Electronic Supplementary Information for Analyst

Electrochemical analysis of novel ferrocene derivative as a potential antitumor drug

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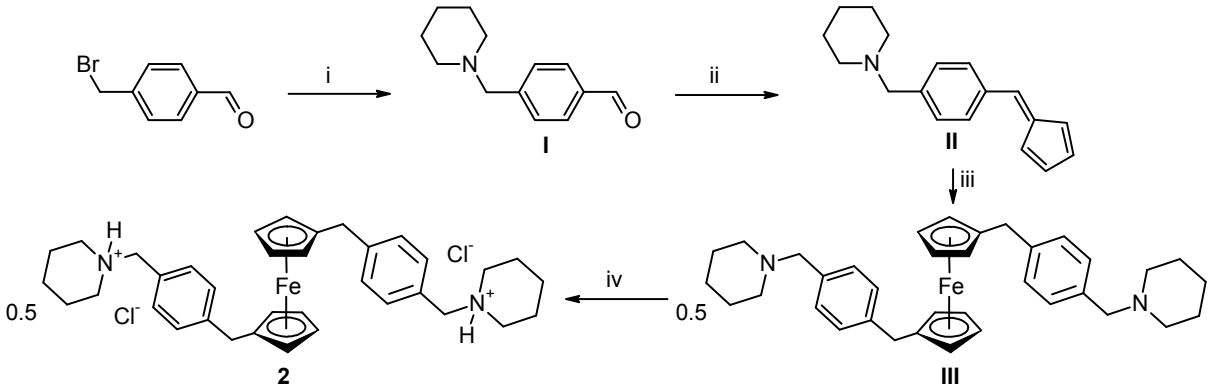
Material

Manipulation of air sensitive compounds was carried out under an argon atmosphere using standard Schlenk techniques. Tetrahydrofuran (THF) was purified by a solvent drying system (Innovative Technology, Inc.) and stored over molecular sieve 3Å. Anhydrous $FeCl_2$ (99.99%), $LiBEt_3H$ (1M solution in THF), hydrogen chloride (1 M solution in diethyl ether), MTT and protease inhibitor cocktail, sodium phosphate monobasic and sodium phosphate dibasic were obtained from Sigma-Aldrich. 4-(bromomethyl)-benzaldehyde and ferrocene **3** were prepared according to the literature.[1,2]

Synthesis and characterization of ferrocene derivative 2

Scheme S1



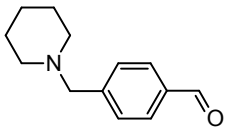
[a] Reagents and conditions: (i) piperidine, K_2CO_3, MeCN; (ii) cyclopentadiene, pyrrolidine, MeOH; (iii) $LiBEt_3H$, THF, then 0.5 eq. $FeCl_2$, THF; (iv) HCl, THF.

Characterization methods

^1H (300.0 MHz) and ^{13}C (75.4 MHz) NMR spectra were recorded on a Varian Mercury 300 spectrometer at 25 °C. Chemical shifts (δ/ppm) are given relative to solvent signals (δ_H/δ_C: $CDCl_3$, 7.26/77.16 ppm; Toluene-d_8; 2.08/137.48; DMSO-d_6, 2.50/39.52). FT-IR spectra of samples in KBr pellets were measured on a Nicolet Avatar FTIR spectrometer over the range 400-4000 cm-1. Electrospray mass spectra (ESI-MS) were measured with a Bruker Esquire 3000 instrument for the complexes dissolved in dichloromethane diluted with large excess of acetonitrile. Melting points were determined on a Koffler block. Elemental analyses were carried out on a FLASH EA1112 CHN-O Automatic Elemental Analyzer (Thermo Scientific). Both organometallic complexes **2** and **III** were characterized by above mentioned spectroscopic methods (^1H and ^{13}C NMR , IR, ESI-MS) and in addition, melting points and elemental analyses were determined. An increased polarity of **2** in comparison to **III** led to its higher solubility in polar solvents (DMSO, water), which was found advantageous in evaluation of biological properties of the complex.

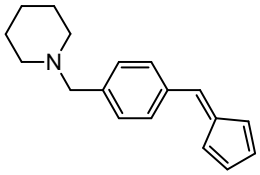
Individual steps during synthesis

4-[(piperidin-1-yl)methyl]-benzaldehyde (**I**)



Anhydrous potassium carbonate (3.4 g, 24.6 mmol) was added to a solution of 4-(bromomethyl)-benzaldehyde[2] (2.13 g, 10.7 mmol) in acetonitrile (33 mL) and the resulting mixture was stirred for 1 h. Piperidine (3 mL, 30.4 mmol) was then added and stirring continued at room temperature overnight. GC/MS indicated consumption of the starting 4-(bromomethyl)-benzaldehyde. The reaction mixture was diluted with water and extracted with diethyl ether (3×). The extracts were combined, dried (Na_2SO_4) and concentrated. Chromatography in ethyl acetate-ethanol 10:1 yielded syrupy 4-[(piperidin-1-yl)methyl]-benzaldehyde **1** (2.025 g, 93 %) which slowly crystallized when left in the refrigerator, Mp 48-49 °C, NMR data agree with those reported for **I** prepared by a different method.[3]

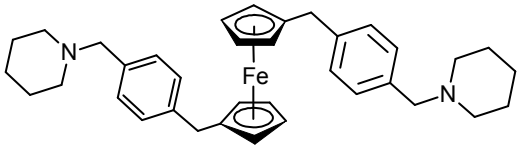
6-[(piperidin-1-yl-methyl)-fenyl]-fulvene (**II**)



Freshly cracked cyclopentadiene (1.68 mL, 20.0 mmol) was added to a stirred solution of 4-[(piperidin-1-yl)methyl]-benzaldehyde **I** (1.952 g, 9.6 mmol) in methanol (11 mL). Pyrrolidine (1.20 mL, 14.4 mmol) was added immediately under stirring. The reaction mixture turned red in about 20 min. Stirring continued at room temperature for 4 h whereupon GC/MS indicated consumption of the starting aldehyde. Glacial acetic acid (1.75 mL, 30.6 mmol) was added to quench the reaction. After 15 min the reaction mixture was alkalized with aqueous 5 M NaOH, diluted with water and extracted with ethyl acetate (5×). The combined extracts were dried (Na_2SO_4) and concentrated to give crude product (2.398 g). Chromatography in ethyl acetate yielded 6-(piperidin-1-yl-methyl-fenyl)-fulvene **II** (1.998 g,

83 %) as a red syrup which slowly crystallized when left in the refrigerator. Mp 47-51 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.45 (dd, $^3J_{HH}$ = 10.9 Hz, $^3J_{HH}$ = 5.0 Hz, 2H, $NCH_2CH_2CH_2$); 1.55-1.62 (m, 4H, $NCH_2CH_2CH_2$); 2.37-2.41 (m, 4H, $NCH_2CH_2CH_2$); 3.49 (s, 2H, $NCH_2C_6H_4$); 6.33 (dt, $^3J_{HH}$ = 5.2 Hz, $^3J_{HH}$ = 1.8 Hz, J_{HH} = 1.8 Hz, 1H, C_5H_4); 6.51 (ddd, $^3J_{HH}$ = 5.2 Hz, $^3J_{HH}$ = 2.0 Hz, J_{HH} = 1.2, Hz, J_{HH} = 2.0 Hz, J_{HH} = 0.6 Hz, 1H, C_5H_4); 6.66 (ddt, $^3J_{HH}$ = 5.3 Hz, $^3J_{HH}$ = 2.1 Hz, $^4J_{HH}$ = 1.5, Hz, J_{HH} = 1.5 Hz, 1H, C_5H_4); 6.72 (ddd, $^3J_{HH}$ = 5.3 Hz, $^3J_{HH}$ = 2.0 Hz, J_{HH} = 1.2, Hz, J_{HH} = 0.6 Hz, 1H, C_5H_4); 7.20-7.21 (m, 1H, $C_5H_4CHC_6H_4$); 7.37, 7.55 (2 × d, $^3J_{HH}$ = 8.1 Hz, 2 × 2H, C_6H_4). ^{13}C NMR (75 MHz, $CDCl_3$): 24.49 ($NCH_2CH_2CH_2$); 26.16 ($NCH_2CH_2CH_2$); 54.73 ($NCH_2CH_2CH_2$); 63.66 ($NCH_2C_6H_4$); 120.42, 127.39 (C_5H_4, *C*H); 129.56 (C_6H_4, *C*H); 130.66 (C_5H_4, *C*H); 130.71 (C_6H_4, *C*H); 135.36 (C_5H_4, *C*H); 135.53 (C_6H_4, C_{ipso}); 138.42 ($C_5H_4CHC_6H_4$); 140.38 (C_6H_4, C_{ipso}); 144.94 (C_5H_4, C_{ipso}). LRMS (EI): *m*/z 251 (M^+ 100%), 250 (69%), 167 (68%), 165 (67%), 152 (60%). The product is somewhat unstable and should be stored at −30 °C and used within weeks.

ferrocene **III**

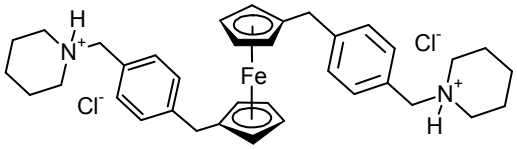


1M solution of $LiBEt_3H$ (2.20 mL, 2.20 mmol) in THF was dropped to a cold (0 ºC) solution of fulvene **II** (0.554 g, 2.21 mmol) in THF (20 mL), which caused the mixture to change color gradually from orange to yellow. The reaction mixture was allowed to warm to a room temperature and stirred for 2h. Solid $FeCl_2$ (0.140 g, 1.10 mmol) was added and the resulting mixture was stirred for 3 days. The formed dark orange mixture was filtered through a small (1.5 × 10 cm) silica column and the column was washed with additional THF (10 mL). The collected THF fractions were evaporated to dryness and the obtained orange solid was washed with methanol (3 × 3 mL). Recrystallization from THF/methanol mixture gave **III** as a yellow solid. Yield was 0.283 g (46 %).

Mp 118-120 ºC. ^{1}H NMR (300 MHz, $CDCl_3$): 1.34-1.46 (m, 4H, $NCH_2CH_2CH_2$); 1.50-1.60 (m, 8H, $NCH_2CH_2CH_2$); 2.28-2.42 (m, 8H, $NCH_2CH_2CH_2$); 3.42 (s, 4H, $NCH_2C_6H_4$); 3.63 (s, 4H, $C_5H_4CH_2C_6H_4$); 4.03 (s, 8H, C_5H_4); 7.11, 7.20 (2 × d, $^3J_{HH}$ = 8.1 Hz, 2 × 4H, C_6H_4). ^{1}H

NMR (300 MHz, Toluene-D_8): 1.33 (dd, $^3J_{HH}$ = 10.7 Hz, $^3J_{HH}$ = 4.1 Hz, 4H, $NCH_2CH_2CH_2$); 1.40-1.56 (m, 8H, $NCH_2CH_2CH_2$); 2.20-2.32 (m, 8H, $NCH_2CH_2CH_2$); 3.32 (s, 4H, $NCH_2C_6H_4$); 3.51 (s, 4H, $C_5H_4CH_2C_6H_4$); 3.88-3.93 (m, 8H, C_5H_4); 7.11, 7.28 (2 × d, $^3J_{HH}$ = 8.0 Hz, 2 × 4H, C_6H_4). ^{13}C NMR (75 MHz, Toluene-D_8): 25.01 ($NCH_2CH_2CH_2$); 26.57 ($NCH_2CH_2CH_2$); 35.91 ($C_5H_4CH_2C_6H_4$); 54.88 ($NCH_2CH_2CH_2$); 63.95 ($NCH_2C_6H_4$); 68.65, 69.74 (C_5H_4, CH); 88.41 (C_5H_4, C_{ipso}); 128.60, 129.14 (C_6H_4, CH); 137.34 (C_6H_4, C_{ipso}); 140.61 (C_6H_4, C_{ipso}). ESI-MS, *m/z* (ESI$^+$): 561 ([M + H]$^+$), 476, 393. IR (KBr, cm^{-1}): 3073 (w), 3046 (w), 3020 (w), 2961 (m), 2930 (sh, s), 2915 (s), 2885 (m), 2849 (m), 2791 (s), 2750 (s), 2716 (m), 2677 (w), 1906 (vw), 1635 (br, vw), 1511 (m), 1439 (sh, m), 1431 (m), 1419 (w), 1389 (w), 1366 (m), 1341 (m), 1317 (w), 1294 (m), 1268 (m), 1193 (vw), 1152 (m), 1113 (m), 1093 (w), 1038 (m), 1021 (m), 995 (m), 961 (vw), 920 (w), 861 (m), 844 (m), 821 (m), 786 (w), 762 (m), 748 (m), 689 (m), 584 (w), 551 (w), 518 (s), 485 (m), 472 (m), 422 (w). Anal. calculated for $C_{36}H_{44}FeN_2$ (560.58): C, 77.13; H, 7.91; N, 5.00. Found: C, 77.28; H, 8.03; N, 4.94.

ferrocene bis(hydrochloride) **2**



To a vigorously stirred ferrocene **III** (0.083 g, 0.15 mmol) solution in THF (3 mL) a 1M solution of hydrogen chloride (0.500 mL, 0.50 mmol) in diethyl ether was dropped, which caused an immediate precipitation of a solid. The solid was isolated, washed with THF (3 × 2 mL) and dried in vacuum to obtain **2** as a yellow powder. Yield was 0.090 g (95 %).

Mp 150 ºC (dec.). ^{1}H NMR (300 MHz, DMSO-D_6): 1.23-2.40 (m, 2H, $NCH_2CH_2CH_2$); 1.60-1.81 (m, 10H, $NCH_2CH_2CH_2$); 2.68-2.87 (m, 4H, $NCH_2CH_2CH_2$); 3.21 (d, $^2J_{HH}$ = 10.8 Hz, 4H, $NCH_2CH_2CH_2$); 3.65 (s, 4H, $C_5H_4CH_2C_6H_4$); 4.06, 4.11 (2 × pseudo t, 2 × 4H, C_5H_4); 4.16 (d, $^3J_{HH}$ = 3.9 Hz, 4H, $NCH_2C_6H_4$); 7.25, 7.48 (2 × d, $^3J_{HH}$ = 7.8 Hz, 2 × 4H, C_6H_4); 10.54 (br s, 2H, NH). ^{1}H NMR (300 MHz, $CDCl_3$): 1.13-1.40 (m, 2H, $NCH_2CH_2CH_2$); 1.59-1.87 (m, 6H, $NCH_2CH_2CH_2$); 2.03-2.28 (m, 4H, $NCH_2CH_2CH_2$); 2.43-2.65 (m, 4H $NCH_2CH_2CH_2$); 3.21-3.40 (m, 4H, $NCH_2CH_2CH_2$); 3.57 (s, 4H, $C_5H_4CH_2C_6H_4$); 3.91-4.05 (m, 12H, C_5H_4 and $NCH_2C_6H_4$); 7.12, 7.44 (2 × d, $^3J_{HH}$ = 7.8 Hz, 2 × 4H, C_6H_4). ^{13}C NMR

(75 MHz, $CDCl_3$): 21.98, 22.47 ($NCH_2CH_2CH_2$); 35.52 ($C_5H_4CH_2C_6H_4$); 52.49 ($NCH_2CH_2CH_2$); 60.53 ($NCH_2C_6H_4$); 68.54, 69.34 (C_5H_4, *C*H); 87.18 (C_5H_4, C_{ipso}); 125.83 (C_6H_4, CCH_2N); 129.00, 131.40 (C_6H_4, *C*H); 143.57 (C_6H_4, $CCH_2C_4H_4$). ESI-MS, *m/z* (ESI^+): 597 ($[M - Cl]^+$), 561 ($[M - Cl - HCl]^+$), 281. IR (KBr, cm^{-1}): 3074 (w), 3031 (w), 3009 (w), 2947 (s), 2862 (m), 2706 (m), 2673 (m), 2625 (m), 2495 (ν(N-H), br, vs), 1614 (w), 1515 (m), 1456 (s), 1429 (s), 1401 (m), 1374 (w), 1327 (w), 1275 (vw), 1218 (w), 1197 (w), 1135 (vw), 1080 (vw), 1062 (w), 1038 (sh, m), 948 (m), 914 (m), 856 (m), 826 (sh, m), 769 (vw), 732 (vw), 700 (vw), 589 (w), 486 (m), 451 (vw). Anal. calculated for $C_{36}H_{46}Cl_2FeN_2$ (633.50): C, 68.25; H, 7.32; N, 4.42. Found: C, 68.07; H, 7.43; N, 4.31.

Cell cultures

Ovarian cancer cell lines A2780 and cisplatin-resistant A2780cis from European Collection of Cell Cultures were maintained in RPMI 1640 medium whereas breast cancer cell line MCF-7 from ATCC was maintained in DMEM. Both media were supplemented with 10% fetal bovine serum (BIOCHROM AG; Berlin, Germany), 100 U/ml penicillin and 0,1 mg/mL streptomycin (Hyclone Laboratories, Utah, USA) and L-glutamine 2 mM (Gibco) in humidified incubator at 37 °C in 5% CO_2 atmosphere. Moreover, each third passage of A2780cis cells was incubated in 1 μM cisplatin containing-medium.

MTT assay

Cytotoxicity was determined using colorimetric assay based on conversion of yellow tetrazole, 3,(4,4-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), to a purple formazan, reflecting mitochondrial activity in living cells. First, A2780 ovarian cancer cells were seeded into 96-well plates at density of 10,000 cells per well and treated with various concentrations of ferrocene derivatives for 24 h. Then, 20 μL of 2.5 mg/mL MTT dissolved in PBS was added into each well and incubated for 3 h at 37 ºC. Resulting formazan crystals were dissolved in 50 μL of DMSO and optical density was measured at 595 nm using Tecan Infinite® 200 PRO series spectrophotometer. The IC_{50} values were determined using GraphPadPrism software version 5.03.

Sample preparation for ferrocene measurement in whole cell lysate

Selected cancer cells were treated with compound **1**, **2**, and **3** (dissolved in DMSO) for 24 h, then centrifuged at 1200 rpm for 5 min at 4 ºC. The cellular pellets were washed twice with a

cold PBS and resuspended in 100 mM sodium phosphate buffer, pH 6.0. Finally, the cells were disrupted by ultrasonication.

Nuclear and cytoplasm fraction extraction

A2780 and MCF-7 cells were treated with compound **2** for 24 h, such treated cells were washed twice with PBS and gently scraped to 1 mL of cold PBS, transferred to a microcentrifuge tube and pelleted at 3000 rpm, 5 min, 4 ºC. The cellular pellet was resuspended in 200 µL of a harvest buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5% Triton X-100 and freshly added 1 mM DTT with protease inhibitor cocktail) and incubated on ice for 10 min. Cells were disrupted by tightly fitting pestle and then pelleted at 1000 rpm, 10 min, 4 ºC. The supernatant was transferred into a new tube, centrifuged at 14 000 rpm, 15 min, 4 ºC and transferred again to a clean tube. At this point, tube contained the cytoplasmic and membrane fraction.

The pellet obtained from centrifugation after disruption step was resuspended in 250 µL of buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and freshly added 1 mM DTT with protease inhibitor cocktail) and centrifuged at 1000 rpm, 10 min, 4 ºC. Two volumes of buffer C (10 mM HEPES pH 7.9, 500 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40 and freshly added 1 mM DTT with protease inhibitor cocktail) was added to the cell pellet, vortexed for 15 min at 4 ºC and centrifuged at 14 000 rpm, 10 min, 4 ºC. The supernatant containing the nuclear fraction was transferred to a new tube. Electrochemical measurements were performed in particular buffers for each fraction.

SDS-PAGE and immunoblotting

This method was carried out as described previously [4] with some differences. Protein concentration was determined by Bradford protein assay (BioRad, USA). Thirty µg of total proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred onto nitrocellulose membranes. Membranes were blocked in 5% milk and 0.1% Tween 20 in PBS and probed overnight with specific monoclonal antibodies anti-PARP (Calbiochem, USA) and α-tubulin (Abcam, USA) diluted to 1 µg/ml. The primary antibodies were washed out using PBS with 0.1 % Tween and membranes were incubated with secondary IgG antibodies conjugated with horseradish peroxidase (Dako, Denmark) for 1 h and then positive signals were visualized with ECL reagents (Amersham Pharmacia Biotech, UK).

Electrochemical measurements

Electrochemical measurements were carried out with Autolab analyzer (Metrohm Autolab, Switzerland), using glassy carbon electrode (GCE) as a working electrode, Ag/AgCl/3 M KCl as a reference electrode and carbon wire as an auxiliary electrode. 100 mM sodium phosphate buffer (pH 6.0) was used as a background electrolyte. All measurements were performed at room temperature using cyclic voltammetry (CV) or differential pulse voltammetry (DPV). GCE surface was regenerated after each measurement by mechanical polishing using 1 μm, 0.3 μm and 0.05 μm alumina slurry, respectively.

Results

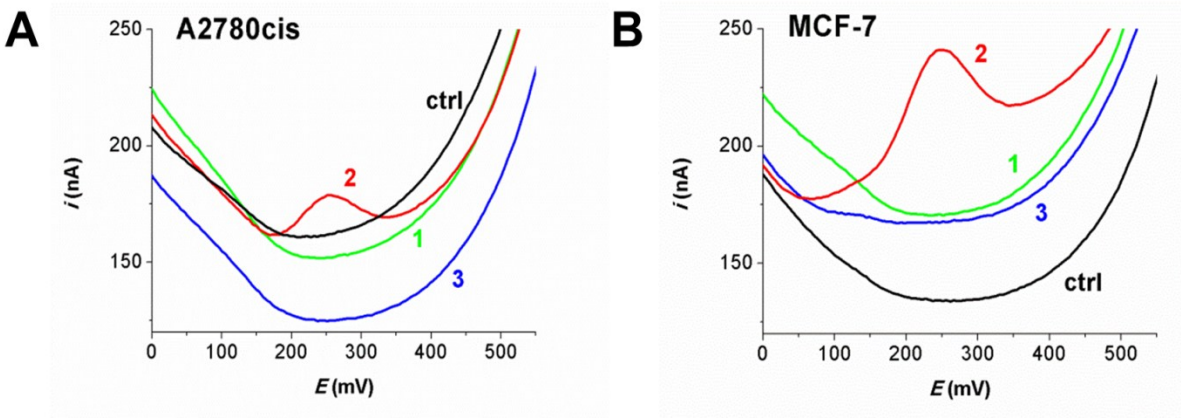


Fig. S1 DPV curves of A2780cis and MCF-7 cell lines treated with compounds **1**, **2** and **3** for 24 h, along with untreated cells (ctrl).

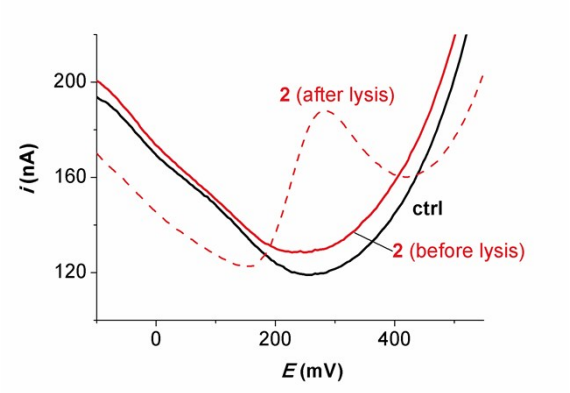


Fig. S2 DPV curves of A2780 cells treated with compound **2** (for 24 h) before and after ultrasonic disruption of the cells**.** Disruption of the cells liberated **2** which yielded oxidation signal at GCE (dashed curve). No signal was obtained from treated cells which were not lysed (solid red curve), indicating sufficient removal by washing. Ctrl = untreated cells.

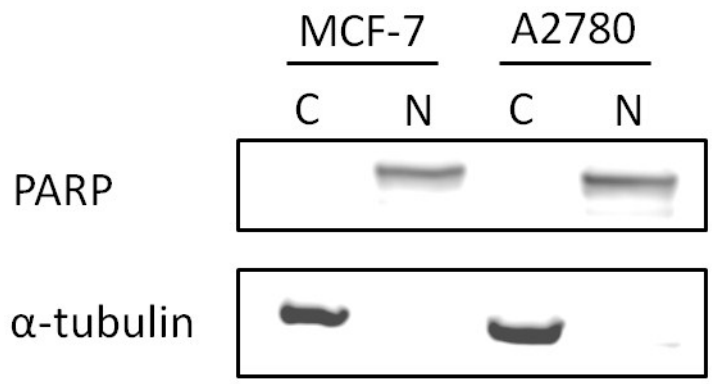


Fig. S3 Demonstration of subcellular fractionation by Western blot analysis. Equivalent amounts of each nuclear or cytoplasmic fraction were subjected to immunoblot with anti-PARP (N= nuclear) and α-tubulin (C= cytoplasm) antibodies.

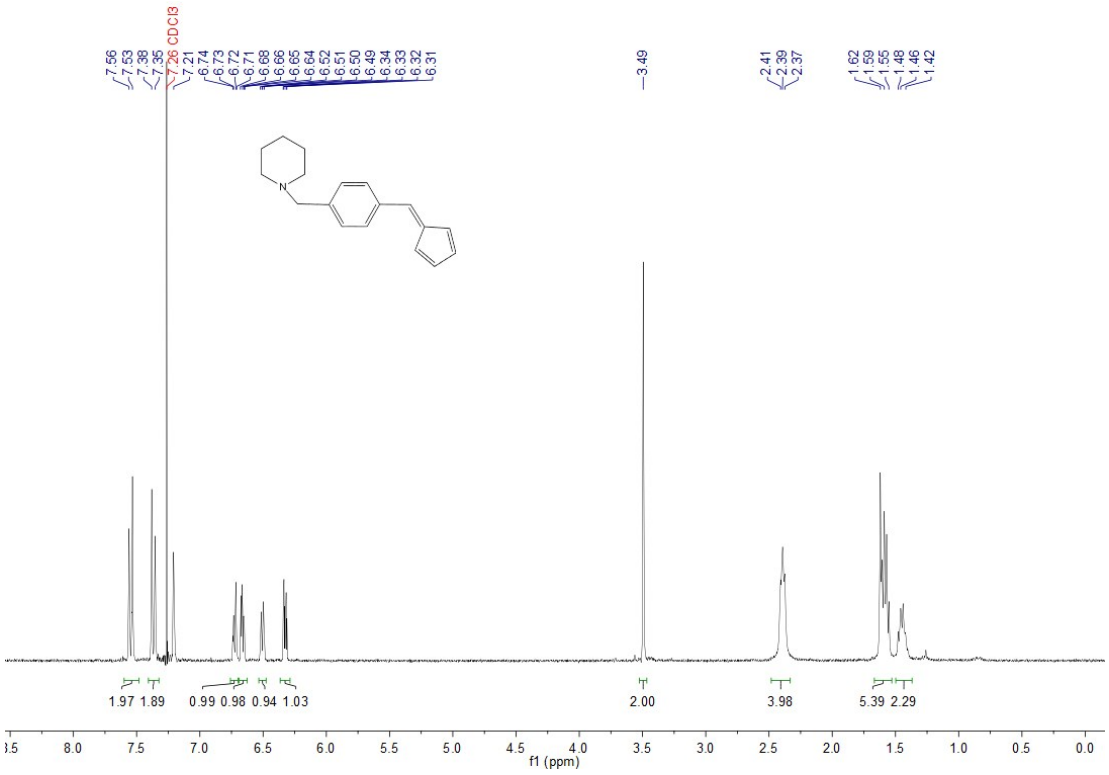


Fig. S4 ^{1}H NMR (300 MHz, 25 ºC) spectrum of **II** in $CDCl_3$ solution.

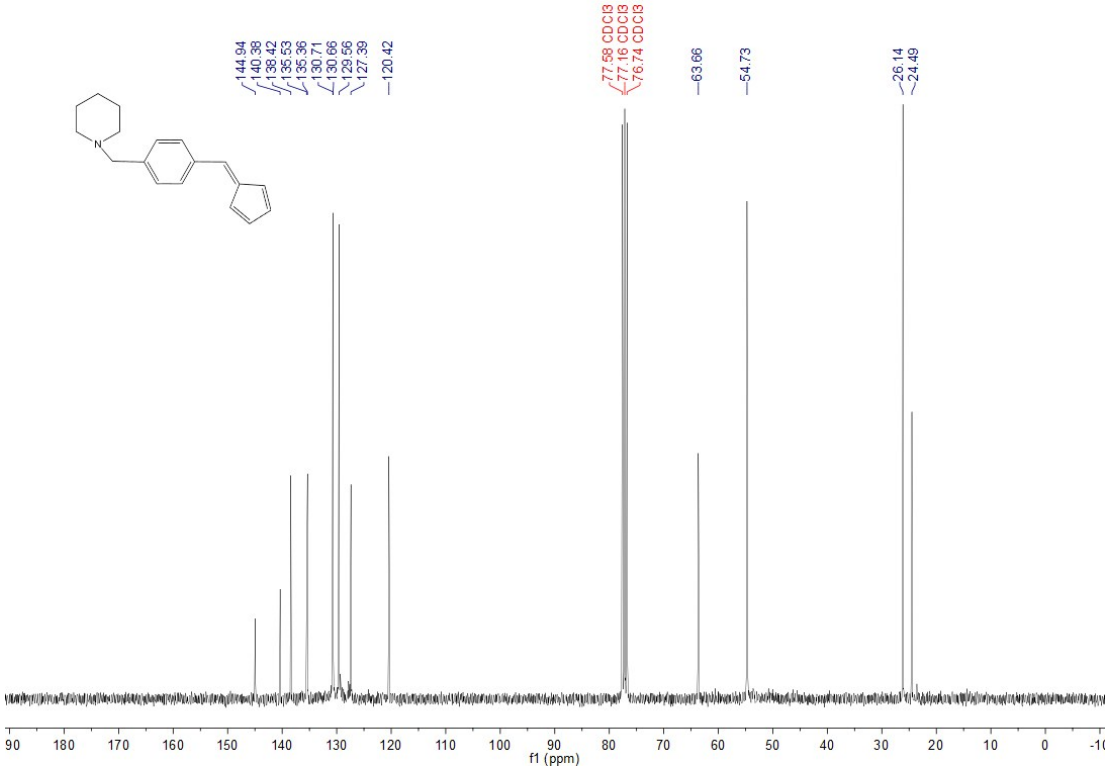


Fig. S5 $^{13}C\{^{1}H\}$ NMR (75 MHz, 25 ºC) spectrum of **II** in $CDCl_3$ solution.

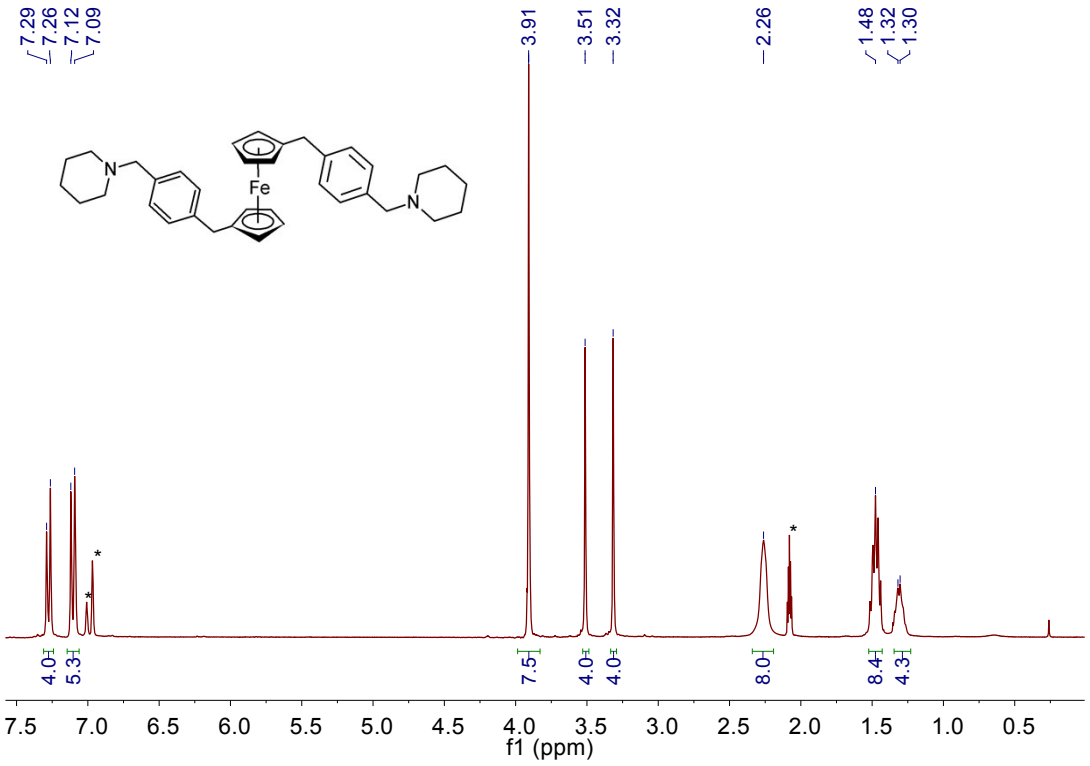


Fig. S6 ^{1}H NMR (300 MHz, 25 ºC) spectrum of **III** in Toluene-D_8 solution. * denotes solvent signals.

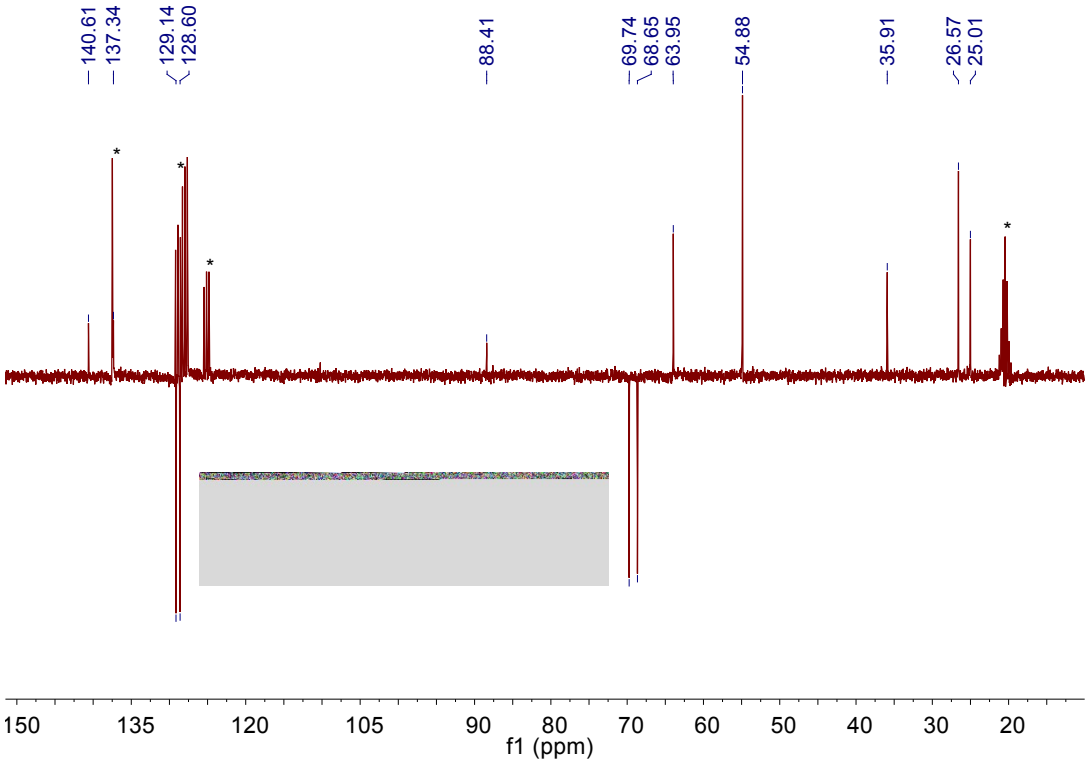


Fig. S7 APT NMR (75 MHz, 25 ºC) spectrum of **III** in Toluene-D_8 solution. * denotes solvent signals.

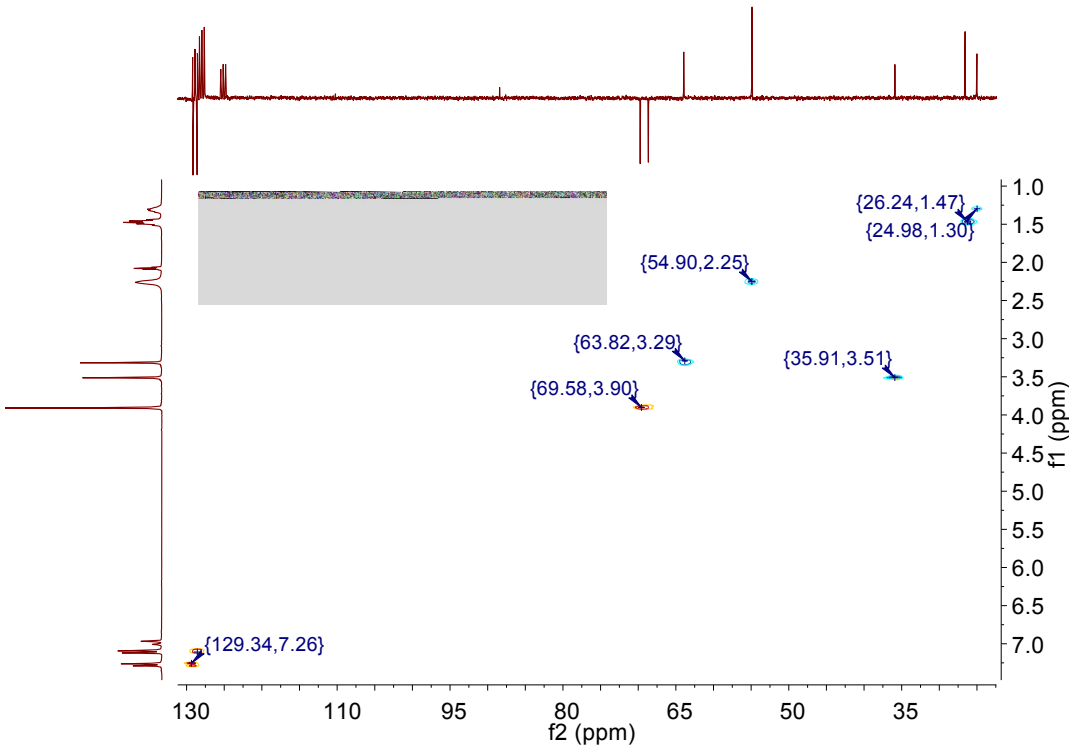


Fig. S8 gHSQC NMR spectrum of **III** in Toluene-D_8 solution.

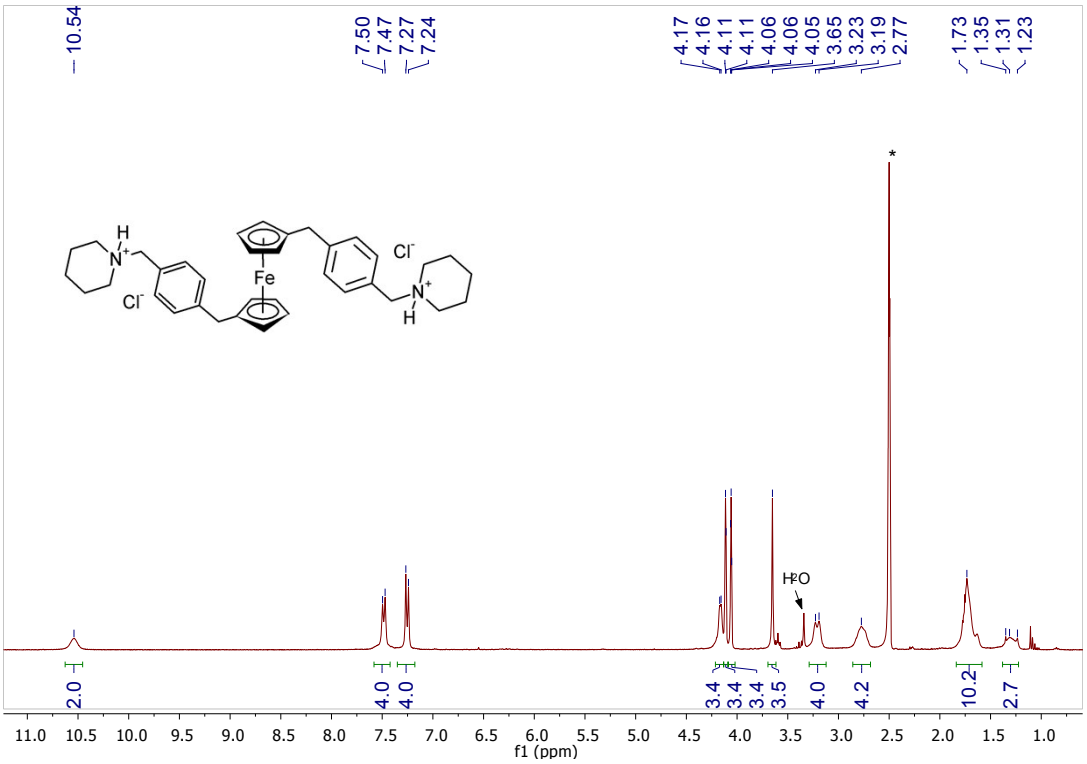


Fig. S9 ^{1}H NMR (300 MHz, 25 ºC) spectrum of **2** in DMSO-D_6 solution. * denotes solvent signal.

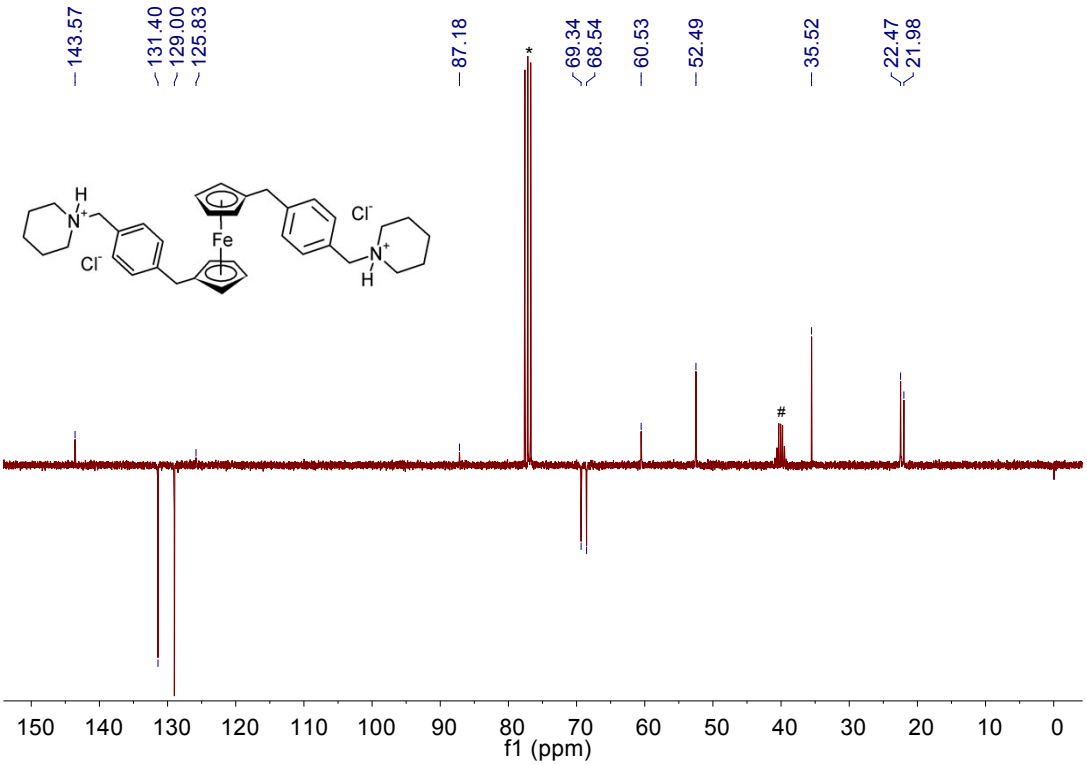


Fig. S10 APT NMR (75 MHz, 25 ºC) spectrum of **2** in $CDCl_3$ solution (75 MHz, 25 ºC). * denotes solvent signals. # denotes DMSO-D_6 added for better solubility of **2**.

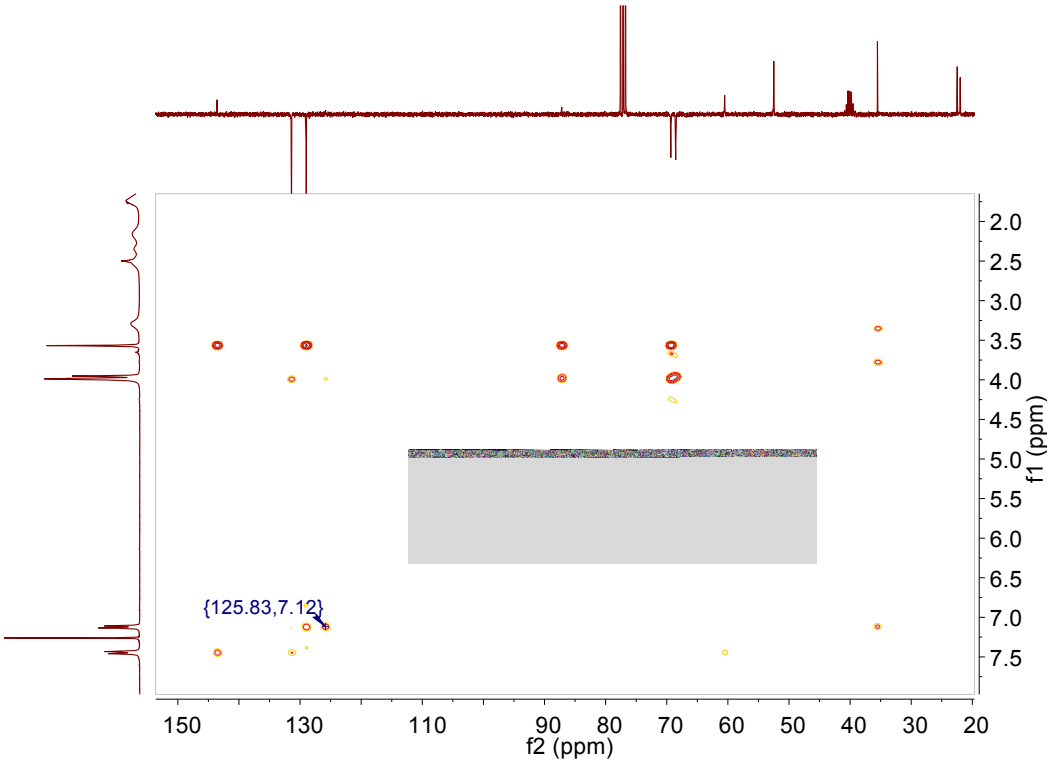


Fig. S11 gHMBC NMR spectrum of **2** in $CDCl_3$ solution, which was used for localization of quarternary carbon *C*CH_2N.

References:

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