Supporting Information

Dual-Emission and Large Stokes Shift Fluorescence Probe for Real-time Discrimination of ROS/RNS and Its Imaging in Living Cells

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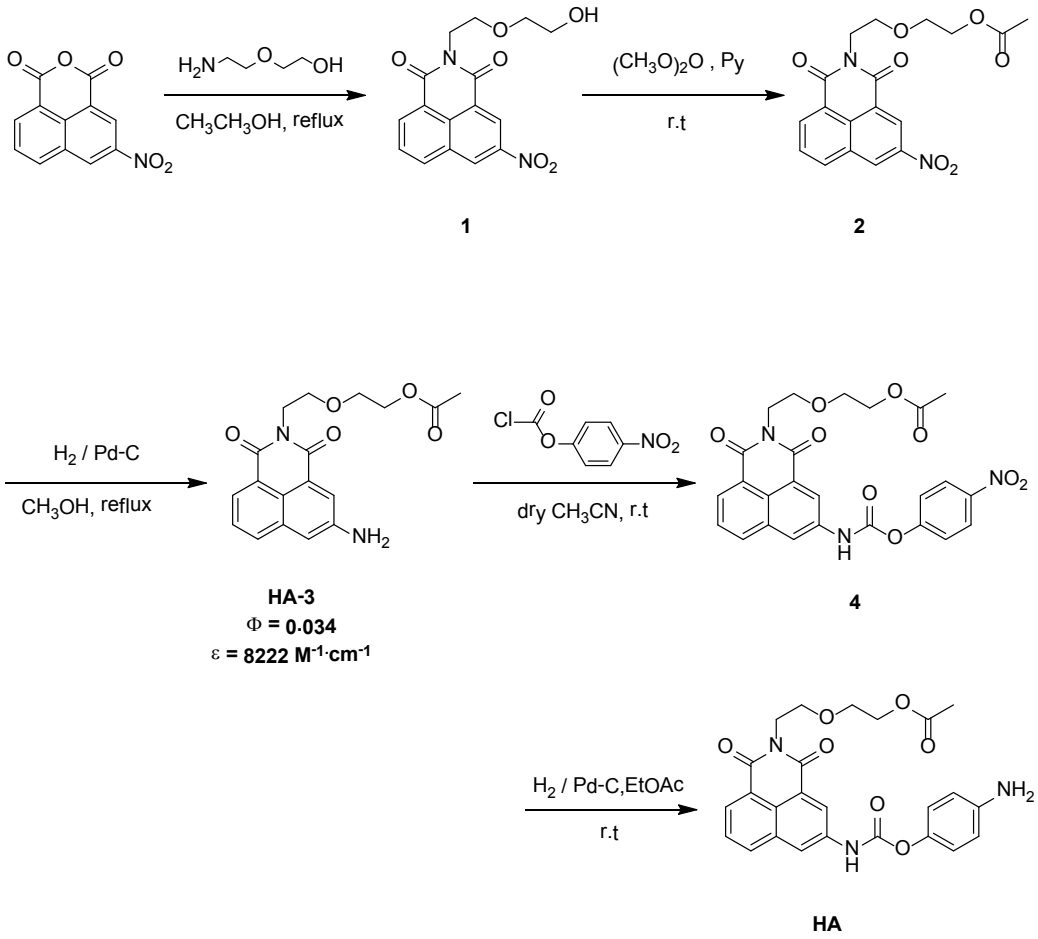
1. Materials and Instruments

Silica gel P60 (Qingdao) was used for column chromatography. All chemicals were purchased from TCI and Aladdin reagent Co without further purification except especial instruction. All the organic solvents were of analytical grade. Acetonitrile were distilled by CaH_2 to remove the water before used. Water was purified by a Milli-Q system.

^{1}H-NMR spectra were collected in $CDCl_3$ and DMSO-d_6 at 25 ºC on a Bruker AV-400 spectrometer at NMR Facility of East China University of Science and Technology (ECUST), which chemical shifts reported in ppm (TMS as internal standard). Mass spectral analyses were carried out at the Analysis and Test Center of East China University of Science and Technology (ECUST).

2. Synthesis



Scheme S1 Synthesis of probe **HA** and reaction product **HA-3**

N-[2-(2-hydroxyethoxy) ethyl]-3-nitro-1, 8-naphthalimide (Compound 1)

A mixture of 3-nitro-1, 8-naphthalimide (500.0 mg, 2.1 mmol) and 2-(2-aminoethoxy) ethanol (310 μL, 2.1mmol) were dissolved in 20 mL ethanol in a single necked flask. The reaction mixture

was heated to reflux for 4 h. After cooling to room temperature, the solution was removed under reduced pressure and the crude product was purified by flash column chromatography on silica gel (100:1 CH_2Cl_2/ CH_3OH) to obtain target compound **1** as a yellow solid (461.0 mg, 68%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 1.12 (s, 1H), 3.67-3.69 (m, 4H), 3.88 (t, *J* = 5.1 Hz, 2H), 4.48 (t, *J* = 5.1Hz, 2H), 7.96 (t, *J* = 7.7 Hz, 1H), 8.44 (d, *J* = 8.2 Hz, 1H), 8.79 (d, *J* = 7.2 Hz, 1H), 9.15 (s, 1H), 9.31 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 39.9, 61.8, 68.2, 72.2, 123.1, 124.4, 124.5, 129.1, 129.2, 130.3, 131.0, 134.7, 135.7, 146.4, 162.8, 163.4.

***N*-[2-(2- acetoxyethoxy) ethyl]-3-nitro-1,8-naphthalimide (Compound 2)**

A mixture of compound **1** (240.0 mg, 0.73 mmol) and acetic anhydride (726 μL, 0.73 mmol) in pyridine (5 mL) was stirred at room temperature for overnight. The solvent was removed under reduced pressure. The crude product was purified by flash column chromatography (silica gel, CH_2Cl_2) to obtain target compound **2** as a yellow solid (189.0 mg, 70%).
^{1}H NMR (400 MHz, $CDCl_3$) δ 1.99 (s, 3H), 3.77 (t, *J* = 4.4 Hz, 2H), 3.88 (t, *J* = 6.0 Hz, 2H), 4.20 (t, *J* = 4.6 Hz, 2H), 4.49 (t, *J* = 5.8 Hz, 2H), 7.97 (t, *J* = 7.8 Hz, 1H), 8.46 (d, *J* = 8.0 Hz, 1H), 8.80 (d, *J* = 7.2 Hz, 1H), 9.16 (d, *J* = 2.0 Hz, 1H), 9.33 (d, *J* = 2.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.9, 39.4, 63.5, 67.7, 68.5, 123.1, 124.3, 124.6, 129.0, 129.1, 130.3, 131.0, 134.5, 135.6, 146.4, 162.6, 163.2, 170.9.

N-[2-(2- acetoxyethoxy) ethyl]-3-amino-1,8-naphthalimide (HA-3)

A mixture of compound **2** (900.0 mg, 2.4 mmol) and Pd/C in methanol (30 mL) were dissolved in a single necked flask. The mixture reaction was heated to reflux under hydrogen for 4 hours. Then, the solution was cooled to room temperature, filtered. The solvent was removed under reduced pressure. The crude product was purified by flash column chromatography (silica gel, 50:1 CH_2Cl_2/ CH_3OH) to obtain target compound **HA-3** as a yellow solid (537.5 mg, 65%).
^{1}H NMR (400 MHz, DMSO-d_6) δ 1.89 (s, 3H), 3.63 (t, *J* = 4.6 Hz, 2H), 3.66 (t, *J* = 6.2 Hz, 2H), 4.06 (t, *J* = 4.8 Hz, 2H), 4.22 (t, *J* = 6.3 Hz, 2H), 6.01 (s, 2H), 7.29 (d, *J* = 2.3 Hz, 1H), 7.62 (t, *J* = 7.8 Hz, 1H), 7.98 (d, *J* = 2.3 Hz, 1H), 8.04 (d, *J* = 8.1 Hz, 1H), 8.08 (d, *J* = 7.2 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 20.9, 38.9, 63.5, 67.3, 68.4, 112.1, 121.1, 122.1, 122.2, 122.9, 125.9, 127.4, 132.0, 134.0, 148.4, 164.1, 164.3, 170.7.

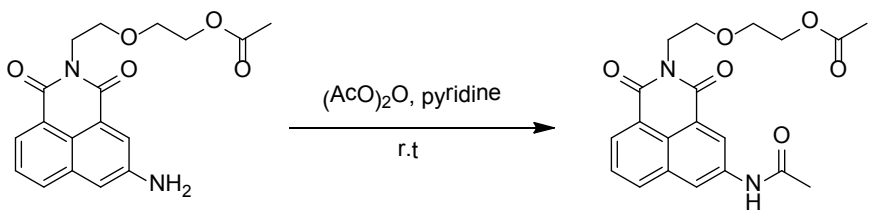
2-(2-(5-(4-nitrophenoxy) carbonyl) amino) - N-[2-(2-acetoxyethoxy) ethyl]-3-amino-1, 8-naphthalimide (Compound 4)

The compound **HA-3** (300 mg, 87.6 mmol) was dissolved in 50 mL anhydrous acetonitrile in a single necked flask. A mixture of 4-nitrophenyl chloroformate (212.0 mg, 105.2 mmol) and 10 mL

anhydrous acetonitrile was added to the reaction solution in 0°C under argon. Then the reaction mixture was stirred at room temperature for overnight. The solution was removed under reduced pressure and the crude product was obtained as a pale yellow solid. The crude product was used without further purification.

2-(2-(5-(4-aminophenoxy) carbonyl) amino) - N-[2-(2-acetoxyethoxy) ethyl]-3-amino- 1, 8-naphthalimide (Probe HA)

To a single necked flask, a mixture of compound **4** (20 mg, 39.4 μmol) and Pd/C were dissolved in AcOEt (50 mL). The mixture reaction was stirred under hydrogen over night at room temperature. The solvent was removed under reduced pressure. The crude product was purified by flash column chromatography (silica gel, 5:1 AcOEt/CH_2Cl_2) to obtain probe **HA** as a yellow solid (12.5 mg, 63%). The target compound was confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.88 (s, 3H), 3.68 (t, J = 9.6 Hz, 2H), 3.69 (t, J = 6.3 Hz, 2H), 4.07 (t, J = 9.2 Hz, 2H), 4.25 (t, J = 6.2 Hz, 2H), 5.06 (s, 2H), 6.60 (d, J = 8.7 Hz, 2H), 6.92 (d, J = 8.7 Hz, 2H), 7.81 (t, J = 7.8 Hz, 1H), 8.34-8.37 (m, 2H), 8.56 (s, 1H), 8.62 (s, 1H), 10.68 (s, 1H). ^{13}C NMR (100MHz, DMSO-d_6) δ 20.9, 39.1, 63.5, 67.2, 68.4, 114.5, 119.7, 120.2, 122.5, 123.3, 123.7, 124.3, 128.1, 129.4, 132.6, 134.1, 138.4, 140.9, 146.9, 153.2, 163.6, 163.9, 170.6. HRMS (ESI): Calcd for $C_{25}H_{23}N_3O_7$ ($M+Na^+$) 500.1434; Found, 500.1432.



Scheme S2 Synthesis of **CA**

2-[2-(dimethylamino) ethyl] - N-[2-(2- acetoxyethoxy) ethyl]-3-amino-1,8-naphthalimide (CA)

The compound HA-3 (100 mg, 0.29 mmol) was dissolved in 4 mL pyridine/acetic anhydride mixture (1:1, v/v) in a single necked flask. The reaction mixture was stirred for 24 hrs at room temperature and then evaporated to dryness under vacuum. The residual yellow oil was dissolved in dichloromethane (50 mL), and the solution was washed with water (2 × 50mL).The organic layer was collected and dried over anhydrous sodium sulfate and evaporated. The crude product was purified by flash column chromatography (silica gel, 1:5 AcOEt/CH_2Cl_2) to obtain compound

CA as a pale yellow solid (91.8 mg, 82%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 1.88 (s, 3H), 2.16 (s, 3H), 3.64 (t, J = 4.6 Hz, 2H), 3.68 (t, J = 6.2 Hz, 2H), 4.06 (t, J = 4.8 Hz, 2H), 4.24 (t, J = 6.2 Hz, 2H), 7.80 (t, J = Hz, 2H), 8.36 (d, J = 3.8 Hz, 1H), 8.59 (d, J = 1.8 Hz, 1H), 8.77 (d, J = 1.8 Hz, 1H), 10.56 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 20.9, 24.59, 39.1, 63.5, 67.3, 68.4, 120.9, 122.2, 123.0, 124.2, 124.4, 128.0, 129.4, 132.6, 134.2, 138.5. HRMS (ESI): Calcd for $C_{20}H_{20}N_2O_6$ ($M+Na^+$) 407.1219; Found, 407.1237.

3. Methods

3.1 Spectroscopic Materials and Methods

Double distilled water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 0.1 M phosphate buffer (pH 7.4, 1% DMF) at room temperature. All pH measurements were made with a Sartorius basic pH-Meter PB-10. Absorption spectra were recorded using a Varian Cary100 Bio UV-Visible spectrophotometer. Fluorescence spectra were recorded using a Varian Cary Eclipse scanning spectrofluorometer equipped with a Xenon flash lamp. Samples for absorption and fluorescence measurements were contained in 1 cm×1 cm quartz cuvettes (3.5 mL volume).

3.2 Determination of Quantum Yield

$$\Phi_1 = \frac{\Phi_B I_1 A_B \lambda_{exB} \eta_1}{I_B A_1 \lambda_{ex1} \eta_B}$$

Where Φ is quantum yield; I is integrated area under the corrected emission spectra; A is absorbance at the excitation wavelength; λex is the excitation wavelength; η is the refractive index of the solution; the subscripts 1 and B refer to the unknown and the standard, respectively.

We chose Quinine sulfate with 0.1 M H_2SO_4 as standard, which has the quantum yield of 0.58.[S1] The quantum yields of **HA-3** and **CA** were calculated as 0.034 and 0.217, respectively.

3.3 Generation of Various ROS and RNS

Generation of OCl^- : The source of NaOCl was commercial bleach.[S2] The concentration of the OCl^- stock solution was determined by measuring the absorbance at 209 nm with a molar extinction coefficient of 350 M^{-1} cm^{-1}.

Generation of peroxynitrite ($ONOO^-$): A mixture of sodium nitrite (0.6 M) and hydrogen peroxide (0.7 M) was acidified with hydrochloric acid (0.6 M), and sodium hydroxide (1.5 M) was added within 1~2 s to make the solution alkaline. [S3] The resulting solution was stored at lower than -18 °C. The solution was thawed immediately before use. The concentration of the $ONOO^-$

stock solution was determined in 0.1 M NaOH by measuring the absorbance at 302 nm with a molar extinction coefficient of 1670 M^{-1} cm^{-1}.

Generation of •OH: Hydroxyl radical (•OH) was generated in the Fenton system from ferrous ammonium sulfate and hydrogen peroxide. [S4]

Generation of H_2O_2: H_2O_2 solution was added directly. The stock H_2O_2 solution was purchased from Sigma-Aldrich. The concentration of H_2O_2 was determined by measuring the absorbance at 240 nm with a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

Generation of $O_2^{\bullet-}$: Solid potassium superoxide was used as superoxide radical anion source. [S5]

Generation of ROO•: A 10 mM stock solution of ^{t}BuOOH was firstly prepared in deionizer water and then added into the probe testing solutions.

3.4 HPLC for Probe HA with Various ROS

HPLC was performed on a ZoRBAX RX-C18 column (Analytical 4.6×250mm 5-Micron, Agilent) with a HP 1100 system. The HPLC solvents employed were 15% acetonitrile and 85% buffer (acetic acid and ammonium acetate pH=6.0). HPLC conditions were as follows: solvent A: solvent B = 0:100 (0 min)-100:0 (20 min), flow rate 2 mL/min, detection by UV (254 nm).

3.5 Cell Culture and Imaging

HeLa cells were obtained from American Type Culture collection, and grown in Dulbecco's modification of Eagle's medium Dulbecco (DMEM/high: with 4500 mg/L Glucose, 4.0 mM L-Glutamine, and 110 mg/L Sodium Pyruvate) supplemented with 10% foetal bovine serum (FBS). Cells were incubated in a 5% CO_2 humidified incubator at 37 ^oC and typically passaged with sub-cultivation ratio of 1:4 every two days. For fluorescence microscopy, HeLa cells were seeded in 35 mm glass bottom dishes in culture medium. After 24 hrs, the cells were first incubated with probe **HA** (50μM) for 20 min at 37 ^oC and washed with phosphate buffer (pH 7.4) to remove excess extracellular dye. Then the cells were treated with or without ClO^- (250 μM) or other ROS for another 30 min at 37 ^oC and washed three times with PBS buffer (pH 7.4). Fluorescence imaging was performed with Nikon Ti-S with Xenon lamp. Blue and green emissions were collected with 4 s exposure time from 430 to 495nm window and with 30 s exposure time from 535-600 nm window, respectively.

Chemical reactions to generate the ROS were performed in 5 mL tubes and were transformed into 35 mm glass bottom dishes. H_2O_2 (250 μM), ROO• (250μM t BuOOH), •OH (250 μM ferrous ammonium sulfate and 2.5 μM hydrogen peroxide), $O_2^{\bullet-}$ (50 μM solid potassium superoxide), $ONOO^-$ (150 μM SIN-1). A saturated solution of KO_2 in DMSO (1 mM) was used as superoxide radical anion source and a saturated solution of SIN-1 in DMF (5 mM) was used as

$ONOO^-$ donor. To avoid inducing cell death, the v/v percentage of DMSO or DMF was below 5 %.

3.6 Live-Cell Fluorescence Measurement Using Microplate Reader

HeLa cells were first incubated with or without probe **HA** (50μM) in 6 cm dish for 20 min at 37 °C and washed with PBS (pH 7.4) three times to remove extracellular probe. Then the cells were harvested by trypsinization and suspended in PBS (contained 25 mM glucose). Aliquots of cells were incubated at 37 °C with different concentration of ClO^- for 30 min in black 96-well plates. Fluorescence readings from the plates were measured on BioTek Synergy 2 multifunction microplate reader (USA). The excitation filter is 360 BP 40 nm and the emission filters are 485 BP 20 nm, 590 BP 35 nm.

4. Data

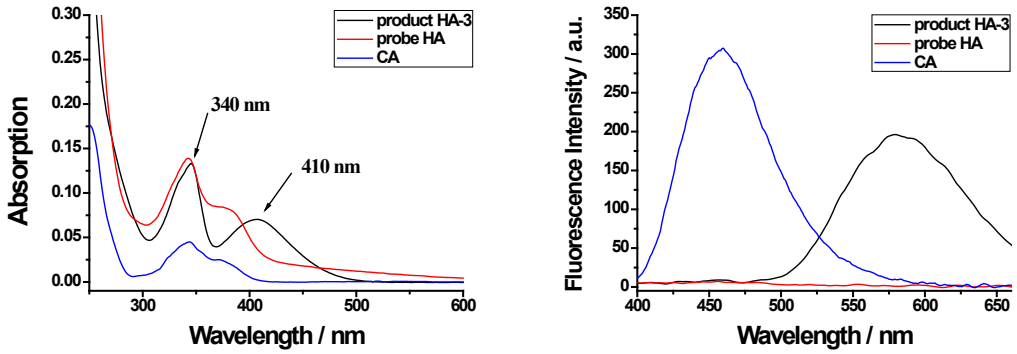


Fig. S1 The UV absorption spectra and fluorescent emission spectra probe **HA** (20 μM, red line), **HA-3** (20 μM, black line) and **CA** (5 μM, blue line) in 0.1 M phosphate buffer (pH 7.4, 1% DMF). Excitation wavelength was 340 nm.

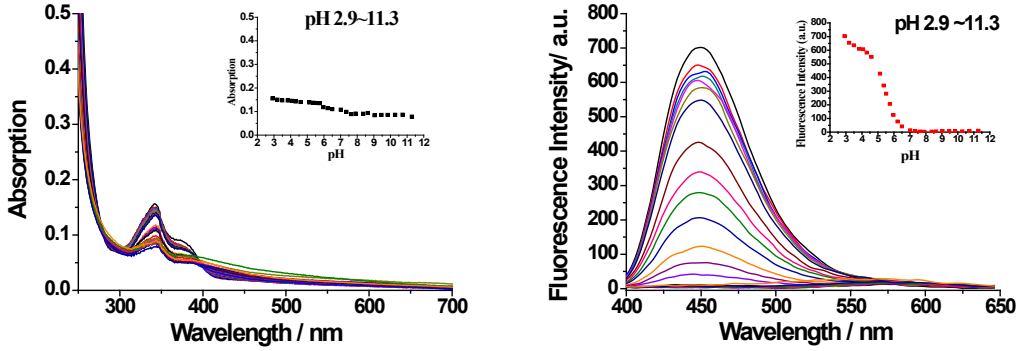


Fig. S2 The pH titration of probe **HA** in H_2O (1% DMF as cosolution).

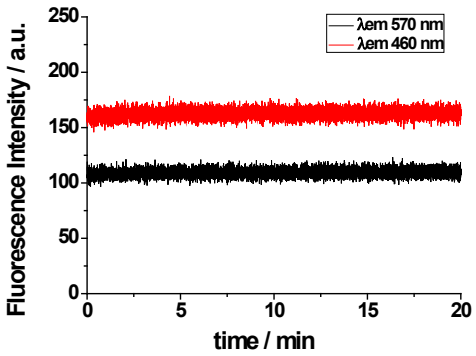


Fig. S3 Reaction–time profile of probe **HA** (20 μM) in the presence of 2 equiv of ClO^- in 0.1 M phosphate buffer (pH 7.4, 1% DMF). Kinetic studies were performed at room temperature. Excitation wavelength was 340 nm.

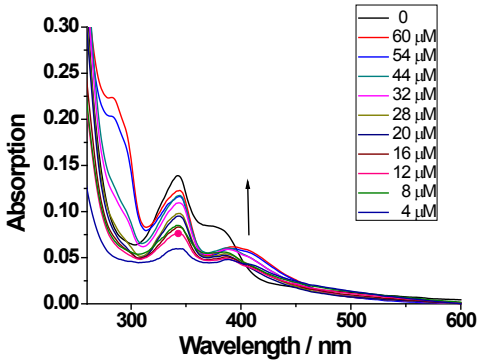


Fig. S4 The UV absorption spectra of probe **HA** (20 μM) upon addition of increasing concentration of hypochlorite anion (0 to 3 equiv) in 0.1 M phosphate buffer (pH 7.4, 1% DMF). Excitation wavelength was 340 nm.

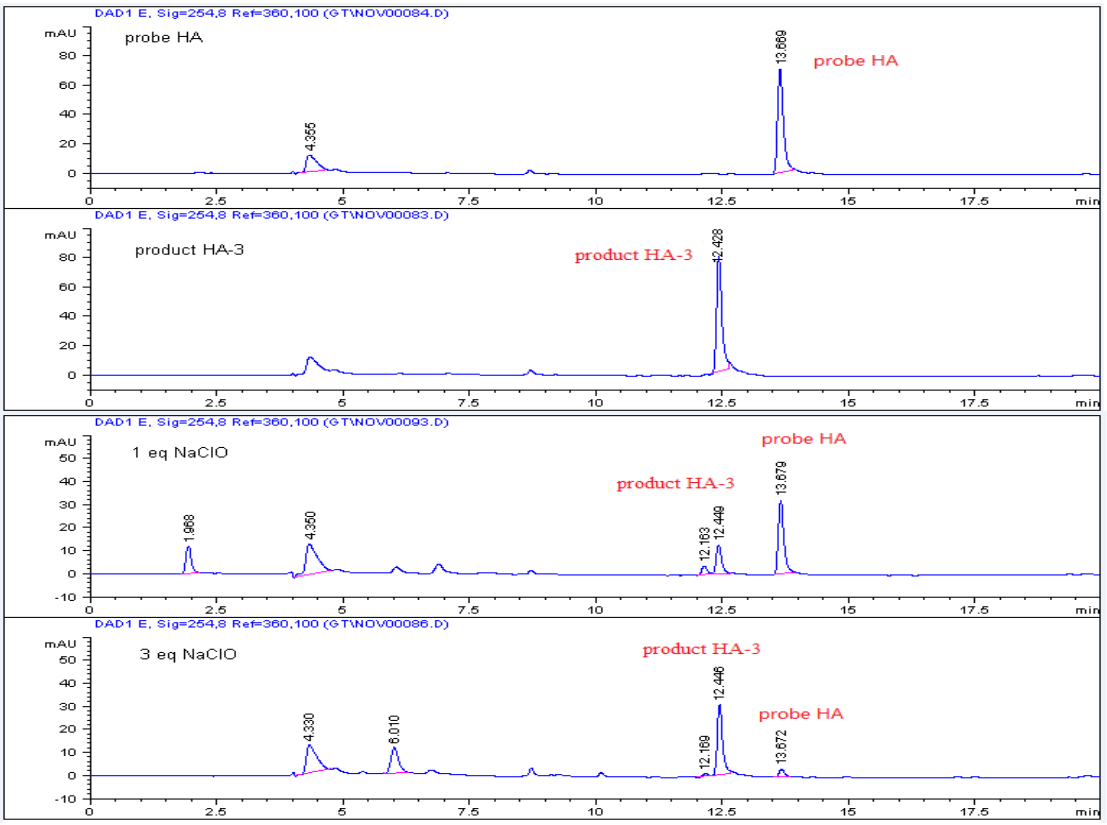


Fig. S5 HPLC chromatogram of probe **HA** (40 μM), after reaction with ClO^- (1eq and 3eq). Chromatogram of dye **HA-3** and probe **HA** are also showed. HPLC profiles were detected by UV at 254 nm.

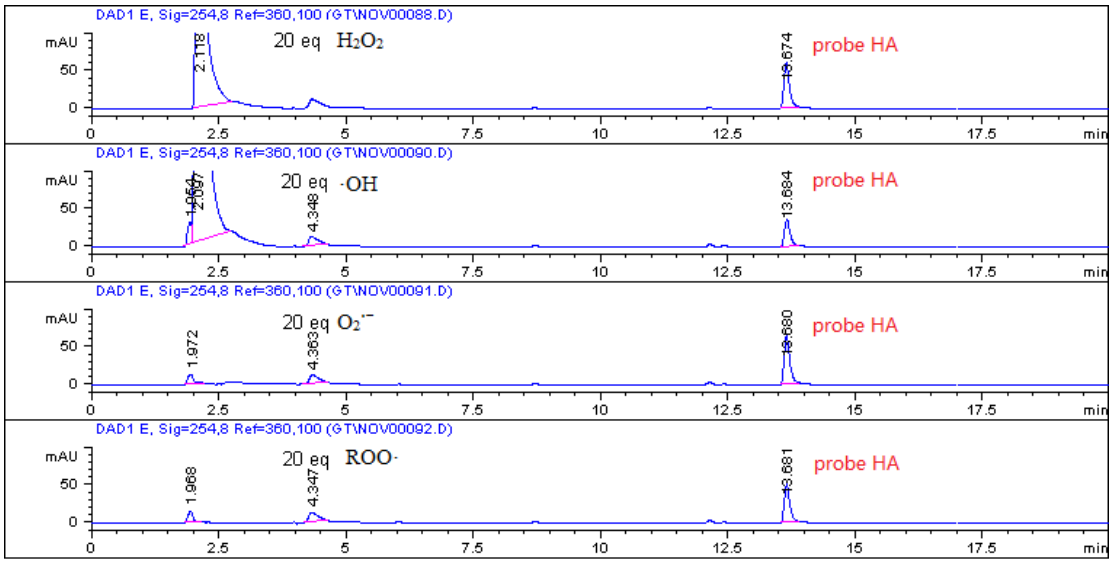


Fig. S6 HPLC chromatogram of probe **HA** (40 μM), after reaction with $O_2^{\bullet-}$, •OH, ROO•, H_2O_2. HPLC profiles were detected by UV at 254 nm.

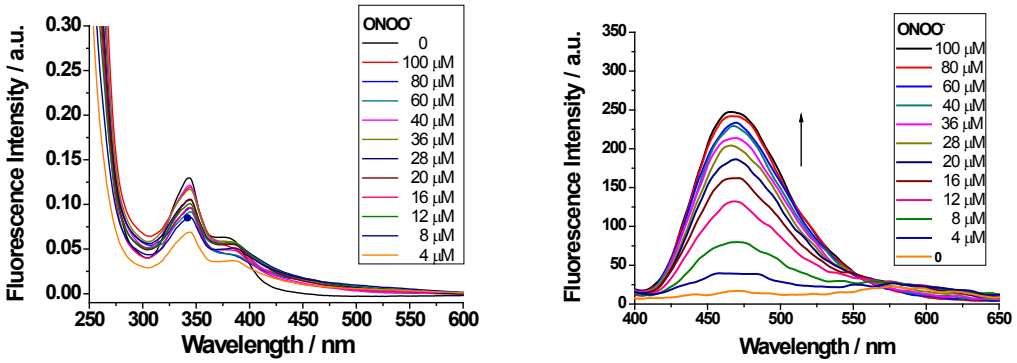


Fig. S7 The UV absorption spectra and fluorescent emission spectra of probe **HA** (20 μM) upon addition of increasing concentration of $ONOO^-$ (0 to 5 equiv) in 0.1 M phosphate buffer (pH 7.4, 1% DMF). Excitation wavelength was 340 nm.

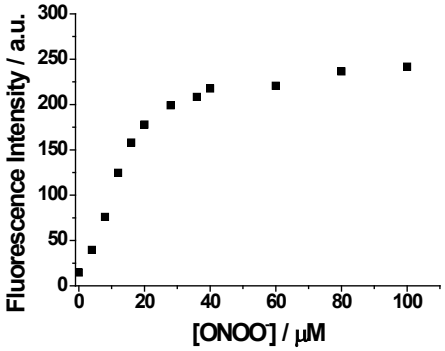


Fig. S8 Fluorescence spectra of probe **HA** (20 μM) with the addition of $ONOO^-$ in phosphate buffer (pH 7.4, 1% DMF). (E_x = 340 nm, E_m = 460 nm)

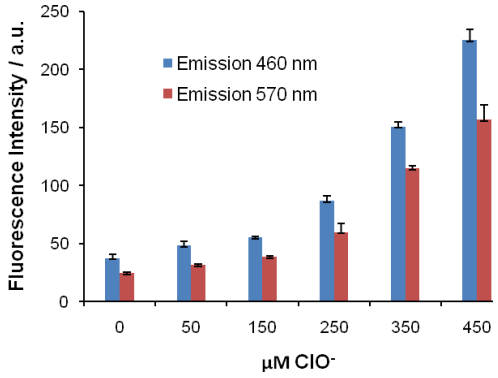


Fig. S9 Fluorescence responses of **HA** with varied concentrations of ClO^-. The fluorescence emission intensities are quantified using a plate reader (n=3). Error bars represent SEM.

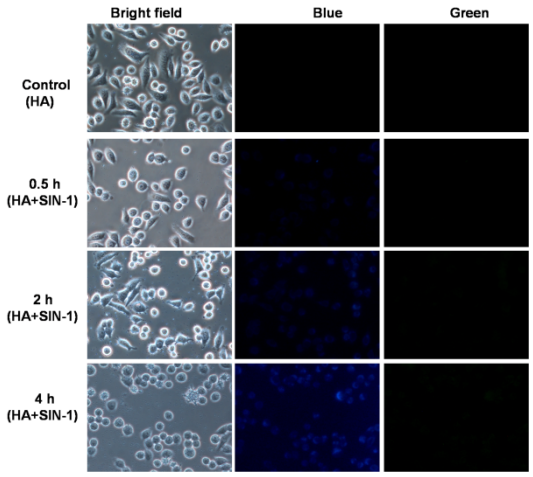


Fig. S10 Fluorescence response of 50 μM **HA** with 150 μM SIN-1 (an $OONO^-$ donor) in HeLa cells.

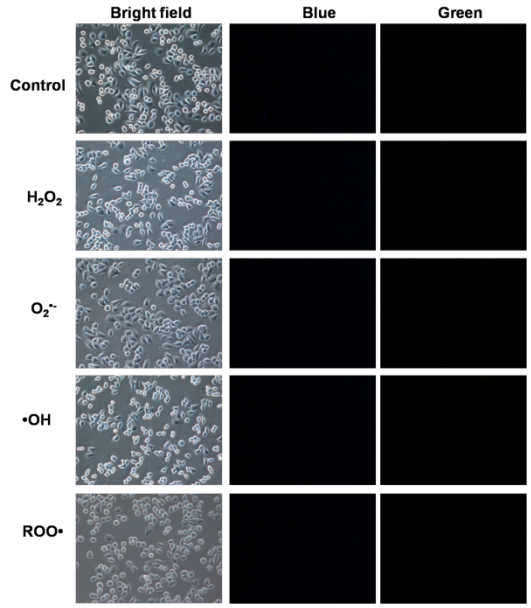


Fig. S11 Fluorescence response of **HA** with various ROS in HeLa cells. Control (50 μM **HA** without ROS).From up to down row, HeLa cells were pre-treated with probe **HA** (50 μM) and then incubated with various ROS. H_2O_2 (250 μM), $O_2^{\bullet-}$ (50 μM solid potassium superoxide), •OH (250 μM ferrous ammonium sulfate and 2.5 μM hydrogen peroxide), ROO• (250 μM ^{t}BuOOH).

5. NMR spectra

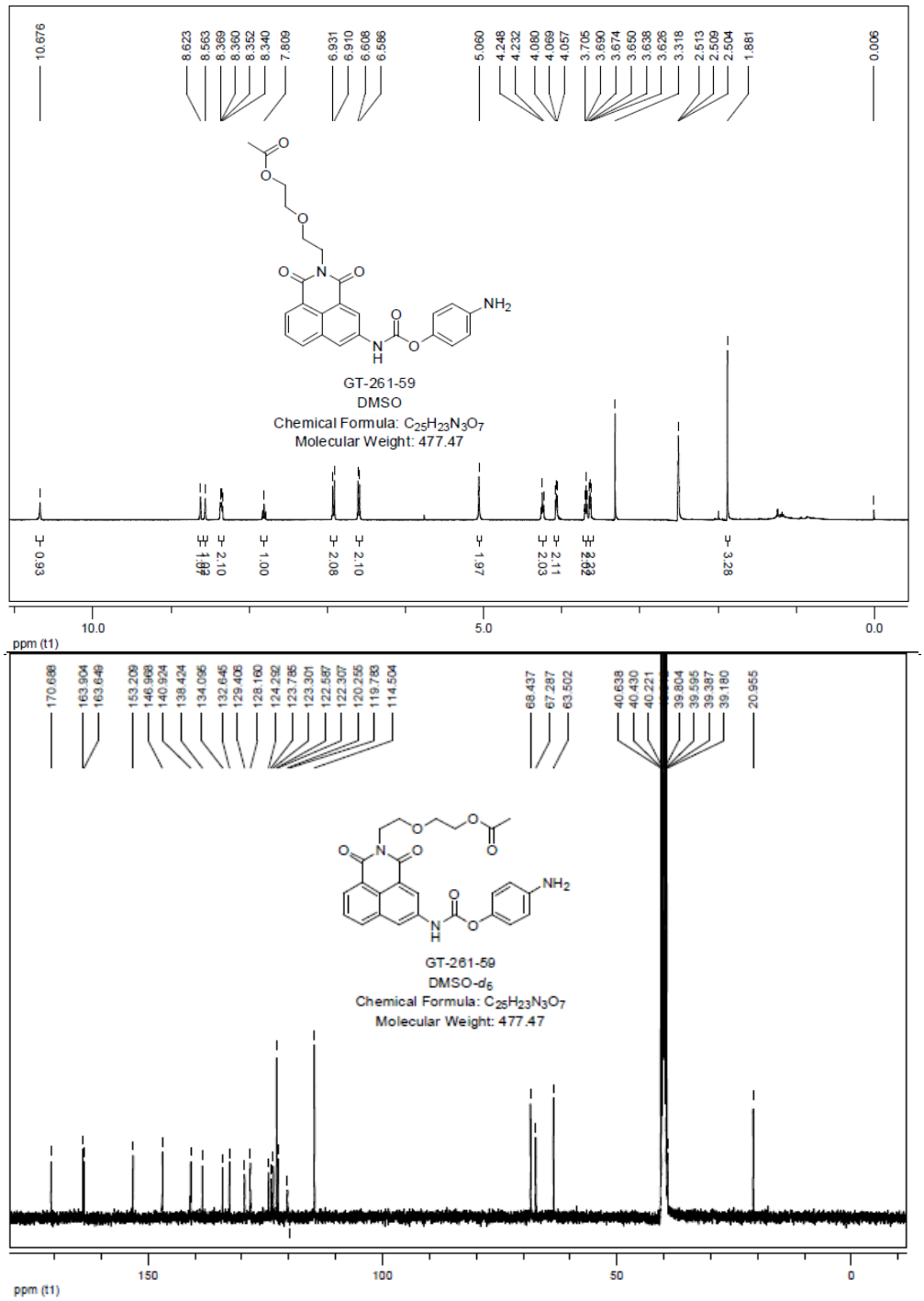


Fig. S12 the ^{1}H-NMR and ^{13}C-NMR spectrum of **HA**

Elemental Composition Report Page 1

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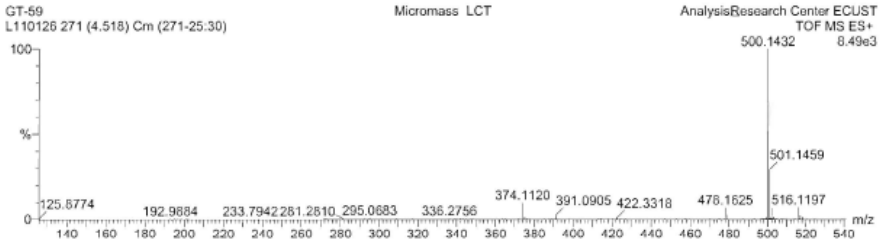
isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
53 formula(e) evaluated with 4 results within limits (up to 50 closest results for each mass)



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479.1648	2.55	479.1648	0.0	0.0	15.5	n/a	12C24 13C 1H24 14N3 16O7
500.1432	100.00	500.1434	-0.2	-0.3	15.5	n/a	12C25 1H23 14N3 16O7 23Na
501.1459	28.86	501.1467	-0.8	-1.6	15.5	n/a	12C24 13C 1H23 14N3 16O7 23Na
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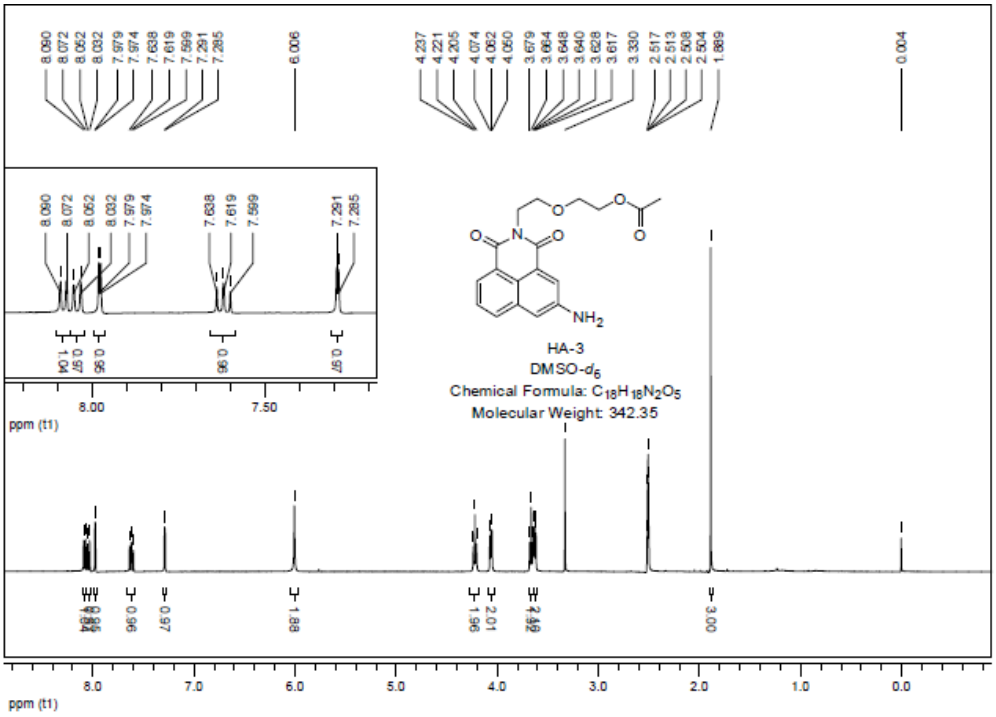
516.1197	6.90	---					

Fig. S13 the HR-MS spectrum of **HA**



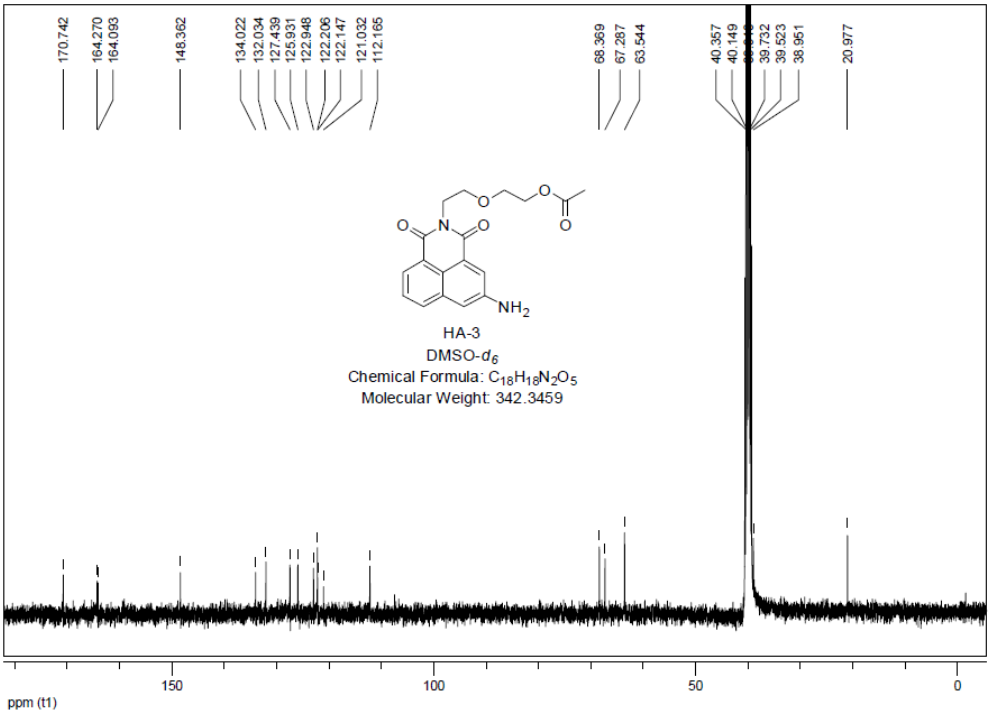
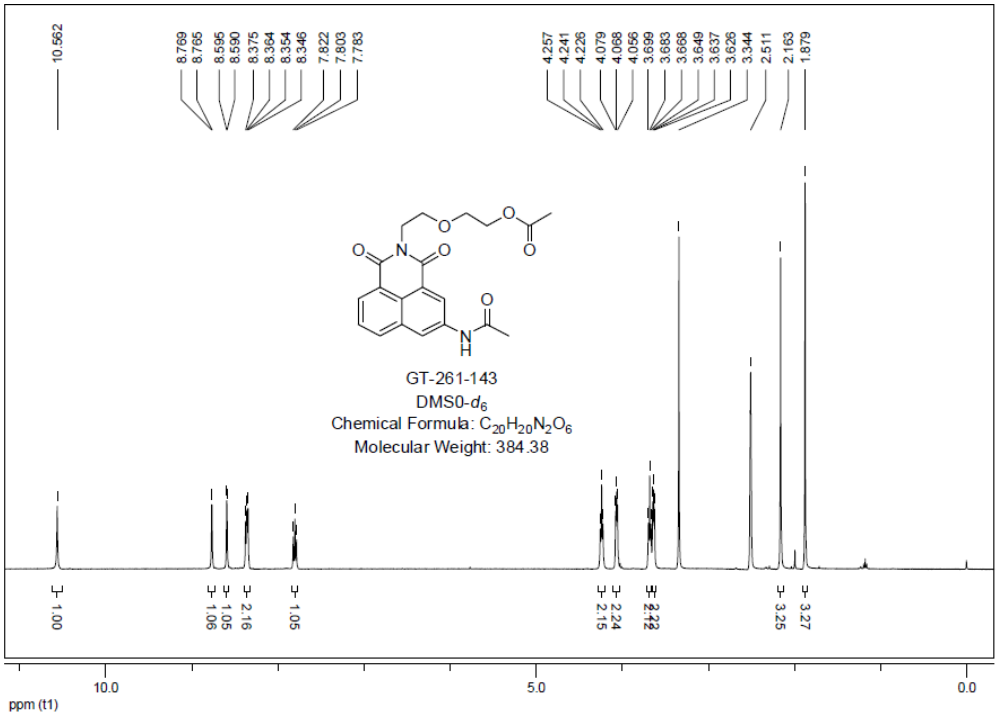


Fig. S14 the ^{1}H-NMR and ^{13}C-NMR spectrum of **HA-3**



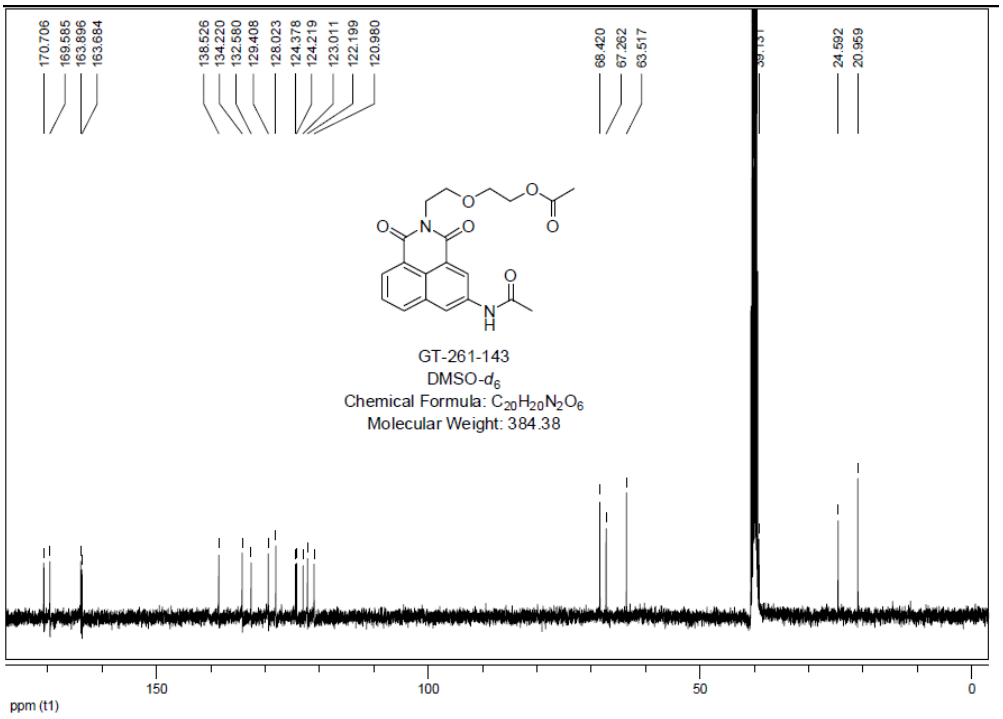


Fig. S15 the ^{1}H-NMR and ^{13}C-NMR spectrum of **CA**

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Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
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GT-261-143
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Micromass LCT

AnalysisResearch Center ECUST
TOF MS ES+
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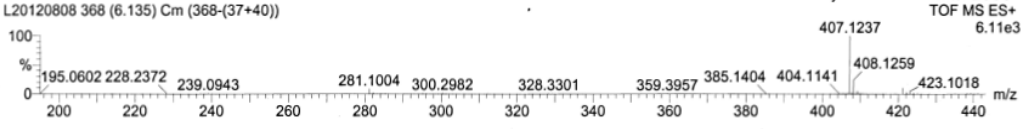

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Maximum:	100.00		5.0	5.0	100.0		
Mass	RA	Calc. Mass	mDa	PPM	DBE	Score	Formula
407.1237	100.00	407.1219	1.8	4.4	11.5	n/a	12C20 1H20 14N2 16O6 23Na
408.1259	23.65	408.1253	0.6	1.6	11.5	n/a	12C19 13C 1H20 14N2 16O6 23Na

Fig. S16 the HR-MS spectrum of **CA**

6. References

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