Electronic Supplementary Information

Observing hydrogen sulfide in the endoplasmic reticulum of cancer cells and zebrafish by using an activity-based fluorescent probe

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1. Materials and instruments

This investigation used only commercially available solvents and chemicals, and no extra cleaning or treatment was required. Thin-layer chromatography (TLC) evaluated the materials on silica gel plates. The silica gel was used to separate the column chromatography samples. The results of electrospray ionization mass spectra (ESI-MS) were recorded in LTQ mass spectrometry. NMR measurements were calibrated against a TMS reference (Bruker 500 MHz). The equipment to test pH was also used to get the results (Model PHS-3C). Dilution was achieved using a Millipore-Q machine (Millipore, USA). Absorption and fluorescence were measured on Agilent Cary 60 Uv-Vis spectrophotometer and an Agilent Cary Eclipse fluorescence spectrophotometer. During the cytotoxicity test, a microplate reader was used to collect an optical density (O.D.) value (Thermo Fisher Scientific, Waltham, MA). A confocal laser scanning microscope was employed to perform fluorescence imaging studies (FV 3000, Olympus).

2. Synthesis of fluorescent probe ER-Nap-NBD

Compounds 1, 2, 3, and ER-NapH were synthesized according to the reported literatures[1, 2]

The synthesis of compound 1 began with the dissolution of 1.45 grams (9 mmol) of tert-butyl (2-aminoethyl) carbamate in 30 milliliters (9 mmol) of 100% ethanol, which was followed by the addition of 2.49 grams (9 mmol) of 4-Bromo-1,8-naphthalic anhydride. For four hours, the solution was heated to the point of reflux. After bringing the temperature of the reaction down to room temperature, the precipitate was filtered, washed with cold ethanol, and then dried in a vacuum drying oven overnight at room temperature in order to get the off-white solid **compound 1**. Column chromatography was applied to clean the compound DCM: MeOH (10:1, v/v) (2.91 grams, 76% yield). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.65 – 8.59 (m, 3H), 8.55 – 8.49 (m, 2H), 8.41 – 8.34 (m, 2H), 8.03 – 7.97 (m, 2H), 7.86 – 7.77 (m, 3H), 4.98 (q, J = 5.9 Hz, 2H), 4.37 – 4.30 (m, 5H), 3.57 – 3.48 (m, 6H), 1.38 – 1.24 (m, 21H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.31, 164.28, 156.44, 133.72, 132.57, 131.74, 131.47, 130.96, 130.74, 129.42, 128.44, 123.31, 122.44, 79.53, 40.44, 39.91, 28.60.

Synthesis of compound 2. Compound 1 (1.25 g, 3 mmol) and excess trifluoroacetic acid (2 mL) were dissolved in 20 mL of CH_2Cl_2. The mixture was stirred at room temperature for 12 h and

then subjected to rotary distillation to obtain the pale-yellow product (0.75 g, 79%). The product was used for the next step without more purification.

The synthesis of compound 3. Compound 2 (0.96 g, 3 mmol) was mixed with 0.8 mL of triethylamine in a volume of CH_2Cl_2 equal to 45 mL. After that, at 0 degrees Celsius, 0.57 grams of *p*-toluenesulfonyl chloride was added to the solution that had been prepared. After allowing the solution to react at room temperature for six hours, the solvent was separated from the mixture. Following purification of the crude product using silica gel column chromatography with dichloromethane/methanol (100: 1, v/v), the desired **chemical 3**, in the form of a white solid (0.93 g and 65% purity), was obtained. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.57 – 8.52 (m, 2H), 8.29 (d, *J* = 7.8 Hz, 1H), 8.02 (d, *J* = 7.9 Hz, 1H), 7.84 (dd, *J* = 8.4, 7.4 Hz, 1H), 7.54 – 7.48 (m, 2H), 6.73 (d, *J* = 8.0 Hz, 2H), 4.31 – 4.23 (m, 2H), 3.48 (dt, *J* = 7.0, 5.3 Hz, 2H), 1.95 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 162.88, 162.82, 142.32, 137.73, 132.46, 131.40, 131.22, 130.78, 129.69, 129.38, 128.98, 128.67, 128.28, 126.26, 122.75, 121.97, 54.85, 40.05, 39.89, 39.72, 39.55, 39.38, 39.22, 39.05, 20.80.

Synthesis of compound ER-NapH. Compound 3 (474 mg, 1.0 mmol), piperazine (860 mg, 10 mmol), and triethylamine (0.50 mL) were sequentially added to the agitated solution of 2-methoxyethanol (15 mL) in a round-bottomed flask while working in an argon atmosphere. The mixture was heated at a temperature of 100 degrees Celsius for 12 hours before being allowed to cool to ambient temperature before being put into diethyl ether. After being filtered and purified using silica gel chromatography with CH_2Cl_2:CH_3OH (v/v, 5:1) as the eluent, the **ER-NapH** in the form of a yellow solid (250 mg, 52%) was obtained. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.46 (t, *J* = 8.1 Hz, 1H), 8.37 (d, *J* = 8.1 Hz, 1H), 7.84 – 7.79 (m, 1H), 7.75 (s, 1H), 7.59 (d, *J* = 8.1 Hz, 2H), 7.35 (t, *J* = 9.7 Hz, 2H), 7.24 (d, *J* = 7.9 Hz, 3H), 3.21 (s, 8H), 3.10 (s, 4H), 2.27 (s, 3H), 1.23 (s, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.59, 168.96, 159.86, 154.24, 152.07, 136.21, 132.98, 131.95, 128.27, 128.05, 127.96, 127.40, 126.38, 123.61, 122.75, 122.08, 119.31, 117.65, 113.99, 113.27, 78.74, 43.42, 39.70, 32.48, 28.46.

Synthesis of fluorescent probe ER-Nap-NBD: N, N-dimethylformamide (25 mL) was used to dissolve compound **ER-NapH** (394 mg, 1 mmol) and N-Ethyl-N-(propane-2-yl)

propane-2-amine (0.2 mL, 1.2 mmol) in a nitrogen environment. At 0 °C, the solution of 4-chloro-7-nitro-1, 2, 3-benzoxadiazole (239.5 mg) in dimethylformamide (10 mL) was added in a stepwise manner to the mixture while it was being stirred. Following overnight stirring at room temperature, the mixture was further purified using silica gel column chromatography with dichloromethane: methanol (v/v, 10:1) eluent resulting in **ER-Nap-NBD** (334.3 mg, yield 60%). 1H and 13C-NMR spectra of **ER-Nap-NBD** are shown in the Supporting Information. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.61 (d, J = 8.5 Hz, 1H), 8.47 (d, J = 7.3 Hz, 1H), 8.38 (d, J = 8.0 Hz, 1H), 7.85 (t, J = 7.9 Hz, 1H), 7.76 (t, J = 6.4 Hz, 1H), 7.60 (d, J = 7.9 Hz, 2H), 7.40 (d, J = 8.1 Hz, 1H), 7.25 (d, J = 7.9 Hz, 2H), 6.76 (d, J = 9.1 Hz, 1H), 3.56 (s, 4H), 3.35 (s, 7H), 3.07 (q, J = 6.6 Hz, 2H), 2.28 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 163.10, 162.54, 154.03, 144.91, 144.39, 144.31, 141.95, 137.10, 135.80, 131.49, 130.17, 130.06, 128.97, 128.73, 125.85, 125.65, 124.69, 122.17, 121.13, 115.65, 114.73, 103.31, 54.39, 51.37, 48.76, 20.37. HRMS (ESI) $[M-H]^-$ m/z: calcd 640.1620, found: 640.1612.

3. Selective test

There was no purification of any of the reagents or solvents obtained from commercial sources. Stock solutions (10 mM) of Al^{3+}, Ca^{2+}, Fe^{2+}, K^+, Zn^{2+}, H_2O_2, ClO^-, NO_2^-, NO, $ONOO^-$, $S_2O_3^{2-}$, SO_3^{2-}, F^-, CO_3^{2-} and Na_2S were prepared for analysis. Several amino acid solutions, including alanine, arginine, glutamine, phenylalanine, threonine, tryptophan, asparagine, histidine, isoleucine, serine, and valine. Homocysteine, cysteine, and glutathione were created.

4. Fluorescence quantum yields calculation

The fluorescence quantum yields of **ER-Nap-NBD** in the presence or absence of H_2S were determined using the reported methods.[3] The quantum yield performances were evaluated in the solvent system of PBS buffer pH=7.4 and Ethanol (1:1, (v/v)), and using Edinburgh instruments (FLS1000) to provide the quantum yield percentage. The probe was incubated in the system of PBS: Ethanol (1:1, (v/v)) and submitted to Edinburgh instruments to evaluate the quantum. The probe was incubated with 1 mM of Na_2S for 70 minutes in the same system before evaluating the quantum yield. λ_{Ex}=400 nm and λ_{Em}=530 nm

5. Cell culture and cytotoxicity assay

Exogenous and endogenous H_2S imaging was performed in live cells using the **ER-Nap-NBD** probe. MCF-7 cells were grown in DMEM, and 4T1 cells in the 1640 medium supplemented with 10% fetal bovine serum and 1% (v/v) penicillin-streptomycin, respectively. The cells were incubated at 37 °C and 5% CO_2 levels.

The cytotoxicity was conducted using the MTT assay. The 4T1 cells were planted in a 96-well plate at a density of 1.5×10^5 per dish and followed with the treatment of different doses of **ER-Nap-NBD** at 37 degrees celsius for 24 hours in an environment containing 5% CO_2, and the blank group was set up without the probe. The MTT solution (0.5 mg/mL) was then applied to each well and incubated for an additional 4 hours. Remove the supernatant and add 100 μL of DMSO to dilute the generated formazan. After gently shaking the 96-well plate for 10 minutes, the absorbance values at 570 nm and 630 nm were recorded using a microplate reader.

Cells viability (%) = $(OD_{dye}\text{-}OD_{Kdye})/(OD_{control}\text{-}OD_{Kcontrol}) \times 100\%$

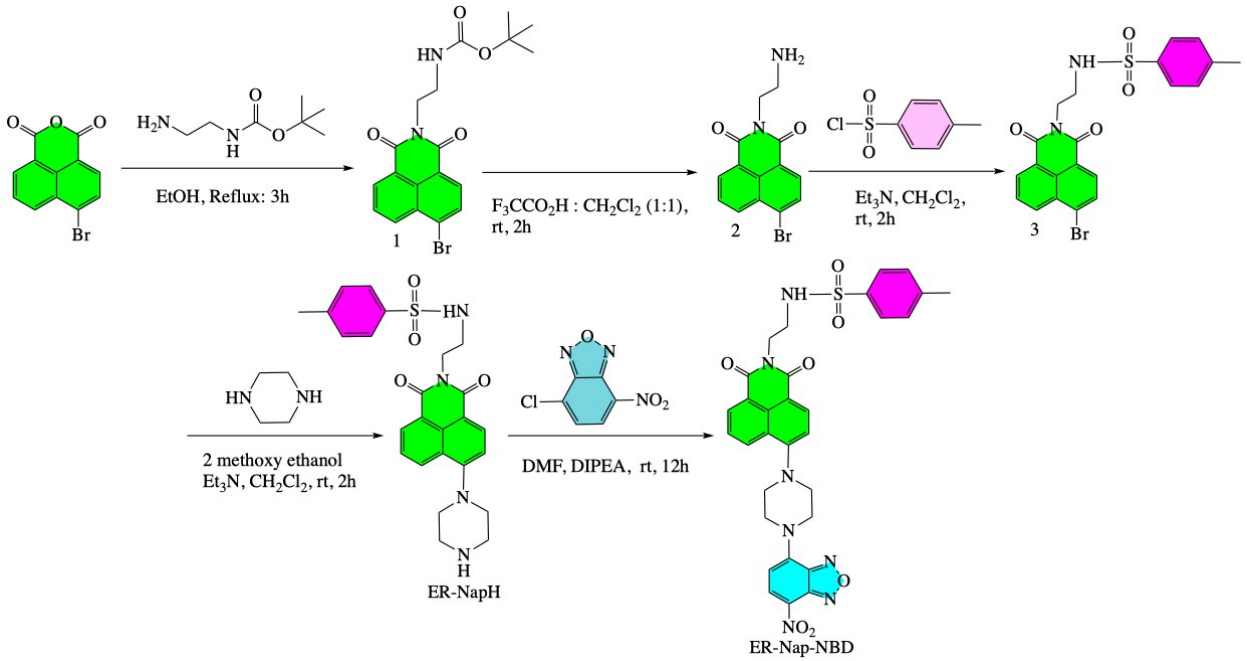
6. Confocal fluorescence imaging for cells

MCF-7 and 4T1 were provided by the Institute of Basic Medical Sciences (IBMS) of the Chinese Academy of Science. In a confocal culture plate, MCF-7 and 4T1 cells were planted and incubated for 24 hours for adherence. The cultures were then maintained under normal conditions for an additional 8 hours at 37 °C. Following cultivation, the probe solution was added to the confocal dish to achieve a final concentration of 10 μM. The cell lines with probes were designated as the control group, the cells with N-ethylmaleimide inhibitors were designated as the negative controls, and the cells treated with L-Cys and Na_2S as the intrinsic and extrinsic positive group. After thirty minutes, the cells were taken from the incubator and the growth media was discarded. The cells were washed three times with PBS. Finally, fluorescence images of cultivated cells were captured using a laser confocal microscope with an excitation light wavelength of 405 nm, and the emission light was collected between 500-600 nm.

7. In vivo imaging of zebrafish

Zebrafish were provided by Nanjing EzeRinka Biotechnology Co., Ltd. All fluorescence images were obtained on a stereomicroscope under the confocal laser scanning microscope. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animal Resources and approved by the Ethics Committee of Dalian University of Technology.

8. Supporting tables and figures



Scheme S1. Synthetic route of fluorescent probe **ER-Nap-NBD**.

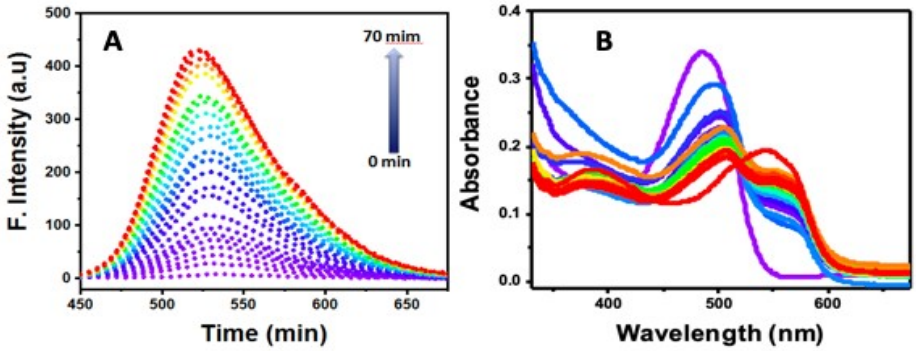


Fig. S1. (A) Time-function fluorescence of **ER-Nap-NBD** 10 μM for the measurement of 1mM Na_2S for 70 min (B) UV-vis absorption spectra of **ER-Nap-NBD** (10 μM) upon addition of 0-1 mM Na_2S in EtOH-PBS (1:1, v/v, pH=7.4) solution. λ_{Ex}=400 nm and λ_{Em}=530 nm.

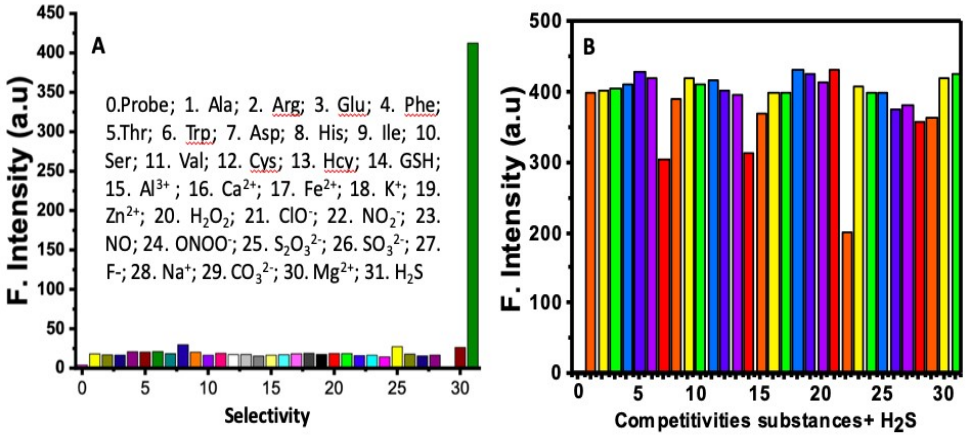


Fig. S2. (A)The selectivity test of probe **ER-Nap-NBD** (10 μM) to measure other chemicals (1 mM). (B) The competitivities fluorescence signals of probe **ER-Nap-NBD** (10 μM) in the presence of 1 mM H_2S and other chemicals (1 mM) in solution.

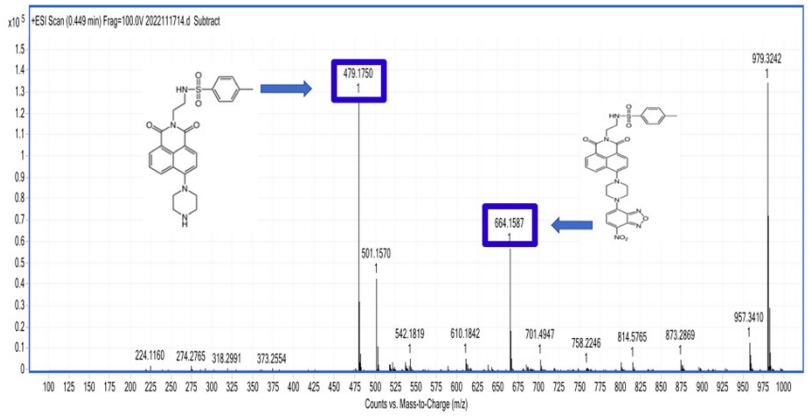


Fig. S3. ESI-HRMS spectrum of probe **ER-Nap-NBD** treated with Na_2S.

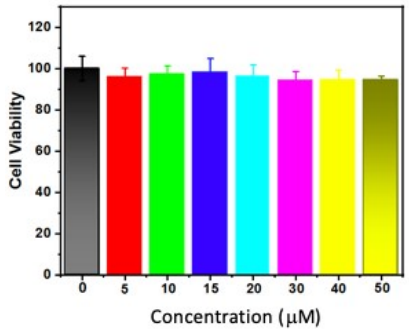


Fig. S4. Cytotoxicity of **ER-Nap-NBD** for 4T1 cells. Cells were incubated with the probe at corresponding concentrations for 24 h. Cell viability was measured by MTT assay, and the results are reported as percentages relative to untreated cells.

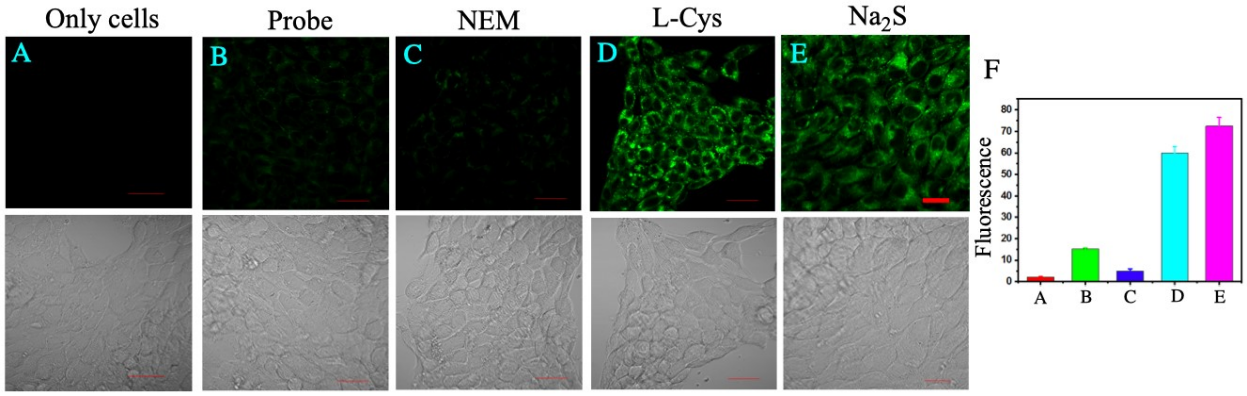


Fig. S5. Fluorescence imaging of 4T1cells. Cells group (A): cells were culture and imaged without any additional compound. Control group probe (B): cells were incubated with 10 μM **ER-Nap-NBD** for 30 min. The NEM group (C): the probe-loaded cells were treated with a concentration of 100 μM NEM for 1 hour and then treated with the **ER-Nap-NBD** (10 μM). The L-Cys group (D): cells were pretreated with a concentration of L-Cys (100 μM) for 2 h and then treated with the **ER-Nap-NBD** (10 μM). The H_2S group (E): the cells were pretreated with the probe for 30 min and then incubated with H_2S 100 μM for another 1h. (F) The relative fluorescence intensity of 4T1 corresponds to pictures A to E, respectively. The scale bar: 20 μm; λ_{ex} = 405 nm/ λ_{em} = 500-600 nm.

^{1}H, ^{13}C NMR, and MS spectra

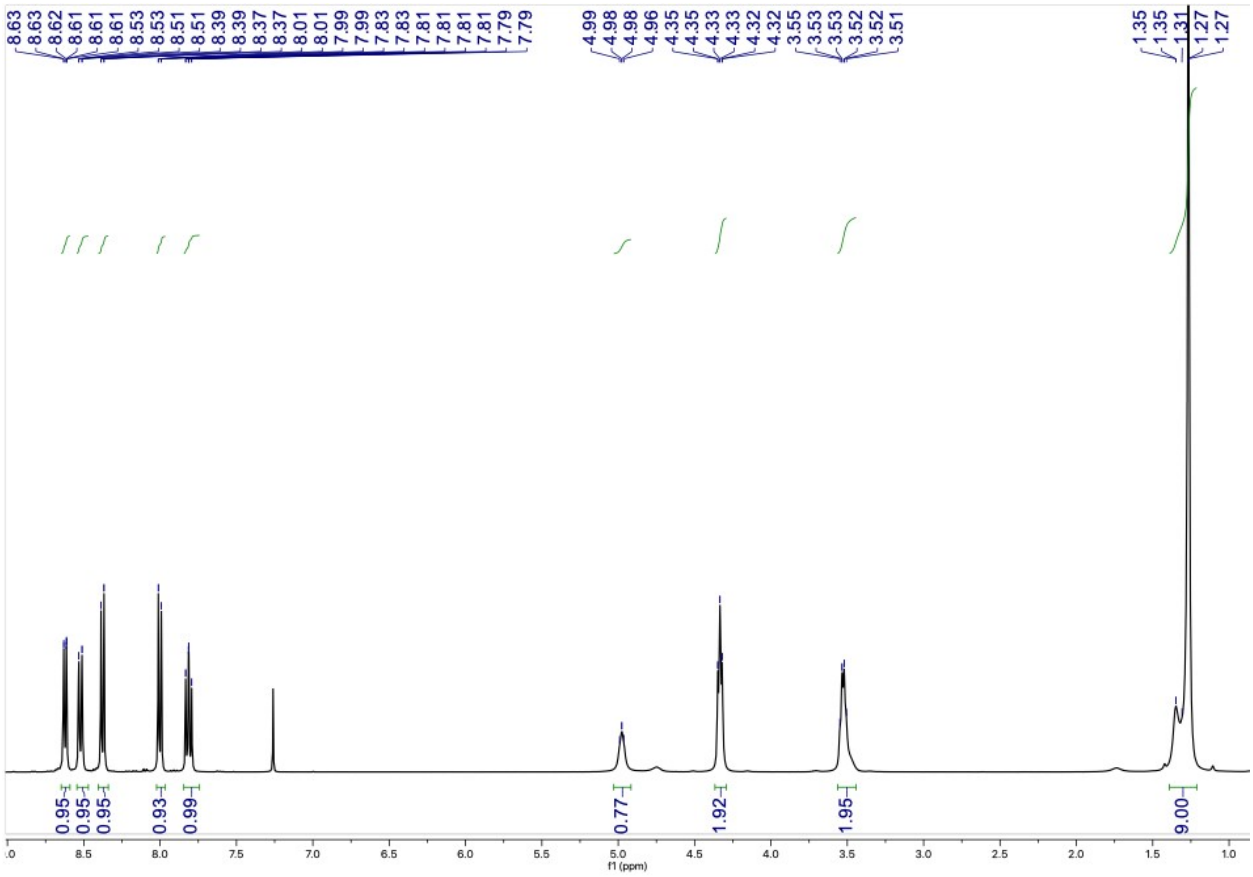


Fig. S6. ^{1}H-NMR spectrum of **compound 1** in $CDCl_3$.

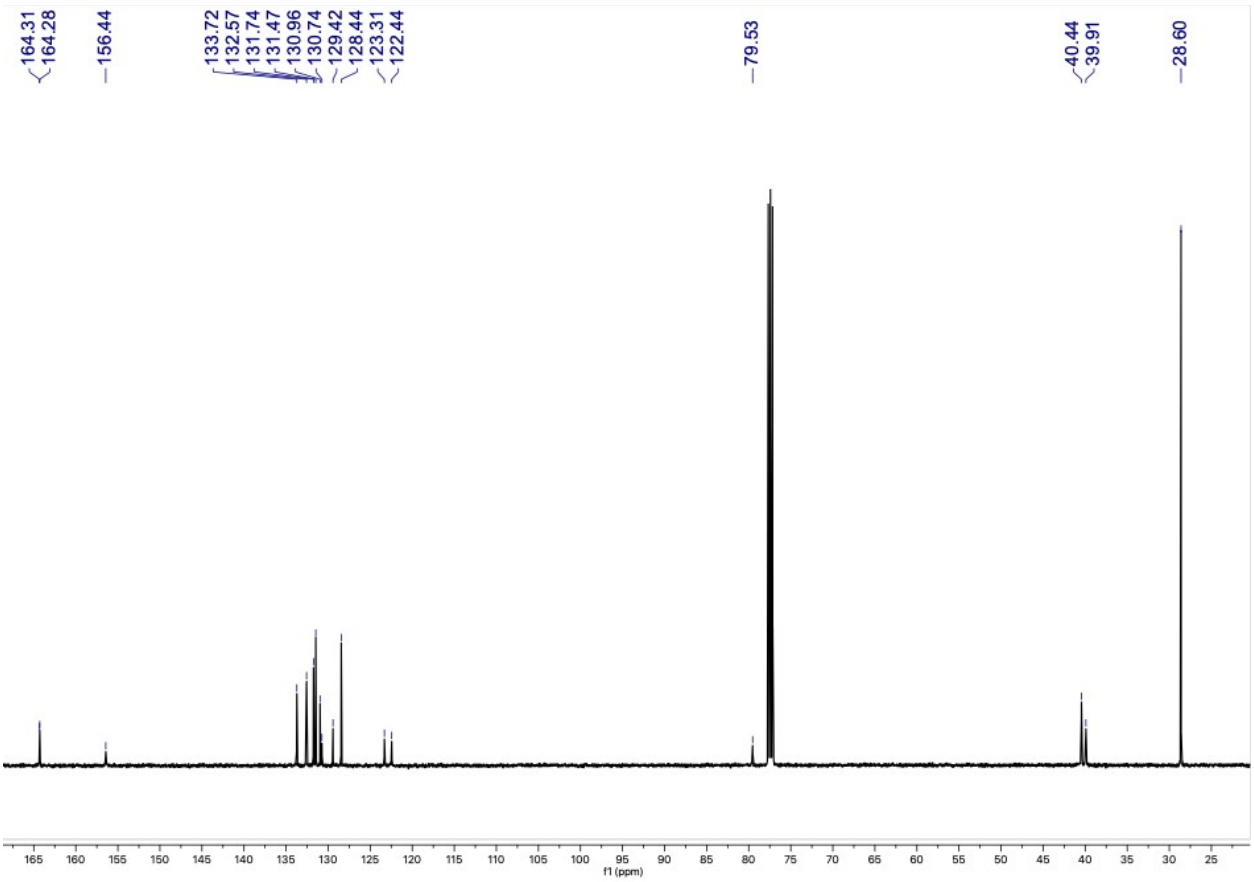


Fig. S7. ^{13}C-NMR spectrum of **compound 1** in $CDCl_3$.

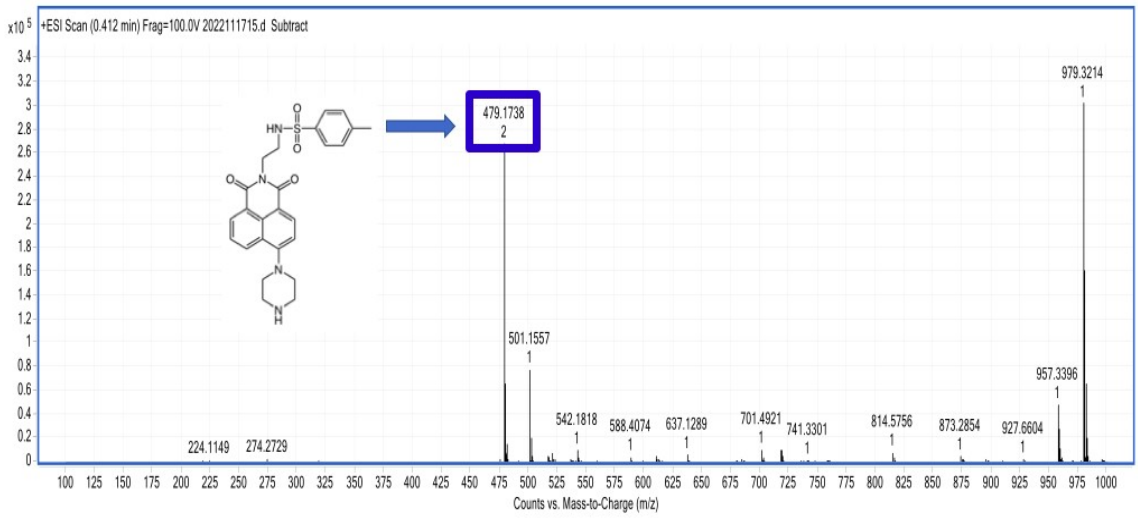


Fig. S8. ESI-HRMS of probe **ER-NapH**.

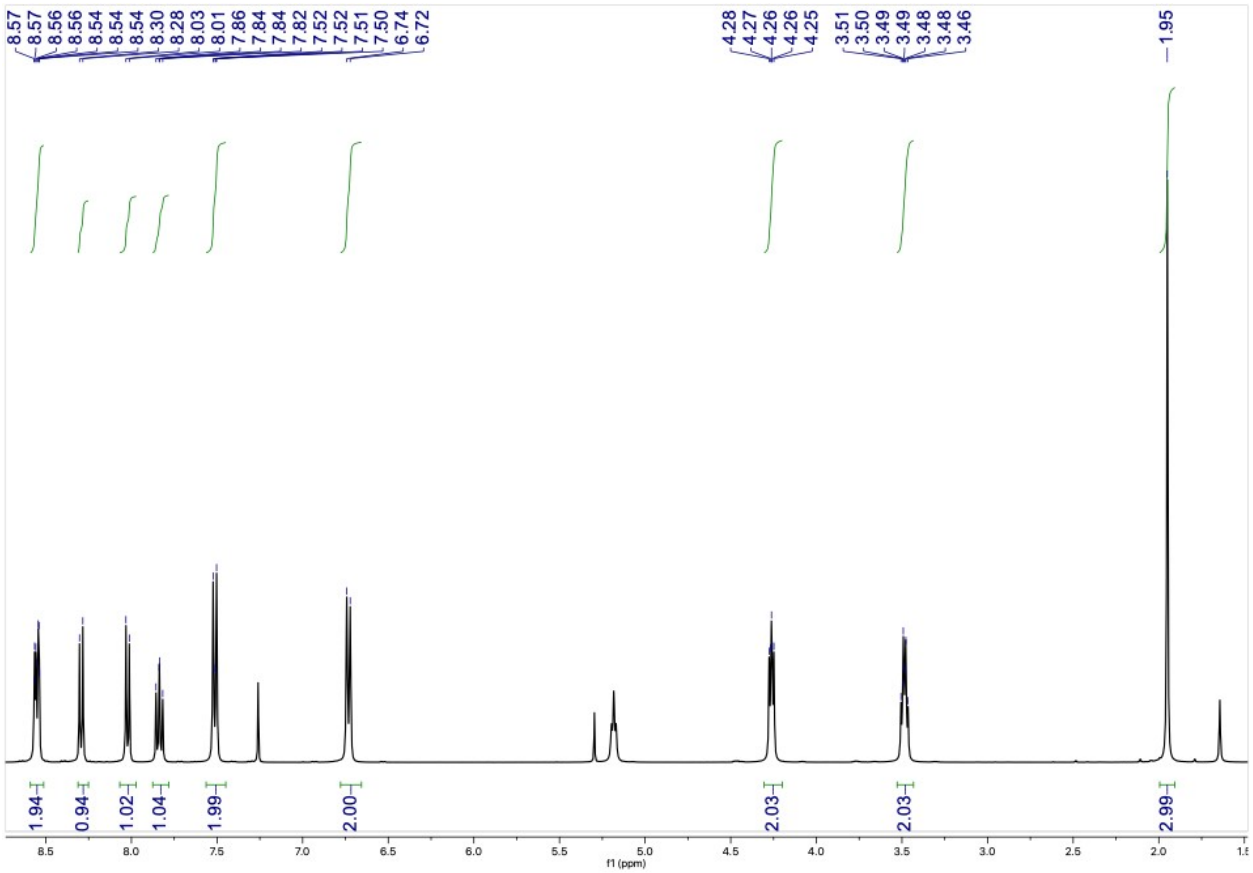


Fig. S9.[1]H-NMR spectrum of **compound 3** in $CDCl_3$.

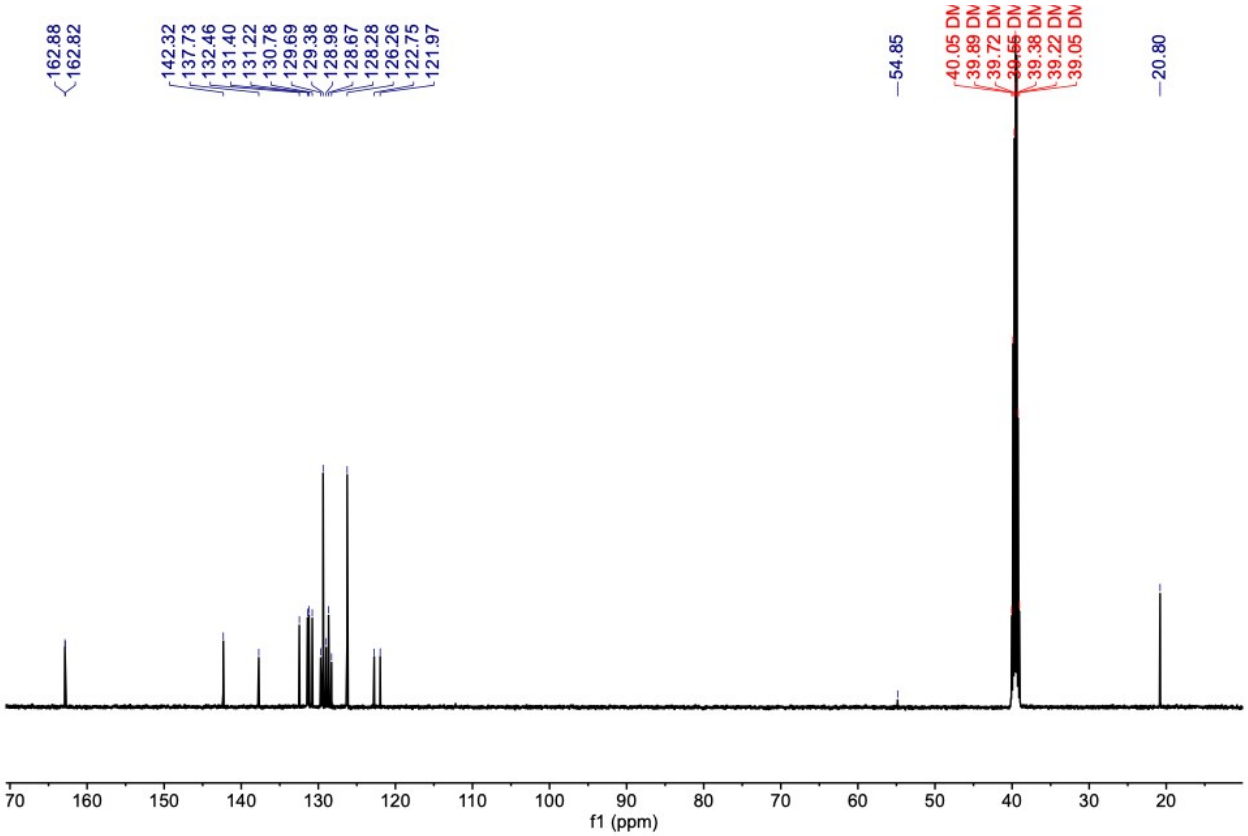


Fig. S10.[13]C-NMR spectrum of **compound 3** in DMSO-d_6.

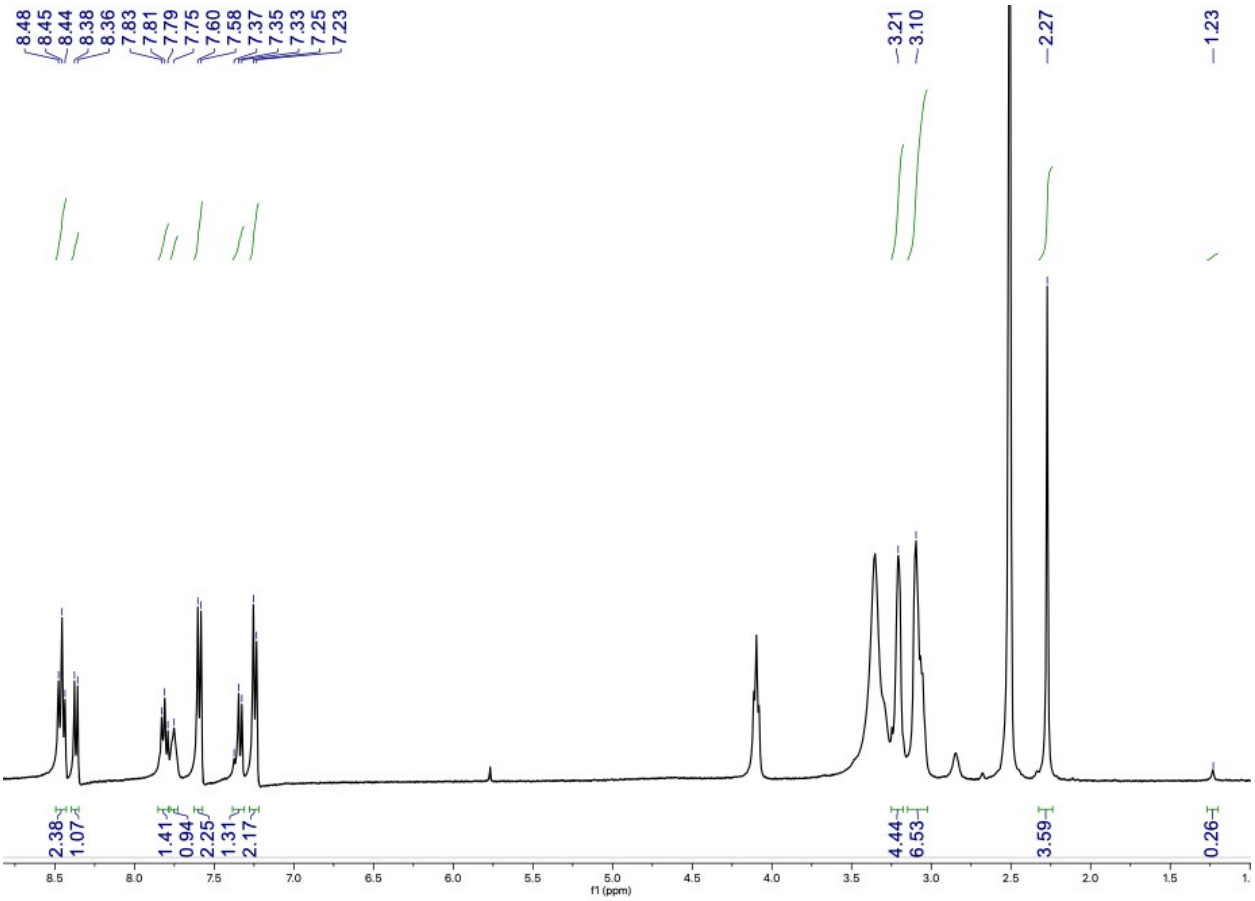


Fig. S11. [1]H-NMR spectrum of **ER-NapH** in DMSO-d_6.

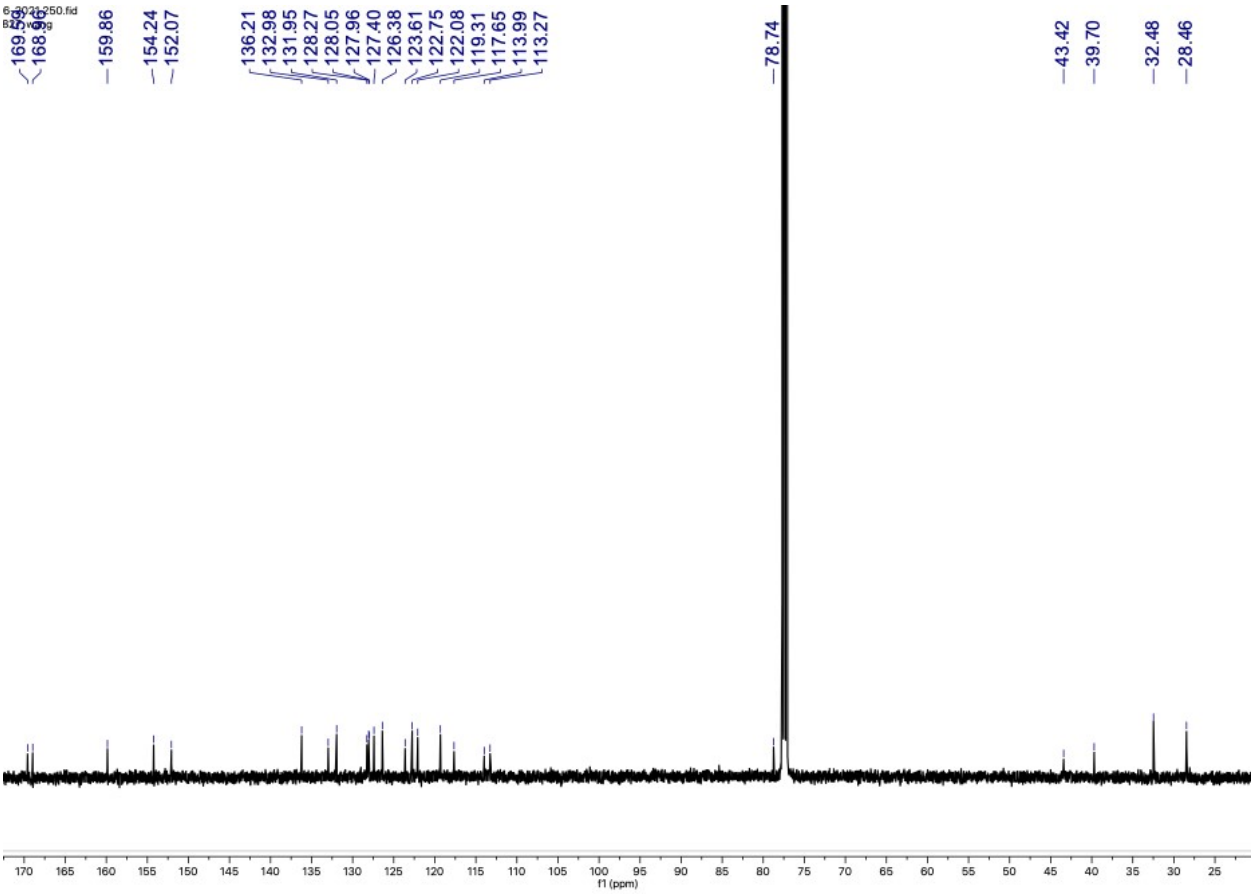


Fig. S12. ^{13}C-NMR spectrum of **ER-NapH** in $CDCl_3$.

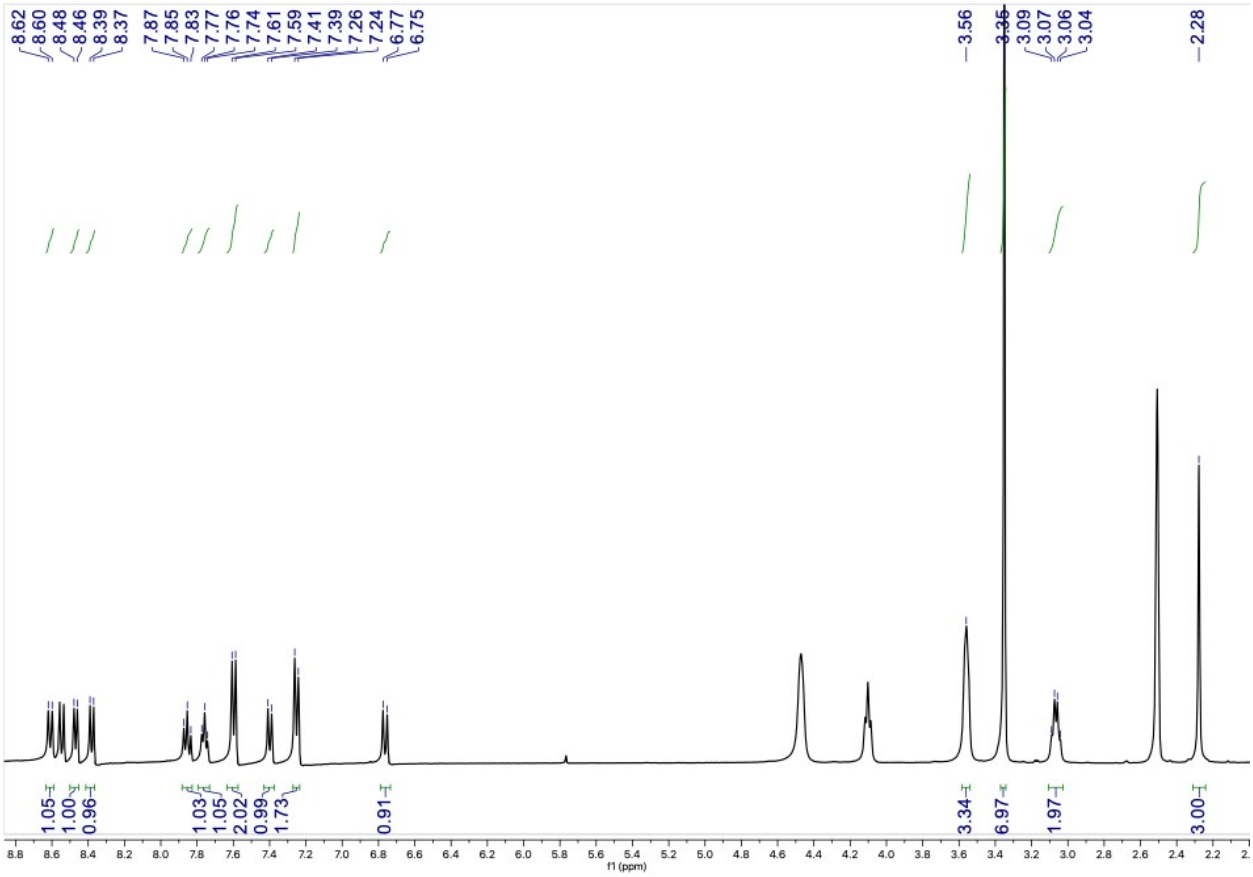


Fig. S13. ^{1}H-NMR spectrum of **ER-Nap-NBD** in DMSO-d_6.

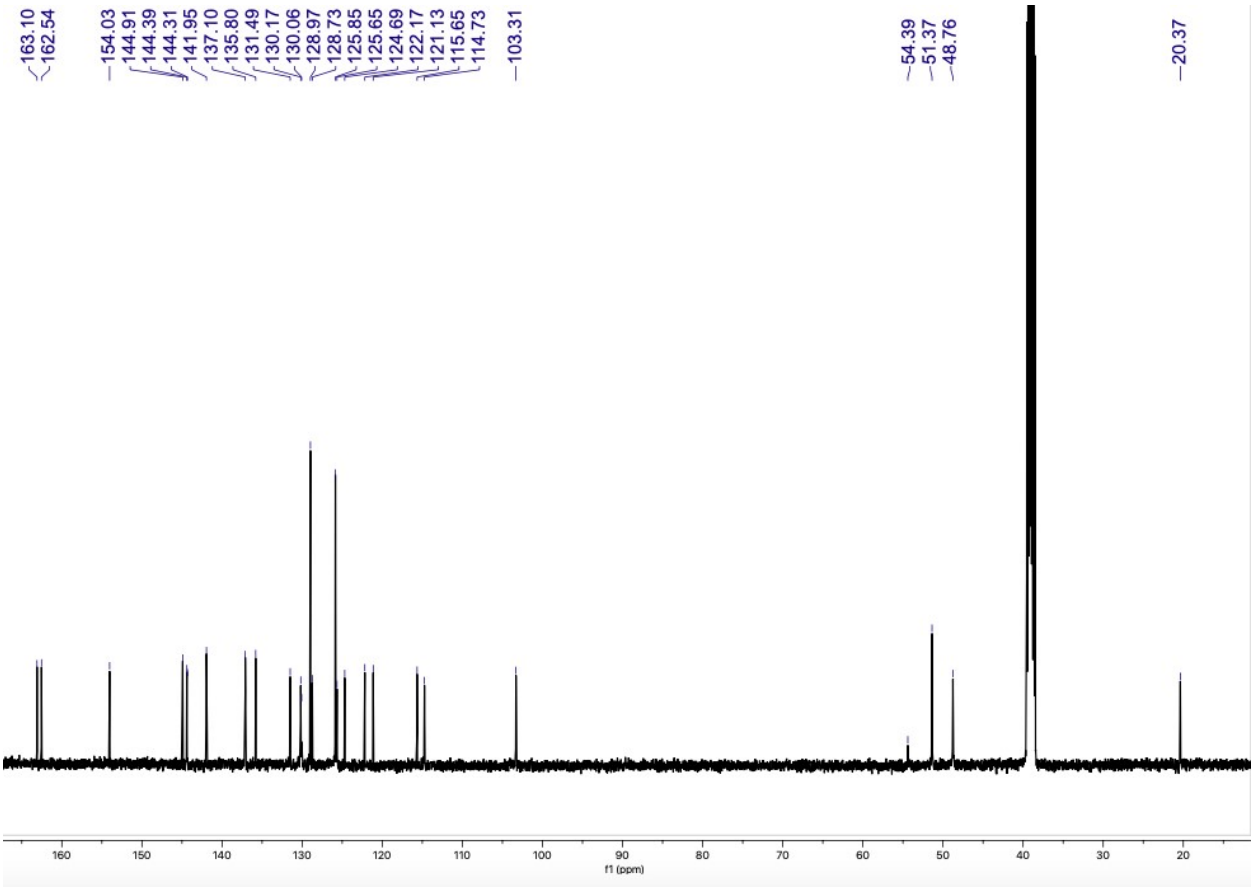


Fig. S14. ^{1}H-NMR spectrum of **ER-Nap-NBD** in DMSO-d_6.

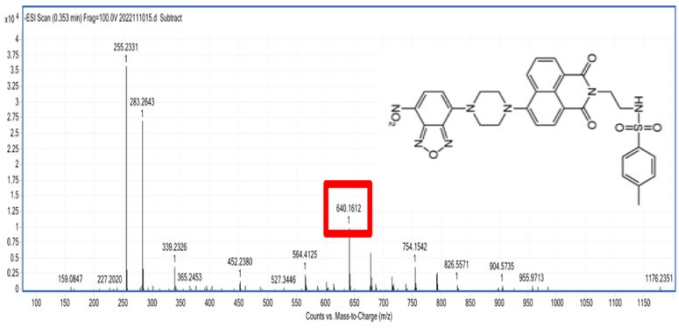
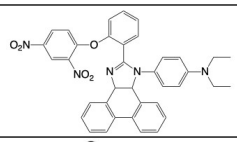
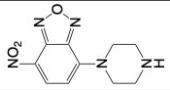
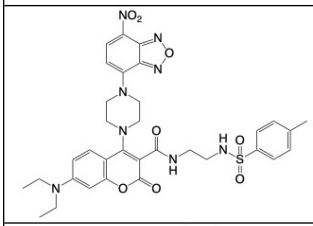
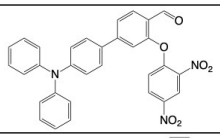
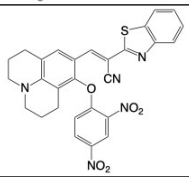
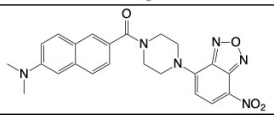


Fig. S15. ESI-HRMS spectrum of **ER-Nap-NBD**.

Table S1 Comparison of probe ER-Nap-NBD and the existed probes for H_2S

Probe	Analytes	$\lambda_{Ex}/\lambda_{Em}$ (nm)	Detection medium	Detection limit	In vitro and in vivo	Ref.
	H_2S	356nm/365nm	PBS (20 mM, pH=7.4, 10% DMSO. V/V)	1.11 μM	Hela	S4
	H_2S	340nm/ 440 and 543nm	PBS (pH=6.64)	57 nM	None	S5
	H_2S	383nm/490nm	PBS (10 mM, pH=7.4, 30% DMSO)	4.9 μM	Hela	S6
	H_2S	380nm/496nm	THF/H2O (2/8, HEPES. 20 mM, pH=7.12	$7.63x10^{-6}$ M	MCF-7	S7
	H_2S	480nm/537nm	HEPES	0.15 μM	EC1	S8
	H_2S	330nm/468nm	PBS (25mM, 20% EtOH. pH=7.4)	24 nM	Hela	S9
This work	H_2S	400nm/530nm	PBS (10mM, 50% EtOH. pH=7.4)	5.2 μM	MCF-7, 4T1 and Zebrafish	

6. References

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Authorship contribution statement

Yves. S. Kafuti: Methodology, Investigation, Data acquisition, Formal analysis, Writing-original draft. Shuang Zeng: Investigation, Data acquisition, Formal analysis. Xiaosheng Liu: Formal analysis. Jingjing Han: Formal analysis, Writing – review & editing. Ming Qian: Formal analysis. Qixian Chen: Resources, Supervision. Jingyun Wang: Resources, Supervision, Characterization, Funding acquisition Xiaojun Peng: Resources, Formal analysis. Juyoung Yoon: Resources, Formal analysis. Funding acquisition, Writing – review & editing. Haidong Li: Resources, Supervision, Characterization, Funding acquisition, Writing – review & editing.