A new fluorescence probe for sensing of biothiols and screening of acetylcholinesterase inhibitors

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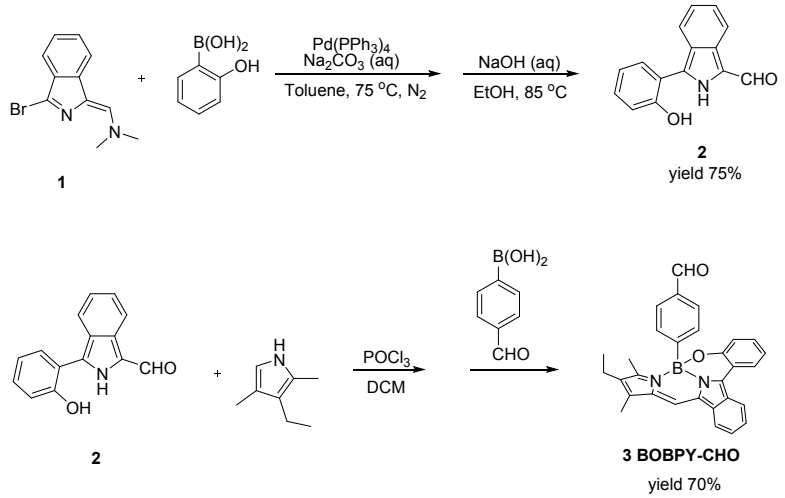
1. Materials and methods

Chemicals were purchased from commercial sources, other reagents were AR grade and used without further purification unless otherwise indicated. Acetylcholinesterase (AChE, cat. No: A8910 220 U/g) was purchased from Solarbio (China), (1U) was defined as the amount of enzyme capable of converting 1μmol substrate acetylcholine per min. High-performance liquid chromatography (Agilent HPLC 1260, USA) and a reverse phase C18 column (250 x 4.6 mm) were used in case needed. Plate reading was performed by a Varioskan LUX plate reader (Thermo Fisher) supplied with SkanIt Software 4.1. UV-vis absorption spectra were recorded on a spectrophotometer TU-1900 (Persee, Beijing). ESI-MS was recorded by an Agilent 6420 LC/MS instrument. ^{1}H and ^{13}C NMR spectra were recorded on AVANCE III HD 400 MHz digital NMR spectrometer (Switzerland). Data was reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, and m = multiplet), coupling constant (*J* values) in Hz and integration. High resolution mass (HRMS) was collected from the Maxis Impact HD Mass Spectrometer (Bruker).

2. **Experimental**

2.1 Syntheses

As described in Scheme S1, the compound **1**, **2** were synthesized according to a reported method.[1] The synthetic route of compound **3** follows a reported procedure.[2]

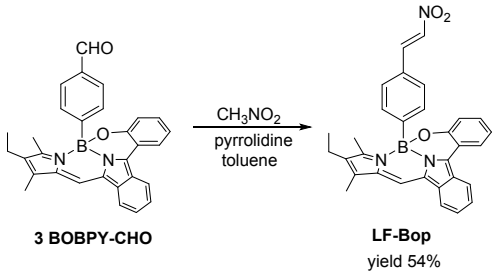


Scheme S1. Synthetic route of intermediate compound **3** (Bodipy-CHO)

Compound **3** (Bodipy-CHO): ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.72 (s, 1H), 8.01 (d, *J* = 8.2 Hz, 1H), 7.88 – 7.86 (m, 1H), 7.81 (d, *J* = 8.1 Hz, 1H), 7.45 (d, *J* = 8.1 Hz, 2H), 7.41 – 7.33 (m, 3H), 7.30 – 7.17 (m, 4H), 6.92 – 6.88 (m, 1H), 2.37 (s, 3H), 2.30 – 2.28 (m, 2H), 2.19 (s, 3H), 0.97 – 0.93 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.83, 156.67, 150.86, 143.42, 135.35, 134.89, 133.81, 133.36, 132.07, 131.96, 130.63, 128.82, 128.68, 126.43, 125.74, 125.68, 123.52, 120.20, 120.09, 119.71, 118.95, 116.09, 17.49, 14.86, 13.06, 9.65. Mass spectrometry (ESI-HRMS, m/z): $[M]^+$ calcd. for $[C30H25BN2O2]^+$ 456.2009; found 456.1982.

Synthesis of probe LF-Bop

A mixture of compound **3** (0.7 mmol), nitromethane (5.39 mmol) and toluene (3 mL) was stirred at room temperature for 5 minutes. After that, pyrrolidine (0.25 mmol) was added and the mixture was stirred overnight. After reaction completed, distilled water was added and extracted with ethyl acetate. The organic layer was separated and dried with Na_2SO_4. After concentrating under vacuum, the crude product was purified by silica gel column (dichloromethane/ethyl acetate/petroleum ether=1:1:50) to get LF-Bop (yield 54%).



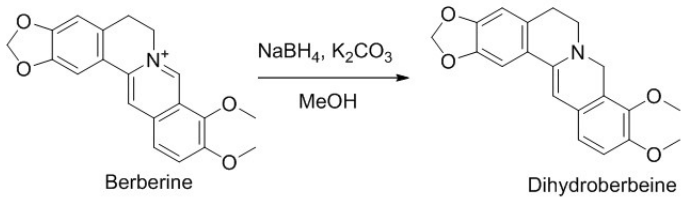
Scheme S2. Synthetic route of LF-Bop.

Probe LF-Bop: yield 54%. ^{1}H NMR (400 MHz, *d6*-DMSO) δ 8.33 (d, *J* = 8.3 Hz, 1H), 8.24 (d, *J* = 8.1 Hz, 1H), 8.15 (d, *J* = 7.0 Hz, 2H), 8.00 – 7.88 (m, 2H), 7.64 – 7.60 (m, 1H), 7.52 – 7.43 (m, 4H), 7.29 (d, *J* = 8.1 Hz, 1H),

7.15 (d, J = 8.0 Hz, 2H), 7.05 – 7.02 (m, 1H), 5.75 (s, 1H), 2.42 (s, 3H), 2.35 (d, J = 7.6 Hz, 2H), 2.29 (s, 3H), 1.01 – 0.97 (m, 3H). ^{13}C NMR (100 MHz, *d6*-DMSO) δ 156.48, 150.72, 142.71, 140.18, 137.48, 135.35, 134.81, 134.12, 132.21, 132.10, 130.51, 129.71, 129.22, 128.89, 128.09, 127.24, 126.62, 125.88, 124.05, 121.05, 120.92, 120.33, 118.74, 118.61, 17.29, 15.20, 13.27, 9.77. Mass spectrometry (ESI-HRMS, m/z): $[M+H]^+$ calcd. for $[C31H27BN3O3]^+$ 500.2145; found 500.2155.

Synthesis of dihydroberberine

Sodium borohydride (54 mg, 1.43 mmol) was dissolved in 5% sodium hydroxide aqueous solution, the solution was then added to the mixture of berberine chloride (450 mg, 1.34 mmol) and potassium carbonate (550 mg, 4.0 mmol) in methanol (18 mL). The reaction mixture is stirred at rt for 15 min. The yellow solution became green, the product was collected by filtration and washed with water and then ethanol/water (30% v/v). The product was further purified by recrystallization against ethanol.[3]



Scheme S3. Synthetic route of dihydroberberine

Dihydroberberine: yield 80%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.08 (s, 1H), 6.65 (s, 2H), 6.49 (s, 1H), 5.86 (d, J = 6.5 Hz, 3H), 4.24 (s, 2H), 3.76 (d, J = 2.1 Hz, 6H), 3.06 – 3.03 (m, 2H), 2.80 – 2.77 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.42, 147.29, 146.70, 144.51, 141.66, 128.77, 128.55, 124.55, 122.15, 118.83, 111.48, 107.87, 103.80, 101.03, 96.35, 60.75, 55.97, 49.36, 49.06, 29.85. ESI $[M+H]^+$ m/z calcd. for C20H20NO4 337.1, found 338.7 $[M+H]^+$.

2.2 Thiol detection

Detection was based on 96-well plate and the use of a Varioskan LUX plate reader. In a typical test, LF-Bop (final concentration 5 μM) was mixed with the analytes in 200 μL reaction solutions. PBS (pH 7.4):DMSO (1:1) solution was used for all the measurements unless otherwise indicated. The thiol-addition reaction was performed at rt for 10 min before measurement. The solutions were excited at 581 nm, the FL spectra or the intensity at 637 nm was recorded. To prepare PBS:DMSO solutions with different pHs, PBS buffers with pH at 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, 11.5 and 12.5 were separately mixed with DMSO at the vol/vol ratio of 1:1.

The lowest limit of detection (LOD) for GSH is estimated by a well established method (S/N=3)[4]. Briefly,

$$LOD = 3\sigma/B \qquad \text{Equation S1}$$

where σ is the standard deviation obtained from three individual measured fluorescence intensity I_{637} in the absence of GSH. B is the slope from the linear fitting of the titration curve. Thus,

$$LOD = 3*0.63/8.78=0.22\ \mu M$$

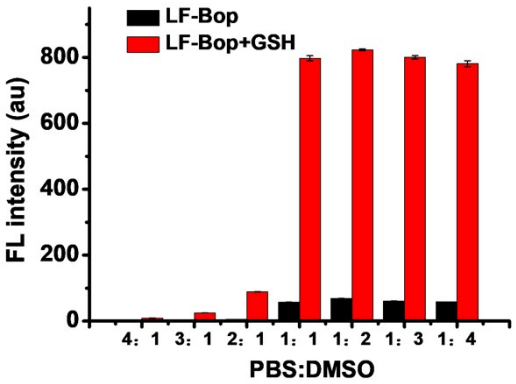


Fig. S1. Fluorescence intensity I_{637} excited at 581 nm from the reaction solution containing 5 μM LF-Bop and 200 μM GSH. PBS (pH 7.4) was mixed with DMSO at different ratios.

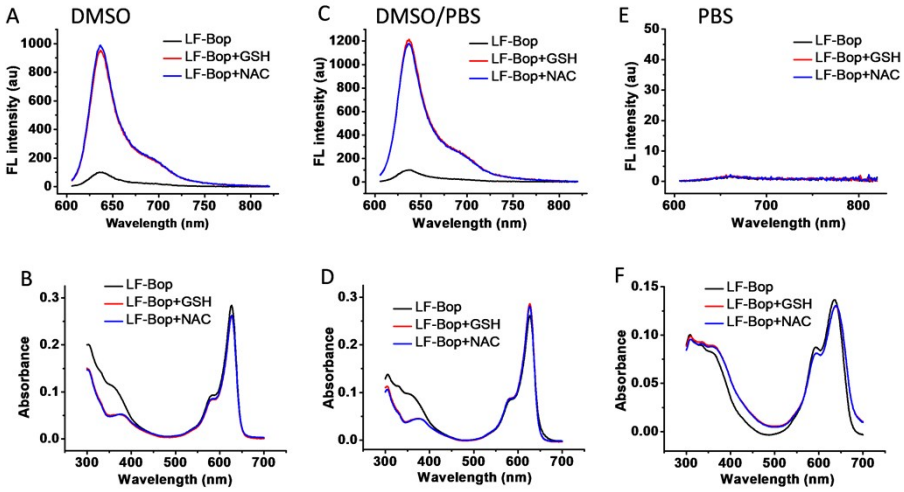


Fig. S2. Fluorescence spectra and absorption spectra of LF-Bop (10 μM) before and after reaction with GSH or NAC（400 μM）.The reaction was performed in different solutions for 10 min: A and B for DMSO (contains 2% PBS), C and D for DMSO/PBS 1:1, E and F for PBS (contains 2% DMSO). Each reaction was conducted in a total 200 μL solution in a 96-well plate. Fluorescence was recorded under 581 nm excitation.

Table S1. Photo-physical properties of LF-Bop under different status.

Solvents	Status	λ_{ab} (nm)	ε ($M^{-1}\cdot cm^{-1}$)	λ_{fl} (nm)	Stokes shift/nm	Quantum yield/φ
DMSO	Probe only	627	51600	637	10	0.046
	+ GSH	627	52800	637	10	0.502
	+ NAC	627	52600	637	10	0.510
DMSO/PBS	Probe only	627	52000	637	10	0.048
	+ GSH	627	50600	637	10	0.584
	+ NAC	627	50600	637	10	0.575
PBS	Probe only	635	15600	--	--	--
	+ GSH	639	14400	--	--	--
	+ NAC	639	14200	--	--	--

Note: Molar extinction coefficient (ε) was calculated based on the absorbance measurements using a quartz cuvette with 10 mm light path. Quantum yields were calculated based on the data from Fig. S2. LF-Bop and its reaction

products in PBS (pH 7.4) are nearly non-fluorescent, so there are no λ_{fl}, stokes shift and quantum yield presented.

Quantum yields measurement: The relative quantum yields of LF-Bop with different status were measured by using the result from Fig. S2 and the well characterized Cresyl violet (φ = 0.54 in ethanol when excited at the wavelength between 540-590 nm) as the reference.[5] The relative quantum yields were calculated by the following equation.

$$\varphi_S = (Abs_R/Abs_S)\times(Area_S/Area_R) \times(n_S/n_R) \times\varphi_R \qquad \text{Equation S2}$$

where the subscripts R and S refer to the reference and samples respectively. Abs, Area and n are the absorbance at the excitation wavelength, area under the fluorescence spectrum and refractive index of the solvent respectively. Refractive indices (n) for ethanol, DMSO, and 1X PBS buffer (pH 7.4) are 1.361, 1.479 and 1.337 respectively. Refractive index of DMSO/PBS (1:1) was estimated to be 1.408 using a simple linear relationship, *J. (Phys. Chem. B* 2015, 119, 33, 10701-10709).

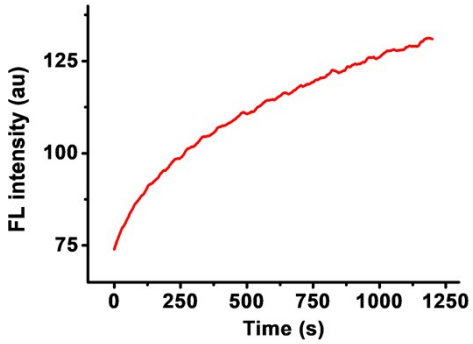


Fig. S3. Kinetic measurement of the fluorescence from LF-Bop (5 μM) in DMSO/PBS 1:1 solution upon reaction with NaHS (200 μM).

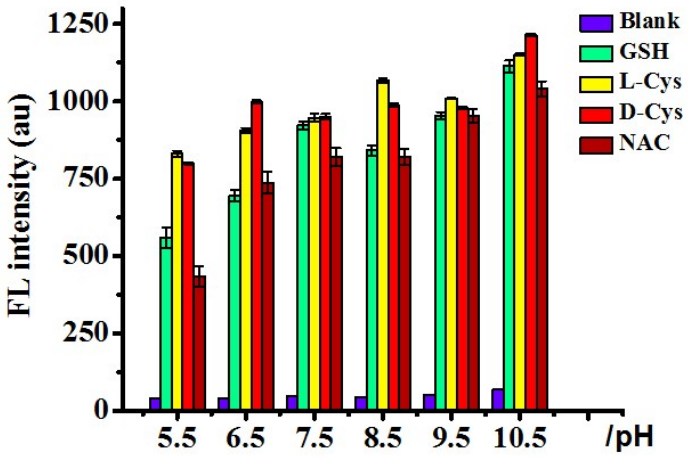


Fig. S4. Fluorescence monitoring thiol detection under different pH conditions in PBS: DMSO 1:1 solutions. LF-Bop was used at 5 μM, all thiols were used at 200 μM, the solution was excited at 581 nm, fluorescence at 637 nm was collected by a plate reader.

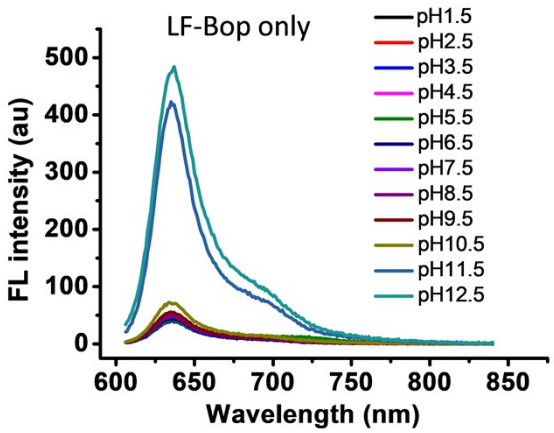


Fig. S5. Fluorescence spectra of LF-Bop (5 μM) in DMSO/PBS 1:1 solution with different pH values.

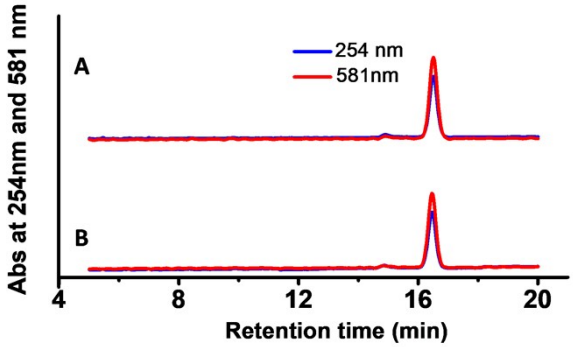


Fig. S6. High-performance liquid chromatography (HPLC) analysis of LF-Bop solution after storage in fridge over three months, A) at 4 ºC in DMOS/PBS (1:1) solution; B) at -20 ºC in pure DMSO. (Agilent HPLC 1260, USA) and a reverse phase C18 column (250 x 4.6 mm) were used, the mobile phase was 90% methanol and 10% water with 0.1% TFA.

2.3 AChE activity evaluation and inhibitor screening

Since enzymes cannot tolerate high concentrations of DMSO, the enzyme reaction was performed in pure aqueous solutions (PBS 7.4) in the presence of LF-Bop (10 μM) and acetylthiocholine iodide (400 μM). The reaction was performed at 37 ºC for 30 min, equal amount of DMSO was added into the reaction mixture before fluorescence measurement. To check the possible influence caused by changing the sequence of reagents addition, kinetic measurement and selectivity study have been re-performed (Fig. S7).

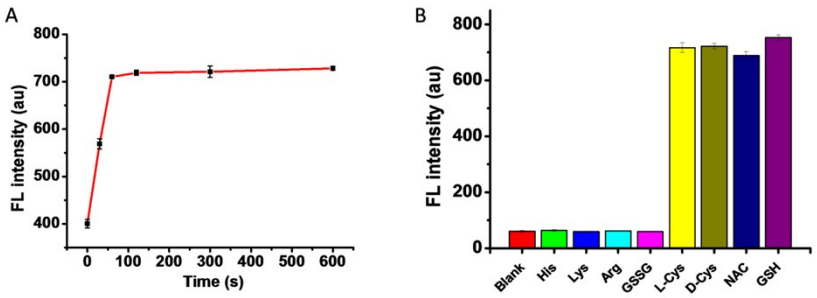


Fig. S7. A) Kinetic measurement of LF-Bop fluorescence upon reaction with GSH; B) LF-Bop exhibits promising selectivity toward thiols. To perform the reaction, LF-Bop (10 μM) was firstly mixed with a thiol (400 μM) in 100

μL PBS buffer (pH 7.4). 100 μL DMSO was then added before plate reading.

There are three basic concepts to choose the candidates for inhibitor screening: First, it should be a basic molecule since most of the inhibitors found for AChE are positively charged under physiological pH conditions; Second, the molecule has been well studied for biomedical purpose; Third, it is commercially available or easy to synthesize. In a typical test, the reaction solution contains LF-Bop (10 μM), acetylthiocholine iodide (400 μM) and AChE 40 mU.

$$\text{Inhibition ratio} = 1-(I_w-I_{blank})/(I_{w/o}-I_{blank})$$

Where, I_w stands for the fluorescence intensity I_{637} in the presence of inhibitors, $I_{w/o}$ stands for I_{637} in the absence of inhibitors, I_{blank} means the background I_{637} from LF-Bop/AChE solution without the substrate acetylthiocholine iodide.

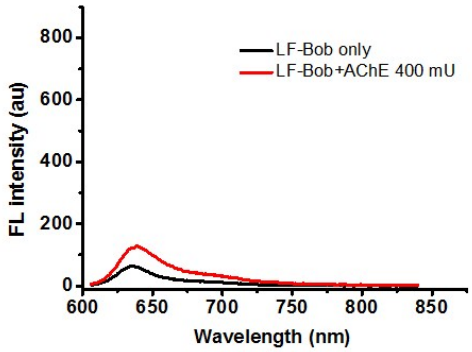


Fig. S8. The fluorescence spectra of LF-Bop (5μM) in the presence and in the absence of 400 mU AChE in PBS (pH 7.4):DMSO 1:1 solution. The solutions were excited at 581 nm.

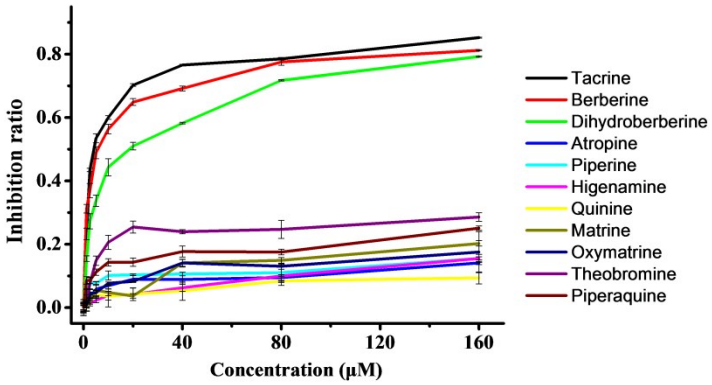


Fig. S9. Evaluation of inhibition effect of different compounds using LF-Bop based fluorescence assay. Inhibition ratio was plotted as the function of inhibitor concentrations.

2.4 Computational molecular docking

Table S2. Molecular docking scores of AcheE binding with BBR and DB

PDB-ID	Molecules	Total score	Crash	polar	C-score
1GQR	BBR	11.12	-0.26	3.91	4
	DB	10.41	-0.46	2.23	3

The docking scoring function Total-Score takes into account factors such as molecular polarity, hydrophobicity, enthalpy, and solvation. A larger value indicates that the complex may be more stable, and generally greater than 7 points are considered active; Crash represents the inappropriate degree of ligand docking into the receptor. The closer to 0, the better. That is, the smaller the absolute value, the better. Polar is a polar function score, when the binding pocket is on the molecular surface, the higher the score, the better. When the binding site is inside the molecule, the lower the score, the better. C-Score is another scoring function, a value close to 5 points is considered to have better activity.[6]

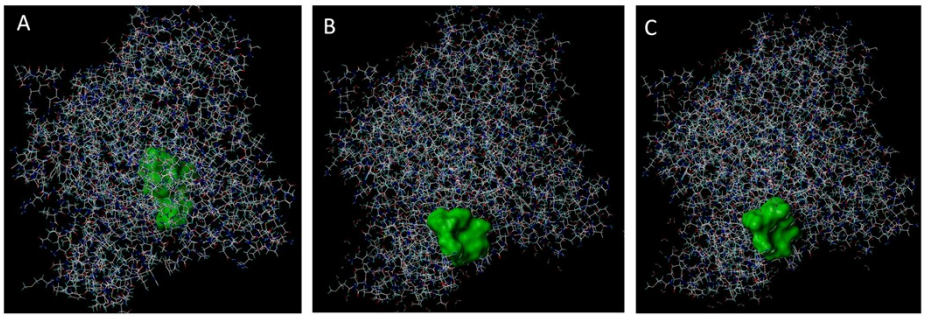


Fig. S10. A) The acetylcholine binding pocket of AChE, the information was adapted from PDB bank; The berberine binding pocket (B) and the dihydroberberine binding pocket (C) in AChE found by molecular docking.

3. References

[1] Chen, N., Zhang, W., Chen, S., Wu, Q., Yu, C., Wei, Y., Xu, Y., Hao, E., and Jiao, L. (2017) Sterically Protected N2O-Type Benzopyrromethene Boron Complexes from Boronic Acids with Intense Red/Near-Infrared Fluorescence, *Org Lett 19*, 2026-2029.

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[3] Rodrigues, C. A. B., Neto, I., Rijo, P., and Afonso, C. A. M. (2018) Synthesizing a Berberine Derivative and Evaluating Antimicrobial Activity To Reinforce with Students the Potential Significance of Small Chemical Structure Changes for Biological Systems, *J Chem Educ 95*, 492-495.

[4] Shrivastava, A., and Gupta, V. (2011) Methods for the determination of limit of detection and limit of quantitation of the analytical methods, *Chron young sci 2*, 21-25.

[5] Brouwer Albert, M. (2011) Standards for photoluminescence quantum yield measurements in solution (IUPAC Technical Report), In *Pure Appl Chem*, p 2213.

[6] SYBYL-X; Tripos Associates Inc.: St. Louis, MO, USA. Available online: http:/www.tripos.com/sybyl , accessed on 16 November 2011.

4. Spectra

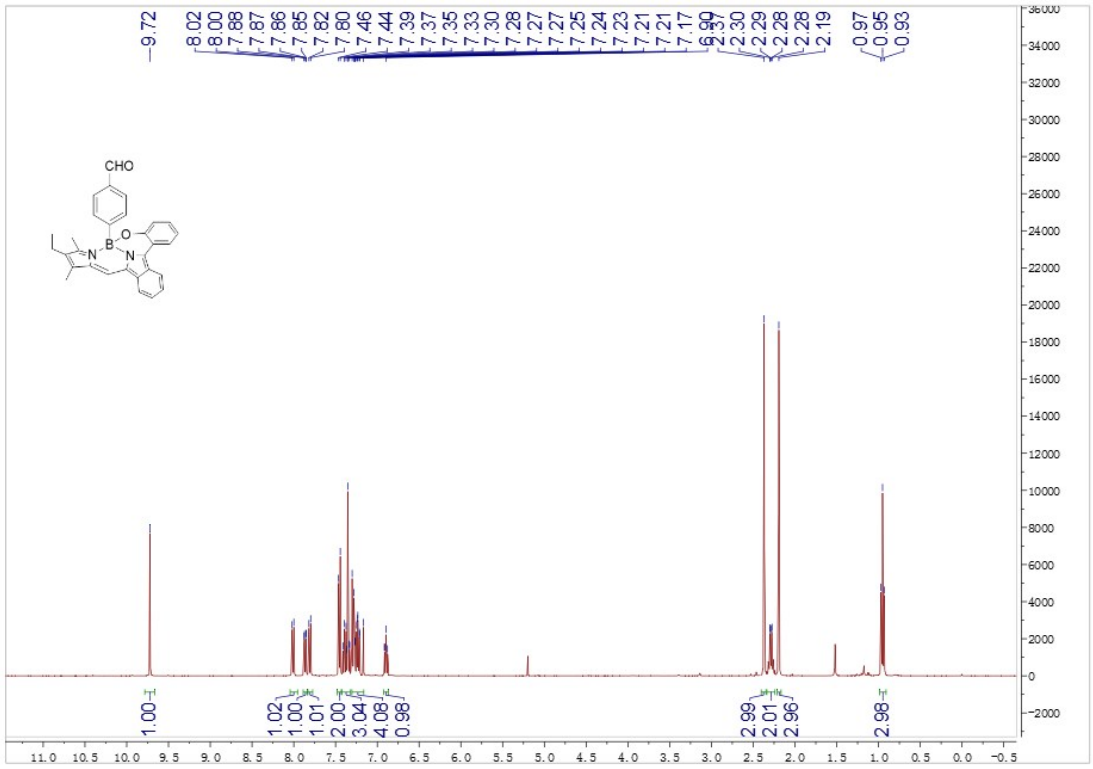

Fig. S11. ^{1}H NMR spectrum of compound **3**

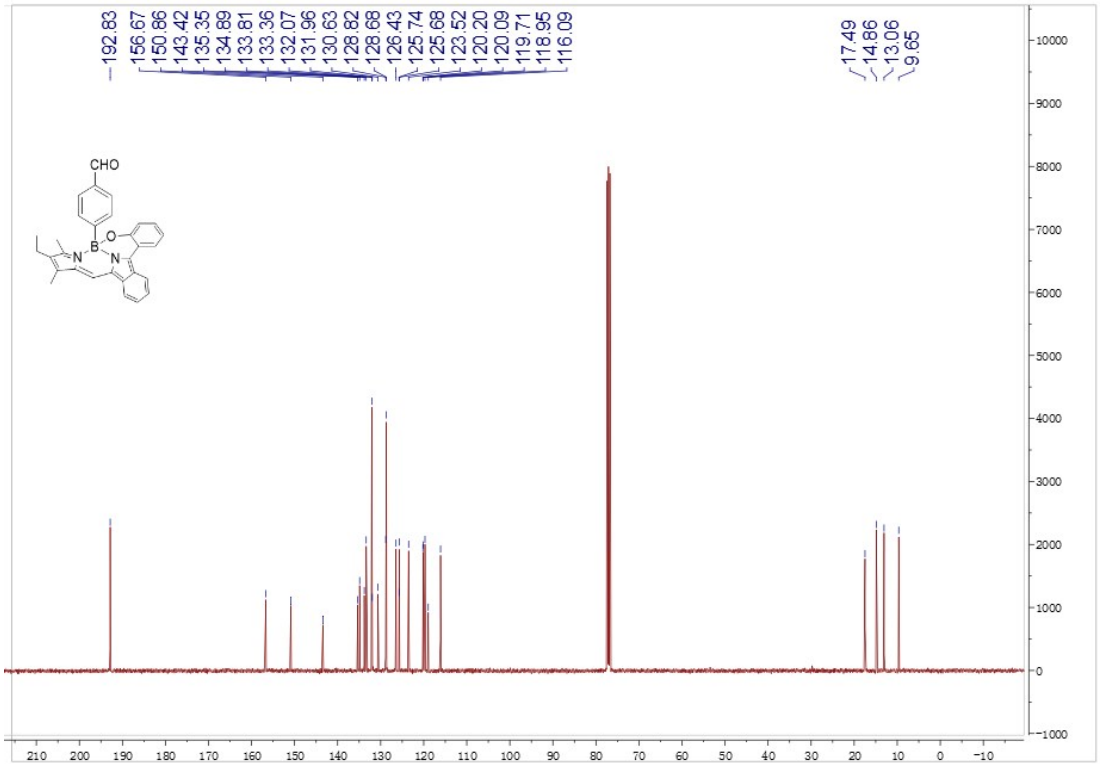

Fig. S12. ^{13}C NMR spectrum of compound **3**

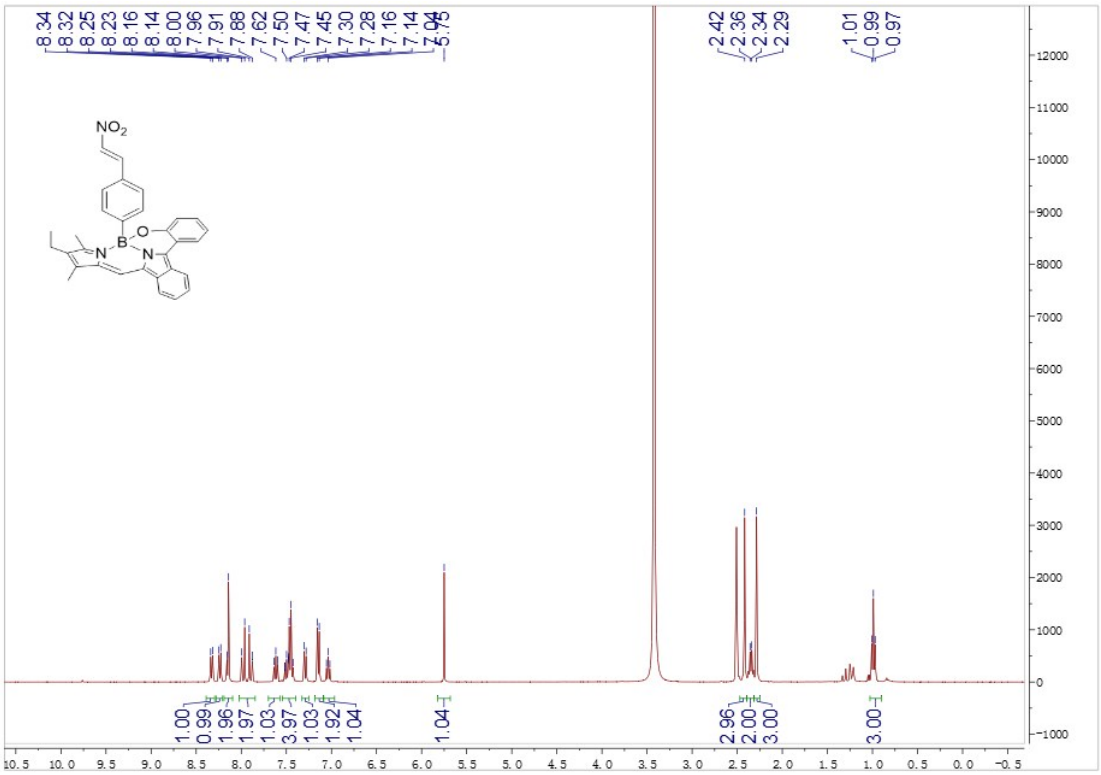

Fig. S13. ^{1}H NMR spectrum of LF-Bop

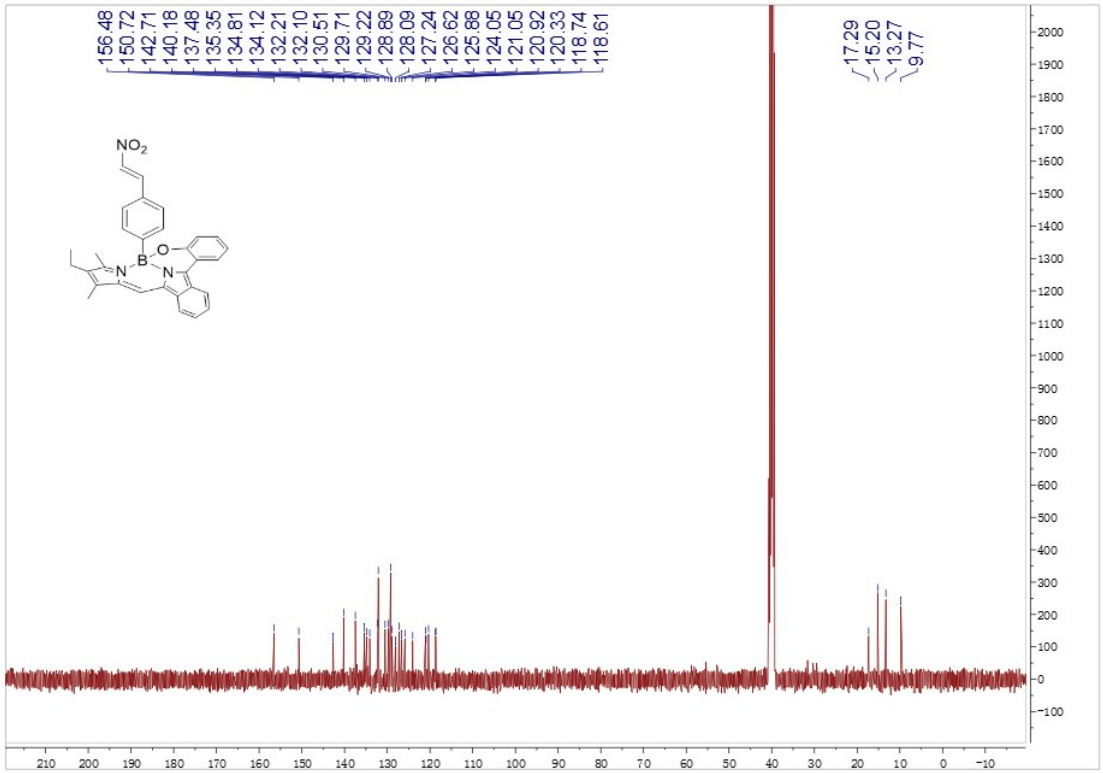

Fig. S14. ^{13}C NMR spectrum of LF-Bop

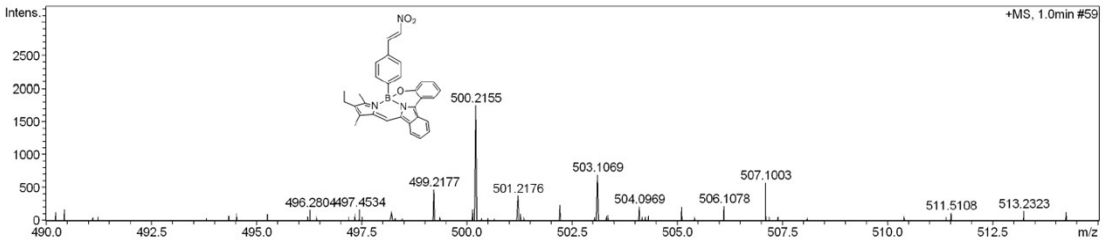

Fig. S15. HRMS spectrum of LF-Bop

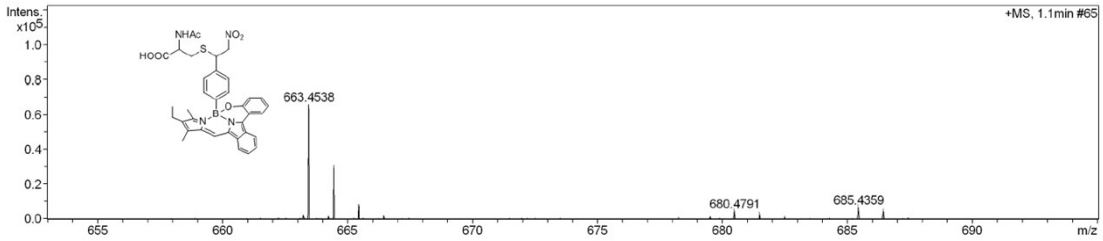

Fig. S16. HRMS spectrum of LF-Bop NAC reaction product

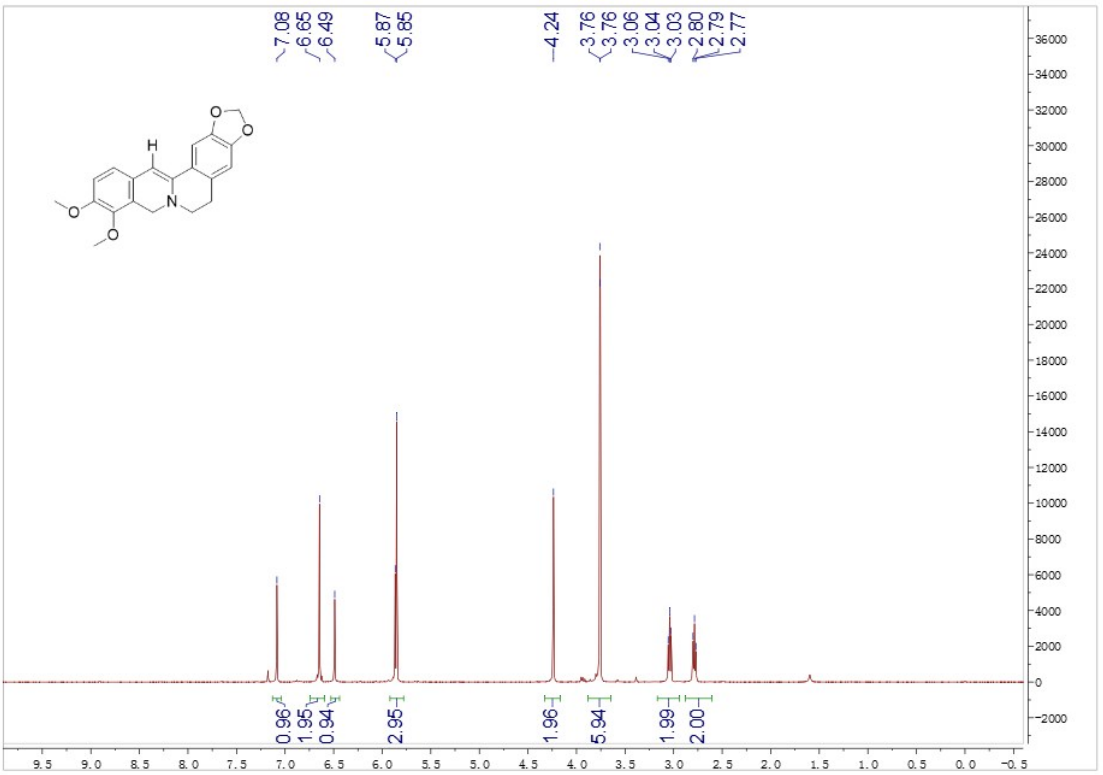

Fig. S17. ^{1}H NMR spectrum of dihydroberberine

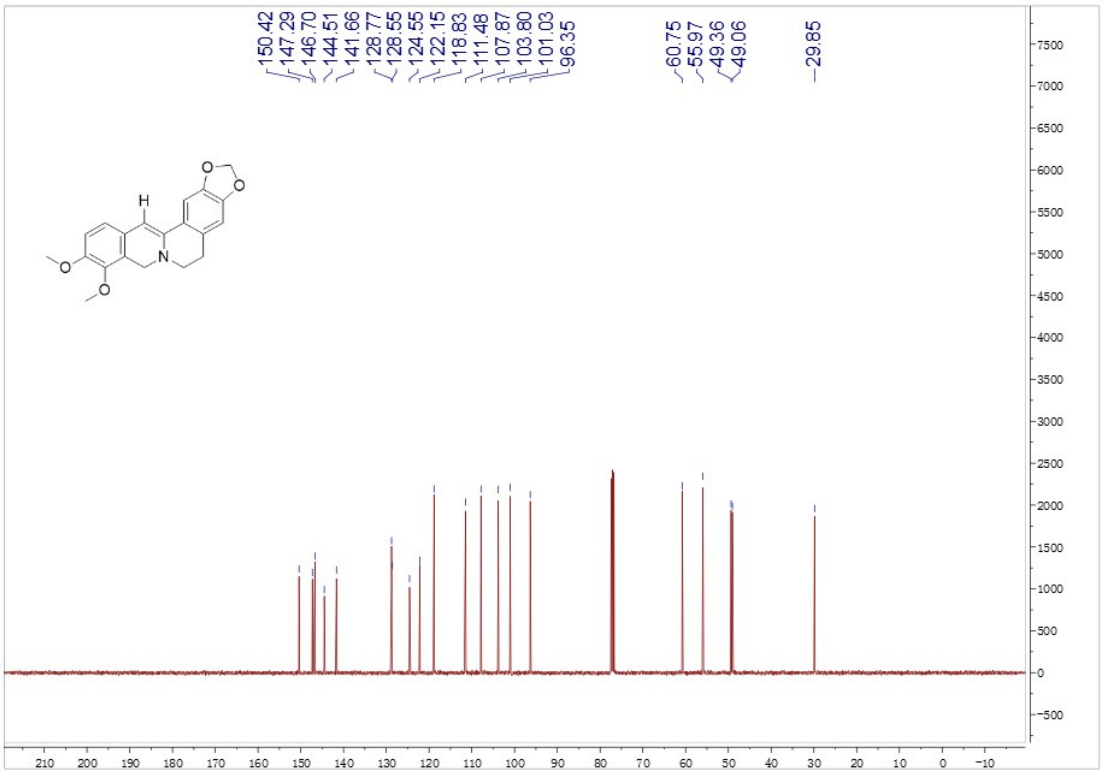

Fig. S18. ^{13}C NMR spectrum of dihydroberberine