Electronic Supplementary Information

for

Anticancer Melatplatin Prodrugs: High Effect and Low Toxicity, MT1-ER-target and Immune Response *In vivo*

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Experimental Details

Materials and Instrumentations. All chemicals were commercially obtained without further purification unless otherwise specified. Cisplatin was purchased from Shandong Platinum Source Pharmaceutical Co., Ltd. (Shandong, China). Melatonin, succinic anhydride, and glutaric anhydride were obtained from Tianjin Heowns Biochem LLC. H_2O_2 (30 wt.% in H_2O), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate (TBTU) and 4-dimethylaminopyridine (DMAP) were purchased from Aladdin. Ascorbic acid (AsA) was purchased from Tianjin Beilian chemicals development Co., Ltd. Fetal bovine serum (FBS) was acquired from Hyclone and Kangyuan Biotechnology Co., Ltd. (Tianjin, China). RPMI1640 medium, DMEM medium, MTT, RNase A, propidium iodide (PI), and BCA protein concentration detection kit were purchased from Solarbio. Annexin V-FITC/PI kit was came from Dojindo. McCoy's 5A powder was obtained from Sigma-Aldrich. DNA Extraction Kit was purchased from Takara. ERα, CyclinD1, and GAPDH monoclonal antibody, β-Tubulin, Bax, p53, γ-H2AX polyclonal antibody, Peroxidase-conjugated Affinipure Goat Anti-Mouse/Rabbit IgG (H + L) and Alexa Fluor 594-conjugated Goat Anti-Mouse/Rabbit IgG (H + L) were obtained from Proteintech. Cells were counted with Countstar cell automatic counter. (Advanced Lab Instrument & Technology Co., Ltd.). ^{1}H and ^{13}C spectra were obtained on 400 MHz Varian Bruker NMR spectrometer. High resolution mass spectra (HR-MS) was determined by an Agilent 6224 ESI/TOF MS instrument. HPLC analysis was performed as on an Shimadzu Prominence HPLC system equipped with a Venusil XBP C18 column (250 × 4.6 mm, 5 μm).

Cell Lines and Cell Culture. The human cancer cells, including epithelial cervical carcinoma cells HeLa, non-small lung carcinoma cells A549, breast carcinoma cells MCF-7, triple negative breast carcinoma cells MDA-MB-231 were obtained from American Type Culture Collection. And the human umbilical vein endothelial cells HUVEC was used as a normal model. All cells were maintained in either DMEM medium (for HeLa, MCF-7, MDA-MB-231, and HUVEC), or RPMI1640 medium (for A549) containing 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C.

The Stability and Reduction of Compounds in PBS. The Pt(IV) compounds in the presence or absence of AsA were investigated by HPLC. Pt(IV) compounds (0.1 mmol) and AsA (1 mmol) were dissolved in PBS/DMF (3:1) and incubated at 37°C in dark. The mobile phase was a gradient elution of methanol/water (95:5, v:v, containing 1% formic acid). Flow rate was 1.0 mL/min. The wavelength of UV detector was 260 nm.

Cyclic Voltammetry Experiments. Cyclic voltammetry (CV) experiments of Pt(IV) prodrugs were conducted in a three-electrode system including a 3.0 mm-diameter glassy carbon disc working electrode, a platinum foil counter electrode and a saturated calomel reference electrode at room temperature. The KCl (0.1 M) was a supporting electrolyte. Pt(IV) compounds was dissolved in DMF/PBS (1:3) and tested as a concentration of 5 mM. CV curves were repeatedly recorded at scan rate of 100 mV s^{-1}, until the signals were stabilized.

MTT Assay. All cells at logarithmic growth phase were seeded into 96-well plates at 3 × 10^3 cells/well, and incubated in complete medium at 37°C to attach the wall. Then, cells were treated with graded concentration of compounds. After 72 h, 10 μL MTT solution (5 mg mL^{-1}) was added and incubated for another 4 h. Finally, after removing

the supernatant, 100 μL DMSO was used to dissolve the formazan precipitate in the plates. The OD values was measured at a wavelength of 570 nm by a microplate reader (Infinite® 200 Pro). And the IC_{50} values were calculated by GraphPad Prism software based on three parallel tests.

Platinum Content in Whole Cells. 1×10^6 cells were seeded into 6-well plates to attach wall. 24 later, the cells were incubated with 10 μM compounds for 5 h at 37°C, respectively. After treatments, all of the cells were digested, collected, washed and lyophilized. Then, the samples were digested by 65% nitric acid (HNO_3) for 4 h and the platinum concentration was detected by ICP-MS which was calibrated with a Pt-containing acid matrix (Thermo iCAP Q).

Cell Cycle Distribution. MCF-7 cells were transferred into 6-well plates at 1×10^6 cells per well. After attachment, the cells were exposed to compounds at 0.1 μM for 24 h in 5% CO_2 at 37°C. Then, cells were collected, and kept in ice-cold 75% ethanol at −20°C for over 48 h. The residual ethanol in the tube should be washed off, then the samples was resuspended by 250 μL PBS containing 2.5 μL RNase A and incubated at 37 °C for 30 minutes before adding the PI dye solution (12.5 μL at density of 1 mg mL^{-1}). After 30 min staining, the results were detected on a flow cytometer (BD FACS Verse) and processed by ModFit LT.

Apoptosis Analysis by Annexin V-FITC/PI Staining. 1×10^6 MCF-7 cells were seeded into 6-well plates at 37°C for 24 h. After attachment, the cells were treated with compounds at 10 μM, respectively. 24 h later, the drug treatment was interrupted, and cells were washed with ice-cold PBS and digested to collect into tubes. The next staining step follows the instructions of the Annexin V-FITC/PI apoptosis detection kit (Solarbio). The apoptosis was detected by a flow cytometry (BD FACS Verse).

Synergistic Analysis. The cytotoxicity of the mixture of CDDP/MT (1:2) was tested, and the synergistic effect between CDDP and MT was calculated by CompuSyn 1.0 software version 1.0 (Ting Chao Chou and Nick Martin, Paramus, NJ, 2005) according to the previous reports.[1,2] And the calculation results were shown in Figure S29 and Table S1.

Western Blot Analysis. The active preferred MCF-7 cells were seeded into 6-well plates until cell density reached 80%. Subsequently, the cells were exposed to compounds (5 μM) at 37℃ for 24 h. Proteins were extracted by lysis buffer (PMSF:RIPA = 1:100) for 0.5 h and stored at −20°C. The protein concentration was quantified using a BCA kit. After the separation of the protein by SDS-PAGE (10% separating gel, 5% concentration shrink plastic), the samples were transferred onto PVDF membrane. The membranes were blocked in 5% (w/v) non-fat milk in TBST for 4 h. After fully washing with TBST, the bands were incubated with primary antibody (1:3000) at 4℃ overnight under gentle shaking. Then, the blots were washed with TBST for three times. In a similar manner, the bands were washing again and further incubated with the secondary antibodies (1:5000) at room time for 1.5 h. After finial washing, the immunoreactive bands were visualized using Pierce ECL Western Blotting Substrate (Thermo) and Tanon automated chemiluminescence imaging analysis system.

Confocal Microscopy. After treatment of compounds (10 μM), MT (10 μM), CDDP (10 μM) and the combination of latter two drugs for different times, the medium containing compounds was abandoned. The MCF-7 cells were fixed in 4% para-formaldehyde at room temperature for 30 min, and then punched with 0.5% Triton X-100 (in PBS) for 30 min at room, respectively. Next, cells were blocked in

5% BSA blocking buffer (albumin bovine V solution in PBS) for 3 h. Then the cells were incubated with the primary antibody at 4°C overnight. After incubation, all slides were fully washed with blocking buffer and stained with the corresponding secondary antibody for 1 h. Then the slides were washed with buffer and mounted with DAPI. Fluorescence images were captured using Olympus FV1000 confocal microscope.

***In vivo* Assay.** In order to further evaluate the anti-tumor activity *in vivo*, thirty 4-week-old female BALB/c nude mice (16 ± 2 g of body weight) were randomly divided into 5 groups. After a week, MCF-7 cells at a density of 1×10^6 cells/100 μL were injected in the right oxter of mice. Until tumor grew to 50−100 mm^3, all animals were administrated *via* intravenous injection (*i.v.*) at a frequency of once every four days. (1) PBS as the control group, (2) CDDP (2 mg-Pt/kg), (3) MT (equal to the mole number of Pt per mouse), (4) the mixture of CDDP (2 mg-Pt/kg) and MT (equal to the mole number of Pt per mouse), and (5) compound **3** (2 mg-Pt/kg). All solutions injected were contained DMF/Tween 80/PBS (16:1:83, v:v:v). The tumor volume and body-weight were measured every 2 days. And the tumor volume calculation referred to previous literature. Three days after the last treatment, all animals were sacrificed, and all tumor and organs were collected and the Pt-content was assessed by ICP-MS. All of the animal experiments were conducted following the protocol approved by the Animal Nursing and Use Committee of Tianjin Medical University.

Molecular Docking of compounds 3–6 and MT. Owing to the lack of a three-dimensional (3D) experimental model of melatonin receptor, we obtained melatonin receptor 1 amino acid sequence is P48039 (in NCBI website http://www.ncbi.nlm.nih.gov/), then we constructed 3D model of melatonin receptor 1 based on the crystal structure of beta1-adrenergic receptor by the homology modeling

package Modeller. The crystal structure of beta1-adrenergic receptor (PDB ID: 2VT4) was downloaded as template from RCSB Protein Data Bank (PDB, http://www.pdb.org). Protein structures were checked for missing amino acid residues and some bad valences, and some of the unnecessary molecules were removed. The structures were used for molecular docking on Autodock 4 software.

Figures and Tables

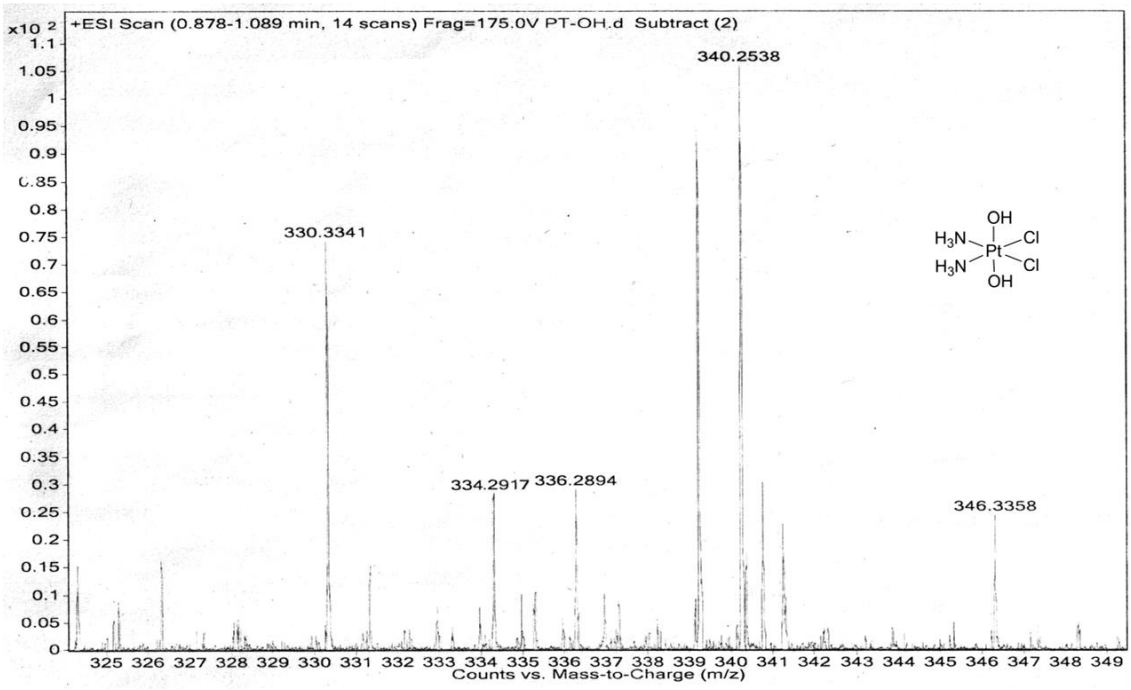


Figure S1. HR-MS spectrum of *c*, *c*, *t*-[$Pt(NH_3)_2Cl_2(OH)_2$].

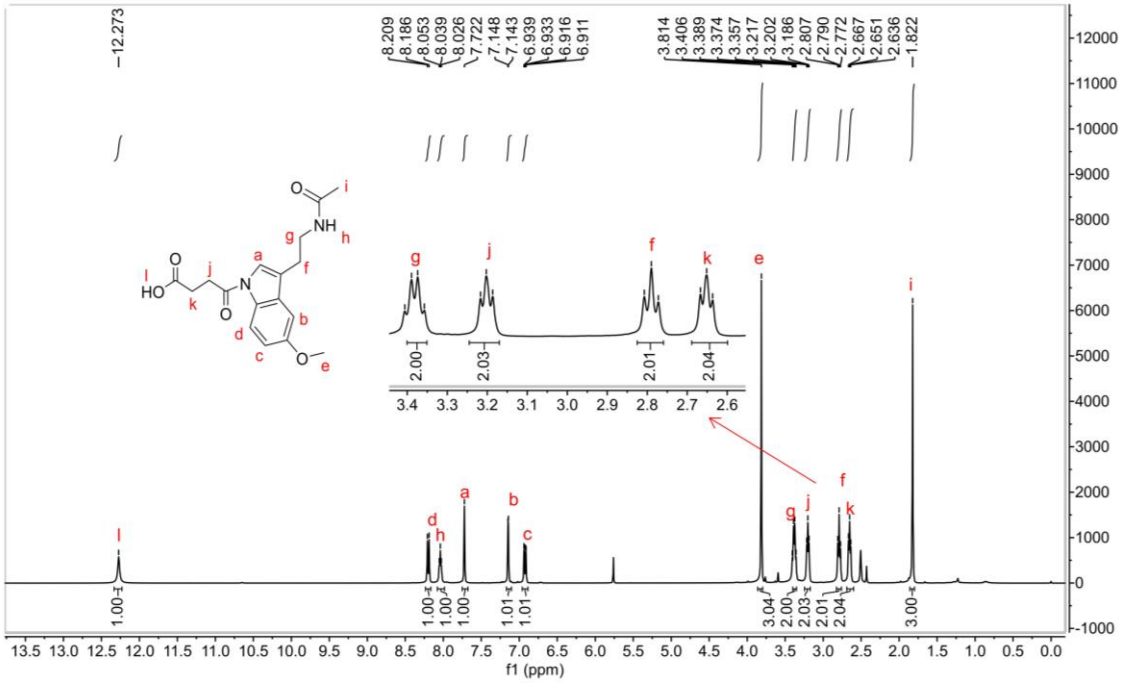


Figure S2. ^{1}H-NMR spectrum of **1** in DMSO-d_6.

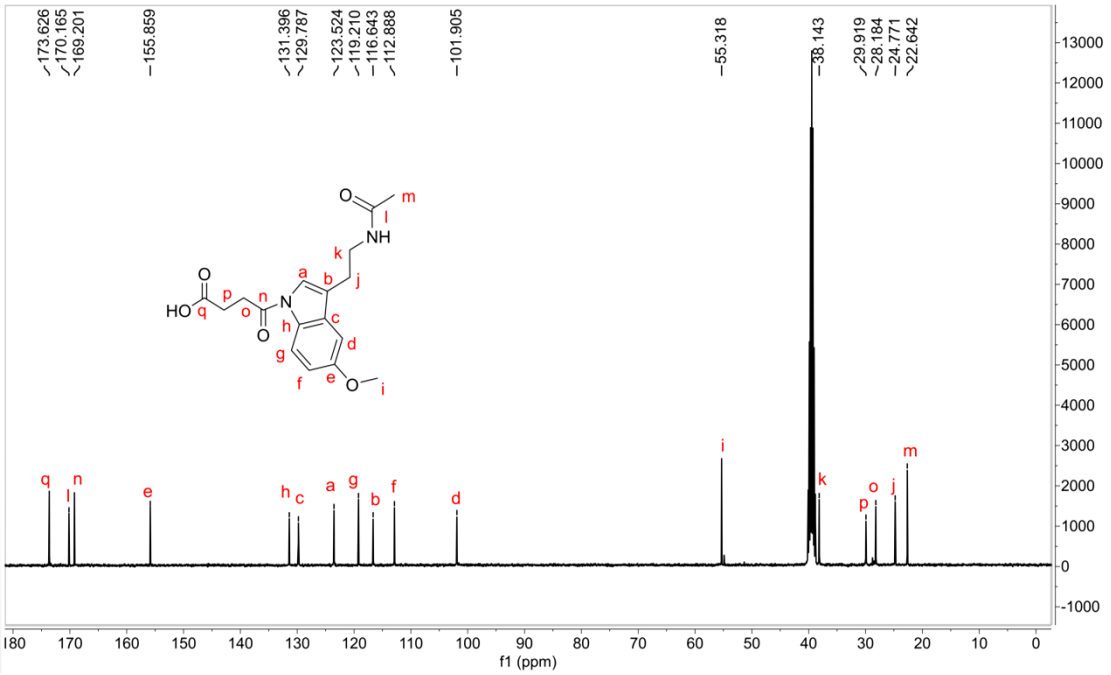


Figure S3. ^{13}C-NMR spectrum of **1** in DMSO-d_6.

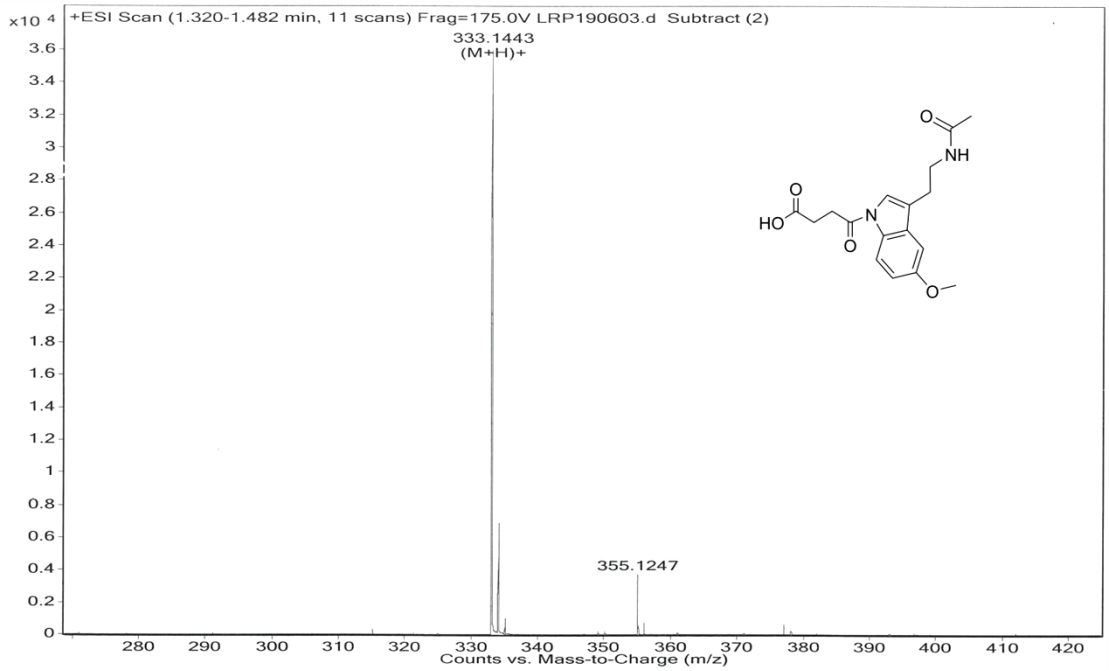
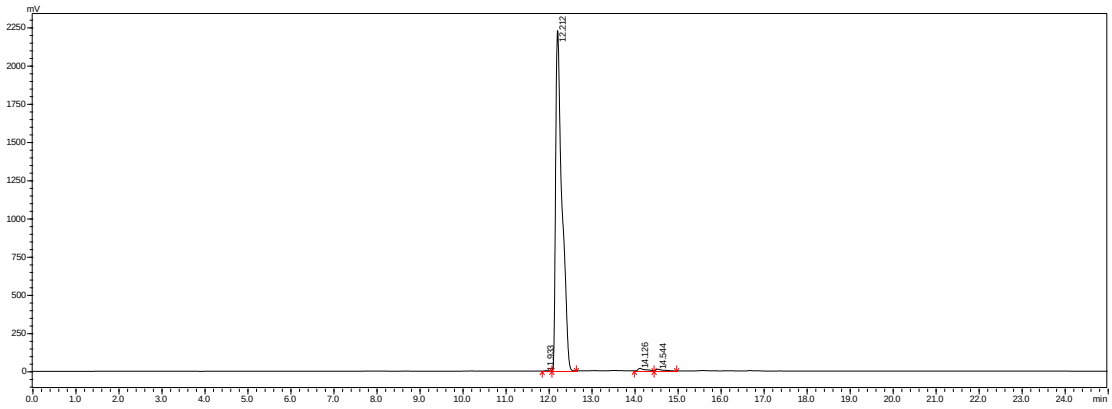


Figure S4. HR-MS of **1** in methanol.



No.	Retention time	Peak area	Concentration (%)
1	11.933	2022	0.066
2	12.212	2225621	98.574
3	14.126	16254	0.811
4	14.544	11002	0.549

Figure S5. HPLC characterization of **1**.

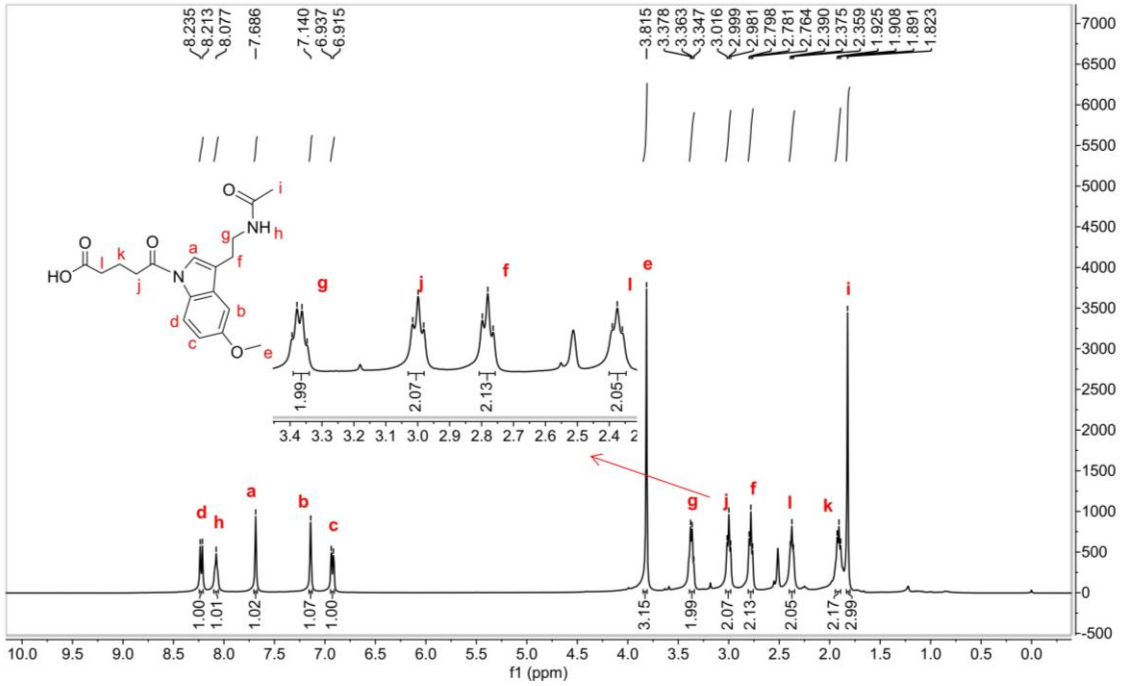


Figure S6. ^{1}H-NMR spectrum of **2** in DMSO-d_6.

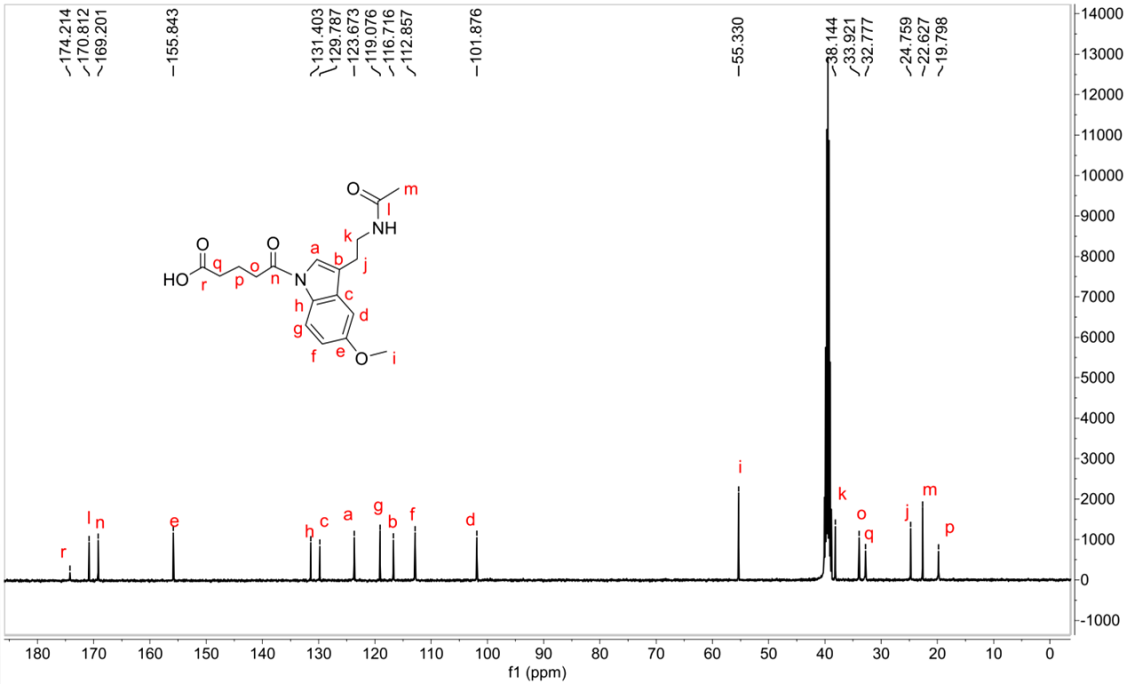


Figure S7. ^{13}C-NMR spectrum of **2** in DMSO-d_6.

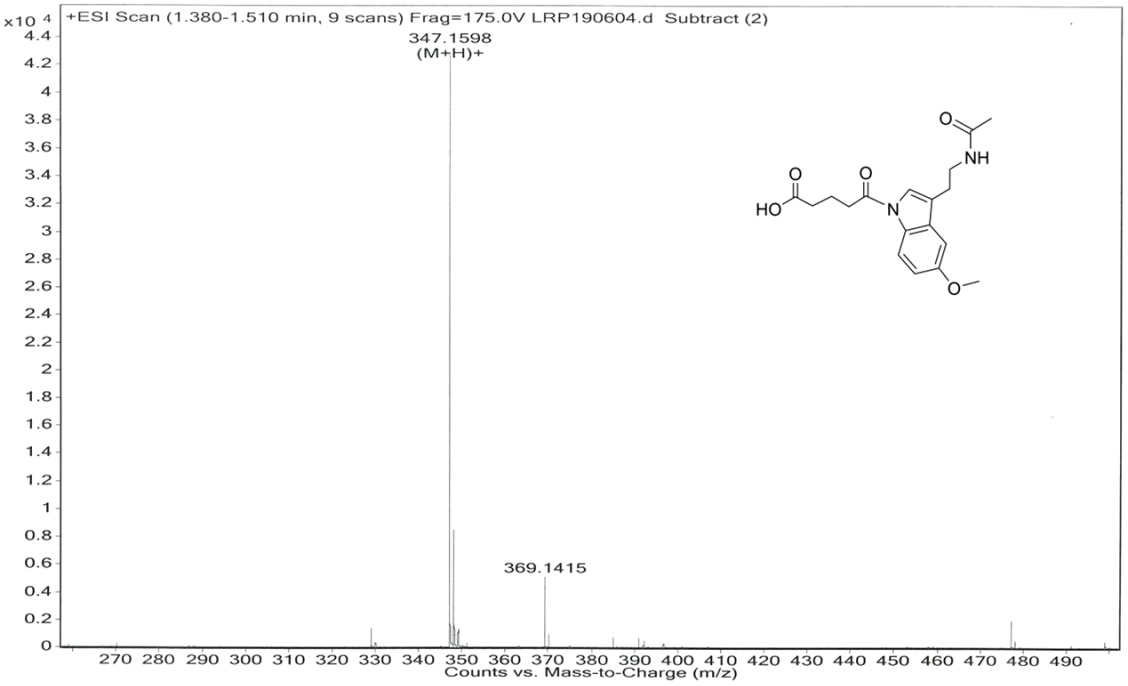
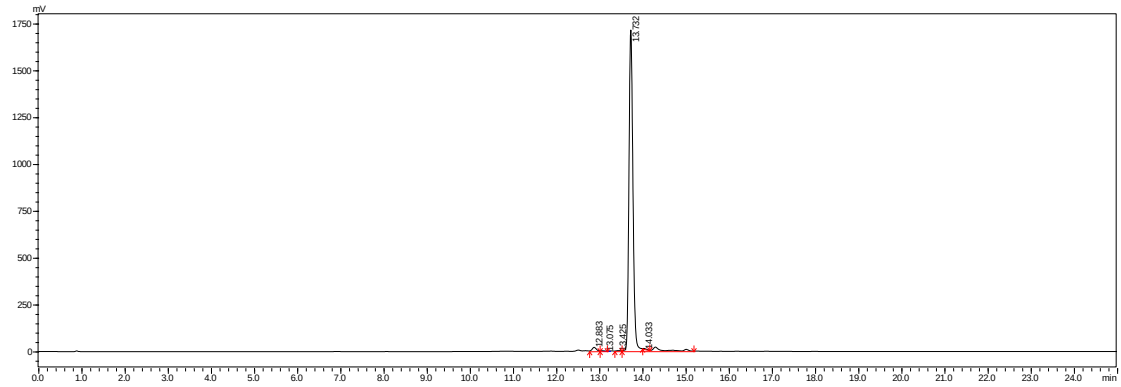


Figure S8. HR-MS of **2** in methanol.



No.	Retention time	Peak area	Concentration (%)
1	12.883	9036	0.525
2	13.075	1308	0.076
3	13.425	1773	0.103
4	13.732	1695053	98.475
5	14.033	14132	0.821

Figure S9. HPLC characterization of **2**.

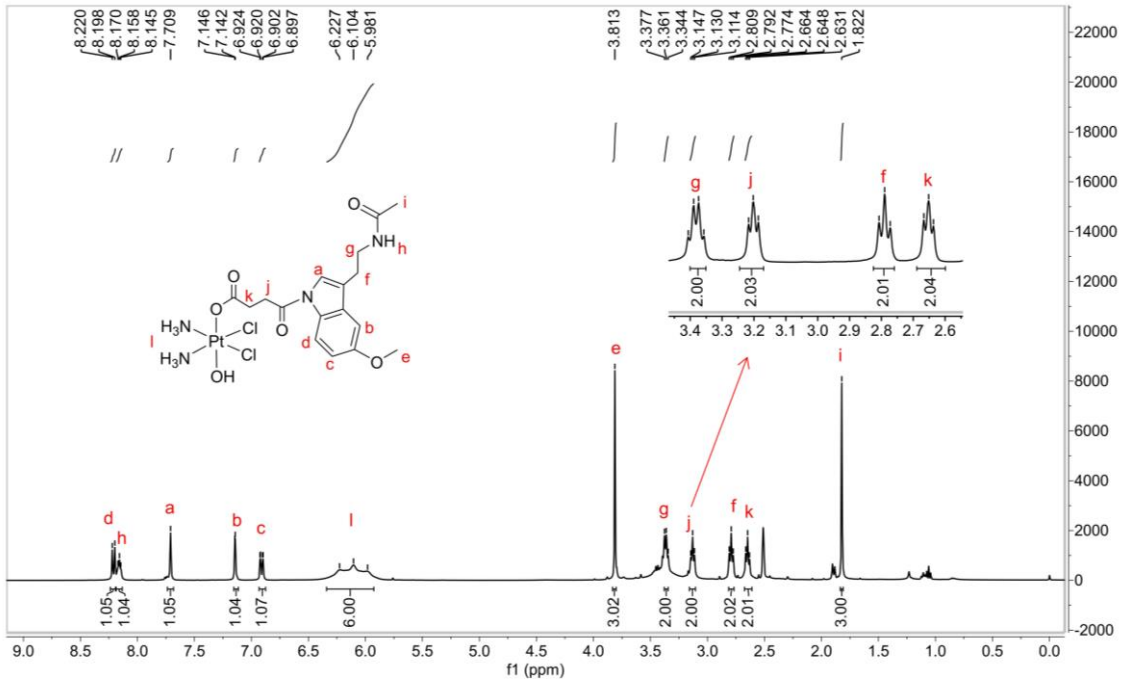


Figure S10. ^{1}H-NMR spectrum of **3** in DMSO-d_6.

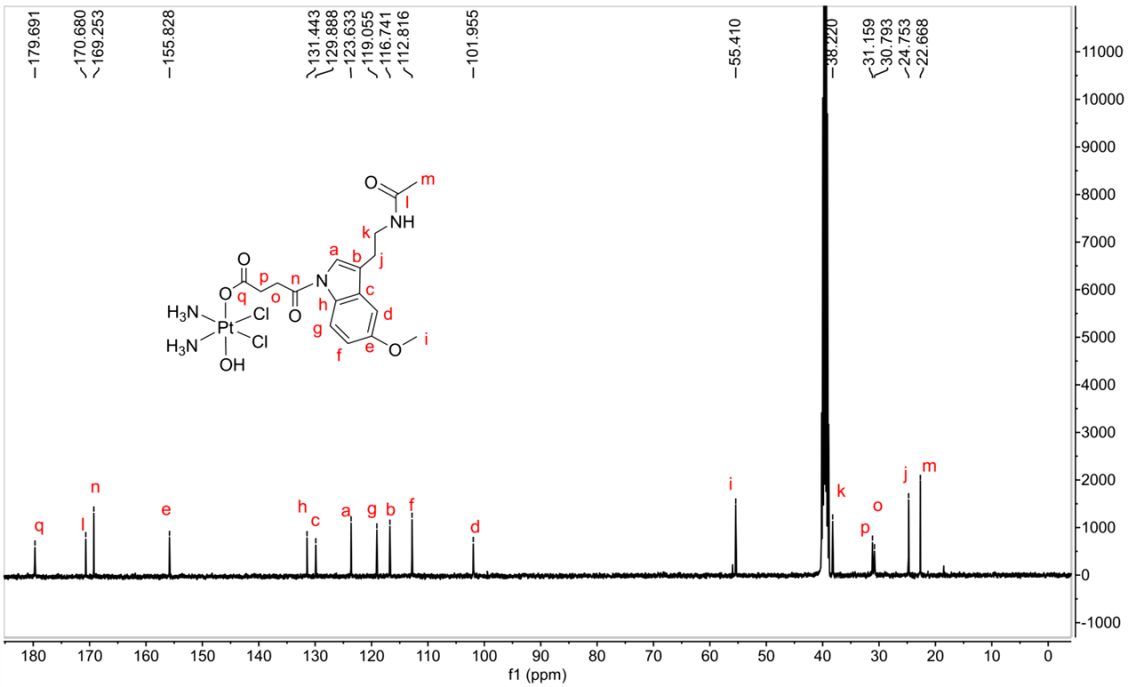


Figure S11. ^{13}C-NMR spectrum of **3** in DMSO-d_6.

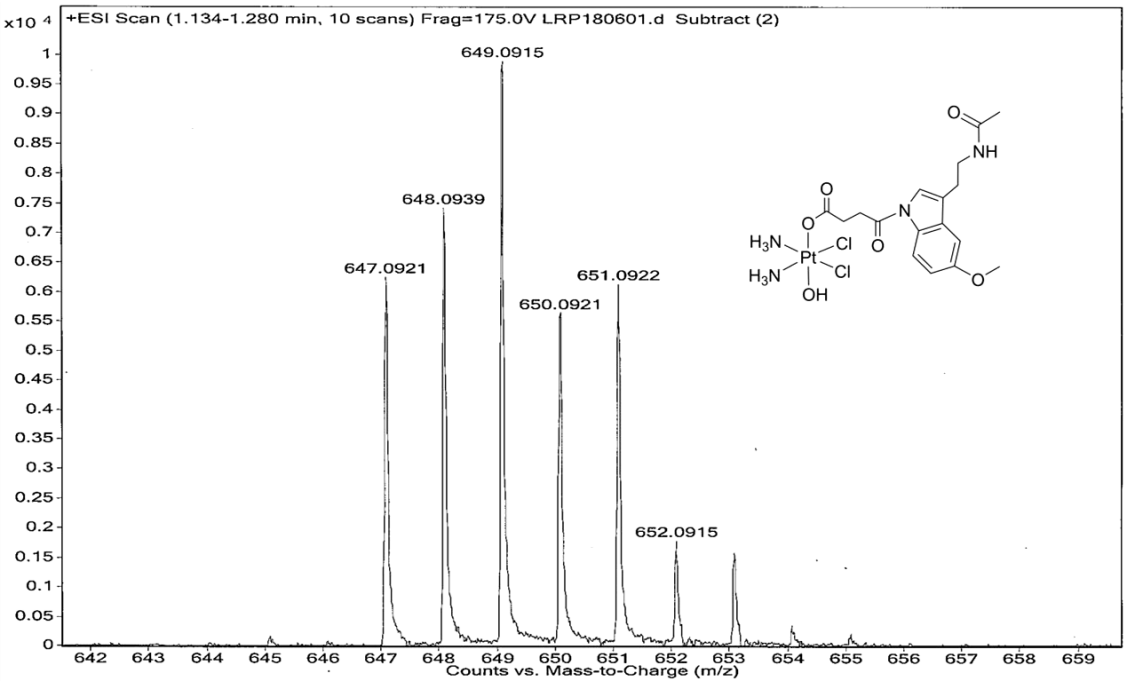
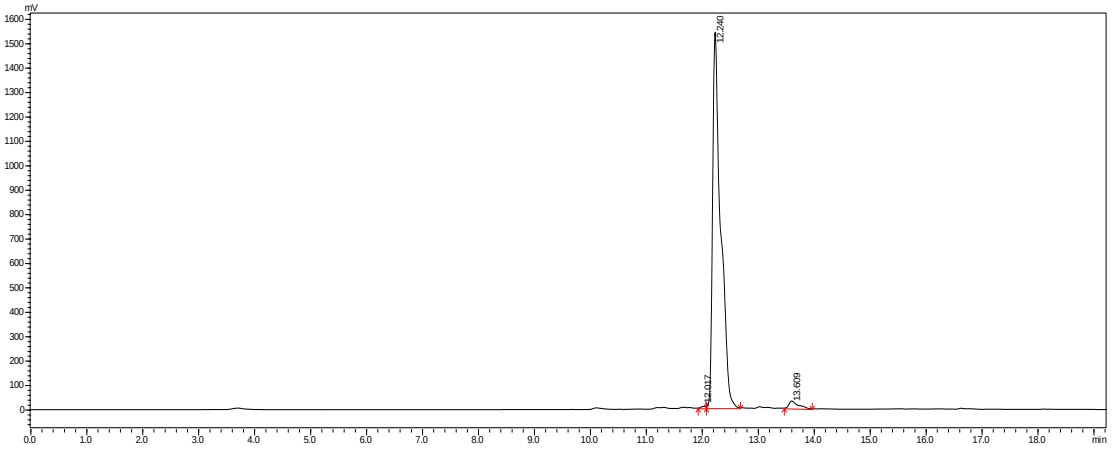


Figure S12. HR-MS of **3** in methanol.



No.	Retention time	Peak area	Concentration (%)
1	12.017	6333	0.248
2	12.240	1539675	97.638
3	13.609	30012	2.114

Figure S13. HPLC characterization of **3**.

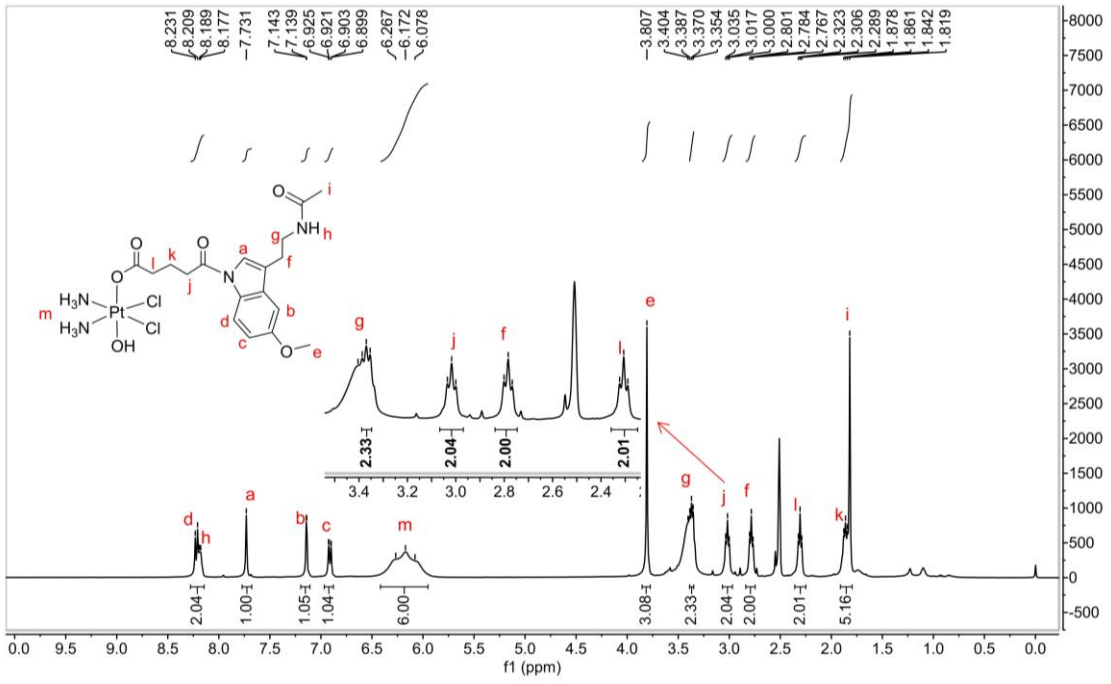


Figure S14. ^{1}H-NMR spectrum of **4** in DMSO-d_6.

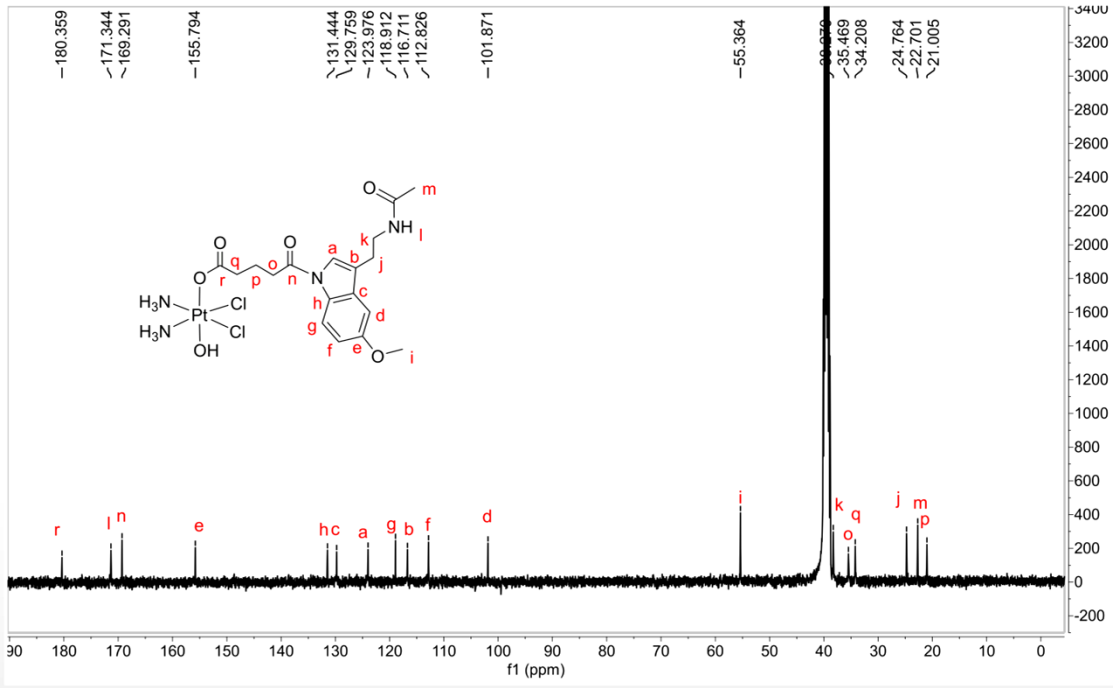


Figure S15. ^{13}C-NMR spectrum of **4** in DMSO-d_6.

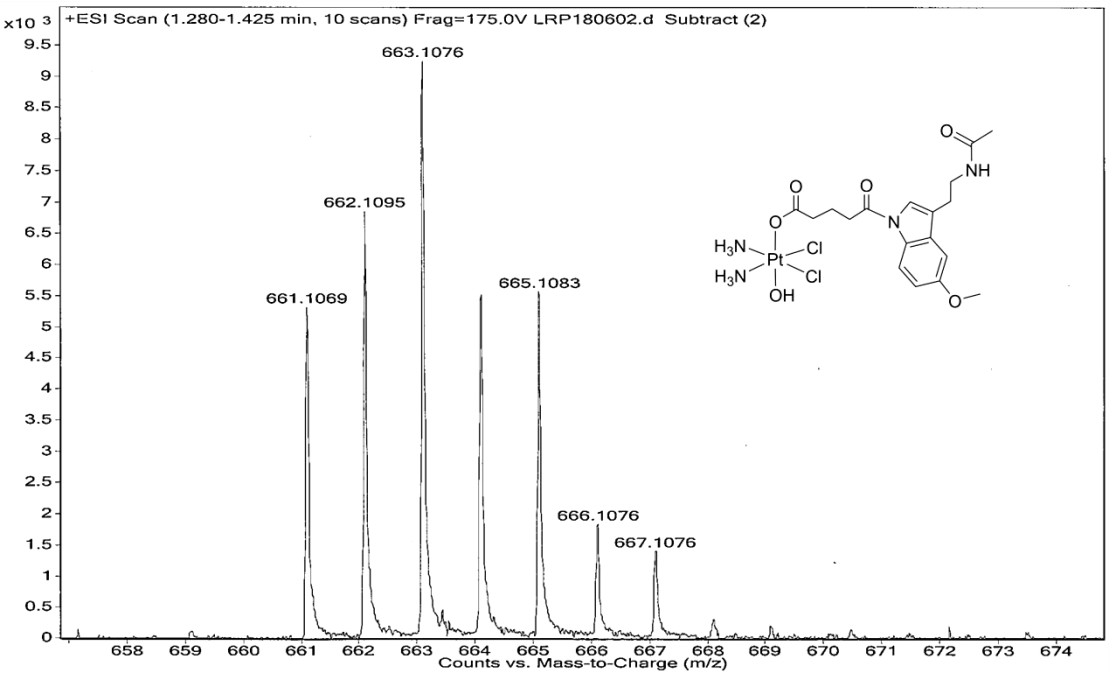
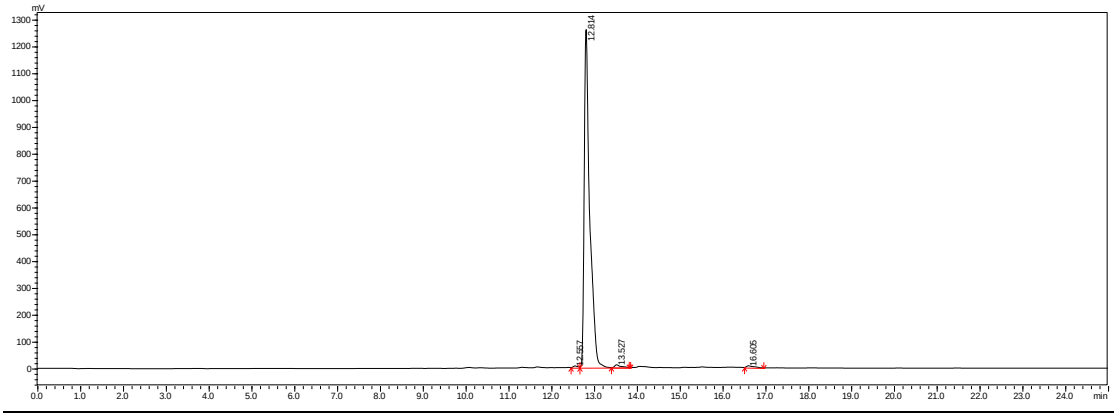


Figure S16. HR-MS of **4** in methanol.



No.	Retention time	Peak area	Concentration (%)
1	12.557	5929	0.356
2	12.814	1259068	98.186
3	13.527	9963	0.788
4	16.605	6586	0.669

Figure S17. HPLC characterization of **4**.

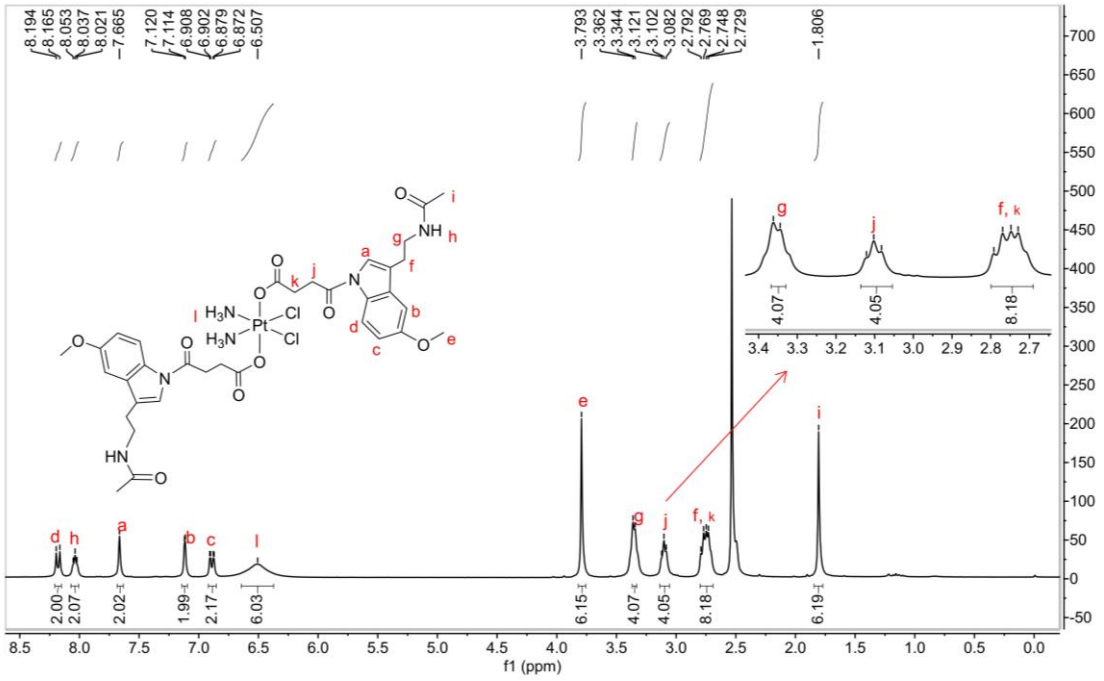


Figure S18. ^{1}H-NMR spectrum of **5** in DMSO-d_6.

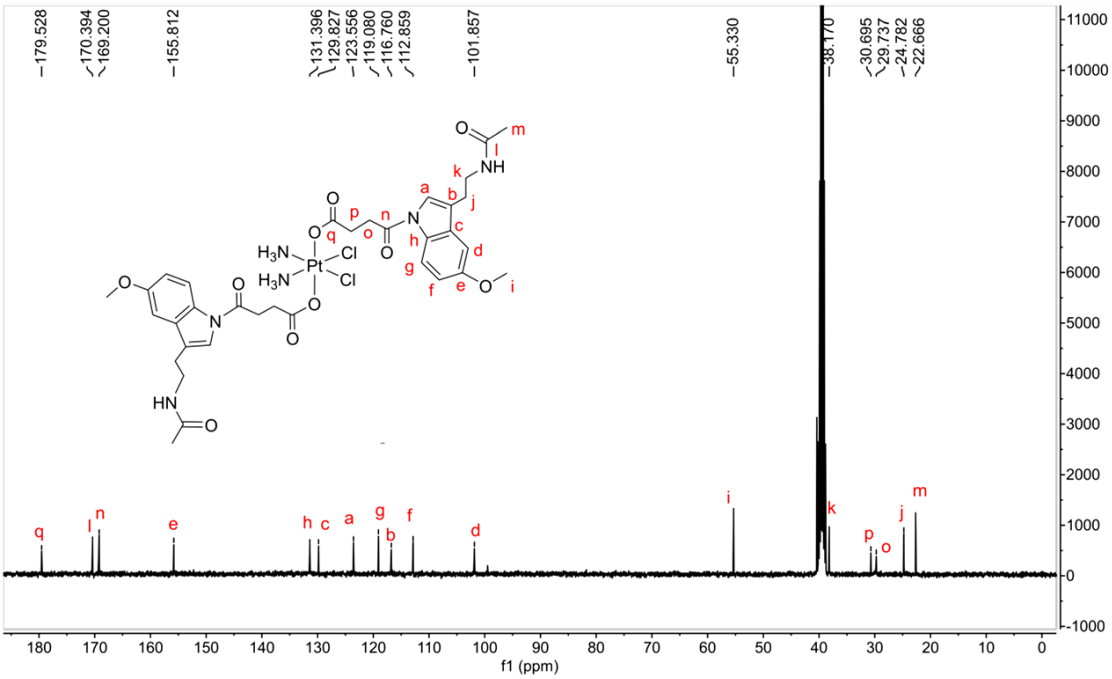


Figure S19. ^{13}C-NMR spectrum of **5** in DMSO-d_6.

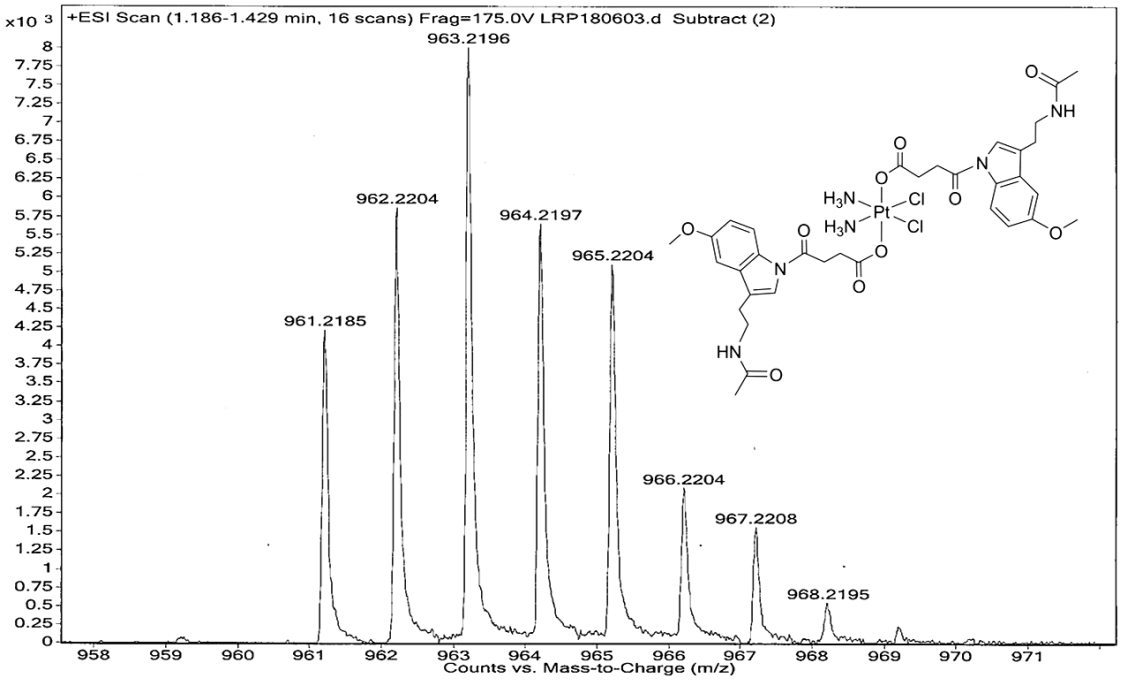
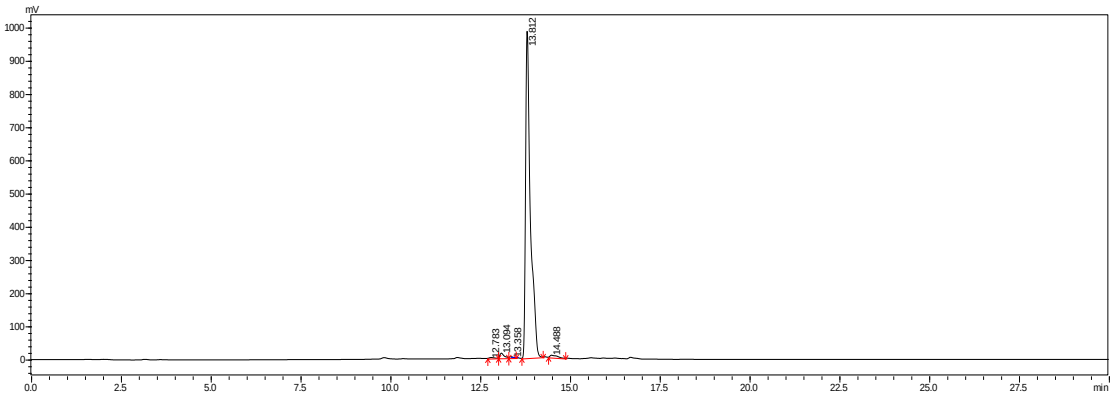


Figure S20. HR-MS of **5** in methanol.



No.	Retention time	Peak area	Concentration (%)
1	12.783	2184	0.222
2	13.094	14997	1.251
3	13.358	3745	0.281
4	13.812	983787	97.256
5	14.488	6898	0.991

Figure S21. HPLC characterization of **5**.

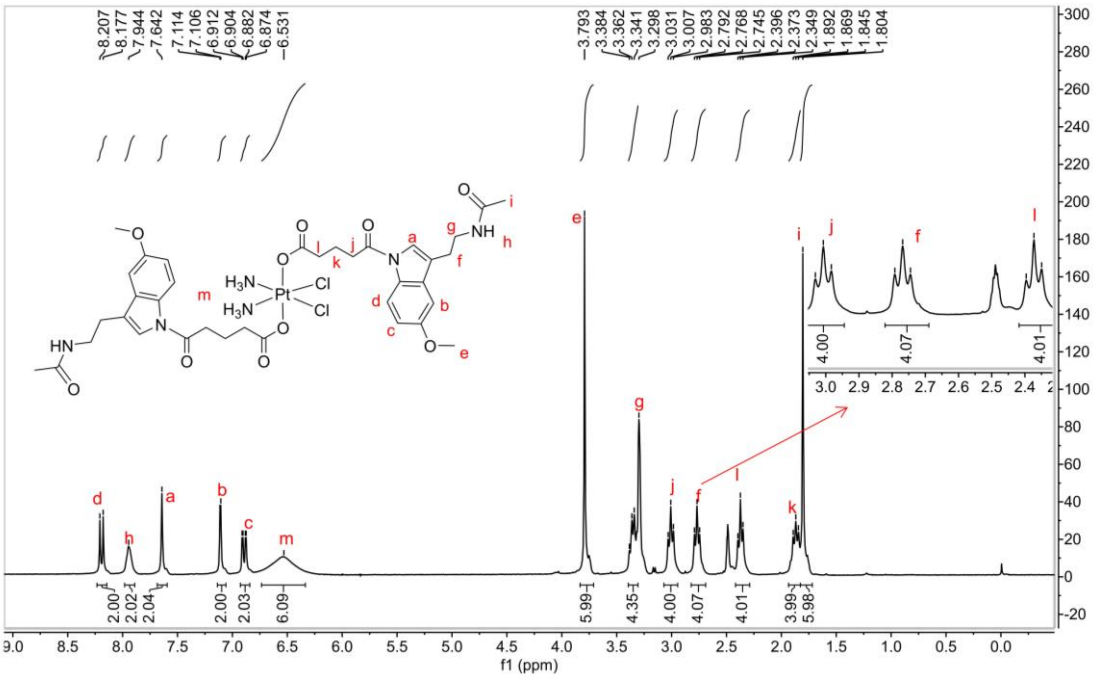


Figure S22. ^{1}H-NMR spectrum of **6** in DMSO-d_6.

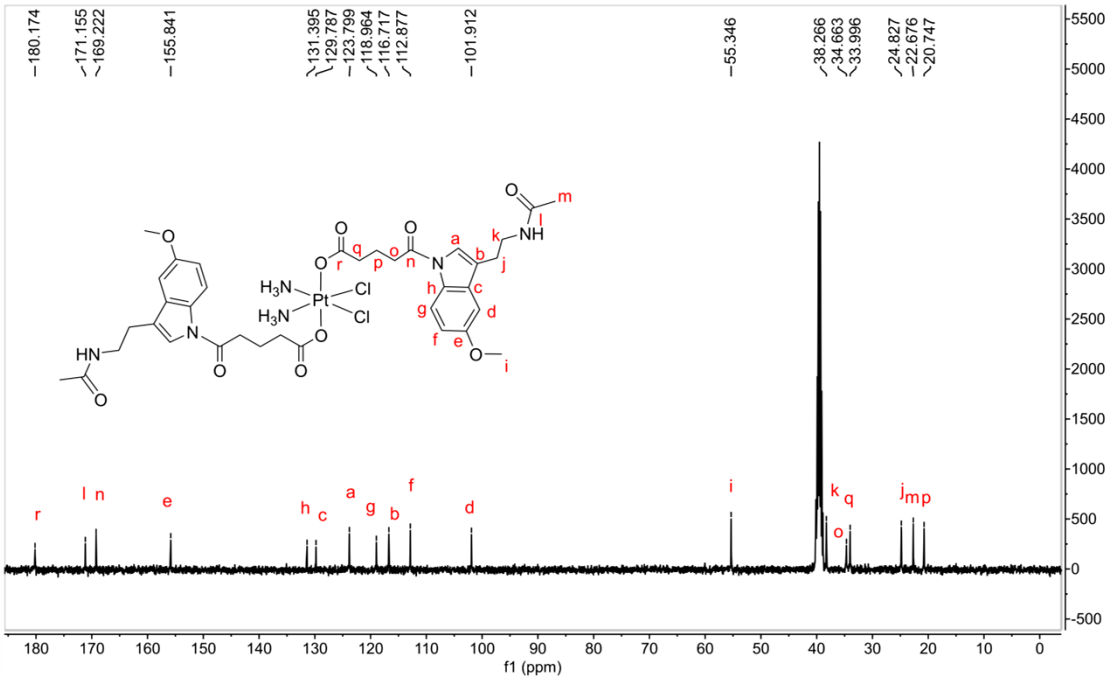


Figure S23. ^{13}C-NMR spectrum of **6** in DMSO-d_6.

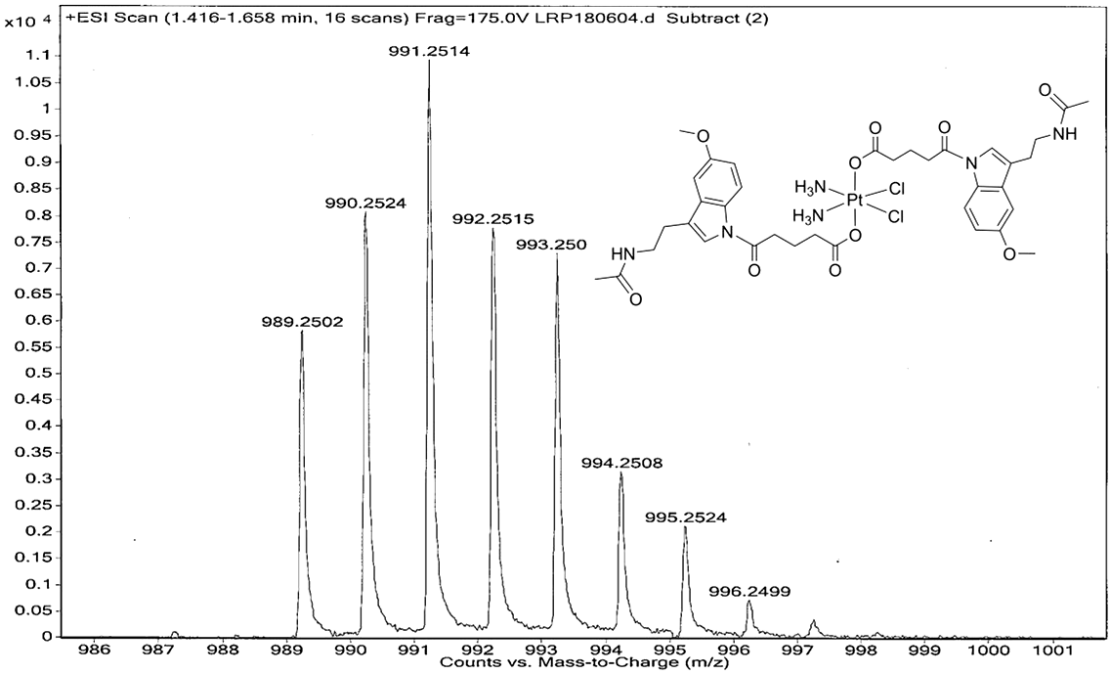
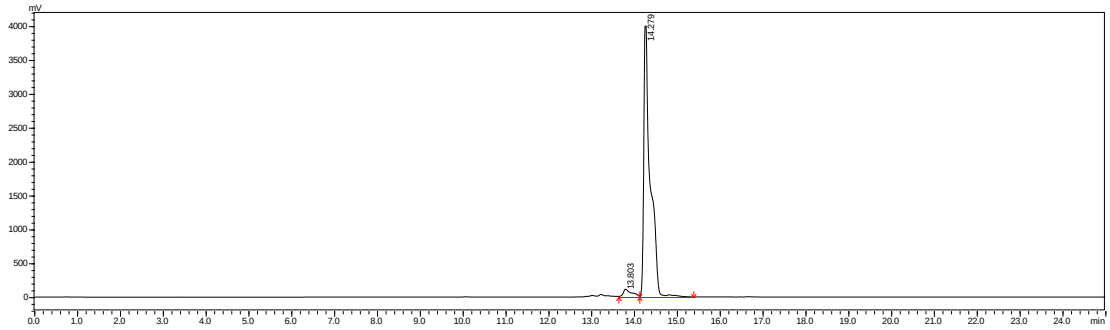


Figure S24. HR-MS of **6** in methanol.



No.	Retention time	Peak area	Concentration (%)
1	13.803	115320	3.699
2	14.279	3997867	96.301

Figure S25. HPLC characterization of **6**.

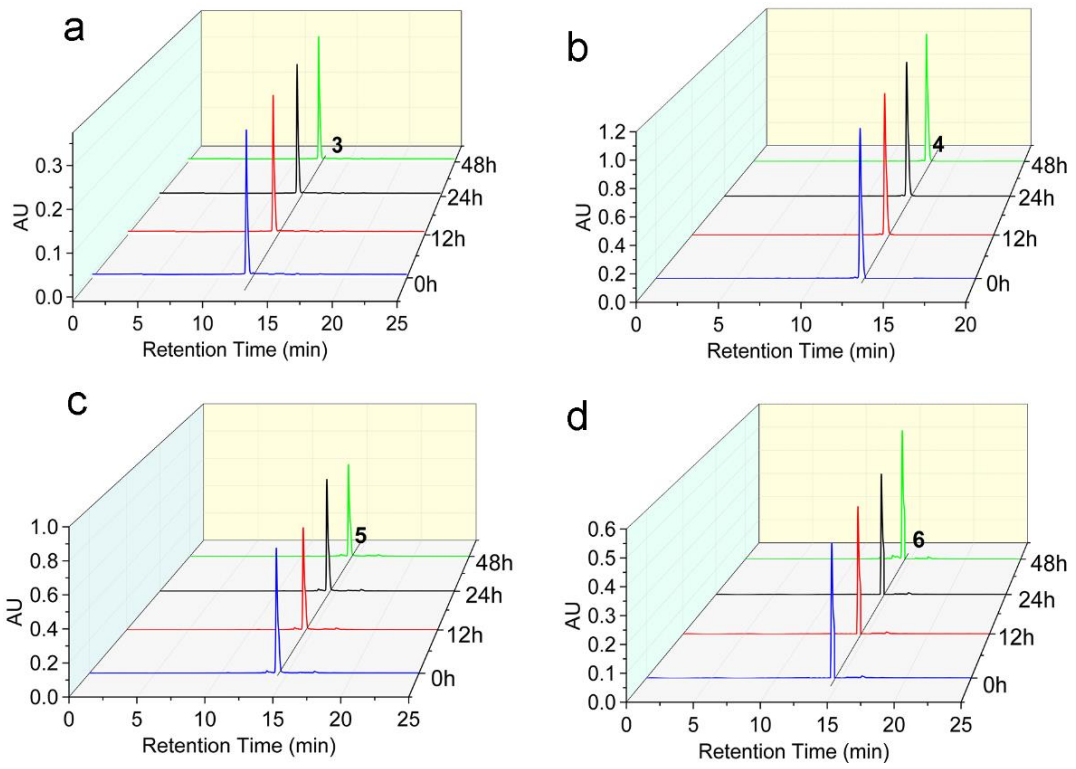


Figure S26. Stability of compounds **3**–**6** at 37°C in PBS/DMF (3:1, v/v, pH 7.4) for 0, 12, 24 and 48 h in dark. (a) Stability of compound **3**. (b) Stability of compound **4**. (c) Stability of compound **5**. (d) Stability of compound **6**.

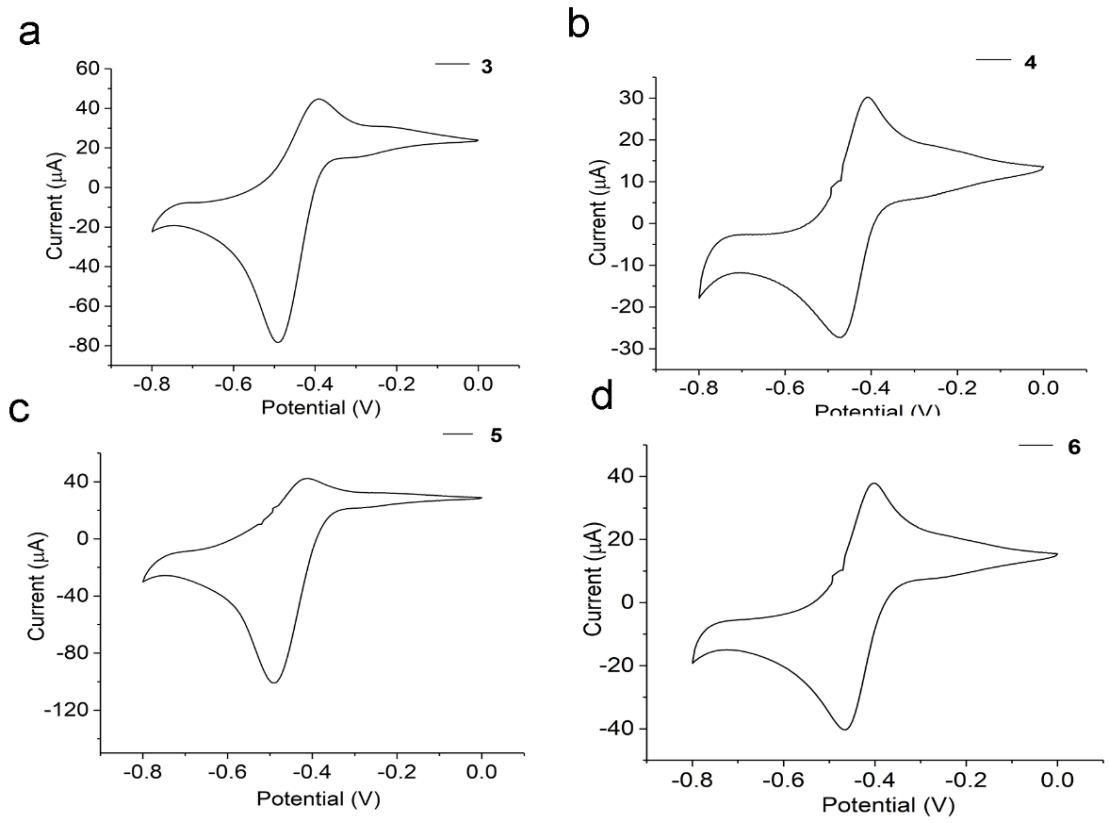


Figure S27. Cyclic voltammetry of Compounds **3**–**6** in PBS/DMF (3:1, v/v, pH 7.4) with 0.1 M KCl as a supporting electrolyte at a scan rate of 100 mV s^{-1}, a 3.0 mm-diameter glassy carbon disc working electrode, a platinum foil counter electrode and a saturated calomel reference electrode at room temperature.

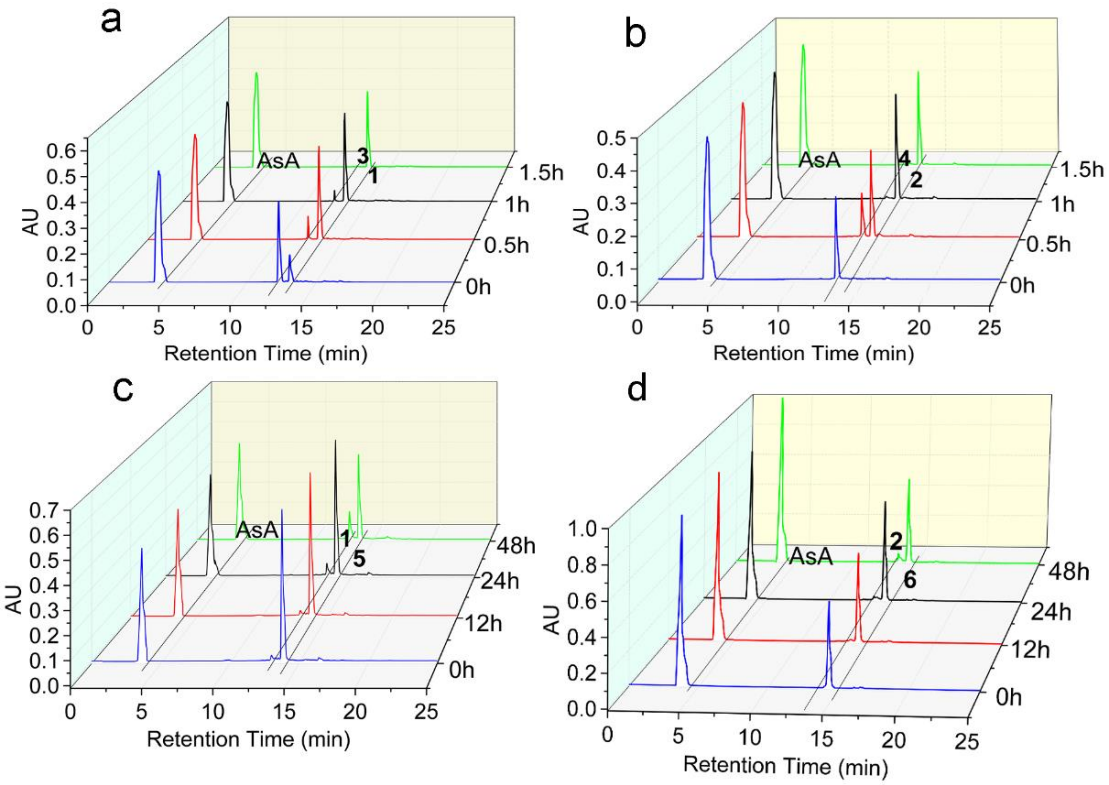


Figure S28. The reduction of compounds **3–6** with AsA in PBS at 37°C for 0, 12, 24 and 48 h in dark. (a) Reduction of compound **3**. (b) Reduction of compound **4**. (c) Reduction of compound **5**. (d) Reduction of compound **6**.

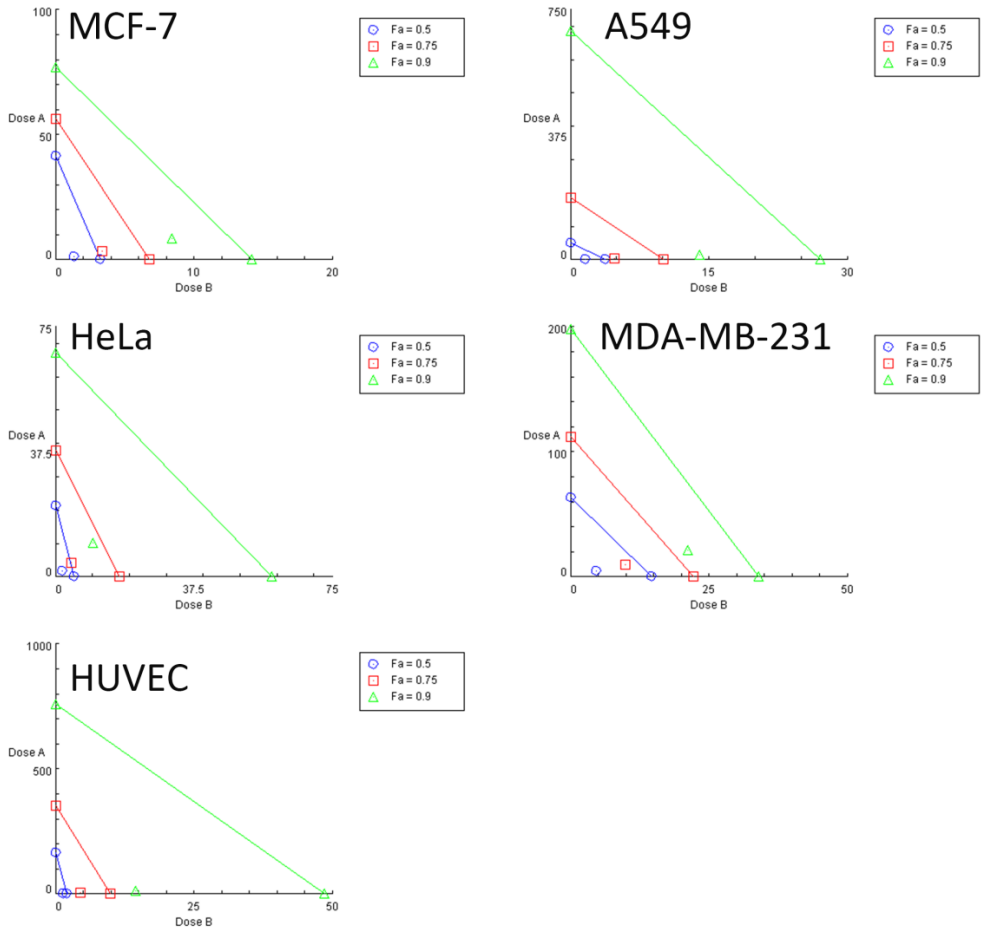


Figure S29. The isobolograms for the combination of CDDP/MT (1:1) in all cell lines tested.

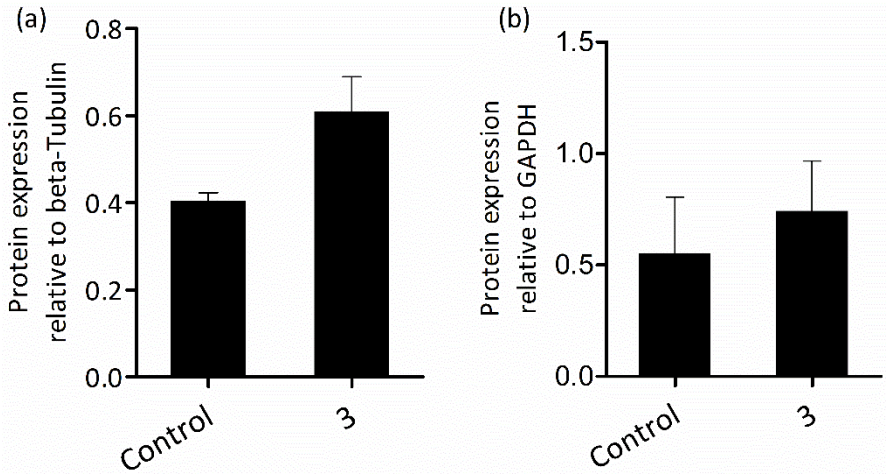


Figure S30. The statistics analysis of protein gray values relative to β-Tubulin or GAPDH. (a) CyclinD1 expression. (b) Bax expression.

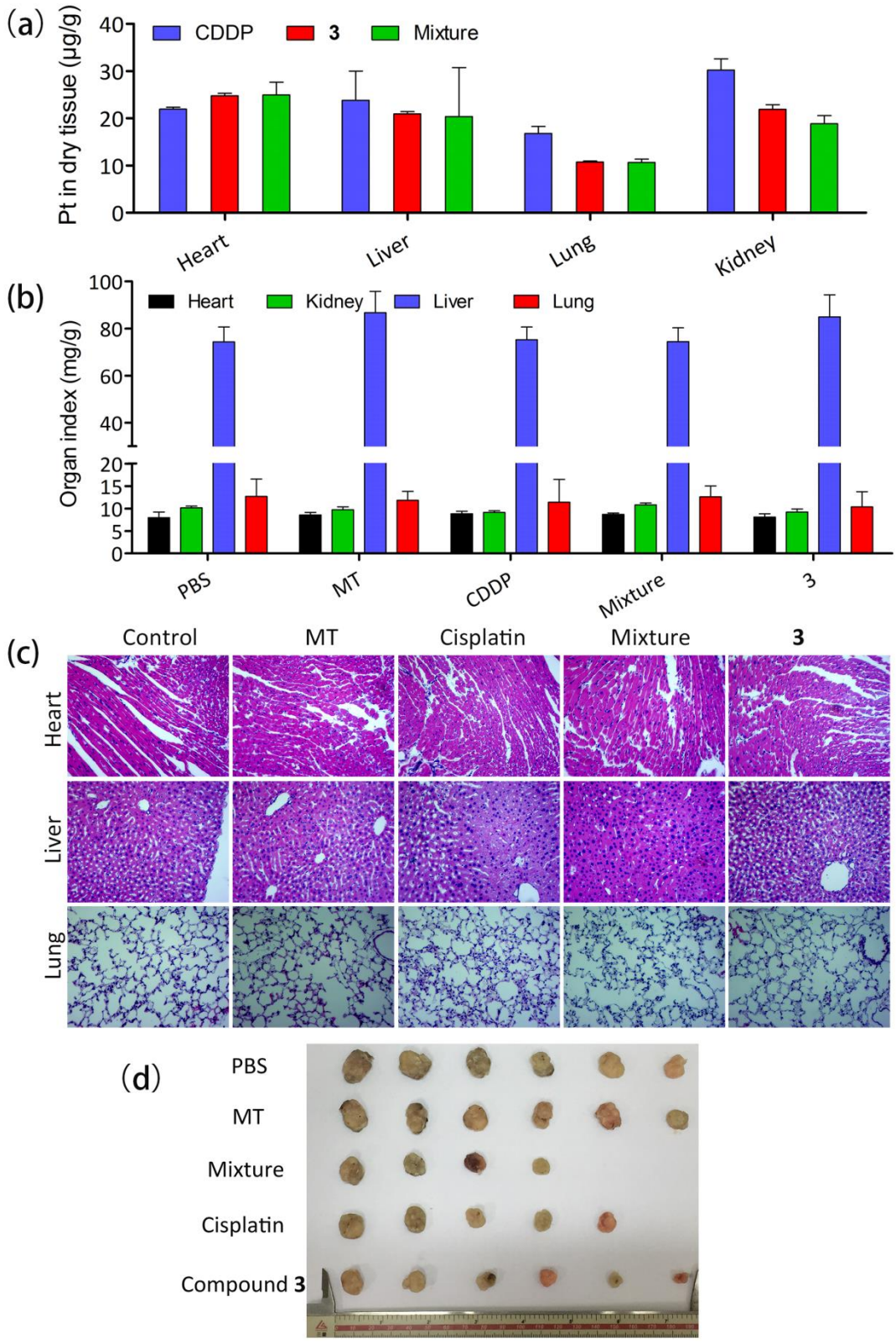


Figure S31. (a) Pt-biodistribution of platinum-based administration groups in MCF-7 bearing mice assessed by ICP-MS. (b) The organ indexes of mice. (c) H&E staining pictures of mice organs (Heart, liver, lung). (d) The image of tumors.

Table S1. Combination index (CI) values for MT/CDDP combination treatment.

Compounds	F_a	CI value				
		MCF-7	A549	HeLa	MDA-MB-231	HUVEC
CDDP + MT	0.50	0.44804	0.46256	0.43051	0.38739	0.70192
	0.75	0.55618	0.49733	0.3565	0.53182	0.46699
	0.90	0.70284	0.53746	0.32281	0.91159	0.31661

References

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