Electronic Supplementary Material (ESI) for Dalton Transactions.

Cancer specific oxaliplatin-releasing prodrug

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Supporting Information

Content

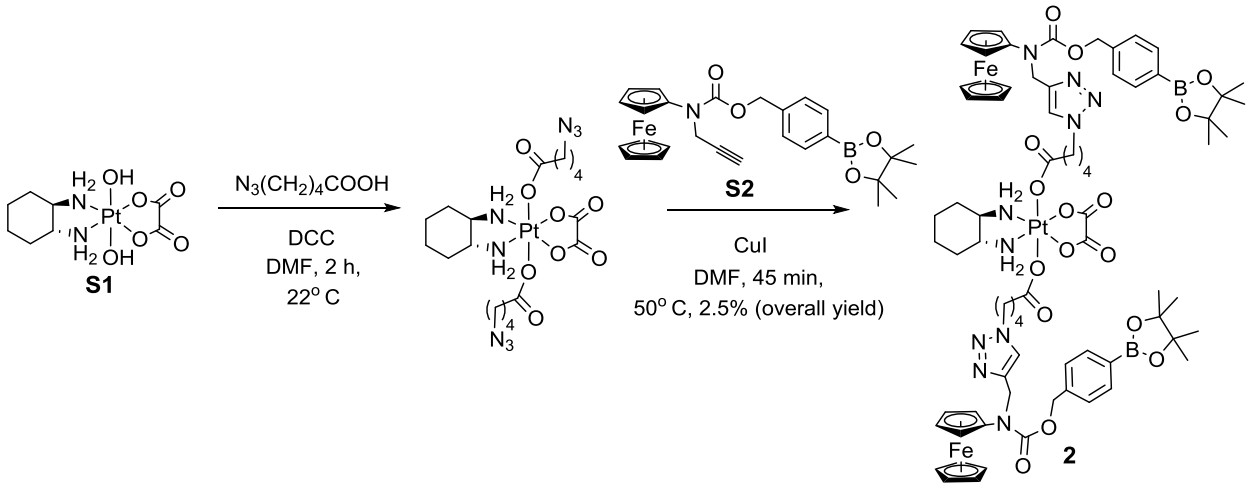
General Information

Commercially available chemicals of the best quality from Sigma-Aldrich (Germany) and Alfa-Aesar (Germany) were obtained and used without purification. NMR spectra were acquired on a Bruker Avance 300. Control compounds **1** and **8** (Figure 1, main text of the paper) were synthesized as reported elsewhere.[1] ESI mass spectra were recorded on a Bruker ESI MicroTOFII. C, H, and N analysis was performed in the microanalytical laboratory of the chemical institute of the Friedrich-Alexander-University of Erlangen-Nürnberg. UV-Vis spectra were measured on a Lambda Bio+ UV/Vis spectrophotometer (Perkin Elmer) Cary 5000 UV-Vis-NIR Spectrophotometer or a Cary 100 UV-Vis Spectrophotometer (Agilent Technologies) by using quartz glass cuvettes (Hellma GmbH, Germany) with a sample volume of 1 mL or micro-cuvettes with a sample volume of 100 µL (BRAND GmbH, Germany). The fluorescence of live cells was quantified using a Guava easyCyteTM 6-2L Flow cytometer from Merck Millipore. The data were processed using the inCyteTM software package (Merck Millipore) and the

ModFIT LTTM software (Verity Software House). The purity of the prodrugs and controls used in the biological assays was determined by C, H, N analysis. According to these data, the purity was greater than 95%. Statistical analysis of the data was conducted using Student's t-test. Two data sets were considered significantly different from each other for $p<0.05$.

Synthesis

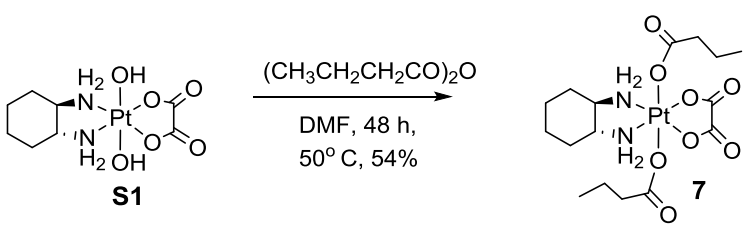
Compound **2.**



The synthesis was conducted using modified procedures from reference 2.

5-Azidopentanoic acid (1,00 g, 7 mmol) and N,N'-dicyclohexylcarbodiimide (DCC, 715 mg, 3.5 mmol) were dissolved in N,N-dimethylformamide (DMF, 10 mL), stirred for 2 hours at 23 ± 2° C and then reaction mixture was filtered. The supernatant was added to the suspension of *trans*-[$Pt(OH)_2$(ox)(R,R-chxn)][3] (**S1**, 600 mg, 1.4 mmol) in DMF (15 mL). The reaction mixture was stirred at 40° C for 48 h and then filtered. The filtrate was collected and the volume of the solution was reduced to ~0.3 - 0.4 mL by evaporation at 50 °C under reduced pressure (10 mbar). Methanol (5 mL) followed by diethyl ether (20 mL) was added to form a yellowish precipitate which was collected by decantation of methanol/diethyl ether fraction, washed with diethyl ether and dried *in vacuo*. The resulting yellowish paste (339 mg, 0.48 mmol) was suspended in DMF (8 mL), to which compound **S2**[4] (1.24 g, 2.48 mmol, 5 eq) and copper iodide (28.5 mg, 0.15 mmol, 0.3

eq) were added. Then the mixture was shaken for 45 min at 50° C. After cooling, the reaction mixture was centrifuged. The supernatant was added to the water (9 mL) and the precipitate was centrifuged and washed with ice-cold water (5 mL), ice-cold ethanol (5 mL) and diethyl ether (10 mL). The crude product was purified by column chromatography eluting with dichloromethane (DCM) / methanol (87:13, v:v, R_f = 0.52) to give **2** (20.9 mg, 0.012 mmol, 0.86 % yield). ^{1}H NMR (300 MHz, DMF-d7), 8.17-8.83 (m, 4 H), 8.02 (s, 2 H), 7.75 (d, *J*=7.9 Hz, 4 H), 7.48 (d, *J*=7.7 Hz, 4 H), 5.28 (s, 4 H), 5.04 (s, 4 H), 4.70 (br. s, 4 H), 4.43 (t, *J*=7.1 Hz, 4 H), 4.16 (s, 10 H), 4.06 (s, 4 H), 2.31 (t, *J*=7.4 Hz, 4 H), 1.80 - 1.95 (m, 4 H), 1.52 - 1.68 (m, 8 H), 1.23 - 1.39 (m, 30 H). ^{13}C NMR (75 MHz, DMSO-d6) δ =180.62, 162.29, 153.62, 144.07, 139.73, 134.51, 134.21, 127.17, 123.10, 83.69, 80.63, 68.92, 64.91, 64.18, 61.99, 49.22, 35.77, 30.75, 29.60, 25.39, 24.67, 15.17. High resolution ESI-mass spectrum (HR-ESI-MS, positive mode): calcd. for $C_{72}H_{90}B_2Fe_2N_{10}O_{16}Pt{\cdot}H_2O$ 1697.5199 $(M\text{-}e^-)^+$; found m/z 1697.5134. Elemental analysis: calcd (%) for $C_{72}H_{90}B_2Fe_2N_{10}O_{16}Pt{\cdot}2CH_3OH$: C 50.96, H 5.66, N 8.03; found: C 50.75, H 5.49, N 7.89.



Compound **7.** Suspension of butyric anhydride (1.1 g, 7 mmol, 5 eq) and compound **S1** (600 mg, 1.4 mmol) in DMF (10 mL) were stirred at 50° C for 48 h and then filtered. The brownish filtrate was collected and the volume of the solution was reduced to ~0.3 - 0.4 mL by evaporation at 50 °C under reduced pressure (10 mbar). Methanol (5 mL) followed by diethyl ether (20 mL) was added to form a brownish precipitate, which was collected by decantation of methanol/diethyl ether fraction and dried *in vacuo*. The crude product was suspended in DCM/CH_3OH (90:10, v:v) mixture and the suspension was loaded to the top of the column and the column was eluted using the eluent mixture CH_3OH/DCM (gradient of MeOH in DCM from 90:10 to 70:30, v:v) to give product **7** (430.0 mg, 0.750 mmol, 54% yield). TLC (SiO_2, eluent DCM/CH_3OH, 87/13, v/v) R_f= 0.62. ^{1}H NMR (300 MHz, DMF-d7), 8.34-9.03 (m, 4 H), 2.81 - 2.94 (m, 2 H), 2.34 (d, *J*=11.1 Hz, 2 H), 2.22 (td, *J*=7.3, 1.3 Hz, 4 H) 1.55 - 1.67

(m, 4 H), 1.48 (sxt, *J*=7.3 Hz, 4 H), 1.22 - 1.34 (m, 2 H), 0.84 (t, *J*=7.3 Hz, 6 H). ^{13}C NMR (75 MHz, DMSO-d6) δ =181.06, 163.32, 61.13, 37.73, 30.91, 23.50, 18.77, 13.43. HR-ESI-MS (positive mode): calcd. for $C_{16}H_{28}N_2O_8Pt$ 572.1568 $(M+H)^+$; found m/z 572.1560. Elemental analysis: calcd (%) for $C_{16}H_{28}N_2O_8Pt{\cdot}2H_2O$: C 31.63, H 5.31, N 4.61; found: C 31.81, H 5.42, N 4.61.

Assays in cell free settings

Solubility determination:

Compounds **2**, **7** and **8** were dissolved in DMF at concentrations of 20 mM, 10 mM, 5 mM, 2 mM, 1.5 mM, 1 mM and 0.2 mM. These solutions (10 µL) were transferred to quartz cuvettes containing 990 µL with either phosphate-buffered saline (PBS: phosphate 10 mM, pH 7, NaCl 154 mM) or Roswell Park Memorial Institute (RPMI) 1640 medium (Biochrom GmbH, Germany) + 5% (Fetal Bovine Serum (FBS)**,** Biochrom GmbH, Germany) to obtain solutions/suspensions of **2**, **7** and **8** at final concentrations of 200 µM, 100 µM, 50 µM, 20 µM, 15 µM, 10 µM and 2 µM. The absorbance at 789 nm of the resulting mixtures was measured (A_{789nm}) and plotted as a function of the concentration of the compounds. Mixtures exhibiting $A_{789nm} < 0.036$ appeared transparent and were considered as true solutions. Those with $A_{789nm} > 0.036$ appeared milky and were considered to be suspensions. Using these data, we determined solubility limits for prodrugs **2, 7** and **8** provided in Table S1.

Table S1. Solubility determination

	Solubility in DPBS, µM	Solubility in FBS, µM
2	43.2 ± 10.5	47.5 ± 14.8
7	90.1 ± 9.7	102.4 ± 6.3
8	>200	>200

Stability of prodrug 2 in different solutions

Compound **2** (25 µM) was incubated for 6 hours at 37^o C in a mixture of DMF and phosphate-buffered saline (PBS: phosphate 10 mM, pH 7, NaCl 154 mM; DMF/PBS

1/99, v/v, pH=7.0±0.2, adjusted with either 0.1 M HCl or 0.1 M NaOH: *mixture 1*) containing either glutathione (GSH, final concentration is 5 mM), N,N,N',N'-ethylenediaminetetraacetic acid (EDTA, final concentration is 1 mM) and Asc (final concentration is 1 mM: *mixture 2*), or H_2O_2 (final concentration is 200 μM) additionally to all components of mixture b: *mixture 3*. Every three hours the aliquot (1mL) was taken from these mixtures, centrifuged and the absorbance at 347 nm was measured. Decomposition of **2** was reflected in the decrease in absorbance at 347 nm (Figure 2A, main text).

Monitoring Generation of ROS in cell free settings

Generation of ROS was monitored using fluorescence spectroscopy as described earlier[5]. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA; 4.9 mg) was dissolved in DMF (100 μL) and mixed with aqueous NaOH (0.1 M, 900 μL). The resulting mixture was incubated for 30 min at 22 °C in the dark to obtain a stock solution of 2′,7′-dichlorofluorescein (DCFH; 10 mM). Next a solution (1 mL) containing DCFH (10 μM), MOPS buffer (100 mM, pH 7.5), EDTA (10 mM), GSH (5 mM), and H_2O_2 (10 mM) was prepared. Monitoring of the fluorescence (λ_{ex} = 501 nm, λ_{em} = 531 nm) of this solution was started. After 5 minutes, prodrugs (final concentration - 24.75 μM except of **8**, for which 49.5 μM was selected for better comparison with prodrug **2** containing two ferrocene moieties per molecule) were added, and the fluorescence monitoring was continued until the fluorescence signal growth was stalled. The fluorescence intensity in this assay correlates with the amount of reactive ROS present in the mixture (Figure 2B, main text).

Determination of octanol-water partition coefficients (LogP values)

Solutions of reference and new compounds were prepared by dissolving them in the mixture of DCM/CH_3OH (1:1, v:v) at the concentration of 0.3-0.5 mg/mL. Commercially available normal phase TLC plates from Macherey-Nagel (Germany) (Polygram Sil G/UV_{254}, stationary phase thickness: 0.2 mm) were used. The plates were manually spotted with approximate volume of 2.0 μL of freshly prepared solutions, at 10 mm distance from the edge of the plate. As the eluent mixture DCM/CH_3OH (87:13, v:v) was used. The solvent migration distance was 4.5 cm. The plates were visually inspected

under the UV-light (254nm) and each zone was clearly marked and its distance was manually measured. All measurements were done in triplicate and average values were used in further calculations. All experiments were performed at ambient temperature (22 ± 2^{o}C). A set of reference compounds was used, for which logP-values were reported: Adenosine (−1.05)[6], 4-aminophenol (0.04)[7], benzylalcohol (1.1).[8] A calibration plot of R_f versus logP, which was obtained based on these data, was used to determine logP values of new prodrugs (Table 1, main text).

Cellular Assays

Cells and Cell Cultures

Cells were cultured according to recommendations of Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). In particular, human ovarian carcinoma cisplatin sensitive A2780 (Sigma-Aldrich, Germany) and cisplatin resistant A2780cis (Sigma-Aldrich, Germany) cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Biochrom GmbH, Germany). Human Dermal Fibroblasts, adult (HdFa, Sigma-Aldrich, Germany) cell line was grown in Dulbecco's modified eagle medium (DMEM, Biochrom GmbH, Germany), supplemented with 10% FBS (Fetal Bovine Serum, Biochrom GmbH, Germany), 1% L-glutamine and 1% penicillin/streptomycin. Detaching of the cells from culture flasks was performed with trypsin (0.025 (A2780, A2780cis) or 0.05 (HdFa) %, w/v) / EDTA (0.02 %, w/v) in PBS. Before the experiments A2780 and A2780cis cell lines were cultivated in 1640 RPMI medium (Roswell Park Memorial Institute medium, Biochrom GmbH, Germany), and HdFa cell line was cultivated in DMEM medium (Dulbecco's Modified Eagle's medium, Biochrom GmbH, Germany), all supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin until they reached 70−80% confluence. Then the medium was removed, and the cells were washed two times with PBS buffer (10 mM phosphate, 154 mM NaCl, pH 7.4), trypsinated, and re-suspended in the desired medium required for the experiment. The resistant cell line A2780cis was treated with 1 μM cisplatin in PBS buffer after every 3 passages[9].

Determination of the viability of A2780, A2780cis, and HdFa cells

The cells were spread in the wells of a 96-well microtiter plate containing RPMI 1640 6.250 cells for A2780, A2780cis and 10.000 for HdFa cell line per well per 100 µL and left standing at 37 °C in the chamber filled with CO_2 (5%) overnight. Stock solutions of prodrugs of different concentrations (1 µL, solvent DMF) were added to the wells and incubated for the time indicated in Table 1 (main text). Four replicants per each concentration of the prodrug were tested. Finally, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 20 µL of the solution prepared by dissolving MTT (5 mg) in PBS buffer (1 mL)) was added to each well, incubated for 3 h, treated with sodium dodecyl sulfate (SDS) solution (90 µL, 10% solution in 0.01 M aqueous HCl), and incubated overnight. Then the intensity of absorbance at 590 nm was measured (MTT is converted to blue dye (λ_{max} = 590 nm) in live cells). The absorbance at 690 nm was taken as a baseline value. Each experiment was repeated at least 3 times. The data obtained were applied to calculate IC_{50} values of the prodrugs (Table 1, main text).

Accumulation of prodrug 2 and control 8 in A2780 cells

Cells were seeded (4 replicants for each condition) in RPMI 1640 medium (5% FBS, 1% L-glutamine, 1% penicillin/streptomycin) in a 6-well microtiter plate at a density of 350 cells/µL (total volume 2 mL) one day before the experiment. On the day of the experiment the cells were washed twice with the PBS buffer and a fresh portion of RPMI 1640 medium (5% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 2 mL) was added. Solutions of prodrugs (20 µL in DMF) were added to the cultured cells and incubated for 1 h. The final concentrations of the prodrugs **2** and **8** in the medium were 25 µM and 50 µM, correspondently. Afterward, the cells were washed twice with the ice-cold PBS buffer (2 × 1000 µL) and treated with concentrated H_2O_2 solution (1 M, 1.5 mL) overnight. After this the suspensions from 4 wells treated at the same condition were combined, the combined suspensions were transferred to microcentrifugation vessels and all volatiles were removed by lyophilization. Dry, lysed suspensions were dissolved in water (300 µL) and the aqueous solutions obtained were acidified with HCl (0.1 M, 0.4 mL). Then these solutions were extracted with 2-ethyl-1,3-hexanediol (10% in $CHCl_3$, v/v, 150 µL,), and portions of the organic phase (90 µL) were mixed with H_2SO_4

and CH_3CO_2H (1:1, v:v, 0.4 mL). Curcumin solution in methyl isobutyl ketone (2 mg in 1 mL of the solvent, 0.25 mL) was added and allowed to react for 24 h. The reaction was quenched by addition of water (1 mL). The light absorbances at 550 and 780 nm of the organic phase were measured. The former value corresponds to the absorbance of the formed curcumin−boron complex, whereas the latter one is taken as a baseline. The baseline corrected absorbance at 550 nm (A(550 nm) − A(780 nm)) was proportional to the concentration of boron in the mixture (Table 1, main text).

Estimation of oxidative stress in live A2780 cells

Cells (A2780) were seeded in their culture medium in a 24-well microtiter plate (total volume 0.5 mL) at a density of 200 cells/µL. They were allowed to attach overnight. On the day of the experiment, the cells were washed twice with PBS buffer and the medium was replaced with Opti-MEM (Reduced-Serum Medium is an improved Minimal Essential Medium (MEM) that allows for a reduction of Fetal Bovine Serum supplementation by at least 50% with no change of growth rate or morphology Biochrom GmbH, Germany). DCFH-DA solution (2.5 mM in DMF, 5 µL) was added (final concentration 25 µM) and incubated in the dark chamber filled with CO_2 (5%) at 37 °C for 10 min. Afterwards the supernatant was removed, the cells were washed with ice-cold PBS buffer and a fresh portion of Opti-MEM (500 µL) was added followed by the prodrugs and reference compounds (5 µL of stock solutions in DMF, final concentration of complexes - 25 µM, except of **8** for which a concentration of 50 µM was selected for better comparison). After 5 h of incubation in the dark chamber filled with CO_2 (5%) at 37 °C, the cells were detached from the culture dish using trypsin/EDTA (0.25% trypsin 100 µL), the medium was removed and the cells were re-suspended in Opti-MEM (150 µL). After that the fluorescence of live cells (λ_{ex} = 488 nm, λ_{em} = 530 nm) in the suspensions was determined by using flow cytometry (Figure 3 A, main text).

Cell cycle

Cells were seeded in a 6-well microtiter plate at a density of 350 cells/µL (total volume 2 mL) one day before the experiment in RPMI 1640 medium (5% FBS, 1% L-glutamine, 1% penicillin/streptomycin). They were allowed to attach overnight. On the next day the cells were washed twice with the PBS buffer and a fresh portion of RPMI 1640 medium

(5% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 2 mL) was added to each well. Solutions of prodrugs/drugs (20 µL in DMF) were added to achieve the final concentration of 2 µM of the prodrugs/drugs in the medium. The incubation time with the compounds was 36 h.

For the measurement of the cell cycle by flow cytometry the cells were detached from the surface by removing the medium, washing the cells with DPBS (2x2 mL) and adding 0.5 mL trypsin (0.05%)/ EDTA (0.02%) solution to each well. Next, they were incubated for 2 min at 22 °C, the trypsin/EDTA solution was removed and the dry cells were incubated for 3 min at 37 °C, 95% air humidity and 5% CO_2. Then, the cells were re-suspended in RPMI 1640 medium (5% FBS, 1% L-glutamine, 1% penicillin/streptomycin) (1 mL), transferred to 1.5 mL Eppendorf tubes and centrifuged for 3 min at 22 °C (1000 rcf). The supernatant was removed and the remaining cell pellet was washed with 0.5 mL DPBS, vortexed gently and centrifuged for 3 min at 22 °C (1000 rcf). The supernatant was removed, 0.4 mL DPBS (4 °C) was added and 0.8 mL pure, ice-cold ethanol was added dropwise over 1 min during gentle shaking of the tubes. The tubes were stored at least for 4 h at 4 °C in the fridge. Then, the tubes were centrifuged again for 3 min at 22 °C (1000 rcf), the supernatant was discarded and the cell pellet washed as previously described. Each cell pellet was re-suspended in 0.2 mL propidium iodide (PI) staining solution (20 µg RNase A, 10 µg PI in DPBS; Sigma-Aldrich) and incubated for 30 min at 37 °C, 95% air humidity and 5% CO_2. 0.2 ml DPBS were added to each tube. The probes were gently vortexed and the fluorescence of the cells was immediately measured via flow cytometry. For fluorescence detection of the nucleic acid dye PI the blue laser (λ_{ex}= 488 nm) was used for excitation and the yellow filter (583/26 nm) for detection of the fluorescence of DNA-bound PI (Figure 3 B, main text).

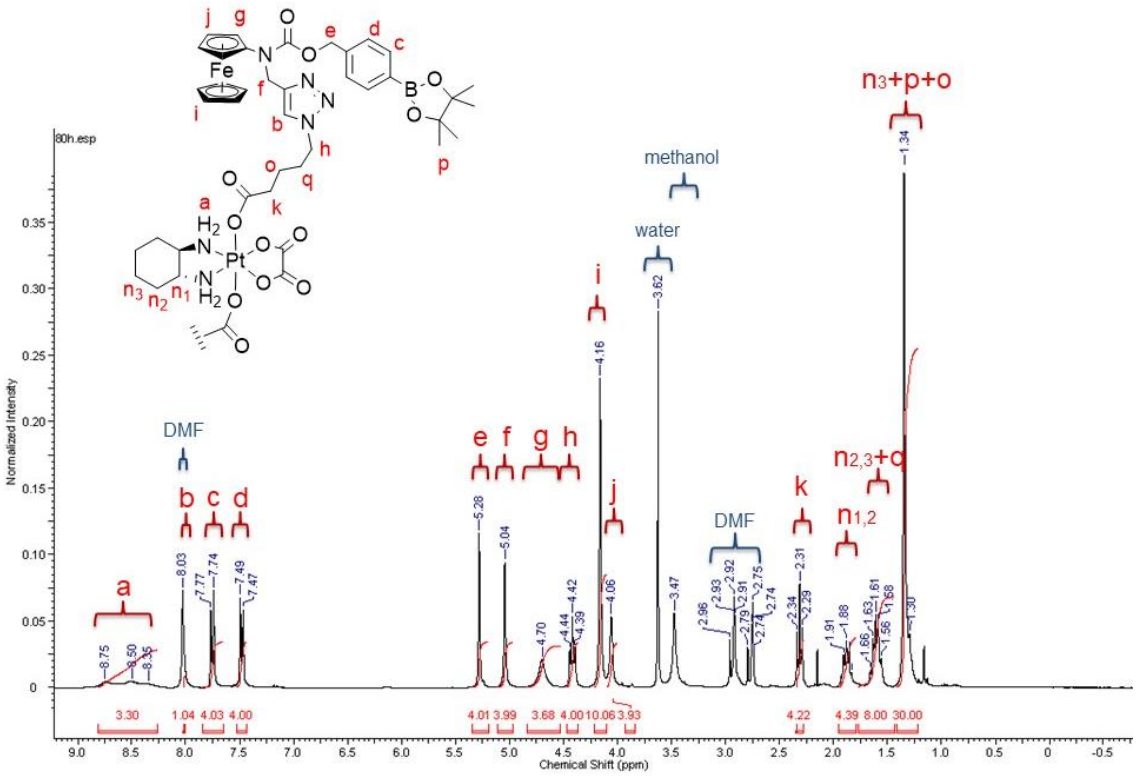


Figure S1. ^{1}H NMR spectrum of prodrug **2** in DMF-d7.

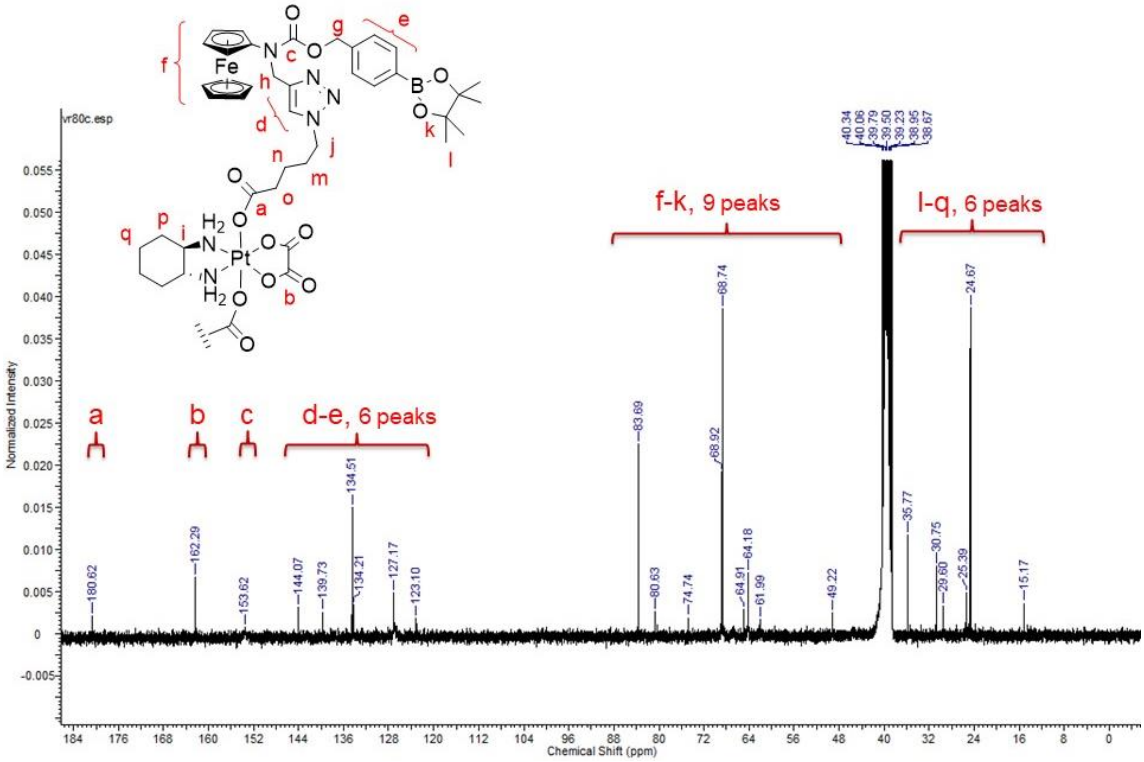


Figure S2. ^{13}C NMR spectrum of prodrug **2** in DMSO-d6.

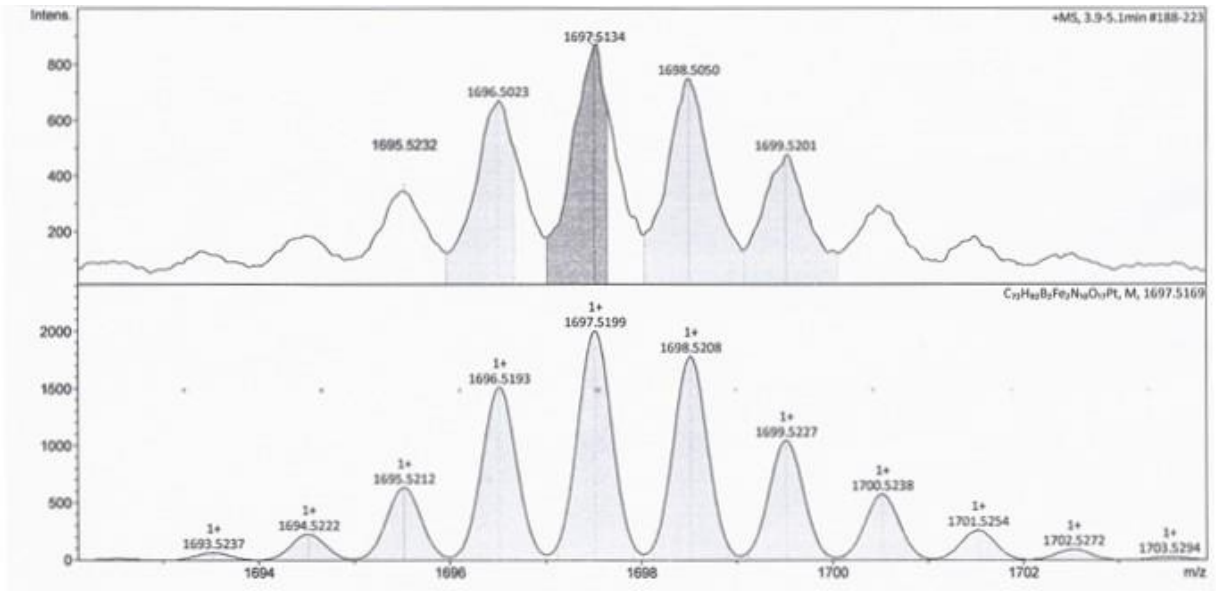


Figure S3. High resolution ESI-TOF mass spectrum of **2**: upper plot – experimental spectrum; bottom plot – theoretical spectrum.

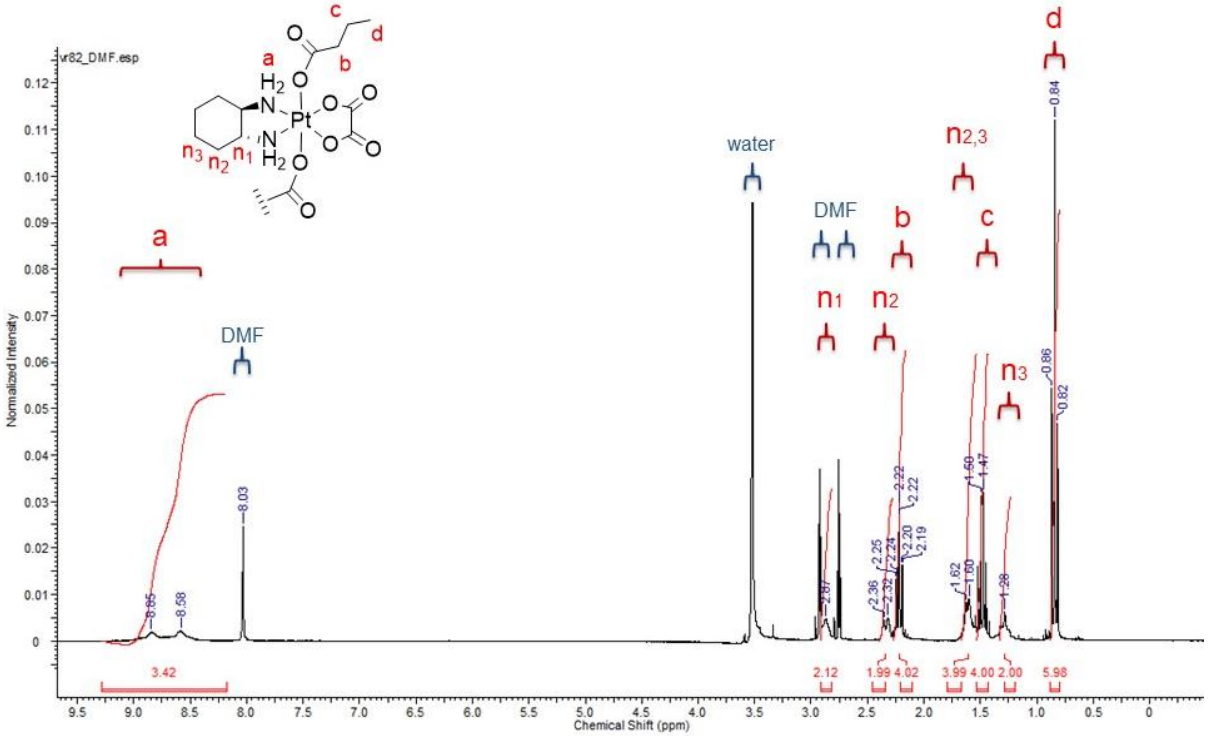


Figure S4. ^{1}H NMR spectrum of prodrug **7** in DMF-d7.

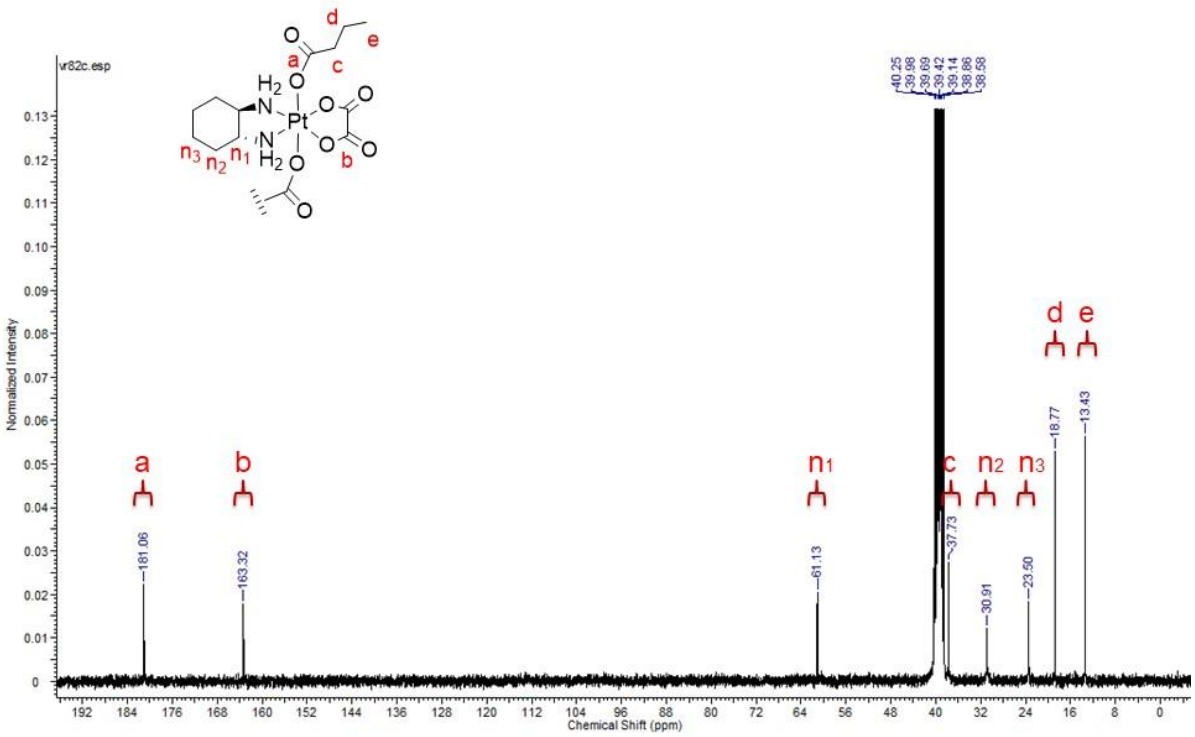


Figure S5. ^{13}C NMR spectrum of prodrug **7** in DMSO-d6.

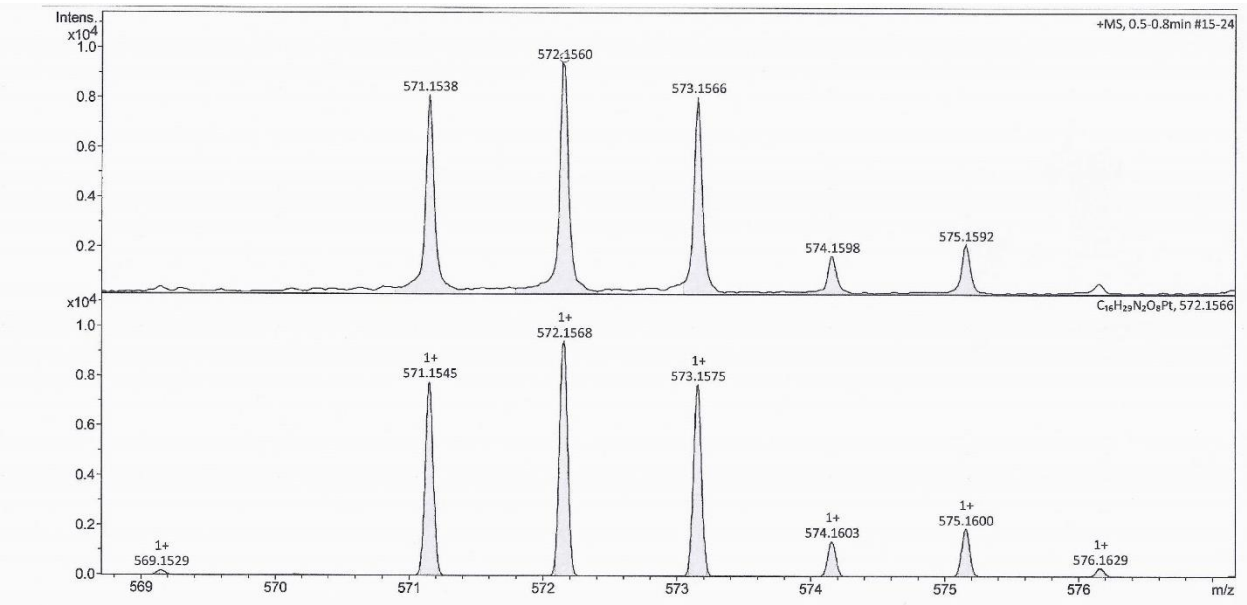


Figure S6. High resolution ESI-TOF mass spectrum of control **7**: upper plot – experimental spectrum; bottom plot – theoretical spectrum.

References

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