Supplementary Information

pKa tuning in quadrupolar-type two-photon ratiometric fluorescent membrane probes

Jonathan Daniel,[a] Cristiano Mastrodonato,[a] Aude Sourdon,[b,c] Guillaume Clermont,[a] Jean-Marie Vabre,[c] Bertrand Goudeau,[a] Hannah Voldoire,[a] Stéphane Arbault,[a] Olivier Mongin[b,c]* and Mireille Blanchard-Desce[a,c]*

[a] Univ. Bordeaux, ISM, UMR 5255 CNRS, F-33400 Talence, France. Phone: +33 (5) 4000 6732; E-mail: mireille.blanchard-desce@u-bordeaux1.fr
[b] Institut des Sciences Chimiques de Rennes (CNRS, UMR 6226), Université de Rennes 1, Campus Scientifique de Beaulieu, Bât 10A, F-35042 Rennes Cedex, France. Phone: +33 (2) 2323 5954; E-mail: olivier.mongin@univ-rennes1.fr
[c] Chimie et Photonique Moléculaires (CNRS, UMR 6510), Université de Rennes 1, Campus Scientifique de Beaulieu, Bât 10A, F-35042 Rennes Cedex, France.

Materials and General procedures:

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 F254 precoated aluminium sheets. Column chromatography: Merck silica gel Si 60 (40–63 mm, 230–400 mesh or 63–200 mm, 70–230 mesh). Melting points were determined on an Electrothermal IA9300 digital melting point instrument or on Mettler Toledo DSC 1. NMR: Bruker ARX 200 (^{1}H: 200.13 MHz, ^{13}C: 50.32 MHz), Avance AV 300 (^{1}H: 300.13 MHz, ^{13}C: 75.48 MHz) or Avance AV 500 (^{1}H : 500.13 MHz, ^{13}C : 125.03 MHz) in $CDCl_3$ solution or CD_2Cl_2 solution; ^{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. High and low resolution mass spectra measurements were performed at the Centre Régional de Mesures Physiques de l'Ouest (C.R.M.P.O., Rennes), using a Micromass MS/MS ZABSpec TOF instrument with EBE TOF geometry; LSIMS (Liquid Secondary Ion Mass Spectrometry) at 8 kV with Cs+ in m-nitrobenzyl alcohol (mNBA); ES+ (electrospray ionization, positive mode) at 4 kV; EI (electron ionization) at 70 eV. Elemental analyses were performed at C.R.M.P.O or at I.C.S.N- C.N.R.S. (Gif-sur-Yvette, France).

Synthesis:

2,7-Diiodo-9*H*-fluorene.[1] A solution of fluorene (20.00 g, 120.38 mmol), acetic acid (100 mL), concentrated sulphuric acid (2.8 mL) and water (7 mL), was heated at 75°C. Then

periodic acid (7.00 g, 30.71 mmol) and iodine (16.00 g, 63.04 mmol) were added. After 1h at 75°C, acetic acid (100 mL), periodic acid (6.44 g, 28.25 mmol) and iodine (16.00 g, 63.04 mmol) were again added, and the mixture was heated at 75°C for 1h, then cooled. CH_2Cl_2 was added and the mixture was neutralized with NaOH (3 M) until basic pH. The solution was washed with a saturated aqueous sodium thiosulfate solution until an orange color appears and extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$, and then evaporated in vacuo. The crude product was recrystallized from heptane to yield 40.23 g (80%) of name as yellow solid. ^{1}H NMR (200.1 MHz, $CDCl_3$) 7.88 (s, 1H), 7.71 (d, J = 8.1 Hz, 2H), 7.50 (d, J = 8.1 Hz, 2H), 3.84 (s, 2H).

9,9-Dibutyl-2,7-diiodo-9*H*-fluorene (3).[2] A solution of *n*-Bu_4NBr (0.93 g, 2.88 mmol) and KOH (8.06 g, 143.76 mmol) in water (8 mL) was heated at 65°C. 2,7-Diiodo-9*H*-fluorene (6.02 g, 14.40 mmol) and 1-bromobutane (9.3 mL, 86.10 mmol) dissolved in toluene (15 mL) were then added to the basic solution and heated at 65°C for 1h. The organic layer was extracted with CH_2Cl_2, dried over $MgSO_4$, filtered and evaporated in vacuo. The residue was purified by column chromatography (heptane) to yield 6.86 g (90%) of **3** as a white solid. ^{1}H NMR (200.1 MHz, $CDCl_3$) 7.65 (dd, J = 8.3 Hz, J = 1.7 Hz, 2H), 7.64 (d, J = 1.7 Hz, 2H), 7.40 (d, J = 8.3 Hz, 2H), 1.90 (m, 4H), 1.08 (m, 4H), 0.68 (t, J = 7.3 Hz, 6H), 0.56 (m, 4H). ^{13}C {^{1}H} NMR (75.5 MHz, $CDCl_3$) 152.5, 139.7, 136.0, 132.0, 121.5, 93.1, 55.4, 39.9, 25.8, 22.9, 13.7.

2,7-Bis(trimethylsilylethynyl)-9,9-dibutyl-9H-fluorene. Air was removed from a solution of **3** (2.00 g, 3.77 mmol) in toluene/Et_3N (1:1, 40 mL) by blowing argon for 20 min. Then CuI (0.03 g, 0.15 mmol), $Pd(PPh_3)_2Cl_2$ (0.11 g, 0.15 mmol) and ethynyltrimethylsilane (1.3 mL, 9.43 mmol) were added. The mixture was stirred at 40 °C for 16 h. The solvents were evaporated and the residue was purified by column chromatography (heptane) to yield 1.40 g (79%) of the title compound. M.p.= 180°C (dec.); ^{1}H NMR (200.1 MHz, $CDCl_3$) 7.64 (d, J = 7.5 Hz, 2H), 7.48 (d, J = 7.5 Hz, 2H), 7.46 (s, 2H), 1.98 (m, 4H), 1.08 (m, 4H), 0.69 (t, J = 7.3 Hz, 6H), 0.55 (m, 4H), 0.33 (s, 18H). ^{13}C {^{1}H} NMR (75.5 MHz, $CDCl_3$) 150.9, 140.9, 131.3, 126.2, 121.8, 119.9, 106.1, 94.3, 55.2, 40.2, 25.8, 23.1, 13.8, 0.1. Anal. Calcd (%) for $C_{31}H_{42}Si_2$ (470.85): C, 79.08, H, 8.99; found: C, 78.88, H, 9.12. HRMS (EI) m/z calcd for $C_{31}H_{42}Si_2$ ($M^{+\cdot}$): 470.2825; found: 470.2848.

9,9-Dibutyl-2,7-diethynyl-9*H*-fluorene (4). To a solution of 2,7-bis(trimethylsilylethynyl)-9,9-dibutyl-9*H*-fluorene (0.86 g, 1.83 mmol) in THF/MeOH (3:1, 44 mL) was added aqueous KOH (1 M, 13 mL), and the mixture was stirred at room temperature for 30 min. CH_2Cl_2 and water were added and the organic layer was separated. The aqueous layer was extracted with CH_2Cl_2, and the combined organic layers were dried over Na_2SO_4. The residue obtained after removal of the solvents was purified by column chromatography using heptane/CH_2Cl_2 (80:20) as eluent to yield 0.50 g (84%) of **4**. M.p.=93 °C; ^{1}H NMR (200.1 MHz, $CDCl_3$) 7.63 (d, J = 8.6 Hz, 2H), 7.48 (d, J = 8.6 Hz, 2H), 7.46 (s, 2H), 3.15 (s, 2H), 1.94 (m, 4H), 1.07 (m, 4H), 0.67 (t, J = 7.2 Hz, 6H), 0.54 (m, 4H). ^{13}C {^{1}H} NMR (50.3 MHz, $CDCl_3$) 151.0, 140.9, 131.2, 126.5, 120.8, 119.9, 84.5, 77.4, 55.1, 40.0, 25.8, 22.9, 13.7. Anal. calcd (%) for $C_{25}H_{26}$ (326.48): C, 91.97; H, 8.03; found: C, 92.17; H, 8.07. HRMS (EI) m/z calcd for $C_{25}H_{26}$ ($M^{+\cdot}$): 326.2035; found: 326.2036.

4,4'-(9,9-Dibutyl-9*H*-fluorene-2,7-diyldi-2,1-ethynediyl)bisbenzaldehyde (5). Air was removed from a solution of **4** (251 mg, 0.77 mmol) and 4-bromobenzaldehyde (354 mg, 1.914 mmol) in toluene/Et_3N (4:1, 10 mL) by blowing argon for 20 min. Then CuI (6 mg, 0.031 mmol) and $Pd(PPh_3)_2Cl_2$ (22 mg, 0.03 mmol) were added, and argon blowing was continued for 10 min. Thereafter the mixture was stirred at 40 °C for 16 h. The solvent was evaporated in vacuo, and the crude product was purified by column chromatography using heptane/CH_2Cl_2 (gradient from 50:50 up to 40:60) as eluent to yield 307 mg (75%) of **5**. M.p.= 199 °C (dec.); ^{1}H NMR (300.13 MHz, $CDCl_3$) δ 9.94 (s, 2H), 7.80 (d, J = 8.4 Hz, 4H), 7.63 (d, J = 8.4 Hz, 4H), 7.62 (d, J = 8.7 Hz, 2H), 7.48 (d, J = 8.7 Hz, 2H), 7.46 (s, 2H), 1.94 (m, 4H), 1.05 (m, 4H), 0.60 (t, J =7.2 Hz, 6H), 0.53 (m, 4H); ^{13}C NMR (75.46 MHz, $CDCl_3$) δ 191.3, 151.2, 141.1, 135.4, 132.0, 131.1, 129.6, 126.2, 121.4, 120.3, 94.5, 89.2, 55.3, 40.2, 25.9, 22.9, 13.8; Anal. calcd (%) for $C_{39}H_{34}O_2$; 0.25 CH_2Cl_2 (555.93): C, 84.80; H, 6.26; found: C, 84.87; H, 6.94. HRMS (ESI) calcd for $C_{39}H_{35}O_2$ [$(M+H)^+$] m/z 535.2637, found 535.2638.

4-(Diphenylphosphinoylmethyl)quinoline To a solution of diisopropylethylamine (1.18 g, 11.70 mmol) in THF (9 mL) and *n*-BuLi (0.68 g, 11.70 mmol) was added dropwise under argon atmosphere at -10 °C. A solution of lepidine (1.52 g, 10.60 mmol) in THF (3 mL) was added dropwise to the previous mixture. The resulting mixture was stirred and warmed to 0°C for 30 minutes then room temperature for 1 h. The solution was stirred at -78°C and chlorodiphenylphosphine was added (2.57 g, 11.70 mmol). The mixture was stirred for 12 h.

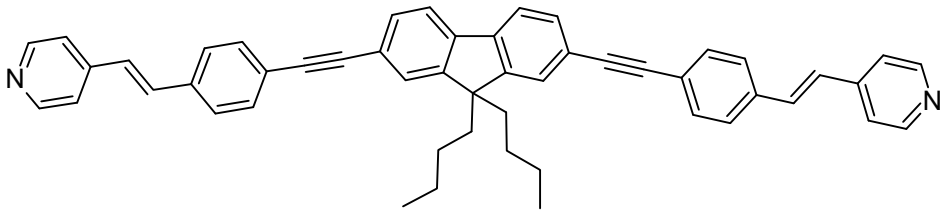
The crude product was first hydrolyzed with water (10 mL) then extracted with CH_2Cl_2, dried over $MgSO_4$, filtered and evaporated in vacuo. The residue was dissolved in heptane and air was bubbled over 12 h. The resulting precipitate was filtered, dried and the organic layer was recrystallized from toluene to yield 1.22 g (33%) of the title compound. M.p. =202.5°C; ^{1}H NMR (300.13 MHz, CD_2Cl_2) δ 8.61 (d, J = 4.4 Hz, 1H), 8.00 (dd, J = 9.4 Hz, J = 8.6 Hz, 2H), 7.66-7.73 (m, 5H), 7.41-7.55 (m, 7H), 7.07 (dd, J = 4.4 Hz, J = 2.9 Hz, 1H), 4.11 (d, J = 14.0 Hz, 2H). ^{31}P NMR (120.05 MHz, CD_2Cl_2) : δ 28.18 (s, 1P). HRMS (ESI) calcd for $C_{22}H_{19}NOP$ [$(M+H)^+$] m/z 344.1204, found 344.1201.

4-(Diphenylphosphinoylmethyl)pyridine. This compound was prepared as reported in lit.[3]

General procedure for the synthesis of compounds 1 and 2.

Air was removed from a solution of **5** (20 mg, 0.037 mmol) and 2.05 equiv of phoshine oxide in THF (5 ml) by blowing argon for 20 min. Then NaH (4 mg, 95%) was added. The mixture was stirred at room temperature for 24 hours after which TLC revealed complete conversion. Water was added to the reaction mixture and extracted with dichloromethane. The organic layer was washed with brine then dried over sodium sulfate, filtered and evaporated in vacuo. The resulting crude yellow solids were repeatedly washed with EtOH then dried under vacuum. 20.8 mg of **1** (81.2%) and 23.3 mg of **2** (79.5%) were obtained.

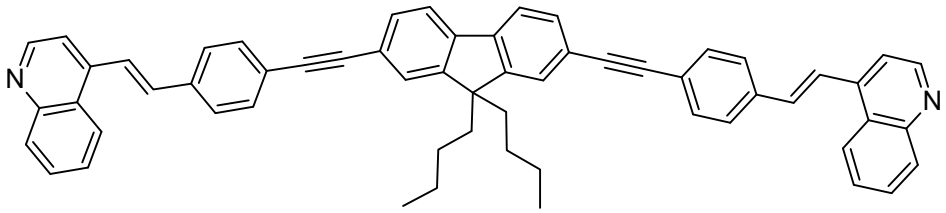
Compound 1



M.p.= 178°C (dec.); ^{1}H NMR (300.13 MHz, $CDCl_3$) δ 8.60 (d, J = 6.2 Hz, 4H), 7.70 (d, J = 7.9 Hz, 2H), 7.62-7.52 (m, 12H), 7.38 (d, J = 6.2 Hz, 4H), 7.31 (d, J = 16.3 Hz, 2H), 7.06 (d, J = 16.3 Hz, 2H), 2.02 (m, 4H), 1.11 (m, 4H), 0.70 (t, J = 7.2 Hz, 6H), 0.61 (m, 4H). ^{13}C NMR (75.48 MHz $CDCl_3$) δ 151.3, 148.8, 141.0, 135.8, 135.7, 133.9, 132.2, 131.0, 127.3, 12 6.3, 126.1, 124.2, 121.9, 121.3, 120.2, 92.4, 89.8, 55.4, 40.3, 26.0, 23.2, 13.9. Anal. calcd (%)

for $C_{51}H_{44}N_2$; 1.5 H_2O (708.90): C, 86.04; H, 6.65; N, 3.93 found: C, 86.42; H, 6.65; N, 3.79. HRMS (ESI) calcd for $C_{51}H_{45}N_2$ [$(M+H)^+$] m/z 685.3577, found 685.3575.

Compound 2



M.p.=140 °C (dec.); ^{1}H NMR (300.13 MHz, $CDCl_3$) δ 8.92 (s, 2H), 8.25 (d, J = 8.4 Hz, 2H), 8.17 (d, J =8.4 Hz, 2H), 7.89 (d, J = 16.2 Hz, 2H), 7.75-7.54 (m, 20H), 7.37 (d, J = 16.2 Hz, 2H), 2.01 (m, 4H), 1.11 (m, 4H), 0.70 (t, J = 7.2 Hz, 6H), 0.60 (m, 4H). ^{13}C NMR (125.03 MHz,$CDCl_3$) δ151.3, 150.2, 148.7, 143.0, 141.0, 136.5, 134.6, 132.2, 131.0, 130.2, 129.6, 12 7.3, 126.8, 126.5, 126.1, 123.9, 123.8, 123.6, 122.0, 120.2, 117.2, 92.5, 90.2, 55.6, 40.7, 26.3, 23.5, 14.3. Anal. calcd (%) for $C_{59}H_{48}N_2$; 0.75 CH_2Cl_2 (848.74): C, 84.56; H, 5.88; N, 3.30 found: C, 84.11; H, 6.35; N, 3.24. HRMS (ESI) calcd for $C_{59}H_{49}N_2$ [$(M+H)^+$] m/z 785.3890, found 785.3895.

Photophysical methods:

All photophysical properties were analyzed with freshly prepared air equilibrated solutions at room temperature (293 K). UV/Vis absorption spectra were recorded using a Jasco V-570 spectrophotometer. Steady-state fluorescence measurements were performed on dilute solutions (1.0 x 10^{-6} M, optical density < 0.1) contained in standard 1 cm quartz cuvettes using an Edinburgh Instruments (FLS920) spectrometer in photon-counting mode. Fully corrected emission spectra were obtained for each compound at $\lambda_{ex} = \lambda_{abs}^{max}$ with an optical density at $\lambda_{ex} \leq 0.1$ to minimize internal absorption. Fluorescence quantum yields were measured according to literature procedures.[4, 5]

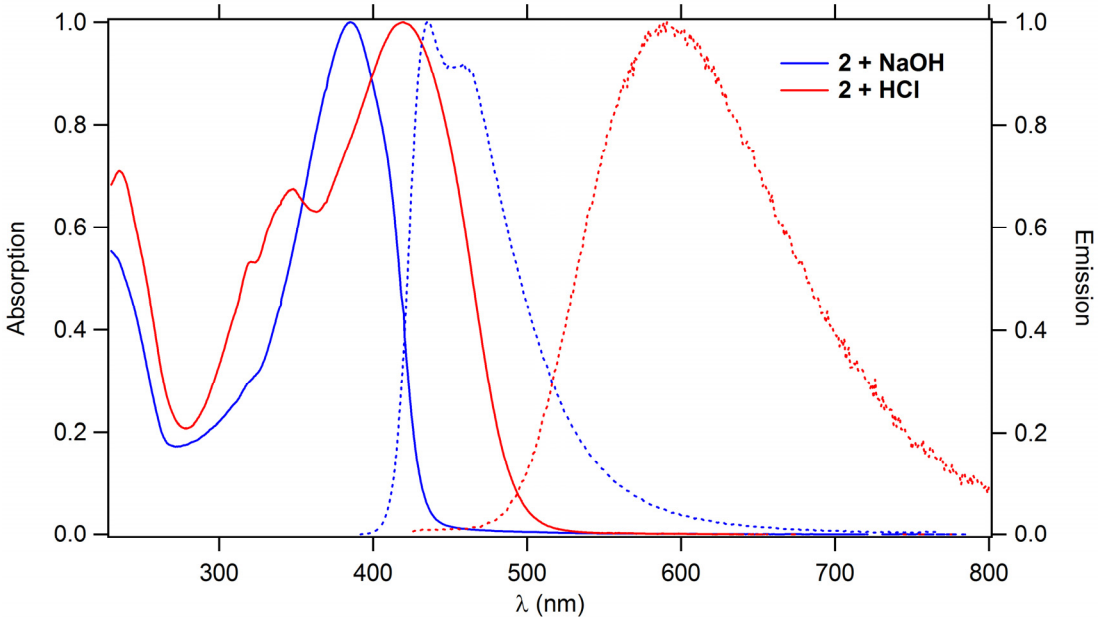


Figure S1. Absorption and emission spectra of compound **2** in basic (blue) and acidic (red) conditions

Detemination of pKa values:

To a solution of probe (1.0 x 10^{-6} M) in a micellar water (SDS/Butanol/water 6:5:89 %wt) were added solutions of HCl (1.0 M) or NaOH (1.0 M) using a microsyringe to reach desired pH (4.6-10.9). The pH values were measured on a Tacussel PHN81 instrument with a glass combined electrode and a saturated KCl electrode as reference. The pK_a values were estimated from the changes in the fluorescence intensity (Figure S1, S2) with pH 4.6-10.9 by using the relationship, $\text{Log}[(I-I_A)/(I_B-I)] = pK_a - pH$, where I_A, I_B and I are the maximum acidic form, basic form and the observed emission intensity at a given pH, respectively. The calculated pK_a values of **1** and **2** measured by fluorescence spectra are 7.0 ± 0.1 and 6.2 ± 0.1 respectively.

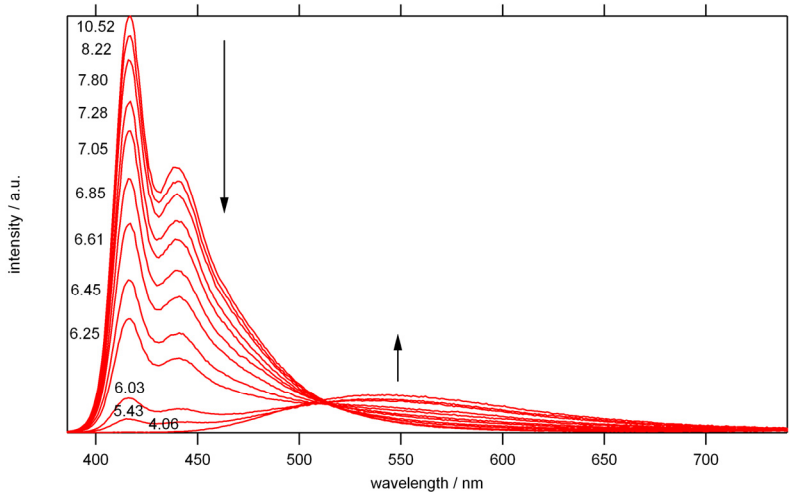


Figure S2. Variation of the emission spectra of **1** in micellar water as a function of pH (λ_{exc} = 380 nm).

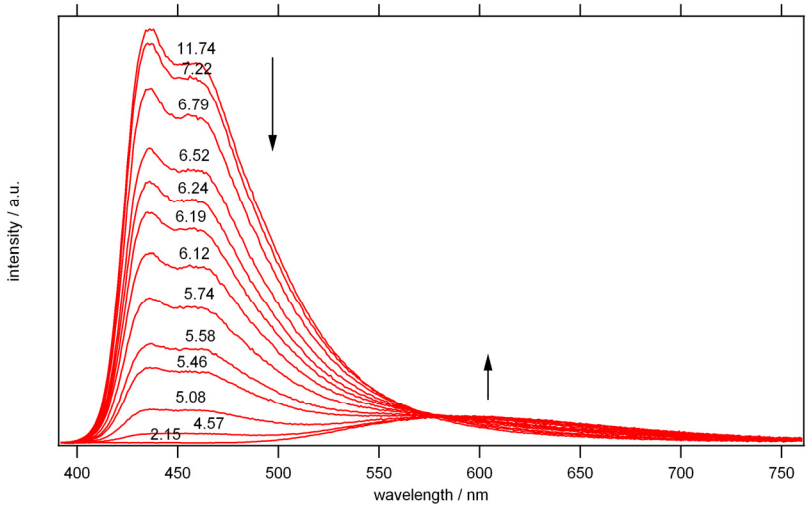


Figure S3. Variation of the emission spectra of **2** in micellar water as a function of pH (λ_{exc} = 386 nm).

Two-photon absorption (TPA):

Two-photon absorption measurements were conducted by investigating the two-photon excited fluorescence (TPEF) of the basic and acidic forms of the fluorophores in micellar buffer SDS/Butanol/water (6:5:89 %wt) at room temperature on air-equilibrated solutions (10^{-4} M) according to the experimental protocol established by Xu and Webb.[6] To span the 700-980 nm range, a Nd:YLF-pumped Ti:sapphire oscillator was used to generate 150 fs pulses at a 76 MHz rate. The excitation was focused into the cuvette through a microscope objective (10x; numerical aperture (NA):0.25). The fluorescence was detected in epifluorescence mode by using a dichroic mirror (Chroma 675dcxru) and a barrier filter (Chroma e650sp-2p) by a compact CCD spectrometer module (BWTek BTC112E). Total fluorescence intensities were obtained by integrating the corrected emission spectra measured by this spectrometer. TPA cross-sections (σ_2) were determined from the two-photon excited fluorescence (TPEF) cross-sections ($\sigma_2\Phi$) and the fluorescence emission quantum yield (Φ). TPEF cross-sections were measured relative to fluorescein in 0.01M aqueous NaOH for 715-980 nm[6, 7] and the appropriate solvent-related refractive index corrections.[8] Data points between 700 and 715 nm were corrected according to the litterature.[9] The quadratic dependence of the fluorescence intensity on the excitation power was checked for each sample and all wavelengths, thereby indicating that the measurements were carried out in intensity regimes in which saturation or photodegradation did not occur.

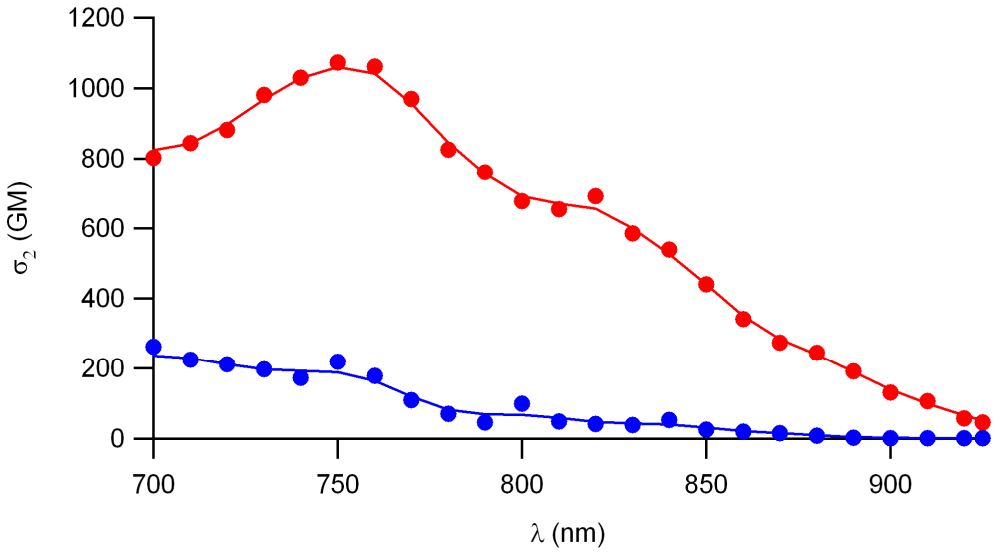


Figure S4. Two-photon absorption spectra of compound **1** in basic (blue) and acidic (red) conditions

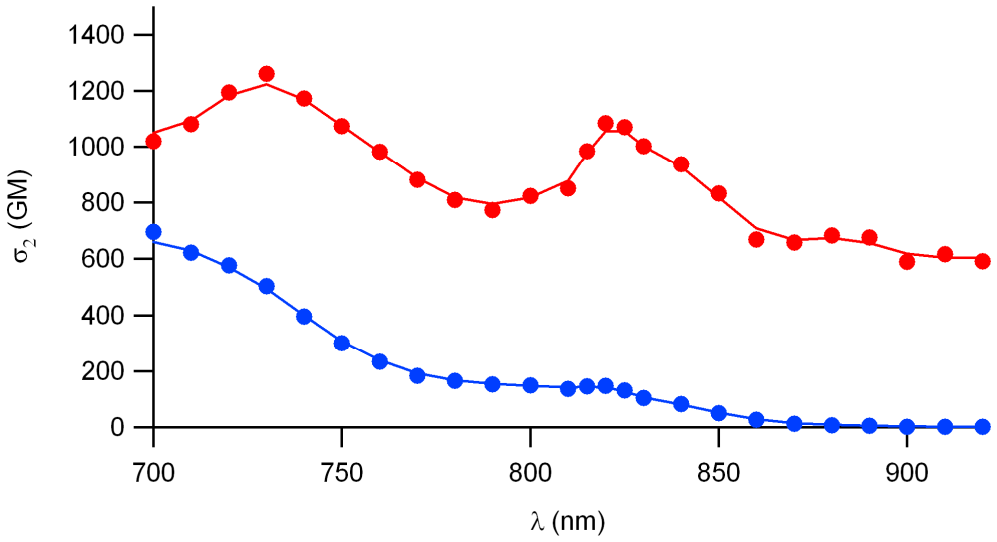


Figure S5. Two-photon absorption spectra of compound **2** in basic (blue) and acidic (red) conditions

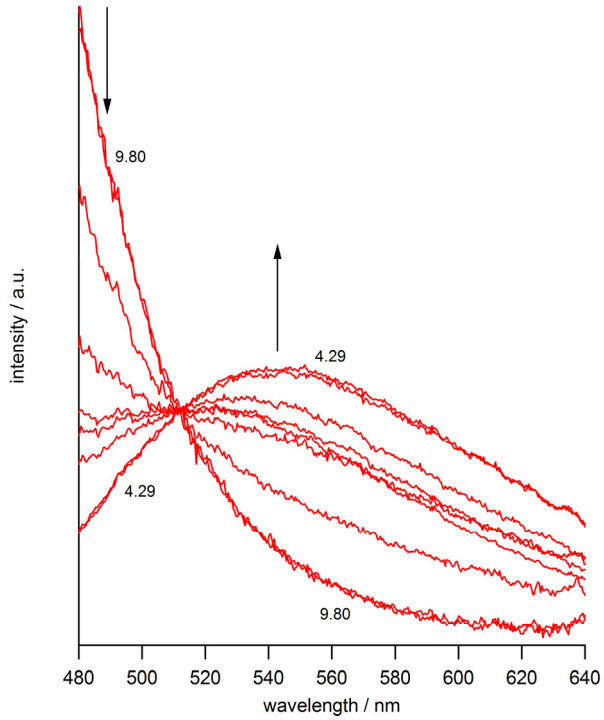


Figure S6. Variation of the two-photon excited emission spectra of **1** in micellar water as a function of pH (λ_{exc} = 750 nm).

Preparation and confocal imaging of GUVs

All reagents and electrodes used herein were purchased form Sigma Inc.. Giant Unillamelar Vesicles (GUV) were electroformed in the presence of compound **1** following a procedure adapted from Pott et al.[10] A solution in chloroform containing 80 % Egg-PC (2 mM) + 20 % cholesterol (0.5 mM) + 0.1 % of **1** (1 mM stock solution in chloroform) was deposited (4 µL volume) with a syringe on the surface of each ITO electrode (Resistivity = 15-25 ohm.square^{-1}). These modified electrodes were let to dry for at least 20 min. in a desiccator under vacuum and mounted into a chamber by using a spacer (3 mm-thickness and 7 cm²-open area) made of polydimethylsiloxane (PDMS). The chamber volume was filled entirely with an aerated PBS solution (10 mM phosphates; 137 mM NaCl; 2.4 mM KCl) at pH7.4 (neutral solution) or adjusted at pH 8.1 (basic solution). Electrodes were connected to an AC generator (Agilent 33210A model) to apply a potential difference ΔE at a fixed frequency of 500 Hz according to the following time-sequence : 5 min. at 0.775 V, 5 min. at 2 V, 15 min. at 3 V, 30 min. at 4 V and 24-36 hours at 5 V. This lead to the formation of numerous 10-40 µm diameter unilammellar vesicles; the latter step could be adjusted to increase the mean GUV size.

The evolution of GUV formation and the incorporation of **1** in their membranes were monitored directly on the surface of electrodes by confocal fluorescence microscopy (SP5 confocal system from Leica Inc., Germany). GUV were imaged with a 20x objective (NA: 0.4) in bright field (differential interference contrast mode) at 633 nm (50 % power) and then in fluorescence at 405 nm (50 % power). Images of Figure 5 were obtained by averaging 3-fold to 8-fold each scan line. No difference was observed in terms of GUV shape, lamellarity and mean diameter with or without compound **1** during the electroformation process.

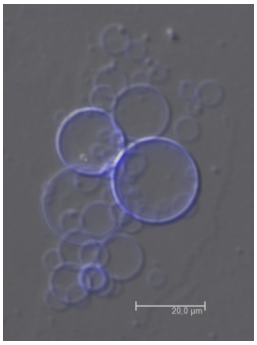


Figure S7. Incorporation of **1** in GUVs at neutral pH, images are the overlap of bright field and fluorescence (λ_{ex} = 405 nm) detections to compare the membrane and chromophore localisations.

Confocal and two-photon cellular imaging

The confocal microscope was a Leica TCS SP5 on an upright stand DM6000 (Leica Microsystems, Mannheim, Germany), using objective HCX IRAPO L 25X water NA 0.95. For confocal microscopy the UV lasers used was a Diode 405 nm. The multiphoton microscopy was done with a tunable pulsed depletion laser Mai Tai HP (Spectra-Physics, Irvine, USA). This laser was tuned at 700 nm (output average power of 0.67W). The system was used with a conventional scanner and with 2 hybrid detectors and 1 PMT for transmission.

Fibroblast-like COS-7 cells were grown in Dulbecco's Modified Eagle Medium without phenol-red (PAN Biotech, P04-01515) supplemented with 10% v/v foetal calf serum (Dominique Dutscher, 500105), 1% v/v penicillin/streptomycin (Dominique Dutscher, P06-07100) in 25 mL flasks (Falcon, 353082) at 37°C, 5% CO_2. They were kept below 90% confluency, at which cells were washed in sterile filtered PBS (PAN Biotech, P04-36500), detached in a small volume of trypsin (PAN Biotech, P10-021100), washed in full warm medium and split 1/10 in a new flask. For imaging, 90% confluent cells were similarly detached with trypsin and diluted 1/10 in full warm medium. Twelve 18 mm-diameter round #1.5 coverslips (Thermo Scientific, DV40008) were placed in a 12-well plate (Cellstar, 665180), each coverslip was incubated in 500 µL spectrograd-rated ethanol (Fluka, 02850-1L) for >10', thoroughly washed twice in PBS, and resuspended in 2 mL full warm medium. Volumes of 25-200 µL of diluted cells were then dropped onto each coverslip. Cells were carefully homogenised by tilting the 12-well plate, and left at 37°C, 5% CO_2 for ~48h.

The cells were incubated in presence of 1µM of **1** in PBS buffer for 90 minutes at 37°C (5% CO_2). Thereafter, the cells were washed three times with PBS buffer in order to remove non stained residual probe **1** prior to imaging. For confocal microscopy the emission of the fluorescence was collected in the range [390 nm – 645 nm] for the fluorescence imaging (S8). The fluorescence resulting from the two-photon excitation for imaging and estimation of the pH was collected in the range [390 nm-688 nm].

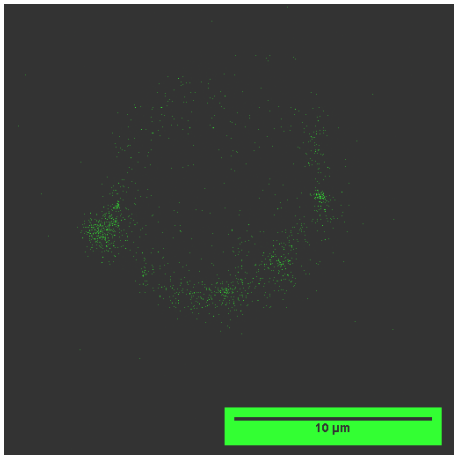


Figure S8. Fluorescence imaging of COS 7 cells stained with **1** (λ_{ex} = 405 nm).

The microscopy was done in the Bordeaux Imaging Center, a service unit of the CNRS-INSERM and Bordeaux University, member of the national infrastructure France BioImaging. The help of Sébastien Marais and is acknowledged.

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