Electronic Supplementary Information

An AceDAN-Porphyrin(Zn) Dyad for Fluorescent Imaging and Photodynamic Therapy via Two-Photon Excited FRET

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Content

Chemicals and instruments

Column chromatography was carried out on silica gel (300-400 mesh, Qingdao Ocean Chemicals) with the indicated eluents. DMF was freshly distilled from CaH_2 under nitrogen. Compounds **2**, **4** and **6** were prepared according to literature methods.[1-3] All other reagents and solvents were used as received. ^1H NMR spectra were recorded on a Bruker DPX 400 MHz spectrometer in $CDCl_3$ and the chemical shifts were reported relative to internal $SiMe_4$. MALDI-TOF mass spectra were measured on a Bruker Microflex™ LRF spectrometer with dithranol as the matrix. Elemental analyses were performed on an Elementar Vario MICRO CUBE elemental analyzer. Electronic absorption spectra were recorded on a Lambda U-750 spectrophotometer. Steady-state fluorescence spectroscopic studies were performed on a Hitachi F4500 fluorophotometer. The singlet oxygen emission spectra were recorded with an Edinburgh Analytical Instruments FLS980 spectrometer equipped with a NIR PMT. A 375 nm laser (EPL) was used as exciting source to measure the excited singlet state lifetimes. The two-photon fluorescence emission spectra were acquired using 100-fs laser pulses with a peak power of 90 mW cm^{-2} from an optical parametric amplifier operating at a 80-MHz repetition rate generated from a mode-locked Ti:sapphire pulsed laser (Spectra-Physics, SP-5W).

Measurement of fluorescence quantum yields (Φ_{em})

The fluorescence quantum yields were determined by the equation $\Phi_{\mathrm{em}}^{\mathrm{s}} = \Phi_{\mathrm{em}}^{\mathrm{r}}(I_{\mathrm{em}}^{\mathrm{s}}A_{\mathrm{r}}n_{\mathrm{s}}^2)/(I_{\mathrm{em}}^{\mathrm{r}}A_{\mathrm{s}}n_{\mathrm{r}}^2)$, where the super/subscripts *s* and *r* refer to the sample and the reference compound, respectively. Φ_{em} is the fluorescence quantum yield, I_{em} is the integrated fluorescence emission intensity, *A* is the absorbance at the excitation wavelength, *n* is the refractive index of the solvent. In all measurements, the steady state fluorescence emission spectra were obtained using an excitation at 370 nm with absorbance ranged between 0.01 and 0.05 in air-saturated $CHCl_3$ solutions at room temperature.

Measurement of two-photon absorption cross section

The two-photon absorption cross section was determined following previously reported method.[4] Dyad **1** (2 μM) was dissolved in $CHCl_3$ and then the two-photon fluorescence intensities were measured at 720-880 nm by using Rhodamine B as the reference.[5] The TPA cross section was calculated by using equation $\delta = \delta_r(F_s\Phi_r n_r c_r)/(F_r\Phi_s n_s c_s)$, where the subscripts s and r stand for the sample and the reference compound, respectively. δ is the two-photon absorption cross section value, the concentration of compound in solution was denoted as c, n is the refractive index of the solution, F is intensity of the signal collected using a CCD detector and Φ is the fluorescence quantum yield.

Measurement of singlet oxygen quantum yields

Measurements were taken at 370 nm excitation in O_2-saturated solutions at room temperature with H_2TPP ($\Phi_\Delta = 0.55$) in $CHCl_3$ as reference,[6] by comparing the integrated intensity of singlet oxygen phosphorescence emission around 1275 nm measured with a FLS980 spectrofluorimeter. The absorption of the photosensitizer (2 μM) at corresponding excitation wavelength was kept between 0.01 and 0.05.[7]

Detection of the singlet oxygen generation by chemical trapping

1,3-diphenylisobenzofuran (DPBF) was chosen as the chemical trap to detect singlet oxygen ($^1O_2$).[8] Oxygen saturated $CHCl_3$ solution of respective photosensitizer (0.5 μM) that contained DPBF (60 μM) was prepared in the dark and irradiated in the Q-bands region (> 490 nm) to prevent the self-bleaching of DPBF. DPBF degradation was monitored by UV-vis absorption spectra ranging from 480 to 360 nm after every 10 s of irradiation (0-100 s).

Two-photon excited imaging depth detection in mock tissue

One- and two-photon fluorescence imaging of dyad **1** in mock tissue was performed by CLSM. Dyad **1** (10 μM) were added into the mock tissue (1% intralipid), and added into 35 mm glass-bottom culture dishes for fluorescence imaging. Two-photon images were excited at 740 nm and one-photon images were excited at 403 nm, and emitted light was collected with red channel of 575-700 nm. An 10× objective lens with numerical aperture (NA) of 0.60 was used to detect the depth in the *z*-plane.

Cell culture

Human lung carcinoma A549 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 U/mL; streptomycin 100 μg/mL). Cells were incubated at 37 °C in a humidified incubator with 5% CO_2. Culture medium was changed prior to exposure to the test compounds. Stock solutions of the test compounds (1 mM) were prepared in DMSO and stored in dark at -20 °C in a refrigerator. Before imaging and MTT assay involving cultured cells, the stock solutions were first restored to r.t., and then diluted to appropriate concentration (2.5 μM, 5 μM, 10 μM, 20 μM) with DMEM. A549 cells were cultured in DMEM supplemented with 10% FBS and then seeded into a 96-well plate or 35 mm glass-bottom culture dishes for 24 h.

Fluorescence imaging

A549 cells were seeded on 35 mm glass-bottom culture dishes and incubated for 24 h at 37 °C under 5% CO_2, then incubated with the solution of compound **1** or **3** (10 μM in DMEM with 1% DMSO and 0.1% Cremophor EL) for 5 min. All cells were rinsed with PBS (pH = 7.4) three times before imaging. **One-photon.** The fluorescence images were obtained on an Olympus FV-1200 confocal laser scanning microscope with excitation at 403 nm (3.5 mW laser power) through 40× objective lens. **Two-photon.** The two-photon induced fluorescent images were obtained on a Nikon ARsiMP-LSM-Kit-Legend Elite-USX confocal microscope. The excitation wavelength was 740 nm from a femtosecond pulsed Ti:Sapphire laser source, and the excitation beam (115 mW, 80 MHz, 140 fs) was focused on the adherent cells through a 40× objective lens with incident power set less than 5% in order to minimize the damage of laser intensity to cells.

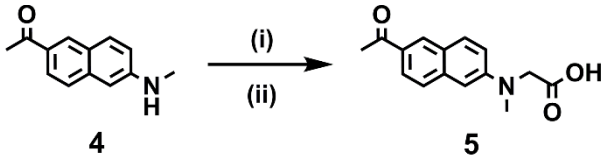
Dark cytotoxicity assay

Exponential growing A549 cells were seeded in flat-bottomed 96-well plates (*ca.* 5×10^3 cells per well) with 100 μL complete culture media in the dark for 24 h. The cells were treated with dyad **1** (2.5-20 μM) for 2 h in dark. The culture medium was replaced with fresh medium and then the cells were incubated for another 24 h. The media was exchanged to 50 μL MTT solution (5 mg/mL) each well and the cells were incubated for 4 hours at 37 °C. The MTT solution was removed carefully and 150 μL DMSO was added to dissolve the formazan crystals. An untreated cell population under the same experimental conditions was used as the reference point to establish 100% cell viability. And the wells treated with DMEM containing 1% DMSO were used as the solvent control groups. The absorbance at 450 nm was read by a 96-well plate reader (Tecan Sunrise™ microplate reader). 6 replicates were collected for each control and test concentration. And the statistical mean and standard deviation (S.D.) were used to estimate the cell viability.

Photocytotoxicity assay

Exponential growing A549 cells were incubated in wells of 96-well plate (*ca.* 5×10^3 cells per well) for 24 h. The cells were treated with compound **1** (2.5-20 μM) for 2 h in dark. The culture medium was replaced with fresh medium and then the cells were exposed to light from a xenon lamp fitted with 490 nm long-pass filter at an intensity of 10 $mW \cdot cm^{-2}$. Cells viability was determined by MTT assay after another 24 h cultivation. The culture medium was exchanged to 50 μL (5 mg/mL) MTT solution each well followed by incubation for 4 h. The formazan crystal formed was dissolved in DMSO (150 μL) and the optical density at 450 nm was measured using a 96-well plate reader (Tecan Sunrise™ microplate reader). 6 replicates were collected for each control and test concentration. And the statistical mean and standard deviation (S.D.) were used to estimate the cell viability.

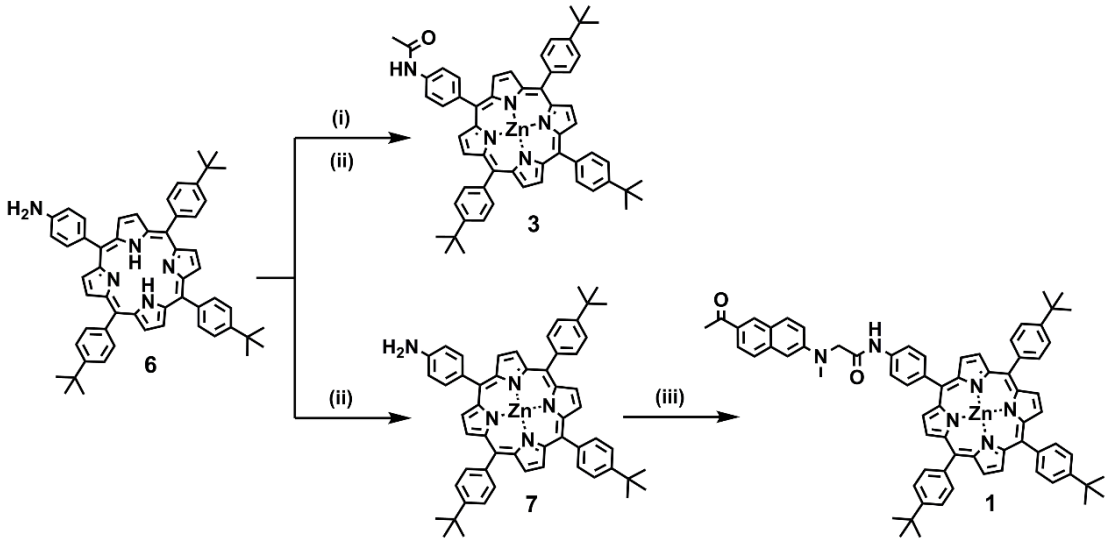
Synthesis and characterization



4 5

Scheme S1 Synthesis of acetylnaphthalene derivatives: (i) Methyl bromoacetate, Na_2HPO_4, NaI, MeCN, reflux; (ii) KOH, EtOH/H_2O, rt.

Synthesis of compound 5. Methyl bromoacetate (400 μL, 4.0 mmol), 2-acetyl-6-methylaminonaphthalene **4** (400 mg, 2.0 mmol), NaI (120 mg, 0.8 mmol) and Na_2HPO_4 (570 mg, 4.0 mmol) were added to a solution of MeCN (16 mL). The mixture was refluxed at 80 °C for 15 h. The product was extracted with ethyl acetate, washed with saturated NaCl and purified by silica gel column chromatography using dichloromethane/petroleum ether = 4:1 as the eluant. After evaporation to dryness, the yellow powder was dissolved in EtOH/H_2O (5:1) (2 mL) containing 150 mg KOH and stirred at room temperature overnight. The resultant solution was diluted with 20 mL of ice water and acidified using concentrated HCl to pH = 3 at 0 °C. The precipitate was filtered, washed with cold water and purified by crystallization from $CHCl_3$/petroleum ether to yield **5** (290 mg, 56%). ^{1}H NMR (CD_3OD, 400 MHz, 293K): δ 8.42 (s, 1H), 7.86-7.89 (m, 2H), 7.67 (d, J = *12.0 Hz*, 1H), 7.21 (dd, J = *8.0 Hz*, J = *2.0 Hz*, 1H), 6.96 (s, 1H), 4.28 (s, 2H), 3.20 (s, 3H), 2.66 (s, 3H). MALDI-TOF-MS: m/z Calcd for $C_{15}H_{15}NO_3$ (M^+) 257.1; Found 257.0. Anal. Calcd for $C_{15}H_{15}NO_3$: C, 70.02; H, 5.88; N, 5.44. Found: C, 70.09; H, 5.92; N, 5.43.



Scheme S2 Synthesis of porphyrin derivatives: (i) Acetic anhydride, acetic acid, CH_2Cl_2, rt; (ii) $Zn(OAc)_2$, $CHCl_3$/MeOH, rt; (iii) Compound **5**, EDCI, DMAP, DMF, rt.

Synthesis of compound 3. To a solution of NH_2-tBu-H_2Por **6** (50 mg, 0.08 mmol) in CH_2Cl_2 (4 mL) was added acetic anhydride (0.2 mL) and acetic acid (0.2 mL). The mixture was stirred for 1 h before being poured into cold water (4 mL). The solution was basified with ammonium hydroxide to pH = 8. The organic layer was washed with water and dried over anhydrous sodium sulfate. The solvent was removed in vacuum to give a purple powder. Then the intermediate product and $Zn(OAc)_2$ (100 mg, 0.55 mmol) were added to a solution of $CHCl_3$/MeOH (2:1) (20 mL). The mixture was stirred at room temperature overnight in the dark and then evaporated to dryness. The residue was dissolved in chloroform, washed with saturated $NaHCO_3$ and water. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed. The crude product was purified by silica gel column chromatography using $CHCl_3$ as the eluant to yield **3** (43.5 mg, 76%). ^{1}H NMR ($CDCl_3$, 400 MHz, 293K): δ 8.90 (d, *J = 4.0 Hz*, 6H), 8.85 (d, *J = 4.0 Hz*, 2H), 8.11 (d, *J = 8.0 Hz*, 8H), 7.94 (s, 1H), 7.83 (d, *J = 8.0 Hz*, 2H), 7.71 (d, *J = 8.0 Hz*, 6H), 2.31 (s, 3H), 1.60 (s, 27H). MALDI-TOF-MS: m/z Calcd for $C_{58}H_{55}N_5OZn$ (M^+) 901.4; Found 901.6. Anal. Calcd for $C_{58}H_{55}N_5OZn$: C, 77.11; H, 6.14; N, 7.75. Found: C, 77.07; H, 6.19; N, 7.69.

Synthesis of compound 7. To a solution of $CHCl_3$/MeOH (2:1) (240 mL) was added NH_2-tBu-H_2Por **6** (480 mg, 0.60 mmol) and $Zn(OAc)_2$ (1.10 g, 6.00 mmol). The mixture was stirred under reflux for 4 hours in the dark and then evaporated to dryness. The residue was dissolved in chloroform, washed with saturated $NaHCO_3$ and water. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed. The crude product was purified by silica gel column chromatography using $CHCl_3$ as the eluant to yield **7** (500 mg, 97%). ^{1}H NMR ($CDCl_3$, 400 MHz, 293K): δ 8.88-8.93 (m, 8H), 8.12 (d, J = *8.0 Hz*, 6H), 7.96 (d, J = *8.0 Hz*, 2H), 7.71 (d, J = *8.0 Hz*, 6H), 6.99 (d, J = *8.0 Hz*, 2H), 2.94 (s, 1H), 2.88 (s, 1H), 1.61 (s, 27H). MALDI-TOF-MS: m/z Calcd for $C_{56}H_{53}N_5Zn$ (M^+) 859.4; Found 859.2. Anal. Calcd for $C_{56}H_{53}N_5Zn$: C, 78.08; H, 6.20; N, 8.13. Found: C, 78.04; H, 6.17; N, 8.08.

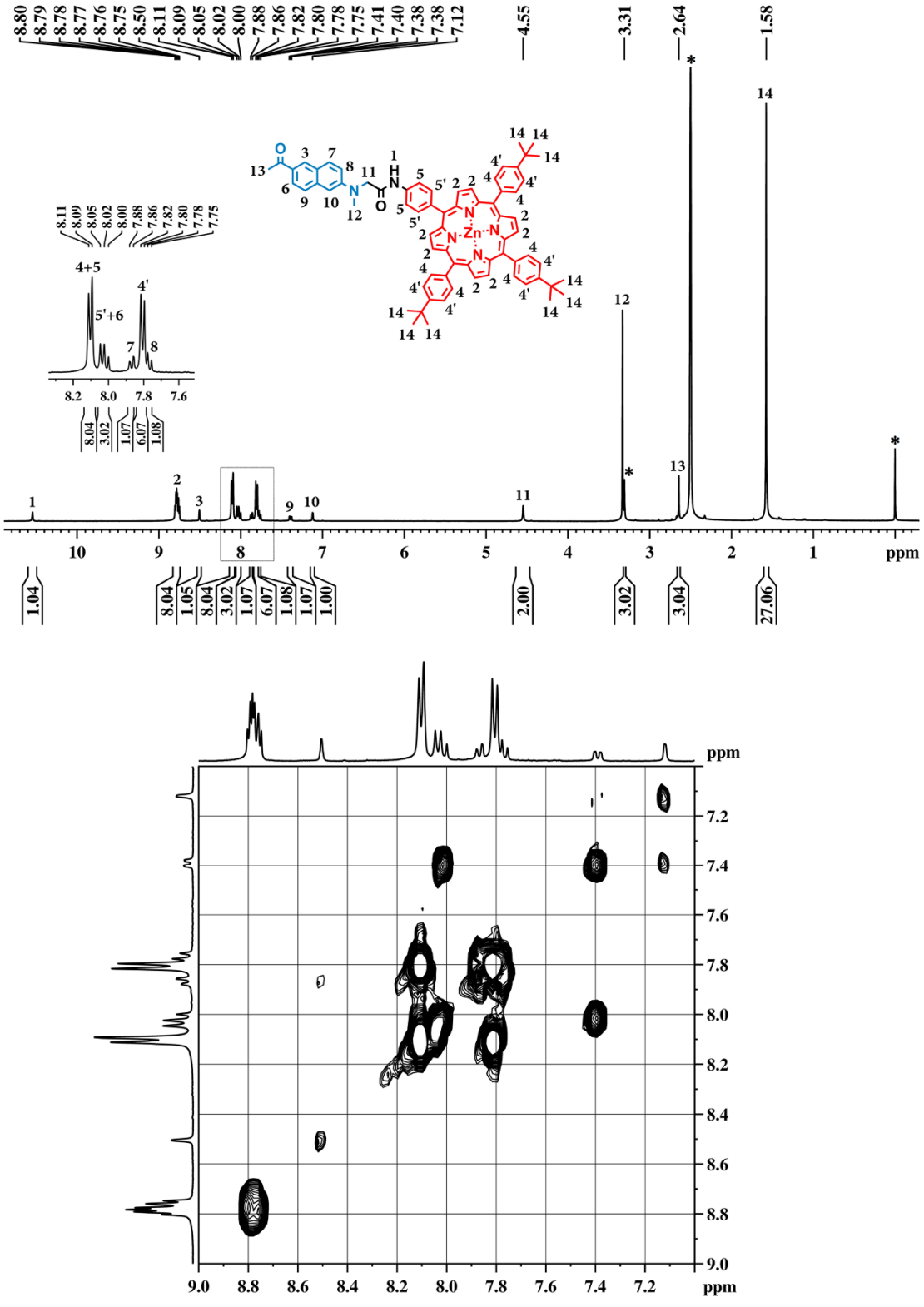


Fig. S1 ^{1}H NMR and ^{1}H-^{1}H COSY spectra of dyad **1** in DMSO at 293 K. * indicate the residual solvent and H_2O signals.

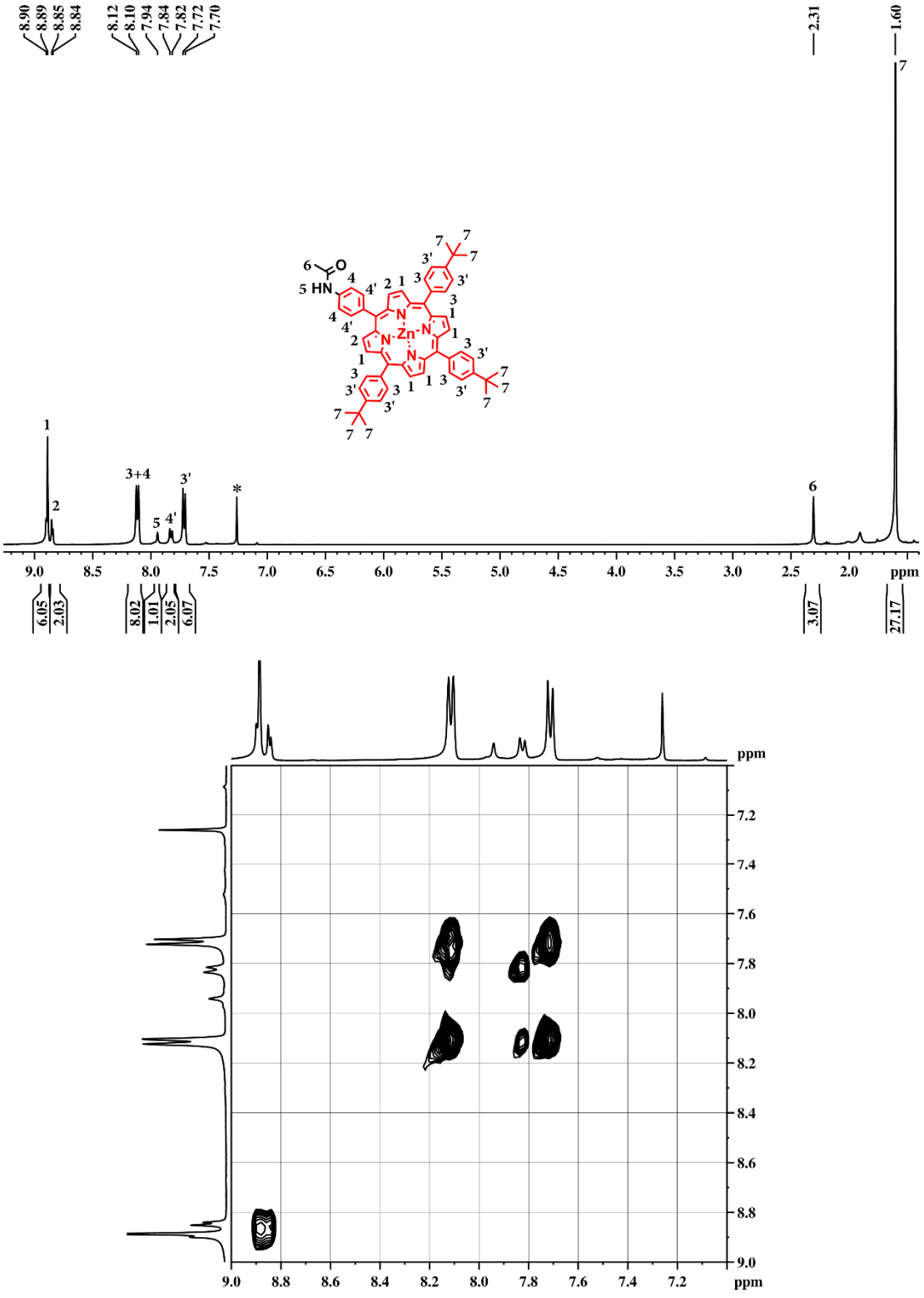


Fig. S2 ^{1}H NMR and ^{1}H-^{1}H COSY spectra of compound **3** in $CDCl_3$ at 293 K. * indicate the residual solvent and H_2O signals.

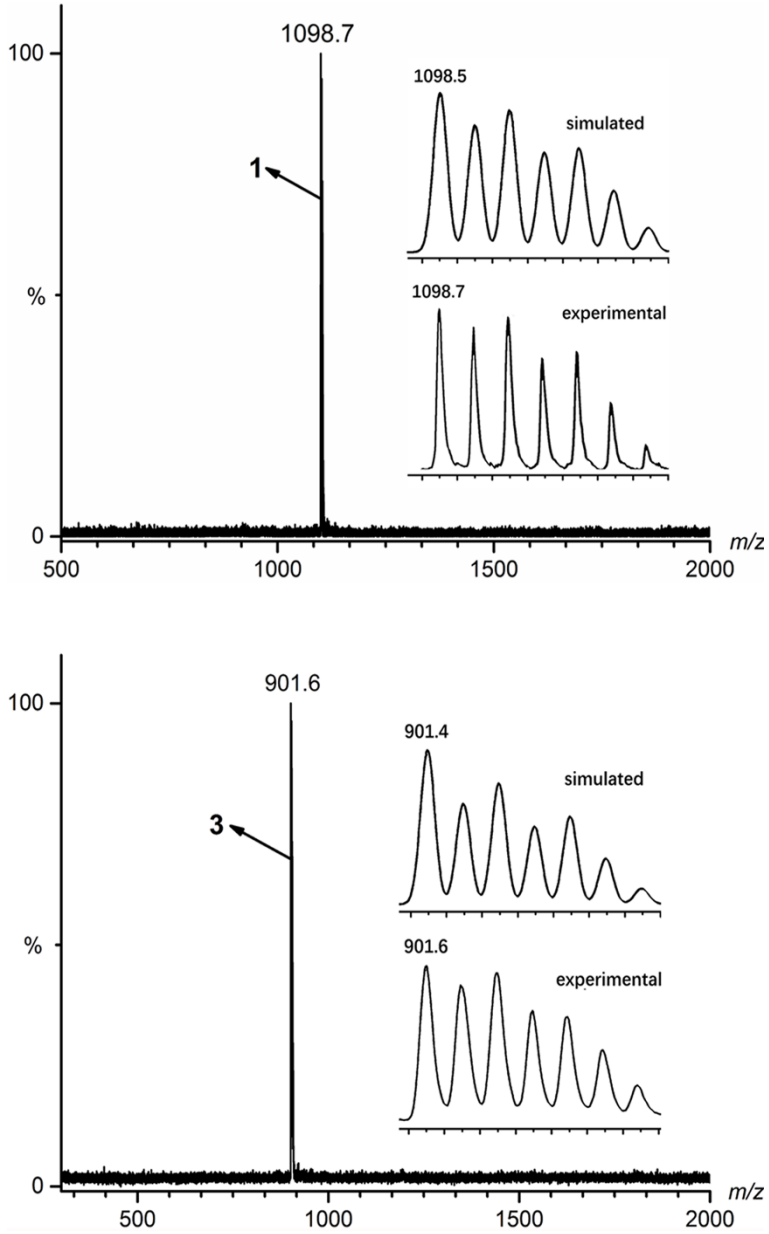


Fig. S3 MALDI-TOF mass spectra of compounds **1** and **3**.

Table S1 Photophysical properties of **1**-**3** in $CHCl_3$ at 298 K.

	UV/Vis absorption λ_{max} [nm] (Log ε [M^{-1} cm^{-1}])				Emission λ_{max} [nm][a] (τ [ns]) (Φ_{em})		η_{EET}[d] (%)	δ_{TPA}[e] [GM]	Φ_{Δ}[f]
1	344 (4.49)	424 (5.56)	552 (4.26)	595 (3.79)	436 (---[n.d.]) (0.011[b])	604, 650 (*1.50*) (0.030[c])	98	82	0.63
2[g]	370 (4.30)				440 (*2.97*) (0.49[b])			120	0.03
3		424 (5.65)	552 (4.22)	595 (3.69)		605, 646 (*1.54*) (0.032[c])		8	0.66

[a] Excitation at 370 nm.

[b] Fluorescence quantum yields were determined by reference to compound **4** in CH_3OH (Φ_{em} = 0.40).[9]

[c] By reference to ZnTPP in benzene (Φ_{em} = 0.033).[10]

[d] The efficiency of energy transfer (η_{EET}) was calculated according to equation: $\eta_{EET} = 1 - \Phi_{em(donor\text{-}in\text{-}dyad)}/\Phi_{em(donor)}$.[11]

[e] Two-photon cross section (GM: 10^{-50} cm^4 s/photon) at 740 nm.

[f] Singlet oxygen quantum yields were obtained by comparison with standard H_2TPP in $CHCl_3$ (Φ_{Δ} = 0.55).[6]

[g] From ref. 1.

[n.d.] Not determined.

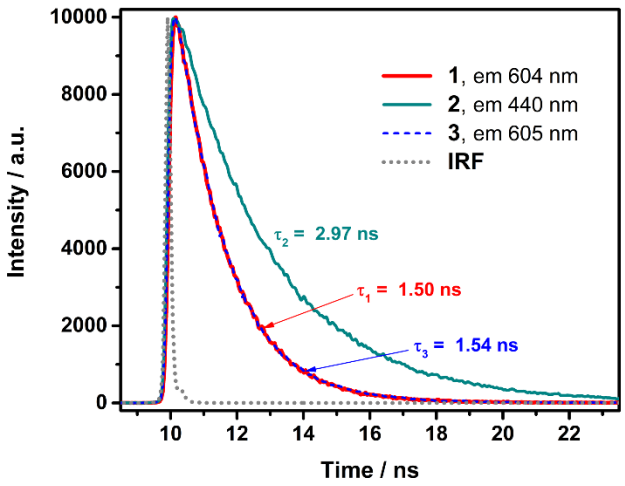


Fig. S4 Fluorescence decay curves of compounds **1-3** in $CHCl_3$. The time profiles of fluorescence decays were obtained with excitation at 375 nm by a EPL laser. Grey dot line is the instrument response function (IRF) of the system.

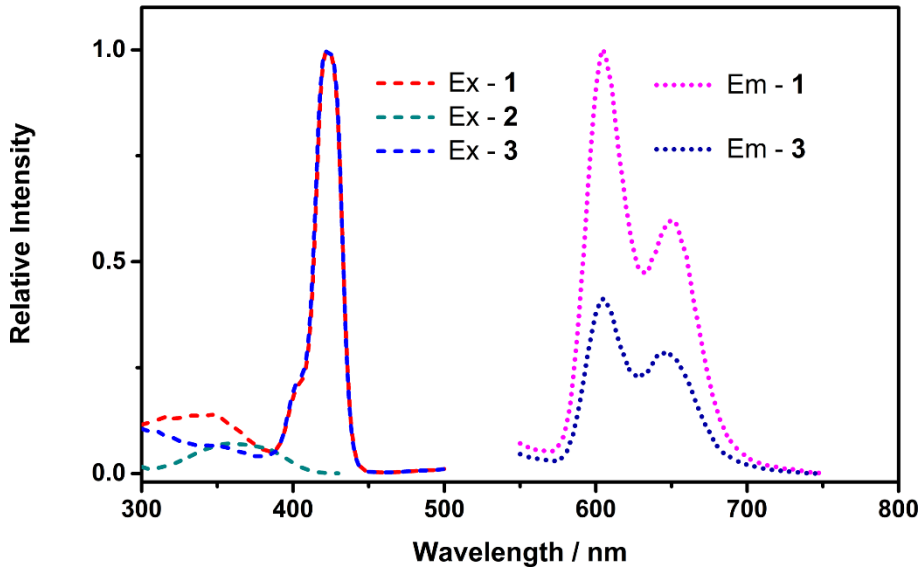


Fig. S5 Excitation spectra (dash line, emission at 605 nm) of compounds **1**-**3** (2 μM) in $CHCl_3$, normalization to the peak intensity of dyad **1**. Emission spectra (dot line, excitation at 370 nm) of compounds **1** and **3** at 2 μM in $CHCl_3$.

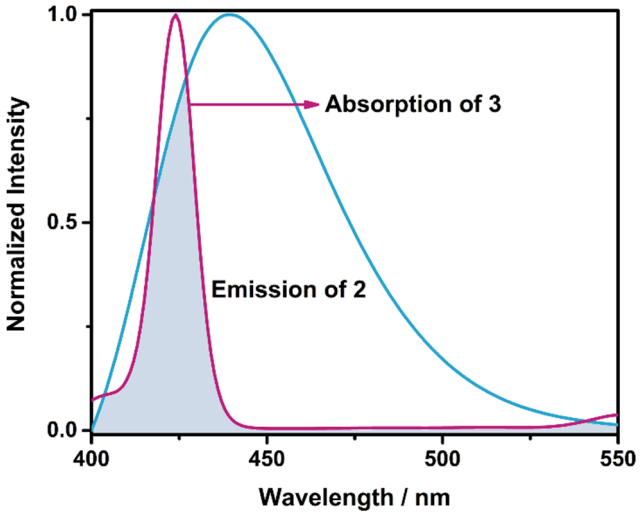


Fig. S6 Normalized spectral overlap between the donor emission (**2**) and the acceptor absorption (**3**).

Density functional theory (DFT) calculation

The DFT calculation was carried out by B3LYP-D3(0) method.[12,13] A mixed basis set, which is a combination of the SDD effective core basis set for zinc atom and 6-31G(d) for C/H/N/O atoms, was chosen.[14,15] And the solvation model based on density (SMD) was used to simulate the chloroform solvent environment.[16] All the calculations were carried out by *Gaussian 09* program.[17] The calculated distance between the two chromophores in AceDAN-Porphyrin(Zn) dyad (**1**) was determined by the geometric centers of them.

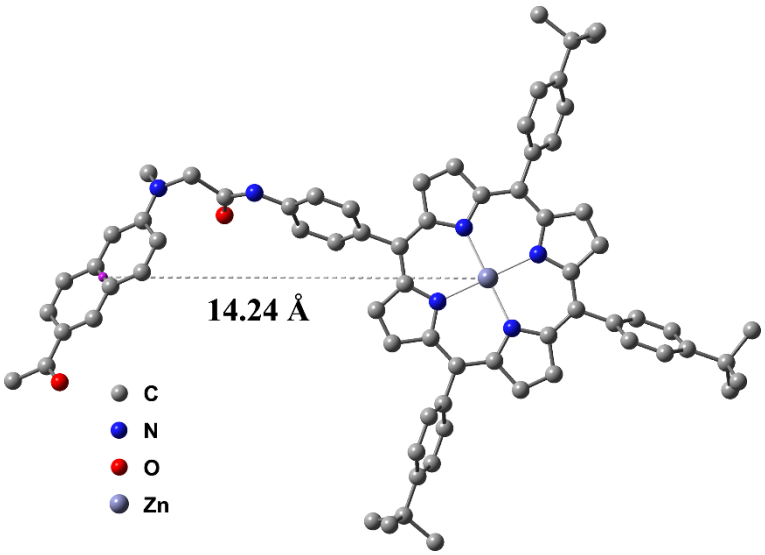


Fig. S7 The DFT optimized molecular structure of AceDAN-Porphyrin(Zn) (**1**). All of the hydrogen atoms have been omitted for clarity.

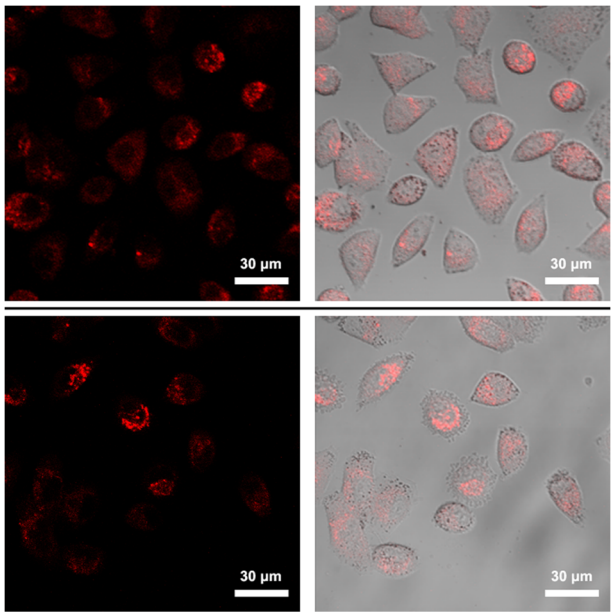


Fig. S8 Fluorescence confocal microscopy images of dyad **1** (10 µM) on A549 cells. Top row: images observed after 5 min incubation of dyad **1**; bottom row: images observed after incubation in fresh DMEM for another 2 h; 40 × magnification, λ_{ex} = 403 nm.

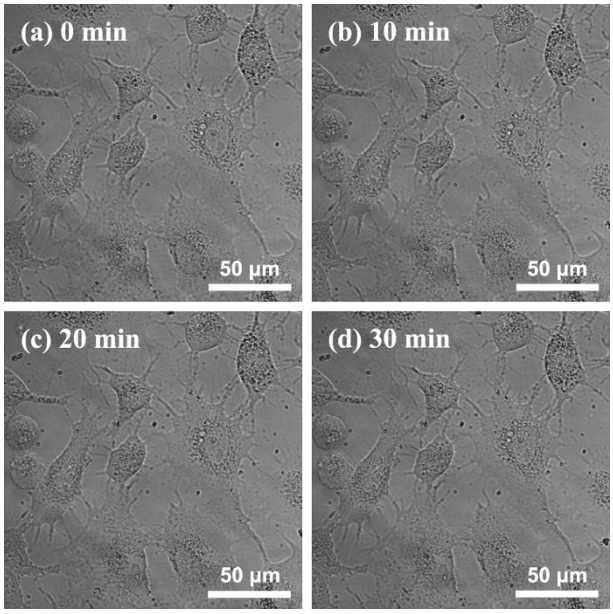


Fig. S9 Confocal microscopic images (bright field) of A549 cells without photosensitizers. Images were obtained after different time (0-30 min) of laser irradiation at 403 nm (P = 3.5 mW).

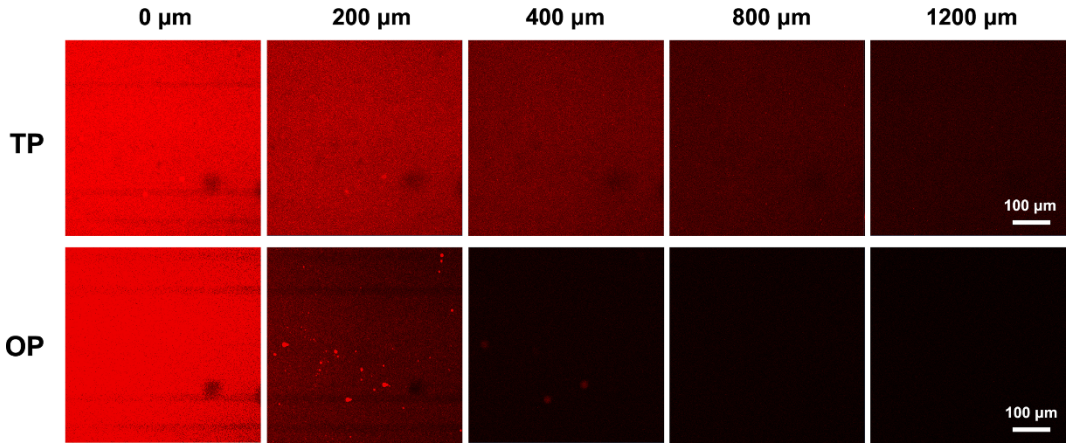


Fig. S10 Confocal microscopic images of dyad **1** (10 μM) at different depths in mock tissue, 10× magnification. Top row: two-photon imaging mode (TP, red channel, λ_{ex} = 740 nm); bottom row: one-photon imaging mode (OP, red channel, λ_{ex} = 403 nm).

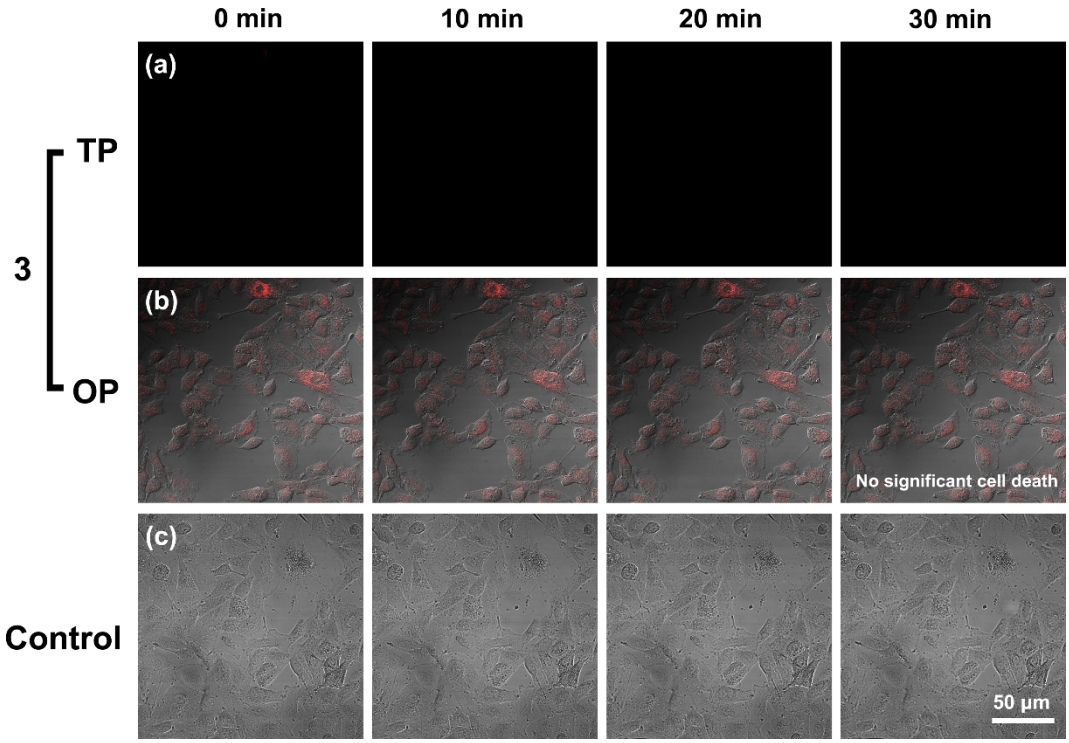


Fig. S11 a) Two-photon (TP, red channel, λ_{ex} = 740 nm) and b) one-photon (OP, red channel, λ_{ex} = 403 nm, merged with bright field) confocal images of A549 cells treated with compound **3**; c) bright field images of A549 cells without photosensitizers (control group). Images were obtained at various time points (0, 10, 20, and 30 min) during two-photon laser irradiation at 740 nm (P = 115 mW).

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