Supporting Information

A Self-Evaluating Photothermal Therapeutic Nanoparticle

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1. Methods

General Methods

All materials were reagent grade or better. All the starting materials were obtained from J&K Chemical Company (Shanghai) or Sangon Biotech. Recombinant human caspase 3 protein (active form, 10 μg, the specific activity is >3,000 pmol/min/μg) was purchased from R & D Systems (Minneapolis, MN). The Casp3 buffer consists of 25 mM 4-(2-hydroxyerhyl)piperazine-1-erhanesulfonic acid (HEPES), 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 10 mM dithiolthreitol (DTT). Alamar Blue, Calcein Acetoxymethyl (AM) /Propidium Iodide (PI) Cell Live/Dead Kit, and LysoTracker Green DND-26 were obtained from Yeasen Biotech Co. Ltd. (China). The anti -active caspase 3 rabbit pAb for immunohistochemical was obtained from Wuhan Servicebio Technology Co., Ltd.. Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb for western blot analysis was obtained from Cell Signaling Technology, Inc.. Ultrapure water (18.2 MΩ. cm) was used throughout the experiment. Electrospray ionization (ESI) mass spectra were obtained on an LTQ Orbitrap mass spectrometer (Thermo Fisher). Matrix-assisted laser desorption ionization-time of flight (MA LDI-TOF) mass spectra were obtained on a time-of-flight Ultrflex II mass spectrometer (Bruker Daltonics). The sonicator is a high-power numerical control ultrasonic cleaner (KQ-400KDE) which was purchased from Kunshan ultrasonic instrument Co., Ltd.. High performance liquid chromatography (HPLC) analyses were performed on an Agilent 1200 HPLC system equipped with a G1322A pump and in-line diode array UV detector using an Agilent Zorbax 300SB-

C18 RP column with CH_3CN (0.1% of trifluoroacetic acid (TFA)) and water (0.1% of TFA) as the eluent. HPLC purification was performed on a Shimazu UFLC system equipped with two LC-20AP pumps and an SPD-20A UV/vis detector using a Shimazu PRC-ODS column. ^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker AV-400 400 MHz and JEOL JNM-ECZ600R 600 MHz spectrometers. Fluorescence spectra were obtained on a Hitachi F-4600 fluorescence spectrophotometer with excitation wavelength set to 785 nm (Hitachi High-Techonologies Corporation, Japan). Transmission electron micrograph (TEM) images were obtained on a JEM-2100F field emission transmission electron microscope operated at an acceleration voltage of 200 kV. Cells were routinely cultured in Dulbecco's modified Eagle'sS2 medium (DMEM, Hycolon) supplemented with 10% fetal bovine serum at 37 °C, 5% CO_2, and humid atmosphere. Fluorescence microscopic images were taken under a fluorescence microscope OLMPUS IX71. The fluorescent intensity of Alamar Blue assay was read by a Thermo Scientific Varioskan Flash 3001 instruments. Given the photothermal performance measurement of the **Cy-CBT-NP**, a 808nm NIR laser (Changchun New Industries Optoelectronics Tech. Co., Ltd., China) was used as a light source, a handy optical power meter (Vega, Ophir, Israel) was used to calibrate the output power and an online thermocouple thermometer (UT-325, Uni-Trend Technology (China) Co., Ltd.) was used to record the temperature changes. BALB/c nude mice (5 week old, 17-19 g) were used for animal experiments. All animals received care according to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the University of Science and Technology of China Animal Care and Use

Committee (Approval of Animal Ethical and Welfare Number: USTCACUC1901023).

Photothermal Studies *In Vitro*

To study the photothermal properties of the **Cy-CBT-NP**, the **Cy-CBT-NP** were dispersed in phosphate-buffered saline (PBS, 10 mM, pH 7.4) with a concentration of 30 μM (calculated in **Cy-CBT**) in a quartz tube. The **Cy-CBT-NP** dispersions were irradiated with an 808nm laser at a power density of 0.4 W cm^{-2} for 5min. The temperature changes were recorded by an online thermocouple thermometer. The infrared images were recorded using an infrared camera (ICI7320, Infrared Camera Inc., Beaumont, Texas, USA) and analyzed using IR Flash thermal imaging analysis software (Infrared Cameras Inc.)

The photothermal conversion efficiency (η) of the **Cy-CBT-NP** can be determined:

$$\eta = \frac{hS(T_{max} - T_{surr}) - Q_{dis}}{I(1 - 10^{(-A_\lambda)})}$$

Where h is the heat transfer coefficient, S is the surface area of the container, T_{max} is the maximum laser-triggered temperature, T_{surr} is the indoor temperature, Q_{dis} is the heat dissipation caused by the quartz sample cell light absorbing, I is the laser power (0.4 W cm^{-2}) and A_λ is the absorbance of the **Cy-CBT-NP** at the wavelength of 808 nm. In this formula, only hS is unknown and can be obtained from the following formula:

$$\tau_s = \frac{m_{buff} \times C_{p,buff}}{hS}$$

Where m_{buff} and $C_{p,buff}$ is the mass and heat capacity of the buffer solution, respectively. τ_s is the time constant of sample system and it can be determined from the cooling period.

$$t = -\tau_s \ln(\theta) \quad \theta = \frac{T - T_{surr}}{T_{max} - T_{surr}}$$

Where T is the solution temperature.

Cell Uptake

The HeLa cells were seeded in five glass bottom cell culture dishes (3.5cm) at a number of five hundred thousand and incubated at 37 ℃ in a CO_2 incubator for 12 h. After that, the HeLa cells were washed for three times with PBS and incubated with 30 μM **Cy-CBT-NP** (calculated in **Cy-CBT**) in DMEM at 37 ℃ or 4 ℃ for 0.5, 1, 2, or 4 h. After that, the HeLa cells were washed for three times with PBS prior to imaging.

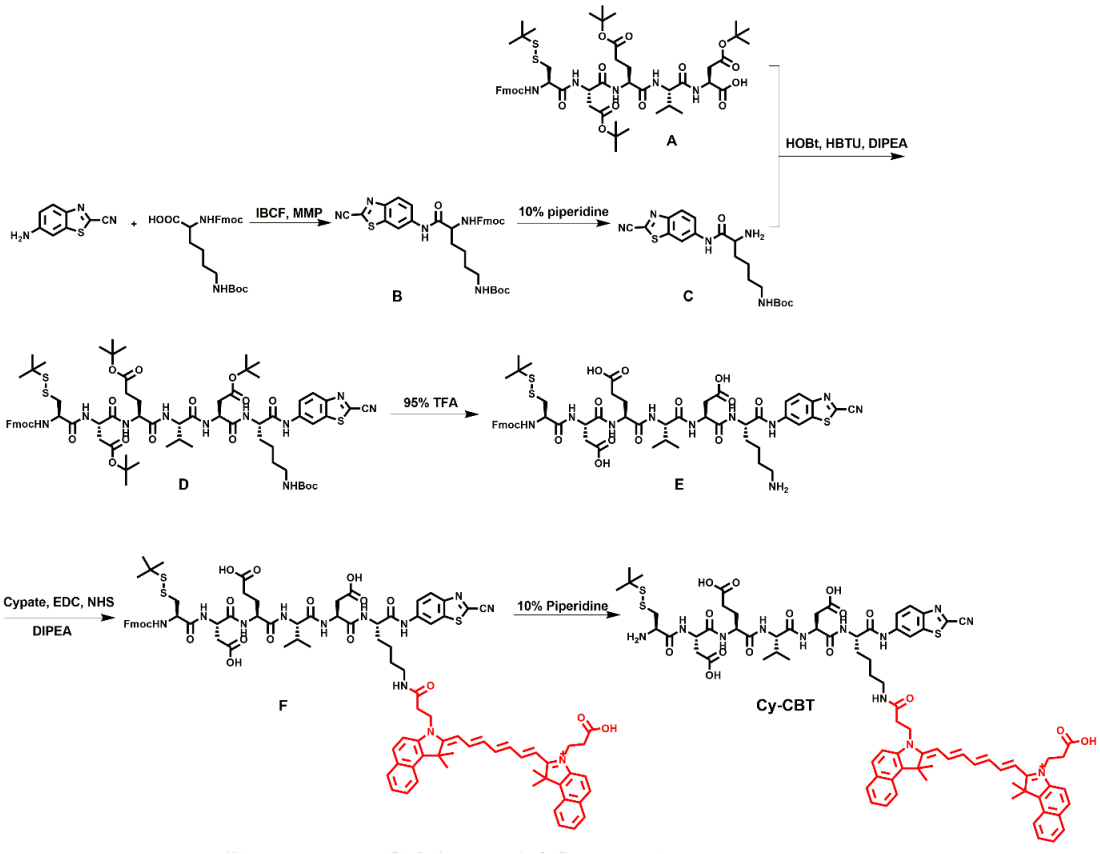
***In Vivo* PTT Treatment**

HeLa cells (2×10^6 cells/each mouse) were subcutaneously injected into the flanks of female BALB/c nude mice (5 week old, 17-19 g) for constructing the HeLa tumor model. Mice were randomly divided into five groups (Saline group, Saline + Laser group, **Cy-CBT-NP** group, **Cy-CBT-NP** + Laser 44 ℃ group, **Cy-CBT-NP** + Laser 49 ℃ group). For the Saline and Saline + Laser groups, a total of 10 μL of saline was injected into each of those tumor-bearing mice through tumor-direct injection. For the **Cy-CBT-NP**, **Cy-CBT-NP** + Laser 44 ℃, and **Cy-CBT-NP** + Laser 49 ℃ groups, a total of 10 μL of **Cy-CBT-NP** at 90 nmol kg^{-1} (calculated in **Cy-CBT**) were injected into each of those tumor-bearing mice through a tumor-direct injection. After 12 h, the mice of the **Cy-CBT-NP** + Laser 44 ℃ group and the **Cy-CBT-NP** + Laser 49 ℃ group were irradiated with an 808nm laser (0.4 W cm^{-2}) until their temperatures reached 44 ℃ and 49 ℃, respectively. The mice of the Saline + Laser group were irradiated with an 808nm laser for 5 min (0.4 W cm^{-2}).

2. Synthesis and characterization of Cy-CBT, Cy-CBT-NP-Cleaved, and Cy-CBT-NP

2-cyano-6-aminobenzothiazole (CBT) was synthesized following the literature method.[1]

Scheme S1. The synthetic route for **Cy-CBT**.



*Synthesis of **B**:* The isobutyl chloroformate (IBCF, 135.4 μL, 1 mmol) was added to a mixture of compound Fmoc-Lys(Boc)-OH (468 mg, 1 mmol) and 4-methylmorpholine (MMP, 224.5 μL, 2 mmol) in THF (2.0 mL) at 0 °C under N_2. The reaction mixture was stirred for 1 h. The solution of 2-cyano-6-aminobenzothiazole (CBT, 192 mg, 1.1 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C. Then the mixture was stirred overnight at room temperature. Compound **B** (462.7 mg, yield: 73.9%) was purified by HPLC using water-methanol mixed with 0.1% TFA as the eluent (from

15:85 to 0:100). Analytical HPLC chromatogram showed a single peak (Figure S1). MS: calculated for **B** $[M+H]^+$: 626.24; obsvd. ESI-MS: *m*/*z* 626.78. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 10.58 (s, 1 H), 8.76 (d, *J* = 1.9 Hz, 1 H), 8.20 (d, *J* = 9.0 Hz, 1 H), 7.90 (d, *J* = 7.5 Hz, 2 H), 7.80 – 7.71 (m, 4 H), 7.42 (t, *J* = 7.5 Hz, 2 H), 7.36 – 7.31 (m, 2 H), 6.80 (t, *J* = 5.4 Hz, 1 H), 4.31 – 4.28 (m, 2 H), 4.24 (d, *J* = 6.3 Hz, 1 H), 4.18 (d, *J* = 5.8 Hz, 1 H), 2.91 (d, *J* = 3.3 Hz, 2 H), 2.55 (s, 2 H), 1.66 (dd, *J* = 15.7, 6.7 Hz, 2 H), 1.41 (s, 2 H), 1.35 (s, 9 H) (Figure S2). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 172.43 (1 C), 156.65 (1 C), 156.04 (1 C), 148.10 (1 C), 144.28 (2 C), 141.19 (2 C), 139.93 (1 C), 137.19 (1 C), 135.51 (1 C), 128.12 (2 C), 127.55 (2 C), 125.79 (2 C), 125.27 (1 C), 121.24 (1 C), 120.59 (2 C), 114.07 (1 C), 111.82 (1 C), 77.81 (1 C), 66.15 (1 C), 56.06 (1 C), 47.11 (1 C), 31.80 (1 C), 29.73 (1 C), 28.72 (3 C), 23.48 (1 C) (Figure S3).

*Synthesis of **C**:* The Fmoc protecting group of compound **B** was cleaved with 10% piperidine in DMF (6 mL) for 10 min at 0 °C and then 540 μL TFA was added to neutralize the alkaline. Thus, the compound **C** (275.5 mg, yield: 92.4%) was obtained after HPLC purification using water-acetonitrile mixed with 0.1% TFA as the eluent (from50:50 to 5:95). Analytical HPLC chromatogram showed a single peak (Figure S4). MS: calculated for **C** $[M+H]^+$: 404.18 obsvd. ESI-MS: *m*/*z* 404.00. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 11.03 (s, 1 H), 8.75 (dd, *J* = 7.6, 2.0 Hz, 1 H), 8.33 (s, 2 H), 8.27 – 8.24 (m, 1 H), 7.82 – 7.78 (m, 1 H), 6.78 (t, *J* = 5.5 Hz, 1 H), 4.01 (d, *J* = 4.9 Hz, 1 H), 2.89 (t, *J* = 6.2 Hz, 2 H), 1.84 (dd, *J* = 14.5, 6.7 Hz, 2 H), 1.45 – 1.26 (m, 13 H) (Figure S5). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 168.62 (1 C), 156.02 (1 C), 148.60 (1

C), 138.89 (1 C), 137.22 (1 C), 136.33 (1 C), 125.52 (1 C), 121.37 (1 C), 114.01 (1 C), 112.58 (1 C), 77.86 (1 C), 53.63 (1 C), 31.30 (1 C), 29.57 (1 C), 28.67 (3 C), 21.88 (1 C) (Figure S6).

*Synthesis of **D**:* Compound Fmoc-Cys(StBu)-Asp(OtBu)-Glu(OtBu)-Val-Asp(OtBu)-OH (**A**) was synthesized through solid phase peptide synthesis (SPPS) (Figure S7). The mixture of compound **A** (127.0 mg, 0.12 mmol), compound **C** (40.3 mg, 0.1 mmol), HBTU (45.5 mg, 0.12 mmol), and HOBt (16.2 mg, 0.12 mmol) in DMF (3 mL) was stirred overnight in presence of DIPEA (248 μL, 0.15 mmol). Compound **D** (83 mg, yield: 57.5%) was obtained after HPLC purification using water-acetonitrile mixed with 0.1% TFA as the eluent (from15:85 to 0:100). Analytical HPLC chromatogram showed a single peak (Figure S8). MS: calculated for **D** $[M+H]^+$: 1443.64 obsvd. ESI-MS: *m*/*z* 1443.26. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 10.45 (s, 1 H), 8.74 (d, *J* = 2.0 Hz, 1 H), 8.44 (d, *J* = 8.0 Hz, 1 H), 8.35 (d, *J* = 7.7 Hz, 1 H), 8.20 (d, *J* = 9.0 Hz, 1 H), 8.07 (d, *J* = 7.3 Hz, 1 H), 7.93 – 7.58 (m, 8 H), 7.42 (t, *J* = 7.2 Hz, 2 H), 7.32 (dd, *J* = 10.3, 4.7 Hz, 2 H), 6.76 (t, *J* = 5.6 Hz, 1 H), 4.61 (ddd, *J* = 36.6, 13.9, 7.9 Hz, 2 H), 4.41 – 4.15 (m, 7 H), 3.06 (dd, *J* = 13.1, 4.2 Hz, 1 H), 2.98 – 2.84 (m, 3 H), 2.76 – 2.64 (m, 2 H), 2.50 – 2.39 (m, 3 H), 2.27 – 2.10 (m, 2 H), 1.98 – 1.82 (m, 2 H), 1.66 (ddd, *J* = 22.8, 19.0, 11.4 Hz, 3 H), 1.32 (dt, *J* = 29.7, 10.5 Hz, 48 H), 0.83 (dd, *J* = 10.3, 6.8 Hz, 6 H) (Figure S9). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 172.19 (1 C), 171.55 (2 C), 171.14 (1 C), 170.54 (1 C), 170.30 (1 C), 169.72 (1 C), 169.64 (2 C), 156.40 (1 C), 155.98 (1 C), 148.15 (1 C), 144.20 (2 C), 141.19 (1 C), 139.77 (2 C), 137.15 (1 C), 135.56 (1 C), 128.13 (2 C), 127.55 (2 C), 125.81 (2 C), 125.25 (1 C), 121.28 (1 C),

120.60 (2 C), 114.05 (1 C), 111.86 (1 C), 80.67 (2 C), 80.01 (1 C), 77.78 (1 C), 66.29 (1 C), 57.76 (1 C), 54.72 (1 C), 54.26 (1 C), 50.07 (1 C), 49.80 (1 C), 48.18 (1 C), 47.06 (1 C), 42.66 (1 C), 37.54 (2 C), 32.00 (1 C), 31.76 (1 C), 31.35 (1 C), 30.03 (3 C), 29.74 (1 C), 28.71 (3 C), 28.14 (12 C), 23.19 (1 C), 19.60 (1 C), 18.49 (1 C) (Figure S10).

*Synthesis of **E**:* The Boc and OtBu protecting groups of compound **D** were removed with dichloromethane (DCM, 1 mL) and triisopropylsilane (TIPS, 200 μL) in TFA (19 mL) for 3 h. Compound **E** (48.7 mg, yield: 72.1 %) was obtained after HPLC purification using water-acetonitrile mixed with 0.1% TFA as the eluent (from 50:50 to 5:95). Analytical HPLC chromatogram showed a single peak (Figure S11). MS: calculated for **E** $[M+H]^+$: 1175.40; obsvd. ESI-MS: *m*/*z* 1175.38. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 10.36 (s, 1 H), 8.75 (d, *J* = 2.0 Hz, 1 H), 8.50 (dd, *J* = 30.1, 7.7 Hz, 1 H), 8.34 (d, *J* = 7.1 Hz, 1 H), 8.19 (dd, *J* = 16.8, 8.3 Hz, 2 H), 7.91 – 7.69 (m, 10 H), 7.42 (t, *J* = 7.2 Hz, 2 H), 7.38 – 7.30 (m, 2 H), 4.64 – 4.52 (m, 2 H), 4.40 – 4.14 (m, 7 H), 3.08 (dd, *J* = 13.1, 4.1 Hz, 1 H), 2.92 (dd, *J* = 13.1, 10.4 Hz, 1 H), 2.82 – 2.68 (m, 4 H), 2.56 (dd, *J* = 15.5, 8.0 Hz, 2 H), 2.34 – 2.15 (m, 2 H), 2.02 – 1.88 (m, 2 H), 1.85 – 1.62 (m, 3 H), 1.54 (dd, *J* = 14.6, 7.0 Hz, 2 H), 1.47 – 1.34 (m, 2 H), 1.29 (s, 9 H), 0.83 (dt, *J* = 17.2, 8.6 Hz, 6 H) (Figure S12). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 174.55 (1 C), 172.38 (1 C), 172.25 (1 C), 171.45 (1 C), 171.37 (1 C), 171.25 (1 C), 170.81 (2 C), 170.70 (1 C), 156.47 (1 C), 148.21 (1 C), 144.22 (2 C), 141.18 (2 C), 139.69 (1 C), 137.14 (1 C), 135.68 (1 C), 128.13 (2 C), 127.57 (2 C), 125.80 (2 C), 125.28 (1 C), 121.36 (1 C), 120.60 (2 C), 114.06 (1 C), 111.98 (1 C), 66.28 (1 C), 57.90 (1 C), 54.71 (1 C), 54.01 (1 C), 52.41 (1 C), 50.15 (1 C), 49.95 (1 C), 49.07 (1 C), 48.18

(1 C), 47.07 (1 C), 42.80 (1 C), 39.12 (1 C), 36.22 (1 C), 31.53 (1 C), 31.15 (1 C), 30.61 (1 C), 30.03 (3 C), 27.70 (1 C), 27.13 (1 C), 22.75 (1 C), 19.59 (1 C), 18.44 (1 C) (Figure S13).

*Synthesis of **F**:* To a cooled solution of Cypate[2] (35.5 mg, 56.79 μmol) in DMF (3 mL) in a three-neck flask filling with N_2, EDC.HCl (13.6 mg, 70.79 μmol) was added. After 20 min, N-hydroxysuccinimide (8.1 mg, 70.04 μmol) was added and further stirred for 24 h at ambient temperature. Then, a mixture of compound **E** (44.5 mg, 37.86 μmol) and DIPEA (298.15 mmol, 70.79 μmol for the neutralization of HCl, 113.58 μmol for the neutralization of COOH of **E,** 56.79 μmol for the neutralization of COOH of Cypate, 56.99 μmol for the condensation reaction) in 1.5 mL DMF was then added under the protection of N_2. The reaction mixture was stirred for 16 h in the dark at room temperature. Compound **F** (23.7 mg, yield: 35.11 %) was purified by HPLC using water-acetonitrile mixed with 0.1% TFA as the eluent (from 40:60 to 5:95). Analytical HPLC chromatogram showed a single peak (Figure S14).MS: calculated for **F** $[M]^+$: 1782.69; obsvd. ESI-MS: *m*/*z* 1782.72. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 10.25 (s, 1 H), 8.73 (s, 1 H), 8.42 (d, *J* = 6.9 Hz, 1 H), 8.30 (d, *J* = 6.4 Hz, 1 H), 8.18 (dt, *J* = 31.6, 15.1 Hz, 3 H), 8.09 – 7.92 (m, 8 H), 7.88 (d, *J* = 7.4 Hz, 2 H), 7.74 (dt, *J* = 26.5, 13.9 Hz, 7 H), 7.63 (d, *J* = 8.2 Hz, 3 H), 7.57 – 7.37 (m, 4 H), 7.29 (dd, *J* = 33.8, 27.0 Hz, 2 H), 6.56 (dd, *J* = 25.4, 12.7 Hz, 2 H), 6.33 (dd, *J* = 51.9, 12.5 Hz, 2 H), 4.58 (dd, *J* = 17.7, 6.4 Hz, 2 H), 4.44 – 4.13 (m, 11 H), 3.18 (s, 3 H), 3.08 (d, *J* = 9.3 Hz, 1 H), 2.92 (d, *J* = 26.6 Hz, 3 H), 2.74 (t, *J* = 10.7 Hz, 4 H), 2.57 (d, *J* = 6.5 Hz, 2 H), 2.32 – 2.16 (m, 2 H), 1.90 (d, *J* = 4.0 Hz, 12 H), 1.75 (d, *J* = 15.5 Hz, 2 H), 1.65 (s, 1 H), 1.55

(s, 1 H), 1.28 (s, 9 H), 1.19 (d, J = 33.8 Hz, 4 H), 0.82 (t, J = 6.7 Hz, 6 H) (Figure S15). ^{13}C NMR (151 MHz, DMSO-d_6) δ(ppm): 174.56 (1 C), 173.46 (1 C), 172.67 (1 C), 172.42 (2 C), 172.27 (1 C), 171.54 (1 C), 171.43 (2 C), 171.20 (1 C), 170.86 (1 C), 170.73 (1 C), 169.55 (1 C), 156.52 (1 C), 148.23 (1 C), 144.32 (1 C), 144.21 (1 C), 141.23 (2 C), 140.15 (1 C), 140.04 (1 C), 139.77 (1 C), 137.14 (2 C), 135.56 (2 C), 133.71 (1 C), 133.35 (1 C), 131.81 (1 C), 131.70 (1 C), 130.64 (1 C), 130.60 (1 C), 130.38 (1 C), 130.25 (1 C), 128.17 (4 C), 128.13 (2 C), 128.01 (2 C), 127.61 (2 C), 126.25 (1 C), 125.83 (2 C), 125.28 (2 C), 125.20 (1 C), 122.70 (1 C), 122.66 (1 C), 121.44 (1 C), 120.61 (2 C), 114.03 (1 C), 112.14 (1 C), 112.11 (1 C), 111.99 (1 C), 104.61 (1 C), 104.03 (1 C), 66.36 (1 C), 58.04 (1 C), 54.84 (1 C), 54.25 (1 C), 52.55 (1 C), 51.03 (1 C), 50.79 (1 C), 50.23 (1 C), 49.95 (1 C), 49.12 (1 C), 48.20 (1 C), 47.15 (1 C), 42.88 (1 C), 39.04 (1 C), 36.31 (2 C), 34.12 (1 C), 32.29 (1 C), 31.78 (1 C), 31.18 (1 C), 30.67 (1 C), 30.08 (3 C), 29.92 (1 C), 29.07 (1 C), 27.75 (1 C), 27.25 (4 C), 23.23 (1 C), 19.62 (1 C), 18.47 (1 C) (Figure S16).

*Synthesis of **Cy-CBT**:* The Fmoc protecting group of compound **F** (63.8 mg, 0.036 mmol) was cleaved with 10% piperidine in DMF (3 mL) at 0 °C for 10 min, then 270 μL TFA was added to neutralize the alkaline, thus compound **Cy-CBT** (39.5 mg, yield: 70.72%) was obtained after HPLC purification using water-acetonitrile mixed with 0.1% TFA as the eluent (from 50:50 to 5:95) and sent for a high-resolution (HR) mass spectrum analysis. Analytical HPLC chromatogram showed a single peak (Figure S17). MS: calculated for **Cy-CBT** $[M]^+$: 1559.6196; obsvd. HR-MALDI-TOF/MS: m/z 1559.4906 (Figure S18). ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 10.28 (s, 1 H), 8.92

(d, *J* = 7.1 Hz, 1 H), 8.73 (s, 1 H), 8.34 (d, *J* = 14.4 Hz, 4 H), 8.25 – 8.21 (m, 2 H), 8.17 (s, 1 H), 8.11 – 8.02 (m, 6 H), 8.01 (s, 1 H), 7.96 (s, 1 H), 7.80 (t, *J* = 9.4 Hz, 3 H), 7.72 (d, *J* = 8.8 Hz, 1 H), 7.63 (t, *J* = 8.6 Hz, 3 H), 7.48 (dd, *J* = 16.7, 7.7 Hz, 2 H), 6.57 (dd, *J* = 27.5, 12.8 Hz, 2 H), 6.43 – 6.35 (m, 2 H), 4.64 – 4.59 (m, 2 H), 4.40 (s, 2 H), 4.34 – 4.29 (m, 2 H), 4.17 – 4.09 (m, 2 H), 3.98 (s, 2 H), 3.18 (dd, *J* = 14.2, 5.0 Hz, 2 H), 3.04 – 2.89 (m, 5 H), 2.77 – 2.69 (m, 4 H), 2.56 (s, 2 H), 2.28 – 2.19 (m, 2 H), 1.91 (s, 12 H), 1.70 (d, *J* = 32.6 Hz, 2 H), 1.65 – 1.58 (m, 1 H), 1.53 (s, 1 H), 1.30 (d, *J* = 9.1 Hz, 9 H), 1.24 (s, 4 H), 0.82 (t, *J* = 7.4 Hz, 6 H) (Figure S19). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 173.90 (1 C), 172.74 (1 C), 172.08 (1 C), 171.77 (2 C), 171.32 (1 C), 170.90 (1 C), 170.75 (2 C), 170.56 (1 C), 169.64 (1 C), 168.87 (1 C), 166.73 (1 C), 147.62 (1 C), 139.53 (1 C), 139.45 (1 C), 139.16 (1 C), 136.53 (2 C), 134.95 (2 C), 133.06 (1 C), 132.76 (1 C), 131.19 (1 C), 131.10 (1 C), 130.08 (1 C), 129.98 (1 C), 129.77 (2 C), 129.57 (1 C), 127.50 (2 C), 127.49 (2 C), 127.40 (1 C), 125.71 (1 C), 124.66 (2 C), 124.59 (1 C), 122.11 (1 C), 122.06 (1 C), 120.82 (1 C), 113.41 (1 C), 111.54 (1 C), 111.47 (1 C), 111.39 (1 C), 103.87 (1 C), 103.39 (1 C), 57.36 (1 C), 53.63 (1 C), 52.03 (1 C), 51.63 (2 C), 50.40 (1 C), 50.19 (1 C), 49.66 (1 C), 49.32 (1 C), 48.01 (2 C), 38.42 (1 C), 35.83 (2 C), 33.49 (1 C), 31.67 (1 C), 31.18 (1 C), 30.61 (1 C), 30.17 (1 C), 29.35 (3 C), 28.91 (1 C), 28.48 (1 C), 27.23 (1 C), 26.65 (4 C), 22.62 (1 C), 19.02 (1 C), 17.85 (1 C) (Figure S20).

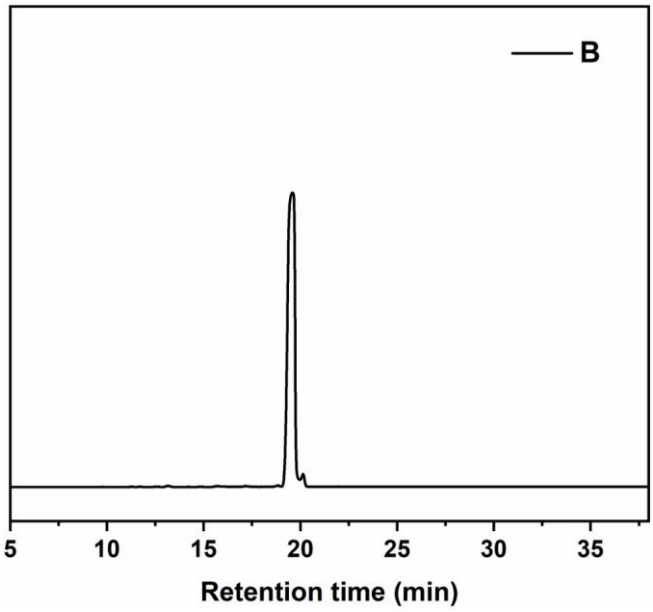


Figure S1. HPLC chromatogram of purified compound **B**.

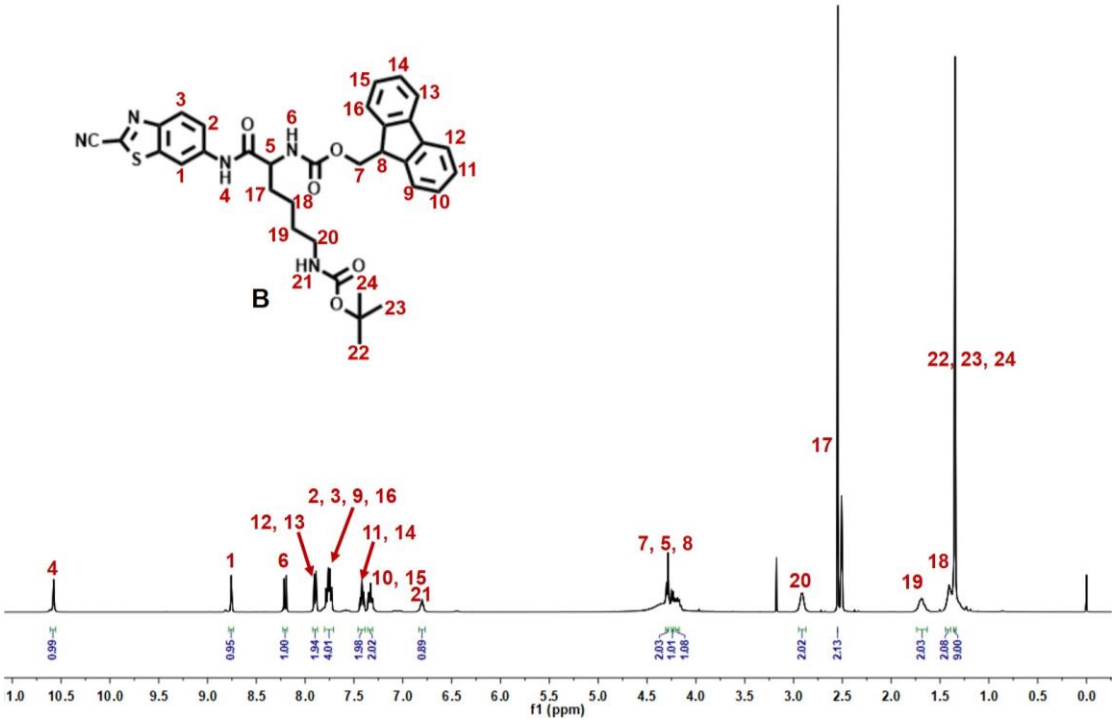


Figure S2. ^{1}H NMR spectrum of **B** in DMSO-d_6.

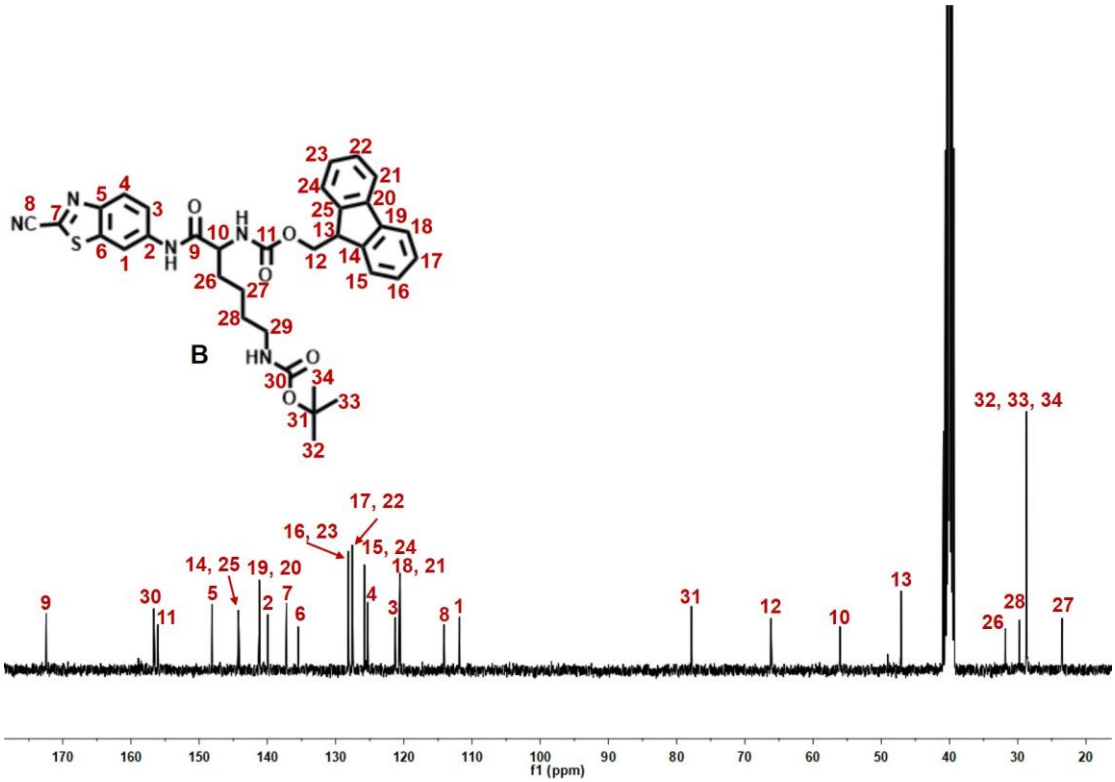


Figure S3. ^{13}C NMR spectrum of **B** in DMSO-d_6.

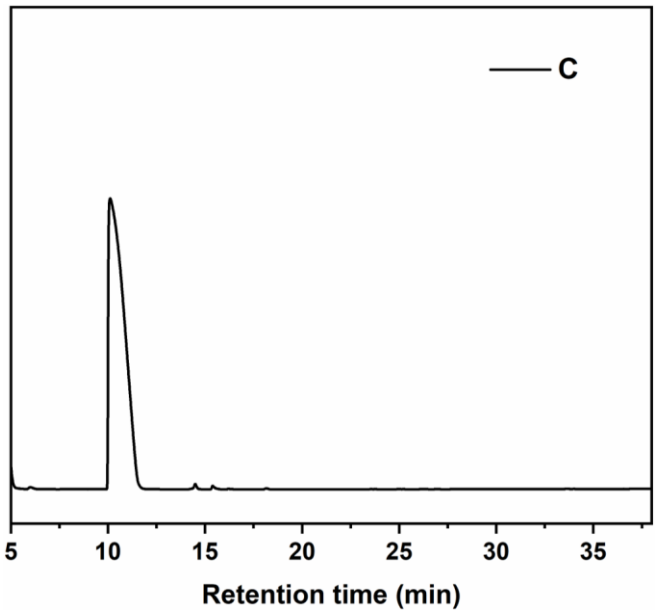


Figure S4. HPLC chromatogram of purified compound **C**.

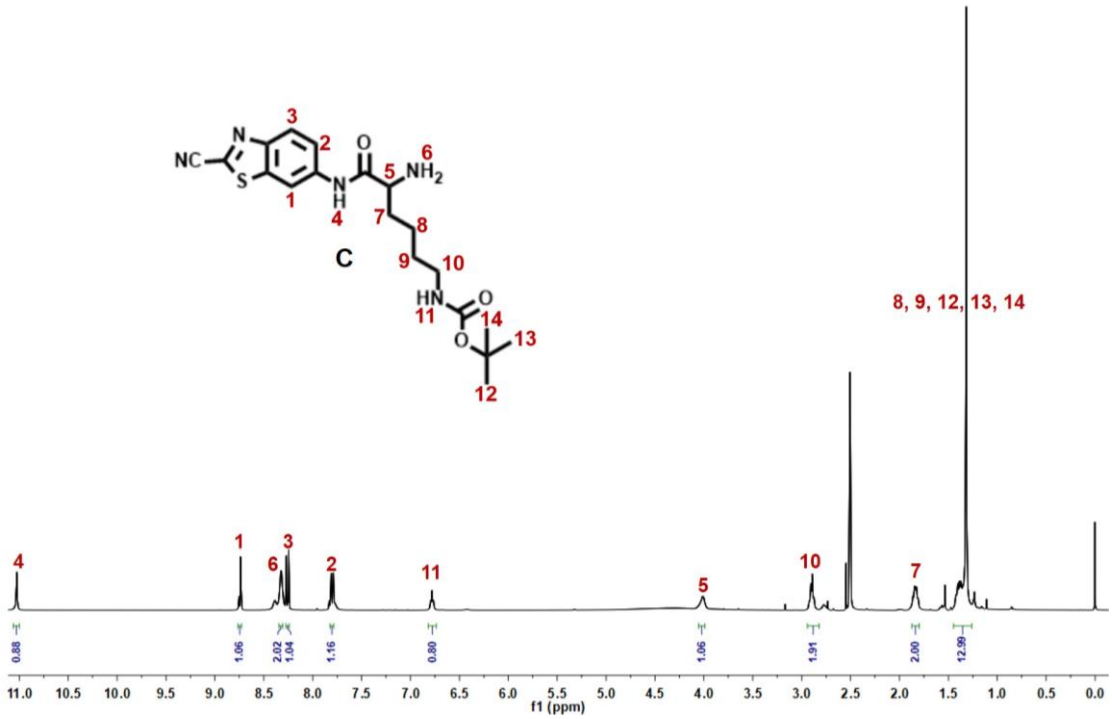


Figure S5. ^{1}H NMR spectrum of **C** in DMSO-d_6.

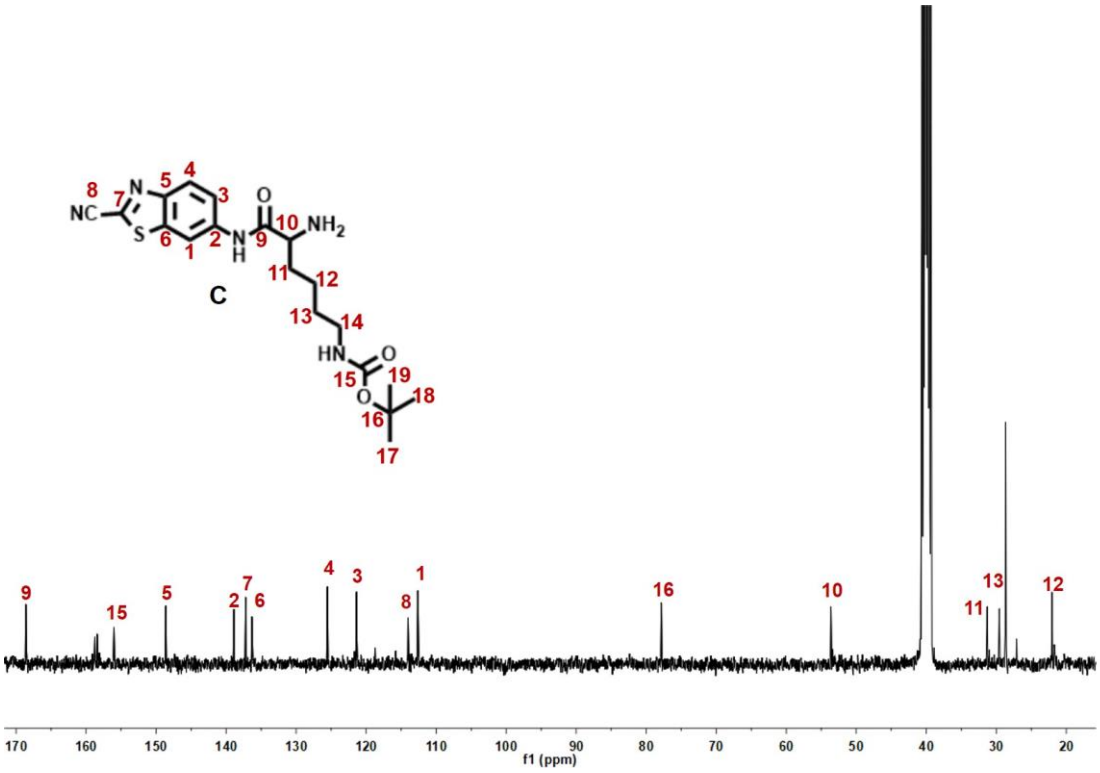


Figure S6. ^{13}C NMR spectrum of **C** in DMSO-d_6.

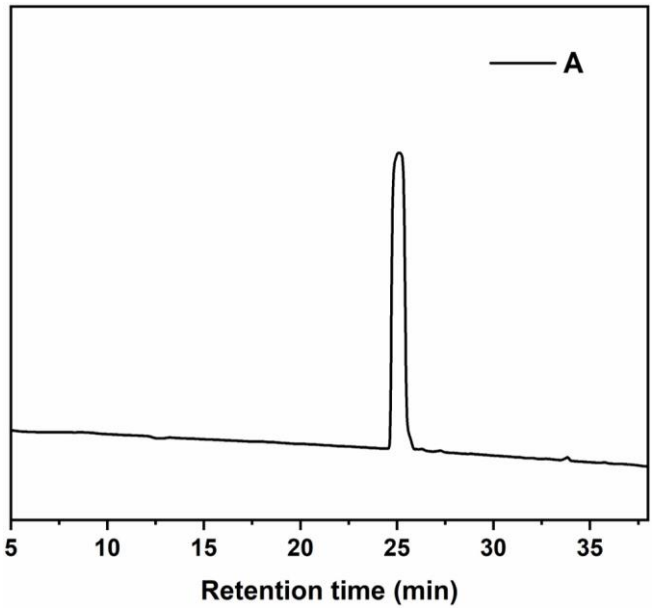


Figure S7. HPLC chromatogram of purified compound **A**.

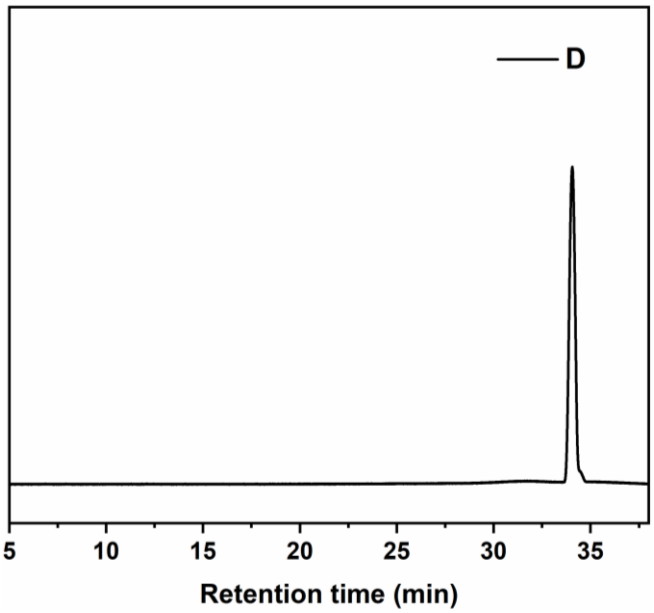


Figure S8. HPLC chromatogram of purified compound **D**.

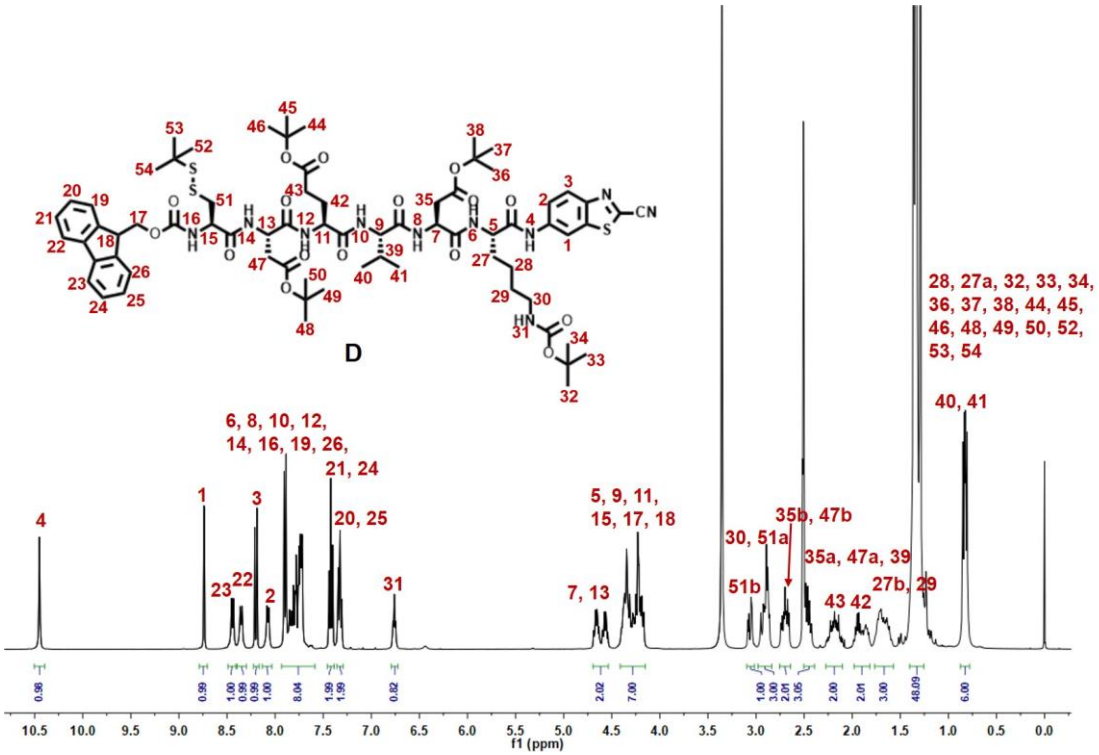


Figure S9. ^{1}H NMR spectrum of **D** in DMSO-d_6.

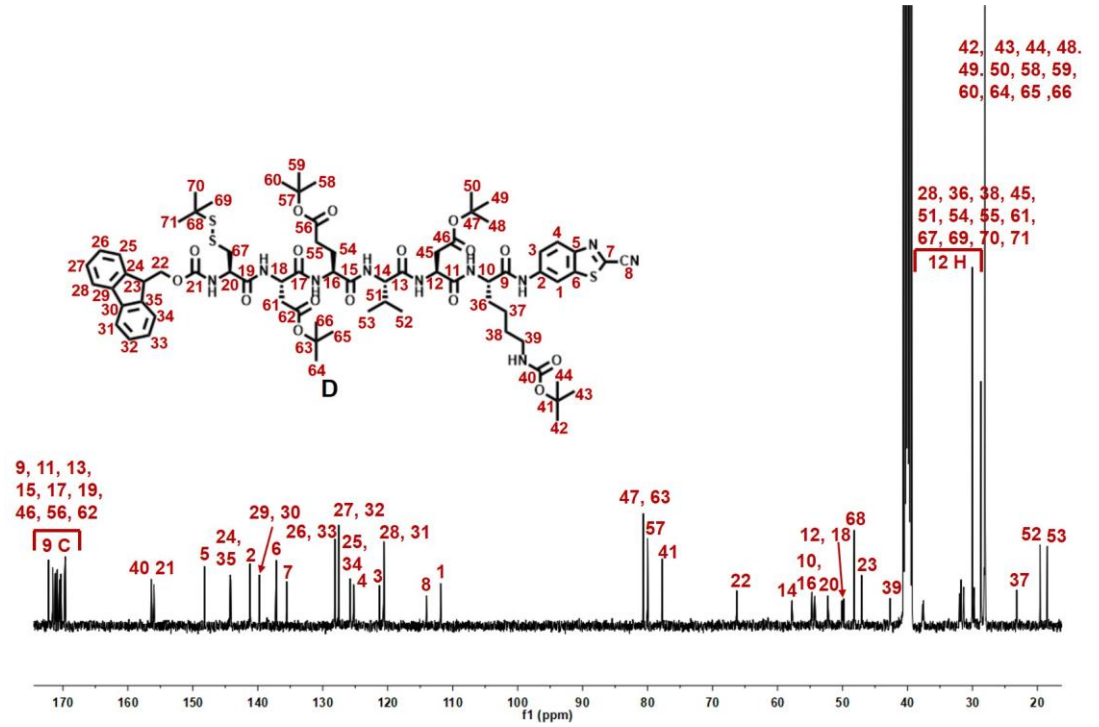


Figure S10. ^{13}C NMR spectrum of **D** in DMSO-d_6.

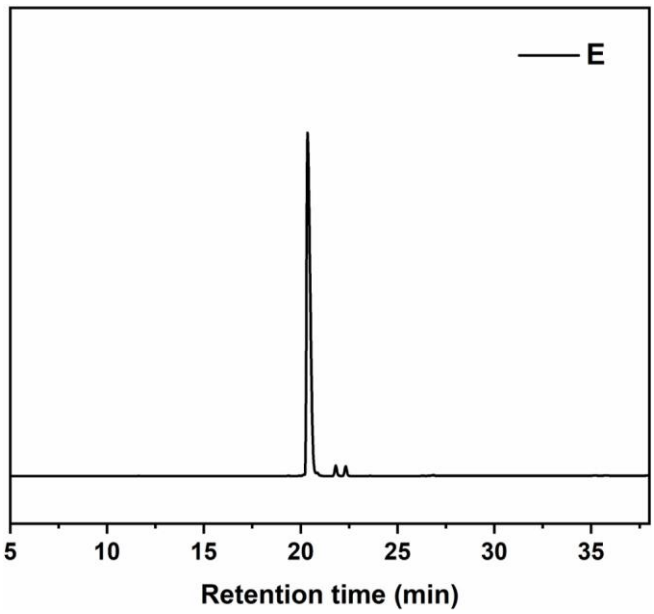


Figure S11. HPLC chromatogram of purified compound **E**.

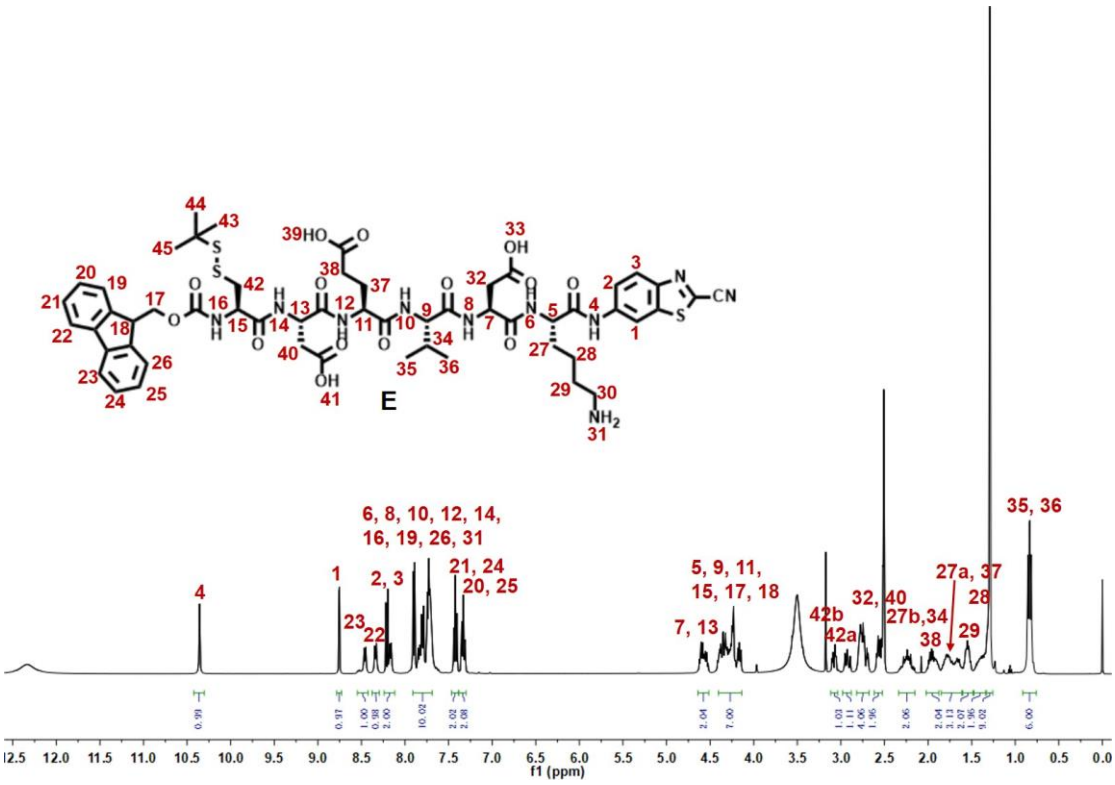


Figure S12. ^{1}H NMR spectrum of **E** in DMSO-d_6.

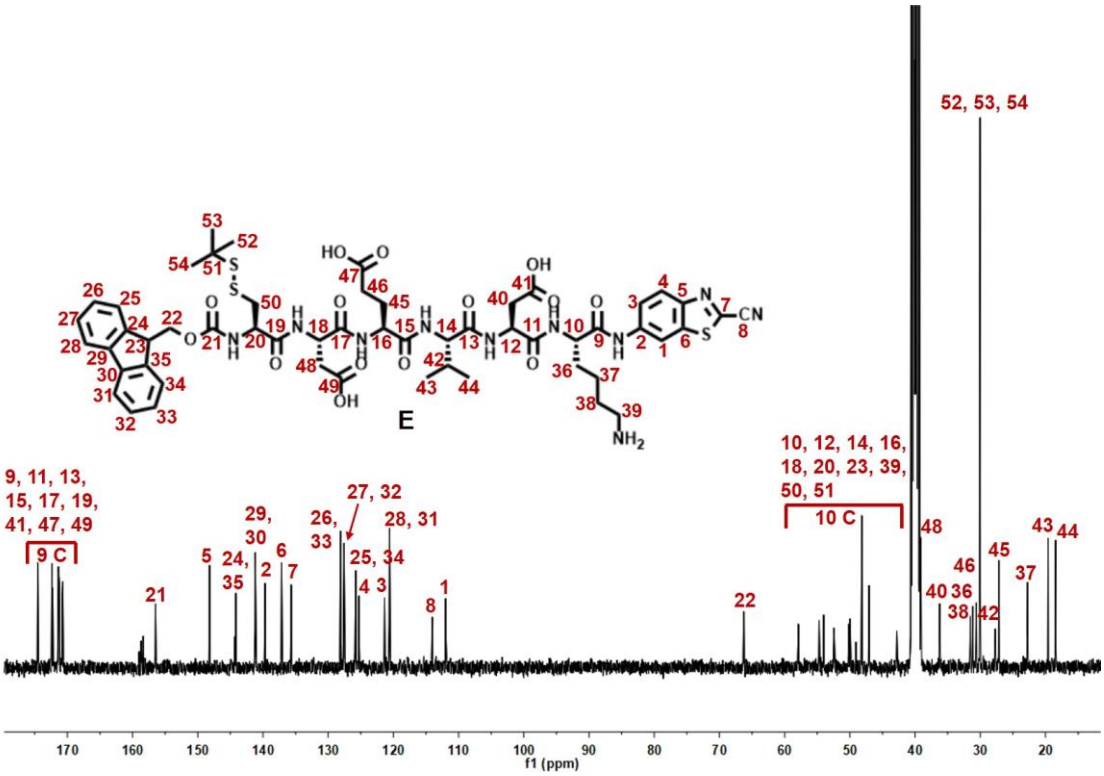


Figure S13. ^{13}C NMR spectrum of **E** in DMSO-d_6.

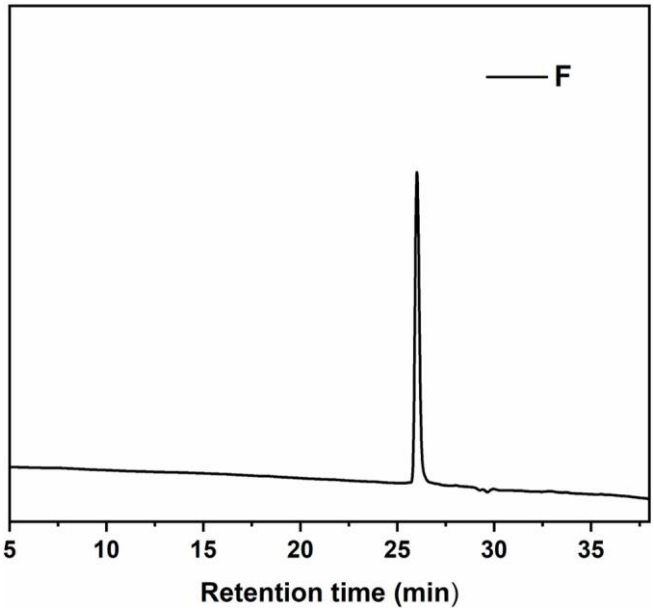


Figure S14. HPLC chromatogram of purified compound **F**.

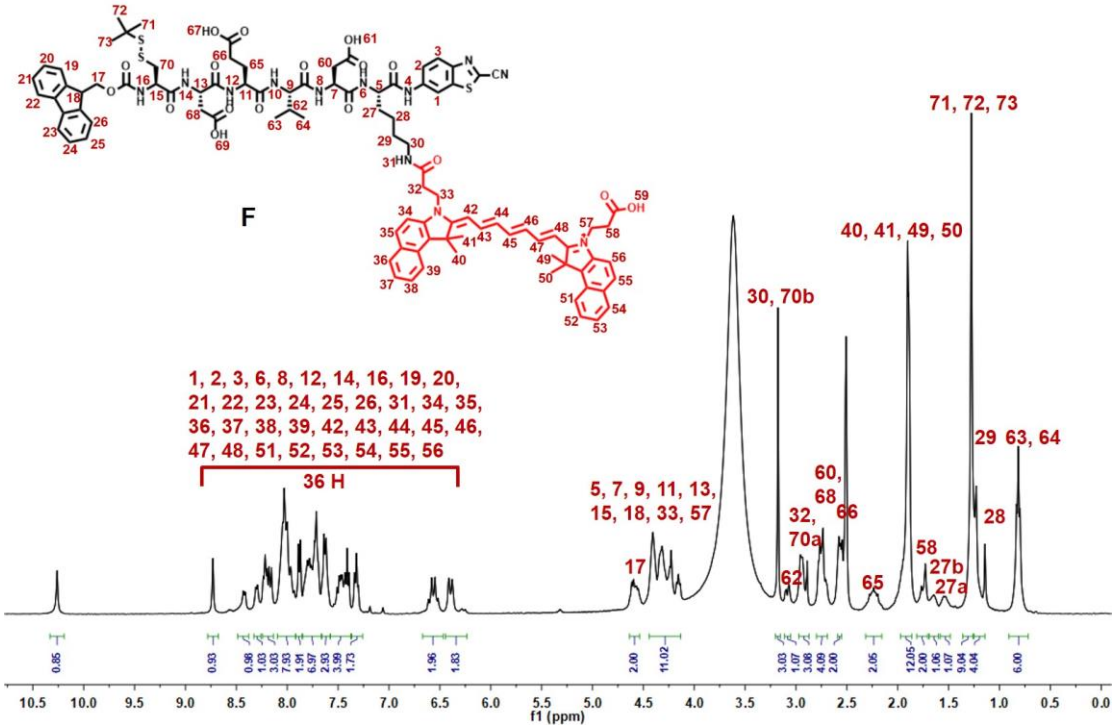


Figure S15. ^{1}H NMR spectrum of **F** in DMSO-d_6.

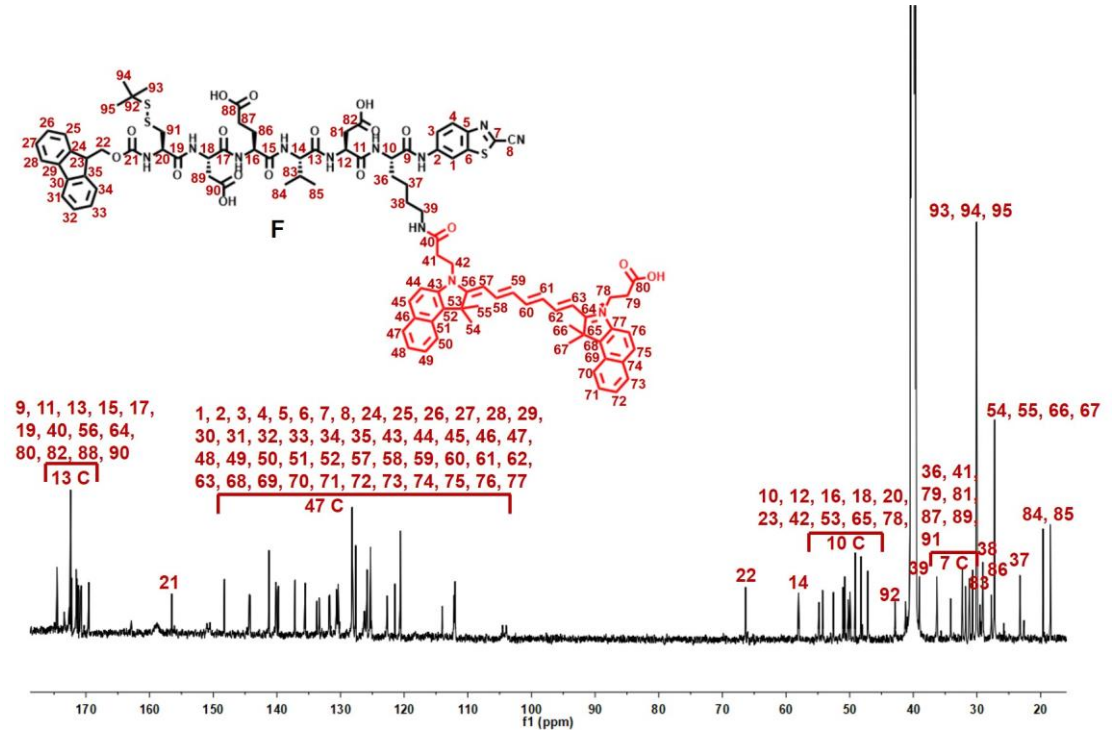


Figure S16. ^{13}C NMR spectrum of **F** in DMSO-d_6.

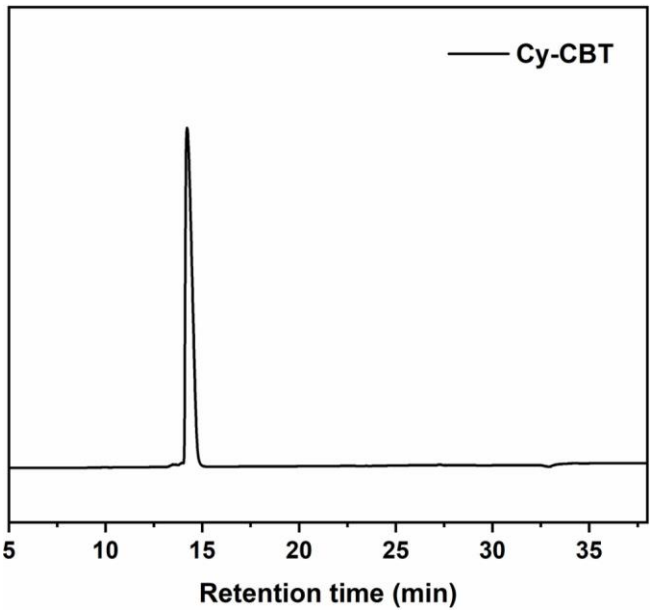


Figure S17. HPLC chromatogram of purified compound **Cy-CBT**.

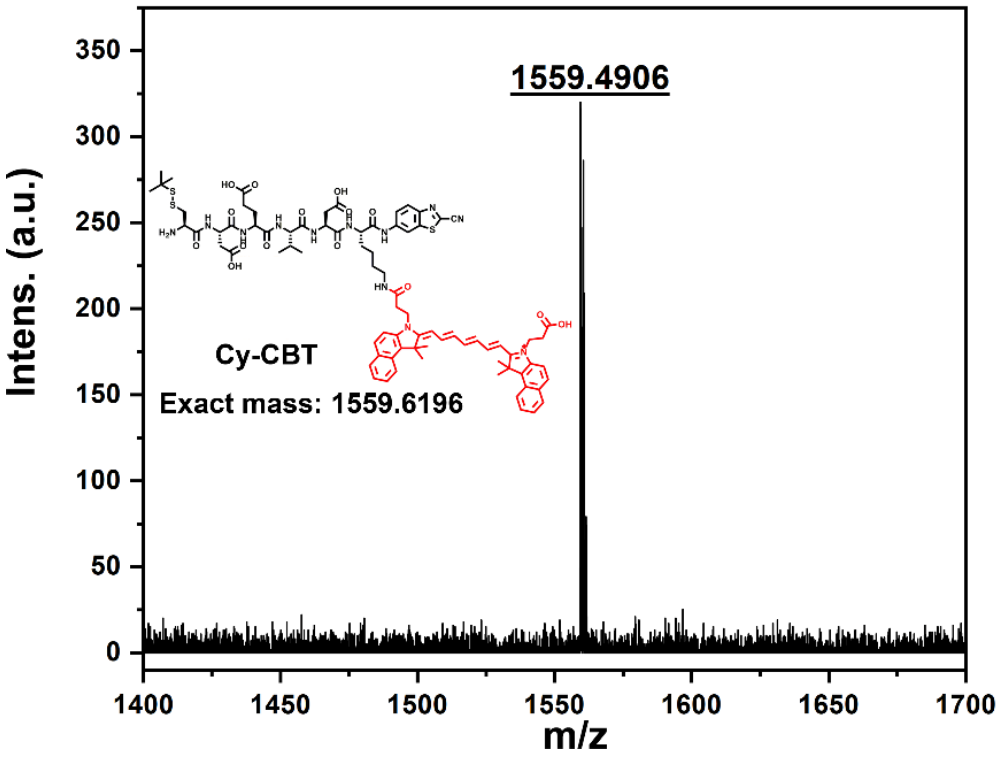


Figure S18. HR-MALDI-TOF/MS spectrum of **Cy-CBT**.

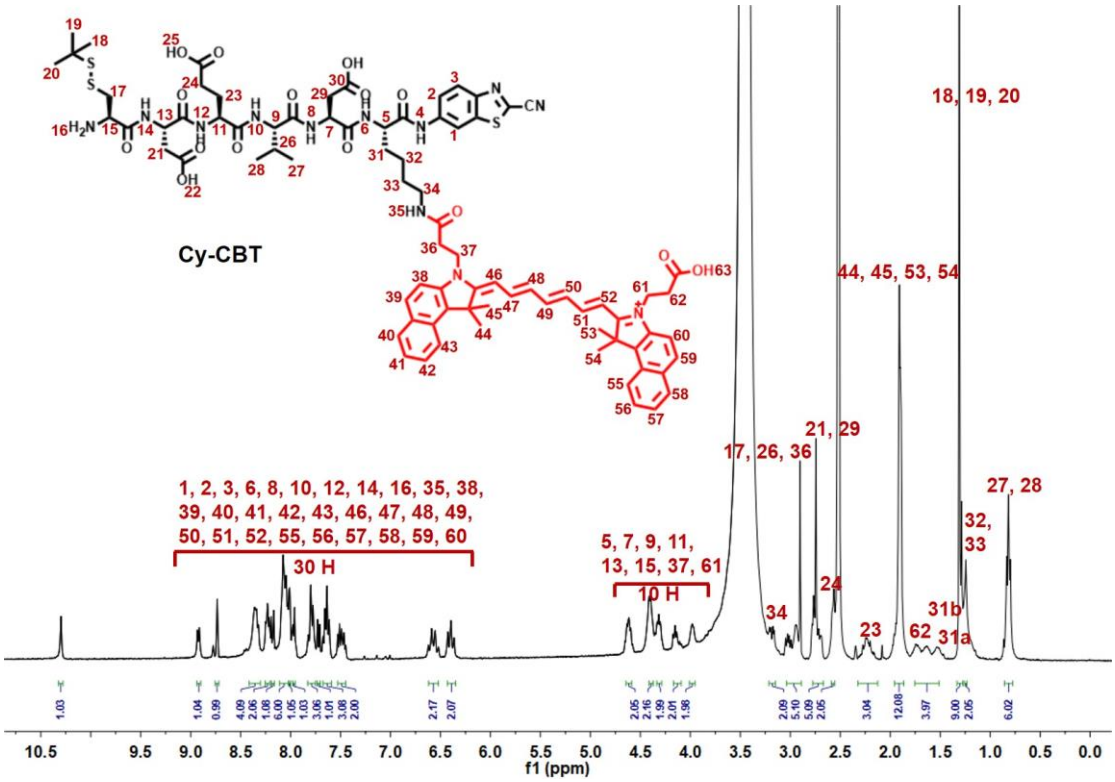


Figure S19. ^{1}H NMR spectrum of **Cy-CBT** in DMSO-d_6.

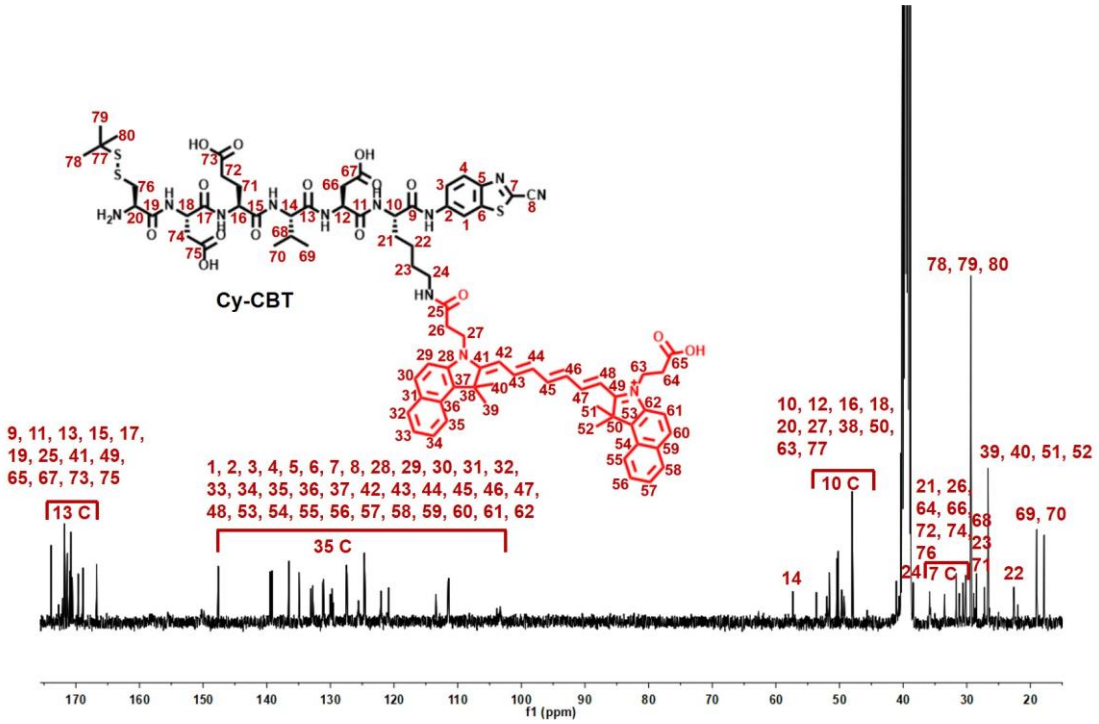
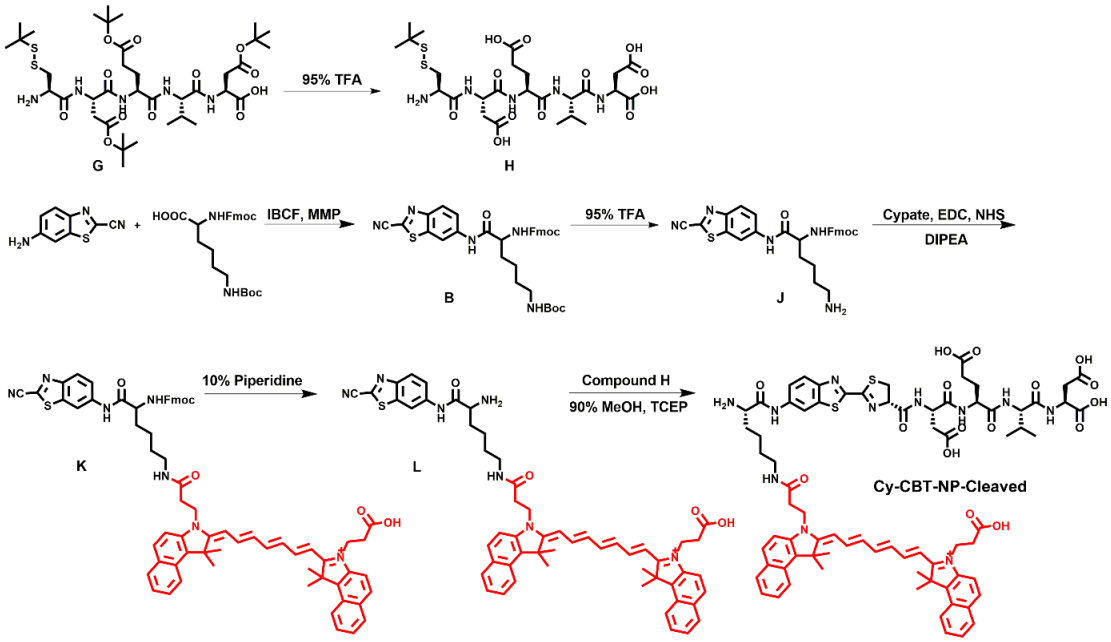


Figure S20. ^{13}C NMR spectrum of **Cy-CBT** in DMSO-d_6.

Scheme S2. The synthetic route for **Cy-CBT-NP-Cleaved**.



*Synthesis of **H**:* Compound NH_2-Cys(StBu)-Asp(OtBu)-Glu(OtBu)-Val-Asp(OtBu)-OH (**G**) was synthesized with solid phase peptide synthesis (SPPS) (Figure S21-23). The OtBu protecting group of compound **G** (300 mg, 0.36 mmol) was removed with dichloromethane (DCM, 1 mL) and triisopropylsilane (TIPS, 200 μL) in TFA (19 mL) for 3 h. Compound **H** (195.4 mg, yield: 81.6%) was purified by HPLC using water-acetonitrile mixed with 0.1% TFA as the eluent (from 99:1 to 35:65). Analytical HPLC chromatogram showed a single peak (Figure S24). MS: calculated for **H** $[M+H]^+$: 668.23; obsvd. ESI-MS: *m*/*z* 668.00. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 8.94 (d, *J* = 7.6 Hz, 1 H), 8.38 (s, 2 H), 8.31 (d, *J* = 7.8 Hz, 1 H), 8.05 (d, *J* = 8.0 Hz, 1 H), 7.73 (d, *J* = 8.8 Hz, 1 H), 4.64 – 4.58 (m, 1 H), 4.52 (dd, *J* = 14.0, 6.7 Hz, 1 H), 4.33 – 4.28 (m, 1 H), 4.19 (dd, *J* = 8.8, 6.2 Hz, 1 H), 3.98 (s, 1 H), 3.22 – 3.15 (m, 1 H), 3.02 (dd, *J* = 13.9, 8.2 Hz, 1 H), 2.76 – 2.65 (m, 2 H), 2.60 – 2.52 (m, 2 H), 2.29 – 2.16 (m, 2 H), 1.99 (dt, *J* = 13.3, 6.7 Hz, 1 H), 1.89 (ddd, *J* = 15.0, 9.3, 5.2 Hz, 1 H), 1.78 – 1.67 (m,

1 H), 1.31 (s, 9 H), 0.83 (dd, *J* = 12.8, 6.8 Hz, 6 H) (Figure S25). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 174.52 (1 C), 172.72 (1 C), 171.97 (2 C), 171.08 (2 C), 170.15 (1 C), 167.30 (1 C), 57.55 (1 C), 52.49 (1 C), 52.13 (1 C), 50.18 (1 C), 48.96 (1 C), 48.60 (1 C), 41.57 (1 C), 36.31 (2 C), 31.34 (1 C), 30.72 (1 C), 29.90 (3 C), 27.84 (1 C), 19.57 (1 C), 18.19 (1 C) (Figure S26).

*Synthesis of **J**:* The Boc protecting groups of compound **B** (200 mg, 0.32 mmol) were removed with dichloromethane (DCM, 1 mL) and triisopropylsilane (TIPS, 200 μL) in TFA (19 mL) for 3 h. Compound **J** (146.2 mg, yield: 86.9%) was purified by HPLC using water-methanol mixed with 0.1% TFA as the eluent (from 30:70 to 0:100). Analytical HPLC chromatogram showed a single peak (Figure S27). MS: calculated for **J** $[M+H]^+$: 526.19; obsvd. ESI-MS: *m*/*z* 526.02. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 10.67 (s, 1 H), 8.78 (t, *J* = 12.8 Hz, 1 H), 8.21 (d, *J* = 9.0 Hz, 1 H), 7.96 – 7.85 (m, 2 H), 7.77 (ddd, *J* = 18.4, 10.8, 4.7 Hz, 6 H), 7.47 – 7.28 (m, 4 H), 4.36 – 4.27 (m, 2 H), 4.24 (d, J = 6.9 Hz, 1 H), 4.19 (dd, *J* = 8.0, 5.1 Hz, 1 H), 2.78 (dt, *J* = 20.6, 10.5 Hz, 2 H), 1.79 – 1.63 (m, 2 H), 1.55 (t, *J* = 12.3 Hz, 2 H), 1.41 (ddd, *J* = 21.9, 14.1, 9.5 Hz, 2 H) (Figure S28). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 172.28 (1 C), 156.68 (1 C), 148.13 (1 C), 144.27 (2 C), 141.22 (2 C), 139.89 (1 C), 137.19 (1 C), 135.58 (1 C), 128.13 (2 C), 127.55 (2 C), 125.76 (2 C), 125.30 (1 C), 121.27 (1 C), 120.62 (2 C), 114.07 (1 C), 111.88 (1 C), 66.13 (1 C), 55.90 (1 C), 47.12 (1 C), 31.50 (1 C), 27.16 (1 C), 23.09 (1 C) (Figure S29).

*Synthesis of **K**:* To a cooled solution of Cypate (40 mg, 0.064 mmol) in DMF (4 mL) in a three-neck flask filling with N_2, EDC·HCl (15.3 mg, 0.08 mmol) was added. After 20

min, N-hydroxysuccinimide (9.1 mg, 0.079 mmol) was added and further stirred for 24 h at ambient temperature. Then, a mixture of **J** (28 mg, 0.053 mmol) and DIPEA (64 μL, 0.37 mmol, 0.08 mmol for the neutralization of HCl, 0.064 mmol for the neutralization of COOH of Cypate) in 1.5 mL DMF was then added under the protection of N_2. The reaction mixture was stirred for 12 h in the dark at room temperature. Compound **K** (11.2 mg, yield: 18.7%) was purified by HPLC using water-acetonitrile mixed with 0.1% TFA as the eluent (from 50:50 to 5:95). Analytical HPLC chromatogram showed a single peak (Figure S30). MS: calculated for **K** $[M]^+$: 1132.48; obsvd. ESI-MS: *m*/*z* 1132.38. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm):10.52 (s, 1 H) 8.74 (d, *J* = 1.9 Hz, 1 H), 8.20 (dd, *J* = 18.3, 8.6 Hz, 3 H), 8.11 – 7.95 (m, 7 H), 7.88 (d, *J* = 7.5 Hz, 2 H), 7.83 – 7.69 (m, 6 H), 7.63 (dt, *J* = 16.2, 8.1 Hz, 3 H), 7.52 – 7.36 (m, 4 H), 7.30 (td, *J* = 7.4, 2.7 Hz, 2 H), 6.56 (dd, *J* = 27.8, 12.7 Hz, 2 H), 6.40 (dd, *J* = 13.7, 7.0 Hz, 2 H), 4.40 (s, 4 H), 4.31 – 4.24 (m, 2 H), 4.23 – 4.19 (m, 1 H), 4.13 (d, *J* = 6.2 Hz, 1 H), 2.96 (d, *J* = 5.3 Hz, 2 H), 2.75 (t, *J* = 7.2 Hz, 2 H), 1.89 (d, *J* = 3.5 Hz, 12 H), 1.56 (s, 2 H), 1.26 (dd, *J* = 14.4, 7.5 Hz, 6 H) (Figure S31). ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 170.30 (1 C), 170.23 (2 C), 167.35 (2 C), 154.49 (1 C), 145.97 (1 C), 142.20 (2 C), 142.07 (2 C), 139.07 (2 C), 137.97 (1 C), 137.89 (1 C), 137.80 (1 C), 135.04 (2 C), 133.35 (1 C), 131.50 (1 C), 131.17 (1 C), 129.62 (1 C), 129.52 (1 C), 128.49 (1 C), 128.45 (1 C), 128.20 (2 C), 125.99 (2 C), 125.91 (2 C), 125.83 (2 C), 125.40 (4 C), 123.63 (1 C), 123.14 (2 C), 123.10 (1 C), 123.02 (1 C), 120.54 (1 C), 119.13 (2 C), 118.46 (3 C), 111.90 (1 C), 109.95 (1 C), 109.70 (1 C), 102,31 (1 C), 101.77 (1 C), 64.00 (1 C), 53.89 (1 C), 48.85 (1 C), 48.63 (2 C), 44.99 (2 C), 36.83 (1

C), 31.97 (1 C), 30.07 (1 C), 29.53 (1 C), 26.92 (1 C), 25.05 (4 C), 21.37 (1 C) (Figure S32).

*Synthesis of **L**:* The Fmoc protecting group of compound **K** was cleaved with 10% piperidine in DMF (1 mL) at 0 °C for 9 min, then 120 μL TFA was added to neutralize the alkaline, thus compound **F** (7.1 mg, yield: 78.6%) was obtained after HPLC purification using water-acetonitrile mixed with 0.1% TFA as the eluent (from 40:60 to 0:100). Analytical HPLC chromatogram showed a single peak (Figure S33). MS: calculated for **L** $[M]^+$: 910.41; obsvd. ESI-MS: *m*/*z* 910.24. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 11.00 (s, 1 H), 8.68 (d, *J* = 15.5 Hz, 1 H), 8.30 (s, 2 H), 8.22 (t, *J* = 9.2 Hz, 3 H), 8.09 (s, 1 H), 8.04 (dd, *J* = 8.2, 4.7 Hz, 2 H), 8.00 (s, 1 H), 7.97 (d, *J* = 11.5 Hz, 2 H), 7.94 (s, 1 H), 7.74 (dd, *J* = 22.4, 8.0 Hz, 3 H), 7.69 – 7.54 (m, 3 H), 7.54 – 7.41 (m, 2 H), 6.71 – 6.45 (m, 2 H), 6.37 (dd, *J* = 30.1, 13.6 Hz, 2 H), 4.37 (d, *J* = 33.1 Hz, 4 H), 3.94 (s, 1 H), 2.88 (s, 2 H), 2.74 (d, *J* = 13.9 Hz, 2 H), 1.89 (d, *J* = 6.4 Hz, 12 H), 1.72 (s, 2 H), 1.24 (d, *J* = 18.4 Hz, 6 H) (Figure S34). ^{13}C NMR (151 MHz, DMSO-d_6) δ(ppm): 172.41 (2 C), 169.55 (2 C), 168.61 (1 C), 148.62 (1 C), 140.13 (1 C), 140.02 (1 C), 139.04 (1 C), 137.22 (2 C), 136.34 (1 C), 133.62 (1 C), 133.45 (1 C), 131.74 (2 C), 130.67 (1 C), 130.54 (1 C), 130.39 (1 C), 130.35 (1 C), 130.22 (1 C), 128.15 (2 C), 128.08 (2 C), 128.01 (1 C), 125.53 (2 C), 125.30 (2 C), 122.68 (2 C), 121.45 (2 C), 113.95 (1 C), 112.63 (1 C), 112.13 (1 C), 112.08 (1 C), 104.56 (1 C), 104.19 (1 C), 53.64 (2 C), 50.97 (2 C), 50.87 (1 C), 38.78 (1 C), 34.06 (1 C), 32.30 (1 C), 31.16 (1 C), 28.97 (1 C), 27.25 (4 C), 22.08 (1 C) (Figure S35).

*Synthesis of **Cy-CBT-NP-Cleaved**:* Tris(2-carboxyethyl)phosphine (TCEP, 35.7 mg,

0.09 mmol) dissolved in water (100 μL) was added to compound **H** (7.6 mg, 0.0113 mmol) dissolved in water (200 μL). A saturated potassium carbonate solution was then added to the mixture and brought to pH of 7.4. The resulting colourless solution was stirred under N_2 for 1 h, and the progress of the reaction was monitored by HPLC. Then compound **J** (5.2 mg, 0.0056 mmol) dissolved in methanol (2 mL) was then added to the mixture above. The saturated potassium carbonate solution was added to pH of 7.4. The blue solution was stirred under N_2 for 2 h, and the progress of the reaction was monitored by HPLC. The methanol was removed in vacuo. Pure ***Cy-CBT-NP-Cleaved*** (5.9 mg, yield: 71.7%) was obtained after HPLC purification with water-acetonitrile as the eluent (from 50:50 to 5:95). Analytical HPLC chromatogram showed a single peak (Figure S36). MS: calculated for **Cy-CBT-NP-Cleaved** $[M]^+$: 1472.5695; obsvd. HR-MALDI-TOF/MS: *m*/*z* 1472.3585 (Figure S37). ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 10.96 (s, 1 H), 8.58 (dd, *J* = 14.3, 2.0 Hz, 1 H), 8.46 (t, *J* = 8.9 Hz, 1 H), 8.32 (d, *J* = 8.0 Hz, 3 H), 8.23 (t, *J* = 8.9 Hz, 2 H), 8.12 (dd, *J* = 8.7, 3.1 Hz, 3 H), 8.02 (dt, *J* = 19.5, 7.6 Hz, 5 H), 7.75 (dd, *J* = 22.8, 11.6 Hz, 3 H), 7.63 (ddd, *J* = 19.9, 15.8, 7.8 Hz, 3 H), 7.54 – 7.42 (m, 2 H), 6.57 (dd, *J* = 28.1, 12.8 Hz, 2 H), 6.40 (t, *J* = 15.1 Hz, 2 H), 5.35 (dt, *J* = 9.8, 6.7 Hz, 1 H), 4.64 (dd, *J* = 13.1, 7.8 Hz, 1 H), 4.56 – 4.51 (m, 1 H), 4.41 (d, *J* = 6.7 Hz, 2 H), 4.34 (dd, *J* = 11.1, 6.4 Hz, 3 H), 4.20 (dd, *J* = 8.7, 6.2 Hz, 1 H), 3.96 (s, 2 H), 3.01 – 2.91 (m, 2 H), 2.80 – 2.54 (m, 8 H), 2.33 – 2.20 (m, 2 H), 2.07 – 1.96 (m, 2 H), 1.90 (d, *J* = 5.2 Hz, 12 H), 1.73 (s, 2 H), 1.26 (d, *J* = 20.6 Hz, 6 H), 0.83 (dt, *J* = 12.8, 6.3 Hz, 6 H). (Figure S38). ^{13}C NMR (151 MHz, DMSO-d_6) δ(ppm): 174.62 (1 C), 172.73 (2 C), 172.42 (2 C), 172.24 (1 C), 172.06 (1 C), 171.27

(1 C), 171.06 (1 C), 170.73 (1 C), 169.76 (1 C), 169.57 (1 C), 168.40 (1 C), 165.03 (1 C), 160.20 (1 C), 149.65 (1 C), 140.14 (2 C), 140.01 (2 C), 137.91 (1 C), 136.90 (2 C), 133.71 (1 C), 133.41 (1 C), 131.77 (2 C), 130.71 (1 C), 130.58 (1 C), 130.40 (2 C), 130.20 (1 C), 128.14 (2 C), 128.09 (2 C), 128.02 (1 C), 125.35 (1 C), 125.25 (1 C), 124.96 (1 C), 122.69 (2 C), 120.44 (1 C), 112.76 (1 C), 112.12 (1 C), 112.08 (1 C), 104.56 (1 C), 104.12 (1 C), 79.38 (1 C), 57.71 (1 C), 53.61 (2 C), 52.58 (1 C), 50.99 (1 C), 50.84 (1 C), 50.34 (1 C), 49.06 (2 C), 38.81 (1 C), 36.39 (1 C), 35.30 (1 C), 34.13 (1 C), 32.29 (1 C), 31.35 (1 C), 31.21 (1 C), 30.67 (1 C), 29.52 (1 C), 28.98 (1 C), 27.78 (1 C), 27.25 (4 C), 22.11 (1 C), 19.62 (1 C), 18.26 (1 C) (Figure S39).

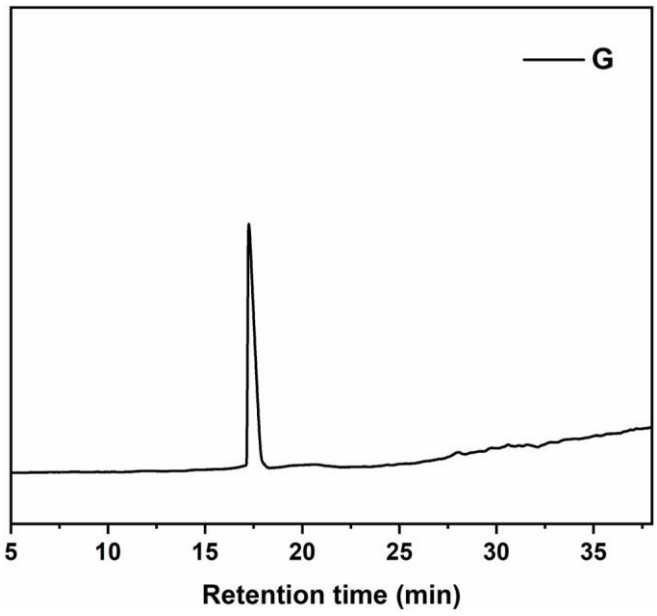


Figure S21. HPLC chromatogram of purified compound **G**.

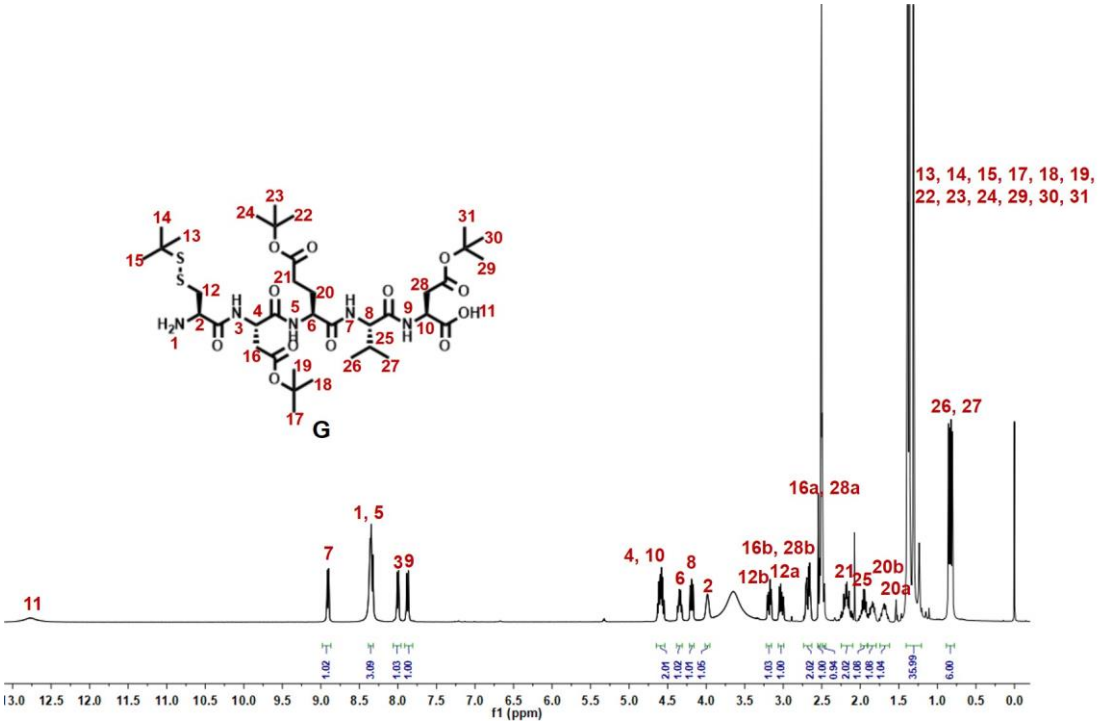


Figure S22. ^{1}H NMR spectrum of **G** in DMSO-d_6.

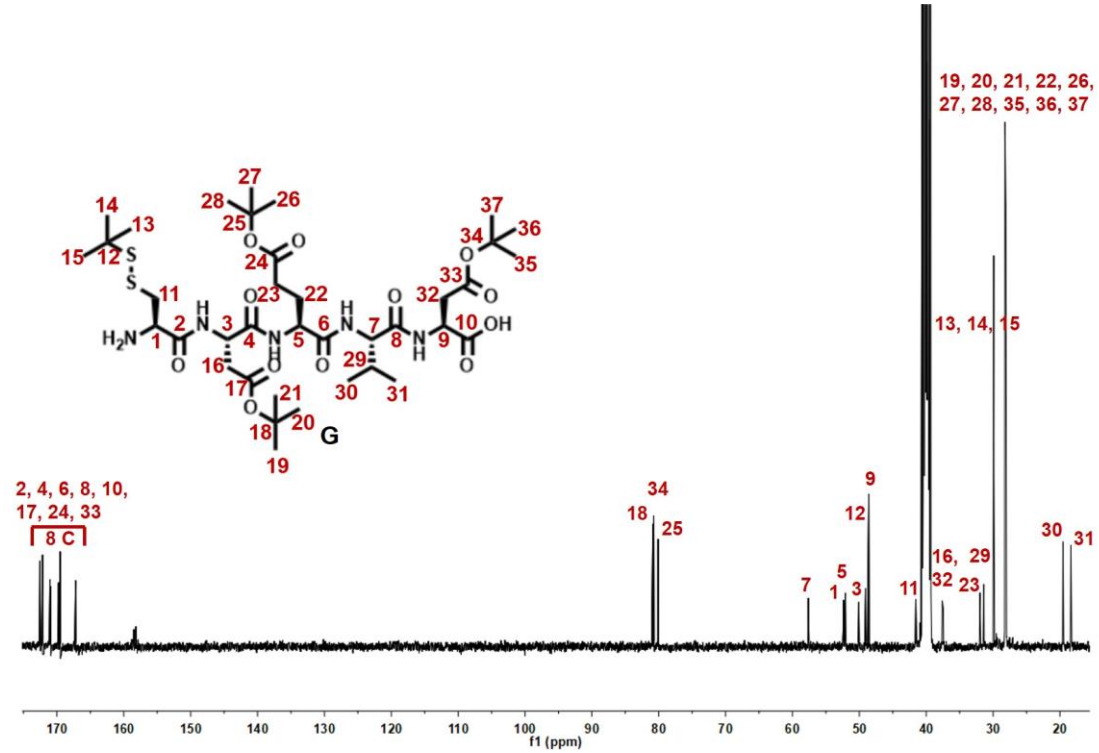


Figure S23. ^{13}C NMR spectrum of **G** in DMSO-d_6.

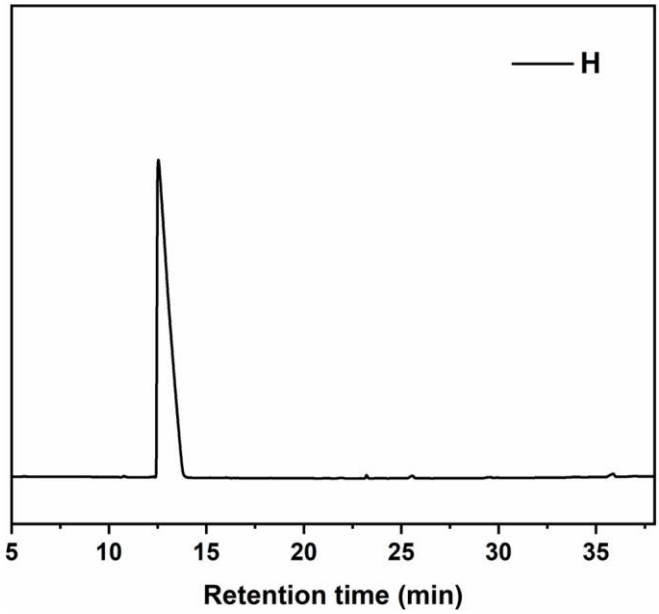


Figure S24. HPLC chromatogram of purified compound **H**.

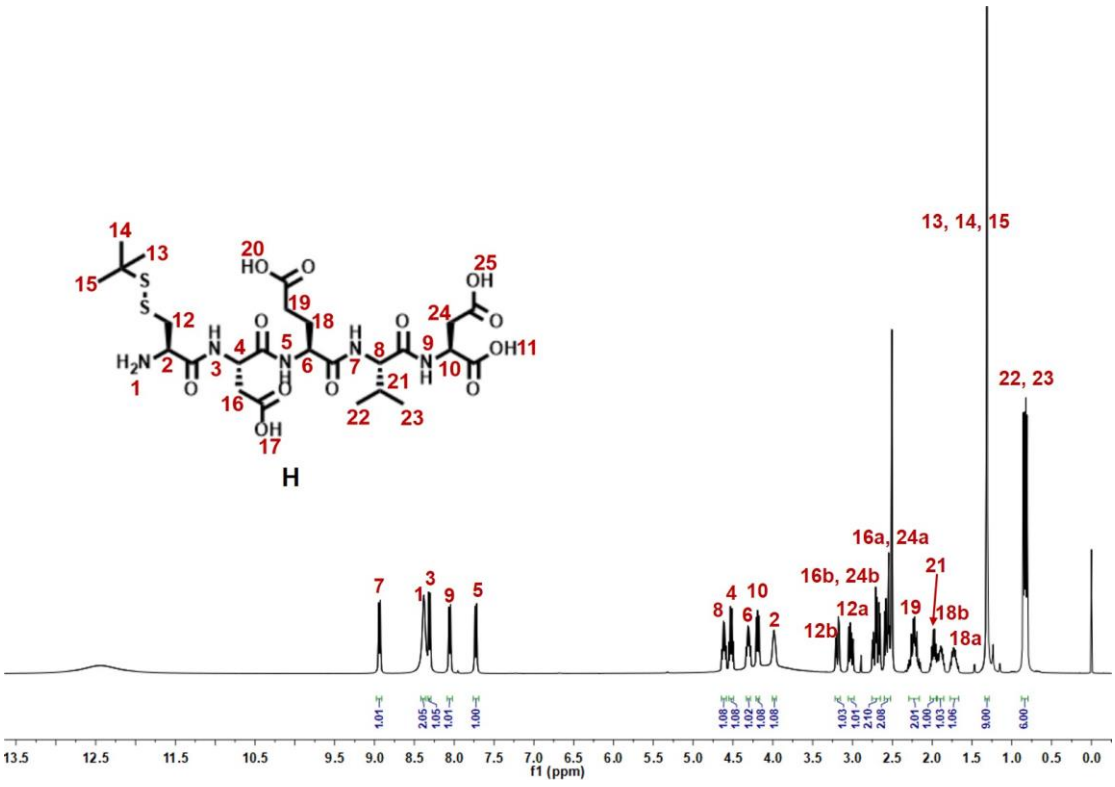


Figure S25. ^{1}H NMR spectrum of **H** in DMSO-d_6.

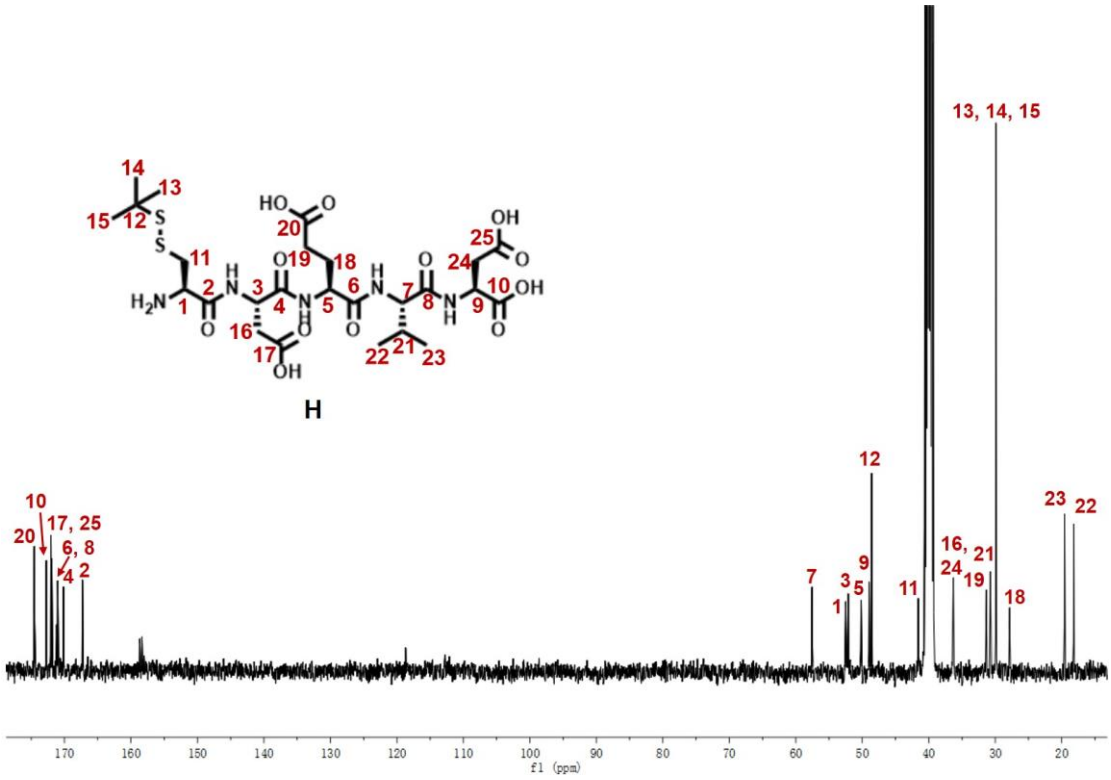


Figure S26. ^{13}C NMR spectrum of **H** in DMSO-d_6.

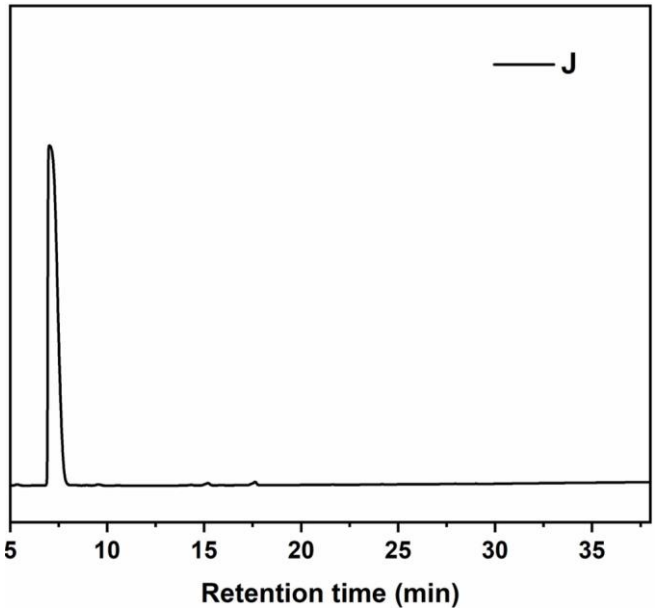


Figure S27. HPLC chromatogram of purified compound **J**.

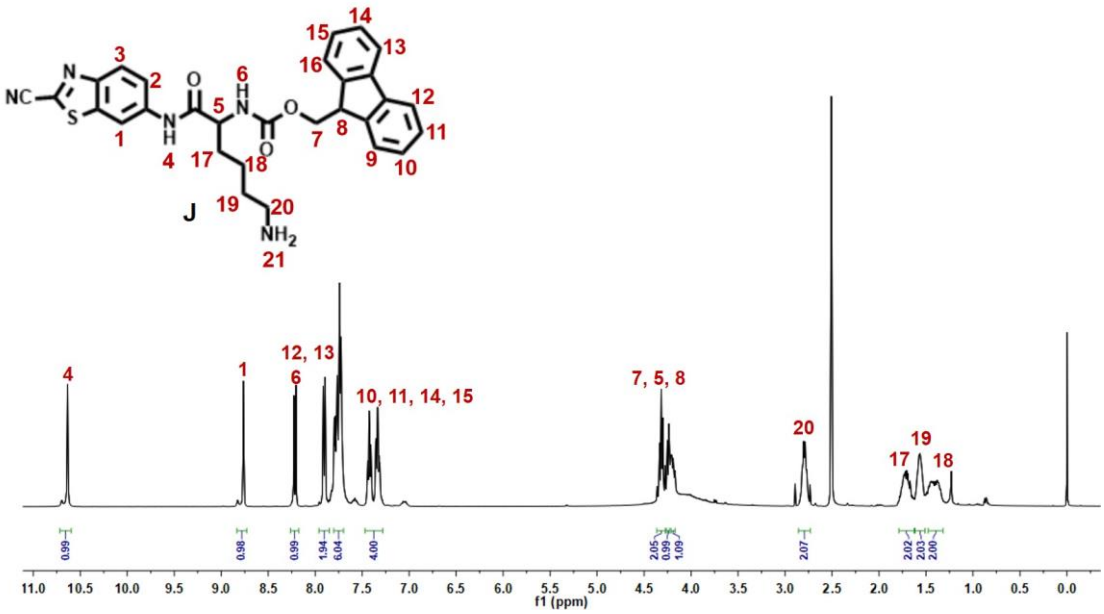


Figure S28. ^{1}H NMR spectrum of **J** in DMSO-d_6.

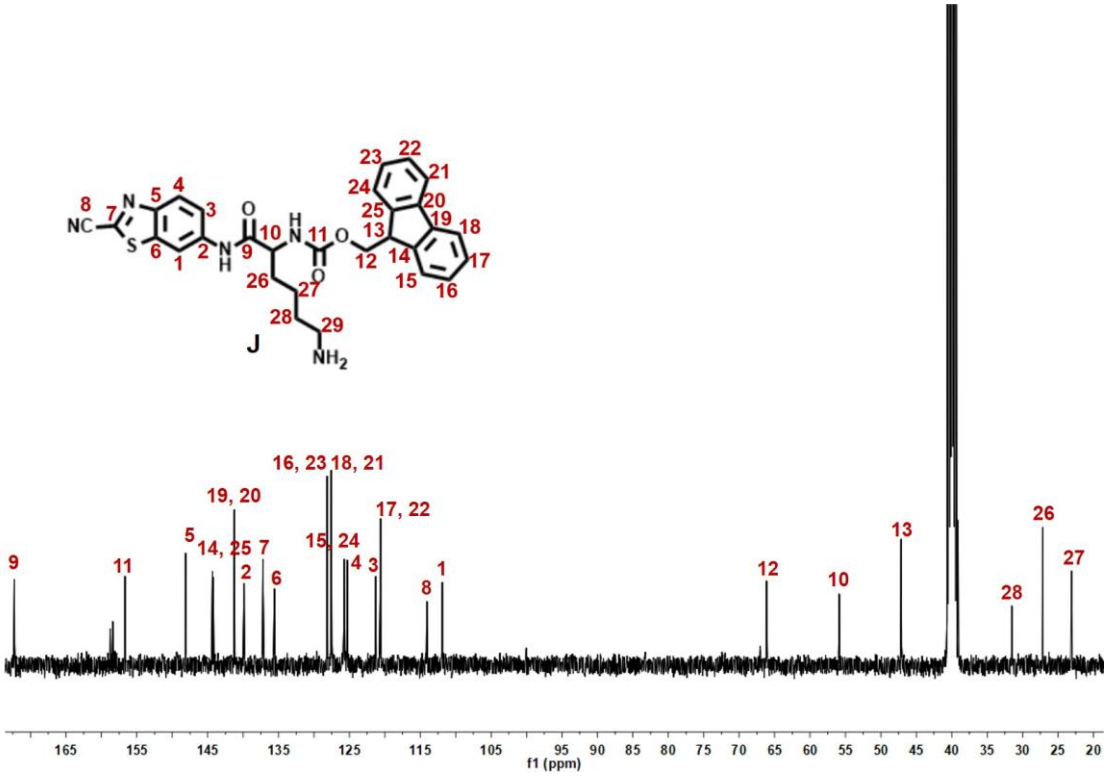


Figure S29. ^{13}C NMR spectrum of **J** in DMSO-d_6.

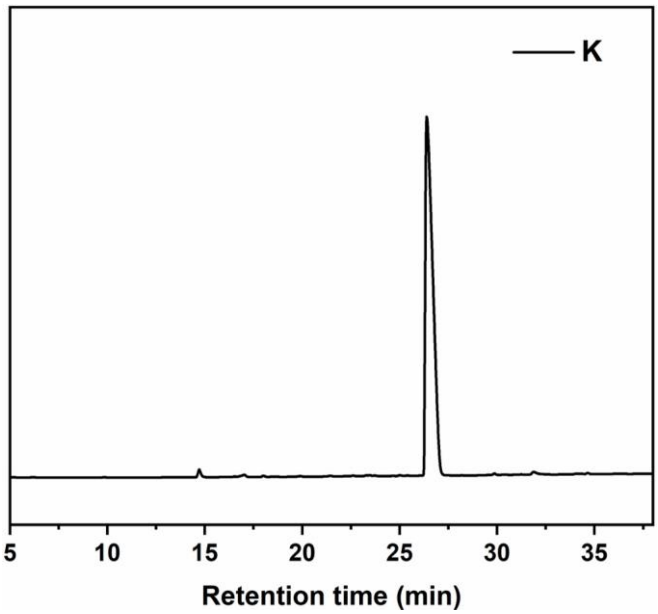


Figure S30. HPLC chromatogram of purified compound **K**.

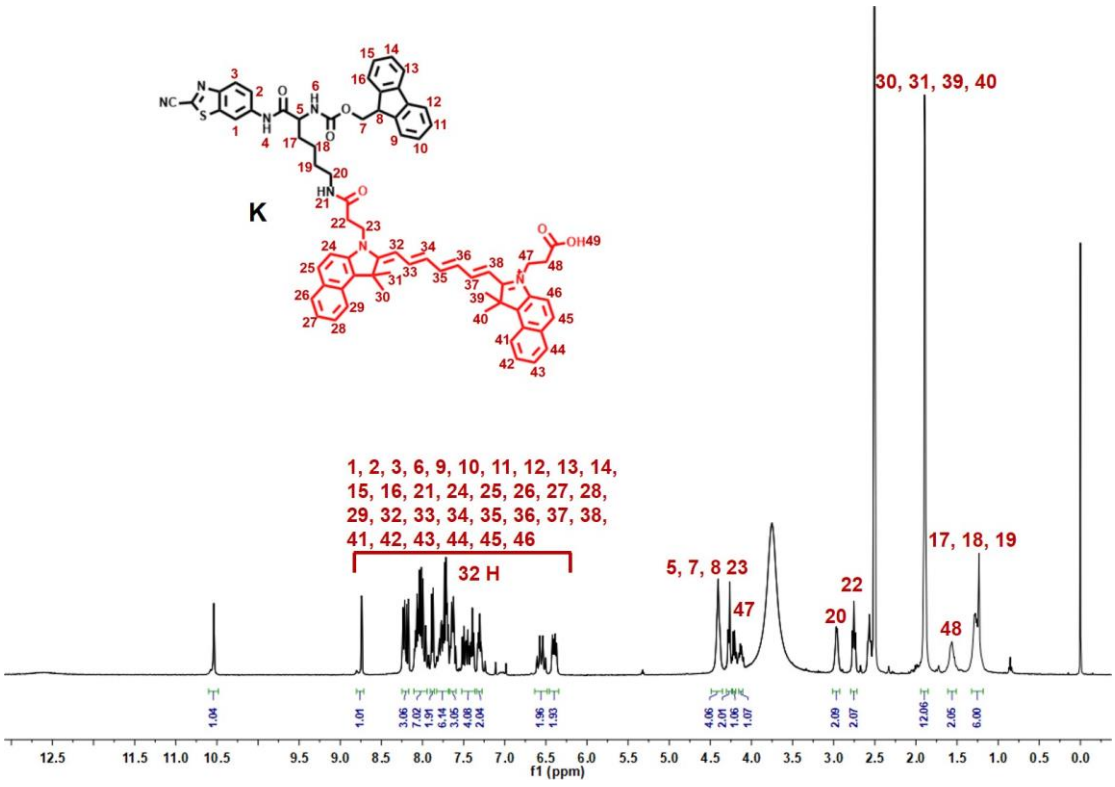


Figure S31. ^{1}H NMR spectrum of **K** in DMSO-d_6.

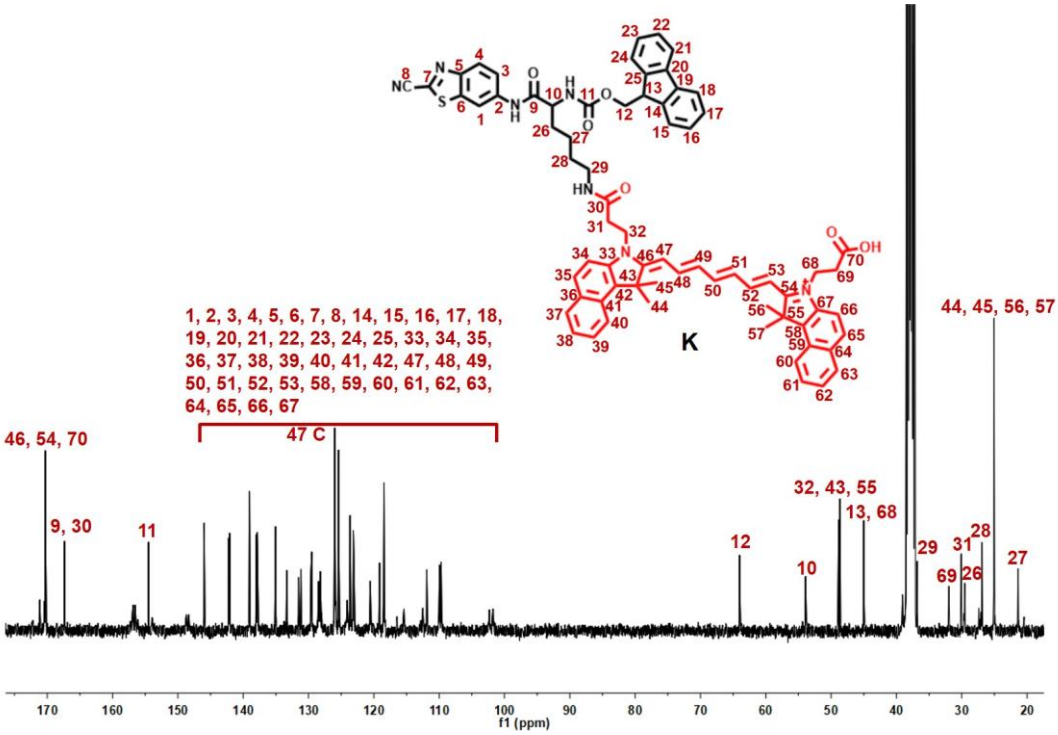


Figure S32. ^{13}C NMR spectrum of **K** in DMSO-d_6.

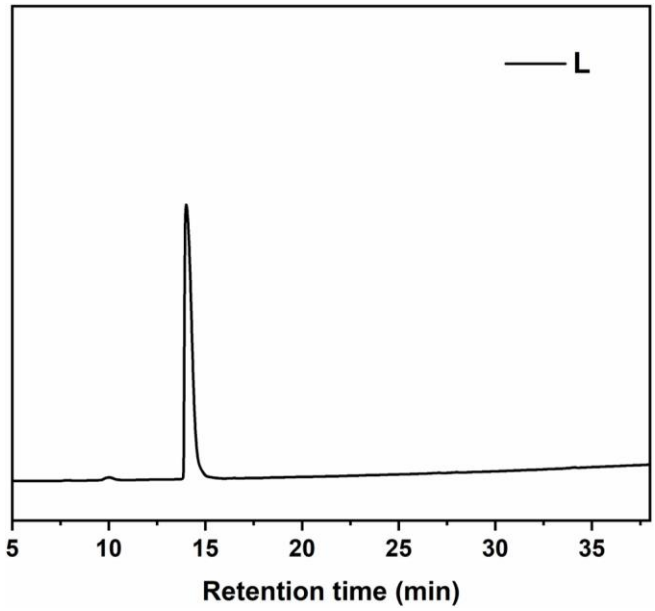


Figure S33. HPLC chromatogram of purified compound **L**.

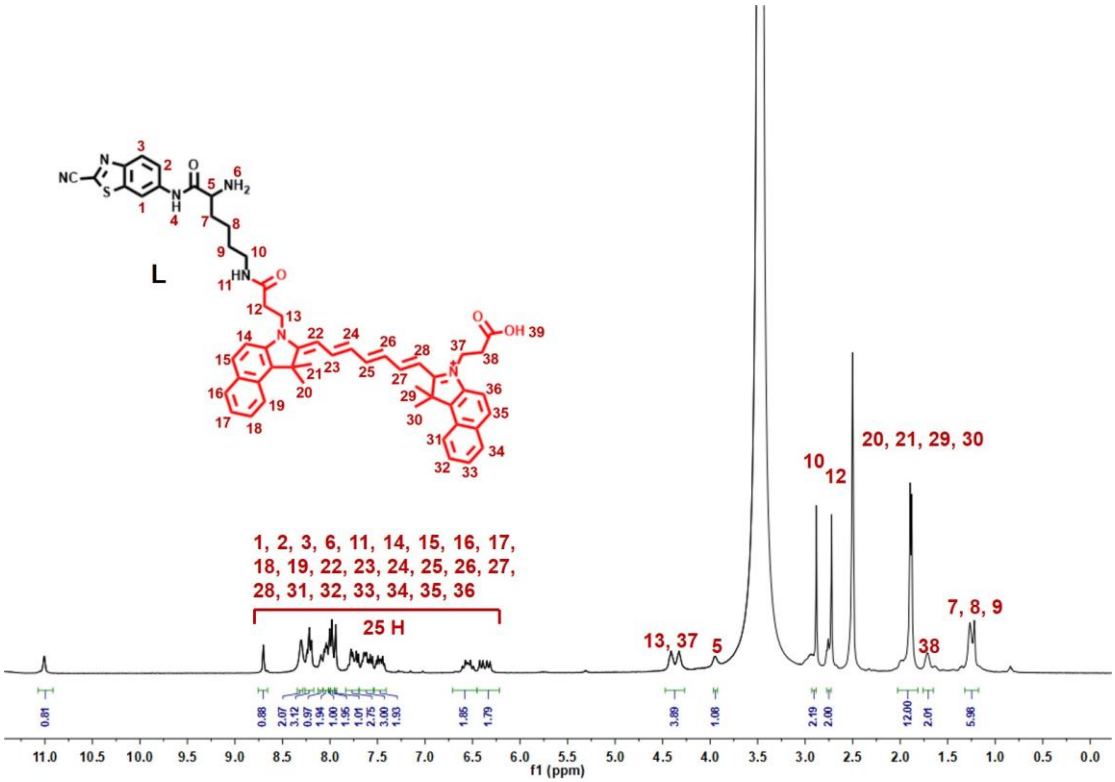


Figure S34. ^{1}H NMR spectrum of **L** in DMSO-d_6.

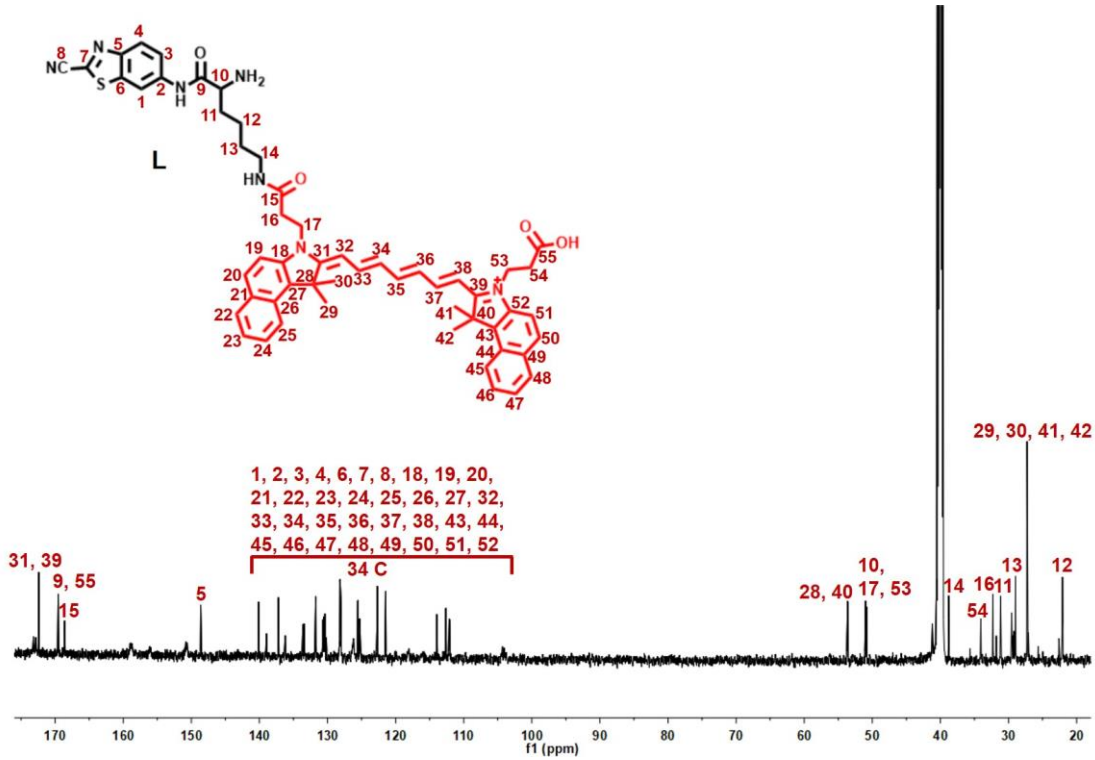


Figure S35. ^{13}C NMR spectrum of **L** in DMSO-d_6.

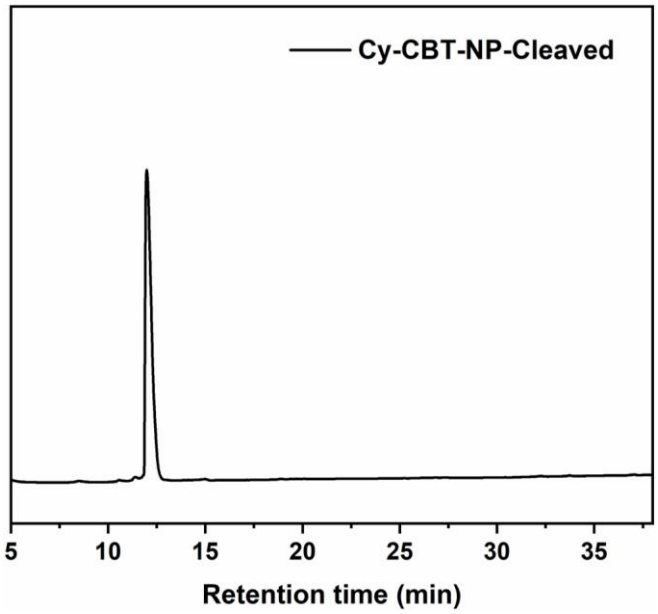


Figure S36. HPLC chromatogram of purified compound **Cy-CBT-NP-Cleaved**.

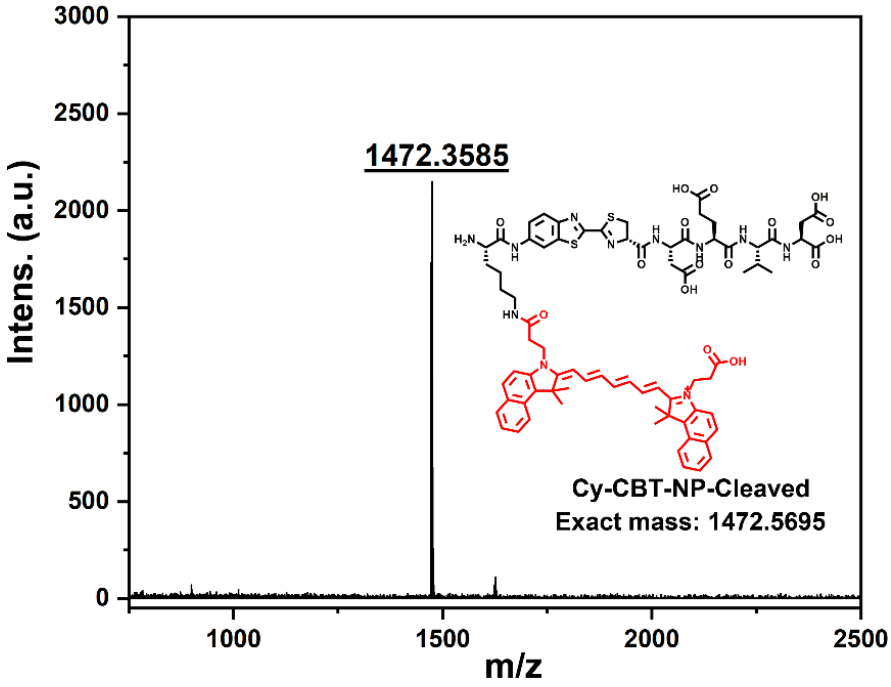


Figure S37. HR-MALDI-TOF/MS spectrum of **Cy-CBT-NP-Cleaved**.

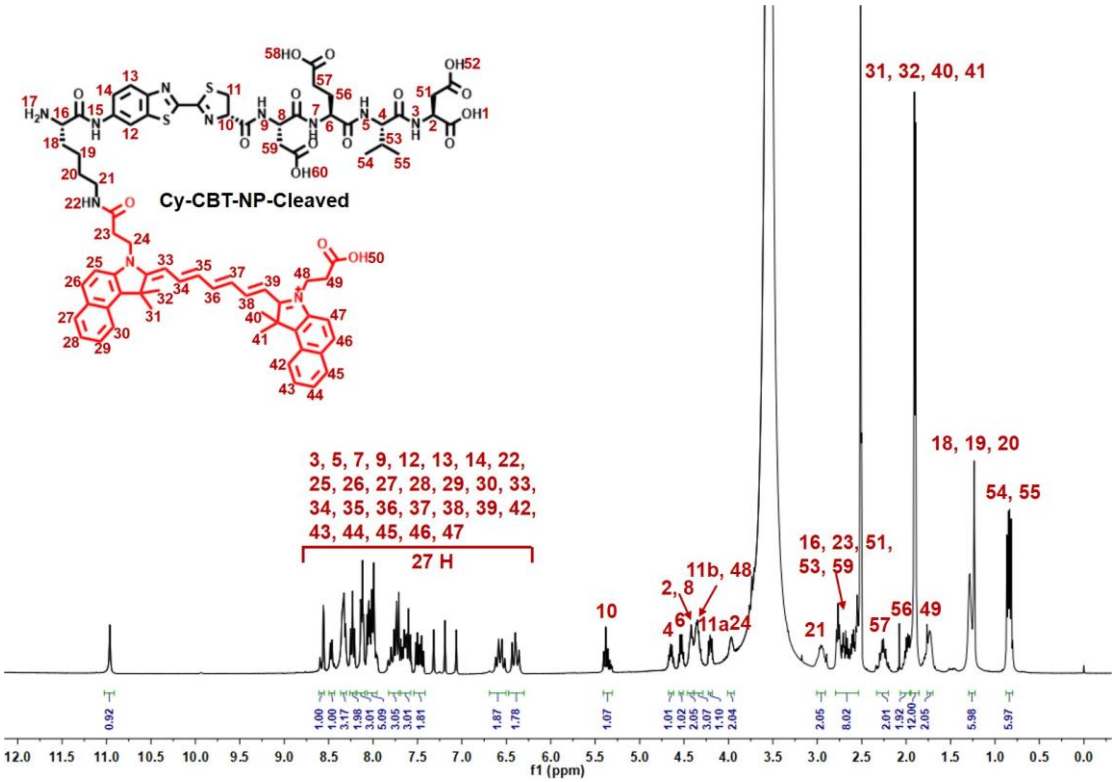


Figure S38. ^{1}H NMR spectrum of **Cy-CBT-NP-Cleaved** in DMSO-d_6.

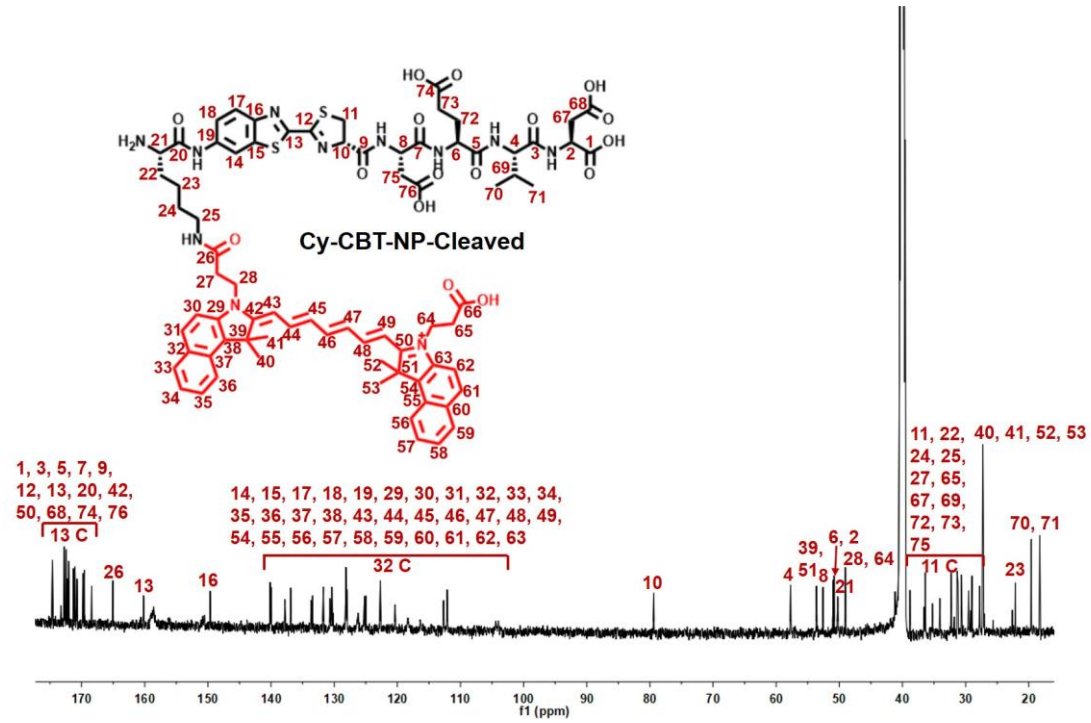
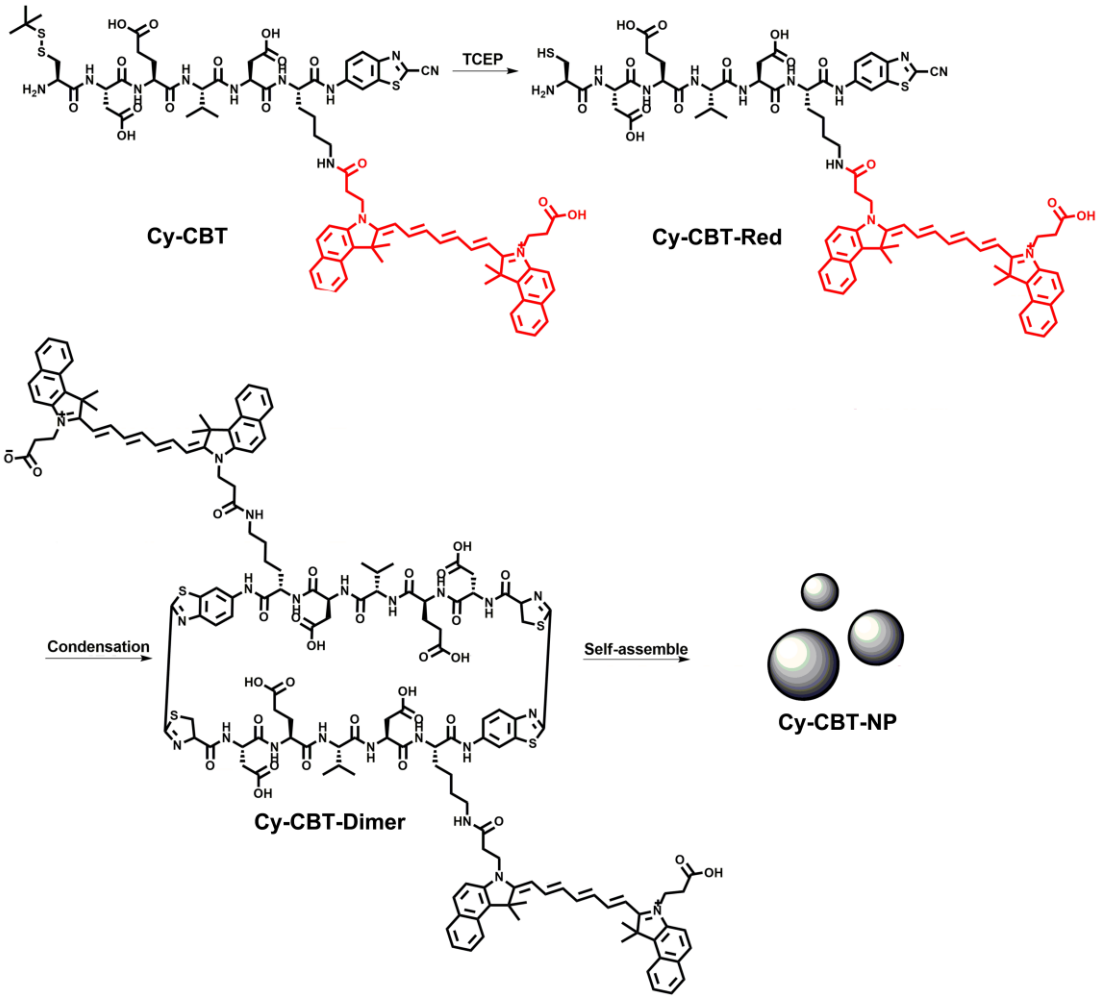


Figure S39. ^{13}C NMR spectrum of **Cy-CBT-NP-Cleaved** in DMSO-d_6.

Scheme S3. Schematic illustration of TCEP-controlled self-assembly of **Cy-CBT-NP** to turn NIR signals "Off".



Synthesis of ***Cy-CBT-Dimer****:* Tris(2-carboxyethyl)phosphine (TCEP, 14.3 mg, 0.05 mmol) dissolved in water (100 μL) was added to compound **Cy-CBT** (7.8 mg, 0.005 mmol) dissolved in water (200 μL). Saturated potassium carbonate solution was then added to the mixture and brought to pH of 7.4. The resulting colourless solution was stirred under N_2 for 3 h, and the progress of the reaction was monitored by HPLC. The methanol was removed in vacuo. Pure **Cy-CBT-Dimer** (2.3 mg, yield: 31%) was obtained after HPLC purification with water-acetonitrile as the eluent (from 50:50 to 5:95). MS: calculated for **Cy-CBT-Dimer** $[M]^+$: 2908.1096; obsvd. HR-MALDI-

TOF/MS: *m*/*z* 2909.407 (Figure S40).

*Preparation of **Cy-CBT-NP**:* The stock solution of **Cy-CBT-Dimer** was prepared in dimethyl sulfoxide (DMSO). Then a certain amount of stock solution of **Cy-CBT-Dimer** was dispersed into the PBS buffer (10 mM, pH 7.4) to a final concentration of 50 μM of **Cy-CBT-Dimer** (calculated in **Cy-CBT**). The amphiphilic **Cy-CBT-Dimer** instantly self-assembles into nanoparticles (*i.e.*, **Cy-CBT-NP**) *via* π−π stacking. After that, an ultrasound was performed on the dispersions for 20 min and left stationary for 10 min to prevent the accumulation of nanoparticles and make the size of nanoparticles more uniform.

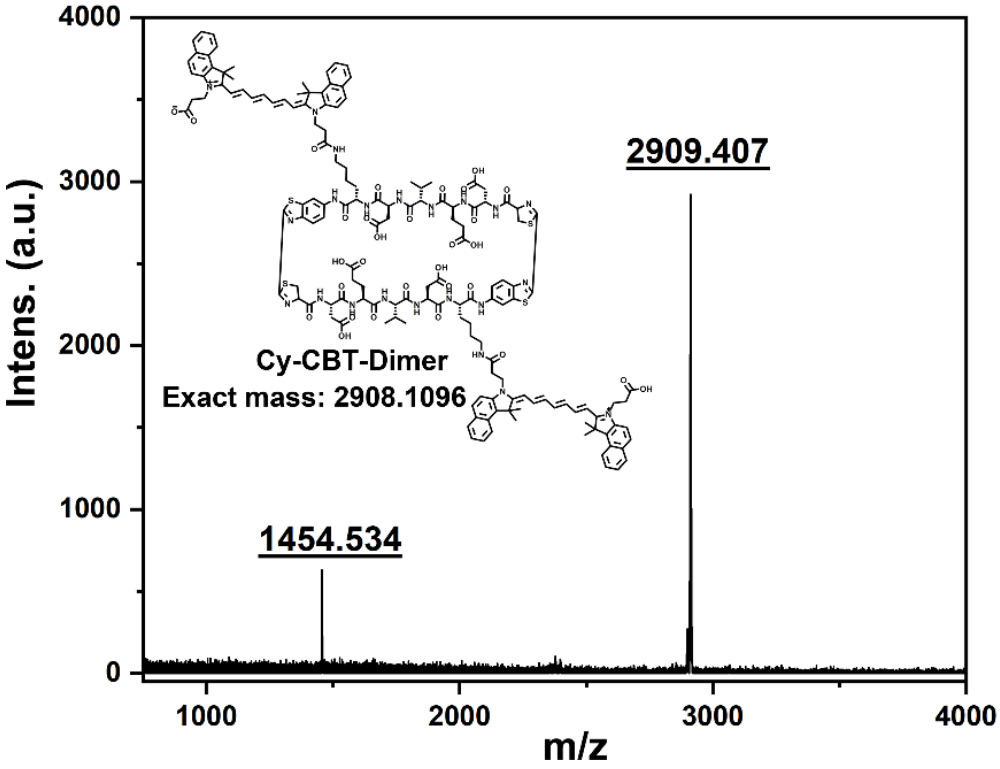


Figure S40. HR-MALDI-TOF/MS spectrum of **Cy-CBT-Dimer.**

3. Supporting Figures and table

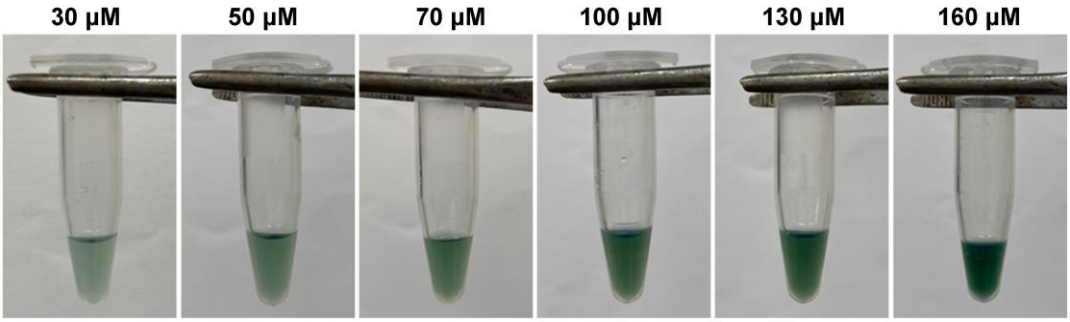


Figure S41. Photographs of **Cy-CBT-NP** at various concentrations in saline.

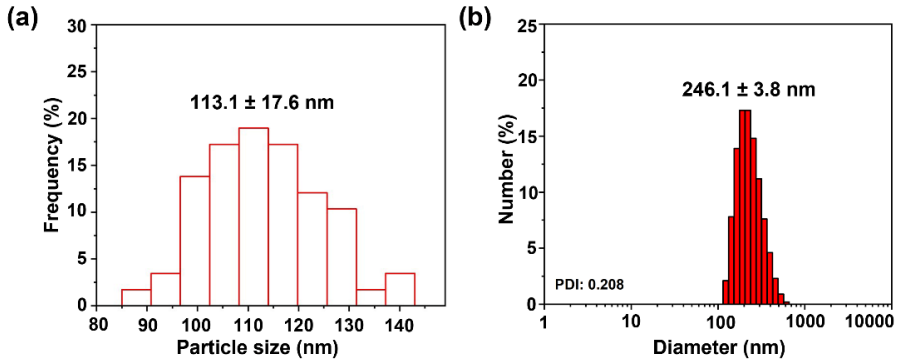


Figure S42. (a) Statistics of size distribution of **Cy-CBT-NP** in Figure 2a. (b) DLS measurement of **Cy-CBT-NP** in PBS.

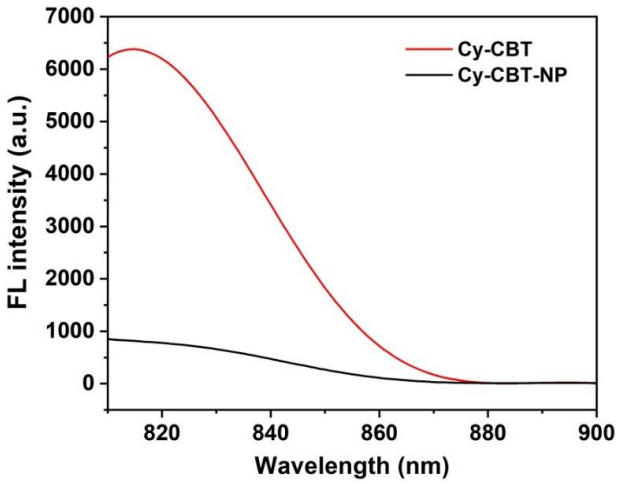


Figure S43. Fluorescence spectra of 15 μM **Cy-CBT** and 15 μM **Cy-CBT-NP** (calculated in **Cy-CBT**) in PBS buffer.

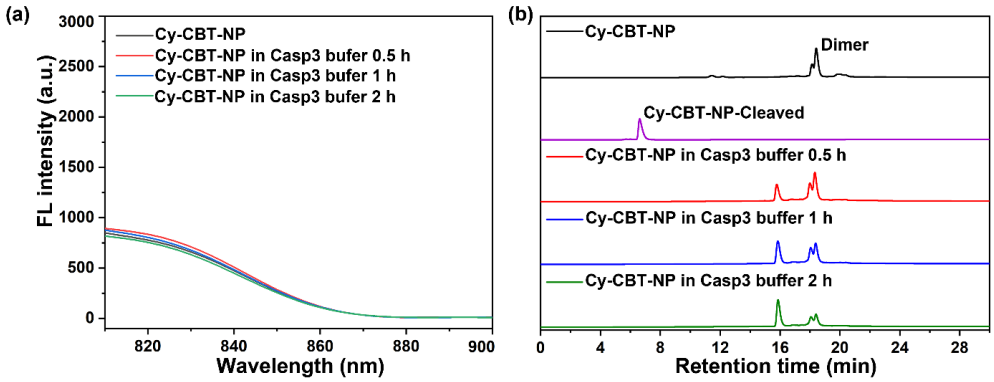


Figure S44. (a) Fluorescence spectra of **Cy-CBT-NP** incubated in Casp3 buffer at 37 ℃ for 0.5, 1, or 2 h. The concentration of samples used for detection is 15 μM diluted from 50 μM **Cy-CBT-NP** (calculated in **Cy-CBT**) after corresponding treatment. (b) HPLC traces of **Cy-CBT-NP**, **Cy-CBT-NP-Cleaved**, 50 μM **Cy-CBT-NP** (calculated in **Cy-CBT**) after incubation in Casp3 buffer at 37 ℃ for 0.5, 1, or 2 h.

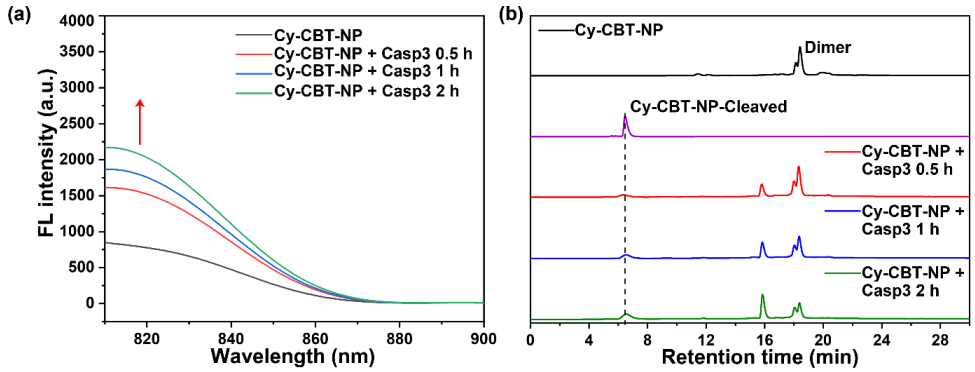


Figure S45. (a) Fluorescence spectra of **Cy-CBT-NP** incubated with 10 μg mL^{-1} Casp3 in Casp3 buffer at 37 ℃ for 0.5, 1, or 2 h. The concentration of samples used for detection is 15 μM diluted from 50 μM **Cy-CBT-NP** (calculated in **Cy-CBT**) after corresponding treatment. (b) HPLC traces of **Cy-CBT-NP**, **Cy-CBT-NP-Cleaved**, 50 μM **Cy-CBT-NP** (calculated in **Cy-CBT**) after incubation with 10 μg mL^{-1} Casp3 in Casp3 buffer at 37 ℃ for 0.5, 1, or 2 h.

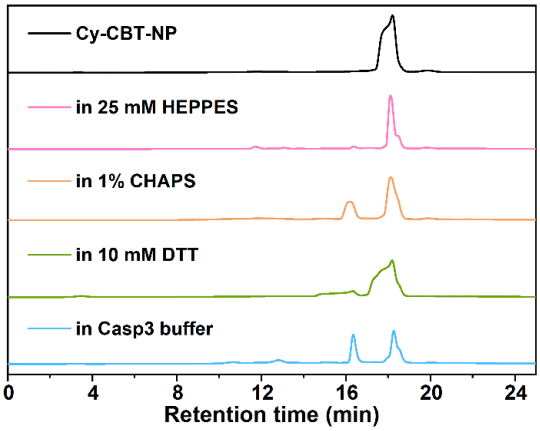


Figure S46. HPLC traces of **Cy-CBT-NP**, **Cy-CBT-NP** in Casp3 buffer, and **Cy-CBT-NP** incubated with the different components in Casp3 buffer for 4 h.

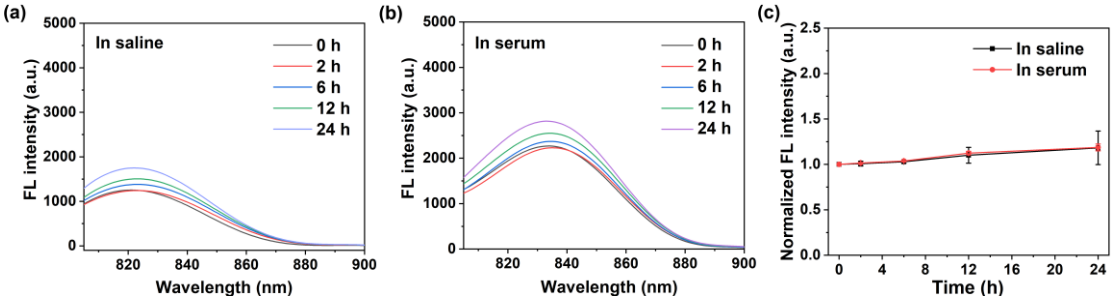


Figure S47. Fluorescence spectra of **Cy-CBT-NP** at different time point during 24 h incubation in saline (a) or mouse serum (b) at 37 °C. λ_{ex} = 785 nm. (c) Time-course normalized fluorescence intensity of **Cy-CBT-NP** in a and b.

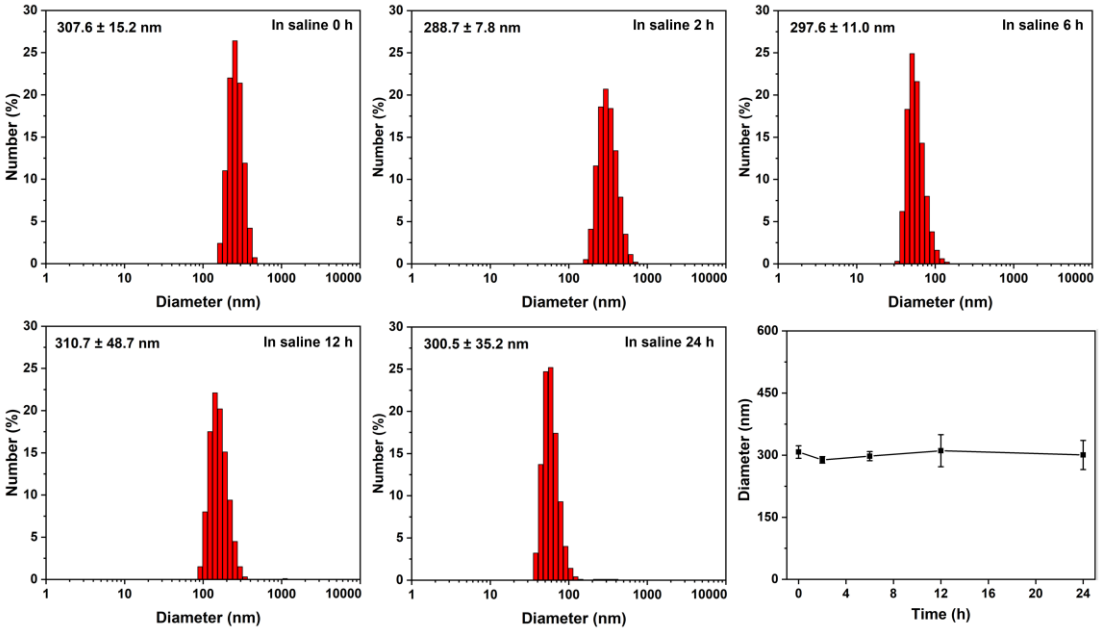


Figure S48. DLS analysis of **Cy-CBT-NP** (50 μM, calculated in **Cy-CBT**) at different time point during 24 h incubation in saline at 37 °C.

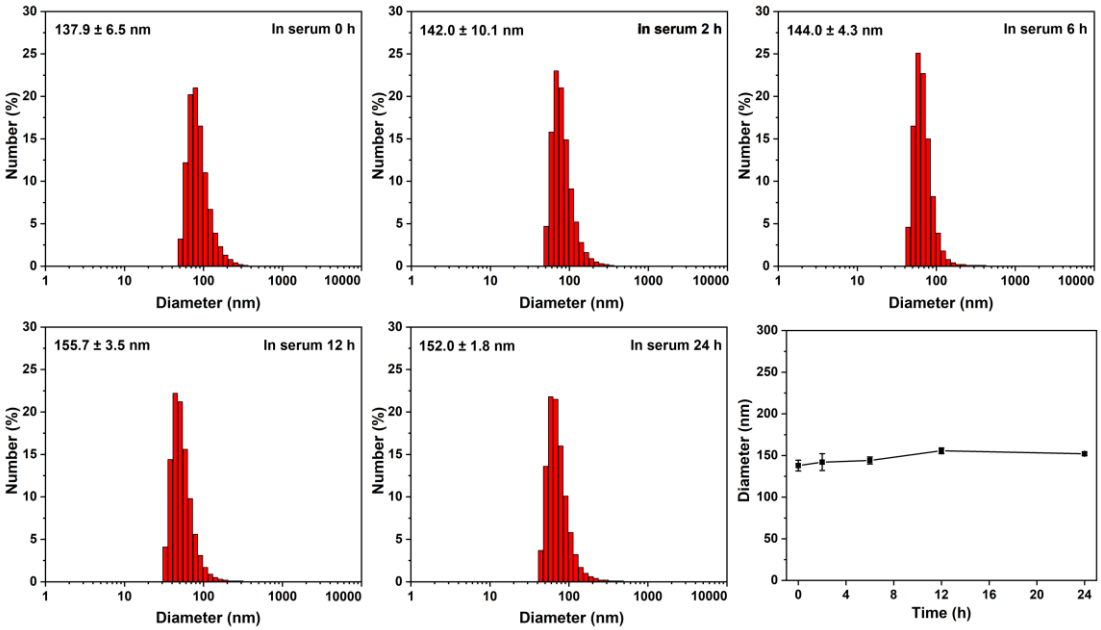


Figure S49. DLS analysis of **Cy-CBT-NP** (50 μM, calculated in **Cy-CBT**) at different time point during 24 h incubation in the mouse serum at 37 °C.

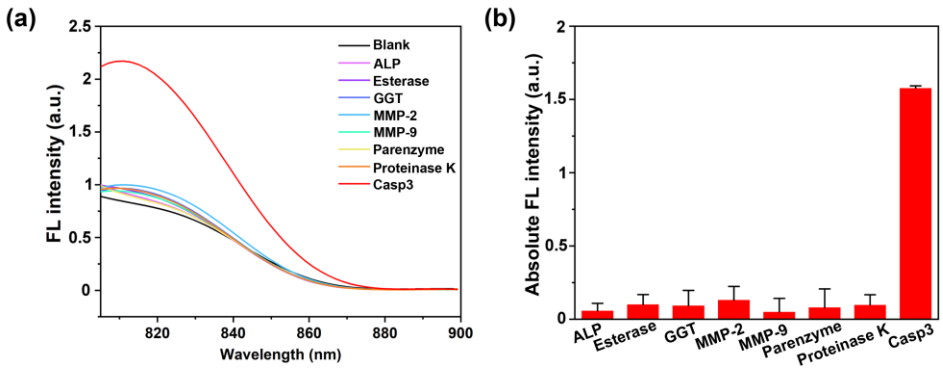


Figure S50. (a) Fluorescence spectra of **Cy-CBT-NP** (15 μM, calculated in **Cy-CBT**) incubated with 10 μg/mL Casp3, ALP, Esterase, GGT, MMP-2, MMP-9, Parenzyme, or Proteinase K at 37 °C for 2 h. λ_{ex} = 785 nm. (b) Absolute fluorescence intensities of **Cy-CBT-NP** in a.

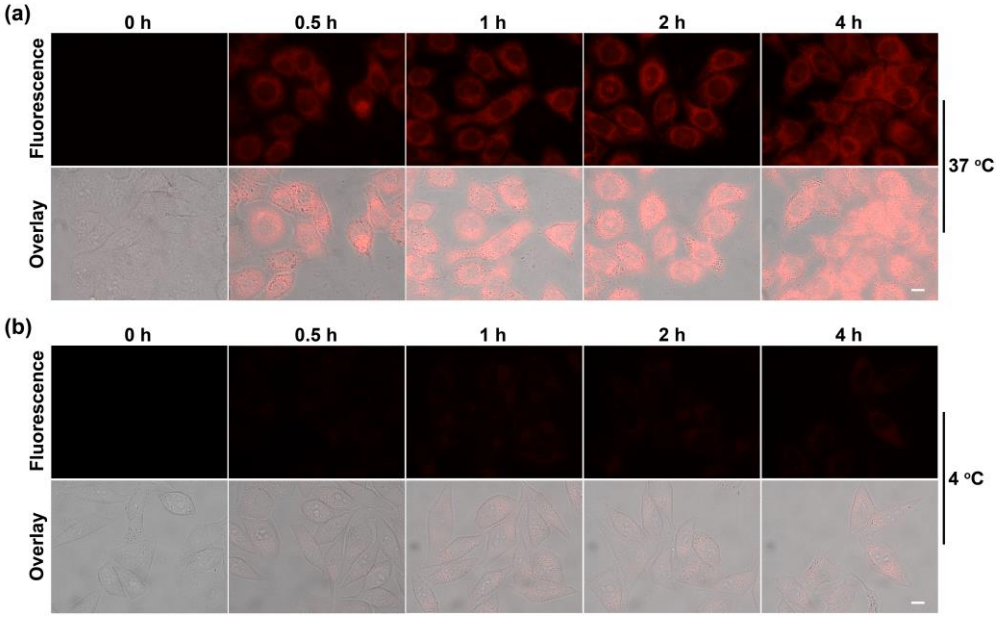


Figure S51. Time-course differential fluorescence images (top row) and overlay images of fluorescence and interference contrasst images (bottom rows) of HeLa cells incubated with 30 μM of **Cy-CBT-NP** (calculated in **Cy-CBT**) in a serum-free DMEM for 0.5, 1, 2, or 4 h at 37 °C (a) or 4 °C (b), washed with PBS for three times prior to imaging, respectively. Scale bar: 10 μm.

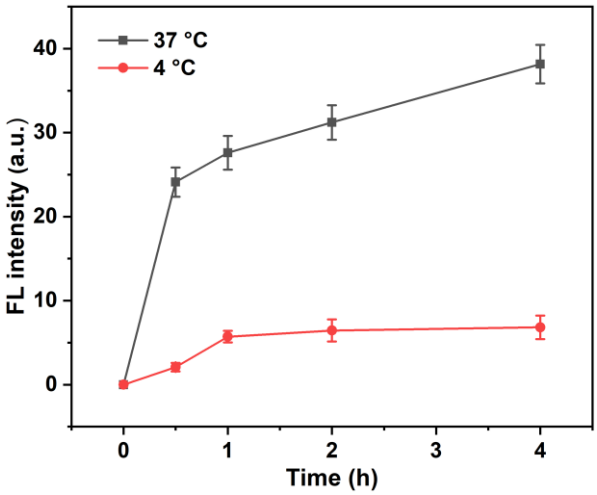


Figure S52. Quantification of the mean flux (photon/s) for the cell images in Figure S51.

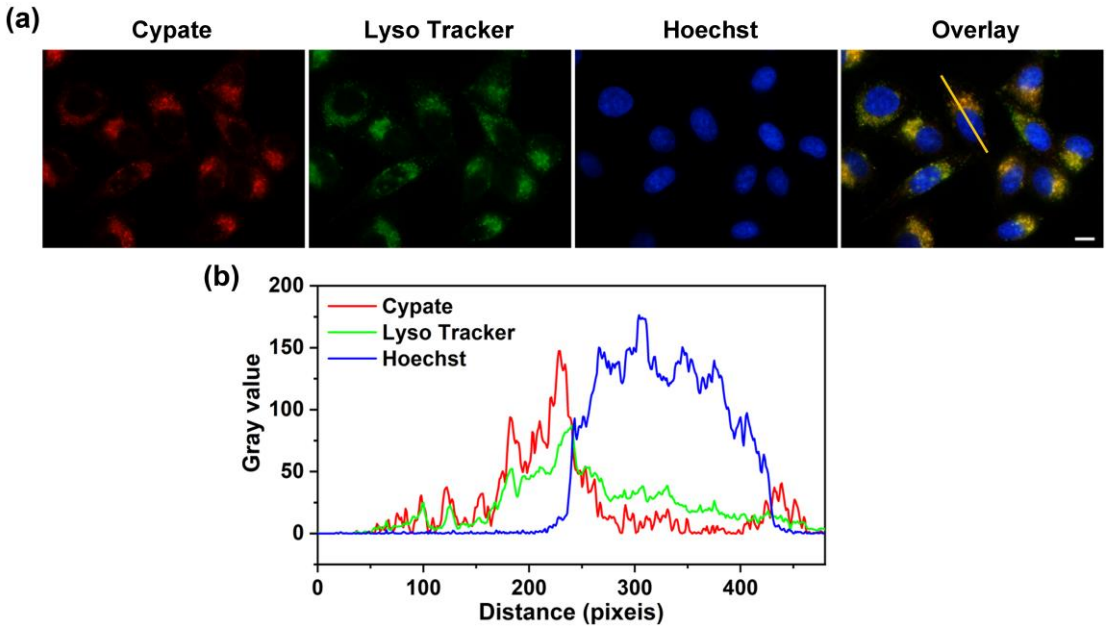


Figure S53. (a) Fluorescence and overlay images of HeLa cells incubated with 30 μM **Cy-CBT-NP** (calculated in **Cy-CBT**) at 37 ℃ for 2 h. Lyso Tracker (green) was used to reveal the location of lysosomes and Hoechst (blue) was used to reveal the location of nucleus. All images have the same scale bar: 10 μm. (b) Intensity scatter plot of red, green, and blue channels.

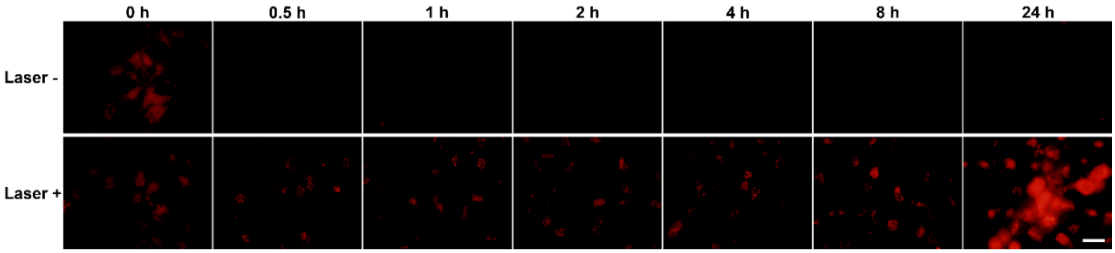


Figure S54. Time-course fluorescent images of HeLa cells after PTT. The HeLa cells were incubated with 30 μM **Cy-CBT-NP** (calculated in **Cy-CBT**) for 2 h, washed, exposed to 808 nm laser irradiation for 5 min (bottom row) or w/o laser irradiation (top row), followed by fluorescence imaging at 0, 0.5, 1, 2, 4, 8, or 24 h. Scale bar: 10 μm.

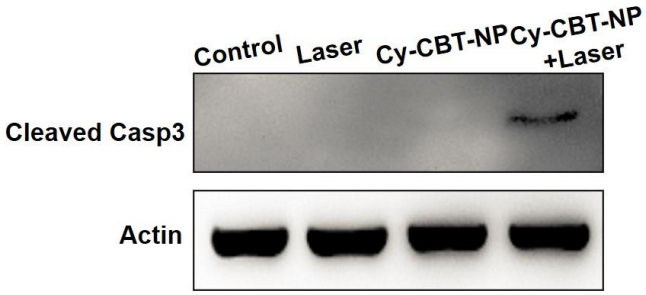


Figure S55. Western blot analysis of expression level of active Casp 3 in HeLa cells incubated with DMEM or 30 μM **Cy-CBT-NP** (calculated in **Cy-CBT**) for 2 h, washed, with (or w/o) 5 min laser irradiation, and followed by another 24 h incubation.

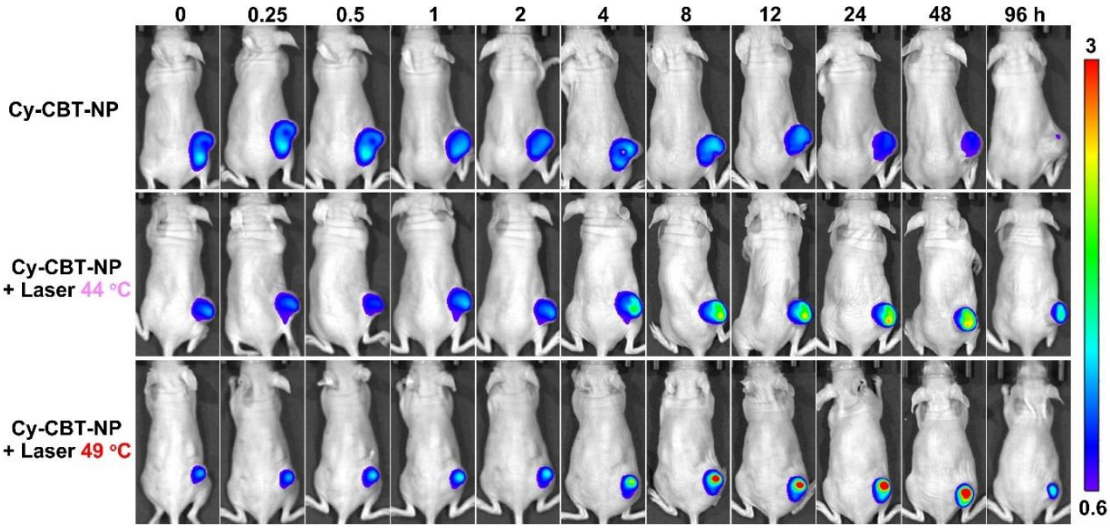


Figure S56. Fluorescence monitoring images of **Cy-CBT-NP**-injected (10 μL, 90 nmol kg^{-1}, calculated in **Cy-CBT**) HeLa tumor-bearing nude mice after irradiation with (middle and bottom rows) (or w/o, top row) 808 nm laser irradiation at 0.4 W cm^{-2} for different times.

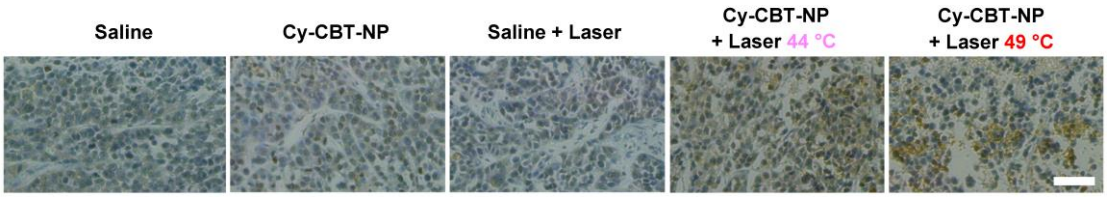


Figure S57. Immunohistochemical staining active Casp3 in tumor tissues from mice sacrificed at 24 h post PTT treatment. Scale bar: 50 μm.

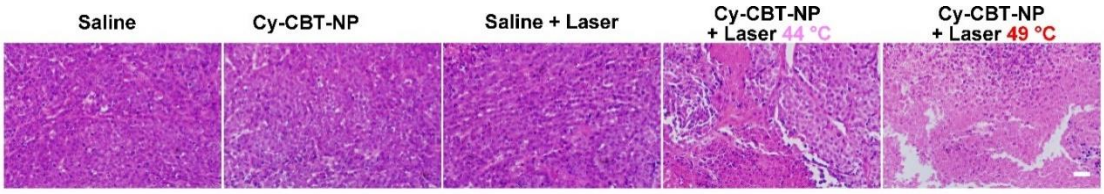


Figure S58. Images of H&E staining of the tumor tissues from mice sacrificed at 24 h post PTT treatment. Scale bar: 100 μm.

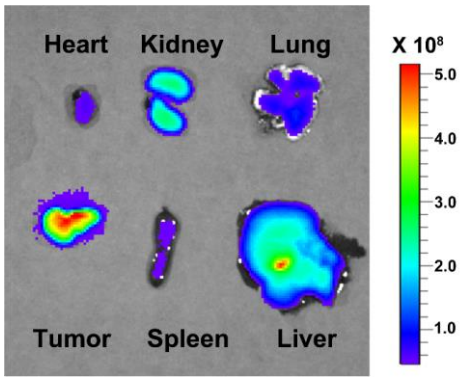


Figure S59. *Ex vivo* fluorescence images of different organs from mice at 48 h post-PTT treatment.

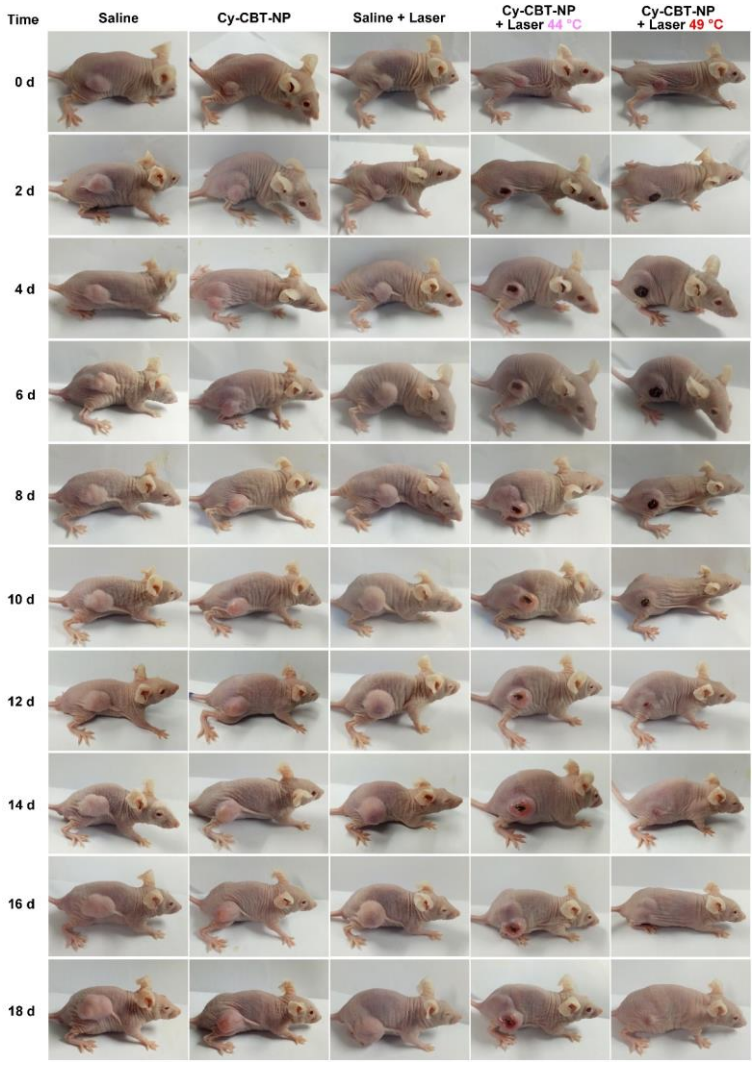


Figure S60. Photographs of one mouse from each group at different time points after PTT treatment.

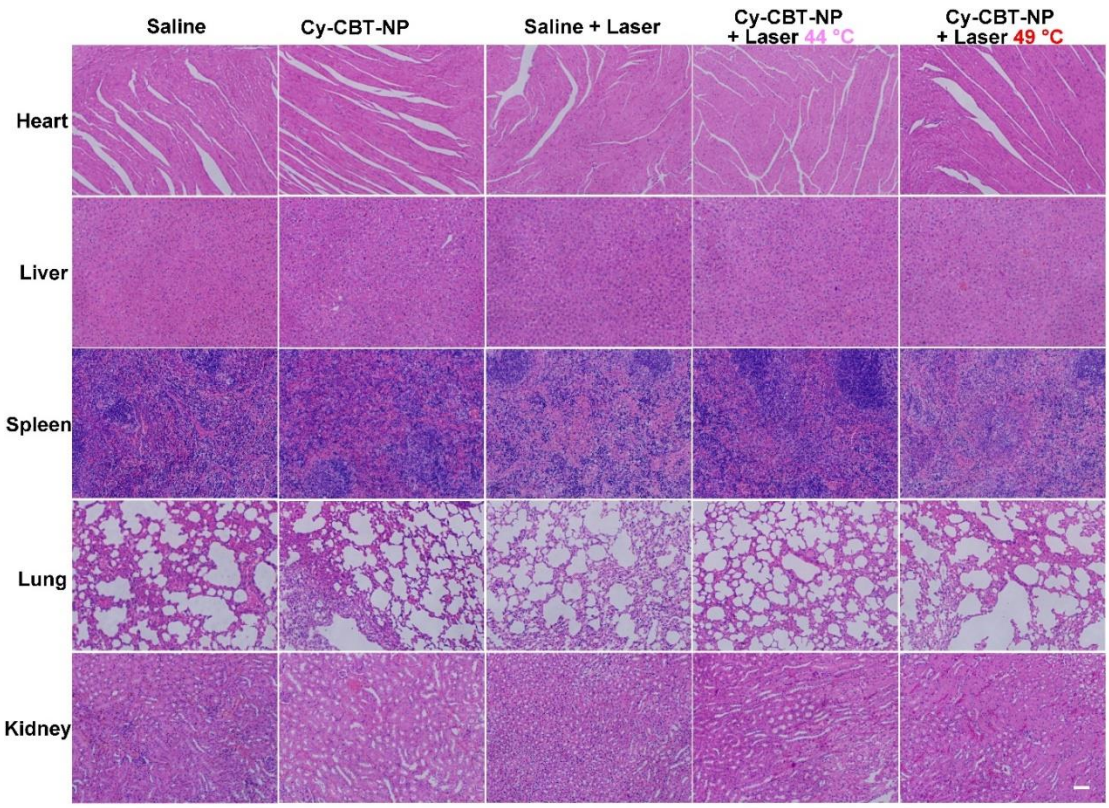


Figure S61. Images of H&E-stained sections of various major organs harvested from five groups of mice at day 18 post irradiation of the tumors. Scale bar: 100 μm.

Table S1. HPLC conditions for the purification of the compounds.

Compound	Mobile phase (A:B, 0.1% TFA)	Method	Flow (mL/min)	total running time (min)	retention time (min)
B	H_2O:CH_3OH	From 15:85 to 0:100	12	40	11.286
C	H_2O:CH_3OH	From 50:50 to 5:95	12	40	15.010
D	H_2O:CH_3CN	From 15:85 to 0:100	12	40	21.216
E	H_2O:CH_3CN	From 50:50 to 5:95	12	40	12.063
F	H_2O:CH_3CN	From 30:70 to 0:100	12	40	14.619
Cy-CBT	H_2O:CH_3CN	From 50:50 to 5:95	3	40	14.648
G	H_2O:CH_3CN	From 70:30 to 10:90	12	40	20.385
H	H_2O:CH_3CN	From 99:1 to 35:65	12	40	20.155
J	H_2O:CH_3OH	From 30:70 to 0:100	12	40	12.551
K	H_2O:CH_3CN	From 35:65 to 0:100	12	40	19.623
L	H_2O:CH_3CN	From 40:60 to 0:100	12	40	9.068
Cy-CBT-NP-Cleaved	H_2O:CH_3CN	From 50:50 to 5:95	3	40	11.782

4. References

1. White, E. H.; Worther, H.; Seliger, H. H.; McElroy, W. D. Amino Analogs of Firefly Luciferin and Biological Activity Thereof. *J. Am. Chem. Soc.* **1966**, *88*, 2015-2019.

2. Ye, Y.; Bloch, S.; Kao, J.; Achilefu, S. Multivalent Carbocyanine Molecular Probes: Synthesis and Applications. *Bioconjugate Chem.* **2005**, *16*, 51-61.