Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Novel hypoxia-targeting Pt(IV) prodrugs

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Contents

1. Materials and Instruments

All chemicals and solvents were of analytical reagent grade and used without further purification, unless noted specifically. c,c,t-[$Pt(NH_3)_2Cl_3(OH)$] and c,c,t-[$Pt(NH_3)_2Cl_2(OH)_2$] as well as YC-1 were prepared according to literature reports.[1]The purity of all compounds used in the biological studies was ≥95%. The GAPDH and HIF-1α antibodies were purchased from Santa Cruz Biotechnology. All cancer cell lines were obtained from Jiangsu KeyGEN BioTECH company (China). ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Bruker 300 MHz spectrometer. ^{195}Pt NMR spectra were measured in DMSO-d_6 with a Bruker 400 MHz spectrometer using a solution of 10 mM potassiumhexachloro- platinate in 95% H_2O/5% D_2O as an external standard. Platinum contents were determined by Inductively Coupled Plasma-Mass Spectrometer (ICP-MS, Optima 5300DV, PerkinElmer, USA). Mass spectra were measured by an Agilent 6224 ESI/TOF MS instrument.

2. Synthesis

2.1 Synthesis of YC-2

To a solution of YC-1 (0.30 g, 1.0 mM) dissolved in dry THF (15 mL) was added succinic anhydride (0.12 g, 1.2 mM) and catalytic amount of DMAP. The reaction mixture was refluxed for 4 h and then evaporated. The crude product was purified by column chromatography (silica gel, methanol/dichloromethane 1 : 9) to give YC-2 as an white solid (0.37 g, yield 93%).^{1}H NMR (300 MHz, Methanol-d_4) δ 8.14 (d, J = 8.2 Hz, 1H), 7.52 (d, J = 8.5 Hz, 1H), 7.42 (t, J = 7.6 Hz, 1H), 7.26 (dt, J = 13.5, 7.6 Hz, 6H), 6.95 (d, J = 3.3 Hz, 1H), 6.64 (d, J = 3.3 Hz, 1H), 5.66 (s, 2H), 5.24 (s, 2H), 2.65 (t, J = 3.8 Hz, 4H), ppm. ESI-MS (m/z): calcd for $C_{23}H_{20}N_2O_5$ $[M-H]^-$: 403.12995, found: 403.12991.

2.2 Synthesis of YCC-1

To a solution of YC-2 (0.49 g, 1.2 mmol), TBTU (0.39 g, 1.2 mmol), and Et_3N (0.12 g, 1.2 mmol) in dry DMF (15 mL), and c,c,t-[$Pt(NH_3)_2Cl_3(OH)$] (0.35 g, 1.0 mmol) was added. The mixture was stirred at room temperature overnight. After completion of reaction, the whole mixture was added to CH_2Cl_2(120 mL), and then extracted twice with water (100 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified on silica gel column eluted DCM/MeOH (50:1) to give the desired product as a yellow solid.^{1}H NMR (300 MHz, DMSO-d_6) δ 8.11 (d, J = 8.1 Hz, 1H), 7.76 (d, J = 8.4 Hz, 1H), 7.46 (t, J = 7.7 Hz, 1H), 7.28 (dt, J = 12.7, 6.9 Hz, 6H), 7.03 (d, J = 3.1 Hz, 1H), 6.72 (d, J = 3.1 Hz, 1H), 5.73 (s, 2H), 5.18 (s, 2H), 2.56 (s, 4H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ 178.88 , 172.03 , 149.11 , 148.42 , 140.26 , 137.19 , 134.94 , 128.51 , 127.49 , 127.17 , 126.84 , 121.62 , 120.94 , 120.22 , 112.55 , 110.21 , 107.92 , 57.63 , 51.92 , 30.95 , 29.70 ppm. ^{195}Pt NMR (129 MHz, DMSO-d_6) δ 544.72 ppm. ESI-MS (m/z): calcd for $C_{23}H_{25}Cl_3N_4O_5Pt$ $[M-H]^-$: 736.04655, found: 736.77424.

2.3 Synthesis of YCC-2

YC-2 (0.49 g, 1.2 mmol), c,c,t-[$Pt(NH_3)_2Cl_2(OH)_2$] (0.33 g, 1.0 mmol), TBTU (0.39 g, 1.2 mmol), and Et_3N (0.12 g, 1.2 mmol) were added into dry DMSO (30 mL). The solution was stirred for 12 h at 60 °C to get a relatively clear orange solution. The solution was filtered to remove the unreacted solid. The clarified solution was added into Et_2O (30 mL) to form a precipitate, and the precipitate was washed with ethyl acetate (30 mL). The final product was obtained as a light yellow solid.^{1}H NMR (300 MHz, DMSO-d_6) δ 8.11 (d, J = 8.0 Hz, 1H), 7.76 (d, J = 8.4 Hz, 1H), 7.46 (t, J = 7.7 Hz, 1H), 7.28 (dt, J = 12.2, 6.6 Hz, 6H), 7.03 (d, J = 3.2 Hz, 1H), 6.72 (d, J = 3.1 Hz, 1H), 5.73 (s, 2H), 5.18 (s, 2H), 1.00 (s, 1H)

ppm. ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.20 , 172.22 , 149.15 , 148.40 , 140.27 , 137.19 , 134.94 , 128.52 , 127.49 , 127.17 , 126.85 , 121.62 , 120.92 , 120.21 , 112.57 , 110.21 , 107.92 , 57.63 , 51.93 , 31.01 , 29.87 ppm. ^{195}Pt NMR (129 MHz, DMSO-d_6) δ 1025.94 ppm. ESI-MS (m/z): calcd for $C_{23}H_{26}Cl_2N_4O_6Pt$ $[M-H]^-$: 718.08044, found: 718.07235.

3. The Stability of YCC-2 in PBS Buffer

The stability of Pt(IV) complexes in a phosphate buffer saline (PBS) was investigated by HPLC. The incubation was generated by adding YCC-2 to PBS buffer, which was performed at 25°C for 0, 3, 6, 12, 24, 36 and 48 h, separately. Reversed phase HPLC was implemented on a 250×4.5 mm ODS column and the HPLC profiles were recorded on UV detection at 210 nm. Mobile phase consisted of acetonitrile/water (80:20, v/v), and flow rate was 1.0 mL/min. The samples were taken for HPLC analysis after filtration by 0.45 μm filter.

4. Released Ability of Pt(IV) Complexes under Reduction with Ascorbic Acid

The released ability of Pt(IV) complexes in a solvent comprising acetonitrile/water (20:80, v:v) was investigated by HPLC. The standard compounds were made by adding ascorbic acid, YC-1, and YCC-2, respectively, to a solvent containing 20% acetonitrile and 80% water. The incubation was generated by adding test compounds (10 mM) to a solvent containing 80% acetonitrile and 20% water in the presence of 15 mM ascorbic acid, which was performed at 25°C for 0, 1, 2, 6, 12, 18 and 24 h, separately. Reversed phase HPLC was implemented on a 250×4.5 mm ODS column and the HPLC profiles were recorded on UV detection at 210 nm. Mobile phase consisted of acetonitrile/water (80:20, v/v), and flow rate was 1.0 mL/min. The samples were taken for HPLC analysis after filtration by 0.45 μm filter.

5. Cell Culture

Human lung (A549), breast (MCF-7), colon (HCT-116), gastric (SGC7901) and cisplatin-resistant gastric (SGC7901/CDDP) cancer cell lines along with human liver cell line (LO2) were maintained in the logarithmic phase at 37°C in a 5% carbon dioxide atmosphere using the following monolayer culture media containing 10% fetal bovine serum (FBS), 100 mg/mL of penicillin and 100 mg/mL of streptomycin.

6. Cytotoxicity Measurement

The growth inhibitory effect towards human cell lines was evaluated by means of MTT assay. Briefly, 1×10^5 cells/well, dependent upon the growth characteristics of the cell line, were seeded in 96-well plates in DMEM medium with 10% FBS and then incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air. After 24 h, the medium was removed and replaced with a fresh one containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After incubation with the target compounds under normoxic condition (20% O_2, 5% CO_2 and 75% N_2, at 37 °C) or hypoxic condition (1% O_2, 5% CO_2 and 94% N_2, at 37 °C) for 72 h, the cells was treated with 10 μL of a 5 mg·mL^{-1} MTT for 5 h additional incubation. The medium was thrown away and replaced by 100 mL DMSO. The inhibition of cell growth induced by the tested complexes was detected by measuring the absorbance of each well at 570/630 nm using enzyme labeling instrument.

7. Cellular Uptake Test

HCT-116 cells were seeded in 6-well plates. After the cells achieved about 80% confluence, 15 μM of cisplatin, oxaliplatin, or YCC-2 was added, respectively. After incubation under normoxic condition (20% O_2, 5% CO_2 and 75% N_2, at 37 °C) or hypoxic condition (1% O_2, 5% CO_2 and 94% N_2, at 37 °C) for 12 h, cells were collected and washed three times with ice-cold PBS, then centrifuged at 1000 × g for 10 min and resuspended in 1 mL PBS. A volume of 100 μL was taken out to determine the cell density. The rest of the cells were spun down and digested at 65°C in 200 μL 65% HNO_3 for 10 h. The Pt level in cells was estimated by ICP-MS.

8. Apoptosis Analysis

HCT-116 cells were seeded at the density of 2×10^6 cells/mL of the DMEM medium with 10% FBS on 6-well plates to the final volume of 2 mL. The plates were incubated for overnight and then treated with equivalent concentrations of cisplatin, oxaliplatin and YCC-2 for 72 h. Briefly, after incubation under normoxic condition (20% O_2, 5% CO_2 and 75% N_2, at 37 °C) or hypoxic condition (1% O_2, 5% CO_2 and 94% N_2, at 37 °C) for 72 h, cells were collected and washed with PBS twice, and then resuspend cells in 1 × Binding Buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) at a concentration of 1×10^6 cells/mL. The cells were stained with 5 μL of FITC Annexin V (BD, Pharmingen) and 5 μL propidium iodide (PI) staining using annexin-V FITC apoptosis kit followed; 100 μL of the solution was transferred to a 5 mL culture tube and incubated for 30 min at room temperature (25°C) in the dark. The apoptosis ratio was quantified by system software (Cell Quest; BD Biosciences).

9. Western Blot

HCT-116 cells were lysed in cell lysis buffer containing PMSF for 30 min at 4 °C. Lysates were collected by centrifugation at 13000 rpm for 20 min at 4 °C. Proteins from cell lysates were separated on the SDS-PAGE and transferred onto polyvinylidine difluoride (PVDF) membrane (Amersham Biosciences). The membrane was blocked with PBST containing 5% non-fat dry milk for 1 h and further incubated with monoclonal anti-human HIF-1α antibody (Santa Cruz Biotechnology, USA) overnight at 4 °C under gentle shaking. After that, the membrane was incubated with the secondary antibody (1:2000) for 1 h at RT (25 °C). Protein blots were detected with chemiluminescence reagent (Thermo Fischer Scientifics Ltd.). Anti-β-a ctin antibody was used as loading control.

10. Antitumor Activity in Vivo

The in vivo cytotoxic activity of YCC-2 was investigated using a human colon cancer cell line in BALB/c nude mice. Five-week-old male BALB/c nude mice (16−18 g) were purchased from Shanghai Ling Chang biotechnology company (China); tumors were induced by a subcutaneous injection in their right armpit region of 10^7 cells in 0.1 mL of sterile PBS. Animals were randomly divided into five groups, and started on the second day. When the tumors reached a volume of 100-150 mm^3 in all mice on day 14, the first group was injected with an equivalent volume of 5% dextrose via a tail vein as the vehicle control mice. No. 2 and No. 3 groups were treated with cisplatin and oxaliplatin at doses of 5 mg/kg. No. 4 and No. 5 groups were treated with YCC-2 at the doses of 5 or 12 mg/kg. Cisplatin and oxaliplatin were dissolved in vehicle. YCC-2 was dissolved in a small amount of DMF, and then diluted with Tween 80 and 5% dextrose injection. The final solution contains DMF: Tween 80: 5% dextrose injection = 10: 2: 88. Tumor volume and body weight were recorded every other day after drug treatment. All mice were sacrificed after 3 weeks of treatment and the tumor volumes were measured with electronic digital calipers and determined by measuring length (A) and width (B) to calculate volume ($V = AB^2/2$).

11. Supplementary Tables

Table S1.Cytotoxicity of cisplatin, YCC-1, YCC-2 against human normal cells and several cancer cells under normoxia

	IC_{50} values (μM)						
Complex	LO2	HCT-116	A549	MCF-7	SGC7901	SGC7901/CDDP	RF[a]
Cisplatin	6.02 ± 0.45	27.10 ± 1.93	19.79 ± 1.52	21.81 ± 1.54	6.84 ± 0.54	13.09 ± 1.21	1.91
YC-1	>80	>80	>80	>80	>80	>80	
YCC-1	7.64 ± 0.03	4.69 ± 0.28	4.58 ± 0.36	13.87 ± 1.11	5.80 ± 0.31	1.45 ± 0.97	0.25
YCC-2	8.03 ± 0.15	3.51 ± 0.19	0.80 ± 0.04	21.64 ± 1.87	5.19 ± 0.27	2.78 ± 0.13	0.54

[a]RF: the ratio between IC_{50} values calculated for the resistant cells and those obtained with the sensitive ones.

Table S2. Cytotoxicity of cisplatin, YCC-1, YCC-2, YC-1 and oxaliplatin against A549 and HCT-116cancer cells under hypoxia

	IC_{50} values (μM)			
	Hypoxia		Normoxia	
Complex	A549	HCT-116	A549	HCT-116
Oxaplatin	nd*	0.984 ± 0.06	nd*	23.89 ± 1.32
Cisplatin	16.617 ± 1.66	5.463 ± 0.26	19.79 ± 1.52	27.10 ± 1.93
YC-1	20.13 ± 1.73	43.26 ± 4.21	>80	>80
YCC-1	5.059 ± 0.17	1.23 ± 0.84	4.58 ± 0.36	4.69 ± 0.28
YCC-2	0.442 ± 0.03	0.059 ± 0.01	0.80 ± 0.04	3.51 ± 0.19

***** not determined.

Table S3. Cellular uptake of cisplatin, oxaliplatin and YCC-2 in HCT-116 cells after 12 h of incubation

	Pt content (ng/10^6 cells)	
	Normoxia	Hypoxia
Complex	HCT-116	HCT-116
Cisplatin	135 ± 16	241 ± 19
Oxaliplatin	236 ± 29	544 ± 28
YCC-2	224 ± 25	992 ± 35

12. Supplementary Figures

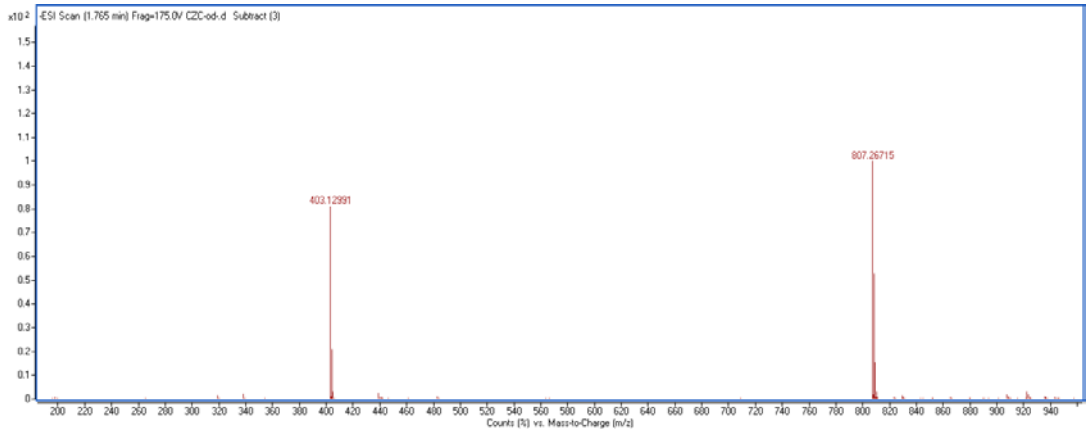


Figure S1. ESI-MS mass spectrum of YC-2.

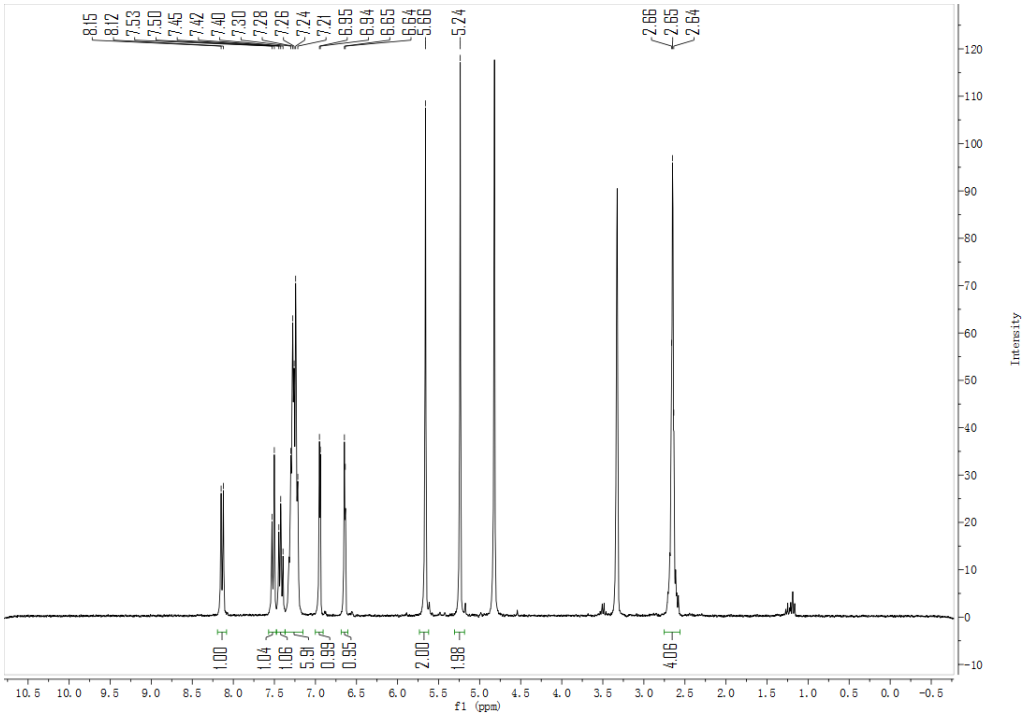


Figure S2.^{1}H NMR spectrum of YC-2 (300 MHz, Methanol-d_4).

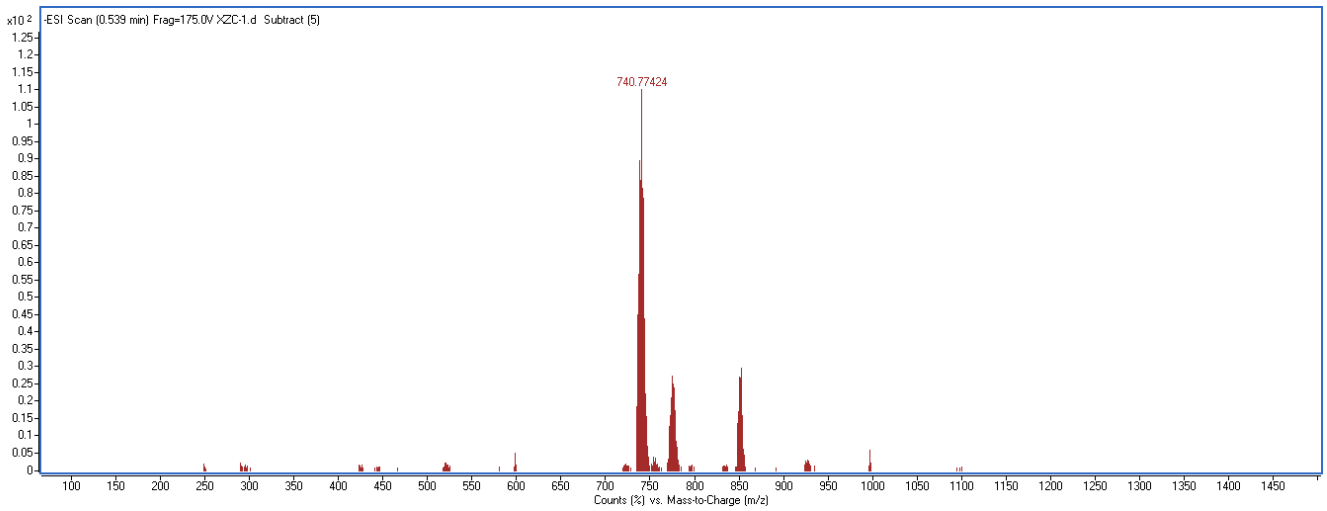


Figure S3. ESI-MS mass spectrum of YCC-1.

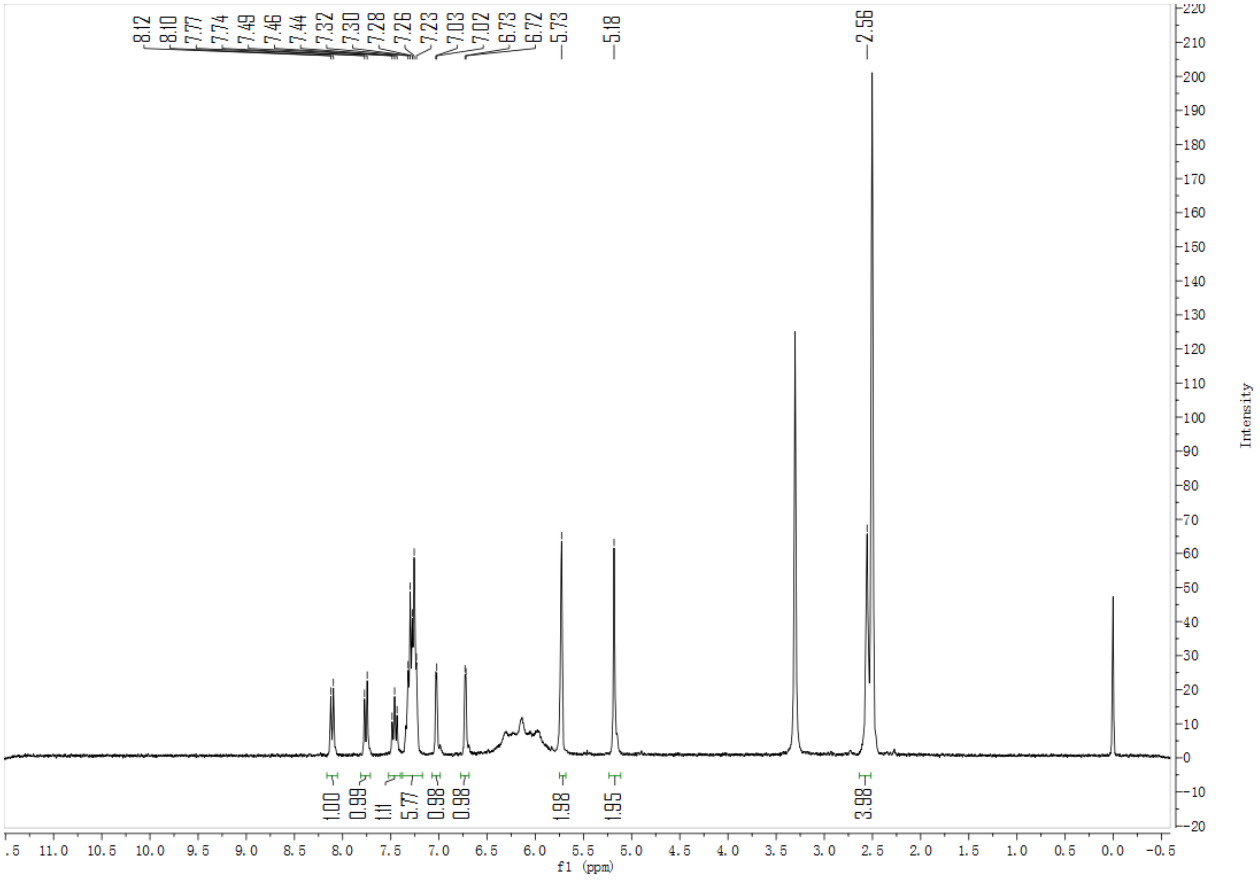


Figure S4. ^{1}H NMR spectrum of YCC-1 (300 MHz, DMSO-d_6).

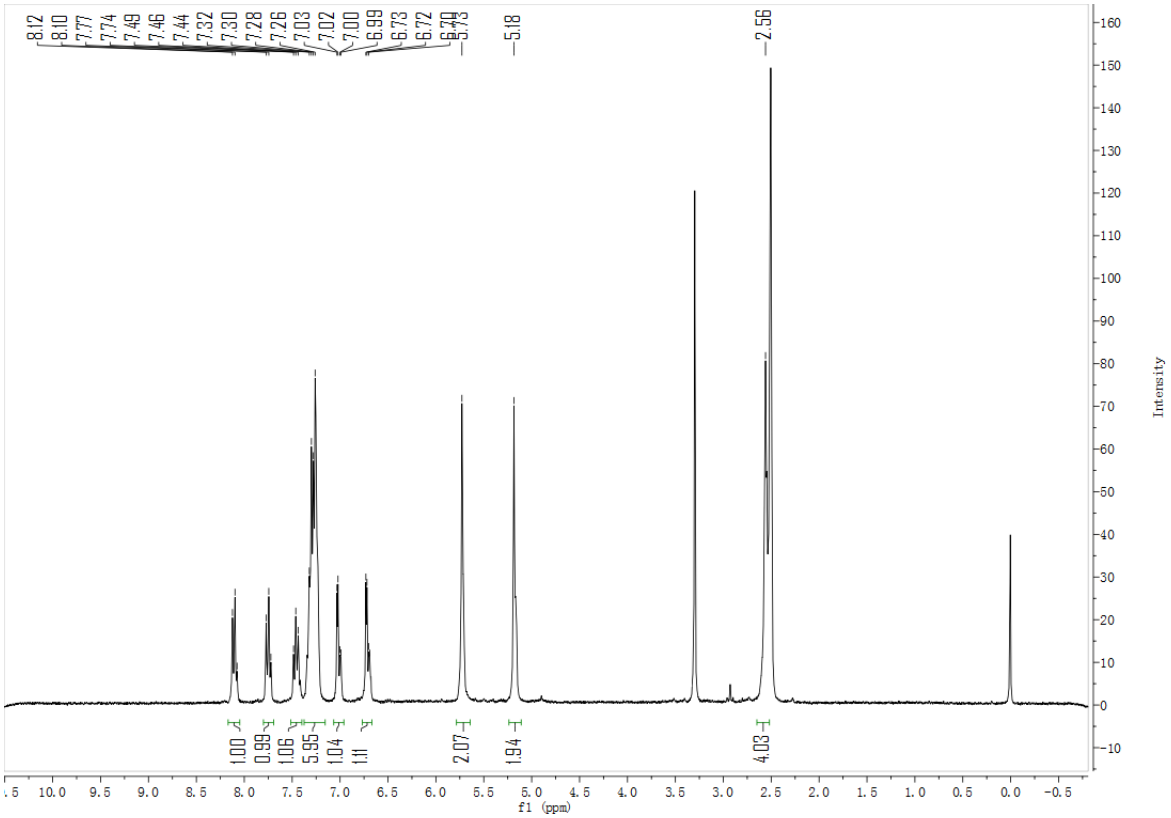


Figure S5. ^{1}H NMR spectrum of YCC-1 (300 MHz, DMSO-d_6 + D_2O).

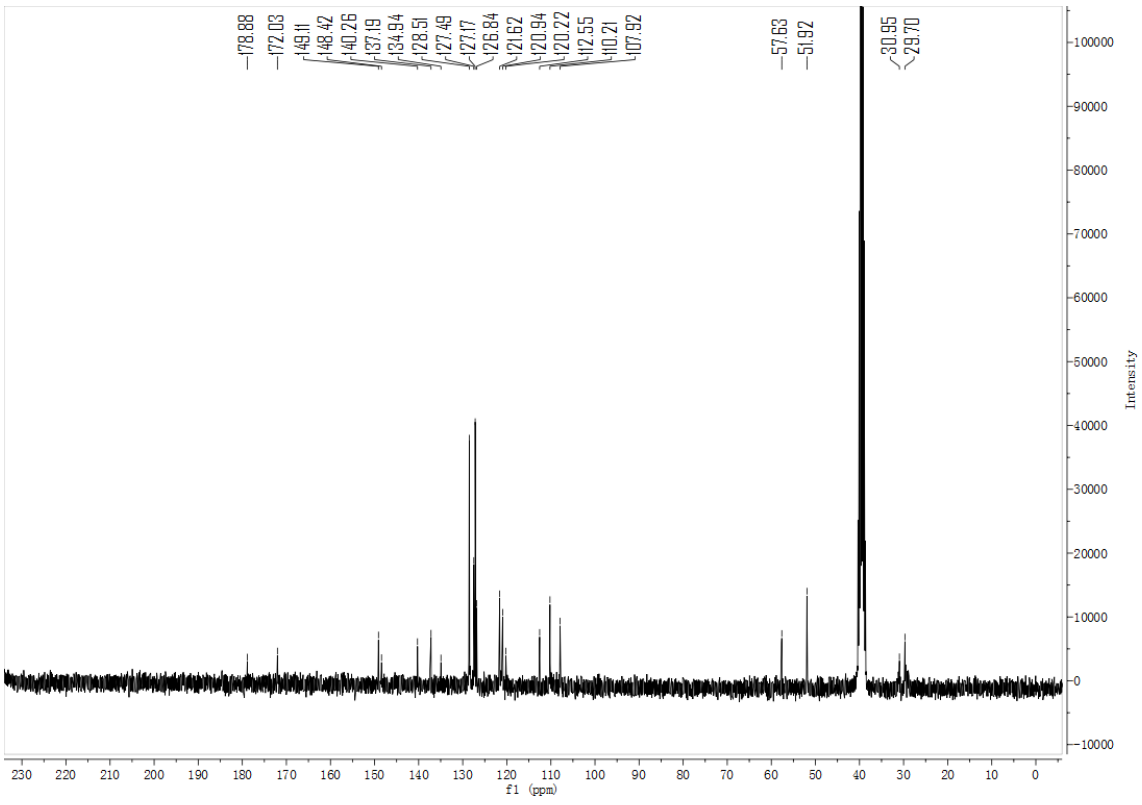


Figure S6. ^{13}C NMR spectrum of YCC-1 (75 MHz, DMSO-d_6).

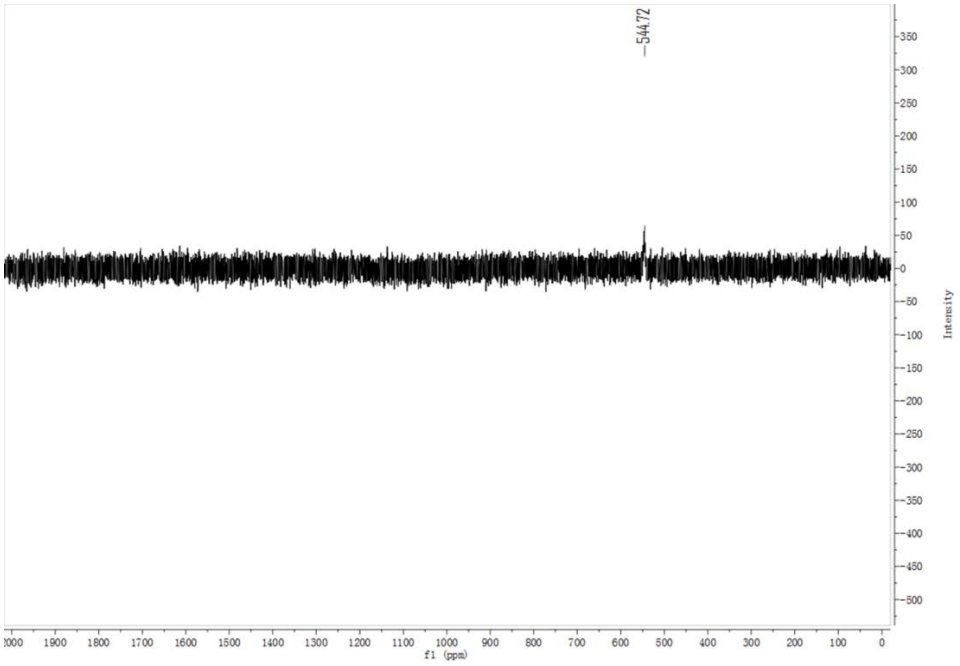


Figure S7.^{195}Pt NMR spectrum of YCC-1 (129 MHz, DMSO-d_6).

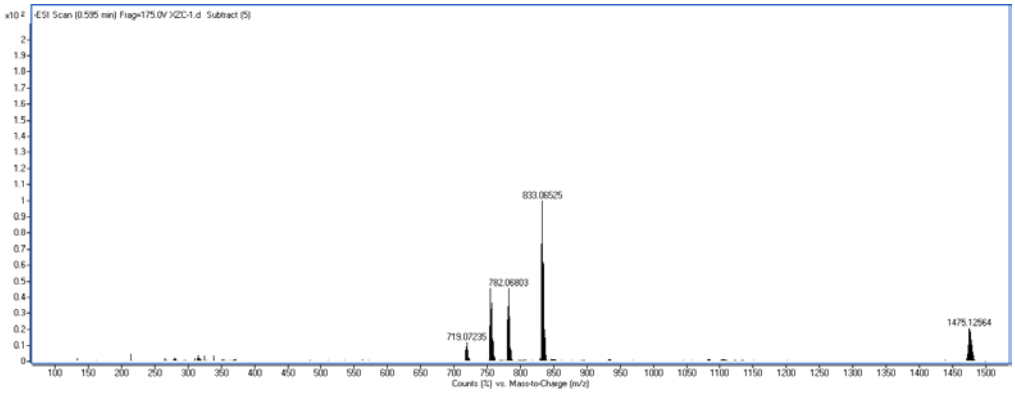


Figure S8.ESI-MS mass spectrum of YCC-2.

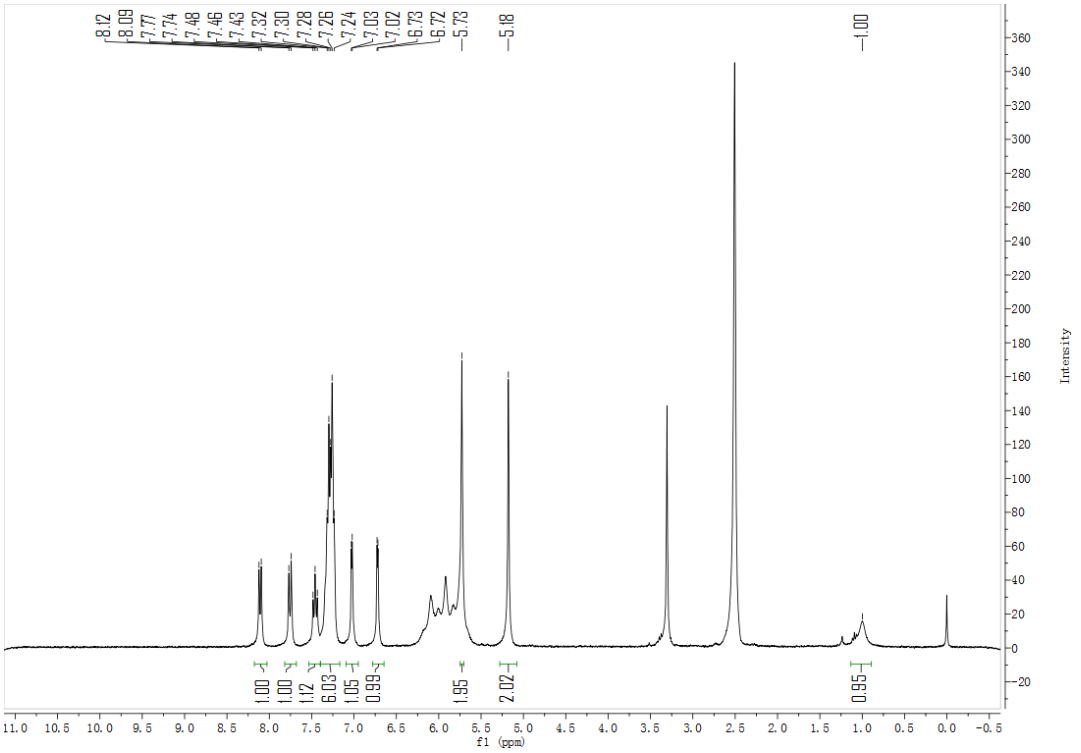


Figure S9. ^{1}H NMR spectrum of YCC-2 (300 MHz, DMSO-d_6).

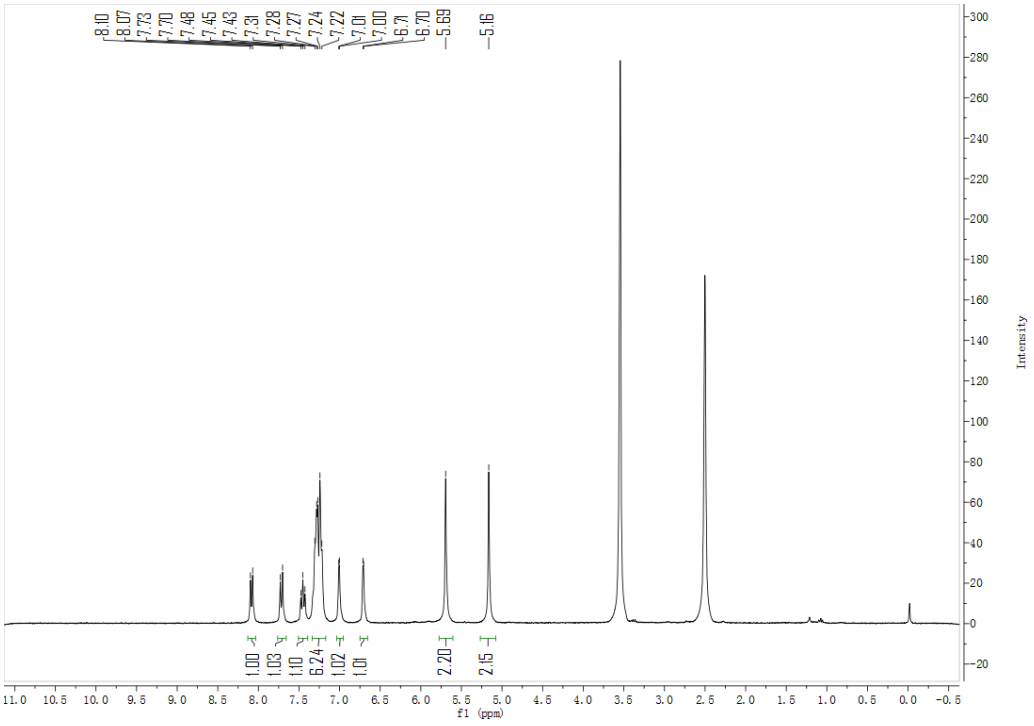


Figure S10. ^{1}H NMR spectrum of YCC-2 (300 MHz, DMSO-d_6 + D_2O).

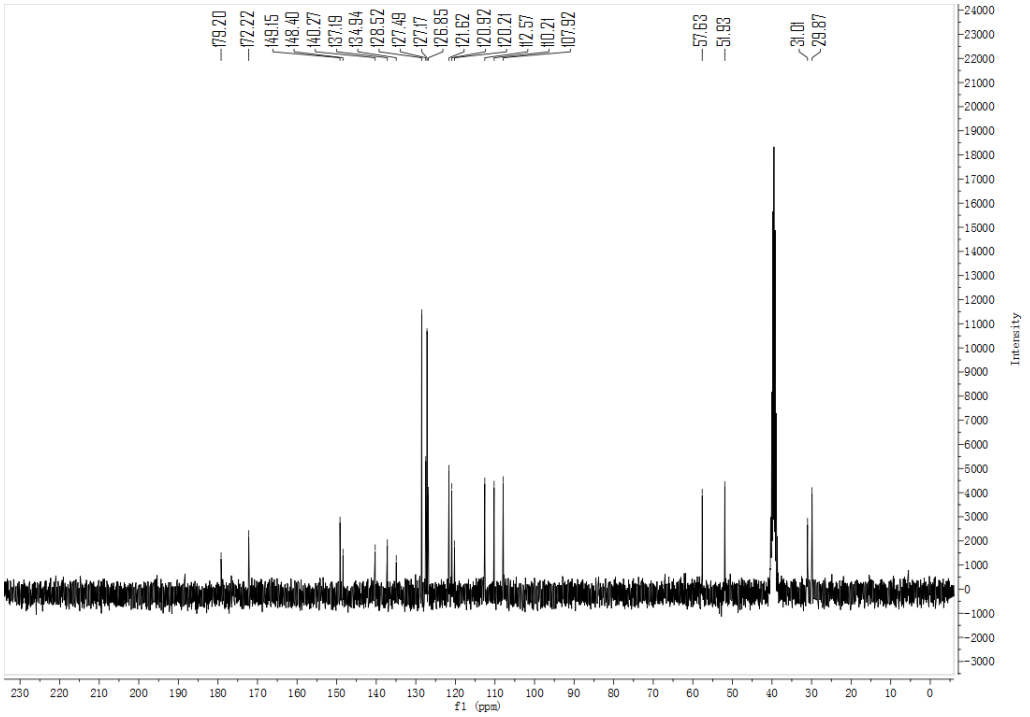


Figure S11. ^{13}C NMR spectrum of YCC-2 (75 MHz, DMSO-d_6).

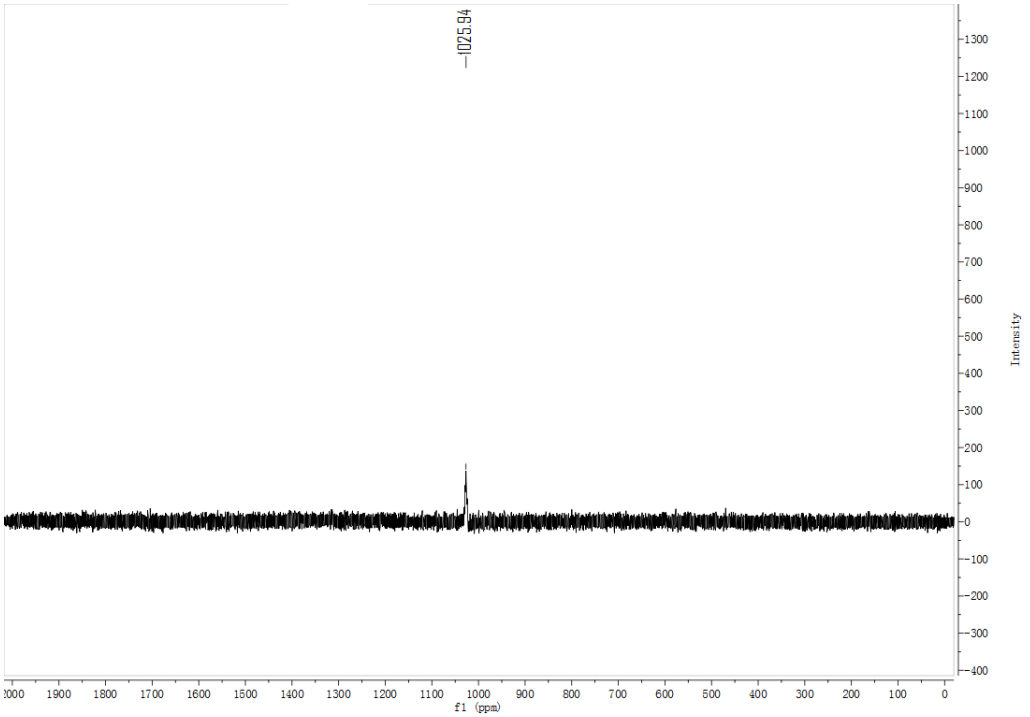
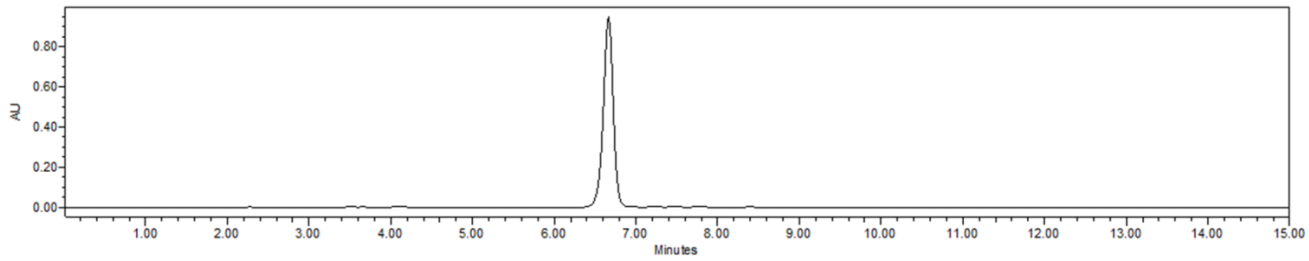
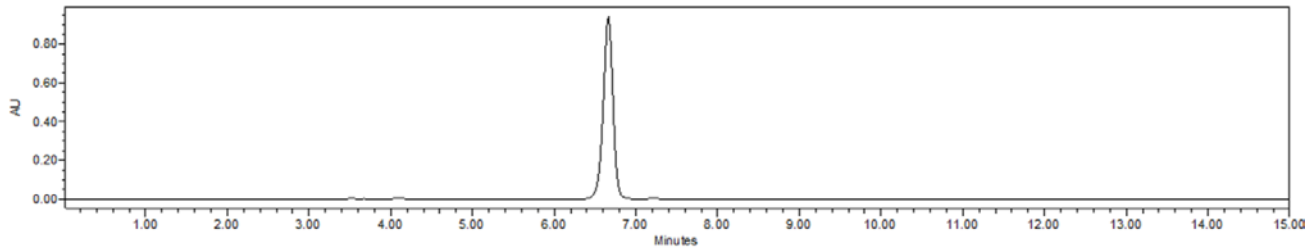


Figure S12. ^{195}Pt NMR spectrum of YCC-2 (129 MHz, DMSO-d_6).

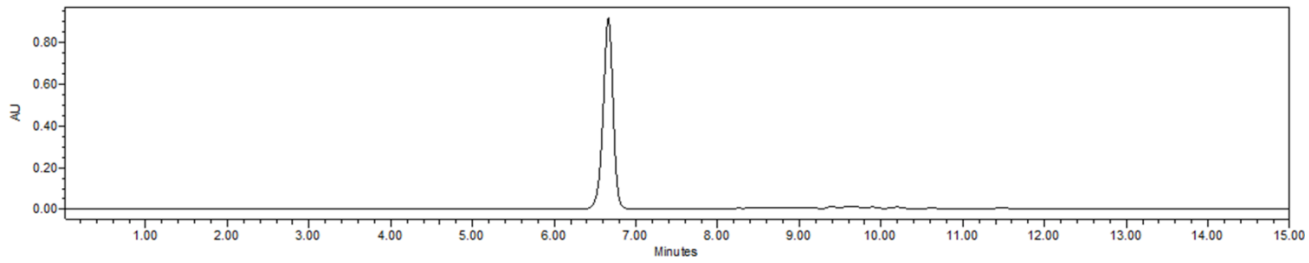
0 h in PBS



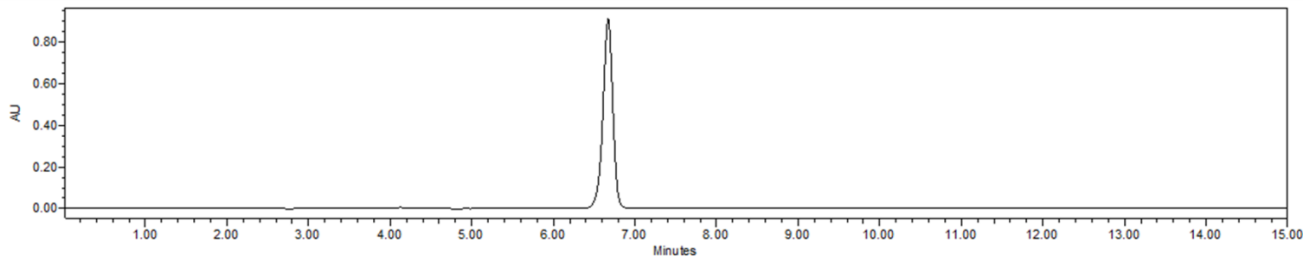
3 h in PBS



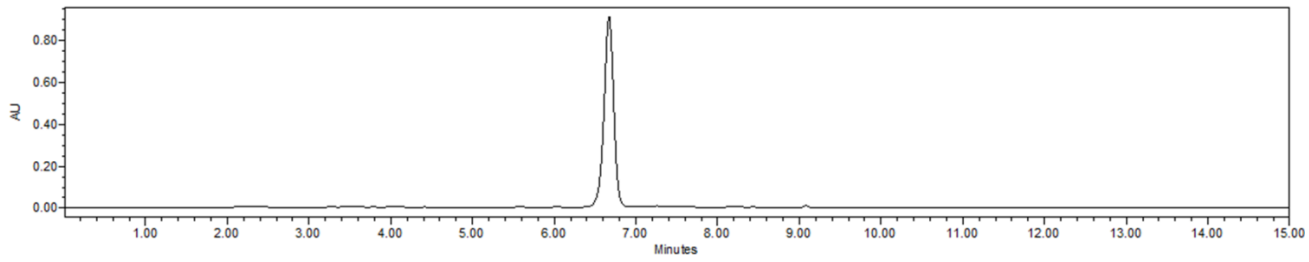
6 h in PBS



12 h in PBS



24 h in PBS



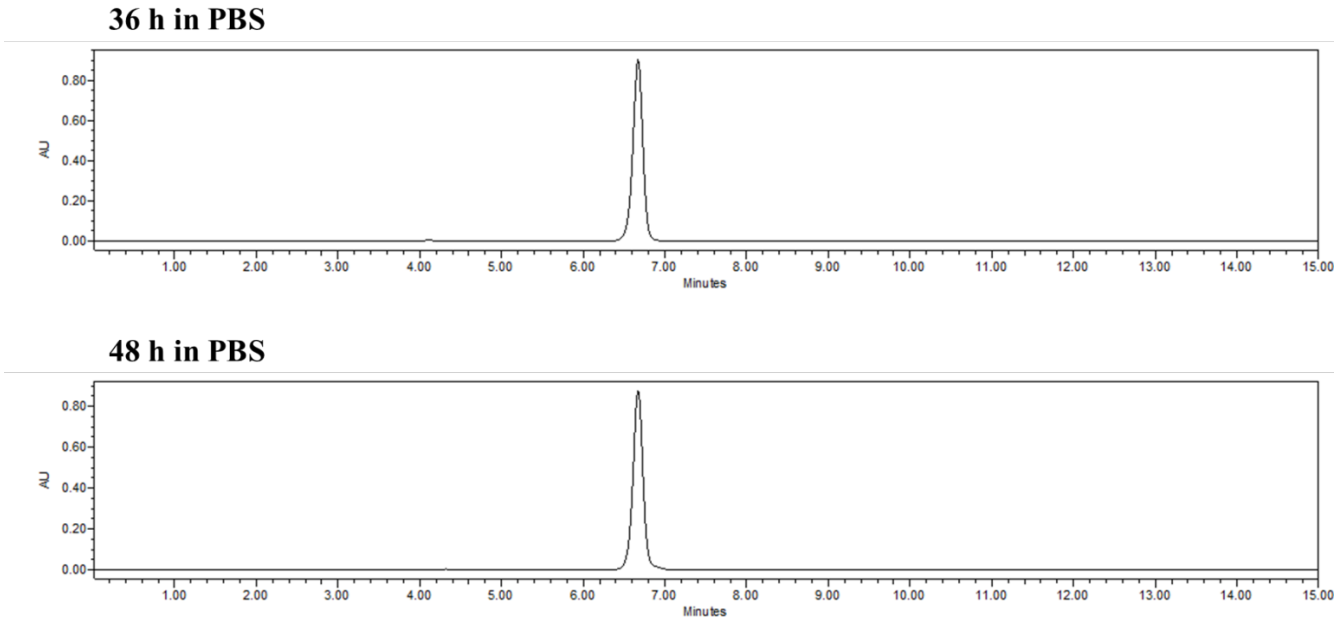
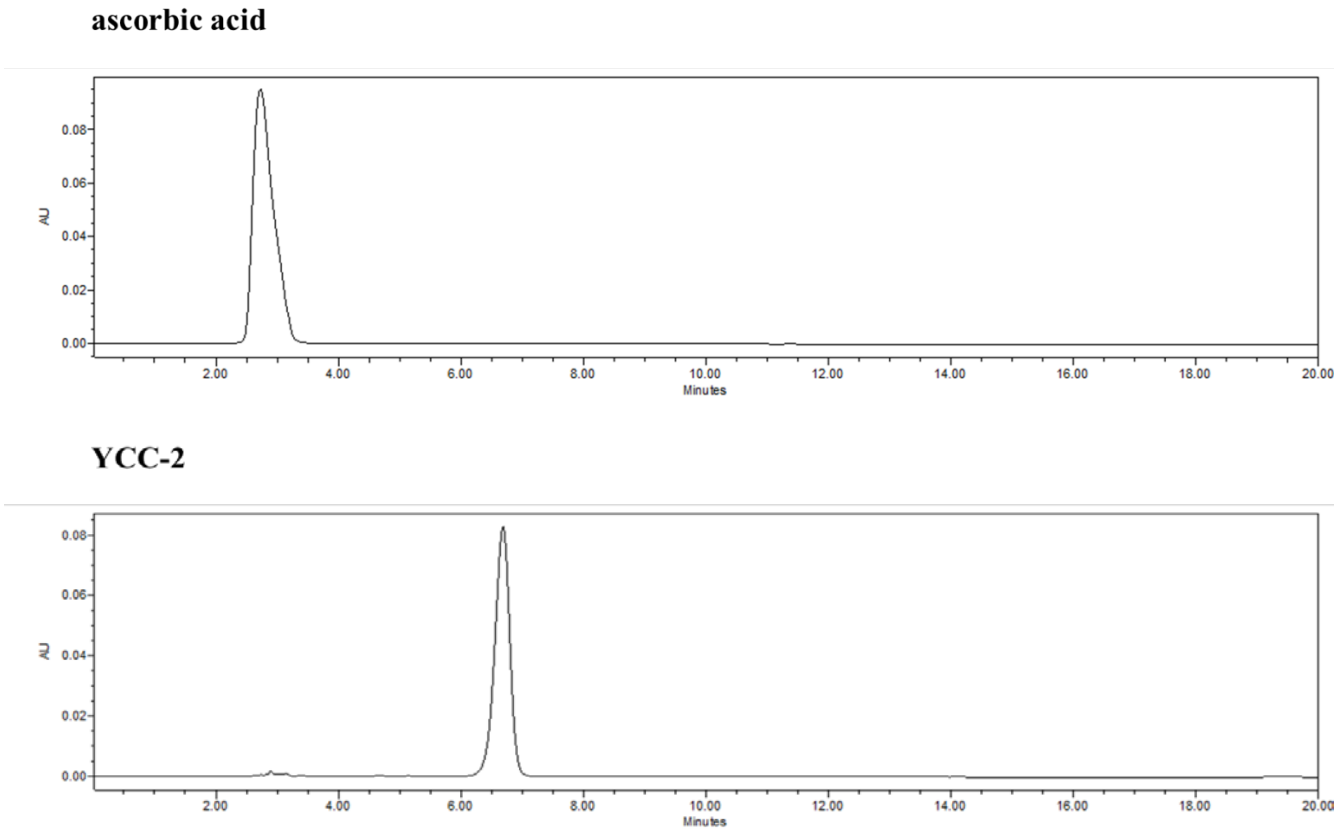
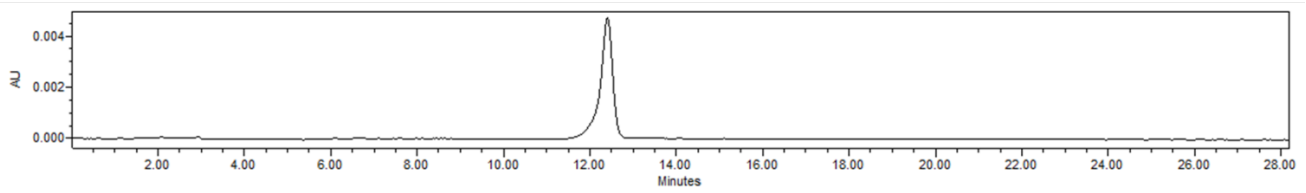


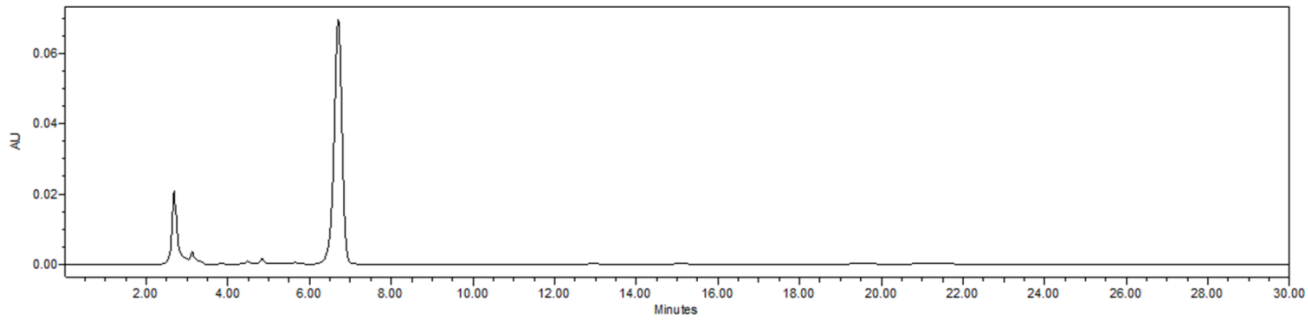
Figure S13. The stability of YCC-2 in PBS buffer.



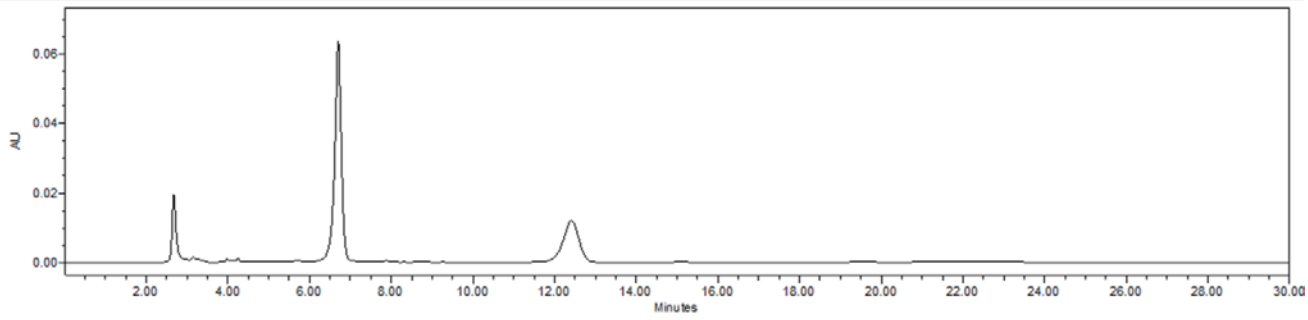
YC-1



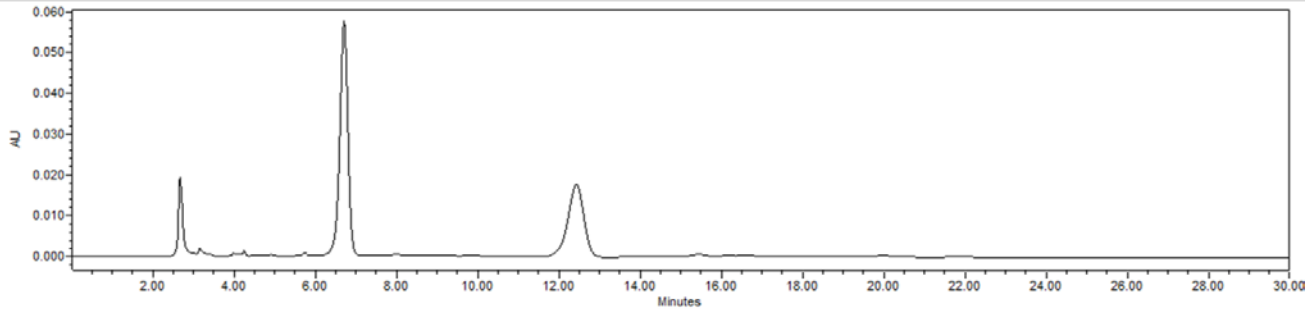
YCC-2 + ascorbic acid 0 h



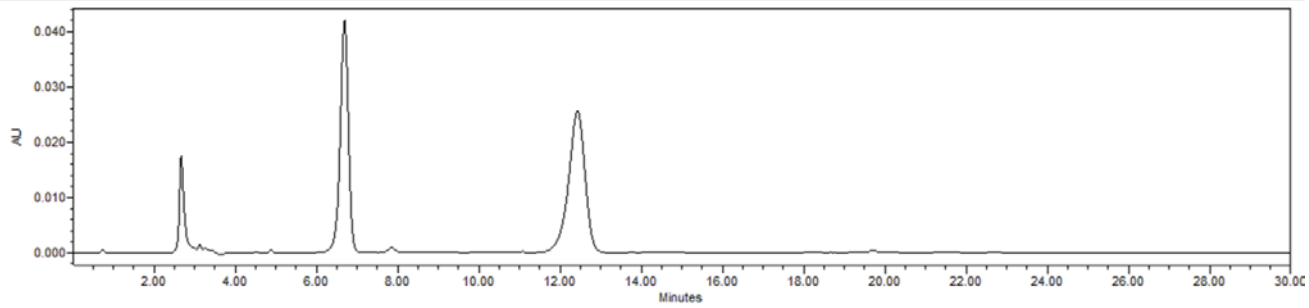
YCC-2 + ascorbic acid 1 h



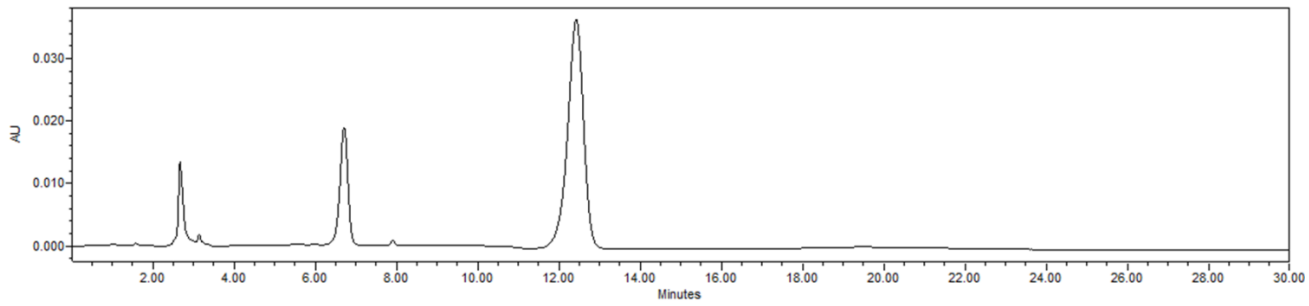
YCC-2 + ascorbic acid 2 h



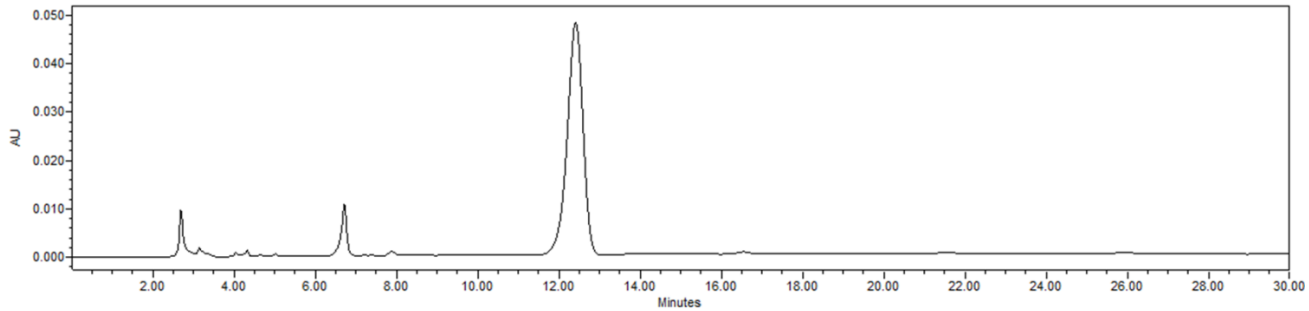
YCC-2 + ascorbic acid 6 h



YCC-2 + ascorbic acid 12 h



YCC-2 + ascorbic acid 18 h



YCC-2 + ascorbic acid 24 h

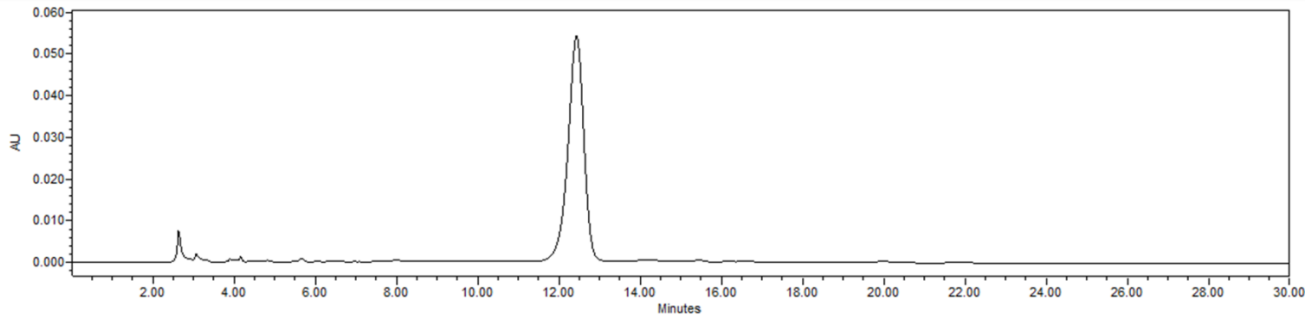
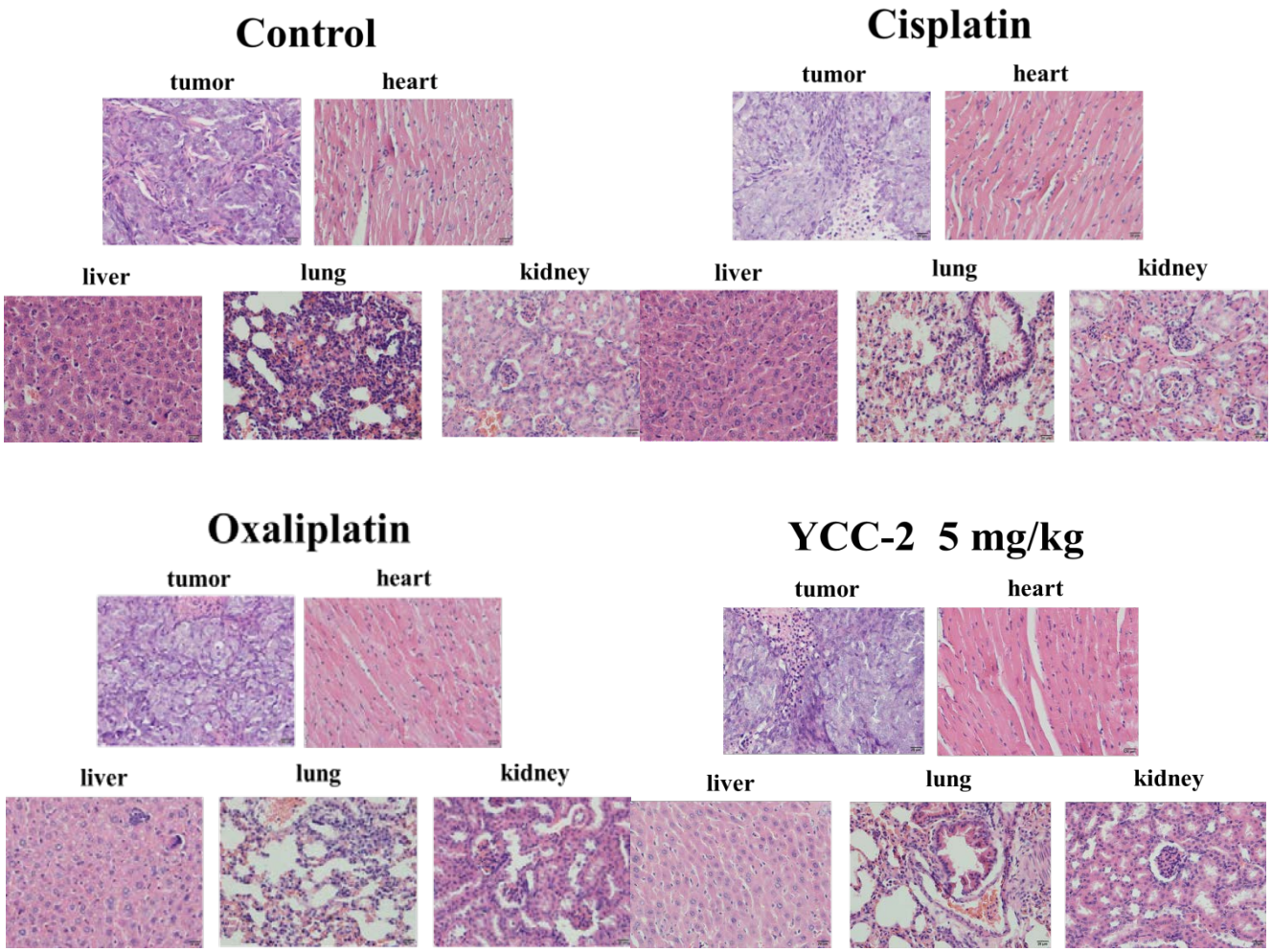


Figure S14. The released ability of YCC-2 under reduction with ascorbic acid.

Figure S15.Images of the mice and tumors after administration with YCC-2 at the dose of 5 and 12 mg/kg, cisplatin at the dose of 5 mg/kg and oxaliplatin at the dose of 5 mg/kg, for 21 days.



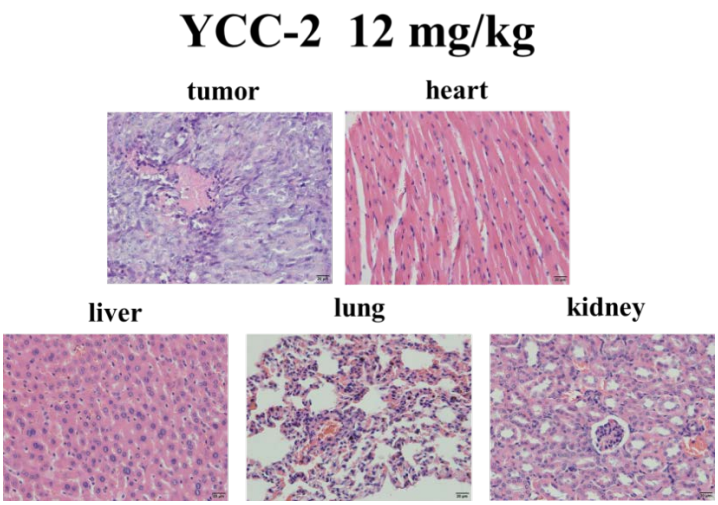


Figure S16. H&E stained images of tumor, heart, liver, lung and kidney sections collected from different groups after treatments.

References

1 (a) M. Ravera, E. Gabano, G. Pelosi, F. Fregonese, S. Tinello and D. Osella, *Inorg. Chem.*, 2014, **53**, 9326-9335;(b) H. An, N. J. Kim, J. W. Jung, H. Jang, J. W. Park and Y. G. Suh, *Bioorg. Med. Chem. Lett.*, 2011, **21**, 6297-6300;(c) J. Xiao, C. Jin, Z. Liu, S. Guo, X. Zhang, X. Zhou and X. Wu, *Org. Biomol. Chem.*, 2015, **13**, 7257-7264.