Electronic Supplementary Material (ESI) for Inorganic Chemistry Frontiers.
This journal is © the Partner Organisations 2020

Electronic Supporting Information

For

A class of Pt(IV) triple-prodrugs targeting nucleic acid, thymidylate synthase and histone deacetylases

Xiao-Jing Ding,[1a] Ran-Zhang.[1a,b] Rui-Ping Liu,[1a] Xue-Qing Song,[a] Xin Qiao,[a] Cheng-Zhi Xie,[a] Xiu-He Zhao,[a] Jing-Yuan Xu,*[a]

[a]Tianjin Key Laboratory on Technologies Enabling Development of Clinical Therapeutics and Diagnostics, School of Pharmacy, Tianjin Medical University, Tianjin 300070, China.

[b]School of Biotechnology, Jiangsu University of Science and Technology, Jiangsu 212018, China

*Corresponding author:

Tel.: +86 22 83336929; E-mail address: xujingyuan@tmu.edu.cn

[1]These authors contributed equally.

The List of Content:

Experimental Details.

Instrumentations and reagents.

Synthesis of **2**.

Synthesis of **7**–**8**.

Synthesis of **9**–**10**.

Cell lines and cell culture.

Antiproliferative activity.

Flow cytometry analysis.

Intracellular accumulation of platinum.

The stability of compound **14**.

Intracellular reduction of compound **14**.

Wound healing assay.

Western blot analysis.

Figure Captions.

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

Fig. S2. HR-MS spectrum of compound **7**.

Fig. S3. ^{1}H-NMR spectrum of compound **7** in MeOD-d_4.

Fig. S4. ^{13}C-NMR spectrum of compound **7** in MeOD-d_4.

Fig. S5. HPLC characterization of compound **7**.

Fig. S6. HR-MS spectrum of compound **8**.

Fig. S7. ^{1}H-NMR spectrum of compound **8** in DMSO-d_6.

Fig. S8. ^{13}C-NMR spectrum of compound **8** in DMSO-d_6.

Fig. S9. HPLC characterization of compound **8**.

Fig. S10. HR-MS spectrum of compound **9**.

Fig. S11. ^{1}H-NMR spectrum of compound **9** in DMSO-d_6.

Fig. S12. ^{13}C-NMR spectrum of compound **9** in DMSO-d_6.

Fig. S13. HR-MS spectrum of compound **10**.

Fig. S14. ^{1}H-NMR spectrum of compound **10** in DMSO-d_6.

Fig. S15. ^{13}C-NMR spectrum of compound **10** in DMSO-d_6.

Fig. S16. HR-MS spectrum of compound **11**.

Fig. S17. ^{1}H-NMR spectrum of compound **11** in DMSO-d_6.

Fig. S18. ^{13}C-NMR spectrum of compound **11** in DMSO-d_6.

Fig. S19. HPLC characterization of compound **11**.

Fig. S20. HR-MS spectrum of compound **12**.

Fig. S21. ^{1}H-NMR spectrum of compound **12** in DMSO-d_6.

Fig. S22. ^{13}C-NMR spectrum of compound **12** in DMSO-d_6.

Fig. S23. HPLC characterization of compound **12**.

Fig. S24. HR-MS spectrum of compound **13**.

Fig. S25. ^{1}H-NMR spectrum of compound **13** in DMSO-d_6.

Fig. S26. ^{13}C-NMR spectrum of compound **13** in DMSO-d_6.

Fig. S27. HPLC characterization of compound **13**.

Fig. S28. HR-MS spectrum of compound **14**.

Fig. S29. ^{1}H-NMR spectrum of compound **14** in DMSO-d_6.

Fig. S30. ^{13}C-NMR spectrum of compound **14** in DMSO-d_6.

Fig. S31. HPLC characterization of compound **14**.

Fig. S32. HR-MS spectrum of compound **15**.

Fig. S33. ^{1}H-NMR spectrum of compound **15** in DMSO-d_6.

Fig. S34. ^{13}C-NMR spectrum of compound **15** in DMSO-d_6.

Fig. S35. HPLC characterization of compound **15**.

Experimental details.

Instrumentations and reagents. The materials were used without further purification in addition to the reaction solvents acetonitrile and triethylamine (TEA) needed to be dried with molecular sieves. Oxaliplatin and cisplatin were obtained from Shandong Platinum Source Pharmaceutical Co., Ltd. TBTU, H_2O_2 reagent and 5-FU were purchased from Tianjin Heowns Biochem LLC. VPA, DMAP (4-dimethylaminopyridine), succinic anhydride solution, 37% formaldehyde, propionic anhydride and glutaric anhydride were obtained from Aladdin. DMEM, RPMI1640 medium and fetal bovine serum (FBS) were obtained from Solarbio. TS, HDAC and β-tubulin antibody were purchased from Proteintech. MTT, PI, Annexin V-FITC/PI assay kit and RNase A were purchased from Solarbio. NMR spectra were tested by a Bruker 400 MHz spectrometer, and analyzed by a software of MestReNova. Elemental analysis was determined on a PerkinElmer analyzer model number 240. High resolution mass spectrometry (HR-MS) was recorded on Agilent 6224 ESI/TOF MS instrument. Confocal photos were obtained using an Olympus FV1000 laser

confocal scanning biomicroscope. The intracellular platinum content was evaluated by Optima 5300 DV ICP-MS (PerkinElmer, USA).

Synthesis of 2. Compound **2** were prepared from cisplatin by oxidation with 30% H_2O_2. H_2O_2 (30% w/v, 10.0 mL) was added dropwise to a suspension of cisplatin or oxaliplatin (3.3 mmol) in H_2O (2 mL) at 70°C. After 5 h, the bright yellow solution was cooled at 4°C overnight to afford yellow crystals. The crystals were collected and washed with cold water, ethanol and ether, and dried in vacuum. Compound **2.** Yield: 83.6%. HR-MS (m/z) calcd for $Cl_2H_9N_2O_2Pt$: $[M + H]^+$, 333.9689; found, 334.2917.

Synthesis of 7–8. 5-FU (1.3 g, 10.0 mmol) was dissolved in 37% formaldehyde solution (1.8 g, 22.2 mmol) and the mixture was refluxed at 60°C for 4 h, giving product **6** (yield 78%) after concentration under vacuum. To the solution of **4** in dry acetonitrile (10 mL) were successively added succinic anhydride or glutaric anhydride (12.8 mmol), and catalytic amount of DMAP. The mixture was kept in oil bath at 50°C overnight and evaporated under vacuum. The residue was purified by silica gel column chromatography to give a white solid. Compound **7**. Yield: 62.0%. ^{1}H-NMR (400 MHz, MeOD-d_4) δ (ppm) 7.89 (d, J = 6.0 Hz, 1H), 5.66 (s, 2H), 2.64–2.61 (m, 4H); ^{13}C NMR (100 MHz, MeOD-d_4) δ (ppm) 175.8, 173.9, 159.7 (d, J = 26.3 Hz), 151.1, 141.4 (d, J = 232.3 Hz), 130.6 (d, J = 34.2 Hz), 71.6, 29.8, 29.5. HRMS (m/z): calcd for $C_9H_9FN_2O_6$ $(M + Na)^+$, 283.0342; found, 283.0337. Elemental analysis (%): calcd for $C_9H_9FN_2O_6$: C, 41.55; H, 3.49; N, 10.77. Found: C, 41.18; H, 3.63; N, 10.83. Compound **8**. Yield: 60.9%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.09 (s, 1H), 11.97 (d, J = 4.9 Hz, 1H), 8.12 (d, J = 6.6 Hz, 1H), 5.57 (s, 2H), 2.39 (t, J = 7.2 Hz,

2H), 2.26 (t, *J* = 7.3 Hz, 2H), 1.77–1.70 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 173.9, 172.2, 157.4 (d, *J* = 25.9 Hz), 149.2, 139.4 (d, *J* = 229.2 Hz), 129.4 (d, *J* = 34.0 Hz), 70.5, 32.4, 32.3, 19.6. HR-MS (m/z): calcd for $C_{10}H_{11}FN_2O_6$ $(M + Na)^+$, 297.0499; found, 297.0487. Elemental analysis (%): calcd for $C_{10}H_{11}FN_2O_6$: C, 43.80; H, 4.04; N, 10.22. Found: C, 43.52; H, 4.23; N, 10.07.

Synthesis of 9–10. To a solution of **7** or **8** (0.36 mmol), TBTU (0.36 mmol), and TEA (0.36 mmol) in dry DMSO (2 mL), after 15 minutes, compound **5** (0.3 mmol) was added in the portions. Then the mixture was stirred at 60°C overnight to form a clear solution. The solution was filtered to remove the unreacted solid. The clarified solution was added into Et_2O (20 mL) to get a yellow precipitate and the precipitate was washed with Et_2O for several times. Compound **9.** Yield: 55.0%. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm) 11.96 (s, 1H), 8.11 (d, *J* = 6.4 Hz, 1H), 6.04–5.78 (m, 6H), 5.56 (s, 2H), 2.54 (s, 1H), 2.47 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 179.1, 172.2, 157.4 (d, *J* = 25.8 Hz), 149.2, 139.4 (d, *J* = 229.5 Hz), 129.5 (d, *J* = 33.9 Hz), 70.4, 30.9, 29.8. HRMS (m/z): calcd for $C_9H_{15}Cl_2FN_4O_7Pt$ $(M + H)^+$, 576.0028; found, 576.0007. Compound **10**. Yield: 46.7%. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.13 (d, *J* = 6.4 Hz, 1H), 6.09–5.85 (m, 6H), 5.57 (s, 2H), 2.40 (d, *J* = 7.2 Hz, 2H), 2.21 (d, *J* = 7.2 Hz, 2H), 1.74–1.67 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 180.2, 172.6, 158.5 (d, *J* = 24.8 Hz), 149.9, 139.5 (d, *J* = 230.3 Hz), 129.1 (d, *J* = 34.4 Hz), 70.6, 35.2, 32.5, 20.7. HR-MS (m/z): calcd for $C_{10}H_{17}Cl_2FN_4O_7Pt$ $(M + H)^+$, 590.1084; found, 590.0156.

Cell lines and cell culture. All cell lines including human cervical cancer cell line (HeLa), breast carcinoma cell line (MCF-7 and MDA-MB-231), non-small cell lung cancer cell line (A549) and normal human umbilical vein endothelial cell (HUVEC) were obtained from the American Type Culture Collection (ATCC) and maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were cultured in DMEM (for HeLa, MCF-7, MDA-MB-231 and HUVEC) or RPMI1640 (for A549) medium containing 10% FBS.

Antiproliferative activity. Four different cancer cells (HeLa, MCF-7, MDA-MB-231 and A549) and normal human umbilical vein endothelial cell (HUVEC) were planted in 96-well plates and cultured at 37°C for 24 h. After treating with the concentration-graded compounds for 72 h, respectively, cells were added 10 μL MTT solution and maintained at 37°C. After 4 h incubation, 100 μL of DMSO were added to dissolve MTT formazan crystal violet after removing the supernatant from each plate. The absorbance was measured at 570 nm using an Enzyme-linked Immunosorbent Assay (ELISA) reader. The IC_{50} values were calculated using GraphPad Prism 5 software, which were based on three parallel experiments.

Flow cytometry analysis. HeLa cells were seeded in 6-well plates at a density of 1×10^6 cells mL^{-1} and grown in DMEM medium with 10% FBS and incubated at 37°C for 24 h., and then treated with CDDP at 10 μM and **14** at varied concentrations (2.5, 5, 10 μM) for 36 h. All cells, including floating and adherent cells, were collected and rinsed with PBS three times. Subsequently, all the samples were fixed with 250 μL 70% cold ethanol at –20°C for 48 h. The cells were centrifuged to remove the ethanol

and washed once with PBS. Thereafter, 250 μL PBS was added to each pellets to resuspend cells. Cells were added 2.5 μL RNase A at 37°C to avoid RNA interference. After 0.5 h, the cells were stained with propidium iodide (50 μg/mL) at 37°C in a dark atmosphere over another 0.5 h. Finally, cell cycle distribution was evaluated immediately using BD FACS Verse flow cytometer. These results were carried out with ModFit 3.1 software.

Apoptosis analysis. The selected cell line (HeLa) were cultured in 6-well plates at the density of 1×10^6 cells mL^{-1} overnight to make them adherent. HeLa cell death was analyzed by fluorescence-activated cell sorting (FACS) using Annexin V-FITC and PI staining assay. Then the cells were treated with CDDP at 10 μM and **14** varied concentrations (2.5, 5, 10 μM) subsequently incubated at 37°C in a 5% CO_2 atmosphere for 36 h. After rinsed with PBS three times, and digested with non-EDTA tryptase, the cells were centrifuged 6 min at 2000 rmp and then removed the supernatant. The cells were resuspended in 300 μL 1 × binding buffer, and stained on the basis of the instruction of AnnexinV-FITC/PI assay kit. Flow cytometer was employed to test the fluorescence and data were analyzed by FlowJo 7.6 and GraphPad Prism 5 softwares.

Intracellular accumulation of platinum. In brief, HeLa cells were cultured in 6-well plates at 10^6 cells/well incubated at 37°C in 5% CO_2 for 24 h. Then, each well was added medium containing cisplatin (10 μM) and **14** (10 μM) for 3, 6 and 9 h. After that, HeLa cells were digested and harvested by centrifugation at 2000 rpm for 4 min. Then, the cells were lyophilized by freeze dry system and digested with HNO_3.

Eventually, ICP-MS was used to quantify the platinum content *via* three parallel experiments.

Stability of compound 14. Compound **14** (1 mM) was kept in a PBS/DMF (99:1) at 37° C in dark and monitored by HPLC. The mobile phase was a gradient elution of methanol and water (95:5, v:v, containing 1% formic acid). Flow rate was 1.0 mL/min. The wavelength of UV detector was 260 nm.

Intracellular release of compound 14. 6×10^6 HeLa cells were cultured in 6-well plates, and then cells were treated with 100 μM 5-FU and **14** for 4 h, the untreated group serving as a control group. Then cells were washed with cold PBS for three times, and collected by centrifugation. The cells were resuspended with a 1 mL mixture of dichloromethane and methanol and added to the grinder for mechanical grinding. The solvent was dried naturally at room temperature and its composition was analysed by HPLC.

Wound healing assay. HeLa cells were translated into 6-well plates (7×10^5 cells/well) and cultivated at 37°C for 24 h to form confluent monolayers. The 200 μL plastic pipette tips were used to wound cells. Then, cells were washed with PBS and cultured in medium containing CDDP and compound **14** at indicated concentration in fresh medium for 24 h. The Motic AE2000 microscope was used to measure the wound closure. The Mtico Images Advanced 3.2. was used to record these pictures.

Confocal microscopy. After incubation with cisplatin (10 μM) and **14** (2.5, 5, 10 μM) for 6 h, HeLa cells were fixed by 70% ethyl alcohol at –20°C for 12 h. Subsequently,

0.5% albumin bovine V solution was added to glass culture dishes to block cells at 20°C for 2 h. After that, cells were co-incubated with γH2AX rabbit polyclonal at 4°C overnight. The cells on the glass bottom of culture dishes were washed and then added Alexa Fluor 594-conjugated Goat Anti-Rabbit IgG(H+L) antibody. After being stained for 2 h, the cells were rinsed with PBS and mounted with DAPI for nucleus staining. These results were evaluated by confocal laser scanning microscope (Olympus FV1000).

Western blot analysis. The active preferred HeLa cells were translated into 6-well plates until the cell density reached 80%. Then the cells were treated with 10 μM compounds and cultured at 37°C for 48 h. Proteins were extracted by lysis buffer and stored at –20°C. The protein concentrations were quantified by BCA Protein Concentration Detection Kit (Solarbio). Then the protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF). The membranes were blocked with 5% non-fat milk in TBST (Tris buffered saline with 0.1% Tween-20) for 4 h and incubated with primary antibodies at 4°C overnight under gentle shaking. Then, the membranes were washed with TBST and further incubated with the secondary antibodies at 37°C for 1.5 h. All membranes were washed with TBST three times for 30 min and protein blots were detected with with a Thermo Pierce ECL Western Blotting Substrate.

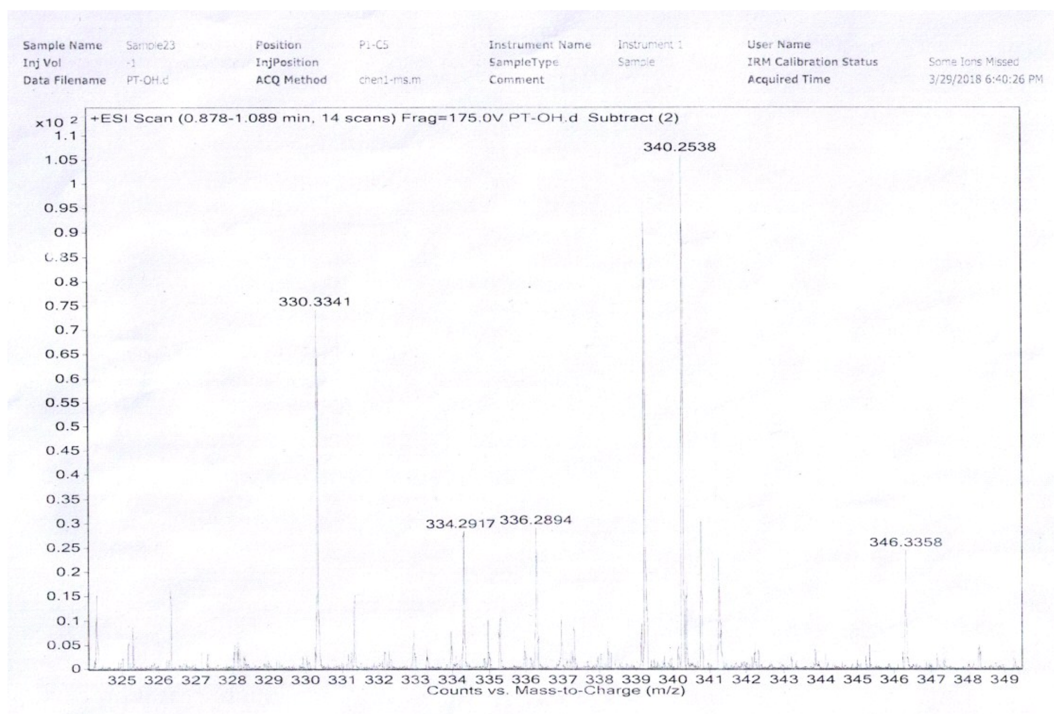


Fig. S1. HR-MS spectrum of *c*,*c*,*t*-$[Pt(NH_3)_2Cl_2(OH)_2]$.

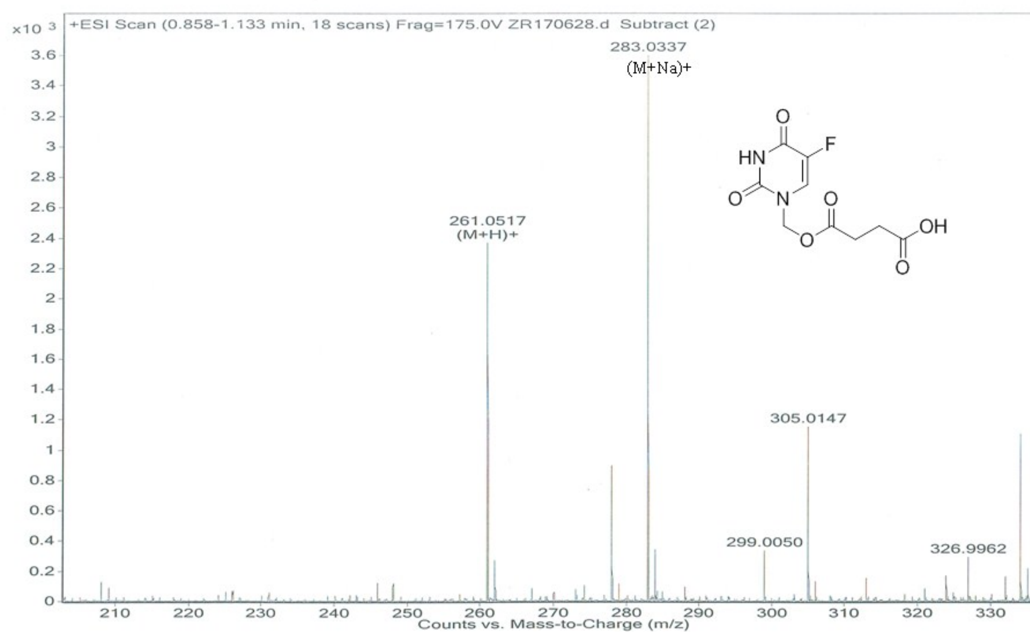


Fig. S2. HR-MS spectrum of compound **7**.

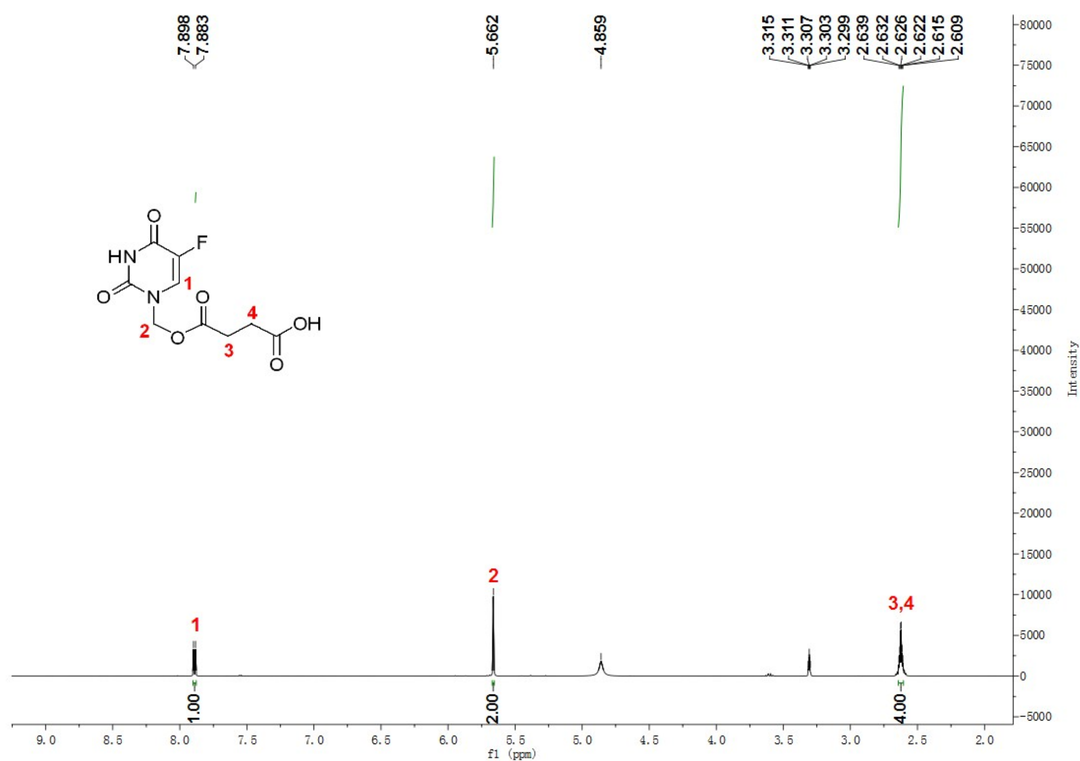


Fig. S3. ^{1}H-NMR spectrum of compound **7** in MeOD-d_4.

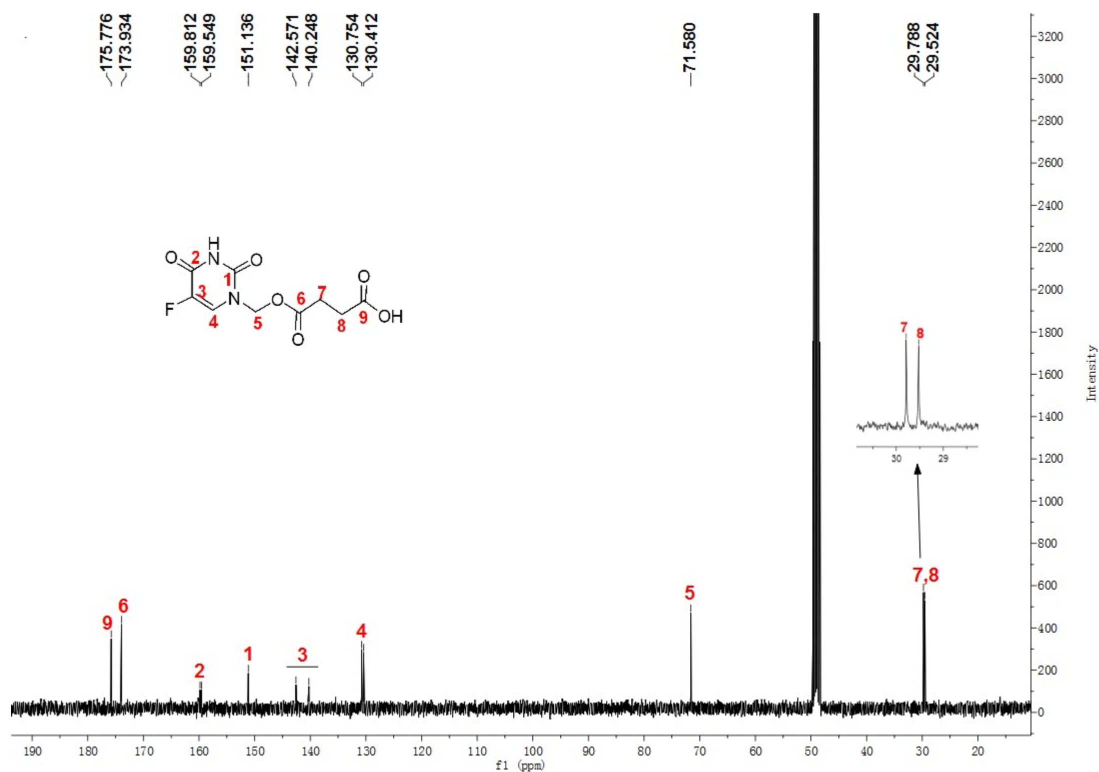
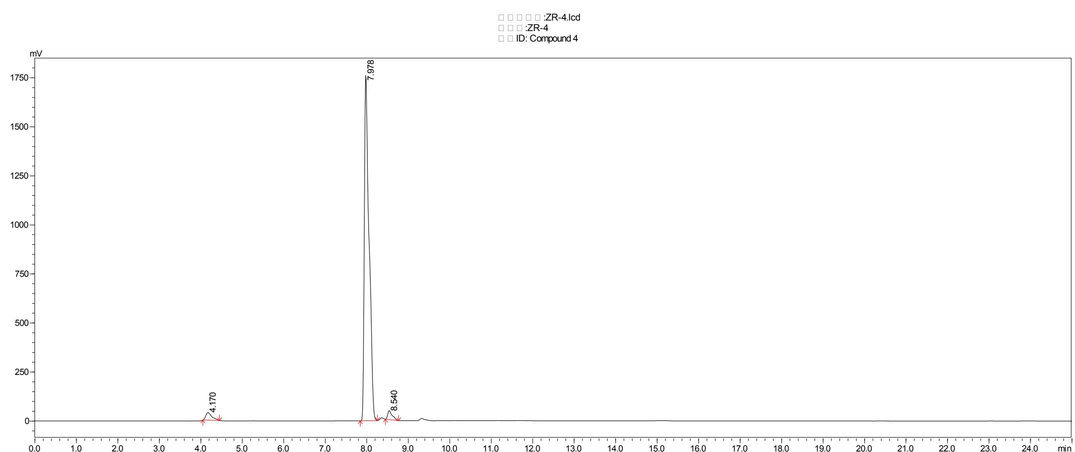


Fig. S4 ^{13}C-NMR spectrum of compound **7** in MeOD-d_4.



	Retention time	Peak area	Concentration (%)
1	4.170	252073	1.729
2	7.978	14126601	96.896
3	8.540	200463	1.375
total		14579143	100.000

Fig. S5. HPLC characterization of compound **7**.

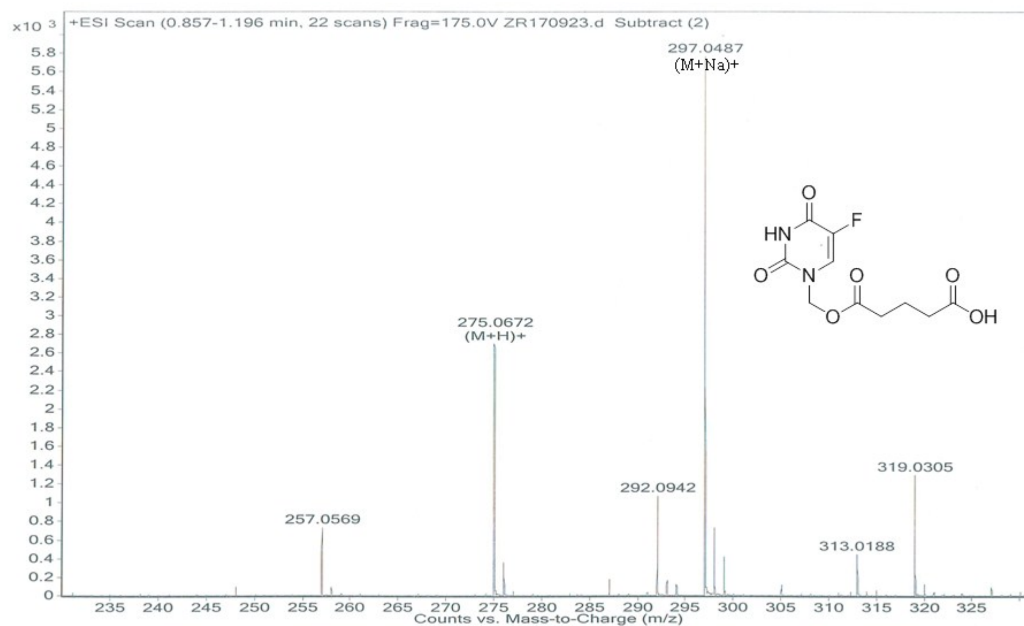


Fig. S6. HR-MS spectrum of compound **8**.

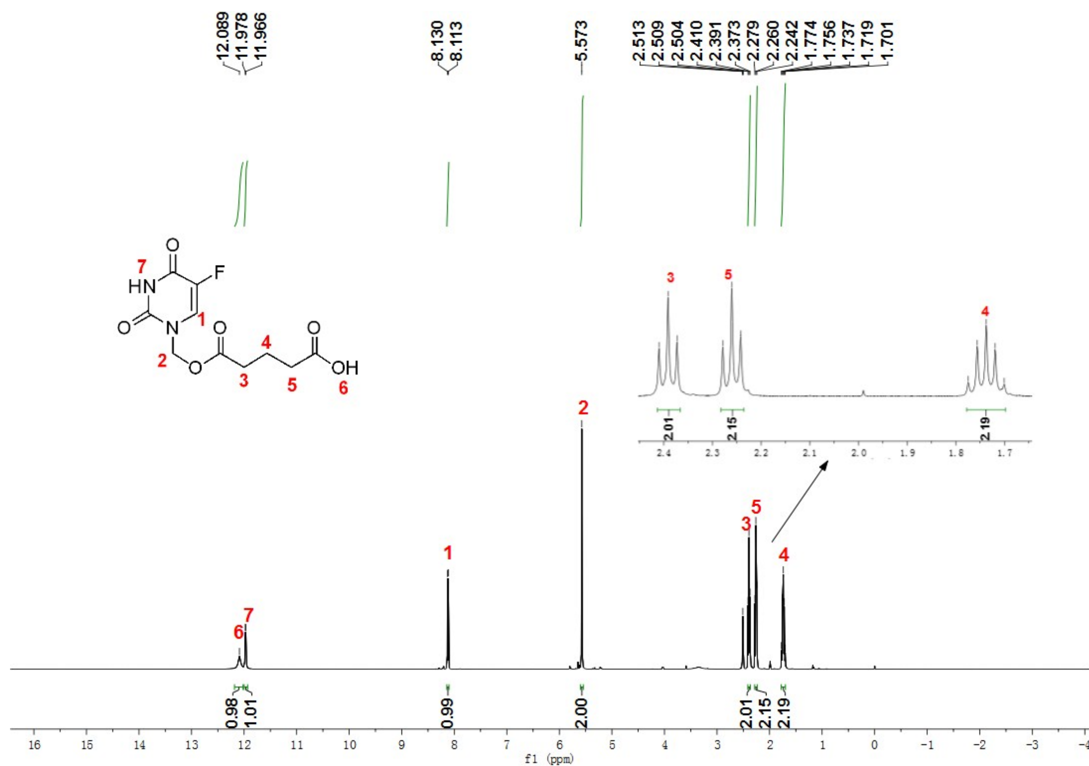


Fig. S7. ^{1}H-NMR spectrum of compound **8** in DMSO-d_6.

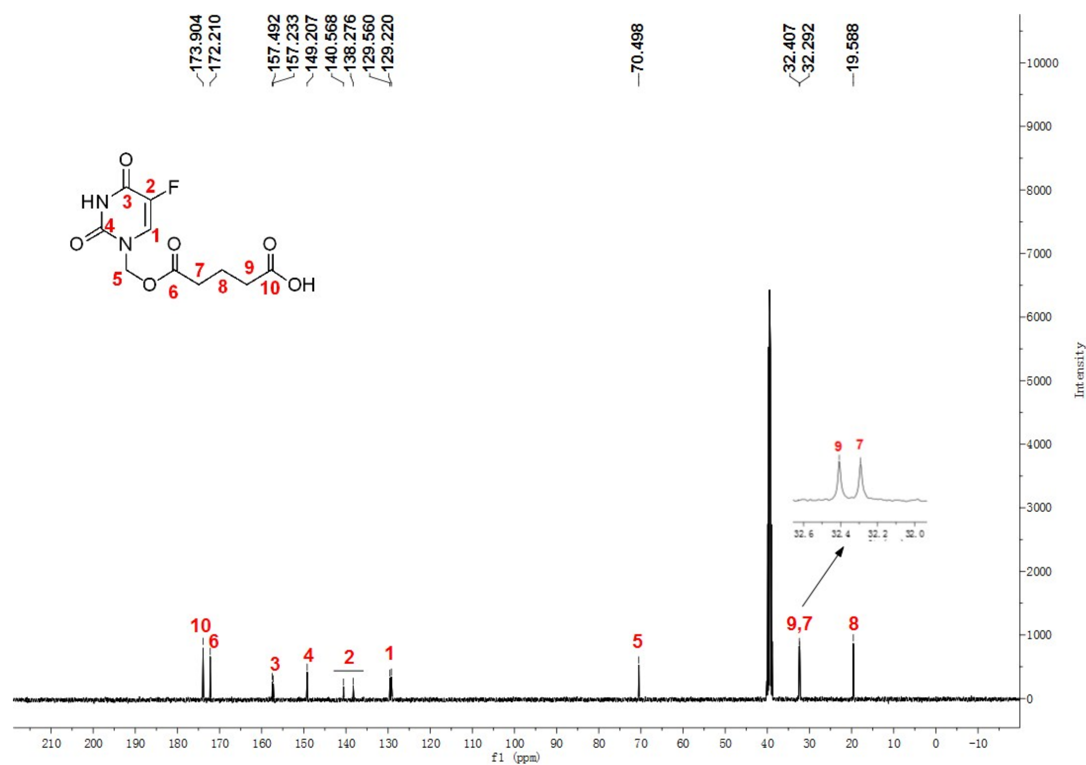
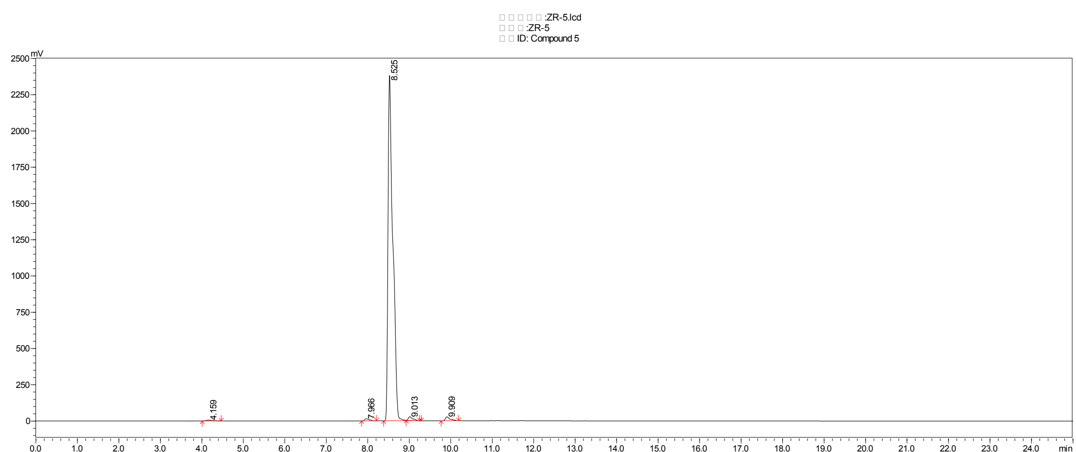


Fig. S8. ^{13}C-NMR spectrum of compound **8** in DMSO-d_6.



	Retention time	Peak area	Concentration (%)
1	4.149	64083	0.324
2	7.966	115711	0.585
3	8.525	19210162	97.038
4	9.013	187456	0.947
5	9.909	219118	1.107
total		19796530	100.000

Fig. S9. HPLC characterization of compound **8**.

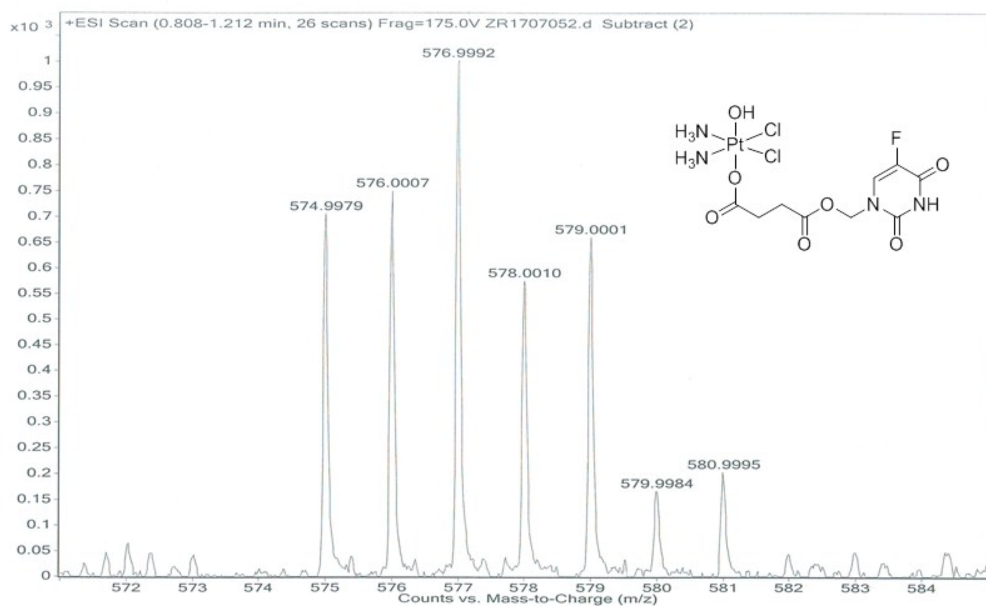


Fig. S10. HR-MS spectrum of compound **9**.

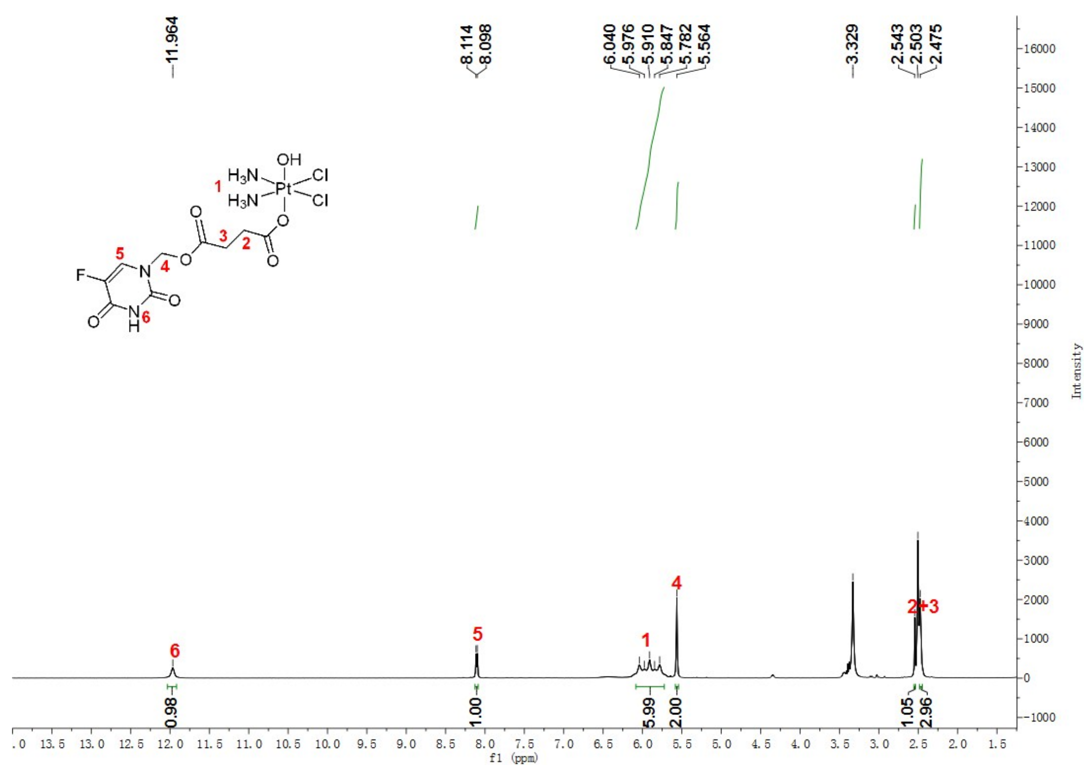


Fig. S11. ^{1}H-NMR spectrum of compound **9** in DMSO-d_6.

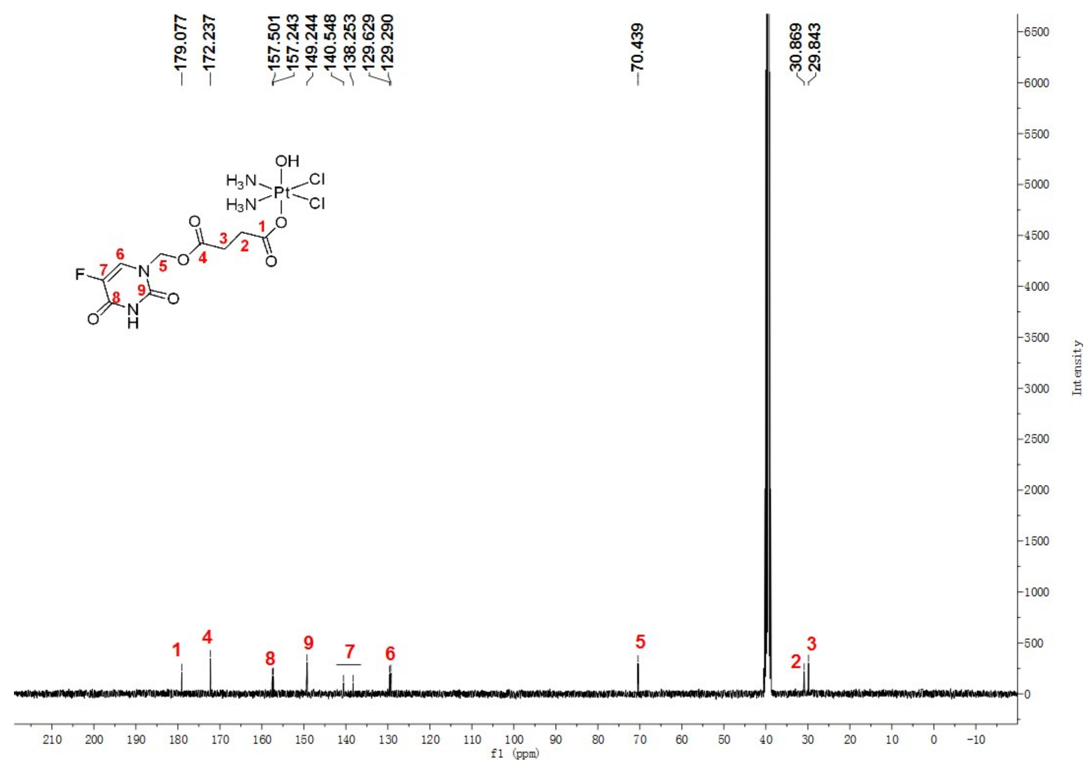


Fig. S12. ^{13}C-NMR spectrum of compound **9** in DMSO-d_6.

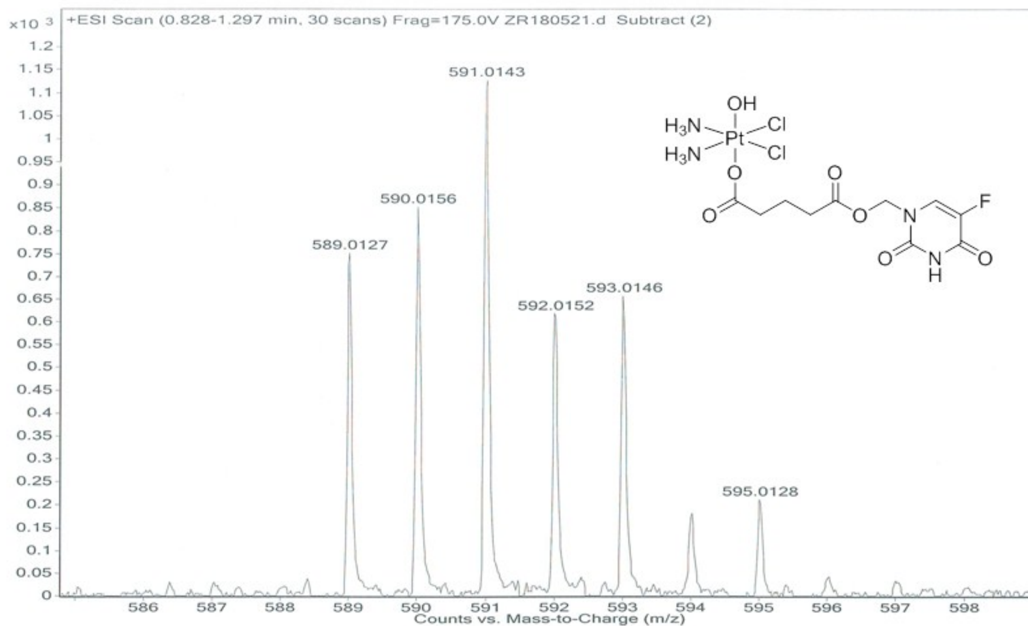


Figure S13. HR-MS spectrum of compound **10**.

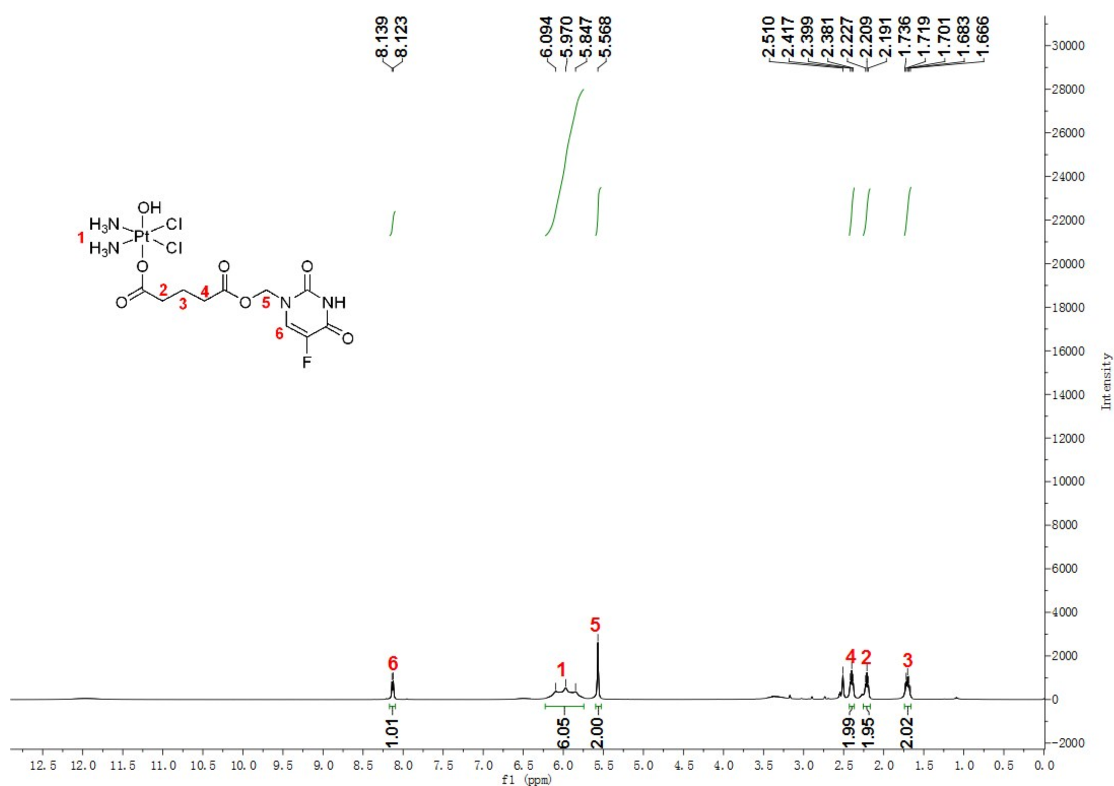


Fig. S14. ^{1}H-NMR spectrum of compound **10** in DMSO-d_6.

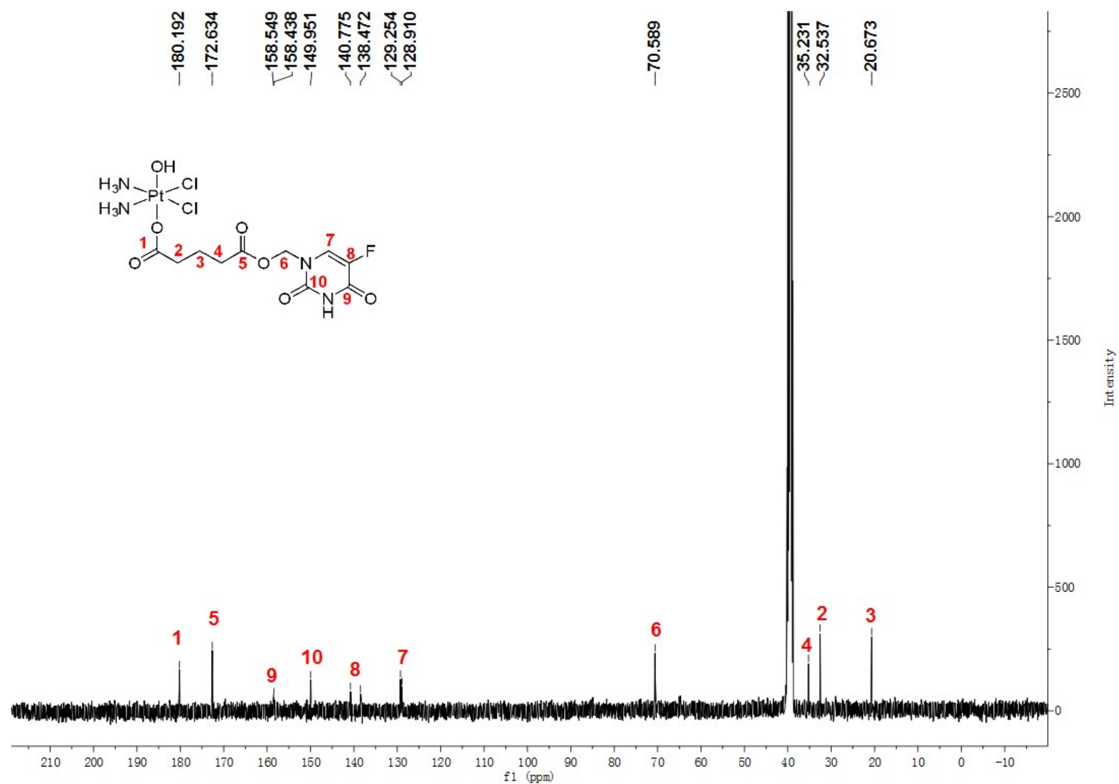


Fig. S15. ^{13}C-NMR spectrum of compound **10** in DMSO-d_6.

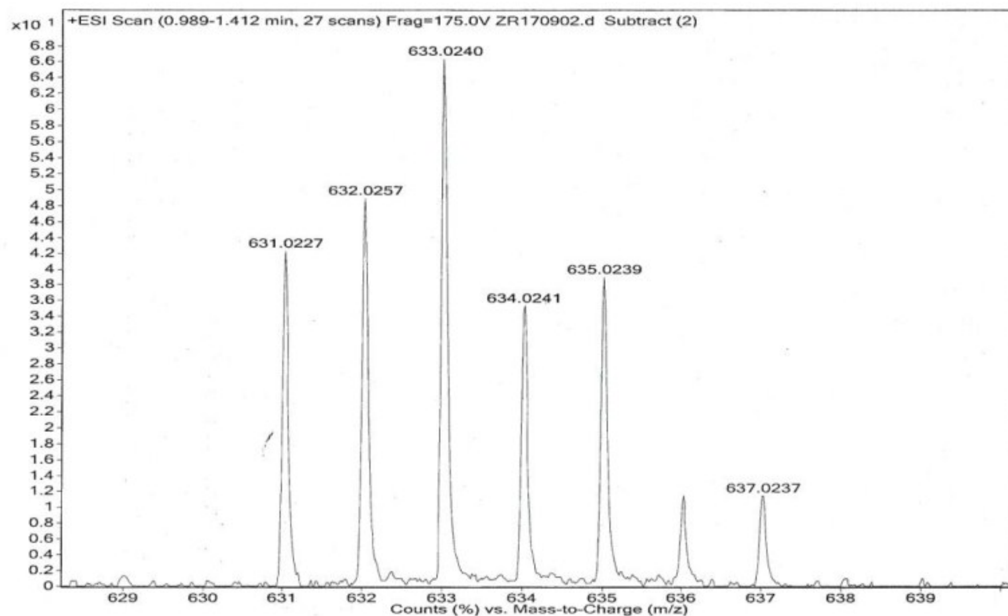


Fig. S16. HR-MS spectrum of compound **11**.

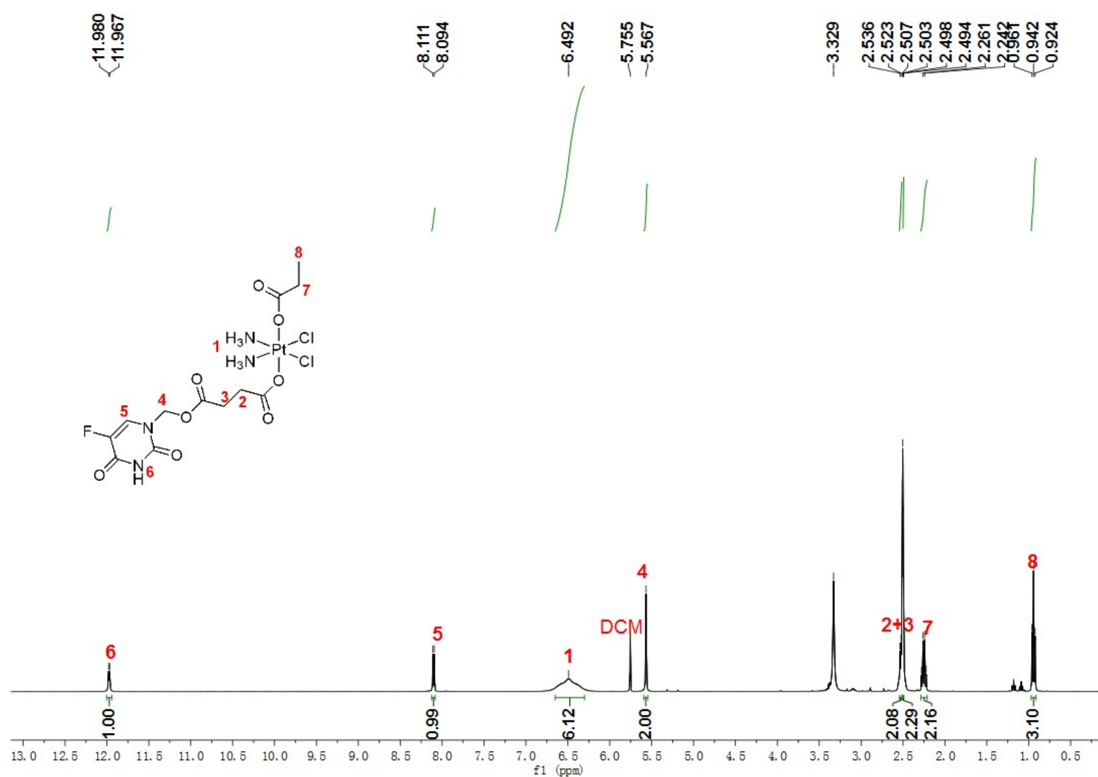


Fig. S17. ^{1}H-NMR spectrum of compound **11** in DMSO-d_6.

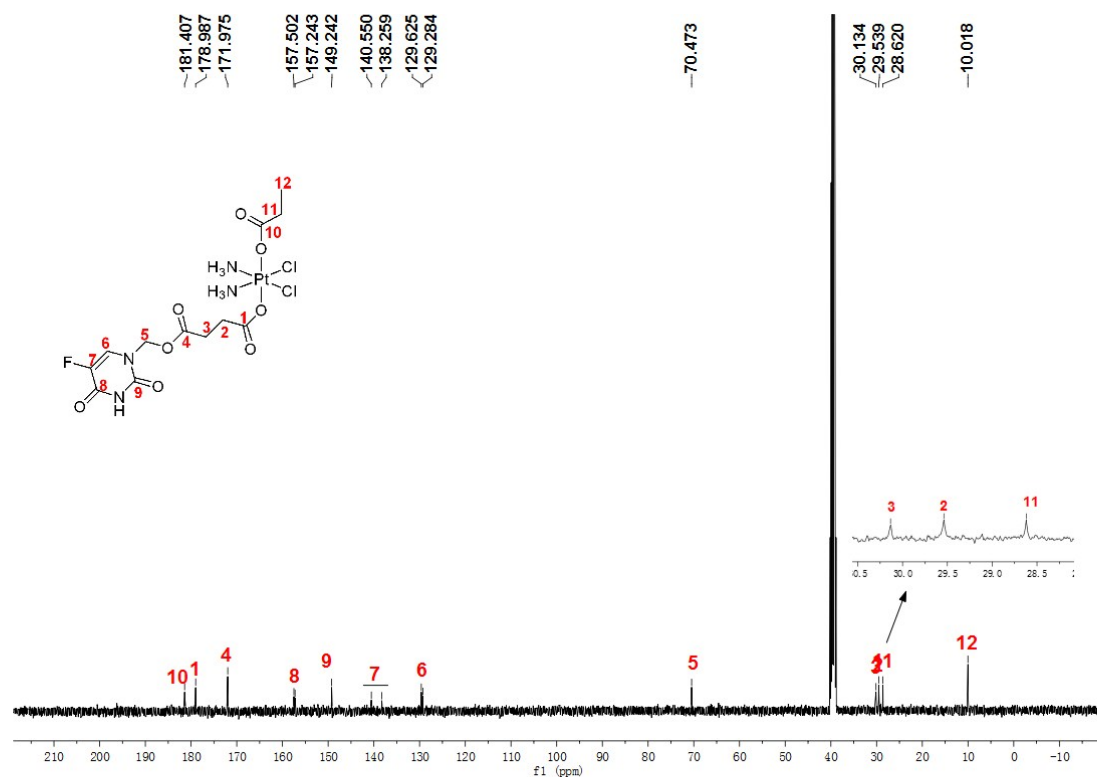
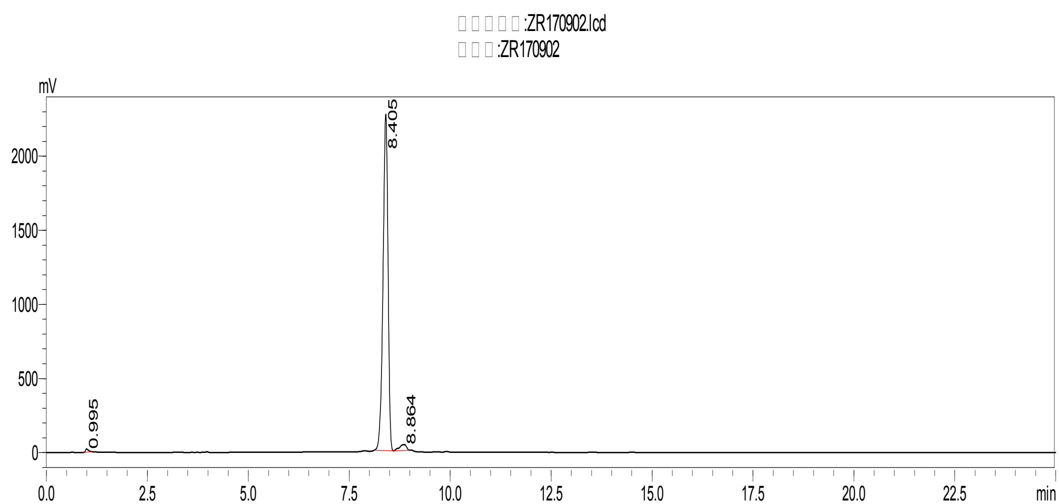


Fig. S18. ^{13}C-NMR spectrum of compound **11** in DMSO-d_6.



	Retention time	Peak area	Concentration (%)
1	0.995	107285	0.538
2	8.405	19369609	97.053
3	8.864	480838	2.409
Total		19957732	100.000

Fig. S19. HPLC characterization of compound **11**.

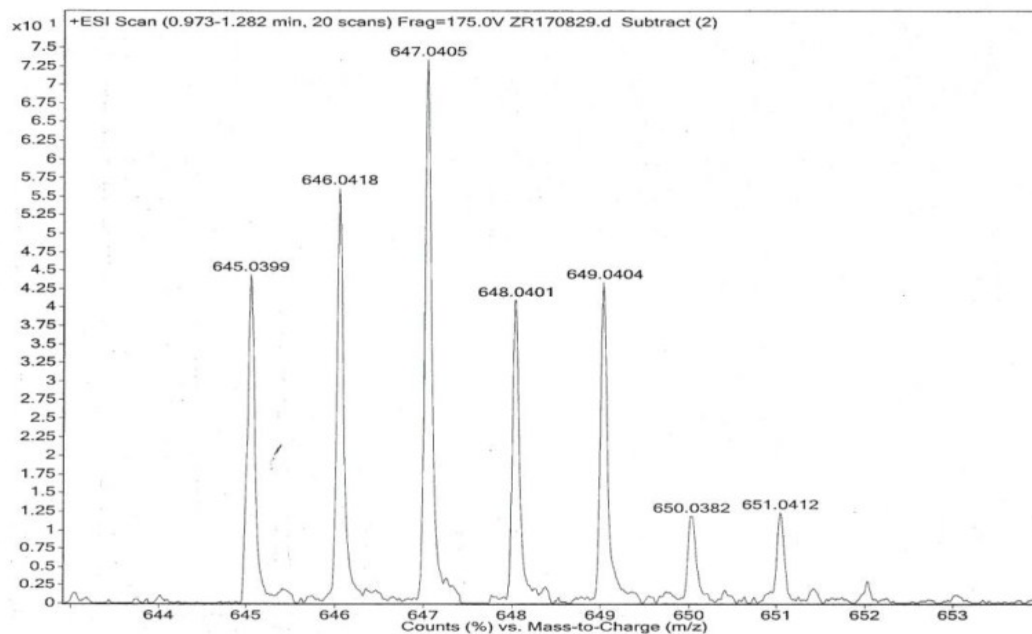


Fig. S20. HR-MS spectrum of compound **12**.

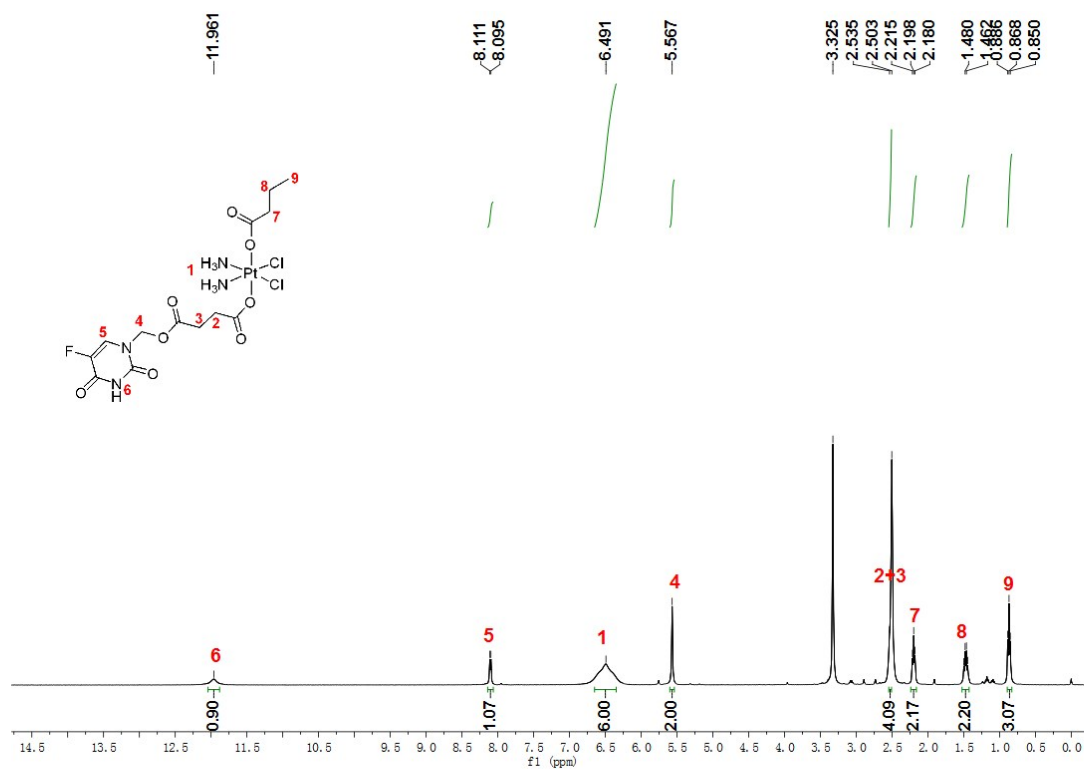


Fig. S21. ^{1}H-NMR spectrum of compound **12** in DMSO-d_6.

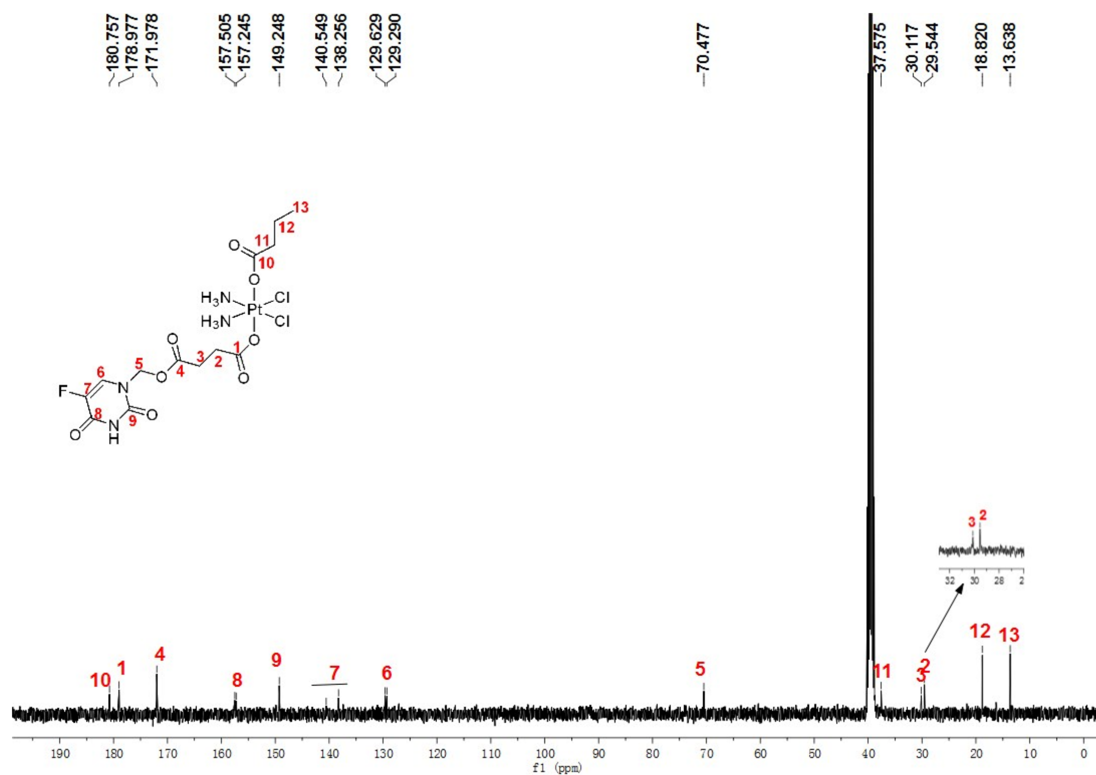
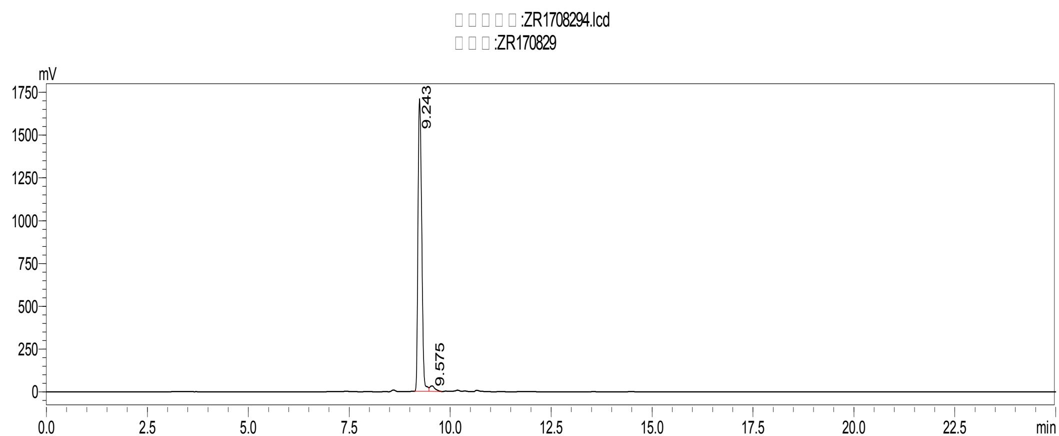


Fig. S22. ^{13}C-NMR spectrum of compound **12** in DMSO-d_6.



	Retention time	Peak area	Concentration (%)
1	9.243	11008646	97.443
2	9.575	288831	2.557
Total		11297277	100.000

Fig. S23. HPLC characterization of compound **12**.

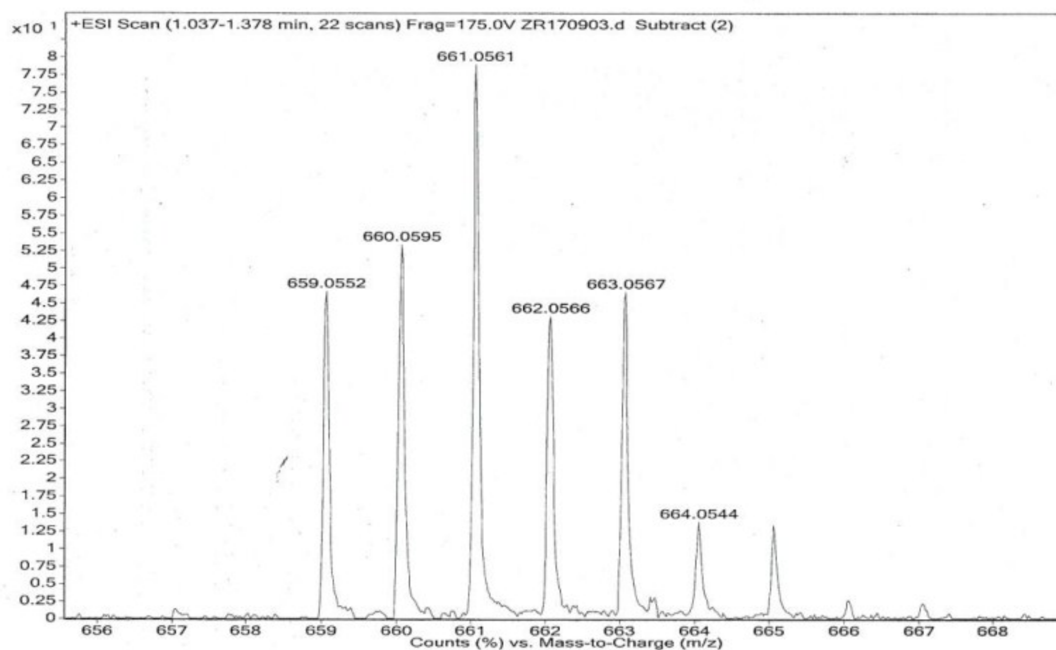


Fig. S24. HR-MS spectrum of compound **13**.

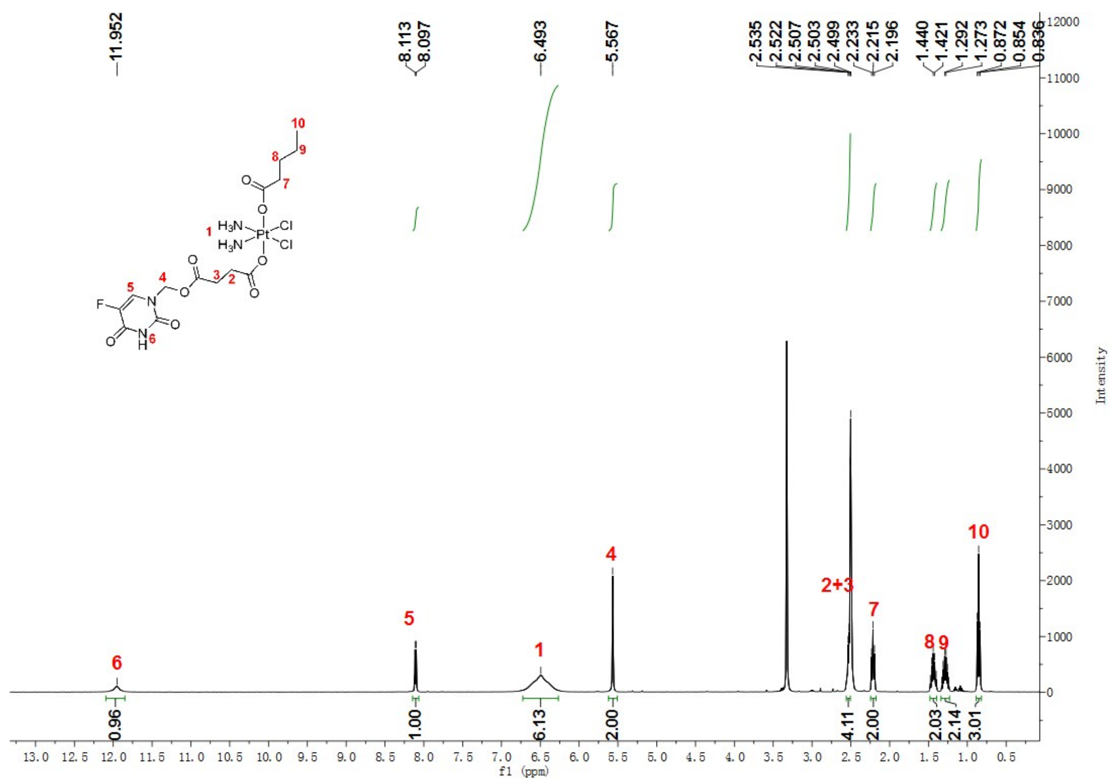


Fig. S25. ^{1}H-NMR spectrum of compound **13** in DMSO-d_6.

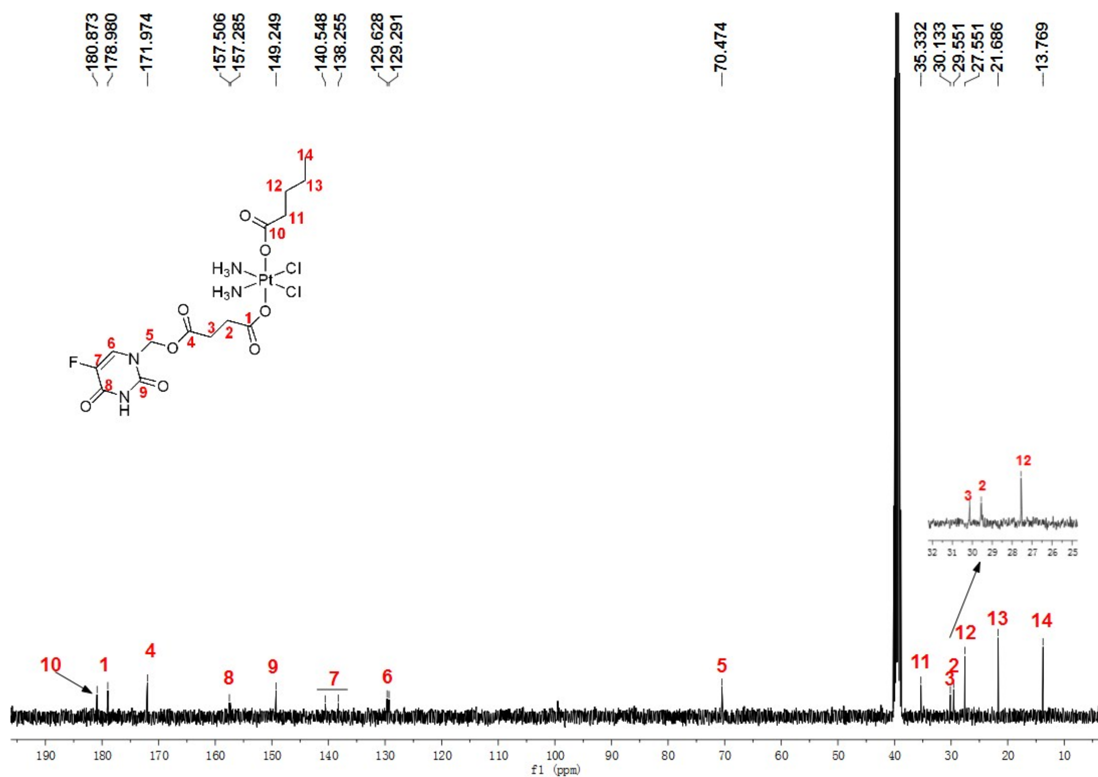
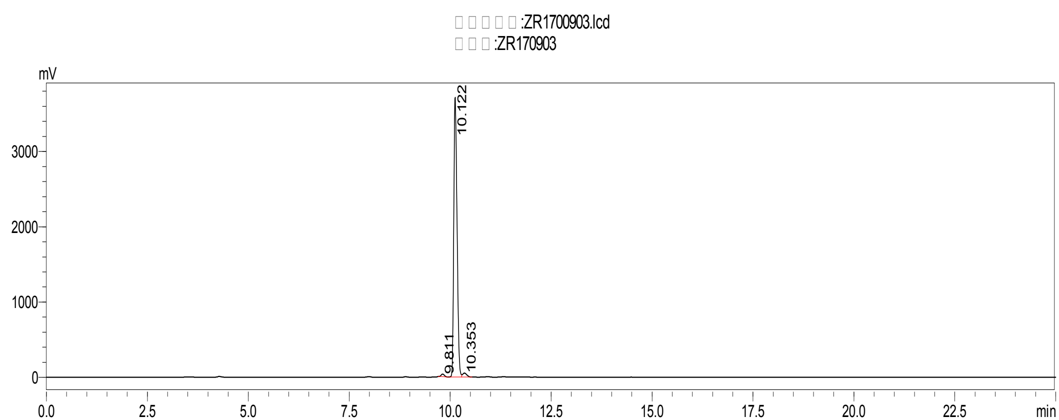


Fig. S26. ^{13}C-NMR spectrum of compound **13** in DMSO-d_6.



	Retention time	Peak area	Concentration (%)
1	9.811	192537	0.893
2	10.122	20998707	97.374
3	10.353	373844	1.734
Total		21191244	100.000

Fig. S27. HPLC characterization of compound **13**.

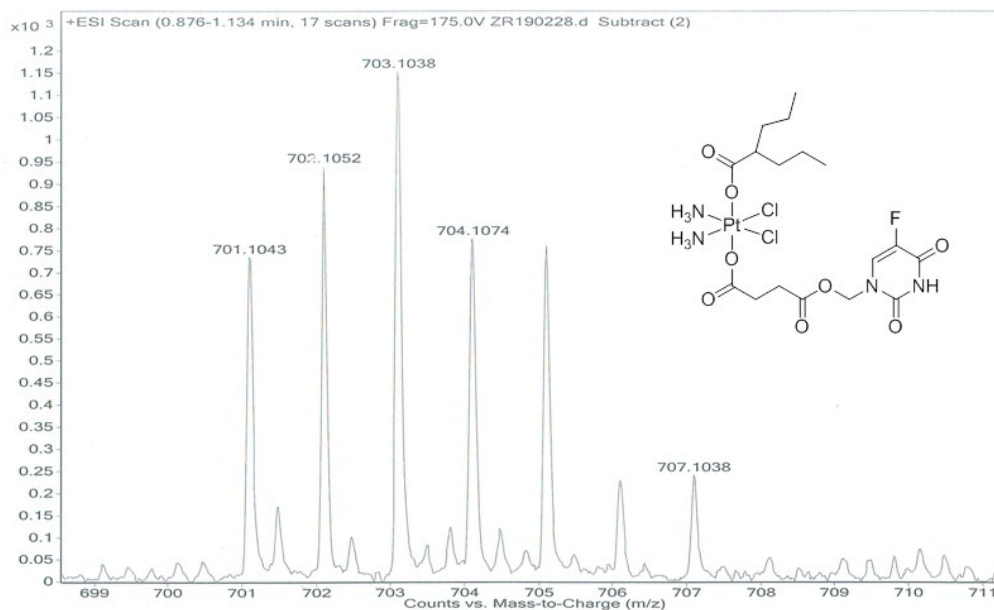


Fig. S28. HR-MS spectrum of compound **14**.

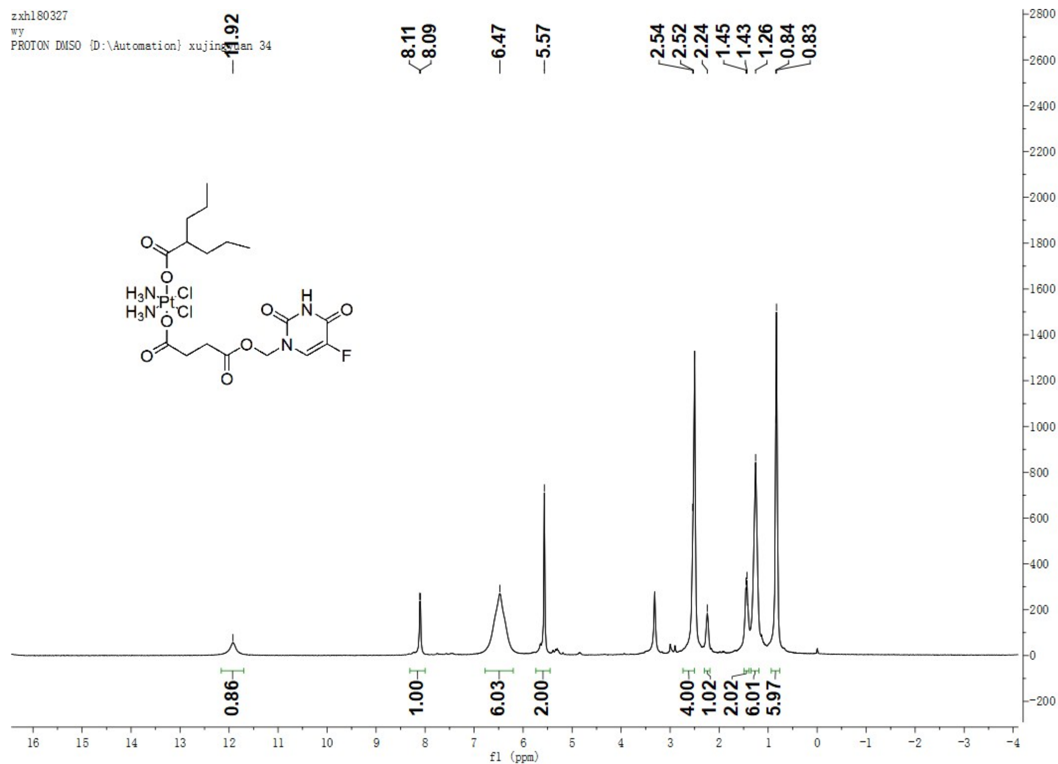


Fig. S29. ^{1}H-NMR spectrum of compound **14** in DMSO-d_6.

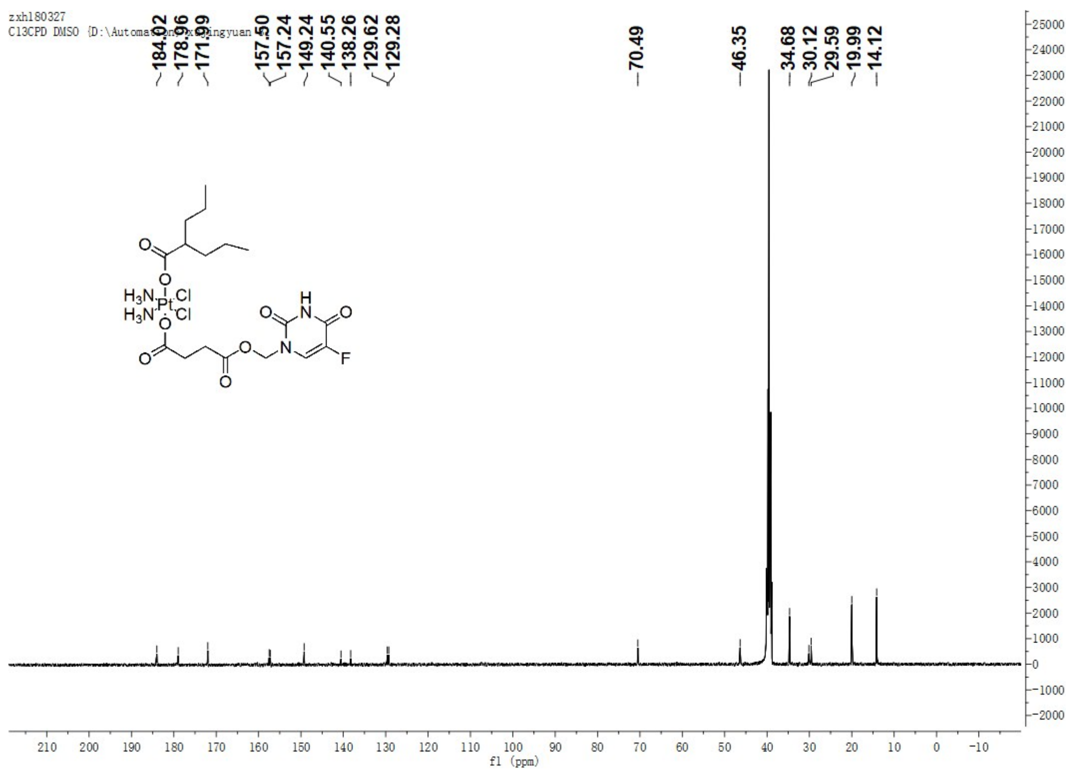
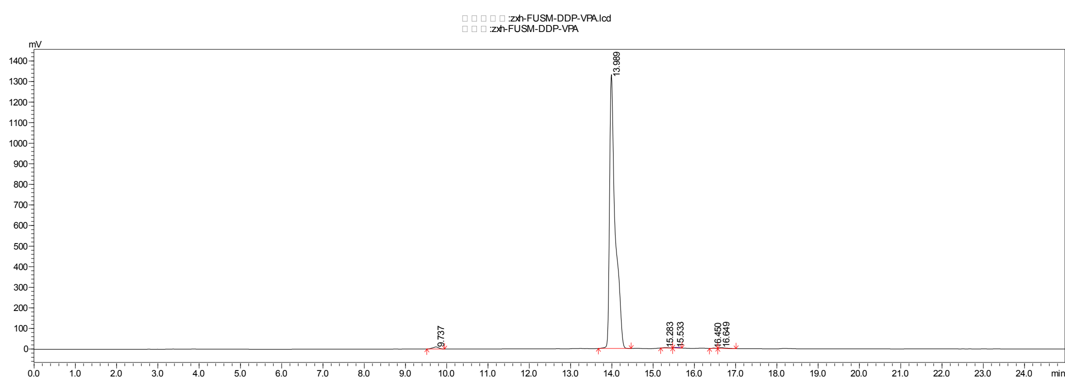


Fig. S30. ^{13}C-NMR spectrum of compound **14** in DMSO-d_6.



	Retention time	Peak area	Concentration (%)
1	9.737	105915	0.820
2	13.989	12703045	98.322
3	15.283	26237	0.203
4	15.533	19253	0.149
5	16.450	13294	0.103
6	16.649	52094	0.403
Total		12919838	100.000

Fig. S31. HPLC characterization of compound **14**.

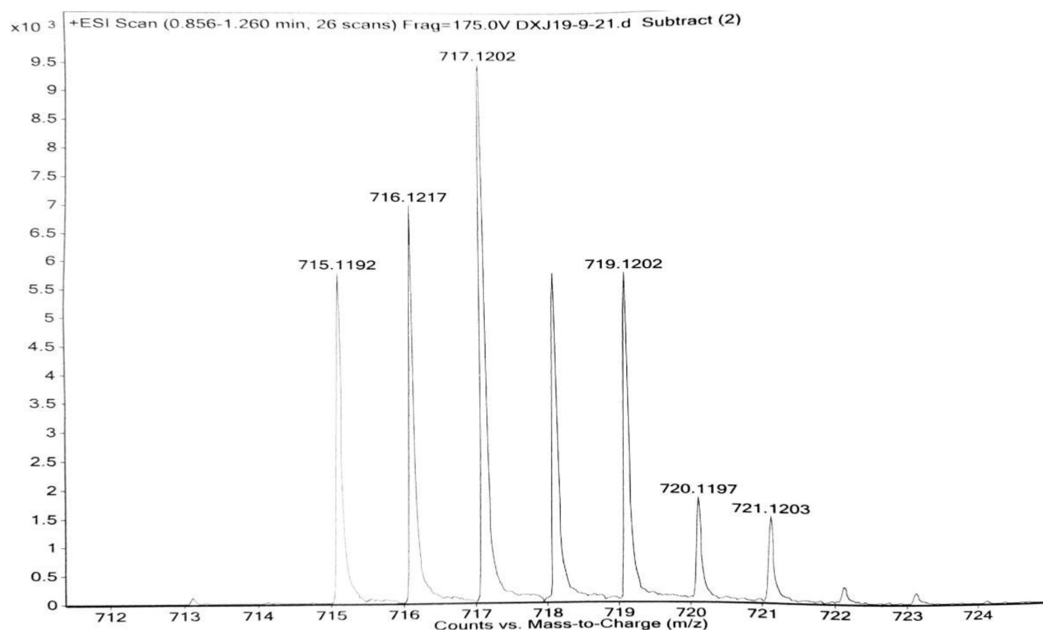


Fig. S32. HR-MS spectrum of compound **15**.

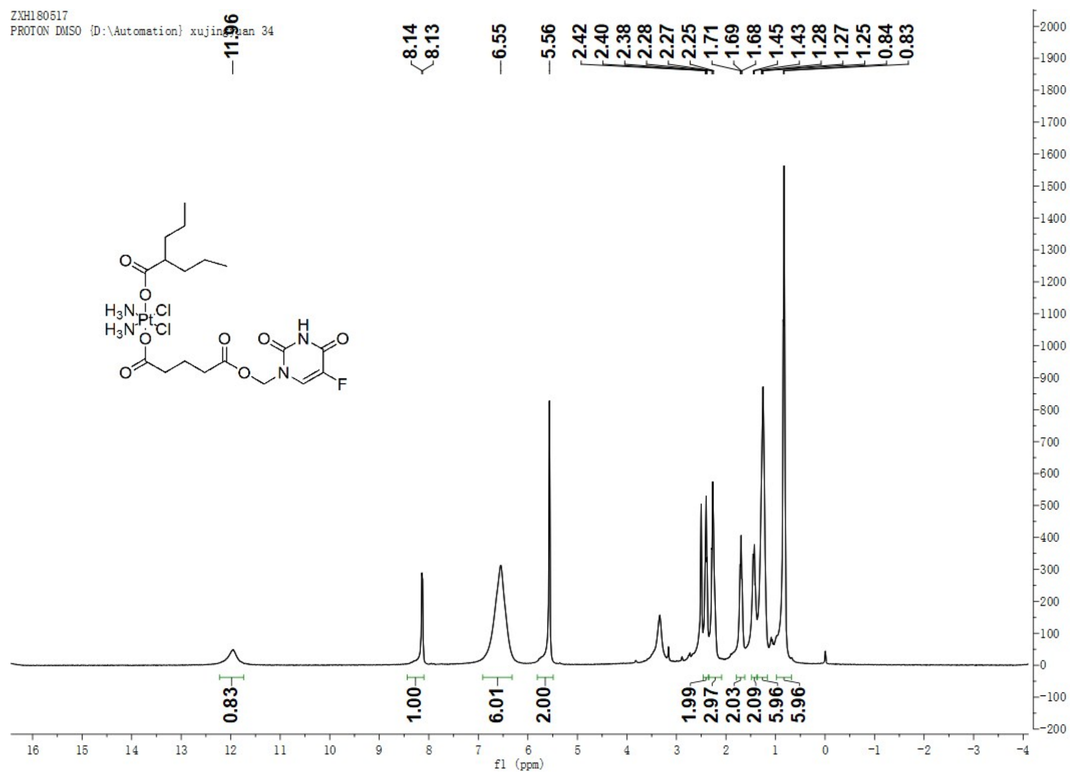


Fig. S33. ^{1}H-NMR spectrum of compound **15** in DMSO-d_6.

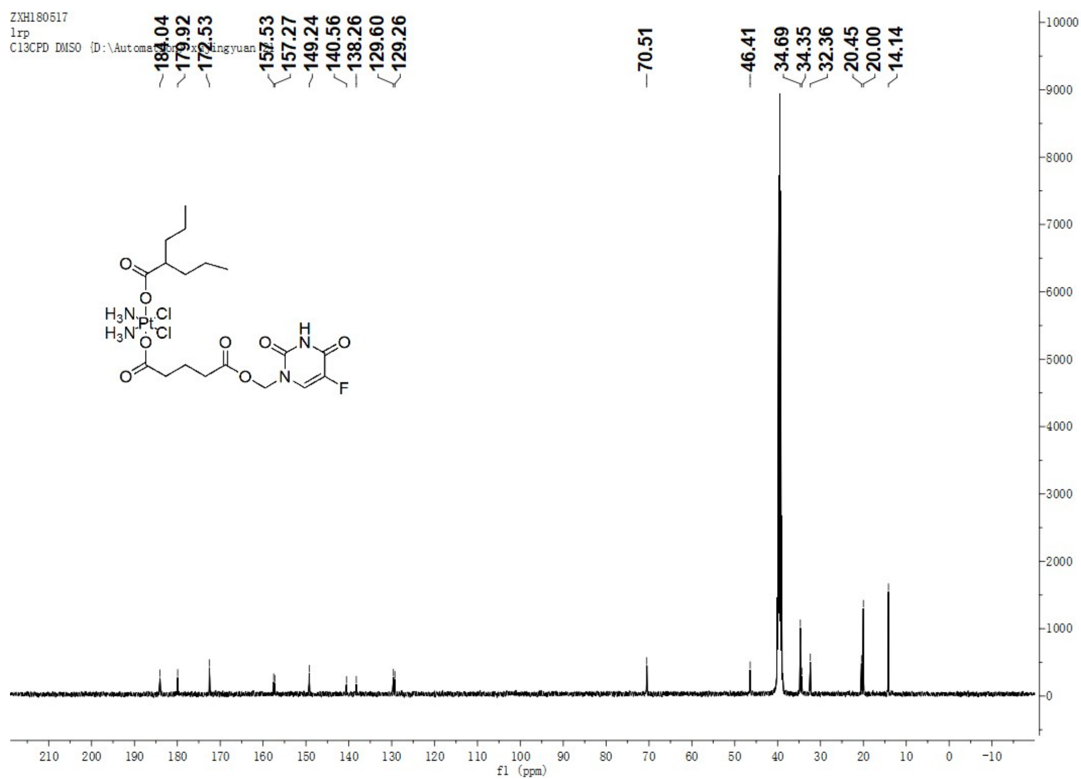
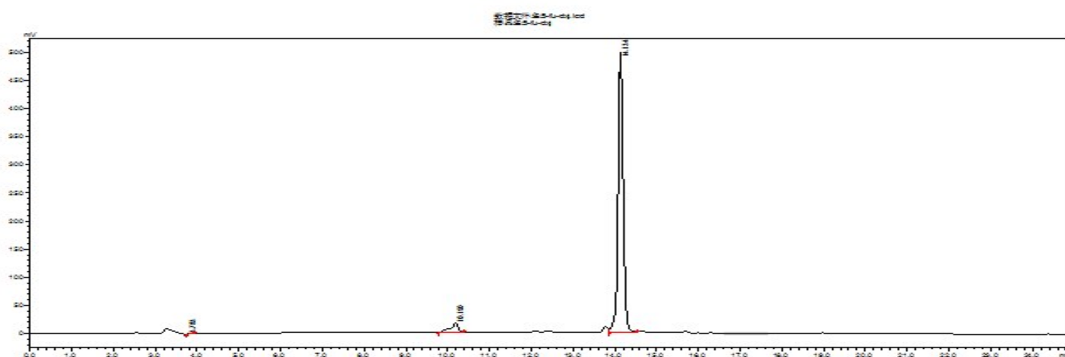


Fig. S34. ^{13}C-NMR spectrum of compound **15** in DMSO-d_6.



	Retention time	Peak area	Concentration (%)
1	3.783	6476	0.144
2	10.190	214705	4.764
3	14.134	4286073	95.093
Total		4507254	100.000

Fig. S35. HPLC characterization of compound **15**.