Supporting Information for:

Overcoming the Limits of Hypoxia in Photodynamic Therapy: A Carbonic Anhydrase IX-Targeted Approach

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- **General information and materials**

UV/Vis and fluorescence were obtained using Shinco S-3100 and Shimadzu RF-5301PC spectrophotometers, respectively. ESI-MS and NMR spectra were collected on Shimadzu LCMS-2020 and Varian 400 MHz NMR or Bruker 500 MHz NMR spectrometers, respectively. All chemicals except for acetazolamide (TCI) and 2-(1H-9 azobenzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (Alfa Aeser) were purchased from Aldrich and used as received. Column chromatography was performed using silica gel 60 (70-230 mesh) as the stationary phase. Analytical thin layer chromatography was performed using 60 silica gel (pre-coated sheets with 0.25 mm thickness).

- **Synthesis of AZ-BPS and BPS**

Compounds **10**[S1], **9**[S2], **8**[S3], **6**[S4] and **5**[S4] were synthesized according to reported procedures.

Compound 7: A mixture of **8** (0.18 g, 0.65 mmol), HATU (0.25 g, 0.65 mmol) and DIPEA (0.11 mL, 0.65 mmol) in DMF (2 mL) was stirred at rt for 20 min. Propagylamine (0.042 mL, 0.65 mmol) and triethylamine (0.18 mL, 1.3 mmol) were then added at rt and the mixture was stirred for an additional 6 h at rt. After removal of the solvents under reduced pressure, the crude product was purified over silica gel using CH_2Cl_2/MeOH (v/v, 95:5) as the eluent to yield **7** as a white solid (0.14 g, 70%): ^{1}H NMR (DMSO-d_6): δ 13.0 (s, 1H), 8.40 (t, J = 5.50 Hz, 1H), 8.33 (s, 2H), 3.85 (m, 2H), 3.10 (s, 1H), 2.75 (t, J = 6.75 Hz, 2 H), 2.51 (m, 2 H). ^{13}C NMR (DMSO-d_6): δ 171.8, 170.5, 164.3, 161.2, 81.2, 73.1, 30.1, 29.2, 27.9. ESI-MS calc. for $C_9H_{11}N_5O_4S_2$ [M-H]$^-$ 316.03, found 315.90.

Compound 4: A mixture of 2,4-dimethyl pyrrole (0.23 g, 2.4 mmol), **10** (0.25 g, 1.1 mmol) and trifluoroacetic acid (1 drop) in DCM was stirred for 6 h at rt. *p*-Chloranil (0.27 g, 1.1 mmol) was then added and the mixture was stirred at rt for 1 h. Then triethylamine (3 mL) and boron trifluoride diethyl etherate (3 mL) were added sequentially and after stirring for 30 min. at rt, the crude product was extracted with DCM, washed with water and brine. The DCM layer was dried over anhydrous $MgSO_4$. After removal of the solvents under reduced pressure, the crude product was purified over silica gel using (n-hexane/EtOAc (v/v, 4:1) as the eluent to yield **4** as an orange solid (0.20 g, 79%): ^{1}H NMR ($CDCl_3$): δ 7.14 (d, J = 8.60 Hz, 2H), 6.99 (d, J = 8.60 Hz,

2H), 5.96 (s, 2H), 4.32 (t, J = 6.72 Hz, 2 H), 3.66 (t, J = 6.72 Hz, 2 H), 2.53 (s, 6H), 1.39 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 158.8, 155.4, 143.2, 141.6, 131.8, 129.4, 127.8, 121.2, 115.3, 68.0, 29.1, 14.6. ESI-MS calc. for $C_{21}H_{22}BBrF_2N_2O$ $[M+H]^+$ 447.10, found 447.15.

Compound 3: To a solution of **4** (0.18 g, 0.40 mmol) in DMF (10 mL) was added sodium azide (0.078 g, 1.2 mmol) and mixture was heated at 100 ºC for 12 h. The product was extracted with Et_2O, washed with water, and dried over anhydrous $MgSO_4$. Evaporation of the organic layer gave a crude product, which was purified over silica gel using n-hexane/EtOAc (v/v, 7:1) as the eluent to yield **3** as an orange solid (0.15 g, 91%): ^{1}H NMR ($CDCl_3$): δ 7.18 (d, J = 8.60 Hz, 2H), 7.03 (d, J = 8.60 Hz, 2H), 5.98 (s, 2H), 4.20 (t, J = 5.12 Hz, 2 H), 3.65 (t, J = 5.12 Hz, 2 H), 2.56 (s, 6H), 1.42 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 159.0, 155.6, 143.4, 141.7, 132.0, 129.6, 128.0, 121.4, 115.4, 67.1, 50.5, 14.9. ESI-MS calc. for $C_{21}H_{22}BF_2N_5O$ $[M+H]^+$ 410.19, found 410.30.

Compound 2: To a solution of **3** (0.14 g, 0.35 mmol) in CH_2Cl_2:DMF (4:1 mL) was added solution of *N*-bromosuccinimide (NBS) (0.12 mg, 0.70 mmol) in CH_2Cl_2 (5 mL). The mixture was then stirred for 1 h at rt. After the extraction and washing with water, the organic layer was dried with $MgSO_4$. After removal of the solvents, the crude product was purified over silica gel using (n-hexane/EtOAc (v/v, 4:1) as the eluent to yield **2** as a pink-orange solid (0.18 g, 90%): ^{1}H NMR ($CDCl_3$): δ 7.17 (d, J = 8.74 Hz, 2H), 7.06 (d, J = 8.74 Hz, 2H), 4.23 (t, J = 4.90 Hz, 2 H), 3.68 (t, J = 4.90 Hz, 2 H), 2.60 (s, 6H), 1.43 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 159.5, 154.1, 142.1, 141.0, 130.9, 129.5, 127.3, 115.6, 112.1, 67.3, 50.4, 14.2, 13.9. ESI-MS calc. for $C_{21}H_{20}BBr_2F_2N_5O$ $[M]^+$ 567.01, found 567.10.

Compound 1: A mixture of **2** (0.18 g, 0.32 mmol), **9** (0.21 g, 0.77 mmol), glacial acetic acid (0.5 mL), piperidine (0.5 mL) and a small amount of $Mg(ClO_4)_2$ in toluene (30 mL) was heated at reflux overnight. The water formed during the reaction was removed with a Dean−Stark apparatus. After removal of the solvents under reduced pressure, the crude product was purified over silica gel using CH_2Cl_2/MeOH (v/v, 97:3) as the eluent to yield **1** as a dark green solid (0.15 g, 44%): ^{1}H NMR ($CDCl_3$): δ 8.10 (d, J = 16.56 Hz, 2H), 7.64-7.58 (m, 6H), 7.17 (d, J = 8.54 Hz, 2H), 7.04 (d, J = 8.54 Hz, 2H), 6.96 (d, J = 9.00 Hz, 4H), 4.25-4.18 (m, 6H), 3.91-3.87 (m, 4H), 3.78-3.76 (m, 4H), 3.72-3.66 (m, 10H), 3.58-3.54 (m, 4H), 3.39 (s, 6H), 1.46 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 160.1, 159.2, 148.4, 141.1, 138.9, 138.7, 132.4, 129.9, 129.8, 129.3, 127.6,

116.2, 115.4, 115.1, 110.2, 72.0, 70.9, 70.7, 70.6, 69.8, 67.6, 67.1, 59.2, 50.3, 14.1. ESI-MS calc. for $C_{49}H_{56}BBr_2F_2N_5O_9$ $[M-H]^-$ 1066.25, found 1065.95.

BPS (BODIPY photosensitizer): A mixture of **5** (0.36 g, 0.71 mmol), **9** (0.46 g, 1.71 mmol), glacial acetic acid (1.2 mL), piperidine (1.2 mL) and a small amount of $Mg(ClO_4)_2$ in toluene (60 mL) was heated at reflux overnight. The water formed during the reaction was removed with a Dean−Stark apparatus. After removal of the solvents under reduced pressure, the crude product was purified over silica gel using CH_2Cl_2/MeOH (v/v, 95:5) as the eluent to yield **BPS** as a dark green solid (0.22 g, 31%): ^{1}H NMR ($CDCl_3$): δ 8.10 (d, J = 16.55 Hz, 2H), 7.64-7.59 (m, 6H), 7.18 (d, J = 8.66 Hz, 2H), 7.04 (d, J = 8.66 Hz, 2H), 6.97 (d, J = 8.89 Hz, 4H), 4.21-4.18 (m, 4H), 3.91-3.88 (m, 7H), 3.78-3.75 (m, 4H), 3.72-3.66 (m, 8H), 3.58-3.55 (m, 4H), 3.39 (s, 6H), 1.47 (s, 6H). ^{13}C NMR ($CDCl_3$): δ 160.7, 160.2, 148.6, 141.3, 139.2, 139.0, 132.6, 130.1, 129.9, 129.5, 127.1, 116.4, 115.2, 115.0, 110.3, 72.2, 71.1, 70.9, 70.8, 69.9, 67.8, 59.3, 55.6, 29.9, 14.2. ESI-MS calc. for $C_{48}H_{55}BBr_2F_2N_2O_9$ $[M-H]^-$ 1011.23, found 1011.00.

AZ-BPS (Acetazolamide-conjugate BODIPY photosensitizer): A mixture of **1** (0.15 g, 0.14 mmol), **9** (0.045 mg, 0.14 mmol), and sodium ascorbate (10 mol %) in DMF:MeOH (2:1 mL) was stirred for 15 min at rt. Then 5 mol % of $CuSO_4$ in 0.5 mL water was added to the reaction mixture, which was stirred for 6 h. After removal of the solvents under reduced pressure, the crude mixture was purified over silica gel using CH_2Cl_2/MeOH (v/v, 95:5) as the eluent to yield **AZ-BPS** as a dark green solid (0.11 g, 57%): ^{1}H NMR ($CDCl_3$+DMSO-d_6): δ 8.44 (t, J = 6.17 Hz, 1H), 8.32 (t, J = 5.83 Hz, 1H), 8.19 (br, 2H), 8.09-8.04 (m, 3H), 7.59 (d, J = 8.95 Hz, 4H), 7.50 (d, J = 16.59 Hz, 2H), 7.29 (d, J = 8.79 Hz, 2H), 7.13 (d, J = 8.79 Hz, 2H), 7.03 (d, J = 16.59 Hz, 4H), 4.81 (t, J = 5.34 Hz, 2H), 4.49 (t, J = 5.34 Hz, 2H), 4.39 (d, J = 5.73 Hz, 2H), 4.21-4.17 (m, 4H), 3.85-3.81 (m, 4H), 3.69-3.65 (m, 4H), 3.63-3.57 (m, 8H), 3.50-3.47 (m, 4H), 3.31 (s, 6H), 2.82 (t, J = 7.18 Hz, 2H), 2.59 (t, J = 7.18 Hz, 2H), 1.46 (s, 6H). ^{13}C NMR ($CDCl_3$+DMSO-d_6): δ 171.3, 171.1, 164.2, 161.4, 159.7, 158.5, 147.8, 144.9, 140.7, 138.4, 131.8, 129.4, 129.2, 128.8, 127.0, 123.2, 115.6, 115.1, 114.6, 109.7, 71.4, 70.4, 70.2, 70.0, 69.2, 67.2, 66.0, 58.5, 49.2, 34.6, 30.6, 29.8, 13.6. ESI-MS calc. for $C_{58}H_{67}BBr_2F_2N_{10}O_{13}S_2$ $[M-H]^-$ 1383.27, found 1382.65.

- **Spectroscopic data**

Stock solutions of **AZ-BPS** and **BPS** (0.5 mM) were prepared in DMSO (dimethyl sulfoxide). For all measurements of the fluorescence spectra, an excitation wavelength of 660 nm was used with the excitation and emission slit widths at 5 nm. All UV/Vis and fluorescence experiments were performed using 5.0 μM solutions of either **AZ-BPS** or **BPS**, with the spectra recorded at rt.

- **Calculation of the fluorescence quantum yield (Φ_f)**

Fluorescence quantum yields were determined by comparing the fluorescence intensity of the sample (*PS*: photosensitizer) with that of a fluorescence standard (R: reference) using the following equation:

$$\Phi_f(PS) = \Phi_f(R) \left(\frac{A_{PS}}{A_R}\right)\left(\frac{{n_{PS}}^2}{{n_R}^2}\right) \qquad \text{----- Eqn. S1}$$

where, A_{PS} and A_R are the integrated area under the corrected fluorescence spectra for the sample (*PS*) and reference (*R*), n_{PS} and n_R are the refractive indexes of the sample (*PS*) and reference (*R*), respectively. The reference used was zinc phthalocyanine for which the fluorescence quantum yield (Φ) in toluene containing 1.0 % pyridine is 0.30.[S5]

- **Calculation of the singlet oxygen quantum yield (Φ_Δ)**

Singlet oxygen quantum yields (Φ_Δ) were calculated according to a reported procedure.[S6] The relative quantum yields were calculated with that of the reference (*R*) (Methylene Blue; MB) in DMSO being 0.52.[S7] The absorbance of 1,3-diphenylisobenzofuran (DPBF) was adjusted by modifying the concentration such that it was around 1.0 in air-saturated DMSO after placing in a cuvette. Then, a DMSO solution of the PS (PS: photosensitizer) was added to the cuvette and DMSO added such that the absorbance of the putative photosensitizer in question was around 0.1. After measurements were taken in the dark, the cuvette was exposed to monochromatic light at the peak absorption wavelength for 1.5 or 3 minutes. The absorbance was collected several times after each irradiation. The recorded data are shown in Figs. 4 and S2. The absorbance maxima of DPBF at 408 nm was then plotted versus time and the slope was determined for each photosensitizer. Singlet oxygen quantum yields were calculated according to the Eqn.S2:[S6]

$$\Phi_\Delta(PS) = \Phi_\Delta(R) \times \left(\frac{m_{PS}}{m_R}\right) \times \left(\frac{F_R}{F_{PS}}\right) \times \left(\frac{PF_R}{PF_{PS}}\right) \qquad \text{----- Eqn. S2}$$

m is the slope of a plot of the change in absorption of DPBF at 408 nm at the irradiation time in question as recorded in the presence of the *PS*, *F* is the absorption correction factor, which can be determined by $F = 1 - 10^{-OD}$ (OD at the irradiation wavelength), and *PF* is an absorbed photonic flux (μ Einstein dm^{-3} s^{-1}).

- **Isothermal titration calorimetry (ITC) analysis**

Isothermal titration calorimetry (ITC) experiments were performed using VP-ITC instrument (Microcal, Inc., USA) with medium containing CAIX (50 μM) in the cell and medium containing **AZ-BPS** (700 μM) in the injection syringe. A typical experiment consisted of 20 injections (10 μL each) within 3 min intervals. Experiments were performed at 37°C using a 10 mM sodium phosphate buffer containing 50 mM NaCl at pH 7.4 and a DMSO concentration of 10% in both the syringe and the cell. The solvent used in both the cell and syringe consisted of 10% DMSO in PBS buffer.

- **Molecular docking studies and hybrid quantum mechanics/molecular mechanics molecular dynamics (QM/MM MD) simulations**

The CAIX structure was obtained from the Protein Data Bank (PDB ID: 5FL4[S8]). A grid box was constructed to cover pharmacophores with spacing of 0.2 Å. Docking parameters were set to 50 runs and 2,500,000 energy evaluations for each cycle by Autodock 4.2 and AutodockTools 1.5.6[S9] using the Lamarckian genetic algorithm. The five dominating binding modes were obtained for further QM/MM MD simulations. The force field of **AZ-BPS** was generated by density functional theory (DFT) calculations employing the M06 exchange function with 6-31G* basis sets, using the Gaussian 09 suite.[S10] In QM/MM MD simulations, M06 with 6-31G was selected for the QM part; the ff14SB force field and Tip3p water model were selected for MM part. Five independent QM/MM MD simulations were calculated using the Amber14 software package.[S11] Gromacs 5.0,[S12] APBS 1.3,[S13] and g_mmpbsa[S14] were used for further free energy analyses. In the simulations, the Zn^{2+}, binding side chain of histidines, and AZ except amide bond, and the boron binding site of **BPS** were treated via QM; the rest of the system was treated using MM. The SHAKE algorithm was applied to constrain bonds, including those involving hydrogen. The Particle-mesh Ewald summation method was used to describe long-range electrostatics. The value

of the MM nonbonded cutoff and the QM cutoff were set as 10 Å. The distance between system and box edge was more than 10 Å. The temperature was controlled using a Langevin thermostat at 298.15 K with a coupling constant of 1.0 ps. The time step was 0.5 ps. The 10 ps QM/MM MD simulation data were collected. The binding free energies were obtained from the last 5 ps converging trajectories using the Molecular Mechanics/Poisson-Boltzmann Surface Area (MM/PBSA) method.

- **Determination of octanol-water partition coefficient (log P_{oct})**

The 1-octanol-water partition coefficient (Log P_{oct}) was calculated according to a reported procedure.[S15] Firstly, Solution A and Solution B were prepared by pre-mixing 1-octanol and PBS buffer (10 mM, pH 7.4) in a ratio of 1:4 and 4:1, respectively. Then, the solutions were stirred at rt for 24 h. Thereafter, small aliquots (10 μL) of a 20 mM probe solution in DMSO was added to Solution A and Solution B (each 5 ml), respectively. The above solutions were then mixed and stirred for 30 min. The concentration of the probe in each layer was measured by UV-Vis spectroscopy using the molar coefficients of the probes. The log P_{oct} value was calculated using following equation:

$\log P_{oct} = \log [probe]_{oct} - \log [probe]_{PBS}$

The log P_{oct} values for **AZ-BPS** and **BPS** were found to be 0.76 and 1.38, respectively, as noted in the main text.

- **Cell culture**

To examine endogenous expression of CAIX, five human carcinoma cell lines; two breast cancer cells (MDA-MB-231 and MCF7), a gastrointestinal carcinoma cell (AGS) and a lung cancer cell (A549), and a cervical cancer cell (HeLa), were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea). Additionally, a neonatal foreskin cell line; human fibroblast cell (BJ), was purchased from Modern Cell & Tissue Technologies (MCTT, Seoul, Republic of Korea). Either Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY, USA) or Roswell Park Memorial Institute medium (RPMI-1640, GIBCO, Grand Island, NY, USA) were used for cell culture analyses after adding 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY, USA), and 1% penicillin and streptomycin (GIBCO, Grand Island, NY, USA). All cultured cells were

maintained at 37 °C in a humidified atmosphere containing 5% of CO_2. Sub-culturing was performed when the cell density reached 60–80% of confluence. Fresh culture medium was added approximately every 2 to 3 days after PBS washing.

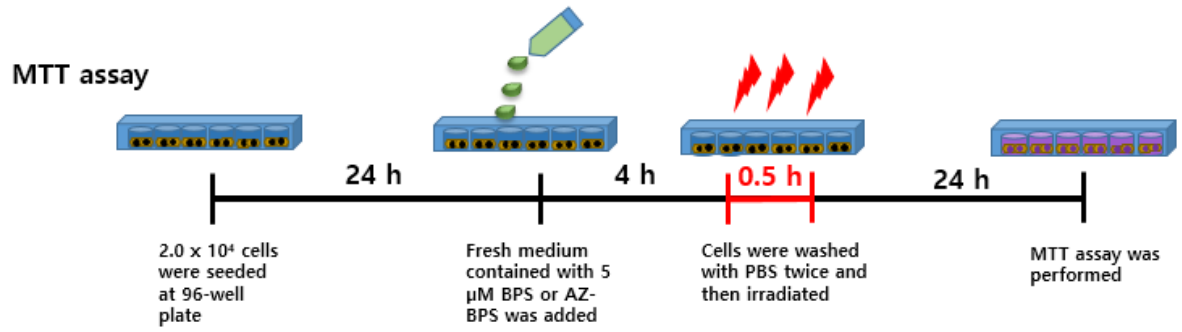
- **Endogenous CAIX expression in various cancer cell lines and CAIX inhibition of AZ-BPS by western blot analysis**

Prior to further analyses, endogenous CAIX expression at the protein level was confirmed by western blotting. The protein extract of cells from all five cancer cell lines (AGS, A549, MDA-MB-231, MCF7, and HeLa) and a normal cell line (BJ) were sampled when the confluence reached 70%. Additionally, CAIX inhibition by AZ-BPS was also validated by means of Western blots. Briefly, the attached cells were washed with ice-cold PBS three times and scraped to collect cell pellets. After removal of the PBS, a radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors provided by the manufacturer (Up-state, Lake Placid, NY, USA) was added into the cell pellets to obtain protein lysates. A Bradford assay was conducted to measure the protein concentration of each cell lines and then protein (30 μg/lane/cell line) from each cell line was loaded onto a sodium dodecyl sulfate poly-acrylamide gel electrophoresis gel (SDS-PAGE) for separating the proteins into bands. The separated protein bands were transferred to nitrocellulose membranes (Schleicher & Schull, Dassel, Germany). The membranes were incubated with GAPDH antibodies (Abcam, Cambridge, UK; ab37168) at 1/100 or CAIX antibodies (Abcam, Cambridge, UK; ab184006) at a 1/1000 dilution overnight at 4°C. The resulting membranes were rinsed with Tris-buffered saline Tween-20 (TBS-T) three times, and incubated with the anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. To detect immune-reactive protein bands, enhanced chemiluminescence reagents (Luminate, Merk Millipore, Billerica, MA, USA) were used according to the manufacturer's instructions. The Western blots were quantified using ImageJ (latest version, National Institute of Health, Bethesda, MD, USA).

- **MTT analysis**

The CAIX-positive cell line (MDA-MB-231), as confirmed by the above analysis, was selected to study the cytotoxicity of **BPS** and **AZ-BPS**. Prior to the cytotoxicity tests, the cells were washed

with phosphate-buffered saline (PBS). The culture medium was exchanged for a FBS-free culture medium. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Life Technologies, Carlsbad, CA, USA) was used to assess cytotoxicity in accord with the manufacturer's instructions. Briefly, 2.0×10^4 cells were seeded in each well in a 96-well plate. Twenty-four hours after seeding, the culture medium was exchanged with fresh medium (80 μl) containing 5 μM of either **BPS** or **AZ-BPS** for 4 h. Cells were then irradiated using a CW laser (COMPACT Laser 660 nm, World Star Tech; 2 W/cm^2 (irradiance), 3600 J/cm^2 fluence) to induce photo-cytotoxicity. Cells were incubated at 37 °C for an additional 24 h and then a 12 mM MTT solution (100 μl) was added to each well. For negative controls, 100 μl of the MTT stock solution or distilled water were added per well in the absence of either compound. After a 24 h incubation period, the culture medium was removed and 50 μl of DMSO were added. After a 15 min-incubation period, the DMSO supernatant from each well was transferred in a 1:1 mapping fashion into a white 96-well plate to measure absorbance using a spectrophotometer at 540 nm (PowerWave XS, Bio-Tek, Winooski, VT, USA).



- **PI and Calcein AM staining**

Fluorescent imaging of cell viability was carried out using the acetoxymethyl diacetyl ester of Calcein AM (Acetoxymethyl ester of calcein; Sigma, Molecular Probe, green) and PI (Propidium iodide; Sigma, red) staining. Cells (1.5×10^4) were seeded in 35-mm glass-bottom confocal dishes (SPL, Seoul, Republic of Korea) 2 days prior to staining. Cells were treated with either medium containing **BPS** or **AZ-BPS** (5 μM, respectively) for 4 h and irradiated by a CW laser (COMPACT Laser 660 nm, World Star Tech; 2.0 W/cm^2 irradiance) over the course of 5 to 20 min to induce photo-cytotoxicity after washing out the **BPS**-based photosensitizer in question with phosphate-buffered saline (PBS) twice. The following day, the cells were incubated with 5 μM PI and 15 μM

of Calcein AM for 30 min. The red PI emission feature (if any) was visualized in the nucleus while Calcein (as an esterase substrate in the cytosol of live cells) was observed as a green signal; a fluorescence microscope (Carl Zeiss) was used for these studies. The experiment was conducted at room temperature.

- ***In vitro* cell imaging**

The five cancer cell lines and the normal cell line noted above were seeded in 35-mm glass-bottom confocal dishes (SPL, Seoul, Republic of Korea) and allowed to stabilize for two days. When the cell density reached 2.0×10^6 in the 35-mm confocal dish, all cells were treated with medium containing **BPS** or **AZ-BPS** (5 μM, respectively) for 4 h at 37 °C in 5% CO_2. Then, the cells were washed with PBS twice and FBS-free RPMI 1640 or DMEM culture medium was added. To obtain tumor spheroids that were considered to mimic *in vivo* tumor growth, 6.0×10^6 cells were seeded in 60 mm Corning ultra-low attachment culture dishes (Sigma). On the second day, the cells were treated with medium containing 5 μM **AZ-BPS** for 24 h. The cells and tumor spheroids were again washed three times with PBS, and florescent images were taken using a confocal laser scanning microscope (Carl-Zeiss LSM 5 Exciter, Oberko, Germany) using excitation and emission wavelengths of 639 nm and 650-750 nm, respectively.

- **Co-localization experiments**

To examine the intracellular localization of **BPS** and **AZ-BPS**, MCF7 and MDA-MB-231 cells were seeded and allowed to grow to 2.0×10^6 per dish in 35-mm glass bottom confocal dishes (SPL). Cells were incubated with medium containing **BPS** or **AZ-BPS** (5 μM, respectively) for 4 h. The cells were then washed three times with PBS. Mito-tracker Green FM (Thermo Fisher, New Hampshire, USA, Ex/Em 490/516 nm), ER-tracker Green (Thermo Fisher, Ex/Em 374/430-640 nm), or Lyso-tracker Green DND-26 (Thermo Fisher, Ex/Em 504/511 nm), 50 μl of each, were then added and the cells incubated for 30 min. The intracellular localization of **BPS** and **AZ-BPS** were inferred by imaging the overlapped fluorescence from the test agent and the dye in question using a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) using excitation and emission wavelengths of 639 nm and 650-750 nm, respectively. The overlapping fluorescent intensity was quantified in the form of a Pearson correlation coefficient

using the ImageJ free-software package (National Institute Health, USA, https://imagej.nih.gov/ij/).

- **Flow cytometry analysis**

Prior to FACS analysis, MDA-MB-231 cells were irradiated using 660 nm laser irradiation (2 W/cm ^{2}, 30 min; 3600 J/cm^2) after incubating for 4 h with medium containing **BPS** or **AZ-BPS** (5 μM, respectively). The cell colonies were then dissociated into single cells by subjecting to 4% paraformaldehyde fixation (Sigma) for 20 min at room temperature. Cells were permeabilized in 0.1% Triton X-100 for 10 min and blocked in a 10% secondary antibody host serum in 0.5% BSA for 1 h. PI (5 μM) was added and the cells incubated for 30 min prior to analyzing using a BD-FACS Caliber Flow Cytometer (FACS Calibur, Becton Dickinson (BD) biosciences, San Jose,CA, USA). Cells not treated with either agent were used as controls. Three independent experiments were performed and the resulting data was analyzed using the CellQuest software (BD).

- **Reverse transcription polymerase chain reaction (RT-PCR)**

To examine the potential mode of action of **AZ-BPS**, the expression of angiogenetic and apoptotic related genes of MCF7 (CAIX negative cell line) and MDA-MB-231 (CAIX positive cell line) cells were measured 4 h after incubation with medium containing **BPS** or **AZ-BPS** (5 μM, respectively) and photo-irradiation with a 660 nm laser (2 W/cm ^{2}, 30 min; 3600 J/cm^2). The total RNA from the cells was isolated using 200 μl of the TRIzol Reagent (Invitrogen). A traditional reverse transcription analysis (Promega, Madison, WI) was carried out according to the manufacture's instruction. Takara Ex Tag DNA polymerase (Takara, Tokyo, Japan) was utilized for the PCR amplification of the different genes, with a program of 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 58°C-60°C for 30 seconds, 72°C for 30 seconds, and extension at 72°C for 10 minutes. The primers and PCR conditions used are shown in Table S1.

Table S1 Primer information used in RT-PCR analysis.

Name of Gene	Forward primer sequence	Reward primer sequence	Annealing Tm (°C) Product size (bp)
CD31	AGACGTGCAGTACACGGAAG	ATCTGCTTTCCACGGCATCA	60(125)
VEGFA	TGA GCT TCC TAC AGC ACA ACA A	AAC GCT CCA GGA CTT ATA CCG	60(137)
TYMP2 v2	GCG AAC CCT GAA CCC TAC G	CAA GGC TGC CAT CGC TCC	60(116)
ANGPT2 v2	AGA ACC AGA CGG CTG TGA TG	GGG AGT GTT CCA AGA GCT GA	60(141)
ANGPT2 v3	GAT GTC CAC ATC AAA CTC AGC	CCA TTC GTG GTG TGT CCT GA	58 (105)
CASP-9	TCGAGGACATCCAGCGGGCA	GCCAGCATGTCCTGGCCTGT	60 (130)
CASP-3	TGGTGTTGATGATGACATGGCGTG	AGCATGGCACAAAGCGACTGGA	60 (130)
Cyto C	CTAAAAAGAGGTGAGAGCGGGT	AGTCCAGGGTCTTCACTCCC	60 (154)
BAD	CGAGTGAGCAGGAAGACTCC	CTGTGCTGCCCAGAGGTT	58 (299)
BAX	CCCTTTTGCTTCAGGGTTTC	GCCACTCGGAAAAAGACCTC	50 (119)
TAIL	GCGCAGCGAGTGGGACAGAG	GGCACTGGGTCCGTGCTGTC	60(130)
FASL	GGATTGGGCCTGGGGATGTTTCA	TTGTGGCTCAGGGGCAGGTTGTTG	58(334)
BCRP	GGTGGAGGCAAATCTTCGTTATTAGA	GAGTGCCCATCACAACATCATCTT	59(154)
GAPDH	TTCAGTGGTGGACCTGACCT	CACCACCCTGTTGCTGTAGC	60(256)

- **Transmission electron microscopy (TEM)**

MDA-MB-231 cells were treated with medium containing **BPS** or **AZ-BPS** (5 μM, respectively) for 4 h and irradiated with a 660 nm laser (2 W/cm ^{2}, 30 min; 3600 J/cm^2). The cells were incubated for 24 h and prepared in accord with the methods described previously.[S16] Briefly, cells were fixed in ice-cold 2.5% glutaraldehyde-2% PFA in 0.1 M phosphate buffer (pH 7.4) for 2 h. The cells were then washed with 0.1 M PBS and post-fixed in 1% OsO_4 in 0.1 M PBS for 1 h in the dark. The post-fixed cells were dehydrated and infiltrated with propylene for embedding in an Epon812 mixture (Sigma). The resulting blocks were cut into ultrathin sections (5-6 μm) using an Ultra-cut UCT (Leica, Germany). The sections were stained with uranyl acetate and lead citrate before being examined using a transmission electron microscope (H-7600; Hitachi, Japan) at an accelerating voltage of 80 kV.

- **Mouse xenograft model**

To examine the efficacy of **BPS** and **AZ-BPS** *in vivo*, male BALB/c nude mice (6- to 8-weeks old) were obtained from Central Lab (Seoul, Republic of Korea). The mice were randomly assigned to control, **BPS**, or **AZ-BPS** groups (n = 6 per treatment group). When the animals were arrived, they were allowed to acclimatize for two days at the animal facility and maintained according to the

Korean University Guide for the Care and Use of Laboratory Animals. This guide is based on the protocols recommended by the National Institutes of Health, USA. All procedures involving animals were approved by the Korea University Institutional Animal Care and Use Committee (IACUC). Approximately 6.0 x 10^6 MDA-MB-231 cells were mixed 1:1 with matrigel (BD, San Jose, California, USA) and injected subcutaneously into the right and left flanks and right and left shoulders (four sites per mouse).

- ***In vivo* diagnostic imaging and photo-cytotoxic efficacy**

All mice were obtained from Jung Ang Laboratory Animal Company, Seoul, Republic of Korea. After they were delivered, at least 3 days of accommodated and randomly assigned to each treatment. In order to evaluate the diagnostic efficacy of **BPS** or **AZ-BPS** *in vivo*, MDA-MB-231-inoculated xenograft mice (BALB/c nude; 6- to 8 weeks old; male) were subject to a single injection (medium containing **BPS** or **AZ-BPS** (500 μM, respectively) in 0.2 ml PBS vs. 0.2 ml PBS only used as a control) prior to subjecting to *in vivo* spectral fluorescent images. Fluorescent images were obtained 3 h after administration using a Maestro™ In-Vivo Fluorescence Imaging System (Maestro, CRi Inc., Woburn, MA, USA). Excitation was effected at 639 nm and the emission detected over the 650 to 750 nm spectral region.

To validate the photo-cytotoxic efficacy, three weeks after inoculation of MDA-MB-231 cells (6.0 x 10^6) into the four sites noted above, the mice (n = 6 per treatment) were treated with medium containing **BPS**, **AZ-BPS** (500 μM, respectively), or PBS control via tail vein injection (three times per week for 3 weeks, total 9 injections). Tumor sites were either subject to 660 nm laser irradiation (2 W/cm ^{2}, 30 min; 3600 J/cm^2) 3 h post injection or kept protected from anything other than ambient light. Tumor volumes were measured and recorded weekly using a clipper. Tumor volumes were calculated using the expression, V = length x (width)^2. Following the 8th week of injection and irradiation, mice were sacrificed using CO_2 gas and the tumor and body weights measured.

- **Immunohistochemical analysis**

The grafted tumor tissues from animals injected with **BPS** or **AZ-BPS** were dissected at the 8-week time point and immediately embedded into optimal cutting temperature compound (OCT

compound, Sakura Finetek, Torrance, USA). Embedded tumor tissues were sectioned (5 μm thick) using a microtome (Leica VT1200S, Wetzlar, Germany). Each section was transferred to an adhesion microscope glass-slide (Paul Mariendeld GmbH & Co.KG, Lauda-Königshofen, Germany) and fixed with absolute ethanol for 3~5 min. To prevent non-specific binding of the antibody and to reduce background interference, the tumor sections were blocked with 10% donkey serum (Sigma) in PBS for 1.5 h. After blocking, the tissue sections were incubated overnight at 4°C with CD31 antibody (BD; 550274) at 1/50 or CAIX antibody (Abcam, Cambridge, UK; ab184006) at a 1/1000 dilution level using 10% donkey serum in PBS. The next day, the tissue sections were washed with PBS and incubated with anti-rabbit secondary antibody (Santa Cruz Biotechnology) at room temperature. After 2 h, each section was washed with PBS three times and then medium containing **AZ-BPS** (10 μM) was added. Nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI; Sigma) for 6 min and then washed with PBS. Finally, the tumor sections were mounted using Immumount (Thermo Fisher). Images were taken using an Axiovert imager M1 fluorescence microscope (Zeiss, Oberkochen, Germany).

- **Statistical analysis**

All data was analyzed using the SAS software (version 8.2, Cary, NC, USA) or the Origin software package (version 9.1, Northampton, MA, USA). All experiment were repeated independently three times. To examine the possible *in vivo* theranostic effects of **BPS** and **AZ-BPS**, six mice per treatment group (n = 6 mice) were studied. The results obtained were subject to one-way analysis of variance (ANOVA) to determine statistical significance. If the ANOVAs results revealed a significant difference, then a two-tailed Student's t-test was carried out. P-values of less than 0.05 were considered as being statistically significant.

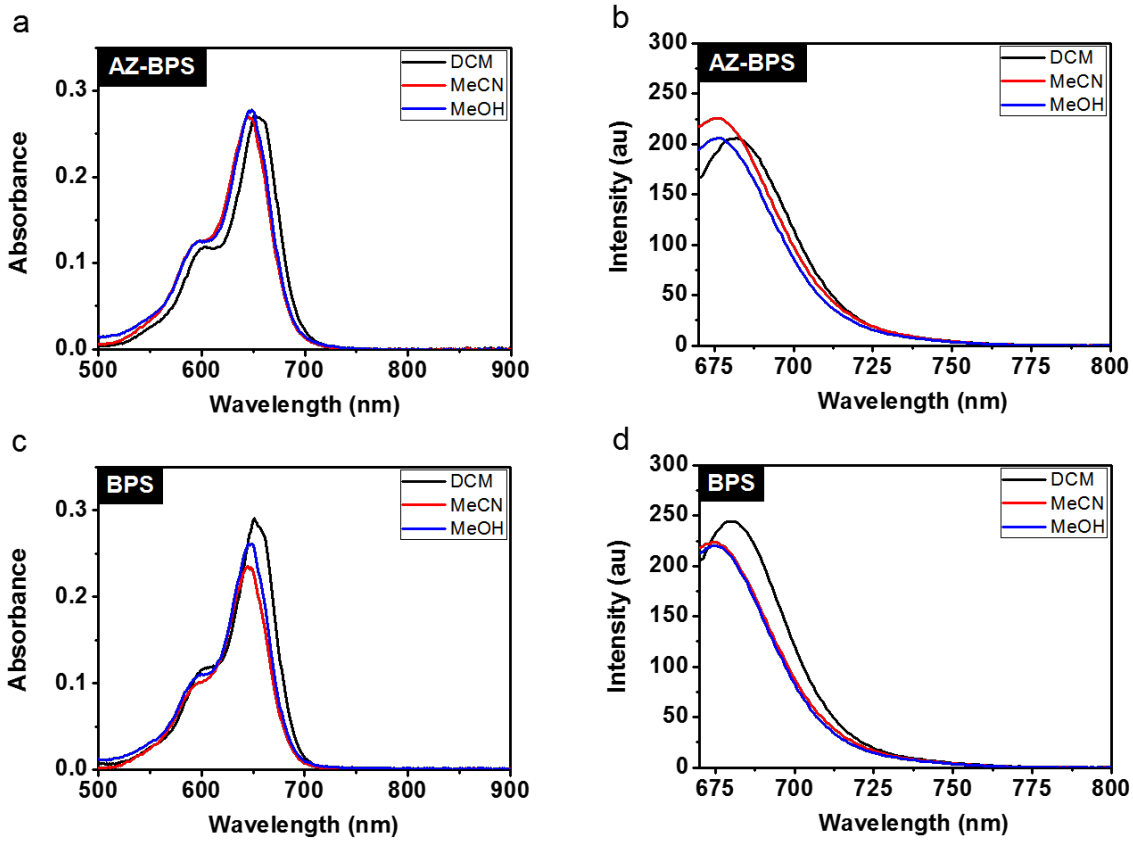


Figure S1. (**a**, **c**) Absorption and (**b**, **d**) emission spectra of **AZ-BPS** (top) and **BPS** (bottom) (5.0 μM, each), respectively, in various solvents (DCM, MeCN and MeOH). The excitation wavelength was 660 nm (slit width = 5/5) in all cases.

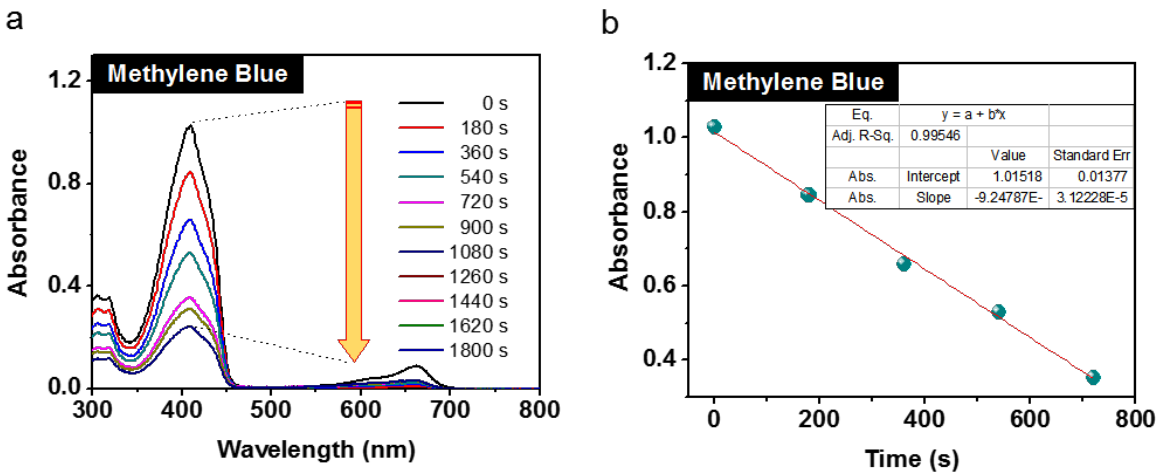


Figure S2. (**a**) Decrease in absorbance intensity of a trap molecule (DBPF) seen in the presence of methylene blue in DMSO upon photo-irradiation. (λ_{ex} = 660 nm, slit width = 15-15, Xe-lamp). (**b**) Plot of the decrease in absorbance of DPBF at 408 nm observed in the presence of methylene blue in DMSO as a function of irradiation time.

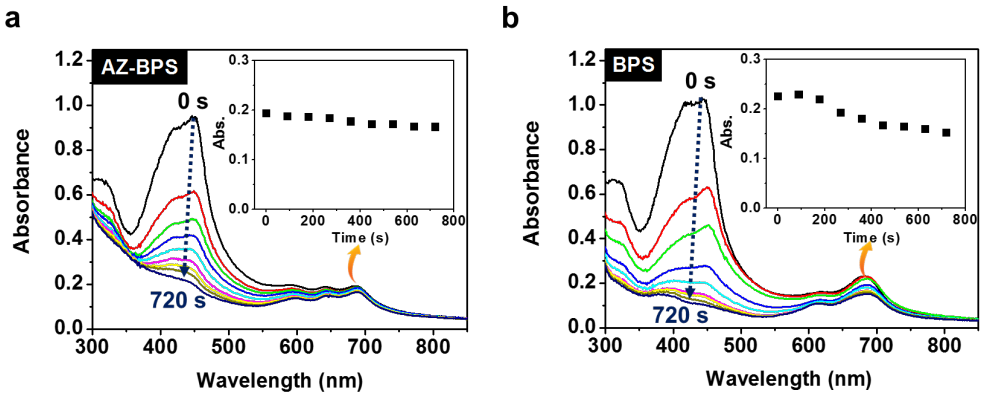


Figure S3. Time-dependent absorption spectral changes seen for 10% DMSO-buffer solutions (pH 7.4, 10 mM PBS) of DPBF and (a) **AZ-BPS** or (b) **BPS** upon irradiation at 660 nm (slit width = 15-15, Xe-lamp). Inset: Plots of the change in the absorption maximum of **AZ-BPS** or **BPS** as a function of time.

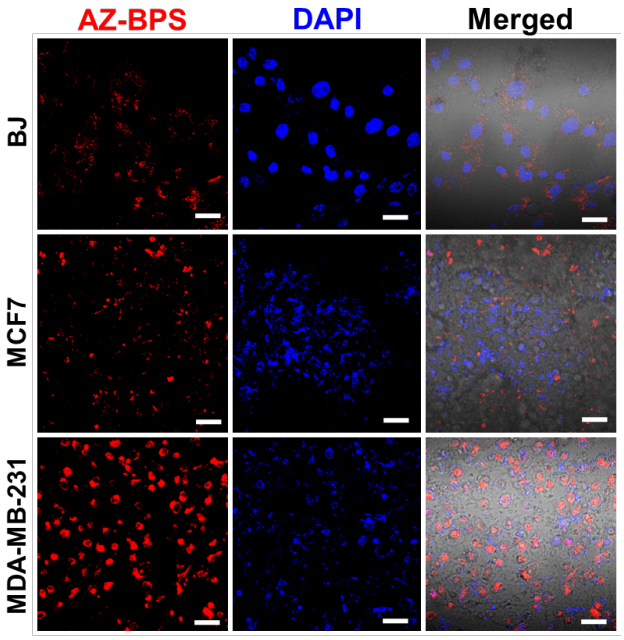


Figure S4. Confocal microscopic images of normal fibroblast (BJ) cells, MCF-7 cells, and MDA-MB-231 cells after incubation with **AZ-BPS** (5 μM) for 4 h.

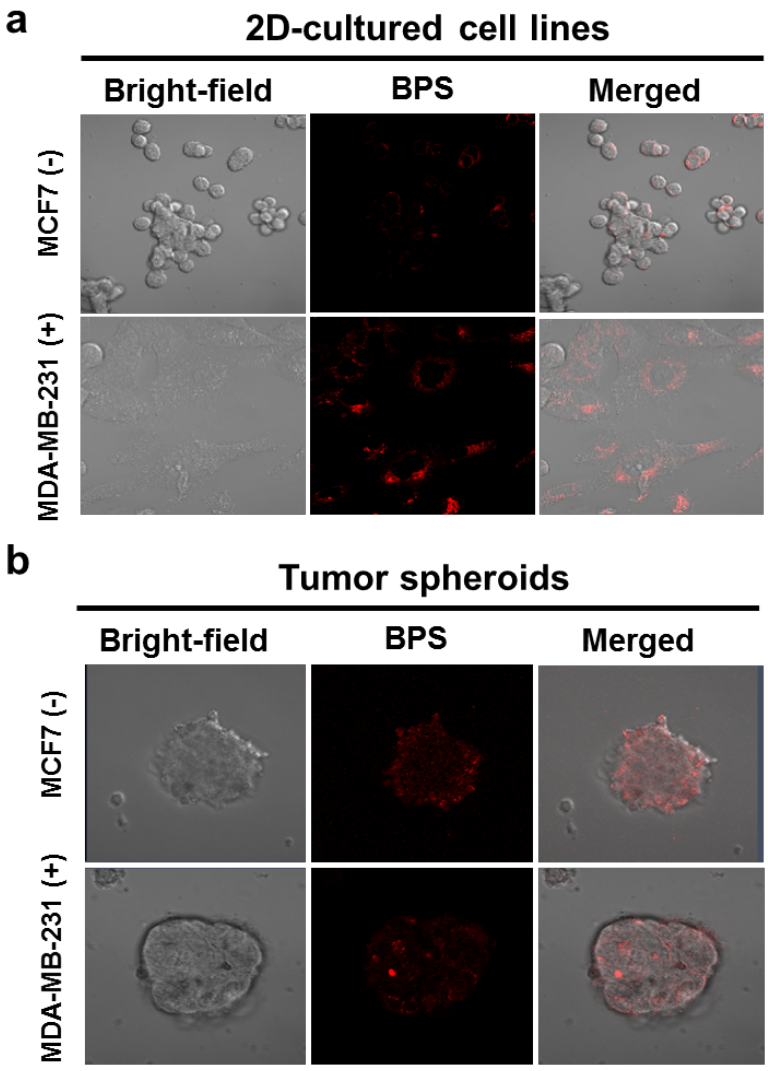


Figure S5. CAIX-dependent specificity of **BPS** in adherent cells and tumor spheroids. (a) Confocal microscopic images of MDA-MB-231 cells and MCF-7 cells after incubation with **BPS** (5 μM) for 4 h. (b) Confocal microscopic images of tumor spheroids formed from MDA-MB-231 cells and CAIX-negative MCF-7 cells after incubation with **BPS** (5 μM) for 4 h. Magnification 400x. To obtain tumor spheroids 6.0×10^6 cells were seeded in 60 mm Corning ultra-low attachment culture dishes (Sigma). **BPS** treatment was commenced on the second day following tumor spheroid formation.

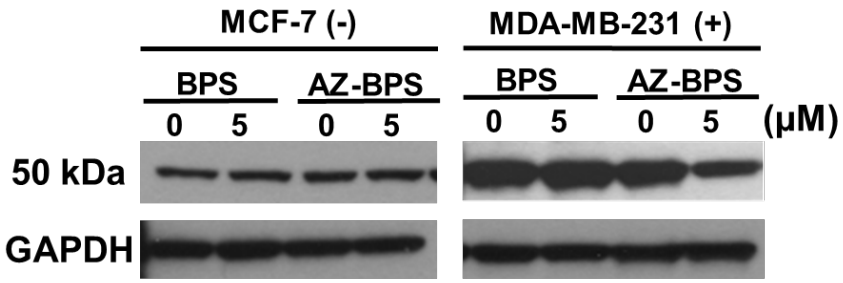


Figure 6. Endogenous CAIX-inhibition observed in the case of **AZ-BPS** and **BPS** in MDA-MB-231 cell lysates. MDA-MB-231 cells were treated 5 μM of **AZ-BPS** and **BPS** for 4 h. Inhibited CAIX expression at the protein level was determined by Western blotting.

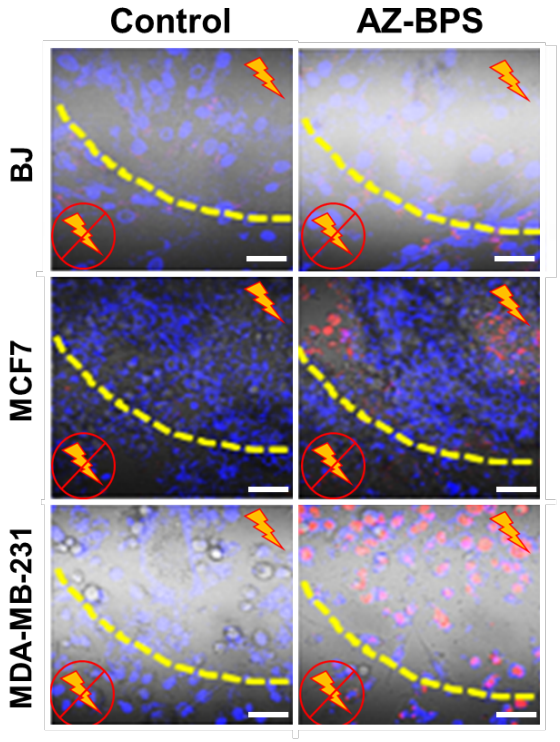


Figure S7. Confocal microscopic images of BJ cells, MCF-7 cells, and MDA-MB-231 cells, incubated with **AZ-BPS** (5 μM) for 4 h, before and after 660 nm laser irradiation (2.0 W/cm^2, 30 min; 3600 J/cm^2). Live/dead cells are blue/red (DAPI/PI), respectively. Dead cells are labeled in red by staining with PI, whereas all cells were visualized using DAPI (blue).

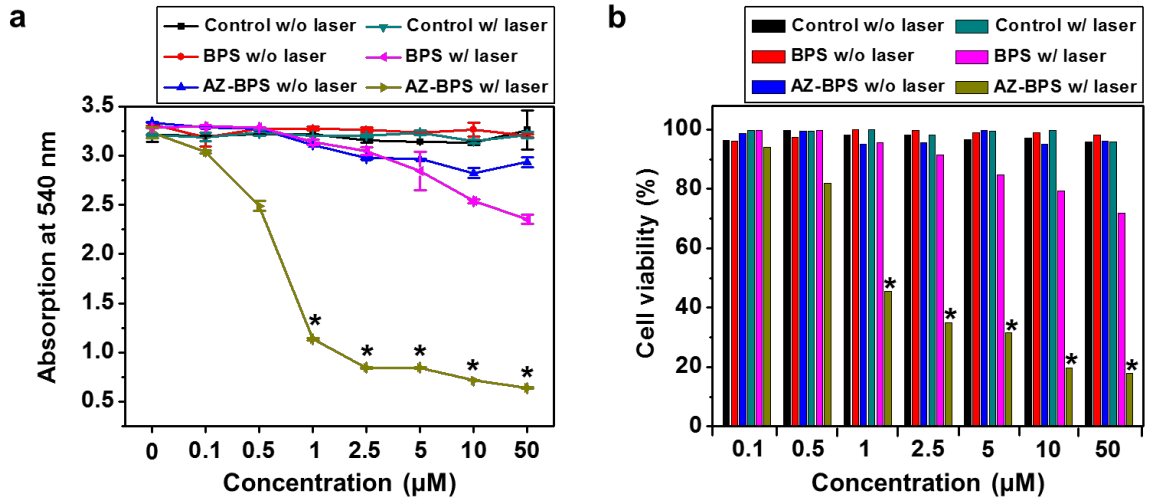


Figure S8. Photo-cytotoxic observed for CAIX-positive MDA-MB-231 cells after treatment with **AZ-BPS** or **BPS**. For the photo-irradiation studies, the cells were irradiated with 660 nm laser (2.0 W/cm^2, 30 min; 3600 J/cm^2). (a) Cytotoxicity of MDA-MB-231 cells. (b) Relative percentage of cell viability (*P < 0.05).

Table S2 IC_{50} values and photo-cytotoxicity indices (PI) for the **BPS** probes of this study.

Name of cell line	IC_{50} (μM)					
	BPS		PI[a]	**AZ-BPS**		PI[a]
	Dark	Light		Dark	Light	
MCF7 (-)	293.2 ± 3.86	255.7 ± 3.22	1.15	297.5 ± 3.18	208.5 ± 4.53	1.43
MDA-MB-231(+)	295.2 ± 3.26	225.7 ± 8.64	1.31	300.5 ± 3.18	1.58 ± 7.53	190

[a] Photo-cytotoxicity index (PI), defined as IC_{50} (dark)/IC_{50} (light), were obtained upon laser irradiation at 660 nm (2.0 W/cm^2, 30 min; 3600 J/cm^2).

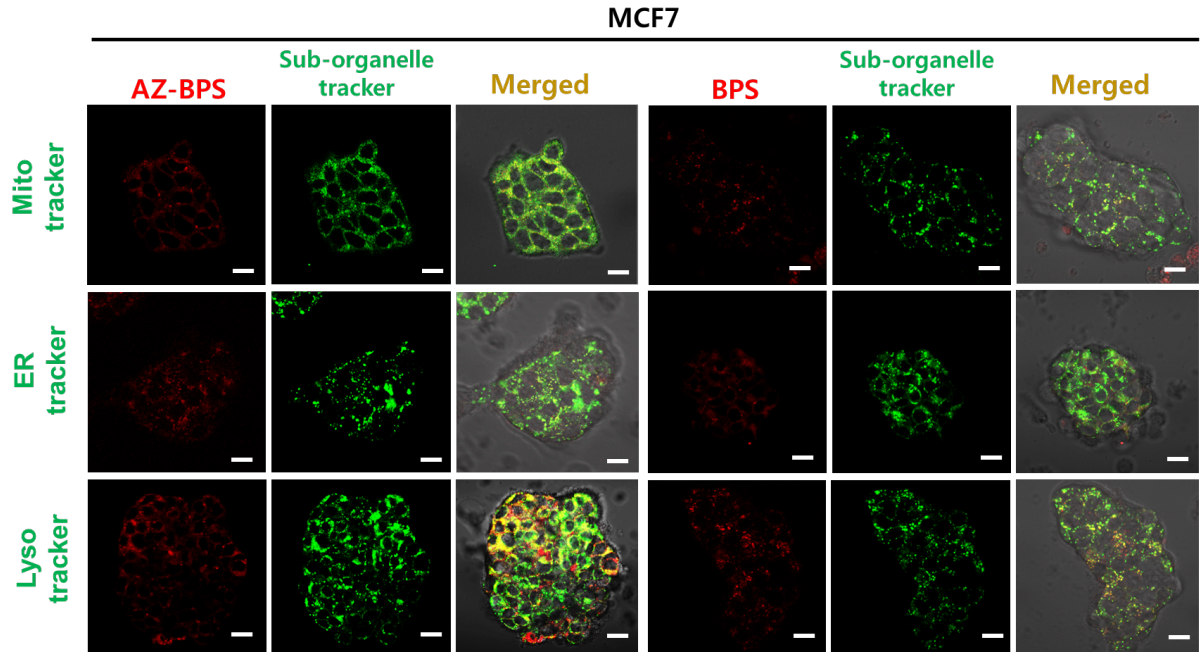


Figure S9. Intracellular co-localization of **AZ-BPS** and **BPS**. MCF-7 cells were incubated with 5 μM of **AZ-BPS** or **BPS** for 4 h. The cells were then washed with PBS. Mito-, ER-, and Lyso-trackers (green) were added and incubated for 30 min. The fluorescent images were recorded after 30 min. Scale bar: 50 μm.

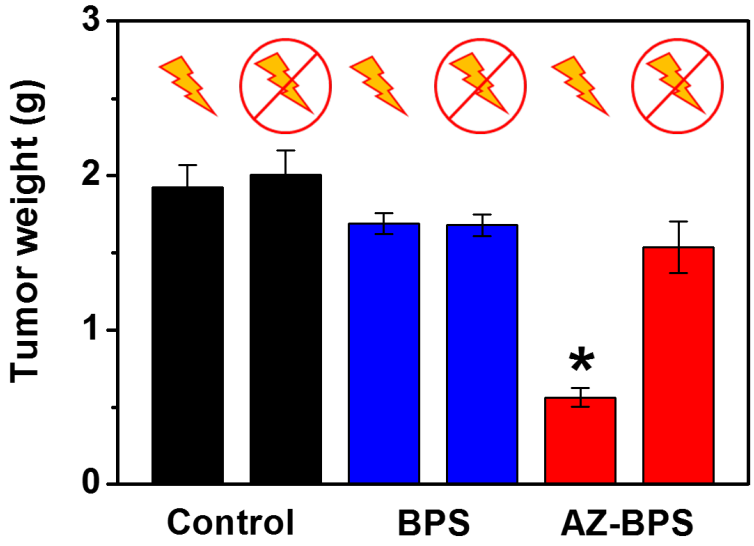


Figure S10. Tumor weights of all MDA-MB-231 xenograft mice in different groups with or without PDT treatment.

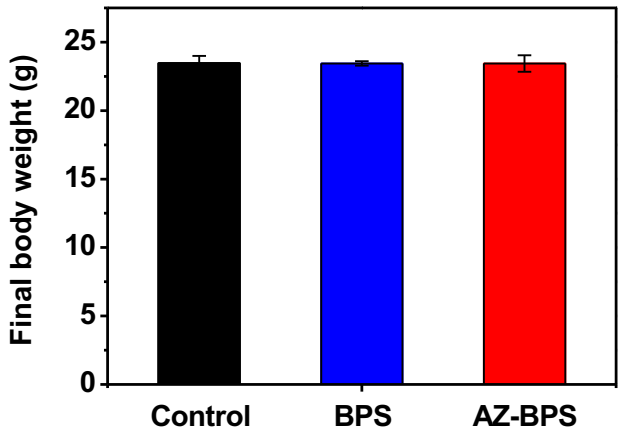


Figure S11. Final body weights of all MDA-MB-231 xenograft mice subject to PDT treatment.

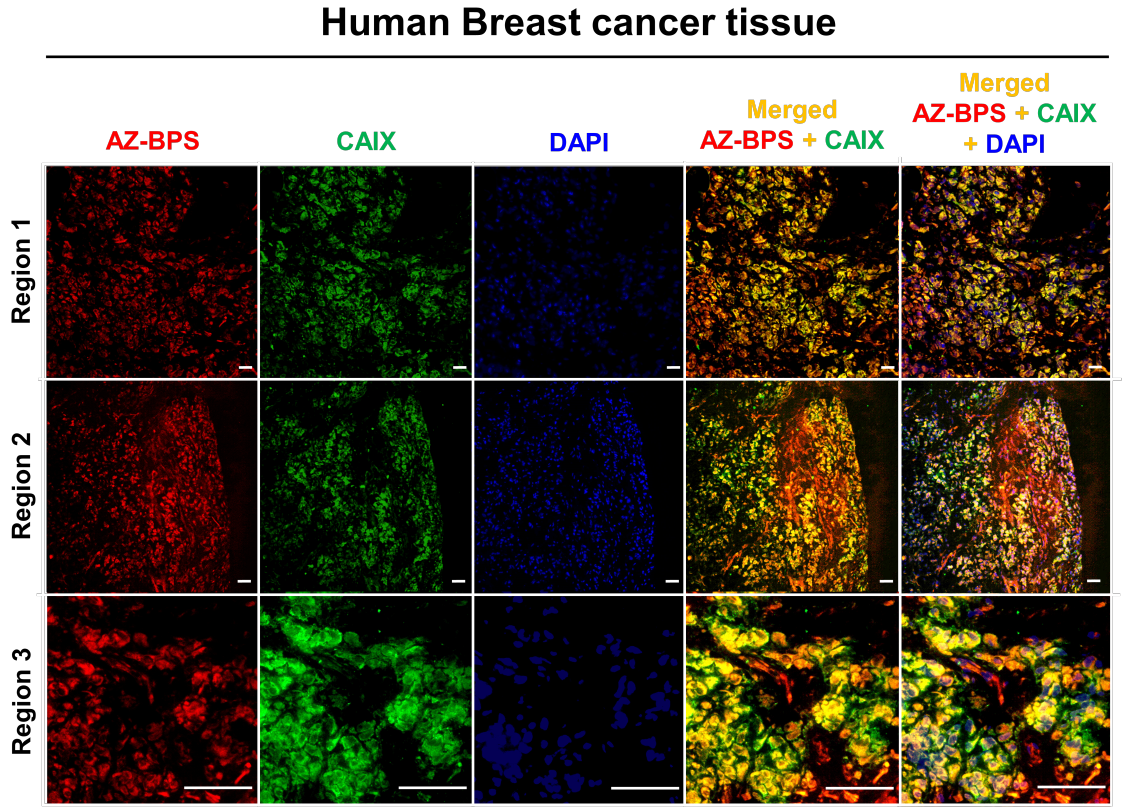


Figure S12. Correlation between CAIX expression (green) and the inherent fluorescent of **AZ-BPS** (red) as observed in cryosectioned tumor tissue taken from a human breast cancer patient. CAIX expression and fluorescent intensity of **AZ-BPS** were found to be correlated as noted in the main text. (Yellow color demarcation: a randomly selected n = 5 area). The magnification for the upper two rows is 100x while that for the lower row is 200x.

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

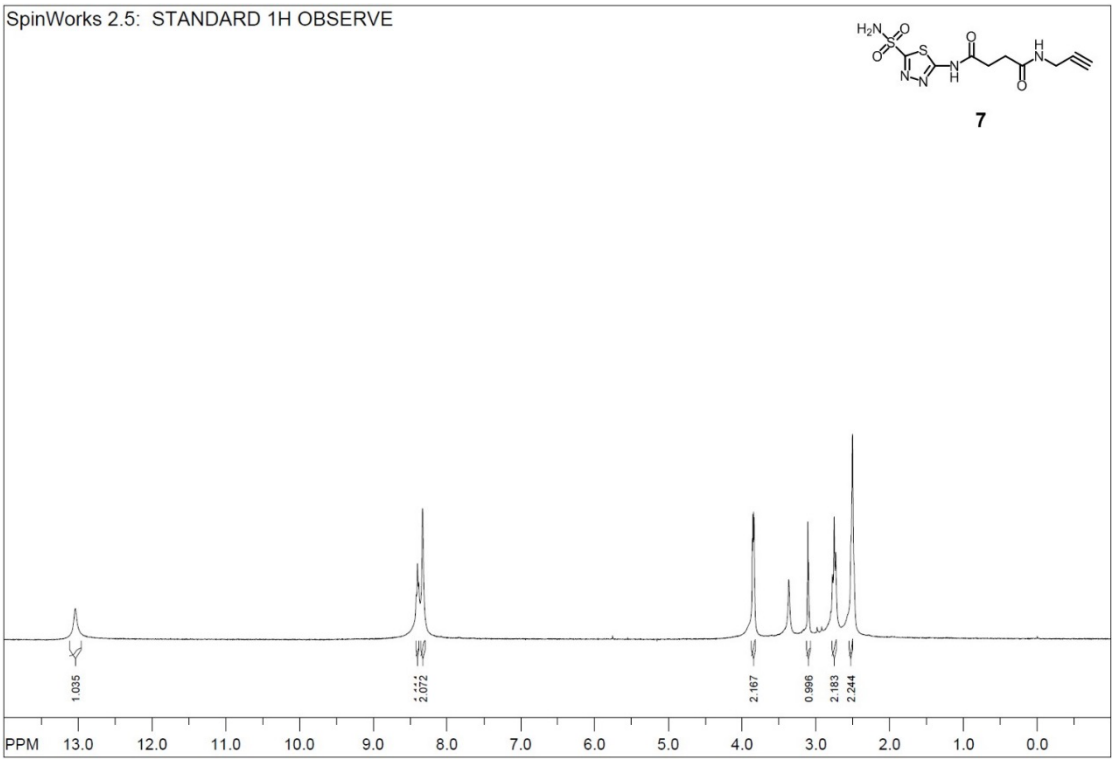


Figure S13. ^{1}H NMR spectra (400 MHz) of **7** in DMSO-d_6.

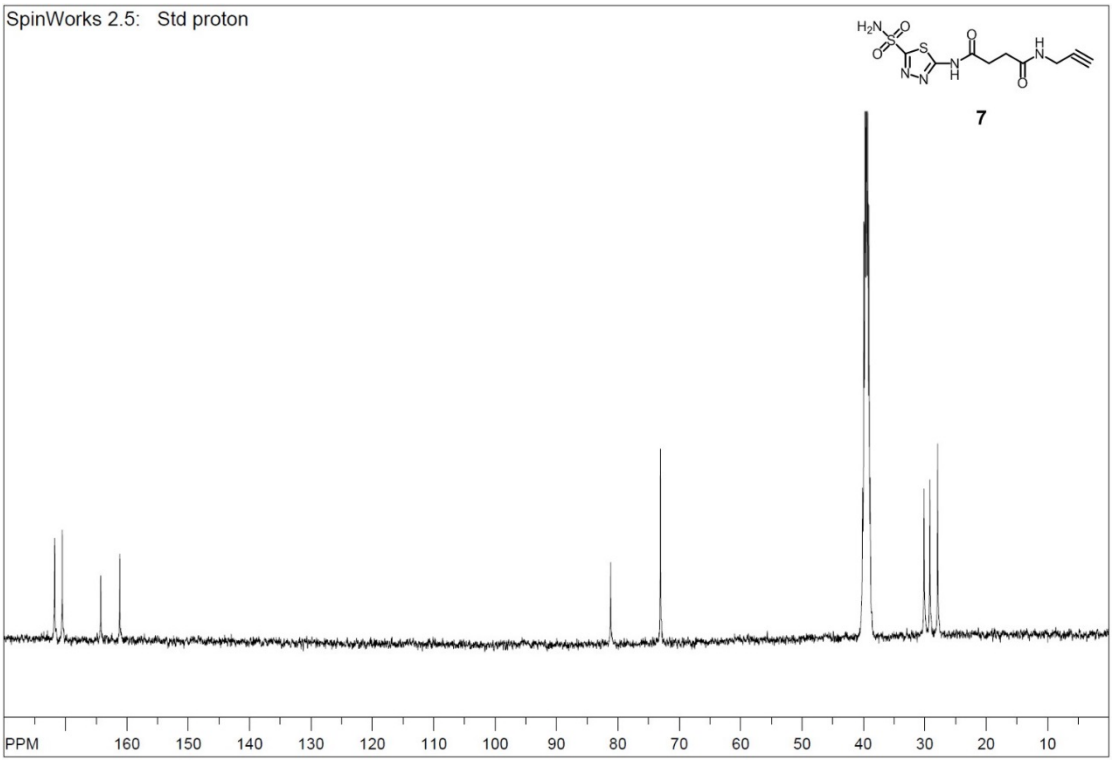


Figure S14. ^{13}C NMR spectra (100 MHz) of **7** in DMSO-d_6.

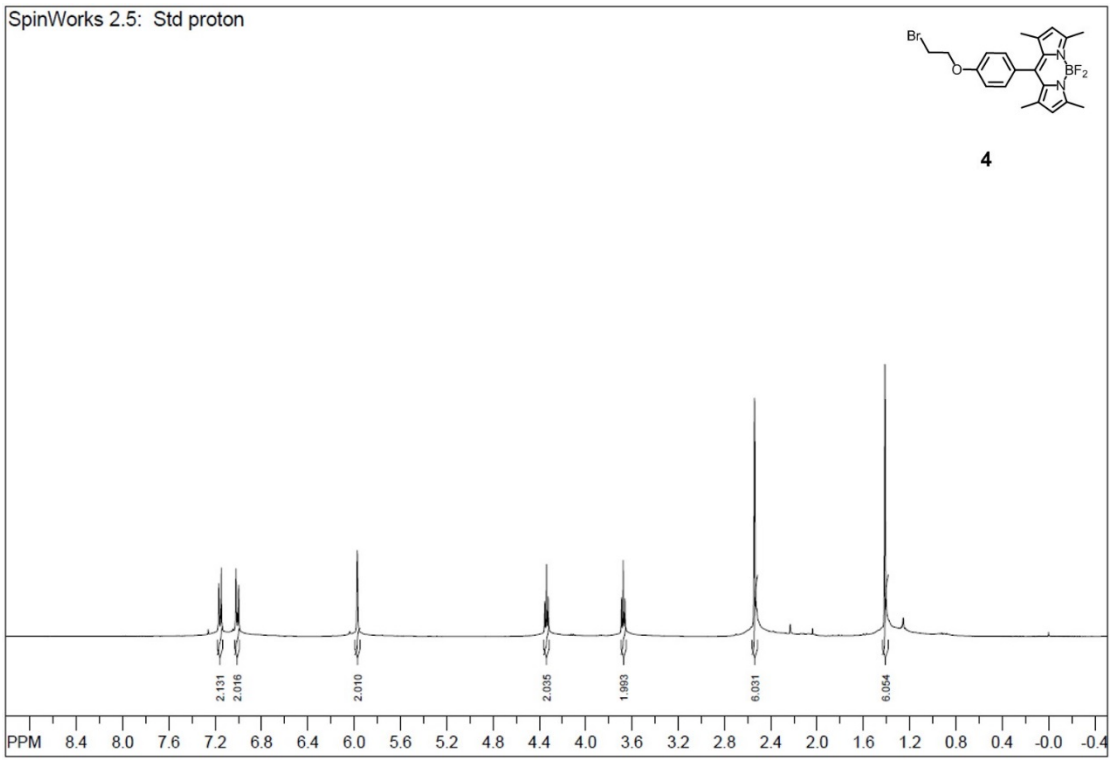


Figure S15. ^{1}H NMR spectra (400 MHz) of **4** in $CDCl_3$.

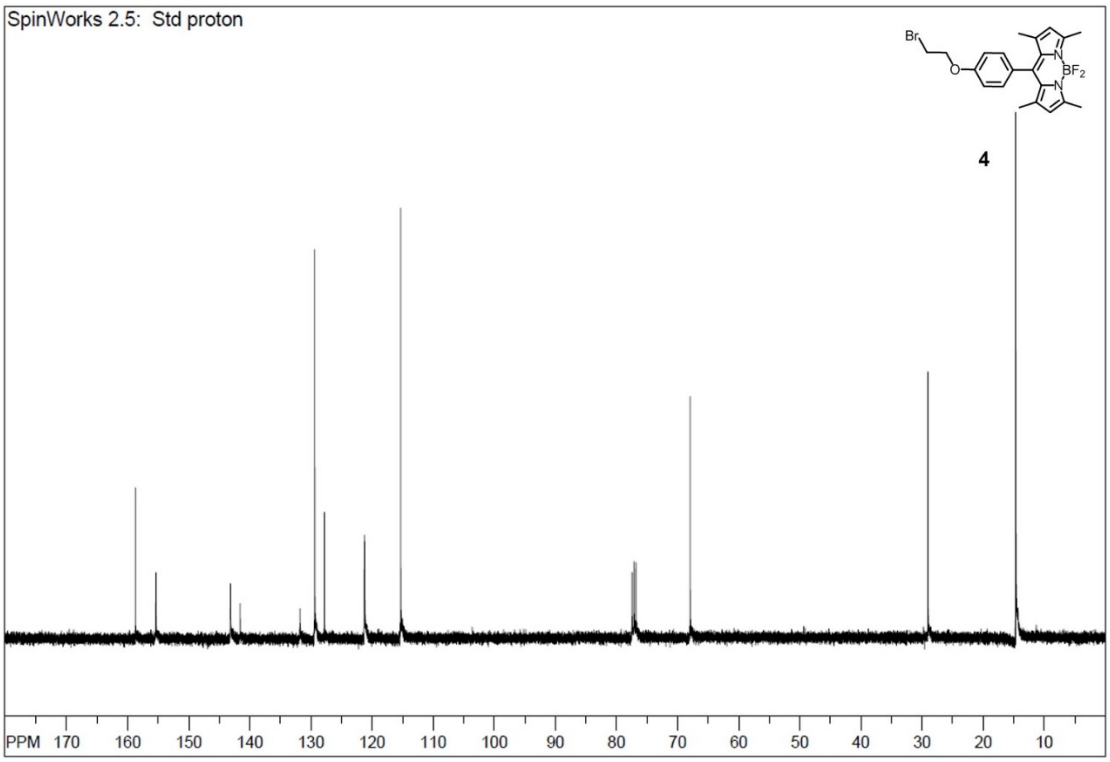


Figure S16. ^{13}C NMR spectra (100 MHz) of **4** in $CDCl_3$.

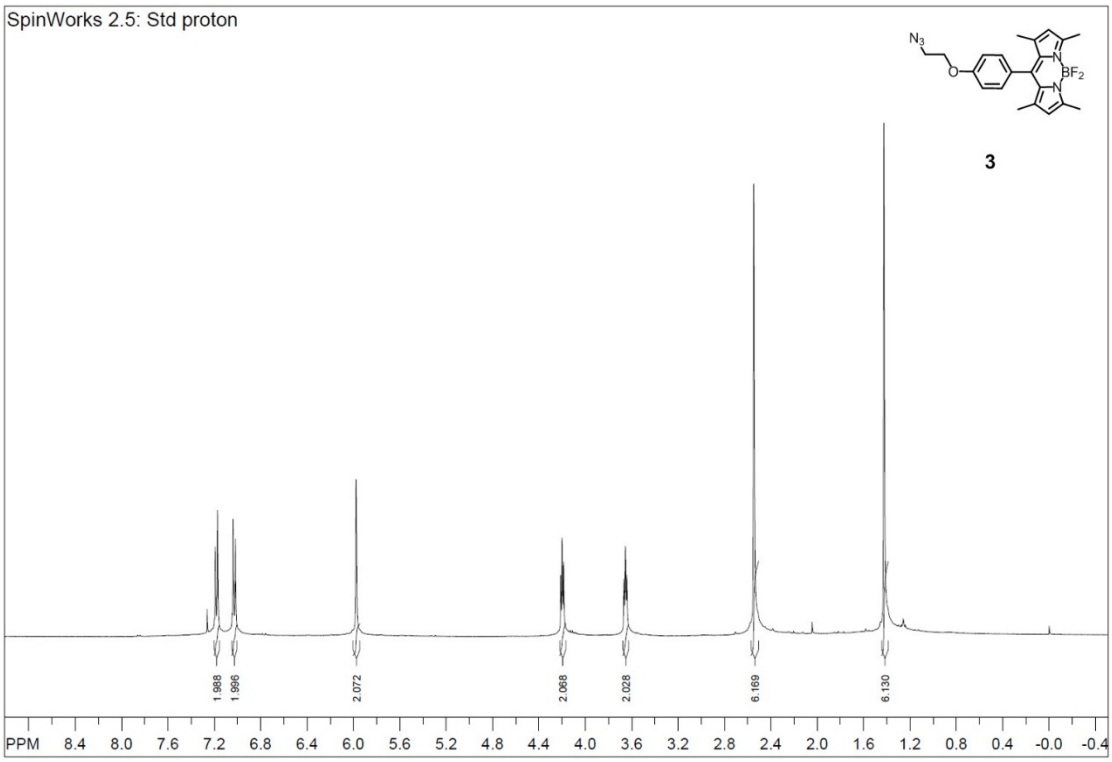


Figure S17. ^{1}H NMR spectra (400 MHz) of **3** in $CDCl_3$.

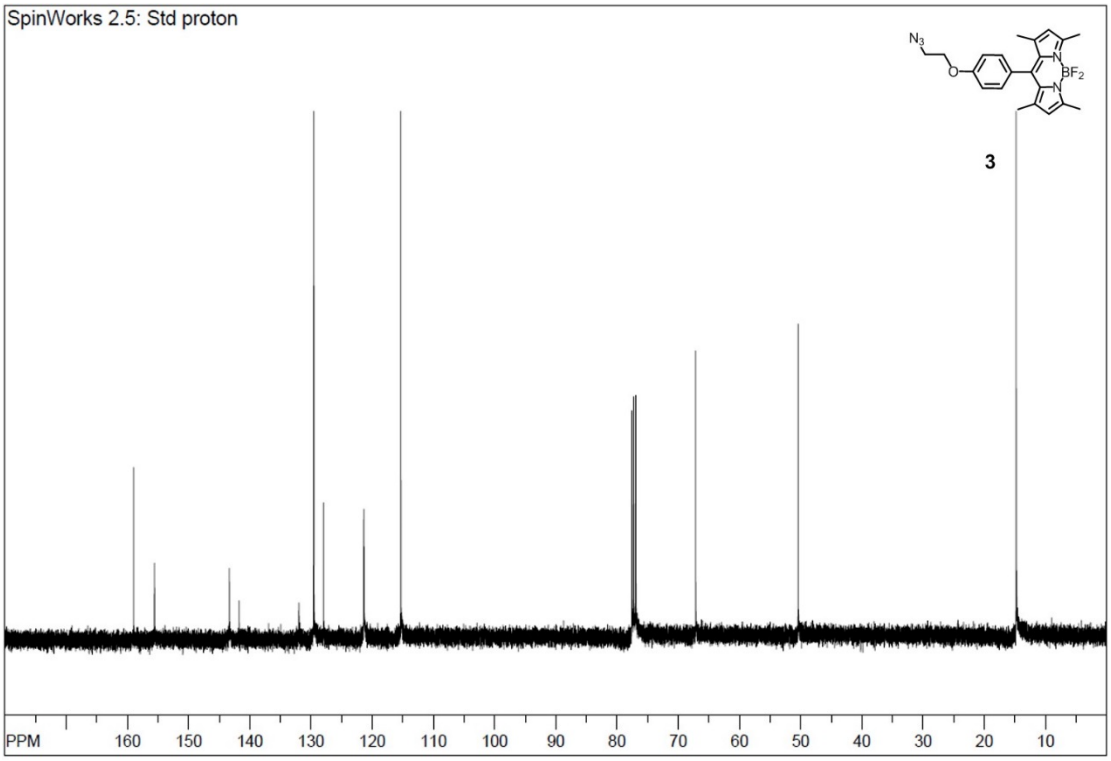


Figure S18. ^{13}C NMR spectra (100 MHz) of **3** in $CDCl_3$.

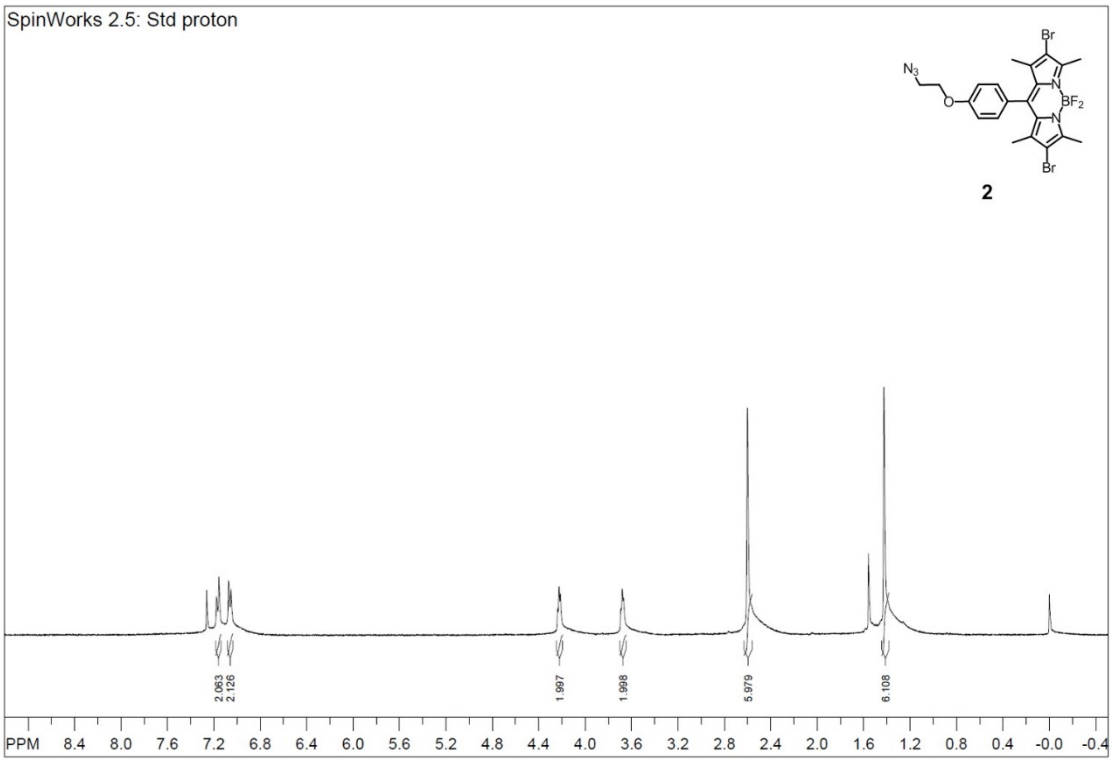


Figure S19. ^{1}H NMR spectra (400 MHz) of **2** in $CDCl_3$.

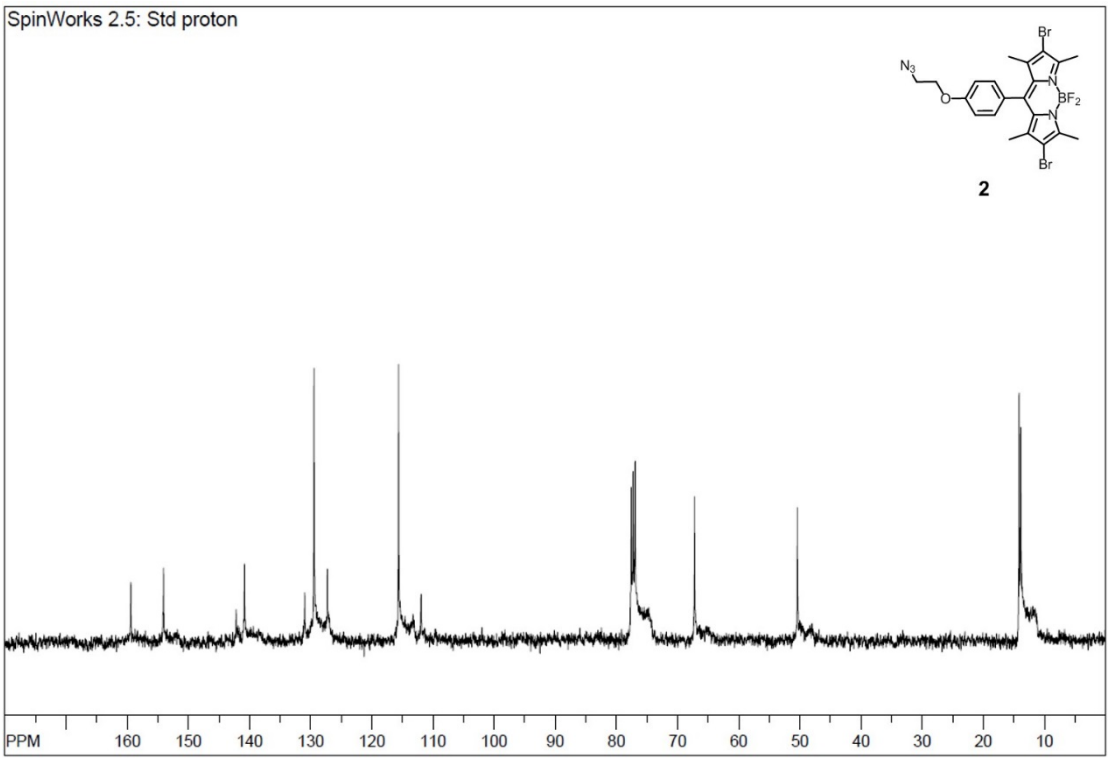


Figure S20. ^{13}C NMR spectra (100 MHz) of **2** in $CDCl_3$.

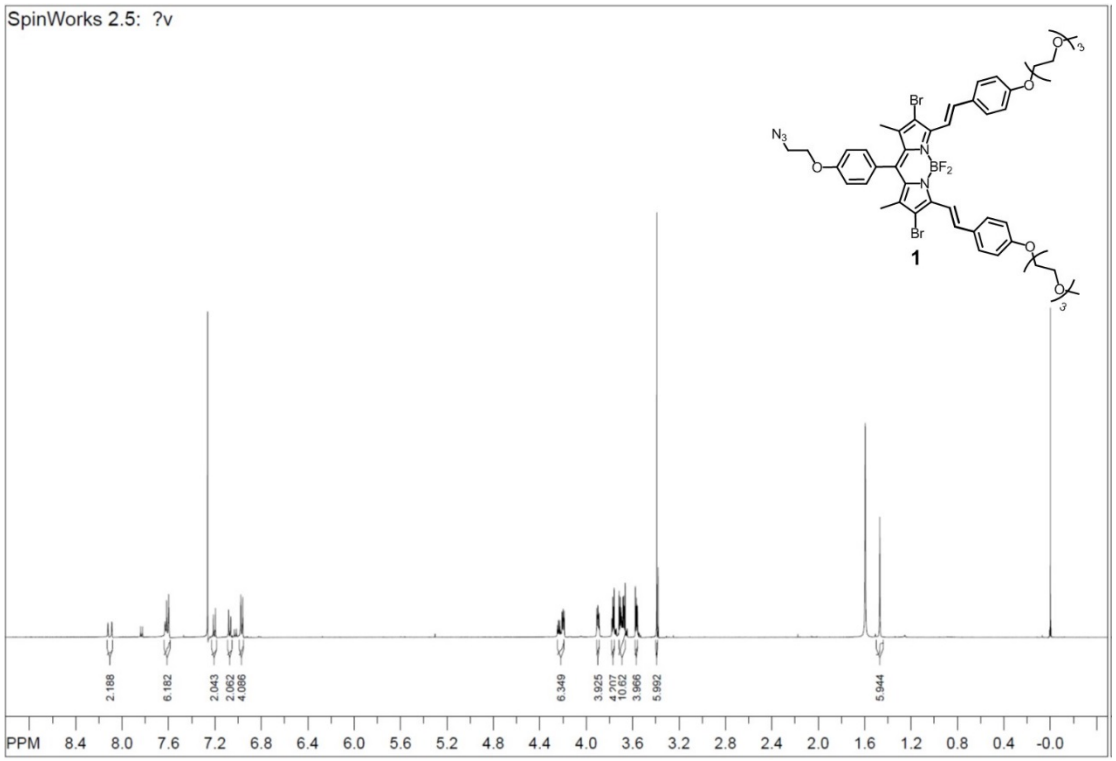


Figure S21. ^{1}H NMR spectra (400 MHz) of **1** in $CDCl_3$.

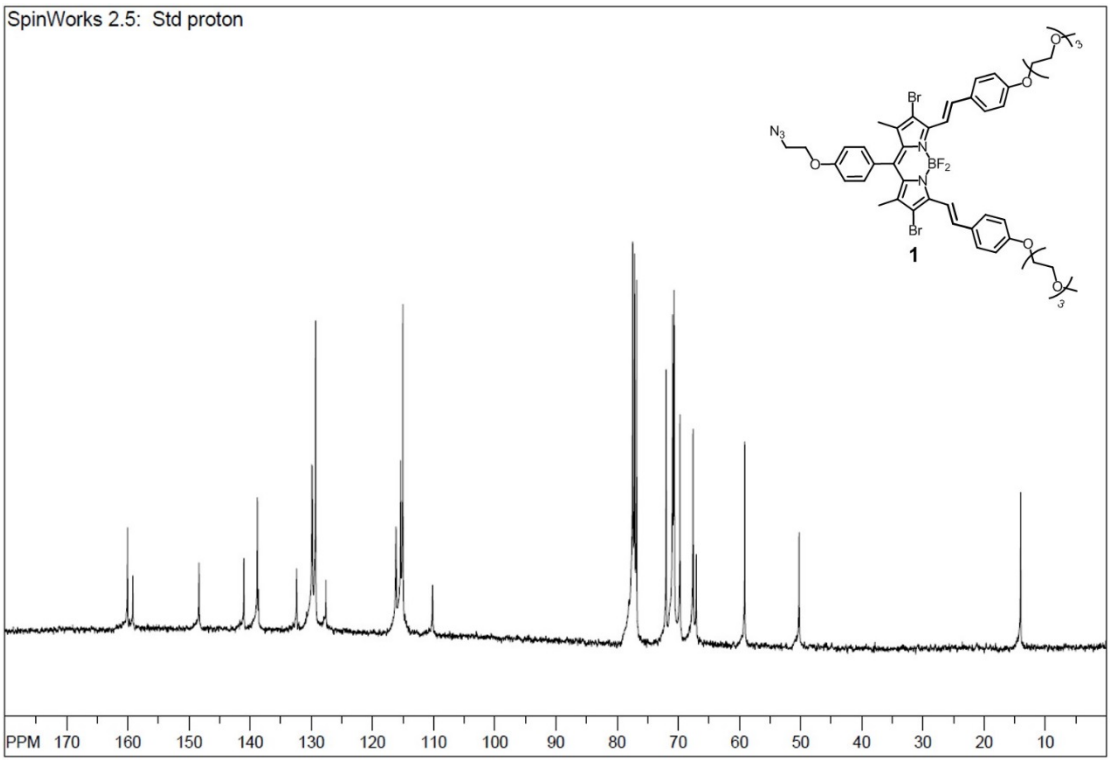


Figure S22. ^{13}C NMR spectra (100 MHz) of **1** in $CDCl_3$.

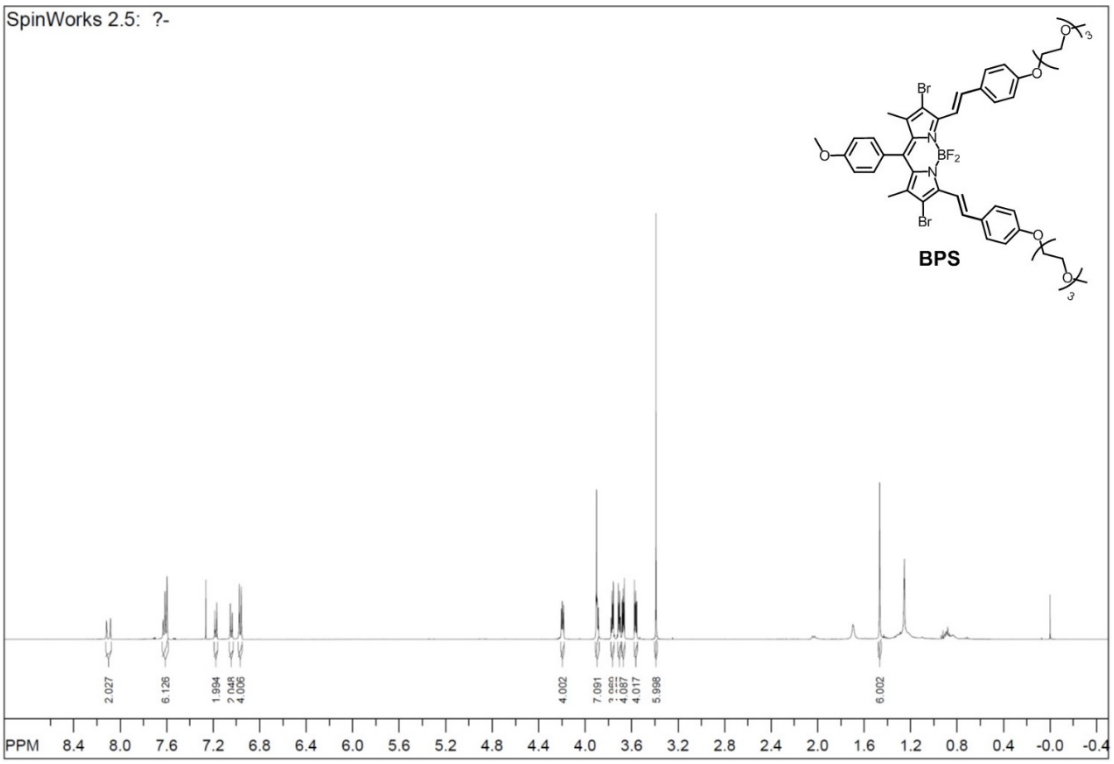


Figure S23. ^{1}H NMR spectra (400 MHz) of **BPS** in $CDCl_3$.

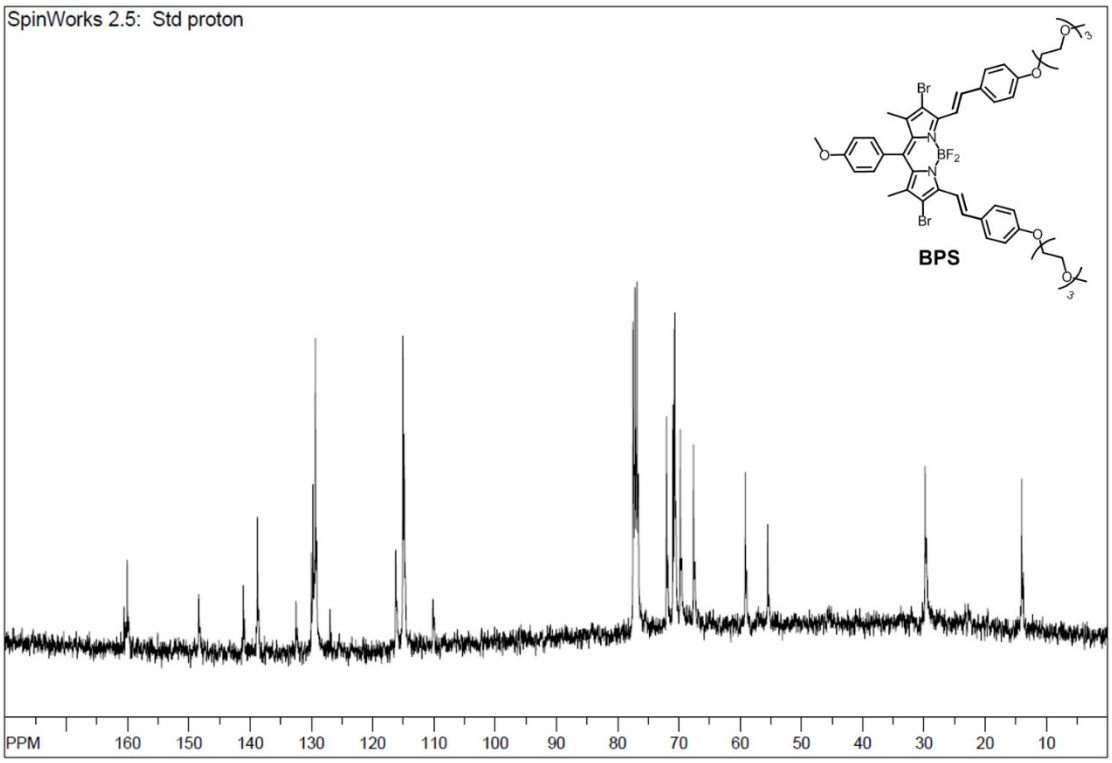


Figure S24. ^{13}C NMR spectra (100 MHz) of **BPS** in $CDCl_3$.

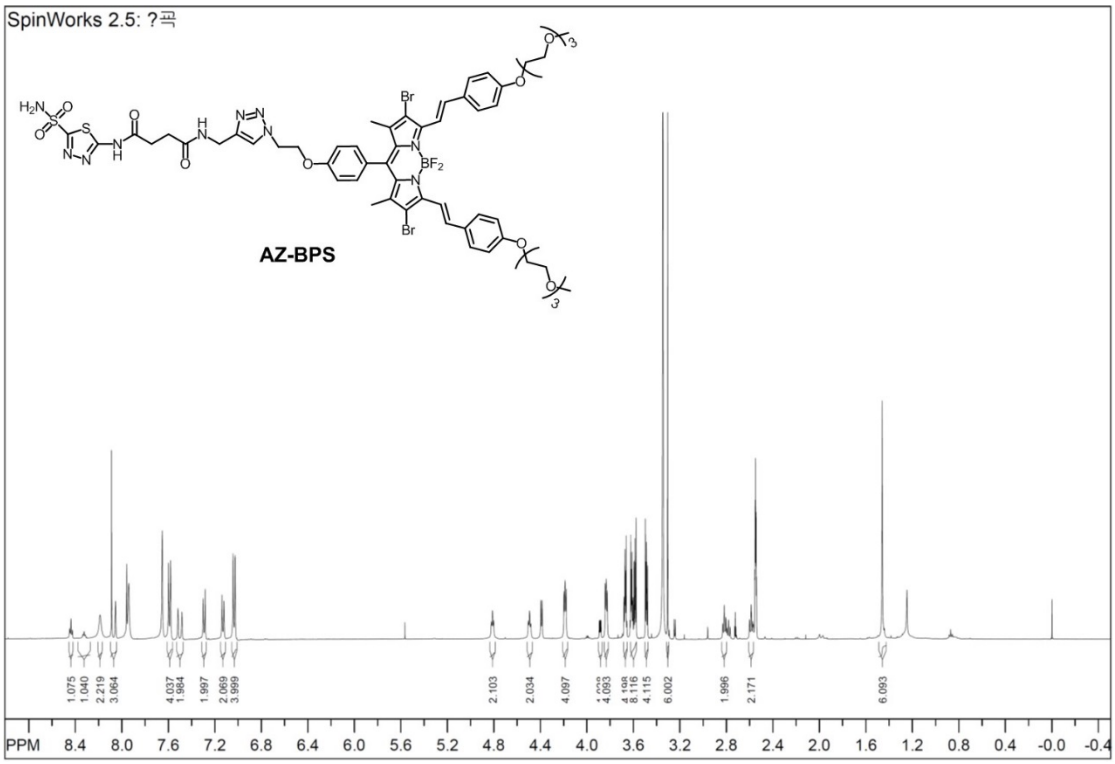


Figure S25. ^{1}H NMR spectra (500 MHz) of **AZ-BPS** in $CDCl_3$/DMSO-d_6 mixed solvents.

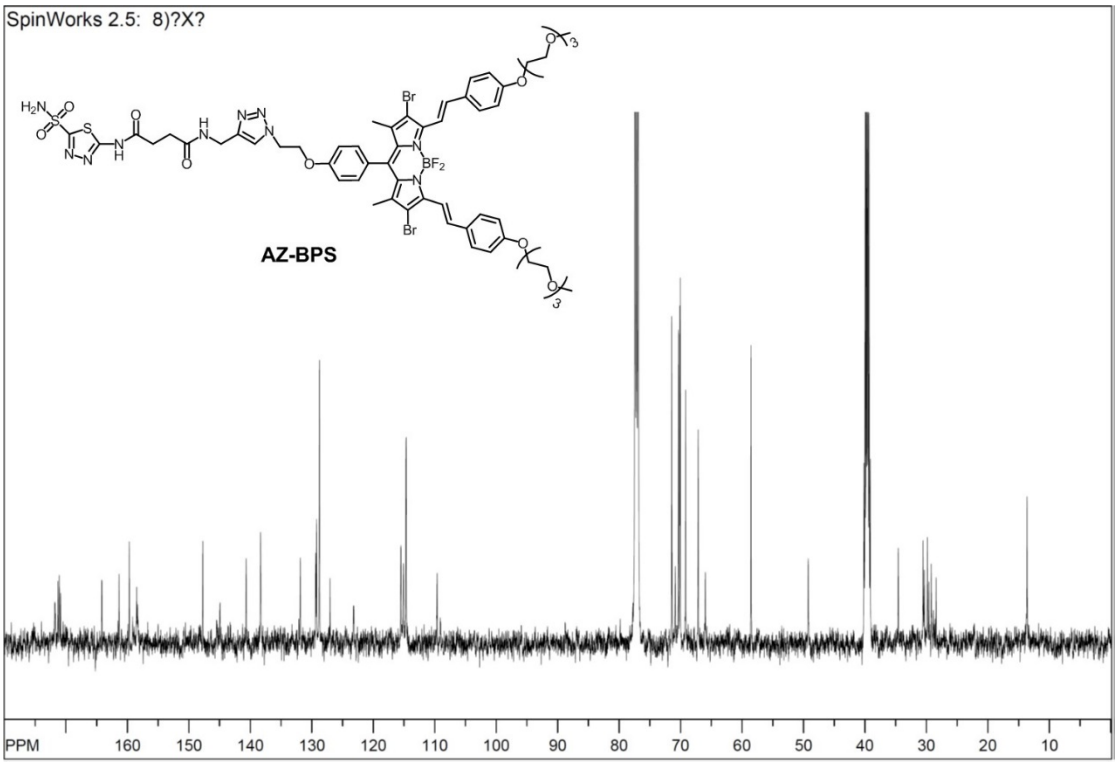


Figure S26. ^{13}C NMR spectra (125 MHz) of **AZ-BPS** in $CDCl_3$/DMSO-d_6.

- **ESI MS data**

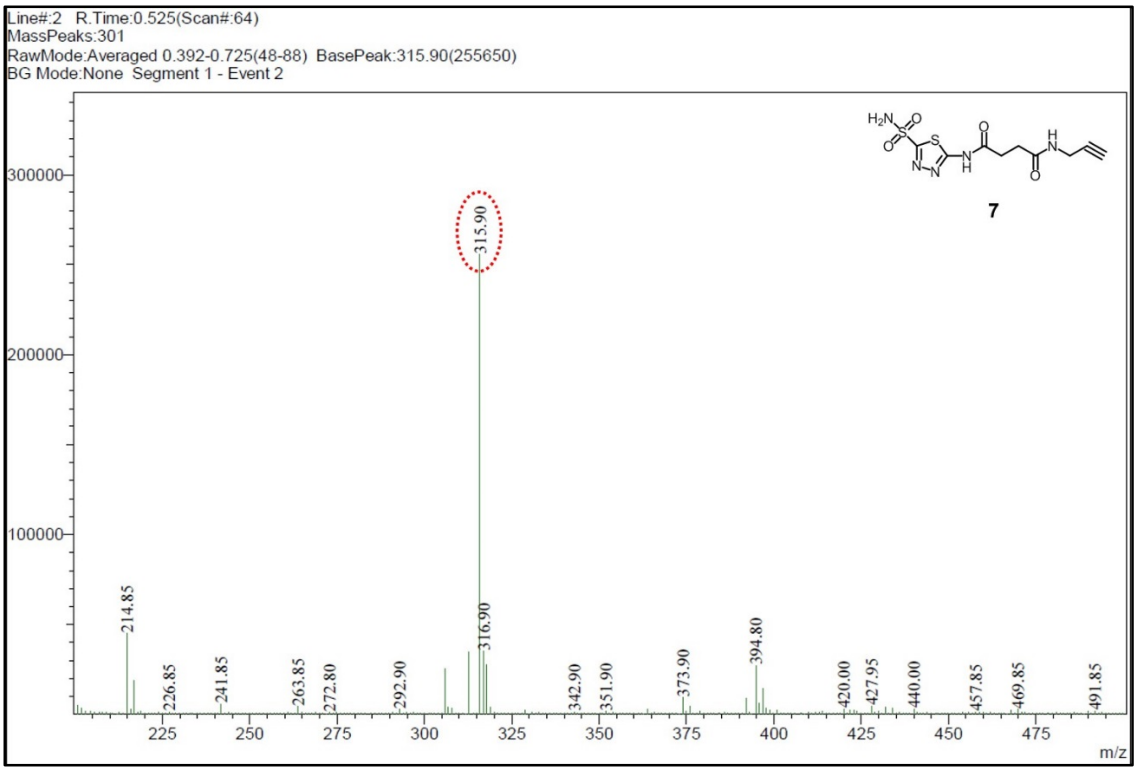


Figure S27. ESI-MS spectra of **7**.

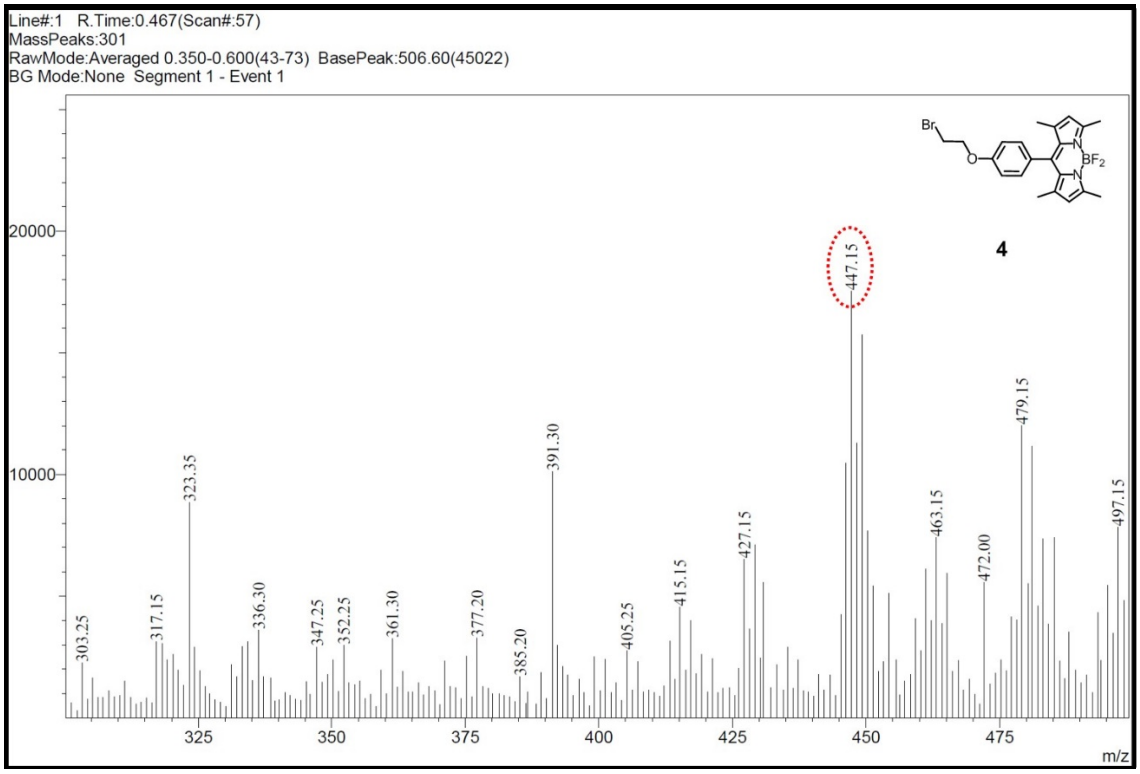


Figure S28. ESI-MS spectra of **4**.

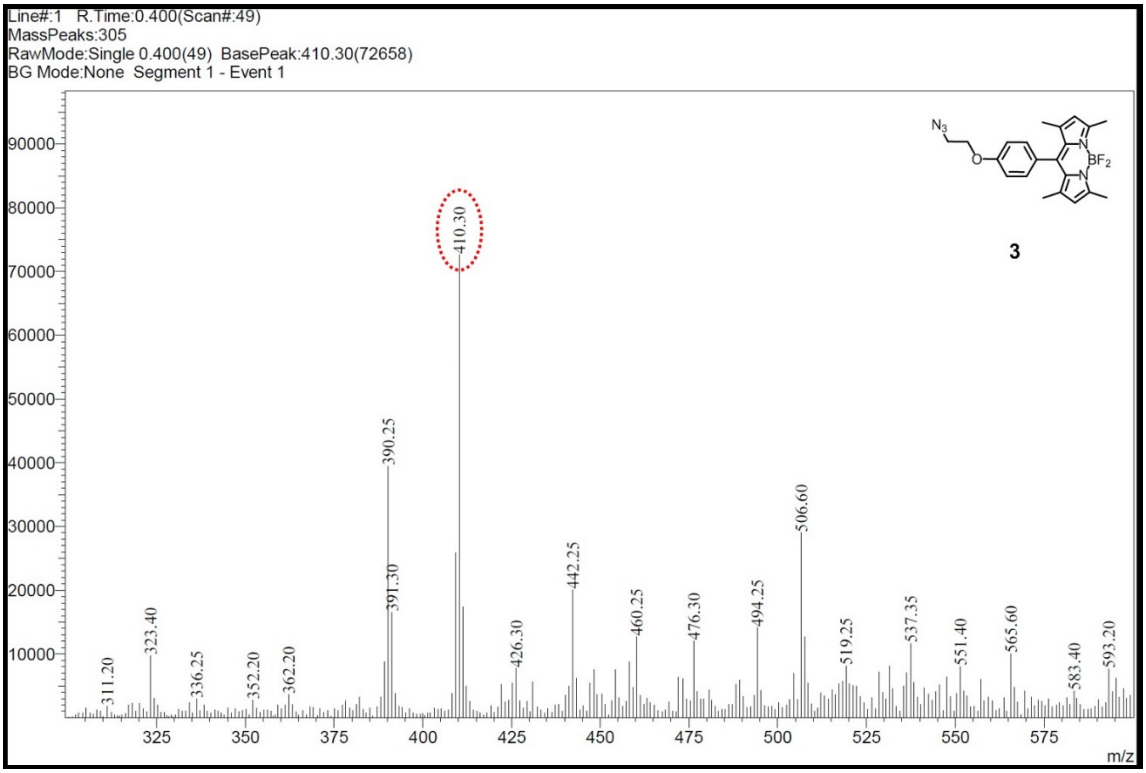


Figure S29. ESI-MS spectra of **3**.

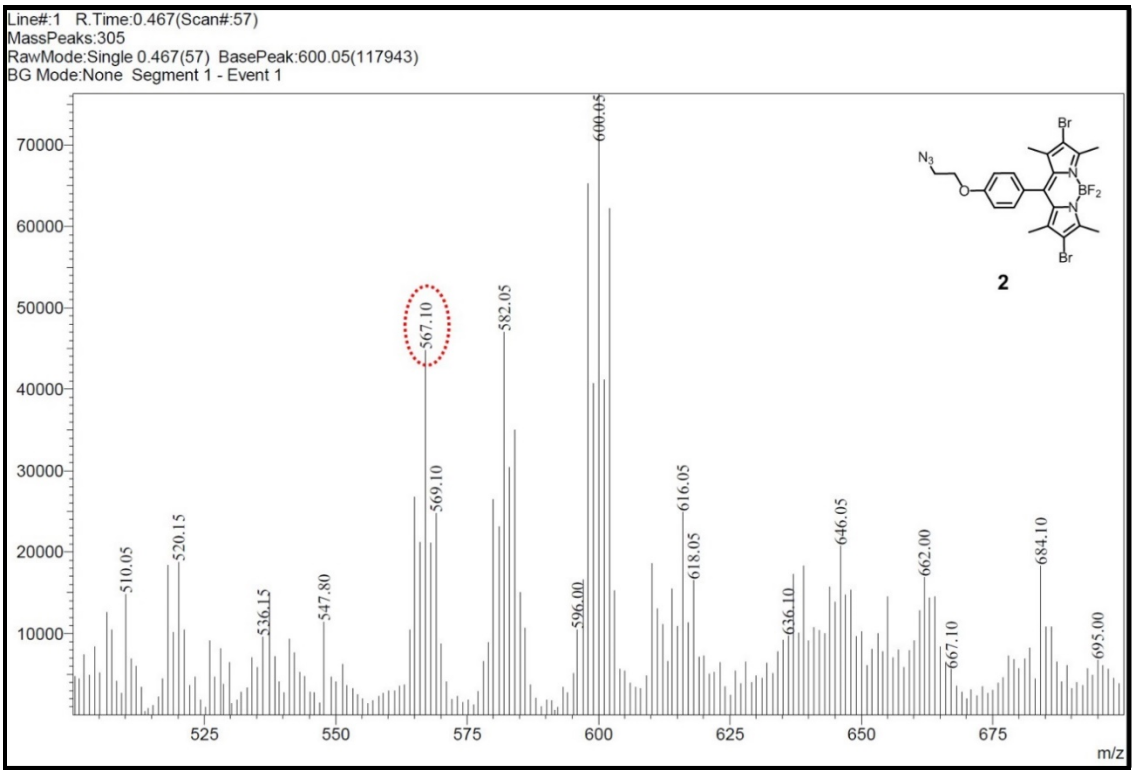


Figure S30. ESI-MS spectra of **2**.

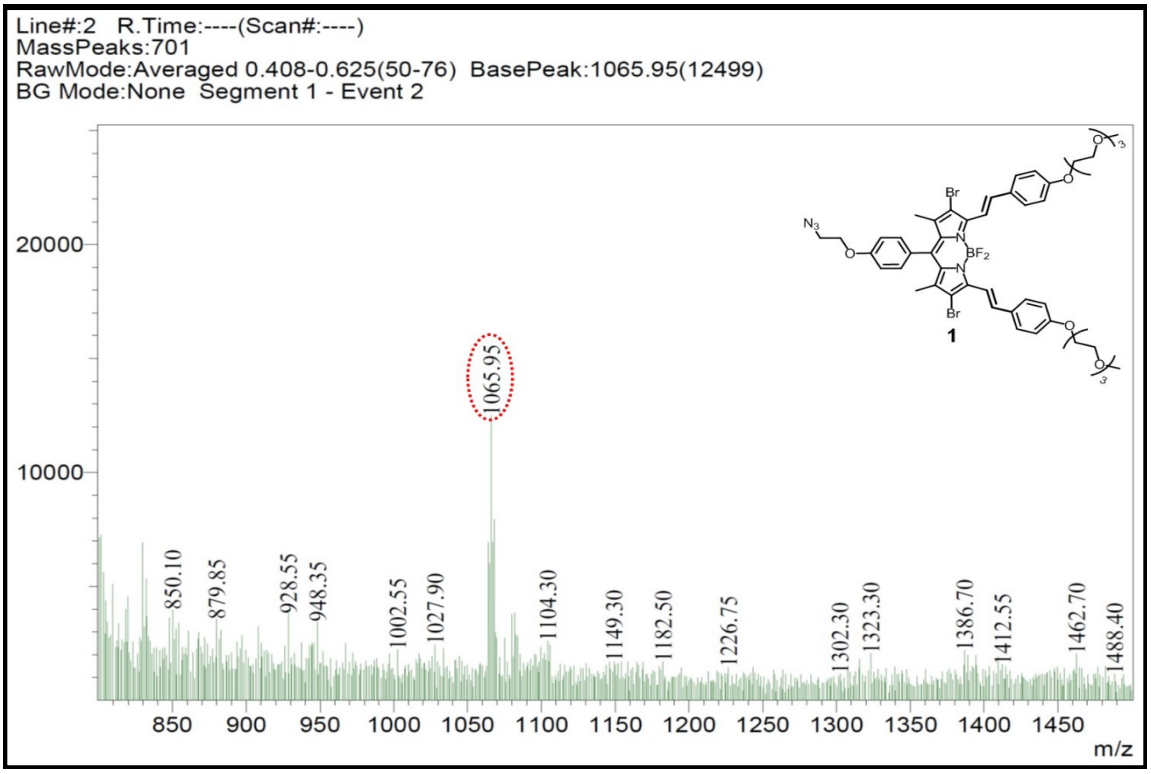


Figure S31. ESI-MS spectra of **1**.

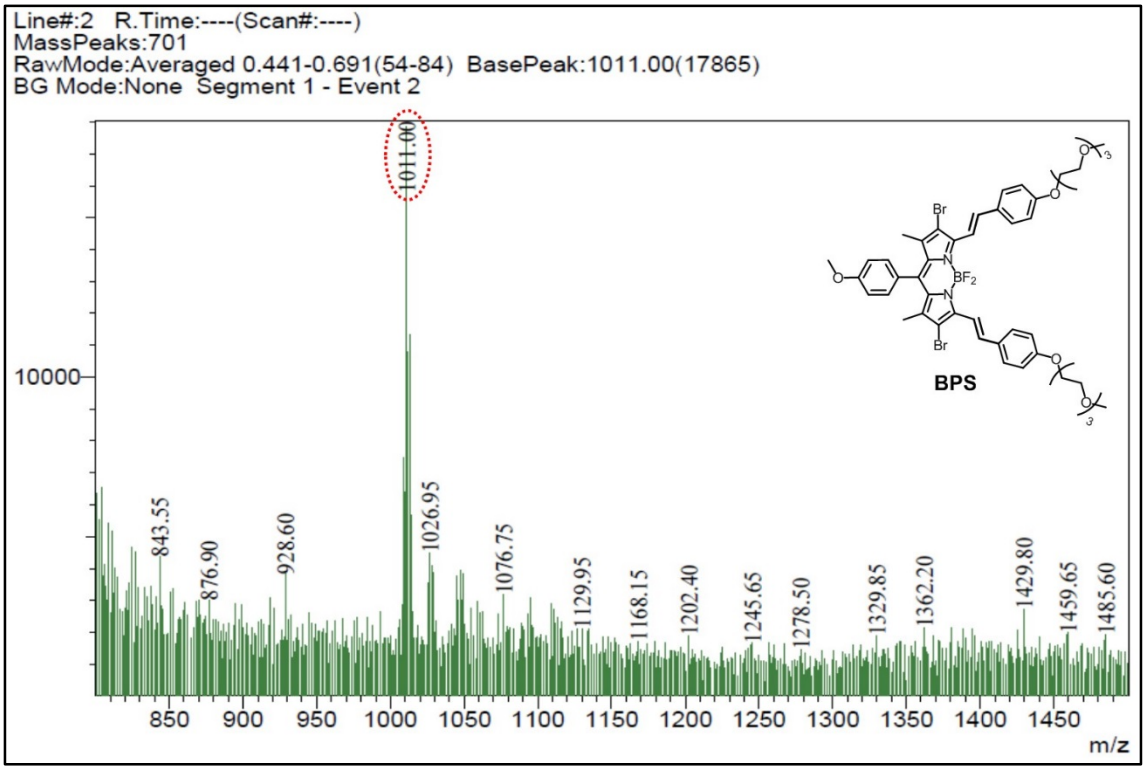


Figure S32. ESI-MS spectra of **BPS**.

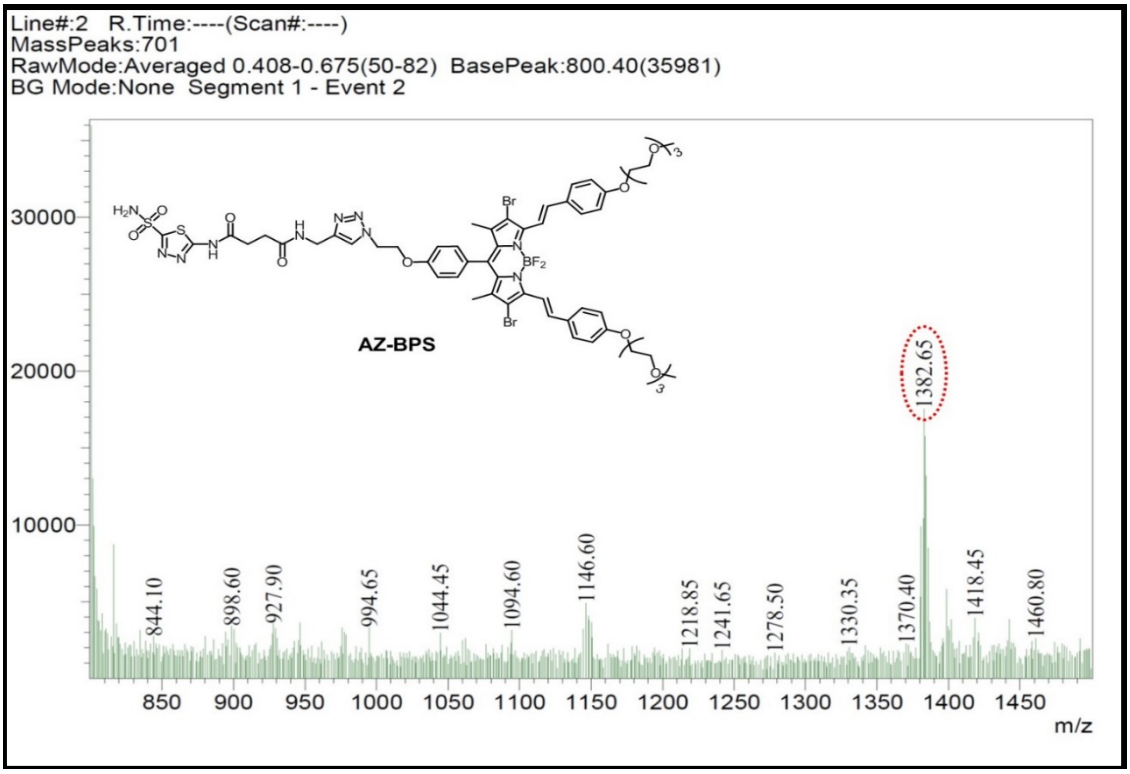


Figure S33. ESI-MS spectra of **AZ-BPS**.

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