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Supporting Information

Clearly Fluorescent Delineating ER+ Breast Tumor Incisal Edge and Identifying Tiny Metastatic Tumor Foci at High Resolution

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1. Materials and instrumentation

Except especially explained, all chemical reagents and solvents used in this study were A.R. grade and directly purchased form manufacturers without further purification. General reagents were purchased form Anergy Chemical or Shanghai Bidet Pharmaceutical Technology Co. The thin layer chromatography (TLC) and silica gel (200 - 300 mesh) in flash column chromatography were brought form Qingdao Ocean Chemicals. Tamoxifen, the inhibitor of ER, was obtained from Macklin. Estrogen Receptor alpha was purchased from Solarbio Co. The Common reagents for cell culture including culture medium, fetal bovine serum (FBS), phosphate buffer (PBS), and penicillin-streptomycin (P/S) were obtained from Gibco. Human normal mammary cells (MCF 10A) and human breast cancer cells (MCF-7 and MDA-MB-231) were purchased from Wuhan Punosai Life Sciences Co.

In TLC analysis, compounds were visualized under irradiation of UV light (254 nm). ^{1}H-NMR, ^{13}C-NMR spectra were acquired on AYANCE NEO 400 spectrometer. Chemical shifts were internally referenced to tetramethylsilane (TMS). All data processing was carried out using MestReNova. Mass spectrometric (ESI-MS) data was detected by LCMS-9030. Absorption and emission spectra for NB-TAM were performed with a HORIBA Canada Inc UV Fluorescence Integrated Spectrometer (Duetta). Fluorescence images were detected by an Olympus FV3000 or a Leica Stellaris 5 WLL confocal laser scanning microscope. Small animals' fluorescence imaging was carried out using NightOWL II LB983 iv vivo imaging system.

2. Experimental methods

2.1. Synthesis of NB-TAM

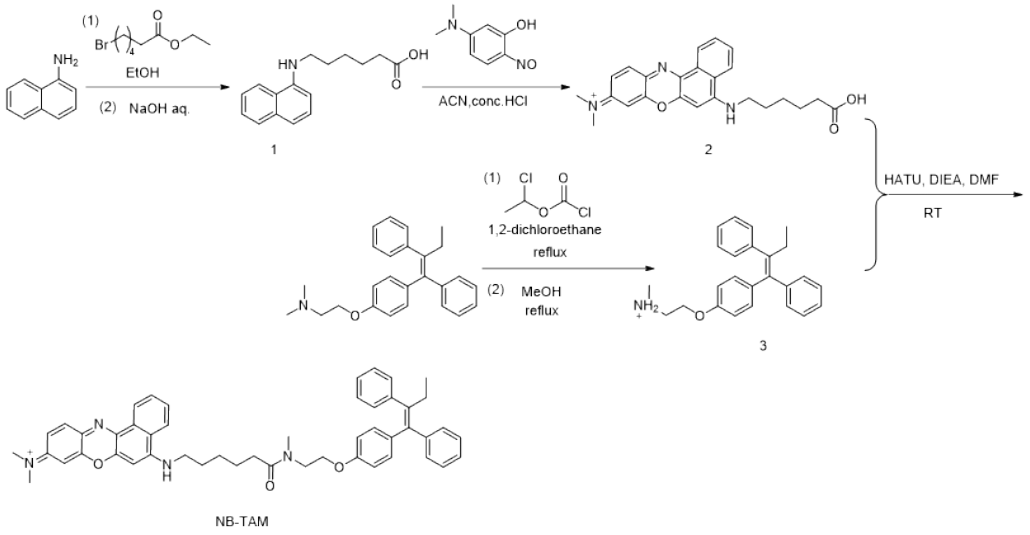

Figure S1. Synthetic routine of NB-TAM.

Synthesis of compound 1

Firstly, ethyl 6-bromohexanoate (38.41 mmol, 8.57 g) was weighed in a 50 mL three-necked flask equipped with a thermometer, anhydrous ethanol (17.5 mL) was added, 1-naphthylamine (34.92 mmol, 5 g) was added at room temperature with stirring. The mixture was refluxed for 12 h under the protection of N_2. The reaction solution turned brown as the reaction progressed. This reaction was monitored through TLC. After the reaction, the mixture was cooled to room temperature, and the solvent was removed to obtain the brown oily crude product.

Then 1,4-dioxane (87.3 mL) was taken to dissolve the brown oily crude (11.5 g) completely, then 2M NaOH (0.175 mol, 6.98 g) aqueous solution was added and stirred at room temperature for 3h, 1,4-dioxane was removed, the pH was adjusted to 2-3, and it was extracted with ethyl acetate, the product was in the ethyl acetate layer. The organic phase was washed twice with saturated saline, dried with anhydrous sodium sulfate, filtered and concentrated by spinning. Purification by column chromatography (DCM: MeOH=20:1, v/v) gave brown solid compound 1 (7.75 g, 86 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 – 7.72 (m, 2H), 7.46 – 7.37 (m, 2H), 7.33 (t, J = 7.9 Hz, 1H), 7.21 (s,

1H), 6.58 (dd, *J* = 7.6, 1.0 Hz, 1H), 3.24 (t, *J* = 7.1 Hz, 2H), 2.37 (t, *J* = 7.4 Hz, 2H), 1.81 – 1.65 (m, 4H), 1.56 – 1.44 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 179.48, 143.43, 134.30, 128.67, 126.63, 125.67, 124.65, 123.37, 119.77, 117.27, 104.33, 70.33, 50.81, 43.96, 33.89, 29.38, 29.03, 26.75, 24.47, 15.17. HRMS: m/z calcd for $[M-H]^-$: 256.1343, found 256.1344.

Synthesis of compound 2

Under an ice bath, compound 1 (3.89 mmol, 1 g) in acetonitrile (10 mL) was added 2-nitroso-3-(dimethylamino) phenol (6.61 mmol, 1.1 g), and then concentrated hydrochloric acid (0.6 mL) was slowly added dropwise to the reaction system. After 10 min, the reaction was warmed up to 85 °C for 24 h. The reaction solution turned blue as the reaction progressed. This reaction was monitored through TLC. After the reaction was completed, the solvent was removed under reduced pressure, and the crude product was purified by silica column chromatography (DCM: MeOH= 10/1, v/v) to obtain dark blue powder (1.13 g, 72%). ^{1}H NMR (400 MHz, MeOD) δ 9.00 (d, *J* = 7.7 Hz, 1H), 8.45 (bs, 1H), 8.06 – 7.84 (m, 3H), 7.31 (d, *J* = 6.9 Hz, 1H), 7.19 – 6.75 (m, 2H), 3.78 (bs, 2H), 3.39 (s, 6H), 2.44 (bs, 2H), 1.99 (bs, 2H), 1.81 (bs, 2H), 1.64 (bs, 2H). ^{13}C NMR (101 MHz, MeOD) δ 177.07, 159.45, 156.88, 153.06, 148.91, 135.35, 133.60, 132.92, 132.34, 131.10, 130.95, 125.62, 124.60, 123.71, 116.14, 96.93, 94.35, 49.85, 45.49, 41.16, 34.47, 29.16, 27.30, 25.33. HRMS: m/z calcd for $[M]^+$: 404.1969, found 404.1968.

Synthesis of compound 3

Tamoxifen (1.16 mmol, 0.43 g) was completely dissolved in 1,2-dichloroethane (8 mL) and 1-chloroethyl chloroformate (1.27 mmol, 0.18 g) was added at 0 °C. The mixture was stirred for 15 min and then heated under reflux for 24 h. The reaction was monitored by TLC, which showed that most of the substance was converted into intermediates with higher R_f. The solvent was removed under reduced pressure and the oily residue was completely dissolved in methanol, then the mixture was refluxed for 4 h. During the reaction, the formation of products with lower R_f values was seen. Methanol was removed and the white solid compound 3 (0.368 g, 89%) was purified by column chromatography (DCM: MeOH=20:1, v/v). ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 9.14 (s, 2H), 7.38 (t, *J* = 7.1 Hz, 2H), 7.29 (d, *J* = 6.9 Hz, 1H), 7.24 – 7.16 (m, 4H), 7.16 – 7.08 (m, 2H), 6.77 (d, *J* = 8.1 Hz, 2H), 6.66 (d, *J* = 8.2 Hz, 2H), 4.11 (s, 2H), 3.21 (s, 2H), 2.54 (s, 3H), 2.37 (dd, *J* = 13.9, 6.5 Hz, 3H), 0.84 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) *δ* 155.62, 143.13, 141.68, 140.90, 137.73, 135.59, 131.34, 129.31, 128.91, 128.27, 127.94, 126.68, 126.20, 113.59, 62.96, 47.16, 32.64, 28.48, 13.28. HRMS: m/z calcd for $[M+H]^+$: 358.2166, found 358.2172.

2.2. Measurement of absolute fluorescence quantum yield

The absolute fluorescence quantum yield of probe NB-TAM in PBS was measured by an absolute luminescence quantum yield spectrometer (HAMAMATSU C11347). The absorbance at 630 nm of NB-TAM was adjusted to be about 0.2 and the excitation wavelength was set as 630 nm.

2.3. Density functional theory simulation

In order to elucidate the "Off-On" mechanism of the probe NB-TAM towards ERs, the ground state structure of the molecular conformation was optimised at the wB97XD/6-31g(d) level of theory using the DFT method, while the excited state structure was optimised at the B3LYP/TZVP level of theory applying the TD-DFT method. All calculations are processed with the polarizable-continuum model (PCM).

2.4. Fluorescence response of NB-TAM to human recombinant protein ERs

To evaluate the selective response of NB-TAM toward estrogen receptor, NB-TAM (5 μM) was incubated with human recombinant protein ERs (45 μg/mL) for 10 min at 37 ºC. As a control, the human recombinant protein ERs (45 μg/mL) was pretreated with ER inhibitor tamoxifen (25 μM) for 30 min before addition of NB-TAM (5 μM). Fluorescent spectra were recorded on a microplate reader with excitation at 630 nm and emission at 655-700 nm.

2.5. Cell culture and MTT assay

Human breast cancer cells (MCF-7, T-47D and MDA-MB-231) were cultured in Dulbecco's modified Eagle's medium Dulbecco with high glucose (DMEM, containing 4.5 g/L glucose, 4.0 mM L-glutamine, and 110 mg/L sodium pyruvate), supplemented with 10% fetal bovine serum (FBS; Gibco), and 1% penicillin/streptomycin (P/S; Gibco). Human breast cancer cells (ZR-75-1) and Human normal breast cells (HBL-100) were cultured in RPMI 1640 medium (containing D-glucose, L-glutamine, and sodium bicarbonate) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (P/S; Gibco). Human normal mammary cells (MCF 10A) were grown in in mammary epithelial cell growth medium (Lonza) supplemented with a mixture of bovine pituitary extract (BPE), human epidermal growth factor (hEGF), insulin, hydrocortisone, and gentamicin sulfate-amphotericin (GA-1000). All cells were cultured in a 5% CO_2 humidified incubator at 37 °C.

The MTT assay was employed to assess the cellular cytotoxicity of NB-TAM. MCF-7, MCF 10A and MDA-MB-231 cells at a density of 1×10^5 cells/mL in 100 μL medium were seeded into 96-well plates and cultured for 24 h. The number of cells was determined by cell counting chamber. After cell attachment, the cells were cultured in medium with various concentration of NB-TAM (2.5, 5, 10, 15, 20 μM) for 24 h. The cells without incubation with NB-TAM were used as control and wells filled with only 100 μL of DMSO was used as the blank. Then 100 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.5 mg/mL) prepared in cell culture medium was added to each well and substituted the previous media. The plates were incubated for another 4 h. The medium was then carefully removed and 100 μL of DMSO was added in each well to dissolve the formazan. Optical density (OD) was determined by a microplate reader (Tristar 5 LB942, Berthold) at 570 nm. The relative cell viability (100%) was calculated using the following equation:

Cell viability (%) = $(OD_{NB\text{-}TAM} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\%$.

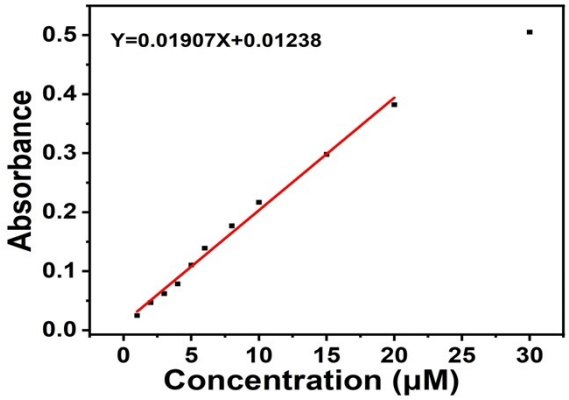

Figure S2. Absorbances at 630 nm of NB-TAM with different concentrations.

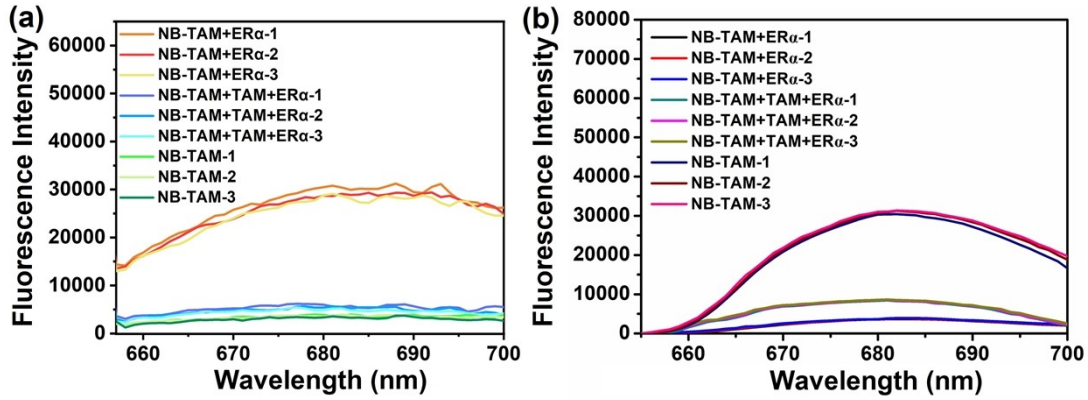

Figure S3. Fluorescence spectra of NB-TAM (5 μM) towards human recombinant protein ERα (a) or ERβ (b) (45 μg/mL) in the presence or absence of tamoxifen (25 μM).

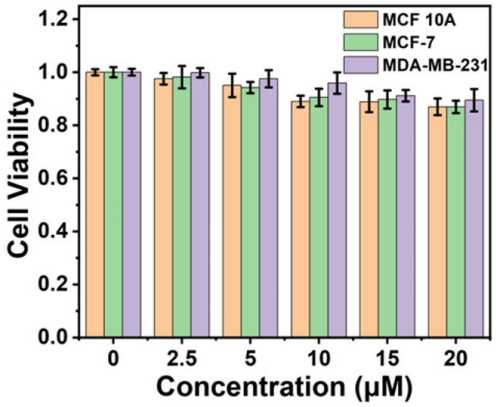

Figure S4. Viabilities of cells MCF 10A, MCF-7 and MDA-MB-231 after treatment with NB-TAM.

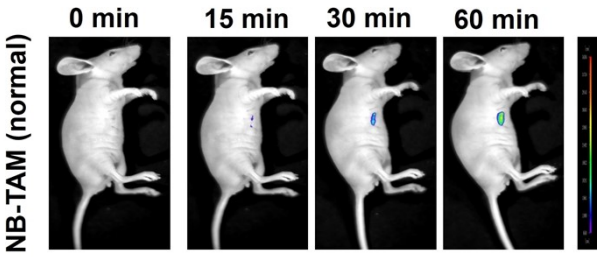

Figure S5. Time-dependent fluorescent images of normal mice not bearing tumor after peritumoral administration of NB-TAM (10 nmol). λ_{ex} = 630 nm, λ_{em} = 680-720 nm.

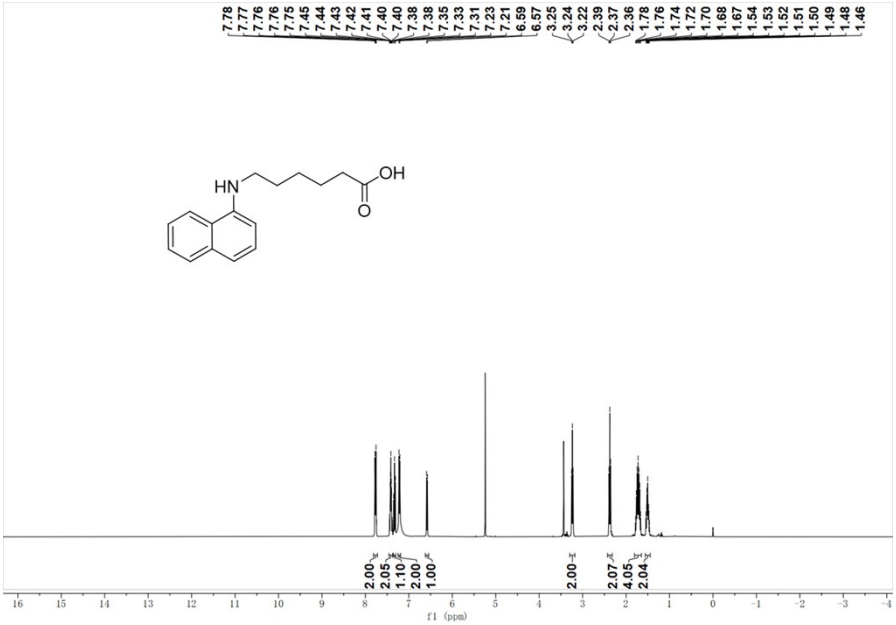

Figure S6. ^{1}H NMR spectrum of Compound 1 (400 MHz, $CDCl_3$).

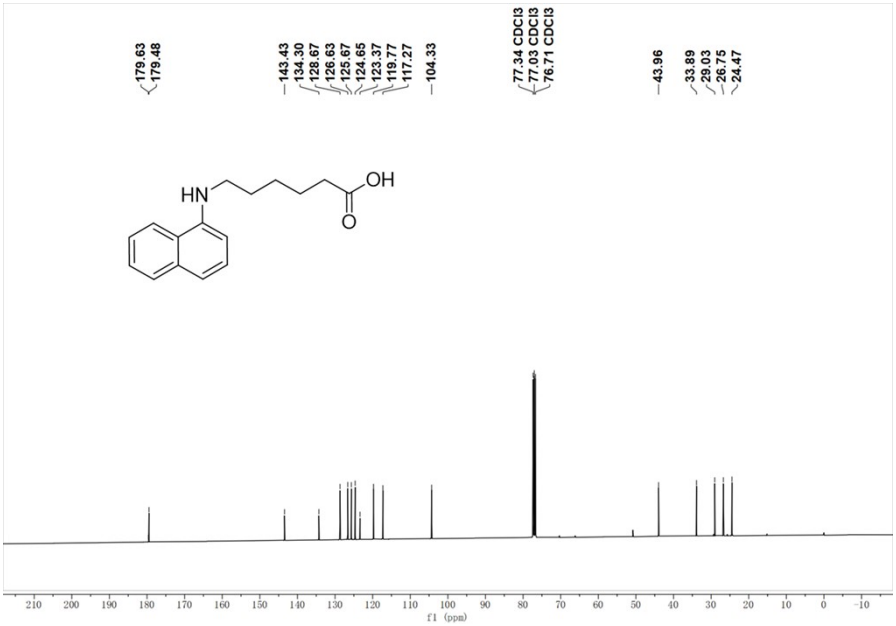

Figure S7. ^{13}C NMR spectrum of Compound 1 (101 MHz, $CDCl_3$).

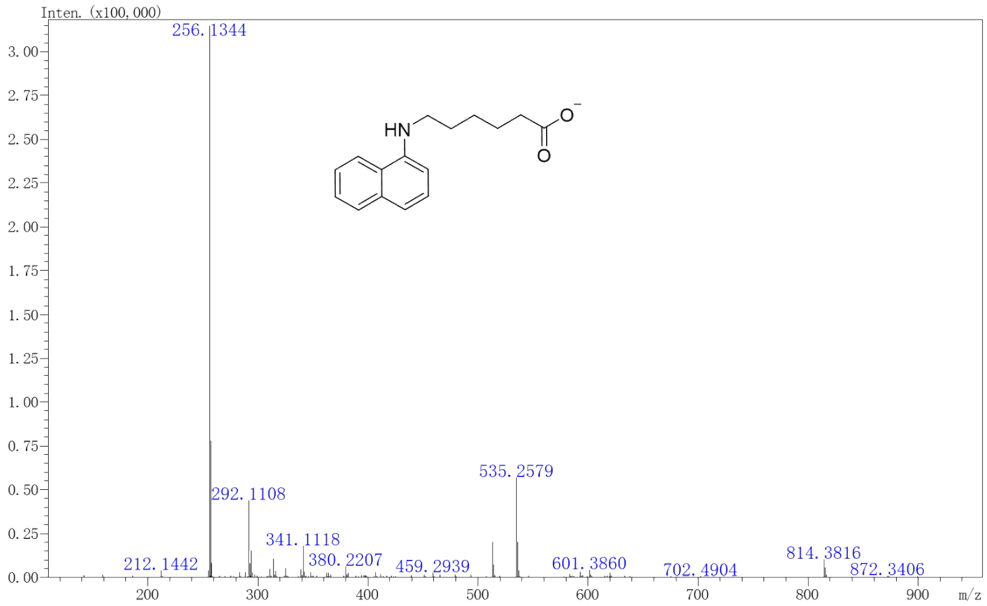

Figure S8. HRMS spectrum of Compound 1.

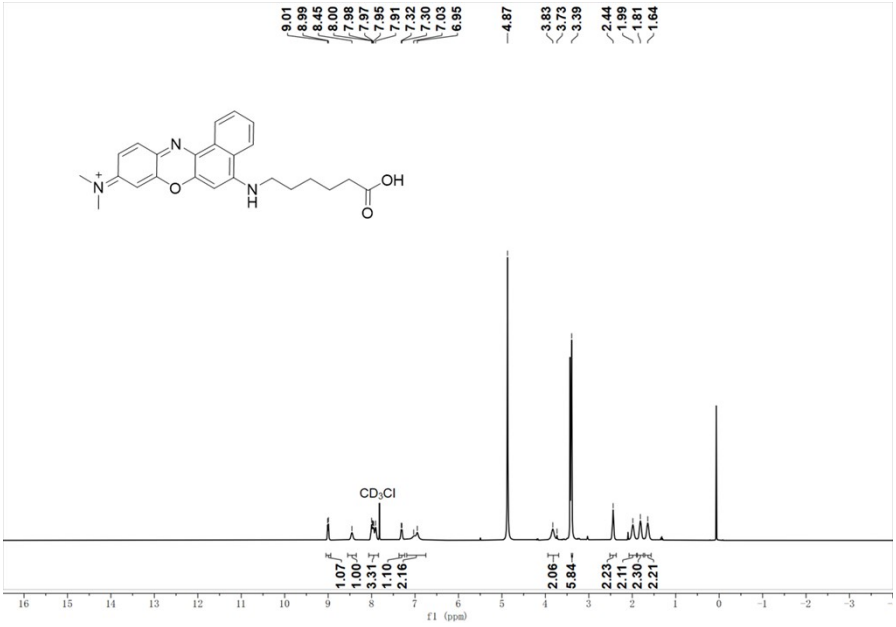

Figure S9. [1]H NMR spectrum of Compound 2 (400 MHz, MeOD).

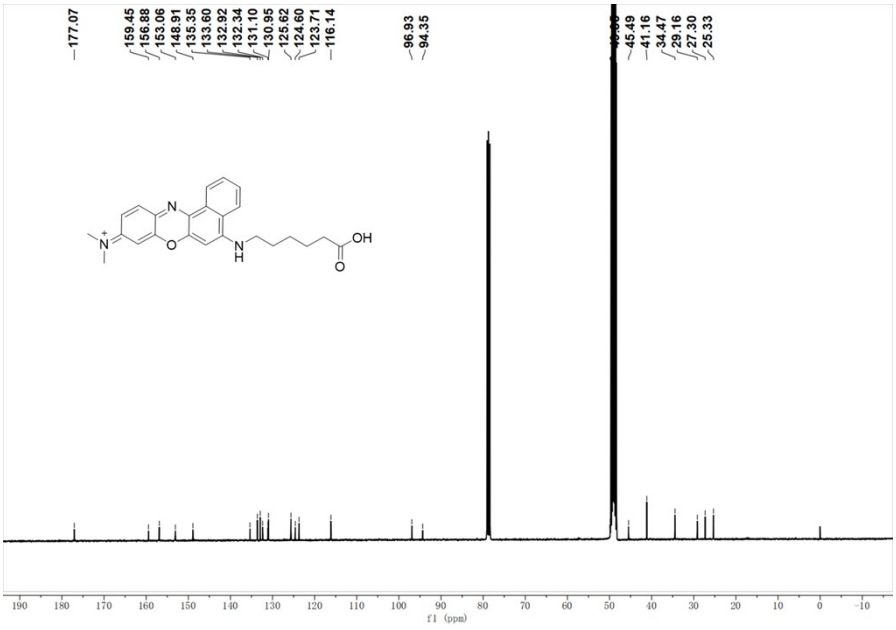

Figure S10. ^{13}C NMR spectrum of Compound 2 (101 MHz, MeOD).

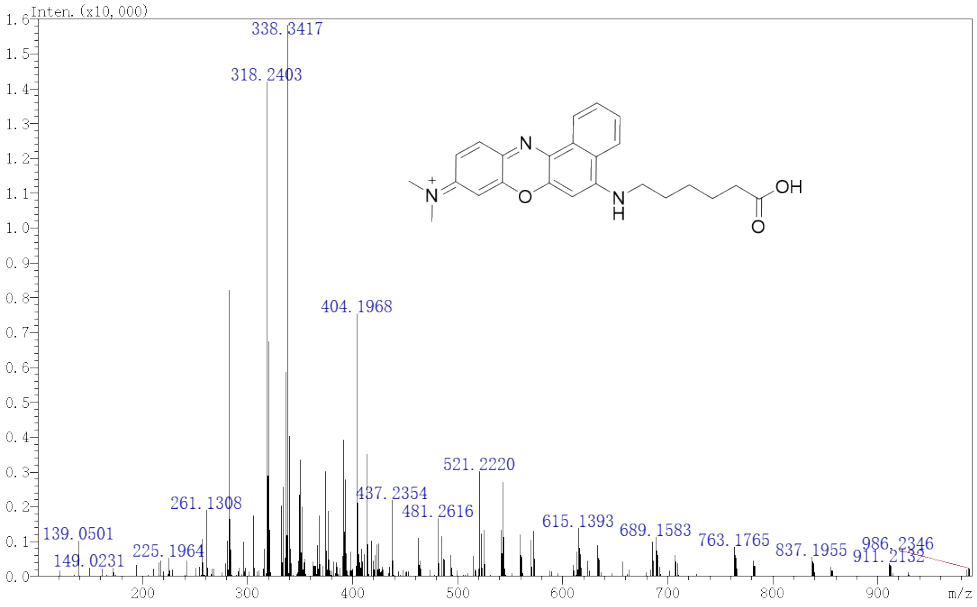

Figure S11. HRMS spectrum of Compound 2.

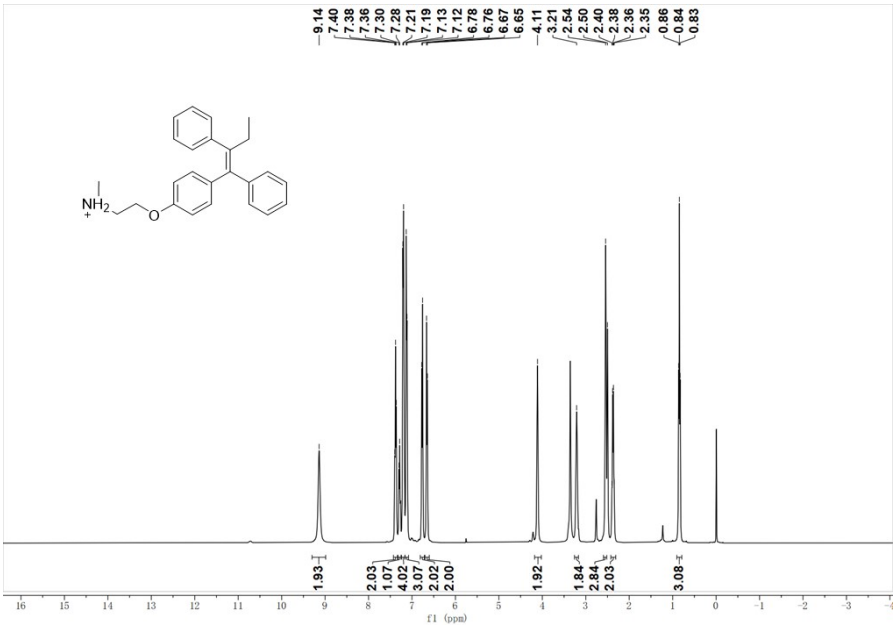

Figure S12. ^{1}H NMR spectrum of Compound 3 (400 MHz, DMSO-d_6).

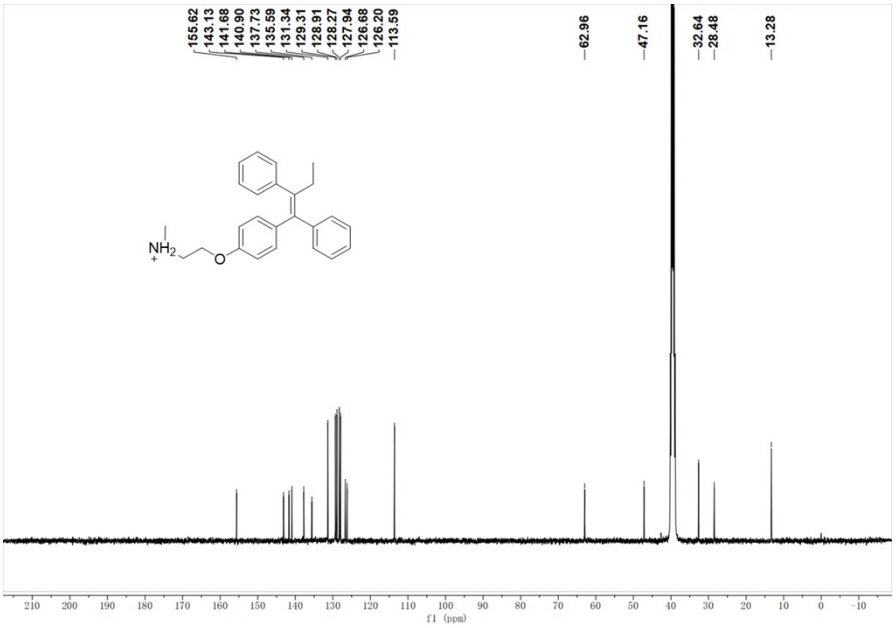

Figure S13. ^{13}C NMR spectrum of Compound 3 (101 MHz, DMSO-d_6).

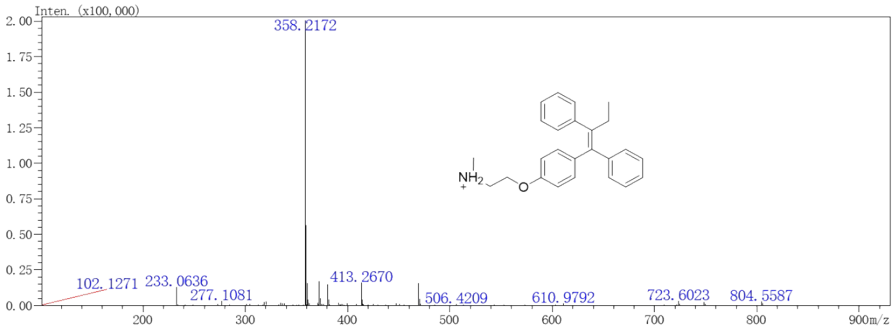

Figure S14. Mass spectrum of Compound 3.

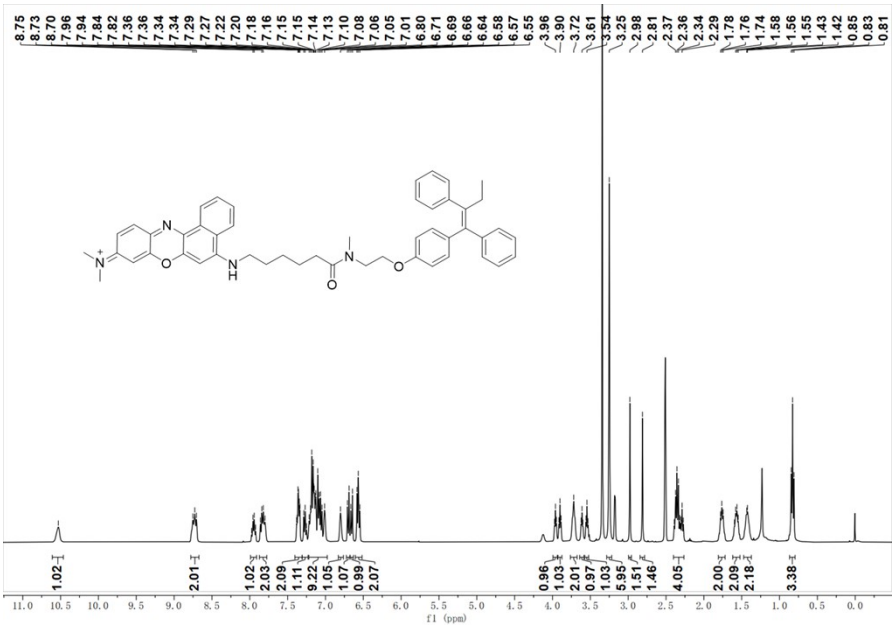

Figure S15. ^{1}H NMR spectrum of NB-TAM (400 MHz, DMSO-d_6).

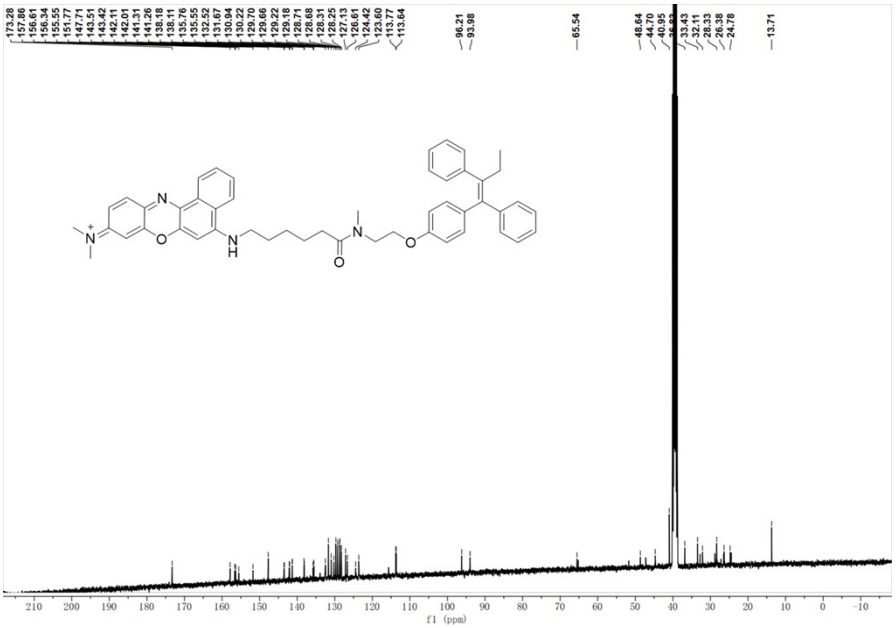

Figure S16. ^{13}C NMR spectrum of NB-TAM (101 MHz, DMSO-d_6).

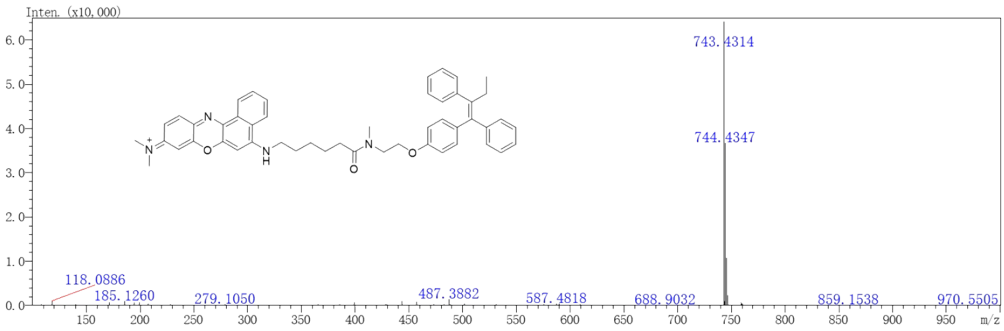

Figure S17. HRMS spectrum of NB-TAM.