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Supporting Information

For

Water-soluble red fluorescent protein dimer for hypoxic two-photon photodynamic therapy

Wan Feng, Ying Qian*

School of chemistry and chemical engineering, Southeast University, Nanjing, 211189, China

Corresponding author: yingqian@seu.edu.cn

Contents

1. Experimental section

Materials

Phenothiazine, 4-(2-chloroethyl)-morpholine hydrochloride, glycine *tert*-butyl ester hydrochloride, *tert*-butanol, 1-ethoxyethylideneammonium chloride, phydroxybenzaldehyde, ether, KOH, NaOH, $POCl_3$ and K_2CO_3. All biomaterials were purchased from Keygen Biotech Co.Ltd. A-549 cells were used in this work from the American Type Culture Collection. Unless stated otherwise, all chemicals with analytical grade were purchased from commercial sources and used directly as received. All the solvents used to investigate the photophysical behaviors of the fluorophores were spectroscopic grade. Column chromatography was performed on silica gel (200-300 mesh).

Measurements and characterization

NMR (^1H and ^{13}C) measurements were recorded on a Bruker ADVANCE III HD 600 NMR spectrometer. UV-vis spectra are recorded on the Shimazdu 2450 UV-visible spectrophotometer. High-resolution mass spectra (HRMS) were acquired on an Ultra flextreme MALDI-TOF/TOF and Agilent Technologies 6530 Accurate-Mass equipment. Steady-state fluorescence excitation and emission spectra were obtained by a time-correlated single photon counting fluorimeter (Fluoromax-4/plus) with a xenon lamp as the light source. The EPR (Electron Paramagnetic Resonance) spectra were recorded on a Bruker Magnettech ESR5000. The power of the customized 460 nm blue LED lamp was 23 $mW \cdot cm^{-2}$. The of singlet oxygen yield (Φ_Δ) and its applications in cells were both performed under the condition of irradiation at 23 $mW \cdot cm^{-2}$ (the irradiation was tested with an Aicevoos-V10 irradiation meter). One-photon fluorescent images of cells were acquired from FLUOVIEW FV3000/FLUOVIEW FV1000MPE OLYMPUS (CLSM) and two-photon fluorescent images of cells were acquired from Olympus multiphoton microscope (FVMPE-RS) equipped with an InSight DS-OL pulsed IR laser system (Spectra-Physics, 80 MHz, 120 fs). The Z-scan two-photon absorption cross section is measured from NLO-Z. The microscope used for 2PE-PDT was an upright

Singlet oxygen quantum yield (Φ_Δ) detection

The Φ_Δ of the photosensitizers FPOH, FP_2R' and FP_2R'' were detected by using 9,10-

Anthracenediyl-bis(methylene)dimalonic acid (ABDA) as the capture agent for $^1O_2$ and the $Ru(bpy)_3Cl_2$ as the reference (Φ_Δ=0.41 in aqueous solution).[1] Keeping the absorbance of the photosensitizers and ABDA were about 0.2 and 1 respectively. The mixed solution was irradiated under 460 nm light, and recorded the absorbance value of the ABDA solution to get the decrease slop. Finally, the Φ_Δ was calculated on the basis of the formula below:[2]

$$\Phi_{\Delta[PS]} = \Phi_{\Delta\left[Ru(bpy)_3cl_3\right]} \times \frac{k_{[PSs]}}{k_{\left[Ru(bpy)_3cl_3\right]}} \times \frac{F_{\left[Ru(bpy)_3cl_3\right]}}{F_{[PSs]}}$$

Where, the k is the decrease slope between the absorbance and its corresponding time. F is absorption correction factor ($F=1-10^{-OD}$), and OD is its maximum absorbance. PSs are FPOH, FP_2R' and FP_2R''.

Theoretical calculations

Molecular structural parameters of the compounds were studied theoretically by quantum chemical computational technique based on the DFT method. The quantum-chemical methods at the B3LYP/6-31G (d) level of theory using Gaussian 16 were employed to calculate the HOMO and LUMO energies of the synthesized compounds.

Two-photon absorption cross section measurement

The two-photon absorption cross sections of compounds FPOH, FP_2R' and FP_2R'' are measured using Z-scan method, and the calculation formula is as follows: [3-4]

$$\delta_{2PA} = \frac{1000 \times h\nu\beta}{N_A c}$$

The δ_{2PA} values are obtained by knowing the coefficient β, where N_A is the Avogadro's number, c is the sample concentration and $h\nu$ is related to the exciting photon energy. (c: 1×10^{-3} M, H_2O, λ_{ex}: 800 nm)

MTT cytotoxicity assay

A-549 cells (5000 cells/well) were seeded into the 96-well plate and incubated for 24 h at 37 °C. Then prepared different concentrations (1 μM, 2 μM, 3 μM, 4 μM, 5 μM, 6 μM and 7 μM) photosensitizers FPOH, FP_2R' and FP_2R'' were added to the 96-well plate incubated for 24 h in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively.

Dark toxicity: Dripping 100 μL/well MTT (5 mg/mL) into the 96-well plate and incubated for 4 h in the dark in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively. Then adding 100 μL/well DMSO solution into well and incubated for 2 h. Finally, the cell viability in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively, were calculated by the absorbance tested by a microplate reader.

Phototoxicity test: Irradiating the 96-well plate in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively, under 460 nm light for 15minutes and incubated for 12 h. Adding 100 μL/well MTT solution (5 mg/mL) into 96-well plate and incubated for 4 h. Then 100 μL/well of DMSO solution were added to 96-well plate and incubated for 2 h. Finally, the cell viability Dark toxicity: Dripping 100 μL/well MTT (5 mg/mL) into the 96-well plate and incubated for 4 h in the dark in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively. Then adding 100 μL/well DMSO solution into well and incubated for 2 h. Finally, the cell viability in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively, were calculated by the absorbance tested by a microplate reader

AO/EB staining

The prepared confocal culture dish containing 2 μM photosensitizer FP_2R'' was irradiated for 5 minutes and 15 minutes under 460 nm light in hypoxic microenvironment (2% O_2) and normoxia microenvironment (21% O_2), respectively. And then placed in the incubator for 2 h. The diluted acridine orange (AO)/ ethidium bromide (EB) stain was added and continued to incubate for 30 minutes. The culture dish was taken out, washed three times with PBS buffer solution. In the end, placed under the confocal laser microscope for imaging. All of the above procedures were done in the dark.

General synthesis procedures for the compounds

Synthesis of Ptz-Lyso. Phenothiazine (2.0 g, 0.01 mol), 4-(2-chloroethyl)-morpholine hydrochloride (2.79 g, 0.015 mol) and KOH (2.24 g, 0.04 mol) were added to DMSO (30 mL). Then, the reaction system was heated up to 70 ℃ and stirred for 10 h. After being cooled down

to room temperature, the reaction mixture was extracted with ethyl acetate and washed with saturated NaCl water solution. The organic layer was dried over anhydrous Na_2SO_4, concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using acetate /petroleum ether *(v/v* = 1/1) as the eluent, in turn, to afford **Ptz-Lyso** as a red solid (yield 85%). ^{1}H NMR (600 MHz, $CDCl_3$, ppm): δ 7.18-7.12 (m, 4H), 6.93 (dd, J = 7.5, 5.4 Hz, 4H), 4.06 (s, 2H), 3.74 (d, J = 3.8 Hz, 4H), 2.80 (s, 2H), 2.57 (s, 4H). ^{13}C NMR (151 MHz, $CDCl_3$, ppm): δ 145.01, 127.55, 127.33, 125.03, 122.67, 115.42, 66.87, 55.92, 53.92, 45.59. HRMS (m/z): $[M+H]^+$ calc. for $C_{18}H_{20}N_2OS$: 313.1330, found 313.1330.

Synthesis of PCHO-lyso. Put DMF (1.8 mL, 22.8 mmol) into a 250 mL round bottom flask, add $POCl_3$ (2.52 mL, 27 mmol) dropwise into DMF solution slowly. After dripping, the reaction mixture was stirred at 0 °C for 1 hour. Add phenothiazine (2.7g, 19 mmol) to the reaction mixture and raise the temperature to 90 ℃. Monitor the progress of the reaction by TLC. After 4 hours, pour the reaction mixture over ice water and neutralize with NaOH. Extract the resultant mixture with dichloromethane and wash with brine solution. Evaporate the combined organic phase under reduced pressure. The crude product was purified by column chromatography on silica gel using acetate /petroleum ether *(v/v* = 1/2) as the eluent, in turn, to afford **PCHO-lyso** as a yellow solid (yield 55%). ^{1}H NMR (600 MHz, $CDCl_3$, ppm): δ 9.80 (s, 1H), 7.65 (dd, J = 8.4, 1.9 Hz, 1H), 7.60 (d, J = 1.8 Hz, 1H), 7.21-7.15 (m, 1H), 7.12 (dd, J = 7.6, 1.4 Hz, 1H), 7.05-6.92 (m, 3H), 4.13-4.01 (m, 2H), 3.77-3.69 (m, 4H), 2.79 (t, J = 6.3 Hz, 2H), 2.56 (s, 4H). ^{13}C NMR (151 MHz, $CDCl_3$, ppm): δ 190.01, 150.43, 143.24, 131.30, 130.16, 128.42, 127.70, 127.66, 125.13, 123.83, 115.93, 114.92, 100.00, 66.90, 55.81, 53.93, 46.40, 0.01. HRMS (m/z): $[M+H]^+$ calc. for $C_{19}H_{20}N_2O_2S$: 341.1249, found 341.1279.

Synthesis of PFP-lyso. Tert-butyl glycinate hydrochloride (0.9 g, 5.4 mmol) and sodium hydroxide (0.2 g, 5.4 mmol) were stirred in tert-butanol at room temperature for 1 h, then PCHO-lyso (0.6 g, 1.8 mmol) was added and stirring continued for 12 h at room temperature, 1.0 equivalent of *tert*-butyl (1-ethoxyethyl)glycinate was added, stirred at 50 ℃ for 12 h, extracted and dried, the crude product was purified by column chromatography on silica gel using acetate /petroleum ether *(v/v* = 1/1) as the eluent, in turn, to afford **PFP-lyso** as orange solid (yield 75%). ^{1}H NMR (600 MHz, $CDCl_3$, ppm): δ 7.98 (d, J = 1.4 Hz, 1H), 7.90 (dd, J =

8.5, 1.6 Hz, 1H), 7.15 (t, J = 7.7 Hz, 2H), 6.99 (s, 1H), 6.96-6.90 (m, 3H), 4.27 (s, 2H), 4.06 (s, 2H), 3.74 (s, 4H), 2.79 (s, 2H), 2.56 (s, 4H), 2.32 (s, 3H), 1.47 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$, ppm): δ 170.01, 166.66, 160.54, 146.47, 143.86, 136.82, 132.18, 130.81, 128.89, 127.54, 127.45, 126.77, 124.62, 124.12, 123.22, 115.53, 115.14, 83.10, 66.87, 60.40, 55.78, 53.91, 46.01, 42.16, 27.99, 21.07, 15.51, 14.22. HRMS (m/z): $[M+H]^+$ calc. for $C_{29}H_{34}N_4O_4S$: 535.23654, found 535.23343.

2. Photophysical properties of the photosensitizers

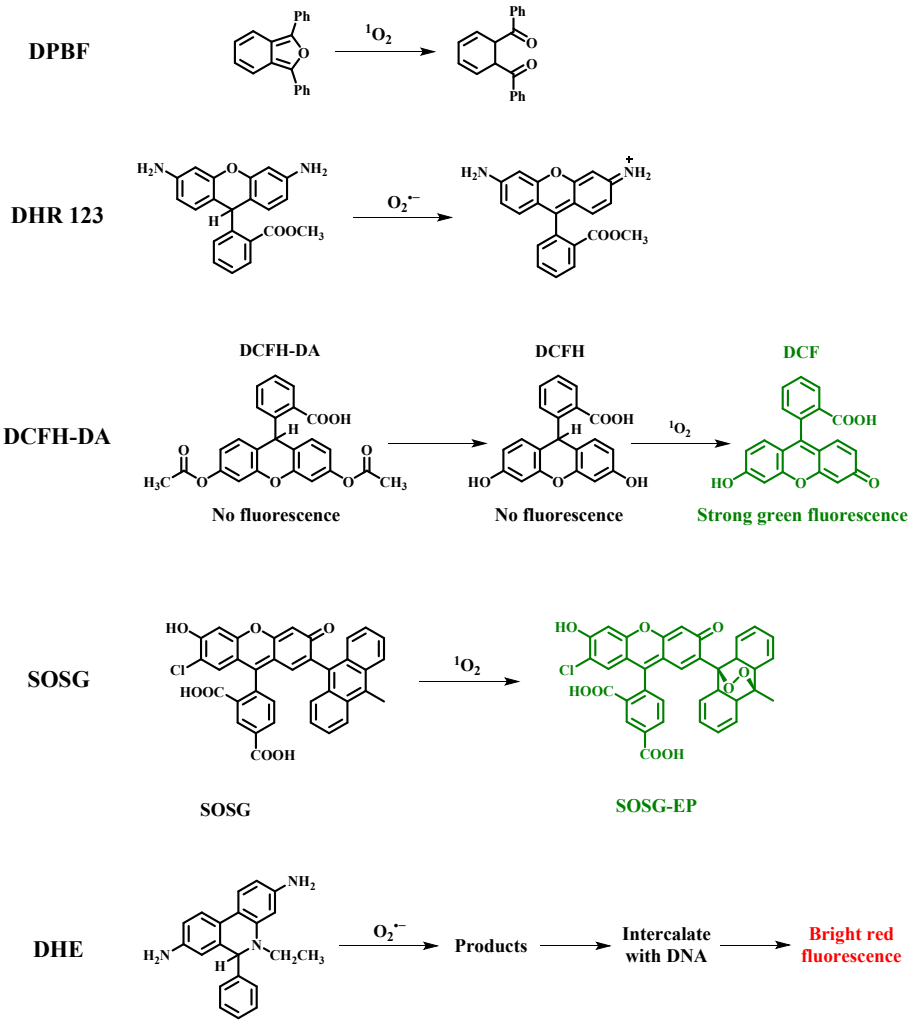


Figure S1. Reaction mechanisms of DPBF, DHR123, DCFH-DA, SOSG and DHE for the detection of general $^1O_2$, $O_2^{\bullet-}$ and ROS.

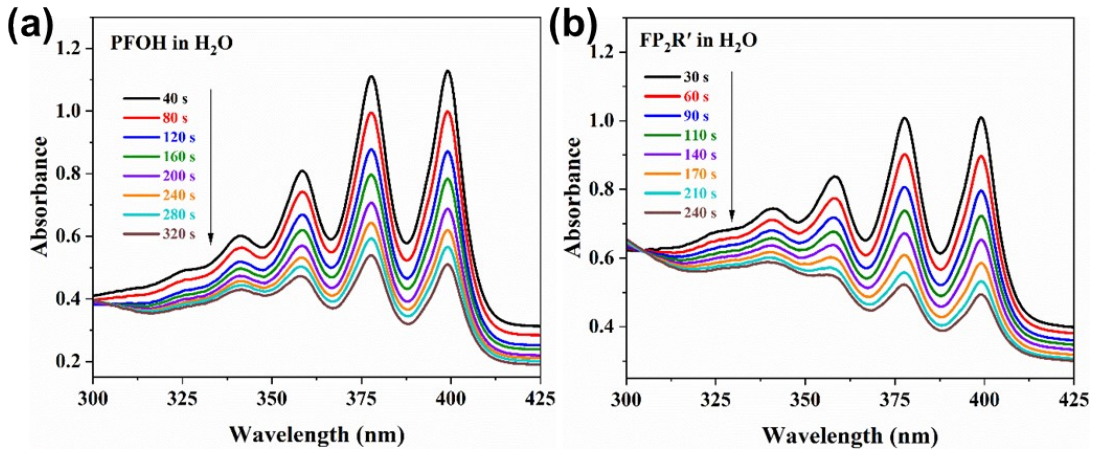


Figure S2. The $^1O_2$ detection of photosensitizer **FPOH** (a) and **FP$_2$R′** (b) in aqueous solution.

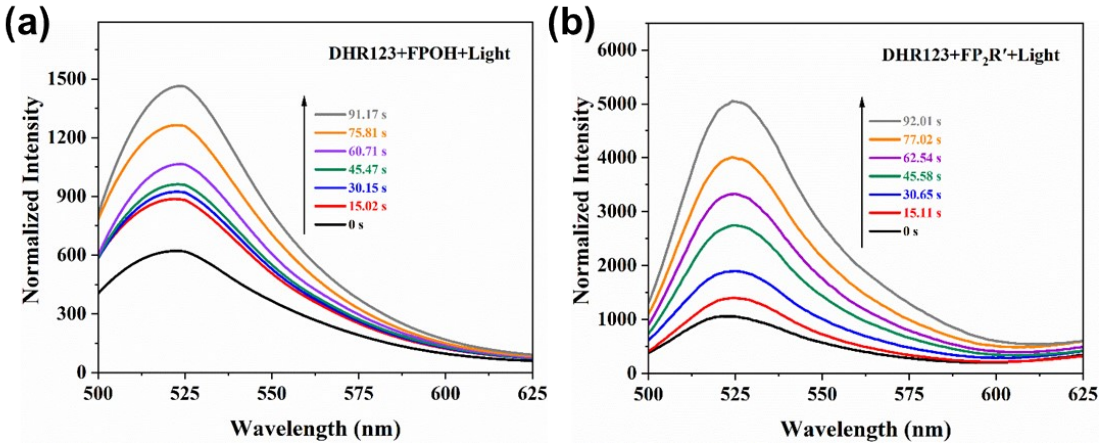


Figure S3. Fluorescence emission spectra of DHR123 for $O_2^{\bullet-}$ detection of photosensitizers **FPOH** (a) and **FP$_2$R′** (b).

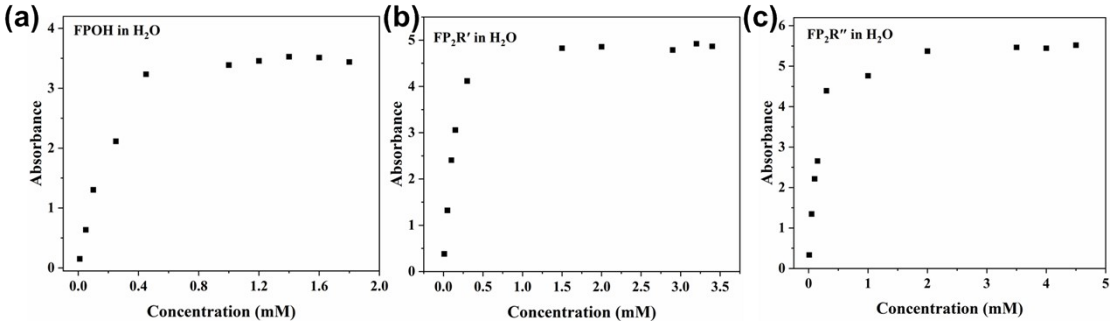
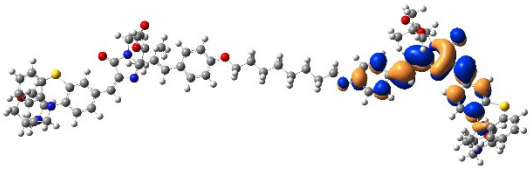
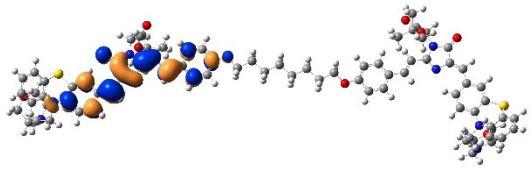
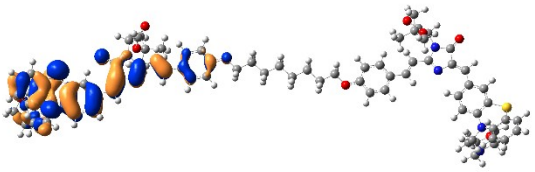
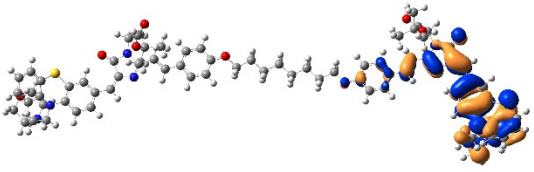
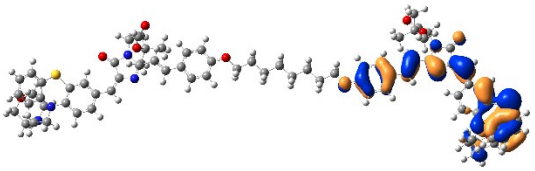


Figure S4. The absorbance of different concentrations of FPOH(a), FP$_2$R′(b) and FP$_2$R″(c) in water.

Table S1. Excited states, symmetry, transitions, wavelengths and oscillator strength of **FP_2R'**.

Excited State	Symmetry	Transitions	Energy (eV)	Wavelength	Osc. Strength (*f*)
S_1	Singlet-A	HOMO→LUMO	2.4090	514.68 nm	0.7879
T_3	Triplet-A	HOMO-5→LUMO-1	2.3054	537.80 nm	0.0000
T_1	Triplet-A	HOMO-1→LUMO	1.3845	895.55 nm	0.0000

Table S2. Calculated S_1/Tn energies of chromophore **FP_2R'** at the TD-CAM-B3LYP/6-31G(d,p).

States	Energy/eV	Electron Delocalization
LUMO-1	2.30	
LUMO	2.25	
HOMO	4.89	
HOMO-1	4.93	
HOMO-5	5.60	

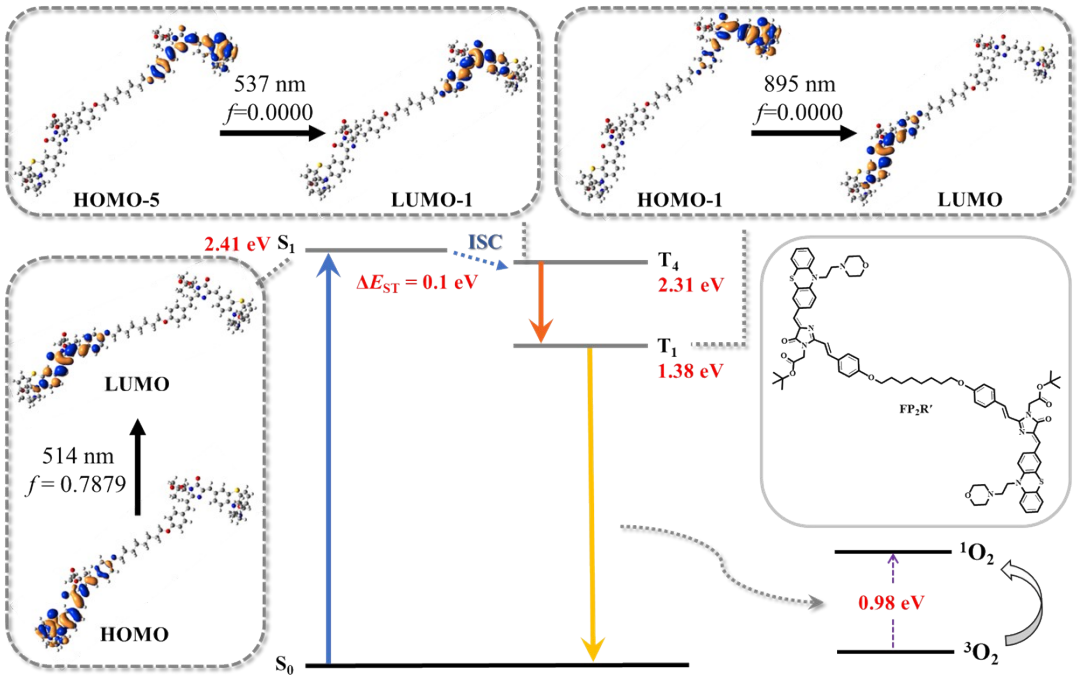
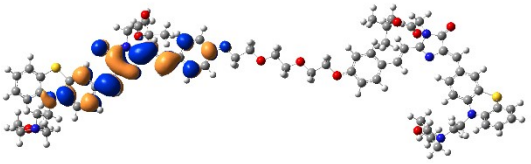
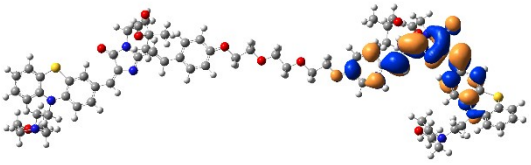
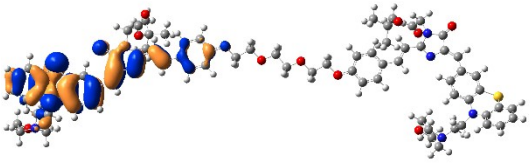
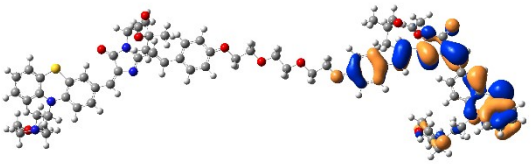


Figure S5. Frontier molecular orbitals and energies (eV) from DFT calculations of photosensitizers **FP_2R'.** (TD-CAM-B3LYP/6-31G(d,p))

Table S3. Excited states, symmetry, transitions, wavelengths and oscillator strength of **FP_2R''**.

Excited State	Symmetry	Transitions	Energy (eV)	Wavelength	Osc. Strength (*f*)
S_1	Singlet-A	HOMO→LUMO	2.4248	511.33 nm	0.8895
T_3	Triplet-A	HOMO-3→LUMO-1	2.3142	535.75 nm	0.0000
T_1	Triplet-A	HOMO→LUMO	1.3832	896.39 nm	0.0000

Table S4. Calculated S1/Tn energies of chromophore **FP$_2$R″** at the TD-CAM-B3LYP/6-31G(d,p).

States	Energy/eV	Electron Delocalization
LUMO	2.24	
LUMO-1	2.28	
HOMO	4.91	
HOMO-3	5.55	

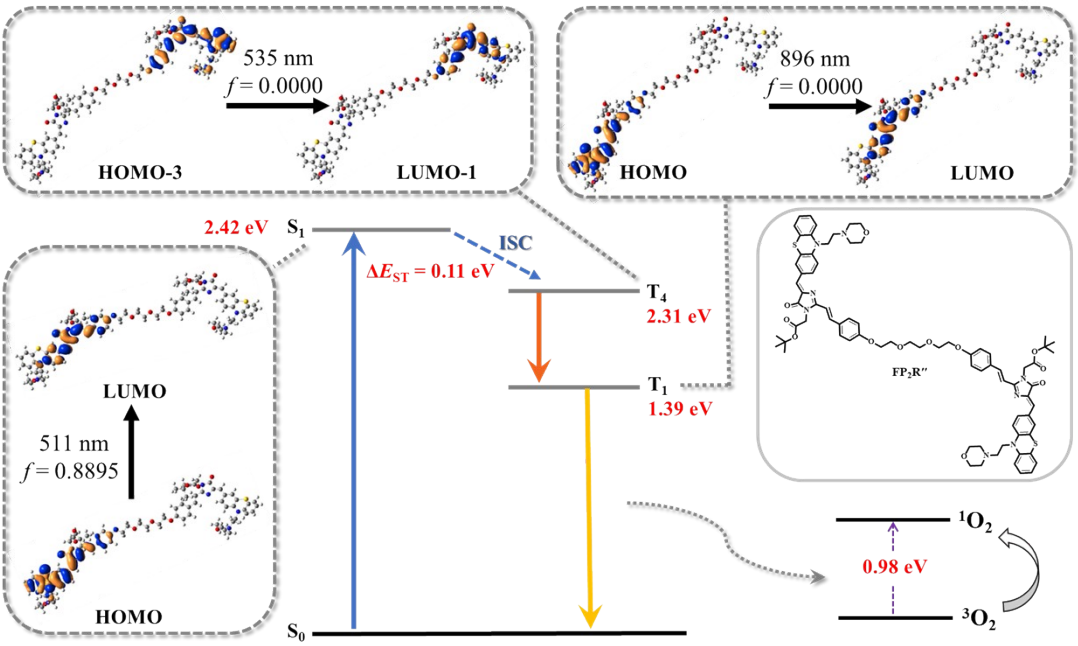


Figure S6. Frontier molecular orbitals and energies (eV) from DFT calculations of **FP$_2$R″.** (CAM-B3LYP/6-31G+g(d) level)

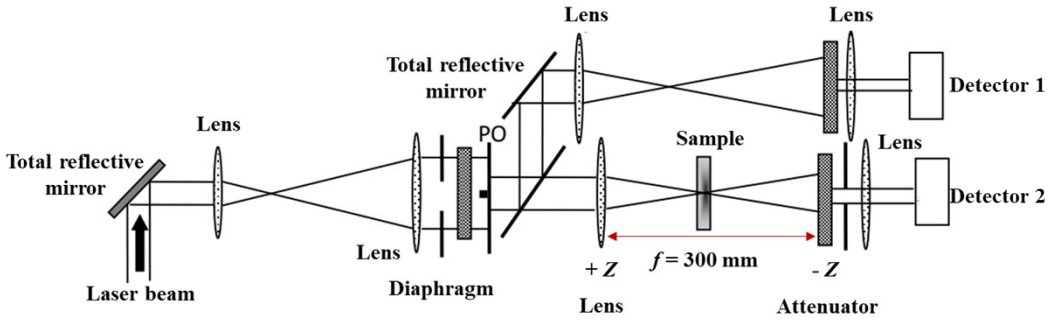


Figure S7. Schematic diagram of femtosecond open-aperture Z-scan setup in 2PA measurements.

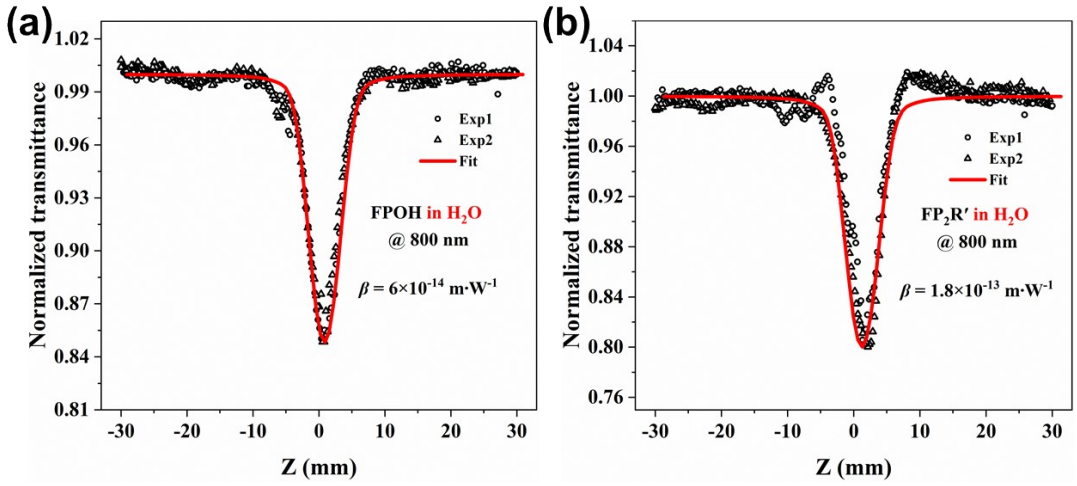


Figure S8. Normalized transmittance curves of photosensitizers 1 mM **FPOH** (a) and **FP_2R'** (b) in H_2O excited at fs-800 nm.

3. The dark/light cytotoxicity test in A-549 cells of photosensitizers

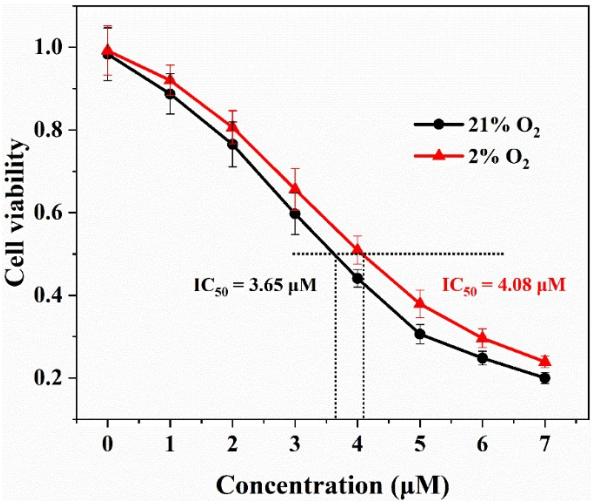


Figure S9. Cell growth curve of A549 cells treated with **FP_2R''** under light irradiation for 10 min (460 nm, 23 $mW \cdot cm^{-2}$).

4. NMR spectra of the compounds

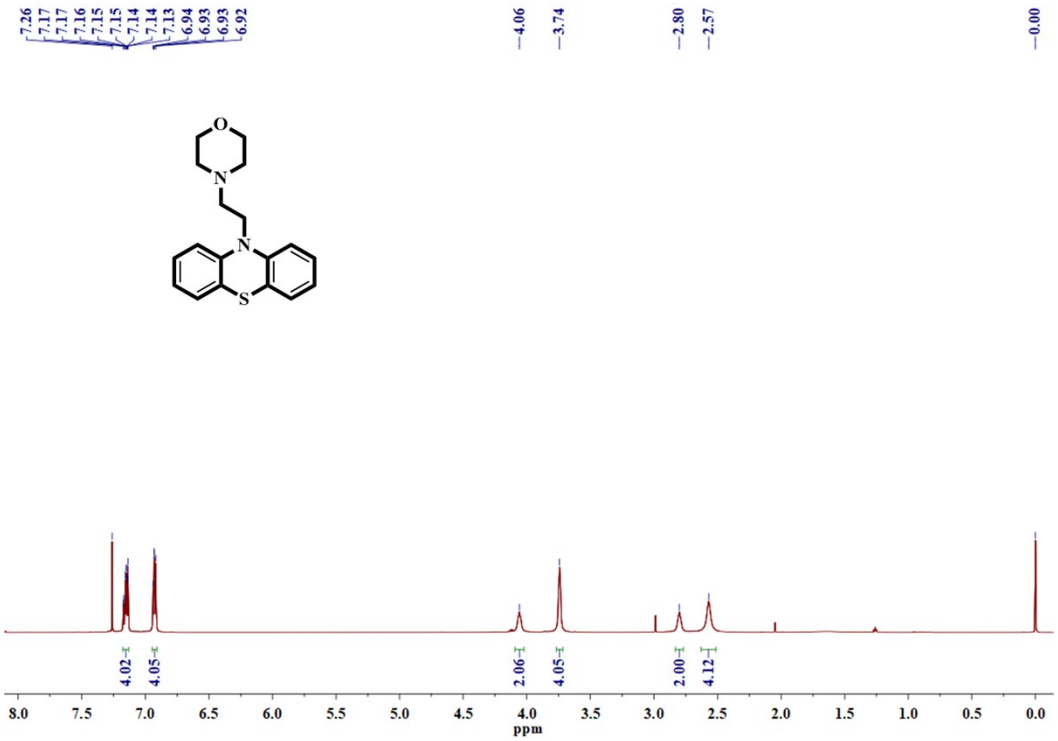


Figure S10. ^{1}H NMR spectrum of compound **Ptz-lyso**.

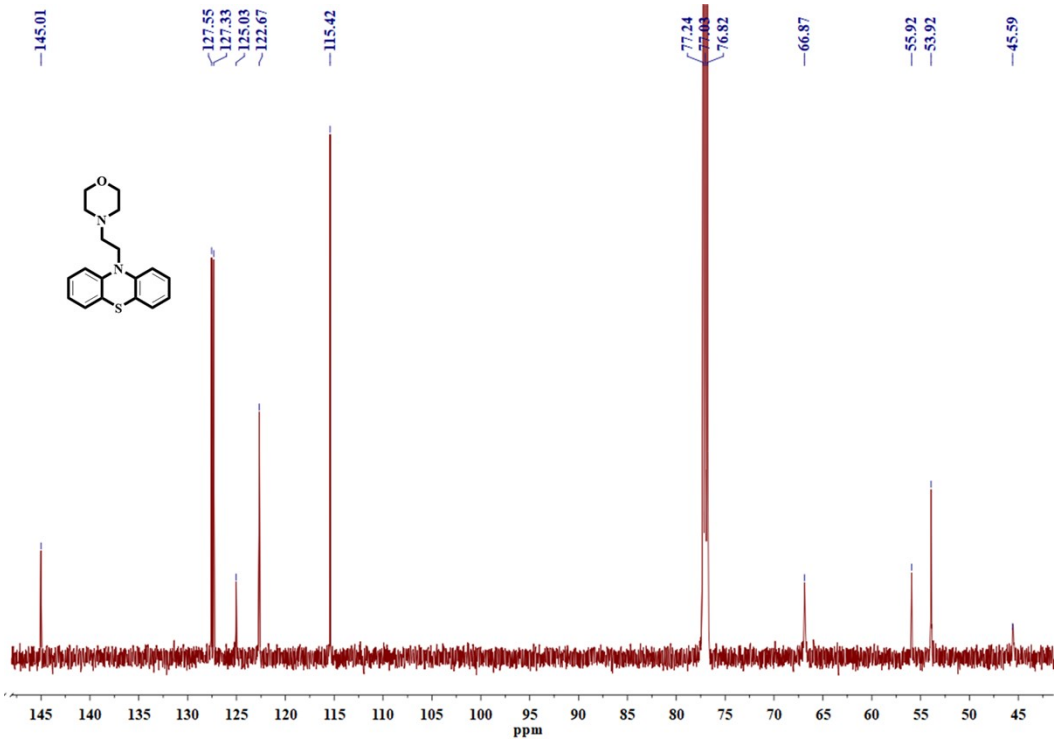


Figure S11. ^{13}C NMR spectrum of compound **Ptz-lyso**.

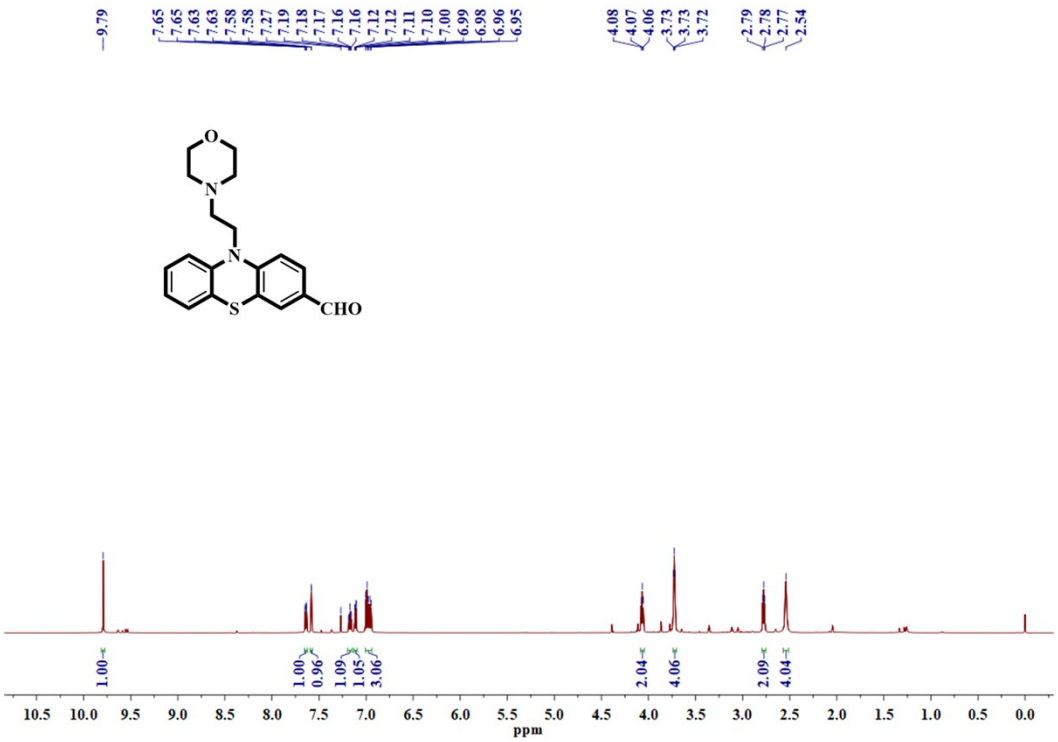


Figure S12. [1]H NMR spectrum of compound **PCHO-lyso**.

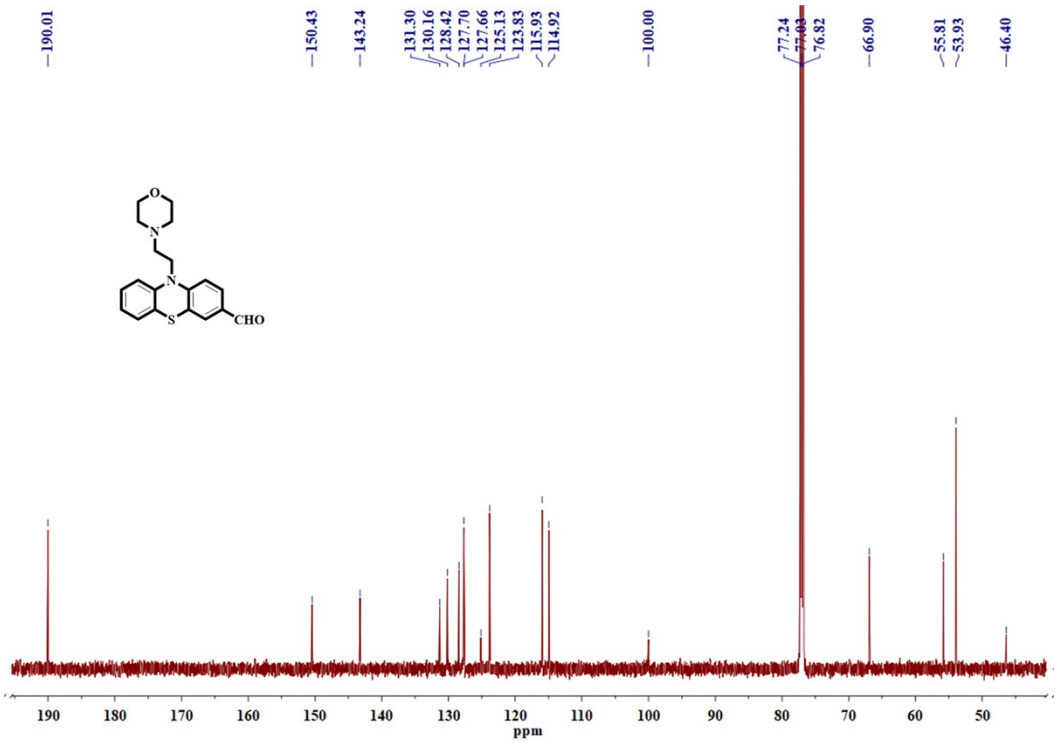


Figure S13. [13]C NMR spectrum of compound **PCHO-lyso**.

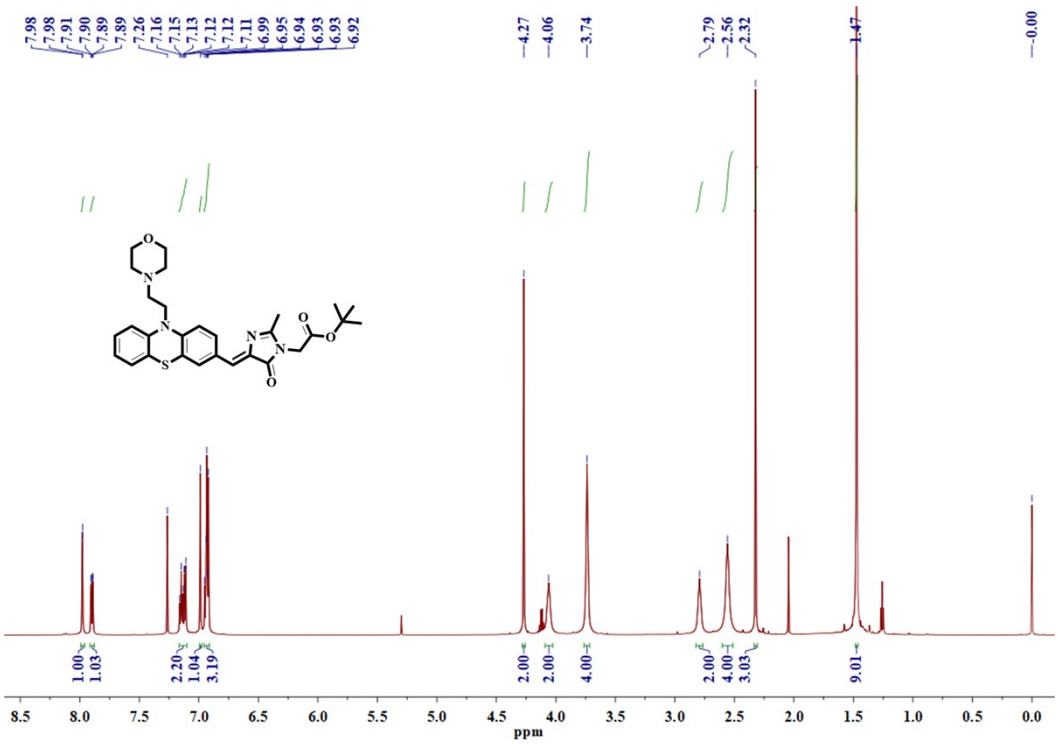


Figure S14. [1]H NMR spectrum of compound **PFP-lyso**.

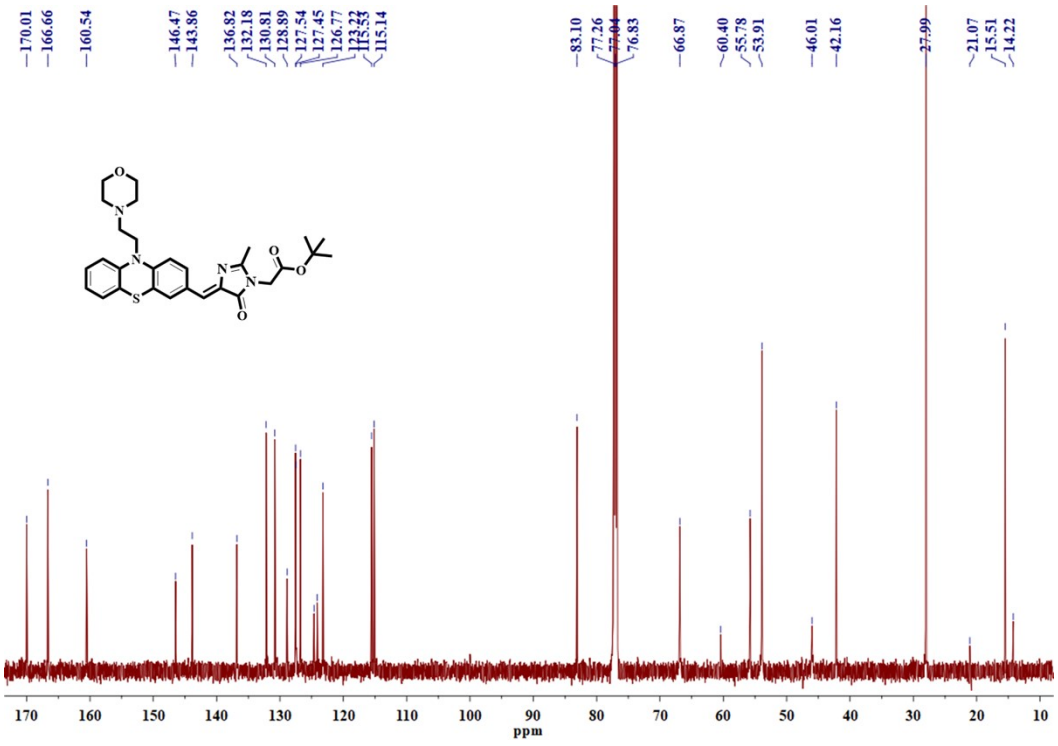


Figure S15. [13]C NMR spectrum of compound **PFP-lyso**.

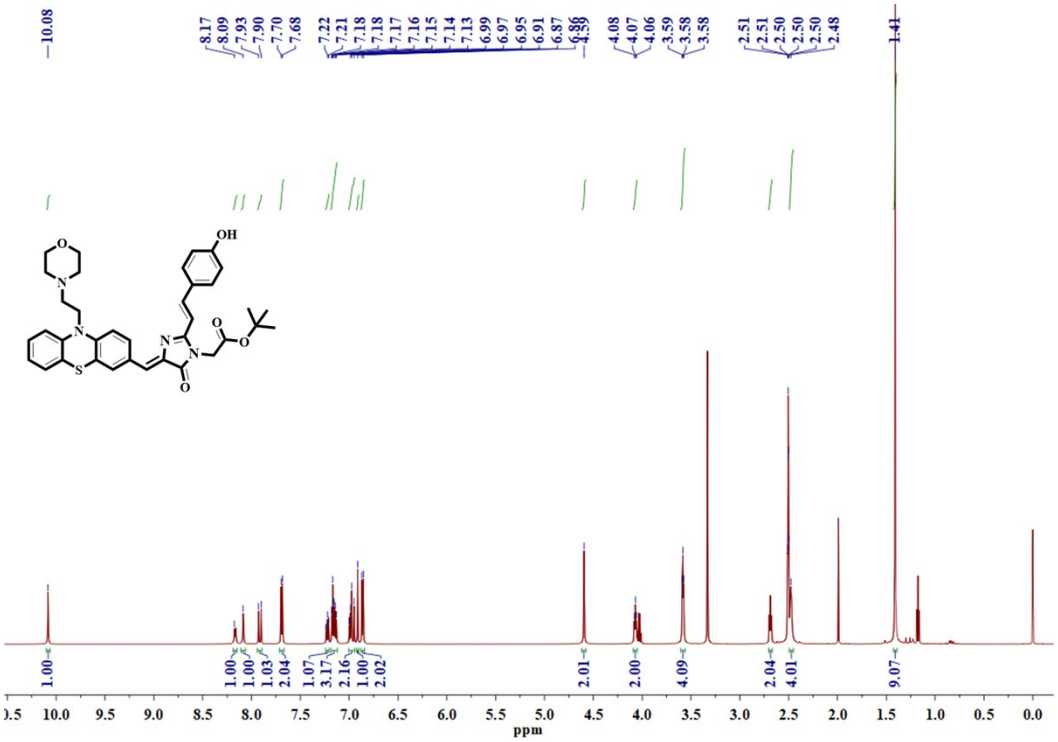


Figure S16. [1]H NMR spectrum of compound **FPOH**.

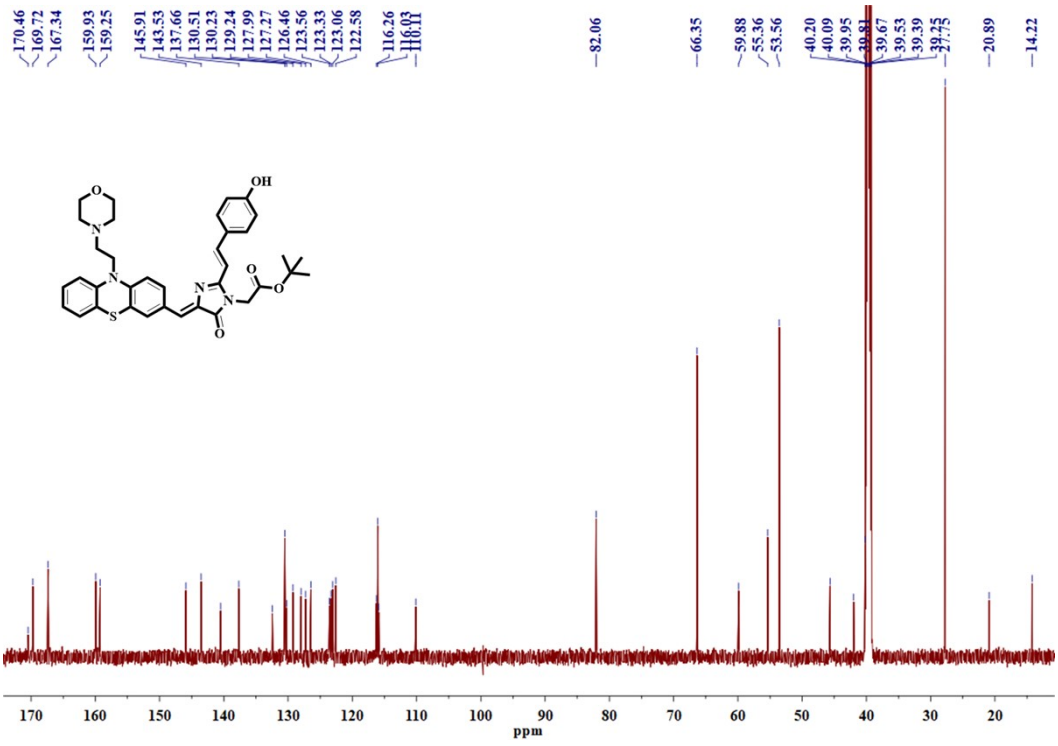


Figure S17. [13]C NMR spectrum of compound **FPOH**.

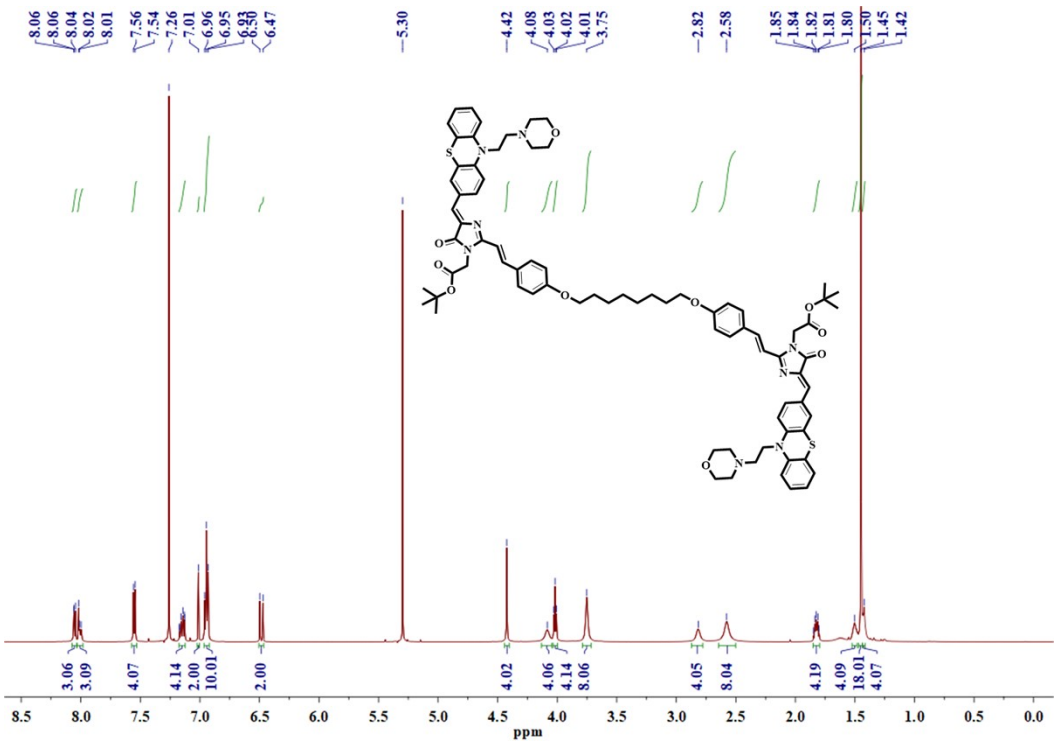


Figure S18. HRMS spectrum of compound **FP_2R'**.

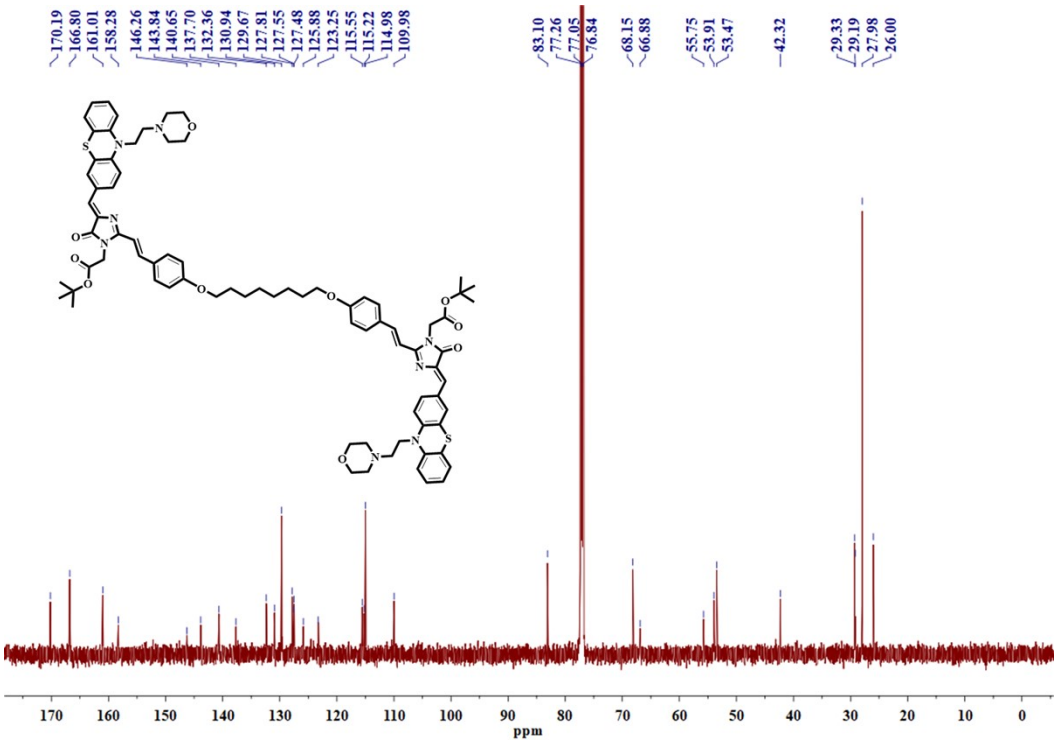


Figure S19. ^{13}C NMR spectrum of compound **FP_2R'**.

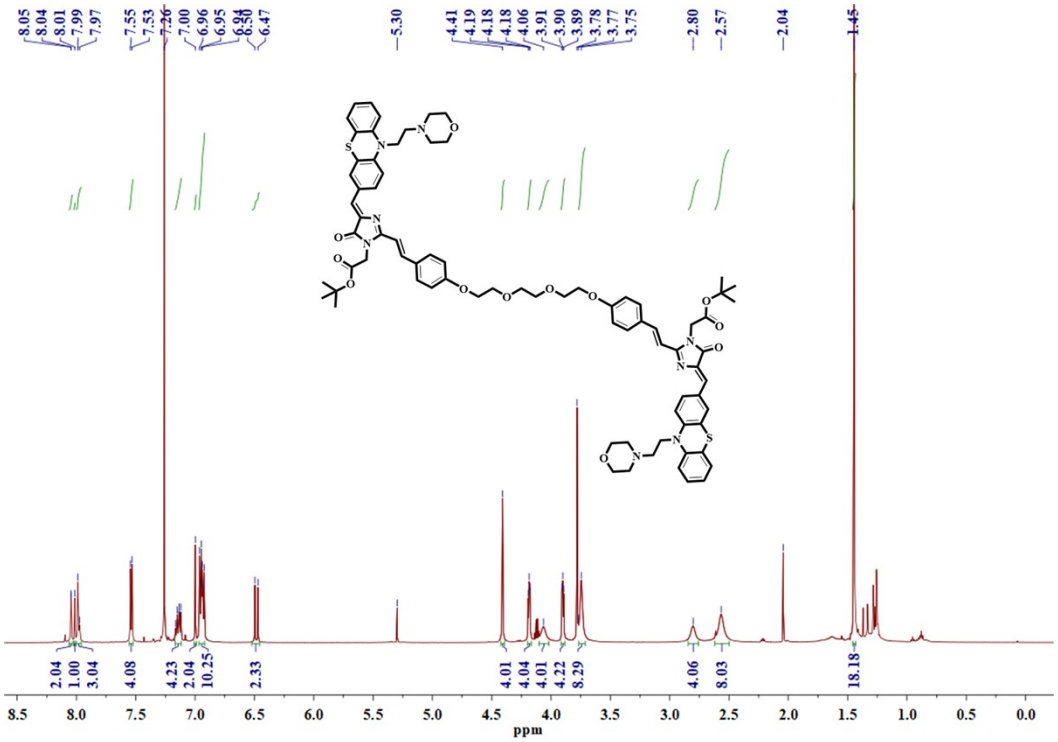


Figure S20. ^{1}H NMR spectrum of compound **FP_2R''**.

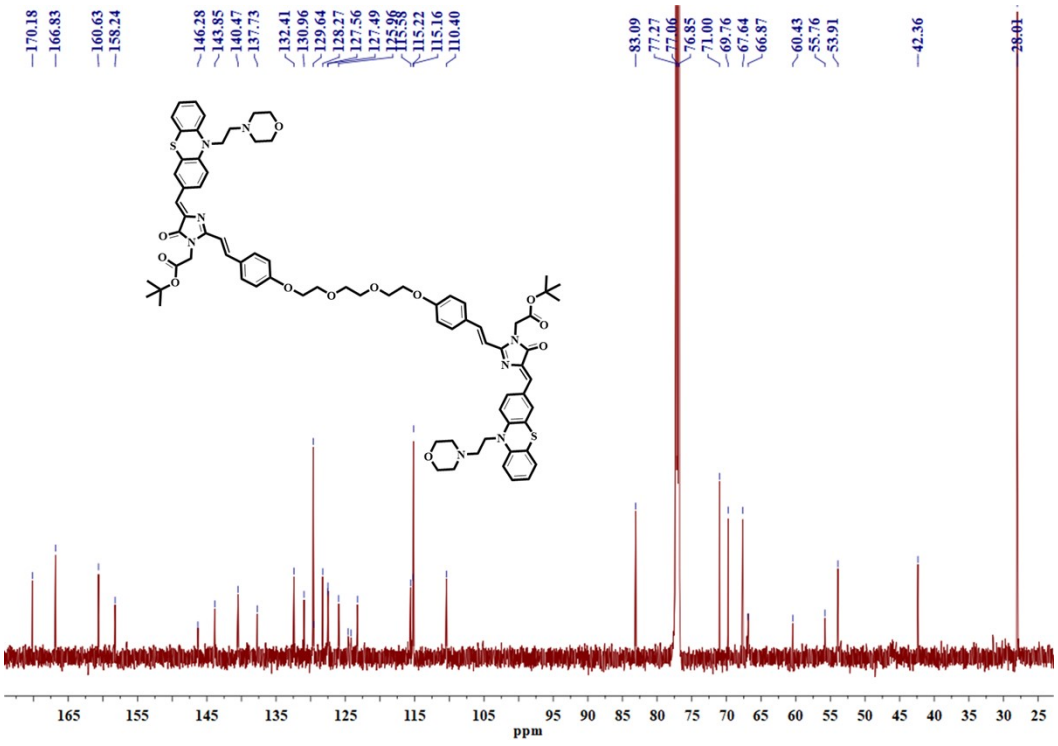


Figure S21. ^{13}C NMR spectrum of compound **FP_2R''**.

5. HRMS spectra of the compounds

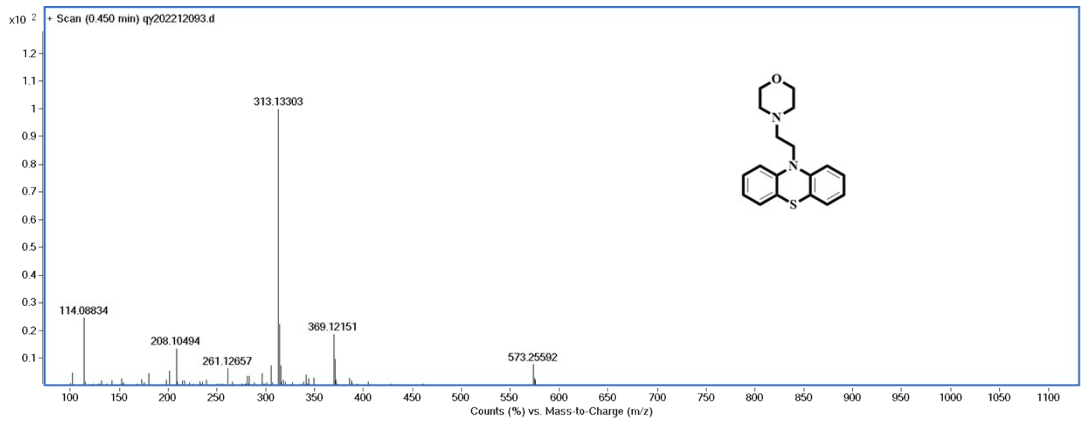


Figure S22. HRMS spectrum of compound **Ptz-lyso**.

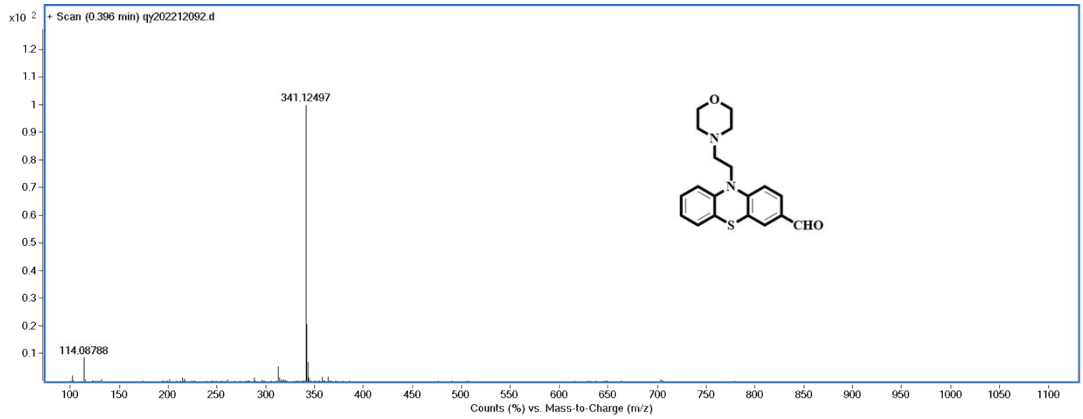


Figure S23. HRMS spectrum of compound **PCHO-lyso**.

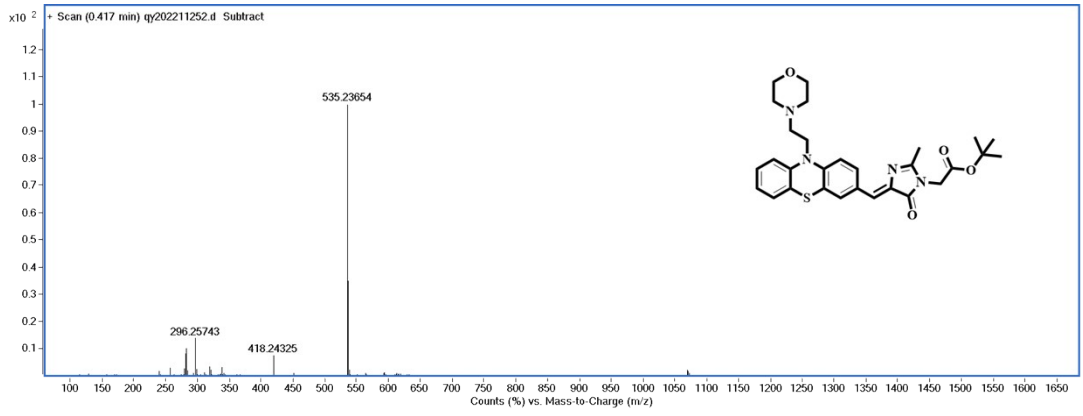


Figure S24. HRMS spectrum of compound **PFP-lyso**.

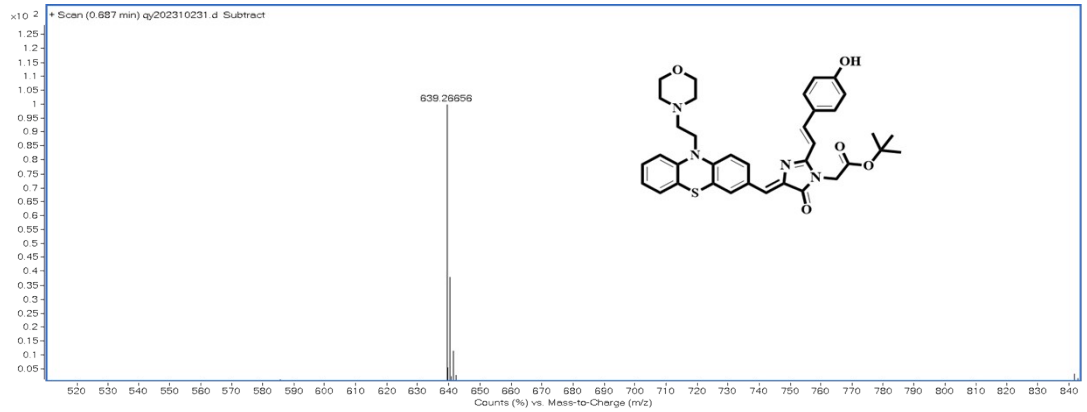


Figure S25. HRMS spectrum of compound **FPOH**.

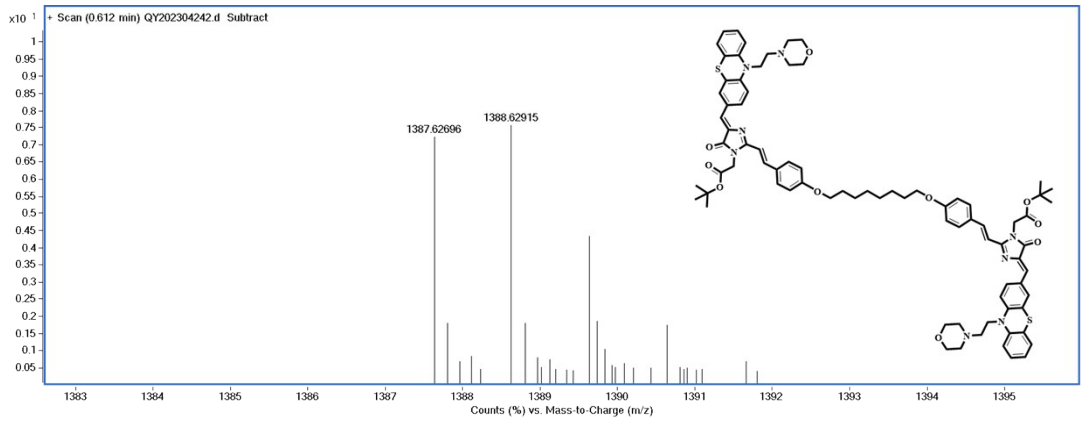


Figure S26. HRMS spectrum of compound **FP$_2$R′**.

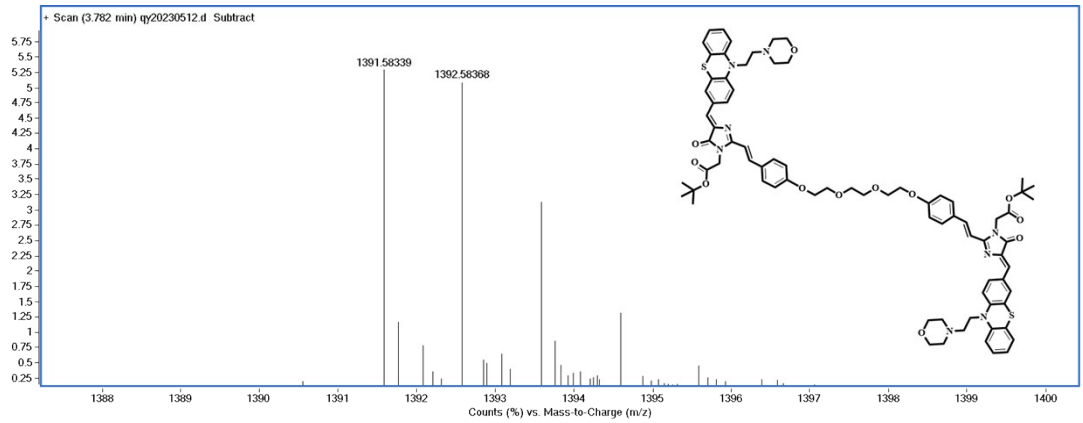


Figure S27. HRMS spectrum of compound **FP$_2$R″**.

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