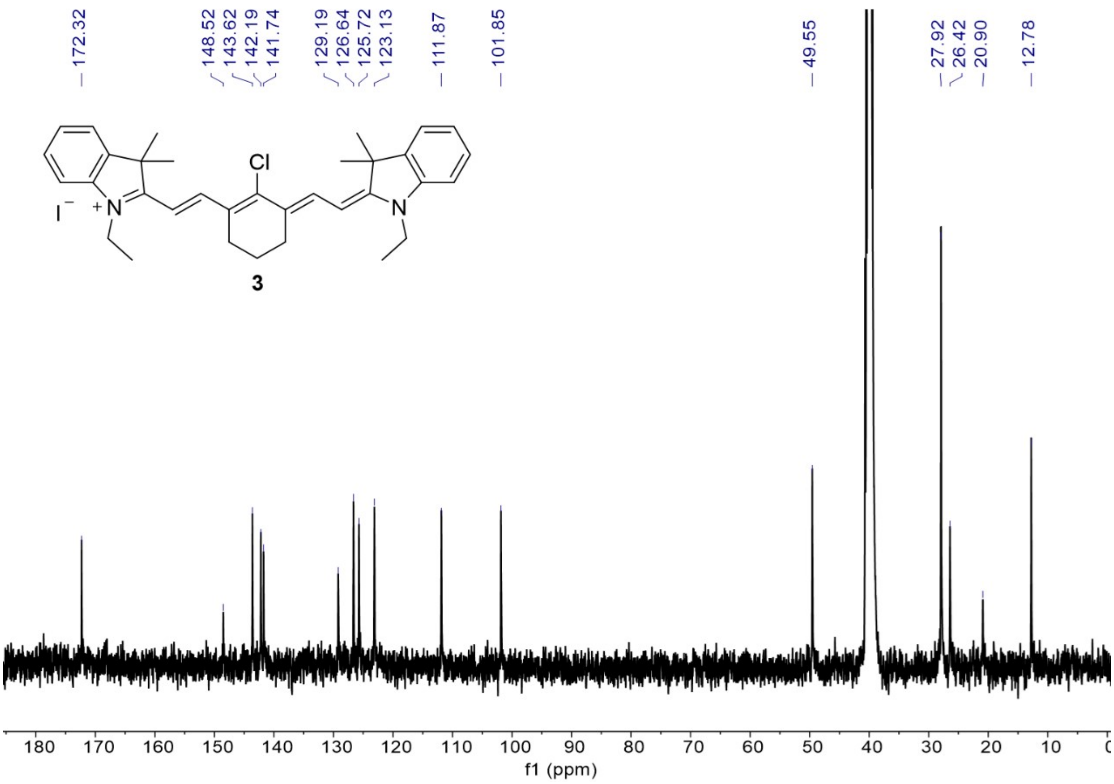


Figure S12. ^{13}C NMR spectrum of compound **3**.

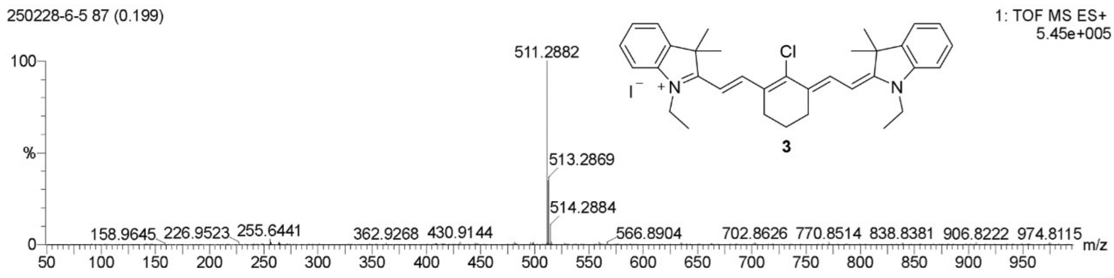


Figure S13. HRMS-ESI spectrum of compound compound **3**.

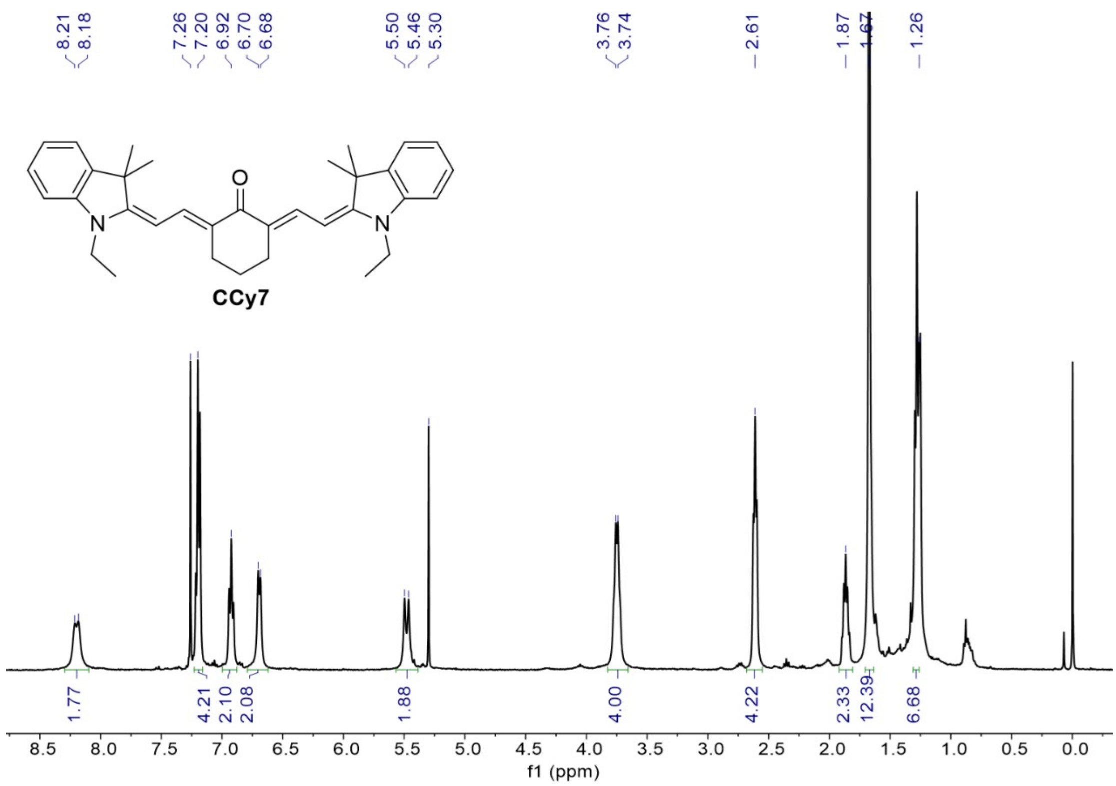


Figure S14. ^{1}H NMR spectrum of compound **CCy7**.

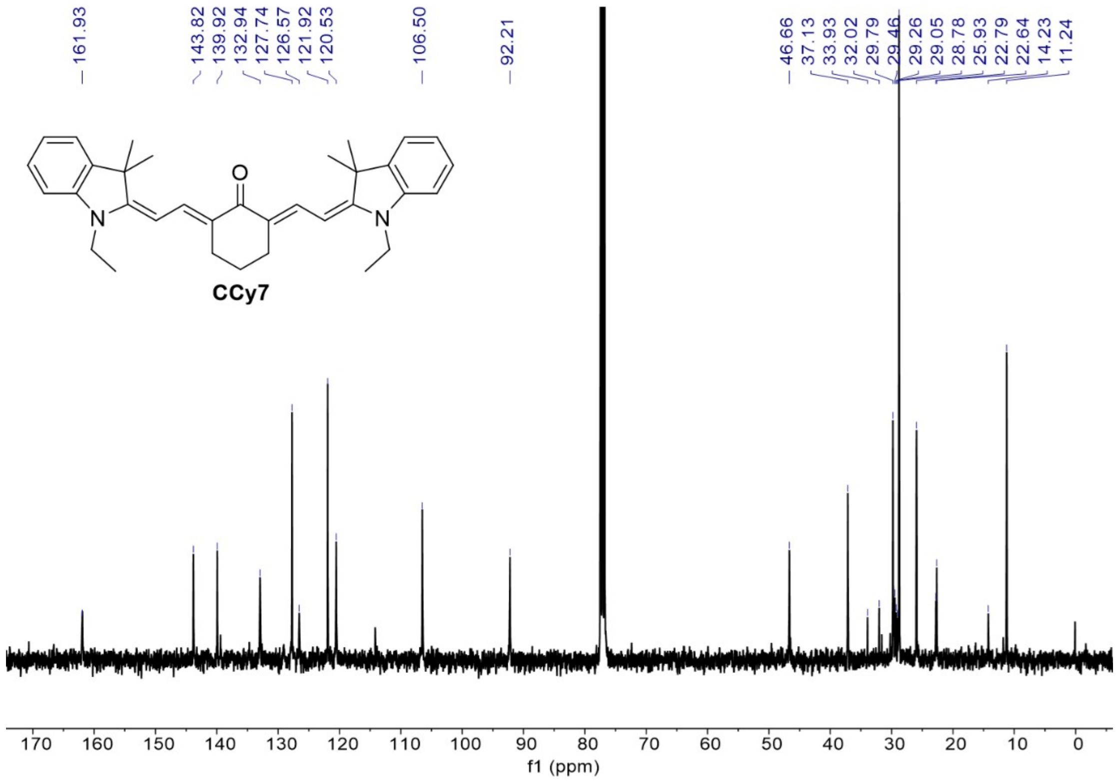


Fig. S15 ^{13}C NMR spectrum of compound **CCy7**.

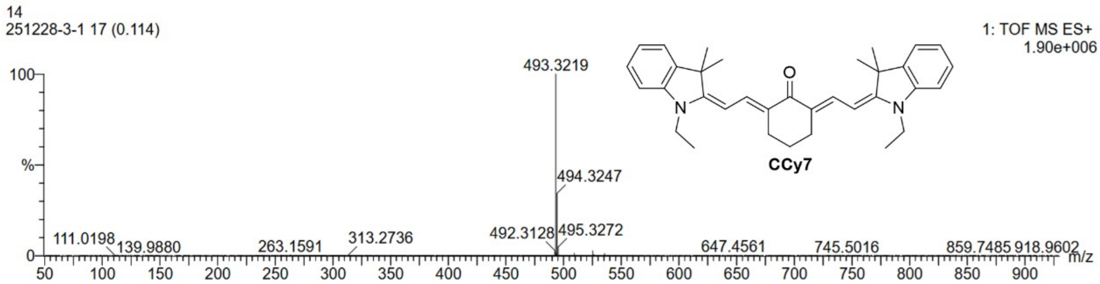


Figure S16. HRMS-ESI spectrum of compound **CCy7**.

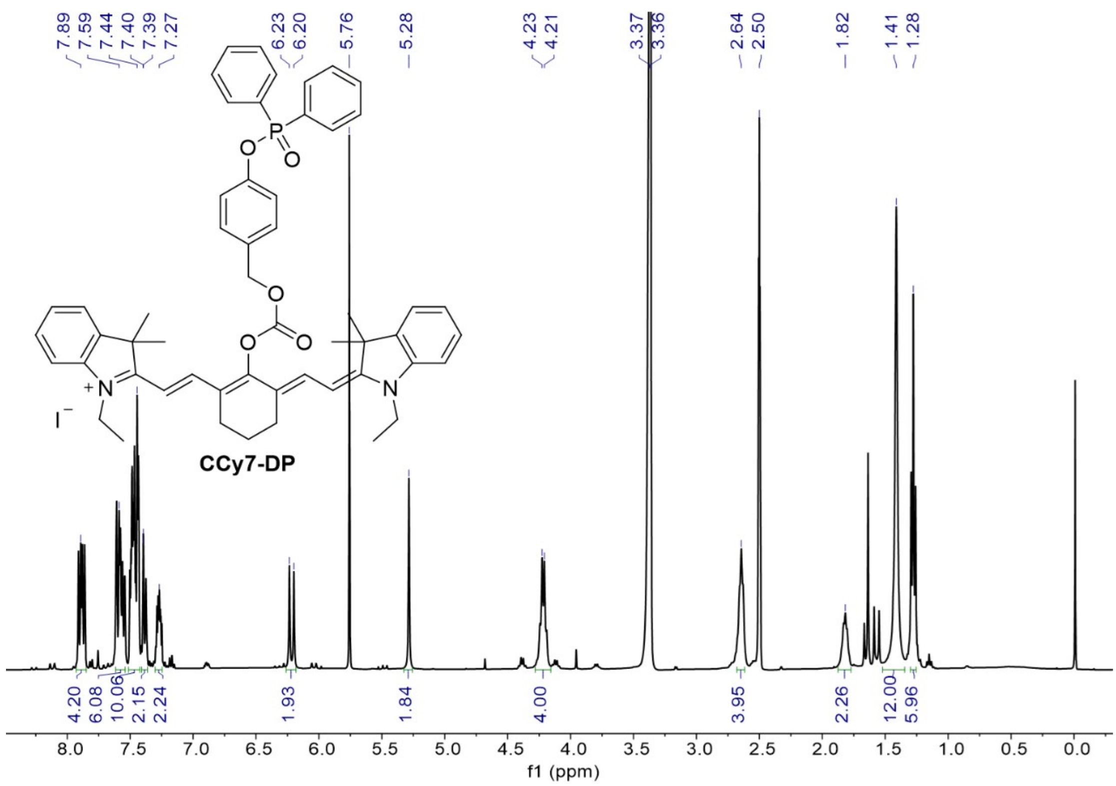


Figure S17. ^{1}H NMR spectrum of compound **CCy7-DP**.

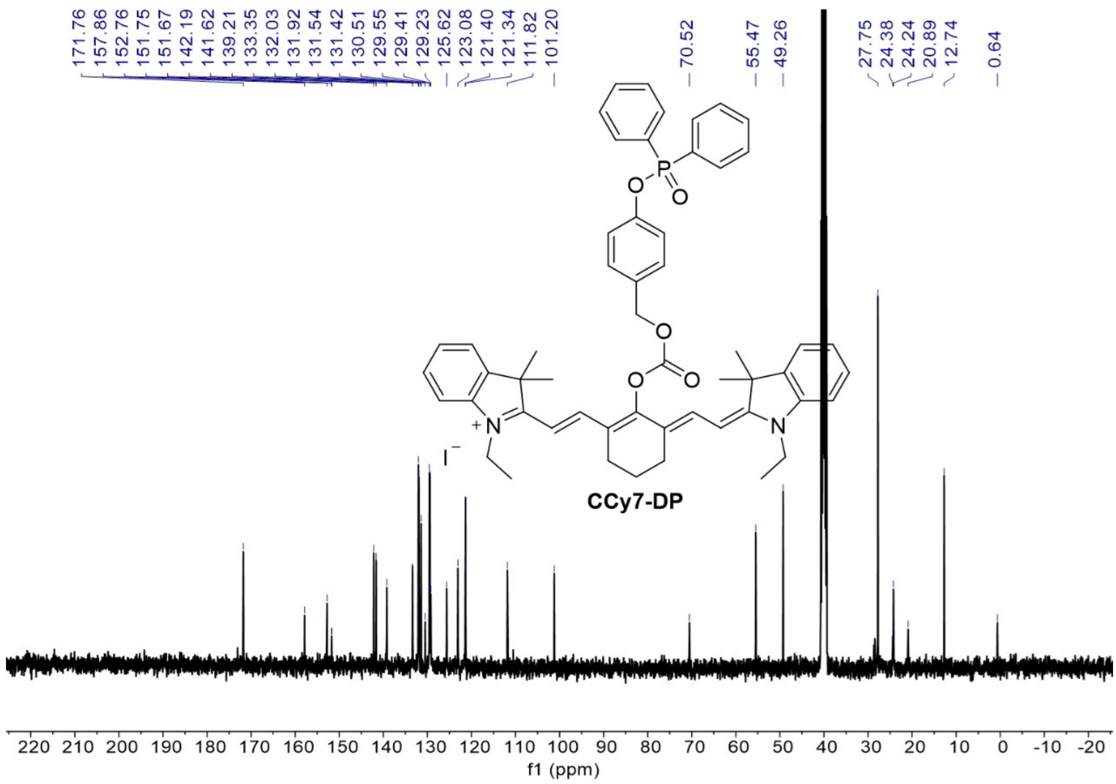


Figure S18. ^{13}C NMR spectrum of compound **CCy7-DP**.

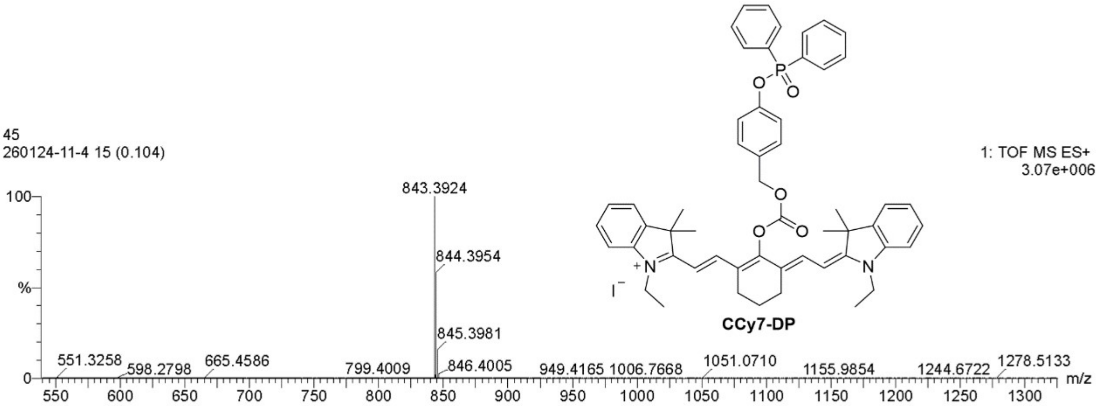


Figure S19. HRMS-ESI spectrum of compound compound **CCy7-DP**.