Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Informations

An Ethylene-Glycol Functionalized Ruthenium(II) Complex for Two-Photon Photodynamic Therapy (PDT)

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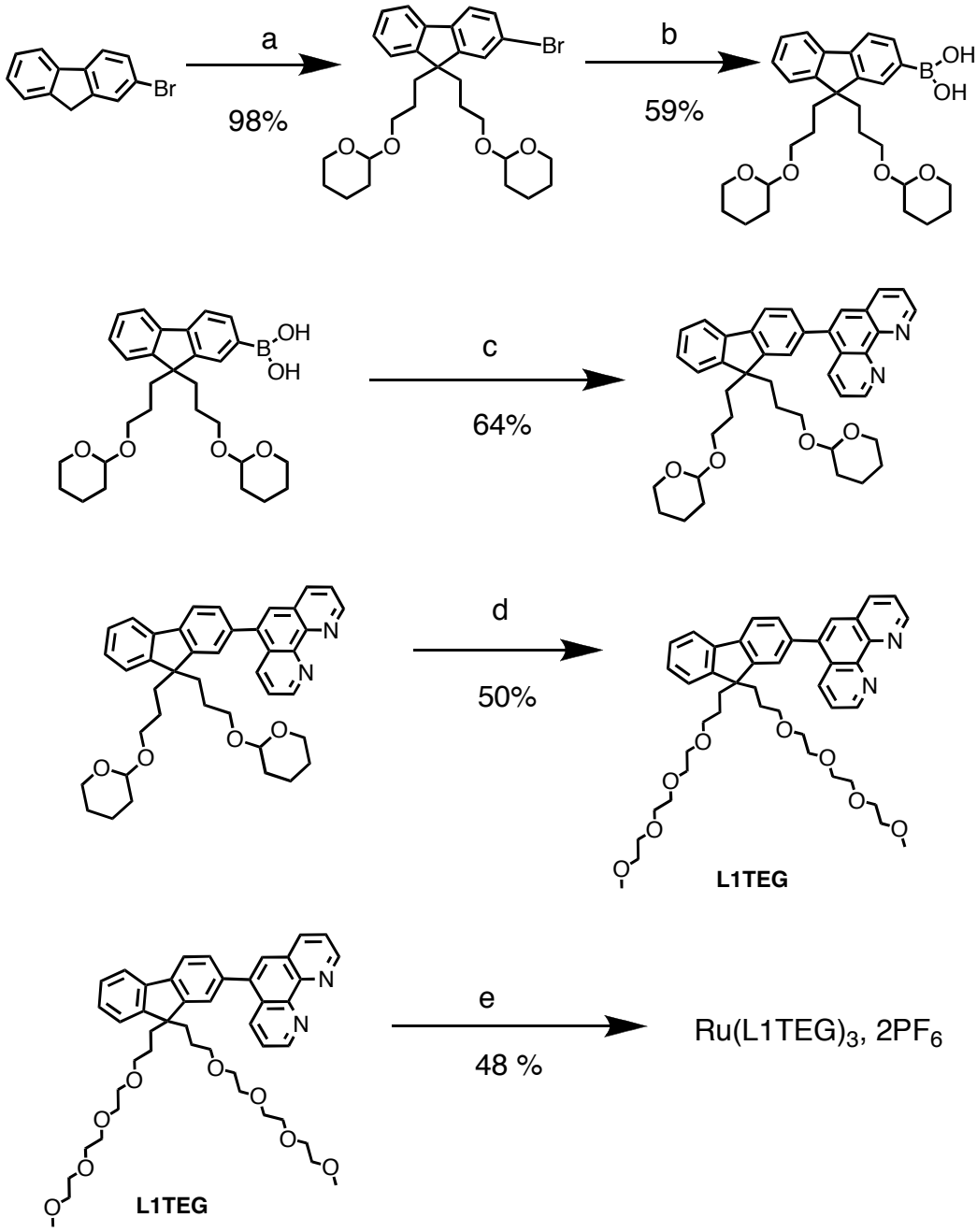
Scheme S1 : Synthesis of **L1TEG** and the related octupolar ruthenium complex **RuL1TEG**



a) 2-(3-bromopropoxy)-tetrahydropyran, NaOH, Et_3BnCl, DMSO, 60°C, 3 h.

b) *n*-BuLi, THF, -78°C, 1 h, then $B(OiPr)_3$, r.t., 14 h.

c) 5-bromo-1,10-phenanthroline, $Pd(PPh_3)_4$, Na_2CO_3 (1 M), toluene, 90°C, 3 days.

d) *i*. HCl, EtOH, r.t. 6 h ; *ii*. NaH, DMF, r.t. 1 h, then $TsO(CH_2CH_2O)_3CH_3$, 60°C for 4 days.

e) $RuCl_2(DMSO)_4$, EtOH, reflux 14 h then NH_4PF_6, r.t., 30 min.

Synthesis.

Commercially available reagents were used without any other purification unless otherwise stated. NMR spectra (^{1}H and ^{13}C) were recorded at room temperature on a Bruker AC 200 operating at 200.13 and 50.32 MHz for ^{1}H and ^{13}C, respectively. Coupling constants are reported in Hz and refer to apparent peak multiplicities (s for singlet, d for doublet, t for triplet and m for multiplet). High resolution mass spectrometry measurements and elemental analyses were performed at the Service Central d'Analyse du CNRS (Vernaison, France). 2-(3-bromopropoxy)-tetrahydropyran,[1] 2-(2-(2-methoxyethoxy)-ethoxy)-ethyl-p-toluenesulfonate[2] and 5-bromo-1,10-phenanthroline[3] were synthesized as previously described in the literature.

2-bromo-9,9-bis(3-(2-tetrahydropyranoxy)-propyl)-fluorene : A 50% NaOH aqueous solution (10.83g, 135.4 mmol) was added to a solution of 2-bromofluorene (4.74 g, 19.3 mmol), 2-(3-bromopropoxy)-tetrahydropyran (10.78 g, 48.3 mmol) and benzyltriethylammonium chloride (264 mg, 1.16 mmol) in DMSO. The mixture was thoroughly stirred at 60°C for 5 h, and was then allowed to cool to room temperature. Ethyl acetate (50 mL) was added and the precipitate obtained was filtered and rinsed. The resulting filtrate was washed with water (2x30 mL) and brine (2x30 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated. The residue was purified using column chromatography on silica (petroleum ether : ethyl acetate 90 : 10 and then 80 : 20) to give a yellow oil (10.05 g, 98%). ^{1}H NMR (200.13 MHz; $CDCl_3$) : δ 7.68-7.63 (m, 1H), 7.55 (d, 1H, ^{3}J = 8.0 Hz), 7.50-7.43 (m. 2H), 7.35-7.27 (m, 3H), 4.39-4.36 (m, 2H), 3.80-3.72 (m, 2H), 3.55-3.38 (m, 4H), 3.19-3.05 (m, 2H), 2.12-2.03 (m, 4H), 1.79-1.49 (m, 12H). 0.96-0.88 (m, 4 H). ^{13}C NMR (50.33 MHz; $CDCl_3$) : δ 152.20 ; 152.15 ; 149.54 ; 140.36 ; 140.24 ; 130.29 ; 127.77 ; 127.33 ; 126.36 ; 123.06; 121.25 ; 119.98 ; 98.78 ; 67.47 ; 62.52 ; 54.87 ; 36.68 ; 30.80 ; 25.53 ; 24.24 ; 19.82 . Elem. anal. calcd for $C_{29}H_{37}BrO_4$: C, 65.78 ; H, 7.04. Found C, 65.00 ; H, 7.04.

9,9-bis(3-(2-tetrahydropyranoxy)-propyl)-2-fluorenyl-boronic acid. A solution of 2-bromo-9,9-bis(3-(2-tetrahydropyranoxy)-propyl)-fluorene (5.00 g, 9.44 mmol) in 100 mL anhydrous THF was cooled to –78°C under argon. *N*-butyllithium (4.53 mL, 11.3 mmol) was added dropwise and the resulting mixture was stirred at –78 °C for 1h. Tri-isopropoxyborane (4.36 mL, 18.9 mmol) was then added dropwise, the solution was left to stir at –78°C for 30 min, then allowed to heat to room temperature and stirred for 14 h. The mixture was hydrolysed with water (150 mL), stirred for 4 hours, and extracted with diethyl ether (3x40 mL). The combined organic layers were dried (Na_2SO_4), filtered, and evaporated. The residue was purified using column chromatography on silica (dichloromethane : THF 90:10 and then 80:20) to give a white solid (2.76 g, 59%). ^{1}H NMR (200.13 MHz; $CDCl_3$) : δ 8.34-8.08 (m, 2H), 7.90-7.72 (m, 2H), 7.46-7.26 (m, 3H), 4.37-4.36 (m, 2H), 3.78-3.70 (m, 2H), 3.53-3.36 (m, 4H), 3.19-3.13 (m, 2H), 2.27-2.15 (m, 4H), 1.75-1.43 (m, 12H), 1.05 – 0.90 (m, 4 H). ^{13}C NMR (50.33 MHz; $CDCl_3$) : δ 150.71; 149.35; 145.84; 141.13; 135.18 ; 129.69 ; 128.41 ; 127.71 ; 127.23 ; 123.06 ; 120.27 ; 119.26 ; 98.78 ; 67.79 ; 62.72 ; 54.62 ; 36.70 ; 30.76 ; 25.44 ; 24.24 ; 19.82. Elem. anal. calcd for $C_{29}H_{39}BO_6$: C, 70.45 ; H, 7.95. Found C, 70.62 ; H, 7.95.

5-((9,9-bis(3-(2-tetrahydropyranoxy)-propyl)-fluoren-2-yl))-1,10-phenanthroline. Air was removed from a solution of 9,9-bis(3-(2-tetrahydropyranoxy)-propyl)-2-fluorenyl-boronic acid (2.50 g, 5.06 mmol) and 5-bromo-1,10 phenanthroline (1.30 g, 5.06 mmol) in toluene by bubbling argon through the solution during 20 min. Then a degased 1M aqueous solution of sodium carbonate (25 mL) and tetrakistriphenylphosphine palladium(0) (292 mg, 0.253 mmol) were added under argon. The mixture was thoroughly stirred at 90 °C for 3 days. After addition of 50 mL toluene and 25 mL water, the aqueous phase was extracted with dichloromethane (3x30 mL). The combined organic layer were dried (Na_2SO_4) and evaporated. The crude was purified using column chromatography on neutral alumina (from ethyl acetate : dichloromethane : triethylamine 70 : 30 : 1 to ethyl acetate : triethylamine 100 : 1) to give a white solid (2.04 g, 64%). ^{1}H NMR (200.13 MHz;

$CDCl_3$) : δ 9.21 (m, 2H), 8.31 (m, 2H), 7.86-7.76 (m, 3H), 7.70-7.44 (m, 4H), 7.39-7.36 (m, 3H), 4.39-4.37 (m, 2H), 3.78-3.69 (m, 2H), 3.58-3.36 (m, 4H), 3.21-3.09 (m, 2H), 2.17-2.09 (m, 4H), 1.70-1.45 (m, 12H), 1.13-1.01 (m, 4 H). ^{13}C NMR (50.33 MHz; $CDCl_3$) : δ 150.50 ; 150.35 ; 150.17 ; ;146.64 ; 145.90 ; 141.36 ; 140.78 ; 139.42 ; 137.82 ; 136.02 ; 134.78 ; 129.17 ; 128.63 ; 128.25; 127.77 ; 127.39 ; 126.61 ; 124.63 ; 123.51 ; 123.19 ; 122.96 ; 120.19 ; 120.02 ; 98.87 ; 67.74 ; 62.57 ; 54.82 ; 36.84 ; 30.82 ; 25.51 ; 24.53 ; 19.82. ESI-HRMS Calcd for $C_{41}H_{45}N_2O_4$ $[M+H]^+$: 629.3374, Found: 629.3358.

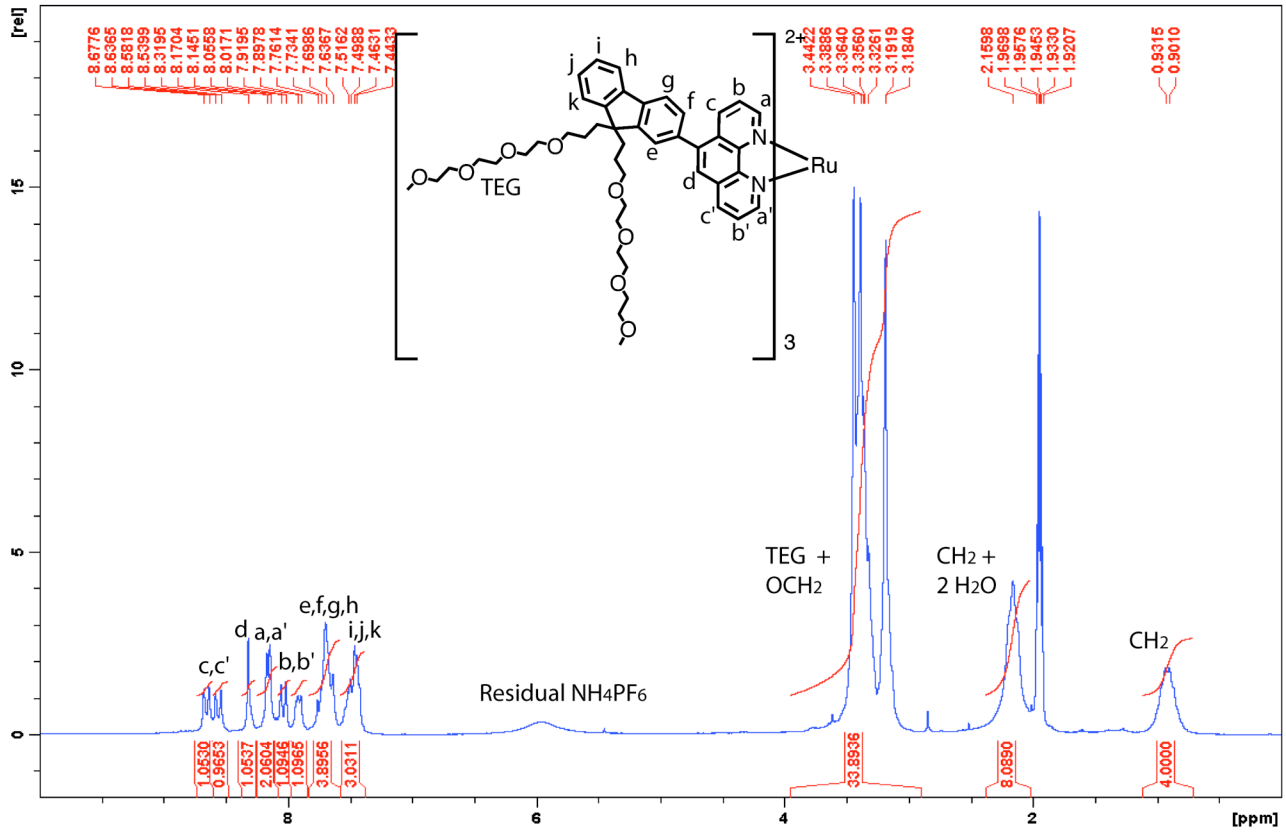
5-((9,9-bis(3-hydroxylpropyl)-fluoren-2-yl))-1,10-phenanthroline. An 10% aqueous solution of HCl (0.25 mL) was added to a solution of 5-((9,9-bis(3-(2-tetrahydropyranoxy)-propyl)-fluoren-2-yl))-1,10-phenanthroline (500 mg, 0.80 mmol) in ethanol (4.75 mL). The mixture was stirred at room temperature for 6 h and then evaporated. After addition of chloroform (50 mL) and water (20 mL), the organic layer was washed with a saturated sodium bicarbonate solution (30 mL), water (2x30 mL) and dried over Na_2SO_4. After evaporation, a white solide was obtained (317 mg, 87%) which was used without any further purification. ^{1}H NMR (200.13 MHz; $CDCl_3$) : δ 9.23-9.20 (m, 2H), 8.36-8.27 (m, 2H), 7.88-7.78 (m, 3H), 7.70-7.57 (m, 4H), 7.53-7.38 (m, 3H), 3.42-3.40 (m, 4H), 2.19-2.11 (t, 4H, ^3J = 8.1 Hz), 1.12-0.97 (m, 4H). ^{13}C NMR (50.33 MHz; $CDCl_3$) : δ 150.41 ; 150.38 ; 150.22 ; 150.07 ; 146.66 ; 145.92 ; 141.29 ; 140.78 ; 139.28 ; 138.01 ; 136.09 ; 134.74 ; 129.32 ; 128.65 ; 128.23 ; 127.91 ; 127.57 ; 126.66 ; 124.57 ; 123.56 ; 123.09 ; 123.03 ; 120.30 ; 120.12 ; 63.15 ; 54.73 ; 36.52 ; 27.60.

5-(9,9-bis(3-(2-(2-(2-methoxyethoxy)-ethoxy)-ethoxy)-propyl)-fluoren-2-yl)-1,10-phenanthroline (L1TEG). A solution of 5-((9,9-bis(3-hydroxypropyl)-fluoren-2-yl))-1,10-phenanthroline (400 mg, 0.87 mmol) in anhydrous DMF (10 mL) was added to a solution of sodium hydride (520 mg 60% in oil, 13.0 mmol) in dry DMF (5 mL). The mixture was stirred at room temperature for 1h and then triethyleneglycol methyl and tosyl ether (1.39g, 4.4 mmol) was added.

The mixture was stirred at 60 °C for 4 days and then evaporated. After addition of dichloromethane (20 mL) and water (20 mL), the aqueous layer was extracted with dichloromethane (3x20 mL). The combined organic layer were washed (saturated NH_4Cl solution 2x20 mL, brine 20mL), dried (Na_2SO_4) and evaporated. The residue was purified using column chromatography on neutral alumina (from dichloromethane : methanol : triethylamine 99 : 1 :1 to 97 : 3 : 1) to give a brown oil (380 mg, 57%). ^{1}H NMR (499.84 MHz; $CDCl_3$) : δ 9.20 (dd, 2H, ^3J = 4.4 Hz, ^4J = 1.6 Hz), 8.30 (dd, 1H, ^3J = 8.4 Hz, ^4J = 1.6 Hz), 8.27 (dd, 1H, ^3J = 8.1 Hz, ^4J = 1.6 Hz), 7.79 (d, 1H, ^3J = 7.7 Hz), 7.78 (s, 1H), 7.72 (d, 1H, ^3J = 6.7 Hz), 7.64 (dd, 1H, ^3J= 8.1 Hz, ^4J = 4.4 Hz), 7.57 (dd, 1H, ^3J = 8.4 Hz, ^4J = 4.3 Hz), 7.46-7.44 (m, 2H), 7.36-7.30 (m, 3H), 3.54-3.36 (m, 24H), 3.29 (s, 6H), 3.18 (t, 4H, ^3J = 6.9 Hz), 2.05 (t, 4H, ^3J = 8.2 Hz), 1.06-0 .94 (m, 4H). ^{13}C NMR (125,68 MHz; $CDCl_3$) : δ 150.23 ; 150.12 ; 149.95 ; 149.92 ; 146.40 ; 145.67 ; 141.11 ; 140.52 ; 139.15 ; 137.61 ; 135.88 ; 134.58 ; 128.99 ; 128.06 ; 127.99 ; 127.57 ; 127.21 ; 126.43 ; 124.34 ; 123.29 ; 122.91 ; 122.79 ; 119.98 ; 119.79 ; 71.79 ; 71.39 ; 70.48 ; 70.44 ; 70.41 ; 70.37 ; 69.79 ; 58.91 ; 54.54 ; 36.40 ; 24.25. ESI-HRMS Calcd for $C_{45}H_{57}N_2O_8$ $[M+H]^+$: 753.4109, Found: 753.4078.

RuL1TEG. To a deoxygenated solution of 5-(9,9-bis(3-(2-(2-(2-methoxyethoxy)-ethoxy)-ethoxy)-propyl)-fluoren-2-yl)-1,10-phenanthroline (200 mg, 0.25 mmol) in absolute ethanol (7 mL), $RuCl_2DMSO_4$ (43,0 mg, 0.08 mmol) was added under argon. The resulting mixture was heated at reflux for 14 h. After cooling to room temperature, a saturated NH_4PF_6 aqueous solution was added dropwise to precipitate an orange solid. It was washed several times with diethyl ether and water to afford a red solid (185 mg; 48%). ^{1}H NMR (200.13 MHz; CD_3CN) : δ 8.66 (d, 1H, ^3J = 8.2 Hz), 8.56 (d, 1H, ^3J = 8.4 Hz), 8.32 (s, 1H), 8.17-8.14 (m, 2H), 8.03 (m, 1H), 7.92-7.89 (m, 1H), 7.76-7.63 (m, 4H), 7.52-7.44 (m, 3H), 3.44-3.17 (m, 34H), 2.22-2.13 (m, 4H), 0.95-0.88 (m, 4 H). IR (KBr) : ν (cm^{-1}) : 3127; 2921; 2875; 1625; 1427; 1402; 1103; 842; 740; 557; 482. Elem. anal. calcd for $C_{135}H_{168}F_{12}N_6O_{24}P_2Ru$ 0.5 NH_4PF_6: C. 59.37 ; H. 6.27 ; N. 3.33 ; Ru. 3.70. Found C. 59.50 ; H. 5.97 ; N. 3.45 ; Ru. 3.66.

^{1}H NMR Spectra of RuL1TEG in CD_3CN (200.13 MHz). Integrations are given for one ligand only, but there are three of them in the complex.



Spectroscopic measurements. Dichloromethane and acetonitrile (spectrometric grade) were employed as solvent for absorption and fluorescence measurements. UV/Vis absorption spectra were recorded on a Jasco V-550 absorption spectrometer. The emission spectra were recorded using a Horiba-Jobin Yvon Fluorolog-3 spectrofluorimeter. The steady-state luminescence was excited by unpolarized light from a 450 W Xenon CW lamp and detected at an angle of 90° for diluted solution (10^{-5} mol.L^{-1}) measurements (10 mm quartz cuvette) by a photomultiplier. Spectra were reference-corrected for both the excitation source light intensity variation and the emission spectral response. Emission quantum yield ϕ_L were determined at 25°C in deoxygenated acetonitrile solutions with a CH_3CN solution of $[Ru^{II}(phen)_3](PF_6)_2$ (ϕ_L^{ref}= 0.03) as a standard, according to equation (1) :

$$\phi_L^S = \frac{I_L^S}{I_L^{Ref}} \frac{(1-10^{-OD^{Ref}})}{(1-10^{-OD^S})} \phi_L^{Ref} \quad (1)$$

where I_L, the emission intensity, was calculated from the spectrum area $\int I(\lambda)d\lambda$, OD represents the optical density at the excitation wavelength. The superscripts "S" and "Ref" refer to the sample and the standard, respectively. The luminescence lifetime of the complexes **RuL1TEG** was measured after irradiation at λ = 337 nm obtained with a 4 ns pulsed N_2 laser (Optilas VSL-337ND-S) and recorded at the emission maximum wavelength (607 nm) using a monochromator and a photomultiplier tube (Hamamatsu R928) coupled with an ultra-fast oscilloscope (Tektronix TDS 520A). The TPA cross-section in the range 700-900 nm were obtained by up-conversion fluorescence using a mode locked Ti:sapphire femtosecond laser (Tsunami Spectra-Physics) with pulse duration 100 fs and at a repetition rate of 82 MHz. The measurements were done at room temperature in dichloromethane and at a concentration of ca. 10^{-5} M. The excitation beam (5 mm diameter) is focalized with a lens (focal length 10 cm) at the middle of the fluorescence cell (10 mm). The fluorescence, collected at 90° to the excitation beam, was focused into an optical fiber (diameter 600 μm) connected to an Ocean Optics S2000 spectrometer. The incident beam intensity

was adjusted to 50 mW in order to ensure an intensity-squared dependence of the fluorescence over the whole spectral range. The detector integration time was fixed to 1 s. Comparison of the spectra was performed with the published 700-900 nm Coumarin-307 two-photon absorption spectrum (Coumarin-307 quantum yield is equal to 0.56 in ethanol).[4] For cell imaging, the ruthenium complex **RuL1TEG** was first diluted in a mixture of ultrapure water and 1% ethanol and then characterized by UV/Vis absorption spectroscopy measurements. The Ti:Sapphire femtosecond laser which was used to record the two-photon absorption spectrum delivered 740 nm pulsed light. The spectrum was taken with an Ocean Optics spectrophotometer through a 100 μm diameter optical fiber. Fluorescence imaging was achieved on a Zeiss Axiovert 200 inverted microscope equipped with a high-pressure mercury lamp and a set of dichroic mirrors (band pass: 546-558 nm for excitation and 575-640 nm for emission). Fluorescence images were collected through a 10X objective, recorded with a color numerical camera of 1 Mega- pixel matrix and procesed by a Zeiss Axiovision software.

Cell line and tissue culture conditions. F98 rat glioma cells were grown as stock cultures by a standard method.[5] Sampling of cells was made from a T25 bottle where cells were attached on more than 90% of the surface. The whole culture process was performed in sterile atmosphere to avoid any contamination of the cells. First, the culture was rinsed with 5 ml phosphate buffered saline (PBS) solution without Ca^{2+} and Mg^{2+} to remove the cellular medium. Then, 500 μL of trypsin-EDTA were added to detach the cells from the surface of the bottle. After trypsinization process, 5 ml of cellular medium supplemented with 4.5 $g.L^{-1}$ D-Glucose pyruvate. 10% fetal calf serum and combined antibiotics (penicillin/streptomycin) were reinjected. From this whole solution, approximately 500 μl were collected and suspended in 60 mm Petri's dishes containing 5 mL of the appropriate growth medium (10% fetal calf serum). When the culture process was completed, the cells were placed in a humidified incubator and kept for 48 hours at 37 °C and 5% CO_2.

Drug and light exposure. When the density of the F98 rat glioma cells on the Petri dish was about 90%. a solution of **RuL1TEG** complex was inserted in the medium containing the cells to obtain a final concentration of $2x10^{-5}$ M. Photo-generation of cytotoxic $^1O_2$ was carried out immediately after injection using for excitation a high-pressure mercury lamp which delivered 450-490 nm light through a 20X objective of a Zeiss Axiovert 200 inverted microscope. The microscope was installed inside an incubator which maintained a 37ºC constant temperature and 5% CO_2 atmosphere, therefore being capable to keep the cells alive during imaging and irradiation process; images were recorded after 2, 6, 8, 10, 15, 20, and 30 minutes. For two-photon therapy model experiments, the samples were irradiated with the femtosecond Ti:Sapphire pulsed laser that delivered an output power of 300 mW at 740 nm and emitted pulses of duration 100 fs at a repetition rate of 82 MHz. The laser beam was focused on the sample through a 10XPh2 objective lens.

Figure S1 : UV-Vis absorption spectrum and emission spectrum of **RuL1PEG** complex in $CHCl_3$ and CH_3CN, respectively.

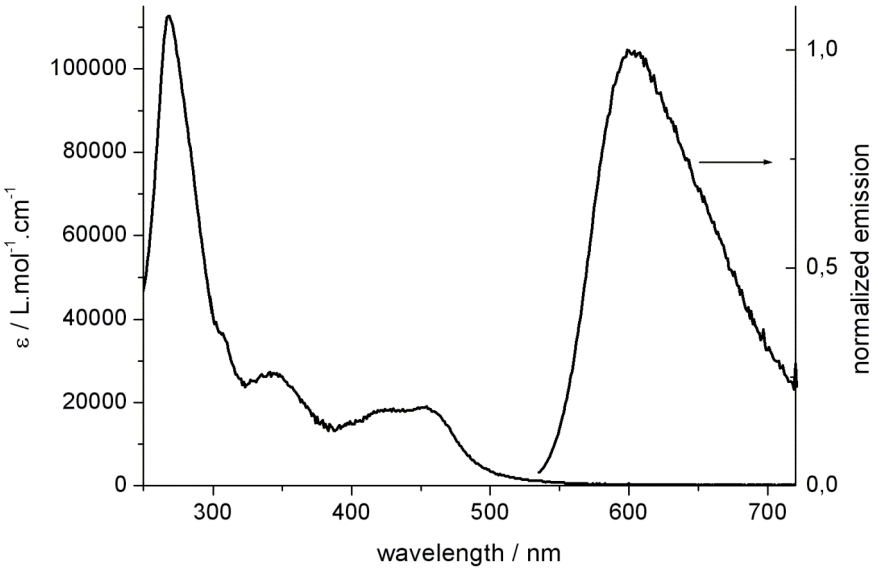


Figure S2 : Fluorescence image of F98 cells loaded with the **RuL1TEG** complex. Excitation/emission wavelengths 546 / 640 nm.

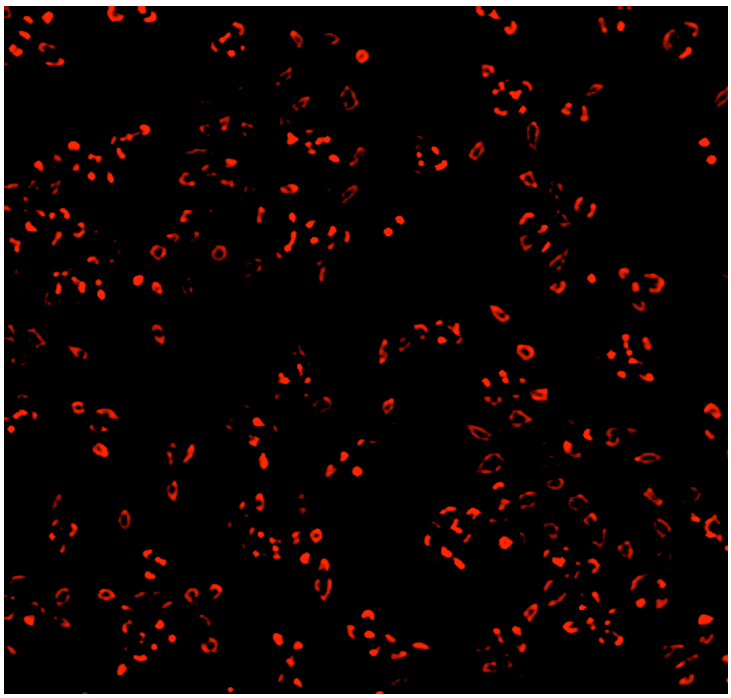


Figure S3 : Transmitted light image of a selected region of the cells before (A) and after (B) a 15 minutes period irradiation with a 450-490 nm light and in presence of the **RuL1TEG** complex

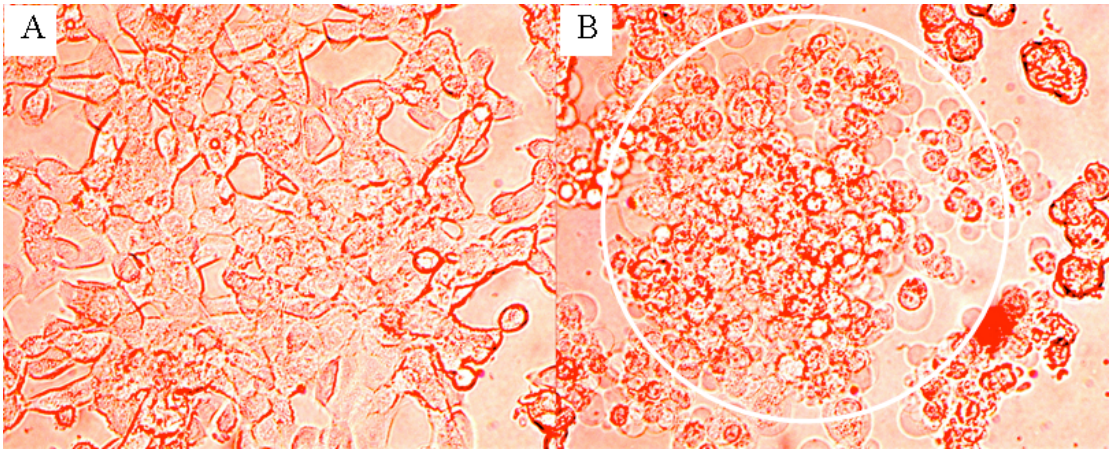
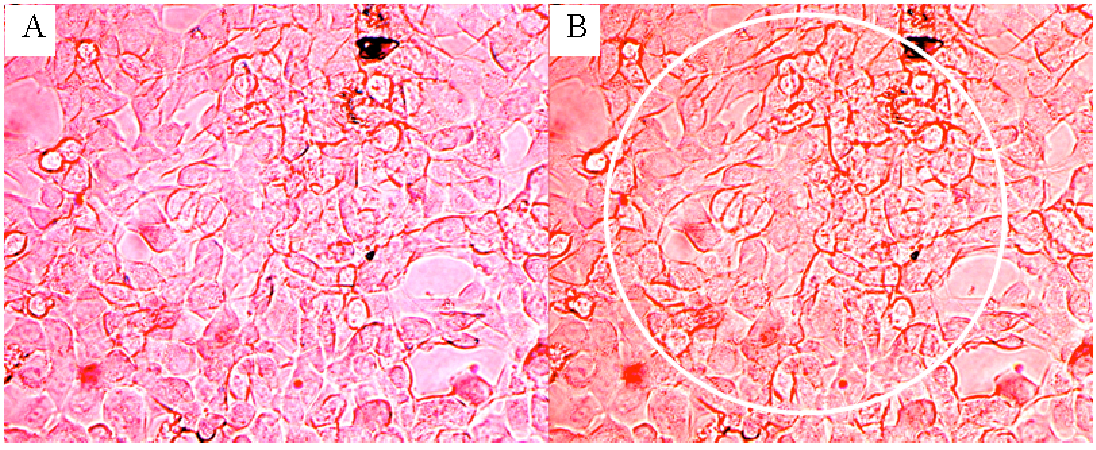


Figure S4 : Transmitted-light image of selected non-irradiated F98 glioma cells (A) *versus* cells irradiated for 10 minutes (B).



The same result was observed whith increasing the irradiation time

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