CONFOCAL FLUORESCENCE MICROSCOPY STUDIES OF A FLUOROPHORE-LABELED DIRHODIUM COMPOUND: VISUALIZING METAL-METAL BONDED MOLECULES IN LUNG CANCER (A549) CELLS

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1. Experimental Section

General Methods

The solvents used were of reagent grade quality. Dichloromethane (EMD Chemicals) was dried over 4Å molecular sieves and diisopropylamine (Alfa Aesar) was dried with CaH_2; both solvents were distilled prior to use. Methanol (EMD Chemicals) and acetone (EMD Chemicals) were used as received without further purification. Standard Schlenk-line techniques (N_2 atmosphere) were used to maintain anaerobic conditions during preparation of the compounds. Analytical thin layer chromatography (TLC) was performed on aluminum-backed sheets coated with silica 60 F254 adsorbent (0.20 mm thickness, EMD Chemicals). Flash chromatography (FC) was carried out with silica gel 60 (40-63 μm, Fluka). The following starting materials were purchased and used without further characterization: $RhCl_3•nH_2O$ (Pressure Chemical Co.), anhydrous DMF (Sigma Aldrich), 1,10-phenanthroline (Alfa Aesar), 3-butyn-1-ol (Sigma Aldrich), $Pd(PPh_3)_2Cl_2$ (Sigma Aldrich), CuI (Spectrum Chemicals), KCN (Alfa Aesar), DMAP (Acros Organics) and EDAC•HCl (AKScientific). The compounds 5-bromo-1,10-phenanthroline,[1] $Rh_2(O_2CCH_3)_4•2MeOH$,[2] $[Rh_2(\mu\text{-}O_2CCH_3)_2(\eta^1\text{-}O_2CCH_3)(L)(H_2O)_3][O_2CCH_3]$ (L= bpy, phen)[3] and bodipy-COOH[4] were prepared according to published procedures.

Instrumentation

^{1}H NMR spectra were recorded on Mercury 300 MHz or Inova 500 MHz spectrometers. Chemical shifts are reported in δ (ppm) and coupling constants (J) in hertz (Hz). The residual solvent peak was used as an internal reference (δ 3.31 for CD_3OD, δ 7.26 for $CDCl_3$). Electrospray mass spectra were acquired on an Applied Biosystems PE SCIEX QSTAR mass spectrometer (MDS Sciex). Elemental analyses were performed by Atlantic Microlab, Inc.

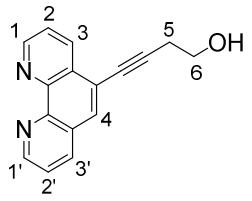
(Norcross, GA). Absorption spectra were recorded on a Shimadzu UVPC-3001 spectrophotometer.

A PTI QuantaMaster series spectrophotometer was used to perform the steady-state fluorescence spectroscopic studies at room temperature. The slit width was set to 1 nm for both excitation and emission, the step size was set to 1 nm and the integration time to 0.1 s. All spectra were corrected (real time correction) to account for variances in the arc lamp intensity and PMT voltage across the range of wavelengths. A long pass filter (455 nm) was placed between the sample and detector before the acquisition of the spectra. The relative fluorescence quantum yields (Φ) were calculated using fluorescein (Sigma aldrich) as reference (Φ_F= 0.95 in 0.1M NaOH) and the following equation:

$$\Phi_x = \Phi_r \left[\left(\frac{I_x}{A_x} \right) / \left(\frac{I_r}{A_r} \right) \right] \left(\frac{\eta_x}{\eta_r} \right)^2$$

where the subscripts "r" and "x" are used to denote the reference and the sample, respectively, I is the integrated sum of the emission intensity, A is the absorbance at the excitation wavelength (λ_{ex} = 496 nm) and η is the refractive index of the solvents used.

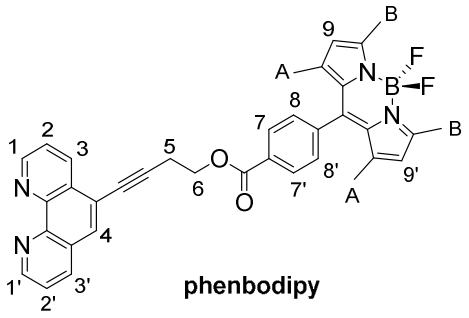
Synthetic procedures



4

4-(1,10-phenanthrolin-5-yl)but-3-yn-1-ol (4). A Schlenk flask was charged with $Pd(PPh_3)_2Cl_2$ (109 mg, 0.15 mmol), CuI (30 mg, 0.16 mmol) and DMF (3 mL) after which 5-bromo-1,10-phenanthroline (400 mg, 1.54 mmol), diisopropyl amine (2 mL) and 3-butyn-1-ol (235 μL, 3.08

mmol) were added. The resulting dark brown solution was heated at 80°C for 2 h. The solvent was removed under reduced pressure to give a dark brown oily residue which was dissolved in MeOH (10 mL). A solution of KCN (100 mg, 1.54 mmol) in H_2O (5 mL) was added which led to an instantaneous color change from dark brown to light yellow. The resulting solution was stirred for 1 h, diluted with H_2O (30 mL) and then extracted with CH_2Cl_2 (5 x 40 mL). The combined organic layers were dried with $MgSO_4$, and reduced to dryness. The residue was purified by FC (SiO_2, CH_2Cl_2/MeOH/Et_3N 91:8:1) to afford **3** as a light beige solid. Yield: 307 mg (80%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.20 (dd, 1H, ^3J = 4.2, ^4J = 1.8, H-1 or H-1'), 9.17 (dd, 1H, ^3J = 4.2, ^4J = 1.8, H-1' or H-1), 8.70 (dd, 1H, ^3J = 8.1, ^4J = 1.5, H-3), 8.17 (dd, 1H, ^3J = 8.1, ^4J = 1.5, H-3'), 7.92 (s, 1H, H-4), 7.68 (dd, 1H, ^3J = 8.1, ^3J = 4.2, H-2 or H-2'), 7.63 (dd, 1H, ^3J = 8.1, ^3J = 4.2, H-2' or H-2), 3.99 (t, 2H, ^3J = 6.3, H-6), 2.89 (t, 2H, ^3J = 6.3, H-5), 2.27 (s, 1H, -OH). HR-MS-ESI (*m/z*) calcd for $C_{16}H_{13}N_2O$ ($[M + H]^+$) 249.1028, found 249.1035.



phenbodipy

phenbodipy. A solution of bodipy-COOH (67 mg, 0.18 mmol) and **4** (55 mg, 0.22 mmol) in CH_2Cl_2 (25 mL) was cooled to 0°C and then EDAC•HCl (62 mg, 0.32 mmol) and DMAP (23 mg, 0.19 mmol) were added. The resulting green-orange solution was stirred at 0°C for 6 h and then slowly warmed to ambient temperature before being stirred for a further 48 h. The solution was washed with 0.1 M HCl (20 mL) and a saturated solution of $NaHCO_3$ (20 mL). The organic

phase was dried with anhydrous $MgSO_4$ and purified by FC (SiO_2, CH_2Cl_2/EtOAc/MeOH/Et_3N 50:46:2:2) to afford phenbodipy as a bright orange solid. Yield: 93 mg (85%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.19 (m, 2H, H-1 and H-1'), 8.74 (d, 1H, ^3J = 8.4, H-3), 8.26 (d, 2H, ^3J = 7.8, H-7, H-7'), 8.19 (d, 1H, ^3J = 8.1, H-3'), 7.99 (s, 1H, H-4), 7.65 (m, 2H, H-2, H-2'), 7.43 (d, 2H, ^3J = 7.8, H-8, H-8'), 5.98 (s, 2H, H-9, H-9'), 4.70 (t, 2H, ^3J = 6.6, H-6), 3.15 (t, 2H, ^3J = 6.6, H-5), 2.56 (s, 6H, CH_3^B), 1.33 (s, 6H, CH_3^A). HR-MS-ESI (*m/z*) calcd for $C_{36}H_{30}BF_2N_4O_2$ ([M + H]$^+$) 599.2430, found 599.2457.

Compound 1. Samples of $Rh_2(O_2CCH_3)_4$•2MeOH (60 mg, 0.12 mmol) and phenbodipy (71 mg, 0.12 mmol) were dissolved in acetone (20 mL). The resulting dark green-orange solution was stirred at ambient temperature with an orange solid precipitating within 30 minutes. The mixture was stirred for a total of 24 h and the bright orange-red solid was collected by filtration, washed with acetone (3 x 10 mL) and then suspended in MeOH (40 mL) with stirring for 24 h at ambient temperature. The resulting dark green-orange solution was concentrated to *ca.* 2 mL and diethyl ether (20 mL) was added slowly with stirring. The resulting hygroscopic brown-orange precipitate was collected by filtration and washed with copious amounts of diethyl ether. Yield: 32 mg (25%). This compound was obtained as a 1:1 mixture of two isomers. ^{1}H NMR (500 MHz, CD_3OD): δ 8.98 (m, 2H, H-1 or H-1'), 8.83-8.68 (m, 4H, H-1' or H-1, H-3 or H-3'), 8.65 (d, 2H, ^3J = 8.5, H-3' or H-3), 8.29 (m, 6H, H-7, H-7', H-4), 8.00 (m, 2H, H-2 or H-2'), 7.95 (m, 2H, H-2' or H-2), 7.51 (d, 2H, ^3J = 8.5, H-8, H-8'), 6.06 (s, 4H, H-9, H-9'), 4.72 (t, 4H, ^3J = 6.0, H-6), 3.21 (m, 4H, H-5), 2.49 (s, 12H, CH_3^B), 2.37 (s, 3H, μ-$O_2CCH_3^-$), 2.36 (s, 3H, μ-$O_2CCH_3^-$), 2.32 (s, 3H, μ-$O_2CCH_3^-$), 2.31 (s, 3H, μ-$O_2CCH_3^-$), 1.88 (s, 6H, $O_2CCH_3^-$), 1.35 (s, 12H,

CH_3^A), 1.06 (s, 3H, η^1-$O_2CCH_3^-$), 1.02 (s, 3H, η^1-O_2CCH_3). Anal. Calcd. for $C_{44}H_{47}BF_2N_4O_{13}Rh_2 \bullet 2H_2O$: C, 46.75; H, 4.55; N, 4.96. Found: C, 46.45; H, 4.47; N, 5.19.

Cell Culture

The A549 cell line, derived from type II pneumocytes (CCL 185) was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM-F12 medium with 10% FBS. Cultures were approximately 80% confluent at the time of analysis. Cell cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

In vitro Cytotoxicity

Cells were plated in 96 well plates and pre-incubated at 37°C for 24 h. Compound **1** was added at different concentration (0–100 μM) and the cells were incubated for another 48 h. Cells were then washed twice with PBS and fixed with methanol for 30 min. Following fixation, Janus green (1 mg/mL) was added to each well and incubated at room temperature for 5 min. Cells were again washed twice with PBS and 100 μL of methanol was added to each well. Janus green signal was then measured using a BioTek Synergy 4 plate reader set to an absorbance of 630 nm. The experiment was performed in triplicate.

Confocal Fluorescence Microscopy Studies

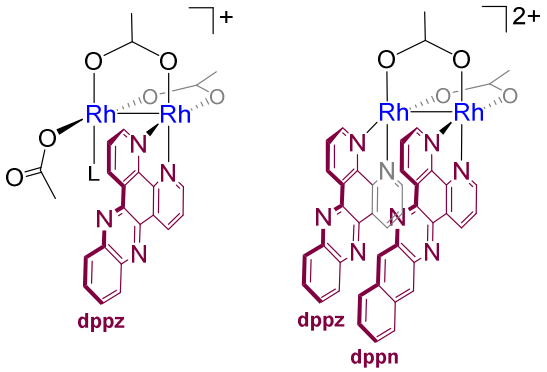
Live cell imaging studies were performed using a Zeiss 510 META NLO multiphoton system consisting of an Axiovert 200 MOT inverted laser scanning confocal microscope (Carl Zeiss Microimaging, Thornwood, NY). A Zeiss Plan-Apochromat 63x/NA=1.4 oil immersion objective was used to acquire the images.

The compounds phenbodipy and **1** were excited with an Ar–ion laser at 488 nm and emission was monitored using a band pass 500–550 filter. To collect Hoechst 33258 (Invitrogen) fluorescence, cells were irradiated with the Chameleon tunable Ti:Sapphire laser (Coherent Inc., Santa Clara, CA) at an excitation wavelength of 740 nm (which is roughly equivalent to 370 nm in single photon excitation with a continuous wavelength laser system) and emission was collected at 430–480 nm. LysoTracker® Red DND-99 (Invitrogen) was excited with a He–Ne laser at 543 nm and emission was monitored using a BP 565–615 filter. Mytotracker® Deep Red FM (Invitrogen) was excited with a He–Ne laser at 633 nm and emission was collected using a BP 650–710 filter. Image acquisition was performed sequentially to reduce the possibility of bleedthrough between channels.

The cellular distribution of phenbodipy and **1** was studied in A549 lung cancer cells. Cells were incubated (37°C) with either phenbodipy or **1** at 1 μM concentration for 2 and 24 h and the cells were washed before collecting the images. In the case of the localization experiments with lysosomes, cells were incubated with **1** (10 and 100 μM) for 5 and 24 h. Cells were then washed with PBS and loaded with 1 μg/mL Hoechst 33258 and 50 nM LysoTracker® Red DND-99 for 30 min. Cells were then washed and images were acquired. At least 10 images were collected per time point per treatment. To analyze the localization with mitochondria, cells were incubated with **1** (10 and 100 μM) for 5 and 24 h. Cells were then washed with PBS and loaded with 1 μg/mL Hoechst 33258 and 100 nM Mytotracker® Deep Red FM for 30 min. Cells were then washed and images were acquired. At least 10 images were collected per time point per treatment. Mander's colocalization coefficients of **1** with either Lysotracker or Mitotracker were determined using the Image J software (National Institutes of Health, USA).

To monitor the cellular uptake of **1**, cells were incubated with the metal complex at 10, 50 and 100 μM for 24 h. Cells were then washed with PBS and at least 8 images per concentration were collected and fluorescence intensities were recorded.

2. Supporting Figures



2 **3**

Figure S1. Molecular structures of $[Rh_2(\mu\text{-}O_2CCH_3)_2(\eta^1\text{-}O_2CCH_3)(dppz)(L)]^+$ (**2**, L = MeOH) and $[Rh_2(\mu\text{-}O_2CCH_3)_2(dppn)(dppz)]^{2+}$ (**3**). The axial solvent molecules have been omitted; dppz = dipyrido[3,2-*a*:2′,3′-*c*]phenazine and dppn = benzo[*i*]dipyrido[3,2-*a*:2,3-*c*]phenazine.

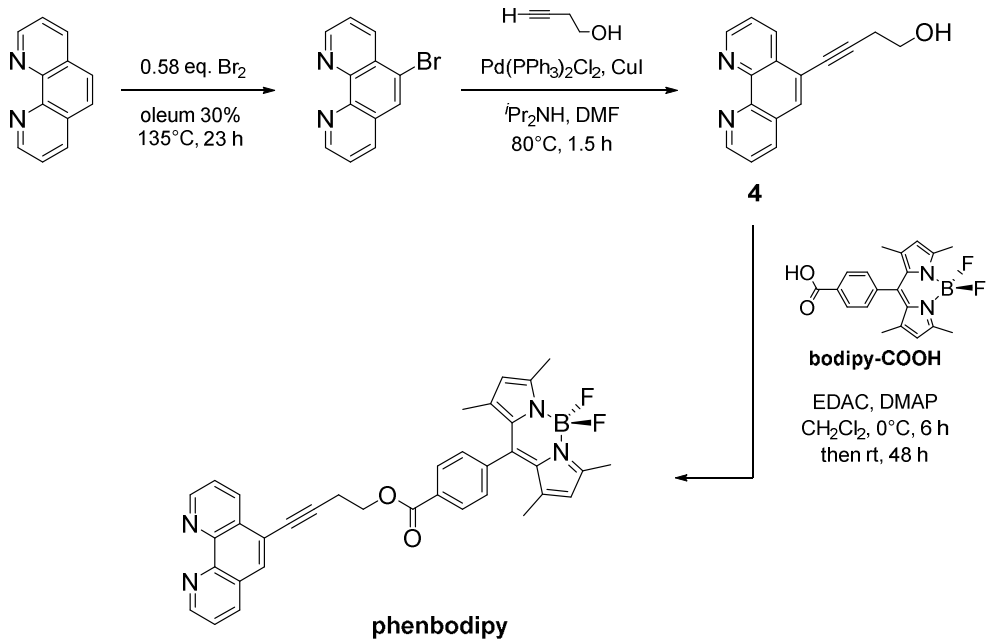


Figure S2. Synthesis of phenbodipy.

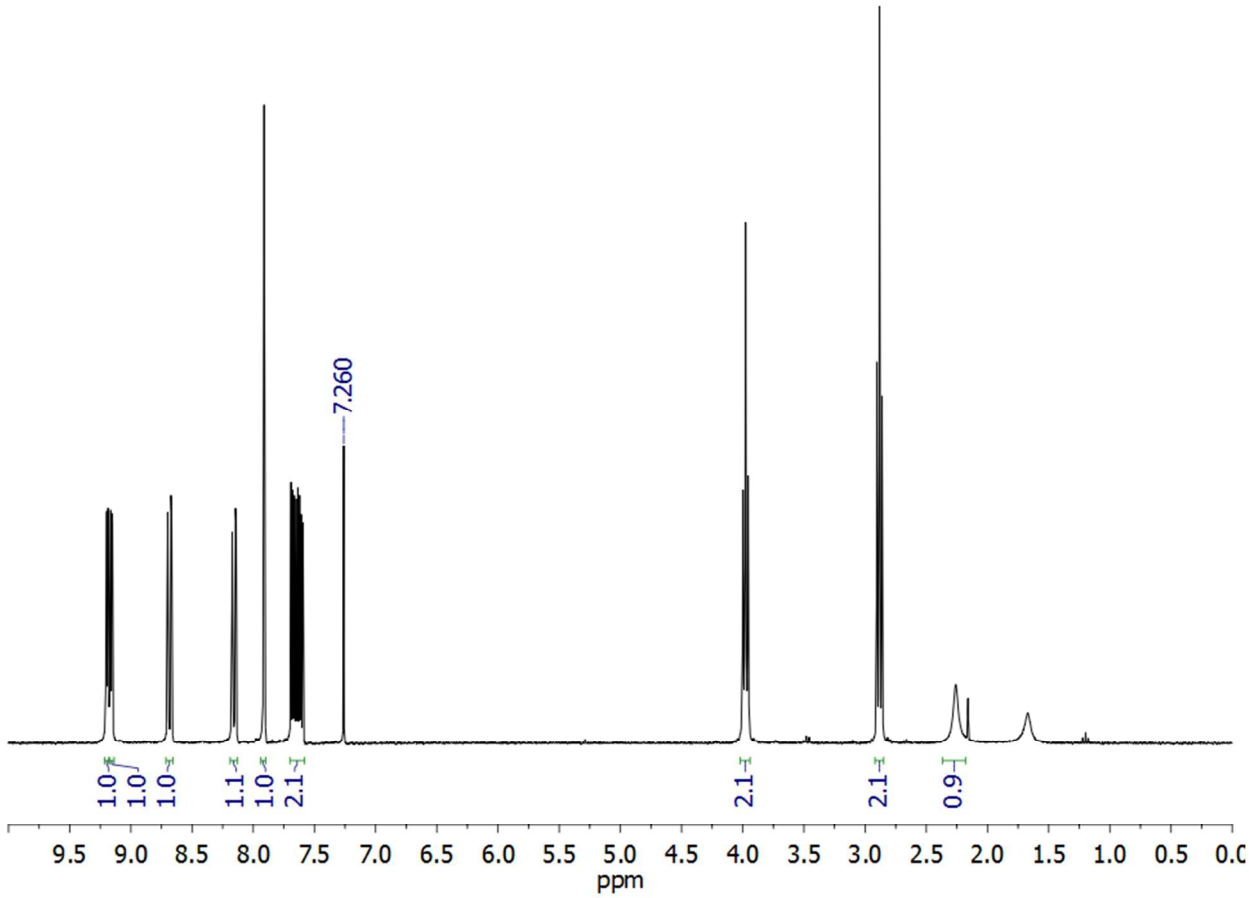


Figure S3. ^{1}H NMR spectrum of **4** (300 MHz, $CDCl_3$).

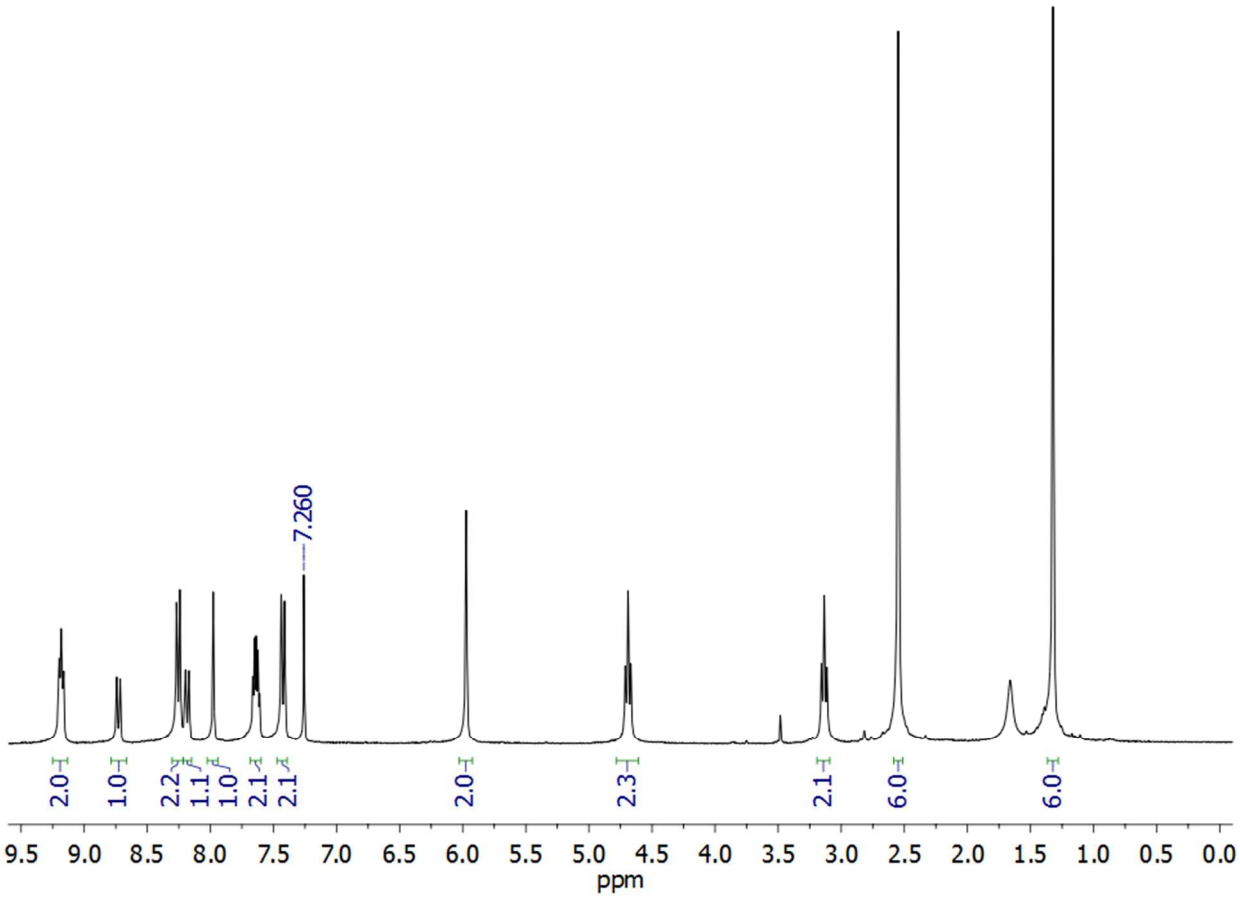


Figure S4. ^{1}H NMR spectrum of phenbodipy (300 MHz, $CDCl_3$).

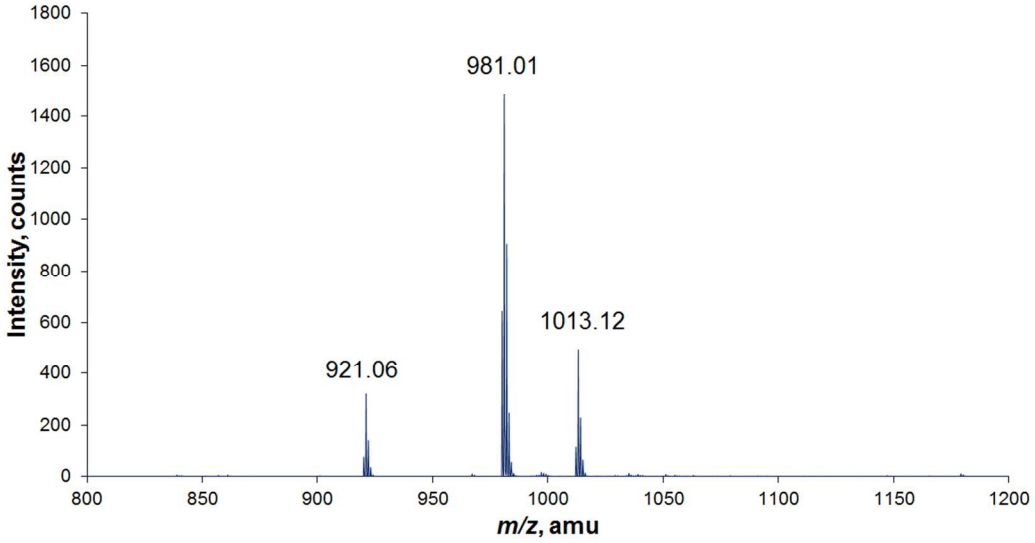


Figure S5. Electrospray ionization (ESI+, methanol) mass spectrum of **1**.

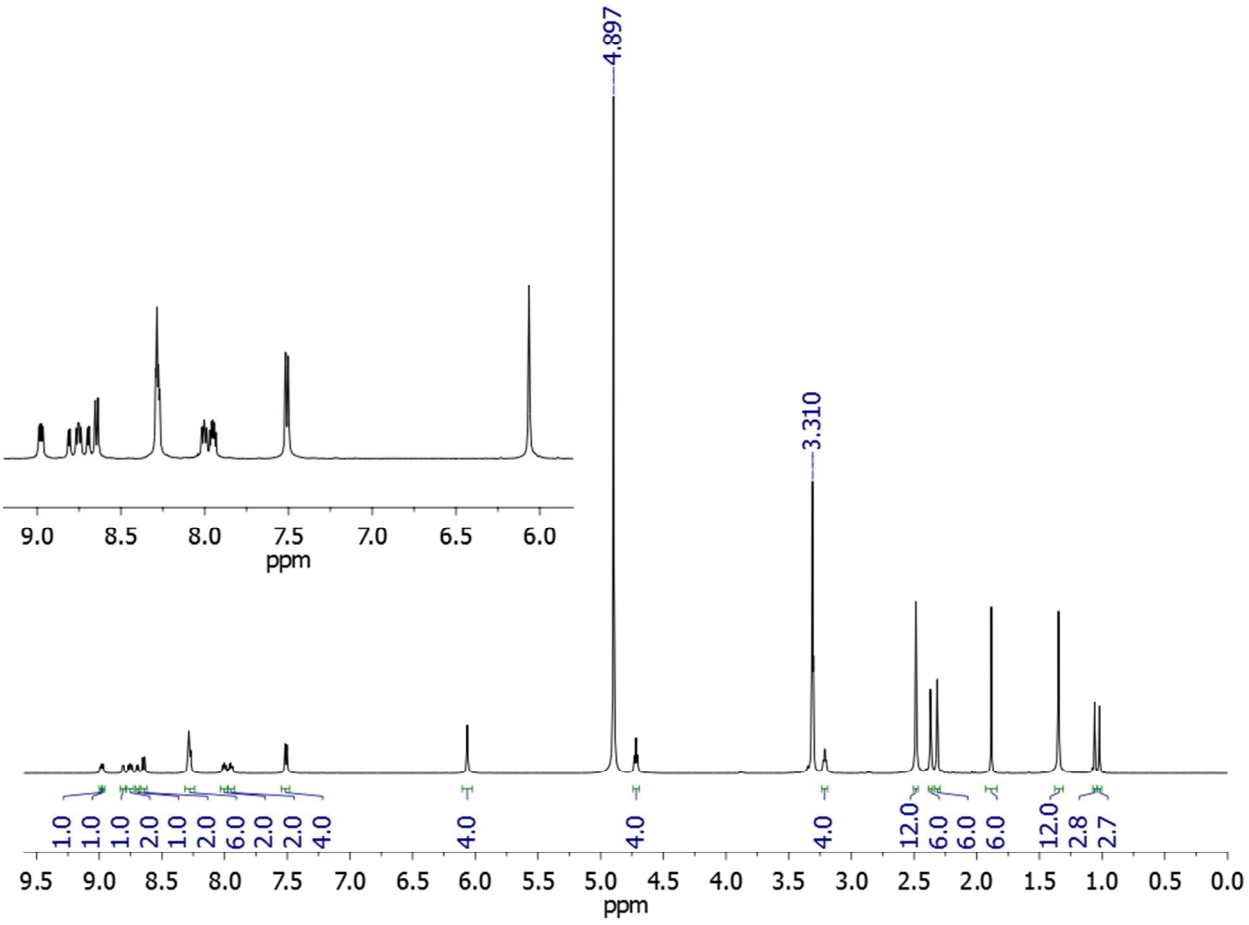


Figure S6. ^{1}H NMR spectrum of **1** (500 MHz, CD_3OD).

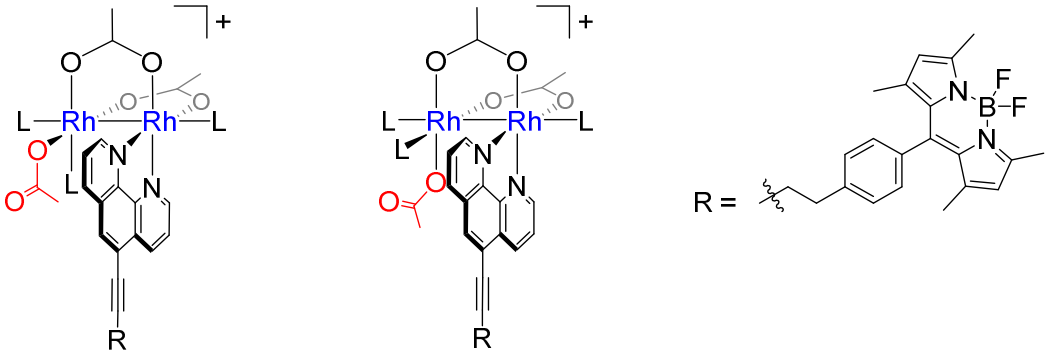


Figure S7. Molecular structures of the two isomers of **1**. L denotes a solvent molecule.

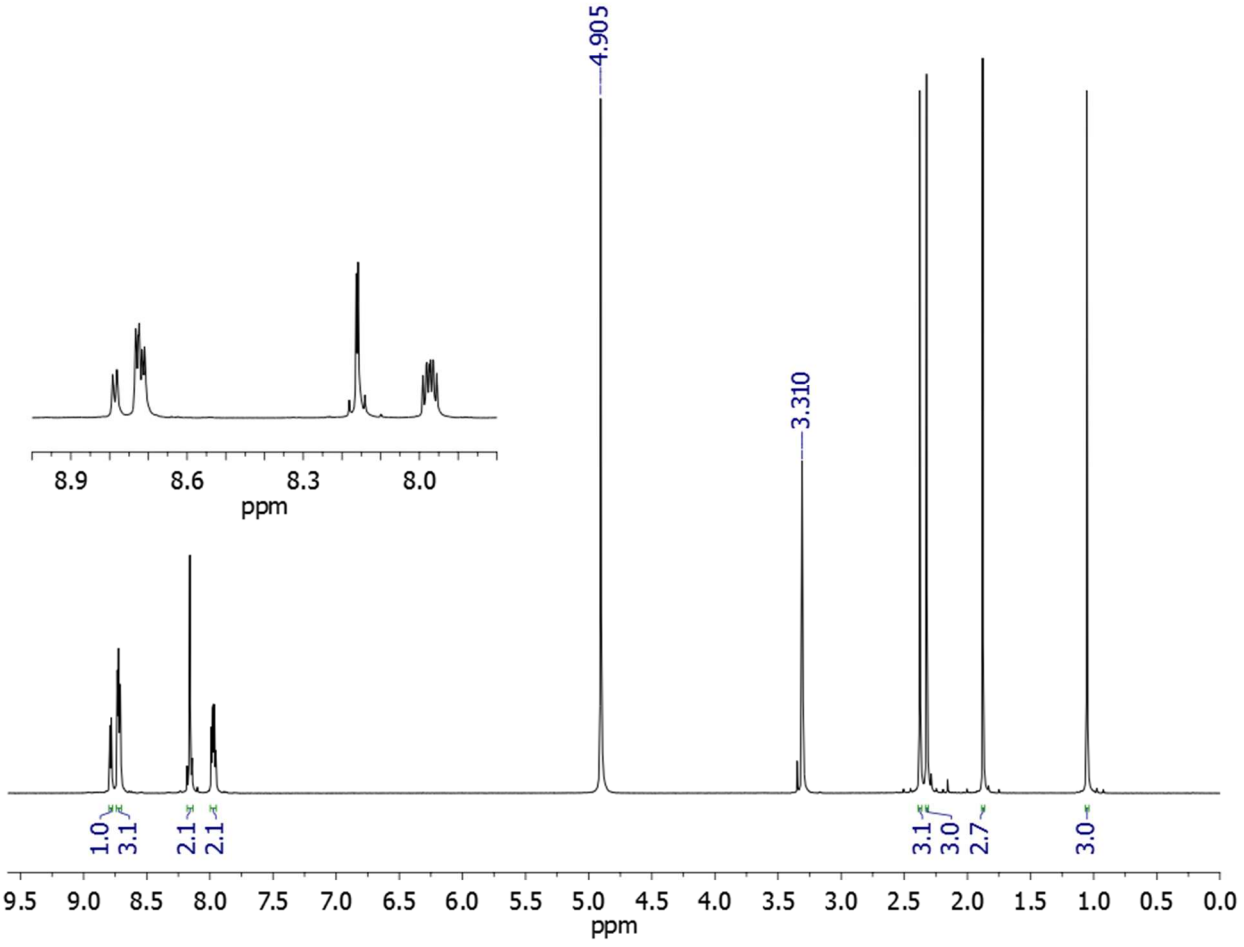


Figure S8. ^{1}H NMR spectrum of $[Rh_2(\mu\text{-}O_2CCH_3)_2(\eta^1\text{-}O_2CCH_3)(phen)(H_2O)_3][O_2CCH_3]$ (Rh_2phen, 500 MHz, CD_3OD).

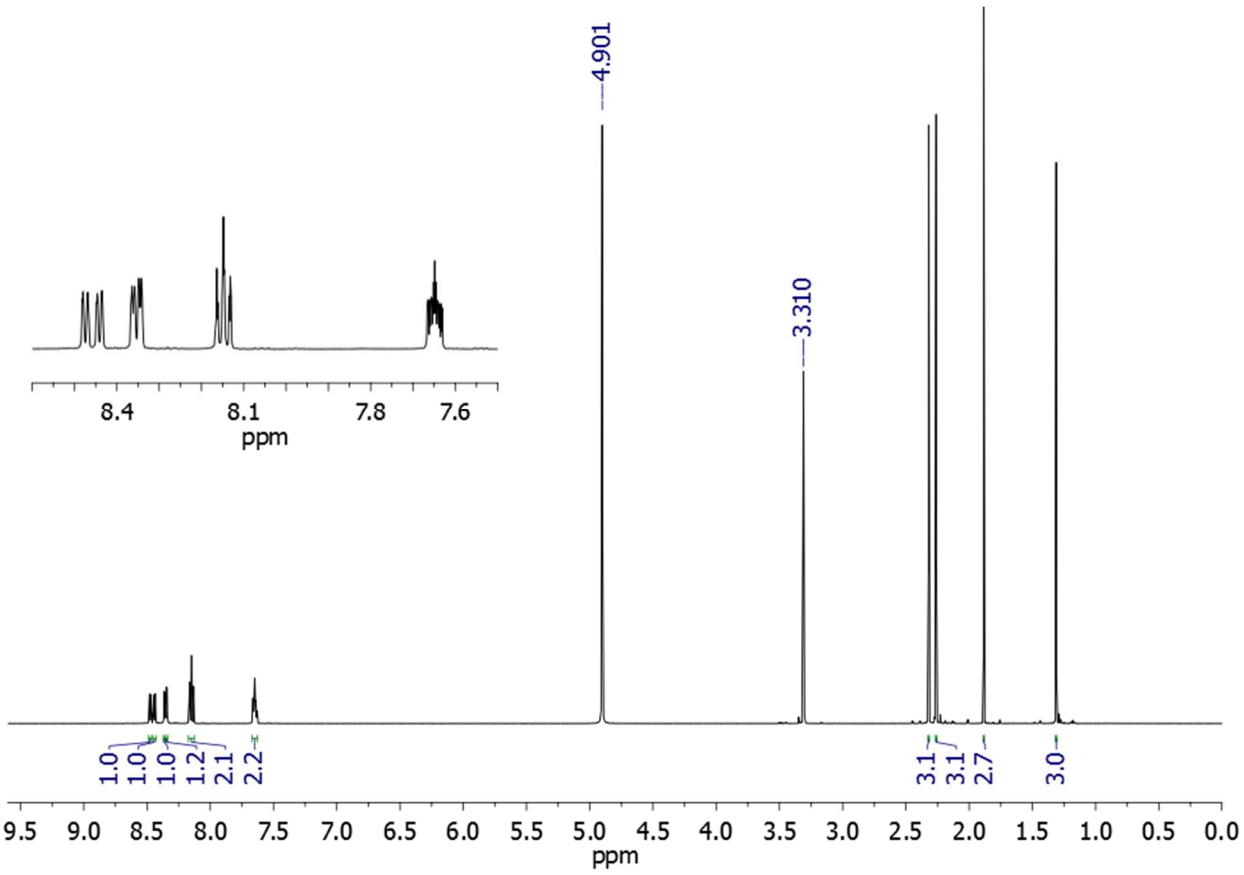


Figure S9. ^{1}H NMR spectrum of $[Rh_2(\mu\text{-}O_2CCH_3)_2(\eta^1\text{-}O_2CCH_3)(bpy)(H_2O)_3][O_2CCH_3]$ (Rh_2bpy, 500 MHz, CD_3OD).

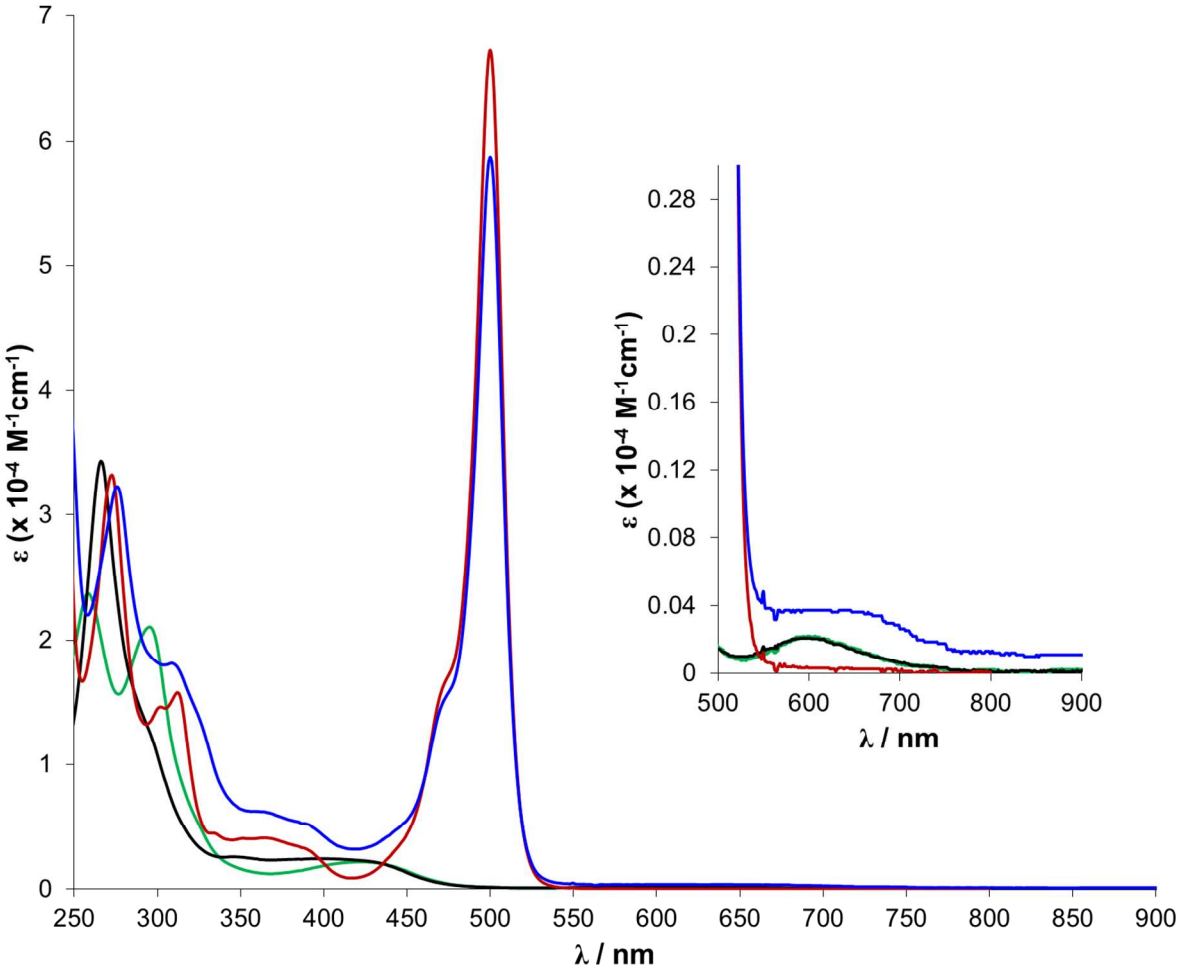


Figure S10. Electronic absorption spectra of phenbodipy (red), **1** (blue), Rh_2phen (black) and (c) Rh_2bpy (green) in MeOH.

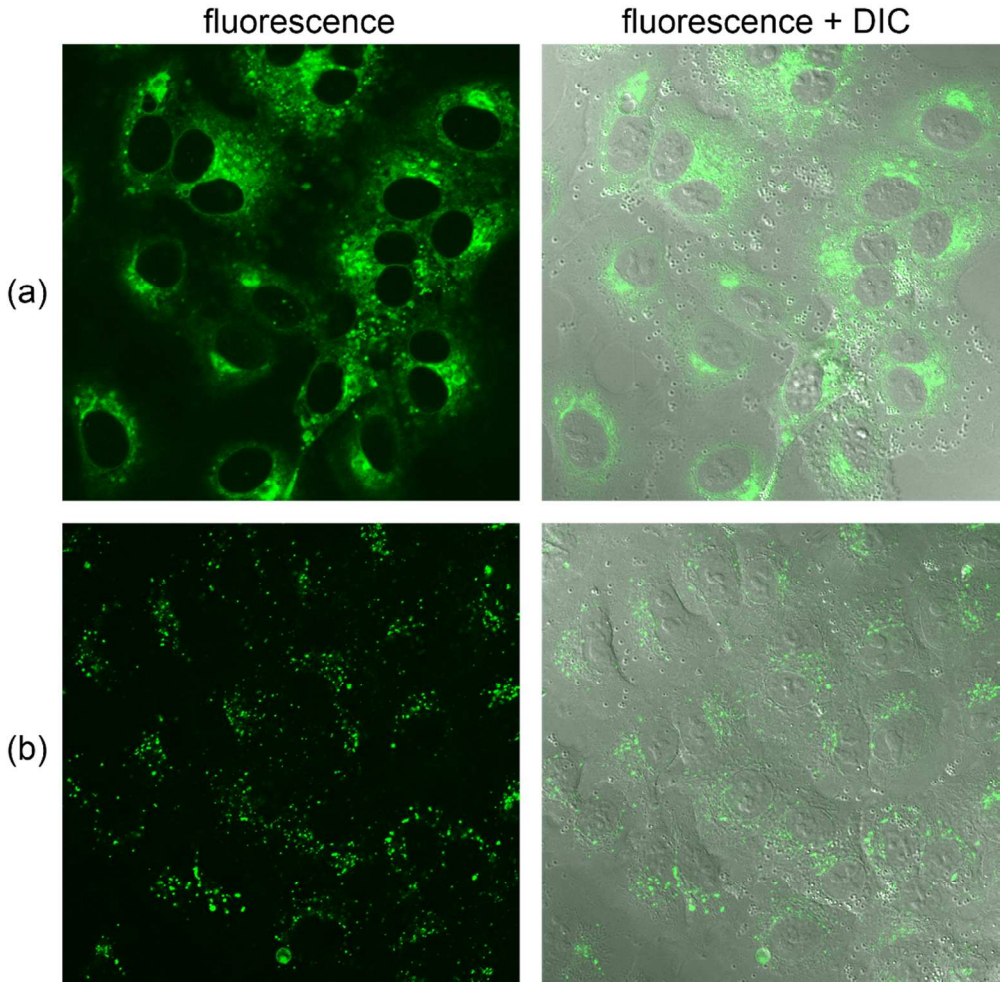


Figure S11. Confocal Fluorescence images of (a) 1 μM phenbodipy and (b) 1 μM **1**, 24 h of incubation. DIC = differential interference contrast. Field of view = 143 μm × 143 μm.

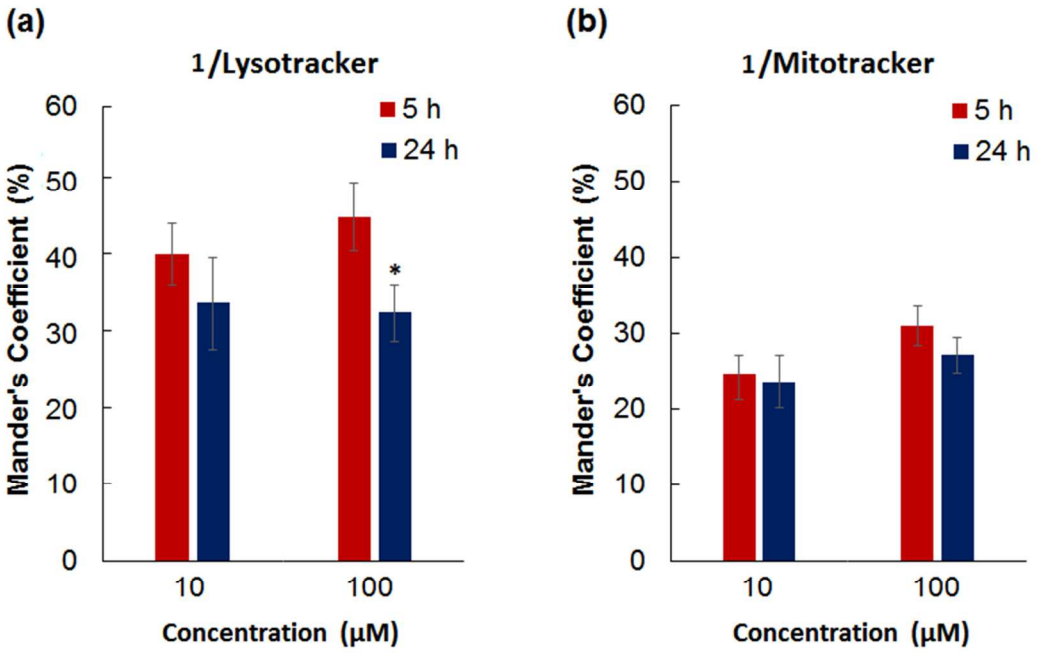


Figure S12. Mander's colocalization coefficients of (a) **1** over Lysotracker signals and (b) **1** over Mitotracker signals. The graphs represent means with standard deviation. The asterisk (*) indicates significant difference (at $p < 0.05$) between the colocalization coefficients at 5 and 24 h for 100 µM **1**.

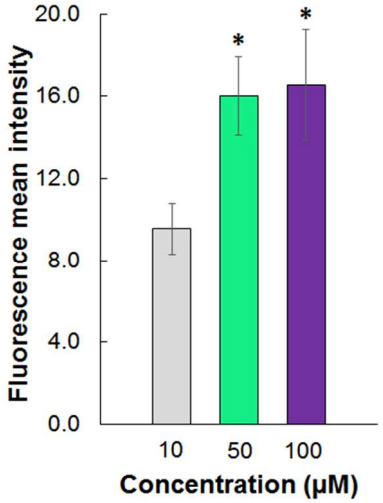


Figure S13. Fluorescence mean intensity of **1** in A549 cancer cells measured after 24 h incubation. The graphs represent means with standard deviation. The asterisk (*) indicates a significant difference compared to the 10 μM concentration at $p < 0.05$.

3. References

1. Hissler, M.; Connick, W. B.; Geiger, D. K.; McGarrah, J. E.; Lipa, D.; Lachicotte, R. J.; Eisenberg, R. *Inorg. Chem.* **2000**, *39*, 447-457.
2. Rempel, G. A.; Legzdins, P.; Smith, H.; Wilkinson, G. *Inorg. Synth.* **1971**, *13*, 90-91.
3. Crawford, C. A.; Matonic, J. H.; Streib, W. E.; Huffman, J. C.; Dunbar, K. R.; Christou, G. *Inorg. Chem.* **1993**, *32*, 3125-3133.
4. Cui, A.; Peng, X.; Fan, J.; Chen, X.; Yunkou Wu; Guo, B. *Photochem. Photobiol. A: Chem.* **2007**, *186*, 85-92.