Coordination-Driven Self-Assembly of a Pt(IV) Prodrug-Conjugated Supramolecular Hexagon

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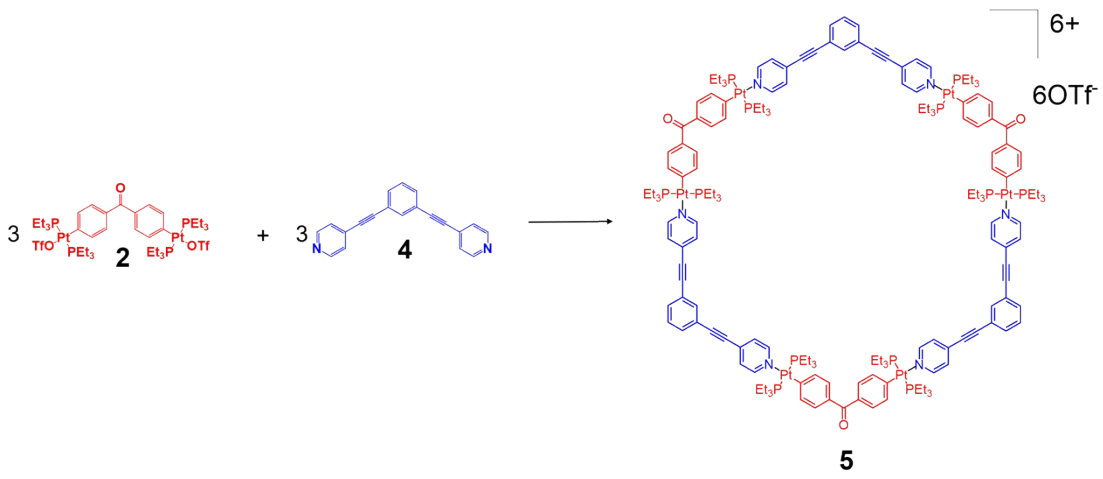
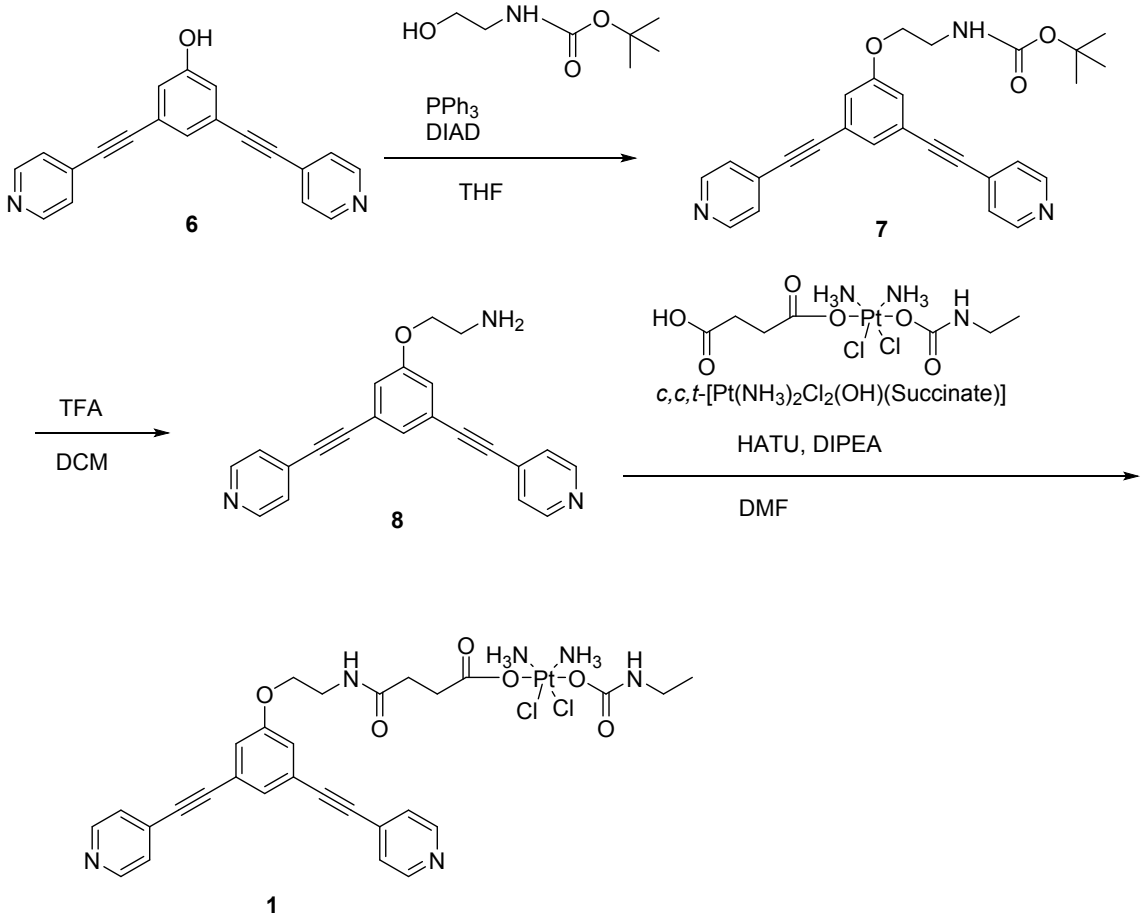
Experimental Section

General information. Compound **2**,[1] **4**,[2] **6**,[1] and *c,c,t*-[$Pt(NH_3)_2Cl_2(OH)$(Succinate)][3] were prepared according the reported literature. The chemical reagents used in this study were purchased from major chemical distributors including Sigma Aldrich, Acros Organic, Alfa Aesar, TCI America, and Matrix Scientific. A Bruker 400 NMR was used for NMR data acquisition (Frequency: 400 M Hz for ^{1}HNMR; 100MHz for ^{13}CNMR) and the plots were generated by TOPSPIN 3.2 software. For the NMR spectra, chemical shifts were given on the δ scale (ppm) and were referenced to the residual solvent signals; Coupling constants J were reported in hertz (Hz). The abbreviations s, d, t, q and m were used for singlet, doublet, triplet, quarlet and multiplet, respectively. For compound **1**, **5** and **6**, the high resolution mass spectra of created ions were recorded on an Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany). Mass spectra were recorded in the positive ionization mode with a scan range of 50–700 m/z, a mass resolving power setting of 140,000, and an automatic gain control (AGC) target value of 1×10^6 ions. To ensure very high mass accuracy (found to be better than 1 ppm) the instrument was mass calibrated daily and a lock mass of m/z 371.10124, due to polysiloxane, was used throughout. Graphite furnace atomic absorption spectroscopic (GFAAS) measurements were taken on a PerkinElmer PinAAcle 900Z spectrometer. Fluorescence images were acquired using an Olympus IX70 inverted epifluorescence microscope equipped with a digital CCD camera (QImaging). Images were processed and intensities were quantified with ImageJ software (NIH). 3D computational modeling was performed using Schrödinger Macromodel Suite based on MMFF force field. Flow cytometry was carried out on a BD Bioscience Accuri C6 flow cytometer. HPLC analysis was performed on Agilent 1100 HPLC. Elemental microanalysis was carried out via the service provided by Atlantic Microlab, inc.



Scheme S1. Coordination-driven self-assembly of the hexagon (**5**) that does not carry Pt(IV) prodrugs as a control for biological studies.



Scheme S2. Synthetic route for preparing the Pt(IV) prodrug-conjugated pyridyl linker **1**.

*Synthesis of tert-butyl 2-(3,5-dibromophenoxy)ethylcarbamate (**7**):* To a mixture of 3,5-bis[2-(4-pyridinyl)ethynyl]-phenol **5** (1.01 g, 3.40 mmol), PPh_3 (1.79 g, 6.83 mmol), and tert-butyl 2-hydroxyethylcarbamate (0.938 g, 5.82 mmol) in dry THF (120 mL), diisopropyl azodicarbonate (1.38 mL, 7.00 mmol) was added at 0 ºC under argon atmosphere. The reaction mixture was stirred at R. T. for 8 h. The reaction mixture was evaporated. The residue was purified by column chromatography on silica gel (gradient elution from DCM : MeOH = 100 : 0 to 100 : 4) to give the desired Compound **7** (0.860 g, 1.97 mmol, yield 58 %) as white powder. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.61(d, J = 3.2 Hz, 4 H), 7.37 (d, J = 4.8 Hz, 1.2 Hz, 4 H), 7.35 (t, J = 1.2 Hz, 4H), 7.08 (d, J = 1.6 Hz, 2H), 5.07 (s, 1H), 4.18 (t, J = 5.0 Hz, 2 H), 3.55 (d, J = 5.2 Hz, 2 H), 1.45 (s, 9 H); ^{13}C NMR ($CDCl_3$, 100 M Hz): δ 158.42, 155.87, 149.78, 131.01, 128.07, 125.59, 123.68, 118.71, 92.56, 87.30, 79.72, 67.61, 40.00, 28.40. HRMS (Positive mode) for $C_{27}H_{26}N_3O_3$: m/z $[M+H]^+$ calcd:440.1974; obsd:440.1969.

*Synthesis of 2-(3, 5-di (pyridin-4-yl)phenoxy)ethanamine (**8**)*. A mixture of tert-butyl 2-(3,5--bis[2-(4-pyridinyl)ethynyl]-phenoxy)ethylcarbamate (**7**) (0.810 g, 1.50 mmol) in trifluoroacetic acid (8 mL) and dichloromethane (16 mL) was stirred at r. t. for 24 h. The reaction mixture was evaporated under reduced pressure to give the desired Compound **8** (TFA salts, 1.21 g, 1.75 mmol, 95% yield) as green powder. ^{1}H NMR (DMSO-d_6, 400 M Hz) δ 8.82 (d, J = 5.2 Hz, 4 H), δ 8.12 (s, 3 H), 7.82 (dd, J = 4.8 Hz, 1.6 Hz, 4 H), 7.60 (t, J = 1.6 Hz, 1 H), 7.41 (d, J = 1.2 Hz, 2 H), 4.30 (t, J = 5.0 Hz, 2H), 3.27 (q, J = 5.1 Hz, 2H). ^{13} C NMR (DMSO-d_6, 100 MHz) δ 158.51, 147.43, 133.89, 128.73, 127.28, 123.20, 120.26, 95.20 87.49, 65.62, 38.68. HRMS (Positive mode) for $C_{22}H_{18}N_3O$: m/z $[M+H]^+$ calcd: 330.1450; obsd:340.1444.

*Syntheses of the Pt(IV) prodrug-conjugated pyridyl linker (**1**)*. The mixture of *c,c,t*-$[Pt(NH_3)_2Cl_2(OH)(Succinate)]$ (0.151g, 0.300 mmol) and HATU (0.118 g, 0.311 mmol) in anhydrous DMF (5.0 mL) was stirred at R. T. for 70 min to give a clear, yellow solution. To a suspension of Compound **8** (0.222 g, 0.322 mmol) in anhydrous DMF (4.0 mL) was added DIPEA (0.20 mL, 1.16 mmol) and stirred at R.T. for 60 min to give a clear, brown solution. Then the solution of Compound **8** was added dropwise to the above mixture. The obtained reaction mixture was stirred at R. T. for 1 hour, following by the addition of DIPEA (0.078 mL, 0.449 mmol). The reaction mixture was stirred at R.T. overnight. Then the reaction mixture was concentrated to about 1 mL under reduced pressure with rotary evaporator. This 1 mL solution was added dropwise into 35 mL ethyl ether under sonication to give a yellow suspension. After centrifugation, the obtained solid was washed with ethyl ether for three times, with water for four times and lyophilized. The obtained solid was suspended in methanol (23 mL), stirred at R. T. for 1 h and filtered. The filtrate was concentrated and dried over night to give the desired product **1** (0.112 g, 45 % yield) as yellow powder. ^{1}HNMR (DMSO-d6, 400 MHz): δ 8.66 (d, *J* = 6.0 Hz, 4 H), 8.10 (t, *J* = 5.6 Hz, 1 H), 7.56 (dd, *J* = 4.4 Hz, 1.6 Hz, 4 H), 7.44 (d, *J* = 1.4 Hz, 1H), 7.29 (d, J = 1.2 Hz, 2 H), 6.90-6.30 (m, 6 H), 4.10 (t, *J* = 5.6 Hz, 2 H), 2.94-2.92 (m, 2 H), 2.47 (t, *J* = 7.4 Hz, 2 H), 2.32 (t, *J* = 7.4 Hz, 2 H), 0.95 (t, *J* = 7.2 Hz, 3 H); ^{13}CNMR (DMSO-d6, 100 M Hz): δ 179.90, 171.86, 163.77, 158.55, 149.90, 129.94, 127.29, 125.51, 123.06, 119.04, 92.28, 87.36, 66.93, 38.07, 35.58, 31.40, 31.24, 15.46. HRMS (Positive mode) for $C_{29}H_{33}Cl_2N_6O_6Pt$: *m/z* [M+ H]+ calcd: 826.1486; obsd: 826.1483.

*Self-assembly of the Pt(IV) prodrug-conjugated supramolecular hexagon (**3**)*. The solution of **1** (12.42 mg, 0.015 mmol) in DMSO (1.8 mL), followed was added dropwise to the solution of **2** (20.11 mg, 0.015mmol) in DMSO (1.8 mL) under good stirring. The reaction mixture was stirred at R.T. for 2 h to give the desired Hexagon **3**. The DMSO was removed via lyophilization to yield **3** in solid (31.98 mg, 98 % yield). ^{1}H NMR (DMSO-d_6, 400 M Hz) δ 9.10-8.70 (s, br, 12 H), 8.20-8.10 (s, br, 3 H), 8.00-7.80 (s, br, 12 H), 7.80-7.30 (m, 33 H), 6.90-6.40 (m, 18 H), 4.13 (s, br, 6 H), 3.65-3.40 (s, br, 6 H), 2.95 (t, *J* = 6.6 Hz, 6 H), 2.48-2.42 (m, 6 H), 2.33 (t, *J* = 5.1 Hz, 6 H), 1.52-1.20 (m, 72 H), 1.20-0.83 (m, 117 H). ^{13}C NMR (DMSO-d_6, 100 M Hz): δ 195.68, 180.33, 164.26, 159.16, 152.97, 150.54, 143.18, 136.40,

132.99, 129.31, 127.88, 125.94, 122.90120.45, 119.54, 96.39, 86.82, 67.43, 36.05, 31.81, 29.48, 15.92, 13.59, 12.36, 7.90. $^{31}P\{^{1}H\}$ NMR: 13.61 ppm (d, $^{1}J_{Pt\text{-}P}$ = 2632 Hz). ESI-TOF-MS: m/z = 934.76 for [**3** – 6OTf]$^{6+}$, m/z = 1151.47 for [**3** – 5OTf]$^{5+}$, m/z = 1476.60 for [**3** – 4OTf]$^{4+}$, and m/z = 2018.44 for [**3** – 3OTf]$^{3+}$. Anal. Calcd for $C_{204}H_{300}Cl_6F_{18}N_{18}O_{39}P_{12}Pt_9S_6{\cdot}(H_2O)_9$: C, 36.76; H, 4.81; N, 3.78. Found: C, 36.57; H, 4.76; N, 3.64.

*Self-assembly of the control hexagon (**5**)*: The solution of **4** (4.8 mg, 0.0172 mmol) in DMSO (1.0 mL) was added dropwise to the solution of **2** (23.0 mg, 0.0172 mmol) in DMSO (1.0 mL) under good stirring. The reaction mixture was stirred at R.T. for 4 h. The DMSO was removed via lyophilization to give the desired Hexagon **5** (27.8 g, 100%). ^{1}H NMR (DMSO-d_6, 400 M Hz) δ 8.95-8.63 (s, br, 12 H), 8.06-8.00 (s, br, 3 H), 7.92-7.81 (m, br, 18 H), 7.78-7.38 (m, 27 H), 1.40-1.25 (m, 72 H), 1.20-1.00 (m, 108 H). ^{13}C NMR (DMSO-d_6, 100 M Hz): δ 195.12, 152.56, 142.73, 135.95, 134.90, 133.69, 132.90, 132.36, 130.08, 129.12, 128.86, 122.18, 121.47, 96.01, 87.09, 11.90, 7.96. ^{31}P NMR: 13.60 ppm. (d, $^{1}J_{Pt\text{-}P}$ = 2446 Hz) Anal. Calcd for $C_{177}H_{240}F_{18}N_6O_{21}P_{12}Pt_6S_6{\cdot}(H_2O)_9$: C, 42.29; H, 5.17; N, 1.67. Found: C, 42.02; H, 5.10; N, 1.62.

MTT Assays. Cytotoxicity profiles of **1**, **2**, the mixture of **1** and **2**, **3**, **5**, and cisplatin against different cell lines (A549, A2780, A2780cis, HT-29, MDA-MB-231 and SKOV-3) were evaluated by the MTT assay. Cells were seeded on a 96 well plate (2000 cells per well) in 200 μL RPMI or DMEM and incubated for 24 h at 37 °C. The following day, solutions of the platinum compounds were freshly prepared in PBS and quantitated by GFAAS. The cells were then treated with the platinum compounds, separately at varying concentrations, and incubated for 72 h at 37 °C. The cells were then treated with 30 μL fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) and incubated for 4 h at 37 °C. The medium was removed, 200 μL of DMSO was added to the cells, and the absorbance of the purple formazan was recorded at 565 nm using a BioTek Elx800 microplate plate reader. Each experiment was performed in triplicate for each cell line.

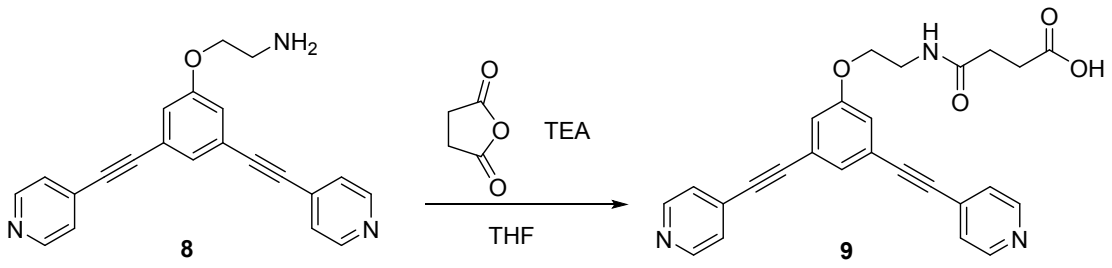
LIVE/DEAD Cell Viability Assay. Characteristic change in apoptotic cells treated with **3** was studied using the LIVE/DEAD cell viability assay (Molecular Probes) in A549 lung cancer cells. A549 cells were cultured on 35 mm sterile glass bottom culture dishes (MATTEK corporation) for 24 h at 37 °C. The cells were then treated with **3** (Pt] = 7 μM) or cisplatin (Pt] = 7 μM) for 48 h at 37 °C and 5% CO_2. Before the assay, cells were washed with 1 mL of PBS to remove serum esterase activity generally present in serum-supplemented growth media. Mix 20 uL Dead Red with 20 uL Live Green. The mixture was added to 10 mL PBS to produce a LIVE/DEAD working solution. A 2 mL aliquot of LIVE/DEAD working solution was carefully added to the petri dishes, which were then incubated at R.T. for 30 min. Subsequently, the medium of the samples was replaced with 1 mL of PBS before examination by fluorescence microscopy.

Cellular Uptake Experiment. One million A549 cells were seeded on 60 mm x 10 mm petri dishes and incubated for 24 h at 37 ^{0}C. These cells were then treated with cisplatin, **1–3**, and **5** ([Pt] = 50 μM) and subsequently incubated for 4 h at 37 °C. Media was then removed and cells were washed with PBS (2 x 1 mL), harvested by trypsinization (1 mL), and washed with 0.5 mL of DMEM. Solutions containing cells were centrifuged at 1400 rpm for 5 min at 4 °C and the platinum content in the whole cell was analyzed by GFAAS upon digestion in 200 μL 70% HNO_3. All experiments were performed in triplicate.

RNA Extraction, cDNA Synthesis, and qPCR. Total RNA was isolated using the ReliaPrep RNA Cell Miniprep System (Promega Corporation). cDNA was synthesized by qScript cDNA SuperMixsuper (Quanta Biosciences). 2 ng/μL of cDNA were mixed with PerfectCTa SYBR Green SupermixPower SYBR Green PCR master mix (Quantabio) and 5 pmol of both

forward and reverse primers (p21 forward: 5'-GAG GCC GGG ATG AGT TGG GAG GAG-3'; p21 reverse: 5'- CAG CCG GCG TTT GGA GTG GTA GAA-3'; p53 forward: 5'- CCC CTC CTG GCC CCT GTC ATC TTC-3'; p53 reverse: 5'- GCA GCG CCT CAC AAC CTC CGT CAT-3'; BAX forward: 5'- TGG AGC TGC AGA GGA TGA TTG-3'; BAX reverse: 5'- GAA GTT GCC GTC AGA AAA CAT G-3'; Apaf-1 forward: 5'- TGC GCT GCT CTG CCT TCT-3'; Apaf-1 reverse: 5'- CCA TGG GTA GCA GCT CCT TCT-3'; GAPDH forward: 5'- ACC ACA GTC CAT GCC ATC AC-3'; GAPDN reverse: 5'- TCC ACC ACC CTG TTG CTG TA-3'). GAPDH was amplified as an internal control. All qPCR performed using SYBR Green was conducted at 95°C for 10 min, and then 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 20 s. The specificity of the reaction was verified by melt curve analysis. The threshold crossing value was noted for each transcript and normalized to the internal control. The relative quantitation of each mRNA was performed using the comparative Ct method. Experiments were performed using a realplex^2 (Eppendorf Mastercycler).

Flow Cytometric Analysis. A549 cells were incubated with or without the test compounds (Pt concentration was 7 μM in both **3** and cisplatin) for 24-72 h at 37 °C. Cells were harvested from adherent cultures by trypsinization. Following centrifugation at 1500 rpm for 5 min, cells were washed with PBS. For the apoptosis experiments, annexin V-FITC early apoptosis detection kit was used. The manufacture's protocol was followed to perform this experiment. Briefly, untreated and treated cells (1×10^5) were suspended in 1× annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), then 5 μL FITC annexin V and 5 μL PI (10 μg/ mL) were added to each sample, gently vortexed and incubated at room temperature in the dark for 15 min, after which, more binding buffer (400 μL) was added. The samples were analysed with the Accuri flow cytometer and 20,000 events per sample were acquired. Cell populations were analyzed using the FlowJo software. For the ɣH2AX analysis, all samples were resuspended in BD fixation/Permeabilization solution (250 μL) for 20 minutes at 4 C. Then the supernatant was removed, the samples were resuspended with BD Perm/Wash Buffer (1 mL) and centrifuged. Added 10% (in volume) of BD Alexa 488-anti ɣH2AX antibody solution and incubated cells in dark for 60 minutes at room temperature. The cells were resuspended with PBS (200 μL) and analyzed by Accuri flow cytometer.



Scheme S3. Synthetic route for preparing the reduction product **9**.

*Synthesis of Compound **9***. The mixture of Compound **8** (0.0186 g, 0.0225 mmol) and TEA (0.5 mL) in anhydrous THF (1.0 mL) was stirred at R. T. for 20 min to give a clear, yellow solution, following by the addition of succinic anhydride (0.0068 g, 0.0675 mmol). The reaction mixture was stirred at R.T. for 21 h. Then the reaction mixture was concentrated to dryness. The residue was re-dissolved in THF (0.3 mL), followed by the addition of water (5.0 mL) under sonication to give a white suspension. The suspension was extracted with DCM for 3 times, dried with anhydrous Na_2SO_4 and filtered. The filtration was concentrated under reduced vacuum with rotavapor. The obtained solid was washed with acetone (1.0 mL) to give the desired product **9** (0.0031 g, 15 % yield) as yellow powder. ^{1}H NMR (DMSO-d^6,

400 MHz): δ 8.65 (dd, *J* = 4.4 Hz, 1.6Hz, 4 H), 8.12 (t, *J* = 5.4 Hz, 1 H), 7.55 (dd, *J* = 4.4 Hz, 1.6 Hz, 4 H), 7.45 (t, *J* = 1.4 Hz, 1H), 7.30 (d, J = 0.8 Hz, 2 H), 4.09 (t, *J* = 5.6 Hz, 2 H), 3.42 (t, *J* = 5.6 Hz, 2 H), 2.43 (t, *J* = 6.2 Hz, 2 H), 2.35 (t, *J* = 7.0 Hz, 2 H); ^{13}CNMR (DMSO-d^6, 100 M Hz): δ 173.85, 171.37, 158.57, 150.06, 129.77, 127.29, 125.45, 123.09, 119.04, 92.15, 87.38, 67.00, 45.72, 29.91,29.08; HRMS (Positive mode) for $C_{26}H_{22}N_3O_4$: *m/z* $[M+H]^+$ calcd: 440.1610; obsd: 440.1605.

*Reduction of the Pt(IV) prodrug-conjugated pyridyl linker (**1**).* Ligand **1** (0.2 mg, 0.25 µmol) was dissolved in 10 µL DMSO and diluted with ddwater 490 µL (previously degassed via sonication for 3 h). Then L(+) ascorbic acid (0.9 mg, 5.0 µmol, 20 eq.) was added. The obtained reaction mixture was stirred at 37 ºC for 15 h and submitted for HPLC analysis. Below is the HPLC gradient method: ("A" solvent is ddwater, and "B" solvent is acetonitrile. The C18 reversed phase column used is a 100x3mm Thermo Scientific Hypersil GOLD HPLC Column. Detection wavelength is 240 nm.) 0-5 min: 2% B; 5-20 min: 2%→98% B; 20-25 min: 98% B; 25-30 min: 98%→2% B. 30-31 min: 2% B. Standard samples of Ligand **1** and the reduction product (**9**) were also employed in HPLC analysis for comparison.

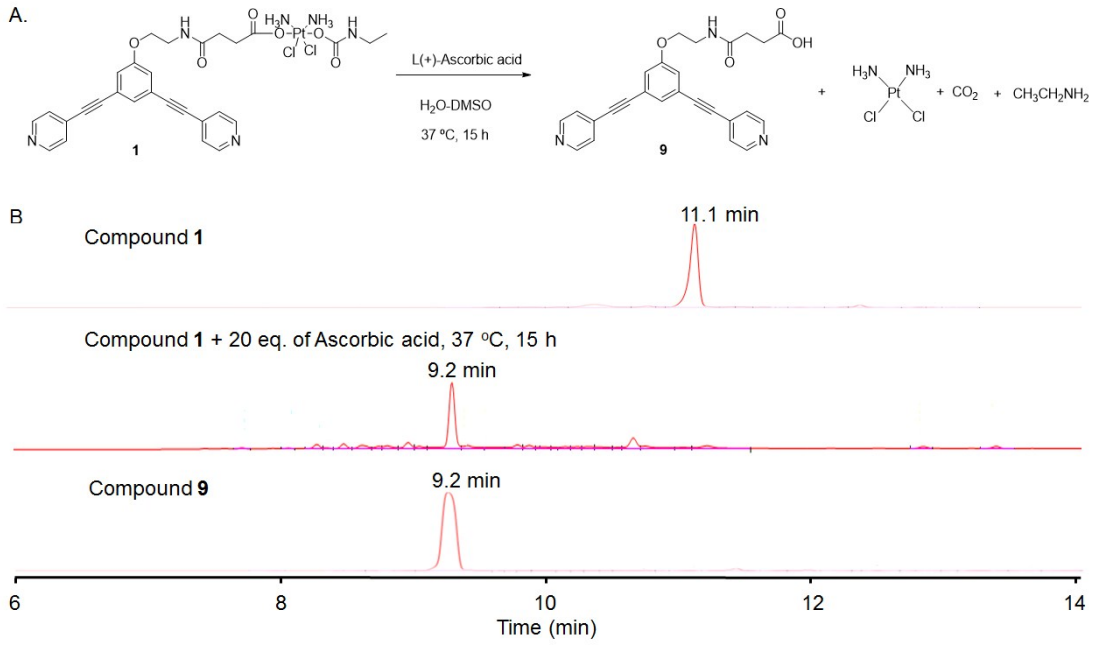


Figure S1. The reduction reaction of Ligand **1** (A) and the corresponding HPLC analysis (B).

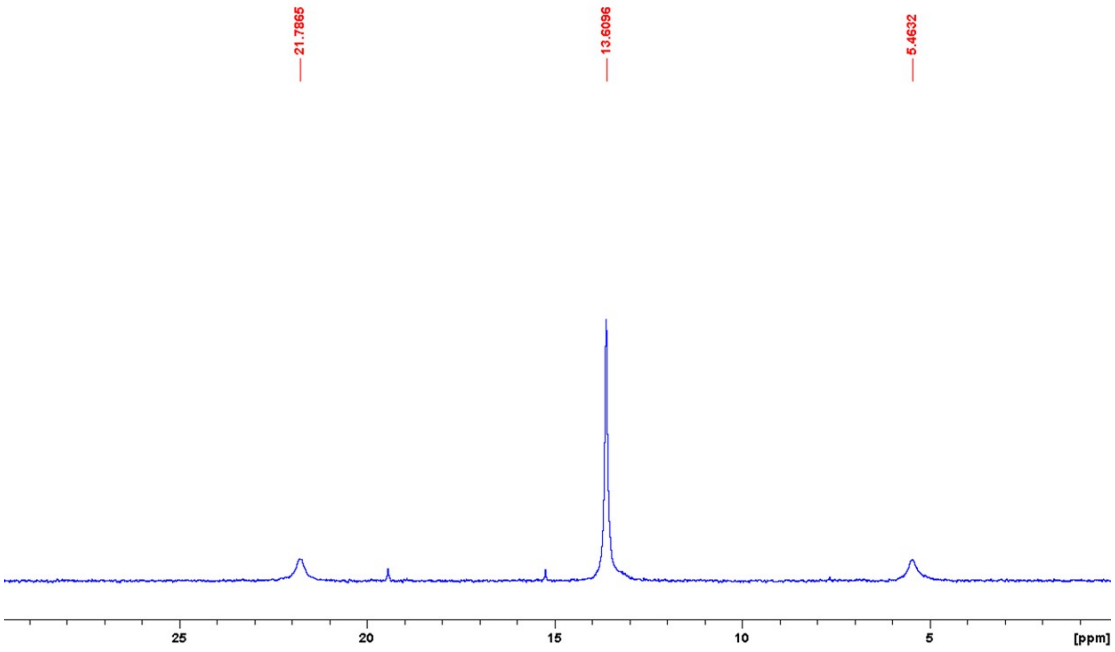


Figure S2. $^{31}P\{^{1}H\}$ NMR spectrum of the supramolecular hexagon (**3**) in DMSO-d_6.

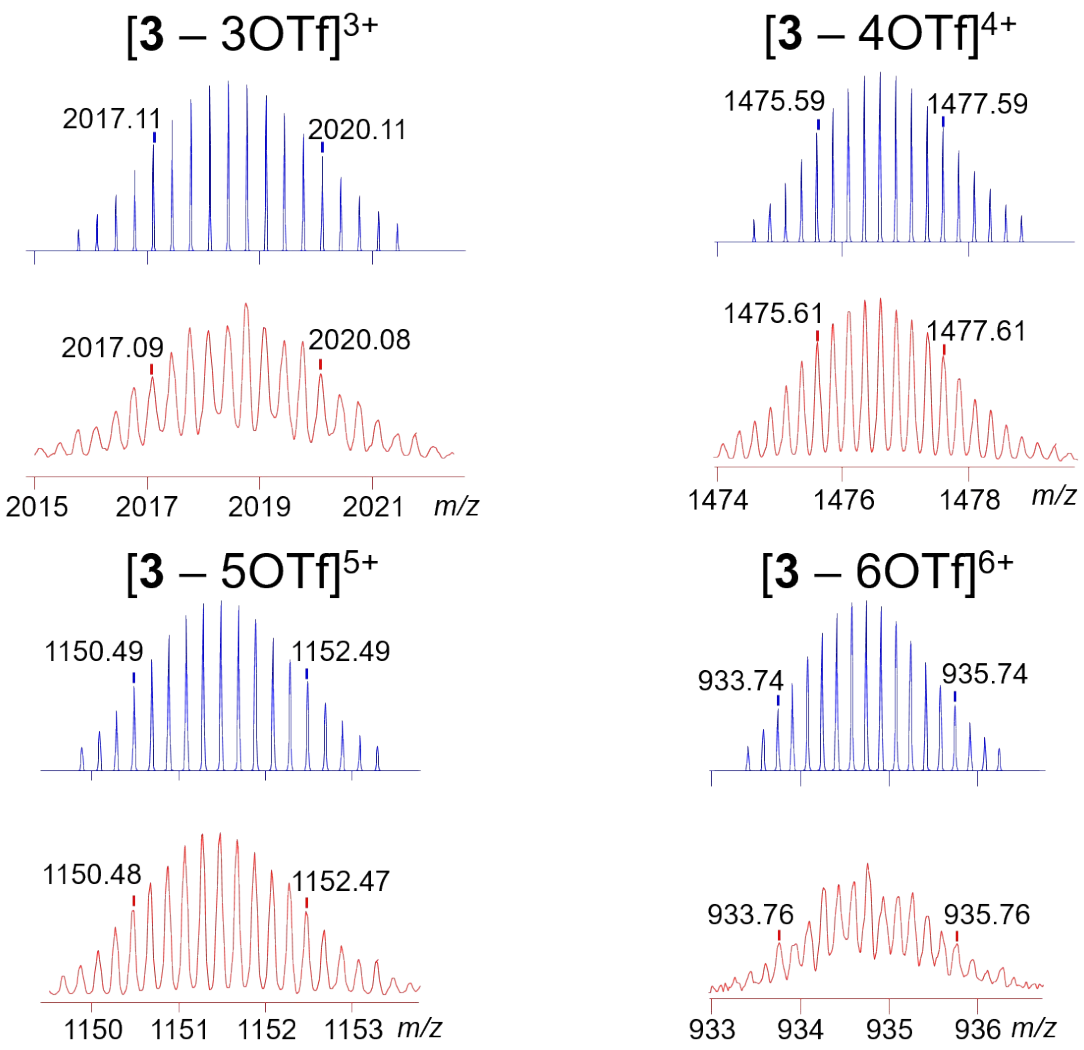


Figure S3. Isotopically resolved ESI-MS spectra of **3**.

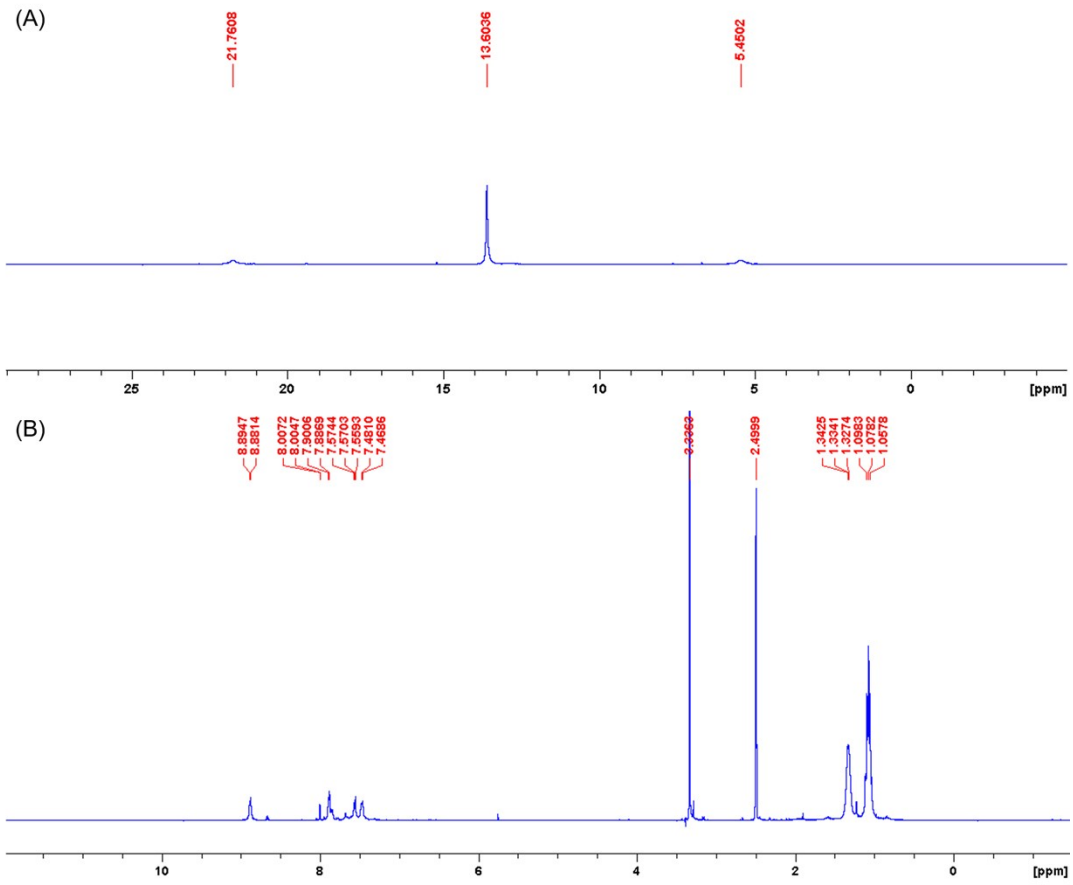


Figure S4. $^{31}P\{^{1}H\}$ and ^{1}H NMR spectrum of the control hexagon (**5**) in DMSO-d_6.

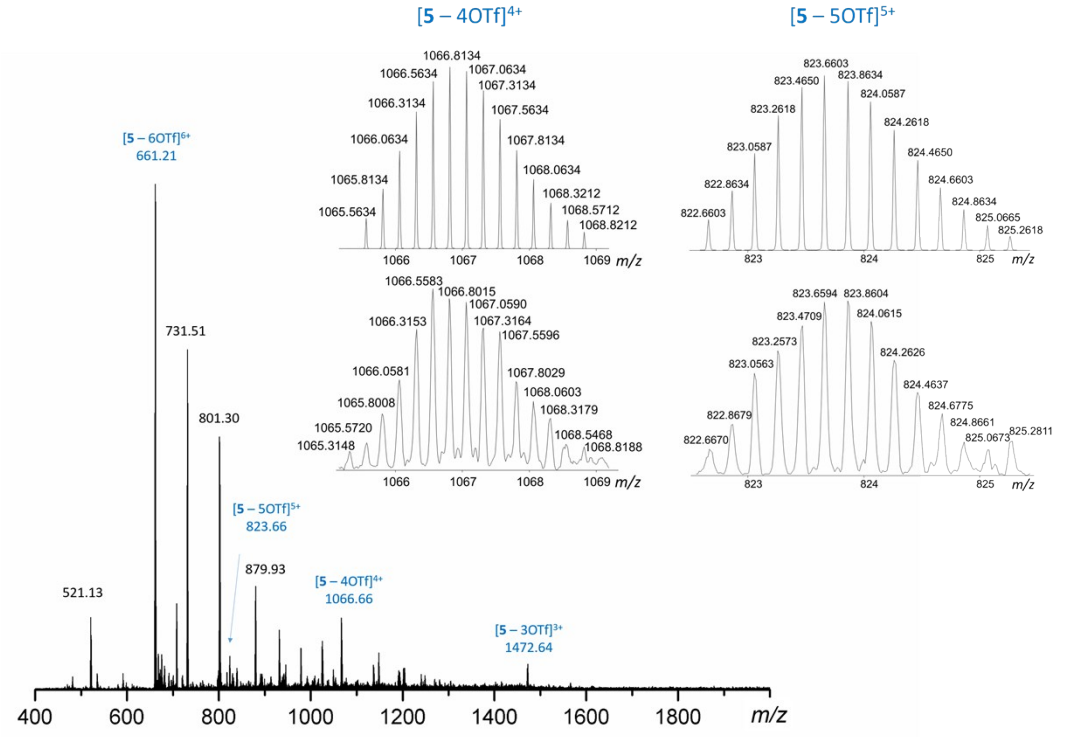


Figure S5. Full and isotopically resolved (top: theoretical distributions; bottom: experimental data) ESI-MS spectra of **5**.

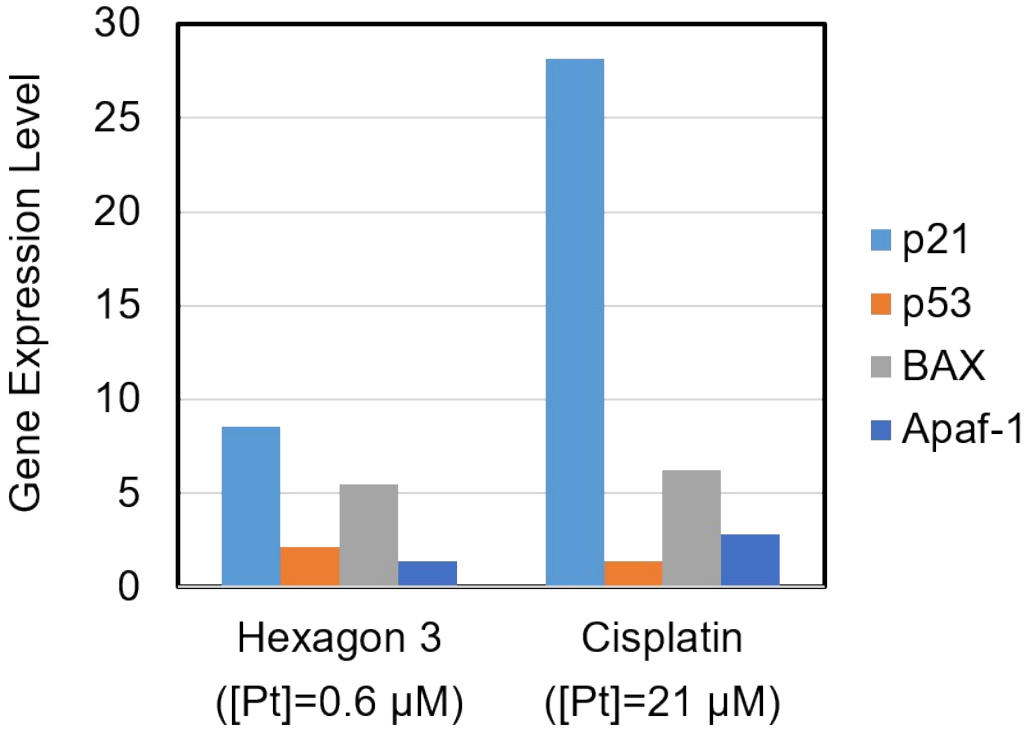


Figure S6. RT-PCR analysis of gene expression of p21, p53, BAX, and Apaf-1 in A549 cells with treatment of Hexagon **3** ([Pt] = 0.6 μM for 48 h) or cisplatin (21 μM for 48 h).

References:

1. Yang, H.-B.; Das, N.; Huang, F.; Hawkridge, A. M.; Muddiman, D. C.; Stang, P. J. *J Am. Chem. Soc.,* **2006**, *128*, 10014-10015.

2. Kikuchi, T.; Murase, T.; Sato, S.; Fujita, M. *Supramol. Chem.* **2008**, *20*, 81-94

3. Zheng, Y. R.; Suntharalingam, K.; Johnstone, T. C.; Yoo, H.; Lin, W.; Brooks, J. G.; Lippard, S. J. *J. Am. Chem. Soc.* **2014**, *136*, 8790−8798.