Three birds one stone: an enzyme-activatable theragnostic agent for fluorescence diagnosis, photodynamic and inhibitor therapies

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1. Experimental Section

1.1 Instruments and materials

^{1}H NMR and ^{13}C NMR spectra were conducted on a Bruker 400 spectrometer. Mass spectra were obtained on a Bruker Daltonics micro-TOF-Q II mass spectrometer. Absorption spectra were recorded with a UV-2450 spectrometer (Shimadzu) and fluorescence spectra were conducted on an F-7600 fluorometer (Hitachi). Fluorescence imaging experiments were conducted on an operetta CLS from the company of PerkinElmer. Unless otherwise stated, all reagents were used as received. Deionized water was used in all experiments.

1.2 Determination of fluorescence quantum yield

The fluorescent quantum yields of photosensitizer **NBS-L-AX** (5.0 μM) in the absence/presence of KIAA1363 (35.0 μg/mL) were tested in Tris (10 mM, pH = 7.4, containing 1% DMSO). The fluorescence quantum yield was calculated according to the equation:

$$\Phi_s = \Phi_r * (A_r/A_s) * (F_s/F_r) * (n_s/n_r)^2$$

where Φ refers to the fluorescence quantum yield; A represents the absorbance; n represents the refractive index of the solvent.[1] The subscripts s and r refer to the sample and the reference, respectively. Cresyl Violet was used as the reference with a fluorescence quantum yield of 0.53 in methanol.[2]

1.3 Singlet oxygen ($^{1}O_2$) measurements

Methylene blue trihydrate (MB) was used as the standard (Φ_Δ = 0.5 in methanol) for $^{1}O_2$ quantum yield (Φ_Δ) measurements.[3] The absorbance of the solution of 1,3-diphenylisobenzofuran (DPBF) in air-saturated methanol was adjusted to 1.0. The solution of the investigated photosensitizer was prepared in a cuvette with an absorbance at 0.2-0.5. The cuvette was illuminated with a 635 nm laser (100 mW/cm^2) at an interval of 2 s. The absorbance was measured after each irradiation. The slope of absorbance of DPBF at 410 nm versus the irradiation time for each photosensitizer was calculated. Φ_Δ was calculated according to a modified equation: $\Phi_\Delta^{ps} = \Phi_\Delta^{ref} * k_{ps}/k_{ref} * (F_{ref}/F_{ps})$, where ps and ref designate the photosensitizer and the reference, respectively. k is the slope of the absorbance of DPBF (410 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.

1.4 In vitro toxicity assay

MTT assays were performed on MCF-7 and HUVEC cells to evaluate the toxicity of photosensitizers with/without a light irradiation.[4] Cells were added to a 96-well plate and incubated at 37 ℃ in 5% CO_2 atmosphere for 24 h. The aqueous solutions of **NBS-L-AX**, **NBS-L**, **AX11890** and **JW480** with a certain concentration were added to each well, respectively, and cells were incubated for 24 h to for MTT assay. For the phototoxicity, cells were incubated with photosensitizer **NBS-L-AX** or **NBS-L** for 30 min, illuminated with a 635 nm laser (100 mW/cm^2) for 20 min, and further incubated for 24 h.

1.5 Colocalization experiments

The subcellular distribution of **NBS-L-AX** in MCF-7 cells was investigated by co-staining commercial staining dyes ER-tracker Green, Mito-tracker Green and Lyso-tracker Green with

photosensitizer **NBS-L-AX**, respectively. MCF-7 cells were incubated with **NBS-L-AX** (5.0 μM) in the culture medium for 20 min at 37 ℃, and then the cells were washed with PBS three times. Then commercial fluorescent dyes 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. In cells, the fluorescence signals of **NBS-L-AX** matched well with those of ER-tracker Green. The colocalization coefficients was calculated to be 0.90, indicating that photosensitizer **NBS-L-AX** could target the endoplasmic reticulum in cancer cells.

1.6 In vitro wound healing assay

Cells were incubated at 37℃ for 24 h to keep about 85-90% density. Cells were divided into two groups for different purposes. Group 1 were used as the control with the treatment with PBS buffer and irradiation with a 635 nm laser (100 mW/cm^2) for 20 min; group 2 were incubated with photosensitizer **NBS-L-AX** (5.0 μM) for 30 min, then irradiated with a 635 nm laser (100 mW/cm^2) for 20 min. Drew a line in the middle of culture dishes with a pipette, washed cells with PBS buffer three times, and incubated cells for 24 h. Inverted fluorescent microscope was used to monitor the wound healing assay.

1.7 Dead/Live cell co-staining.

Propidium iodide (PI) and calcein AM were used as co-staining reagents to differentiate dead and live cells, respectively. PI is a staining reagent for dead cells with a red fluorescence and calcein AM can stain live cells to give off a green fluorescence. The excitation wavelength was between 460-490 nm, and the emission signals were collected from green (500-550 nm) and red (570-650 nm) channels, respectively. Cells were incubated with photosensitizer **NBS-L-AX** (5.0 μM) at 37 ℃ for 30 min, and were exposed to the light illumination for 20 min under a 635 nm laser (100 mW/cm^2). **NBS-L-AX**-treated cells without light illumination were used as a control.

1.8 In vivo imaging.

The 4T1 tumor-bearing mice model was employed for the *in vivo* experiments. The mice were purchased from Hunan SJA Laboratory Animal Co., Ltd. All related animal experiments were performed according to guidelines approved by the ethics committee of Hunan Normal University. All mice were kept anesthetized by using 10% chloral hydrate during the treatment. To construct the model, 3×10^6 4T1 cells (100.0 μL) were injected into the right flank of the mice via subcutaneous injection (BALB/c, male, 6 weeks).

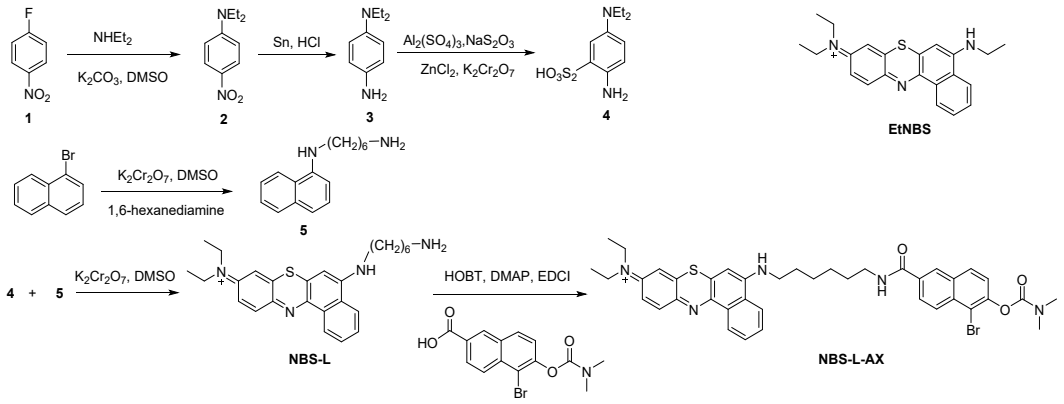
Fluorescent imaging experiments on the mice were performed on the IVIS Lumina II *in vivo* Imaging System. When the tumor volume reached 100-120 mm^3, the mice were divided into three groups with injection of different compounds: 1) PBS; 2) **NBS-L-AX**; 3) **JW480** + **NBS-L-AX**. 4 h after the injection was completed, the fluorescence images were captured by the IVIS Lumina II (excitation wavelength: 640 nm; emission wavelength: 695-770 nm). For group 3, the time interval between the two injections was 2 h.

1.9 In vivo photodynamic therapy.

The photodynamic therapy experiments were carried out when the volume of the 4T1 tumors grew to ~ 50 mm^3. The mice were divided into six groups (n = 4 per group) with different treatments: 1)

PBS injection; 2) PBS injection and light irradiation; 3) **NBS-L-AX** injection; 4) **NBS-L-AX** injection and light irradiation; 5) **JW480** + **NBS-L-AX** injection; 6) **JW480** + **NBS-L-AX** injection and light irradiation. The injected volume of photosensitizer **NBS-L-AX** (5.0 μM) or PBS was 100.0 μL. For groups 5 and 6, the mice were intratumorally preinjected with **JW480** (10.0 μL, 500.0 μM) to inhibit KIAA1363 enzyme activity, and waited for 2 h before the treatment with photosensitizer **NBS-L-AX**. 4 h after the injections were completed, the irradiation with a 635 nm laser (100 mW/cm^2) for 20 mins was performed on the tumor sites of groups 2, 4 and 6, respectively. Tumor volumes and body weights of all mice were measured each day for 14 days. At the end of the experiments, the mice were euthanized, and tumor tissues were harvested for histological analysis by means of hematoxylin-eosin (H&E) staining.

2. Synthesis



Scheme S1. Synthetic route of photosensitizer **NBS-L-AX** and chemical structure of **EtNBS**.

Synthesis of compounds 2-5 and NBS-L. Compounds **2-5** and **NBS-L** were prepared according to the literature methods.[5]

Synthesis of photosensitizer NBS-L-AX. To a solution of **NBS-L** (70.0 mg, 0.15 mmol) and **AX11890** (45.0 mg, 0.31 mmol) in DMF (10 mL) under an argon atmosphere were added EDCI (36.0 mg, 0.19 mmol), HOBT (30.0 mg, 0.23 mmol), and DMAP (23.0 mg, 0.19 mmol). The solution was stirred for 10 h at room temperature, and 50 mL brine was added to quench the reaction. The obtained mixture was extracted with CH_2Cl_2 three times (30 mL x 3). The organic layers were collected, washed with brine twice and dried over anhydrous Na_2SO_4. Removed the organic solvent to give a residue, which was subjected to column chromatography purification (silica gel, MeOH/CH_2Cl_2 as eluent, v/v from 1:20 to 1:10) to afford photosensitizer **NBS-L-AX** as a blue solid (37.0 mg, 33%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.8 (d, J = 8.0 Hz, 1H), 8.2 (d, J = 7.7 Hz, 1H), 8.0 (d, J = 8.8 Hz, 1H), 7.8 (t, J = 9.5 Hz, 2H), 7. 5 (dt, J = 15.1, 6.9 Hz, 4H), 7.3 – 7.2 (m, 2H), 7.1 (s, 1H), 7.0 (s, 1H), 3.6 (d, J = 6.7 Hz, 6H), 3.5 (s, 2H), 3.2 (s, 3H), 3.2 – 3.1 (m, 3H), 1.8 – 1.7 (m, 5H), 1.4 (dd, J = 14.9, 7.4 Hz, 3H), 1.4 (d, J = 11.3 Hz, 4H), 1.3 – 1.3 (m, 8H), 1.0 (t, J = 7.3 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD) δ 154.3, 153.9, 150.6, 148.1, 140.4, 137.2, 133.6, 131.5, 130.7, 130.4, 129.7, 127.9, 127.1, 125.8, 125.3, 123.9, 123.1, 116.2, 104.4, 102.5, 77.4, 77.3, 77.1, 76.8, 53.4, 45.6, 44.2, 39.7, 36.8, 36.7, 31.3, 30.1, 29.6, 28.8, 28.4, 26.1, 26.0, 12.6. HRMS (ESI) m/z: calcd for $C_{40}H_{43}BrN_5O_3S$ $[M]^+$: 754.2244, found 754.2243.

3. Supplemental Figures

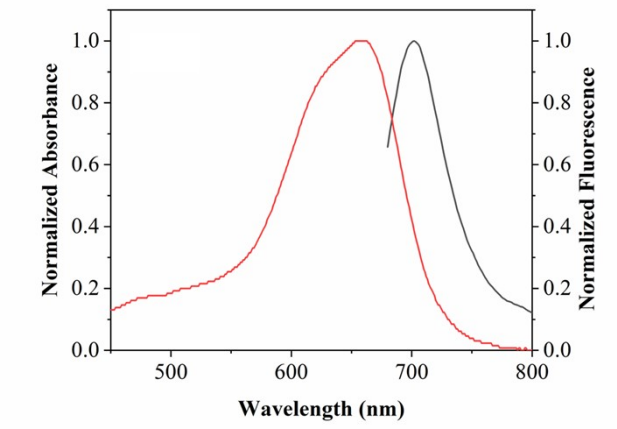


Fig. S1 Absorption (red line) and emission (black line) spectra of **NBS-L** in Tris buffer (10 mM, pH = 7.4, containing 1% DMSO).

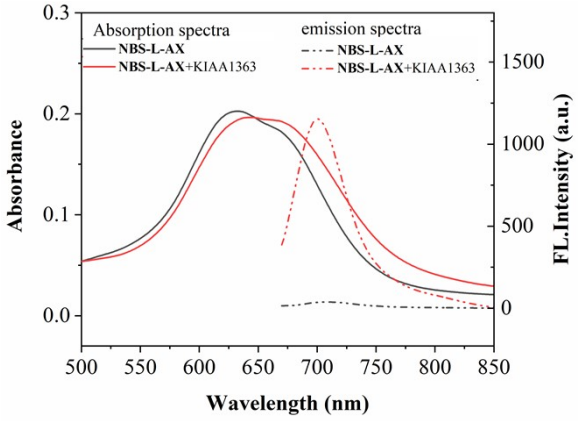


Fig. S2 Absorption (solid lines) and emission (dashed lines) spectra of photosensitizer **NBS-L-AX** (5.0 μM) (black lines) and photosensitizer **NBS-L-AX** (5.0 μM) with enzyme KIAA1363 (50.0 μg/mL) (red lines) in Tris buffer (10 mM, pH = 7.4, containing 1% DMSO).

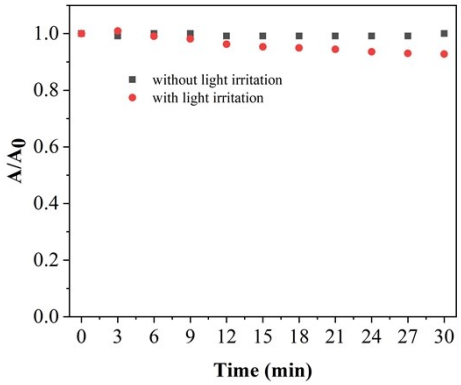


Fig. S3 Time-dependent absorbance changes of photosensitizer **NBS-L-AX** (10.0 μM) with/without light irradiation (635 nm, 100 mW/cm^2) in Tris buffer (10 mM, pH = 7.4, containing 1% DMSO).

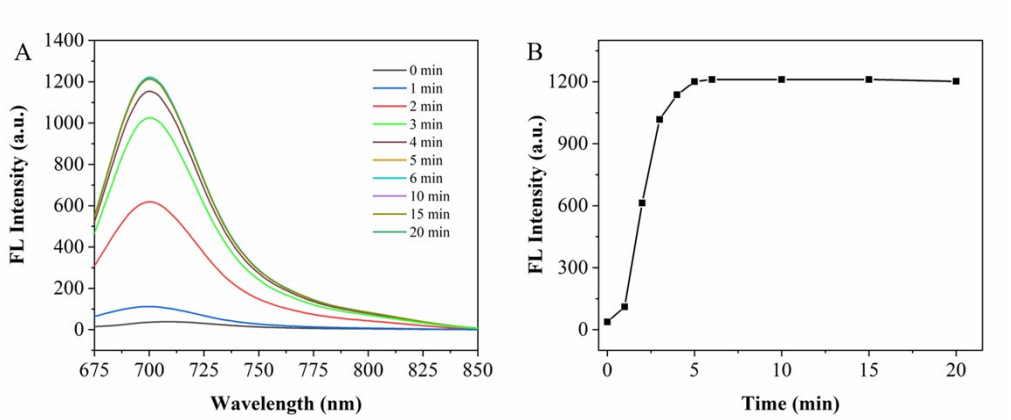


Fig. S4 (A) Time-dependent emission spectra of photosensitizer **NBS-L-AX** (5.0 μM) with enzyme KIAA1363 (50.0 μg/mL); (B) plot of the fluorescence intensity against the reaction time of photosensitizer **NBS-L-AX** (5.0 μM) with enzyme KIAA1363 (50.0 μg/mL). Excitation wavelength: 655 nm.

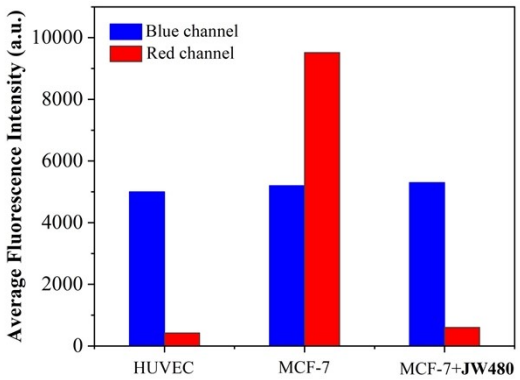


Fig. S5 Quantitative image analysis of the average fluorescence intensity of cells in Fig. 2. Blue channel (Hoechst 33258): λ_{ex} = 355-385 nm, λ_{em} = 430-500 nm; red channel (**NBS-L-AX**): λ_{ex} = 650-675 nm, λ_{em} 685-760 nm.

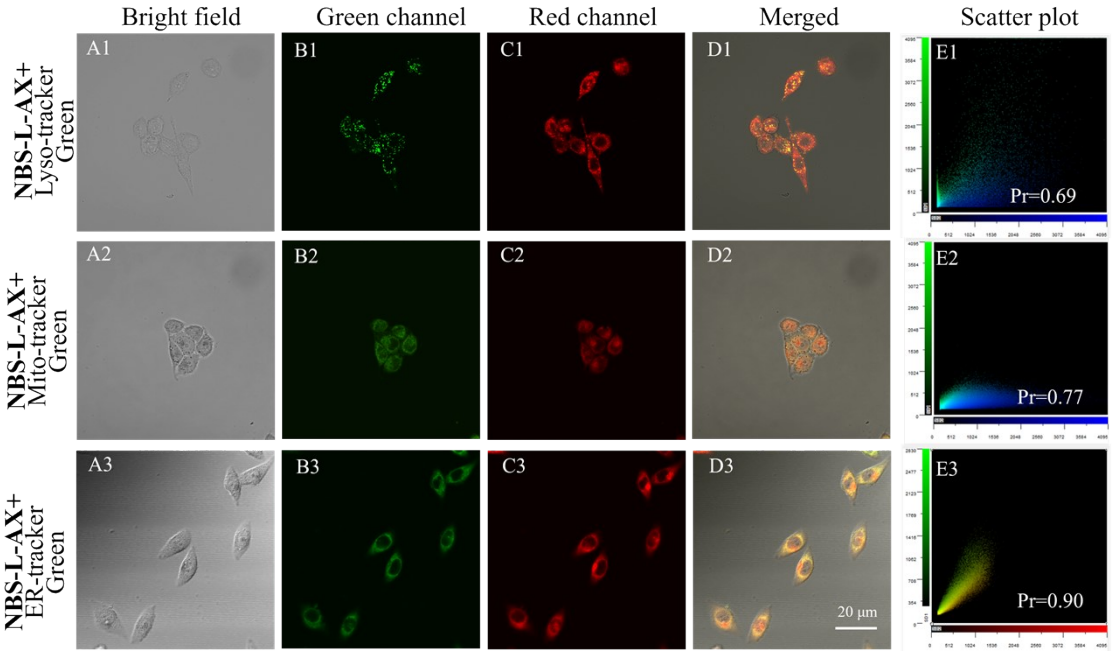


Fig. S6 Images of MCF-7 cells incubated with photosensitizer **NBS-L-AX** (5.0 μM) for 30 min, followed by organelle-selective staining dyes (1.0 μM) for 30 min. Green channel: λ_{ex} = 488 nm, λ_{em} = 500-540 nm; Red channel: λ_{ex} = 640 nm, λ_{em} = 650-750 nm. Scale bar: 20 μm.

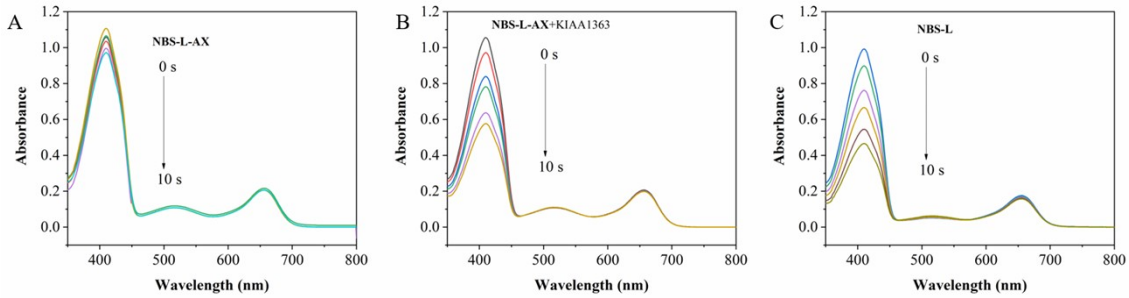


Fig. S7 Absorption spectra of the mixture of DPBF with respective compounds in MeOH under the irradiation of a 635 nm laser (100 mW/cm^2) for different times: (A) **NBS-L-AX** (5.0 μM), (B) **NBS-L-AX** (5.0 μM) and KIAA1363 (40.0 μg/mL), and (C) **NBS-L** (5.0 μM).

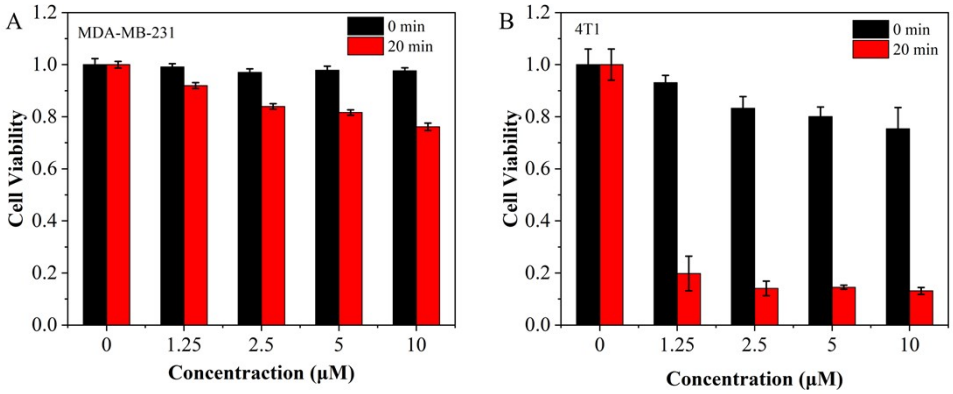


Fig. S8 Cytotoxicity to MDA-MB-231 (A) and 4T1 (B) cells incubated with photosensitizers **NBS-L-AX** at different concentrations with/without light irradiation, respectively. Light source: a 635 nm laser with a power of 100 mW/cm^2.

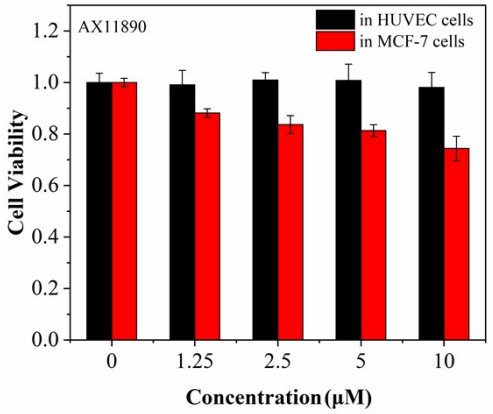


Fig. S9 Cytotoxicity to HUVEC and MCF-7 cells after treatment with different concentrations of **AX11890** (0.0, 1.25, 2.5, 5.0, 10.0 μM) under the dark.

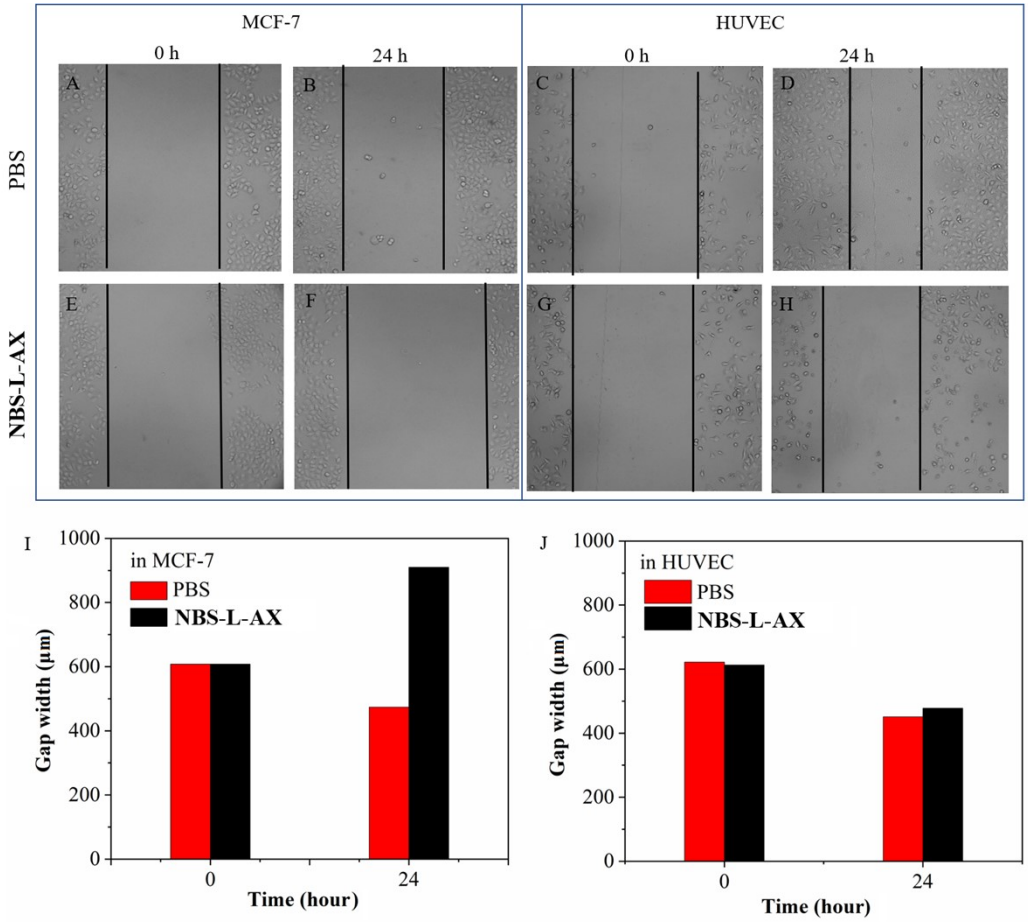


Fig. S10 The wound healing assay for MCF-7 and HUVEC cells after the irradiation with a 635 nm laser (100 mW/cm^2) for 20 min. (A-D) PBS; (E-H) photosensitizer **NBS-L-AX** (5.0 μM); (I and J) wound gap width in cells measured by Image J at different times.

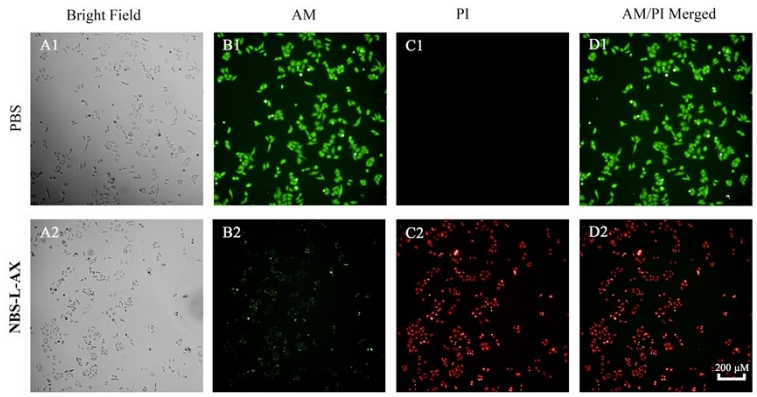


Fig. S11 Bright field and fluorescence images of MCF-7 cells incubated without (top row) and with (bottom row) photosensitizer **NBS-L-AX** (5.0 μM). Cells irradiated under a 635 nm laser (100 mW/cm^2) for 20 min and then stained with calcein AM and PI for 30 min. Excitation wavelengths: 460-490 nm; emission wavelengths: 500-550 nm (green channel) and 570-650 nm (red channel). Scale bar: 200 μm.

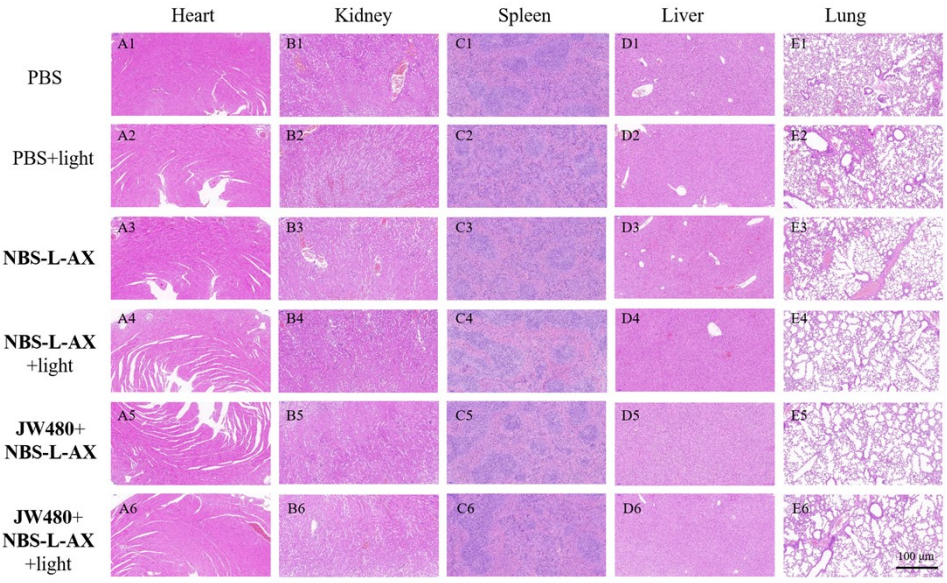


Fig. S12 *In vivo* biosafety assay. H&E staining of major organs of mice from different treatment groups on 14 d. Scale bar = 100 μm.

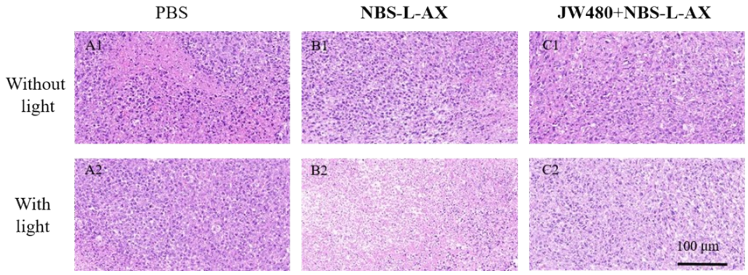


Fig. S13 H&E staining of tumor tissues excised from different treatment groups on day 14. Scale bar = 100 μm.

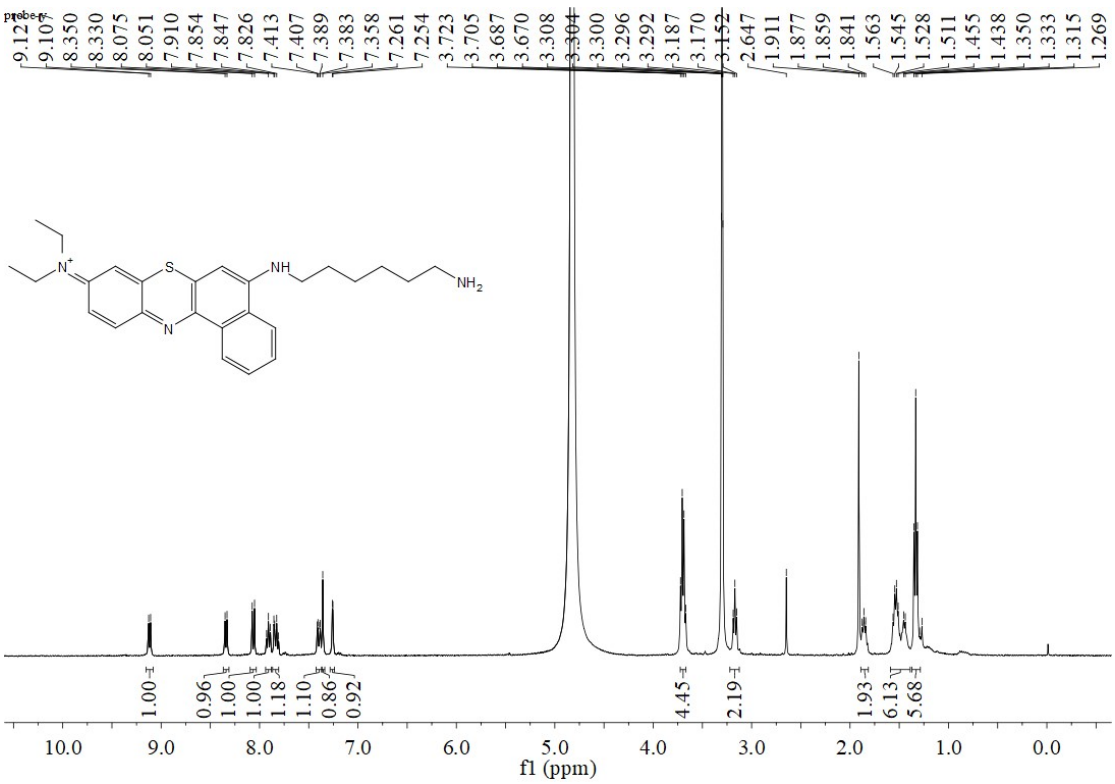


Fig. S14 ^{1}H NMR spectrum of **NBS-L** in CD_3OD.

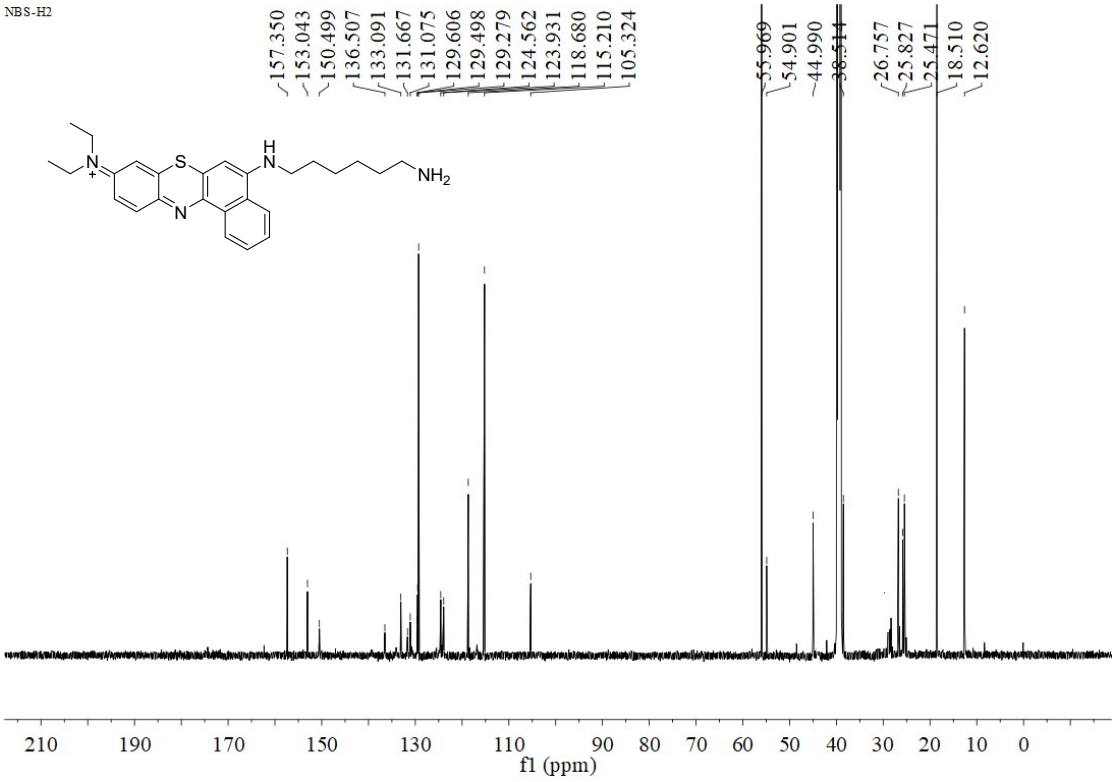


Fig. S15 ^{13}C NMR spectrum of **NBS-L** in DMSO-d_6.

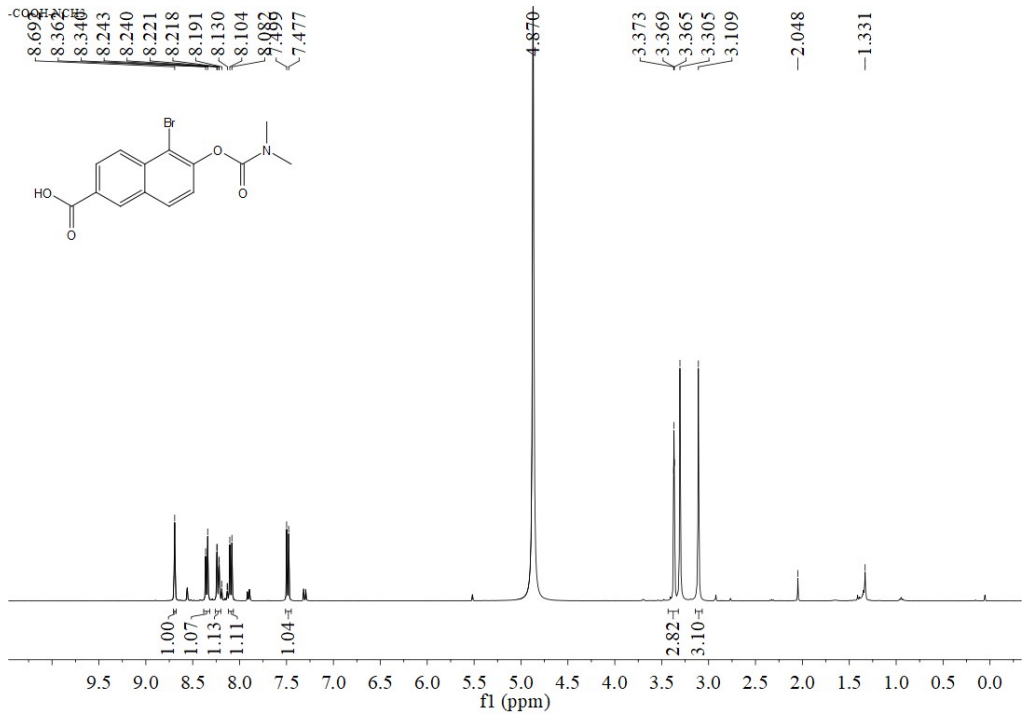


Fig. S16 ^{1}H NMR spectrum of compound **AX11890** in $CDCl_3$.

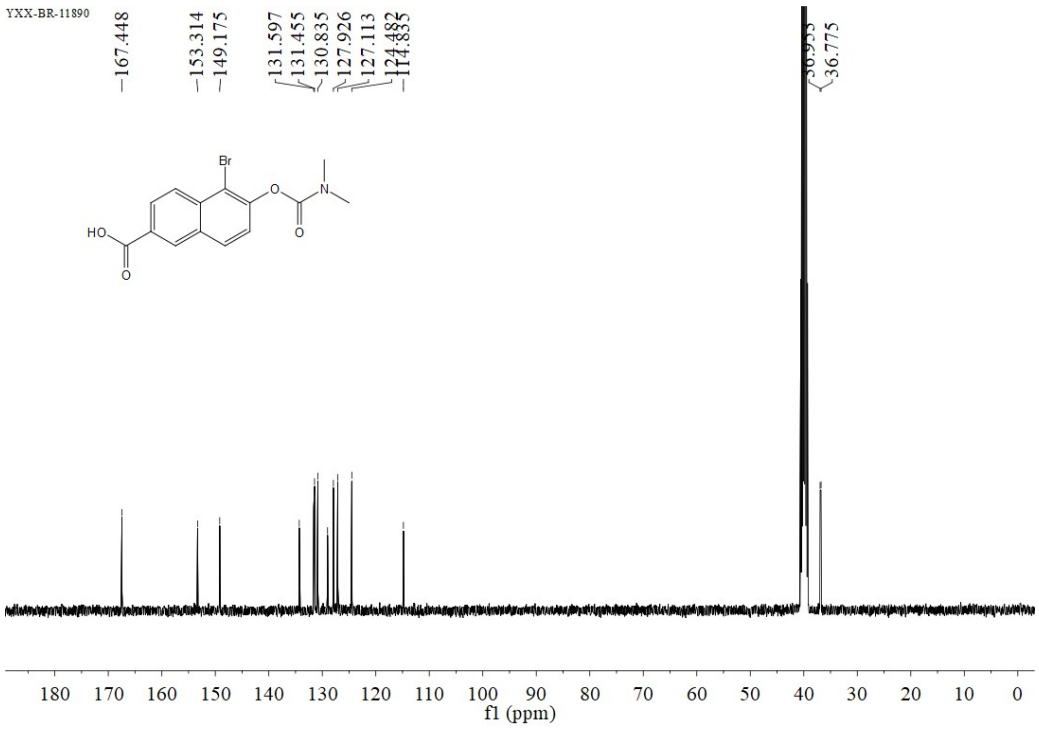


Fig. S17 ^{13}C NMR spectrum of compound **AX11890** in DMSO-d_6.

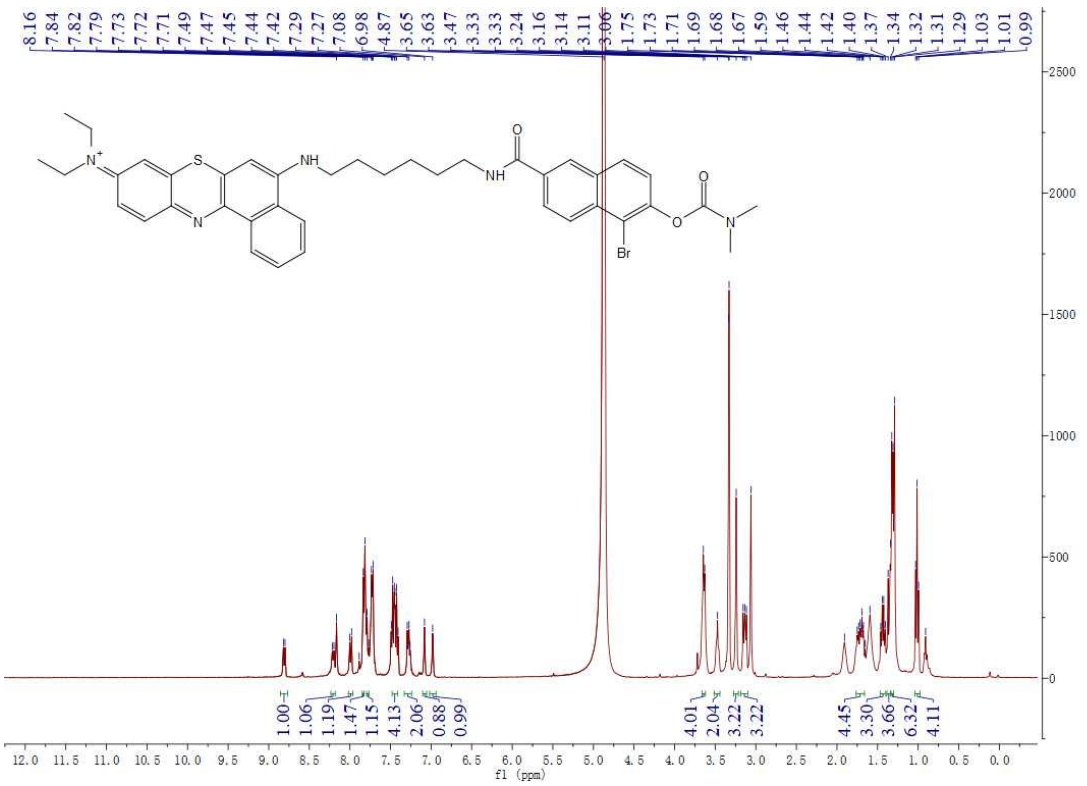


Fig. S18 ^{1}H NMR spectrum of photosensitizer **NBS-L-AX** in CD_3OD.

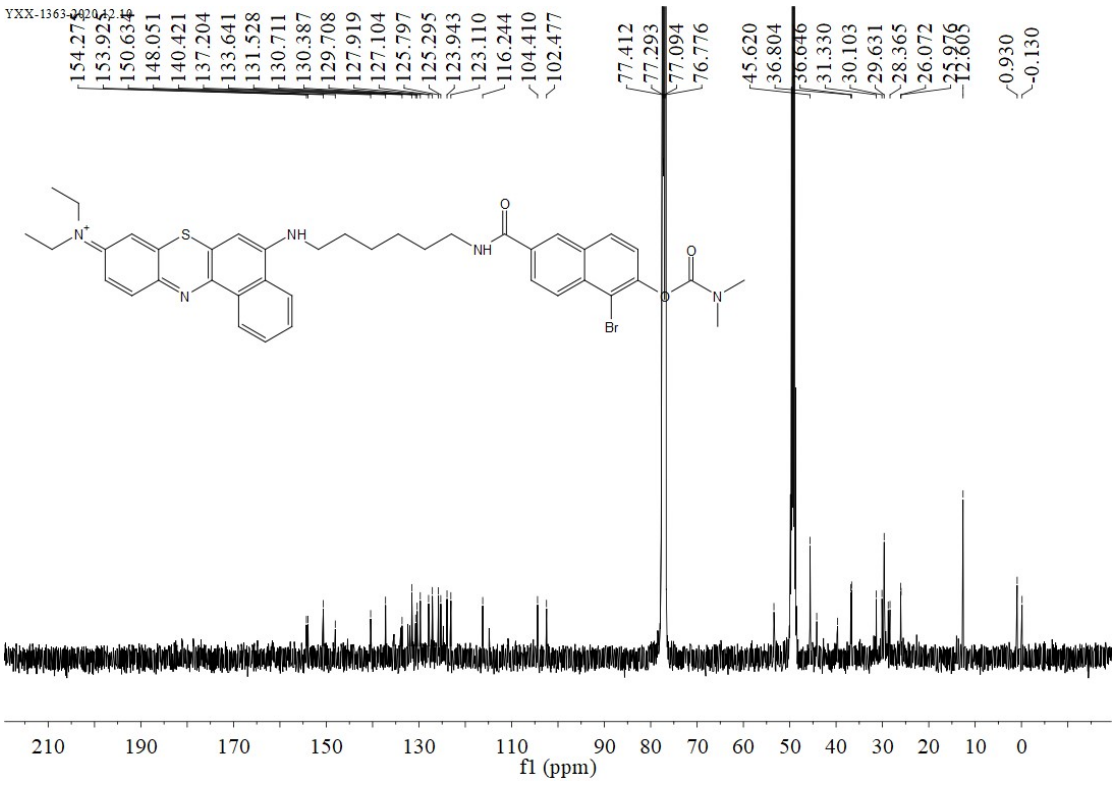


Fig. S19 ^{13}C NMR spectrum of photosensitizer **NBS-L-AX** in $CD_3OD/CDCl_3$.

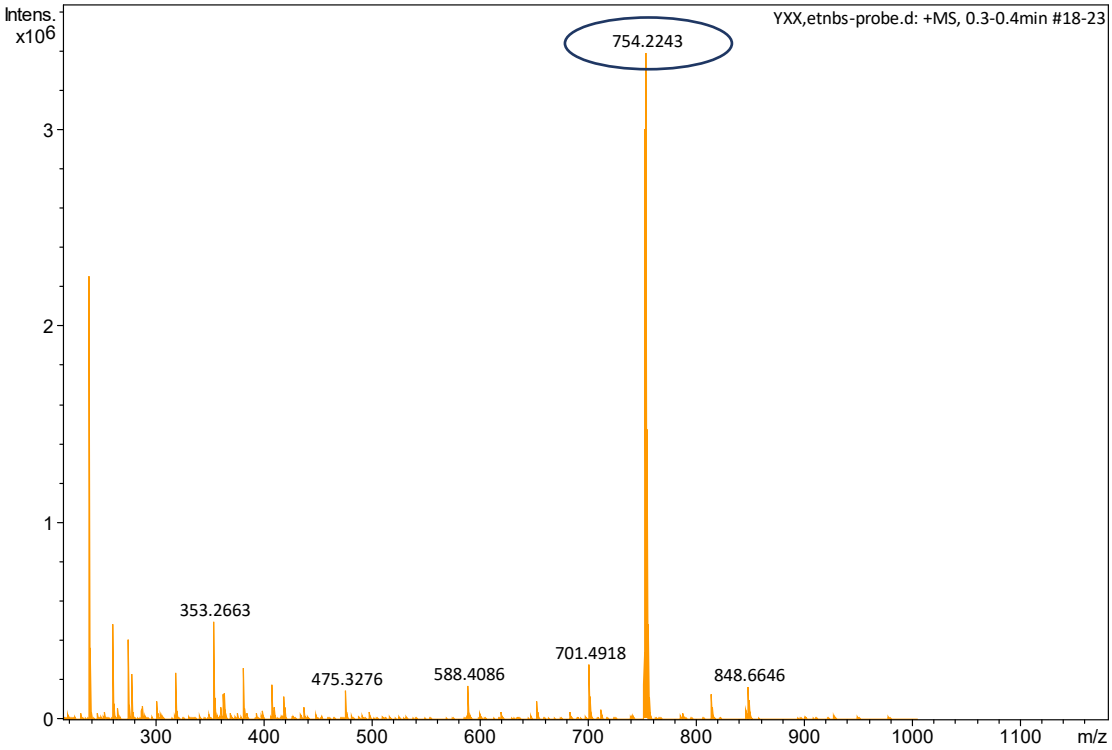


Fig. S20 HRMS spectrum of photosensitizer **NBS-L-AX**.

4. References

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