Supporting Information

Construction of a novel highly selective NIR probe for monitoring the changes of glutathione levels in drug-induced liver injury

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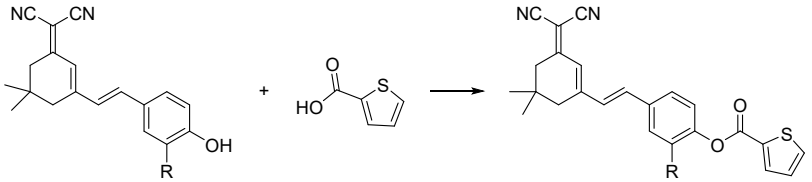
I. Materials and Instruments

All reagents and solvents used in this study were purchased from commercial sources. Tumour cells were obtained from the First Hospital of Lanzhou University, and mice were obtained from the Animal Medical Experiment Center of Lanzhou University. Fluorescence spectroscopy studies were performed using a LUMINA fluorescence spectrophotometer (Thermo Fisher Scientific) and a UV spectrophotometer EVOLUTION 220 (Thermo Fisher Scientific). The slit width for both excitation and emission in the fluorescence spectra was 20 nm. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AVANCE NEO 600 using the solvent $CDCl_3$. The fluorescence imaging of the cells was done using an Olympus FV3000 laser confocal microscope. Fluorescence images of mice were captured using an in vivo imager (Boluten Aniview 100). HPLC was recorder on Shimadzu LCMS-2020 system with a Wondasil C18 Superb reversed-phase column (5 μm, 4.6 × 150 μm).

All procedures for *in vivo* experiments were carried out in accordance with the institutional guidelines (Guidance of the Care and Use of Laboratory Animals) and all *in vivo* experiments were approved by the Ethics Committee of Lanzhou University, China.

II. Chemical Synthesis



The Flu-R series fluorophores were synthesized based on previously reported literature. [1]

General steps for synthesis of compound **AH-R**. 2-Thiophenecarboxylic acid (156 mg, 1.2 mmol, 1.2 eq) was dissolved in 5 mL of sulfoxide chloride and reacted at reflux for 2 h. At the end of the reaction, the system was concentrated and dissolved in 10 mL of tetrahydrofuran, and the raw materials of the differently substituted fluorophores were dissolved in the tetrahydrofuran and added to the reaction vial, at which time TEA (150 mg, 1.5 eq) and a catalytic amount of DMAP were added and a catalytic amount of DMAP were added at this time, then the ice bath was removed and stirring was continued for 2 h at room temperature. After the reaction was completed, the reaction solution was concentrated and the product was purified by chromatography on silica gel column (PE/EA = 4:1). The target product was obtained.

Synthesis of compound **AH-F**. Following the general procedure, (83%, Yellow solid). ^{1}H NMR (600 MHz, Chloroform-d) δ 8.02 (d, *J* = 2.5 Hz, 1H), 7.72 (d, *J* = 3.6 Hz, 1H), 7.35 (d, *J* = 10.9 Hz, 1H), 7.33 (m, 2H), 7.22 (m, 1H), 7.01 (m, 2H), 6.87 (s, 1H), 2.61 (s, 2H), 2.46 (s, 2H), 1.09 (s, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 169.19, 159.48, 155.42, 153.76, 153.07, 135.60, 134.45, 131.58, 130.65, 128.37, 124.50, 123.90, 115.36, 115.23, 113.43, 112.64, 43.10, 39.33, 32.19, 29.84, 28.16. HRMS: $[M]^-$ calculated for $C_{24}H_{19}FN_2O_2S^-$: 417.1151; found: 417.1058.

Synthesis of compound **AH-Cl**. Following the general procedure, (79%, Yellow solid). ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.04 (d, *J* = 2.5 Hz, 1H), 7.72 (d, *J* = 3.7 Hz, 1H), 7.62 (d, *J* = 2.1 Hz, 1H), 7.46 (dd, *J* = 8.5, 2.1 Hz, 1H), 7.33 (d, *J* = 8.4 Hz, 1H), 7.23 (m, 1H), 6.97 (s, 2H), 6.87 (s, 1H), 2.61 (s, 2H), 2.46 (s, 2H), 1.09 (s, 6H).^{13}C NMR (151 MHz, Chloroform-*d*) δ 169.17, 159.56, 153.06, 147.59, 135.58, 135.19, 134.46, 134.44, 131.75, 130.71, 129.21, 128.38, 128.08, 126.75, 124.52, 113.43, 112.63, 43.10,

39.31, 32.19, 32.06, 29.84, 28.16. HRMS: $[M]^-$ calculated for $C_{24}H_{19}ClN_2O_2S^-$: 433.0856; found: 417.0774.

Synthesis of compound **AH-Br**. Following the general procedure, (81%, Yellow solid). ^{1}H NMR (600 MHz, Chloroform-d) δ 8.05 (s, 1H), 7.79 (s, 1H), 7.73 (d, *J* = 4.9 Hz, 1H), 7.51 (d, *J* = 8.4 Hz, 1H), 7.33 (d, *J* = 8.4 Hz, 1H), 7.22 (t, *J* = 4.5 Hz, 1H), 6.97 (s, 2H), 6.87 (s, 1H), 2.62 (s, 2H), 2.46 (s, 2H), 1.09 (s, 6H). ^{13}C NMR (151 MHz, Chloroform-d) δ 169.16, 159.57, 153.05, 148.83, 135.61, 135.45, 134.46, 134.34, 132.30, 131.88, 130.72, 128.39, 127.42, 124.54, 124.47, 117.22, 112.63, 43.11, 39.32, 32.20, 29.85, 28.17. HRMS: $[M]^-$ calculated for $C_{24}H_{19}BrN_2O_2S^-$: 477.0351; found: 477.0264.

Synthesis of compound **AH-H**. Following the general procedure, (84%, Yellow solid). ^{1}H NMR (600 MHz, Chloroform-d) δ 8.00 (d, J = 2.4 Hz, 1H), 7.69 (d, *J* = 3.6 Hz, 1H), 7.57 (d, *J* = 4.6 Hz, 2H), 7.27 (d, *J* = 8.7 Hz, 2H), 7.22 (m, 1H), 7.06 (d, *J* = 16.1 Hz, 1H), 6.97 (d, *J* = 16.1 Hz, 1H), 6.85 (s, 1H), 2.61 (s, 2H), 2.48 (s, 2H), 1.09 (s, 6H). ^{13}C NMR (151 MHz, Chloroform-d) δ 169.36, 160.44, 153.71, 151.67, 135.98, 135.11, 134.02, 133.65, 132.60, 129.52, 128.77, 128.29, 123.91, 122.49, 113.59, 112.80, 43.13, 39.34, 32.19, 28.17. HRMS: $[M]^-$ calculated for $C_{24}H_{20}N_2O_2S^-$: 399.1245; found: 399.1161.

Synthesis of compound **AH-Me**. Following the general procedure, (87%, Yellow solid). ^{1}H NMR (600 MHz, Chloroform-d) δ 8.00 (d, *J* = 2.4 Hz, 1H), 7.70 (d, *J* = 3.6 Hz, 1H), 7.43 (s, 1H), 7.39 (d, *J* = 8.3 Hz, 1H), 7.21 (m, 2H), 7.03 (d, *J* = 16.1 Hz, 1H), 6.96 (d, *J* = 16.1 Hz, 1H), 6.85 (s, 1H), 2.61 (s, 2H), 2.47 (s, 2H), 2.28 (s, 3H), 1.09 (s, 6H).^{13}C NMR (151 MHz, Chloroform-d) δ 169.39, 160.20, 153.86, 150.35, 136.26, 135.05, 133.93, 133.79, 132.44, 131.35, 130.36, 129.33, 128.31, 126.43, 123.75, 122.87, 113.62, 112.83, 43.11, 39.31, 32.17, 28.15, 16.49. HRMS: $[M+Na]^+$ calculated for $C_{25}H_{22}N_2O_2S+Na^+$: 437.1299; found: 437.1314.

Synthesis of compound **AH-MeO**. Following the general procedure, (87%, Yellow solid). ^{1}H NMR (600 MHz, Chloroform-d) δ 8.00 (d, *J* = 2.4 Hz, 1H), 7.68 (d, *J* = 6.4 Hz, 1H), 7.19 (t, *J* = 6.5 Hz, 2H), 7.15 (s, 1H), 7.12 (s, 1H), 7.04 (d, *J* = 16.1 Hz, 1H), 6.96 (d, *J* = 16.1 Hz, 1H), 6.87 (s, 1H), 3.90 (s, 3H), 2.61 (s, 2H), 2.48 (s, 2H), 1.09 (s, 6H). ^{13}C NMR (151 MHz, Chloroform-d) δ 169.39, 160.07, 153.69, 151.95, 140.99, 136.38, 135.09, 134.95, 133.80, 129.54, 128.20, 123.97, 123.66, 121.10, 113.56,

112.87, 110.55, 56.19, 43.11, 39.33, 32.21, 28.17. HRMS: $[M]^-$ calculated for $C_{25}H_{22}N_2O_3S^-$: 429.1351; found: 429.1264

III. Experimental Methods

Experimental preparation procedure.

AH-F was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution, which was then diluted with DMSO to achieve the desired concentration. GSH was dissolved directly in PBS buffer. The probe was subsequently added to the diluent (which had a final composition of 50% DMSO) for GSH detection. The probe was incubated with different concentrations of analytes in PBS buffer at 37°C, and the absorption and fluorescence spectra of the samples were recorded. The fluorescence enhancement ratio (F/F_0) was normalized to the initial fluorescence intensity of the probe.

Spectral response of **AH-F** to GSH

Fluorescence spectra were measured using a fluorescence spectrometer. The fluorescence of **AH-F** between 560 nm and 800 nm was collected under excitation at 538 nm. **AH-F** (10 μM) and GSH (1 mM) were incubated in PBS buffer at 37°C and scanned at 5-minute intervals for a total of 50 minutes to determine the responsivity of **AH-F** to GSH. To record the response to different concentrations of GSH, **AH-F** was incubated with varying concentrations of GSH in PBS buffer at 37°C for 50 minutes, and the fluorescence change at 688 nm was measured.

pH-dependent fluorescence response of **AH-F** to GSH

AH-F (10 μM) and a mixture of **AH-F** (10 μM) with GSH (1 mM) were incubated in buffer solutions with varying pH levels PBS (pH range 6.0-8.0) and sodium carbonate buffer (pH range 9.0-11.0) at 37°C to assess their fluorescence responses. The fluorescence at 688 nm was collected under excitation at 538 nm, and each experiment was repeated at least three times.

Selectivity of **AH-F** for GSH

In this experiment, AH-F (10 μM) was incubated with various test substances, including 1, Phe; 2, Glu; 3, His; 4, Pro; 5, Ala; 6, Tyr; 7, Ser; 8, Val; 9, Lys; 10, Gly; 11, Trp; 12, Leu; 13, Ile; 14, CuI; 15, $FeCl_3$; 16, $CaCl_2$; 17, $FeSO_4$; 18, KCl; 19, $ZnCl_2$; 20, NaClO; 21,$ONOO^-$; 22,KO_2;23,Na_2S; 24, Cys; 25, Hcy; 26, NAC and 27, GSH, in PBS buffer at 37°C for 40 minutes. The concentrations of small molecules and mercaptides were 100 eq and 10 eq, respectively. After co-incubation, the fluorescence intensity of **AH-F**

was measured at 688 nm following excitation at 538 nm. Each experiment was repeated at least three times.

Cytotoxicity test.

The toxicity of **AH-F** on HepG2 and HeLa cells was detected using the CCK-8 assay. 1×10^4 cells and **AH-F** (1-20 μM) were cultured in 96-well plates at 37°C in triplicate, with a final volume of 100 μL. Cells treated with medium only served as controls. At the end of the treatment period (24 hours), 10 μL of CCK-8 was added to each well and incubated at 37°C for 2 hours. Absorbance was measured at 450 nm using a Thermo Scientific spectrophotometer.

Cells imaging.

HepG2 cells were seeded into imaging dishes at a density of 100,000 cells per dish in 2 mL of medium, and then imaged at 37°C using **AH-F** (10 μM) every 15 minutes for a total of four times. After washing the cells three times with PBS buffer, fluorescence images were captured using a laser confocal microscope (FV-3000).

Mouse imaging.

The Kunming mice used in this study were obtained from the Animal Medical Experiment Center of Lanzhou University and housed in the animal facilities of the School of Basic Medical Sciences at Lanzhou University. All animal experiments were approved by the Ethics Committee of Lanzhou University. First, **AH-F** was dissolved in DMSO and then diluted to a 500 μM solution with PBS. After anesthetizing the mice with isoflurane, 100 μL of **AH-F** was injected into the abdominal cavity of the mice, and the mice were imaged at various time points. The imaging instrument used was a Bolton Aniview100, and images of the mice were acquired with 580 nm excitation and 650-750 nm NIR emission wavelengths.

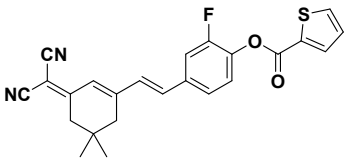
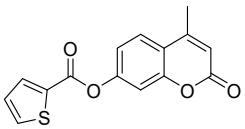
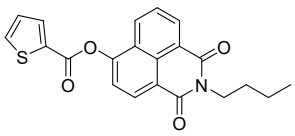
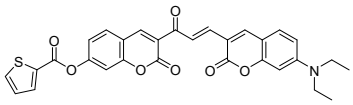
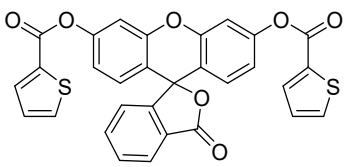
Pathological model

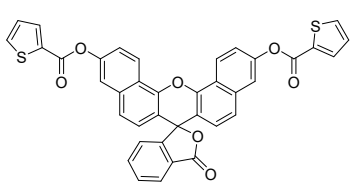
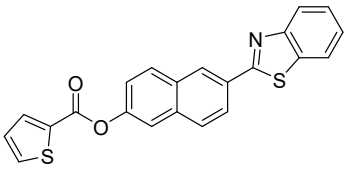
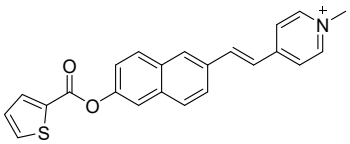
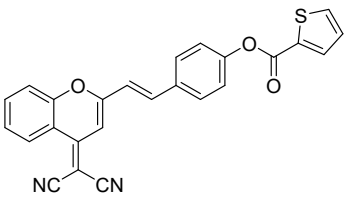
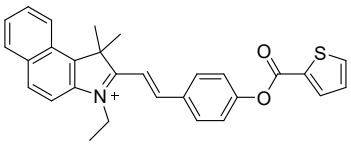
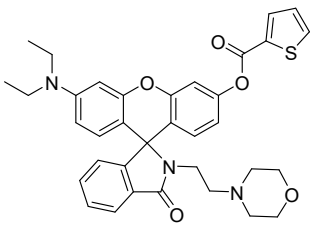
Here we used 4-week-old Kunming male mice weighing 18-22 g. A total of 10 mice were purchased from the Laboratory Animal Center of Lanzhou University. The mice were acclimatized and fed for 1 week before the experiment, and the experimental protocol was approved by the Laboratory Animal Ethics Committee of Lanzhou University. All mice were fasted 12 h before the experiment, and APAP (300 mg/kg)

was injected intraperitoneally. 8 h later, mice in the treatment group were injected intraperitoneally with DDB (100 mg/kg), NAC (100 mg/kg), and UDCA (100 mg/kg), respectively, and were imaged 4 h after the administration of drugs in the treatment group.

IV. Supplementary Figures

Table S1. Previously reported related work

Probe structure	responsive substance	$\lambda_{ex}/\lambda_{em}$ (nm) (values for product for ratio metric probes)	F/F_0 (values for product for turn-on probes), Time	Solvent system for spectroscopic studies	Applicati-ons	Referenc e
	GSH	538/688	357, 40 min	PBS (pH 7.4) with DMSO (50 %)	Live cells，*in vivo*	**This work**
	H_2S	307/446	N.R, 30 min	PBS (pH 5.0) with DMSO (25 %)	Food	[2]
	GSH	450/555	N.R, 2 h	PBS buffer (10 mM, pH 7.4, 1 mM CTAB)	Live cells，*in vivo*	[3]
	H_2S	500/585	N.R, 35 s	PBS buffer (pH 7.4, 30% DMSO).	Live cells, Plants	[4]
	H_2S	450/542	N.R, 15 min	PBS buffer (20 mM, pH 7.4, 1% CH_3COCH_3	Live cells, *in vivo*	[5]

	H_2S	550/680	N.R, 10 min	DMSO-PBS (1 %, pH 7.4)	Live cells, Food	**[6]**
	H_2S	307/504	N.R, 30 min	PBS; pH 7.4 with DMSO (25 %)	Food	**[7]**
	H_2S	410/590	6, 30 min	HEPES buffer	Live cells, Food	**[8]**
	H_2S	500/680	N. R, 10 min	EtOH-PBS buffer (1 %, pH 7.4)	Live cells, Materials	**[9]**
	H_2S	500/620	N. R, 30 min	HEPES (pH 7.4, 10 mM)	Live cells, *in vivo*	**[10]**
	H_2S	273/550	N. R, 15 min	PBS buffer (pH = 7.4)	Live cells	**[11]**

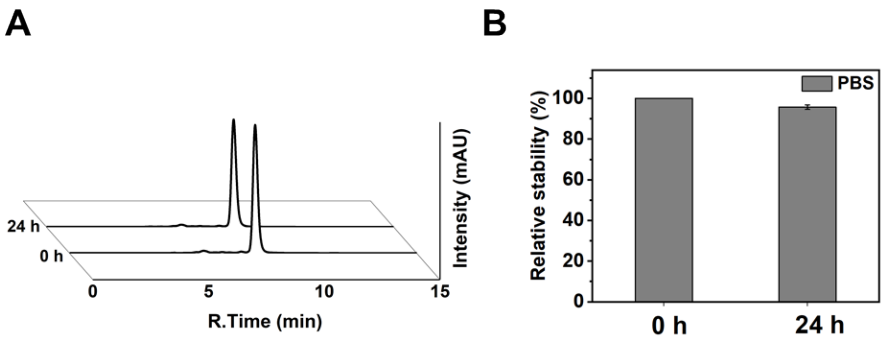


Fig. S1. (A) HPLC monitoring of 24 h stability of AH-F (10 μM). HPLC mobile phase was as follows: H_2O/MeOH = 10/90 (v/v); flow rate: 0.4 mL min^{-1}. (B) Integral area statistics of absorption peaks at 0 h and 24 h in HPLC results.

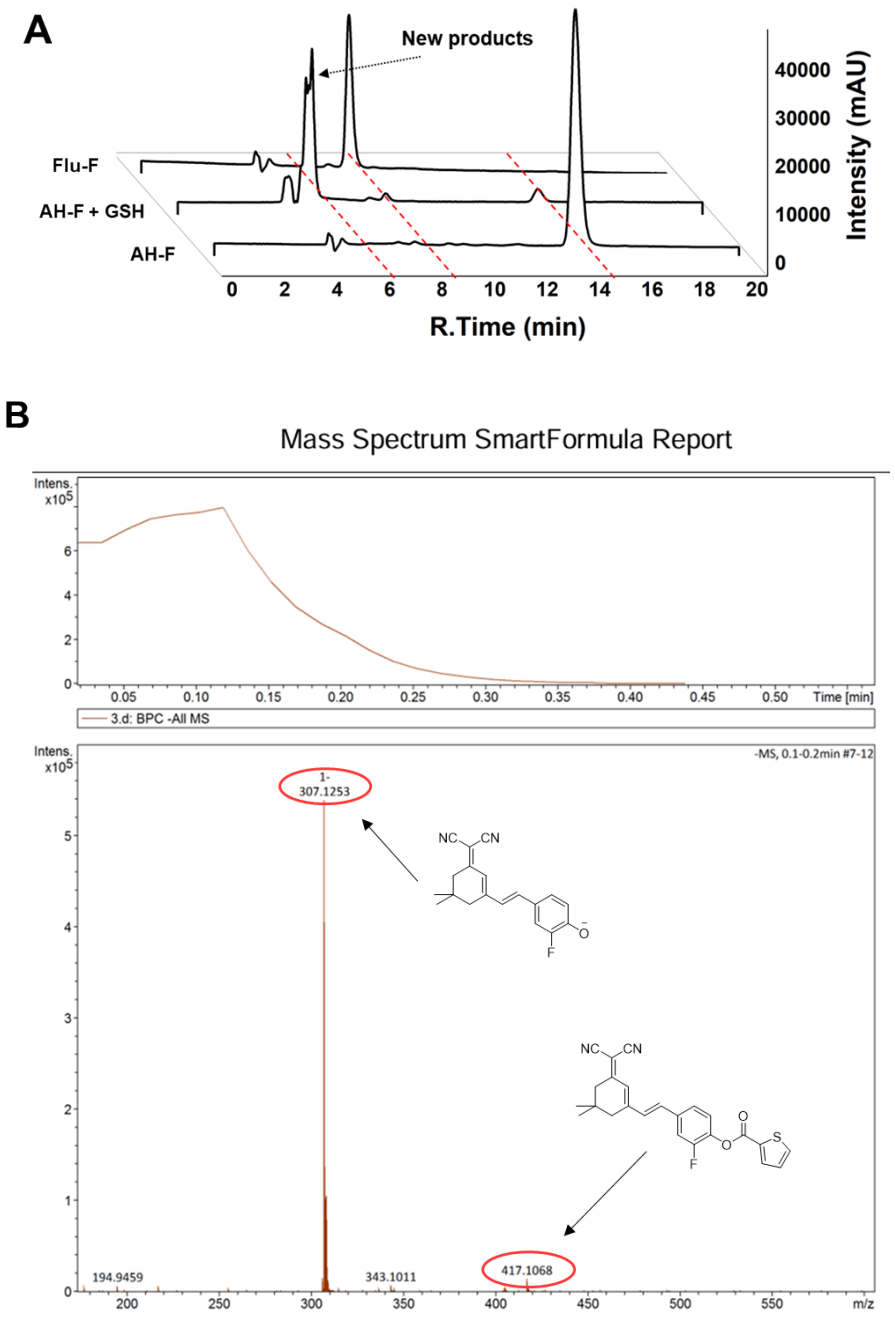


Fig. S2. (A) HPLC experiment of AH-F (100 μM) reacted with GSH (1 mM) for 40 min. HPLC mobile phase was as follows: H_2O/MeOH = 20/80 (v/v); flow rate: 0.4 mL min^{-1}. (B) HR-MS monitoring of AH-F (10 μM) incubated with GSH (1 mM) for 10 min.

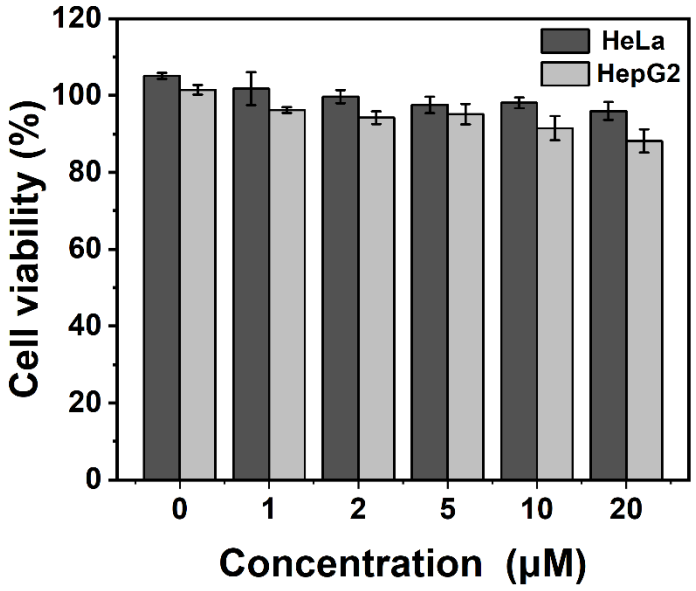


Fig. S3. The viability testing of AH-F in cells. HepG2 and HeLa cells were incubated with different concentrations of AH-F for 24 h. The cell viability was assessed using the CCK-8 assay.

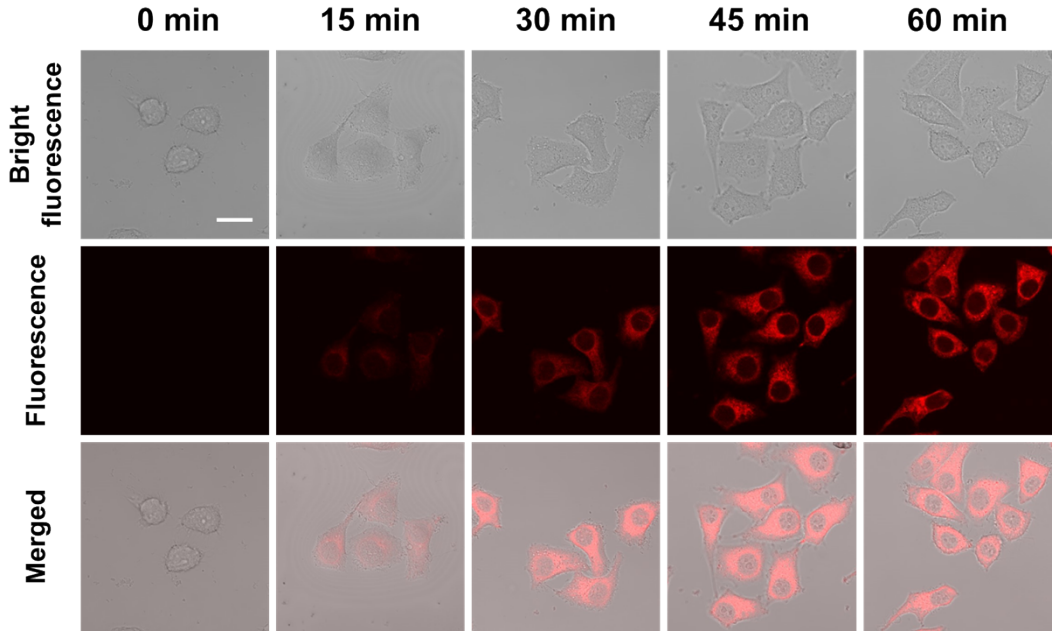


Fig. S4. Changes in the intracellular fluorescence intensity of AH-F over time. AH-F (10 μM) was co-incubated with HeLa cells and photographed separately for different incubation times. Scale bars: 20 μm.

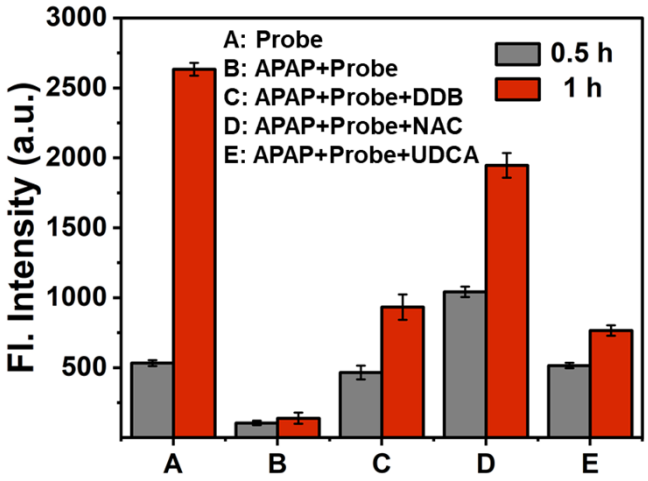


Fig. S5. Quantitative analysis of imaging results in different groups of mice.

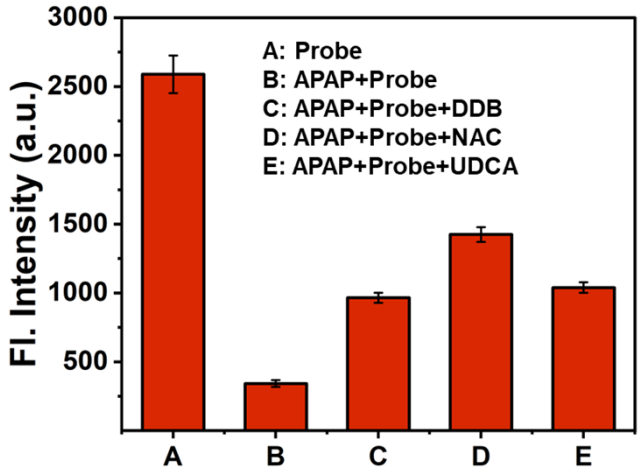
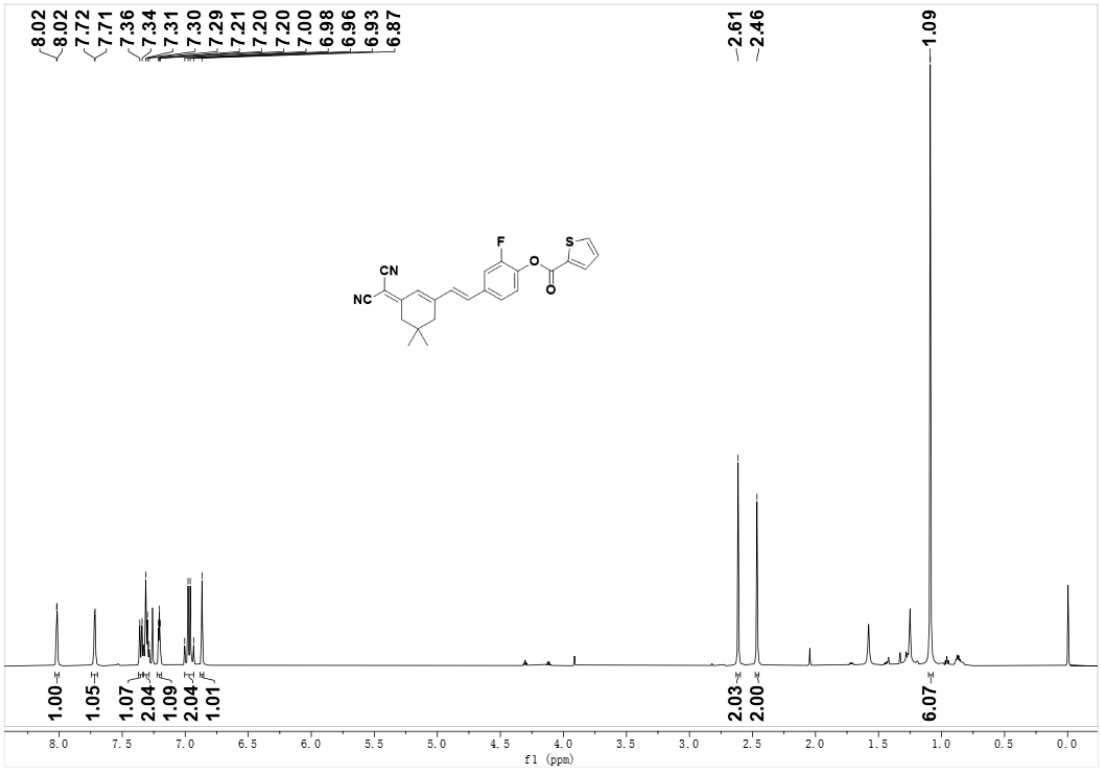
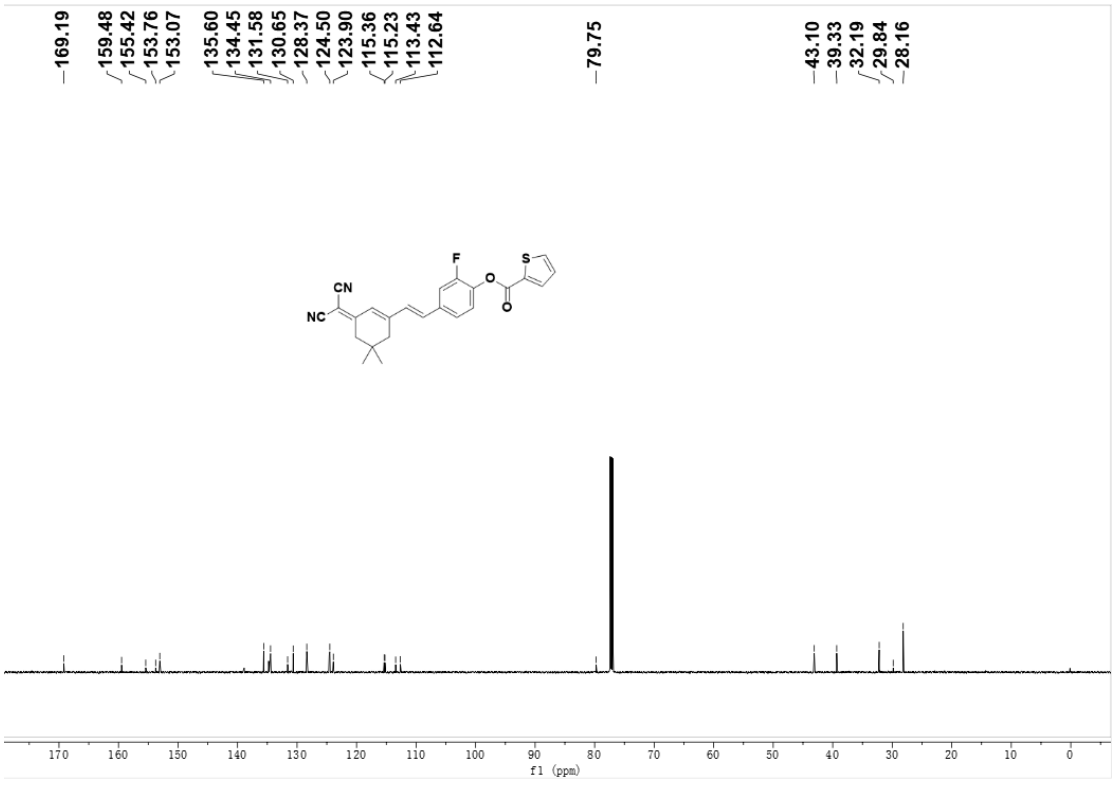


Fig. S6 Quantitative analysis of organ imaging results in different groups of mice.

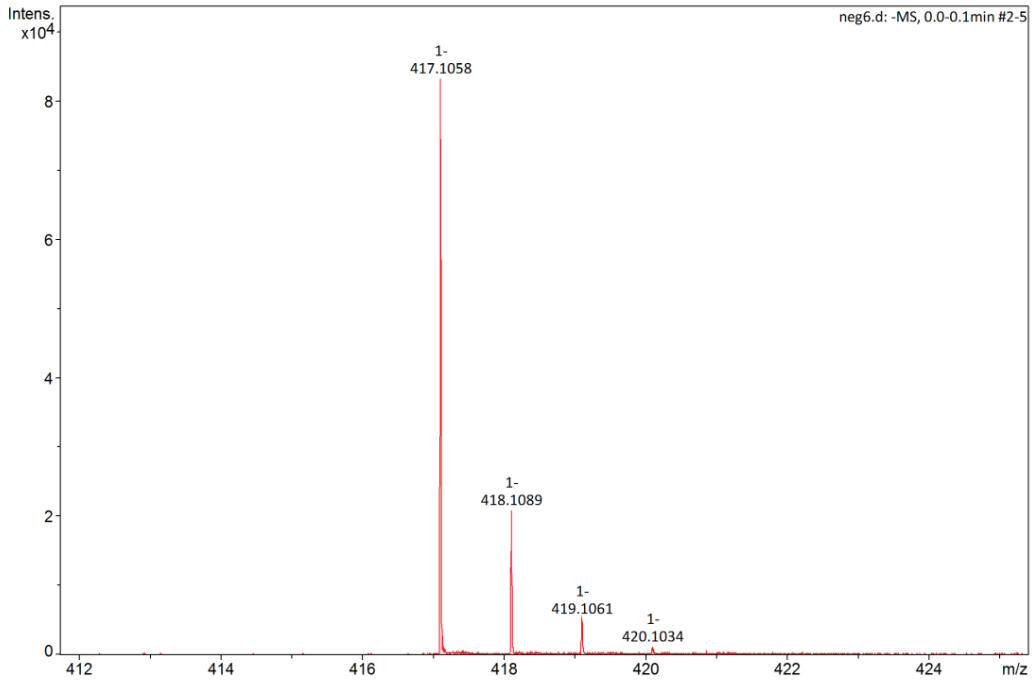
V. Supporting Figures



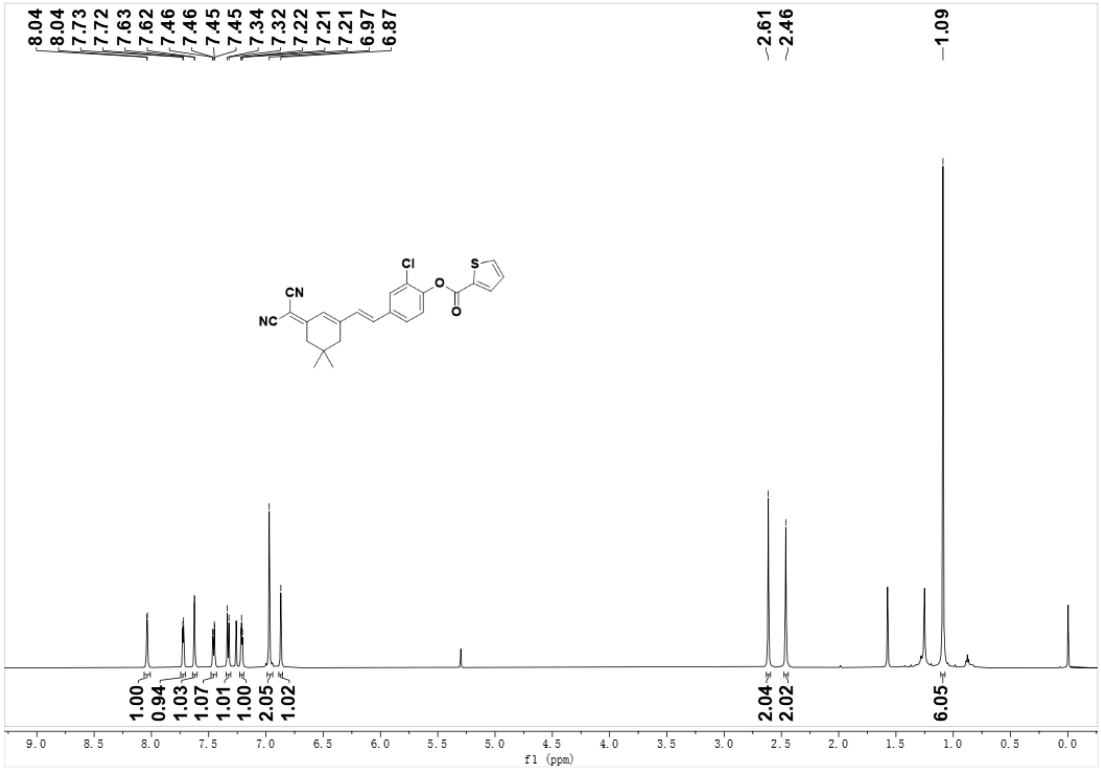
^{1}H NMR (600 MHz, $CDCl_3$-*d*) of compound **AH-F**.



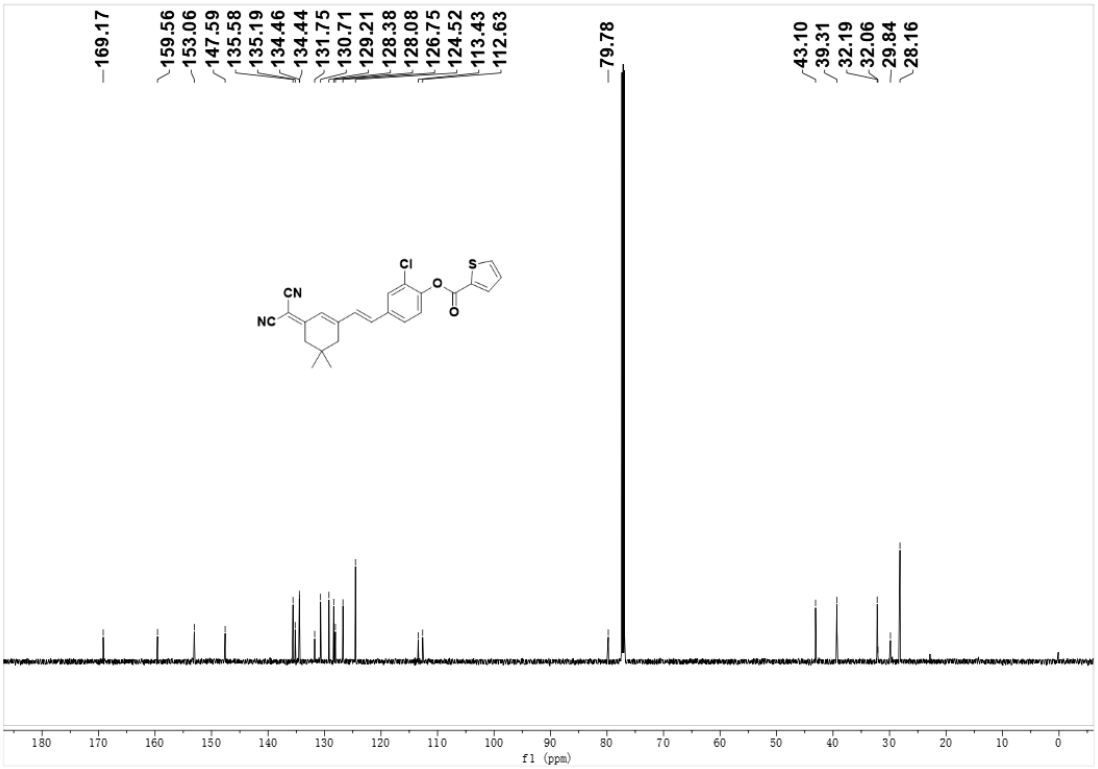
^{13}C NMR (151 MHz, $CDCl_3$-*d*) of compound **AH-F**.



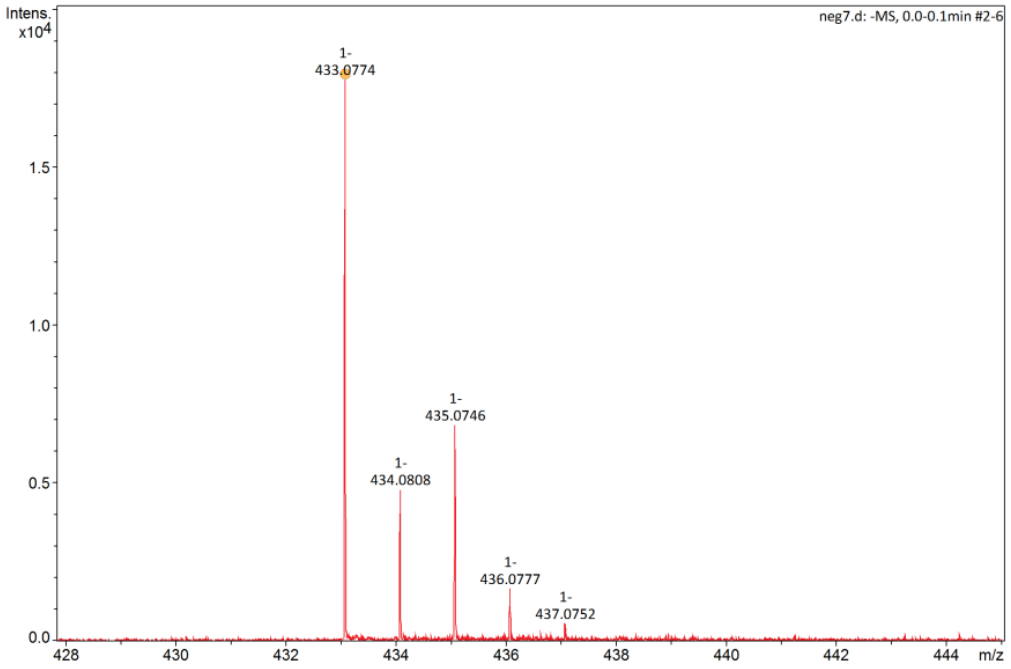
ESI-Mass spectrum of **AH-F** (ESI-MS).



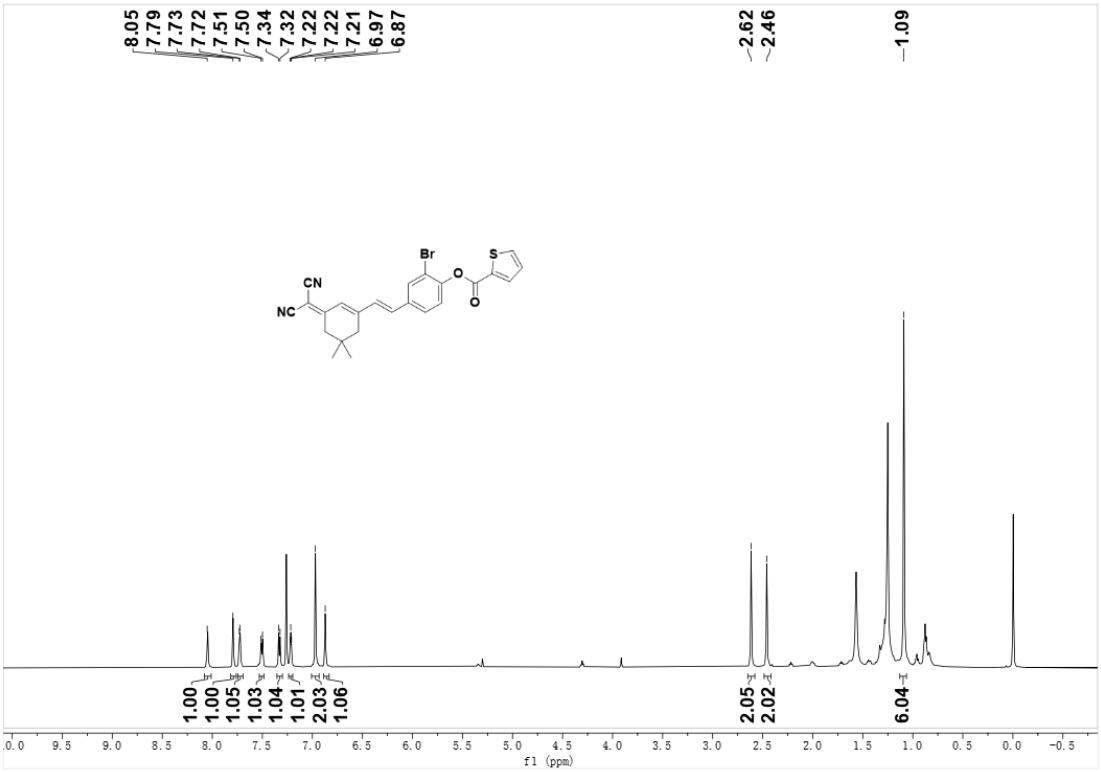
^{1}H NMR (600 MHz, $CDCl_3$-*d*) of compound **AH-Cl**.



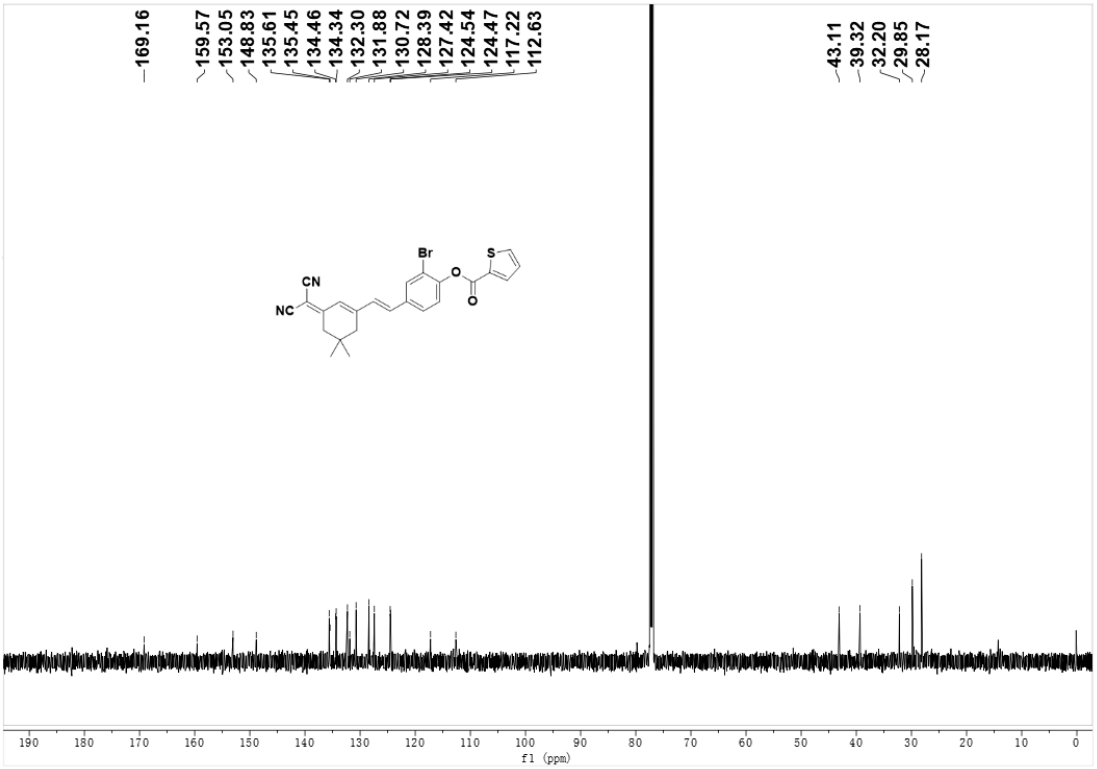
^{13}C NMR (151 MHz, $CDCl_3$-*d*) of compound **AH-Cl**.



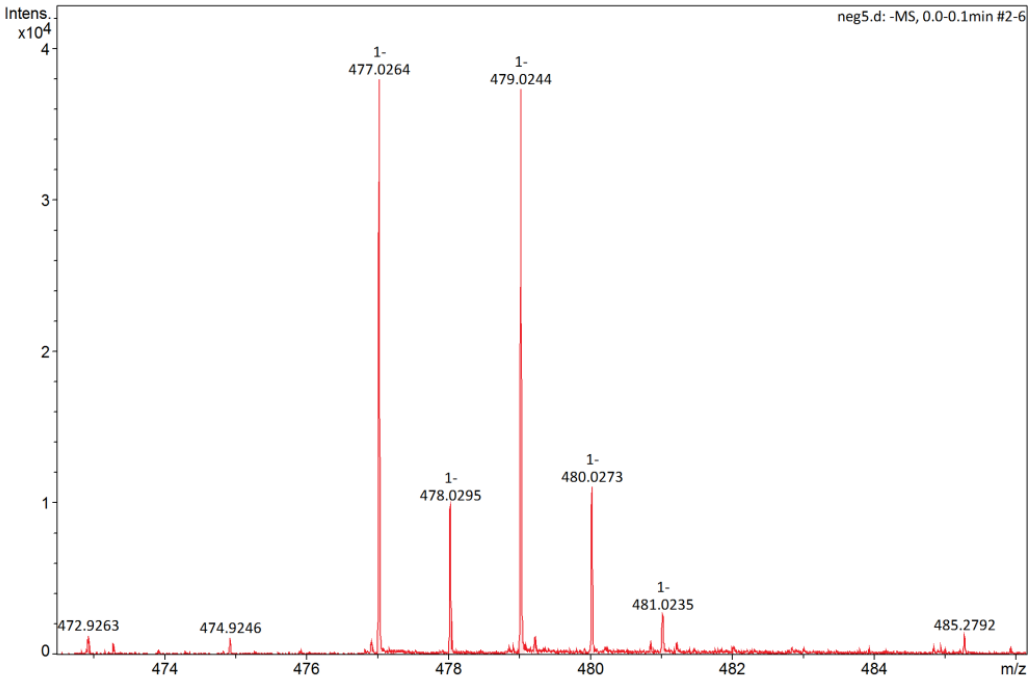
ESI-Mass spectrum of **AH-Cl** (ESI-MS).



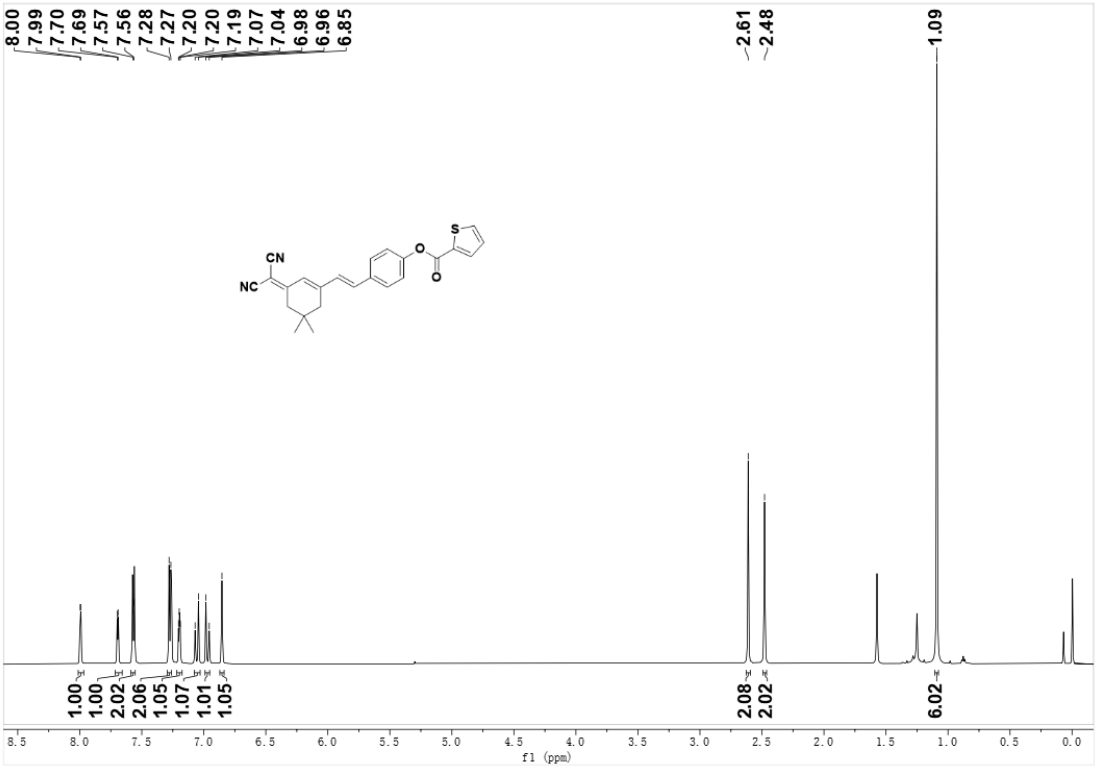
^{1}H NMR (600 MHz, $CDCl_3$-*d*) of compound **AH-Br**.



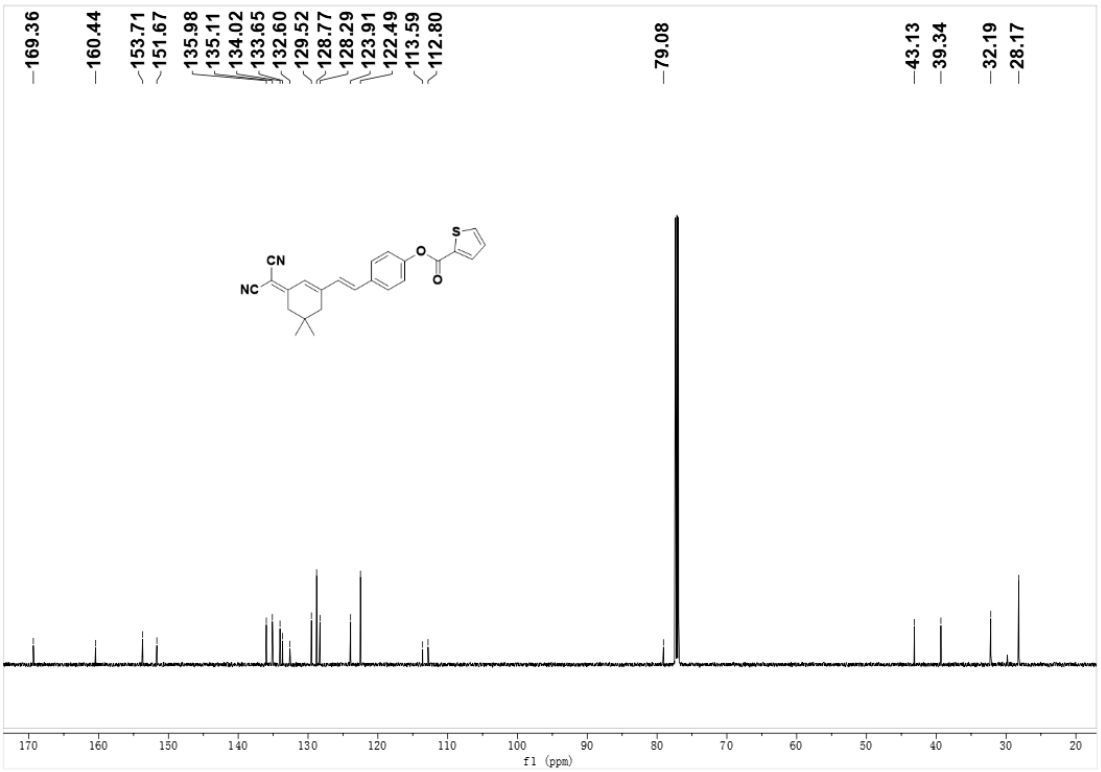
^{13}C NMR (151 MHz, $CDCl_3$-*d*) of compound **AH-Br**.



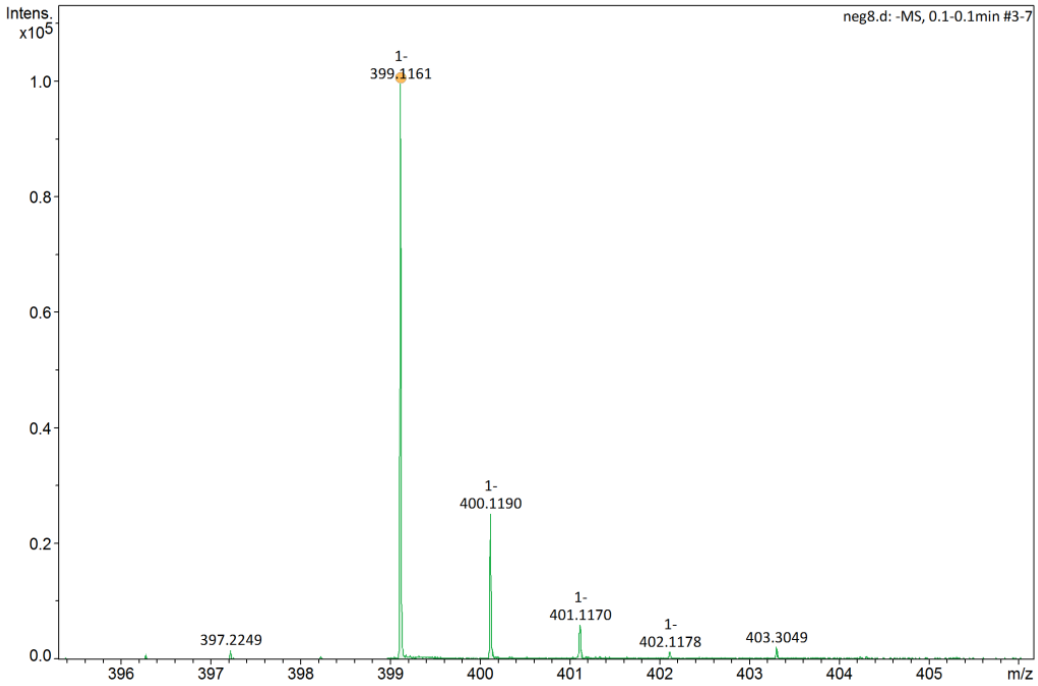
ESI-Mass spectrum of **AH-Br** (ESI-MS).



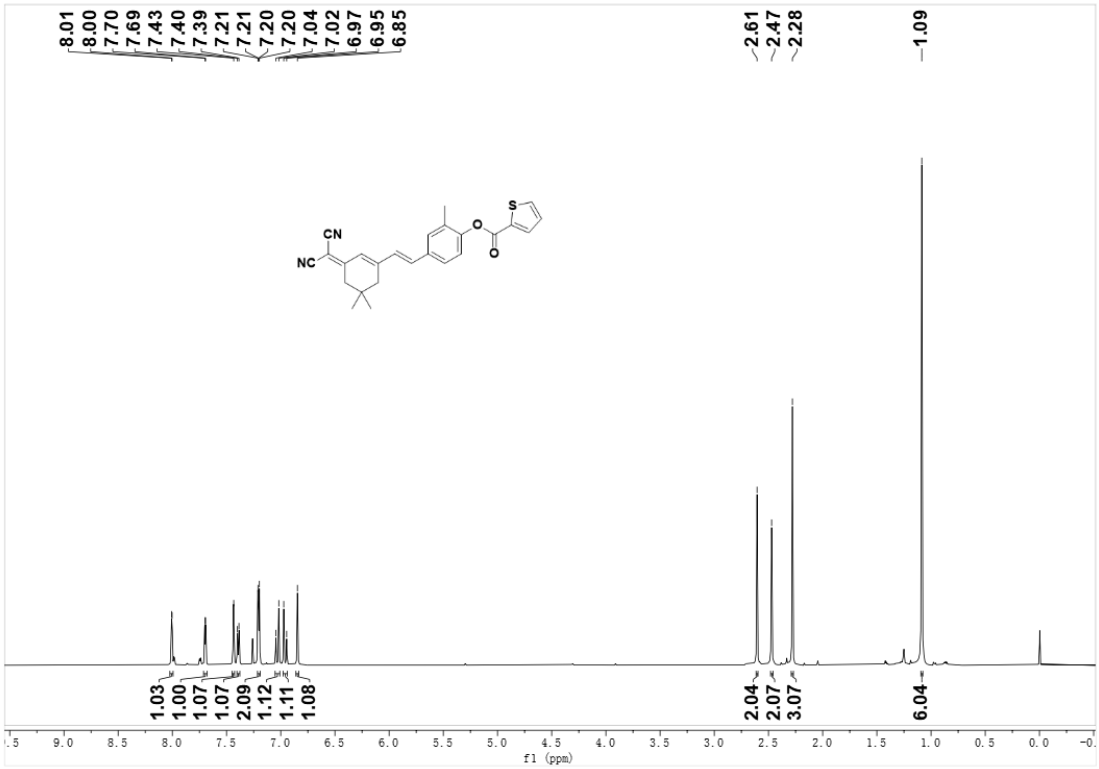
^{1}H NMR (600 MHz, $CDCl_3$-*d*) of compound **AH-H**.



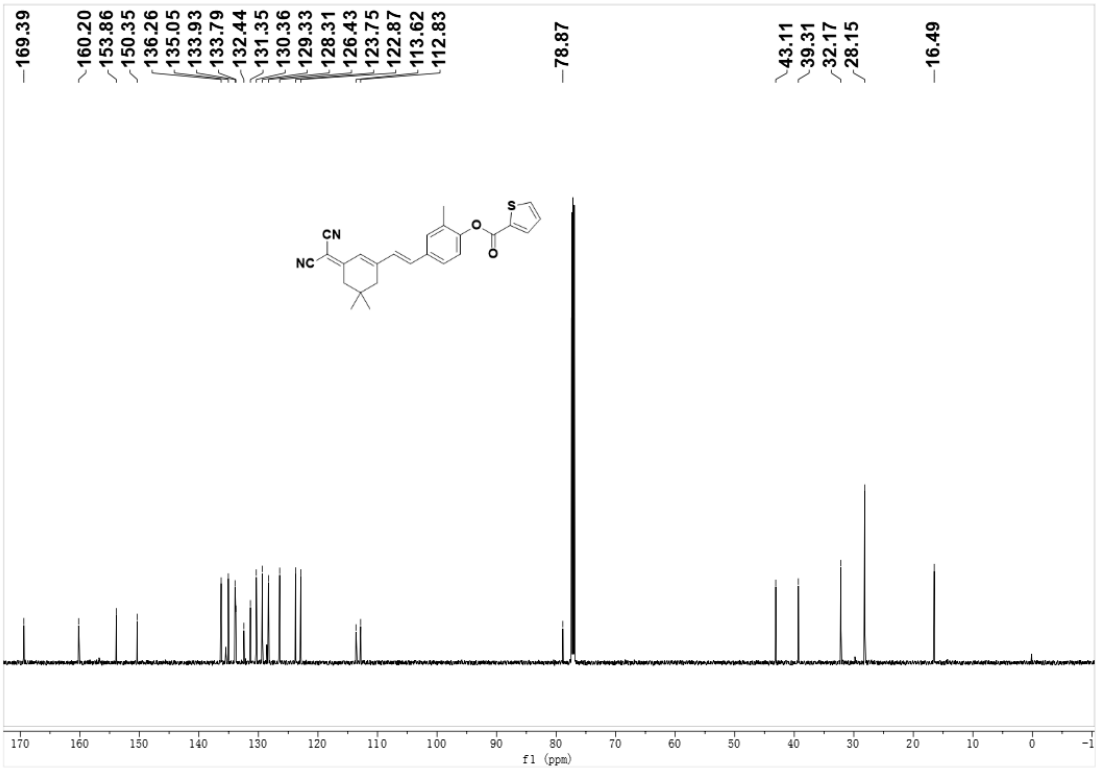
^{13}C NMR (151 MHz, $CDCl_3$-*d*) of compound **AH-H**.



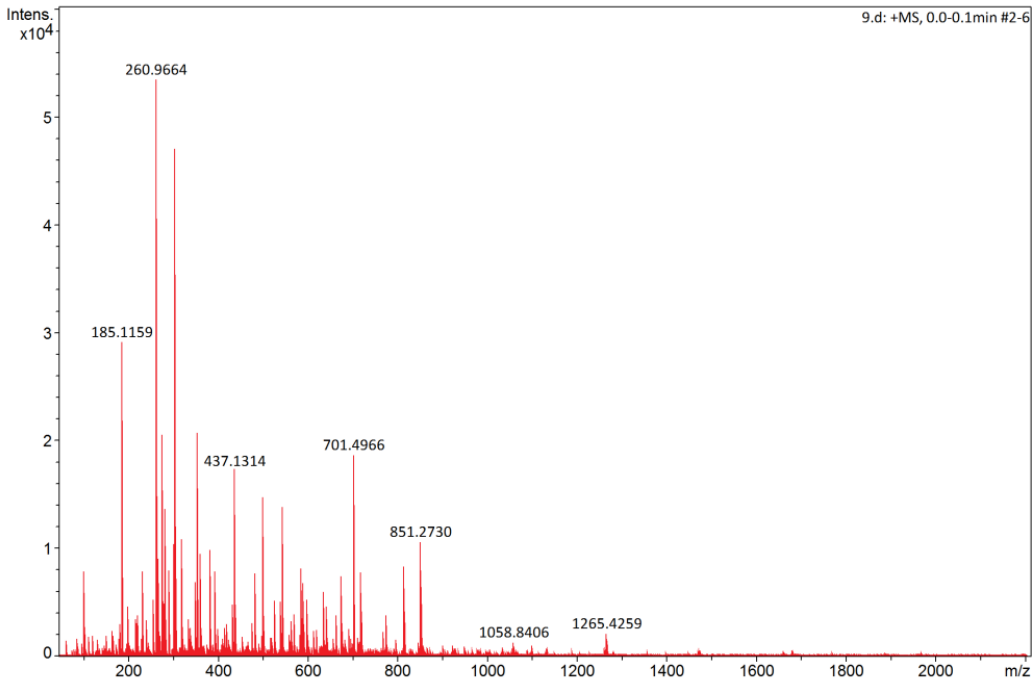
ESI-Mass spectrum of **AH-H** (ESI-MS).



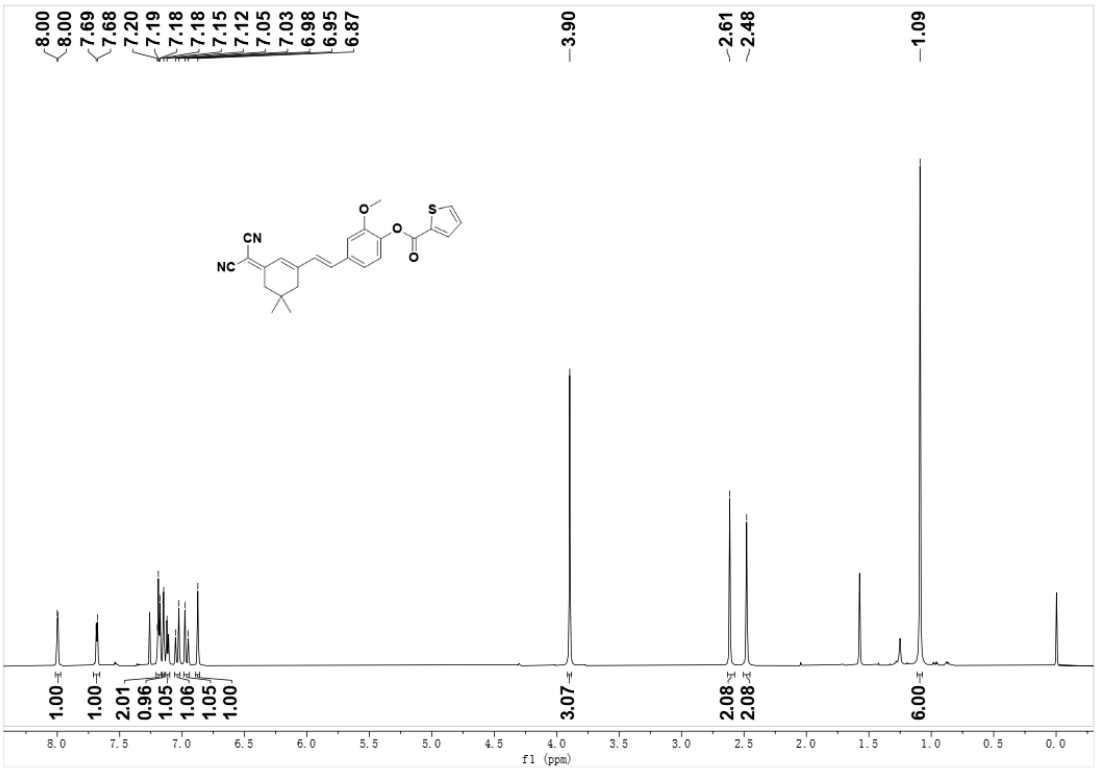
^{1}H NMR (600 MHz, $CDCl_3$-*d*) of compound **AH-Me**.



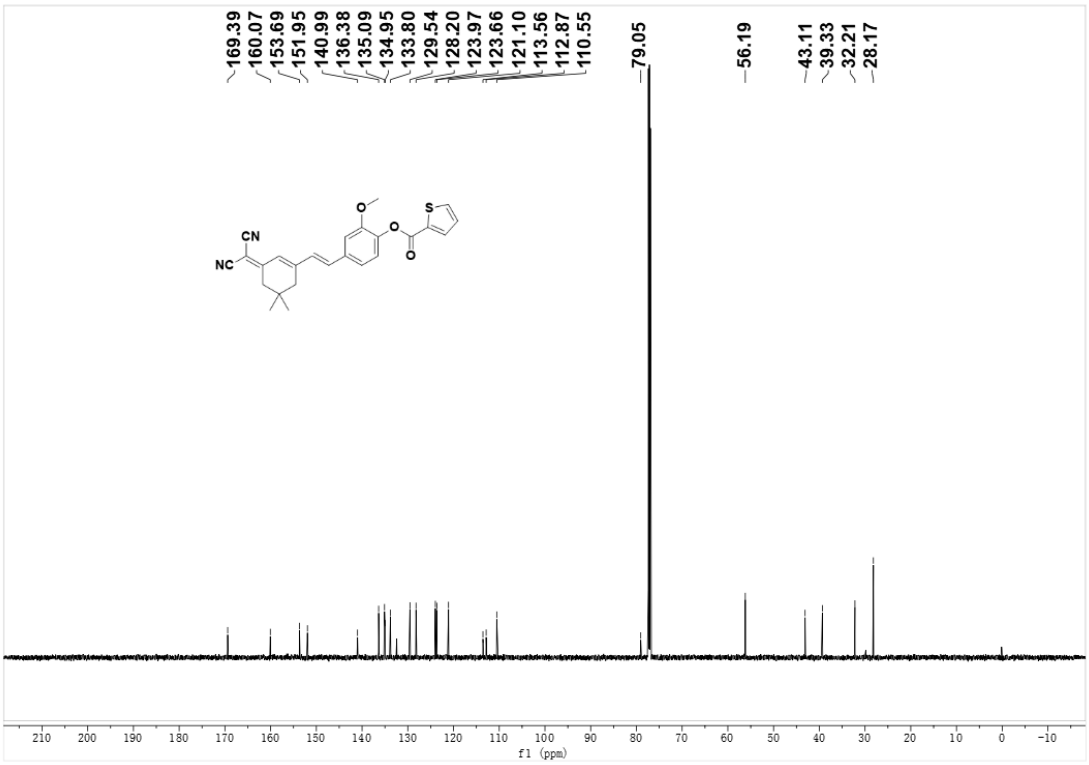
^{13}C NMR (151 MHz, $CDCl_3$-*d*) of compound **AH-Me**.



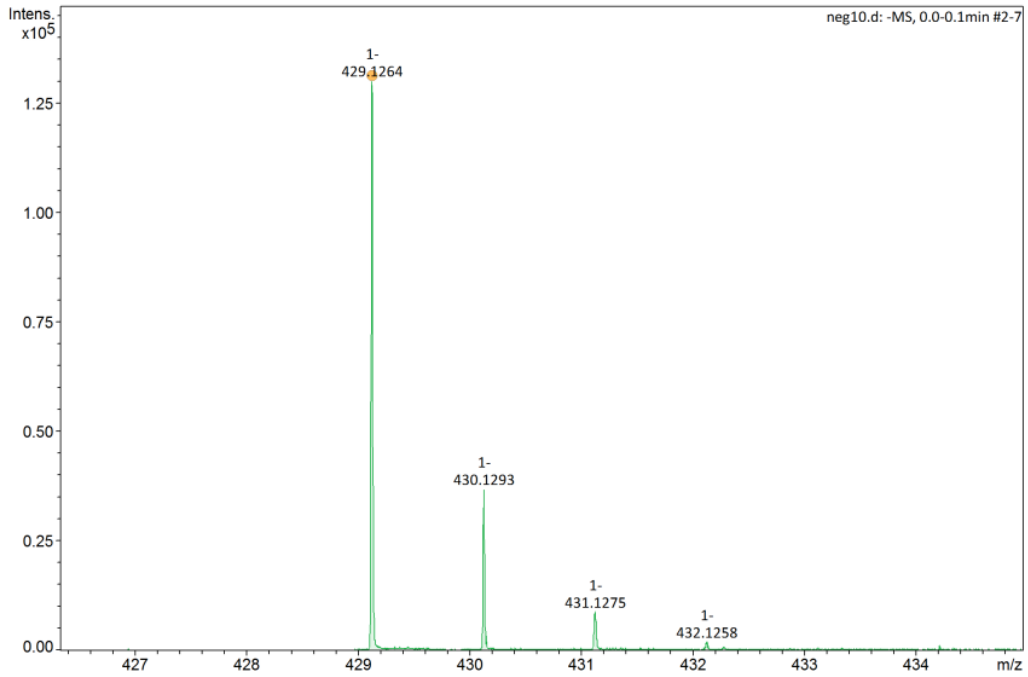
ESI-Mass spectrum of **AH-Me** (ESI-MS).



^{1}H NMR (600 MHz, $CDCl_3$-*d*) of compound **AH-MeO**.



^{13}C NMR (151 MHz, $CDCl_3$-*d*) of compound **AH-MeO**.



ESI-Mass spectrum of **AH-MeO** (ESI-MS).

VI. References

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