Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information for:

A molecular rotor for measuring viscosity in plasma membranes of live cells

Ismael López-Duarte,[a#] Thanh Truc Vu,[a#] M. Angeles Izquierdo,[a] James A. Bull [a] and Marina K. Kuimova [a*]

Department of Chemistry, Imperial College London, Exhibition Road, London, SW7 2AZ, United Kingdom

Email: m.kuimova@imperial.ac.uk

[#] These authors contributed equally.

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1. Synthesis and compound characterisation.

1.1 General Materials and Methods

The manipulation of all air and/or water sensitive compounds was carried out using standard high vacuum techniques. All chemicals were used as received from commercial sources without further purification. Anhydrous solvents were used as received from commercial sources. Analytical thin layer chromatography (TLC) was carried out on Merck® aluminium backed silica gel 60 GF254 plates and visualization when required was achieved using UV light or I_2. Column chromatography was performed on silica gel 60 GF254 using a positive pressure of nitrogen. Where mixtures of solvents were used, ratios are reported by volume. ^{1}H, ^{13}C and ^{19}F NMR spectra were recorded at ambient probe temperature using a Brucker DPX400 (400 MHz). Chemical shifts are quoted as parts per million (ppm) relative to the internal signal of the solvent (chloroform/methanol). ^{19}F NMR spectra were recorded with complete proton decoupling. Chemical shifts are reported in parts per million referenced to the standard hexafluorobenzene: −164.9 ppm. Mass spectra were carried out using ElectroSpray Ionization (ESI), and only molecular ions are reported.

1.2 Synthetic procedures

4-(6-Iodohexyloxy)benzaldehyde (4). 1,6-Diiodohexane (6.1 mL, 37 mmol) was added to a mixture of 4-hydroxybenzaldehyde (1.0 g, 8.2 mmol) and potassium carbonate (3.38 g, 25 mmol) in dry *N,N*-dimethylformamide (15 mL). The reaction mixture was stirred at 70 °C for 4 h, then cooled and diluted with CH_2Cl_2 (150 mL). The organic solution was washed with H_2O (3 x 100 mL), dried over anhydrous $MgSO_4$, filtered and the solvents were removed by rotary evaporation. The crude product was purified by flash chromatography on silica gel (7 petrol ether / 1 ethyl acetate) to give a colorless oil. Yield: 0.95 g (36%).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 9.84 (s, 1H), 7.79 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 8.8 Hz, 2H), 4.01 (t, J = 6.3 Hz, 2H), 3.17 (t, J = 6.7 Hz, 2H), 1.8 (m, 4H), 1.4 (m, 4H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ_C 190.80, 164.15, 131.99, 129.84, 114.74, 68.13, 33.29, 33.18, 29.38, 28.86, 6.76

BODIPY 3. Aldehyde **4** (0.95 g, 2.86 mmol) was dissolved in freshly distilled pyrrole (10 mL, 144 mmol) and the resulting solution was degassed by sparging with N_2 for 20 minutes before the addition of TFA (0.05 mL, 0.6 mmol). The mixture was stirred for 45 minutes at

room temperature, diluted with CH_2Cl_2 (100 mL) and then washed consecutively with H_2O (100 mL), $NaHCO_3$ (100 mL, 0.5M) and H_2O (100 mL). The organic extracts were dried over anhydrous $MgSO_4$, filtered and evaporated using a rotary evaporator. The excess pyrrole was removed using high vaccum to give the dipyrromethane as a dark viscous oil. The crude dipyrromethane was purified by flash chromatography in silica gel (2 CH_2Cl_2 / 1 petrol ether) to give a green viscous oil. Yield: 1.1 g (85%). The dipyrromethane (1.1 g, 2.43 mmol) was dissolved in CH_2Cl_2 (60 mL) and DDQ (0.55 g, 2.43 mmol) was added. The reaction mixture was stirred at room temperature shielded from light for 45 min. Then, Et_3N (1 mL, 7.14) was added, followed immediately by the addition of $BF_3{\cdot}(OEt_2)_2$ (0.75 mL, 6.1 mmol) and the reaction mixture was stirred at room temperature overnight. The organic solution was washed with H_2O (100 mL), NH_4Cl (100 mL, 0.5 M), $NaHCO_3$ (100 mL, 0.5 M) and finally H_2O (100 mL), and then dried over anhydrous $MgSO_4$, filtered and evaporated to give a black viscous oil which was purified by column chromatography on silica gel (6 petrol ether / 1 ethyl acetate) to give **BODIPY 3** as a red-orange sticky solid. Yield: 396 mg (33%).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.92 (br s, 2H), 7.54 (d, J = 8.3 Hz, 2H), 7.01 (d, J = 8.3 Hz, 2H), 6.98 (d, J = 6.4 Hz, 2H), 6.55 (dd, J = 4.5, 1.6 Hz, 2H), 4.06 (t, J = 6.5 Hz, 2H), 3.22 (t, J = 6.4 Hz, 2H), 1.87 (m, 4H), 1.55 (m, 4H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ_C 161.63, 147.49, 143.35, 134.84, 132.45, 131.34, 126.20, 118.24, 114.51, 68.07, 33.33, 30.21, 28.96, 25.06, 6.86; **^{19}F NMR** (377.5 MHz, CD_3OD) δ_F –145.85;
MS (ESI) m/z 475.0865 ($C_{21}H_{22}BF_2IN_2O$, $[M\text{-}F]^+$, requires 475.0854, 100%).

BODIPY 2. To a solution of **BODIPY 3** (200 mg, 0.4 mmol) in 20 mL of THF was added *N,N,N′,N′*-tetramethyl-1,3-propanediamine (7 mL, 44.5 mmol). The resulting mixture was stirred at room temperature overnight, during which time a dark-red waxy compound precipitated out from the reaction mixture. The solvent and excess of *N,N,N′,N′*-tetramethyl-1,3-propanediamine were removed by evaporation under reduced pressure and the crude product was washed several times with diethyl ether. This mono-charged intermediate, which is used without further purification, was dissolved in DMF (5 mL) and iodomethane (2 mL, 32 mmol) was added to the solution. After stirring the reaction mixture at room temperature overnight, the solvent was evaporated under reduced pressure to give a dark-red crude product which was purified by column chromatography on silica gel (methanol, and then a mixture of 3 methanol/1 NH_4Cl 0.5M). Fractions were evaporated at 30 °C to give a mixture of **BODIPY 2** and NH_4Cl which was further dissolved in methanol and successively filtered to remove the excess of NH_4Cl. Finally, the crude product was passed through a

Dowex 1x8 200 mesh ion-exchange column (H_2O). Fractions were evaporated to dryness (at 30 °C) to give **BODIPY 2** as a red-orange wax. Yield: 112 mg (48%).

^{1}H NMR (400 MHz, CD_3OD) δ_H 7.94 (br s, 2H), 7.59 (d, J = 8.6 Hz, 2H), 7.13 (d, J = 8.6 Hz, 2H), 7.05 (d, J = 4.3 Hz, 2H), 6.64 (dd, J = 4.3,1.6 Hz, 2H), 4.13 (t, J = 6.5 Hz, 2H), 3.47 (m, 6H), 3.26 (s, 9H), 3.21 (s, 6H), 2.39 (m, 2H), 1.89 (m, 4H), 1.66 (m, 2H), 1.52 (m, 2H); **^{13}C NMR** (100 MHz, CD_3OD) δ_C 161.95, 147.56, 143.14, 134.59, 132.38, 131.17, 125.85, 118.12, 114.38, 67.77, 64.91, 62.49, 60.19, 52.65, 50.05, 28.63, 25.70, 25.35, 22.21, 17.31; **^{19}F NMR** (377.5 MHz, CD_3OD) δ_F –145.85;

MS (ESI) m/2z 256.1618 ($C_{29}H_{43}BF_2N_4OCl_2$, $[M\text{-}2Cl^-]^{2+}$, requires 256.1743, 100%).

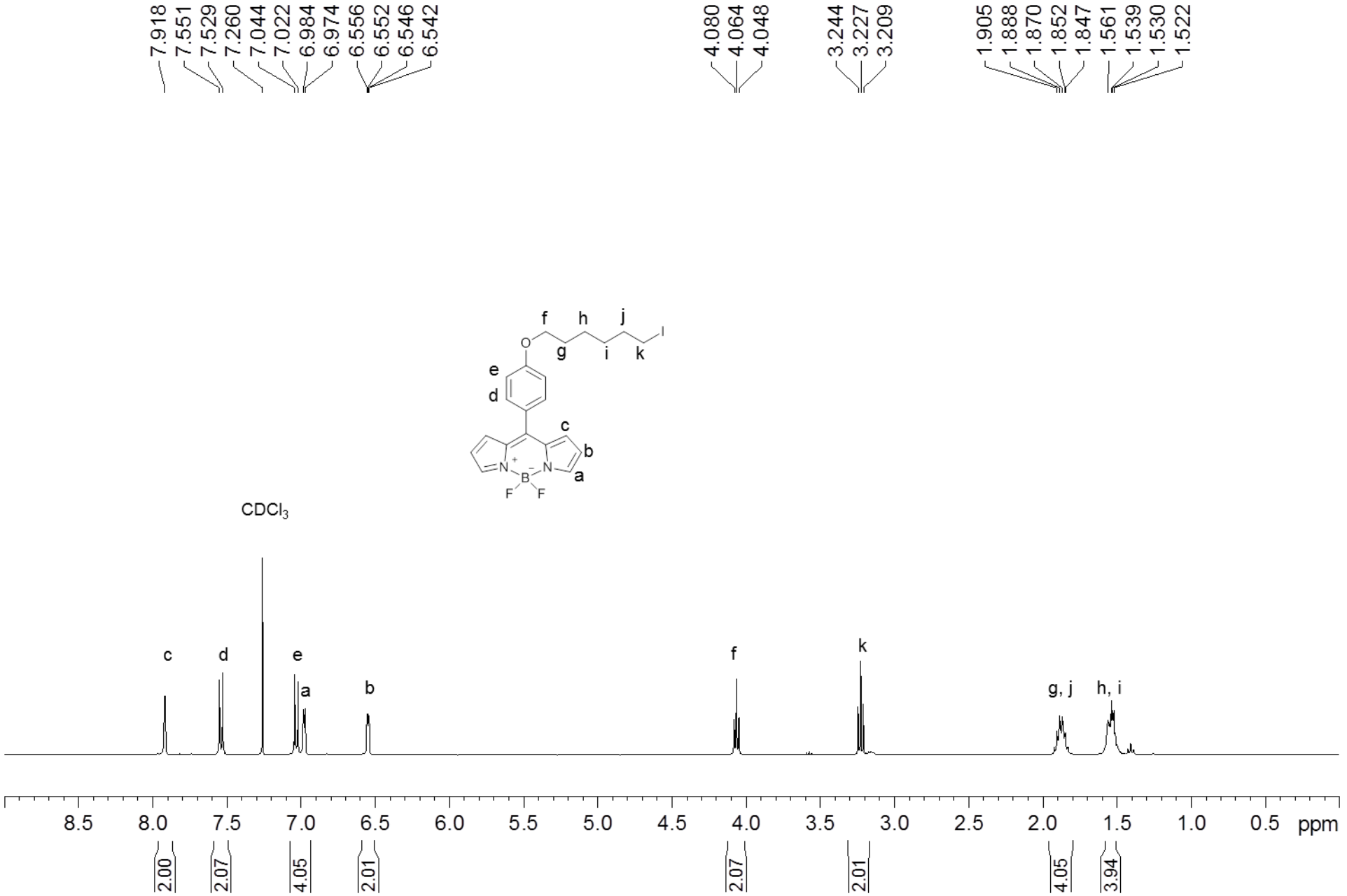

Figure S1. ^{1}H NMR spectrum of **BODIPY 3** (400 MHz, CD_3OD).

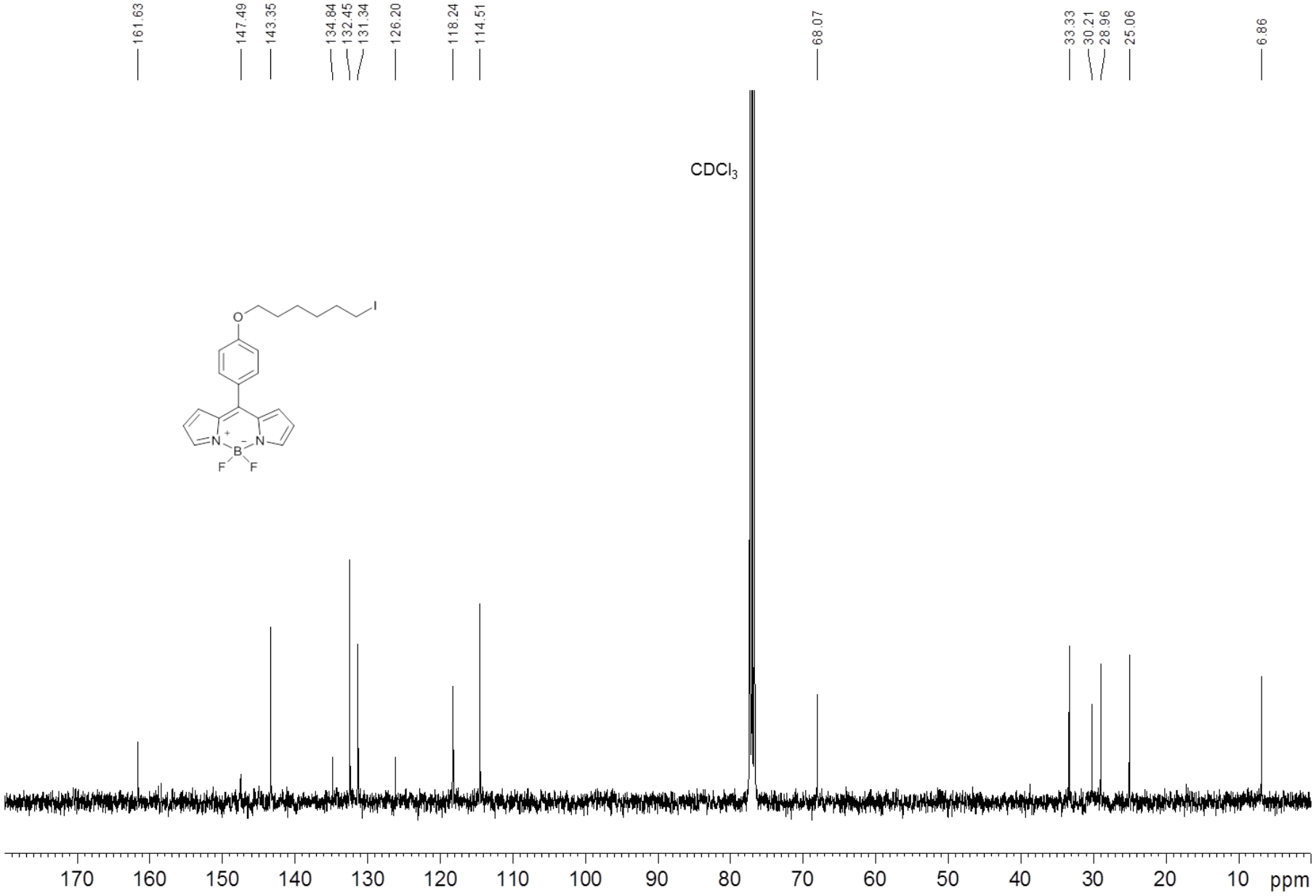

Figure S2. ^{13}C NMR spectrum of **BODIPY 3** (100 MHz, CD_3OD).

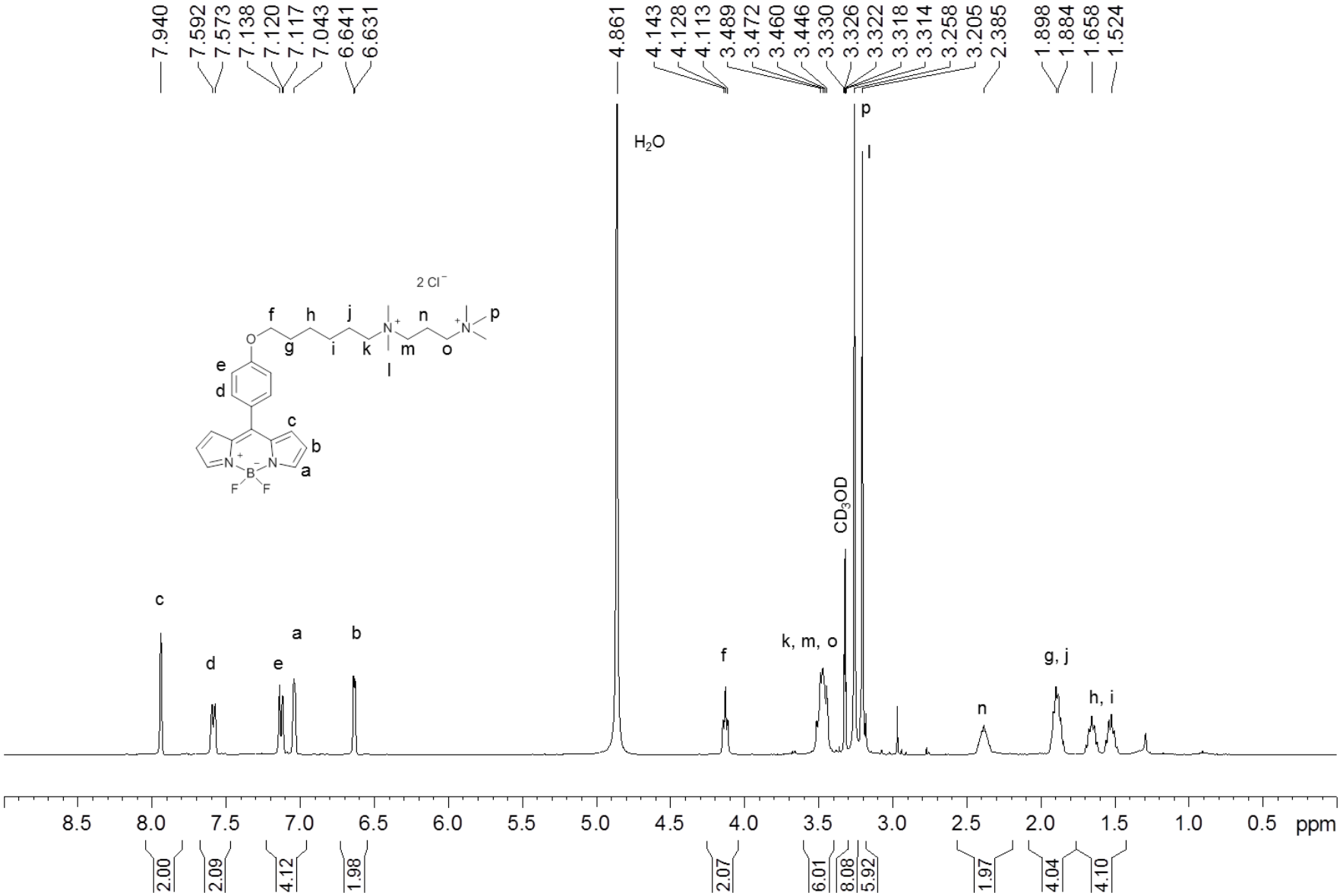

Figure S3. ^{1}H NMR spectrum of **BODIPY 2** (400 MHz, CD_3OD).

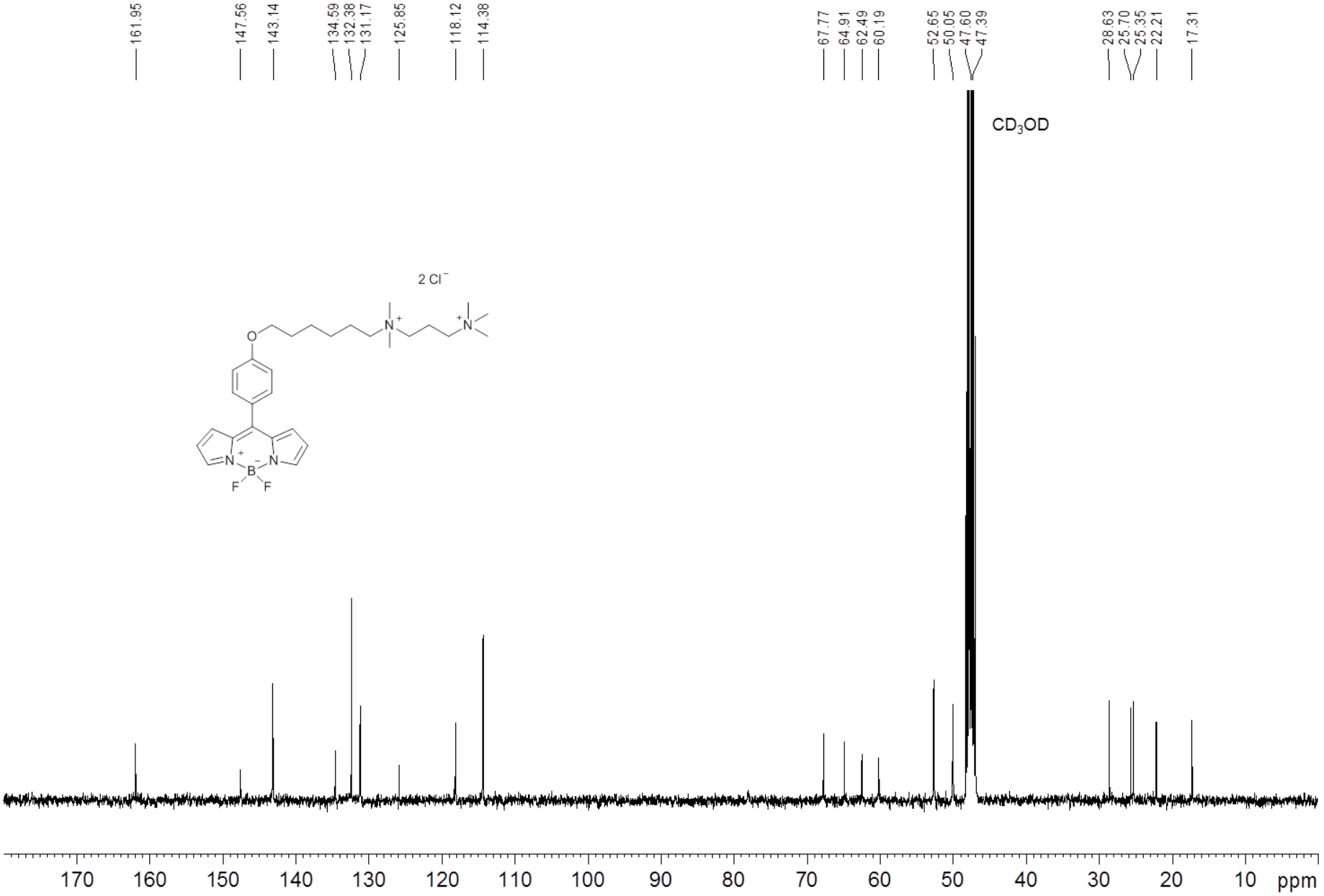

Figure S4. ^{13}C NMR spectrum of **BODIPY 2** (100 MHz, CD_3OD).

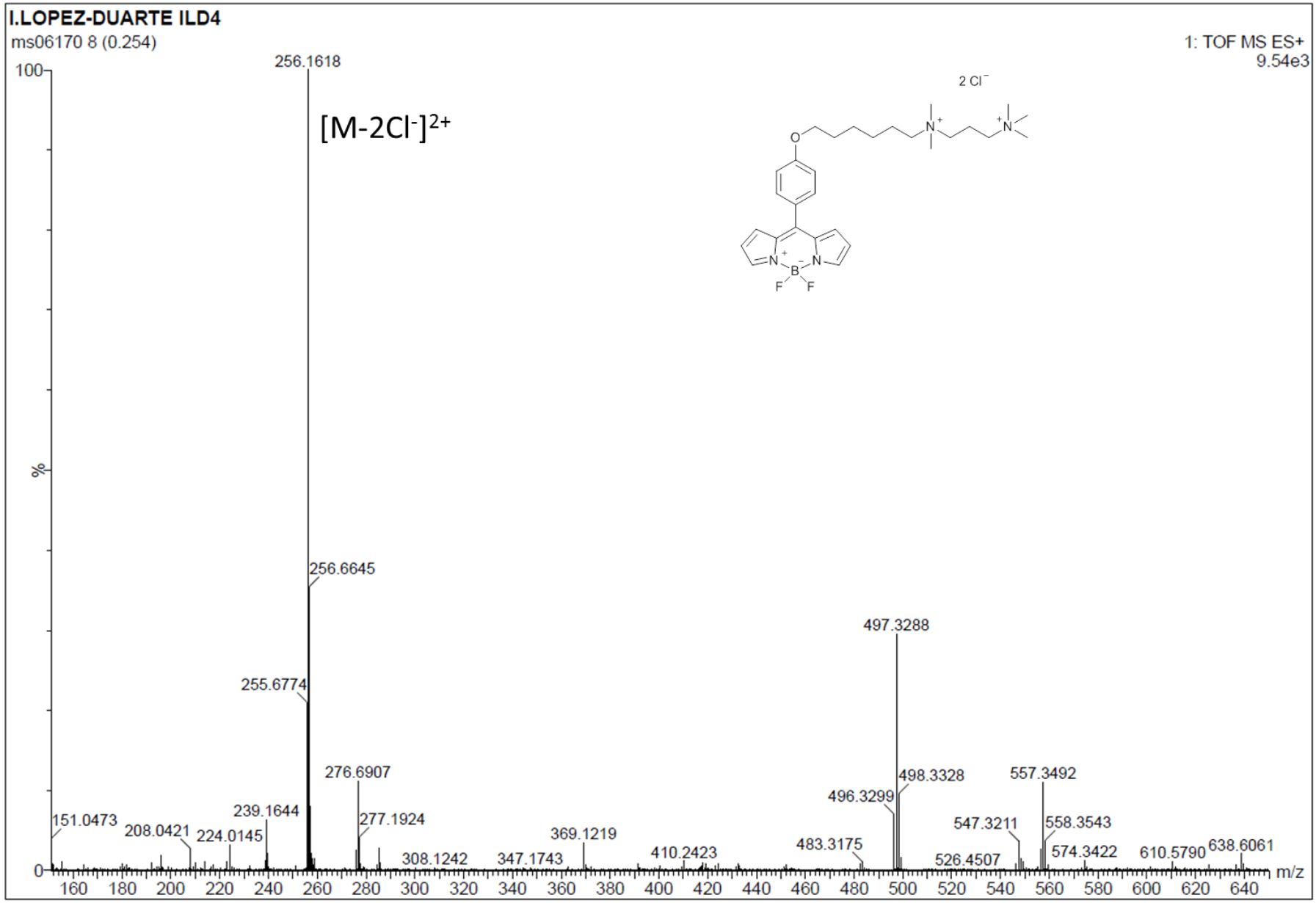

Figure S5. ESI+ mass spectrum of **BODIPY 2**.

2. Spectroscopic characterisation and procedures

Sample preparation

Spectroscopic grade methanol–glycerol mixtures were prepared at 10 concentrations between 0% and 100% (vol/vol) glycerol and maintained at room temperature. Dissolution of compound **2** was achieved by mechanical stirring for mixtures of % glycerol below or equal 50 and with further sonication at *ca* 40 ºC (for at least 20 minutes) for mixtures of % glycerol above 50. The dynamic viscosity (η) of each binary mixture was measured using a Stabinger viscometer (SVM 3000, Anton Paar) with an accuracy and precision of ±0.35% and ±0.1%, respectively. The concentration of compound **2** in methanol-glycerol mixtures was adjusted so the maximum absorbance is below 0.1 for all measurements to avoid reabsorption artefacts.

Steady-State Spectroscopy in Solution

Spectra of compound **2** were recorded in 10 mm path quartz cuvettes.

UV-visible absorption spectra were measured on an Agilent 8453 single-beam spectrophotometer and fluorescence spectra on a Fluoromax-4 (Horiba Jobin-Yvon). The quantum yields were measured using 485 nm excitation wavelength and fluorescein in 0.1M NaOH as a standard (Φ_f = 0.95).[1]

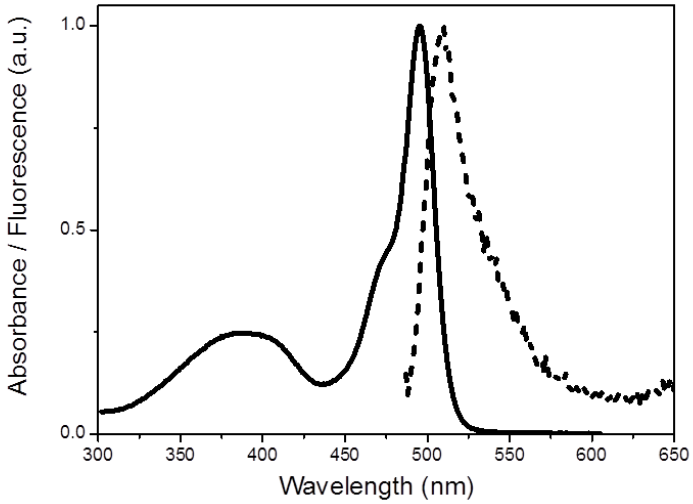

Figure S6. Absorption (solid) and fluorescence (dotted) spectra of **2** in methanol, λ_{ex} = 485 nm.

Fluorescence Lifetime of compound 2 in different solvents (solvatochromism study)

The fluorescence decay traces were collected from 10mm path quartz cuvettes using a DeltaFlex Time-Correlated Single Photon Counting system (Horiba). The samples were excited at 467 nm using a pulsed NanoLED excitation source (IRF ca. 300ps). Low absorption was maintained at the excitation wavelength (A < 0.1). Fluorescence was collected at the emission maximum.

Viscosity Calibration of Fluorescence Lifetime of compound 2

Fluorescence decay traces were recorded in untreated 8-well coverglass chambers (Lab-TekTM, Nunc) using a Leica TCS SP5 inverted scanning confocal microscope coupled with a TC SPC module (SPC830, Becker&Hickl GmbH) and internal FLIM detector (PMH-100, Becker&Hickl GmbH). The module was synchronized to a Ti-Sapphire pulsed laser source (680-1080 nm, 80 MHz, 140 fs, Chameleon Vision II, Coherent Inc.). Lifetime acquisition was carried out with a 10× (N.A. 0.3) HC PL

FLUOTAR dry objective lens. Two-photon excitation was performed at 990 nm and emission captured at a band pass of 507 – 517 nm. The number of photons in all traces is above 100 000 counts. The temperature was checked after a decay acquisition with a thermocouple for each sample.

Temperature effects

We confirmed that **2** acts as a molecular rotor by measuring fluorescence emission spectra in methanol-glycerol mixtures of varied viscosity, Fig. 2A (main text). We performed the measurements in several mixtures (50, 90 and 100% glycerol) at a range of temperatures between 5-100 °C to extend the viscosity range available to us and to verify that the data recorded for samples at different temperatures and compositions but equal viscosity display identical quantum yields. We observed a good overlap between different temperature and composition data, and concluded that the main determinant of the photophysical properties of **2** is, indeed, viscosity.

At the same time it is useful to plot the quantum yield data according to the Equation S1[2], which takes the temperature into account:

$$\Phi_f = z\left(\frac{\eta}{T}\right)^{\alpha} \qquad \text{(S1)}$$

where *z* and *α* are constants, η is viscosity and *T* is temperature.

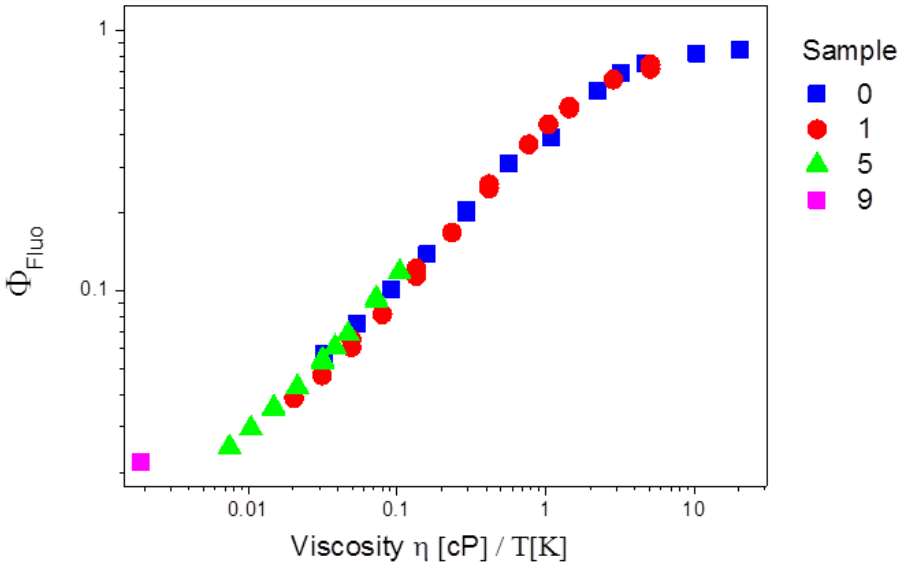

Figure S7. Fluorescence quantum yield of **2** recorded in methanol/glycerol mixtures at varied temperatures 5-100 °C; % glycerol: ■ 100; ● 90; ▲ 50; ■ 0.

While the temperature adjusted data, Figure S7, fall slightly better on a straight line *log (ϕ) vs log (η),* we would like to emphasise that the temperature effect is small and **2** retains its calibration at different temperatures, as demonstrated by the excellent linear fit to the combined temperature/composition data in Fig 2C (inset).

Polarity effects

Polarity of the environment is known to affect the properties of TICT molecular rotors.[3] We have verified that the solvent polarity does not have a significant effect on the spectra or fluorescence lifetime of **2**. Bodipy **2** shows no solvatochromism in either absorption or fluorescence spectra. Fluorescence lifetime at low viscosities decreases slightly upon ε increase from 5 to 47, Figure S8. However, this effect is significantly smaller than that for TICT rotors.[3, 4]

Table S1. Spectroscopic properties of **2** in several solvents of varied polarity; $\lambda_{max,\ abs}$ and $\lambda_{max,\ f}$ are the maxima of absorption and fluorescence, respectively.

Solvent	$\lambda_{max,\ abs}$ (nm)	$\lambda_{max,\ f}$ (nm)	τ_f (ps)
Chloroform	500	513	752
Dichloromethane	499	513	494
Ethanol	497	510	380
Acetonitrile	495	508	232
Dimethyl sulfoxide	500	516	370

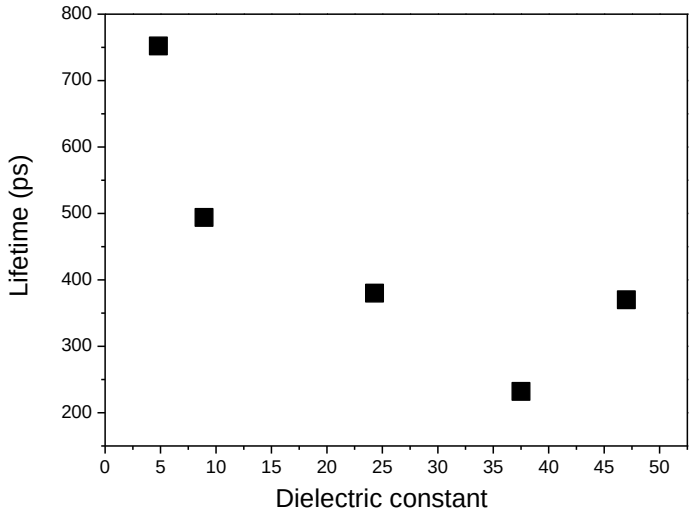

Figure S8. Fluorescence lifetime of **2** as a function of solvent polarity.

3. Microscopy characterisation and procedures

Cell culture

The human ovarian carcinoma cell line SK-OV-3 was obtained from the European Collection of Cell Cultures (ECACC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum and passaged when 70-90 % confluent in 25 cm^3 flasks grown at 37 ºC in 10% CO_2. For imaging, SK-OV-3 cells were seeded at 15000-10000 cells/well in 0.4 mL of culture medium in untreated 8-well coverglass chambers (Lab-TekTM, Nunc) and allowed to grow to confluence for 24 h.

Incubation of SK-OV-3 with BODIPY 2

The uptake of **2** in live cells was tested using the protocol suggested by Molecular Probes for the plasma membrane labelling of styryl derivatives.[5] Ice cold Hank's balanced salt solution (HBSS) without magnesium or calcium was used for cell incubation to induce selective staining of the plasma membrane. At these conditions endocytosis is not favourable.[6] The chambers loaded with cells were placed on ice. The culture media was replaced with cold HBSS without calcium, magnesium and phenol red before the addition of the dye. The ice-cold SK-OV-3 cells were loaded with cold staining solution containing **2** (8.9 µM, 0.1 % DMSO) and the chamber was placed on the microscope stage for imaging at room temperature (22 °C). The samples were measured in staining solution without washing. The stock solution of **2** was prepared in DMSO (8.9 mM).

Control experiments were carried out to test the potential of compound **2** containing a double positive charge to stain the plasma membrane under standard staining conditions when endocytosis can occur. In this case, the culture media was replaced with HBSS containing calcium and magnesium at room temperature (22 °C). The cells were loaded with the staining solution containing **2** (8.9 µM, 0.1 % DMSO) and the chamber was placed on the microscope stage for imaging at room temperature (22 °C). Confocal images of the cells incubated with dye **2** using the two different experimental conditions are shown in Figure S9 along with the fluorescence intensity profiles across a single cell. These results demonstrate the potential of the new dye **2** to selectively stain the cell membrane, although staining of the intracellular organelles is observed in HBSS at 22 °C.

Fluorescence confocal imaging

Confocal fluorescence imaging of live SK-OV-3 cells stained with **2** was performed using a confocal laser scanning microscope (Leica TCS SP5). Imaging was carried out with a 63× (N. A. 1.2) HCX PL APO CS water immersion objective lens with correction collar (11506279, Leica Microsystem, Ltd). Samples were excited at 496 nm with an argon ion laser and emission intensity was recorded at 510-700 nm.

Fluorescence lifetime imaging

FLIM images of 256 × 256 pixels were obtained using a Leica TCS SP5 inverted scanning confocal microscope coupled with a TCSPC module (SPC830, Becker&Hickl GmbH) and internal FLIM detector (PMH-100, Becker&Hickl GmbH). The module was synchronized to a Ti-Sapphire pulsed laser source (680-1080 nm, 80 MHz, 140 fs, Chameleon Vision II, Coherent Inc.). Imaging was carried out with a 63× (N. A. 1.2) HCX PL APO CS water immersion objective lens with correction collar (11506279, Leica Microsystem, Ltd).

Two-photon excitation was performed at 990 nm and emission captured at a band pass of 510-700 nm. The laser power was maintained at <120 mV before entering the microscope to avoid cell damage. The acquisition time was 400 s for each image.

FLIM Data were analysed in TRI2 software (Paul Barber, Oxford[7]) where a monoexponential model was fitted, using a Levenberg-Marquardt algorithm, to the fluorescence decay in each pixel. Pixels were binned to maintain a minimum peak count of 100 counts per pixel and thresholding was used to remove background noise. A false colour scale was assigned to each fluorescence lifetime value (blue for a short lifetime and red for long lifetime) to provide lifetime maps.

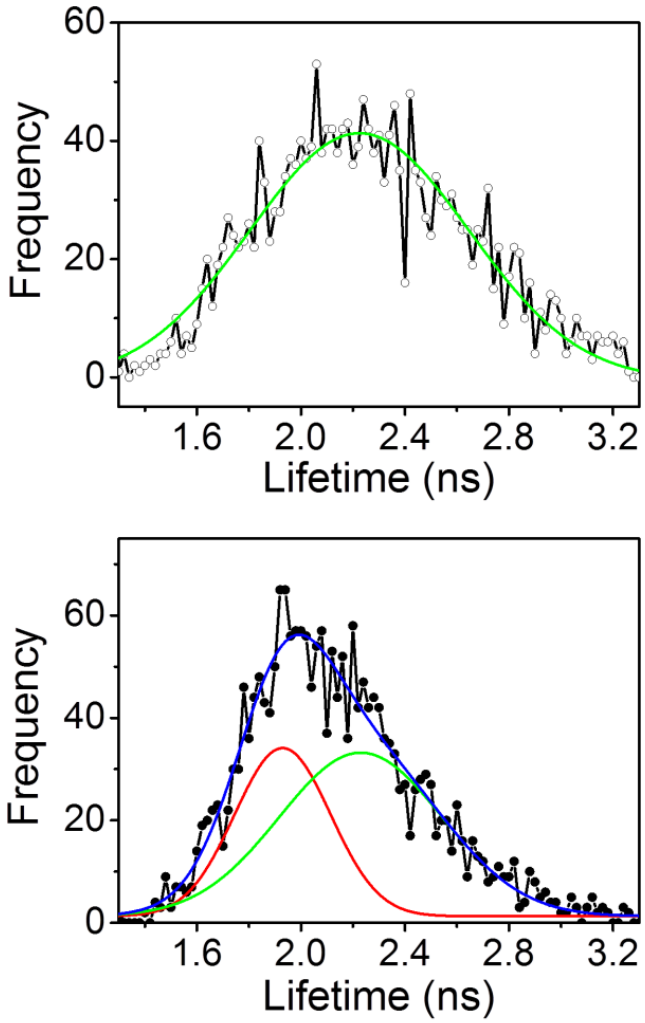

Figure S9. Lifetime histograms obtained from FLIM images of SK-OV-3 cells incubated with **2** for 9 min (top) and 40 min (bottom). 40 min histogram is more adequately fitted with a bimodal Gaussian peak fit, individual peaks are centred at 1.9 ns and 2.2 ns. We tentatively assign the higher lifetime peak (2.2 ns maximum, shown in green in both images) to the plasma membrane fraction of **2**.

We have also performed FLIM of several methanol/glycerol solutions, to confirm that the large FWHM of the histograms of live cells (Figure S9) are, indeed, due to a large heterogeneity of micro-viscosities in the intracellular environment, and not to an intrinsically low signal/noise in our images. The data shown in Table S1 and Figure S10 clearly demonstrate that the homogeneous solutions display extremely narrow histograms. We interpret this data to demonstrate that the cellular environment is, indeed, very heterogeneous.

Table S2. FLIM histogram parameters (peak maxima and FWHM) obtained for several glycerol/methanol mixtures and cells. To allow the comparison, the total amount of fluorescent counts in the image was kept approximately constant for all samples.

Sample	Peak maximum (ns)	FWHM (ns)
MeOH:glycerol (8:2)	0.38	0.04
MeOH:glycerol (5:5)	0.64	0.03
MeOH:glycerol (2:8)	2.47	0.09
MeOH:glycerol (1:9)	3.30	0.23
Cells	2.22	0.99

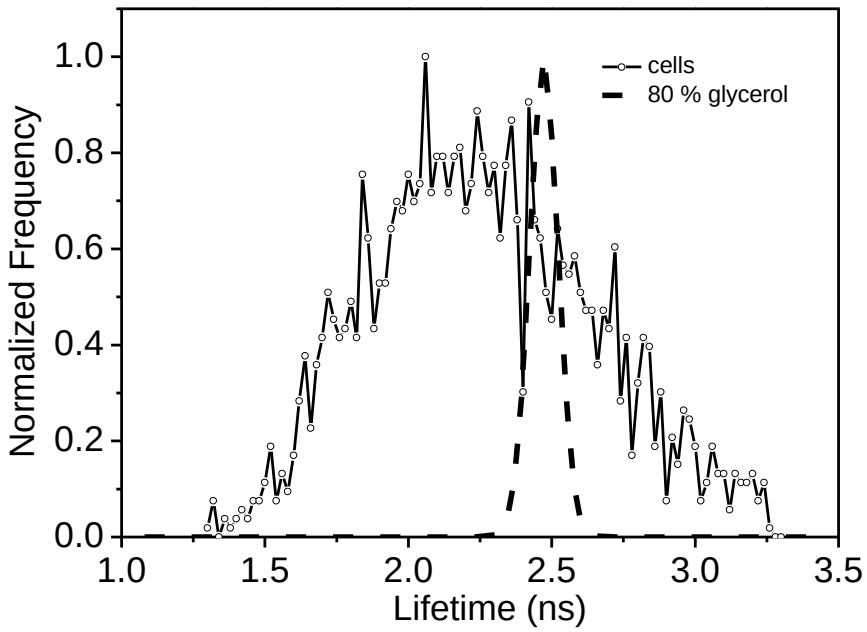

Figure S10. Lifetime histograms obtained from FLIM images of SK-OV-3 cells incubated with **2** for 9 min and a 8:2 solution glycerol/methanol at room temperature. It is clear that the histogram of the homogeneous solution is considerably narrower than that of a cellular membrane, in spite of a similar peak maximum and similar total counts.

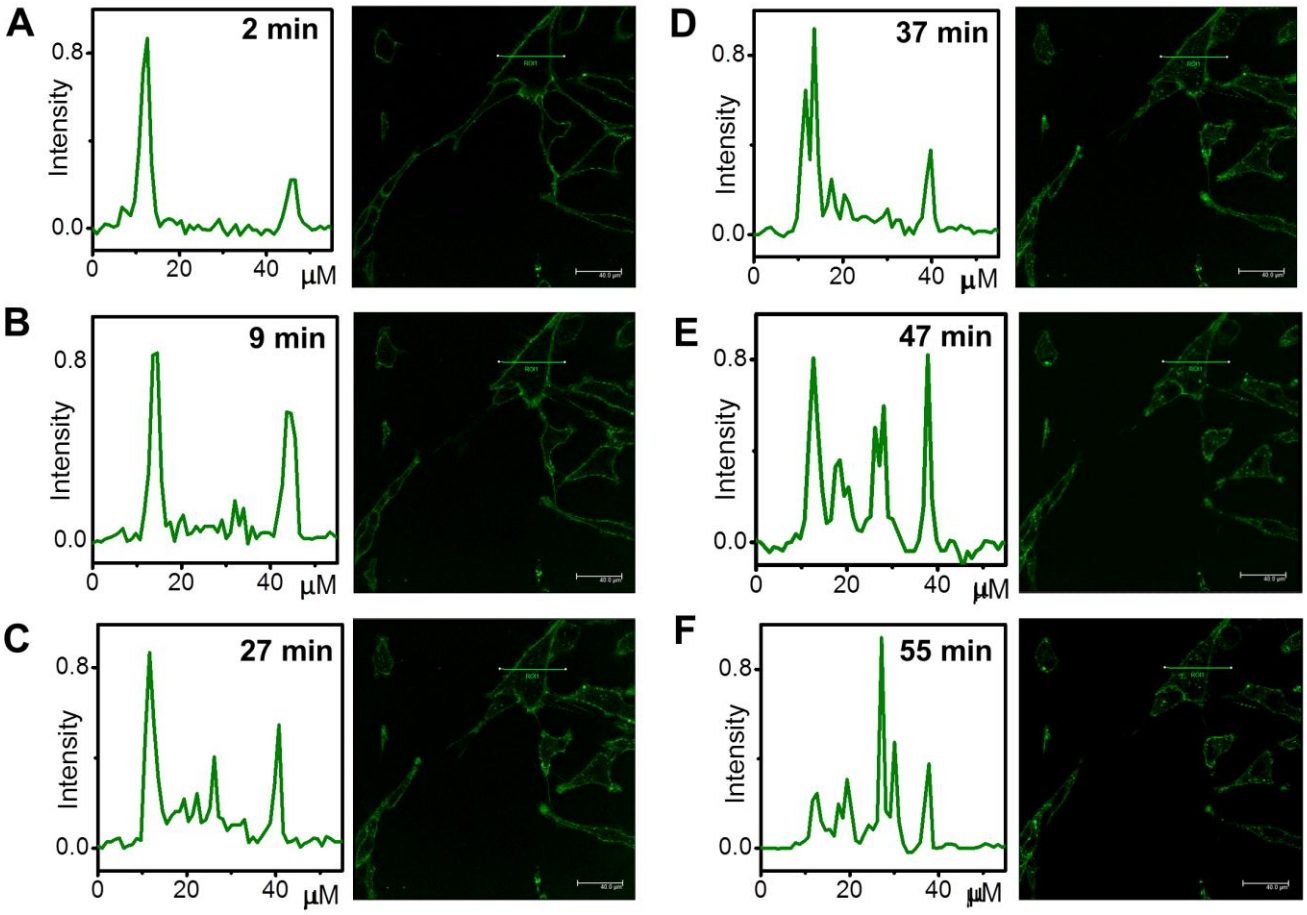

Figure S11. Confocal images of SK-OV-3 cells recorded 2 min (A), 9 min (B), 27 min (C),37 min (D), 47 min (E), and 55 min (F) following the incubation of a fresh layer of cells with 8.9 μM solution of **2** at the conditions preventing endocytosis. Panels on the left show the fluorescence intensity profiles across a single cell indicated by a bar in the image.

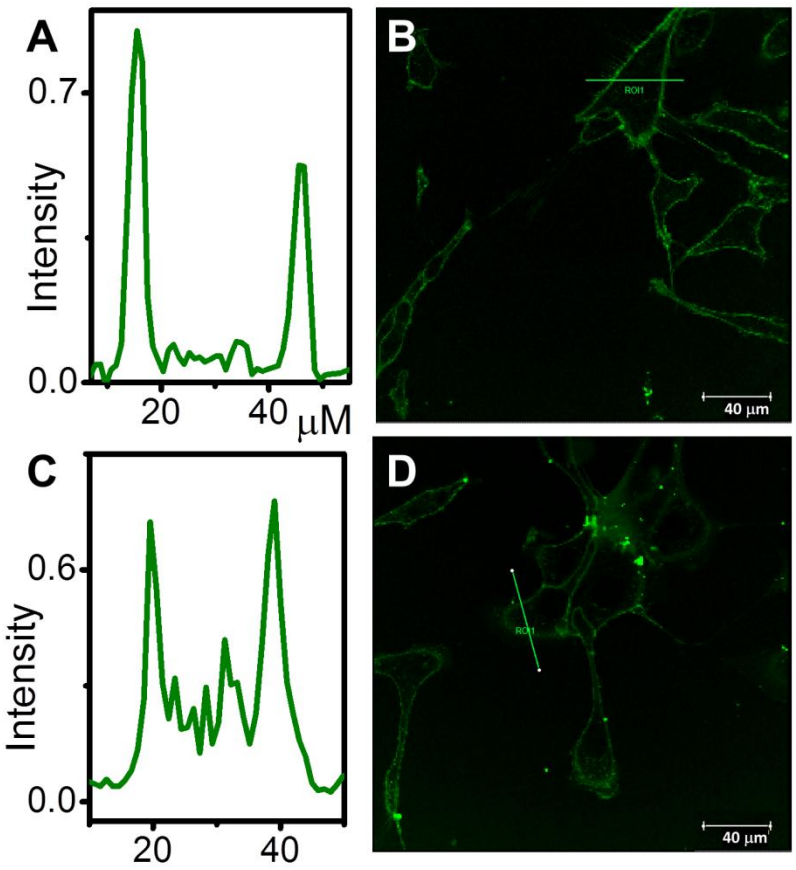

Figure S12. Confocal images of SK-OV-3 cells recorded 9 min following the incubation of a fresh layer of cells with 8.9 μM solution of **2** in HBSS without calcium and magnesium on ice (B) and in HBSS with calcium and magnesium at room temperature 22 °C (D). Panels on the left show the fluorescence intensity profiles across a single cell indicated by a bar in the image. We conclude that even at standard incubation conditions **2** works as a plasma membrane stain.

4. References

1. G. Weber, F. W. J. Teale, *Trans. Faraday Soc.* **1957**, 53, 646 – 655

2. B. Valeur, Molecular Fluorescence, Wiley-VCH, Weinheim, 2002

3. M. A. Haidekker and E. A. Theodorakis, *Org. Biomol. Chem.*, 2007, **5**, 1669-1678.

4. A. Battisti, S. Panettieri, G. Abbandonato, E. Jacchetti, F. Cardarelli, G. Signore, F. Beltram and R. Bizzarri, *Anal. Bioanal. Chem.*, 2013, **405**, 6223-6233.

5. www.lifetechnologies.com

6. W. Wu, J. Xu, X. S. Wu, L. G. Wu, *J. Neurosci*. **2005,** 25, 11676-11683

7. P. R. Barber et al. *J Royal Soc. Interface*, **2009**, 6(Suppl 1):S93–S105.