Electronic Supplementary Information

Tumor-targeted near-infrared fluorescent probe for HNO and its application to real-time monitoring of HNO release *in vivo*

Ziyin Chai,[ab] Diankai Liu,[a] Xiaoyi Li,[a] Yanyan Zhao,[a] Wen Shi*[ab], Xiaohua Li[a] and Huimin Ma*[ab]

[a] *Beijing National Laboratory for Molecular Sciences, Key Laboratory of Analytical Chemistry for Living Biosystems, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China.*

[b] *University of Chinese Academy of Sciences, Beijing 100049, China.*

E-mail: shiwen@iccas.ac.cn; mahm@iccas.ac.cn

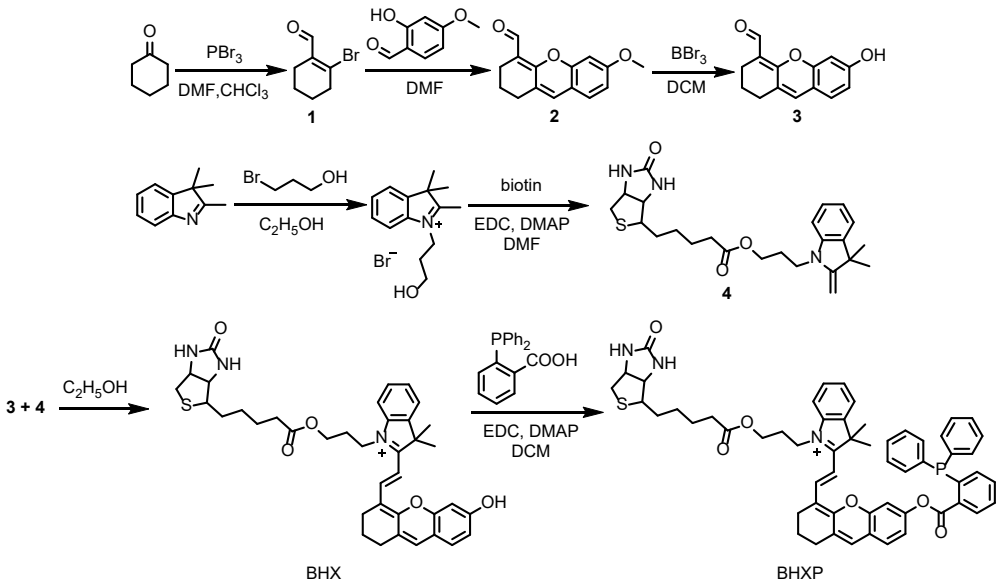
Contents

1. Apparatus and reagents

^{1}H-NMR and ^{13}C-NMR spectra were measured with a Bruker DMX-400 spectrometer and a Bruker AVANCE-600 spectrometer. Electrospray ionization mass spectra (ESI-MS) were recorded with a Shimadzu LC-MS 2020A. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were measured on an APEX IVFTMS instrument (Bruker, Daltonics). UV-vis absorption spectra were made by a UV-2600 UV-Vis spectrophotometer (Shimadzu). Fluorescence spectra were tested on an F-4600 spectrophotometer (Hitachi). MTT analyses were made on a SpectraMax I3 microplate reader (Molecular Devices). Confocal fluorescence images were recorded on an FV 1200-IX83 confocal laser scanning microscope (Olympus, Japan). In-vivo imaging was carried out on In-vivo Master (Grand-imaging Technology, China).

Cyclohexanone, phosphorus tribromide, 2-hydroxy-4-methoxybenzaldehyde, boron tribromide, 2,3,3-trimethylindolenine, 3-bromo-1-propanol, biotin, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), 4-dimethylamino-pyridine (DMAP), and 2-(diphenylphosphino) benzoic acid were obtained from Beijing Innochem Science & Technology Co., Ltd. Angeli's salt (AS), diethylamino NONOate (NO donor), S-nitrosoglutathione (GSNO), cysteine, N-acetyl cysteine (NAC), glucose, glutathione (GSH), vitamin C and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. KCl, $CaCl_2$, $CuCl_2$, $ZnCl_2$, $MgCl_2$, $FeCl_2$, $NaNO_2$ and KNO_3 were obtained from Beijing Chemicals, Ltd. Dulbecco's modified Eagle's media (DMEM), Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 (DMEM/F12), HeLa, HepG2, COS-7 and HK-2 cell lines were purchased from KeyGEN BioTECH Co., Ltd. Reactive oxygen species (ROS) including •OH, H_2O_2, $^{1}O_2$, ClO^-, $•O_2^-$ and $ONOO^-$ were prepared following the reported methods.[1] Ultrapure water (over 18 MΩ·cm) produced by a Milli-Q reference system (Millipore) was used throughout the experiments.

2. Synthesis of probe BHXP



Scheme S1 Synthetic route of BHXP

Probe BHXP was synthesized according to the route shown in Scheme S1. Compounds **1** and **2** were synthesized following the reported procedure.[2] Compound **3** was prepared according to the literature.[3]

Synthesis of compound 4. 2,3,3-Trimethylindolenine (5 g, 31.4 mmol) and 3-bromo-1-propanol (7 g, 50.3 mmol) were dissolved in ethanol (15 mL). The mixture was refluxed for 8 h. After removal of the solvent, the residue was extracted with water and the water extracts were washed with dichloromethane for 3 times, followed by evaporation. The obtained residue was dispersed in DMF (20 mL), followed by addition of biotin (1.22 g, 5 mmol), EDC (2.88 g, 15 mmol) and DMAP (660 mg, 5 mmol). The mixture was stirred at room temperature for another 12 h, accompanied by the solution changing from turbid to transparent. Then DMF was removed under reduced pressure and the residue was extracted with dichloromethane and washed with brine water for 3 times. The product was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH=100/1, v/v) to give compound **4** as colorless crystal (780 mg, 35% yield). ^{1}H-NMR (400 MHz, 298 K, $CDCl_3$; Fig. S1): δ 7.13-7.07 (m, 2H), 6.76 (t, J=14.8 Hz, 1H), 6.52 (d, J=7.6 Hz, 1H), 5.60 (s, 1H), 5.23 (s, 1H), 4.94 (t, J=12.8 Hz, 1H), 4.31 (t, J=12.0 Hz, 1H), 4.11 (t, J=12.4 Hz, 2H), 3.86 (d, J=10.4 Hz, 2H), 3.60 (t, J=13.6 Hz, 2H), 3.16 (q, J=19.6 Hz, 1H), 2.90 (dd, J=17.6 Hz, 1H), 2.73 (d, J=12.8 Hz, 1H), 2.34 (t, J=15.2 Hz, 2H), 2.01-1.95 (m, 2H), 1.75-1.66 (m, 4H), 1.53-1.48 (m, 2H), 1.33 (s, 6H). ^{13}C-NMR (100 MHz, 298 K, $CDCl_3$; Fig. S2): δ 173.5, 163.3, 161.5, 145.7,

137.5, 127.5, 121.9, 118.5, 104.9, 73.4, 62.0, 61.9, 60.1, 55.3, 44.2, 40.5, 38.9, 33.9, 30.1, 28.4, 28.3, 25.5, 24.8. HR-ESI-MS: m/z calcd for compound **4** ($C_{24}H_{34}N_3O_3S^+$, $[M+H]^+$), 444.2314; found, 444.2314 (Fig. S3).

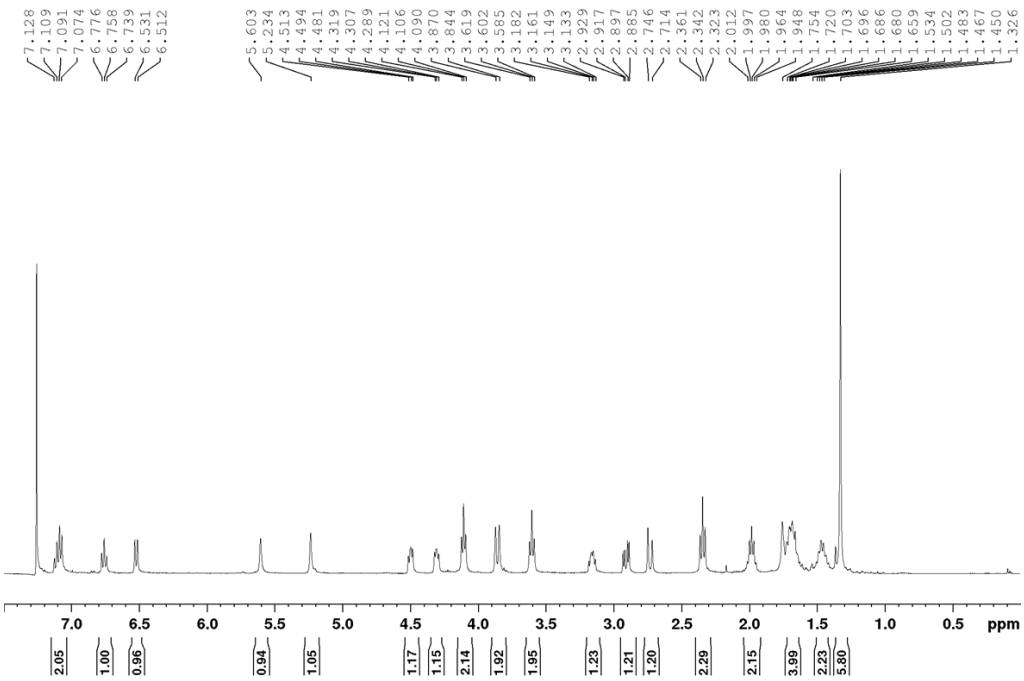


Fig. S1 ^{1}H-NMR spectrum of compound **4** (400 MHz, 298K, $CDCl_3$).

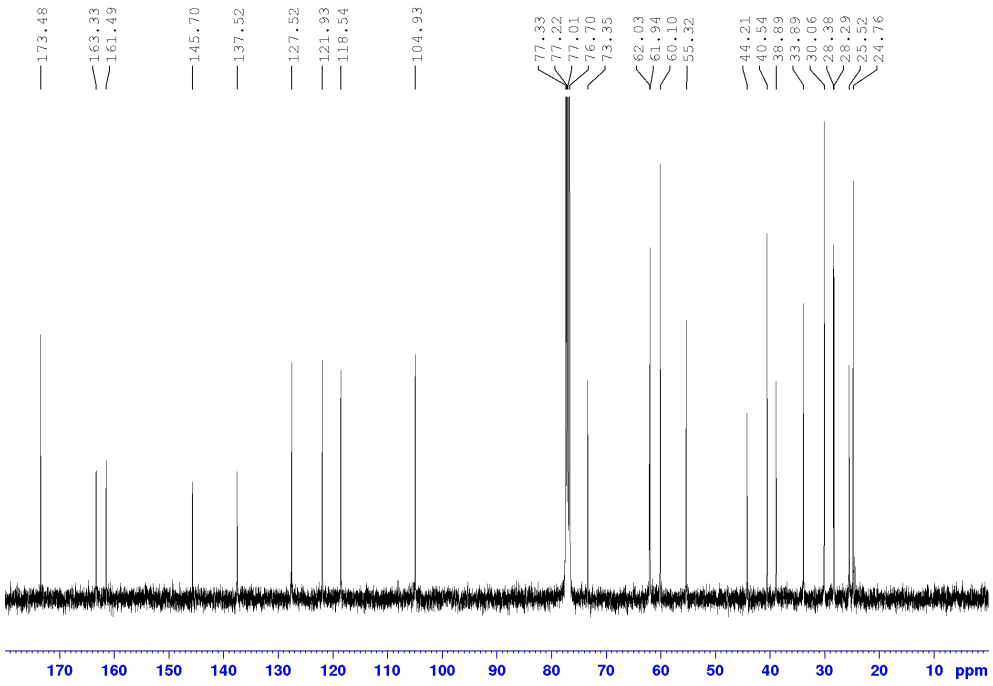


Fig. S2 ^{13}C-NMR spectrum of compound **4** (100 MHz, 298K, $CDCl_3$).

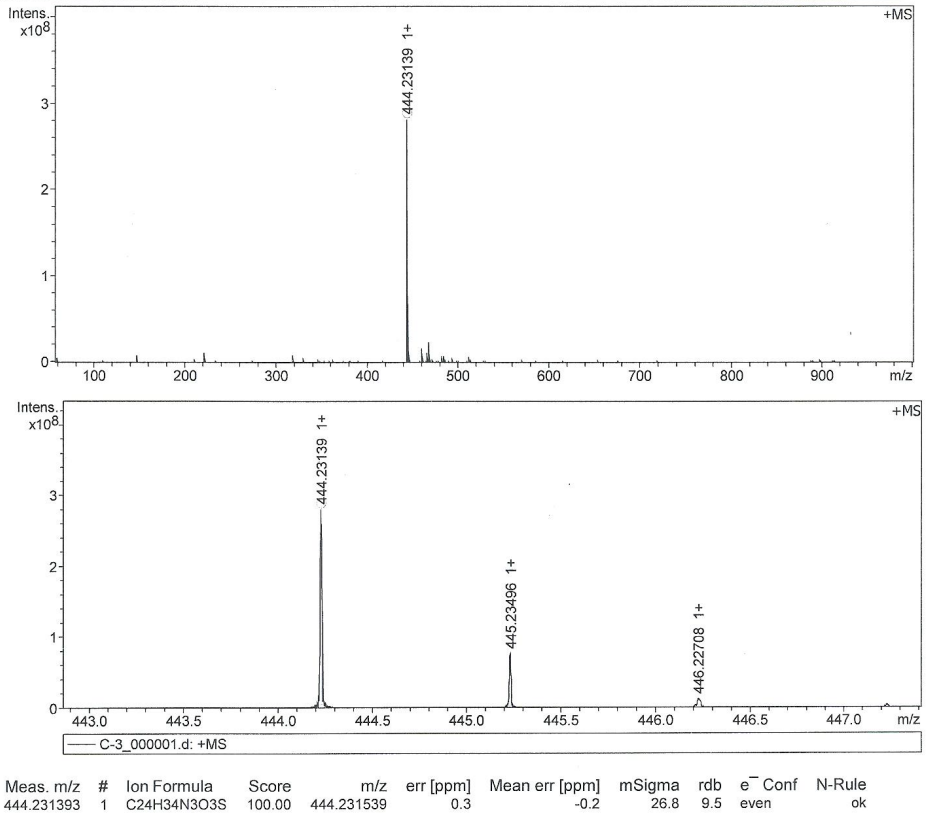


Fig. S3 HR-ESI-MS spectrum of compound **4**.

Synthesis of BHX. Compound **3** (228 mg, 1 mmol) and compound **4** (444 mg, 1 mmol) were dissolved in ethanol (15 mL) and the mixture was refluxed for 2 h. The deep blue solution obtained was concentrated and the resulting residue dissolved in dichloromethane and washed with brine water. The organic layer was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH=25/1, v/v) to give BHX as dark blue powder (600 mg, 80% yield). ^{1}H-NMR (400 MHz, 298 K, CD_3OD; Fig. S4): δ 8.77 (d, J= 14.8 Hz, 1H), 7.65 (d, J=7.2 Hz, 1H), 7.66-7.51 (m, 3H), 7.49-7.43 (m, 2H), 6.87 (t, J=10.0 Hz, 2H), 6.47 (d, J=14.4 Hz, 1H), 4.85-4.42 (m, 3H), 4.27 (t, J=7.6 Hz, 1H), 4.23 (t, J=12.8 Hz, 2H), 3.16 (t, J= 8.4Hz, 1H), 2.87 (dd, J=18 Hz, 1H), 2.81 (t, J=11.2 Hz, 2H), 2.74 (t, J=12.0 Hz, 2H), 2.66 (d, J=12.8 Hz, 1H), 2.27 (t, J=14.4 Hz, 4H), 1.96 (t, J=11.6 Hz, 2H), 1.84 (s, 6H), 1.62-1.55 (m, 5H), 1.41 (m, 2H). ^{13}C-NMR (100 MHz, 298 K, CD_3OD; Fig. S5): δ 177.0, 173.5, 164.6, 162.7, 162.6, 155.0, 145.3, 141.7, 135.6, 129.2, 128.8, 126.6, 126.2, 122.4, 115.1, 114.8, 114.4, 112.0, 102.3, 101.6, 62.0, 61.4, 60.2, 55.6, 41.9, 39.6, 33.3, 28.5, 28.4, 28.2, 27.1, 26.4, 24.4, 23.8, 20.4. HR-ESI-MS: m/z calcd for BHX ($C_{38}H_{44}N_3O_5S^+$, $[M]^+$), 654.2992; found, 654.2992 (Fig. S6).

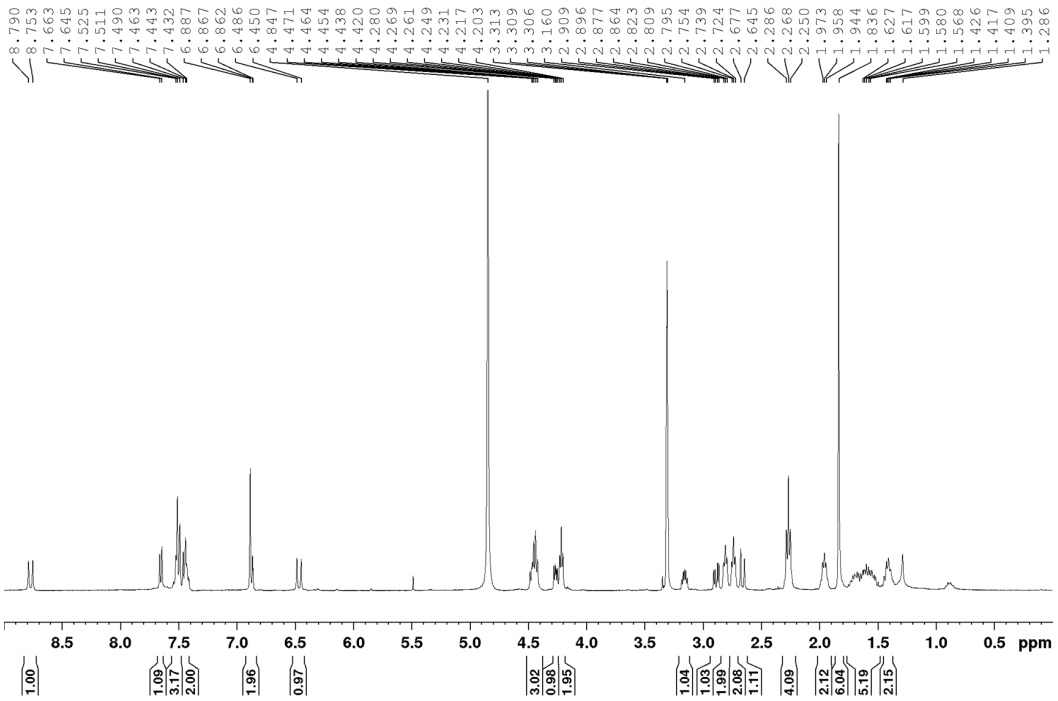


Fig. S4 ^{1}H-NMR spectrum of BHX (400 MHz, 298K, CD_3OD).

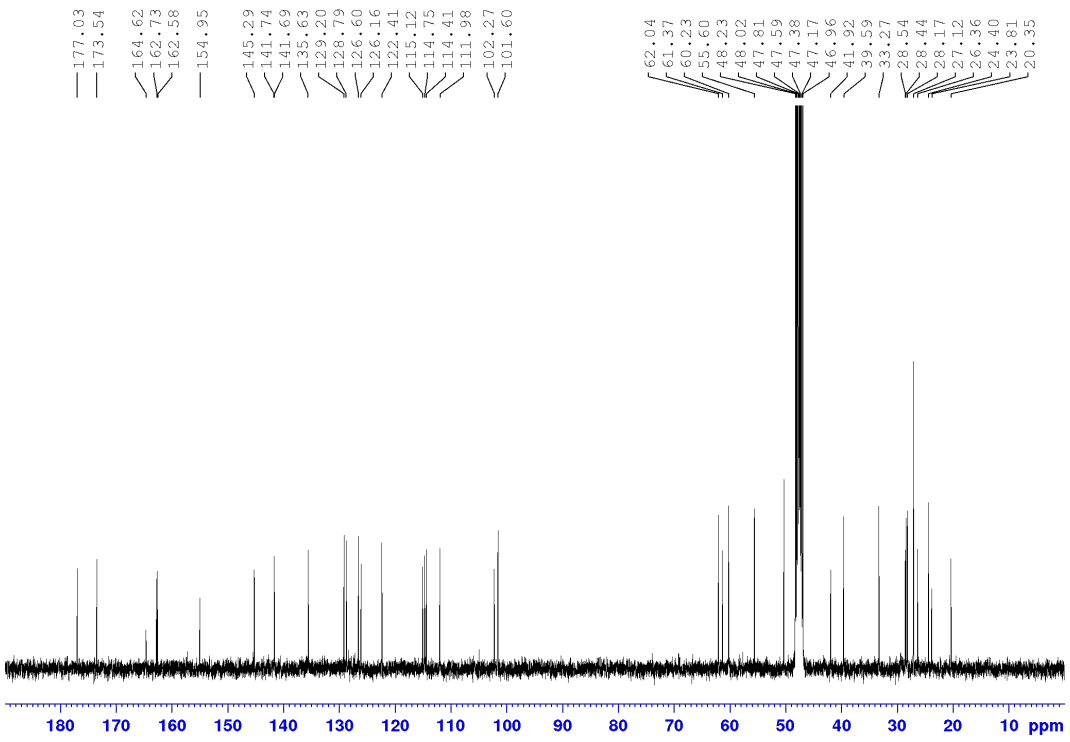
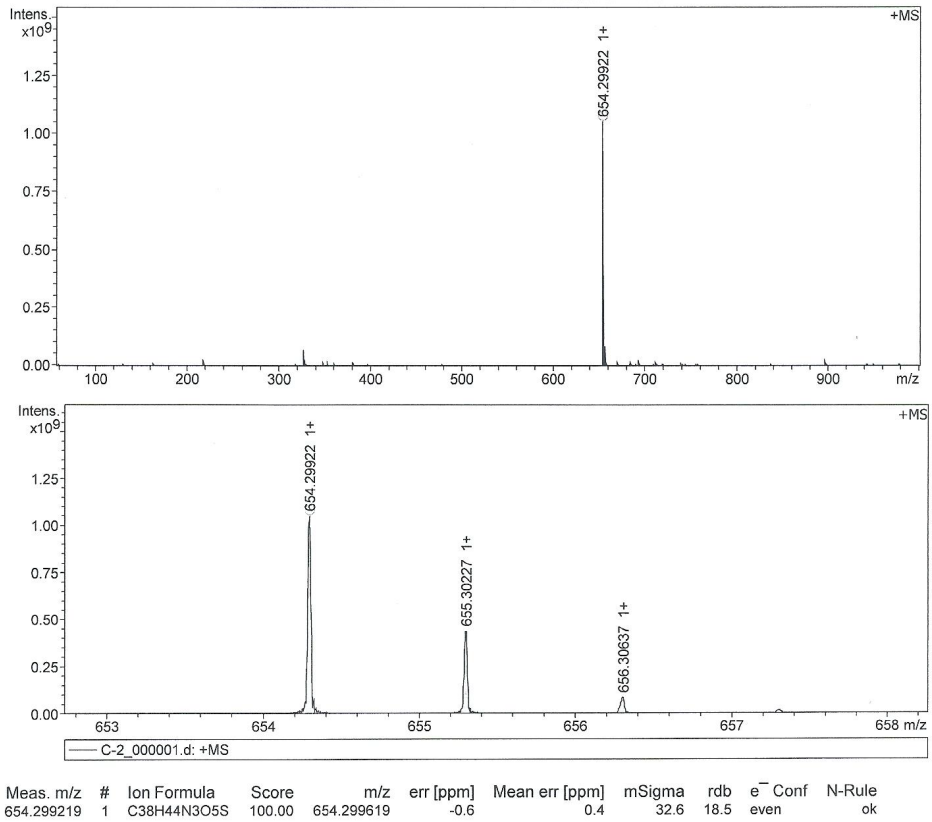


Fig. S5 ^{13}C-NMR spectrum of BHX (100 MHz, 298K, CD_3OD).



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
654.299219	1	C38H44N3O5S	100.00	654.299619	-0.6	0.4	32.6	18.5	even	ok

Fig. S6 HR-ESI-MS spectrum of BHX.

Synthesis of probe BHXP. BHX (367 mg, 0.5 mmol), 2-(diphenylphosphino)-benzoic acid (168 mg, 0.55 mmol), EDC (288 mg, 1.5 mmol) and DMAP (61 mg, 0.5 mmol) were dissolved in dry dichloromethane (10 mL). The reaction mixture was stirred at room temperature for 20 h under N_2 atmosphere. Then, the mixture was poured into 1 N HCl and extracted with $CHCl_3$. The organic layer was washed with water for 3 times and then with brine water for 3 times. The residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH=40/1, v/v) to give BHXP as dark purple powder (250 mg, 49% yield). ^{1}H-NMR (600 MHz, 298 K, CD_2Cl_2; Fig. S7): δ 8.72 (d, J= 13.8 Hz, 1H), 8.31 (d, J=3.6 Hz, 1H), 7.54 (t, J=13.8 Hz 4H), 7.48 (t, J=15Hz, 1H), 7.44 (d, J=8.4 Hz, 1H), 7.39-7.34 (m, 7H), 7.29-7.25 (m, 5H), 7.19 (s, 1H), 7.05 (q, J=7.3 Hz, 1H), 6.94 (dd, J=9.6 Hz, 1H), 6.42 (d, J=15.0 Hz, 1H),5.90 (s, 1H), 4.59 (s, 1H), 4.37 (s, 3H), 4.20 (s, 2H), 3.20 (s, 1H), 2.93 (d, J=9.0 Hz, 1H), 2.79 (s, 2H), 2.73 (d, J=12.6 Hz, 1H), 2.68 (s, 2H), 2.32 (t, J=13.8 Hz, 2H), 2.27 (s, 2H), 1.96 (s, 2H), 1.81 (s, 6H), 1.70-1.64 (m, 5H), 1.45 (s, 2H). ^{13}C-NMR (100 MHz, 298 K, CD_2Cl_2; Fig. S8): δ 178.3, 173.0, 161.5, 153.2, 153.1, 146.9, 142.0, 141.9, 141.1, 137.6, 137.4, 134.7, 134.1, 133.8, 132.9, 131.4, 130.1, 129.4, 128.9, 128.7, 128.6, 128.4, 127.9, 122.7, 119.9,

119.8, 115.3, 112.5, 109.8, 104.2, 62.4, 60.9, 55.3, 51.0, 42.9, 40.4, 33.5, 29.7, 29.3, 28.3, 28.2, 28.1, 26.9, 24.6, 24.1, 20.2. HR-ESI-MS: m/z calcd for BHXP ($C_{57}H_{57}N_3O_6PS^+$, $[M]^+$), 942.3703; found, 942.3703 (Fig. S9).

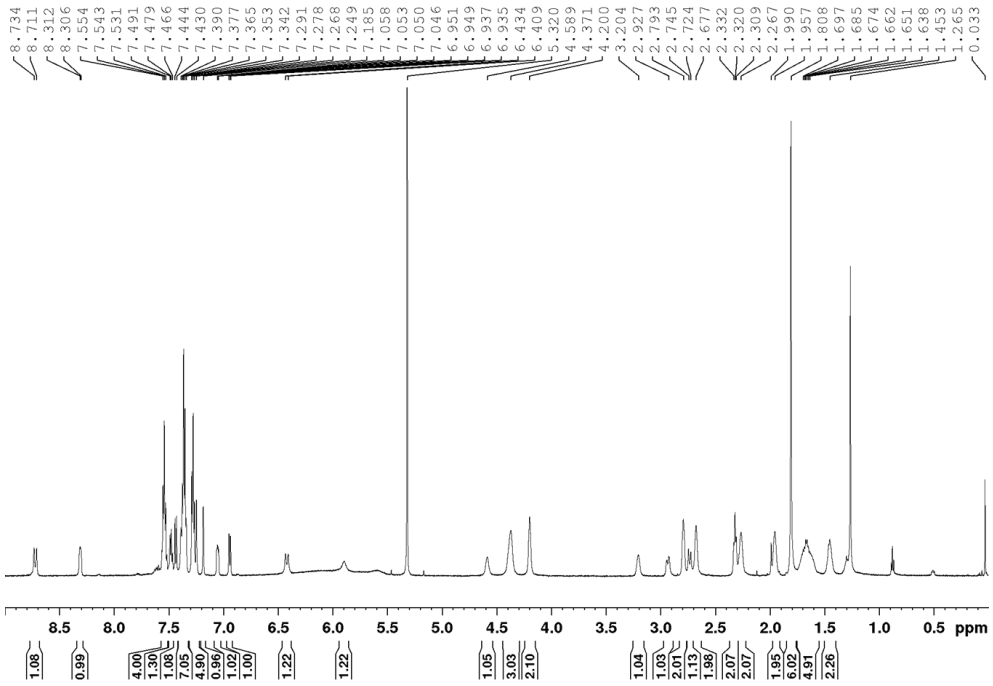


Fig. S7 ^{1}H-NMR spectrum of BHXP (600 MHz, 298K, CD_2Cl_2).

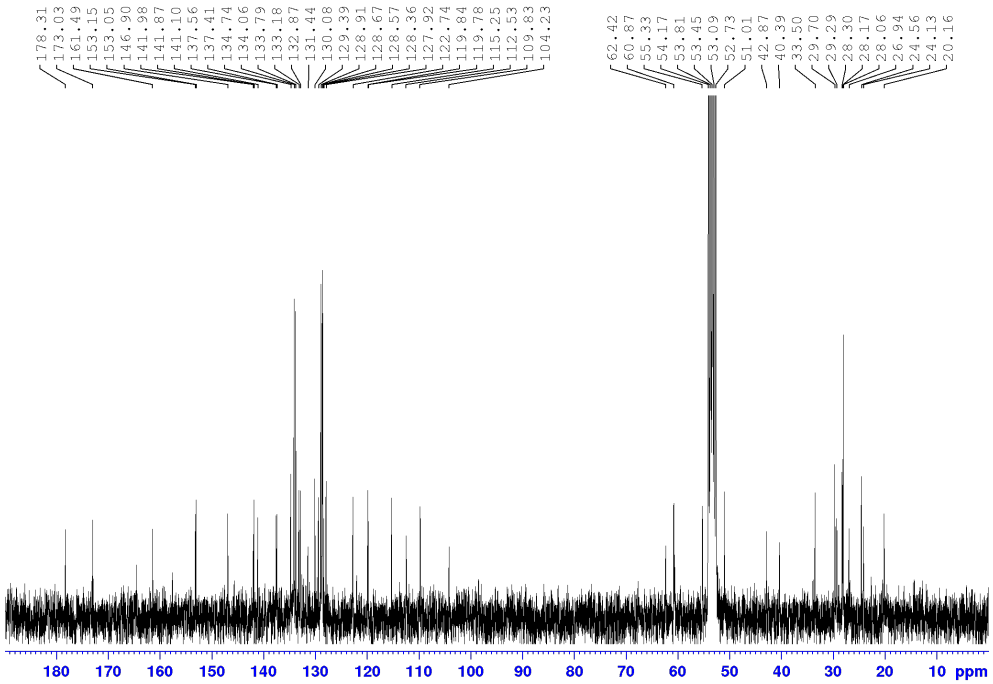


Fig. S8 ^{13}C-NMR spectrum of BHXP (100 MHz, 298K, CD_2Cl_2).

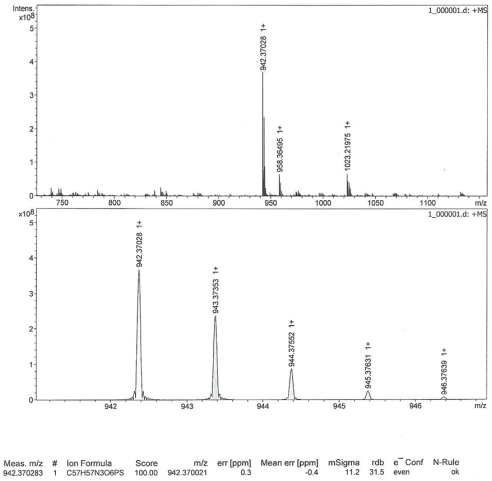


Fig. S9 HR-ESI-MS spectrum of BHXP.

3. Supplementary methods

General procedure for fluorescence assay. Unless otherwise noted, all the spectral measurements of BHXP (10 μM) reacting with AS were measured in phosphate buffer saline (PBS, pH 7.4). In a test tube, 0.9 mL of PBS and 10 μL of 1 mM stock solution of BHXP in DMSO were mixed, followed by adding appropriate volume of 10 mM stock solution of AS in water. The final volume was adjusted to 1 mL with PBS. After reaction at 37 ℃ for 30 min in a shaker incubator, the reaction solution was transferred to a 1 cm quartz cell to measure fluorescence with $\lambda_{ex/em}$ = 670/708 nm. Meanwhile, a solution without AS was prepared and determined for comparison under the same conditions. Data are expressed as mean ± standard deviation (n = 3).

Determination of fluorescence quantum yield. Fluorescence quantum yield (Φ) was determined by using ICG (Φ = 0.13 in DMSO) as a standard.[4]

Cytotoxicity. The cytotoxicity of BHXP on HeLa cells, HepG2 cells, COS-7 cells and HK-2 cells were all evaluated by the MTT assay, as described previously.[5]

Cell culture and fluorescence imaging in cells. HeLa, HepG2 and COS-7 cells were cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin at 37 ℃ in a humidified 5% CO_2 incubator. HK-2 cells were cultured in DMEM/F12 containing 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin in a similar manner.

For fluorescence imaging, the cells were incubated with 10 μM BHXP in DMEM at 37 ℃ for 30 min. After incubated with probe, cells were washed twice with DMEM to remove the free probe, and then incubated with AS for 30 min. Before imaging, cells were also washed twice with DMEM. The fluorescence was collected with the

excitation of 635 nm and emission of 700 - 800 nm.

***In vivo* imaging of mice.** Five-week-old female BALB/c nude mice with HeLa tumor were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal care and experimental protocols complied with the Animal Management Rules of the Ministry of Health of the People's Republic of China and were approved by the Institute of Process Engineering, Chinese Academy of Sciences.

Before *in vivo* imaging, all the mice were anesthetized by isoflurane. BHXP (200 μM, 100 μL) and AS (400 μM, 100 μL) were used for intratumoral injection, while BHXP, BHX, HXPI (500 μM, 200 μL) and AS (1mM, 200 μL) were used for intravenous injection. Excitation light was provided by a 680 nm diode laser. The emission light was filtered by a 730 nm bandpass filter.

4. Supplementary figures

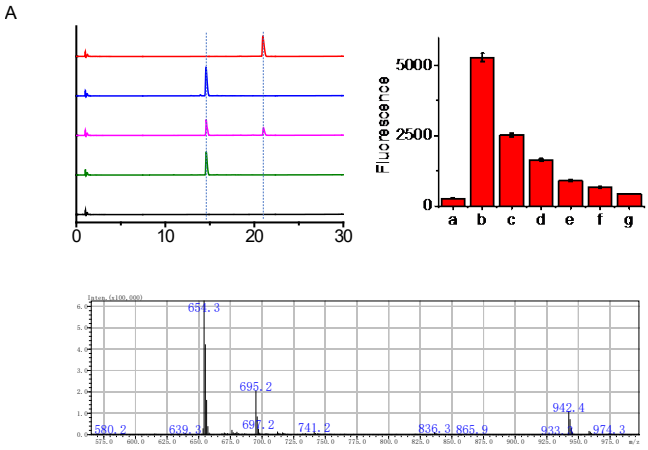


Fig. S10 (A) Chromatograms of different reaction systems: (a) BHXP (50 μM); (b) BHX (50 μM); (c) reaction solution of BHXP (50 μM) with AS (50 μM); (d) reaction solution of BHXP (50 μM) with AS (100 μM). (e) PBS (blank). The assignments of the peaks: (1) t_R = 14.6 min, BHX; (2) t_R = 21.0 min, BHXP. HPLC analyses were made using two LC-20AD pumps, SPD-M20A diode array detector (Shimadzu, Japan) and Shim-pack GIST-HP C18 column (3 μm, 2.1 mm×150 mm, Shimadzu, Japan) with CH_3CN and H_2O as eluents [elution program: 0-30 min, 5% to 100% CH_3CN (flow rate, 0.5 mL/min)]. The chromatography peaks were monitored by the absorbance at 630 nm. (B) Fluorescence responses of BHXP (10 μM) to AS (20 μM) with various concentration of NAC: (a) BHXP only (control); (b) BHXP + AS (another control); (c-g) BHXP + AS + NAC (20, 50, 100, 500, 1000 μM, respectively). $\lambda_{ex/em}$ = 670/708 nm. (C) ESI-MS of the reaction system. The peak at m/z =654.3 $[M]^+$ indicates the generation of BHX.

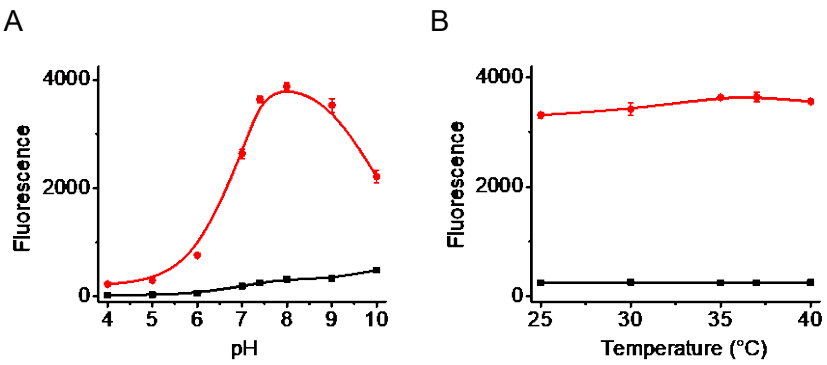


Fig. S11 Effects of (A) pH and (B) temperature on the fluorescence intensity of BHXP (10 μM) in the absence (black) and presence (red) of AS (10 μM). $\lambda_{ex/em}$ = 670/708 nm.

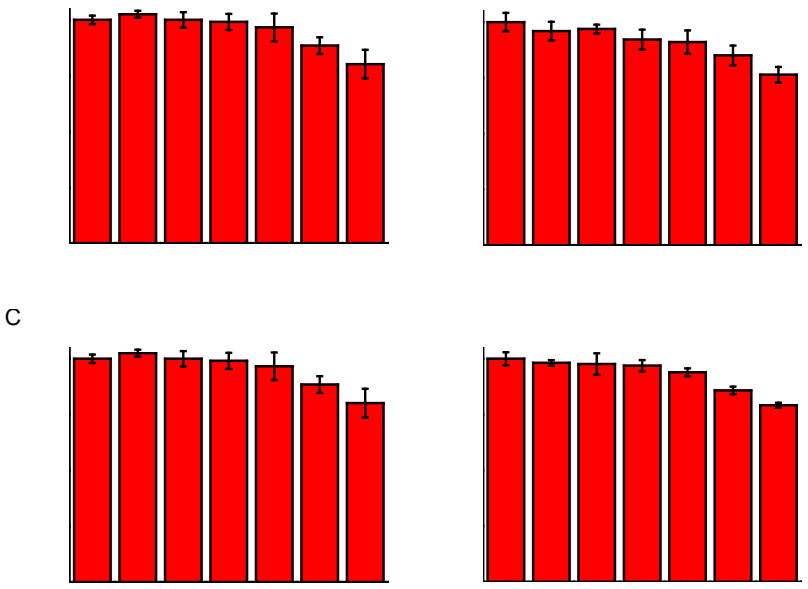


Fig. S12 Effects of BHXP on the viability of (A) HeLa cells, (B) HepG2 cells, (C) COS-7 cells and (D) HK-2 cells. The viability of cells without BHXP is defined as 100%. The results are expressed as the mean ± standard deviation of five separate measurements at each probe concentration.

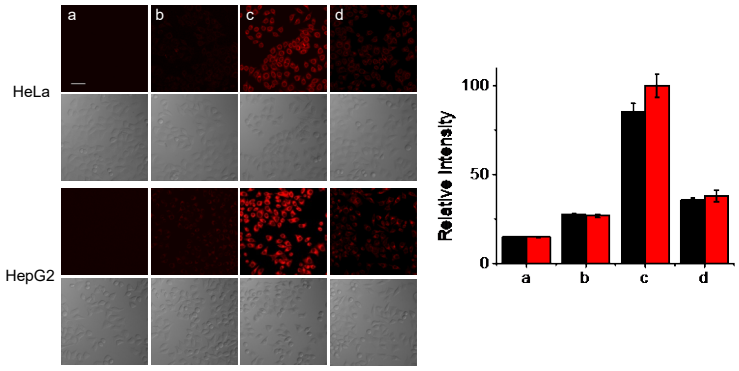


Fig. S13 (A) Fluorescence and differentiation interference contrast (DIC) images of HeLa cells and HepG2 cells. (a) Cells + biotin (50 μM, control); (b) system (a) + BHXP (10 μM); (c) cells + BHXP (10 μM) + AS (20 μM); (d) system (b) + AS (20 μM). The incubation time for each step was 30 min. (B) Relative intensity of HeLa (black) and HepG2 (red) cells in panel (A). The intensity at least from ten cells in the fluorescence image was measured and averaged. Scale bar = 50 μm.

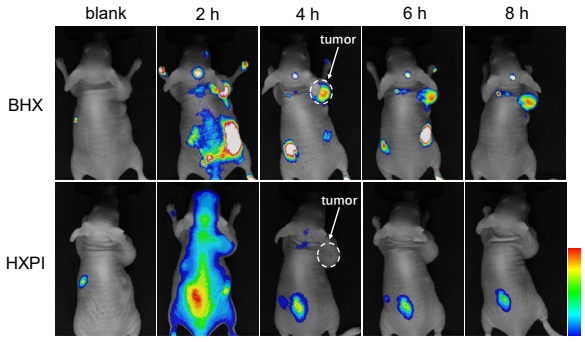


Fig. S14 Representative *in vivo* fluorescence images of tumor-bearing mice. The mice were intravenously administrated with BHX (500 μM in 200 μL saline) or HXPI (500 μM in 200 μL saline), respectively, and imaged at different time points.

5. Supplementary table

Table S1 Comparison of BHXP with other fluorescence probes for HNO

name	$\lambda_{ex/em}$ (nm)	detection limit (nM)	target site	ref.
TEMPO-9-AC	361/430	100	-	6a
ER-HNO	530/585	2000	endoplasmic reticulum	6b
R2	450/555	32	endoplasmic reticulum	6c
Lyso-JN	690/700	60	lysosome	6d
Mito-1	398/452	18	mitochondria	6e
MitoHNO	690/727	60	mitochondria	6f
Mito-HNO	488/545	170	mitochondria	6g
Mito-JN	680/730	50	mitochondria	6h
BHXP	670/708	57	tumor	this work

6. References

1. (a) X. Y. Zhu, M. Y. Xiong, H. W. Liu, G. J. Mao, L. Y. Zhou, J. Zhang, X. X. Hu, X. B. Zhang and W. H. Tan, *Chem. Commun.*, 2016, **52**, 733-736; (b) R. M. Uppu, *Anal. Biochem.*, 2006, **354**, 165-168; (c) J. Zhou, L. H. Li, W. Shi, X. H. Gao, X. H. Li and H. M. Ma, *Chem. Sci.*, 2015, **6**, 4884-4888.
2. J. A. Richard, *Org. Biomol. Chem.*, 2015, **13**, 8169-8172.
3. Y. Chen, Y. M. Wang, X. H. Wu, B. Liu and J. F. Zhang, *Dyes Pigm.*, 2021, **186**, 109015.
4. K. Licha, B. Riefke, V. Ntziachristos, A. Becker, B. Chance and W. Semmler, *Photochem. Photobiol.*, 2000, **72**, 392-398.
5. X. F. Wu, L. H. Li, W. Shi, Q. Y. Gong, X. H. Li and H. M. Ma, *Anal. Chem.*, 2016, **88**, 1440-1446.
6. (a) M. R. Cline and J. P. Toscano, *J. Phys. Org. Chem.*, 2011, **24**, 993-998; (b) F. Ali, S. Sreedharan, A. H. Ashoka, H. K. Saeed, C. G. W. Smythe, J. A. Thomas and A. Das, *Anal. Chem.*, 2017, **89**, 12087-12093; (c) S. X. Peng, Z. P. Li, Y. R. Zhang, W. B. Cao, J. F. Liu, W. M. Zhu and Y. Ye, *Sens. Actuators B Chem.*, 2020, **317**, 128211; (d) X. T. Jing, F. B. Yu and L. X. Chen, *Chem. Commun.*, 2014, **50**, 14253-14256; (e) K. Sunwoo, K. N. Bobba, J. Y. Lim, T. Park, A. Podder, J. S. Heo, S. G. Lee, S. Bhuniya and J. S. Kim, *Chem. Commun.*, 2017, **53**, 1723-1726; (f) X. Y. Gong, X. F. Yang, Y. G. Zhong, Y. H. Chen and Z. Li, *Dyes Pigm.*, 2016, **131**, 24-32; (g) M. G. Ren, B. B. Deng, K. Zhou, J. Y. Wang, X. Q. Kong and W. Y. Lin, *J. Mater. Chem. B*, 2017, **5**, 1954-1961; (h) Y. Huang, X. Zhang, N. He, Y. Wang, Q. Kang, D. Z. Shen, F. B. Yu and L. X. Chen, *J. Mater. Chem. B*, 2019, **7**, 305-313.