Supporting Information for

A single molecule fluorescent probe for visualizing viscosity and hypoxia in lysosomes and zebrafish embryos

Jingchao Wang[a†], Lina Zhou[a†], Zekun Jiang[a], Haiyan Wu[b*] and Xiuqi Kong[a*]

[a]School of Chemistry and Chemical Engineering, University of Jinan, Jinan, Shandong 250022, P.R. China.

[b]Department of Pharmacy, Central Hospital Affiliated to Shandong First Medical University, Shandong Jinan 250013, P.R. China.

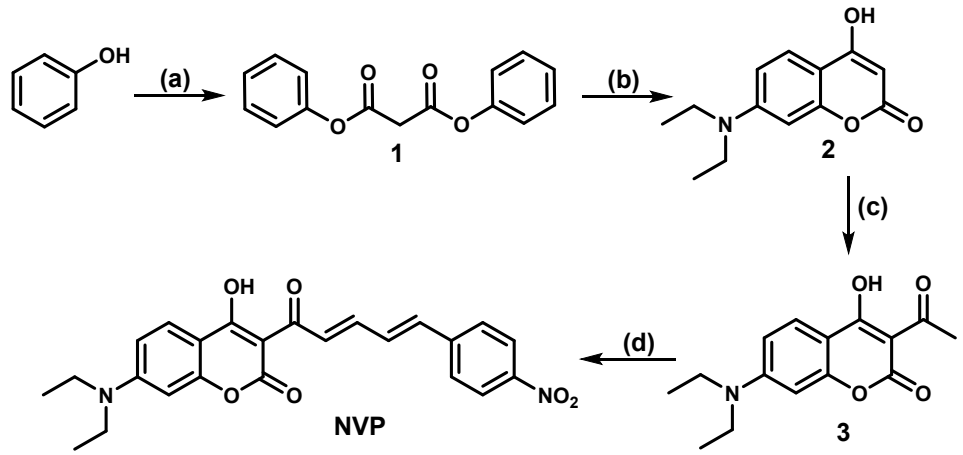
[*]Corresponding author: wuhaiyanangel@163.com; ifp_kongxq@ujn.edu.cn

Table of contents

1. Materials and instruments

All chemicals and materials with analytical grade were directly used in this work without further treatment. Double distilled water was used throughout all experiments. The thin layer chromatography (TLC) was used to monitor the organic synthesis reactions. All compounds were purified through silica gel (200-300 mesh) column (Qingdao Ocean Chemicals). All the intermediate compounds were analyzed by NMR and high-resolution mass spectrometry (HRMS). ^{1}HNMR and ^{13}CNMR results were got using an AVANCE III 400 MHz Digital NMR spectrometer with tetramethylsilane (TMS) as an internal standard. The UV-Vis absorption spectra were obtained with a Shimadzu UV-2700 spectrophotometer. Fluorescence spectra were measured using a Hitachi F-4600 Spectrofluorometer. Cellular imaging experiments were achieved with a Nikon A1 fluorescence microscopy.

2. The synthesis of compounds



Scheme S1. The synthetic routes of the probe **NVP**. Reagents and conditions: (a) malonic acid，$POCl_3$, 110℃, 3 h; (b) 3-Diethylaminophenol, toluene, 115℃, 7 h; (c) acetic anhydride, pyridine, 120℃, 6 h; (d) 4-Nitrocinnamaldehyde, EtOH, piperidine, reflux, overnight.

Synthesis of compound 1

Phenol (1.88 g, 20 mmol), malonic acid (2.08 g, 20 mmol) were dissolved in $POCl_3$ (20 mL) at 0℃. The resulting mixture was stirred at 110℃ for 3 h. After being cooled to room temperature, the reaction mixture was poured into 100 mL water and extracted with dichloromethane (3×100 mL). The organic layer was separated and washed successively with brine and water, dried over anhydrous Na_2SO_4 then vacuum

evaporated, the crude product do not require further refinement to afford compound **1** (3.32g, 65 %).

Synthesis of compound 2

Compound **1** (1.02 g, 4 mmol) and 3-diethylaminophenol (660 mg, 4 mmol) were dissolved in 20 mL toluene. The resulting mixture was stirred at 115 ℃ for 7 h. After the reaction was finished, the system was cool to room temperature, a large amount of yellow precipitate appeared. The precipitate was filtered under reduced pressure, and the filter cake was washed with ethanol for 2-3 times. Finally, the crude product was obtained by vacuum drying (470 mg, 50%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.54 (d, J = 8.9 Hz, 1H), 6.66 (d, J = 9.0 Hz, 1H), 6.45 (s, 1H), 5.26 (s, 1H), 3.41 (d, J = 7.0 Hz, 4H), 1.11 (t, J = 6.9 Hz, 6H).

Synthesis of compound 3

Compound **2** (275.1mg, 1.0 mmol) and acetic anhydride (2.08 g, 20 mmol) were dissolved in 20 mL pyridine. The reaction were stirred at 120 ℃ for 6 h. The mixture cool to room temperature, and pale yellow precipitates appeared. The cake was washed three times with ethanol and dried in vacuum to obtain coarse product, and then purified by column chromatography (dichloromethane: methanol = 50:1 v/v) to obtain pure compound **3**(178 mg, 68%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (d, J = 9.1 Hz, 1H), 6.65 (dd, J = 9.1, 2.4 Hz, 1H), 6.42 (d, J = 2.4 Hz, 1H), 3.47 (q, J = 7.1 Hz, 4H), 2.73 (s, 3H), 1.26 (t, J = 7.1 Hz, 6H).

3. Preparation before probe NVP spectroscopy test

Unless otherwise stated, working solutions were prepared according to the following procedures to test. The probe **NVP** was dissolved in DMSO to prepare for stock solution (5 mM). Both nitroreductase (NTR) and NADH were dissolved using double distilled water (ddH_2O) at concentration of 200 U/mL and 50 mM, respectively; Methanol and glycerol solutions with different volume ratios were used as the viscosity response test systems, and the specific viscosity values of the different

systems were determined by NDJ-8S rotational viscometer. Common anions, cations, biothiols, amino acids, NTR, and glycerol were selected for selective interference experiments. The excitation wavelengths were 380 nm and 440nm, respectively. The excitation and emission slit widths were 5/5 nm for all tests.

4. Cell culture and imaging

HeLa cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% FBS (fetal bovine serum). Cells were seeded in 96-well plates at a density of 1×10^4 cells per well.

4.1 Cytotoxicity Studies and Co-localization experiments

HeLa cells were seeded in 20 mm glass confocal dishes and incubated at 37°C for 24 h detected by standard CCK-8 assay. After the cells were completely adhered to the bottom of 96-well plates, different concentrations of **NVP** (0−40 μM) were added to culture for 24 h continuly. After the incubation,10 μL CCK-8 was injected into each well and treated for 24 h. Finally, the absorbance of methylzan crystal was determined by microplate reader.

HeLa cells were pre-seeded into 20-mm glass dishes with the 80% confluence. Cells were firstly incubated wtih 2% O_2 condition for 6 h，then treated with 10 μM **NVP** for 30 min, finally Lyso Deep Red was added and treated for another 10 min. λ_{ex}(NVP) = 488 nm, λ_{em}(NVP) = 500-550 nm; λ_{ex}(Lyso Deep Red) = 647 nm, λ_{em} (Lyso Deep Red) = 663-740 nm.

4.2 Cellular imaging with the treatment of chloroquine using probe NVP

HeLa cells were pre-seeded into 20-mm glass dishes with the 80% confluence. Cells were pre-incubated with Chlor (Chloroquin，30 μM) for 1 h and 2 respectively. Then they were treated with 10 μM **NVP** for 30 min; Blue channel: λ_{ex} = 405 nm, λ_{em} = 425-475 nm; Green channel: λ_{ex} = 488 nm, λ_{em} = 500-550 nm.

4.3 Cellular imaging using probe NVP under hypoxia conditions

HeLa cells were pre-seeded into 20-mm glass dishes with the 80% confluence. Cells were treated under normoxic conditions (20% O_2, 5% O_2 and 2% O_2) for 6 h, and further incubated with **NVP** (10 μM) for 30 min; Green channel: λ_{ex} = 488 nm,

λ_{em} = 500-550 nm.

4.4 Cellular imaging with the treatment of dicoumarin using probe NVP

HeLa cells were pre-seeded into 20-mm glass dishes with the 80% confluence. Cells were firstly incubated with hypoxic conditions (2% O_2) for 6 h, then treated the 100 μM dicoumarin and **NVP** (10 μM) for 30 min successively. Green channel: λ_{ex} = 488 nm, λ_{em} = 500-550 nm.

4.5 Cellular imaging with the treatment of chloroquine and hypoxia conditions

HeLa cells were pre-seeded into 20-mm glass dishes with the 80% confluence. Cells were incubated with hypoxic conditions (2% O_2) for 6 h, and then treated with 30 μM Chlor for 1 h and treated with **NVP** (10 μM) for 30 min successively. Green channel: λ_{ex} = 488 nm, λ_{em} = 500-550 nm.

4.6 imaging with the treatment of chloroquine using probe NVP

Six zebrafish were pre-incubated with Chlor (Chloroquin, 30 μM) for 1 h and 2 h in 6 cm cell culture dishes, respectively. Then they were exposed with 10 μM **NVP** for 30 min. Before imaging, zebrafish were dechorionated with droppers in a Petri dish coated with 1% (w/v) agarose. Blue channel: λ_{ex} = 405 nm, λ_{em} = 425-475 nm.

4.7 Zebrafish embryos imaging under hypoxia conditions using probe NVP

Six zebrafish embryos were incubated under hypoxic conditions (5% O_2, 2% O_2) for 6 h, and then treated with **NVP** (10 μM) for 30 min. Before imaging, zebrafish were dechorionated with droppers in a Petri dish coated with 1% (w/v) agarose.Green channel: λ_{ex} = 488 nm, λ_{em} = 500-550 nm.

5. Figures

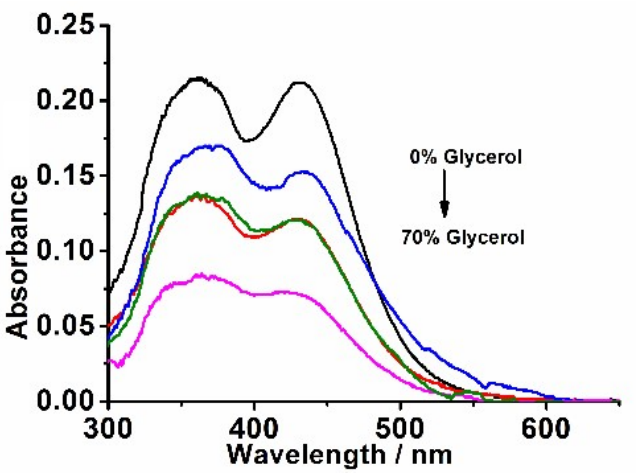


Fig.S1. UV-Vis absorption spectra of **NVP** (10 μM) in mixture containing different fractions of methanol and glycerol.

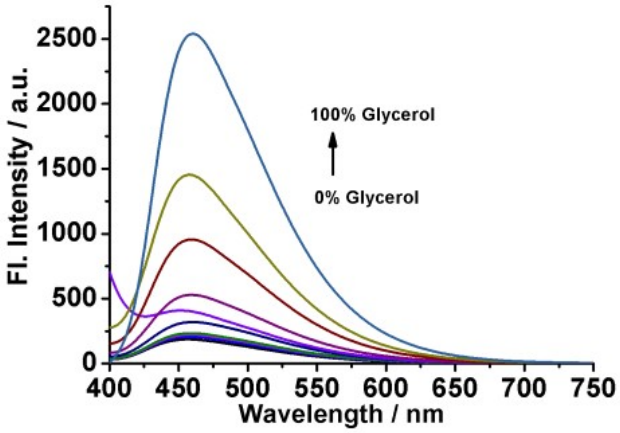


Fig.S2. Fluorescence spectra of compound **2** in viscosity mixture containing different fractions of methanol and glycerol (0-100%, v/v), λ_{ex}=380 nm.

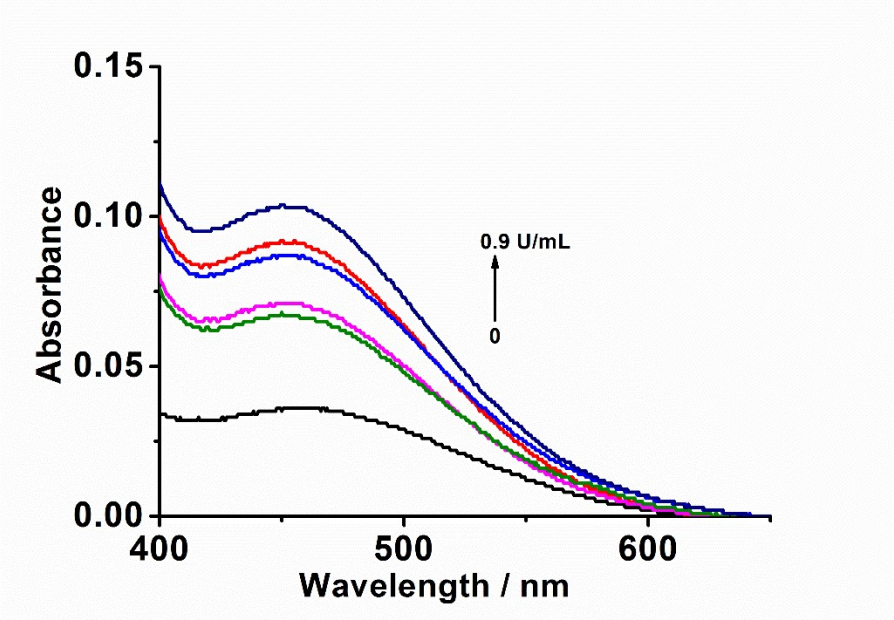


Fig.S3. (a) UV-Vis absorption spectra of **NVP** (10 μM) in the presence of NTR (0-0.9 U/mL) coexisting NADH (100 μM) in PBS buffer.

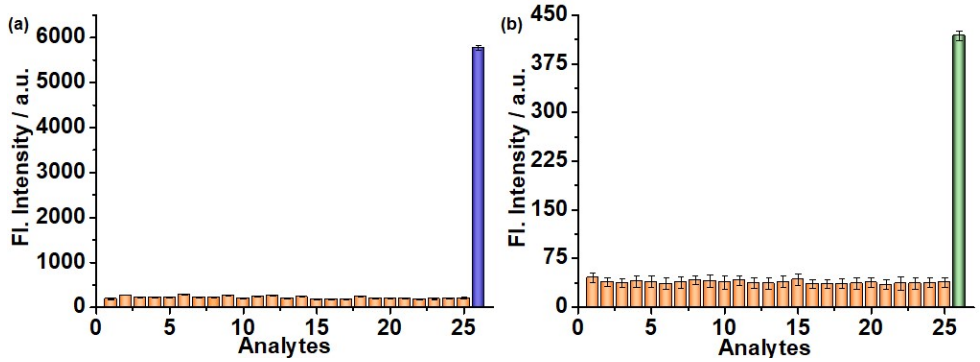


Fig. S4. (a) Fluorescence response of **NVP** (10 μM) to 30 μM various analytes in pure MeOH buffer, λ_{ex}=380 nm(a) and PBS buffer in the being of NADH (100 μM), λ_{ex}=380 nm (b) The numbers 1-26 are: free NVP、Ba^{2+}、Mg^{2+}、Na^{+}、Zn^{2+}、Cu^{2+}、F^{-}、CO_3^{2-}、SO_3^{2-}、HCO_3^{-}、HS^{-}、NO_3^{-}、ClO^{-}、$Na_2S_2O_3$、H_2O_2、H_2O、Cys、Ace、Ser、Lys、Esterase (0.9 U/mL)、Cholinesterase (0.9 U/mL)、Catalase (0.9 U/mL)、Trypsin(0.9 U/mL)、Glucose oxidase (0.9 U/mL)、Glycerol (a) or NTR (b).

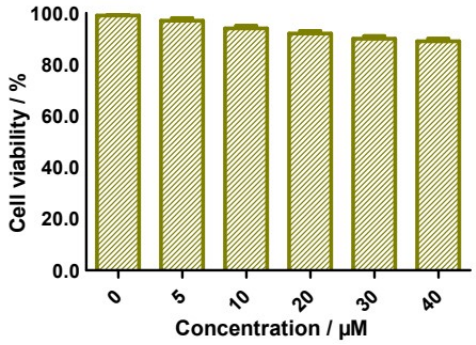


Fig. S5. Cell viability of HeLa cells incubated with different concentrations of probe **NVP**.

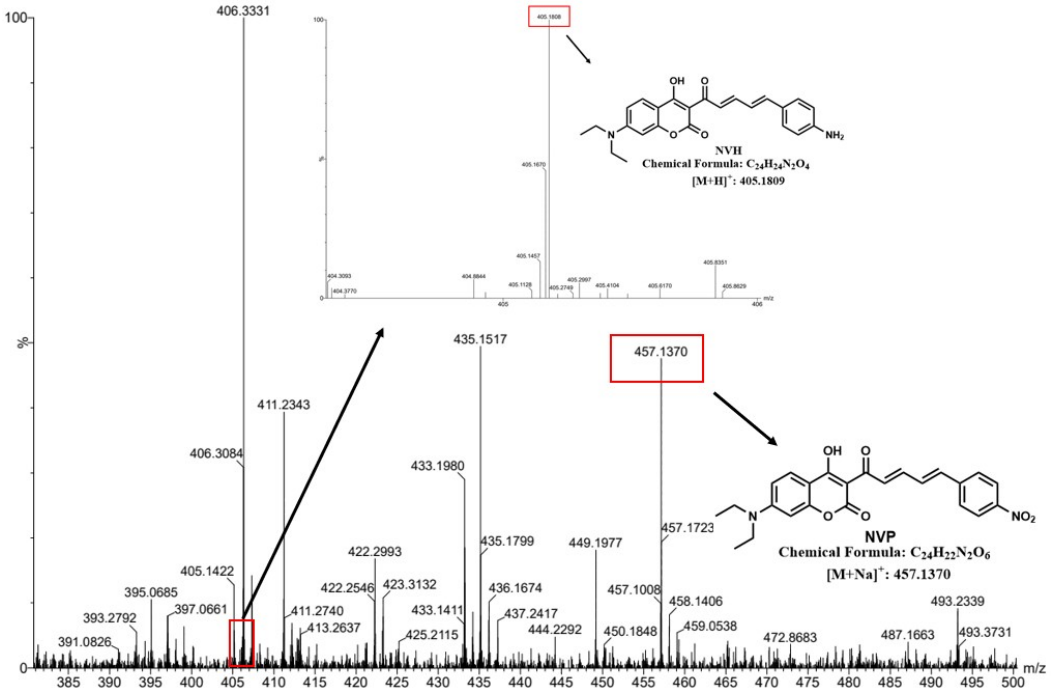


Fig. S6. The HR-MS spectrum of **NVP** after the addition of NTR.

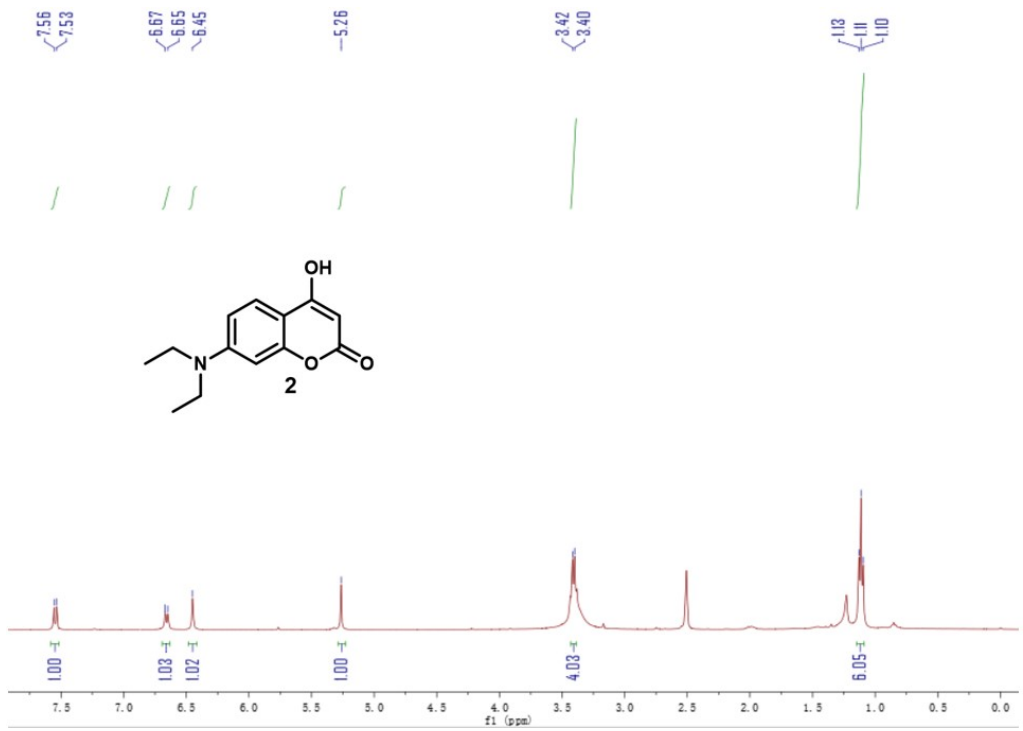


Fig. S7. The [1]HNMR spectrum of compound **2** in DMSO-d_6.

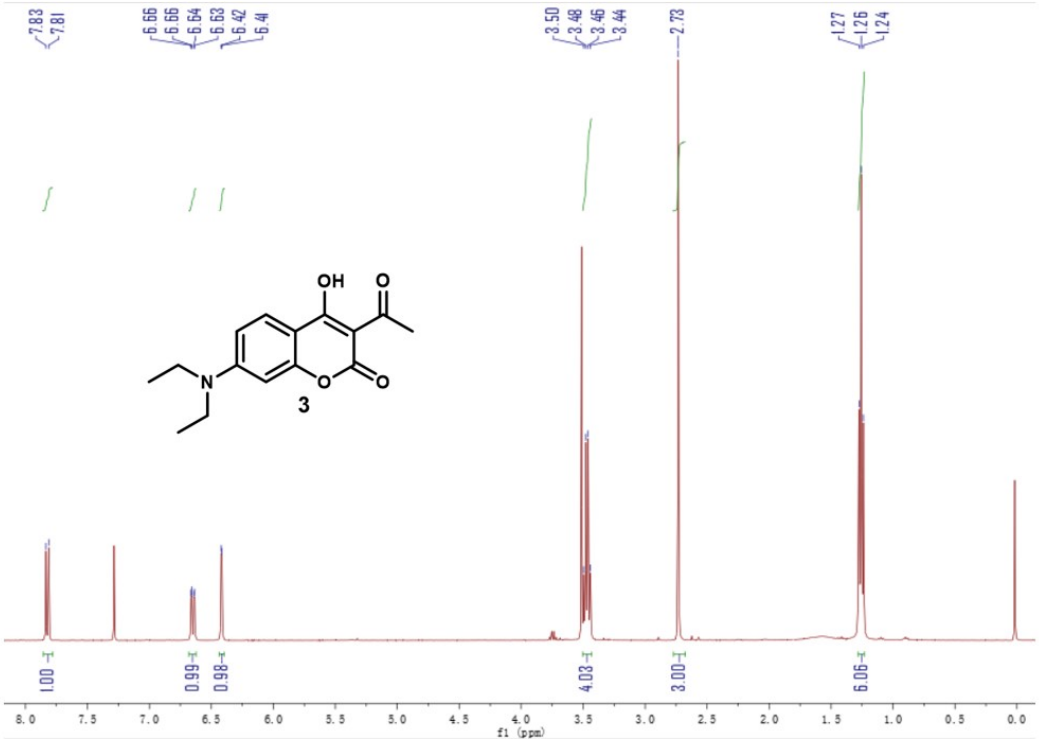


Fig. S8. The [1]HNMR spectrum of compound **3** in $CDCl_3$

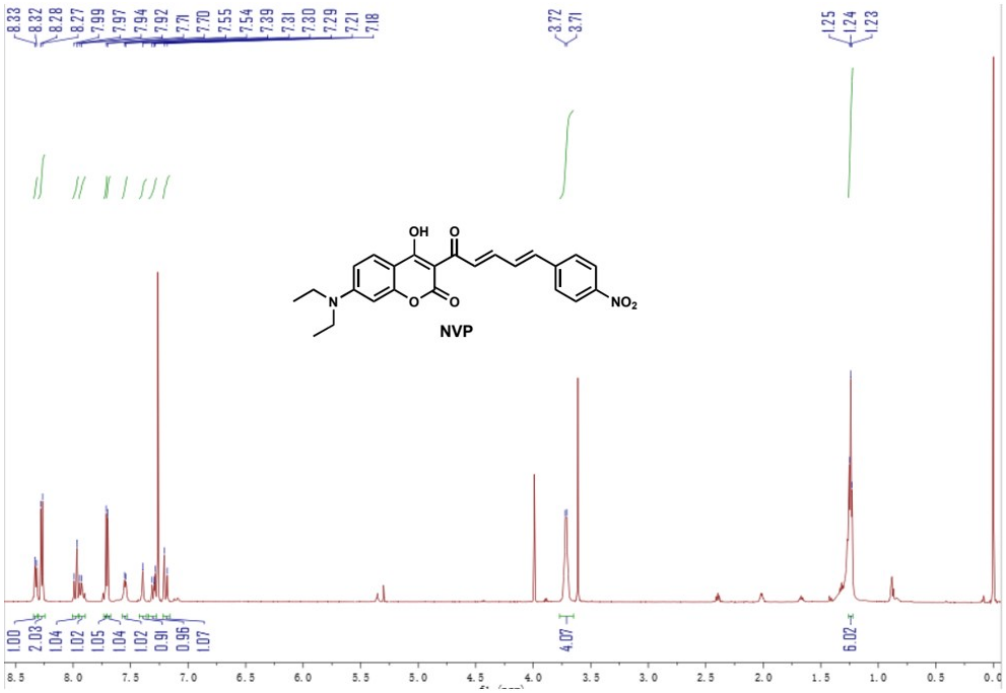


Fig. S9. The [1]HNMR spectrum of probe **NVP** in $CDCl_3$

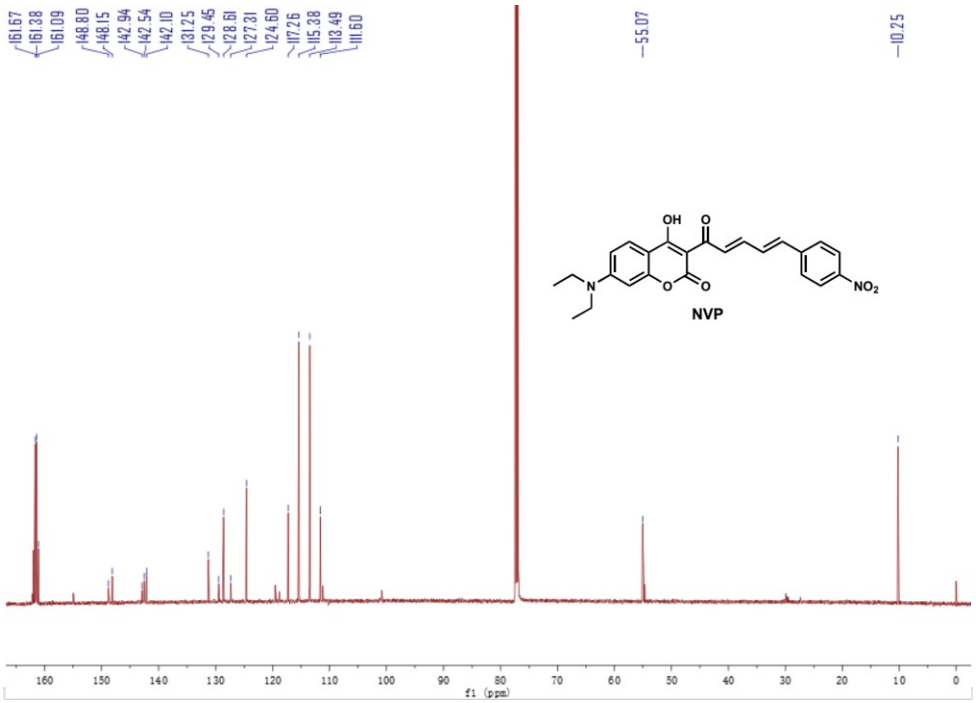


Fig. S10. The [13]C NMR spectrum of probe **NVP** in $CDCl_3$

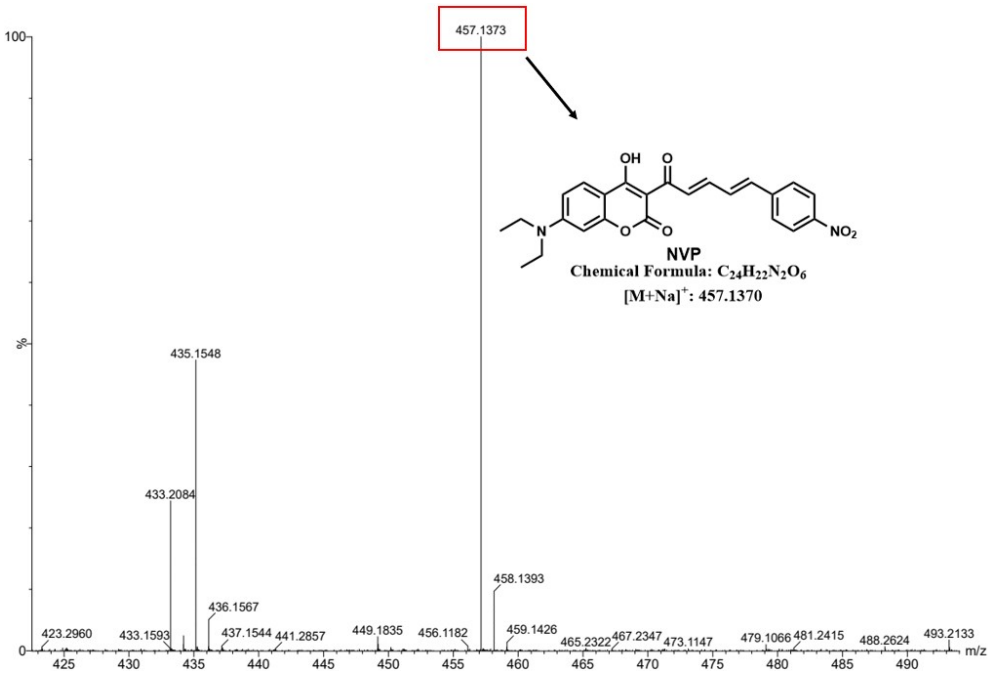


Fig.S11. The HRMS spectrum of **NVP**

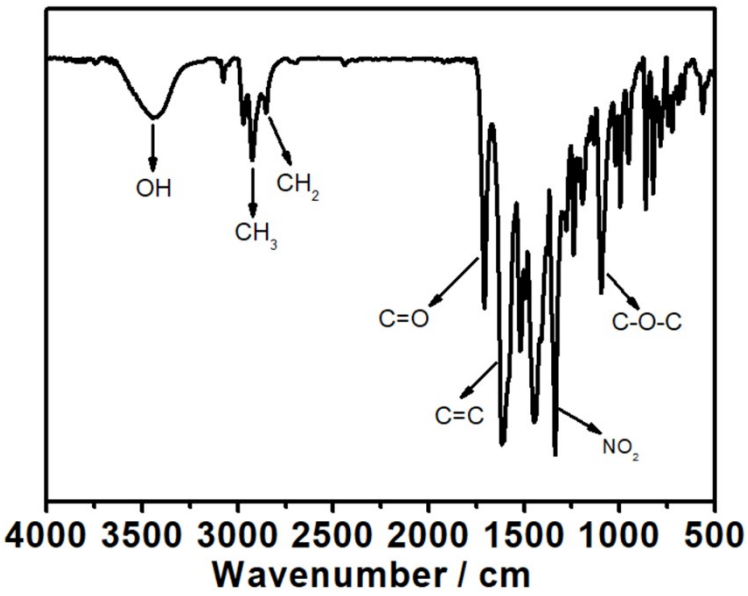


Fig S12. FR-IR spectrum of **NVP**