Supporting Information to Accompany

"Development of a Small Molecule-Based Two-Photon Photosensitizer for Targeting Cancer Cells"

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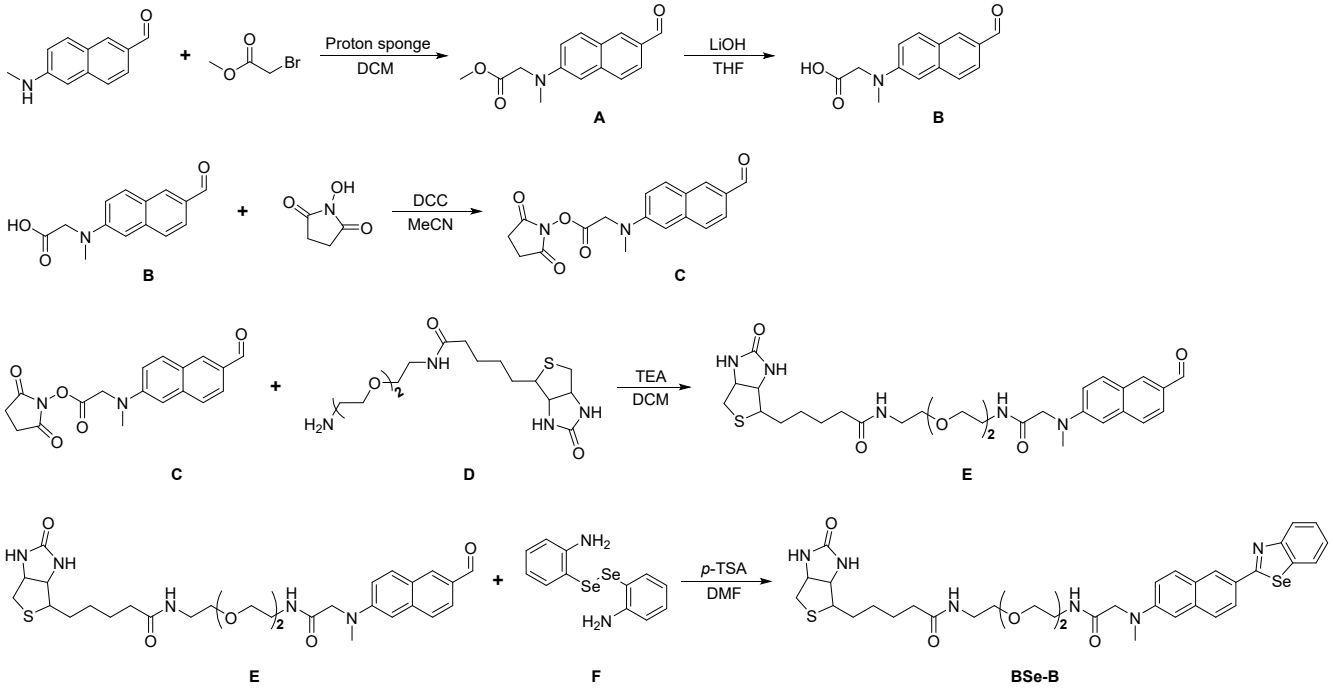
Materials and General Method. All compounds used for synthesis were purchased from Sigma-Aldrich. The reaction progress was monitored using thin layer chromatography (TLC) plates (TLC Silica gel 60 F254, 1057150001, Merck). The products were purified using medium pressure liquid chromatography (MPLC, AI-580S, YAMAZEN). 1H-NMR and 13C-NMR spectra were obtained using a 600MHz NMR spectrometer (JNM-ECZ600R, JEOL). Mass data was obtained using HRMS system (Accela UHPLC/LTQ-Orbitrap XL, Thermo Fisher Scientific) and Q-TOF system (Dionex UHPLC/TripleTOF 5600+, ABSCIX) from Gyeonggi do Business & Science Accelerator (GBSA, Korea).

Synthesis of BSe-B. Compound **A**, **B**, **D**, and **F** were prepared by the literature methods [1–3] and synthesis of **C**, **E** and **BSe-B** are described below.



Scheme S1. Synthesis of **BSe-B**

Synthesis of **BSe-B**

Compound **C**. To a round bottom flask compound **B** (115 mg, 0.5 mmol) and N, N′-dicyclohexylcarbodiimide (DCC; 98 mg, 0.5 mmol) were added and dissolved by acetonitrile (10 ml). The mixture was stirred at 0 °C for 1h and solution of N-hydroxysuccinimide (55 mg, 0.5 mmol) in acetonitrile (5 ml) was added to the mixture. After it was stirred for 0.5 h at 0 °C, mixture was stirred for 24 h at room temperature. The end of reaction was confirmed via TLC. After reaction, the organic solvent of reaction mixture was eliminated by evaporation under vacuo. Through column chromatography (5 % methanol in DCM was used as eluent), the product compound **C** could be obtained. Yield: 86 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ(ppm) 10.01 (s, 1H), 8.15 (s, 1H), 7.87 (d, J = 9.0 Hz, 1H), 7.84 (dd, J = 9.0, 1.4 Hz, 1H), 7.74 (d, J = 8.3 Hz, 1H), 7.17 (dd, J = 9.0, 2.8 Hz, 1H), 6.99 (s, 1H), 4.54 (s, 2H), 3.24 (s, 3H), 2.79 (s, 4H).

Compound **E**. To a solution of compound **C** (240 mg, 0.7 mmol) and **D** (264 mg, 0.7 mmol) in DCM triethylamine (TEA; 285 mg, 2.8 mmol) was added. The mixture was stirred for 24 h at room temperature. The end of reaction was confirmed via TLC. After reaction, the organic solvent of reaction mixture was eliminated by evaporation under vacuo. Through column chromatography (8 % methanol in DCM was used as eluent), the product compound **E** could be obtained. Yield: 63 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ(ppm) 9.90 (s, 1H), 8.06 (s, 1H), 7.76 (d, J = 9.6 Hz, 1H), 7.71 (dd, J = 9.0, 1.4 Hz, 1H), 7.59 (d, J = 9.0 Hz, 1H), 7.12 (s, 1H), 7.04 (dd, J = 9.3, 2.4 Hz, 1H), 6.86 (s, 1H), 6.74 (s, 1H), 6.41 (s, 2H), 4.37 (s, 1H), 4.16 (s, 1H), 4.01 (s, 2H), 3.42-3.37 (m, 6H), 3.31 (q, J = 4.8 Hz, 4H), 3.25 (d, J = 5.5 Hz, 2H), 3.11 (s, 3H), 3.00 (s, 1H), 2.75 (d, J = 8.3 Hz, 1H), 2.62 (d, J = 12.4 Hz, 1H), 2.07 (s, 2H), 1.57-1.33 (m, 6H)

Compound **BSe-B**. To a solution of compound **F** (163 mg, 0.48 mmol) in DMF in round bottom flask **E** (190 mg, 0.32 mmol) and p-toluenesulfonic acid (60 mg, 0.32 mmol) were added. The mixture was stirred at 120 °C for 1 day under nitrogen atmosphere. After cooling to room temperature, mixture was poured to DCM and washed with water. Extracted organic layer was dried over Na_2SO_4 and evaporated. The product **BSe-B** could obtained by further purification via column chromatography ($CHCl_3$: MeOH 9 : 1). Yield: 32 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ(ppm) 8.30 (s, 1H), 8.08 (d, J = 8.3 Hz, 1H), 8.01 (dd, J = 8.6, 1.7 Hz, 1H), 7.93 (d, J = 7.6 Hz, 1H), 7.82 (d, J = 9.6 Hz, 1H), 7.70 (d, J = 8.3 Hz, 1H), 7.47 (t, J = 7.6 Hz,

1H), 7.29 (t, J = 7.6 Hz, 1H), 7.11-7.08 (m, 2H), 6.94 (s, 1H), 6.57 (s, 1H), 6.40 (s, 1H), 5.51 (s, 1H), 4.32 (t, J = 6.2 Hz, 1H), 4.12 (t, J = 5.9 Hz, 1H), 4.03 (s, 2H), 3.48 (dd, J = 10.7, 3.8 Hz, 4H), 3.41 (t, J = 4.5 Hz, 2H), 3.36 (t, J = 5.2 Hz, 2H), 3.33 (q, J = 3.0 Hz, 2H), 3.30-3.29 (m, 2H), 3.15 (s, 3H), 2.99 (dd, J = 12.1, 7.2 Hz, 1H), 2.76 (dd, J = 13.1, 4.8 Hz, 1H), 2.66-2.61 (m, 1H), 2.11 (t, J = 7.6 Hz, 2H), 1.63-1.53 (m, 4H), 1.32 (q, J = 7.6 Hz, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ(ppm) 173.5, 172.8, 170.4, 164.0, 156.0, 148.3, 138.1, 136.3, 130.5, 130.3, 128.0, 127.2, 126.9, 126.5, 125.7, 125.2, 124.9, 124.5, 116.6, 106.9, 70.1, 69.9, 69.8, 61.8, 60.2, 58.2, 55.6, 40.5, 40.0, 39.1, 39.1, 35.9, 29.8, 28.2, 28.1, 25.6. HRMS (ESI$^+$) $[C_{36}H_{45}Se_1N_6O_5S_1]^+$: 753.2259, found: 753.2337

Spectroscopic Experiments. Absorption spectra and fluorescence spectra were obtained using a UV-Vis spectrophotometer (S-3100) and fluorescence spectrophotometer (FluoroMate FS-2), respectively. The relative fluorescence quantum yield was measured with Coumarin 307 (Φ = 0.95 in methanol) as the reference [4]. Cell viability tests were performed using a multiple detection microplate reader (Varioskan Flash, Thermo Fisher Scientific) and 96-well microplates for fluorescence (164588, Thermo Fisher Scientific).

Measurement of Two-Photon Cross Section. The two-photon absorption cross-sectional area (δ) was measured by the following published method [5]. Probes (1.0 μM) was dissolved in ethanol and aqueous buffer (10 mM PBS, pH 7.4). The characteristics of rhodamine 6G for a two-photon cross-section were referenced in the literature and used as a reference [6]. Two-photon fluorescence was excited by an ultrafast Ti:sapphire laser (Mai Tai HP-067, Spectra Physics). Two-photon excited fluorescence was detected by CCD system ((Monora 320i monochromator with DV401A-BV detector, DONGWOO OPTRON, Korea). The TP cross section was calculated using □□= □$_r$ (S_s □$_r$ □$_r$ c_r)/(S_r □$_s$ □$_s$ c_s), s : sample, r : reference, S : intensity of signal, □□□□fluorescence quantum yield, □□□□overall fluorescence collection of the experimental system, c : concentration of each sample and □$_r$: two-photon cross section of reference.

ROS generation study in cuvette. ROS generation was confirmed in phosphate-buffered saline (PBS) (10 mM, pH 7.4) by adding non-fluorescent dihydrorhodamine 123 (DHR123) and probes together. A cuvette containing 5 μM probe and 10

μM DHR123 was treated under LED or 730 nm TP conditions for 30 min. The fluorescence emission of DHR123 was measured using a fluorescence spectrophotometer (FluoroMate FS-2).

Singlet oxygen generation study in cuvette. To measure singlet oxygen production, 2,2'-[9,10-anthracenediylbis(methylene)]dimalonic acid (ABDA) and probes were used together. A cuvette containing 70 μM ABDA and 5 μM probe in PBS was treated under LED or 730 nm TP conditions for 30 min. ABDA absorption-decomposition was measured using a UV-Vis spectrophotometer (S-3100).

ROS inhibition study in cuvette. To confirm the specific ROS produced by BSe-B, various ROS scavengers, including sodium azide (for $^1O_2$), D-mannitol (for ·OH), tiron (for O_2 •−), ebselen (for $ONOO^-$), and sodium pyruvate (for H_2O_2), were used. A cuvette containing DHR123 (5 μM), BSe-B (5 μM) and (100 μM) ROS inhibitor was treated under LED or 730 nm TP conditions for 30 min. The fluorescence emission of DHR123 was measured using a fluorescence spectrophotometer (Fluorometer FS-2).

One- and two-Photon Fluorescence Microscopy. One- and two-photon fluorescence microscopy Cell and tissue images were acquired with spectral confocal and multiphoton microscopy (Leica TCS SP8 MP) in a 40x oil, numerical aperture (NA) = 1.30. The fluorescence microscopy images were obtained with a DMI6000B Microscope (Leica) by exciting the probes with a 488 nm laser and Ti:sapphire laser source (Mai Tai HP; Spectra Physics) set at wavelength 730 nm and average power in the focal plane 1.6 mW. Living cell imaging was performed using a cell incubator (Chamlide IC system, Live Cell Instrument, Korea) to maintain a stable cell environment.

Cell Culture. HeLa, A549, OVCAR-3, WI 38 and L-929 (Korean Cell Line Bank) cells were cultured in glass bottom dishes (NEST) for 2 days before imaging. Stored in a CO_2 incubator (Water Jacketed 3010, Thermo Fisher Scientific) under a humidified atmosphere of 5 / 95 (v / v) CO_2 / air at 37°C. For staining, the growth medium was removed and replaced with a glucose-free medium. The BSe-B was dissolved in DMSO to make a 10 mM stock solution for the cell experiments.

Then, the cells were incubated with each 2.0 μM probe for 30 minutes or 1 h. They were then washed twice with PBS (Gibco, Thermo Fisher Scientific) and then imaged. The growth medium of each cell is as follows. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (WelGene, Korea) supplemented with 10 % FBS (WelGene, Korea), 100 unit/mL penicillin and 100 μg/mL streptomycin. A549, OVCAR-3, WI 38 and L-929 cells were cultured in RPMI medium (WelGene, Korea) supplemented with 10 % FBS (WelGene, Korea), 100 unit/mL penicillin, and 100 μg/mL streptomycin.

ROS generation study in cells. HeLa cells were stained with each 2 μM probe and 10 μM DCFH-DA for 30 min. After incubation, the cells were washed twice with serum-free DMEM. At the same location, 730 nm of TP light was irradiated with 100 scans. The imaging conditions for DCF-DA were 488 nm excitation and 500–550 nm emission window.

Cell Viability. Cytotoxicity was evaluated by performing CCK8 kit (Dojindo) assay. HeLa cells were cultured in 96-well plates for 24 h, and then probes at different concentrations were added. Incubated for 24 h in the dark and after irradiation with LED light. Add 10 % CCK8 to the culture medium and incubate for another 4 h. Absorbance was measured at 450 nm using a microplate reader.

Hoechst33342/PI assays. HeLa cells were stained with each 2 μM probe and 2 μM Hoechst 33342 for 30 min. After incubation, cells were washed twice with serum-free DMEM, and TP excitation sources of 730 nm were irradiated. Then, the cells were stained with 10 μM PI for 30 min. Image conditions were 730 nm excitation and 400–450 nm emission windows for Hoechst 33342 and 488 nm excitation and 650–700 nm emission windows for PI.

Fluorescence Activated Cell Sorting (FACS) analysis. HeLa cells are stained with 10 μM **BSe-B** for 1 h. After irradiating TP, stain with 5 μL annexin v and 1 μL PI. And measured using FACS flow cytometry. FACS flow cytometry data were obtained using a FACSAria III (Becton Dickinson And Company(BD), USA) with laser sources of 488 nm and 633 nm wavelengths.

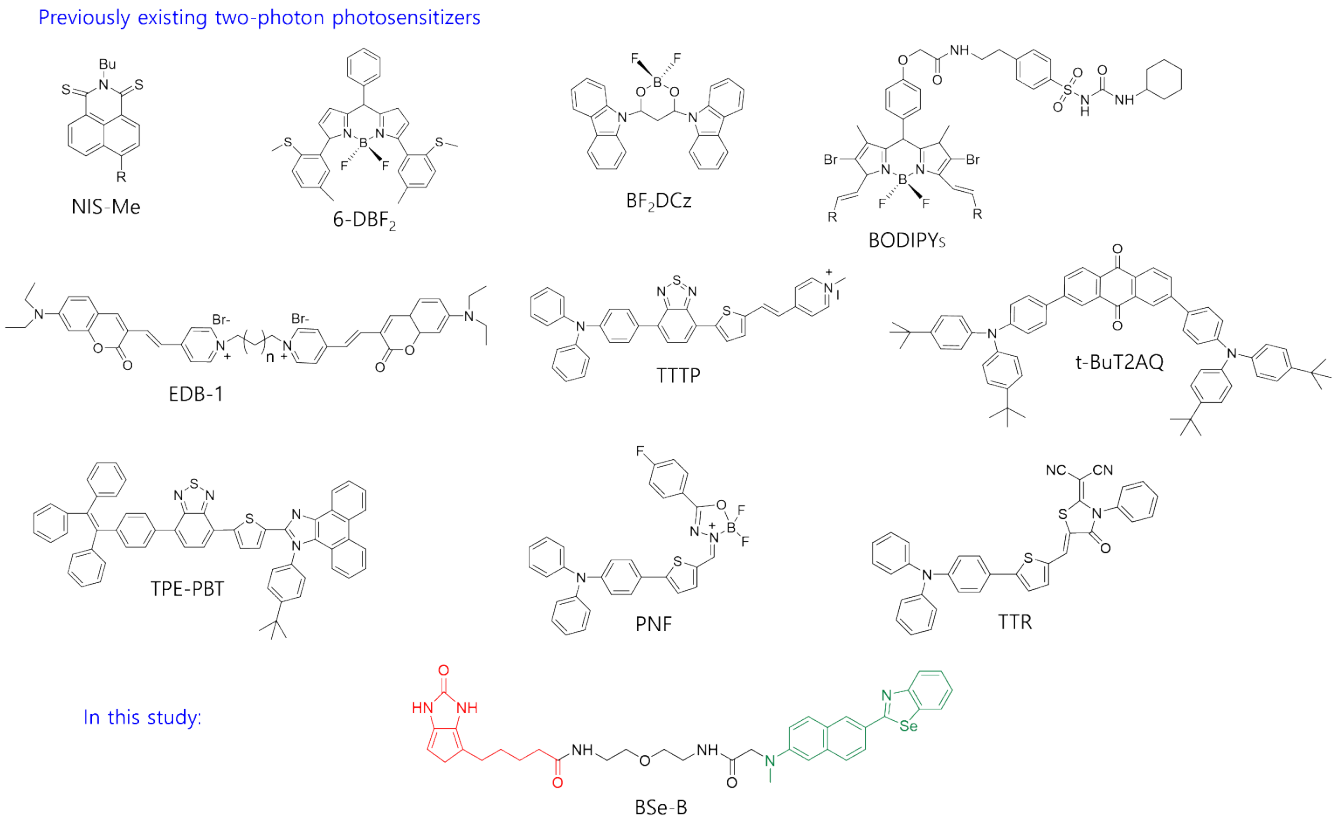
3D Spheroid fluorescence Image of BSe-B. To make spheroids, HeLa and WI38 cells were incubated in 3D cell culture dishes (MicroFIT) for 48 h. Cell culture medium was replaced with serum-free DMEM or RPMI and each 3D spheroid was incubated with 10 μM **BSe-B** for 3 h. The difference in **BSe-B** uptake for each 3D spheroid was confirmed through the fluorescence intensity after incubation. Then, the z-axis was set and the TP was irradiated. To observe apoptosis, 10 μM PI was stained for 24 h. More than 100 cross-sectional images were acquired using xyz mode to obtain 3D spheroid fluorescence images, and then each cross-sectional image was combined using the following LAS-X program. Image conditions were 730 nm excitation and 480–520 nm emission windows for **BSe-B** and 488 nm excitation and 650–700 nm emission windows for PI.

Fluorescence images of BSe-B in human colon tissue. We measured the cancer-selective uptake of **BSe-B** using live normal and cancerous colon tissues. Colon normal and cancer tissues were obtained from three patients by biopsy forceps during a colonoscopy from Ajou University Medical Center (approval no. AJIRB-BMR-SMP-17-164). Each tissue piece was incubated with 10 μM **BSe-B** and DHR123 for 3 h at 37 °C and 5 % CO_2 conditions. To compare **BSe-B** uptake in human normal and cancer colon tissues, fluorescence images were acquired in xyz mode at depths of up to 60 μm within a 480–520 nm emission window. After establishing the xyz coordinates for the ROS generation study, TP was irradiated. DHR123 fluorescence intensity after TP treatment was measured using fluorescence images of whole normal and cancer tissue slices within a 500–550 nm emission window.

***In vivo* biosafety measurements of BSe-B.** All animal experiments were conducted in strict accordance with the guidelines established by the Animal Care and Use Committee of Hubei University, ensuring compliance with ethical standards for animal research. The experiment was conducted with male BALB/c mice aged 5 weeks with a weight range of 20–25 g. To investigate the biocompatibility of BSe-B, the 4T1 tumor-bearing mice were divided into two groups as follows: (1) Control, (2) BSe-B (n = 3). When the volume of the tumors reached ~100 mm^3, the mice were administered with 5μl of PBS and BSe-B (2.5 mg/ml,) via intravenous injection. After complete treatment of 14 days, the mice were sacrificed, and tumors were dissected from the mice. To investigate the biosafety of treatment, the major organs (heart, liver, spleen, lung, and kidney) of each mice were also excised, and H&E staining was performed.

Table S1. Comparison of properties of existing two-photon photosensitizers (TP-PSs).

TP-PSs	Abs./Emission Wavelength (nm)	TP-PDT condition	Cancer cells/organelle selectivity	ROS type	References
NIS-Me	422/476	770 nm, 6.7 mW, 1 min	no	Type I + II	8
6DBF2	541/595	820 nm, 100 mW, 10 min	yes	Type II	9
BF_2DC_Z	380/497	800 nm, 4 mW, 30 sec.	no	Type II	10
BODIPY-derived	669/692	800 nm, 40 mW, 10 min	ER-Targeted	Type II	11
EDB-1	489/633	808 nm, 200 mW/cm2	Cell-membrane targeted	Type I + II	12
TTTP	432/630	700 nm, 80 mW/cm2	Mitochondria targeted	Type II	13
tBuT2AQ	465/658	940 nm, 50 mW, 3 min	no	Type I	14
TPE-PTB	495/600	800nm, 450 mW cm^{-2}, 5 min	no	Type I + II	15
PNF	486/630	800 nm, 50mW cm^{-2}, 6min	no	Type I	16
TTR	504/704	704nm, 60 W/cm2, 5 min	Lysosome-targeted	Type I	17
BSe-B	370/487	730 nm, 1.4 mW, 5 min	Cancer cells targeting	Type I	



Scheme S2. Structures of previously existing two-photon photosensitizers and **BSe-B**.

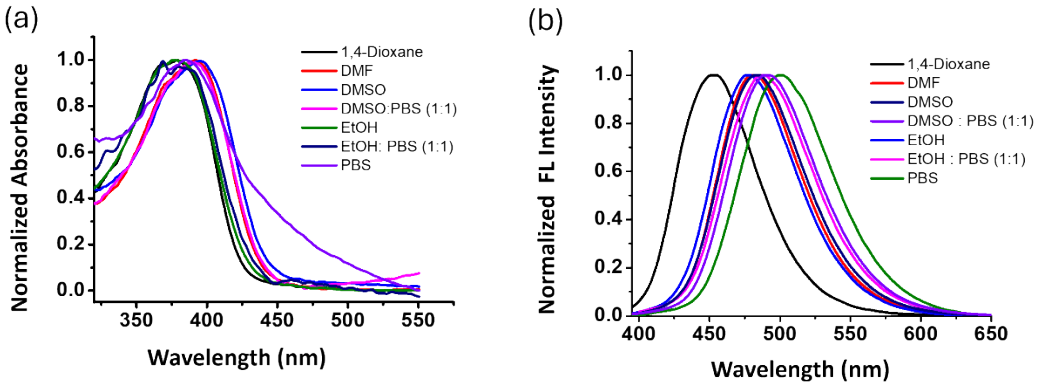
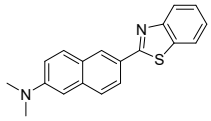


Figure S1. Photophysical characteristics of **BSe-B**. (a) Normalized absorption spectrum and (b) Normalized fluorescence spectrum.

Table S2. Photophysical characteristics of **BSe-B** for each solvent.

Probe	Solvent	λ_{max}^{abs}	λ_{max}^{fl}	Φ
BSe-B	1,4-Dioxane	378	453	0.24
	DMF	392	481	0.45
	DMSO	395	483	0.65
	DMSO:PBS (1:1)	388	490	0.67
	EtOH	378	477	0.47
	EtOH:PBS (1:1)	370	487	0.46
	PBS	385	502	0.35
BTDAN [Ref.7]	PBS	362	497	0.24



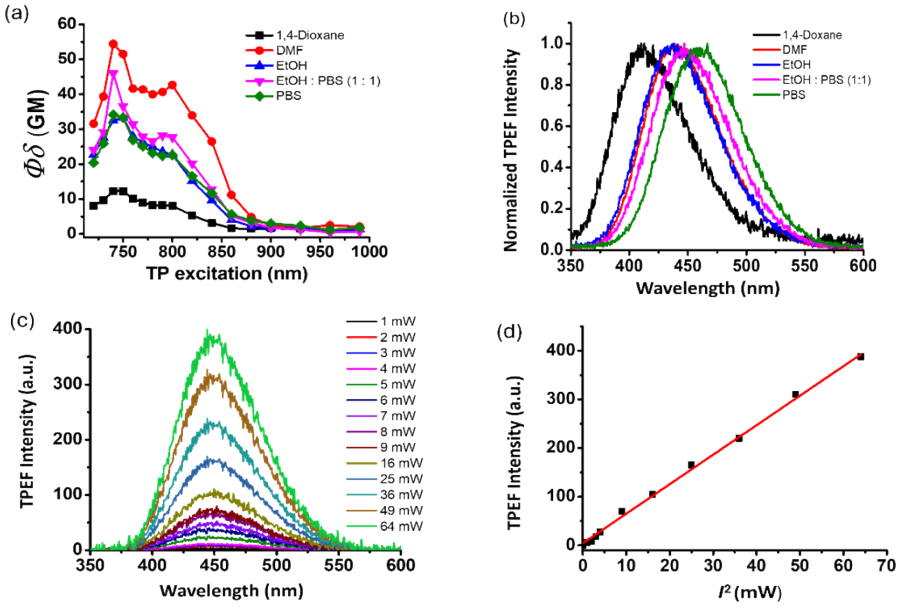


Figure S2. (a) Two-photon action cross section spectrum, (b) Normalized Two-photon emission fluorescence spectra of BSe-B under 730 nm femtosecond laser irradiation in different solvents, (c) TPEF intensity of BSe-B in EtOH: PBS (1:1), under 730 nm femtosecond laser irradiation at varying power levels, (d) Excitation-energy dependent TPEF of (10μM) BSe-B in EtOH: PBS (1:1) solvent at 730 nm.

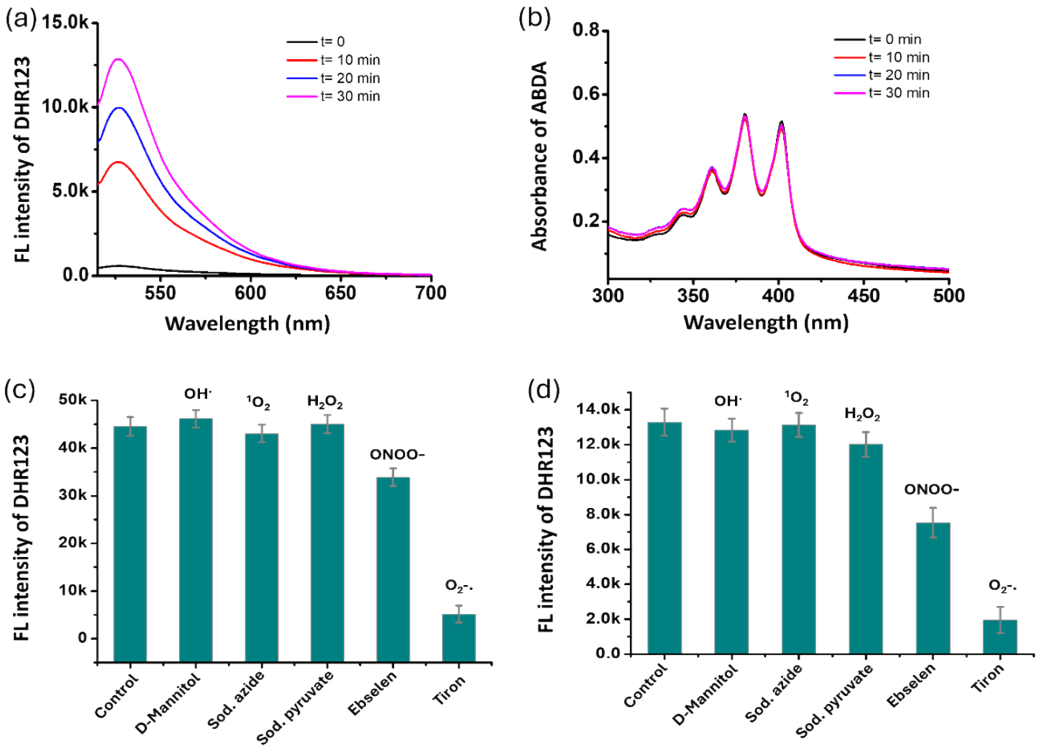


Figure S3. (a) Fluorescence spectra of DHR 123 (5μM) with BSe-B (5μM) after 30 min irradiation at 730 nm (TP), (b) Absorption spectra of (20 μM) ABDA containing BSe-B (5μM) after 30 min TP irradiation at 730 nm. (c) Fluorescence spectra of DHR 123 (5μM) with BSe-B (5μM) after 30 min under white LED irradiation, and (d) Fluorescence spectra of DHR 123 (5μM) with BSe-B (5μM) with 30 min TP irradiation at 730 nm, in the presence of (100 μM) ROS inhibitors; D-mannitol (for •OH), Sodium azide for ($^1O_2$), Sodium pyruvate (for H_2O_2), Ebselen for (ONOO−), and Tiron for (O_2•-) in PBS solution (pH 7.4).

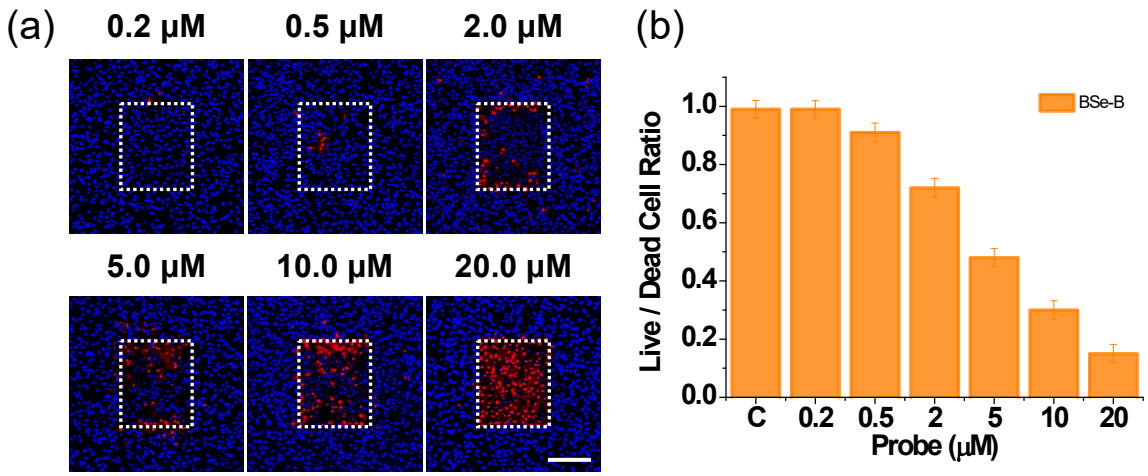


Figure S4. (a) Fluorescence image of HeLa cells. HeLa cells were stained with **BSe-B** at each concentration and then irradiated with TP at 730 nm. (b) Live / dead cell ratio at each concentration. Excitation: 488 nm (PI), 730 nm (Hoechst 33342, TP). Emission: 650 – 700 nm (PI), 400 – 450 nm (Hoechst 33342). Scanning laser: 730 nm, 1.4 mW, 1.3 s per scan, the scanning number: 100 scans. scale bar: 200 μm. (Mean ± SD, n = 3).

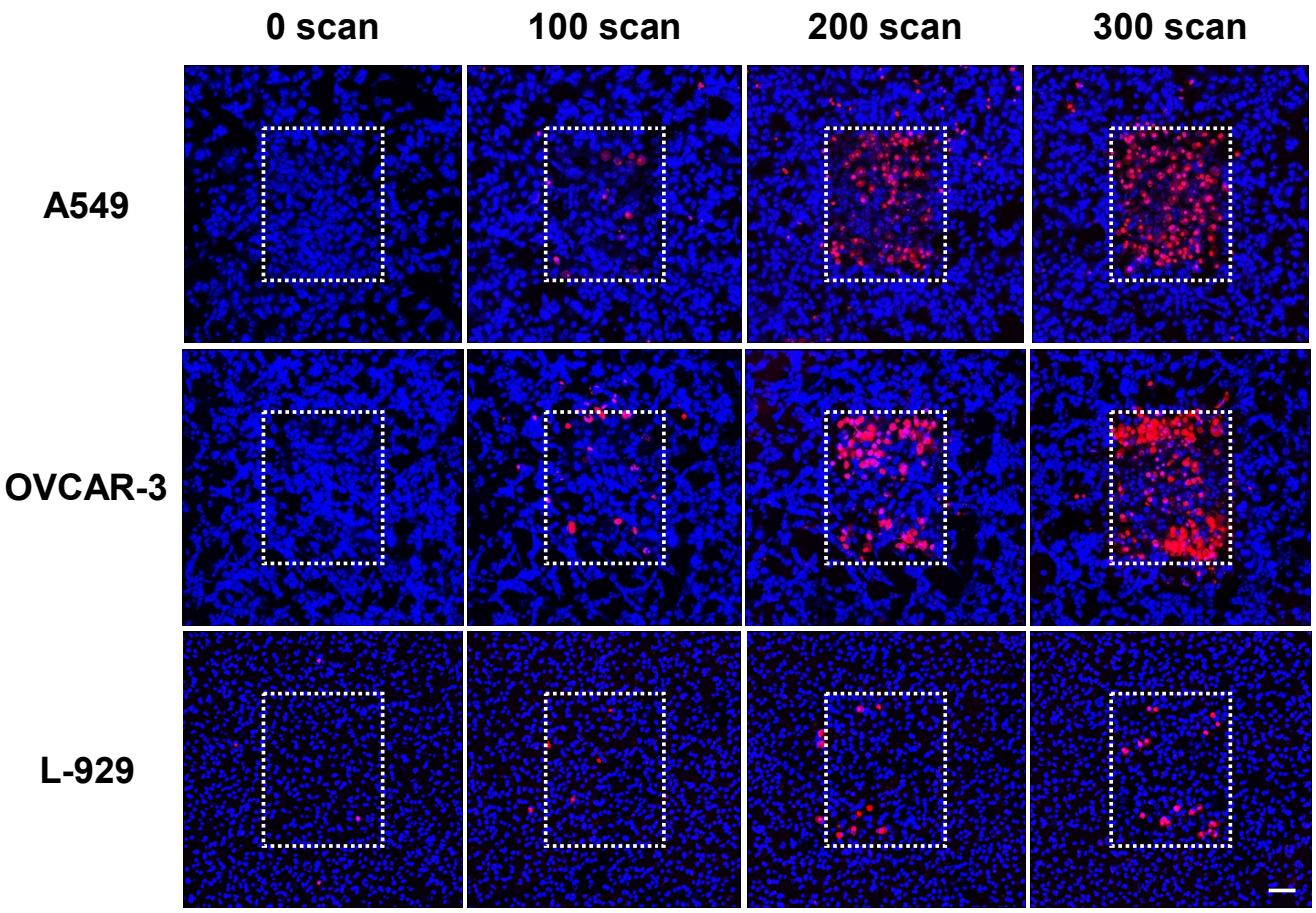


Figure S5. Fluorescence images of A549, OVCAR-3 cancer cells and L-929 normal cells. The cells were treated with **BSe-B** (2 μM, 1 h incubation). After probe staining, white dash line box of cells was irradiated under two photon laser at 730 nm. Excitation: 488 nm (PI), 730 nm (Hoechst 33342, TP). Emission: 400–450 nm (Hoechst 33342), 650–700 nm (PI). Scanning laser: 730 nm, 1.4 mW, 1.3 s per scan. scale bar: 80 μm.

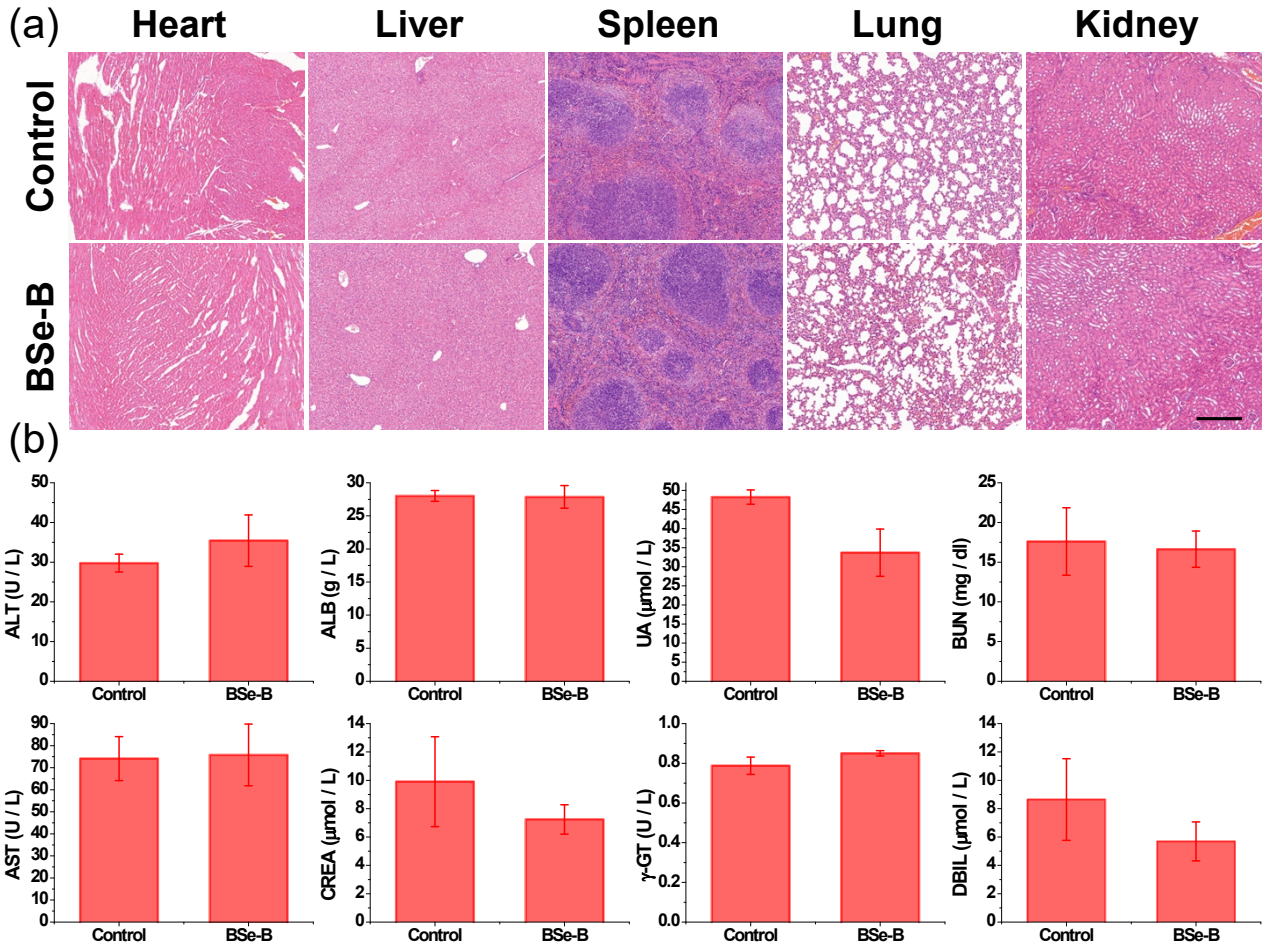


Figure S6. (a) H&E staining images for each organ for biocompatibility testing of **BSe-B**. (b) Bar graph of biochemical blood analysis results for **BSe-B**. Bar graph shows comparison of ALT (alanine aminotransferase), ALB (albumin), UA (uric acid), BUN (urea nitrogen), AST (aspartate aminotransferase), CREA (creatinine), γ-GT (gamma-glutamyltransferase), and DBIL (biochemical parameters). There was little difference between the control and **BSe-B** groups, suggesting that there were no serious adverse effects on drug-induced liver and kidney function. Scale bar: 200 μM. (Mean ± SD, n = 3).

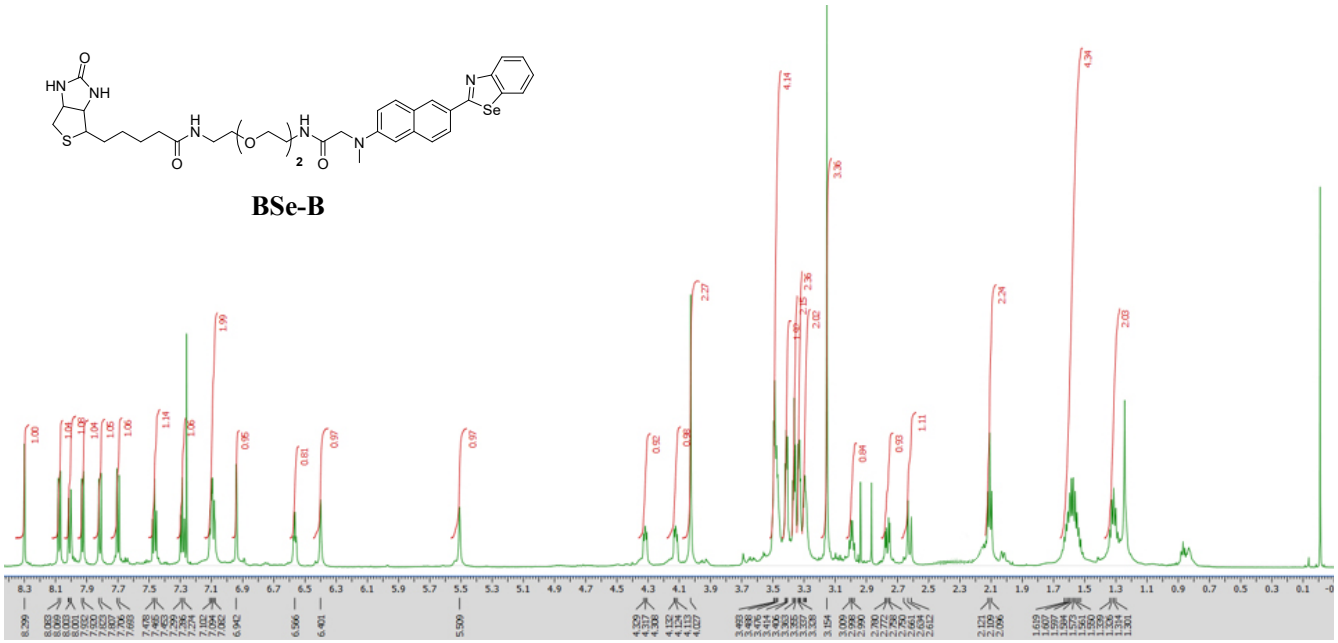


Figure S7. ^{1}H-NMR spectrum (600 MHz) of **BSe-B** in $CDCl_3$.

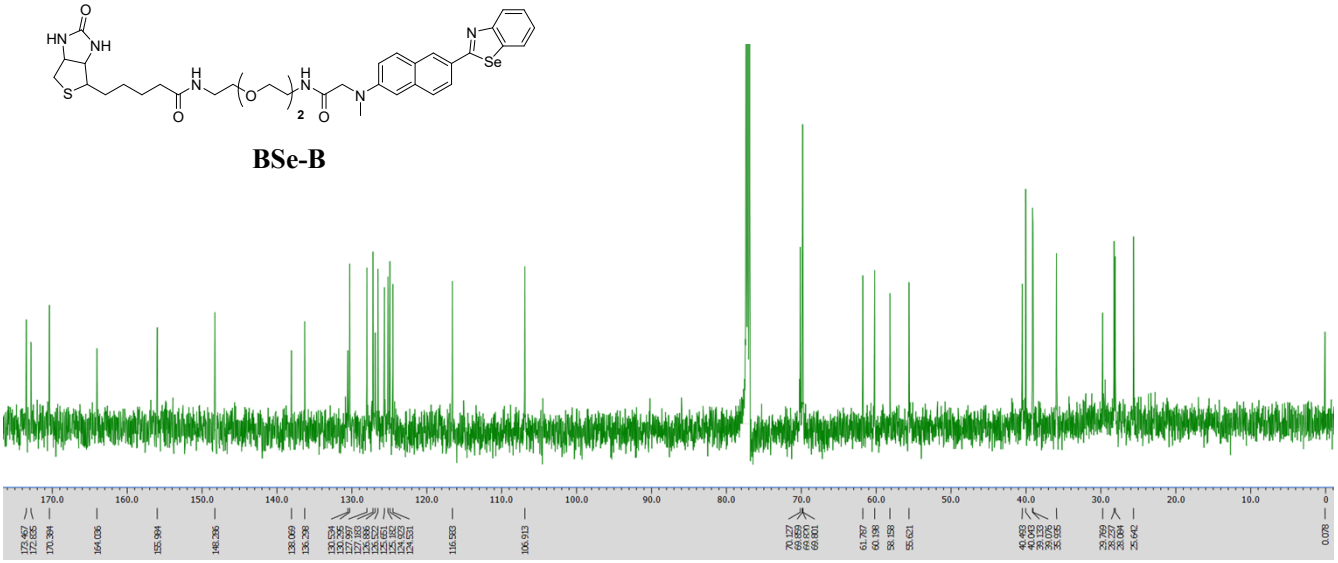


Figure S8. ^{13}C-NMR spectrum (150 MHz) of **BSe-B** in $CDCl_3$.

[3] MS spectrum (Biotin BSe)

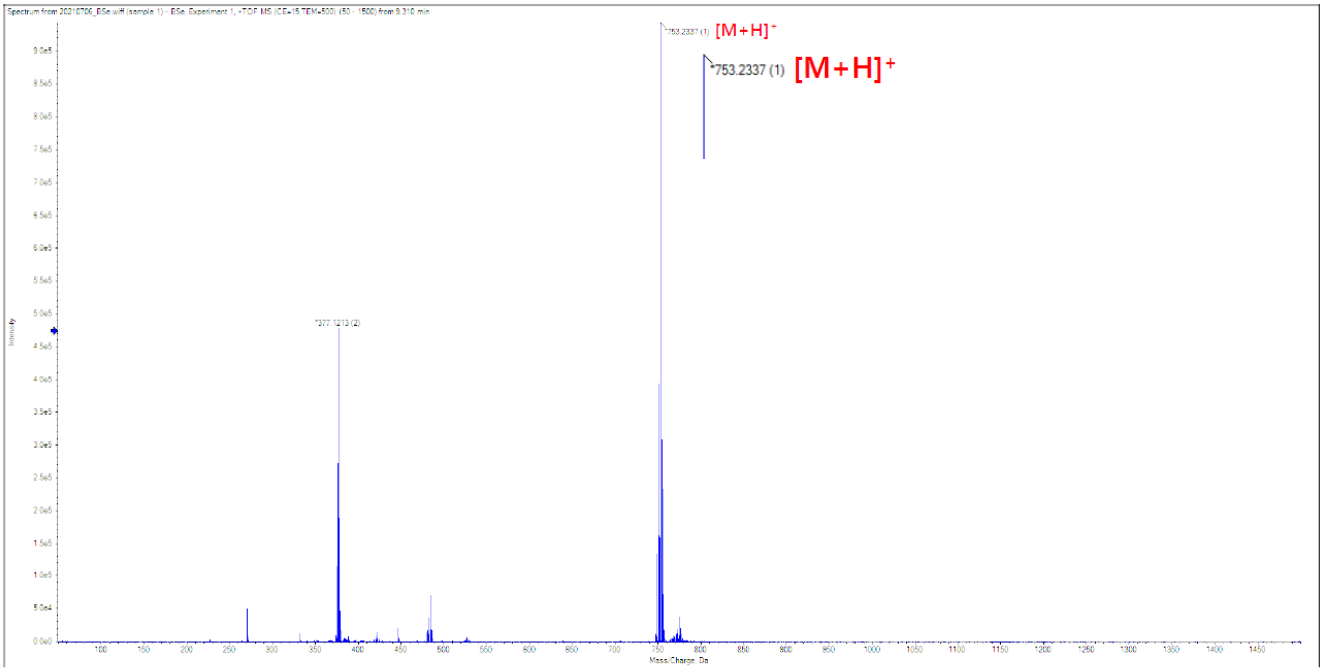


Figure S9. HRMS spectrum of **BSe-B**.

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