Electronic Supplementary Information

A pH-responsive fluorescent probe and photosensitiser based on a self-quenched phthalocyanine dimer

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Contents

Experimental Section

All the reactions were performed under an atmosphere of nitrogen. *N*,*N*-dimethylformamide (DMF) was dried over barium oxide and distilled under reduced pressure. Dichloromethane was distilled from calcium hydride. Chromatographic purifications were performed on silica gel (Macherey-Nagel, 70-230 mesh) columns with the indicated eluents. All other solvents and reagents were of reagent grade and used as received. Compounds **3**[R1] and **4** [R2] were prepared as described.

^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were recorded on a Bruker AVANCE III 400 spectrometer (^{1}H, 400; ^{13}C, 100.6 MHz) in pyridine-d_5. Spectra were referenced internally by using the residual solvent [^{1}H: δ = 8.70 (for the most downfield signal)] or solvent [^{13}C: δ = 149.6 (for the most downfield signal)] resonances relative to $SiMe_4$. Electrospray ionisation (ESI) mass spectra were recorded on a Thermo Finnigan MAT 95 XL mass spectrometer. Elemental analyses were performed by the Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, China.

UV-Vis and steady-state fluorescence spectra were taken on a Cary 5G UV-Vis-NIR spectrophotometer and a Hitachi F-7000 spectrofluorometer respectively. The fluorescence quantum yields (Φ_F) of the samples (in DMF) were determined by

the equation: $\Phi_{F(sample)} = (F_{sample}/F_{ref})(A_{ref}/A_{sample})(n^2_{sample}/n^2_{ref})\Phi_{F(ref)}$,[R3] where F, A and n are the measured fluorescence (area under the emission peak), the absorbance at the excitation position (610 nm) and the refractive index of the solvent respectively. Unsubstituted zinc(II) phthalocyanine (ZnPc) in DMF was used as the reference [$\Phi_{F(ref)} = 0.28$].[R4] UV-Vis and fluorescence spectra were also recorded in citrate buffer solutions with pH at 5.0, 5.5, 6.0, 6.5, 7.0 and 7.4. Compounds **1**-**3** were dissolved in DMF to give 1.0 mM solutions, which were then diluted to 2 μM with the buffer solutions in the presence of Cremophor EL. The final solutions contained 0.2% DMF and 0.05% Cremophor EL (both by volume).

The singlet oxygen quantum yields (Φ_Δ) were measured in DMF by the method of chemical quenching of 1,3-diphenylisobenzofuran (DPBF) by using ZnPc as the reference ($\Phi_\Delta = 0.56$).[R5] To mimic the biological environment, the singlet oxygen generation efficiency of **1**-**3** was also examined in citrate buffer solutions with pH at 5.5 and 7.4 using DPBF as the quencher. Compounds **1**-**3** were dissolved in DMF to give 1.0 mM solutions, which were then diluted to 2 μM (for **1** and **2**) or 4 μM (for **3**) with the buffer solutions in the presence of Cremophor EL. The solutions, which contained 0.2-0.4% DMF and 0.05-0.10% Cremophor EL (both by volume), were incubated for 10 h. These solutions (3 mL) were then mixed with a solution of DPBF in DMF (40 mM, 6 μL) in a quartz cell. The mixtures were irradiated with red light

and then the absorbance of DPBF at 417 nm was recorded immediately for every 20 sec. The light source consisted of a 100 W halogen lamp and a colour glass filter (Newport) cut-on 610 nm. The rates of decay of DPBF induced by these compounds were monitored over a total irradiation time of 180 sec.

Dimer 1. A mixture of **3** (88.7 mg, 86.3 μmol), ketyl diamine **4** (7.0 mg, 43.1 μmol), 1-hydroxybenzotriazole (HOBt) (17.5 mg, 129.5 μmol), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU) (42.3 mg, 111.5 μmol) and diisopropylethylamine (DIPEA) (1 mL) in CH_2Cl_2 (4 mL) was stirred at room temperature for 30 h. The mixture was diluted with CH_2Cl_2 (15 mL), and then washed with water (15 mL × 2). The organic layer was dried over anhydrous $NaSO_4$ and evaporated to dryness under reduced pressure. The residue was chromatographed with silica gel, which was pre-treated with 1% Et_3N in CH_2Cl_2, using CH_2Cl_2/MeOH (25:2 v/v) as the eluent. The crude product was dissolved in CH_2Cl_2 (4 mL) and precipitated with ethyl acetate (10 mL). The precipitate was filtered and dried *in vacuo* to afford a dark-green solid (40 mg, 42%). ^{1}H NMR: δ 9.43-9.49 (m, 12 H, Pc-H_α), 9.24 (t, J = 5.2 Hz, 2 H, NH), 8.38 (s, 2 H, triazole-H), 8.14-8.22 (m, 8 H, Pc-H_β), 8.05- 8.07 (m, 4 H, Pc-H_β), 7.37 (s, 4 H, Pc-H_β), 5.50 (s, 4 H, CH_2), 4.93-4.96 (m, 8 H, CH_2), 4.81 (s, 4 H, CH_2), 4.49-4.55 (m, 8 H, CH_2), 4.15-4.17 (m, 8 H, CH_2), 3.86-3.91 (m, 8 H, CH_2), 3.77-3.78 (m, 8 H, CH_2), 3.68 (t, J

= 4.8 Hz, 4 H, CH_2), 3.48-3.56 (m, 12 H, CH_2), 3.22 (s, 6 H, CH_3), 1.15 (s, 6 H, CH_3). $^{13}C\{^1H\}$ NMR: δ 166.4, 153.7, 153.6, 153.5, 153.4, 153.3, 152.8, 150.6, 145.2, 139.2, 138.8, 138.6, 129.3, 129.2, 129.0, 127.3, 127.2, 125.7, 122.8, 115.5, 99.7, 72.1, 71.3, 71.2, 71.0, 70.9, 70.8, 70.7, 70.6, 69.9, 69.5, 64.7, 59.5, 58.4, 52.6, 39.9, 24.5 (some of the signals are overlapped). MS (ESI): an isotopic cluster peaking at *m*/*z* 1091 (100%, $[M+2H]^{2+}$). HRMS (ESI) calcd. for $C_{107}H_{110}N_{24}O_{20}Zn_2$ $[M+2H]^{2+}$ 1091.3448, found 1091.3442. Anal. Calcd for $C_{107}H_{108}N_{24}O_{20}Zn_2$: C, 58.93; H, 4.99; N, 15.41. Found: C, 58.45; H, 4.85; N, 15.15.

Dimer 2. According to the above procedure, **3** (83.2 mg, 81.0 μmol) was treated with diamine **5** (6.0 mg, 40.5 μmol), HOBt (16.4 mg, 121.4 μmol), HBTU (44.4 mg, 117.1 μmol) and DIPEA (1 mL) in CH_2Cl_2 (3 mL). The crude product was purified by silica gel column chromatography using $CHCl_3$/MeOH (10:1 v/v) as the eluent followed by reprecipitation with CH_2Cl_2 (4 mL) and ethyl acetate (10 mL) (22 mg, 25%). ^1H NMR: δ 9.49-9.58 (m, 12 H, Pc-H_α), 9.23 (t, *J* = 5.6 Hz, 2 H, NH), 8.28 (s, 2 H, triazole-H), 8.14-8.24 (m, 8 H, Pc-H_β), 8.07- 8.11 (m, 4 H, Pc-H_β), 7.44 (s, 4 H, Pc-H_β), 5.43 (s, 4 H, CH_2), 4.95-4.98 (m, 8 H, CH_2), 4.76 (s, 4 H, CH_2), 4.50-4.54 (m, 8 H, CH_2), 4.15-4.18 (m, 8 H, CH_2), 3.86-3.89 (m, 8 H, CH_2), 3.73 (s, 8 H, CH_2), 3.66-3.69 (m, 4 H, CH_2), 3.54 (t, *J* = 4.8 Hz, 4 H, CH_2), 3.49 (t, *J* = 4.8 Hz, 8 H, CH_2), 3.39 (s, 4 H, CH_2), 3.21 (s, 6 H, CH_3). $^{13}C\{^1H\}$ NMR: δ 166.1, 153.7, 153.6, 153.5,

153.4, 153.3, 152.7, 150.5, 145.2, 139.2, 139.1, 138.8, 138.6, 129.3, 129.2, 129.1, 127.2, 125.4, 122.7, 115.5, 72.0, 71.2, 70.9, 70.8, 70.7, 70.6, 70.2, 69.8, 69.6, 69.4, 64.6, 58.4, 52.5, 39.8 (some of the signals are overlapped). MS (ESI): isotopic clusters peaking at *m*/*z* 1084 (100%, $[M+2H]^{2+}$) and *m*/*z* 2168 (28%, $[M+H]^{+}$). HRMS (ESI) calcd. for $C_{106}H_{108}N_{24}O_{20}Zn_2$ $[M+2H]^{2+}$ 1084.3369, found 1084.3362. Anal. Calcd for $C_{106}H_{112}N_{24}O_{23}Zn_2$ (**2**·$3H_2O$): C, 57.32; H, 5.08; N, 15.14. Found: C, 57.32; H, 5.06; N, 15.04.

Cell lines and culture conditions. The HT29 cells (ATCC, no. HTB-38) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, no. 10313-021) supplemented with fetal calf serum (10%), penicillin-streptomycin (100 units mL^{-1} and 100 μg mL^{-1} respectively), L-glutamine (2 mM) and transferrin (10 μg mL^{-1}). Approximately 3×10^4 cells per well in the medium were inoculated in 96-multiwell plates and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere.

Cellular uptake (determined by extraction). About 1.0×10^6 HT29 cells in DMEM (2 mL) were seeded on each of the wells of a 6-multiwell plate and incubated overnight at 37 °C under 5% CO_2. The medium was removed and the cells were rinsed with phosphate buffered saline (PBS) (2 mL). The cells were incubated with a solution of **1** or **2** in the medium [1 μM, with 0.06% DMF and 0.006% Cremophor EL

(both by volume), 2 mL] for 2 h under the same conditions. The solution was then removed and the cells were rinsed with PBS (2 mL) and then harvested by 0.25% trypsin-EDTA (Invitrogen, 500 μL) followed by quenching the trypsin with the medium (500 μL). The solution was transferred to 1.5 mL centrifuge tubes and centrifuged at 3000 rpm for 3 min. The pellet was then washed with PBS (1 mL) and the suspension was centrifuged again. After removing the PBS, the cells were lysed with DMF (1.2 mL). The mixture was sonicated for 20 min and then centrifuged again. The supernatants were transferred for UV-Vis spectroscopic measurements. The absorbance at 688 nm was compared with the respective calibration curves to give the uptake concentrations.

Cellular uptake (determined by confocal microscopy). About 1.2×10^5 HT29 cells in DMEM (2 mL) were seeded on a coverslip and incubated overnight at 37 °C under 5% CO_2. The medium was then removed and the cells were incubated with a solution of **1** or **2** in the medium [5 μM, with 0.3% DMF and 0.03% Cremophor EL (both by volume), 2 mL] for 2 h under the same conditions. The cells were then rinsed with PBS and viewed with a Leica SP5 confocal microscope equipped with a 633 nm helium neon laser. The compounds were excited at 633 nm and monitored at 650-750 nm. The images were digitised and analysed using the Leica Application Suite Advanced Fluorescence. The intracellular fluorescence intensities (for a total of 50

cells for each sample) were also determined.

pH-Dependent intracellular fluorescence studies. About 1.2×10^5 HT29 cells in DMEM (2 mL) were seeded on a coverslip and incubated overnight at 37 ^oC under 5% CO_2. The medium was then removed and the cells were incubated with nigericin (Sigma) in PBS (25 μM, 2 mL) at pH 5.0 or 7.4 for 30 min under the same conditions. The cells were then rinsed with PBS and incubated with a solution of dimer **1** in the medium [5 μM, with 0.3% DMF and 0.03% Cremophor EL (both by volume), 2 mL] for 1 h. The cells were viewed with a Leica SP5 confocal microscope equipped with a 633 nm helium neon laser. The emission signals from 650-750 nm were collected and the images were digitised and analysed by the Leica Application Suite Advanced Fluorescence. The intracellular fluorescence intensities (for a total of 25 cells for each pH solution) were also determined.

Photocytotoxicity assay. Dimers **1** and **2** were dissolved in DMF to give 1.6 mM solutions, which were diluted to 80 μM with an aqueous solution of Cremophor EL (Arcos, 0.5% by volume in these 80 μM solutions). The solutions were then diluted with the culture medium to various concentrations. The cells, after being rinsed with PBS, were incubated with 100 μL of these phthalocyanine solutions for 4 h at 37 ^oC under 5% CO_2. The cells were then rinsed again with PBS and refilled with 100 μL of the culture medium before being illuminated at ambient temperature. The light source

consisted of a 300 W halogen lamp, a water tank for cooling and a colour glass filter (Newport) cut-on 610 nm. The fluence rate (λ > 610 nm) was 40 mW cm^{-2}. Illumination of 20 min led to a total fluence of 48 J cm^{-2}.

Cell viability was determined by means of the colourimetric MTT assay.[R6] After illumination, the cells were incubated at 37 ^{o}C under 5% CO_2 overnight. An MTT (Sigma) solution in PBS (3 mg mL^{-1}, 50 μL) was added to each well followed by incubation for 2 h under the same environment. A solution of sodium dodecyl sulfate (Sigma, 10% by weight, 50 μL) was then added to each well. The plate was incubated in an oven at 60 ^{o}C for 30 min, then 80 μL of *iso*-propanol was added to each well. The plate was agitated on a Bio-Rad microplate reader at ambient temperature for 10 sec before the absorbance at 540 nm for each well was taken. The average absorbance of the blank wells, which did not contain the cells, was subtracted from the readings of the other wells. The cell viability was then determined by the following equation: % viability = $[(A_i/A_{control} \times 100)]/n$, where A_i is the absorbance of the ith data (i = 1, 2, ..., n), $A_{control}$ is the average absorbance of the control wells in which the phthalocyanine was absent and n (= 4) is the number of the data points.

References

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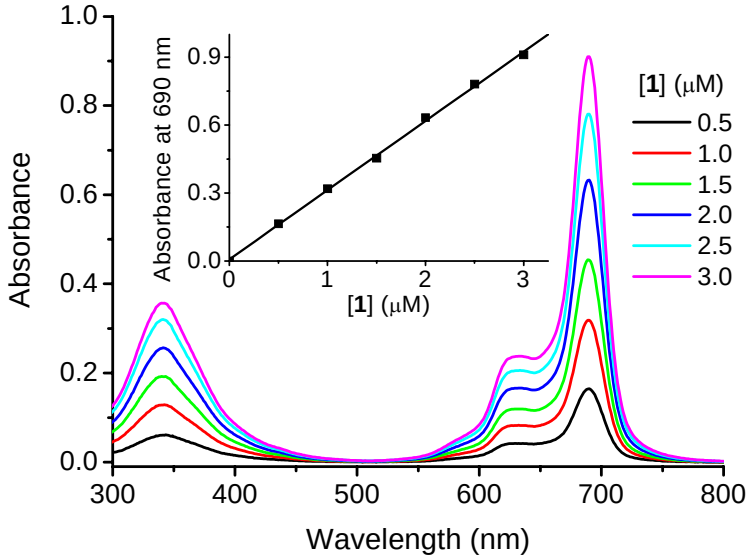
R6 H. Tada, O. Shiho, K. Kuroshima, M. Koyama and K. Tsukamoto, *J. Immunol. Methods*, 1986, **93**, 157.

(a)



(b)

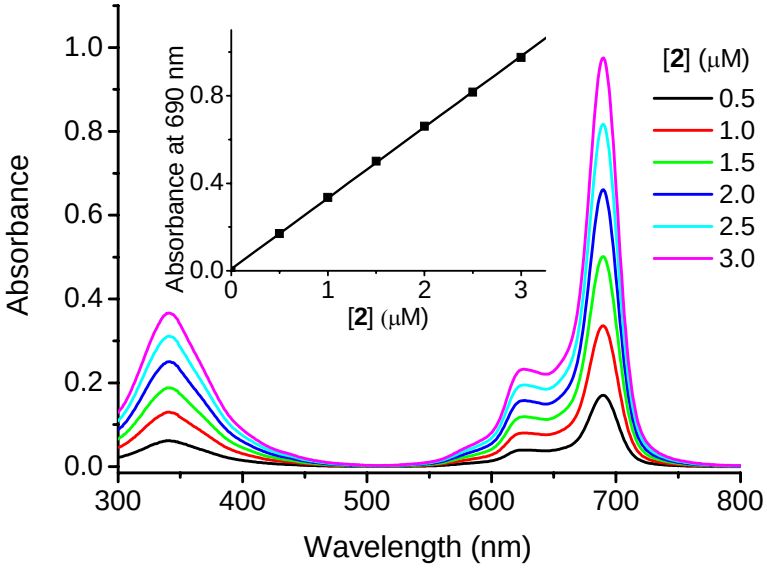
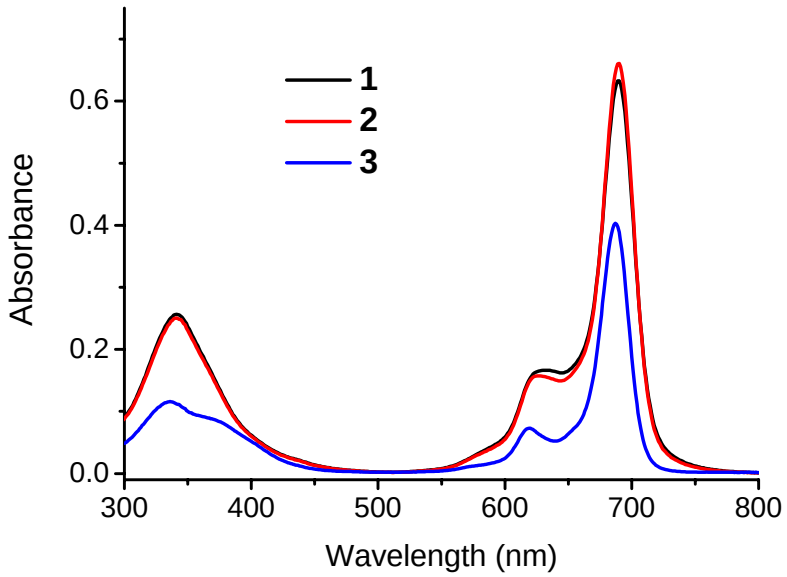


Fig. S1 UV-Vis spectra of **1** and **2** at different concentrations in DMF.

(a)



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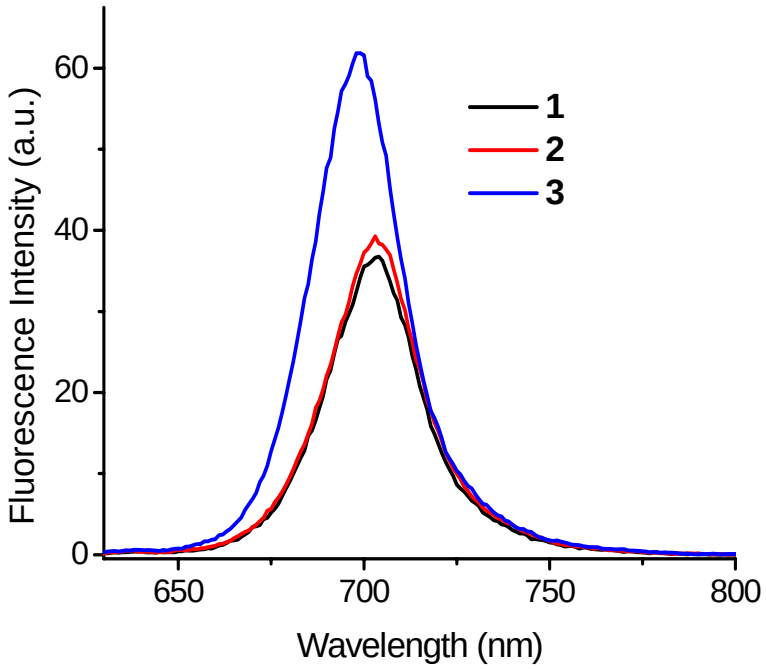


Fig. S2 Comparison of the (a) UV-Vis and (b) fluorescence spectra (λ_{ex} = 610 nm) of **1-3** (all at 2 μM) in DMF.

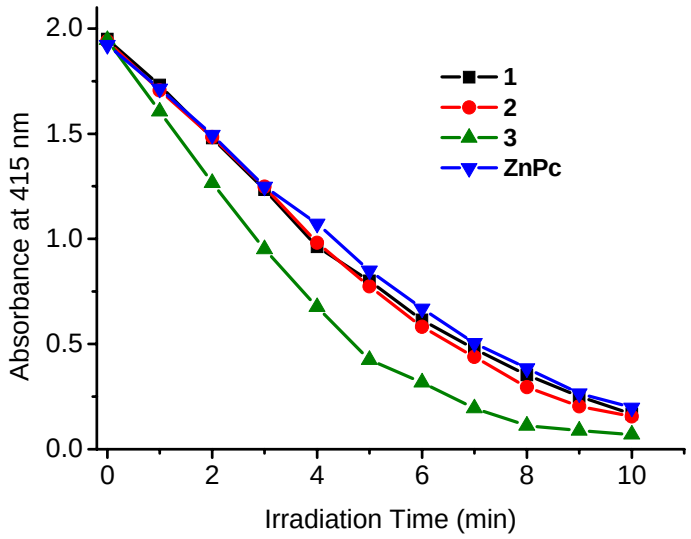
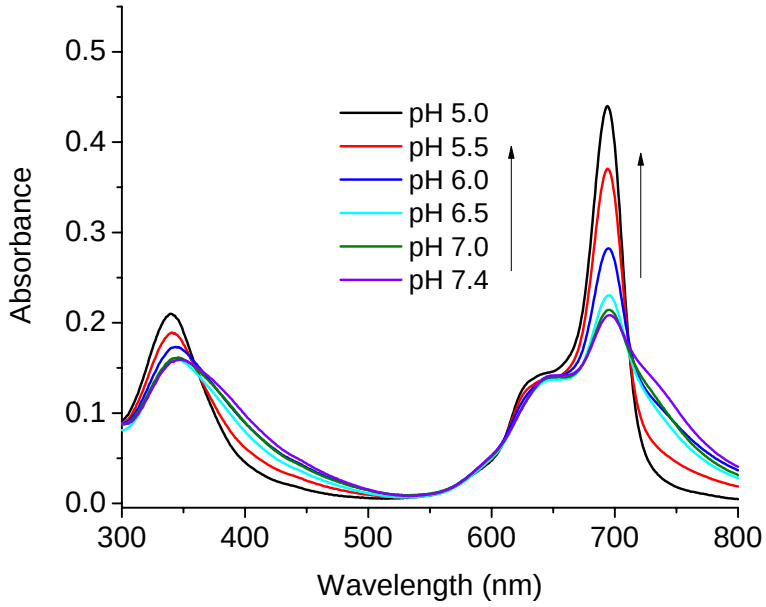


Fig. S3 Comparison of the rates of photodegradation of DPBF in DMF using compounds **1-3** and ZnPc as the photosensitisers. The Q-band absorbance was adjusted to 0.8 for all the cases.

(a)



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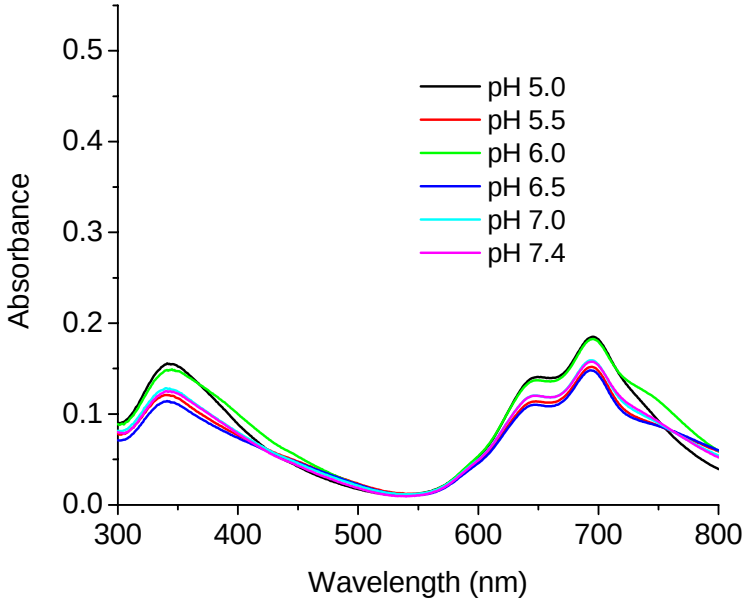


Fig. S4 UV-Vis spectra of (a) **1** and (b) **2** (both at 2 μM) after incubation for 8 h in citrate buffer solutions with different pH.

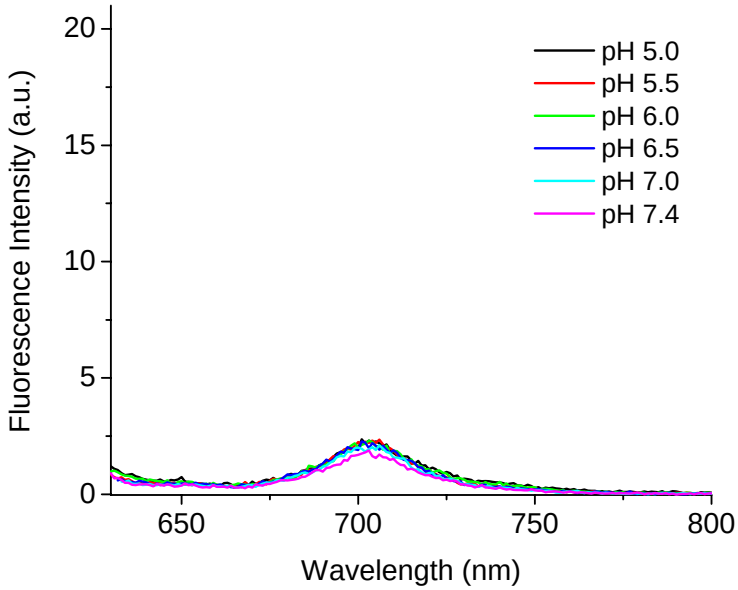
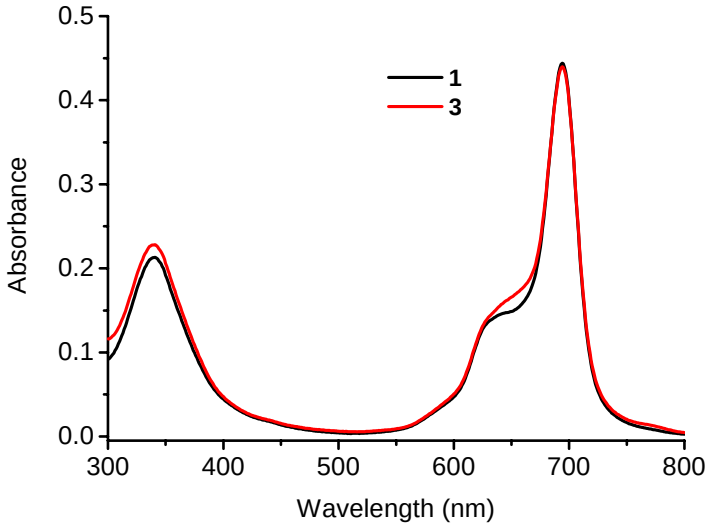


Fig. S5 Fluorescence spectra (λ_{ex} = 610 nm) of **2** (2 μM) after incubation for 8 h in citrate buffer solutions with different pH.

(a)



(b)

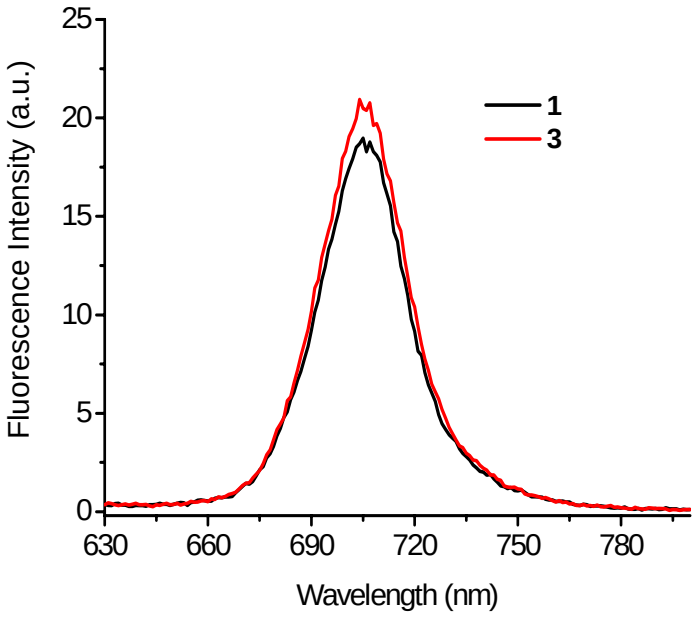


Fig. S6 Comparison of the (a) UV-Vis and (b) fluorescence spectra (λ_{ex} = 610 nm) of **1** (2 μM) and **3** (4 μM) after incubation for 8 h in a citrate buffer solution at pH 5.0.

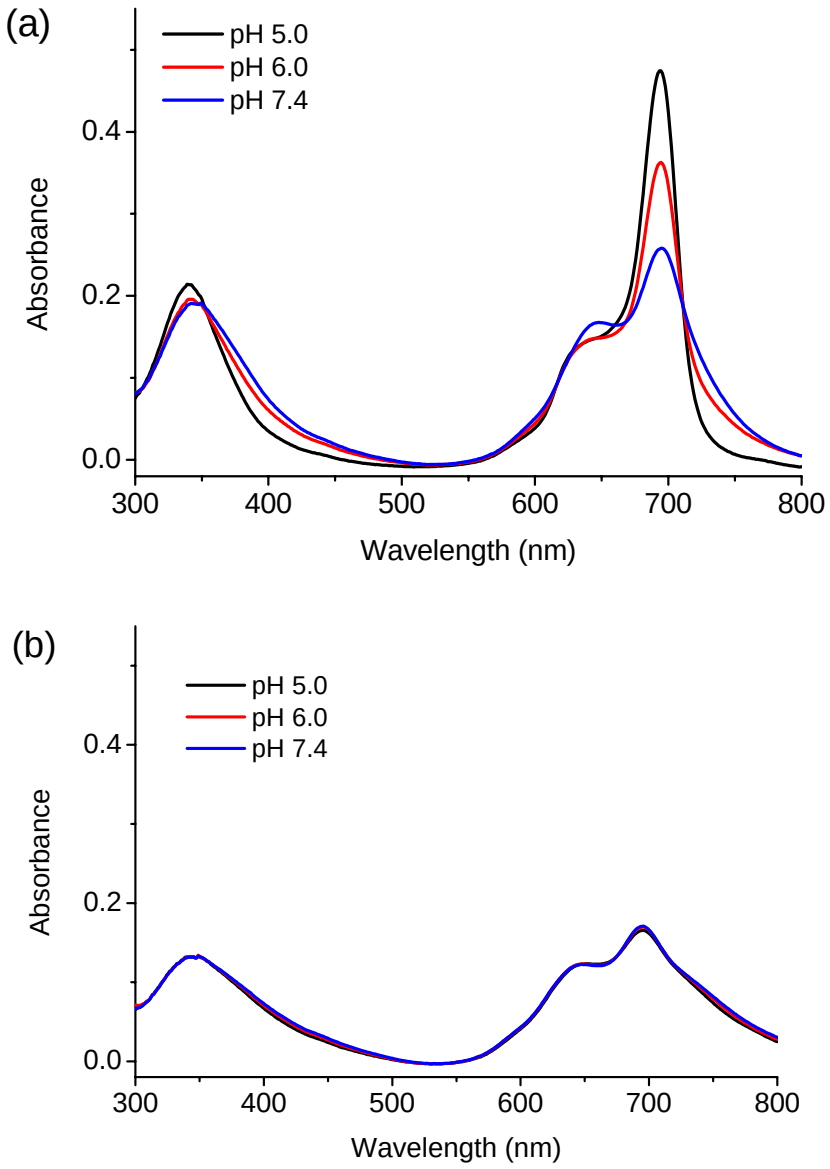


Fig. S7 UV-Vis spectra of (a) **1** and (b) **2** (both at 2 μM) after incubation for 8 h in phosphate buffered saline with different pH.

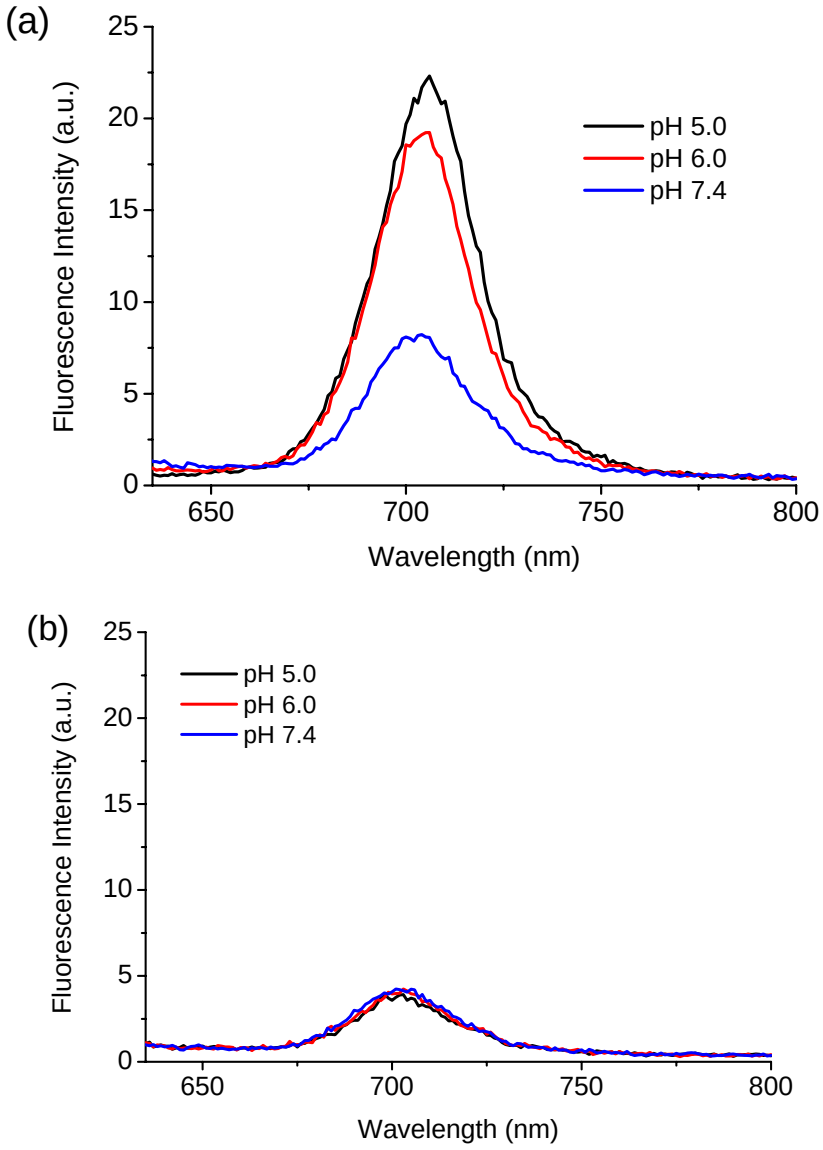


Fig. S8 Fluorescence spectra (λ_{ex} = 610 nm) of (a) **1** and (b) **2** (both at 2 μM) after incubation for 8 h in phosphate buffered saline with different pH.

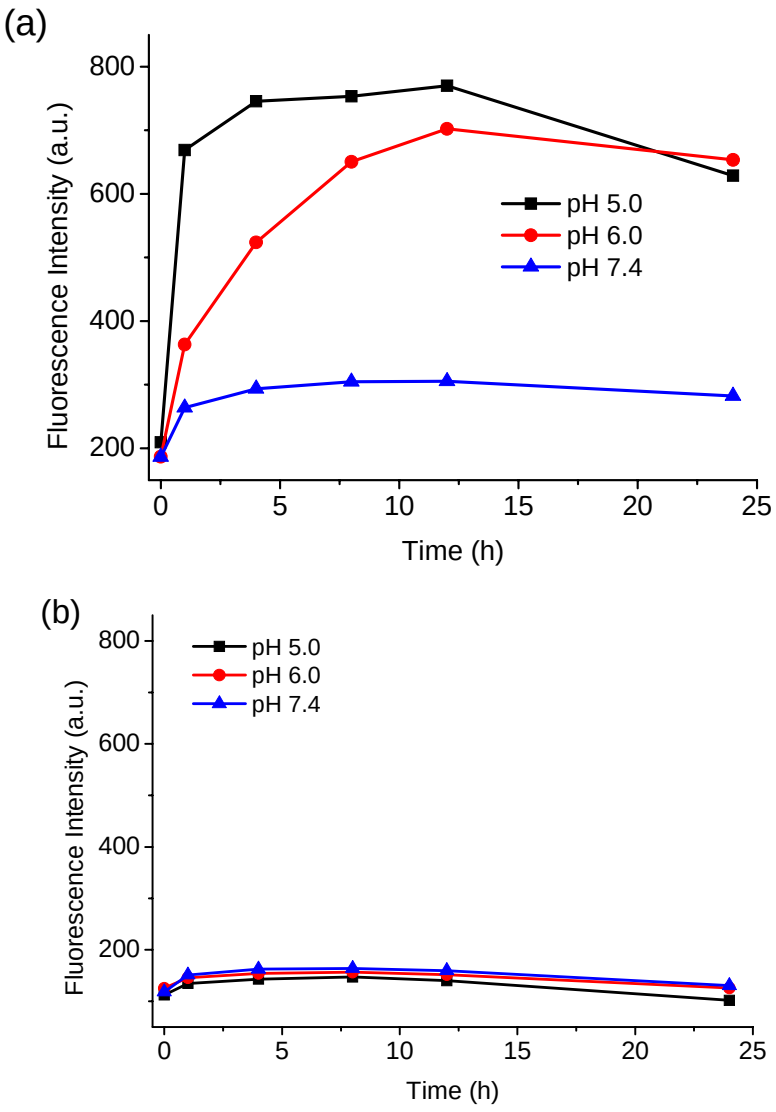


Fig. S9 Changes in fluorescence intensity of (a) **1** and (b) **2** (both at 2 μM) in phosphate buffered saline with different pH at different time intervals.

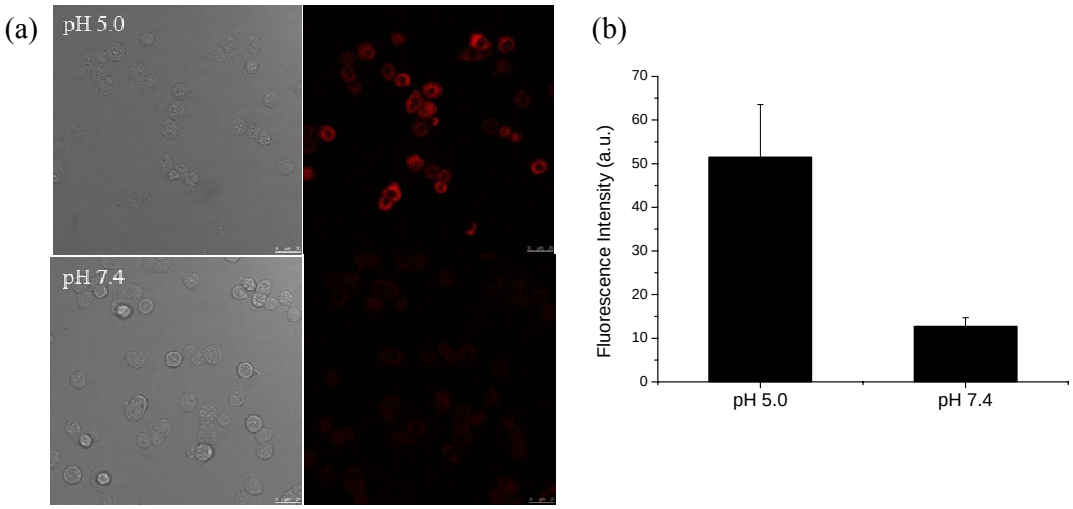


Fig. S10 (a) The bright field (left column) and fluorescence (right column) images of HT29 cells after incubation with nigericin (25 μM) at pH 5.0 or 7.4 for 30 min, followed with **1** (5 μM) for 1 h. (b) Comparison of the intracellular fluorescence intensities of **1** at pH 5.0 and 7.4. Data are expressed as the mean ± standard deviation (number of cells = 25).

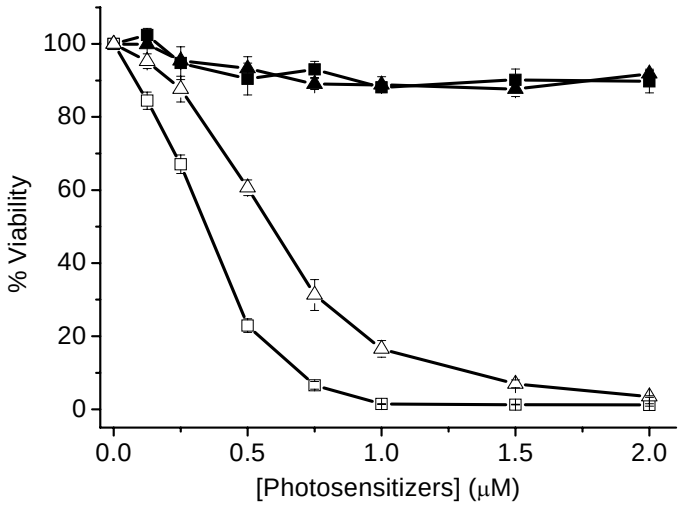


Fig. S11 Comparison of the cytotoxic effects of **1** (squares) and **2** (triangles) on HT29 cells in the absence (closed symbols) and presence (open symbols) of light (λ > 610 nm, 40 mW cm^{-2}, 48 J cm^{-2}). Data are expressed as the mean ± standard error of the mean of three independent experiments, each performed in quadruplicate.

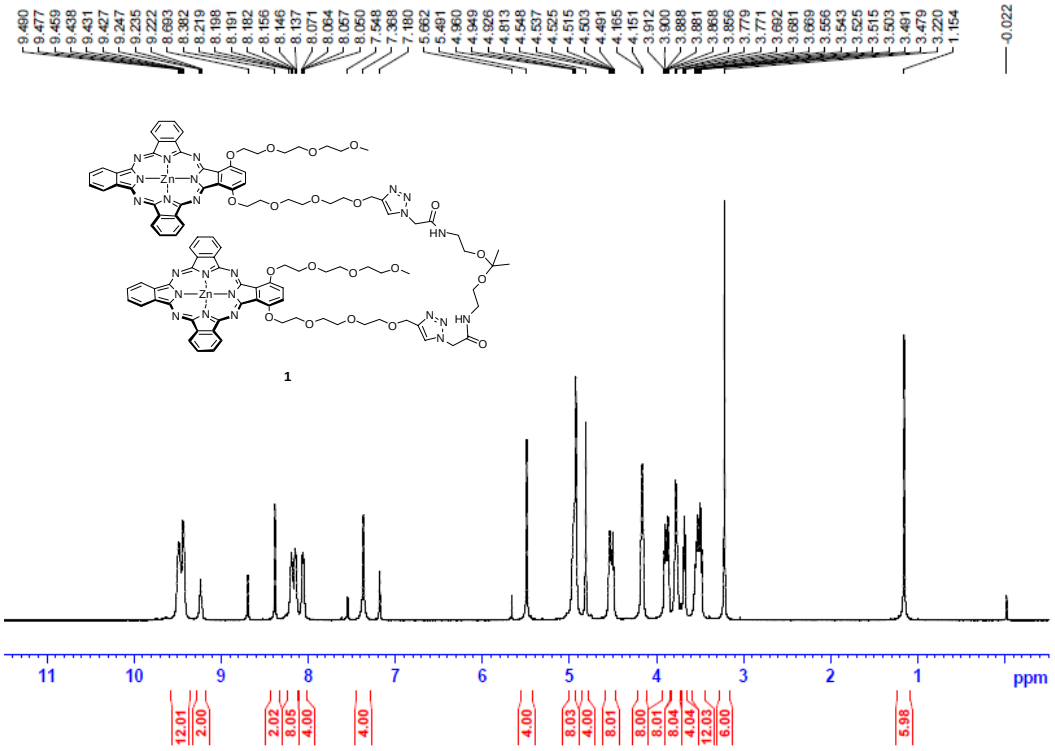


Fig. S12 ^{1}H NMR spectrum of **1** in pyridine-d$_5$.

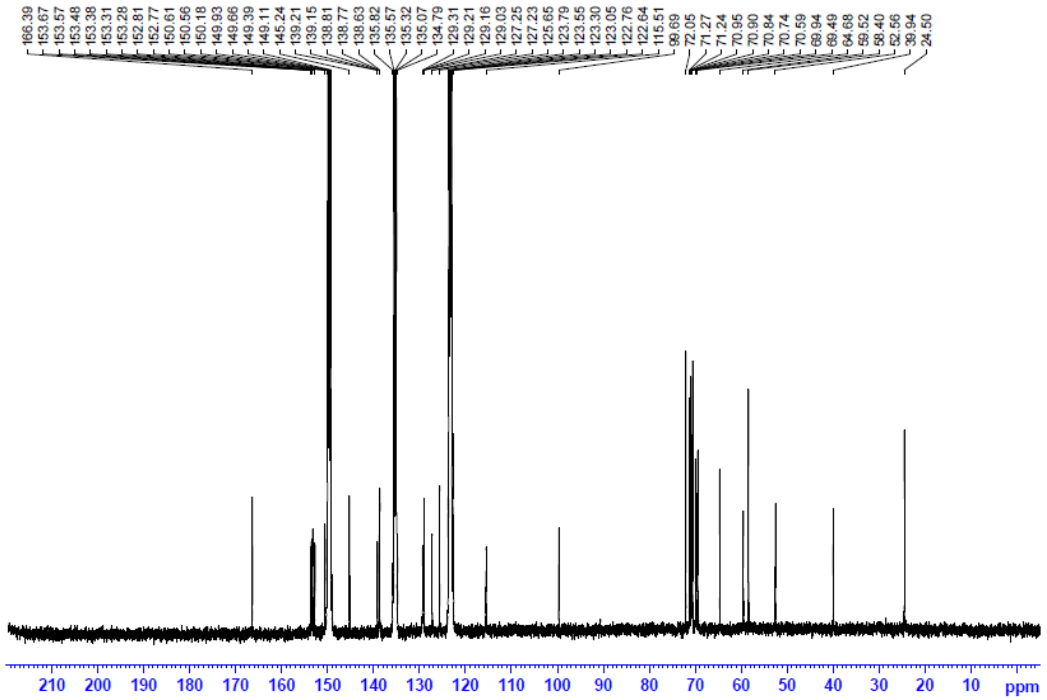


Fig. S13 ^{13}C{^{1}H} NMR spectrum of **1** in pyridine-d$_5$.

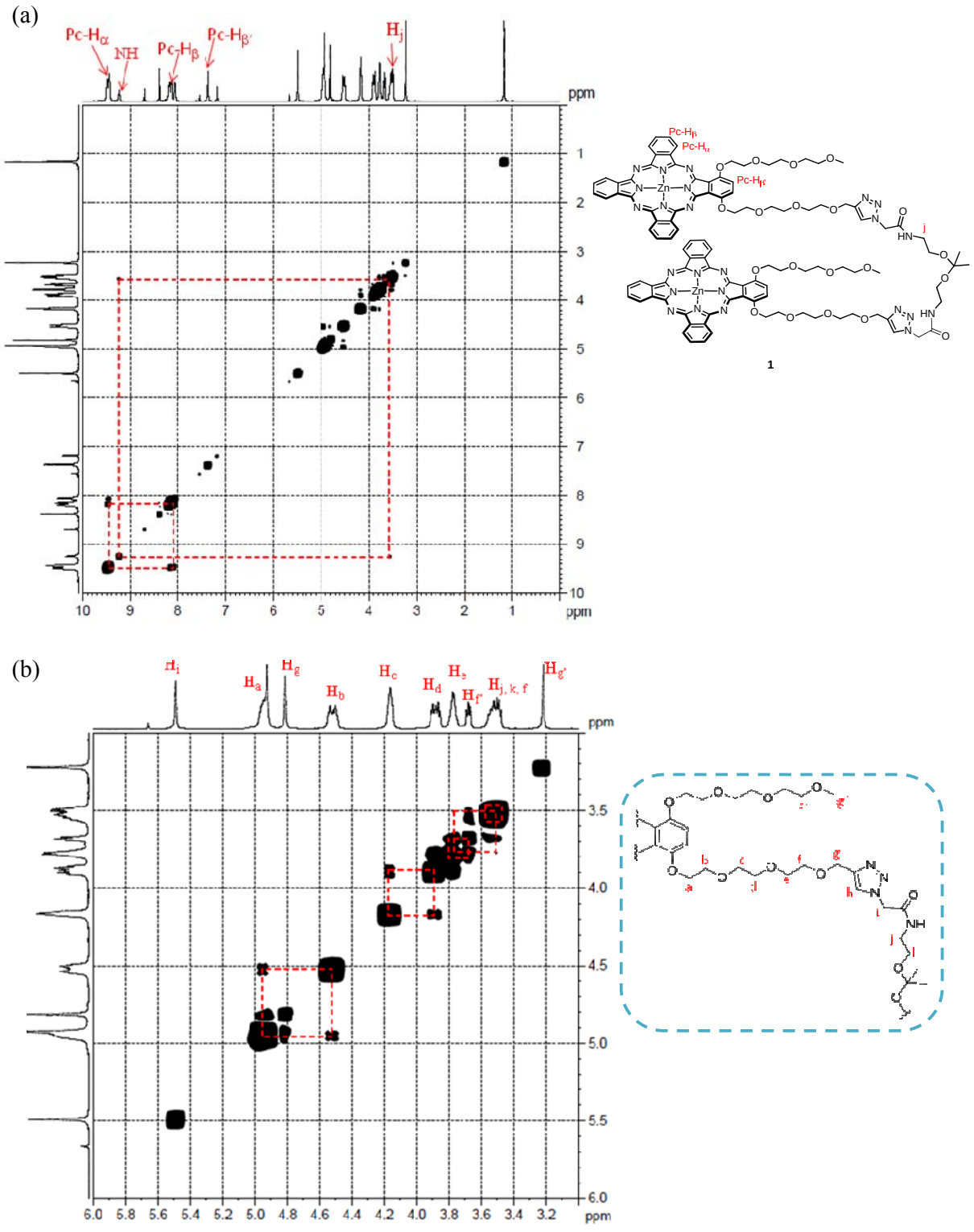


Fig. S14 ^{1}H-^{1}H COSY spectrum of **1** in pyridine-d_5. An enlarged spectrum is shown in (b).

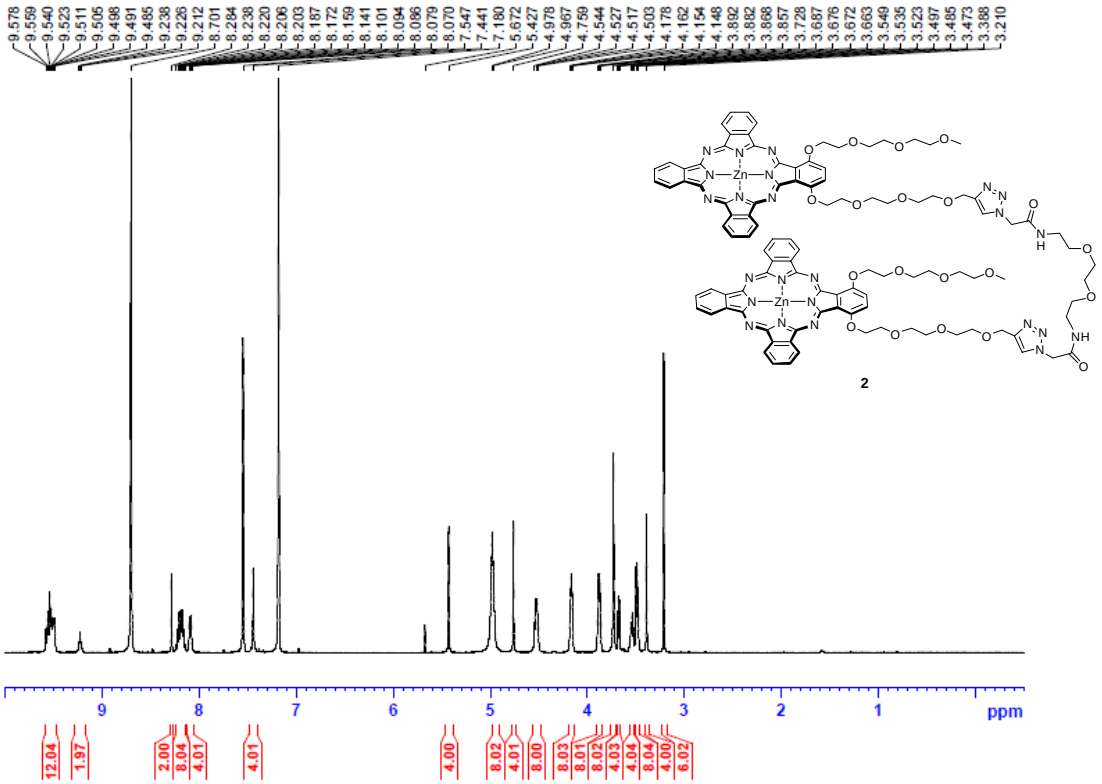


Fig. S15 ^{1}H NMR spectrum of **2** in pyridine-d_5.

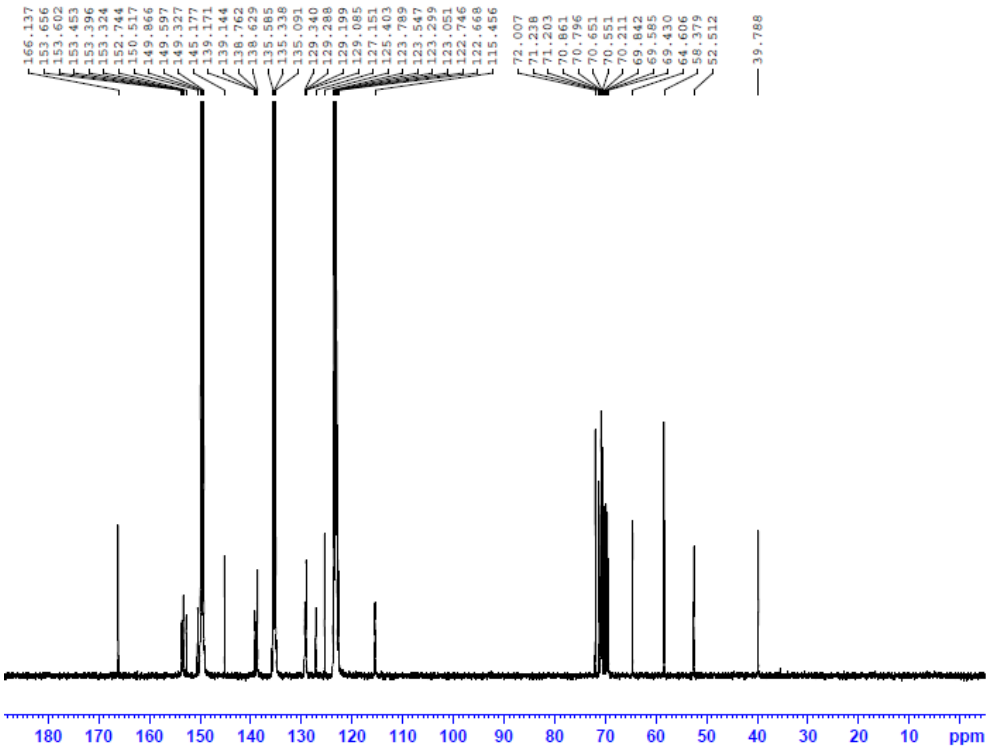


Fig. S16 ^{13}C{^{1}H} NMR spectrum of **2** in pyridine-d_5.

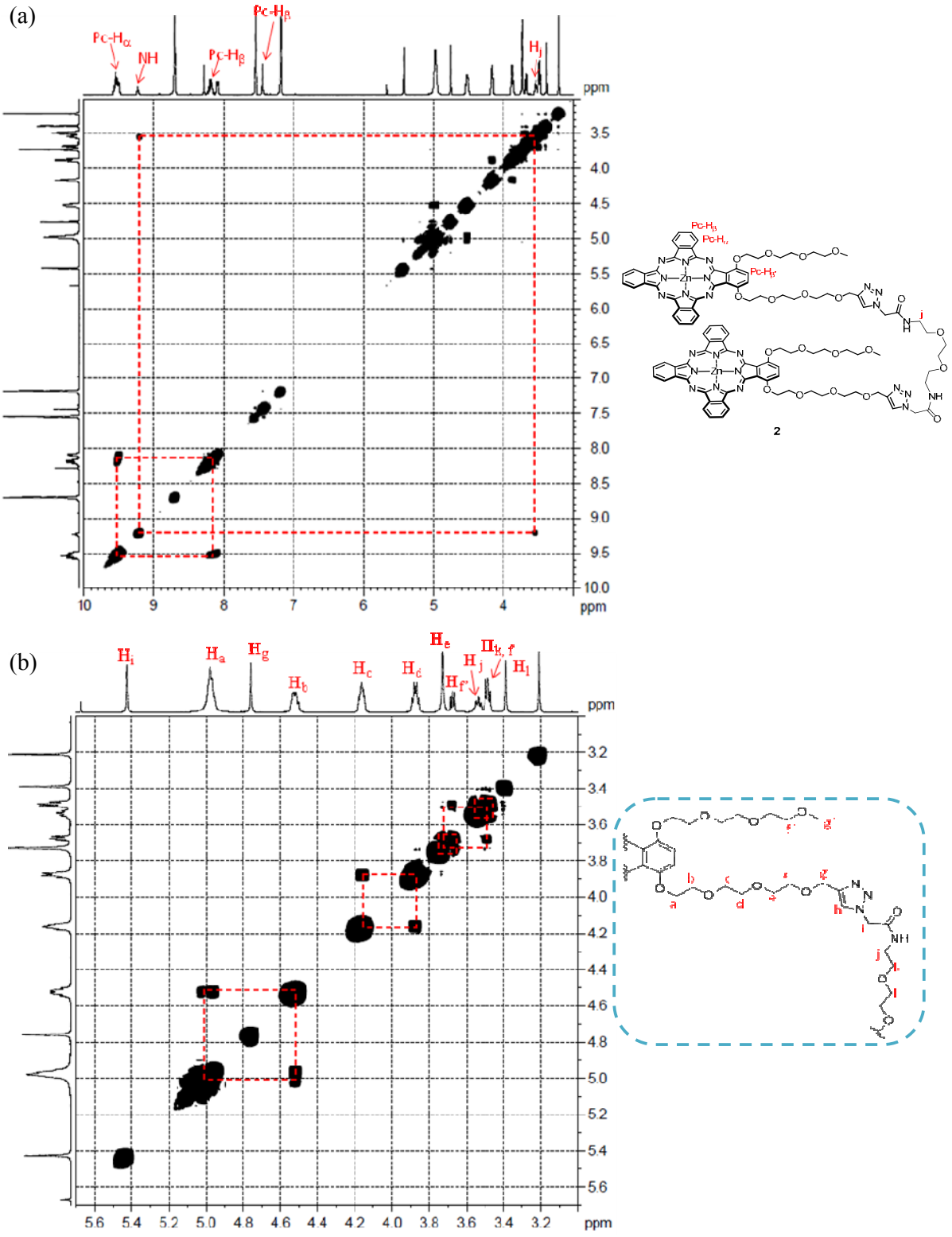


Fig. S17 ^{1}H-^{1}H COSY spectrum of **2** in pyridine-d_5. An enlarged spectrum is shown in (b).