

Supporting Information

Rational design of type I photosensitizers based on Ru(II) complexes for effective photodynamic therapy under hypoxia

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1. Cell culture

HeLa cells lines were obtained by the Institute of Biochemistry and Cell Biology, SIBS, CAS (China). The cells were cultured in DMEM supplemented with FBS (10%, fetal bovineserum), 100 mg mL⁻¹ streptomycin and 100 U mL⁻¹ penicillin at 37 °C in humidified incubator with 5% CO₂.

2. Hypoxia cellular assay

Hela cells were planted on confocal petri dish and allowed to adhere for 24 h. Next, the Hela cells were treated with Ru(II) complexes. Then, the Hela cells were washed twice with DMEM. After, 1 mL fresh DMEM was added for imaging in Hela cells. Cell imaging experiments, flow counters were used to control the oxygen (O₂) concentrations at 5%. To control a certain oxygen concentration during imaging, the live cell station equipped with two flow counters which could control the flow of O₂ and nitrogen (N₂), respectively, was used. During the irradiation period, cells were incubated in the live cell station under 5% O₂ atmosphere.

3. ROS detection in HeLa cells

The HeLa cells were cultured with Ru(II) complexes (5 μM) for 2 h and treated with DCFH-DA (10 μM) for another 20 min at 37 °C. Then, cells were irradiated by white light with a xenon lamp (400-800 nm, 30 mW cm⁻²). The fluorescence of DCF was monitored by confocal microscopy ($\lambda_{\text{ex}} = 488 \text{ nm}$ and $\lambda_{\text{em}} = 500\text{-}540 \text{ nm}$).

4. Hydroxyl radicals detection in HeLa cells.

The HeLa cells were incubated with Ru(II) complexes (5 μM) for 0.5 h and treated with 10 μM HPF for 60 min at 37 °C. Then, cells were washed with PBS and then irradiated by white light with a xenon lamp (400-800 nm, 30 mW cm⁻²) for 10 min. The green fluorescence was monitored by confocal microscopy ($\lambda_{\text{ex}} = 488 \text{ nm}$ and $\lambda_{\text{em}} = 500\text{-}540 \text{ nm}$).

5. Cell viability in dark and during PDT process

HeLa cells were harvested and seeded in 96-well plates at $4\text{-}7 \times 10^4$ cells/well under 100% humidity, and were cultured at 37 °C with 5% CO₂ for 24 h. Ru(II) complexes at concentrations of 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 μM were respectively added into the wells. The dark viability of cells incubated with Ru(II) complexes was evaluated in dark at 37 °C and 5% CO₂ atmosphere for 24 h. The cells were further incubated for an additional 24 h under 5% O₂ or 21% O₂. Besides, PDT treatment was performed with white light (400-800 nm, 30 mW cm⁻²) for 10 min. Then, the cells were further incubated for 24 h under 5% O₂ or 21% O₂. MTT (1 mg mL⁻¹, 150 μL per well) was added to the wells and cells was incubated for another 4 h and then the medium was replaced with dimethyl sulfoxide (DMSO) (100 μl) per well, and OD570 was monitored by an enzyme-linked immunosorbent assay (ELISA) reader. After three parallel tests, the following formula was used to calculate the inhibition of cell growth: Cell viability (%)

= (mean of Abs. value of treatment group/mean Abs. value of control) × 100%. Then, the SPSS software was used to calculate the values of IC₅₀ through three parallel experiments. Complexes **Ru1-Ru4** were dissolved in DMSO and diluted to different concentrations with medium (the final concentration of DMSO should be less than 0.4%), and others procedure were consistent with means of MTT.

6. Analysis of mitochondrial membrane potential

HeLa cells were seeded and incubate at 37 °C for 12 h. The cells were incubated with Ru(II) complexes for 2 h with 5% CO₂ and then irradiated by white light (400-800 nm, 30 mW cm⁻²). After 15 min, the cells were stained with JC-1 (5 mg mL⁻¹) for 20 min. Cells were monitored by confocal microscopy in the red channel for J-aggregates ($\lambda_{\text{ex}} = 515 \text{ nm}$, $\lambda_{\text{em}} = 580\text{-}640 \text{ nm}$) and the green channel for JC-1 monomers ($\lambda_{\text{ex}} = 515 \text{ nm}$, $\lambda_{\text{em}} = 530\text{-}560 \text{ nm}$).

7. Calcein-AM/PI assays

To evaluated the viability of cells, the cells were incubated with Ru(II) complexes via Calcein-AM/propidium iodide (PI) staining. Cells were seeded at 100% humidity 37 °C with 5% CO₂ for 24 h. Then, Ru(II) complexes (5 μM) were added to the medium and cells were incubated for additional 2 h under 5% or 21% O₂. Then the cells were stained with PI (5 μL) and Calcein-AM (10 μL) in dark for 10 min and then irradiated with white light (400-800 nm, 30 mW cm⁻²) with a xenon lamp for 10 min. The confocal microscopy was used to monitor the images of cells in green channel for Calcein-AM ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 500\text{-}560 \text{ nm}$) and red channel for PI ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 600\text{-}680 \text{ nm}$) after 2 h.

8. Statistical Analysis

Statistical analysis was obtained by Student's t-test for three groups, and one-way analysis of variance for multiple groups. All results were expressed as the mean ± s.d. unless otherwise noted. A value of p < 0.05 was considered statistically significant.

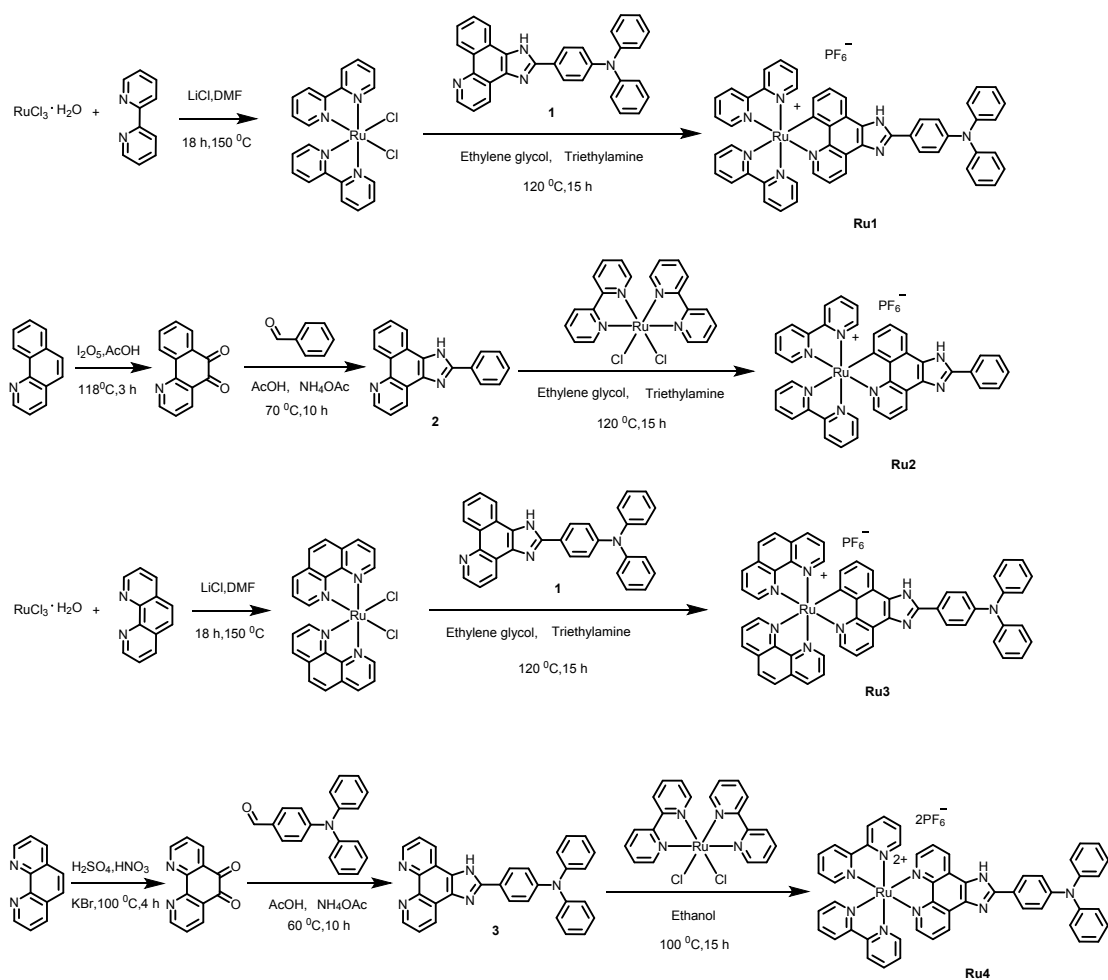


Figure S1 The synthetic route of Ru(II) complexes.

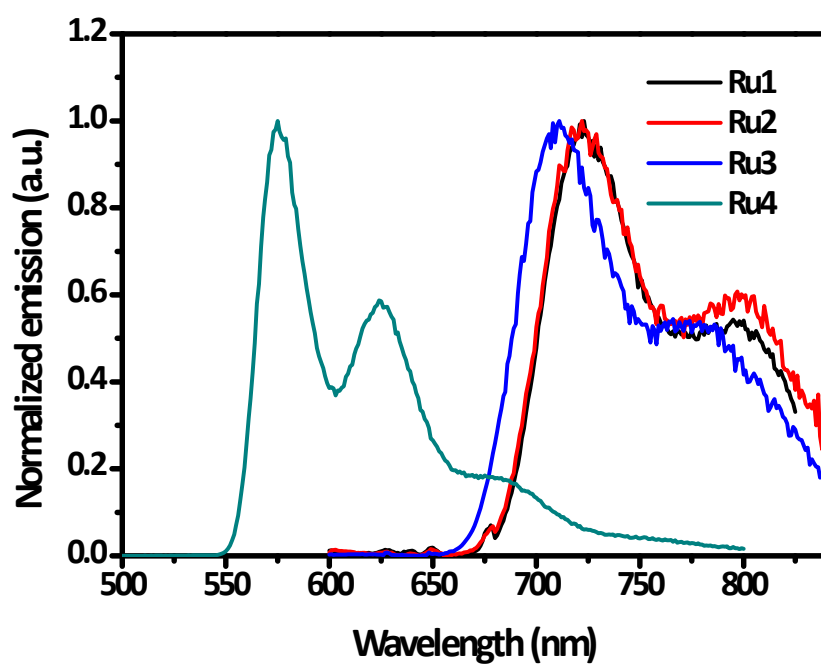


Figure S2. Normalized emission spectra of Ru1, Ru2, Ru3 ($\lambda_{\text{ex}} = 365$ nm) and Ru4 ($\lambda_{\text{ex}} = 475$ nm) in 2-methyltetrahydrofuran at 77 K.

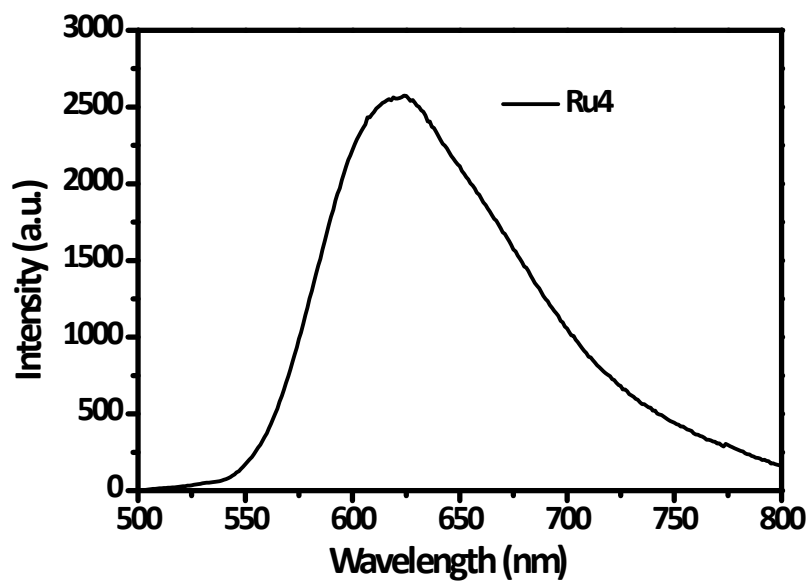


Figure S3. Emission spectrum of Ru4 ($\lambda_{\text{ex}} = 475$ nm) in MeCN at 298 K

Table S1. Selected calculated singlet excited-state transitions for **Ru1-Ru4**

Complex	Trans	Energy (eV)	<i>f</i>
Ru1	$S_0 \rightarrow S_1$	2.1460 (577.75 nm)	0.0052
Ru2	$S_0 \rightarrow S_1$	2.1506 (576.50 nm)	0.0041
Ru3	$S_0 \rightarrow S_1$	2.1957 (564.68 nm)	0.0064
Ru4	$S_0 \rightarrow S_1$	2.5318 (489.71 nm)	0.1156

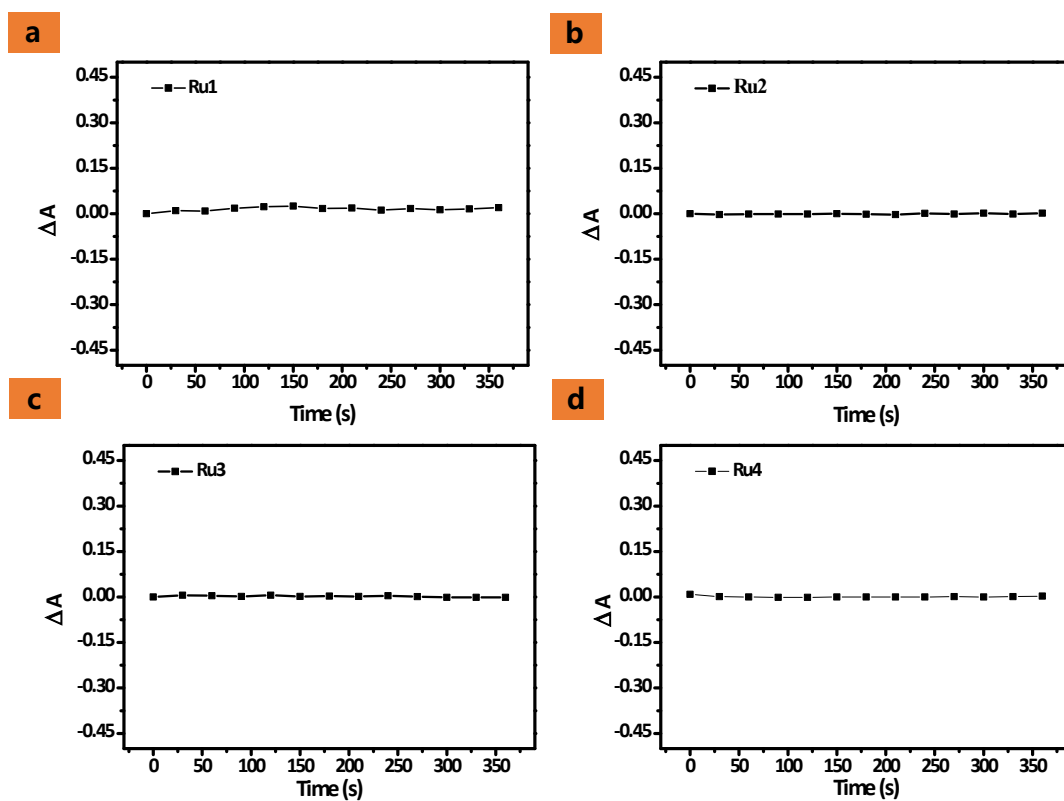


Figure S4. Absorption changes of **Ru1** (a), **Ru2** (b), **Ru3** (c) and **Ru4** (d) in methanol solution under different irradiation time (400-800 nm, 100 mW cm⁻²) at 25 °C.

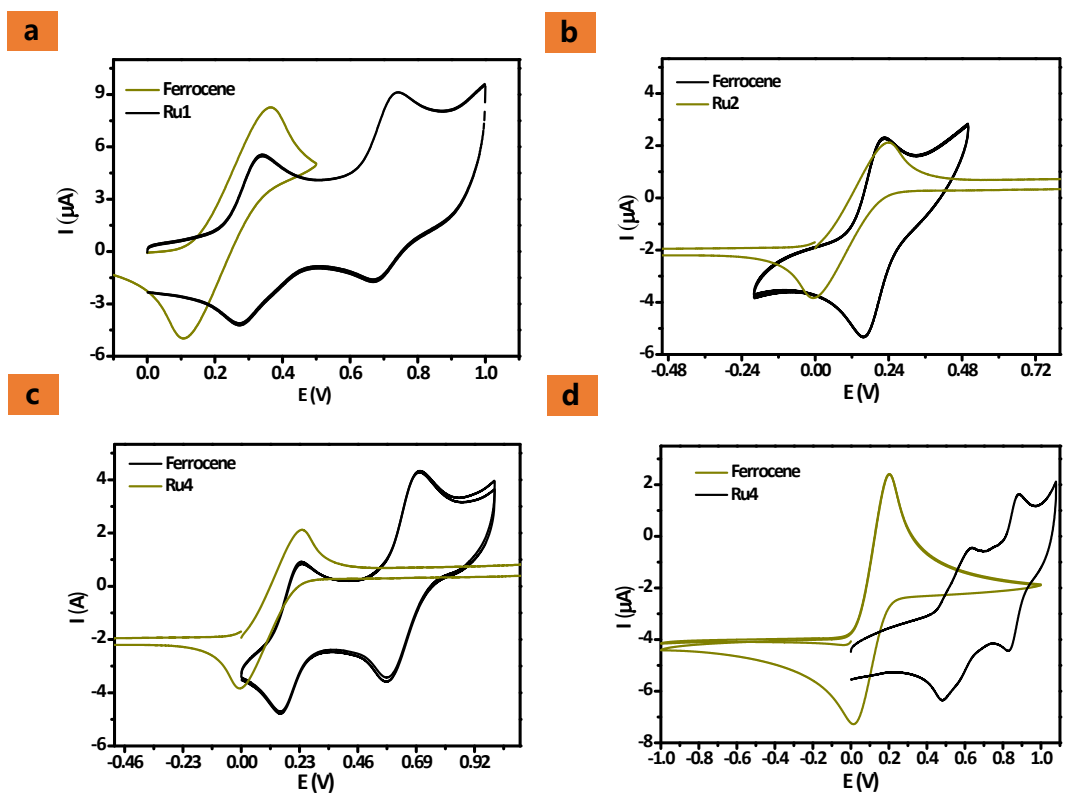


Figure S5. Cyclic voltammograms of **Ru1** (a), **Ru2** (b) and **Ru3** (c) **Ru4** (d) in CH₃CN with 0.01 M TBAPF₆ as the electrolyte at room temperature. Data were collected using the standard three electrode setup with a glassy carbon working electrode, platinum wire counter electrode and Ag/AgNO₃ reference electrode.

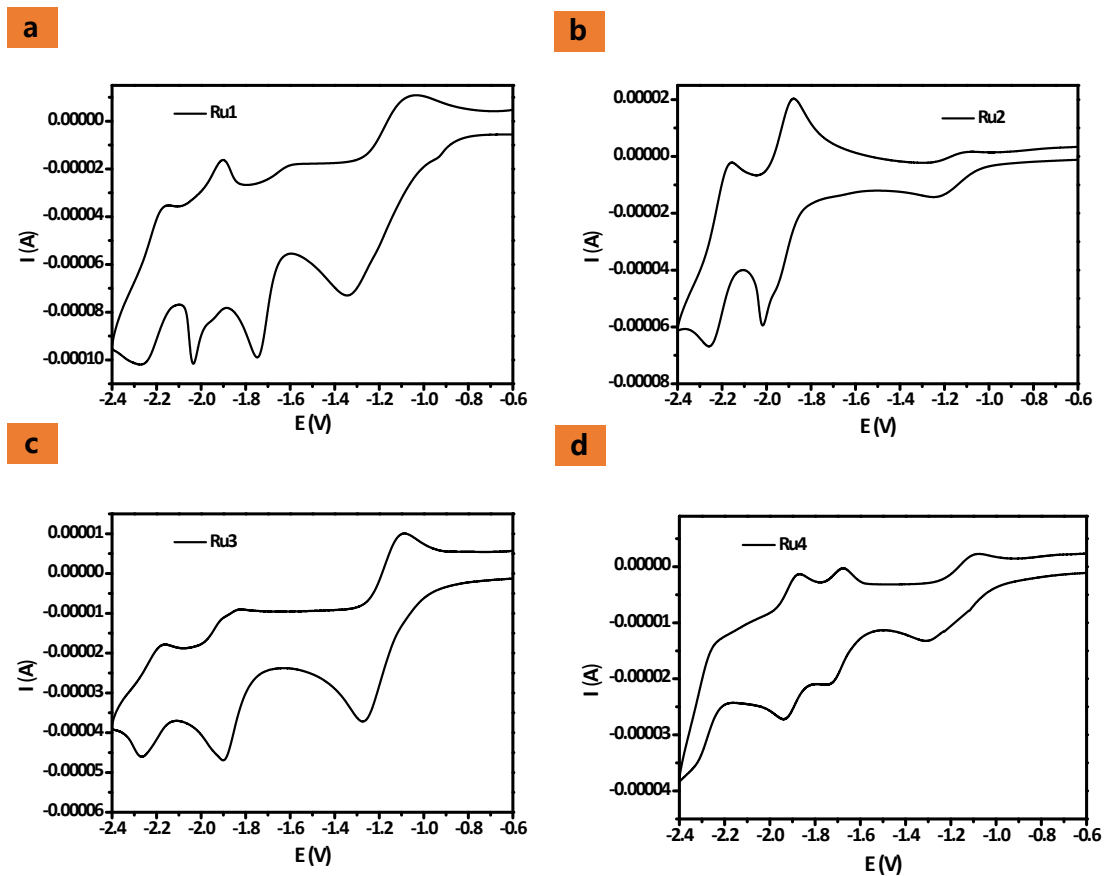


Figure S6. Cyclic voltammograms of **Ru1** (a), **Ru2** (b) and **Ru3** (c) **Ru4** (d) in CH_3CN with 0.01 M TBAPF_6 as the electrolyte at room temperature. Data were collected using the standard three electrode setup with a glassy carbon working electrode, platinum wire counter electrode and Ag/AgNO_3 reference electrode.

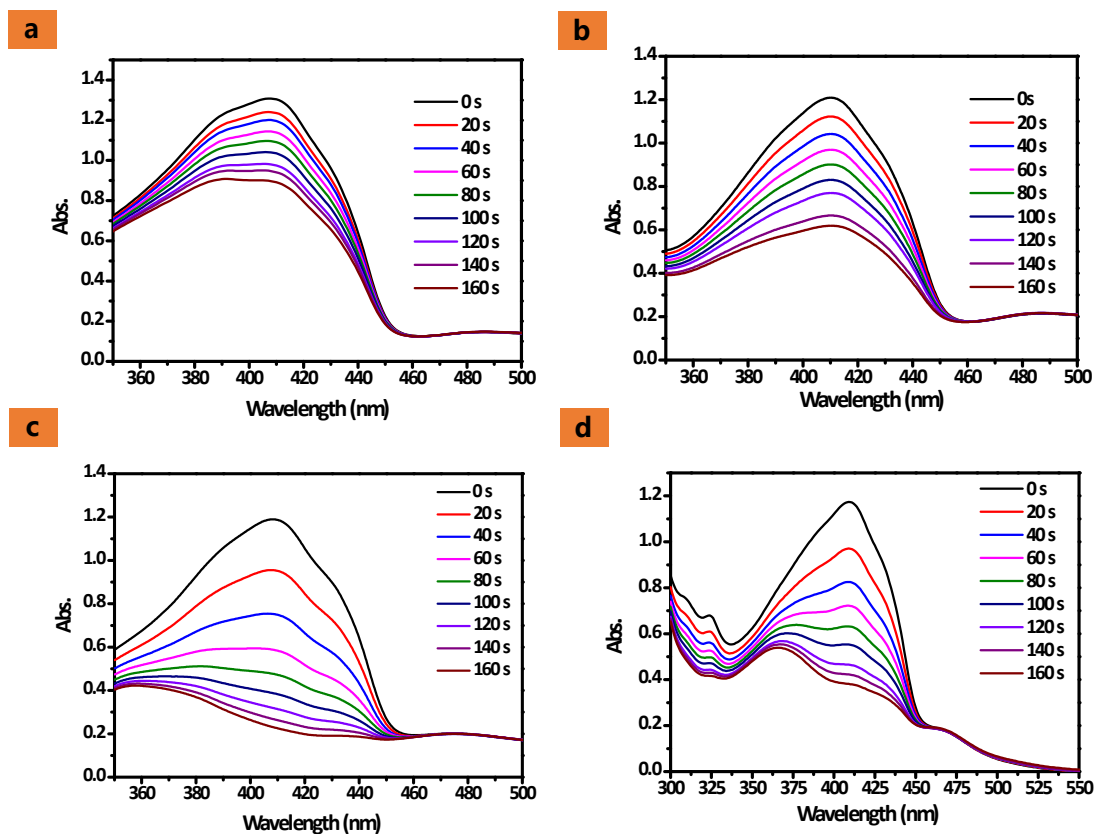


Figure S7. Absorption spectra of DPBF at different irradiated time in the presence of **Ru1**(a), **Ru2** (b), **Ru3** (c) and **Ru4** (d), respectively. The time interval was 20 s. The light source is a xenon lamp with a 475 ± 20 nm output (4.0 mW cm^{-2}).

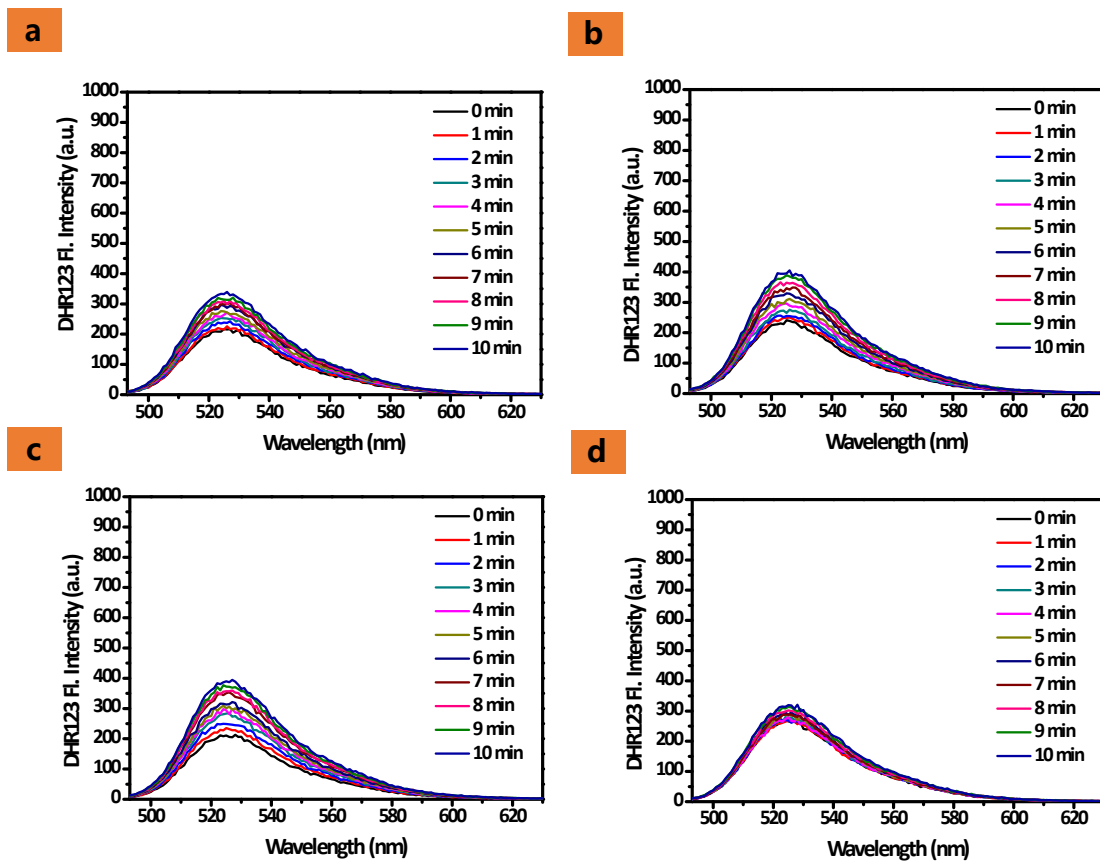


Figure S8. Fluorescence spectra for $O_2^{\cdot-}$ using DHR123 as fluorescence probe. The light source is a xenon lamp with a white light (400-800 nm) output (10.0 mW cm^{-2}). All groups (**Ru1** (a), **Ru2** (b), **Ru3** (c) and black) were in the 21% O_2 solution.

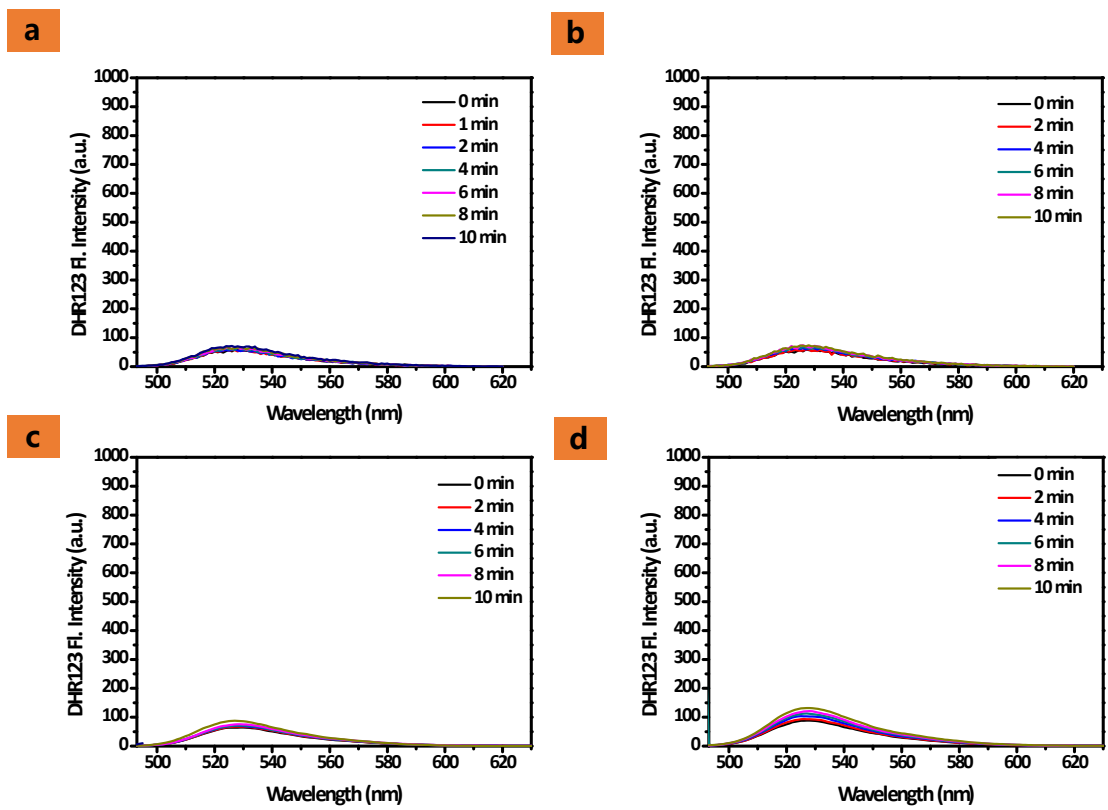


Figure S9. Fluorescence spectra for $O_2^{\bullet-}$ using DHR123 as fluorescence probe. The light source is a xenon lamp with a white light (400-800 nm) output (10.0 mW cm^{-2}). All groups (Ru1 (a), Ru2 (b), Ru3 (c) and black) were in the 5% O_2 solution.

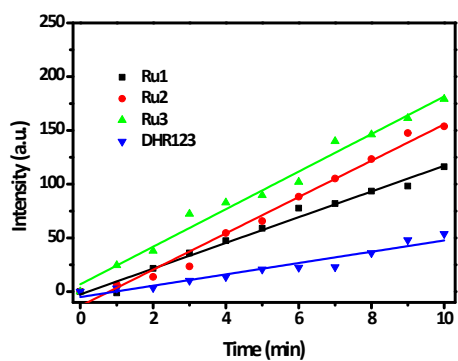
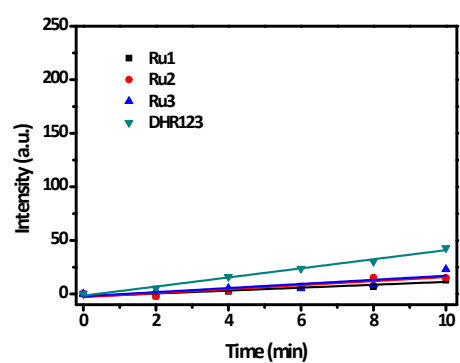
a**b**

Figure S10. Change of DHR123 fluorescence spectra at 525 nm as a function of irradiation time in the presence of Ru(II) complexes. All group were in the 21% O₂ solution(a), 5% O₂ solution(b).

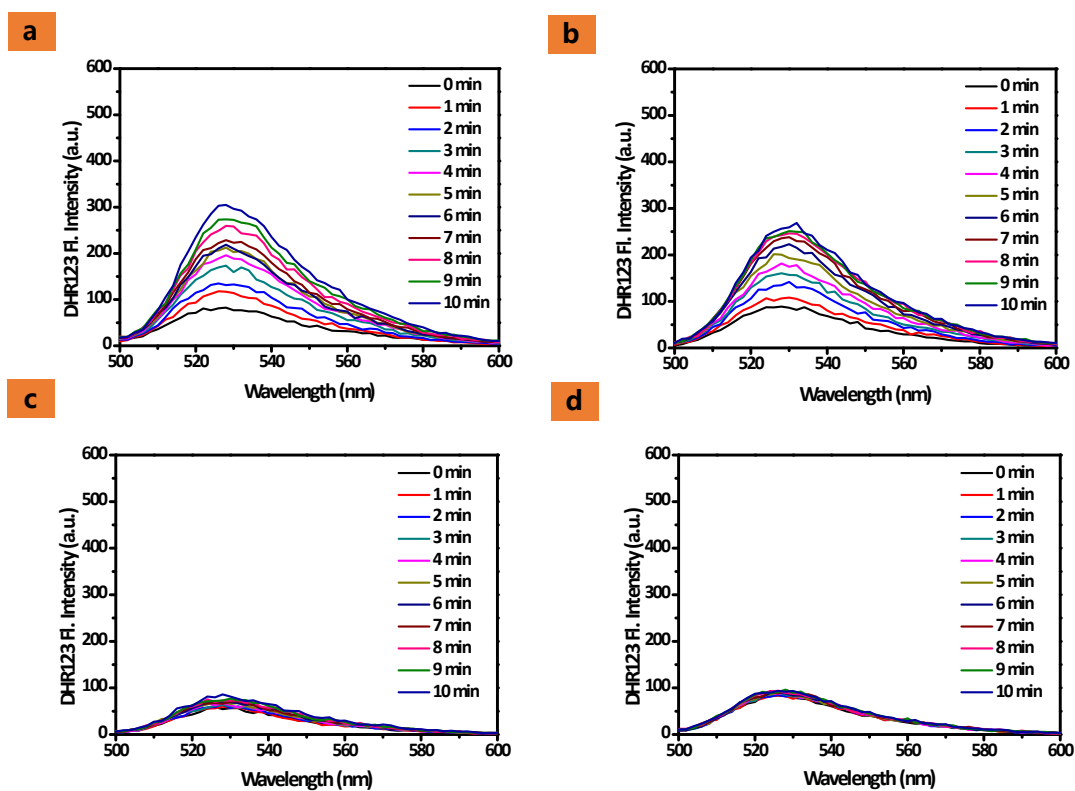


Figure S11. Fluorescence spectra for O_2^- using DHR123 as fluorescence probe. The light source is a xenon lamp with a white light (400-800 nm) output (10.0 mW cm^{-2}). **Ru4** (a), **Ru1** (c), and black (d) were in the 21% O_2 solution and **Ru4** (b) were in the 5% O_2 solution.

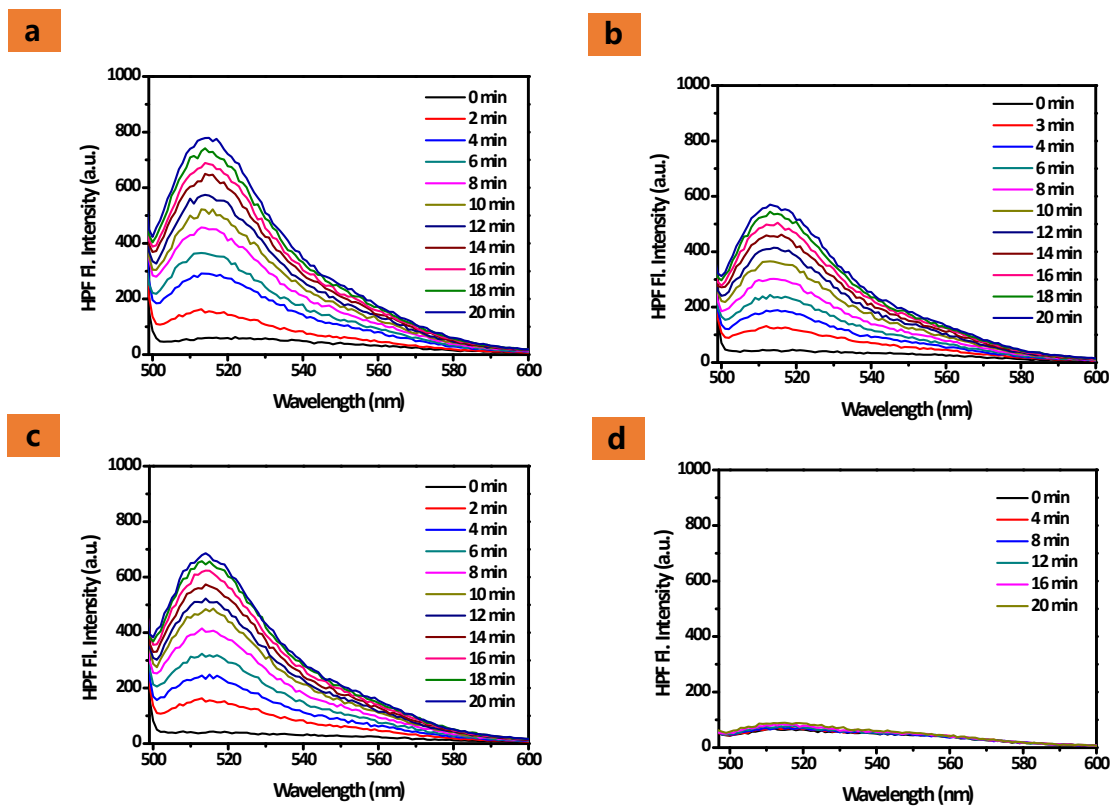


Figure S12. Fluorescence spectra for HPF of irradiation time in the presence of Ru(II) complexes. All groups (**Ru1** (a), **Ru2** (b), **Ru3** (c) and black (d)) were in the 21% O₂ solution.

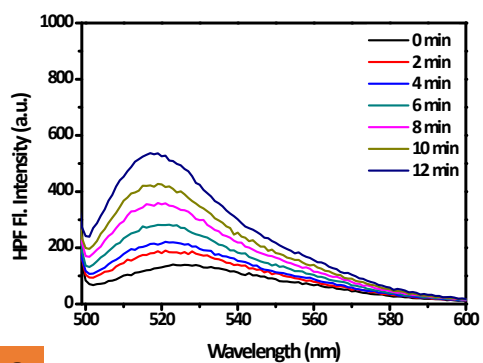
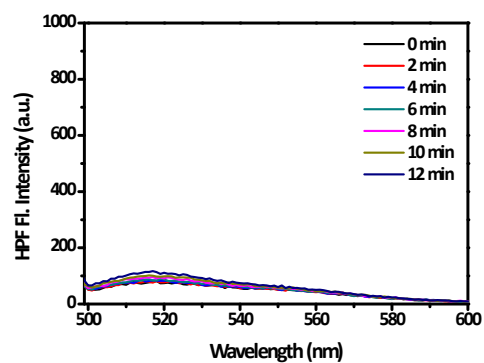
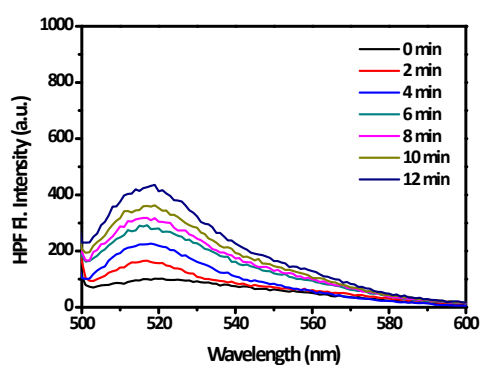
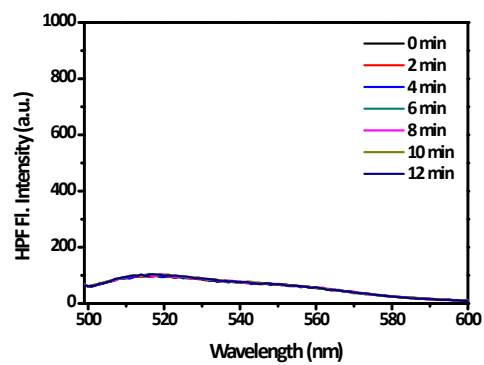
a**b****c****d**

Figure S13. Fluorescence spectra for HPF of irradiation time in the presence of Ru(II) complexes. All groups (Ru1 (a), Ru2 (b), Ru3 (c) and black (d)) were in the 5% O₂ solution.

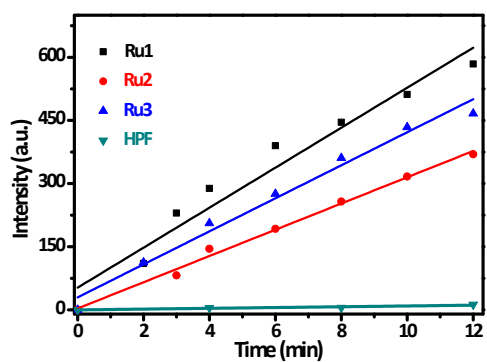
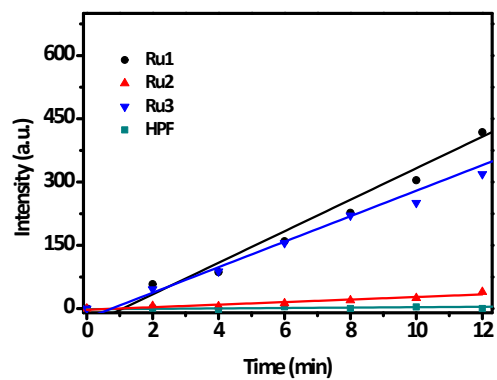
a**b**

Figure S14. Change of HPF fluorescence spectra at 515 nm as a function of irradiation time in the presence of Ru(II) complexes. All group were in the 21% O₂ solution(a), 5% O₂ solution(b).

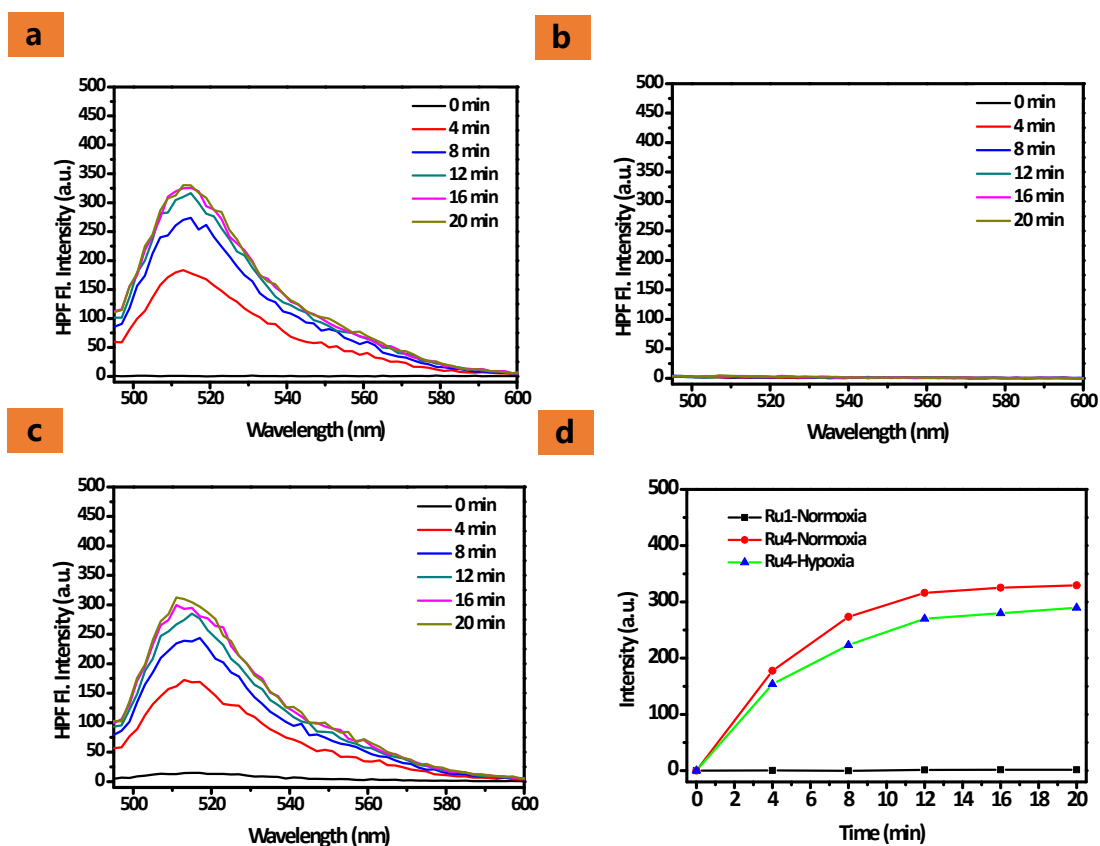


Figure S15. Fluorescence spectra for •OH using HPF as fluorescence probe. The light source is a xenon lamp with a white light (400-800 nm) output (10.0 mW cm⁻²). **Ru4** (a), **Ru1** (b) were in the 21% O₂ solution and **Ru4** (c) were in the 5% O₂ solution. (d) Change of HPF fluorescence spectra at 515 nm as a function of irradiation time.

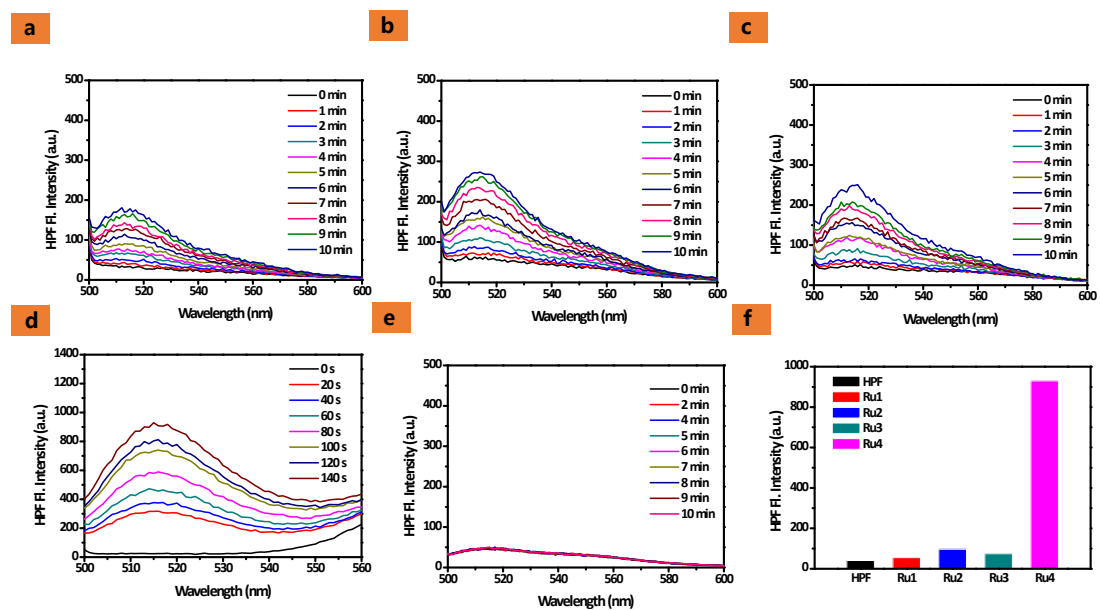


Figure S16. Fluorescence spectra for $\bullet\text{OH}$ using HPF as fluorescence probe. The light source is a xenon lamp with a white light (400-800 nm) output (10.0 mW cm^{-2}). Ru1 (a), Ru2 (b), Ru3 (c), Ru4 (d), blank (e) were in the N_2 solution. (f) Fluorescence intensity of HPF at 515 nm after white light irradiation for 140 s in the N_2 solution.

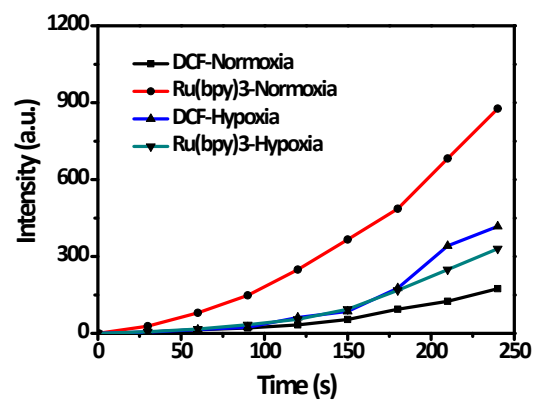


Figure S17. DCF emission intensity at 520 nm as a function of light irradiation time in aqueous solution in the presence $[\text{Ru}(\text{bpy})_3]^{2+}$ and black. The concentration of the complex is 0.5 μM .

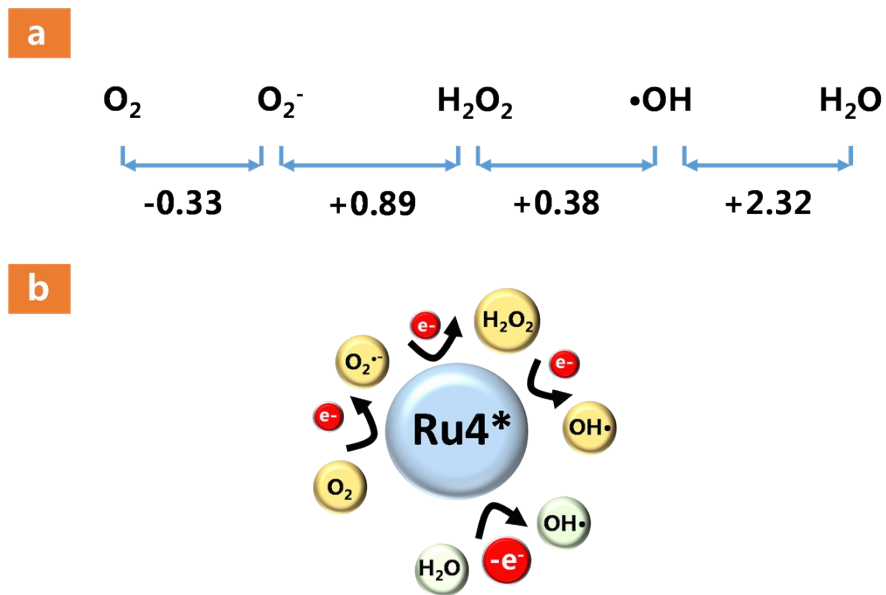


Figure S18. (a) Potential diagram for oxygen at pH 7. (b) Proposed mechanisms of **Ru4** for the ROS generation.

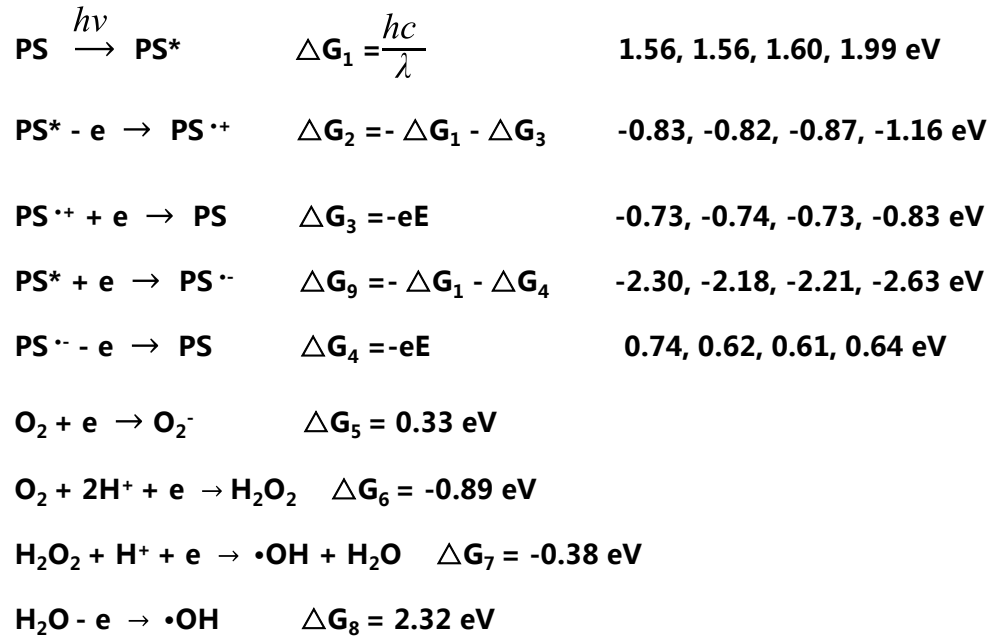


Figure S19. Reactions in Figure 3 at pH 7.

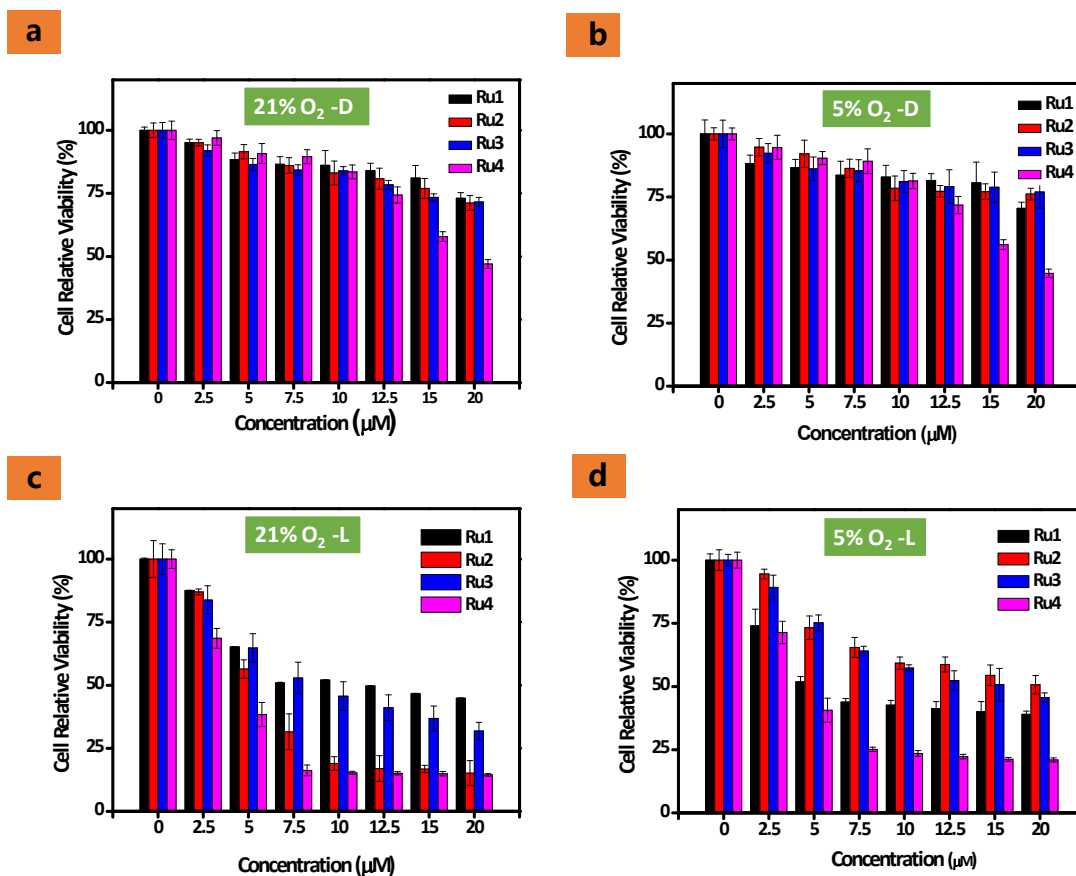


Figure S20. Dose-dependent curves for cell viability of HeLa cells treated with **Ru1-Ru4** using a typical MTT assay under light irradiation (L) or in the dark (D).

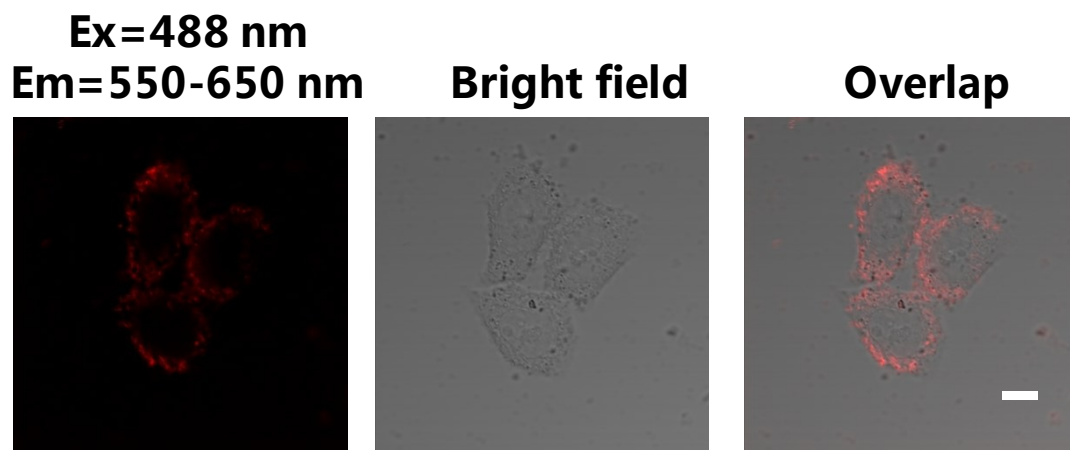


Figure S21. Confocal fluorescence images of Hala cells treated with 5 μ M **Ru4**. The images share the same scale bar of 20 μ m.

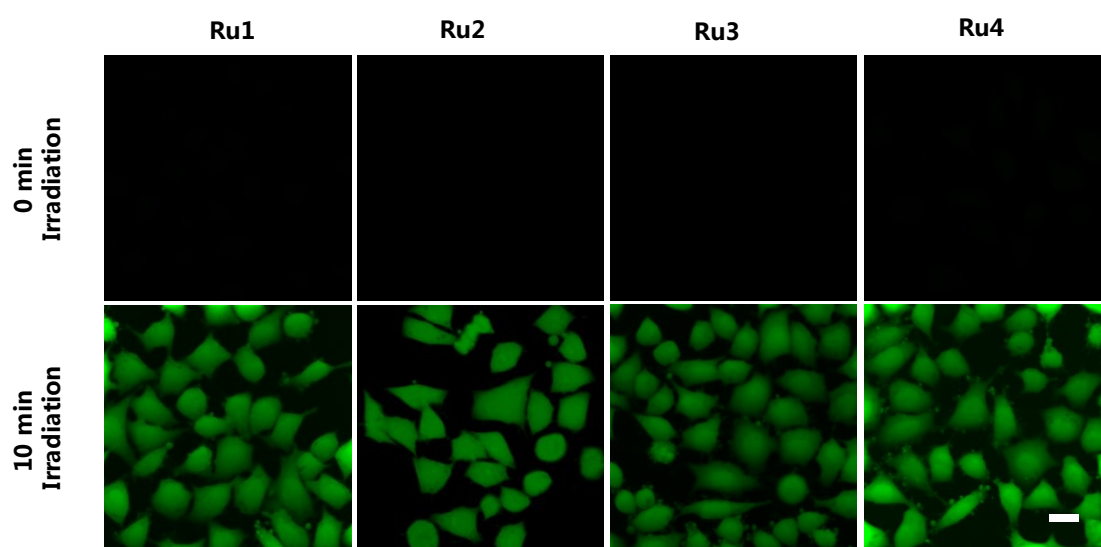


Figure S22. Confocal fluorescence images of ROS generation in cells incubated with Ru(II) complexes (5 μM), cells were treated with light irradiation (400-800 nm, 30 mW cm^{-2}) for 10 min under normoxia. The images share the same scale bar of 20 μm .

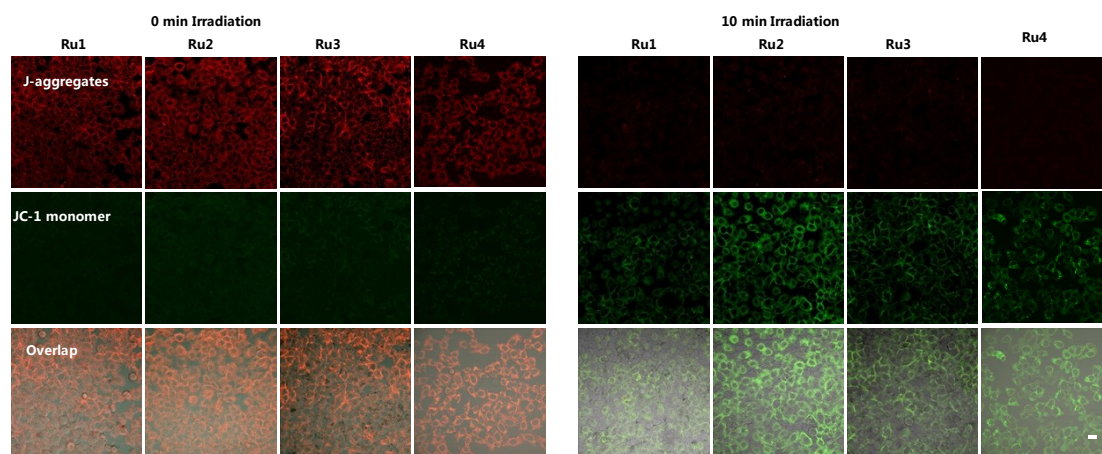


Figure S23. Fluorescence images of JC-1 stained HeLa cells. Cells were incubated with Ru(II) complexes and protected from light and then stained with JC-1 under normoxia. Cells were viewed in red channel for J-aggregates ($\lambda_{\text{ex}} = 515 \text{ nm}$, $\lambda_{\text{em}} = 580\text{-}640 \text{ nm}$) and green channel for JC-1 monomer ($\lambda_{\text{ex}} = 515 \text{ nm}$, $\lambda_{\text{em}} = 530\text{-}560 \text{ nm}$), respectively. All the images share the same scale bar of $60 \mu\text{m}$.

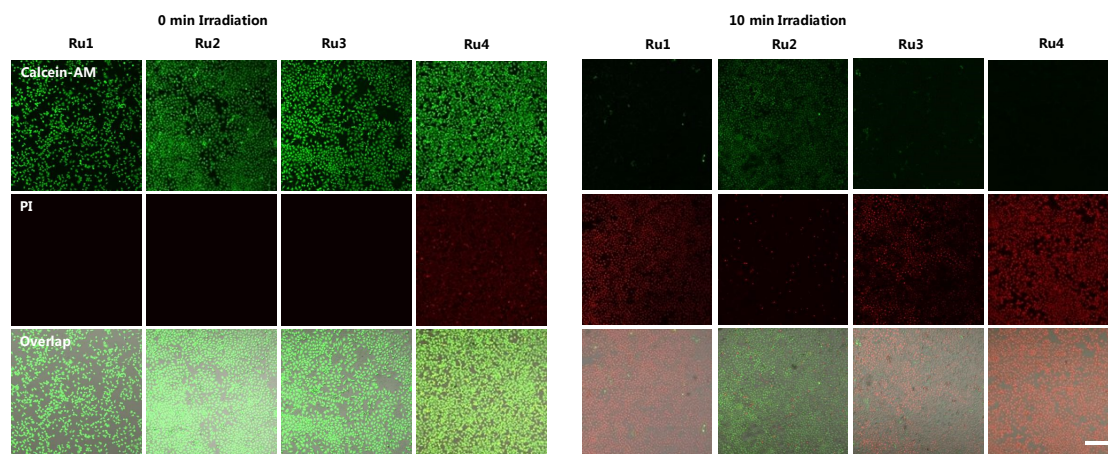


Figure S24. Fluorescence images of **Ru1-Ru4** (5 μM) loaded HeLa cells after 10 min light (400-800 nm, 30 mW cm^{-2}) irradiation under normoxia. The cells were incubated with Ru1-Ru4 at 37 $^{\circ}\text{C}$ for 2 h under normoxia and then stained Calcein-AM /PI. Cells were viewed in green channel for Calcein-AM ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 500\text{-}560 \text{ nm}$) and red channel for PI ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 600\text{-}680 \text{ nm}$), respectively. The images were taken at 3 h after the irradiation. All the images share the same scale bar of 240 μm . Images were taken at 25 $^{\circ}\text{C}$.

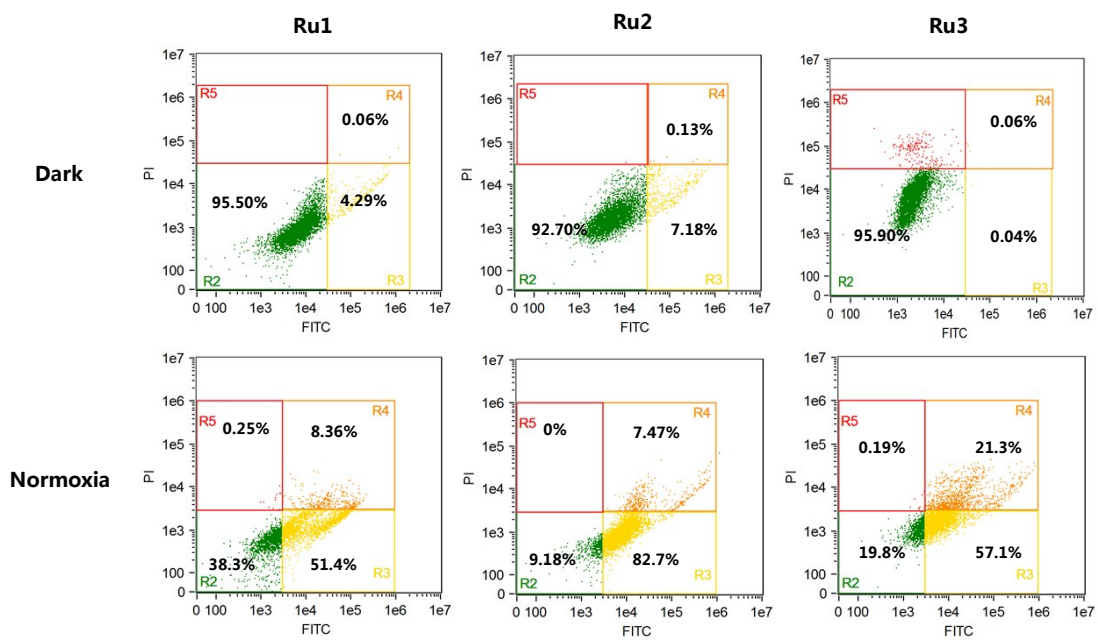


Figure S25. The ratios of PDT cell viability to dark cell viability. Cells were irradiated with white light (400-800 nm, 30 mW cm⁻², 10 min). Flow cytometric assay of cell death induced by **Ru1**, **Ru2** and **Ru3** (5 μM) mediated PDT.

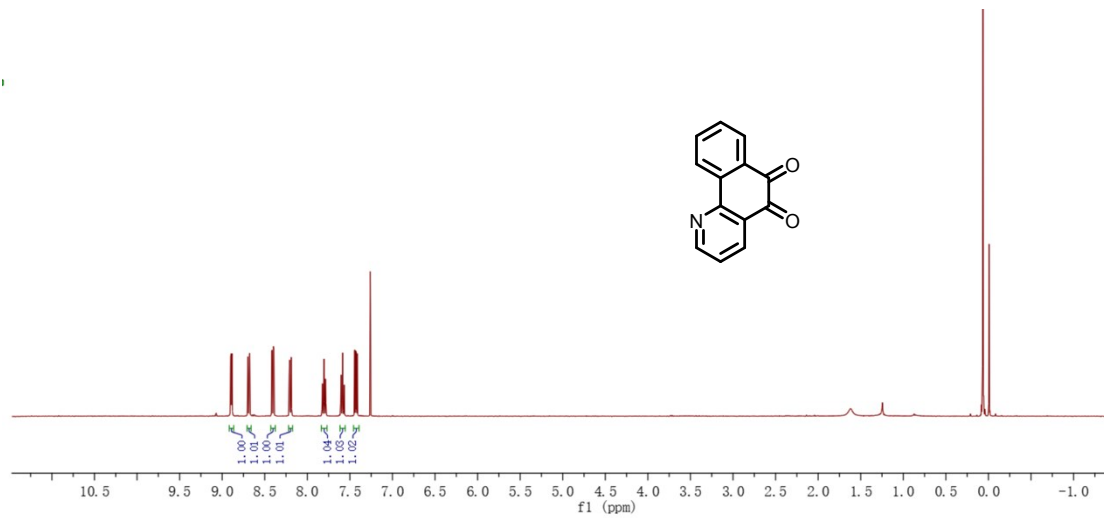


Figure S26. ¹H NMR spectrum of Benzo[h]quinoline-5,6-dione in CDCl₃.

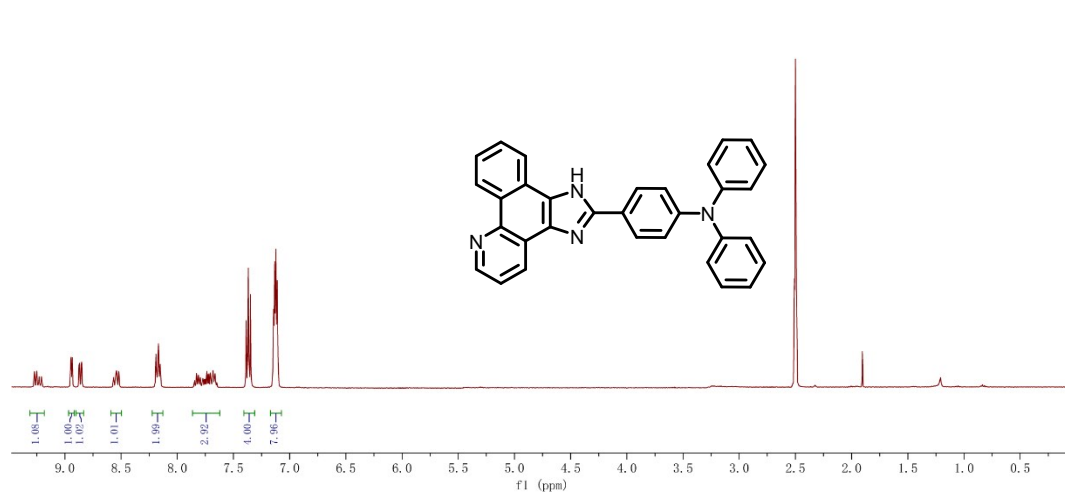


Figure S27. ¹H NMR spectrum of 4-(1H-benzo[h]imidazo[4,5-f]quinolin-2-yl)-N,N-diphenylaniline. in DMSO-d₆.

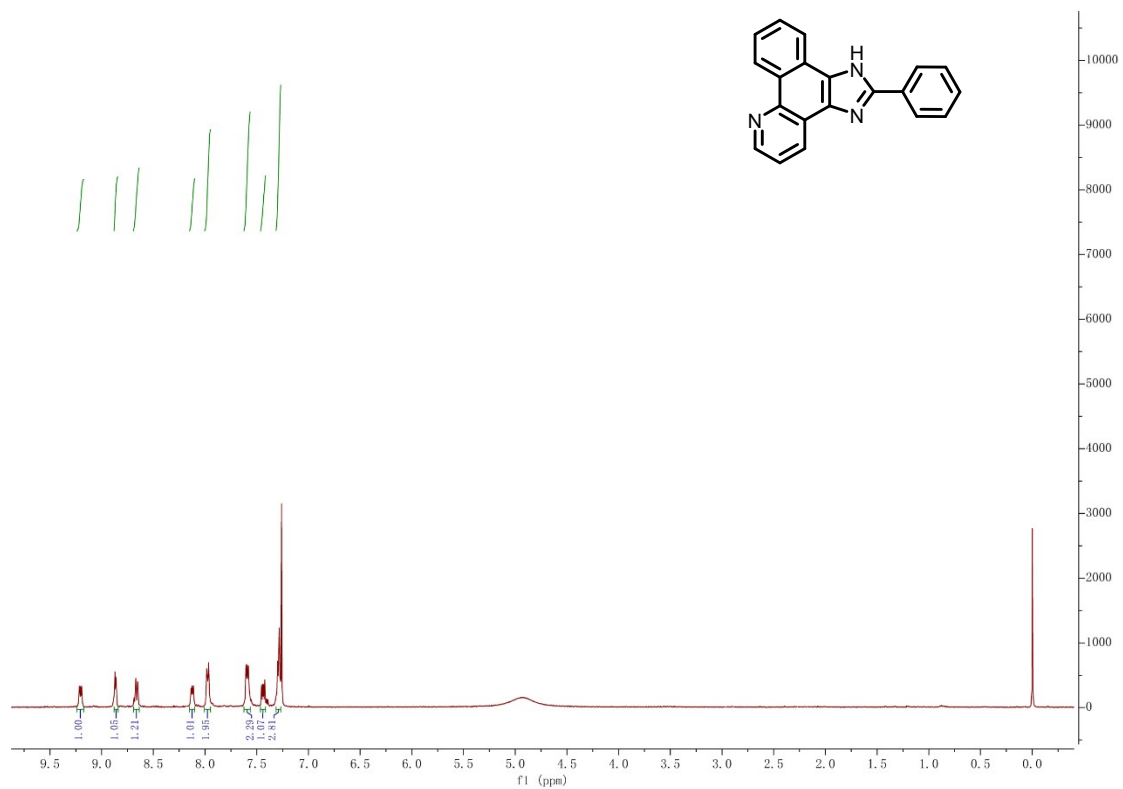


Figure S28. ^1H NMR spectrum of 2-phenyl-1H-benzo[h]imidazo[4,5-f]quinoline in DMSO-d_6 .

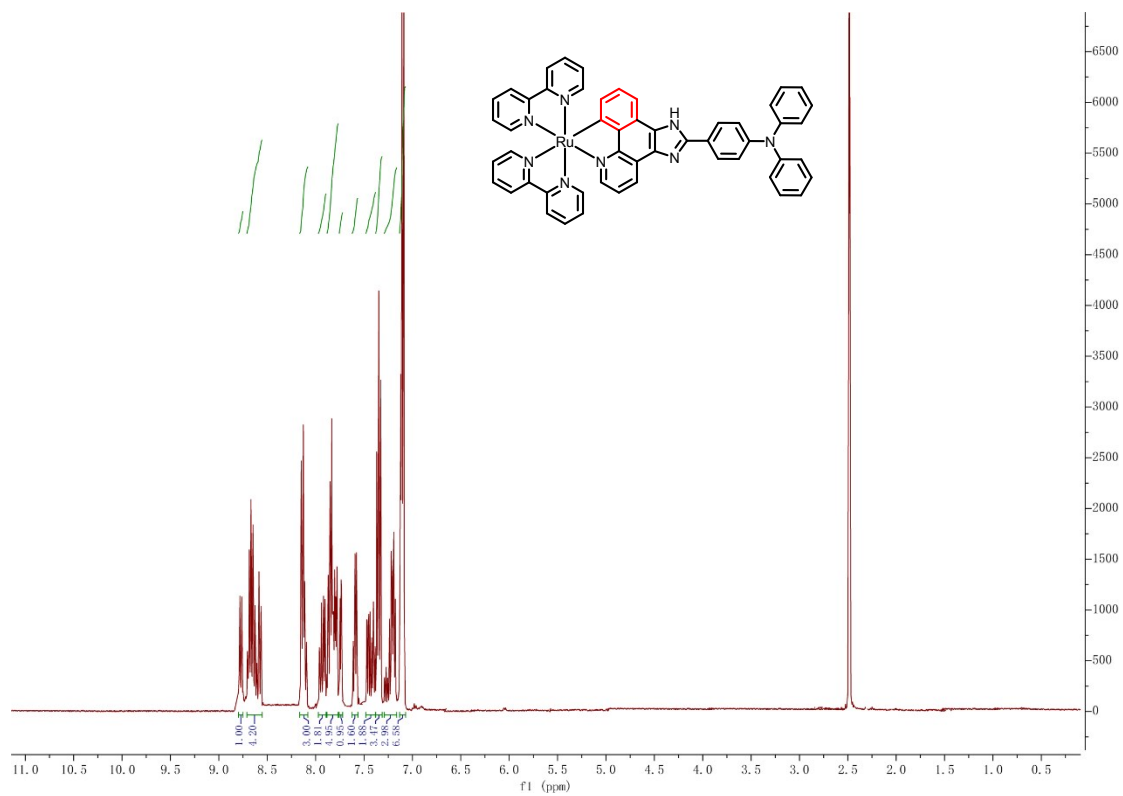


Figure S29. ^1H NMR spectrum of Ru1 in DMSO-d_6 .

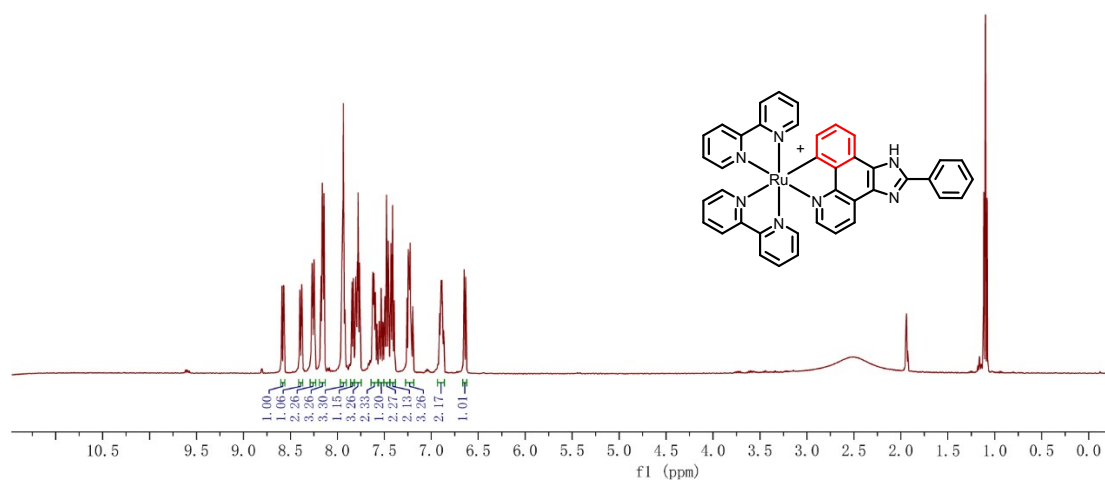


Figure S30. ^1H NMR spectrum of Ru2 in CD_3CN .

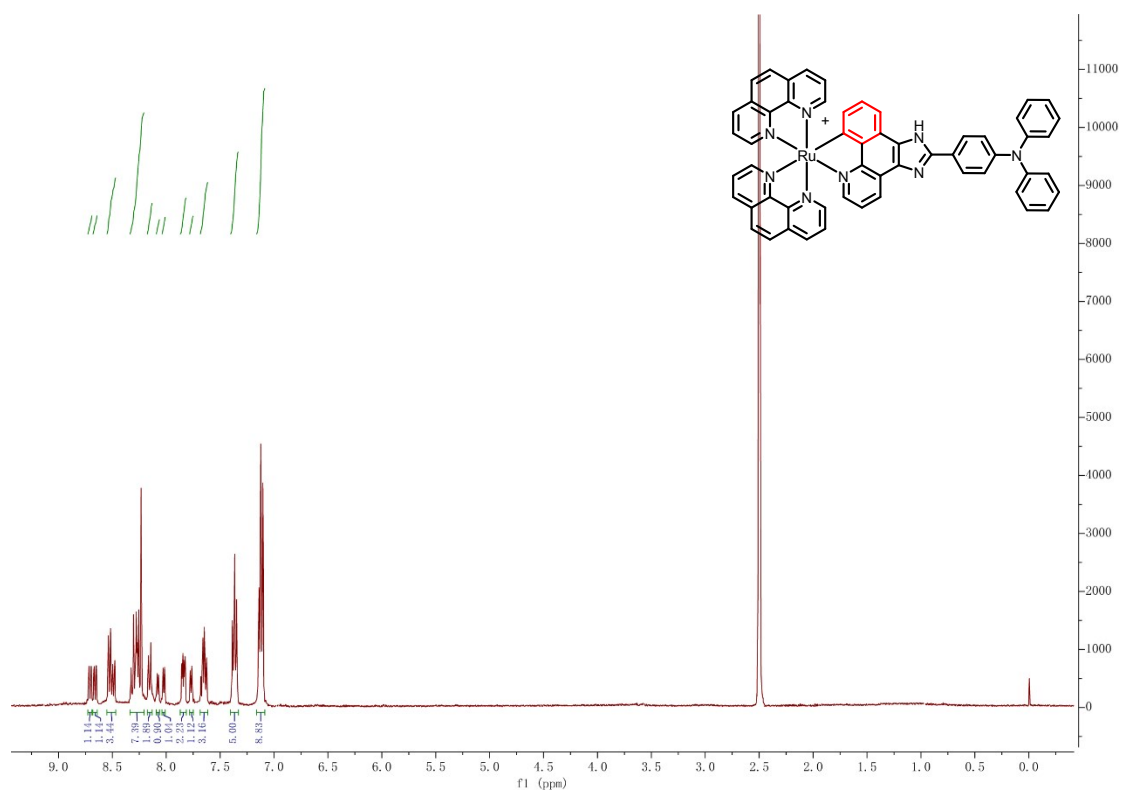


Figure S31. ^1H NMR spectrum of Ru3 in DMSO- d_6 .

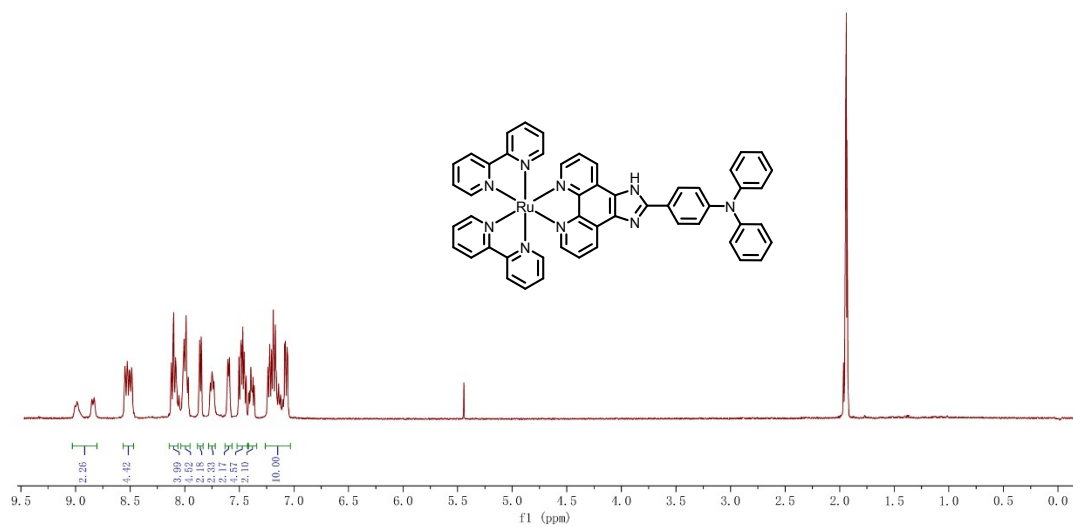


Figure S32. ¹H NMR spectrum of Ru4 in CD₃CN.

Comment 1

Comment 2

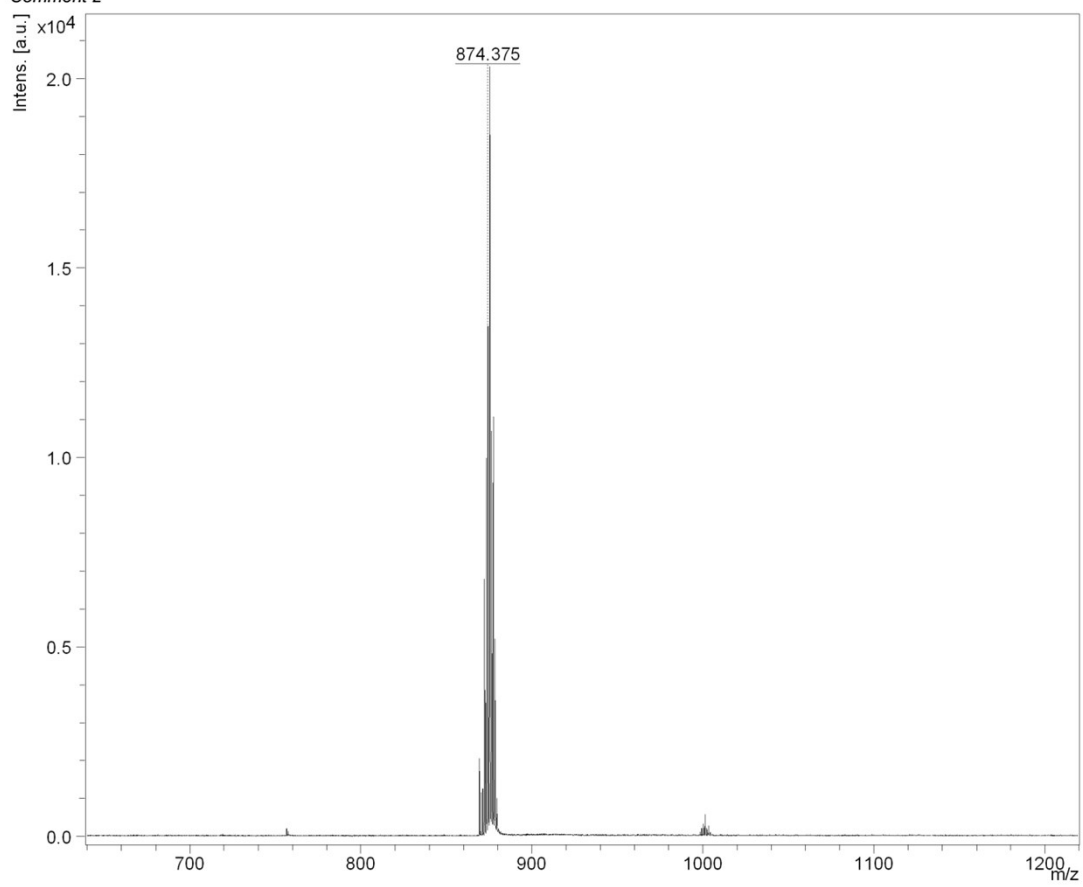


Figure S33. MS spectrum of Ru1.

Comment 2

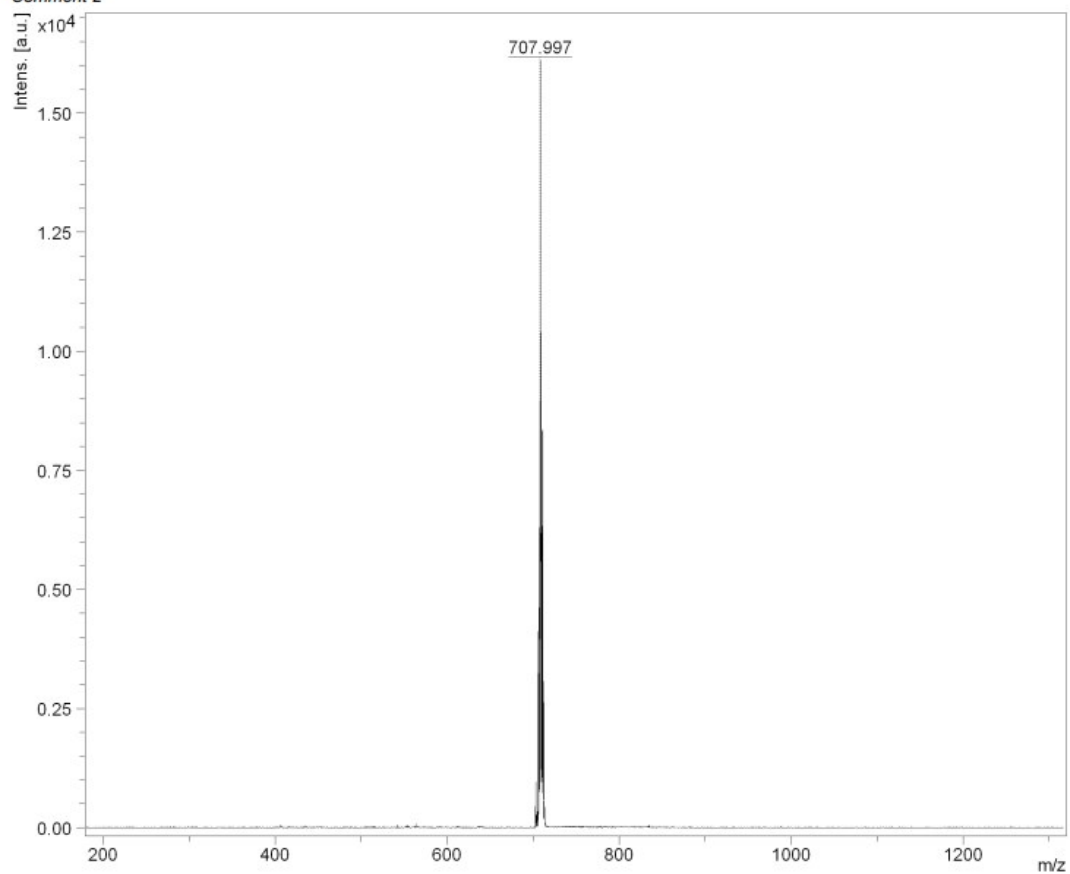


Figure S34. MS spectrum of Ru2.

Comment 1

Comment 2

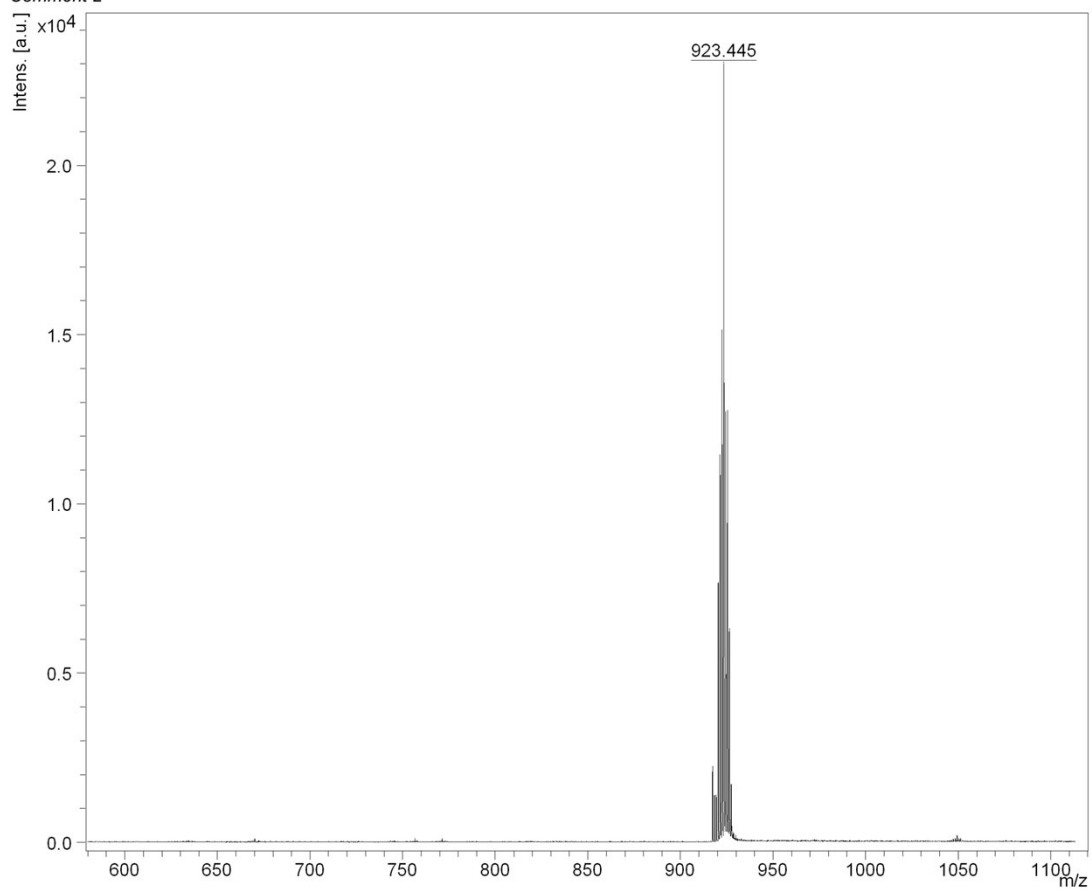


Figure S35. MS spectrum of Ru₃.

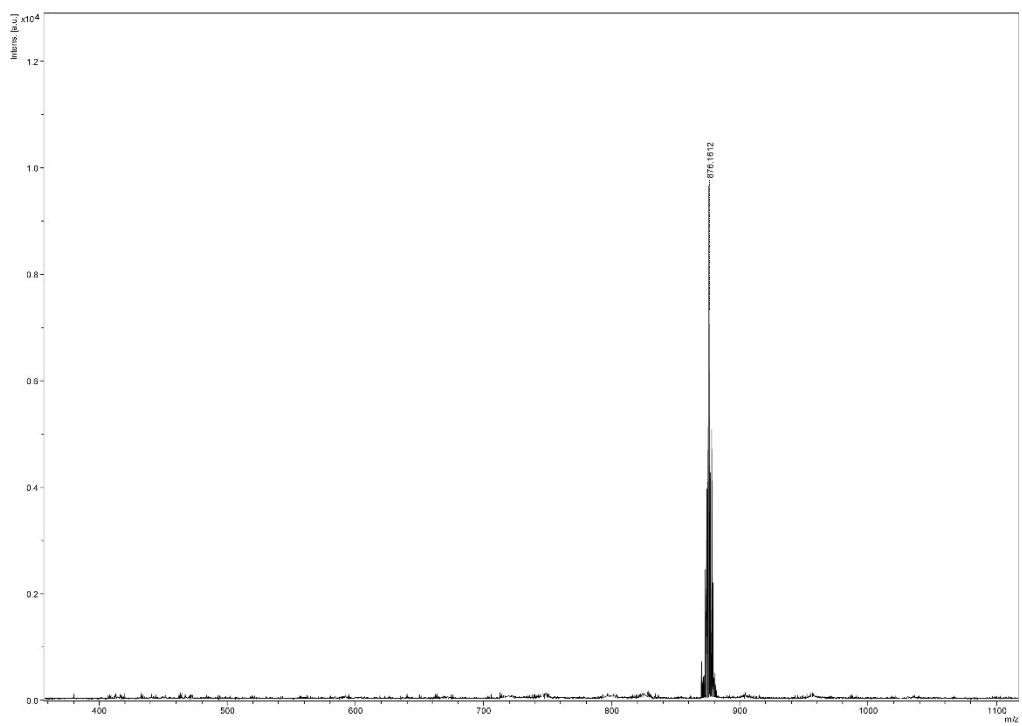


Figure S36. MS spectrum of Ru4.

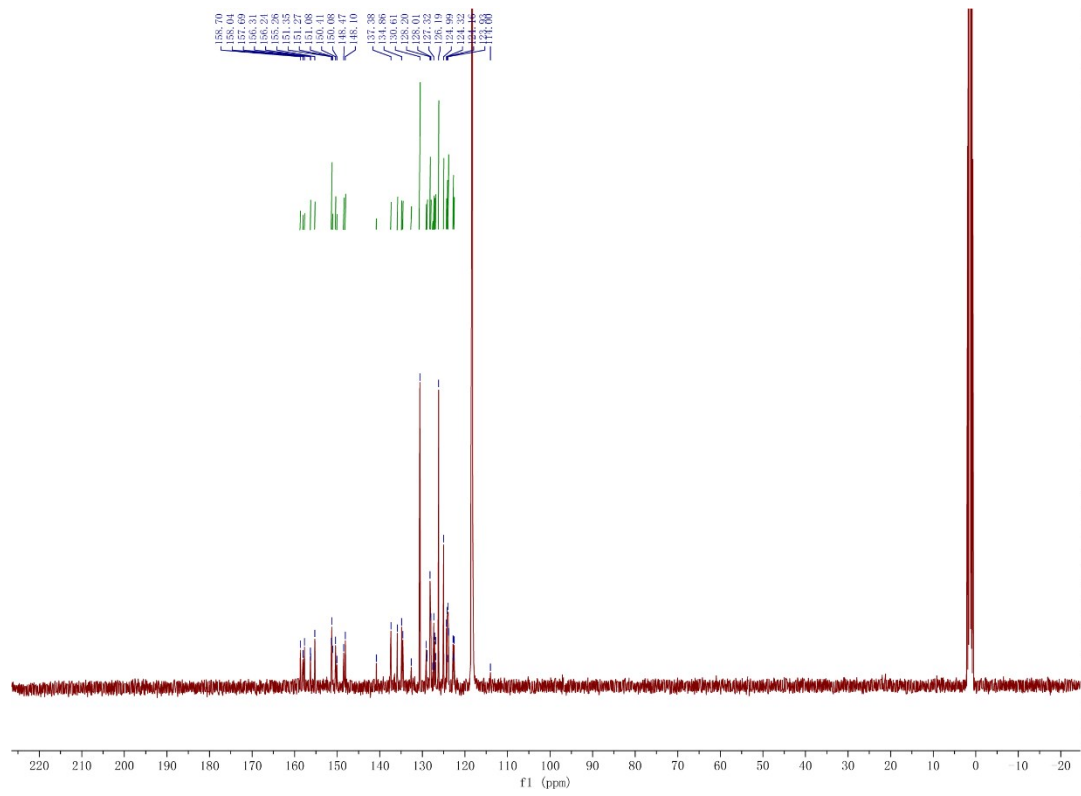


Figure S37. ^{13}C NMR spectrum of Ru1 in CD_3CN .

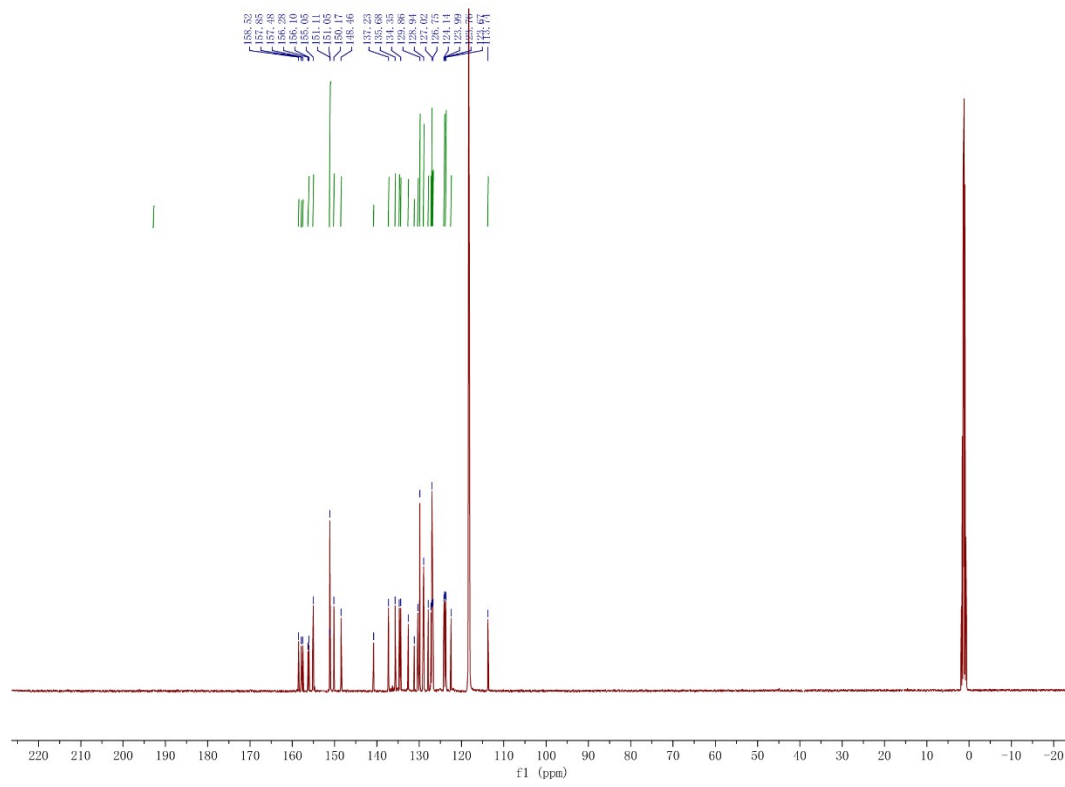


Figure S38. ¹³C NMR spectrum of Ru2 in CD₃CN.

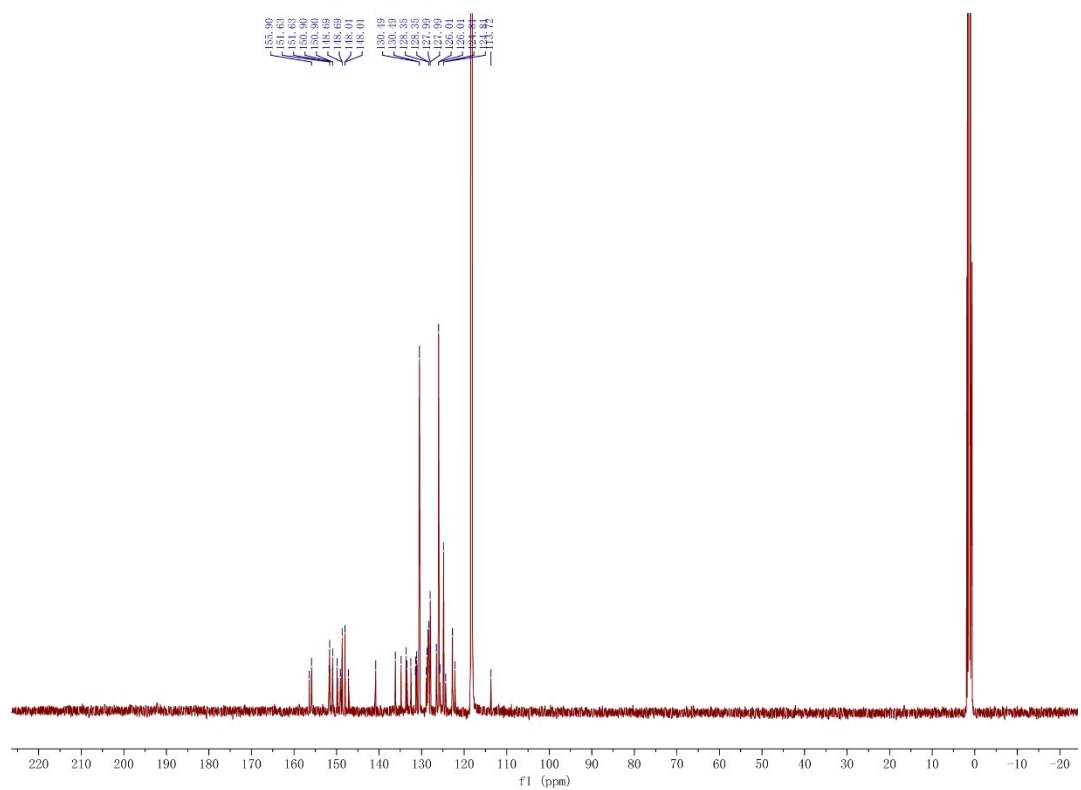


Figure S39. ¹³C NMR spectrum of Ru3 in CD₃CN.