Electronic Supplementary Information (ESI)

Cyclometalated Ru(II)-NHC Complexes with Phenanthroline Ligand Induce Apoptosis Mediated by Mitochondria and Endoplasmic Reticulum Stress in Cancer Cells

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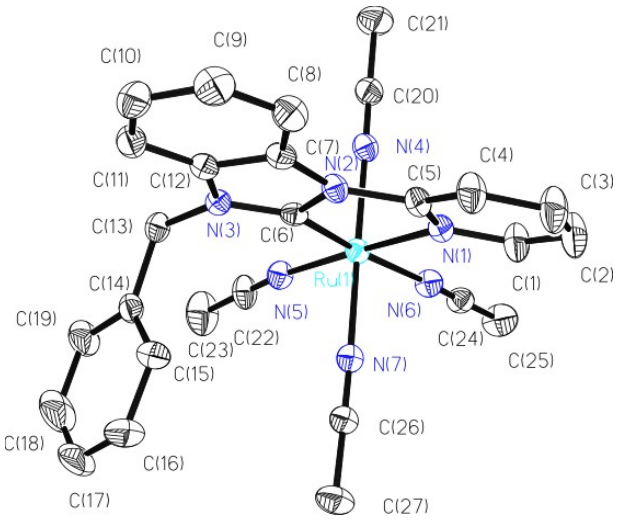
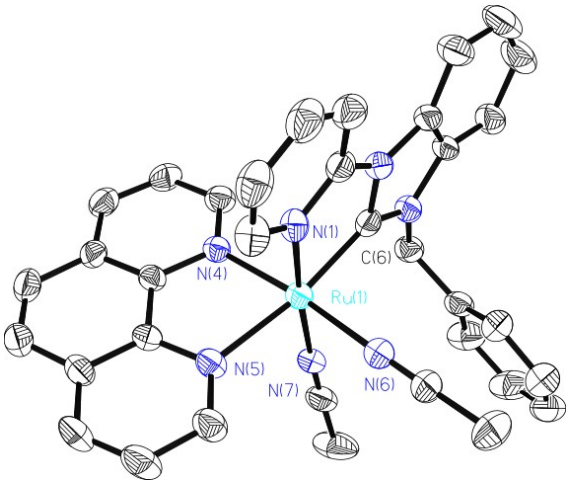
A. ORTEP drawing of **Ru1**-**Ru4** and selected bond lengths and angles.



ORTEP drawing of **Ru1** showing atomic numbering scheme at 50% probability ellipsoids. Selected bond lengths (Å) and angles(deg): Ru(1)-C(6) 1.993(5), Ru(1)-N(7) 2.041(4), Ru(1)-N(4) 2.049(5), Ru(1)-N(5) 2.057(5), Ru(1)-N(1) 2.075(4), Ru(1)-N(6) 2.152(5), C(6)-Ru(1)-N(7) 89.97(17), C(6)-Ru(1)-N(4) 90.63(17), N(7)-Ru(1)-N(4) 179.37(15), C(6)-Ru(1)-N(5) 101.20(17), N(7)-Ru(1)-N(5) 88.61(15), N(4)-Ru(1)-N(5) 91.49(16), C(6)-Ru(1)-N(1) 78.27(16), N(7)-Ru(1)-N(1) 89.21(14), N(4)-Ru(1)-N(1) 90.70(15), N(5)-Ru(1)-N(1) 177.76(16), C(6)-Ru(1)-N(6) 174.30(17), N(7)-Ru(1)-N(6) 91.19(17), N(4)-Ru(1)-N(6) 88.20(17), N(5)-Ru(1)-N(6) 84.42(15), N(1)-Ru(1)-N(6) 96.15(15).



ORTEP drawing of **Ru2** showing atomic numbering scheme at 50% probability ellipsoids. Selected bond lengths (Å) and angles(deg): Ru(1)-C(6) 1.975(5), Ru(1)-N(7) 2.028(5), Ru(1)-N(6) 2.041(5), Ru(1)-N(1) 2.053(4), Ru(1)-N(4) 2.063(4), Ru(1)-N(5) 2.141(4), C(6)-Ru(1)-N(7) 99.88(19), C(6)-Ru(1)-N(6) 90.53(19), N(7)-Ru(1)-N(6) 88.16(18), C(6)-Ru(1)-N(1) 78.5(2), N(7)-Ru(1)-N(1) 175.35(16), N(6)-Ru(1)-N(1) 87.51(18), C(6)-Ru(1)-N(4) 95.44(18), N(7)-Ru(1)-N(4) 90.70(17), N(6)-Ru(1)-N(4) 174.03(17), N(1)-Ru(1)-N(4) 93.78(16), C(6)-Ru(1)-N(5) 170.61(19), N(7)-Ru(1)-N(5) 87.69(17), N(6)-Ru(1)-N(5) 95.29(17), N(1)-Ru(1)-N(5) 94.40(17), N(4)-Ru(1)-N(5) 78.81(16).

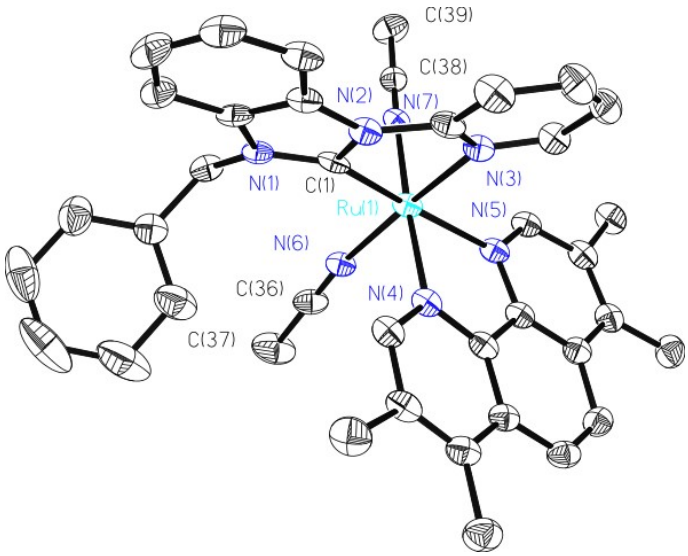


Fig. S3. ORTEP drawing of **Ru3** showing atomic numbering scheme at 50% probability ellipsoids. Selected bond lengths (Å) and angles(deg): Ru(1)-C(1) 1.977(4), Ru(1)-N(7) 2.038(4), Ru(1)-N(6) 2.039(4), Ru(1)-N(4) 2.055(4), Ru(1)-N(3) 2.062(3), Ru(1)-N(5) 2.134(4), C(1)-Ru(1)-N(7) 92.76(15), C(1)-Ru(1)-N(6) 100.25(16), N(7)-Ru(1)-N(6) 90.48(14), C(1)-Ru(1)-N(4) 94.55(15), N(7)-Ru(1)-N(4) 172.59(13), N(6)-Ru(1)-N(4) 87.01(14), C(1)-Ru(1)-N(3) 78.39(16), N(7)-Ru(1)-N(3) 94.47(14), N(6)-Ru(1)-N(3) 174.91(14), N(4)-Ru(1)-N(3) 88.20(14), C(1)-Ru(1)-N(5) 169.20(16), N(7)-Ru(1)-N(5) 94.16(13), N(6)-Ru(1)-N(5) 87.98(14), N(4)-Ru(1)-N(5) 78.79(14), N(3)-Ru(1)-N(5) 92.79(14).

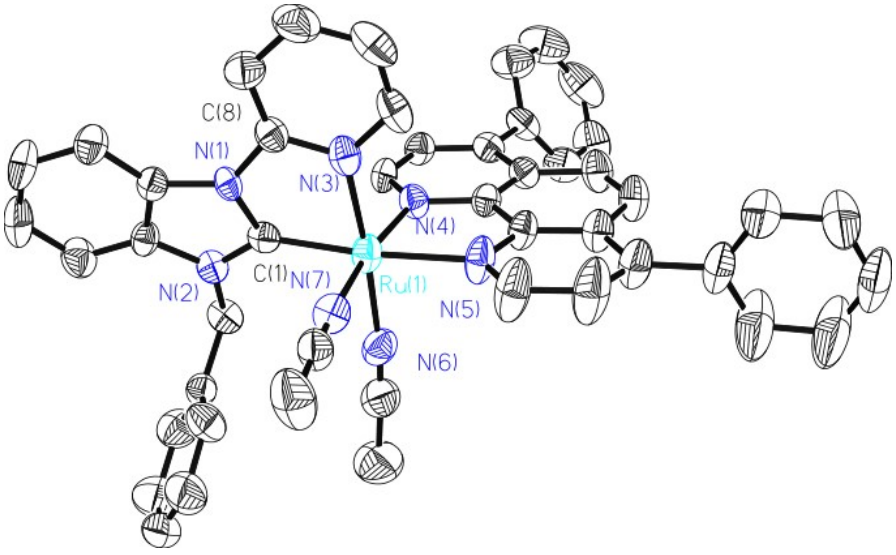
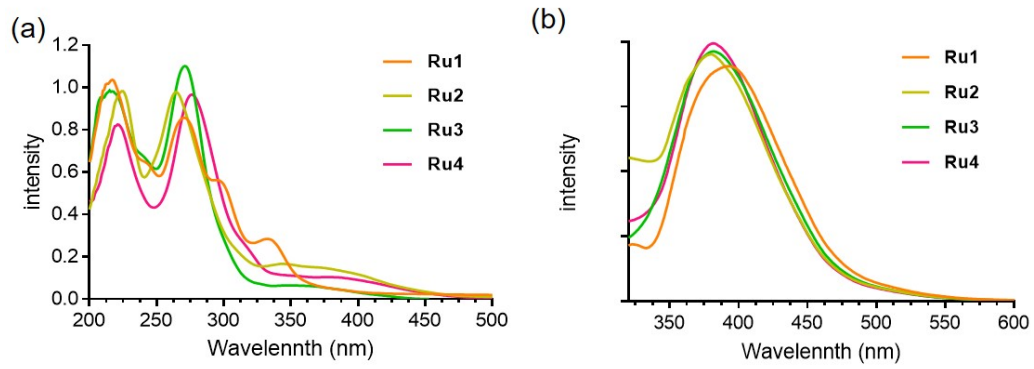


Fig. S4. ORTEP drawing of **Ru4** showing atomic numbering scheme at 50% probability ellipsoids. Selected bond lengths (Å) and angles(deg): Ru(1)-C(1) 1.983(6), Ru(1)-N(6) 2.035(6), Ru(1)-N(7) 2.042(6), Ru(1)-N(4) 2.056(5), Ru(1)-N(3) 2.078(5), Ru(1)-N(5) 2.129(5), C(1)-Ru(1)-N(6) 97.6(2), C(1)-Ru(1)-N(7) 88.1(2), N(6)-Ru(1)-N(7) 88.8(2), C(1)-Ru(1)-N(4) 100.1(2), N(6)-Ru(1)-N(4) 89.8(2), N(7)-Ru(1)-N(4) 171.84(19), C(1)-Ru(1)-N(3) 78.7(2), N(6)-Ru(1)-N(3) 175.5(2), N(7)-Ru(1)-N(3) 88.6(2), N(4)-Ru(1)-N(3) 93.34(19), C(1)-Ru(1)-N(5) 173.9(2), N(6)-Ru(1)-N(5) 88.4(2), N(7)-Ru(1)-N(5) 93.0(2), N(4)-Ru(1)-N(5) 78.91(18), N(3)-Ru(1)-N(5) 95.4(2).

B. Table S1. X-ray crystallographic data of Ru1-Ru4.

	Ru1	Ru2	Ru3	Ru4
formula	$C_{29}H_{30}F_{12}N_8P_2Ru$	$C_{35}H_{29}F_{12}N_7P_2Ru$	$C_{39}H_{37}F_{12}N_7P_2Ru$	$C_{47}H_{37}F_{12}N_7P_2Ru$
Fw.	881.62	938.66	994.77	1090.85
crystal system	Monoclinic	Monoclinic	Triclinic	Triclinic
space group	*P*2(1)/c	*P*2(1)/c	*P*-1	*P*-1
a/Å	10.494(7)	17.9763(9)	11.7572(16)	11.1717(6)
b/Å	15.943(10)	11.8371(6)	13.3532(18)	12.5812(7)
c/Å	22.151(15)	18.6576(12	15.822(2	18.4997(6)
β/deg	92.845(12)	107.175(6)	70.759(2)	93.676(3)
V/Å^3	3701(4)	3793.1(4)	2334.6(5)	2454.0(2)
Z	4	4	2	2
D_{calcd}, Mg/m^3	1.582	1.644	1.415	1.476
Refls collected	18573	17302	29121	31388
Independent reflections, R_{int}	6519, 0.0569	6677, 0.0520	8163, 0.0171	8632 , 0.0861
Goodness-of-fit on F^2	1.069	1.056	1.042	1.051
*R*1, *wR*2 [$I > 2\sigma(I)$]	0.0495, 0.1316	0.0596, 0.1501	0.0557, 0.1448	0.0710, 0.1859
*R*1, *wR*2 (all data)	0.0758, 0.1615	0.0864, 0.1797	0.0656, 0.1511	0.1119, 0.2117
Largest diff. peak and hole (e. Å^{-3})	0.847 and -0.657	0.759 and -0.708	1.274 and -1.030	0.937 and -0.533

C. UV-vis spectra and emission spectra of Ru1-Ru4 in CH_3CN



FigS1. (a) **UV-vis spectra of Ru1-Ru4** in CH_3CN. (b) Room-temperature emission spectra of **Ru1-Ru4** in CH_3CN (excitation wavelength at 300 nm).

D. MTT curves of Ru1-Ru4, ***cis*****-Pt,** and $HL{\cdot}PF_6$ **against cancer cells**

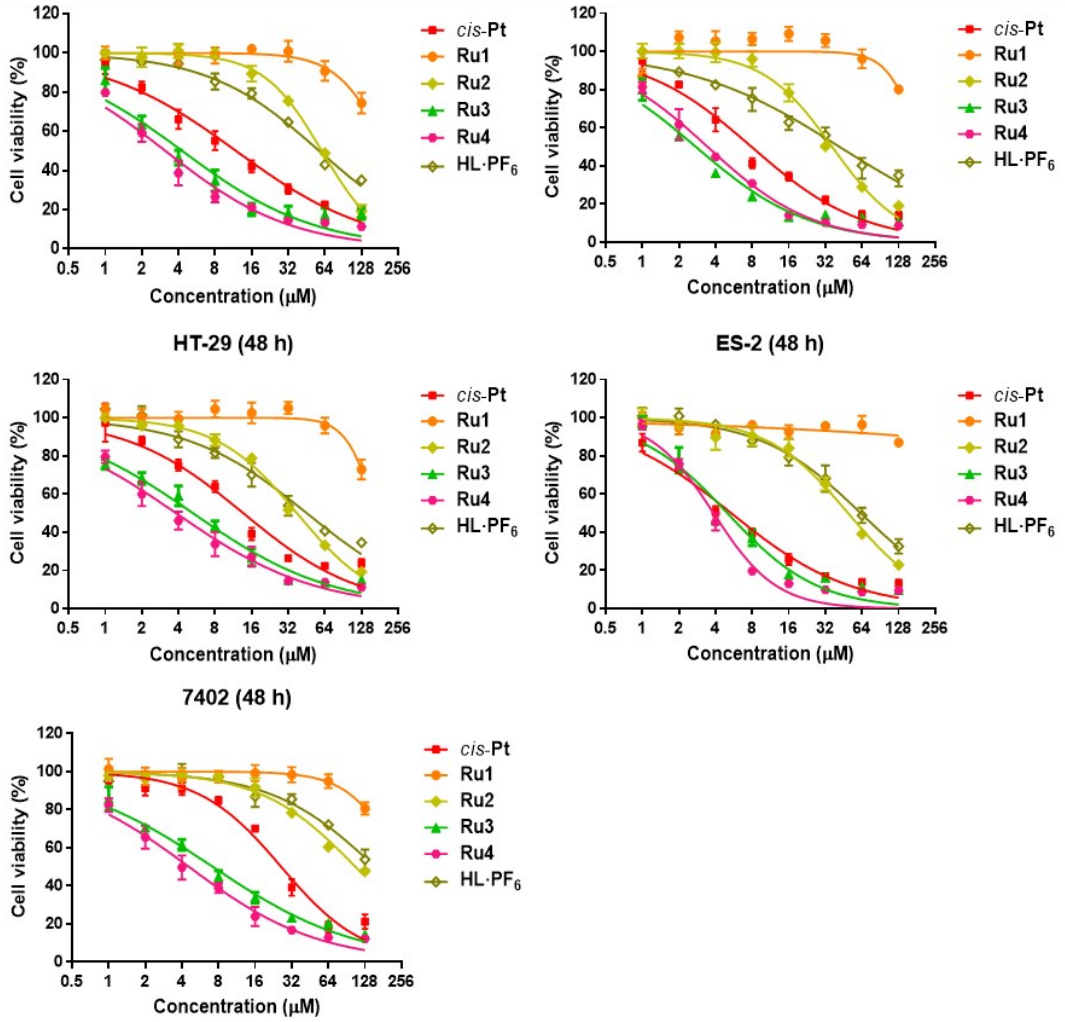


Fig.S2 The *in vitro* cytotoxic activities of **Ru1-Ru4,** ***cis*****-Pt,** and $HL{\cdot}PF_6$ against cancer cells

E. Cell experiment

Cells were cultured in 1640 medium containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin), and were maintained in a humid atmosphere at 37 °C with 5% CO_2. Drug solution preparation: **Ru1**-**Ru4** were first dissolved in DMSO to form a 32 mM solution. Then take 4 μL of the solution and dilute it in 1 ml of 1640 medium to obtain a drug solution of 128 μM. The concentrations of 64 μM, 32 μM, 16 μM, 8 μM, 4 μM, 2 μM and 1 μM were obtained by multiple dilution.

MTT viability assay

The *in vitro* cytotoxicity of the **Ru1**-**Ru4** complexes and cisplatin were measured by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The cells were plated in 96-well plates (4000-5000 cells per well) and incubated at 37 °C overnight. Then, cells were treated with a serial dilution of **Ru1**-**Ru4** and cisplatin in various concentrations for an additional 48 h. Following exposure, 30 μL MTT solution (5 mg/mL in PBS) was added to each well. The MTT solution was removed from the wells after 4 h and the purple MTT-formazan crystals were then dissolved by the addition of DMSO (100 μL). The absorbance in each well was measured at 490 nm using a microplate reader (Multiskan FC, Thermo Scientific).

Cell proliferation as determined by EdU incorporation

HeLa cells were plated in 48-well plates (2×10^4 cells per well) and incubated at 37 °C overnight. Then, **Ru1-Ru4** and cisplatin (4 μM) were added to the cells and then incubated for 24 h at 37 °C. DNA synthesis was quantified at the end of the drug treatment using a Click-iT EdU Alexa Fluor 488 Assay Kit (Invitrogen) according to the manufacturer's protocol. Finally, the cells were imaged by fluorescence microscopy (Olympus, IX72, Japan), $n \geq 5$ regions with 1500-2000 total cells were counted to assess the presence of cell proliferation

Wound healing assay

HUVEC cells were seeded into 6-well plates and reached 90% confluence after 24 h of incubation at 37 °C. A pipette tip (200 μL) was used to make a line across the cell monolayer. After washing with PBS, cells were cultured in serum-free culture medium containing **Ru3, Ru4** and cisplatin (4 μM). Images of wounds were acquired by optical microscopy (Olympus, IX72, Japan) at 0 and 24 h of the incubation. The migration ratio (%) = change in width value of each group at each time / avenge width

of initial wound × 100%.

Measurement of mitochondrial membrane potential

HeLa cells were seeded in 6-well plates (2×10^5 cells/well) overnight. After 12 h of treatment with **Ru3, Ru4,** or cisplatin (4 μM), the cells were incubated with 5 μM of 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) for another 30 min. After incubation, the cells were washed twice with prewarmed PBS, harvested, and analyzed with a flow cytometer (Cytoflex, Beckman Coulter, American). The JC-1 fluorescence data were recorded and analyzed with the CytExpert software. A parallel batch of treated cells was stained with JC-1 and washed with PBS before visualization under a fluorescence microscope (Olympus, IX72, Japan).

Intracellular Reactive Oxygen Species (ROS) detection

For this assay, HeLa cells were seeded in a 6-well plate (2×10^5 cells/well). After 12 h of growth, the cells were treated with **Ru3, Ru4,** or cisplatin (4 μM) for 12 h, harvested, and washed twice with PBS. Then, the cells were resuspended in 1 mL of 1640 solution with 5 μM DCFH-DA and incubated for 30 min at 37 °C. After incubation, the samples were washed with PBS and analyzed for DCF fluorescence in a flow cytometer (Cytoflex, Beckman Coulter, American) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The fluorescence data were recorded and analyzed with the CytExpert software with 1×10^4 cells in each sample. ROS generation was expressed in terms of the percentage of cells with DCF (green) fluorescence.

Apoptosis analysis by flow cytometry

HeLa cells were seeded at a density of 1×10^6 cells in each well and allowed to grow overnight at 37 °C.Then, the cells were incubated with **Ru3, Ru4** and cisplatin (4 μM) for another 24 h. The untreated cells were used as the control. After drug treatment, cells were centrifuged and washed with cold PBS repeatedly. The cell apoptosis analysis was determined by an Alexa Fluor 488 annexin V/PI apoptosis detection kit according to the manufacturer's protocol. Briefly, cells (1×10^5) were collected and resuspended in 1× annexin V binding buffer (500 μL) with 5 μL Alexa Fluor 488 annexin V and 1 μL PI (100 μg mL^{-1}) in each sample. After incubation for 15 min,

the stained cells were subjected to flow cytometry (Cytoflex, Beckman Coulter, American), and the result was analyzed by CytExpert software.

Western Blot Analysis

HeLa cells were recovered following treatment with **Ru3, Ru4** and cisplatin (24 h, 4 μM) and lysed in RIPA Lysis Buffer (Beyotime)-containing protease inhibitor phenylmethanesulfonyl fluoride (PMSF). A bicinchoninic acid protein assay was used to determine the protein concentration. Samples with equivalent amounts of protein were separated on 10% SDS-PAGE gels and then transferred to PVDF membranes. After incubation with specific antibodies, including cleaved PARP, cleaved Caspase 3 cleaved Caspase 9, PERK, P-PERK, eIF2α, p-eIF2α and CHOP (Cell Signaling technology), HRP-linked secondary antibodies were added and the bands were visualized using the chemiluminescent substrate on Biorad.

F. $LogP_{o/w}$ of Ru1-Ru4 and Intracellular accumulation of Ru1-Ru4 in HeLa cells

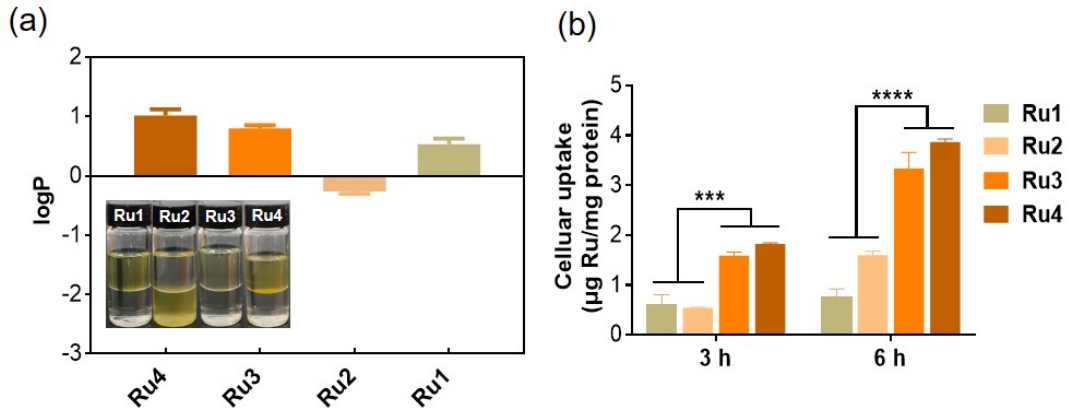
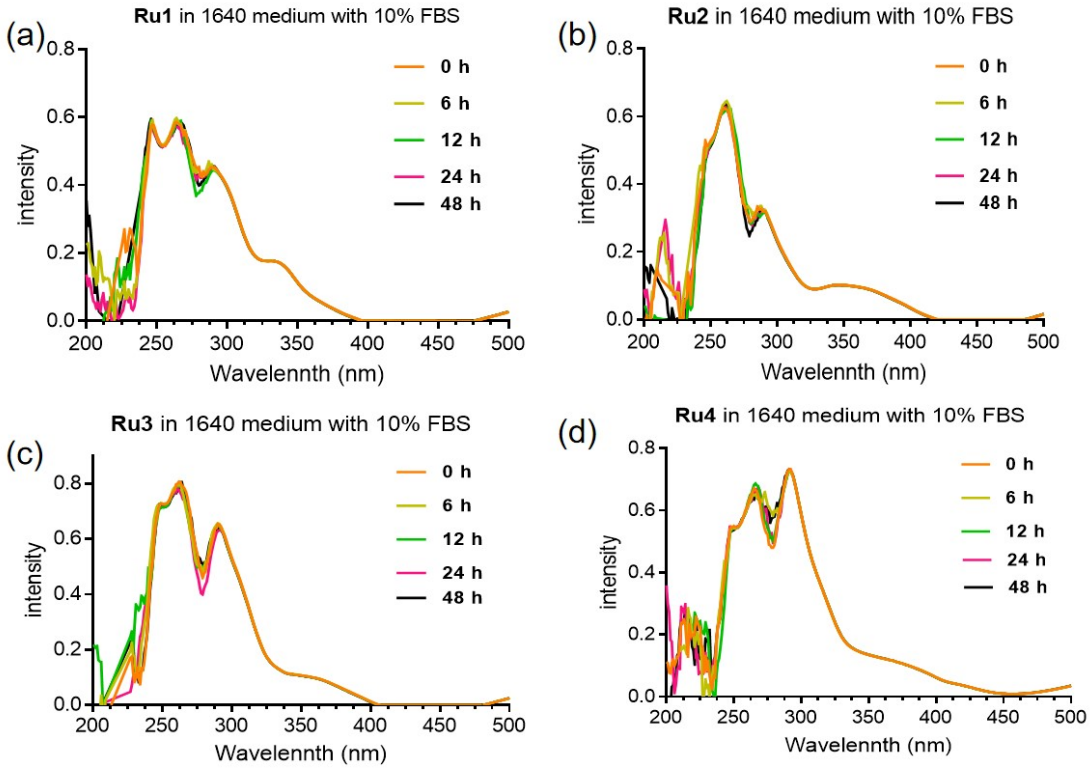


Fig.S3 (a) Lipophilicity of **Ru1-Ru4** by using oil-water distribution coefficient determination; (b) Intracellular accumulation of **Ru1-Ru4** in HeLa cells. Cells were treated with drugs at a concentration of 32 μM.

HeLa cells were seeded in 6-well plates at a density of 1.5×10^5 per well and incubated at 37 °C for 12 h. Then the cells were treated with cisplatin and **Ru1-Ru4** with a final concentration of 32 μM and incubated at 37 °C for 3 h or 6 h. The cells were washed with cold PBS three times, trypsinized, and centrifuged at 1000×g for 3 min to afford cell pellets. Subsequently, 100 μL cell lysis buffer (pH 7.6, 25 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% Deoxycholic acid) containing 1% protease

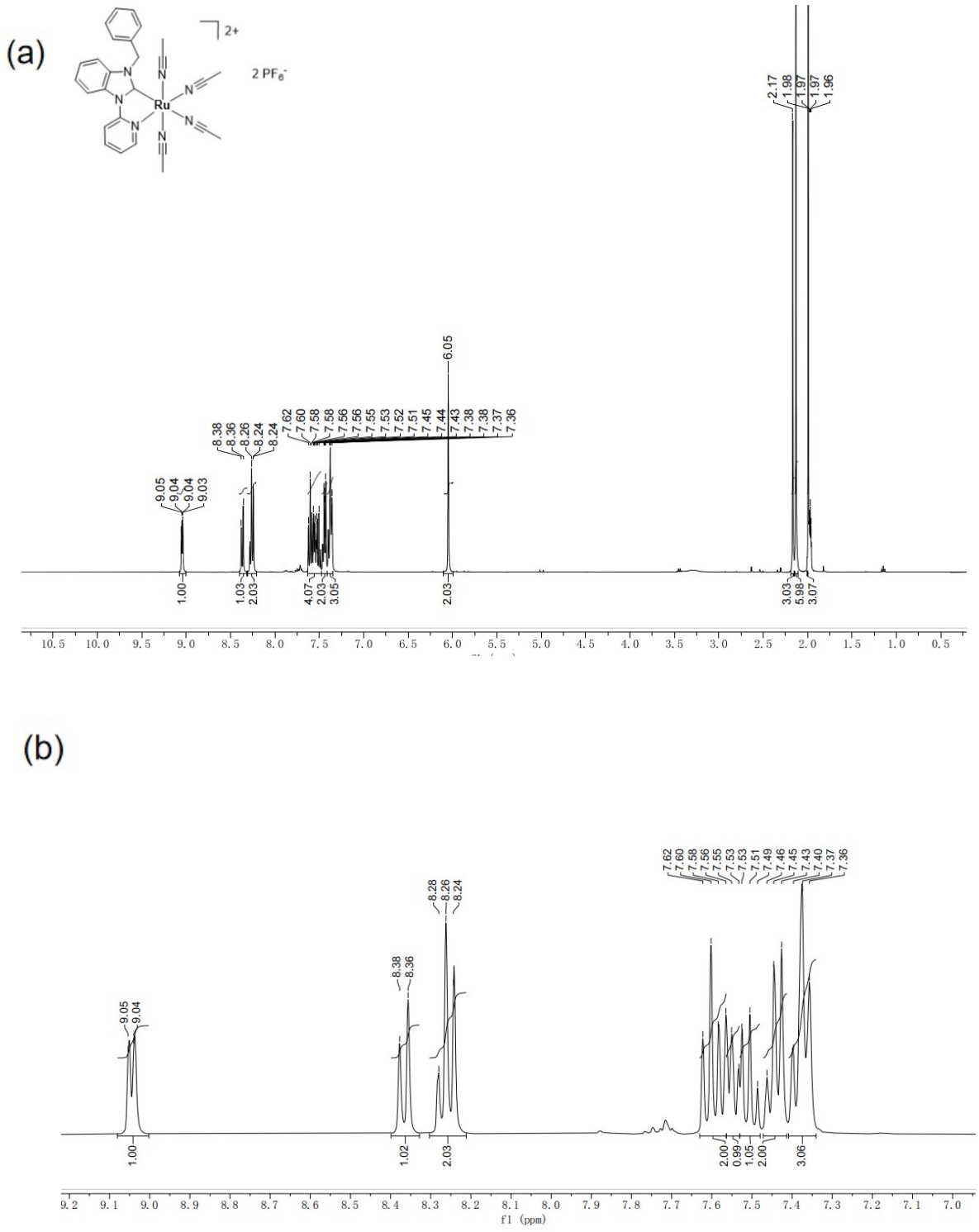
inhibitor cocktail set III (Novagen) was added to each sample to obtain the cell lysis solution. 80 μL cell lysate was diluted with 4 mL DI water and then the samples were subjected to ICP-MS for measurement of ruthenium contents. Another 20 μL cell lysate was used to determine the protein content using bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, USA)

G. **UV-vis spectra change of Ru1-Ru4 in 1640 medium**

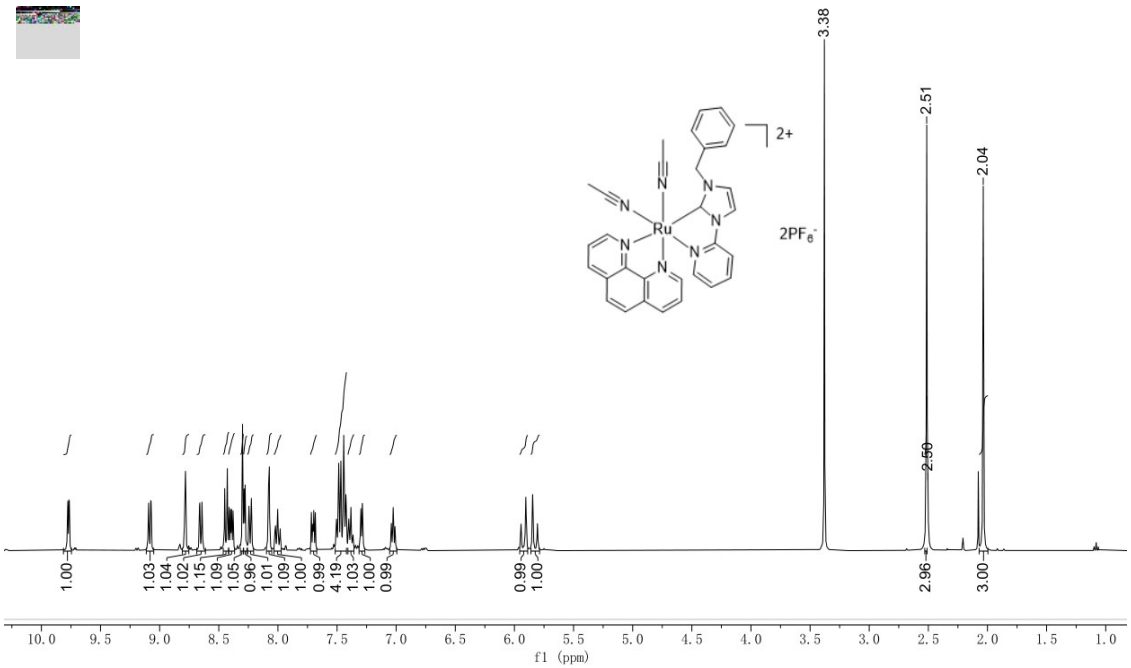


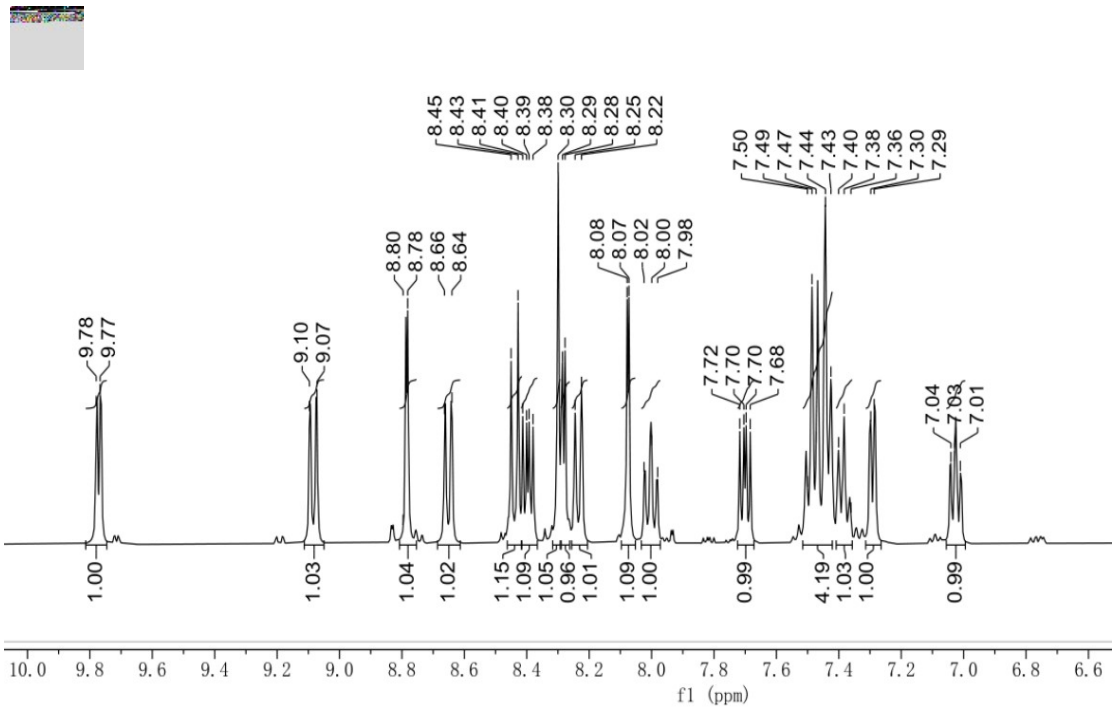
FigS4. (a) UV-vis spectra of **Ru1**-**Ru4** in 1640 medium with 10% FBS (v/v) after 6, 12, 24, and 48 h incubation at 37℃.

H. ^{1}H and ^{13}C NMR Spectrum of Ru1-Ru4

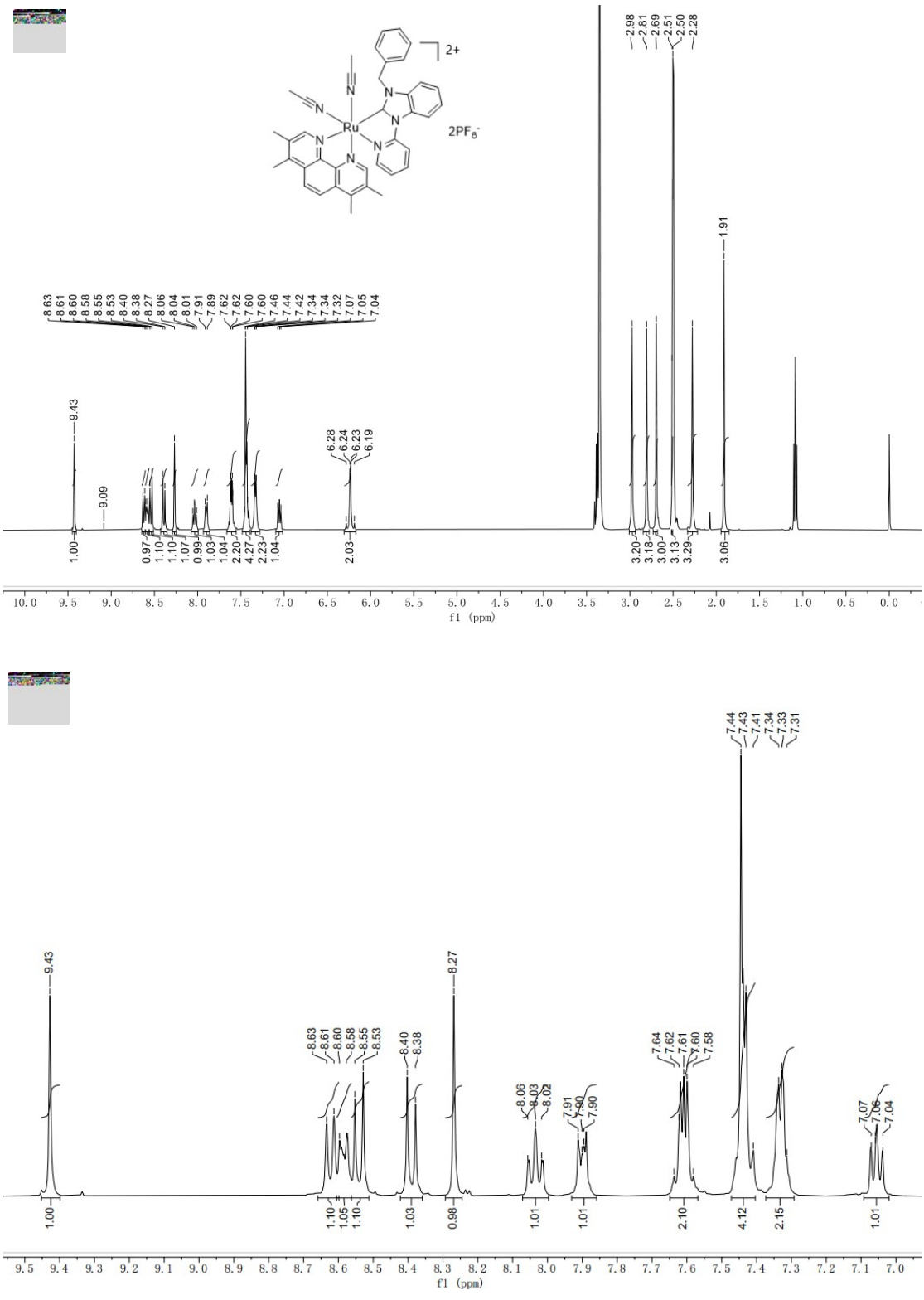


(a) ^{1}H NMR (CD_3CN) of **Ru1,** (b) aromatic region ^{1}H NMR of **Ru1**

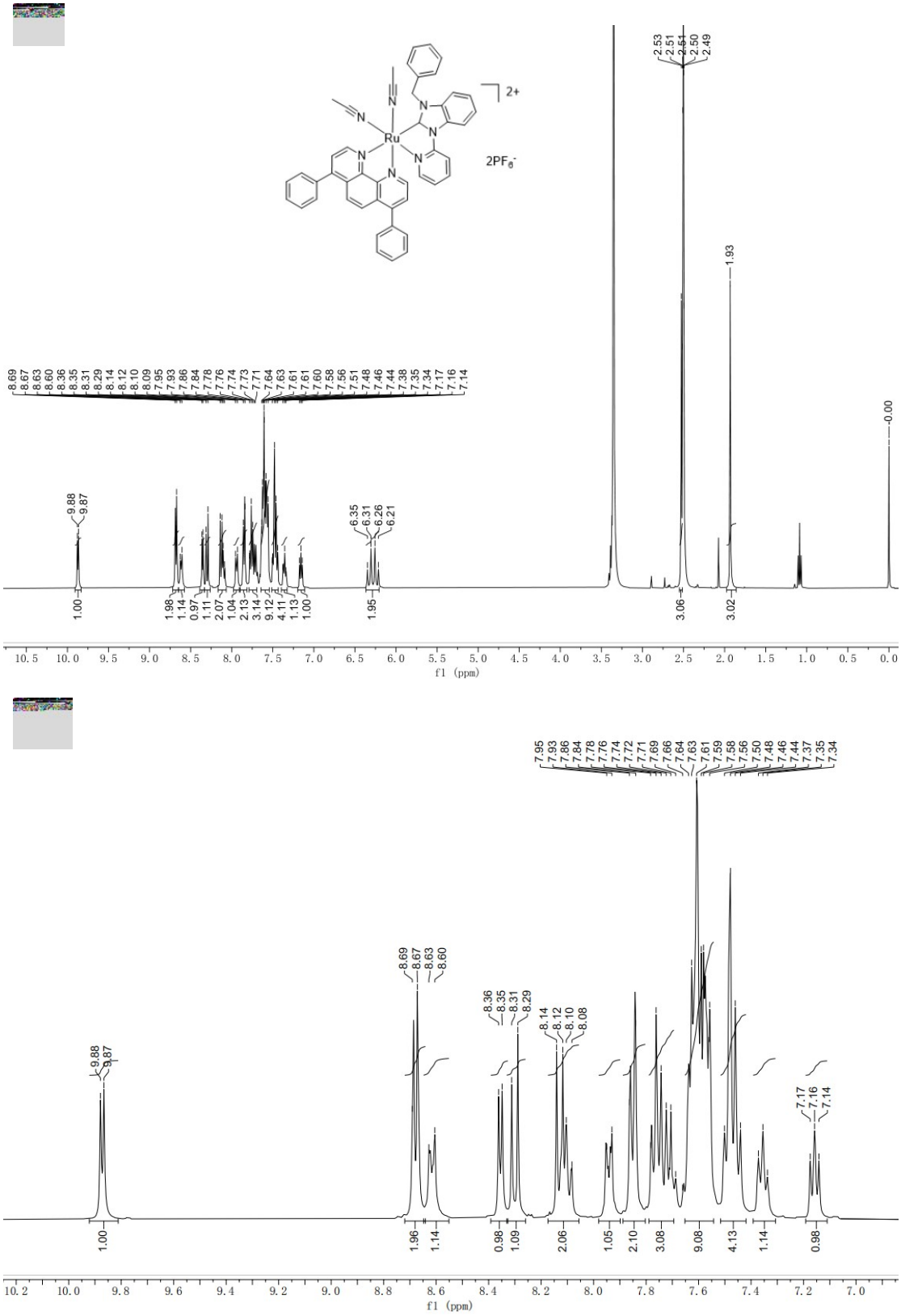




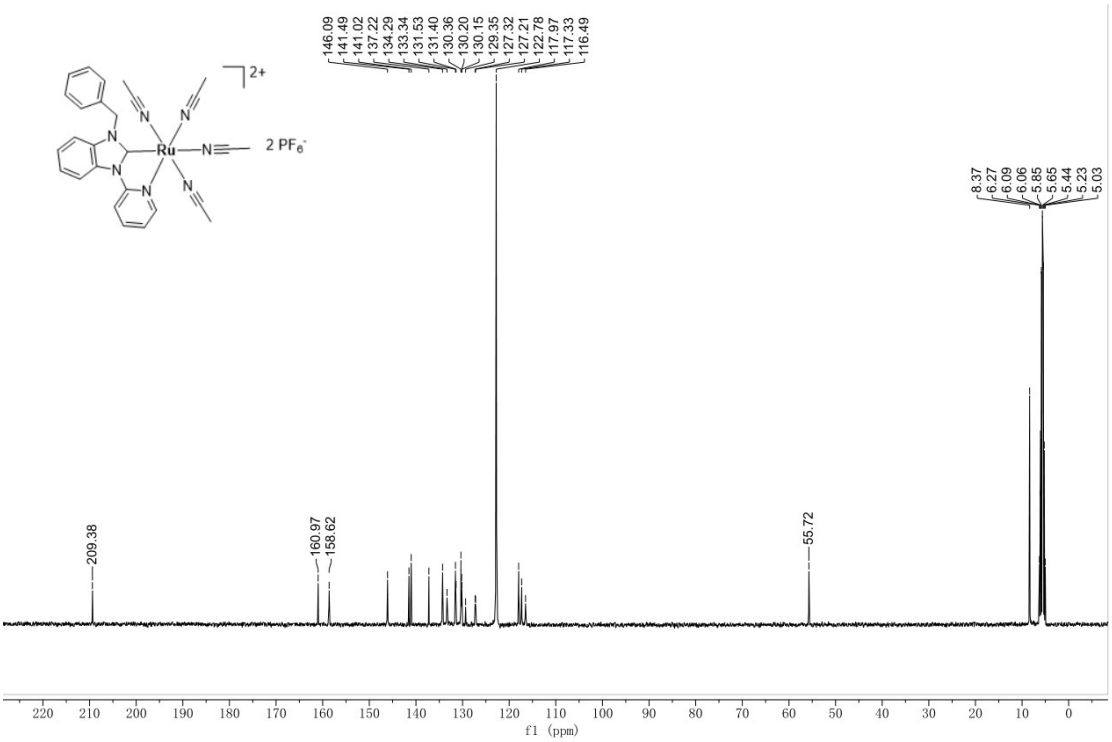
(a) ^{1}H NMR (DMSO) of **Ru2,** (b) aromatic region ^{1}H NMR of **Ru2**



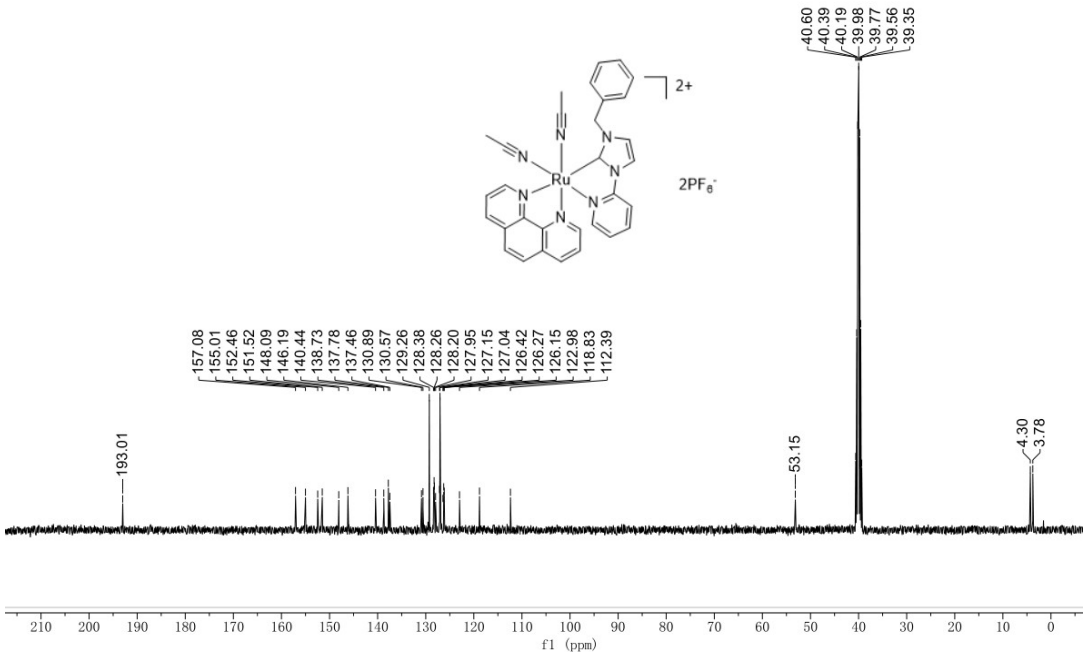
(a) ^{1}H NMR (DMSO) of **Ru3,** (b) aromatic region ^{1}H NMR of **Ru3**



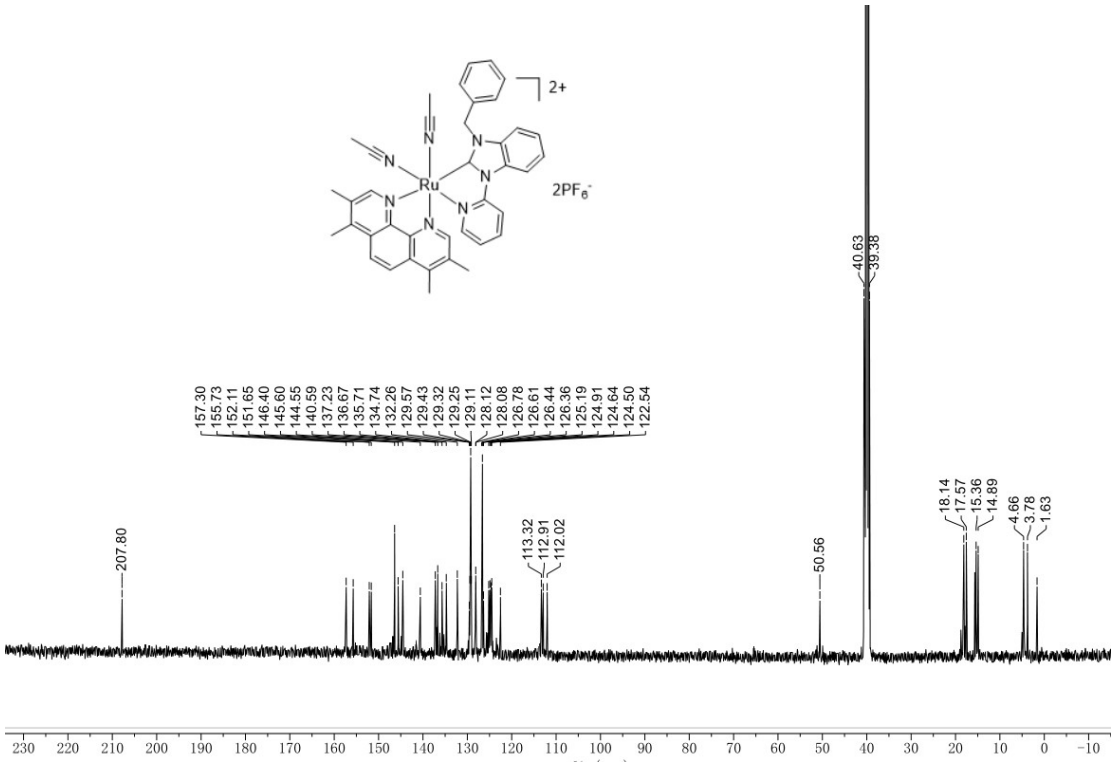
(a) ^{1}H NMR (DMSO) of **Ru4,** (b) aromatic region ^{1}H NMR of **Ru4**



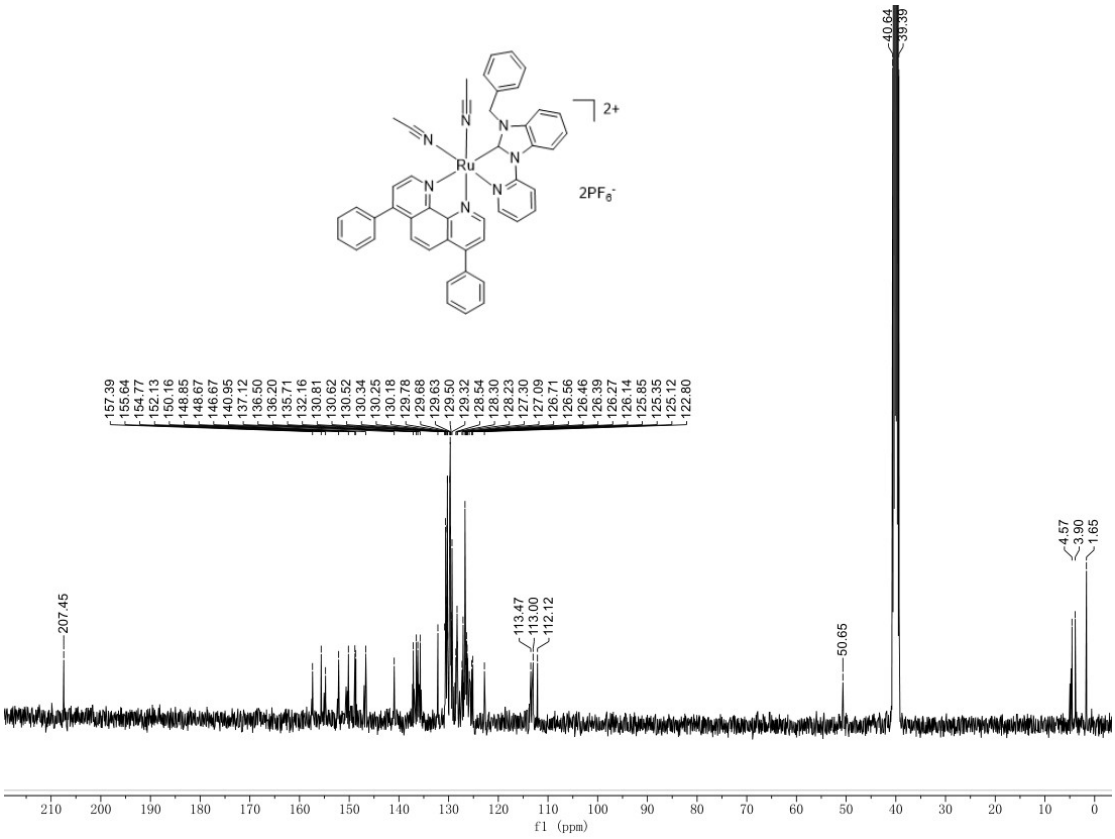
^{13}C NMR (CD_3CN) of **Ru1**



^{13}CNMR of **Ru2** (DMSO)



^{13}C NMR of **Ru3** (DMSO)



^{13}C NMR of **Ru4** (DMSO)