Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

SIWV tetrapeptide and ROS-responsive prodrug conjugate for advanced glioblastoma therapy

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This file includes:

1. Materials and Methods

1.1. Materials

The chemical reagents were purchased from Acros Organics (Geel, Flanders, Belgium), Alfa Aesar (Haverhill, MA, USA), Sigma-Aldrich (St. Louis, MO, USA), and TCI (Tokyo, Japan). Dimethyl sulfoxide (DMSO, anhydrous, Product No. 1.02952.1000) and hydrogen peroxide (H_2O_2) were purchased from MilliporeSigma, Inc. (Burlington, MA, USA). Deionized water (D.I H_2O, Ultra370, Younglin Co., Gyeonggi-do, Rep. of Korea) was used to prepare aqueous solutions. Cell counting Kit-8 (CCK-8, Product No. CK04-13, Dojindo Molecular Tech. Inc, Tokyo, Japan) was used to confirm the cellular toxicity. Phosphate buffered saline (PBS pH 7.4 (10×), Product No. 70011044), Penicillin streptomycin (Product No. 15140-122), and Trypsin-EDTA (Product No. 25200-056) were purchased from Gibco (Carlsbad, CA, USA). 7-Ethyl-10-hydroxycamptothecin (SN-38, Product No. FE29579) was purchased from Carbosynth (Berkshire, UK). Ethanol (Ethyl alcohol (94.5%), Product No. E0223) was purchased from Samchun Chemicals (Seoul, Rep. of Korea). Species used to perform the screening of metal ions and amino acids; $FeCl_2$, $ZnCl_2$, $CuCl_2$, $CdCl_2$, AlCl, $CoCl_2$, NaCl, $MgCl_2$, KCl, $CaCl_2$, L-cysteine, DL-homocysteine, L-glutathione, L-glycine, and L-lysine. Fetal bovine serum (FBS, Product No. SH30084.03), Dulbecco's modified eagle's medium (DMEM, Product No. SH30243.01), and Dulbecco's phosphate-buffered saline (DPBS, Product No. SH30028.02) were purchased from Hyclone (Logan, UT, USA). Cell culture dishes (96-well plate and 100-Ø dish) were purchased from SPL Life Science (Product No. 30096, 20100, Gyeonggi-do, Rep. of Korea).

1.2. Instrumentations

^1H NMR spectra were measured with a JNM-ECZ500R (500 MHz, Tokyo, Japan). CLSM images were observed using a confocal laser scanning microscope (CLSM, LSM 800, Carl Zeiss, Jena, Germany). Fourier transform infrared (FTIR) spectroscopy was analyzed using Thermo Scientific Nicolet™ iS™ 5 FT-IR spectrometer instrument (32 scans, Waltham, MA, USA). 55 mM Tert-Butyl Hydrogen Peroxide (TBHP, Product No. ab113851) was purchased from (Abcam, Cambridge, UK). ESI-MS spectra were measured at the Korea Basic Science Institute (western Seoul, Rep. of Korea).

1.3. UV/Vis absorption and spectroscopic emission methods

UV/Vis absorption spectra were detected using a spectrophotometer (Agilent, California, USA). Fluorescence spectra were obtained using a spectro-fluorophotometer (SHIMADZU CORP. RF-6000, Kyoto, Japan). Materials were dissolved in dimethyl sulfoxide to prepare the stock solution (100 μM). The stock solutions were diluted into PBS (pH 7.4) (final concentration: 10 μM). **SIWV-PB-SN** was dissolved in PBS (10 μM, pH 7.4) and transferred to a 1 cm standard quartz cuvette to acquire UV/Vis absorption and emission spectra.

1.4. Synthesis of compound 1

[See Fig. S1] 3,4-dihydroxybenzaldehyde (13.8 g, 100 mmol) and NaH (4.8 g, 200 mmol) were dissolved in anhydrous dimethyl sulfoxide (50 mL). The mixture was stirred and cooled. While cooling the mixture to 0 °C, 3,4-dihydroxybenzaldehyde (13.8 g, 100 mmol) and anhydrous dimethyl sulfoxide (60 mL) were added dropwise. This mixture was stirred at room temperature for 30 min. Then, the mixture was added with propargyl bromide (11.1 mL 100 mmol) dropwise and stirred at room temperature overnight. After completion of the reaction (as monitored by TLC), the final mixture was poured onto

ice, neutralized carefully with 1 M HCl solution, extracted with ethyl acetate (3 × 250 mL), and dried over anhydrous sodium sulfate. The organic extracts were washed with brine (5 × 250 mL), and the organic layer was dried over anhydrous sodium sulfate. After filtration, the residue was purified with flash chromatography on silica gel (ethyl acetate/n-hexane/acetic acid = 20:80:1), which produced compound **1**.[1]

1.5. Synthesis of compound 2

[See Fig. S1] Compound **1** (1.0 g, 5.68 mmol), K_2CO_3 (1.57 g, 11.36 mmol) and 4-bromomethyl phenylboronic acid pinacol ester (1.68 g, 5.68 mmol) were dissolved in acetonitrile (20 mL). The mixture was stirred at 80 °C overnight. After completion of the reaction (as monitored by TLC), the final mixture was cooled, filtered, and concentrated under vacuum. Flash chromatographic purification on silica gel (eluent: 1:4 (*v/v*) ethyl acetate:n-hexane) produced compound **2** (1.78 g, 80% yield) as a light-yellow viscous liquid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.81 (s, 1H), 7.82 (d, *J* = 7.54 Hz, 2H), 7.55 (t, *J* = 1.74 Hz, 1H), 7.44-7.40 (m, 3H), 6.96 (dd, *J* = 8.28, 1.62 Hz, 1H), 5.24 (s, 2H), 4.81 (d, *J* = 2.4 Hz, 2H), 2.55 (t, *J* = 2.4 Hz, 1H), 1.33 (s, 12H). ^{13}C NMR (125 MHz, $CDCl_3$): 190.63, 154.00, 147.62, 138.93, 135.05, 130.01, 127.00, 126.18, 112.95, 83.78, 77.85, 76.35, 70.67, 56.75, 24.76 ppm.

1.6. Synthesis of compound 3

[See Fig. S1] Compound **2** (1.60 g, 4.08 mmol) and $NaBH_4$ (0.31 g, 8.16 mmol) were dissolved in methanol (10 mL). The mixture was stirred at room temperature for 2 h. After completion of the reaction (as indicated by TLC), the final mixture was concentrated under vacuum and purified by column chromatography on silica gel (ethyl acetate/n-hexane) = 1:4 to 1:2 as eluent), which produced compound **3** (1.29 g, 85% yield) as a light-yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.82-7.79 (m, 2H), 7.44-7.41 (m, 2H), 7.08 (d, *J* = 1.85 Hz, 1H), 6.87 (dd, *J* = 8.25, 1.90 Hz, 1H), 6.83 (d, *J* = 8.25 Hz, 1H), 5.16 (s, 2H), 4.77 (d, *J* = 2.40 Hz, 2H), 4.58 (d, *J* = 4.60 Hz, 2H), 2.51 (t, *J* = 2.40 Hz, 1H), 1.86 (br s, 1H), 1.34 (s, 12H). ^{13}C NMR (125 MHz, $CDCl_3$): 148.33, 147.45, 140.14, 134.95, 134.13, 126.28, 120.91, 114.55, 114.49, 83.78, 78.70, 75.73, 70.99, 64.97, 57.04, 24.80 ppm.

1.7. Synthesis of compound 4

[See Fig. S1] Compound **3** (1.20 g, 3.04 mmol) and PBr_3 (0.41 g, 1.52 mmol) were dissolved in dichloromethane (30 mL) at 0 °C. The mixture was warmed to room temperature and stirred overnight. After completion of the reaction (as monitored by TLC), the final mixture was cooled to 0 °C, neutralized carefully with $NaHSO_4$, extracted with dichloromethane (3 x 30 mL), and dried over anhydrous sodium sulfate. The concentrated residue was purified with flash chromatography on silica gel (ethyl acetate/n-hexane = 1:4 as eluent), which produced compound **4** (1.25 g, 90% yield) as a pale-yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.82-7.79 (m, 2H), 7.44-7.41 (m, 2H), 7.10 (d, *J* = 2.10 Hz, 1H), 6.93 (dd, *J* = 8.30, 2.10 Hz, 1H), 6.81 (d, *J* = 8.3 Hz, 1H), 5.17 (s, 2H), 4.79 (d, *J* = 2.35 Hz, 2H), 4.47 (s, 2H), 2.53 (t, *J* = 2.35 Hz, 1H), 1.34 (s, 12H). ^{13}C NMR (125 MHz, $CDCl_3$): 149.00, 147.22, 139.80, 134.93, 130.56, 126.20, 122.99, 116.24, 114.11, 83.72, 78.38, 75.95, 70.79, 57.04, 33.88, 24.76 ppm.

1.8. Synthesis of B-SN

[See Fig. S2] SN-38 (0.10 g, 0.25 mmol) and Cs_2CO_3 (0.10 g, 0.30 mmol) were dissolved in dry dimethylformamide (10 mL). The mixture was stirred at room temperature and added with compound **4** (0.34 g, 0.75 mmol) after 15 min. The resulting mixture was stirred overnight at room temperature.

After the overnight reaction, ethyl acetate (50 mL) was added and the final mixture was washed with brine (50 mL), and the organic layer was dried over anhydrous sodium sulfate. After the concentration process, the residue was purified with flash chromatography on silica gel (methanol/dichloromethane = 1:99 to 2:99), which produced **B-SN** (0.19 g, 69% yield) as a light-yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.13 (d, *J* = 9.25 Hz, 1 H), 7.81 (d, *J* = 8.0 Hz, 2H), 7.60 (s, 1H), 7.52 (dd, *J* = 9.25, 2.65 Hz, 1H), 7.43 (d, *J* = 7.9 Hz, 2H), 7.21 (d, *J* = 1.9 Hz, 1H), 7.37 (d, *J* = 2.65 Hz, 1H), 7.03 (dd, *J* = 8.30, 1.80 Hz, 1H), 6.91 (d, *J* = 8.30 Hz, 1H), 5.75 (d, *J* = 16.15 Hz, 1H), 5.30 (d, *J* = 16.20 Hz, 1H), 5.23 (s, 2H), 5.19 (s, 2H), 5.17 (s, 2H), 4.80 (d, *J* = 2.40 Hz, 2H), 3.74 (br s, 1H), 3.10 (q, *J* = 7.65 Hz, 2H), 2.45 (t, *J* = 2.35 Hz, 1H), 1.95-1.82 (m, 2H), 1.34 (s, 12H), 1.33 (t, *J* = 7.70 Hz, 3H), 1.03 (t, *J* = 7.38 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): 173.80, 157.70, 157.57, 150.22, 149.47, 148.95, 147.56, 147.00, 145.13, 143.73, 140.01, 135.10, 135.04, 132.01, 132.00, 129.09, 127.99, 127.25, 126.31, 122.67, 121.77, 118.00, 115.07, 114.47, 103.15, 97.63, 83.86, 78.63, 75.99, 72.96, 70.94, 70.20, 66.20, 57.16, 49.40, 31.61, 24.90, 23.14, 13.55, 7.91 ppm.

1.9. Synthesis of PB-SN

[See Fig. S2] **B-SN** (15 mg, 0.02 mmol) and azido-PEG-NHS (M.W. = 2,000 g/mol, 39 mg, 1 equiv.) were dissolved in dichloromethane/methanol (2:1, total = 10 mL) solution, and sodium ascorbate (10 mol%) was added. The mixture was degassed for 30 min by purging Ar gas whilst continuously stirring. In the meantime, $CuSO_4.5H_2O$ (5 mol%) was dissolved in methanol (0.5 mL) in an Eppendorf tube and degassed by purging Ar gas. After 30 min, the degassed $CuSO_4.5H_2O$ solution was added to the reaction mixture, and the stirring continued for an additional 8 h. After completion of the reaction (indicated by TLC), the solvent was evaporated in vacuo, and the resulting crude was directly passed through a concise silica gel column (methanol/dichloromethane = 1:19 to 2:8) to produce **PB-SN**.

1.10. Synthesis of SIWV-PB-SN

[See Fig. S2] **PB-SN** (2 mg, 1 equiv.) and SIWV-ahx6-K (0.51 mg, 1 equiv.) were dissolved in D.I H_2O (0.5 mL). The mixture was stirred at 4 °C for 4 h. **SIWV-PB-SN** was used in this study without further purification.

1.11. Cell culture

U87MG (human malignant glioma cell line) and Huh7 (human hepatocellular carcinoma cell line) cells were incubated in DMEM containing 10% (*v/v*) FBS and 1% (*v/v*) penicillin-streptomycin in humidified cell culture incubator (5% CO_2 air condition, 37 °C).

1.12. Cell cytotoxicity assay

The U87MG and Huh7 cells (5 × 10^3 cells per well) were seeded on 96-well plates and incubated for 24 h. After incubation, each cell was treated with **SIWV-PB-SN** (0–50 μM) for 48 h. The cells were then washed with Dulbecco's phosphate-buffered saline (DPBS, 3 times) and treated with CCK-8 solution (100 μL) following the manufacturer's instructions.

1.13. Intracellular H_2O_2 responsiveness of SIWV-PB-SN

The U87MG cells (2 × 10^5 cells per confocal dish) were incubated for 24 h at 37 °C. After 24 h incubation, the cells were pre-treated with TBHP (150 μM) for 3 h and then treated with 10 μM of **SIWV-PB-SN** for 6 h at 37 °C. After 6 h incubation, the cells were washed three times with DPBS and

then fixed with 4% paraformaldehyde (10 min). Excitation and emission channel selection: **SIWV-PB-SN** (405/422 nm, 400–500 nm, detector Gain: 900 V, laser power: 2.00%), Free SN (405/553 nm, 500–700 nm, detector Gain: 900 V, laser power: 2.00%, detector Gain: 900 V, laser power: 2.00%).

1.14. Confocal laser scanning microscopy (CLSM) analysis for intracellular uptake study

The U87MG and Huh7 cells (2×10^5 cells/mL) were seeded on a confocal dish and incubated for 24 h at 37 °C. The cells were then treated with 10 μM of **SIWV-PB-SN** and further incubated for 12 h at 37 °C. After 12 h incubation, the cells were washed three times with DPBS and then fixed with 4% paraformaldehyde (10 min). Excitation and emission channel selection: **SIWV-PB-SN** (405/422 nm, 400–500 nm, detector Gain: 900 V, laser power: 2.00%), Free SN (405/553 nm, 500–700 nm, detector Gain: 900 V, laser power: 2.00%, detector Gain: 900 V, laser power: 2.00%).

2. Supporting Figures

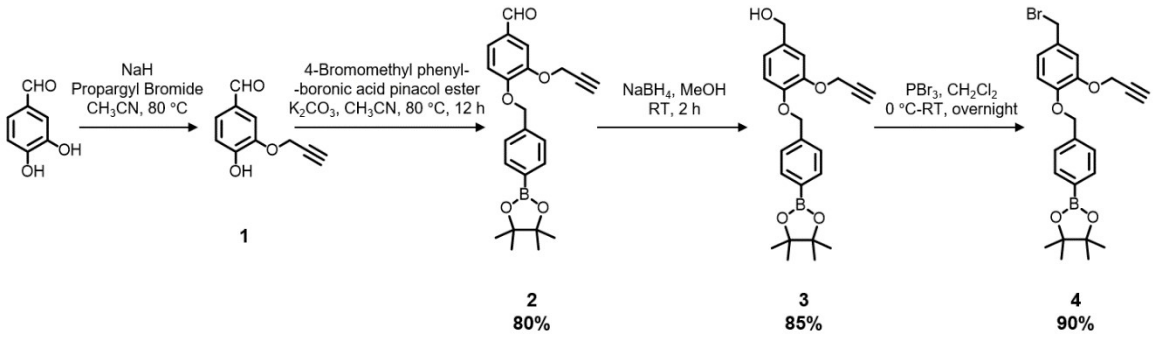

Fig. S1. Synthetic scheme of compounds **1**–**4**.

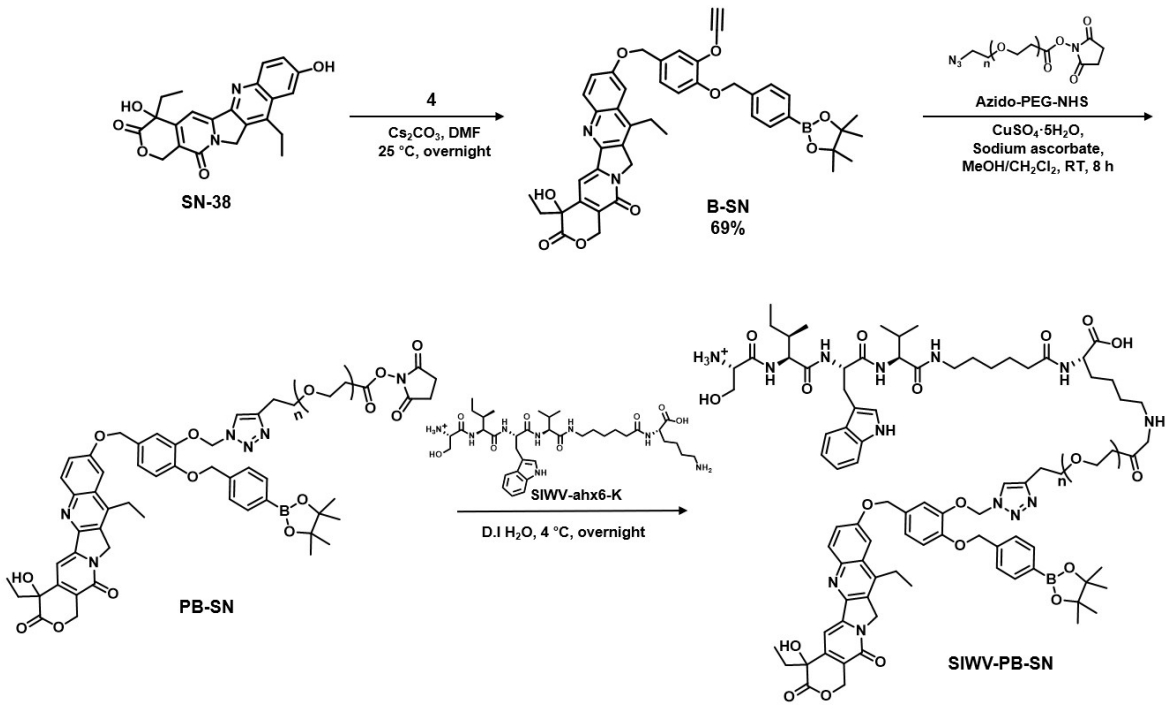

Fig. S2. Synthetic scheme of compounds **B-SN**, **PB-SN**, and **SIWV-PB-SN**.

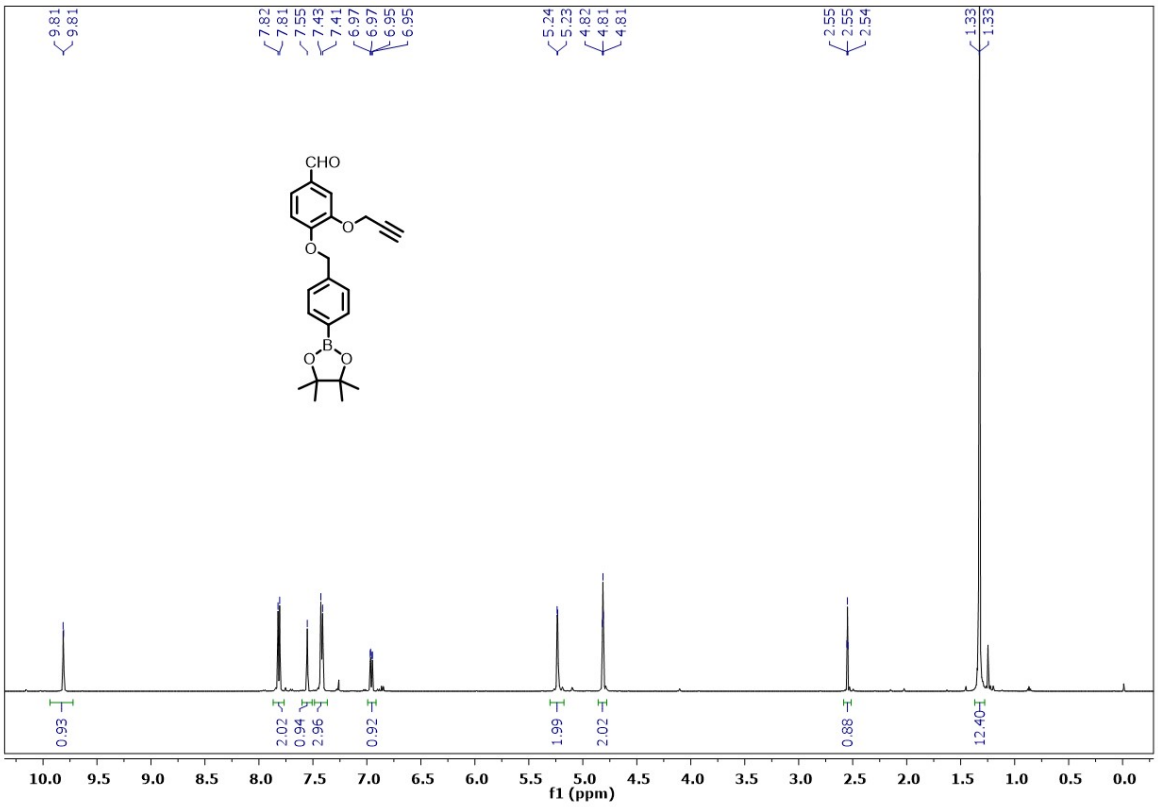

Fig. S3. ^{1}H NMR (500 MHz) spectrum of compound **2** in $CDCl_3$.

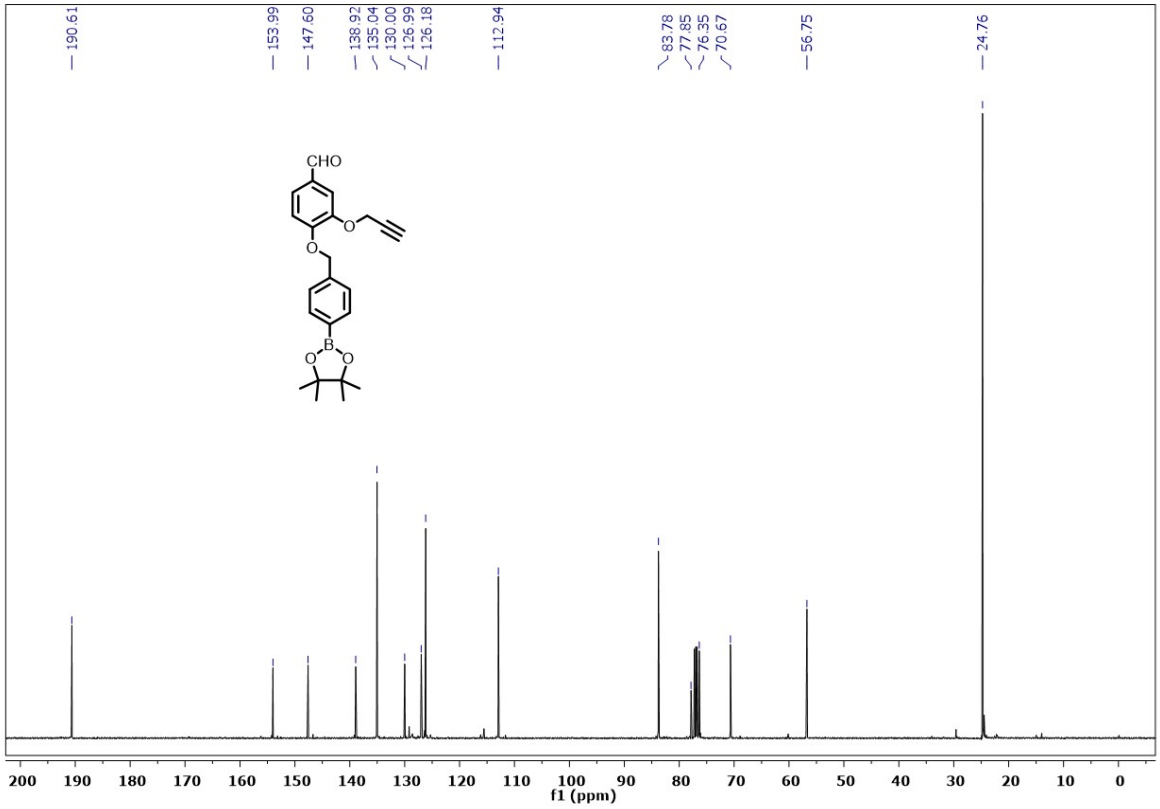

Fig. S4. ^{13}C NMR (125 MHz) spectrum of compound **2** in $CDCl_3$.

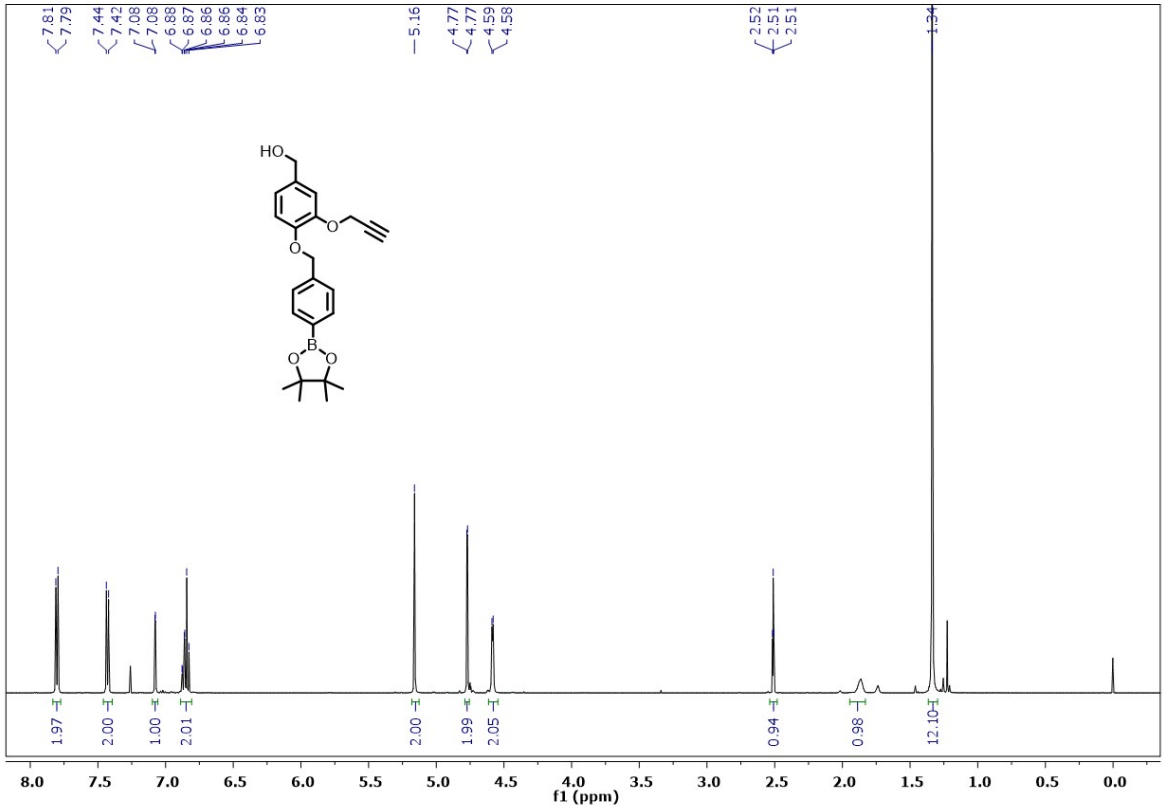

Fig. S5. ^{1}H NMR (500 MHz) spectrum of compound **3** in $CDCl_3$.

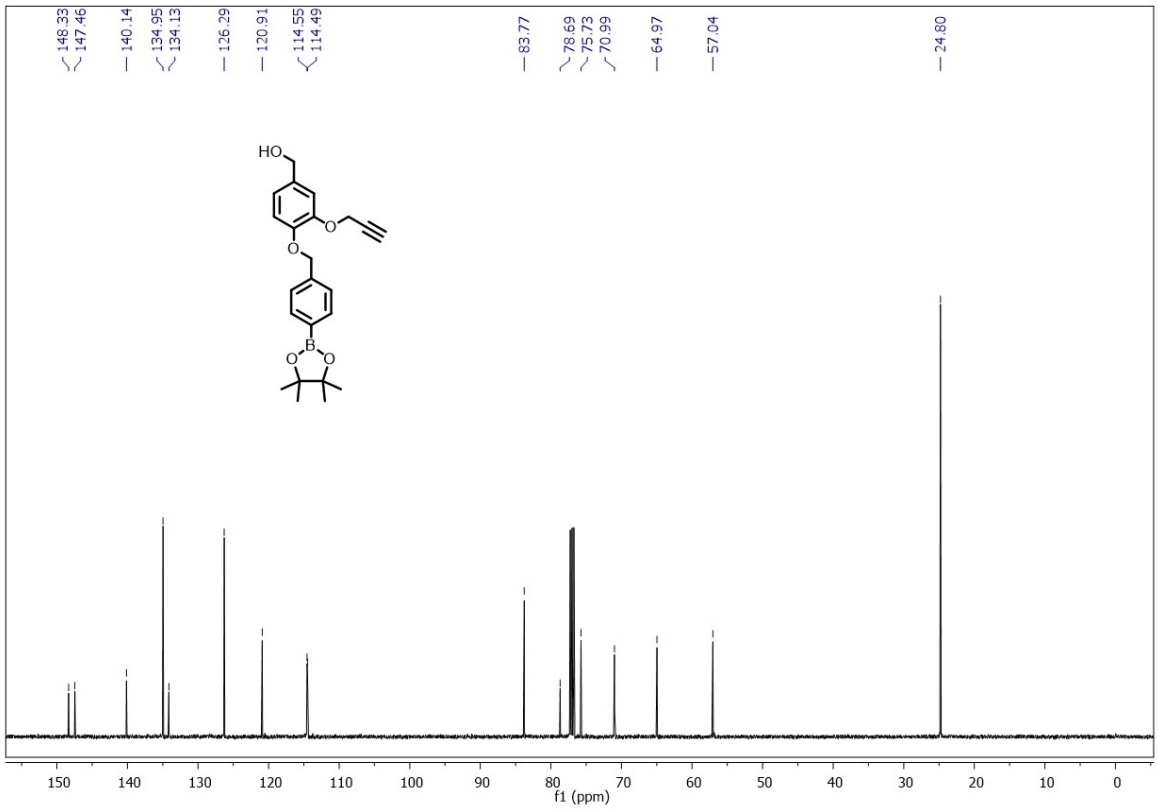

Fig. S6. ^{13}C NMR (125 MHz) spectrum of compound **3** in $CDCl_3$.

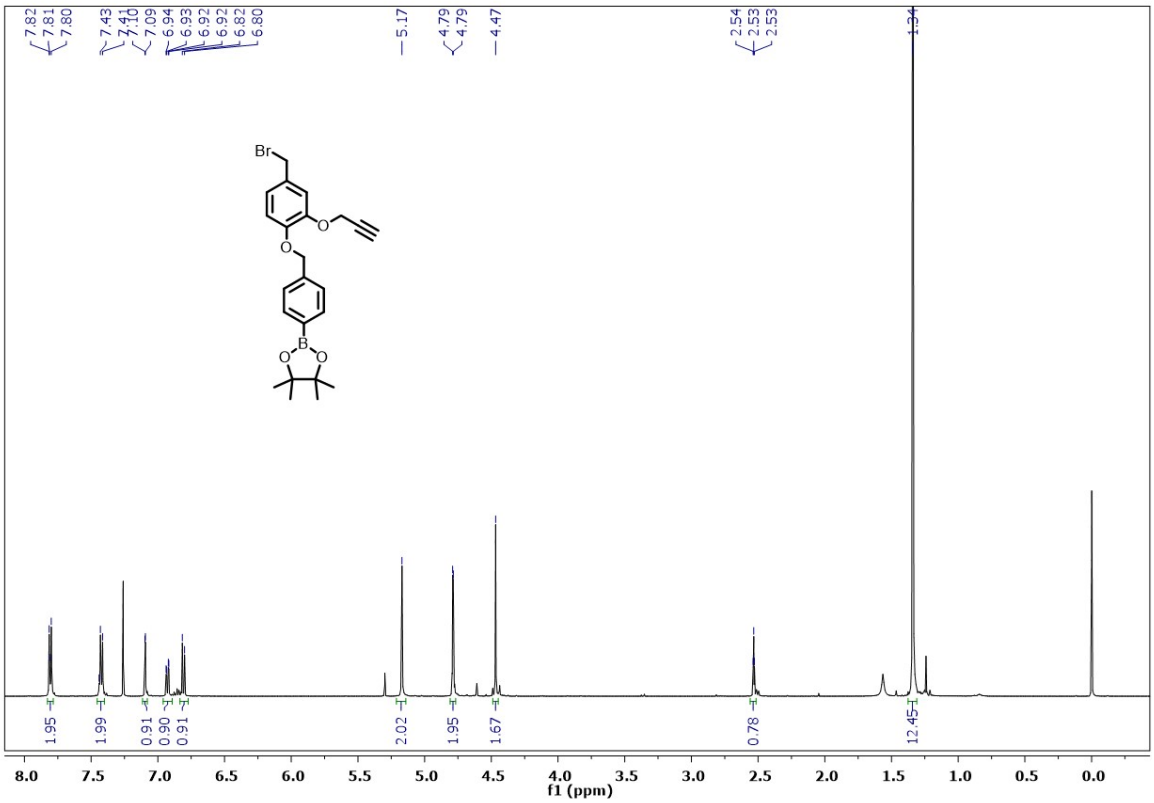

Fig. S7. ^{1}H NMR (500 MHz) spectrum of compound **4** in $CDCl_3$.

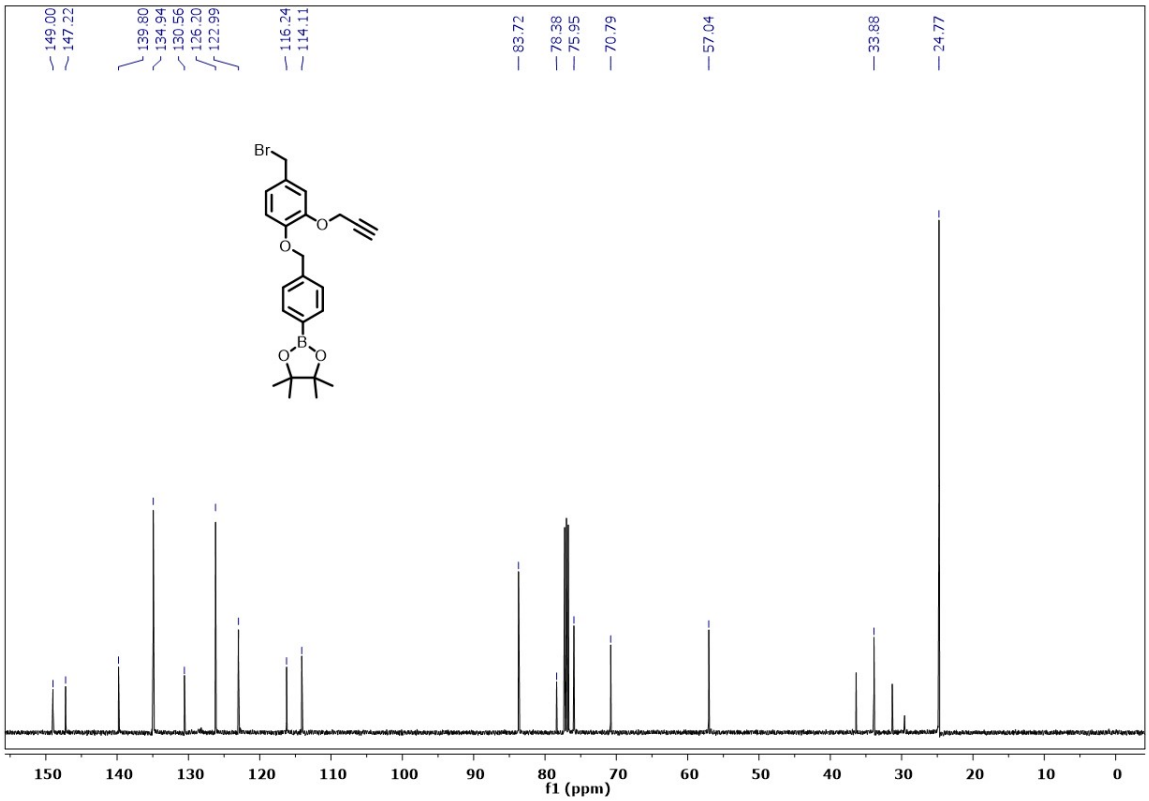

Fig. S8. ^{13}C NMR (125 MHz) spectrum of compound **4** in $CDCl_3$.

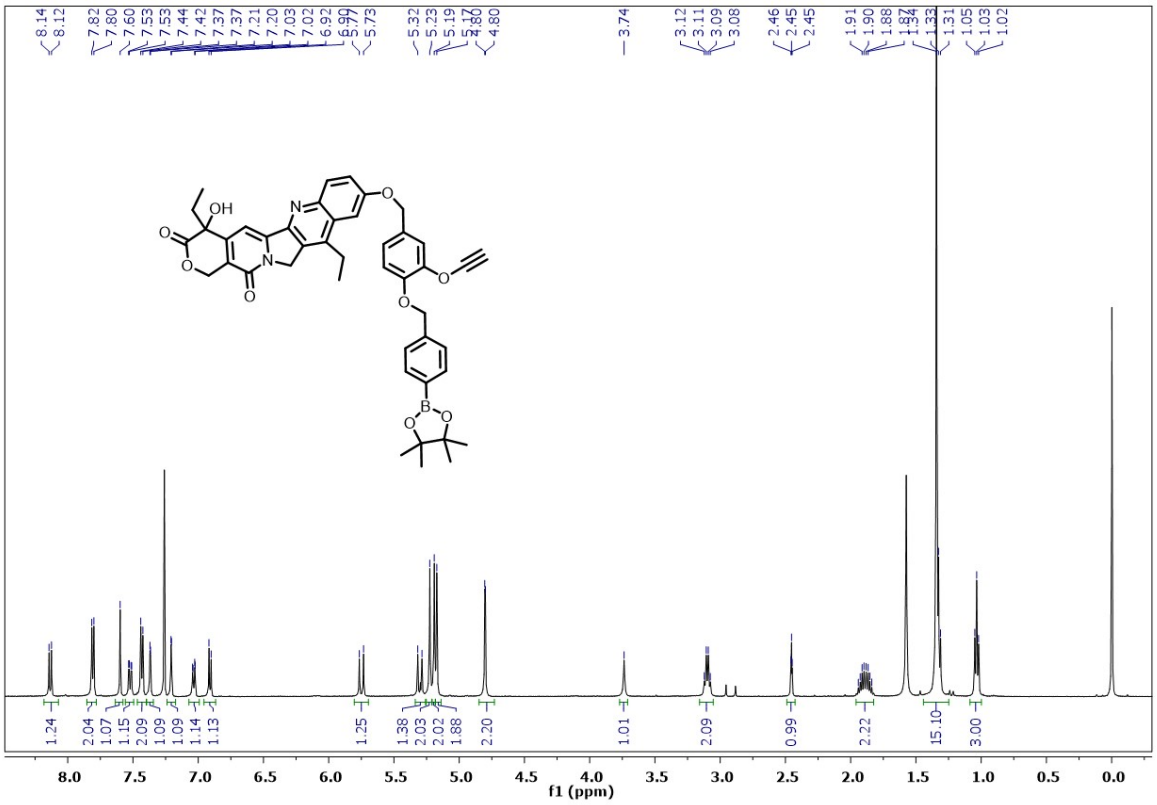

Fig. S9. ^{1}H NMR (500 MHz) spectrum of **B-SN** in $CDCl_3$.

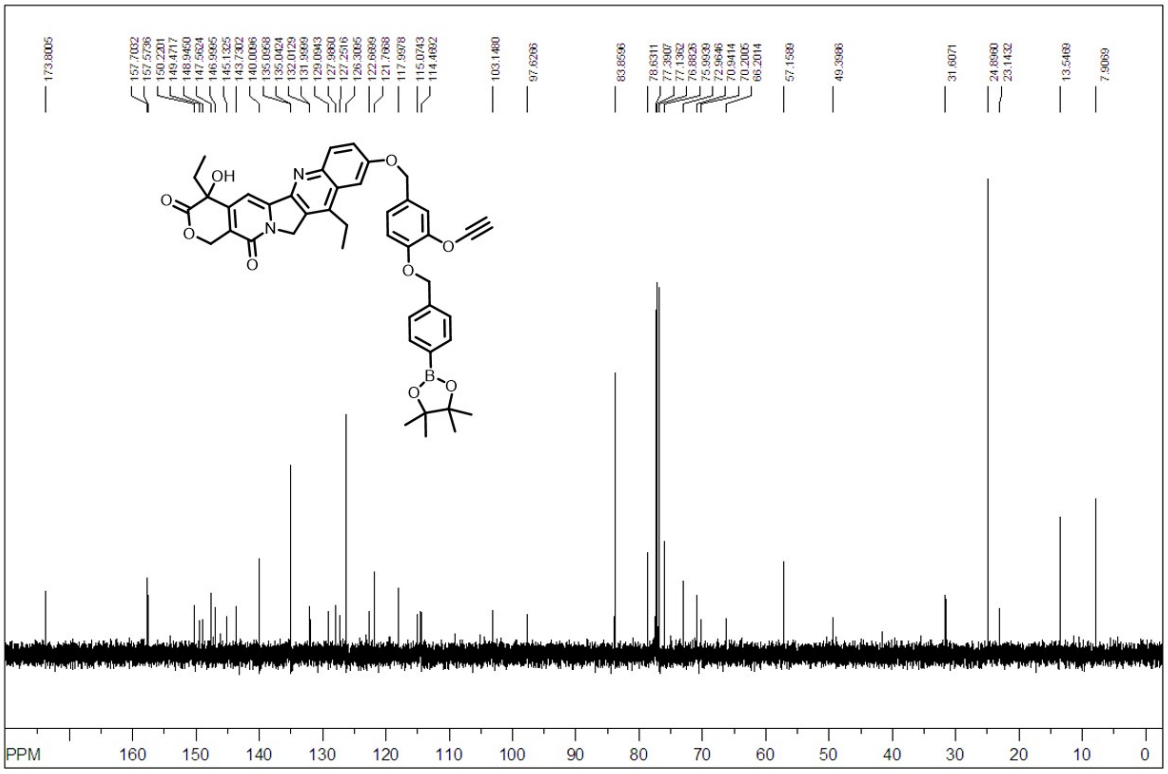

Fig. S10. ^{13}C NMR (125 MHz) spectrum of **B-SN** in $CDCl_3$.

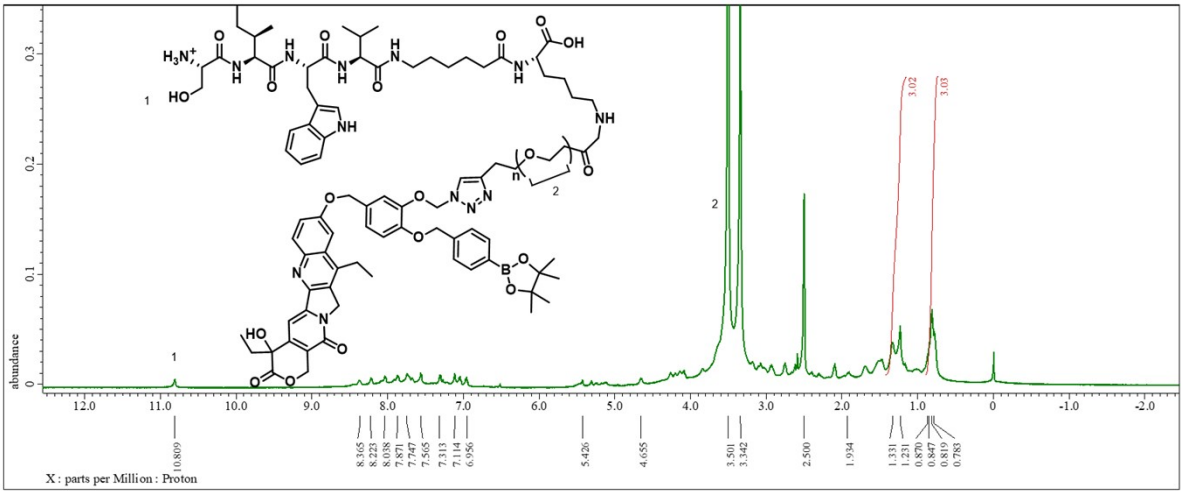

Fig. S11. ^{1}H NMR (500 MHz) spectrum of **SIWV-PB-SN** in DMSO-d_6.

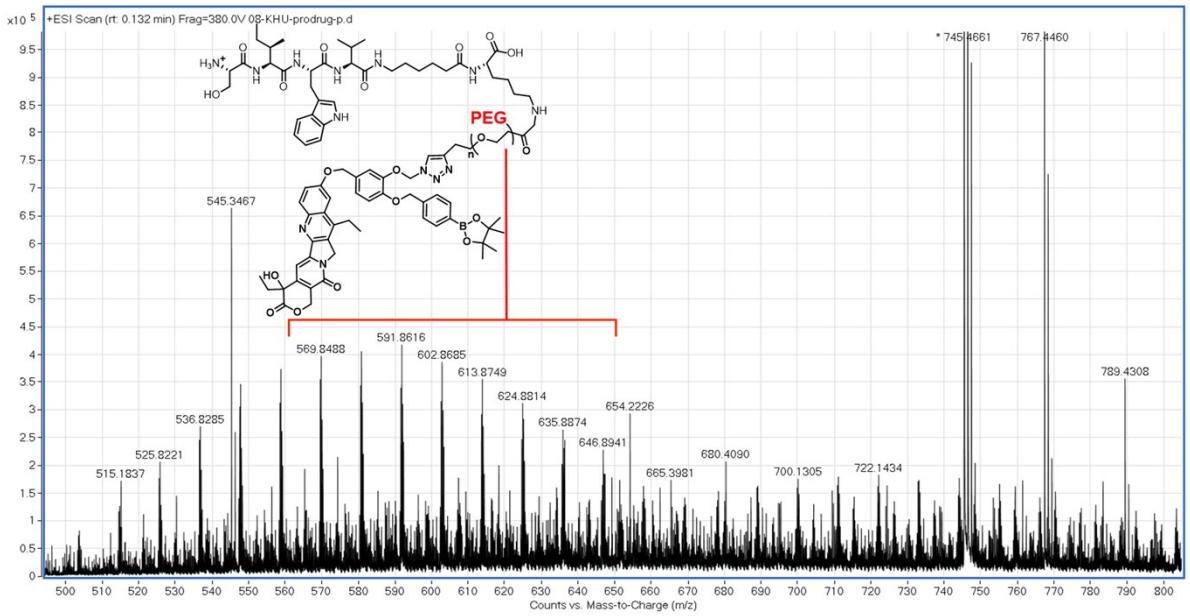

Fig. S12. ESI-MS spectrum of **SIWV-PB-SN** (positive).

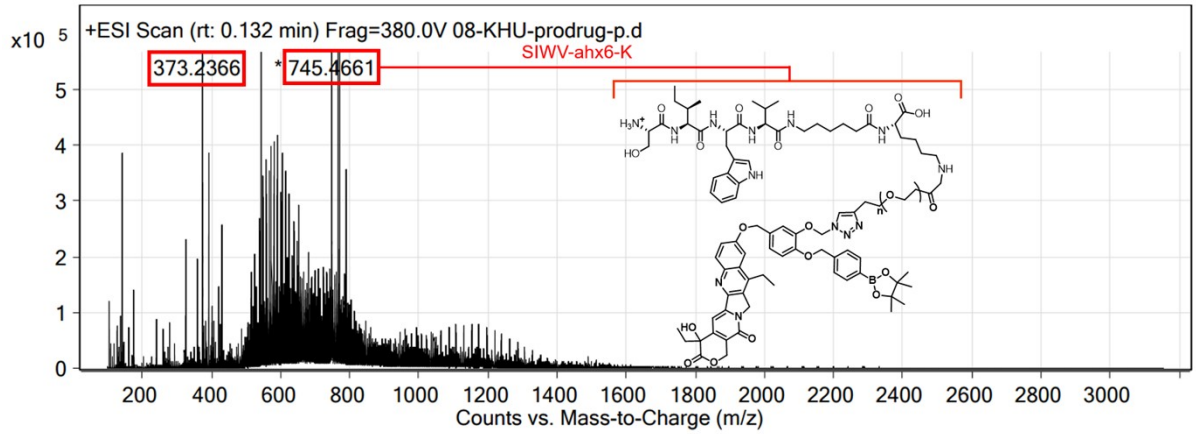

Fig. S13. ESI-MS spectrum of **SIWV-PB-SN** (positive).

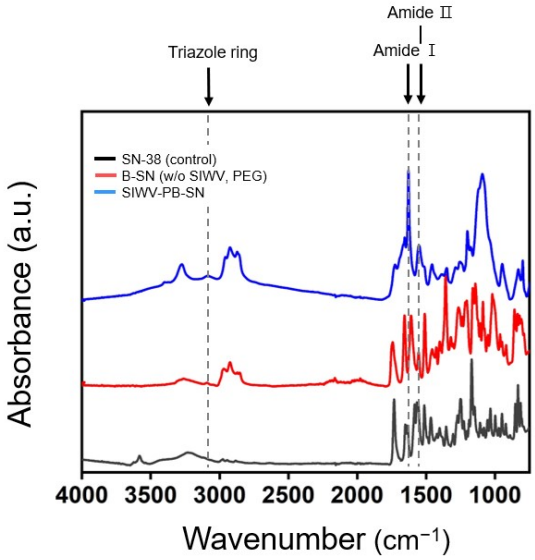

Fig. S14. Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectra of SN-38, **B-SN** (without SIWV and PEG), and **SIWV-PB-SN**.[2-4]

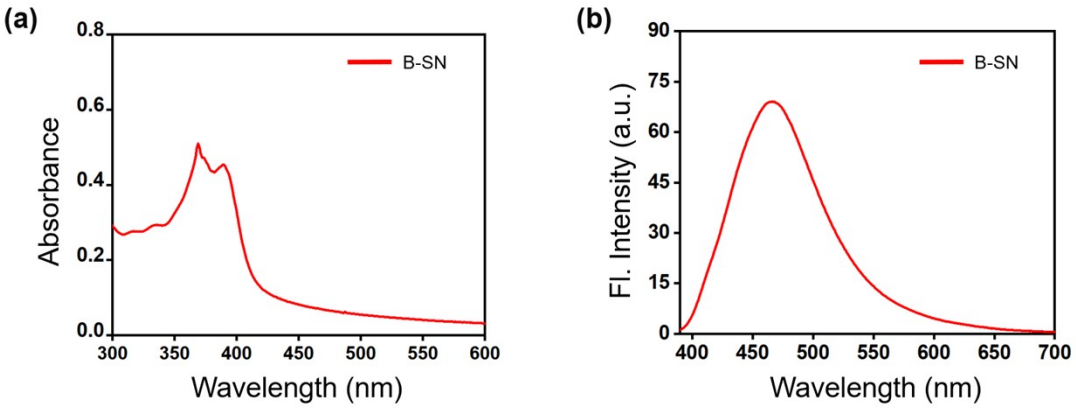

Fig. S15. (a) UV/Vis absorption and (b) emission spectra of **B-SN** (without SIWV and PEG) in PBS (pH 7.4, 10 μM).

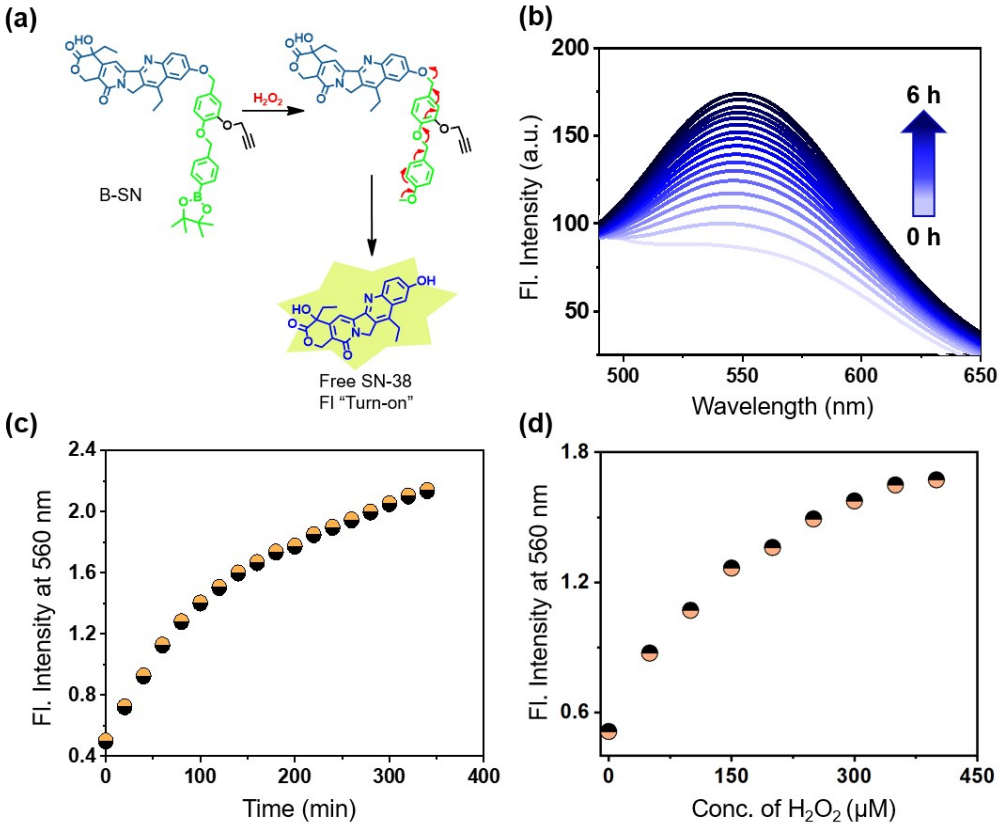

Fig. S16. (a) The drug activation mechanism of **B-SN** in the presence of H_2O_2 releases free SN-38 (SN). (b) Time-dependent fluorescence intensity change of **B-SN** (1 μM) after incubation with H_2O_2 (0.1 mM) in PBS (37 °C, λ_{ex} = 370 nm, slit width: 5/5). (c) Fluorescence intensity enhancement of **B-SN** (1 μM, PBS solution, 37 °C) at 548 nm after incubation with H_2O_2. (d) Fluorescence intensity enhancement of **B-SN** (1 μM, PBS solution, 37 °C) at 560 nm after incubation with different concentrations of H_2O_2. (Fluorescence was recorded for every sample 1.5 h after adding H_2O_2. Excitation wavelength: 370 nm).

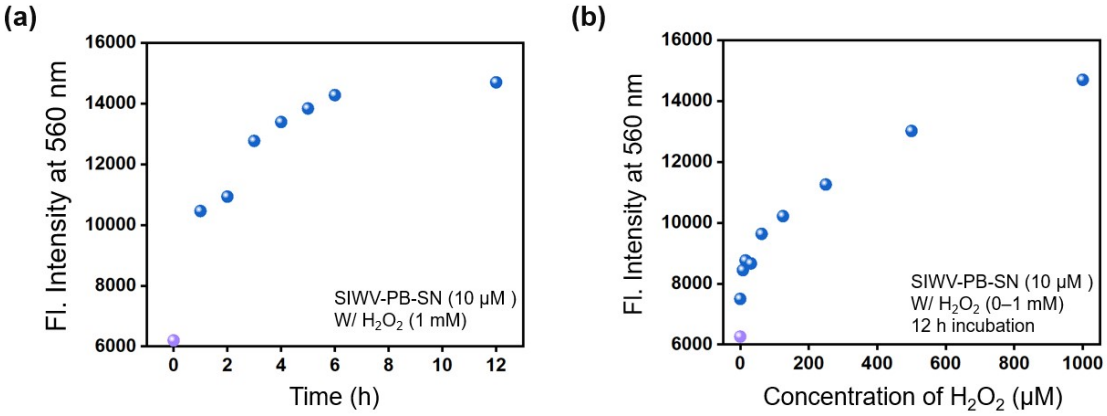

Fig. S17. (a) Time (0, 1, 2, 3, 4, 5, 6, and 12 h)-dependent fluorescence intensity (560 nm) changes of **SIWV-PB-SN** (10 μM) in the presence of H_2O_2 (1 mM) at 37 °C over a 12 h incubation period. (b) H_2O_2 (0, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, and 1000 μM) concentration-dependent emission intensity changes of **SIWV-PB-SN** (10 μM). Excitation wavelength: 370 nm.

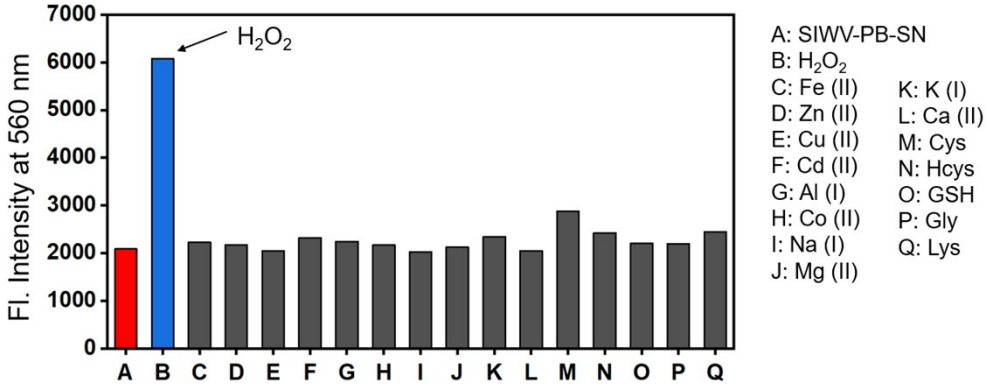

Fig. S18. Emission intensity plot (at 560 nm) of **SIWV-PB-SN** (10 μM) after treatment with metal ions (10 eq) and biomolecules (10 eq) and 1 h incubation at 37 °C.

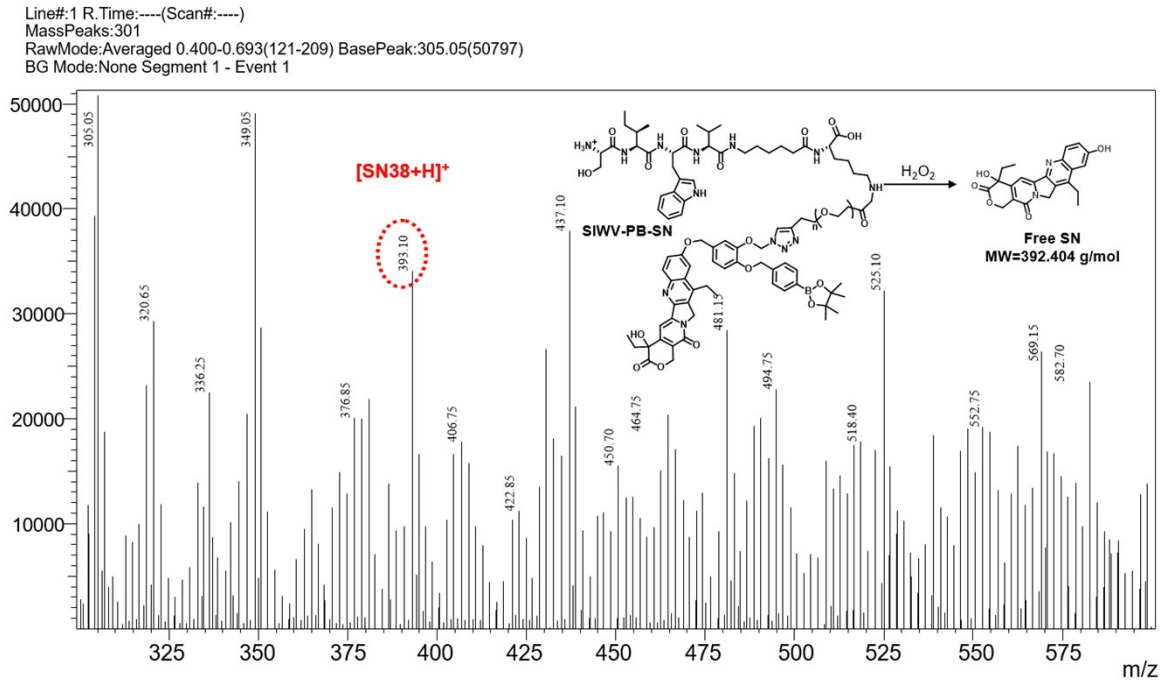

Fig. S19. ESI-MS spectrum of **SIWV-PB-SN** after treatment with H_2O_2 (0.1 mM) and 4 h incubation at 37 °C.

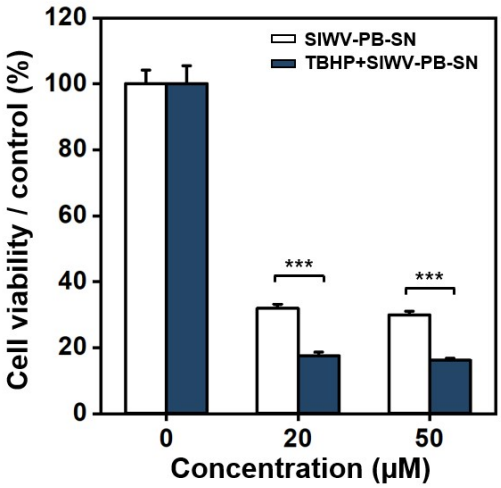

Fig. S20. Cell viability of the U87MG cells after treatment with **SIWV-PB-SN** and TBHP with **SIWV-PB-SN** for 48 h incubation at 37 °C. Error bars represent the mean ± standard error of the mean (SEM) of individual experiments conducted in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

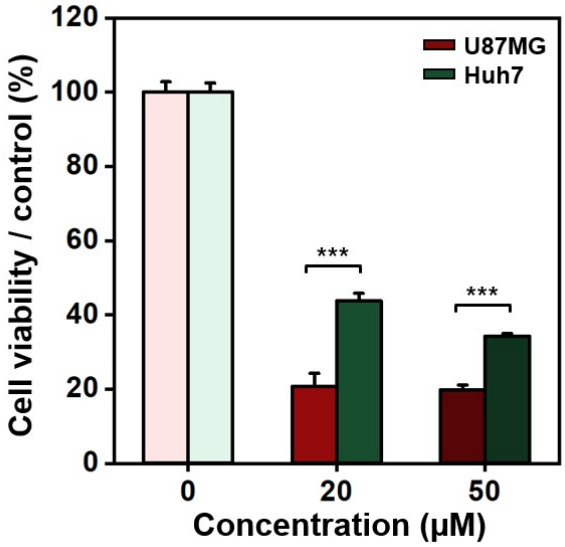

Fig. S21. Cell viability of the U87MG Huh7 cells after treatment with **SIWV-PB-SN** and 48 h incubation at 37 °C. Error bars represent the mean ± standard error of the mean (SEM) of individual experiments conducted in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3. Reference

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