Electronic Supplementary Material (ESI) for Dalton Transactions.

Supplementary Material for

Localization-controlled two-color luminescence imaging via environmental modulation of energy transfer in a multichromophoric species

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INDEX

MATERIALS AND METHODS

General

Solvents were purified according to standard procedures. All reactions were monitored by TLC on commercially available precoated plates (Aldrich silica gel 60 F254 and Alugram® Alox N/UV 254) eluted with Hexane/EtOAc, DCM/MeOH and AcCN/H_2O, and the products were visualized with vanillin [1 g dissolved in MeOH (60 mL) and conc. H_2SO_4 (0.6 mL)] and UV light. Flash column chromatography was performed on Silica gel Aldrich 60 and Aluminum oxide neutral Fluka. ^1H and ^{13}C NMR spectra were recorded in acetone-d_6 and dimethylsulfoxide-d_6 with a Varian Mercury 300 spectrometer at 300 and 75 MHz, and with a Varian 500 spectrometer at 500 and 125 MHz, respectively; the attributions are supported by Heteronuclear Single Quantum Coherence (HSQC) experiments and Correlation spectroscopy (COSY). Elemental analysis were determined by a FISON EA1108; in %.

UV/Vis absorption spectra were taken on a Jasco V-560 spectrophotometer. For steady-state luminescence measurements, a Jobin Yvon-Spex Fluoromax 2 spectrofluorimeter was used, equipped with a Hamamatsu R3896 photomultiplier. The spectra were corrected for photomultiplier response using a program purchased with the fluorimeter. For the luminescence lifetimes, an Edinburgh OB 900 time-correlated single-photon-counting spectrometer was used. As excitation sources, a Hamamatsu PLP 2 laser diode (59 ps pulse width at 408 nm) and/or the nitrogen discharge (pulse width 2 ns at 337 nm) were employed. Emission quantum yields for deaerated solutions were determined using the optically diluted method.[1] As luminescence quantum yield standards, we used a trimethylammonium-phenylstyryl BODIPY species (ϕ=0.69 in ACN). [2]

Time-resolved transient absorption experiments were performed using a pump-probe setup based on the Spectra-Physics MAI-TAI Ti:sapphire system as the laser source and the Ultrafast Systems Helios spectrometer as the detector. The output of laser beam was split to generate pump and probe beam pulses with a beam splitter (85 and 15%). The pump pulse (400 nm, 1-4 μJ) was generated with a Spectra-Physics 800 FP OPA and was focused onto the sample cuvette. The probe beam was delayed with a computer controlled motion controller and then focused into a 2-mm sapphire plate to generate a white light continuum (spectral range 450-800 nm). The white light was then overlapped with the pump beam in a 2-mm quartz cuvette containing the sample. The effective time resolution was ca. 200 fs, and the temporal chirp over the white-light 450–750 nm range ca. 150 fs; the temporal window of the optical delay stage was 0-3200 ps. Please note that all the transient

spectra shown in the present paper are chirp corrected. The chirp correction was done by using the pump-induced absorption signals themselves in the same conditions (same cuvette, solvent, temperature, stirring frequency…) used for each single experiment. The time-resolved data were analyzed with the Ultrafast Systems Surface Explorer Pro software.

Evaluation of antiproliferative activity

Human cell lines A2780 (ovary, adenocarcinoma), MDA-MB-231 and MCF7 (breast carcinoma), A549 (lung carcinoma), SHMel28 (melanoma), and SHSY5Y (neuroblastoma) were plated at the desired concentrations (range: 1600-3000/well) into flat-bottomed microtiter plates. After 6–8 h cells were treated with 20 μl containing 10X concentrations of 1:10 fold serial dilutions of compounds **1** (starting concentration, 30 μM) and **2** (starting concentration, 100 μM). After 72 hours cells were stained with MTT (Sigma, St. Louis, MO, USA) as described elsewhere.[3]

IC_{50}s were calculated on the basis of the analysis of single concentration–response curves, each final value being the mean of 4-7 independent experiments.

Reagents for cell experiments

The stock solutions of our complexes were prepared in DMSO (30 and 100 mM, 3 μl) and then frozen at -20 °C. Before use aliquots were added 3 μl DMSO and 294 μl distilled water in order to achieve the final concentration of 300 and 1000 μM. The final concentration of DMSO in the cell culture medium did not exceed 0.2%.

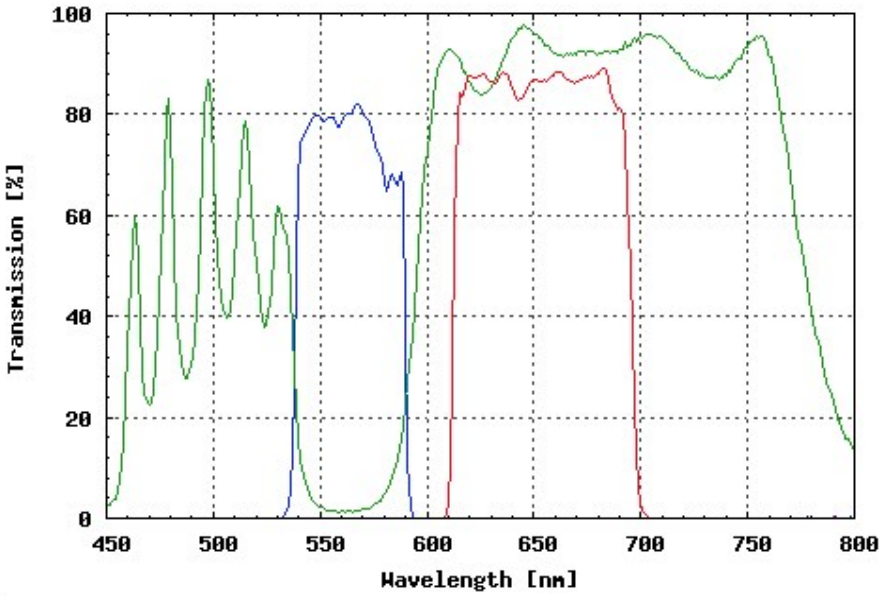
Epifluorescence analysis

Cells were seeded and grown on glass coverslip in growth medium for 16 h in 24 multiwells plates for cell culture. Thereafter, the growth medium was discarded and fresh medium, supplemented with the indicated compound at 50μM final concentration, was added in each well. After 1 h of incubation in a humidified incubator at 37°C and in a 5% O_2 atmosphere, the medium was discarded and cells were fixed with 3.7% paraformaldehyde in phosphate buffered saline (PBS) containing 2% sucrose (fixing solution) for 5 min at room temperature. After a single wash with PBS, coverslip were either mounted on glass slides with Permafluor Aqueous Mounting Medium (Lab Vision, Thermoscientific, Waltham, MA USA) or subjected to incubation with acetone at -20°C for 5min and a further PBS wash before mounting or processed for immunofluorescence analysis.

Immunofluorescence staining was performed after fixation with fixing solution. Cells were permeabilized with 20 mM HEPES pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X- 100 for 5 min at 0°C. Non-specific binding was prevented by incubation with pure Goat serum for 30 min at 0°C. Coverslips were incubated with the anti calreticulin antibody PA3-900 (Thermoscientific) for 2 hrs at 0°C. An alexa-594 conjugated anti-rabbit antibody (Thermoscientific) was used to reveal the anti-calreticulin antibody. Nuclei were stained with Hoechst 33258 in PBS for 5 min at 0°C. Coverslips were mounted on glass slides as described above. Epifluorescent microscopy analysis was performed using a Zeiss Axio Imager M1 microscope (Carl Zeiss, Jena, Germany) equipped with the fluorescent filter sets: Zeiss 49, Zeiss 10 and Omega XF102-2 (Omega Optical, Brattleboro, VT, USA). Optical sections along the z axis were acquired by structured epifluorescent illumination performed by using an Apotome module, a MRm camera and the Axiovision software (Carl Zeiss).

The spectral details for the used Zeiss 49, Zeiss 10 and Omega XF102-2 sets, are reported below.

Omega Set XF102-2:



FILTER SET COMPONENTS	
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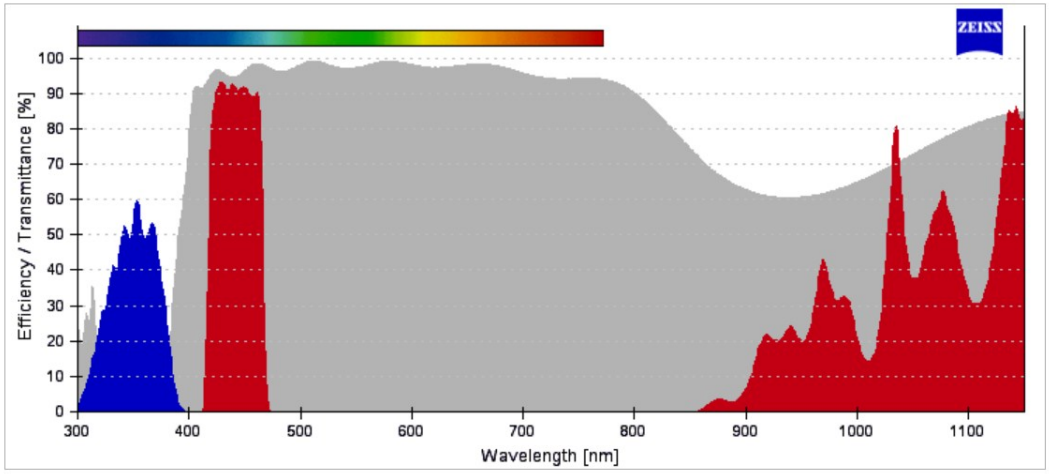
Dichroic:	XF2029 (595DRLP)
Emitter:	XF3081 (645AF75)

For the Zeiss 49:

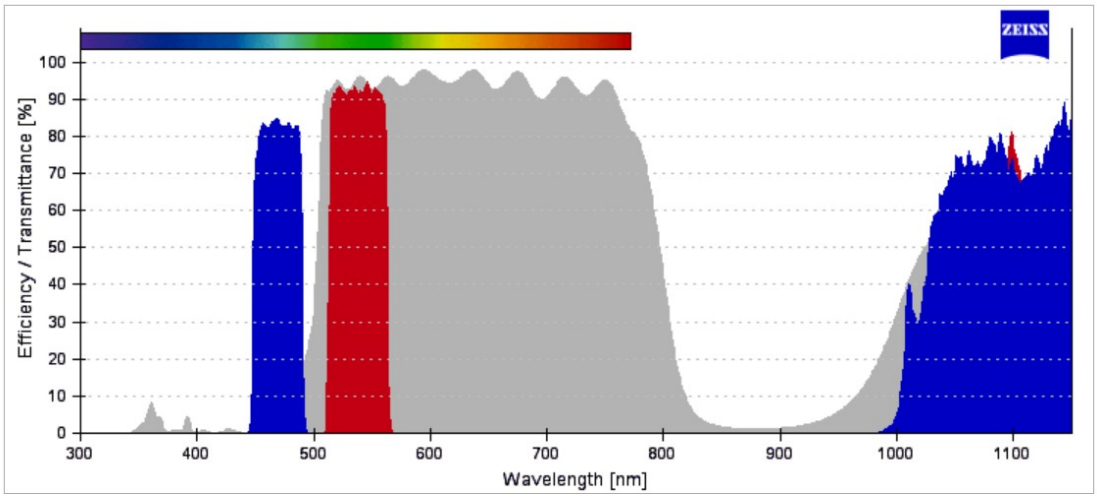


Show total efficiency

Note:
Additional components can influence efficiency.

Filter Set	49
Excitation	G 365
Beam Splitter	FT 395
Emission	BP 445/50

For the Zeiss 10:



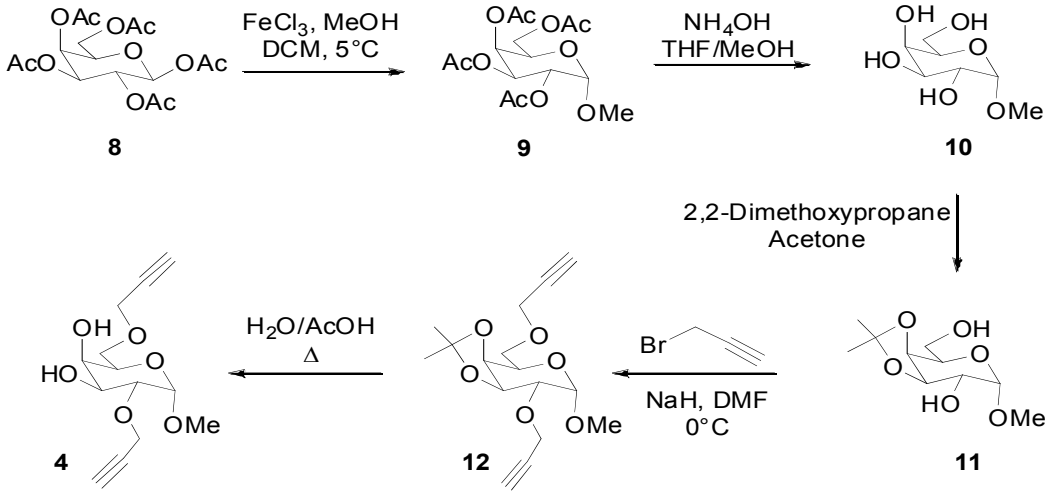
Show total efficiency

Note:
Additional components can influence efficiency.

Filter Set	10
Excitation	BP 450-490
Beam Splitter	FT 510
Emission	BP 515-565

SYNTHESIS

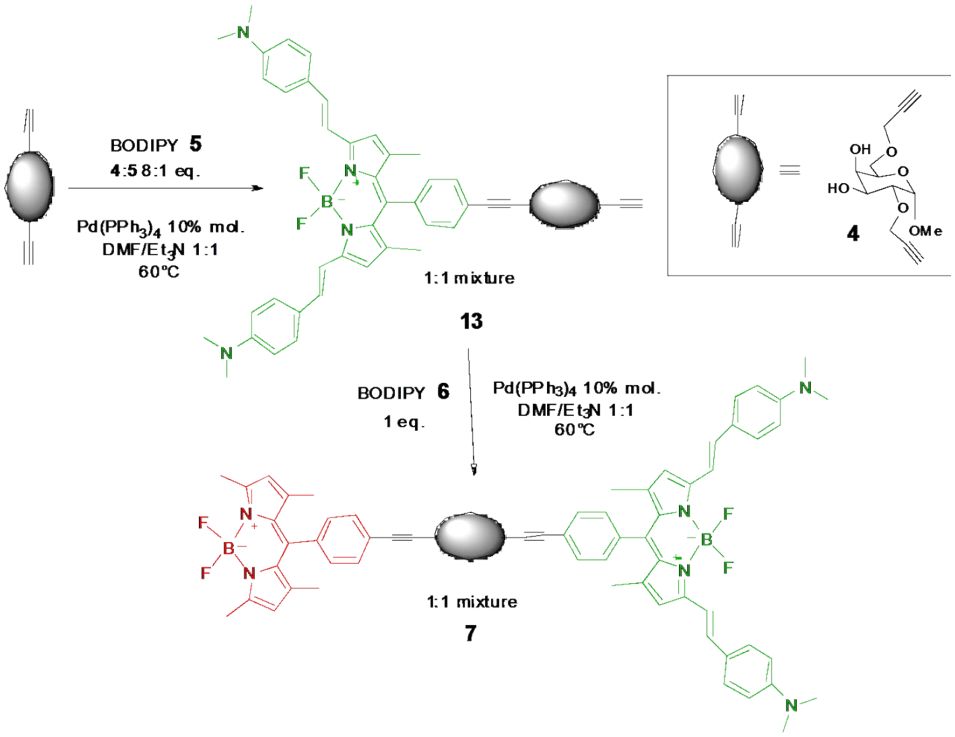
Scheme 1 illustrates the synthesis of the D-galactose platforms used to assemble luminophores with different photophysical properties. Although methyl-α-D-galactopyranoside **10** is commercially available, it was easily obtained from β-Dgalactopyranoside pentacetate **8** in two steps.[4,5] C-4 and C-3 of the pyranose ring of **10** were protected with acetone in 2,2-dimethoxypropane[6] and propagyl bromide was linked to C-6 and C-2 of the D-galactoderivative **11**.[7] Hydrolysis in acidic conditions of D-galactopyranoside **12** afforded the D-galactose platform **4**.[8]



Scheme 1

Methyl 2,6-di-*O*-propargyl-α-D-galactopyranoside (4): ^{1}H NMR: (500 MHz, CD_3COCD_3) δ 4.85 (d, 1H, *J* = 3.4 Hz, H_1), 4.35 (d, 2H, *J* = 2.4 Hz, *CH*$_2$-C≡CH), 4.20 (d, 2H, *J* = 2.4 Hz, *CH*$_2$-C≡CH), 4.03 (broad s, 1H, OH), 3.89-3.65 (m, 7H, OH, H_2, H_3, H_4, H_5 and 2xH_6), 3.33 (s, 3H, OCH_3), 2.93 (t, 2H, C≡*CH*);^{13}C NMR (150 MHz, CD_3COCD_3): δ 99.8, 82.2, 81.5, 77.7, 76.4, 76.2, 71.4, 70.9, 70.7, 70.5, 59.5, 59.0, 55.7.

Scheme 2 illustrates the synthetic steps for the preparation of compound **7**. Compound **13** was obtained from the first copper-free Sonogashira cross-coupling, purified and then involved in the second copper-free Sonogashira reaction.



Scheme 2

General procedure for the copper free Sonogashira cross-coupling. [$Pd(PPh_3)_4$] (10% mmol) was added to a degassed solution of BODIPY **5** or **6** (1 eq.) and methyl 2,6-di-*O*-propargyl-α-D-galactopyranoside (**4**) (8 or 1 eq.) in DMF/TEA (0.02M, 1:1). The mixture was then heated, at 60°C, under argon, for 3-4 h, until the disappearance of the starting compounds was observed by TLC. The solvent was removed under reduced pressure.

Compound 13: BODIPY **5** and the galactopyranoside **4** (ratio 1:8) were reacted following the general procedure of Sonogashira cross-coupling. Compound **13** was obtained as a 1:1 mix of isomers and purified by chromatography column using as eluants Hexane/EtOAc (50:50 up to 20:80). Purification led to a deep green solid with a 70% yield. TLC: *Rf* 0.55 (EtOAc 100%). Mp 175-185°C. ^{1}H NMR (500 MHz, CD_3COCD_3): δ 7.71-7.39 (m, 24H), 6.87 (d, *J* = 7.8 Hz, 8H), 6.81 (s, 4H), 4.97 e 4.87 (d, 2H, *J* = 2.9 Hz, 2xH_1), 4.61 and 4.49 (s, 4H, 2xCH_2-C≡C), 4.33 and 4.21 (d, 4H, *J* = 2.0 Hz, 2xCH_2-C≡CH), 4.15 and 4.06-3.71 (m, 16H, 4 OH, 2xH_2, 2xH_3, 2xH_4, 2xH_5 and 2x2H_6), 3.36 (s, 6H, 2xOCH_3), 3.06 (s, 24H, 4xN$(CH_3)_2$), 2.94 (m, 2H, 2 x -C≡*CH*), 1.49 (s, 12H);^{13}C NMR (150 MHz, CD_3COCD_3): δ 154.5, 152.9, 142.2, 138.3, 137.2, 133.3, 132.5, 130.9, 130.3, 126.2, 125.0, 119.0, 116.0, 114.1, 99.9, 92.4, 88.5, 86.7, 81.7, 78.0, 77.7, 77.3, 76.4, 76.2, 71.1, 70.9, 68.1, 60.2, 59.5, 58.6, 55.8, 41.1, 15.6. Anal. Calcd for $C_{50}H_{53}BF_2N_4O_6$ (854,79): C 70.26, H 6.25, N 6.55. Found: C 70.40, H 6.26, N 6.53.

Compound 7: Compound **13** was reacted with BODIPY **6** (ratio 1:1) following the general procedure of copper-free Sonogashira cross-coupling. Compound **7** was purified by flash chromatography as a 1:1 mixture of isomers using as eluants Hexane/EtOAc 30:70 gradient to EtOAc 100% and obtained as deep green solid with a yield of 77%. TLC (Hexane/EtOAc 1:1) *Rf*: 0.27. Mp 175-185°C. ^{1}H NMR (500 MHz, CD_3COCD_3): δ 7.70-7.39 (m, 32H), 6.82 (d, *J* = 8.8 Hz, 8H), 6.79 (s, 4H), 6.11 (s, 4H), 4.99 (m, 2H, 2xH_1), 4.61 and 4.50 (s, 8H, 4xCH_2-C≡C), 4.13 (m, 2H, 2xOH), 3.97-3.82 (m, 14H, 2xOH, 2xH_2, 2xH_3, 2xH_4, 2xH_5 and 2x2H_6), 3.39 (s, 6H, 2xOCH_3), 3.05 (s, 24H, 4xN$(CH_3)_2$), 2.49 (s, 12H), 1.48 and 1.43(s, 24H); ^{13}C NMR (150 MHz, CD_3COCD_3): δ 157.0, 154.5, 152.9, 144.5, 142.8, 142.2, 138.4, 137.2, 136.6, 134.0, 133.9, 133.7, 132.5, 130.9, 130.3, 130.0, 126.2, 125.3, 125.0, 122.8, 118.9, 115.7, 113.7, 99.9, 89.3, 88.7, 86.7, 86.5, 78.0, 71.6, 71.2, 70.8, 70.6, 60.2, 59.8, 55.9, 40.9, 15.6, 15.3, 15.2. Anal. Calcd for $C_{69}H_{70}B_2F_4N_6O_6$ (1176,95): C 70.41, H 5.99, N 7.14. Found: C 70.62, H 6.00, N 7.16.

Compound 1: A large excess of CH_3I (500 eq.) was added to a solution of compound **7** (1 eq.) in acetonitrile (0.01M). The resulting mixture was stirred at RT for three days. The course of the reaction was followed by TLC on aluminium oxide neutral using a mixture of AcCN/H_2O (80:20). After disappearance of the starting material, the deep-blue solution was evaporated to dryness. The reaction crude was purified by flash column chromatography on aluminium oxide neutral using as eluants AcCN/H_2O 90:10. Compound **1** was obtained as a deep blue solid (1:1 mixture of isomers) in quantitative yield. TLC (AcCN/H_2O 9:1) *Rf*: 0.35. Mp 170-175°C. ^{1}H NMR (500 MHz, CD_3COCD_3): δ 8.32 (d, 8H, *J* = 9.3 Hz), 7.93 (d, 8H, *J* = 9.3 Hz), 7.83-7.44 (m, 24H), 6.96 (s, 4H), 6.11 (s, 4H), 4.92 (d, 2H, *J* = 3.4 Hz, H_1), 4.65, 4.64, 4.53 and 4.52 (s, 8H, 4xCH_2-C≡CH), 4.00 (s, 36H, 4xN$(CH_3)_3$), 3.99-3.82 (m, 12H, 2xH_2, 2xH_3, 2xH_4, 2xH_5 and 2x2H_6), 3.40 (s, 6H, OCH_3), 2.48 (s, 12H), 1.55 and 1.44 (s, 24H); ^{13}C NMR (150 MHz, CD_3COCD_3): δ 157.0, 153.7, 148.8, 144.5, 140.2, 138.1, 136.6, 135.6, 134.0, 130.3, 130.1, 125.2, 123.0, 120.3, 113.6, 99.5, 88.8, 86.6, 73.2, 72.8, 72.0, 71.7, 70.9, 68.1, 60.2, 59.1, 58.6, 56.2, 15.7, 15.6, 15.3. Anal. Calcd for $C_{71}H_{76}B_2F_4I_2N_6O_6$ (1458,81): C 58.38, H 5.24, N 5.75. Found: C 58.26, H 5.25, N 5.73.

Compound 2: It was synthesized from BODIPY **6** (1 eq.) and the galactopyranoside **4** (1 eq.) following the general procedure of Sonogashira cross-coupling. Purification (eluants Toluene/AcCN 60:40) of the crude mixture gave compound **2** as an orange solid (1:1 mix of isomers) with a 50% yield. TLC: *Rf* 0.50 (Toluene/AcCN 40:60). Mp 90-95°C. ^{1}H NMR (500 MHz, CD_3COCD_3): δ 7.69 (d, *J* = 7.9 Hz, 4H), 7.45 (d, 4H), 6.13 (s, 4H), 4.95 and 4.87 (d, 2H, *J* = 2.9 Hz, 2xH_1), 4.60 and 4.49 (s, 4H, 2xCH_2-C≡C), 4.32 and 4.20 (d, 4H, *J* = 1.5 Hz 2xCH_2-C≡CH), 4.15-3.68 (m, 16H, 4 OH, 2xH_2, 2xH_3, 2xH_4, 2xH_5 and 2x2H_6), 3.35 (s, 6H, 2xOCH_3), 2.94 (m, 2H, 2 x -C≡*CH*), 2.50 (s, 12H), 1.49 (s, 12H); ^{13}C NMR (150 MHz, CD_3COCD_3): δ 157.0, 144.5, 142.9, 136.6, 137.2, 133.9, 132.5, 130.1, 130.0, 129.7, 125.3, 122.9, 122.7, 99.9, 99.8, 89.3, 88.7, 86.5, 86.2, 82.2, 77.7, 76.4, 76.2, 71.4, 71.1, 70.6, 60.2, 59.8, 59.5, 59.1,

55.9, 15.3. Anal. Calcd for $C_{32}H_{35}BF_2N_2O_6$ (592,44): C 64.87, H 5.95, N 4.73. Found: C 64.83, H 5.96, N 4.72.

Anions exchange procedures

Hexafluorophosphate salt

15 mg of **1** were dissolved in 2 ml of water. An excess of solid NH4PF6 have been added and the mixture was stirred for 30 min to induce the precipitation of the dyad as hexafluorophosphate salt.

The retentate was washed with H2O, dissolved in a small amount of acetonitrile and purified by Sephadex G 15 flash chromatography.

Chloride salt

10 mg of the dyad as hexafluorophosphate salt have been dssolved in 3 mL of Acetone. An excess of solid Tetrabutylammonium chloride have been added and the mixture was stirred for 1 h to induce the precipitation of the dyad as chloride salt. The retentate was washed with acetone, dissolved in a small amount of water and purified by Sephadex G 15 flash chromatography.

Supplementary Figures

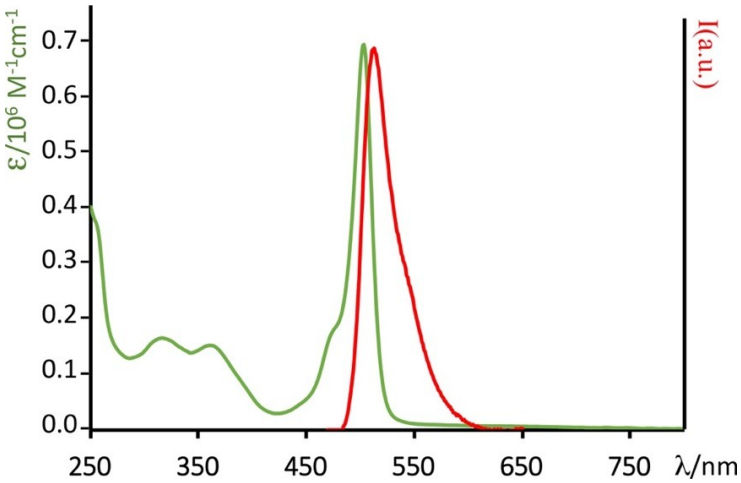


Figure S1. Absorption (green line) and emission (red line) spectra of **2** in acetonitrile. The excited state lifetime in acetonitrile is 3.1 ns, whereas the luminescence quantum yield is 0.45.

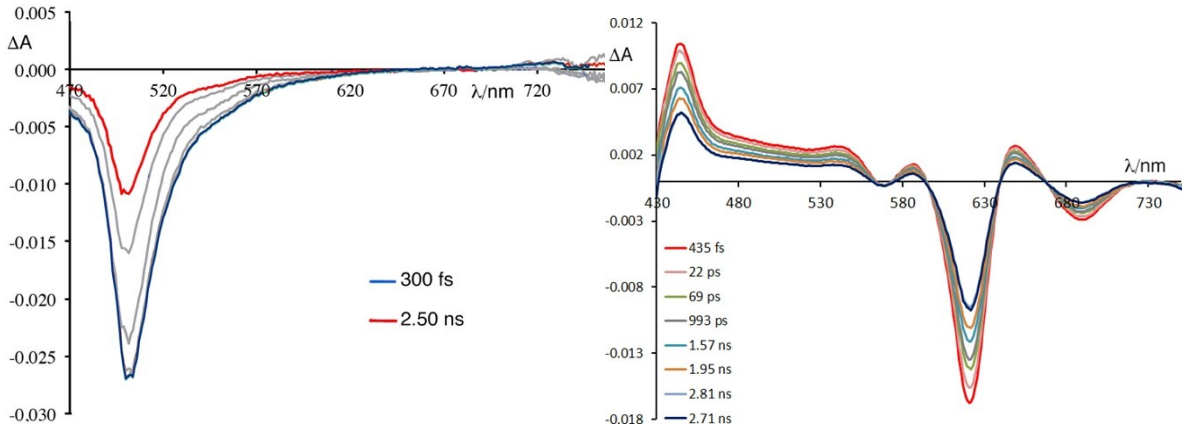


Figure S2. Left: Transient absorption spectra at different delays of **2** in acetonitrile. Right: Transient absorption spectra at different delays of **3** in acetonitrile. Please note the apparent bleaching band, in the range 660 nm -700 nm that is due to stimulated emission.

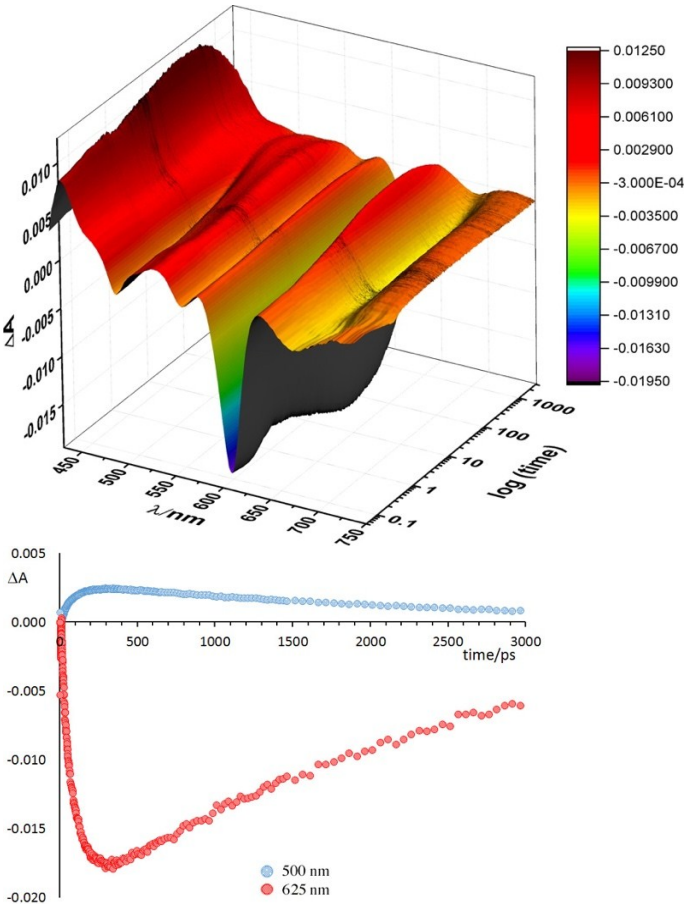


Figure S3. Transient absorption matrix of **1** in acetonitrile. Please note that the data are reported in the logarithmic time scale to highlight the disappearing of the bleaching at around 500 nm (spectral fingerprint of the BODIPY donor subunit, see left panel in Figure S2) and, with the same rate, the increasing of the bleaching at around 620 nm (spectral region of the Bodipy acceptor subunit see right panel in Figure S2), see kinetic traces on the right. Infact, in the transient spectra matrix of **1** a bleaching at 500 nm is clearly evident at early times after the excitation laser pulse (120 fs), which disappears within 80 ps and in the same time scale (see kinetics) the bleaching at 620 nm increases, so indicating the occurrence of ultrafast energy transfer in **1**, with an estimated rate constant of 1.31 x 10^{13} s^{-1}.

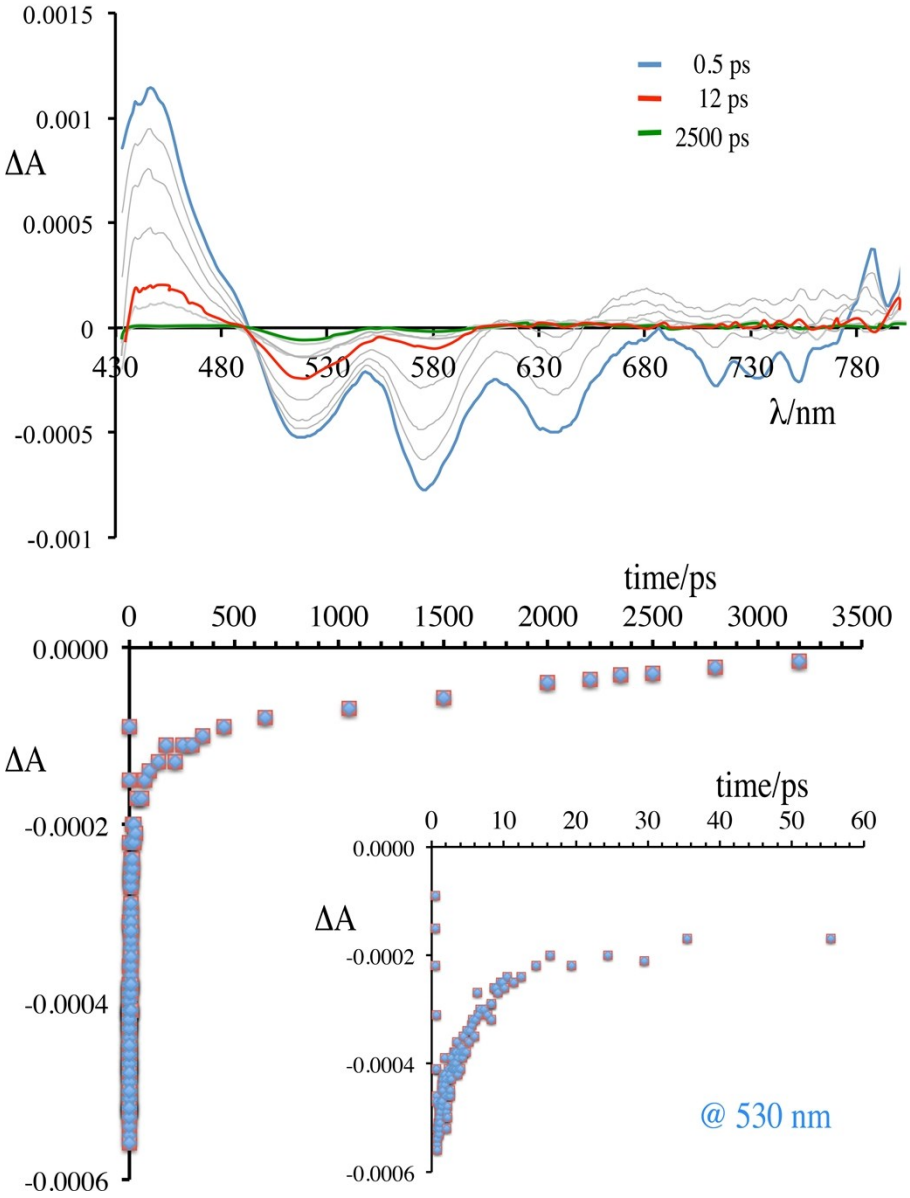


Figure S4. Transient absorption spectra (top panel) and decay at 530 nm (bottom panel) of **1** in oil solution. At early times (500 fs after the excitation pulse @400 nm -120 fs), the TAS shows an absorption band centered at 450 nm typical of the acceptor moiety and a structured bleaching at lower energy. The first bleaching band at around 510 nm is attributed to the donor moiety and the 580 nm band to the stimulated emission of this subunit. The bleach at 630 nm and the broad band at lower energy, by comparison with the absorption spectrum in oil, it attributed to the acceptor dye. This transient absorption spectrum decays biphasically, with a fast component (time constant of a few ps) followed by a slower component (about 3 ns) practically identical to the luminescence lifetime of **1** in oil. The fast component is due to the acceptor directly excited at 400 nm, which non-radiatively decays independently from the donor chromophore.

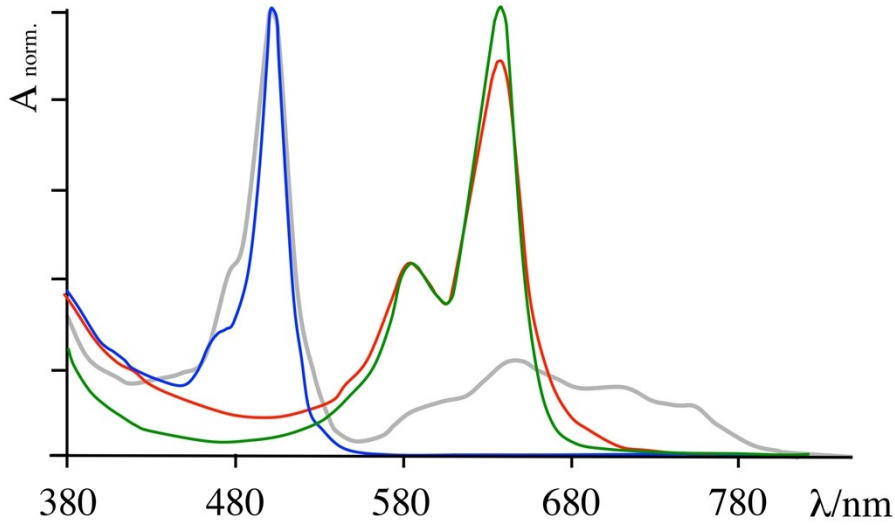


Figure S5. Absorption spectra of **1** (grey line), **2** (blue line) and **3** (red line) in oil. For comparison the absorption spectrum of **3** in acetonitrile is also shown.

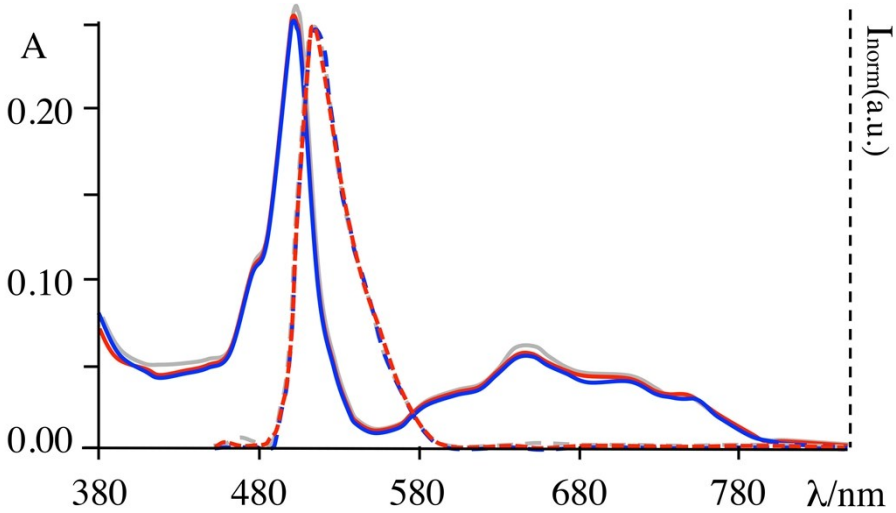


Figure S6. Absorption and emission spectra of **1** (grey lines) in oil compared to the chloride (orange lines) and hexafluorophosphate (blue lines) salts.

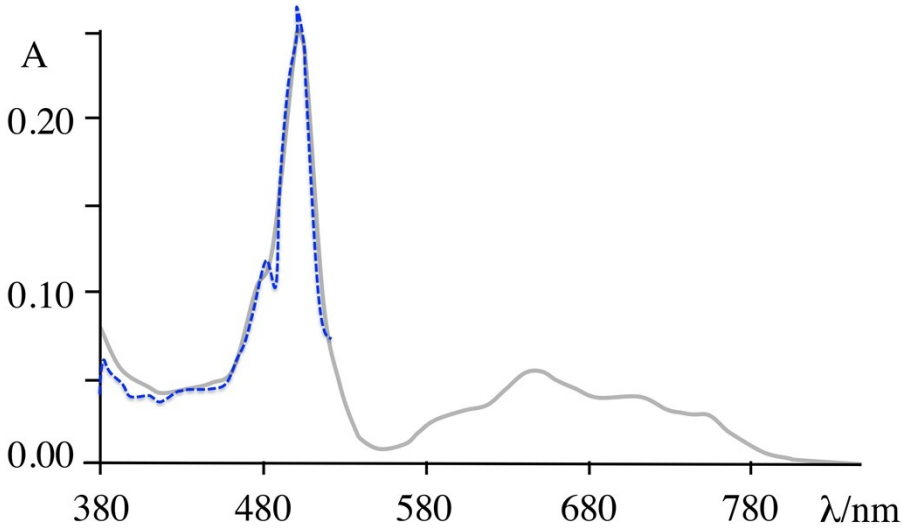


Figure S7. Absorption (grey line) and excitation (blue dashed line) spectra of **1** in oil**.** The excitation spectrum has been registered at 530 nm (green emission).

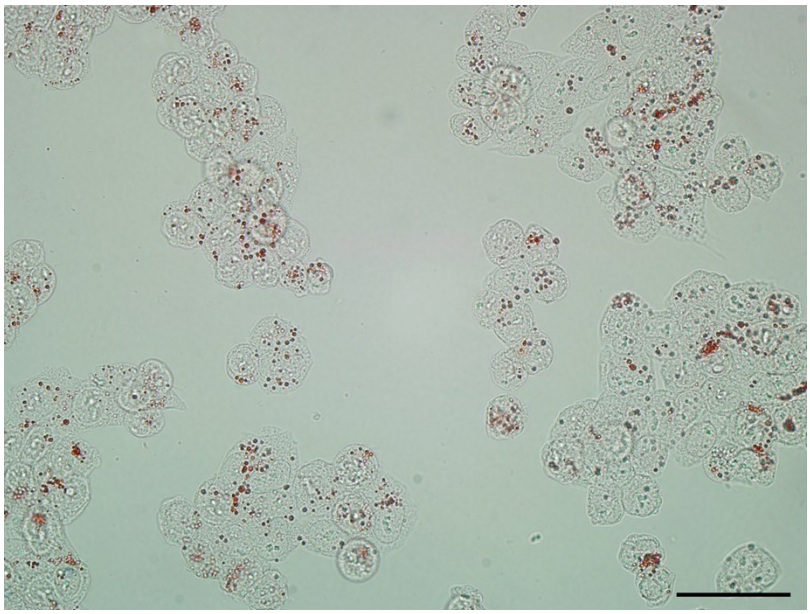


Figure S8. A2780 cells stained using standard procedures with Oil Red O, a neutral lipid selective staining. Lipid droplets are stained in red. Bar = 50µm.

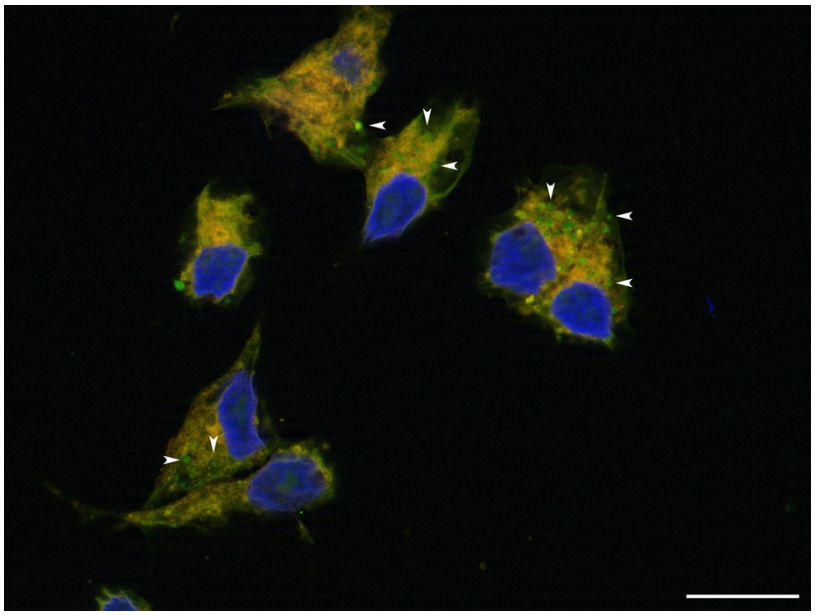


Figure S9. Epifluorescent microscopy analysis of cells labeled with **1**. Optical sections of A2780 cells incubated with **1** for 1 h and treated with PFA and DAPI to visualize nuclei and imaged 6 months later to show persistence staining of cellular structures. Arrow heads indicate lipid droplets. Bar = 20 µm.

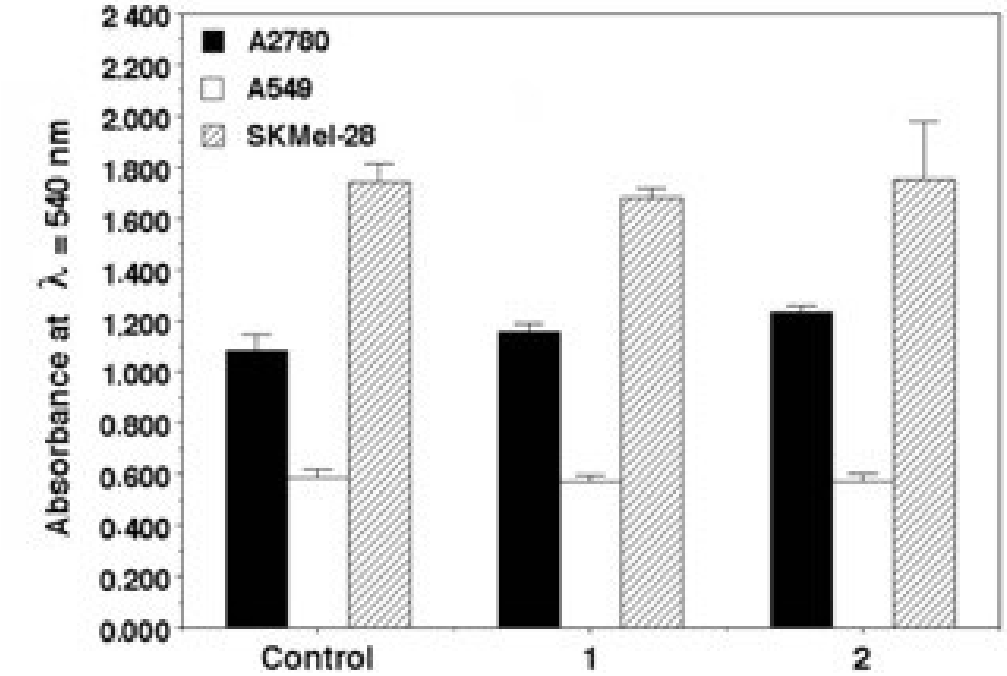
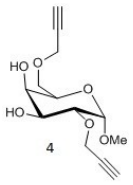


Figure S10. Antiproliferative activity after 1 hour exposure of cells to compounds **1** and **2**. These experiments were performed in order to exclude any loss of viability of the cells treated with 50 μM of compounds **1** and **2** for 1 hour. The A549, A2780 and SKMel28 cell lines were utilized in this series of experiments (n=3). In these conditions no variations of cell viability were observed .

NMR Spectra (compound formulae shown in graphics)



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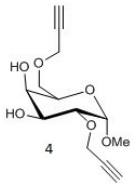
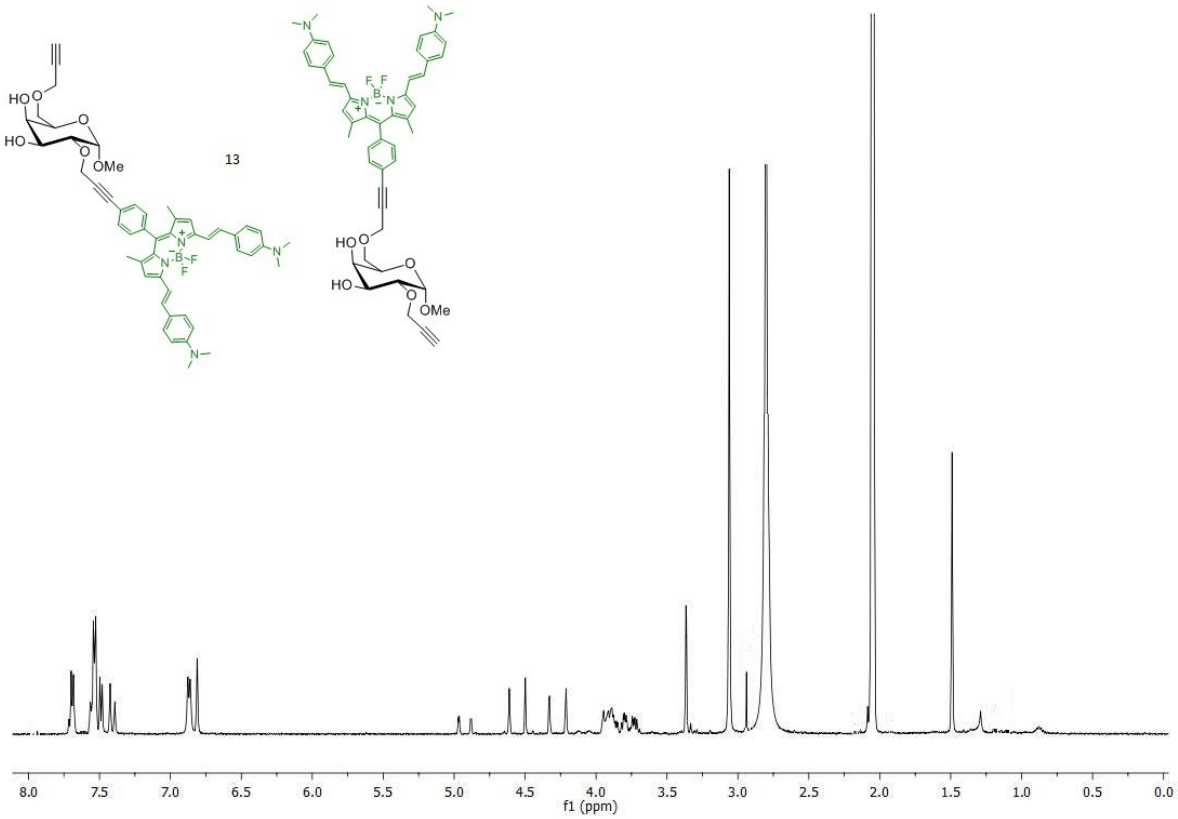
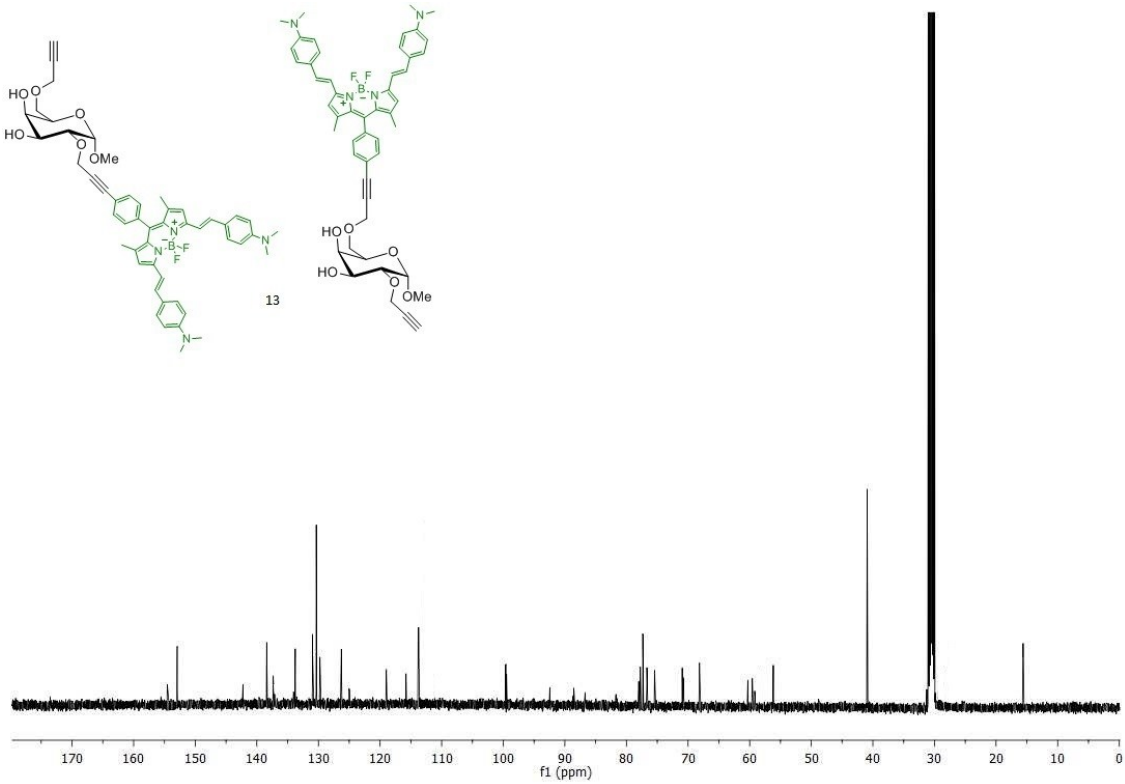
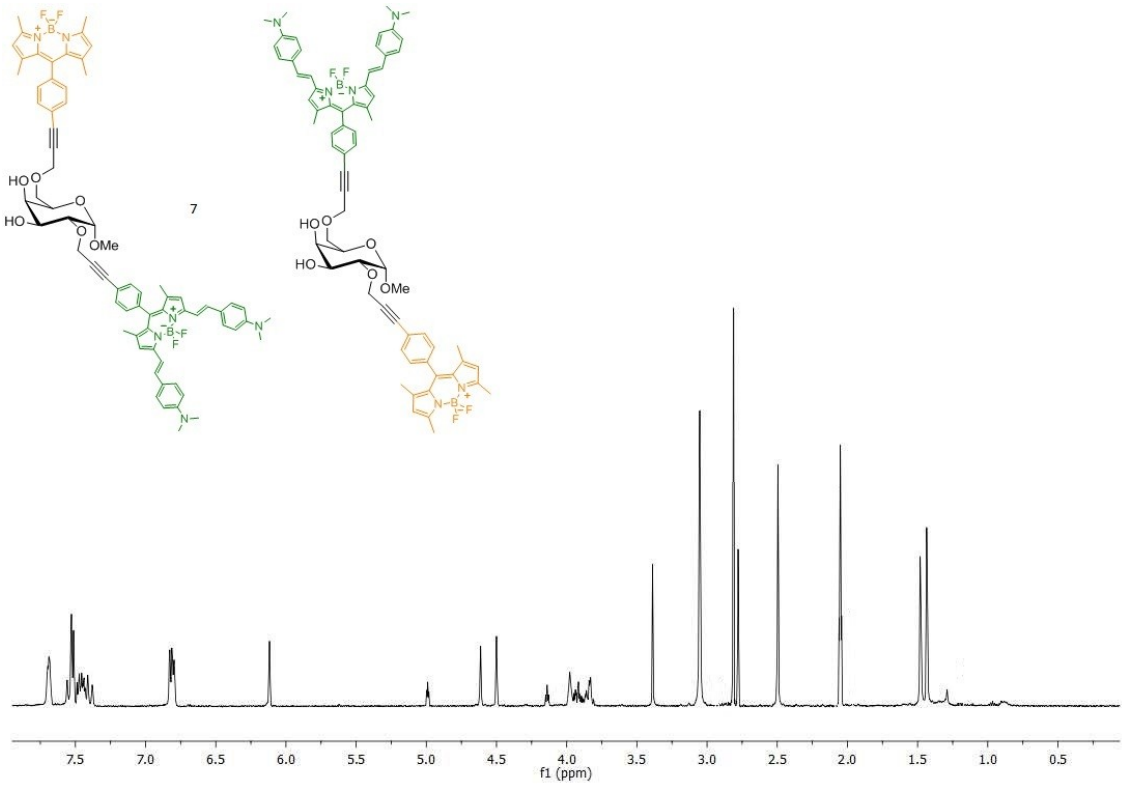
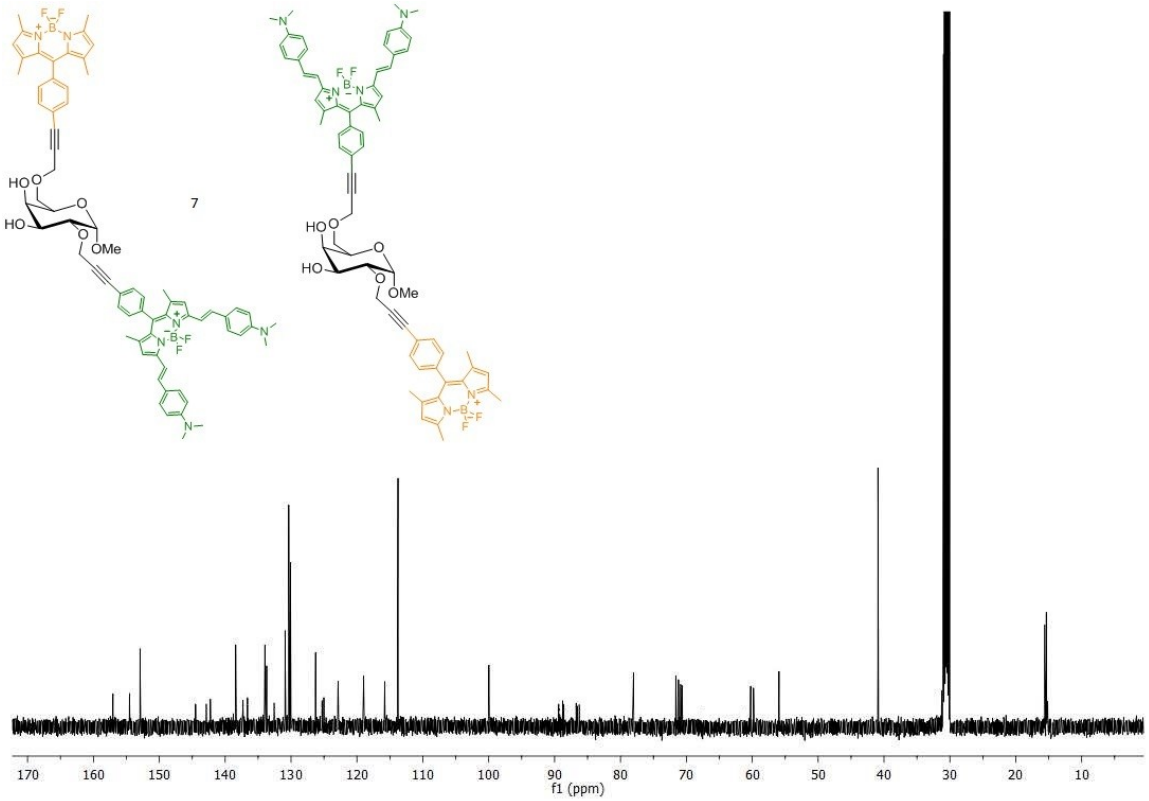


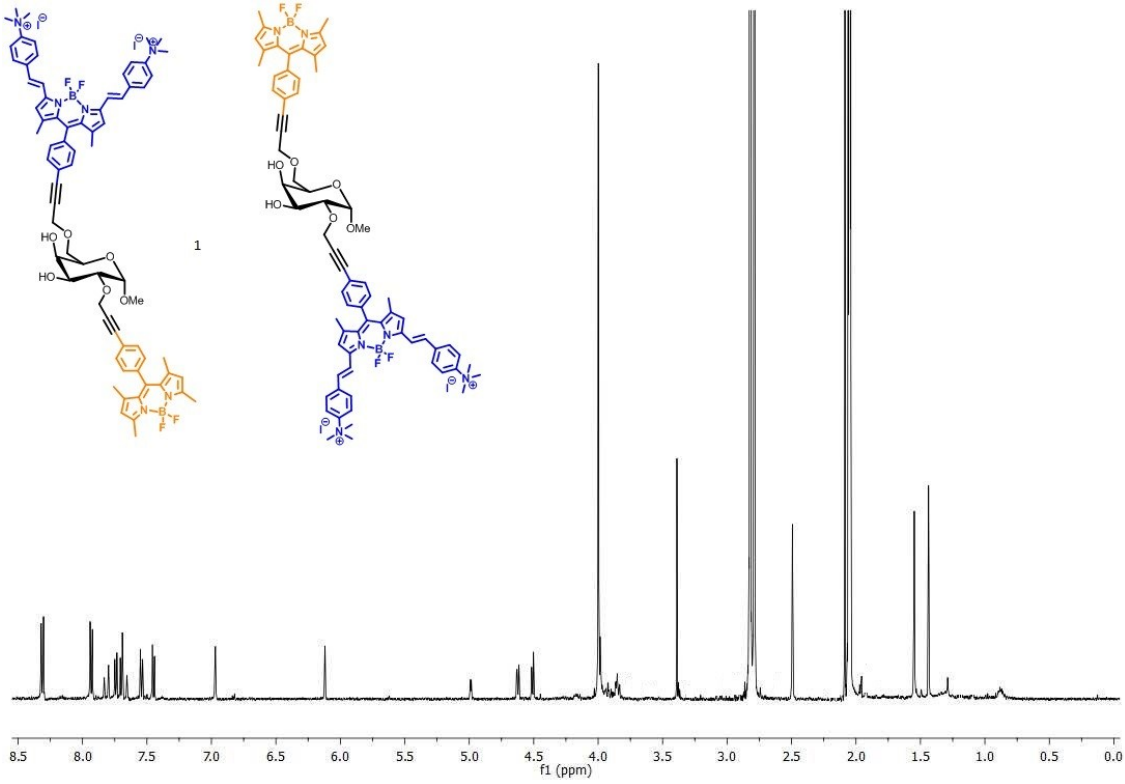
4

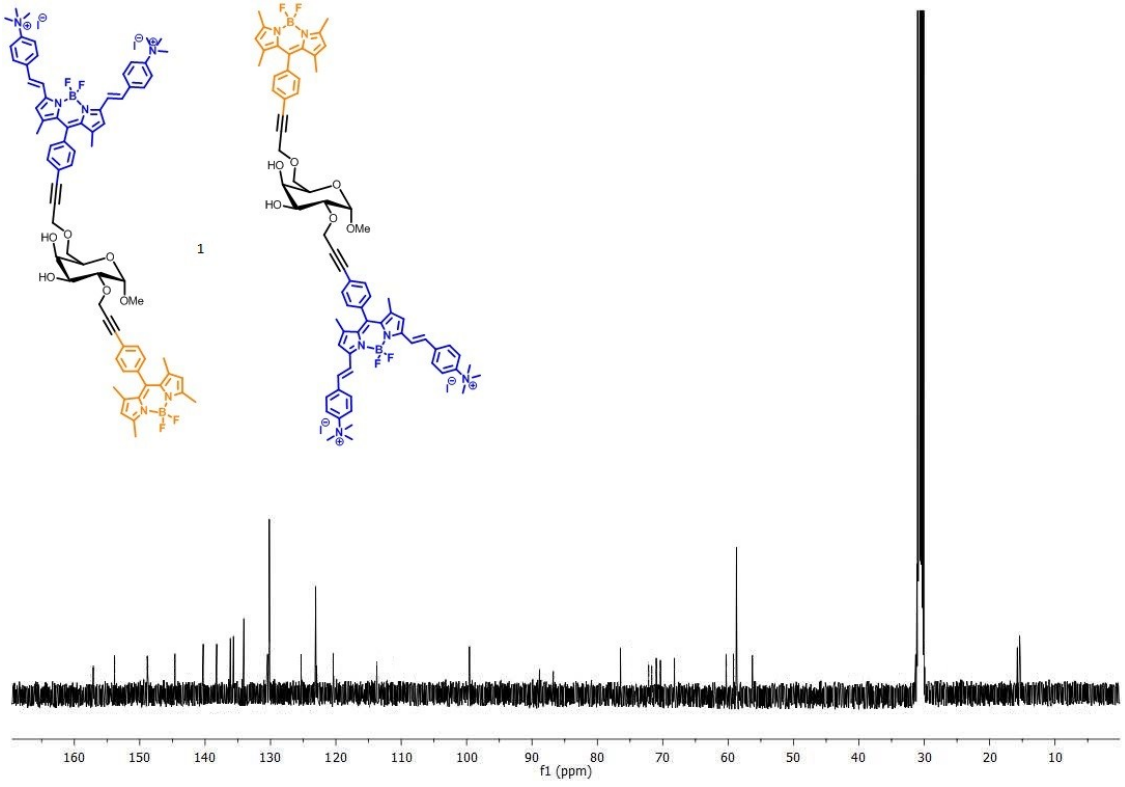

13
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

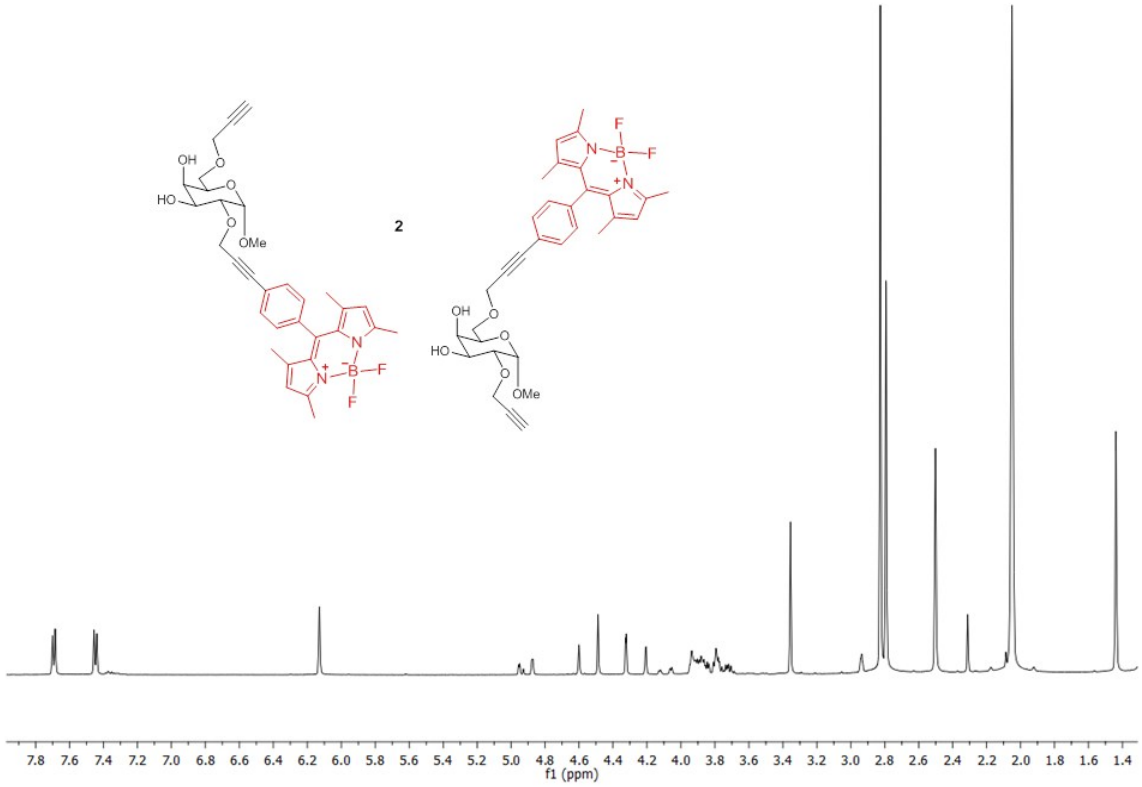

13
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

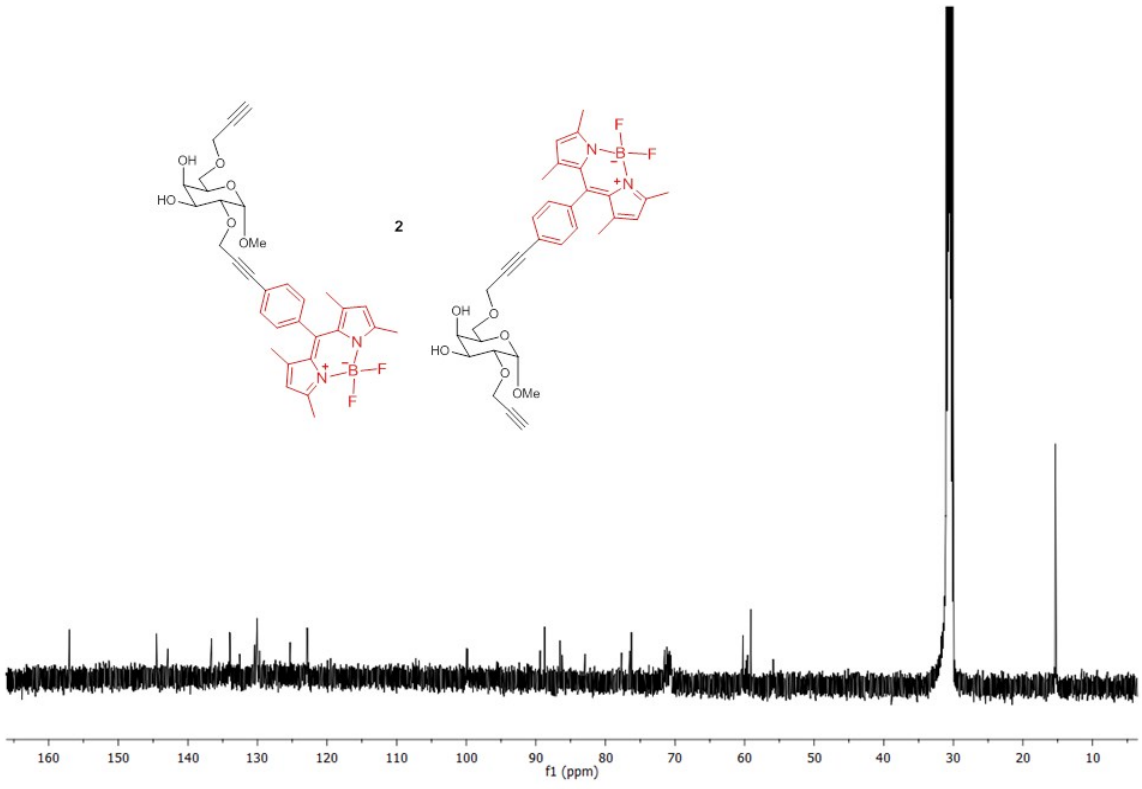

7
HO
HO
OMe
F
B
N
+
-
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)


7
HO
HO
OMe
F
B
N
+
-
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


1
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


1
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)


2
7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 6.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4
f1 (ppm)


2
160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

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