Supporting Information

Biocompatible Conjugated Fluorenylporphyrins for Two-photon Photodynamic Therapy and Fluorescence Imaging

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Including:

1. General Experimental Details

General. All air or water-sensitive reactions were carried out under dry argon. Solvents were generally dried and distilled prior to use. Reactions were monitored by thin layer chromatography on Merck silica gel 60 F_{254} precoated aluminum sheets, or by NMR. Column chromatography: Merck silica gel Si 60 (40-63 μm, 230-400 mesh or 63-200 μm, 70-230 mesh). NMR: Bruker Avance AV 300 (^{1}H: 300.13 MHz, ^{13}C: 75.48 MHz) or Bruker Ascend 400, in $CDCl_3$ solutions; ^{1}H chemical shifts (δ) are given in ppm relative to TMS as internal standard, J values in Hz and ^{13}C chemical shifts relative to the central peak of $CDCl_3$ at 77.0 ppm, ^{31}P chemical shifts relative to 85% H_3PO_4. High resolution mass spectra measurements were performed at the Centre Regional de Mesures Physiques de l'Ouest (C.R.M.P.O, Rennes) using a Bruker MicrOTOF-Q II, a Thermo Fisher Scientific Q-Exactive in ESI positive mode and a Bruker Ultraflex III MALDI Spectrometer. Elemental analyses were performed at C.R.M.P.O.

Spectroscopic Measurements. All photophysical properties have been performed with freshly-prepared air-equilibrated solutions at room temperature. UV-Vis absorption spectra were recorded on a Jasco V-570 spectrophotometer. Steady-state fluorescence measurements were performed on dilute solutions (*ca.* 10^{-6} M, optical density < 0.1) contained in standard 1 cm quartz cuvettes using an Edinburgh Instrument (FLS920) spectrometer in photon-counting mode. Fully corrected emission spectra were obtained, for each compound, after excitation at the wavelength of the absorption maximum, with $A_{\lambda ex}$ < 0.1 to minimise internal absorption. Porphyrins **1b** and **2b-c** were dissolved in water by adding 0.1 mL of a ~5×10^{-3} M stock solution in DMSO to 20 mL of water (HPLC grade) and sonicated.

Measurements of singlet oxygen quantum yield (Φ_Δ). Measurements were performed on a Fluorolog-3 (Horiba Jobin Yvon) fluorimeter, using a 450 W Xenon lamp. The emission at 1272 nm was detected using a liquid nitrogen-cooled Ge-detector model (EO-817L, North Coast Scientific Co). Singlet oxygen quantum yields Φ_Δ were determined in dichloromethane solutions, using tetraphenylporphyrin (TPP) in dichloromethane as reference solution (Φ_Δ [TPP] = 0.60) and were estimated from $^1O_2$ luminescence at 1272 nm.

Two-Photon Absorption Experiments. To span the 790-920 nm range, a Nd:YLF-pumped Ti:sapphire oscillator (Chameleon Ultra, Coherent) was used generating 140 fs pulses at a 80 MHz rate. The excitation power is controlled using neutral density filters of varying optical density mounted in a computer-controlled filter wheel. After five-fold expansion through two achromatic doublets, the laser beam is focused by a microscope objective (10x, NA 0.25, Olympus, Japan) into a standard 1 cm absorption cuvette containing the sample. The applied average laser power arriving at the sample is typically between 0.5 and 40 mW, leading to a time-averaged light flux in the focal volume on the order of 0.1–10 mW/mm^2. The fluorescence from the sample is collected in epifluorescence mode, through the microscope objective, and reflected by a dichroic mirror (Chroma Technology Corporation, USA; ''red'' filter set: 780dxcrr). This makes it possible to avoid the inner filter effects related to the high dye concentrations used (10^{-4} M) by focusing the laser near the cuvette window. Residual excitation light is removed using a barrier filter (Chroma Technology; ''red'': e750sp–2p). The fluorescence is coupled into a 600 μm multimode fiber by an achromatic doublet. The fiber is connected to a compact CCD-based spectrometer (BTC112-E, B&WTek, USA), which measures the two-photon excited emission spectrum. The emission spectra are corrected for the wavelength-dependence of the detection efficiency using correction factors established through the measurement of reference compounds having known fluorescence emission spectra. Briefly, the set-up allows for the recording of corrected fluorescence emission spectra under multiphoton excitation at variable excitation power and wavelength. 2PA cross sections (σ_2) were determined from the two-photon excited fluorescence (2PEF) cross sections ($\sigma_2.\Phi_F$) and the fluorescence emission quantum yield (Φ_F). 2PEF cross sections of 10^{-4} M dichloromethane solutions were measured relative to fluorescein in 0.01 M aqueous NaOH using the well-established method described by Xu and Webb[1] and the appropriate solvent-related refractive index corrections.[2] The quadratic dependence of the fluorescence intensity on the excitation power was checked for each sample and all wavelengths.

[1] C. Xu and W. W. Webb, *J. Opt. Soc. Am. B: Opt. Phys.*, 1996, **13**, 481-491.

[2] M. H. V. Werts, N. Nerambourg, D. Pélégry, Y. Le Grand and M. Blanchard-Desce, *Photochem. Photobiol. Sci.*, 2005, **4**, 531-538.

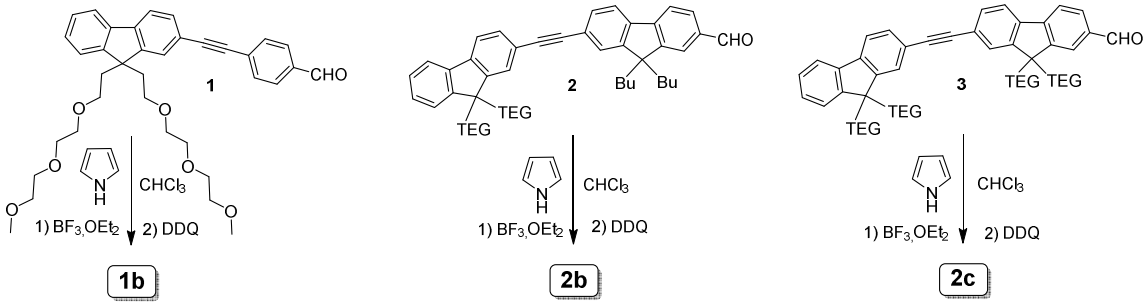
Cell culture. Human breast cancer cells MCF-7 (purchased from ATCC) were cultured in DMEM F12 Media - GlutaMAX™-I (containing 4,5 $g.L^{-1}$ of D-glucose) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and allowed to grow in humidified atmosphere at 37 °C under 5 % CO_2.

Cytotoxicity study. MCF-7 breast cancer cells were seeded into 96-well plates at 2000 cells per well in 200 µL culture medium and allowed to grow for 24 h. Increasing concentrations of porphyrins were added in culture medium of MCF-7 cells. Three days after treatment, a MTT assay was performed to determine the cell viability. Briefly, cells were incubated for 4 h with 0.5 $mg.mL^{-1}$ of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Promega) in media. The MTT/media solution was then removed and the precipitated crystals were dissolved in EtOH/DMSO (v/v). The solution absorbance was read at 540 nm in a microplate reader.

Two-photon photodynamic therapy. MCF-7 breast cancer cells were seeded in a 384 multi-well plate with 0.17 mm glass bottom at 500 cells per well in 50 µL of culture medium and allowed to grow for 24 h. Then, cells were incubated for 24 h with porphyrins at a concentration of 25 µg mL^{-1}. After incubation, cells were washed, maintained in fresh culture medium and then submitted or not to laser irradiation. Two-photon irradiation was performed on living cells with a LSM 780 LIVE confocal microscope (Carl Zeiss, Le Pecq, France) equipped with tunable Chameleon Ultra II laser (Coherent, 680-1080 nm) generating 140 fs wide pulses at 80 MHz rate. The laser beam was focused with a microscope objective lens (10×/0.3) at maximum laser power (3 W input, 900 mW cm^{-2} output before the objective). Half of the well were irradiated at 790 nm by 3 scans of 1.57 s duration in 4 different areas of the well. The scan size does not allow irradiating more areas without overlapping. After 2 days, a cell death quantification assay was performed in which cells were incubated in the presence of Thiazolyl Blue Tetrazolium Bromide (MTT) (0.5 $mg.mL^{-1}$) for 4 h to determine the mitochondrial enzyme activity. Then, supernatant was removed, and 150 µL of EtOH/DMSO (1:1) was added to dissolve the MTT precipitates. Absorbance was measured at 540 nm with a microplate reader. The value obtained was corrected according to the following formula: Abs “no laser” – 2 × (Abs “no laser” – Abs “laser”).

Two-photon fluorescence imaging. Cells were seeded onto bottom glass dishes (World Precision Instrument, Stevenage, UK) at a density of 10^6 cells cm^{-2} in culture medium during 24 h. Then, the cells were incubated overnight with different porphyrins at a concentration of 25 µg mL^{-1}. For cell membrane staining, 15 minutes before the end of incubation, cells were loaded with Cell Mask orange (Invitrogen, Cergy Pontoise, France) at a final concentration of 5 µg mL^{-1}. Before imaging, cells were washed twice with culture medium. Fluorescence imaging was performed on living cells with a LSM 780 LIVE confocal microscope (Carl Zeiss, Le Pecq, France), at 790 nm for porphyrins, and 561 nm for cell membranes. All images were performed with a high magnification (63×/1.4 OIL DIC Plan-Apo).

2. Synthesis of the Porphyrins 1b and 2b-c



Porphyrin 1b. Boron trifluoride etherate (9.6 µL) was added to a solution of aldehyde **1** (180 mg, 0.31 mmol) and pyrrole (22.3 µL, 0.31 mmol) in $CHCl_3$ (8 mL). The solution was stirred for 4 hours at room temperature under argon atmosphere. DDQ (52.2 mg, 0.23 mmol) was added and stirring was continued for 1 hour. Solvent was evaporated, and the residue was purified by column chromatography (petroleum ether /THF 1:1), affording **1b** as a purple powder (35 mg, 18%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.03 (s, 4H), 8.93 (s, 4H), 8.29 (d, J = 8.1 Hz, 8H), 8.02 (d, J = 8.1 Hz, 8H), 7.85-7.66 (m, 16H), 7.57-7.31 (m, 12H), 3.61-3.25 (m, 88H), 2.90-2.78 (m, 16H), 2.52-2.41 (m, 16H), -2.69(s, 2H). ^{13}C{^{1}H} NMR (75 MHz, $CDCl_3$): δ = 149.32 (s), 149.21 (s), 140.91 (s), 139.82 (s), 137.07 (s), 134.65 (s), 131.19 (s), 129.99 (s), 127.92 (s), 127.50 (s), 126.53 (s), 123.27 (s), 121.87 (s), 120.21 (s), 119.90 (s), 91.44 (s), 89.76 (s), 71.92 (s), 70.48 (s),

70.06 (s), 67.06 (s), 59.01 (s), 51.35 (s), 39.74 (s), 29.69 (s). HRMS-ESI (CH_2Cl_2): m/z = 2536.2570 $[M+H]^+$ (calcd for $C_{160}H_{175}N_4O_{24}$: 2536.25908).

Porphyrin 2b. Boron trifluoride etherate (8 μL) was added to a solution of aldehyde **2** (200 mg, 0.25 mmol) and pyrrole (18.4 μL, 0.25 mmol) in $CHCl_3$ (5 mL). The solution was stirred for 3 hours at room temperature under argon atmosphere. DDQ (43.2 mg, 0.19 mmol) was added and stirring was continued for 1 hour. Solvent was evaporated, and the residue was purified by column chromatography (ethyl acetate/THF 9:1), affording **2b** as a purple powder (78.8 mg, 37%). ^{1}H NMR (300 MHz, $CDCl_3$): δ= 8.97 (s, 8H), 8.28 (t, *J* = 7.2 Hz, 8H), 8.13 (d, *J* = 7.6 Hz, 4H), 8.00 (d, *J* = 8.2 Hz, 4H), 7.76-7.61 (m, 24H), 7.49-7.34 (m, 12H), 3.61-3.49 (m, 32H), 3.46 (t, *J* = 4.5 Hz, 16H), 3.37 (s, 24H), 3.28 (t, *J* = 4.2 Hz, 16H), 2.84 (t, *J* = 7.8 Hz, 16H), 2.47 (t, *J* = 6.9 Hz, 16H), 2.21 (d, *J* = 9.1 Hz, 16H), 1.31-1.19 (m, 16H), 1.13-0.91 (m, 16H), 0.82 (q, *J* = 13.8, 6.9 Hz, 24H), -2.52 (s, 2H). ^{13}C{^{1}H} NMR (75 MHz, $CDCl_3$, ppm): δ= 151.35 (s), 149.60 (s), 149.17 (d, *J* = 9.1 Hz), 141.35 (d, *J* = 12.5 Hz), 140.67 (s), 140.13 (s), 139.84 (s), 133.83 (s), 130.99 (s), 129.42 (s), 129.42 (s), 127.87 (s), 127.48 (s), 126.40 (s), 126.19 (s), 123.24 (s), 122.02 (d, *J* = 7.2 Hz), 120.72 (s), 120.15 (s), 119.85 (s), 118.23 (s), 109.51 (s), 91.01 (s), 90.49 (s), 71.87 (s), 70.44 (d, *J* = 1.6 Hz), 70.00 (s), 67.03 (s), 58.98 (s), 55.44 (s), 51.28 (s), 40.34 (s), 39.71 (s), 29.70 (s), 26.39 (s), 23.14 (s), 20.71 (s), 14.01 (d, *J* = 3.2 Hz). HRMS-ESI (CH_3OH/CH_2Cl_2 90:10): m/z = 3336.8783 $[M+H]^+$ (calcd for $C_{220}H_{255}O_{24}N_4$: 3336.88508). Anal. Calcd. (%) for $C_{220}H_{254}O_{24}N_4$, 2 CH_2Cl_2: C, 76.00; H, 7.41; N, 1.60. Found: C, 75.94; H, 7.53; N, 1.68.

Porphyrin 2c. Boron trifluoride etherate (6.8 μL) was added to a solution of aldehyde **3** (200 mg, 0.22 mmol) and pyrrole (15.7 μL, 0.21 mmol) in $CHCl_3$ (5 mL). The solution was stirred for 12 hours at room temperature under argon atmosphere. DDQ (36.9 mg, 0.16 mmol) was added and stirring was continued for 1 hour. Solvent was evaporated, and the residue was purified by column chromatography (petroleum ether /THF 4:6), affording **2c** as a purple powder (69 mg, 31%). ^{1}H NMR (300 MHz, $CDCl_3$): δ= 9.02 (s, 4H), 8.92 (s, 4H), 8.43-8.24 (m, 8H), 8.13 (t, *J* = 4.5 Hz, 4H), 7.98 (t, *J* = 7.8 Hz, 4H), 7,81 (s, 4H), 7.73 (m, 16H), 7.64 (d, *J* = 7.8 Hz, 4H), 7.49-7.34 (m, 12H), 3.58-3.24 (m, 176H), 2.84 (m, 24H), 2.61 (s, 16H), 2.46 (t, *J* = 7.5 Hz, 24H), -2.55 (s, 2H). ^{13}C{^{1}H} NMR (75 MHz, $CDCl_3$): δ= 149.94 (s), 149.27 (s), 149.14 (s), 148.20 (s),

141.71 (d, J = 2.6 Hz), 140.75 (s), 140.53 (s), 139.80 (s), 139.44 (s), 131.30 (d, J = 3 Hz), 131.07 (s), 129.68 (s), 127.86 (s), 127.47 (s), 126.68 (s), 126.30 (s), 123.26 (s), 122.34 (d, J = 3.4 Hz), 122.03 (s), 120.44 (s), 120.28-120.15 (m), 119.87 (s), 118.44 (s), 90.75 (d, J = 16.2 Hz), 71.84 (d, J = 6.7 Hz), 70.44 (t, J = 2.5 Hz), 70.09 (d, J = 10.7 Hz), 67.49 (d, J = 6.5 Hz), 67.01 (s), 58.94 (d, J = 7.3 Hz), 51.75 (d, J = 10.5 Hz), 51.29 (s), 39.71 (s). HRMS-MALDI (DCTB): m/z = 4056.13136 $[M]^{+\cdot}$ (calcd for $C_{244}H_{302}N_4O_{48}$: 4056.13081). Anal. Calcd. (%) for $C_{244}H_{302}O_{48}N_4$, 1.5 CH_2Cl_2: C, 70.44; H, 7.34; N, 1.34. Found: C, 70.34; H, 7.37;N, 1.39.

3. Excitation spectra of the Porphyrins 1b and 2b-c in THF

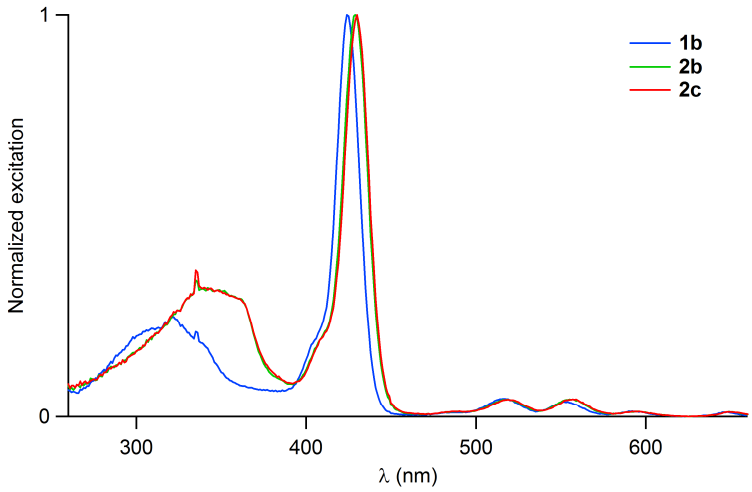


Figure S1. Excitation spectra of porphyrins **1b** and **2b-c** in THF through emission at 670 nm.

4. UV-Visible Absorption and Fluorescence of the Porphyrins 1b and 2b-c in water

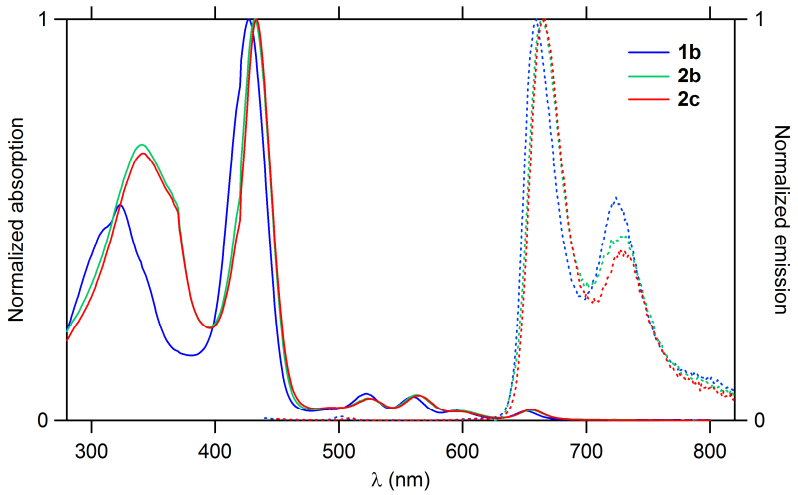


Figure S2. Normalised UV-visible absorption and fluorescence emission spectra of **1b**, **2b** and **2c** in water.

Table S1. Photophysical properties of porphyrins **1b** and **2b-c** in water.

Cpnd	λ_{abs} (UV band) (nm)	λ_{abs} (Soret) (nm)	λ_{abs} (Q bands) (nm)	λ_{em} (nm)	Φ_F [b]
1b [a]	324	427	521, 559, 595, 651	660, 724	0.03
2b [a]	343	432	524, 563, 598, 655	664, 731	0.03
2c [a]	342	433	525, 564, 597, 655	667, 731	0.03

[a] Absorption and emission spectra were measured at a concentration of ~2.5×10^{-6} M and ~4×10^{-7} M, respectively. No sign of turbidity or cloudiness was observed by naked eye, and the solutions are stable for weeks. Baselines of the UV-visible spectra do not show light scattering (which would be indicative of aggregates), which was confirmed by DLS (Dynamic Light Scattering) measurements. [b] Fluorescence quantum yield, using H_2TPP in toluene ($\Phi_F = 0.11$) as standard, upon excitation at Soret band.

5. Two-Photon Absorption Spectra of the Porphyrins 1b and 2b-c and Corresponding Dependence of Intensity *vs.* Fluence

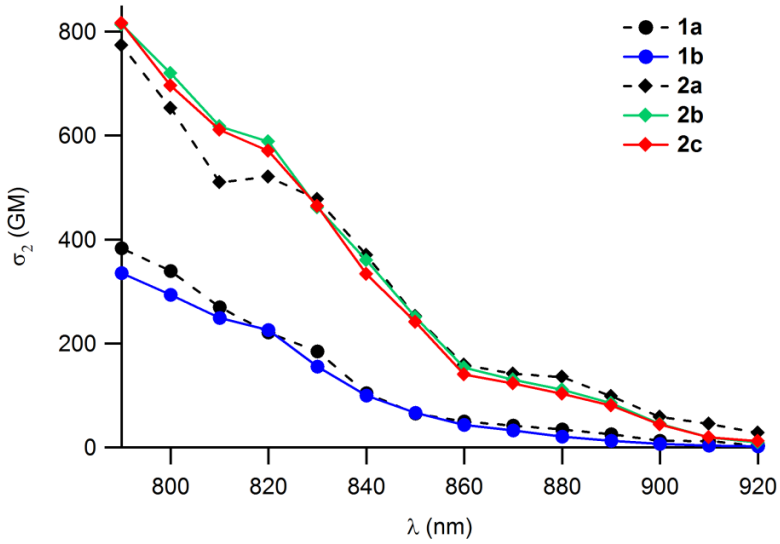


Figure S3. Two-photon absorption spectra of **1-2a** in dichloromethane and of **1b** and **2b-c** in THF (obtained two-photon excited fluorescence measurements in the femtosecond regime).

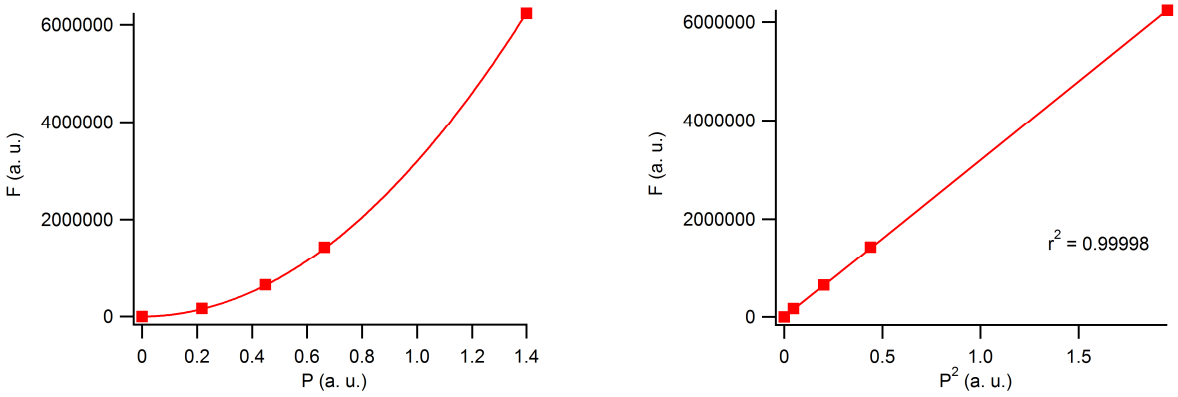


Figure S4. Left: quadratic dependence of the emission intensity (F) on laser excitation power (P) for compound **1b** at 790 nm. Right: dependence of F on P^2.

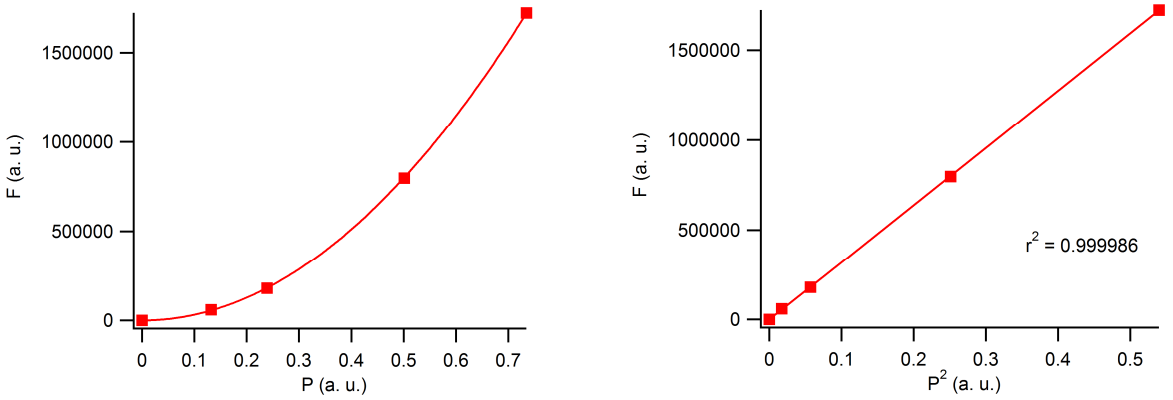


Figure S5. Left: quadratic dependence of the emission intensity (F) on laser excitation power (P) for compound **2b** at 790 nm. Right: dependence of F on P^2.

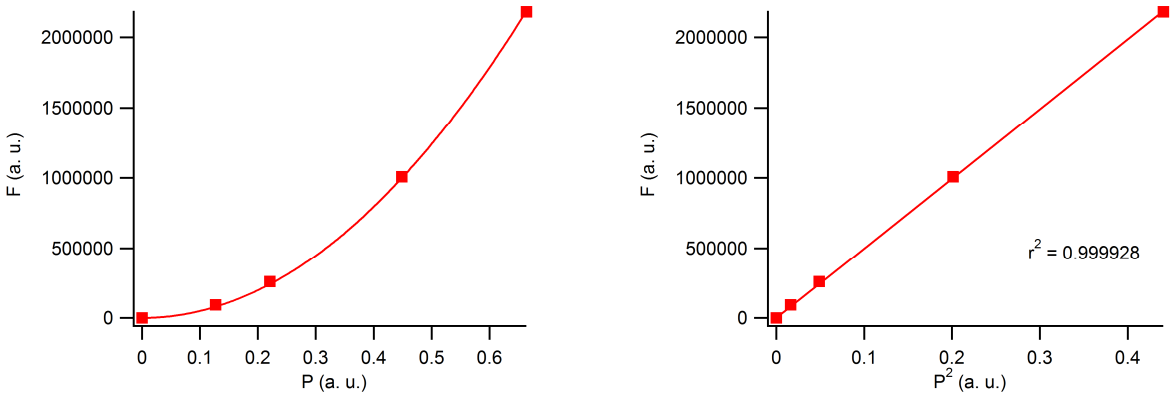


Figure S6. Left: quadratic dependence of the emission intensity (F) on laser excitation power (P) for compound **2c** at 790 nm. Right: dependence of F on P^2.

6. Two-Photon Excited Fluorescence Emission Spectra of the Porphyrins 1b and 2b-c

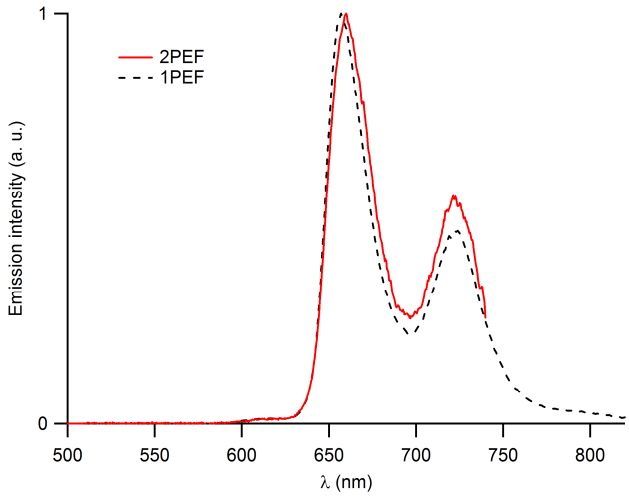


Figure S7. Red solid line: two-photon excited fluorescence (2PEF) emission spectrum of compound **1b** in THF (excitation at 790 nm); black dotted line: corresponding one-photon excited fluorescence (1PEF) emission spectrum (excitation at 425 nm).

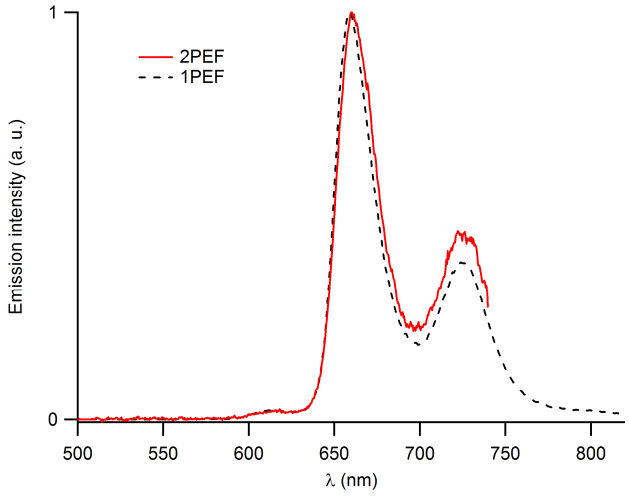


Figure S8. Red solid line: two-photon excited fluorescence (2PEF) emission spectrum of compound **2b** in THF (excitation at 790 nm); black dotted line: corresponding one-photon excited fluorescence (1PEF) emission spectrum (excitation at 430 nm).

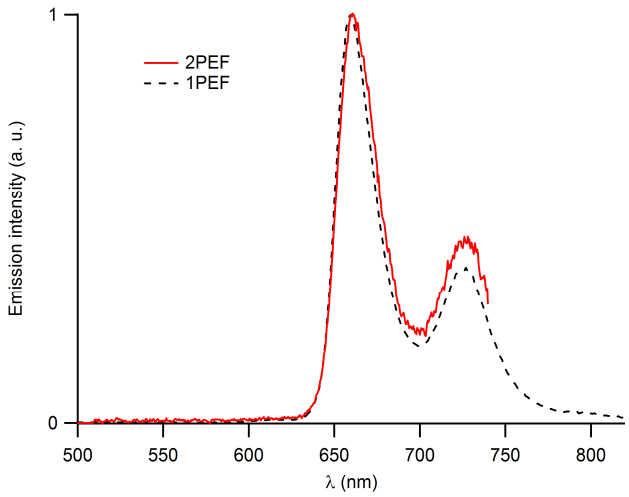


Figure S9. Red solid line: two-photon excited fluorescence (2PEF) emission spectrum of compound **2c** in THF (excitation at 790 nm); black dotted line: corresponding one-photon excited fluorescence (1PEF) emission spectrum (excitation at 430 nm).

7. Biocompatibility analysis for the Porphyrins (1b and 2b-c)

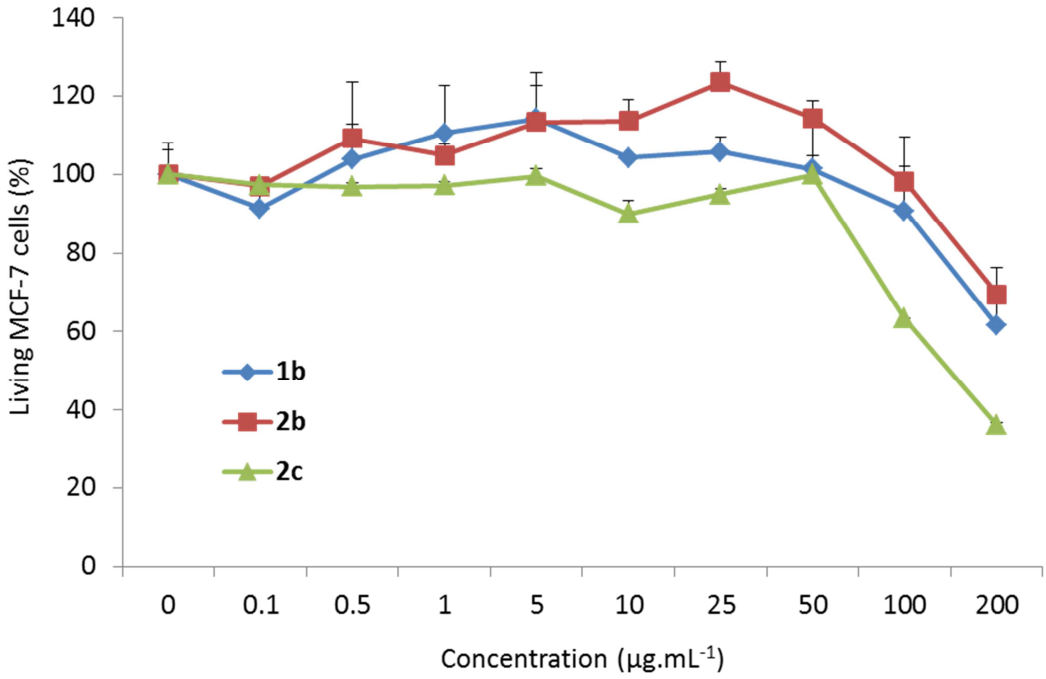


Figure S10. Cytotoxic study of human breast cancer cells (MCF-7) incubated for 72 h with porphyrins at a range (from 0.1 to 200 µg mL^{-1}). The percentage of living cells was measured by a MTT assay performed at the end of the incubation time. Values are means ± standard deviations of 3 experiments. This experiment of dark cytotoxicity analysis aimed at determining the safe concentration of porphyrin that could be used without inducing any cytotoxicity in the absence of irradiation. After 72 h incubation time with increasing doses of the three porphyrins, no cell death was detected up to 50 µg.mL^{-1}. This suggested their biocompatibility at this concentration, and to avoid any cell death without irradiation, we decided to treat the cells with 25 µg.mL^{-1} for 2P-PDT experiments.

8. Localisation Experiments of the Porphyrins (1b and 2b-c) in MCF-7 cells

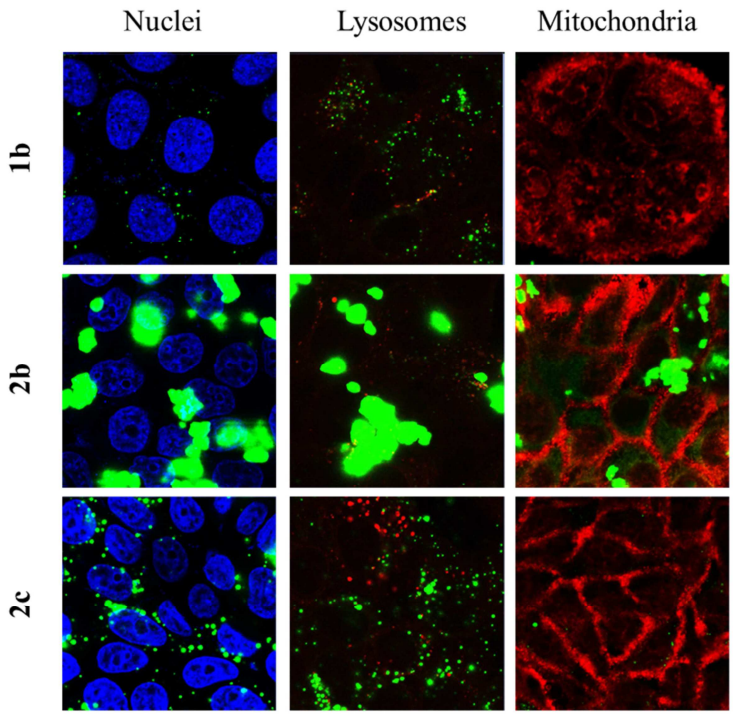


Figure S11. Localisation experiments of porphyrins **1b** and **2b-c** in MCF-7 cells. Nuclei stained with Hoechst 33342 (λ_{exc} = 405 nm) are visualised in blue (left column), lysosomes stained with LysoTracker (λ_{exc} = 488 nm) are visualised in red (middle column), mitochondria stained with MitoTracker (λ_{exc} = 561 nm) are visualised in red (right column), and porphyrins (λ_{exc} = 405 nm) **1b** and **2b-c** appear in green. Porphyrins were not localised in the nuclei (which is not surprising), but they are also neither localised in lysosomes nor in mitochondria, which suggests their diffusion in the cytoplasm.