A Hypoxia-Activated NO Donor for the Treatment of Myocardial Hypoxia Injury

Wen Zhou,[ab] Wanxiang Yang,[ab] Keyu Fan,[ab] Wuyang Hua, [ab] and Shaohua Gou*[ab]

a. Jiangsu Province Hi-Tech Key Laboratory for Biomedical Research, Southeast University, Nanjing, 211189, China

b. Pharmaceutical Research Center and School of Chemistry and Chemical Engineering, Southeast University, Nanjing 211189, China

Corresponding Author:

2219265800@qq.com (Shaohua Gou)

Electronic Supplementary Information

Table of Contents

Experimental Procedures

Synthesis of the Compound

All reagents were of analytical grade and used without further purification. NMR spectra were performed on a Bruker 600 MHz NMR spectrometer and reported as parts per million (ppm) from TMS. The final product was characterized by ^{1}H– and ^{13}C–NMR spectra together with high-resolution mass spectrum by an Agilent 6224 ESI/TOF MS instrument.

Compound Hano was prepared as follows.

Intermediate 1: To a solution of metol (1.722 g, 10.0 mmol) in acetic acid (50 ml) in an ice-water bath, sodium nitrite (3.450 g, 50.0 mmol) in water (10 ml) was slowly dropped in dark. The reaction kept under ice-water bath for 3 h, then 50 ml water was added to the reaction solution. After adjusting the pH to 7 with saturated sodium bicarbonate aqueous solution, the solution was extracted with ethyl acetate. The organic phase was separated and dried over anhydrous sodium sulfate. After removing the solvent by a rota-vapor, the product was obtained with a yield of 96.0% (1.46 g). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 9.79 (s, 1H), 7.41 (d, J = 8.8 Hz, 2H), 6.89 (d, J = 8.8 Hz, 2H), 3.38 (s, 3H) ppm.

Intermediate 2: To a mixture of 4-nitrobenzyl alcohol (0.168 g, 1.1 mmol) and triphosgene BTC (0.237 g, 0.8 mmol) in 20 ml anhydrous tetrahydrofuran, added was N,N-diisopropylethylamine DIPEA (0.194 g, 1.5 mmol) in an ice water bath under nitrogen atmosphere. Half an hour later, removed the ice-water bath and kept the solution at room temperature for 5 h until the reaction completed. The resulting solution was spin-dried, excess phosgene was removed by adding anhydrous tetrahydrofuran. After that, anhydrous dichloromethane (20 ml) was added and used for the next reaction.

Compound Hano: Intermediate 1 (0.152 g, 1.0 mmol) and DIPEA (0.258 g, 2.0 mmol) were firstly mixed in anhydrous dichloromethane (20 ml) and placed in an ice-water bath, then intermediate 2 in anhydrous dichloromethane solution (20 ml) was slowly dropped. After addition, removed the ice-water bath, and kept the reaction for 5 h at room temperature. The resulting solution was washed 3 times with diluted hydrochloric acid, water, and saturated brine, respectively. After concentration, raw product was filtered out, pale yellow granular crystals were obtained by recrystallization using EA/PE with a yield of 86.0% (285 mg). ^{1}H-NMR (600 MHz, DMSO-d_6): δ 8.30 (d, J = 8.6 Hz, 1H), 7.75 (d, J = 8.6 Hz, 1H), 7.72 (d, J = 8.9 Hz, 1H), 7.47 (d, J = 8.9 Hz, 1H), 5.46 (s, 1H), 3.44 (s, 2H). ^{13}C-NMR (150 MHz, DMSO-d_6): δ 153.18, 150.03, 147.87, 143.09, 140.39, 129.33, 124.17, 122.82, 121.22, 69.03, 32.16 ppm. HRMS (ESI) m/z calcd for $C_{15}H_{14}N_3O_6$ $[M+H]^+$ 332.2800, found 332.0881.

Cell culture and cellular modeling

Myocardial cells H9c2 and coronary artery smooth muscle cells HCASMC were cultured in Dulbecco's Minimum Essential Medium (DMEM) containing 10% fetal bovine serum (FBS). Blank group cells were commonly cultured under normal oxygen. Model group cells were cultured under hypoxia condition. In hypoxia treatment, cells were starved 2h in DMEM without FBS and glucose in cell incubator (37°C, 21% O_2, 5% CO_2), treated with or without drugs for 2h, and finally subjected to hypoxic conditions (37°C, 1% O_2, 5% CO_2) for 4h. The cells were rapidly placed on ice to stop cell metabolism.

Cell viability assay

After hypoxia treatment or normal cell culture with drugs, H9c2 cell viability was assessed with Cell Counting Kit-8 (CCK-8 kit, Beyotime Institute of Biotechnology, Shanghai, China). The CCK-8 kit contains 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazoliumsodiumsalt (WST-8), and in the presence of the electron carrier 1-methoxy phenazine methosulfate (1-Methoxy PMS). WST-8 is reduced by intracellular dehydrogenase to generate water-soluble orange-yellow formazan which can be dissolved in the tissue culture medium, and the amount of formazan is proportional to the number of living cells. Cells were harvested and stained with working solution WST-8 in a microplate with 96 wells. The absorbance intensity at 450 nm was measured with a microplate reader. The cell viability was calculated as number of unstained cells divided by total number of cells and expressed as a percentage.

NO release in cells

NO release in both H9c2 and HCASMC cells was measured with griess reagents in NO Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). After hypoxia treatment, cell lysis solutions were collected. Griess reagents were added following the manufacturer's instructions. The absorbance at 560 nm is detected by the microplate reader. The samples were further detected by ultraviolet (UV) spectrophotometer. The trend of absorption intensity at 560 nm was determined from the UV absorption peak.

NO fluorescent probe DAF-FM DA was also used to detect intracellular NO levels *in situ* (Ex: 495 nm, Em: 515 nm). DAF-FM DA enters the cell and could be catalyzed by esterase to form membrane-impermeable DAF-FM. DAF-FM has weak autofluorescence. After combining with NO, the fluorescence intensity of DAF-FM increases significantly. Therefore, the fluorescence intensity can be used to determine NO level in cells. Cells incubated with DAF-FM DA were collected for fluorescence spectroscopy detection, which was further used to analyze NO levels in different cell groups.

Cell apoptosis detection assay

In this study, Annexin V-FITC or Hoechst 33258 apoptosis detection kit was used to detect cell apoptosis or necrosis caused by hypoxia. Annexin V labeled with a green fluorescent probe FITC, Annexin V-FITC, can be used to detect phosphatidyserine eversion by laser scanning confocal microscope or flow cytometry, which is an important feature of cell apoptosis. Hoechst 33258 could also be used to detect cell apoptosis with blue fluorescence. PI can stain dead cells or cells that have lost their membrane integrity late in apoptosis, presenting red fluorescence. H9c2 cells were cultured in a six-well plate overnight. After the cells adhered, compounds (10 μmol/L) were added and incubated for 4h under hypoxia condition. Cells were washed with PBS for 3 times and resuspended in 1 mL cell staining buffer, added Annexin V-FITC (5 μL), Propidium iodide (PI, 10 μL) and incubated for 30 min at r.t., and then detected by flow cytometry. The results of cell double staining test were analyzed with FCS Express V3 software. Cells were also detected by laser scanning confocal microscope. Microscopy was performed on a Leica TCS NT confocal scanner equipped with an ArKr-Laser on the Leica DM IRBE inverted microscope (lens: HCX PlanApo 63x oil/NA1.32).

ATP production detection

ATP production in H9c2 cells was measured using ATP Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's instructions. The Kit is based on the fact that firefly luciferase catalyzes the fluorescence production of fluorescein, which requires ATP to provide energy, and the fluorescence is proportional to the concentration of ATP, thereby detecting ATP concentration. After hypoxia treatment, the culture dish was placed on ice rapidly, and all metabolic activities of the cells were stopped. The culture solution was aspirated and the cell lysate was added, cell lysis solution was collected, centrifuged at 4 degrees 12000 g for 5 min, and the supernatant was taken for detection. The RLU value (The fluorescence intensity) was measured by multimode reader (luminometer). Solutions were evaluated in triplicate.

ROS level

DCFH-DA was used as a fluorescent probe to detect the content of ROS in H9c2 cells. DCFH-DA is not fluorescent. It easily penetrates into the cell, and is hydrolyzed to DCFH by the esterase. Non-fluorescent DCFH can no longer penetrate the cell membrane, so DCFH-DA is easily loaded as fluorescence probe. ROS in cells can oxidize DCFH to fluorescent DCF, and the fluorescence intensity can indicate the level of ROS content in cells. Rosup was used as a positive control group. ROS level was detected by flow cytometry.

Nitroreductase (NTR) activity assay

Nitroreductase (NTR) activities were measured by NTR Elisa kit (Nanjing Boyan Biotechnology Co. Ltd, China), following the kit instruction. The specimen or standard substance, biotinylated antibody against this index and HRP-labeled avidin are successively added into the microporous plate. After thorough washing, the substrate TMB is used for color development. The depth of the color was positively correlated with the index in the sample. The absorbance (OD) was measured at 450 nm with a microplate analyzer, and the NTR concentration was calculated according to the standard curve and OD values.

Protein-Protein interaction analysis by string database

String is a database of known and predicted protein-protein interactions (https://string-db.org). The interactions include direct (physical) and indirect (functional) associations; they stem from computational prediction, from knowledge transfer between organisms, and from interactions aggregated from other (primary) databases.

Western blot of cell protein

Cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer, supplemented with 1 mmol/L PMSF to extract protein. The lysates was centrifuged at 12000 g for 15 min at 4°C, and the concentration was quantified by Bicinchoninic Acid Protein Assay kit (Biosky Biotechnology Corporation, Nanjing, China). Equivalent amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking at room temperature for 3h, the membranes were immunoblotted with primary antibody (anti-TSP1, proteintech, 1:500, 18304-1-AP; anti-VEGF, proteintech, 1:500, 19003-1-AP; anti-ACE, proteintech, 1:500, 24743-1-AP; anti-mTORC1, Abcam, 1:1000, ab120224; anti-TSC2-P, Abgent, 1:1000, A-AP3825a; anti-GAPDH, Bioworld

Technology, 1:1500, AP0063) respectively at 4°C overnight, followed by incubation with HRP-conjugated secondary antibody (Goat Anti-Rabbit IgG (H+L) HRP, Proteintech, 1:5000, SA00001-2, or Goat Anti-Mouse IgG (H+L) HRP, Proteintech, 1:5000, SA00001-1). Equal loading was verified by incubation with anti-GAPDH. The blots were visualized using ECL, and the signals were quantified by densitometry (Image-Pro Plus 6.0). Relevant band intensities were quantified after normalization to the amount of loading control protein.

Electron clouds distribution simulation

Calculate the charge of each atom in compounds with Chem3D Ultra 10.0 software, assign a surface to the compound, spread the calculated charges on the surface, and distinguish the quantity of electric charges by colors to get the electron cloud distribution of compounds Nno, Hano and Hano reduction product Hano-NH_2.

HPLC and LC-MS assay

In the NTR enzymatic reaction, Hano (0.5 mM) was dissolved in DMSO (10 μL) and H_2O (2 mL), NTR (30 U/mL) and NADH (500 μM, 1 μL) were added at 37 °C. The reduction of Hano to Hano-NH_2 was tracked by HPLC and two compounds at 10.4 and 18.0 min were identified as Hano-NH_2 and Hano by LC-MS. The methanol gradient in mobile phase (methanol/water) increased from 10% to 100%, and the methanol increase gradient was 4% per minute.

Coronary heart disease mouse modeling

Kunming male mice (4–6 weeks) were purchased from Shanghai Jiesijie Laboratory Animal Co., LTD. The animal care and experimental procedures were approved by Animal Ethics Committee of Southeast University.

Male kunming mice were randomly divided into four groups, six in each group, respectively. Blank group (normal feeding), model group (ISO modeling, intraperitoneal injection), two medicated groups (20 mg/kg Hano or Nno and ISO, intraperitoneal injection). The hypoxia modeling method was as follows: In the pre-experimental stage, both high and low concentrations were used for modeling, results showed that 20 mg/kg continuous administration for three days could not cause significant myocardial hypoxia injury, and 85 mg/kg showed obvious effect. 85 mg/kg isoproterenol (ISO) was injected subcutaneously once a day for three consecutive days. Isoproterenol (ISO) is a β-adrenergic agonist that could induces severe stress to cardiomyocyte, which can lead to the loss of myocardial integrity through oxygen deficit.[1] ISO-induced myocardial injury serves as a well standardized model to study the beneficial effects of drugs and cardiac function.[2] In the blank group, an equal amount of 0.5% sodium carboxymethyl cellulose was injected. In the medicated groups, drugs were given 20 mg/kg 2h before the injection of ISO. After the ISO injection on the third day for 1 h, the mice were sacrificed by cervical spine, the heart was quickly removed, detected by TTC and HE staining, or stored at −80°C for subsequent western blot experiments, or placed in 4% paraformaldehyde for Immunohistochemical (IHC) detection. Myocardial tissue protein was attracted for WB detection. Blood was taken for the detection of myocardial enzymes.

TTC staining

The mice hearts were isolated followed by 1% triphenyltetrazolium chloride (TTC) staining to

test infarct size. TTC is a proton receptor of the pyridine-nucleoside structure enzyme system in the respiratory chain. It reacts with dehydrogenases in normal tissues and turns red, while dehydrogenase activity in ischemic tissues decreases and turns white. The heart was washed by PBS and cut into 5 pieces, and then stained in TTC solution with a water bath at 37°C for 15−30 min. Pictures were taken under the microscope to calculate the percentage of myocardial infarction area.

HE staining

Hematoxylin-eosin (HE) staining was used to observe the pathological changes of myocardial tissue. The hearts of mice were fixed by soaking in 4% paraformaldehyde, and paraffin sections were prepared for staining. They were observed by light microscope and images were collected for analysis.

NO level of heart homogenate

After finishing the modeling and medicating experiments, the hearts of mice were extracted, and tissue homogenization was carried out with a homogenizer at 4°C. The supernatant was extracted by centrifugation (12000 rpm for 15 min at 4°C) for NO detection. NO release was also measured with griess reagents in NO Assay Kit.

NO level variations in mouse serum at 0–12h

NO level variations in mouse serum were measured with griess reagents in NO Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). NO as a chemically active agent can be quickly converted to nitrate NO_3^- and NO_2^- in vivo, while NO_2^- is further converted to NO_3^-. In this method, the griess reagent was used to reduce NO_3^- to NO_2^- that has a characteristic absorption at 560 nm. After mice were injected 10 mg/kg Hano or Nno, serum was collected from 0 to 12h by eyeball blood collection. Griess reagents were added following the manufacturer's instructions. The absorbance at 560 nm is detected by the microplate reader.

Immunohistochemisty (IHC) assay

The hearts were isolated from mice of each group and fixed with 4% paraformaldehyde. After dehydration and the hearts were embedded in paraffin and then cut into thickness and affixed onto the slides. After blocking with 3% peroxide-methanol at room temperature for 25 min, the slides were incubated with primary antibody (anti-mTORC1, Abcam, 1:50, ab120224) overnight at 4°C, followed by the incubation with HRP-conjugated secondary antibody for 1h at room temperature. Nuclei were stained with DAPI. After dehydration and stabilization, staining was detected by NanoZoomer 2.0-RS (Hamamatsu, Japan).

Western blot of heart tissue protein

Heart tissue protein preparation: After the end of the administration and modeling experiments, quickly remove the heart, cut out the ventricular tissue, weigh a certain amount of tissue and cut it, and add the lysate containing the protease inhibitor at a ratio of 1:9 (mg:μL). Grind on ice for 40 times using a glass homogenizer. The homogenate was collected, centrifuged at 12000 rpm for 15 min at 4°C, and the supernatant was collected. A small amount of the supernatant was collected for protein quantification according to the BCA protein content

determination kit. Add the remaining supernatant to 1/2 volume of 6× loading buffer, mix well, boil in boiling water for 10 min, cool it for WB detection, or put it in −80°C refrigerator for future use.

Myocardial enzyme level in mouse serum

Creatine kinase MB isoenzyme (CK-MB) is one of the most important indicators of diagnosing myocardial injury through detecting enzyme levels in serum. Cardiac troponin I (cTNI) is a specific antigen of cardiomyocytes, which is degraded from the myocardial fiber when the cardiomyocytes are injured, and the content significantly increases. cTNI is another marker of myocardial injury. The contents of CK-MB and cTNI were detected by the corresponding kit and the automatic biochemical instrument.

Statistical analysis

The results were expressed as the mean ± S.D.. The significance of differences was analyzed by one-way ANOVA followed by the Bonferroni correction. A value of $P < 0.05$ was considered statistically significant.

Results and Discussion

Compound Hano and intermediate 1 were characterized by ^{1}H-NMR, ^{13}C-NMR and ESI/TOF MS (Figure S1-Figure S4). All characterization results indicate that the target compound Hano coincides with the structure as designed. The purity of Hano was 98.37 % detected by HPLC (Figure S5, Table S2).

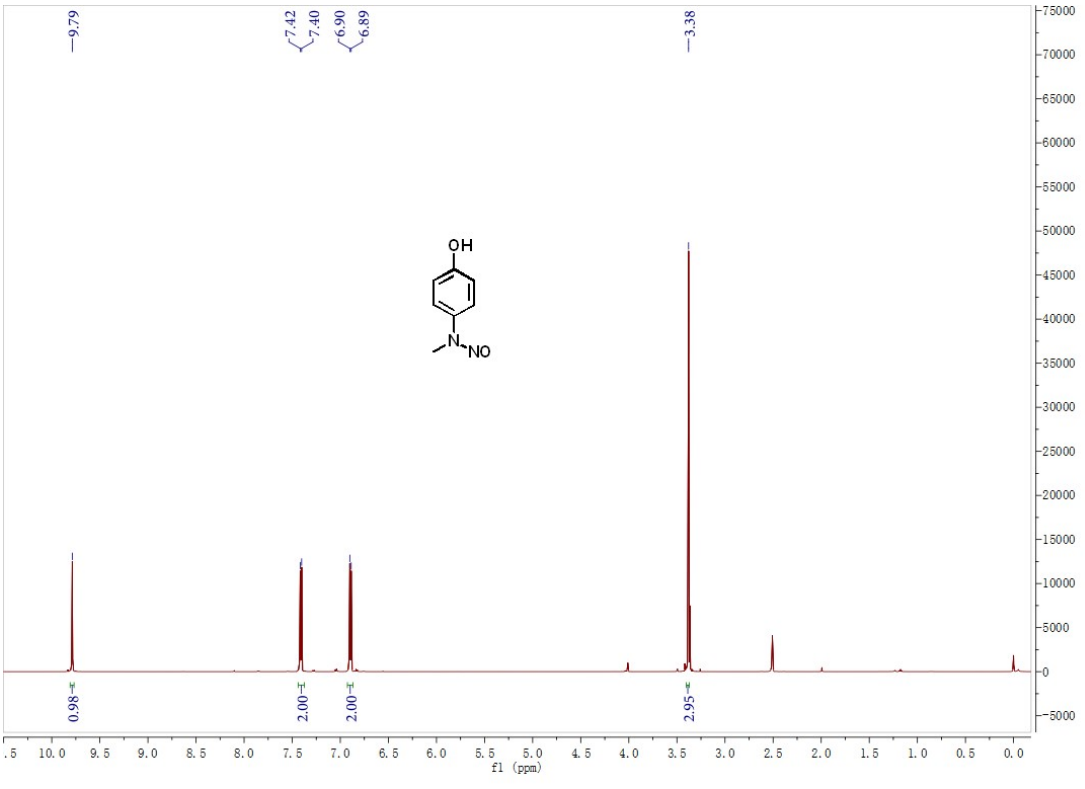


Figure S1. ^{1}H-NMR spectrum of Intermediate 1.

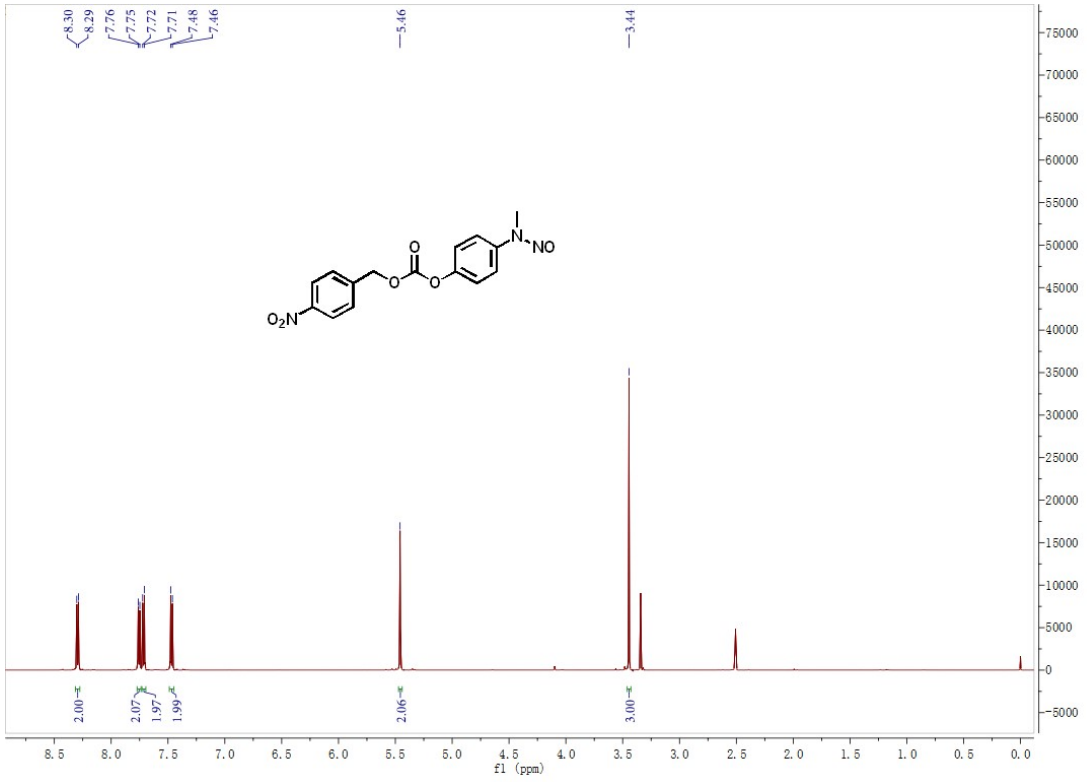


Figure S2. ^{1}H-NMR spectrum of Hano.

Figure S3. ^{13}C-NMR spectrum of Hano.

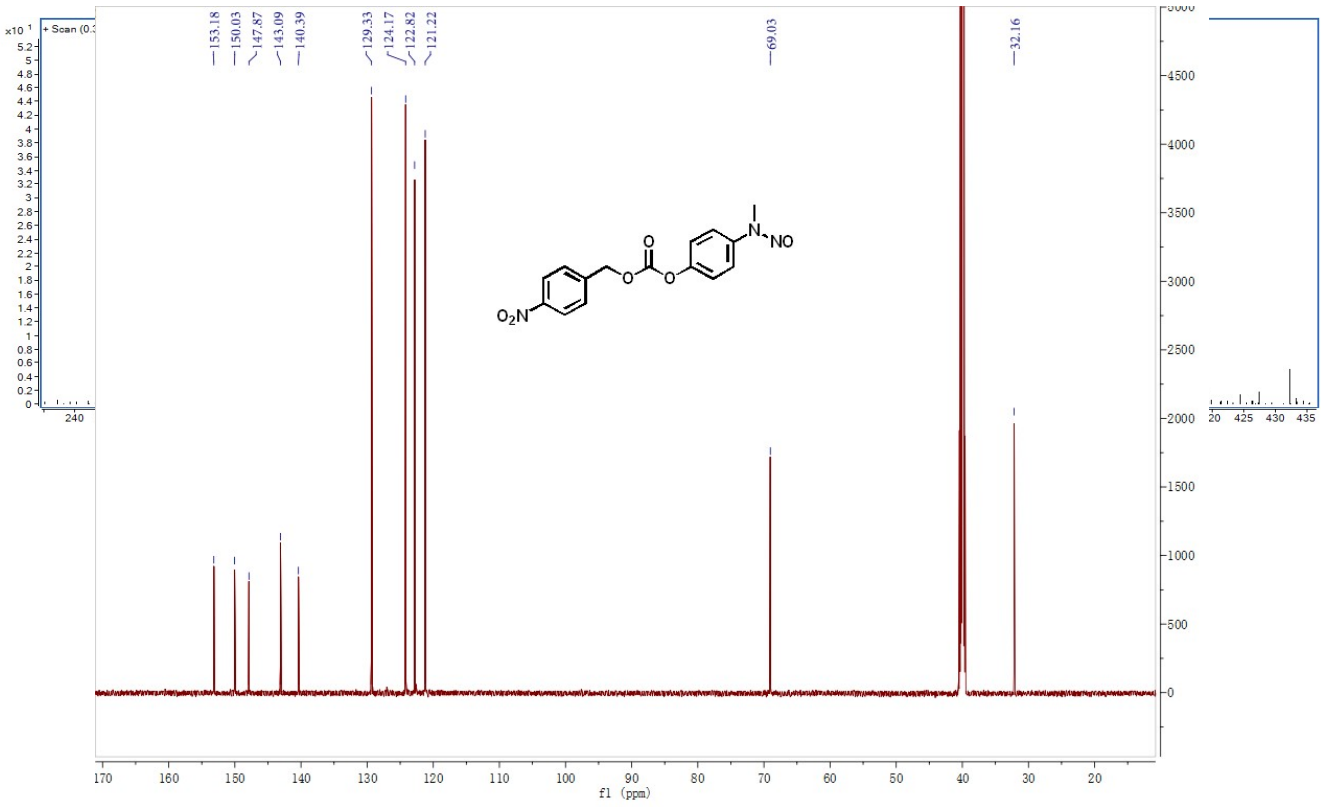


Figure S4. ESI/TOF MS spectrum of Hano.

Table S1. Assignments of the observed peaks in the ESI/TOF Ms spectrum of Hano.

Species	Formula	Observed m/z	Calculated m/z
$[Hano]H^+$	$C_{15}H_{14}N_3O_6$	332.08812–333.51257	332
$[Hano](C_2H_5OH)H^+$	$C_{17}H_{20}N_3O_7$	375.16313–378.63574	378
$[Hano](C_2H_5OH)(H_2O)H^+$	$C_{17}H_{22}N_3O_8$	396.25582–397.14479	396

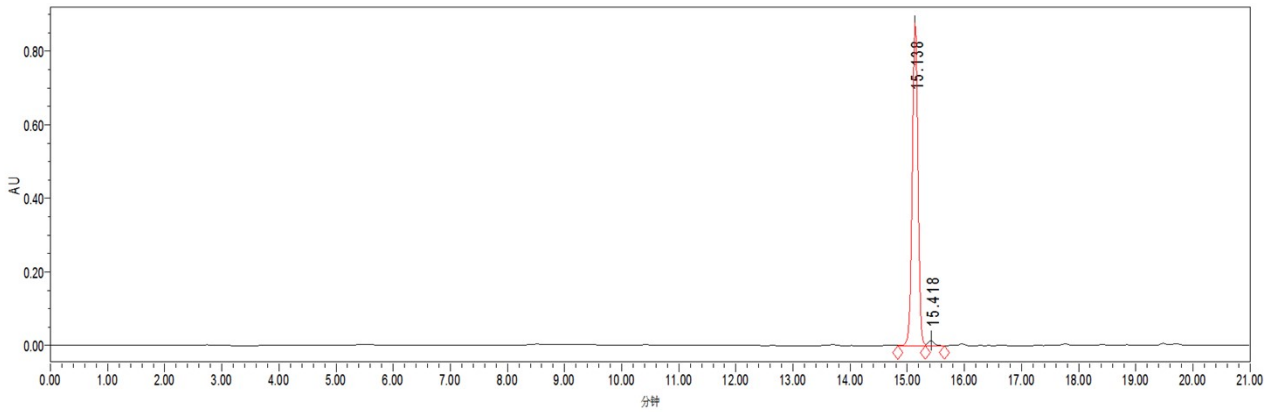
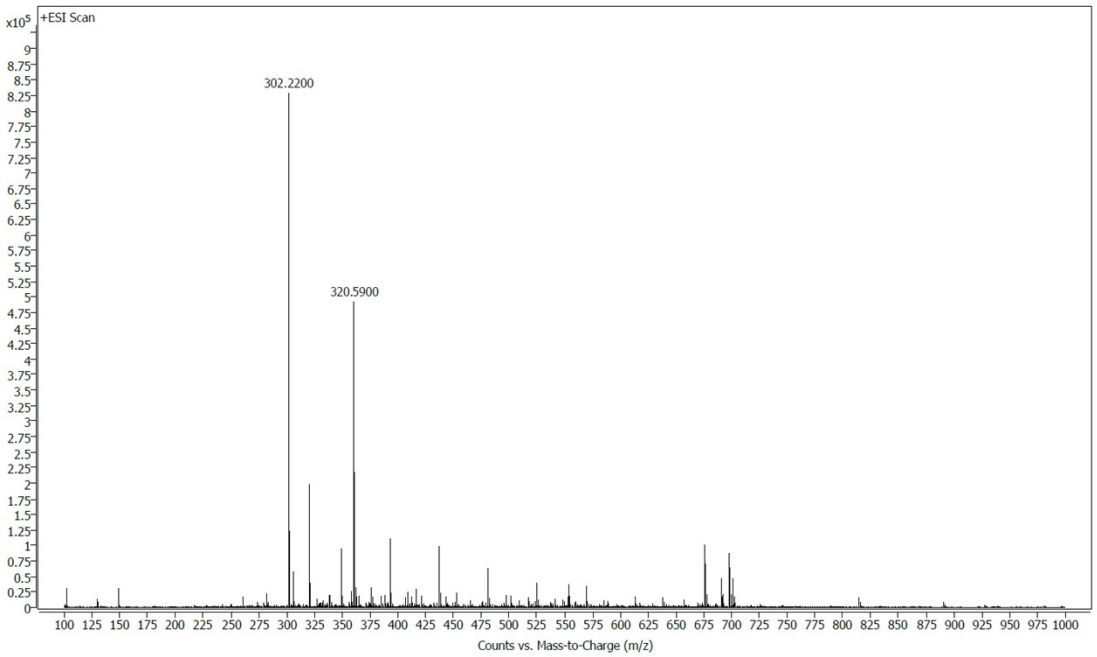


Figure S5. The purity of Hano detected by HPLC.

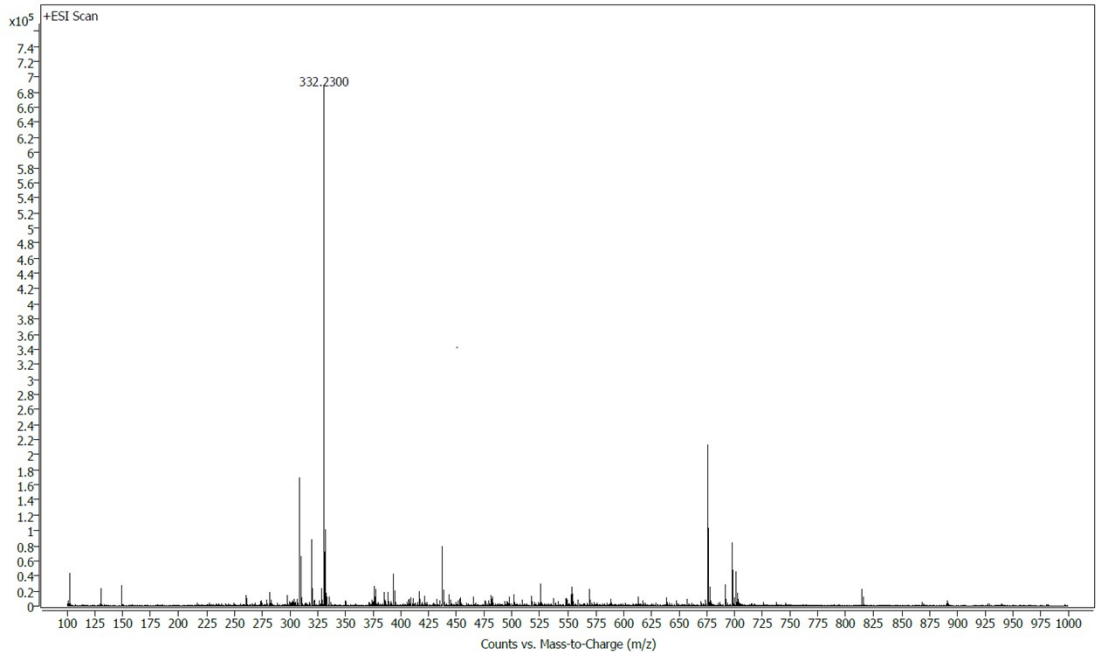
Table S2. Retention time and areas of the observed peaks in the HPLC diagram of Hano.

	RT (min)	Area	% Area	Height	% Height
1	15.138	6103866	98.21	876619	98.37
2	15.418	111080	1.79	14537	1.63



Species	Formula	Observed m/z	Calculated m/z
$[Hano\text{-}NH_2]H^+$	$C_{15}H_{16}N_3O_4$	302.11281–303.16452	302.2200
$[Hano\text{-}NH_2](H_2O)H^+$	$C_{15}H_{18}N_3O_5$	320.33167–321.28497	320.5900

Figure S6. MS spectrum of the compound at 10.4 min detected by LC-MS and peak assignments.



Species	Formula	Observed m/z	Calculated m/z
$[Hano]H^+$	$C_{15}H_{14}N_3O_6$	332.08812–333.51257	332.2300

Figure S7. MS spectrum of the compound at 18.0 min detected by LC-MS and peak assignments.

References

[1] O. E. Osadchii, *Heart Fail. Rev.* **2007**, 12, 66–86.

[2] P. Allawadhi, A. Khurana, N. Sayed, P. Kumari, C. Godugu, *Phytother. Res.* **2018**, *32*, 1908–1932.