

ESIPT-based ratiometric fluorescence probe for the intracellular imaging of peroxynitrite

Luling Wu^{a‡}, Yang Wang^{b‡}, Maria Weber^a, Liyuan Liu^a, Adam C. Sedgwick^{*a}, Steven D. Bull^{*a}, Chusen Huang^{*b} and Tony D. James^{*a,c}

^aDepartment of Chemistry, University of Bath, Bath, BA2 7AY, UK.

^bThe Education Ministry Key Laboratory of Resource Chemistry, Shanghai Key Laboratory of Rare Earth Functional Materials, and Shanghai Municipal Education Committee Key Laboratory of Molecular Imaging Probes and Sensors, Department of Chemistry, Shanghai Normal University, 100 Guilin Road, Shanghai 200234, China.

^cDepartment of Materials and Life Sciences, Faculty of Science and Technology, Sophia University, 7-1 Kioi-cho, Chiyoda-ku, Tokyo 102-8554 Japan

[‡]Equal contributions

Email: huangcs@shnu.edu.cn; t.d.james@bath.ac.uk, a.c.sedgwick@bath.ac.uk, s.d.bull@bath.ac.uk

Table of Contents

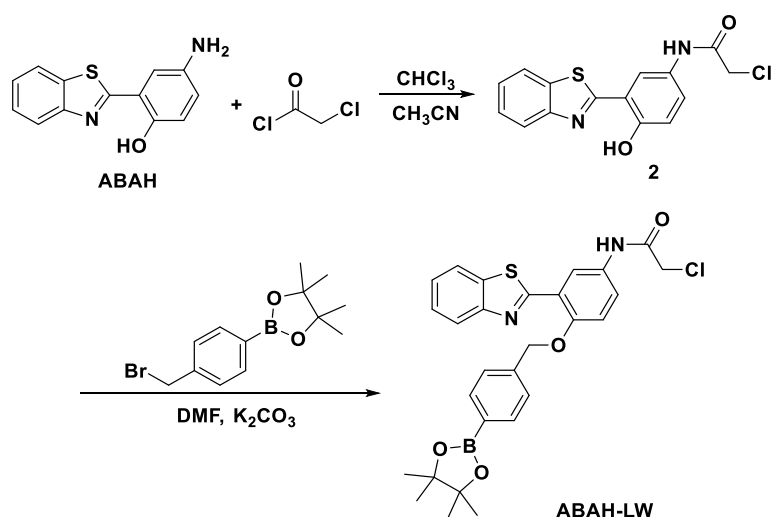
- 1. Experimental**
- 2. Generation of various ROS**
- 3. The detailed mechanism of the reaction between ABAH-LW and peroxynitrite**
- 4. Detailed protocols for characterization of ABAH-LW performance towards peroxynitrite**
- 5. Detailed protocols for cell culture**
- 6. Fluorescence imaging in live cell with ABAH-LW**
- 7. References**
- 8. NMR spectrum**

1. Experimental

1.1. Synthetic experiments: material and apparatus

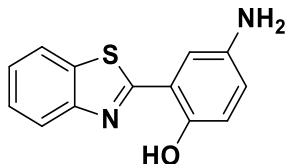
All chemical reagents and solvents were purchased from commercial sources and used without further purification. Thin-layer chromatography (TLC) was performed on silica gel plates and visualized by UV. Column chromatography was performed using silica gel (Sigma-Aldrich) 200-400 mesh. ^1H and ^{13}C NMR spectra were recorded employing a Bruker AV-500 spectrometer with chemical shifts expressed in parts per million (DMSO- d_6 , CDCl_3 , Me_4Si as internal standard). Electrospray ionization (ESI) mass spectrometry was performed in a Bruker MicrTOF spectrometer.

1.2. Synthetic experiments: synthetic procedures



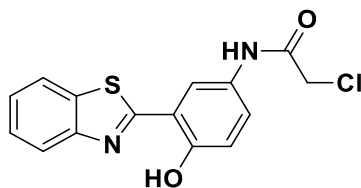
Scheme S1. Synthesis of ABAH-LW.

4-Amino-2-(benzo[d]thiazol-2-yl)phenol (ABAH).



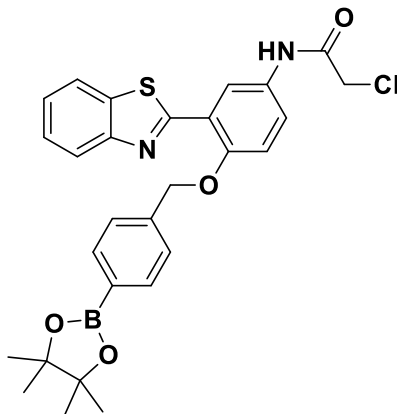
ABAH was synthesized according to the similar procedures.^{1,2} A mixture of 2-aminothiophenol (2.00 g, 16.0 mmol) and 5-aminosalicylic acid (2.45 g, 16.0 mmol) was stirred in 50 mL polyphosphoric acid (PPA) at 185 °C for 4 h. Then the reaction mixture was poured into cold water to give a yellow precipitate. After filtering, the yellow precipitate was washed by 10% Na₂CO₃ and the product changes colour to green (2.83 g, 73% yield). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.11 (d, *J* = 8.0 Hz, 1H), 8.02 (d, *J* = 8.1 Hz, 1H), 7.72 (d, *J* = 15.1 Hz, 1H), 7.52 (t, *J* = 7.7 Hz, 1H), 7.42 (t, *J* = 7.6 Hz, 1H), 7.01 (d, *J* = 8.5 Hz, 1H), 6.95 (d, *J* = 8.4 Hz, 1H). ¹³C NMR (125 MHz, DMSO-*d*₆) δ 165.64 (s), 151.94 (s), 150.46 (s), 134.77 (s), 126.88 (s), 125.44 (s), 122.80 (s), 122.45 (s), 122.39 (s), 118.77 (s), 118.14 (s), 117.18 (s), 115.56 (s). HRMS (ES⁺): calc. for C₁₃H₁₀N₂OS [M+H]⁺ 243.0587, found 243.0577.

***N*-3-(Benzo[d]thiazol-2-yl)-4-hydroxyphenyl)-2-chloroacetamide (2)**



ABAH (1.26 g, 5.2 mmol) was dissolved in mixture solvent of CHCl_3 and CH_3CN (1/1, v/v), NaHCO_3 saturated solution (120 μL) and chloroacetyl chloride (1.65 ml, 20.8 mmol) were added to the solution and then stirred for 1 h. The solvent was evaporated in vacuum. Then the residue was washed with dichloromethane, the organic layer was extracted with aqueous NaHCO_3 and washed with H_2O . The organic layer was dried over anhydrous MgSO_4 . The solvent was evaporated in vacuum to present the residue, which was purified by flash column chromatography eluting with petroleum ether/EtOAc (20:1) to afford pure product as white solid (0.45 g, 27% yield). ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 11.36 (s, 1H), 10.34 (s, 1H), 8.48 (d, $J = 2.6$ Hz, 1H), 8.13 (d, $J = 7.9$ Hz, 1H), 8.05 (d, $J = 8.1$ Hz, 1H), 7.63 (dd, $J = 8.8, 2.7$ Hz, 1H), 7.53 (ddd, $J = 8.3, 7.3, 1.2$ Hz, 1H), 7.47 – 7.40 (m, 1H), 7.05 (d, $J = 8.8$ Hz, 1H), 4.24 (s, 2H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 164.81 (s), 164.74 (s), 153.04 (s), 151.88 (s), 135.06 (s), 131.27 (s), 126.90 (s), 125.51 (s), 124.65 (s), 122.60 (s), 122.48 (s), 119.56 (s), 118.81 (s), 117.62 (s), 43.97 (s). HRMS (ES^+): calc. for $\text{C}_{15}\text{H}_{11}\text{ClN}_2\text{O}_2\text{S}$ $[\text{M}+\text{H}]^+$ 319.0303 found 319.0302.

N-(3-(benzo[d]thiazol-2-yl)-4-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)oxy)phenyl)-2-chloroacetamide (ABAH-LW)



To a solution of **2** (0.14 g, 0.44 mmol) in dry DMF 4-bromomethylphenylboronic acid pinacol ester (0.13 g, 0.44 mmol) was added. The suspension was stirred at room temperature for 1.5 h, and reaction mixture was concentrated under reduced pressure. The crude product was then purified by chromatography on silica gel (petrol ether: EtOAc, from 50:1 to 20:1, v/v) to afford a pale yellow solid (56 mg, 25% yield). ^1H NMR (500 MHz, CDCl_3) δ 8.46 – 8.37 (m, 2H), 8.11 (d, $J = 8.1$ Hz, 1H), 8.02 (dd, $J = 9.0, 2.7$ Hz, 1H), 7.89 (dd, $J = 16.0, 7.9$ Hz, 3H), 7.57 – 7.48 (m, 3H), 7.39 (t, $J = 7.6$ Hz, 1H), 7.10 (d, $J = 9.0$ Hz, 1H), 5.35 (s, 2H), 4.22 (s, 2H), 1.37 (s, 12H). ^{13}C NMR (125 MHz, CDCl_3) δ 163.99 (s), 163.64 (s), 162.26 (s), 153.53 (s), 151.53 (s), 138.78 (s), 135.11 (s), 130.70 (s), 127.06 (s), 126.15 (s), 124.88 (s), 124.17 (s), 122.67 (s), 122.63 (s), 121.43 (s), 121.12 (s), 113.74 (s), 83.92 (s), 71.41 (s), 42.85 (s), 24.89 (s). HRMS (ES^+): calc. for $\text{C}_{28}\text{H}_{28}\text{BClN}_2\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 535.1629 found 535.1639.

2. Generation of various ROS

ROO•

ROO• was generated from 2,2'-azobis (2-amidinopropane) dihydrochloride. AAPH (2, 2' azobis (2-amidinopropane) dihydrochloride, 1 M) was added into deionizer water, and then stirred at 37 °C for 30min.

⁻O₂

Superoxide was generated from KO₂. KO₂ and 18-crown-6 ether (2.5 eq) was dissolved in DMSO to afford a 0.25 M solution.

•HO

Hydroxyl radical was generated by the Fenton reaction. To prepare •OH solution, hydrogen peroxide (H₂O₂, 10 eq) was added to Fe(ClO₄)₂ in deionised water.

ONOO⁻

Simultaneously, 0.6 M KNO₂, 0.6 M in HCl, 0.7 M in H₂O₂ was added at to a 3 M NaOH solution at 0 °C. The concentration of peroxyntirite was estimated by using extinction coefficient of 1670 cm⁻¹M⁻¹ at 302 nm in 0.1 M sodium hydroxide aqueous solutions.

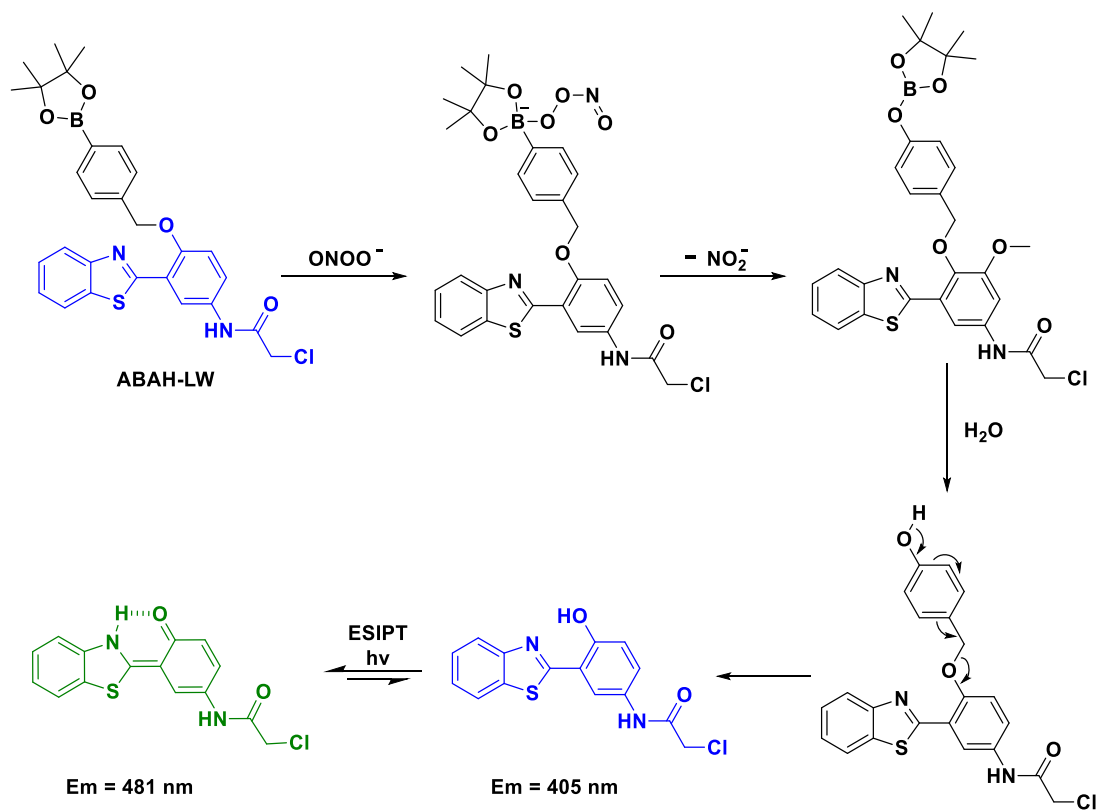
⁻OCl

The concentration of ⁻OCl was determined from the absorption at 292 nm ($\epsilon = 350 \text{ M}^{-1} \text{ cm}^{-1}$).

H₂O₂

The concentration of H₂O₂ was determined from the absorption at 240 nm ($\epsilon = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$).

3. Mechanism of ONOO⁻ with ABAH-LW



Scheme S2. Detailed mechanism of reaction between ABAH-LW and peroxyntrite.

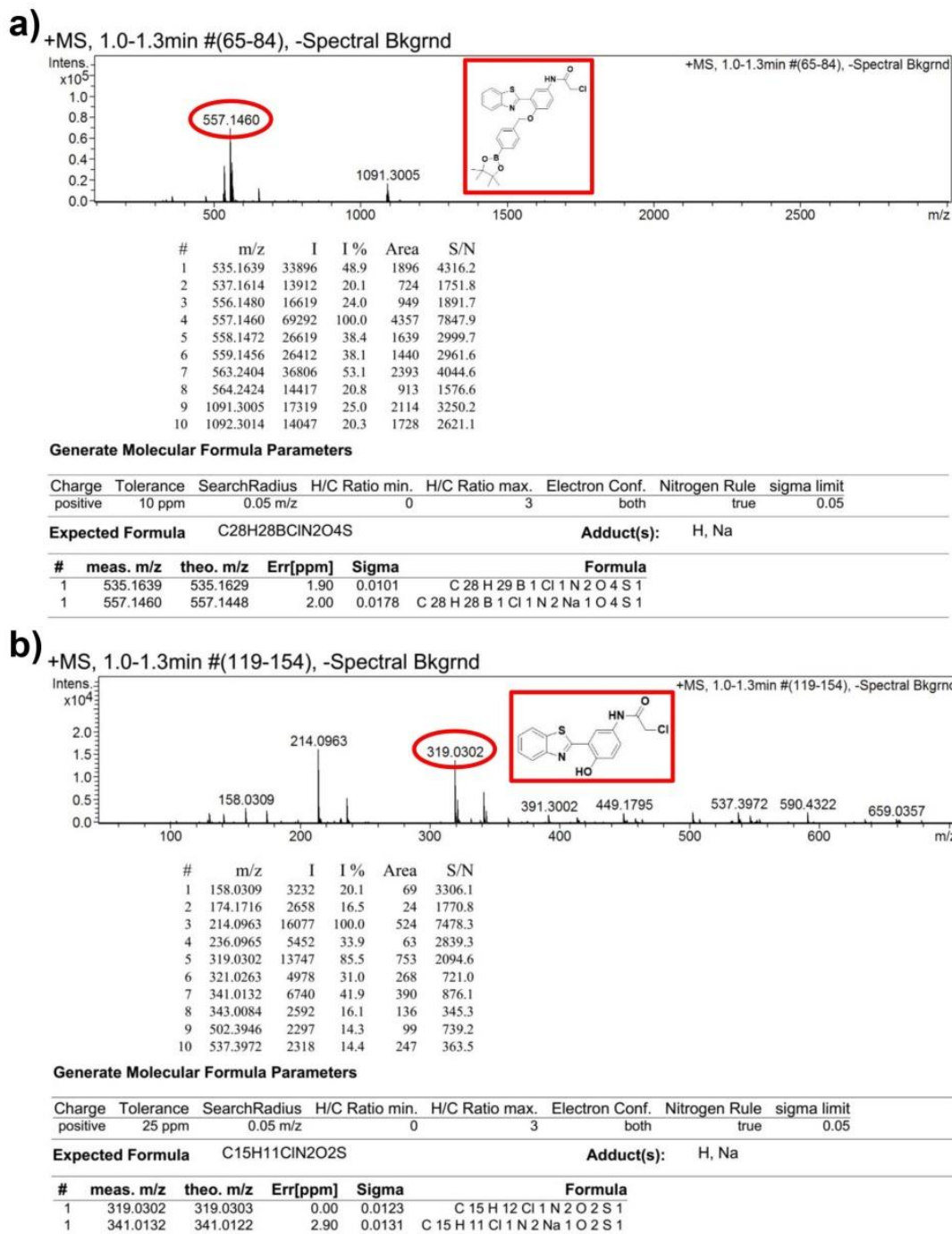


Figure S1. Detection mechanism was explored by mass spectrometry analysis. (a) Mass spec of compound ABAH-LW; (b) mass spec of a sample of ABAH-LW after addition of ONOO⁻.

4. UV and fluorescence analysis

Fluorescence measurements were determined on a perkin-elmer LS50 scanning spectrophotometer. Excitation and emission slit widths were modified to adjust the fluorescence intensity to a suitable range. Absorption spectra were measured on a CARYWINUV UV-Visible spectrophotometer. The detection limit (CDL) was calculated by IUPAC assay. $CDL = 3 \text{ sbm}^{-1} \text{ sb}$ is the ratio of signal and noise, m is the slope of linear equation). The sb was determined through standard deviation (11 times) of F_{481}/F_{405} for ABAH-LW at $3 \mu\text{M}$ without addition of peroxyinitrite.

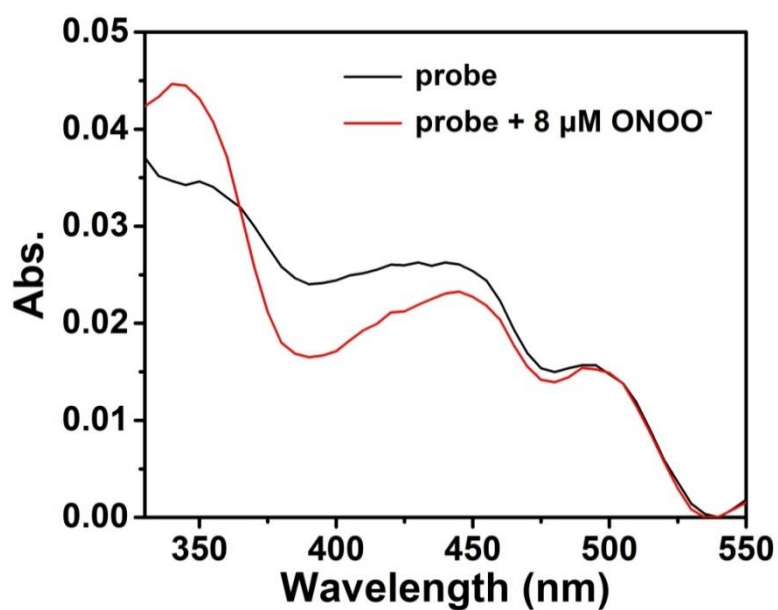


Figure S2. UV spectra of probe ABAH-LW ($3 \mu\text{M}$), with and without ONOO^- ($8 \mu\text{M}$) in pH 8.2 buffer solution (pH 8.2, containing 8% DMSO, 1 mM CTAB).

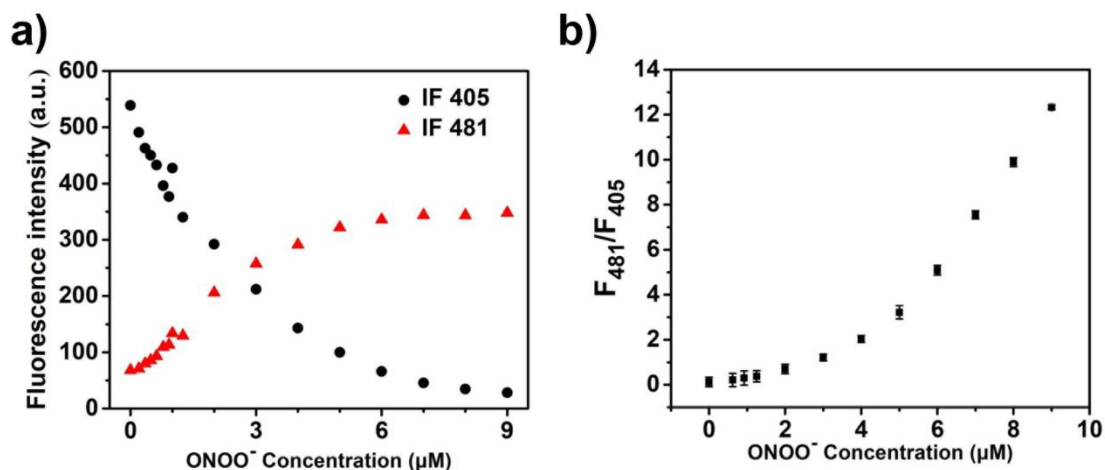


Figure S3. (a) Emission at 405 and 481 nm of **ABAH-LW** (3 μM) with increasing addition of ONOO⁻ (from 0 to 9 μM) in PBS buffer (pH 8.2, containing 8% DMSO, 1 mM CTAB) after 1 min. $\lambda_{\text{ex}} = 370$ nm. (b) A plot of fluorescence intensity ratio changes (based on the peak heights at the maxima, 405 and 481 nm respectively) depending on ONOO⁻ concentration. Error bar represents s.d.. $\lambda_{\text{ex}} = 370$ nm. Slit widths: ex = 5 nm, em = 6 nm.

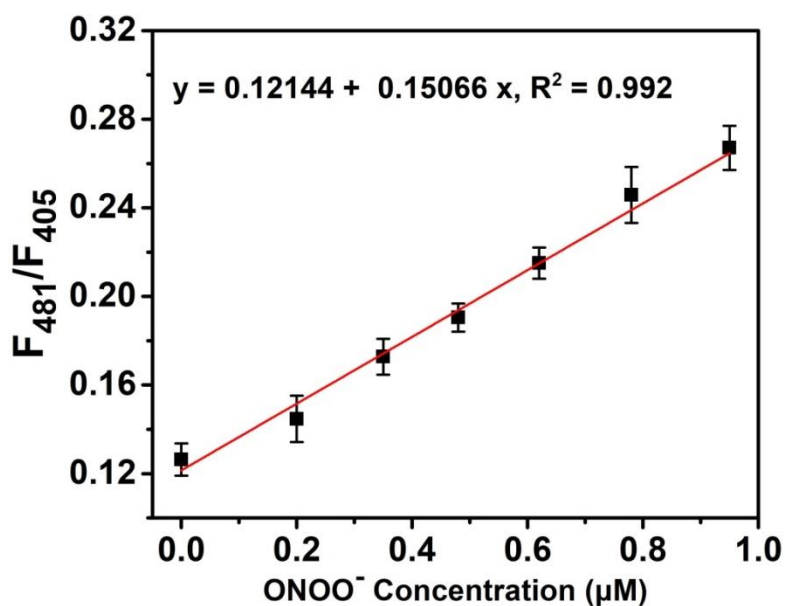


Figure S4. Fluorescence intensity versus concentration of ONOO⁻ for the calculation of the limit of detection for **ABAH-LW** in PBS pH 8.2, containing 8% DMSO, 1 mM CTAB at 25 °C. Fluorescence intensities were measured with $\lambda_{\text{ex}} = 370$ nm. Error bar represents s.d.. LOD is 21.4 nM.

5. Detailed protocols for cell culture

HeLa cells were obtained from American Type Culture collection, and grown in DMEM (High glucose) medium supplemented with 10% FBS. Cells were incubated in a 5% CO₂ humidified incubator at 37 °C and typically passaged with sub-cultivation ratio of 1:4 every two days.

6. Fluorescence imaging in live cells with ABAH-LW

Intracellular fluorescence imaging with ABAH-LW. HeLa cells were grown on confocal petri dishes in DMEM containing 10% FBS and then incubated in a humidified 37 °C, 5% CO₂ incubator. The cells were attached after 12 h, and washed with PBS three times before they are incubated with 2 mL of 1 (50 μM) for another 40 min. Before use, the cells were washed with PBS. ABAH-LW was excited at 405 nm, and the corresponding emissions were collected at 410-460 nm (channel 1, blue) and 480-550 nm (channel 2, green).

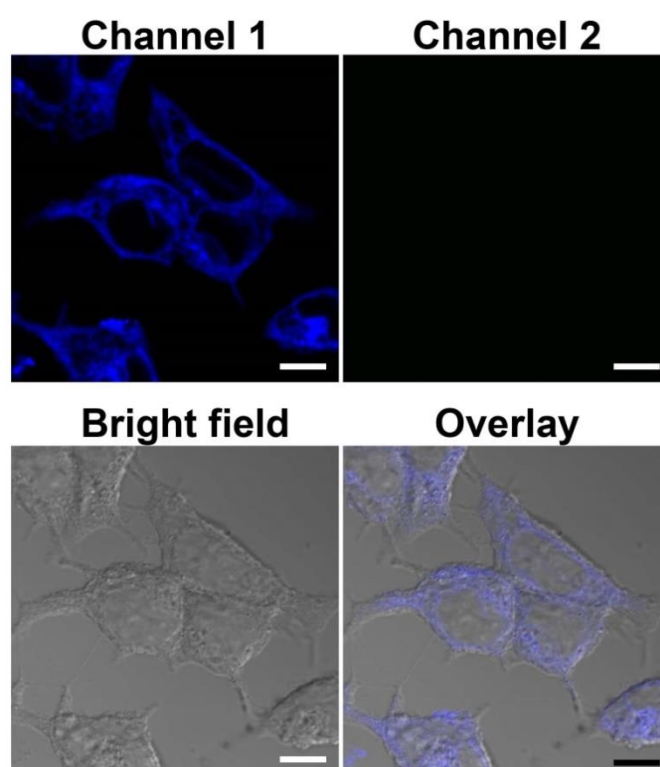


Figure S5. Fluorescence imaging of HeLa cells loaded with ABAH-LW (50 μM). λ_{ex} = 405 nm; Channel 1 (green): λ_{em} = 410-460 nm; Channel 2 (green): λ_{em} = 480-550 nm. Overlay: the merged images of Channel 1, Channel 2 and bright field. Scale bar: 10 μm.

Co-localization of ABAH-LW with commercial endoplasmic reticulum probe ER-Tracker™ Red dye. The HeLa cells were attached on confocal Petri dishes in complete medium under standard culture conditions. Then the cells were washed with PBS for three times, and were co-incubated with 2 mL of ER-Tracker™ Red dye (1 μM) and ABAH-LW (50 μM) for 40 min at 37 °C. Fluorescence imaging experiments were implemented by a Olympus FV1200 laser scanning microscopy excitations at 405 nm (ABAH-LW) and 568 nm (for ER-Tracker™ Red dye) and the emissions were collected in the range of 410-460 nm (channel 1, blue), 480-550 nm (channel 2, green) and 575-675 nm (red).

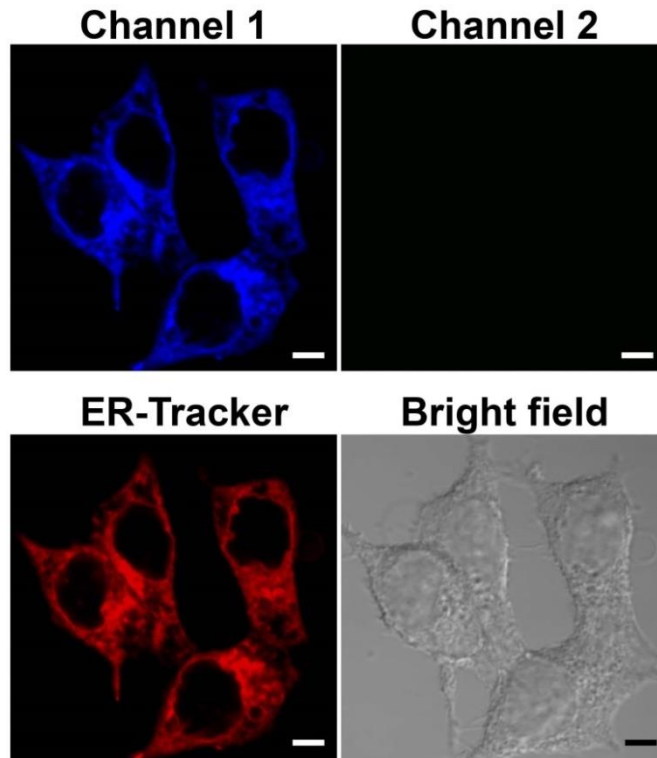


Figure S6. Enlarged images of HeLa cells loaded with **ABAH-LW** and ER-Tracker for co-localization assay. Scale bar: 5 μm .

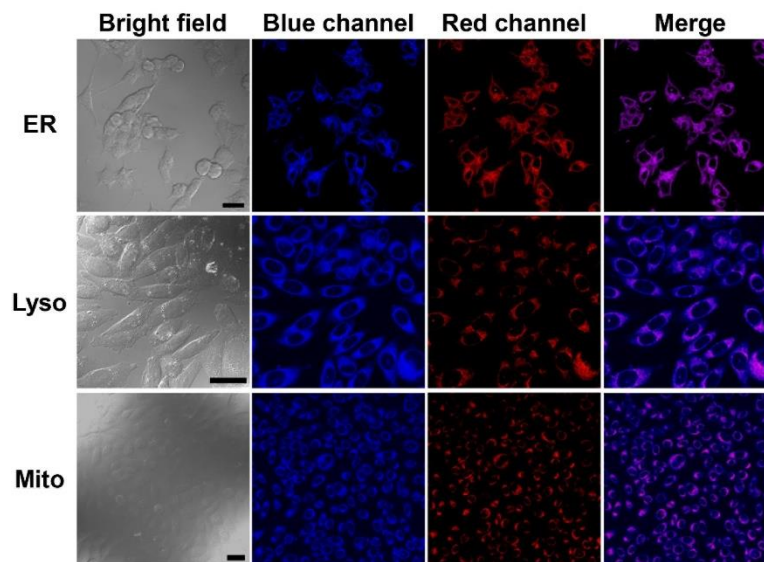


Figure S7. Confocal fluorescence images for intracellular localization of **ABAH-LW** in HeLa cells were treated with 50 μM **ABAH-LW** for 0.5 h and stained (0.5 h) with 1 μM ER-Tracker Red, 65 nM Lyso-Tracker Red or 300 nM Mito-Tracker Red. Blue channel at 410-460 nm. Red channel at 575-675 nm for ER-Tracker Red 650-800 nm for Lyso-Tracker Red, and 655-700 nm for Mito-Tracker Red.

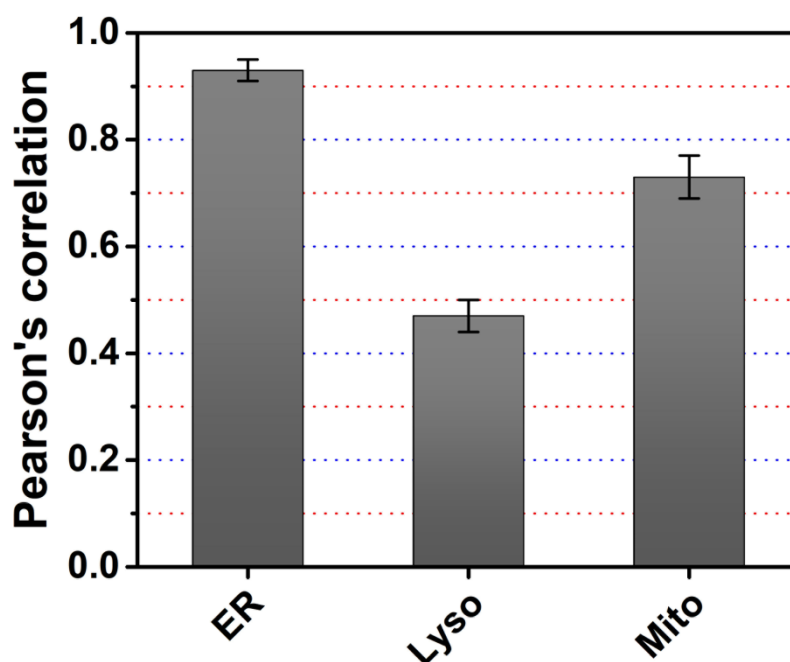


Figure S8. Pearson's correlation coefficient of three subcellular organelles. Pearson's correlation coefficient of ER, Lyso, and Mito is 0.93 ± 0.02 , 0.47 ± 0.03 , and 0.73 ± 0.04 , respectively.

The HeLa cells were attached on confocal Petri dishes in complete medium under standard culture conditions. To produce ONOO⁻ exogenously, the cells were first incubated with **ABAH-LW** (50 μM) for 30 min, followed by incubation with Sin-1 (0.5 and 1.2 mM) for 30 min. To quench the exogenously produced ONOO⁻, the cells were incubated with **ABAH-LW** (50 μM) for 30 min, then ebselen (200 μM) for 1 h, and finally Sin-1 (1.2 mM) for 30 min. To simulate endogenous peroxynitrite, lipopolysaccharide (1.2 μg mL⁻¹) was first added to HeLa cells. After 16 hours, the cells were incubated with 70 ng mL⁻¹ interferon-γ for 4 h. Then the cells were incubated with **ABAH-LW** (50 μM) for 30 min. To quench the endgenously produced ONOO⁻, LPS (1.2 μg mL⁻¹) was incubated with HeLa cells then IFN-γ (70 ng mL⁻¹) was added, HeLa cells were incubated with 150 μM ebselen for 1 h. HeLa cells were washed with PBS (phosphate buffered saline) three times before imaging. The fluorescence images were recorded using a FV1200 Laser Scanning Microscope (Olympus, Japan). **ABAH-LW** was excited at 405 nm, and the corresponding emissions were collected at 410-460 nm (channel 1, blue) and 480-550 nm (channel 2, green).

7. References

1. Cui, L.; Zhu, W.; Xu, Y.; Qian, X., A novel ratiometric sensor for the fast detection of palladium species with large red-shift and high resolution both in aqueous solution and solid state. *Analytica Chimica Acta* 2013, 786, 139-145.
2. Zhao, Y.; Xue, Y.; Li, H.; Zhu, R.; Ren, Y.; Shi, Q.; Wang, S.; Guo, W., An excited state intramolecular proton transfer dye based fluorescence turn-on probe for fast detection of thiols and its applications in bioimaging. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 2017, 175, 215-221.

8. NMR spectrum

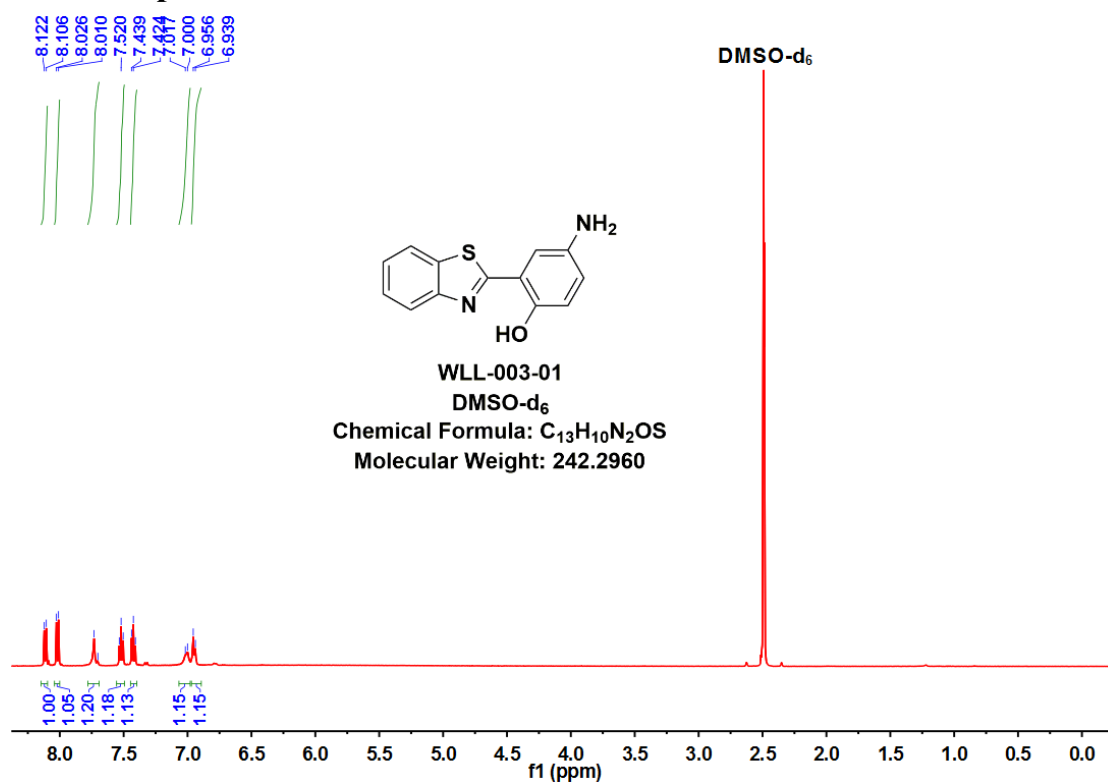


Figure S9. ¹H NMR spectrum of compound ABAH.

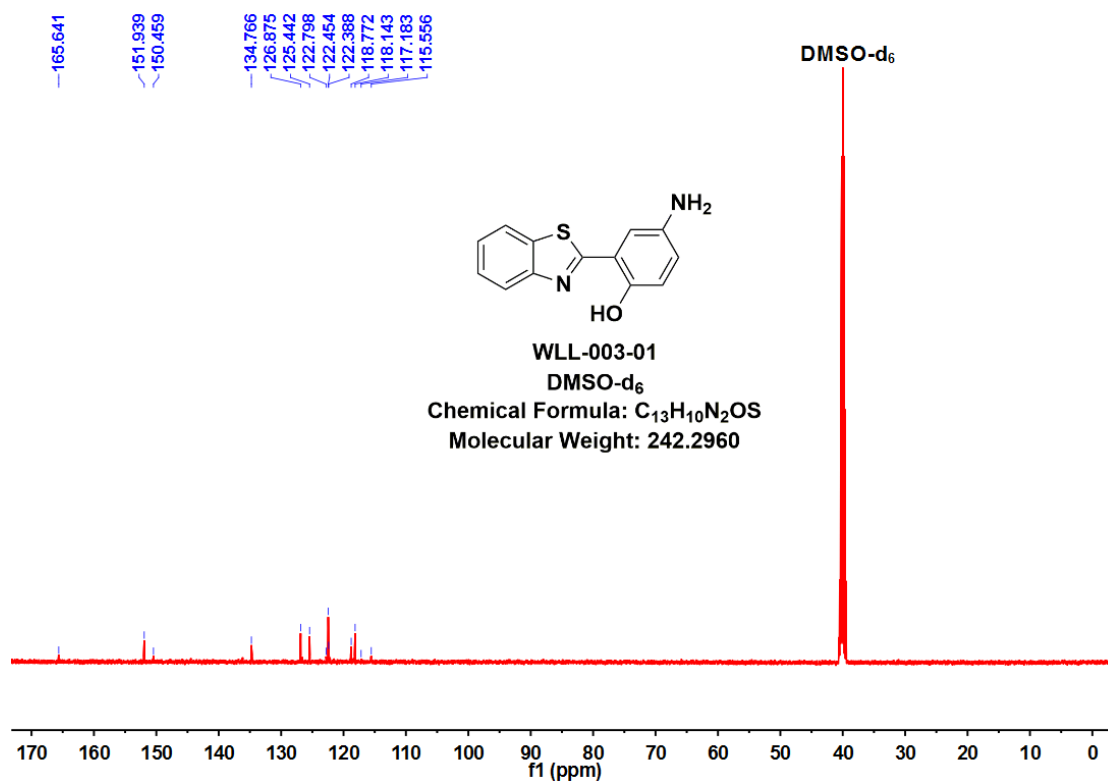


Figure S10. ¹³C NMR spectrum of compound ABAH.

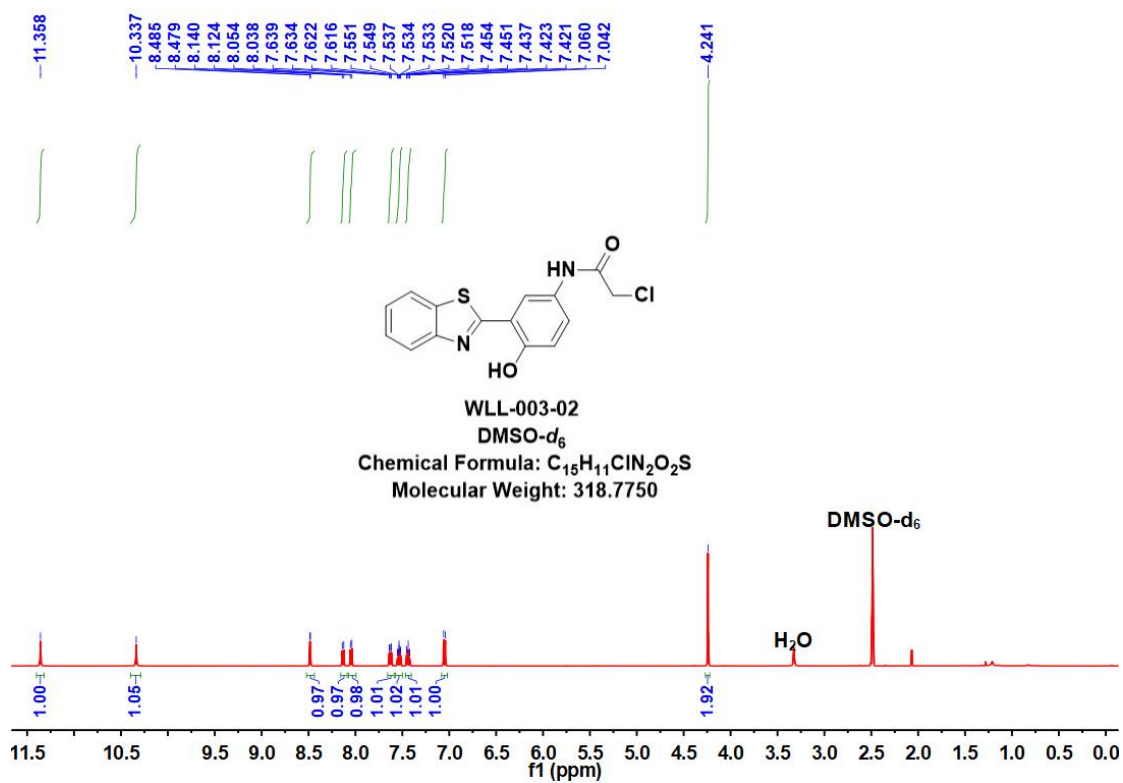


Figure S11. ¹H NMR spectrum of compound 2.

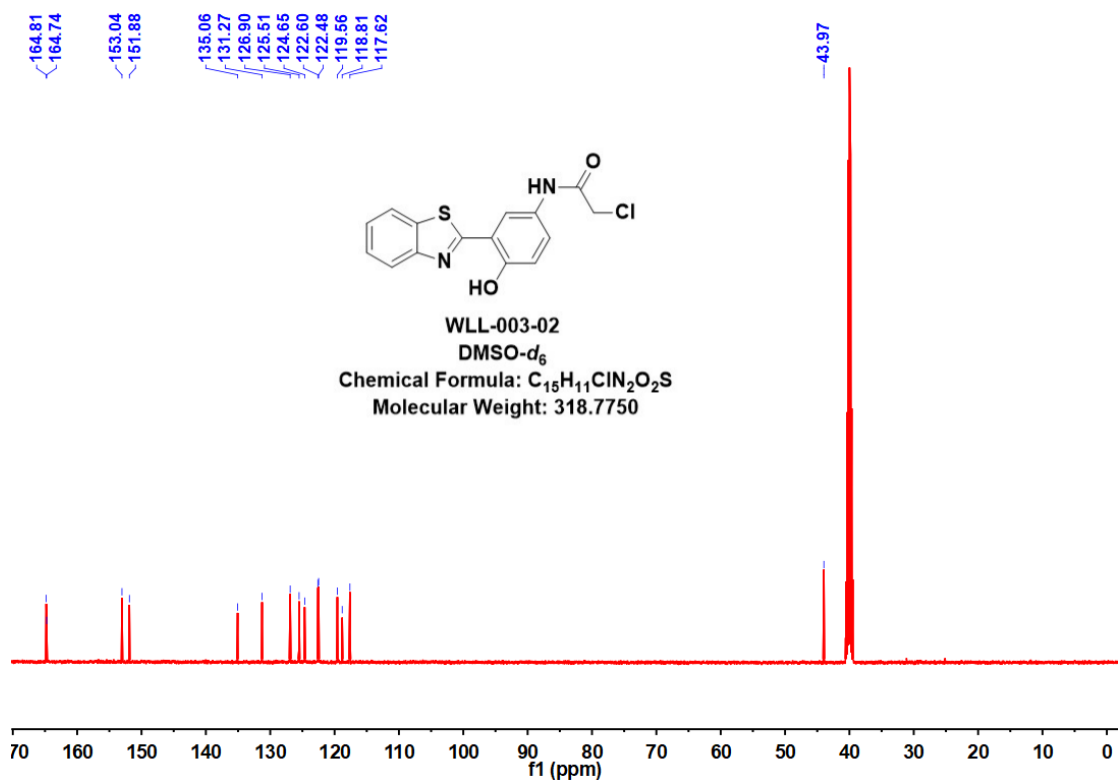


Figure S12. ¹³C NMR spectrum of compound 2.

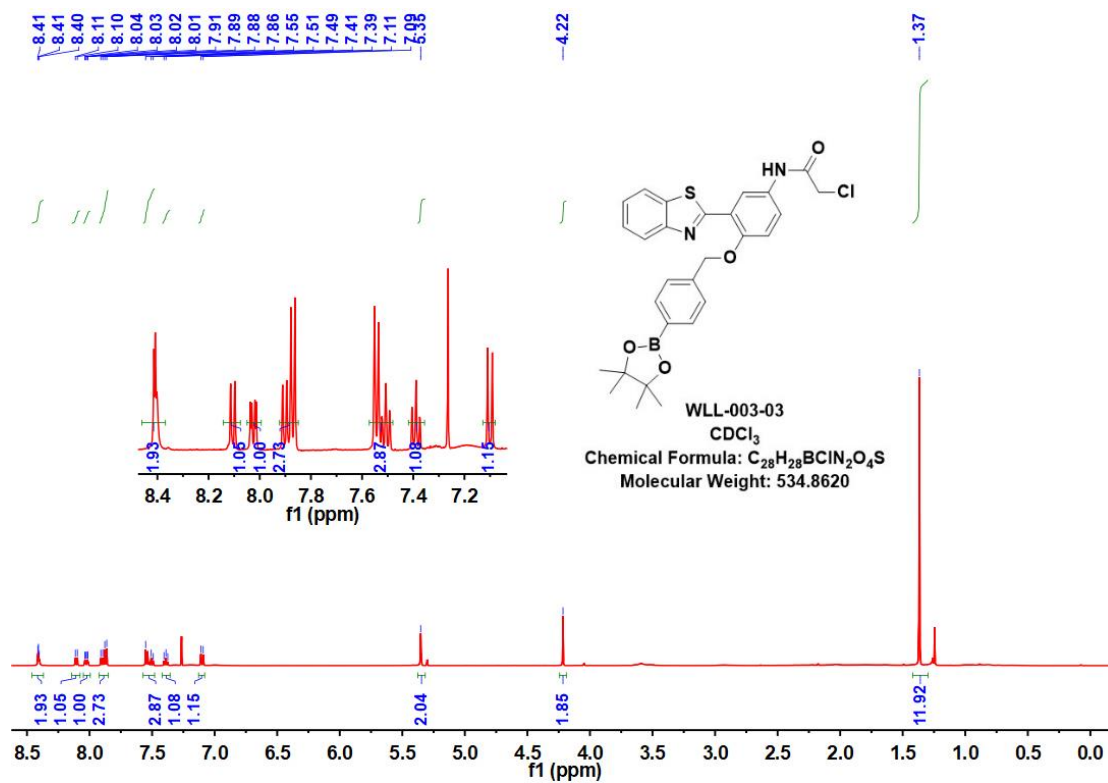


Figure S13. ^1H NMR of target probe ABAH-LW.

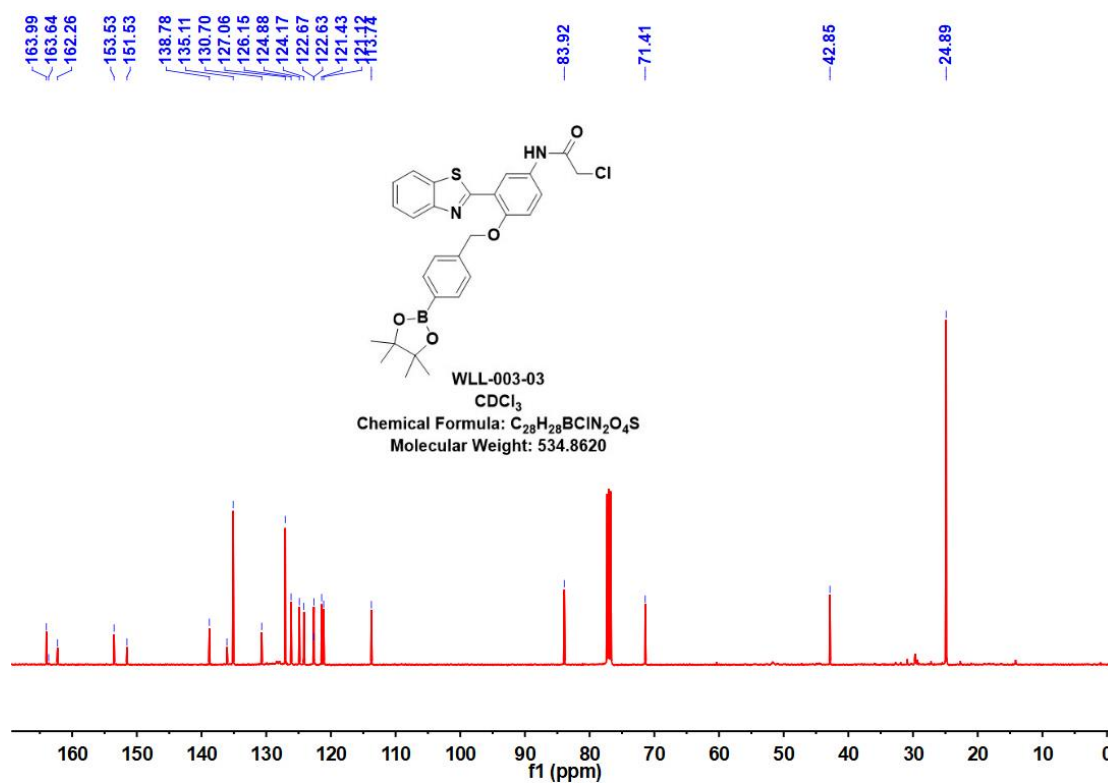


Figure S14. ^{13}C NMR spectrum of target probe ABAH-LW.