Supporting Information

A 4-aminonaphthalimide-based fluorescent traceable prodrug with excellent photoinduced cytotoxicity

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Materials and instruments

4-Bromo-1,8-naphthalic anhydride was purchased from Liaoning Liangang Dye Chemical Co. Ltd. LysTracker Red (LysRed, a lysosome dye) was purchased from beyotime Ltd. MitoTracker Red (MTRed, a mitochondrion dye), Endoplasmic Reticulum Tracker Red (ER-Red, a endoplasmic reticulum dye), and Hoechst 33342 (a nuclear dye) were purchased from KeyGEN BioTECH Ltd. N, N-dimethyl-1,3-diaminopropane and 1-Hydroxybenzotriazole were purchased from J&K. L-lysine was purchased from Beijing XinJingKe Biotechnology Ltd. 10-hydroxycamptothecin was purchased from energy chemical Co. Ltd. Ethyl-3-(3-(dimethylamino) propyl) carbodimide hydrochloride (EDC) was purchased from GL Biochem. Annexin V-Alexa Fluor 647 apoptosis detection kit was purchased from Beijing Solarbio Science & Technology Co., Ltd. All other reagents were purchased from Beijing Chemical Plant. HeLa (human cervical cancer cell line), PC3 (Human prostate cancer cell line), A549T (Human non-small cell lung cancer cell line), and LoVo (Human colon cancer cell line) were purchased from the Cell Bank of Shanghai Bioscience Center, Chinese Academy of Sciences.

^{1}H nuclear magnetic resonance (NMR) spectra were recorded on Avance III 500WB and 500WB nuclear magnetic resonance spectrometer (Bruker). High-resolution mass spectra (HR-MS) were measured on a solarix FT-ICR mass spectrometer (Bruker). Electropray ionization mass spectrometry (ESI-MS) was recorded on an LC-MS 2010A system (Shimadzu). Absorption spectra were recorded on a UH5300 spectrophotometer (Hitachi). Fluorescence spectra were collected on a F-4600 fluorescent spectrophotometer (Hitachi). Fluorescence images were recorded on an FV1000-IX83 confocal microscope (Olympus). Liquid Chromatography were recorded on a LC-20A high performance liquid chromatography (Shimadzu). The absorbance for cell counting kit-8 (CCK-8) analysis were recorded on a SpectraMax M2e Reader (Molecular Devices). Flow cytometry analysis and cell apoptosis experiments were completed on the flow cytometer (FACScalibur, Becton Dickinson, USA).

Experimental section

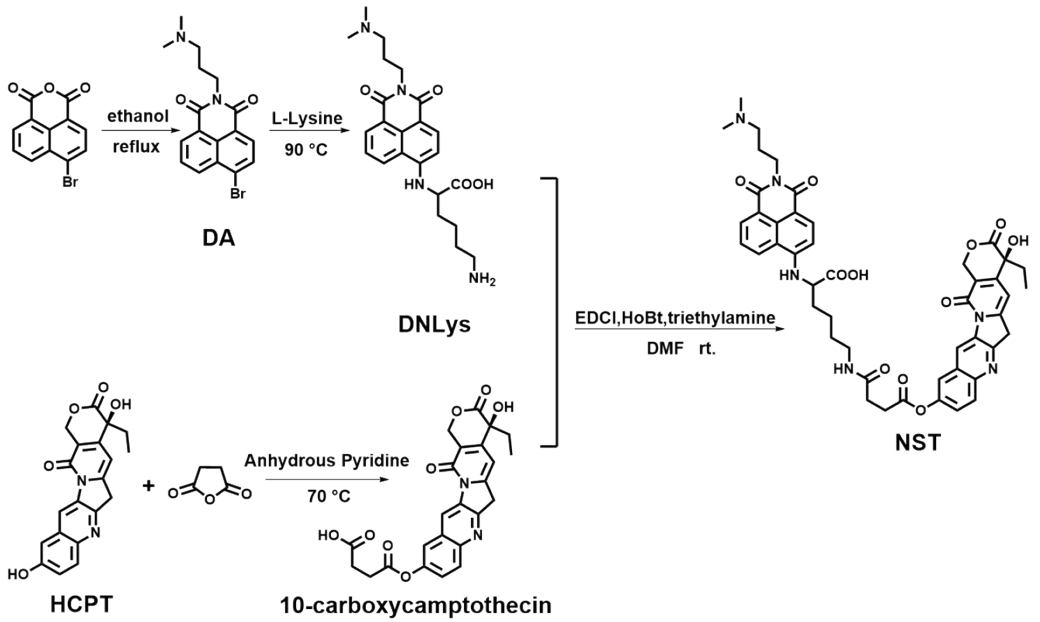
Synthesis of NST (Scheme 1)

3.5 g (12.6 mmol) of 4-bromo-1,8 naphthalenedihydride mixed with 300 mL of ethanol and heated to reflux. After refluxing for 10 minutes, cooled to 50 °C and slowly added 1.7 mL of dimethyl-1,3-diaminopropane (13.3 mmol). The mixed solution was heated under reflux for 1 h, cooled to room temperature, precipitated by adding a large amount of water, and filtered to obtain a precipitate. The precipitate was filtered and washed with water and ethanol three times, and then dried under vacuum to obtain a white solid (DA) with a yield of 89%. MS (ESI): m/z calcd for $C_{17}H_{17}BrN_2O_2$ $[M+H]^+$: 361.05; found: 361.10.

500.0 mg (1.39 mmol) of compound DA, 200.0 mg (1.45 mmol) of K_2CO_3, added into 10 mL DMSO solvent and stirred at 50 °C. After 0.5 h, 550.0 mg (3.02 mmol) of L-lysine was dissolved in 3 mL NaOH (2 M) aqueous solution and dropped into the reaction solution, then heated to 90 °C and stirred for 24 hours. After cooling to room temperature, the solvent was removed under reduced pressure. The solid was purified on a silica gel column with dichloromethane, methanol and ammonia (4: 1: 0.2) to obtain a yellow solid (DNLys) with a yield of 36.9%. MS (ESI): m/z calcd for $C_{23}H_{30}N_4O_4$ $[M+H]^+$: 427.23; found: 427.20.

200.0 mg of HCPT (0.55 mmol) and 600.0 mg (6.0 mmol) of succinic anhydride dissolved in 5.0 mL of anhydrous pyridine and reacted at 70 °C for 8 h. After cooling to room temperature, the solvent was removed and the product was purified on a silica gel column with dichloromethane and methanol (7: 1) to obtain a yellowish solid of 10-carboxycamptothecin. MS (ESI): m/z calcd for $C_{24}H_{20}N_2O_8$ $[M+H]^-$: 463.43; found: 463.11.

120.0 mg (0.26 mmol) of 10-carboxycamptothecin was mixed with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCl), 1-hydroxybenzotriazole (HoBt), and triethylamine at a molar ratio of 1: 2: 1: 1.2, and magnetically stirred for 2 h in an ice bath. Then 1.2 equivalents of DNLys were added and reacted at room temperature for 20 h. The product was purified on a silica gel column with dichloromethane / methanol / ammonia (v / v / v, 15: 1: 0.1) as eluent gave pure NST. ^{1}H NMR (500 MHz, DMSO) δ 8.76 (dd, J = 37.3, 8.6 Hz, 1H), 8.35 (s, 1H), 8.31 (d, J = 6.9 Hz, 1H), 8.19 (dd, J = 22.9, 8.5 Hz, 1H), 7.97 (d, J = 9.1 Hz, 1H), 7.92 (d, J = 5.4 Hz, 1H), 7.62 – 7.56 (m, 1H), 7.54 (d, J = 6.5 Hz, 1H), 7.42 (dd, J = 9.1, 2.5 Hz, 1H), 7.23 (d, J = 2.5 Hz, 1H), 7.00 (s, 1H), 6.64 (dd, J = 45.3, 8.7 Hz, 1H), 5.44 (s, 2H), 5.16 (q, J = 19.2 Hz, 2H), 4.00 (d, J = 6.1 Hz, 4H), 3.03 (s, 2H), 2.81 – 2.60 (m, 4H), 2.41 (s, 6H), 2.19 – 2.02 (m, 2H), 1.96 – 1.77 (m, 4H), 1.42 (t, J = 13.1 Hz, 4H), 1.23 (s, 2H), 0.90 (t, J = 7.4 Hz, 3H). (Fig. S11) HR-MS (ESI): m/z calcd for $C_{47}H_{48}N_6O_{11}$ $[M+H]^+$: 873.3381; found: 873.3447. (Fig. S12)



Scheme S1. Synthesis route of NST.

UV−vis absorption and fluorescence spectra

Stock solution of NST was prepared in DMSO with a concentration of 10.0 mM. The stock solution was diluted to 10.0 μM by different solvents, and the absorption and fluorescence spectra were recorded.

In order to confirm that the NST molecule was dissociated by blue light irradiation, 10.0 μM NST in methanol was irradiated under a blue LED (465-470 nm, 7.96 mW / cm^2) for different time. And then the fluorescence spectra were collected respectively.

High performance liquid chromatography (HPLC) analysis

To further study the photocleaved products of NST molecule, 100.0 μM of NST was irradiated under a blue LED (7.96 mW / cm^2) for 0 min, 15 min, 30 min, 60 min, 90 min, 120 min, 150 min, and 180 min, respectively. And then the different solutions were analyzed by HPLC. The stationary phase used is a

C18 reversed phase chromatographic column (Promosil C18 5μm 4.6 × 250 mm, Agela Technologies). The products were separated by gradient elution at a flow rate of 1.0 mL/min.

Cell culture and imaging

HeLa cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 IU/mL penicillin-streptomycin in a humid atmosphere at 37 °C, 5% CO_2. Prior to imaging, cells were seeded and cultured in confocal dishes for 24 h. To study the photocleveable effect, cells were incubated with 10.0 μM NST in fresh medium without FBS and irradiated for 1 h (465-470 nm, 11.15 mW/cm^2), then further incubated (without washing) for different times according to the experimental needs. Before imaging, the cells were washed 3 times with PBS. Fluorescence images of HCPT and 4-aminonaphthalimide were collected in the range of 430-470 nm and 500-570 nm with 405 nm excitation respectively.

For location studies, cells were incubated with NST (10.0 μM), HCPT or DNNH in fresh medium without FBS and irradiated under a blue LED (11.15 mW / cm^2) for 60 min firstly, then further incubated (without washing) for 60 and 180 min respectively. After washing with PBS, HeLa cells further incubated with different commercial dyes, LysRed, MTRed, ER-Red, or Hoechst 33342 for 30 min, respectively. After washed 3 times with PBS, the fluorescence images were collected immediately. Fluorescence images of LysRed and MTRed were collected in the range of 570-620 nm under 561 nm excitation.

Cytotoxicity

HeLa cells were seeded into 96-well plates (approximately 5000 cells per well) and cultured for 24 hours, then various concentrations of NST, DNNH, and HCPT (0, 1.25, 2.5, 5.0, 10.0, 15.0, and 20.0 μM) were added, respectively. For dark cytotoxicity, the cells were further incubated for 48 h. For photo cytotoxicity, the cells were irradiated under a blue LED (465-470 nm, 11.15 mW/cm^2) for 0, 10, 30, 60 min respectively, and then further incubated for 48 h. The cytotoxicity was detected using a CCK-8 method. Cytotoxicity studies for HEK293, PC3, A549T, and LoVo cells were also carried out without or with 60 min of irradiation respectively.

Flow cytometry

Flow cytometry analysis was carried out to study the uptake of NST and its photocleaved products by the cells. Typically, HeLa cells were seeded and cultured in 6-well plates for 24 h, then added 10.0 μM NST and exposed to a blue LED (465-470 nm, 11.15 mW/cm^2) for 60 min. After further cultured for different times (30, 60, 90, 120, 150 min), the cells were digested and washed three times with PBS, then analyzed by flow cytometry.

Cell apoptosis analysis

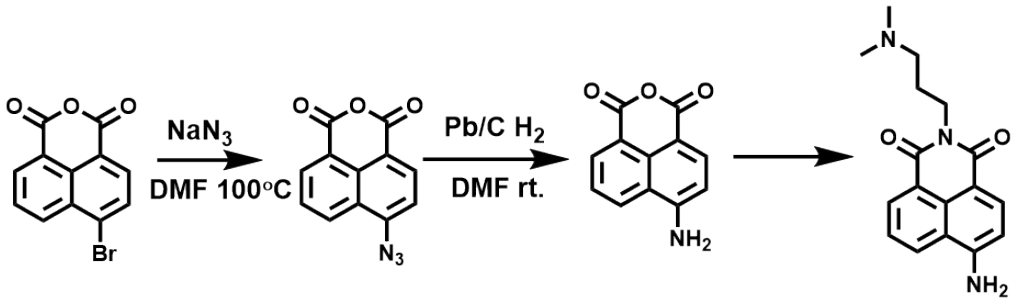
HeLa cells were seeded in different culture dishes at a density of 1×10^6 cells/dish for 24 h. The cells were added with NST, DNNH, or HCPT and treated with or without irradiation under a blue LED (11.15 mW / cm^2) for 1 h. Then the cells were further cultured for 1, 5, 12, 24 h respectively. Finally, the cells were digested by 5.0 mM EDTA in PBS, collected and washed with PBS for three times. After double-stained with Annexin V-Alexa Fluor 647 and PI, the cells were analyzed by flow cytometry. In addition, cells only treated with drugs/without irradiation and with irradiation/without drugs were used as control. The detailed list of all experimental conditions was shown in Table S1.

Table S1. Cell apoptosis protocol.

Sample Name	Reagent type (10.0 μM)	Irradiation time (h)	Incubation time (h)
A1	NST	0	0
A2	NST	0	1
A3	NST	0	5
A4	NST	0	12
A5	NST	0	24
A6	NST	1	0
A7	NST	1	1
A8	NST	1	5
A9	NST	1	12
A10	NST	1	24
A11	none	1	0
A12	none	1	1
A13	none	1	5
A14	none	1	12
A15	none	1	24

Sample Name	Reagent type (10 μM)	Irradiation time (h)	Incubation time (h)
B1	HCPT	0	0
B2	HCPT	0	1
B3	HCPT	0	5
B4	HCPT	0	12
B5	HCPT	0	24
B6	HCPT	1	0
B7	HCPT	1	1
B8	HCPT	1	5
B9	HCPT	1	12
B10	HCPT	1	24

Sample Name	Reagent type (10 μM)	Irradiation time (h)	Incubation time (h)
C1	DNNH	0	0
C2	DNNH	0	1
C3	DNNH	0	5
C4	DNNH	0	12
C5	DNNH	0	24
C6	DNNH	1	0
C7	DNNH	1	1
C8	DNNH	1	5
C9	DNNH	1	12
C10	DNNH	1	24



Scheme S2. Synthesis of DNNH. (DNNH was synthesized according to the previous reported synthesis method.[1])

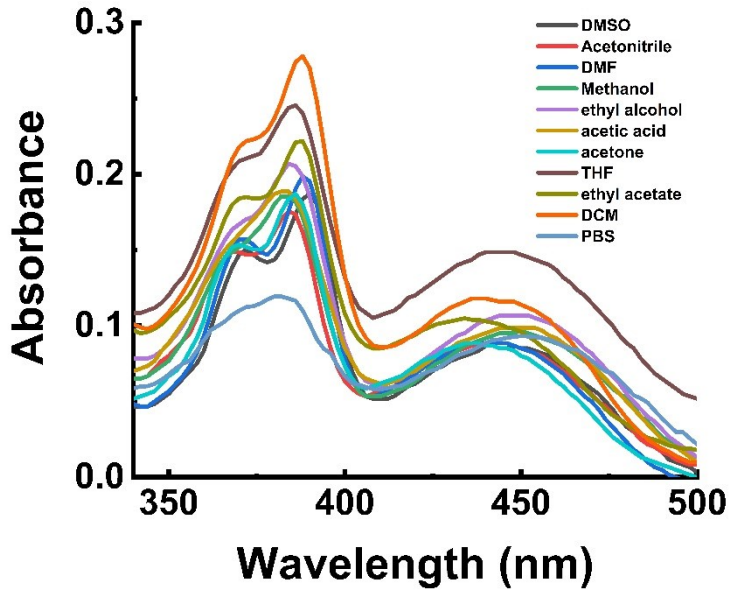


Fig. S1 UV-Vis absorption spectra of NST in different solvents.

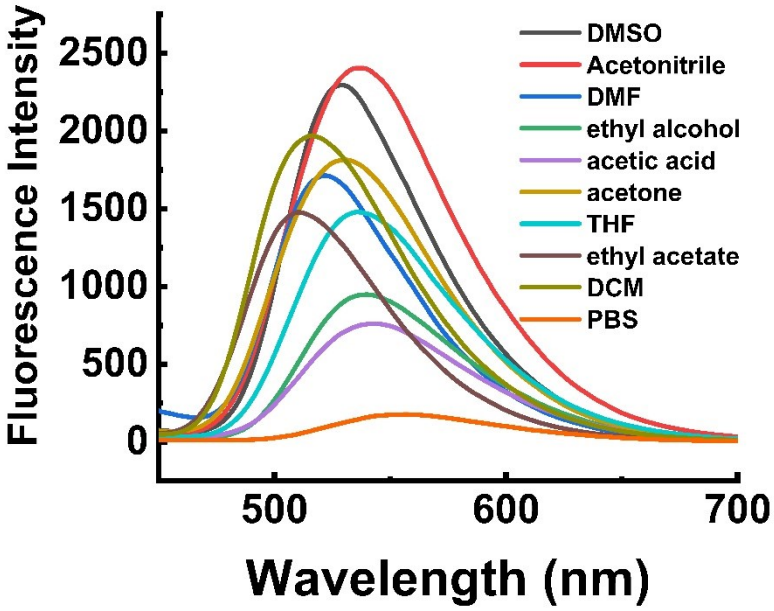


Fig. S2 Fluorescence spectra (λ_{ex} 385nm) of NST in different solvents.

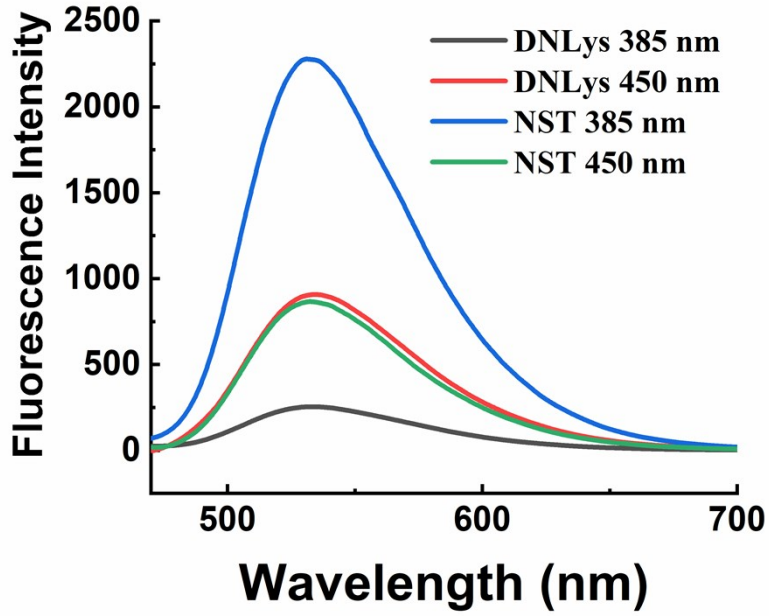


Fig. S3 Fluorescence spectra of NST and DNLys (10.0 μM) under different excitation wavelength.

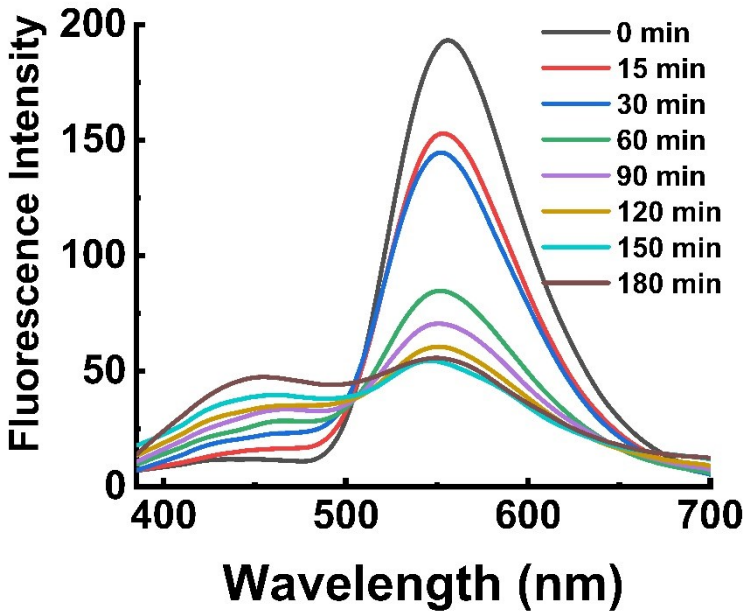


Fig. S4 Fluorescence spectra of NST dissolved in PBS after irradiation for different time under a blue LED (7.96 mW/cm^2, λ_{ex} 385 nm).

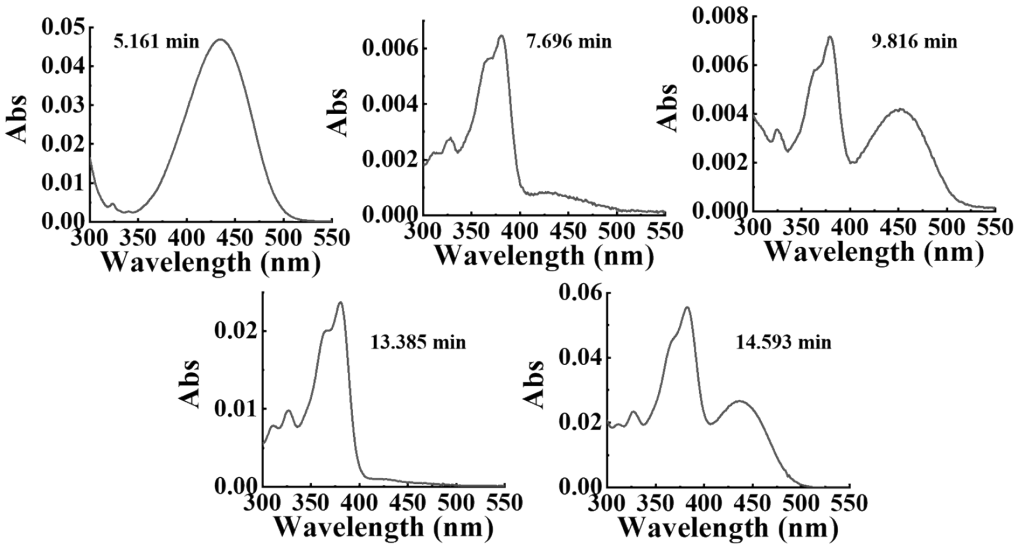


Fig. S5 Corresponding absorption spectra of NST and its photocleavage products at different retention time.

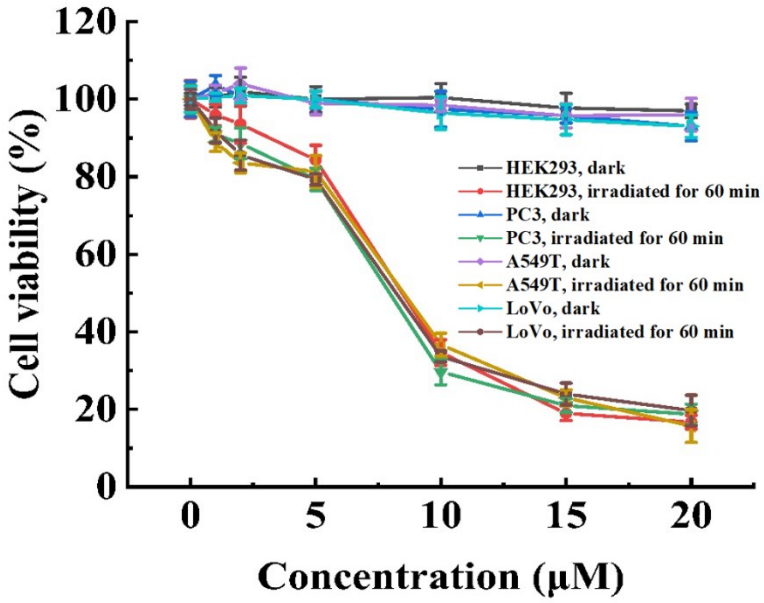


Fig. S6 Cytotoxicity of NST to HEK293, PC3, A549T, and LoVo cells with or without irradiation. After irradiation (60 min, 11.15 mW/cm^2), cells were further cultured for 48 h.

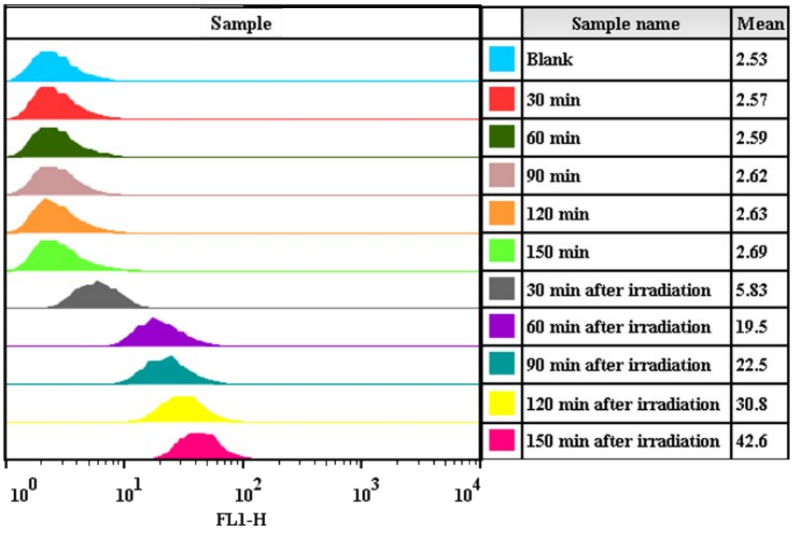


Fig. S7 Flow cytometry analysis of the interaction between NST and HeLa cells before and after light irradiation.

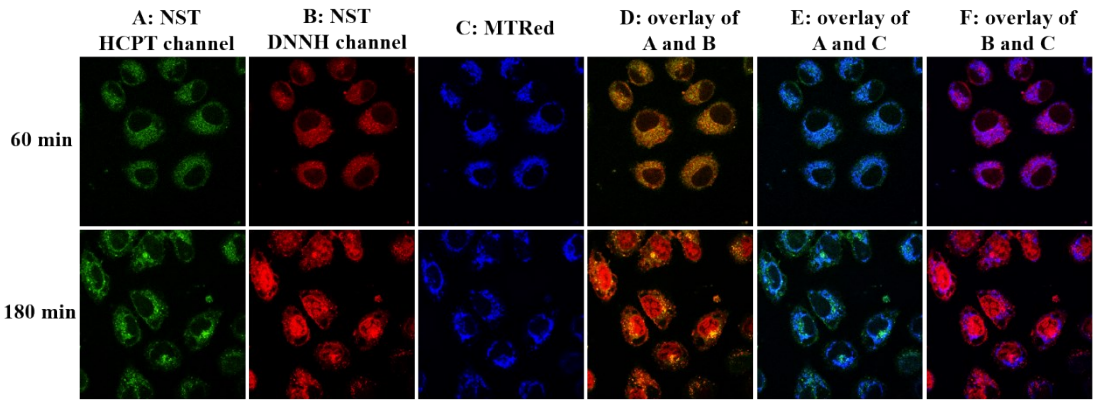


Fig. S8 Confocal images of HeLa cells co-stained by the photocleaved products of NST and MTRed. Irradiated for 1 h and then further incubated for 60 and 180 min respectively. Pseudo green fluorescence (HCPT channel, λ_{ex} 405 nm); pseudo red fluorescence (DNNH channel, λ_{ex} 405 nm); pseudo blue fluorescence (MTRed channel, λ_{ex} 561 nm).

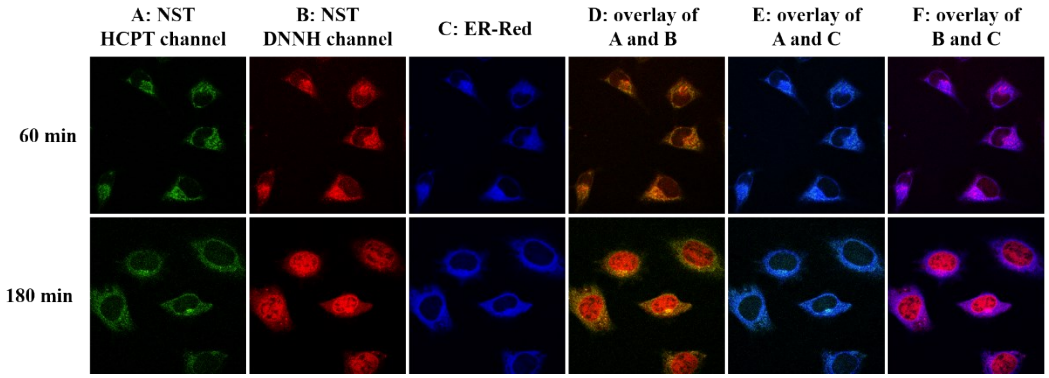


Fig. S9 Confocal images of HeLa cells co-stained by the photocleaved products of NST and ER-Red. Irradiated for 1 h and then further incubated for 60 and 180 min respectively. Pseudo green fluorescence (HCPT channel, λ_{ex} 405 nm); pseudo red fluorescence (DNNH channel, λ_{ex} 405 nm); pseudo blue fluorescence (ER-Red channel, λ_{ex} 561 nm).

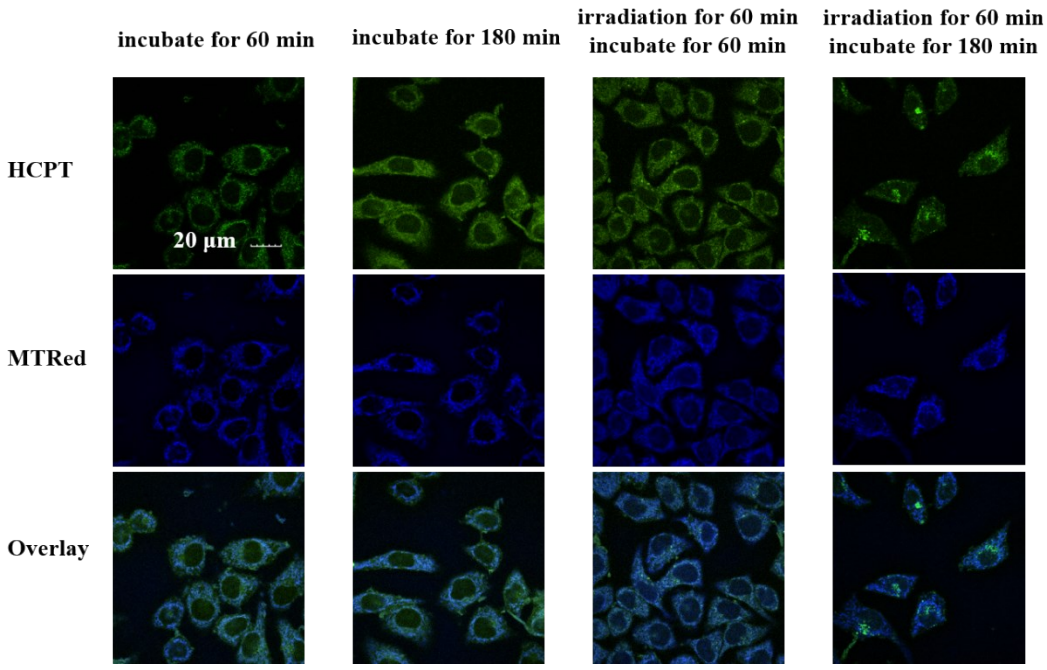


Fig. S10 Confocal images of HeLa cells co-stained by HCPT and MTRed. Two columns on the left: incubated with HCPT 60 and 180 min without irradiation; two columns on the right: irradiated for 1 h and then further incubated for 60 and 180 min respectively; pseudo green fluorescence (HCPT channel, λ_{ex} 405 nm); pseudo blue fluorescence (MTRed channel, λ_{ex} 561 nm).

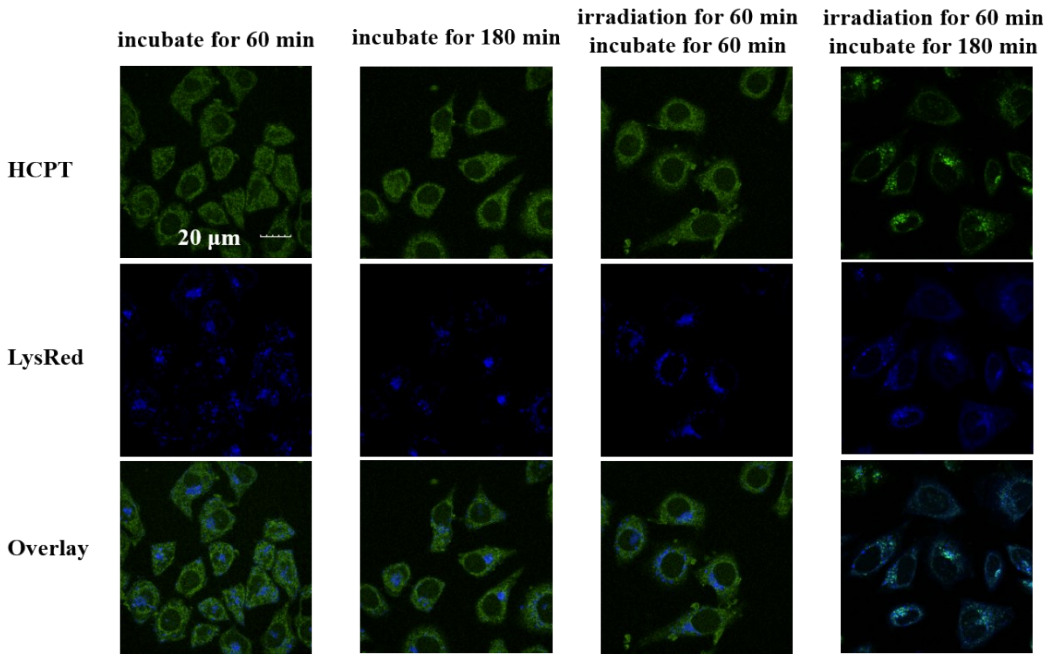


Fig. S11 Confocal images of HeLa cells co-stained by HCPT and LysRed. Two columns on the left: incubated with HCPT 60 and 180 min without irradiation; two columns on the right: irradiated for 1 h and then further incubated for 60 and 180 min respectively; pseudo green fluorescence (HCPT channel, λ_{ex} 405 nm); pseudo blue fluorescence (LysRed channel, λ_{ex} 561 nm).

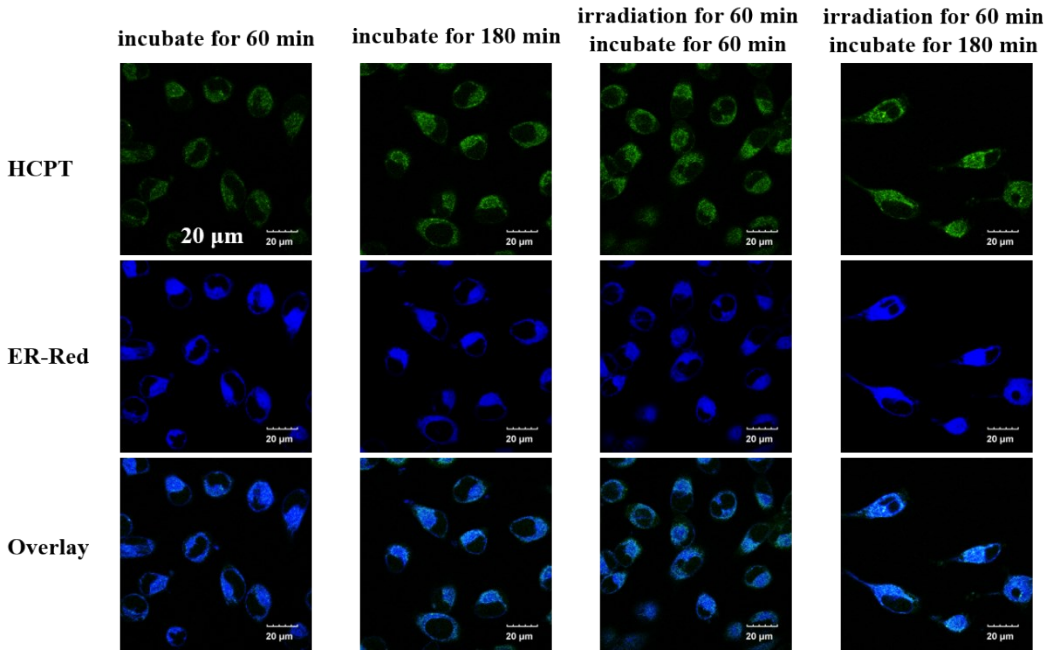


Fig. S12 Confocal images of HeLa cells co-stained by HCPT and ER-Red. Two columns on the left: incubated with HCPT 60 and 180 min without irradiation; two columns on the right: irradiated for 1 h and then further incubated for 60 and 180 min respectively; pseudo green fluorescence (HCPT channel, λ_{ex} 405 nm); pseudo blue fluorescence (ER-Red channel, λ_{ex} 561 nm).

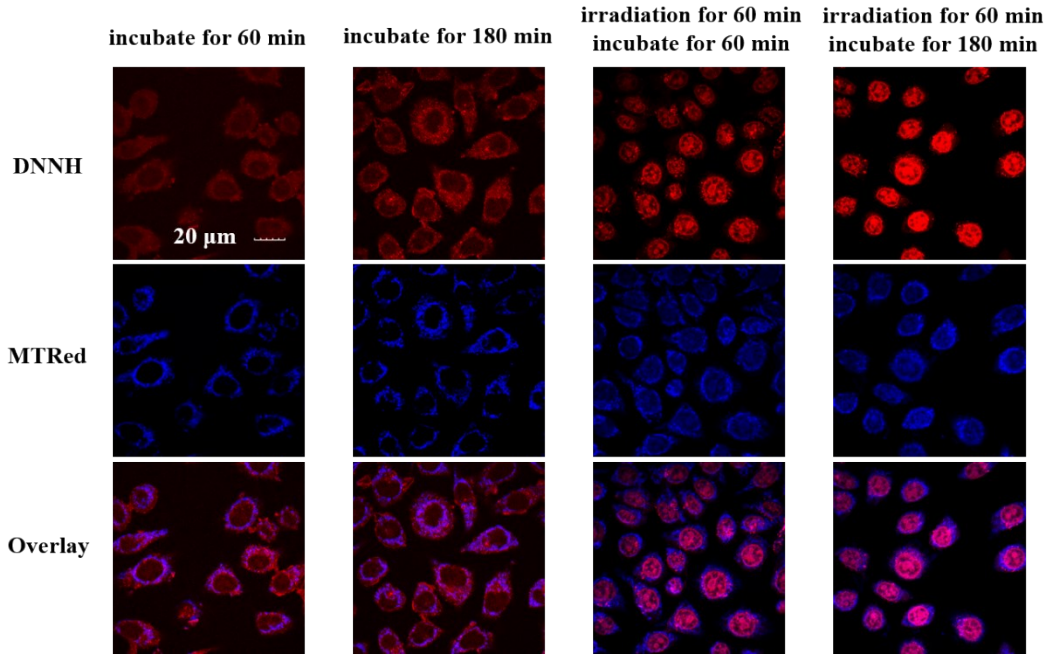


Fig. S13 Confocal images of HeLa cells co-stained by DNNH and MTRed. Two columns on the left: incubated with DNNH 60 and 180 min without irradiation; two columns on the right: irradiated for 1 h and then further incubated for 60 and 180 min respectively; pseudo red fluorescence (DNNH channel, λ_{ex} 405 nm); pseudo blue fluorescence (MTRed channel, λ_{ex} 561 nm).

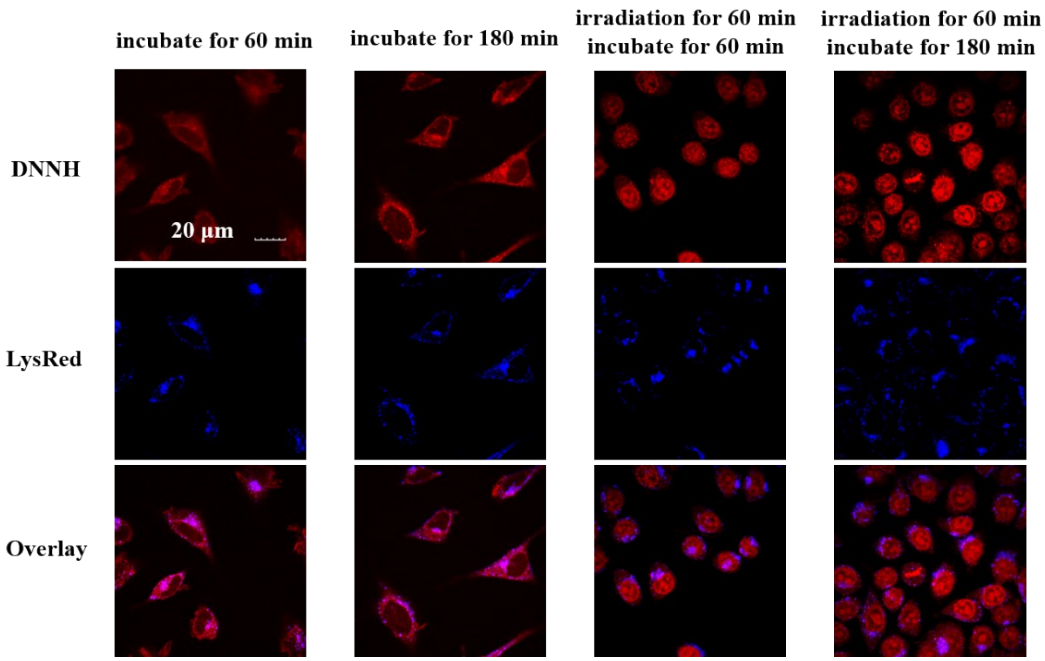


Fig. S14 Confocal images of HeLa cells co-stained by DNNH and LysRed. Two columns on the left: incubated with DNNH 60 and 180 min without irradiation; two columns on the right: irradiated for 1 h and then further incubated for 60 and 180 min respectively; pseudo red fluorescence (DNNH channel, λ_{ex} 405 nm); pseudo blue fluorescence (LysRed channel, λ_{ex} 561 nm).

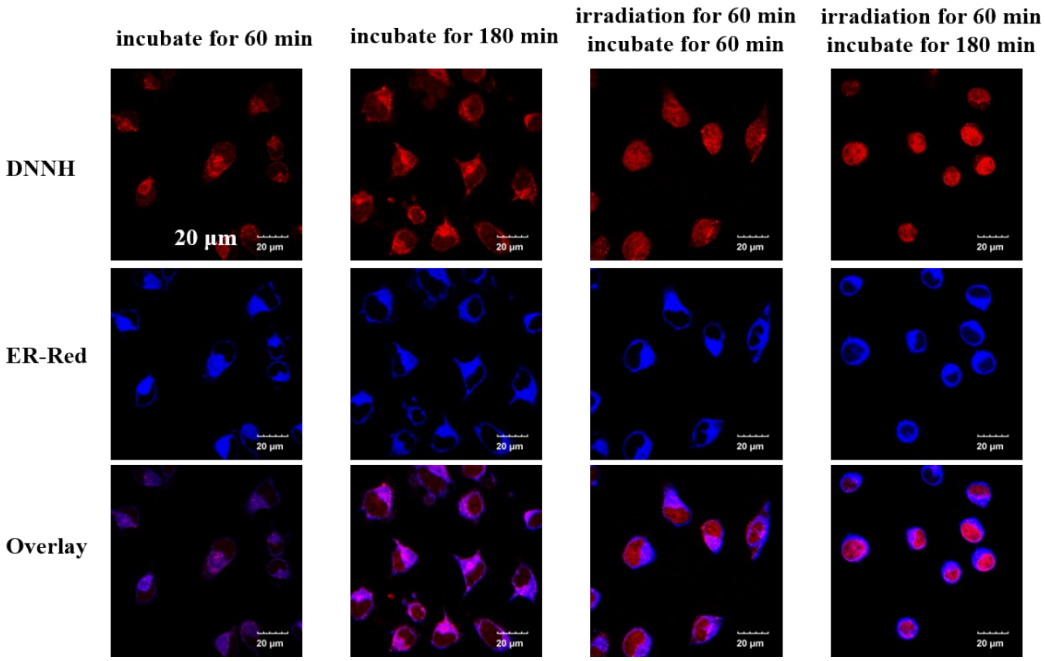


Fig. S15 Confocal images of HeLa cells co-stained by DNNH and ER-Red. Two columns on the left: incubated with DNNH 60 and 180 min without irradiation; two columns on the right: irradiated for 1 h and then further incubated for 60 and 180 min respectively; pseudo red fluorescence (DNNH channel, λ_{ex} 405 nm); pseudo blue fluorescence (ER-Red channel, λ_{ex} 561 nm).

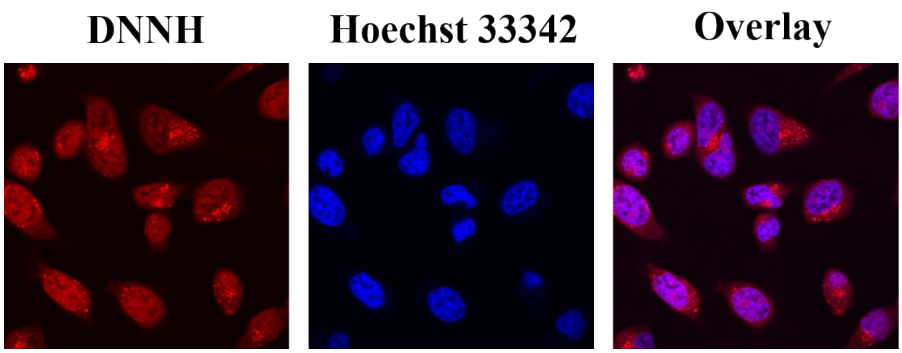


Fig. S16 Confocal images of HeLa cells co-stained by DNNH and Hoechst 33342. Irradiated for 1 h and then further incubated for 60 min. Pseudo red fluorescence (DNNH channel, λ_{ex} 405 nm); pseudo blue fluorescence (Hoechst 33342 channel, λ_{ex} 405 nm).

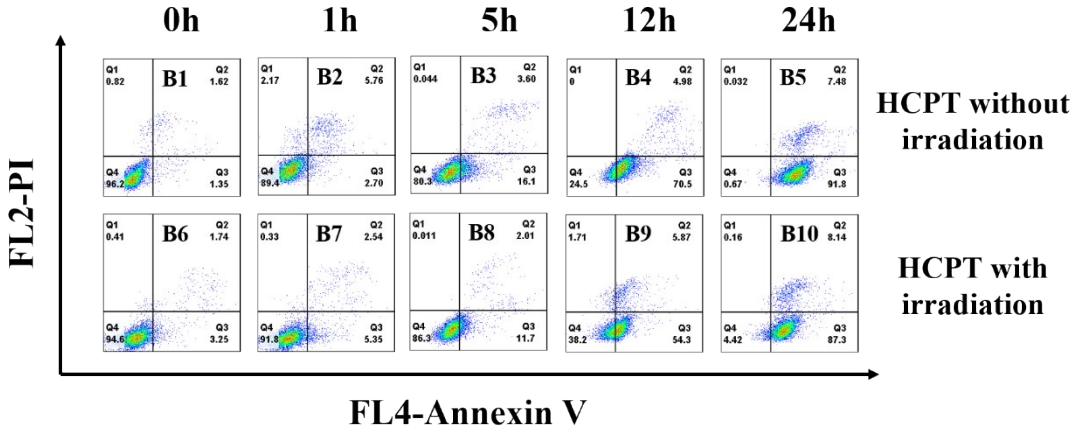


Fig. S17 Apoptosis analysis of HeLa cells treated by HCPT.

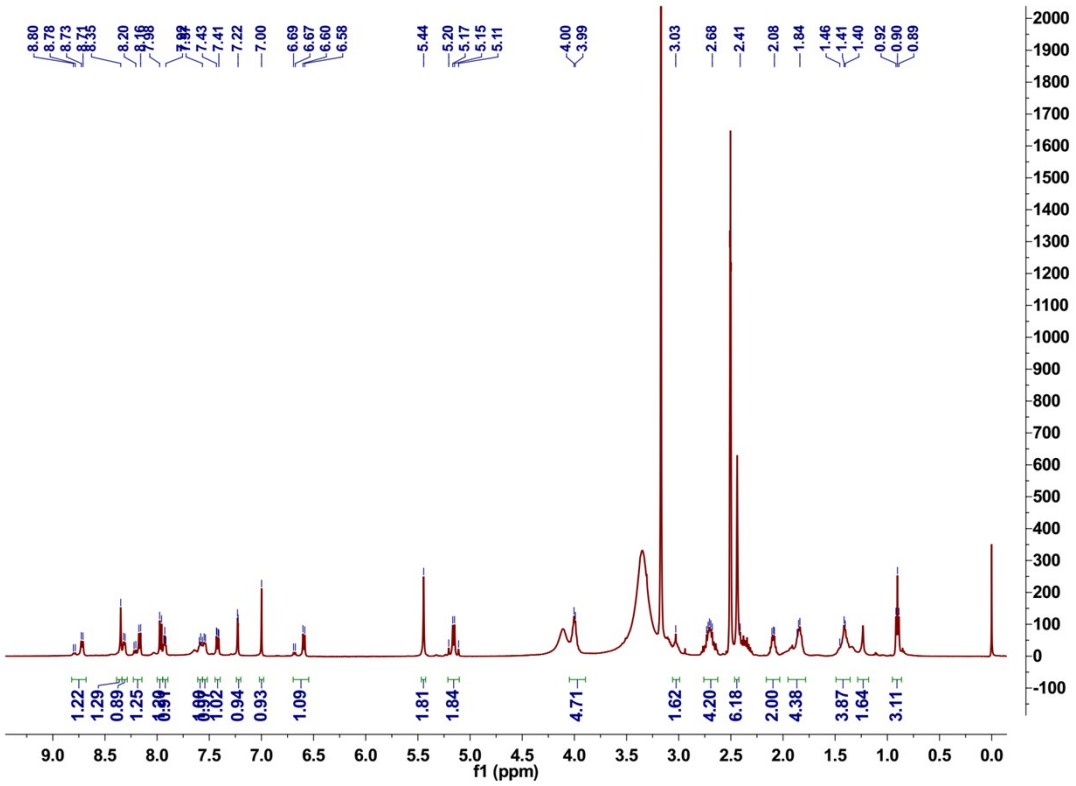
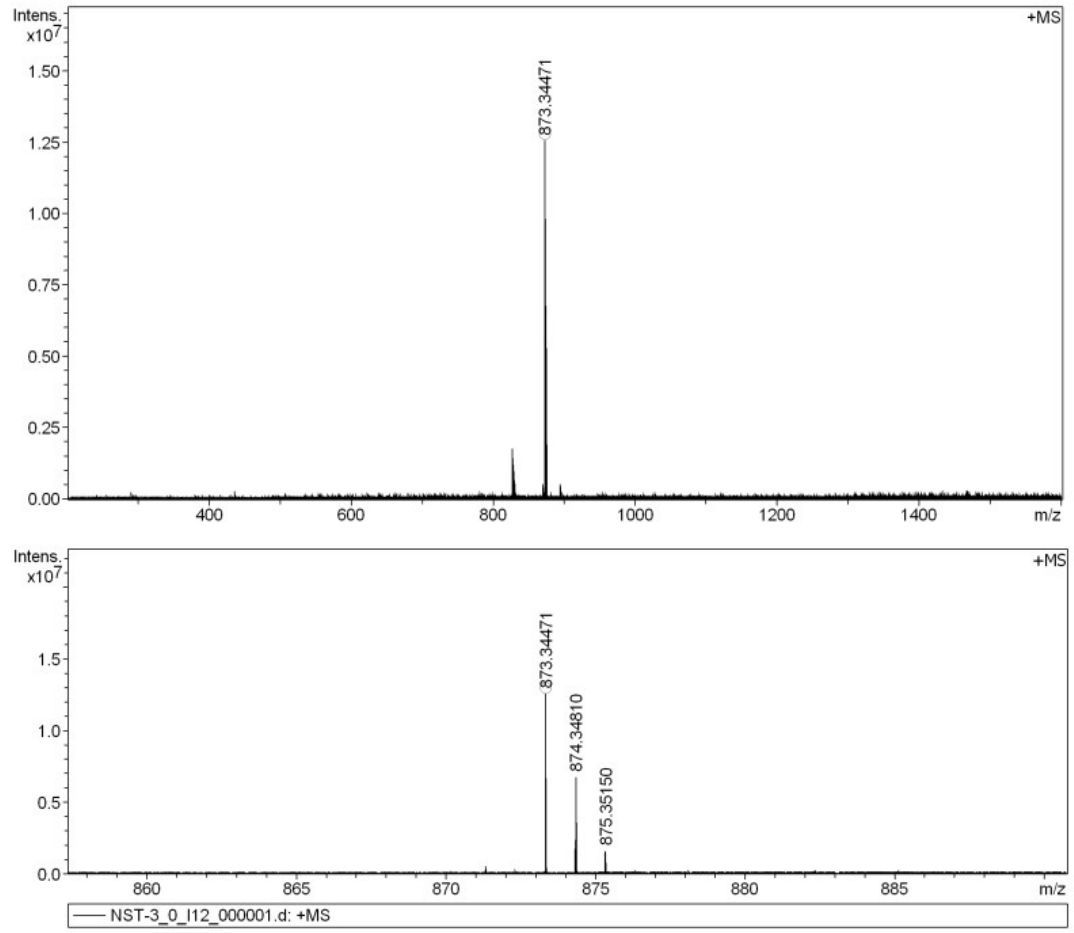


Fig. S18 ^{1}H NMR of NST.

Acquisition Parameter

Acquisition Mode	Single MS	Acquired Scans	2	Calibration Date	Thu Nov 19 06:05:11
Polarity	Positive	No. of Cell Fills	1	Data Acquisition Size	2097152
Broadband Low Mass	202.1 m/z	No. of Laser Shots	10	Data Processing Size	4194304
Broadband High Mass	1600.0 m/z	Laser Power	28.0 lp	Apodization	Sine-Bell Multiplication
Source Accumulation	0.001 sec	Laser Shot Frequency	0.020 sec		
Ion Accumulation Time	0.100 sec				



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e¯ Conf	N-Rule
873.344707	1	C47H49N6O11	100.00	873.345383	-0.8	0.6	23.7	26.5	even	ok

Fig. S19 HR-MS (ESI) of NST.

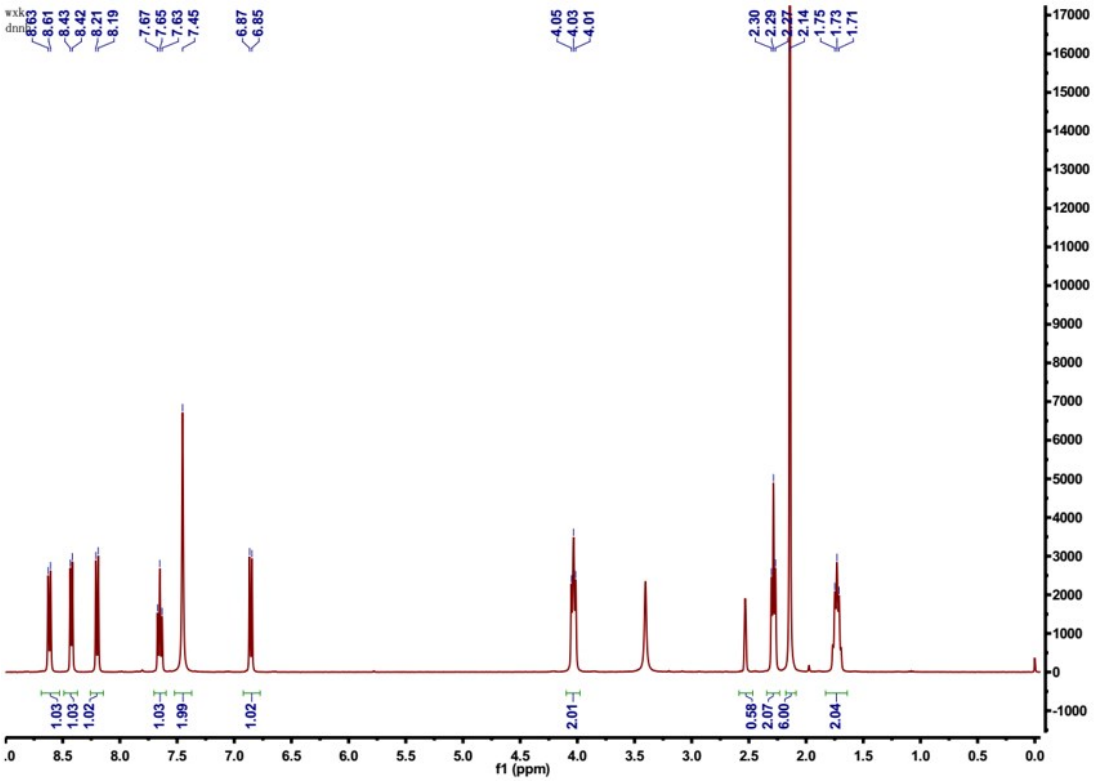


Fig. S20 ^{1}H NMR of DNNH.

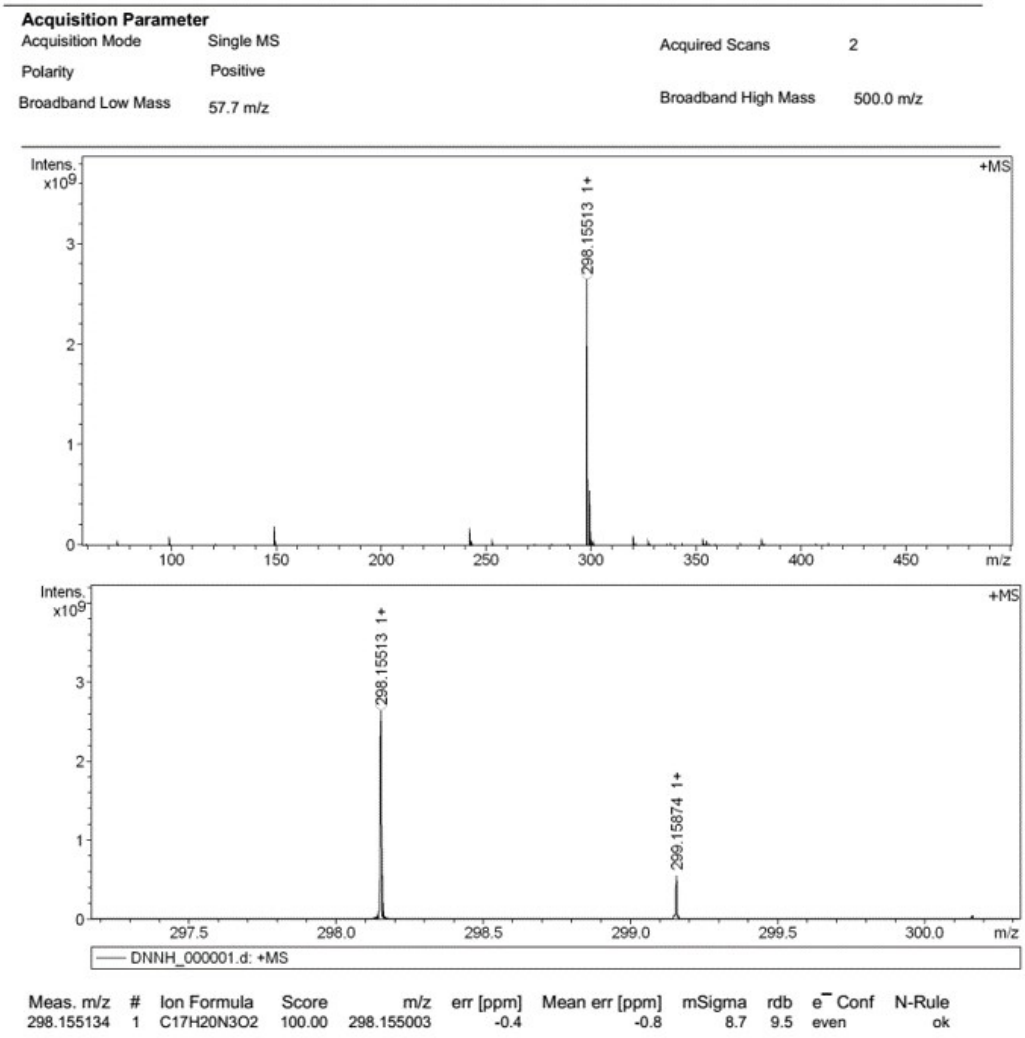


Fig. S21 HR-MS (ESI) of DNNH.

Notes and references

1. J. Zhou, C. Fang, Y. Liu, Y. Zhao, N. Zhang, X. Liu, F. Wang and D. Shangguan, *Org. Biomol. Chem.*, 2015, **13**, 3931-3935.