

Supporting Information

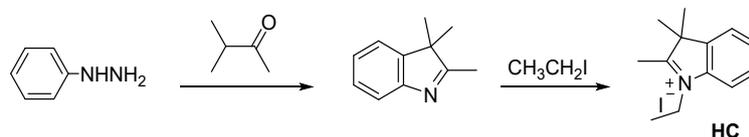
Novel Turn-on Fluorescent Probe for Endogenous Peroxynitrite Detection Based on Selective Oxidation of Nitrobenzoyl Formate Ester

Experimental

Materials and general methods

Unless otherwise stated, all reagents were purchased from commercial suppliers and were used with no purification. All samples were prepared at room temperature. UV-vis spectra were recorded using a Lambda-900 spectrophotometer (Perkin-Elmer). Fluorescence spectra were recorded using a LS-55 fluorescence spectrometer (Perkin-Elmer). Infrared spectral data were obtained using a WQT-200 FT-IR spectrometer with KBr. NMR spectra were recorded on Bruker Avance II (500 MHz). High resolution mass spectra were recorded on an Agilent 1260 LC-MS equipped with an Agilent 6530 Q-TOF. Melting points were recorded using a melting point apparatus WPS-2A Optimelt (Shanghai Precision & Scientific Instrument) and are uncorrected. The geometries for structures were optimized by density functional theory (DFT) calculation at the B3LYP theory level performed on Gaussian 16 program package. The fluorescence microscopy experiment was carried out on a Zeiss Microscopy Colibri 7.

Synthesis of probe

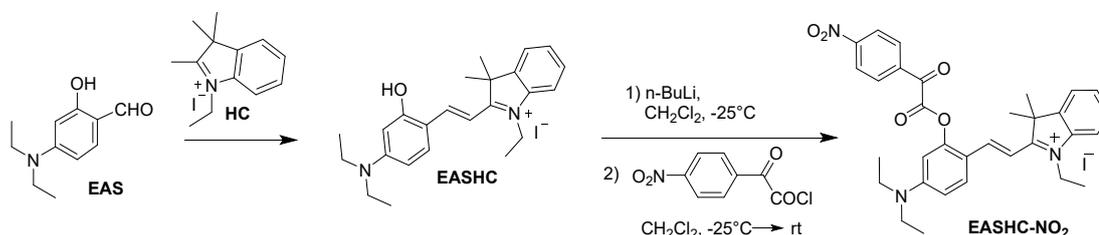


Scheme S1. Synthesis of Compounds **HC**.

Phenylhydrazine (2.50 g, 23.1 mmol) and 3-methyl-2-butanone (4.00 g, 46.2 mmol) were dissolved in anhydrous ethanol (25 mL). The mixture was heated under

reflux for 30 minutes under nitrogen atmosphere. After concentrating under reduced pressure, acetic acid (25 mL) was added and reflux was continued for 4 hours with nitrogen protection. The reaction was monitored by TLC. Upon completion, saturated NaHCO₃ solution (100 mL) was added to the mixture. The product was extracted with dichloromethane (30 mL × 3) and the combined organic phases were dried over anhydrous MgSO₄. After solvent removal under reduced pressure, 2,3,3-trimethyl-3H-indole was obtained as a light red oil, which was used directly in the next step without further purification.

The obtained intermediate (3.42 g, 21.4 mmol) and iodoethane (6.70 g, 42.9 mmol) were dissolved in acetonitrile (15 mL). The reaction mixture was heated under reflux for 6 hours under nitrogen protection. The reaction progress was monitored by TLC. After completion, the mixture was cooled to room temperature and allowed to stand, resulting in the precipitation of a solid. The product was collected by suction filtration to afford 1-ethyl-2,3,3-trimethyl-3H-indol-1-ium (Compound **HC**) as an orange crystalline solid (5.12 g, yield: 76%). MP: 153.8 ~ 155.4°C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.00 – 7.90 (m, 1H), 7.86 – 7.79 (m, 1H), 7.64 – 7.56 (m, 2H), 4.47 (q, *J* = 8.0 Hz, 2H), 2.81 (s, 3H), 1.51 (s, 6H), 1.42 (t, *J* = 8.0 Hz, 3H); ¹³C NMR (128 MHz, DMSO) δ 196.84, 149.65, 140.25, 127.34, 124.11, 120.19, 110.50, 46.18, 42.02, 26.49, 13.57, 8.40. HRMS (APCI) calcd for C₁₃H₁₈N⁺ [M+H]⁺ 188.1434, found *m/z* 188.1437.



Scheme S2. Synthesis of Compound **EASHC** and probe **EASHC-NO₂**.

Compound **EAS** (193.1 mg, 1.00 mmol) and compound **HC** (188.2 mg, 1.00 mmol) were dissolved in anhydrous ethanol (20 mL). The mixture was heated under

reflux overnight under nitrogen protection. The reaction progress was monitored by TLC. After completion, the reaction solution was cooled and allowed to stand, resulting in the formation of purple-black crystals. The solid compound (E)-2-(4-(dimethylamino)-2-hydroxybenzylidene)-1-ethyl-3,3-dimethyl-3H-indol-1-ium (EASHC) was obtained by suction filtration (228.8 mg, yield: 63%). MP: 232.2 ~ 234.6°C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.07 (s, 1H), 8.42 (d, *J* = 16.0 Hz, 1H), 7.98 (s, 1H), 7.72 (d, *J* = 7.5 Hz, 1H), 7.63 (d, *J* = 8.0 Hz, 1H), 7.50 (t, *J* = 8.0 Hz, 1H), 7.40 (t, *J* = 8.0 Hz, 1H), 7.16 (s, 1H), 6.51 (d, *J* = 8.0 Hz, 1H), 6.23 (s, 1H), 4.39 (d, *J* = 8.0 Hz, 2H), 3.49 (q, *J* = 8.0 Hz, 4H), 1.69 (s, 6H), 1.35 (t, *J* = 4.0 Hz, 3H), 1.17 (t, *J* = 10.0 Hz, 6H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 181.02, 160.67, 151.92, 141.27, 140.91, 130.90, 127.63, 123.12, 113.12, 109.92, 102.10, 97.95, 54.63, 47.20, 44.57, 28.45, 17.73, 14.89. HRMS (APCI) calcd for C₂₄H₃₁N₂O⁺ [M+H]⁺ 363.2431 found *m/z* 363.2432.

p-Nitrobenzoylformic acid (100 mg, 0.52 mmol) was dissolved in anhydrous dichloromethane (5 mL). Thionyl chloride (5 mL, 0.78 mol) was added dropwise, followed by two drops of DMF. The mixture was refluxed for 1 hour under a nitrogen atmosphere. After the reaction was confirmed to be complete by TLC, the volatiles were removed under reduced pressure to afford **p**-nitrobenzoylformyl chloride as a pale-yellow solid.

Compound EASHC (123 mg, 0.34 mmol) was dissolved in anhydrous dichloromethane (8 mL) under a nitrogen atmosphere. The solution was cooled to -25 °C with stirring, and **n**-butyllithium (0.1 mL, 0.85 mmol) was added dropwise over 10 minutes. **p**-Nitrobenzoylformyl chloride, dissolved in anhydrous dichloromethane (5 mL), was then injected into the reaction mixture via syringe. The resulting mixture was allowed to warm to room temperature and stirred for 2 hours. After the reaction was confirmed to be complete by TLC, the mixture was concentrated under reduced pressure. Purification by silica gel column chromatography (ethyl acetate/petroleum ether = 1:5, v/v) afforded the brick-red solid probe, 3,3-dimethyl-1-ethyl-2-{2-[4-(diethylamino)-2-((4-nitrobenzoyl)formyloxy)phenyl]ethenyl}-3*H*-indol-1-ium

(EASHC-NO₂, 108.2 mg, yield: 58%). MP: 271.2 ~ 274.7°C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.40 – 8.36 (m, 3H), 8.20 – 8.15 (m, 3H), 7.72 (d, *J* = 8.0 Hz, 1H), 7.62 (d, *J* = 8.0 Hz, 1H), 7.50 (t, *J* = 8.0 Hz, 1H), 7.40 (t, *J* = 7.5 Hz, 1H), 6.51 (d, *J* = 8.0 Hz, 1H), 4.38 (d, *J* = 8.0 Hz, 2H), 3.49 (d, *J* = 8.0 Hz, 6H), 1.69 (s, 6H), 1.35 (t, *J* = 7.5 Hz, 3H), 1.17 (t, *J* = 8.0 Hz, 6H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 185.85, 73.94, 160.37, 159.82, 154.50, 149.02, 140.30, 138.45, 136.80, 133.73, 128.86, 128.65, 125.84, 122.88, 116.27, 114.43, 108.42, 97.28, 51.69, 46.39, 25.77, 13.41. HRMS (APCI) calcd for C₃₂H₃₄N₃O₅⁺ [M+H]⁺ 540.2493, found *m/z* 540.2452.

Preparation of the test solution

H₂O₂ was diluted with 30% solution in water. ClO⁻ was obtained by diluting commercial NaClO solution with 0.1 mol/L NaOH aqueous solution. The concentrations of both solutions were determined by measuring their absorbance at 240 nm. Hydroxyl radical ([•]OH) was obtained according to the Fenton reaction.^{S1} ONOO⁻ prepared according to previous literature and the concentration was determined by the absorbance at 302nm.^{S2} ¹O₂ was produced by mixing HClO and H₂O₂ solutions. [•]O²⁻ was prepared from KO₂ in dry DMSO. NO was generated from sodium nitroferricyanide (III) dihydrate. Other ions were dissolved in twice-distilled water using their corresponding sodium salts, including NO₂⁻, F⁻, Cl⁻, I⁻, HCO₃⁻, CO₃²⁻, SO₃²⁻, SO₄²⁻, HSO₄⁻, HSO₃⁻, HPO₄²⁻, H₂PO₄⁻, OH⁻. Prepare solutions of GSH, Cys, and Hcy in distilled water.

Cell culture and fluorescence imaging

MCF-7 cells were cultured in MEM Medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% *L*-glutamine, and 1% penicillin/streptomycin at 37 °C in a humidified incubator containing 5% CO₂. Before sensing, the cells were washed three times with PBS. For ONOO⁻ sensing imaging, the cells were first loaded with the probe EASHC-NO₂ (10μM) in PBS at 37 °C in a humidified incubator containing 5% DMSO for 30 minutes, then washed 3 times with PBS solution (pH 7.4) to remove the excess

EASHC-NO₂, and then treated with ONOO⁻ at 37 °C.

RAW 264.7 cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10 % FBS and 1% antibiotics at 37 °C in humidified environment of 5% CO₂. Before sensing, the cells were washed three times with PBS. For endogenous ONOO⁻ sensing imaging, the cells were activated with LPS (1 µg/mL) and INF-γ (50 ng/mL) for 6 h, and then loaded with 10 µM probe **EASHC-NO₂** (10µM) for 30 min.

Supporting figures

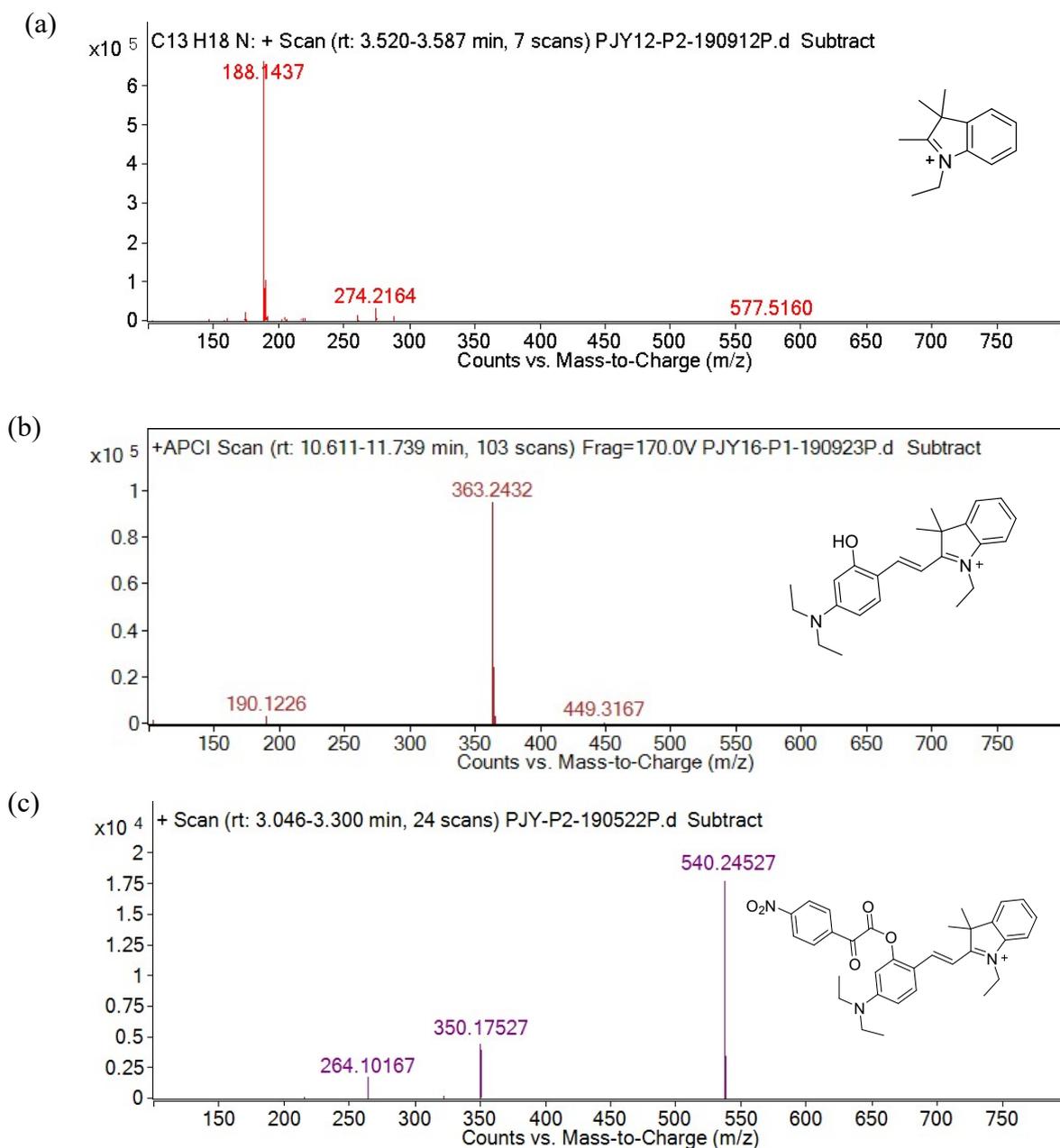


Figure S1. HRMS (ESI) measurements. (a) HC, (b) EASHC and (c) EASHC-NO₂.

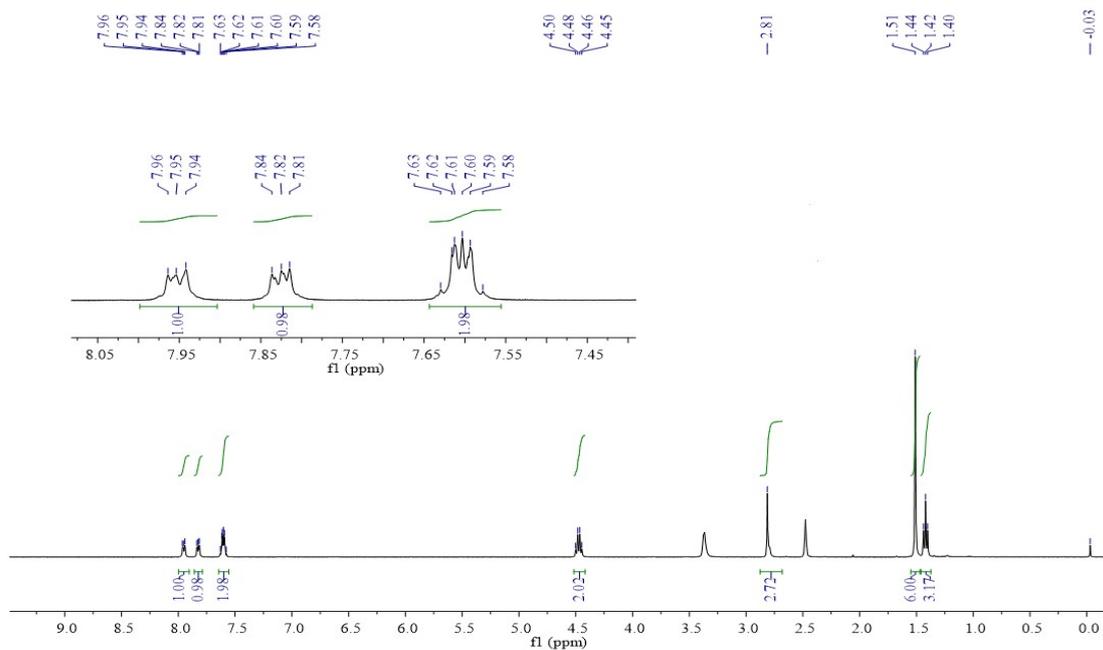


Figure S2. ^1H NMR spectrum of HC.

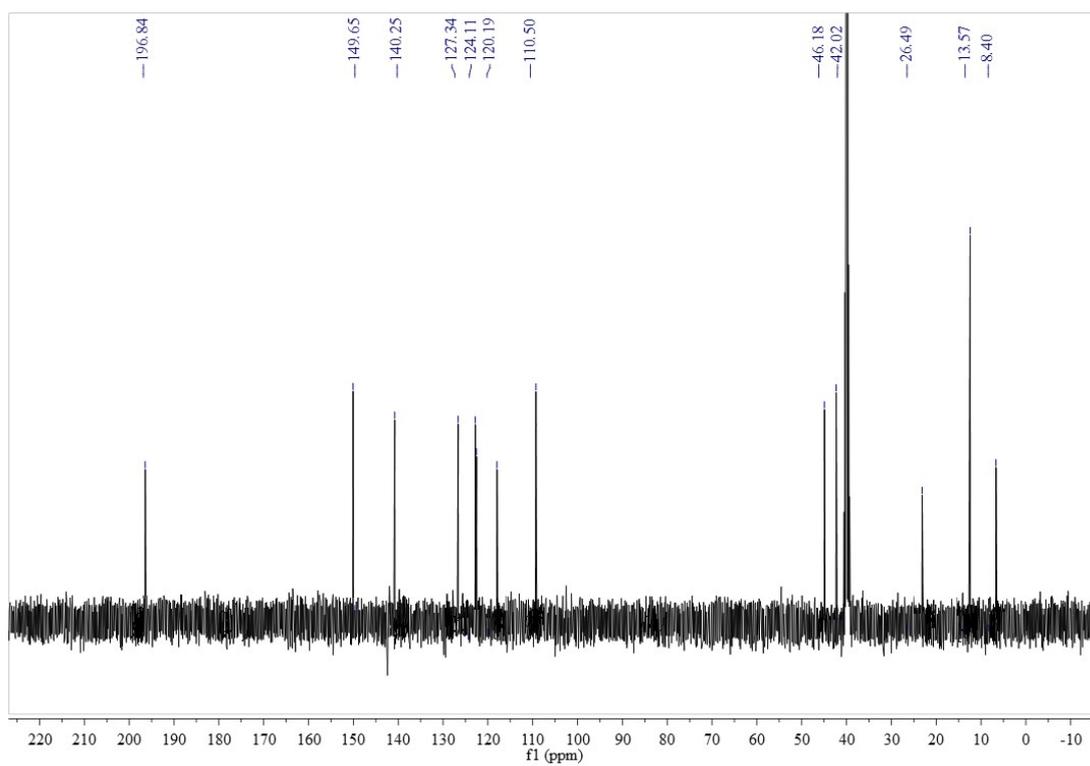


Figure S3. ^{13}C NMR spectrum of HC.

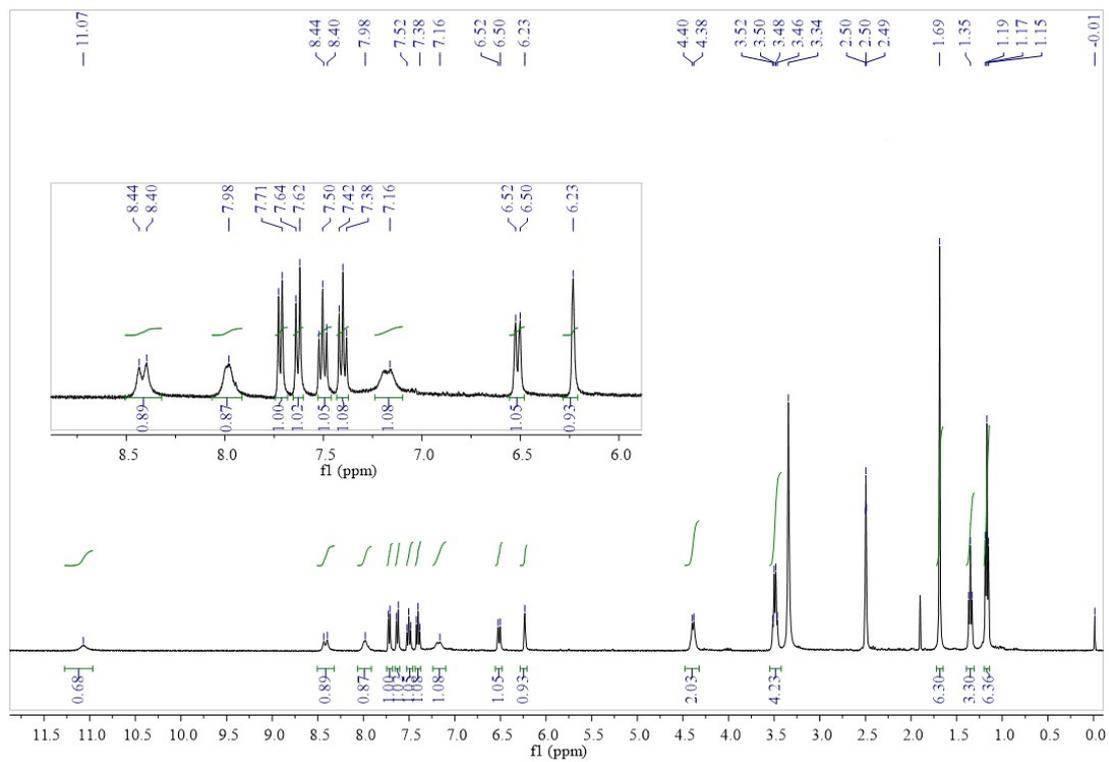


Figure S4. ^1H NMR spectrum of EASHC.

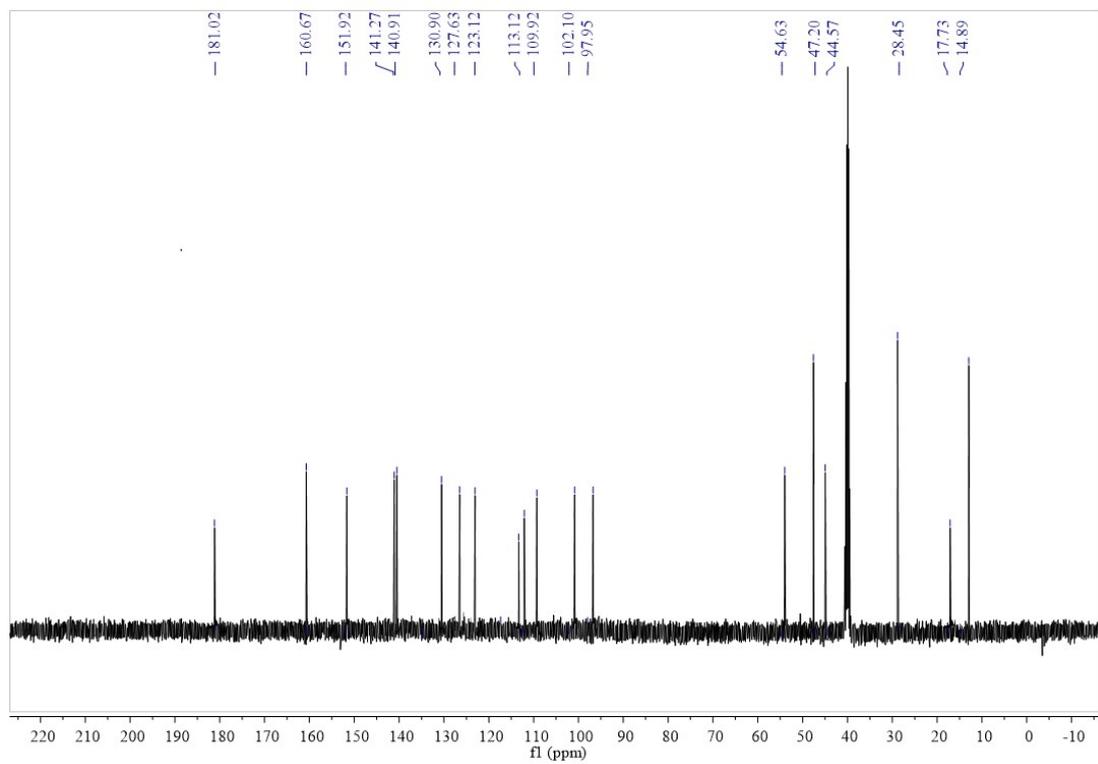


Figure S5. ^{13}C NMR spectrum of EASHC.

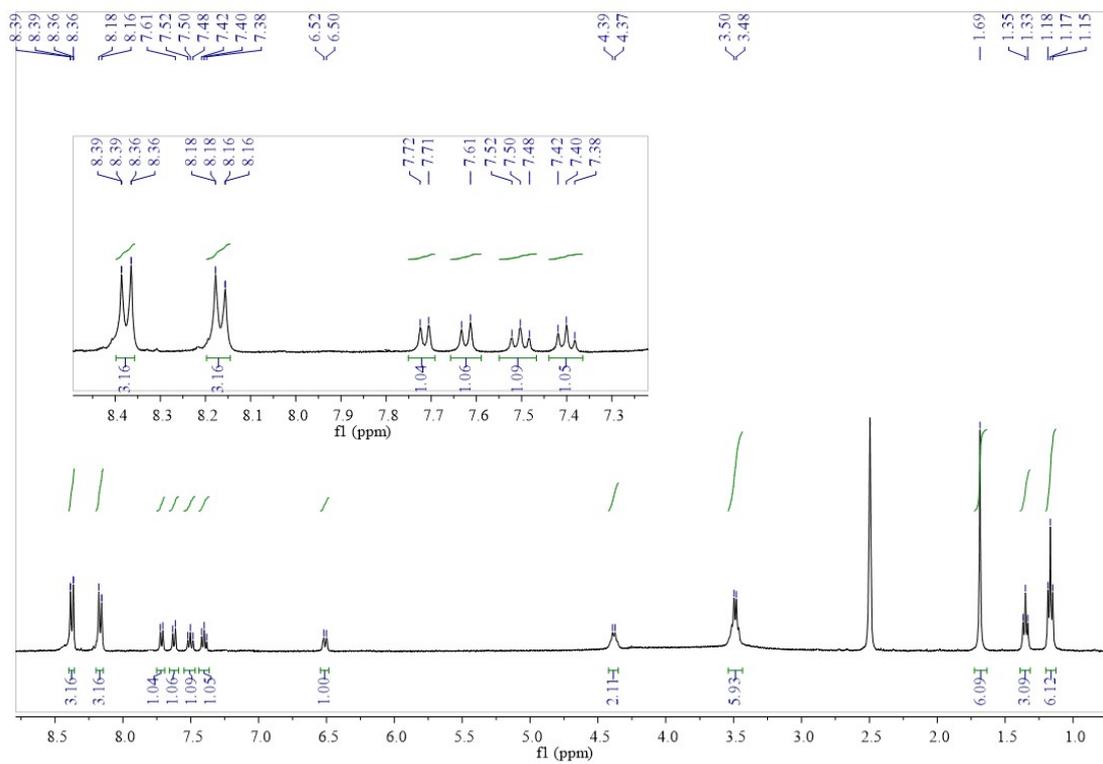


Figure S6. ^1H NMR spectrum of EASHC- NO_2 .

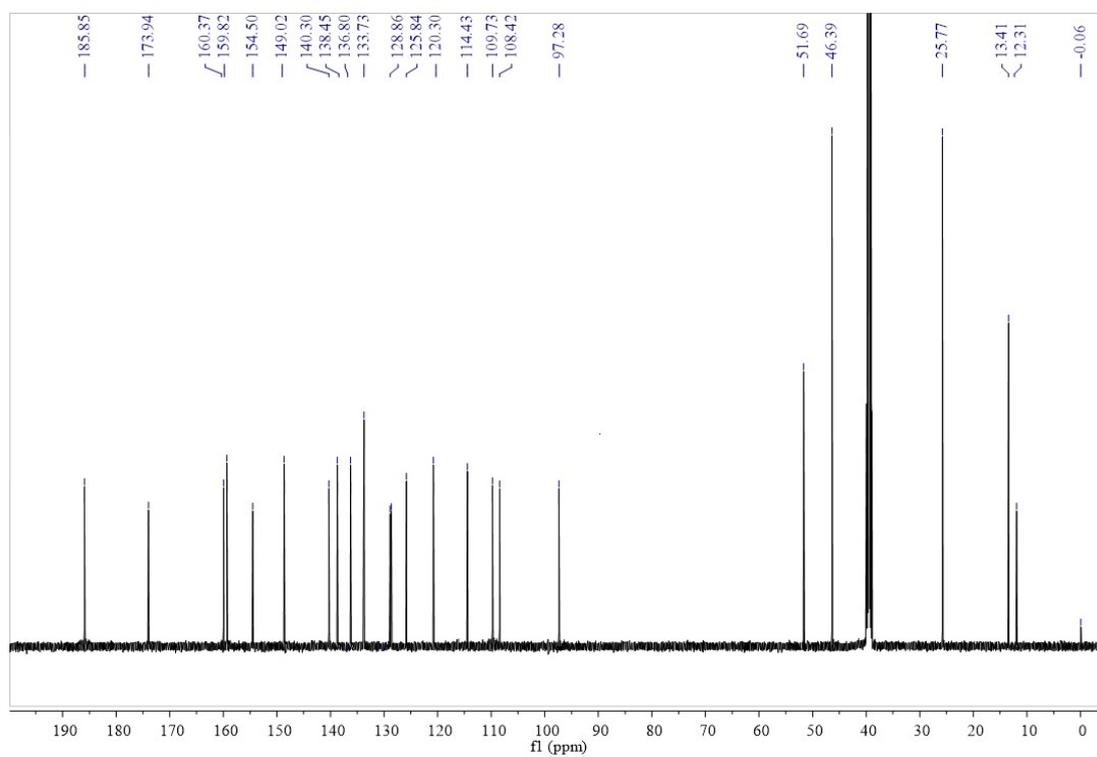


Figure S7. ^{13}C NMR spectrum of EASHC- NO_2 .



Figure S8. The color of the solution under the light the probe **EASHC-NO₂** buffer was reacted with 30 μ M different substances for 5 min.

(1. blank; 2. NO; 3. ClO⁻; 4. NO₂⁻; 5. ·OH; 6. H₂O₂; 7. ·O₂⁻; 8. ¹O₂; 9. F⁻; 10. Cl⁻; 11. I⁻; 12. HCO₃⁻; 13. CO₃²⁻; 14. SO₃²⁻; 15. SO₄²⁻; 16. HSO₄⁻; 17. HSO₃⁻; 18. HPO₄²⁻; 19. H₂PO₄⁻; 20. OH⁻; 21. GSH; 22. Cys; 23. Hcy; 24. ONOO⁻)

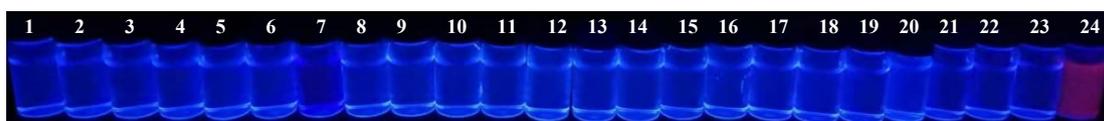


Figure S9. The color of the solution under the UV light the probe **EASHC-NO₂** buffer was reacted with 30 μ M different substances for 5 min.

(1. blank; 2. NO; 3. ClO⁻; 4. NO₂⁻; 5. ·OH; 6. H₂O₂; 7. ·O₂⁻; 8. ¹O₂; 9. F⁻; 10. Cl⁻; 11. I⁻; 12. HCO₃⁻; 13. CO₃²⁻; 14. SO₃²⁻; 15. SO₄²⁻; 16. HSO₄⁻; 17. HSO₃⁻; 18. HPO₄²⁻; 19. H₂PO₄⁻; 20. OH⁻; 21. GSH; 22. Cys; 23. Hcy; 24. ONOO⁻)

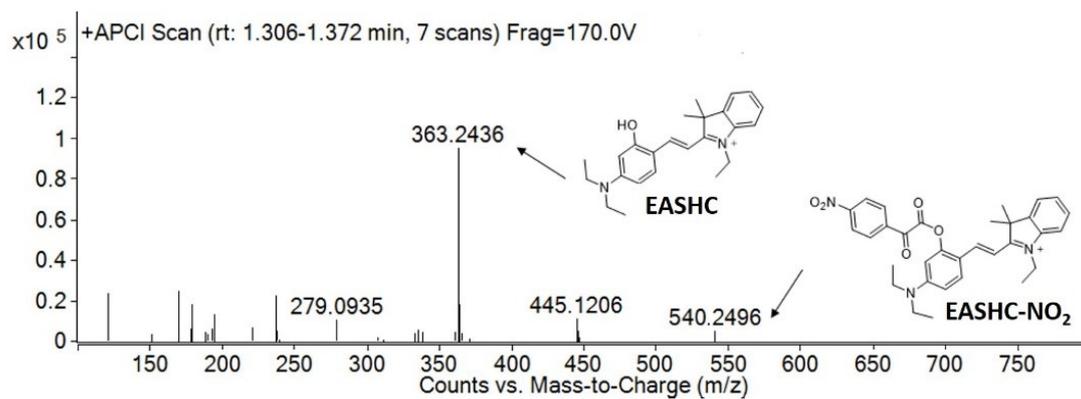


Figure S10. HRMS spectrum of EASHC and EASHC-NO₂.

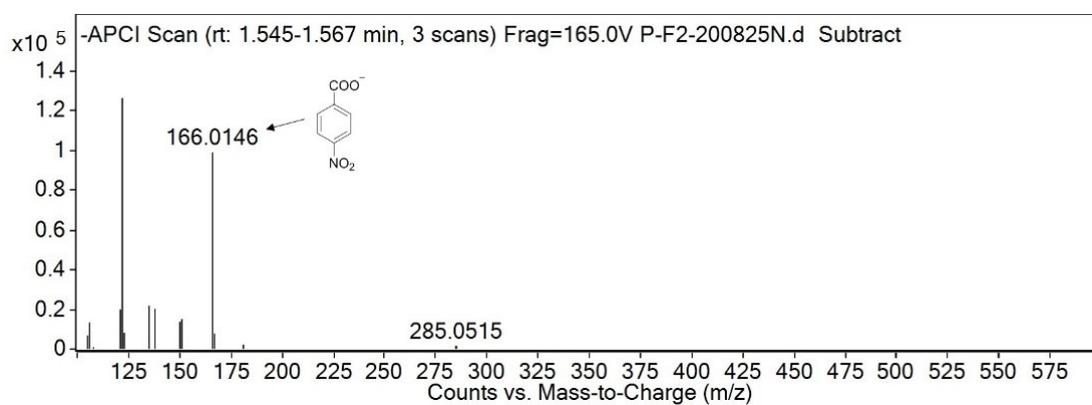


Figure S11. HRMS spectrum of *p*-nitrobenzoic acid.

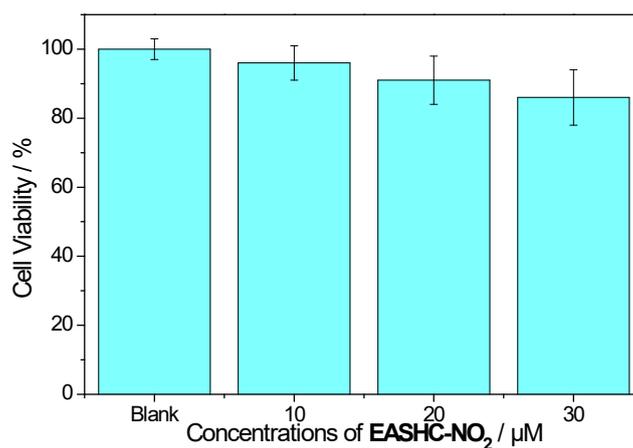


Figure S12. Cell viability of MCF-7 treated with different concentrations of EASHC-NO₂.

References

- S1. C. Mitsui, M. Yamagishi, R. Shikata, H. Ishii, T. Matsushita, K. Nakahara, M. Yano, H. Sato, A. Yamano, J. Takeya, T. Okamoto, *Bull. Chem. Soc. Jpn.*, 2017, **90**, 931–938.
- S2. X. Zhou, Y. Kwon, G. Kim, J.-H. Ryu, J. Yoon, *Biosens. Bioelectron.*, 2015, **64**, 285–291.