A promising family of fluorescent water-soluble aza-BODIPY dyes for in vivo molecular imaging

Jacques Pliquett[a,b], Adrien Dubois[a], Cindy Racoeur[b], Nesrine Mabrouk[b], Souheila Amor[a], Robin Lescure[a], Ali Bettaïeb[b], Bertrand Collin[c], Claire Bernhard[a], Franck Denat[a], Pierre Simon Bellaye[c], Catherine Paul[b*], Ewen Bodio[a*], Christine Goze[a*]

AUTHOR ADDRESS [a] Department of Chemistry, ICMUB, 9 avenue Alain Savary, 21000 Dijon, France. [b] Laboratoire d'Immunologie et Immunothérapie des Cancers, EPHE, PSL Research University, 75000 Paris ; LIIC, EA7269, Université de Bourgogne Franche Comté, 21000 Dijon, France. [c] Centre Georges François Leclerc, Service de médecine nucléaire, 1 rue Professeur Marion, BP77980, 21079 Dijon Cedex

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1 Methods and Materials

1.1 Materials

Unless stated, reactions were carried out in technical grade solvents under normal atmosphere. Dry solvents were non-stabilized, purchased from Carlo Erba and dried using a MB-SPS-800 (MBraun) or PureSolv-MD-5 (Inert®). All reagents were purchased from SigmaAldrich or ACROS Organics™ and used as received without further purification. Column chromatography was carried out using silica gel (SigmaAldrich; 40-63 µm 230-400 mesh 60Å). Analytical thin-layer chromatography was performed with Merck 60 F254 silica gel (precoated sheets, 0.2 mm thick). Reactions were monitored by thin-layer chromatography, RP-HPLC-MS. Ion exchange was executed using a DOWEX basic ion-exchange resin.

1.2 Instrumentation

NMR spectra: (^{1}H, ^{13}C, ^{11}B, ^{19}F) were recorded at 300K on Bruker 500 Avance III or Bruker Avance III HD 600 MHz spectrometer (equipped with double resonance broad band probes). Chemical shifts are given relative to TMS (^{1}H, ^{13}C), BF_3*Et_2O (^{11}B), $CFCl_3$ (^{19}F) and were referenced to the residual solvent signal. High-resolution mass spectra were recorded on a Thermo LTQ Orbitrap XL ESI-MS spectrometer. NMR and Mass-analyses were performed at the "Plateforme d'Analyse Chimique et de Synthèse Moléculaire de l'Université de Bourgogne" (PACSMUB).

Spectroscopic properties: UV-Visible absorption spectra were recorded obtained on a Varian Cary 50 scan (single-beam). Data are reported as absorption maximum wavelength ([λ_{max}] = nm) and molar absorption coefficient at the absorption maximum wavelength ([ε] = L mol^{-1} cm^{-1}). The steady - state fluorescence emission spectra were obtained using a HORIBA Jobin Yvon Fluorolog spectrofluorometer (software FluorEssence). All fluorescence spectra were corrected from apparatus response. Quartz cuvettes (1.5 mL) with a path length of 1 cm were used. All solutions were prepared with spectroscopic grade solvents. Three different measurements (i.e. different solutions) were performed for each compound. The sample concentrations were chosen to obtain a maximum absorbance between 0.3 and 1.0 for UV spectra and between 0.035 and 0.1 at excitation wavelength for quantum yield measurements. Relative quantum efficiencies were obtained by comparing the areas under the corrected emission spectrum. All measurements were performed in DMSO (Sigma Aldrich, spectroscopic grade ≥ 99.9 %) and PBS at 298K. Aza-BODIPY **1** (Φ = 0.36 in $CHCl_3$, λ_{ex} = 670 nm) was used as the standard. In all Φ_f determinations, correction for the solvent refractive index (η) was applied [DMSO: η = 1.477, $CHCl_3$: η = 1.4459, PBS: η=1.337]. The equation $\Phi x = \Phi_{st} (I_x/I_{st}) (A_{st}/A_x) (\eta_x^2/\eta_{st}^2)$ was used to calculate the quantum yield of the sample, in which Φ_{st} is the reported quantum yield of the standard, I is the integrated emission spectrum, A is the absorbance at excitation wavelength, and η is the refractive index of the solvent used. The X subscript denotes unknown, st denotes standard.

Analytical HPLC: HPLC-MS analyses were performed on a Thermo-Dionex Ultimate 3000 instrument (pump + autosampler at 20 °C + column oven at 25 °C) equipped with a diode array detector (Thermo-Dionex DAD 3000-RS) and a MSQ Plus single quadrupole mass spectrometer equipped with a Phenomenex Kinetex® column (2.6 µm C18 100 Å, LC Column 50 x 2.1 mm).

The employed gradient for analyses is the following:

Table S 1: analytical HPLC experimental conditions

Time [min]	**% H_2O+0.1%TFA**	**% ACN+0.1%TFA**	**Flow** [mL/min]
0	95	5	0.5
5	0	100	0.5
6.5	0	100	0.5
6.6	95	5	0.5
8.5	95	5	0.5
8.51	95	5	0.05

Semi preparative chromatography: Semi preparative separations were executed on a HPLC-system from Shimadzu that is equipped with 2 LC-20AT pumps, a SPD-20A UV/Vis detector, a FRC-10A fraction collector, a SIL-10AP sampler and a CBM-20A control unit. The column was a Shim-Pack GIST 5 µm C18 10x250 mm column. The gradient (gradient A) that was used for purifications (unless mentioned otherwise) is the following:

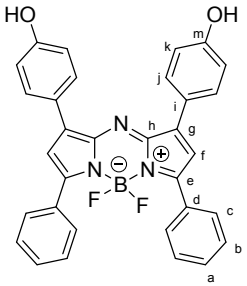
Table S 2: semi-preparative LC experimental conditions

Time [min]	**% H_2O+0.1%TFA**	**% ACN+0.1%TFA**	**Flow** [mL/min]
0	95	5	5
10	0	100	5
13	0	100	5
13.1	95	5	5
15	95	5	5

MALDI: Matrix-assisted laser desorption ionization/time of flight (MALDI/TOF) mass spectra were obtained on a Bruker DALTONICS Ultra flex II (Bruker Daltonics, Bremen, Germany) mass spectrometer using sinapinic acid as matrix (Sigma-Aldrich, St. Quentin Falavier, France) mass spectrometer using sinapinic acid as matrix (Sigma-Aldrich, St. Quentin Falavier, France).

2 Synthetic procedures

2.1 Aza-BODIPY A1:



A1 was synthesized at gram-scale (1-2 g) in 40% overall yield following established synthetic protocols (*doi 10.1002/chem.201204317*).

^{1}H NMR (300 MHz, CD_3CN) δ (ppm)= 6.93 (d, ^{3}J = 8.9 Hz, 4H, H_k), 7.07 (s, 2H, H_f), 7.50 (dd, ^{3}J = 5.3, ^{4}J = 1.9 Hz, 6H, H_a, H_b), 8.00 (m, 8H, H_c, H_j)

^{19}F NMR (282 MHz, CD_3CN) δ (ppm)= -130.2 (q, $J_{F-}{}^{11}{}_{B}$ = 31.3 Hz)

^{11}B NMR (96 MHz, CD_3CN) δ (ppm)= 0.8 (t, J_{B-F} = 31.3 Hz)

^{13}C{^{1}H}NMR (75 MHz, DMSO-D_6) δ (ppm) = 113.8, 115.3, 124.5, 126.5, 128.6, 129.4, 130.2 – 130.4 (m), 131.5, 142.0, 148.7, 154.74, 158.0.

HR-MS (ESI) (Da): calculated for $C_{32}H_{22}N_3O_2B_1F_2Na_1$ $[M+Na]^+$ 552.16654; found 552.16550.

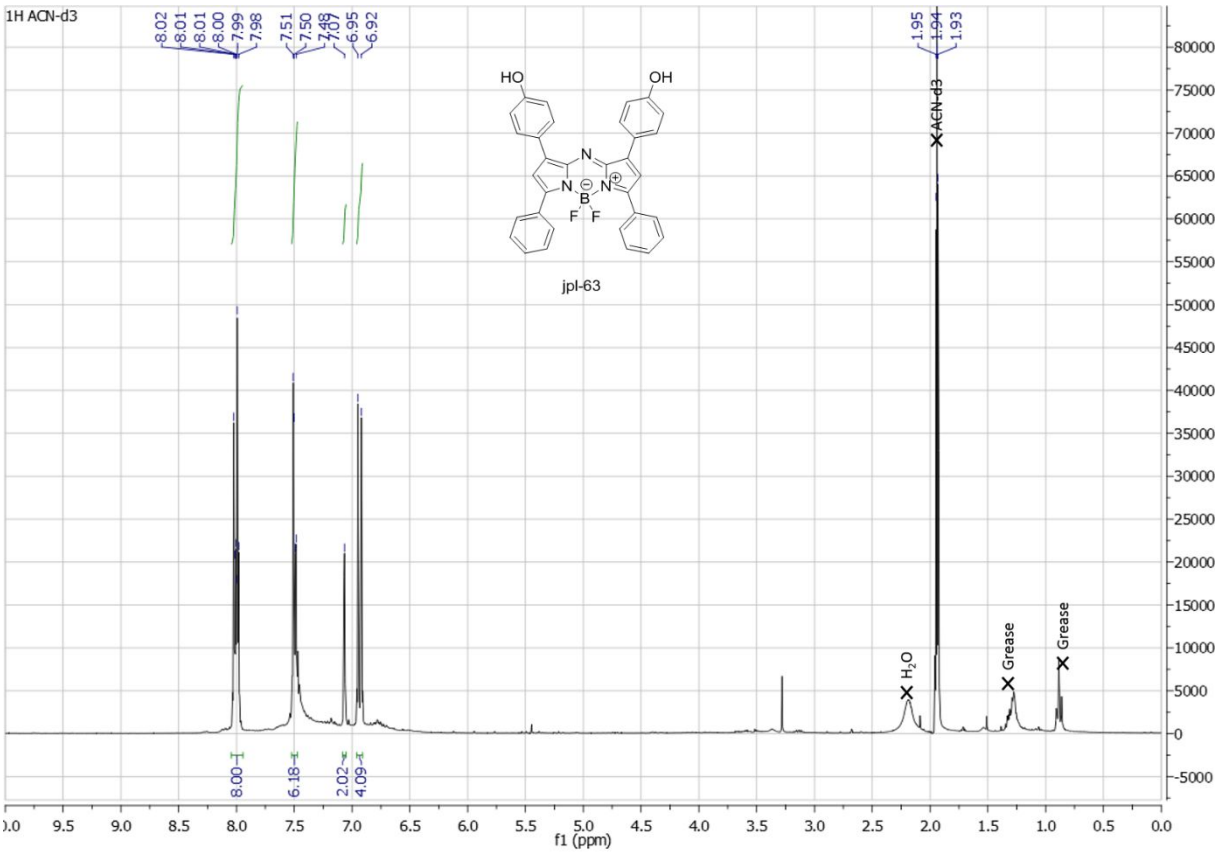


Figure S 1: ^{1}H NMR spectrum of aza-BODIPY A1 (300 MHz, CD_3CN)

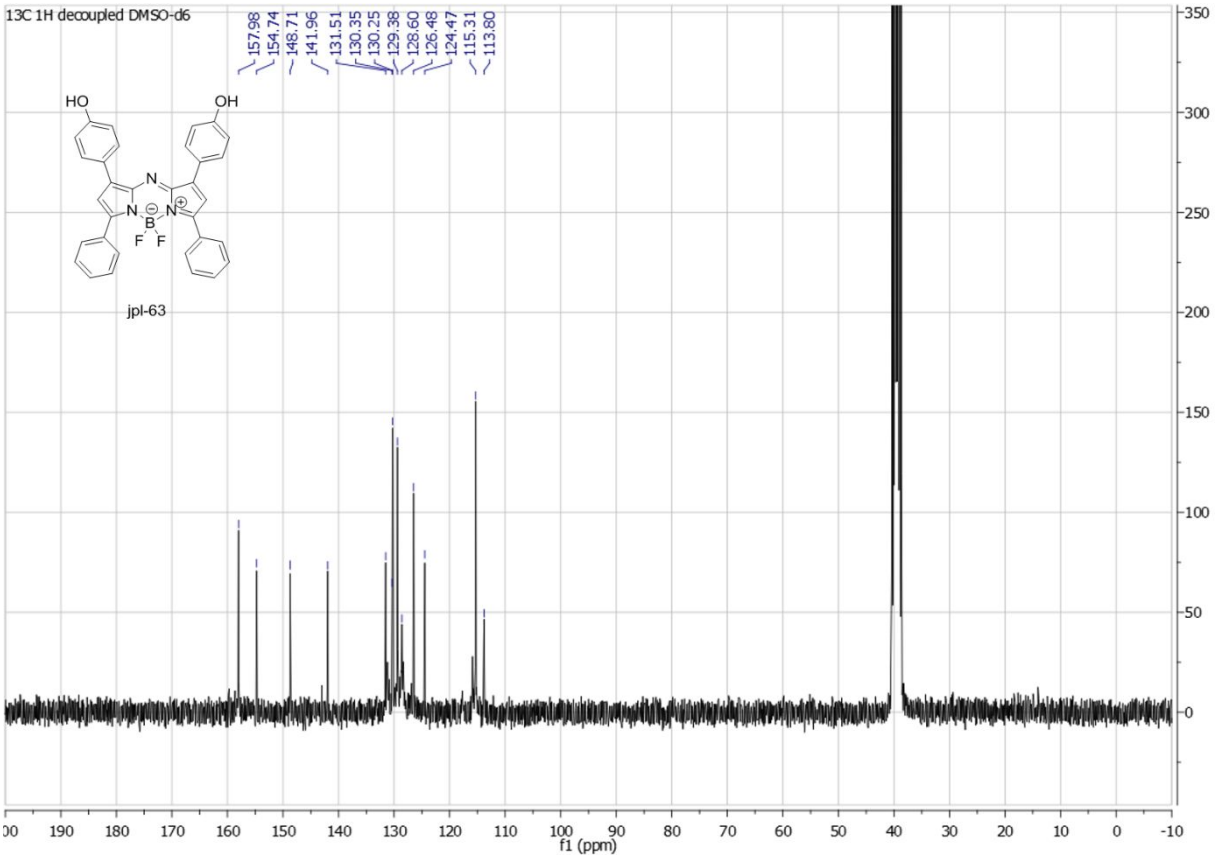


Figure S 2: $^{13}C\{^{1}H\}$ NMR of aza-BODIPY A1 (75 MHz, DMSO-D_6)

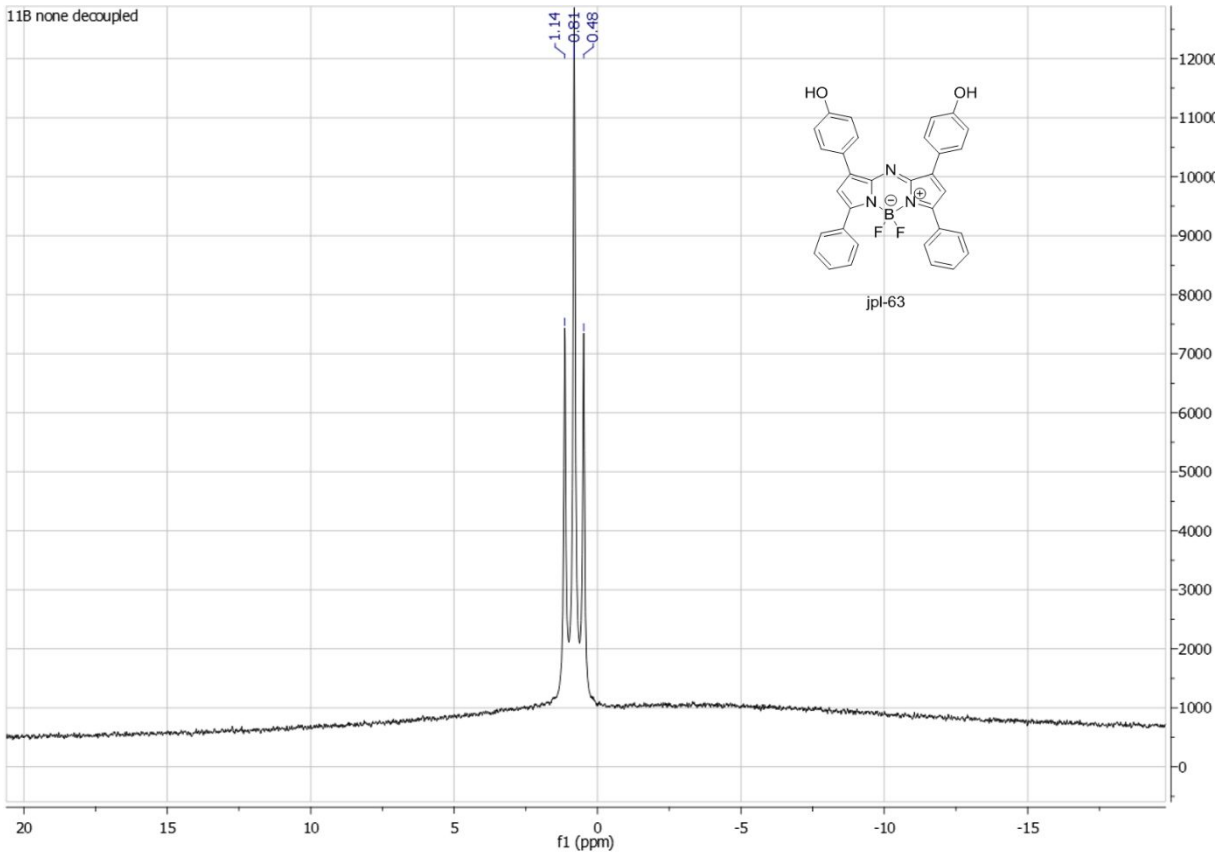


Figure S 3: ^{11}B NMR of aza-BODIPY A1 (96 MHz, CD_3CN)

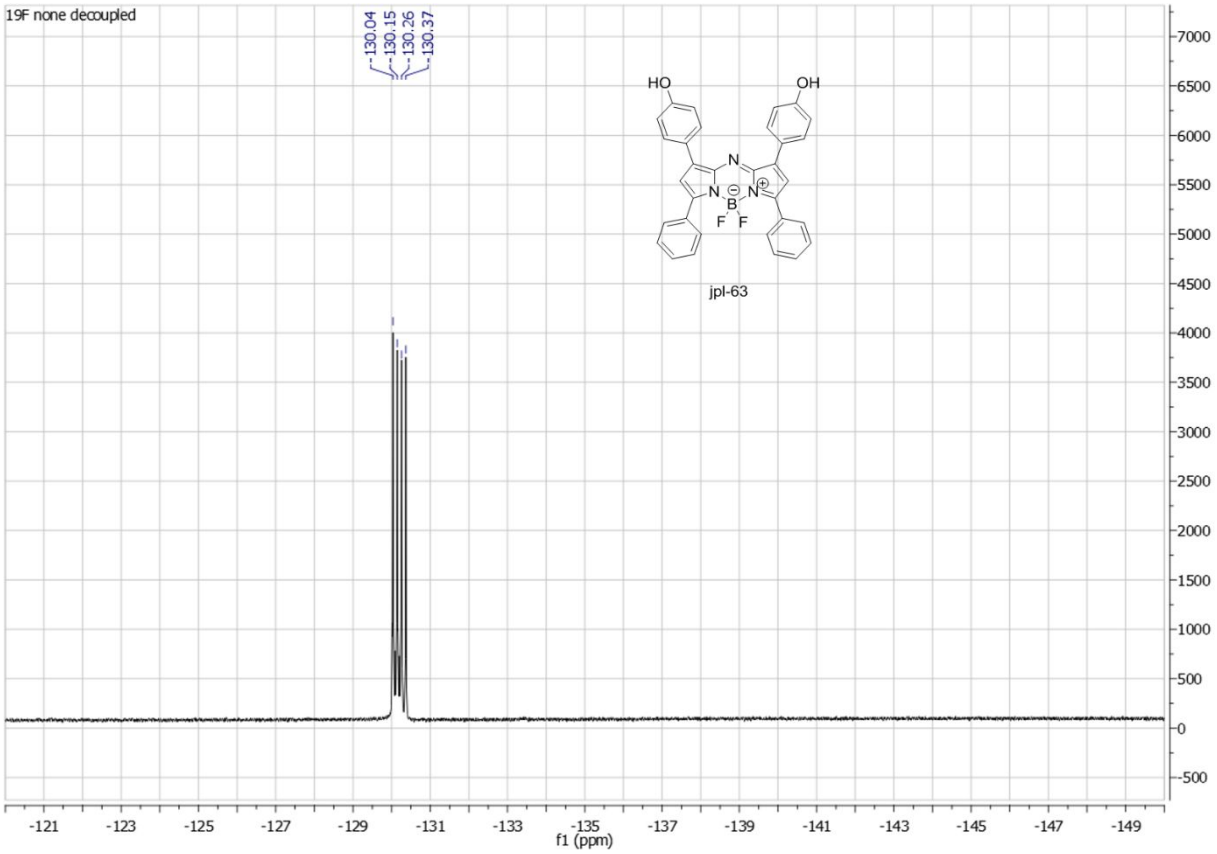
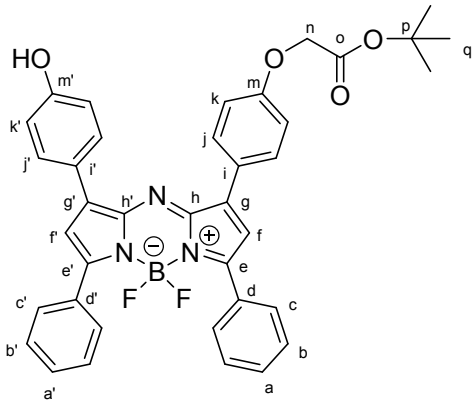


Figure S 4: ^{19}F NMR of aza-BODIPY A1 (282 MHz, CD_3CN)

2.2 Aza-BODIPY A2:



500 mg (945 µmol, 1 eq) of **A1** were dissolved in 4mL of dry DMSO into a 10 mL flask. 574 mg (3.78 mmol, 4 eq) of CsF were added. The resulting mixture was stirred at room temperature for 10 min. 279 µL (1.889 mmol, 2 eq) *t*-Butyl-bromoacetic acid were added. After 2.5 minutes at room temperature the reaction was quenched by addition of 50 mL EtOAc and poured into 60mL PBS-buffer. The organic layer was washed three times with 60 mL of PBS-buffer, then with brine. Organic layer was dried over anhydrous $MgSO_4$ and evaporated under reduce pressure. The crude product was then purified by silica gel column chromatography (Heptane 7: 3 EtOAc) to obtain a dark blue substance. Precipitation from EtOAc/Pentane yields 318 mg of pure **A2** (494 µmol, 52% yield).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) = 1.52 (s, 9H, H_q), 4.59 (s, 2H, H_n), 6.91 (d, J = 7.7 Hz, 4H, H_f, $H_{f'}$, H_k), 6.97 (d, J = 8.9 Hz, 2H, $H_{k'}$), 7.45 – 7.49 (m, 6H, H_j, $H_{j'}$, H_a, $H_{a'}$), 7.97 (d, J = 8.6 Hz, 2H, H_c), 7.99 – 8.04 (m, 6H, H_b, $H_{c'}$, $H_{b'}$).

^{13}C{^{1}H} NMR (75 MHz, $CDCl_3$) δ (ppm) = 28.2, 65.8, 82.9, 114.9, 115.9, 117.7, 125.4, 126.3, 128.7, 129.7, 130.8, 130.9, 131.0, 131.3, 131.90, 131.94, 143.6, 157.4, 159.2, 159.5, 168.0.

^{11}B NMR (96 MHz, $CDCl_3$) δ (ppm) = 0.9 (t, $J_{B\text{-}F}$ = 31.2 Hz)

^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) = -130.9 (q, $J_{F\text{-}^{11}B}$ = 31.2 Hz)

HR-MS (ESI) (Da): calculated for $C_{38}H_{32}B_1F_2N_3O_4Na_1$ $[M+Na]^+$ 666.23461; found 666.23338

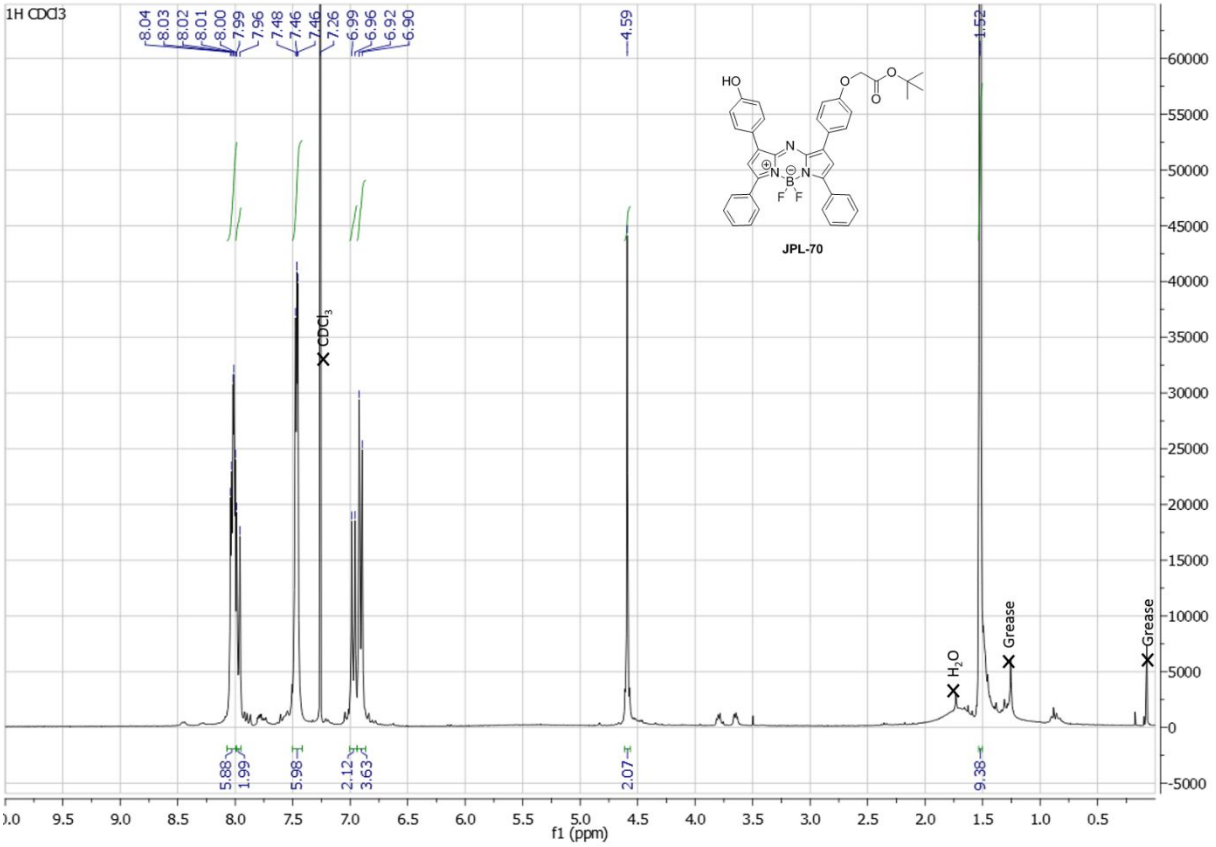


Figure S 5: ^{1}H NMR spectrum of aza-BODIPY A2 (300 MHz, $CDCl_3$)

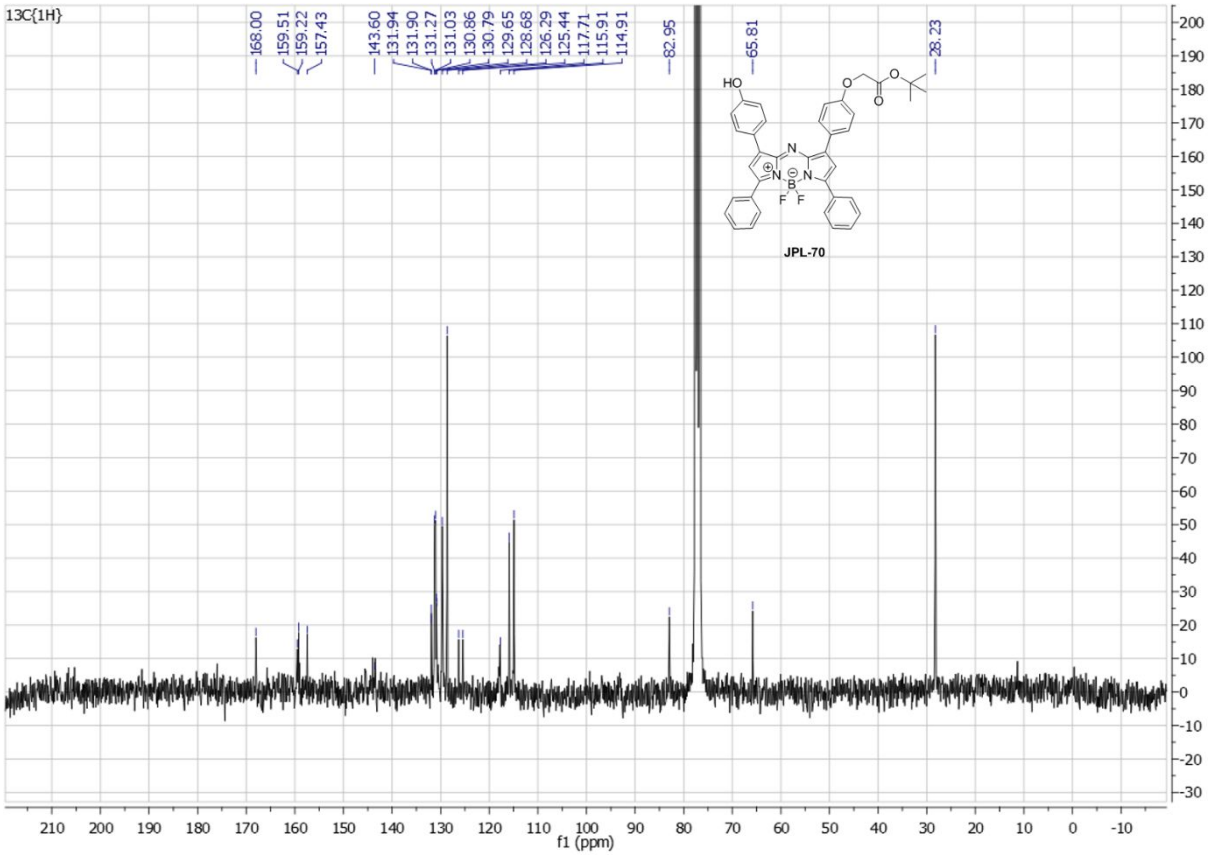


Figure S 6: $^{13}C\{^{1}H\}$ of aza-BODIPY A2 (75 MHz, $CDCl_3$)

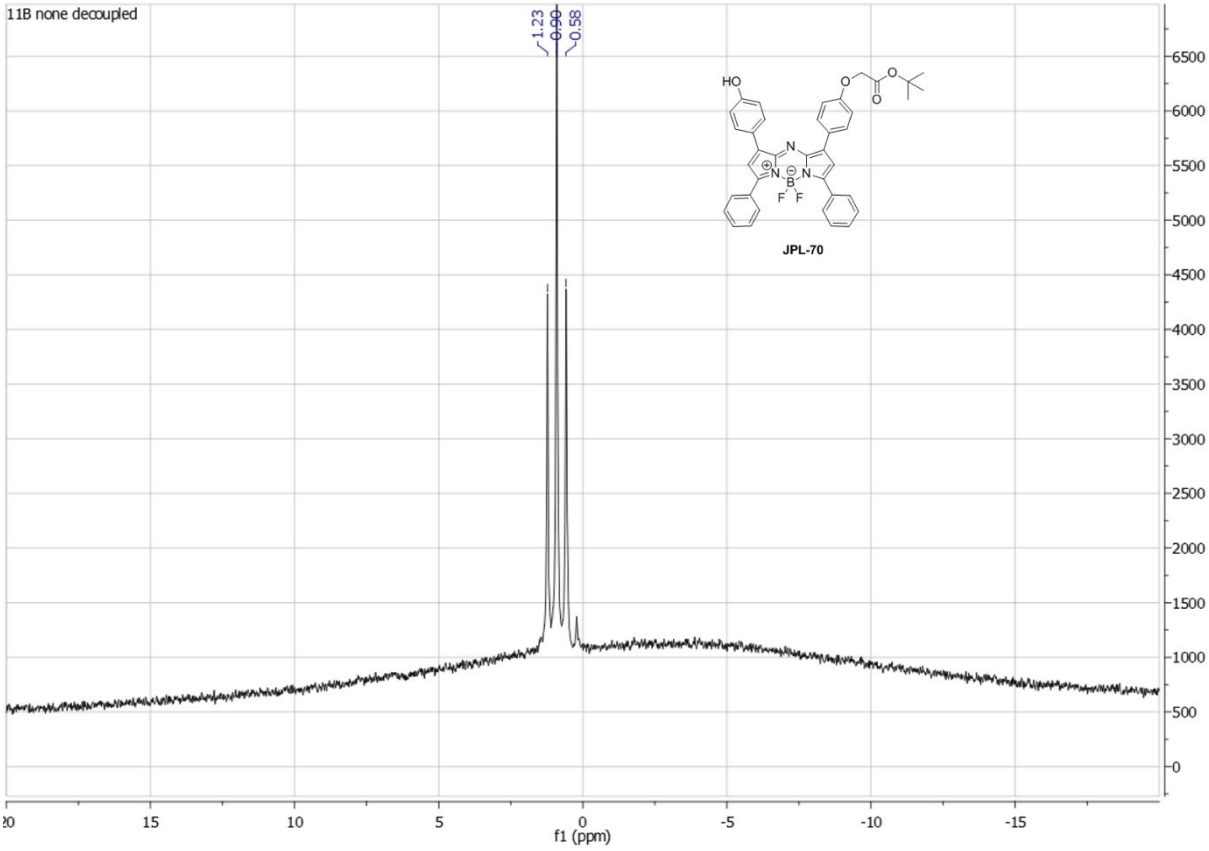


Figure S 7: ^{11}B NMR of aza-BODIPY A2 (96 MHz, $CDCl_3$)

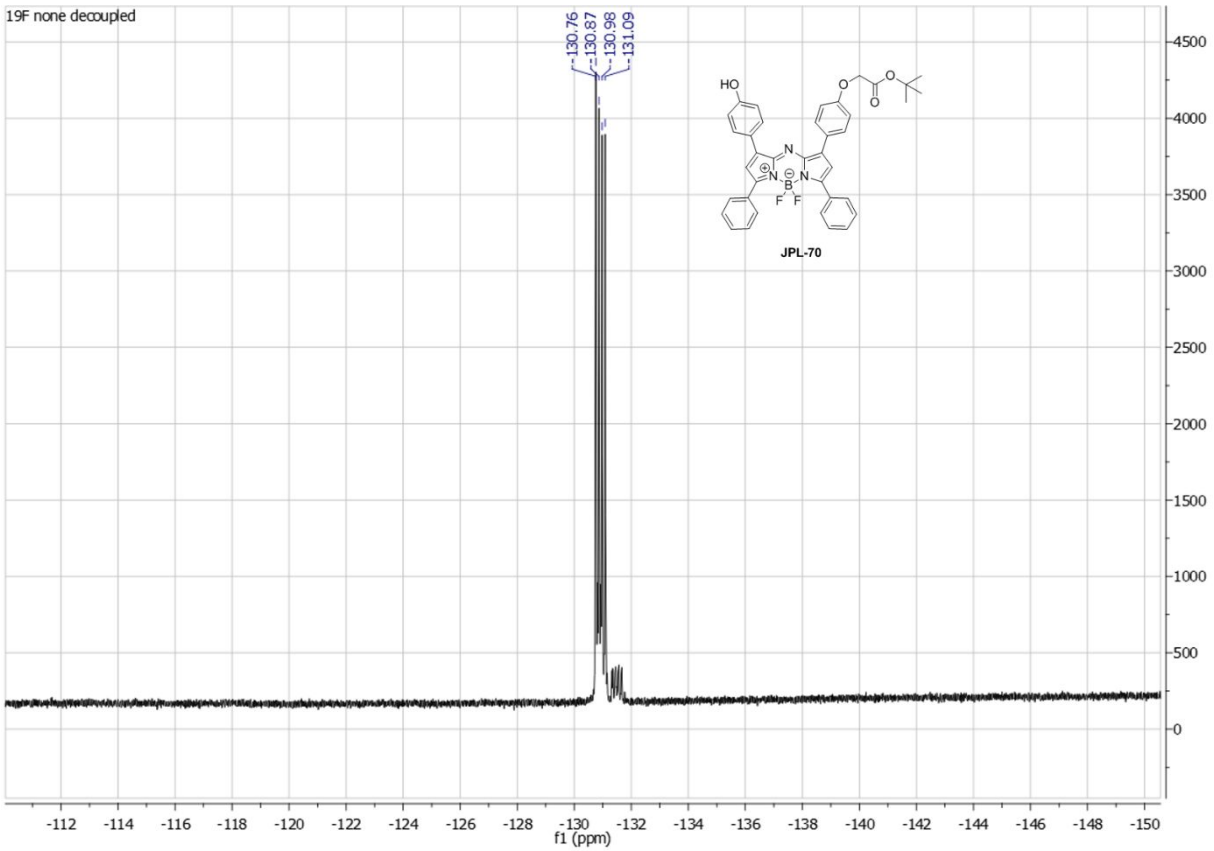
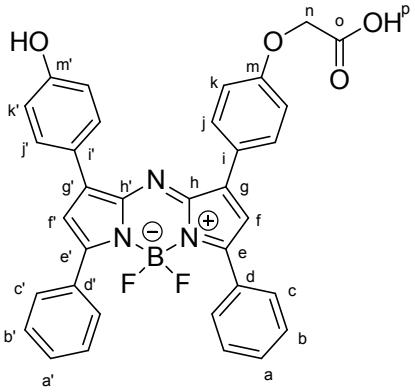


Figure S 8: ^{19}F NMR of aza-BODIPY 1 A2 (282 MHz, $CDCl_3$)

2.3 Aza-BODIPY A3:



141mg (0.219 mmol) of **A2** were dissolved in 4 mL of THF. 2 mL of a solution of 6 M HCl in water were added and the solution was stirred at room temperature upon completion (7 h, followed by TLC). The solvents were evaporated under reduced pressure. The crude product was purified by RP-Flash-chromatography using a gradient of an aqueous, 50 mM TEAB (triethylammonium bicarbonate) solution and acetonitrile (10%ACN→100%ACN in 10 min, followed by 10 min 100% ACN). The collected product fractions were evaporated to dryness to yield 89mg (152 µmol, 69% yield) of the target compound **A3** as a dark blue solid.

^{1}H NMR (600 MHz, DMF-d_7) δ (ppm) = 4.95 (s, 2H, H_n), 7.08 (d, J = 8.8 Hz, 2H, $H_{k'}$), 7.21 (d, J = 9.0 Hz, 2H, H_k), 7.49 (s, 1H, $H_{f'}$), 7.51 (d, J = 0.8 Hz, 1H, H_f), 7.55 – 7.59 (m, 6H, H_b, $H_{b'}$, H_a, $H_{a'}$), 8.17 – 8.21 (m, 4H, H_c, $H_{c'}$), 8.25 (d, J = 8.8 Hz, 2H, $H_{j'}$), 8.29 (d, J = 9.0 Hz, 2H, $H_{j'}$), 10.40 (s, 1H, H_p).

^{13}C{^{1}H} NMR (151 MHz, DMF-d_7) δ (ppm) = 66.0, 116.1, 117.2, 119.1, 119.5, 124.6, 126.7, 129.7, 129.7, 130.7, 130.8, 131.9, 132.1, 132.1, 132.5, 132.9, 133.0, 144.1, 145.6, 146.0, 146.6, 159.3, 160.7, 160.9, 161.6, 171.1.

^{11}B NMR (96 MHz, CD_3CN) δ (ppm)= 0.8 (t, $J_{B\text{-}F}$ = 31.2 Hz).

^{19}F NMR (282 MHz, CD_3CN) δ (ppm)= -130.3 (q, $J_{F\text{-}}{}^{11}{}_{B}$ = 31.3 Hz)

HR-MS (ESI) (Da): calculated for $C_{35}H_{25}B_1F_2N_3O_4$ $[M+H]^+$ 588.19007; found 588.19042

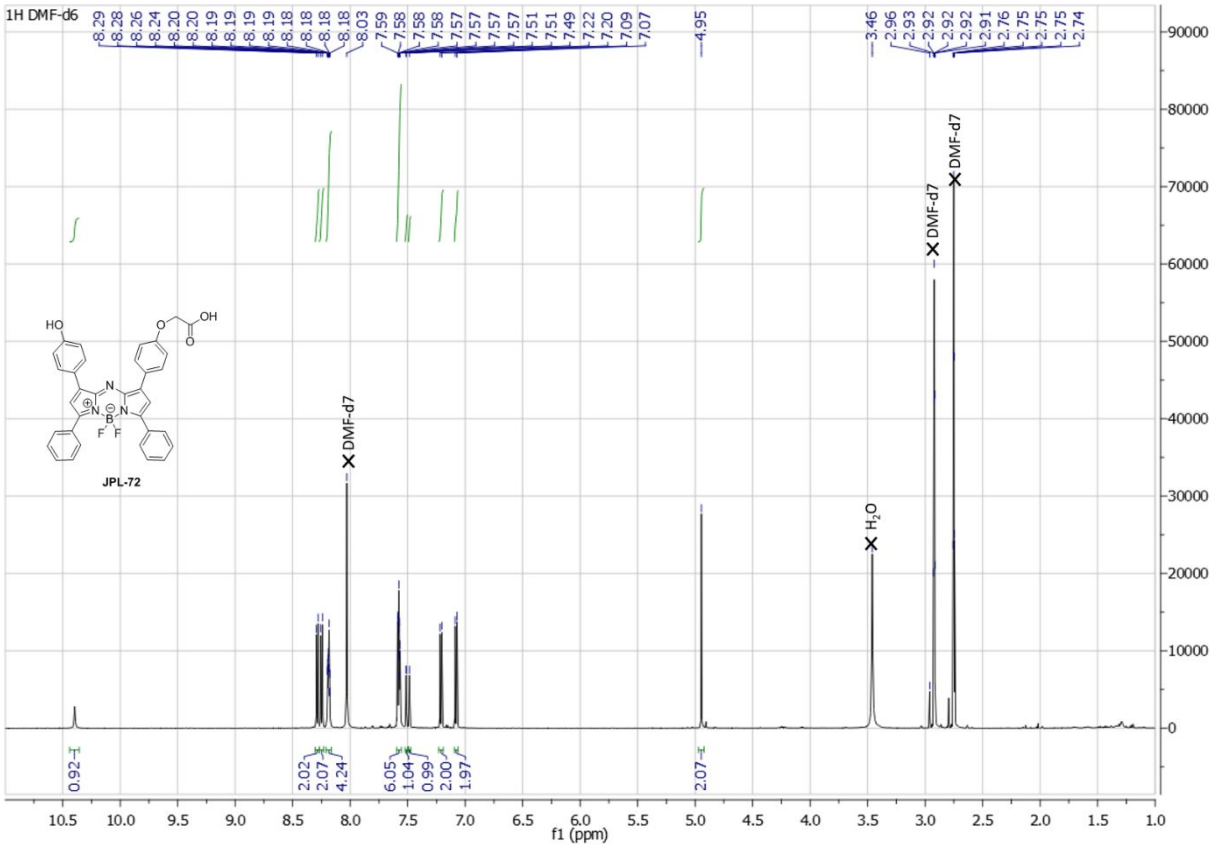


Figure S 9: ^{1}H NMR spectrum of aza-BODIPY A3 (600 MHz, DMF-d_7)

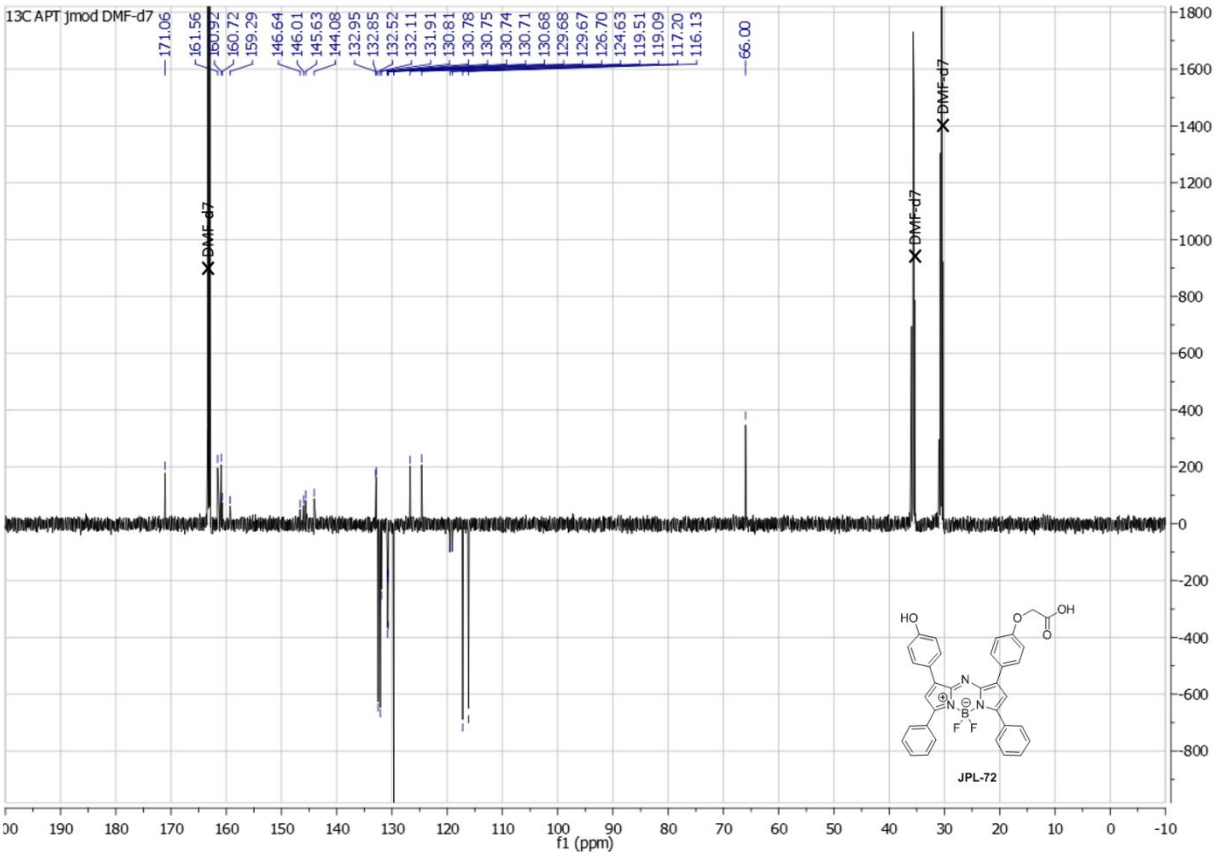


Figure S 10: $^{13}C\{^{1}H\}$ NMR of aza-BODIPY A3 (151 MHz, DMF-d_7)

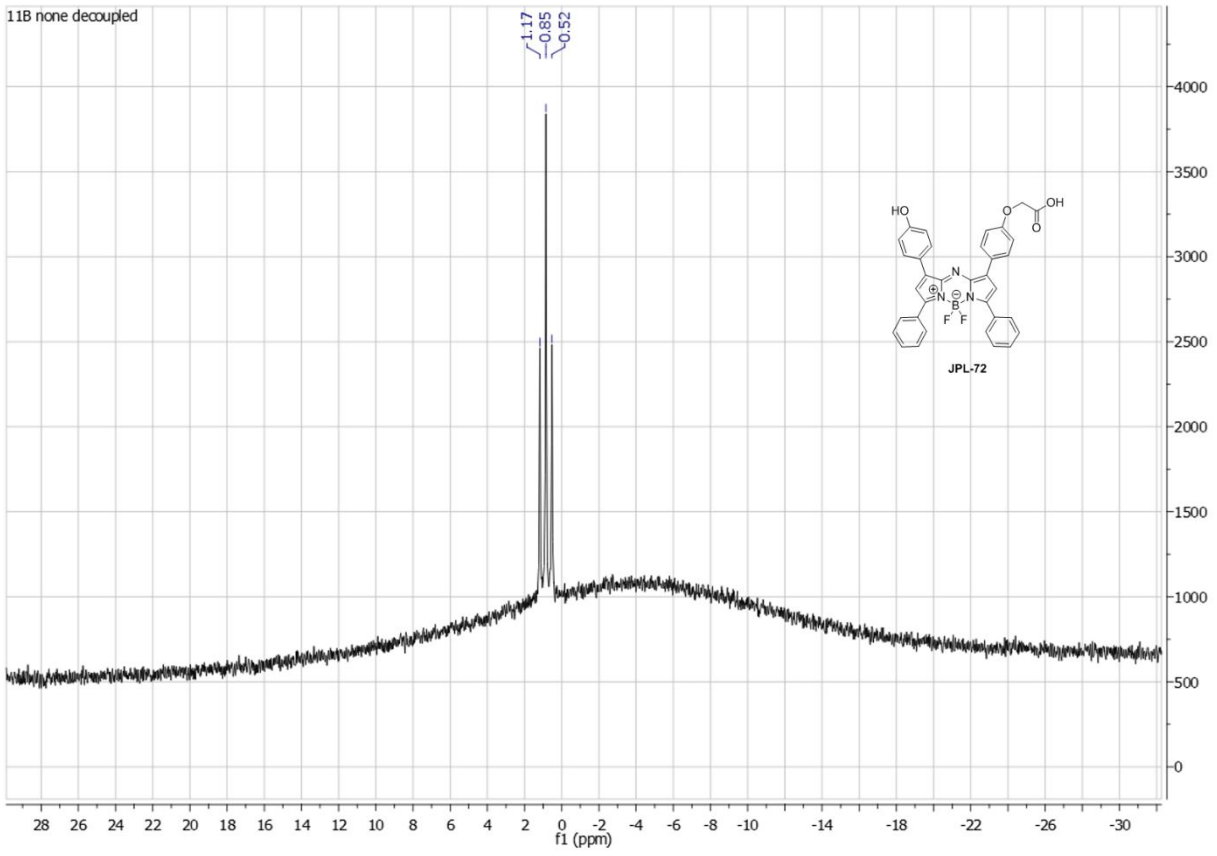


Figure S 11: ^{11}B NMR of aza-BODIPY A3 (96 MHz, CD_3CN)

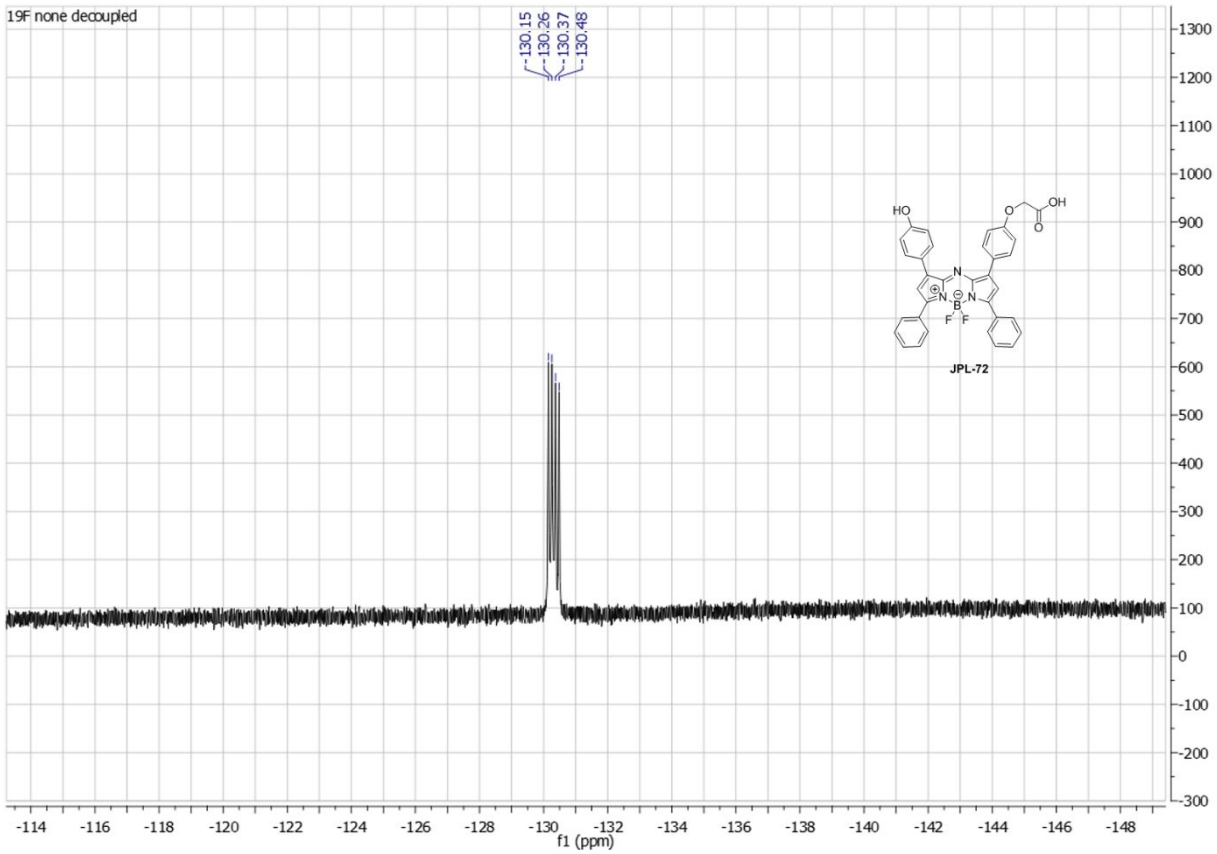
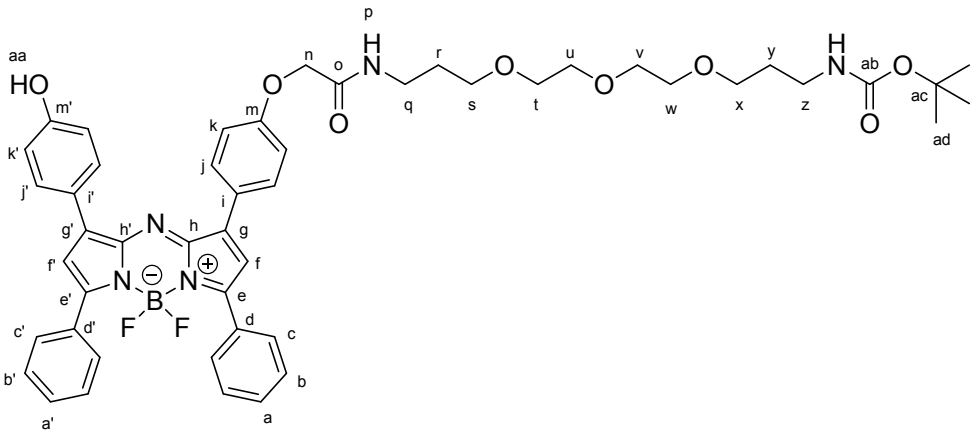


Figure S 12: ^{19}F NMR of aza-BODIPY A3 (282 MHz, CD_3CN)

2.4 Aza-BODIPY A4:



20 mg (34 µmol, 1eq) of aza-BODIPY-acid **A3** were dissolved in 3 mL of DCM. Two droplets (m_{theo}=5mg, v_{theo}=3.5µL, 1.2eq) of oxalylchloride and one droplet of DMF were added. After the development of gas has seized, the reaction was stirred for one hour at room temperature. The solution was evaporated to dryness and the resulting residue was dissolved in DMF (1 mL). 13 mg (41 µmol, 1.2eq) of TOTA-Boc were dissolved in 1mL of DMF and added to the Aza-BODIPY solution, followed by addition of 16 mg (41 µmol, 1.2eq) of HBTU in 1 mL of DMF and 0.1 mL of DIPEA. The reaction

was further stirred for 16 h at room temperature (reaction control by HPLC). After total conversion of the starting material, the solvents were evaporated under reduced pressure. The crude product was washed three times with diluted HCl (pH=3) and neutralized by washing with $NaHCO_3$ solution. The organic fractions were gathered, dried over Na_2SO_4 and solvents removed *in vacuo*. The product was dissolved in DCM and precipitated by addition of pentane. The resulting product was dried, resulting in pure **A4** as dark blue solid in 73% yield (22 mg, 25 µmol).

^{1}H NMR (300 MHz, CD_3CN) δ (ppm)= 1.37 (s, 9H, H_{ad}), 1.61 (q, ^3J = 6.4 Hz, 2H, H_r), 1.74 (q, ^3J = 6.4 Hz, 2H, H_y), 3.05 (dd, ^3J = 12.8 Hz, ^4J = 6.7 Hz, 2H, H_q), 3.33 (dd, ^3J = 12.7 Hz, ^4J = 6.6 Hz, 2H, H_z), 3.40 (t, ^3J = 6.1 Hz, 2H, H_s), 3.56 – 3.42 (m, 10H, H_t, H_u, H_v, H_w, H_x), 4.50 (s, 2H, H_n), 5.38 (s, 1H, H_{aa}), 6.96 (d, ^3J = 8.9 Hz, 2H, H_k), 7.06 (d, ^3J = 9.0 Hz, 2H, $H_{k'}$), 7.10 (s, 2H, H_f, $H_{f'}$),7.26 (t, J = 5.7 Hz, 1H, H_p), 7.57 – 7.42 (m, 6H, H_a, $H_{a'}$, H_b, $H_{b'}$), 8.04 – 7.95 (m, 6H, H_c, $H_{c'}$, H_j), 8.08 (d, J = 9.0 Hz, 2H, $H_{j'}$).

^{13}C{^{1}H} NMR (75 MHz, CD_3CN) δ (ppm) = 28.6, 30.1, 37.4, 57.4, 68.1, 69.6, 69.8, 70.8, 70.9, 71.0, 75.4, 116.0, 116.8, 124.9, 126.8, 129.4, 129.4, 130.0, 131.7, 131.9, 132.2, 132., 132.7, 144.4, 159.9, 160.3, 160.6, 168.6.

^{11}B NMR (96 MHz, CD_3CN) δ (ppm)= 0.8 (t, $J_{B\text{-}F}$ = 31.2 Hz)

^{19}F NMR (282 MHz, CD_3CN) δ (ppm) = -131.3 (q, $J_{F\text{-}^{11}B}$ = 31.2 Hz)

HR-MS (ESI) (Da): calculated for $C_{49}H_{54}B_1F_2N_5O_8Na_1$ $[M+Na]^+$ 912.39257; found 912.39411

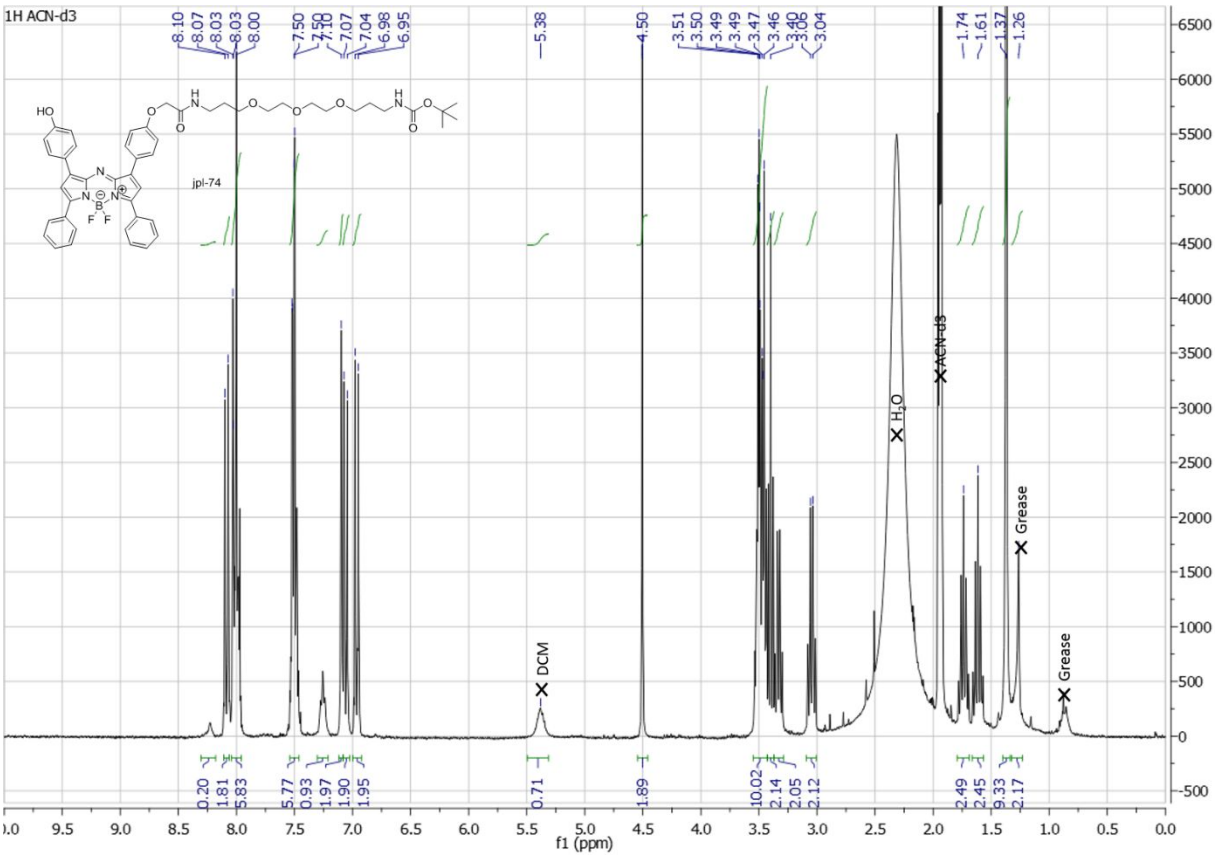


Figure S 13: ^{1}H NMR spectrum of aza-BODIPY A4 (300 MHz, CD_3CN)

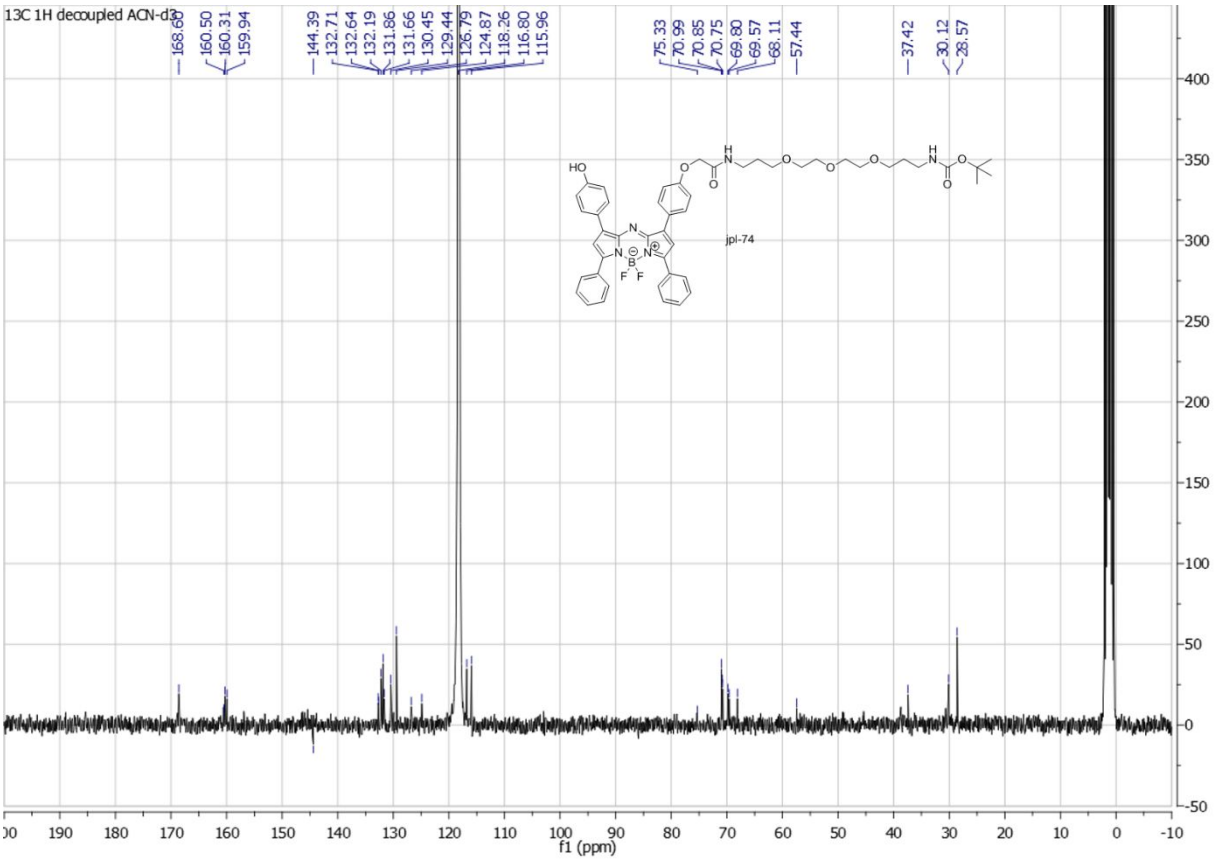


Figure S 14: $^{13}C\{^{1}H\}$ NMR of aza-BODIPY A4 (75 MHz, CD^3CN)

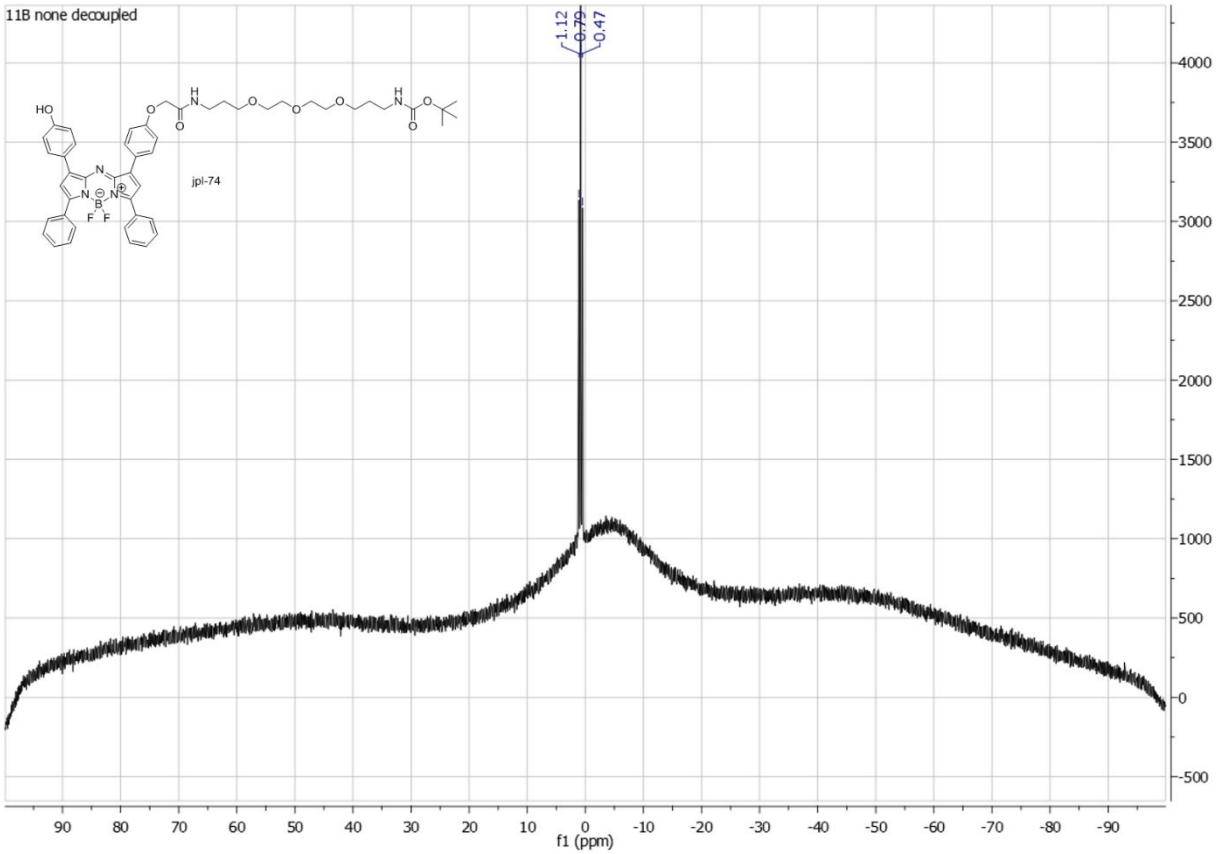


Figure S 15: ^{11}B NMR of aza-BODIPY A4 (96 MHz, CD_3CN)

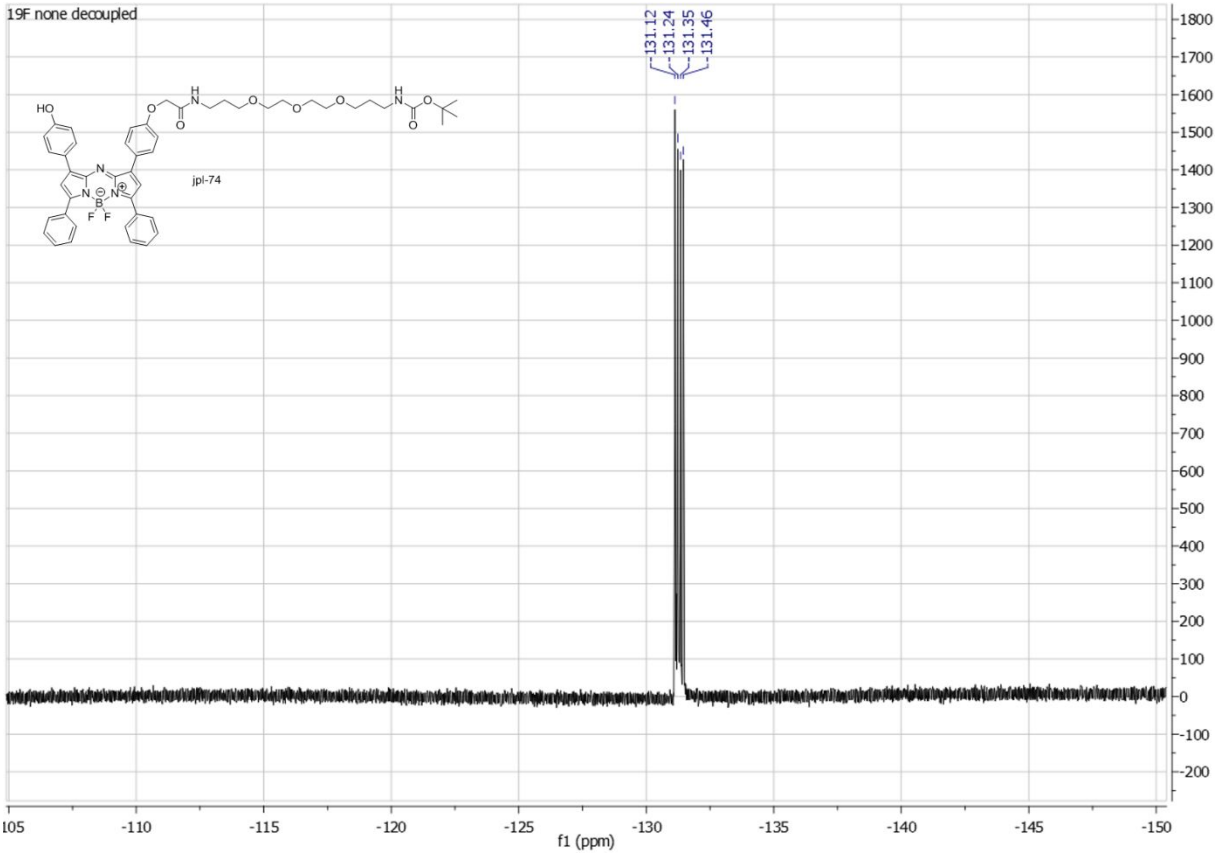
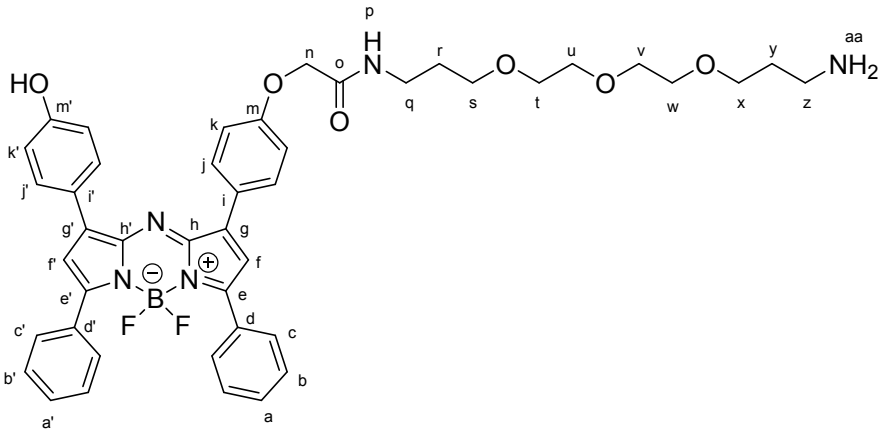


Figure S 16: ^{19}F NMR of aza-BODIPY A4 (282 MHz, CD_3CN)

2.5 Compound A:



21 mg (24 µmol) of azaBODIPY-TOTA-Boc **A4** were dissolved in THF (3 mL). 3 mL of 3 M HCl were then added and the reaction was stirred for 18h at room temperature, followed by 4 h at 35°C. The resulting crude product was evaporated to dryness at 30°C and purified by semi-preparative HPLC using as eluent, the following gradient: H_2O+0.1% formic acid and ACN+0.1% formic acid (30%ACN + 0.1FA to 90%ACN + 0.1%FA in 20 min). The fractions corresponding to the targeted product were evaporated to dryness and precipitated from DCM/Pentane to obtain 16 mg (20 µmol, 83%) of pure **A** as a dark blue powder.

^{1}H NMR (500 MHz, CD_2Cl_2) δ (ppm)= 1.80 (s, 2H, H_r), 1.97 (s, 2H, H_y), 3.23 (s, 2H, H_q), 3.43 (m, 2H, H_z), 3.49 – 3.68 (m, 14H, H_t, H_u, H_v, H_w, H_x, H_{aa}), 4.56 (s, 2H, H_n), 6.94 (s, 2H, H_f, $H_{f'}$), 7.03 (t, ^{3}J = 8.7 Hz, 4H, $H_{k'}$, H_k), 7.42 – 7. 52 (m, 6H, H_b, H_a, $H_{b'}$, $H_{a'}$), 7.54 (s, br, H_p), 7.94-8.04 (m, 6H, H_c, $H_{c'}$, H_j), 8.06 (d, J = 8.5 Hz, 2H, $H_{j'}$)

^{13}C{^{1}H} NMR (151 MHz, DMF-d_6) δ (ppm) = 28.5, 37.4, 38.6, 39.2, 68.5, 69.3, 69.7, 70.9, 71.0, 71.1, 71.3, 116.3, 117.2, 119.1, 119.5, 124.5, 126.9, 129.7, 129.7,130.7, 130.8, 131.9, 132.1, 132.2, 132.5, 132.8, 132.9, 143.8, 145.8, 160.7, 161.7, 168.8.

^{11}B NMR (160 MHz, MeOD) δ (ppm)= 0.89 (t, $J_{B\text{-}F}$ = 31.3 Hz).

^{19}F NMR (470 MHz, CD_2Cl_2) δ (ppm)= -130.57 (q, $J_{F\text{-}^{11}B}$ = 31.3 Hz).

HR-MS (ESI) (Da): calculated for $C_{44}H_{47}B_1F_2N_5O_6$ $[M+H]^+$ 790.35820; found 790.35768.

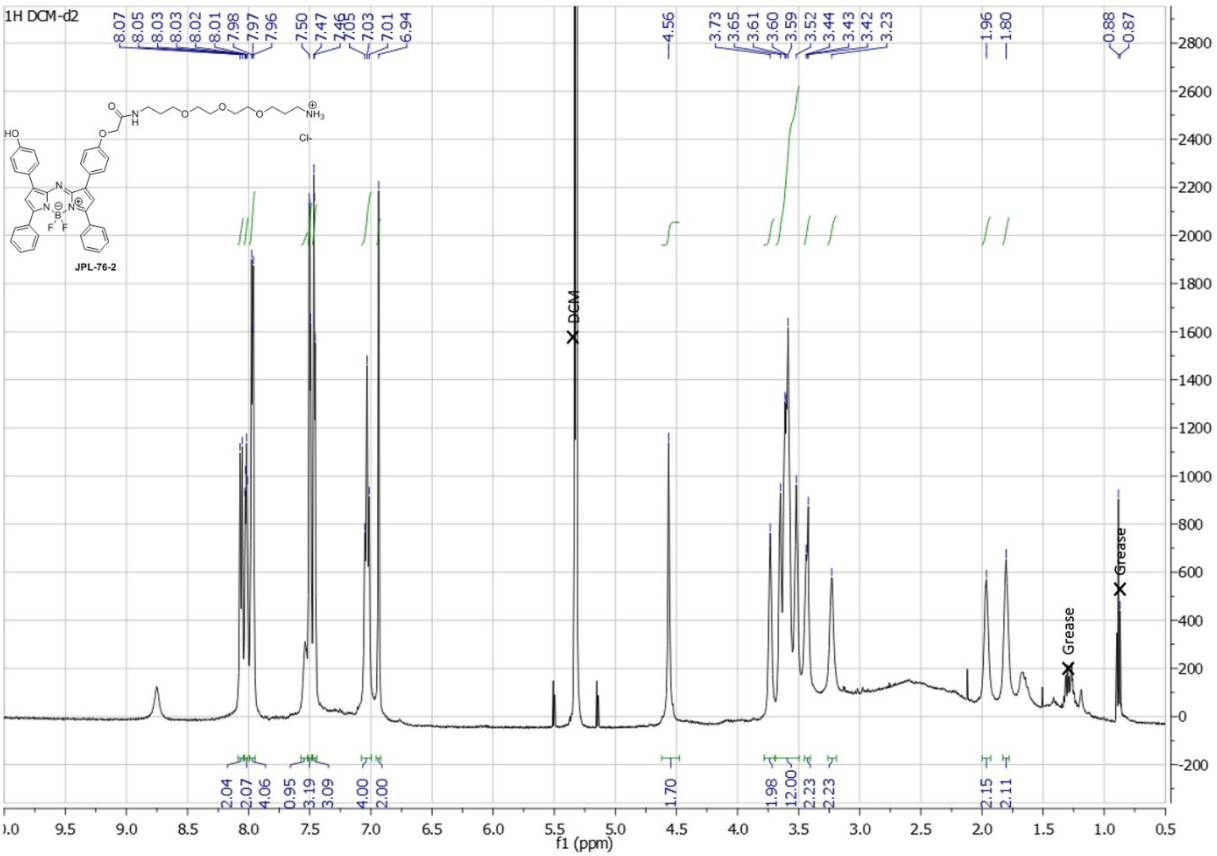


Figure S 17: ^{1}H NMR spectrum of aza-BODIPY A (500 MHz, CD_2Cl_2)

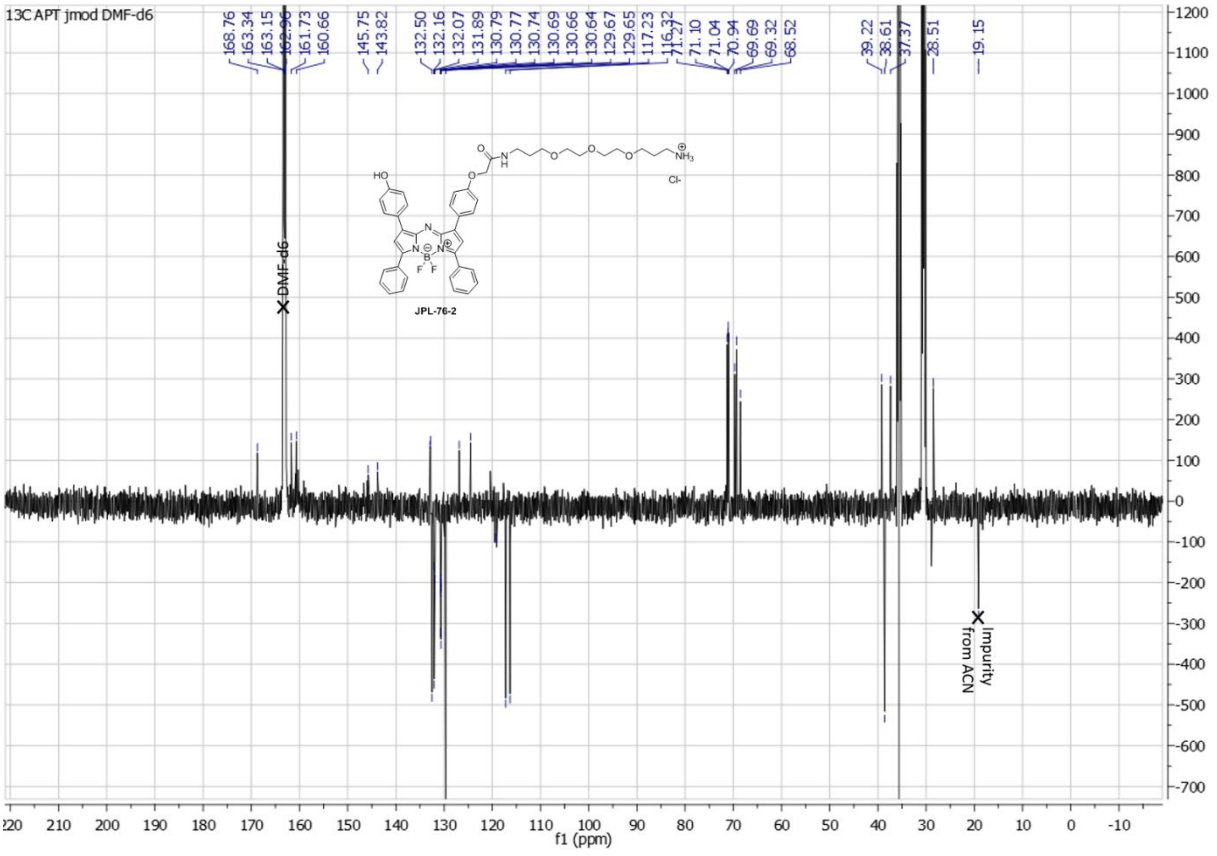


Figure S 18: ^{13}C APT NMR of aza-BODIPY A (151 MHz, DMF-d_7)

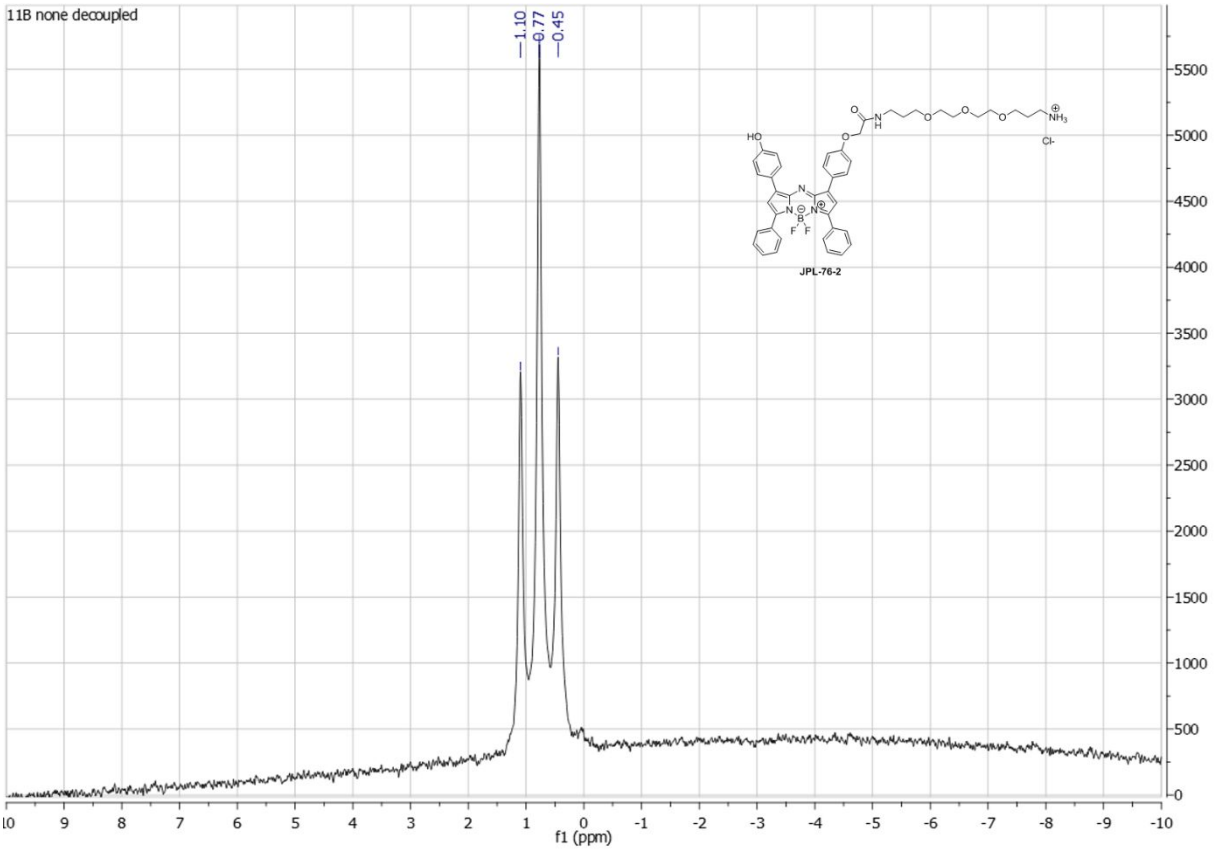


Figure S 19: ^{11}B NMR of aza-BODIPY A (160 MHz, MeOD)

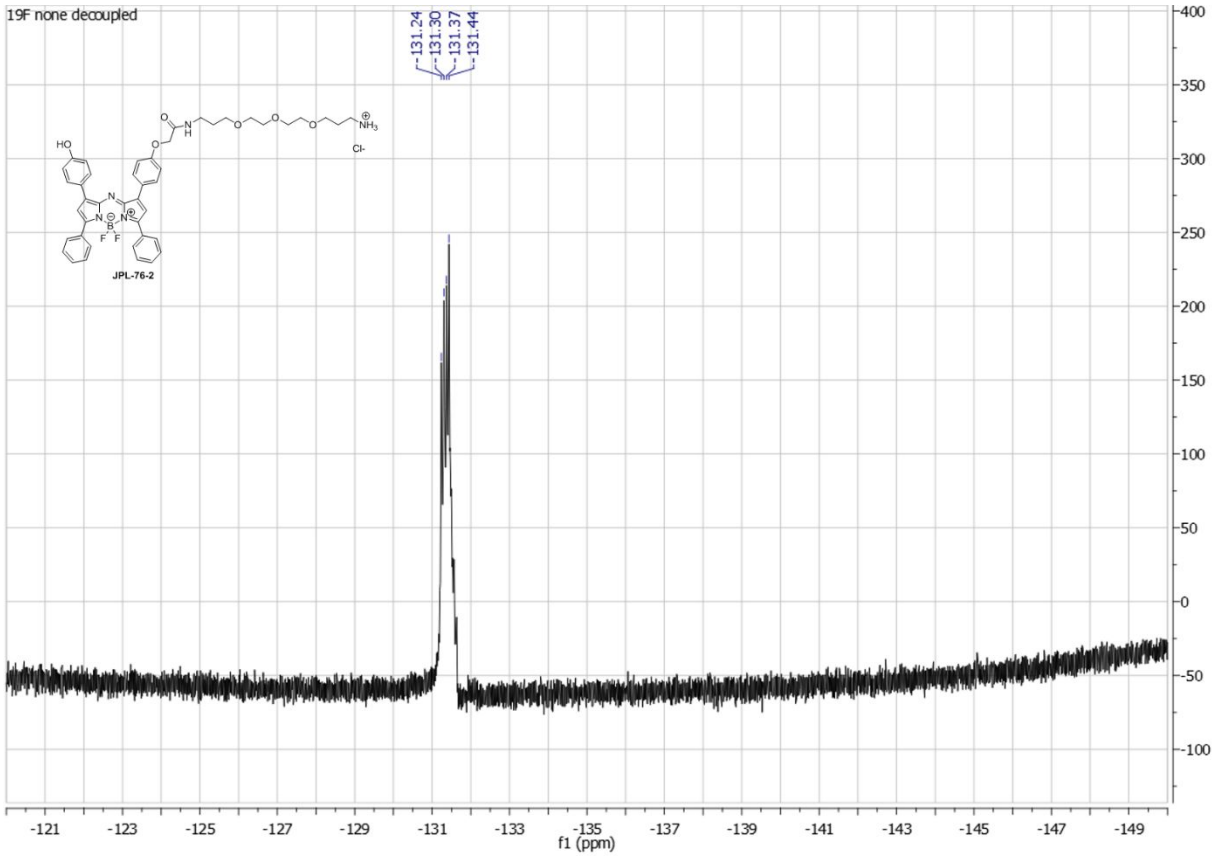
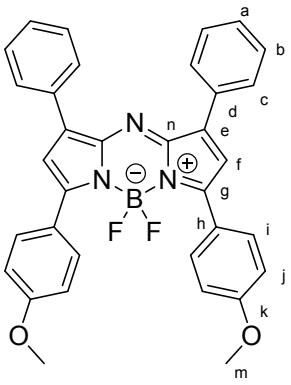


Figure S 20: ^{19}F NMR of aza-BODIPY A (470 MHz, CD_2Cl_2)

2.6 Aza-BODIPY 1:



Aza-BODIPY **1** was synthesized following a reported procedure (*DOI 10.1021/jo402160b*).

^{1}H NMR (500 MHz, DMSO-D$_6$) δ (ppm)= 3.89 (s, 6H, H$_m$), 7.18 – 7.12 (m, 4H, H$_j$), 7.48 (t, *J* = 7.3 Hz, 2H, H$_a$), 7.54 (t, *J* = 7.4 Hz, 4H, H$_b$), 7.61 (s, 2H, H$_f$), 8.18 (t, *J* = 8.4 Hz, 8H, H$_c$, H$_i$).

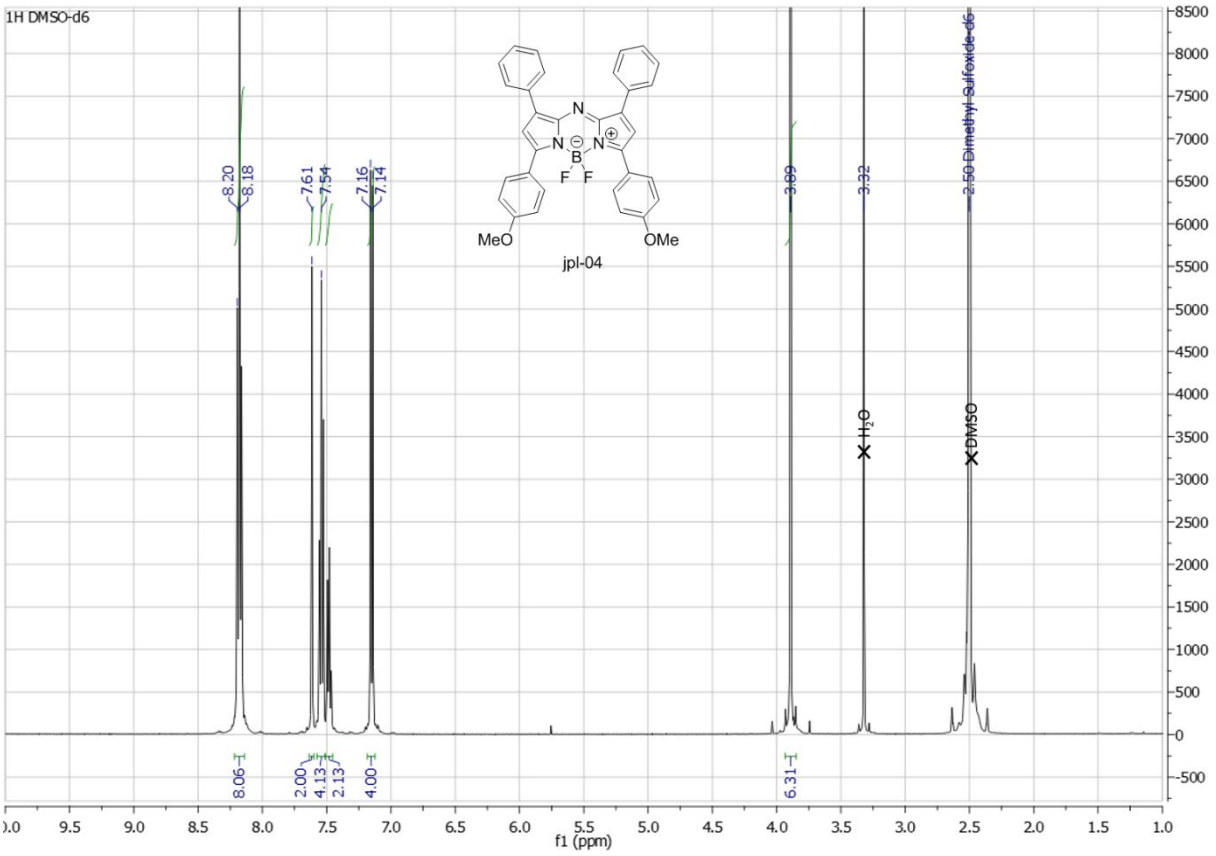
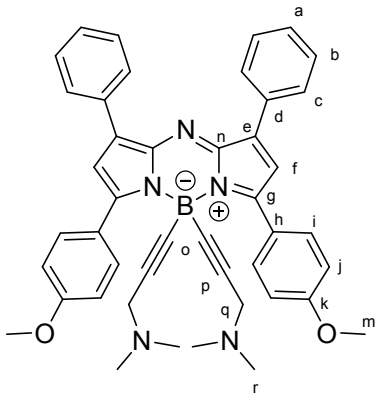


Figure S 21: ^{1}H NMR spectrum of aza-BODIPY 1 (500 MHz, DMSO-D$_6$)

2.7 Aza-BODIPY 2:



In a dried Schlenktube, 386 µL (3.89 mmol, 2eq) of 3-dimethylpropyne were dissolved in dry THF (3 mL). 4.503 mL (2.1eq) of freshly dosed ethylmagnesiumbromide were added and the resulting solution was refluxed for 45 min. In a separate Schlenktube, 1 g (1.79 mmol, 1eq) of aza-BODIPY **1** was dissolved in dry THF (15 mL) and added to the Grignard-solution *via* cannula. The mixture was refluxed for another 45 min. EtOH (5 mL) was then added, the solvents were evaporated and the crude product dissolved in EtOAc and washed twice with diluted bicarbonate aqueous solution. Organic fractions were collected, evaporated to dryness, adsorbed onto silica gel and purified using a short silica gel plug (eluent: EtOAc, followed by DCM/MeOH 80:20). Product fractions were collected, evaporated and precipitated once from DCM/pentane, yielding pure aza-BODIPY 2 (1.1 g, 1.61 mmol, 90% yield) as a dark green powder.

^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm)= 2.10 (s, 12H, H_r), 3.05 (s, 4H, H_q), 3.87 (s, 6H, H_m), 6.97 (s, 2H, H_f), 6.99 (s, 4H, H_j), 7.40 (t, J = 7.3 Hz, 2H, H_a), 7.45 (t, J = 7.3 Hz, 4H, H_b), 8.06 (d, J = 7.3 Hz, 4H, H_c), 8.31 (d, J = 8.7 Hz, 4H, H_i).

^{13}C NMR (126 MHz, $CDCl_3$) δ (ppm)= 29.8, 43.0, 48.2, 55.6, 91.7, 113.5, 119.4, 125.2, 128., 129.1, 129., 132.7, 132.8, 142.2, 143.3, 157.8, 161.4.

^{11}B NMR (160 MHz, $CDCl_3$) δ (ppm)= -12.3 (very broad singlet)

HR-MS (ESI) (Da): *m/z* = calculated for $C_{44}H_{43}B_1N_5O_2$ $[M+H]^+$ 684.35043; found 684.34847.

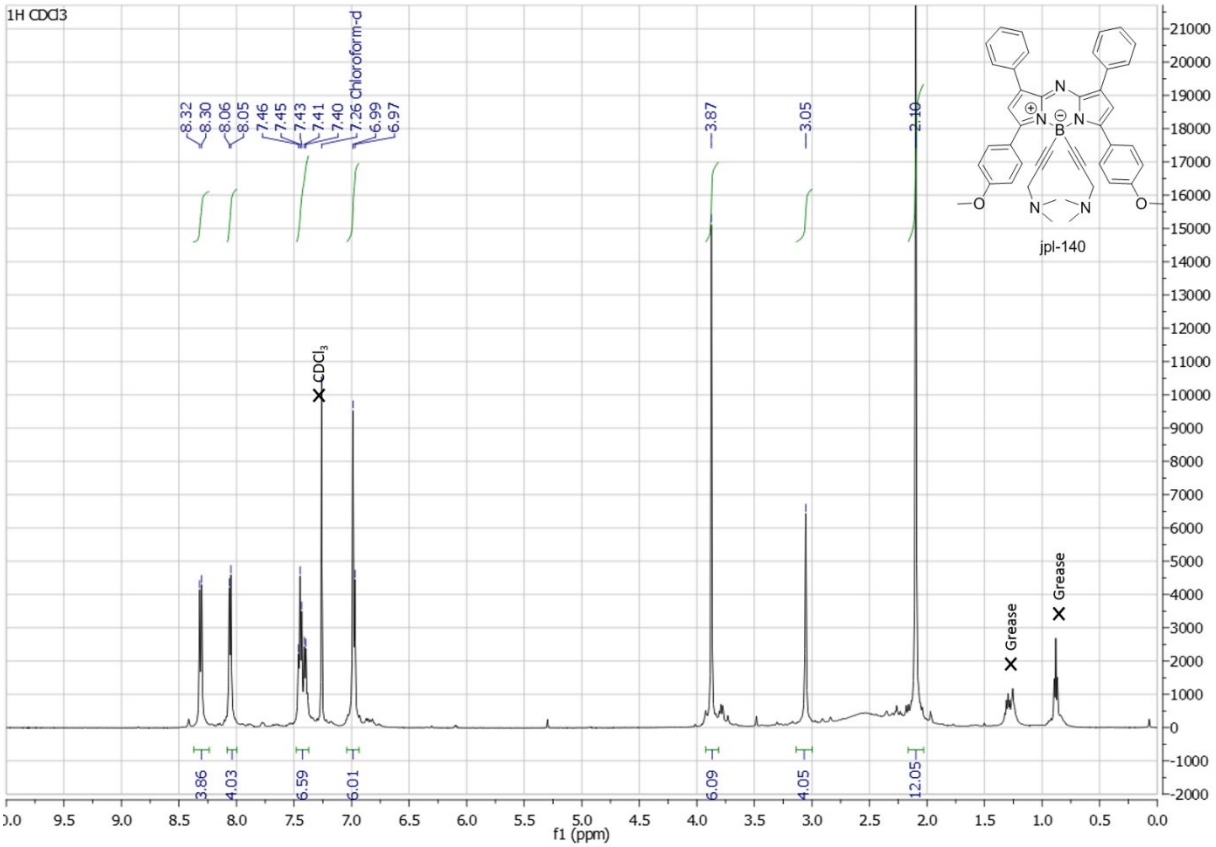


Figure S 22: ^{1}H NMR spectrum of aza-BODIPY 2 (500 MHz, $CDCl_3$)

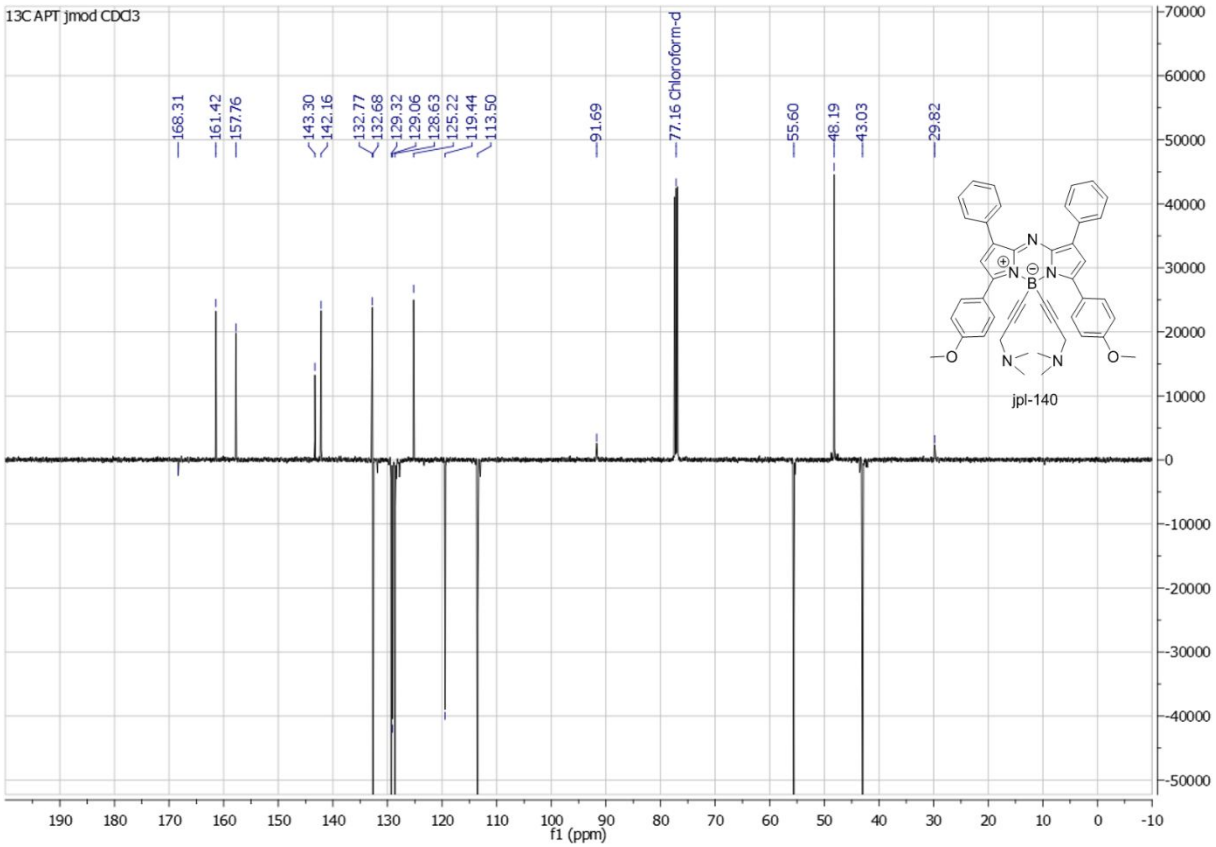


Figure S 23: ^{13}C-APT jmod NMR of aza-BODIPY 2 (126 MHz, $CDCl_3$)

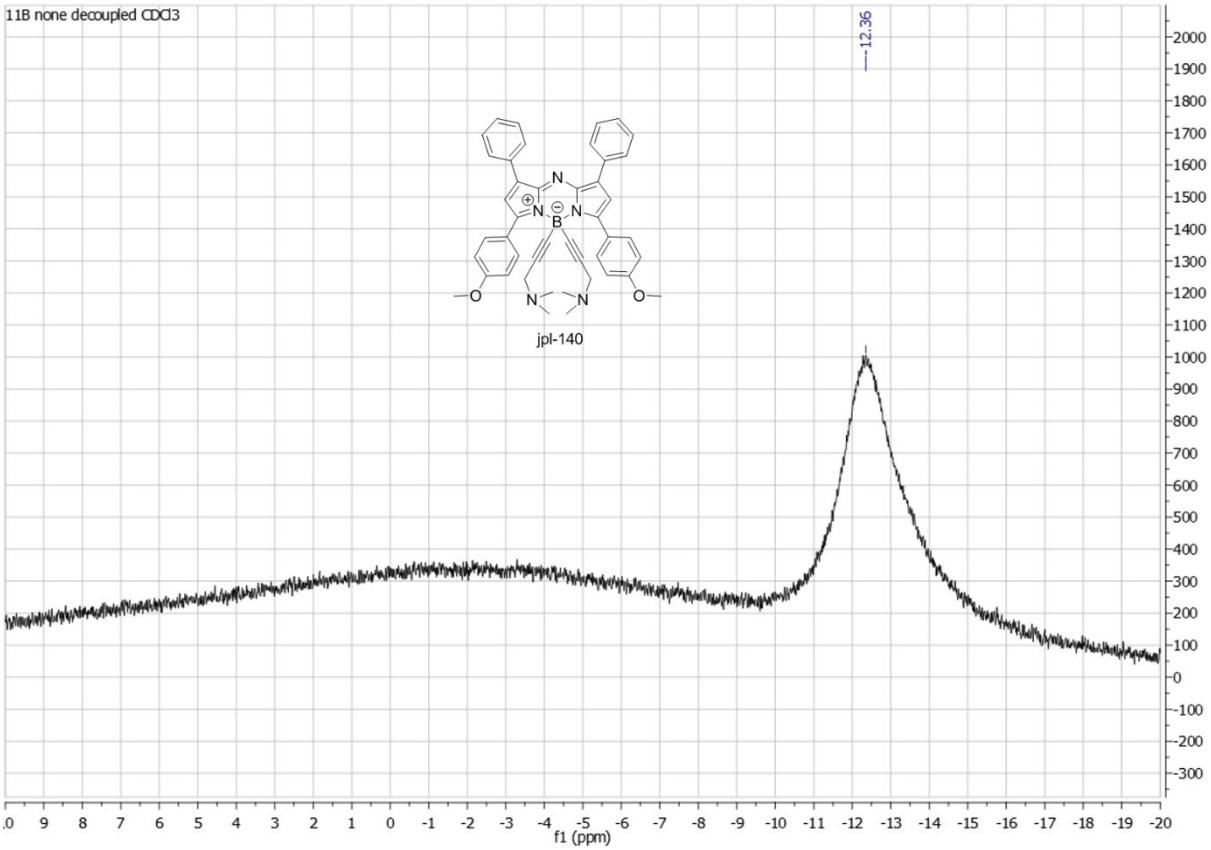
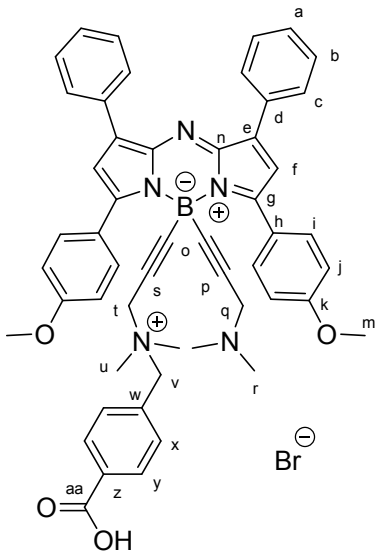


Figure S 24: ^{11}B NMR of aza-BODIPY 2 (160 MHz, $CDCl_3$)

2.8 Aza-BODIPY 3:



194 mg (0.284 mmol, 1eq) of aza-BODIPY **2** were dissolved in 200 mL of dry THF. 67 mg (0.312 mmol, 1.1eq) of 4-(Bromomethyl) benzoic acid were added and the resulting solution was stirred overnight. The stirring was stopped and the supernatant decanted once all solids had sedimented. The sediment was washed three times with hot THF (supernatants contain Aza-BODIPY **3**). All supernatants were gathered, evaporated to dryness and purified by column chromatography on a short silica gel column. Leftover starting material is eluted with toluene → Tol:MeOH 8:2, followed by 100% MeOH to isolate

Aza-BODIPY **3**. Product fractions of monoacid were reunited and evaporated to dryness. The product was dissolved in a minimum amount of MeOH, diluted in DCM and precipitated by addition of pentane to obtain 83 mg (91 µmol 32%) of the target compound **3** as a dark green powder. Overall yield 58%, leftover starting material can be isolated and re-used.

^{1}H NMR (600 MHz, MeOD) δ (ppm)= 2.62 (d, J = 1.3 Hz, 6H, H_r (protonated form)), 2.68 (s, 6H, H_u), 3.58 (s, 2H, H_q), 3.86 – 3.76 (m, 8H, H_m+H_t), 3.99 (s, 2H, H_v), 7.10 (d, J = 8.8 Hz, 4H, H_j), 7.32 (dd, J = 12.3, 4.2 Hz, 4H, H_f, H_x), 7.51 – 7.40 (m, 6H, H_a, H_b), 8.04 – 7.97 (m, 2H,H_y), 8.14 – 8.08 (m, 4H, H_c), 8.42 (dd, J = 8.8, 2.4 Hz, 4H, H_i).

^{11}B NMR (160 MHz, MeOD) δ (ppm)= -12.1 (very broad singlet).

^{13}C{^{1}H} NMR (151 MHz, MeOD) δ (ppm)= 43.0, 50.6, 55.0, 56.2, 66.6, 87.9, 90.2, 115.1, 121.1, 125.6, 129.7, 130.4, 130.5, 131.1, 131.2, 133.5, 133., 133.9, 138.4, 143.9, 144.2, 159.1, 163.5, 171.4.

HR-MS (ESI) (Da): *m/z* = calculated for $C_{52}H_{49}B_1N_5O_4$ $[M]^+$ 828.38721; found 828.38718

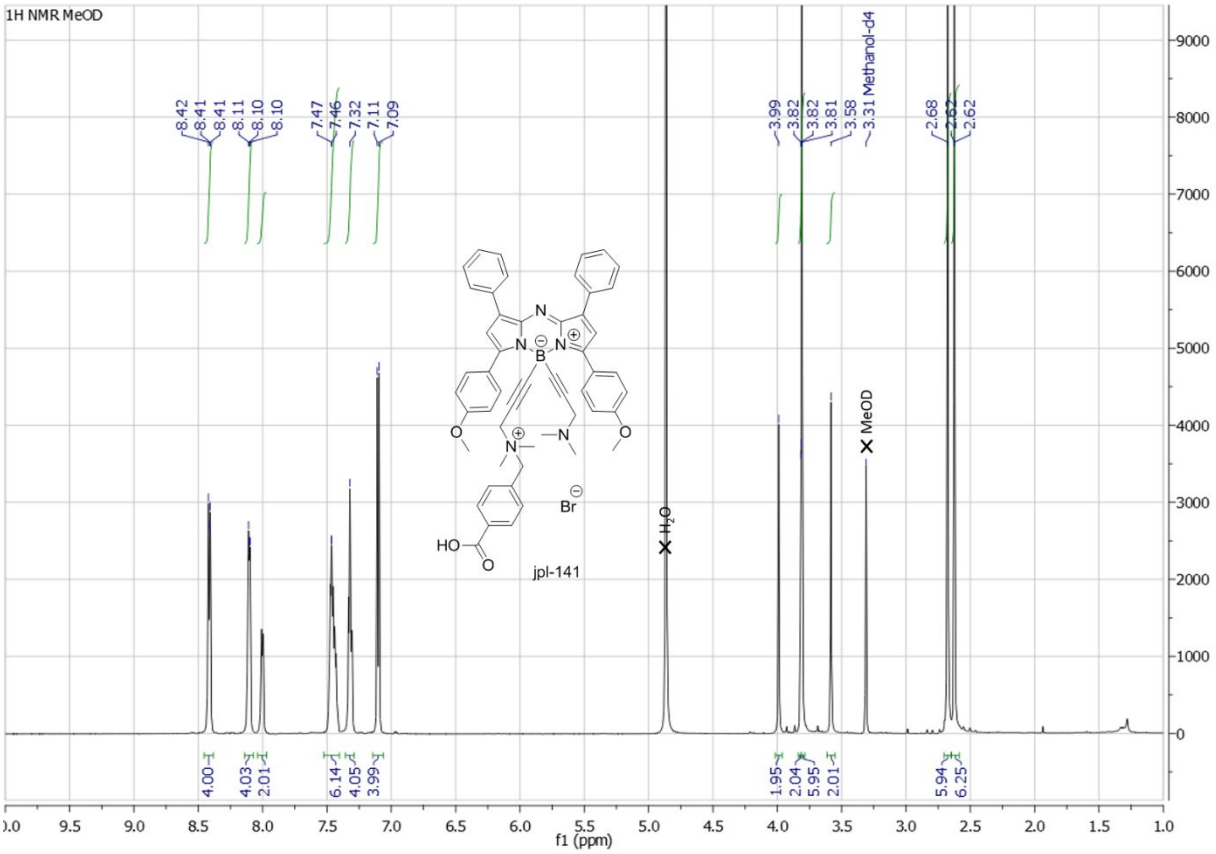


Figure S 25: ^{1}H NMR spectrum of aza-BODIPY 3 (600 MHz, MeOD)

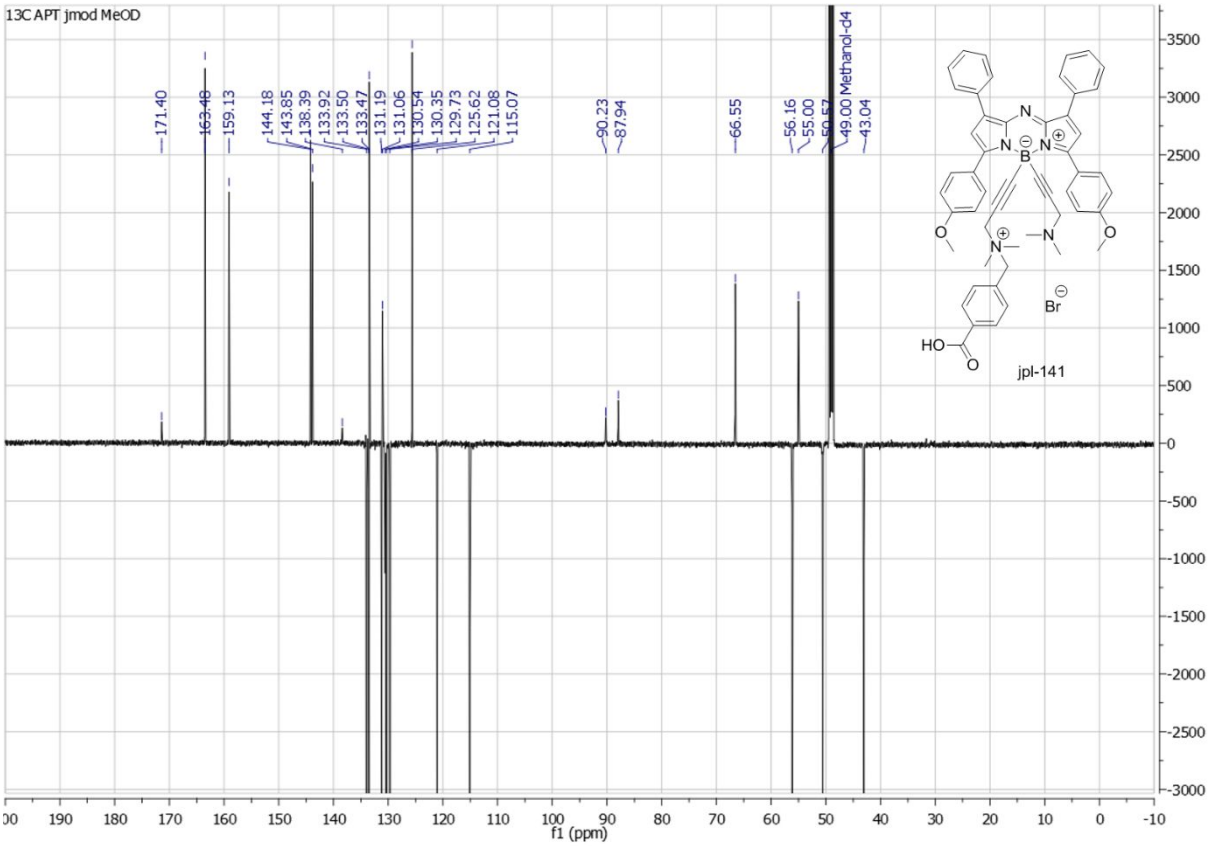


Figure S 26: [13]C APT NMR of aza-BODIPY 3 (160 MHz, MeOD)

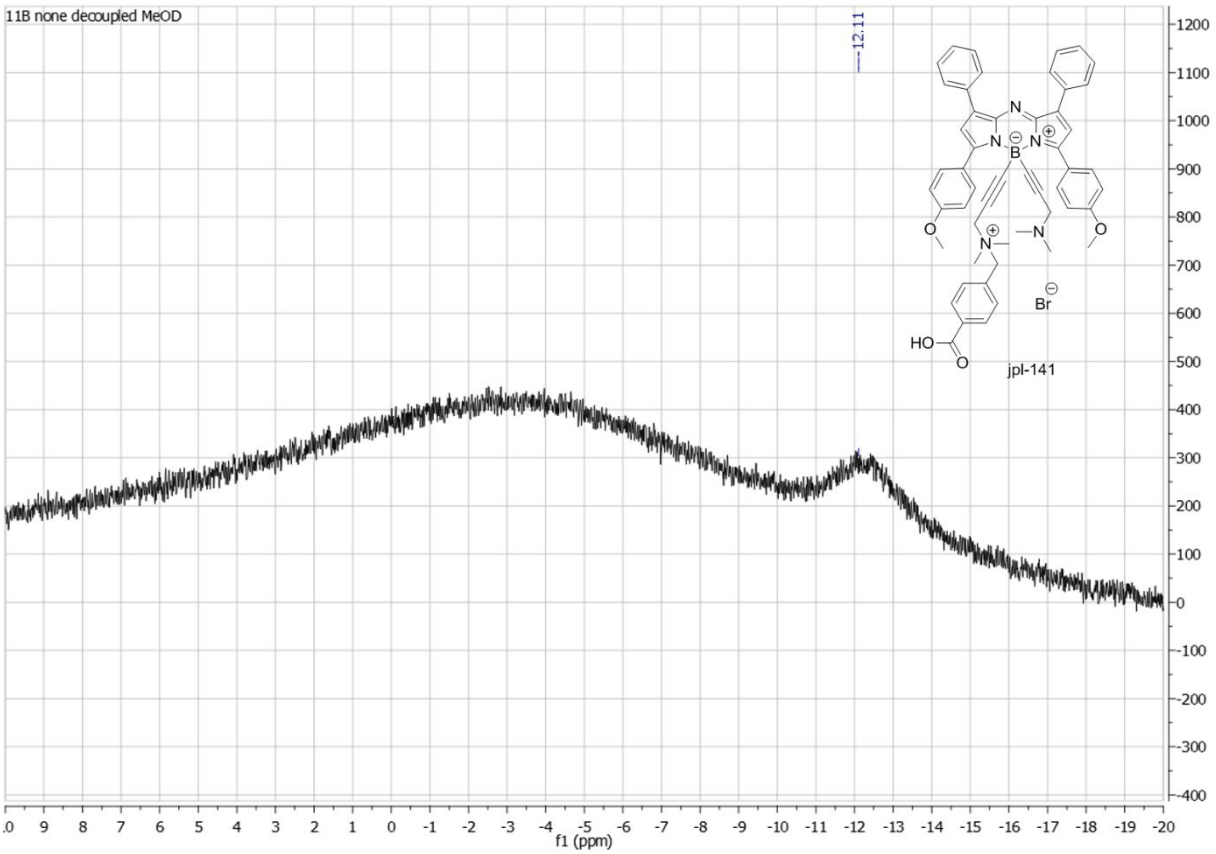
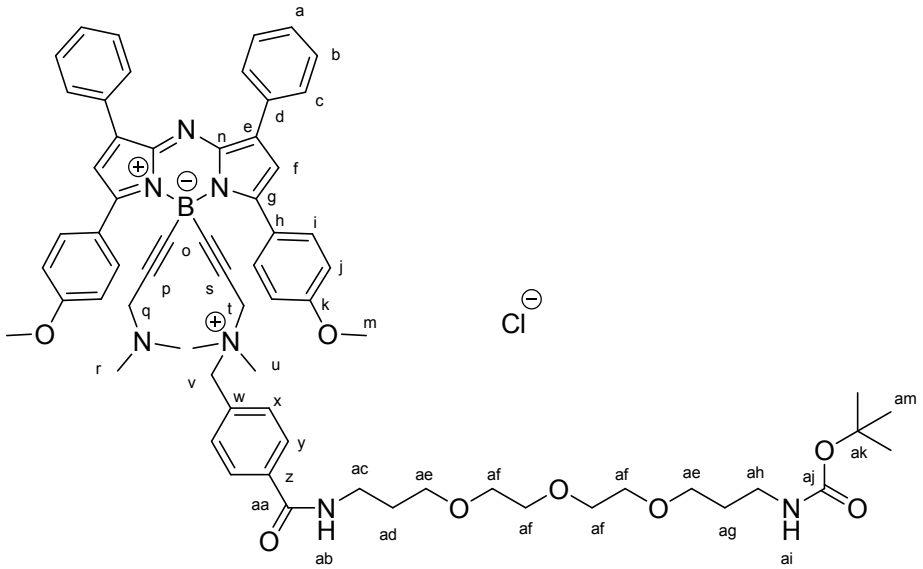


Figure S 27: [11]B NMR of aza-BODIPY 3 (151 MHz, MeOD)

2.9 Aza-BODIPY 4:



50 mg (56 µmol, 1eq) of **3** were placed in a round bottom flask and dissolved in dry DMF (5 mL). HBTU (25 mg, 67 µmol, 1.2eq) and DIPEA (0.3 mL, 1.8 mmol, 33eq) were added and the mixture was stirred for 1 h at 30°C. 21 mg (67 µmol, 1.2eq) of TOTA-Boc in DMF (1 mL) were added, and the solution stirred at 30°C for 4 h (reaction control by HPLC). Once finished, the solvents were evaporated to dryness and the crude product purified by column chromatography on 5% deactivated alumina, using DCM: MeOH 100→97:3 as eluent. Product fractions were collected and evaporated. The product was dissolved in ACN/H_2O (10 mL) and ion-exchange on a DOWEX basic resin performed. The eluate was evaporated and precipitated once from DCM/pentane to obtain the target compound **4** as a dark green solid. (20 mg, 17 µmol, 31% yield).

^{1}H NMR (500 MHz, MeOD) δ (ppm)= 1.30 (t, *J* = 7.3 Hz, 6H), 1.40 (s, 9H, H_{am}), 1.66 (dt, *J* = 12.9, 6.5 Hz, 2H, H_{ag}), 1.87 (p, *J* = 6.4 Hz, 2H, H_{ad}), 2.41 – 2.34 (m, 6H, H_r), 2.72-2.67 (m, 6H, H_u), 3.01 - 3.09 (m, 4H, H_{ae}), 3.41 - 3.52 (m, 8H, $2H_q$, $2H_{ah}$, $2H_{ac}$, H_{ai}, H_{ab}), 3.54 (s, 2H, H_t), 3.56 - 3.64 (m, 8H, H_{af}), 3.82 (s, 6H, H_m), 3.96 (s, 2H, H_v), 7.09 (d, *J* = 8.8 Hz, 4H, H_j), 7.33 (s, 2H, H_f), 7.36 (d, *J* = 8.1 Hz, 2H, H_x), 7.52 – 7.44 (m, 6H, H_a, H_b), 7.86 (d, *J* = 8.1 Hz, 2H, H_y), 8.15 – 8.11 (m, 4H, H_c), 8.46 (d, *J* = 8.8 Hz, 4H, H_i).

^{13}C NMR (151 MHz, MeOD) δ (ppm) = 28.8, 30.3, 30.9, 38.7, 38.9, 42.6, 48.7, 50.6, 55.1, 56.2, 66.5, 69.8, 70.3, 71.2, 71.3, 71.5, 79.8, 88.1, 88.5, 115.1, 121.1, 125.5, 128.9, 129.1, 129.8, 130.4, 130.7, 131.2, 133.4, 133.9, 133.9, 138.2, 144.0, 144.2, 159.1, 162.0, 163.6, 168.6.

^{11}B NMR (160 MHz, MeOD) δ (ppm) = -12.8 (very broad singlet).

HR-MS (ESI) (Da): *m/z* = calculated for $C_{67}H_{79}B_1N_7O_8$ $[M]^+$ 1120.60777; found 1120.60905.

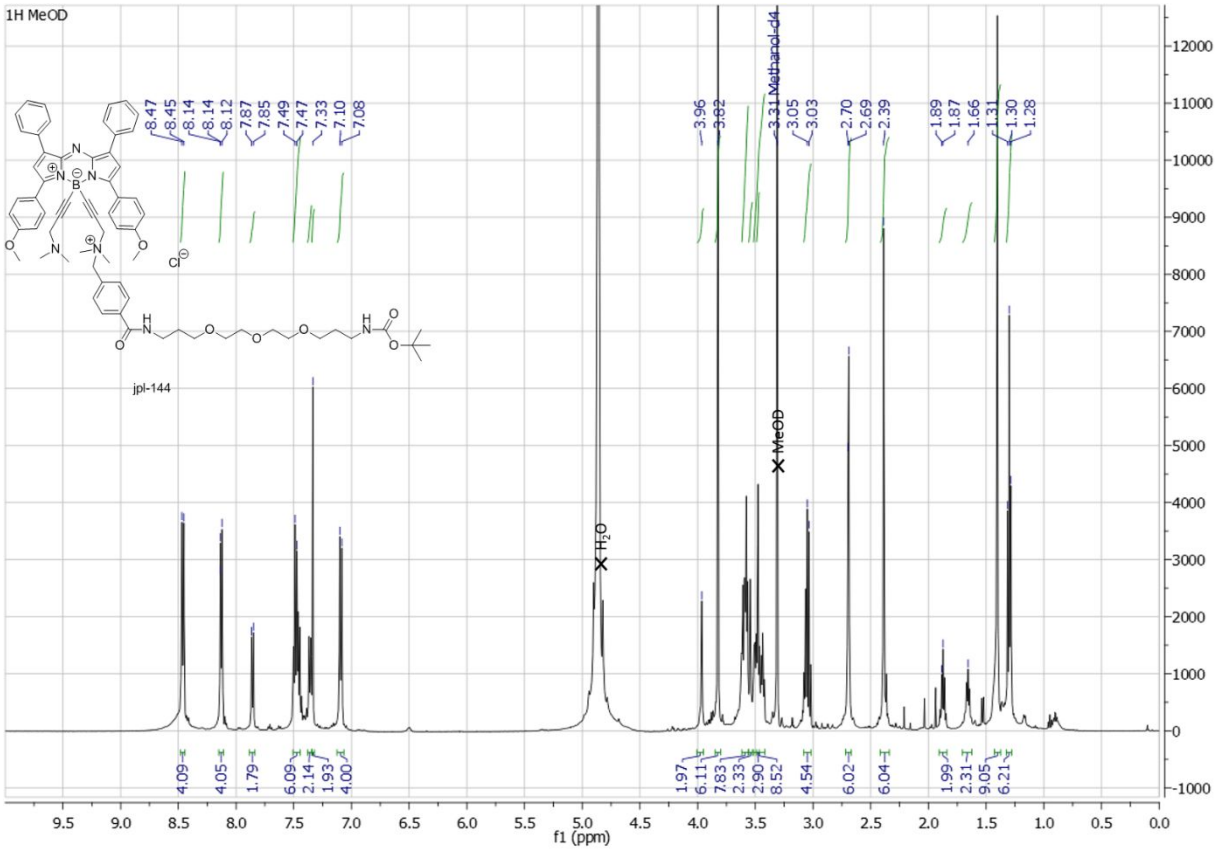


Figure S 28: ^{1}H NMR spectrum of aza-BODIPY 4 (500 MHz, MeOD)

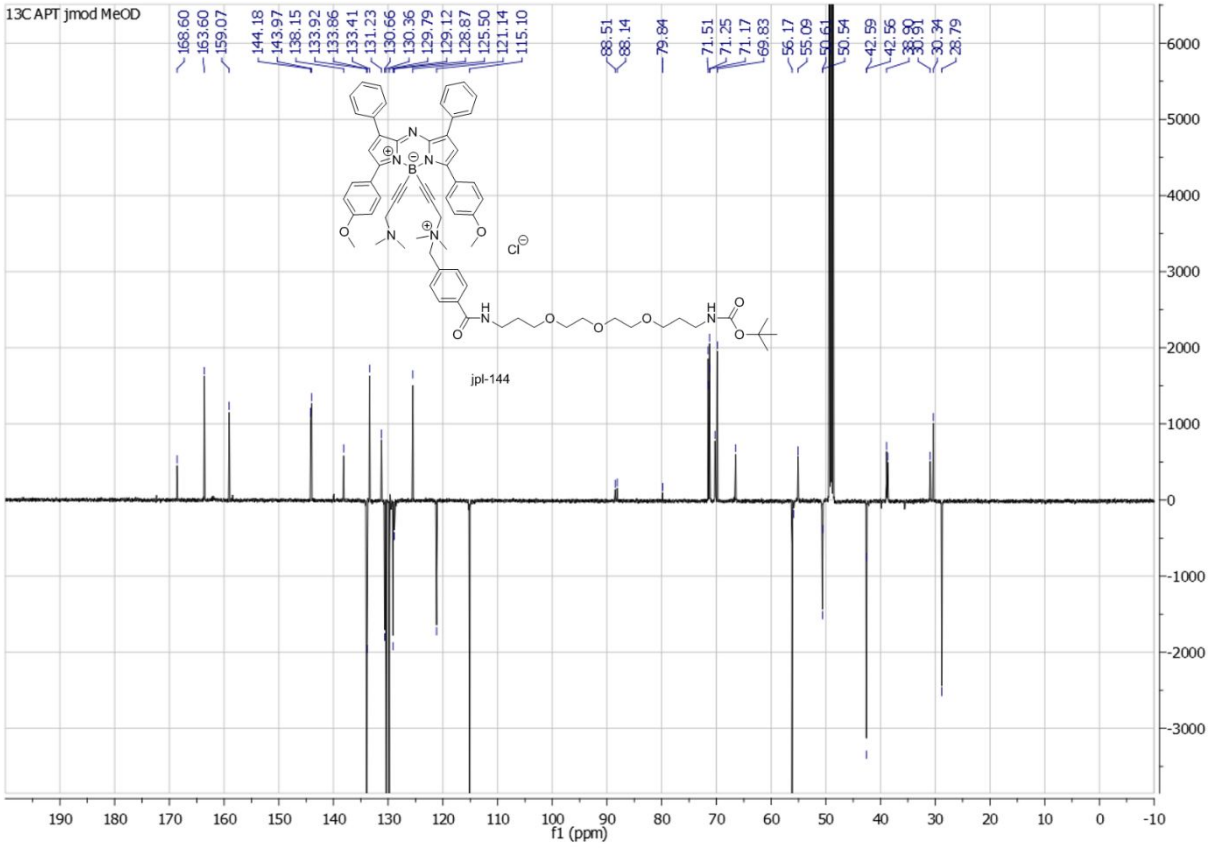


Figure S 29: ^{13}C NMR APT of aza-BODIPY 4 (151 MHz, MeOD)

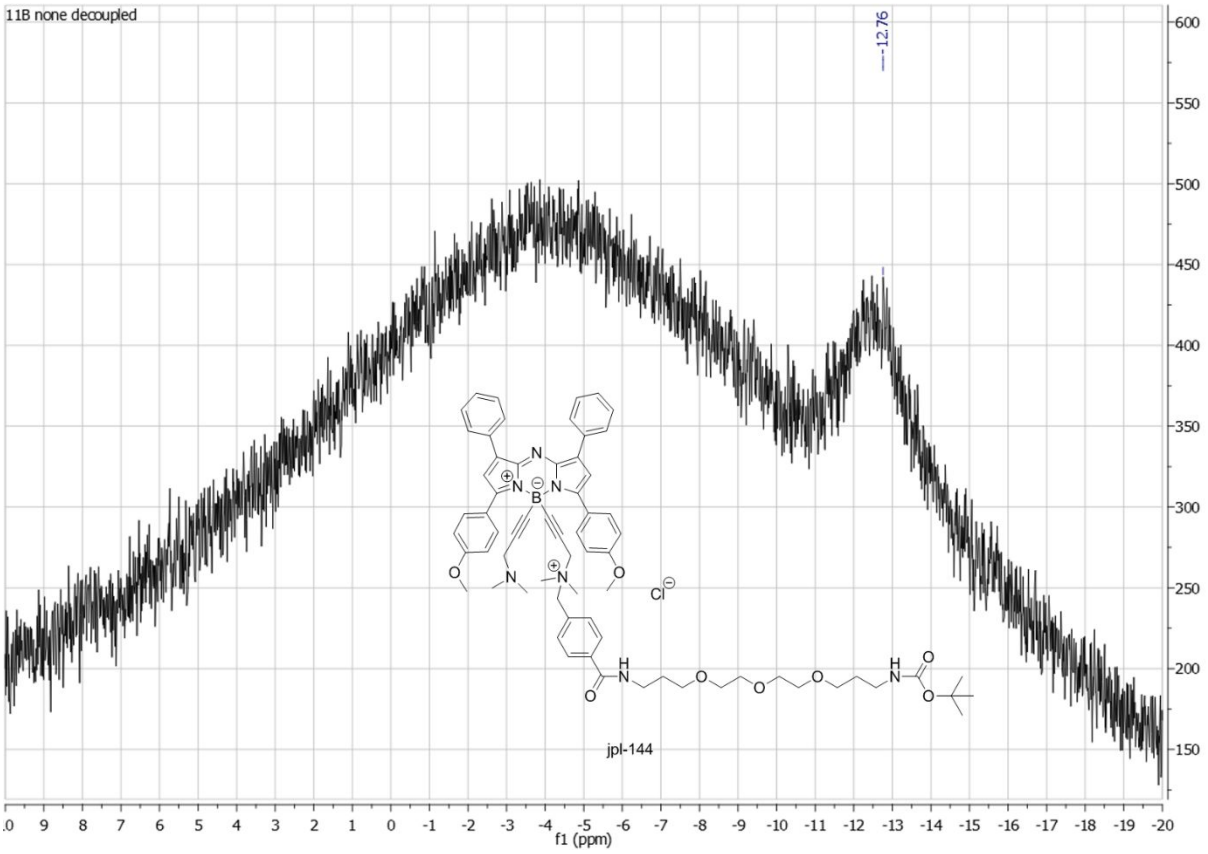
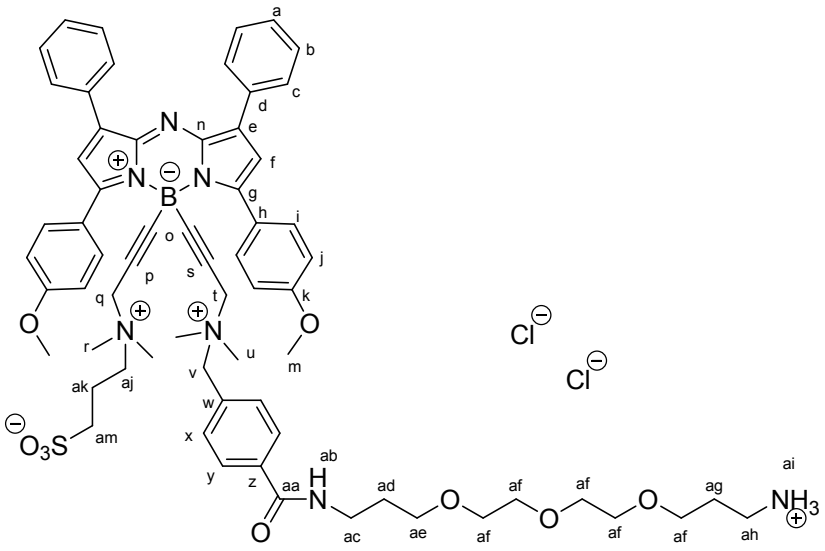


Figure S 30: 11B NMR of aza-BODIPY 4 (160 MHz, MeOD)

2.10 Wazaby1:



40 mg (34 µmol, 1eq) aza-BODIPY **4** were placed in a round bottom flask and dissolved in 6mL of CH_3CN. 21 mg (172 µmol, 5eq) of 1,3-propane sultone and 14 mg (172 µmol, 5eq) of $NaHCO_3$ were added and the reaction refluxed overnight. After cooling the solution, 1M HCl (10 mL) was added and the reaction was stirred at 30-35°C until complete hydrolysis. Solvents were evaporated and the crude

product was purified by semi preparative HPLC using gradient A. Product fractions were collected and underwent ion exchange (Dowex basic resin) before lyophilisation to yield 15 mg (12 µmol, 38% yield) of the target compound **Wazaby1** as a strongly hygroscopic dark green solid.

^{1}H NMR (600 MHz, MeOD-d_4) δ (ppm) = 1.89 (dd, *J* = 13.0, 6.5 Hz, 2H, H_{ad}), 1.93 (dt, *J* = 12.9, 6.6 Hz, 2H, H_{ag}), 2.23 – 2.16 (m, 2H, H_{ak}), 2.85 (s, 6H, H_u), 2.91 – 2.89 (m, 2H, H_{am}), 2.93 (s, 6H, H_r), 3.12 (t, *J* = 6.3 Hz, 2H, H_{ah}), 3.49 (t, *J* = 7.0 Hz, 2H, H_{ac}), 3.55 – 3.51 (m, 2H, H_{aj}), 3.58 (t, *J* = 6.0 Hz, 2H, H_{ae}), 3.68 – 3.62 (m, 13H, H_{af}, H_{ai}), 3.88 (s, 6H, H_m), 3.91 (s, 2H, H_t), 4.01 (s, 2H, H_q), 4.27 (s, 2H, H_v), 7.25 (d, *J* = 8.7 Hz, 4H, H_j), 7.42 (s, 2H, H_f), 7.53 – 7.46 (m, 8H, H_x, H_a, H_b), 7.89 (d, *J* = 8.0 Hz, 2H, H_y), 8.14 (d, *J* = 7.5 Hz, 4H, H_c), 8.48 (d, *J* = 8.7 Hz, 4H, H_i).

^{13}C NMR (151 MHz, MeOD-d_4) δ (ppm) = 20.1, 28.1, 30.5, 38.5, 40.1, 49.1, 51.0, 51.0 55.5, 56.0, 56.4, 63.7, 66.9, 69.9, 70.3, 70.4, 71.0, 71.1, 71.4, 87.5, 88.0, 115.5, 121.2, 125.3, 129.1, 129.8, 130.4, 130.6, 131.5, 133.5, 134.0, 138.0, 143.8, 144.1, 158.9, 163.7, 168.9 .

^{11}B NMR (160 MHz, MeOD-D_4) δ (ppm) = -12.4 (broad singlet).

HR-MS (ESI) (Th): calculated for $C_{65}H_{77}B_1N_7O_9S_1Na_1$ $[M-H+Na]^{2+}$ 582.77416; found 582.77359
Analytical HPLC: T_R = 4.287 min

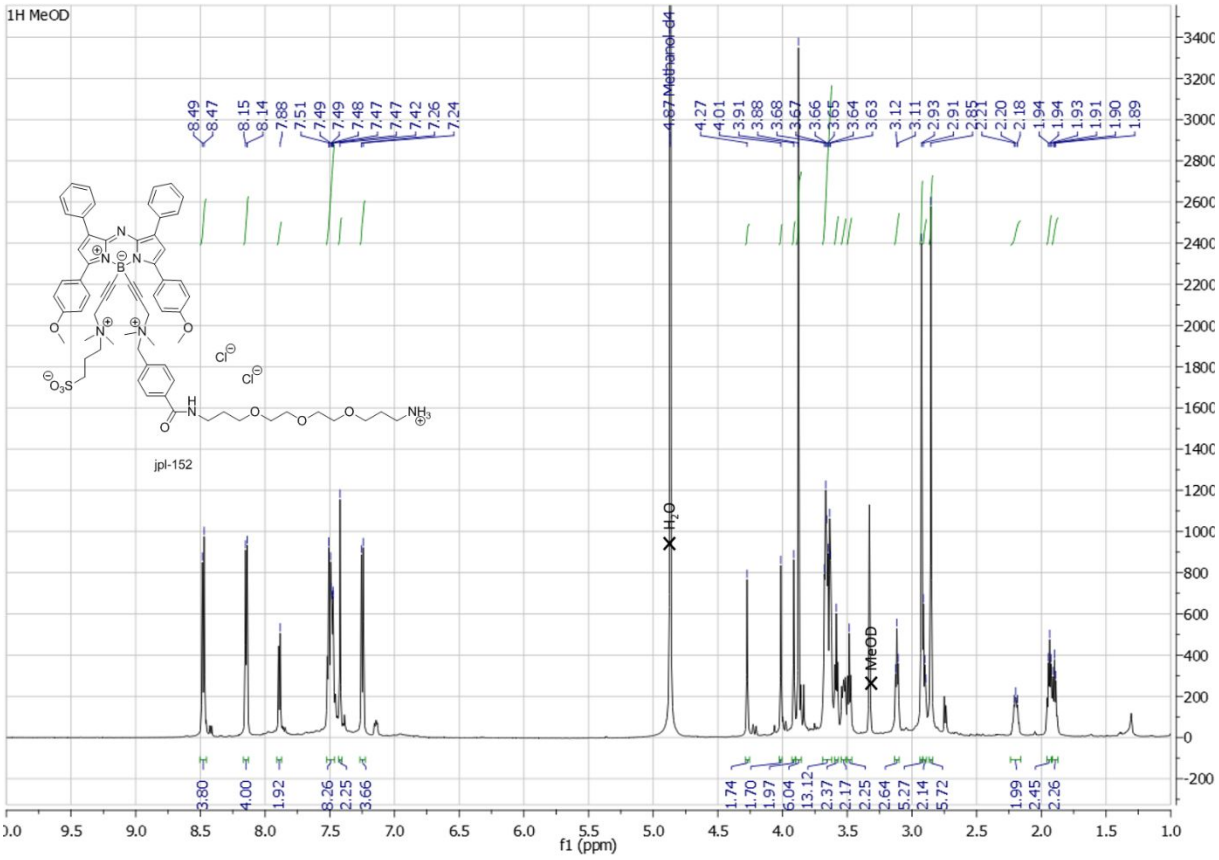


Figure S 31: 1H NMR spectrum of Wazaby1 (600 MHz, MeOD)

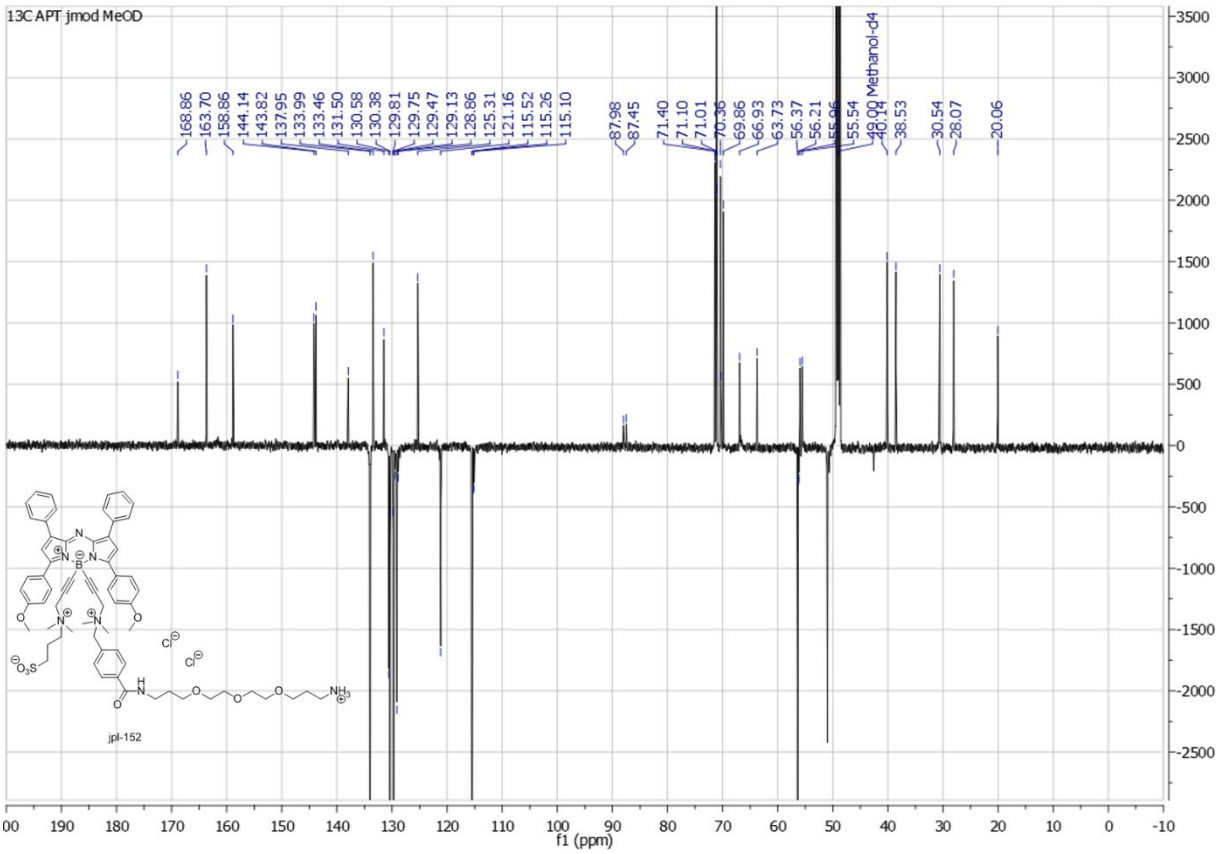


Figure S 32: ^{13}C NMR of Wazaby1 (151 MHz, MeOD)

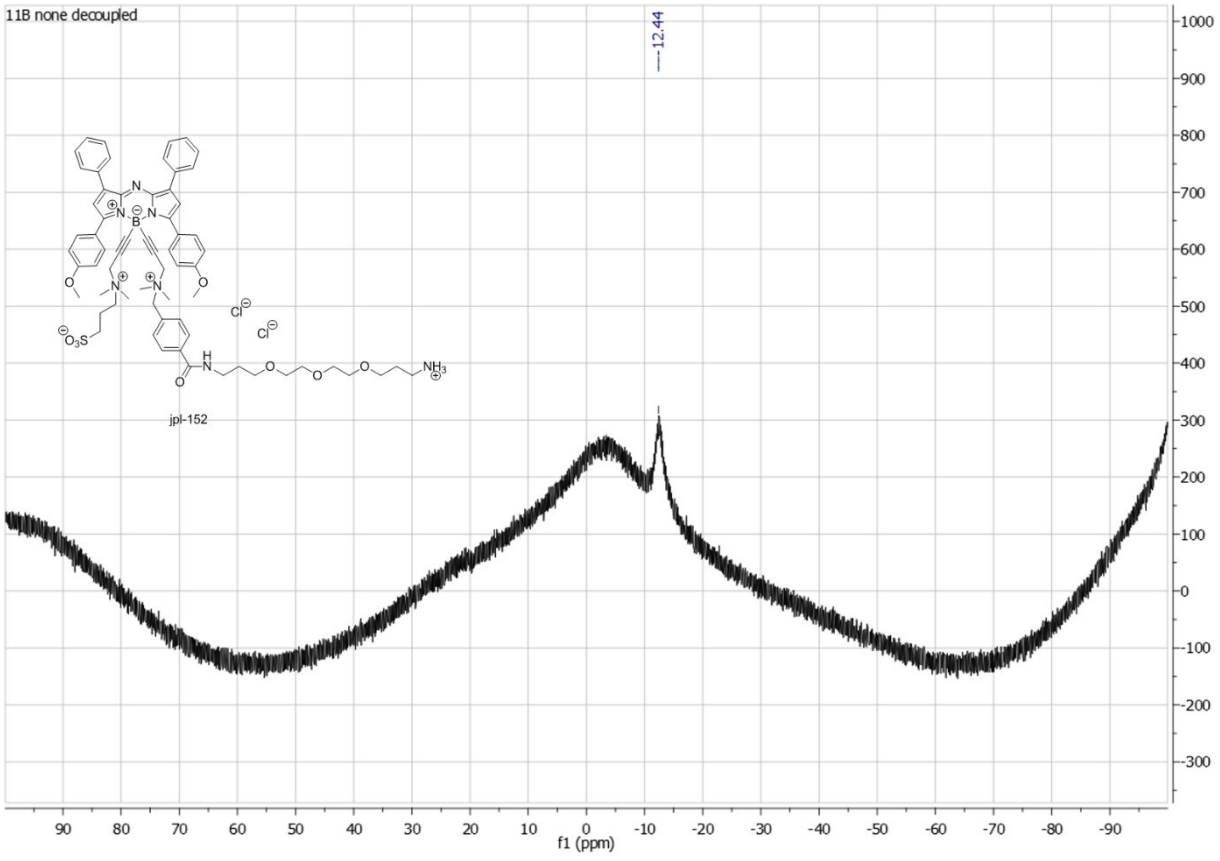


Figure S 33: ^{11}B NMR of Wazaby1 (96 MHz, $CDCl_3$)

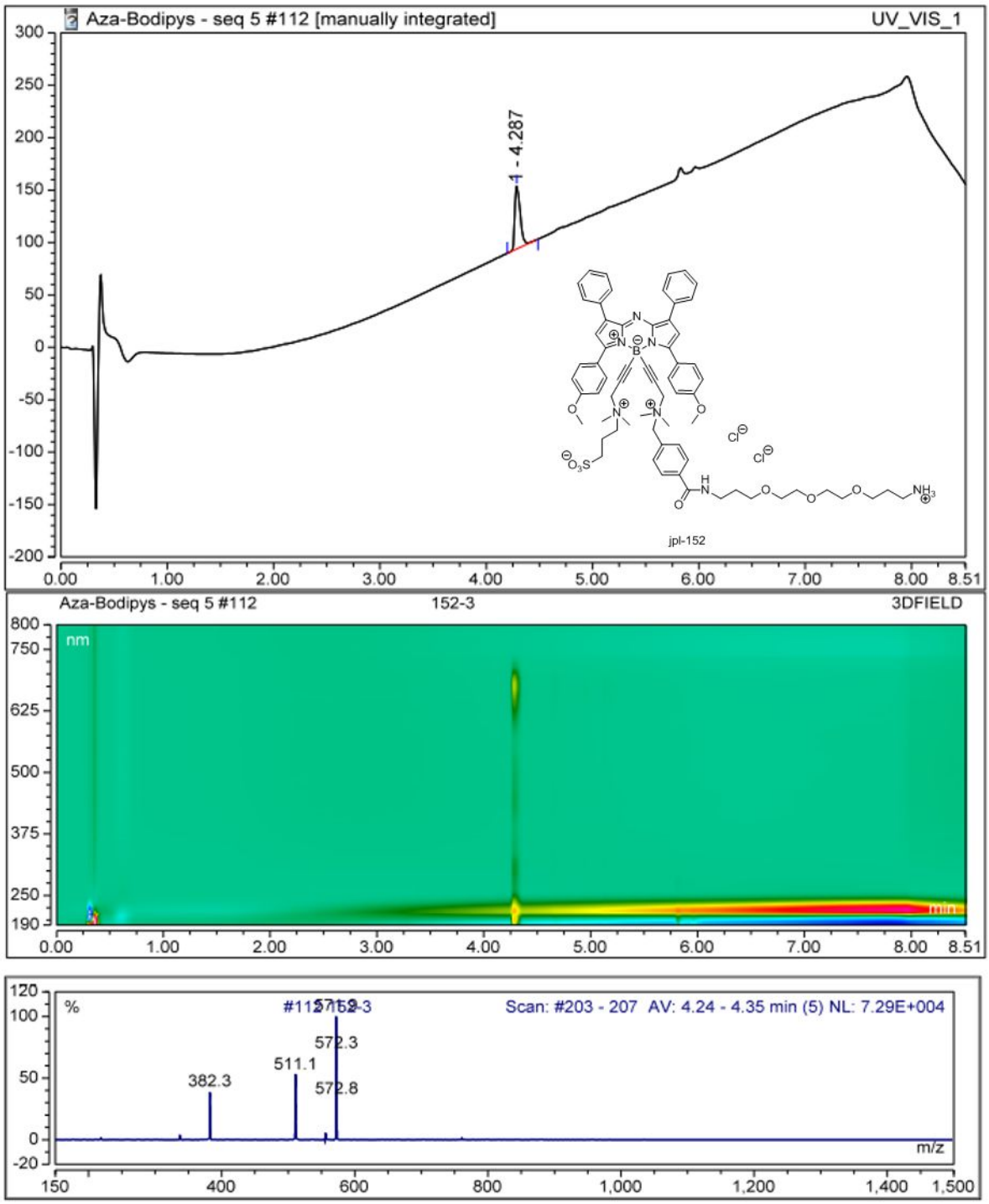
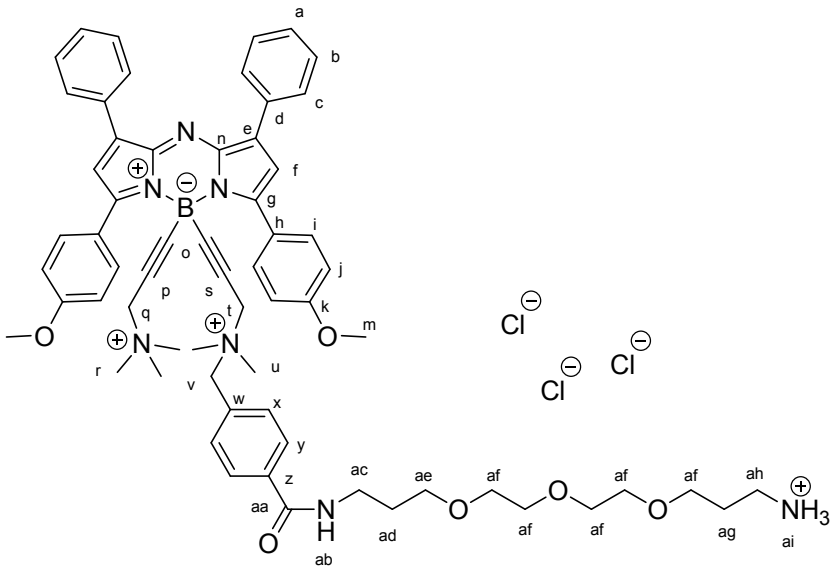


Figure S 34: HPLC of Wazaby1. Top: UV-Channel at 190 nm. Middle: 3D-field (190 nm-800 nm). Bottom: LR-MS(ESI)-channel

2.11 Wazaby2:



30 mg (26 µmol) of **4** were dissolved in DCM (5 mL). 0.5mL of MeI were added and the solution was stirred at room temperature for 1h. The solution was evaporated to dryness and the residue was dissolved in ACN (8 mL). 16 mL of 1M HCl were added and the reaction was stirred for 8 h at 30-35°C. Solvents were then evaporated and the crude product was purified by semi preparative HPLC using gradient A. Product fractions were collected, evaporated to dryness/lyophilized and ion exchange was performed. The resulting solution was lyophilized, yielding **Wazaby2** (Cl-salt) (23 mg, 20 µmol, 78% yield) as a crystalline dark green solid.

^{1}H NMR (500 MHz, MeOD) δ (ppm) = 1.88 (dt, *J* = 12.7, 6.0 Hz, 2H, H_{ad}), 1.92 (dt, *J* = 12.0, 6.5 Hz, 2H, H_{ag}), 2.84 (s, 6H, H_u), 2.94 (s, 9H, H_r), 3.10 (t, *J* = 6.4 Hz, 2H, H_{ah}), 3.46 (t, *J* = 7.1 Hz, 2H, H_{ac}), 3.57 (t, *J* = 6.1 Hz, 2H, H_{ae}), 3.67 – 3.59 (m, 10H, H_{af}), 3.87 (s, *J* = 6.4 Hz, 6H, H_m), 3.90 (s, 2H, H_t), 4.13 (d, *J* = 3.6 Hz, 2H, H_q), 4.21 (s, 2H, H_v), 7.16 (d, *J* = 9.0 Hz, 4H, H_j), 7.40 (s, 2H, H_f), 7.53 – 7.42 (m, 8H, H_x, H_a, H_b), 7.87 (d, *J* = 8.3 Hz, 2H, H_y), 8.13 (dd, *J* = 8.1, 1.4 Hz, 4H, H_c), 8.41 (d, *J* = 8.9 Hz, 4H, H_i).

^{13}C NMR (126 MHz, MeOD) δ (ppm)= 28.1, 30.5, 38.5, 40.1, 50.7, 53.0, 55.5, 56.3, 58.1, 66.7, 69.9, 70.4, 71.0, 71.1, 71.4, 87.9, 88.1, 115.3, 121.3, 125.4, 129.2, 129.8, 130.4, 130.7, 131.4, 133.4, 133.9, 134.0, 138.0, 144.0, 144.2, 159.1, 163.7, 168.8.

^{11}B NMR (160 MHz, MeOD) δ (ppm) = -12.4 (broad singlet).

HR-MS (ESI) (Th): *m/z* = calculated for $C_{63}H_{75}B_1N_7O_6$ $[M\text{-}3Cl]^{3+}$ 345.52851; found 345.52954.

Analytical HPLC: T_R = 3.970 min

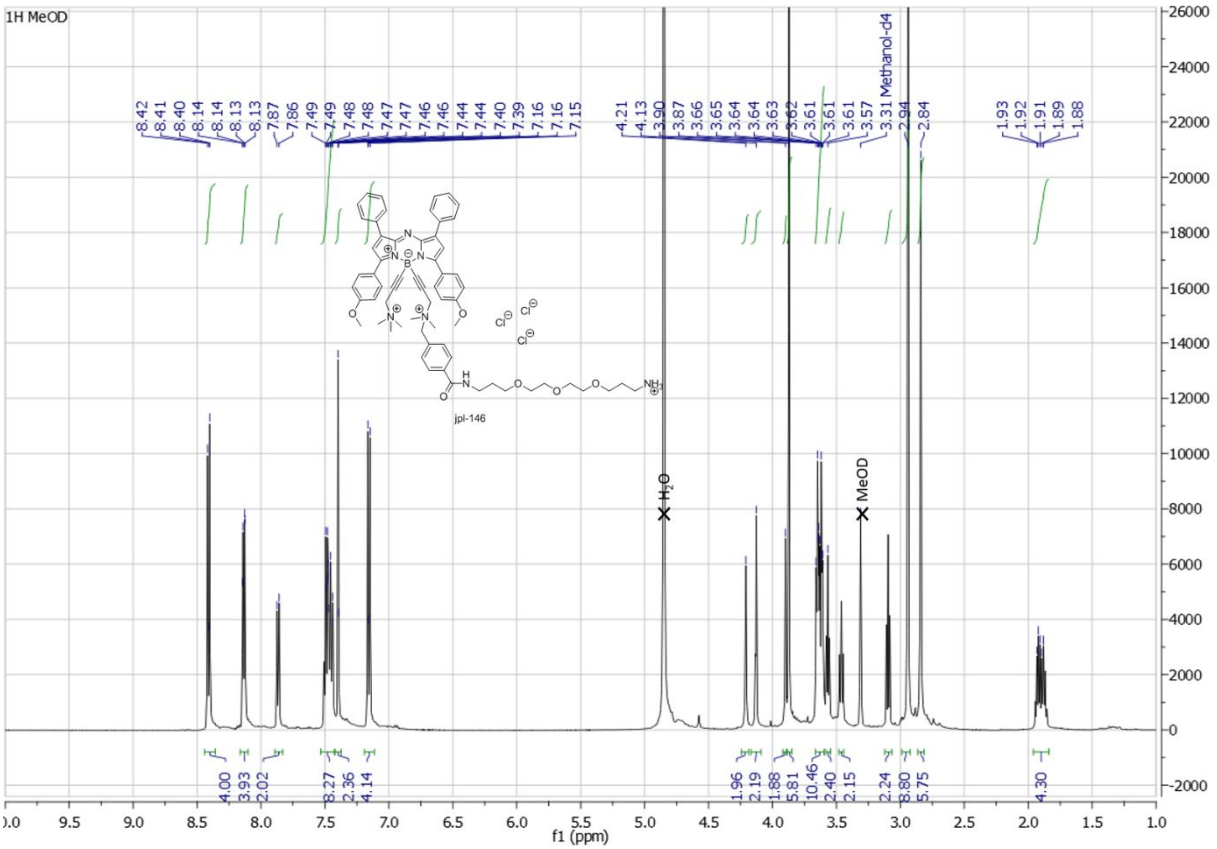


Figure S 35: [1]H NMR spectrum of Wazaby2 (500 MHz, MeOD)

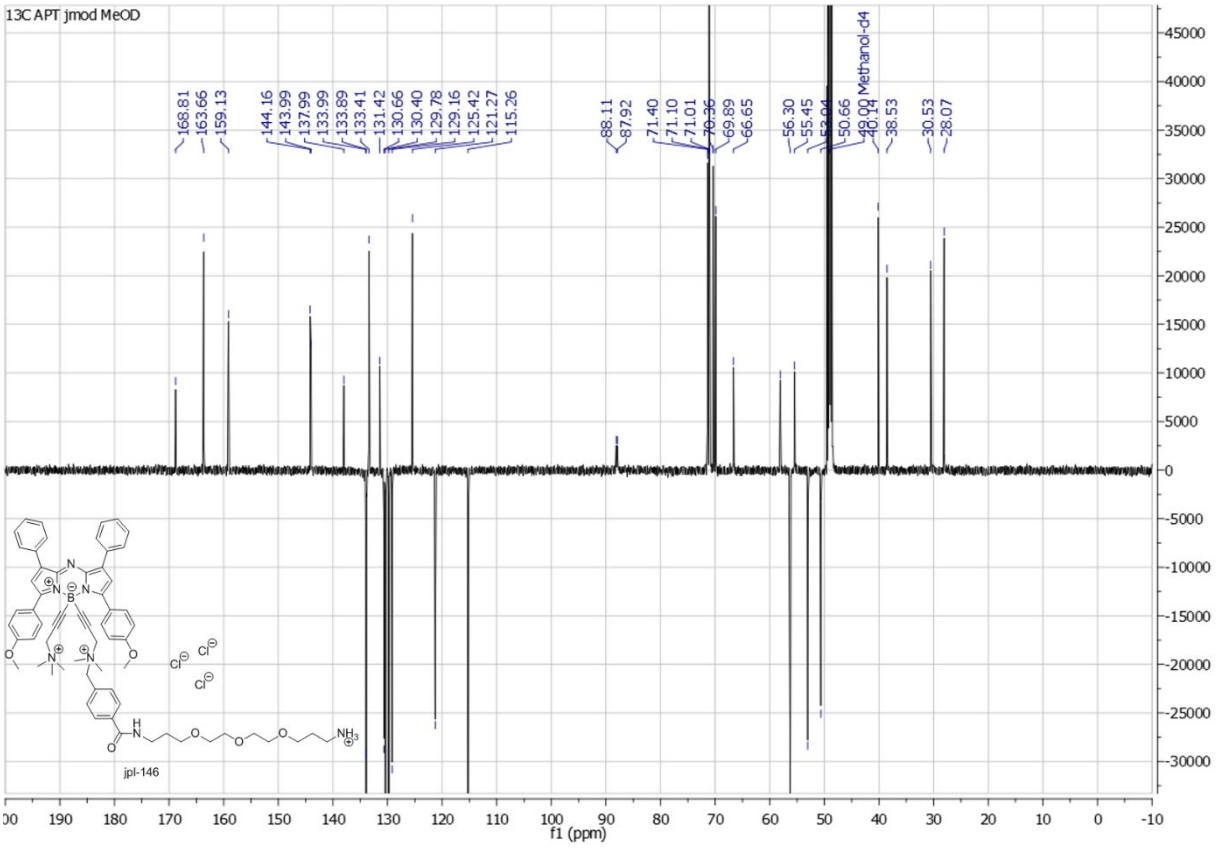


Figure S 36: [13]C APT NMR of Wazaby2 (126 MHz, MeOD)

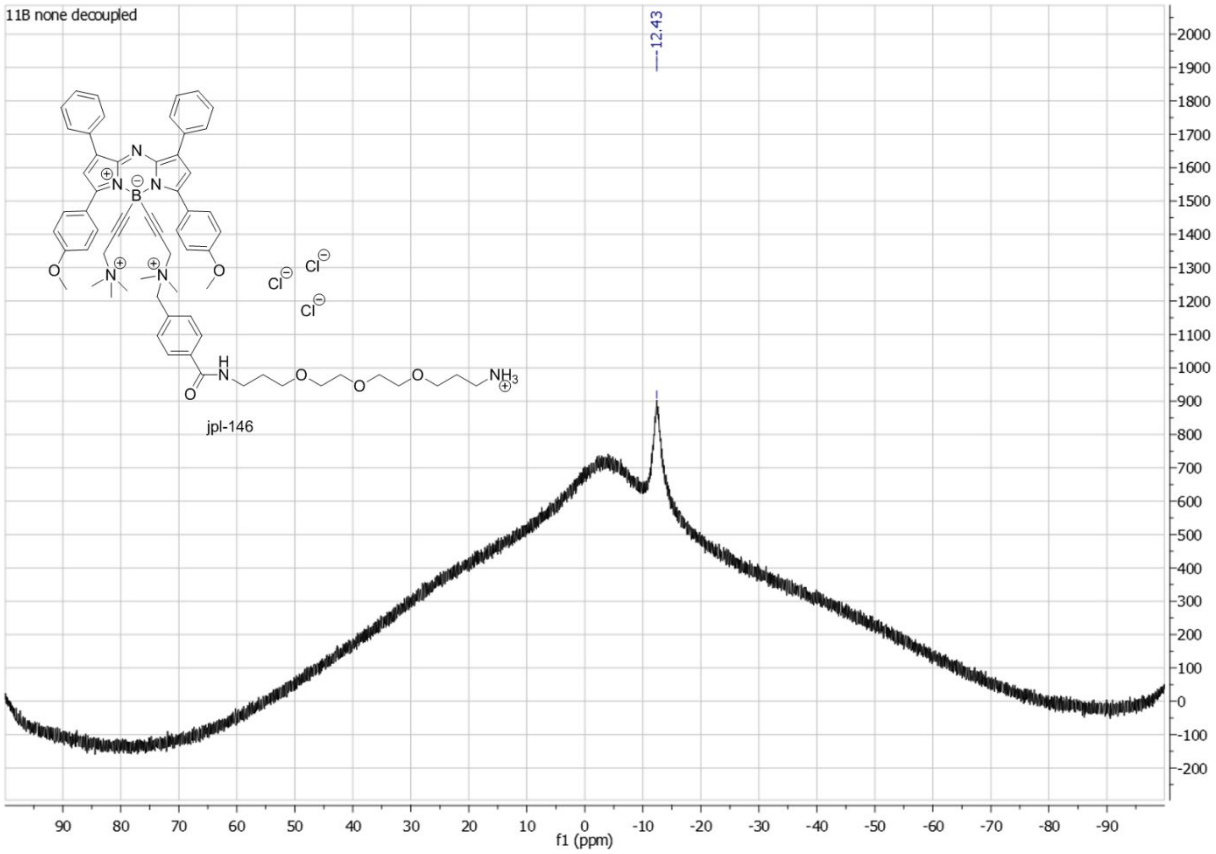


Figure S 37: ^{11}B NMR of Wazaby2 (160 MHz, MeOD)

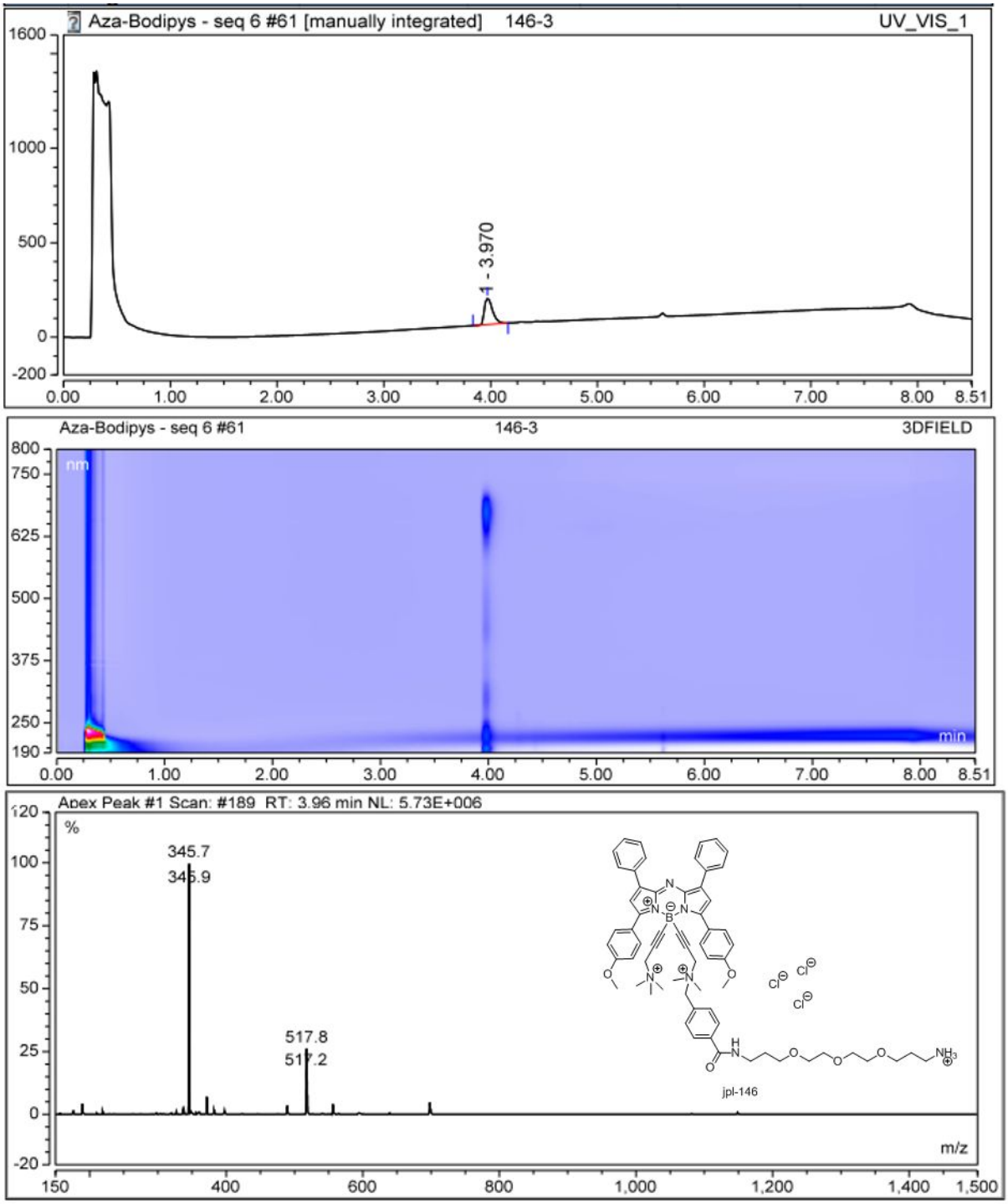
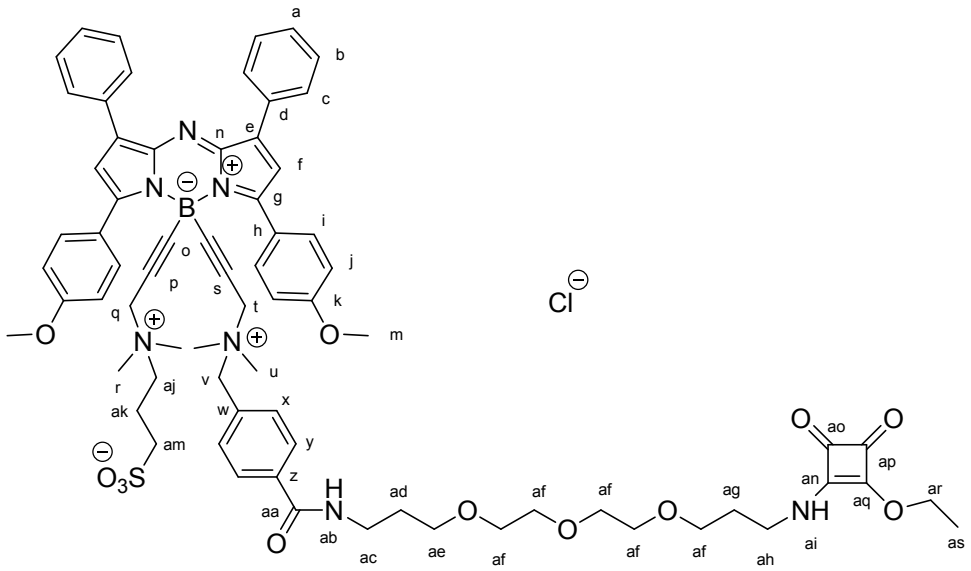


Figure S 38: HPLC of Wazaby2. Top: UV-Channel at 190 nm. Middle: 3D-field (190 nm-800 nm). Bottom: LR-MS(ESI)-channel

2.12 Wazaby3:



16 mg (1eq. 13 µmol) of **Wazaby1** were dissolved in 1 mL of EtOH. After addition of 20 µL (10eq, 135 µmol) of diethyl squarate and 20 µL (9eq. 118 µmol) DIPEA the reaction was stirred at 30-35°C for 5 h. The solvents were evaporated and the crude product was purified by semi preparative HPLC using gradient A. Product fractions were collected and lyophilized, followed by ion-exchange and subsequent lyophilization to yield 15 mg (11 µmol, 85%) of the target compound **Wazaby3** as a fluffy dark green solid (Cl-salt).

^{1}H NMR (600 MHz, CD_3CN) δ (ppm) = 1.36 (t, *J* = 7.2 Hz, 3H, H_{as}), 1.73-1.78 (m, 2H, H_{ad}), 1.82 (dt, *J* = 6.2, 12.5 Hz, 2H, H_{ag}), 2.10-2.16 (m, 2H, H_{ah}), 2x 2.78 (s, 2x6H, H_r, H_u), 3.43 (dd, *J* = 6.5, 12.5 Hz, 10H, H_{af}), 3.52 – 3.58 (m, 6H, H_{aj}, H_{ac}, H_{ae}), 3.64 (s, 2H, H_t), 3.79 (s, 6H, H_m), 3.88 (s, 2H, H_q), 4.06 (s, 2H, H_v), 4.64 (m, 2H, H_{ar}), 7.16 (d, *J* = 8.8 Hz, 4H, H_j), 7.32 (s, 2H, H_f), 7.38 (d, *J* = 7.9 Hz, 2H, H_x), 7.46 (t, *J* = 7.3 Hz, 2H, H_a), 7.51 (t, *J* = 7.4 Hz, 4H, H_b), 7.87 (s, br, 2H, H_y), 8.11 (d, *J* = 7.2 Hz, 4H, H_c), 8.34 (t, *J* = 8.6 Hz, 4H, H_i).

^{13}C NMR (151 MHz, CD_3CN) δ (ppm) = 18.6, 20.0, 30.1, 31.0, 38.6, 43.0, 48.8, 51.1, 51.2, 54.9, 55.9, 56.5, 63.7, 66.3, 69.1, 69.1, 70.1, 70.7, 70.8, 70.9, 71.0, 86.8, 87.4, 115.2, 119.4, 121.4, 124.9, 128.8, 129.7, 130.2, 130.5, 130.6, 133.0, 133.6, 133.7, 137.8, 143.2, 143.8, 158.7, 163.1, 166.8, 173.8 .

^{11}B NMR (160 MHz, CD_3CN) δ (ppm) = -12.6 (very broad singlet).

HR-MS (ESI) (Da): *m/z* = calculated for $C_{71}H_{81}B_1N_7O_{12}S_1$ $[M]^+$ 1266.57515; found 1266.57764.

Anaytical HPLC: T_R = 4.577 min

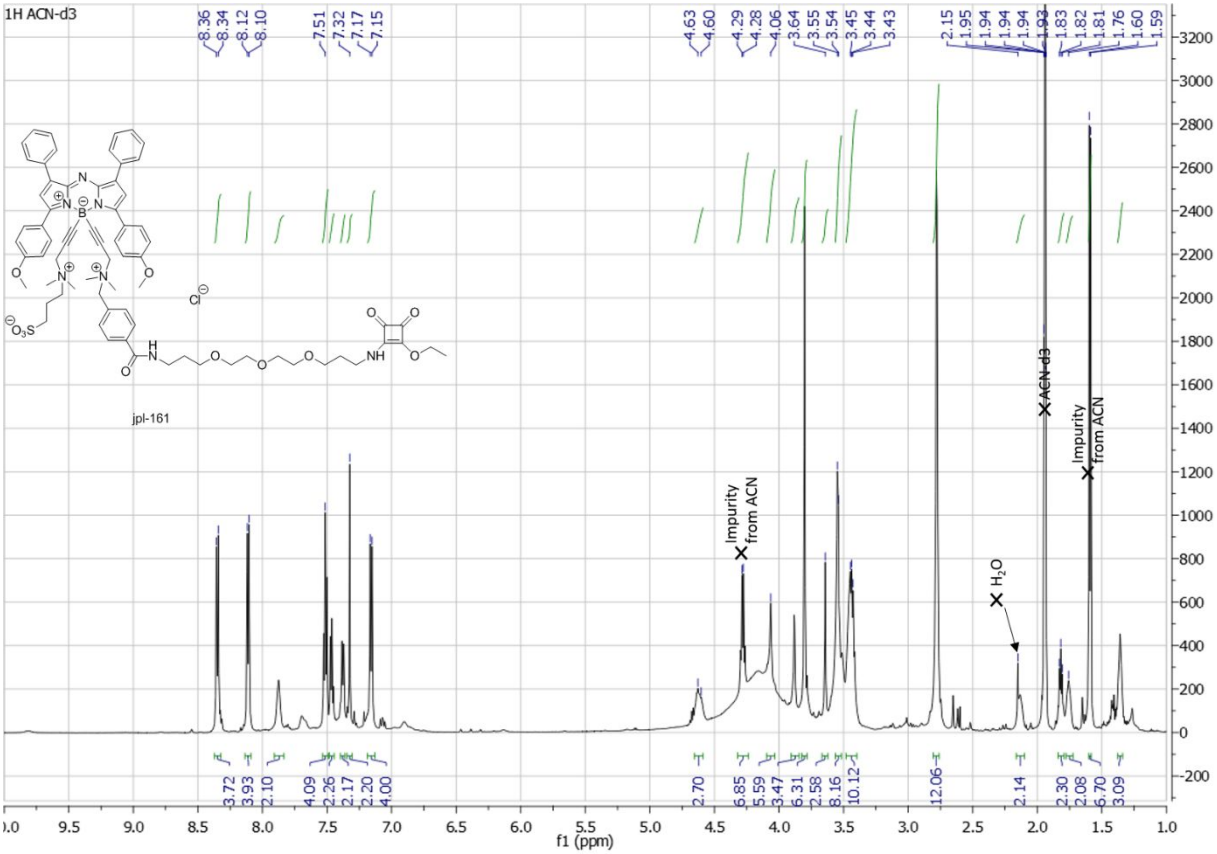


Figure S 39: ^{1}H NMR spectrum of Wazaby3 (600 MHz, CD_3CN)

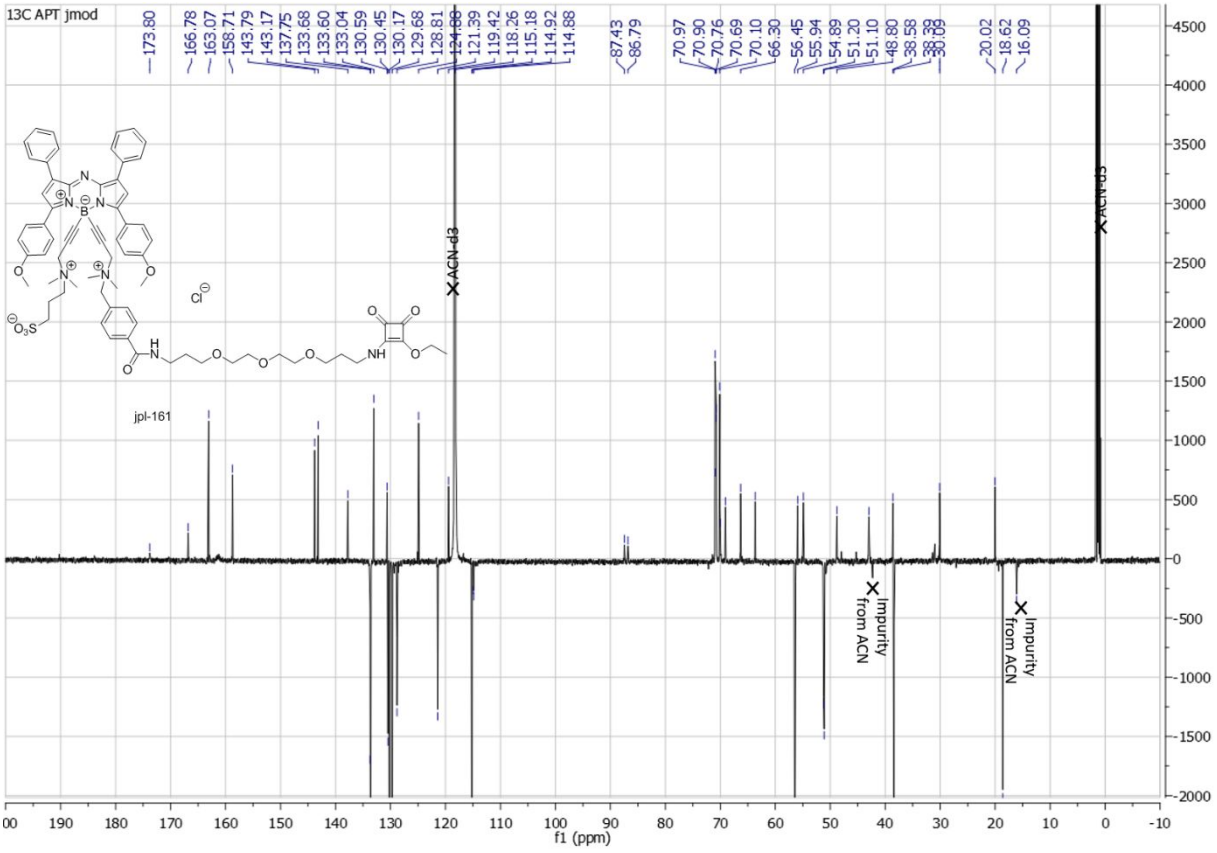


Figure S 40: ^{13}C NMR of Wazaby3 (151 MHz, CD_3CN

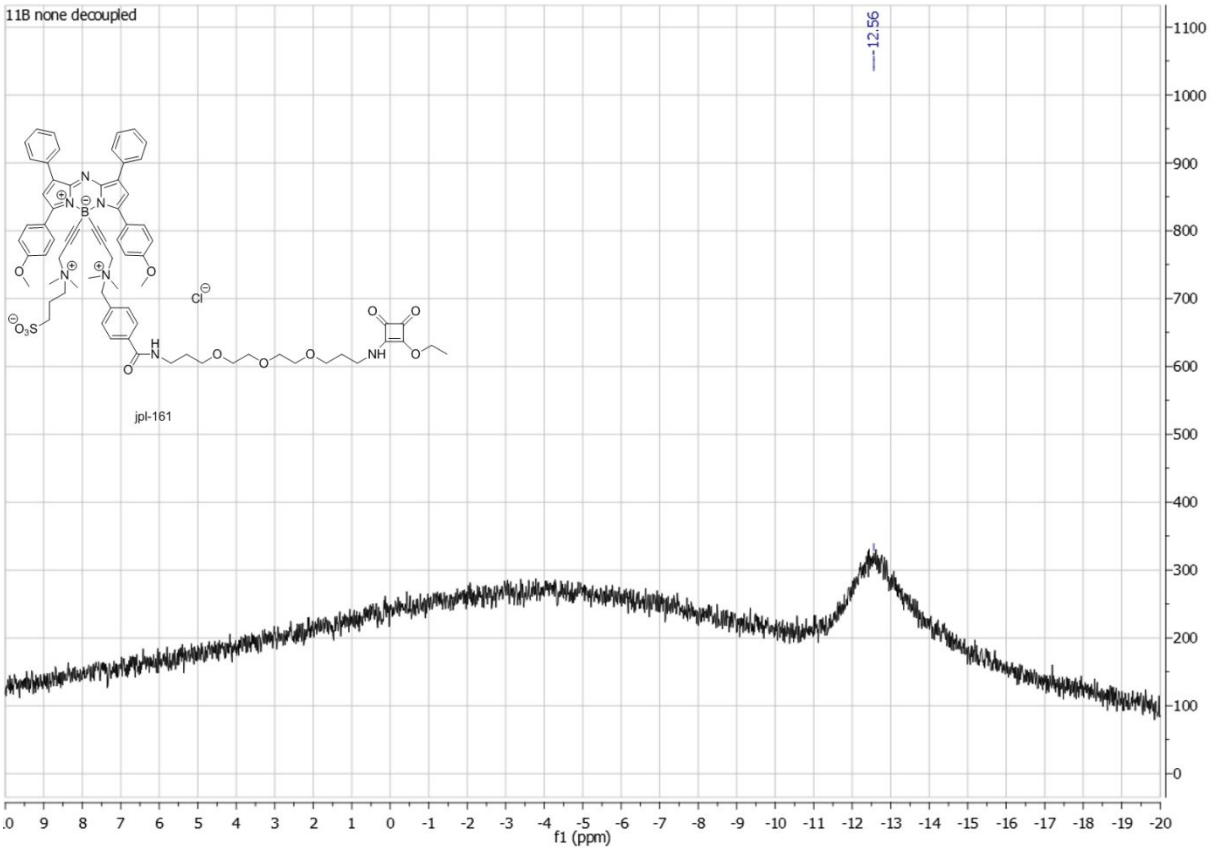


Figure S 41: ^{11}B NMR of Wazaby3 (600 MHz, CD_3CN)

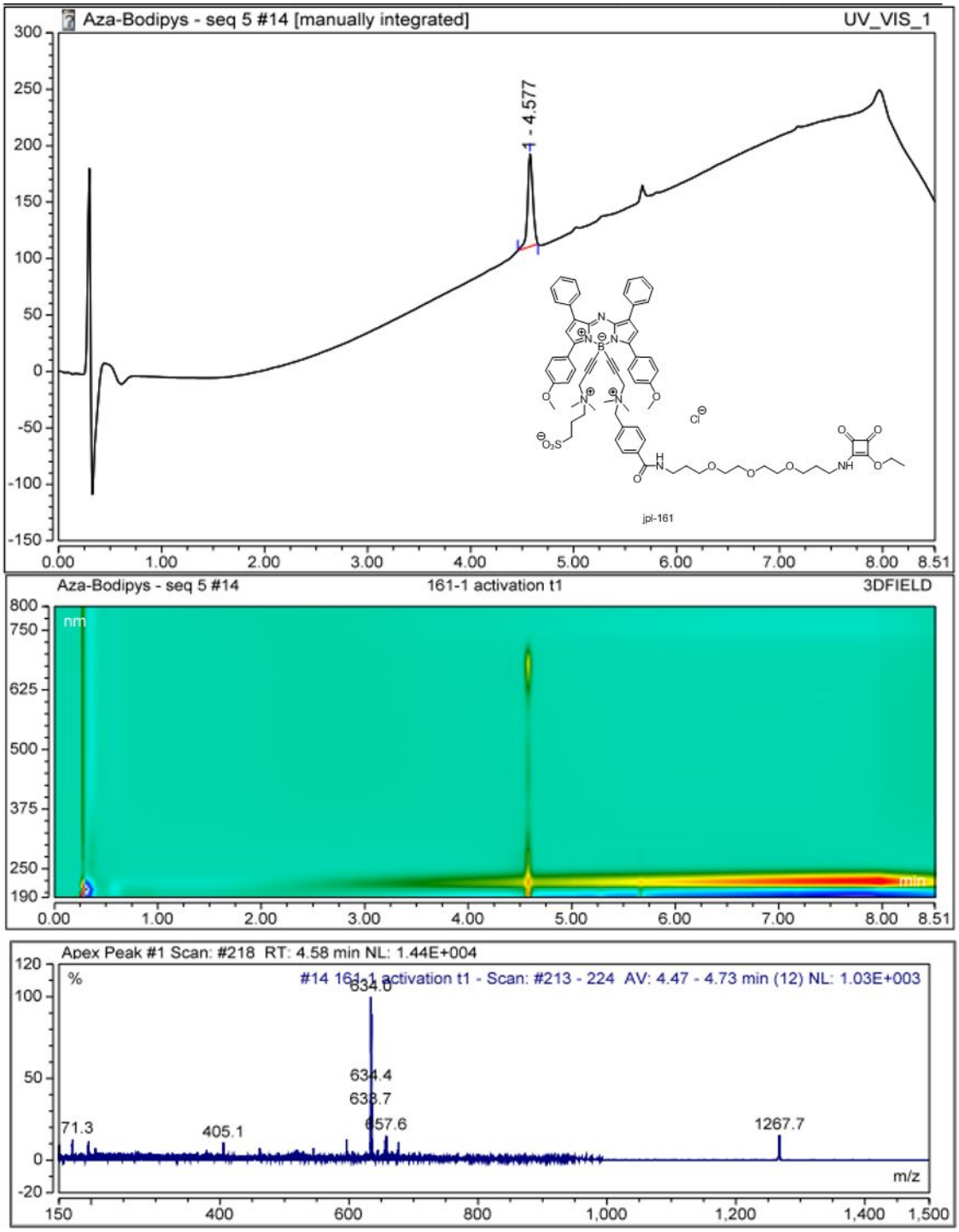
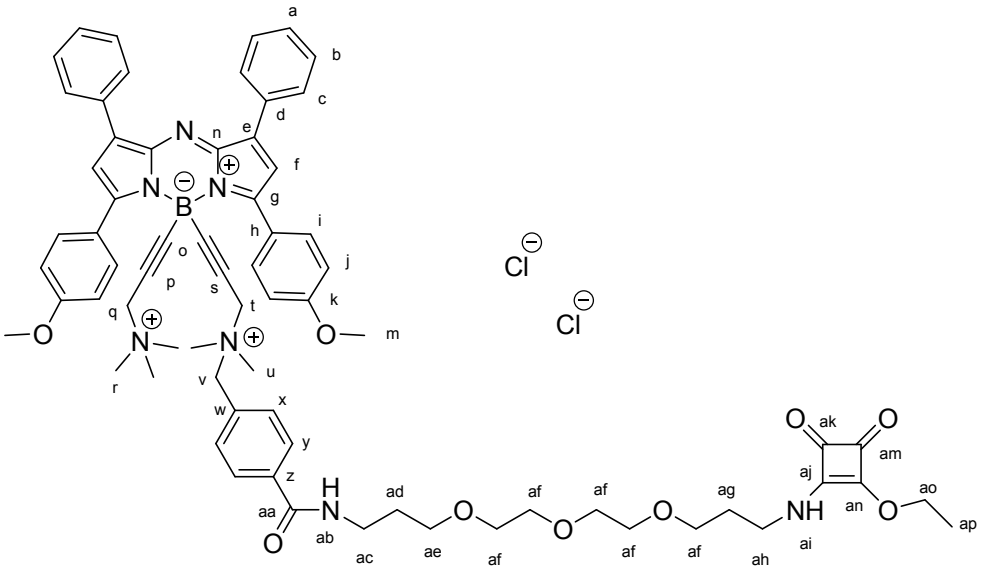


Figure S 42: HPLC of Wazaby3. Top: UV-Channel at 190 nm. Middle: 3D-field (190 nm-800 nm). Bottom: LR-MS(ESI)-channel

2.13 Wazaby4:



17 mg (1eq., 14 µmol) of **Wazaby2** were dissolved in 2 mL of EtOH. After addition of diethyl squarate (12 mg, 5eq., 73 µmol) and 20 µL (8eq., 118 µmol) DIPEA the reaction was stirred for 5 h at 30-35°C. The solvents were evaporated and the crude product purified by semi preparative HPLC using gradient A. Product fractions were collected and lyophilized, followed by ion exchange and lyophilization to yield 15 mg (12 µmol, 84%) of the target compound **Wazaby4** as a fluffy green solid (Cl-salt).

^{1}H NMR (600 MHz, MeOD) δ (ppm) = 1.39 (td, *J* = 4.0, 7.0 Hz, 3H, H_{ap}), 1.75 (dt, *J* = 6.2, 12.6 Hz, 1H, H_{ag}), 1.81 (dt, *J* = 6.1, 12.2 Hz, 1H, H_{ag}), 1.86 (p, *J* = 6.4 Hz, 2H, H_{ad}), 2.83 (s, 6H, H_{u}), 2.87 (s, 2H, H_{ah}), 2.92 (s, 9H, H_{r}), 3.44-3.47 (m, 4H, H_{ac}), 3.52 (t, *J* = 6.0 Hz, 2H, H_{ae}), 3.54 – 3.63 (m, 10H, H_{af}), 3.85 (s, 2H, H_{t}), 3.87 (s, 6H, H_{m}), 3.93 (s, 1H, H_{ai}), 4.01 (s, 1H), 4.08 (s, 2H, H_{q}), 4.16 (d, *J* = 10.4 Hz, 2H, H_{v}), 4.64 (dq, *J* = 7.0, 19.4 Hz, 2H, H_{ao}), 7.14 (d, *J* = 9.0 Hz, 4H, H_{j}), 7.40 (s, 2H, H_{f}), 7.40-7.42 (m, 1H, H_{ab}), 7.45 – 7.52 (m, 8H, H_{a}, H_{b}, H_{x}), 7.82 (d, *J* = 7.7 Hz, 2H, H_{y}), 8.14 (d, *J* = 6.9 Hz, 4H, H_{c}), 8.41 (d, *J* = 8.8 Hz, 4H, H_{i}).

^{13}C NMR (151 MHz, MeOD) δ (ppm) = 16.1 (d, *J* = 15.6 Hz), 30.3 (d, *J* = 22.2 Hz), 31.6 (d, *J* = 59.7 Hz), 38.9 (d, *J* = 40.2 Hz), 43.0 (d, *J* = 51.3 Hz), 50.7 (s), 53.0 (s), 55.3 (s), 56.2 (s), 58.1 (s), 66.7 (s), 69.2 (d, *J* = 25.2 Hz), 70.1 (s), 70.4 (s), 70.6 (d, *J* = 6.3 Hz), 71.1 (s), 71.2 (s), 71.5 (s), 87.9 (s), 88.2 (s), 115.1 (d, *J* = 11.3 Hz), 121.2 (d, *J* = 13.7 Hz), 125.5 (s), 129.1 (s), 129.8 (s), 130.4 (s), 130.7 (s), 131.3 (s), 133.4 (s), 133.8 (s), 133.9 (d, *J* = 6.3 Hz), 138.1 (s), 144.0 (s, vbr) 144.2 (s), 159.1 (s), 163.7 (s), 168.6 (s), 174.6 (s).

^{11}B NMR (193 MHz, MeOD) δ (ppm) = -12.28 (s, br).

HR-MS (ESI) (Th): *m/z* = calculated for $C_{69}H_{78}B_1N_7O_9$ $[M]^{2+}$ 579.79716; found 579.79812.

Analytical HPLC: T_R = 4.323 min

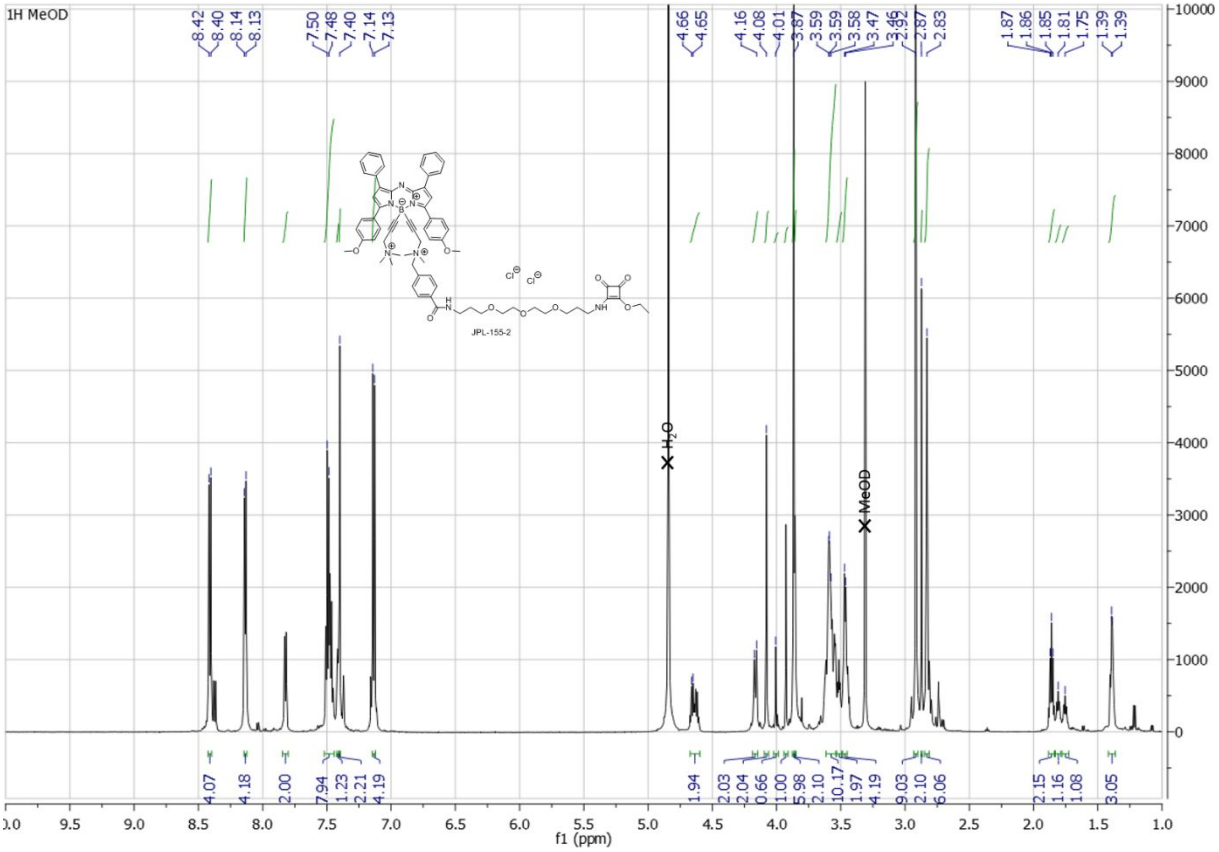


Figure S 43: [1]H NMR of Wazaby4 (600 MHz, MeOD)

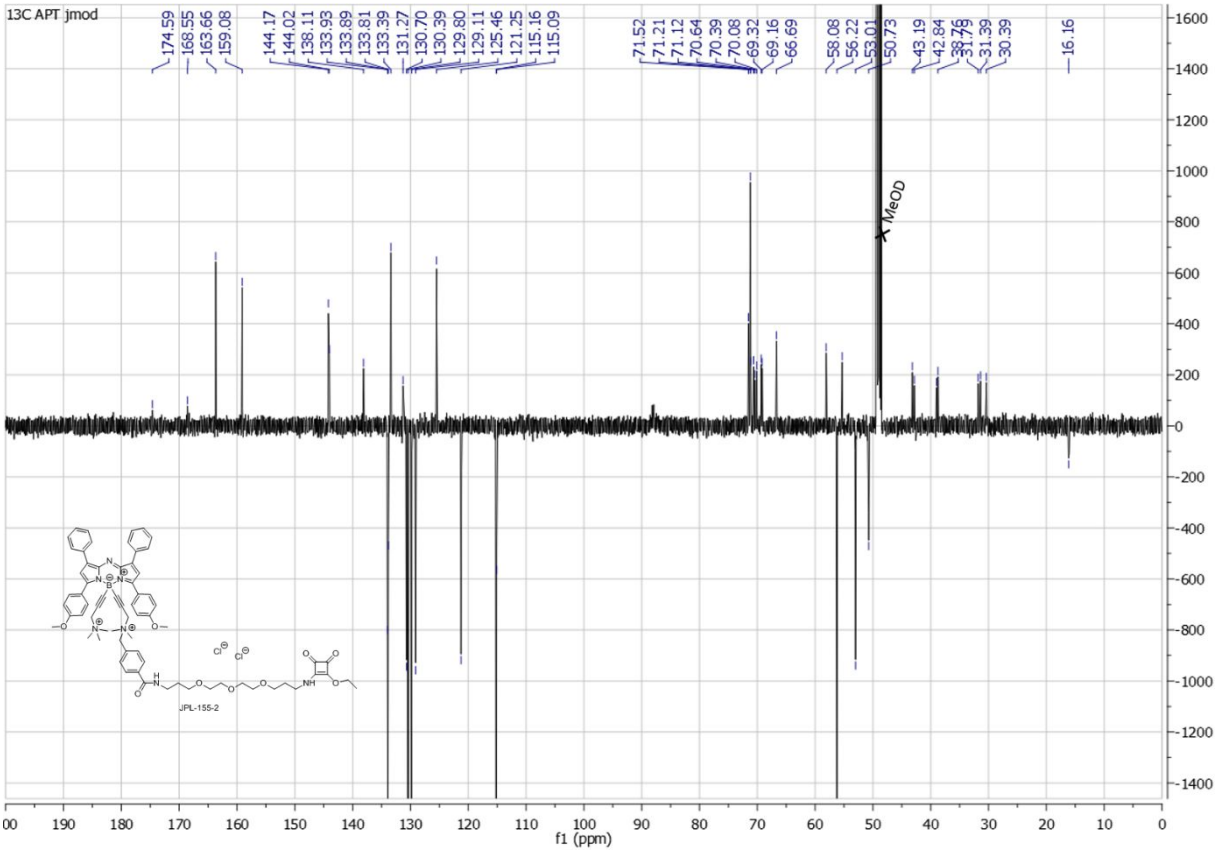


Figure S 44: [13]C APT NMR of Wazaby4 (151 MHz, MeOD)

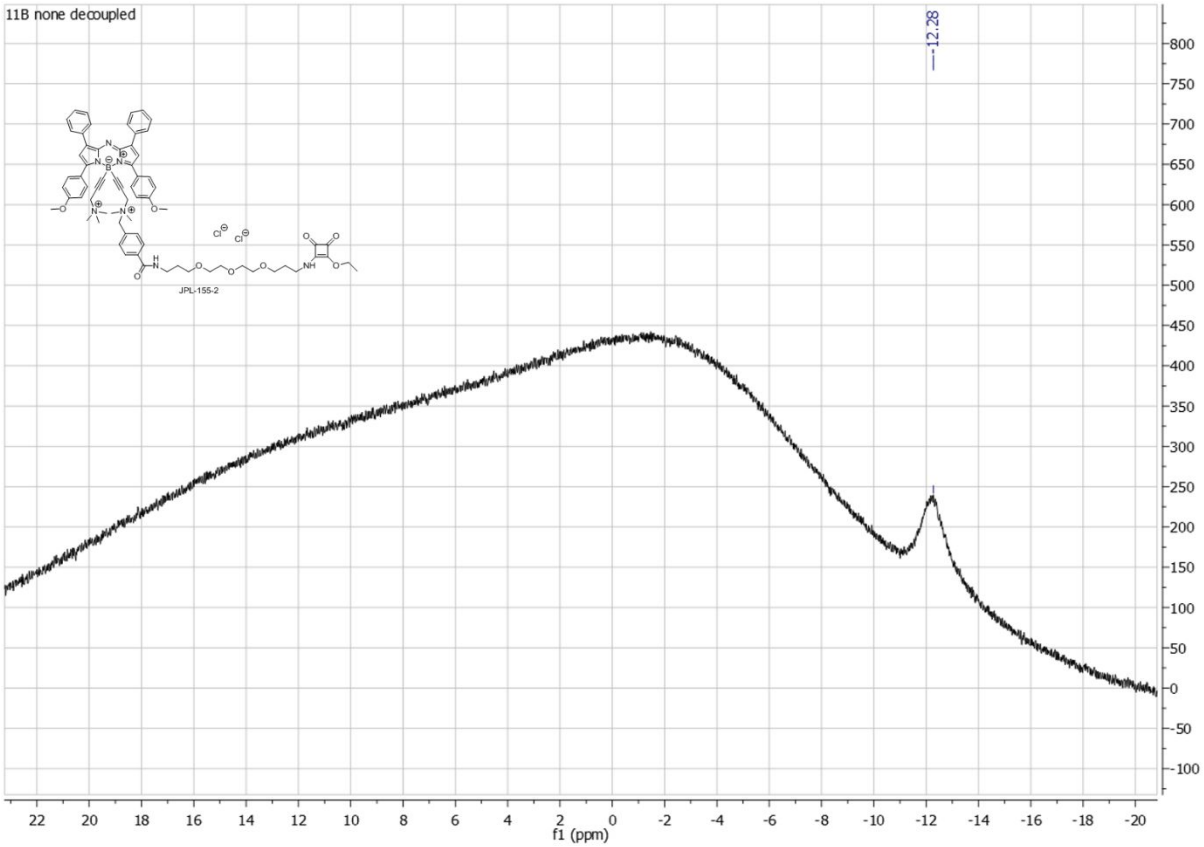


Figure S 45: ^{11}B NMR of Wazaby4 (193 MHz, MeOD)

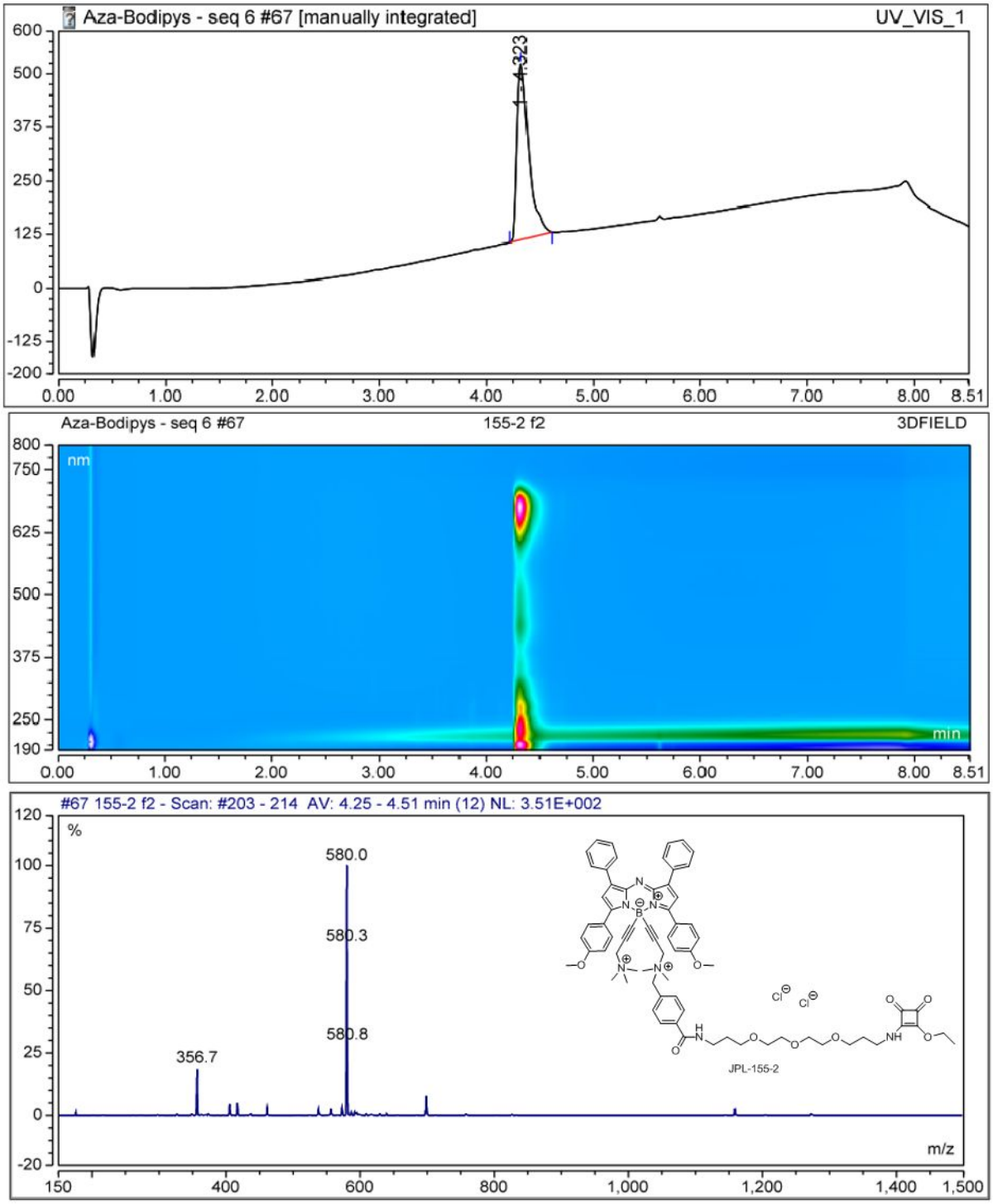


Figure S 46: HPLC of Wazaby4. Top: UV-Channel at 190 nm. Middle: 3D-field (190 nm-800 nm). Bottom: LR-MS(ESI)-channel

3 Spectra of obtained compounds

3.1 Aza-BODIPY **A1**

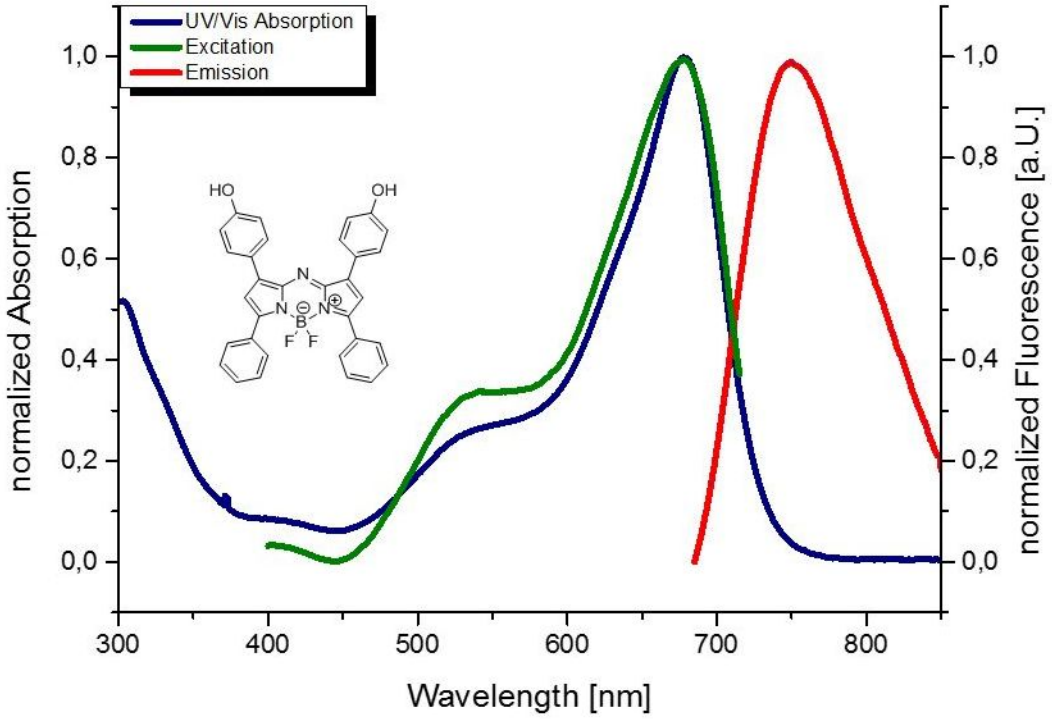


Figure S 47: Normalized Absorption, Emission and Excitation spectra of azaBODIPY A1 in DMSO

3.2 Aza-BODIPY A2

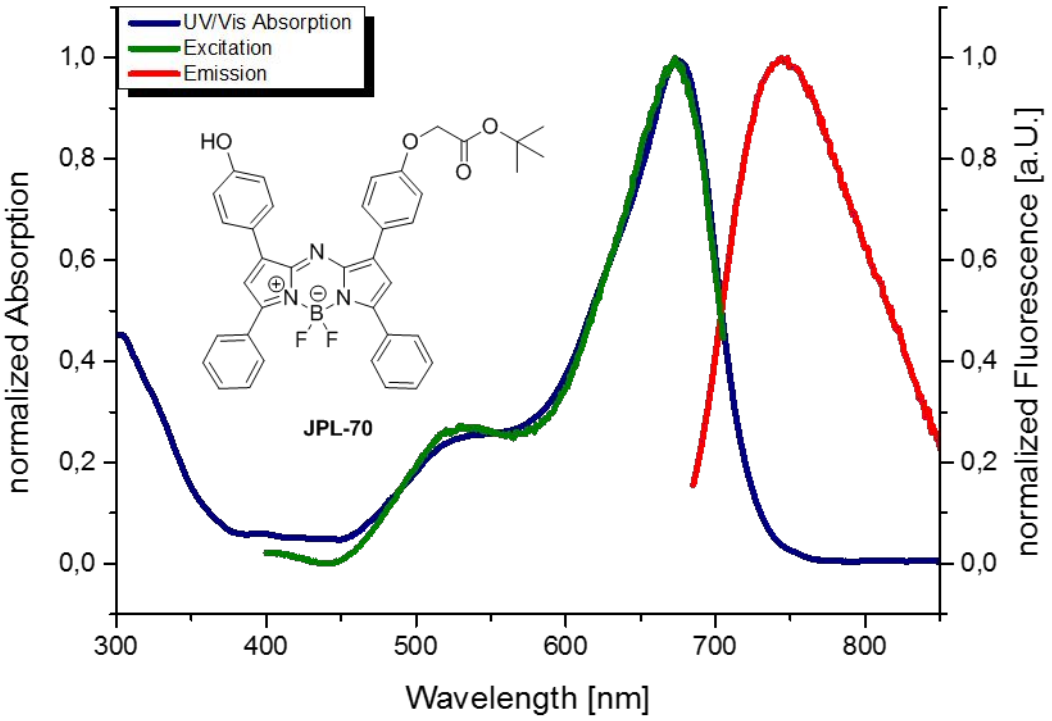


Figure S 48: Normalized Absorption, Emission and Excitation spectra of azaBODIPY A2 in DMSO

3.3 Aza-BODIPY A3

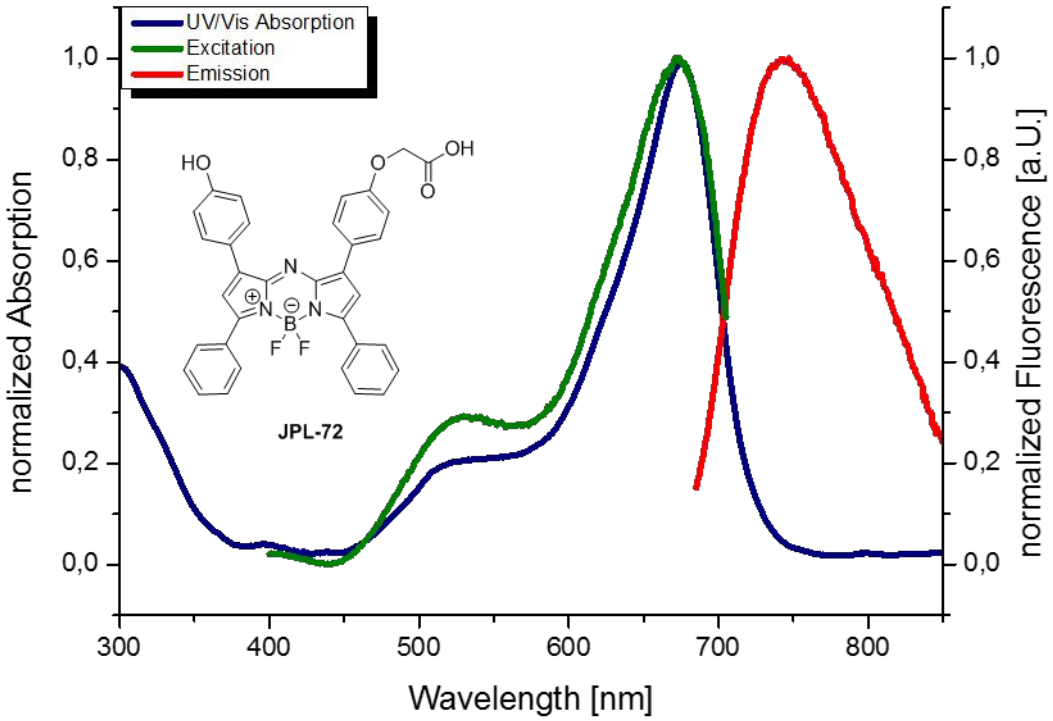


Figure S 49: Normalized Absorption, Emission and Excitation spectra of azaBODIPY A3 in DMSO

3.4 Compound A

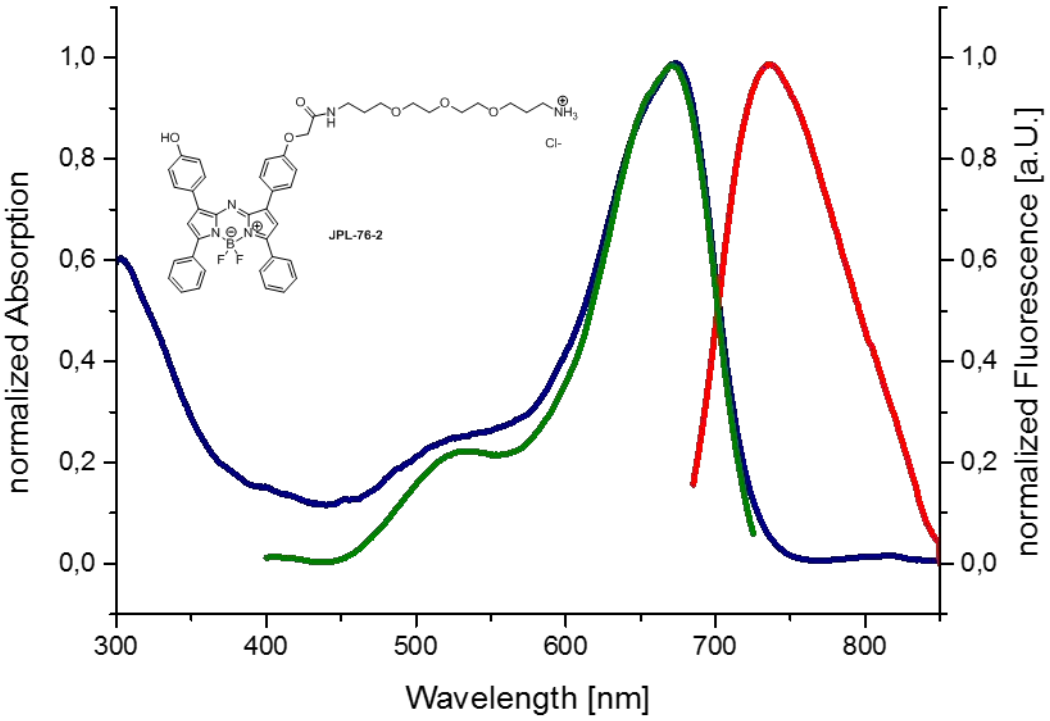


Figure S 50: Normalized Absorption, Emission and Excitation spectra of Compound A in DMSO

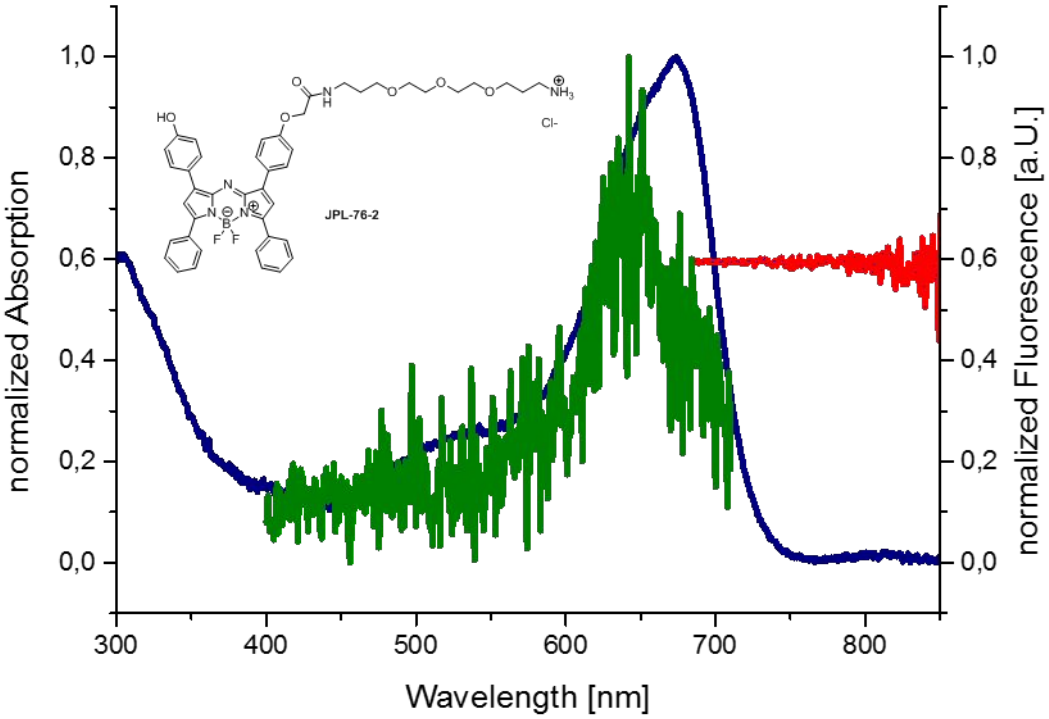


Figure S 51: Normalized Absorption, Emission and Excitation spectra of Compound A in PBS. Blue: Absorption spectrum, Red: Fluorescence spectrum, Green: Excitation spectrum

3.5 AzaBODIPY 1

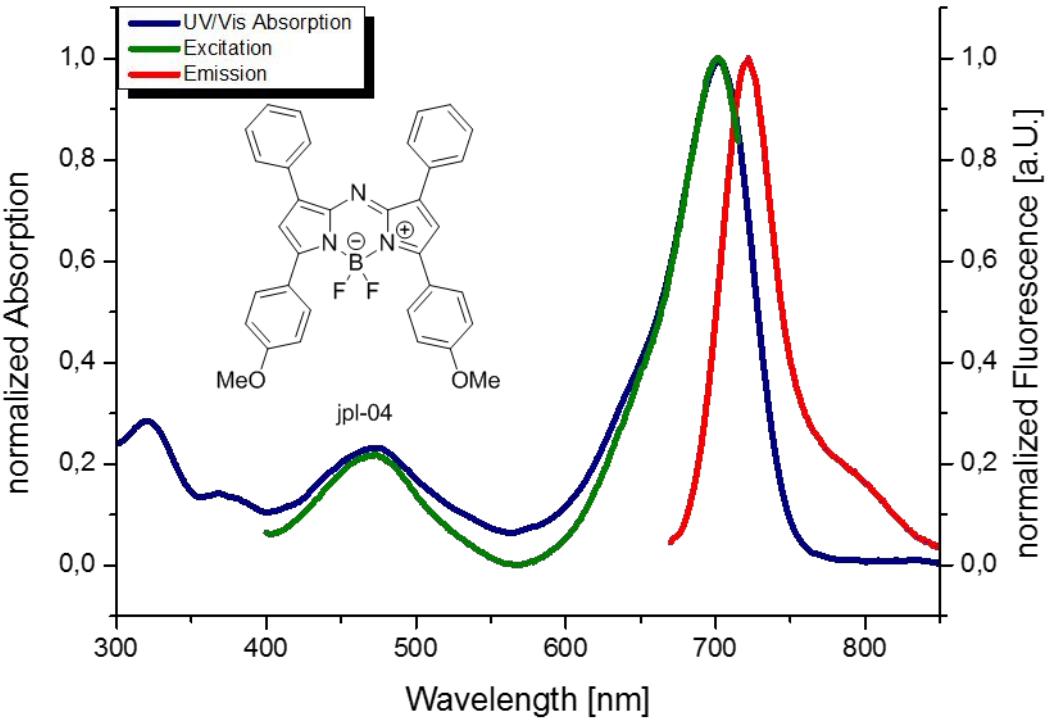


Figure S 52: Normalized Absorption, Emission and Excitation spectra of Aza-BODIPY 1 in DMSO

3.6 Aza BODIPY **2**

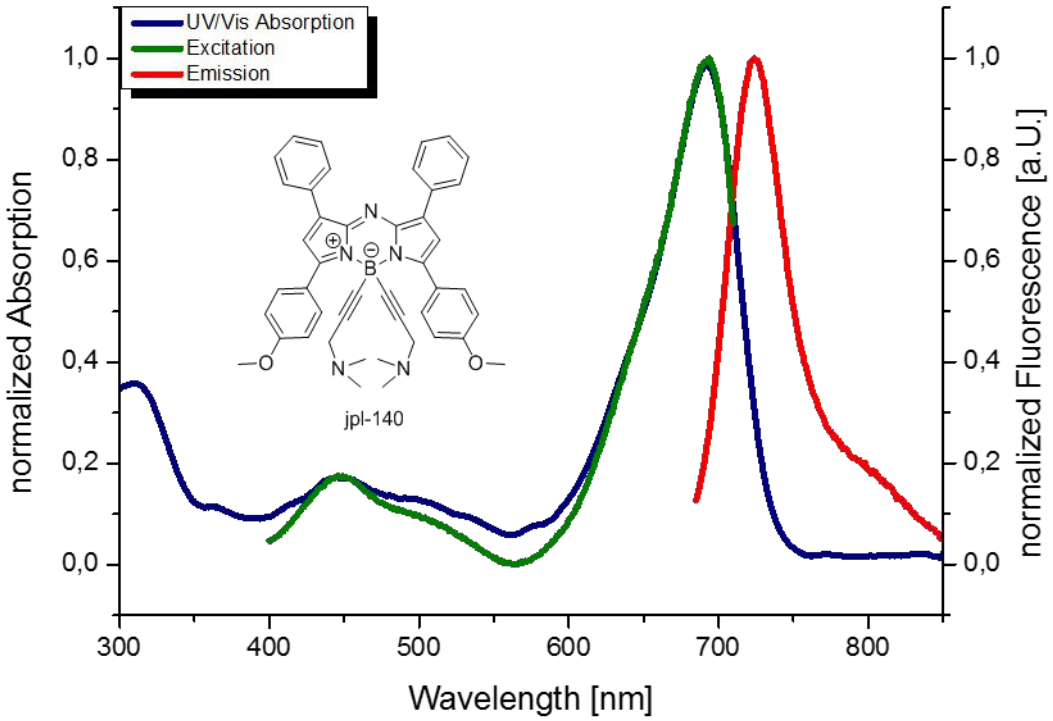


Figure S 53: Normalized Absorption, Emission and Excitation spectra of Aza-BODIPY 2 in DMSO

3.7 Aza-BODIPY **3**

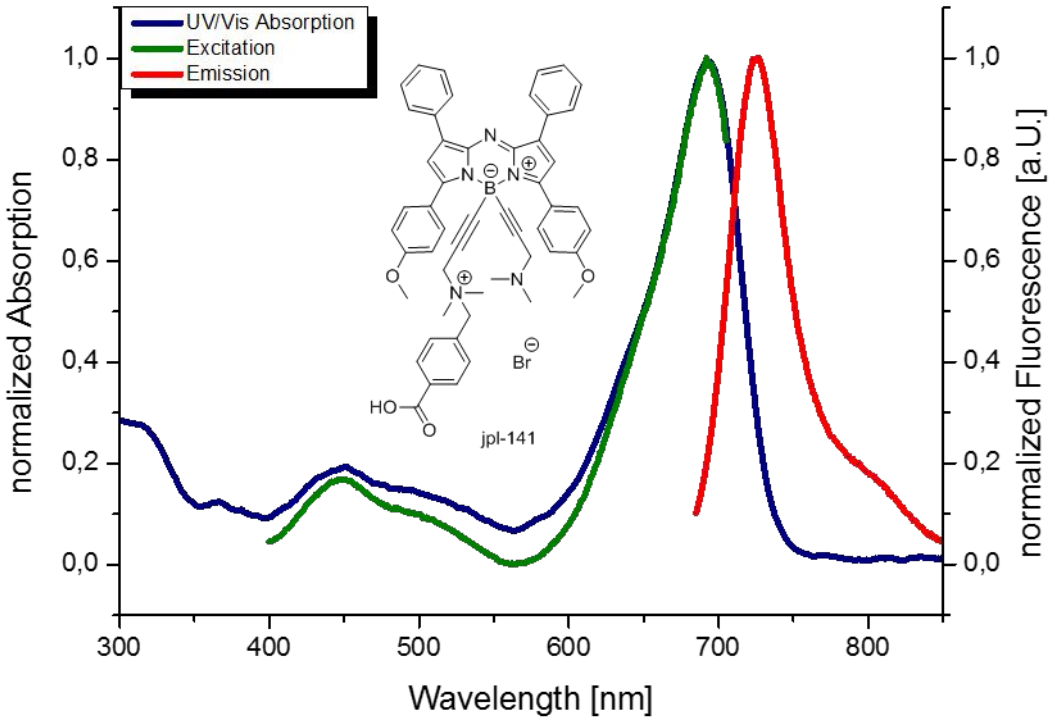


Figure S 54: Normalized Absorption, Emission and Excitation spectra of Aza-BODIPY3 in DMSO

3.8 Aza-BODIPY **4**

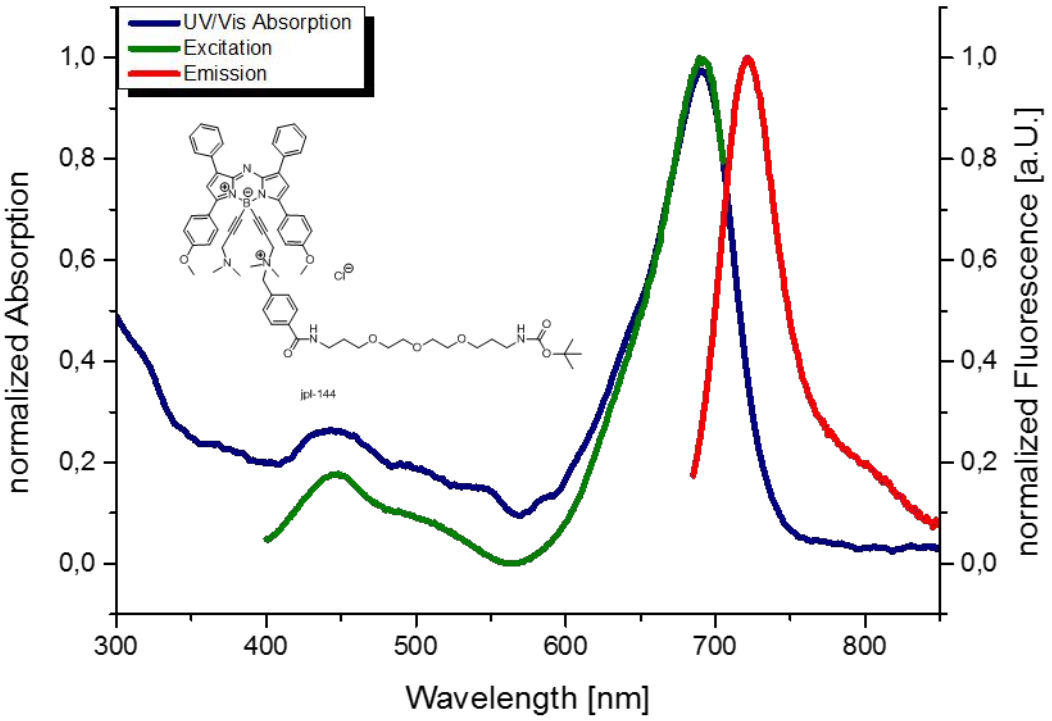


Figure S 55: Normalized Absorption, Emission and Excitation spectra of Aza-BODIPY 4 in DMSO

3.9 Wazaby1

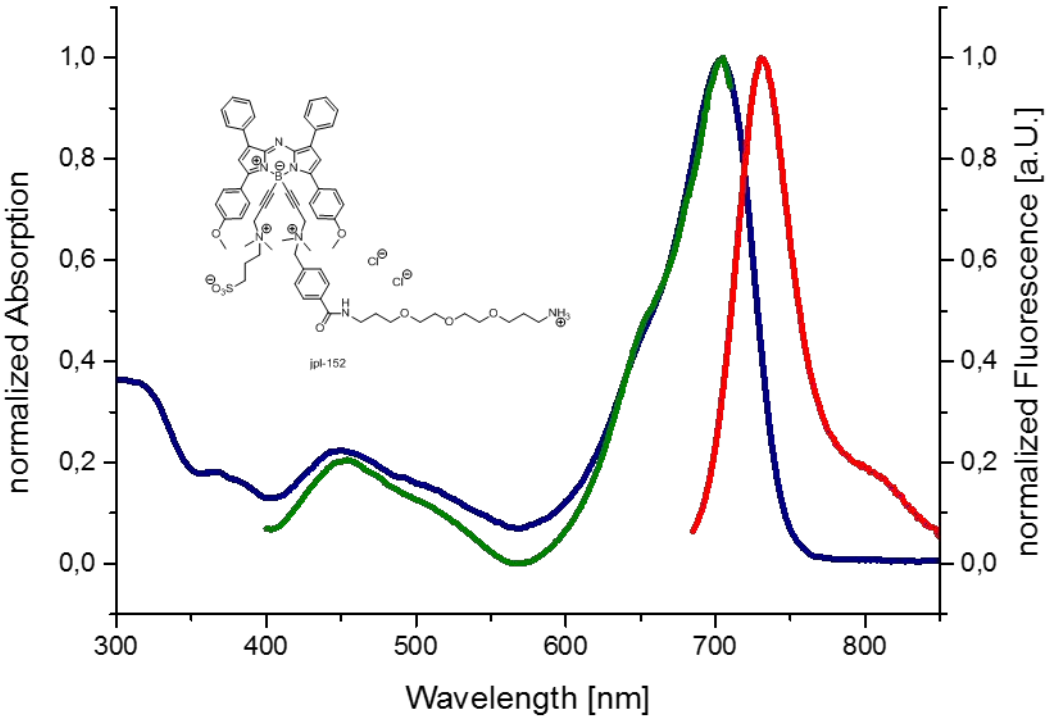


Figure S 56: Normalized Absorption, Emission and Excitation spectra of Wazaby1 in DMSO

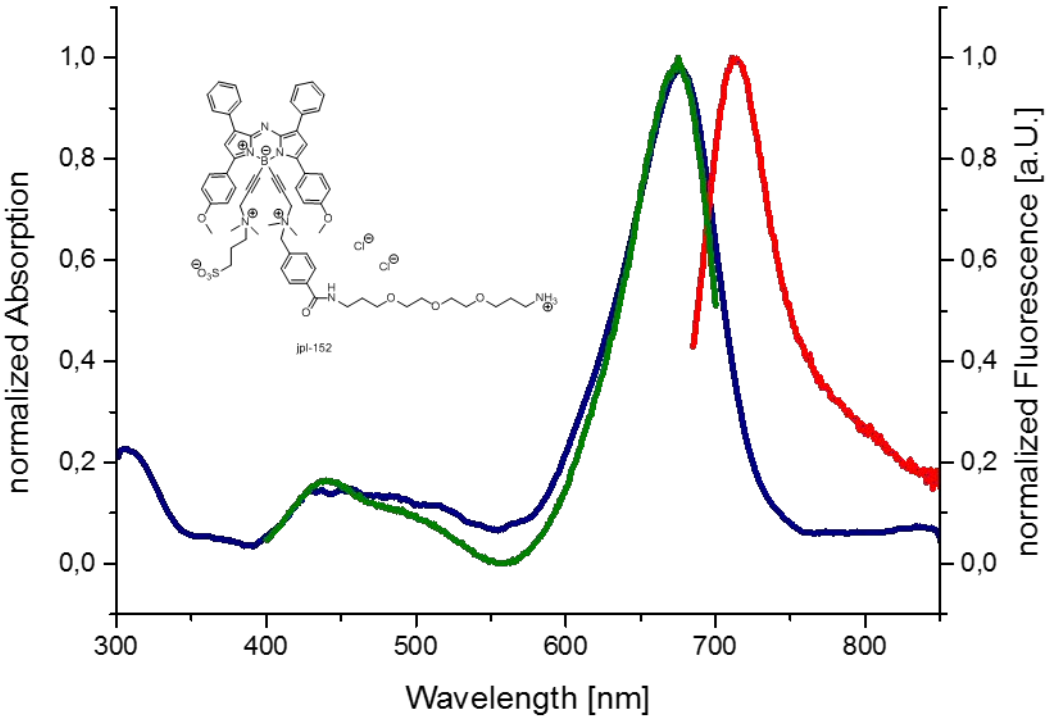


Figure S 57: Normalized Absorption, Emission and Excitation spectra of Wazaby1 in PBS

3.10 Wazaby2

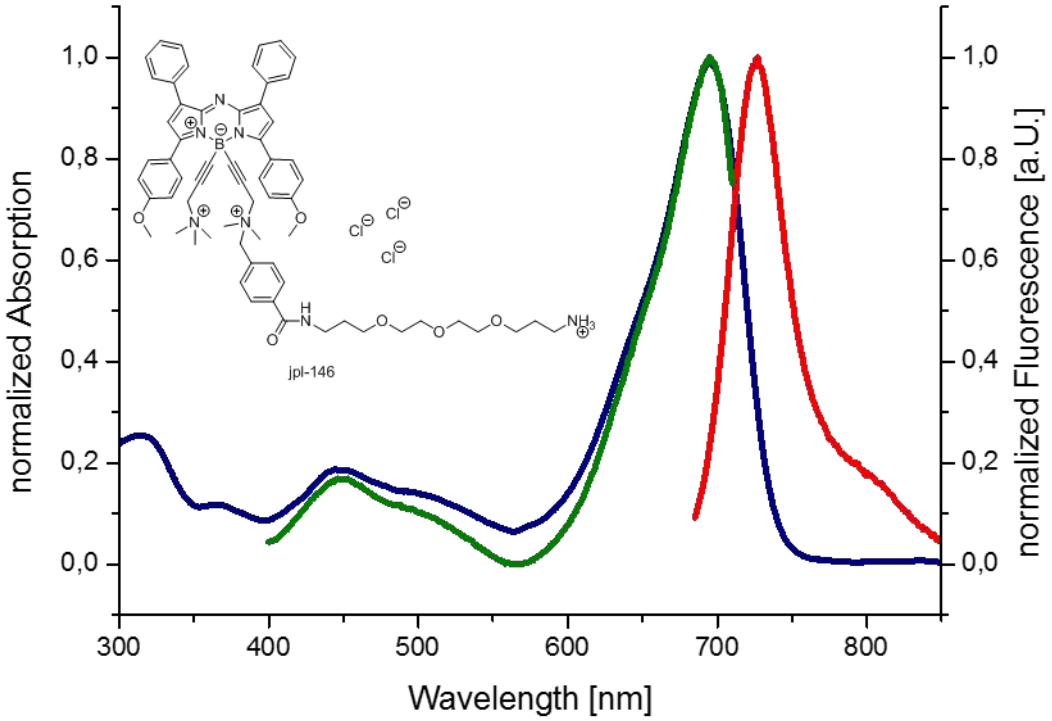


Figure S 58: Normalized Absorption, Emission and Excitation spectra of Wazaby2 in DMSO

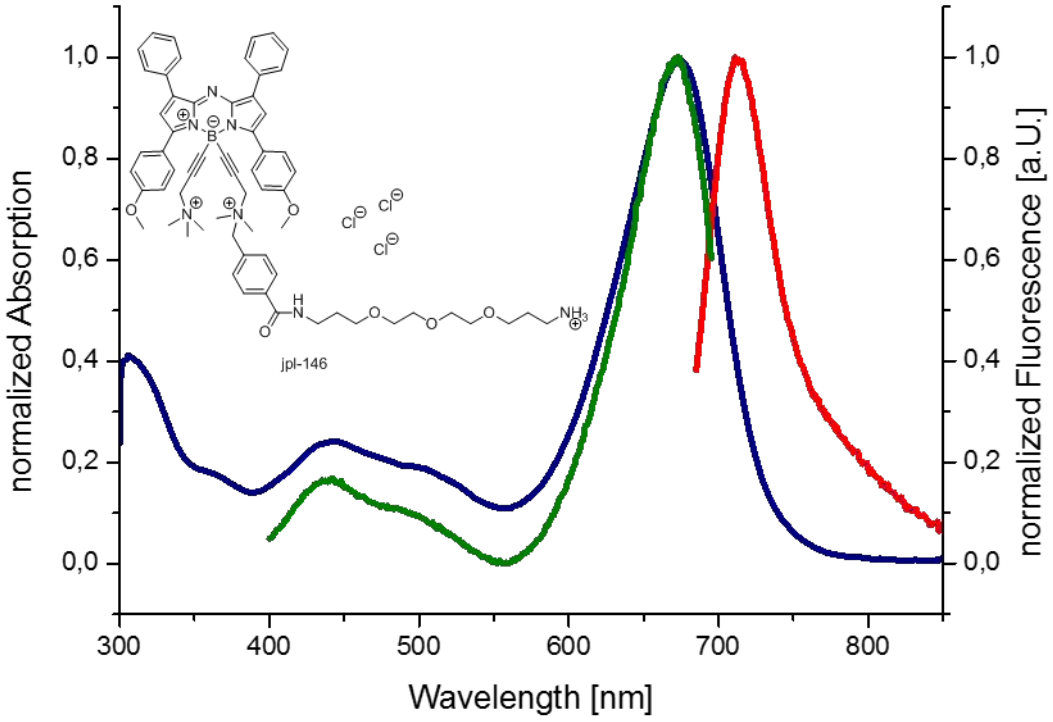


Figure S 59: Normalized Absorption, Emission and Excitation spectra of Wazaby2 in PBS

3.11 Wazaby3

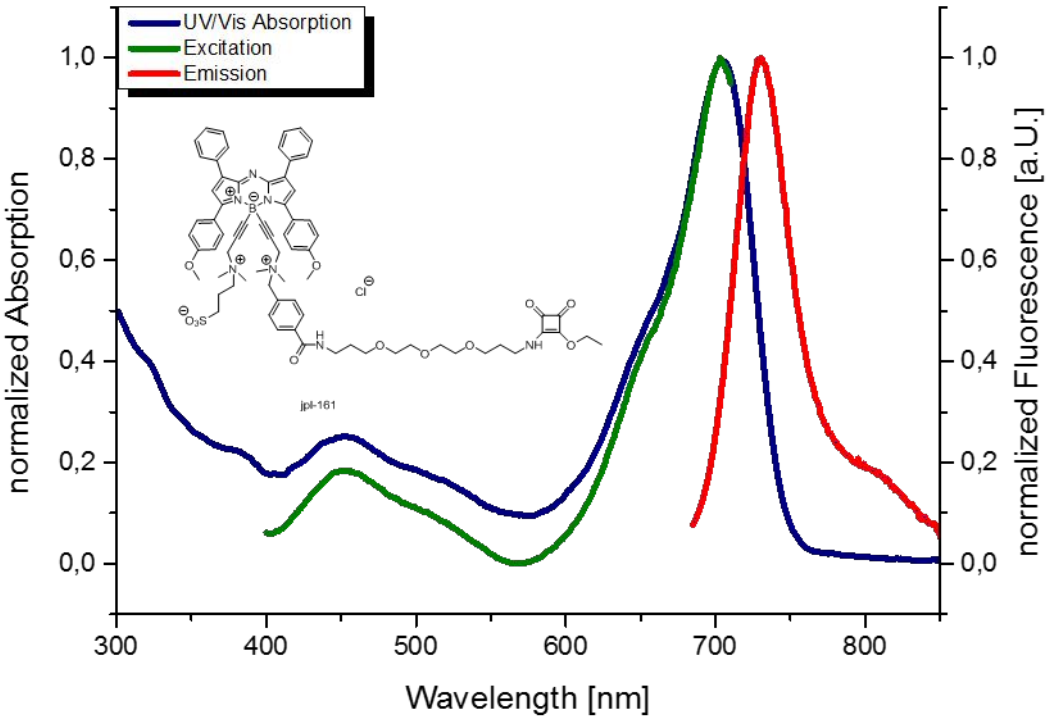


Figure S 60: Normalized Absorption, Emission and Excitation spectra of Wazaby3 in DMSO

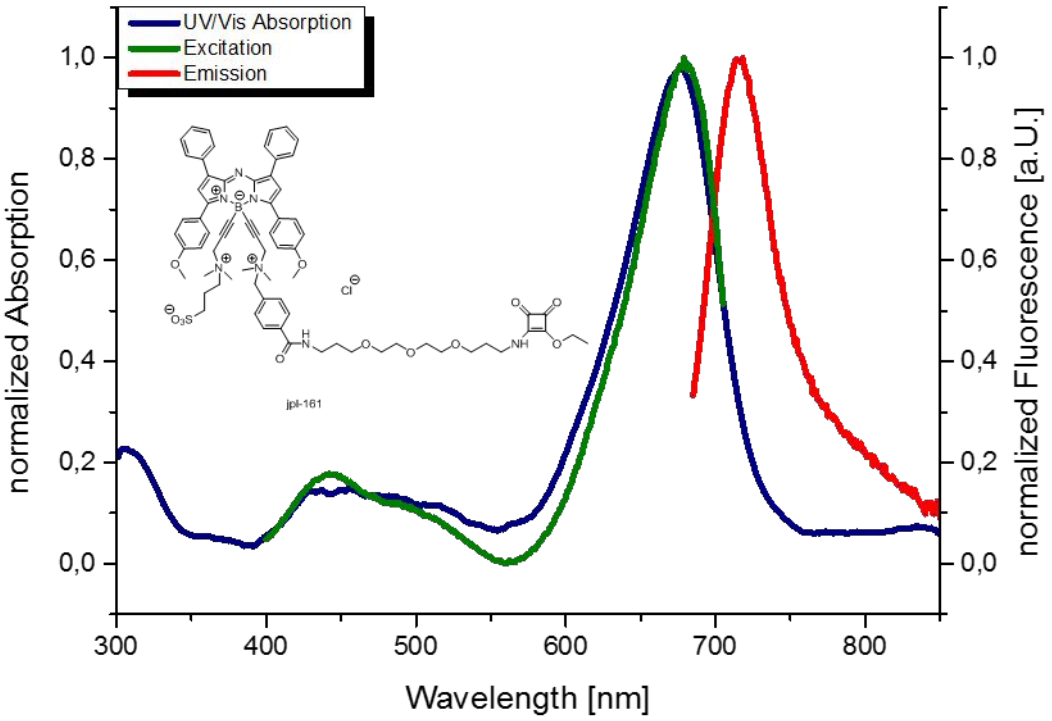


Figure S 61: Normalized Absorption, Emission and Excitation spectra of Wazaby3 in PBS

3.12 Wazaby4

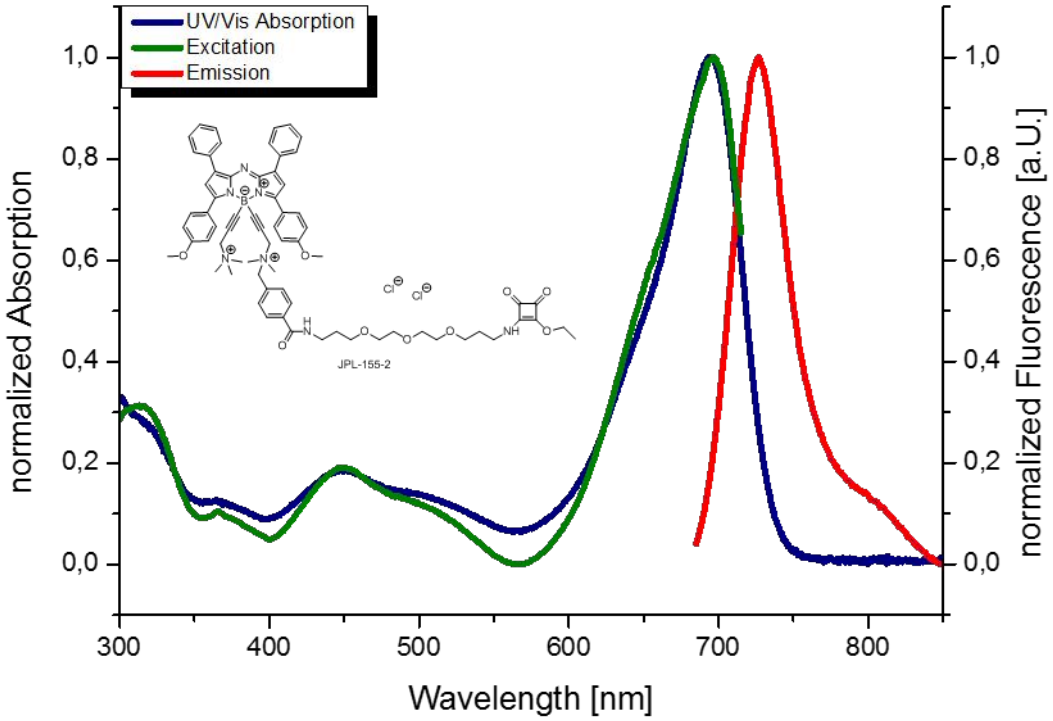


Figure S 62: Normalized Absorption, Emission and Excitation spectra of Wazaby4 in DMSO

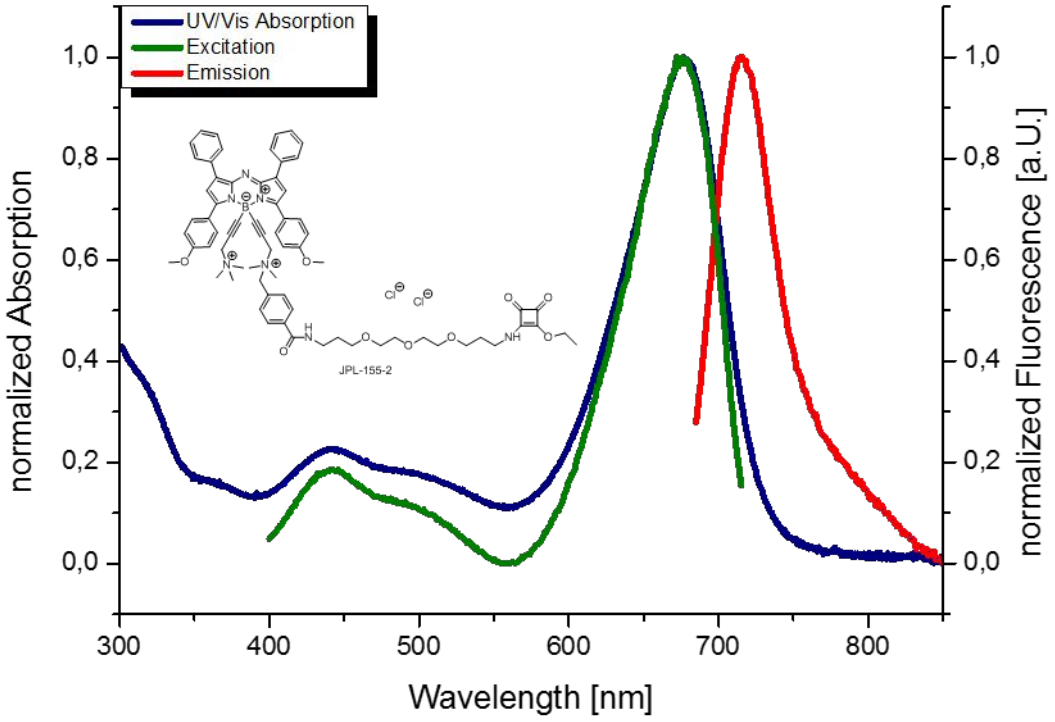


Figure S 63: Normalized Absorption, Emission and Excitation spectra of Wazaby4 in PBS

4 Bioconjugation

10eq of a 2 mM stock solution of **Wazaby4** in DMSO or 4eq of a 14.7 mM Cy5-NHS stock solution was added to a 3 mg/mL solution of antibody in $NaHCO_3$ buffer (0.2 M, pH 8.4). The amount of DMSO in the final aqueous reaction mixture was 10%. The solution was stirred overnight (**Wazaby4**) or for 4h (Cy5) in a thermomixer (900 rpm, 37 °C). The bioconjugate was then purified by FPLC (Äkta Pure 25M chromatography system, GE Healthcare Life Sciences) on a HiTrap® Protein G HP column (MabSelect resine, Protein G, cross-linked agarose, column I.D 7 mm, bed dimensions 7 x 25 mm, bed volume 1 mL). Excess of probe was removed with 20 mM phosphate buffer (pH 7.3) as eluent and 0.1% Tween 80 in 20 mM phosphate buffer (pH 7.3). Thereafter, the solution was transferred to an ultra-centrifugal filter device (Amicon Ultra 2 mL, Ultracel cut-off 30 kDa from Merck Millipore) and centrifuged at 4000 rpm for 3x30 min at 4°C in order to condition the mixture in 20 mM phosphate-buffered saline (pH 7.4). The recovered yields, concentrations and masses are listed below in Table S 3. The degree of labeling (amount of probe per IgG) was determined by MALDI-TOF mass spectrometry measurements.

Table S 3:Yields, Degree's of Labelling (DOL) and concentrations of obtained bioconjugates

Bioconjugate	DOL	IgG engaged	conjugate mass (yield)	concentration
Wazaby4-anti-PD-L1	2.9	1.8mg	772 µg (43%)	1.233mg/mL
Cy5-anti-PD-L1	2.9	1.8mg	1109 µg (62%)	2.209mg/mL

5 *In vitro* and *in vivo* studies

Bioconjugation assay:

The anti-PD-L1 antibodies (2 µg) uncoupled (UC) or coupled with cyanin5 (Cy5) or Wazaby4 (Wazaby4) were analyzed by electrophoresis on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE analysis, 10% acrylamide gel) in non-reducing (loading buffer without dithiothreitol (DTT), or reducing conditions (with loading buffer with DTT and heating at 95°C during 5 minutes). The loading buffer was constituted of 60 mM Tris-HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.001% Bromophenol blue +/- 0.05 M DTT and the protein ladder used was a PageRuler™ Prestained Protein Ladders, 10 to 180 kDA (Thermofisher Scientific). After protein migration (50 V during 30 min and 90 V during 2 h) in a running buffer (25 mM Tris Base, 0.1% SDS, 192 mM Glycine), the fluorescence analysis of the gels was performed on Odissey CLx Infrared Imaging System (LI-COR Biosciences) in pair filter mode (685/700 nm). After Coomassie blue staining (Instant Blue, Sigma) during 1 h, the gels were visualized on a Chemidoc XRS+ analyzer (BioRad).

Stability assay:

25 µl of **Wazaby4-anti-PD-L1** antibody (1 mg/ml) were incubated at 37°C, in a dark, with a gentle agitation with 25 µl of mice plasma. After 24 or 48h the reaction was stopped with the addition of loading buffer and 2 µg of antibody were loaded on a SDS polyacrylamide gel (7%), in non-reducing conditions (without DTT and heating), as previously described. The fluorescence analysis of the gel was performed on Odissey CLx Infrared Imaging System (LI-COR Biosciences) in pair filter mode (685/700 nm). After Coomassie blue staining (Instant Blue, Sigma) during 1h, the gel was visualized on a Chemidoc XRS+ analyzer (BioRad). The protein ladder used was a PageRuler™ Prestained Protein Ladders, 10 to 180 kDA (Thermofisher Scientific).

Antiproliferative assay:

Murine colon cancer cell (CT26, ATCC) were grown at a density of 1x104 cells per well in 96-well cell culture plates (Dutscher) in DMEM with 4.5 g/l glucose supplemented with 10% FBS and antibiotics (Dutscher, Brumath, France). After 24h at 37°C, cells were treated during 48h with different concentrations of **Wazaby1**. Thereafter, 10 µL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Promega, Charbonnieres, France) was added in 200 µL of medium and absorbance at 490 nm was measured after 3 h incubation at 37°C. DMSO at comparable concentrations did not show any effects on CT26 cell cytotoxicity. IC50 was

calculated using GraphPad7.0 Prism software. The compound display a low and similar cytotoxicity, with 50.41 µM and 49.93 µM IC50 for Wasaby2 and the bioconjugate compounds respectively.

In vitro confocal microscopy experiments:

CT26 Cells were seeded on chambered coverglasses (24 well-plate) and allowed to recover. Cells were incubated with 10 µM **Wazaby2** or 0.08mg/mL **Wazaby4-anti-PD-L1** 1 h and then fixed with PAF 4%. Cells were then mounted with Prolong® with DAPI. Confocal imaging was performed using a confocal laser-scanning microscope (Leica TCS SP8) with a × 63 HCX PL APO oil immersion (ON 1.4) objective lens that allowed to obtain fluorescent images (1024 pixels × 1024 pixels), and LASX software (Leica Microsystems, Ltd). The samples were excited using internal microscope lasers and emission intensity was recorded at the appropriate emission wavelength. Fluorescence images were sequentially acquired. For co-localization experiments, AzaBODIPY compounds (red) were excited at 638 nm and their emission was recorded from 643 to 800 nm, whereas the nuclear staining DAPI (blue), was excited at 405 nm and its emission was recorded from 410nm to 550 nm. Image processing and analyses were carried out using Fiji/ImageJ.

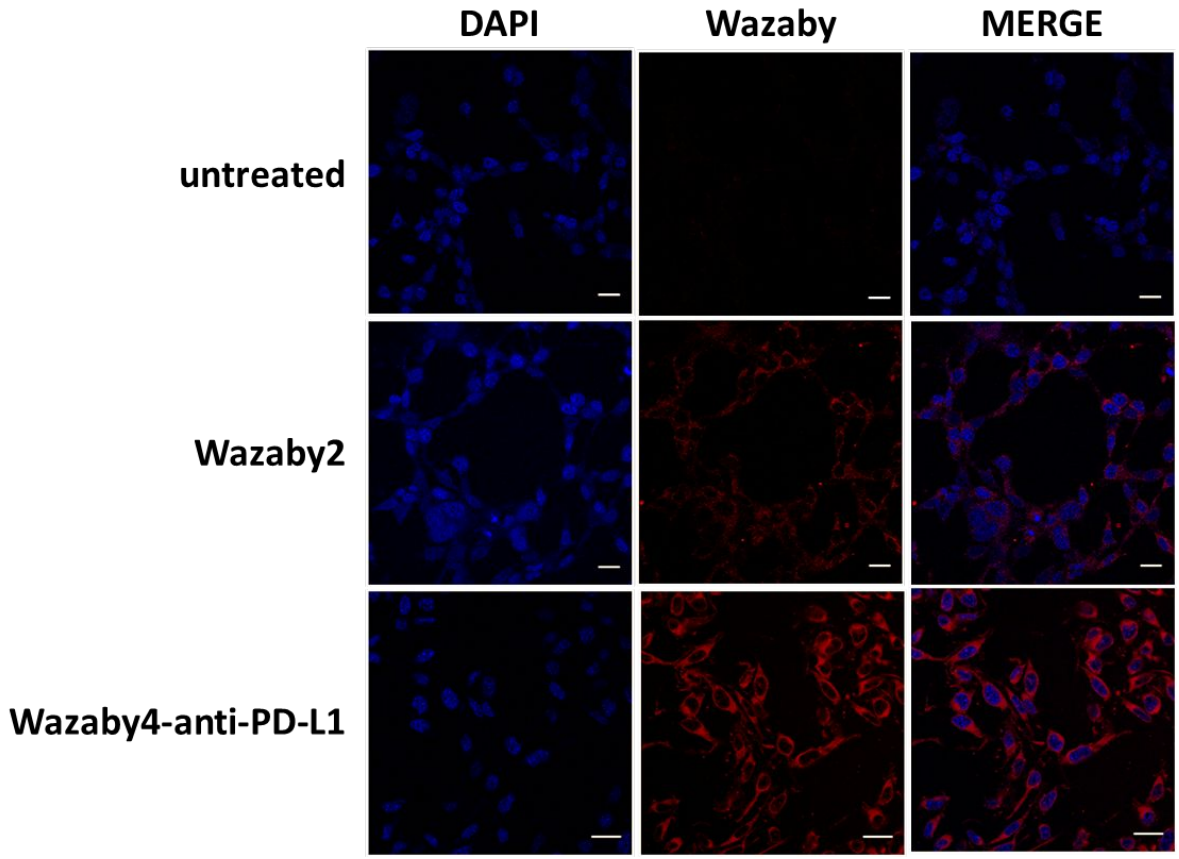


*Figure S 64: Confocal immunofluorescent analysis of CT26 cells labelled with **Wazaby2** or **Wazaby4-anti-PD-L1**. Cells are incubated or not with 10 µM **Wazaby2** or with 0.08 mg/mL of **Wazaby4-anti-PD-L1** (red) for 1h at 37°C, then fixed, permeabilized with 4% paraformaldehyde. The nuclei are counterstained with DAPI (blue, fluorescent DNA dye) (Scale bare 20µm).*

In vivo studies:

A syngeneic mouse model of colorectal cancer was used. Briefly, colorectal tumor cells CT26 ($1x10^6$) were resuspended in non-supplemented RPMI culture medium and injected subcutaneously (100 µl, 27G needle) on the right flank of the Balb/c mice (8 weeks old, n=6). Tumor volume was monitored daily. Imaging was performed at day 12 with tumor ranging from 200 to 400 mm^3. Mice were randomized into 2 groups with 1/ mice receiving **Wazaby4-anti-PD-L1** (100 µl, 50 µg, n=3) and 2/ mice receiving **Cy5-anti-PD-L1** (100µl, 50 µg, n=3). Fluorescent conjugates (**Wazaby4-anti-PD-L1** or **Cy5-anti-PD-L1**) were administered by intravenous injection in the mouse tail vein under anaesthesia (Isofluorane 2%). Whole animal imaging was performed at 1h, 6h, 24h and 48h post-injection by acquisition of the fluorescence on IVIS Lumina III In Vivo Imaging System (PerkinElmer) in pair filter mode (660/710 nm or 620/660 nm). After the last imaging (48h post-injection) mice were euthanized and organs were collected for *ex vivo* fluorescence imaging.

6 Additives used for preventing aggregation in reported water-soluble aza-BODIPY

Table S 4: list of the previous publications reporting water soluble aza-BODIPYs and the corresponding surfactant(s), which was (were) added to prevent aggregation phenomena.

References	Surfactant
O'Shea and coll. *Chem. Comm.*, **2002**, 1862 – 1863	CrEL
O'Shea and coll. *Org. Lett.*, **2009**, *11*, 5386–5389	CrEL
Kim and coll. *Chem. Comm.*, **2013**, *49*, 7141	DMSO+SDS and CrEL (4%DMSO+0,4%CrEl)
Chen and coll. *Chem Commun.*, **2014**, *50*, 14253–14256	Tween 80 (0.5%DMSO, 0.5% Tw80)
O'Shea and coll. *Eur. J. Org. Chem.*, **2014**, 6841–6845	SDS (1%SDS)
Chen and coll. *Analyst*, **2015**, *140*, 3766–3772	Tween 80 (0.4% Tw80)

Burgess and coll. *Chem. Commun.*, **2015**, *51*, 10664–10667	CrEL (0.1% CrEl)
O'Shea and coll. *Nat. Commun.*, **2016**, *7*, 10855	PEG5000, DMEM+ 10%FBS
O'Shea and coll. *Eur. J. Med. Chem.*, **2017**, *135*, 392–400	CrEL
O'Shea and coll. *Tetrahedron Lett.*, **2017**, *58*, 4468–4472	SDS (1%SDS)
O'Shea and coll. *Org. Biomol. Chem*., **2018**, *16*, 1144–1149	PEG5000, aggregation in PBS (no surfactant but aggregation can be seen on the spectra)