Supplementary Information

for

O^2-3-Aminopropyl diazeniumdiolates suppress progression of highly metastatic triple-negative breast cancer by inhibition of microvescile formation via nitric oxide-based epigenetic regulation

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Chemical Synthesis

General informations.

All commercially available compounds were used without further purification, unless otherwise noted. Anhydrous solvents (DMF, diethyl ether and THF) were used as commercially available. Analytical and preparative TLC was performed on silica gel (200 - 300 mesh) GF/UV 254 plates, and the chromatograms were visualized under UV light at 254 and 365 nm. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) spectrometer at 303 K, using TMS as an internal standard. MS spectra were recorded with a Mariner mass spectrometer (ESI) and high resolution mass spectrometry (HRMS) spectra on an Agilent Technologies LC/MSD TOF instrument. Infrared (IR) spectra (KBr) were recorded on IRAffinity-1 (KBr pellet). HPLC was performed on a Shimadzu Series (LC-20AT) using a WondaSil 5 μm C18 column (4.6 × 150 mm). Solutions after reactions and extractions were concentrated using a rotary evaporator operating at a reduced pressure of ~20 Torr. Individual compounds with a purity of > 95% were used for biological experiments.

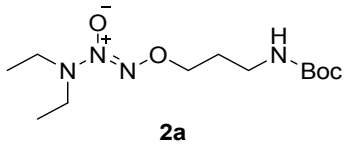
General synthesis of compounds 1a-f.

Sodium diazeniumdiolates **1a-f** were synthesized using a general procedure as previously published.[1] A solution of a certain secondary amine (0.39 mol) in 200 mL of anhydrous ether was placed in a Parr bottle. The solution was treated with 19 mL (0.38 mol) of 30% sodium methoxide in methanol. The system was evacuated, charged with 50 psi of NO (gas), and kept at 25 °C for 48 h. The white crystalline product was collected by filtration and washed with anhydrous ether. Diazeniumdiolate **1** was obtained by drying under vacuum.

General synthesis of compounds 2a-f.

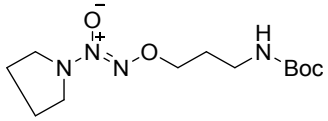
Compounds **2a-f** were synthesized using a general procedure. Diazeniumdiolate **1** (1.37 mmol) was added to a mixture of sodium bicarbonate (33 mg, 0.39 mmol) and 18-crown-6 (2 drops) in anhydrous DMF (2 mL) and anhydrous THF (2 mL) at room temperature with stirring during 10 min. *N*-Boc-3-bromopropylamine (300 mg, 1.26 mmol) was added drop wise to the mixture obtained above, and the reaction was allowed to stir at room temperature for 12 h in nitrogen

atmosphere. Then the reaction mixture was diluted with saturated sodium bicarbonate ($NaHCO_3$) aqueous solution (50 mL) and extracted with EtOAc (50 ml × 3). The combined organic layer was washed with brine, dried with anhydrous sodium sulfate, filtered, and evaporated in vacuum. The obtained crude product was purified by silica column chromatography to yield compound **2**.



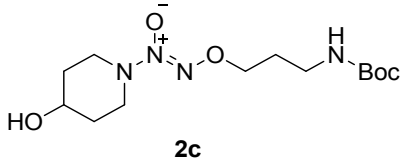
2a

O^2-(*N*-Boc-3-aminopropyl) diethylamine diazen-1-ium-1,2-diolate (**2a**) was obtained in 20% yield as a pale yellow oil; TLC (petroleum ether: AcOEt, 4:1 v/v): R_f = 0.28; IR (KBr): 3351.07, 2983.30, 2923.11, 2893.76, 2852.46, 1700.91, 1659.38, 1637.41, 1495.55, 1468.61, 1371.59; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.33 (t, *J* = 6.4 Hz, 2H), 3.26 (t, *J* = 5.9 Hz, 2H), 3.09 (q, *J* = 7.1 Hz, 4H), 2.00 - 1.92 (m, 2H), 1.44 (s, 9H),1.09 (t, *J* = 7.1 Hz, 6H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.39, 78.34, 71.18, 48.06, 36.56, 29.01, 27.73, 10.92 ppm; ESI-MS m/z: 313 $[M + Na]^+$.



2b

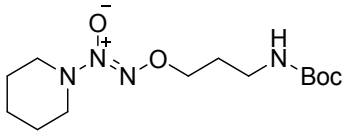
O^2-(*N*-Boc-3-aminopropyl) pyrrolidine diazen-1-ium-1,2-diolate (**2b)** was obtained in 19% yield as a pale yellow oil; TLC (petroleum ether: AcOEt, 4:1 v/v): R_f = 0.3; IR (KBr): 3350.08, 2961.73, 2928.13, 2870.32, 2849.27, 1701.22, 1673.28, 1600.31, 1475.26, 1460.17, 1362.87; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.37 (t, *J* = 6.1 Hz, 2H), 3.64 - 3.50 (m, 4H), 3.21 (t, *J* = 6.1 Hz, 2H), 2.41 - 2.32 (m, 2H), 2.00 - 1.88 (m, 4H), 1.44 (s, 9H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.55, 79.09, 71.68, 50.01, 36.58, 29.18, 27.89, 22.62, 22.18 ppm; ESI-MS m/z: 311 $[M + Na]^+$.



2c

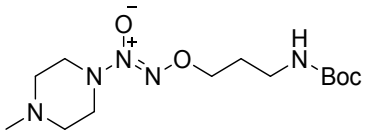
O^2-(*N*-Boc-3-aminopropyl) 4-hydroxypiperidine diazen-1-ium-1,2-diolate (**2c**) was obtained in 13% yield as a pale yellow oil; TLC (petroleum ether: AcOEt, 1:2 v/v): R_f = 0.22; IR (KBr): 3442.78, 3350.14, 2961.33, 2928.56, 2917.19, 2870.11, 2849.72, 1706.79, 1674.71, 1651.82,

1478.37, 1462.28, 1361.39; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.28 (t, *J* = 6.1 Hz, 2H), 3.90 - 3.88 (m, 1H), 3.67 (t, *J* = 6.9 Hz, 2H), 3.30 - 3.23 (m, 4H), 2.05 - 1.93 (m, 4H), 1.83 - 1.74 (m, 2H), 1.44 (s, 9H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.52, 78.83, 71.06, 65.15, 48.06, 36.94, 31.92, 28.76, 27.89 ppm; ESI-MS m/z: 341 $[M + Na]^+$.



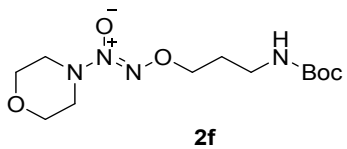
2d

O^2-(*N*-Boc-3-aminopropyl) piperidine diazen-1-ium-1,2-diolate (**2d**) was obtained in 16% yield as a pale yellow oil; TLC (petroleum ether: AcOEt, 4:1 v/v): R_f = 0.32; IR (KBr): 3360.00, 2954.95, 29225.16, 2850.79, 1653.00, 1681.93, 1673.28, 1635.64, 1471.34, 1456.26, 1338.60; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.28 (t, *J* = 6.3 Hz, 2H), 3.33 (t, *J* = 5.5 Hz, 4H), 3.23 (t, *J* = 6.2 Hz, 2H), 1.99 - 1.91 (m, 2H), 1.80 - 1.72 (m, 4H), 1.55 - 1.49 (m, 2H), 1.44 (s, 9H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.49, 78.87, 71.09, 52.29, 36.99, 29.19, 27.89, 24.27, 22.87 ppm; ESI-MS m/z: 325 $[M + Na]^+$.



2e

O^2-(*N*-Boc-3-aminopropyl) *N*-methylpiperazine diazen-1-ium-1,2-diolate (**2e**) was obtained in 16% yield as a pale yellow oil; TLC (petroleum ether: AcOEt, 1:3 v/v): R_f = 0.23; IR (KBr): 3353.01, 2960.14, 2923.56, 2881.67, 2852.11, 1749.44, 1649.09, 1646.87, 1478.70, 1462.74, 1337.76; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.27 (t, *J* = 6.1 Hz, 2H), 3.48 (s, 3H), 3.23 (t, *J* = 5.8 Hz, 2H), 2.64 (t, *J* = 5.1 Hz, 4H), 2.36 (t, *J* = 5.1 Hz, 4H), 1.97 - 1.92 (m, 2H), 1.44 (s, 9H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.48, 78.67, 71.08, 53.03, 50.25, 44.91, 36.88, 28.69, 27.86 ppm; ESI-MS m/z: 318 $[M + H]^+$, 340 $[M + Na]^+$.

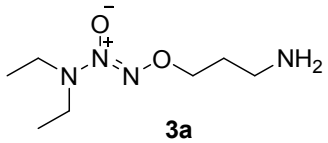


2f

O^2-(*N*-Boc-3-aminopropyl) morpholine diazen-1-ium-1,2-diolate (**2f**) was obtained in 14% yield as a pale yellow oil; TLC (petroleum ether: AcOEt, 5:1 v/v): R_f = 0.32; IR (KBr): 3350.01, 2960.24, 2926.45, 2871.73, 2854.11, 1702.19, 1543.17, 1517.39, 1458.63, 1349.37, 1323.69; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.28 (t, *J* = 6.3 Hz, 2H), 3.85 (t, *J* = 4.7 Hz, 4H), 3.43 (t, *J* = 4.7 Hz, 4H), 3.25 (t, *J* = 5.9 Hz, 2H), 2.00 - 1.91 (m, 2H), 1.44 (s, 9H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.42, 78.08, 70.90, 64.85, 50.85, 36.52, 28.47, 27.67 ppm; ESI-MS m/z: 327 $[M + Na]^+$.

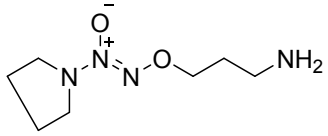
General synthesis of compounds 3a-f.

Compounds **3a-f** were synthesized using a general procedure. In a 25 mL round bottle flask filled with 10 mL of water, compound **2** (0.25mmol) was added. Then the flask topped with a condenser was dipped in a 120 °C oil bath. TLC was used to monitor the progress of the reaction. After completed, the reaction mixture was cooled to the room temperature, and was extracted with ethyl acetate (60 mL × 3). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. Flash chromatograph of the residue afforded the target compound **3**.



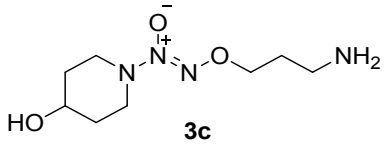
3a

O^2-(3-Aminopropyl) diethylamine diazen-1-ium-1,2-diolate (**3a**) was obtained in 68% yield as a yellow oil; TLC (CH_2Cl_2:MeOH, 7:1 v/v): R_f = 0.40; IR (KBr): 3444.87, 3363.86, 2964.59, 2927.94, 2873.81, 2853.20, 1659.03, 1636.34, 1609.26, 1457.21, 1450.41, 1384.89; ^{1}H NMR (300 MHz, CD_3OD): δ 4.37 (t, *J* = 6.2 Hz, 2H), 3.12 (q, *J* = 7.0 Hz, 4H), 2.90 (t, *J* = 7.1 Hz, 2H), 2.04 - 1.96 (m, 2H), 1.08 (t, *J* = 7.1 Hz, 6H) ppm; ^{13}C NMR (75 MHz, CD_3OD): δ 71.96, 49.28, 38.06, 27.93, 11.78 ppm; ESI-MS m/z: 191 $[M + H]^+$, 213 $[M + Na]^+$; HRMS (ESI, m/z): $[M + H]^+$ calcd. for $C_7H_{18}N_4O_2$, 191.1430, found 191.1508, ppm error 2.1; HPLC purity: 96.39%.



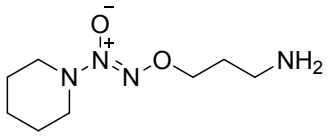
3b

O^2-(3-Aminopropyl) pyrrolidine diazen-1-ium-1,2-diolate (**3b**) was obtained in 80% yield as a yellow oil; TLC (CH_2Cl_2:MeOH, 8:1 v/v): R_f = 0.34; IR (KBr): 3439.08, 3361.93, 2926.01, 2852.72, 1654.92, 1635.64, 1608.63, 1456.26; ^{1}H NMR (300 MHz, CD_3OD): δ 4.24 (t, *J* = 5.3 Hz, 2H), 3.52 (t, *J* = 5.0 Hz, 4H), 2.76 (t, *J* = 6.5 Hz, 2H), 1.96 - 1.92 (m, 4H), 1.90 - 1.83 (m, 2H) ppm; ^{13}C NMR (75 MHz, CD_3OD): δ 71.36, 51.60, 38.04, 27.34, 23.45 ppm; ESI-MS m/z: 189 $[M + H]^+$; HRMS (ESI, m/z): $[M + H]^+$ calcd. for $C_7H_{16}N_4O_2$, 189.1273, found 189.1352, ppm error 2.1; HPLC purity: 96.48%.



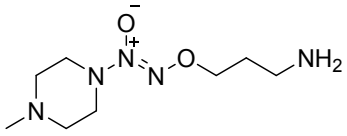
3c

O^2-(3-Aminopropyl) 4-hydroxypiperidine diazen-1-ium-1,2-diolate (**3c**) was obtained in 75% yield as a yellow oil; TLC (CH_2Cl_2:MeOH, 6:1 v/v): R_f = 0.36; IR (KBr): 3525.01, 3445.08, 3362.13, 2926.02, 2818.83, 1663.12, 1658.37, 1638.17, 1465.76, 1387.33; ^{1}H NMR (300 MHz, CD_3OD): δ 4.34 (t, *J* = 5.8 Hz, 2H), 3.83 - 3.78 (m, 1H), 3.72 - 3.67 (m, 2H), 3.29 - 3.24 (m, 2H), 3.08 (t, *J* = 6.2 Hz, 2H), 2.11 - 2.08 (m, 2H), 1.98 - 1.92 (m, 2H), 1.72 - 1.64 (m, 2H) ppm; ^{13}C NMR (75 MHz, CD_3OD): δ 71.98, 66.30, 49.45, 38.36, 33.17, 27.82 ppm; ESI-MS m/z: 219 $[M + H]^+$; HRMS (ESI, m/z): $[M + H]^+$ calcd. for $C_8H_{18}N_4O_3$, 218.1379, found 219.145, ppm error 1.8; HPLC purity: 95.53%.



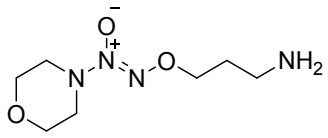
3d

O^2-(3-Aminopropyl) piperidine diazen-1-ium-1,2-diolate (**3d**) was obtained in 71% yield as a yellow oil; TLC (CH_2Cl_2:MeOH, 8:1 v/v): R_f = 0.32; IR (KBr): 3445.23, 3362.74, 2924.97, 2876.34, 1674.31, 1645.12, 1612.53, 1471.27; ^{1}H NMR (300 MHz, CD_3OD): δ 4.34 (t, *J* = 6.1 Hz, 2H), 3.39 (t, *J* = 5.5 Hz, 4H), 2.97 (t, *J* = 7.1 Hz, 2H), 2.08 - 1.99 (m, 2H), 1.79 - 1.71 (m, 4H), 1.59 - 1.51 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD): δ 71.88, 53.39, 38.31, 27.77, 25.69, 24.42 ppm; ESI-MS m/z: 203 $[M + H]^+$, 225 $[M + Na]^+$; HRMS (ESI, m/z): $[M + H]^+$ calcd. for $C_8H_{18}N_4O_2$, 203.1430, found 203.1508, ppm error 2.0; HPLC purity: 99.73%.



3e

O^2-(3-Aminopropyl) *N*-methylpiperazine diazen-1-ium-1,2-diolate (**3e**) was obtained in 70% yield as a yellow oil; TLC (CH_2Cl_2:MeOH, 7:1 v/v): R_f = 0.40; IR (KBr): 3439.08, 3361.93, 2956.87, 2935.66, 2924.09, 2912.56, 1647.95, 1631.78, 1602.85, 1502.55, 1458.18, 1387.85; ^{1}H NMR (300 MHz, CD_3OD): δ 4.30 (t, *J* = 6.0 Hz, 2H), 4.14 - 4.09 (m, 2H), 3.62 - 3.57 (m, 2H), 3.42 - 3.30 (m, 4H), 3.01 (t, *J* = 6.7 Hz, 2H), 2.88 (s, 3H), 2.08 - 2.01 (m, 2H) ppm; ^{13}C NMR (75 MHz, CD_3OD): δ 72.47, 53.15, 48.99, 43.47, 38.27, 27.79 ppm; ESI-MS m/z: 218 $[M + H]^+$, 240 $[M + Na]^+$; HRMS (ESI, m/z): $[M + H]^+$ calcd. for $C_8H_{19}N_5O_2$, 218.1539, found 218.1617, ppm error 2.3; HPLC purity: 99.60%.



3f

O^2-(3-Aminopropyl) morpholine diazen-1-ium-1,2-diolate (**3f**) was obtained in 73% yield as a yellow oil; TLC (CH_2Cl_2:MeOH, 8:1 v/v): R_f = 0.36; IR (KBr): 3442.94, 3361.93, 2978.09, 2875.86, 1673.20, 1645.53, 1606.70, 1469.97; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.34 (t, *J* = 6.0 Hz, 2H), 3.83 (t, *J* = 4.7 Hz, 4H), 3.43 (t, *J* = 4.6 Hz, 4H), 2.93 (t, *J* = 7.1 Hz, 2H), 2.05 - 1.96 (m, 2H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ 72.05, 66.73, 64.97, 53.37, 52.75, 38.27, 27.77 ppm; ESI-MS m/z: 205 $[M + H]^+$, 227 $[M + Na]^+$; HRMS (ESI, m/z): $[M + H]^+$ calcd. for $C_7H_{16}N_4O_3$, 205.1222, found 205.1301, ppm error 2.4; HPLC purity: 99.60%.

Materials and General Methods

Cell lines. Metastatic triple-negative breast cancer cells (MDA-MB-231, MDA-MB-468 and MDA-MB-436), metastatic melanoma B16F10 cells, metastatic prostate cancer PC-3 cells, metastatic glioma U251 cells，poorly invasive breast cancer MCF-7 cells and human mammary epithelial MCF10A cells were purchased from American Tissue Culture Collection. MDA-MB-231, MDA-MB-468 and MDA-MB-436 cell

lines were maintained in L-15 medium, B16F10 and U251 cells were maintained in DMEM medium, MCF-7 and MCF10A cell lines were maintained in EMEM medium, and PC-3 cell line was maintained in F12 medium. Both media were supplemented with 10% fetal bovine serum, and antibiotics [100 IU/mL penicillin and 100 IU/mL streptomycin (Amresco)]. All of the cell lines were grown at 37 °C in a 5% CO_2 atmosphere.

MTT. The inhibitory effects on cell proliferation of test compounds were investigated by the MTT method. MDA-MB-231, MDA-MB-436, MDA-MB-468, B16F10, PC-3, U251, MCF-7 or MCF10A cells at a final density were placed in 96-well cell plates and treated with or without different concentrations of test compounds for 72 h. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, 20 μL, 5 mg/mL) was added into each well, the cells were incubated for additional 4 h, and the resulting formazan crystals were dissolved in 150 μL of DMSO and measured using a spectrophotometer at a test wavelength of 570 nm. Experiments were conducted in triplicate. Inhibition rate (%) = [(Acontrol − Atreated)/Acontrol] × 100%.

In addition, MDA-MB-231 cells at a final density were placed in 96-well cell plates and pretreated with 0, 0.5 and 1 μM of BAPN, Carboxy-PTIO and NAC for 1 h, respectively. Subsequently, the cells were treated with **3f** (1 μM) for 72 h. The inhibition ratios of **3f** at 1 μM were obtained by MTT assay as mentioned above and the resluts was shown in Fig. 3a, 3b and S9.

RT-PCR. MDA-MB-231, MDA-MB-436, MDA-MB-468, B16F10, PC-3, U251, MCF-7 and MCF10A cell lines (1×10^6cells/well) were cultured in six-well plates at 37 °C in a 5% CO_2 atmosphere. Total RNA was extracted from individual groups of cells and reversely transcribed into cDNA using 5 × iScript reaction mix、iScript reverse transcriptase and Nuclease-free water following the manufacturer's instruction. The relative levels of LOX mRNA transcripts to the control GAPDH in individual groups of cells were analyzed by RT-PCR using LOX-specific primers[2]: LOX (forward: 5'-TGGGCGAAGGTACAGCATAC-3'; reverse: 5'-TGACAACTGTGCCATTCCCA-3'), GAPDH (forward: 5'-ACCACAGTCCATGCCATCAC-3'; reverse: 5'-TCCACCACCCTGTTGCTGTA -

3'). For each primer pair, annealing temperature was optimized by gradient PCR. The LOX expression in each cell, normalized to GAPDH, was calculated based on the threshold cycle (Ct) as $E = 2^{-\Delta(\Delta Ct)}$, where ΔCt = Ct(target) – Ct(18S) and $\Delta(\Delta Ct) = \Delta Ct(20\%) - \Delta Ct(1\%)$. The resluts was shown in Fig. S1.

MDA-MB-231 cells were treated with **3f** (20 nM) for 24 h in the presence or absence of pretreatment of a NO scavenger of PITO (40 nM) for 1 h, transfection with the miR-203 specific inhibitor (5'-TAGTGGTCCTAAACATTTCA-3') [3], or cultured cells in a hypoxia condition (1% O_2). Then, total RNA was extracted from cells using TRIzol and treated with DNase I. 20 μL of total RNA was used for first-strand DNA synthesis using the iScript cDNA Synthesis kit according to manufacturer's instructions. PCR amplification was performed with *RAB22A*- and miR203-specific primers respectively. For each primer pair, annealing temperature was optimized by gradient PCR. The expression of each target mRNA ware calculated based on the threshold cycle (Ct) as $E = 2^{-\Delta(\Delta Ct)}$, where ΔCt = Ct(target) – Ct(18S) and $\Delta(\Delta Ct) = \Delta Ct(20\%) - \Delta Ct(1\%)$. Primer sequences are as follows: miR-203 RT (5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTGT-3'), miR-203 primer amplification sequence (Sense: 5'-CTCGCTTCGGCAGCACA-3'; Anti-sense: 5'-AACGCTTCACGAATTTGCGT-3'), *RAB22A* (Sense: 5'-CACCTTACCGTCTCTGTGATTG-3'; Anti-sense: 5'-GTCTGTGGCCATGTGTCTTTA-3') and GAPDH (Sense: 5'-GGAGCGAGATCCCTCCAAAAT-3'; Anti-sense: 5'-GGCTGTTGTCATACTTCTCATGG-3'). The resluts was shown in Fig. 8a, 8b and 8c.

Western blotting analysis. MDA-MB-231, MDA-MB-436, MDA-MB-468, B16F10, PC-3, U251, MCF-7 and MCF10A cell lines (1×10^6 cells/well) were cultured in six-well plates at 37 °C in a 5% CO_2 atmosphere. The cells were harvested and lysed at 4 °C for 30 min in a lysis buffer. The cell lysates were centrifuged at 12000 - 16000 g for 5 min at 4 °C, and the supernatants were collected. The protein concentration in the cell lysates was determined by bicinchonininc acid assay. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (7.5% gel, 20 μg per lane) and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking, the PVDF

membranes were washed three times with TBST at room temperature and incubated with primary antibody for LOX at 4°C overnight. After extensive washing, membranes were incubated with secondary antibody for 1 h. After washing four times for 15 min with TBST at room temperature once more, the bands were detected by a Tanon 6000. The flms were scanned and quantitation was carried out with Image pro plus 6.0.

Stability assay. An appropriate amount of **3f** (1.5 mM) was initially added in a 2 mL 0.1 M PBS (pH 7.4) solution and its stability in PBS were monitored at 37 °C. Then the mixture was monitored by HPLC at the indicated time points. The HPLC conditions utilized consisted of a 70:30 methanol /water mixture with 5 mM triethylamine and a flow rate of 1 mL/min. Detection wavelengths were 241 nm for **3f**.

Rat plasma was then collected from adult male Sprague-Dawley rats. The plasma was stored at - 20 °C until it was used. The in vitro stability of **3f** was determined with an initial concentration of 0.28 mM in 80% of rat plasma (dilute with PBS) at 37 °C. A 200 μL portion of plasma was aliquoted from the incubation solution at 0, 1, 2, 3, 4, 5, 6 and 7 h time points followed by addition of equal volumes of acetonitrile to terminate the reaction. The mixture was centrifuged at 12000 for 5 min and the supernatant samples (20 μL each) were injected into the HPLC system as described above.

Enzymatic decomposition and NO releasing. A 0.1 M PBS (pH 7.4) mixture containing 30 μg/ml LOX (Immuno Clone Bioscience Co., NY, USA) and 150 μM individual test compounds were allowed to react at 37 °C. A 150 μL portion of PBS mixture was aliquoted from the incubation solution at 0, 30, 60, 90 and 120 min time points followed by solid phase extraction using 150 μL MeCN (equivalent amount). Then, 100 μL of the extract was divided into three parts in parallel to inject into the HPLC system as described above to monitor the decomposition of **3f**. The levels of NO generated from enzymatic decomposition of **3f** were determined by the colorimetric assay using the nitrite colorimetric assay kit.[4] The rest of 150 μL extract was divided into three parts in parallel, and then mixed with 50 μL Griess reagent respectively for 10 min at 37 °C followed by measurement at 540 nm by a spectrophotometer. 0.1 M PBS (pH 7.4) treated with equivalent amount of Griess reagent were used as negative controls for the background levels of nitrite production, while sodium nitrite at different

concentrations was prepared as the positive control for the establishment of a standard curve.

Detection of intracellular NO releasing. MDA-MB-231, MCF-7 and MCF10A cells in 96-well plates were used as above, and treated with or without different concentrations of **3f** for 72 h. After being loaded with 1.5ml of 5 μM DAF-FM DA[5] at 37 °C for 20 min and washed three times with PBS buffer to remove excess dye. The fluorescence intensity, used as a fluorescent indicator of intracellular NO, was recorded in a plate reader and measured with the flow cytometer with an excitation/emission (Ex/Em) frequency of 485/515 nm.

MDA-MB-231 cells in 96-well plates were used as above, and treated with 2 μM of **3a-f** for 12 h. The intracellular NO levels were measured by DAF-FM DA as mentioned above and the resluts was shown in Fig. S6a.

MDA-MB-231 cells were pretreated with or without BAPN (2 μM) for 1 h and then treated with 2 μM of **3f** for 72 h. The intracellular NO levels were measured by DAF-FM DA as mentioned above and the resluts was shown in Fig. S6b.

In addition, we have conducted additional experiments to examine whether there is any interference of DAF-FM-DA with acrolein during NO detection. As we can see Fig. S7, 1.5 ml of DAF-FM DA (5 μM) were randomly divided into five groups. The fluorescence intensity, used as a fluorescent indicator of intracellular NO, was recorded in a plate reader for cell free with an excitation/emission (Ex/Em) frequency of 485/515 nm. While obvious fluorescent signal was observed when treated with **1f**, but very weak fluorescent signal was detected when treated with acrolein. Furthermore, the intensity of NO released from **1f** was not enhanced or decreased in the presence of acrolein. These results indicated that there is no interference of DAF-FM-DA with acrolein during NO detection.

Detection of intracellular acrolein releasing. A fluorometric method[6] was used to measure intracellular evaluation of acrolein through its reaction with *m*-aminophenol in an acid media. Briefly, MDA-MB-231 cells (1×10^4/well) were treated with **3f** (0, 0.5, 1, 2 μM) for 12 h. Furthermore, some experiments were performed after pretreatment of cells with 2 μM of a LOX inhibitor of BAPN for 1 h. Subsequently, the

cells were harvested and mixed with a 1 M HCl mixture (1ml) containing hydroxylamine hydrochloride (3.5×10^{-2} mmol/ml) and *m*-aminophenol (1.5×10^{-2} mmol/ml) and then incubated at 98 °C water bath for 20min after treatment by ultrasonic processor. Then, the fluorescence intensity was immediately measured in a plate reader with an excitation/emission (Ex/Em) frequency of 350/459 nm.

Detection of intracellular H_2O_2 releasing. MDA-MB-231 cells were pretreated with, or without BAPN (2 μM) for 1 h and treated with the **3f** (0, 0.5, 1, 2 μM) for 12 h. After being loaded with 1.5ml of 5 μM BES-H_2O_2-Ac[7] at 37 °C for 20 min and washed three times with PBS buffer to remove excess dye. The fluorescence intensity, used as a fluorescent indicator of intracellular H_2O_2, was recorded in a plate reader and measured with the flow cytometer with an excitation/emission (Ex/Em) frequency of 505/545 nm.

Cell migration, invasion, lateral migration and adhesion assay. Migration assay: MDA-MB-231 cells were cultured in the upper chambers of 24-well transwell plates (8 μm pore, Corning Costar) and the bottom chambers were added in triplicate with medium alone or containing TGF-β[8] (10 ng/ml) alone or combined with **3f** (5, 10, 20 nM) for 48 h. The cells on the upper surface of the membrane were removed and the migrated cells on the bottom surface of the membranes were stained with crystal violet, followed by photoimaging. The cells in ten fields of each well were counted in blinded manner.

Invasion assay: MDA-MB-231 cells were seeded on matrigel-coated chambers. **3f** (5, 10, 20 nM) was treated on the lower surface for 48 h with or without incubation with TGF-β[8] (10 ng/ml). Cells that migrated through the matrigel-coated chambers were stained with crystal violet. Representative images were captured and the cells were counted from three independent experiments.

Lateral migration: MDA-MB-231 cells were cultured in 48-well plates until 95-100% of confluency. The monolayer of cells was wounded using a plastic tip and after being measured for the wounded areas, the cells were cultured in triplicate in medium alone, medium containing TGF-β[8] (10 ng/ml) alone or combined with **3f** (5, 10, 20 nM) for 48 h, followed by photoimaging. The healed areas in individual group of cells were calculated. Experiment was confirmed for three independent times.

Adhesion assay: Fluorescence-based analysis was used to evaluate effects of **3f** on the hetero-adhesion. HUVECs grown to confluence in 24-well tissue culture plates were pretreated with IL-1β[9] (1 ng/ml) for 1 h. Rhodamine 123-labeled MDA-MB-231 cells were cocultured with the HUVECs monolayers in each well, followed by treatment with **3f** (20 nM) for 1 h. After incubation, non-adhered MDA-MB-231 cells were removed by washing three times (drop-to-drop) with 1 mL PBS. We randomly selected 20 visual fields for each well and took pictures under a fluorescence microscope.

Acute toxicity assay. All animal experimental protocols were approved by the Administration Committee of Experimental Animals in Jiangsu Province and the Ethics Committee of China Pharmaceutical University. Female BALB/c nude mice at 5 - 6 weeks old were housed in an individual ventilated cage under controlled environmental conditions in an AAALAC-accredited facility. Groups of BALB/c nude mice were treated intravenously with a single dose of **3f** at 80.00, 111.33, 154.92, 215.58 and 300.00 mg/kg according to our pilot study, and the survival of the mice was monitored up to 14 days postinjection. While treatment with **3f** with a large dose (300.00 mg/kg) killed almost all mice, injection with 80.00 mg/kg of **3f** did not cause any death or abnormality in eating, drinking, body weight, or activity throughout the observation period. The LD_{50} value of **3f** was calculated to be 140.20 mg/kg for this strain of mice.

***In vivo* anti-proliferative activity.** All animal experimental protocols were approved by the Administration Committee of Experimental Animals in Jiangsu Province and the Ethics Committee of China Pharmaceutical University. The female BALB/c nude mice at 6 - 7 weeks old were inoculated subcutaneously with 2×10^6 MDA-MB-231 cells in the left flank. After establishment of solid tumors at an average volume of 100 mm^3, the animals were randomly divided into four groups with eight mice in each group. The groups with compound **3f** treatment received three dosages (2, 4 and 8 mg/kg) every other day by tail intravenous injection. At the end of the experiment, the mice were sacrificed, their tumors were dissected, and the tumor size and weight were measured. The tumor inhibitory ratio was calculated by the following formula: tumor inhibitory ratio (%) = $[(W_{control} - W_{treated})/W_{control}] \times 100\%$. $W_{treated}$ and $W_{control}$ were the average

tumor weights of the treated and control mice, respectively. The tumor diameters were measured with calipers, and the tumor volume was calculated by the formula V (mm^3) = d2 × D/^{2}, where D is the largest diameter and d the smallest diameter.

Anti-TNBC lung metastases test *in vivo*. All animal experimental protocols were approved by the Administration Committee of Experimental Animals in Jiangsu Province and the Ethics Committee of China Pharmaceutical University. MDA-MB-231 cells (1×10^6) were injected into the lateral tail vein of female BALB/c nude mice. The animals were randomly divided into four groups with eight mice in each group. The groups with compound **3f** treatment received three dosages (2, 4 and 8 mg/kg) every other day by tail intravenous injection for 6 weeks. Their body weights were measured every three days. At the end of the experiment, their lungs were dissected and the lung sections (100 μm) were stained with H&E. The numbers and areas of metastatic nodules in three sections of each mouse were examined in a blinded manner.

Analysis of MVs by fluorescence microscopy. MDA-MB-231 cells plated on 6-well plates with polylysine-coated slides were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton, and blocked with 5% BSA. The cells were then incubated with primary antibody (monoclonal mouse anti-human transglutaminase 2 antibodies, TMG2) followed by incubation with secondary antibody (FITC-conjugated goat anti-mouse IgG2a). Alexa Fluor 568-conjugated phalloidin was used to label budding MVs on the surface of the cells and 4,6-diamidino-2-phenylindole (DAPI) was used to stain nuclei. The samples were analyzed by immunofluorescence.[10]

Nanoparticle tracking analysis. When MDA-MB-231 cells grown in a 96-well plate reached 70% confluence, they were treated with **3f** (20 nM) for 24 h in the presence or absence of pretreatment of a NO scavenger of PITO (40 nM) for 1 h, transfection with the miR-203 specific inhibitor (5'-TAGTGGTCCTAAACATTTCA-3'), or cultured cells in a hypoxia condition (1% O_2). Then the media was carefully removed from the flasks and centrifuged consecutively at 500 × g for 10 min, 2,000 × g for 20 min, and 10,000 × g for 70 min. The resulting pellet was washed and resuspended in PBS. Samples of MVs (the number of MV particles per 10^7 cells) were analyzed in real time using the NanoSight NS500 instrument equipped with a green laser (532 nm) and

Nanoparticle Tracking Analysis software version 2.3, Build 0033 (NanoSight). Postacquisition settings were based on the manufacturer's recommendations and kept constant between samples. Each video was analyzed to obtain particle size distribution profiles and concentration measurements.[10]

Focal adhesion assays. Parental MDA-MB-231 cells were exposed to the **3f** (20 nM) for 24 h in the presence or absence of pretreatment of a NO scavenger of PITO (40 nM) for 1 h and shed MVs were collected from the conditioned media. Naïve MDA-MB-231 cells were incubated in serum-free L15, either alone [Ctrl] or supplemented with MVs from the indicated groups for 24 h, and the cells were stained with Alexa Fluor 568-conjugated phalloidin to detect F-actin stress fibers, vinculin antibody to detect focal adhesions (FAs), and DAPI to detect nuclei. (Scale bar inall panels, 10 μm.) Image analysis was performed to determine the number of FAs and total FAs area. [10]

Supplementary Results

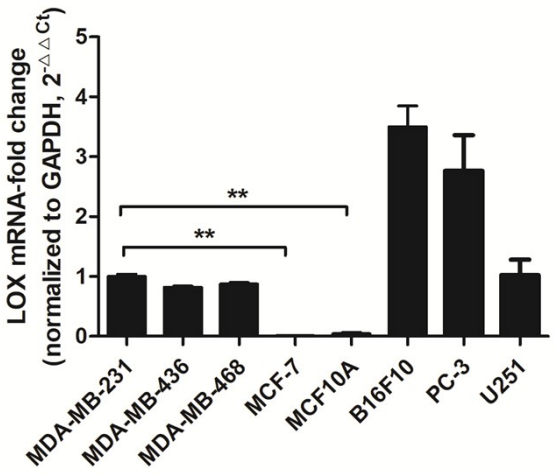


Figure S1. Expression of LOX mRNA in various cell lines. RT-PCR analysis of LOX mRNA expression in highly invasive/metastatic TNBC MDA-MB-231, TNBC MDA-MB-436, TNBC MDA-MB-468, melanoma B16F10, prostate carcinoma PC-3 and glioma U251 cells, and poorly invasive breast cancer MCF-7 cells, as well as non-cancer breast MCF10A cells by using LOX-specific PCR primers. GAPDH-specific primers were used as a control for equal loading. $^{**}P < 0.01$ vs. the MDA-MB-231 group. Data are present as mean of LOX mRNA-fold change ± SD of each cells from three independent experiments.

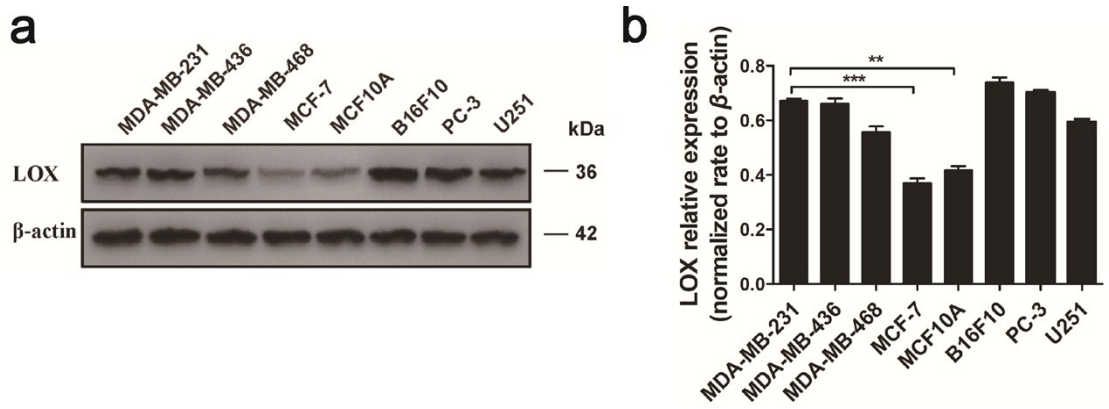


Figure S2. LOX protein expression of MDA-MB-231, MDA-MB-436, MDA-MB-468, B16F10, PC-3, U251, MCF-7 and MCF10A cells were determined by western blot. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the MDA-MB-231 group. Data shown here were representative of three different experiments. Data are presented as means ± SD (n = 3).

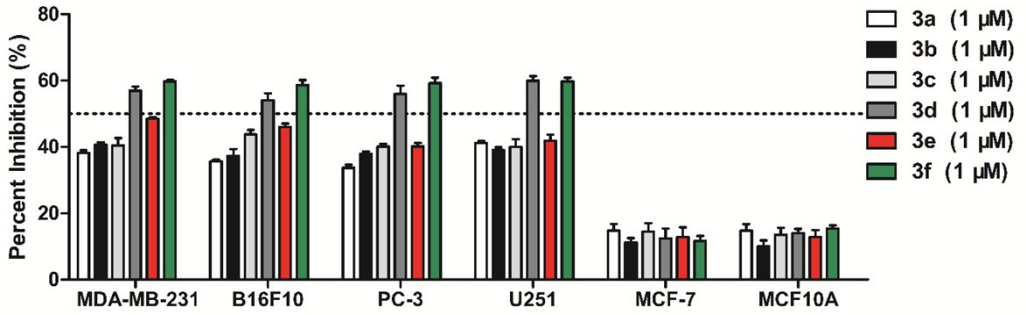


Figure S3. Compounds **3a-f** inhibited the proliferation of MDA-MB-231, B16F10, PC-3, U251, MCF-7 and MCF10A cells. Cells were treated with the indicated compounds at 1 μM for 72 h and the cell proliferation was measured by MTT. The percentage inhibition (%) of each compound was determined. Data are present as the mean (%) ± SD of each compound from three independent experiments.

Table S1. IC_{50} values (μM) of **3d** and **3f** against four cancer cell lines with high levels of LOX expression. The values of IC_{50} for each compound were calculated and data are expressed as mean IC_{50} (μM) ± SD from three independent experiments.

Compounds	MDA-MB-231	B16F10	PC-3	U251
3d	0.85 ± 0.10	1.12 ± 0.34	0.99 ± 0.23	1.13 ± 0.14
3f	0.68 ± 0.10	0.45 ± 0.03	0.67 ± 0.02	0.63 ± 0.04

Table S2. IC_{50} values (μM) of **3f** and cisplatin against TNBC cells, breast cancer MCF-7 cells and non-tumor MCF10A cells. The values of IC_{50} for each compound were calculated and data are expressed as mean IC_{50} (μM) ± SD from three independent experiments.

Compounds	**3f**	cisplatin
MDA-MB-231	0.68 ± 0.10	1.87 ± 0.16
MDA-MB-436	0.90 ± 0.09	1.98 ± 0.15
MDA-MB-468	0.98 ± 0.08	2.10 ± 0.18
MCF-7	17.90 ± 1.63	3.18 ± 0.30
MCF10A	20.41 ± 2.20	17.50 ± 1.64

Table S3. IC_{50} values (μM) of selected compounds. The values of IC_{50} for each compound were calculated and data are expressed as mean IC_{50} (μM) ± SD of from three independent experiments.

Compounds	MDA-MB-231
1f	5.18 ± 0.56
acrolein	13.25 ± 1.27
H_2O_2	26.01 ± 1.46
1f + acrolein (1:1)	5.18 ± 0.56
1f + H_2O_2 (1:1)	1.52 ± 0.08
H_2O_2 + acrolein (1:1)	10.50 ± 0.67
1f + acrolein + H_2O_2 (1:1:1)	0.83 ± 0.05
3f	0.68 ± 0.10

Table S4. IC_{50} values (μM) of H_2O_2, acrolein and **1f** in combination at equimolar dose on breast cancer MCF-7 cells and non-tumor MCF10A cells as compared with **3f**. The values of IC_{50} for each compound were calculated and data are expressed as mean IC_{50} (μM) ± SD from three independent experiments.

Compounds	**3f**	**1f** + acrolein + H_2O_2 (1:1:1)
MCF-7	17.90 ± 1.63	0.90 ± 0.05
MCF10A	20.41 ± 2.20	1.48 ± 0.09

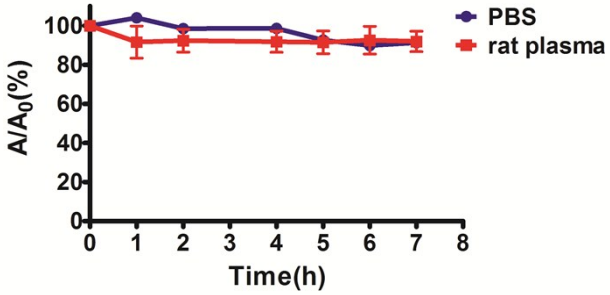


Figure S4. Stability of **3f** in rat plasma (red) and in PBS (blue) (pH = 7.4). Data are presented as means ± SD from three independent experiments.

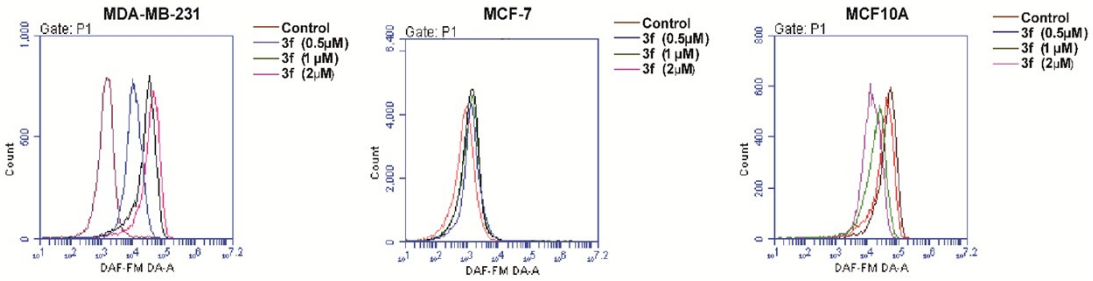


Figure S5. NO released from **3f** in MDA-MB-231, MCF-7 and MCF10A cells determined by an NO probe (DAF-FM DA). The cells were treated with the indicated concentrations of **3f** for 72 h. Peak shift after cell being stained with DAF-FM DA analyzing by FACS.

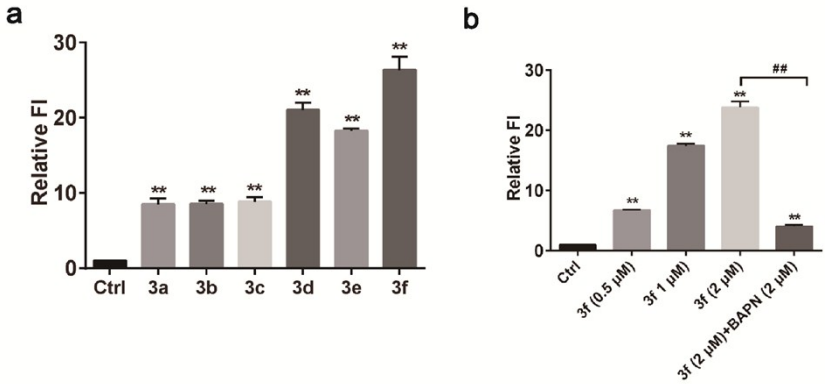


Figure S6. (a) MDA-MB-231 cells were treated with **3a-f** compounds for 12 h, and then stained with DAF-FM DA and analyzed by microplate reader. (b) MDA-MB-231 cells were pretreated with

or without BAPN, and then treated with **3f** for 72h. Cells were stained with DAF-FM DA and analyzed by Microplate reader. Data shown here were representative of three different experiments. Data are presented as means ± SD (n = 3). $^{**}P < 0.01$, vs. the control group, $^{\#\#}P < 0.01$ vs. the **3f** + BAPN group.

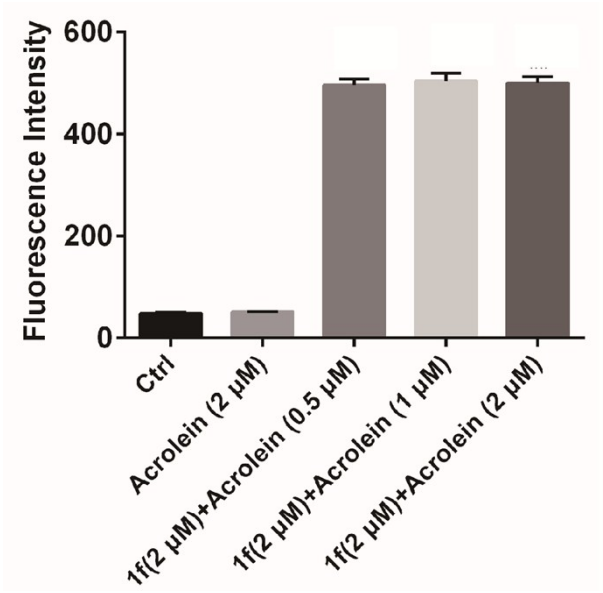


Figure S7. Different compounds promoted NO release in cell-free. Data are presented as means ± SD (n = 3).

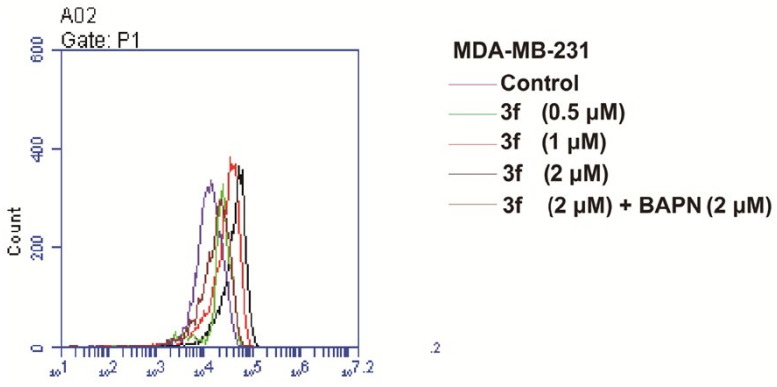


Figure S8. H_2O_2 released from **3f** in MDA-MB-231 determined by an H_2O_2 probe (BES-H_2O_2-Ac). MDA-MB-231 cells were treated with the indicated concentrations of **3f** for 12 h with, or without LOX inhibitor BAPN (2μM), and then stained with BES-H_2O_2-Ac and analysed by FACS. Data

shown here were representative of three different experiments. Data are presented as means ± SD (n = 3). $^{**}P < 0.01$ vs. control group, $^{\#\#}P < 0.01$ vs. the **3f** + BAPN group.

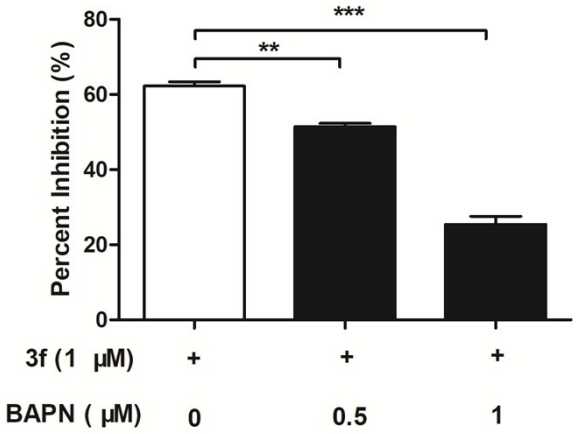


Figure S9. MDA-MB-231 cells were pre-treated with or without an LOX inhibitor (BAPN) for 1 h, and then treated with **3f** (1 μM) for 72 h. BAPN diminished inhibitory activity of **3f** against MDA-MB-231 cells, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the control group.

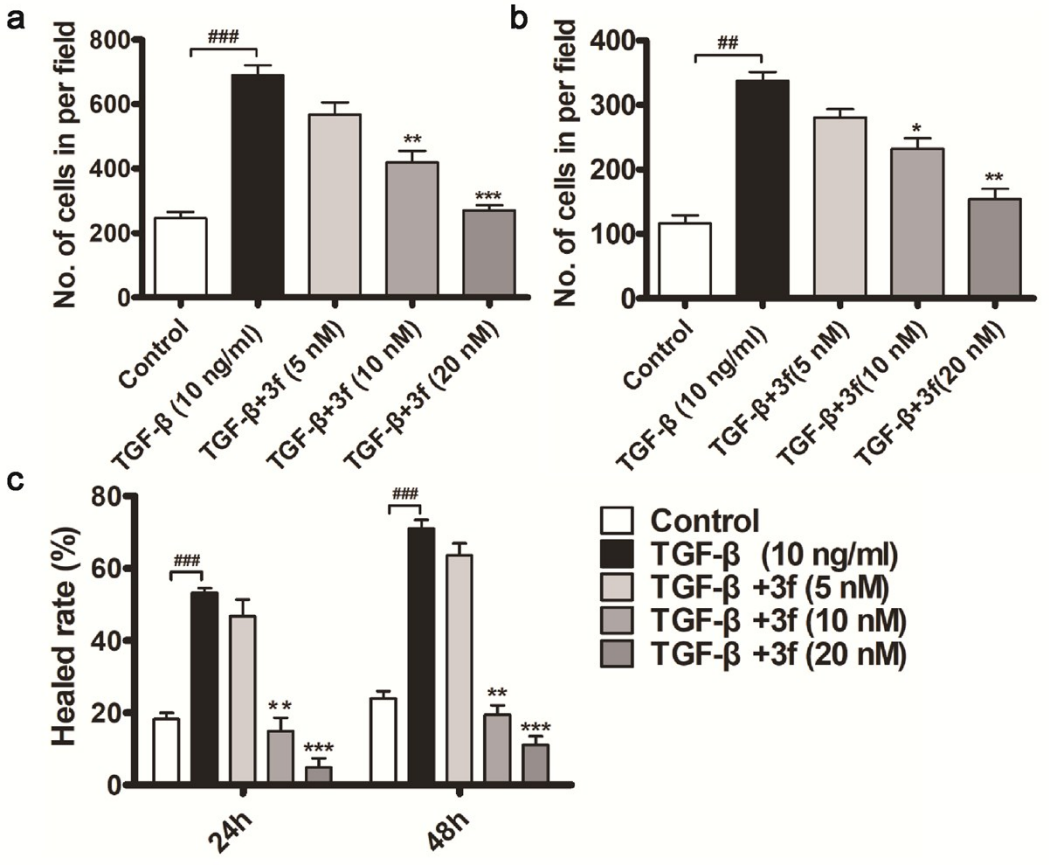


Figure S10. **3f** inhibited migration, invasion and lateral migration of TNBC cells in vitro. (a): **3f** inhibited migration of MDA-MB-231 cells. The MDA-MB-231 cells were seeded on chambers and incubated with the indicated factors for 48 h. Cells that migrated through the chambers were stained with crystal violet. Representative images were captured. $^{**}P < 0.01$, $^{***}P < 0.001$ vs the TGF-β (10 ng/ml) group; $^{###}P < 0.001$. (b): **3f** inhibited invasion of MDA-MB-231 cells. The MDA-MB-231 cells were seeded on chambers and incubated with the indicated factors for 48 h. Cells that migrated through the matrigel-coated chambers were stained with crystal violet. Representative images were captured. $^{*}P < 0.05$, $^{**}P < 0.01$ vs the TGF-β (10 ng/ml) group; $^{##}P < 0.01$. (c): **3f** inhibited lateral migration of MDA-MB-231 cells. The MDA-MB-231 cells were seeded on 48-well plates. After 24 or 48 h incubation with the indicated factors, representative images of wound were captured, and the healed rate is presented. Experiment was confirmed for three independent times. $^{**}P < 0.01$, $^{***}P < 0.001$ vs respective TGF-β (10 ng/ml) group; $^{###}P < 0.001$. Data were expressed as the means ± SD of each group of cells.

Table S5. Acute toxicity of **3f** in mice.

Dose (mg/kg)	80.00	111.33	154.92	215.58	300.00
No. of mice	10	10	10	10	10
Total mortality	0	3	5	9	10

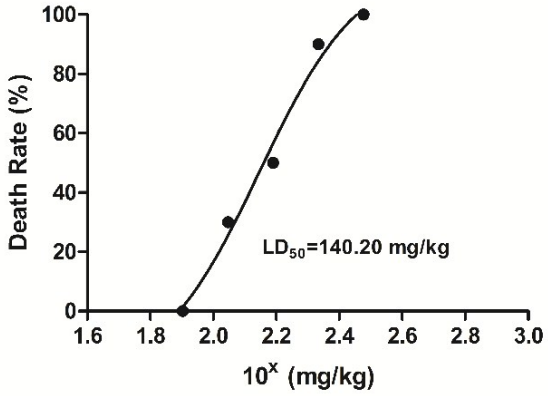


Figure S11. The LD_{50} of **3f** in mice was determined.

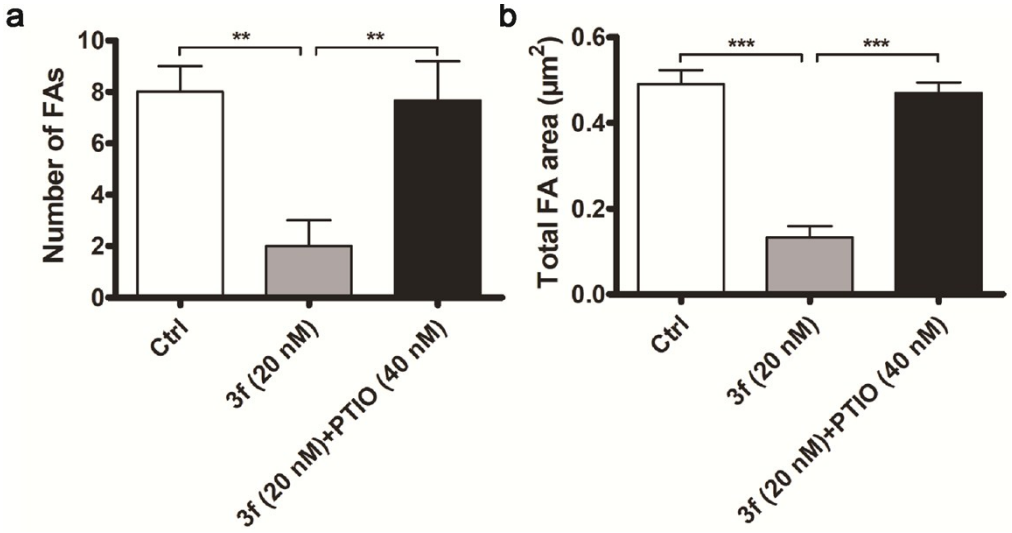


Figure S12. **3f** inhibited the MVs-mediated focal adhesion (FAs) in MDA-MB-231 cells. Image analysis was performed to determine the numbers of FAs (a) and total FAs area (b). Mean ± SD of three independent experiments are shown. $^{**}P < 0.01$, $^{***}P < 0.001$.

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NMR and HRMS Spectra

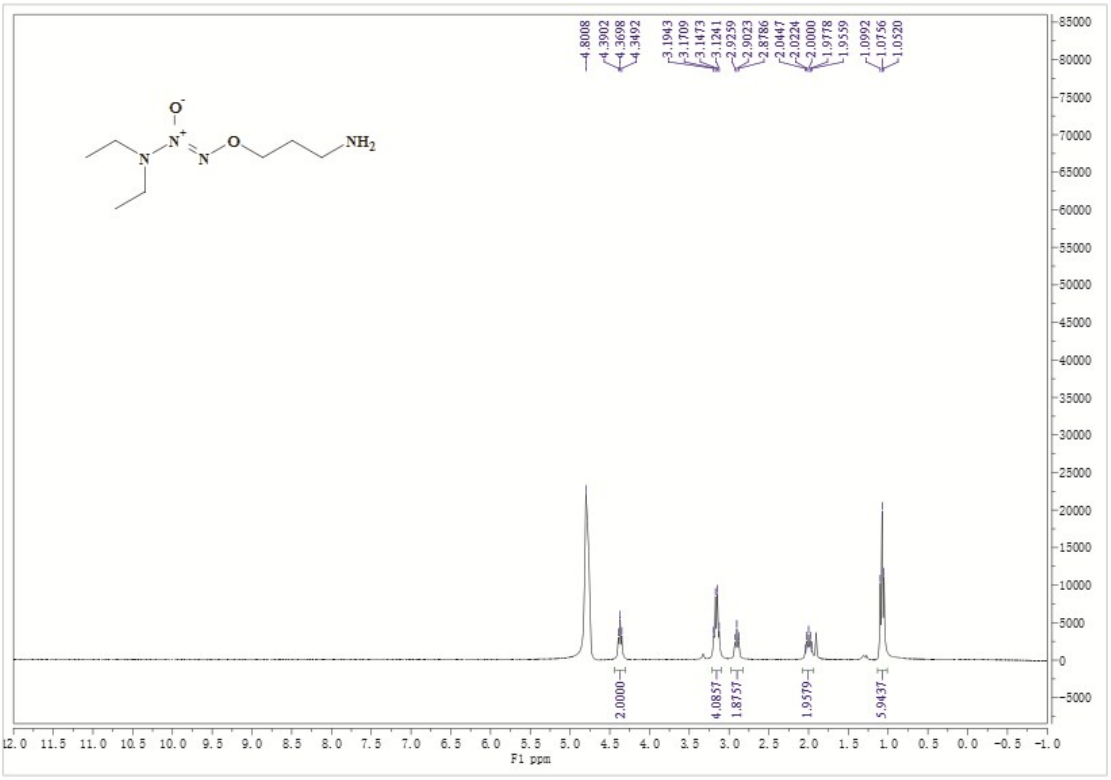

Figure S13. **^{1}H NMR of 3a**.

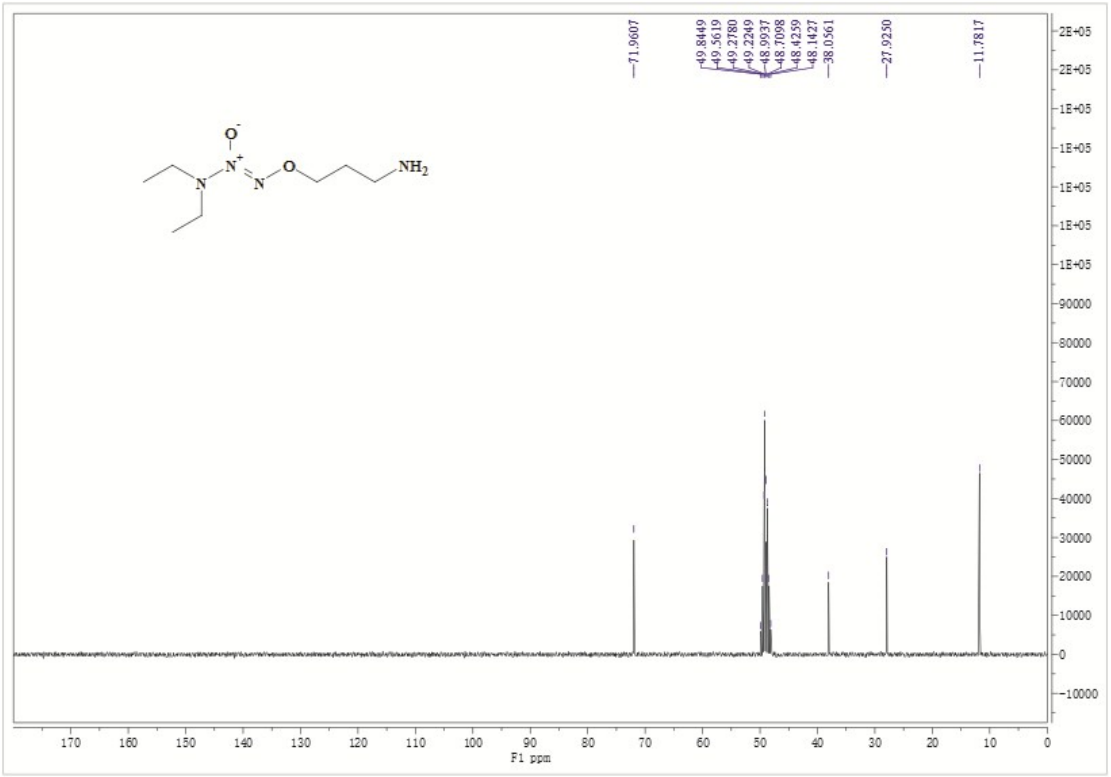

Figure S14. **^{13}C NMR of 3a**.

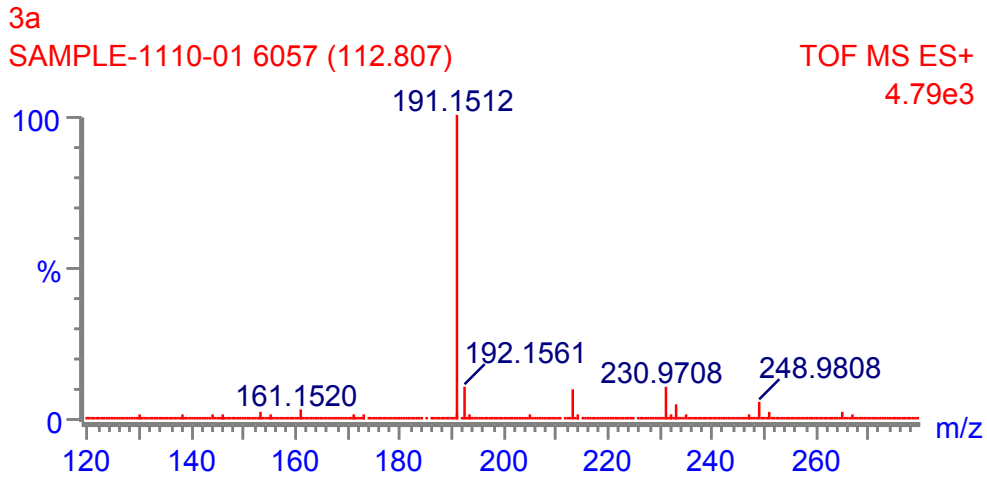

Figure S15. **HRMS of 3a**.

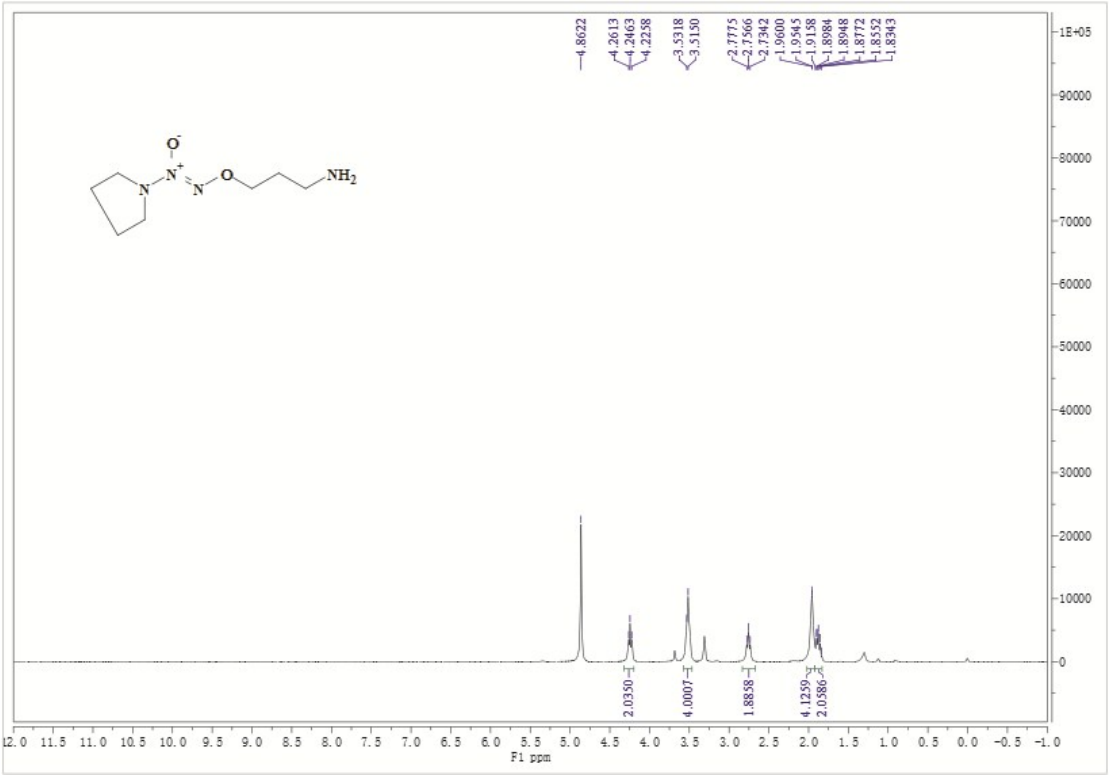

Figure S16. **^{1}H NMR of 3b**.

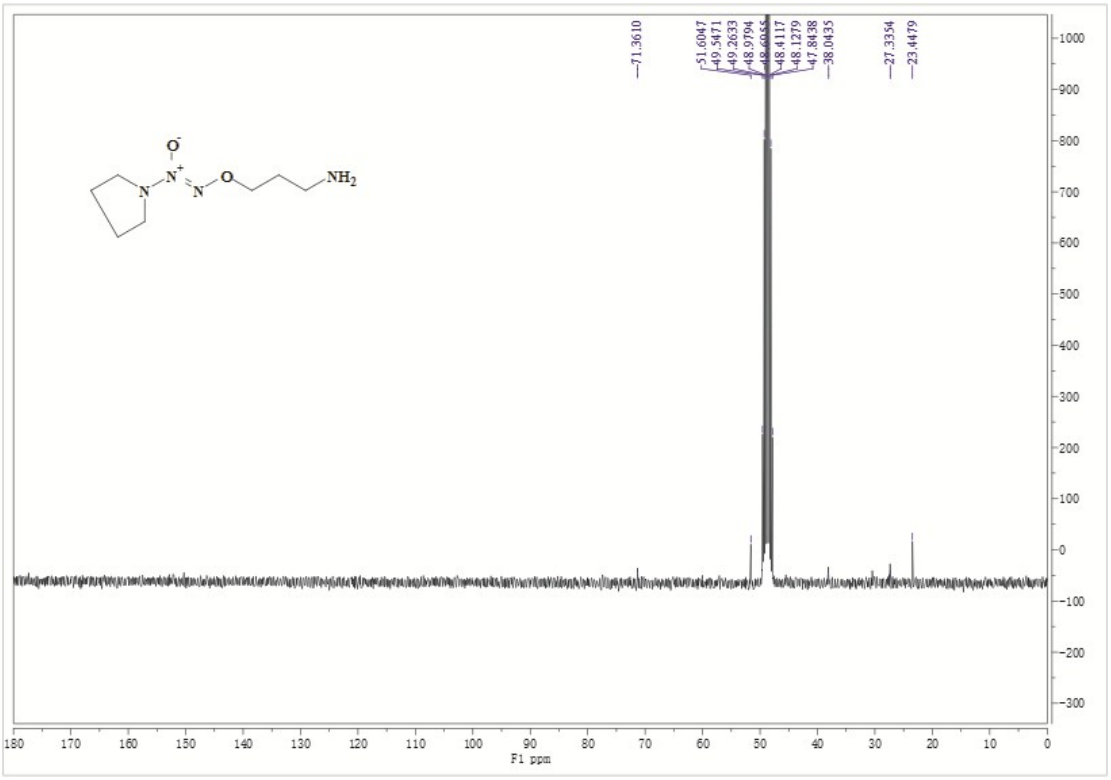

Figure S17. **^{13}C NMR of 3b**.

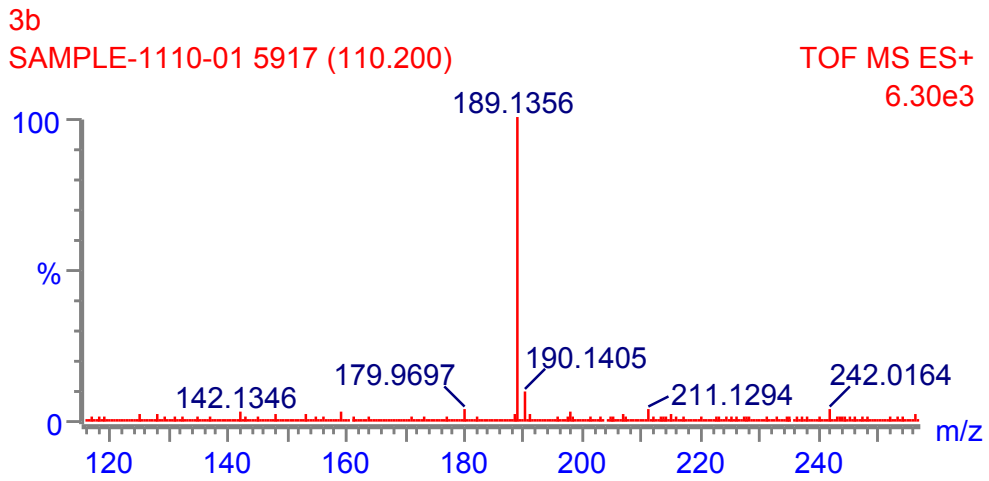

Figure S18. **HRMS of 3b**.

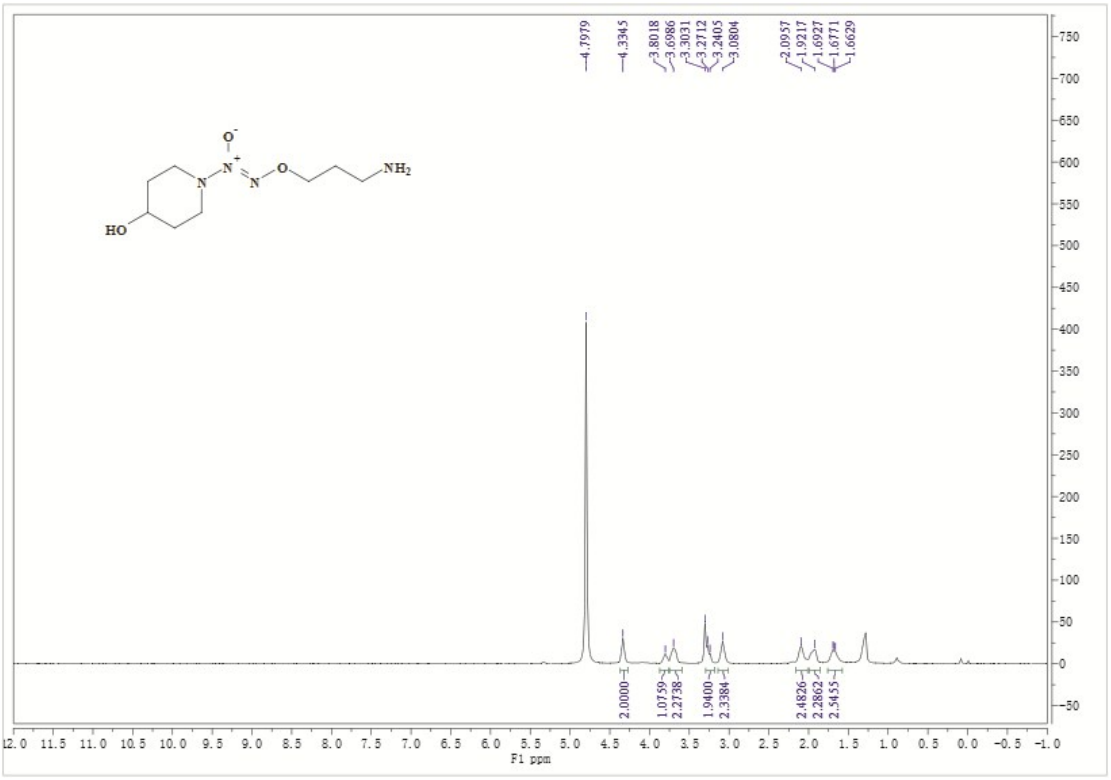

Figure S19. **^{1}H NMR of 3c**.

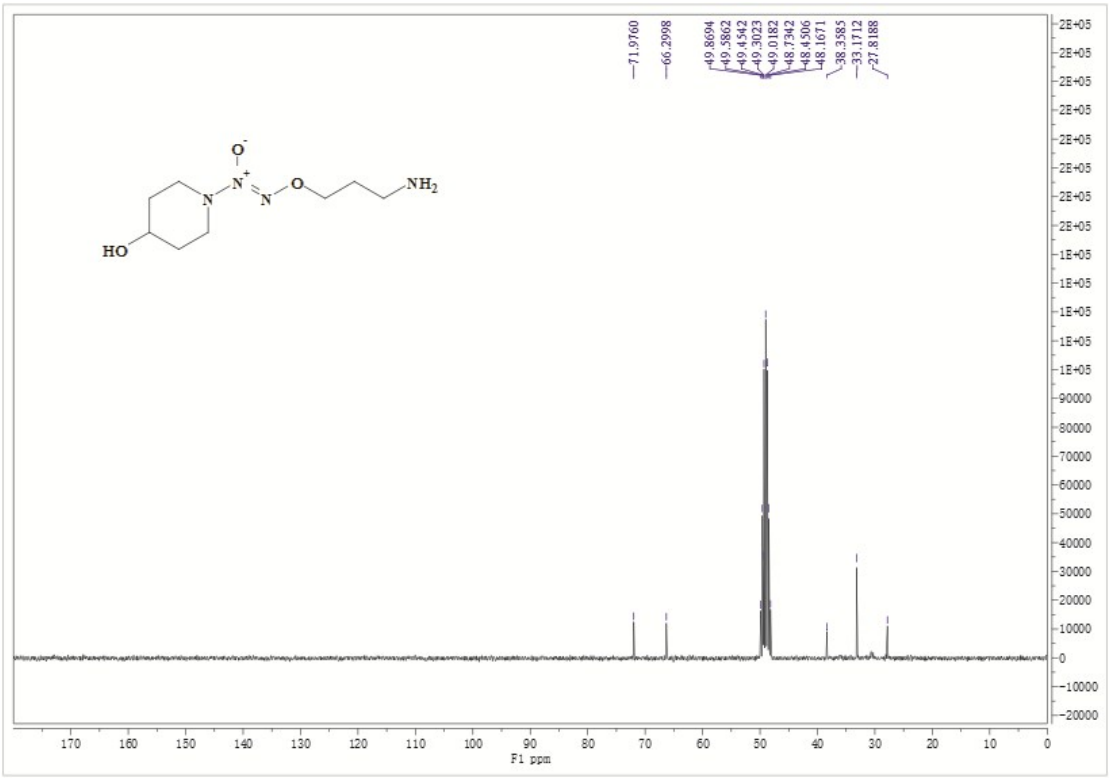

Figure S20. **^{13}C NMR of 3c**.

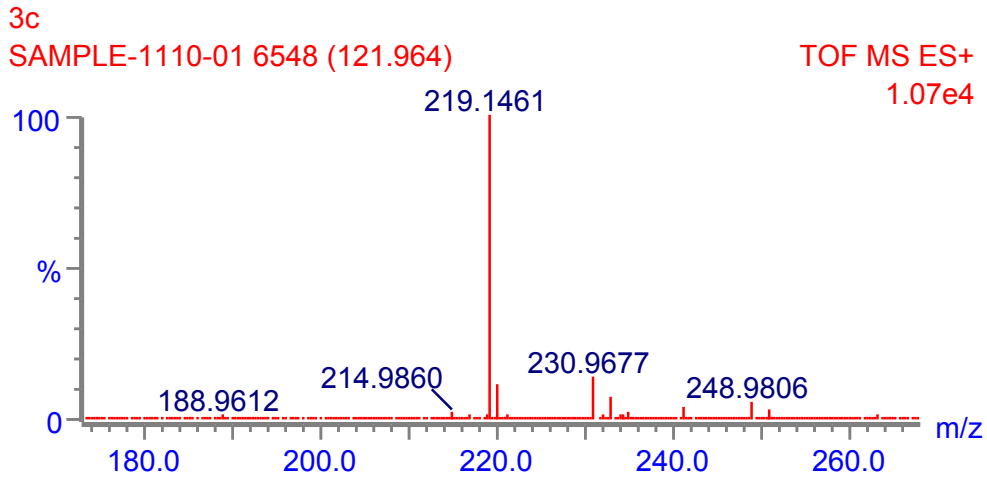

Figure S21. **HRMS of 3c**.

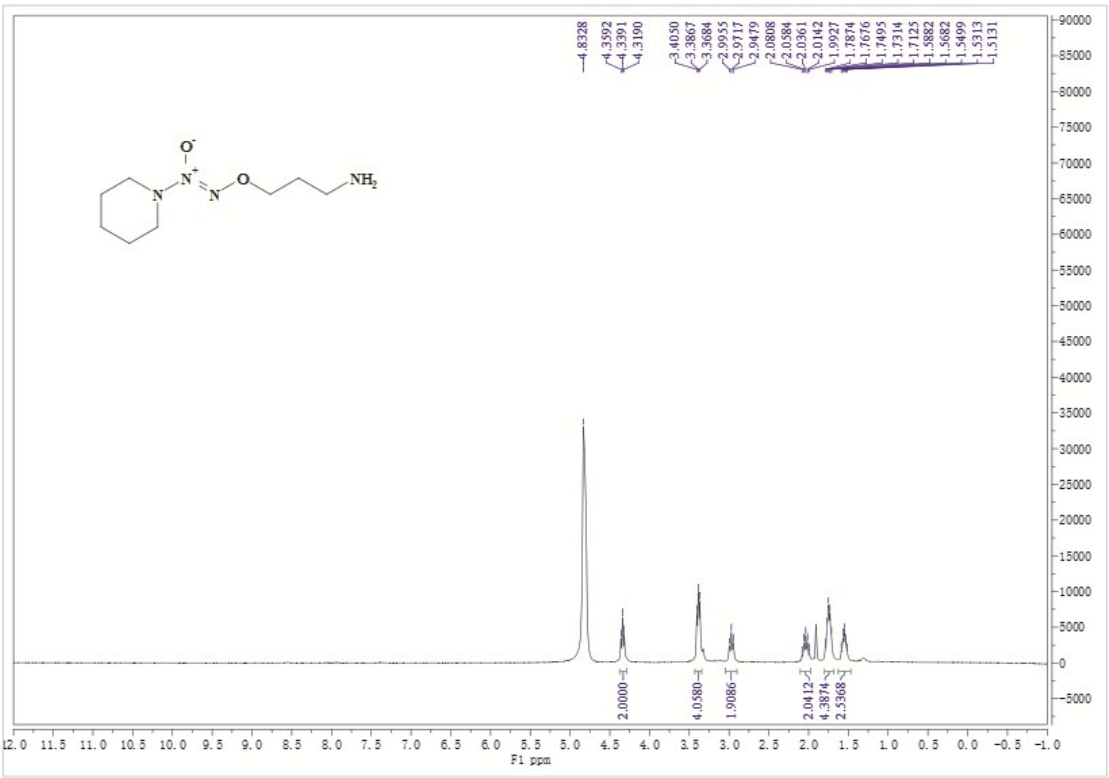

Figure S22. **^{1}H NMR of 3d**.

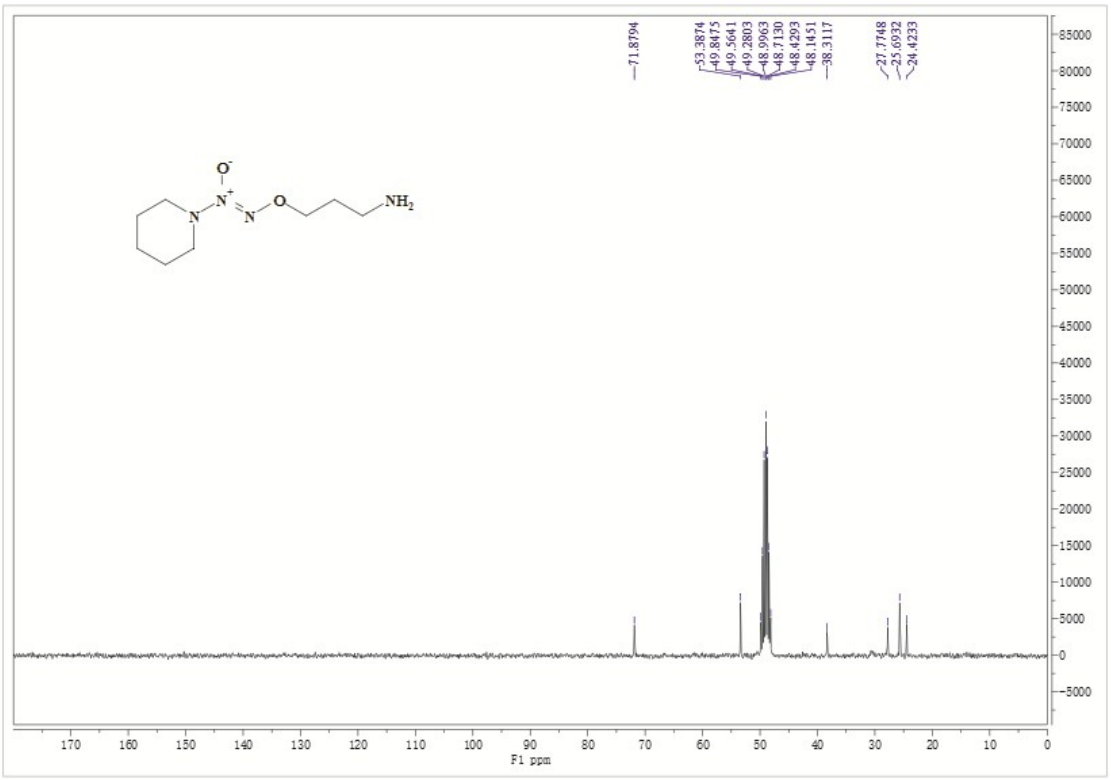

Figure S23. ^{13}C NMR of **3d**.

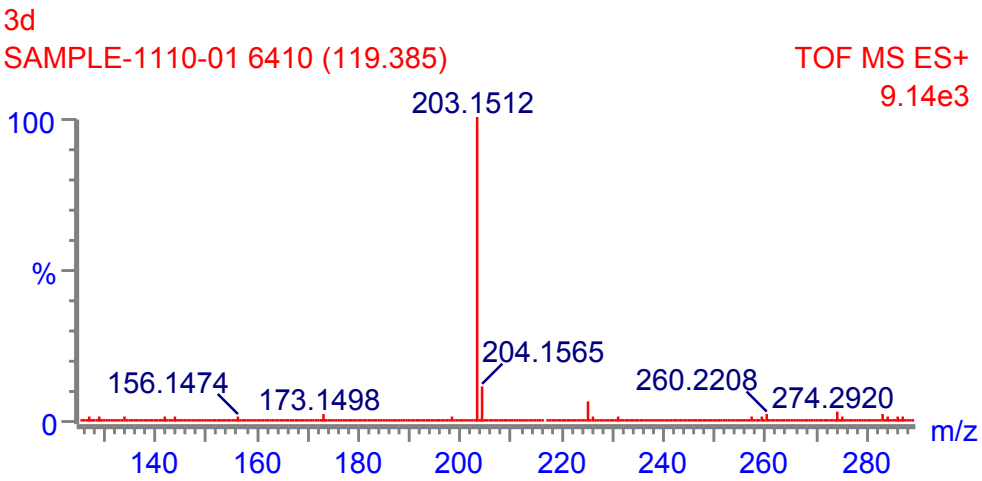

Figure S24. HRMS of **3d**.

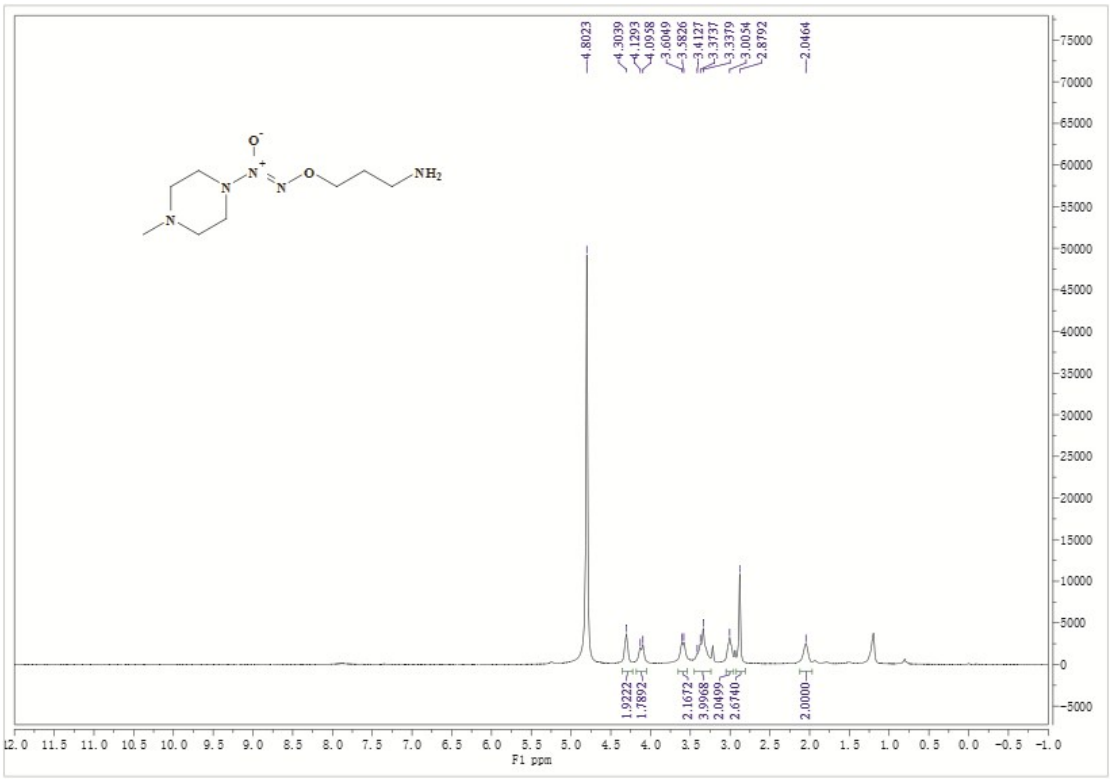

Figure S25. **^{1}H NMR of 3e**.

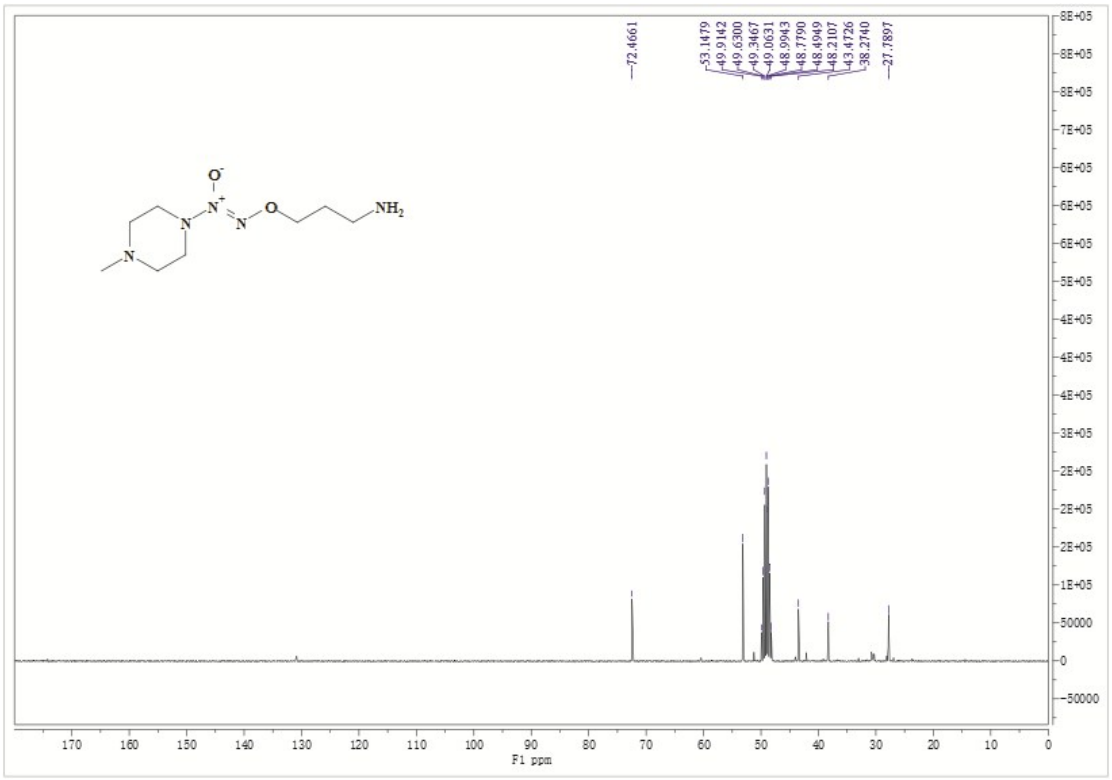

Figure S26. **^{13}C NMR of 3e**.

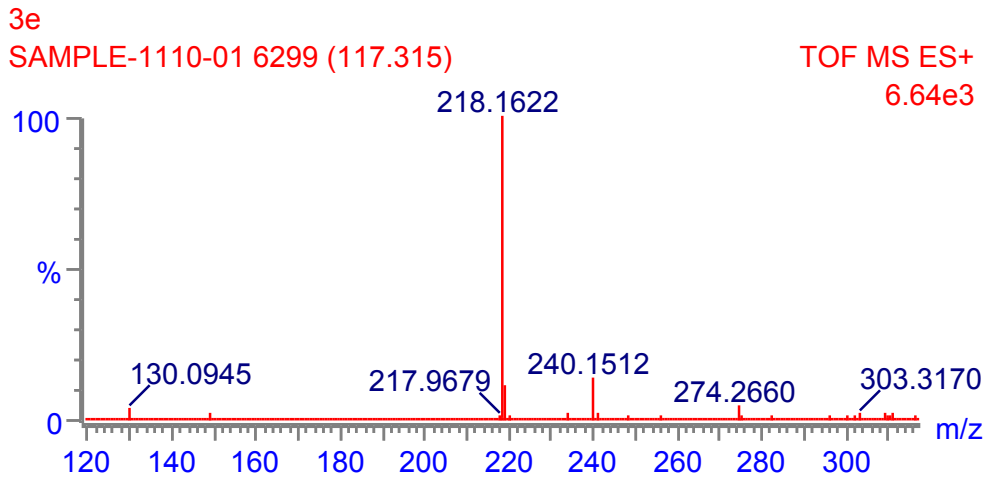

Figure S27. **HRMS of 3e**.

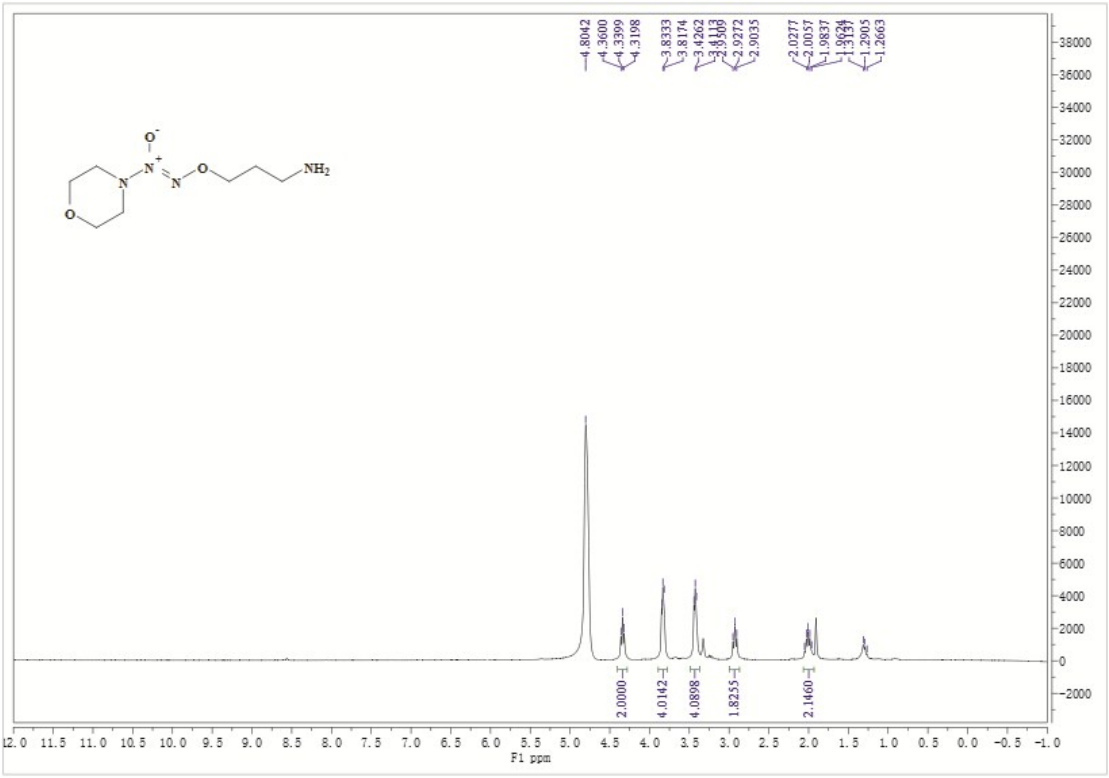

Figure S28. **^{1}H NMR of 3f**.

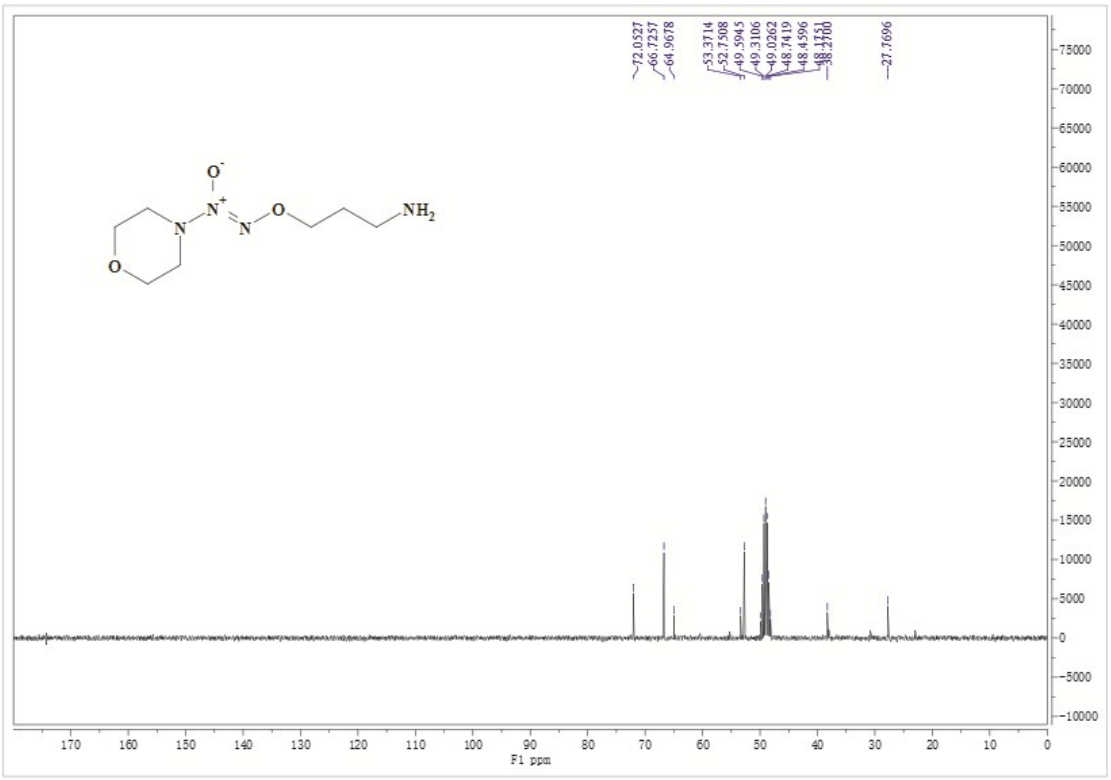

Figure S29. ^{13}C NMR of 3f.

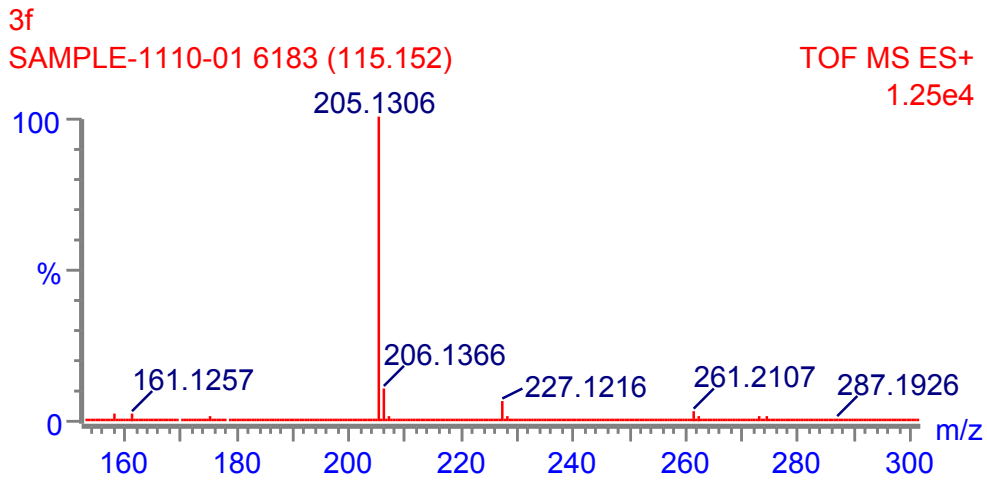

Figure S30. HRMS of 3f.

HPLC Assessment of Compounds Purity

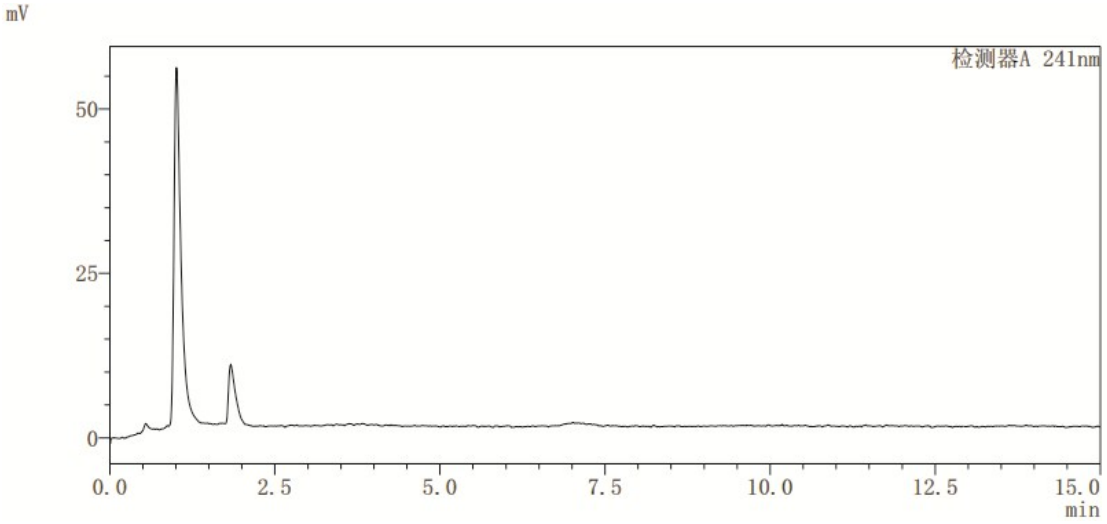

Figure S31. **Solvent peak (MeOH)**: column: Wondasil C18 (150 mm × 4.6 mm × 5 μm), mobile phase: methanol (5 mM TEA): water = 30:70 (v/v); Rate: 1 mL/min; Temperature: 25 °C.

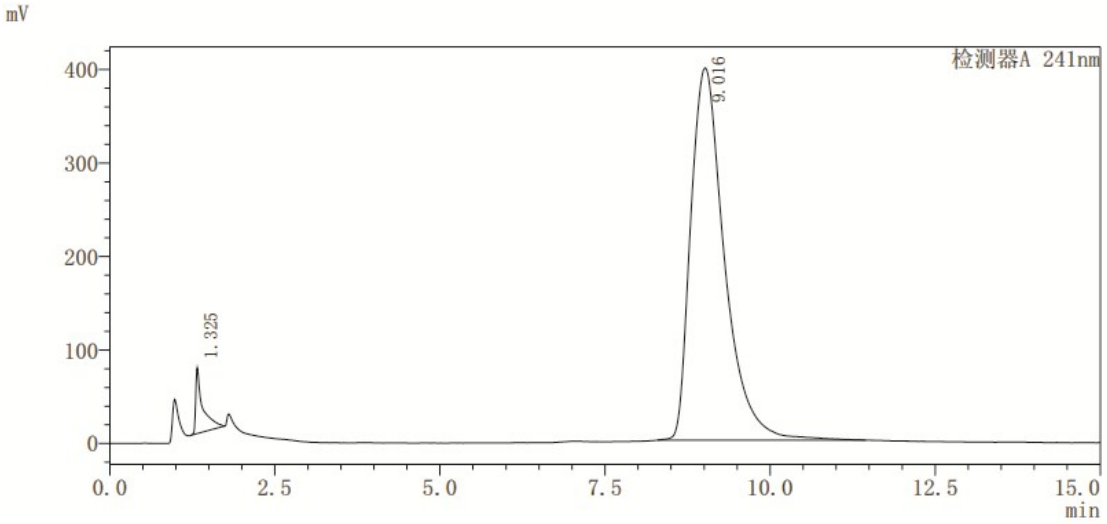

Figure S32. **Compound 3a**: 96.39%, column: Wondasil C18 (150 mm × 4.6 mm × 5 μm), mobile phase: methanol (5 mM TEA): water = 30:70 (v/v); Rate: 1 mL/min; Temperature: 25 °C.

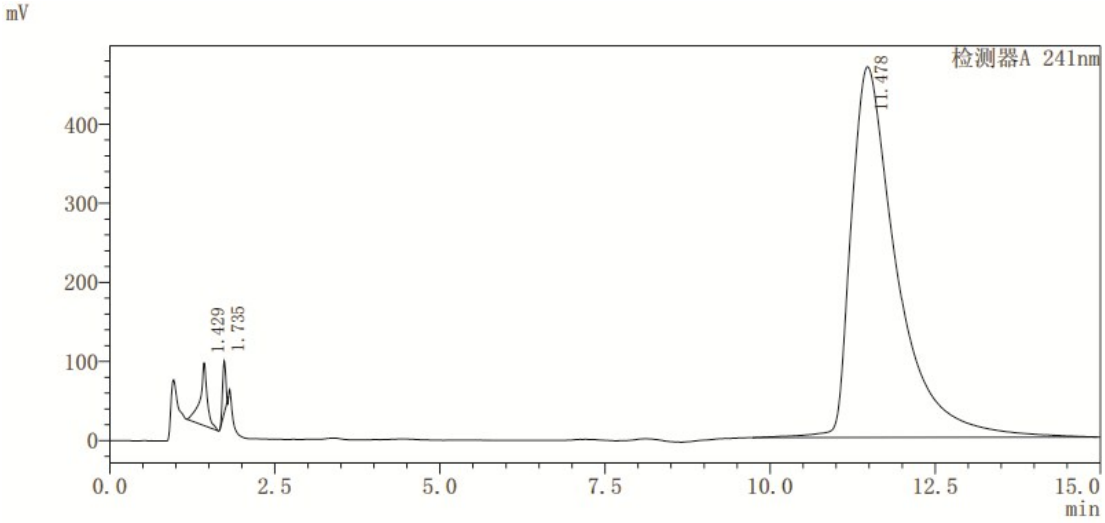

Figure S33. **Compound 3b**: 96.48%, column: Wondasil C18 (150 mm × 4.6 mm × 5 μm), mobile phase: methanol (5 mM TEA): water = 30:70 (v/v); Rate: 1 mL/min; Temperature: 25 °C.

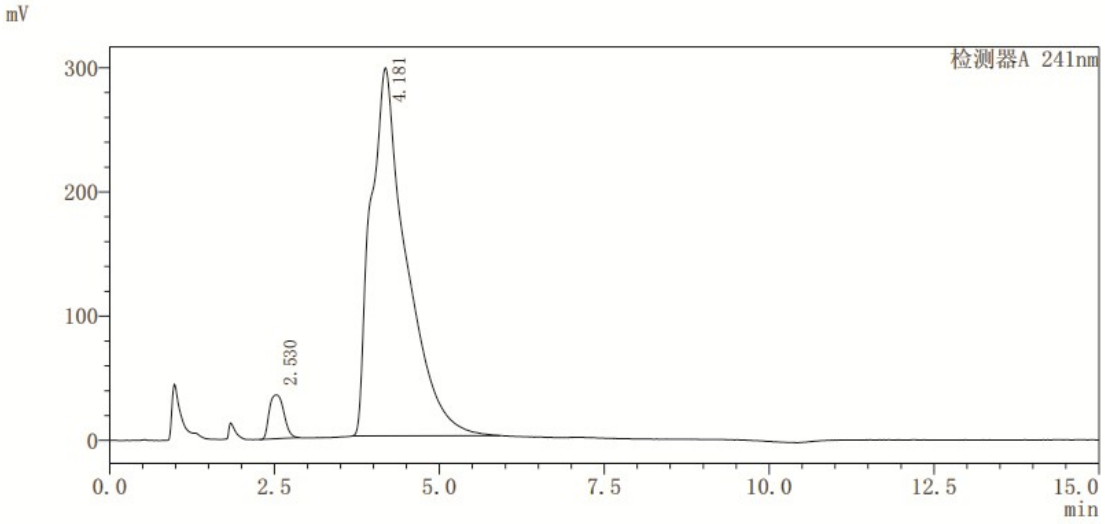

Figure S34. **Compound 3c**: 95.53%, column: Wondasil C18 (150 mm × 4.6 mm × 5 μm), mobile phase: methanol (5mM TEA): water = 20:80 (v/v); Rate: 1 mL/min; Temperature: 25 °C.

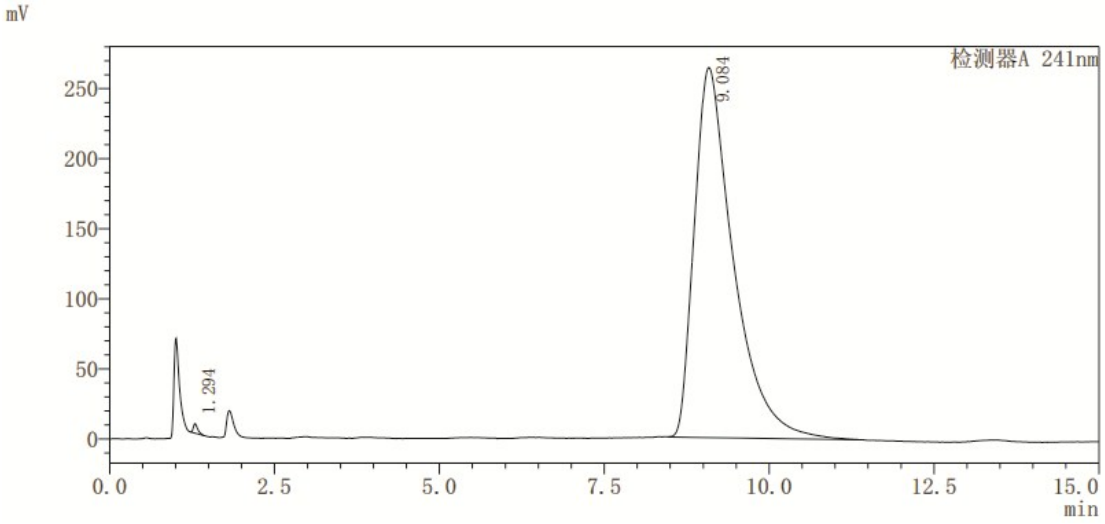

Figure S35. **Compound 3d**: 99.73%, column: Wondasil C18 (150 mm × 4.6 mm × 5 μm), mobile phase: methanol (5 mM TEA): water = 30:70 (v/v); Rate: 1 mL/min; Temperature: 25 °C.

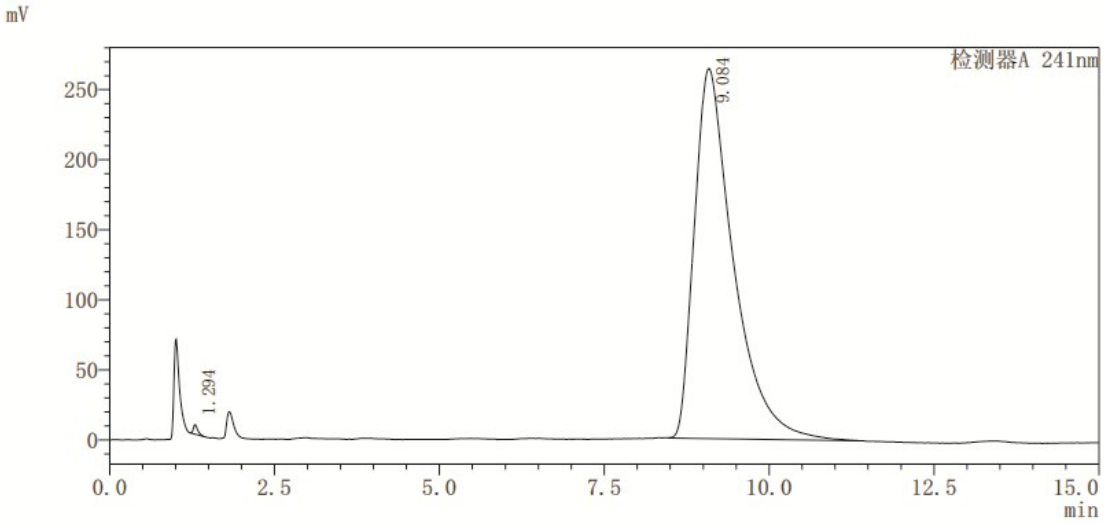

Figure S36. **Compound 3e**: 99.60%, column: Wondasil C18 (150 mm × 4.6 mm × 5 μm), mobile phase: methanol (5 mM TEA): water = 20:80 (v/v); Rate: 1 mL/min; Temperature: 25 °C.

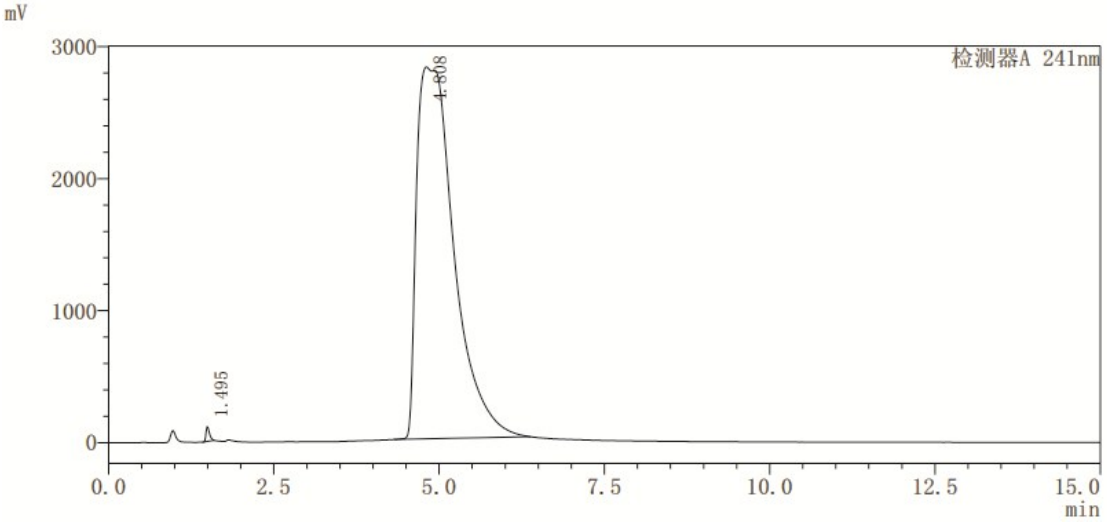

Figure S37. **Compound 3f**: 99.60%, column: Wondasil C18 (150 mm × 4.6 mm × 5 μm), mobile phase: methanol (5 mM TEA): water = 20:80 (v/v); Rate: 1 mL/min; Temperature: 25 °C.