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An Estradiol-Functionalized Red-Emissive Photosensitizer for Enhanced and Precise Photodynamic Therapy of Breast Cancers

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1. Experimental Section

Materials and instruments. Chemicals and reagents were purchased from the supplier and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker 400 spectrometer with chemical shifts reported in ppm (TMS as an internal standard). Mass spectra were obtained on a Bruker Daltonics micro-TOF-Q II mass spectrometer. Emission and UV-vis absorption spectra were recorded on a Hitachi F-7000 fluorometer and Agilent UV-2450 spectrophotometer, respectively. Fluorescence imaging experiments *in vitro* were conducted on the Operetta CLS high-content analysis system (PerkinElmer). Fluorescence imaging experiments *in vivo* were carried out on the IVIS Lumina XR *in vivo* imaging system (PerkinElmer). Cells were provided by the State Key Laboratory of Chemo/Biosensing and Chemometrics, Hunan University, China. A 635 nm laser with a power of 0.1 W/cm^2 was used as the light source for the irradiation on cells and tumors.

Measurements of optical spectroscopic properties. Unless otherwise noted, all the measurements were carried out according to the following procedure. A 1.0 mM stock solution of photosensitizer was prepared in DMSO for the UV-vis and fluorescence spectral analysis. The fluorescence spectra were recorded with an excitation wavelength at 655 nm. All the spectral measurements were carried out at room temperature.

Measurement of octanol-water partition coefficient. Prepared a solution of photosensitizer **NBS-ER** in water with an optical density at 1.0. Placed 3.0 mL of this aqueous solution in a 20.0 mL test tube, and then added 3.0 mL of *n*-octanol. The test tube was shaken vigorously for 5 min and then stood for 3 h. The aqueous layer was separated and its optical density was determined to be 0.15. Since the optical density of photosensitizer **NBS-ER** is proportional to its concentration, we determined the water/octanol partition coefficient (K_{ow}) by the following equation:

$$K_{ow} = (A_0 - A)/A$$

where A_0 refers the initial optical density of photosensitizer **NBS-ER** in water and A represents the final optical density of photosensitizer **NBS-ER** in water from the

equilibrium mixture of water/octanol. K_{ow} is calculated to be 5.67.

Singlet oxygen detection. 1,3-Diphenylisobenzofuran (DPBF) was used as a reagent for the capture of $^1O_2$. The $^1O_2$ quantum yields (Φ_Δ) of the photosensitizers were measured in methanol using methylene blue trihydrate (MB) as a standard (Φ_Δ = 0.5 in methanol).[1] The solution containing DPBF and the photosensitizer was prepared in methanol, and the absorbances of DPBF and photosensitizer were adjusted to 1.0 and 0.2-0.5, respectively. The cuvette was irradiated with the laser for different times. The slope of the absorbance of DPBF at 411 nm versus the irradiation time for each photosensitizer was calculated. Φ_Δ was determined from the following equation:

$$\Phi_{\Delta sample} = \Phi_{\Delta ref} * (k_{sample}/k_{ref}) * (F_{ref}/F_{sample})$$

where *sample* and *ref* designate the photosensitizer and the reference, respectively; k is the slope of the absorbance of DPBF (411 nm) versus the irradiation time, and F is the absorption correction factor, which is given by $F = 1\text{-}10^{-O.D.}$; O.D. is the optical density of the solution at the irradiation wavelength.

Measurement of Photostability. The stability of **NBS-ER** and **NBS-N_3** in PBS buffer (10.0 mM, pH = 7.4) or cell media was measured by observing the absorbance change of the solution. The solution of **NBS-ER** or **NBS-N_3** was placed under a 635 nm laser irradiation (0.1 mW/cm^2) at a 10 cm distance.

Cell culture and fluorescence imaging experiments. Cells were incubated on the cell culture plate in DMEM (Dulbecco's Modified Eagle's Medium) at 37 ℃ under 5% CO_2 for 24 h. Next, **NBS-ER** or **NBS-N_3** was added into the cell culture plate and cells were incubated for 20 min and subsequently washed three times with PBS. For the inhibition study of 17β-estradiol, MCF-7 cells were treated with 17β-estradiol (40.0 μM) for 24 h, then incubated with photosensitizer **NBS-ER** for another 20 min. Before conducting the imaging experiments, cells were washed with PBS three times. The excitation wavelength was between 650-675 nm, and the emission signals were collected between 685-760 nm.

Colocalization experiments. MCF-7 cells were incubated with **NBS-ER** (5.0 μM) in the culture medium for 20 min at 37 ℃, and then the cells were washed with PBS three times. Commercial fluorescent dyes (ER-tracker Green, Mito-tracker Green and Lyso-

tracker Green) were added and co-incubated for another 30 min, and cell imaging was then carried out after washing the cells with PBS three times. Green channel: λ_{abs} = 488 nm, λ_{em} = 500-540 nm; Red channel: λ_{abs} = 640 nm, λ_{em} = 650-750 nm.

Cell viabilities. The toxicity of photosensitizers on MCF-7, 4T1, MDA-MB-231 and HUVEC cells with/without light irradiation was evaluated by determining the cell viability. Cells were incubated in 96-well plates at 37 ℃ in 5% CO_2 for 24 h. The solution of the respective photosensitizer was added into each well and cells were incubated for 20 min. After cells were washed with PBS three times, cells were irradiated by a 635 nm laser (0.1 W/cm^2) for 1 min, and further incubated for 24 h. Then, the cell viabilities were determined by a standard MTT protocols.[2]

Live/dead cell co-staining experiments. Calcian AM and propidium iodide were used as co-staining agents for live and dead cells. Green channel: λ_{ex} = 460-490 nm, λ_{em} = 500-550 nm; red channel: λ_{ex} = 520-560 nm, λ_{em} = 570-650 nm. MCF-7 cells were incubated for 24 h in the culture plate and divided into five groups with different treatments: (1) cells were incubated with PBS for 20 min and then irradiated with light for 1 min; (2) cells were incubated with photosensitizer **NBS-N_3** (0.15 μM) for 20 min in the dark; (3) cells were incubated with photosensitizer **NBS-N_3** (0.15 μM) for 20 min and then irradiated with light for 1 min; (4) cells were incubated with photosensitizer **NBS-ER** (0.15 μM) for 20 min in the dark; (5) cells were incubated with photosensitizer **NBS-ER** (0.15 μM) for 20 min and then irradiated with light for 1 min. Then, each group of cells were stained with calcian AM and propidium iodide.

Real-time cellular uptake of photosensitizers. MCF-7 cells were incubated with photosensitizer **NBS-ER** (0.15 μM) or **NBS-N_3** (0.15 μM) for 20 min, washed with PBS three times and re-suspended in PBS. Then, the fluorescence images were captured at 0, 20, 40 min after the re-suspension. λ_{ex} = 650-675 nm, λ_{em} = 685-760 nm.

Tumor model. Mice were purchased from Hunan SJA Laboratory Animal Co., Ltd (No. SYXK (Xiang) 2020-0012). The mice (BALB/c, male, 6 weeks) bearing 4T1 tumor model were employed for *in vivo* experiments (license number: SCXK2019-0004). All related animal experiments were performed according to guidelines approved by the ethics committee of Hunan Normal University. To construct the model,

3 × 10^6 4T1 cells (100.0 μL) were injected into the right flank of the mice via subcutaneous injection. All mice were shaved in the tumor area.

Image-guided PDT efficiency *in vivo*. When the tumors of 4T1 tumor-bearing mice reached approximately 100 mm^3, 100.0 μL aqueous solution of PBS or photosensitizer (0.3 mg/kg) was injected via the tail vein of the mice. Next, *in vivo* fluorescence imaging was performed on the IVIS Lumina XR imaging system (λ_{ex} = 640 nm, λ_{em} = 695-770 nm) at different time points (0, 2, 4, 8, 24 h).

The mice with a tumor size of approximately 50 mm^3 were randomly divided into six groups (n = 3). Each group of mice were anesthetized with 0.8 μL Chloral hydrate (10%, intraperitoneally) and injected with aqueous solution of PBS or photosensitizer (0.3 mg/kg) via the tail vein of the mice. At 8 h post-injection, tumors of groups 2, 4 and 6 were irradiated with a 635 nm laser (0.1 W/cm^2) for 10 min for PDT treatment, and the distance between the light source and the tumor site is 10 cm.

Table S1. Tumors of mice with different treatments.

Group / Treatment	1	2	3	4	5	6
Injection compound	PBS	PBS	**NBS-N_3**	**NBS-N_3**	**NBS-ER**	**NBS-ER**
Light irradiation	-	yes	-	yes	-	yes

The body weight and tumor volume of the mice were measured over a period of 14 days. Then the maximum longitudinal diameter (length) and the maximum transverse diameter (width) were used to calculate the tumor volume. Tumor volume = width × width × length/2. After 14 days of treatment, the mice were euthanized. Tumor tissues of mice were obtained and analyzed histologically by hematoxylin & eosin (H&E) staining.

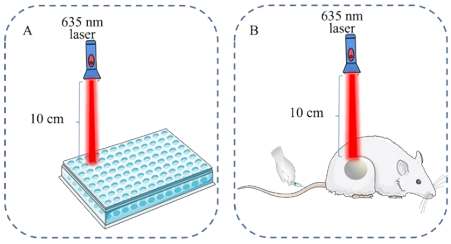
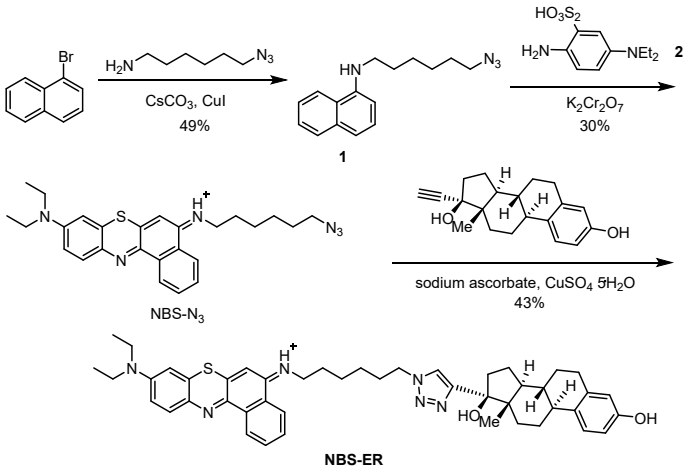


Figure S1. Schematic diagram illustrating the irradiation system *in vitro* and *in vivo*.

2. Synthesis



Scheme S1. Synthetic route of photosensitizer **NBS-ER**.

Synthesis of compounds 1 and 2. Compounds **1** and **2** were prepared according to the reported methods.[3]

Synthesis of NBS-N_3. To a solution of compounds **1** (680.0 mg, 2.50 mmol) and **2** (512.0 mg, 1.85 mmol) in DMSO (5.0 mL), potassium dichromate (600.0 mg, 2.00 mmol) was added and the resultant mixture was stirred at room temperature for 30 min. Then 50.0 mL methanol and 5.0 mL aqueous HCl (2.0 M) were sequentially added into the reaction mixture. Stirred the reaction mixture for another 1 h. After removing the excessive methanol under a reduced pressure, the remaining mixture was slowly poured into 100.0 mL brine to precipitate out a solid. The solid was collected by filtration, and was purified by silica gel column chromatography (DCM/MeOH = 20:1, *v/v*) to give **NBS-N_3** (255.0 mg) as a blue powder. Yield, 30%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.1 (s, 1H), 8.9 (d, J = 7.9 Hz, 1H), 8.6 (d, J = 8.0 Hz, 1H), 7.9 (dd, J = 15.2, 8.3 Hz, 2H), 7.9 (t, J = 7.4 Hz, 1H), 7.6 (s, 1H), 7.4 (d, J = 9.1 Hz, 2H), 3.7 (dd, J = 12.8, 6.5 Hz, 6H), 3.3 (d, J = 6.5 Hz, 2H),1.8 (s, 2H), 1.6 – 1.5 (m, 2H), 1.4 (s, 4H), 1.3 – 1.2 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 153.6, 151.3, 137.3, 134.2, 133.7, 132.5, 131.8, 130.1, 125.2, 124.0, 117.8, 105.9, 103.7, 51.1, 45.6, 44.4, 28.8, 28.6, 26.4, 26.4, 13.1. HRMS (ESI) m/z: $[M]^+$ *Calcd*. for $C_{26}H_{31}N_6S$ 459.2326; Found 459.2312.

Synthesis of photosensitizer NBS-ER. The solution of **NBS-N_3** (69.0 mg, 0.15 mmol), ethinylestradiol (30.0 mg, 0.10 mmol), sodium ascorbate (24.0 mg, 0.12 mmol), and

$CuSO_4 \cdot 5H_2O$ (15.0 mg, 0.06 mmol) in a mixed solvent (14.0 mL, $CHCl_3$: EtOH: H_2O = 12: 1: 1, v/v/v) was stirred at room temperature for 24 h. After removing the solvent, the residue was purified by silica gel column chromatography (DCM/MeOH = 10: 1, *v/v*) to give **NBS-ER** (33.0 mg) as a blue solid. Yield, 43%. ^{1}H NMR (400 MHz, CD_3OD) δ 9.1 (d, *J* = 8.1 Hz, 1H), 8.3 (d, *J* = 8.3 Hz, 1H), 8.1 (d, *J* = 9.5 Hz, 1H), 7.9 (t, *J* = 7.6 Hz, 1H), 7.8 (dd, *J* = 13.7, 7.1 Hz, 3H), 7.4 (d, *J* = 9.6 Hz, 1H), 7.2 – 7.2 (m, 1H), 7.2 (s, 1H), 6. 7 (d, *J* = 8.4 Hz, 1H), 6.3 (s, 1H), 6.2 (d, *J* = 8.4 Hz, 1H), 4.6 (s, 8H), 3.7 (q, *J* = 6.9 Hz, 4H), 3.6 (t, *J* = 7.2 Hz, 2H), 2.5 (dd, *J* = 17.3, 12.4 Hz, 2H), 2.15 – 1.78 (m, 9H), 1.7 – 1.5 (m, 6H), 1.4 (s, 2H), 1.3 (s, 6H), 1.0 (s, 3H), 0.7 (t, *J* = 11.0 Hz, 1H). ^{13}C NMR (100 MHz, $CD_3OD/CDCl_3$) δ 154.2, 151.4, 140.3, 137.3, 137.2, 134.3, 133.8, 133.1, 131.9, 130.9, 130.7, 129.5, 125.3, 122.7, 122.0, 117.4, 114.5, 112.2, 104.7, 101.5, 81.9, 49.6, 48.4, 48.2, 47.0, 45.5, 43. 8, 43.4, 39.4, 37.0, 33.0, 31.7, 30.6, 29.5, 29.4, 29.1, 28.3, 27.2, 26.1, 25.9, 25.5, 23.3, 22.4, 13.5, 13.2, 11.8. HRMS (EI) m/z: $[M]^+$ *Calcd.* for $C_{46}H_{56}N_6O_2S$ 755.4107; Found 755.4094.

3. Supplemental Figures

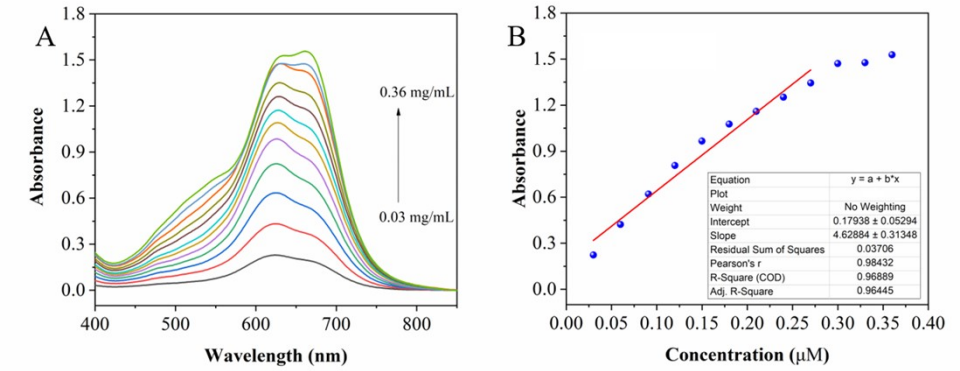


Figure S2. (A) UV-vis absorption spectra of photosensitizer **NBS-ER** at different concentrations in water. (B) The linear correlation between the absorbance and the concentration of photosensitizer **NBS-ER**.

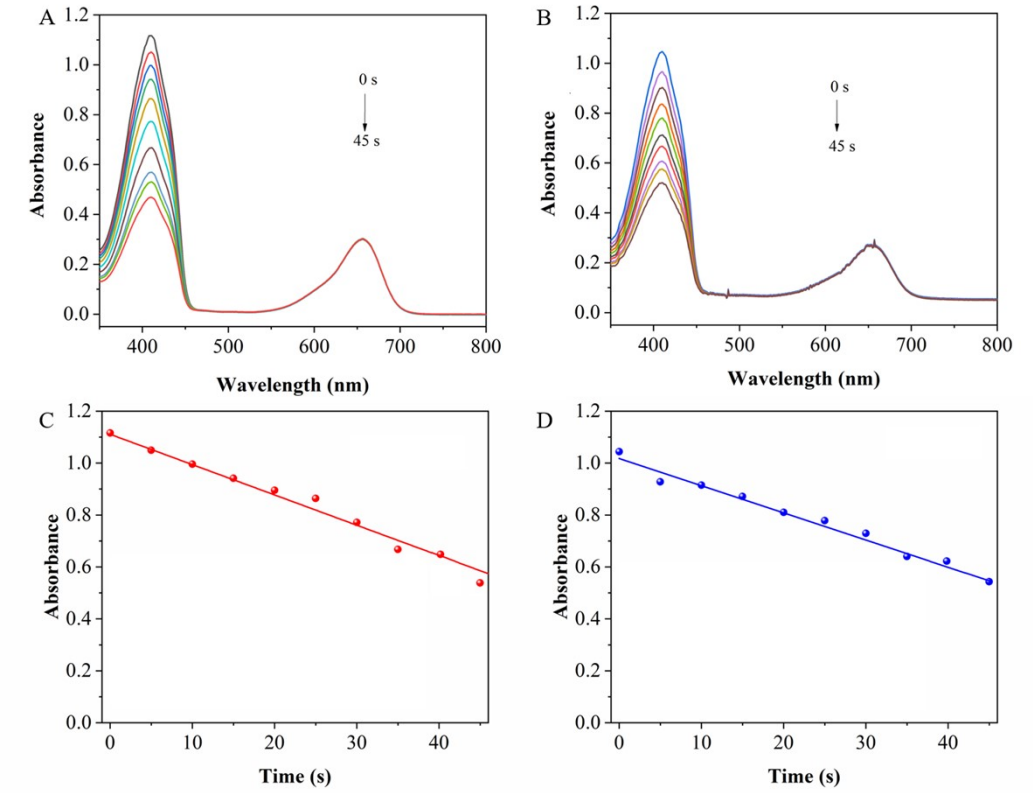


Figure S3. Top row: UV-vis spectra of DPBF with photosensitizers **NBS-ER** (A) and **NBS-N_3** (B) in methanol before and after 635 nm laser irradiation for different time. Bottom row: The linear correlation between absorbance at 411 nm of DPBF with photosensitizers **NBS-ER** (C) and **NBS-N_3** (D) in methanol and the irradiation time (0-45 s). Light source: a 635 nm laser with a power of 0.1 W/cm^2.

Photosensitizers	λ_{abs}/nm	λ_{em}/nm	Stokes shifts/nm	Φ_Δ (%)
NBS-ER	655	710	55	5.9
NBS-N_3	655	710	55	5.0

Table S2. Photophysical properties of photosensitizers **NBS-ER** and **NBS-N_3** in PBS buffer.

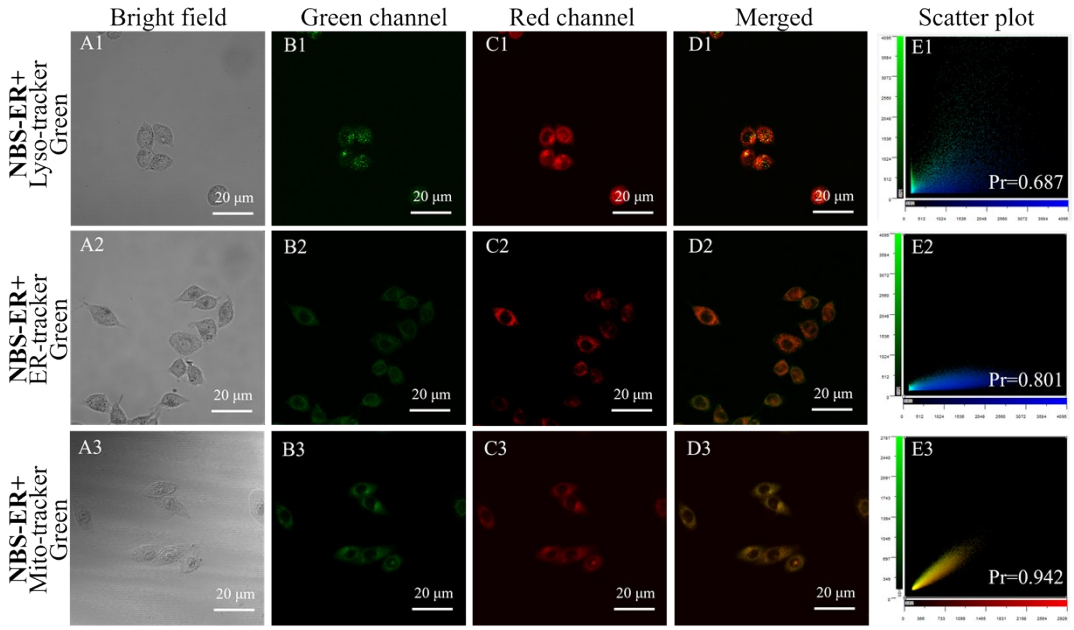


Figure S4. Confocal images of MCF-7 cells incubated with **NBS-ER** (5.0 μM) for 30 min, followed by ER-tracker Green (1.0 μM) (A1-E1), Mito-tracker Green (1.0 μM) (A2-E2) and Lyso-tracker Green (1.0 μM) (A3-E3) for 30 min, respectively. Green channel: λ_{ex} = 488 nm, λ_{em} = 500-540 nm; Red channel: λ_{ex} = 640 nm, λ_{em} = 650-750 nm. Scale bar: 20 μm.

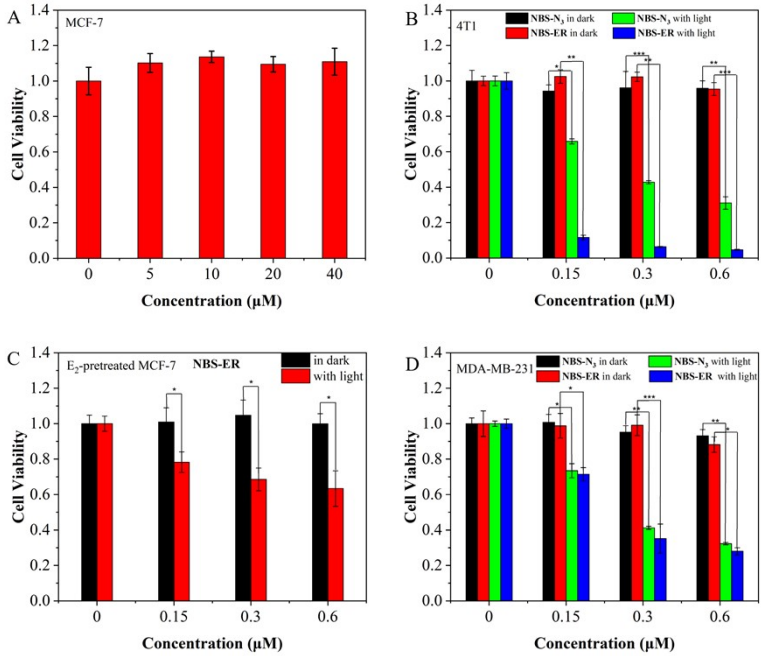


Figure S5. (A) Cytotoxicity of 17β-estradiol on MCF-7 cells under dark condition. (B) Cytotoxicity of photosensitizers **NBS-ER** and **NBS-N_3** on 4T1 cells with and without light irradiation, respectively. (C) Cytotoxicity of photosensitizer **NBS-ER** on E_2-pretreated MCF-7 cells with or without light irradiation. (D) Cytotoxicity of photosensitizers **NBS-ER** and **NBS-N_3** on MDA-MB-231 cells with and without light irradiation, respectively. Light source: a 635 nm laser with a power of 0.1 W/cm^2. Irradiation time: 1 min. Data are expressed as means ± SD (n = 6), *P < 0.05, **P < 0.01 and ***P <0.001.

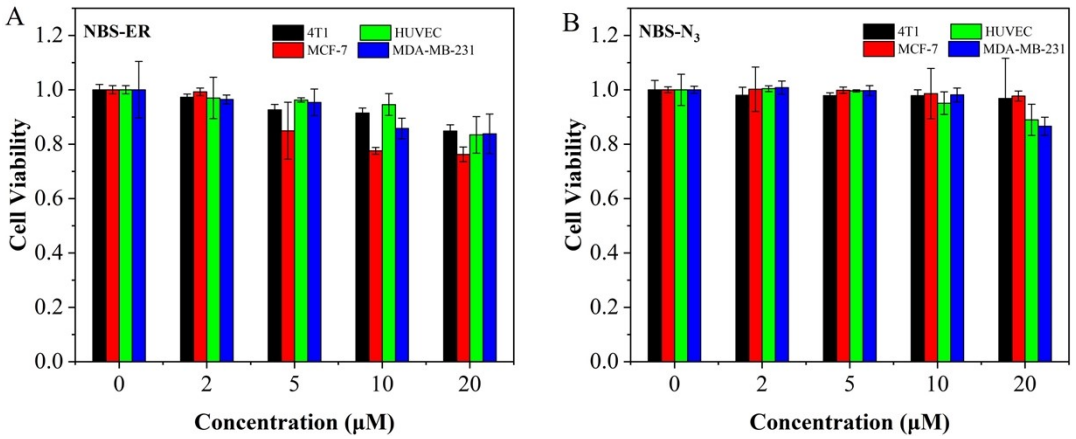


Figure S6. Cytotoxicity of photosensitizers **NBS-ER** (A) and **NBS-N_3** (B) on different cells without light irradiation.

Table S3. IC_{50} values and phototoxicity indexes (PI) of photosensitizers **NBS-N_3** and **NBS-ER** in different cells with and without light irradiation.

Photo-sensitizers	MCF-7 cells			4T1 cells			MDA-MB-231 cells			HUVEC cells		
	IC_{50} (μM)		PI	IC_{50} (μM)		PI	IC_{50} (μM)		PI	IC_{50} (μM)		PI
	light	dark		light	dark		light	dark		light	dark	
NBS-N_3	0.25	56	20	0.26	69	19	0.34	56	15	0.35	67	14
NBS-ER	0.08	48	59	0.07	72	73	0.27	55	18	0.28	56	17

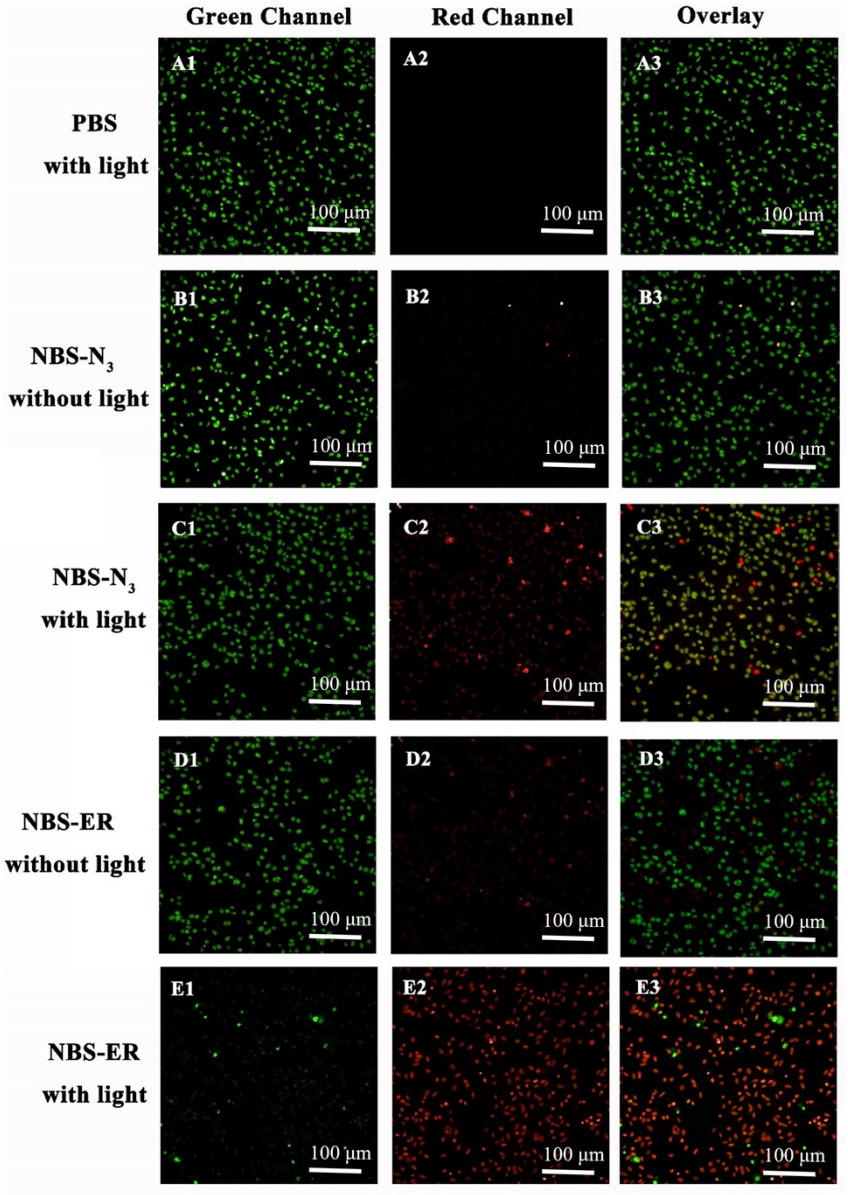


Figure S7. Fluorescence images of calcein-AM and PI labelled MCF-7 cells with different treatments. Green channel (living cells): λex = 460-490 nm, λem = 500-550 nm; red channel (dead cells): λex = 530-560 nm, λem = 570-650 nm. Light source: a 635 nm laser with a power of 0.1 W/cm2. Irradiation time: 1 min. Scale bar: 100 μm.

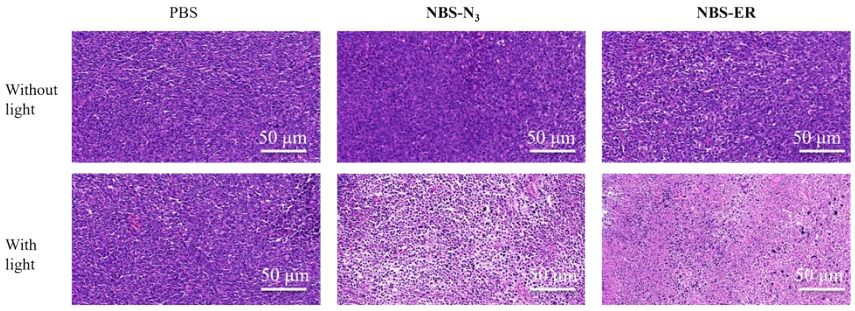


Figure S8. Analysis of hematoxylin & eosin (H&E) staining of tumor sections. Scale bar: 50 μm.

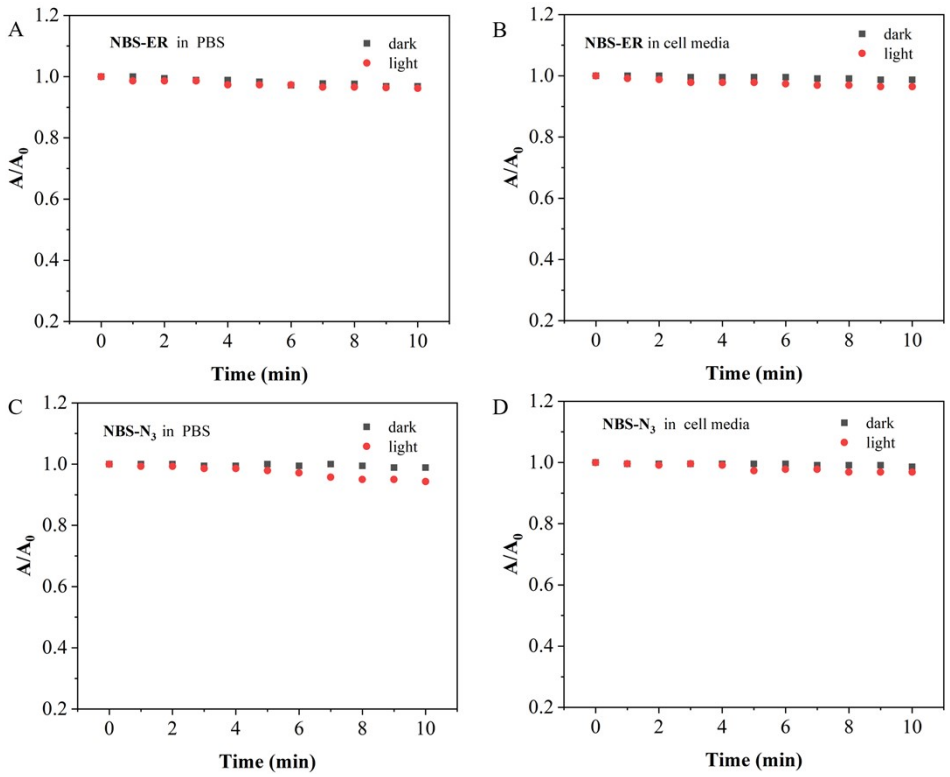


Figure S9. Photobleaching of photosensitizers with/without light irradiation (635 nm, 100 mW/cm^2) for different time (0-10 min). (A) **NBS-ER** in PBS; (B) **NBS-ER** in cell media; (C) **NBS-N$_3$** in PBS; and (D) **NBS-N$_3$** in cell media.

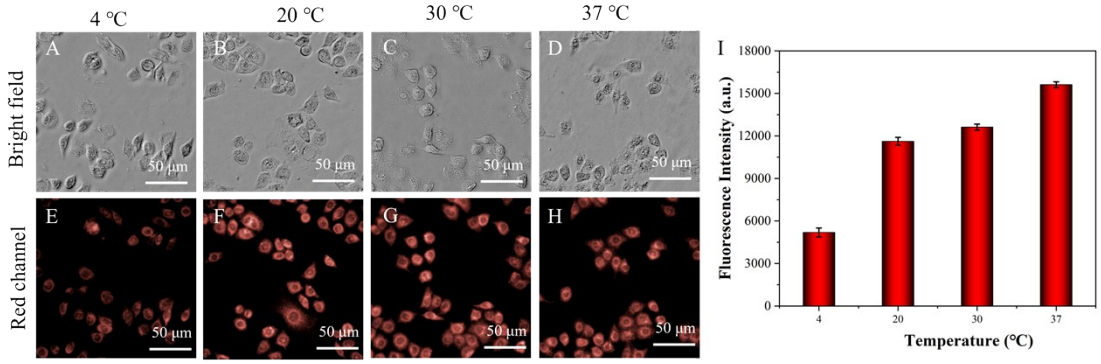


Figure S10. Images of MCF-7 cells incubated with photosensitizer **NBS-ER** at different temperature. (A-D) Bright field; (E-H) Red channel; (I) Quantitative image analysis of the average fluorescence intensity. Red channel: λ_{ex} = 650-675 nm, λ_{em} = 685-760 nm. Scar bar: 50 μm.

4. ^{1}H NMR, ^{13}C NMR and HRMS Spectra

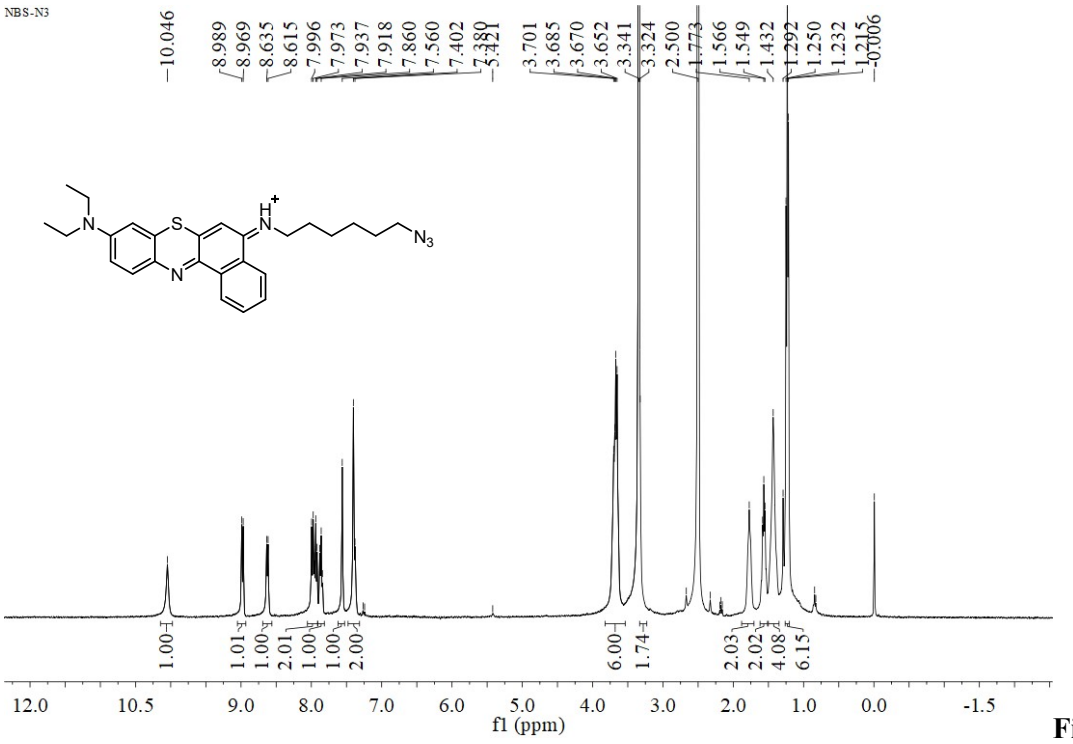


Figure S11. ^{1}H NMR spectrum of photosensitizer **NBS-N$_3$** in DMSO-d_6.

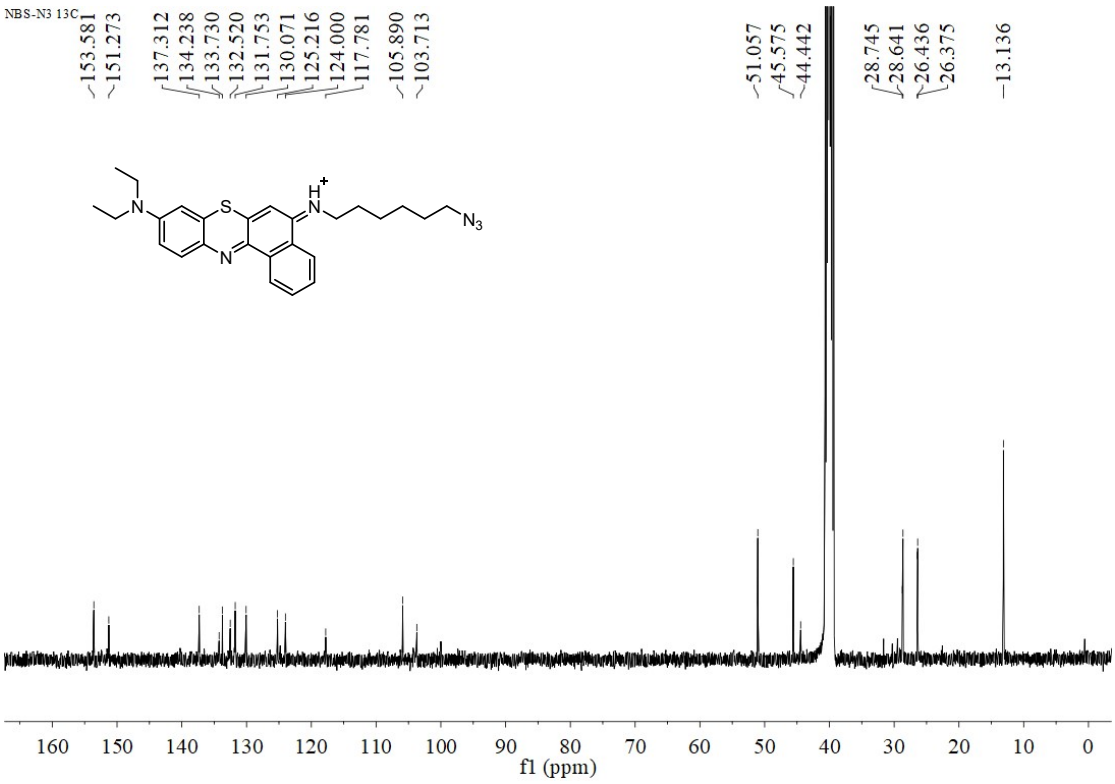


Figure S12. ^{13}C NMR spectrum of photosensitizer **NBS-N$_3$** in DMSO-d_6.

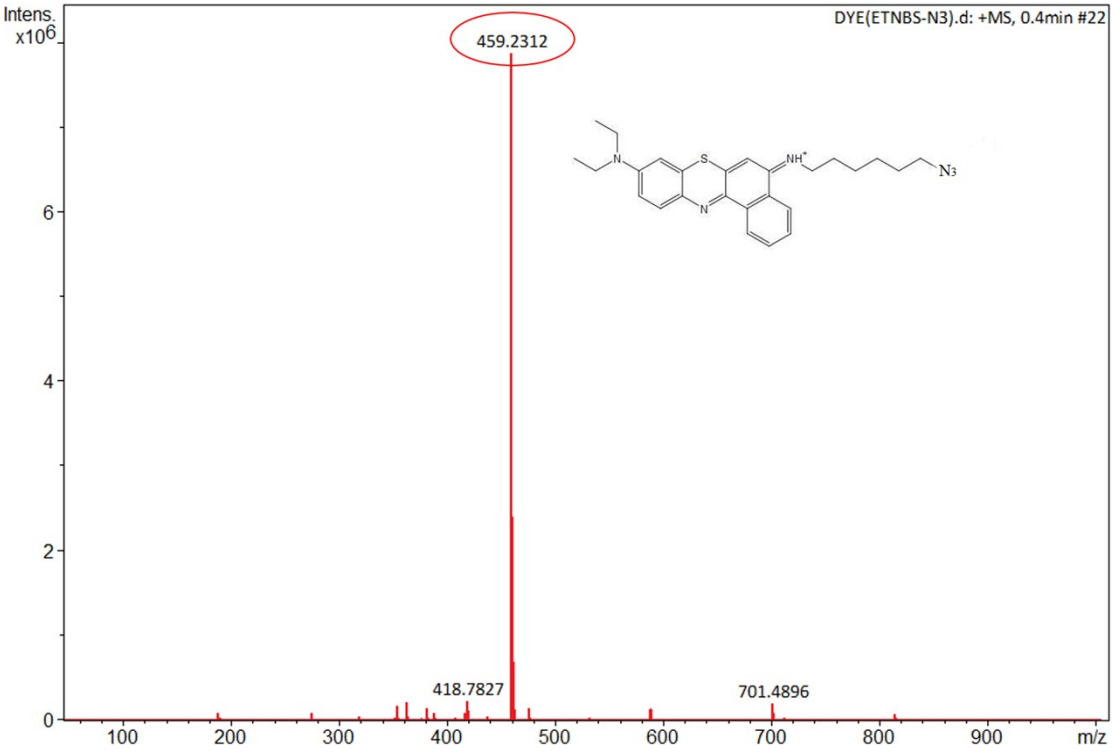


Figure S13. HRMS spectrum of photosensitizer **NBS-N_3**.

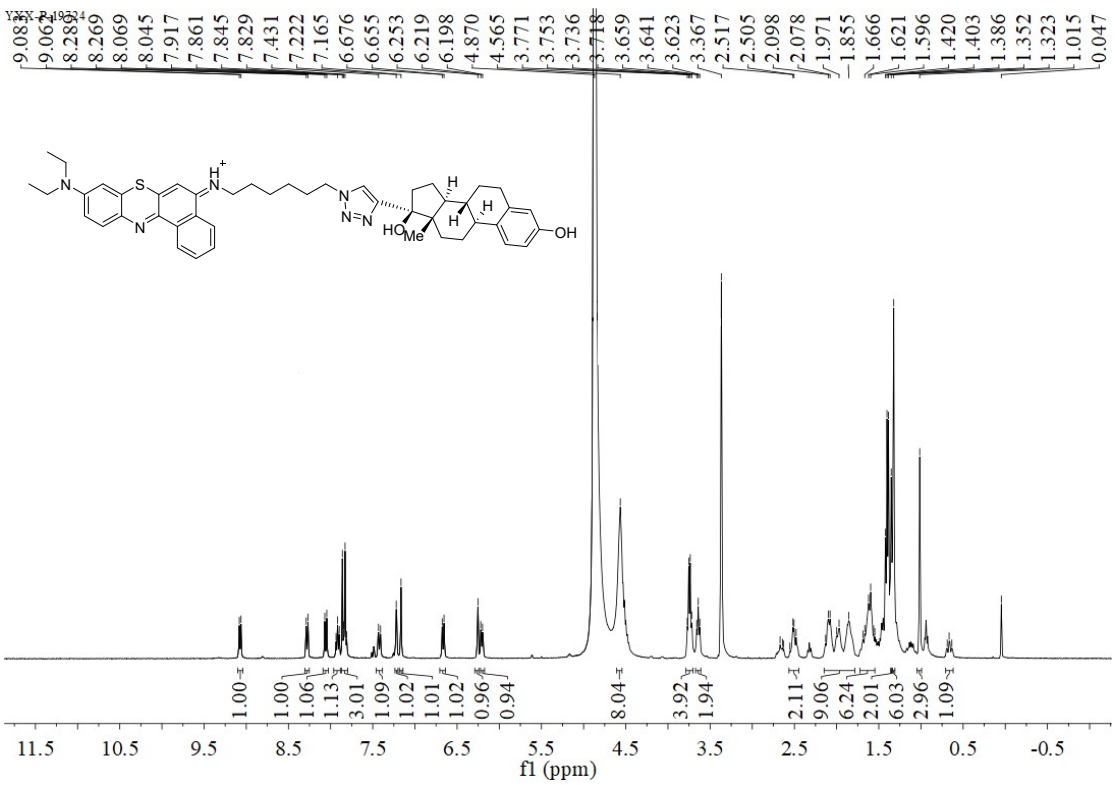


Figure S14. ^{1}H NMR spectrum of photosensitizer **NBS-ER** in CD_3OD.

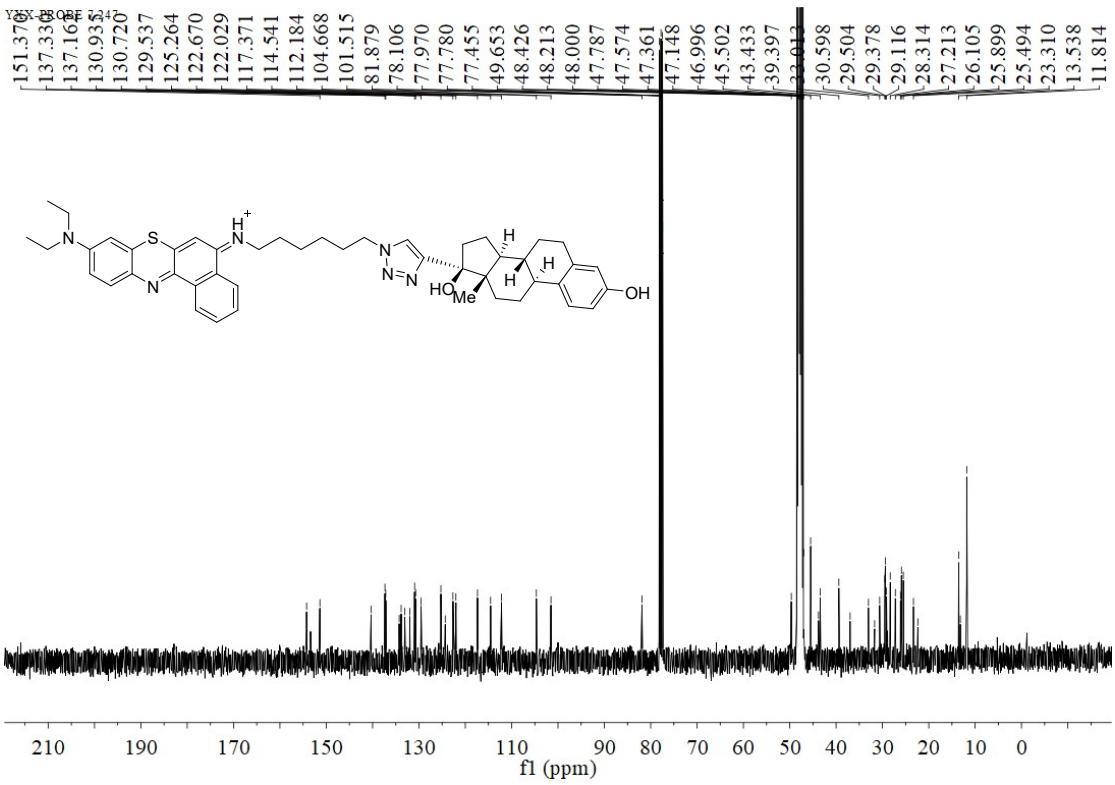


Figure S15. ^{13}C NMR spectrum of photosensitizer **NBS-ER** in $CD_3OH/CDCl_3$.

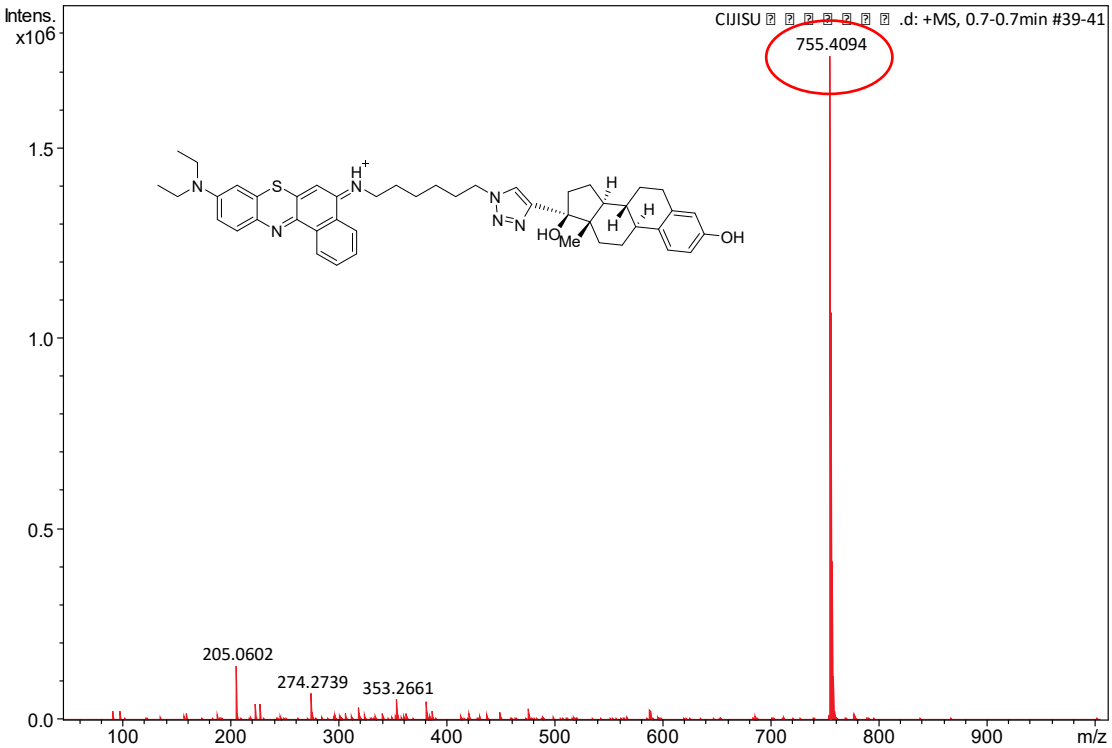


Figure S16. HRMS spectrum of photosensitizer **NBS-ER**.

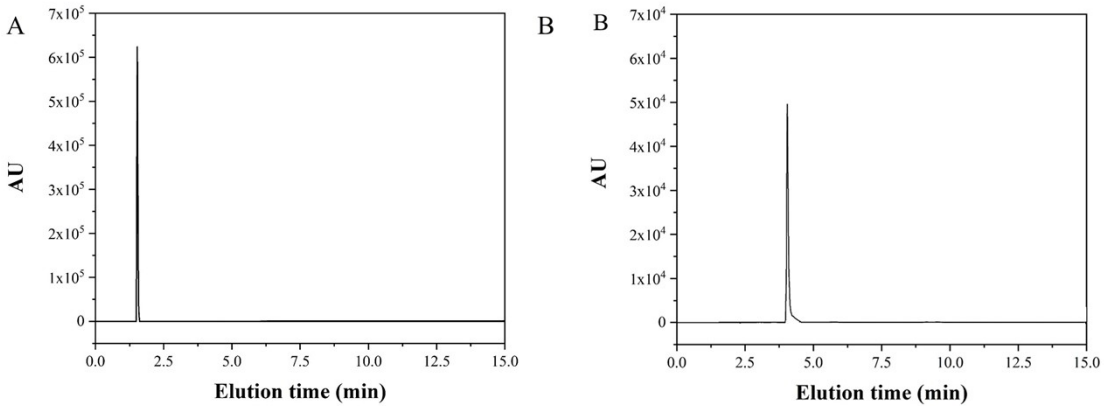


Figure S17. Liquid chromatography of (A) **NBS-ER** and (B) **NBS-N_3**. Conditions: eluent, MeOH; flow rate, 1.0 mL/min; detection wavelength, 635 nm; injection volume, 10.0 μL.

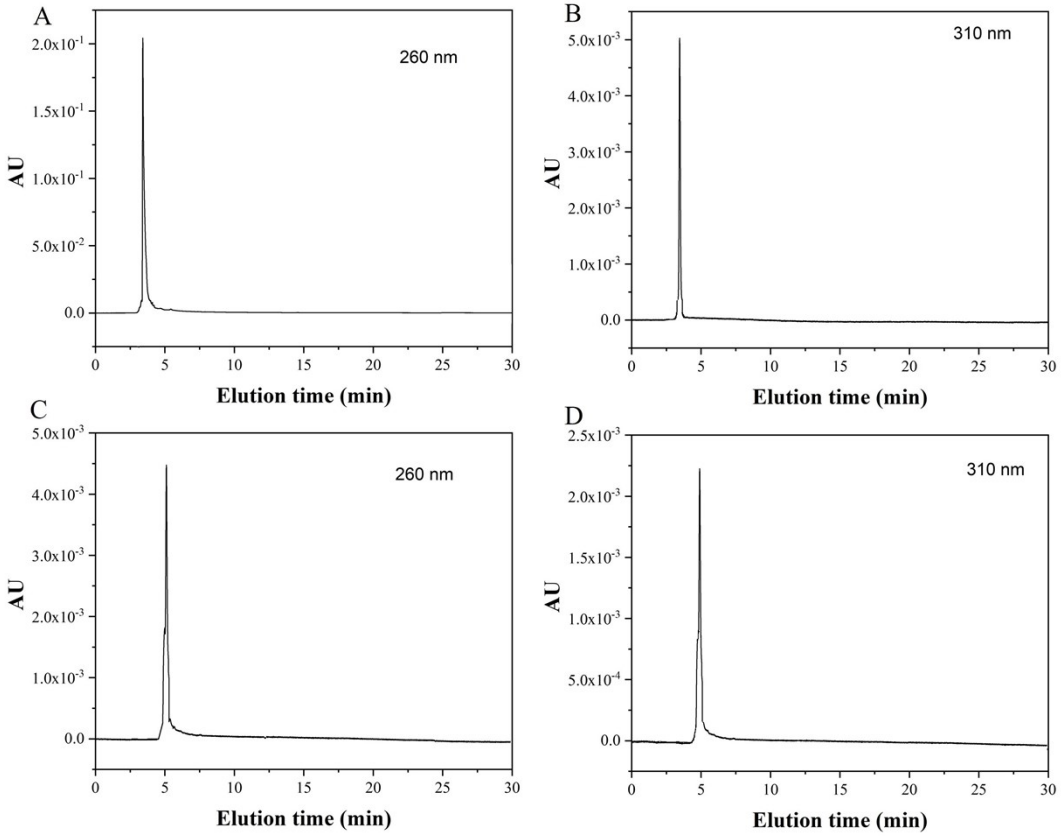


Figure S18. Liquid chromatography of **NBS-ER** (A and B) and **NBS-N_3** (C and D). Conditions: eluent, CH_3CN; flow rate, 1.0 mL/min; detection wavelength: 260/310 nm; injection volume, 5.0 μL.

5. References

1. Detty, M. R., Merkel, P. B., *J. Am. Chem. Soc.* 1990, ***112***, 3845-3855.

2. Cincotta, L., Foley, J. W., Cincotta, A. H., *Photochem. Photobiol.* 1987, ***46***, 751-758.

3. (a) Verma, S., Sallum, U. W., Athar, H., Rosenblum, L., Foley, J. W., Hasan, T., *Photochem. Photobiol.* 2009, ***85***, 111-118; (b) Li, M., Shao, Y., Kim, J. H., Pu, Z., Zhao, X., Huang, H., Xiong, T., Kang, Y., Li, G., Shao, K., Fan, J., Foley, J. W., Kim, J. S., Peng, X., *J. Am. Chem. Soc.* 2020, ***142***, 5380-5388.