Electronic Supporting Information

Anticancer application of half-sandwich iridium(III) ferrocene-thiosemicarbazide Schiff base complexes

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[+] *X. C. Liu and A. Lv have equivalent contribution.*

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Experimental Section

X-ray Crystallography

All diffraction data were obtained on a Bruker Smart Apex CCD diffractometer equipped with graphite-monochro mated Mo *Kα* radiation. Absorption corrections were applied using SADABS program. SQUEEZE option was used to remove the non-localized electron density at the final step of structure refinement. The structures were solved by direct methods using SHELXS (TREF) with additional light atoms found by Fourier methods. Complexes were refined against *F2* using SHELXL, and hydrogen atoms were added at calculated positions and refined riding on their parent atoms. X-ray crystallographic data for IrFc2 are available in Tables S1 and S2 and has been deposited in the Cambridge Crystallographic Data Centre under the accession numbers CCDC 2259306. IrFc2 was grown by slow diffusion of hexane into a saturated dichloromethane solution. X-ray crystallographic data in CIF format are available from the Cambridge Crystallographic Data Centre.

NMR Spectroscopy

^{1}H NMR spectra were acquired in 5 mm NMR tubes at 298K on Bruker DPX 500 (^{1}H =500.13 MHz) spectrometers. ^{1}H NMR chemical shifts were internally referenced to $(CHD_2)(CD_3)_2SO$ (2.50 ppm) for DMSO-d_6 and $CHCl_3$ (7.26 ppm) for chloroform-d_1. Additional, organic solvents were utilized in the synthesis and purification, which were maintained in ^{1}H NMR, including methanol (3.32 ppm), dichloromethane (5.30 ppm), and *n*-hexane (0.88 ppm) All data was carried out using XWIN-NMR version 3.6 (Bruker UK Ltd.).

UV-Vis Spectroscopy

The UV-Vis spectra of these compounds were recorded by TU-1901 UV spectrophotometer with 1 cm path-length quartz cuvettes (3 mL). Spectra were processed using UV Winlab software. Experiments were carried out at 298 K unless otherwise stated.

Stability Studies

The hydrolysis characteristics of IrFc1~IrFc6 (ca.250 μM) in a 20% DMSO/80% H_2O (*v/v*) solution at 298 K for 8 h were evaluated by UV-Vis absorption spectra, and the presence of DMSO ensured the solubility of these complexes.

Reaction with NADH

The reaction of IrFc1~IrFc6 (ca. 1 μM) with NADH (ca. 100 μM) in 20% MeOH/80% H_2O (*v/v*) was monitored by UV-Vis at 298 K after various time intervals. TON was calculated from the difference in NADH concentration after 8 h divided by the concentration of iridium catalyst. The concentration of NADH was obtained using the extinction coefficient $\varepsilon_{339} = 6220\ M^{-1}\ cm^{-1}$.

Cell Culture

Both human cancer cells (cervical carcinoma HeLa cells and lung cancer A549 cells) were obtained from Shanghai Institute of Biochemistry and Cell Biology (SIBCB) and were grown in Dubelco's Modified Eagle Medium (DMEM). All media were supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin solution. All cells were grown at 310 K in a humidified incubator under 5% CO_2 atmosphere.

Anti-proliferative assay (MTT assay)

After plating 5000 cells per well in 96-well plates, the cells were preincubated in drug-free media at 310 K for 24 h before adding different concentrations of the compounds to be tested. In order to prepare the stock solution of the drug, the solid compound was dissolved in DMSO. This stock was further diluted using cell culture medium until working concentrations were achieved. The drug exposure period was 24 h. Subsequently, 15 μL of 5 mg mL^{-1} MTT solution was added to form a purple formazan. Afterwards, 100 μL of dimethyl sulfoxide (DMSO) was transferred into each well to dissolve the purple formazan, and results were measured using a microplate reader (DNM-9606, Perlong Medical, Beijing, China) at an absorbance of 570 nm. Each well was triplicated and each experiment repeated at least three times. IC_{50} values quoted are mean ± SEM.

Measurement of Lipophilicity (logPo/w)

The octanol-water partition coefficients ($P_{o/w}$) of complexes were determined using a shake-flask method. Water (50 mL, distilled after milli-Q purification) and 1-octanol (50 mL, vacuum distilled) were shaken together using a laboratory shaker, for 72 h to allow saturation of both phases. Stock solutions of IrFc7 and IrFc8 (50.0 μM) were prepared in the aqueous phase and aliquots (5 mL) of each of these stock solutions were then added to an equal volume of the 1-octanol phase. The resultant biphasic solutions were mixed for 2 h and then centrifuged (3000×g, 5 min) to separate the phases. The concentrations of IrFc7 and IrFc8 in the organic and aqueous phases were then determined using UV-Vis. Log$P_{o/w}$ was defined as the logarithm of the ratio of the concentrations of the compound in the organic and aqueous phases (Values reported are the means of three separate determinations).

ROS Determination

Flow cytometry analysis of ROS generation in A549 cells caused by exposure to iridium compounds were carried out using the Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the supplier's instructions. Briefly, 1.5×10^6 A549 cancer cells per well were seeded in a six-well plate.

Cells were preincubated in drug-free media at 310 K for 24 h in a 5% CO_2 humidified atmosphere, and then drugs were added at concentrations of $1.0\times IC_{50}$ and $2.0\times IC_{50}$. After 24 h of drug exposure, cells were washed twice with PBS and then incubated with the DCFH-DA probe (10 μM) at 310 K for 30 min, and then washed triple immediately with PBS. The fluorescence intensity was analyzed by flow cytometry (ACEA NovoCyte, Hangzhou, China). Data were processed using NovoExpress™ software.

Cell Cycle Analysis

A549 cancer cells at 1.5×10^6 per well were seeded in a six-well plate. Cells were preincubated in drug-free media at 310 K for 24 h, after which IrFc8 was added at concentrations of $0.5\times IC_{50}$, $1.0\times IC_{50}$ and $2.0\times IC_{50}$ against A549 cells. After 24 h of exposure, supernatants were removed by suction and cells were washed with PBS. Finally, cells were harvested using trypsin-EDTA and fixed for 24 h using cold 70% ethanol. DNA staining was achieved by suspending the cell pellets in PBS containing propidium iodide (PI) and RNAse. Cell pellets were washed and suspended in PBS before being analyzed in a flow cytometer (ACEA NovoCyte, Hangzhou, China) using excitation of DNA-bound PI at 488 nm, with emission at 585 nm. Data were processed using NovoExpress™ software. The cell cycle distribution is shown as the percentage of cells containing G1, S and G2 DNA as identified by propidium iodide staining.

Induction of Apoptosis

Flow cytometry analysis of apoptotic populations of the cells caused by exposure to IrFc8 was carried out using the Annexin V-FITC Apoptosis Detection Kit (Beyotime Institute of Biotechnology, China) according to the supplier's instructions. Briefly, A549 cells (1.5×10^6/2 mL per well) were seeded in a six-well plate. Cells were preincubated in drug-free media at 310 K for 24 h, after which IrFc8 was added at concentrations of $1.0\times IC_{50}$, $2.0\times IC_{50}$ and $3.0\times IC_{50}$ of IrFc8 against A549 cancer cells. After 24 h of drug exposure, cells were collected, washed once with PBS, and suspended in 195 μL of Annexin V-FITC binding buffer which was then added to 5 μL of Annexin V-FITC and 10 μL of PI, and then incubated at room temperature in the dark for 15 min. Subsequently, the buffer placed in an ice bath in the dark. The samples

were analyzed by a flow cytometer (ACEA NovoCyte, Hangzhou, China).

Mitochondrial Membrane Assay

Analysis of the changes of mitochondrial potential in cells after exposure to IrFc8 was carried out using the mitochondrial membrane potential assay kit with JC-1 (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, 1.5×10^6 A549 cancer cells were seeded in six-well plates left to incubate for 24 h in drug-free medium at 310 K in a humidified atmosphere. Drug solutions, with the concentration changed from $0.5\times IC_{50}$ to $3.0\times IC_{50}$ of IrFc8 against A549 cells, were added in triplicate, and the cells were left to incubate for a further 24 h under similar conditions. Supernatants were removed by suction, and each well was washed with PBS before detaching the cells using trypsin-EDTA. Staining of the samples was done in flow cytometry tubes protected from light, incubating for 30 min at ambient temperature. The samples were immediately analyzed by a flow cytometer (ACEA NovoCyte, Hangzhou, China). For positive controls, the cells were exposed to carbonyl cyanide 3-chlorophenylhydrazone, CCCP (5 μM), for 20 min. Data were processed using NovoExpress™ software.

Cellular Localization and Uptake Mechanism assay

Two Photon Laser Scanning Microscope (*/LSM/880NLO) is produced at Carl Zeiss AG, Germany. LTDR (Life Technologies, USA), MTDR (Life Technologies, USA), CCCP (Sigma Aldrich, USA), chloroquine (Sigma Aldrich, USA) was used as received. A549 cells were seeded into 35 mm dishes (Greiner, Germany) for confocal microscopy. After cultured overnight, the cells were incubated with IrFc8 ($1.0\times IC_{50}$) for 1 h. The treated cells were observed immediately under a confocal microscope with excitation at 488 nm. For colocalization studies, the cells were incubated with IrFc8 ($1.0\times IC_{50}$) for 1 h. Subsequently, the medium was replaced with staining medium containing MTDR/LDTR and stained for another 20 min and 1 h. The cells were washed twice with PBS, and then viewed immediately under a confocal microscope. Investigation of drug entry pattern: Cells were incubated with IrFc8 ($1.0\times IC_{50}$) for 1 h, after which media was replaced with staining medium containing CCCP (10 μM, 1 h)

chloroquine (50 μM, 1 h) and re-stained for 15 min. The cells were washed twice with PBS, and then viewed immediately under a confocal microscope.

Lysosomal damage assay

A549 cells seeded into six-well plate (Corning) were exposed to IrFc8 at the indicated concentrations for 12 h. The cells were then washed twice with PBS and incubated with AO (5 μM) at 310 K for 15 min. The cells were washed twice with PBS and visualized by confocal microscopy (LSM/880NLO). Emission was collected at 510±20 nm (green) and 625 ± 20 nm (red) upon excitation at 488 nm.

Synthesis

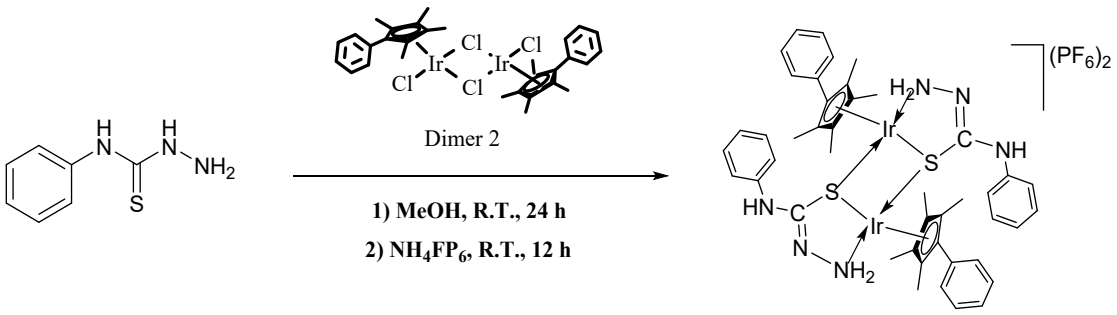
Synthesis of [(η^5-Cp)Ir(μ-Cl)Cl]$_2$ (**Dimer1**).*

$IrCl_3 \cdot 3H_2O$ (0.50 g, 1.7 mmol) was dissolved in methanol (20 mL) in a microwave vial, pentamethyl cyclopentadiene (0.69 g, 5.0 mmol) was added and reacted at 400K, 400 psi for 30 min in a microwave instrument. The reaction mixture was allowed to cool to ambient temperature and the dark green precipitate was filtered off. The volume of the dark red filtrate was reduced to ca. 15 mL on a rotary evaporator. Upon cooling to ambient temperature, an orange precipitate appeared and was collected by filtration. The product was washed with methanol and diethyl ether, dried in air, and pure Dimer 1 was obtained. Yield 0.40 g (65%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.60 (s, J = 1.4 Hz, 30H, Cp-CH_3).

*Synthesis of [(η^5-Cp^{xph})Ir(μ-Cl)Cl]$_2$ (**Dimer2**).*

Dimer 2 was synthesized using phenyltetramethyl cyclopentadiene (0.84 g, 5.0 mmol) and $IrCl_3 \cdot 3H_2O$ (0.50 g, 1.67 mmol) according to the method of Dimer 1. Yield 0.37 g (58.5%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.58 (m, 4H, Ar-H), 7.35 (m, 6H, Ar-H), 1.72 (s, 12H, Cp-CH_3), 1.63 (s, 12H, Cp-CH_3).

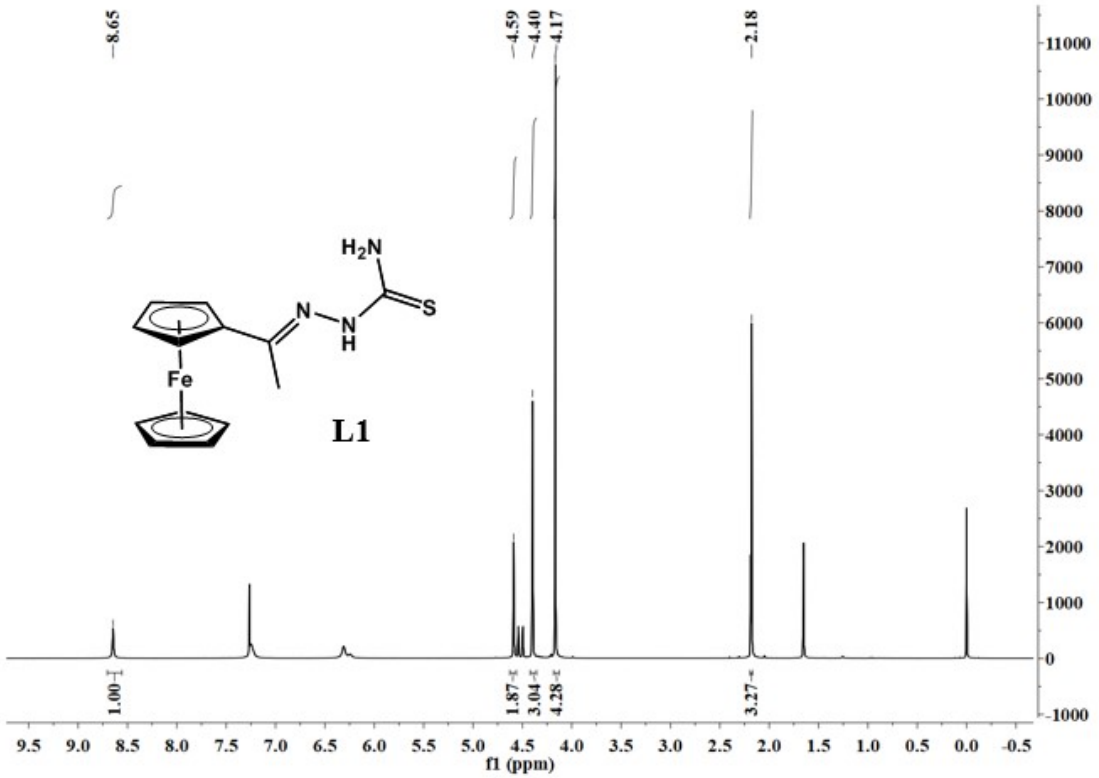
*Synthesis of ferrocene-unappended half-sandwich Ir^{III} TSC complexes (**IrPh**)*



Scheme S1. Design of ferrocene-unappended half-sandwich Ir^{III} TSC complexes (**IrPh**)

Dimer 2 (92.0 mg) and 4-Phenylthiosemicarbazide ligands (33.4 mg) were added to a Schlenk flask and dissolved in 20 mL methanol. The reaction was carried out in nitrogen and room temperature for 24 h. Then ammonium hexafluorophosphate (130.4 mg, 0.80 mmol) was added and reacted for 6 h. The solvent was removed by vacuum distillation, 3 mL dichloromethane was added and filtered, then product was obtained after *n*-hexane added for diffusion. The ^{1}H NMR and ESI-MS of IrPh were presented in Figures S5. Yield 106.3 mg (75.8%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.64 (s, 2H), 7.50 (d, J = 7.6 Hz, 4H, Ar-H), 7.42 (d, J = 8.2 Hz, 8H, Ar-H), 7.36 (t, J = 7.4 Hz, 4H, Ar-H), 7.26 (d, J = 6.9 Hz, 4H, Ar-H), 7.20 (t, J = 7.1 Hz, 2H), 2.10 (s, 6H, Cp-CH_3), 2.06 (s, 6H, Cp-CH_3), 2.05 (s, 6H, Cp-CH_3), 1.68 (s, 6H, Cp-CH_3). ESI-MS (*m/z*): calcd for $C_{44}H_{50}N_6F_{12}P_2S_2Ir_2$, 1400.2057; Found 556.1425, $[M\text{-}2PF_6]_{1/2}^+$. Elem. anal. calcd for $C_{44}H_{50}N_6F_{12}P_2S_2Ir_2$: C, 37.71; H, 3.60; N, 6.00%; Found C, 37.56; H, 3.98; N, 5.74%.

2. Figures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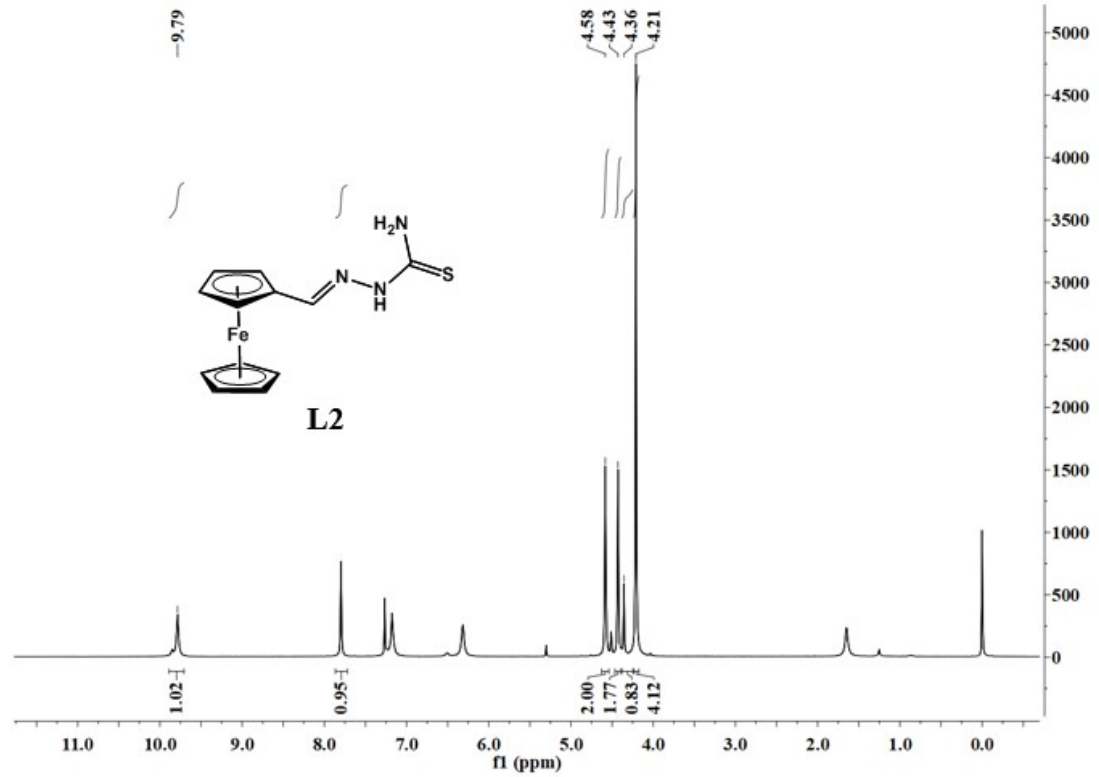
L1
1.00
1.87
3.04
4.28
3.27
f1 (ppm)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4.36
4.21
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1.02
0.95
2.00
1.77
0.83
4.12
f1 (ppm)

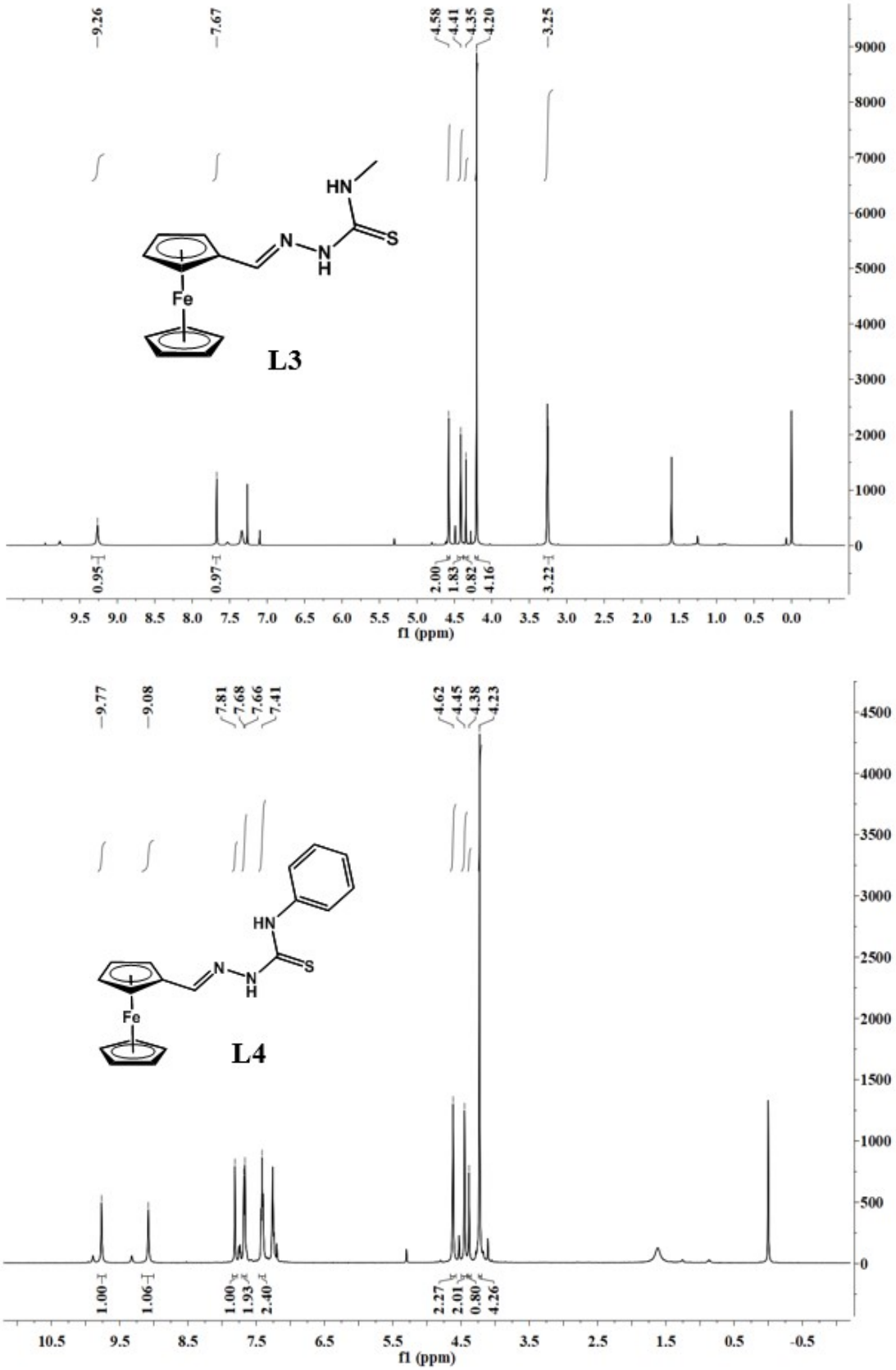
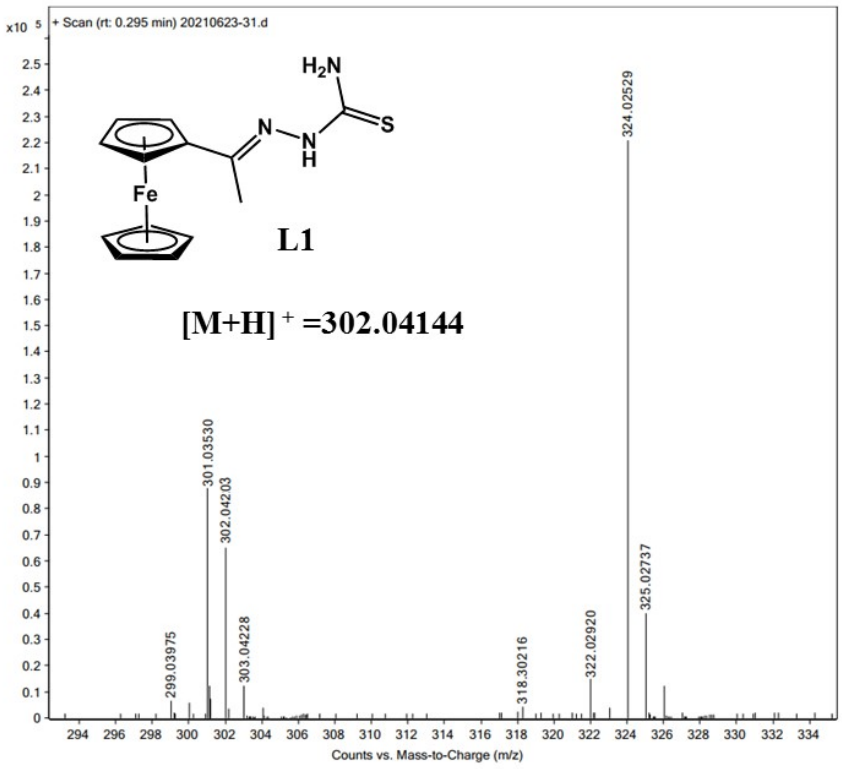
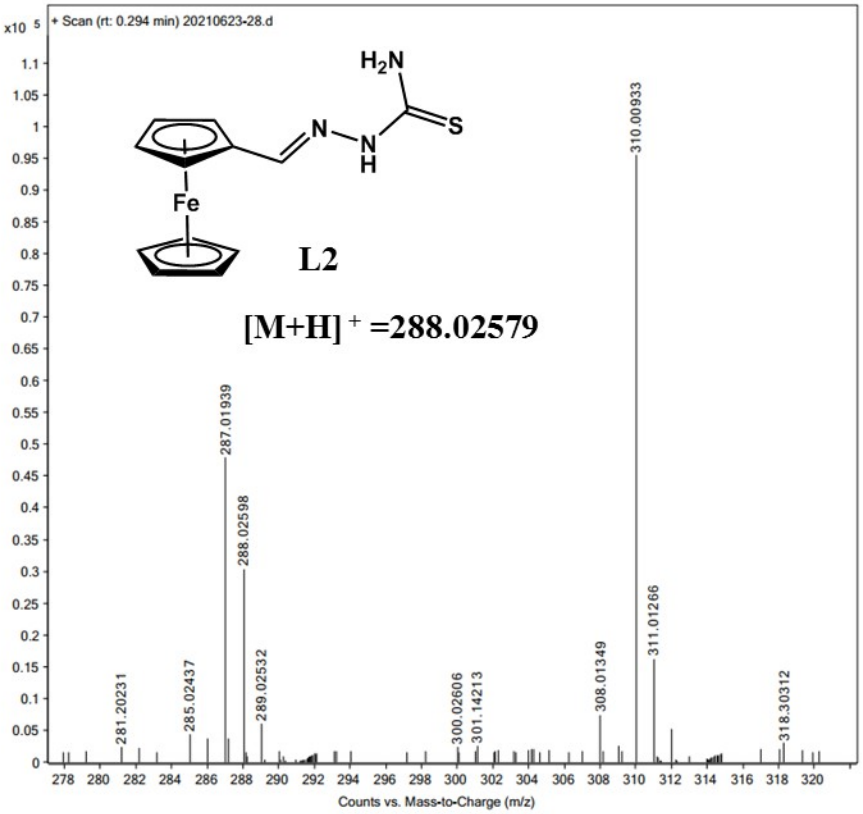


Figure S1 ^{1}H NMR spectra of L1- L4 in $CDCl_3$.


+ Scan (rt: 0.295 min) 20210623-31.d
x10 5
Counts vs. Mass-to-Charge (m/z)
L1
[M+H]+ =302.04144
324.02529
301.03530
302.04203
325.02737
299.03975
303.04228
318.30216
322.02920


+ Scan (rt: 0.294 min) 20210623-28.d
x10 5
Counts vs. Mass-to-Charge (m/z)
L2
[M+H]+ =288.02579
310.00933
287.01939
288.02598
311.01266
289.02532
281.20231
285.02437
308.01349
300.02606
301.14213
318.30312

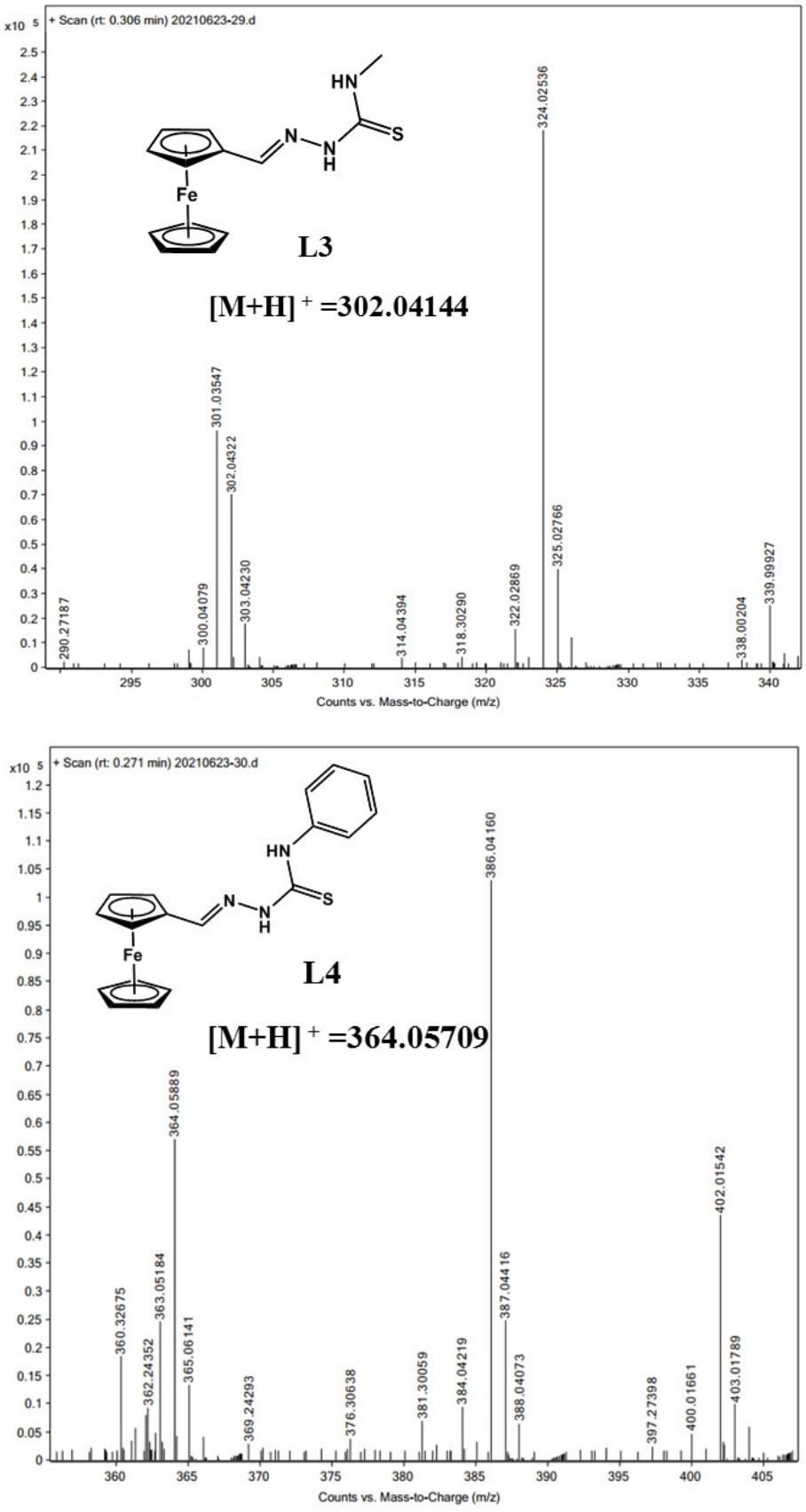
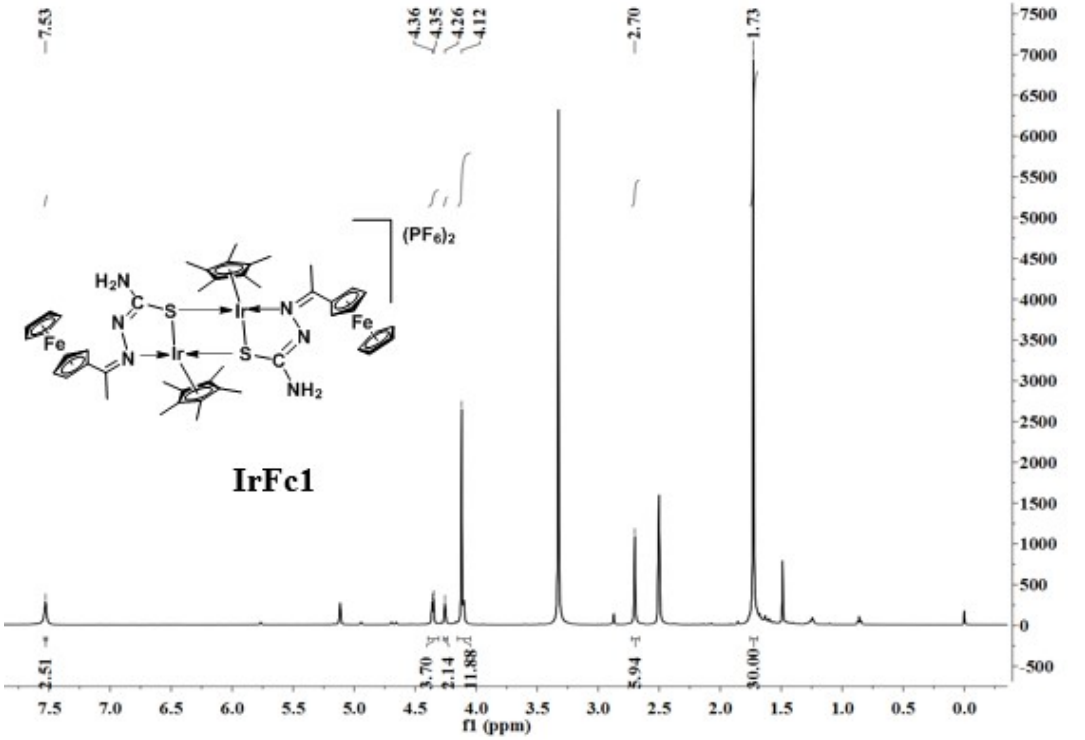
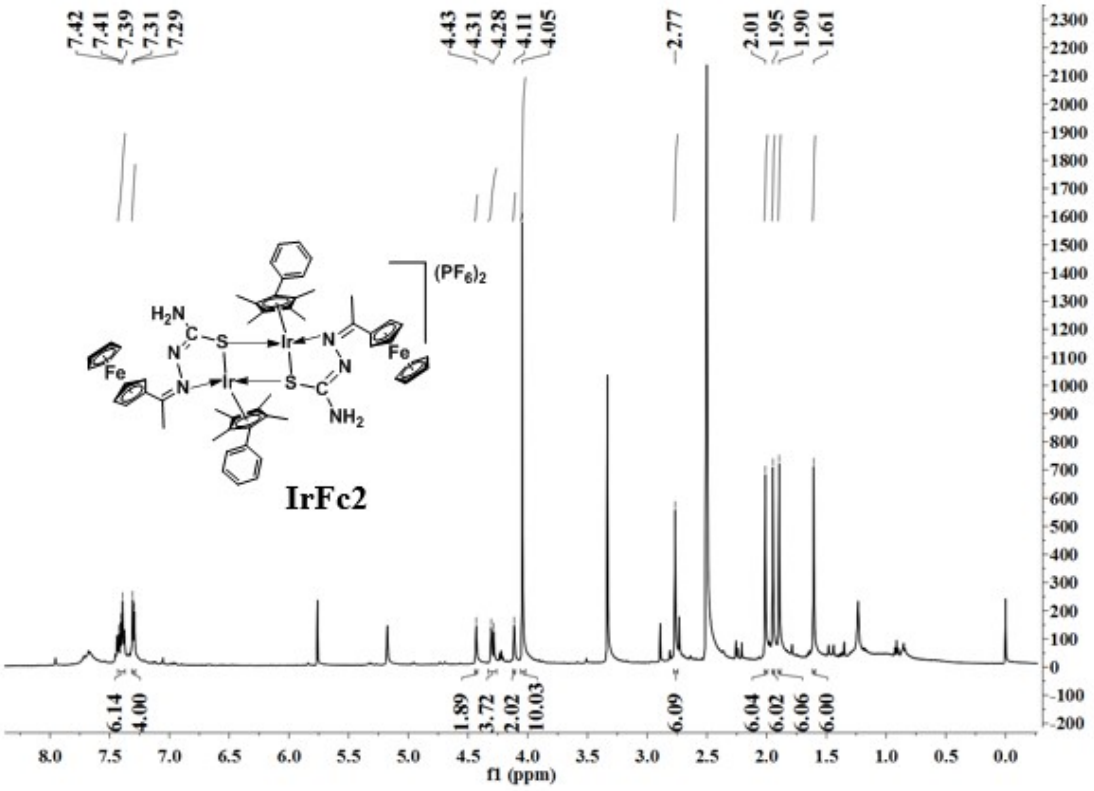
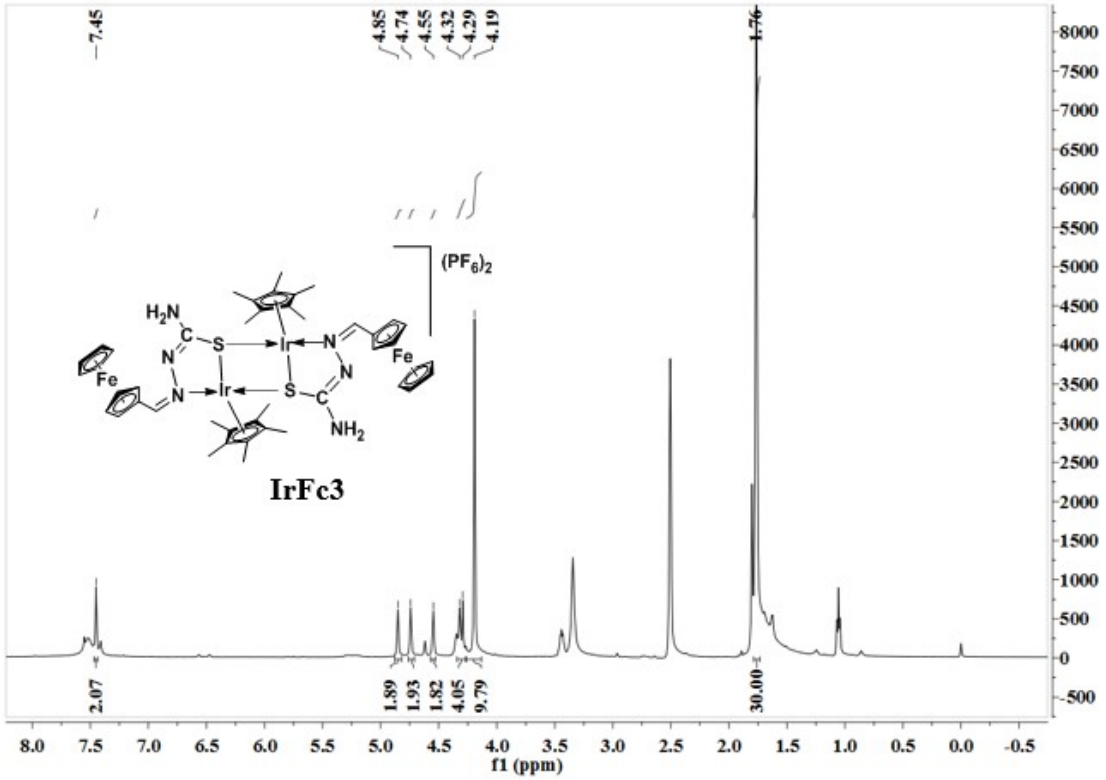


Figure S2. HR-MS of L1-L4.


7.53
4.36
4.35
4.26
4.12
2.70
1.73
(PF6)2
IrFc1
2.51
3.70
2.14
11.88
5.94
30.00
f1 (ppm)


7.42
7.41
7.39
7.31
7.29
4.43
4.31
4.28
4.11
4.05
2.77
2.01
1.95
1.90
1.61
(PF6)2
IrFc2
6.14
4.00
1.89
3.72
2.02
10.03
6.09
6.04
6.02
6.06
6.00
f1 (ppm)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4.85
4.74
4.55
4.32
4.29
4.19
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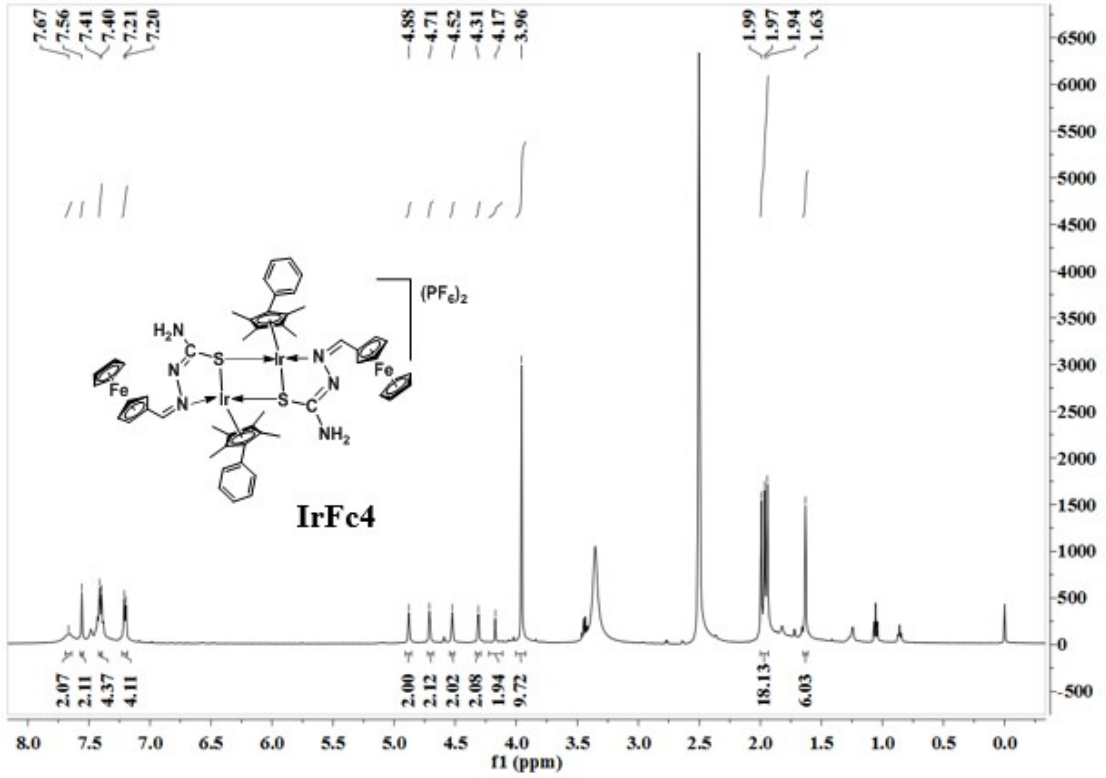
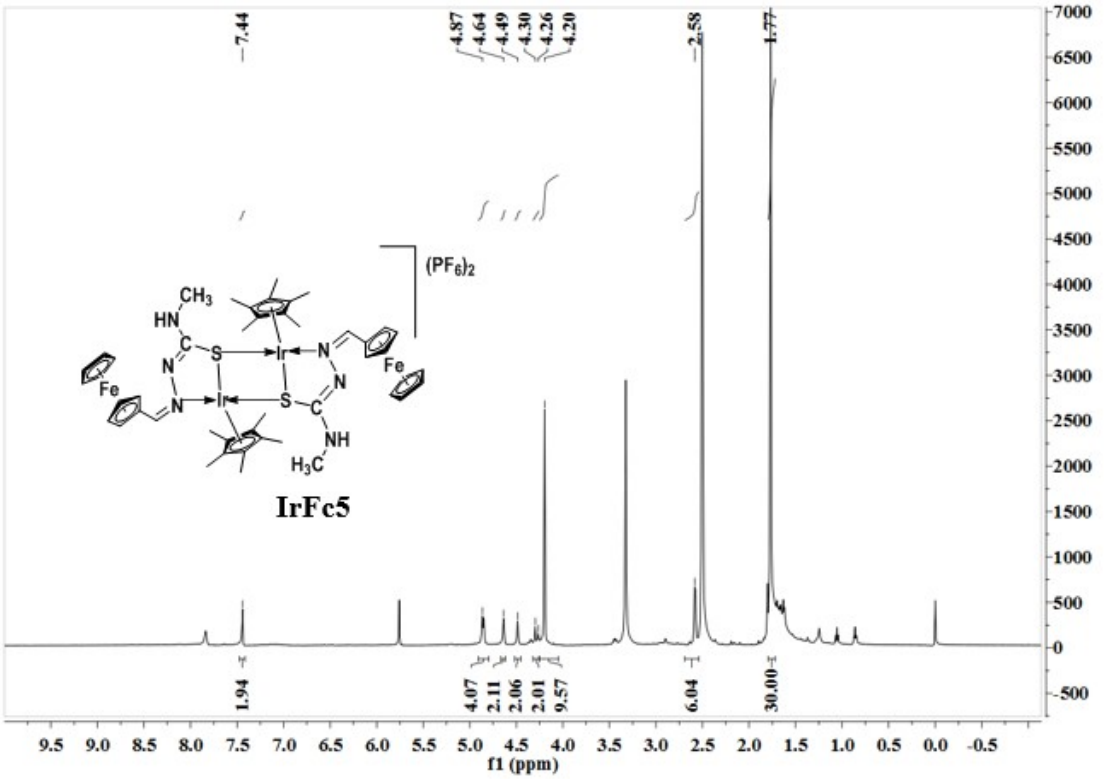
1.93
1.82
4.05
9.79
30.00
f1 (ppm)


7.67
7.56
7.41
7.40
7.21
7.20
4.88
4.71
4.52
4.31
4.17
3.96
1.99
1.97
1.94
1.63
(PF6)2
IrFc4
2.07
2.11
4.37
4.11
2.00
2.12
2.02
2.08
1.94
9.72
18.13
6.03
f1 (ppm)


7.44
4.87
4.64
4.49
4.30
4.26
4.20
2.58
1.77
(PF6)2
IrFc5
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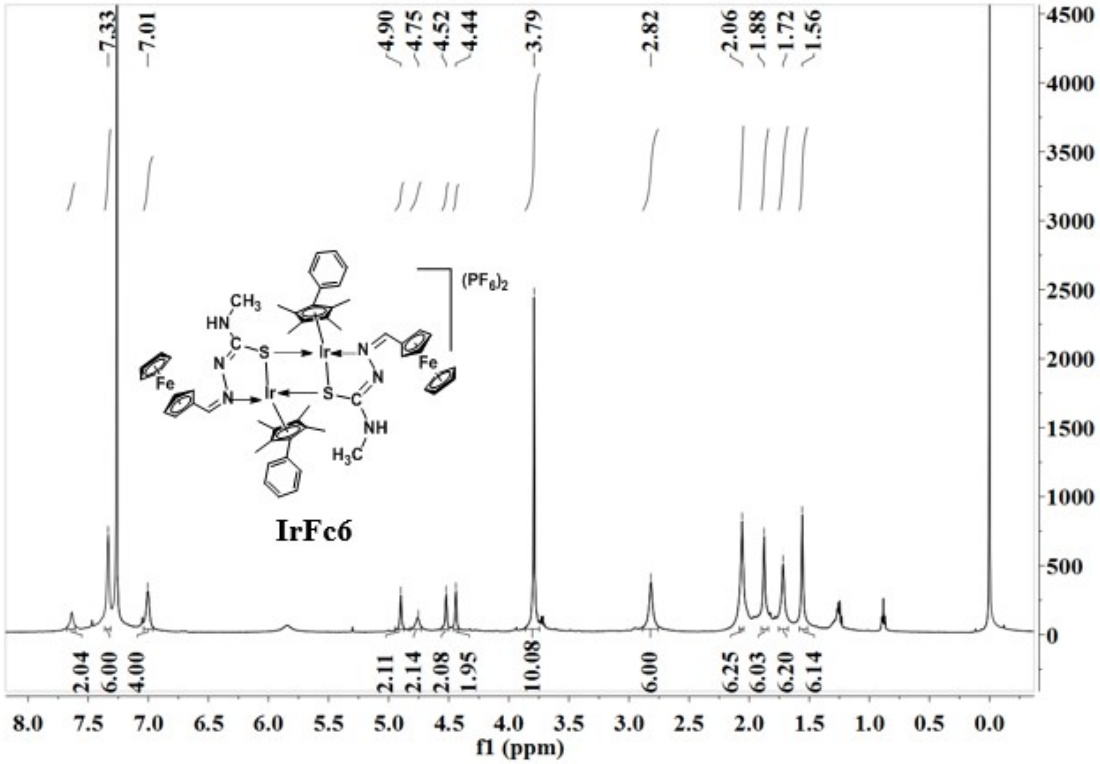
2.06
2.01
9.57
6.04
30.00
f1 (ppm)


7.33
7.01
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4.75
4.52
4.44
3.79
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2.06
1.88
1.72
1.56
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IrFc6
2.04
6.00
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6.25
6.03
6.20
6.14
f1 (ppm)

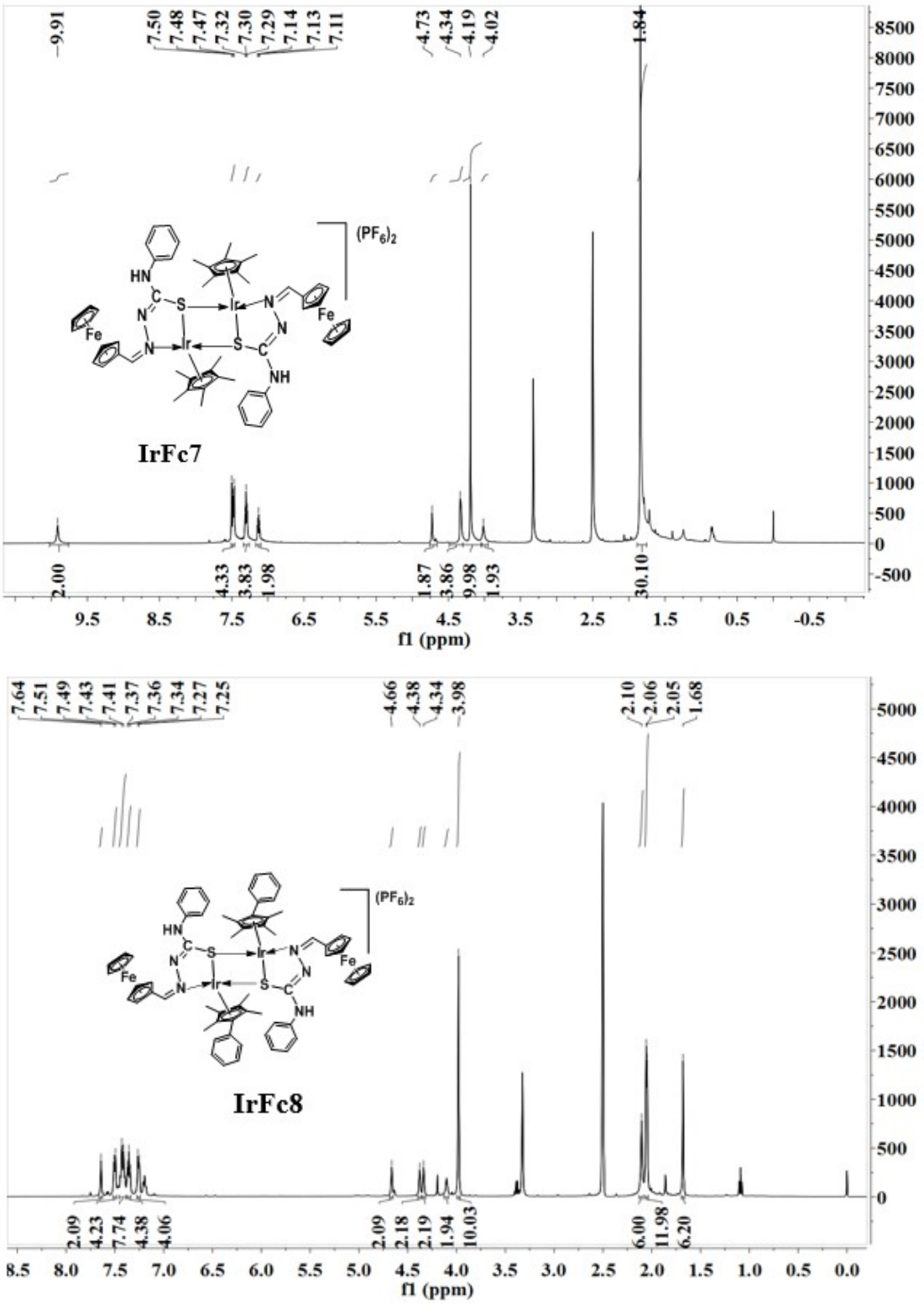
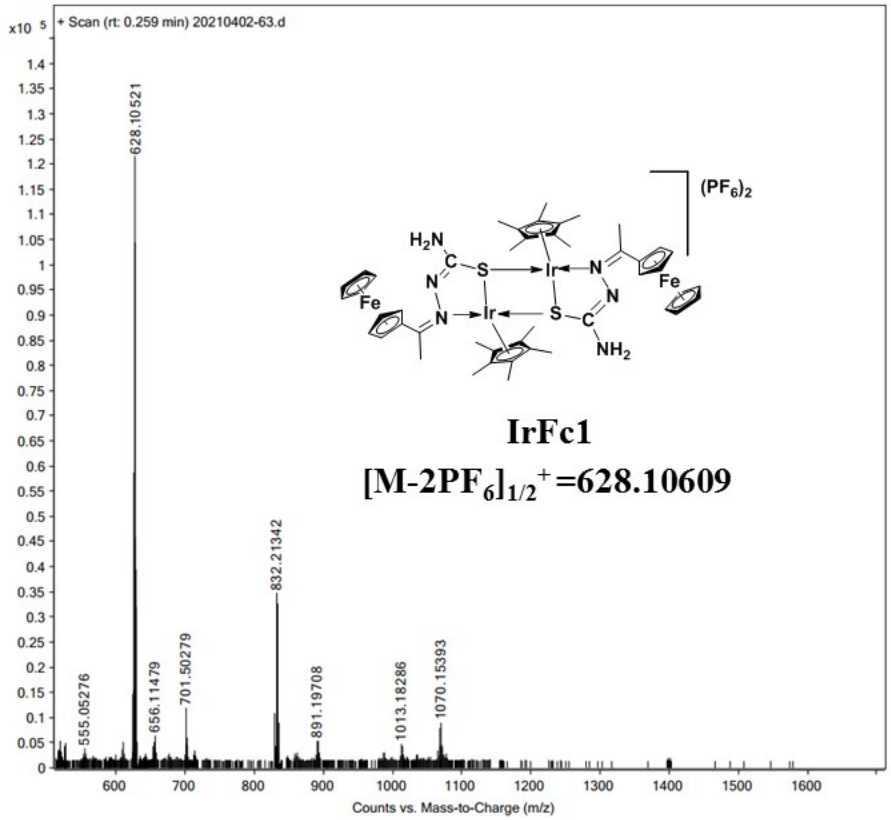
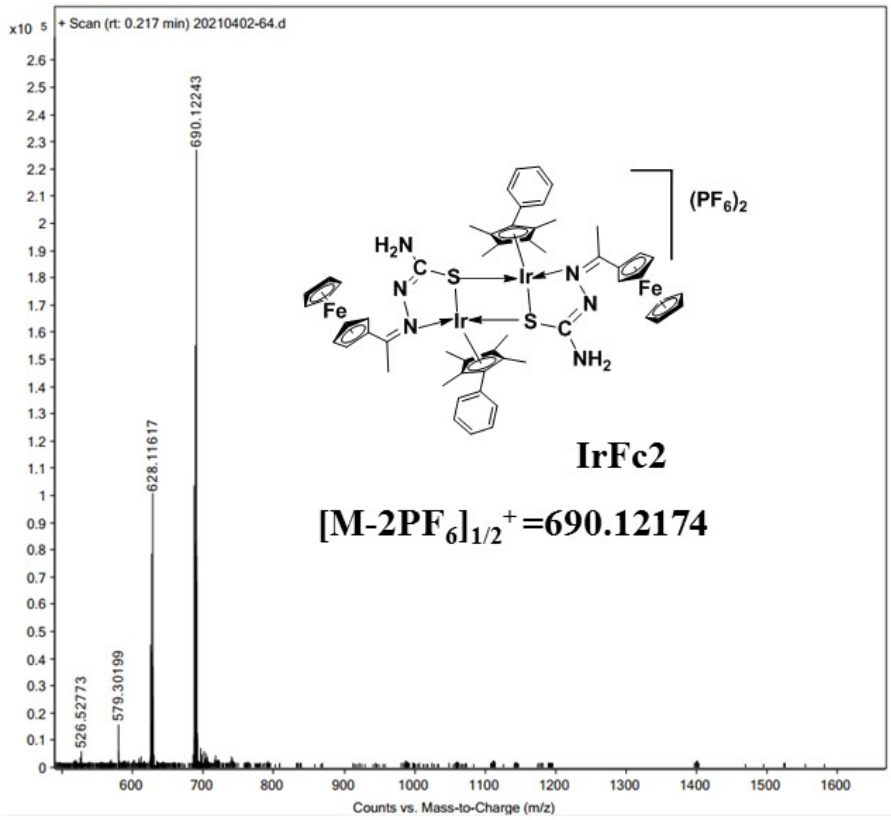


Figure S3 ^{1}H NMR spectra of IrFc1~IrFc8 in $CDCl_3$/ DMSO-d_6.



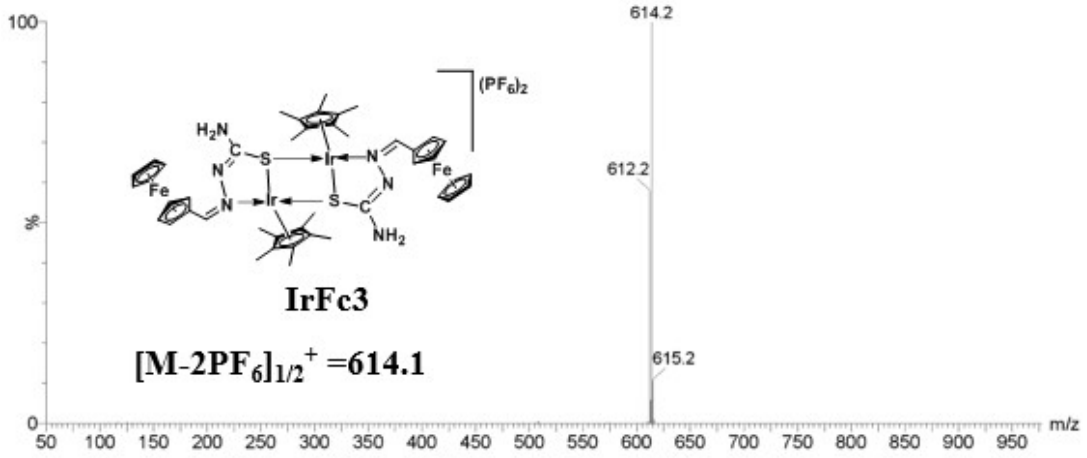
IrFc1

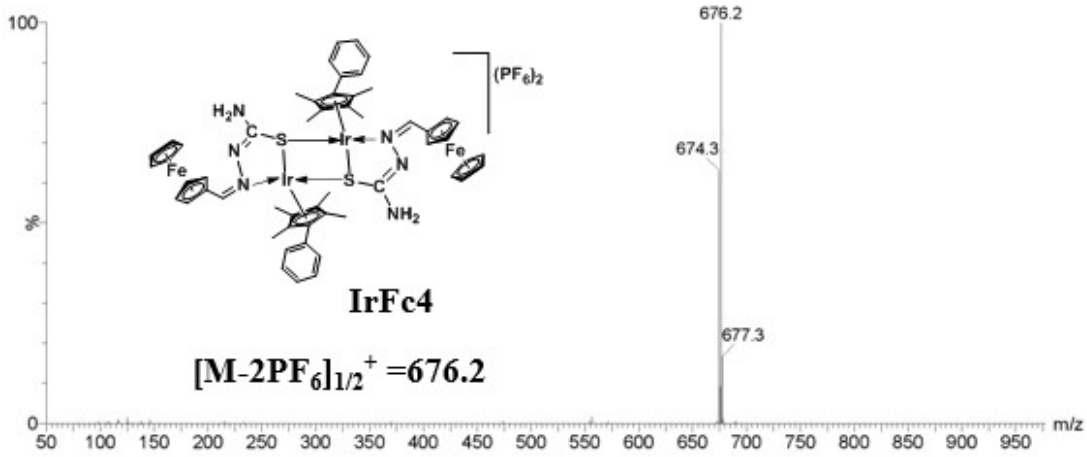
$[M-2PF_6]_{1/2}^{+}$=628.10609

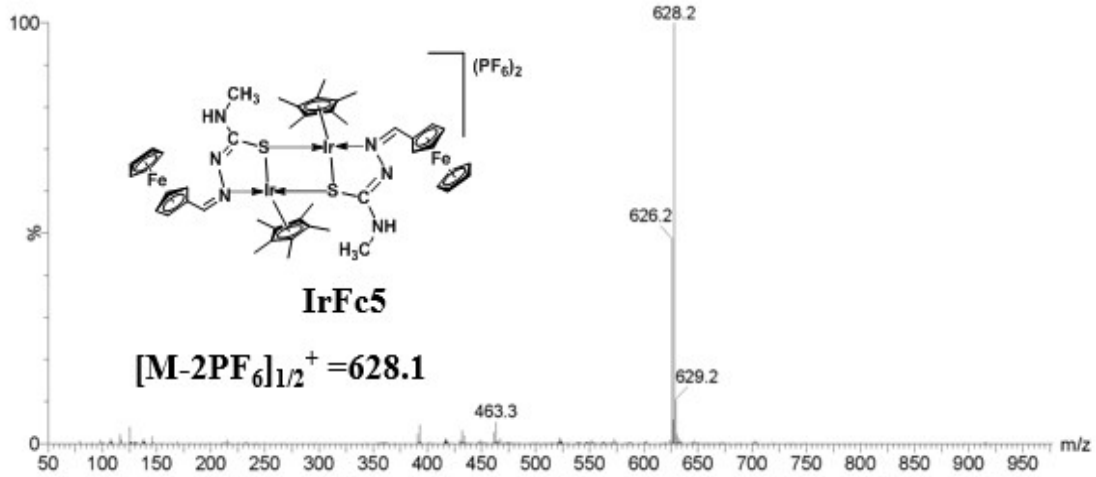


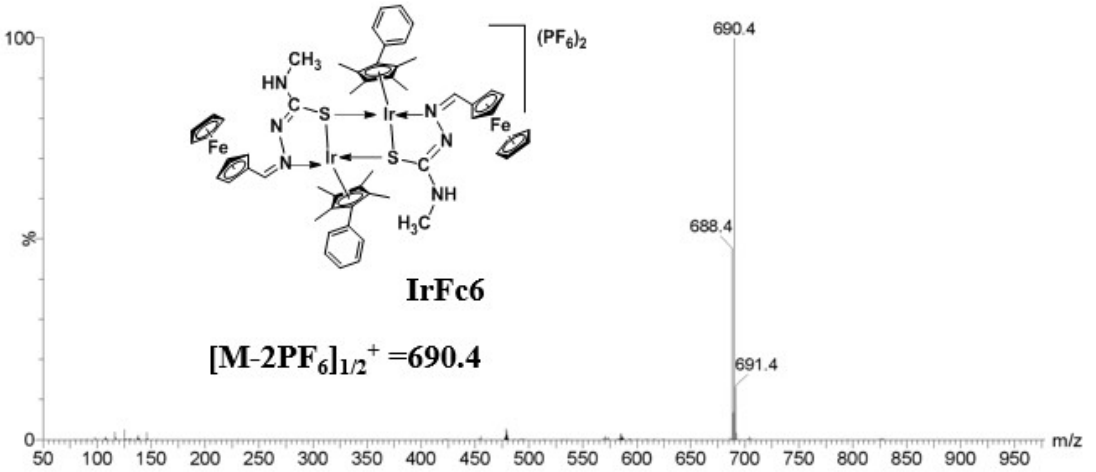
IrFc2

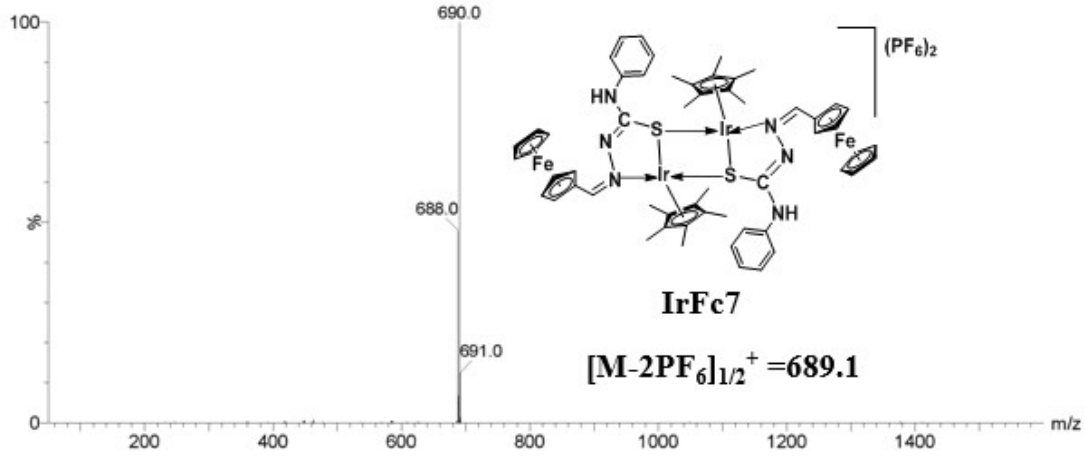
$[M-2PF_6]_{1/2}^{+}$=690.12174


614.2
612.2
615.2
(PF6)2
IrFc3
[M-2PF6]1/2+ =614.1
100
%
0
m/z
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950


676.2
674.3
677.3
(PF6)2
IrFc4
[M-2PF6]1/2+ =676.2
100
%
0
m/z
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950


628.2
626.2
629.2
463.3
(PF6)2
IrFc5
[M-2PF6]1/2+ =628.1
100
%
0
m/z
50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950





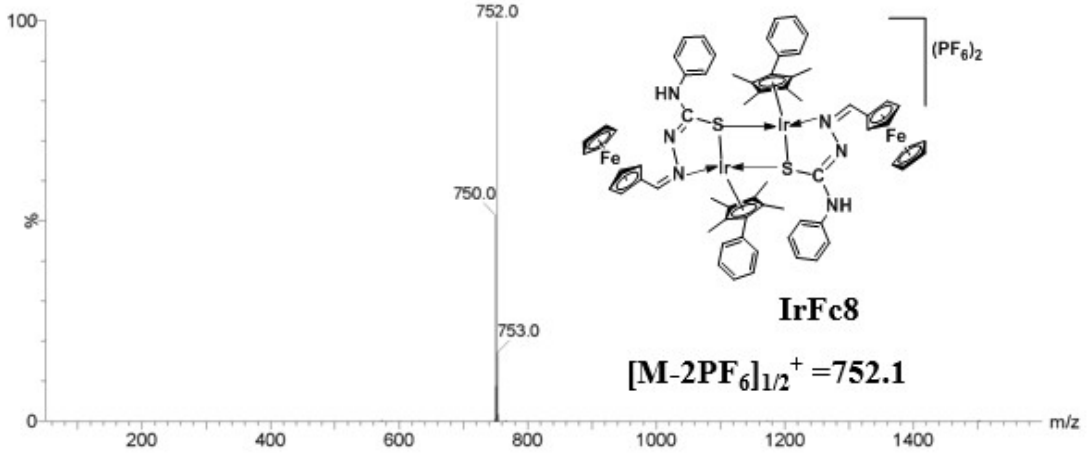


Figure S4 HR-MS and ESI-MS of IrFc1~IrFc8

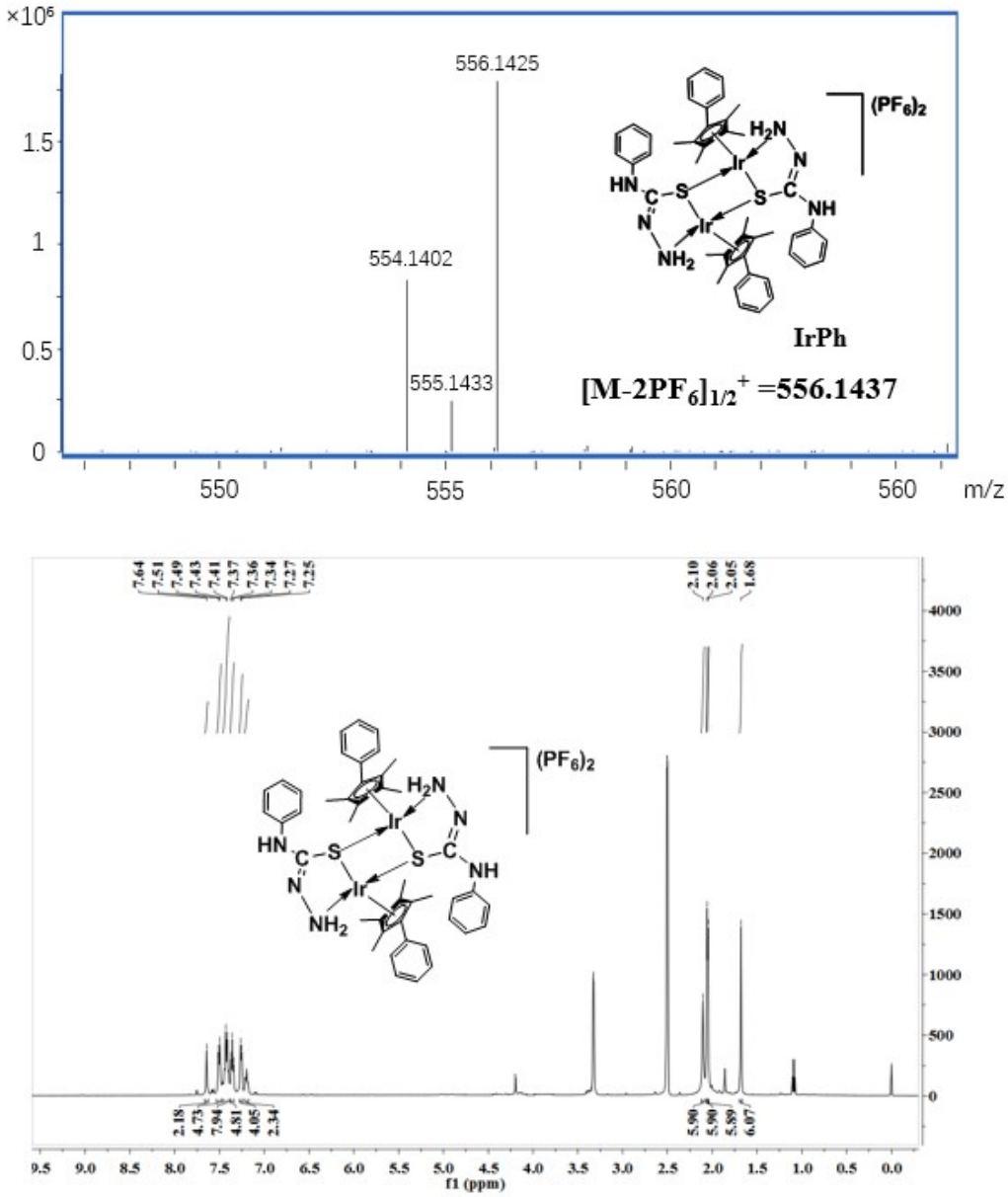


Figure S5 ESI-MS and ^{1}H NMR spectra in $CDCl_3$ of IrPh.

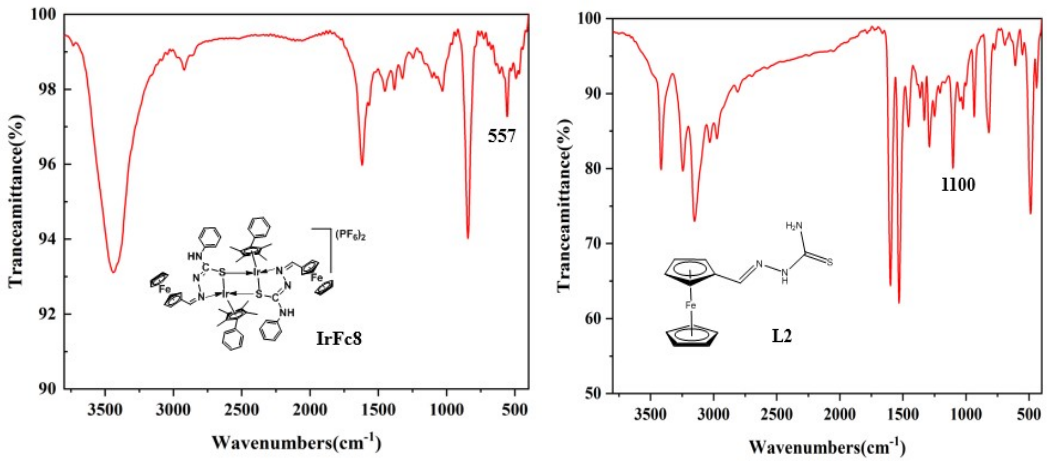


Figure S6 FT-IR of IrFc8 and L2.

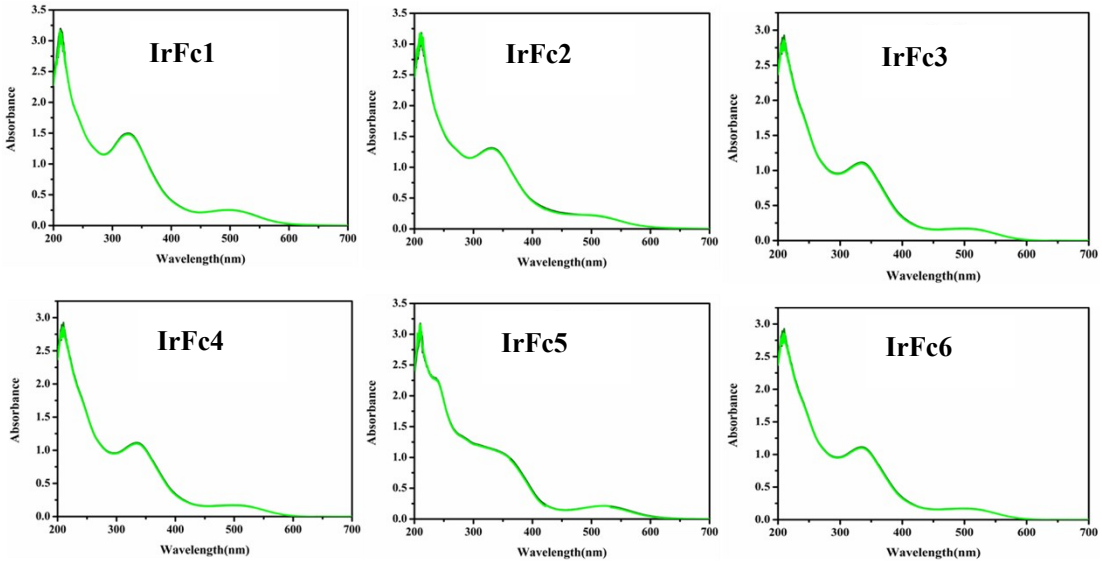


Figure S7 The stability of IrFc1-IrFc6 in 20% DMSO/80% H_2O (*v/v*) solution at 298 K were recorded by UV-Vis spectra over 8 h.

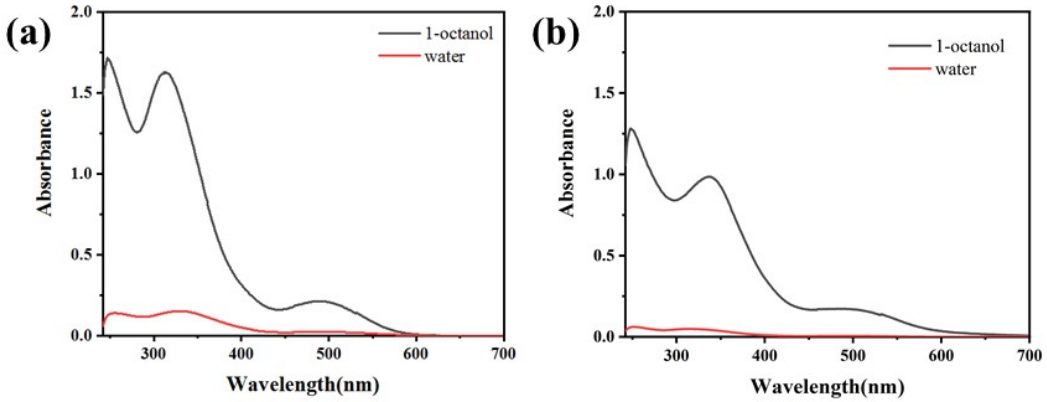


Figure S8 UV-Vis spectra of IrFc7 (a) and IrFc8 (b) in the 1-octanol and water solution.

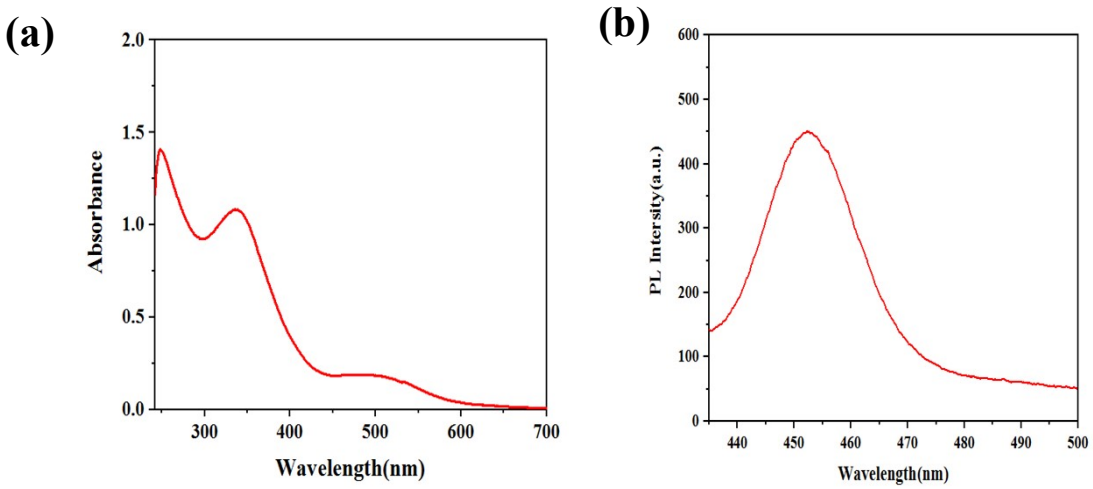


Figure S9 UV-Vis (a) and fluorescence spectra (b) of IrFc8 (20 μM) in DMSO solution.

3. Tables

Table S1 IC_{50} values of L1-L4 and IrPh towards A549, Hela cells after 24 h of incubation.

Complexes	IC_{50}（μM）	
	A549 cells	Hela cells
IrPh	>100	>100
L1	92.91±1.32	＞100
L2	＞100	＞100
L3	＞100	＞100
L4	＞100	＞100

Table S2 Crystallographic data and structure refinement for IrFc2.

Empirical formula	$C_{56}H_{62}F_{12}Fe_2Ir_2N_6P_2S_2$	Density (mg/m^3)	3.349
Formula weight	373.11	Z	37
F(000)	6105	Cryst size (mm)	0.46×0.20×0.04
Volume (Å^3)	6844.4(17)	Wavelength (Å)	0.71073
Theta range for data collection (deg)	2.023 to 26.000	Crystal system	Monoclinic
Limiting indices	-13≤h≤14, -36≤k≤36, -24≤l≤24	Space group	P21/n
Reflections collected/unique	13450/13450 [R(int) = 0.0293]	a (Å)	11.6402(16)
Data/restraints/parameters	13450/2/715	b (Å)	29.210(4)
Goodness-of-fit on F^2	1.064	c (Å)	20.134(3)
Final R indices [I>2sigma(I)]	R1=0.0544, wR2=0.1650	α (°)	90
R indices (all data)	R1=0.0687, wR2=0.1791	β (°)	91.112(5)
		γ (°)	90

Table S3 Flow cytometry analysis to determine the percentages of apoptotic cells, using Annexin V-FITC/PI staining, after exposing A549 cells to IrFc8.

	Concentration	Population (%)			
		Viable	Early apoptosis	Late apoptosis	Non-viable
Control		79.1±0.2	0.68±0.5	8.85±0.1	11.37±0.1
IrFc8	$1.0\times IC_{50}$	70.7±0.9	0.78±0.2	18.4±0.7	10.12±0.03
	$2.0\times IC_{50}$	62.8±3.3	1.33±0.3	31.0±2.0	4.87±0.07
	$3.0\times IC_{50}$	21.3±3.3	1.65±0.3	68.3±2.0	8.75±0.07

Table S4 Cell cycle analysis carried out by flow cytometry using PI staining after exposing A549 cells to IrFc8.

	Concentration	Population (%)		
		G_1 phase	S phase	G_2 phase
Control		65.52±0.1	10.28±0.5	24.20±2.3
IrFc8	$0.5\times IC_{50}$	65.91±2.7	10.61±0.8	23.48±1.3
	$1.0\times IC_{50}$	66.45±0.7	10.06±0.4	23.49±0.8
	$2.0\times IC_{50}$	77.32±1.3	6.47±0.1	16.20±0.7

Table S5 The mitochondrial membrane polarization of A549 cells induced by IrFc8.

	Concentration	Population (%)	
		JC-1 Aggregates	JC-1 Monomers
IrFc8	$0.5\times IC_{50}$	88.25±1.0	11.75±1.0
	$1.0\times IC_{50}$	85.68±1.5	14.31±0.8
	$2.0\times IC_{50}$	84.00±1.2	16.00±0.3
	$3.0\times IC_{50}$	82.99±0.9	17.01±0.7
Negative Control		89.86±0.2	10.14±0.2
Positive Control		27.67±1.4	72.33±2.4

Table S6 14-Day body weight (g)

Group	Animals	0d	2d	4d	6d	8d	10d	12d	14d
Cisplatin	194	18.93	19.10	19.43	19.76	20.08	20.29	20.75	21.39
	195	17.82	18.33	18.75	19.06	19.44	19.80	20.43	20.92
	196	18.38	18.66	19.03	19.27	19.56	19.92	20.31	20.88
IrFc8	189	18.30	18.67	19.05	19.72	20.24	20.68	21.03	21.35
	191	18.50	18.82	19.25	19.69	20.05	20.60	21.03	21.49
	192	17.87	18.05	18.34	18.95	19.33	19.56	20.01	20.43

Table S7 Tumor weight (mm^3)

Group	Animals	Tumor weight (g)
Cisplatin	195	1.4635
	196	1.0113
	198	1.3462
IrFc8	189	0.4671
	191	0.4483
	192	0.2615

Table S8 14-day tumor volume (mm^3)

Group	Animals	0d	2d	4d	6d	8d	10d	12d	14d
Cis-platin	194	111.01	190.78	268.32	425.92	647.15	1164.06	1779.06	2572.80
	195	98.78	158.76	231.89	332.80	516.91	829.99	1094.40	1468.53
	196	90.40	138.60	183.79	255.79	404.60	569.92	896.72	1335.10
IrFc8	189	98.84	143.65	181.30	241.88	305.94	400.95	497.66	622.91
	191	113.13	141.31	173.40	208.25	237.14	272.81	306.13	400.00
	192	91.85	111.01	129.60	159.74	205.80	247.50	294.40	349.27