Supporting information

A ratiometric fluorescent probe monitoring pH fluctuations during autophagy in living cells

Shan Wang,[a,c,*] Bingya Wang,[c] Lei Zhu,[a] Ji-Ting Hou[c,*] and Kang-Kang Yu[b,*]

[a] *School of Chemistry and Materials Science, Hubei Engineering University, Xiaogan, 432000, PR China. E-mail: smallcoral@live.cn (S. Wang)*

[b] *Key Laboratory of Bio-resources and Eco-environment (Ministry of Education), College of Life Sciences, Sichuan University, Chengdu 610064, PR China. E-mail: kangkangyu@scu.edu.cn (K.-K. Yu)*

[c] *College of Chemistry and Chemical Engineering, Xinyang Normal University, Xinyang, 464000, PR China. E-mail: houjiting2206@163.com (J.-T. Hou)*

Experimental Section

General remarks for experimental

^{1}H NMR, ^{13}C NMR spectra were measured on a 600 MHz NMR spectrometer (JEOL ECZ600R/S3) equipped with a 14.09 T superconducting magnet and a 5.0 mm 600MHz broadband Z-gradient high resolution ROYAL probe (JEOL RESONANCE Inc., Japan). Proton Chemical shifts of NMR spectra were given in ppm relative to internals reference TMS (1H, 0.00 ppm). Mass spectra were measured on a HP-1100 LC-MS spectrometer. UV-vis spectra were recorded on UV-3900 spectrometer. Fluorescence spectra were recorded on FLS 1000 fluorimeter. Confocal microscopy fluorescence images were acquired on a ZEISS LSM 780 confocal laser scanning microscope (CLSM). The pH values were determined by a PHS-3C pH meter, which was purchased from the Shanghai Yoke Instrument Co., Ltd. The solvents used for UV-vis and fluorescence measurements were of HPLC grade. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. All the solvents were dried according to the standard methods prior to use. All of the solvents were either HPLC or spectroscopic grade in the optical spectroscopic studies.

The preparation of B-R buffer

Aqueous NaOH was added into the mixture of phosphoric acid, acetic acid and boric acid (10 mM for each acid) in 100 mL of distilled water. The buffer solutions with varying pH values were determined using pH meter.

Fluorescence analysis.

Stock solution of the probe (5 mM) was prepared in HPLC grade DMSO. Stock solutions of analytes were prepared in twice-distilled water. For spectral measurements, the probe was diluted to 10 μM in 10 mM B-R buffer solution. 3.0 mL probe solution was placed in a quartz cell of 1 cm optical path length each time. All spectroscopic experiments were carried out at room temperature.

Cell culture

SKOV-3 cells (Perking Union Medical College, China) were cultured in RPMI Medium 1640, supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA) and penicillin (100 units/mL)-streptomycin (100 μg/mL) liquid (Invitrogen Corp.) at 37 °C in a humidified incubator

containing 5% CO_2 in air. Before imaging experiments, cells were seeded in confocal plates (2 × 104 cells/mL).

Intracellular pH calibration.

SKOV-3 cells were firstly incubated with 2.5μM **pH-Ap-1** for 30 min at 37 ℃. Then the cells were washed with PBS (10 mM, pH 7.4) and respectively incubated with high K^+ buffer at various pH values (pH 4.95, 5.46, 6.05, 6.41, 7.06, 7.46, and 8.02) in the presence of nigericin (10.0 μM). The fluorescence images were tested with the confocal microscope under the same condition.

Drug stimulation.

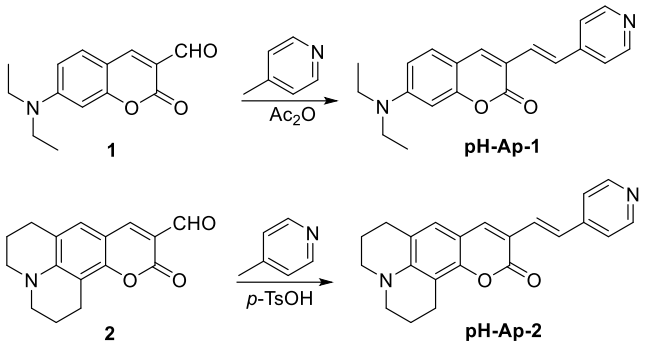
SKOV-3 cells were respectively stimulated with 200 μM verapamil for 12 h, 5 μM loperamide for 12 h, and 500 nM rapamycin for 18 h, 1 μM bafilomycinA1 for 12 h. Meanwhile, a group of SKOV-3 cells were incubated in FBS free medium for 12 h. Then the cells were incubated 2.5 μM **pH-Ap-1** for 30 min, respectively. The fluorescence images were tested before washing.

The staining of autophagy blue (Autophagy Assay Kit).

The Autophagy Assay Kit was brought from Shanghai Yihui Biotechnology Co., Ltd. SKOV-3 cells were respectively stimulated with 200 μM verapamil for 12h, 5μM loperamide for 12 h, and 500 nM rapamycin for 18h. Meanwhile, a group of SKOV-3 cells were starved for 12h. Then the cells were incubated with autophagy blue followed by the protocol of Autophagy Assay Kit, respectively.

Cytotoxicity assay

SKOV-3 was prepared into a density of 10^6 cell / mL with culture medium, and then the cells were seeded into 96-well plates. 100ul of medium was added to each well and placed in a cell culture incubator (5% CO_2/95% Air). Medium culture, after attachment is completed, add a series of probe solutions (0, 1.25, 2.5, 5, 10, 20 μM) to 96-well plates, and place them in the cell culture incubator (5% CO_2/95% Air) Continue incubating for 24h, aspirate the culture medium from the 96-well plate, add 100 μl culture medium, and then add 10 μL CCK-8 solution to each well, and continue in the cell incubator (5 % CO_2/95% Air) for 1-4 h, and then take out the absorbance at 450nm on a microplate reader.



Scheme S1. The preparation of the probes.

Preparation of pH-Ap-1

Compounds **1** and **2** were synthesized according to our previous reports.[1] To a solution of compound **1** (219 mg, 0.87 mmol) in 5 mL Ac_2O, 4-methylpyridine (0.15 mL, 1.56 mmol) was added and the mixture was heated to reflux 12 h. Then, the solvent was removed under reduced pressure, and the crude product was purified on silica column using CH_2Cl_2: EtOAc = 30: 1 (V/V) as eluent to afford a red solid. (87 mg, 31.3%) ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.52 – 8.47 (m, 2H), 8.09 (s, 1H), 7.50 – 7.40 (m, 4H), 7.33 (d, *J* = 16.2 Hz, 1H), 6.71 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.53 (d, *J* = 2.4 Hz, 1H), 3.42 (q, *J* = 7.0 Hz, 4H), 1.10 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (150 MHz, $CDCl_3$-*d*) δ 161.13, 156.07, 151.11, 150.00, 145.34, 140.59, 129.33, 128.07, 127.24, 120.91, 116.38, 109.40, 108.91, 97.14, 45.02, 12.57. ESI-MS: m/z 321.1607 $[M + H]^+$: (calcd 321.1598).

Preparation of pH-Ap-2

To a solution of compound **2** (587.5 mg, 2.2 mmol) and 4-methylpyridine (0.26 mL, 2.64 mmol) in 5 mL dry DMF, *p*-TsOH (568 mg, 3.3 mmol) was added and the mixture was stirred at 90 ^{o}C for 4 h. Then, the reaction mixture was poured into ice water (4 mL), and stirred for 20 min. CH_2Cl_2 was added for extraction. The organic phase was successively washed with water (20 mL x 3) and brine (20 mL x 3), and was dried over Na_2SO_4. The solvent was removed under reduced pressure, and the crude product was purified on silica column using CH_2Cl_2: EtOAc = 30: 1 (V/V) as eluent to afford a red solid. (75 mg, 9.9%). ^{1}H NMR (600 MHz, $CDCl_3$-*d*) δ 8.53 (d, *J* = 5.4 Hz, 2H), 7.63 (s, 1H), 7.40 (d, *J* = 16.3 Hz, 1H), 7.37 – 7.34 (m, 2H), 7.25 (d, *J* = 16.4 Hz, 1H), 6.89 (s, 1H), 3.34 – 3.24 (m, 4H), 2.90 (t, *J* = 6.5 Hz, 2H), 2.80 – 2.74 (m, 2H), 2.00 – 1.96 (m, 4H). ^{13}C NMR (150 MHz, $CDCl_3$-*d*) δ 161.41, 151.14, 149.95, 146.53, 145.48, 140.71, 128.33, 126.51, 125.43, 120.86, 119.03, 115.40, 108.81, 106.31, 50.19, 49.80, 27.59, 21.46, 20.52, 20.33. ESI-MS: m/z 345.1578 $[M + H]^+$: (calcd 345.1598)

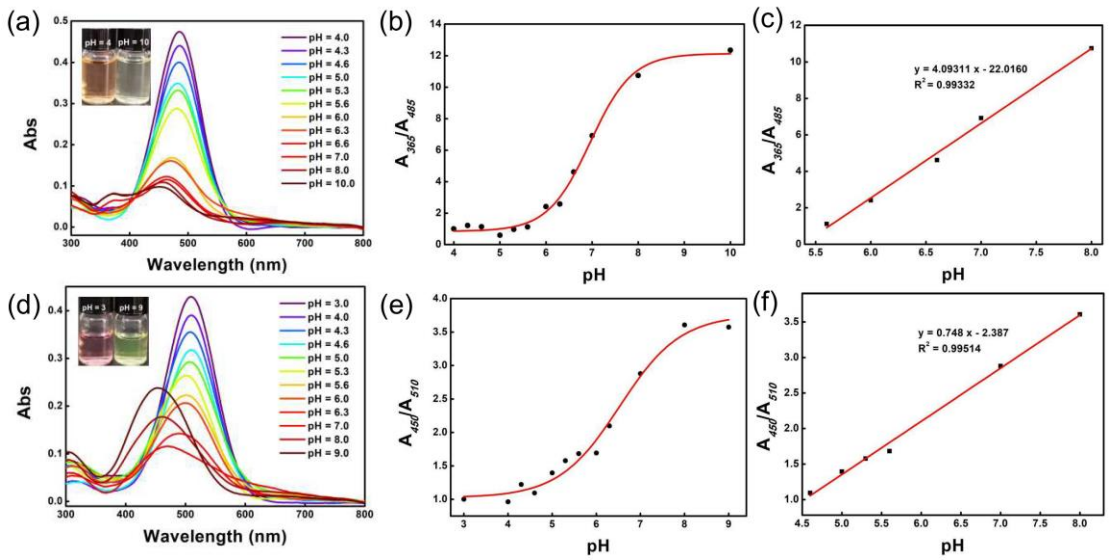


Fig. S1. (a) The changes of Uv-vis absorption spectra of **pH-Ap-1** (15 μM) in B-R buffer solutions with different pH values; (b) Uv-vis titration plot of **pH-Ap-1** in B-R buffer solutions with different pH values; (c) Linear relationship between the absorbance of **pH-Ap-1** in B-R buffer solutions with different pH values; (d) Uv-vis titration of **pH-Ap-2** (15 μM) in B-R buffer solutions with different pH values; (e) Uv-vis titration plot of **pH-Ap-2** in B-R buffer solutions with different pH values; (f) Linear relationship between the absorbance of **pH-Ap-2** in B-R buffer solutions with different pH values.

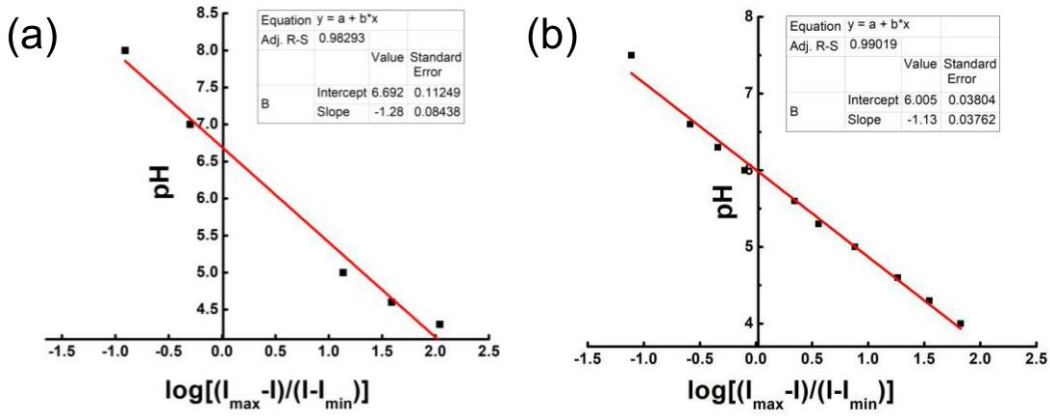


Fig. S2. (a) Plot for **pH-Ap-1** of pH vs log[(I_{max} -I)/(I-I_{min})]. The y-intrecept is the pK_a (6.69±0.11) value of **pH-Ap-1**; (b) Plot for **pH-Ap-2** of pH vs log[(I_{max} -I)/(I-I_{min})]. The y-intrecept is the pK_a (6.00±0.04) value of **pH-Ap-2**.

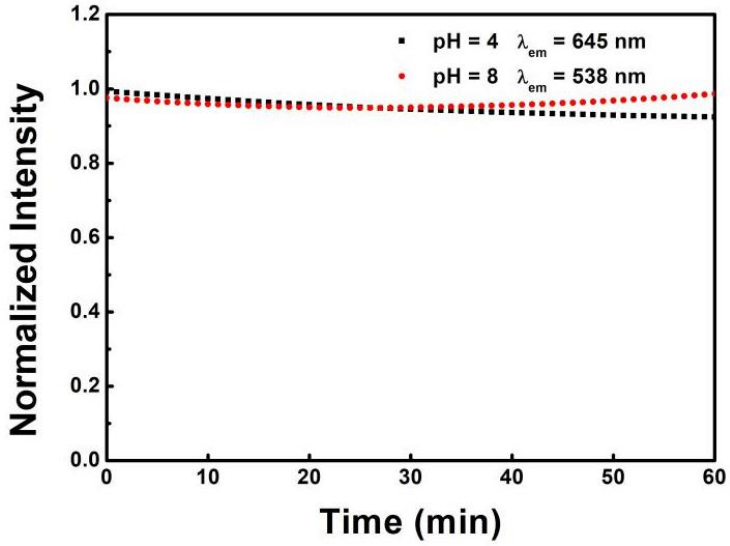


Fig. S3. Dynamic emission intensity changes of **pH-Ap-1** (10 μM) in B-R buffer solution at pH 4 and pH 8. λ_{ex} = 475 nm.

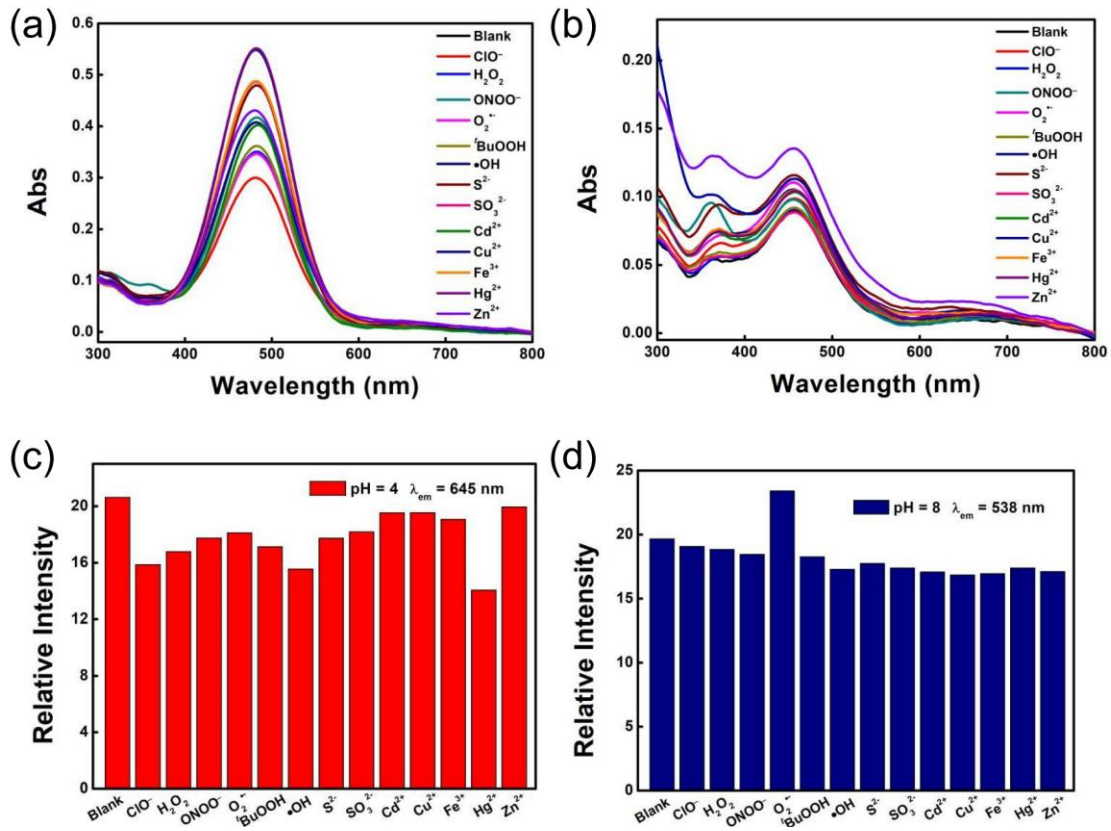


Fig. S4. Uv-vis absorption spectra of **pH-Ap-1** (15 μM) before and after addition of various species in B-R buffer solution at pH 4 (a) and pH 8 (b), respectively; The intensity changes of **pH-Ap-1** (10 μM) before and after addition of various species in B-R buffer solution at pH 4 (c) and pH 8 (d), respectively.

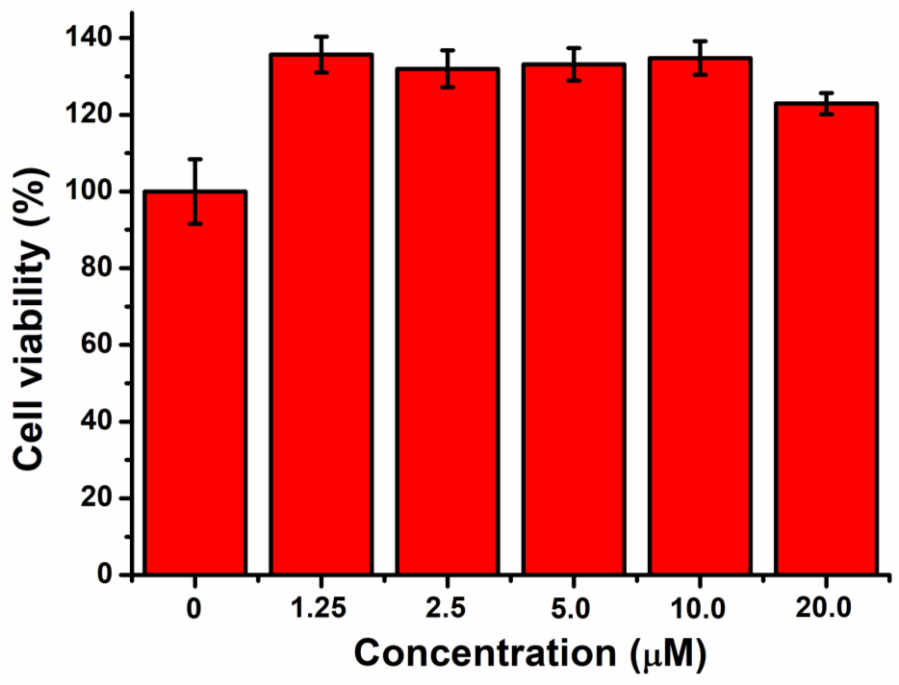


Fig. S5. The viability of SKOV-3 cells after incubation with various concentrations of **pH-Ap-1** for 24 h.

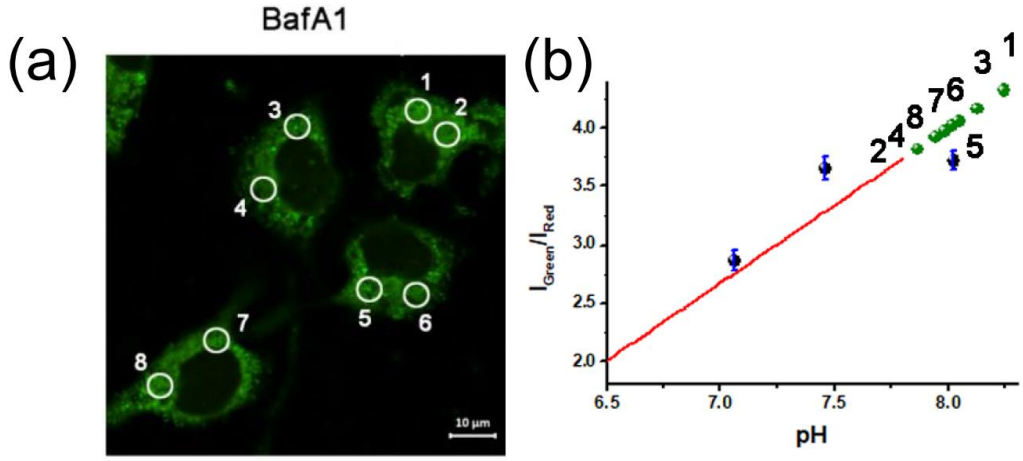


Fig. S6. (a) Confocal fluorescent images of **pH-Ap-1** (2.5 μM) stained SKOV-3 cells that marked with ROI. The cells were stimulated with 1 μM BafA1 for 12 h. (b) The quantitative calculation of pH in the stimulated cells.

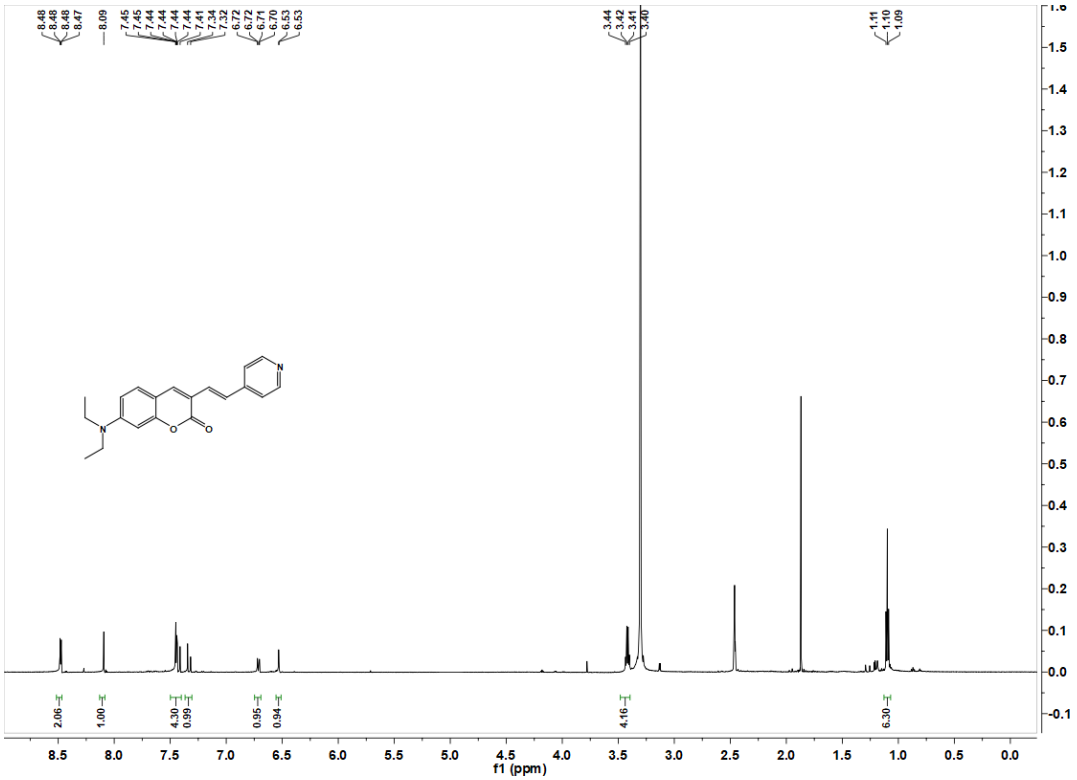


Fig. S7. The ^{1}H NMR spectrum of **pH-AP-1** in DMSO-d_6.

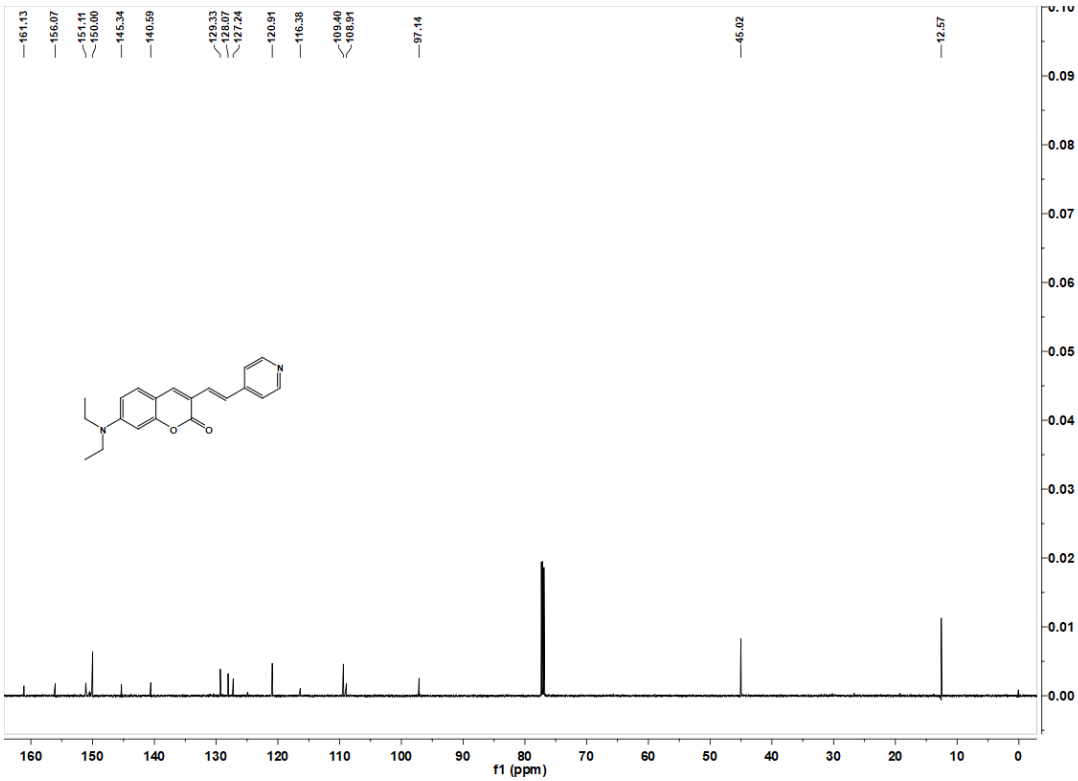


Fig. S8. The ^{13}C NMR spectrum of **pH-AP-1** in $CDCl_3$.

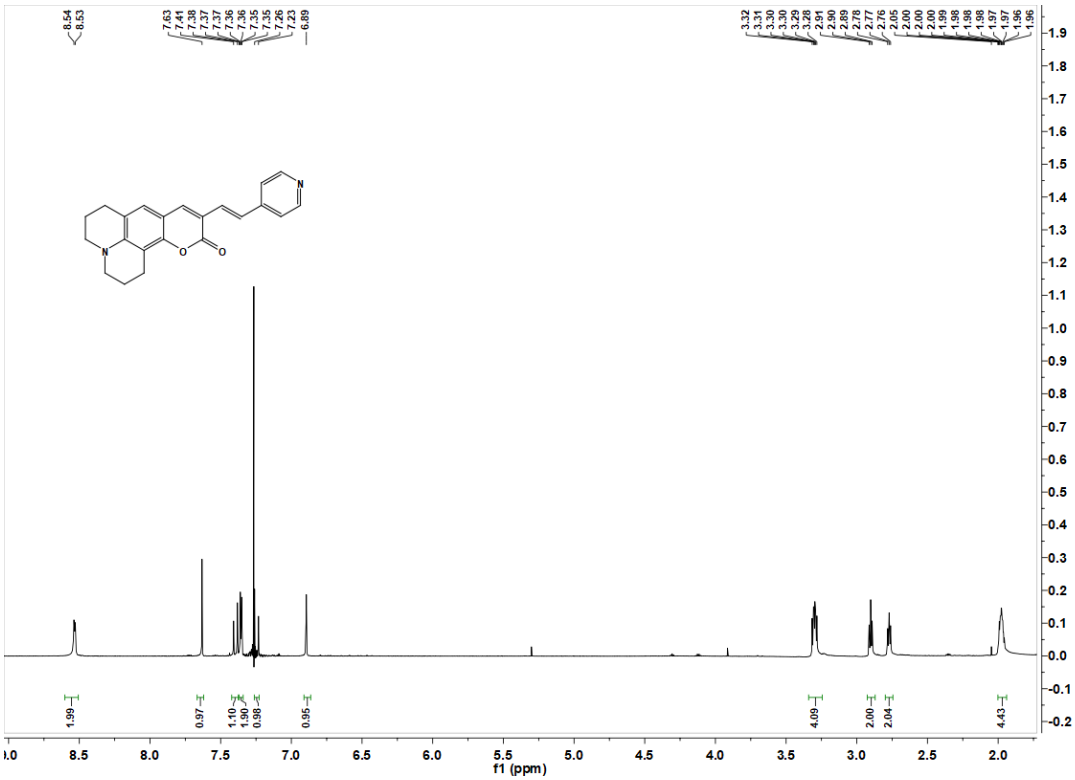


Fig. S9. The ^{1}H NMR spectrum of **pH-AP-2** in $CDCl_3$.

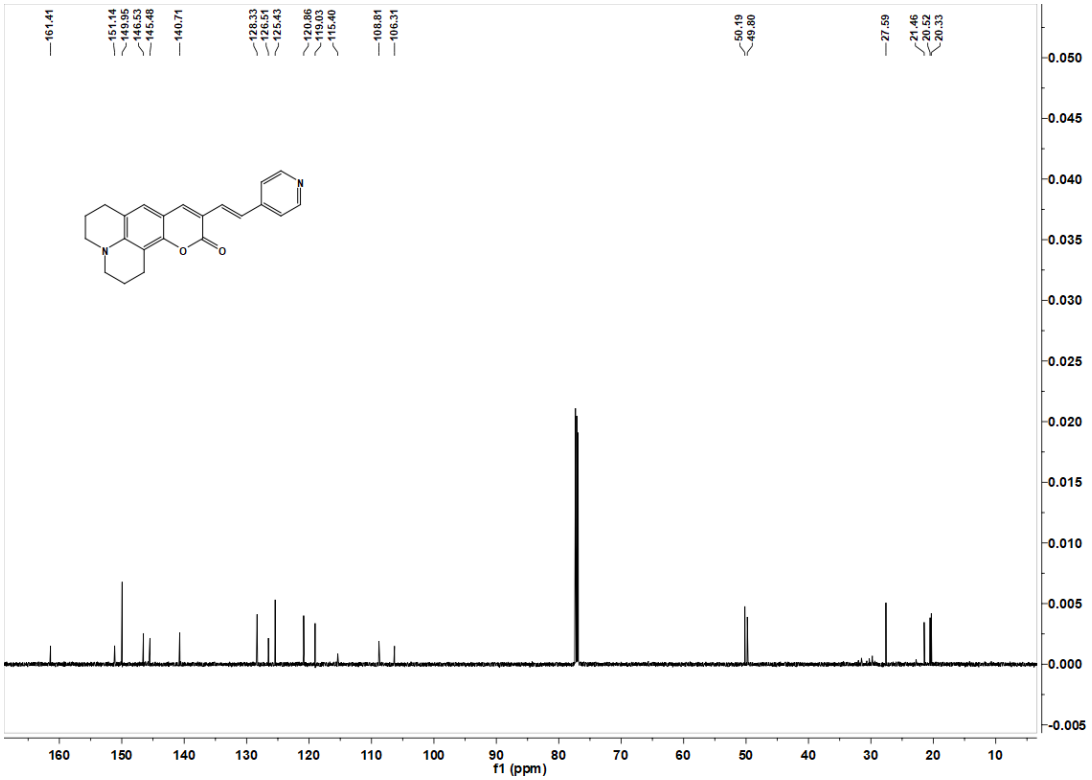


Fig. S10. The ^{13}C NMR spectrum of **pH-AP-2** in $CDCl_3$.

Reference:

1. J.-T. Hou, J. Yang, K. Li, Y.-X. Liao, K.-K. Yu, Y.-M. Xie and X.-Q. Yu, *Chem. Commun.*, 2014, **50**, 9947-9950