Design, synthesis, and biological evaluation of novel SN38-sulfonamide conjugates as Topo I/CA IX dual inhibitors inducing ferroptosis in colorectal cancer

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1. Biological assays

1.1 Antiproliferative activity

The cancer cell lines HCT-116, SW480, and HT-29 and human renal cortical proximal tubular epithelial cell line HK-2 were obtained from the Shanghai Cell Bank at the Chinese Academy of Sciences. HCT-116, SW480, and HT-29 and HK-2 cell lines were grown on 96-well microtiter plates at a density of 1×10^4 cells/well in DMEM with 10% FBS. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air overnight. The cells were then exposed to different concentrations of target compounds, SN38 and SLC-0111 and incubated for another 48 h. The cells were stained with 10 μl of MTT at incubator for approximately 4 h. The medium was discarded and replaced by 100 mL DMSO. The O.D. value was read at 570/630 nm with a spectrophotometer.

1.2 Antiproliferative activity under hypoxic conditions

The cytotoxic effects of selected compounds were evaluated under hypoxic conditions. Cells were seeded into 96-well plates and cultured for 12 h to allow attachment, followed by incubation with complete medium containing 200 μM $CoCl_2$ for 24 h to induce hypoxic stress. The cells were then treated with various concentrations of the test compounds, with untreated wells serving as negative controls and SLC-0111 as a positive control. After 48 h, cell viability was assessed using the MTT assay as described above, and IC_{50} values were calculated accordingly.

1.3 CA inhibitory assay

Enzyme inhibition assays of CAIX and CAII were carried out as previous reported method. This assay spectrophotometrically measured the *p*-nitrophenol, a yellow color product which is formed by the hydrolysis of p-nitrophenyl acetate (4-PNA) catalyzed by CA. Absorbance was measured at 400 nm with the help of UV/visible spectrophotometer (Shimadzu UV2600) equipped with temperature regulator. The absorption data was analyzed with the help of Graph Pad Prism (Version 6.0) and the IC_{50} values for tested compounds were determined.

1.4 Agarose gel electrophoresis assay on DNA binding

The pBR322 DNA was incubated with compound **4f** at a range of concentrations in TAE buffer for 3 h. Then, the reaction was terminated by the addition of loading buffer. The samples were electrophoresed in a 1% agarose gel and stained with ethidium bromide before detection. The images of the DNA gel were captured using a Bio-Rad imaging system under a UV-Vis transilluminator.

1.5 Agarose gel electrophoresis assay

In Topo I inhibition experiments, Topo I, Topo I buffer, 1% BSA, and the gradient concentrations of compound **4f** were mixed with DNA brought to 10 μL by TAE buffer, and then all samples were incubated at 37°C in darkness for 30 min. Following that the samples were separated by electrophoresis and images of the DNA gel were obtained using an imaging system after staining with EB staining reagent. The images of the DNA gel were captured using a Bio-Rad imaging system under a UV-Vis transilluminator.

1.6 Comet assay

DNA breaks in HCT-116 cells following treatment with compound **4f** for 4 h were evaluated using the neutral single-cell gel electrophoresis (comet) assay. Briefly, 600 cells were resuspended in 0.5% low-

melting-point agarose. After solidification, the slides were lysed for 2 h at 4 °C in lysis buffer consisting of 2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA, 1% sodium sarcosinate, 10% DMSO, and 1% Triton X-100 (pH 8.5). The slides were then placed in ice-cold neutral buffer (1× TAE buffer, pH 8.5) at 4 °C for 20 min to allow DNA unwinding. Electrophoresis was performed at room temperature for 20 min at 20 V (1 V/cm). Subsequently, the slides were stained with acridine orange (10 μg/mL) for 5 min. Fluorescent comet patterns were examined using a fluorescence microscope under 100× magnification.

1.7 Immunofluorescence assay

HCT-116 cells were seeded onto coverslips. Following treatment, the cells were washed with PBS and fixed with 3% paraformaldehyde for 15 min. The cells were then permeabilized with 0.5% Triton X-100 in PBS for 5 min and blocked with 5% goat serum for 1 h at room temperature. Subsequently, the cells were incubated with the primary antibody (C2035S-4) overnight at 4 °C, followed by incubation with Alexa Fluor 488 conjugated secondary antibody (C2035S-5) for 20 min at 37 °C. After washing with PBS, the cell nuclei were counterstained with DAPI. Fluorescence signals were examined using confocal microscopy.

1.8 Molecular docking

All docking studies were carried out in Sybyl-X 2.1 on a Windows workstation. The crystal structure of the CAIX proteins was retrieved from the RCSB Protein Data Bank (CAIX: 3IAI) [2]. Compound **4f** were selected for the docking studies. The 3D structures of these selected compounds were first built with Sybyl-X 2.1 sketch, and this was followed by energy minimization with the MMFF94 force field and Gasteiger-Marsili charges. We used Powell's method for optimizing the geometry with a distance dependent dielectric constant and a termination energy gradient of 0.005 kcal/mol. All selected compounds were automatically docked into the binding pocket of CAIX through an empirical scoring function and a patented search engine in the Surflex docking program. Before the docking process, the natural ligand was extracted, and the water molecules were removed from the crystal structure. Subsequently, the protein was prepared with the Biopolymer module implemented in Sybyl. The polar hydrogen atoms were added, and other parameters were established by default to estimate the binding affinity characterized by the Surflex-Dock scores in the software. The Surflex-Dock total scores, which were expressed in -log10 (K_d) units to represent binding affinities, were applied to estimate the ligand-receptor interactions of newly designed molecules.

1.9 Western blot assay

Total cell lysates from cultured HCT-116 cells after treatment with compound **4f** as mentioned earlier were obtained by lysing the cells in ice-cold RIPA buffer with protease and phosphatase inhibitor and stored at -20°C for future use. The protein concentrations were quantified by the Bradford method (Bio-Rad) using a Multimode Varioscan instrument (Thermo Fischer Scientific). Equal amounts of protein per lane were applied to 12% SDS polyacrylamide gel for electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). After the membrane was blocked at room temperature for 2 h in blocking solution, primary antibody was added and incubated at 4°C overnight. BAX, Bcl-2, CAIX, GPX4, NCOA4, and xCT antibodies were purchased from Imgenex, USA. After three TBST washes, the membrane was incubated with corresponding horseradish peroxidase-labeled secondary

antibody (1:2,000) (Santa Cruz) at room temperature for 1 h. Membranes were washed with TBST three times for 15 min and the protein bands were detected with chemiluminescence reagent (Thermo Fischer Scientific). The X-ray films were developed using developer and fixed with fixer solution.

1.10 Apoptosis assay

Apoptosis induction by compound **4f** in HCT-116 cells was evaluated using an Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instructions. Briefly, HCT-116 cells (1×10^5) were treated with compound 4f at its IC_{50} concentration for 24 h. The cells were then harvested by trypsinization, and 0.5×10^6 cells were washed twice with PBS. The cells were subsequently stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) in 1× binding buffer for 15 min at room temperature in the dark. Annexin V-FITC binding was analyzed using a flow cytometer (FACS Calibur), and the data were processed with the accompanying software.

1.11 Hoechst 33258 staining

HCT-116 cells in the logarithmic growth phase were seeded into 6-well plates at a density of 1×10^5 cells per well and cultured at 37 °C in a 5% CO_2 incubator for 24 h to allow attachment. The cells were then treated with compound 6a and incubated for an additional 24 h. Following treatment, the cells were washed with PBS. Hoechst 33258 staining solution (diluted 1:100 in PBS) was added dropwise to the cells and incubated for 5 min. The cells were then washed three times with PBS (5 min per wash) and immediately examined using a fluorescence microscope.

1.12 ROS measurement

HCT-116 cells in the logarithmic growth phase were seeded into 6-well plates at a density of 1×10^5 cells per well and cultured at 37 °C in a 5% CO_2 incubator for 24 h to allow attachment. The cells were then treated with various concentrations of compound **4f** for 24 h, washed with PBS, and incubated with 10 μM DCFH-DA at 37 °C for 30 min. Subsequently, the cells were washed again with PBS and immediately examined using a fluorescence microscope or flow cytometer.

1.13 Assessment of mitochondrial membrane potential

HCT-116 cells were seeded onto 96-well plates and incubated overnight. Following treatment with varying concentrations of **4f** for 24 h, the cells were stained with JC-1 (Beyotime C2006). The cells were incubated at 37 °C for 30 min and analyzed using a fluorescence spectrometer at 590 nm (for red fluorescence) and 520 nm (for green fluorescence). The fluorescence ratio (590/520 nm) represented the change in mitochondrial membrane potential.

1.14 Wound healing assay

HCT-116 cells (5×10^5 cells/well) were cultured in 6 well plates as confluent monolayers for 24 h. Then artificial scratch on the monolayers was created with 200 mL sterile pipette tip and washed twice with PBS to remove non-adherent cells. The media containing 0.3 μM, 0.6 μM and 1.2 μM of compound 4f or without compound 4f were added to each well. The migration of cells across the scratched area were photographed by using phase contrast microscope (Nikon) at 0 h (immediately) and 24 h time interval after treatment in three or more randomly selected fields.

1.15 Transwell assay

Cell invasion was assessed using a Transwell Boyden chamber system. Briefly, 500 μL of DMEM supplemented with 10% fetal bovine serum (FBS) was added to the lower chamber. HCT-116 cells in the exponential growth phase were harvested, and 5×10^4 cells suspended in 300 μL of serum-free medium containing various concentrations of compound **4f** (0.3, 0.6, or 1.2 μM) were seeded into the upper chamber. Cells were allowed to migrate through the polyethylene terephthalate (PET) membrane toward the lower chamber. Following incubation for 24 h at 37 °C in a 5% CO_2 incubator, non-migrated cells on the upper surface of the membrane were removed with cotton swabs. Cells that had migrated to the lower side of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution for 10 min. Invasion was quantified by counting the stained cells under a light microscope at 200× magnification. For each insert, migrated cells were counted in three randomly selected fields, and the mean value was calculated. All experiments were performed in triplicate for each condition.

1.16 Lipid Peroxidation Assay

After treatment with various concentrations of compound **4f** for 24 h, HCT-116 cells were washed with PBS and then incubated with 10 μM C11-BODIPY581/591 at 37 °C for 30 min. The cells were subsequently washed with PBS and divided into two groups for detection. In the first group, cells were directly examined using a confocal microscope. In the second group, cells were lysed in cell lysis buffer at 4 °C. The lysates were centrifuged at 10,000 g for 10 min at 4 °C, and the resulting cell suspension was filtered through a 40 μm cell strainer. The supernatant was then collected and analyzed by flow cytometry.

1.17 TEM assay

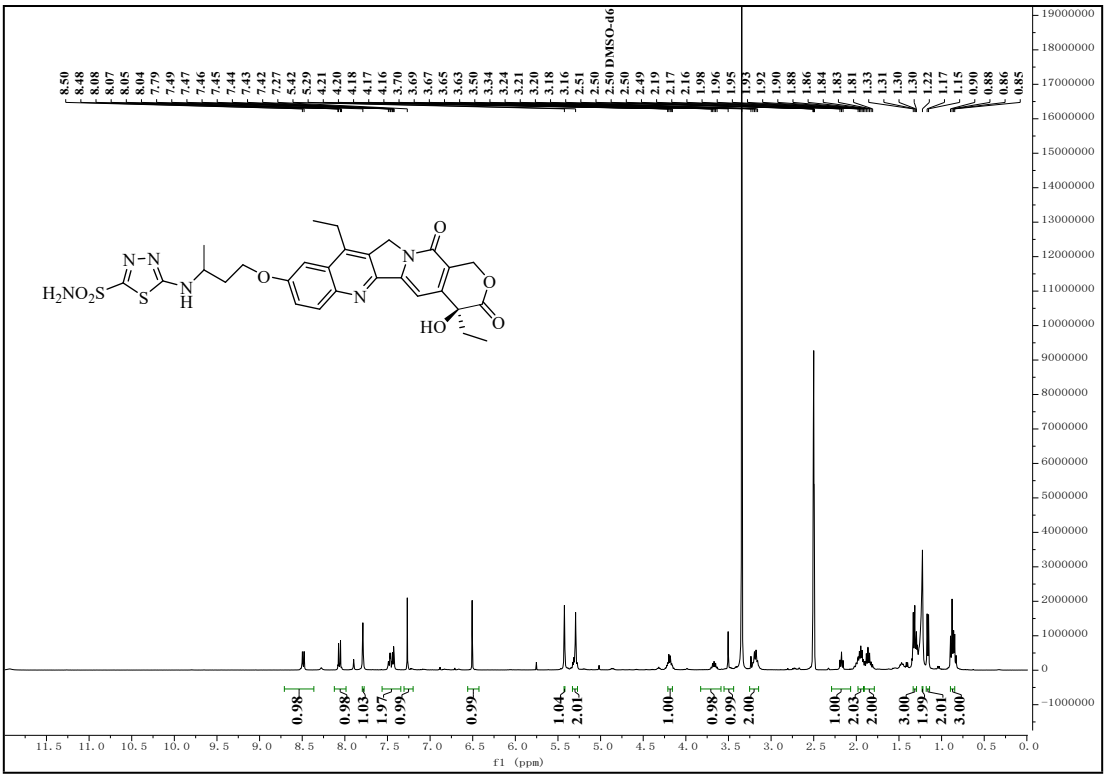
HCT-116 cells were seeded in six-well plates (3×10^5 cells/well) and incubated in the presence or absence of compound **4f** (1.2 μM) for 24 h. After treatment, the cells were washed with PBS, trypsinized, and fixed in 2% glutaraldehyde for 4 h. Then, the samples were secondarily fixed with 1% osmium tetroxide for 1 h at 4 °C, dehydrated with an ethanol solution gradient (50%, 70%, 80%, 90%, and 95%), and finally embedded in Epon. Samples were then sliced into semithin sections and stained with lead citrate. Images were acquired with a transmission electron microscope (JEM-1400FLASH, JEOL, Japan).

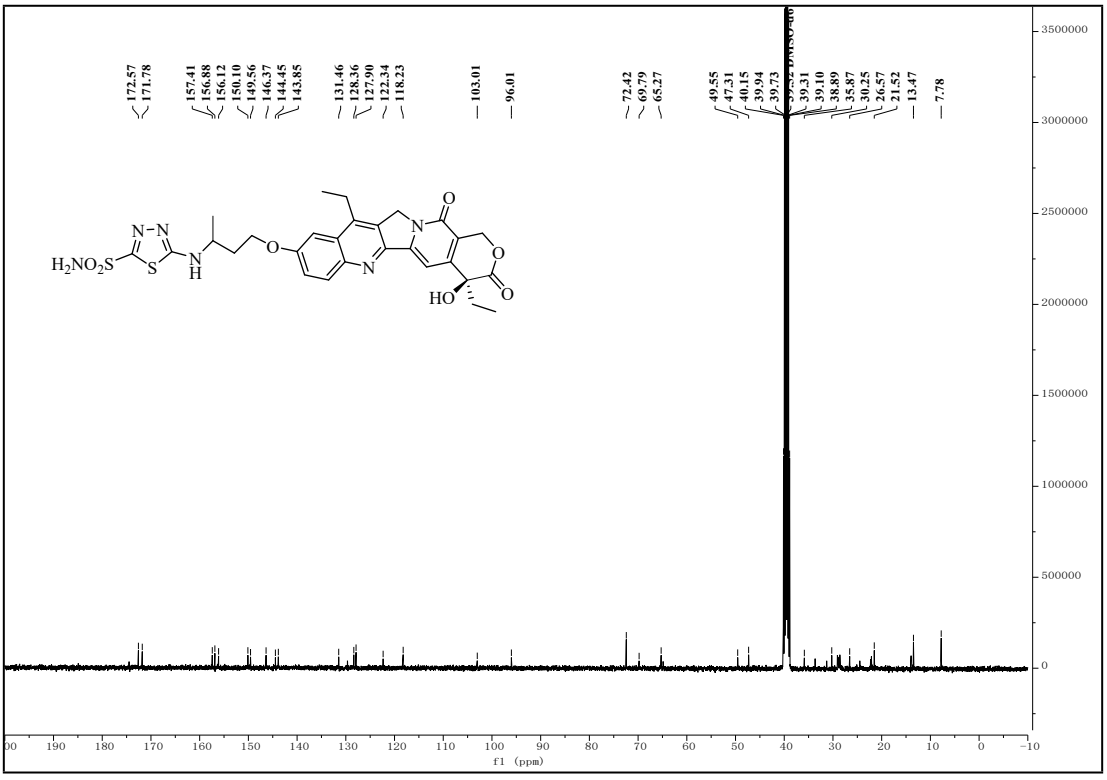
1.18 Statistical analysis

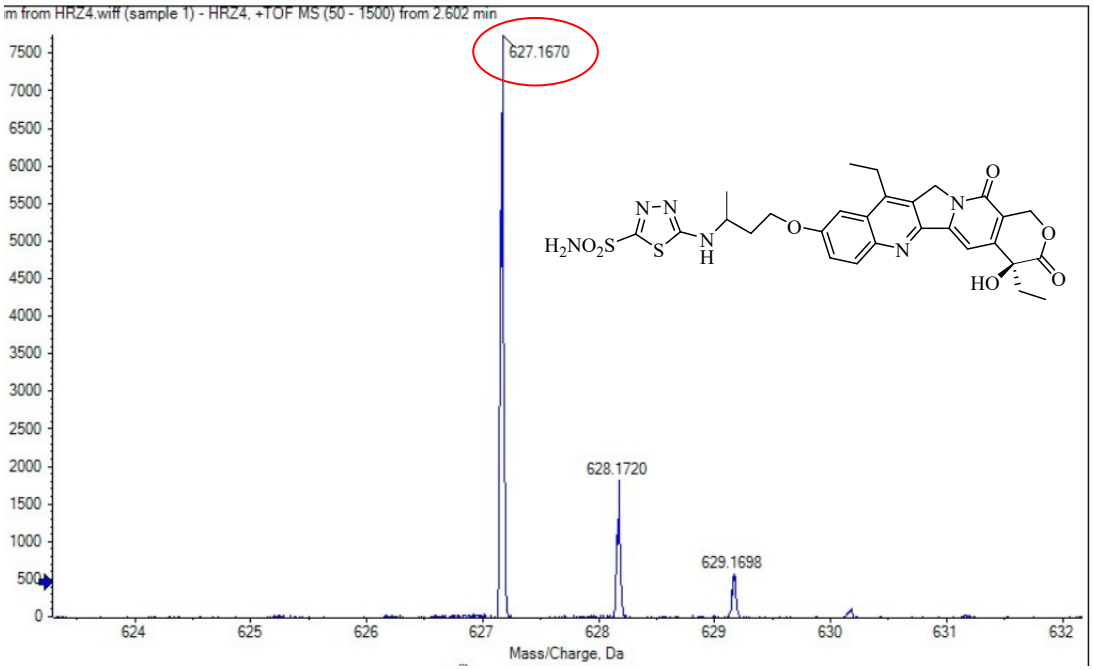
Data were analyzed using Prism Software 5.0. All data are presented as mean ± standard deviation (SD) unless otherwise indicated. Statistical significance was determined using one-way analysis of variance (ANOVA), with a threshold of $P<0.05$ considered statistically significant.

^{1}H NMR, ^{13}C NMR and HR-MS of compounds**:**

4a



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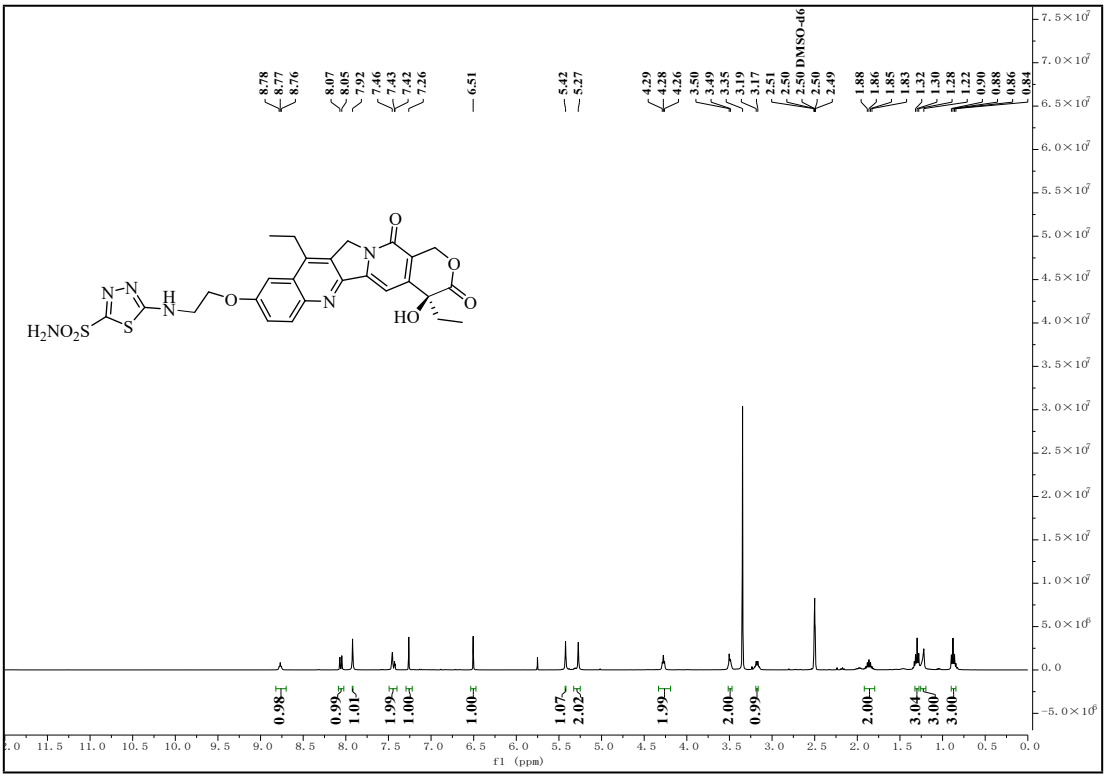
Mass/Charge, Da

4b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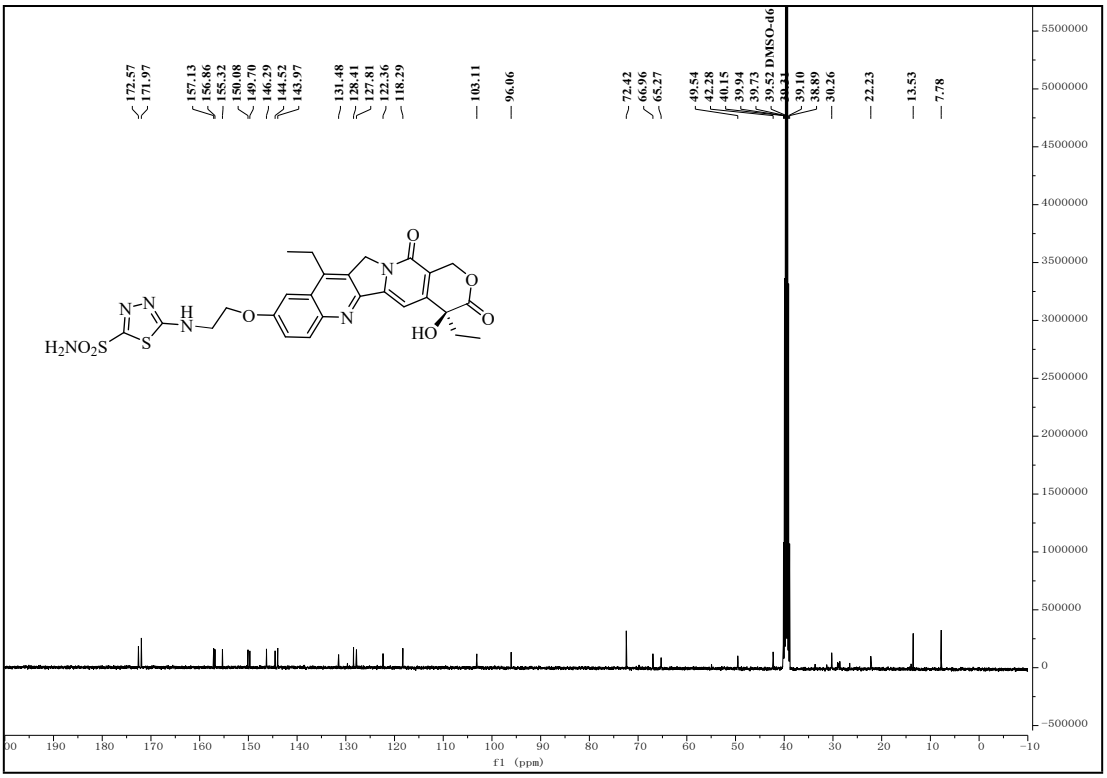
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118.29
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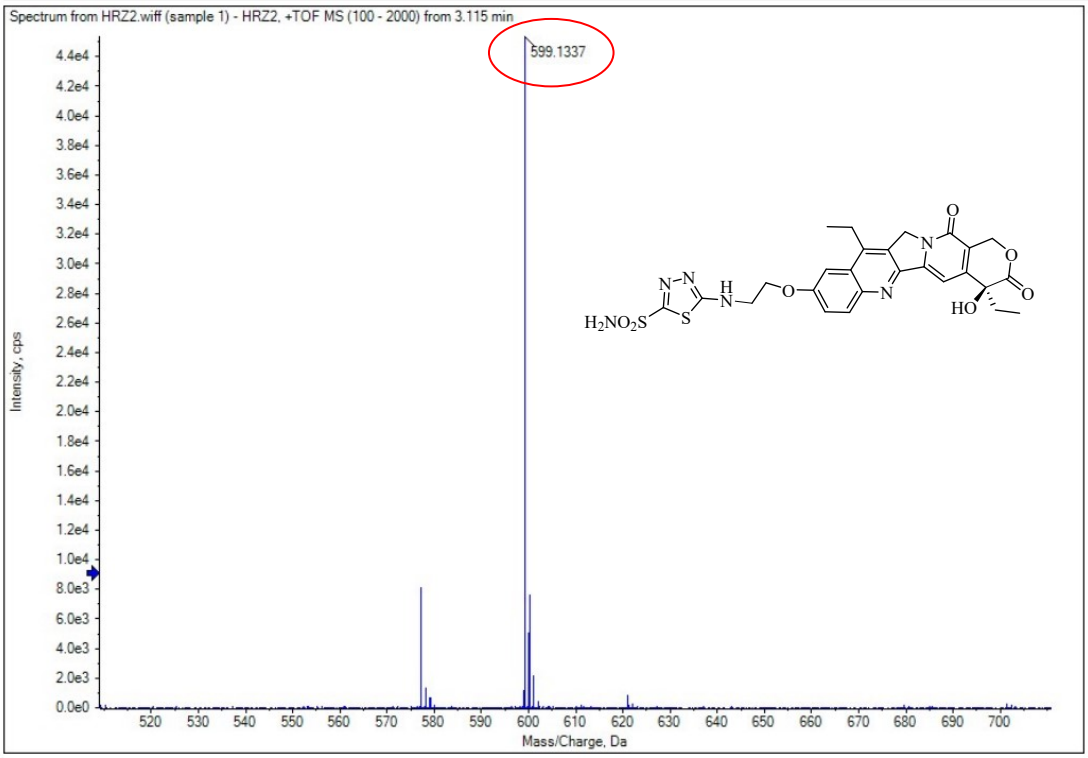
f1 (ppm)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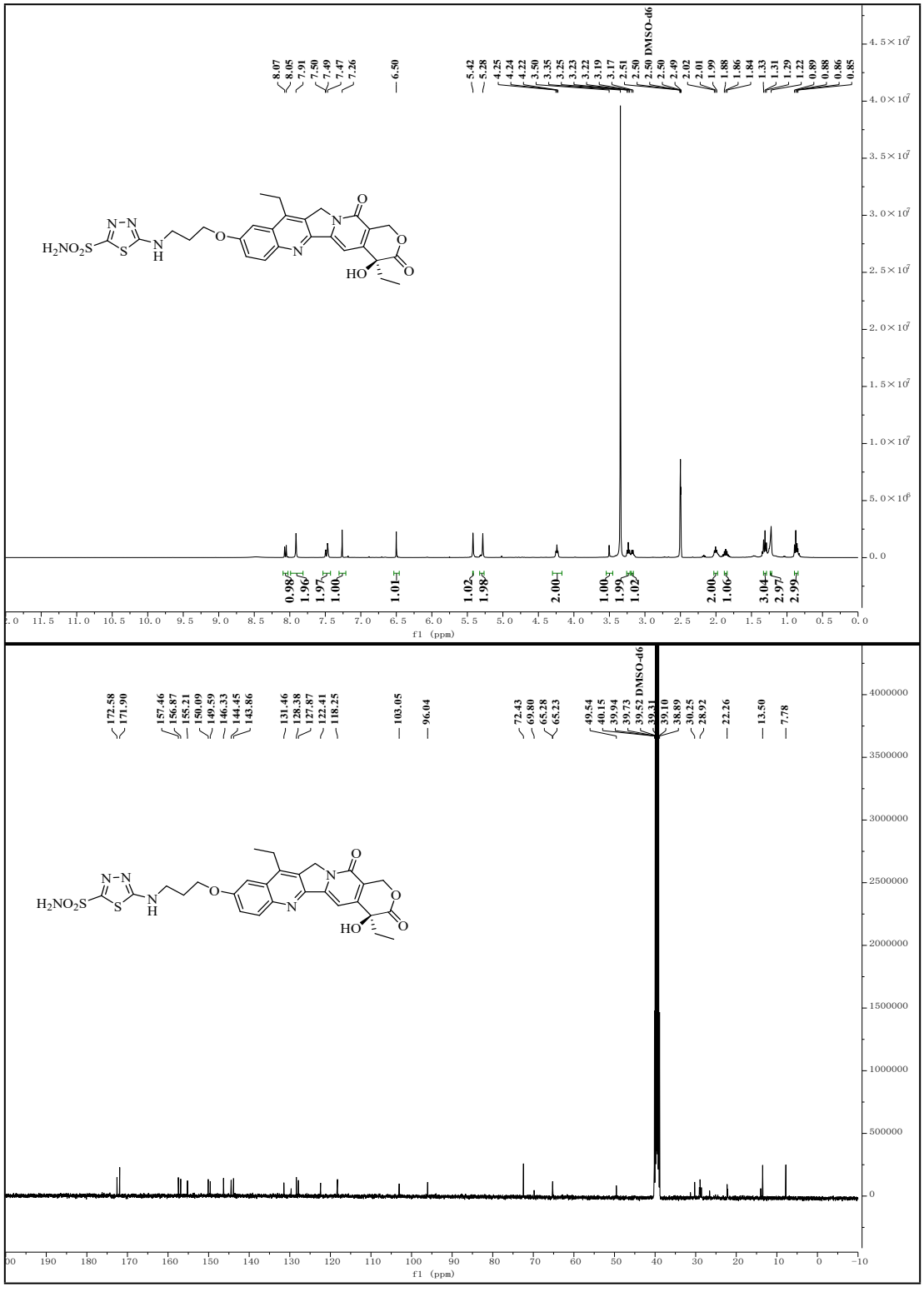
Mass/Charge, Da

4c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2.01
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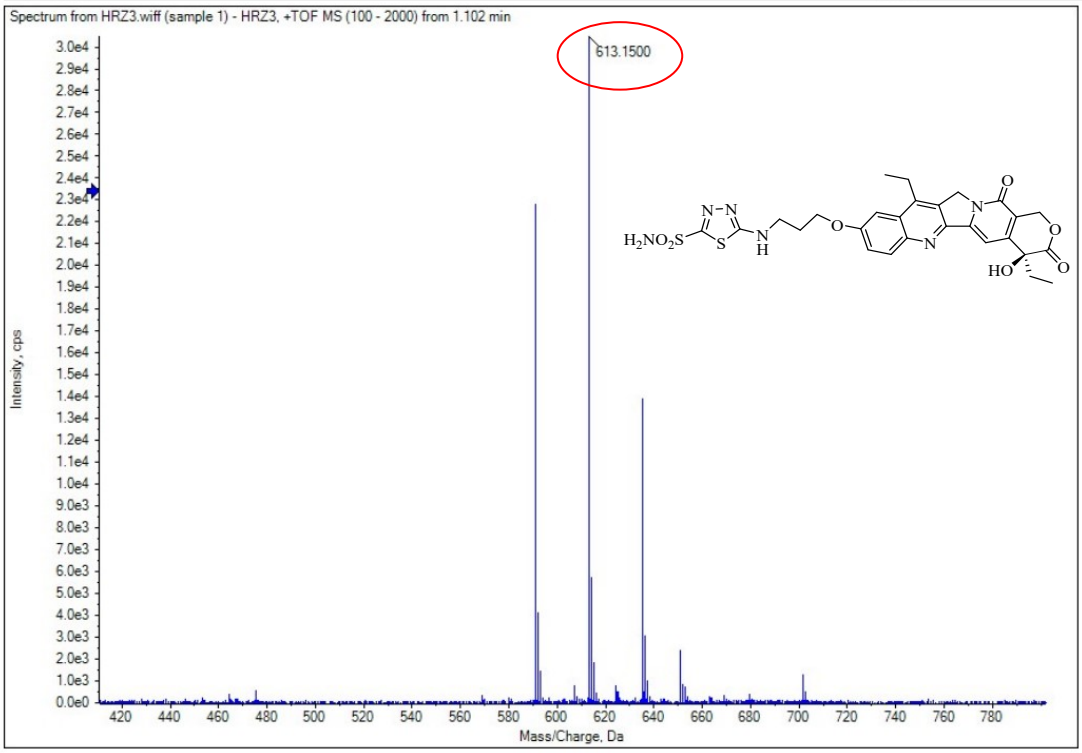
7.78
f1 (ppm)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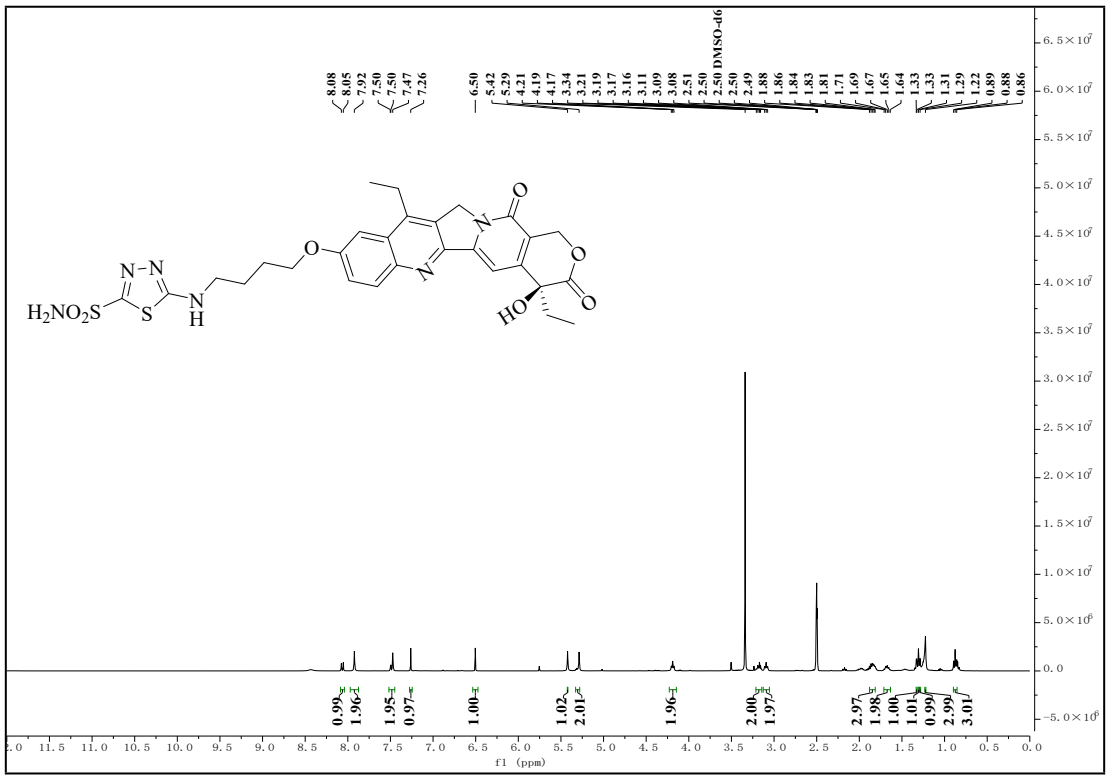
Mass/Charge, Da

4d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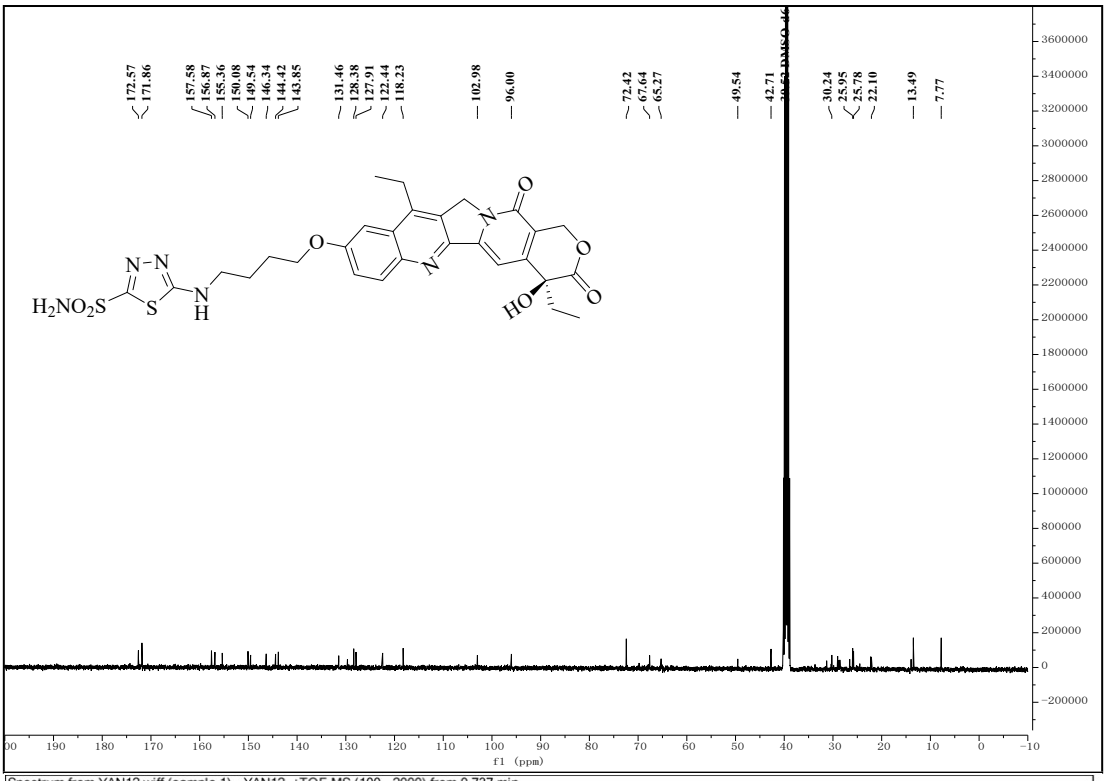
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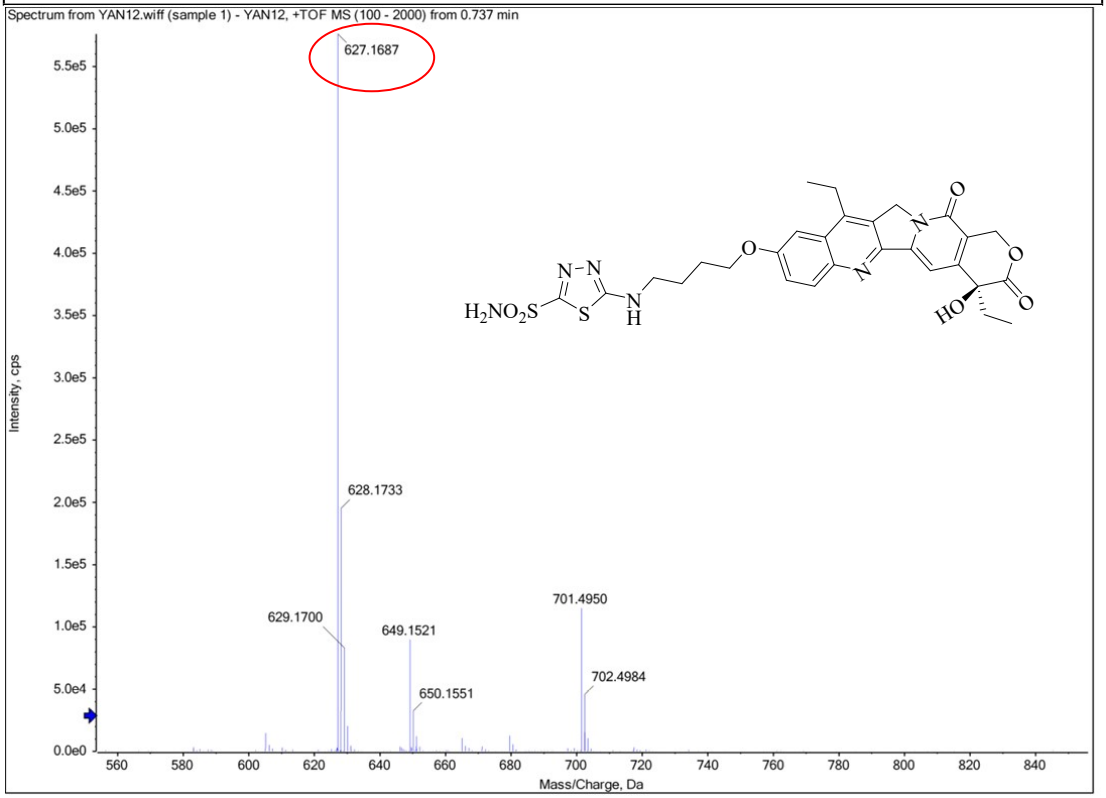
f1 (ppm)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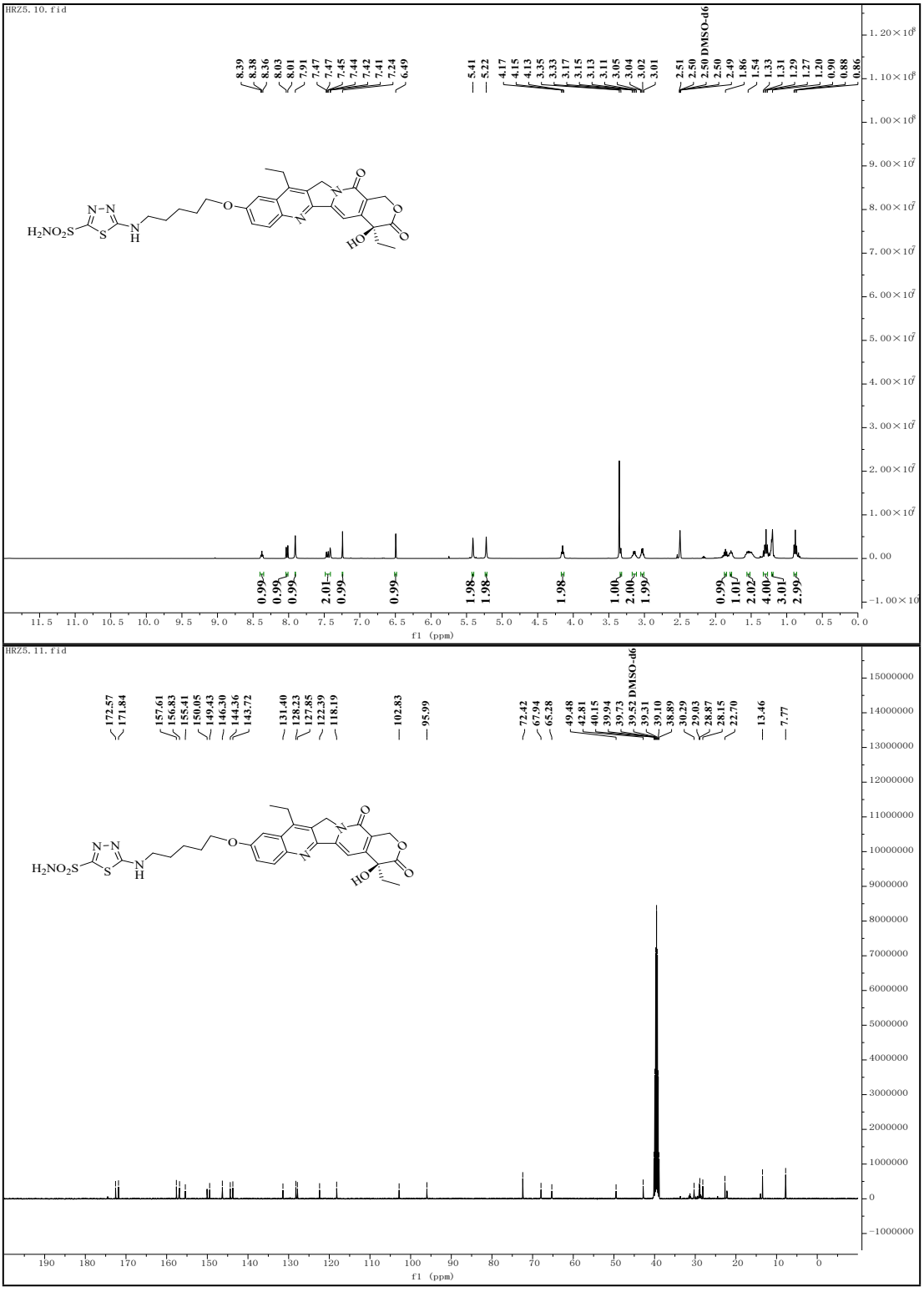
Intensity, cps
Mass/Charge, Da

4e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f1 (ppm)
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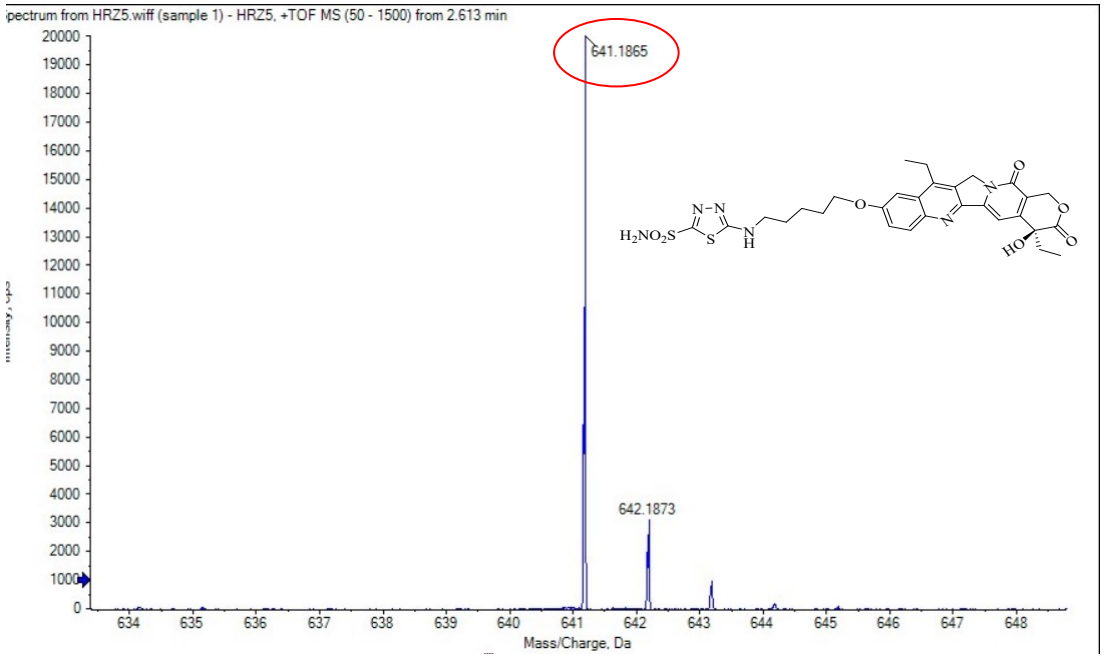
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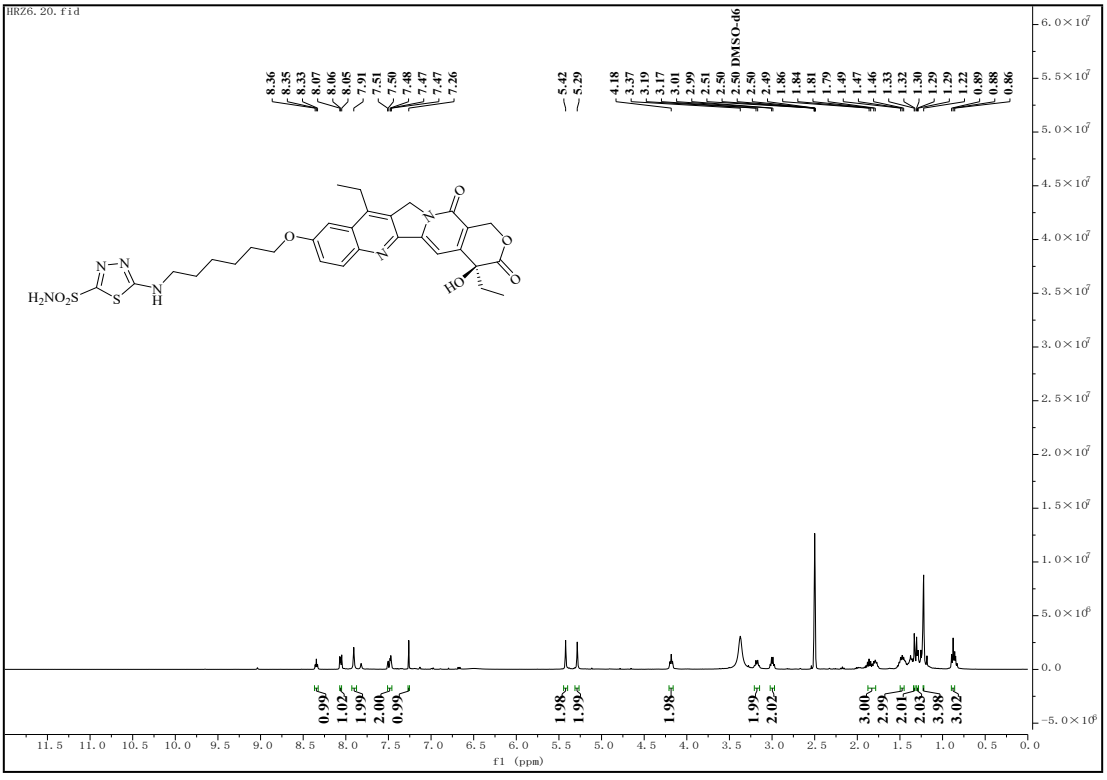
Mass/Charge, Da

4f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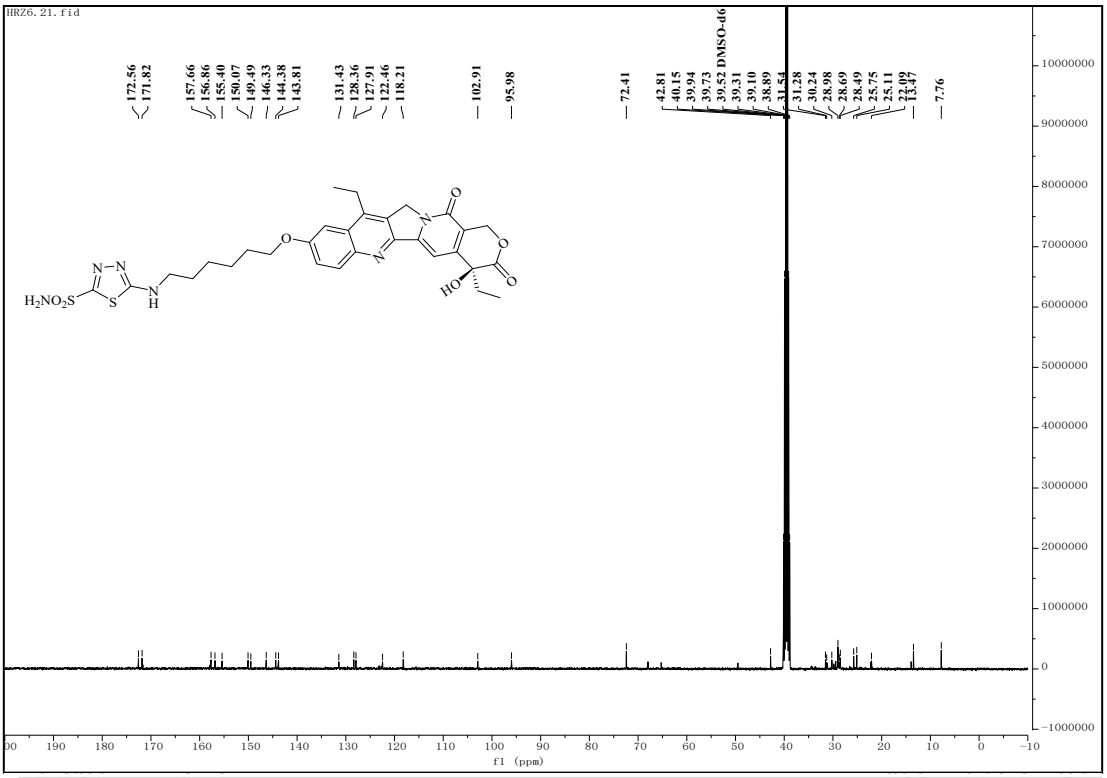
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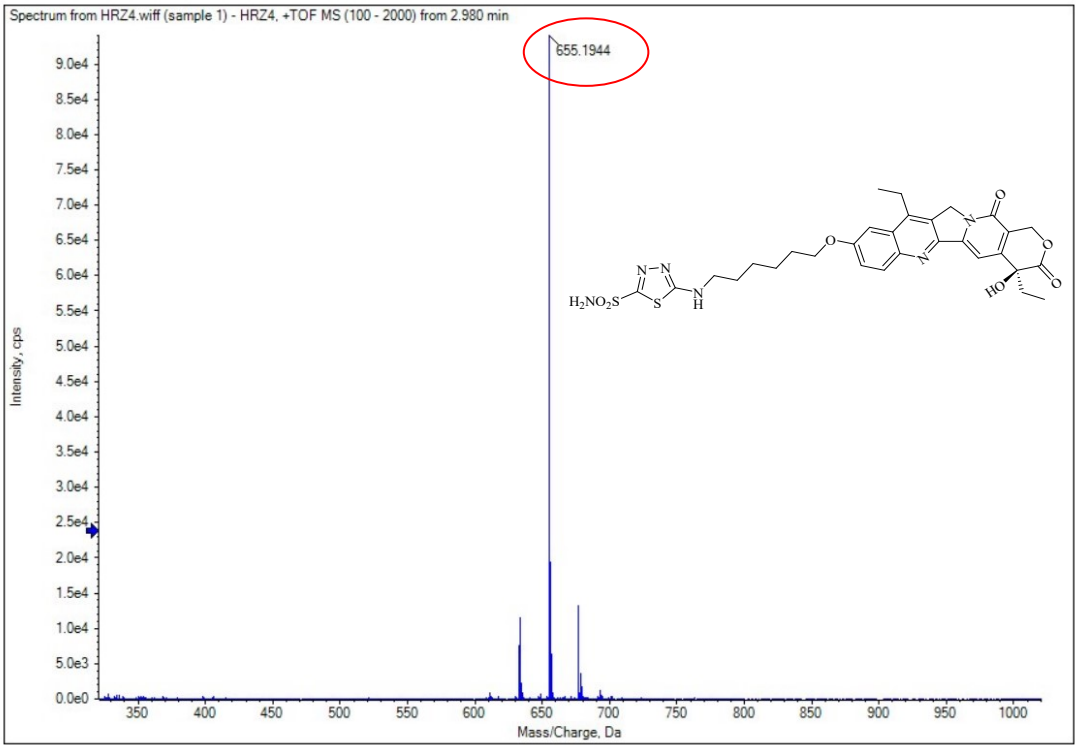
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Mass/Charge, Da

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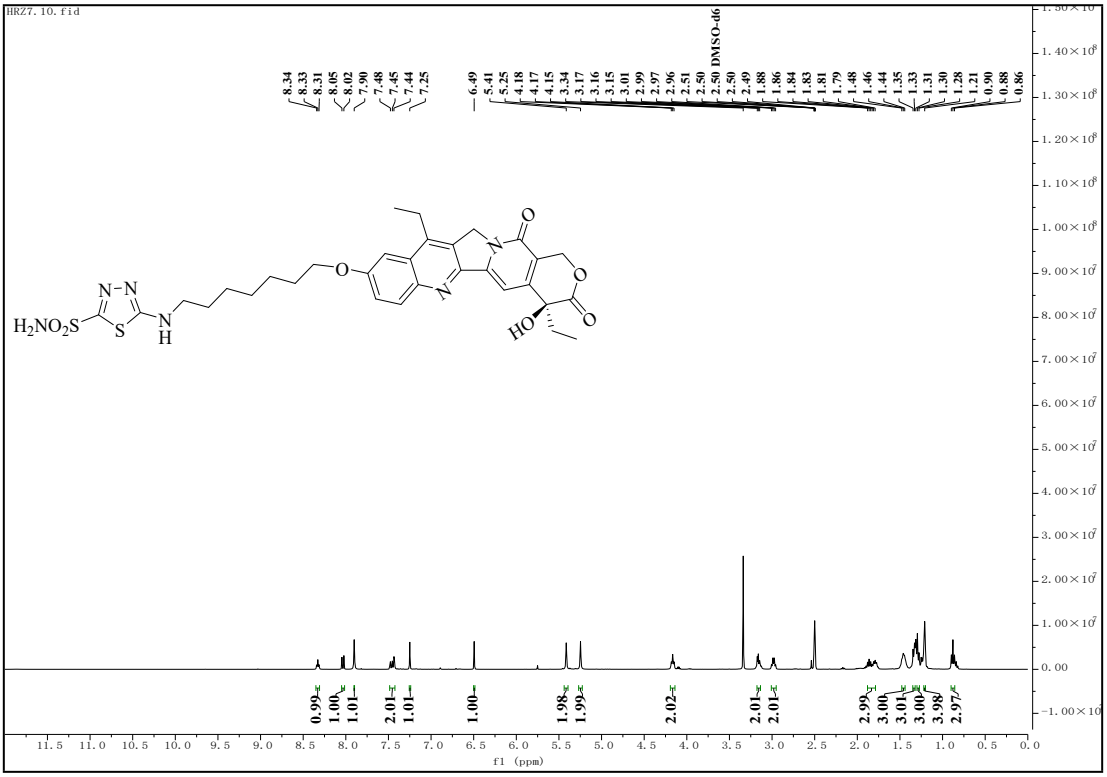
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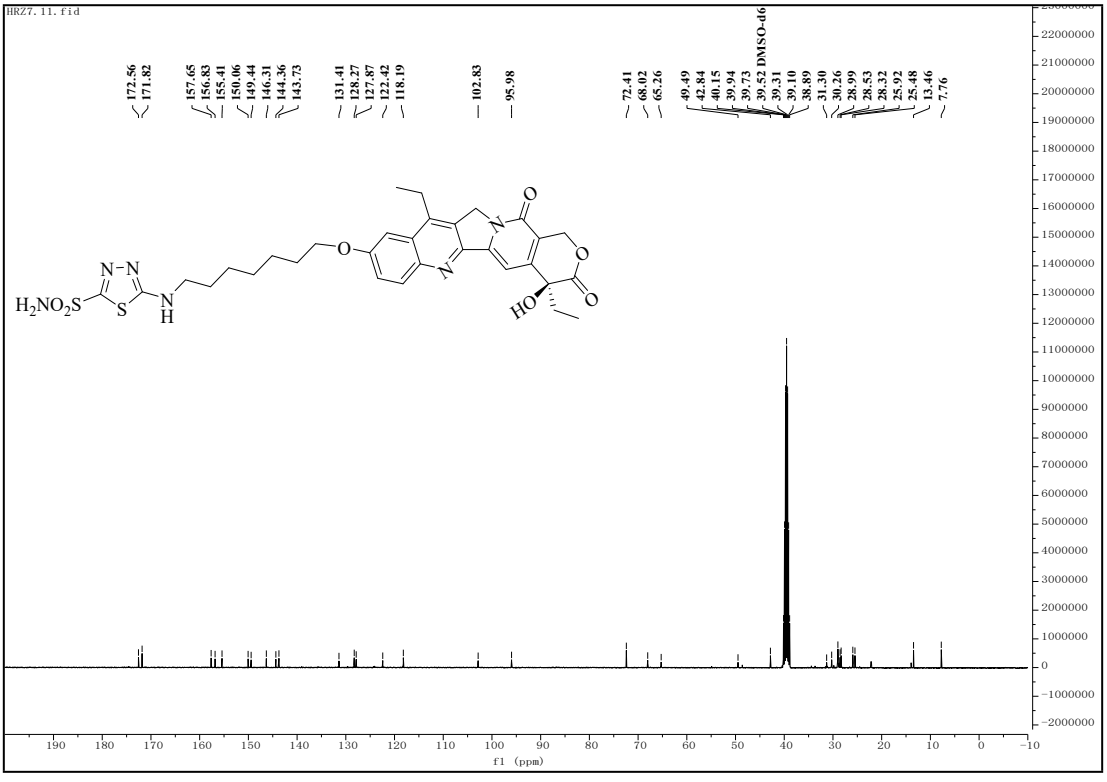
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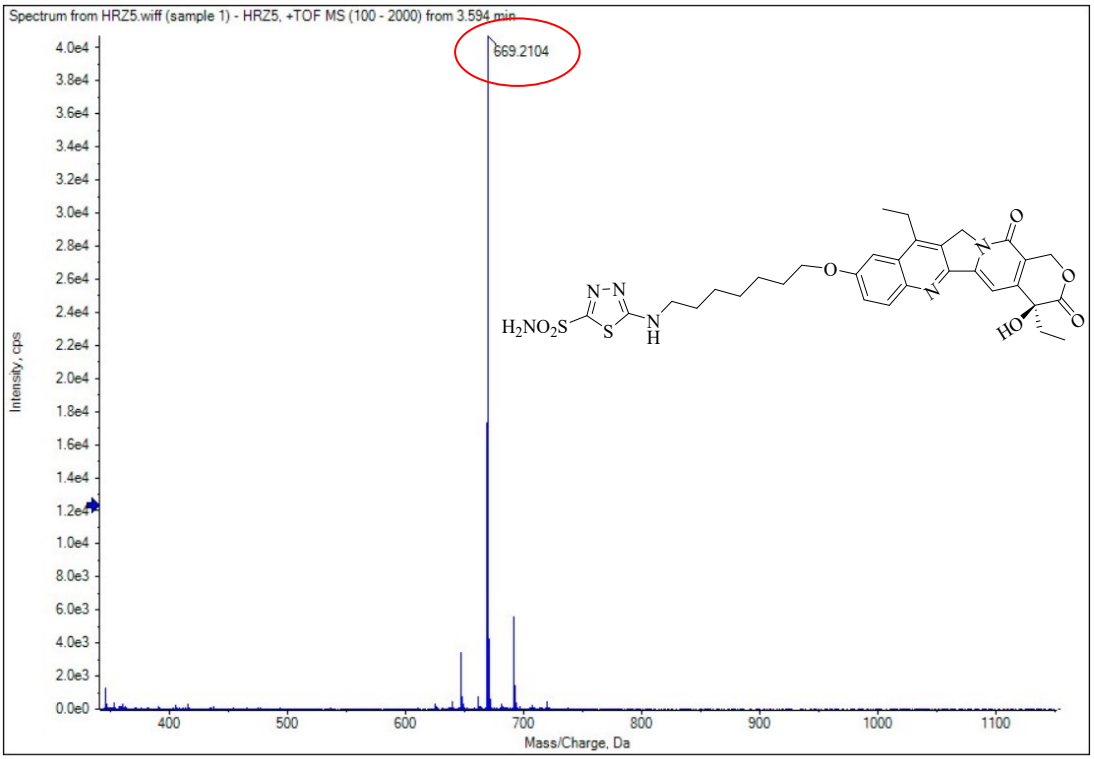
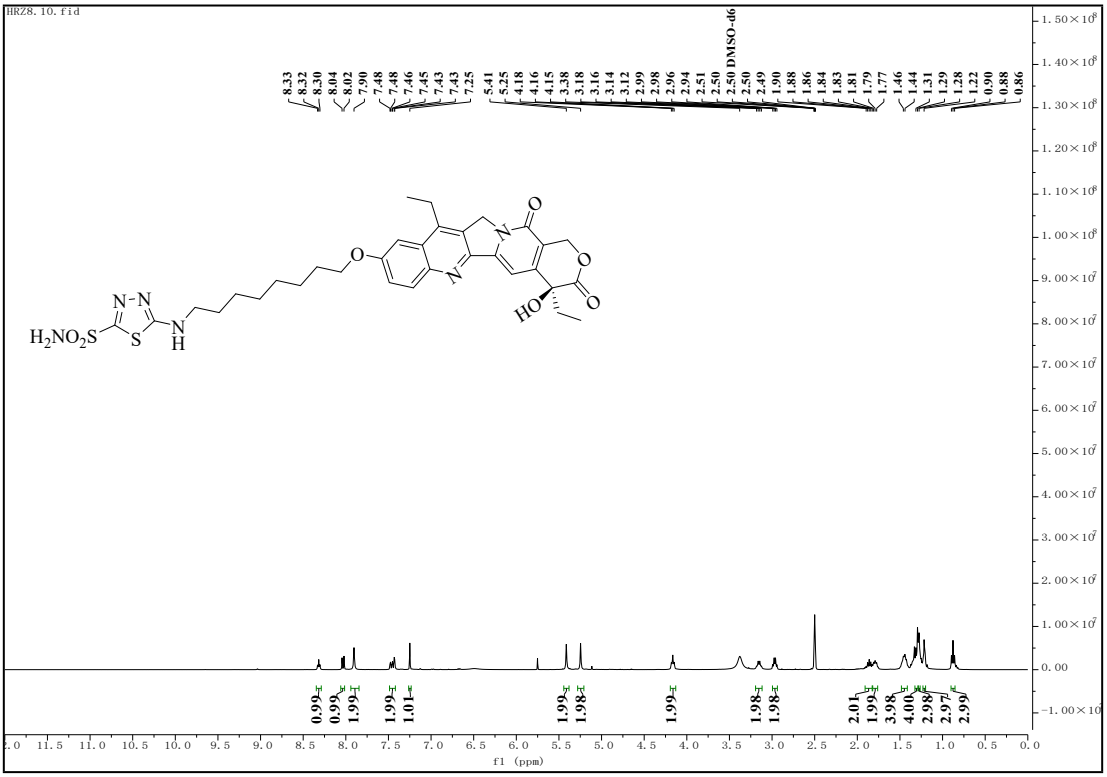
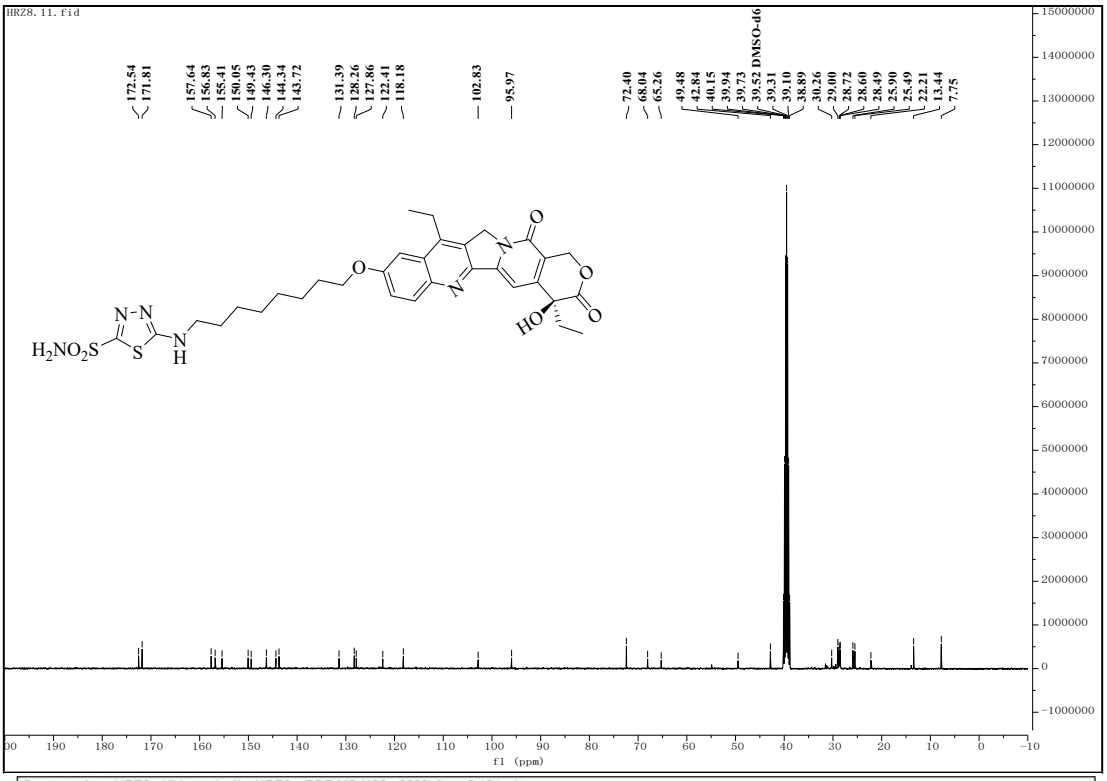
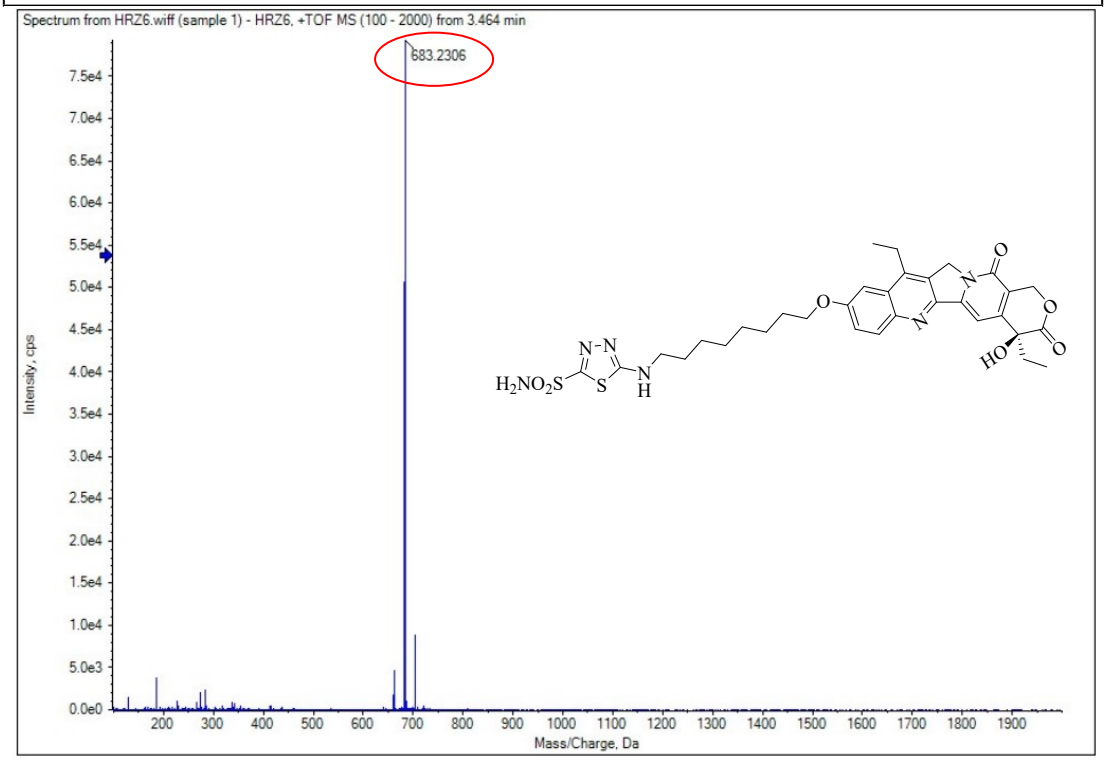
7.76
f1 (ppm)


Spectrum from HRZ5.wiff (sample 1) - HRZ5, +TOF MS (100 - 2000) from 3.594 min
669.2104
Intensity, cps
Mass/Charge, Da

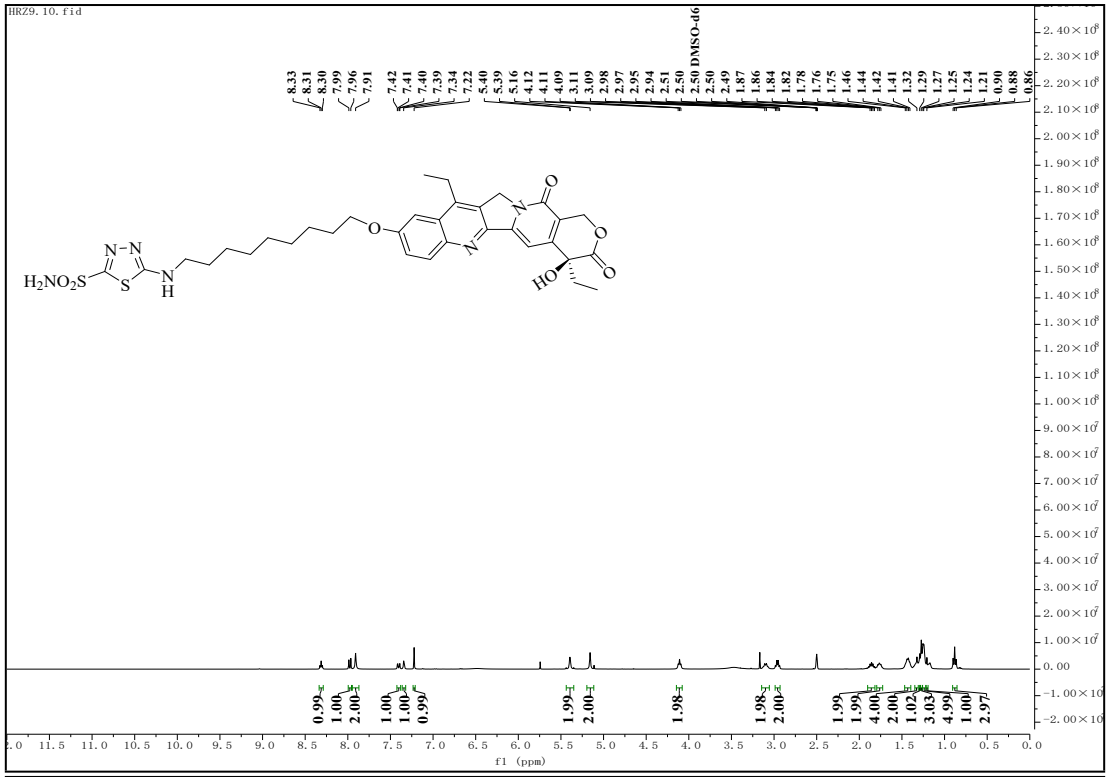
4h

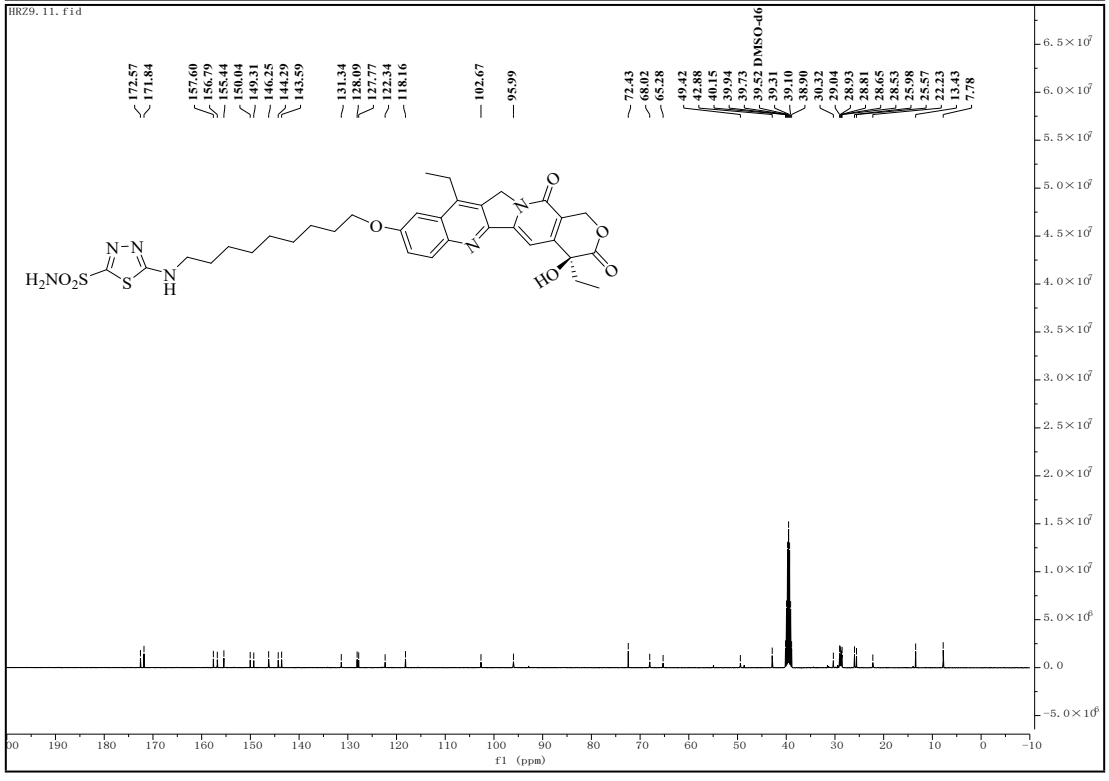

HRZ8. 10. fid
DMSO-d6
f1 (ppm)

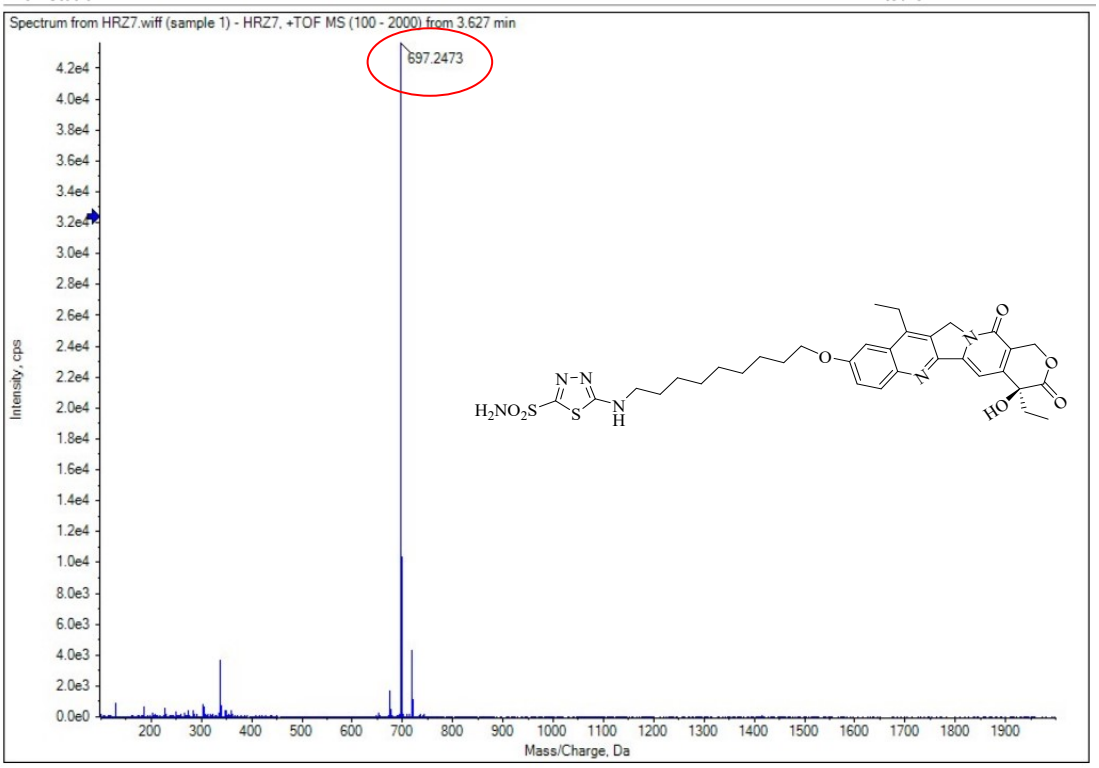

HRZ8. 11. fid
172.54
171.81
157.64
156.83
155.41
150.05
149.43
146.30
144.34
143.72
131.39
128.26
127.86
122.41
118.18
102.83
95.97
72.40
68.04
65.26
49.48
42.84
40.15
39.94
39.73
39.52 DMSO-d6
39.31
39.10
38.89
30.26
29.00
28.72
28.60
28.49
25.90
25.49
22.21
13.44
7.75
f1 (ppm)


Spectrum from HRZ6.wiff (sample 1) - HRZ6, +TOF MS (100 - 2000) from 3.464 min
683.2306
Intensity, cps
Mass/Charge, Da

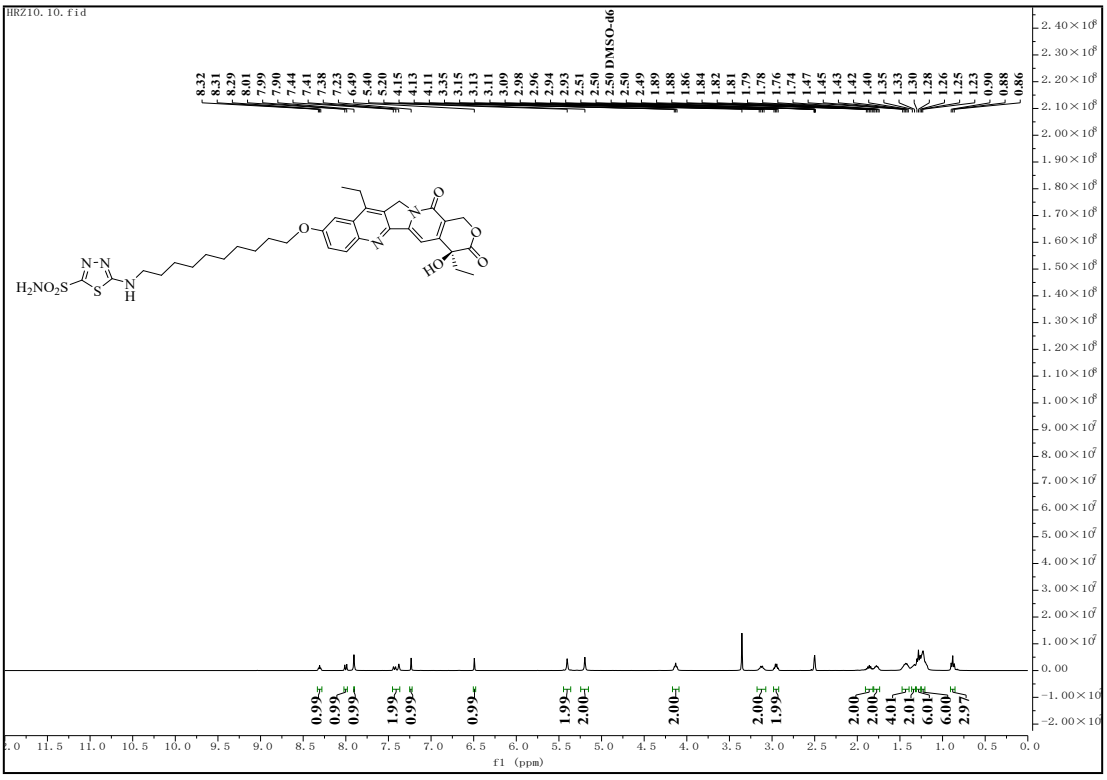
4i

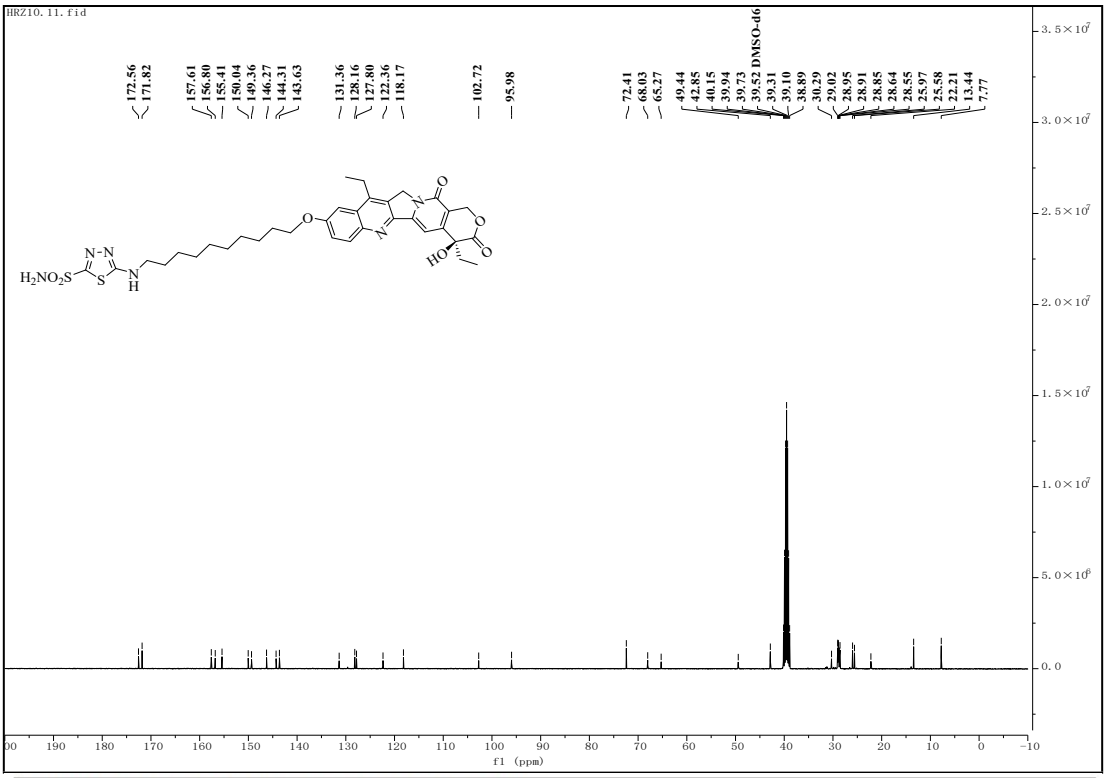

HRZ9.10.fid
8.33
8.31
8.30
7.99
7.96
7.91
7.42
7.41
7.40
7.39
7.34
7.22
5.40
5.39
5.16
4.12
4.11
4.09
3.11
3.09
2.98
2.97
2.95
2.94
2.51
2.50
2.50 DMSO-d6
2.50
2.49
1.87
1.86
1.84
1.82
1.78
1.76
1.75
1.46
1.44
1.42
1.41
1.32
1.29
1.27
1.25
1.24
1.21
0.90
0.88
0.86
0.99
1.00
2.00
1.00
1.00
0.99
1.99
2.00
1.98
1.98
2.00
1.99
1.99
4.00
2.00
1.02
3.03
4.99
1.00
2.97
f1 (ppm)

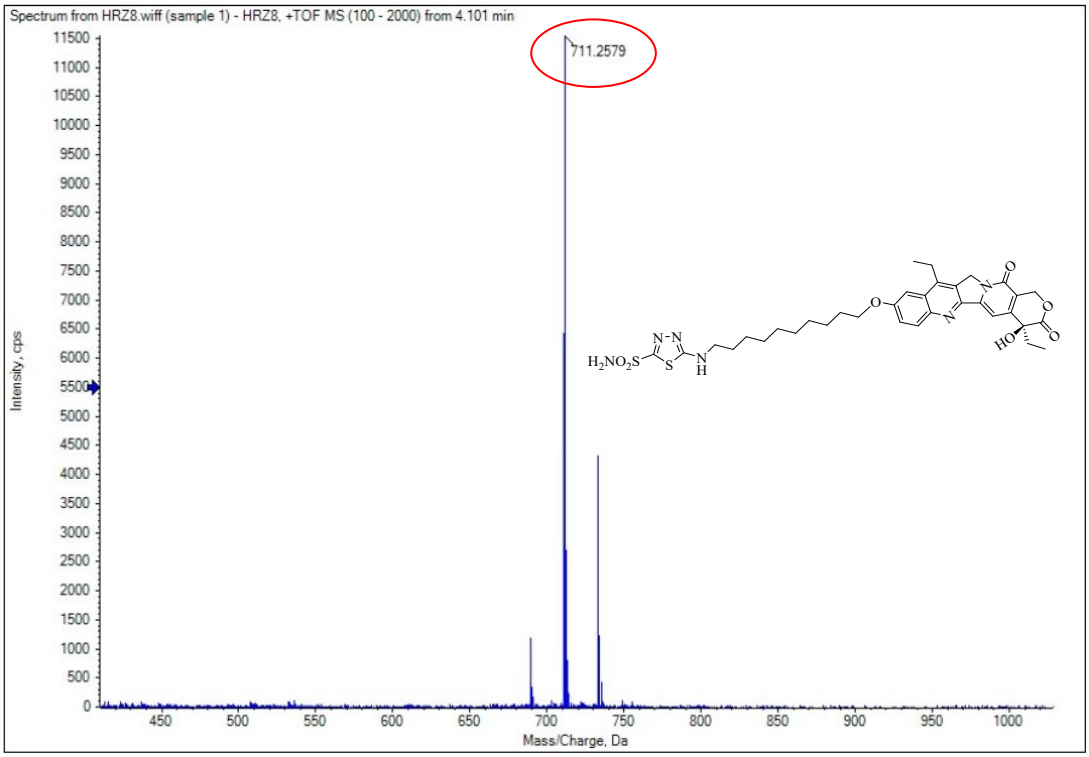

HRZ9.11.fid
172.57
171.84
157.60
156.79
155.44
150.04
149.31
146.25
144.29
143.59
131.34
128.09
127.77
122.34
118.16
102.67
95.99
72.43
68.02
65.28
49.42
42.88
40.15
39.94
39.73
39.52 DMSO-d6
39.31
39.10
38.90
30.32
29.04
28.93
28.81
28.65
28.53
25.98
25.57
22.23
13.43
7.78
f1 (ppm)


Spectrum from HRZ7.wiff (sample 1) - HRZ7, +TOF MS (100 - 2000) from 3.627 min
697.2473
Intensity, cps
Mass/Charge, Da

4j

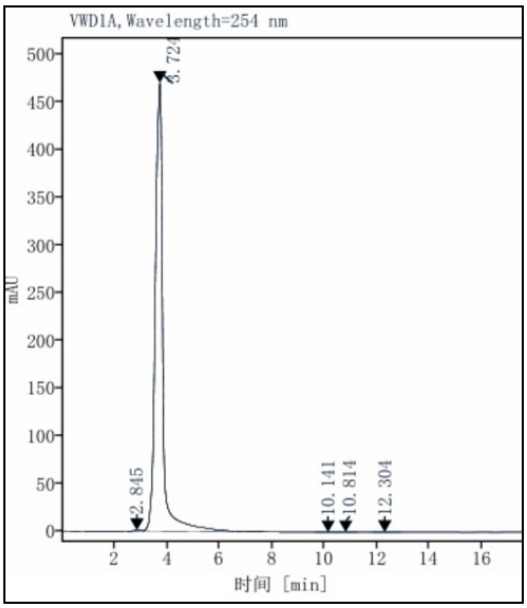

HRZ10. 10. fid
DMSO-d6
f1 (ppm)


HRZ10.11.fid
172.56
171.82
157.61
156.80
155.41
150.04
149.36
146.27
144.31
143.63
131.36
128.16
127.80
122.36
118.17
102.72
95.98
72.41
68.03
65.27
49.44
42.85
40.15
39.94
39.73
39.52 DMSO-d6
39.31
39.10
38.89
30.29
29.02
28.95
28.91
28.85
28.64
28.55
25.97
25.58
22.21
13.44
7.77
f1 (ppm)


Spectrum from HRZ8.wiff (sample 1) - HRZ8, +TOF MS (100 - 2000) from 4.101 min
711.2579
Intensity, cps
Mass/Charge, Da

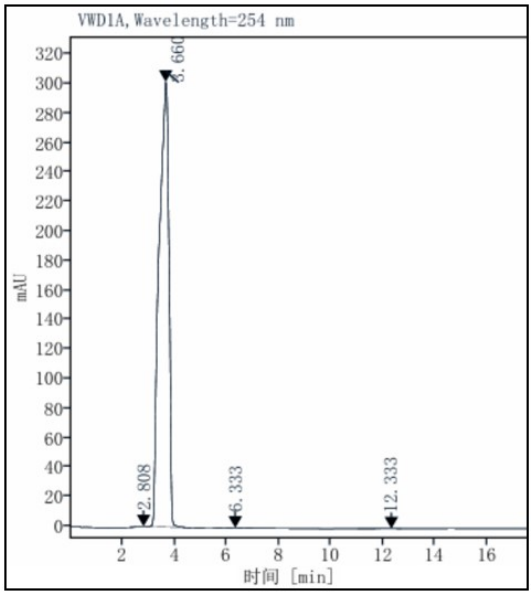
HPLC of compounds**:**

4a



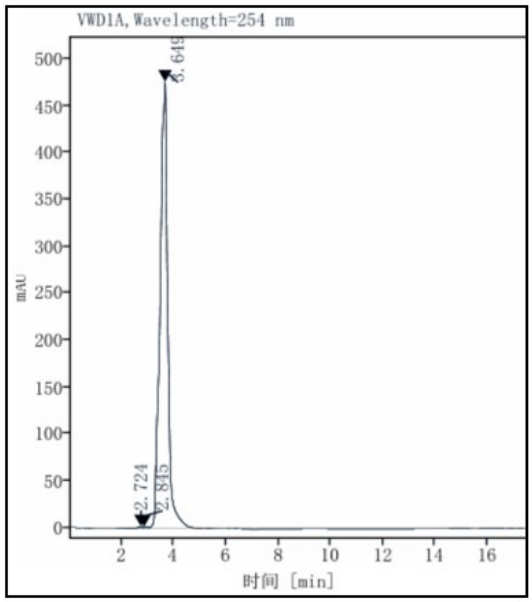
No.	RT (min)	Area	High (mAU)	%Area
1	2.845	17.1321	0.9032	0.1798
2	3.724	9510.6519	470.3148	99.8022
3	10.141	0.5025	0.0226	0.0053
4	10.814	0.6046	0.0237	0.0063
5	12.304	0.6083	0.0265	0.0064

4b



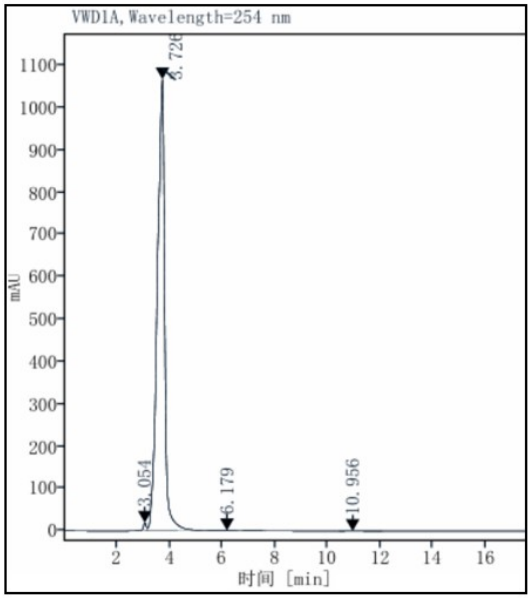
No.	RT (min)	Area	High (mAU)	%Area
1	2.808	9.2330	0.2916	0.1172
2	3.660	7851.2609	301.8411	99.6864
3	6.333	11.8657	0.3391	0.1507
4	12.333	3.5974	0.0519	0.0457

4c



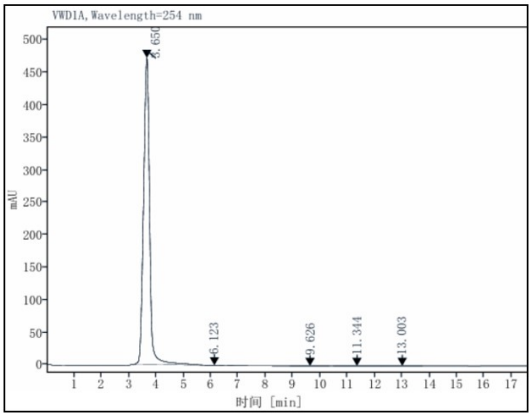
No.	RT (min)	Area	High (mAU)	%Area
1	2.724	26.7993	2.1764	0.2728
2	2.845	19.7985	1.8778	0.2015
3	3.649	9777.4198	476.5995	99.5257

4d



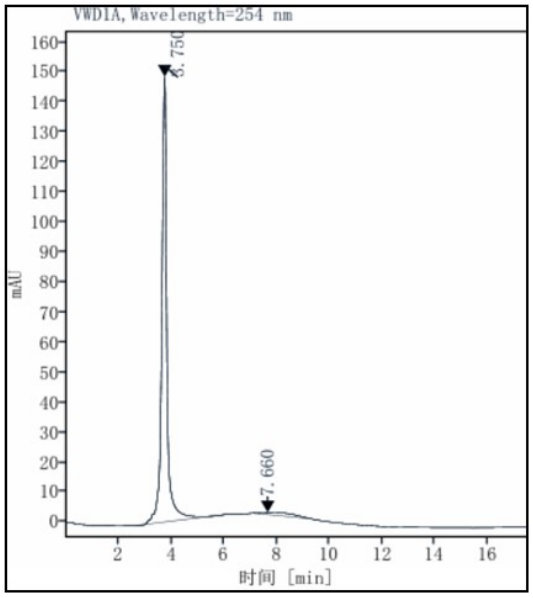
No.	RT (min)	Area	High (mAU)	%Area
1	3.054	159.3019	19.5097	0.7534
2	3.726	20967.6062	1068.1099	99.1583
3	6.179	10.8508	0.3626	0.0513
4	10.956	7.8270	0.1678	0.0370

4e



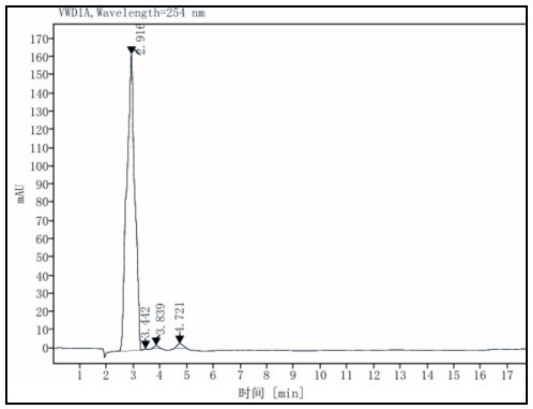
No.	RT (min)	Area	High (mAU)	%Area
1	3.650	7302.4800	472.6063	99.9061
2	6.123	2.0329	0.1704	0.0278
3	9.626	0.3519	0.0291	0.0048
4	11.344	2.1370	0.0465	0.0292
5	13.003	2.3440	0.0560	0.0321

4f



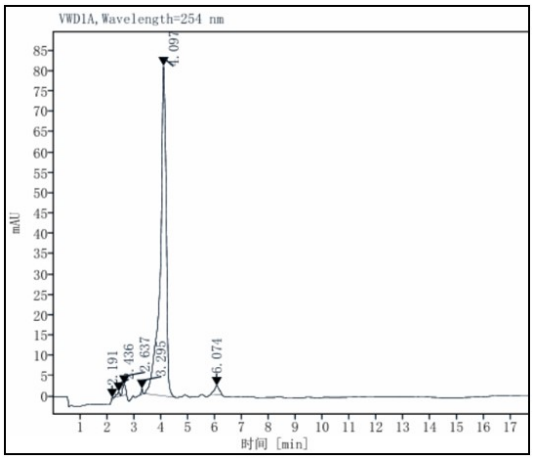
No.	RT (min)	Area	High (mAU)	%Area
1	3.750	2015.4527	148.4317	96.0085
2	7.660	83.7902	0.7316	3.9915

4g



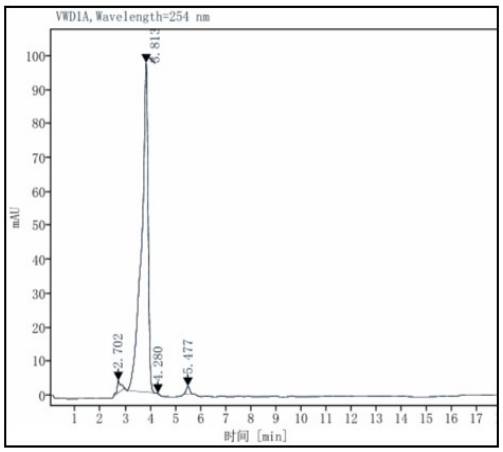
No.	RT (min)	Area	High (mAU)	%Area
1	2.916	3431.2522	162.6654	98.2678
2	3.442	2.6430	0.3348	0.0757
3	3.839	19.4948	1.6502	0.5583
4	4.721	38.3463	2.4926	1.0982

4h



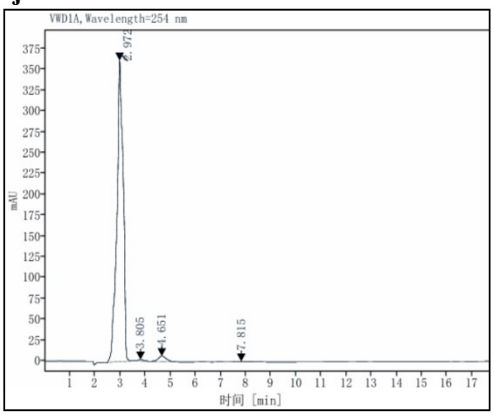
No.	RT (min)	Area	High (mAU)	%Area
1	2.191	2.4743	0.5352	0.1870
2	2.436	7.5775	1.1885	0.5728
3	2.637	20.2076	2.6793	1.5275
4	3.295	4.8753	1.3698	0.3685
5	4.097	1258.1751	81.1274	95.1077
6	6.074	29.5856	2.2468	2.2364

4i



No.	RT (min)	Area	High (mAU)	%Area
1	2.702	36.7659	3.5881	1.9971
2	3.813	1779.6662	97.0838	96.6708
3	4.280	1.6576	0.2592	0.0900
4	5.477	22.8665	2.3653	1.2421

4j



No.	RT (min)	Area	High (mAU)	%Area
1	2.972	6266.6201	361.1092	97.0725
2	3.805	27.2598	1.8003	0.4223
3	4.651	148.3569	6.5853	2.2981
4	7.815	13.3685	0.6009	0.2071