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Supplementary Information

HydroFlipper membrane tension probes: Imaging membrane hydration and mechanical compression simultaneously in living cells

José García-Calvo,‡ Javier López-Andarias,‡ Jimmy Maillard, Vincent Mercier, Chloé Roffay, Aurelien Roux, Alexandre Fürstenberg, Naomi Sakai, and Stefan Matile*

School of Chemistry and Biochemistry and National Centre of Competence in Research (NCCR) Chemical Biology, University of Geneva, Geneva, Switzerland

Stefan.Matile@unige.ch

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1. Materials and methods

As in ref. S1 and S2. Briefly, reagents for synthesis were purchased from Fluka, Sigma-Aldrich, TCI and Across. PEG linker **11** was purchased from BroadPharm. Salts of the best grade available from Fluka or Sigma-Aldrich were used as received. Egg sphingomyelin (SM), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-choline (DPPC) and Mini-extruder were purchased from Avanti Polar Lipids, cholesterol (CL) was purchased from Sigma-Aldrich. Phosphate buffered saline (PBS, pH = 7.4), DMEM (GlutaMAX, 4.5 g/L D-glucose, with phenol red) medium, FluoroBrite DMEM (high D-Glucose) medium, Leibovitz's L-15 medium, Opti-MEM reduced serum medium with GlutaMAX supplement, Penicillin-Streptomycin, Fetal Bovine Serum, TrypLE Express Enzyme, Lipofectamine 2000, MitoTracker™ Red, LysoTracker™ Red and ER-Tracker™ Red were obtained from Thermo Fisher Scientific. 35 mm glass-bottom dishes were obtained from MatTek (P35G-0.170 14-C); 96-well μ-plates and μ-Slide 8-Well Glass Bottom was obtained from Ibidi. Column chromatography was carried out on silica gel 60 (SilicaFlash P60, 40-63 μm). Analytical (TLC) and preparative thin layer chromatography (PTLC) were performed on silica gel 60 (Merck, 0.2 mm) and silica gel GF (SiliCycle, 1 or 0.25 mm), respectively.

Fluorescence cellular imaging was performed using Leica SP5 confocal equipped with 63x oil immersion objective lens, or an IXM-C automated microscope from ImageXpress equipped with a Lumencor Aura III with solid-state light sources, bandpass filters and a 40x objective. Sample preparation and washing on 96-well plates was performed using a Plate washer Biotek EL406®. Fluorescence lifetime imaging microscopy (FLIM) was performed on a Nikon Eclipse Ti A1R microscope upgraded with a FLIM kit from PicoQuant, equipped with a laser at 485 nm (PicoQuant, LDH-D-C-485) at 20 MHz and a 100x oil immersion objective lens, and collecting the fluorescence between 550 and 650 nm.

Melting points (Mp) were measured on a Melting Point M-565 (BUCHI). IR spectra were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometer (ATR, Golden Gate) and are reported

as wavenumbers υ in cm^{-1} with band intensities indicated as s (strong), m (medium), w (weak), br (broad). All ^{1}H and ^{13}C NMR spectra were recorded (as indicated) on a Bruker 300 MHz, 400 MHz or 500 MHz spectrometer 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). ESI-HRMS was measured on Xevo G2-S Tof (Waters). All mass data are reported as mass-per-charge ratio *m/z* (intensity in %, [assignment]).

Abbreviations. CL: Cholesterol; CLSM: Confocal laser scanning microscopy; DMSO: Dimethyl sulfoxide; DOPC: Dioleyl-*sn*-glycero-3-phosphocholine; DTT: Dithienothiophene; FLIM: Fluorescence lifetime imaging; GFP: Green fluorescent protein; GUVs: Giant unilamellar vesicles; LP: Laser power; LUVs: Large unilamellar vesicles; MOI: Membrane of interest; ROI: Region of interest; SM: Egg sphingomyelin; TBTA: Tris((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)amine; TFA: Trifluoroacetic acid.

2. Flipper synthesis

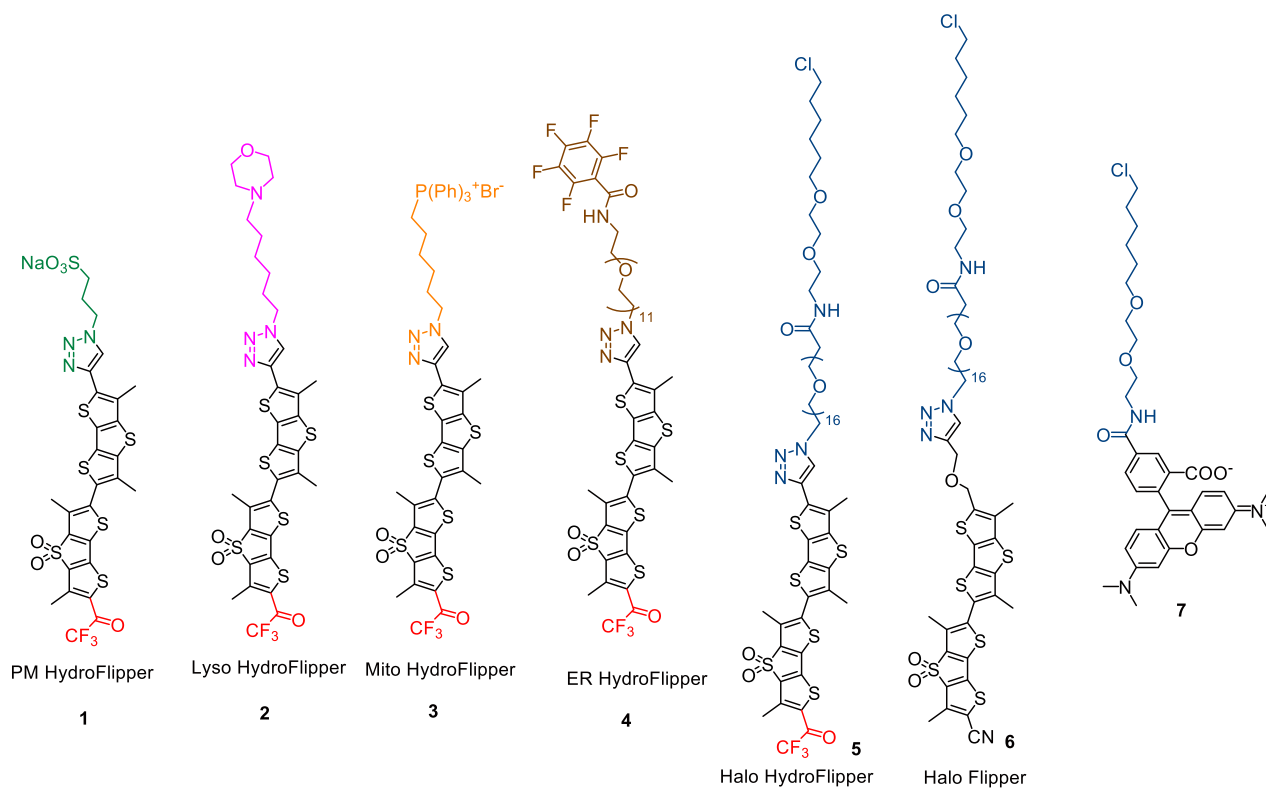


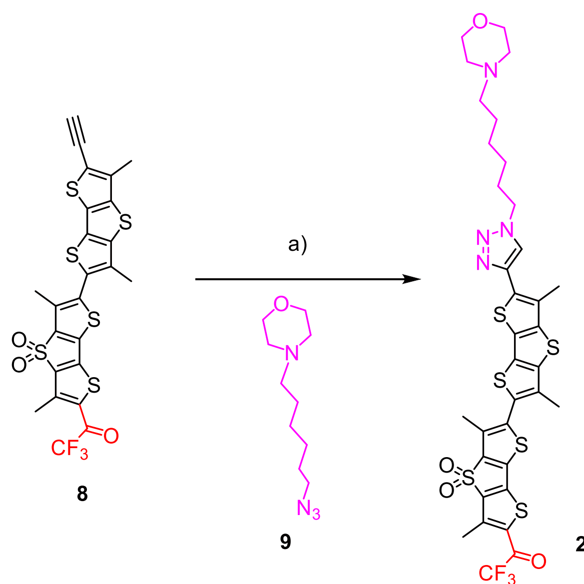
Fig. S1 Molecular structure of all the molecules used in this research.

Compound 1 was synthesized and purified according to procedures described in ref. S2.

Compound 6 was synthesized and purified according to procedures described in ref. S1.

Compound 7 was synthesized and purified according to procedures described in ref. S3.

2.1. Synthesis of Lyso-HydroFlipper



Scheme S1 (a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, TBTA, CH_2Cl_2, H_2O, DMSO, rt, 2 h, 43%.

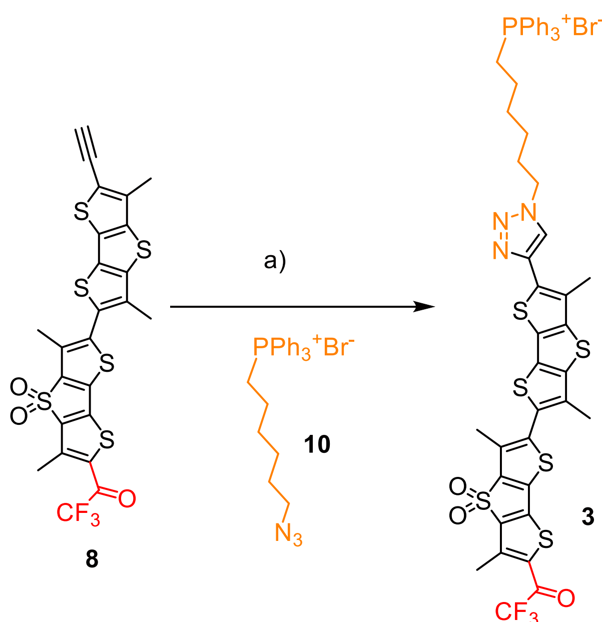
Compound 8 was synthesized and purified according to procedures described in ref. S2.

Compound 9 was synthesized and purified according to procedures described in ref. S4.

Compound 2. To a solution of **8** (5.4 mg, 9.0 µmol) in CH_2Cl_2 (2 mL), **9** (3.8 mg, 18.0 µmol), $CuSO_4.5H_2O$ (2.3 mg, 9.2 µmol), TBTA (3.8 mg, 7.2 µmol), sodium ascorbate (1.8 mg, 0.9 µmol), DMSO (10 µL) and water (200 µL) were subsequently added. After 10 minutes, to a solution of $CuSO_4.5H_2O$ (1.2 mg, 4.5 µmol) in water (200 µL) sodium ascorbate (0.9 mg, 0.5 µmol) was added; the resulting yellow mixture was immediately added to the reaction mixture. After 2 h at rt, the crude mixture was diluted with of CH_2Cl_2 (10 mL), washed with water (3×10 mL), dried with Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash chromatography (SiO_2, CH_2Cl_2 to CH_2Cl_2/MeOH 20:1) to yield **2** as a deep red solid (3.1 mg, 43%). R_f (CH_2Cl_2/MeOH 20:1): 0.65; Mp: decomposed > 120 °C; IR (neat): 2923 (s), 2854 (s), 2348 (w), 1678 (s), 1445 (m), 1408 (m), 1378 (m), 1309 (s), 1192 (s), 1143 (s), 1094 (w), 751 (w); ^{1}H NMR (400 MHz, $CDCl_3$): 7.73 (s, 1H), 4.46 (t, $^3J_{H\text{-}H}$= 7.2 Hz, 2H), 3.76 (s, 4H), 2.84 (s, 3H), 2.59 (s, 3H), 2.48 – 2.37 (m, 11H), 2.02 (t, $^3J_{H\text{-}H}$ = 7.2 Hz, 2H), 1.64 (s, 2H), 1.48 – 1.40 (m, 4H); ^{13}C NMR (126 MHz, $CDCl_3$): 171.0 (q, $^2J_{C\text{-}F}$ =

37.5 Hz, C=O), 170.8 (C), 143.7 (C), 143.6 (C), 141.7 (C), 141.2 (C), 140.4 (C), 140.1 (C), 139.9 (C), 137.1 (C), 130.1 (C), 129.6 (C), 129.0 (C), 126.9 (C), 126.4 (C), 126.0 (C), 125.2 (C), 123.3 (C), 117.6 (CH), 113.6 (q, $^{1}J_{C\text{-}F}$ = 290.7 Hz, C), 68.1 (CH_2), 64.6 (CH_2), 56.2 (CH_2), 51.1 (CH_2), 48.0 (CH_2), 27.7 (CH_2), 27.3 (CH_2), 24.3 (CH_2), 23.9 (CH_2), 12.5 (CH_3), 12.1 (CH_3), 11.7 (CH_3), 10.0 (CH_3); ^{19}F NMR (282 MHz, $CDCl_3$): -73.11; HRMS (ESI+) calcd. for $C_{34}H_{35}F_3N_4O_5S_6$: 829.0957 ($[M+H]^+$), found: 829.0976.

2.2. Synthesis of Mito-HydroFlipper



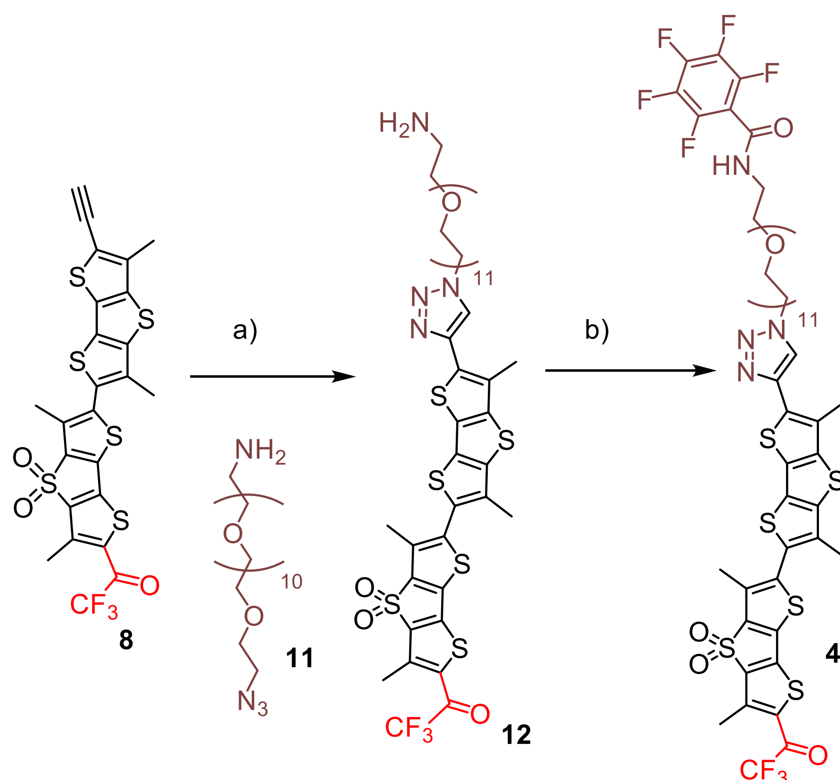
Scheme S2 (a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, TBTA, CH_2Cl_2, DMSO, H_2O, rt, 2 h, 18%.

Compound 10 was synthesized and purified according to procedures described in ref. S4.

Compound 3. To a solution of **8** (6.2 mg, 10 μmol) in CH_2Cl_2 (2 mL), **10** (9.7 mg, 21 μmol), $CuSO_4 \cdot 5H_2O$ (2.6 mg, 10 μmol), TBTA (4.5 mg, 8 μmol), sodium ascorbate (2.1 mg, 10 μmol), and water (300 μL) were subsequently added. After 10 minutes, in a solution of $CuSO_4 \cdot 5H_2O$ (1.3 mg, 5.0 μmol) in water (200 μL), sodium ascorbate (1 mg, 5 μmol) was added; the resulting yellow mixture was immediately added to the reaction mixture. The reaction was stirred for 2 h at rt, diluted with of CH_2Cl_2 (5 mL), washed with water (3×10 mL), dried with Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash chromatography (SiO_2, CH_2Cl_2 to CH_2Cl_2/MeOH 9:1).

Subsequently, the residue was dissolved in DMSO (1 mL) and further purified by reverse phase flash chromatography (Biotage® SNAP Ultra C_{18} 12 g, CH_3CN/H_2O 20 – 100% gradient with 0.1% TFA). The solid obtained was redissolved in CH_2Cl_2 (2 mL) and water (2 mL), and KBr (500 mg) was added. The mixture was stirred vigorously for 1 h. The organic phase was separated, dried over Na_2SO_4, filtered, and the solvents were evaporated to yield **3**, as a deep red solid (2 mg, 18%). R_f (CH_2Cl_2/MeOH 10:1): 0.32; Mp: decomposed > 110 °C; IR (neat): 3500 (br), 2924 (s), 2856 (s), 1677 (s), 1439 (m), 1306 (m), 1201 (m), 1178 (s), 1138 (s), 831 (w), 800 (w), 721 (m), 691 (w); ^{1}H NMR (500 MHz, acetone-d_6 + 10% D_2O): 8.36 (s, 1H), 7.88 – 7.71 (m, 15H), 4.45 (t, $^3J_{H-H}$ = 6.9 Hz, 2H), 3.48 – 3.42 (m, 2H), 2.46 (s, 3H), 2.45 (s, 3H), 2.34 (s, 3H), 2.31 (s, 3H), 1.91 – 1.88 (m, 2H), 1.68 (t, $^3J_{H-H}$ = 7.2 Hz, 2H), 1.60 (t, $^3J_{H-H}$ = 8.1 Hz, 2H), 1.33 (t, $^3J_{H-H}$ = 7.2 Hz, 2H); ^{13}C NMR (126 MHz, acetone-d_6 + 10% D_2O): 144.4 (C), 143.0 (C), 142.2 (C), 142.0 (C), 135.8 (CH), 135.8 (d, $^4J_{C-P}$ = 3.7 Hz, CH), 135.5 (C), 135.4 (C), 134.9 (C), 134.2 (d, $^3J_{C-P}$ = 10.2 Hz, CH), 132.6 (C), 132.6 (C), 132.2 (C), 131.0 (d, $^2J_{C-P}$ = 13.1 Hz, CH), 129.8 (C), 128.6 (C), 127.9 (C), 127.2 (C), 123.9 (q, $^1J_{C-F}$ = 289.6 Hz, C), 122.1 (CH), 119.0 (d, $^1J_{C-P}$ = 85.8 Hz, C), 50.6 (CH_2), 30.4 – 29.9 (m, CH_2), 25.8 (CH_2), 22.5 (d, $^3J_{C-P}$ = 3.7 Hz, CH_2), 21.9 (d, $^1J_{C-P}$ = 51.0 Hz, CH_2), 14.2 (CH_3), 13.7 (CH_3), 12.1 (CH_3), 12.0 (CH_3); ^{19}F NMR (282 MHz, acetone-d_6 + 10% D_2O): -84.4; HRMS (MeOH hemiacetal, ESI+) calcd. for $C_{49}H_{44}BrF_3N_3O_4PS_6$: 1018.1341 ($[M-Br]^+$), found: 1018.1390.

2.3. Synthesis of ER-HydroFlipper



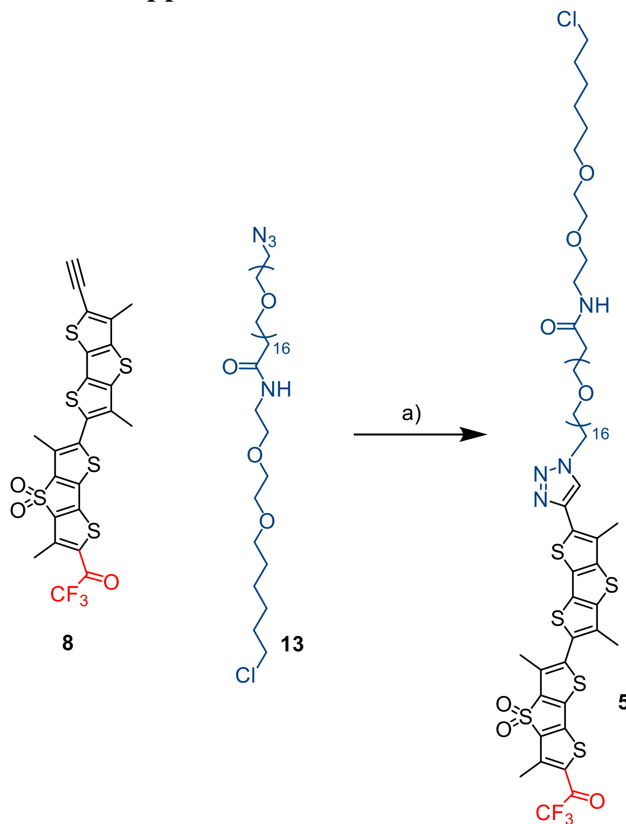
Scheme S3 (a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, TBTA, CH_2Cl_2, DMSO, H_2O, rt, 2 h; (b) Pentafluorobenzoyl chloride, Et_3N, THF, rt, 4 h, 46%.

Compound 11. To a solution of **8** (8.0 mg, 13 µmol) in CH_2Cl_2 (2 mL), **11** (15.4 mg, 27.8 µmol), $CuSO_4 \cdot 5H_2O$ (3.4 mg, 14 µmol), TBTA (5.7 mg, 10 µmol), sodium ascorbate (2.7 mg, 13 µmol), DMSO (20 µL) and water (250 µL) were subsequently added. After 10 minutes, to a solution of $CuSO_4 \cdot 5H_2O$ (1.7 mg, 6.5 µmol) in water 250 µL, sodium ascorbate (1.3 mg, 6.5 µmol) was added; the resulting yellow mixture was immediately added to the reaction mixture. The reaction was stirred for 90 min at rt then, diluted with 10 mL of CH_2Cl_2, washed with water (3×15 mL), dried with Na_2SO_4 and concentrated *in vacuo*. The crude solid was used in the next step without further purification.

Compound 4. To a solution of **12** (13 µmol) in THF (2 mL), triethylamine (3.0 µL, 19 µmol) and pentafluorobenzoyl chloride (2.3 µL 16 µmol) were added and the reaction was stirred at rt for 4 h. The solvents were removed under reduced pressure to yield a crude red wax that was purified by flash chromatography (SiO_2, CH_2Cl_2 to CH_2Cl_2/MeOH 10:1) to yield **4** as a deep red solid (8.4 mg,

46%). R_f (CH_2Cl_2/MeOH 10:1): 0.52; Mp: decomposed > 161 °C; IR (neat): 2921 (m), 1683 (m), 1503 (m), 1314 (m), 1142 (s), 992 (w), 751 (m); ^{1}H NMR (400 MHz, acetone-d_6 + 10% D_2O): 8.41 (s, 1H), 4.67 (t, $^3J_{H-H}$ = 5.1 Hz, 2H), 3.95 (t, $^3J_{H-H}$ = 5.1 Hz, 2H), 3.82 – 3.21 (m, 42H), 2.50 (s, 3H), 2.44 (s, 3H), 2.31 (s, 3H), 2.30 (s, 3H), 2.27 – 2.23 (m, 2H); ^{13}C NMR (126 MHz, acetone-d_6 + 10% D_2O): 158.1 (C=O), 144.4 (C), 142.9 (C), 142.1 (C), 141.9 (C), 135.6 (C), 135.4 (C), 134.9 (C), 132.6 (C), 132.5 (C), 132.1 (C), 131.1 (C), 129.8 (C), 129.4 (C), 129.3 (C), 128.6 (C), 128.0 (C), 127.1 (C), 124.0 (q, $^1J_{C-F}$ = 287.4 Hz, C), 123.1 (CH), 93.0 (q, $^2J_{C-F}$ = 33.5 Hz, $C(OH)_2$), 70.7 – 70.2 (m, 20 CH_2), 69.6 (CH_2), 69.4 (CH_2), 50.8 (CH_2), 40.3 (CH_2), 14.2 (CH_3), 13.8 (CH_3), 12.1 (CH_3), 12.0 (CH_3); ^{19}F NMR (282 MHz, acetone-d_6 + 10% D_2O): -84.1 (s, 3F), -143.0 (m, 2H), -154.8 (m, 1H), -162.8 (m, 2F); HRMS (MeOH hemiacetal, ESI+) calcd. for $C_{56}H_{66}F_8N_4O_{16}S_6$: 1395.2744 ($[M+H]^+$), found: 1395.2744.

2.4. Synthesis of Hydro-HaloFlipper



Scheme S4 (a) $CuSO_4 \cdot 5H_2O$, Na-ascorbate, TBTA, CH_2Cl_2, H_2O, rt, 90 min, 39%.

Compound 13. was synthesized and purified according to procedures described in ref. S1.

Compound 5. To a solution of **8** (10 mg, 17 µmol) in CH_2Cl_2 (2 mL), **10** (9.4 mg, 26 µmol), $CuSO_4.5H_2O$ (4.8 mg, 17 µmol), TBTA (1.2 mg, 3.4 µmol, and sodium ascorbate (3.8 mg, 17 µmol), DMSO (20 µL) and water (250 µL) were subsequently added. After 10 minutes, to a solution of $CuSO_4{\cdot}5H_2O$ (2.4 mg, 8.5 µmol) in water (250 µL). sodium ascorbate (1.9 mg, 8.5 µmol) was added and the resulting yellow mixture was immediately added to the reaction mixture, which was stirred for 90 min at rt then, diluted with of CH_2Cl_2 (10 mL), washed with water (3×15 mL), dried with Na_2SO_4 and concentrated *in vacuo*. The residue was purified by reverse phase column chromatography (Claricep C18, H_2O + 0.1% TFA/MeCN + 0.1% TFA 20 – 90% gradient). The fractions containing product were combined and diluted with CH_2Cl_2. The aqueous phase was extracted two more times with CH_2Cl_2 and the combined organic layers were dried over Na_2SO_4, concentrated under reduced pressure and triturated with pentane to yield **5** as a dark red wax (7.5 mg, 39%). R_f (CH_2Cl_2/MeOH 20:1): 0.35; IR (neat): 2923 (s), 2854 (s), 1721 (s), 1654 (s), 1457 (s), 1376 (m), 1307 (m), 1100 (m), 948 (w), 720 (w); ^1H NMR (500 MHz, acetone-d_6 + 10% D_2O): 8.42 (s, 1H), 4.68 (t, $^3J_{H-H}$ = 4.7 Hz, 2H), 3.96 (t, $^3J_{H-H}$ = 4.7 Hz, 2H), 3.68 (t, $^3J_{H-H}$ = 6.3 Hz, 2H), 3.64 – 3.62 (m, 2H), 3.59 – 3.51 (m, 60H), 3.49 (t, $^3J_{H-H}$ = 5.6 Hz, 2H), 3.41 (t, $^3J_{H-H}$ = 6.7 Hz, 2H), 3.31 (t, $^3J_{H-H}$ = 5.6 Hz, 2H), 2.51 (s, 3H), 2.48 – 2.39 (m, 5H), 2.34 (s, 3H), 2.32 (s, 3H), 1.78 – 1.65 (m, 2H), 1.57 – 1.47 (m, 2H), 1.42 – 1.26 (m, 4H); ^{13}C NMR (126 MHz, acetone-d_6 + 10% D_2O): 172.9 (C), 144.5(C), 143.0 (C), 142.9 (C), 142.2 (C), 141.9 (C), 135.6 (C), 135.4 (C), 134.9 (C), 132.6 (C), 132.2 (C), 131.2 (C), 130.0 (C), 129.5 (C), 128.7 (C), 128.0 (C), 127.4 (C), 127.2 (C), 125.1 (C), 123.1 (CH), 122.8 (C), 120.5 (q, $^1J_{C-F}$ = 287.4 Hz, C), 93.0 (m, $C(OH)_2$), 71.4 (CH_2), 70.7 – 69.6 (m, 36 CH_2), 67.6 (CH_2), 50.9 (CH_2), 45.8 (CH_2), 39.6 (CH_2), 36.9 (CH_2), 32.9 (CH_2), 27.0 (CH_2), 25.7 (CH_2), 14.3 (CH_3), 13.8 (CH_3), 12.1 (CH_3), 12.0 (CH_3); ^{19}F NMR (282 MHz, acetone-d_6 + 10% D_2O): -84.1; HRMS (ESI+) calcd. for $C_{69}H_{105}ClF_3N_4O_{23}S_6$ ($[M+H+Na]^{2+}$): 832.2511, found: 832.2517.

3. Fluorescence lifetime measurements in solution

Fluorescence lifetime experiments in dioxane. 1 µM solutions of Flipper **1b** (4 µL of 0.5 mM in DMSO) were prepared in a fluorescence cuvette containing 2 mL dioxane or dioxane/water mixture. Fluorescence decays were acquired under magic-angle conditions by using the time-correlated single-photon counting technique on a previously described setup (SX). Excitation at 480 ± 15 nm was provided by a tuneable pulsed picosecond laser (NKT-Photonics). Fluorescence emission was collected into an optical fibre, spectrally filtered to 600 ± 10 nm with a spectrograph (Horiba), and detected on a fast avalanche photodiode (PicoQuant). The instrument response function of the setup has a full width at half-maximum shorter than 100 ps.[S5]

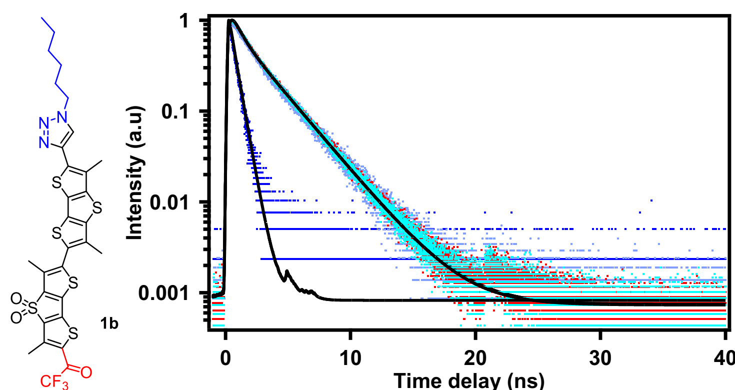


Fig. S2 Fluorescence Lifetime decays of **1b** in dioxane (red) and dioxane:water mixtures (0.2% water, light blue; 0.5% water, blue; 20% water, dark blue).

Table S1. Lifetimes (τ_1, τ_2, τ_3) and relative amplitudes (RA_1, RA_2, RA_3) extracted from a biexponential fit to the fluorescence decay of **1b** in water-dioxane solvent mixtures.

Cpd[a]	Water (%)[b]	τ_1 (ns)	RA_1	τ_2 (ns)	RA_2	τ_3 (ns)	RA_3
1b	0	0.7	0.4	2.7	0.6		
1b	0.2	0.7	0.4	2.7	0.6		
1b	0.5	0.6	0.4	2.6	0.6		
1b	20	0.5	0.3			< 0.1	0.7

[a]Compound. [b]Water percentage in volume.

4. Cell lines and DNA transfections

HeLa Kyoto cells. The cells were cultured in 25 cm^2 cell culture flasks with a vent cap and grew in DMEM (with GlutaMAX) + 10% FBS + 1% Pen/Strep. For microscopy experiments, the cells were seeded at 8 $\times 10^4$ cells/mL in DMEM + 10% FBS + 1% Pen/Strep on 35 mm glass bottom dishes and incubated overnight.

HeLa cells stably expressing the HaloTag-GFP-Mito fusion protein (HGM): As described in ref. S1. The cells were originally designed by the Chenoweth lab[S6] and cultured as described in S7.

General procedure for microscopy experiments. Cells were seeded at 8 $\times 10^4$ cells/mL in DMEM (with GlutaMAX) + 10% FBS + 1% Pen/Strep on 35 mm glass bottom dishes (2 mL per dish) or 8-well glass bottom µ-slides (µL per well) for confocal microscopy or in FluoroBrite DMEM + 10% FBS on 96-well ibiTreat sterile µ-plates (150 µL per well), and kept at 37 °C with 5% CO_2 overnight.

General procedure for transfection. As described in ref. S1. HeLa cells were seeded at 5 $\times 10^4$ cells/mL in DMEM + 10% FBS + 1% Pen/Strep on 35 mm glass bottom dishes (2 mL per dish) or 8-well glass bottom µ-slides (µL per well), and kept at 37 °C with 5% CO_2 overnight. The next day, transient transfection was performed by diluting the cells with Lipofectamine® 2000 – DNA complexes prepared as follows (conditions per well, multiplied by a factor of 6 for a glass bottom dish): Lipofectamine® 2000 (0.5 µL) was diluted with Opti-MEM/GlutaMAX reduced serum medium (25 µL) and left incubate at rt for 5 min. DNA (amount stated in Table S2) was diluted with Opti-MEM/GlutaMAX reduced serum medium (25 µL), gently mixed and added to the diluted Lipofectamine solution. The resulting solution was gently mixed and incubated for 20 min at rt. From each well of µ-slides, half of the original medium was removed. The solution of DNA-Lipofectamine complex (50 µL each) was added to each well. The cells were kept at 37 °C with 5% CO_2. After 4 h, the medium was exchanged with fresh DMEM + 10% FBS + 1% Pen/Strep or FluoroBrite DMEM + 10% FBS and the cells were incubated overnight.

Plasmids were kind gifts of several research groups as summarized in Table S2.[S1]

Table S2. Overview of Plasmids Used in Presented Study

Organelle[b]	Fusion protein[c]	Origin[d]	m^e (µg)
Peroxisomes	PEX3-GFP-HaloTag	Lampson group[S8] (Addgene)	0.25 – 0.5
Endo-lysosomes	LAMP1-HaloTag-meGFP	Hensel group[S9]	0.25 – 0.5
Golgi apparatus	GTS-HaloTag-meGFP	Hensel group[S9]	0.25
Golgi apparatus	ST-HaloTag-HA	Toomre group[S10]	0.125 – 0.25
Endoplasmic reticulum	HaloTag-Sec61B	See Ref S1	0.125 – 0.25

[a]Plasmid. [b]Organelle targeted by using given plasmid. [c]The constructs: protein(s) of interest fused to the HaloTag protein. [d]Plasmid prepared/provided by. [e]Amount of plasmid used for transfection as discussed above.

5. Confocal laser scanning microscopy (CLSM) in cells

Colocalization studies for unmodified HK cells.[S4] The day of the experiment, seeded cells were washed with Leibovitz's medium (3 × 1 mL) and incubated in 2 mL of Leibovitz's medium containing the probe and the appropriate tracker at the desired concentration and time (see figure captions in section 5.1 and 5.2). Then, cells were washed again with Leibovitz's medium (3 × 1 mL) and proceed to imaging. Fluorescence distribution was analyzed without fixing. Sequential scans were performed using two lasers (λ_{ex1} = 488 nm, 40 % LP, λ_{ex2} = 561 nm, 5% LP) and the fluorescence was collected between 500 – 590 nm for the flipper probes and 620 – 675 nm for their respective tracker LysoTracker® Red, ER-Tracker™ Red and MitoTracker® Red; avoiding any bleedthrough.

Colocalization studies for HGM and transfected HK cells.[S1] The day of the experiment, seeded HGM or transiently transfected HeLa cells were washed with Leibovitz medium (3 × 200 µL

per well) and incubated with **5** (25 nM) in Leibovitz medium (150 μL) for 15 min at 37 °C under 5% CO_2. The cells were washed with Leibovitz's medium (3 × 200 μL) and finally kept in clean Leibovitz's medium. Distribution of fluorescent compounds was analyzed without fixing. Argon laser was used as light source with excitation wavelength 488 nm and emission 618 – 737 nm in the flipper channel, LP: 30% and 492 – 534 nm for the GFP channel, LP: 10%.

Pearson Correlation Coefficient. ROIs were selected and the PCCs (without threshold) were calculated using ImageJ for each of them similar to previous publications.[S1, S4]

Assay Protocol and Analysis for CAPA. As in ref. S1, the day of the experiment, seeded HGM cells were washed with PBS (3 × 3 mL/well) and the media was exchanged to Leibovitz's (4 × 150 μL/well) using a plate washer, keeping a final volume of 135 μL/well. Then, serial dilutions of probe **5** and control HaloFlipper **6** in PBS were prepared in a 96-well V-bottom plate and added to the μ-Plate containing the cells (15 μL/well, 10x final concentration in PBS) to reach a final volume of 150 μL/well (0 to maximum 1 μM). Duplicates were performed for each condition. Cells were incubated for 45 min at 37 °C with 5% CO_2. After this, cells were washed again and **7** was added (15 μL/well, 50 μM in PBS) to reach a final volume of 150 μL/well (5 μM), except for the control wells, where only PBS was added (15 μL/well). After 15 min of incubation at 37 °C with 5% CO_2, the plate was washed again. Then, Hoechst 33342 was added (15 μL/well, 170 μM in PBS) to reach a final volume of 150 μL/well (17 μM). After 15 min of incubation at 37 °C with 5% CO_2, the plate was washed one last time and the cells were kept in clean Leibovitz's media.

For signal subtraction of the residual fluorescence of the probes in the red channel (rhodamine channel), the same experiment was carried out, skipping the addition of **7** and incubating instead with clean media.

During imaging, samples were kept at 37 °C with 5% CO_2. A total of 16 images/well at 40x were recorded, using three channels: blue (excitation filter: 377/50 nm, emission filter: 477/60 nm, exposure time: 10 ms), green (excitation filter: 475/34 nm, emission filter: 536/40 nm, exposure time: 20 ms) and red (excitation filter: 531/40 nm, emission filter: 593/40 nm, exposure time: 40 ms).

As in ref. S11, nuclei segmentation of the blue channel image and cell body segmentation and top-hat transform of the green channel image are applied. Mitochondria mask is applied to extract the integrated intensity value for each condition in the red channel image, which is corrected by subtraction of the integrated intensity value without addition of **7**. The corrected integrated intensity values for each condition were then normalized.

The resulting dependence of the relative intensity values (I_{HRO}) to the concentration of probe **5** or **6** (c) was plotted and fitted with Equation (S1) to retrieve the half maximal effective concentration (EC_{50}) value.

$$I_{HRO} = 1 / (1 + (c / EC_{50})) \qquad (S1)$$

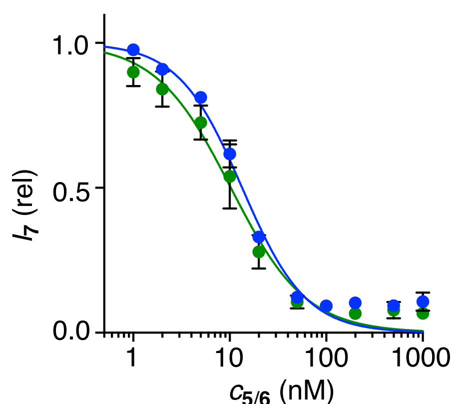


Fig. S3 CAPA dose-response curves after 45 min incubation of **5** (green) and **6** (blue). Duplicates were performed for each condition.

Table S3. Calculated EC_{50} values extracted from plots in Fig. S3.

Cpd	EC_{50} (nM)
5	10.3 ± 0.8
6	13 ± 1

5.1. CLSM in unmodified HK Cells

5.1.1. Lysosomes colocalization

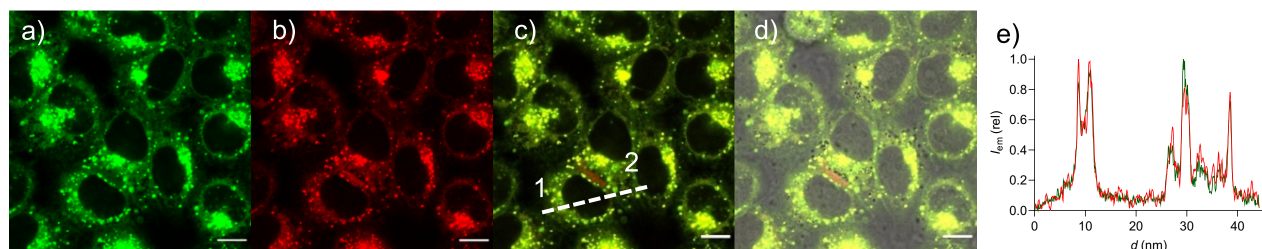


Fig. S4 CLSM images of HK cells after incubation with **2** (2 µM)**,** 10 min, and LysoTracker® Red (200 nM), 10 min. a) Green channel: LysoTracker® Red; b) red channel: Flipper **1**; c) green+red: merged channels; d) green+red+brightfield; e) emission profile (from 1 to 2) normalized, green and red channels. Brightness and contrast of the fluorescent signal were adjusted to comparable values. Scale bar: 10 µm. PCC 0.88.

5.1.2. Mitochondria colocalization

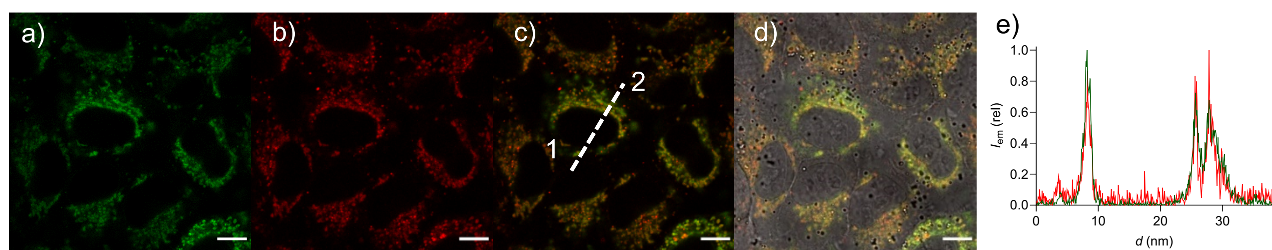


Fig. S5 CLSM images of HK cells after incubation with **3** (4 µM)**,** 20 min, and MitoTracker® Red (100 nM), 10 min. a) Green channel: MitoTracker® Red; b) red channel: **3**; c) green+red: merged channels; d) green+red+brightfield; e) emission profile (from 1 to 2) normalized, green and red channels. Brightness and contrast of the fluorescent signal were adjusted to comparable values. Scale bar: 10 µm. PCC 0.84.

5.1.3. ER colocalization

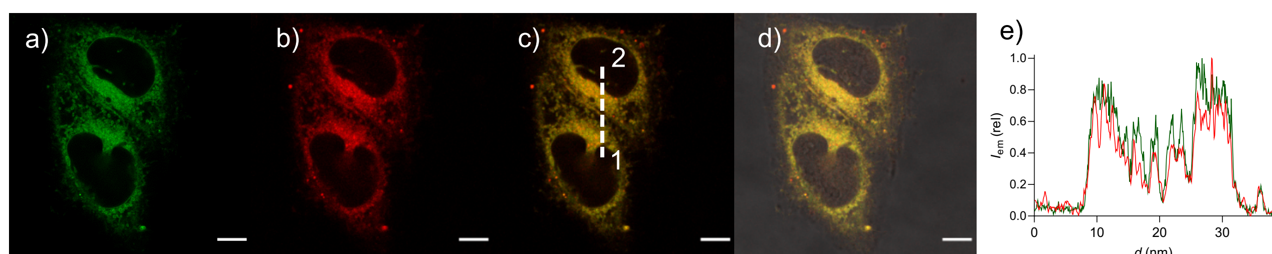


Fig. S6 CLSM images of HK cells after incubation with **4** (4 µM)**,** 20 min, and ER-Tracker^{TM} Red (100 nM), 10 min. a) Green channel: ER-Tracker^{TM} Red; b) red channel: **4**; c) green+red: merged channels; d) green+red+brightfield; e) emission profile (from 1 to 2) normalized, green and red channels. Brightness and contrast of the fluorescent signal were adjusted to comparable values. Scale bar: 10 µm. PCC 0.84.

5.2. CLSM in HGM cells

*5.2.1. Colocalization with **5***

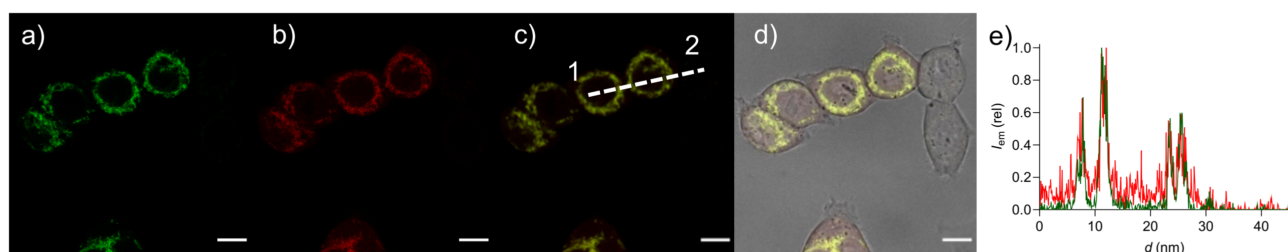


Fig. S7 CLSM image of HGM cells after incubation with **5** (25 nM, 45 min). a) Green channel: GFP; b) red channel: Flipper **5**; c) green+red: GFP+**5**; d) green+red+brightfield; e) emission profile (from 1 to 2) normalized, green and red channels (covering a non-transfected cell, a transfected cell and a cell-free background). Scale bar: 10 µm. PCC = 0.80.

*5.2.2. Colocalization with **3***

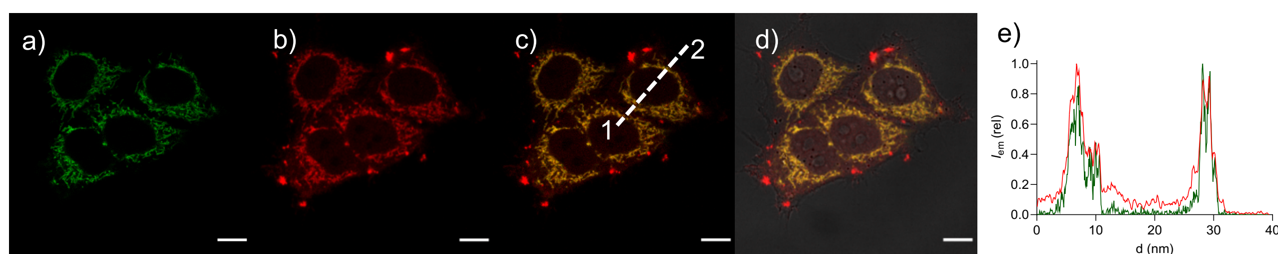


Fig. S8 CLSM image of HGM cells after incubation with **3** (4 µM, 20 min). a) Green channel: GFP; b) red channel: Flipper **3**; c) green+red: GFP+**3**; d) green+red+brightfield; e) emission profile (from 1 to 2) normalized, green and red channels (covering a non-transfected cell, a transfected cell and a cell-free background). Scale bar: 10 µm. PCC = 0.86.

5.3. CLSM in transfected HK cells expressing GFP in different organelles

5.3.1. Colocalization in peroxisomes

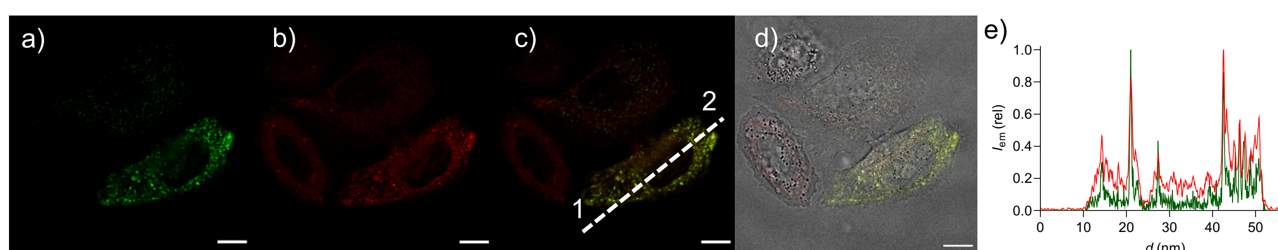


Fig. S9 CLSM image of transfected HK cells with PEX3-GFP-HaloTag after incubation with **5** (25 nM, 45 min). a) Green channel: GFP; b) red channel: Flipper **5**; c) green+red: GFP+**5**; d) green+red+brightfield; e) emission profile (from 1 to 2) normalized, green and red channels. Scale bar: 10 µm. PCC = 0.76.

5.3.2. Colocalization in lysosomes

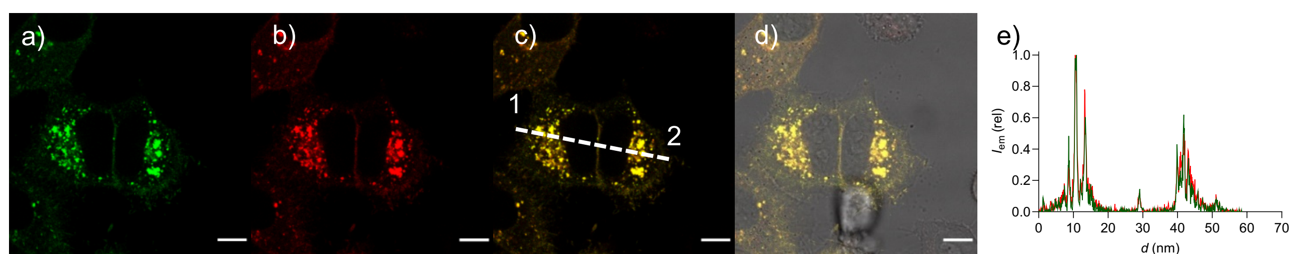


Fig. S10 CLSM image of transfected HK cells with p2 (LAMP1-HaloTag-meGFP) after incubation with **5** (25 nM, 45 min). a) Green channel: GFP; b) Red channel: Flipper **5**; c) green+red: GFP+**5**; d) green+red+brightfield; e) emission profile (from 1 to 2) normalized, green and red channels. Scale bar: 10 µm. PCC = 0.90.

5.3.3. Colocalization in Golgi apparatus

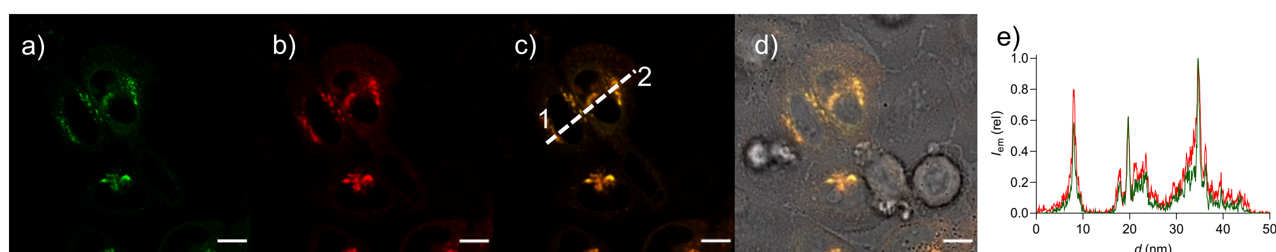


Fig. S11 CLSM image of transfected HK cells with p3 (GTS-HaloTag-meGFP) after incubation with **5** (25 nM, 45 min). a) Green channel: GFP; b) red channel: Flipper **5**; c) green+red: GFP+**5**; d) green+red+brightfield; e) emission profile (from 1 to 2) normalized, green and red channels. Scale bar: 10 µm. PCC = 0.85.

6. FLIM studies

6.1. FLIM experiments in GUVs

Experiments were performed as described in reference S2.

6.2. FLIM experiments in cells

Measurements under isotonic conditions. The day of the experiment, seeded (and transfected, if required) HeLa Kyoto cells were washed with Leibovitz's medium (3 × 1 mL) and incubated with probe **1** (4 μM, 15 min), **2–4** (4 μM, 30 min) or **5** (25 nM, 45 min) in Leibovitz's medium (1 mL) at 37 ºC under 5% CO_2. The cells were then washed with Leibovitz's medium (2 × 1 mL) and finally kept in clean medium (1 mL). The washing step was not required for probe **1**. Several images were acquired in different positions of the dish.

Measurements after hypertonic shock. Samples previously recorded under isotonic conditions were then treated with an aq. sucrose solution (1 M, 1 mL) to reach a final volume of 2 mL (0.5 M sucrose). After 10 min of equilibration, images were acquired in the same previous positions of the dish.

Measurements after cholesterol depletion. The day of the experiment, seeded HeLa Kyoto cells were washed with Leibovitz's medium (3 × 1 mL) and incubated with a solution of MβCD in Leibovitz's medium (5 mM, 1 mL) for 60 min. For the experiments with probe **1**, cells were washed with Leibovitz's medium (1 × 1 mL), the probe was added (4 μM) and, after 10 min of incubation, several images were directly acquired in different positions of the dish. For the experiments with probe **4**, 30 min after addition of MβCD, the probe was added (4 μM) and the cells were incubated for extra 30 min. Then, the cells were washed with Leibovitz's medium (2 × 1 mL) and finally kept in medium containing MβCD (5 mM, 1 mL). Several images were acquired in different positions of the dish.

As controls, same experiments were performed replacing medium containing MβCD with clean Leibovitz's medium.

6.3. FLIM analysis

In all cases, a pixel binning to two points was performed to increase the number of photons per pixel and improve the fitting. A threshold was also applied in each image to exclude background pixels during the analysis.

The obtained fluorescence decay curves were fit using n-exponential reconvolution model (n = 3).

The values of τ_1 and τ_2 reported in Table S4 are those directly obtained from the fitted curve. Then, a FLIM fit of the image was performed, fixing the "shift IRF", "background IRF" and "background decay" to zero. Since lifetime values of the components, accounting for the hydration and planarization of the probe, depend on its local environment, the three fitted lifetime values (τ_1–τ_3) and their corresponding intensities were not fixed during the analysis.

The updated lifetime frequency histogram containing the three contributions was then used to calculate the dehydration factor dh, using the total integrated counts from τ_1 and τ_2, $\Sigma\tau_1$ and $\Sigma\tau_2$, respectively, as in Equation (S2):

$$dh = \text{area } \Sigma\tau_1 / \text{area } \Sigma\tau_2 \qquad \text{(S2)}$$

The reported values in Table S4 represent the mean ± standard deviation from at least three pictures containing at least 6 cells each. To guide eye, reconvoluted histograms were fitted using Gaussian function.

Table S4. Dehydration factors and lifetimes of HydroFlippers.

	probe[a]	dh_i[b]	dh_h[c]	τ_{1i} (ns)[d]	τ_{2i} (ns)[e]	τ_{1h} (ns)[f]	τ_{2h} (ns)[g]
1	**1** (PM)	6.3 ± 0.8	6.5 ± 0.2	4.79 ± 0.01	1.78 ± 0.06	4.40 ± 0.02	1.8 ± 0.1
2	**1** (+CL)[h]	6.6 ± 1	-	4.96 ± 0.02	2.8 ± 0.2	-	-
3	**1** (-CL)[i]	6.1 ± 0.1	-	4.80 ± 0.03	2.5 ± 0.1	-	-
4	**2** (Lyso)	2.94 ± 0.01	2.81 ± 0.06	4.42 ± 0.01	1.59 ± 0.01	3.98 ± 0.02	1.52 ± 0.04
5	**3** (Mito)	2.3 ± 0.1	1.9 ± 0.1	4.40 ± 0.02	1.48 ± 0.06	4.03 ± 0.02	1.43 ± 0.08
6	**4** (ER)	1.8 ± 0.1	1.5 ± 0.1	4.33 ± 0.02	1.45 ± 0.01	3.68 ± 0.01	1.35 ± 0.01
7	**4** (+CL)[h]	1.8 ± 0.3	-	4.48 ± 0.03	2.21 ± 0.03	-	-
8	**4** (-CL)[i]	1.11 ± 0.07	-	4.05 ± 0.06	1.88 ± 0.04	-	-
9	$\mathbf{5}_G$ (GA)[j]	2.5 ± 0.1	2.3 ± 0.3	4.18 ± 0.02	1.53 ± 0.03	3.75 ± 0.03	1.50 ± 0.01
10	$\mathbf{5}_E$ (ER)[k]	1.7 ± 0.1	1.2 ± 0.1	3.82 ± 0.01	1.36 ± 0.02	3.65 ± 0.04	1.41 ± 0.04
11	**1** (L_o)[l]	11 ± 3	-	5.16 ± 0.05	1.9 ± 0.1	-	-
12	**1** (L_d)[m]	1.21 ± 0.09	-	3.42 ± 0.06	1.29 ± 0.03	-	-

[a]Flipper (target MOI). [b]dh_i = area $\Sigma\tau_{1i}$ / area $\Sigma\tau_{2i}$ in FLIM histogram under iso-osmotic (i) conditions. [c]dh_h = area $\Sigma\tau_{1h}$ / area $\Sigma\tau_{2h}$ in FLIM histogram under hyper-osmotic (h) conditions. [d]Fluorescence lifetime value of the slowest component from the fitted fluorescence decay under iso-osmotic (i) conditions. [e]Same as *d*, for the fluorescence lifetime value of the intermediate component. [f]Same as *d*, under hyperosmotic (h) conditions. [g]Same as *e*, under hyperosmotic (h) conditions. [h]Control experiment without cholesterol (CL) removal in PM (entry 2) or ER (entry 7). [i]Measured after cholesterol (CL) removal from cells with MβCD in PM (entry 3) or ER (entry 8). [j]Measured in transiently transfected HeLa cells with ST-HaloTag-HA expressed on GA. [k]Measured in transiently transfected HeLa cells with HaloTag-Sec61B expressed on ER. [l]Measured in SM/CL GUVs as described previously,[S2] and newly analyzed. [m]Measured in DOPC GUVs as described previously,[S2] and newly analyzed.

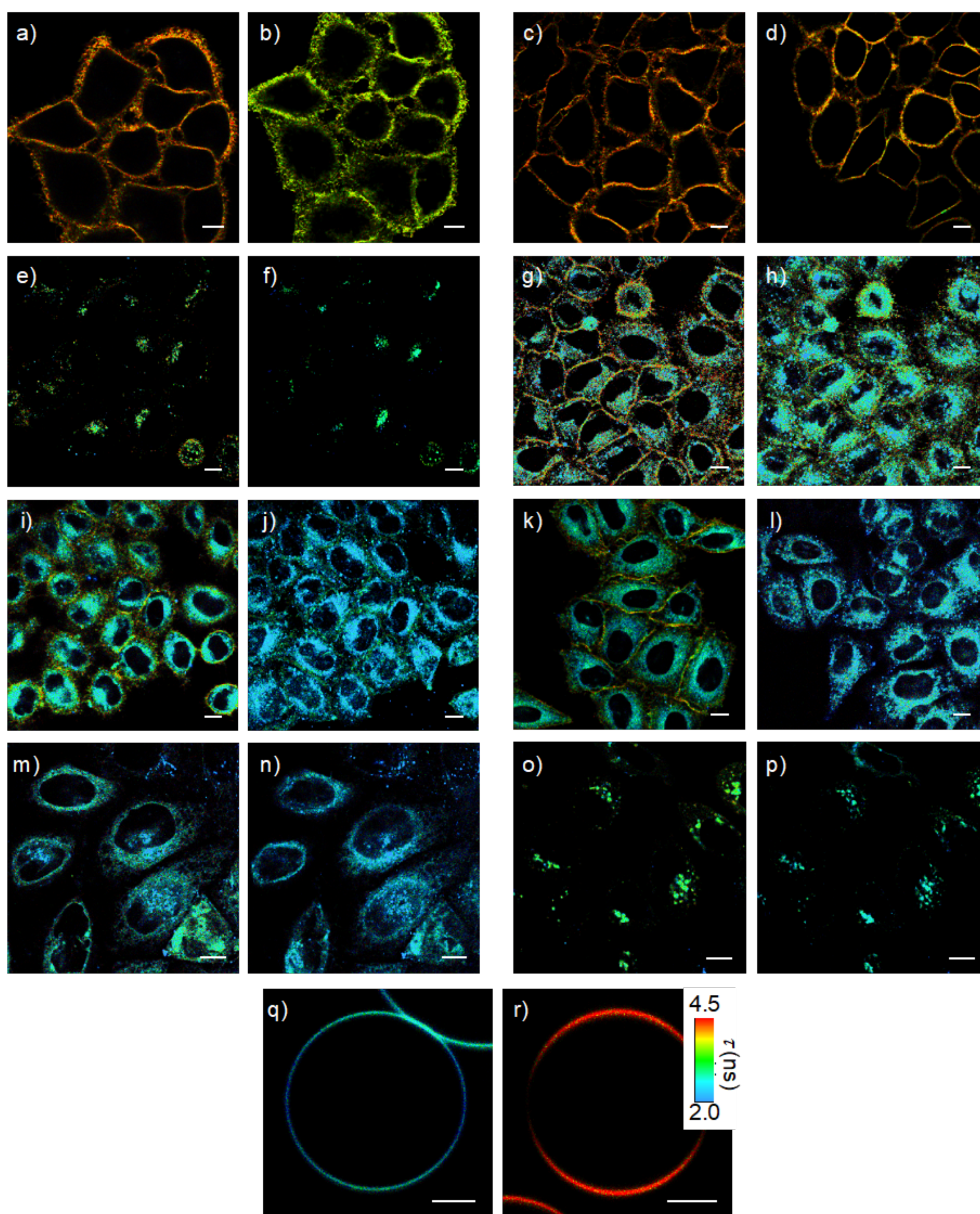


Figure S12. FLIM images showing τ (average) in non-transfected HK cells (a-l), HK cells transfected with HaloTag-Sec61B plasmid (m,n), HK cells transfected with ST-HaloTag-HA (o,p), DOPC GUVs (q) and SM/CL GUVs (r) after incubation with **1** (4 µM, 15 min, a-d; 2 µM, < 1 min, q-r), **2** (4 µM, 30 min, e-f), **3** (4 µM, 30 min, g-h), **4** (4 µM, 30 min, i-l) and **5** (25 nM, 45 min, m-p) under isotonic conditions (a, c, e, g, i, k, m, o), hypertonic conditions (0.5 M sucrose, b, f, h, j, n, p) or after cholesterol depletion (5 mM MβCD, d, l). Scale bar: 10 µm. (q, r) From reference S2.

7. NMR spectra

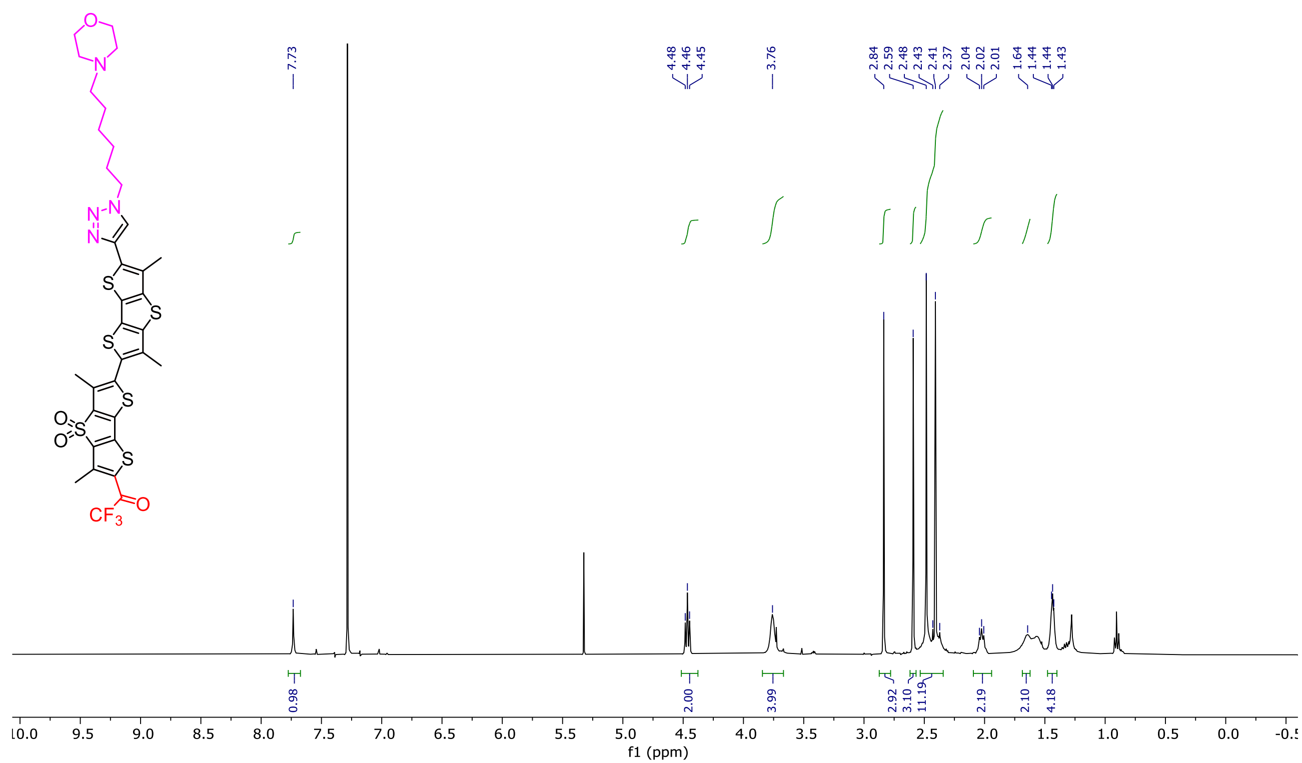


Figure S13. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound **2**.

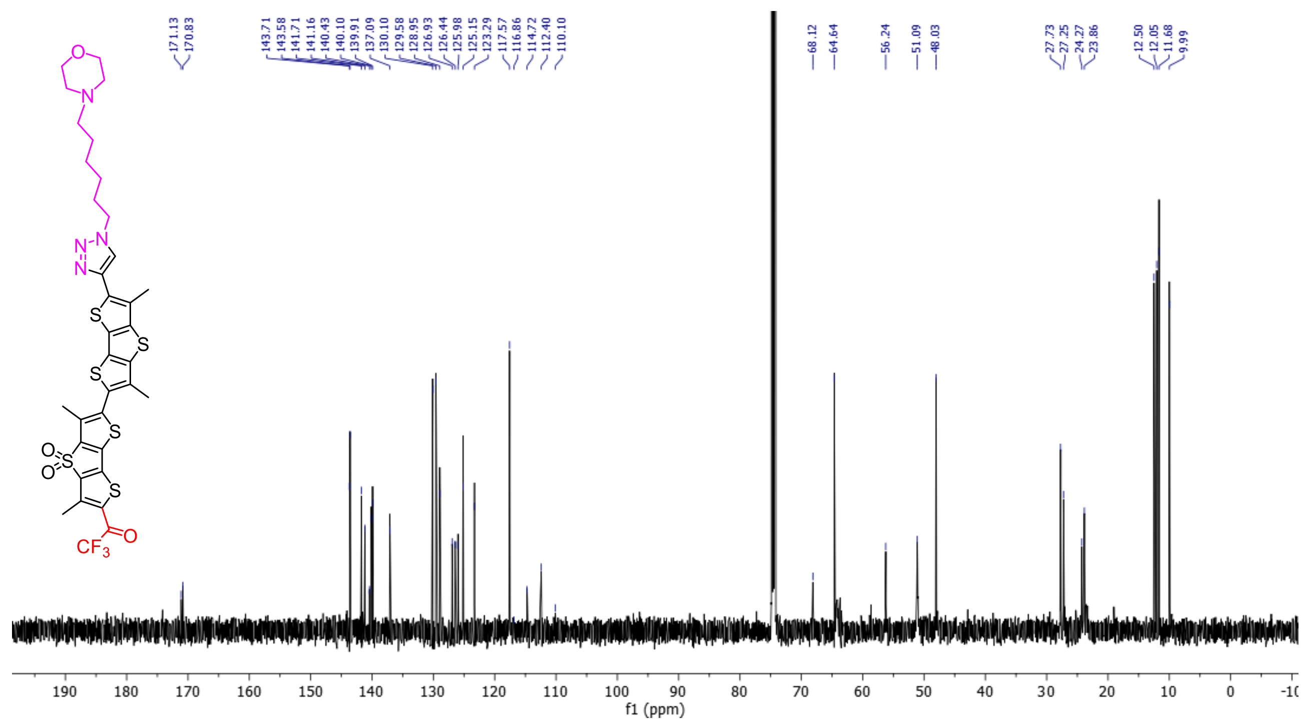


Figure S14. ^{13}C NMR (126 MHz, $CDCl_3$) spectrum of compound **2**.

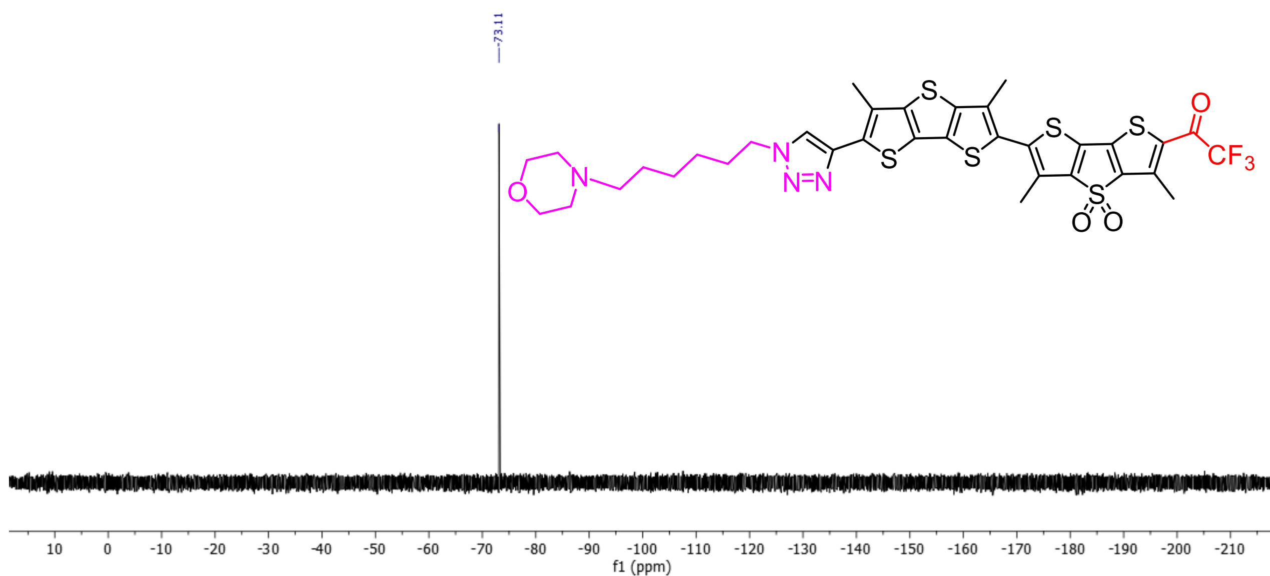


Figure S15. ^{19}F NMR (282 MHz, $CDCl_3$) spectrum of compound **2**.

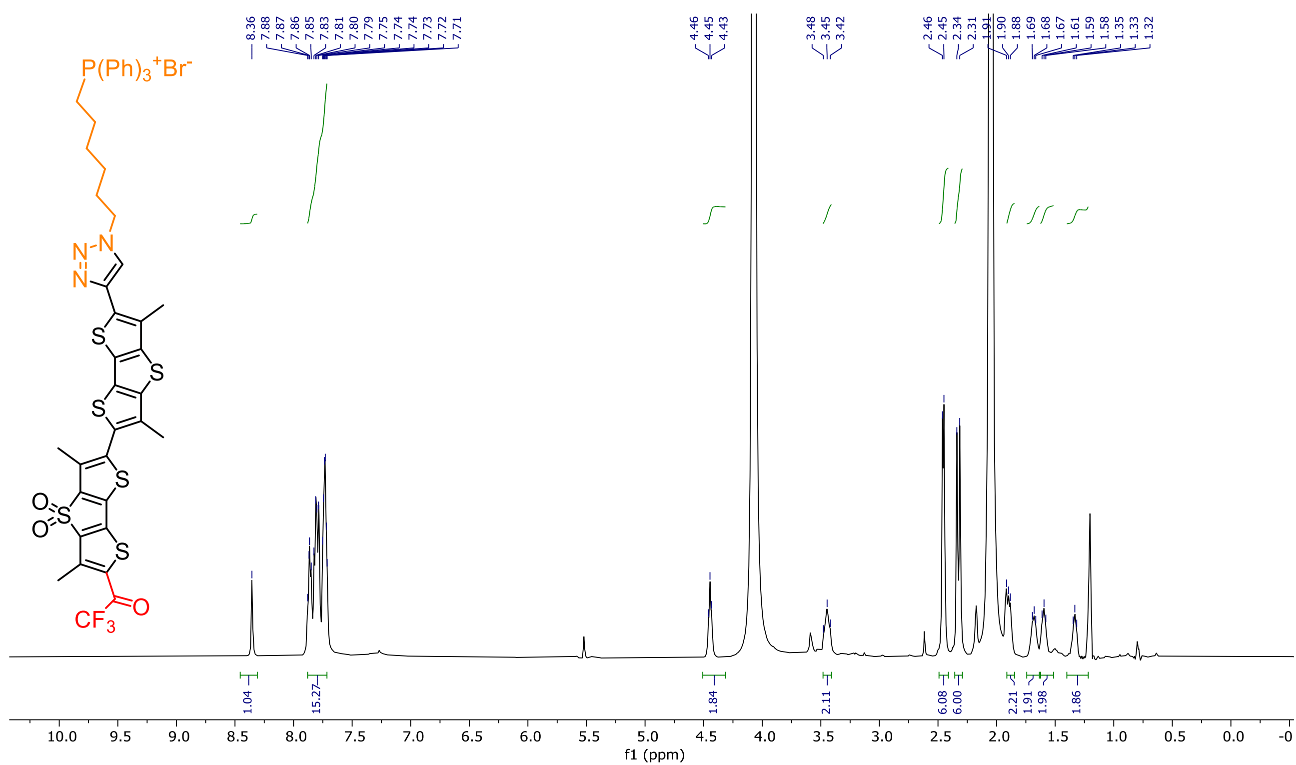


Figure S16. ^{1}H NMR (500 MHz, acetone-d_6 + 10% D_2O) spectrum of compound **3**.

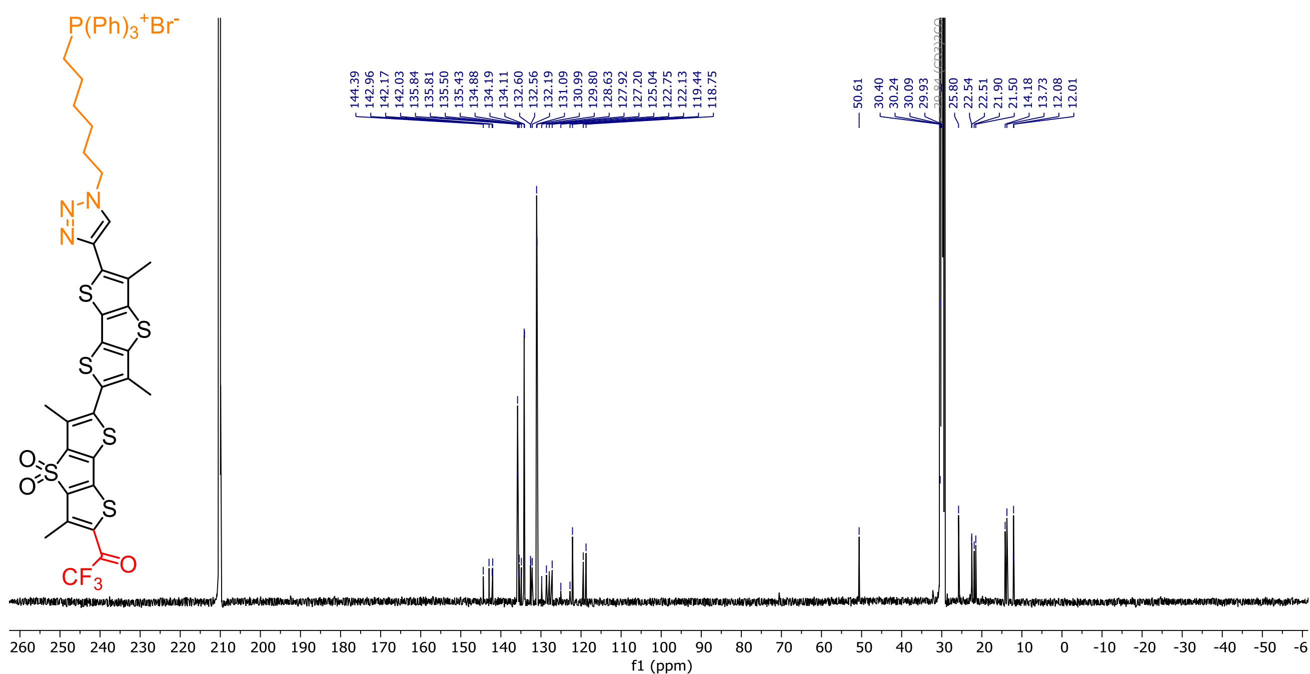


Figure S17. ^{13}C NMR (126 MHz, acetone-d_6 + 10% D_2O) spectrum of compound **3**.

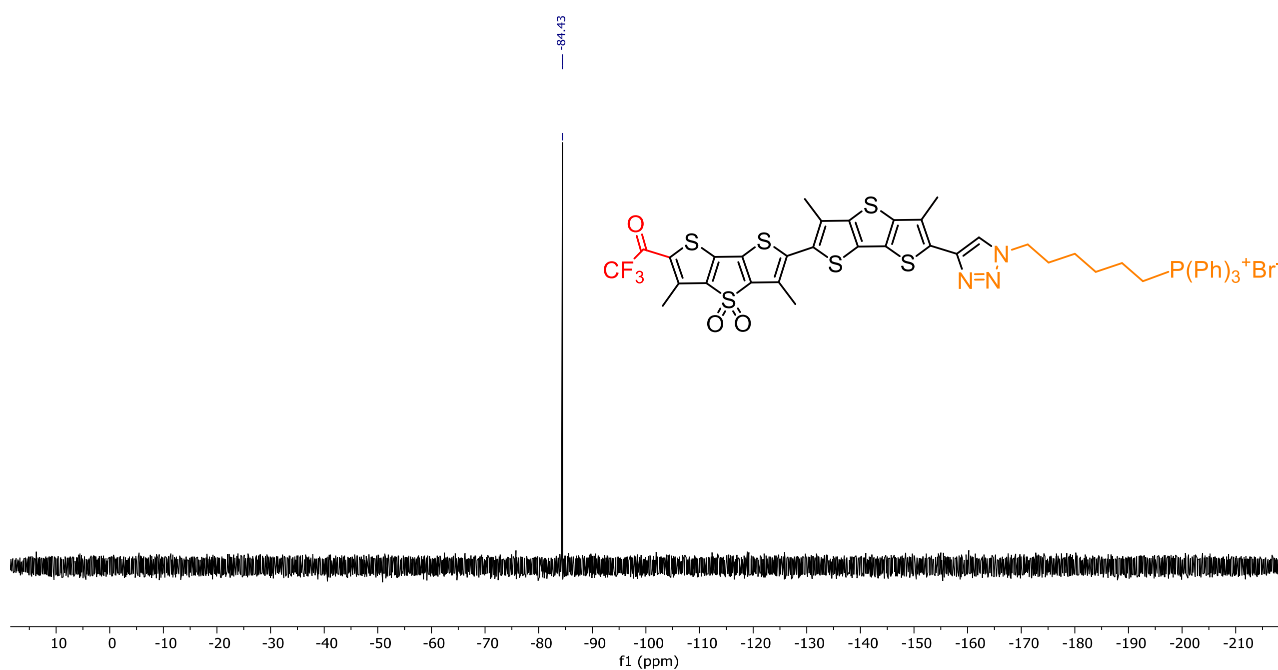


Figure S18. ^{19}F NMR (282 MHz, acetone-d_6 + 10% D_2O) spectrum of compound **3**.

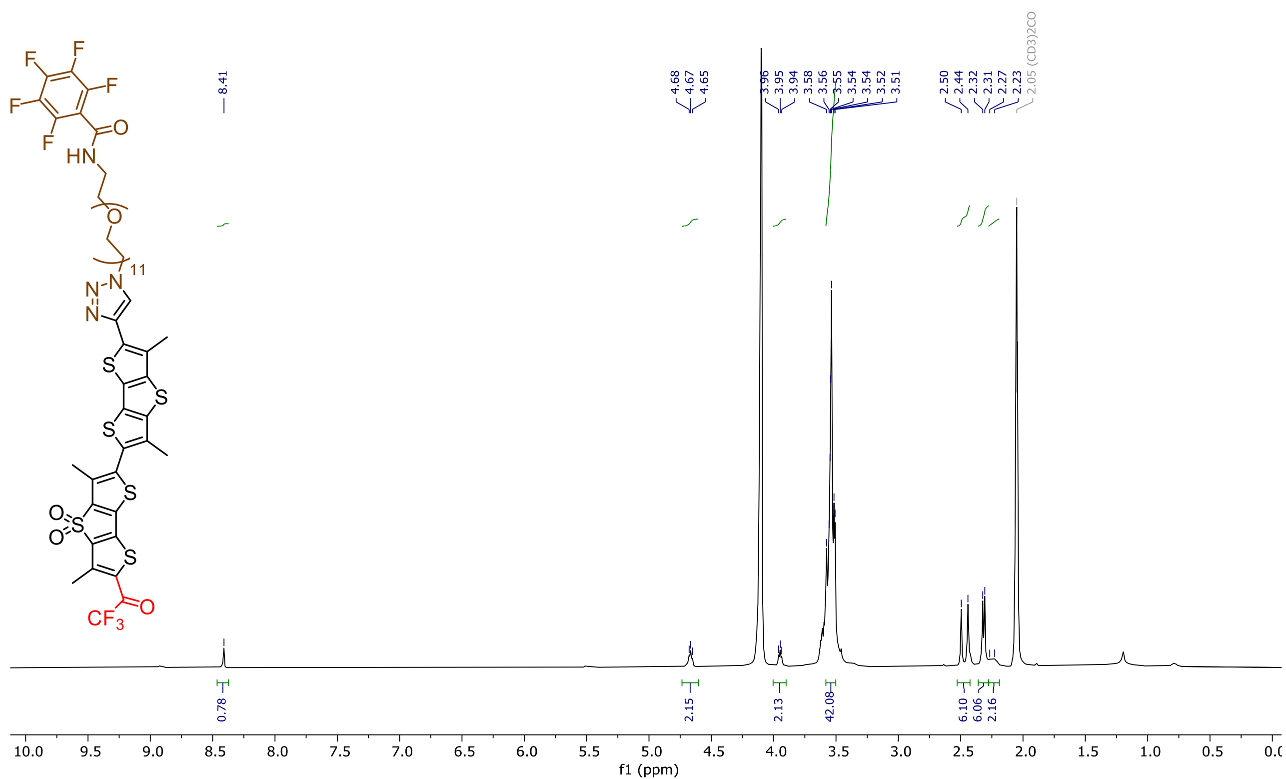
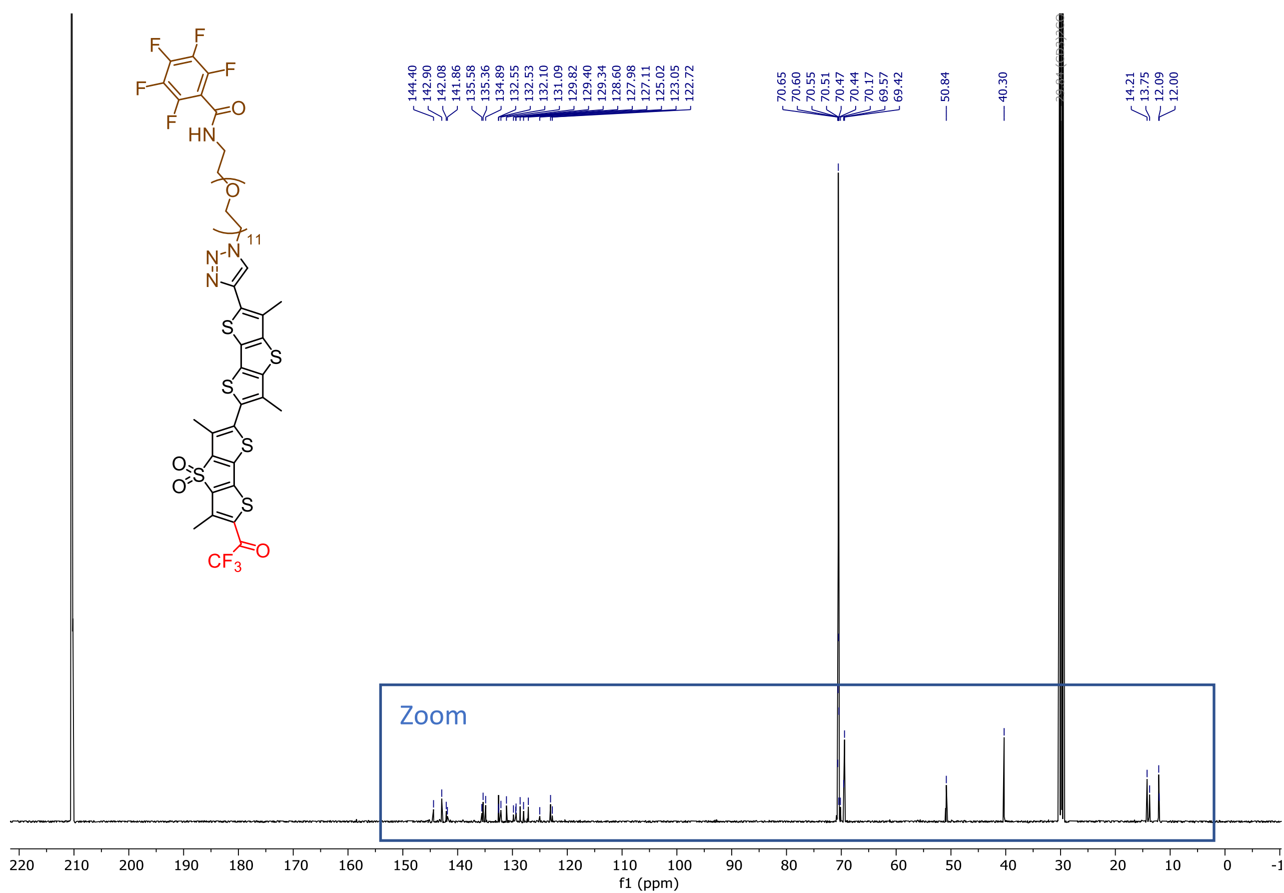


Figure S19. ^{1}H NMR (400 MHz, acetone-d_6 + 10% D_2O) spectrum of compound **4**.



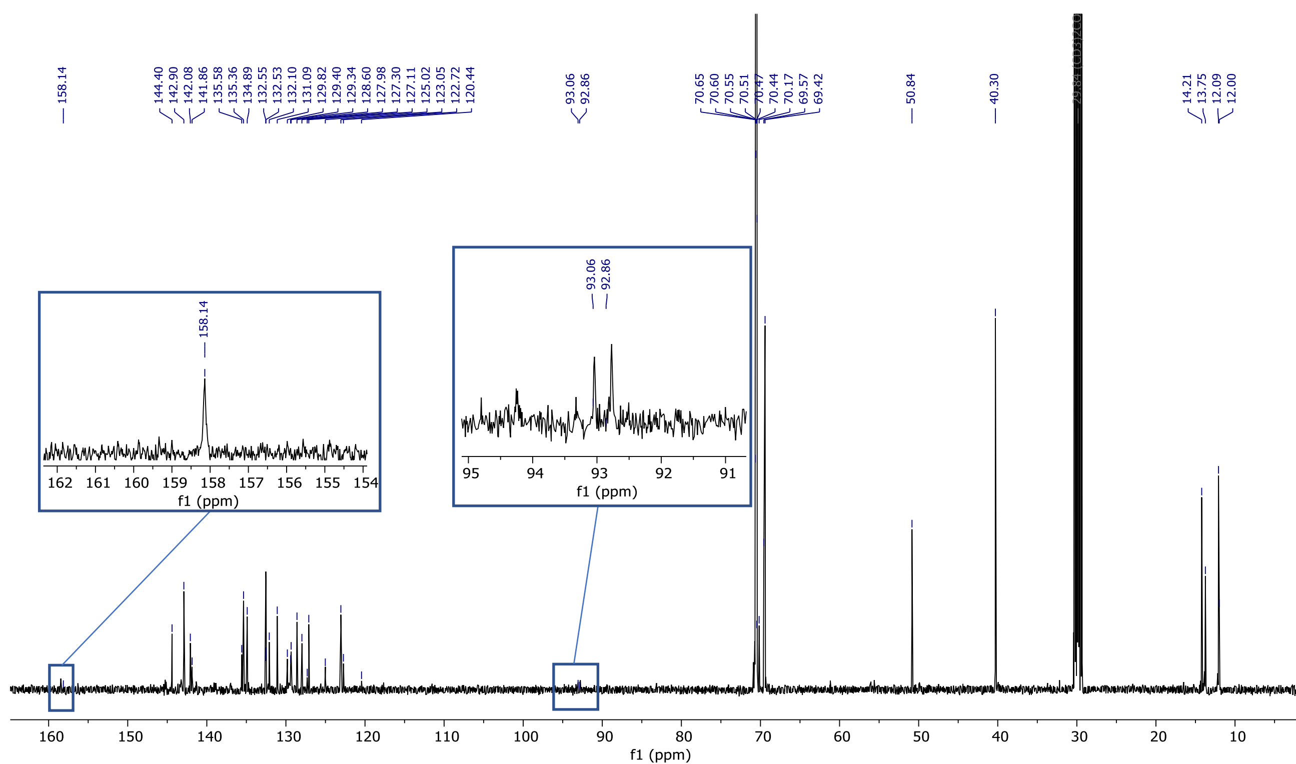


Figure S20. ^{13}C NMR (126 MHz, acetone-d_6 + 10% D_2O) spectrum of compound **4**.

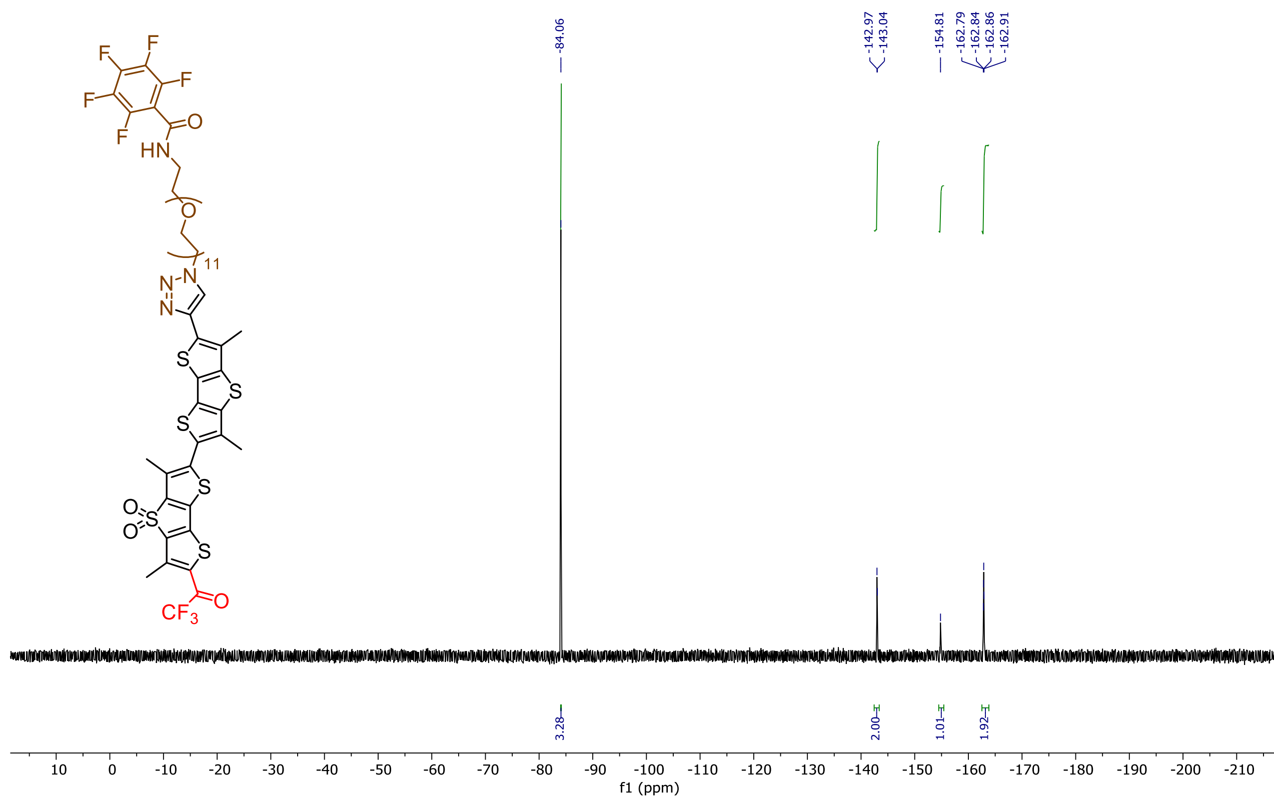


Figure S21. ^{19}F NMR (282 MHz, acetone-d_6 + 10% D_2O) spectrum of compound **4**.

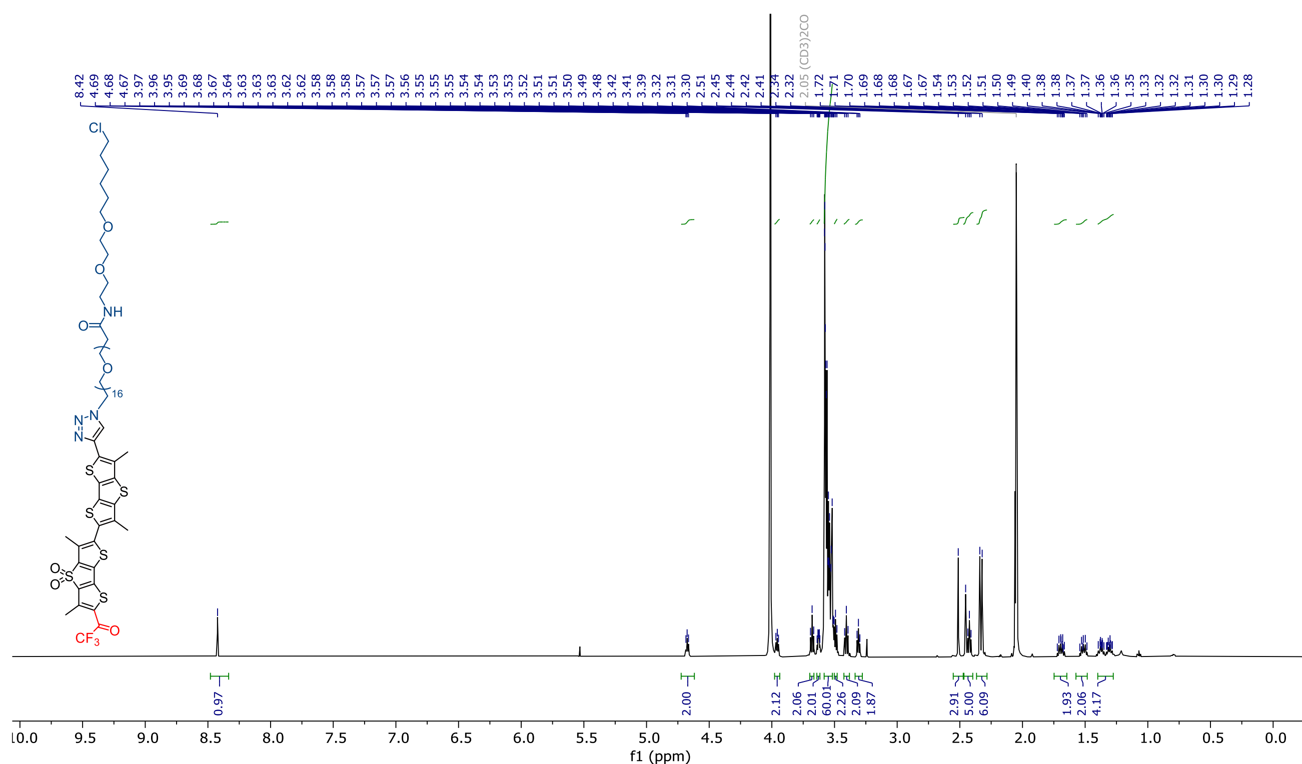
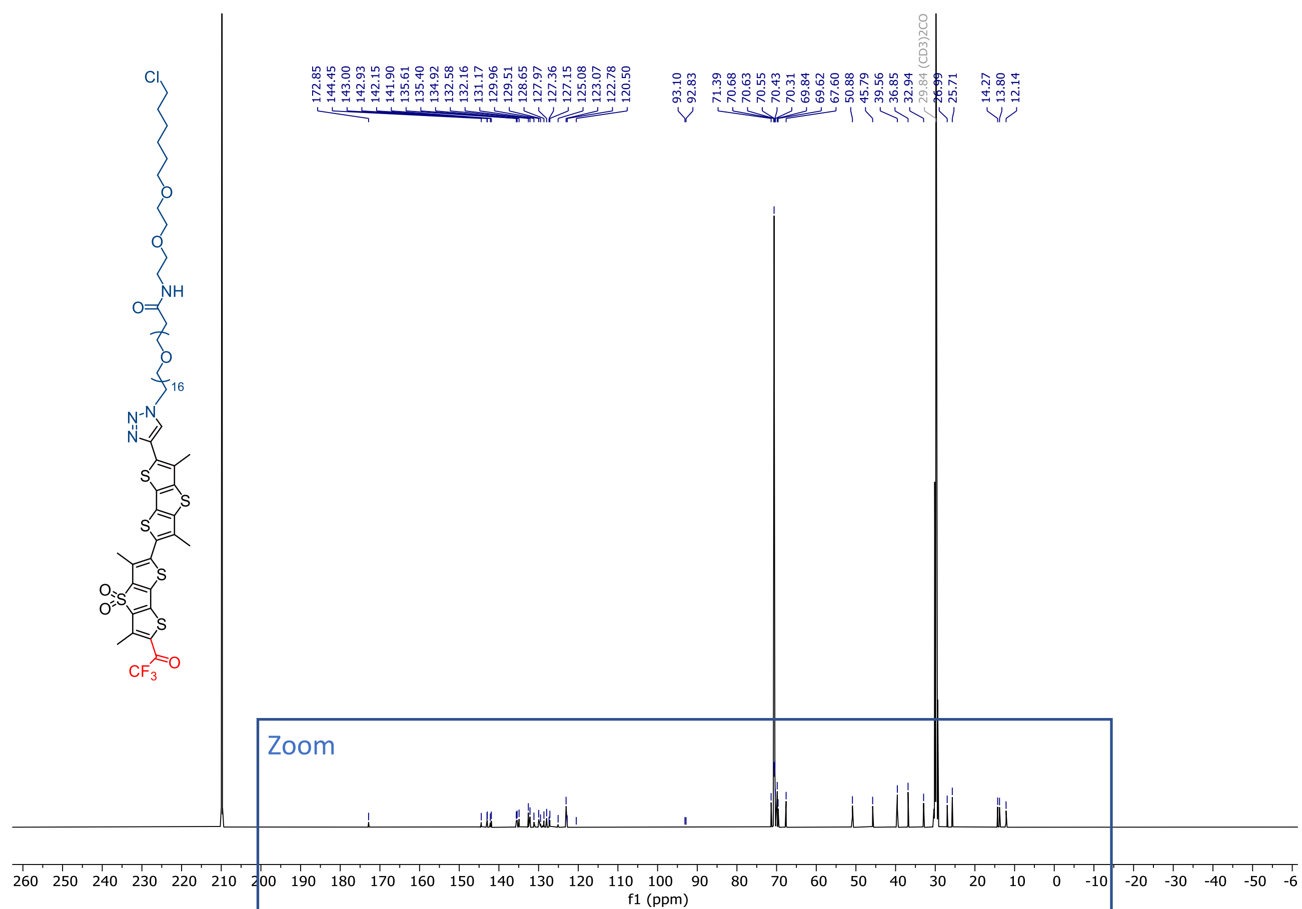


Figure S22. ^{1}H NMR (500 MHz, acetone-d_6 + 10% D_2O) spectrum of compound **5**.



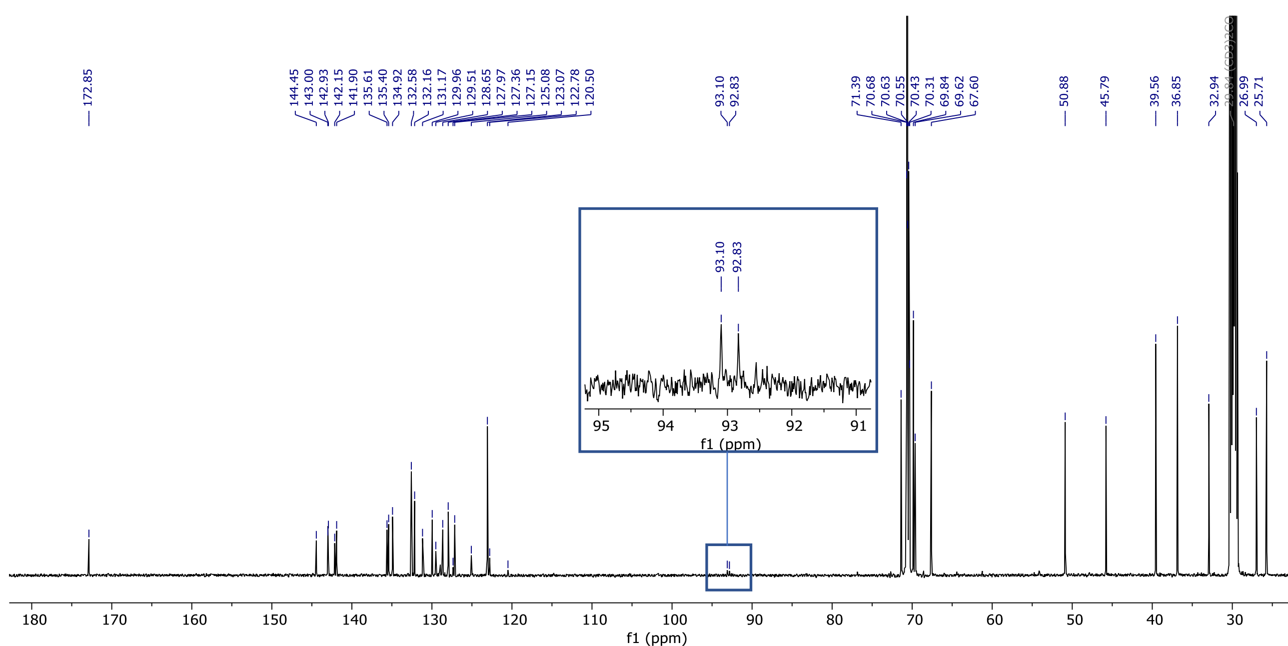


Figure S23. ^{13}C NMR (126 MHz, acetone-d_6 + 10% D_2O) spectrum of compound **5**.

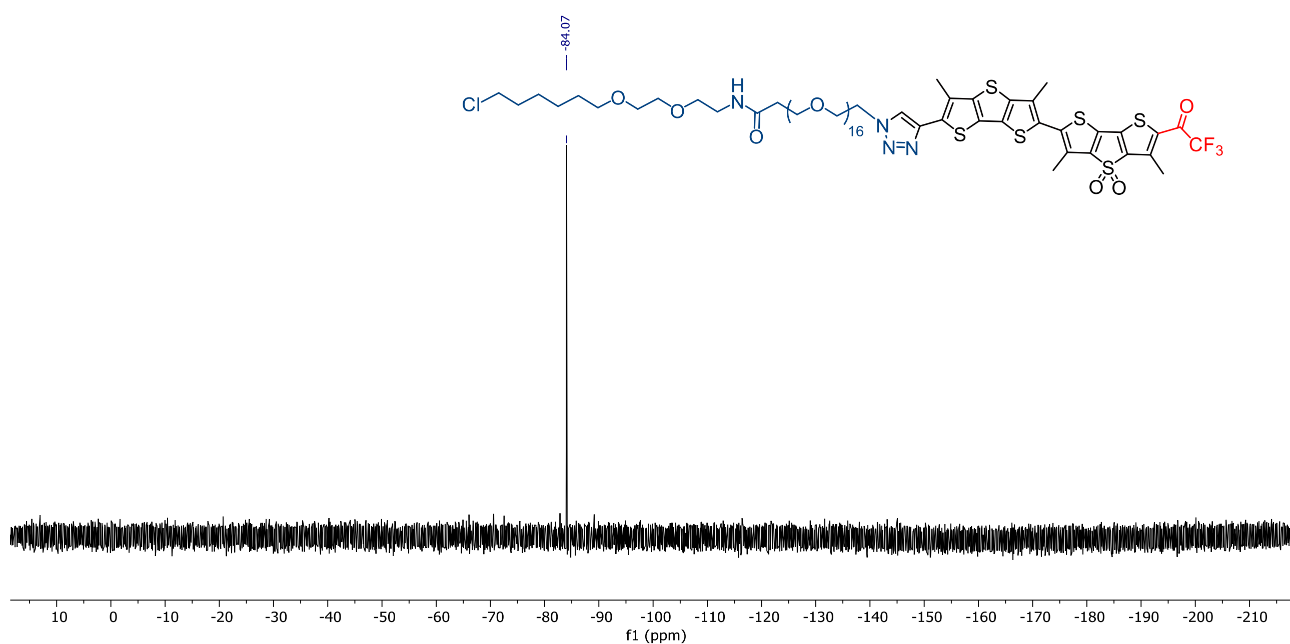


Figure S24. ^{19}F NMR (282 MHz, acetone-d_6 + 10% D_2O) spectrum of compound **5**.

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The original data can be found at: https://doi.org/10.5281/zenodo.5801916