Confronting Molecular Rotor and Self-Quenched Dimer as Fluorogenic BODIPY Systems to Probe Biotin Receptors in Cancer Cells

Supporting information

Experimental

Chemicals were purchased from Sigma Aldrich, Alfa Aesar or TCI and used as received.

Analytical characterization

NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer. Mass spectra were obtained using an Agilent QTOF 6520 mass spectrometer.

Spectroscopy. Absorption spectra were recorded on a Cary 5000 spectrophotometer (Varian) and fluorescence spectra on a FS5 (Edinburgh Instruments) spectrofluorometer. The fluorescence signal was corrected for the lamp intensity fluctuations and for wavelength-dependent sensitivity of the detector. Relative fluorescence quantum yields were measured using standard (Fluorescein in 0.1 M NaOH).

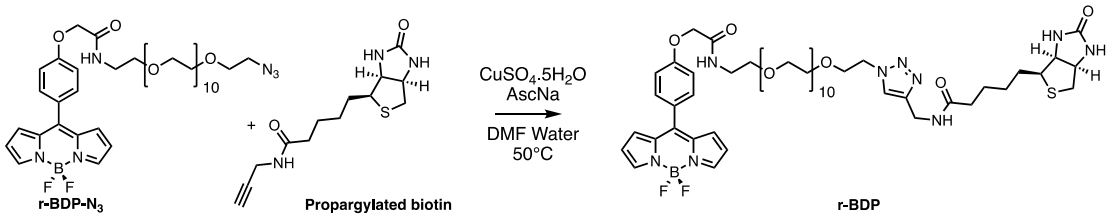
Microscopy imaging. Cells were grown at 37° C in humidified atmosphere containing 5% CO_2: KB cells (ATCC® CCL-17) in Dulbecco's Modified Eagle Medium without phenol red (DMEM, Gibco-Invitrogen) with 10% fetal bovine serum (FBS, Lonza), 1% non-essential amino acids (Gibco-Invitrogen), 1% MEM vitamin solution (Gibco-Invitrogen), 1% L-Glutamine (Sigma Aldrich) and 0.1% antibiotic solution (gentamicin, Sigma-Aldrich); HEK293T (ATCC® CRL-3216™) and HeLa (ATCC® CCL-2™) in DMEM without phenol red supplemented with 10% FBS (Lonza), 1% L-Glutamine (Sigma Aldrich) and 1% antibiotic solution (Penicillin-Streptomycin, Sigma-Aldrich); NIH/3T3 (ATCC® CRL-1658™) in DMEM without phenol red supplemented with 10% bovine calf serum, iron fortified (Sigma Aldrich), 1% L-Glutamine (Sigma Aldrich) and 1% antibiotic solution (Penicillin-Streptomycin, Sigma-Aldrich). Cells were seeded onto a chambered coverglass (IBiDi®) 24 h before the microscopy measurement. For imaging, the culture medium was removed, the attached cells were washed with Hank's Balanced Salt Solution (HBSS, Gibco-Invitrogen) and incubated with solution of a probe (0.2 μM) or at indicated concentration. In competition experiment, KB cells were pre-treated with competitor (100 μM) for 30 min prior to incubation with d-BDP probe. Images were taken with Nikon Ti-E inverted microscope, equipped with CFI Plan Apo × 60 oil (NA = 1.4) objective, X-Light spinning disk module (CresOptics) and a Hamamatsu Orca Flash 4 sCMOS camera, was used. The microscopy settings were: Hoechst (ex. 405 nm, em. 510±42 nm), BODIPY (exc. 488 nm, em. 531±40 nm), MemBright Cy5 (ex. 638 nm, em. 705±36 nm). The images were recorded using NIS Elements and then processed with Icy open source imaging software.

Flow cytometry. Cells were grown at 37°C in humidified atmosphere containing 5% CO_2 in 25 cm^2 (Nunc™ EasYFlask, ThermoFisher). On the day of the analysis, the cells were washed and harvested. The cell suspension ($3x10^5$ cells/mL) was incubated with corresponding probe (0.2 μM) for 5 or 30 min at room temperature and analyzed immediately using flow cytometry (MACSQuant VYB, Miltenyi Biotec).

Cytotoxicity assay. Cytotoxicity of the dyes was quantified by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). A total of $1x10^4$ KB cells/well were seeded in a 96-well plate 24 h prior to the cytotoxicity assay in growth medium and were incubated in a 5% CO_2 incubator at 37°C. After medium removal, an amount

of 100 µL DMEM containing 5 µM, 1 µM, 0.2 µM or 0 µM (DMSO control) of a probe was added to the KB cell and incubated for 24 h at 37°C (5% CO_2). Next, cells were incubated with MTT (0.5 mg/mL) in DMEM solution for 3 h at 37°C. Then, 75 µL of the mix was replaced by 50 µL of DMSO (100%) and gently shaken for 15 min at room temperature in order to dissolve the insoluble purple formazan reduced in living cells. The absorbance at 540 nm was measured. Data were shown as the mean value (n=6) plus a standard deviation (±SD). For each concentration, we calculated the percentage of cell viability in reference of the 0 µM (DMSO control).

Synthesis of rBDP-B

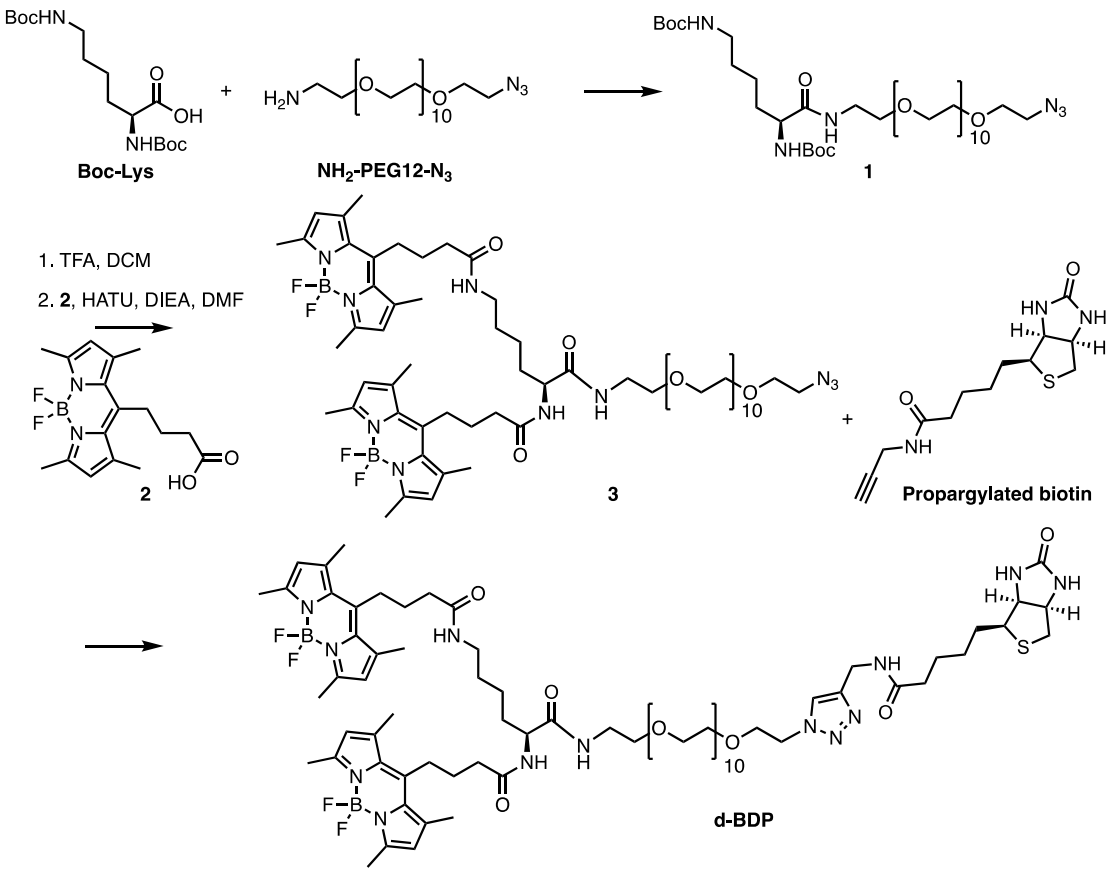


The synthesis of **r-BDP-N_3** has already been reported by our group.[1]

Propargylated biotin was synthesized according to a reported protocol.[2]

r-BDP. To a solution of **r-BDP-N_3** (15 mg, 16.77 µmol) and **propargylated biotin** (7 mg, 24.91 µmol, 1.5 eq) in DMF (2 mL) added a heterogeneous solution of $CuSO_4.5H_2O$ (2 mg, 8.03 µmol, 0.48 eq) and sodium ascorbate (2 mg, 10.10 µmol, 0.6 eq) in water (300 µL). The solution was allowed to stir at 50°C overnight. The solvent was evaporated, the crude was solubilized in DCM and washed with an aqueous solution of EDTA (1 mM). The organic phase was dried over anhydrous $MgSO_4$, filtered, evaporated and the crude was purified by column chromatography on silica gel (DCM/MeOH: 9/1) to obtain 13 mg of **r-BDP** as a yellow oil (Yield = 66%). Rf= 0.42 (DCM/MeOH: 9/1). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.95 (s, 2H, H Ar), 7.77 (s, 1H, H triazol), 7.60-7.57 (m, 3H, H Ar), 7.13-7.10 (m, 2H, H Ar), 6.97 (d, *J* = 4.2 Hz, 2H, H Ar), 6.72 (s, 1H, NH), 6.58 (dd, *J* = 4.1, 1.6 Hz, 2H, H Ar), 6.08-6.08 (m, 1H, NH), 4.63 (s, 2H, CH_2-CO), 4.57-4.51 (m, 4H), 4.44 (d, *J* = 5.6 Hz, 2H), 3.87 (t, *J* = 5.1 Hz, 2H), 3.66-3.59 (m, 46H, CH_2-PEG), 2.92 (d, *J* = 4.9 Hz, 1H), 2.78 (dd, *J* = 12.7, 0.2 Hz, 1H), 2.26-2.25 (m, 3H), 1.71-1.67 (m, 4H, CH_2-biotin). HRMS (ESI+) calculated for $C_{54}H_{80}BF_2N_9O_{15}S$ $[M+H]^+$ 1176.5556, found 1176.5671.

Synthesis of d-BDP



1. To a solution of NH_2-PEG12-N_3 (162 mg, 284 μmol, 1 eq) and **Boc-Lys** (128 mg, 370 μmol, 1.3 eq) in DMF (10 mL) were added under argon: HATU (140 mg, 370 μmol, 1.3 eq) andDIEA (148 μL, 851 μmol, 3 eq) . The reaction mixture was allowed to stir at room temperature overnight under Argon. The solvent was removed under vacuum, the crude was dissolved in DCM, then washed with brine solution, dried over $MgSO_4$ filtered and concentrated. The crude was purified by column chromatography on silica gel (DCM/MeOH: 9/1) to obtain 220 mg of **1** as viscous colorless oil. (Yield = 86%). R f= 0.4 (DCM/MeOH: 9/1). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.80 (s, 1H), 5.33 (s, 1H), 4.84 (s, 1H), 3.97 (s, 1H), 3.58 – 3.49 (m, 38H), 3.42 (s, 2H), 2.98 (s, 2H), 2.69 (s, 2H), 1.73 (s, 1H), 1.53 (s, 1H), 1.44 – 1.36 (m, 2H), 1.32 (s, 19H), 1.28 – 1.18 (m, 2H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 172.11, 156.06, 72.28 – 69.44 (m), 54.28, 50.58, 39.97, 39.14, 38.52, 32.45, 29.53, 28.39, 28.28. HRMS (ESI+) calculated for $C_{40}H_{78}N_6NaO_{16}$ $[M+Na]^+$ 921.5372, found 921.5509, HRMS (ESI+) calculated for $C_{40}H_{78}KN_6O_{16}$ $[M+K]^+$ 937.5111, found 937.5248.

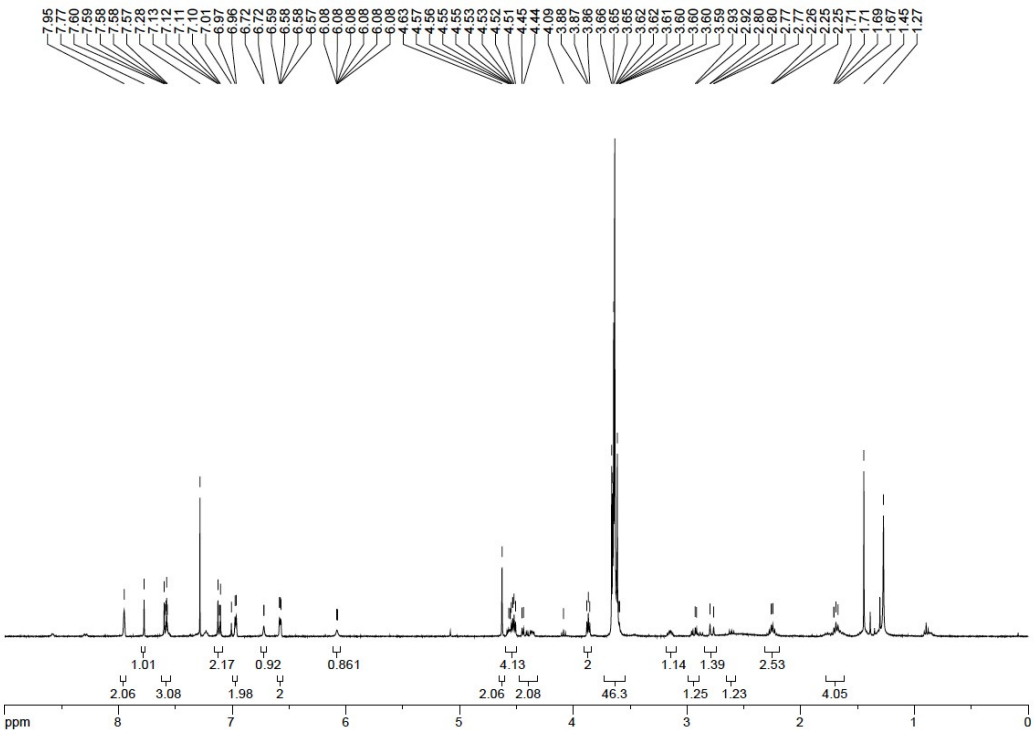
Deprotected 1. **1** was dissolved in DCM and TFA was added dropwise. The mixture was allowed to stir for 1.5 h and concentrated to dryness. **Deprotected 1** was used without further purification.

The synthesis of **2** has already been reported by our group.[3]

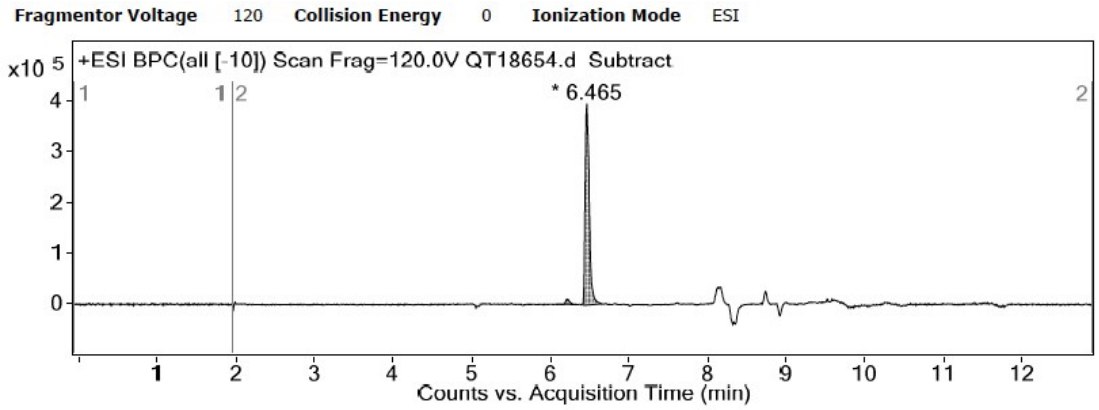
3. To a solution of **deprotected 1** (205 mg, 307 μmol, 1 eq) and **2** (58 mg, 614 μmol, 2 eq) in DMF (5 mL) was added HATU (303 mg, 799 μmol, 2.6 eq) and DIEA (376 μL, 2.15 mmol, 7 eq). The reaction mixture was allowed to stir at room temperature for 2 h under Argon. The solvent was removed under vacuum, the crude was dissolved in DCM, then washed with water, then with brine, dried over anhydrous $MgSO_4$ filtered and concentrated. The crude was purified by column chromatography on silica gel (DCM/MeOH: 95/5) to obtain 259 mg of **3** as an orange solid (Yield = 63%). Rf = 0.6 (DCM/MeOH: 9/1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.83 – 6.76 (m, 1H, NH), 6.64 – 6.57 (m, 1H, NH), 6.16 – 6.10 (m, 1H, NH), 6.01 (s, 4H, H Pyrrole), 4.39 – 4.32 (m, 1H), 3.71 – 3.47 (m, 44H, H PEG), 3.43 – 3.33 (m, 4H), 3.22 – 3.13 (m, 2H), 2.93 (m, 4H), 2.47 (s, 12H, CH_3 Pyrrole), 2.36 (s, 14H, CH_3 Pyrrole), 2.27 (t, J = 7.1 Hz, 2H), 1.95 – 1.84 (m, 4H), 1.76 (m, 1H), 1.64 (m, 1H), 1.48 (m, 2H), 1.31 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.86, 171.65, 171.61, 154.05, 153.99, 145.44, 145.23, 140.53, 140.47, 131.44, 121.77, 70.68, 70.65, 70.62, 70.56, 70.54, 70.49, 70.44, 70.12, 70.01, 69.47, 52.85, 50.68, 39.28, 38.92, 36.19, 36.13, 33.06, 28.86, 27.54, 27.48, 27.36, 22.01, 16.38, 16.33, 14.46, 14.43, 14.41. ^{19}F NMR (376 MHz, $CDCl_3$) δ -146.31. ^{11}B NMR (128 MHz, $CDCl_3$) δ 0.78, 0.52, 0.26. HRMS (ESI+) calculated for $C_{64}H_{104}B_2F_4N_{11}O_{14}$ $[M+NH_4]^+$ 1348.7886, found 1348.7897.

d-BDP. To a solution of **3** (110 mg, 83 μmol, 1 eq) and propargylated biotin (26 mg, 91 μmol, 1.1 eq) in DMF (3 mL) added 200 μL of an aqueous solution of $CuSO_4.5H_2O$ (26 mg, 107 μmol, 1.3 eq) and ascorbic acid (22 mg, 124 μmol, 1.5 eq). The reaction mixture was left to stir at 50°C overnight. The solvent was evaporated, the crude was dissolved in DCM and washed with an aqueous solution of $NaHCO_3$. The organic phase was dried over anhydrous $MgSO_4$ filtered and evaporated. The crude was purified by column chromatography on silica gel (DCM/MeOH: 95/5) to obtain 84 mg of **d-BDP** as an orange oil (Yield = 63 %). Rf = 0.4 (DCM/MeOH: 9/1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (s, 1H, H triazole), 7.55 – 7.50 (m, 1H, NH), 7.40 – 7.34 (m, 2H, $(NH)_2CO$ Biotin), 6.76 (s, 1H, NH), 6.45 (s, 1H, NH), 6.02 (2s, 4H, H Pyrrole), 5.95 (s, 1H, NH), 4.52 – 4.42 (m, 5H), 4.42 – 4.34 (m, 1H, H Biotin), 4.32 – 4.25 (m, 1H, H biotin), 3.83 (t, J = 5.1 Hz, 2H), 3.68 – 3.48 (m, 44H, H PEG), 3.48 – 3.05 (m, 6H), 3.01 – 2.91 (m, 4H), 2.91 – 2.84 (m, 1H), 2.72 (d, J = 12.8 Hz, 1H), 2.48 (s, 12H, CH_3 Pyrrole), 2.38 (d, J = 6.0 Hz, 15H, CH_3 Pyrrole), 2.31 (t, J = 7.1 Hz, 2H), 2.16 (q, J = 7.1 Hz, 2H), 1.97 – 1.85 (m, 4H), 1.78 – 1.44 (m, 9H), 1.40 – 1.29 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.15, 172.43, 172.11, 171.95, 153.25, 145.62, 145.45, 141.08, 131.45, 121.73, 77.26, 70.53, 70.49, 70.47, 70.45, 70.40, 70.36, 70.05, 69.60, 69.34, 61.85, 60.17, 55.75, 53.11, 50.69, 50.30, 40.62, 39.22, 38.86, 36.19, 36.08, 35.49, 34.57, 32.12, 29.69, 28.68, 28.11, 28.06, 27.58, 27.49, 25.36, 25.29, 22.62, 16.40, 15.80, 14.44. ^{19}F NMR (376 MHz, $CDCl_3$) δ -146.32. ^{11}B NMR (128 MHz, $CDCl_3$) δ 0.54 (t, J = 33.0 Hz). HRMS (ESI+) calculated for $C_{77}H_{120}B_2F_4N_{13}O_{16}S$ $[M+H]^+$ 1612.8819, found 1612.8826.

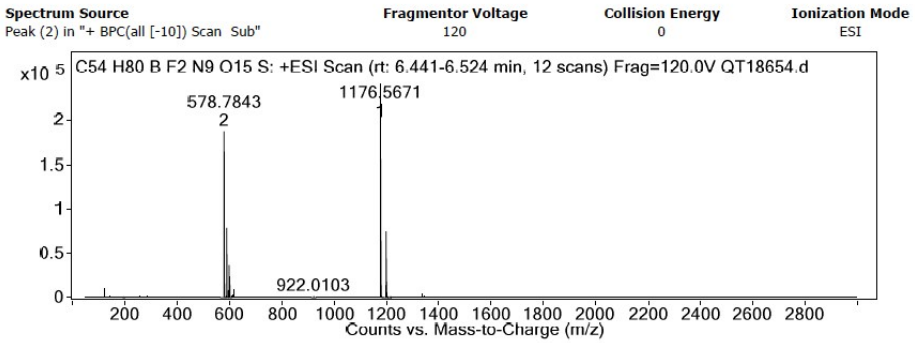
NMR and mass spectra



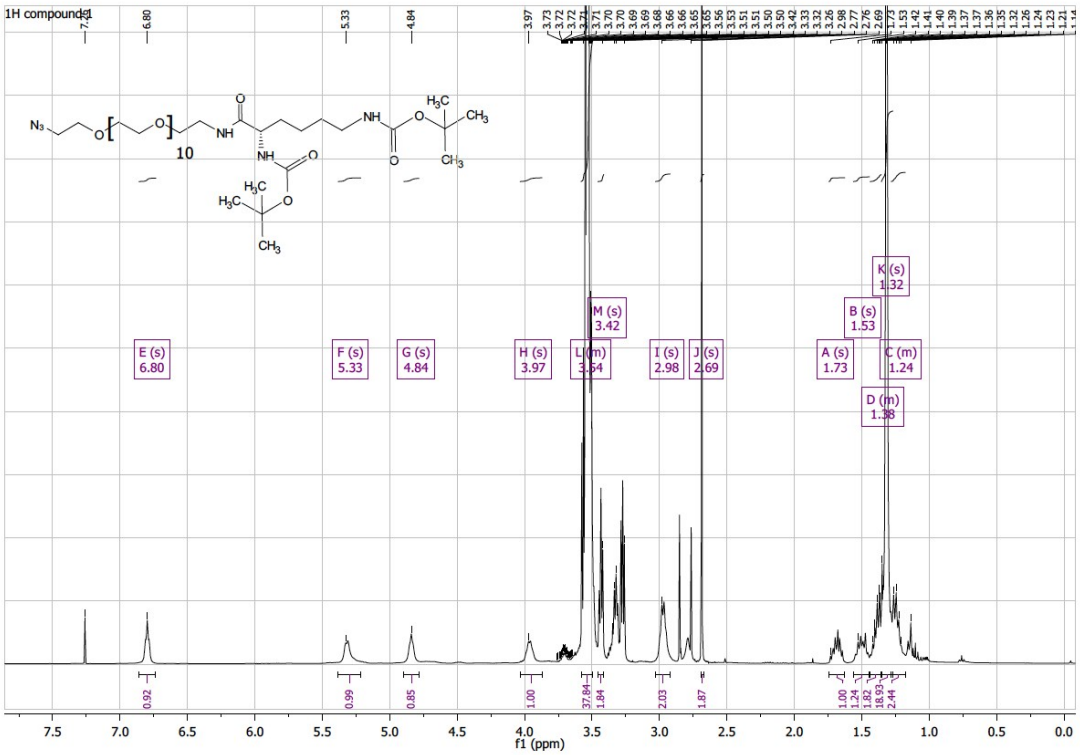
^{1}H NMR spectrum of **r-BDP**



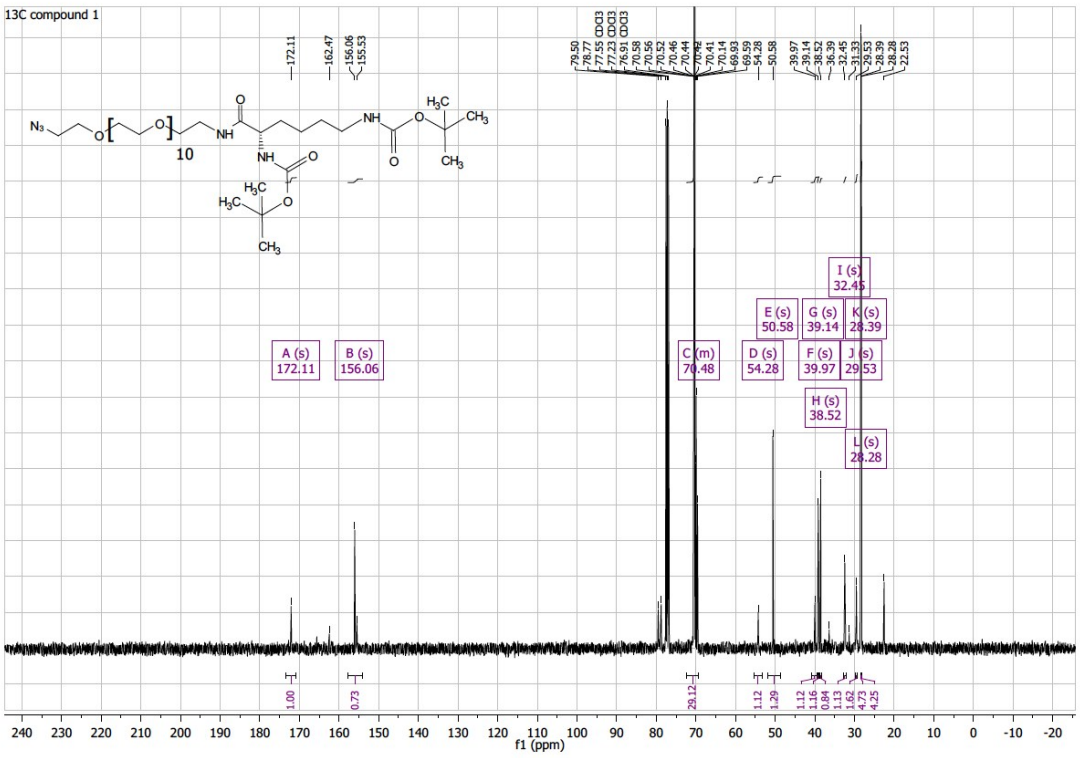
HPLC trace of **r-BDP**



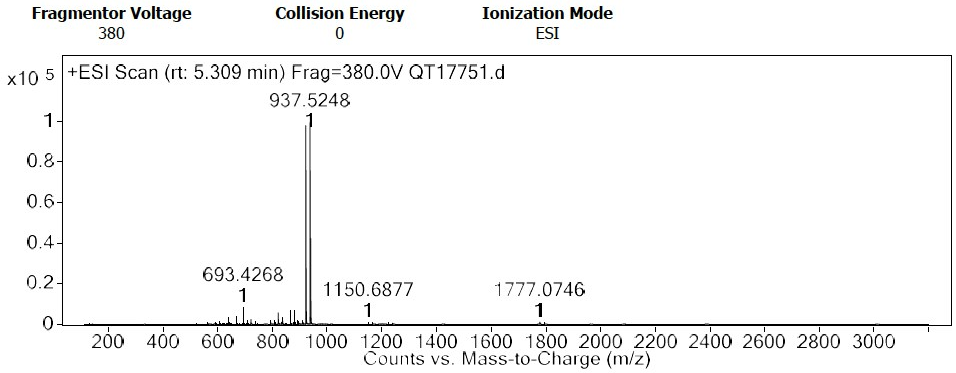
HRMS spectrum of **r-BDP**



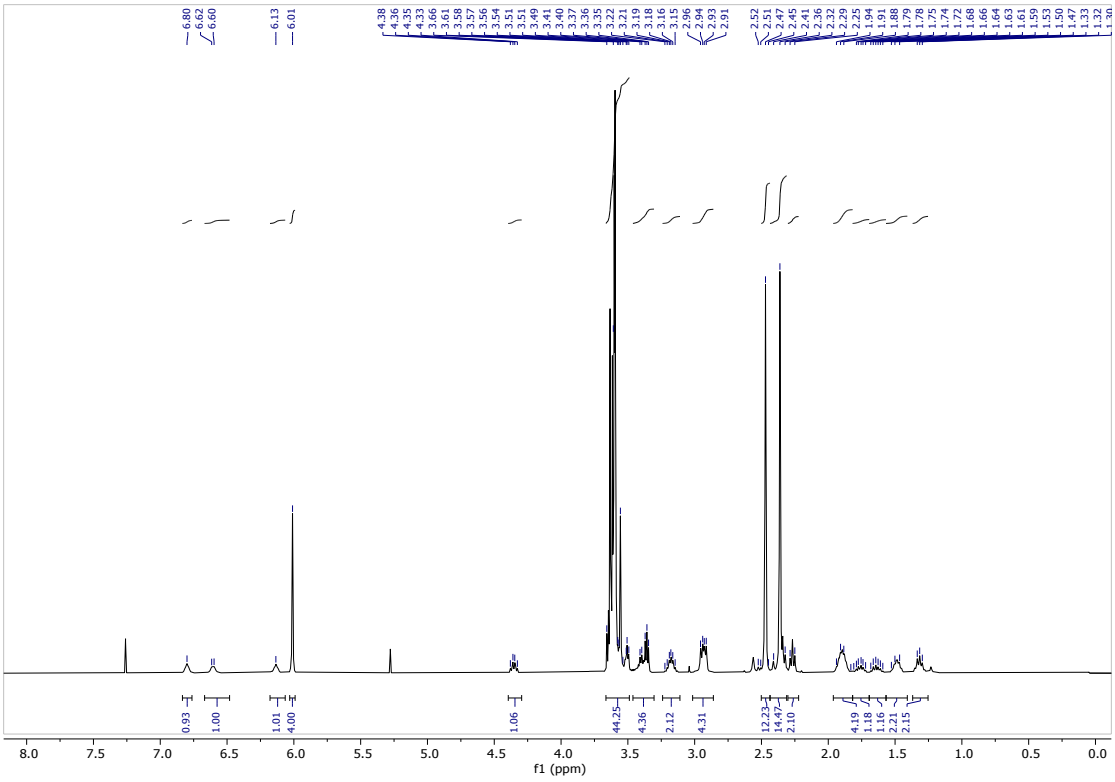
^{1}H NMR spectrum of **1**



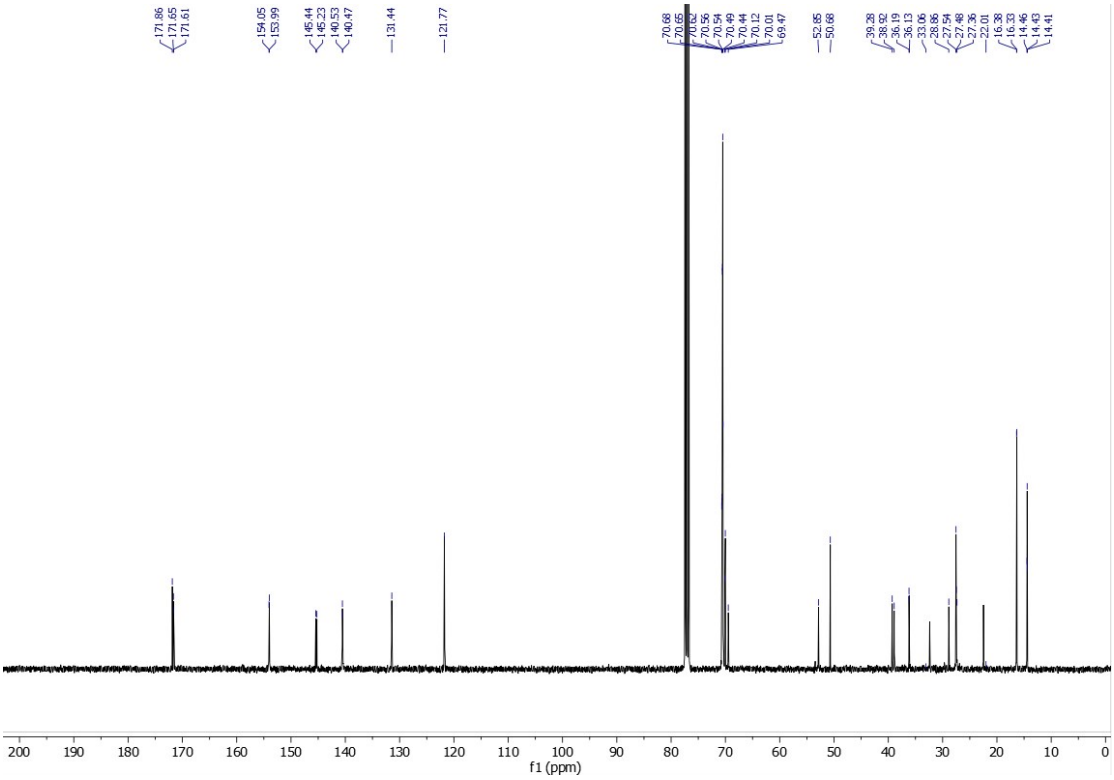
^{13}C NMR spectrum of **1**



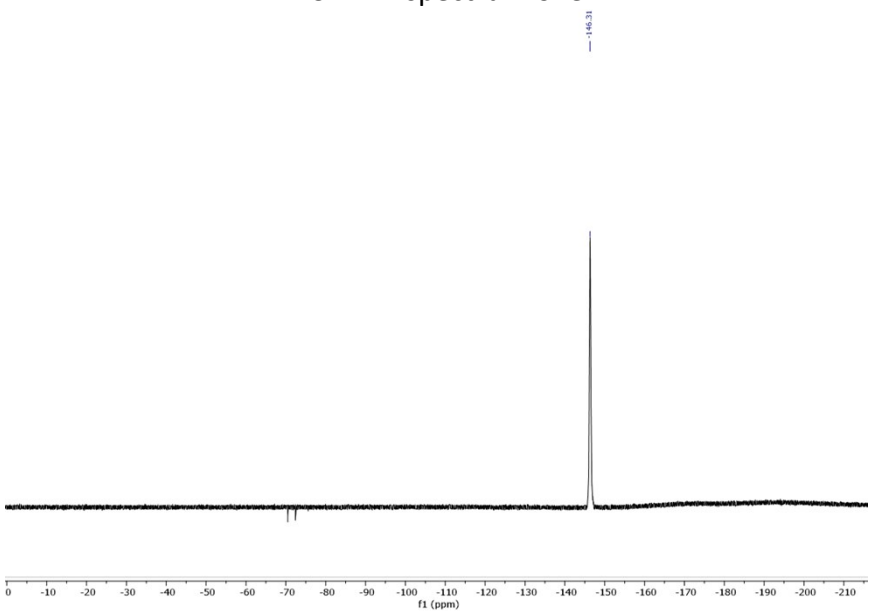
HRMS spectrum of **1**



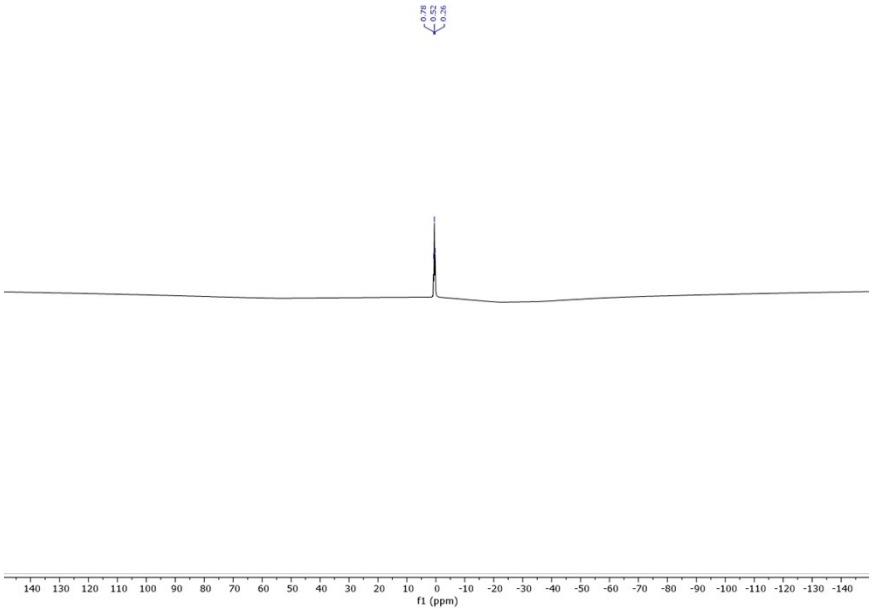
^{1}H NMR spectrum of **3**



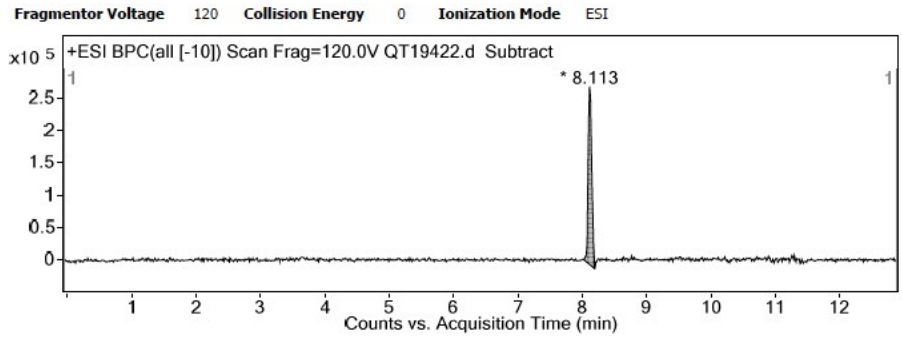
^{13}C NMR spectrum of **3**



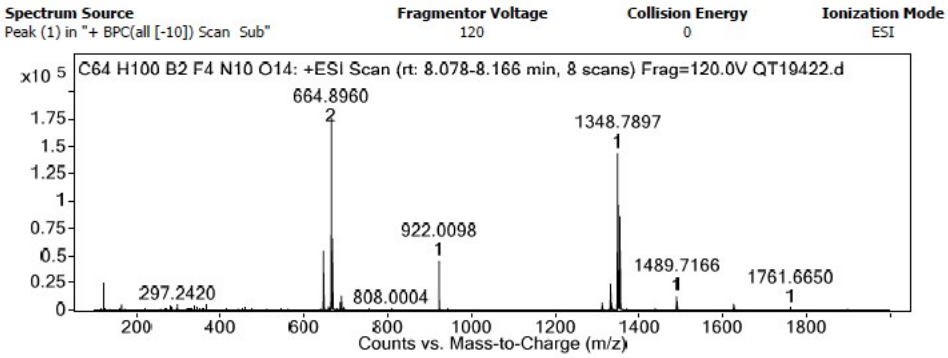
^{19}F NMR spectrum of **3**



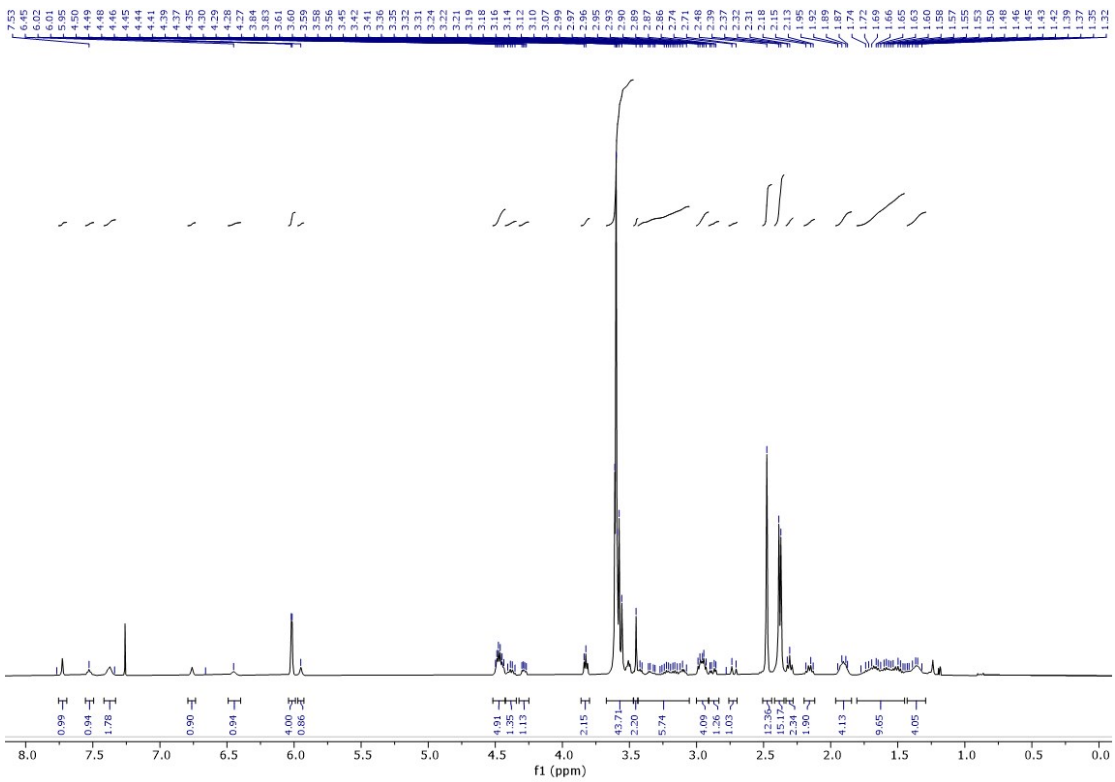
^{11}B NMR spectrum of **3**



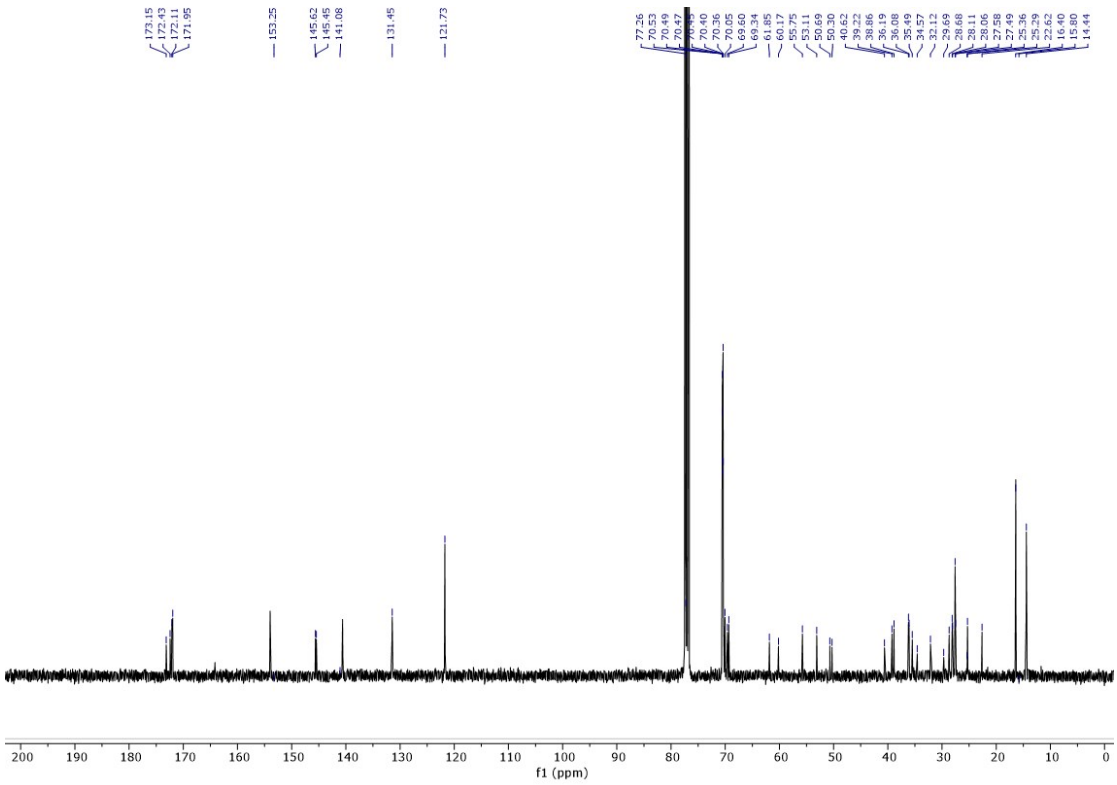
HRMS spectrum of **3**



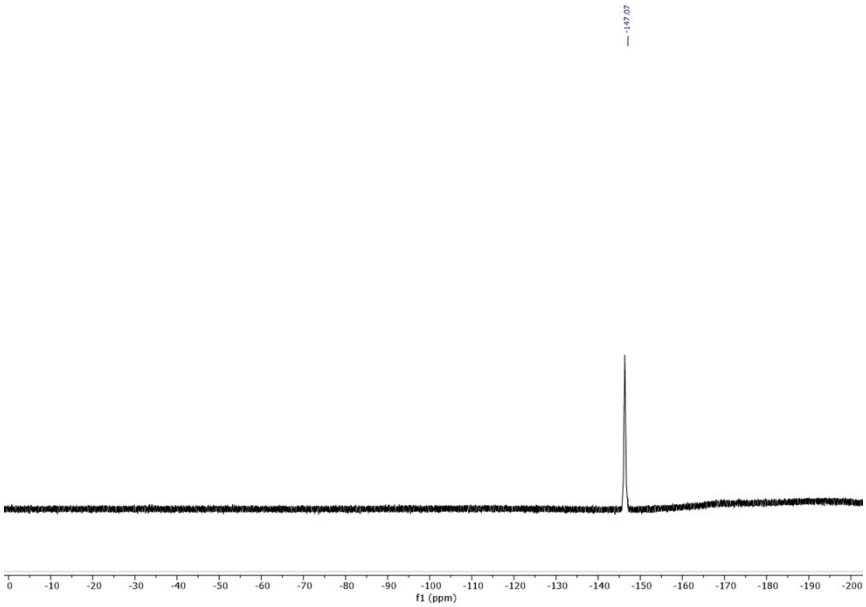
HPLC trace of **3**



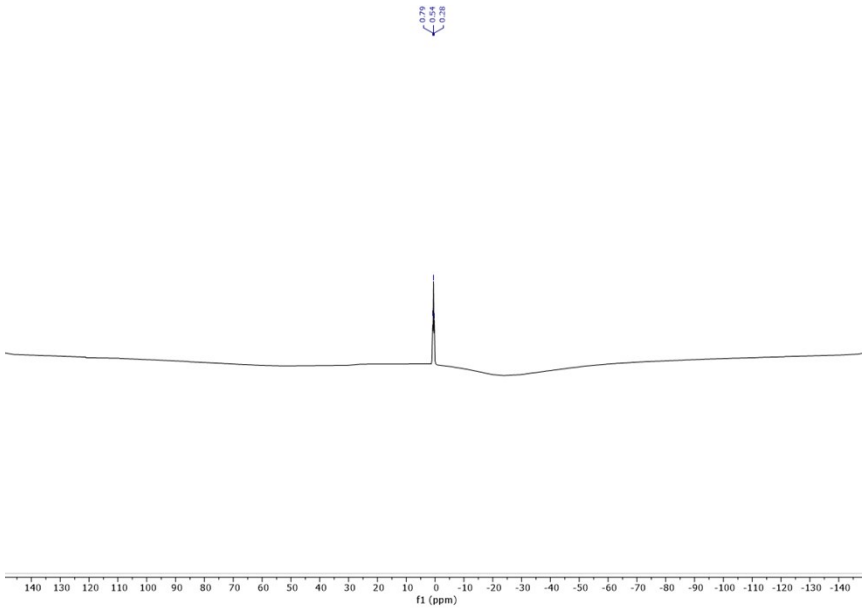
^{1}H NMR spectrum of **d-BDP**



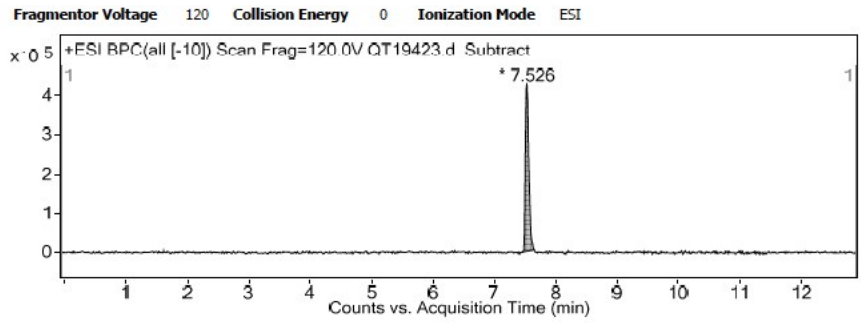
^{13}C NMR spectrum of **d-BDP**



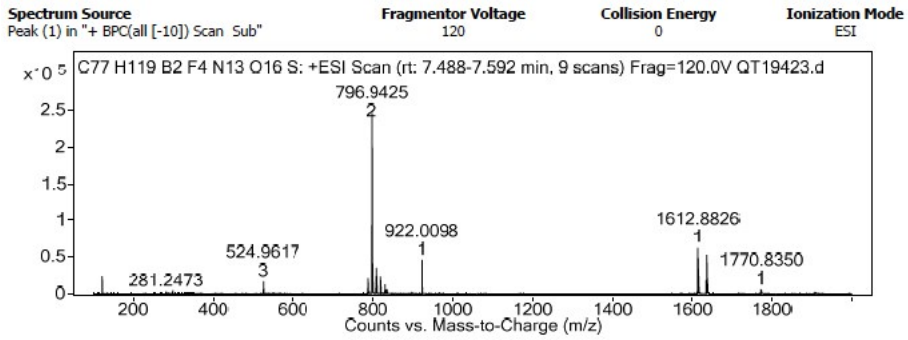
^{19}F NMR spectrum of **d-BDP**



^{11}B NMR spectrum of **d-BDP**



HPLC trace of **d-BDP**



HRMS spectrum of **d-BDP**

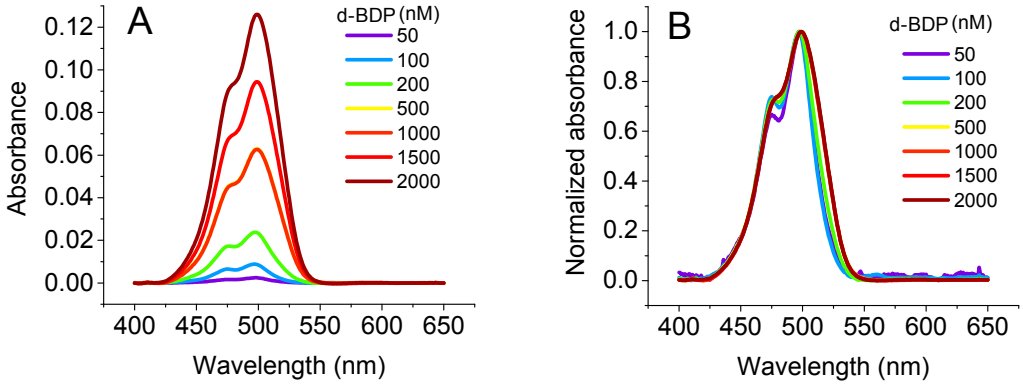


Figure S1. Intramolecular H-aggregation of d-BDP in water. (A) Absorption spectra of d-BDP at various concentrations. (B) Normalized absorption spectra of d-BDP at various concentrations showing formation of intramolecular *H*-aggregates.

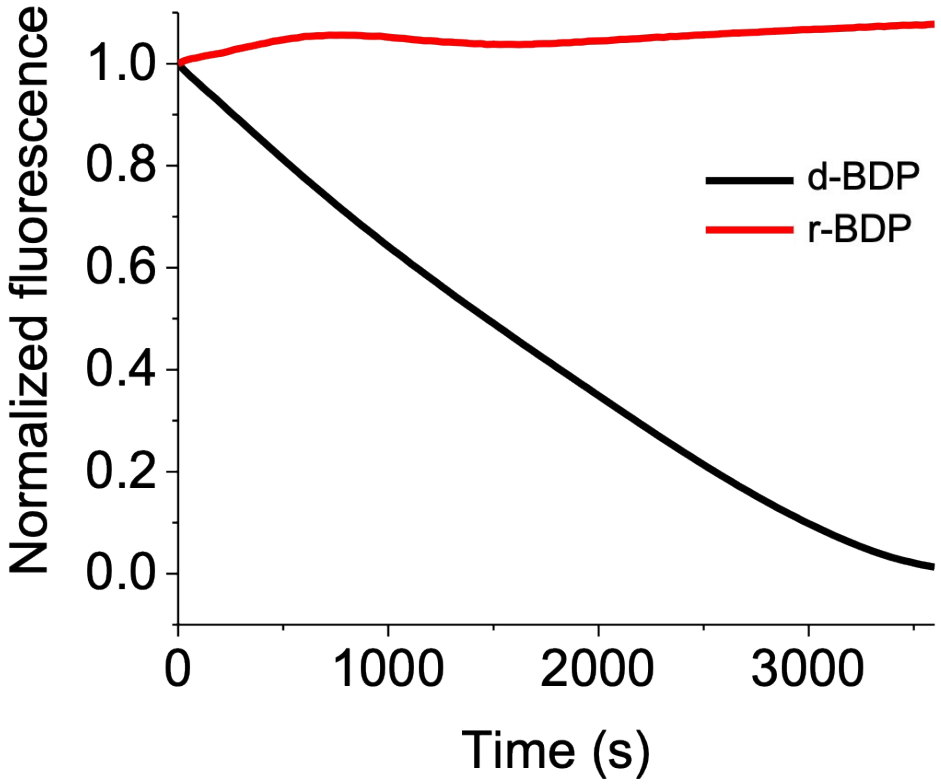


Figure S2. Florescence decay of **d-BDP** (1 µM) in MeOH and **r-BDP** (1 µM) in glycerol. Excitation at 456 nm, emission monitored at 505 nm.

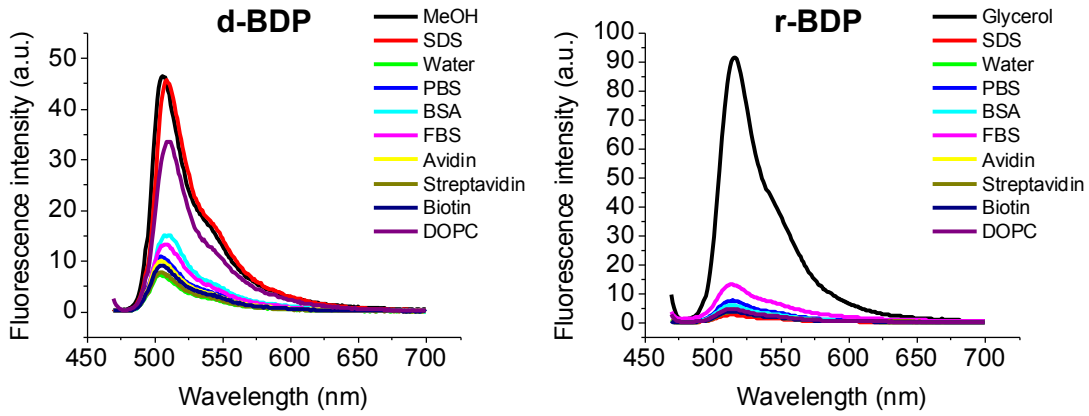


Figure S3. Emission spectra of biotin BDP probes in various conditions to assess the non-specific fluorogenicity. SDS (2 mg/mL), BSA (0.1 mg/mL), FBS (0.1 mg/mL), Avidin (0.1 µM), Streptavidin (0.1 µM), Biotin (100 µM). DOPC: Large unilamellar vesicles were prepared (100 nm diameter); DOPC concentration was 20 µM. Excitation was at 460 nm.

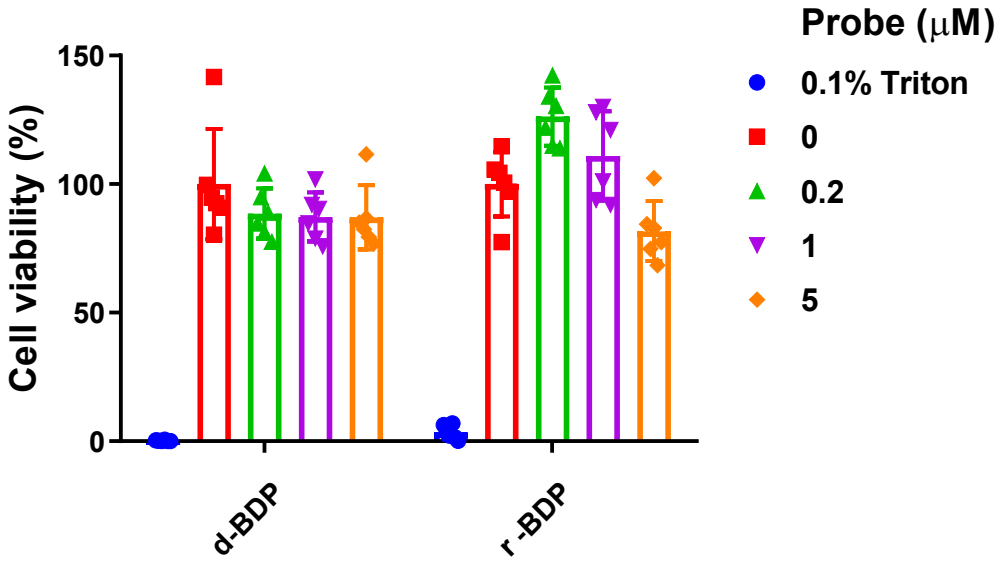


Figure S4. Cell viability test using the MTT assay on KB cells incubated for 24h with the BDP probes at indicated concentrations. Triton 0.1% was used as a negative control to provoke cell death.

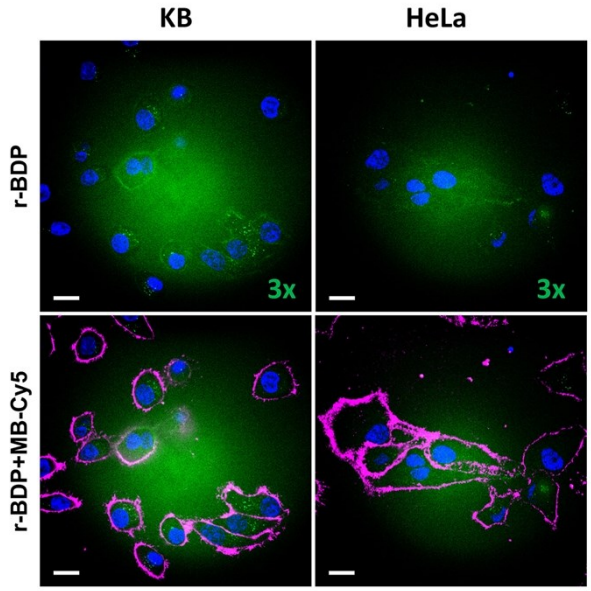


Figure S5. Confocal images of BR positive KB and Hela cells in the presence of r-BDP (200 nM) in green. The nucleus was stained with Hoechst (5 μg/mL, blue color) and the plasma membrane with MemBright Cy5 (20 nM, magenta color). The % laser power was increased by 3-fold for the green channel compared to Figure 2. Scale bar is 20 μm.

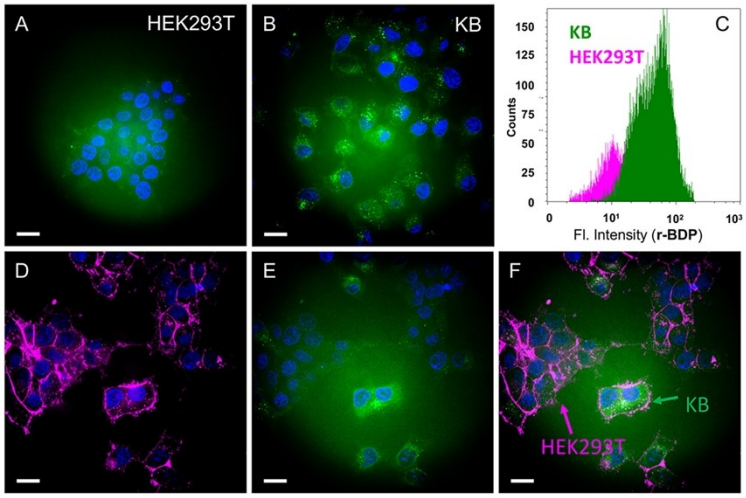


Figure S6. Optimized conditions for r-BDP. Confocal images of non-cancer HEK293T cells and cancer KB cells preincubated with **r-BDP** (1 μM) for 30 min. The nucleus was stained with Hoechst (5 μg/mL, blue color) and the plasma membrane with MemBright Cy5 (20 nM, magenta color). The % laser power was increased by 3-fold for the green channel compared to Figure 2. Scale bar is 20 μm.

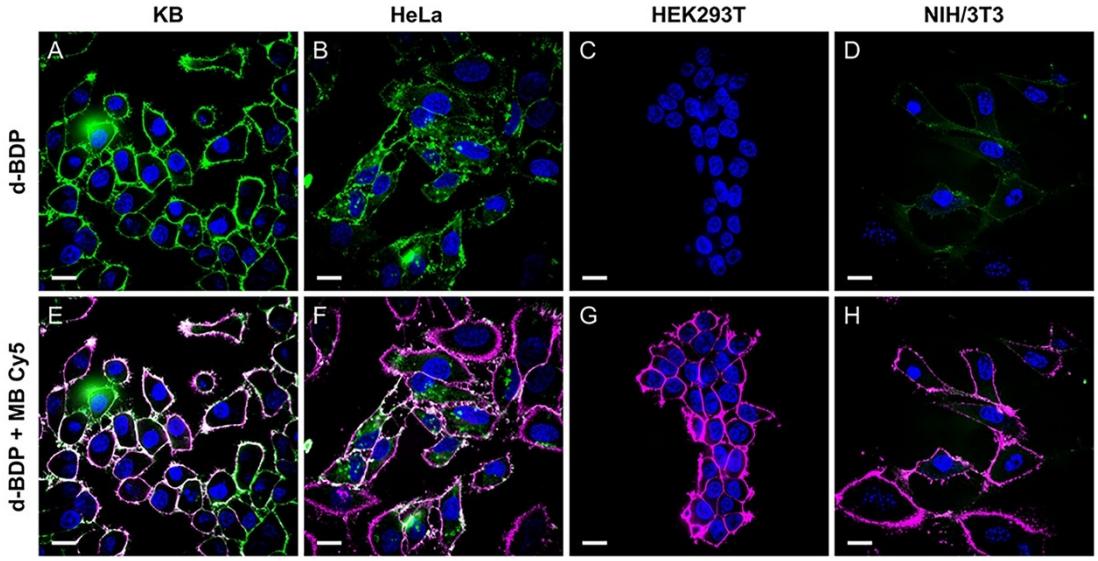


Figure S7. Confocal images of cancer HeLa (A, E) and KB (B, F) cells and non-cancer HEK293T (C, G) and NIH/3T3 (D, H) cells in the presence of **d-BDP** (200 nM). The nucleus was stained with Hoechst (5 μg/mL, blue color) and the plasma membrane with MemBright Cy5 (20 nM, magenta color). Scale bar is 20 μm.

References

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