Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Target Identification and Occupancy Measurement of Necrosis Avid Agent rhein using Bioorthogonal chemistry-enabling probes

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1. Materials and Methods

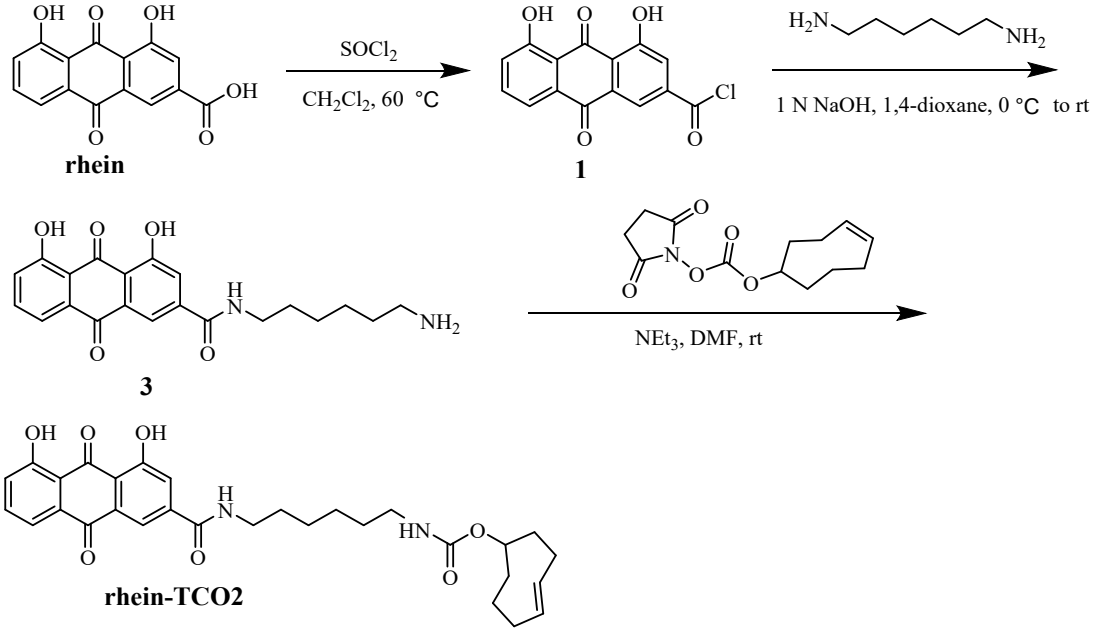
1.1 General

Unless otherwise stated, all reactions were carried out in anhydrous solvents under N_2 atmosphere and were monitored by thin-layer chromatography (TLC) on silica gelprecoated glass plates, and spots were visualized with UV light. All solvents were purchased from commercial suppliers and were purified according to standard procedures. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 400 spectrometers in DMSO-d_6 (^{1}H NMR δ 2.50, ^{13}C NMR δ 39.52) at room temperature, and tetramethylsilane (TMS) was used as an internal reference. Chemical shifts were reported in parts per million (ppm, δ units), and coupling constants (J) were expressed in hertz. Electrospray-ionization mass spectrometry (ESI-MS) was carried out on an HP1100 mass spectrometer (Agilent, Santa Clara, CA, USA). Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Waters Alliance 2695 Separations module equipped with Waters 2998 UV/visible detector (Waters Cooperation, Milford, MA, USA) and Berthold HERM LB500 radiometric detector (Millipore, Billerica, MA, USA). The RP-HPLC column was GRACE Alltima C18 analytical column (250 mm × 4.6 mm, 5 μm). Sodium iodide's ($Na^{131}I$) radionuclidic purity was >99% and specific activity was 370 MBq/mL, which was supplied by HTA Co., Ltd. (Beijing, China). Calf thymus DNA (Ct-DNA) and ethidium bromide (EB) were purchased from Sigma Aldrich, USA. Plasmid DNA (pUC19, 2686 bp) was purchased from Thermo Fisher Scientific Co., USA. The human lung cancer A549 cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) incubated in 5% CO_2 humidified atmosphere at 37 °C. Kunming mice (male, 24–28 g) and Sprague-Dawley rats (male, 260–280 g) were provided by the Experimental Animal Center of Academy of Military Medical Sciences. The Institutional Animal Care and Use Committee approved the project. The care and treatment of all animals were maintained in accordance with NIH publication No.85–23 (revised in 1996) on "Principles of

laboratory animal care".

1.2 Chemicals

Procedure for the Synthesis of compound **rhein-TCO2**.

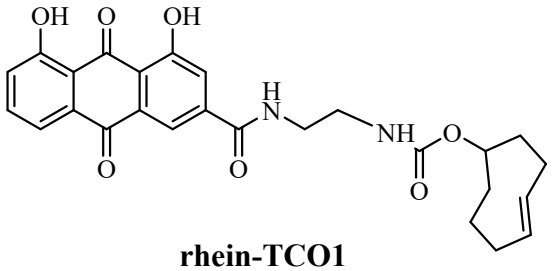


To the solution of rhein (1.0 g, 3.52 mmol) in dry CH_2Cl_2 (80 mL), $SOCl_2$ (20.0 mL) was added dropwise at 0 °C. The resulting mixture was stirred at 60 °C under N_2 atmosphere overnight. After evaporation, the obtained acyl chloride **1** was directly used in the next reaction. Subsequently, to a solution of 1,6-hexamethylenediamine (4.1 g, 35.2 mmol) and 1 N NaOH (10.0 mL) in 1,4-dioxane (80 mL). Then temperature of the reaction system was cooled to 0 °C and was added acyl chloride **1** (dissolved in 40 mL 1,4-dioxane) dropwise. The resulting solution was stirred at room temperature for 3 h. The solvent was evaporated to give purple oil amide **2**.

The amide **2** (0.20 g, 0.52 mmol) was dissolved in DMF (5 ml). To this solution was added (*E*)-cylooct-4-en-1-yl (2,5-dioxopyrrolidin-1-yl) carbonate (0.14 g, 0.52 mmol) and triethylamine (0.16 g, 1.56 mmol). The mixture stirred at room temperature for 3 h. The mixture was added water (10 mL) and extracted with CH_2Cl_2 (5 mL × 3 times). The organic layer was dried over $MgSO_4$, filtered, and concentrated under vacuum. The residue was purified by chromatography (CH_2Cl_2/MeOH = 80:1) to afford compound **rhein-TCO2** (0.21 g, 11% yield), MS m/z: 535.2$[M+H]^+$, yellow solid. ^1H

NMR (400 MHz, DMSO-d_6) δ 11.87 (d, *J* = 4.2 Hz, 2H, OH), 8.88 (t, *J* = 5.5 Hz, 1H, NH), 8.09 (d, *J* = 1.4 Hz, 1H, Ar-H), 7.80 (t, *J* = 7.9 Hz, 1H, NH), 7.72 (dd, *J* = 13.5, 4.4 Hz, 2H, Ar-H), 7.38 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.00 (t, *J* = 5.6 Hz, 1H, Ar-H), 5.67-5.55 (m, 2H, CH), 4.58-4.50 (m, 1H, OCH), 3.27 (dd, *J* = 12.8, 6.7 Hz, 2H, NCH_2), 2.94 (dd, *J* = 12.8, 6.5 Hz, 2H, NCH_2), 2.28 (d, *J* = 8.9 Hz, 1H, CH), 2.10 (dd, *J* = 13.2, 6.8 Hz, 2H, CH_2), 2.06-1.98 (m, 1H, CH), 1.77 (ddd, *J* = 14.4, 9.3, 3.6 Hz, 2H, CH_2), 1.65-1.59 (m, 1H, CH), 1.56-1.43 (m, 5H, CH+CH_2), 1.42-1.36 (m, 2H, CH_2), 1.32-1.26 (m, 4H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 191.9, 181.5, 164.2, 161.9, 161.6, 156.3, 142.3, 138.0, 133.9, 133.7, 130.1, 129.9, 124.9, 122.8, 119.9, 118.1, 117.8, 116.5, 74.7, 34.2 (2C), 29.9, 29.3, 26.7, 26.5 (3C), 25.6, 25.0, 22.4. HRMS calcd for $C_{30}H_{35}N_2O_7$ $[M+H]^+$ 535.2444, found 535.2436.

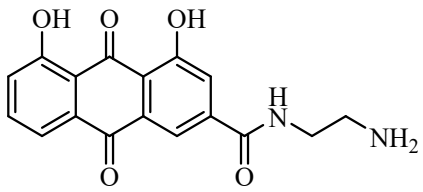
Compound **rhein-TCO1**



rhein-TCO1

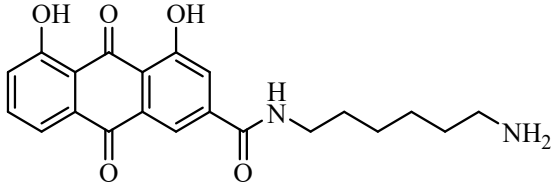
Compound **rhein-TCO1** was prepared from the general procedure of compound **rhein-TCO2** and purified via column chromatography with CH_2Cl_2/MeOH = 80:1 as the eluent. Yellow solid (0.50 g, 15%), MS m/z: 477.5$[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.89 (t, *J* = 5.4 Hz, 1H, NH), 8.08 (s, 1H, Ar-H), 7.80 (d, *J* = 8.1 Hz, 1H, NH), 7.72 (d, *J* = 7.0 Hz, 2H, Ar-H), 7.38 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.15 (t, *J* = 5.8 Hz, 1H, Ar-H), 5.68-5.54 (m, 2H, CH), 4.56 (dd, *J* = 12.9, 5.2 Hz, 1H, OCH), 3.32 (d, *J* = 5.9 Hz, 2H, NCH_2), 3.19-3.14 (m, 2H, NCH_2), 2.31-2.22 (m, 1H, CH), 2.11-2.06 (m, 2H, CH_2), 2.04-1.97 (m, 1H, CH), 1.84-1.70 (m, 2H, CH_2), 1.66-1.61 (m, 1H, CH), 1.56-1.40 (m, 3H, CH+CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 191.9, 181.6, 164.7, 161.8, 161.6, 156.5, 142.2, 138.0, 133.9, 133.7, 130.1, 129.9, 125.0, 123.0, 119.9, 118.1, 117.9, 116.5, 75.1, 34.2 (2C), 34.1, 25.6 (2C), 25.0, 22.4. HRMS calcd for $C_{26}H_{26}N_2O_7Na$ $[M+23]^+$ 501.1638, found 501.1631.

Compound **2**



Known compound, ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.04 (s, 1H, NH); 8.18 (s, 1H, Ar-H); 7.84 (dd, 1H, J = 5.3, 7.9 Hz, NH); 7.74-7.71 (m, 2H, Ar-H); 7.45 (d, 1H, J = 5.3 Hz, Ar-H); 3.58 (t, 2H, J = 3.6 Hz, NCH_2); 3.07 (t, 2H, J = 3.8 Hz, NCH_2). MS m/z: 328.1$[M+H]^+$.

Compound **3**



Known compound, ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.90(s, 2H, OH); 8.92(s, 1H, NH); 8.17(s, 1H, Ar-H); 7.88(dd, 1H, J = 6.1, 7.5 Hz, NH); 7.77-7.79(m, 2H, Ar-H); 7.42 (d, 1H, J = 7.4 Hz, Ar-H); 3.59(m, 2H, NCH_2); 2.83(m, 2H, NCH_2); 1.60(m, 4H, CH_2); 1.37(m, 4H, CH_2). MS m/z: 383.2$[M+H]^+$.

1.3 Radiochemistry and in Vitro Stability

Considering the high detection sensitivity and convenience of nuclear medicine procedures, radioisotope iodine-131 was used to label rhein, rhein-TCO1 and rhein-TCO2 for the evaluation of necrosis affinity in vitro and in vivo as we reported previously. The radiochemical purity was determined by HPLC using methanol/0.1% phosphoric acid in water (80:20, v/v) at a flow rate of 1 mL/min under 30 °C. Iodogen (1, 2, 4, 6-tetrachloro-3a, 6a-diphenylglycouracil; Sigma) was dissolved in dichloromethane and deposited on the wall of tubes as a thin film. Radioiodination was initiated by adding dimethylsulfoxide (DMSO) solutions of rhein, rhein-TCO1 and rhein-TCO2 (1mg/mL) and $Na^{131}I$ solutions (4:1, v/v) into Iodogen-coated tube, adjusting pH with phosphate buffered saline (PBS, pH 6.8).

For in vitro serum stability analysis, both tracers were intact for 72 h at 37 °C in rat serum, suggesting that they had very good stability in vitro.

1.4 In Vitro Cell Uptake Assay

The necrosis affinity of ^{131}I-rhein, ^{131}I-rhein-TCO1 and ^{131}I-rhein-TCO2 were assessed in vitro with Human lung carcinoma necrotic A549 cell models. A549 cells were seeded into 6-well plates with 2 × 10^5 cells/well for 24h. Necrotic cells were induced under intense hyperthermia at 57 °C for 1 h according to Pereket al. Nontreated cells were used as a control. The percentage of necrotic cells reached in the range of 75~85% as determined by flow cytometry.

Normal A549 cells and Necrotic A549 cells were incubated with ^{131}I-rhein, ^{131}I-rhein-TCO1 and ^{131}I-rhein-TCO2 (37 KBq/mL) for 15 min and washed twice with PBS. The culture supernatant and cells were collected by centrifuging at 12000 rpm for 15 min, respectively. The radioactivity was counted using an automated gamma counter. The data were expressed as the percentage uptake per 10^5 cells (%uptake/10^5 cells). Experiments were performed in triplicate.

1.5 Biodistribution Studies and Blocking Experiment in Mice

Muscular necrosis mice model. All animals were given 0.12% potassium iodide in drinking water from 3 days before the experiment till the end of experiment to protect the thyroid gland from taking up free ^{131}I. Each mouse was intramuscularly injected with 0.1 mL absolute alcohol in the left hind limb to induce muscular necrosis.

For biodistribution studies, Kunming mice (n = 5/group) were intravenously administered with ^{131}I-rhein, ^{131}I-rhein-TCO1 and ^{131}I-rhein-TCO2 (3.7 MBq/kg, 0.2 mg/kg) under anesthesia and sacrificed at 6 h p.i., respectively. Organs of interest were removed, weighed, and counted for radioactivity using a γ counter. Uptake of the tracers was calculated as the percentage of the injected dose per gram of tissue (% ID/g) with the values expressed as mean ± standard deviation (SD). The decay-corrected activity per mass of tissue was calculated.

To verify whether ^{131}I-rhein-TCO2 with the azide-containing linker has effect on necrosis affinity of rhein, blocking experiment were also performed in necrotic muscle bearing mice. For the blocking studies, mice (n = 5) were injected with excess rhein (10

mg/kg) 1 h prior to injection of ^{131}I-rhein-TCO2 and sacrificed at 6 h p.i. of tracer. Biodistribution studies were then performed as described for other mice groups.

1.6 Autoradiography and Histochemical Staining in Vitro

For autoradiography studies, representative tissues of necrotic muscle and normal muscle at 6h p.i. of tracer were obtained and washed thoroughly with 0.9% saline (4°C) to remove blood pool activity. Sections of 10 μm were cut using a cryostat microtome (Shandon Cryotome FSE; Thermo Fisher Scientific Co., MA) at −20 °C and were puted on glass slides. Autoradiographs of these slides were obtained by exposing the sections for 6−24 h to a high performance phosphor screen (Cyclone; Canberra-Packard, Ontario, Canada). After the exposure, the screen was read using a Phosphor Imager scanner and analyzed using Optiquant software (Cyclone; Canberra-Packard, Meriden, CT). Relative tracer concentration was estimated by regions of interest (ROI) analysis for the necrotic and viable tissues on all autoradiographs. The slides were stained with hematoxylin−eosin (H&E) using a conventional procedure and digitally photographed to confirm the presence or absence of necrosis. Photomicrographs were obtained from an optical microscope (Axioskop; Zeiss, Oberkochen, Germany) with magnification at ×200.

1.7 Confocal Microscopy imaging

A549 cells were seeded into 2 mL of medium per dishs (Thermo Fisher Scientific Co., USA) with 2×10^5 cells·mL^{-1} suspended, incubated 24h. For necrosis A549 groups, A549 cells were induced after incubation of the cells for 1 h under intense hyperthermia at 57 °C, and then treated with rhein-TCO1 or TCO2 (50 μM) for 3 h. After rinsing with chilled PBS (3×), cells were fixed by treatment with 3.7% formaldehyde solution (in PBS, pH 7.4) for 15 min at room temperature and subsequently washed with a 3% solution of bovine serum albumin (BSA; Sigma) in PBS (pH 7.4) twice for 10 min. Cells were permeabilized by treatment with 0.5% Triton X-100 (in PBS, pH 7.4) at room temperature for 20 min, and quenched with 3% BSA in PBS (2 × 10 min). Then, cells were incubated with 250 μL of Cy5-Tz (5 μM) solution at room temperature for

30 min. Cells were subjected to extensive washes: 1) 3% BSA in PBS (5 min); 2) 0.5% Triton X-100 in PBS (2 × 10 min); 3) PBS for (3 × 10 min). Nuclei were stained by 1 μg·mL-1 DAPI (Sigma) in PBS for 15 min. Three final PBS washes were performed immediately prior to image capture. For no-rhein-TCO2 control, the rhein-TCO2 solution was replaced with PBS. For normal A549 groups, necrotic A549 cells were replaced with normal A549 cells.

Images were collected using Olympus FV3000 confocal microscope (Olympus, Japan) using either a 600x objective lens. All images were acquired in multi-track configuration mode to minimize excitation cross talk and emission bleed-through. We utilized a 405 nm laser line for DAPI and a 647 nm laser line for Cy5.

1.8 In gel fluorescence imaging

PUC19 DNA (2686 bp, Keygentec, Nanjing, China) was purchased and linearized with restriction endonuclease BamH I (Keygentec, Nanjing, China) at 37 °C overnight. BamH I enzyme was inactivated at 65 °C for 1 h, and the DNA was purified with the Steady Pure PCR DNA Purification Kit (Accurate Biotechnology, Hunan, China). DNA concentrations and purity were determined spectrophotometrically at 260 nm and 280nm. Samples preparation for group A: the linearized pUC19 DNA (50 μM) was used without rhein-TCO2 and Cy5-Tz for control. Samples preparation for group B and C: the linearized pUC19 DNA (50 μM) was incubated with rhein-TCO2 (50 μM) for 12 h, and then reacted with Cy5-Tz (5 μM) for 30min. The reaction mixture were purified with the Steady Pure PCR DNA Purification Kit (Accurate Biotechnology, Hunan, China) to remove remove small molecules that don't attached to DNA. Samples preparation for group D: the linearized pUC19 DNA (50 μM) was incubated with Cy5-Tz (5 μM) for 30min in absence of rhein-TCO2 for control, and the mixture were purified with the Steady Pure PCR DNA Purification Kit before used. All the samples were stained with Cyber Green and analyzed by agarose gel (1 %) electrophoresis and visualized through in-gel fluorescence using Sapphire biomolecular imager (Azone biosystems, California, USA). Fluorescence images were collected with exciting at a 488 nm and a 647nm.

1.9 Absorption spectra study of DNA, RNA and rhein, rhein-TCO2

Ct-DNA or RNA were purchased from sigma and prepared freshly in Tris-HCl buffer (50mM, pH 7.4). The purity of the DNA or RNA was checked by observing the ratio of the absorbance at 260/280 nm. The solution gave the A260/A280 in the range of 1.8–1.9:1, which indicated that DNA or RNA were sufficiently free of protein. The concentration of Ct-DNA or RNA was determined spectrophotometrically by employing an extinction coefficient of 6600 M^{-1} cm^{-1} (for DNA) and 7700 M^{-1} cm^{-1} (for RNA) at 260 nm. DNA and RNA solutions were stored at 4°C. Rhein and rhein-TCO2 were dissolved in DMSO.

The UV spectra were recorded with a Cary 60 UV-visible spectrophotometer (Agilent Technologies Inc., California, USA) using a 1 cm × 1 cm quartz cuvettes. The spectra of rhein-TCO2 and Ct-DNA complex were recorded in the wavelength range of 200 – 800 nm. Experiment was carried out in the presence of fixed concentration of rhein-TCO2 in a total volume of 2 mL and titrated with varying concentration of DNA. The spectra of rhein and RNA complex were also recorded in the wavelength range of 200 – 800 nm and carried out in the presence of fixed concentration of rhein in a total volume of 2 mL and titrated with varying concentration of RNA. The binding constant (Kb) was determined from the spectroscopic titration data using the following equation:

$$[\text{DNA or RNA}] / (\varepsilon a - \varepsilon f) = [\text{DNA or RNA}] / (\varepsilon b - \varepsilon f) + 1/\text{Kb}\ (\varepsilon b - \varepsilon f)$$

Where [DNA or RNA] is the concentration of DNA or RNA, the apparent absorption coefficient (ε_a) was obtained by calculating A_{obsd}/[compound]. The terms ε_f and ε_b correspond to the extinction coefficient of free (unbound) and the fully bound compounds, respectively.

1.10 Molecular Modeling

The X-ray cocrystal structures of the double-stranded DNA and RNA were retrieved from the Protein Data Bank (DNA PDB code: 282D; RNA PDB code: 1O15). Prior to docking, the compounds were prepared using the Ligand Preparation Wizard in Maestro with default settings to enumerate chiralities, tautomers, and protonation

states. The DNA or RNA was prepared following the standard procedure. And the Glide Receptor Grid generation with default settings was used to generate a grid for docking (version 10.2). The conformational ensembles were docked flexibly using Glide with standard settings in both standard and extra precision mode. Only poses with low energy conformations and good hydrogen bond geometries were considered.

2. Supplementary Tables and figures

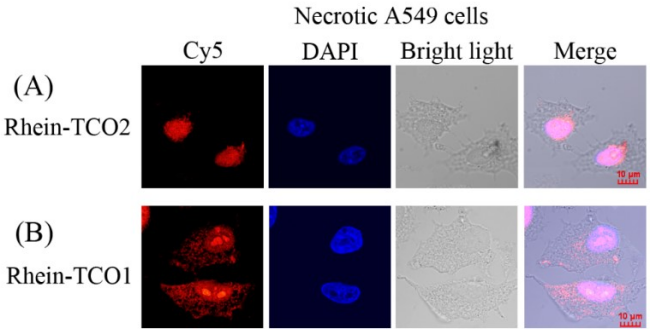


Figure S1. Confocal imaging of necrotic A549 cancer cells co-stained with DAPI (blue). Cy5 (red), DAPI (blue), corresponding bright field and the merged images are shown. (**A**) Necrotic A549 cells treated with **rhein-TCO2** (50 μM, 3 h) and subsequent reacted with Cy5-Tz for 30 min; (**B**) Necrotic A549 cells treated without **rhein-TCO1** and then reacted with Cy5-Tz for 30 min.

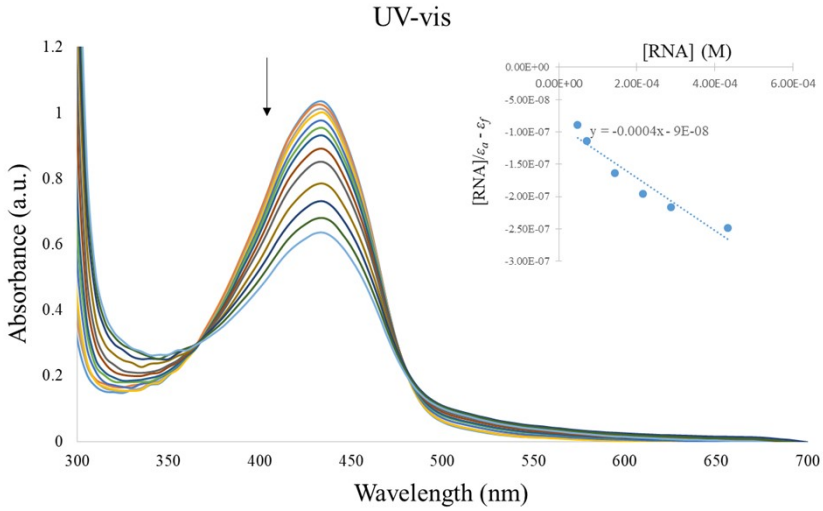


Figure S2. (A) Absorption spectra of rhein (1.0×10^{-4} M) varying with concentrations of RNA (up to down: 0, 2.4×10^{-5}, 4.8×10^{-5}, 7.2×10^{-5}, 1.44×10^{-4}, 2.16×10^{-4}, 2.88×10^{-4}, 4.32×10^{-4}, 5.76×10^{-4}, 8.64×10^{-4}, 1.15×10^{-3}, 1.44×10^{-3}, and 1.73×10^{-3} M, respectively). The arrow shows the

absorbance change upon increasing the RNA concentration. Inset: plot of [RNA]/(ε_a - ε_f) versus [RNA].

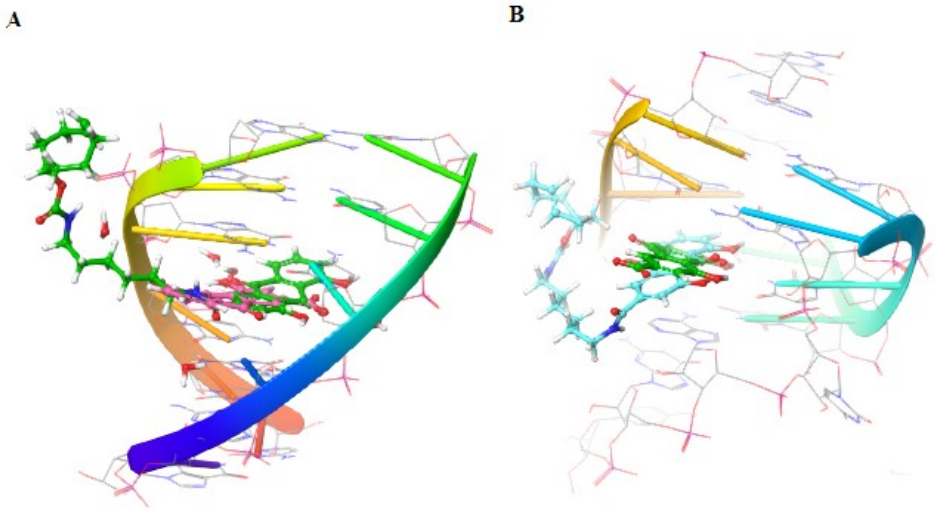


Figure S3. (A) Predicted binding modes of **rhein** (pink) and **rhein-TCO2** (green) with DNA (PDB code: 282D); (B) Predicted binding modes of **rhein** (green) and **rhein-TCO2** (blue) with RNA (PDB code: 1O15).

Table S1. Accumulation of ^{131}I-rhein and ^{131}I-rhein-TCO2 in Necrotic A549 cells and Live A549 cells[a]

	^{131}I-rhein	^{131}I-rhein-TCO1	^{131}I-rhein-TCO2	rhein blocking
Necrotic A549 cells	0.688±0.009	0.485±0.029	0.619±0.012	0.163±0.012
Live A549 cells	0.219±0.011	0.227±0.011	0.201±0.002	0.116±0.009
Ratio of Necrotic/live cells	3.14	2.14	3.08	1.41

[a] Data were displayed as average percentage uptake per 10^5 cells plus or minus standard deviation (%uptake/10^5 cells).

Table S2. Biodistribution and the Necrotic-to-Normal Tissue Ratios of ^{131}I-rhein, ^{131}I-rhein-TCO2 and rhein blocking in Muscular Necrosis Mice Models (n = 6/Group)[a]

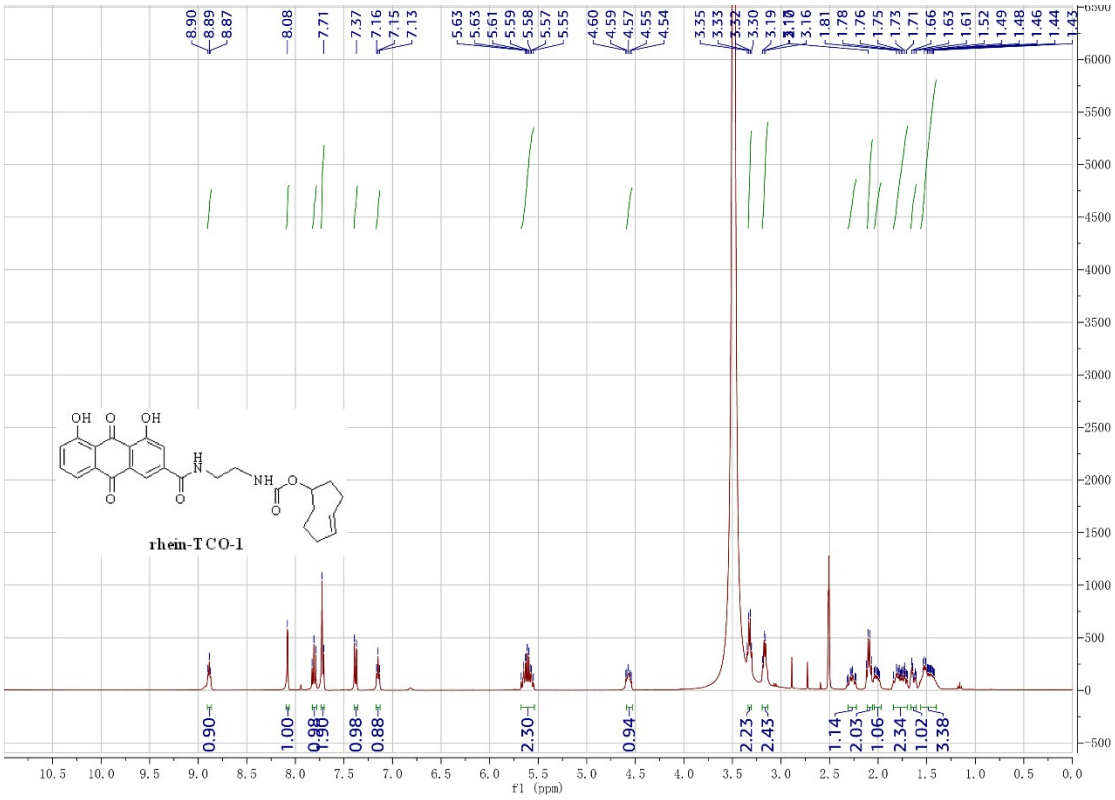
	^{131}I-rhein	^{131}I-rhein-TCO2	rhein Blocking
Blood	2.23±0.35	1.76±0.40	0.59±0.16
Brain	0.16±0.04	0.12±0.03	0.08±0.02
Thyroid	1.82±0.27	1.05±0.30	0.21±0.15
Lung	0.34±0.11	0.37±0.06	0.13±0.02

Heart	0.28±0.18	0.19±0.09	0.12±0.02
Liver	0.61±0.24	0.90±0.31	0.28±0.05
Spleen	0.51±0.17	0.43±0.16	0.21±0.06
Stomach	1.69±0.41	1.21±0.35	1.04±0.09
Pancreas	0.87±0.16	0.49±0.27	0.19±0.04
Small intestine	0.61±0.33	0.47±0.15	0.26±0.05
Large intestine	0.77±0.55	1.67±0.42	0.38±0.14
Kidney	1.31±0.47	1.53±0.22	0.45±0.15
Bladder	4.43±0.39	2.95±0.55	1.49±0.23
Normal muscle	0.42±0.11	0.29±0.09	0.20±0.03
Necrotic muscle	2.29±0.51	1.56±0.14	0.42±0.03
N/V Ratio[b]	5.49	5.38	2.12

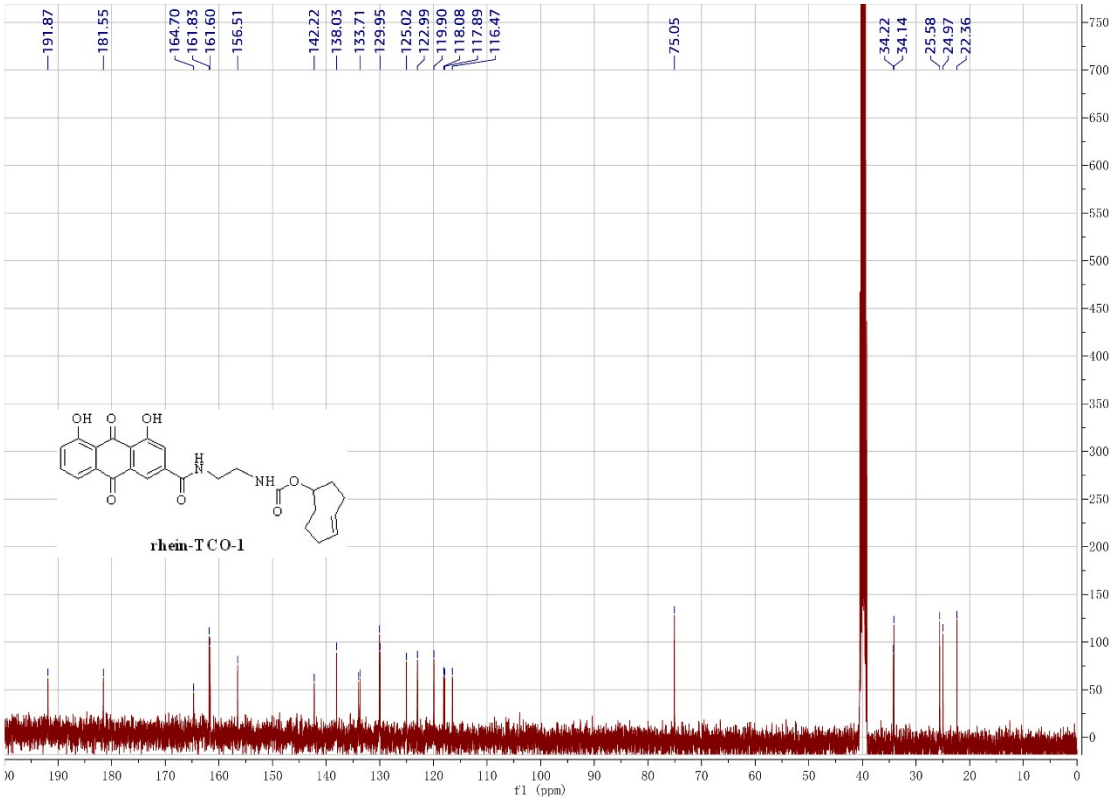
[a] Data are presented as % ID/g tissue ± SD (n = 6) after intravenous injection of the tracer (3.7 MBq) at 6 h (n = 5). [b] N/V ratio, % ID/g of necrotic muscle/normal muscle.

3. Copies of NMR spectra for products rhein-TCO1, rhein-TCO2, 2 and 3.

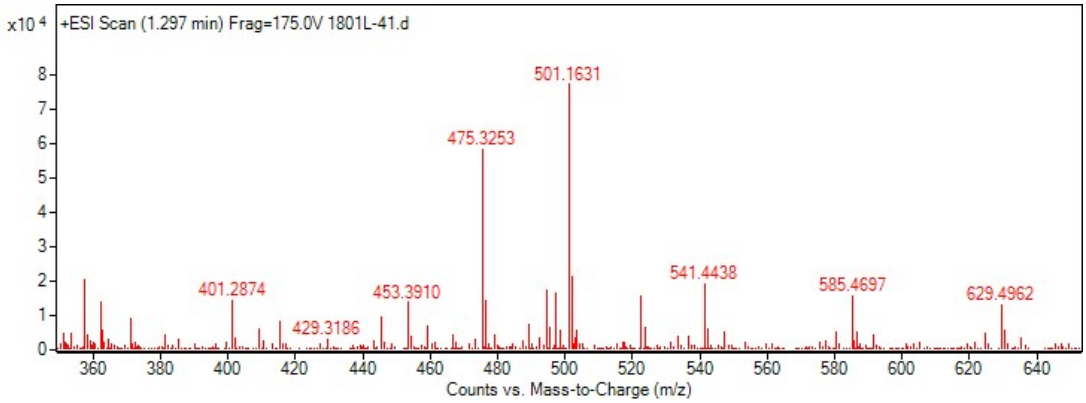
Rhein-TCO1 HNMR



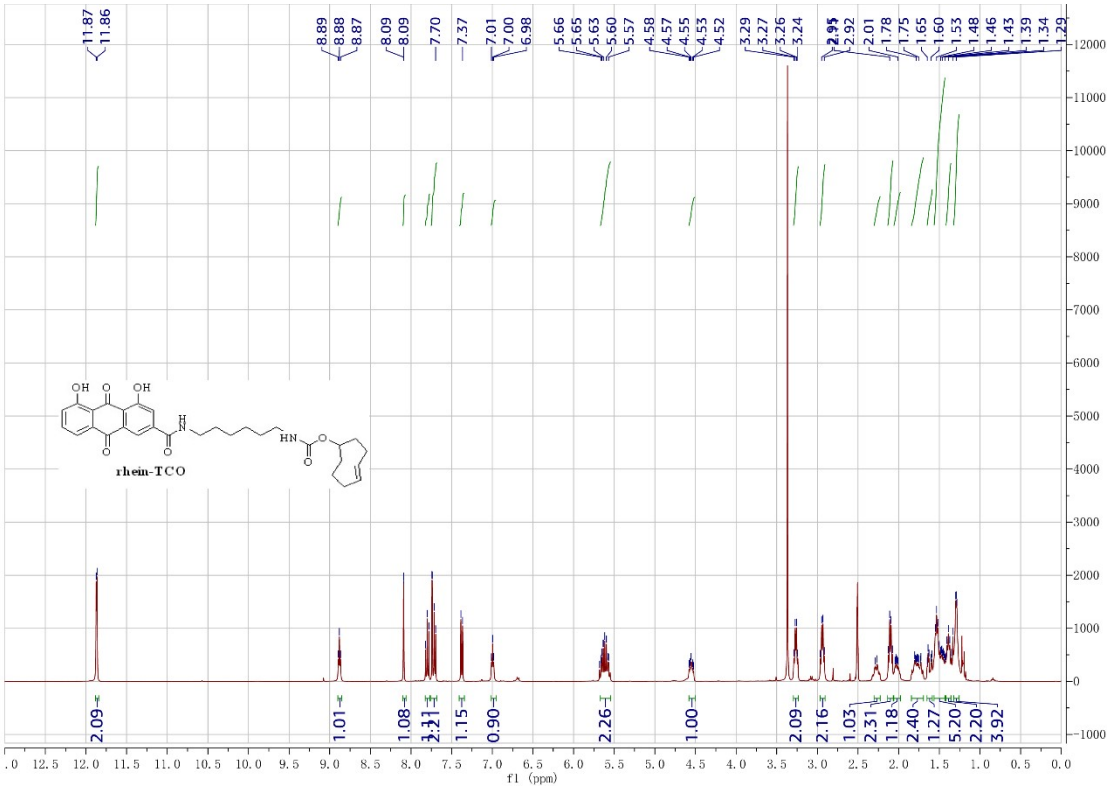
Rhein-TCO1 CNMR



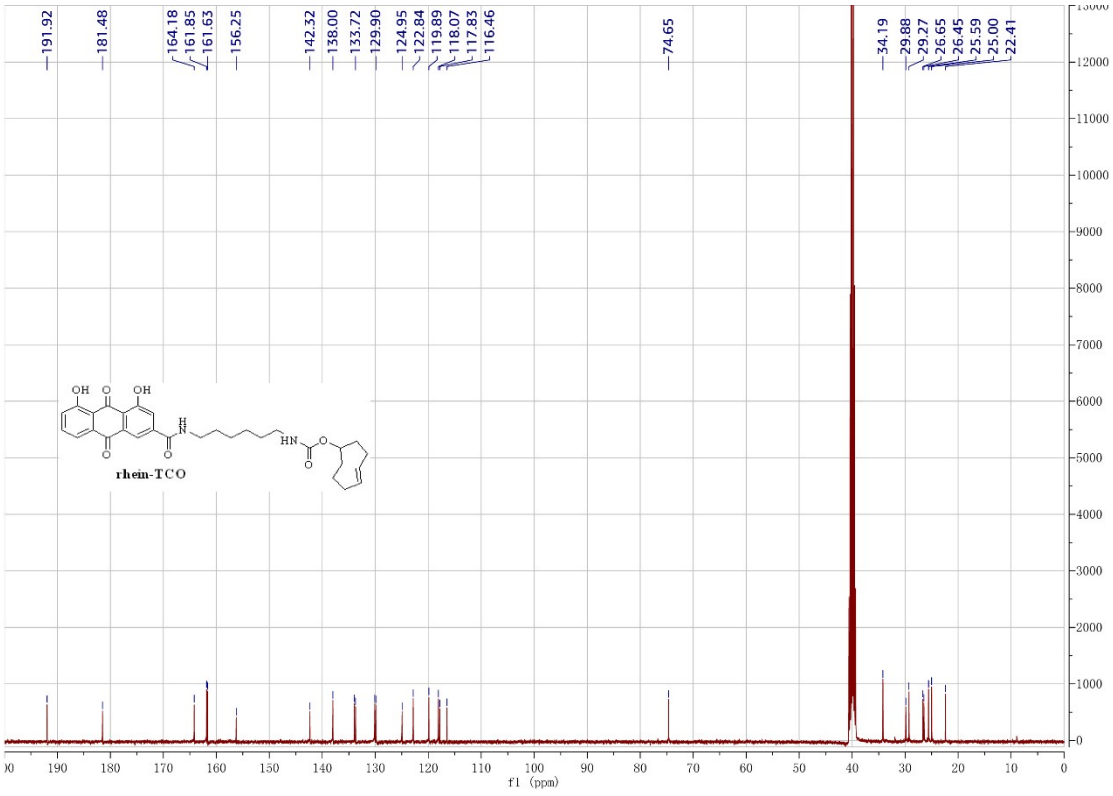
Rhein-TCO1 HRMS



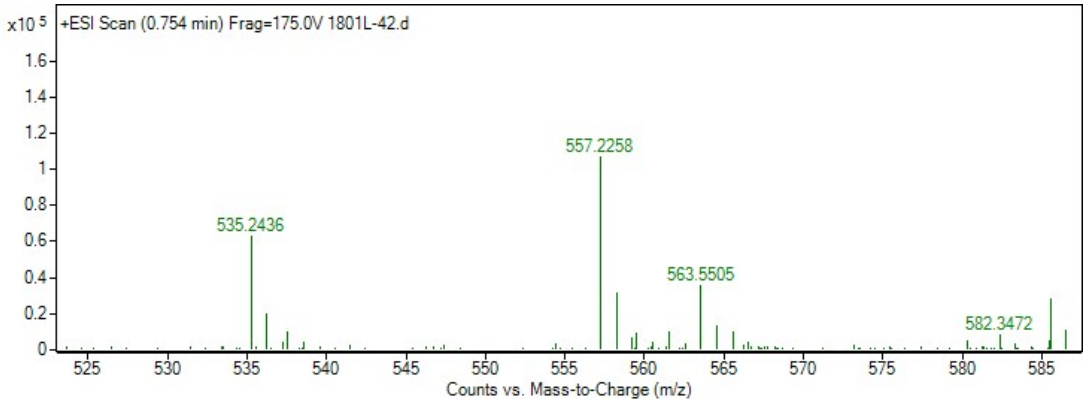
Rhein-TCO2 HNMR



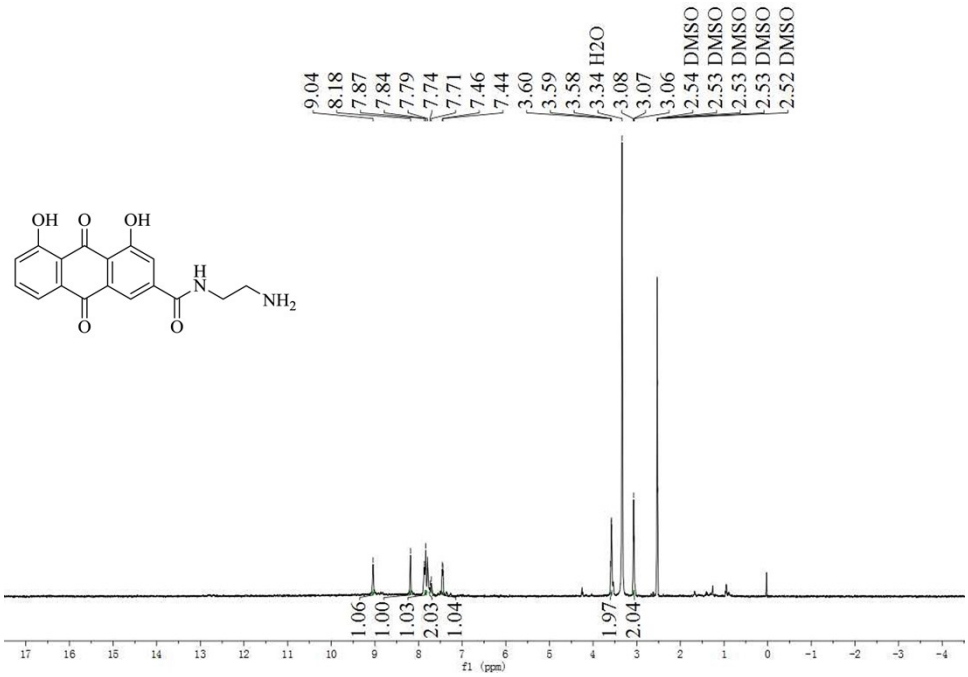
Rhein-TCO2 CNMR

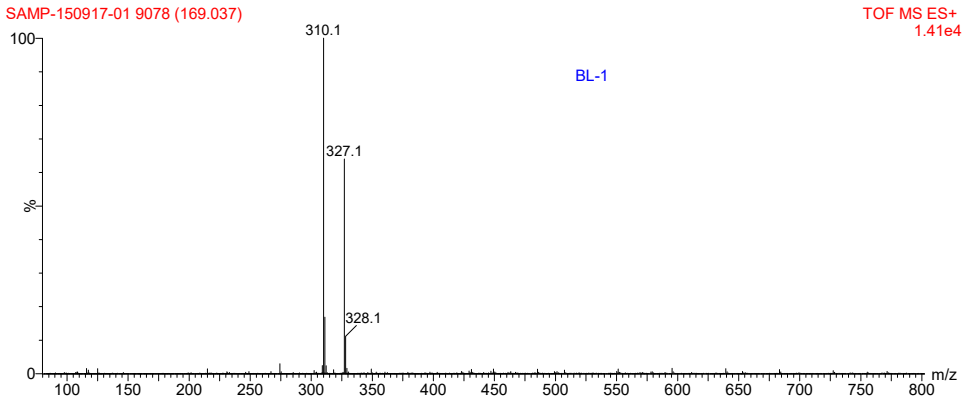


Rhein-TCO2 HRMS

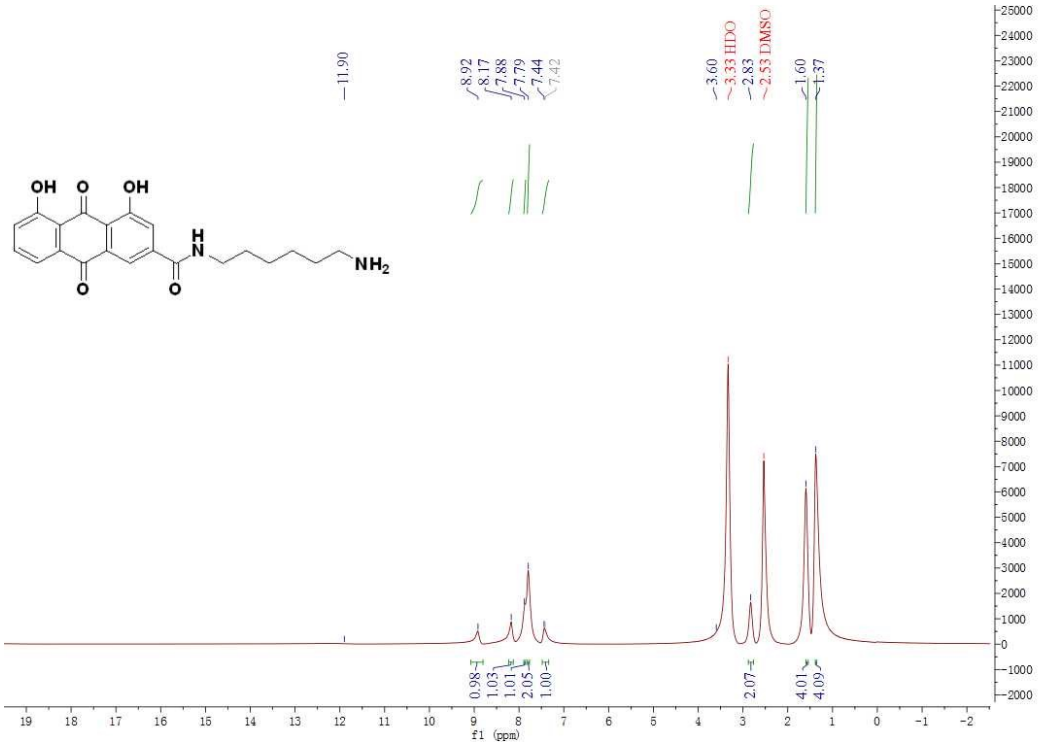


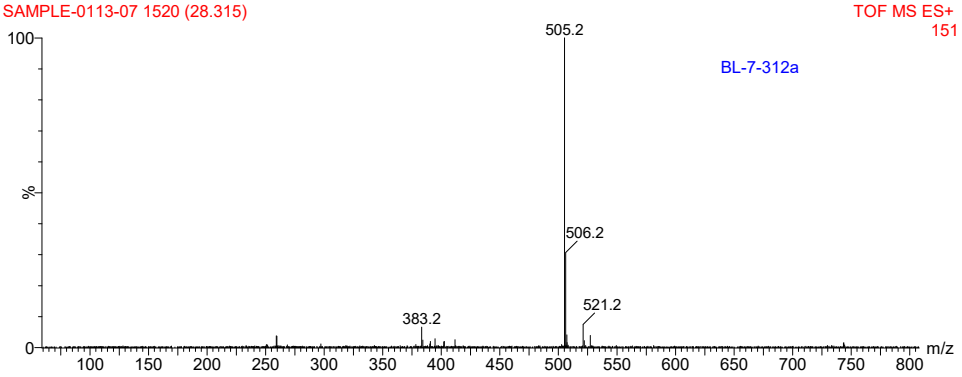
Known compound 2





Known compound 3


OH
O
OH
H
N
NH2
O
O
11.90
8.92
8.17
7.88
7.79
7.44
7.42
3.60
3.33 HDO
2.83
2.53 DMSO
1.60
1.37
0.98
1.03
1.01
2.05
1.00
2.07
4.01
4.09
f1 (ppm)


SAMPLE-0113-07 1520 (28.315)
TOF MS ES+
151
BL-7-312a
505.2
506.2
521.2
383.2
100
%
0
m/z