Supporting Information

Fluorescent protein chromophore modified with aromatic heterocycles for photodynamic therapy and two-photon fluorescence imaging

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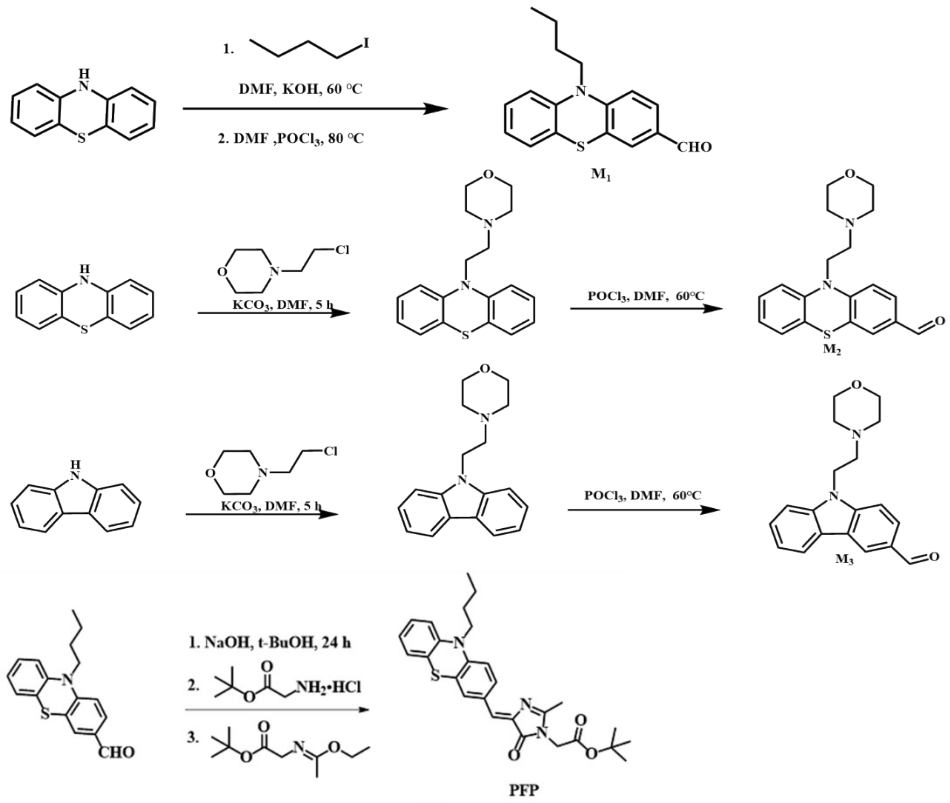
1. The synthesis of PFP, M_1, M_2 and M_3

The intermediate PFP was prepared according to the methods of published literature[1].

Intermediate M_1 was prepared from reference[1,2], phenothiazine and n-butyl iodide, heated to reflux at 60 °C, the solvent was concentrated by rotary distillation and set aside. Freezing salts were prepared using trichlorophosphorus with DMF in an ice water bath, subsequently the above concentrate was added to the freezing salts and refluxed at elevated temperature. After completion of the reaction the pH was adjusted until the solid was precipitated. The green solid was obtained by further column chromatographic purification..

The reaction process of intermediate M_2 was obtained with reference to the preparation of M_1. Dimethyl sulfoxide, 4-(2-chloroethyl)morpholine hydrochloride, phenothiazine and potassium carbonate were added to a round-bottomed flask and heated to reflux at 100 ℃. After the reaction was completed, the solution was extracted with ethyl acetate and brine, and the solvent was concentrated by rotary distillation, and was set aside. The prepared lyophilized salt was mixed with the solution and refluxed at elevated temperatures. After completion of the reaction, the pH was adjusted until the solid precipitated. The yellow-green solid was obtained by further column chromatographic purification..

The synthesis of intermediate M_3 was identical to that of M_2 and the milky white solid was prepared by using carbazole instead of phenothiazine.



Scheme S1. Synthesis route of PFP, M_1, M_2 and M_3

2. The experiment procedure of singlet oxygen yield test. [3,4]

The singlet oxygen production of the photosensitizer was determined by using 1,3-Diphenylisobenzofuran (DPBF) as the scavenger for singlet oxygen and the reference $Ru(bpy)_3Cl_2$ as the standard. First, the absorbance of the photosensitizer was adjusted to 0.2-0.3. Then, DBPF was added to the above solution to adjust the absorbance to about 1. The mixed solution was irradiated under 460 light (20 mW/cm^2), and the change in the absorbance of the solution at 415 nm was recorded. Finally, the slope of singlet oxygen yield was obtained by taking the decreased value of absorbance at 415 nm as horizontal and the time interval as vertical coordinates. The singlet oxygen yield is calculated according to the following formula.

$$\Phi_{\Delta} = \Phi_{\Delta\,(ref)} \times \frac{K_{PSs}}{K_{ref}} \times \frac{F_{ref}}{F_{PSs}}$$

Φ_{Δ} represents the singlet oxygen yield. $\Phi_{\Delta\,(ref)}$ represents the singlet oxygen quantum yield of the reference $Ru(bpy)_3Cl_2$. k represents the slope between the absorbance and its corresponding time. F is the absorption correction factor and $F=1-10^{-OD}$ and OD is the absorbance of the solution at the irradiation wavelength (460 nm). PSs are dyes. Ref is the reference $Ru(bpy)_3Cl_2$.

3. The experiment procedure of the MTT assay test. [1,3,4]

A549 cells were used in this work, and they came from the American Type Culture Collection (ATCC). The medium is changed every other day. For MTT, cells should be kept in 96-well plates with about 1×10^4 cells per well, and cultured for 24 hours at 37°C (standard culture condition) in 95% air and 5% CO_2. For confocal laser scanning microscope (CLSM) analysis, cells were divided into 35 mm Petri dishes. Then the cells were incubated for 24 h under standard culture conditions, and the density should be kept at about 1.0×10^5 cells per dish. For the wound scratch test, cells were planted in 6-well plates under standard culture conditions, and the density of each well plate was maintained at about 1×10^5 cells in each Petri dish.

A549 cells (5000 cells/well) were seeded into the 96-well plate and placed in an incubator containing a 5 % CO_2 atmosphere for 24 h at 37 °C. Take out the old medium and add freshly cultured medium. Then the prepared photosensitizers were added to the 96-well plate at concentrations of 1, 2, 3, 4, 5, and 6 μM and incubated for 24 h.

Dark toxicity: In the dark, MTT solution (100 μL/well) with a concentration of 5 mg/mL was added to the 96-well plate and incubated for 4 h. Then 100 μL of DMSO solution was added to each well and incubated for 2 h. Finally, the absorbance was tested by a microplate reader, and the cell viability was calculated.

Phototoxicity test: The 96-well plate was irradiated under 460 nm light (20 mW/cm^2) for 10 min respectively, and incubated for 12 h. MTT solution (100 μL/well) with a concentration of 5 mg/mL was added to the 96-well plate and incubated for 4 h. Then 100 μL of DMSO solution was added to each well and incubated for 2 h. Finally, the absorbance was tested by a microplate reader, and the cell viability was calculated.

Cell viability (%) = A_1/A_2, here A_1 represents the experimental absorbance value at 490 nm, and A_2 represents the control absorbance value at 490 nm.

4. The experiment procedure of fluorescence imaging in cells and zebrafish. [1.3]

In the cell fluorescence imaging, the A549 cells were seeded into a petri dish with 2 mL 1640 culture medium. After 24 h of cell cultivation, 10 μL photosensitizer (10^{-3} M) solution was added to the petri dish and incubated with cells for 4 h. Then, the fluorescence imaging was performed on Olympus FV3000 laser scanning confocal microscope.

The purchased larval zebrafish were incubated in a water bath at room temperature for two days. Then zebrafish were evenly divided into each confocal dish (6-7 fish/dish). Before taking fluorescence imaging, a small amount of anesthetic was added to the confocal dish to inhibit zebrafish movement. Then fluorescence imaging was taken by confocal laser scanning microscopy (laser: Ti: Sapphire laser, pulse duration: 100 fs, focal plane of 800 nm: 40 mW/cm^2).

5. The experimental procedures of singlet oxygen capture in cells and zebrafish.[3, 4]

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was used as the intracellular $^1O_2$ indicator, which can be converted to 2',7'-Dichlorofluorescein (DCF) and emit bright green fluorescence in the presence of $^1O_2$, A 549 cells cultured in 1640 incomplete medium were inoculated in a 20 mm confocal dish. The cells containing photosensitizer (1 μM)) were incubated for 1 h in the dark and treated with DCFH-DA, and incubated for another 30 minutes. The medium was discarded and washed three times with PBS buffer. Here, a controlled experiment was performed to exclude the effects of the presence of light and DCFH-DA alone. Then use a confocal laser scanning microscope (CLSM, DCFH-DA: E_x: 488 nm, E_m: 488-520 nm) to image cells in the green and red channels. Experiments in the zebrafish system were similar to the cellular steps.

6. The experiment procedure of AO/EB staining test and lysosomal localization.[5,6]

The prepared confocal culture dish containing 2 μM photosensitizer was irradiated for 5 min and 15 min under 460 nm light (20 mW/cm^2), respectively. And then placed in the incubator for 2 h. The diluted AO/EB stain was added and continued to incubate for 30 min. The culture dish was taken out and washed three times with PBS buffer solution. In the end, it was placed under the confocal laser microscope for imaging. All of the above procedures were done in the dark. After 24 hours of incubation, A549 cells were stained with dye (2 μM) and Lyso-tracker Blue (1 μM), and different fluorescent colors were given to show the position of lysosomes. Cells were evenly inoculated on confocal Petri dishes (3×10^5 cells/dish). Cells with dye (2 μM) were incubated in the dark for 40 minutes. Lyso-Tracker Blue (0.3 microns) was then added to the culture medium. Incubate for another 20 minutes and wash with PBS buffer solution three times. Then, confocal laser scanning microscope (CLSM, Lyso-Tracker Blue) was used to image the cells in the blue and red channels. Mitochondrial localization follows the same method, using Mito-tracker Green instead.

7. ^{1}H NMR, ^{13}C NMR and HRMS spectra of photosensitizer PFPT

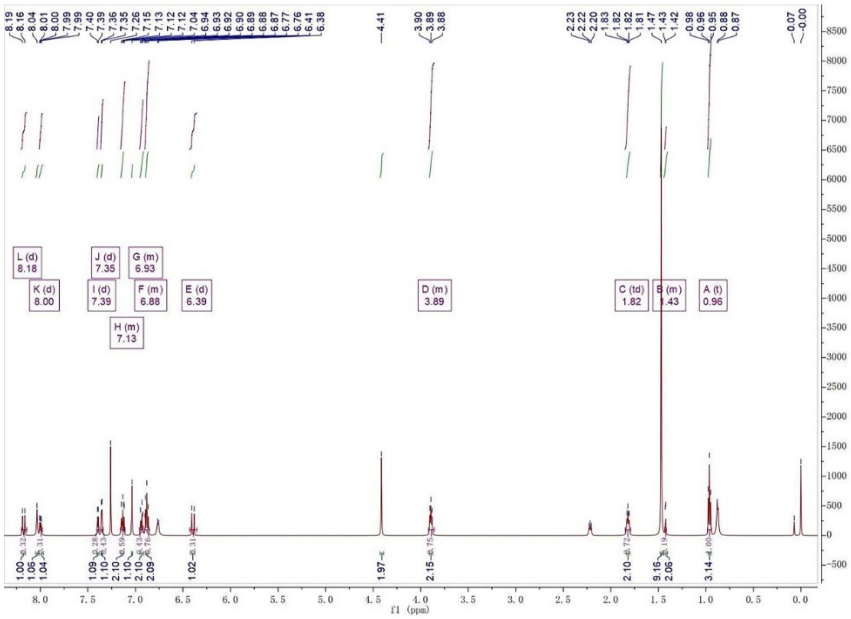


Fig. S1 ^{1}H NMR spectra of photosensitizer PFPT

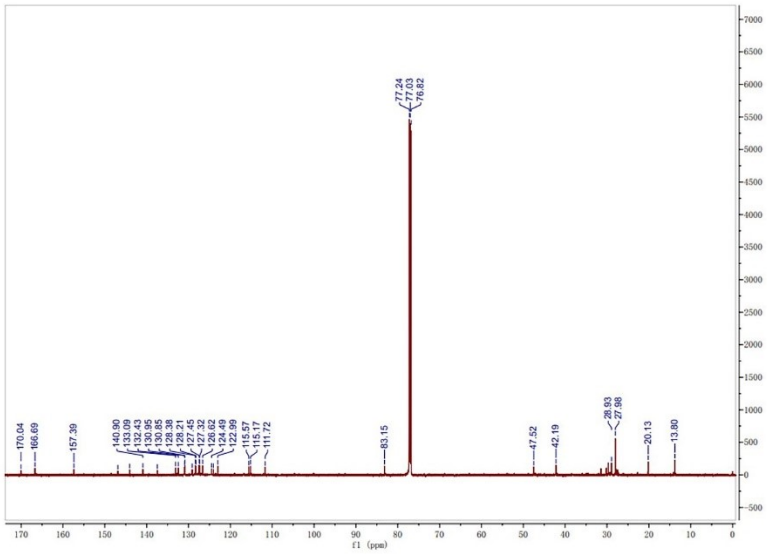


Fig. S2 ^{13}C NMR spectra of photosensitizer PFPT

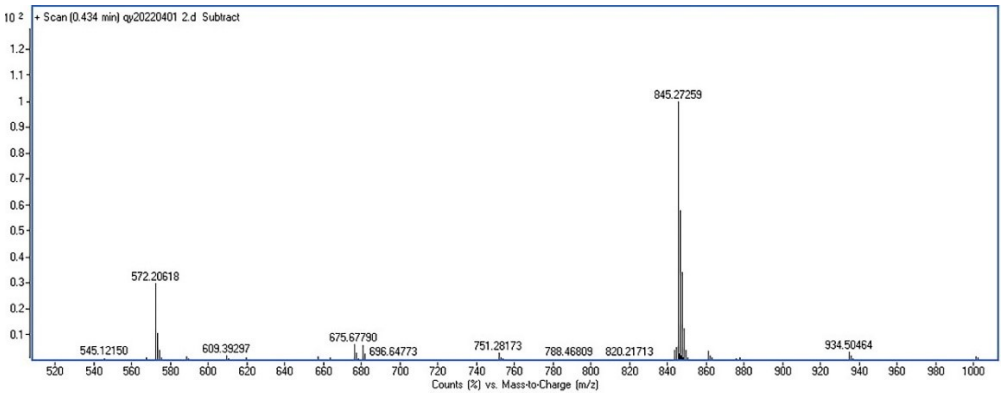


Fig. S3 HRMS spectra of photosensitizer PFPT

8. ^{1}H NMR, ^{13}C NMR and HRMS spectra of photosensitizer PFPP

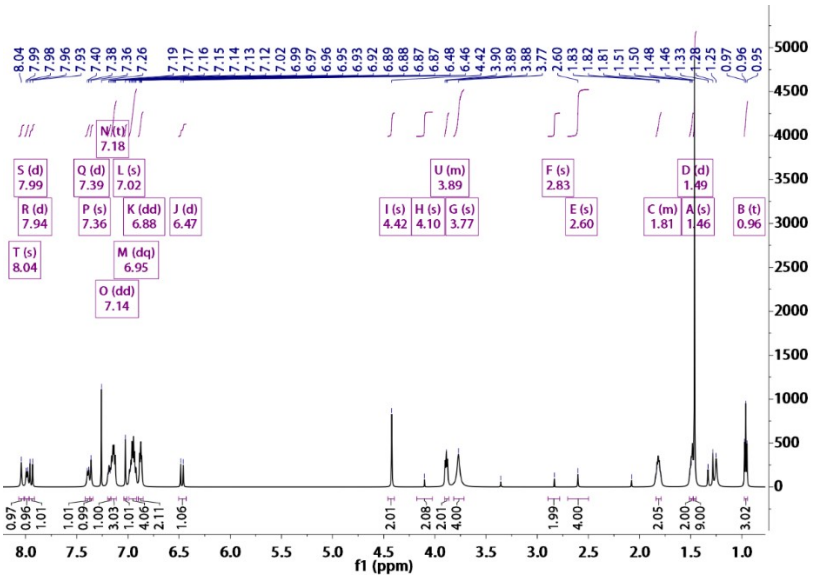


Fig. S4 ^{1}H NMR spectra of photosensitizer PFPP

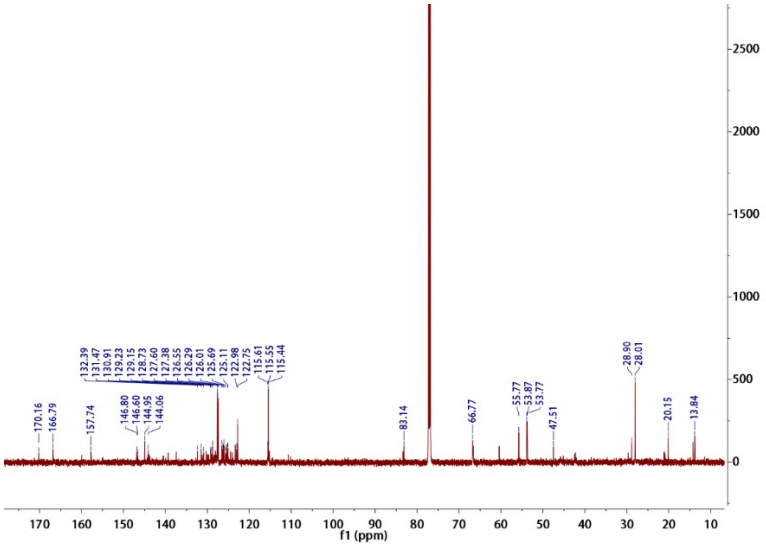


Fig. S5 ^{13}C NMR spectra of photosensitizer PFPP

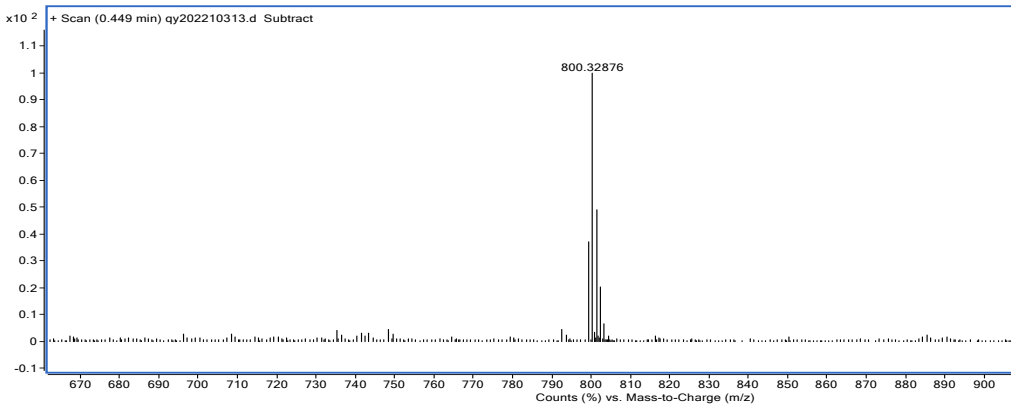


Fig. S6 HRMS spectra of photosensitizer PFPP

9. ^{1}H NMR, ^{13}C NMR and HRMS spectra of photosensitizer PFPC

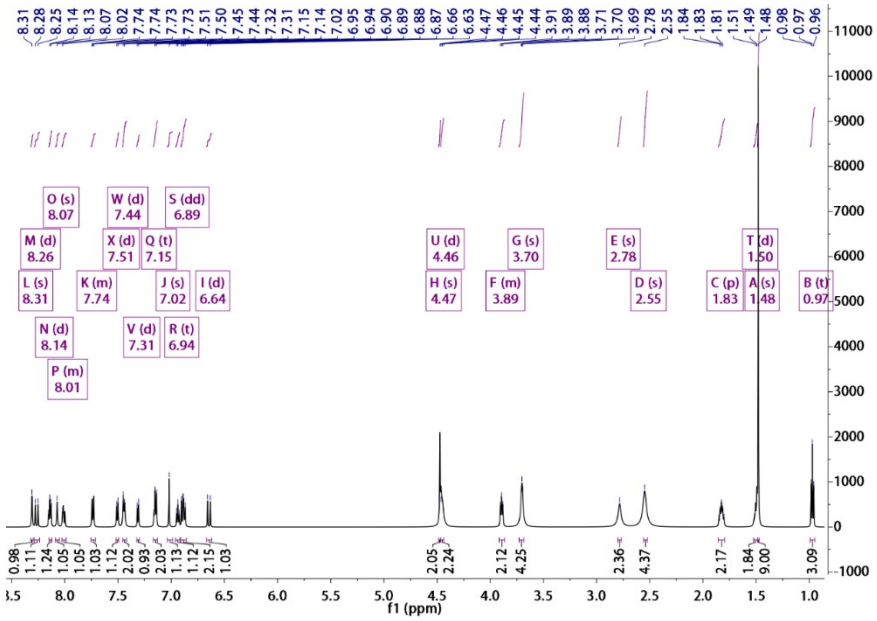


Fig. S7 ^{1}H NMR spectra of photosensitizer PFPC

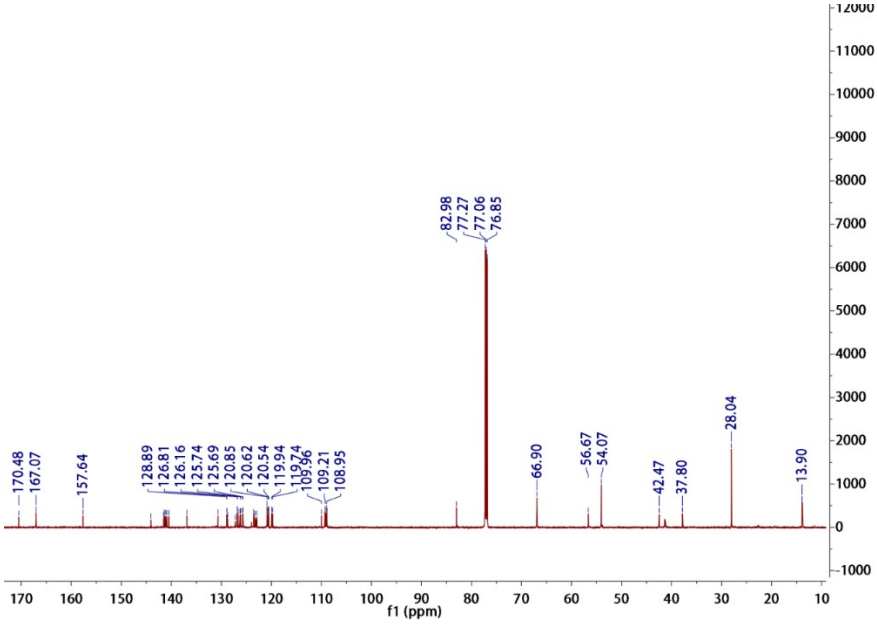


Fig. S8 ^{13}C NMR spectra of photosensitizer PFPC

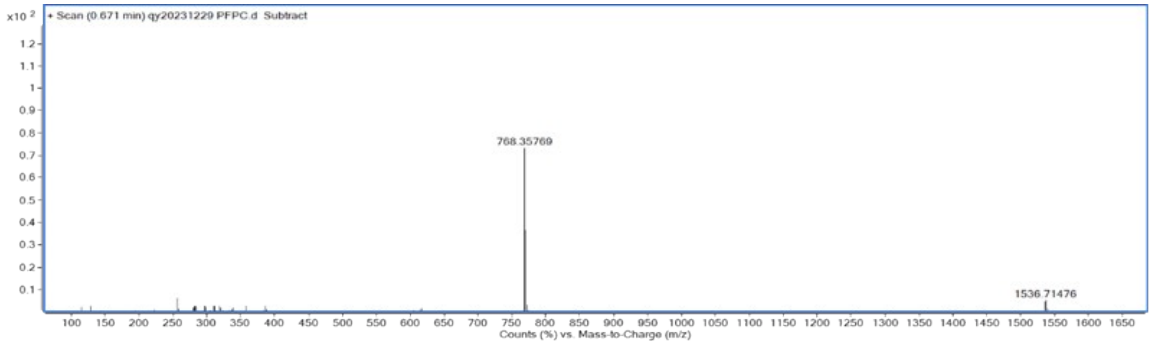


Fig. S9 HRMS spectra of photosensitizer PFPC

10. Theoretical calculations of photosensitizers

DFT calculations were conducted to obtain optimized structure and excited states energy of photosensitizers. Calculations were performed by Gaussian 09 software. The optimized structure of photosensitizers was obtained by the DFT//cam-B3LYP/6-31G(d) method and the excited states energy of photosensitizers was calculated by TD-DFT//cam-B3LYP/6-31G (d,p) method. The detailed data of excited states is displayed in **Table S1**.

Table S1 Detailed data in DFT calculation of photosensitizers

Compound	Excited states		Energy (eV)/λ (nm)	f[a]	Composition[b]	$\triangle E_{ST}$
PFP	Singlet	S_1	3.50/353	0.7525	H→L	0.36eV
		S_2	4.14/299	0.2456	H-1→L	
	Triplet	T_1	1.77/698	0.0000	H→L	
		T_2	3.16/392	0.0000	H-3→L	
		T_3	3.26/368	0.0000	H-1→L	
PFPT	Singlet	S_1	2.93/422	0.6925	H→L	0.15 ev
		S_2	3.71/334	0.2288	H-1→L	
	Triplet	T_1	1.11/1117	0.0000	H→L	
		T_2	2.78/567	0.0000	H-3→L	
		T_3	2.98/416	0.0000	H-1→L	
PFPC	Singlet	S_1	2.85/508	0.6913	H→L	0.12ev
		S_2	3.54/442	0.3214	H-1→L	
	Triplet	T_1	1.38/905	0.0000	H→L	
		T_2	2.73/589	0.0000	H→L+1	
		T_3	2.91/420	0.0000	H-1→L	
PFPP	Singlet	S_1	2.29/539	0.7512	H→L	0.10ev
		S_2	3.42/451	0.2245	H-1→L	
	Triplet	T_1	1.35/912	0.0000	H→L	
		T_2	2.19/564	0.0000	H-1→L	
		T_3	2.99/413	0.0000	H-2→L	

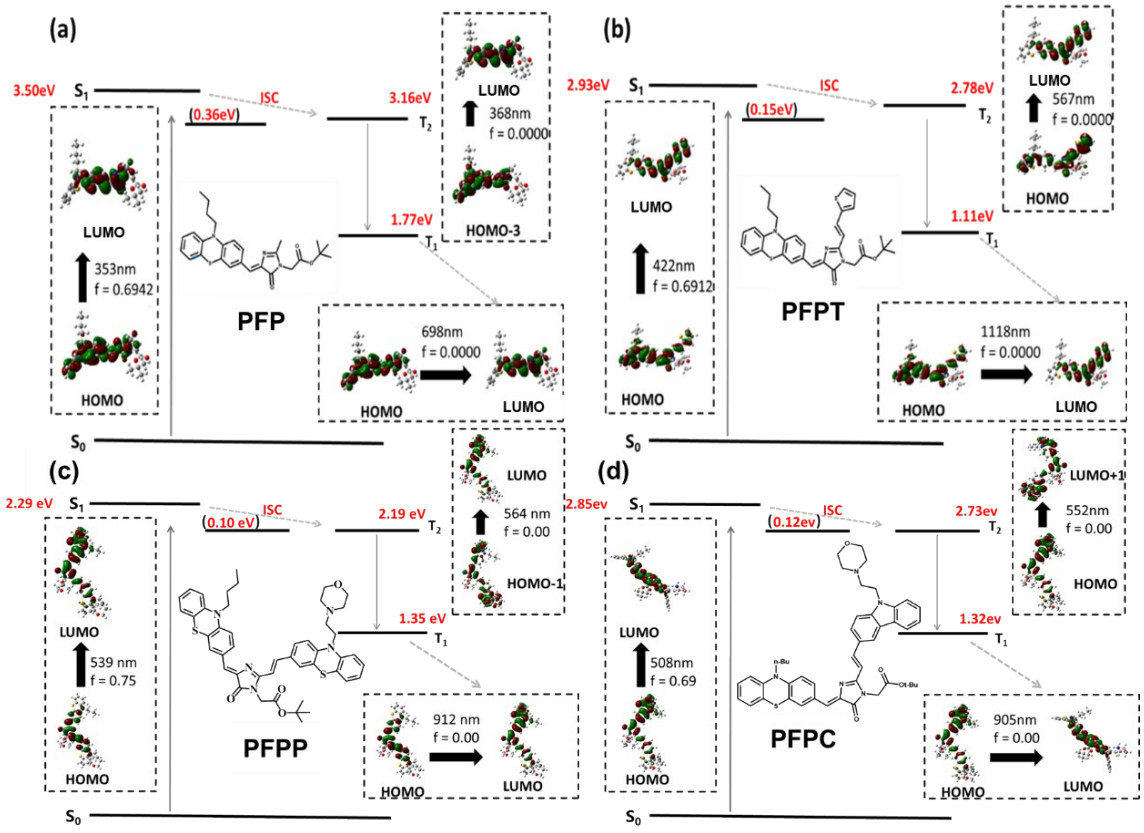


Fig. S10 (a) Excited state energy diagram of PFP obtained by TD-DFT//cam-B3LYP/6-31G (d, p) method based on optimized structure calculated by DFT//cam-B3LYP/6-31G(d) method. (b) Excited state energy diagram of PFPT obtained by TD-DFT//cam-B3LYP/6-31G (d, p) method based on optimized structure calculated by DFT//cam-B3LYP/6-31G(d) method. (c) Excited state energy diagram of PFPC obtained by TD-DFT//cam-B3LYP/6-31G (d, p) method based on optimized structure calculated by DFT//cam-B3LYP/6-31G(d) method. (d) Excited state energy diagram of PFPP obtained by TD-DFT//cam-B3LYP/6-31G (d, p) method based on optimized structure calculated by DFT//cam-B3LYP/6-31G(d) method.

11. UV-vis absorption and fluorescence emission spectra of PFP.

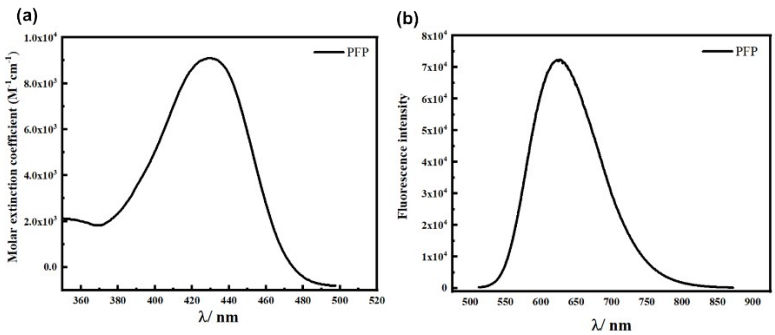


Fig. S11 (a) The UV-Vis absorption of photosensitizers PFP; (b) The fluorescence emission spectra of photosensitizers PFP. (1.0×10^{-5} M in methanol).

12. Determination of singlet oxygen in photosensitizers PFPT and PFPC

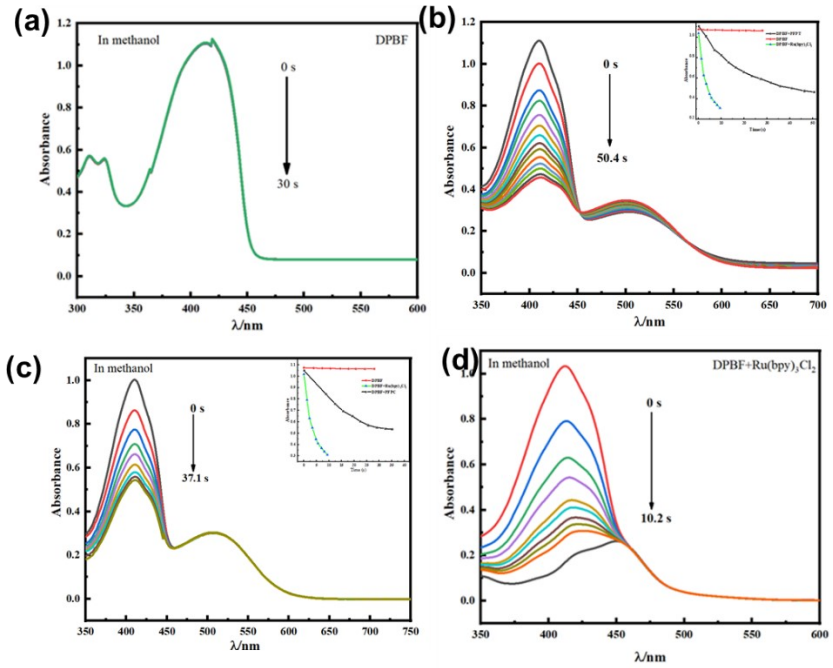


Fig.S12. (a) UV-visible spectra of DPBF solution under light radiation; The inset is the plot of change in absorption of DPBF at 415 nm versus time under light radiation (DPBF, DPBF+PFPT, DPBF+$Ru(bpy)_3Cl_2$). (b) UV-visible spectra of DPBF in PFPT solution under light radiation. (c) UV-visible spectra of DPBF in PFPC solution under light radiation; The inset is the plot of change in absorption of DPBF at 415 nm versus time under light radiation (DPBF, DPBF+PFPC, DPBF+$Ru(bpy)_3Cl_2$). (d) UV-visible spectra of DPBF in $Ru(bpy)_3Cl_2$ solution under light radiation. (Light: 460 nm, 20 mW/cm^2, Solvent: methanol).

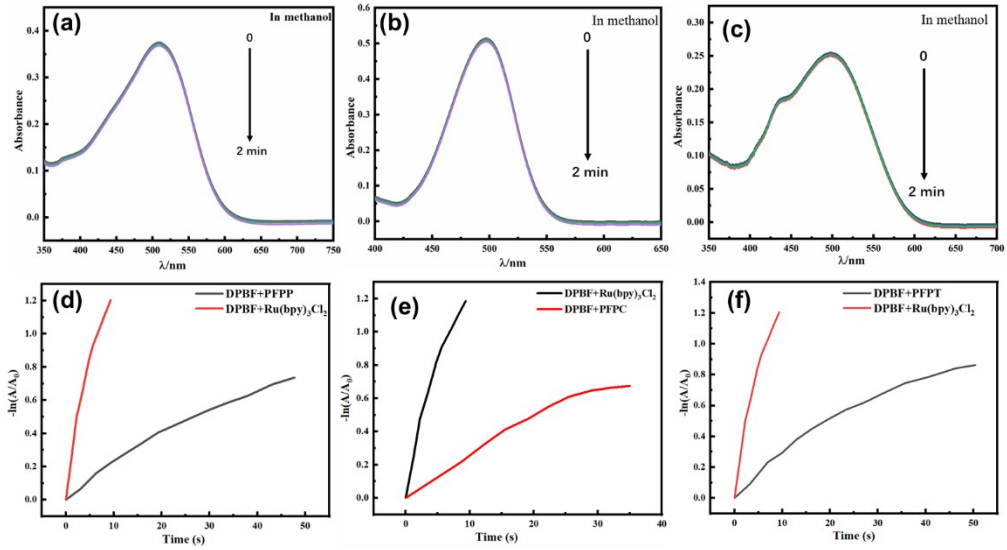


Fig.S13. UV-Vis absorption curves of photosensitizers under light radiation(a: PFPP, b: PFPC, c: PFPT Solvent: methanol). The plot of the variation of -ln(A/A_0) with time of light radiation (d: PFPP, e: PFPC, f: PFPT)

13. Determination of absolute fluorescence quantum yield of photosensitizers

Table S2 Fluorescence quantum yield of photosensitizers in different solutions

Compound	Fluorescence quantum yield (Φ_F) /%		
	MeOH	DMF	Toluene
PFP	0.11	0.21	0.22
PFPP	0.39	2.41	2.74
PFPC	0.21	1.52	1.73
PFPT	0.17	0.91	1.51

The solution concentrations are 1.0×10^{-5} M. Instrument information: (Absolute Fluorescence Quantum Yield Measuring Meter, C11347-11, HAMAMATSU). Scanning range: 300-950 nm; excitation wavelength range: 250-850 nm.

14. Co-localization of photosensitizer PFPC in subcellular organelles

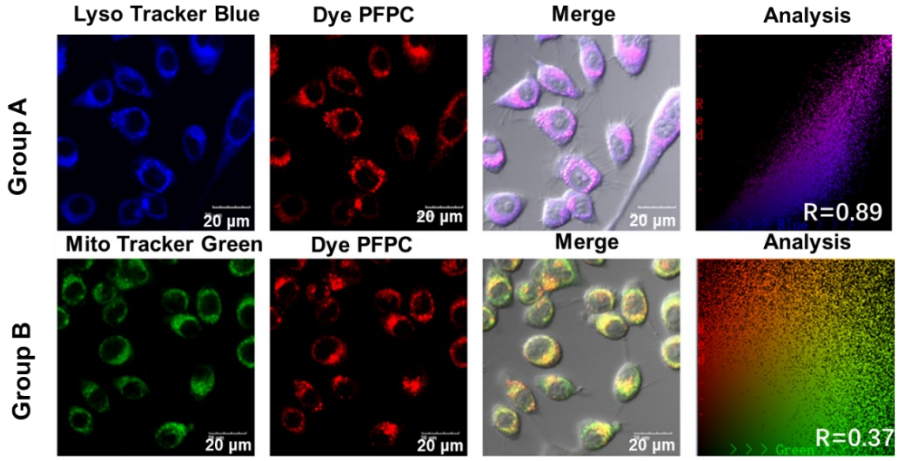


Fig. S14. Photosensitizer PFPC co-located imaging in A549 cells. Group A Lyso-tracker Blue Kit, fluorescence imaging of dye PFPC and fluorescence scatter analysis diagram; Group A Mito-tracker Green and dye PFPC fluorescence imaging and fluorescence scatter analysis diagram.

15. Single crystal data of photosensitizer PFP

Table S3 Crystal data and structure refinement for PFP.

Identification code	PFP
Empirical formula	$C_{27}H_{31}N_3O_3S_1$
Formula weight	477.2
Temperature/K	292.99(10)
Crystal system	monoclinic
Space group	Pc
a/Å	14.9798(7)
b/Å	5.5917(3)
c/Å	15.3856(7)
α/°	90.00
β/°	105.311(5)
γ/°	90.00
Volume/Å^3	1243.00(10)
Z	5
ρ_{calc}g/cm^3	1.276
μ/mm^{-1}	0.164
F(000)	508.0
Crystal size/mm^3	
Radiation	Mo Kα (λ = 0.71073)
2Θ range for data collection/°	5.46 to 61
Index ranges	-18 ≤ h ≤ 21, -6 ≤ k ≤ 7, -16 ≤ l ≤ 21
Reflections collected	7767
Independent reflections	4897 [R_{int} = 0.0211, R_{sigma} = 0.0330]
Data/restraints/parameters	4897/2/313
Goodness-of-fit on F^2	1.044
Final R indexes [I>=2σ (I)]	R_1 = 0.0428, wR_2 = 0.1136
Final R indexes [all data]	R_1 = 0.0491, wR_2 = 0.1191
Largest diff. peak/hole / e Å^{-3}	0.65/-0.25
Flack parameter	-0.04(7)

16. Reference

[1]. W. Xiang, B. Liu and Y. Qian, *Dyes Pigm.*, 2022, **205**, 110524.

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