Supporting Information for

Engineering of a Bioluminescent Probe for Imaging Nitroxyl in Live Cells and Mice

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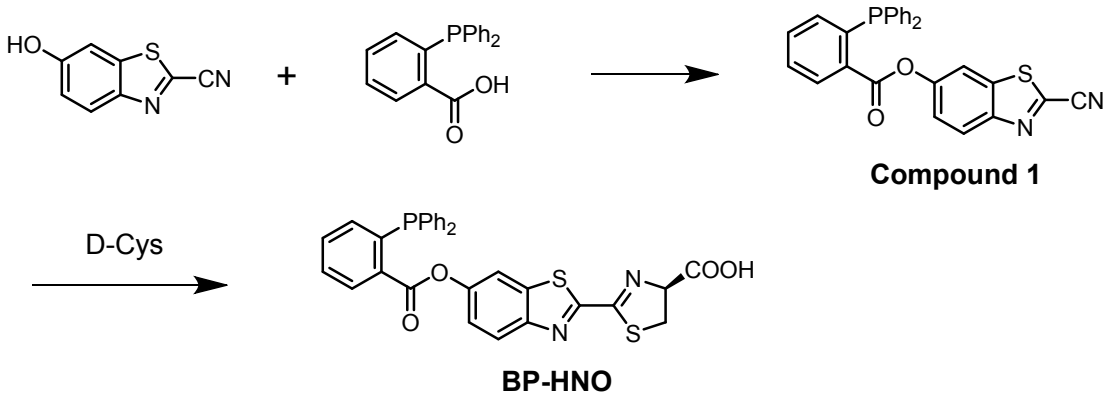
Reagents and Apparatus

All chemicals were obtained from Sigma-Aldrich or Aladdin. Ultrapure water was purified by a Milli-Q reference system (Millipore) and was used to prepare all solutions. A stock solution of probe **BP-HNO** (10.0 mM) was obtained by dissolving an appropriate amount of probe into DMSO. Human serum samples were collected from healthy people and stored at -20 °C before use. Luminescence emission spectra were obtained using a HORIBA Fluoromax-4 spectrofluorometer (JobinYvon) with the Xe lamp shut off and emission slits set at 10.0 nm. UV-vis absorption spectra were performed on a Shimadzu UV-2450 spectrophotometer. Thin layer chromatography (TLC) analysis was performed on silica gel plates, and the compounds were isolated by column chromatography on silica gel (200-300 mesh) columns, both the silica gel and plates were obtained from Yantai Jiangyou Silica Gel Development Co., Ltd. (Shandong, China). High-performance liquid chromatography (HPLC) analyses were conducted over an Agilent 1200 HPLC system, and the eluent were CH_3CN (0.1% of trifluoroacetic acid (TFA)) and water (0.1% of TFA). ^{1}H NMR, ^{31}P NMR and ^{13}C NMR spectra were plotted on a Bruker DRX-400 spectrometer (Bruker). Mass spectra were obtained on a LCQ Advantage ion trap mass spectrometer (Thermo Finnigan). The bioluminescent images were plotted using an IVIS Lumina XR Imaging System (Caliper, U.S.A.) with a cooled charge coupled device (CCD) camera equipped. Circular ROIs were drawn over the areas and signals were quantified by Lumina XR Living Image software, version 4.3.

Synthesis of Compound 1 and probe BP-HNO

Compound 1 and probe **BP-HNO** were synthesized according to previous literatures.[1] The synthetic route is as follows：



Synthesis of Compound 1. A mixture of 2-(diphenylphosphino) benzoic acid (153.0 mg, 0.5 mmol), EDCI (96.0 mg, 0.5 mmol) and DMAP (31.0 mg, 0.25 mmol) was dissolved in dry DCM (10 mL), and the reaction solution was stirred at room temperature for 30 min. Compound 6-hydroxy-benzothiazole-2-carbonitrile (88.0 mg, 0.5 mmol) was then added to the mixture, and the reaction was stirred at room temperature overnight. The mixture was concentrated under reduced pressure, and the residue was purified by silica gel chromatography to yield a white solid (155.5 mg, 67% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 – 8.28 (m, 1 H), 8.17 (d, J = 9.0 Hz, 1 H), 7.58 (d, J = 1.9 Hz, 1 H), 7.55 – 7.49 (m, 2 H), 7.42 – 7.30 (m, 10 H), 7.25 (dd, J = 9.0, 2.1 Hz, 1 H), 7.08 – 7.02 (m, 1 H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.89, 164.87, 150.64, 149.96, 141.73, 141.46, 137.25, 137.15, 136.62, 135.98, 134.61, 134.17, 133.96, 133.03, 132.74, 132.56, 131.49, 131.47, 129.00, 128.73, 128.66, 128.50, 125.77, 122.84, 114.61, 112.78. ^{31}P NMR (162 MHz, $CDCl_3$) δ -3.76. MS (ESI): $[M+H]^+$ found, 465.1; calcd for $C_{27}H_{18}N_2O_2PS^+$, 465.1.

Synthesis of BP-HNO. Compound 1 (46.4 mg, 0.1 mmol) was dissolved in 2 mL of DCM/MeOH (1:1, v/v) and the mixture of D-cysteine hydrochloride (D-Cys, 35.1 mg, 0.20 mmol) and potassium carbonate (27.6 mg, 0.20 mmol) dissolved in 2 mL of H_2O/MeOH (1:1, v/v) was added. The reaction was stirred vigorously for 10 min at room temperature. The organic solutions were removed under reduced pressure, and then 0.1 M aqueous HCl solution was added until the pH became 2-3. The precipitate was filtered off and washed with ice water. The crude material was dried in vacuo then purified by silica gel chromatography to provide **BP-HNO** as a white solid (23.3 mg, 41% yield). ^{1}H NMR (400 MHz, DMSO) δ 8.29 (dd, *J* = 8.5, 4.1 Hz, 1 H), 8.18 (d, *J* = 8.9 Hz, 1 H), 7.90 (d, *J* = 2.1 Hz, 1 H), 7.67 – 7.62 (m, 2 H), 7.40 (d, *J* = 3.7 Hz, 6 H), 7.27 (dd, *J* = 8.9, 2.3 Hz, 1 H), 7.22 (dd, *J* = 9.2, 5.5 Hz, 4 H), 6.95 (dd, *J* = 8.5, 4.2 Hz, 1 H), 5.45 (t, *J* = 9.1 Hz, 1 H), 3.77 (dt, *J* = 11.0, 6.8 Hz, 2 H). ^{13}C NMR (101 MHz, DMSO) δ 172.49, 171.54, 165.18, 164.65, 161.74, 151.01, 149.50, 140.94, 140.67, 137.59, 137.48, 136.42, 134.39, 134.11, 133.90, 133.62, 133.29, 133.10, 131.70, 129.49, 129.31, 129.23, 125.18, 122.30, 116.26, 78.81, 35.39. ^{31}P NMR (162 MHz, DMSO) δ -5.83. MS (ESI): $[M+H]^+$ found, 569.0; calcd for $C_{30}H_{22}N_2O_4PS_2^+$, 569.1. HRMS (ESI): $[M-H]^-$ found, 567.0602; calcd for $C_{30}H_{20}N_2O_4PS_2^-$, 567.0680.

Spectrophotometric Experiments

Both the luminescence and UV-vis absorption measurements were performed in PBS (10 mM, pH 7.4) with 10 mM $MgCl_2$ at 37 °C. A 20 μM final concentration solution of **BP-HNO** in PBS (10 mM, pH 7.4) was prepared by diluting a 10.0 mM DMSO stock solution of **BP-HNO** into prewarmed PBS (37 °C) in a tube. Angeli's

salt (AS) was stored at -20 °C. Stocks of AS were added as a 10 mM solution in 10 mM NaOH (for stability). AS solution was added into the tube. The luminescence emission spectra (λ_{em} = 450-700 nm) were immediately collected at 50 μL of luciferase (10 μg/mL) containing an added 2 mM ATP, with emission slits set at 10 nm.

***In Vitro* BL Measurements**

The **BP-HNO** solution was diluted to a final concentration of 20 μM in PBS (10 mM, pH 7.4) with 10 mM $MgCl_2$ and incubated for 30 min at room temperature with various concentrations of AS. Subsequently, 50 μL of luciferase containing 2 mM ATP was added. The BL was immediately measured at auto integration time. Circular ROIs were drawn over the each well, and luminescent signals were quantified by Living Image software, version 4.3.

MTT Cytotoxicity Assay

The cytotoxicity of **BP-HNO** to MDA-MB-231 cells was determined by MTT assays (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide). In 96-well plates, MDA-MB-231 cells were seeded at 1×10^5 cells per well and grown for 24 h. Then, these cells were incubated with various concentrations of **BP-HNO** (0, 12.5, 25, 50, 100, 200 μM) and cultured for a further 24 h or 48 h. Next, 20 μL of MTT solution was added to each well. After 4 h of incubation at 37 °C, the medium was removed and 100 μL of dimethyl sulfoxide was added to fully dissolve the crystals. The absorbance at 570 nm was recorded by a microplate reader (BioTek). 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.

Living Cell BL Imaging

All living cells and animal operations were conformed to the regulations of cells and animal use and care, according to protocol No. SYXK (Xiang) 2008-0001, approved by the Laboratory Animal Center of Hunan. The MDA-MB-231 cells were obtained from the biomedical engineering center of Hunan University (Changsha, China). And the MDA-MB-231 cells expressing firefly luciferase were obtained by fLuc-transfected MDA-MB-231 cells. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere in a 5% CO_2 incubator. We treated cells in black 96-well plates (4×10^5 cells per well). After 24 h incubation period, cells were treated with various concentrations of AS (0, 50, 100 μM). Then, the same concentrations of **BP-HNO** (20 μM) were added to each well，the BL and fluorescence intensity was measured using an IVIS Lumina XR Imaging System. Circular ROIs were drawn over the each well, and luminescent signals were quantified by Living Image software.

***In Vivo* BL Imaging**

Mice were purchased from Hunan SJA Laboratory Animal Co.，Ltd. (Changsha, China). To generate tumour xenografts in mice, a total of 2×10^6 fLuc-transfected MDA-MB-231 were subcutaneously implanted into each nude mouse. The mice were single or group housed on a 12:12 light-dark cycle at 22 °C with free access to food and water. Mice bearing fLuc-transfected MDA-MB-231 subcutaneous tumours were

anesthetized with isoflurane and intratumorally injected with 25 μL of **BP-HNO** at 100 μM, or 25 μL of AS at 1 mM followed by 25 μL of **BP-HNO** at 100 μM in saline. Subsequently, the anesthesia mice were imaged with an IVIS Spectrum. The pseudocolored BL images (in photons/s/cm^2/steradian) were superimposed over the gray scale photographs of the animals. Circular ROIs were drawn over the tumour and quantified by Living Image software. The results were reported as total photon flux within an ROI in photons per second.

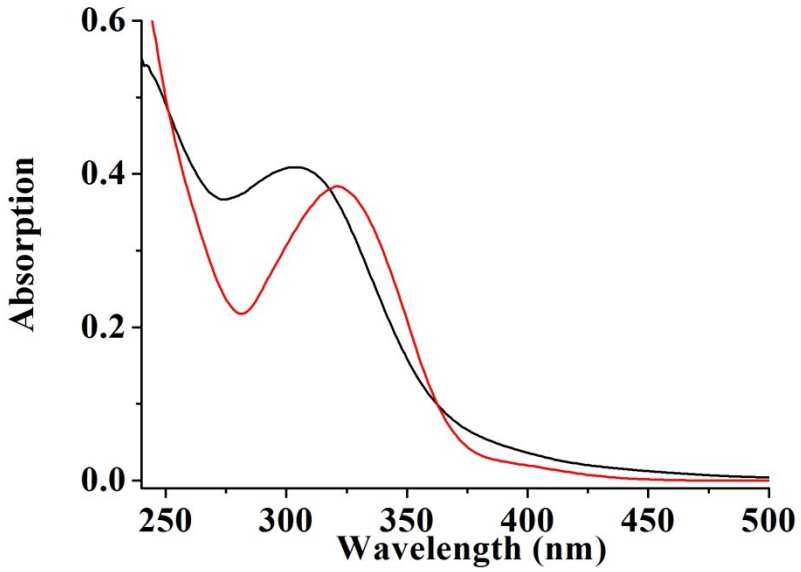


Fig. S1 UV-vis spectra of **BP-HNO** (black) and the reaction product (red) of **BP-HNO** with AS after incubation of them for 30 min at 37 °C in PBS (10 mM, pH 7.4).

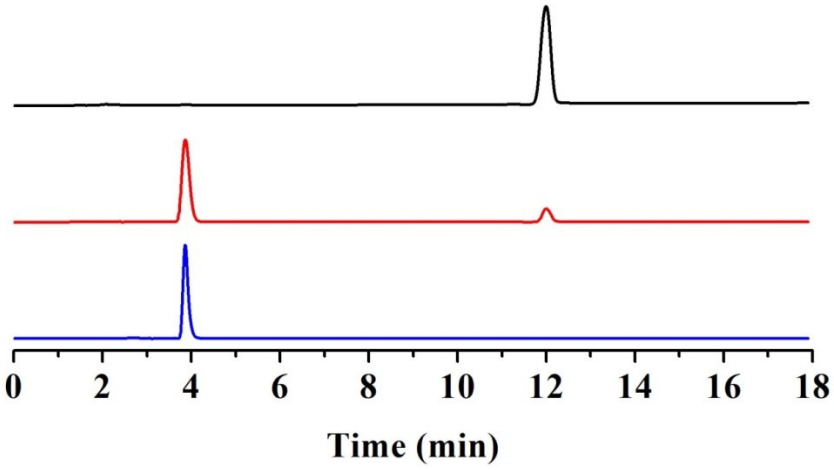


Fig. S2 HPLC traces of **BP-HNO**, D-luciferin, and reaction product of **BP-HNO** with HNO after incubation of them for 30 min at 37 °C in PBS (10 mM, pH 7.4) solution. The black, blue, and red lines represent **BP-HNO**, D-luciferin, and the reaction product of **BP-HNO** with HNO, respectively. Wavelength for detection: 320 nm.

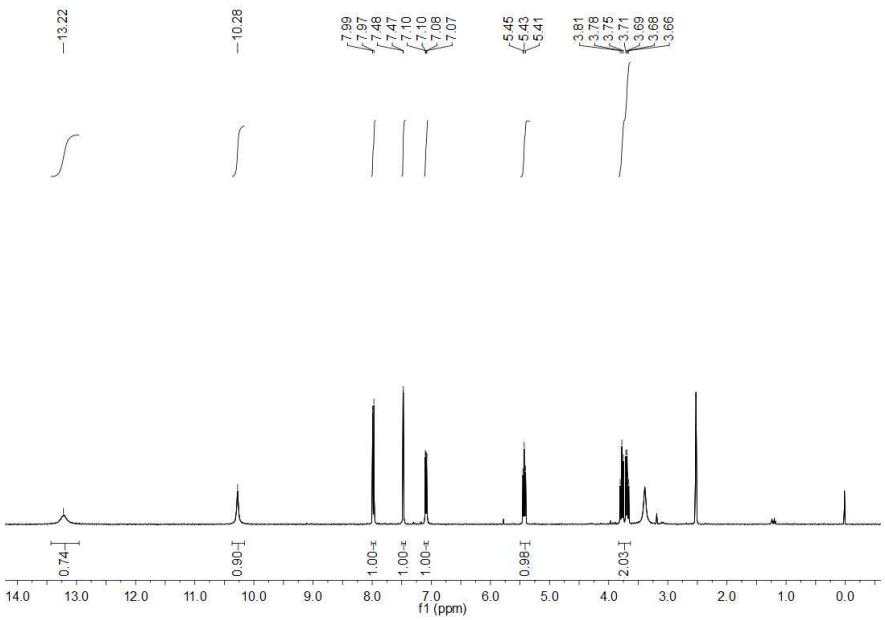


Fig. S3 ^{1}H NMR spectrum of **BP-HNO** with HNO. ^{1}H NMR spectrum showing D-luciferin was produced by the reaction of **BP-HNO** with HNO. ^{1}H NMR (400 MHz, DMSO) δ 13.22 (s, 1H), 10.28 (s, 1H), 7.98 (d, J = 8.9 Hz, 1H), 7.47 (d, J = 2.3 Hz, 1H), 7.09 (dd, J = 8.9, 2.3 Hz, 1H), 5.49 – 5.33 (m, 1H), 3.83 – 3.63 (m, 2H).

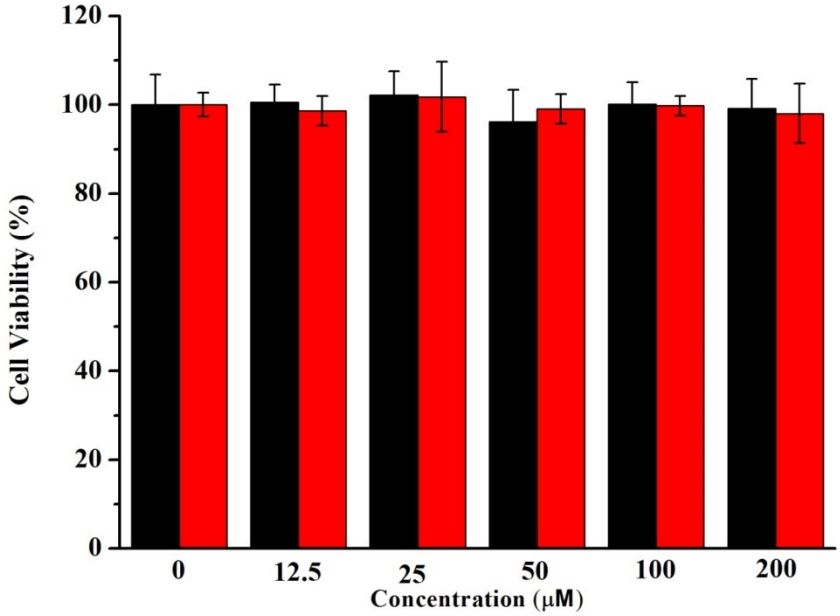


Fig. S4 MTT assay of probe **BP-HNO** on MDA-MB-231 cells (non-luciferase transfected). The cells were incubated for 24 h (black bar) or 48 h (red bar). The results are the mean ± standard deviation of five separate measurements.

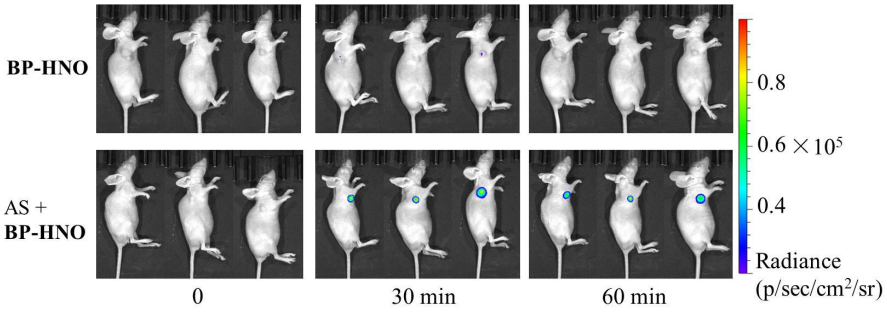


Fig. S5 Time-course BL imaging of HNO in nude mice. Mice were xenografted with fLuc-transfected MDA-MB-231 tumour and intratumorally injected with 25 μL of **BP-HNO** at 100 μM (top row), 25 μL of AS at 1 mM followed by 25 μL of **BP-HNO** at 100 μM (bottom row) in saline at 0, 30, and 60 min.

Table S1. HPLC condition.

Time (min)	Flow (mL/min)	H_2O % (0.1 % TFA)	CH_3CN % (0.1 % TFA)
0	1.0	50	50
3	1.0	50	50
35	1.0	10	90
37	1.0	10	90
38	1.0	50	50
40	1.0	50	50

References

1. A. S. Cohen, E. A. Dubikovskaya, J. S. Rush and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2010, **132**, 8563-8565.

NMR spectrum and MS of all the new compounds

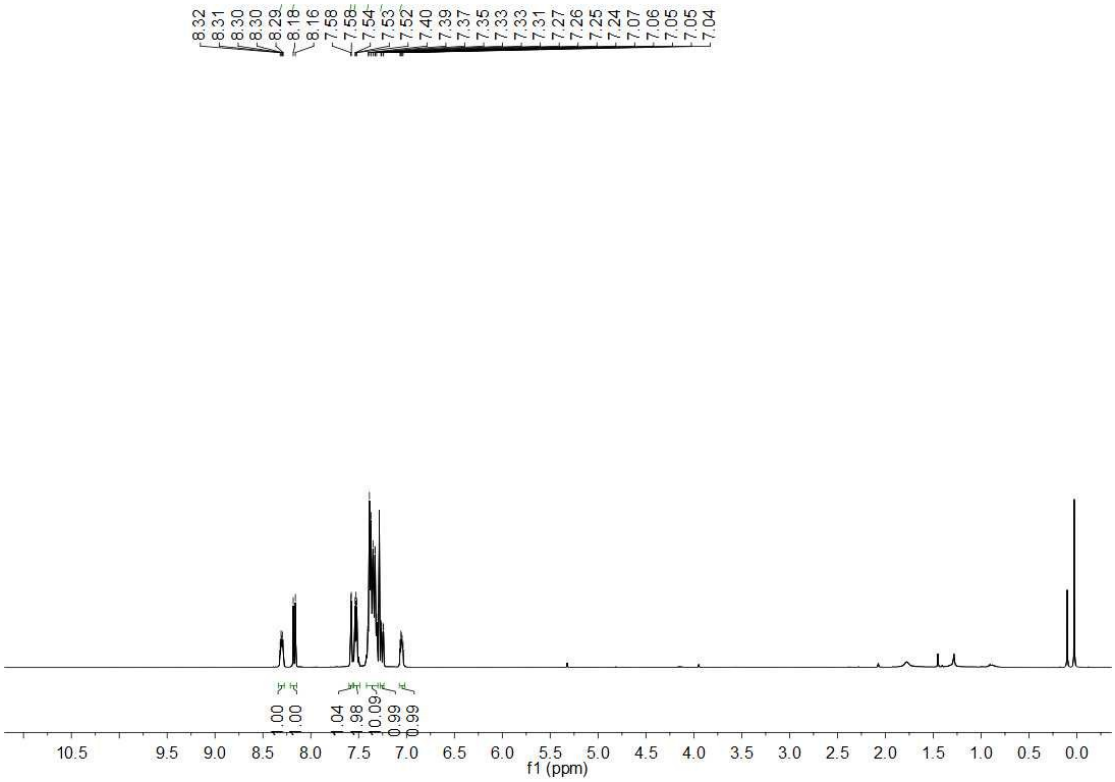


Fig. S6 ^{1}H NMR spectrum of the compound **1**

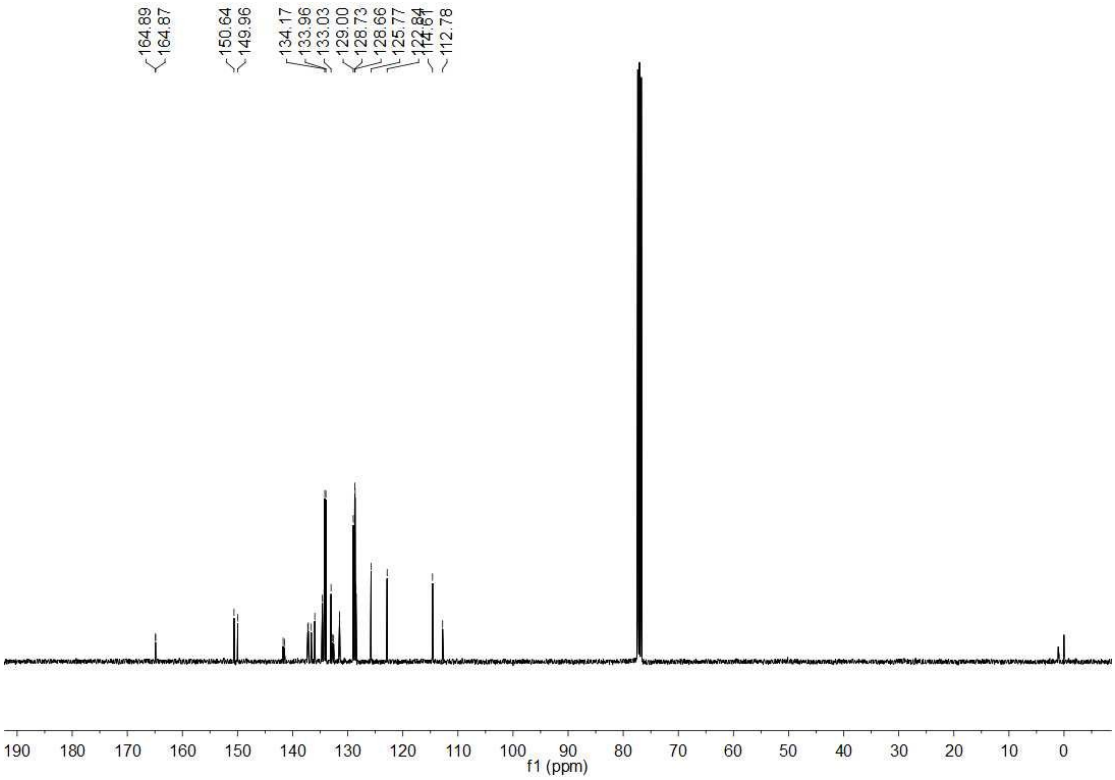


Fig. S7 ^{13}C NMR spectrum of the compound **1**

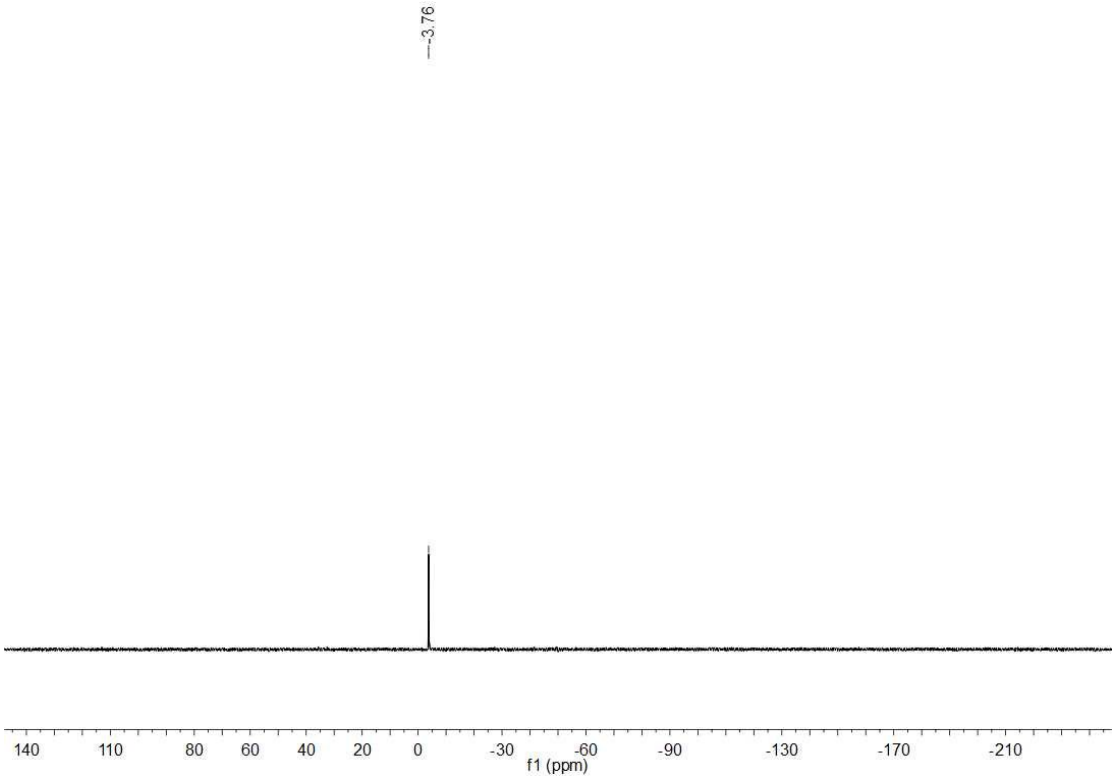


Fig. S8 ^{31}P NMR spectrum of the compound **1**

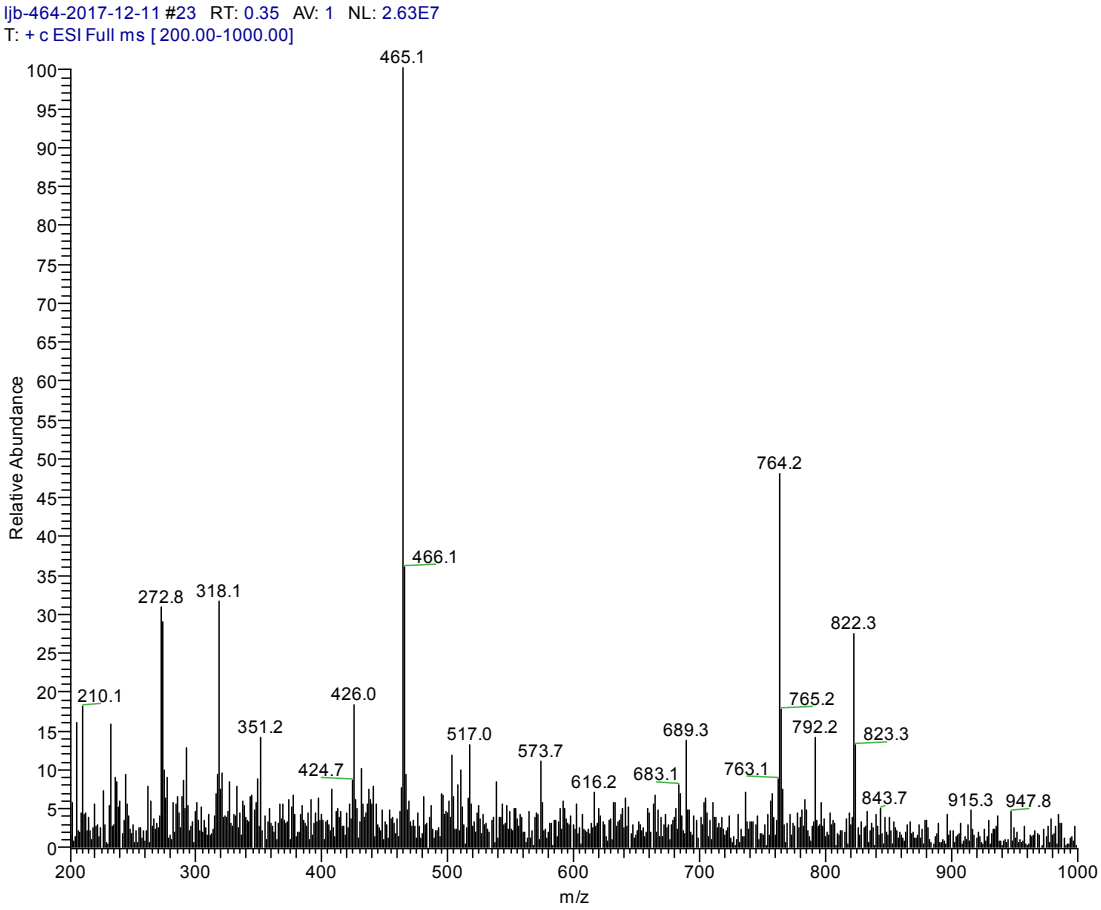


Fig. S9 ESI mass spectrum of the compound **1**

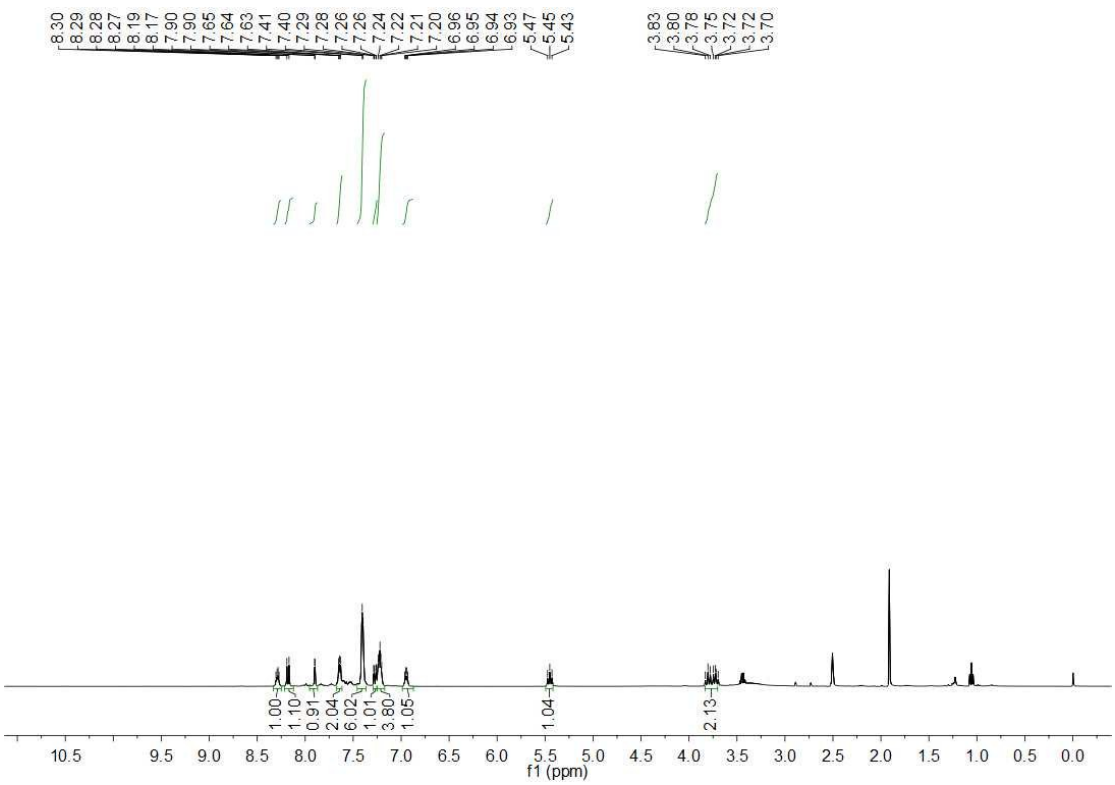


Fig. S10 ^{1}H NMR spectrum of the compound **BP-HNO**

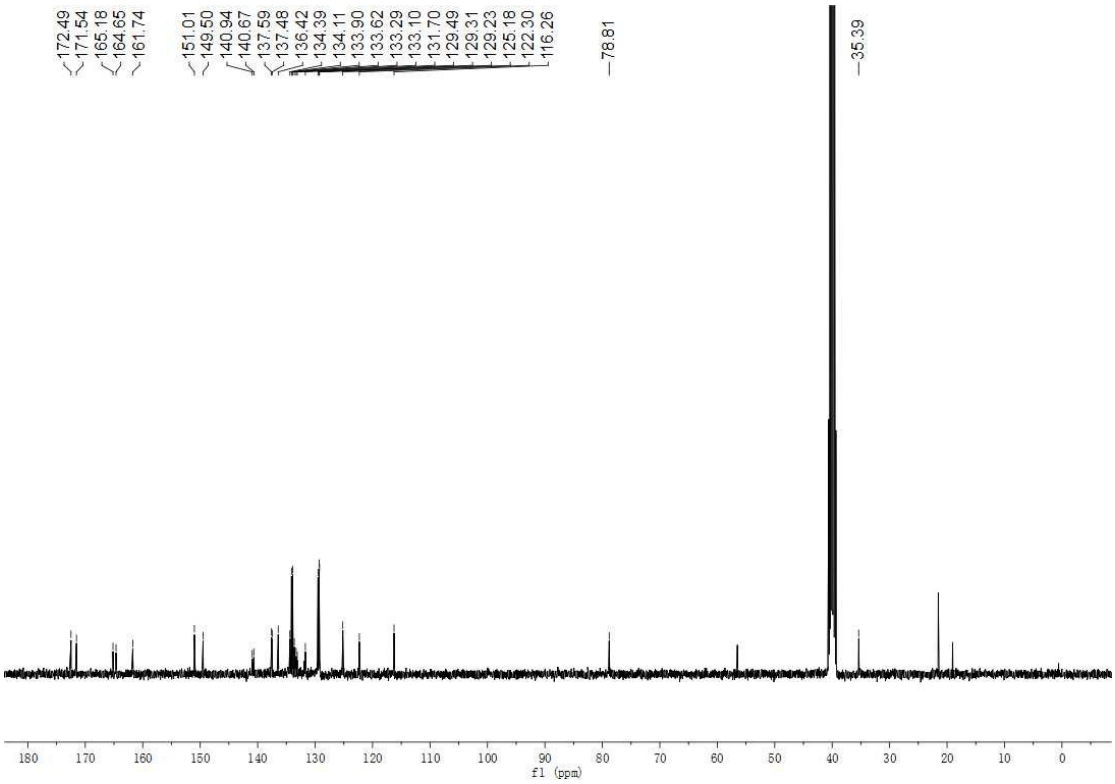


Fig. S11 ^{13}C NMR spectrum of the compound **BP-HNO**

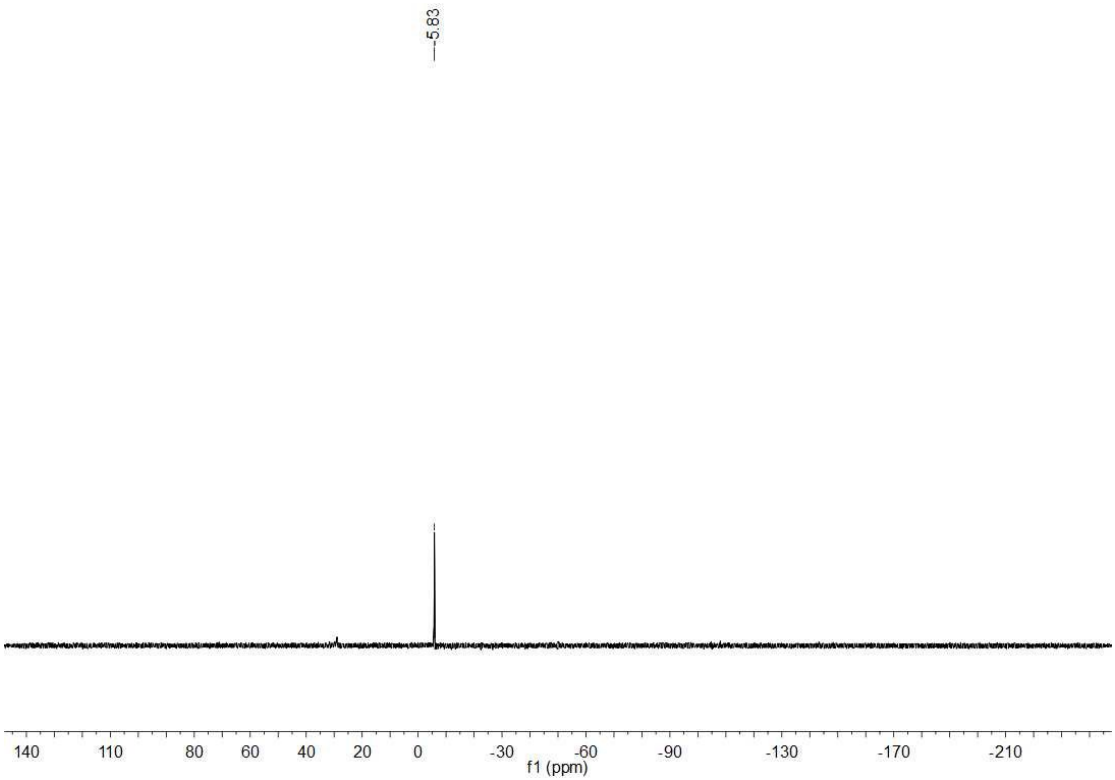


Fig. S12 ^{31}P NMR spectrum of the compound **BP-HNO**

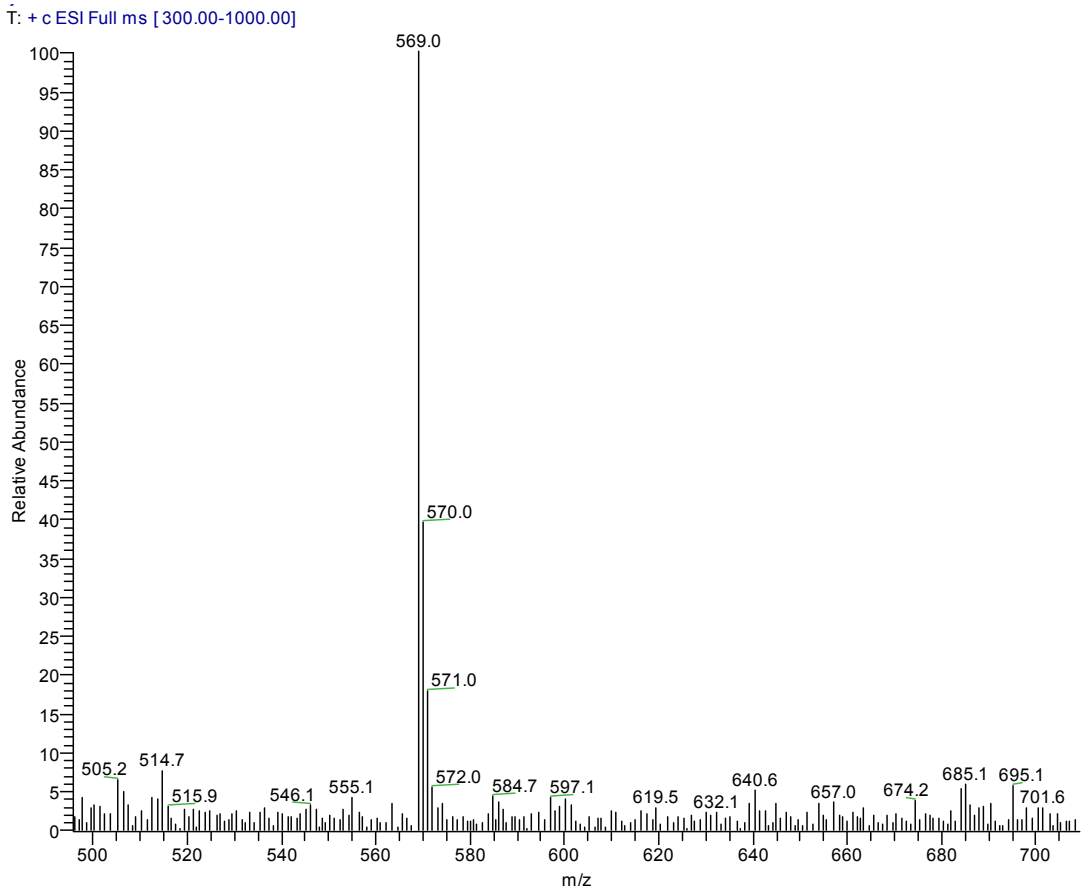


Fig. S13 ESI mass spectrum of the compound **BP-HNO**

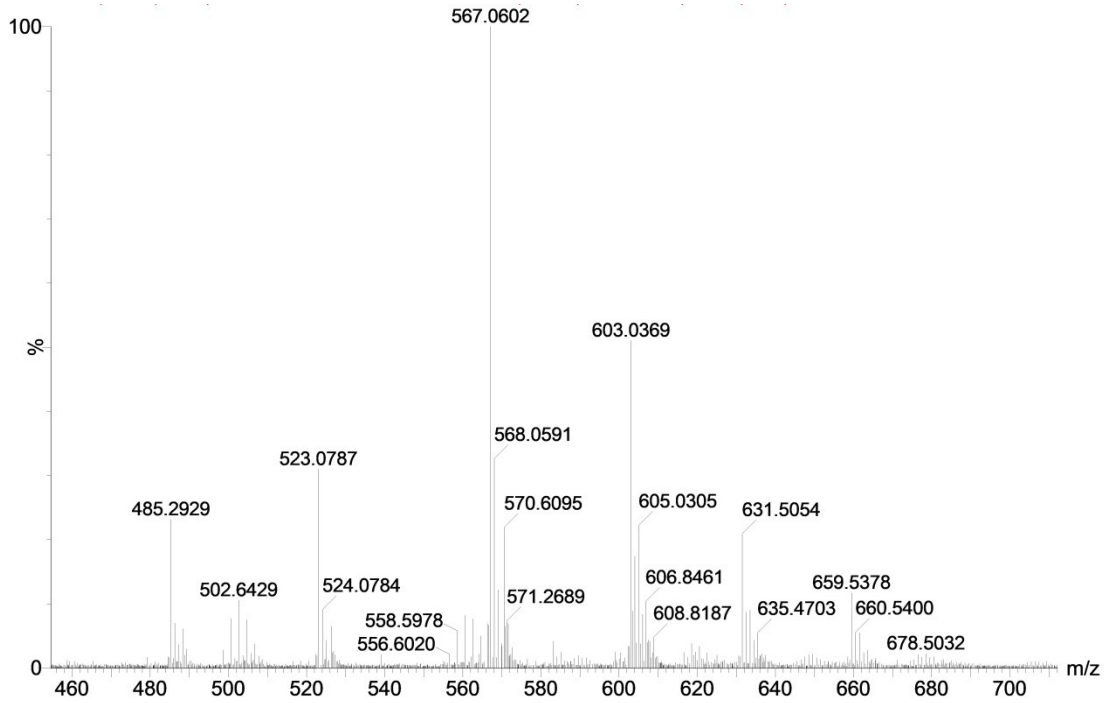


Fig. S14 HRMS of the compound **BP-HNO**