Supporting Information

PEGylated Aza-BODIPY Derivatives as NIR Probes for Cellular Imaging

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General Experimental

Instruments. All photophysical measurements performed in solutions were carried out using dilute solutions with absorbance around 0.1 at the maximum absorption wavelength in 1 cm path length quartz cells and in air-equilibrated solutions at room temperature. The UV–Vis absorption spectra were recorded with a HP 8452A Diode Array Spectrophotometer spectrophotometer. The fluorescence spectra were recorded with a JASCO FP-750 Spectrofluorometer. Fluorescence quantum yields were determined using Rhodamine B (Φ_F = 0.53 in EtOH) as a reference and corrected for the corresponding refractive index.[1] Proton and carbon nuclear magnetic resonance spectra (^{1}H and ^{13}C NMR) of the newly synthesized compounds were recorded on a Bruker Advance III 400 MHz Ascend instrument and were referenced to the signal of the residual solvent. (^{1}H NMR, $CHCl_3$ at 7.26 ppm, CD_3OD at 3.34 ppm; ^{13}C NMR, $CDCl_3$ at 77.0 ppm, CD_3OD at 49.9 ppm).[2] Data for ^{1}H NMR are reported as follows: chemical shift (ppm), integration, multiplicity (s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet) and coupling constants, *J*, in (Hz). The HRMS data were recorded on a Bruker MicroTOF mass spectrometer.

Materials. All reagents (including the starting compound **5a**) used in the synthetic procedures were commercially available and were purchased in the highest purity available (≥ 98%). Aldehydes **4a**[3]**-4b**[4], acetophenones **5b**[5]**-5c**[6], aza-BODIPY **9**[7] and compound **8**[8] were synthesized according to previously reported procedures. Unless otherwise noted, all reagents and chemicals were used without further purification. Chromatography on silica gel was performed using silica gel 60 (230-400 mesh). Thin layer chromatography (TLC) was performed on silica gel 60 plates. Visualization of spots on TLC plates was accomplished with UV light (254 nm). Solvents for synthesis and NMR were used without further purification. The overall pH of the PBS buffer was 7.4.

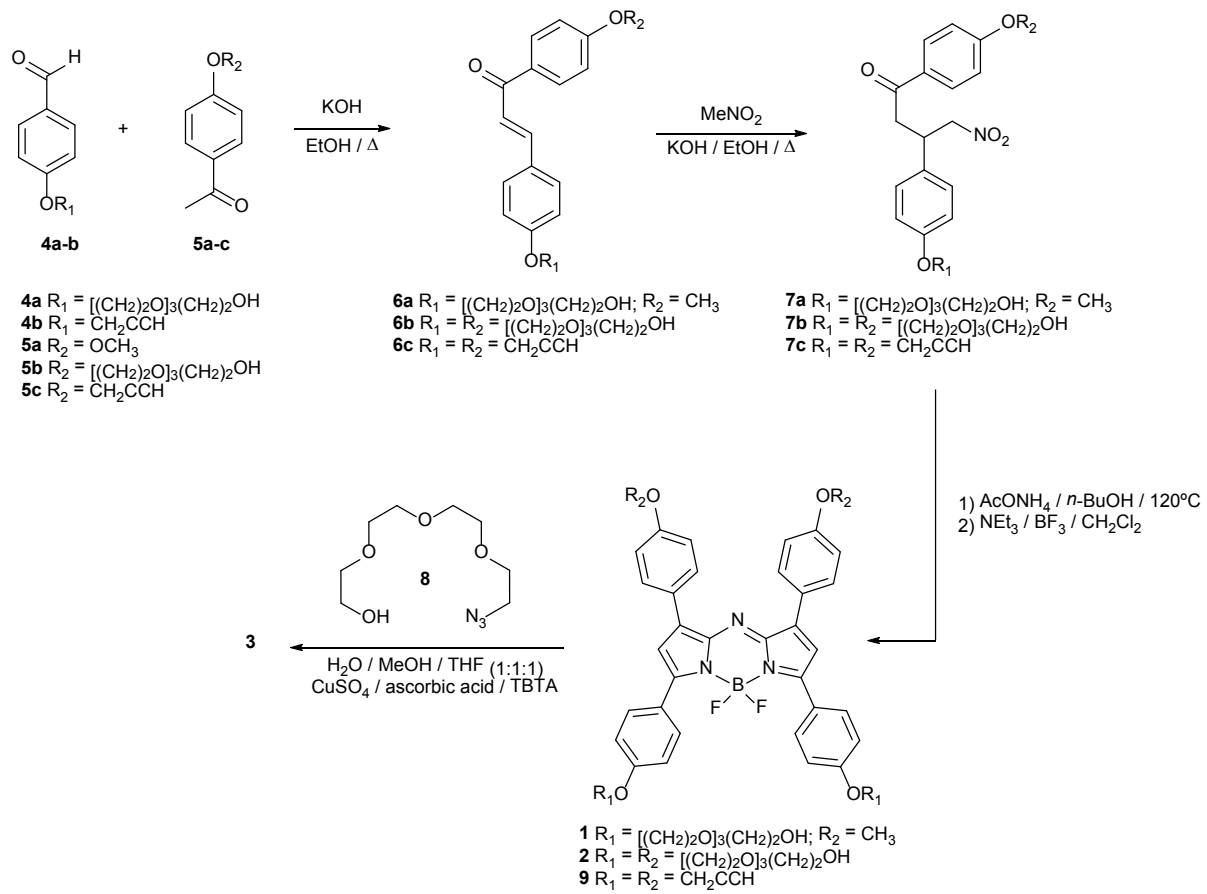
Cell Culture and aza-BODIPY incubations. 8-well chambered coverglass slides were seeded with the HeLa human tumor cell line. Cells were cultured at 37ºC with 5% CO_2 in RPMI (1640 medium supplemented with L-Glutamine and Fetal Bovine Serum until they reached 50-70% confluence, 1-2 days). HeLa cell-containing wells were incubated for 1 h with 200µl of fresh, pre-heated RPMI 1640 culture medium containing different concentrations of aza-BODIPY plus a negative control. After incubation, dye-containing media was removed from each well, washed two times with preheated PBS to remove unbound aza-BODIPY, and then fixed using 4% paraformaldehyde for 10 min at room temperature. Finally cells were washed with PBS three times and kept at 4ºC until analysis.

Confocal Microscopy. The fluorescence properties of aza-BODIPY **2** in HeLa cells were analyzed using an inverted confocal microscope. 3D image series containing 7 images were captured at ~1µm intervals. Aza-BODIPY fluorescence was visualized using excitation at 633 nm and detection of emissions between 643nm–758nm. Non-confocal transmitted light images were obtained simultaneously. All samples were analyzed using identical imaging conditions. Emission spectra for cell and background Regions of Interest (ROIs) were calculated using the manufacturer´s software. The 633 nm emission spectrum was measured using a dynamic 20 nm wide emission detection window moving in 20 steps between 650 nm and 795 nm.

Computational Methods. All calculations were achieved using the Gaussian 09 package.[9] The ground-state (S_0) geometrical parameters have been determined with the density functional level of theory, employing the PBE0 functional[10,11] and the 6-311G(2d,p) basis set, and within C2 point group, as recommended.[12] The nature of the minima was confirmed by the absence of a negative frequency in the vibrational analysis. We have replaced the PEG chains by methoxy groups, as it is expected that the length of these chains has no substantial influence on the optical properties. Restricted formalism was applied for the singlet electronic states and unrestricted formalism for the triplets states. Absorption

spectra were computed as vertical electronic excitations from the minima of the ground-state structures by using time-dependent density functional response theory[13] employing the PBE0 functional, and with the 6-311+G(2d,p) basis set.[12,14] The solvent effect has been accounted for with the polarizable continuum model (PCM),[15] and water as solvent.

Experimental procedures



Scheme S1. General route to generate aza-BODIPYs **1**, **2** and **3**.

General procedure for the synthesis of chalcones 6a, 6b and 6c[7]

A solution of aromatic ketone (10 mmol) in ethanol (10 mL) was added gradually to an aqueous solution of 10% KOH (30 mL) at 0ºC. After stirring for 15 min, aromatic aldehyde (10 mmol) was added and stirred at 0ºC for 15 min. The mixture was then allowed to attain room temperature and stirred for 4h. The solvent was removed *in vacuo*, the residue diluted with CH_2Cl_2 (100 mL), washed with water (2 x 15 mL) and dried with anhydrous $MgSO_4$. The organic layer was concentrated, and the residue was purified by chromatography on a silica gel column (AcOEt/MeOH 20/1) to give the title product.

(E)-3-(4-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethoxy)phenyl)-1-(4-ethoxyphenyl)prop-2-en-1-one), ***(6a)***. Colorless solid, (232 mg, 60%); δ_H(400 MHz, $CDCl_3$) 8.00 (2H, d, J = 9.2 Hz), 7.74 (1H, d, J = 15.4 Hz), 7.56 (2H, d, J = 9.2 Hz), 7.40 (1H, d, J = 15.4 Hz), 6.98-6.91 (4H, m), 4.17 (2H, t, J = 4.3 Hz), 3.87-3.84 (4H, m), 3.70-3.56 (14H, m); δ_C(100 MHz, $CDCl_3$) 188.3, 162.4, 160.6, 144.0, 131.6, 130.1, 129.3, 127.4, 119.5, 114.4, 114.2, 72.4, 70.7, 70.5, 70.4, 70.1, 69.5, 69.4, 67.5, 67.4, 61.5, 55.6; HRMS (ESI) *m/z* calcd for $C_{24}H_{30}O_7$+H^+, 431.2070 [M+H^+]; found, 431.2074.

(E)-1,3-bis(4-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethoxy)phenyl)prop-2-en-1-one, ***(6b)***. Colorless oil, (408 mg, 72%); δ_H(400 MHz, $CDCl_3$) 8.00 (2H, d, J = 8.6 Hz), 7.75 (1H, d, J = 16.0 Hz), 7.57 (2H, d, J = 8.6 Hz), 7.40 (1H, d, J = 16.0 Hz), 7.00-6.90 (4H, m), 4.23-4.14 (6H, m), 3.89-3.83 (6H, m), 3.71-3.57 (22H, m); δ_C(100 MHz, $CDCl_3$) 188.7, 162.3, 160.5, 143.7, 131.3, 130.6, 130.0, 127.9, 119.5, 114.9, 114.3, 72.5, 70.7, 70.5, 70.4, 70.1, 69.5, 69.4, 67.5, 67.4, 61.6; HRMS (ESI) *m/z* calcd for $C_{31}H_{44}O_{11}+H^+$, 593.2962 [M+H^+]; found 593.2965.

General procedure for the synthesis of nitro derivatives 7a, 7b and 7c[7]

A solution of chalcone **6a-c** (5 mmol) in EtOH (50 mL) was treated with KOH (25 mmol) and nitromethane (25 mmol) and heated under reflux for 24 h. After cooling to room temperature, the solvent was removed *in vacuo* and the oily residue obtained was dissolved in CH_2Cl_2 (75 mL) and washed with water (2 x 50 mL). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and concentrated to give the target compound, which could be used directly in the next step without further purification.

3-(4-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethoxy)phenyl)-1-(4-methoxyphenyl)-4-nitrobutan-1-one, ***(7a)***. Brown oil, (240 mg, 90%); δ_H(400 MHz, $CDCl_3$) 7.87 (2H, d, J = 9.1 Hz), 7.16 (2H, d, J = 8.5 Hz), 6.92-6.82 (4H, m), 4.83-4.53 (2H, m), 4.16-4.05 (4H, m), 3.85-3.78 (5H, m), 3.69-3.57 (14H, m); δ_C(100 MHz, $CDCl_3$) 195.4, 163.7, 158.1, 131.3, 130.3, 129.4, 128.4, 114.9, 113.6, 79.8, 72.6, 70.8, 70.6, 70.5, 70.4, 70.1, 70.0, 69.7, 69.5, 67.2, 66.5, 61.5, 55.4, 41.1, 38.7; HRMS (ESI) *m/z* calcd for $C_{25}H_{33}NO_9+H^+$, 492.2234 [M+H^+]; found, 492.2238.

1,3-bis(4-(2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethoxy)phenyl)-4-nitrobutan-1-one, ***(7b)***. Brown oil, (148 mg, 72%); δ_H(400 MHz, $CDCl_3$) 7.86 (2H, d, J = 8.5 Hz), 7.16 (2H, d, J = 8.5 Hz), 6.92 (2H, d, J = 9.1 Hz), 6.84 (2H, d, J = 9.1 Hz) 4.83-4.53 (2H, m), 4.17-4.08 (6H, m), 3.88-3.79 (5H, m), 3.75-3.56 (26H, m); δ_C(100 MHz, $CDCl_3$) 188.7, 162.3, 160.5, 160.2, 143.7, 131.3, 130.6, 130.0, 127.8, 119.5, 114.9, 114.3, 72.5, 70.7, 70.5, 70.4, 70.1, 69.5, 69.4, 67.4, 67.3, 61.5, 41.3, 39.1; HRMS (ESI) *m/z* calcd for $C_{32}H_{47}NO_{13}+H^+$, 654.3126 [M+H^+]; found 654.3130.

Synthesis of aza-BODIPY 1-2

Ammonium acetate (10 mmol) was added to a stirred solution of **7a-c** (0.5 mmol) in *n*-BuOH (20 mL). The mixture was heated at 120 ºC for 24 h. The solvent was removed *in vacuo*, the residue diluted with CH_2Cl_2 (100 mL), washed with water (2 x 15 mL) and dried with anhydrous $MgSO_4$. The organic layer was concentrated *in vacuo*. The resulting solid was subsequently dissolved in dry CH_2Cl_2 (10 mL), cooled to 0ºC, and treated with triethylamine (5 mL) followed by slow addition of $BF_3{\cdot}OEt_2$ (5 mL). The mixture was stirred for 24 h. The reaction was quenched with crushed ice and extracted with CH_2Cl_2 (2 x 30 mL). The organic layer was dried over anhydrous $MgSO_4$ and evaporated to dryness. Purification by column chromatography on silica gel (AcOEt/MeOH 8/2) gave the title compound.

Aza-BODIPY ***1***. Purple solid (56 mg, 12%). δ_H(400 MHz, $CDCl_3$) 8.13 (4H, d, J = 8.5 Hz), 7.72-7.64 (6H, m), 7.06-6.98 (8H, m), 4.20 (4H, t, J = 4.3 Hz), 3.90-3.86 (10H, m), 3.74-3.57 (26H, m); δ_C(100 MHz, $CDCl_3$) 160.4, 159.4, 156.8, 149.4, 132.4, 131.7, 128.3, 128.2, 115.2, 115.1, 113.9, 72.5, 70.8, 70.6, 70.5, 69.6, 70.2, 67.4, 61.7, 55.3; HRMS (MALDI) *m/z* calcd for $C_{50}H_{58}BF_2N_3O_{12}+H^+$, 942.4160 [$M$+$H^+$]; found, 942.4166.

Aza-BODIPY ***2***. Purple solid (43 mg, 15%). δ_H(400 MHz, $CDCl_3$) 8.10 (4H, d, J = 8.5 Hz), 7.71-7.63 (6H, m), 7.04-6.99 (8H, m), 4.20-4.17 (8H, m), 3.87-3.85 (8H, m), 3.74-3.57 (52H, m); δ_C(100 MHz, $CDCl_3$) δ 159.5, 142.7, 136.2, 131.4, 130.7, 128.3, 115.1, 114.7, 72.5, 70.8, 70.6, 70.5, 70.3, 69.7, 67.6, 61.7; HRMS (MALDI) *m/z* calcd for $C_{64}H_{86}BF_2N_3O_{20}+H^+$, 1266.5944 [$M$+$H^+$]; found 1266.5950.

Synthesis of aza-BODPIPY 3

Aza-BODIPY **9** (116 mg, 0.29 mmol) was suspended in a 2:1:1 mixture of MeOH:H_2O:THF (6 mL), to which was added **8** (325 mg, 0.61 mmol), $CuSO_4 \cdot 5H_2O$ (184 mg, 2.90 mmol), and TBTA (3.1 mg, 0.006 mmol). The reaction mixture was allowed to stir vigorously at room temperature for 24 h after which it was diluted with CH_2Cl_2, and then filtered through a bed of Celite to remove the excess $CuSO_4$. The filtrate was dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to yield the crude material. The crude material was purified by reversed-phase column chromatography using MeOH for the elution to yield aza-BODIPY **3** as a blue solid.

Blue solid (34 mg, 78%); δ_H(400 MHz, CD_3OD) 7.94 (4H, d, J = 8.5 Hz), 7.41-7.30 (10H, m), 7.11 (4H, d, J = 8.5 Hz), 6.97 (4H, d, J = 8.8 Hz), 5.29 (8H, s), 3.92-3.90 (8H, m), 3.74-3.50 (60H, m); δ_C(100 MHz, CD_3OD) δ 177.7, 160.8, 145.3, 135.4, 132.3, 130.2, 128.7, 128.2, 127.7, 125.5, 125.1, 124.1, 114.8, 114.5, 114.4, 72.3, 70.3, 70.2, 70.1, 70.0, 69.9, 69.8, 69.7, 68.9, 60.8, 50.4; HRMS (MALDI) *m/z* calcd for $C_{76}H_{98}BF_2N_{15}O_{20}+H^+$, 1590.7252 [$M+H^+$]; found 1590.7260.

Additional figures

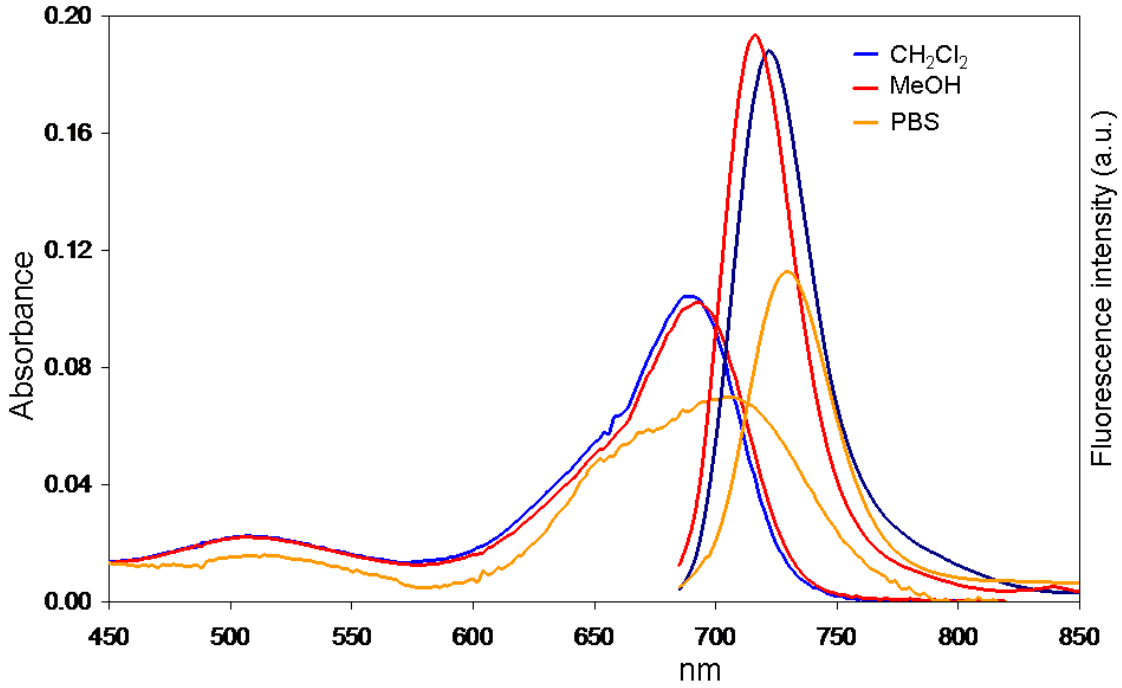


Figure S1. Absorption and emission spectra (λ_{exc} = 680 nm) for aza-BODIPY **3** in different solvents.

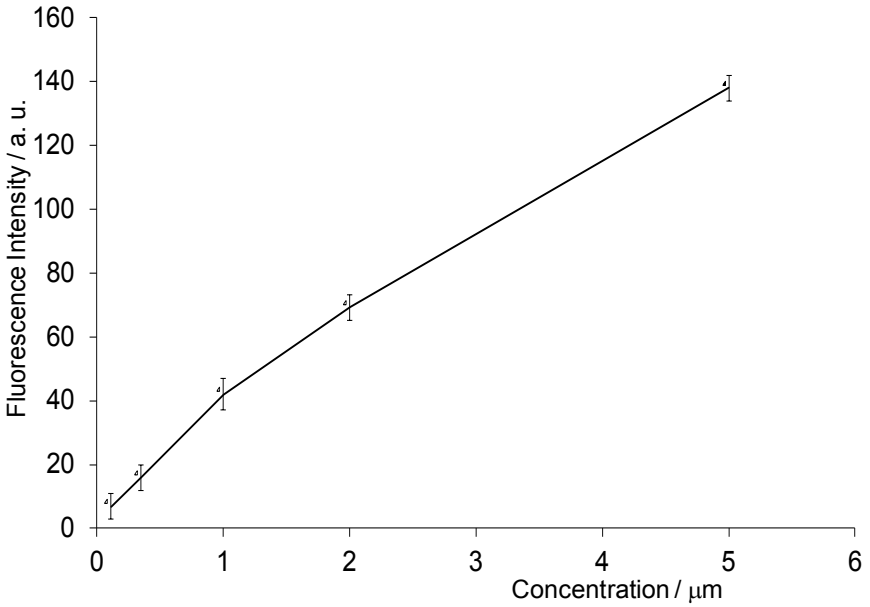


Figure S2. Uncorrected fluorescence intensities of aza-BODIPY **2** (λ_{exc} = 680 nm) *versus* concentration in PBS.

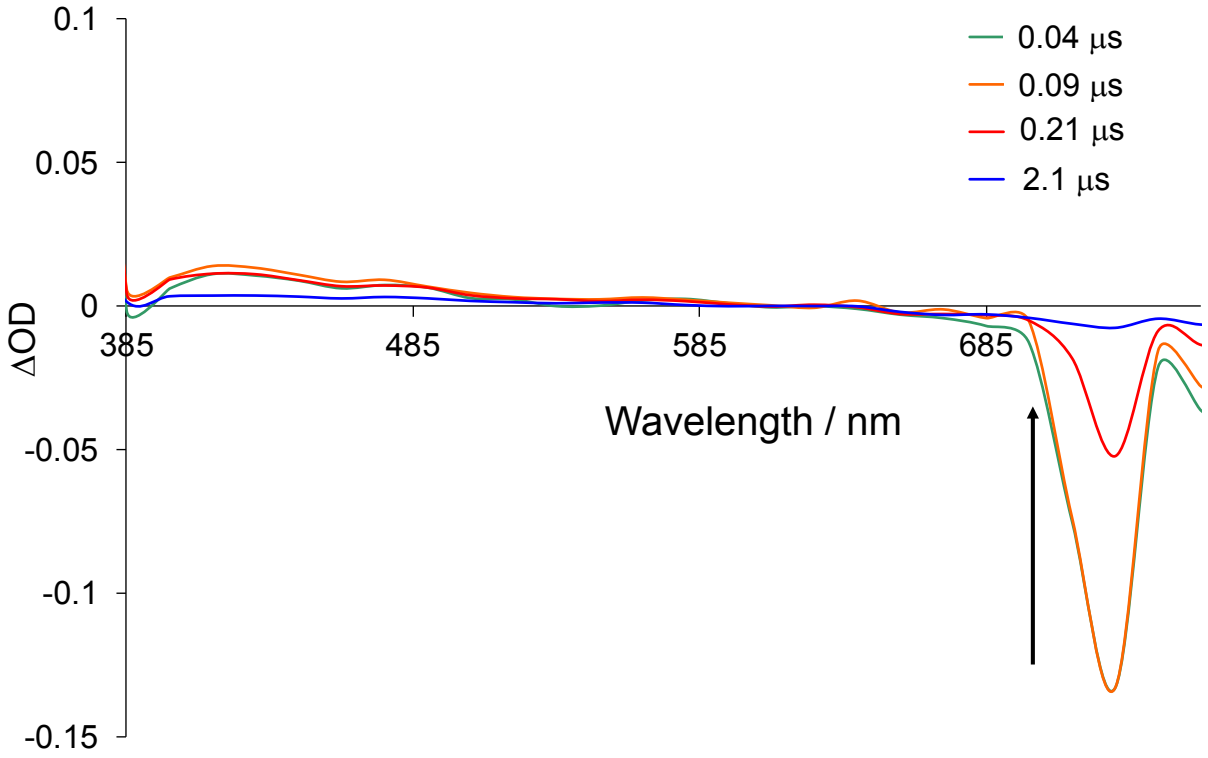
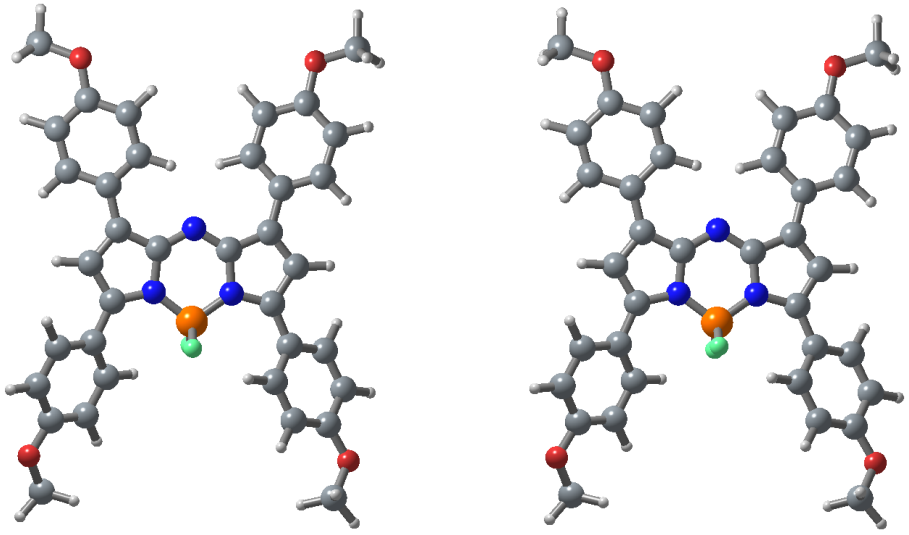


Figure S3. Transient absorption spectra of aza-BODIPY **2** following 355 nm laser pulse excitation.



S_0 geometry S_1 geometry

Figure S4. Ground and excited state geometries for the model aza-BODIPY obtained at the PCM-(TD)-PBE0/6-311G(2d,p) level.

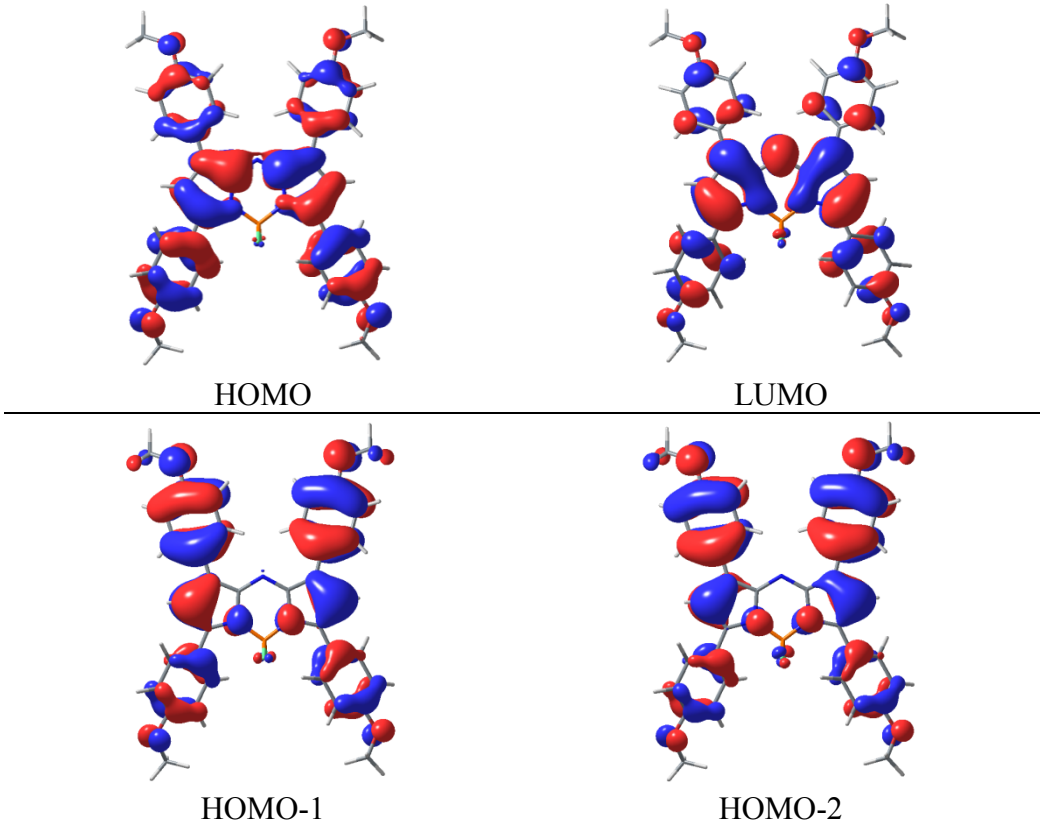


Figure S5. Computed molecular occupied and virtual orbitals for the model aza-BODIPY obtained at the PCM(H_2O)-TD-PBE0/6-311G+(2d,p) level using an isosurface value of 0.02.

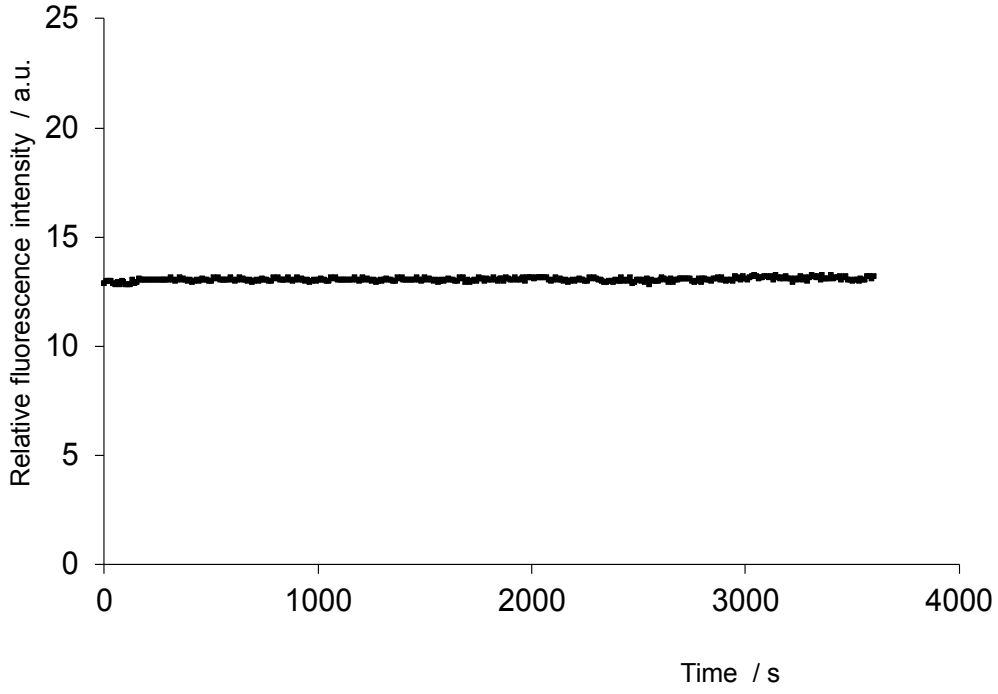


Figure S6. Plot of change in fluorescence emission of solution of aza-BODIPY **2** in PBS at 720 nm *versus* irradiation time (absorbance 0.1, λ_{exc} = 680 nm, 8 W, 20 nm/5 nm excitation/emission slit widths).

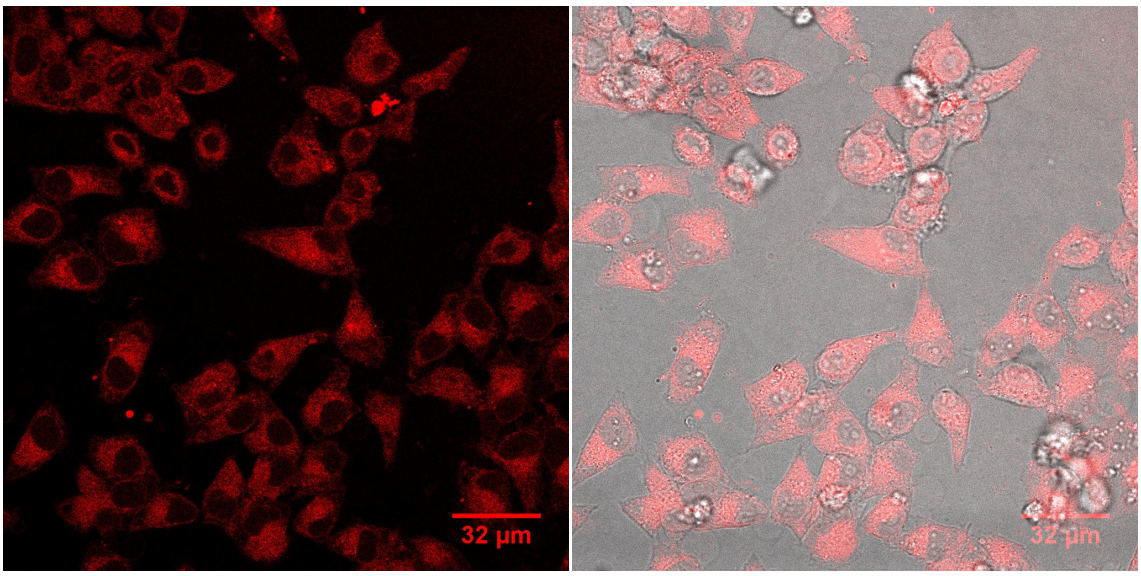


Figure S7. Lower magnification confocal fluorescence images of HeLa cells with aza-BODIPY **2** (6 µM) following 1 h incubation, washing, and fixation (laser excitation λ = 633 nm).

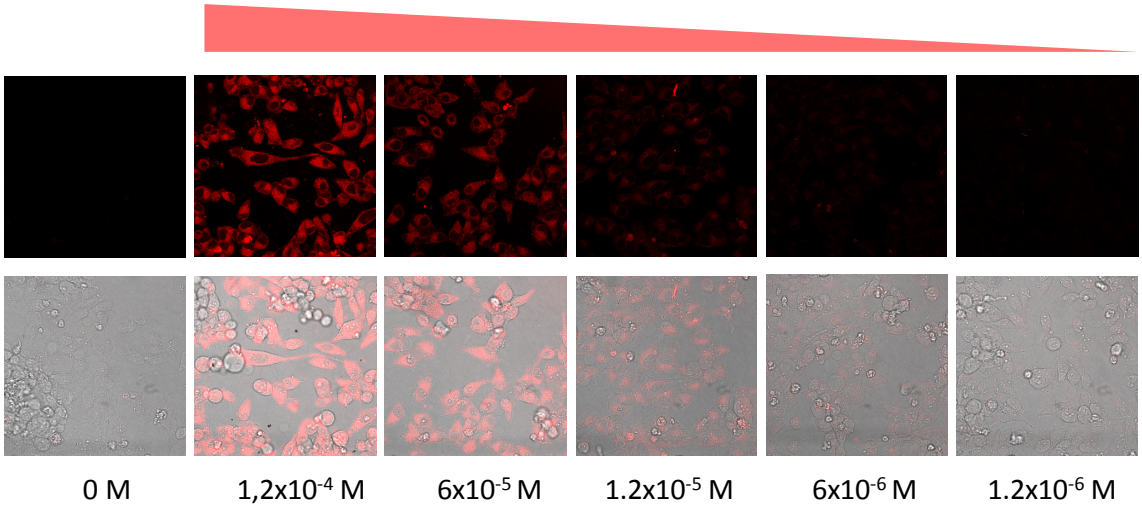


Figure S8. Confocal fluorescence images of HeLa cells with aza-BODIPY **2** following 1 h incubation at different concentration, washing, and fixation.

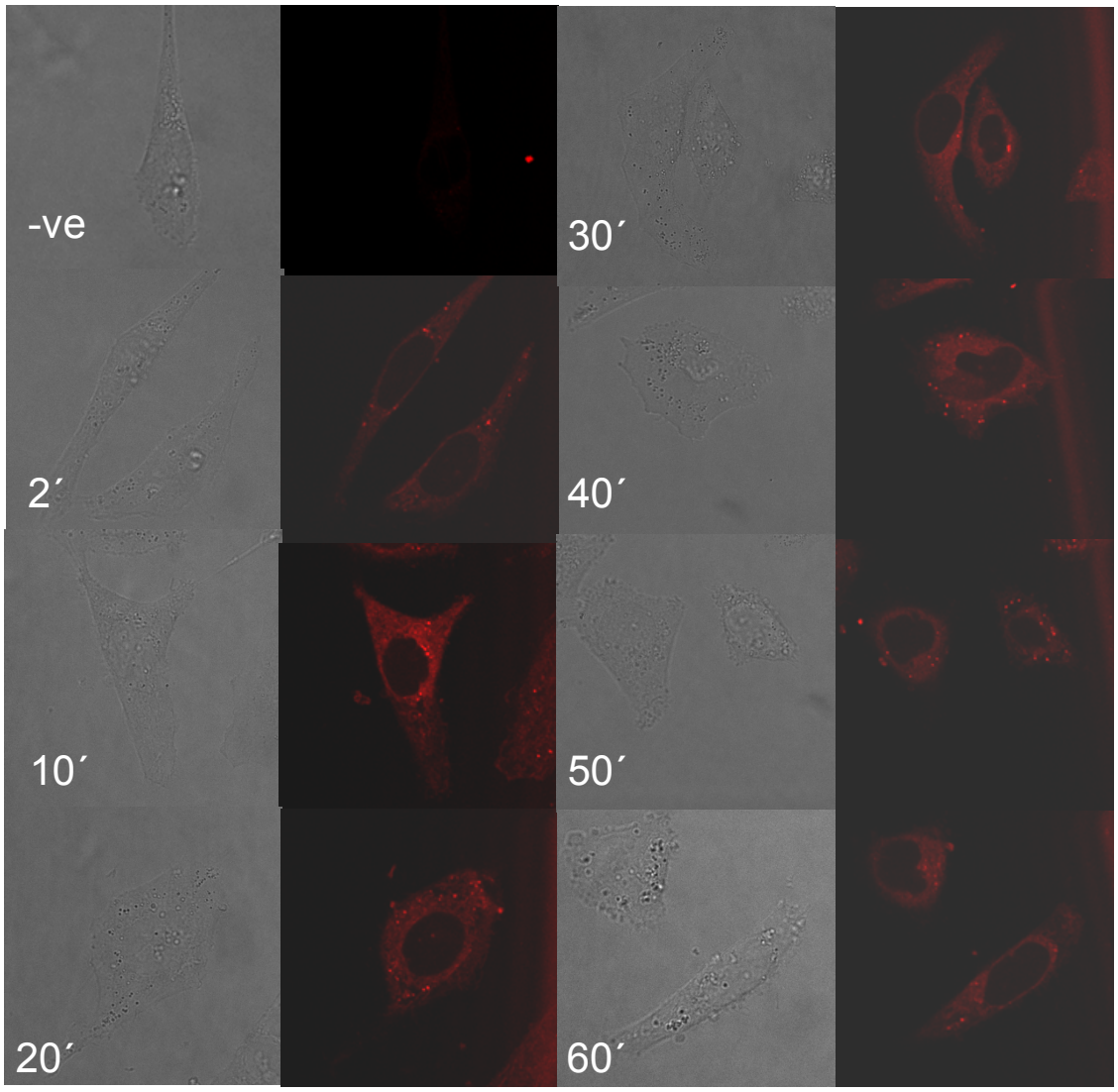


Figure S9. Confocal fluorescence images of HeLa cells with aza-BODIPY **2** at different incubation times at $6x10^{-5}$M, washing, and fixation.

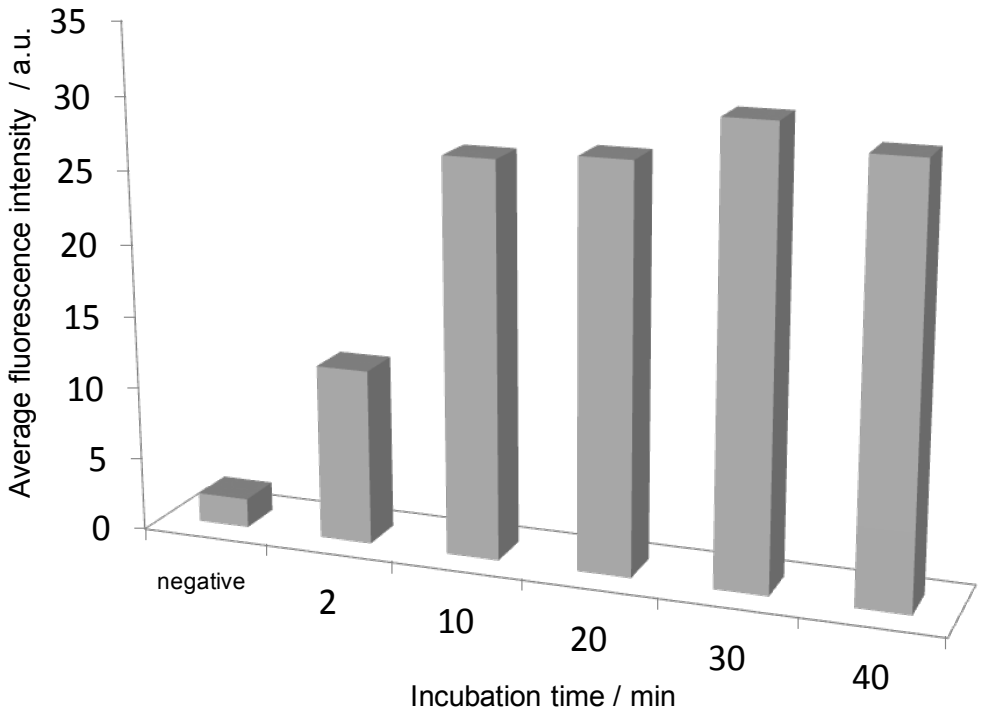


Figure S10. Average of fluorescence emission intensity obtained from confocal microscopy images following different aza-BODIPY **2** incubation times ($6x10^{-5}$M).

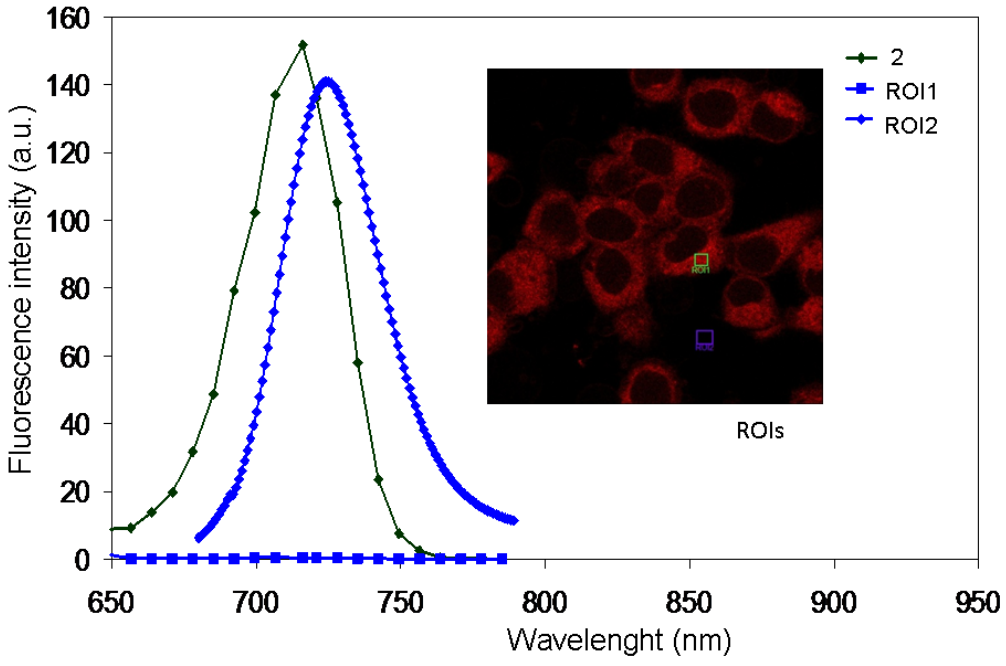


Figure S11. Localized emission spectrum of cytoplasmic HeLa cell fluorescence treated with aza-BODIPY **2** (ROI1) compared to fluorescence emission in PBS solution of compound **2** and background fluorescence (ROI2).

Table S1. Total energies and singlet-triplet energetic gaps (ΔE) for the model aza-BODIPY at PCM(H_2O)-TD-UPBE0/6-311+G(2d,p) level.

Electronic state	Total energy (hartrees)	ΔE (eV)
^1A	-2077.9907	-
^3A	-2077.9634	0.74

Geometry: the computed structural parameters in water solution for both ground (S_0) and excited state (S_1) geometries of the model aza-BODIPY are represented in Table S2. The bond lengths and plane angles slightly change in the excited state S_1. The main modifications involve the dihedral angles (ϕ_1, ϕ_2, ϕ_3, ϕ_4) describing the spatial orientation of the phenyl rings relative to the aza-BODIPY core. In the state S_1 these angles are closed to 180º and the molecule is then more planar.

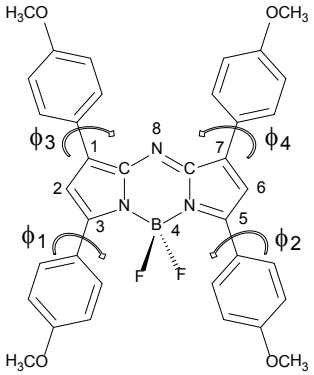


Table S2. Selected geometric parameters for PCM(H_2O)-(TD)-PBE0 optimized geometric structures of ground (S_0) and excited (S_1) states of the model aza-BODIPY.

	S_0	S_1
bond lengths [Å]		
B-F	1.392	1.400
B-N	1.554	1.546
N^{4a}-C^{7a}	1.388	1.370
C^{7a}-N^8	1.313	1.328
C-C_{Phenyl}	1.455	1.445
C_{Phenyl}-O	1.346	1.344
valence angles [º]		
B-N^{4a}-C^{7a}	121.9	121.6
F-B-F	110.3	109.6
C^{7a}-N^8-C^{8a}	120.4	118.9
torsion angles [º]		
ϕ_1	149.8	156.7
ϕ_2	149.8	156.7
ϕ_3	156.0	163.1
ϕ_4	156.0	163.1

Table S3. Selected excitation energies (ΔE), oscillator strengths (f) and transition coefficients computed, at PCM(H_2O)-TD-PBE0/6-311+G(2d,p) level, for the model aza-BODIPY. All electronic states belong to ^1A.

Excited state	Transition character	Weight	ΔE (eV)	f
1	HOMO→LUMO	0.707	1.91	0.858
2	HOMO-1→LUMO	0.698	2.37	0.015
3	HOMO-2→LUMO	0.702	2.44	0.605
Exp. **2** (in H_2O)			1.76	

^{1}H and ^{13}C NMR spectra

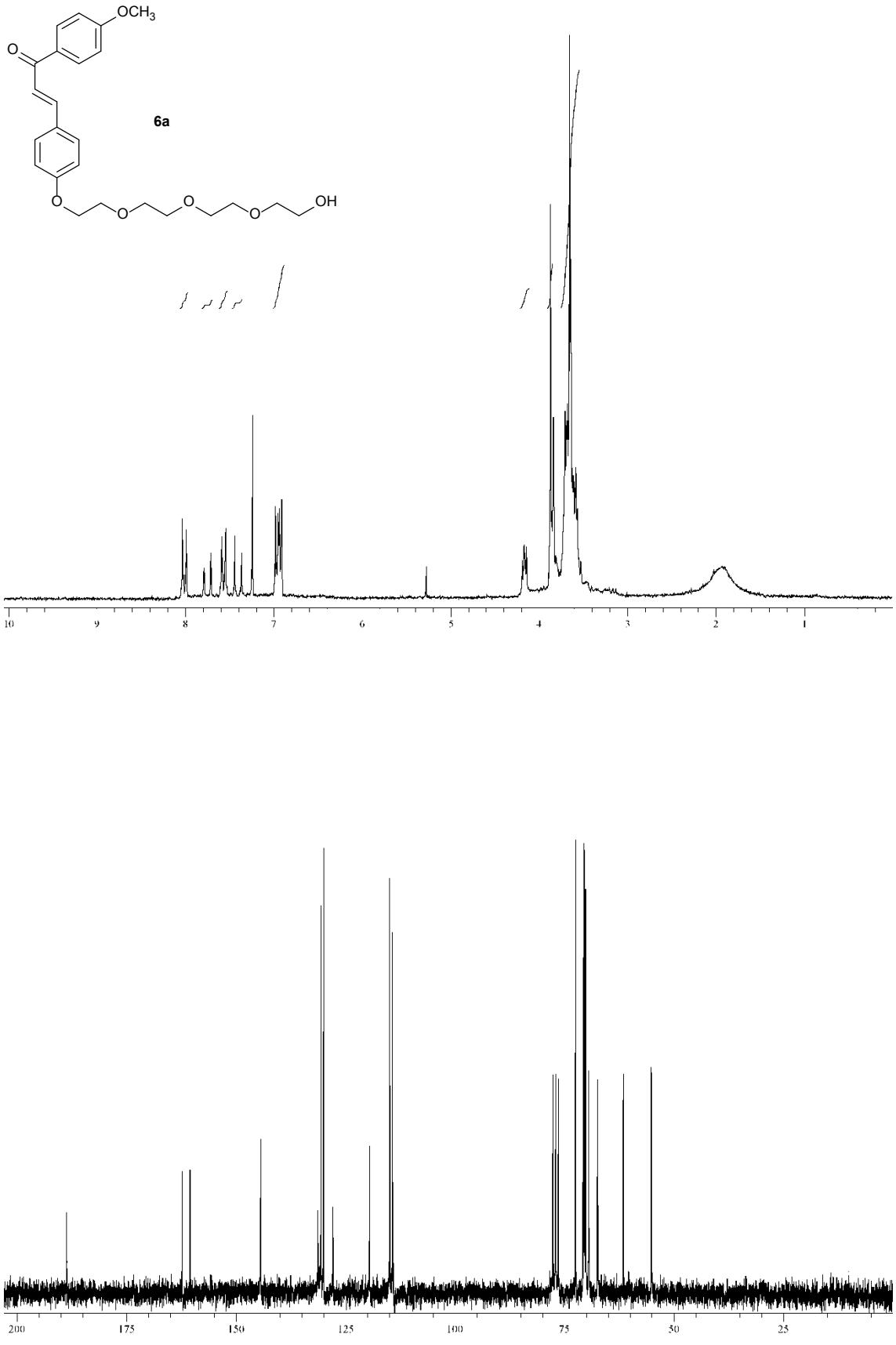


Figure S1. ^{1}H (top) and ^{13}C NMR (bottom) spectra of **6a** in $CDCl_3$.

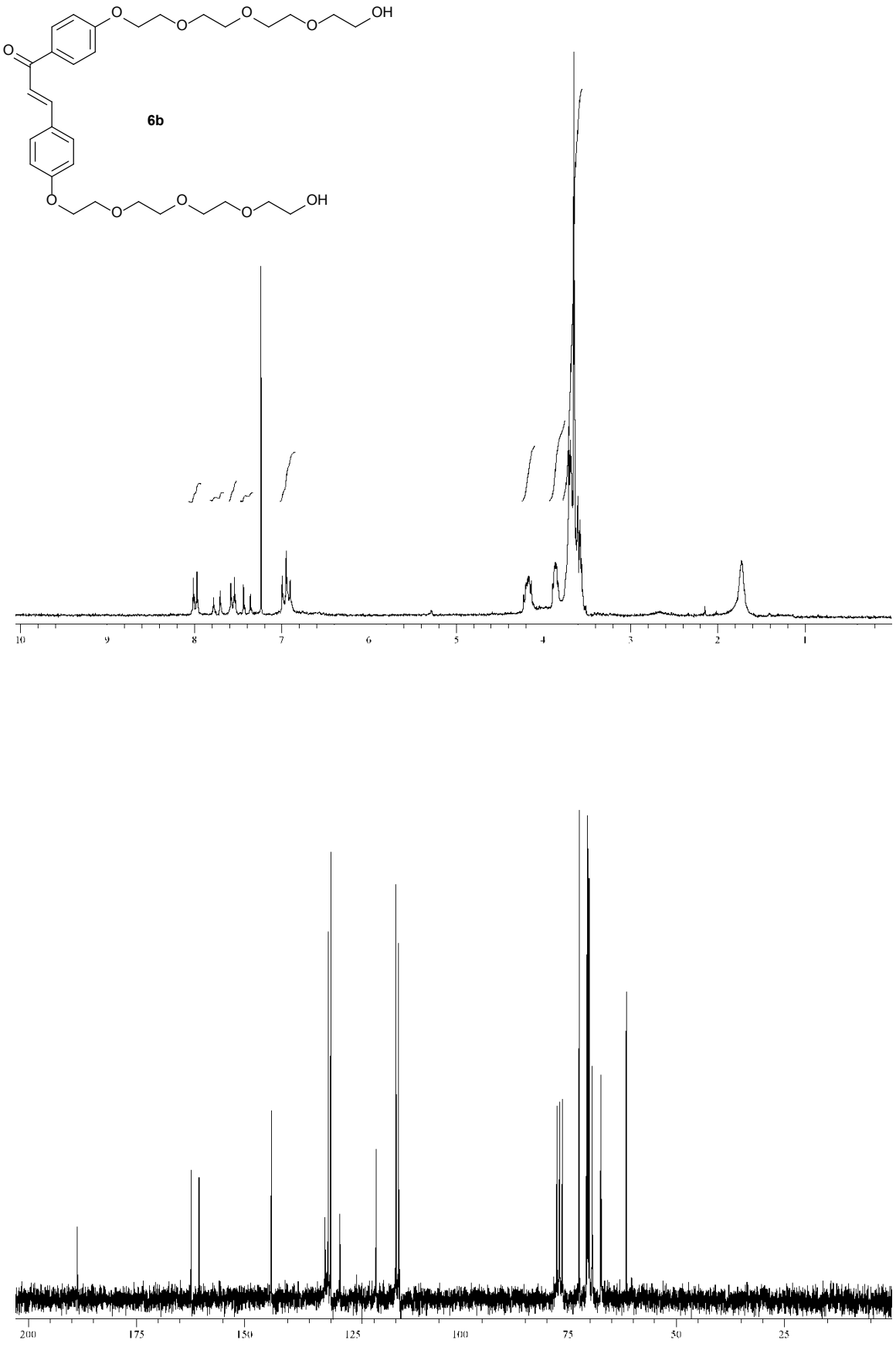


Figure S2. ^{1}H (top) and ^{13}C NMR (bottom) spectra of **6b** in $CDCl_3$.

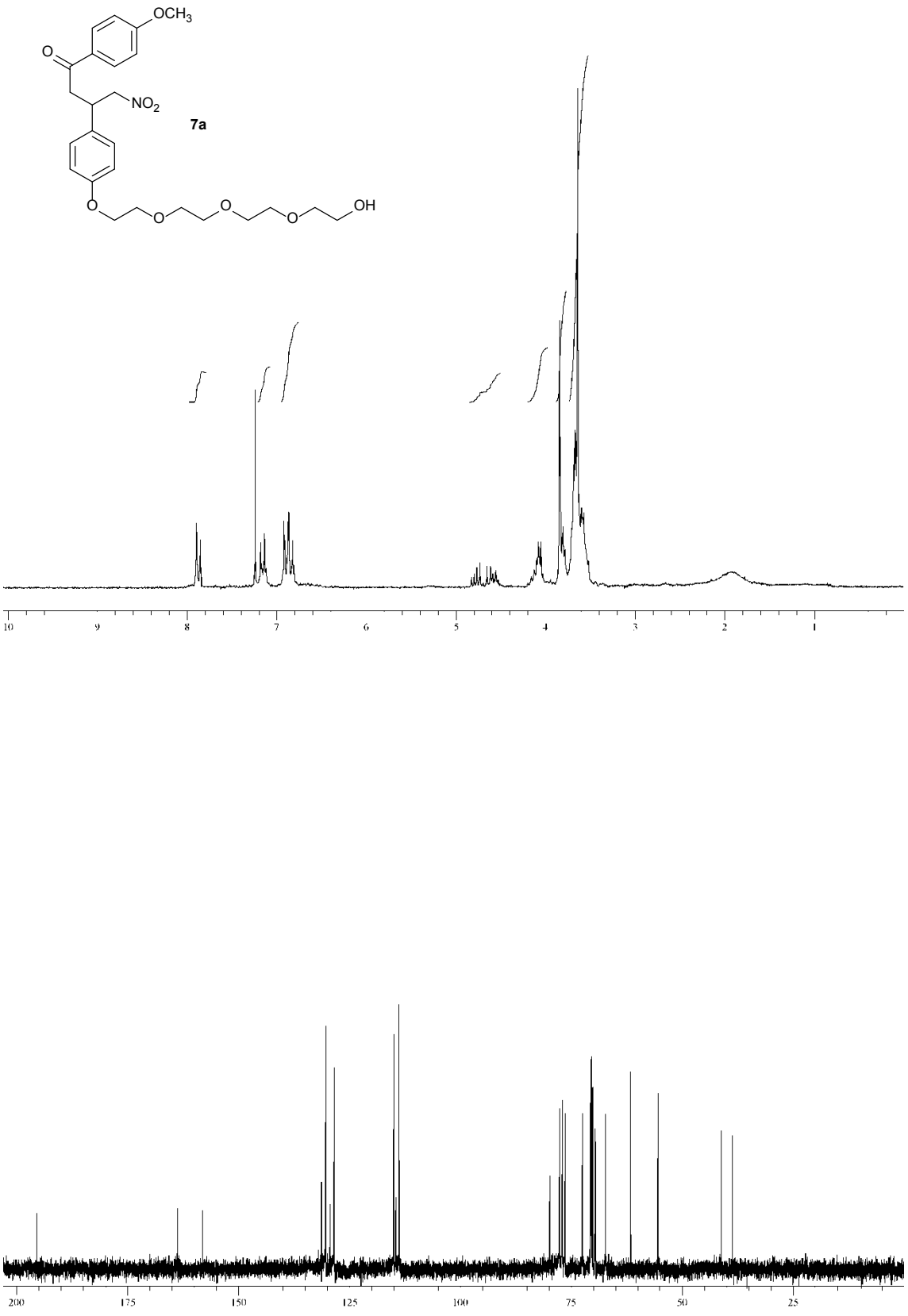


Figure S3. ^{1}H (top) and ^{13}C NMR (bottom) spectra of **7a** in $CDCl_3$.

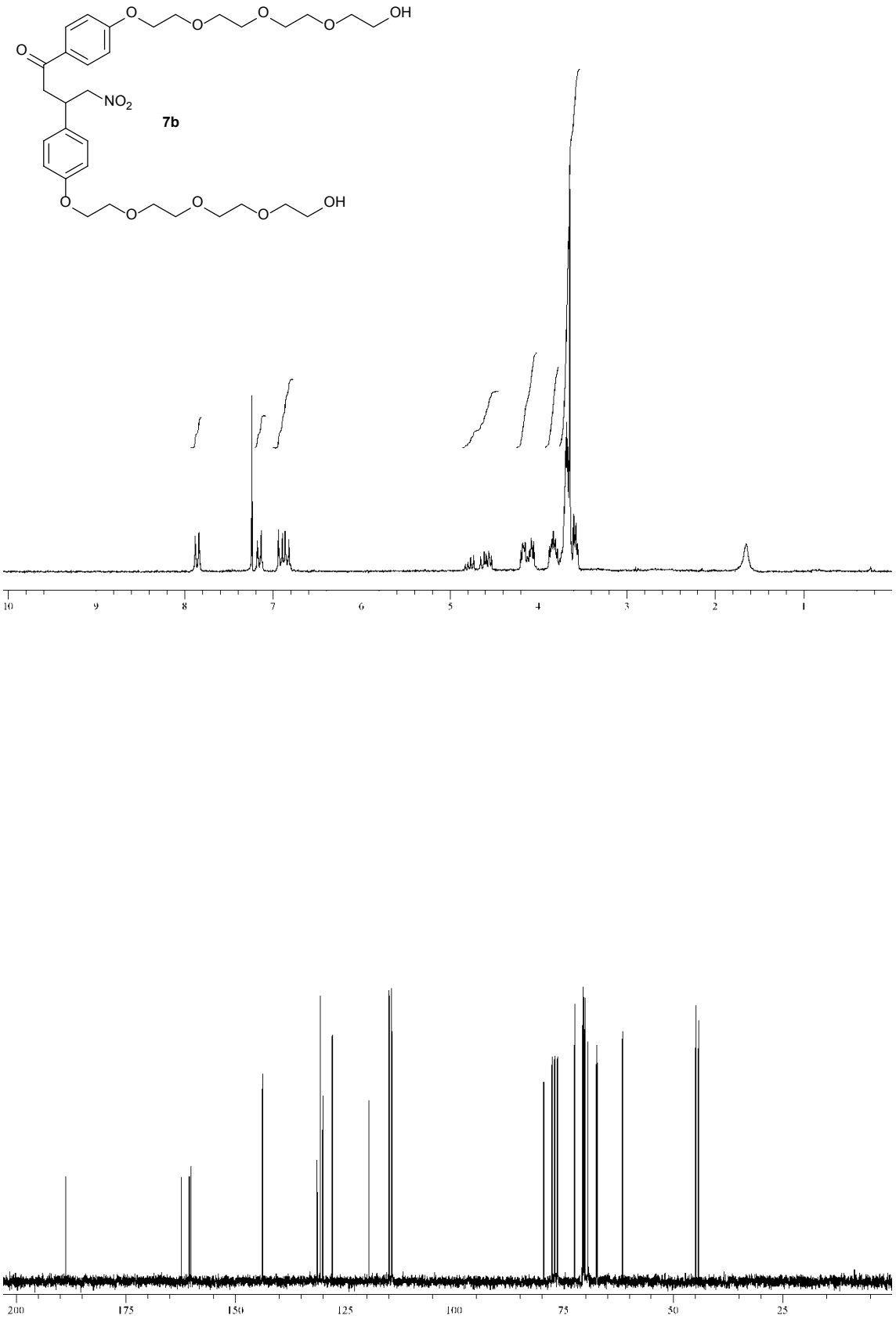


Figure S4. ^{1}H (top) and ^{13}C NMR (bottom) spectra of **7b** in $CDCl_3$.

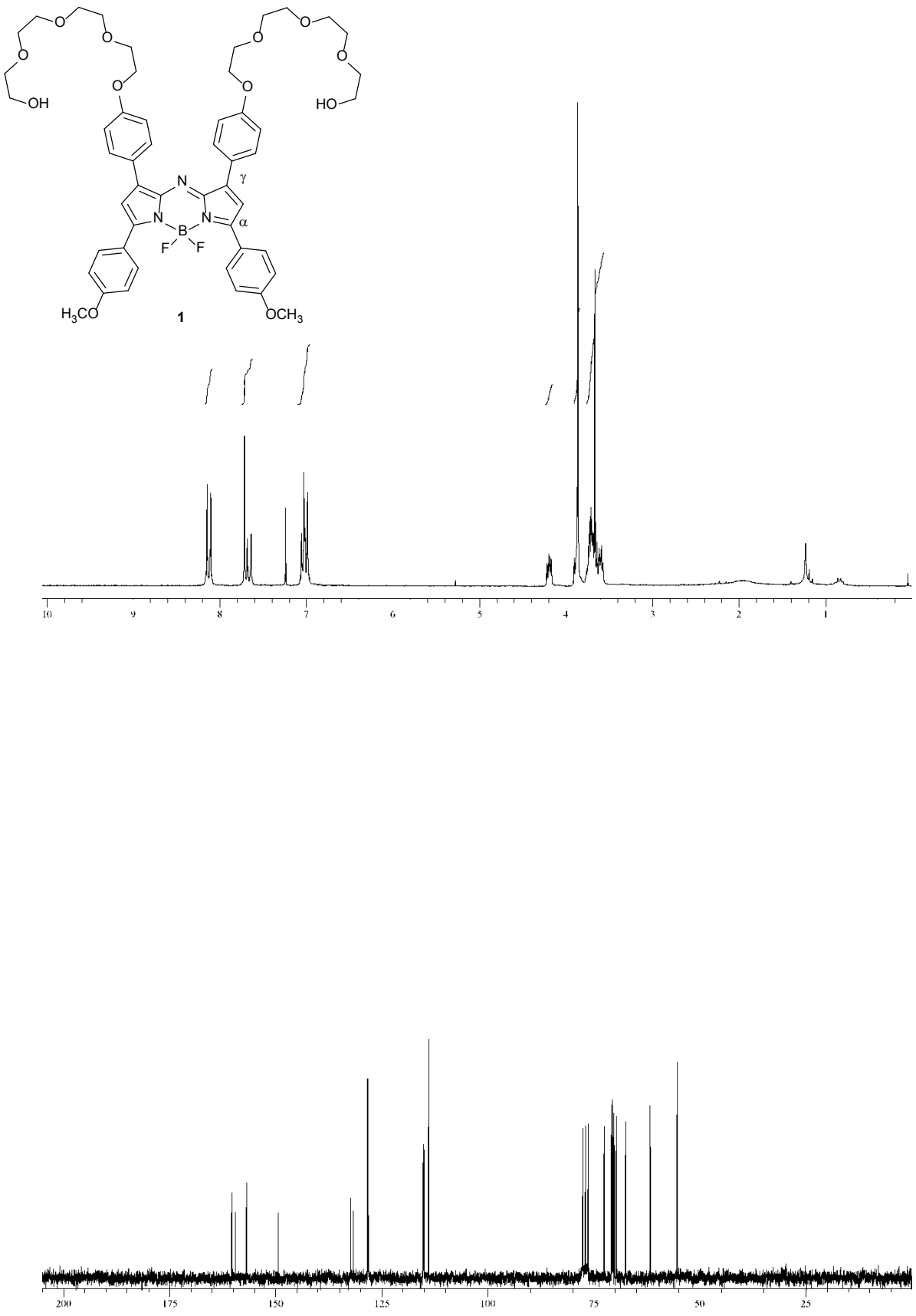


Figure S5. ^{1}H (top) and ^{13}C NMR (bottom) spectra of **1** in $CDCl_3$.

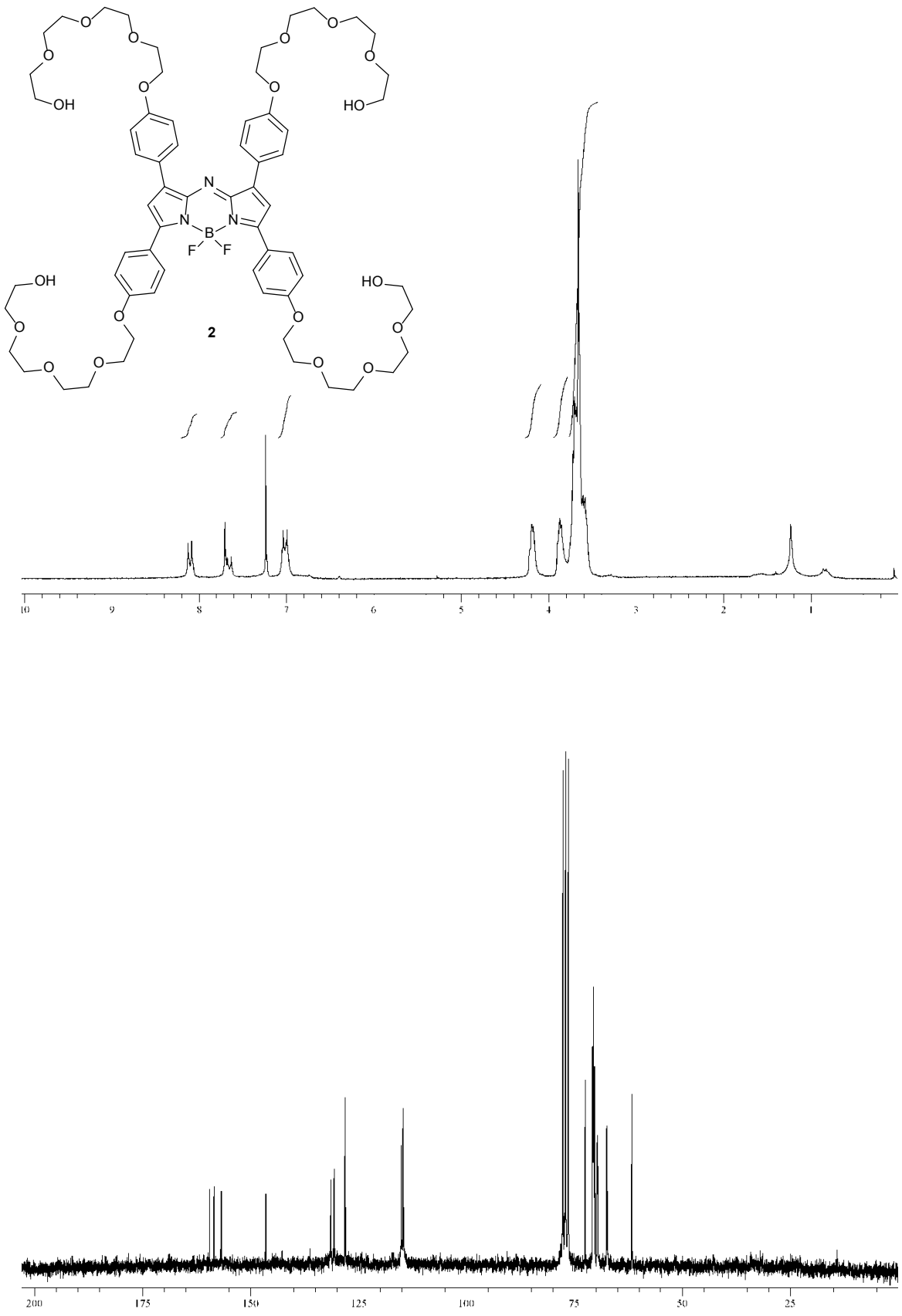


Figure S6. ^{1}H (top) and ^{13}C NMR (bottom) spectra of **2** in $CDCl_3$.

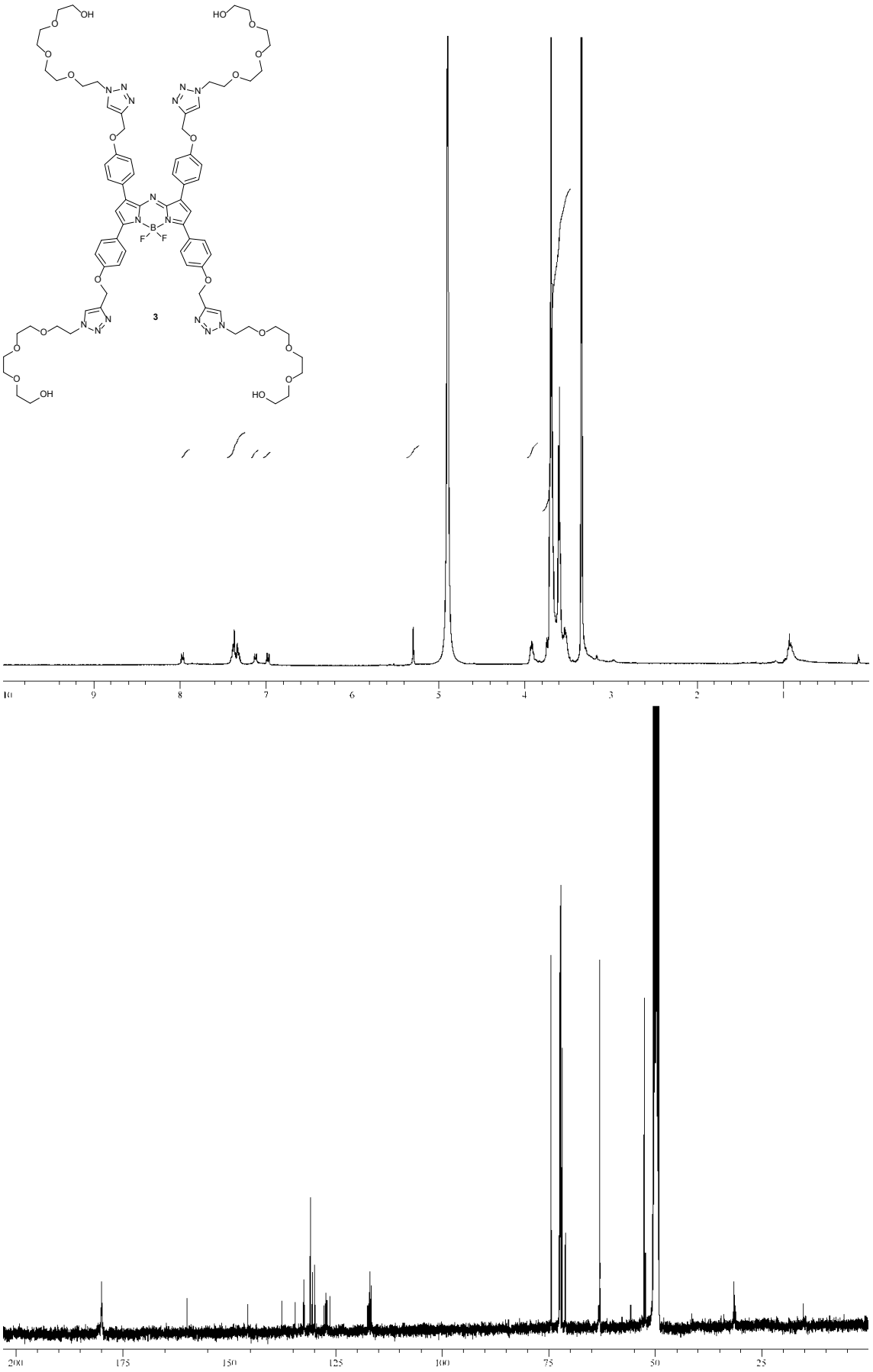


Figure S7. ^{1}H (top) and ^{13}C NMR (bottom) spectra of **3** in CD_3OD.

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