Supporting Information

Conformation-Dependent Bifunctional Fluorescent Sensor for Viscosity-pH Monitoring in Oxidative Stress

Lifang Guo,[†*] Yuxi Ding,[†] Haijiao Wu, Qianxin Long, Zhiyun Zhang, and Jianhua Su

Key Laboratory for Advanced Materials and Joint International Research Laboratory of Precision Chemistry and Molecular Engineering, Feringa Nobel Prize Scientist Joint Research Center, East China University of Science & Technology, 130 Meilong Road, Shanghai 200237, China. Email: guolf@ecust.edu.cn.

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1. Materials and Methods

General

All the original reagents were purchased from the market and were all of analytical grade, without further purification. The solvents used in the synthesis were all ultra-dry solvents (or solvents containing molecular sieves), and aniline was purified by vacuum distillation.

The nuclear magnetic resonance hydrogen spectrum (^{1}H NMR) and nuclear magnetic resonance carbon spectrum (^{13}C NMR) used for compound characterization. The spectrometer was produced by the Swiss manufacturer Bruker, with models AVANCE III 400 and Ascend 600. The solvents were deuterated dimethyl sulfoxide (DMSO-d_6) and deuterated dichloromethane (Methylene Chloride-d_2), and the internal standard was tetramethylsilane (TMS, δ = 0 ppm). The following are the multiple attributes and abbreviations: single peak (s), double peak (d), triple peak (t), quadruple peak (q), and multiple peak (m). High-resolution mass spectrometry (HRMS) was obtained through real-time direct analysis mass spectrometry (DART) and mass spectrometry (ESI). The fluorescence emission spectrum was measured by Shimadzu Spectro Fluorophotometer RF-6000, and the ultraviolet absorption spectrum (UV-Vis) was measured by Shimadzu UV-2600 ultraviolet-visible spectrophotometer. The solvents used during the testing process were all of spectral purity grade or obtained through re-distillation.

Spectroscopic measurements

Unless otherwise stated, all tests are conducted according to the following method. A certain amount of the probe PPAC-AOH is dissolved in a spectral-grade DMSO solution to prepare a 1 mM probe stock solution. The pH spectroscopy test system is an ethanol-water mixture solution (60% ethanol), with the final probe concentration of 10 μM, and the pH test is achieved by adding a small amount of sodium hydroxide solution or hydrochloric acid solution to the test solution to change the pH value. The viscosity spectroscopy test system is a glycerol-ethanol mixture solution, with the final probe concentration of 10 μM, and the viscosity test is achieved by adjusting the proportion of ethanol and glycerol in the mixed solution to change the viscosity.

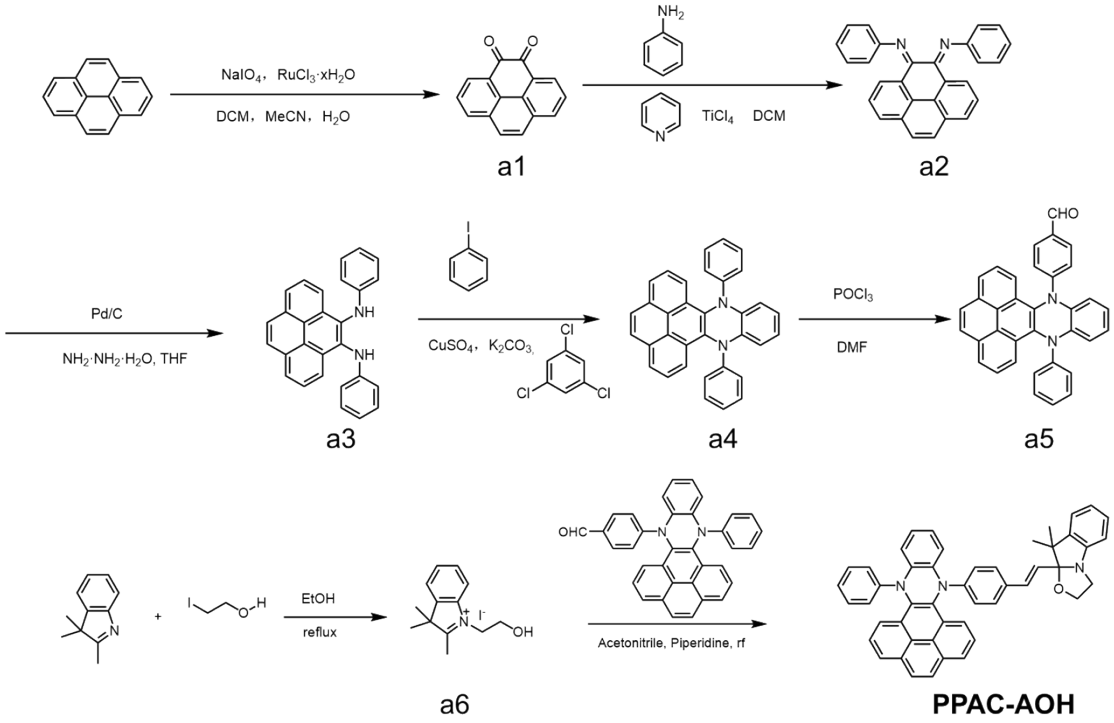
Nuclear magnetic titration test solution preparation: Using a 10 μL pipette with a range of 10 μL, 10 μL of deuterated trifluoroacetic acid containing TMS is transferred to 0.1 mL of spectral-grade DMSO containing TMS, and a deuterated trifluoroacetic acid titration solution with a concentration of 0.16 g/mL is prepared. Each equivalent of H^+

is taken 2.16 μL of the deuterated trifluoroacetic acid titration solution.

Spectroscopic test conditions for the probe **PPAC-AOH**: λ_{ex} = 365 nm.

2. Synthesis details

Scheme S1. The synthesis routes of PPAC-AOH.



9,14-Diphenyl-9,14-dihydrodibenzo[a,c]phenazine (**a4, PPAC**) was synthesized according to a previous literature report.[1-4]

Preparation of Intermediate a5: 4-(14-phenylphenanthro[4,5-abc]phenazin-9(14H)-yl)benzaldehyde

The clean magnetic stir bar was placed in a three-necked flask. The gas was evacuated, the flask was placed in an ice bath, and nitrogen was introduced. 4 mL of ultra-dry N,N-dimethylformamide solvent was added under these conditions, followed by the dropwise addition of 4 mL of ultra-dry trichlorophosphine. The mixture was stirred at ice bath for 2 hours. Then, the raw material a4 (1.240 g, 7.80 mmol) was added, and 16 mL of ultra-dry N,N-dimethylformamide solvent was added. The ice bath was removed, and the mixture was stirred at room temperature for 30 minutes. The mixture was heated to 70 °C, and the reaction was carried out by condensation reflux for 8 hours. After the reaction was completed, the reaction mixture was poured into an ice-water mixture to quench, the precipitate was obtained, filtered, the ethanol was used to pulp the precipitate, and then filtered and dried to obtain the crude product. The crude product

was purified by silica gel column chromatography using an eluent of petroleum ether: dichloromethane = 1:2. The white product of 0.98 g was obtained, with a yield of 74.8%.^{1}H NMR (600 MHz, DMSO-d_6) δ 9.70 (s, 1H), 8.39 (ddd, J = 15.0, 7.6, 1.1 Hz, 2H), 8.29 (d, J = 1.3 Hz, 2H), 8.24 (ddd, J = 8.9, 7.8, 1.1 Hz, 2H), 8.12 (t, J = 7.7 Hz, 1H), 8.10 – 8.08 (m, 1H), 8.06 – 8.02 (m, 2H), 7.63 – 7.60 (m, 2H), 7.53 – 7.50 (m, 2H), 7.17 (dt, J = 7.2, 1.2 Hz, 2H), 7.11 (dd, J = 8.9, 7.1 Hz, 4H), 6.88 (td, J = 7.2, 1.1 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 190.28, 146.04, 143.94, 141.51, 138.31, 135.92, 130.63, 130.54, 128.59, 128.44, 127.13, 127.10, 127.02, 126.35, 126.27, 126.24, 126.20, 125.96, 125.53, 125.31, 122.80, 121.69, 121.04, 120.35, 117.56, 114.23. HRMS for $C_{35}H_{22}N_2O$ m/z $[M+H]^+$ calcd.: 487.1810, found: 487.1770.

Preparation of Intermediate a6: 1-(2-hydroxyethyl)-2,3,3-trimethyl-3H-indol-1-ium

The clean magnetic stir bar was placed in a three-necked flask. The gas was evacuated three times. Then, 1.28 g of iodine ethanol (7.44 mmol) was added, and the solution was dissolved in ultra-dry ethanol. Next, 0.992 g of 2,3,3-trimethylindole (6.22 mmol) was added. The temperature was raised to 90 °C and the reaction was carried out overnight. After the reaction, the solvent was removed by rotary evaporation. Then, the mixture was ground with ethanol and hexane, and the crystals were filtered to obtain 1.25 g of a light yellow crystal, with a yield of 60.68%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 7.95 (dd, J = 5.82, 3.25 Hz, 1H), 7.88 – 7.83 (m, 1H), 7.62 (dd, J = 5.77, 3.14 Hz, 2H), 4.60 (t, J = 5.06 Hz, 2H), 3.88 (t, J = 5.12 Hz, 2H), 3.46 (s, 1H), 2.82 (s, 3H), 1.55 (s, 6H). ^{13}C NMR (101 MHz, DMSO): δ (pp HR-MS calculated for $C_{13}H_{18}NO^+$ *m*/*z* 204.1383 found 204.1389.

Preparation of target compound PPAC-AOH: (E)-9,9-dimethyl-9a-(4-(14-phenylphenanthro[4,5-abc]phenazin-9(14H)-yl)styryl)-2,3,9,9a-tetrahydrooxazolo[3,2-a]indole

Put the raw materials a5 (0.10g, 0.21 mmol) and a6 (0.10g, 0.30 mmol) into a three-necked flask. Exhaust air three times, then add 10 mL of ultra-pure acetonitrile and 100 μL of analytical-grade piperidine. After the reaction, remove the solvent by rotary evaporation, pulp the ethanol, filter and dry it. Perform column chromatography separation using the eluent of dichloromethane: ethanol = 30:1. Purify the product to obtain a yellow solid of 70 mg, with a yield of 41.7%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.33 (dt, J = 7.7, 1.2 Hz, 2H), 8.26 – 8.19 (m, 4H), 8.04 (td, J = 7.8, 2.5 Hz, 2H), 7.97 (dt, J = 7.5, 3.8 Hz, 2H), 7.45 (dd, J = 6.0, 3.4 Hz, 2H), 7.29 – 7.25 (m, 2H), 7.12 – 7.09 (m, 4H), 7.06 (dd, J = 8.3, 6.9 Hz, 4H), 6.89 – 6.79 (m, 3H), 6.61 (d, J = 15.9 Hz, 1H),

6.08 (d, J = 16.0 Hz, 1H), 3.61 (dtt, J = 11.4, 7.3, 3.7 Hz, 2H), 3.42 (q, J = 7.9, 7.1 Hz, 1H), 1.29 (s, 3H), 1.25 – 1.20 (m, 1H), 0.98 (s, 3H), 0.88 – 0.82 (m, 1H). ^{13}C NMR (101 MHz, Methylene Chloride-d_2) δ 151.53, 148.22, 147.77, 140.40, 138.90, 132.09, 132.07, 130.12, 129.41, 128.66, 128.56, 128.06, 128.02, 127.98, 127.88, 126.69, 126.19, 126.06, 126.01, 125.97, 124.07, 122.81, 122.37, 122.20, 121.88, 121.83, 117.55, 117.15, 112.37, 110.46, 63.86, 50.49, 48.24, 28.64, 20.56.HRMS (ESI, *m/z*): $[M+H]^+$ calcd for $C_{38}H_{37}N_3O$, 672.3015; found, 672.3014.

3. Photophysical properties.

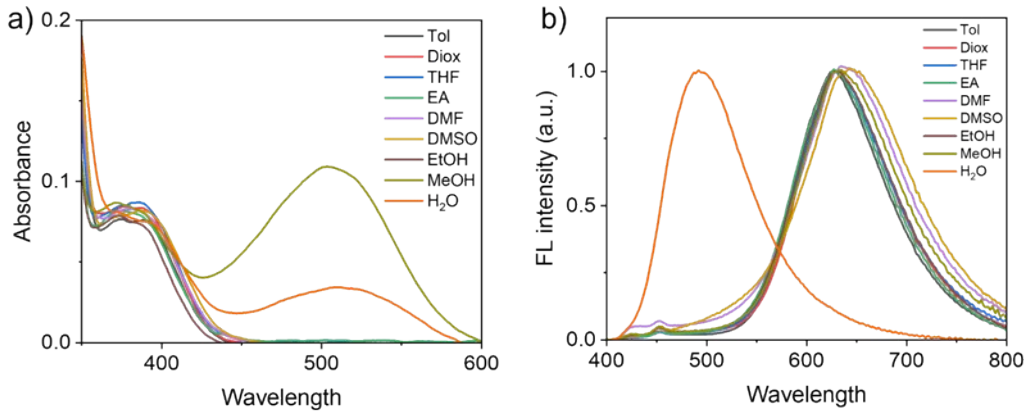


Figure S1. Photophysical properties of **PPAC-AOH** in different Solvents. (a) UV-Vis absorption and (b) fluorescence spectra. λ_{ex} = 365 nm.

Table S1. The photophysical properties of **PPAC-AOH** in different solvents.

Solvents	λ_{abs}[a] (nm)	ε[b] ($M^{-1}\cdot cm^{-1}$)	λ_{em}[c] (nm)	Φ_f[d] (%)
Tol	388	4344	629	12.59
Diox	388	4773	631	12.80
THF	385	4969	630	11.61
EA	375	4685	627	8.67
DMF	381	4745	635	6.49
DMSO	388	4673	637	5.39
EtOH	373	4512	633	6.22
MeOH	371, 504	4965, 6238	635	3.18
H_2O	393, 510	4255, 1962	492	4.27

a, Wavelength of absorption maxima, in nm. b, the dielectric constants of the solvents; c, Wavelength of emission maxima, in nm. d, the fluorescence quantum yields of the probe **PPAC-AOH** in different solvents with 0.5 M quinine sulfate (Φ_s = 55%) as reference standard.

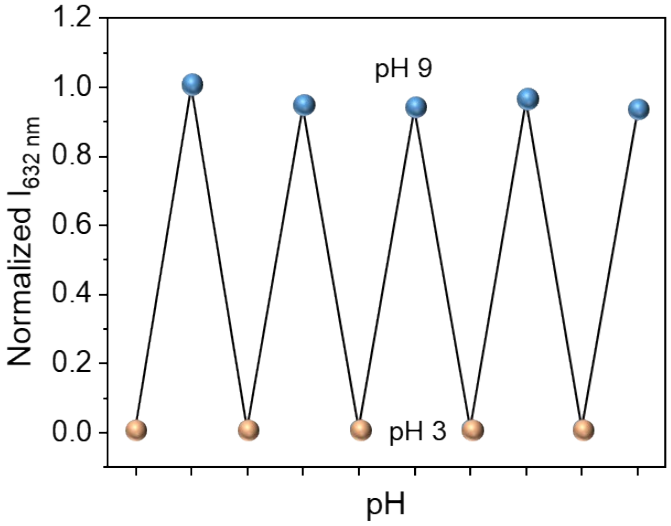


Figure S2. Reversible I_{632} changes of **PPAC-AOH** between pH3 and pH9. λ_{ex} = 365 nm, concentration = 10 μM.

4. Cell culture and fluorescence imaging

Cell culture

Hela cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin and placed in a 5% CO_2 incubator at 37 °C. In each experiment, cells treated with vehicle control (1% DMSO) were kept as the control group.

Biotoxicity evaluation

The cytotoxicity of **PPAC-AOH** on Hela cells were carried out using the standard MTT assay. Hela cells growing in the logarithmic phase were seeded into 96-well plates (ca. 1 × 10^4 cells/well) and allowed to adhere for 24 h. Probe **PPAC-AOH** (100 μL/well) of different concentrations was added into the wells of the treatment group for different time, and 100 μL/well DMSO diluted in DMEM at the same concentration to the negative control group. Then 10 μL of MTT was added to each well. After incubation for 4 h, the culture medium in each well was removed, and DMSO (100 μL) was added to dissolve the purple crystals. After 15 min, the absorbance was measured at 570 nm with a microplate reader. Finally, the cell survival rate can be calculated using the following equation:

Survival rate = $(A_{Sample} - A_{Blank}) / (A_{DMSO} - A_{Blank})$

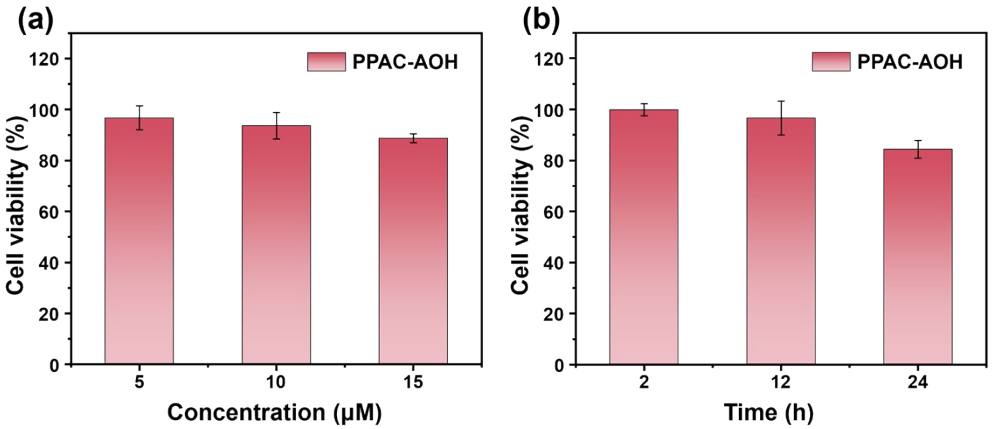


Figure S3. MTT results of Hela cells incubated with **PPAC-AOH** for different concentrations (a) and different time (b).

Photostability test

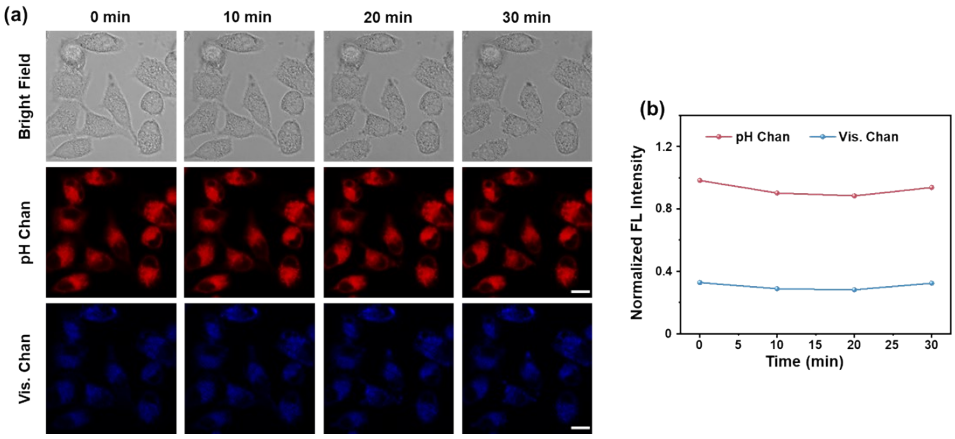


Figure S4. (a) The photostability test of **PPAC-AOH** under constant laser irradiation of 405 nm during 30 min, and (b) the corresponding fluorescent intensity at different time points. Scale bar = 20 μm.

Co-localizaiton experiments

Co-localization experiments were investigated by co-incubation of **PPAC-AOH** (10 μM) and commercial dyes (Mito-Tracker Deep Red: MTDR, BODIPY 493/503: LDs-Bodipy, Lyso-Tracker Red: LTR, ER-Tracker Red: ER Red, CytoTrace™ Red: CTR) to the cells for 30 min, respectively. After that, the culture media were removed, and the cells were washed with PBS three times. The cell images were observed by confocal fluorescence microscopy (Leica TCS SP8).

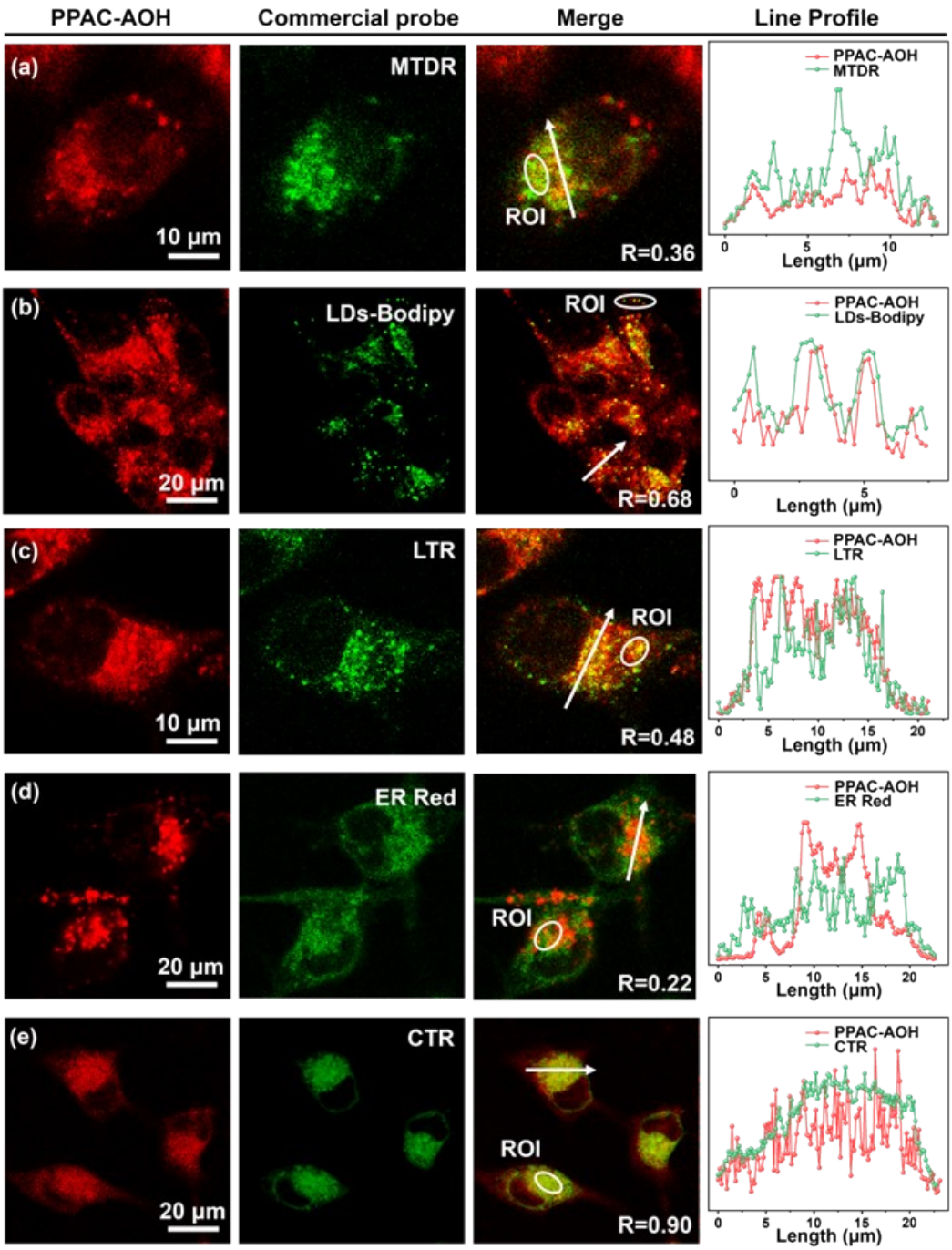


Figure S5. Confocal images of HeLa cells treated with **PPAC-AOH** and then with MTDR (a), LDs-Bodipy (b), LTR (c), ER Red (d), and CTR (e), as well as corresponding intensity profiles. **PPAC-AOH**: 10 μM, 24 h; λ_{ex} = 405 nm, λ_{em} = 420-520 nm or λ_{em} = 600-700 nm; MTDR: 500 nM, 30 min; λ_{ex} = 623 nm, λ_{em} = 630-660 nm. LDs-Bodipy: 10 μM, 30 min; λ_{ex} = 494 nm, λ_{em} =495-520 nm; LTR: 500 nM, 30 min; λ_{ex} = 580 nm, λ_{em} = 600-700 nm. ER Red: 2 μM, 30 min; λ_{ex} = 587 nm, λ_{em} =596-630 nm. CTR: 10 μM, 30 min; λ_{ex} = 570 nm, λ_{em} =590-650 nm.

Intracellular pH imaging

HeLa cells were incubated with **PPAC-AOH** (10 μM) for 12 h and washed with PBS three times. Then, the cells were treated with high K+ buffer (30 mM NaCl, 120 mM KCl, 1mM $CaCl_2$, 0.5 mM $MgSO_4$, 1 mM NaH_2PO_4, 5 mM glucose, 20 mM HEPES, and 20 mM NaOAc) at various pH values (4.5-9) in the presence of 10 μM nigericin. After 30 min, the red channel images were recorded with the confocal microscope. Red channel: λ_{ex} = 405 nm, λ_{em} = 600-700 nm.

Intracellular viscosity imaging.

HeLa cells were incubated with **PPAC-AOH** (10 μM) for 12 h and washed with PBS three times. Then, the cells were treated with nystatin (Nys, 10 μM) for 0, 3, 6, 9, 12, and 24 h to induce increasing intracellular viscosity. Fluorescence imaging was recorded with the confocal microscope. Blue channel: λ_{ex} = 405 nm, λ_{em} = 420-520 nm; red channel: λ_{ex} = 405 nm, λ_{em} = 600-700 nm.

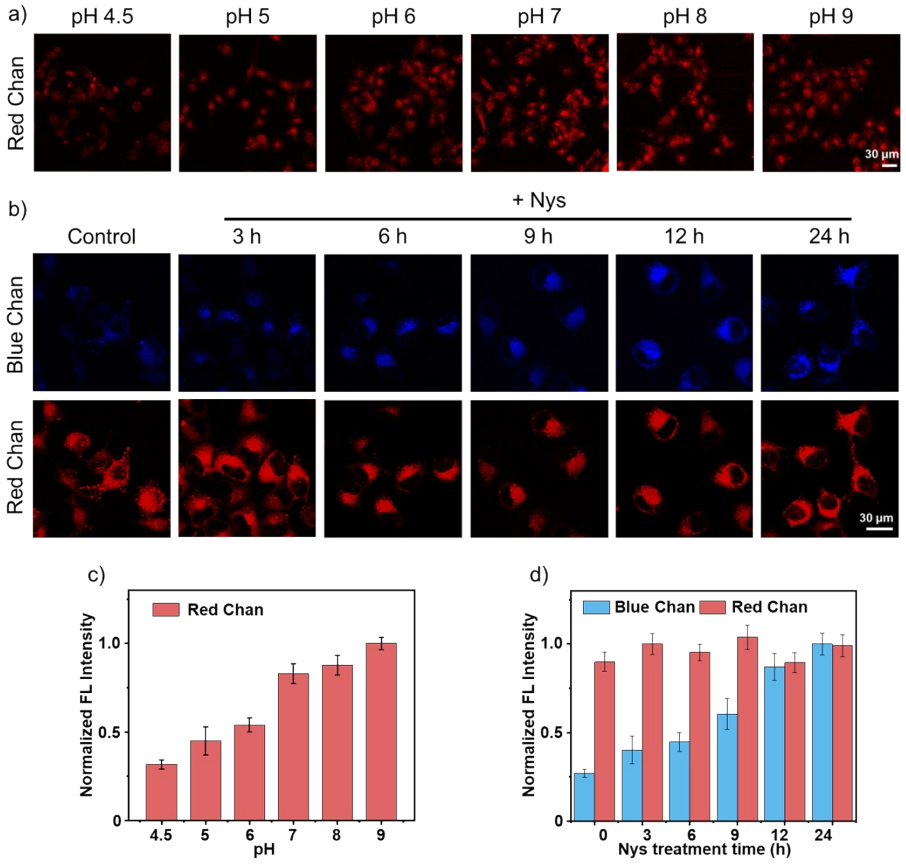


Figure S6. pH and viscosity responses in living cells. (a) Confocal images of **PPAC-AOH** in Hela cells incubated with buffers of different pH and (b) Nys for different times, and corresponding fluorescence intensity changes (c, d). **PPAC-AOH**: 10 μM, 24 h; λ_{ex} = 405 nm, λ_{em} = 420-500 nm or λ_{em} = 600-700 nm. Nys: 10 μM.

Monitoring pH/viscosity change under oxidative stress.

HeLa cells were incubated with **PPAC-AOH** (10 μM) for 12 h and washed with PBS three times. Then, the cells were incubated with DCFH-DA (10 μM) to indicate total ROS. After 30 min, the culture media were removed and washed with PBS three times, and the cells were treated with H_2O_2 (0, 25,50,75,100, and 150 μM) for 1 h to induce increasing oxidative stress. For the antioxidant-treated control groups for reverse validation, cells were treated with H_2O_2 (150 μM) and N-acetyl-L-cysteine (NAC, 500 μM) for 1 h. The fluorescence images were recorded with the confocal microscope. Blue channel: λ_{ex} = 405 nm, λ_{em} = 420-520 nm; red channel: λ_{ex} = 405 nm, λ_{em} = 600-700 nm; DCFH channel: λ_{ex} = 488 nm, λ_{em} = 500-560 nm.

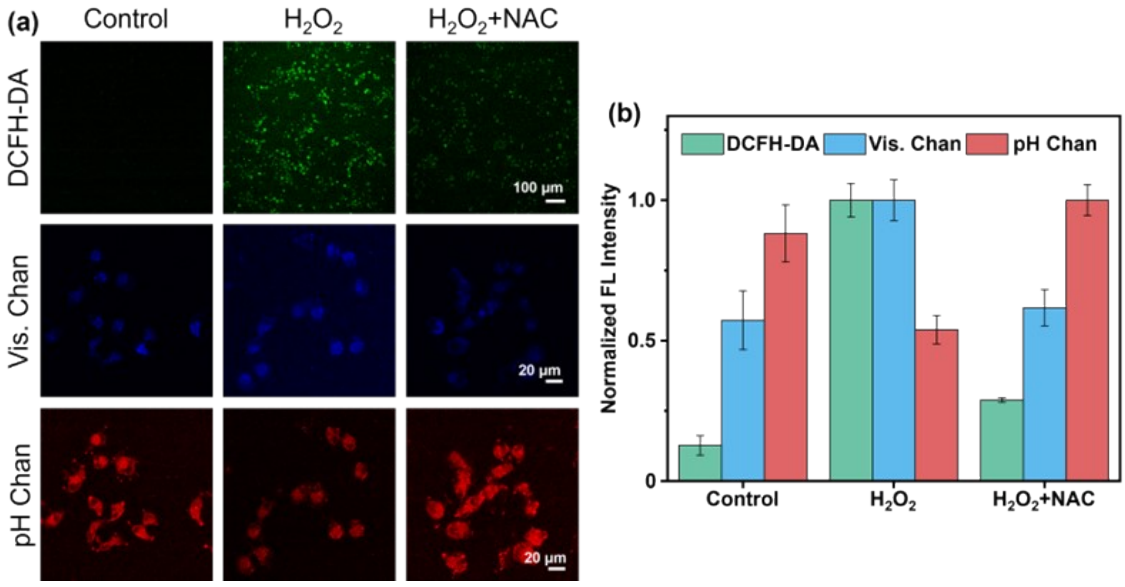
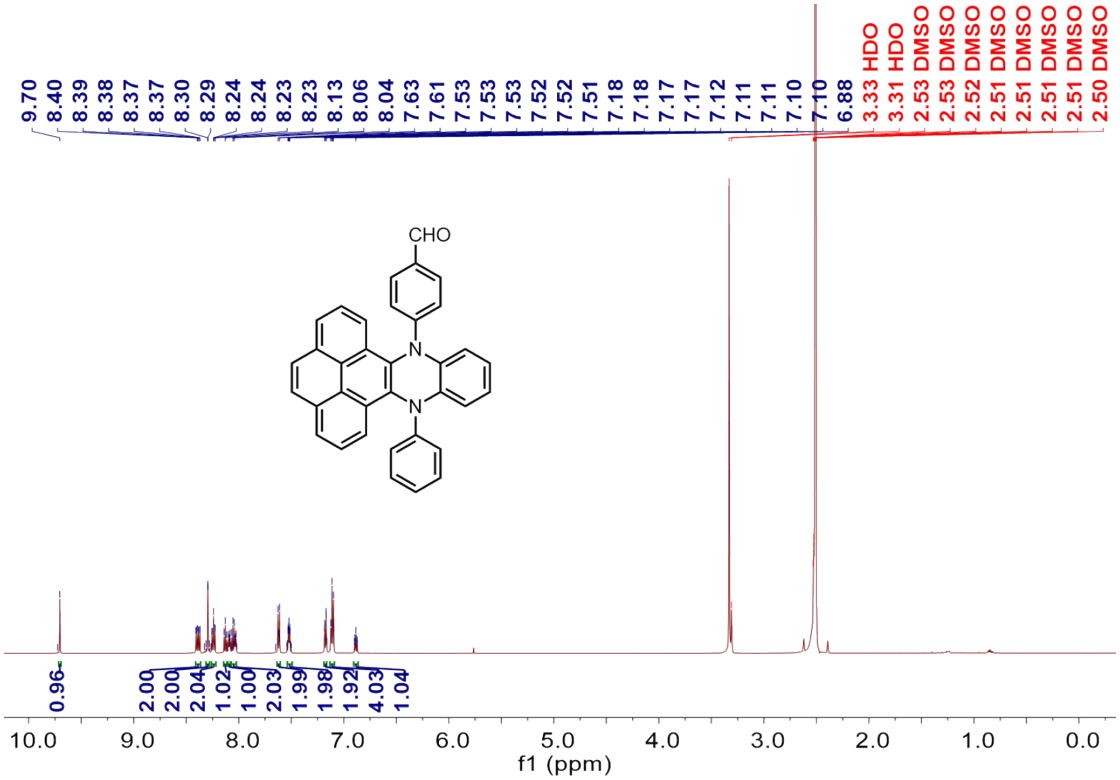
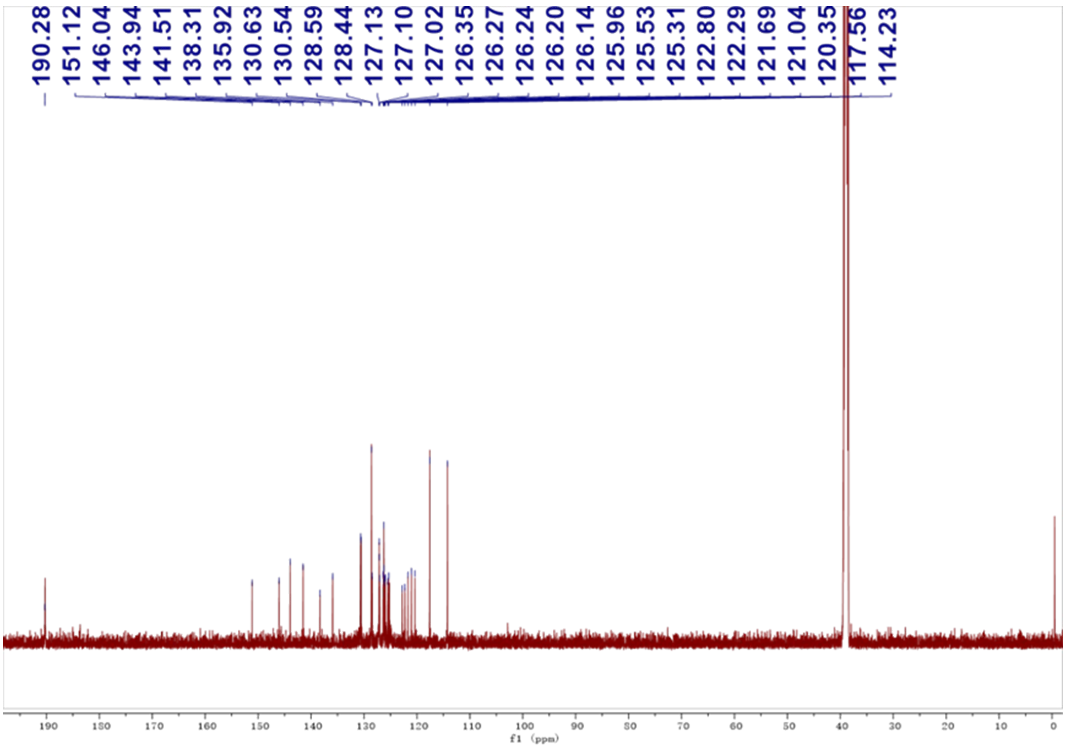


Figure S7. Viscosity and pH variations in the oxidative stress model. (a) Confocal images of DCFH-DA and **PPAC-AOH** in HeLa cells treated with H_2O_2 and H2O2+NAC, and (b) corresponding fluorescence intensity changes. DCFH-DA: 10 μM, 30 min; λ_{ex} = 488 nm, λ_{em} = 500-560 nm. **PPAC-AOH**: 10 μM, 12 h; λ_{ex} = 405 nm, λ_{em} = 420-500 nm or λ_{em} = 600-700 nm.

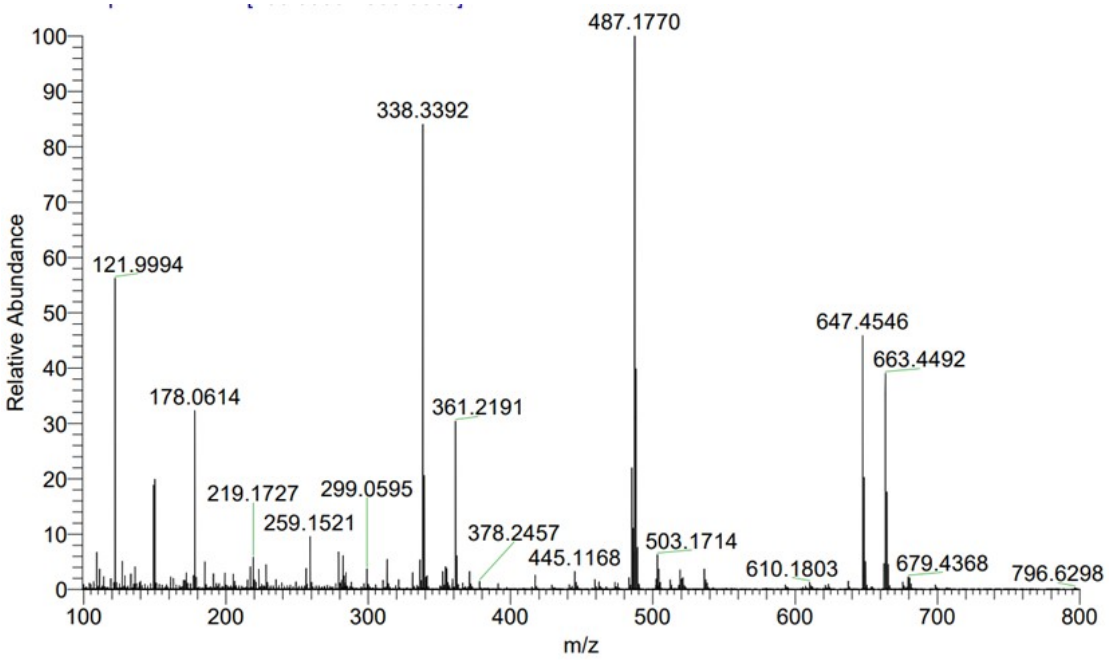
5. NMR and HR-MS



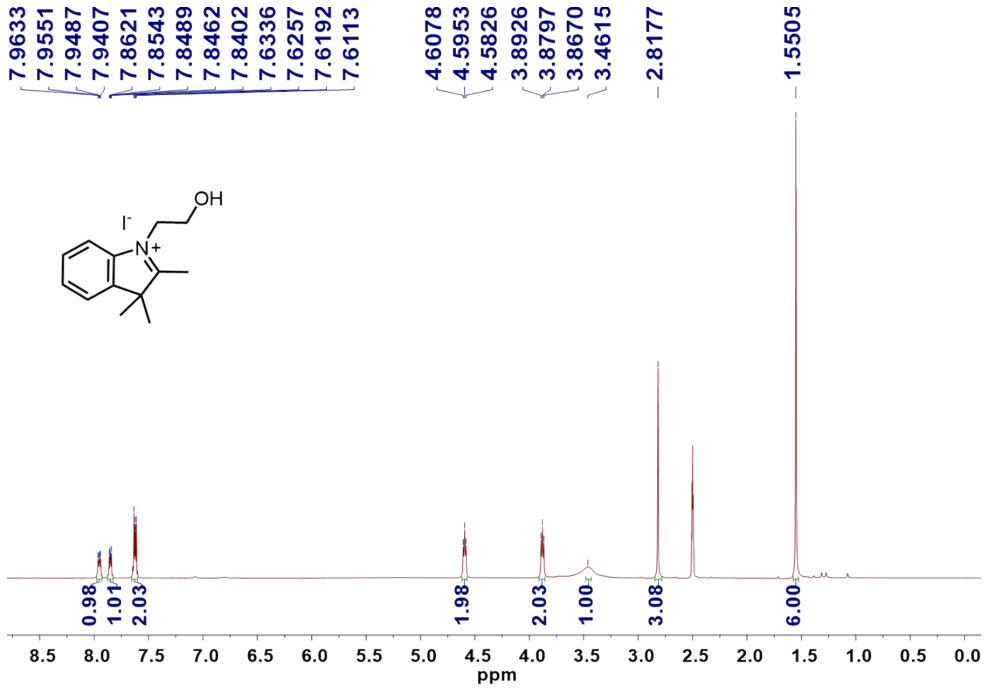
^{1}H NMR spectrum of **a5** in DMSO-d_6.



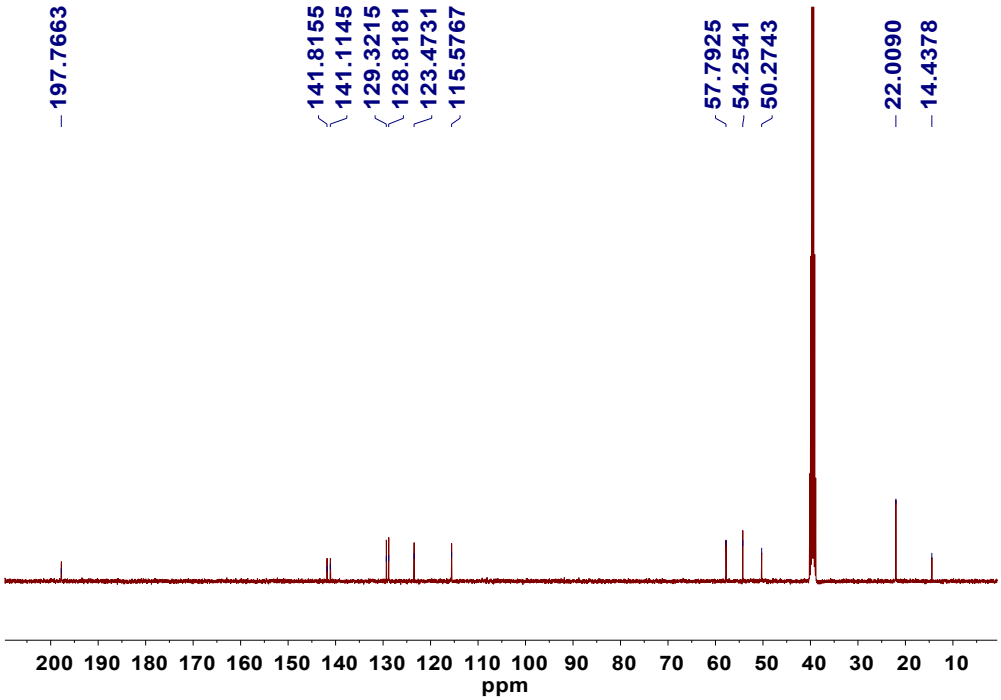
^{13}C NMR spectrum of **a5** in DMSO-d_6.



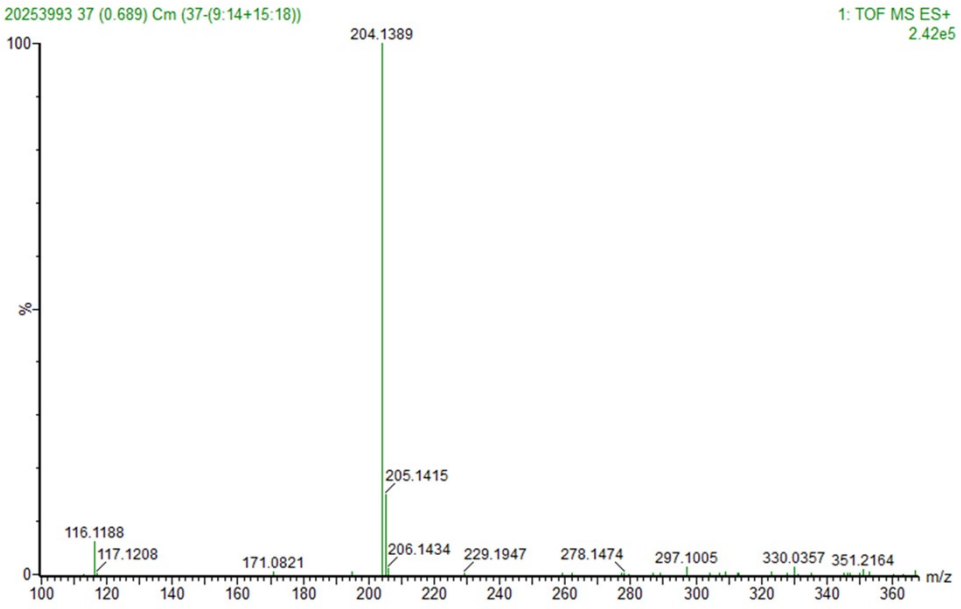
HRMS of **a5**.



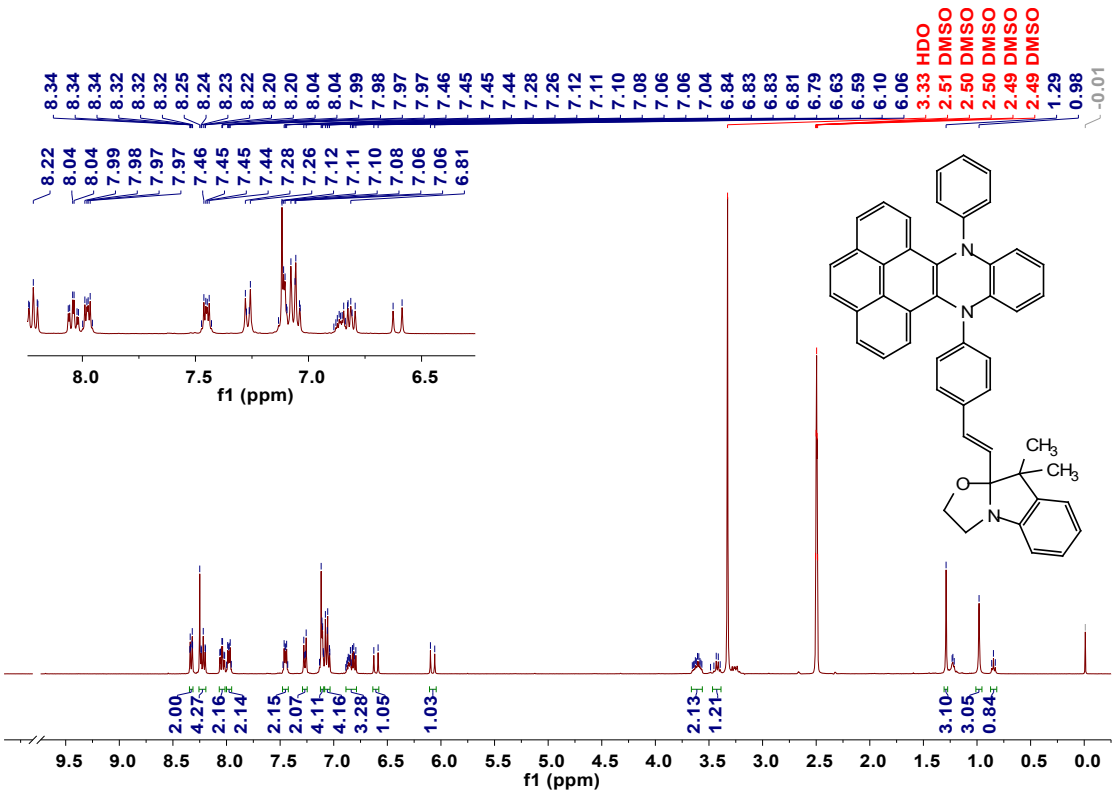
^{1}H NMR spectrum of **a6** in DMSO-d_6.



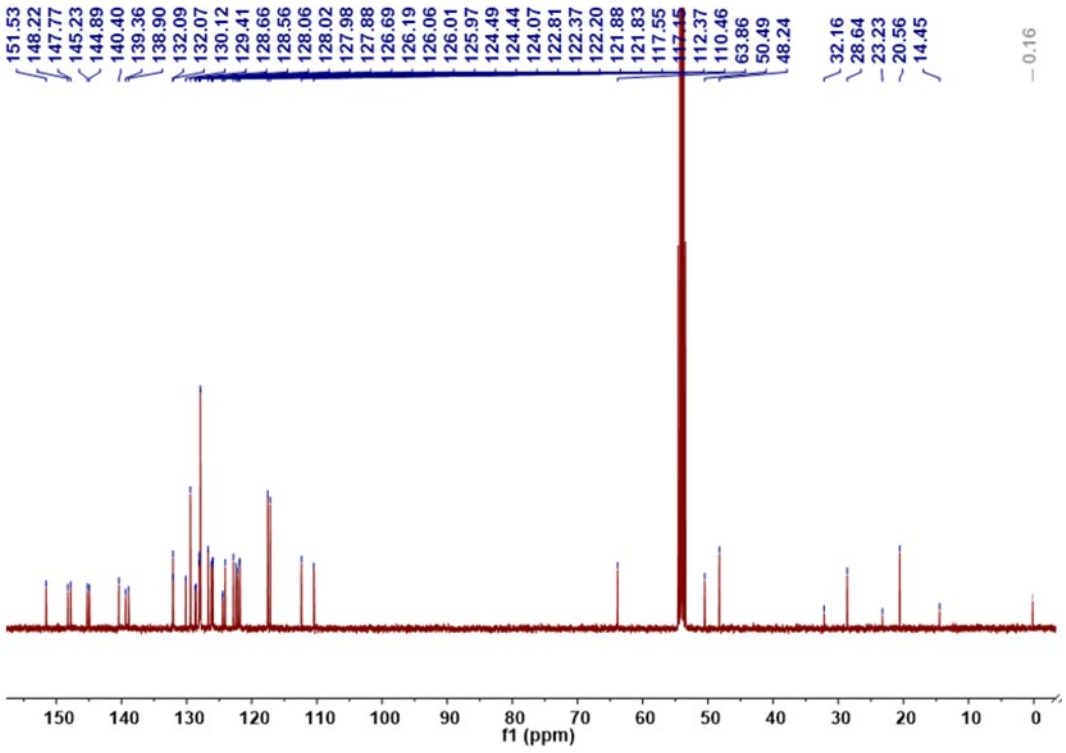
^{13}C NMR spectrum of **a6** in DMSO-d_6.



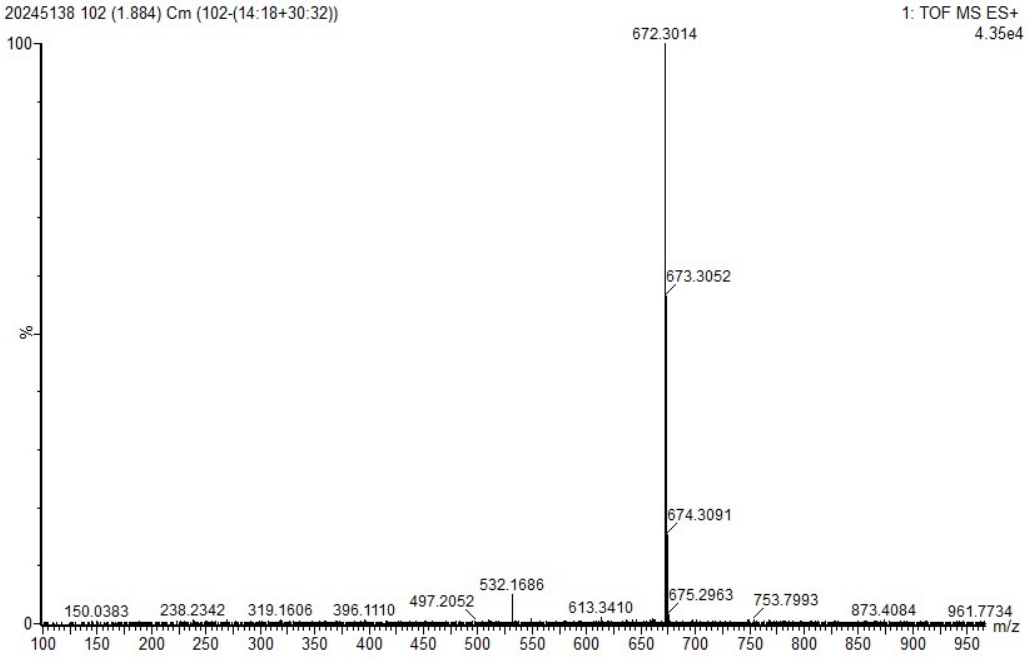
HRMS of **a6**.



^{1}H NMR spectrum of **PPAC-AOH** in DMSO-d_6.



^{13}C NMR spectrum of **PPAC-AOH** in Methylene Chloride-d_2.



HRMS of **PPAC-AOH**.

References

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