Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

Supplementary information

Subphthalocyanine-flipper dyads for selective membrane staining

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1. Materials and methods

Chemical reagents and solvents were purchased from Merck-Sigma Aldrich, BLD-pharmaceuticals, TCI and Across and were used without further purification. Egg sphingomyelin (SM) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids, cholesterol (CL) was purchased from Sigma-Aldrich. Phosphate buffered saline (PBS, pH = 7.4), FluoroBrite DMEM (high D-Glucose, 3.7 g/L Sodium Bicarbonate, without phenol red) medium, Penicillin-Streptomycin, Fetal Bovine Serum, TrypLE Express Enzyme were obtained from Thermo Fisher Scientific. 35 mm glass-bottom dishes were obtained from MatTek (P35G-0.170 14-C). Large unilamellar vesicles (LUVs) were prepared by the extrusion method using Mini-Extruder (Avanti Polar Lipids). Giant unilamellar vesicles (GUVs) were prepared using Nanion Vesicle prep pro.

Monitoring of the reactions has been carried out by thin layer chromatography (TLC), employing aluminum sheets coated with silica gel type 60 F254 (0.2 mm thick, E. Merck). Purification and separation of the synthesized products was performed by column chromatography, using silica gel (230–400 mesh, 0.040- 0.063 mm, Merck).

Mass Spectrometry (MS) and High-Resolution Mass Spectrometry (HRMS) spectra were recorded employing Electrospray Ionization (ESI Positive TOF_MS) mass spectra using a MAXIS II spectrometer, or Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) using a Bruker Ultraflex III TOF/TOF spectrometer, with a nitrogen laser operating at 337 nm, or with a NdYAG laser operating at 335 nm. The different matrixes employed are indicated for each spectrum. Mass spectrometry data are expressed in m/z units. All MS experiments were carried out at the "Servicio Interdepartamental de Investigación" (SIdI) of the Universidad Autónoma de Madrid. ^{1}H NMR and ^{13}C NMR were recorded on Bruker XRD-300 (300 MHz) and/or Bruker XRD-500 (500 MHz) instruments at room temperature (25 °C) and are reported as chemical shifts (δ) in ppm relative to TMS (δ = 0). Spin multiplicities are reported as a singlet (s), doublet (d), triplet (t) and quartet (q)

with coupling constants (*J*) given in Hz, or multiplet (m). Broad peaks are marked as br. ^{1}H and ^{13}C resonances were assigned with the aid of additional information from 1D and 2D NMR spectra (H,H-NOESY, H,H-COSY, DEPT 135, HSQC and HMBC). Deuterated solvents employed are indicated in each spectrum. IR spectra were recorded on an Agilent Technology Cary 630 FT-IR spectrometer (ATR) and are reported as wavenumbers υ in cm^{-1} with band intensities indicated as s (strong), m (medium), w (weak), br (broad). Analytical HPLC analysis were performed in a Varian Prostar with ternary pump and a diode array detector. Spectroscopic and photophysical characterization UV-vis spectra were recorded on a JASCO-V660 UV-vis spectrophotometer using spectroscopic grade solvents and 10x10mm quartz cuvettes with a Jasco Peltier ETCS-761 temperature controller incorporated, or in a double beam UV-Vis-NIR Varian Cary 6000i spectrophotometer (Varian, Palo Alto, CA, USA). Fluorescence spectra were recorded with a JASCO FP-8600 spectrophotometer using spectroscopic grade solvents and quartz cuvettes (1cm) with a Jasco Peltier ETCS-761 temperature controller incorporated, or in a Spex Fluoromax-4 spectrofluorometer (Horiba Jobin-Yvon, Edison, NJ, USA) equipped with a stirrer and a temperature controller. All Fluorescence spectra were corrected for lamp intensity fluctuations, background, and the wavelength-dependent response function of the detector. Fluorescence lifetime imaging microscopy (FLIM) images were obtained using Leica Stellaris 8 FALCON, at 20 MHz, with a 63x oil immersion objective lens.

Abbreviations: Cholesterol (CL); 1,4-Diazabicyclo[2.2.2]octane (DABCO); Dioleyl-*sn*-glycero-3-phosphocholine (DOPC); Fluorescence lifetime imaging (FLIM); Giant unilamellar vesicles (GUVs), Large unilamellar vesicles (LUVs); Egg sphingomyelin (SM); Subphthalocyanine (SubPc).

2. Synthesis and characterization

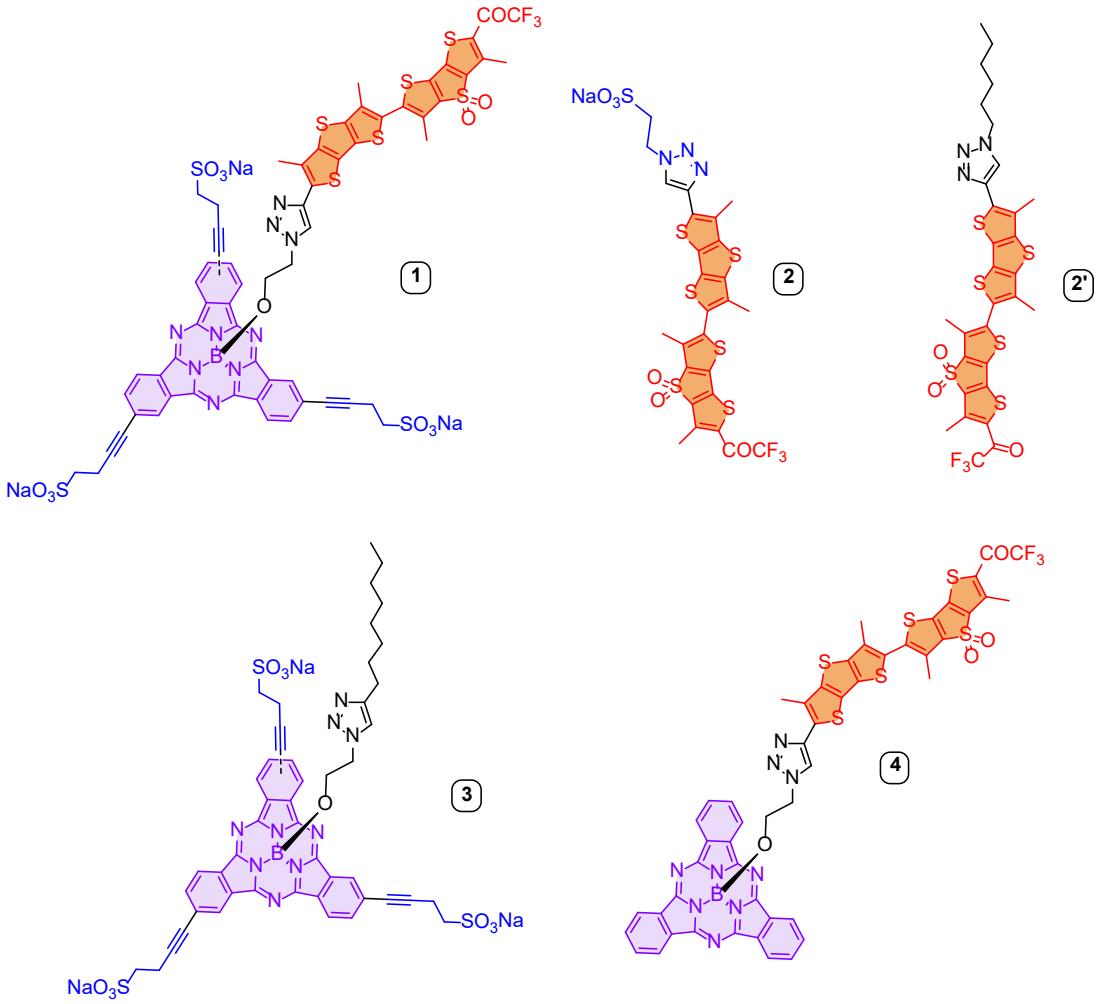
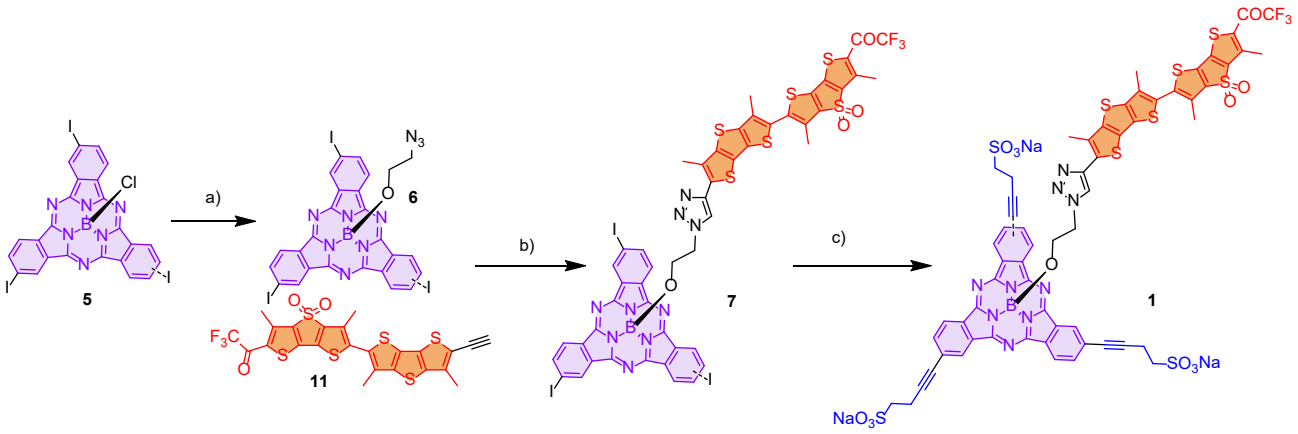


Fig. S1 Structures of the final compounds used in this study, **SubPc-flipper amphiphile** (**1**), **Flipper** (**2**),[S1] **Flipper** (**2'**),[S1] **SubPc amphiphile** (**3**) and **SubPc-Flipper** (**4**).

Compound 2 and 2' were prepared following the reported procedure.[S1]

2.1. Synthesis of SubPc-Flipper amphiphile (1)



Scheme S1 (a) 2-Hydroxyethyl-1-azide, DBU, toluene, 84%; (b) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, CH_2Cl_2/THF/H_2O, rt, 12 h, 42%; (c) but-3-yne-1-sulfonate, $PdCl_2(PPh_3)_2$, CuI, Et_3N, THF, rt, 48 h, 19%.

Compound 5 was prepared following the reported procedure.[S2-S3]

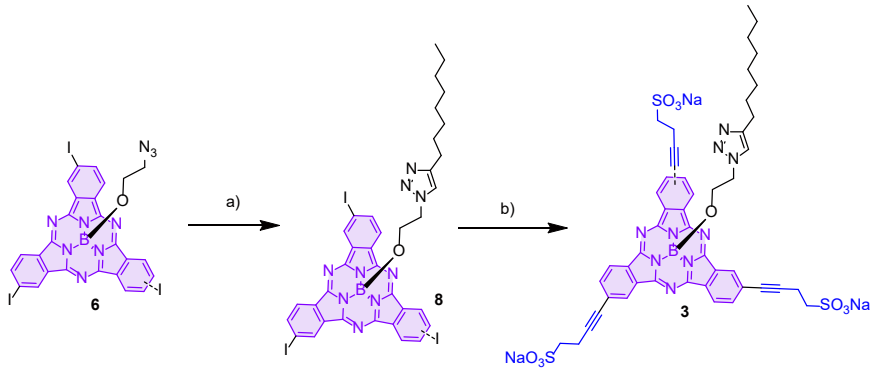
Compound 6. In a 50 mL two neck flask SubPc **5** (60 mg, 74 µmol) was dissolved in toluene (2 mL) and 2-hydroxyethyl-1-azide (65 mg, 0.74 mmol) and 10 µL of DBU were added to the solution. After stirring overnight at 110 ºC, the mixture was concentrated under vacuum and purified by column chromatography (SiO_2, toluene/EtOAc 20:1, R_f = 0.20) to obtain compound **6** as a purple solid (54 mg, 84%). IR (neat): 3063 (m), 2922 (m), 2856 (m), 2099 (s), 1548 (m), 1461 (m), 1443 (m), 1427 (m), 1291 (m), 1222 (s), 1176 (s), 1128 (m), 1040 (m), 818 (s), 759 (s), 704 (s); ^{1}H NMR (300 MHz, $CDCl_3$): 9.19 (s, 3H), 8.54 (d, $^3J_{H\text{-}H}$ = 8.3 Hz, 3H), 8.20 (d, $^3J_{H\text{-}H}$ = 8.3 Hz, 3H), 2.33 (t, $^3J_{H\text{-}H}$ = 5.2 Hz, 2H), 1.64 (d, $^3J_{H\text{-}H}$ = 5.2 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): 151.6 (C), 150.5 (C), 138.9 (CH), 132.4 (CH), 131.5 (C), 130.0 (C), 123.7 (CH), 96.7 (C), 58.6 (CH_2), 51.3 (CH_2); HRMS (MALDI, DCTB$^+$) calcd. for $C_{26}H_{13}BI_3N_9O$: 858.8469 ([M]$^+$), found: 858.8487.

Compound 11 was prepared following the reported procedure.[S1]

Compound 7. To a mixture of **6** (7.2 mg, 8.4 μmol), **10** (5 mg, 8.4 μmol) and $CuSO_4 \cdot 5H_2O$ (2.1 mg, 8.4 μmol) in 1 mL of CH_2Cl_2; 200 μL of THF, 100 μL of water and sodium ascorbate (1.7 mg, 8.4 μmol) were subsequently added. After 5 min, $CuSO_4 \cdot 5H_2O$ (2.1 mg, 8.4 μmol) was diluted in 100 μL of water and sodium ascorbate (1.7 mg, 8.4 μmol) was added to this solution, which was immediately poured into the reaction mixture. After 12 h, water (10 mL) was added, and the mixture was extracted with CH_2Cl_2 (2 x 5 mL). The organic solvents were dried with $MgSO_4$, evaporated *in vacuo* and the crude mixture was purified by column chromatography (SiO_2, CH_2Cl_2, R_f = 0.34) to give **7** (5.1 mg, 42%) as a red solid, checked by ^{1}H NMR and used in the next reaction without further purification. ^{1}H NMR (300 MHz, $CDCl_3$): 9.19 – 9.05 (m, 3H), 8.56 – 8.44 (m, 3H), 8.18 (dd, $^3J_{H\text{-}H}$ = 8.3, $^4J_{H\text{-}H}$ = 1.5 Hz, 3H), 6.91 (s, 1H), 3.71 – 3.74 (m, 2H), 2.89 (s, 3H), 2.65 (s, 3H), 2.58 (s, 3H), 2.53 (s, 3H), 1.99 – 1.93 (m, 2H).

Compound 1. In a 10 mL Schlenk, **7** (5.0 mg, 3.4 μmol), sodium but-3-yne-1-sulfonate (4.0 mg, 25 μmol), $PdCl_2(PPh_3)_2$ (0.3 mg, 1.0 μmol) and CuI (0.1 mg, 0.7 μmol) were dissolved in dry THF (1 mL) under Ar atmosphere, dry Et_3N (0.2 mL) was added, and the reaction stirred at rt. After 24 h, the progress of the reaction was checked and further $PdCl_2(PPh_3)_2$ (0.3 mg, 1.0 μmol) and sodium but-3-yne-1-sulfonate (2 mg, 12 μmol) were added. After additional 24 h, the reaction mixture was concentrated *in vacuo*. The crude product was purified by column in Sephadex™ LH-20 (GE Healthcare) (CH_3OH, 30 cm, 2 cm diameter) to obtain a **1** (1 mg, 19%) as red solid. IR (neat): 3453 (br), 2921 (m), 2361 (w), 1614 (br), 1434 (m), 1183 (s), 1148 (s), 1049 (s), 800 (m), 791 (m), 740 (m), 611 (m); ^{1}H NMR (500 MHz, CD_3OD, hemiacetal): 8.71 – 8.68 (m, 3H), 7.92 (s, 1H), 7.83 – 7.79 (m, 6H), 3.68 – 3.71 (m, 2H), 3.11 – 3.08 (m, 6H), 2.96 – 2.93 (m, 6H), 2.56 (s, 3H), 2.54 (s, 3H), 2.50 – 2.47 (m, 3H), 2.39 (s, 3H), 2.23 – 2.18 (m, 3H), 2.06 – 2.03 (m, 2H). ^{19}F NMR (471 MHz, CD_3OD): -83.85; HRMS (ESI-) calcd for $C_{62}H_{38}BF_3N_9O_{13}S_9$: 490.6716 ($[M]^{3-}/3$), found: 490.6710.

2.2. Synthesis of SubPc amphiphile (3)

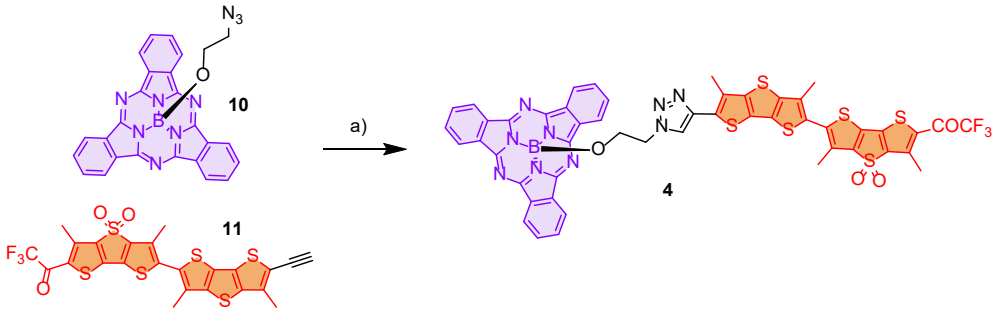


Scheme S2. (a) Sodium 1-decyne, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, CH_2Cl_2/H_2O, rt, 2 h, 79%; (b) but-3-yne-1-sulfonate, $PdCl_2(PPh_3)_2$, CuI, Et_3N, THF, rt, 36 h, 27%.

Compound 8. To a mixture of **6** (10.0 mg, 12 µmol) and $CuSO_4 \cdot 5H_2O$ (2.9 mg, 12 µmol) in 2 mL of CH_2Cl_2, 100 µL of water, 1-decyne (4.5 µL, 23 µmol) and sodium ascorbate (2.3 mg, 12 µmol) were subsequently added. After 5 min, $CuSO_4 \cdot 5H_2O$ (1.5 mg, 6 µmol) was diluted in 100 µL of water and sodium ascorbate (1.2 mg, 6 µmol) was added to this solution, which was immediately poured into the reaction mixture. After 12 h, the mixture was diluted with CH_2Cl_2 (5 mL) and washed with water (2 × 5 mL). The organic solvents were dried with Na_2SO_4, evaporated *in vacuo* and the crude mixture was purified by column chromatography (SiO_2, CH_2Cl_2/Heptane 1:1, $R_f = 0.25$) to give **8** (9.2 mg, 79%) as a purple solid. IR (neat): 2956 (s), 2924 (s), 2854 (s), 2353 (w), 1728 (m), 1603 (m), 1462 (m), 1443 (m), 1380 (w), 1291 (m), 1220 (s), 1177 (m), 1040 (m), 819 (m), 759 (m), 704 (m); ^{1}H NMR (300 MHz, $CDCl_3$): 9.17 (s, 3H), 8.52 (d, $^3J_{H\text{-}H}$ = 8.2 Hz, 3H), 8.20 (d, $^3J_{H\text{-}H}$ = 8.2 Hz, 3H), 6.42 (s, 1H), 3.44 (t, $^3J_{H\text{-}H}$ = 4.9 Hz, 2H), 2.55 (t, $^3J_{H\text{-}H}$ = 7.8 Hz, 2H), 1.79 (t, $^3J_{H\text{-}H}$ = 5.2 Hz, 2H), 1.64 – 1.57 (m, 2H), 1.36 – 1.33 (m, 10H), 0.91 (d, $^3J_{H\text{-}H}$ = 7.0 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): 151.6 (C), 150.5 (C), 148.0 (C), 139.0 (CH), 132.3 (C), 131.5 (CH), 129.8 (C), 123.6 (CH_2), 121.0 (CH_2), 96.4 (C), 58.5 (CH_2), 50.7 (CH_2), 32.1 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 25.8 (CH_2), 22.9 (CH_2), 14.3 (CH_3); HRMS (MALDI, DCTB$^+$) calcd. for $C_{36}H_{31}BI_3N_9O$: 996.9879 ([M]$^+$), found: 996.9864.

Compound 3. In a 10 mL Schlenk, **8** (7.0 mg, 7.0 µmol), sodium but-3-yne-1-sulfonate (4.0 mg, 25 µmol), $PdCl_2(PPh_3)_2$ (1.5 mg, 2.1 µmol) and CuI (0.3 mg, 1.4 µmol) were dissolved in dry THF (1 mL) under Ar atmosphere, Et_3N (0.2 mL) was added, and the reaction stirred at rt. After 36 h, the mixture was concentrated *in vacuo*. The crude product was purified by column in Sephadex™ LH-20 (GE Healthcare) (MeOH, 30 cm, 2 cm diameter) to give three purple fractions from which the product is the last one eluted **3** (2.5 mg, 27%) as a purple solid. IR (neat): 3436 (br), 2924 (s), 2853 (m), 2359 (w), 1617 (w), 1480 (w), 1454 (w), 1378 (m), 1351 (s), 1301 (m), 1179 (m), 1143 (s), 1073 (m), 858 (w); ^{1}H NMR (500 MHz, CD_3OD): 8.84 (s, 3H), 8.78 – 8.74 (m, 3H), 7.97 (d, $^3J_{H\text{-}H}$ = 8.2 Hz, 3H), 6.84 (s, 1H), 3.54 – 3.48 (m, 2H), 3.16 (t, $^3J_{H\text{-}H}$ = 7.9 Hz, 6H), 3.01 (t, $^3J_{H\text{-}H}$ = 7.9 Hz, 6H), 2.51 – 2.46 (m, 2H), 1.86 – 1.83 (m, 2H), 1.56 (s, broad, 2H), 1.41 – 1.39 (s, 10H), 0.97 (t, $^3J_{H\text{-}H}$ = 6.9 Hz, 3H). HRMS (ESI-) calcd. for $C_{48}H_{43}BN_9Na_2O_{10}S_9$: 1058.2197 ($[M]^-$) and $C_{48}H_{43}BN_9NaO_{10}S_9$: 517.6152 ($[M]^{2-}/2$), found: 1058.2203 and 517.6149.

2.3. Synthesis of SubPc-Flipper (4)



Scheme S3 (a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, CH_2Cl_2/H_2O, rt, 12 h, 29%.

Compound 10 was prepared following the reported procedure.[S3]

Compound 4. To a mixture of **10** (3.6 mg, 7.5 µmol), **11** (5.0 mg, 7.5 µmol) and $CuSO_4 \cdot 5H_2O$ (1.9 mg, 7.5 µmol) in 1.5 mL of CH_2Cl_2, 100 µL of water, sodium ascorbate (1.5 mg, 7.5 µmol) was subsequently added. After 5 min, $CuSO_4 \cdot 5H_2O$ (1.0 mg, 4 µmol) was diluted in 100 µL of water and

sodium ascorbate (0.8 mg, 4 μmol) was added to this solution, which was immediately poured into the reaction mixture. After 2 h, the mixture was diluted with CH_2Cl_2 (5 mL) and washed with water (2 × 5 mL). The organic solvents were dried with $MgSO_4$, evaporated *in vacuo* and the crude mixture was purified by column chromatography (SiO_2, CH_2Cl_2, R_f = 0.35) to give **4** (3.1 mg, 53%) as a bright red solid. IR (neat): 2955 (s), 2923 (s), 2852 (s), 2358 (m), 2341 (m), 1728 (m), 1682 (m), 1493 (w), 1408), 1378 (m), 1320 (m), 1284 (m), 1212 (m), 1193 (m), 1145 (s), 1094 (m), 1025 (w); ^{1}H NMR (500 MHz, $CDCl_3$): 8.81 (dd, $^3J_{H-H}$ = 5.9, 3.0 Hz, 6H), 7.88 (dd, $^3J_{H-H}$ = 5.9, 3.0 Hz, 6H), 7.69 (s, 1H), 3.60 (t, $^3J_{H-H}$ = 4.9 Hz, 2H), 2.85 (s, 3H), 2.52 (s, 3H), 2.47 (s, 3H), 2.42 – 2.40 (m, 3H), 1.92 (t, $^3J_{H-H}$ = 4.9 Hz, 2H); ^{13}C NMR (126 MHz, $CDCl_3$): 146.3 (C), 146.1 (C), 144.2 (C), 143.8 (C), 142.6 (C), 142.4 (C), 139.8 (C), 132.7 (C), 132.0 (C), 131.8 (C), 129.90 (C), 129.5 (C), 127.4 (C), 125.7 (C), 122.1 (CH), 92.7 (C). 58.4 (CH_2), 51.3 (CH_2), 14.9 (CH_3), 14.4 (CH_3), 14.2 (CH_3), 12.5 (CH_3); ^{19}F NMR (471 MHz, $CDCl_3$): -73.1; HRMS (MALDI, DCTB$^+$) calcd. for $C_{50}H_{29}BF_3N_9O_4S_6$: 1079.0715 ([M]$^+$), found: 1079.0693.

3. Solvatochromism

The absorption and fluorescence emission spectra (λ_{ex} = 470 nm or 520 nm) of **1**, **3** and **4** were recorded at 25 ºC in solvents of different polarity (*c* (**1, 3**) = 3 μM; *c* (**4**) = 2.5 μM). The absorbance of the solutions was < 0.1.

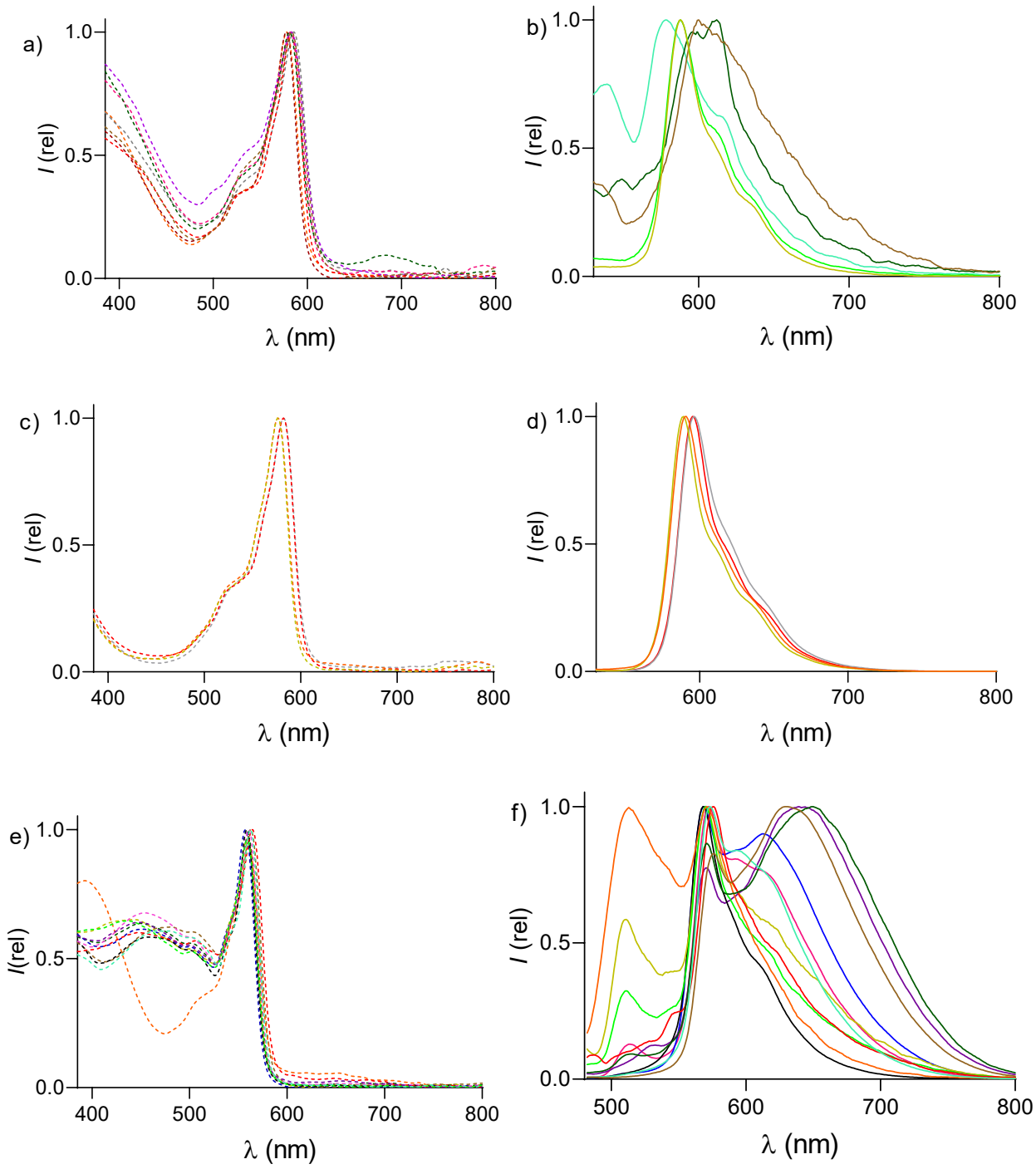


Fig. S2 Normalized absorption (dashed; a, c and e) and emission spectra (solid; b, d and f) of **1** (a and b; λ_{ex} = 520 nm), **3** (c and d; λ_{ex} = 520 nm), **4** (e and f; λ_{ex} = 470 nm). In water (grey), DMSO (red), MeCN (dark yellow), MeOH (orange), $CHCl_3$ (brown), acetone (light green), THF (dark green), EtOAc (purple), dioxane (pink), toluene (light blue), Et_2O (dark blue) and methylcyclohexane (black).

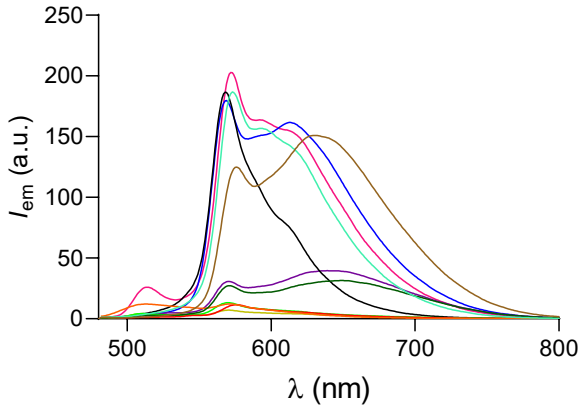


Fig. S3 Emission spectra of **4** λ_{ex} = 470 nm in DMSO (red), MeCN (dark yellow), MeOH (orange), $CHCl_3$ (brown), acetone (light green), THF (dark green), EtOAc (purple), dioxane (pink), toluene (light blue), Et_2O (dark blue) and methylcyclohexane (black).

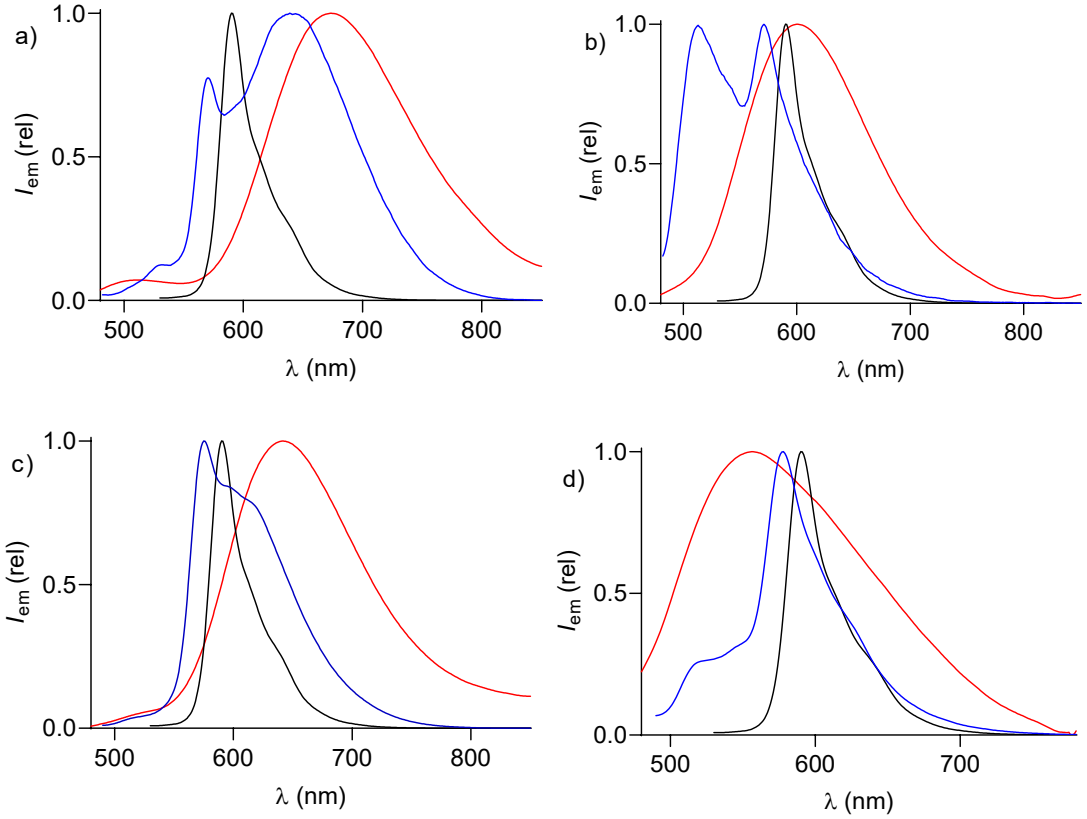


Fig. S4 Normalized emission spectra (λ_{ex} = 425 nm) from an and **1** (blue) and aliphatic flipper **2'** (red, data from reference S1), in (a) EtOAc "ketone" (b) DMSO "hydrate" (c) dioxane "ketone" and (d) dioxane:water "hydrate" (20:1). Compound **3** (black) was represented dissolved in CH_3OH for comparison.

4. Fluorescence quantum yields and molar extinction coefficients

Fluorescence quantum yields were calculated for solutions with absorbance < 0.1. They were evaluated based on a standard, whether Rhodamine 6G (EtOH, Φ_R = 95%, λ_{ex} = 480 nm) or Rhodamine B (EtOH, Φ_R = 70%, λ_{ex} = 510 nm), using equation (S1):

$$\Phi = \Phi_R \frac{Int}{Int_R}\left(\frac{OD_R}{OD}\right)\frac{n^2}{n_R^2} \quad \text{(S1)}$$

$$OD = 1 - 10^{-A} \quad \text{(S2)}$$

Φ_R is the quantum yield of the standards, *Int* is the area of the emission intensity of the sample, Int_R is the area of the emission intensity of the standard, *A* is the absorbance, A_R is the absorbance of the standard, *OD* is the optical density of the sample, OD_R is the optical density of the standard, *n* is the refractive index of the sample and n_R is the refractive index of the standard.

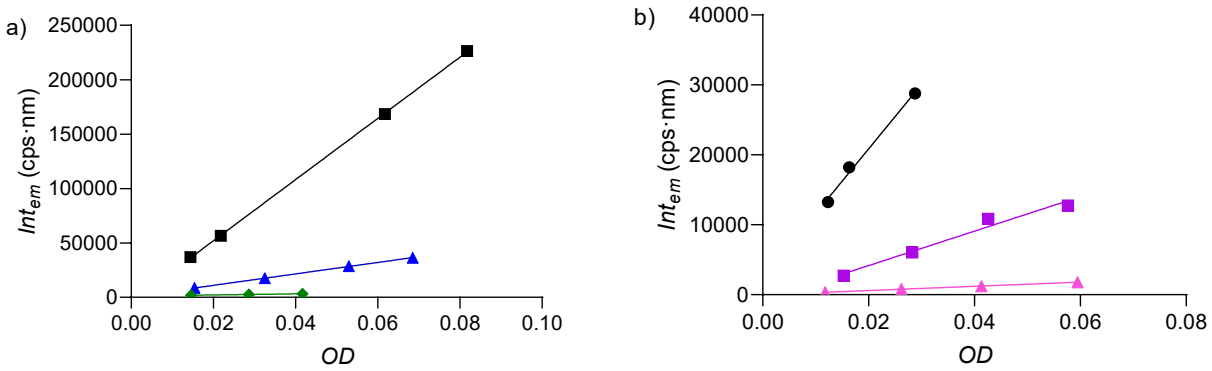


Fig. S5 *OD* against integral of the emission. (a) Rhodamine B (black, Φ_F (EtOH) = 70%), **1** (green, Φ_F (CH_3OH) = 2%) and **3** (blue, Φ_F (CH_3OH) = 14%); (b) Rhodamine 6G (black, Φ_F (EtOH) = 95%,) and **4** (purple, Φ_F ($CHCl_3$) = 17%; pink Φ_F (CH_3OH) = 2%).

Molar extinction coefficients (ε). They were calculated and expressed as the average of the different absorbance / concentration; the error was calculated from a t-student test with a confidence interval of 95 %.

5. Dynamic-covalent ketone chemistry

Kinetics of hydrate formation. A 2 µM solution of **4** was prepared in dioxane and water (1.5%) was added. The effect over fluorescence for the formation of the diol was studied against time; 25 ºC were maintained for all the experiment.

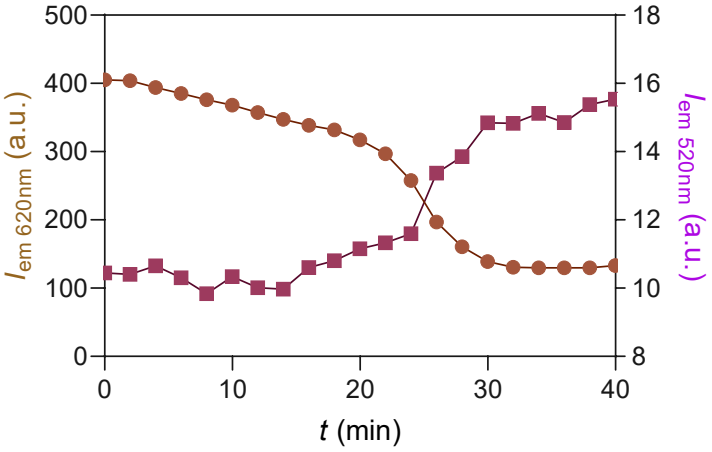


Fig. S6 Emission against time of **4**, brown (λ= 620 nm) to purple (λ= 520 nm), after the addition of 1.5 % water.

6. Fluorescence measurements in LUVs

6.1. Preparation of LUVs

DOPC LUVs. As described in S4. A thin lipid film was prepared by evaporating a solution of DOPC (23 mg, 31 µmol) in $CHCl_3$ (1 mL), followed by drying under vacuum overnight. The resulting film was hydrated with a buffer solution (1.0 mL, 10 mM Tris-HCl, 100 mM NaCl, pH 7.4) for 30 min at rt, subjected to freeze-thaw cycles (5×, liquid N_2, 40 °C water bath) and extruded (15×) through a polycarbonate membrane (pore size, 100 nm) using Mini-Extruder.

SM/CL LUVs were prepared similarly using SM (15 mg, 21 µmol) and CL (4 mg, 9 µmol). Hydration and extrusion were performed at 65 ºC.

6.2. Fluorescence spectra measurements in LUVs

General procedure. A solution of the probe (0.5 – 4 μL of 0.12 mM **1**, 0.10 mM **2**, or 0.15 mM **3**, in DMSO) was added to a stirred dispersion of LUVs (75 μM lipids) in buffer (2.0 mL, 10 mM Tris, 100 mM NaCl, pH 7.4) at 25 °C in a disposable PMMA cuvette. Emission spectra were recorded upon excitation at 480 nm with excitation/emission slit widths of 5/5 nm and a long-pass filter (cut on wavelength 520 nm) in the emission path, or upon excitation at 560 nm with excitation/emission slit widths of 5/5 nm and a long-pass filter (cut on wavelength 580 nm) in the emission path. Excitation spectra were recorded for emission at 650 nm with excitation/emission slit widths of 2/20 nm, and a long-pass filter (cut on wavelength 540 nm) in the emission path. Spectra obtained under the same conditions without the probe but with the same quantity of DMSO were used as backgrounds and subtracted from the spectra of probes.

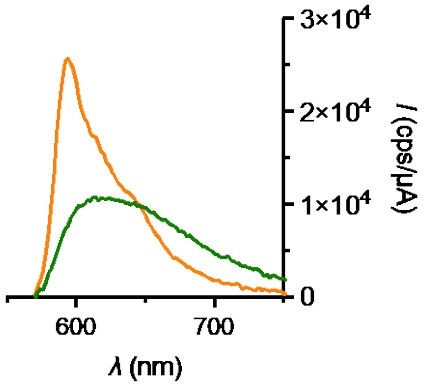


Fig. S7 Emission spectra (λ_{ex} = 560 nm) of **1** (200 nM) in SM/CL (7:3, green), or DOPC LUVs (orange) at 25 °C.

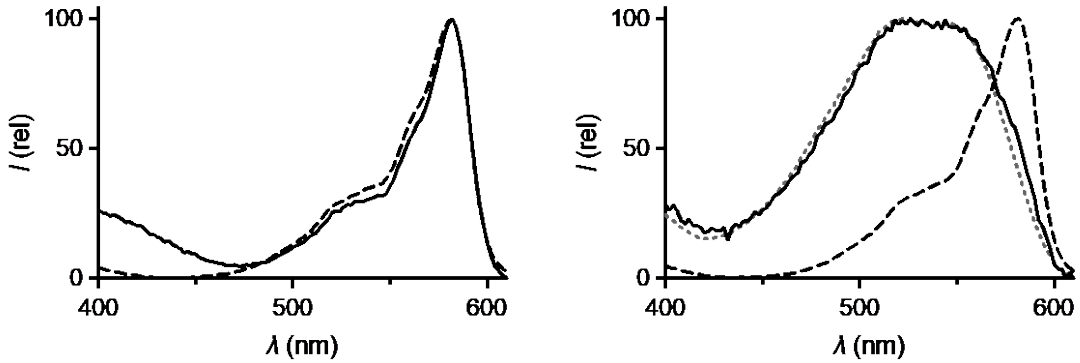


Fig. S8 Normalized excitation spectra (λ_{em} = 650 nm) of **1** (solid), **2** (dotted), and **3** (dashed), in DOPC (left) or SM/CL LUVs (right). The excitation spectrum of **2** in DOPC LUVs is featureless, thus not shown.

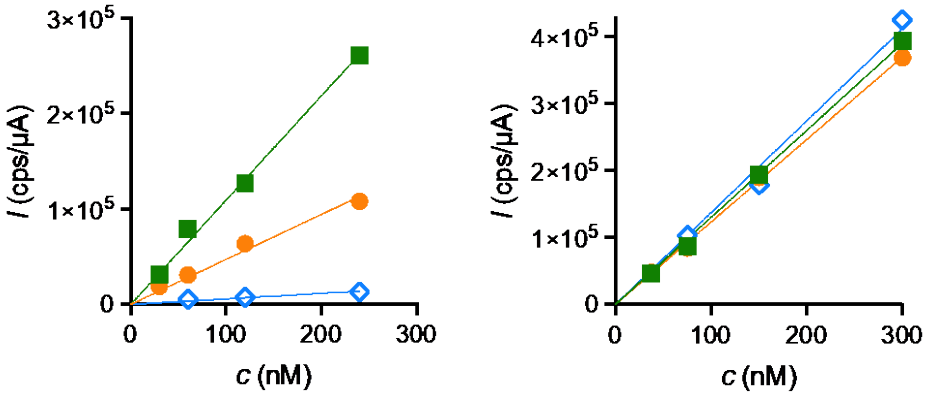


Fig. S9 Fluorescence intensities of **1** (left) and **3** (right) at λ_{ex} = 530 nm, λ_{em} = 650 nm in SM/CL (green squares), or DOPC LUVs (orange circles) or in buffer (blue diamonds) at 25 °C.

7. Fluorescence lifetime imaging microscopy (FLIM)

7.1. Preparation of GUVs

GUVs were prepared by electroformation method using a Nanion Vesicle Prep Pro following the reported procedures.[S5]

DOPC GUVs. A thin lipid film was prepared by evaporating a solution of DOPC (10 mM) in $CHCl_3$ (20 µL) on the conductive side of an ITO electrode and further drying under vacuum for 1.5 h. An o-ring covered in silicon grease was placed around the film, and 250 µL of an aqueous sucrose solution (250 mM) was added to the film. The second ITO electrode was placed on top of the first one, conductive side toward the joint and the sucrose solution. The electrodes were placed in Vesicle Prep Pro and an electric field of 1.2 V at 10 Hz was applied for 2 h at rt. This resulting stock solution of GUVs was used without further purification.

SM/CL GUVs. A thin lipid film was prepared by evaporating a preheated (55 °C) solution of

SM/CL (7:3, 10 mM) in $CHCl_3$ with a drop of MeOH (20 µL) on the conductive side of a preheated ITO electrode (55 °C) and further drying under vacuum at 55 °C for 1.5 h. The obtained electrode was assembled in Vesicle Prep Pro as above, and an electric field of 1.2 V at 10 Hz was applied for 2 h at 55 °C. This resulting stock solution of GUVs was used without further purification.

Phase-separated GUVs were prepared analogously to SM/CL GUVs using a solution of DOPC/SM/CL (3:2:1) in $CHCl_3$ with a drop of MeOH.

7.2. FLIM in GUVs

General procedure. 20 µL of stock solutions of GUVs were added to 200 µL of Tris buffer on a 35 mm glass bottom dish (Mattek Corporation, P35G-1.5-14-C, pre-treated with Casein) followed by the addition of **1**, **2**, or **3** (0.4 µL of 0.5 (**2**) or 1 mM (**1** and **3**) stock solutions in DMSO). Images were recorded immediately at rt. FLIM images were analyzed using Leica LAS X. The presented images are of Fast FLIM. The reported average fluorescence lifetimes (τ_{av}) are the intensity averaged lifetimes, estimated by fitting the fluorescence decay of overall images to bi- or tri-exponential functions. τ_2 and τ_3 where associated to the emission from the fluorescent probes (flipper and/or SubPc). τ_1 comes from the background fluorescence, a low intensity/lifetime component that is insensitive to any change in membrane tension (see for example ref. S5).

Table S1 Fluorescence lifetimes of compounds **1–3** in GUVs.[a]

P[b]	DOPC			SM/CL		
	t_1 (ns)[c]	t_2 (ns)[d]	t_{av} (ns)[e]	t_1 (ns)[c]	t_2 (ns)[d]	t_{av} (ns)[e]
1	0.7 (0.66)	1.8 (0.33)	1.4	0.9 (0.2)	5.1 (0.8)	4.9
2[f]	–	3.4	2.1	–	5.2	4.7
3	0.5 (0.1)	1.9 (0.9)	1.9	–[g]	–[g]	–[g]

[a]Lifetimes (t) are of overall images. Triexponential fits were used. Standard errors of lifetimes are ≤ 0.1 ns. [b]Probes. [c]The shortest fluorescence lifetimes and the corresponding fractional amplitudes in parentheses. [d]The intermediate fluorescence lifetimes and the corresponding fractional amplitudes in parentheses. [e]The longest

fluorescence lifetimes and the corresponding fractional amplitudes in parentheses. [f]Intensity averaged lifetimes.

Table S2. Fluorescence lifetimes of compounds **1–3** in phase-separated GUVs.[a]

P[b]	DOPC/SM/CL			
	t_1 (ns)[c]	t_2 (ns)[d]	t_3 (ns)[e]	t_{av} (ns)[f]
1	0.5 (0.47)	1.3 (0.51)	4.4 (0.02)	1.4
2	0.5 (0.24)	2.2 (0.33)	4.7 (0.43)	4.0
3	0.6 (0.07)	1.8 (0.91)	3.1 (0.02)	1.8

[a]Lifetimes (t) are of overall images. Triexponential fits were used. Standard errors of lifetimes are ≤ 0.1 ns. [b]Probes. [c]The shortest fluorescence lifetimes and the corresponding fractional amplitudes in parentheses. [d]The intermediate fluorescence lifetimes and the corresponding fractional amplitudes in parentheses. [e]The longest fluorescence lifetimes and the corresponding fractional amplitudes in parentheses. [f]Intensity averaged lifetimes.

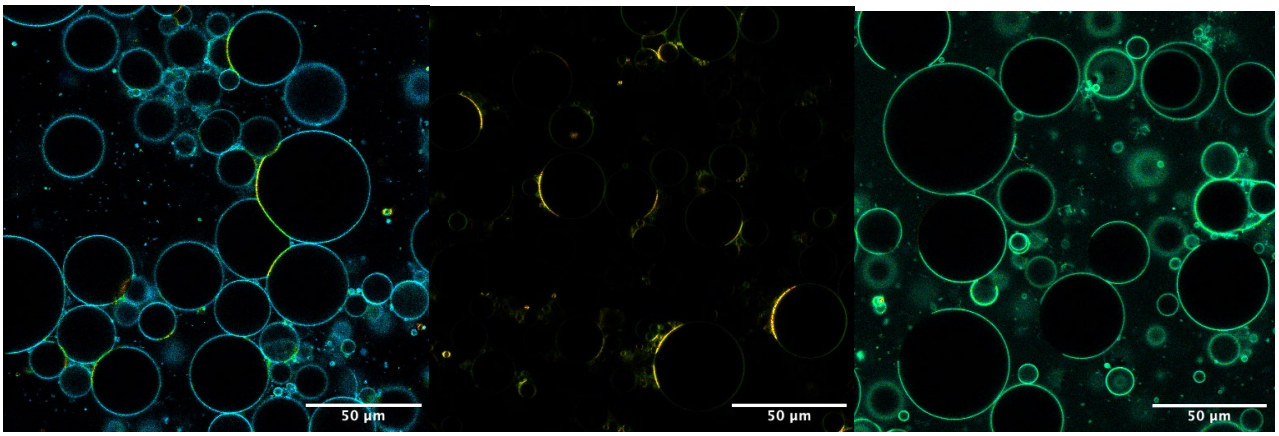


Fig. S10 Representative FLIM images of **1** (left), **2** (middle), and **3** (right) in DOPC/SM/CL (3:2:1) GUVs.

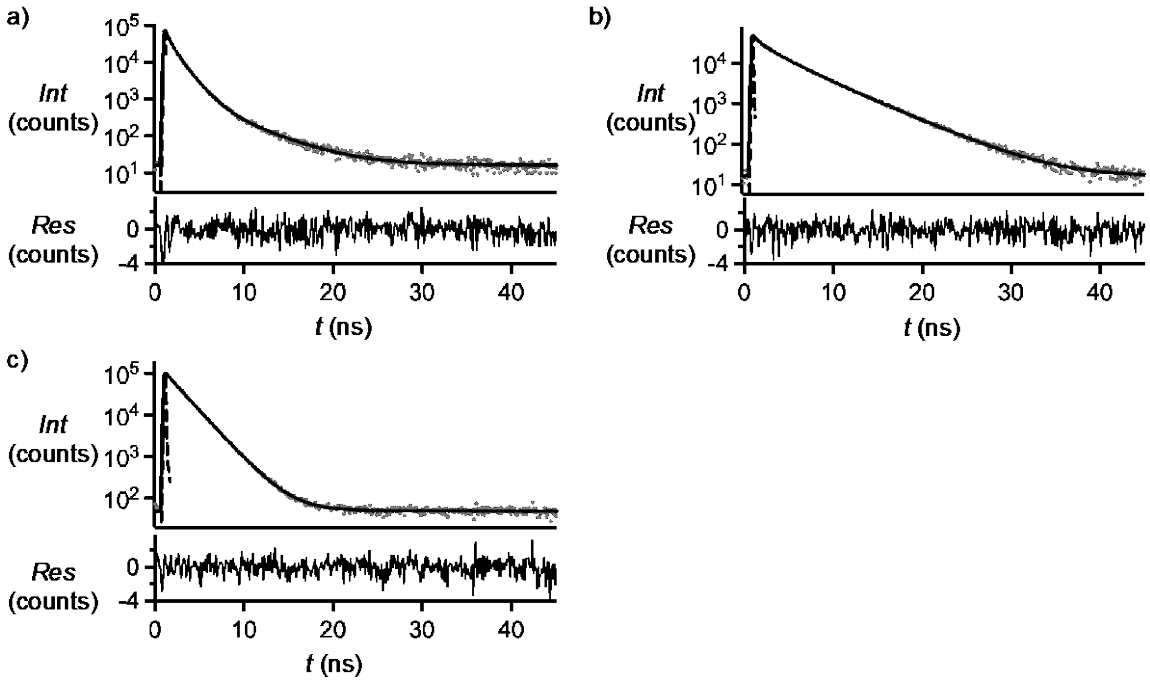


Fig. S11 Representative lifetime traces of a) **1**, b) **2**, and c) **3** in DOPC/SM/CL (3:2:1) GUVs. Above: measured intensity counts (grey points), IRF (dashed lines), and fit curves (black solid lines). Below: residual counts.

7.3. Imaging in cells

Cell culture. HeLa Kyoto cells were cultured in FluoroBrite DMEM (high D-Glucose, without phenol red) medium containing 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (PS) and 1% Glutamine in 25 cm^2 cell culture flasks with a vent cap. Tissue culture flasks were kept under 5% CO_2 in a humidified atmosphere at 37 °C. The cells were detached by adding 1.0 mL of TrypLE Express at 37 °C for 3 min, followed by the addition of 1 mL of DMEM (same as above). For the microscopy experiment the cells were seeded at 8×10^4 cells/mL in 35 mm glass bottom dishes and kept at 37 °C at 5% CO_2 overnight.

FLIM imaging in HeLa Kyoto cells. The cells were washed (3 x 1 mL) with PBS buffer and incubated with DMEM medium containing the corresponding probe (3 μM, 1 mL) for 10 minutes

at 37 °C at 5 % CO_2. The FLIM images were acquired without exchanging the incubation medium using the laser at 530 nm (white light laser) and photon collection between 550 and 650 nm. The hypertonic shock was achieved by adding 1 mL of 1 M sucrose medium containing the probe (3 µM) in the dish containing 1 mL isotonic medium for 5 min.

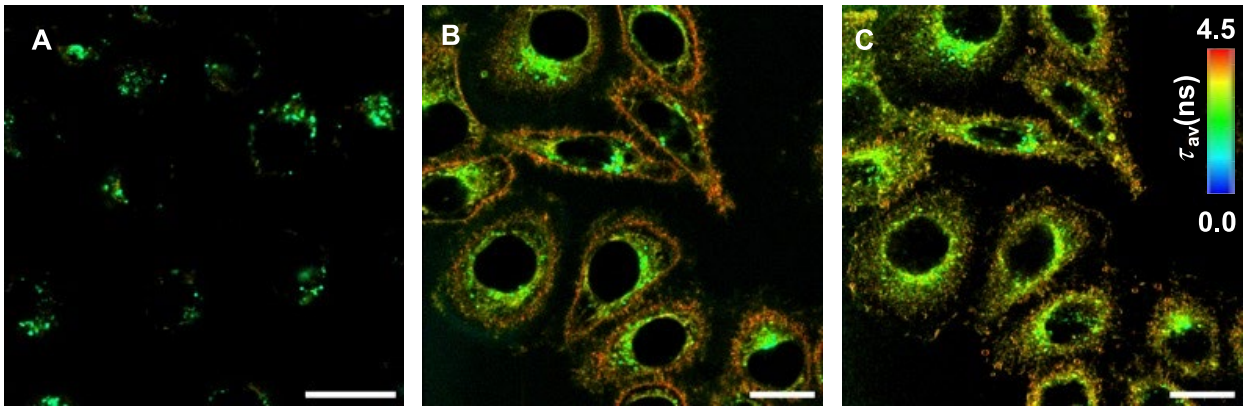


Fig. S12 FLIM images of **3** (3 µM, A) and **1** (3 µM, B and C) in HK cells under isotonic conditions (A, B), and 5 minutes after hypertonic shock (C). Scale bars = 20 µm

8. Characterization, NMR spectra and HRMS

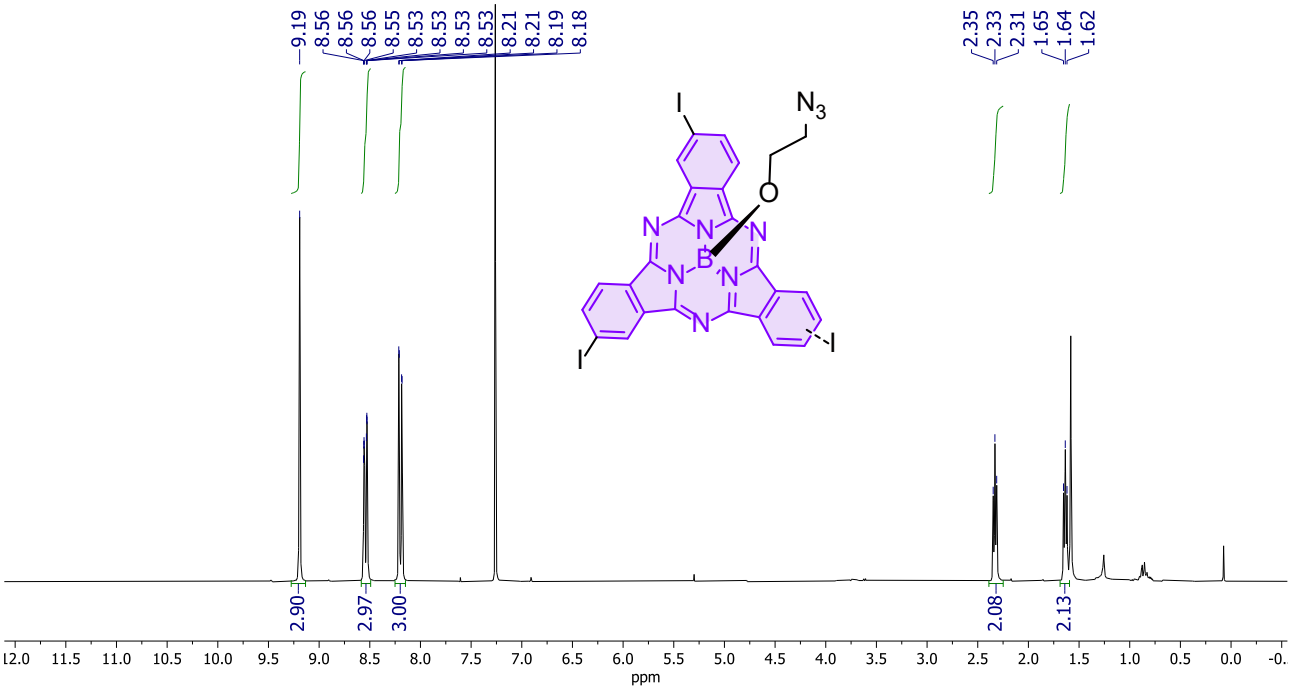


Fig. S13 ^{1}H NMR (300 MHz) spectrum of compound **6** in $CDCl_3$.

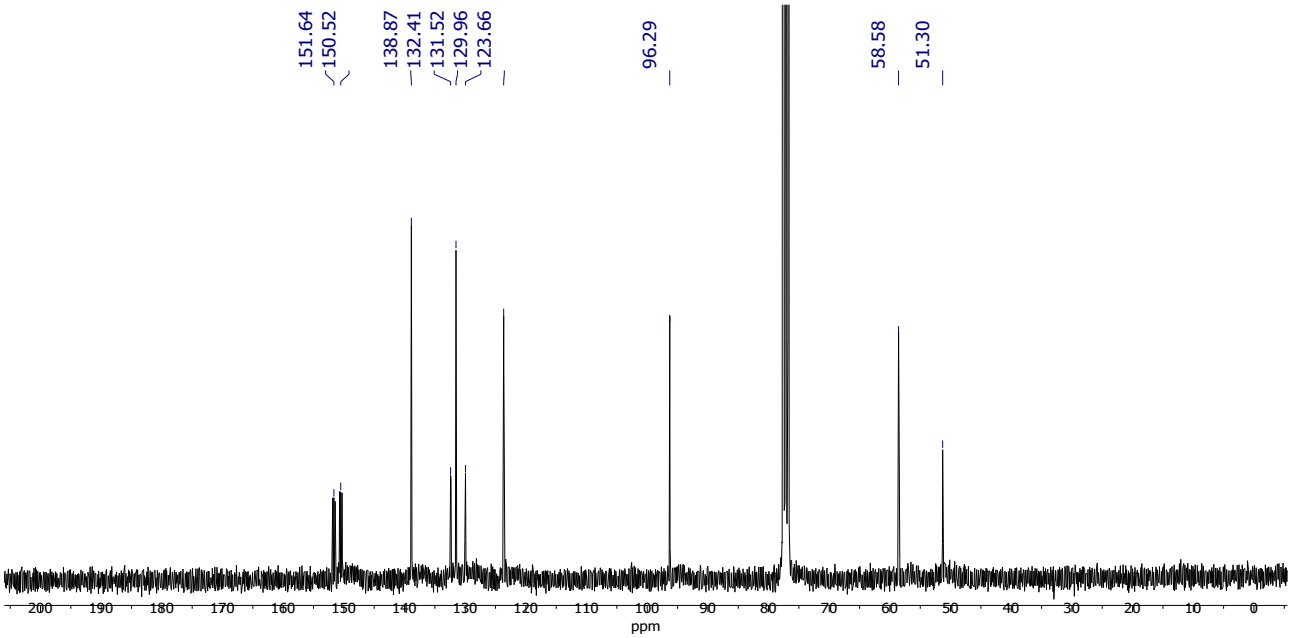


Fig. S14 ^{13}C NMR (75 MHz) spectrum of compound **6** in $CDCl_3$.

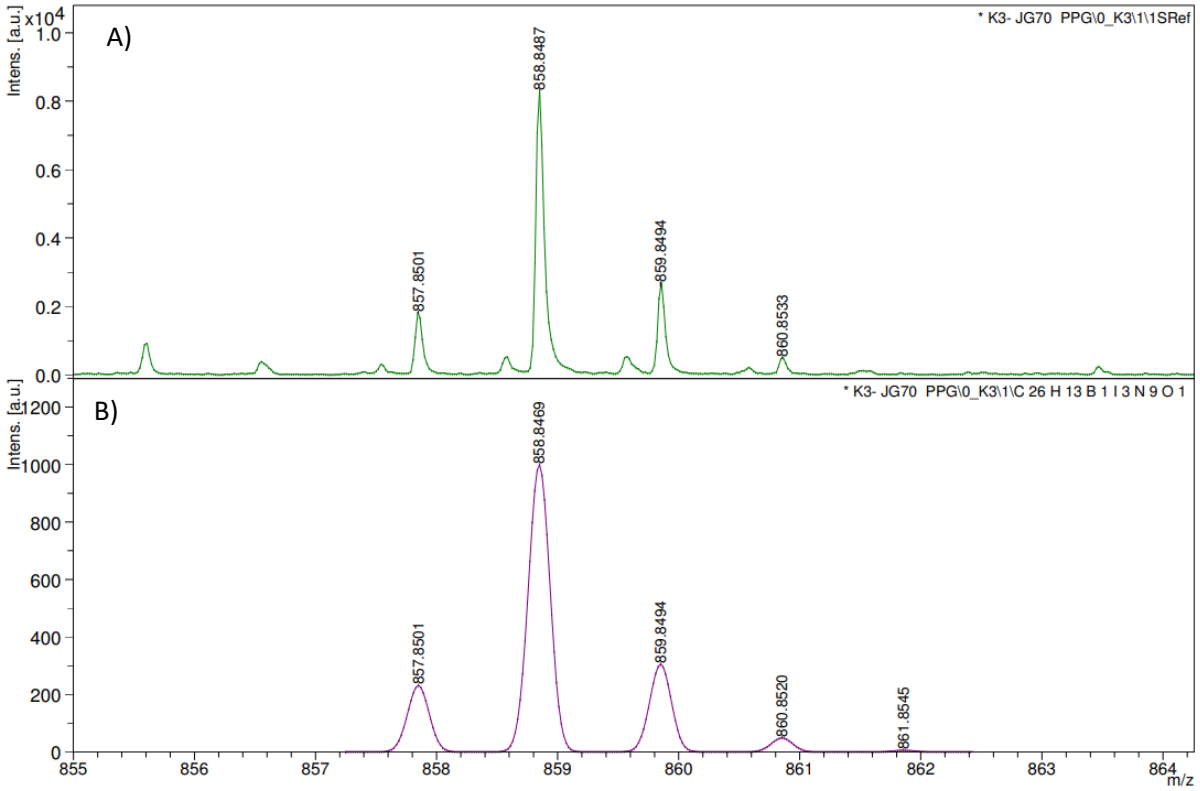


Fig. S15 HRMS (MALDI, $DCTB^+$) profile of compound **6** (A obtained, B theoretical).

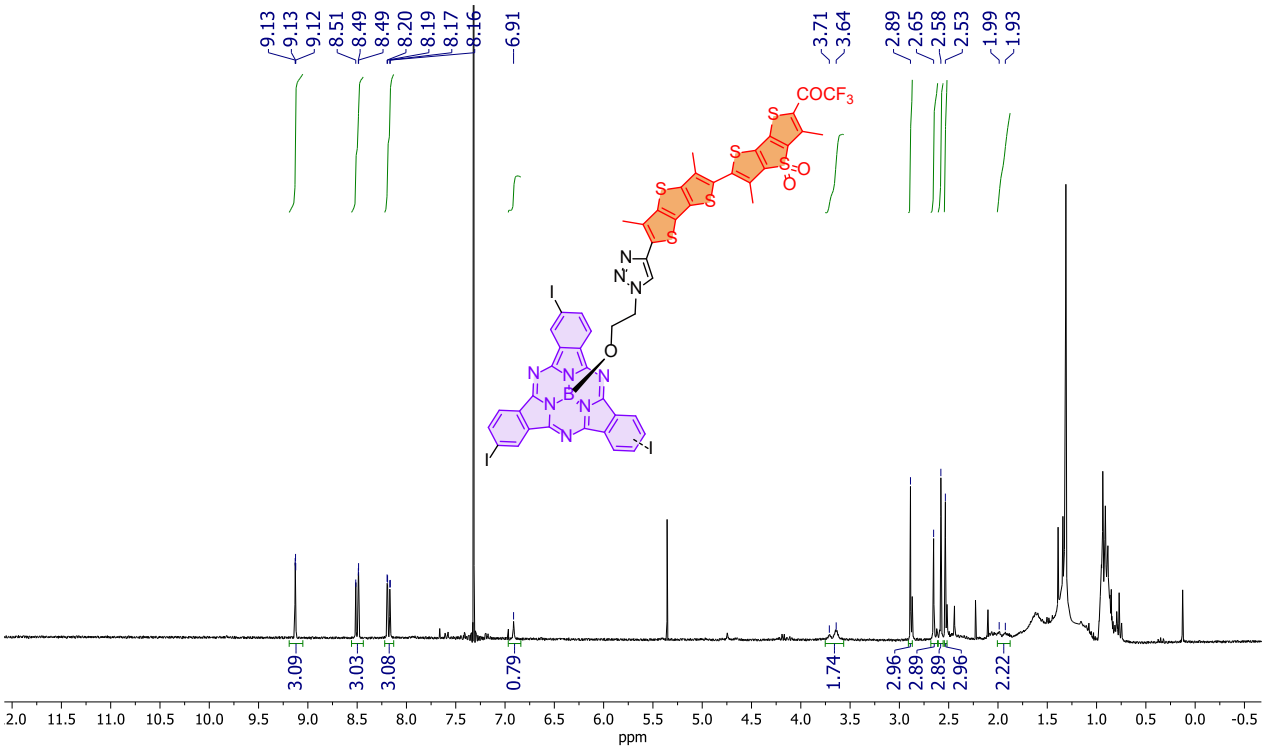


Fig. S16 ^{1}H NMR (300 MHz) spectrum of compound **7** in $CDCl_3$.

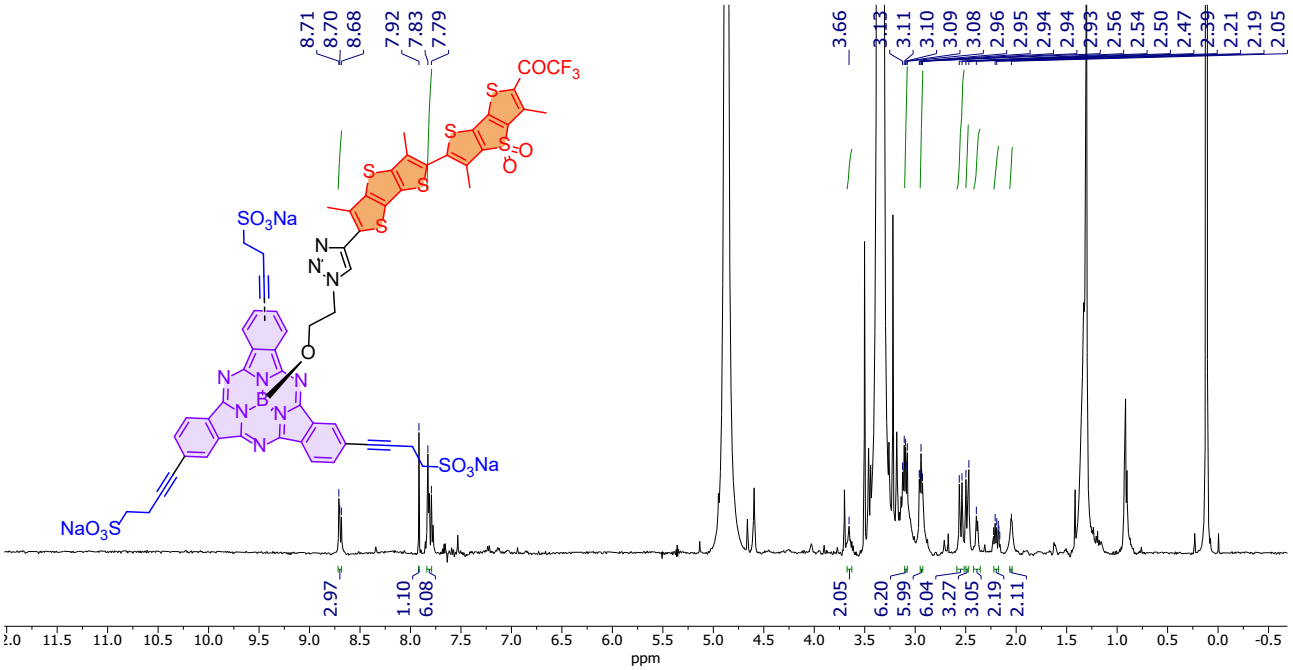


Fig. S17 ^{1}H NMR (500 MHz, hemiacetal) spectrum of compound **1** in CD_3OD.

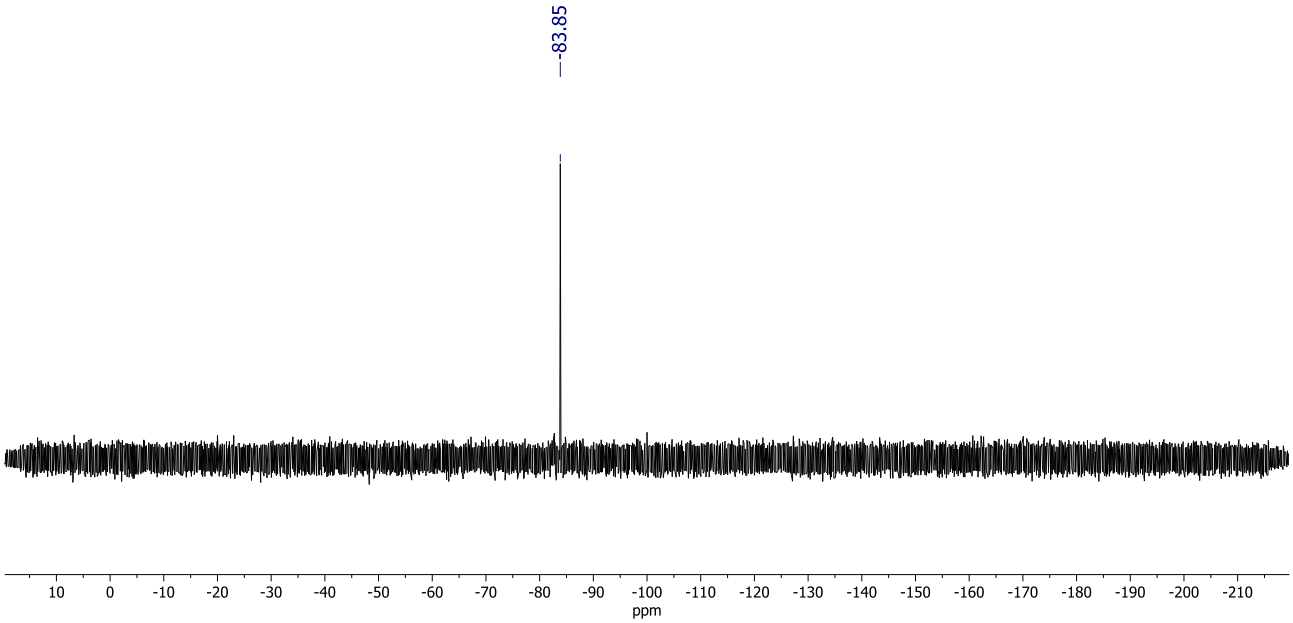


Fig. S18 ^{19}F NMR (471 MHz, hemiacetal) spectrum of compound **1** in CD_3OD.

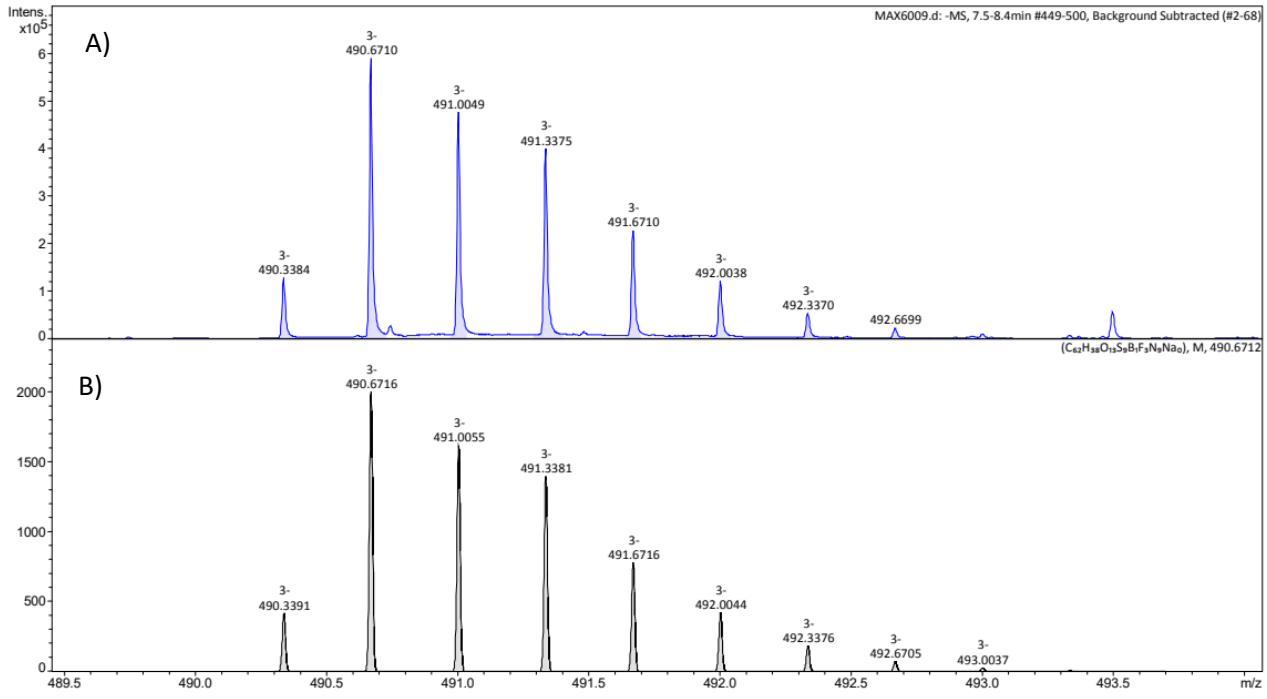


Fig. S19 HRMS (ESI^{3-}) profile of compound **1** (A obtained, B theoretical).

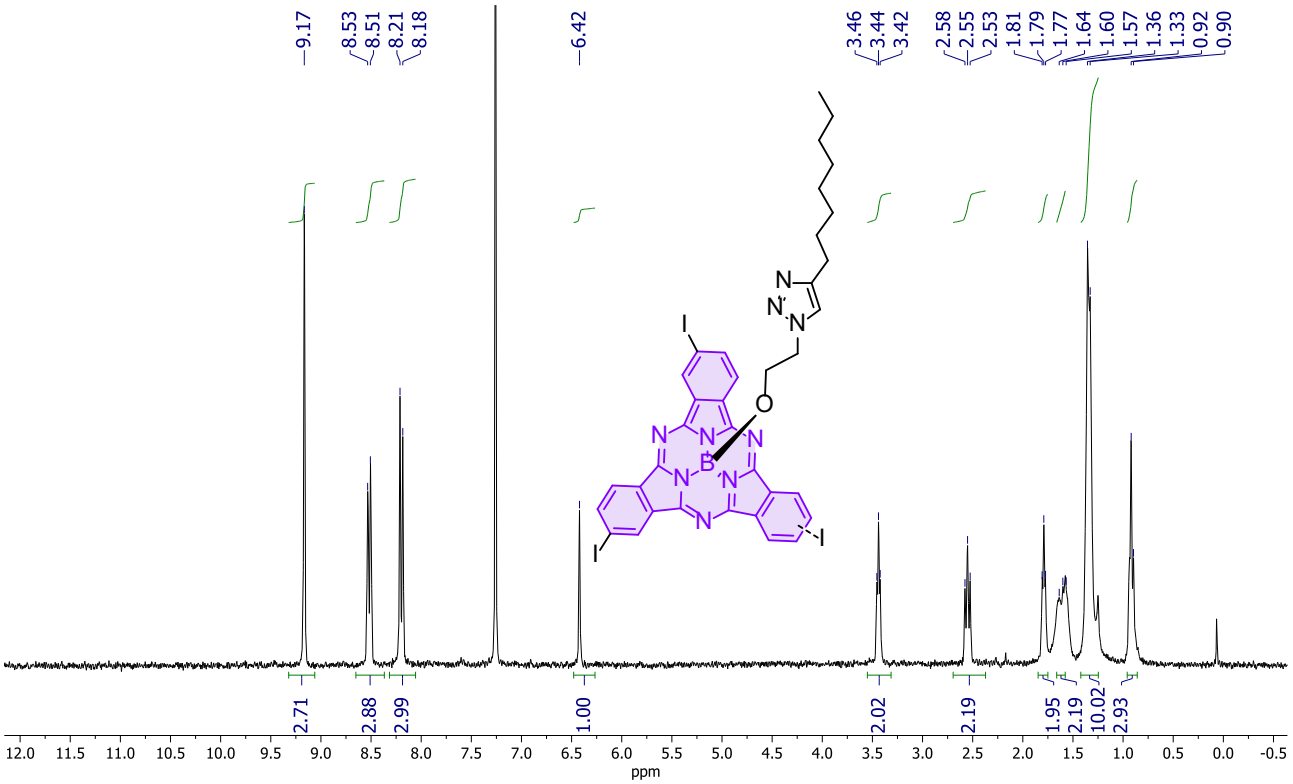


Fig. S20 ^{1}H NMR (300 MHz) spectrum of compound **8** in $CDCl_3$.

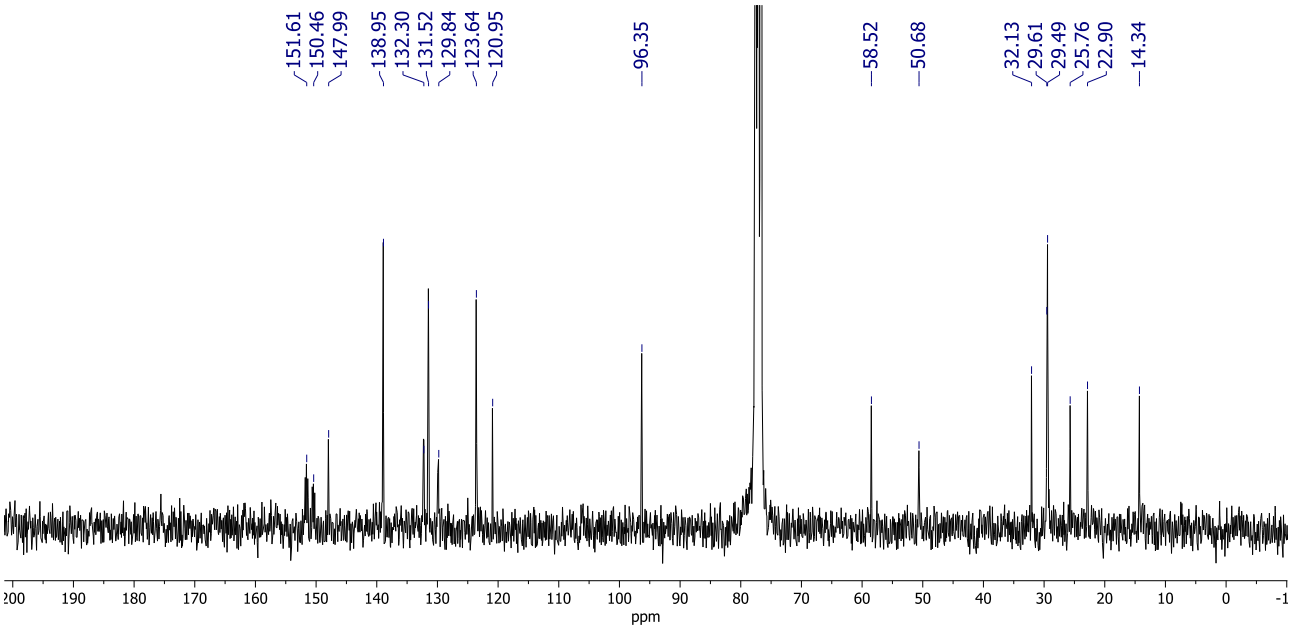


Fig. S21 ^{13}C NMR (75 MHz) spectrum of compound **8** in $CDCl_3$.

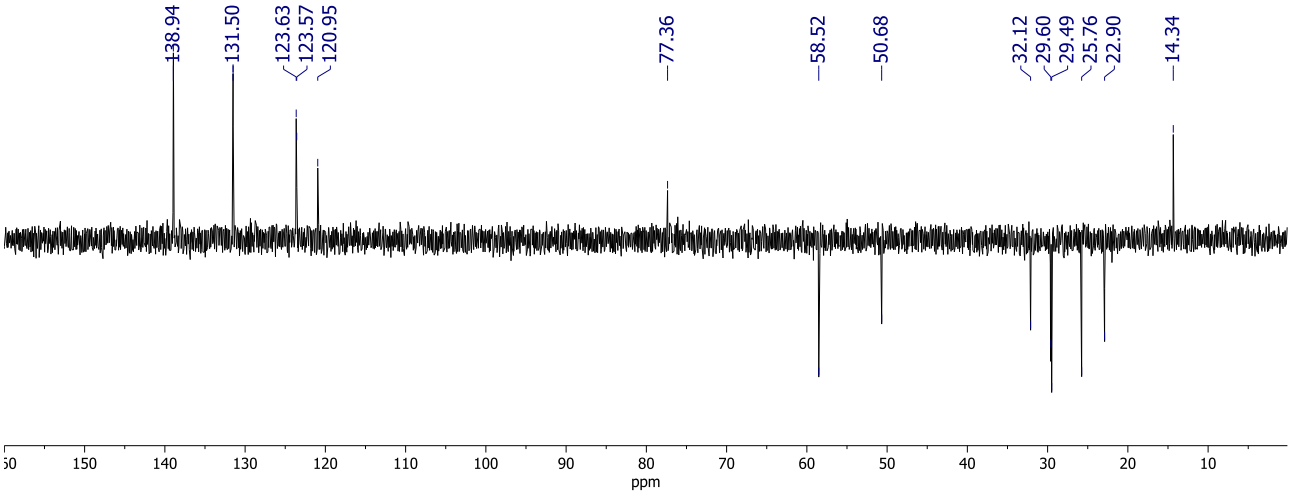


Fig. S22 DEPT (75 MHz) spectrum of compound **8** in $CDCl_3$.

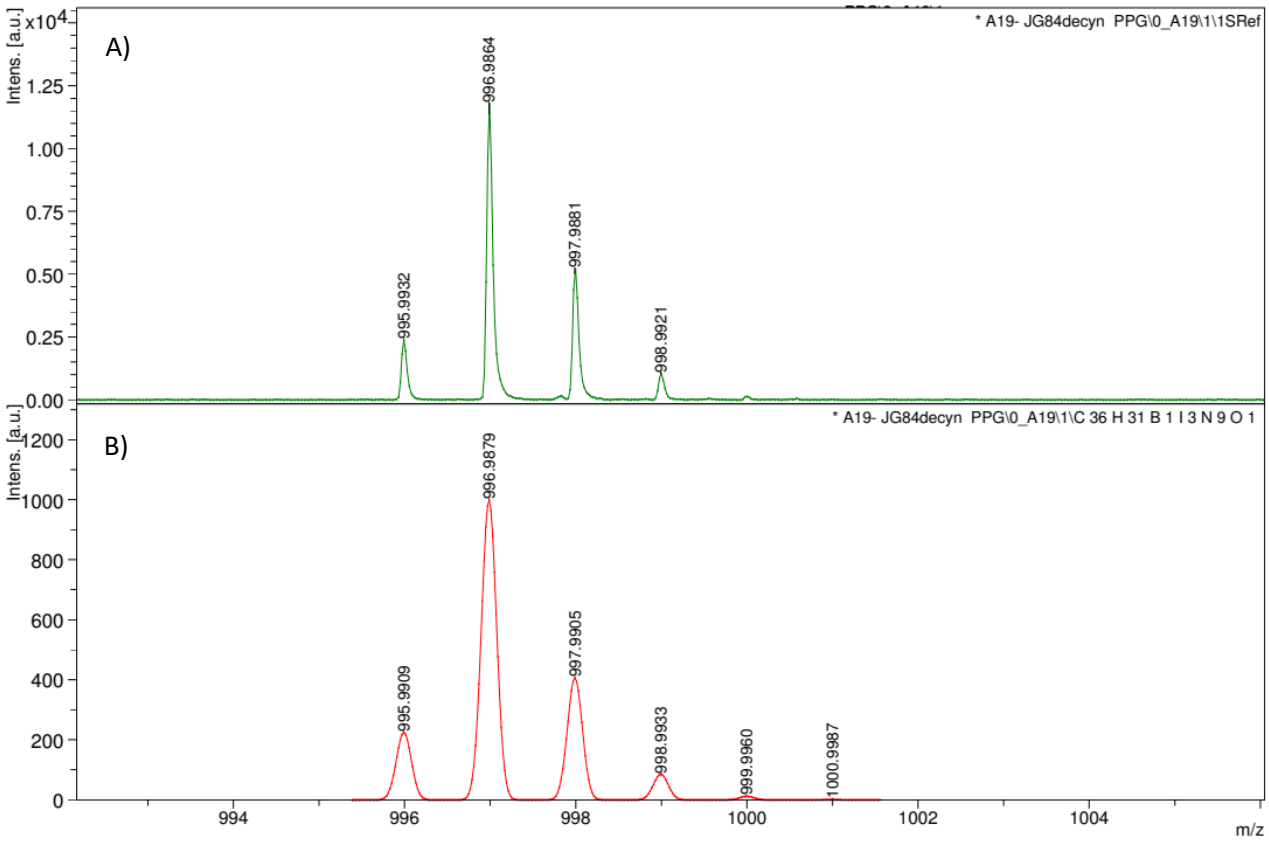


Fig. S23 HRMS (MALDI, $DCTB^+$) profile of compound **8** (A obtained, B theoretical).

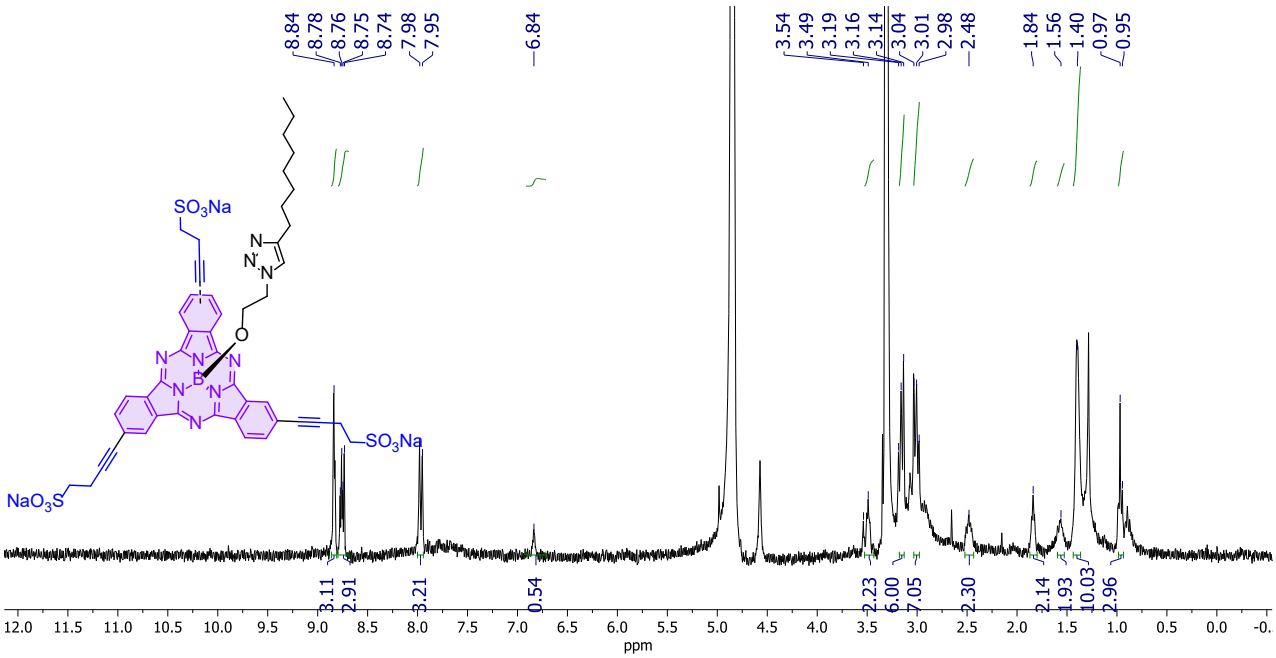


Fig. S24 ^{1}H NMR (500 MHz) spectrum of compound **3** in CD_3OD.

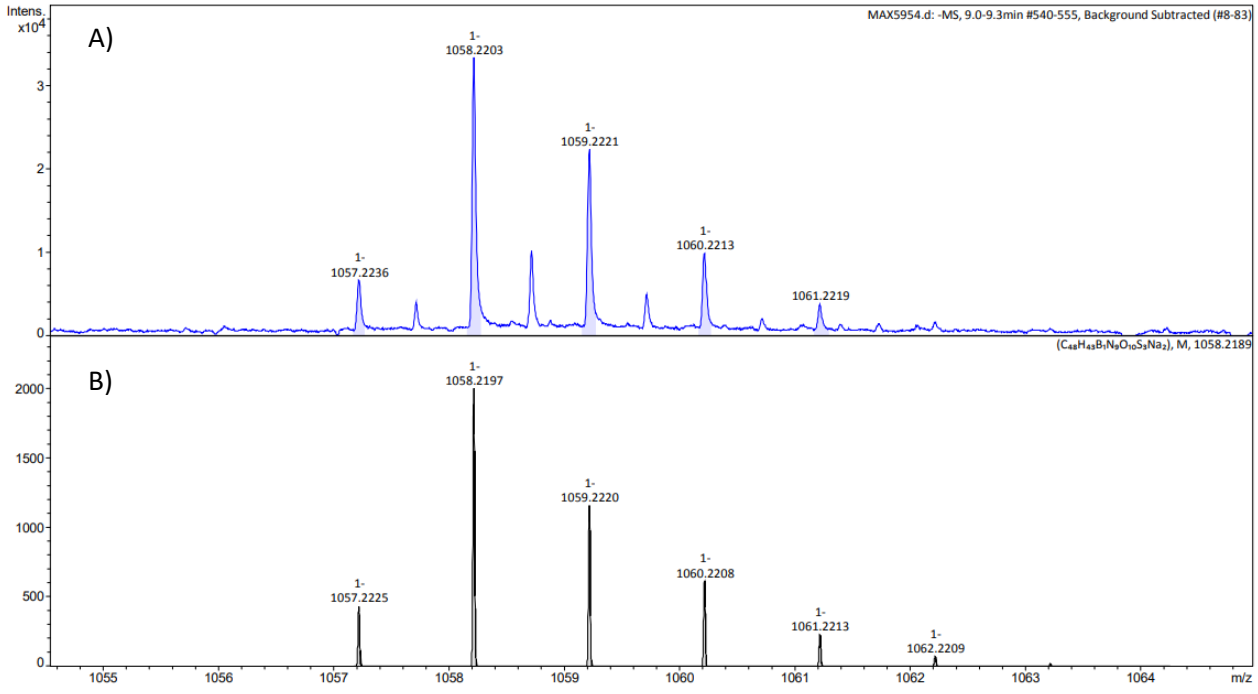


Fig. S25 HRMS (ESI$^-$) profile of compound **3** (A obtained, B theoretical).

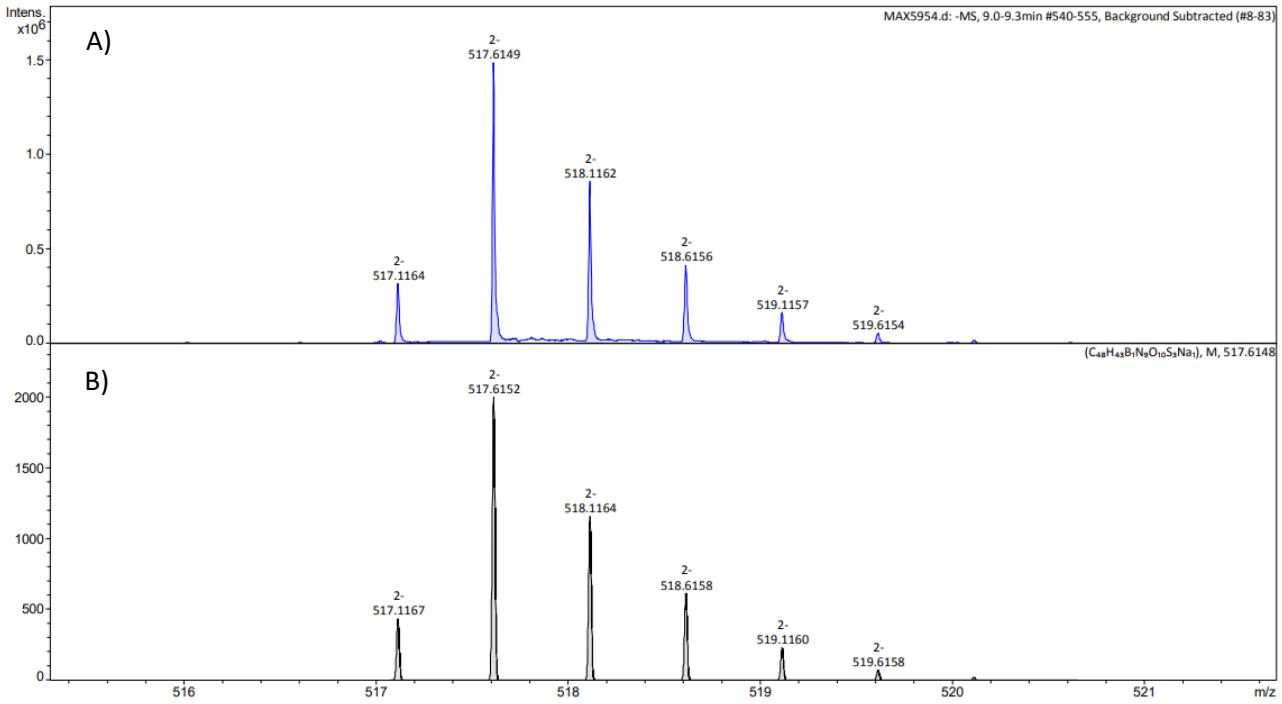


Fig. S26 HRMS (ESI^{2-}) profile of compound **3** (A obtained, B theoretical).

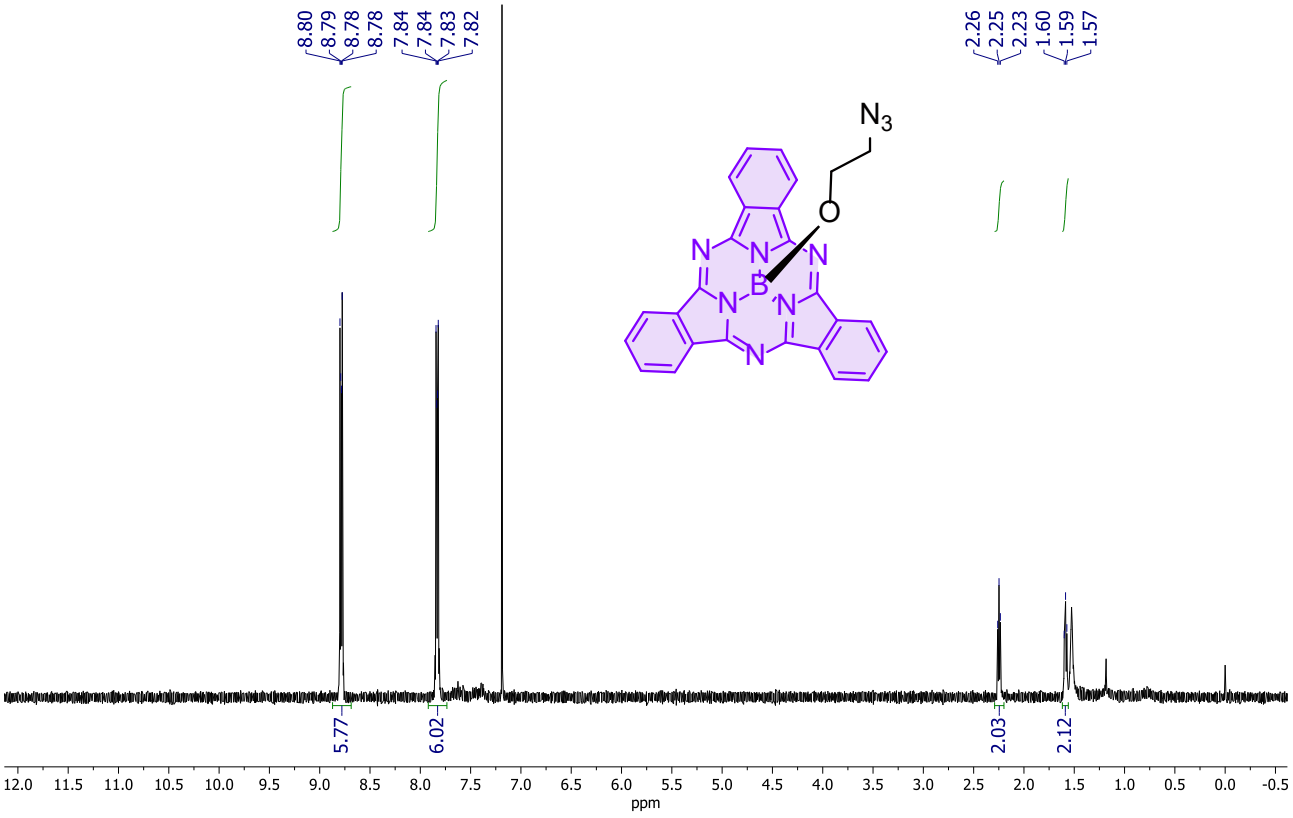


Fig. S27 ^{1}H NMR (300 MHz) spectrum of compound **10** in $CDCl_3$.

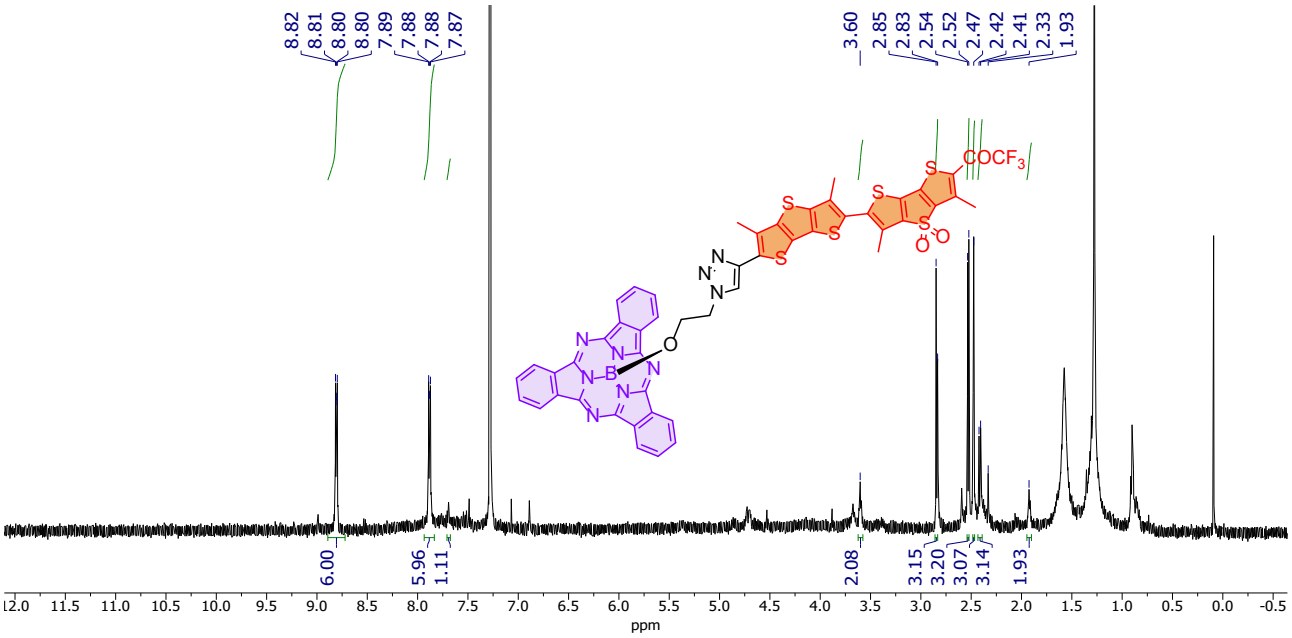


Fig. S28 ^{1}H NMR (500 MHz) spectrum of compound **4** in $CDCl_3$.

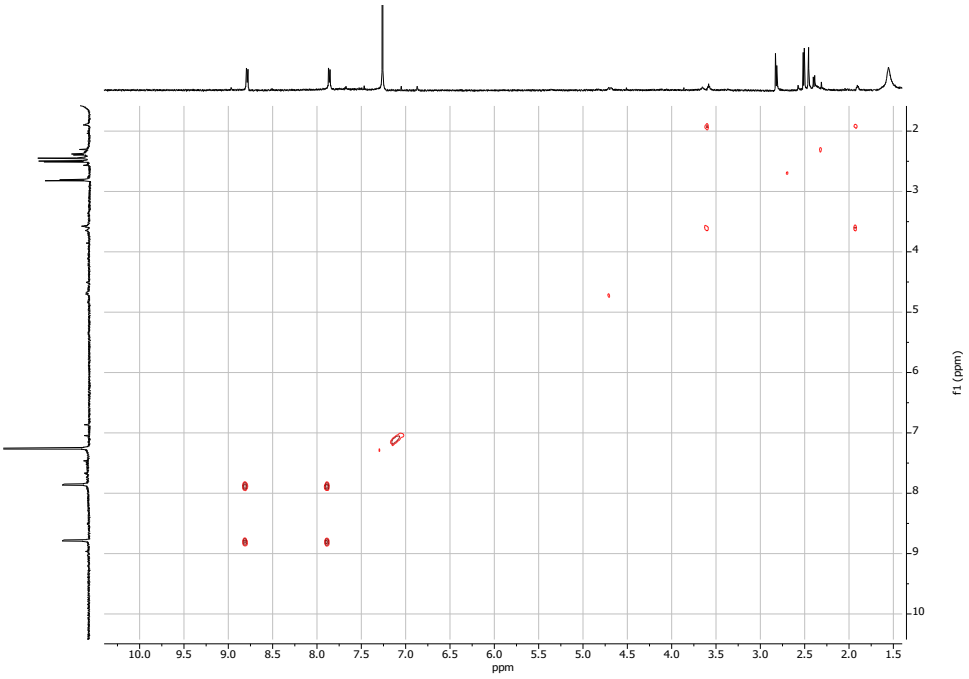


Fig. S29 COSY NMR (500 MHz) spectrum of compound **4** in $CDCl_3$.

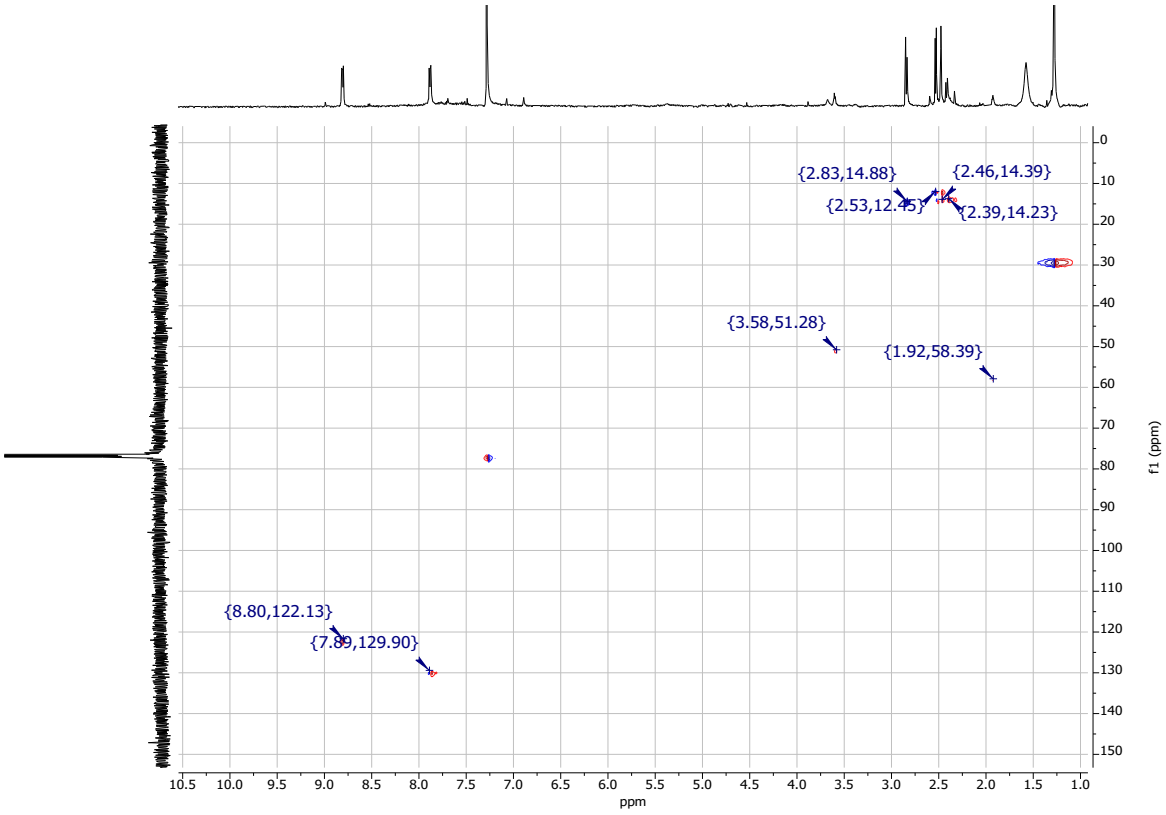


Fig. S30 HMQC (500 MHz) spectrum of compound **4** in $CDCl_3$.

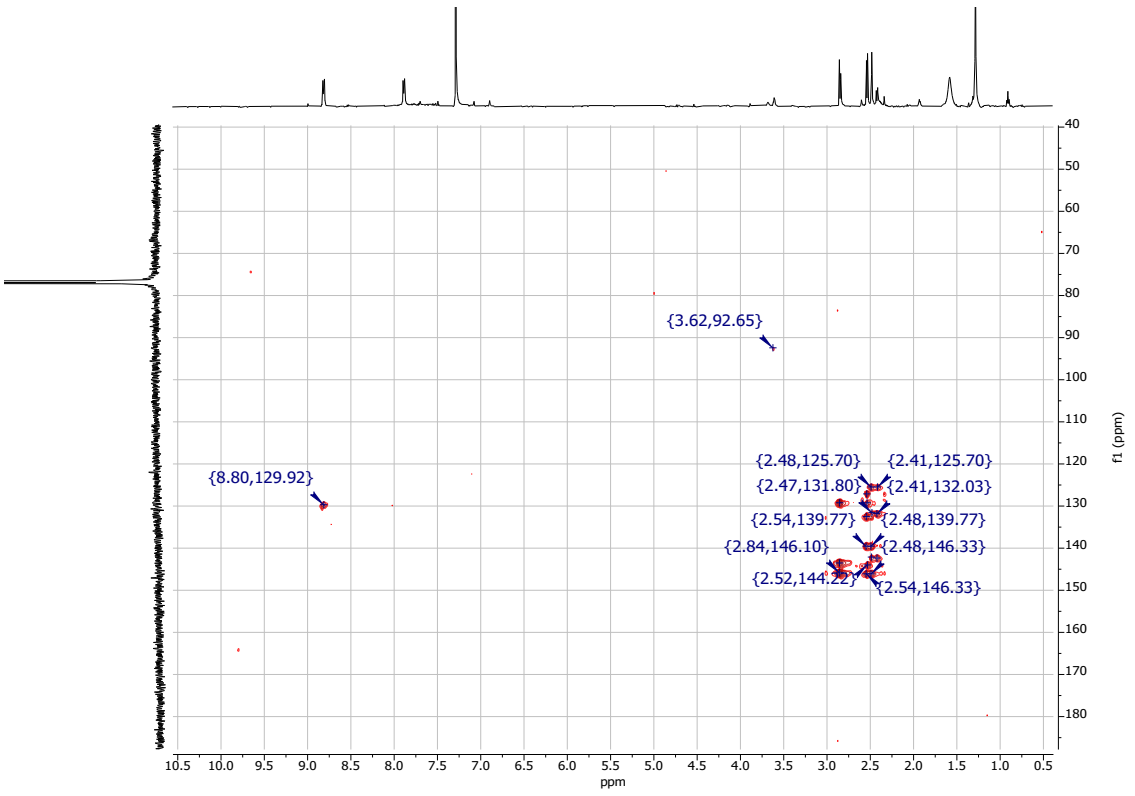


Fig. S31 HMBC (500 MHz) spectrum of compound **4** in $CDCl_3$.

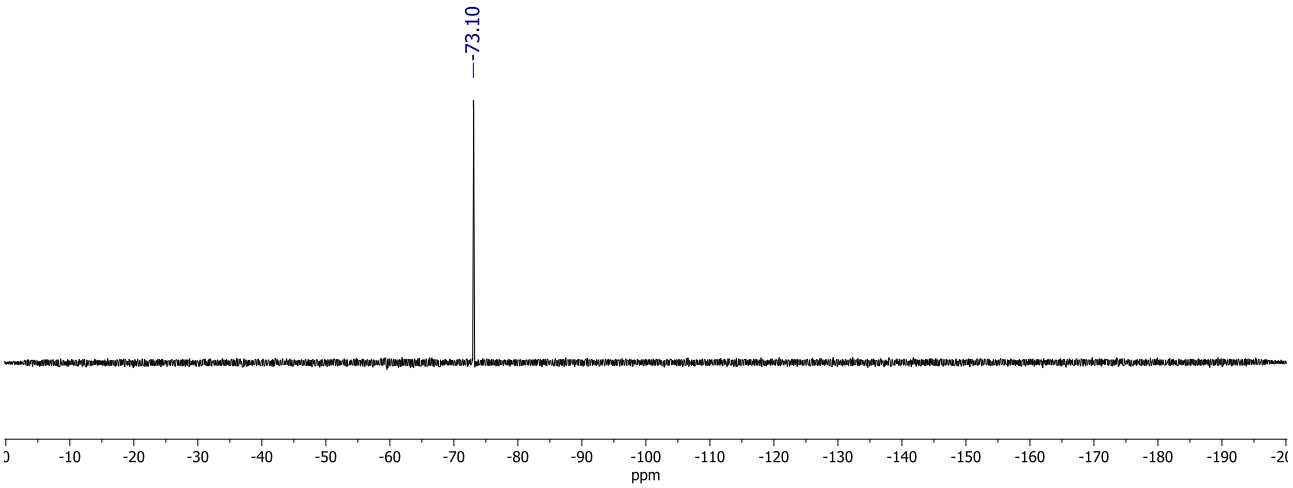


Fig. S32 ^{19}F NMR (471 MHz) spectrum of compound **4** in $CDCl_3$.

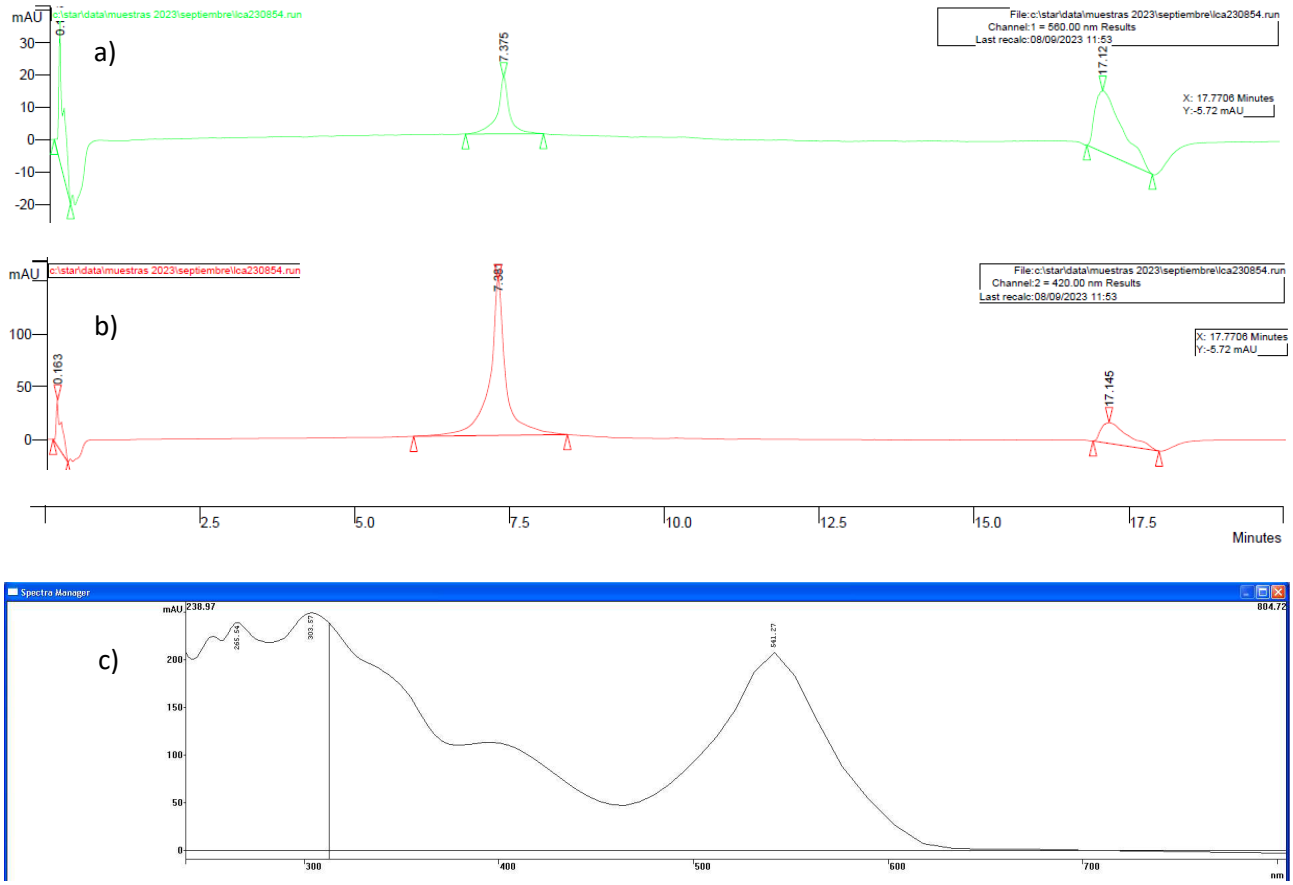


Fig. S33 HPLC analysis of compound **4** in water:CH_3CN (7:3); a) chromatogram at 560 nm, b) chromatogram at 420 nm and c) absorption spectra registered at 7.4 minutes.

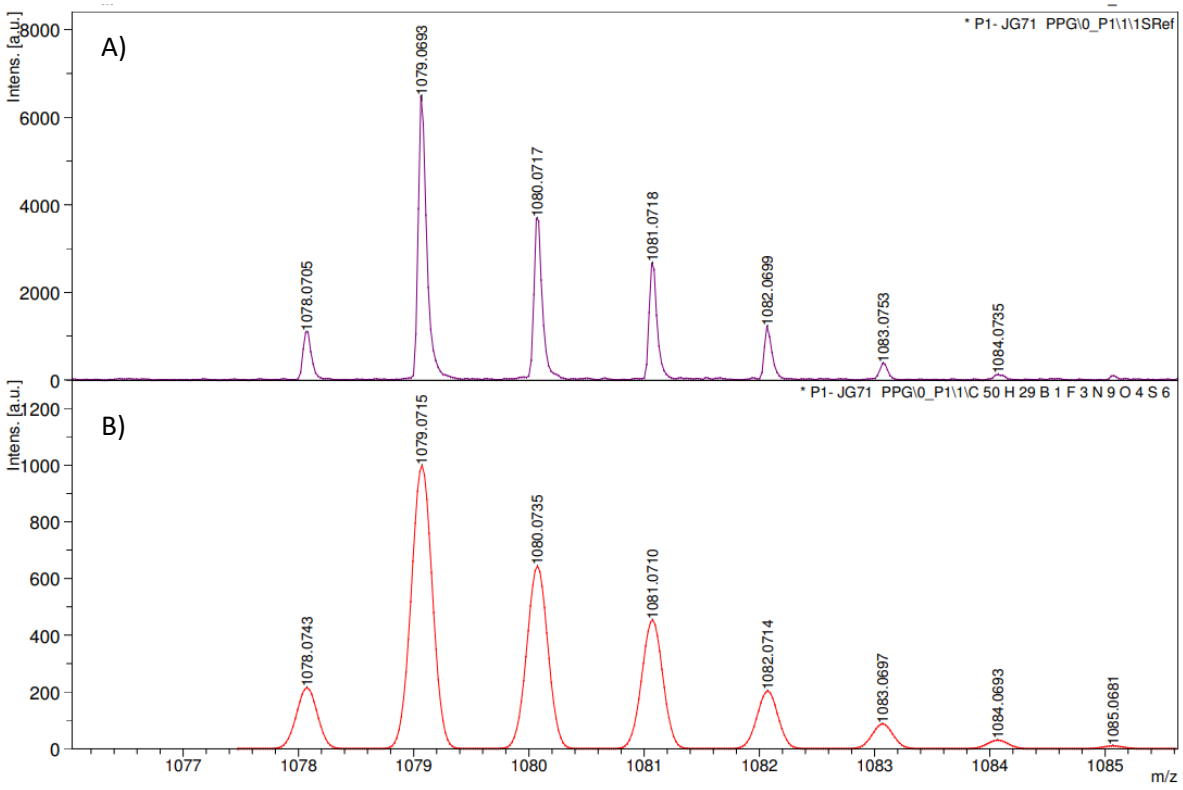


Fig. S34 HRMS (MALDI, DCTB$^+$) profile of compound **4** (A obtained, B theoretical).

9. Supplementary references

[S1] J. García-Calvo, J. Maillard, I. Fureraj, K. Strakova, A. Colom, V. Mercier, A. Roux, E. Vauthey, N. Sakai, A. Fürstenberg and S. Matile, *J. Am. Chem. Soc.*, 2020, **142**, 12034–12038.

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[S2] Ö. Göktuğ, C. Göl and M. Durmuş, *J. Porphyrins Phthalocyanines*, 2017, **21**, 539–546.

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The original data can be found at:

https://doi.org/10.5281/zenodo.10304798.