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A novel water-soluble BODIPY dye as red fluorescent probe for imaging hypoxic status of human cancer cells

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Materials and chemicals

Unless otherwise noted, all commercially available reagents and solvents were used as received and without further purification. TLC was carried out on Merck Millipore DC Kieselgel 60 F-254 aluminum sheets. The spots were visualized directly or through illumination with dual wavelength UV lamp (λ = 254 and 365 nm). Column chromatography was performed using silica gel, purchased from Sigma-Aldrich (technical grade, 63–200 μm) or Merck Millipore (Geduran® Si 60, 40–63 μm), for purification of **NBB** probe. Toluene (HPLC-grade) and CH_2Cl_2 (HPLC-grade) were dried over alumina cartridges immediately before use, by means of solvent purification system PureSolv PS-MD-5 model from Innovative Technology. Anhydrous DMF was purchased from Carlo Erba and stored over 3 Å molecular sieves. Piperidine and TFA was provided by Iris Biotech GmbH. The HPLC-gradient grade CH_3CN used for HPLC-MS analyses was obtained from Carlo Erba or VWR. Formic acid (FA, puriss p.a., ACS reagent, reag. Ph. Eur., ≥98%). NTR and NADH were provided by Sigma-Aldrich. PBS used in this work and aq. mobile phase for HPLC were prepared using water purified with a PURELAB Ultra system from ELGA (purified to 18.2 MΩ cm).

Instruments and methods

^{1}H, ^{11}B, ^{13}C and ^{19}F NMR spectra were recorded on a Bruker Avance NEO 500 MHz spectrometer equipped with a 5 mm BBOF iProbe (Bruker, Wissembourg, France). Chemical shifts are given in parts per million (ppm) using the residual non-deuterated solvent peak as a reference.[S1] Splitting patterns are designated as s for singlet, d for doublet, t for triplet, q for quartet, m for multiplet, dd for doublet of doublet and ddd for doublet of doublets of doublets. Coupling constants (*J*) are expressed in Hertz. 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. The following chromatographic systems were used for the analytical experiments: <u>*System A*</u>: RP-HPLC (Phenomenex Kinetex C_{18} column, 2.6 μm, 2.1 × 50 mm) with CH_3CN (+ 0.1% FA) and 0.1% aq. formic acid (aq. FA, pH 2.5) as eluents [5% CH_3CN (0.1 min) followed by a linear gradient from 5% to 100% (5 min) of CH_3CN, then 100% CH_3CN (1.5 min)] at a flow rate of 0.5 mL/min. UV-visible detection was achieved at four distinct wavelengths of 220, 260, 500 and 670 nm (+ diode array detection in the range 220–800 nm). Low resolution ESI-MS detection in the positive/negative mode (full scan, 100–2000 a.m.u., peaking format: centroid, needle voltage: 3.0 kV, probe temperature: 350 °C, cone voltage: 75 V, detector voltage: 1153 V and scan time: 1 s). Low-resolution mass spectra

(LRMS) were recorded on this Thermo Scientific MSQ Plus single quadrupole spectrometer equipped with an electrospray (ESI) source (HPLC-MS coupling mode). High-resolution mass spectra (HRMS) were obtained on Bruker MicroTOF-Q spectrometer equipped with an ESI source. Melting points (mp) were measured using a Boetius capillary melting point apparatus and are uncorrected. UV–VIS absorption spectra and steady-state fluorescence emission spectra for photophysical properties were recorded with a FLUORAT-02-Panorama spectrofluorometer (Lumex Instruments, Russia) using a rectangular quartz cell (KU-1, KV, Pushchino' Optical Plant, Russia, 45×12.5×12.5 mm, path length: 10 mm, chamber volume: 3.5 mL). Fluorescence spectroscopic studies with NTR (emission/excitation spectra and kinetics) were performed with an HORIBA Jobin Yvon Fluorolog spectrophotometer (software: FluoEssence) with a standard fluorometer cell (Labbox, LB Q, 10 mm).

Abbreviations

The following abbreviations are used throughout the text: CH_2Cl_2, dichloromethane; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DMF, *N*,*N*-dimethylformamide; EtOAc, ethyl acetate; HPLC-MS, high-performance liquid chromatography coupled to mass spectrometry; MeOH, methanol; NADH, nicotinamide adenine dinucleotide; p-NB, *para*-nitrobenzyl; PB, phosphate buffer; PBS, phosphate buffered saline; ; TFA, trifluoroacetic acid; FA, formic acid; TsOH, *p*-toluenesulfonic acid; rt, room temperature; TLC, thin layer chromatography.

Synthesis of NBB probe

4-(4-Bromobutoxy)benzaldehyde ***2*** *[72621-19-3].* 4-Hydroxybenzaldehyde (1.2 g, 10.0 mmol, 1.0 equiv.), anhydrous K_2CO_3 (2.1 g, 15.0 mmol, 1.5 equiv.) and 1,4-dibromobutane (4.3 g, 20.0 mmol, 2.0 equiv.) were dissolved in dry DMF (20 mL). The resulting reaction mixture was stirred at rt for 24 h. Thereafter the mixture was filtered over cotton. The filtrate was diluted with EtOAc (50 mL) and washed with deionized water (30 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting residue was purified by column chromatography over silica gel [eluent: pentane–EtOAc, (10 : 1 v/v)] to provide benzaldehyde **2** as colorless crystals (1.14 g, yield 44%), mp 44–43 °C (literature 43 °C)[S2]; ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.90 (s, 1H, C*H*O), 7.86 (d, *J* = 8.4 Hz, 2H, H-Ar), 7.01 (d, *J* = 8.4 Hz, 2H, H-Ar), 4.07 (t, *J* = 6.8 Hz, 2H, -C*H*$_2$O), 3.51 (t, *J* = 6.8 Hz, 2H, -C*H*$_2$Br), 2.12–2.04 (m, 2H, -C*H*$_2$-), 2.03–1.95 (m, 2H, -C*H*$_2$-); LRMS (ESI+, HPLC-MS coupling): *m/z* = 258.2 $[M + H]^+$, calcd for $C_{11}H_{14}BrO_2^+$ 258.1 and 256.1. All other spectroscopic data are identical to those reported by Sreenath *et al*.[S3]

*2,8-Diethyl-1,3,7,9-tetramethyl-9-(4-hydroxyphenyl)-BODIPY dye **3** [1230026-26-2]*. 3-Ethyl-2,4-dimethylpyrrole (also known as kryptopyrrole, 2.7 mL, 20.0 mmol, 2.7 equiv.) and 4-hydroxybenzaldehyde (1.0 g, 8.0 mmol, 1.0 equiv.) were dissolved in dry CH_2Cl_2 (600 mL). TFA (50 μL, 0.7 mmol, 10 mol %) was added and the resulting reaction mixture was stirred at rt for 50 min. Then, DDQ (1.9 g, 8.0 mmol, 1.0 equiv.) was added and the mixture was stirred for further 50 min followed by the addition of Et_3N (16 mL, 120.0 mmol, 15.0 equiv.). The mixture was stirred for 30 min. Then, $BF_3{\cdot}OEt_2$ (16 mL, 128.0 mmol, 15.0 equiv.) was added, and the mixture was stirred for 10 h at rt. Thereafter, the mixture was sequentially washed with 1.0 M aq. $KHSO_4$ (3 × 200 mL) and deionized water, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by column chromatography over silica gel (eluent: step gradient of EtOAc in pentane from 9% to 17%). The phenol-based BODIPY dye was obtained as a red amorphous powder (763 mg, yield 22%). R_f [pentane–EtOAc (5 : 1 v/v)] = 0.55; mp 288–290 °C (literature 290 °C) [S4]; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.04 (d, J = 8.5 Hz, 2H, H-Ar), 6.87 (d, J = 8.5 Hz, 2H, H-Ar), 2.45 (s, 6H, 2 × C$\underline{H}_3$), 2.23 (q, J = 7.6 Hz, 4H, 2 × C$\underline{H}_2$), 1.27 (s, 6H, 2 × C$\underline{H}_3$), 0.91 (t, J = 7.6 Hz, 6H, 2 × C$\underline{H}_3$), signal of the phenolic proton was not observed; LRMS (ESI+, LC-MS coupling): *m/z* = 377.2 $[M - F]^+$ (100) and 397.2 $[M + H]^+$ (40), calcd for $C_{23}H_{28}BF_2N_2O^+$ 397.3; LRMS (ESI-, LC-MS coupling): *m/z* = 395.1 $[M - H]^-$ (100), calcd for $C_{23}H_{26}BF_2N_2O^-$ 397.2. All other spectroscopic data are identical to those reported by Dumas-Verdes *et al.*[S4]

*Distyryl phenol-based BODIPY dye **4**.* 4-(4-Bromobutoxy)benzaldehyde **2** (648 mg, 2.5 mmol, 5.0 equiv.), piperidine (1 mL), and TsOH (10 mol %) were sequentially added to a stirred solution of the *meso*-(4-hydroxyphenyl)-BODIPY dye (200 mg, 0.5 mmol, 1.0 equiv.) in dry toluene (60 mL). The resulting mixture was concentrated to dryness through heating to boiling point without condenser. Then, further amount of dry toluene (10 mL) was added and the mixture was again heated to boiling point without condenser. Thereafter, the resulting solid was dissolved in CH_2Cl_2, sequentially washed with 1.0 M aq. $KHSO_4$ (3 × 10 mL) and deionized water (2 × 10 mL), and finally dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure. The resulting residue was purified by column chromatography over silica gel [eluent: DCM–MeOH (100 : 1 v/v)] to give the targeted distyryl phenol-based BODIPY dye as a deep-blue amorphous powder (30 mg, yield 2%). R_f [CH_2Cl_2–MeOH (100 : 1 v/v)] = 0.23; mp 281–283 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.59 (d, J = 8.8 Hz, 4H, H-Ar), 7.48 (d, J = 7.3 Hz, 2H, H-Ar), 7.27 (d, J= 7.3 Hz, 2H, H-Ar), 7.16 (d, J = 8.4 Hz, 2H, 2 × C$\underline{H}$=), 7.04 (d, J = 8.8 Hz, 4H, H-Ar), 6.96 (d, J = 8.4 Hz, 2H, 2 × C$\underline{H}$=), 4.12–4.08 (m, 4H, 2 × C$\underline{H}_2$), 3.09–3.13 (m, 5H), 2.88 (q, J = 7.0 Hz, 4H, 2 × C$\underline{H}_2$), 2.68–2.56 (m, 4H, 2 × C$\underline{H}_2$), 1.88–1.60 (m, 23H, 14 ×

C<u>*H*</u>$_2$), 1.42 (s, 6H, 2 × C<u>*H*</u>$_3$), 1.05 (t, J = 7.0 Hz, 6H, 2 × C<u>*H*</u>$_3$), signal of the phenolic proton was not observed; ^{13}C NMR (126 MHz, DMSO-d_6): δ 159.8 (2 × C), 158.6 (C), 158.3 (2 × C), 149.8 (C), 139.7 (4 × CH), 139.4 (2 × C), 135.7 (C), 133.2 (2 × CH), 130.0 (2 × CH), 129.0 (2 × CH), 125.6 (2 × C), 122.4(4 × CH), 118.3 (2 × C), 117.5 (2 × C), 115.5 (2 × CH), 56.5 (2 × CH_2), 56.0 (4 × CH_2), 52.5 (2 × CH_2), 40.5 (2 × CH_2), 36.9 (2 × CH_2), 26.2 (2 × CH_2), 23.0 (2 × CH_2), 21.8 (2 × CH_2), 20.8 (2 × CH_2), 14.4 (2 × CH_3), 11.9 (2 × CH_3); ^{11}B NMR (160 MHz, DMSO-d_6): δ 1.01 (t, $J_{B\text{-}F}$ = 67.5 Hz, 1B, <u>*B*</u>F$_2$); ^{19}F NMR (470 MHz, DMSO-d_6): δ -136.69 (q, $J_{F\text{-}B}$ = 67.5 Hz, 2F, B<u>*F*</u>$_2$); HPLC (system A): t_R = 4.8 min (purity 91% at 260 nm and >99% at 670 nm); LRMS (ESI+, HPLC-MS coupling): *m/z* = 442.4 $[M + 2H]^{2+}$ (100) and 883.6 $[M + H]^+$ (45), calcd. for $C_{55}H_{70}BF_2N_4O_3^+$ 883.7.

*NTR-responsive BODIPY-based **NBB** probe.* Distyryl *meso*-(4-hydroxyphenyl)-BODIPY dye **4** (15 mg, 0.014 mmol, 1.0 equiv.) and Et_3N (7 μL, 0.05 mmol, 3.0 equiv) were dissolved in dry CH_2Cl_2 (5 mL) and the solution was cooled to 0 °C with an ice-water bath. Then, solid 4-nitrobenzyl chloroformate (11 mg, 0.05 mmol, 3.0 equiv.) was added and the resulting reaction mixture was stirred at 0 °C for 30 min. Thereafter, the mixture was diluted with CH_2Cl_2 (10 mL), sequentially washed with 1 M aq. $KHSO_4$ (10 mL), brine (10 mL), and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and the resulting residue was purified by column chromatography over silica gel (eluent: step gradient of MeOH in DCM from 1% to 9%) to give **NBB** probe as a deep-blue amorphous powder (5 mg, yield 40%), mp 277–279 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.21 (d, J = 8.8 Hz, 2H, H-Ar), 7.62 (d, J = 8.8 Hz, 2H, H-Ar), 7.52 (d, J = 7.2 Hz, 2H, H-Ar), 7.48 (d, J = 8.7 Hz, 4H, H-Ar), 7.24 (d, J = 8.7 Hz, 2H, 2 × C<u>*H*</u>=), 7.18 (d, J = 7.2 Hz, 2H, H-Ar), 6.91 (d, J = 8.7 Hz, 4H, H-Ar), 6.82 (d, J = 8.8 Hz, 2H, 2 × C<u>*H*</u>=), 5.34(s, 2H, C<u>*H*</u>$_2$-pNB), 4.08–4.02 (m, 4H, 2 × C<u>*H*</u>$_2$),3.11–3.02 (m, 4H, 2 × C<u>*H*</u>$_2$), 2.85 (q, J = 7.0, 4H, 2 × C*H*$_2$), 1.92–1.61 (m, 28H, 14 × C<u>*H*</u>$_2$), 1.33 (s, 6H, 2 × C<u>*H*</u>$_3$), 1.05 (s, J = 7.0 Hz, 6H, 2 × C<u>*H*</u>$_3$); ^{13}C NMR (126 MHz, DMSO-d_6): δ 159.9 (C=O), 158.4 (C), 158.2 (2 × C), 156.2 (C), 154.5 (2 × C) 152.8 (C), 152.3 (C), 147.9 (C), 143.1 (2 × C), 139.2 (2 × C), 137.8 (4 × CH), 136.1 (2 × CH), 129.9 (2 × CH), 129.5 (2 × CH), 129.2 (2 × C), 125.1 (2 × CH), 124.2 (2 × C), 122.8 (4 × CH), 118.5 (2 × CH), 115.6 (2 × CH), 69.1 (CH_2), 56.6 (2 × CH_2), 56.0 (4 × CH_2), 52.6 (2 × CH_2), 40.6 (2 × CH_2), 37.0 (2 × CH_2), 26.3 (2 × CH_2), 23.0 (2 × CH_2), 21.8 (2 × CH_2), 20.8 (2 × CH_2), 14.4 (2 × CH_3), 11.8 (2 × CH_3); ^{11}B NMR (160 MHz, DMSO-d_6): δ 1.07 (t, $J_{B\text{-}F}$ = 66.8 Hz, 1B, <u>*B*</u>F$_2$); ^{19}F NMR (470 MHz, DMSO-d_6): δ -136.74 (q, J = 66.8 Hz, 2F, B<u>*F*</u>$_2$); HPLC (system A): t_R = 5.2 min (purity 96% at 260 nm and 100% at 670 nm); LRMS (ESI+, HPLC-MS coupling): *m/z* = 531.8 $[M + 2H]^{2+}$, calcd for $C_{63}H_{75}BF_2N_5O_7^+$1062.6; HRMS (ESI-Q-TOF, positive mode): calcd. for ($C_{63}H_{76}BF_2N_5O_7^{2+}$) 531.8085; found 531.7872 $[M+ 2H]^{2+}$.

UV–VIS absorption and Fluorescence measurements

UV–VIS absorption spectra and steady-state fluorescence emission spectra were recorded using 3 mL quartz cuvettes with a path length of 1 cm. The relative fluorescence quantum yield of **NBB** was determined at 25 °C, using sulforhodamine 101 (Φ_F = 95% in EtOH) as a standard[S5] and the following equation:

$$\Phi_F(x) = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2 \Phi_F(s)$$

where A is the absorbance (in the range of 0.01–0.1 A.U.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in the measurements, and the subscripts s and x represent the standard and unknown, respectively. The following refractive index values were used: 1.361 for EtOH and 1.346 for water–DMSO mixture (9:1 v/v).[S6]

Detection of NTR through* in vitro *fluorescence-based assay

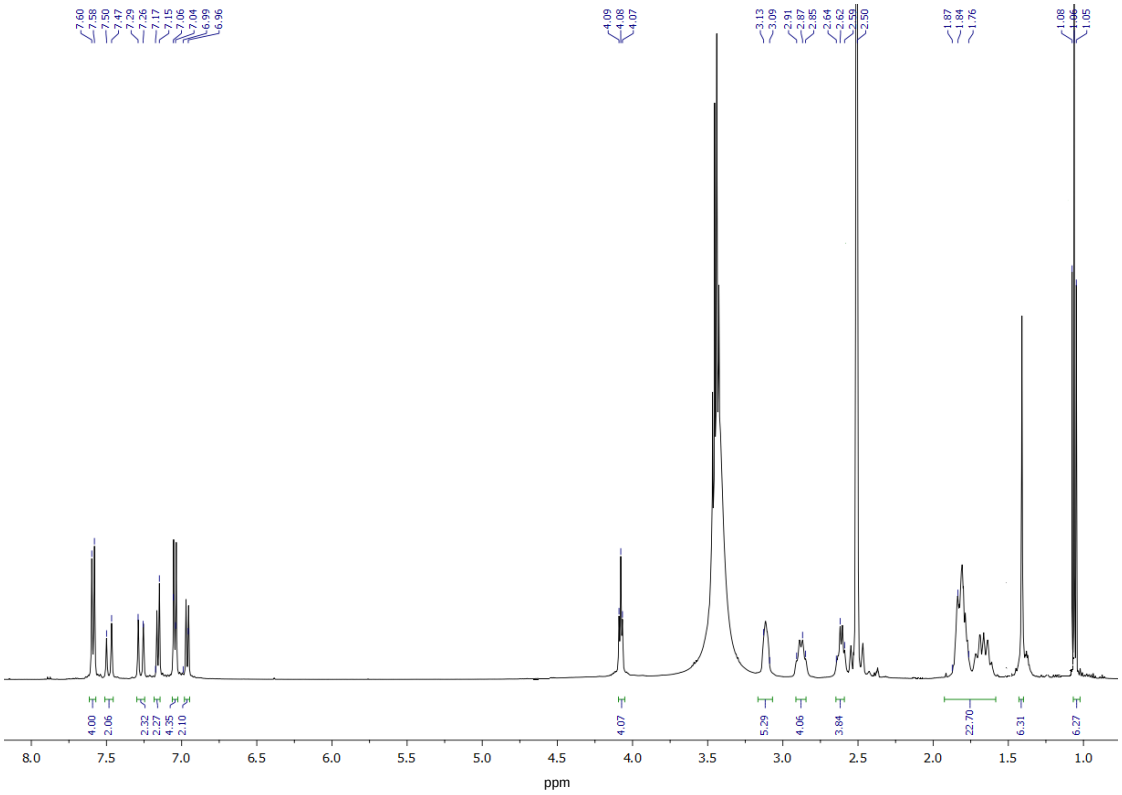
Freeze-dried sample of commercial nitroreductase from *E. coli* (NTR, Sigma-Aldrich, #N9284, 0.1 U/μg) was dissolved in ultrapure water containing NADH (140 mM final concentration) to the final nitroreductase concentration of 1 mg/mL. A probe solution (2 μL) was dissolved in DMSO (final concentration: 1 μM) and added to PBS (100 mM, pH 7.4, 2997 μL) followed by the addition of NTR/NADH solution (1 μL). This solution was incubated at 37 °C for the indicated time periods.

Real-time monitoring of NTR in the A549 lung cancer cell line

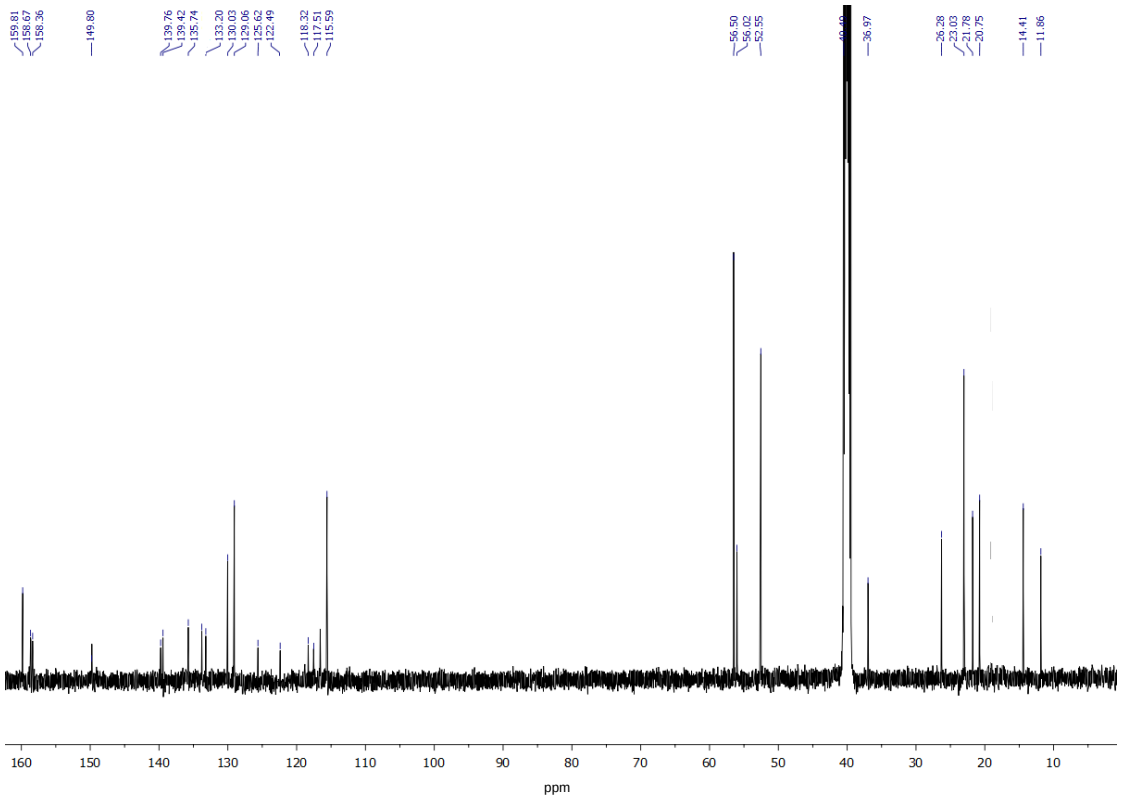
The probe distribution within cells was evaluated using a Carl Zeiss Axiovert 200 fluorescence microscope. The A549 lung cancer cell line was obtained from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle medium (DMEM; HyClone) supplemented with 10% fetal bovine serum (HyClone) and 50 U/mL gentamicin (PanEco) at 37 °C, 5% CO_2 and a relative humidity of 80–85%. The cells were harvested in the late logarithmic growth phase. Hypoxia was simulated in a Binder multi-gas incubator with the possibility of lowering the oxygen content in the atmosphere to 1%. **NBB** probe was dissolved in a DMSO–PBS mixture (1:1 v/v) at a concentration of 2.5 mM and kept until use at -20 °C. The cells were seeded into 24-well cell culture plates (150 000 per well) and kept for 24 h. Then, one plate was transferred to an incubator under hypoxia, and the second one was left in an incubator under standard normoxic conditions. After 6 h, the plates were simultaneously removed from the incubators and the culture medium was changed in all wells for a serum-free medium (900 μL). Then, **NBB** probe was added to the cells, previously dissolved in the medium (100 μL). After

incubation with probe for 1 h, the medium was removed, the cells were twice washed with the serum-free medium, and the analysis was performed using a microscope and a fluorescence reader. For microscopy, the cells were placed in the serum-free medium (200 μL) and analyzed in the red region on a Carl Zeiss Axiovert 200 fluorescence microscope. Prior to analysis using the reader, the medium was removed and the cells were lysed in a 10% PBS–90% DMSO solution (300 μL) as described previously.[S7,S8] The resulting samples (200 μL) were transferred to black 96-well plates and the measurements were taken using a SpectraMax M reader at $\lambda_{ex}/\lambda_{em}$=610/680 nm (cut-off λ = 665 nm). The autofluorescence of the control cells was subtracted from the fluorescence of the probe-containing samples.

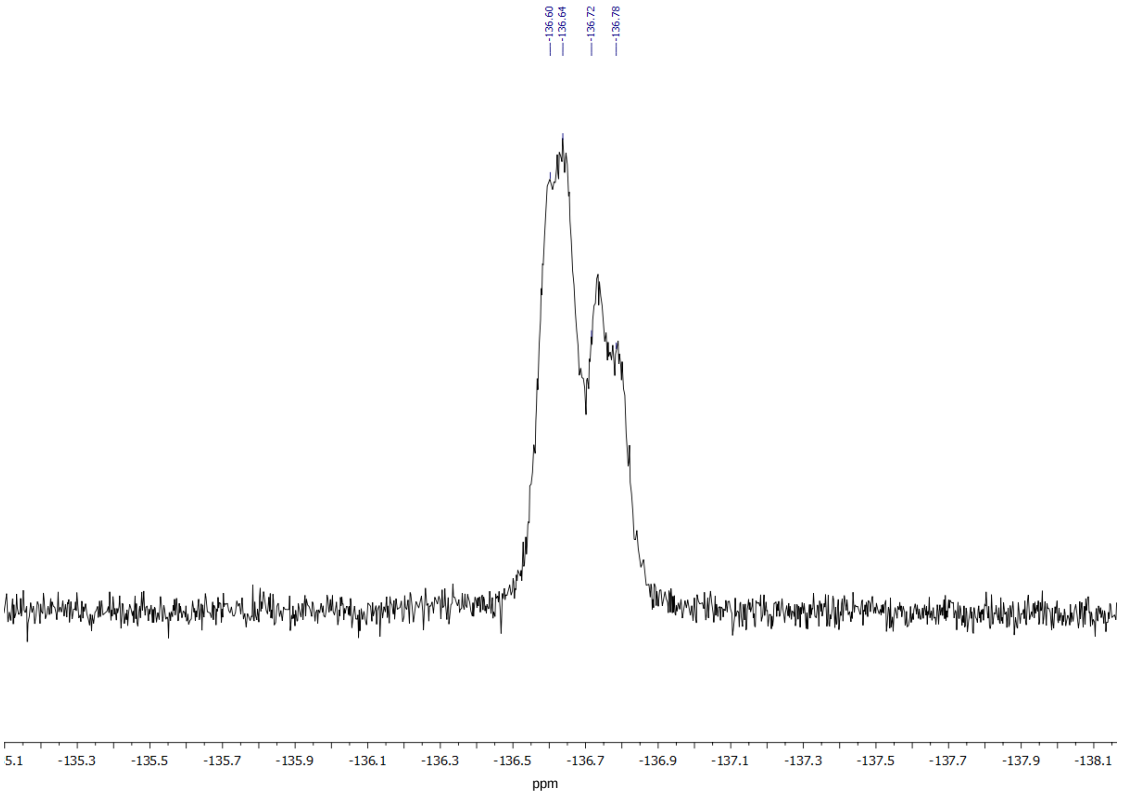
Copies of ^{1}H, ^{13}C, ^{11}B and ^{19}F NMR spectra for compound 4 and NBB



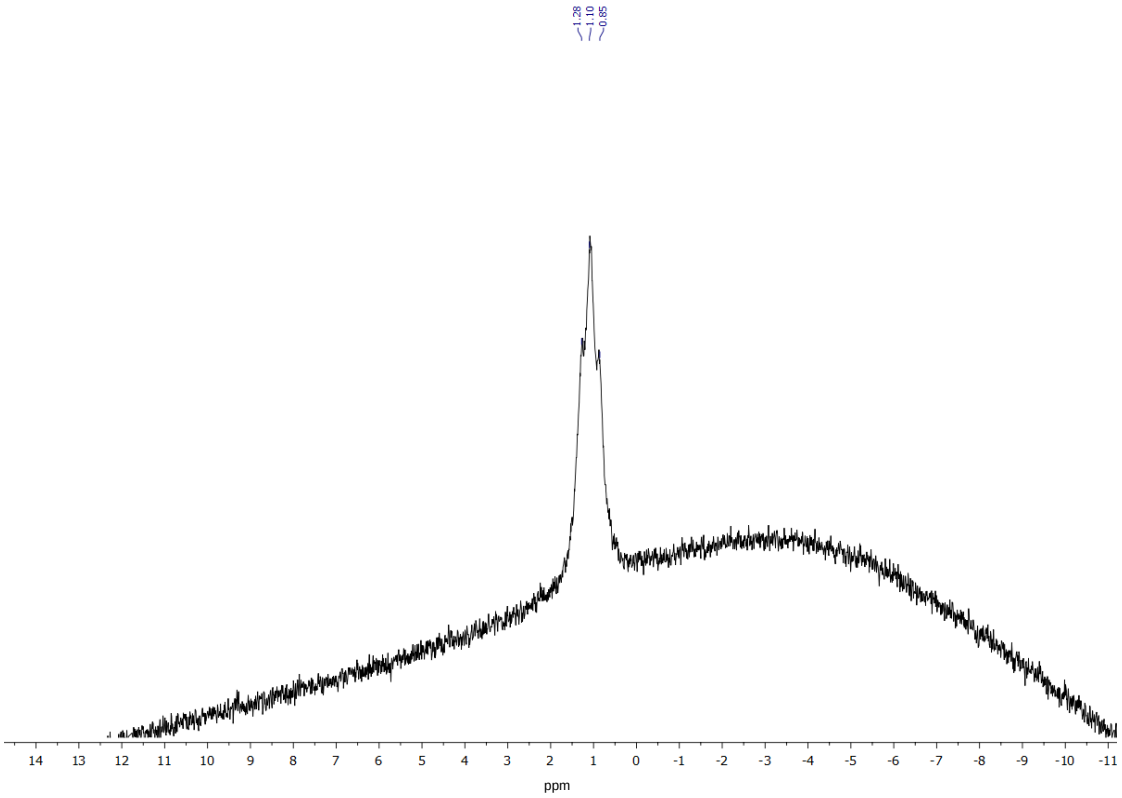
^{1}H NMR (500 MHz, DMSO-d_6) spectrum of distyryl phenol-based BODIPY dye **4**.



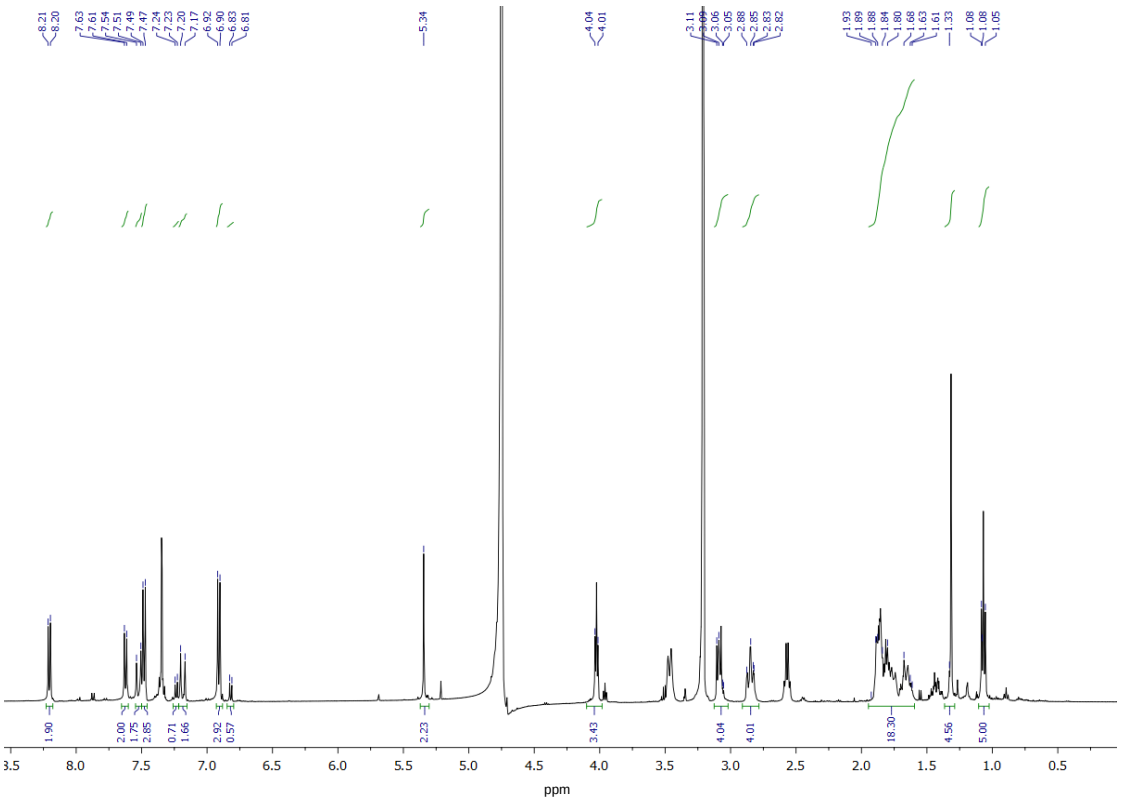
^{13}C NMR (126 MHz, DMSO-d_6) spectrum of distyryl phenol-based BODIPY dye **4**.



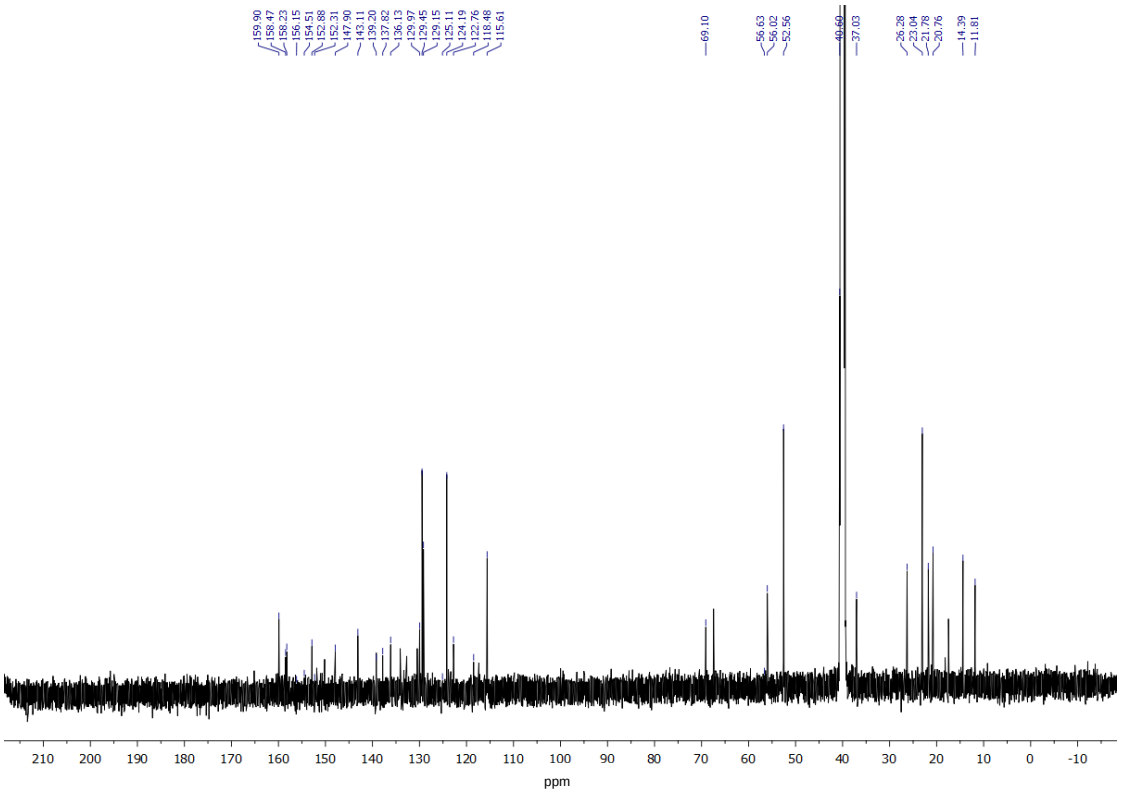
^{19}F NMR (470 MHz, DMSO-d_6) spectrum of distyryl phenol-based BODIPY dye **4**.



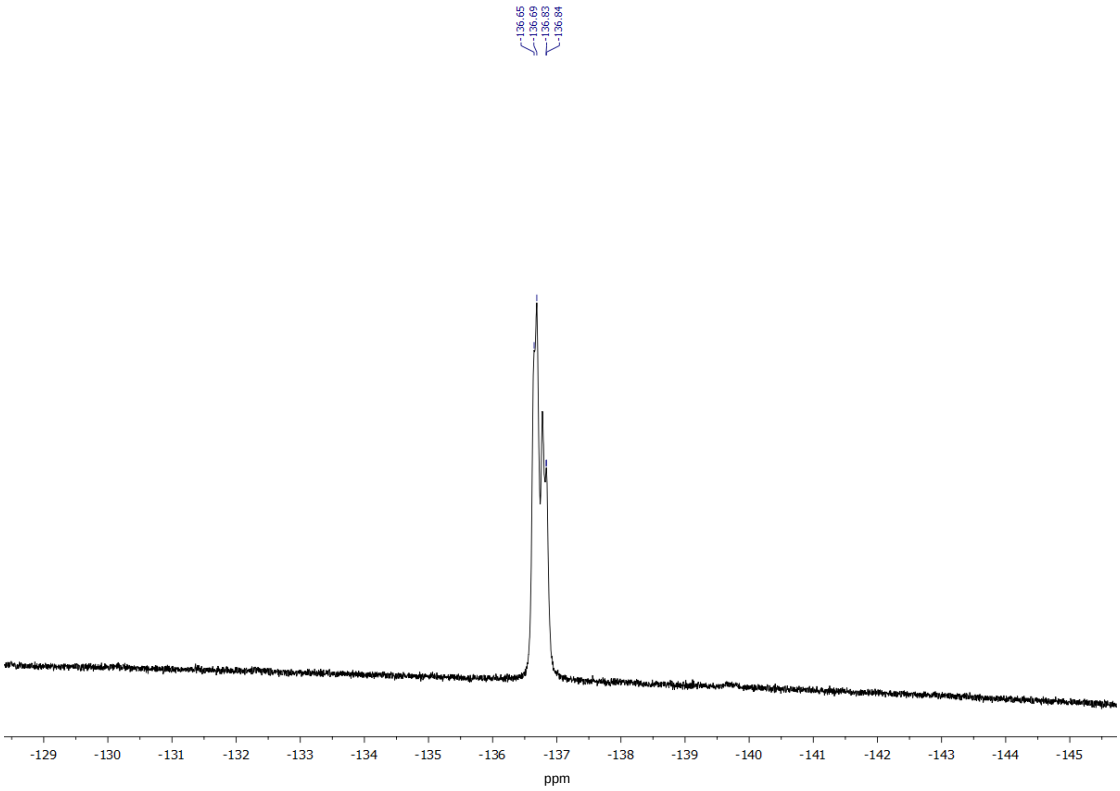
^{11}B NMR (160 MHz, DMSO-d_6) spectrum of distyryl phenol-based BODIPY dye **4**.



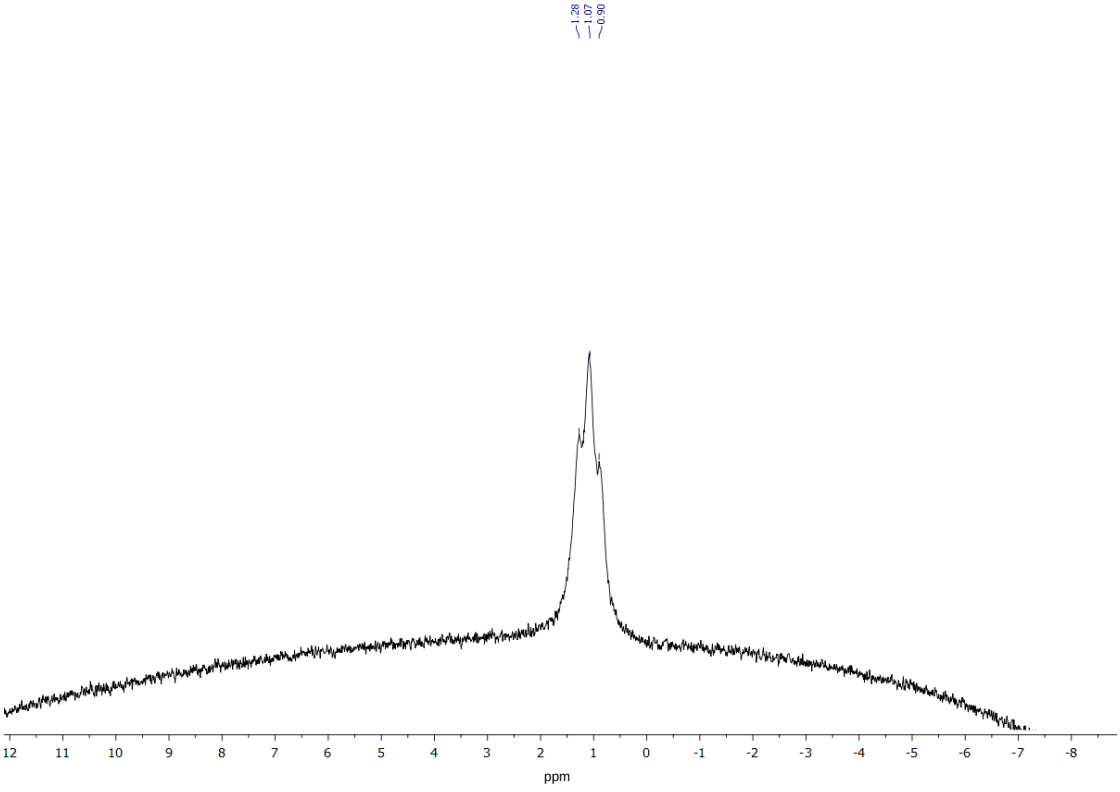
^{1}H NMR (500 MHz, DMSO-d_6) spectrum of **NBB**.



^{13}C NMR (126 MHz, DMSO-d_6) spectrum of **NBB**.



^{19}F NMR (470 MHz, DMSO-d_6) spectrum of **NBB**.



^{11}B NMR (160 MHz, DMSO-d_6) spectrum of **NBB**.

Photophysical properties of **NBB**

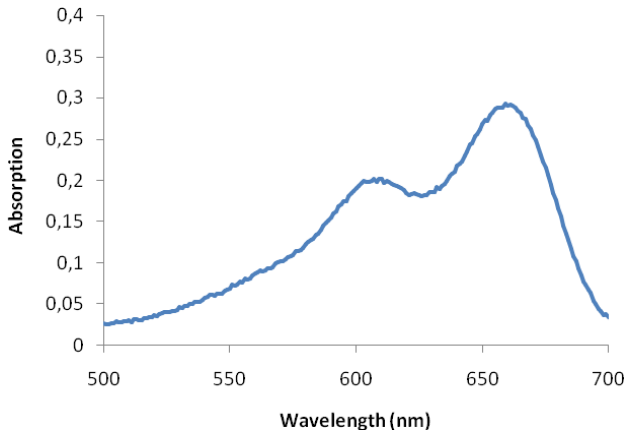


Figure S1 UV–VIS absorption spectrum of **NBB** in water–DMSO (9 : 1 v/v) (concentration: 1.5×10^{-5} M) at 25 °C.

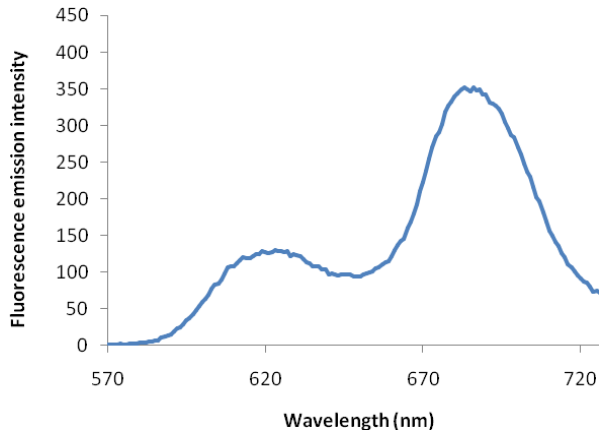


Figure S2 Fluorescence emission spectrum (λ_{ex} = 610 nm) of **NBB** in water–DMSO (9 : 1 v/v) (concentration: 1.5×10^{-5} M) at 25 °C.

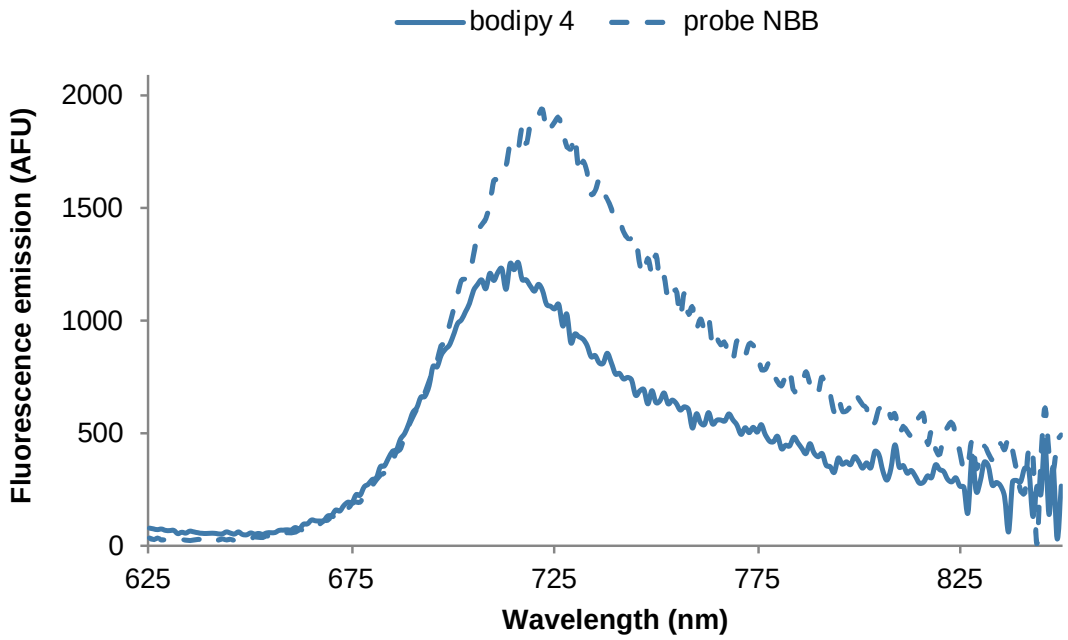


Figure S3 Fluorescence emission spectra (λ_{ex} = 610 nm, ex/em slit = 5 nm) of BODIPY **4** and **NBB** probe recorded in PBS (pH 7.6) (concentration: 2.4×10^{-6} M) at 25 °C.

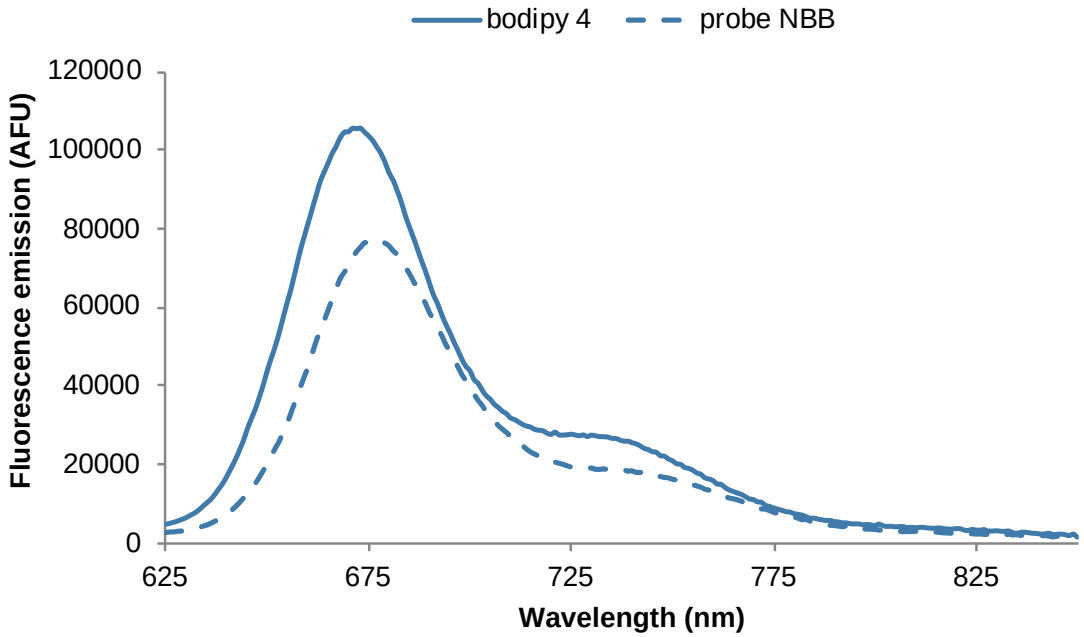


Figure S4 Fluorescence emission spectra (λ_{ex} = 610 nm, ex/em slit = 5 nm) of BODIPY **4** and **NBB** probe recorded in CH_3CN (concentration: 3×10^{-7} M) at 25 °C.

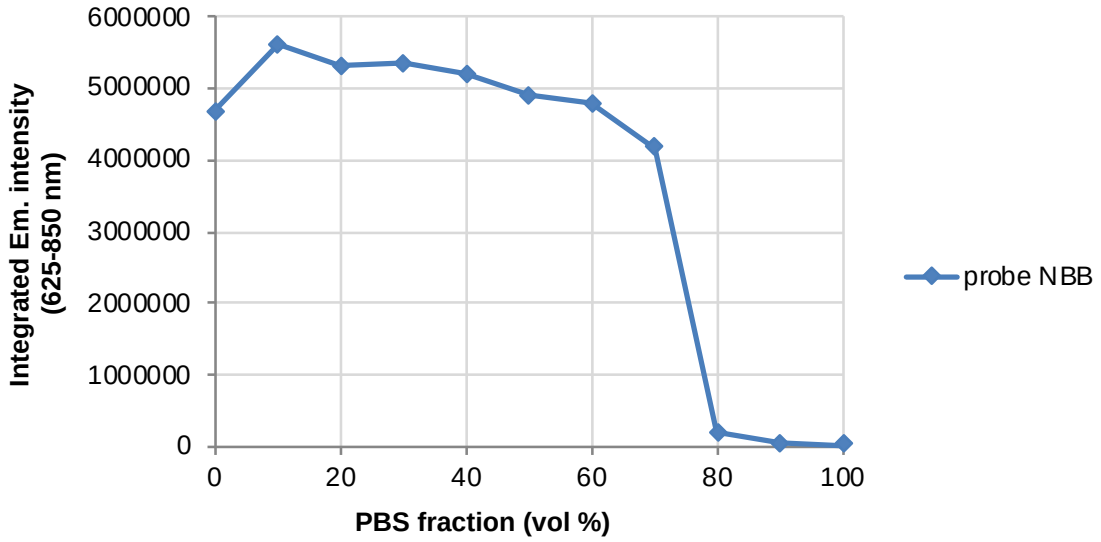


Figure S5 Fluorescence emission (integrated between 625 nm and 850 nm) of **NBB** probe *vs.* PBS fraction in CH_3CN–PBS mixtures (concentration: 0.3 μM, λ_{ex} = 610 nm, ex/em slit = 5 nm).

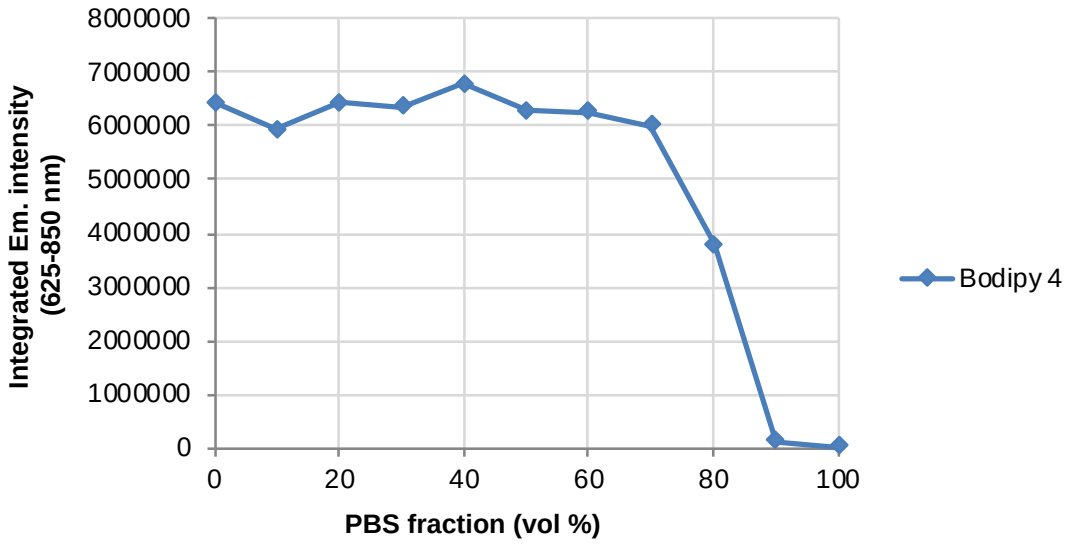


Figure S6 Fluorescence emission (integrated between 625 nm and 850 nm) of compound **4** *vs.* PBS fraction in CH_3CN–PBS mixtures (concentration: 0.4 μM, λ_{ex} = 610 nm, ex/em slit = 5 nm)

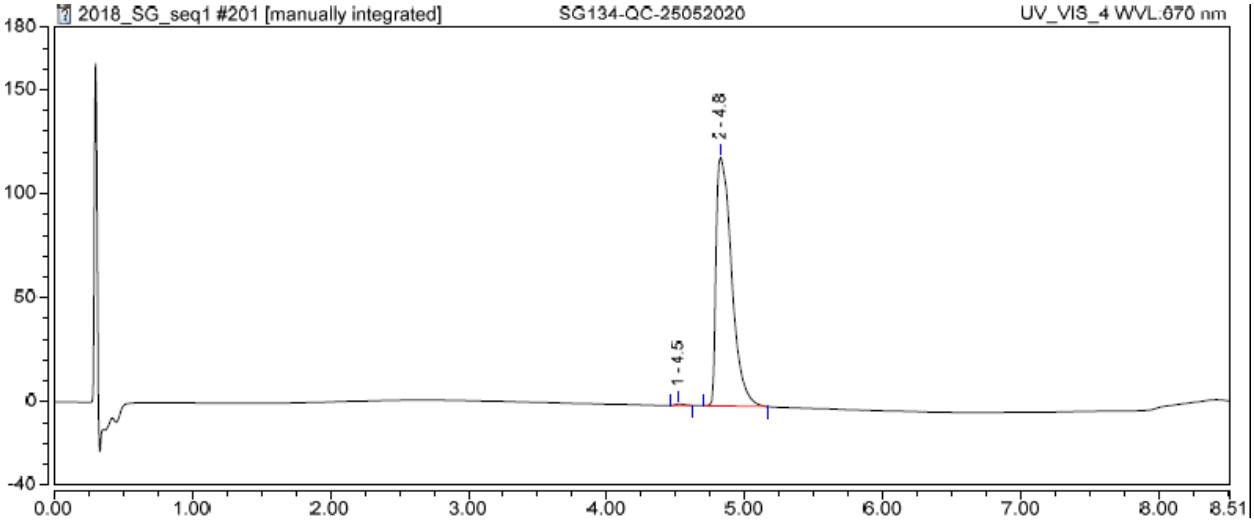


Figure S7 RP-HPLC elution profile (system A) of compound **4** at 670 nm.

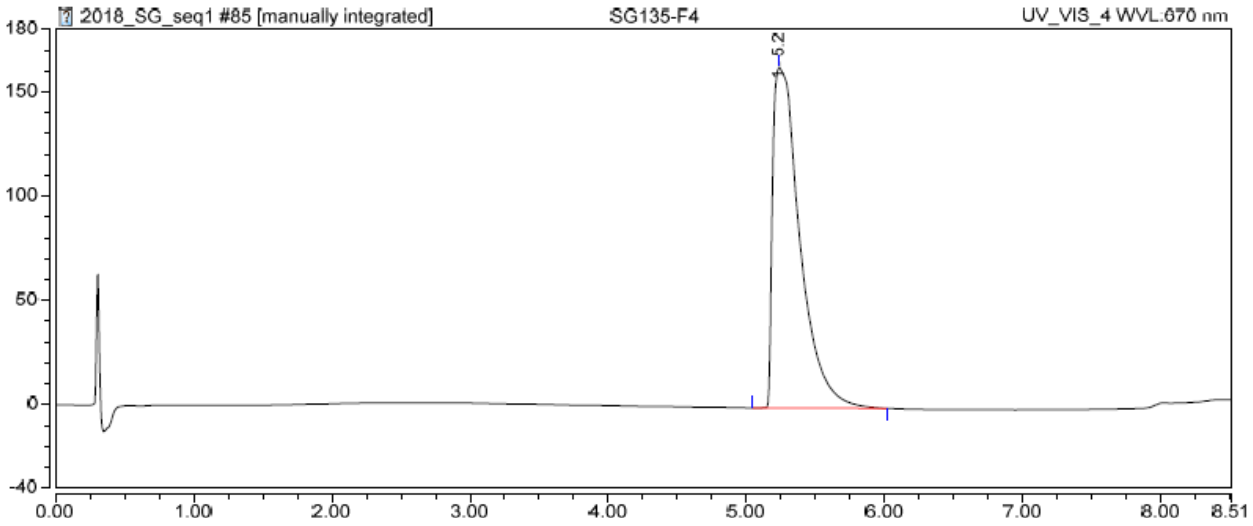


Figure S8 RP-HPLC elution profile (system A) of **NBB** at 670 nm.

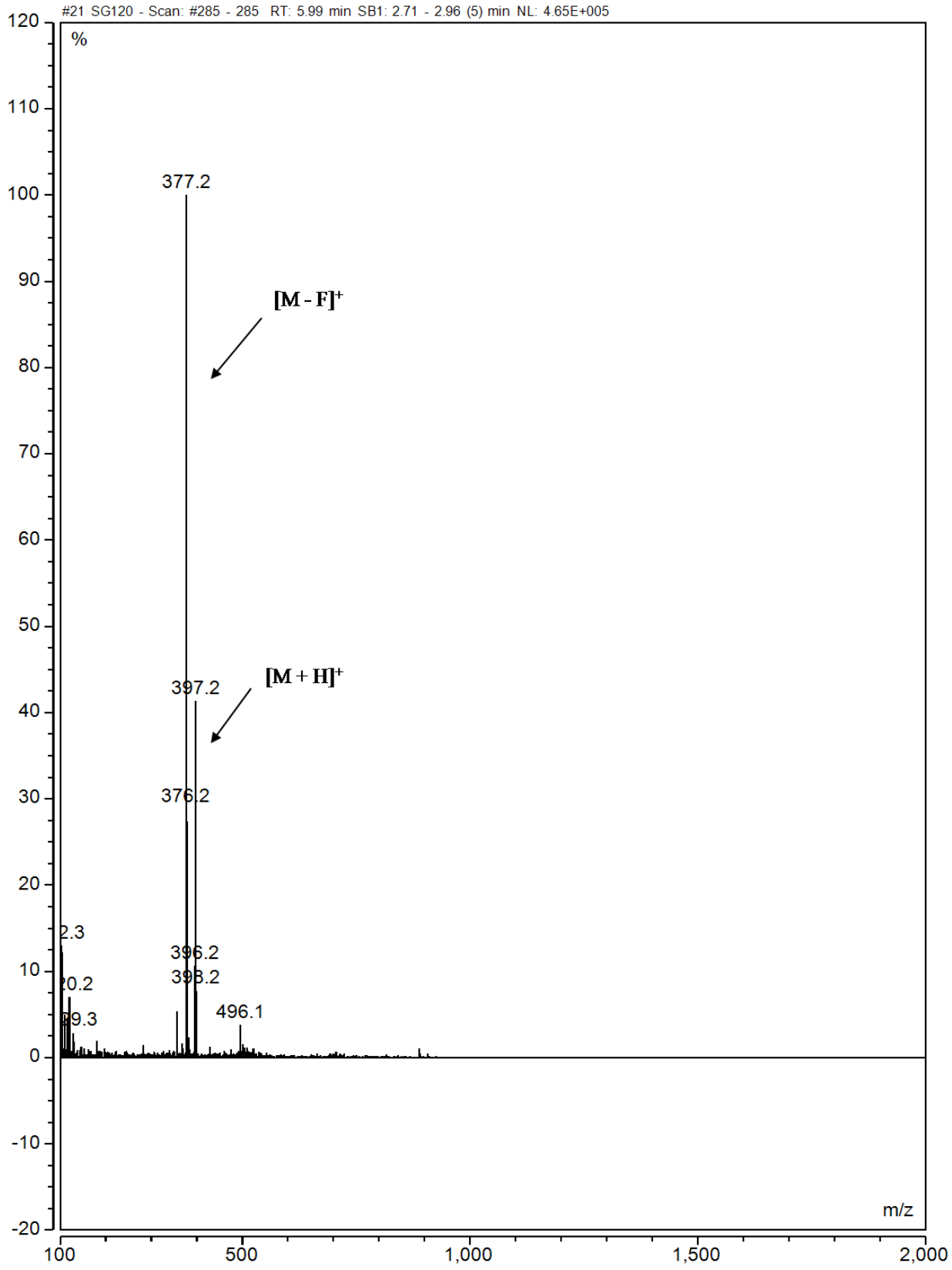


Figure S9 ESI mass spectrum (low resolution) of compound **3.**

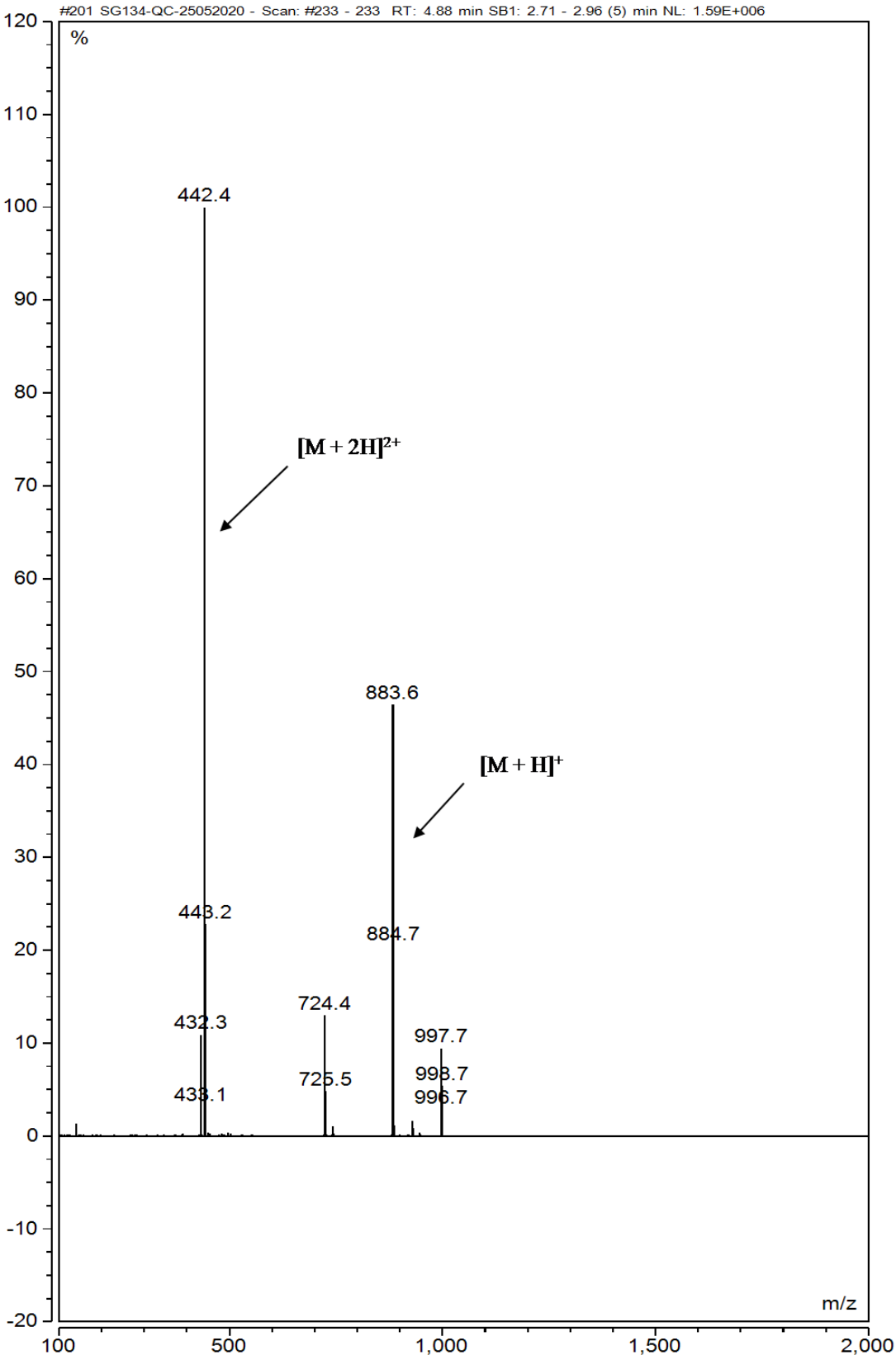


Figure S10 ESI$^+$ mass spectrum (low resolution) of compound **4**.

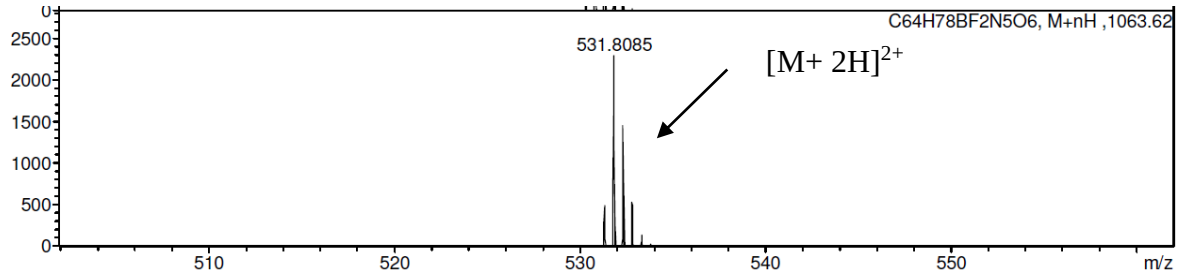


Figure S11 ESI$^+$ mass spectrum (high resolution) of **NBB**.

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