Supplementary Material for Dalton Transactions

Synthesis, structures, cellular uptake studies and apoptosis-inducing properties of highly cytotoxic ruthenium-Norharman complexes

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Supplemental experimental procedures

Cytotoxicity assays

The cytotoxicity of the listed compounds against the indicated cell lines was determined by MTT assay, as previously described.[1] The percentage of cell viability was calculated using the equation: (mean OD of treated cells / mean OD of control cells) × 100%. Cells treated with vehicle (1% DMSO) were used as controls. Data were presented as means of three independent experiments ± standard deviations and the duration of treatment was 48 h.

Cellular uptake studies

For live cell confocal microscopy, HeLa cells were seeded into 35 mm glass-bottom dishes (Corning) and grown to approximately 80% confluence. Complex **3** (5 μM) was added to the culture medium (final DMSO concentration: 1% v/v) and incubated for varying amounts of time at 37 °C. The cells were then washed twice with PBS (2 × 500 μL) and visualized under a confocal laser scanning microscope (TCS SP5, Leica, Wetzlar, Germany) with excitation at 350 nm and emission at 440–460 nm.

For GF-AAS, HeLa cells were seeded in 60 mm culture dishes (Corning) and grown to approximately 90% confluence. The cells were then treated with the **1** (100 μM), **2** (100 μM) and **3** (10 μM) for 2 h (final DMSO concentration: 1% v/v). The cells were collected by trypsinization and washed twice with PBS, and ruthenium content determined by GF-AAS was presented as ng ruthenium per mg cellular protein as described in the literature.[2]

Cell cycle analysis and apoptosis assays

Cell cycle analysis

Cell Cycle analysis was performed as previously described.[3] Briefly, HeLa cells were treated with Ru(II) complexes (**1**: 5 μM; **2**: 2.5 μM; **3**: 0.5 μM) for various time intervals. The cells were harvested and centrifuged (5 min at 800 g) and fixed in 2 mL of 70% aqueous ethanol (v/v). After an incubation period of at least 12 h at –20 ^{o}C, cells were centrifuged (15 min at 800 g) and washed twice with ice-cold PBS. The cells were resuspended in 200 μL staining solution containing PI (10 μg/mL) and DNase-free RNase (100 μg/mL) and analyzed by a BD FACSCalibur^{TM} cytometer (Becton Dickinson, Heidelberg, Germany). The number of cells analyzed for each sample was 10, 000, and the experiments were repeated at least three times under identical conditions. Data were collected by BD CellQuest™ Pro software and analyzed by ModFit LT 2.0 software.

Morphological studies

HeLa cells were seeded into 24-well plates (Corning) and grown to approximately 40% confluence. The growth medium was removed, and the cells in each well were exposed to 500 μL of culture medium containing **3** (2 μM) for 24 h or 48 h. The cell images were acquired by an inverted microscope (Axio Observer Z1, Carl Zeiss, Germany) at a magnification of 10×.

Hoechst staining

HeLa cells were grown on chamber slides to about 50% confluence. Ru(II) complexes (**1**: 10 μM; **2**: 10 μM; **3**: 2 μM) were then added into the wells and incubated for various time intervals. After fixed with 4% paraformaldehyde for 30 min and washed with ice-cold PBS, the cells were labeled with Hoechst 33342 (5 μg/mL). The nuclear morphology was visualized under a fluorescence microscope (Axio Imager Z1, Carl Zeiss, Germany). The rate of abnormal (condensed or fragmented) nuclei was determined in three independent experiments counting at least 10 fields of view and 300 cells per sample. Data were expressed as a percentage of apoptotic nuclei compared to total number of cells.

Annexin V assay

Annexin V staining of the apoptotic membranes was performed by using the annexin V-FITC apoptosis detection kit following the manufacturer's protocol (Sigma-Aldrich). For confocal microscopy, HeLa cells seeded into 35 mm glass-bottom dishes (Corning) were treated with **3** (2 μM) for 24 h. After washed twice with PBS, the cells were stained with annexin V-FITC for 10 min and observed immediately under a confocal laser scanning microscope (TCS SP5, Leica, Wetzlar, Germany) with excitation at 488 nm and emission at 500–560 nm.

For flow cytometry analysis, HeLa cells cultured with Ru(II) were trypsinized and washed twice with PBS, after which the cells were stained with annexin V-FITC for 10 min. Annexin-fluorescence intensity was measured by a BD FACSCalibur^{TM} cytometer (Becton Dickinson, Heidelberg, Germany) with an excitation wavelength of 488 nm and an emission wavelength of 530 ± 15 nm (FL-1 channel). Annexin-V positive Cells were considered apoptotic.

Activation of caspase-3/7

For detection of caspase-3/7 activition, cells cultured in 96-well plates were treated with **1**−**3** at the concentrations indicated in the figure legends for 6 h and analyzed using Caspase-Glo Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

DNA binding studies

Absorption spectral studies

Absorption titration experiments were performed in Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, pH 7.1) following the literature method.[4] Briefly, the concentration of the complexes was kept constant ([Ru] = 10 μM), and the absorbance (A) was recorded after successive additions of CT-DNA ([DNA] = 0–200 μM). The intrinsic binding constants K_b illustrating the binding strength of the complexes with CT-DNA were determined according to the literature method.[5,6]

Competitive binding with EB

The experiments of competitive binding between EB were carried out in Tris-HCl buffer (5 mM Tris-HCl, 50 mM NaCl, pH 7.1) by keeping [DNA]/[EB] = 5 and varying the concentrations of Ru(II) complexes as described in literature.[1] The fluorescence spectra of EB were measured using an excitation wavelength of 537 nm and the emission range was set between 550 and 750 nm. The spectra were analyzed according to the classical Stern–Volmer equation, $F_0/F = 1 + K_{SV}[Q]$, where F_0 and F were fluorescence intensities at 590 nm in the absence and presence of Ru(II), respectively, K was the linear Stern–Volmer quenching constant, [Q] was the ratio of the total concentration of the complexes to that of DNA ([Ru]/[DNA]).

Fluorescence titration

The determination of the intrinsic binding constants K_b was carried out following the literature method.[7-10] Briefly, the concentration of Ru(II) was kept constant (1 μM), and increasing amounts of

DNA were added (0.25~20 μM). The fluorescent intensity was monitored at 450 nm with an excitation wavelength set at 350 nm. The binding constants were calculated using the equations given below:

$C_b = C_t[(F_0 - F)/(F_0 - F_f)]$ (**1**)

$C_f = C_t - C_b$ (**2**)

$r/C_f = K_b(1 - nr)[(1 - nr)/(1 - (n - 1)r)]^{n-1}$ (**3**)

where C_t was the total concentration of [Ru], F was the fluorescence intensity at the given DNA concentration, F_0 was the fluorescence intensity in the absence of DNA, F_f was the fluorescence intensity when an equilibrium was obtained, r was the binding ratio C_b/[DNA], and C_b and C_f represented the bound and free concentration of [Ru], respectively. The binding constants were obtained using the modified Scatchard equation (**3**), given by McGhee and Von Hippel. [11,12]

Supplemental figures and tables

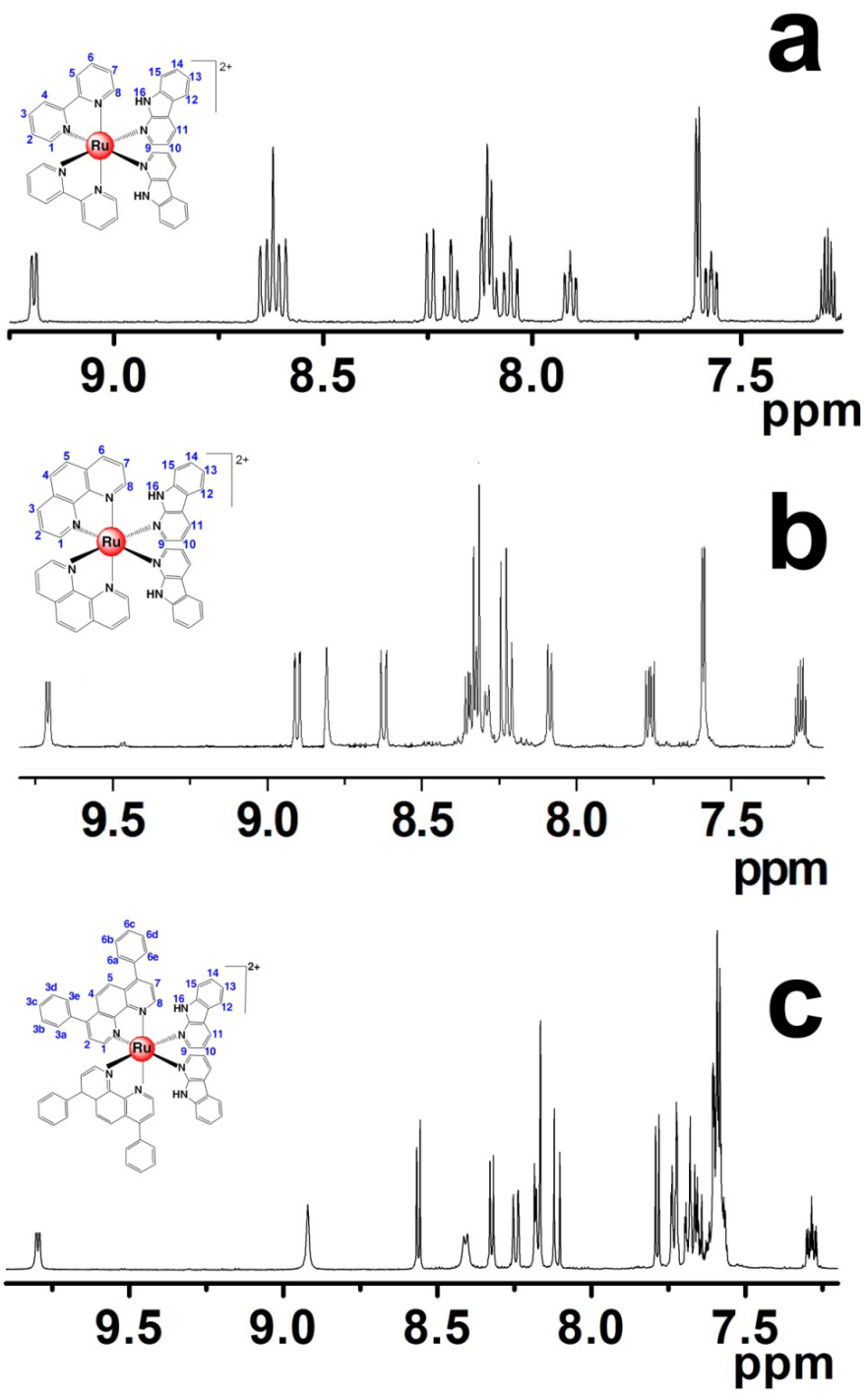

Fig. S1. ^{1}H NMR spectra and the numbering schemes used for NMR assignments of **1** (**a**), **2** (**b**) and **3** (**c**).

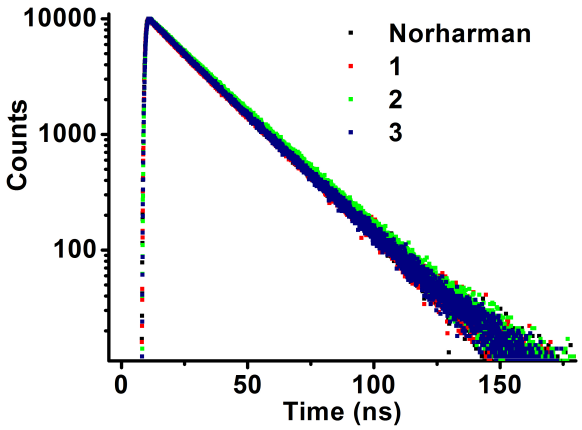

Fig. S2: Time-resolved fluorescence decays of Norharman, **1**, **2** and **3** in Tris-HCl Buffer (λ_{ex} = 350 nm; λ_{em} = 450 nm).

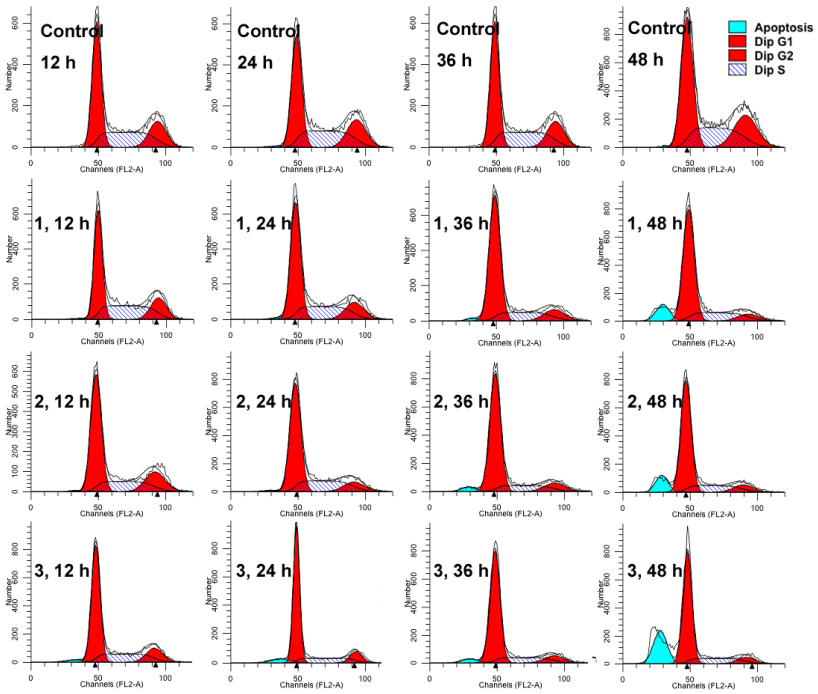

Fig. S3. Effect of **1** (5 μM), **2** (2.5 μM) and **3** (0.5 μM) on the distribution of HeLa cells in cell cycle phases after treatment for 12, 24, 36 and 48 h (Dip G1: G0/G1 phase cells; Dip S: S-phase cells; Dip G2: G2/M-phase cells; apoptosis: apoptotic cells).

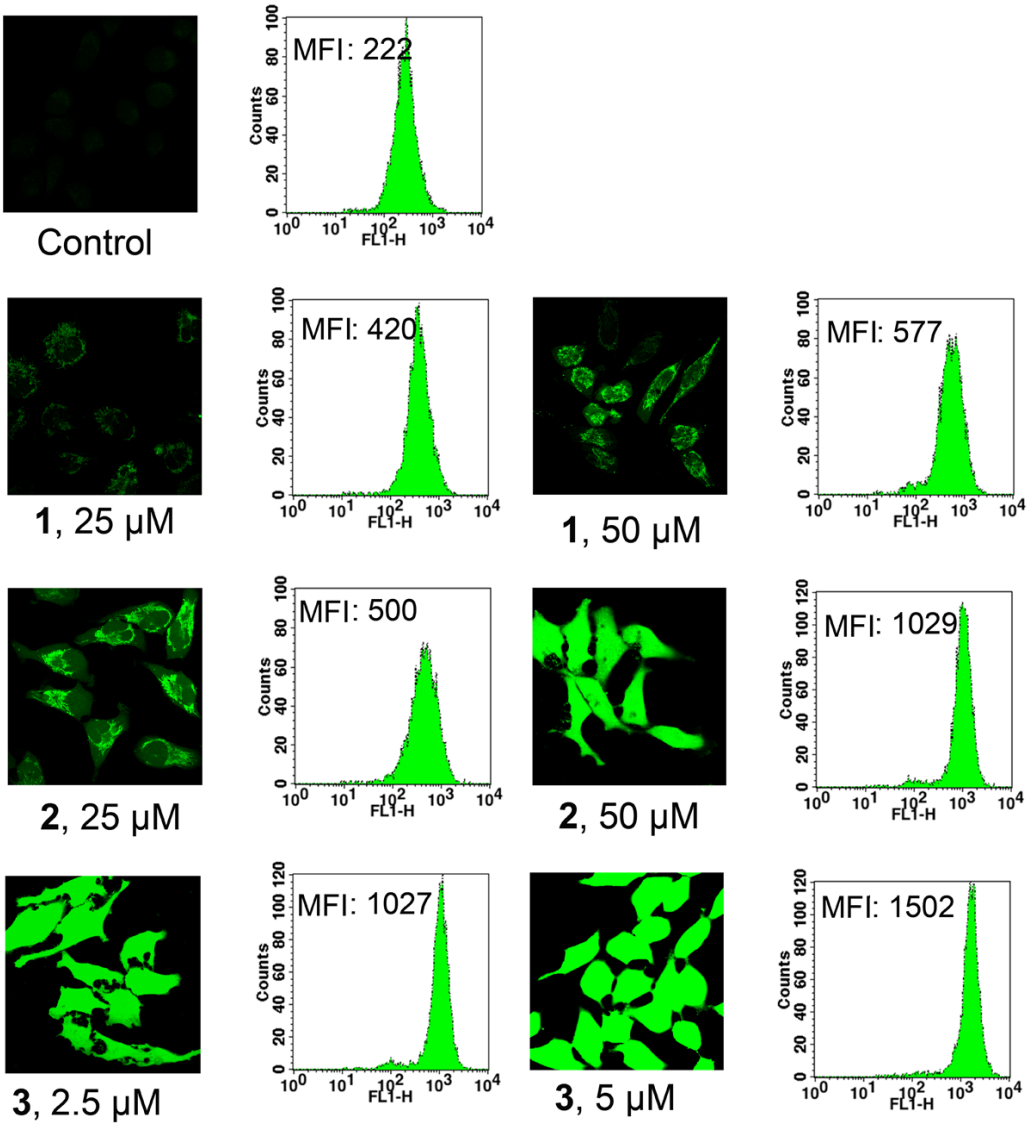

Fig. S4. Analysis of ROS production after HeLa cells were treated with Ru(II) analyzed by confocal microscopy and flow cytometry at the indicated concentrations for 6 h. Left: The intracellular ROS level was detected by confocal microscope (excitation at 488 nm and emission at 500–560 nm); Right: ROS level is expressed as MFI measured by flow cytometry (FL-1 channel, excitation at 488 nm and emission at 530 ± 15 nm).

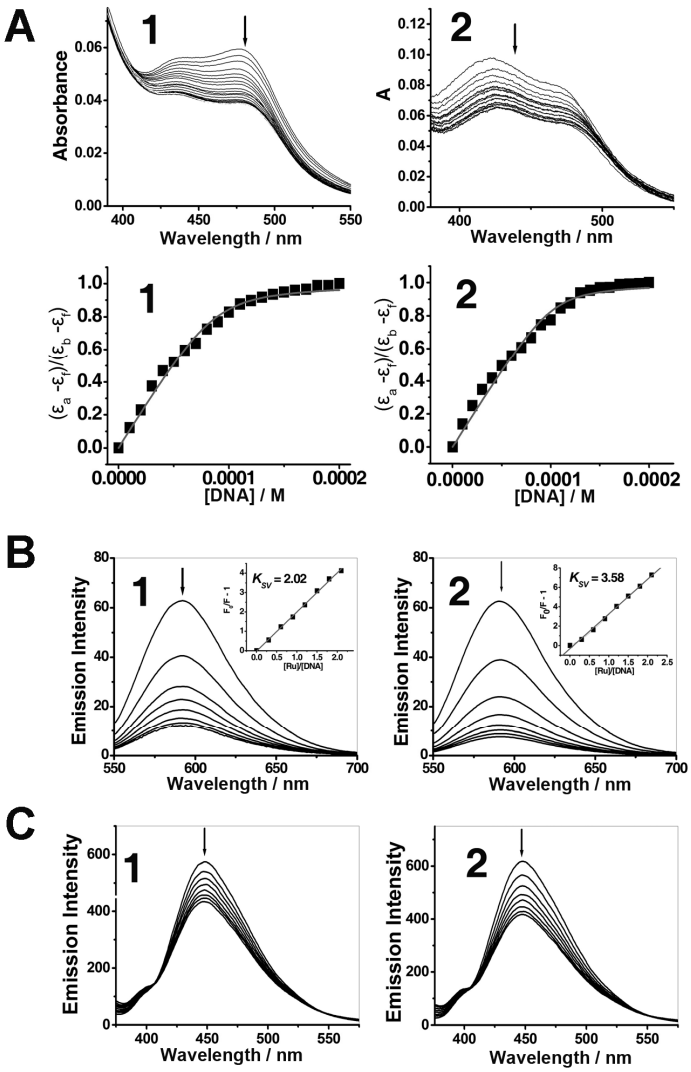

Fig. S5. DNA binding properties of complexes **1** and **2**. (**A**) Upper: the absorbance changes in the ^{1}MLCT bands of **1**and **2** in Tris-HCl buffer at 25 ºC in the absence and presence of increasing amounts of CT-DNA. [Ru] = 10 μM, [DNA] = 0–200 μM from top to bottom. Arrows indicate the changes in absorbance upon increasing the DNA concentration. Below: plots of $(\varepsilon_a - \varepsilon_f)/(\varepsilon_b - \varepsilon_f)$ *vs* [DNA] and the nonlinear fit for the titration of the complexes with DNA. (**B**) Emission spectra of EB bound to DNA in the presence of Ru(II). [EB] = 2 μM; [DNA] = 10 μM; [Ru]/[DNA] = 0–2.10; λ_{ex} = 537 nm. The arrows show the intensity changes upon increasing concentrations of the complexes. Inset: plots of $F_0/F - 1$ *vs* [Ru]/[DNA] with ■ for experimental data points and full line for linear fitting of the data. (**C**) Fluorescence spectra of **1**and **2** (1 μM) with increasing concentrations of CT-DNA (0–10 μM) measured in Tris-HCl buffer at 25 ºC.

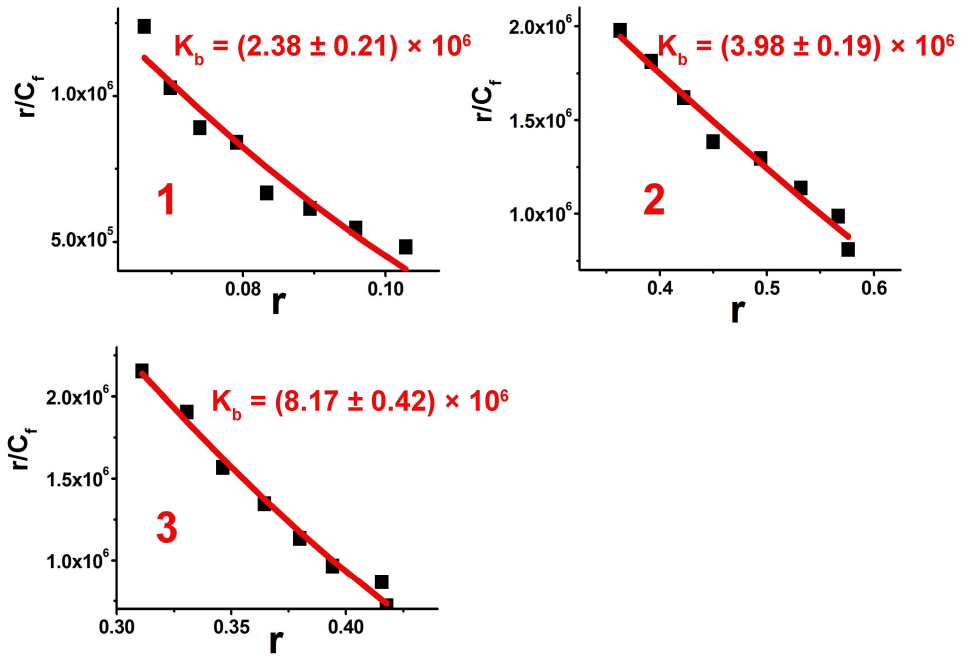

Fig. S6. Scatchard plots of the fluorescence titration data of Ru(II) complexes.

Table S1 Summary of crystal data and data collection parameters for **1**•$2C_6H_5CH_3$ and **2**•$3H_2O$

Complexes	**1**•$2C_6H_5CH_3$	**2**•$3H_2O$
formula	$C_{58}H_{48}F_6N_8O_6RuS_2$	$C_{48}H_{38}F_6N_8O_9RuS_2$
formula weight	1232.23	1150.05
T (K)	173(2)	173(2)
crystal system	Monoclinic	Monoclinic
space group	C2/c	P2(1)/c
a (Å)	20.372(2)	13.7259(16)
b (Å)	18.564(2)	21.933(2)
c (Å)	16.544(2)	17.942(2)
α (deg)	90.00	90.00
β (deg)	119.485(2)	109.690(2)
γ (deg)	90.00	90.00
volume (Å^3)	5446.3(11)	5085.6(10)
Z	4	4
ρ_{calc} (g cm^{-3})	1.503	1.502
μ (mm^{-1})	0.445	0.475
crystal size	0.41 × 0.34 × 0.29	0.31 × 0.22 × 0.21
total reflections	12793	28214
unique reflections (R_{int})	5295 (0.0346)	9957 (0.0428)
GOF^b	1.052	1.293
Final R^a indexes [I>2σ (I)]	R_1 = 0.0436, wR_2 = 0.1055	R_1 = 0.1039, wR_2 = 0.2933

$^a R1 = \sum \| |F_0| - |F_c| \| / \sum |F_0|, wR2 = \left\{ \sum \left[w\left(F_0^2 - F_c^2\right)^2 \right] / \sum \left[w\left(F_0^2\right)^2 \right] \right\}^{1/2}$

$^b GOF = \left\{ \sum \left[w\left(F_0^2 - F_c^2\right)^2 / (n-p) \right] \right\}^{1/2}$ where n is the number of data and p is the number of parameters refined.

Table S2 Electronic absorption spectral data of **1**−**3** at 298 K

Complexes	Solvent	λ_{abs}/nm (ε/L mol^{-1} cm^{-1})
1	CH_3OH	290 (70 502), 352 (26 849), 472 (8742)
	CH_3CN	287 (62 190), 346 (20 544), 440 (7756)
	CH_2Cl_2	292.5 (69 292), 355 (29 907), 449 (8414)
	Tris-HCl Buffer	289 (56 897), 357 (24 210), 457 (7097)
2	CH_3OH	265 (105 278), 351.5 (20 714), 450 (12 384)
	CH_3CN	264 (104 015), 346 (20 528), 450 (11 983)
	CH_2Cl_2	265 (120 886), 359 (25 069), 421 (14 455)
	Tris-HCl Buffer	259 (86 316), 359 (20 393), 450 (9599)
3	CH_3OH	279 (120 238), 353 (26 991), 440 (23 026)
	CH_3CN	279 (131 865), 358 (24 893), 439 (25 069)
	CH_2Cl_2	281 (129 628), 350 (29 147), 444 (24 995)
	Tris-HCl Buffer	281 (109 169), 350 (30 663), 445 (23 361)

Supplemental references

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