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Supporting Information

Highly selective ratiometric molecular probe for imaging peroxynitrite during drug-induced acute liver injury

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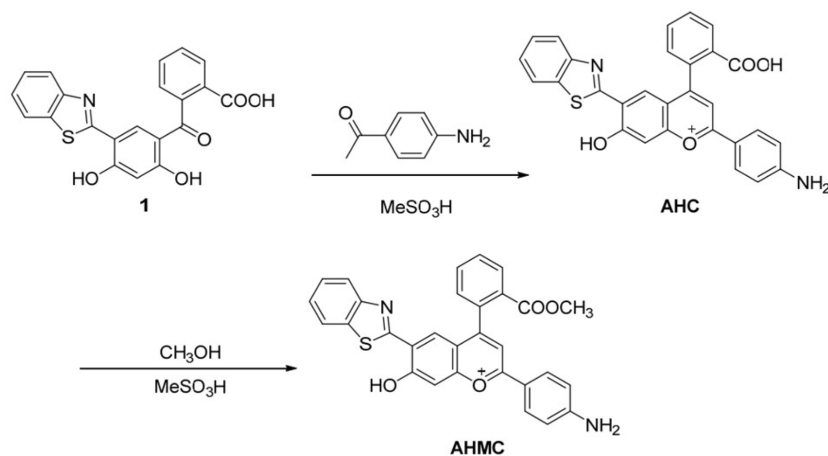
1. Materials and General Experimental Methods

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Mass spectra were performed using an LCQ advantage ion trap mass spectrometer from Thermo Finnigan. NMR spectra were recorded on a Bruker-400 spectrometer, using TMS as an internal standard. High-resolution electronspray (ESI-HRMS) mass spectra were obtained from The Thermo Fisher Scientific LTQ FT Ultra (Shanghai Institute of Organic Chemistry Chinese Academic of Sciences). Photoluminescent spectra were recorded at room temperature with a HITACHI F4600 fluorescence spectrophotometer (1 cm standard quartz cell). The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter; the fluorescence images were acquired with a confocal laser scanning microscope (Nikon, Japan); TLC analysis was performed on silica gel plates and column chromatography was

conducted over silica gel (mesh 200-300), both of which were obtained from the Yantai Jiangyou Silica Gel Development Company Limited.

2. Synthesis and Characterization



Scheme S1. Synthetic routes of probes **AHC** and **AHMC**.

Synthesis of compound 1. Compound **1** was prepared by reported method.[1]

3. Detection Limit Calculation.

The detection limit was calculated based on the fluorescence titration. The detection limit is calculated using the following equation:

$$\text{Detection limit} = 3\sigma/k \quad (1)$$

where σ is the standard deviation of blank measurements, k is the slope between the emission ratios versus sample concentration.

4. Spectrometric Studies

Measurement of photophysical properties. For photophysical characterization, probe **AHC** was dissolved in EtOH to make the stock solutions (500 μM), which were diluted to 5 μM as the testing solutions with PBS buffer solution (25 mM, 20% EtOH, pH 7.4). Absorption and fluorescence spectroscopic studies were performed on a Shimadzu UV-2700 power spectrometer; a Hitachi F-4600 fluorescence spectrophotometer.

Sources for different ROS/RNS are described as follows. Specifically, H_2O_2 solution was purchased from Sigma-Aldrich and added into the probe solution directly. Superoxide ($O_2•^-$) was generated from KO_2 was dissolved in DMSO. The source of NaOCl was commercial bleach. Hydroxyl radical (•OH) was generated by Fenton reaction. Briefly, ferrous chloride ($FeCl_2$) was added in the presence of 10 equiv. of H_2O_2. The concentration of •OH was equal to the Fe(II) concentration. Peroxynitrite ($ONOO^-$) solution was synthesized according to literature report.[2] Briefly, a mixture of sodium nitrite (0.6 M) and hydrogen peroxide (0.7 M) was acidified with

hydrochloric acid (0.6 M), and sodium hydroxide (1.5 M) was added within 1-2 seconds to make the solution alkaline. The excess hydrogen peroxide was removed by passing the solution through a short column of manganese dioxide. The concentration of peroxynitrite was determined by measuring the absorption of the solution at 302 nm. The extinction coefficient of peroxynitrite solution in 0.1 M NaOH is 1,670 M^{-1} cm^{-1} at 302 nm.[3]

5. Fluorescence Microscopic Studies

Cell culture. RAW264.7 and L02 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, BI), and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Hyclone) at 37 °C and 5% CO_2. Cells were carefully harvested and split when they reached 80% confluence to maintain exponential growth.

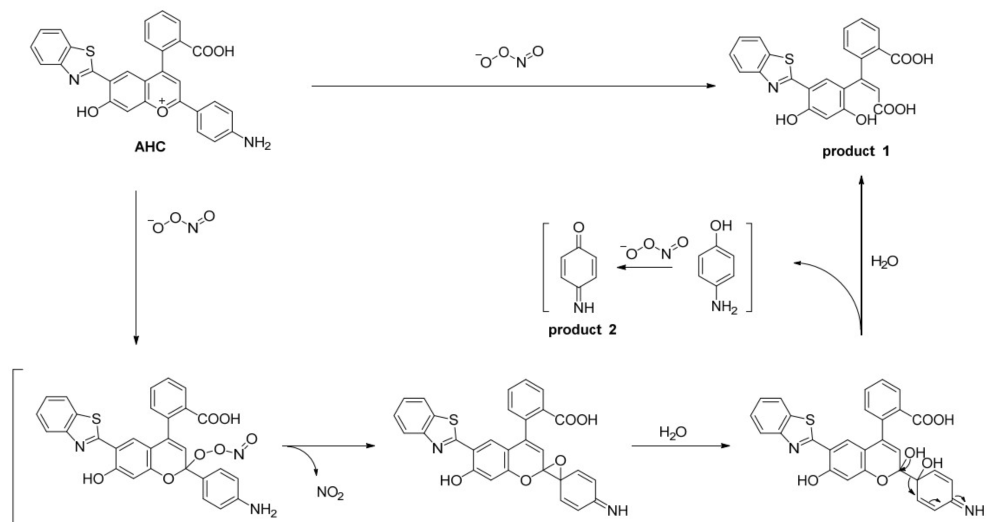
Fluorescence microscopic imaging. All the experiments were conducted in live cells.

For the cell specific experiment, the cell experiment can be divided into five groups. The first group is that RAW264.7 cells were incubated with 10 μM **AHMC** for 30 min. Then the cells were washed by PBS buffer before imaging. In the second group, RAW264.7 cells were pre-incubated with lipopolysaccharide (LPS, 2 μg/mL) and interferon-gamma (IFN-γ, 100 ng/mL) for 12 hours, and 10 μM **AHMC** was further added for 30 min. Then the cells were washed by PBS prior to imaging. In the third group, RAW264.7 cells were incubated with LPS (1 μg/mL) and IFN-γ (50 ng/mL) in the presence of aminoguanidine (AG, 5 mM) for 12 hours, respectively, and then incubated with 10 μM **AHMC** for 30 min prior to imaging. In the forth and fifth group, RAW264.7 cells were incubated with 10 μM **AHMC** for 30 min, then washed, and incubated with 150 μM H_2O_2 or 50 μM NaOCl respectively for 30 min prior to imaging. The microscopic imaging uses Nikon confocal microscope with an excitation filter of 405 nm and the collection wavelength range is from blue channel at 425-475 nm and red channel at 560-750 nm, respectively.

Cell cytotoxicity in MTT assay. Cells were plated in 96-well flat-bottomed plates at 1×10^5 cells per well and allowed to grow overnight prior to exposure to **AHMC**. Then the MTT (0.5 mg/mL) reagent was added for 4 hours at 37 °C and DMSO (100 μL/well) was further incubated with cells to dissolve the precipitated formazan violet crystals at 37 °C for 15 min. The absorbance was measured at 490 nm by a multidetection microplate reader. The following formula was used to calculate the viability of cell growth: Cell viability (%) = (mean of A value of treatment group / mean of A value of control) × 100.

Determining the subcellular location of probes. Live RAW264.7 and L02 cells cultured in 35-mm glass bottom culture dishes were pre-treated with 10 μM **AHMC** for 30 min. After 30 min treatment at 37 °C, Mito-tracker Green (1.0 μM) or Lyso-tracker Green (1.0 μM) were added for another 10 min. Then the cells were washed by PBS prior to imaging.

6. Supplemental Figures



Scheme S2. The proposed reaction mechanism of probe **AHC** and $ONOO^-$.

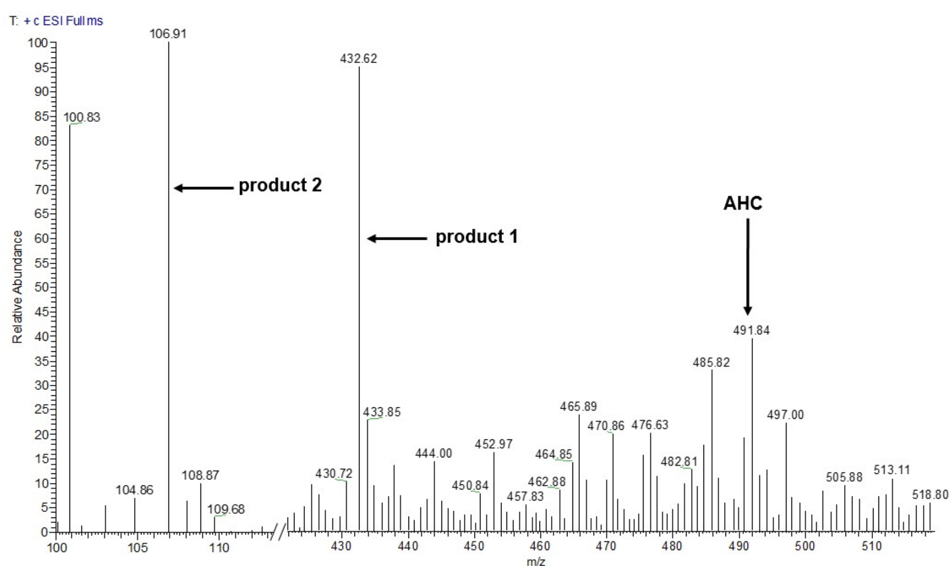


Figure S1. The ESI mass spectrum of probe **AHC** in the presence of $ONOO^-$.

Table S1. Properties of representative ratiometric fluorescent probes for $ONOO^-$

Probe	$\lambda_{ex}/\lambda_{em}$(nm)	Detection limit
PNCy3Cy5[4]	530/560,660	0.65 nM
F_{482}[5]	480/510, 606	150.54 nM
MITO-CC[2]	420/473,651	11.3 nM

RTFP[6]	405/469,703	4.1 nM
ABAH-LW[7]	370/481,405	21.4 nM
AHC (this work)	380/462,626	1.8 nM

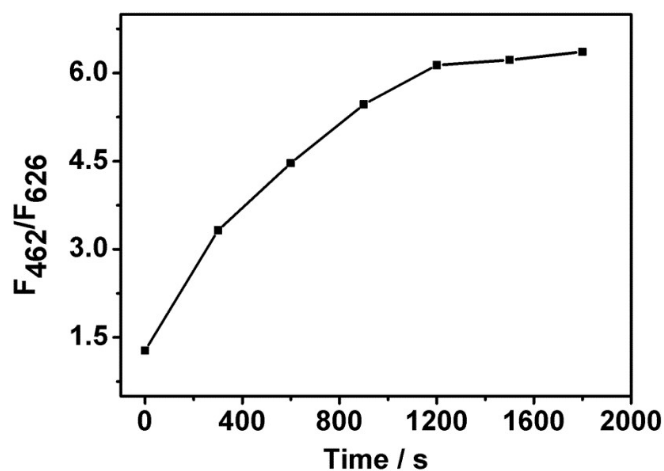


Figure S2. Time profile of probe **AHC** (5 μM) at F_{462} /F_{626} with $ONOO^-$ (5.4 μM) in pH 7.4 PBS/EtOH (v/v, 8/2).

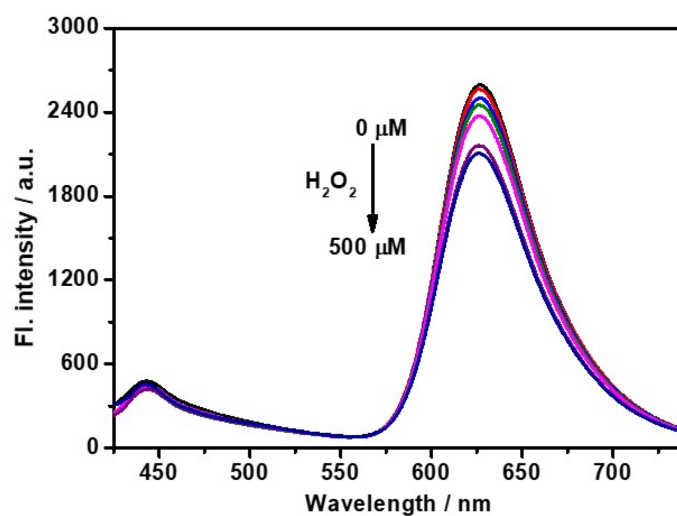


Figure S3. Fluorescence spectrum of probe **AHC** (5 μM) upon addition of H_2O_2 (0-500 μM) in pH 7.4 PBS/EtOH (v/v, 8/2). λ_{ex}: 380 nm.

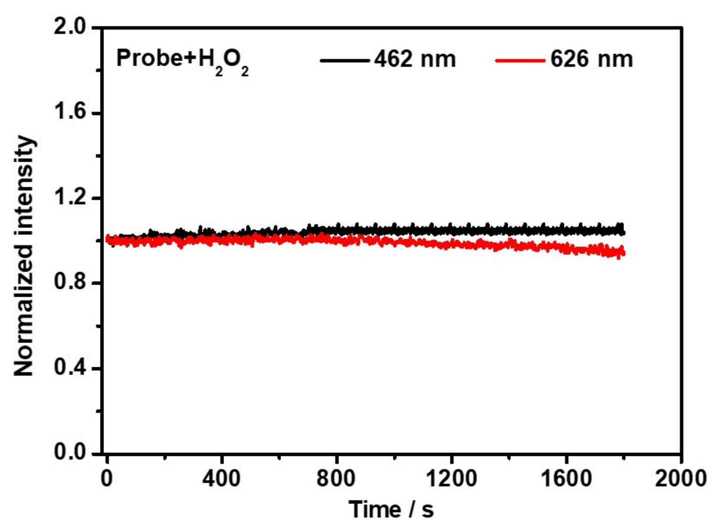


Figure S4. Reaction time profile of probe **AHC** (5 µM) at 626 nm and 462 nm with H_2O_2 (500 µM) respectively in pH 7.4 PBS/EtOH (v/v, 8/2).

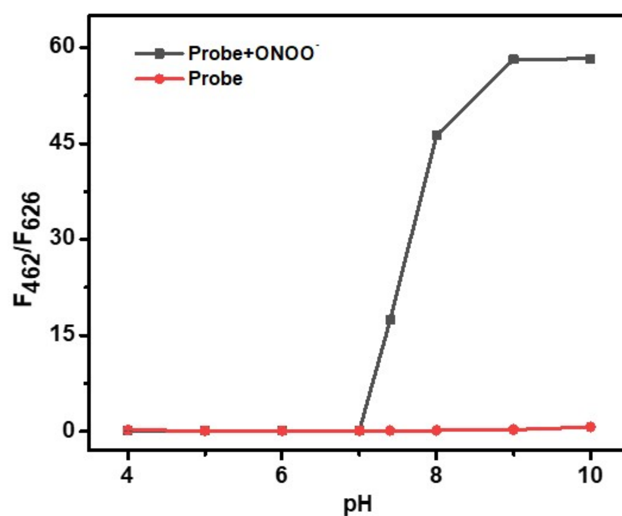


Figure S5. pH-dependent fluorescence intensity changes of **AHC** (5 µM) toward $ONOO^-$ (6 µM) in PBS buffer solution (25 mM, 20% EtOH, pH = 4.0-10.0), λ_{ex}: 380 nm.

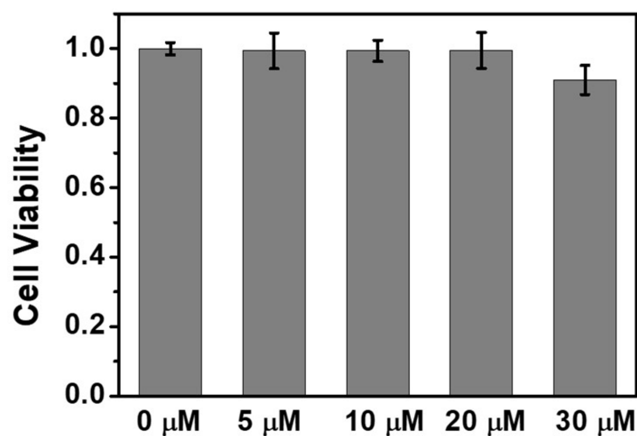


Figure S6. Cytotoxicity of **AHMC** for L02 cells. Cells were incubated with the probe at corresponding concentrations for 24 hours. Cell viability was measured by MTT assay and the results are reported as percentage relative to untreated cells (mean ± SD).

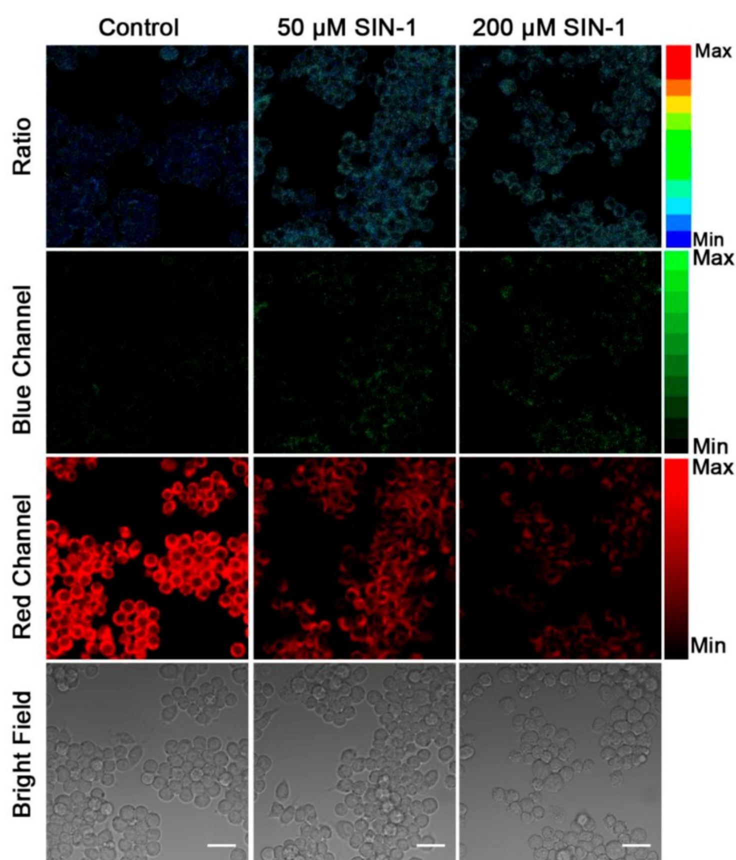


Figure S7. Fluorescence images of probe **AHMC** in RAW264.7 cells under different conditions by confocal fluorescence images. The first column, cells were incubated with **AHMC** (10 μM, 30 min), then imaged. Cells were prestimulated with SIN-1 (50 μM for the second column or 200 μM for the third column) for 2 h and then with **AHMC** (10 μM) for 30 min, then imaged. Blue channel: 425-475 nm; red channel: 560−750 nm. Excitation: 405 nm. Ratio images were from blue/red channel. Scale bar = 20 μm.

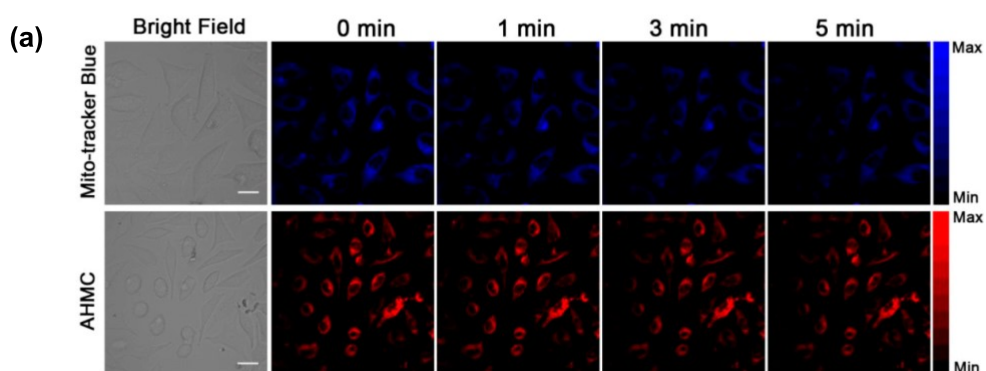


Figure S8. (a) Confocal fluorescence images of living L02 cells cultured with **AHMC** (5 μM) and **Mito-tracter Blue** (5 μM) with continuous irradiation using confocal microscope with the same parameters. (b) Quantification of the relative mean fluorescence levels of cells from the images of **AHMC (**λ_{ex} = 405 nm, λ_{em} = 560-750 nm**)** and **Mito-tracter Blue (**λ_{ex} = 405 nm, λ_{em} = 425-475 nm**)**. Scale bar = 20 μm.

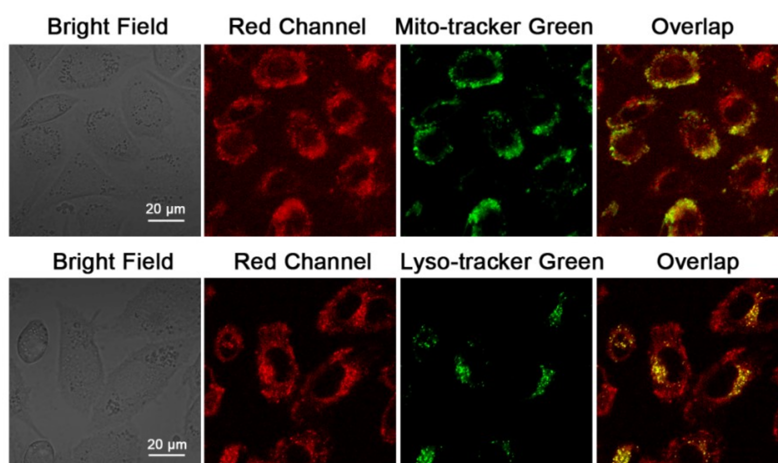


Figure S9. Co-localization images of **AHMC** in L02 cells. Green channel at 500-550 nm for Mito-tracker Green and Lyso-tracker Green (λ_{ex}= 488 nm); red channel at 560-750 nm for probe (λ_{ex} = 405 nm). Overlap: the merged images from green and red channel. Scale bar: 20 μm.

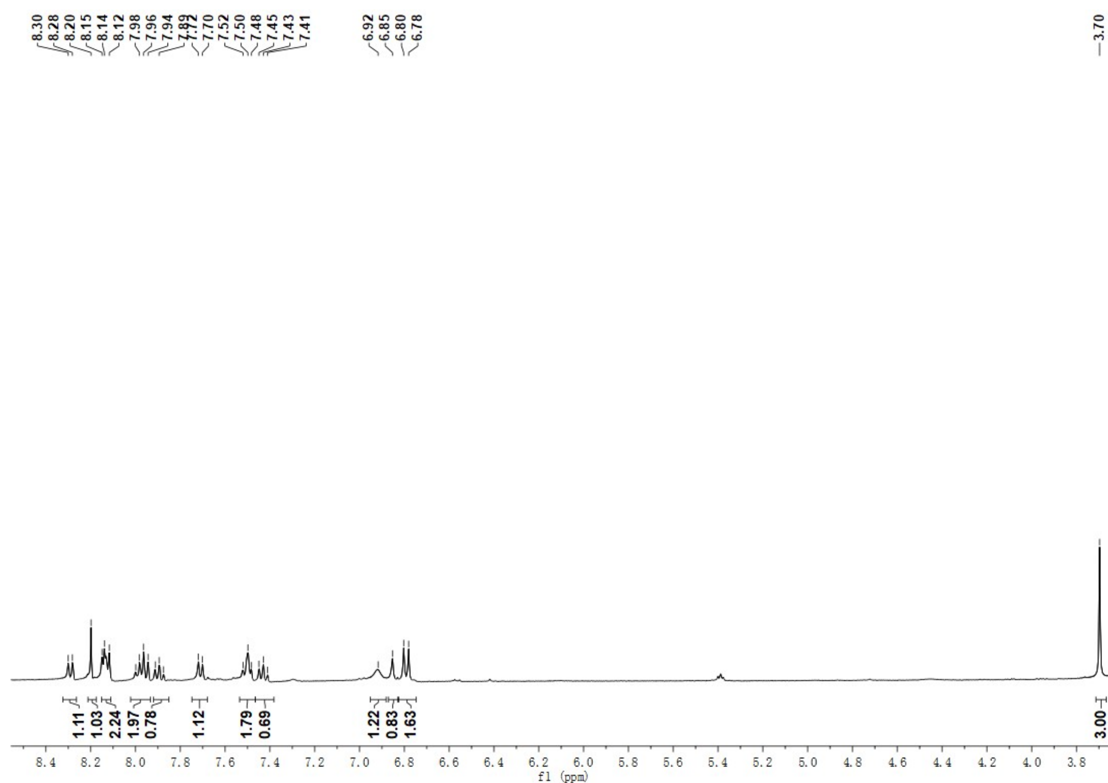


Figure S10. The ^{1}HNMR spectrum of probe **AHMC** (DMSO-d_6)

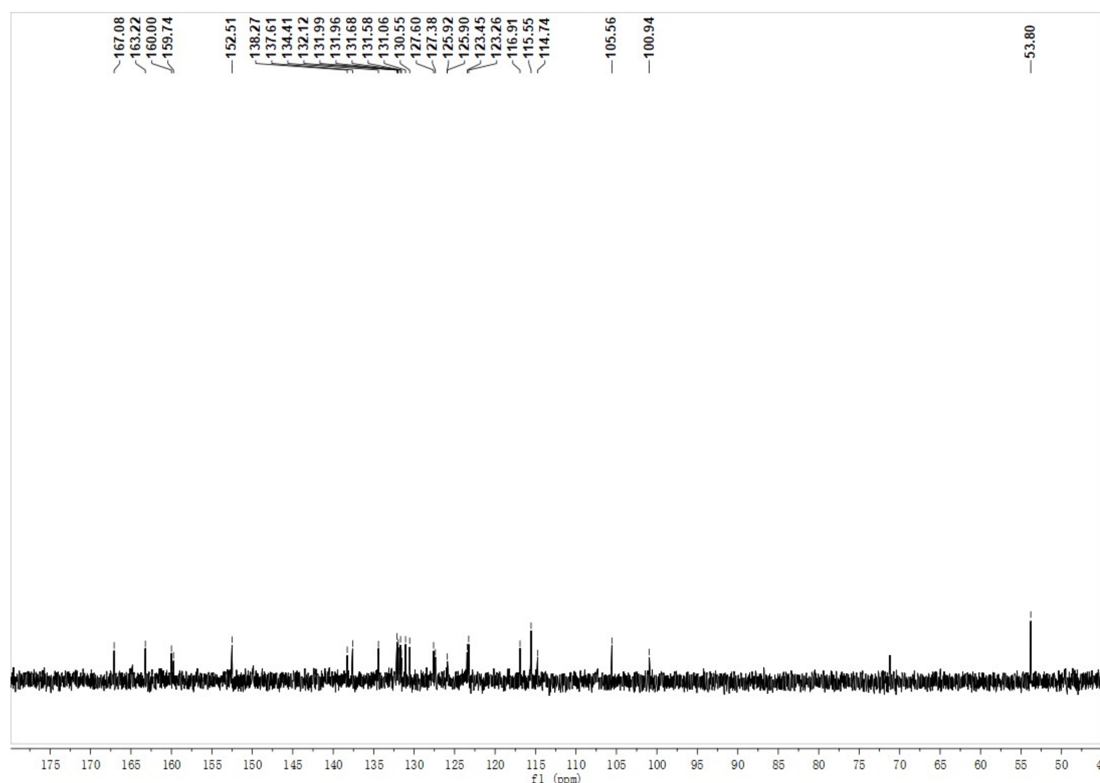


Figure S11. The ^{13}CNMR spectrum of probe **AHMC** (DMSO-d_6)

7. References

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