Supporting Information

Asymmetric small organic molecule-based NIR-II fluorophores for high performance tumor phototheranostics

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1. General information and experimental procedure

General information

Compound 1[S1] and compound 4[S2] were prepared according to previous protocols. F127 was obtained from Shanghai Yare Biotech, Inc. All starting materials used in the synthesis of DTPT were purchased from Sigma-Aldrich. Calcein AM/PI stain kit, NIH-3T3 cells, Hela cells and HeLa tumor-bearing mice were purchased from Jiangsu KeyGEN BioTECH Corp., Ltd. The morphology and size of NPs were determined by a HT7700 transmission electron microscope (TEM) and a particle size analyzer (Brookhaven Instruments), respectively. Absorption and emission spectra were obtained using a UV3600 UV/vis/NIR spectrophotometer (Shimadzu) and a FLSP920 fluorescence spectrophotometer (Edinburgh), respectively. The MTT experiments were conducted using a PowerWave XS/XS2 microplate reader (BioTek).

Experimental procedure

Preparation of DTPT NPs: DTPT NPs were prepared through one-step nanoprecipitation. In short, DTPT (1 mg) and F127 (10 mg) were dissolved in THF. Then the solution was quickly injected into the water under sonication. THF was removed by nitrogen flow. Then DTPT NPs were obtained after filtration and centrifugation.

Measurement of photothermal/photodynamic performance of NPs: To assess the photothermal performance, the various concentrations of DTPT NPs were exposed to 808 nm laser (1 W cm^{-2}). Besides, 100 μg/mL DTPT NPs was exposed to 808 nm laser with different power densities. The temperature changes and real-time thermal imaging of DTPT NPs were recorded by an infrared thermal imaging camera.

DPBF was chosen as a probe to monitor the $^1O_2$ generation. Briefly, the mixtures of DPBF and DTPT NPs in water were illuminated by 808 nm laser with various power densities. The changes of absorbance of DPBF were detected by using UV-vis-NIR spectrophotometer.

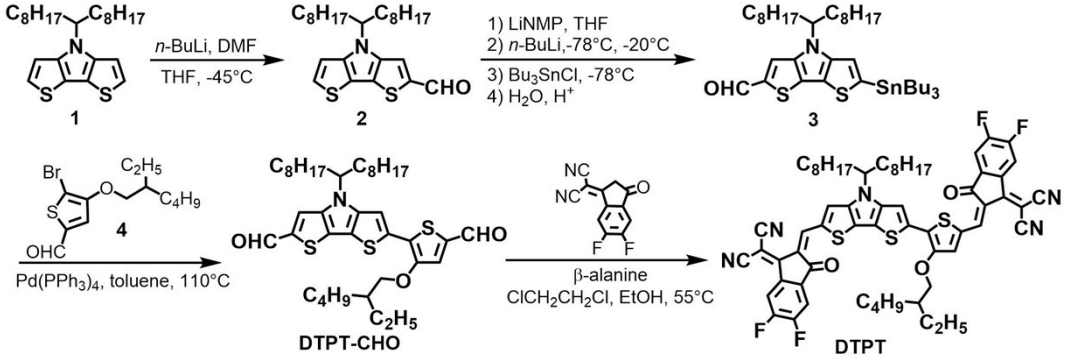
MTT assay: After 24 h of NIH-3T3 or Hela cells cultivation in DMEM medium with 10% FBS, various concentrations of DTPT NPs were co-cultured with the NIH-

3T3 or Hela cells for another 24 or 4 h. Moreover, one group of Hela cells was treated without laser illumination. Another group was treated with 808 nm laser (1 W/cm^2, 4 min) illumination. After further incubation of the cells with fresh culture medium, MTT assay was carried out. The absorbance was determined with a microplate reader.

In vivo NIR-II imaging: The in vivo NIR-II imaging performance of DTPT NPs was studied using Hela subcutaneous xenograft tumor mouse model by tail intravenous injection of NPs (2 mg/mL, 150 μL). At specific time points, NIR-II images were acquired utilizing an in vivo NIR-II imaging system (E_x = 808 nm).

In vivo tumor treatment: All in vivo mice therapy investigations were studied with the permit from the Animal Ethics Committee of Simcere BioTech Corp., Ltd., and executed in Jiangsu KeyGEN BioTECH Corp., Ltd. Four groups (4 mice each) of mice were given differently treatments: (1) PBS (150 μL), (2) PBS (150 μL) and 808 nm laser irradiation (1 W/cm^2, 10 min), (3) DTPT NPs (1 mg/mL, 150 μL), (4) DTPT NPs (1 mg/mL, 150 μL) and 808 nm laser (1 W/cm^2, 10 min). The changes of tumor volume, tumor weight, and mice body weight were monitored to evaluate the in vivo phototherapeutic effect of DTPT NPs. After treatment, H&E staining of major organs and tumor were carried out by using standard techniques for histology studies.

2. Synthetic procedures



Scheme S1. Synthesis of DTPT.

Synthesis of Compound **2**:

Compound **1** (3.0 g, 7.2 mmol) in anhydrous THF (80 mL) was placed at -45°C under nitrogen for 10 min, then *n*-BuLi (3.0 mL, 7.2 mmol) was added slowly. After stirring for 1.5 h, N,N-dimethylformamide (1.1 g, 14.4 mmol) was then added. The

solution was further stirred for 1 h at room temperature. After extraction and washing, the solvent was evaporated, and compound **2** could be obtained via column chromatography as a yellow solid (3.0 g, 93%). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.88 (s, 1H), 7.67 (s, 1H), 7.36 (d, 1H), 7.03 (d, 1H), 4.25 (m, 1H), 2.02 (m, 2H), 1.86 (m, 2H), 1.25-1.14 (m, 22H), 1.08-1.00 (m, 2H), 0.84 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 183.20, 148.68, 143.56, 140.12, 128.46, 123.68, 120.35, 115.30, 111.85, 60.41, 35.29, 31.95, 29.47, 29.40, 29.32, 26.74, 22.80, 14.27.

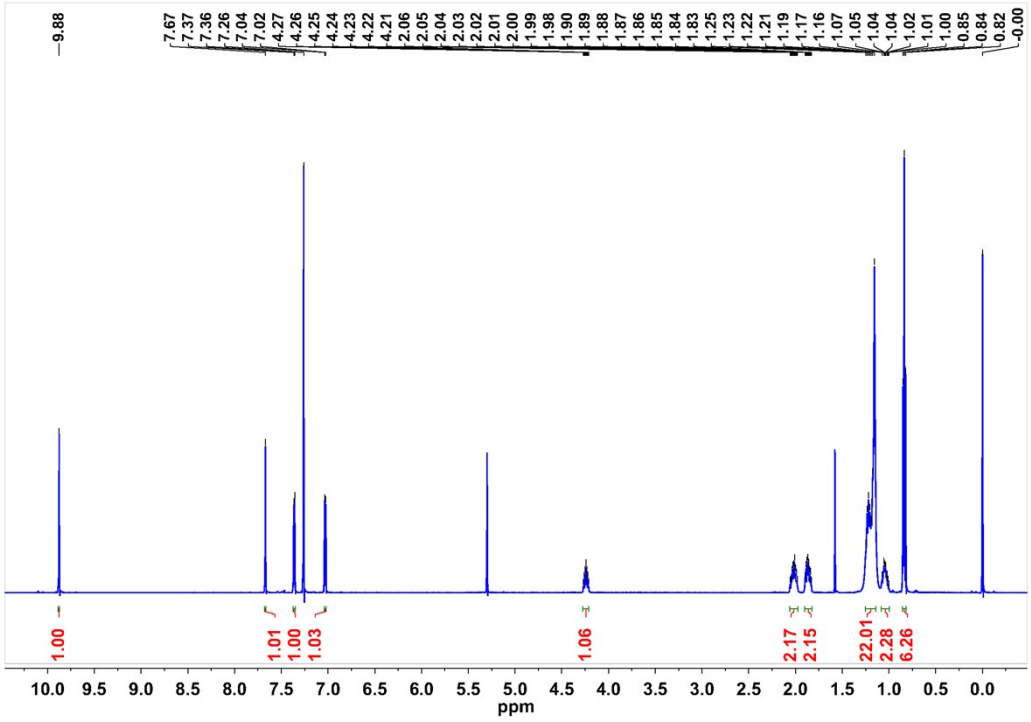


Fig. S1 ^{1}H NMR spectrum of **2**.

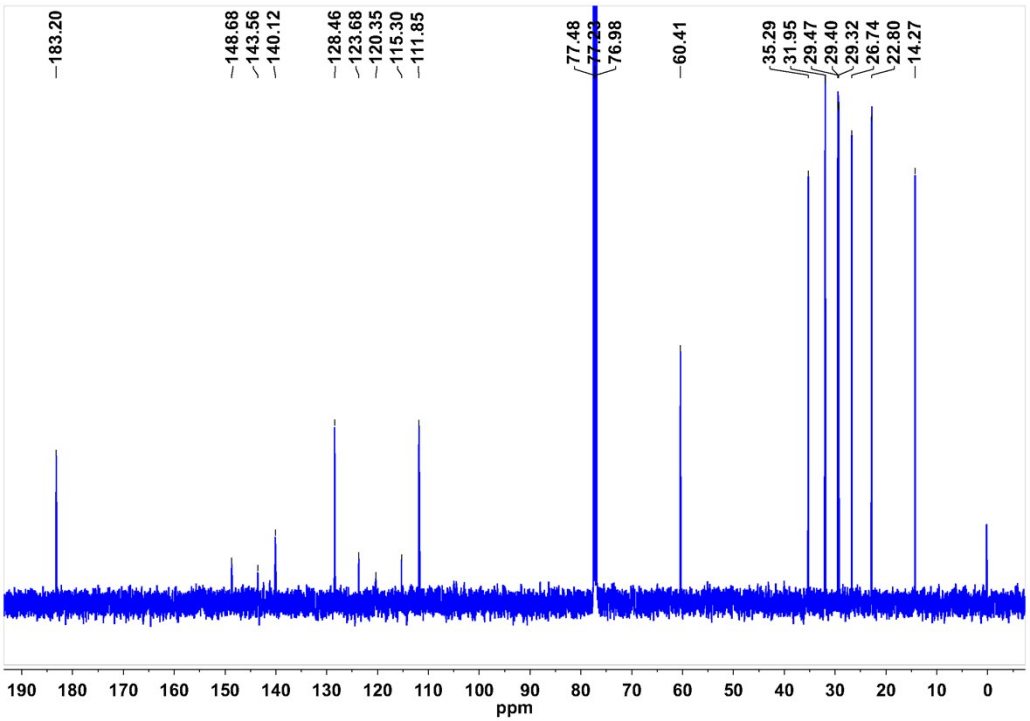


Fig. S2 ^{13}C NMR spectrum of **2**.

Synthesis of Compound **3**:

N-methylpiperazine (858.6 mmL, 7.7 mmol) in anhydrous THF (80 mL) was placed at -78°C under nitrogen for 10 min, then *n*-BuLi (3.2 mL, 7.7 mmol) was added slowly. After stirring for 30 min, compound **2** (3 g, 6.7 mmol) was then added. After further stirring for 30 min, *n*-BuLi (3.2 mL, 7.7 mmol) was added. After stirring for 2 h at -20°C, Bu_3SnCl (2.2 mL, 8.1 mmol) in anhydrous THF (3 mL) was added slowly at -78°C. The solution was further stirred for 2 h at room temperature. Then HCl (50 mL) was added at 0°C for 2 min and neutralized with Na_2CO_3 solution. After extraction and washing, the solvent was evaporated, and a viscous liquid was obtained, and directly used in the following step without further purification.

Synthesis of **DTPT-CHO**:

Compound **3** (1.2 g, 1.6 mmol), compound **4** (625.7 mg, 2.0 mmol), $Pd(PPh_3)_4$ (37.8 mg, 32.7 μmol) were added in anhydrous toluene (12 mL). The solution was stirred for 6 h at 110°C under nitrogen, and the solvent was evaporated. DTPT-CHO could be obtained via column chromatography as an orange viscous liquid (950 mg, 85%). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.87 (s, 1H), 9.77 (s, 1H), 7.65 (s, 1H), 7.50 (s, 1H), 7.44 (s, 1H), 4.24 (m, 1H), 4.16-4.10 (m, 2H), 2.02 (m, 2H), 1.88 (m, 3H), 1.60

(m, 4H), 1.40-1.32 (m, 4H), 1.23-1.13 (m, 22H), 1.06 (m, 2H), 0.99 (m, 3H), 0.92 (m, 3H), 0.80 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 182.98, 181.81, 154.08, 147.99, 144.24, 140.93, 136.74, 136.68, 129.09, 126.71, 123.50, 123.32, 116.27, 109.29, 74.46, 60.49, 39.93, 35.18, 31.88, 30.73, 29.42, 29.34, 29.27, 29.26, 26.73, 24.16, 23.19, 22.73, 14.25, 14.18, 11.36.

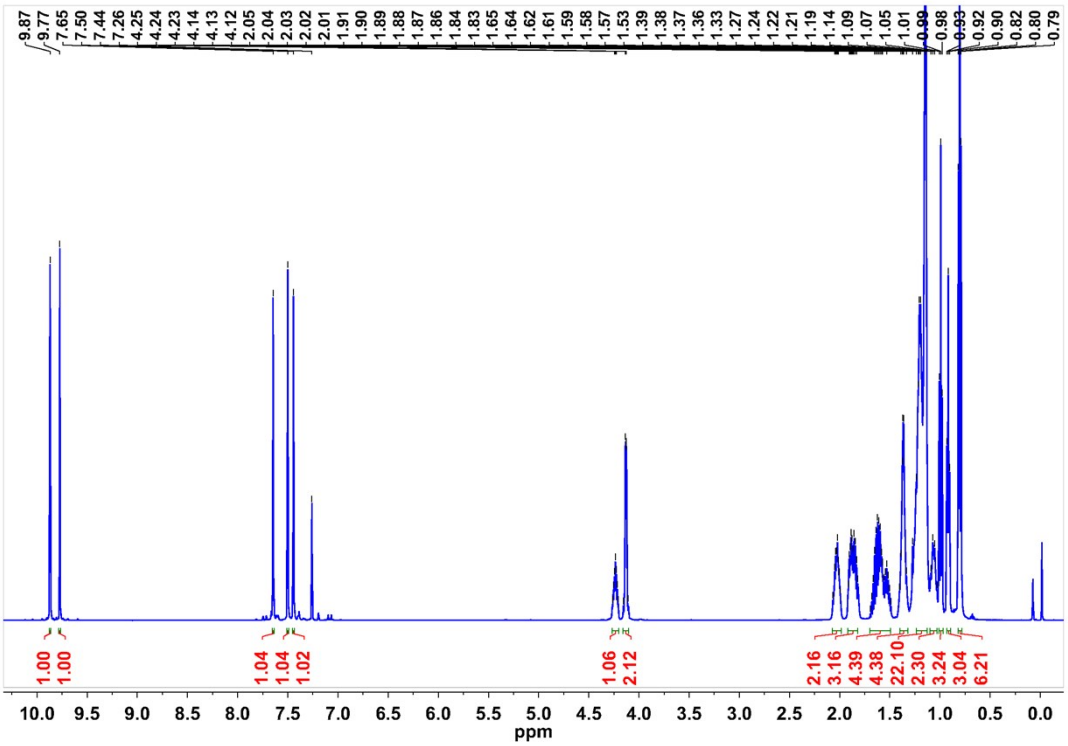


Fig. S3 ^{1}H NMR spectrum of **DTPT-CHO**.

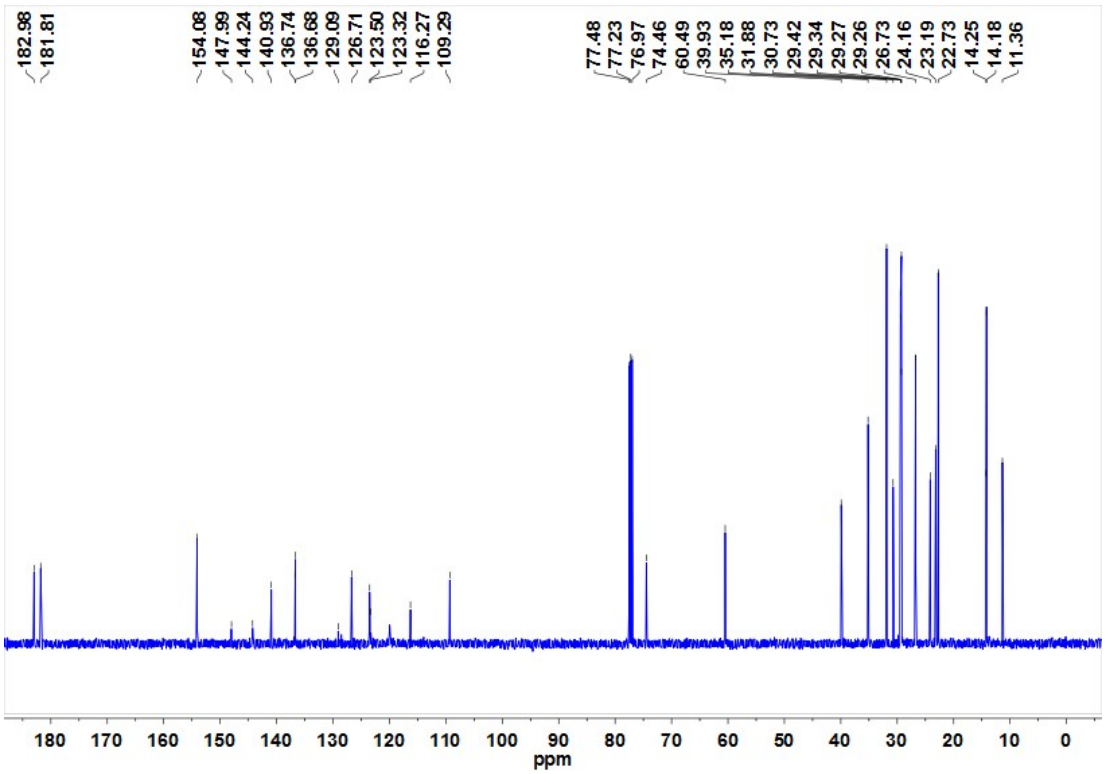


Fig. S4 ^{13}C NMR spectrum of **DTPT-CHO**.

Synthesis of **DTPT**:

Compound **DTPT-CHO** (200 mg, 292 μmol), 1,2-dichloroethane (20 mL), β-alanine (2.6 mg, 29.2 μmol), EtOH (4 mL), and 2-(5,6-difluoro-3-oxo-2,3-dihydro -1H-inden-1-ylidene) malononitrile (269 mg, 1.2 mmol) were mixed and stirred at 55°C. Then the solvent was evaporated. **DTPT** could be obtained via column chromatography as a dark-blue solid (250 mg, 77%). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 8.87 (s, 1H), 8.73 (s, 1H), 8.51 (m, 2H), 7.79-7.63 (m, 3H), 7.54 (d, 2H), 4.30-4.18 (m, 3H), 2.07 (m, 2H), 1.94 (m, 3H), 1.67 (m, 4H), 1.45-1.38 (m, 4H), 1.30-1.17 (m, 22H), 1.12 (m, 2H), 1.05 (m, 3H), 0.96 (m, 3H), 0.81 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 186.30, 185.90, 158.52, 158.50, 157.78, 157.77, 157.75, 156.10, 153.44, 140.66, 138.50, 137.93, 136.99, 136.97, 136.91, 136.74, 136.71, 136.67, 136.34, 134.87, 134.64, 134.62, 134.61, 134.58, 133.35, 129.82, 125.97, 122.55, 120.45, 119.82, 115.36, 115.19, 114.98, 114.92, 114.83, 114.37, 114.33, 112.86, 112.70, 112.54, 110.12, 75.15, 70.42, 68.07, 61.25, 40.03, 35.12, 31.98, 30.75, 29.61, 29.52, 29.37, 29.36, 27.08, 24.30, 23.28, 22.82, 14.35, 14.26, 11.49. MALDI-TOF MS (*m*/*z*): $[M+H]^+$ calcd. for $C_{34}H_{35}NO_4S_4$, 1107.3873; found, 1107.3861.

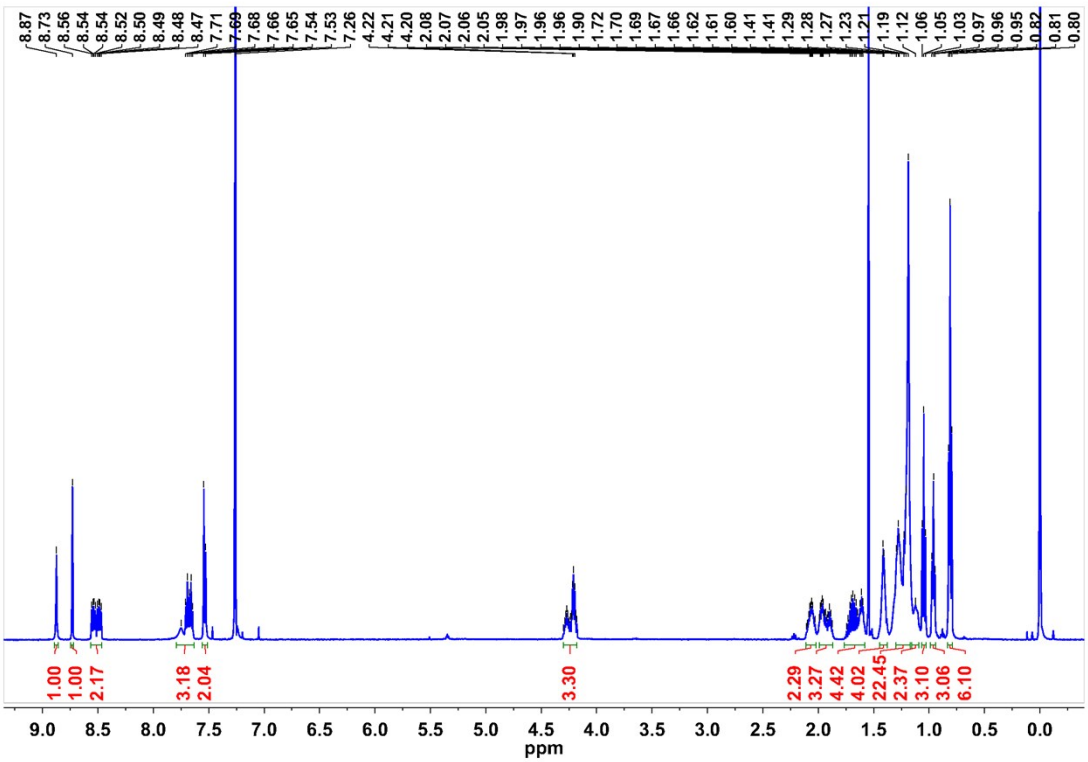


Fig. S5 ^{1}H NMR spectrum of **DTPT**.

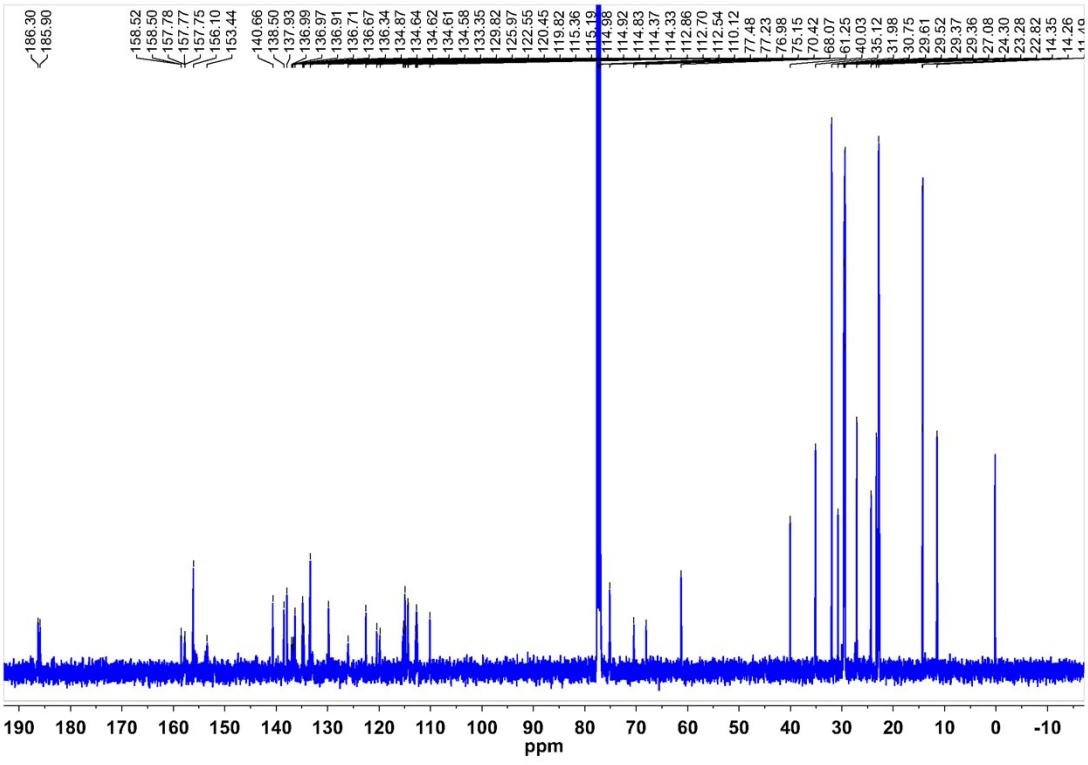


Fig. S6 ^{13}C NMR spectrum of **DTPT**.

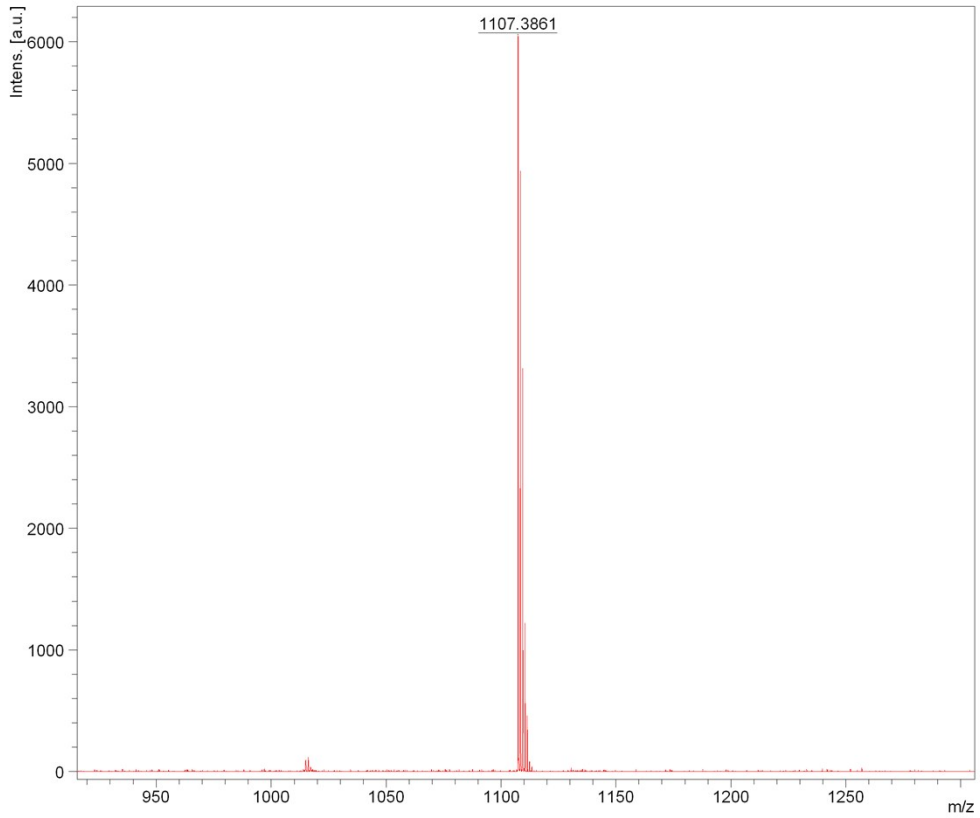


Fig. S7 MALDI-TOF MS plot of **DTPT**.

3. *Molar absorption coefficient of DTPT NPs*

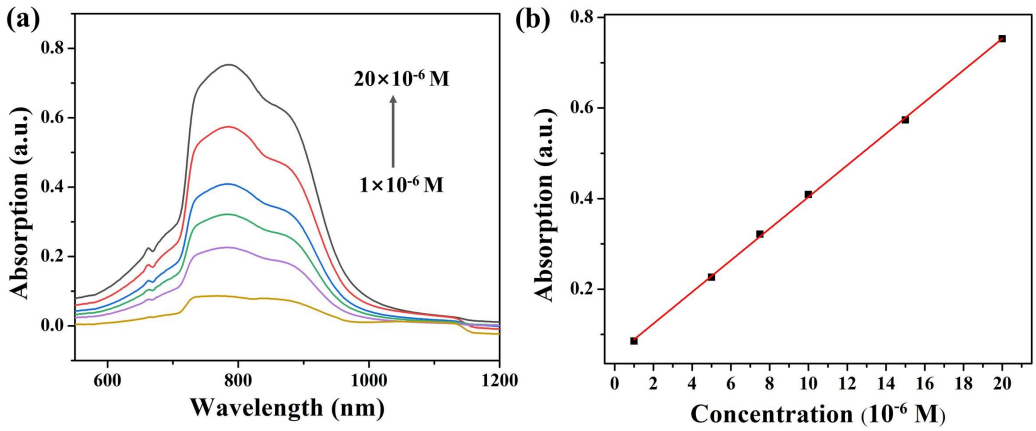


Fig. S8 (a) Absorption curves of DTPT NPs aqueous solution at different concentrations. (b) Linear absorbance versus concentration obtained from (a).

4. *Fluorescence quantum yield measurements of DTPT NPs*

The fluorescence quantum yield (QY) of DTPT NPs was measured utilizing dye IR-26 as a reference, whose QY has been reported as 0.5% in 1,2-dichloroethane

(DCE)[S3]. The QY value of DTPT NPs was measured based on four concentrations with OD of 0.09105, 0.07323, 0.05896 and 0.04891 at 808 nm. The integrated total emission intensity was in the 900-1400 nm region. Two slopes, one obtained for the IR-26 reference and the other from DTPT NPs, were used in the calculation of the quantum yield of DTPT NPs in water, according to the following equation[S4]:

$$QY_{\text{sample}} = QY_{\text{ref}} \times \frac{slope_{\text{sample}}}{slope_{\text{ref}}} \times \frac{\eta^2_{\text{sample}}}{\eta^2_{ref}}$$

where η_{sample} and η_{ref} are the refractive index of water and DCE, respectively.

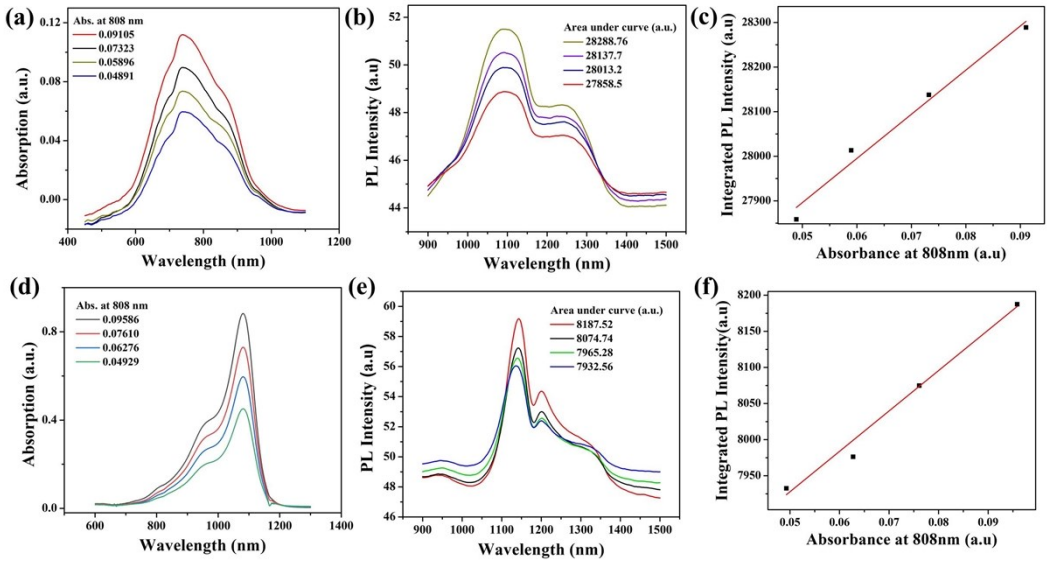


Fig. S9 (a) UV-Vis-NIR absorption spectra of DTPT NPs in water with different concentrations. (b) NIR emission spectra of DTPT NPs in water with different concentrations under 808 nm excitation. Area under curve (AUC) in the emission spectra for each solution was then calculated and listed in the right of the figure. (c) For all DTPT NPs solutions, their absorbance values were then plotted versus AUC, and fitted into a linear function. (d) UV-Vis-NIR spectra of IR-26 in DCE with different concentrations. (e) NIR emission spectra of IR-26 in DCE with different concentrations under 808 nm excitation. AUC in the emission spectra for each solution was then calculated and listed in the right of the figure. (f) For all IR-26 DCE solutions, their absorbance values were then plotted versus AUC, and fitted into a

linear function.

5. Photothermal conversion efficiency measurement of DTPT NPs

For calculating the photothermal conversion efficiency (PCE) of DTPT NPs, DTPT NPs was irradiated by an 808 nm laser for 10 min, then the laser was shut off. After reaching a plateau temperature, the solution was naturally cooled to room temperature without NIR laser irradiation. And the temperature was recorded by IR thermal camera. Deionized water of the same volume was used as control.

The PCE was calculated by equation[S5]:

$$PCE = \frac{\mathrm{hs}(T_{\max} - T_{\mathrm{surr}}) - Q_0}{I(1-10^{-A_\lambda})} \qquad (1)$$

hs can be calculated by the follow equation:

$$\mathrm{hs} = \frac{\sum m_i C_{P.i}}{\tau_s} \qquad (2)$$

$$\tau_s = \frac{\mathrm{t}}{-\ln\theta} \qquad (3)$$

$$\theta = \frac{T - T_{\mathrm{surr}}}{T_{\max} - \mathrm{T}_{\mathrm{surr}}} \qquad (4)$$

$$Q_0 = hs(T_{\max} - T_{surr}) \qquad (5)$$

h represents the heat transfer coefficient,

s represents the sample container surface area,

T_{max} represents the steady-state maximum temperature,

T_{surr} represents the ambient room temperature,

T represents instantaneous temperature during cooling,

t represents the time it takes by T cooled to room temperature,

C approximate to the specific heat capacity of water,

m represented the mass of the solution (g),

Q_0 represents the energy input by the same solvent without NPs in the same quartz cuvette after same laser irradiation.

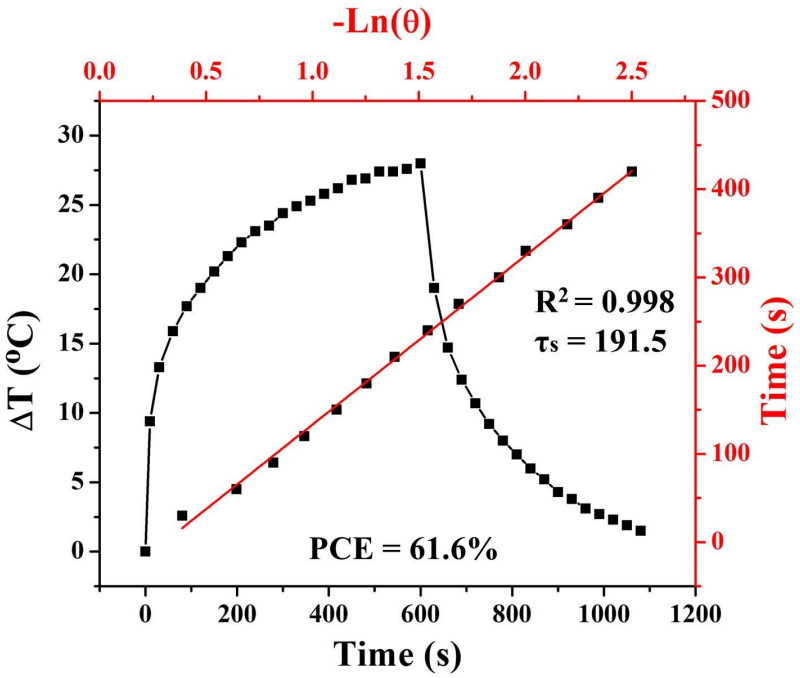


Fig. S10 PCE measurement of DTPT NPs.

6. *Photodynamic measurement of DTPT NPs*

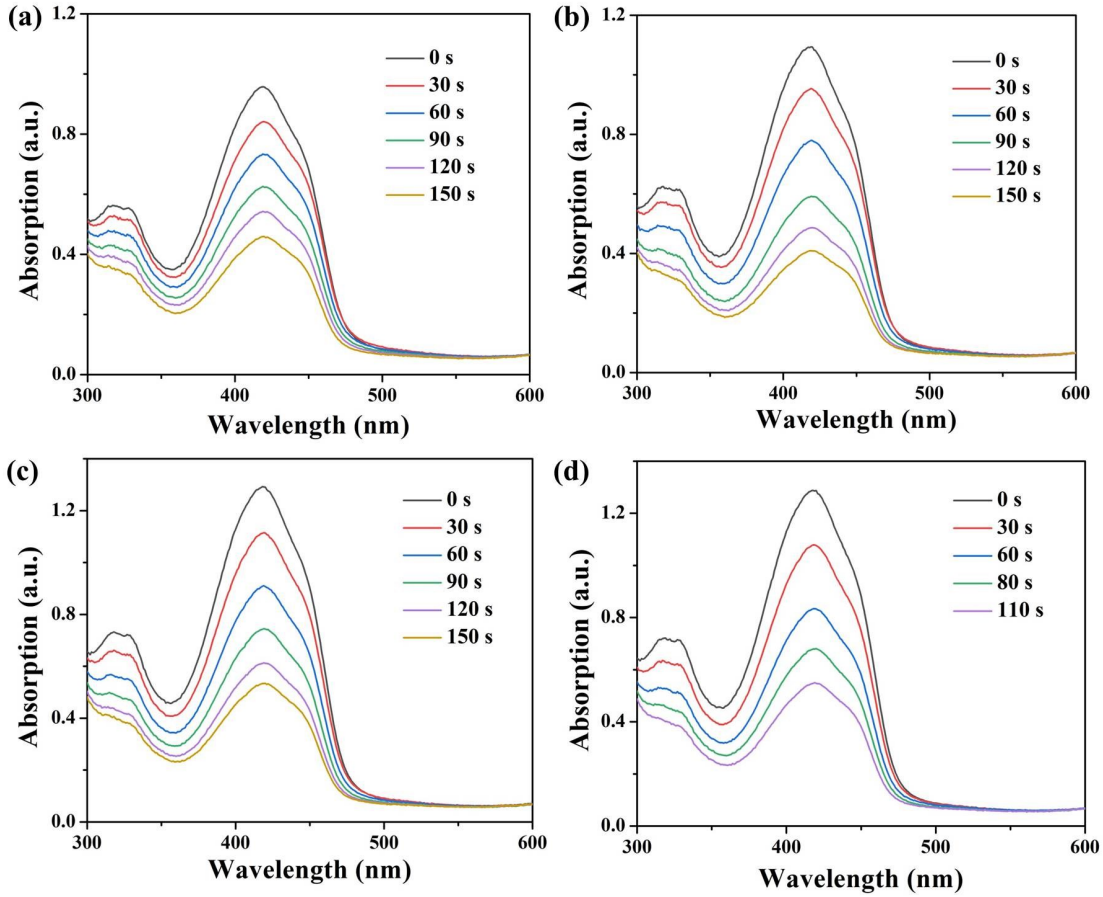


Fig. S11 Absorption spectra of the mixed aqueous solution of DTPT NPs and DPBF

under 808 nm laser irradiation with different power densities (a) 0.25, (b) 0.5, (c) 0.75 and (d) 1 W/cm^2.

7. The blood metabolism of NPs

The DTPT NPs (2 mg/mL, 300 μL) were intravenously injected into healthy mice (n = 3). At 10 min, 30 min, 1, 3, 6, 12, 24, and 48 h p.i., 500 μL of blood was collected and mixed with 200 μL of 10 mM EDTA solution. The DTPT concentration in plasma was measured by comparing its absorbance spectrum to a predetermined standard curve. The blood circulation half-life curve was fitted with a single-compartment pharmacokinetic model.

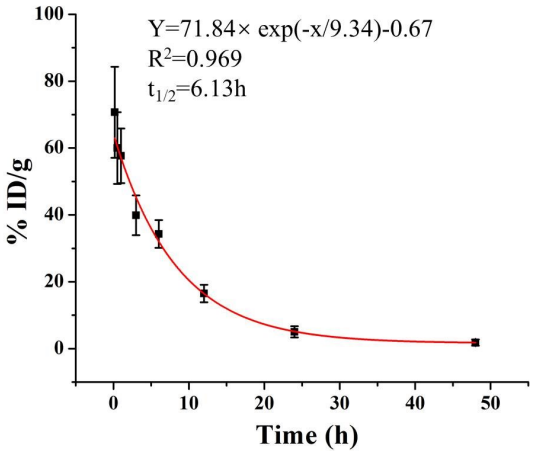
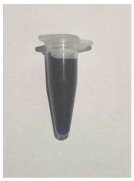


Fig. S12 The blood metabolism of NPs.

8. Penetration depth measurement of DTPT NPs



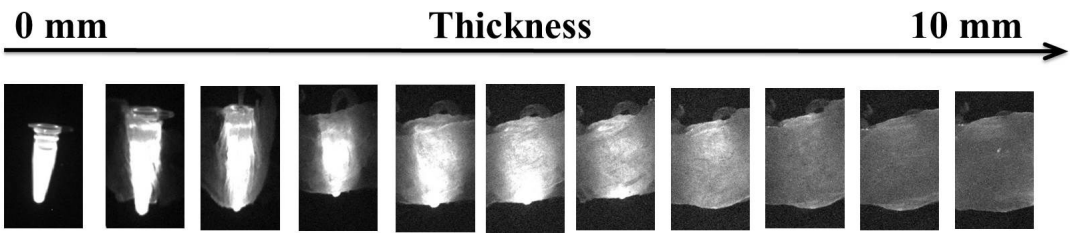


Fig. S13 Penetration depth measurement of DTPT NPs (1 mg/mL) in a simulated deep-tissue setting (chicken-breast tissues).

9. Ex vivo NIR-II fluorescence quantification of tumors and major organs

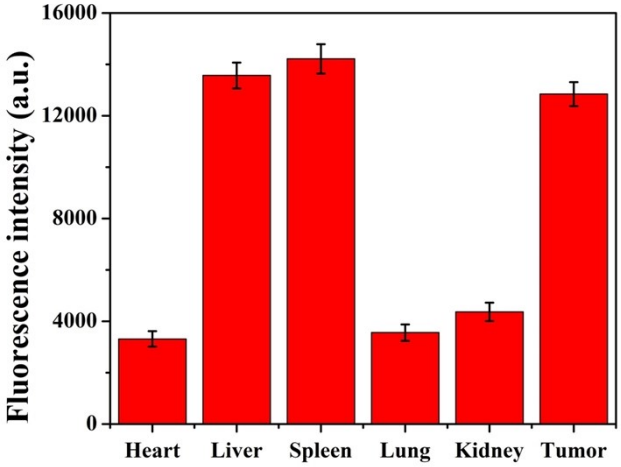


Fig. S14 Semi-quantitative biodistribution analysis based on fluorescence intensity of tumors and major organs.

10. Temperature profiles of tumor under laser illumination

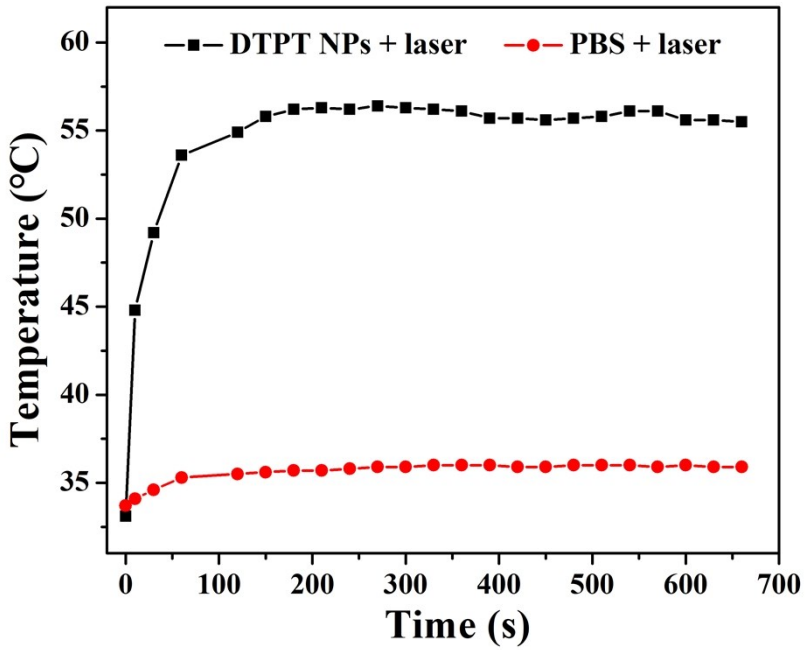


Fig. S15 Temperature profiles of tumor under laser illumination.

11.H&E stained images of tumors

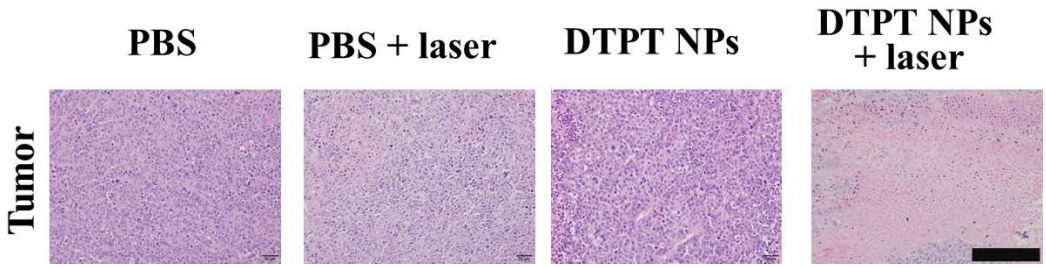


Fig. S16 H&E stained images of tumors (Scale bar: 200 μm).

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