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Nitroxyl-responsive near-infrared fluorescent chemosensor for visualizing H_2S/NO crosstalk in biological systems

Mingwang Yang[a], Jiangli Fan*[a,b], Wen Sun[a,b], Jianjun Du[a,b], Saran Long[a,b], Kun Shao[a,b], Xiaojun Peng[a,b]

[a] State Key Laboratory of Fine Chemicals, Dalian University of Technology, 2 Linggong Road, Dalian, 116024, P.R. China.
[b] Research Institute of Dalian University of Technology in Shenzhen, Gaoxin South fourth Road, Nanshan District, Shenzhen 518057, China

Email: fanjl@dlut.edu.cn

Table of Contents

Table S1 Comparation of reported probes for recognition of HNO **S3**

1. Materials and Instruments.....**S4**

2. Synthesis and characterization of probe **S4**

3. Spectrophotometric experiments **S5**

4. Determination of detection limits **S5**

5. Cell culture and cytotoxicity experiments **S6**

6. Fluorescence imaging of living cells and tissues.....**S6**

7. Fluorescence imaging in live mice **S6**

8. The test spectra and images of **NR-HNO**.....**S7**

Figure S1 Normalized absorption and fluorescence spectra of probe **S7**

Figure S2 The fluorescence comparation of compound 3 and **NR-HNO** **S7**

Figure S3 The HRMS of probe treated with AS.....**S8**

Figure S5 The stability of time dependence of **NR-HNO**.....**S8**

Figure S6 The fluorescence spectra of **NR-HNO** to various analytes **S9**

Figure S7 Effect of pH on the fluorescence of **NR-HNO**. **S9**

Figure S8 MTT assay of probe **S10**

Figure S9 The fluorescence images for detecting exogenous HNO in HepG2 cells.. .....**S10**

Figure S10 The fluorescence imaging of endogenous HNO **S12**

Figure S11 The fluorescence imaging of mouse kidney slices.....**S12**

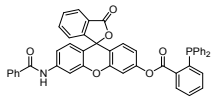
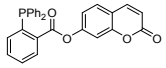
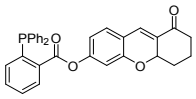
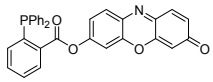
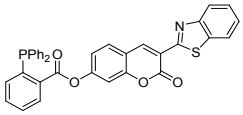
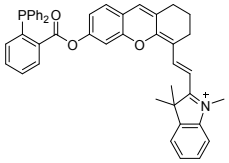
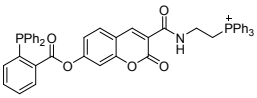
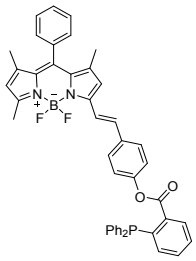
Figure S12 The colocalization imaging of **NR-HNO**.. .....**S11**

Figure S13 The control imaging of H_2S/NO crosstalk.. .....**S13**

Figure S14 The fluorescence image of tumor tissue.....**S13**

9. The NMR and HR-MS spectra of all compounds. **S13**

Table S1 Comparation of reported probes for recognition of HNO

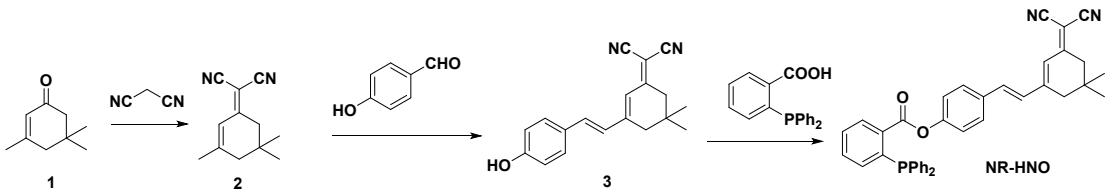
Probe	$\lambda_{em}/\lambda_{em}$(nm)	Stokes shift (nm)	Detection limit	Response time	Imaging application
 *J. Am. Chem. Soc.* ,2013, **135**, 12690	491/526	35	Not mentioned	20 min	A549 cells
 *Chem. Commun.*, 2014, **50**, 5790	370/450	80	20 nM	30 min	Not mentioned
 *Chem. Commun.*, 2015, **51**, 5754	405/512	107	0.590 μM	~30 min	HeLa cells
 *RSC Adv.*, 2015, **5**, 84543	565/590	25	0.02 μM	30 min	CHO cells
 *J. Mater. Chem. B,* 2016, **4**, 1263	454/490	36	0.648 μM	45 min	HeLa cells
 *J. Mater. Chem. B,* 2016, **4**, 1263	670/710	40	0.622 μM	45 min	A549, HeLa, mice
 *Chem. Commun.,* 2017, **53**, 1723	405/452	47	18 nM	20 min	HeLa
 *Anal. Chem.*, 2017, ***89***, 12087	530/586	56	2 μM	Not mentioned	RAW 264.7
This work	545/676	131	0.09 μM	~5 min	MCF-7 and HepG2 cells; Kidney and liver tissues; Live mice

1. Materials and Instruments

Unless otherwise stated, all the chemicals were purchased from commercial supplies and used without further purification. The solvents were purified by conventional methods before used. Water was purified and doubly distilled by using a Milli-Q system. Hoechst 33342, Lyso-Tracker Green, Mito-Tracker Green and Golgi-Tracker Green were purchased from Invitrogen. Isophorone (**Compound 1**) and malononitrile were purchased from Energy Chemical. The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Company. The Angeli's salt (AS), usually HNO donor, was purchased from Cayman. Silica gel (200-300 mesh) used for flash column chromatography was purchased from Qingdao Haiyang Chemical Co., Ltd.^{1}HNMR and ^{13}CNMR spectra were determined by 400 MHz and 100 MHz using Bruker NMR spectrometers. Chemical shifts (δ) were expressed as parts per million (ppm, in $CDCl_3$ or DMSO, with TMS as the internal standard). Meanwhile, high-resolution mass spectrometry was achieved with ESI-TOF and FTMS-ESI instrument.

Fluorescence measurements were performed on an Agilent Technologies CARY Eclipse fluorescence spectrophotometer, and absorption spectra were measured on a PerkinElmer Lambda 35 UV-vis spectrophotometer. The pH values of sample solutions were measured with a precise pH-meter pHS-3C. Fluorescence quantum yield was achieved from a C11347-11 Absolute PL Quantum Yield Spectrometer. MTT assays were conducted on the Varioskan LUX Multimode Microplate Reader. The instrument used for imaging living cells and tissues of mice was an Olympus FV 3000 confocal microscopy purchased from Olympus.

2. Synthesis and characterization of probe NR-HNO



Scheme S1 Synthesis of probe NR-HNO

Compound 2: Isophorone (2.07g, 15.0 mmol), malononitrile (660 mg, 10.0 mmol), piperidine (0.1 mL, 1.0 mmol), and glacial acetic acid (50 mg, 0.8 mmol) were dissolved in 80 mL EtOH under argon atmosphere. The mixture was refluxed overnight. After the reaction completed, the solvent was removed under reduced pressure and the residue was dissolved with CH_2Cl_2 and washed with water, the organic solvent was dried over Na_2SO_4. Finally, the solvent was removed under reduced pressure and the residue was purified by column chromatography with dichloromethane: petroleum (5:1) as the eluent to afford the desire product as a white solid (1.33 g, yield 71.2%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 6.62 (s, 1H), 2.51 (s, 2H), 2.17 (s, 2H), 2.03 (s, 3H), 1.01 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ (ppm): 170.35, 159.75, 120.58, 113.17, 112.39, 77.29, 77.03, 76.78, 45.68, 42.63, 32.35, 27.80, 25.29.

Compound 3: Compound **2** (930 mg, 5.0 mmol), p-hydroxybenzaldehyde (610 mg, 5.0 mmol) and some piperidine were dissolved in 20 mL CH_3CN under argon atmosphere. The mixture was refluxed 6 h and the solvent was removed under reduced pressure and the residue was dissolved

with CH_2Cl_2 and washed with water, the organic solvent was dried over Na_2SO_4. Finally, the solvent was removed under reduced pressure and the residue was purified by column chromatography with dichloromethane: methanol (50:1) as the eluent to afford the desire product as a yellow solid (587 mg, yield 40.5%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.96 (s, 1H), 7.56 (d, J = 8.5 Hz, 2H), 7.21 (d, J = 5.2 Hz, 2H), 6.79 (d, J = 8.1 Hz, 3H), 2.60 (s, 2H), 2.53 (s, 2H), 1.01 (s, 6H). ^{13}C NMR (126 MHz, DMSO) δ (ppm): 170.20, 159.32, 156.64, 138.24, 129.83, 127.09, 126.21, 121.34, 115.85, 114.09, 113.27, 74.80, 42.29, 40.01, 39.84, 39.77, 39.68, 39.51, 39.34, 39.18, 39.01, 38.17, 31.62, 27.41.

NR-HNO: Compound **3** (290 mg, 1.0 mmol), 2-(diphenylphosphanyl) benzoic acid (306 mg, 1 mmol), EDCI (192 mg, 1.0 mmol) and DMAP (12.2 mg, 0.1 mmol) were dissolved in 15 mL CH_2Cl_2 under argon atmosphere. The mixture was stirred overnight and diluted with 10 mL water, then extracted with CH_2Cl_2, the organic solvent was dried over Na_2SO_4. Finally, the solvent was removed under reduced pressure and the residue was purified by column chromatography with dichloromethane: methanol (100:1) as the eluent to afford the probe as a yellow solid (227 mg, yield 39.3%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.28 – 8.22 (m, 1H), 7.49 – 7.41 (m, 4H), 7.32 (ddd, J = 9.2, 7.0, 1.5 Hz, 10H), 7.06 – 6.96 (m, 4H), 6.92 (d, J = 16.1 Hz, 1H), 6.84 (s, 1H), 2.60 (s, 2H), 2.46 (s, 2H), 1.08 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 169.18, 164.98, 164.96, 153.61, 151.59, 137.53, 137.42, 135.96, 134.51, 134.15, 133.95, 133.32, 133.15, 132.69, 131.37, 131.34, 129.21, 128.85, 128.63, 128.56, 128.49, 128.38, 123.68, 122.32, 113.45, 112.67, 78.89, 43.02, 39.24, 32.04, 28.03, 1.02. ^{31}P NMR (162 MHz, $CDCl_3$) δ (ppm): -4.45. HRMS (ESI-TOF): calculated for C38H32N2O2P$^+$, [M+H]$^+$, m/z , 579.2201, found: 579.2208.

3. Spectrophotometric experiments

The probe **NR-HNO** was dissolved in DMSO to form a concentration of 2 mM solution as a stock solution. A certain amount of AS was placed in a volumetric flask and made up to volume with 0.01 M NaOH solution as a stock solution. This stock solution should be stored in a 4°C refrigerator and the concentration is determined by the absorption at 248 nm ($\varepsilon = 8.2\times10^3$ $M^{-1}\cdot cm^{-1}$). The following solutions (50 mM) were prepared in deionized water: amino acids (His, Asp, Val, Phe, Tyr, Ala, Ser, Leu, Arg, Pro, Thr, Trp, Lys), and ions (Na^+, Ag^+, Mg^{2+}, Cl^-, SO_3^{2-}, OAc^-, HCO_3^-, SCN^-, F^-). The other analytes were obtained by traditional methods. For spectroscopic measurement, the probe stock solution was diluted to 10 μM in 10 mM PBS buffer (containing 10% DMF) solutions of different pH values as needed, which was treated with testing species and then kept at 37 °C in an incubator for 12 min. After specified time, the fluorescence spectra were recorded. Unless otherwise stated, the excitation wavelength is 545 nm, and the excitation/emission slit widths are 10/10 nm for all measurements.

4. Determination of detection limits

According to the fluorescence titration data, a linear relationship between the fluorescence intensity (F 676 nm) and AS concentrations was observed, the detection limit was calculated with the following equation: Detection limit = 3σ/k, where σ is the standard deviation of blank measurements (n=10), k is the slop between the fluorescence intensity versus the concentrations of AS.

5. Cell culture and cytotoxicity experiments

Human breast cancer cells (MCF-7) cells, liver hepatocellular carcinoma (HepG2) and HeLa cells were purchased from Institute of Basic Medical Sciences (IBMS) of the Chinese Academy of Medical Sciences. HeLa and MCF-7 cells were cultured in 90% Dulbecco's Modified Eagle Medium (DMEM, Gibico) supplemented with 10% FBS (Gibico) and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Hyclone) in an atmosphere of 37 °C and 5% CO_2. The MTT method was employed to assess the cellular cytotoxicity of **NR-HNO**. Before experiments, HeLa and MCF-7 cells at a density of 1×104 cells/well were seeded into 96-well plates and cultured for 24 h. Then the fresh culture contained **NR-HNO** over a range of concentrations (0-25 μM) (n = 6) to substitute the previous media, and further incubation for 24 h. After that, 10 μL of MTT (5 mg/mL in PBS) was added into per well and incubated another 4 h. Finally, 100 μL of DMSO was then added to dissolve formazan. The absorbance at 490 nm was measured in a microplate reader, and the cell viability (%) was calculated according to the following equation: Viability (%) = [OD490 (sample) - OD490 (blank)] / [OD490 (control) - OD490 (blank)] × 100.

6. Fluorescence imaging of living cells and tissues

One day before imaging, the cells were detached and were replanted on glass-bottomed dishes and allowed to adhere for 24 hours. For imaging of exogenous HNO in living cells, the MCF-7 and HepG2 cells were stained with probe **NR-HNO** (10 μM) for 30 min, then washed three times with PBS and further incubated with free-FBS DMEM containing different concentration of AS. For inhibition tests, cells were treated with AS for 30 min and DMEM containing 1 mM N-acetylcysteine (NAC) for 1 hours, then incubated with **NR-HNO** (10 μM) for 30 min before imaging.

For tracking endogenous HNO that generated by the reaction of H_2S and NO, the MCF-7 cells were incubated with NR-HNO for 30 min under different conditions: (a) only Na_2S; (b) only SNP; (c) only **NR-HNO**; (d) in the presence of 10 μM **NR-HNO** and 500 μM Na_2S; (e) in the presence of 10 μM **NR-HNO** and 500 μM SNP; (f) in the presence of 10 μM **NR-HNO**, 500 μM Na_2S and 500 μM SNP; (g) the cells were pretreated with NAC, further incubated with 500 μM Na_2S and 500 μM SNP then with 10 μM **NR-HNO**. Then the cells were imaged with confocal laser scanning microscopy with a 561 nm laser.

For tissue imaging, the rat kidney tissue slices were pretreated with or without 100 μM AS, the incubated with probe for another 30 min before imaging. For detecting endogenous HNO in liver tissue slices, the slices were incubated with **NR-HNO** for 30 min under different conditions: (a-d) only probe **NR-HNO**; (e-h) in the presence of 10 μM **NR-HNO**, 1 mM Na_2S and 1 mM SNP; (i-l) the cells were pretreated with NAC, further incubated with 1 mM Na_2S and 1 mM SNP. Fluorescence imaging operations for tissue slices were similar as cellular ones. λ_{ex} = 561 nm, collected: 610-700 nm.

7. Fluorescence imaging in live mice

The BALB/c mice were purchased from Dalian Medical University. All the animal experiments were carried out in accordance with the relevant laws and guidelines approved by Dalian Medical

University Animal Care and Use Committee. For the in vivo fluorescence imaging experiments, the mice were anesthetized by intraperitoneal injection of 5% chloral hydrate, then given a subcutaneous injection of 1 mM AS, followed by the subcutaneous injection of 50 μM **NR-HNO**. After 30 min, the mice were imaging using NightOWL II LB983 small animal imaging system with an excitation filter of 590 nm and an emission filter of 700 nm.

8. The test spectra and images of NR-HNO

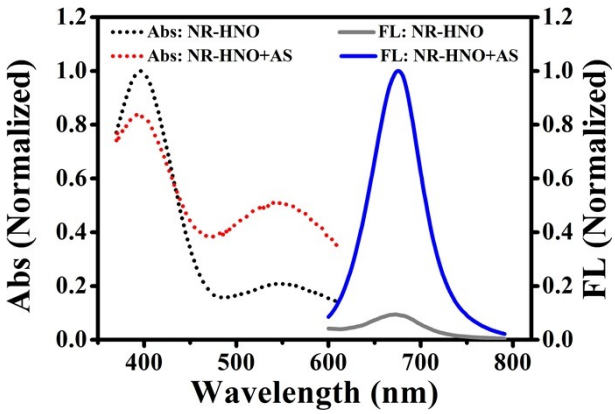


Figure S1 Normalized absorption and fluorescence spectra of **NR-HNO** (10 μM) in absence and presence of AS (90 μM) in PBS (pH = 7.4, 10 mM, 10% DMSO)

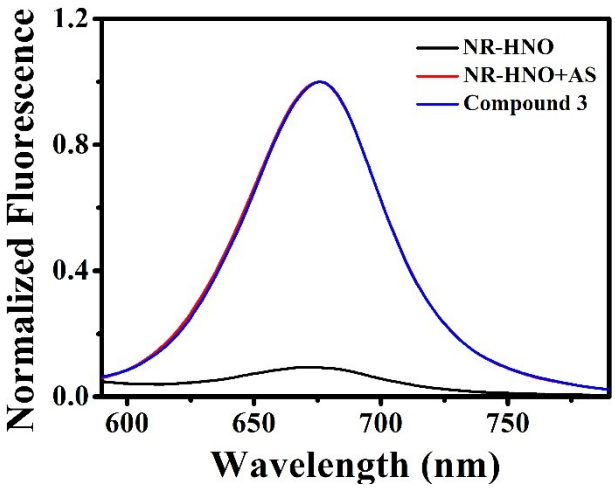


Figure S2 The fluorescence comparation of compound 3 and **NR-HNO** in presence and absence of AS (90 μM). λ_{ex} = 545 nm

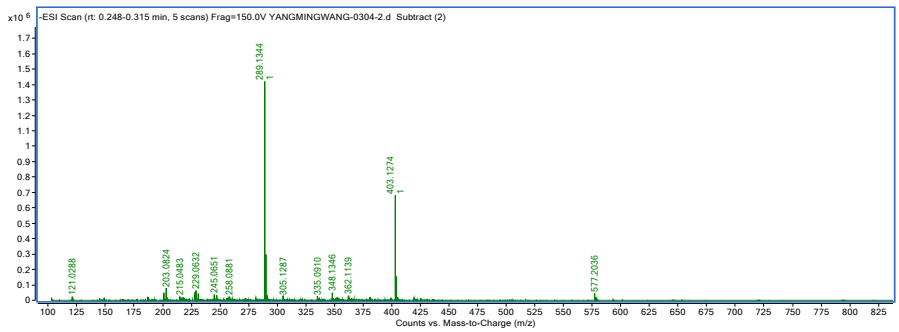


Figure S3 The HRMS of probe **NR-HNO** treated with AS.

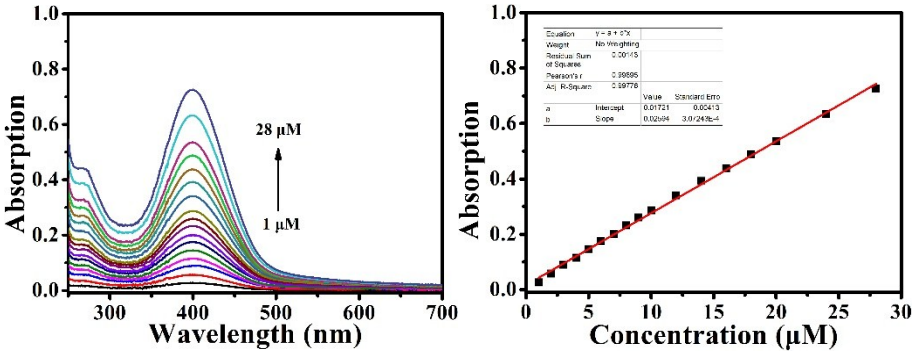


Figure S4 The absorption spectra of **NR-HNO** (1- 28 μM) in PBS and its linear relationship

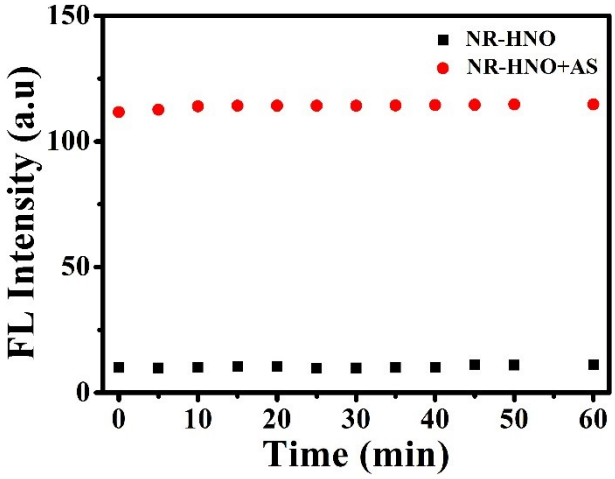


Figure S5 The stability of time dependence of **NR-HNO** in absence and presence of 90 μM AS. λ_{ex} = 545 nm

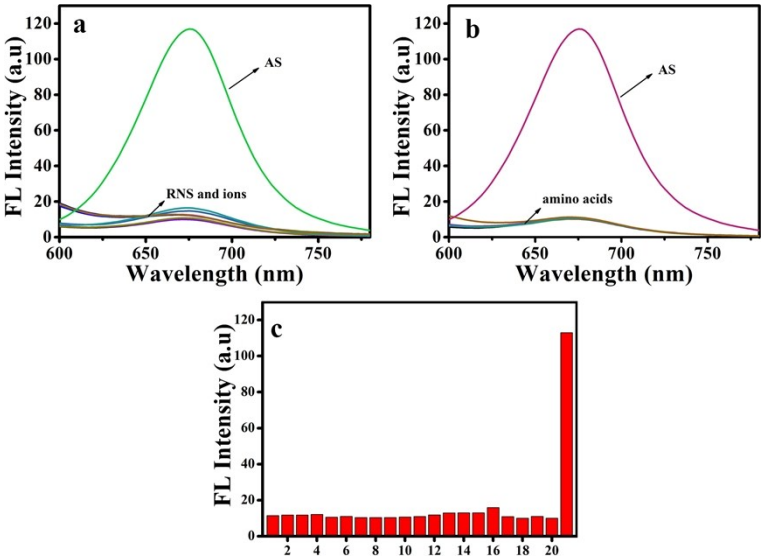


Figure S6 (a, b) The fluorescence spectra of **NR-HNO** to various RNS, ions and amino acids. (c) The fluorescence changes of probe with incubated with diffrent analytes for 9 min. 1, Ala; 2, Tyr; 3, Phe; 4, Val; 5, Asp; 6, His; 7, Trp; 8, Thr; 9, Pro; 10, Arg; 11, Leu; 12, Ser; 13, Ag^{+}; 14, Mg^{2+}; 15, Cl^{-}; 16, F^{-}; 17, Na^{+}; 18, AS. λ_{ex} = 545 nm

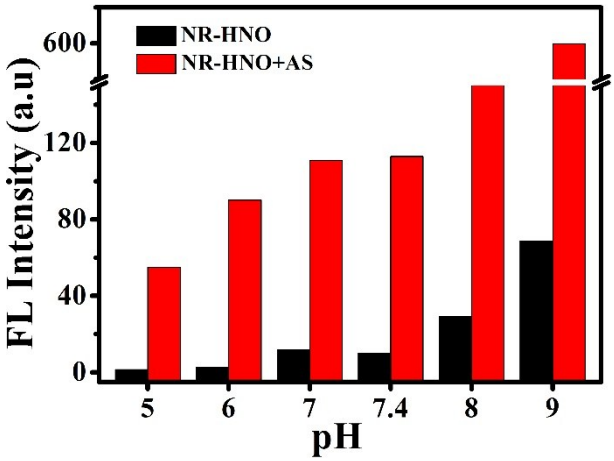


Figure S7 Effect of pH on the fluorescence of **NR-HNO** (10 μM) before and after reacting with AS (90 μM) in PBS (10 mM, 10% DMSO). λ_{ex} = 545 nm.

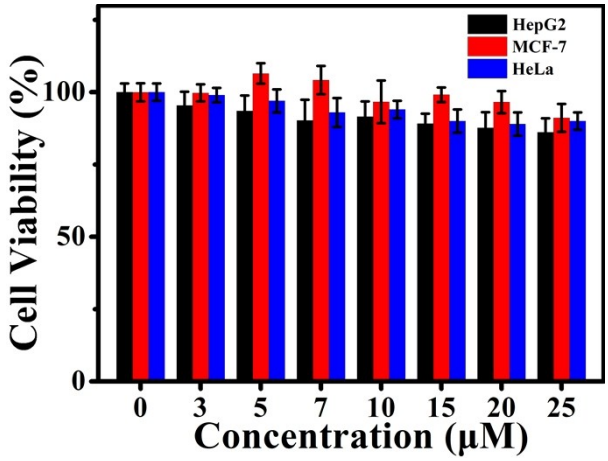


Figure S8 MTT assay of MCF-7, HepG2 and HeLa cells in different concentration of **NR-HNO** (0-25 μM) for 24 h

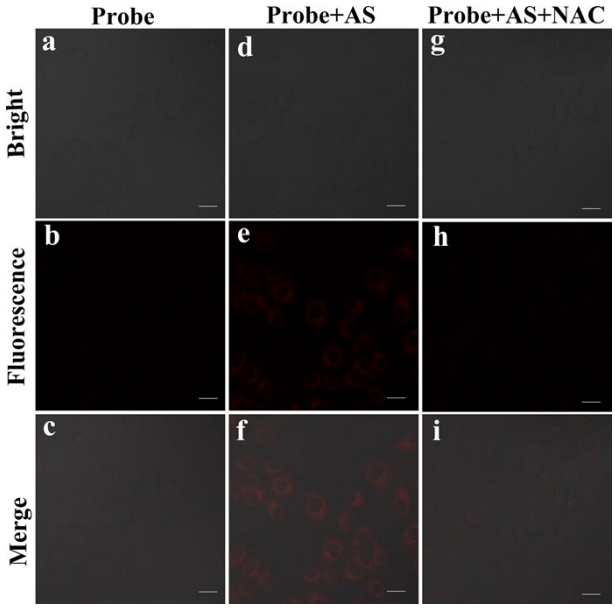


Figure S9 Confocal fluorescence images for detecting exogenous HNO in HepG2 cells. The cells were incubated with **NR-HNO** (10 μM) for 30 min firstly, then incubated with (a-c) 0 μM; (d-f) 30 μM AS for 30 min; (g-i) Cells were pretreated with AS (30 μM) and NAC (1 mM) sequentially, then with **NR-HNO** (10 μM) for 30min before imaging. λ_{ex} = 561 nm. Scale bar: 20 μm.

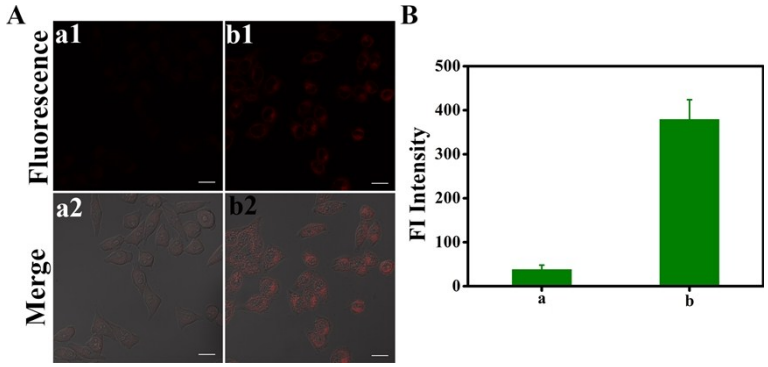


Figure S10 (a)The MCF-7 cells incubated with only probe. (b) The cells pretreated with 2 mM NaASc and 500 μM DEA NONOate, then with 10 μM probe. (B) Relative fluorescence intensities output of group (A). λ_{ex} = 561 nm, collected: 610-700 nm. Scale bar: 20 μm.

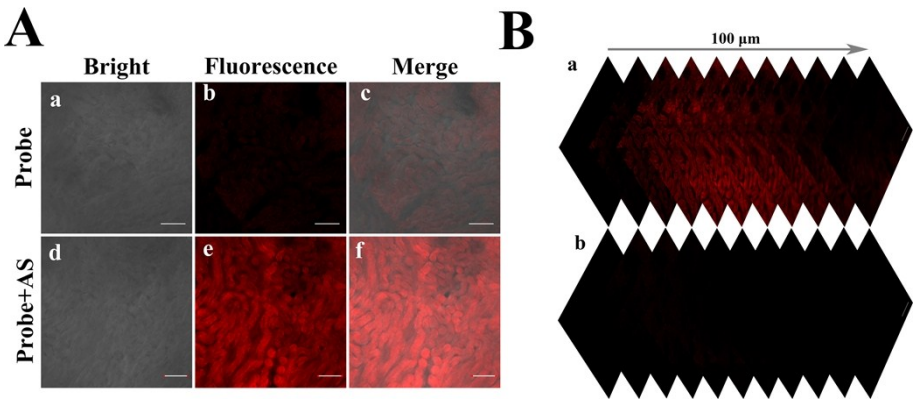


Figure S11 Confocal fluorescence imaging of mouse kidney slices. (A) The tissue was stained with **NR-HNO** (10 μM) for 30 min in the absence or presence of 100 μM AS. (B) The fluorescence imaging of slices at different depth (0-100 μm) and conditions corresponding to (A). λ_{ex} = 561 nm, collected: 610-700 nm. Scale bar: 100 μm.

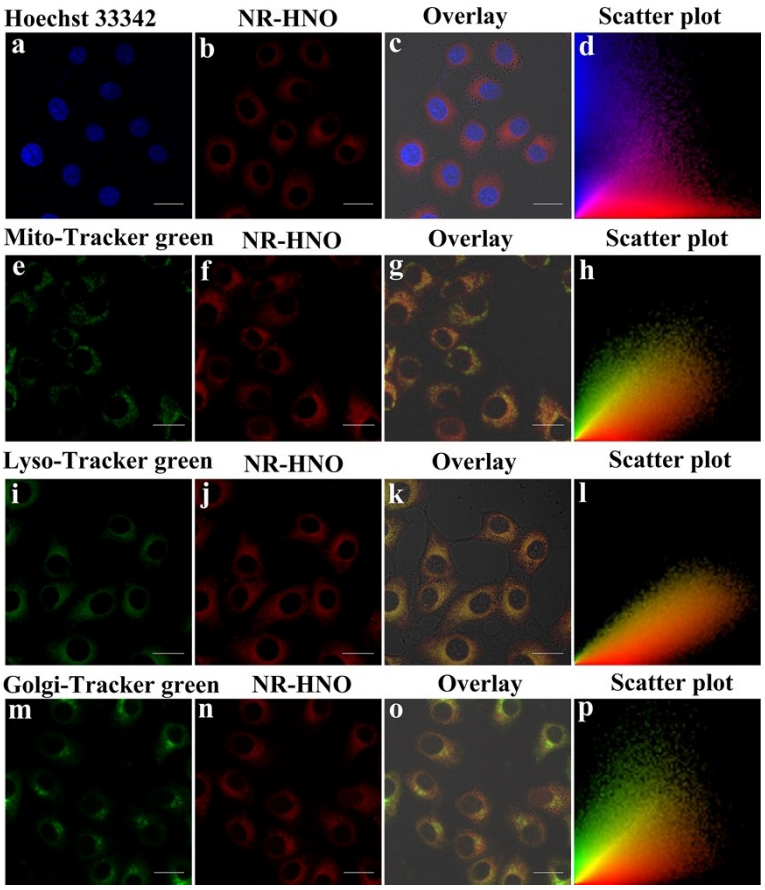


Figure S12 The colocalization imaging of MCF-7 cells stained with **NR-HNO** (10 μM, λ_{ex} = 561 nm, collected: 610-700 nm) with organelle dyes, including Hoechst 33342 (500 nM, λ_{ex} = 405 nm, collected 420-480 nm); Mito-Tracker Green (100 nM, λ_{ex} = 635 nm, collected 640-690 nm); Lyso-Tracker Green (100 nM, λ_{ex} = 559 nm, collected 580-610 nm); Golgi-Tracker Green (500 nM, λ_{ex} = 559 nm, collected 590-640 nm). Scale bar: 20 μm

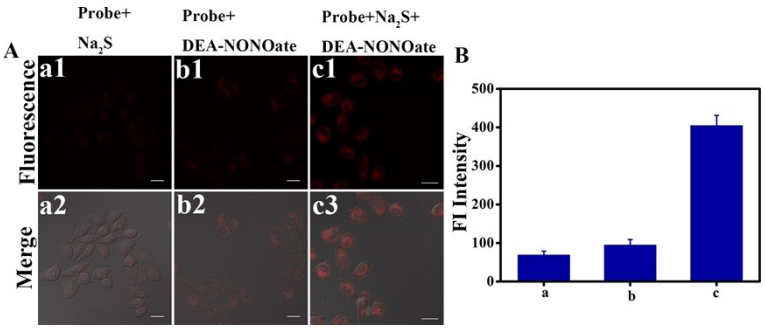


Figure S13 The fluorescence images of H_2S/NO crosstalk in MCF-7 cells using **NR-HNO** (10 μM) under different conditions. (a) in the presence of 10 μM **NR-HNO** and 500 μM Na_2S; (b) in the presence of 10 μM **NR-HNO** and 500 μM DEA-NONOate; (d) in the presence of 10 μM **NR-HNO**, 500 μM Na_2S and 500 μM SNP. (B) Relative fluorescence intensities output of group (A).λ_{ex} = 561 nm, collected: 610-700 nm. Scale bar: 20 μm.

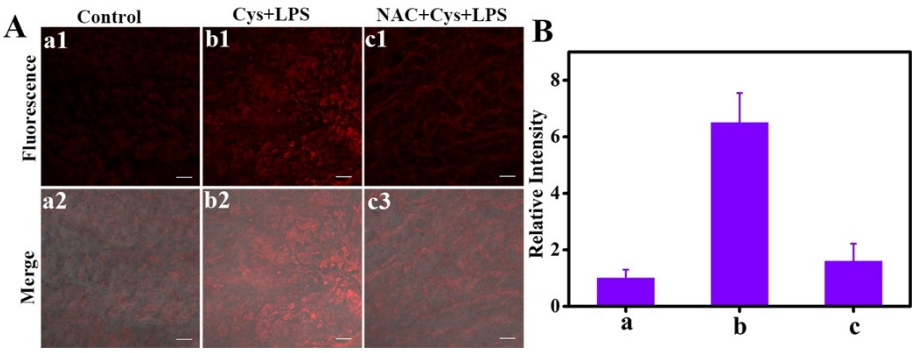
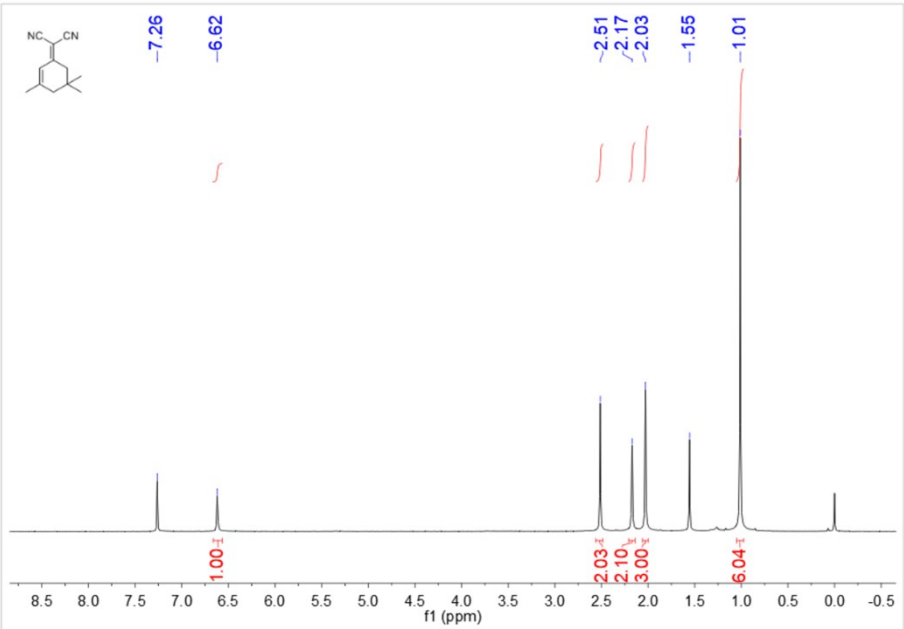
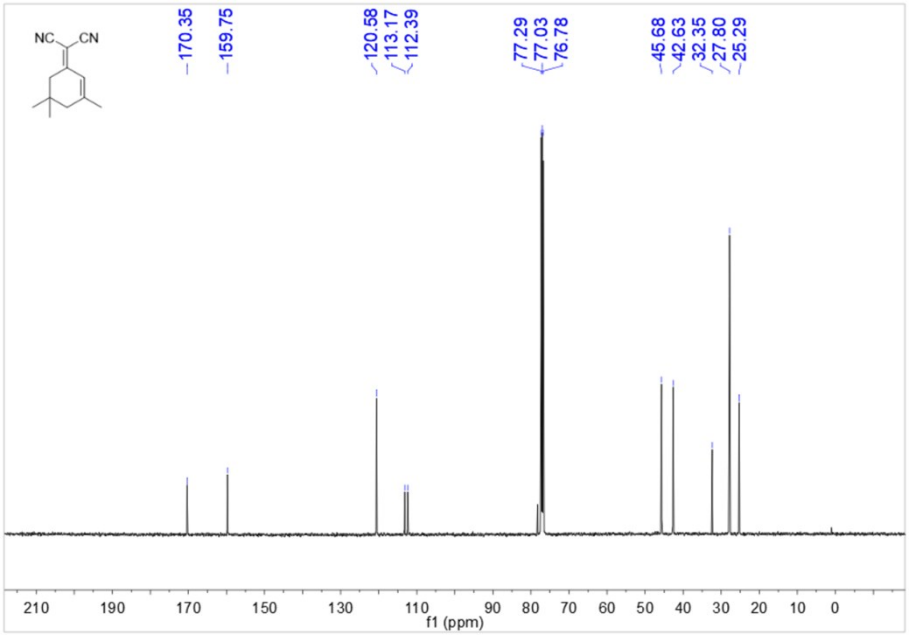


Figure S14 The fluorescence images of endogenous H_2S/NO crosstalk in tumor tissue using **NR-HNO** (a) only probe **NR-HNO**; (b) in the presence of **NR-HNO**, Cys and LPS; (c) the tumor were pretreated with NAC, further with Cys and LPS then with probe; (B) Relative intensity of group A. λ_{ex} = 561 nm, collected: 610-700 nm. Scale bar: 20 μm.

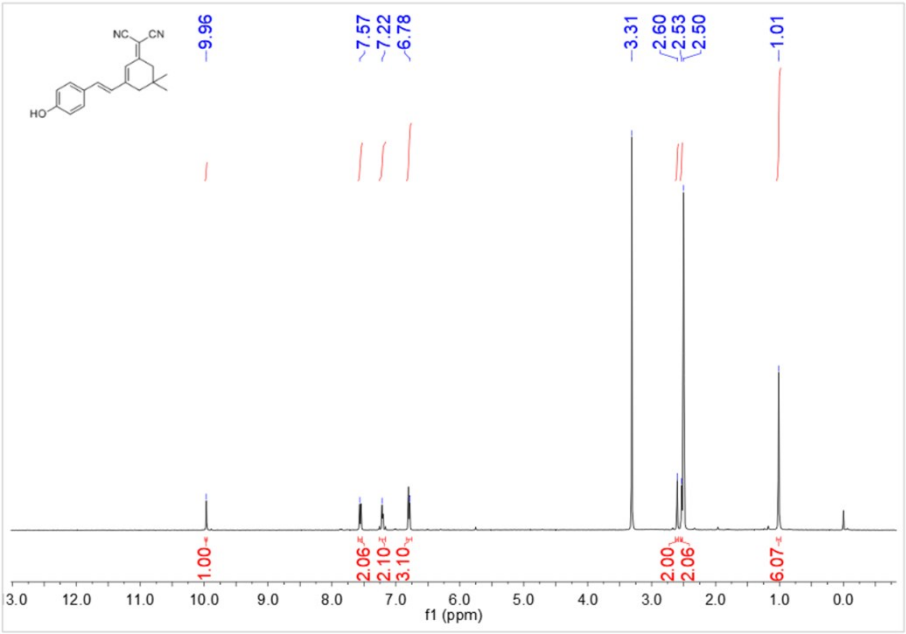
9. The NMR and HR-MS spectra of all compounds.



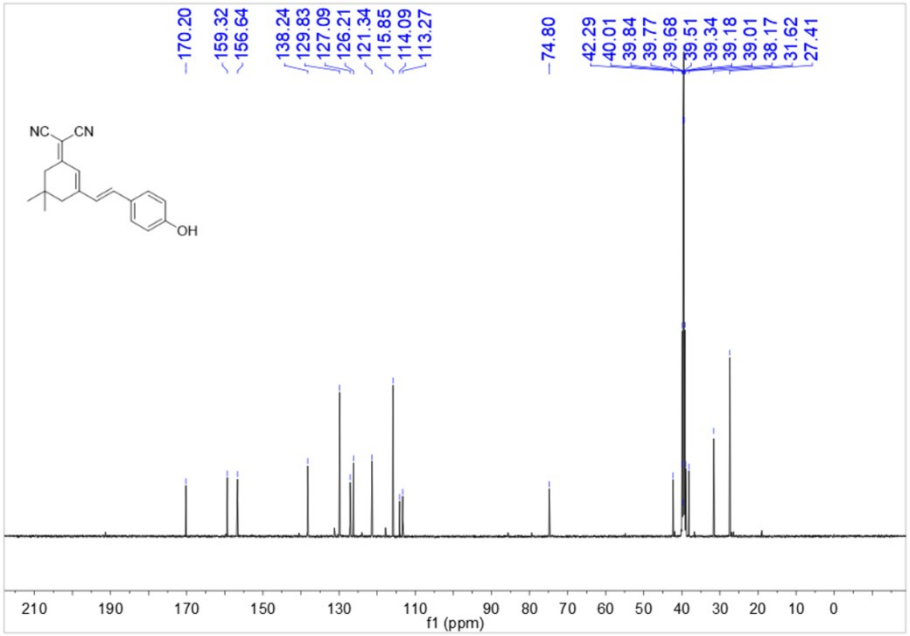
^{1}H NMR of compound **2** in $CDCl_3$



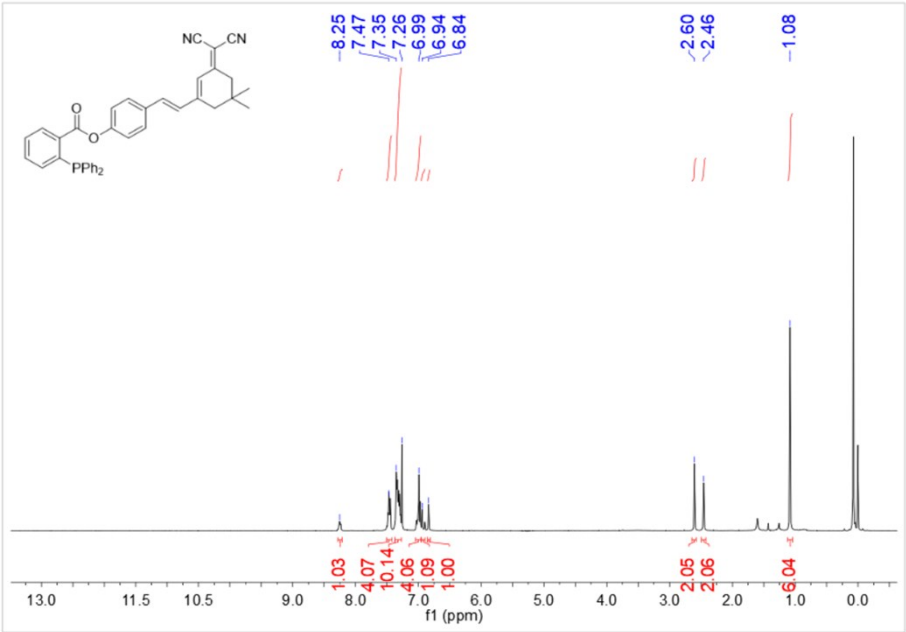
^{13}C NMR of compound **2** in $CDCl_3$



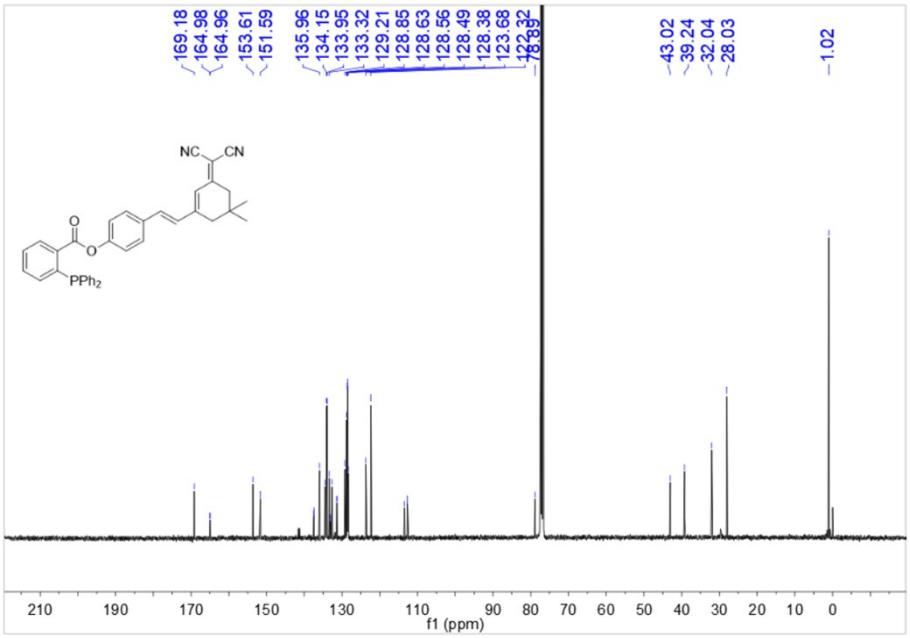
^{1}H NMR of compound **3** in DMSO-d_6



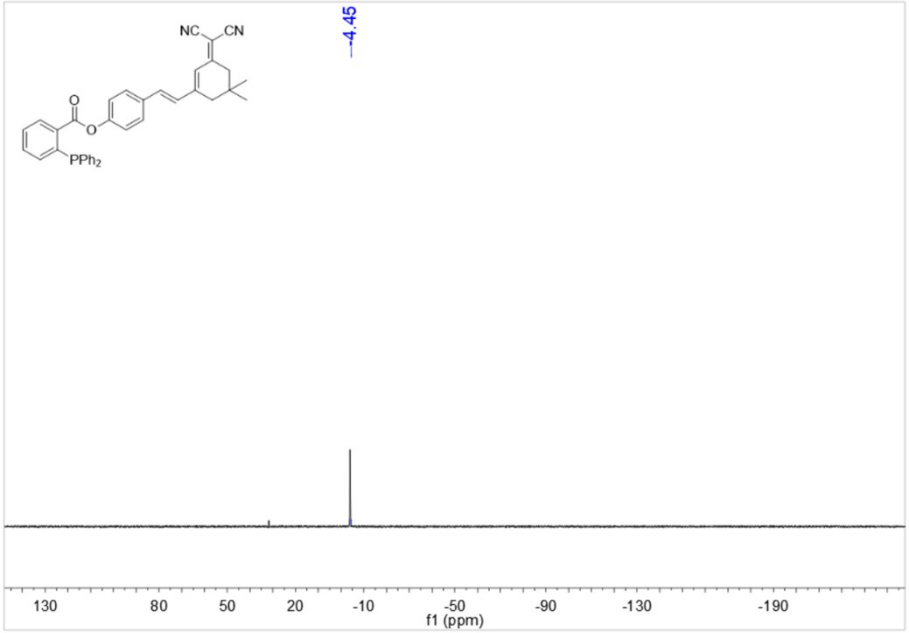
^{13}C NMR of compound **3** in DMSO-d_6



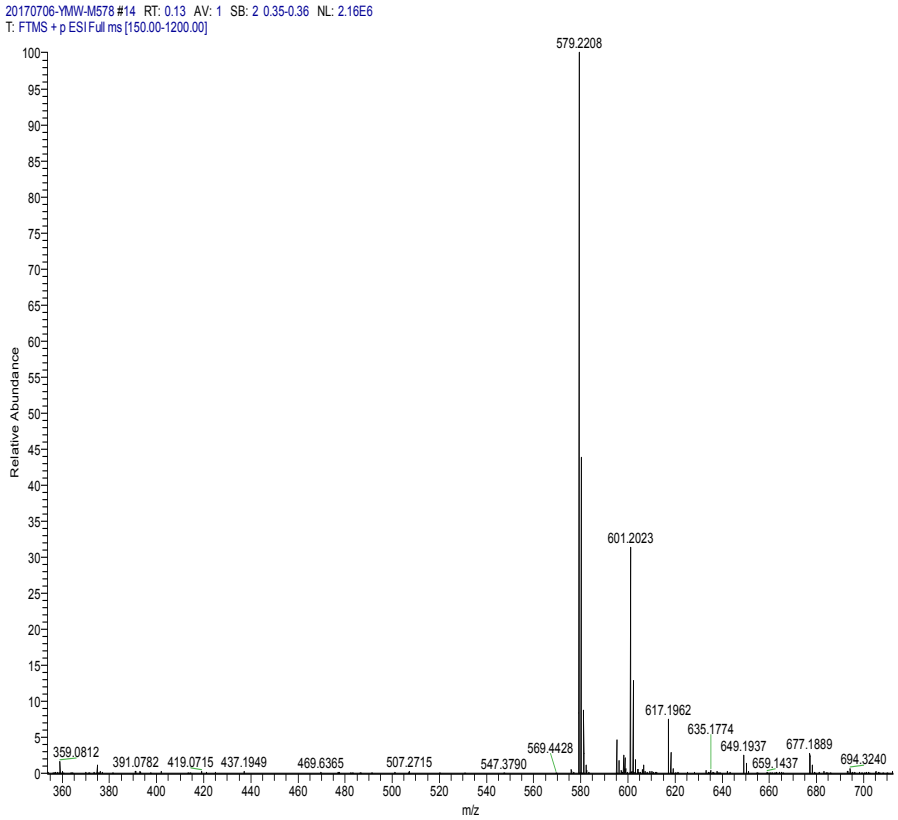
^{1}H NMR of probe **NR-HNO** in $CDCl_3$



^{13}C NMR of probe **NR-HNO** in $CDCl_3$



^{31}P NMR of probe **NR-HNO** in $CDCl_3$



HRMS of probe **NR-HNO**