

Supplementary information

Optimising the synthesis, polymer membrane encapsulation and photoreduction performance of Ru(II)- and Ir(III) bis(terpyridine) cytochrome c bioconjugates

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4-nitro-2'-azachalcone To a solution of 4-nitrobenzaldehyde (2.08 g, 13.8 mmol) and aqueous sodium hydroxide (1 M, 13.75 mL) in methanol (60 mL) was added 2-acetylpyridine (1.60 mL, 14.3 mmol). The solution was stirred for 1 h, filtered, and the collected precipitate washed with coldmethanol, dissolved in dichloromethane, washed with water, dried over anhydrous sodium sulphate and the solvent removed *in vacuo*. The resulting yellow solid wasrecrystallised from ethanol to give 4-nitro-2'-azachalcone as yellow crystals (1.01 g, 29%). These results are in agreement with those reported in the literature.¹

1-(2-oxo-2-(2-pyridyl)ethyl)pyridinium iodide To a stirred solution of iodine (7.78 g, 30.7 mmol) in dry pyridine (20 mL) under nitrogen at 60 °C was added 2-acetylpyridine (3.68 g, 30.4 mmol). The resulting mixture was stirred at 100 °C for 2 h, cooled, then the crystals filtered and washed with chloroform and diethyl ether to yield 1-(2-oxo-2-(2-pyridyl)ethyl)pyridinium iodide as black crystals (7.84 g, 79%). These results are in agreement with those reported in the literature.²

4'-(4-nitrophenyl)-2,2':6',2''-terpyridine A solution of 4-nitro-2'-azachalcone (0.870 g, 3.42 mmol), 1-(2-oxo-2-(2-pyridyl)ethyl)pyridinium iodide (1.13 g, 3.47 mmol) and ammonium acetate (3.75 g, 48.6 mmol) in dry methanol (60 mL) was refluxed for 20 h. The crystals were cooled, filtered and washed with cold methanol (6 × 50 mL) to yield terpyridine 4'-(4-nitrophenyl)-2,2':6',2''-terpyridine as a purple solid (0.91 g, 75%). These results are in agreement with those reported in the literature.¹

4'-(4-aminophenyl)-2,2':6',2''-terpyridine (2) A solution of compound 4'-(4-nitrophenyl)-2,2':6',2''-terpyridine(891.8 mg, 2.52 mmol) in absolute ethanol (60 mL) was refluxed for 45 min in the presence of 10% Pd/charcoal catalyst(120.6 mg). Hydrazine monohydrate (4.75 mL, 98.0 mmol) in absolute ethanol (60 mL) was then added drop-wise to the reaction mixture and refluxed for 3 h. The solution was filtered over celite and washed with dichloromethane (150 mL). The organic phase was washed with water (4 × 100 mL), dried over anhydrous sodium sulphate and solvent removed *in vacuo* to yield terpyridine 4'-(4-aminophenyl)-2,2':6',2''-terpyridine as yellow crystals (658.4 mg, 81%). These results are in agreement with those reported in the literature.³

4-(hydroxymethyl)benzaldehyde A solution of terephthalaldehyde (8.50 g, 63.0 mmol) in tetrahydrofuran (80 mL) was cooled to 0 °C. Sodium borohydride (0.85 g, 22.0 mmol) was added and the solution was allowed to warm to room temperature. After 1 h, the solvent was removed *in vacuo* and the resulting residue was dissolved in ethyl acetate (70 mL), washed with water (2 × 75 mL) and brine (75 mL), and dried over anhydrous magnesium sulphate. The crude product was purified by column chromatography (silica, hexane/ethyl acetate, a gradient from 2:1 to 1:1 v/v) and isolated as a pale yellow oil that solidified overnight to give 4-(hydroxymethyl)benzaldehyde as an off-white solid (1.81 g, 21%). ¹H NMR (300 MHz, CDCl₃): δ 10.01 (s, 1H), 7.88 (d, 2H, *J* = 8.3 Hz), 7.54 (d, 2H, *J* = 8.3 Hz), 4.81 (s, 2H). MS (ESI): *m/z* 137.01([M + H]⁺, C₈H₉O₂⁺ requires 137.06). This data is in agreement with reported literature.⁶

4'-(4-hydroxymethylphenyl)-2,2':6'2''-terpyridine To a mixture of crushed sodium hydroxide (1.00 g, 25.0 mmol) and poly(ethylene glycol) (PEG-400) (15 mL) at 0 °C was added 2-acetylpyridine (3.78 g, 31.0 mmol). After 5 min, 4-(hydroxymethyl)benzaldehyde (1.80 g, 13.0 mmol) was added and stirred at 0 °C for 2 h. Aqueous ammonia (15 mL, 25%) was then added and heated to 100 °C for an additional 2 h. The resulting precipitate was collected by filtration and washed with water and cold ethanol. Recrystallisation from ethanol gave 4'-(4-hydroxymethylphenyl)-2,2':6'2''-terpyridine as an off-white solid (1.50 g, 34%). mp 203–204 °C; ¹H NMR (200 MHz, CDCl₃): δ 8.74–8.66 (m, 6H), 7.93–7.84 (m, 4H), 7.51 (d, 2H, *J* = 8.5 Hz), 7.35 (ddd, 2H, *J* = 7.2, 5.5, 1.7 Hz), 4.80 (d, 2H, *J* = 5.4 Hz), 1.84 (br s, 1H). MS (ESI): *m/z* 340.18([M + H]⁺, C₂₂H₁₈N₃O⁺ requires 340.15). This data is in agreement with reported literature.⁴

[Ru(tpy)]Cl₃ (Ru(II) 1) A solution of terpyridine (418.9 mg, 1.795 mmol), and ruthenium trichloride hydrate (722.0 mg, 3.481 mmol) in absolute ethanol (50 mL) was refluxed for 23 h under nitrogen. The product was collected by filtration and washed with absolute ethanol, water, and diethyl ether yielding [Ru(tpy)]Cl₃ as a black solid (695 mg, 88%). These results are in agreement with those reported in the literature.^{5,6}

[Ir(tpy)]Cl₃ (Ir(III) **1**) Iridium trichloride hydrate (212 mg, 0.548 mmol) and terpyridine (131 mg, 0.562 mmol) were crushed together with a glass rod in a round-bottomed flask. To this, ethylene glycol was added (10 mL), and the reaction mixture was degassed with three freeze-thaw cycles, heated to 160 °C under nitrogen in the dark, and stirred at this temperature for 15 min. The mixture was cooled and the precipitate centrifuged, washed with water (4 × 5 mL), absolute ethanol (3 × 5 mL), and diethyl ether (3 × 5 mL) to yield [Ir(tpy)]Cl₃ (170 mg, 58%) as a red solid. ¹H NMR (300 MHz, DMSO) δ 9.21 (dd, *J* = 5.6, 0.9 Hz, 2H), 8.82 – 8.65 (m, 4H), 8.32 – 8.17 (m, 3H), 7.96 (ddd, *J* = 7.7, 5.6, 1.4 Hz, 2H). MS (ESI) *m/z*: 230.10 ([M-2Cl])²⁺, C₁₅H₁₁ClN₃Ir²⁺ requires 230.52. These results are in agreement with those reported in literature.⁷

In-gel tryptic digest Bands of interest from a gel electrophoresis experiment were excised, placed in 1.5 mL plastic eppendorf tubes, and incubated in 100 mM ammonium bicarbonate/acetonitrile (1:1) for 24 h to remove gel stain. The protein was then incubated in 100 mM dithiothreitol/ultrapure water/100 mM ammonium bicarbonate (1:3:1) at 37 °C for 1 h to reduce any disulfide bonds and alkylated by the addition of iodoacetamide (20 μL, 200 mM). The alkylation/reduction solution was decanted and acetonitrile added and decanted to wash away dithiothreitol and iodoacetamide before the addition of 20 ng of Promega trypsin in ammonium bicarbonate (40 μL, 10 mM). Samples were left to digest overnight, followed by the addition of 0.1% formic acid (40 μL) and acetonitrile (100 μL). Sample solutions were transferred to plastic vials and solvent removed *in vacuo*. Peptides were redissolved in 0.1 % heptafluorobutyric acid, 1.0% formic acid in ultrapure water (5 μL) and analysed by tandem mass spectrometry (LTQ FT ULTRA, Thermo Scientific).

Photoreduction of Ru(II)-cyt *c* The reduction rate of the heme group in cytochrome *c* bioconjugate Ru(II)-cyt *c* was monitored using UV-Vis spectroscopy from 350 nm to 650 nm as shown in Figure S1 and the degree of reduction was calculated based on absorbance changes at 550 nm according to Equation 1:

$$\text{Reduction(\%)} = \frac{A_t - A_{ox}}{A_{red} - A_{ox}} \times 100 \quad (1)$$

where A_t is the absorbance after reduction time t , A_{ox} is the original absorbance of oxidised cytochrome *c* or bioconjugate and A_{red} is the final absorbance after complete reduction.

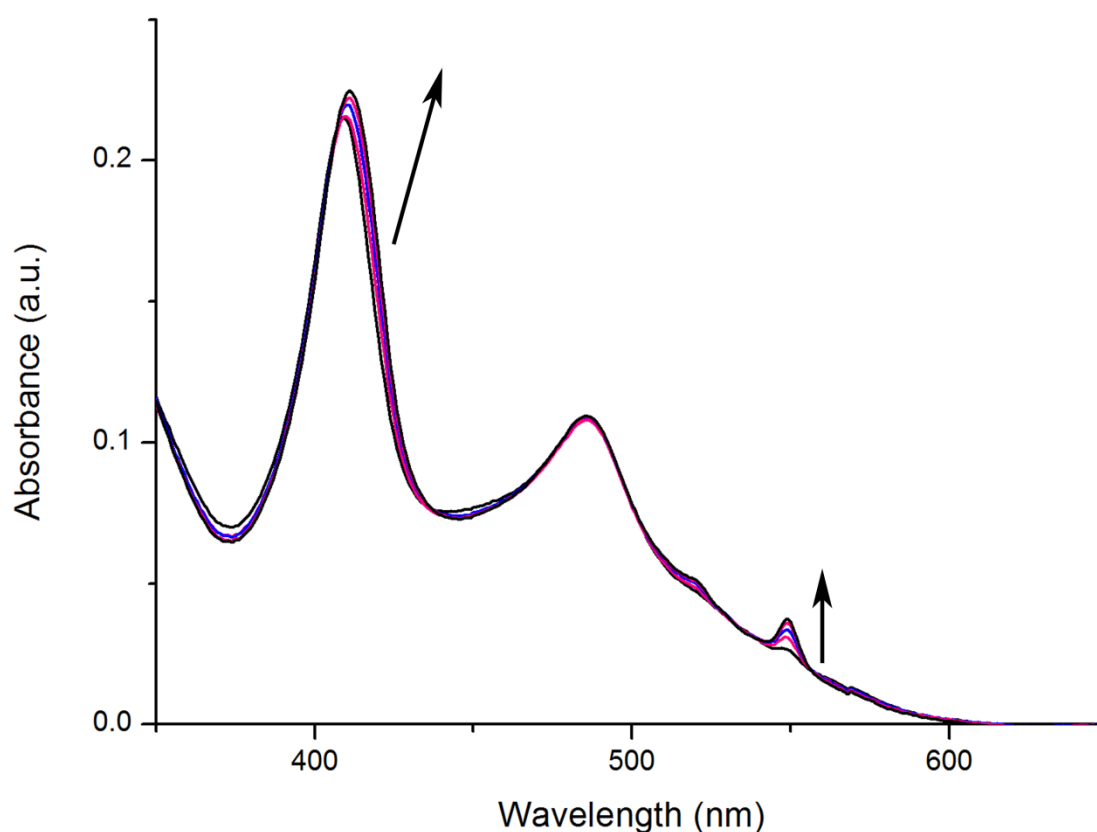


Figure S1. UV-Vis absorbance spectra showing an increase in 550 nm absorbance band over time corresponding to photoreduction of Ru(II) bioconjugate Ru(II)-cyt *c* ($2.3 \pm 0.1 \mu\text{M}$) in 5 mM phosphate buffer, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0. Samples irradiated with $20 \pm 2.3 \text{ mW/cm}^2$ of 465 nm light.

Optical density correction The number of photons absorbed by a chromophore can be estimated based on transmittance using the formula $A = -\log T$ where A is absorbance and T is transmittance.⁸

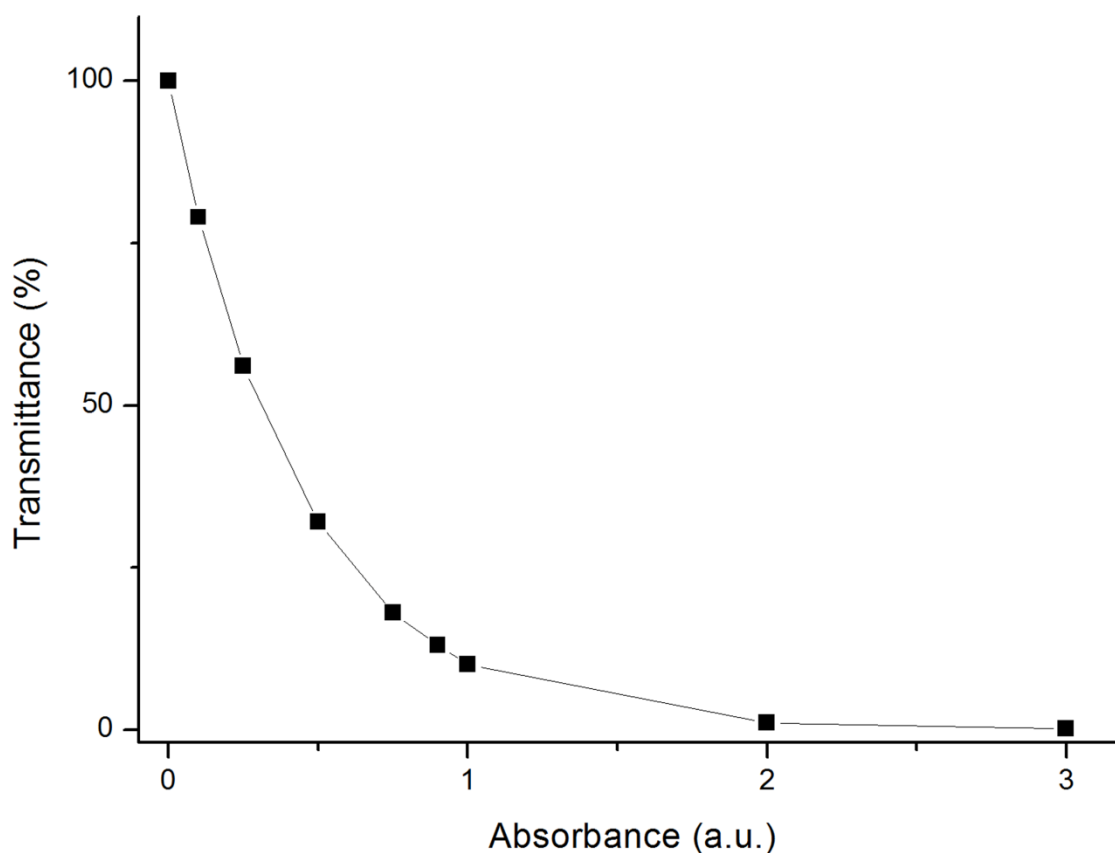


Figure S2. *Transmittance of light based on absorbance of chromophore absorption maxima.*

In a typical calculation, for a Ru(II)-bis(terpyridine) chromophore with an absorbance of 0.0115 a.u., an equivalent 97.9% transmittance of photons is observed. Therefore, 2.1% of incident photons are absorbed by the complex participating in photoexcitation, which is the quantity used for subsequent quantum efficiency calculations.

Quantum efficiency of photoreduced Ru(II)-cyt *c* bioconjugates In a typical calculation, assume that the initial rate of heme reduction is within the linear region.

$$\Delta\text{Absorbance}_{\text{photoreduction}} = 0.007373 \text{ a.u. } (\epsilon_{550} = 24.3 \text{ mM}^{-1}\text{cm}^{-1})$$

$$\text{Abs} = \epsilon b[\text{Ru(II)-cyt } c]$$

$$[\text{Ru(II)-cyt } c] = 3.03 \times 10^{-7} \text{ M (in an 80 } \mu\text{L solution)}$$

$$\text{moles of reduced 2-cyt } c = 2.427 \times 10^{-11} \text{ mol}$$

$$= 2.427 \times 10^{-11} \times N_A \text{ electrons in 1860 s}$$

$$= 1.46 \times 10^{13} \text{ electrons in 1860 s}$$

Therefore, rate of heme reduction is 7.85×10^9 electrons/s

%efficiency = (rate of heme reduction/incident photons) \times 100% ($\gamma_{\text{incident}} = 1.4 \times 10^{16}$ photons/s)

$$= (7.85 \times 10^9 / 1.4 \times 10^{16}) \times 100\%$$

$$= 5.59 \times 10^{-5} \%$$

$$\Phi = \frac{\text{rate of heme reduction}}{(\text{incident photons} \times \text{optical density correction})} \times 100\%$$

$\Phi = (7.85 \times 10^9 / [1.4 \times 10^{16} \times 0.095]) \times 100\%$ (For 0.046 a.u., 9.5% photons contributing to excitation)

$$= 5.9 \times 10^{-4}\%$$

Ruthenium-heme electron transfer distance The bioconjugate Ru(II)-cyt *c* was analysed *via* molecular modelling to estimate the long range electron transfer distance from Ru(II) complex to heme as shown in Figure S3. The donor-acceptor distance was estimated by summation of the linear bond distance between the heme group and cysteine (CYS102) in the crystal structure of iso-1 cytochrome *c* (Fe-S: 11 Å) and the maximum distance between the ruthenium to thiol (CYS102) in a protein attached complex Ru(II) **4** (Ru-S: 21 Å), resulting in an estimated maximum distance between ruthenium and heme centre of ≤ 32 Å.

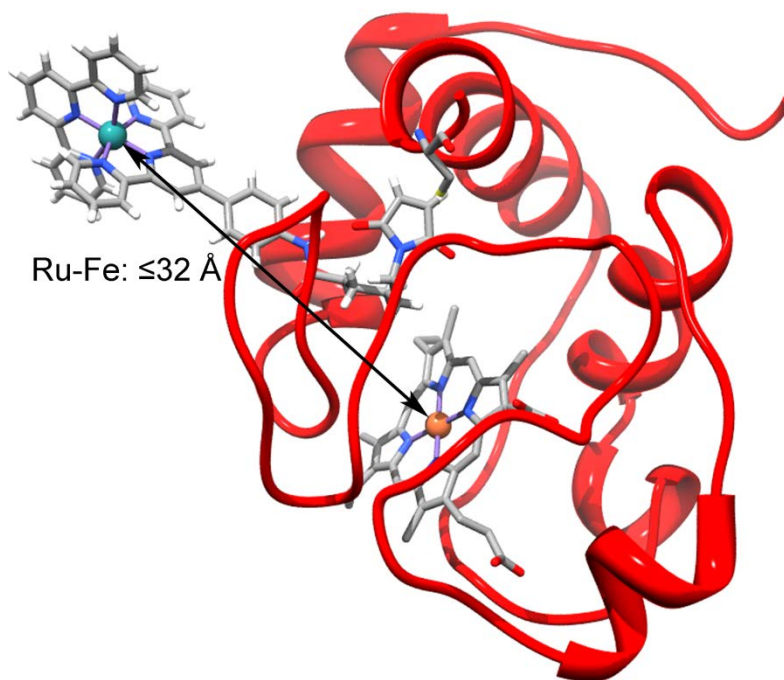


Figure S3. A model of Ru(II)-cyt *c* generated from the X-ray crystal structure of yeast iso-1 cytochrome *c* (cyt *c*) attached to the X-ray structure of ruthenium(II)-bis(terpyridine) complex Ru(II) **4** via the CYS102 residue. Maximum distance between Ru-Fe estimated as ≤ 32 Å. Cytochrome *c* structure derived from the protein data bank file '1YCC'.⁹

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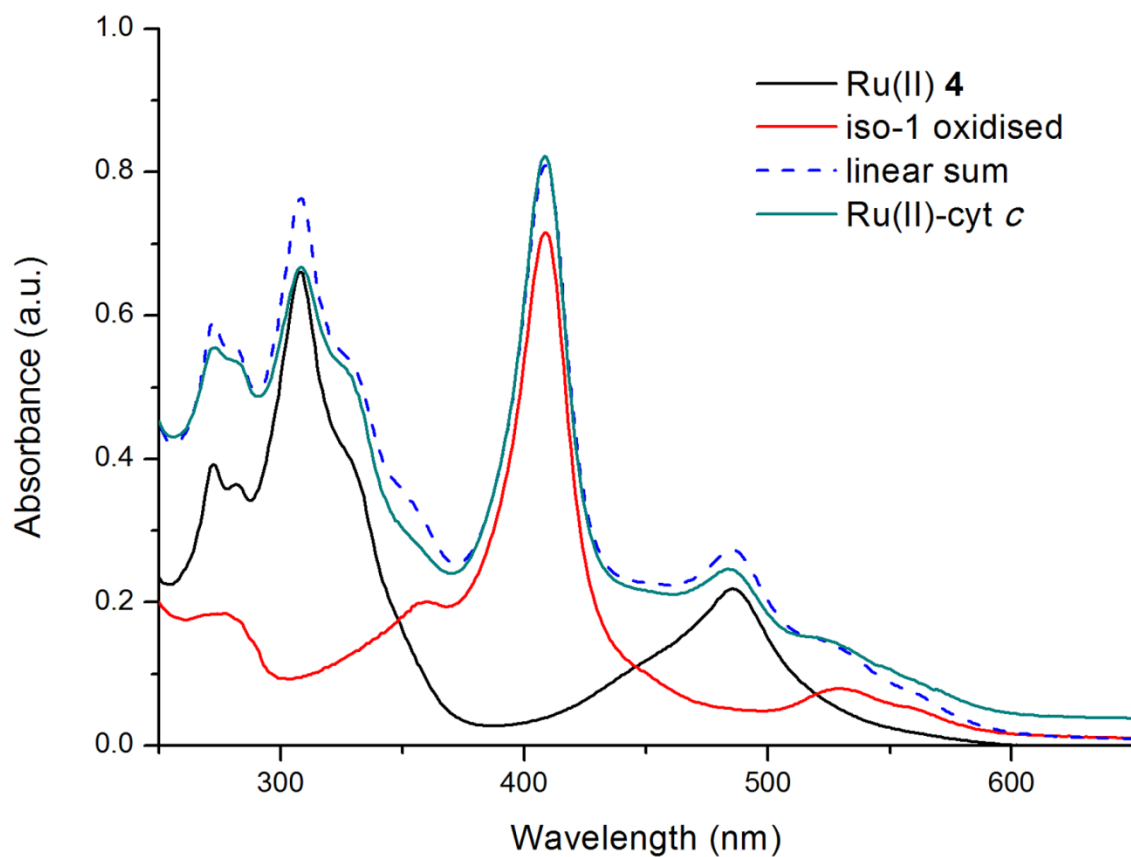


Figure S4. UV-Vis spectra of Ru(II)-cyt *c* (green, H₂O) and approximated by the linear sum (blue dashed) of oxidised iso-1 cytochrome *c* (red, H₂O) and complex Ru(II) **4** (black, CH₃CN).

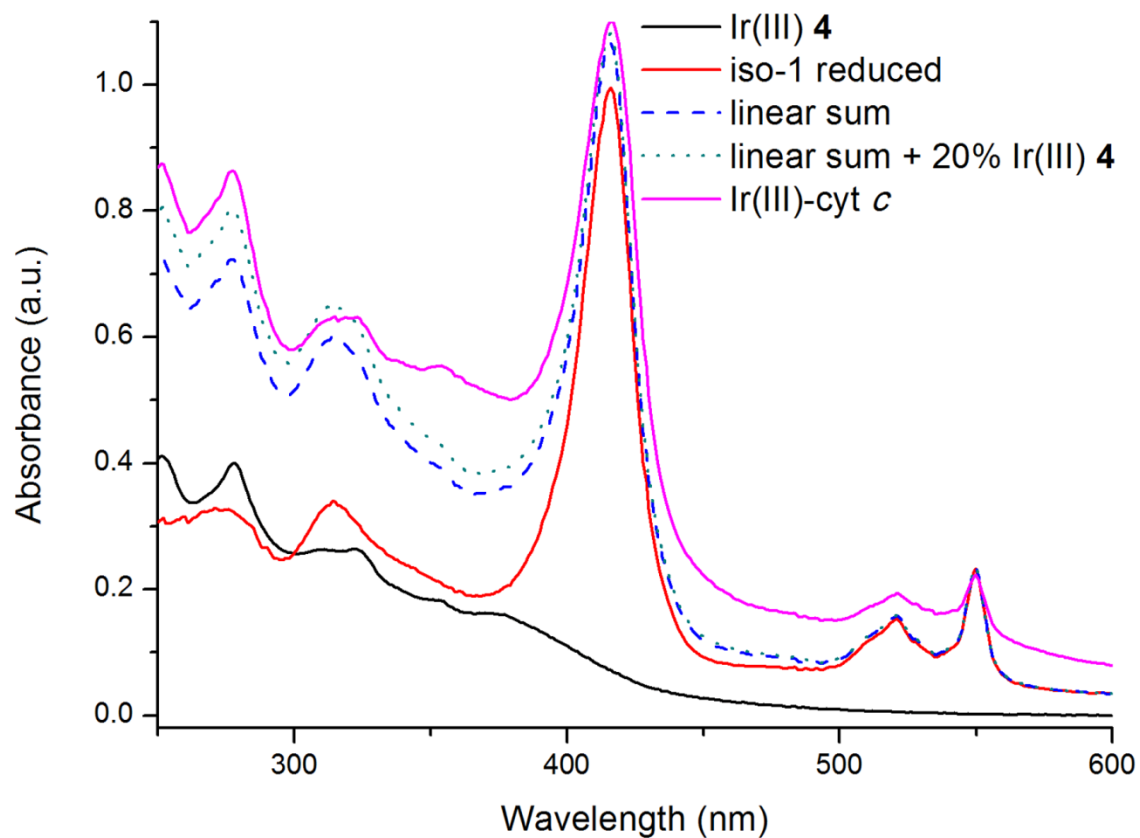


Figure S5. UV-Vis spectra of Ir(III)-cyt *c* (pink, H₂O) and approximated by the linear sum (blue dashed) and linear sum + 20% complex Ir(III) **4** (green dotted) of reduced iso-1 cytochrome *c* (red, H₂O) and complex Ir(III) **4** (black, H₂O).

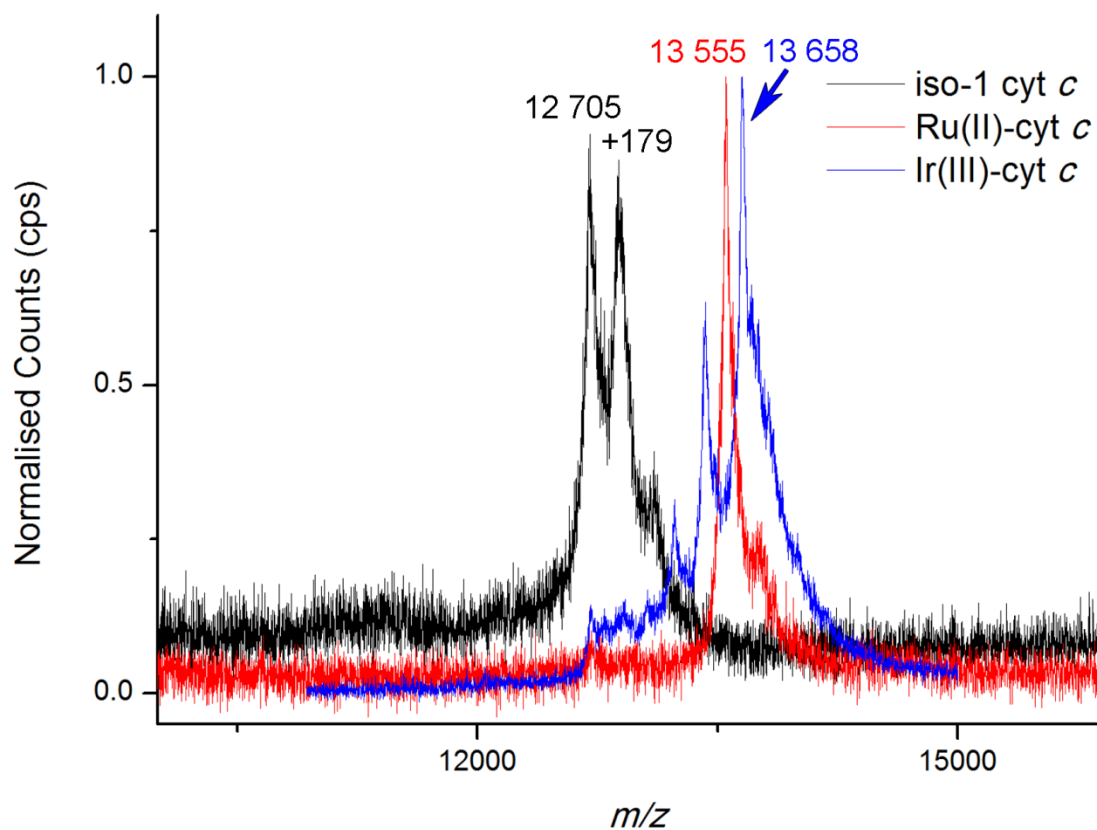
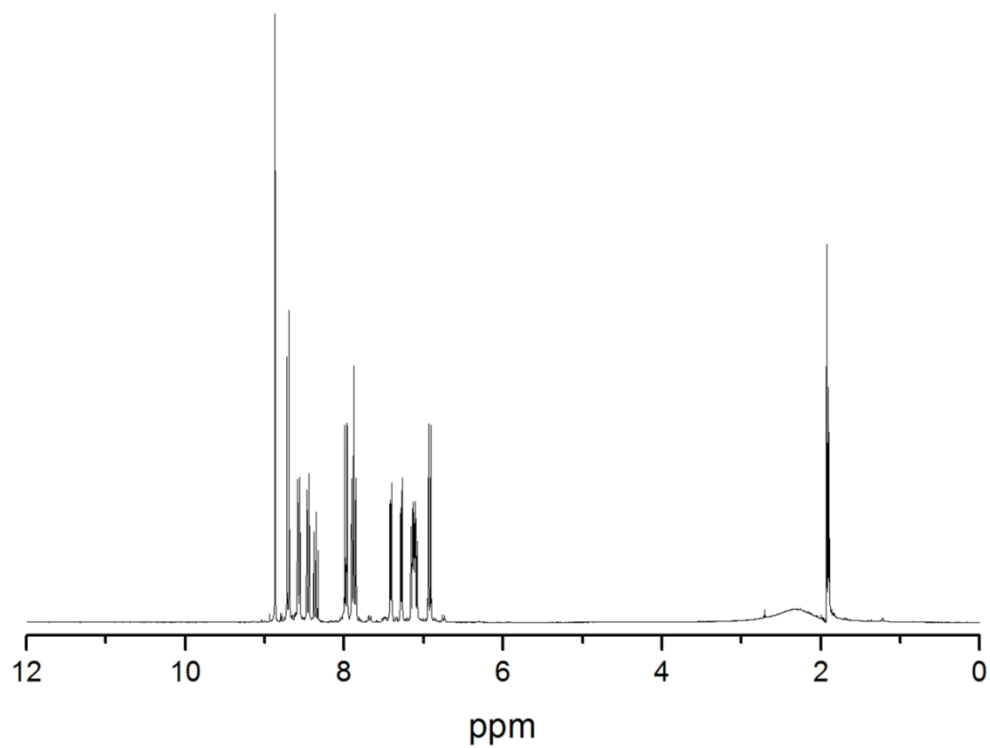
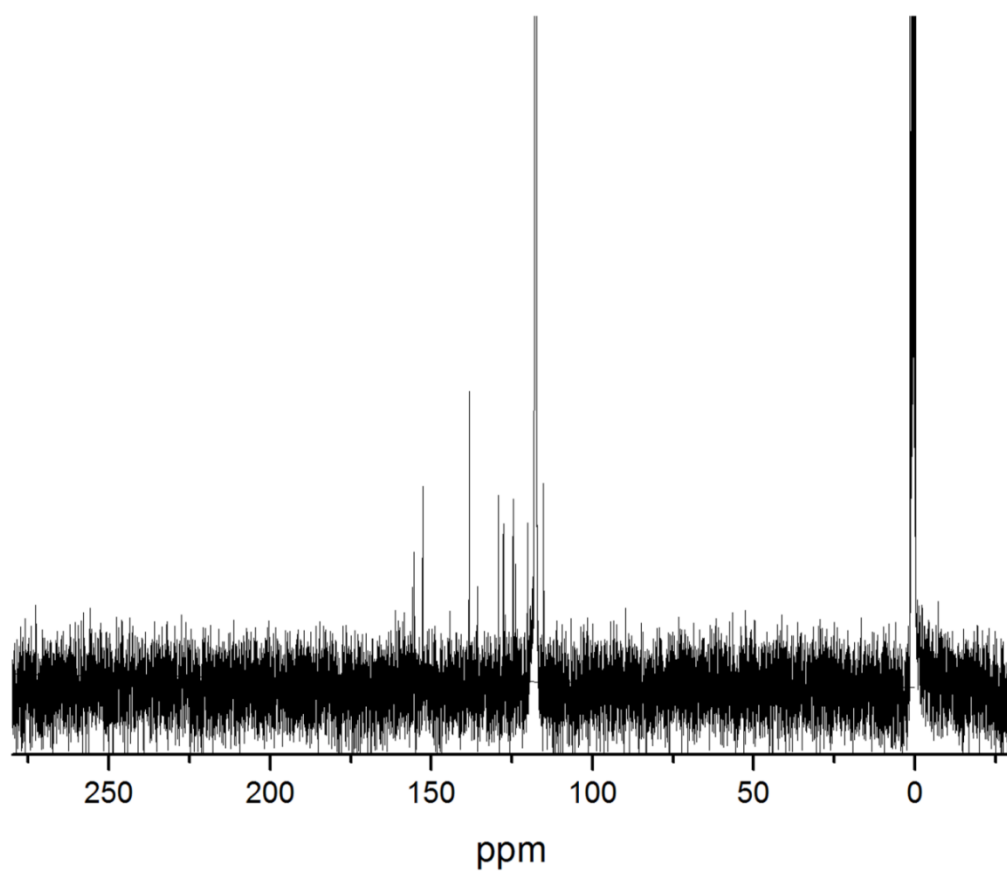


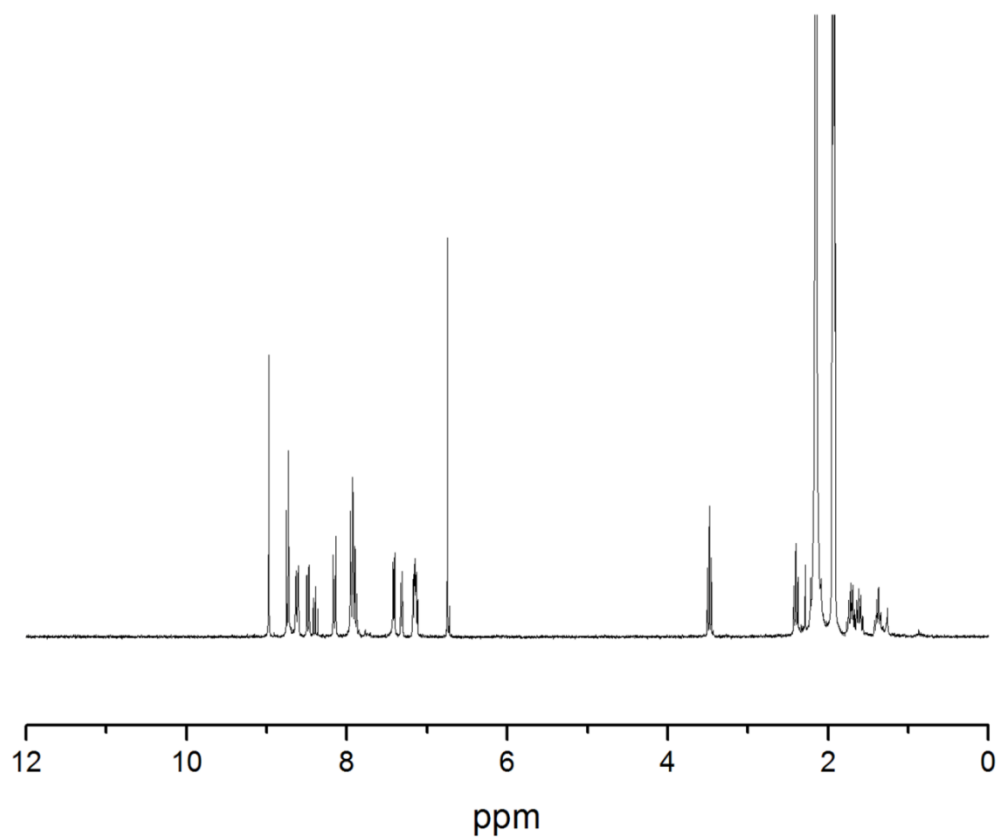
Figure S6. MALDI-TOF mass spectra of iso-1 cytochrome c (black), Ru(II)-cyt c (red) and Ir(III)-cyt c (blue). Peaks corresponding to the calculated masses 12 706, 13 559 and 13 650 Da, respectively, were detected. Spectra were baseline corrected.



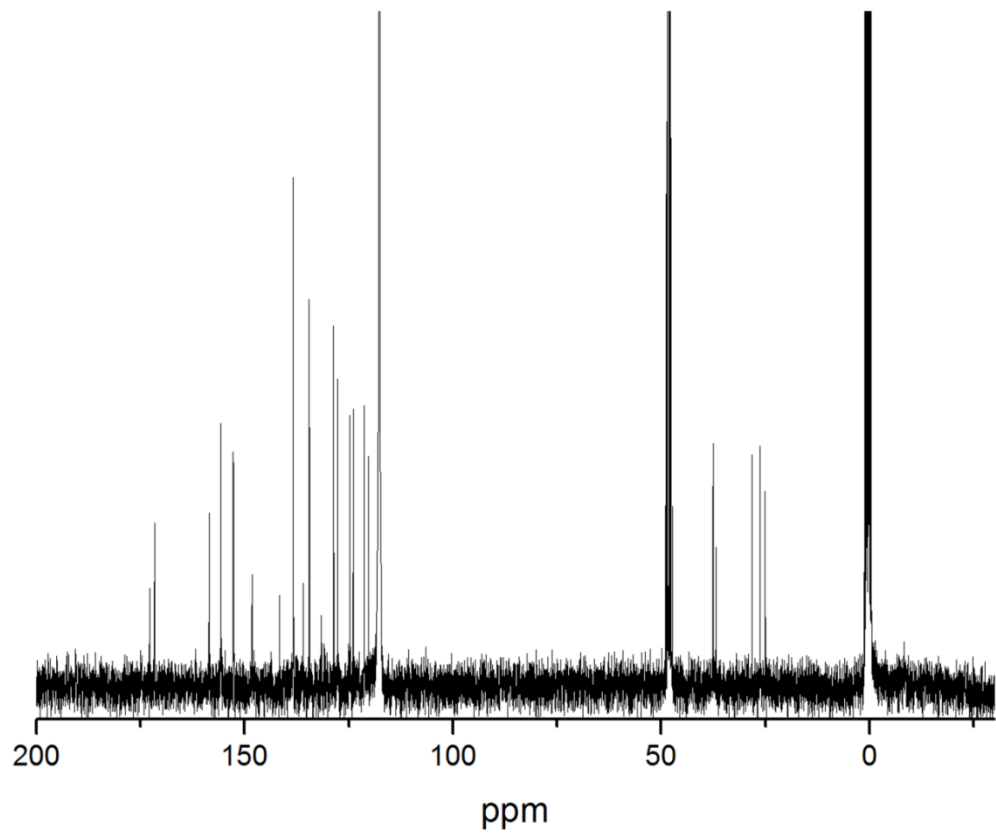
¹H NMR spectrum of compound Ru(II) **3** in CD₃CN at 300 MHz



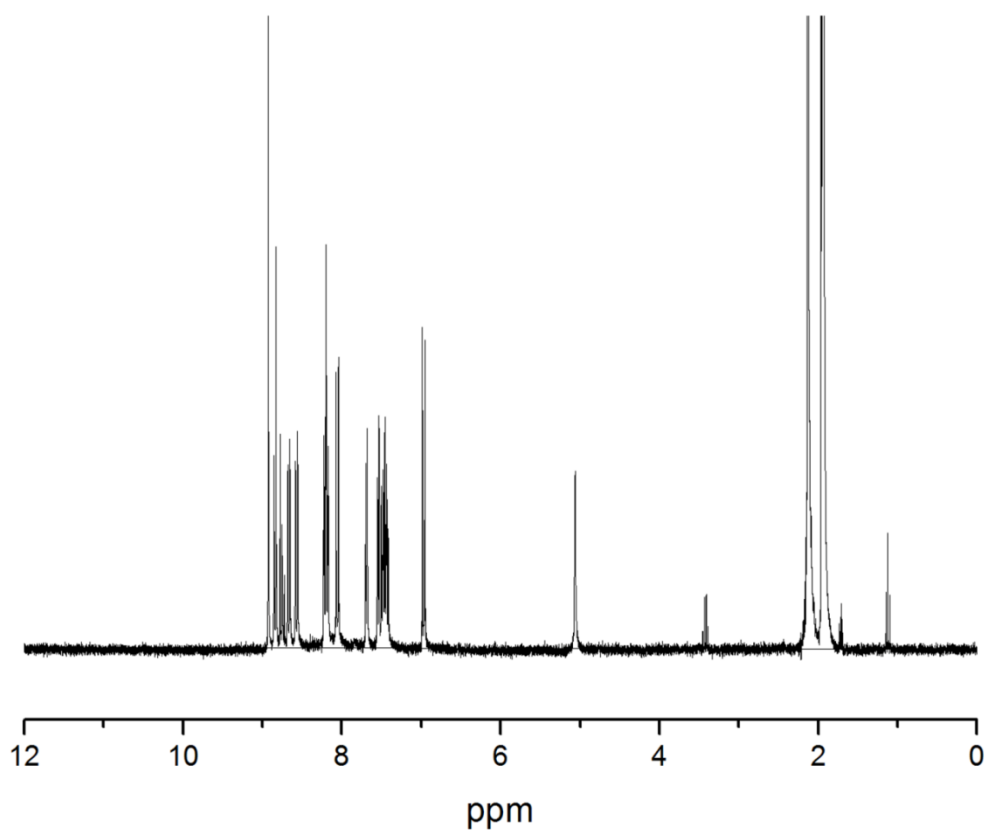
¹³C NMR spectrum of compound Ru(II) **3** in CD₃CN at 75 MHz



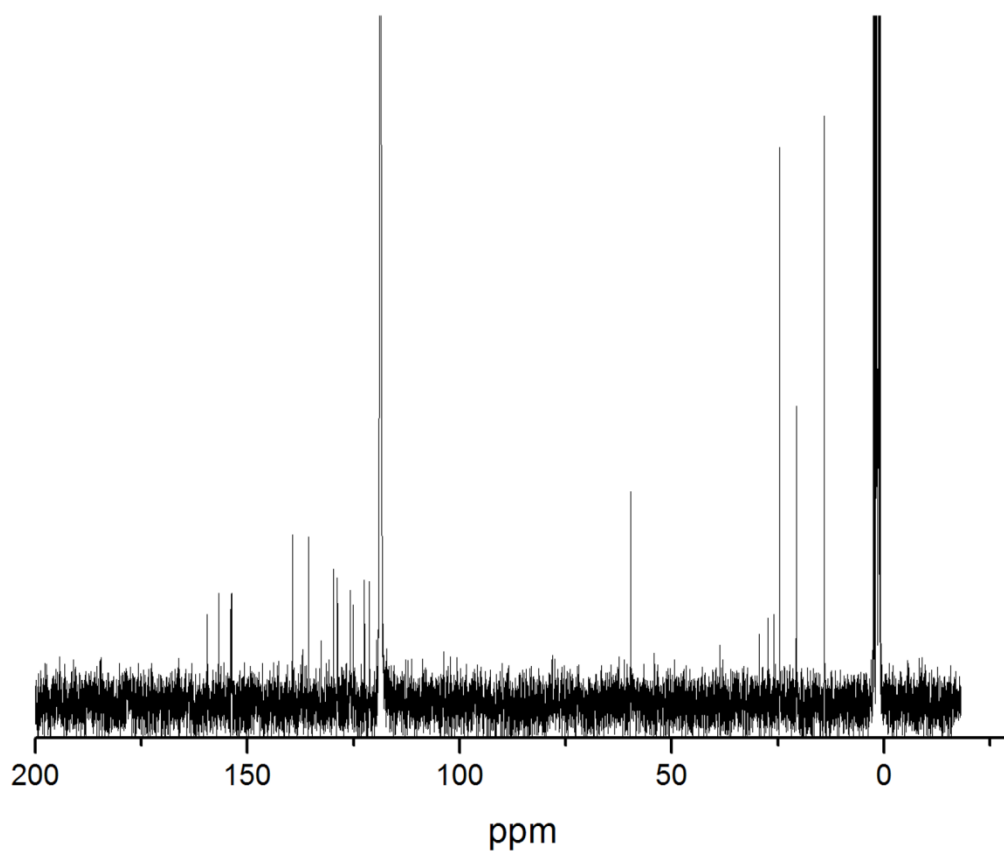
¹H NMR spectrum of compound Ir(III) **3** in CD₃CN at 300 MHz



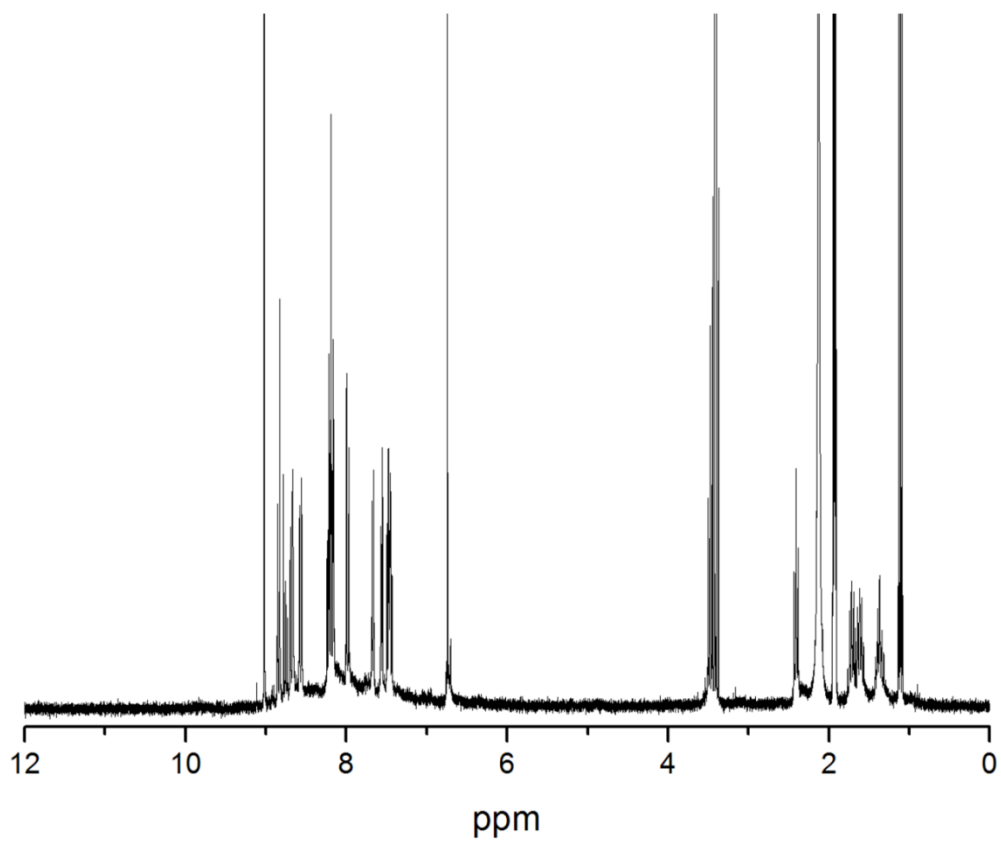
¹³C NMR spectrum of compound Ir(III) **3** in CD₃CN at 75 MHz



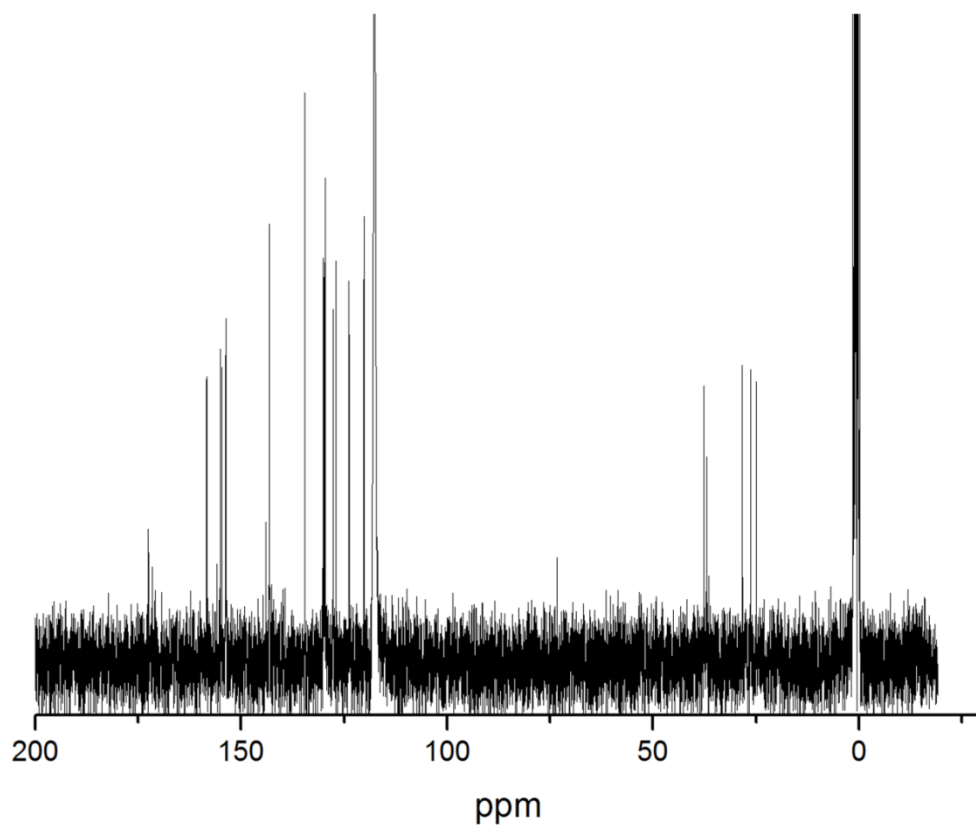
^1H NMR spectrum of compound Ru(II) **4** in CD_3CN at 300 MHz



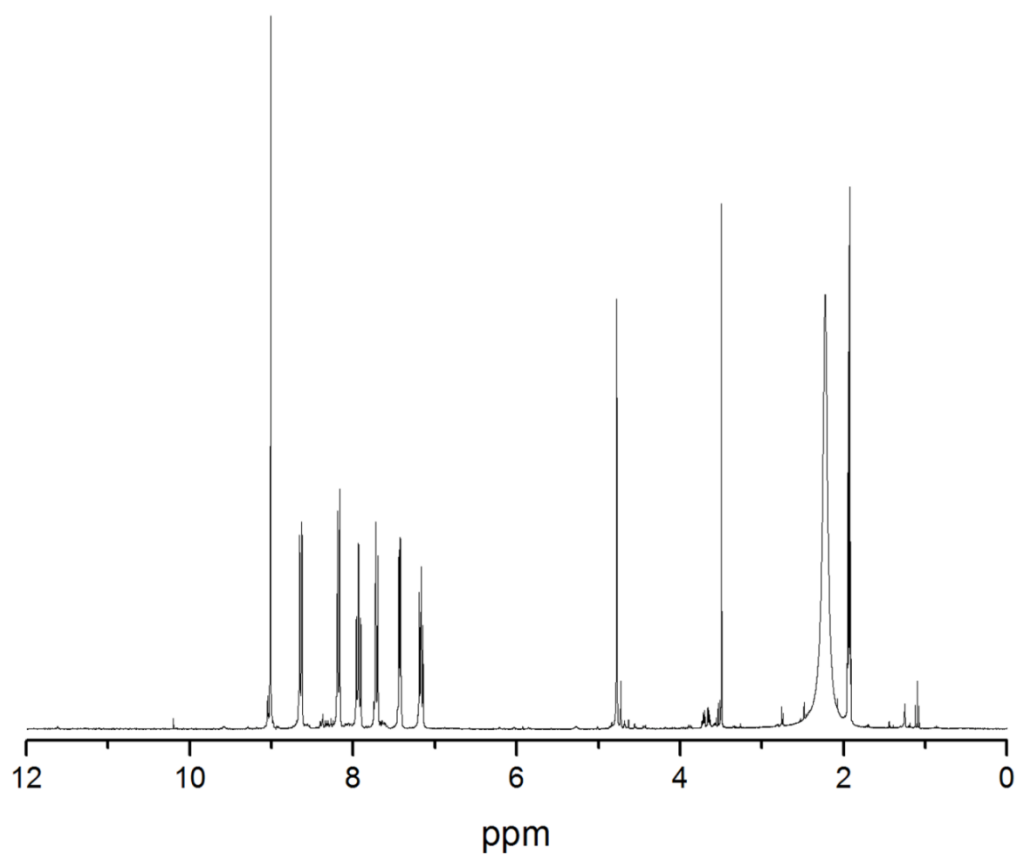
^{13}C NMR spectrum of compound Ru(II) **4** in CD_3CN at 75 MHz



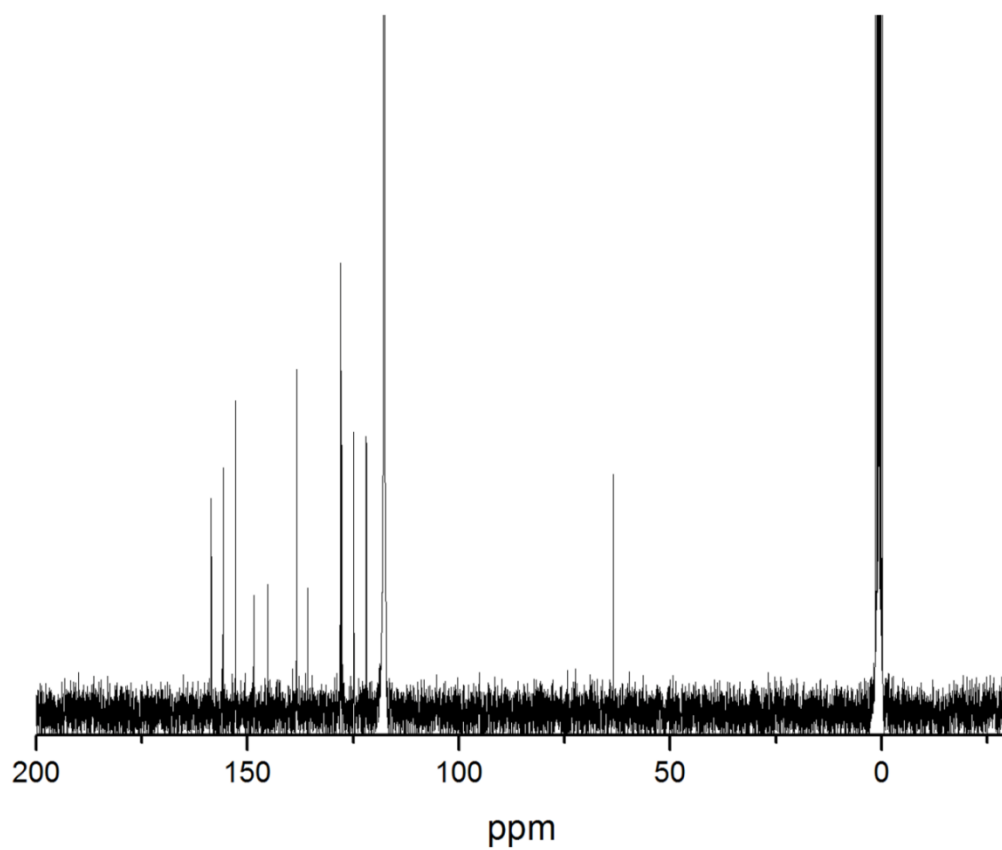
¹H NMR spectrum of compound Ir(III) **4** in CD₃CN at 300 MHz



¹³C NMR spectrum of compound Ir(III) **4** in CD₃CN at 75 MHz



^1H NMR spectrum of compound Ir(III) **6** in CD_3CN at 300 MHz



^{13}C NMR spectrum of compound Ir(III) **6** in CD_3CN at 75 MHz

Table S1 Selected distances (Å) and angles (deg) for Ru(II) **3** and Ru(II) **4**

| | Ru(II) 3 ^a | Ru(II) 4 ^a |
|--------------------------------|------------------------------|------------------------------|
| Ru-N1A | 2.061(3) | 2.076(4) |
| Ru-N2A | 1.980(3) | 1.976(4) |
| Ru-N3A | 2.061(3) | 2.068(4) |
| Ru-N1B | 2.061(3) | 2.061(4) |
| Ru-N2B | 1.971(3) | 1.985(4) |
| Ru-N3B | 2.065(3) | 2.060(4) |
| C5A-C6A | 1.463(4) | 1.458(5) |
| C5B-C6B | 1.472(4) | 1.481(5) |
| C10A-C11A | 1.466(4) | 1.467(5) |
| C10B-C11B | 1.483(4) | 1.462(5) |
| C8B-C16B | 1.469(5) | 1.486(6) |
| N2A-Ru-N3A ^b | 78.8(5) | 78.7(9) |
| C7B-C8B-C16B-C17B ^c | 29.1(2) | 23.9(8) |

^a Crystallisation solvent: DMF-Et₂O. ^b Angle between terpyridine rings and Ru. ^c Dihedral angles between terpyridine and aryl group.