Supplementary Information (SI) for Analytical Methods.
This journal is © The Royal Society of Chemistry 2026

Supporting Information (SI)

Rational design of environment-sensitive fluorescent probes for butyrylcholinesterase and its application in biological imaging

Lulian Shao, †[a] Feng Wang, †[a, d] Yu Wang,[a] Jiahao Du,[a] Wenting Cao,[a] Qingwen Zhang,[a] Fenqin Zhao,[a] Junfeng Wang,[e] Wenhui Fan,[b] Yuemin Zhou,[f] Bingxin Wang,*[a] Xiaobin Pang,*[a c] Lin Yan,*[a]

[a] *State Key Laboratory of Antiviral Drugs, School of Pharmacy, Henan University, Kaifeng, Henan, 475004, China. E-mail: wbx@henu.edu.cn; pxb@vip.henu.edu.cn; yanlin@henu.edu.cn*

[b] *College of Chemistry and Molecular Sciences, Henan University, Kaifeng, Henan 475004, China.*

[c] *Department of Anaesthesiology, Huaihe Hospital of Henan University, Kaifeng, Henan, 475004, China.*

[d] *Department of pharmacy, Nanyang Medical College, Nanyang, Henan, 473004, China.*

[e] *Gordon Center for Medical Imaging, Department of Radiology, Massachusetts General Hospital, Harvard medical school, 125 Nushua St, Boston, MA, 02149, USA.*

[f] *The Second Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450014, China.*

† These authors contributed equally to this work

Content

1. Synthesis of ESP series probes

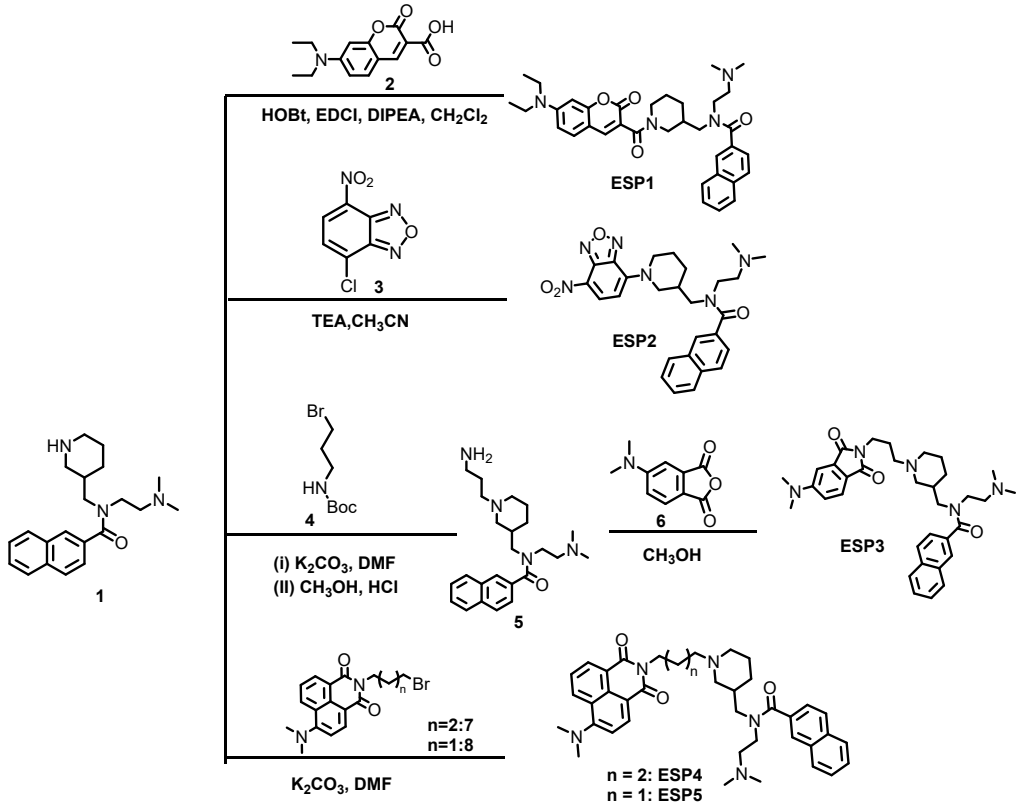

Fig. S1 The synthesis process of **ESP** series probes.

Synthesis of compound 1, 2, 6, 7, and 8

Compound **1**, **2, 6**, **7** and **8** were synthesized according to the method reported in the literature [1-5].

Synthesis of probe ESP1

Compound **2** (400 mg, 1.53 mmol) was dissolved in CH_2Cl_2. Subsequently, HOBt (282 mg, 1.84 mmol) and EDCI (353 mg, 1.84 mmol) were added. The reaction mixture was stirred for 30 min, followed by the addition of a mixture containing compound **1** (625 mg, 1.84 mmol) and DIPEA (593 mg, 4.59 mmol). After reaction for 24 h at room temperature, the reaction mixture was transferred to a separatory funnel and washed successively with water, 0.5 M hydrochloric acid, saturated aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4. Subsequently, the solvent was removed under reduced pressure by distillation. The resulting product was purified by silica gel column chromatography using a CH_2Cl_2 and methanol mixture (15:1, v/v) as the elution

solvent, yielding **ESP1** (453 mg, green solid, 50% yield).

ESP1: ^{1}H NMR (300 MHz, Chloroform-*d*) δ ppm 8.00-7.74 (m, 6H, Ar-H), 7.60-7.47 (m, 3H, Ar-H), 6.65-6.44 (m, 2H, Ar-H), 4.71-4.09 (m, 2H, CH_2), 3.85-3.26 (m, 11H, -N(CH_3)$_2$, CH_2, CH), 2.87-2.47 (m, 5H, CH_2), 2.23-1.99 (m, 4H, CH_2), 1.96-1.67 (m, 3H, CH_2), 1.35-1.22 (m, 6H, CH_3). ^{13}C NMR (75 MHz, Chloroform-*d*) δ ppm 172.6, 165.2, 159.3, 157.1, 151.6, 133.5, 132.7, 129.9, 128.5, 127.8, 126.8, 124.1, 109.4, 107.9, 97.0, 48.3, 45.9, 44.9, 42.9, 29.7, 29.3, 28.5, 12.4. HRMS (ESI) calcd. for $C_{35}H_{43}N_4O_4$, $[M+H]^+$ 583.3279, found 583.3304.

Synthesis of probe ESP2

Compound **1** (261 mg, 0.77 mmol) and compound **3** (153 mg, 0.77 mmol) were mixed in acetonitrile, and subsequently, a few drops of triethylamine were added. The content was heated at 85 ℃ for about 8 h. After cooling the reaction mixture to room temperature, it was concentrated under vacuum. Water was then added, and the mixture was extracted with CH_2Cl_2. The organic layer was washed with brine and dried over anhydrous sodium sulfate. Subsequently, the solvent was removed under reduced pressure by distillation. The resulting residue was purified by silica gel column chromatography using CH_2Cl_2 and methanol mixture (50:1, v/v) as the elution solvent, yielding **ESP2** (280 mg, orange solid, 72% yield).

ESP2: ^{1}H NMR (300 MHz, Chloroform-*d*) δ ppm 8.36 (d, J = 9.1 Hz, 1H, Ar-H), 8.13-7.85 (m, 4H, Ar-H), 7.68-7.50 (m, 3H, Ar-H), 6.28 (d, J = 9.2 Hz, 1H, Ar-H), 4.01-3.78 (m, 1H, CH_2), 3.64-3.24 (m, 5H, CH_2), 2.50-2.23 (m, 4H, CH_2), 2.15-2.10 (m, 1H, CH), 2.03 (s, 6H, -N(CH_3)$_2$), 1.93-1.75 (m, 2H, CH_2), 1.63-1.29 (m, 2H, CH_2). ^{13}C NMR (75 MHz, Chloroform-*d*) δ ppm 172.9, 144.8, 135.6, 133.8, 133.5, 132.7, 128.5, 127.8, 127.1, 126.8, 126.3, 124.1, 102.3, 57.9, 55.1, 50.4, 47.8, 45.6, 36.8, 29.7, 28.7, 25.1. HRMS (ESI) calcd. for $C_{27}H_{31}N_6O_4$ $[M+H]^+$ 503.2401, found 503.2384.

Synthesis of compound 5

Compound **1** (800 mg, 2.35 mmol) and compound **4** (560 mg, 2.35 mmol) were dissolved in DMF, and K_2CO_3 (1.62 g, 11.75 mmol) was added. The mixture was

stirred at 80 °C for 10 h. After cooling the reaction mixture to room temperature, it was concentrated under vacuum. Water was added, and the mixture was extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated under vacuum again. The crude product was dissolved in hydrochloric acid and methanol (1:3, v/v) and stirred at room temperature for 3 h, water was added, and the mixture was extracted with ethyl acetate. Then, the organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated. Compound **5** was finally purified by silica gel column chromatography (elution solvent: dichloromethane: methanol = 10:1, v/v).

Synthesis of probe ESP3

Compound **5** (170 mg, 0.43 mmol) and compound **6** (82 mg, 0.43 mmol) were stirred into methanol. The mixture was heated at 70 °C for 8 h. Then, the solvent was removed, and the product was purified by silica gel column chromatography (CH_2Cl_2: CH_3OH = 15:1, v/v) to give compound **ESP3** (152 mg, yellow solid, yield: 62%).

ESP3: ^{1}H NMR (300 MHz, Chloroform-*d*) δ ppm 8.00-7.80 (m, 4H, Ar-H), 7.66 (d, J = 8.5 Hz, 1H, Ar-H), 7.59-7.44 (m, 3H, Ar-H), 7.09 (s, 1H, Ar-H), 6.80 (d, J = 8.6 Hz, 1H, Ar-H), 3.85-3.57 (m, 4H, CH_2), 3.53-3.38 (m, 2H, CH_2), 3.12 (s, 6H, -$N(CH_3)_2$), 2.88-2.66 (m, 3H, CH_2, CH), 2.48-2.28 (m, 6H, CH_2), 2.02 (s, 6H, -$N(CH_3)_2$), 1.86-1.67 (m, 4H, CH_2), 1.42-1.33 (m, 2H, CH_2). ^{13}C NMR (75 MHz, Chloroform-*d*) δ ppm 164.7, 164.2, 163.4, 155.5, 134.3, 131.1, 130.3, 128.4, 128.3, 127.8, 127.1, 126.8, 123.2, 117.4, 115.9, 115.1, 106.6, 57.7, 56.4, 45.5, 40.8, 38.6, 31.9, 31.5, 29.8, 29.5, 29.4, 25.2, 24.7, 22.6. HRMS (ESI) calcd. for $C_{34}H_{44}N_5O_3$, $[M+H]^+$ 570.3439, found 570.3433.

Synthesis of probe ESP4

Compound **1** (800 mg, 2.35 mmol) and compound **7** (879mg, 2.35 mmol) were dissolved in DMF. After the addition of K_2CO_3 (1.62 g, 11.75 mmol), the mixture was stirred at 80 °C for 10 h. The reaction solution was then cooled to room temperature, concentrated under vacuum, and subsequently diluted with water. The aqueous solution

was extracted with ethyl acetate, washed with brine, dried over anhydrous Na_2SO_4, and once again concentrated under vacuum. The resulting residue was purified by column chromatography on silica gel using a dichloromethane:methanol mixture (15:1) as the eluent to obtain **ESP4** as a yellow solid (840 mg, yield: 56%).

ESP4: ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.60 (d, J = 7.4 Hz, 1H, Ar-H), 8.49 (t, J = 10.1 Hz, 2H, Ar-H), 7.93-7.87 (m, 4H, Ar-H), 7.69 (t, J = 7.6 Hz, 1H, Ar-H), 7.59-7.52 (m, 3H, Ar-H), 7.15 (d, J = 8.2 Hz, 1H, Ar-H), 4.19-4.10 (m, 2H, CH_2), 3.77-3.63 (m, 2H, CH_2), 3.58-3.44 (m, 2H, CH_2), 3.14 (s, 6H, -N(CH_3)$_2$), 2.40-2.24 (m, 6H, CH_2), 2.08 (s, 6H, -N(CH_3)$_2$), 2.01-1.90 (m, 4H, CH_2), 1.86-1.76 (m, 3H, CH_2, CH), 1.46-1.34 (m, 4H, CH_2). ^{13}C NMR (75 MHz, Chloroform-*d*) δ ppm 164.6, 164.1, 156.9, 134.4, 133.4, 132.7, 132.6, 131.1, 131.0, 130.2, 128.4, 128.3, 127.8, 126.8, 126.6, 125.3, 124.9, 123.1, 115.1, 113.3, 58.9, 45.7, 44.8, 40.0, 29.7, 28.4, 26.3, 24.9, 24.4, 14.2. HRMS (ESI) calcd. for $C_{39}H_{48}N_5O_3$, $[M+H]^+$ 634.3752 found 634.3731.

Synthesis of probe ESP5

Compound **ESP5** was synthesized in the same way as **ESP4**. Finally, the yellow solid compound **ESP5** was obtained with yields of 56 %.

ESP5: ^{1}H NMR (300 MHz, Chloroform-*d*) δ ppm 8.60 (d, J = 7.5 Hz, 1H, Ar-H), 8.55-8.41 (m, 2H, Ar-H), 7.98-7.81 (m, 4H, Ar-H), 7.79-7.65 (m, 1H, Ar-H), 7.60-7.44 (m, 3H, Ar-H), 7.20-7.09 (m, 1H, Ar-H), 4.36-4.14 (m, 2H, CH_2), 3.75-3.39 (m, 4H, CH_2), 3.14 (s, 6H, -N(CH_3)$_2$), 2.65-2.31 (m, 6H, CH_2), 2.03 (s, 6H, -N(CH_3)$_2$), 1.95-1.82 (m, 5H, CH, CH_2), 1.51-1.35 (m, 4H, CH_2). ^{13}C NMR (75 MHz, Chloroform-*d*) δ ppm 164.6, 164.0, 156.9, 134.3, 133.4, 132.7, 132.6, 131.1, 131.0, 130.3, 128.4, 128.3, 127.8, 126.8, 126.5, 125.3, 124.9, 123.2, 115.1, 113.4, 57.7, 56.4, 45.5, 44.8, 38.6, 31.9, 31.5, 29.7, 29.5, 29.4, 25.1, 24.7, 22.7. HRMS (ESI) calcd. for $C_{38}H_{46}N_5O_3$, $[M+H]^+$ 620.3595 found 620.3580.

2. Optical properties of ESP series probes

2.1 UV absorption and fluorescence emission of probe ESP series

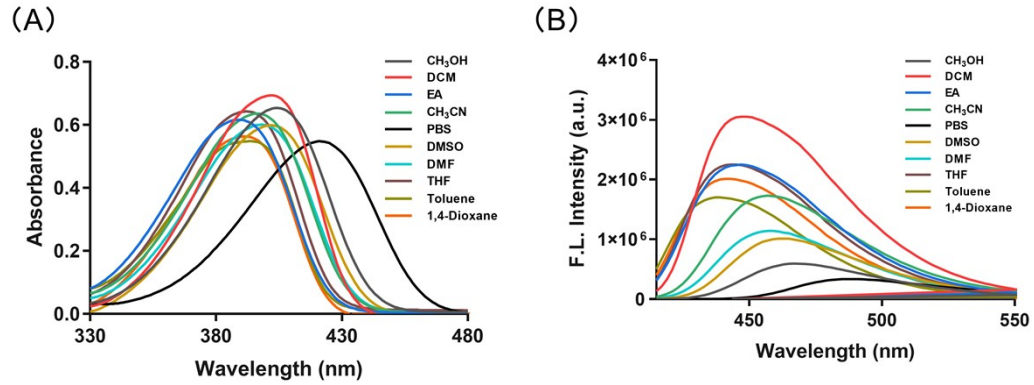

Fig. S2 UV absorption (A) and fluorescence emission (B) of probe **ESP1** (10 μM) in different solvents.

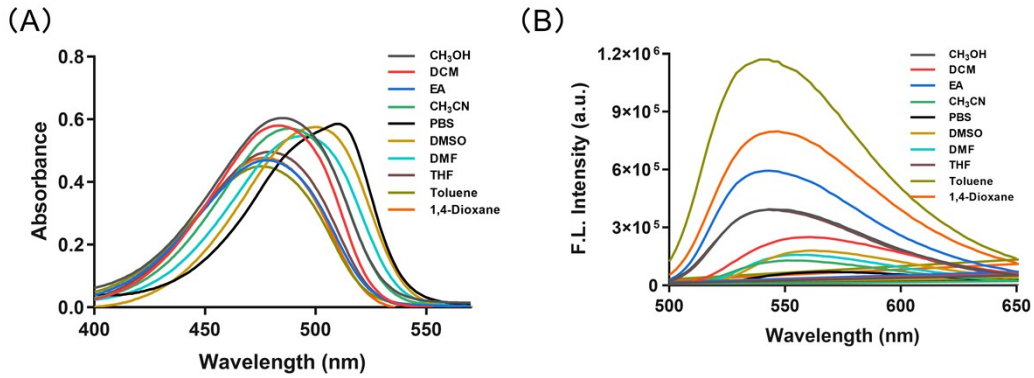

Fig. S3 UV absorption (A) and fluorescence emission (B) of probe **ESP2** (10 μM) in different solvents.

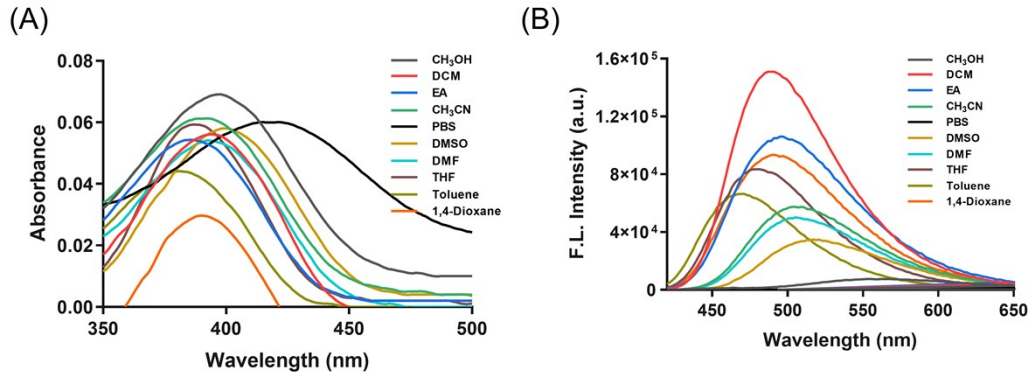

Fig. S4 UV absorption (A) and fluorescence emission (B) of probe **ESP3** (10 μM) in different solvents.

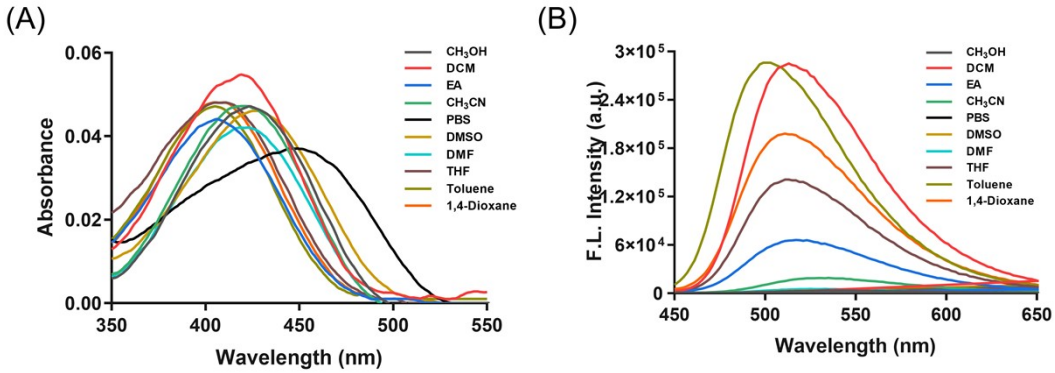

Fig. S5 UV absorption (A) and fluorescence emission (B) of probe **ESP4** (10 μM) in different solvents.

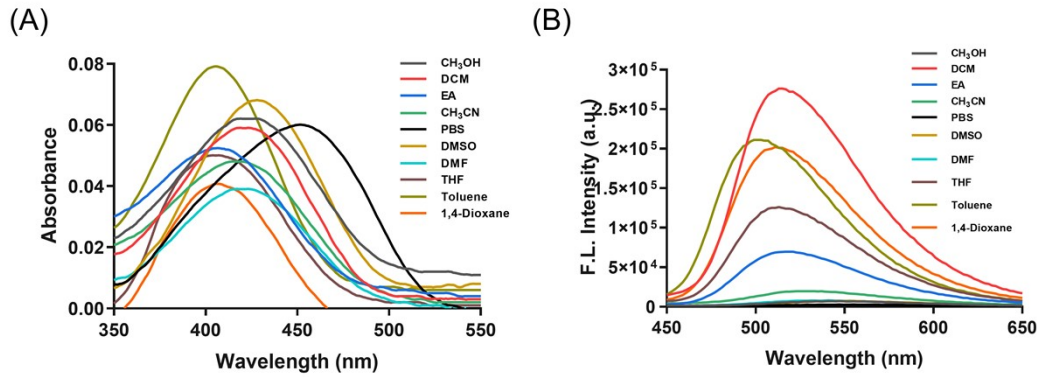

Fig. S6 UV absorption (A) and fluorescence emission (B) of probe **ESP5** (10 μM) in different solvents.

2.2 Fluorescence properties of ESP series probes combined with BChE

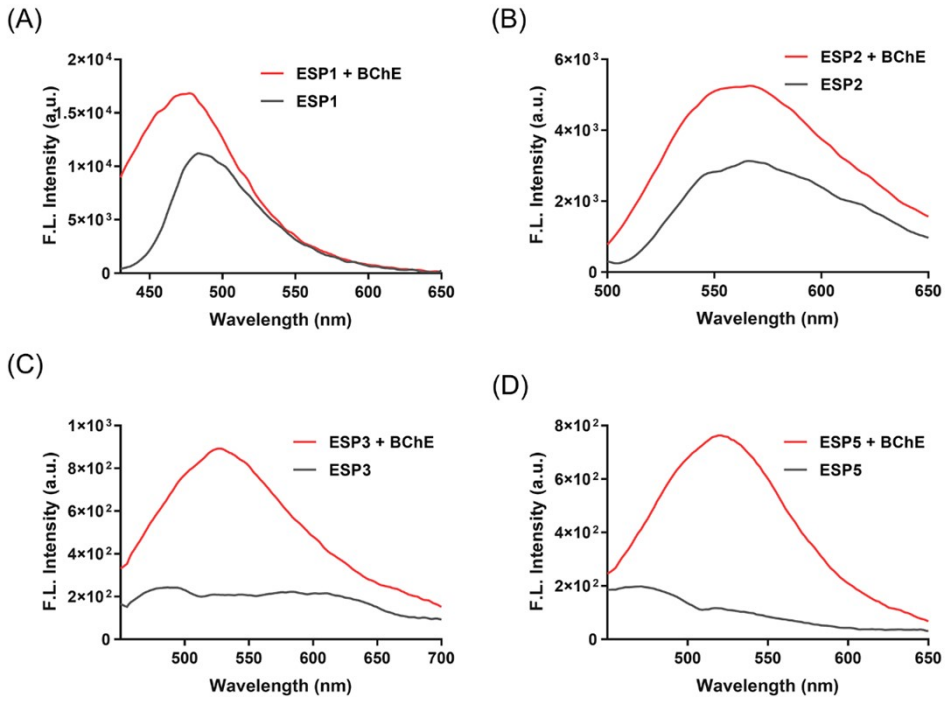

Fig. S7 (A) Fluorescence spectra of **ESP1** (1 μM) before (black line) and after (red line) treated with 1 μM BChE for 10 min at 37 ℃ in PBS buffer of pH 7.4. (B) Fluorescence spectra of **ESP2** (1 μM) before (black line) and after (red line) treated with 1 μM BChE for 10 min at 37 ℃ in PBS buffer of pH 7.4. (C) Fluorescence spectra of **ESP3** (1 μM) before (black line) and after (red line) treated with 1 μM BChE for 10 min at 37 ℃ in PBS buffer of pH 7.4. (D) Fluorescence spectra of **ESP5** (1 μM) before (black line) and after (red line) treated with 1 μM BChE for 10 min at 37 ℃ in PBS buffer of pH 7.4.

2.3 Photophysical properties of the probes

Table. S1. Photophysical properties of the probes

Probe	λ_{ex} (nm)	λ_{em} (nm)	SS[a] (nm)	ε^b (10^4M^{-1} cm^{-1})	Φ^c	Φ^d	Φ^e
ESP1	420	490	70	6.91	0.31	0.029	0.033
ESP2	510	570	60	5.83	0.22	0.011	0.018
ESP3	430	520	90	0.55	0.35	/	/
ESP4	450	520	70	0.59	0.53	0.001	0.012
ESP5	450	520	70	0.57	0.51	/	/

[a] SS: Stokes shift; ε^b: molar absorption coefficient in CH_2Cl_2; Φ^c: absolute quantum yield in CH_2Cl_2; Φ^d: absolute quantum yield in PBS; Φ^e: absolute quantum yield in PBS with BChE

3. Fluorescence properties of ESP4 probe

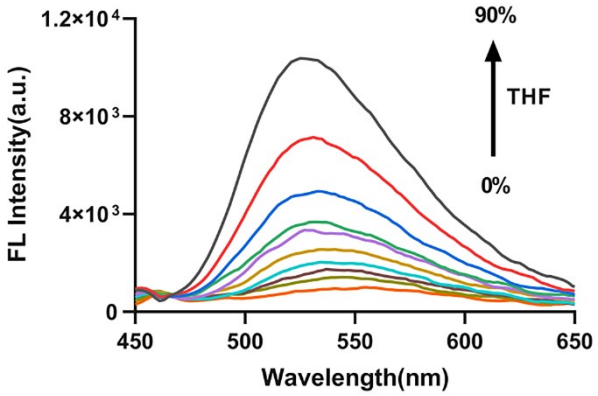

Fig. S8 Fluorescence spectra of 10 μM **ESP4** probe in PBS/ THF mixture with different volume fractions. (λ_{ex} = 450 nm and λ_{em} = 521 nm)

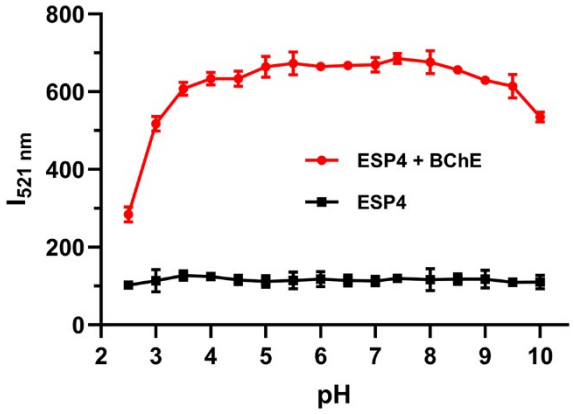

Fig. S9 The pH dependent fluorescence intensity of **ESP4** (500 nM) at 521 nm before (black line) and after (red line) treated with BChE (500 nM) for 10 min at 37 ℃ in PBS buffer at pH 2.5 to 10. (λ_{ex} = 450 nm and λ_{em} = 521 nm)

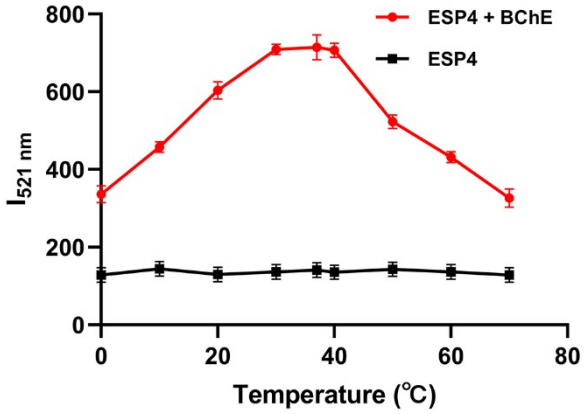

Fig. S10 The temperature dependent fluorescence intensity of **ESP4** (500 nM) at 521 nm before (black line) and after (red line) treated with BChE (500 nM) in PBS buffer at 0 ℃ to 70 ℃. (λ_{ex} = 450 nm and λ_{em} = 521 nm)

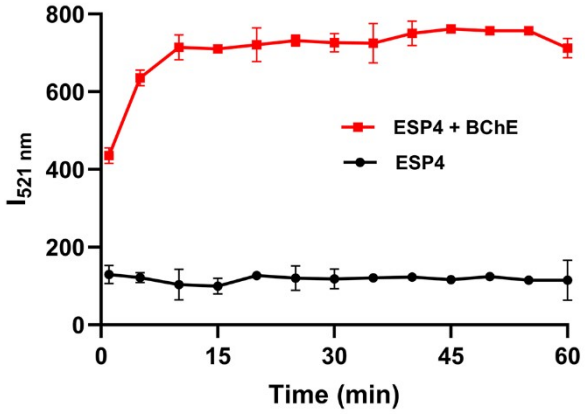

Fig. S11 The time-dependent fluorescence intensity of **ESP4** (500 nM) at 521 nm before (black line) and after (red line) treatment with BChE (500 nM) at 37 ℃ in PBS buffer. (λ_{ex} = 450 nm and λ_{em} = 521 nm)

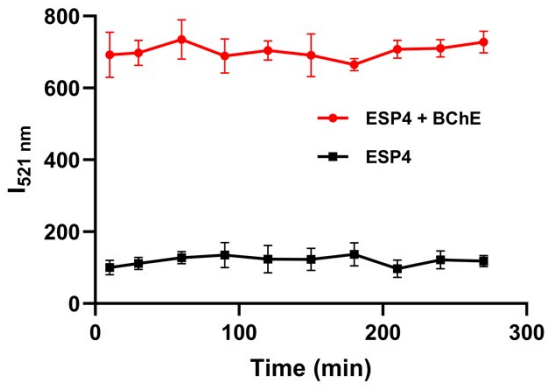

Fig. S12 $I_{521\ nm}$ of **ESP4** (500 nM) in the absence (black line) or presence (red line) of BChE (500 nM) under continuous excitation light irradiation at 37 ℃ in PBS buffer. (λ_{ex} = 450 nm and λ_{em} = 521 nm)

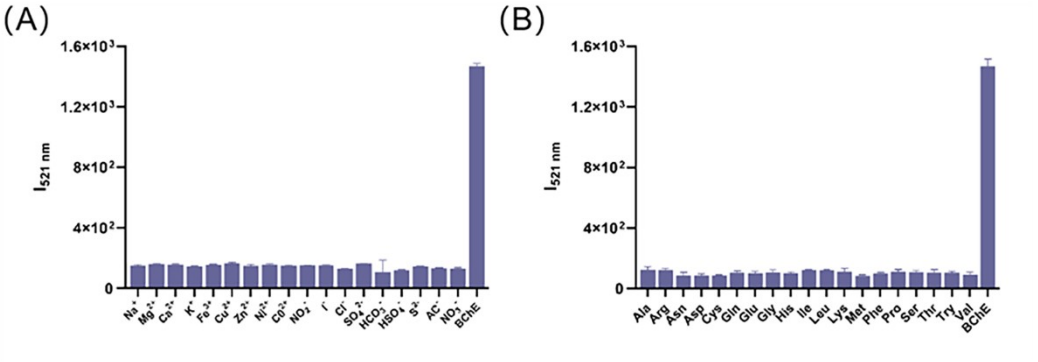

Fig. S13 (A) Fluorescence intensity of **ESP4** (1 μM) at 521 nm for different cations and anions (1 μM). (cations: Na^+, Mg^{2+}, Ca^{2+}, K^+, Fe^{3+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}; anions: NO_2^-, I^-, Cl^-, SO_4^{2-}, HCO_3^-, HSO_4^-, S^{2-}, Ac^-, NO_3^-). (B) Fluorescence intensity of **ESP4** (1 μM) at 521 nm for different amino acids (1 μM) (Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Try, Val). (λ_{ex} = 450 nm and λ_{em} = 521 nm).

4. Determination of fluorescence quantum yield

Fluorescence quantum yields were measured using fluorescein in 0.1 M NaOH solution as a standard (Φ = 0.92, 5 μM). The formula is

$$\Phi_X = \Phi_S\ (A_S/A_X)\ (F_X/F_S)\ (\eta_X/\eta_S)^2$$

Where subscript X and S are the sample and the standard, respectively, Φ is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, η is the solvent refractive index, F is the area under the corrected emission curve.

5. Cytotoxicity study

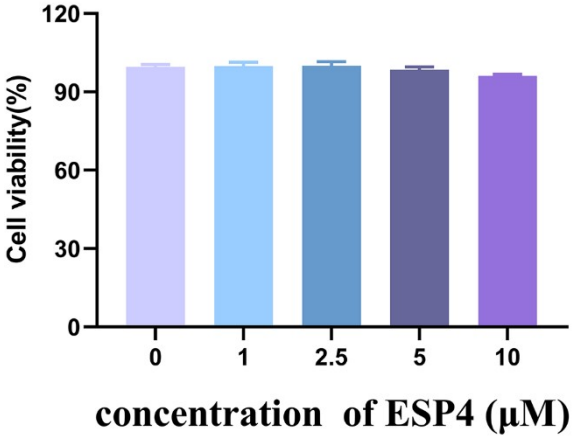

Fig. S14 Assessment of cytotoxicity of **ESP4** on SH-SY5Y cells by MTT assay.

6. Real-time fluorescence imaging of SH-SY5Y cells

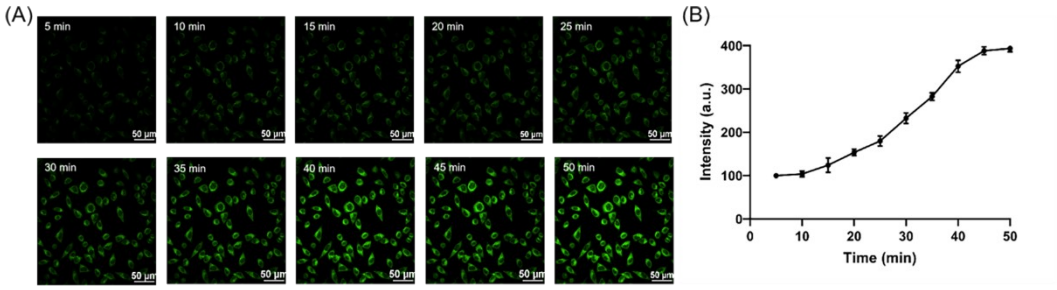

Fig. S15 (A) Real-time fluorescence imaging of SH-SY5Y cells incubated with 5 μM **ESP4** for 0 to 50 min. Fluorescence channels are 480-540 nm, λ_{ex} = 450 nm. (B) Average intensity found in (A). Scale bar: 50 μm.

7. Preparation of $A\beta_{1-42}$ fibril

The configuration methods of different states of $A\beta_{1-42}$ species have been reported in our previous work [6]. $A\beta_{1-42}$ peptide (1 mg) was purchased from Glbiochem. $A\beta_{1-42}$ monomer: 1 mg of lyophilized powder was dissolved in hexafluoro-2-propanol (HFIP, 0.6 mL) to form the stock solution, which was ultrasonically solubilized and then left at room temperature for 4 h. After that, the HFIP was removed by depressurization to form $A\beta_{1-42}$ peptide film. The prepared peptide membrane was solubilized by adding 44 μL of DMSO to obtain the 5 mM of $A\beta_{1-42}$ stock solution, which was then diluted

with pre-cooled PBS solution and centrifuged at 16000 × g for 15 min to obtain 100 μM of $A\beta_{1-42}$ monomer. $A\beta_{1-42}$ fibril:100 μM of $A\beta_{1-42}$ monomer was incubated at 37 ℃ for 36 h to obtain fibril.

8. Western blotting

SHSY5Y cells were seeded onto a six-well plate at a density of 2 × 10^6 cells per well. Following this, they were stimulated with 20 μM of $A\beta_{1-42}$ for 36 h. Afterward, the SHSY5Y cells were harvested, and their proteins were lysed and extracted using RIPA lysis buffer. The protein concentration was determined using a BCA protein assay kit. The proteins were then separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane. The PVDF membrane was placed in a solution containing the primary antibody and incubated overnight at 4 °C. The primary antibodies used were anti-BCHE antibody CY7021 (1:1,000 dilution, Shanghai Powan Biotechnology Co., Ltd.) and anti-GAPDH antibody (1:1,000 dilution, Wuhan Shanying Biotechnology Co., Ltd.). Subsequently, the PVDF membrane was incubated with HRP-Goat-Anti-Rabbit-IgG (1:10,000 dilution) for 2 h at room temperature. The protein bands were developed, saved, and exported. Grayscale analysis was performed using Image J.2 software.

9. Real-time fluorescence imaging of Zebrafish

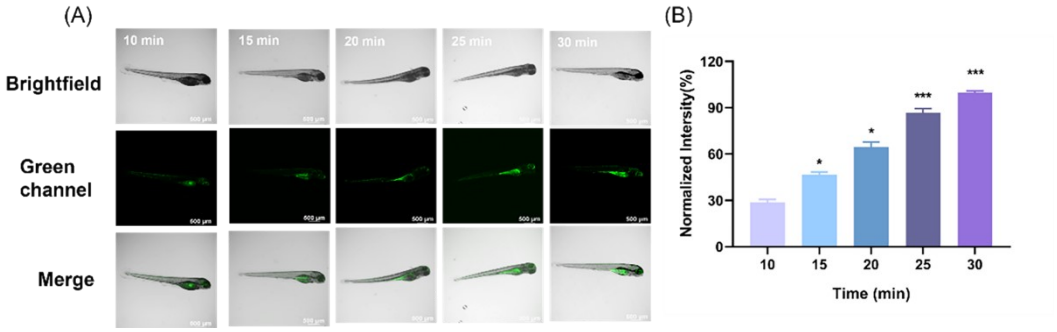

Fig. S16 (A) Real-time fluorescence imaging of zebrafish incubated with 2 μM **ESP4** for 0-30 minutes. (B) See (A) for average strength. ($^{*}P < 0.05$, $^{***}P < 0.001$, one-way analysis of variance (One-way ANOVA) was used for statistical analysis, mean ± standard deviation, n = 3 independent experiments.) Fluorescence: λ_{ex} = 450 nm, λ_{em} = 480-540 nm. Scale = 500 μm.

10. ESP4 was injected into the lateral ventricle of mice

Remove the hair from the top of the mouse with depilatory cream. Each mouse was anesthetized by injecting 50mg/kg pentobarbital sodium. After the eyelid reflex disappeared, the mice were fixed on the stereotactic device in prone position. Disinfect the surgical site with 75% alcohol, make a sagittal incision about 1.5cm in the center of the mouse head with a scalpel, and corrode the skull membrane with 3% hydrogen peroxide to expose the anterior and posterior fontanels. Aim the syringe needle at the front fontanel (AP: 0.00 mm, mL: 0.0 mm, DV: 0.00 mm), and adjust the needle positioning coordinate to (AP:-0.50 mm; Ml: 1.00 mm), drill holes with skull drill. Finally, the injection coordinates of the lateral ventricle were determined as (AP:-0.50 mm, mL: 1.00 mm, DV:-2.50 mm), 5 μL of **ESP4** was extracted with a 5 μL micro syringe, and the needle was slowly inserted 2.50 mm (DV:-2.50 mm), and **ESP4** was slowly injected into the lateral ventricle at a rate of 1 μL/min. After injection, use a small animal imager to capture the fluorescence signal in the mouse brain in time.

11. Molecular docking analysis

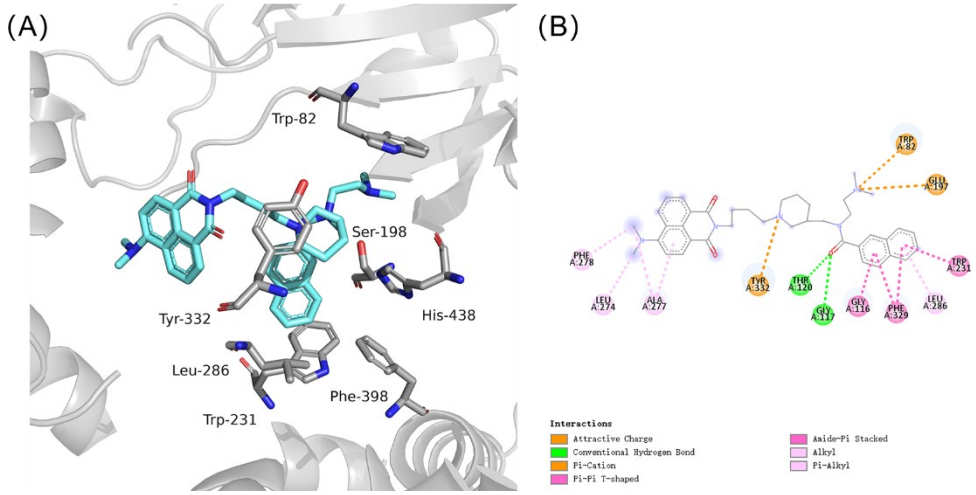

Fig. S17 (A) The crystal structure of the complex of **ESP4** and hBChE (PDB: 5NN0). **ESP4** (light blue) and the key residues and catalytic residues of the binding hBChE (His438, Ser198, Trp231, Phe398, etc.) are displayed in a rod-shaped manner. (B) Hydrophobic interactions bonds and π-

kation interactions between **ESP4** and hBChE.

12. NMR and ESI-HRMS spectra

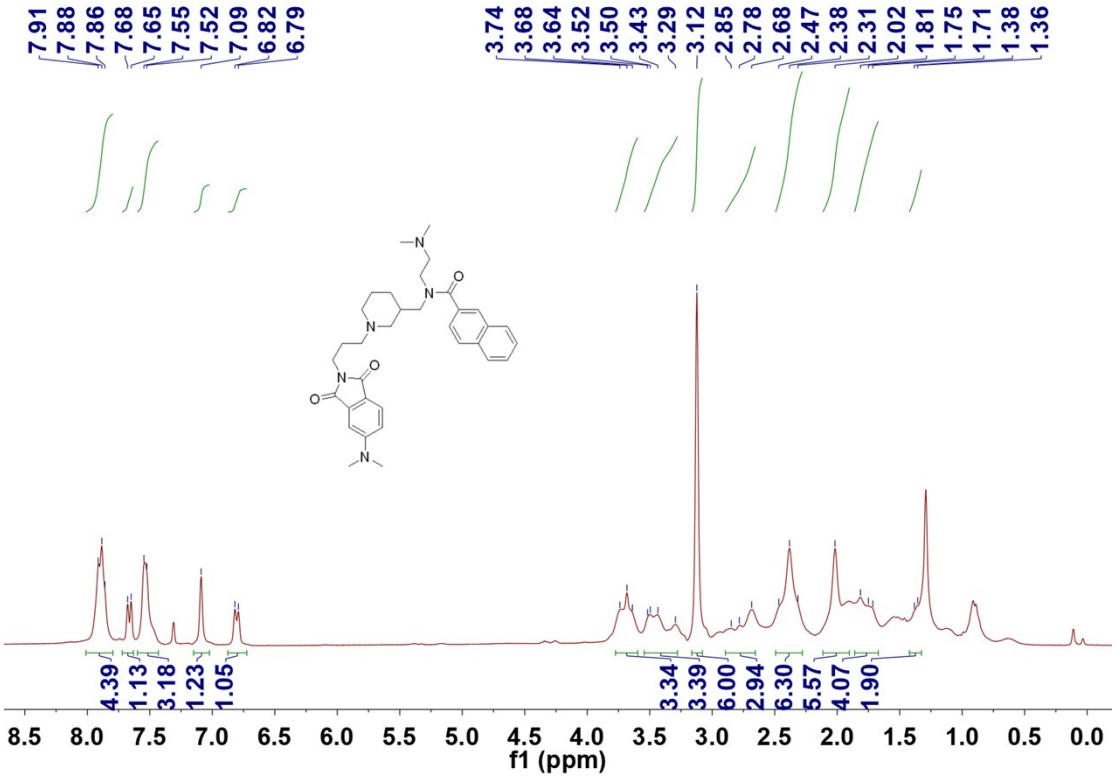

Fig. S18 ^{1}H NMR of **ESP3**

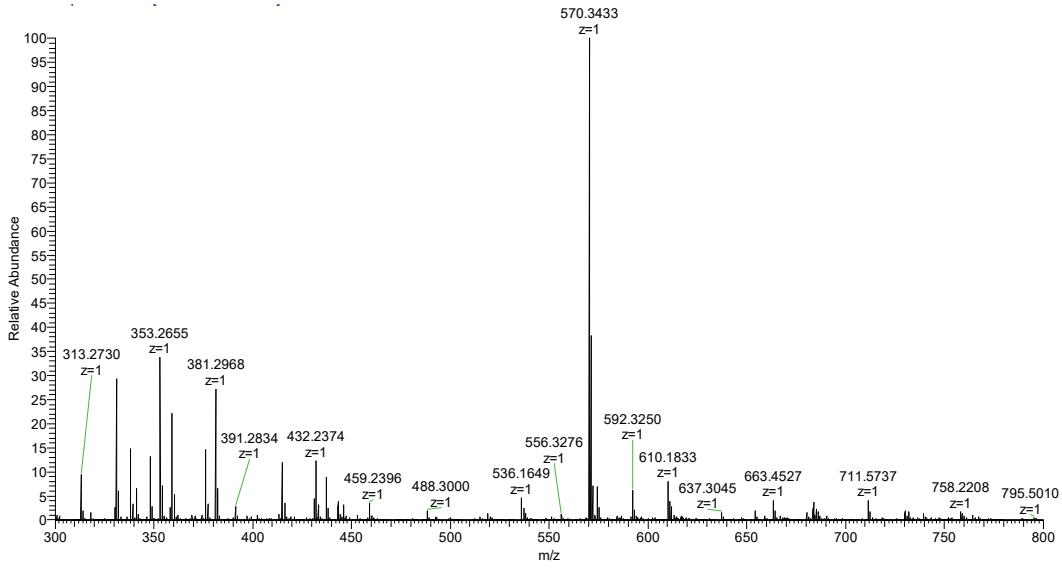

Fig. S19 ESI-HRMS of **ESP3**

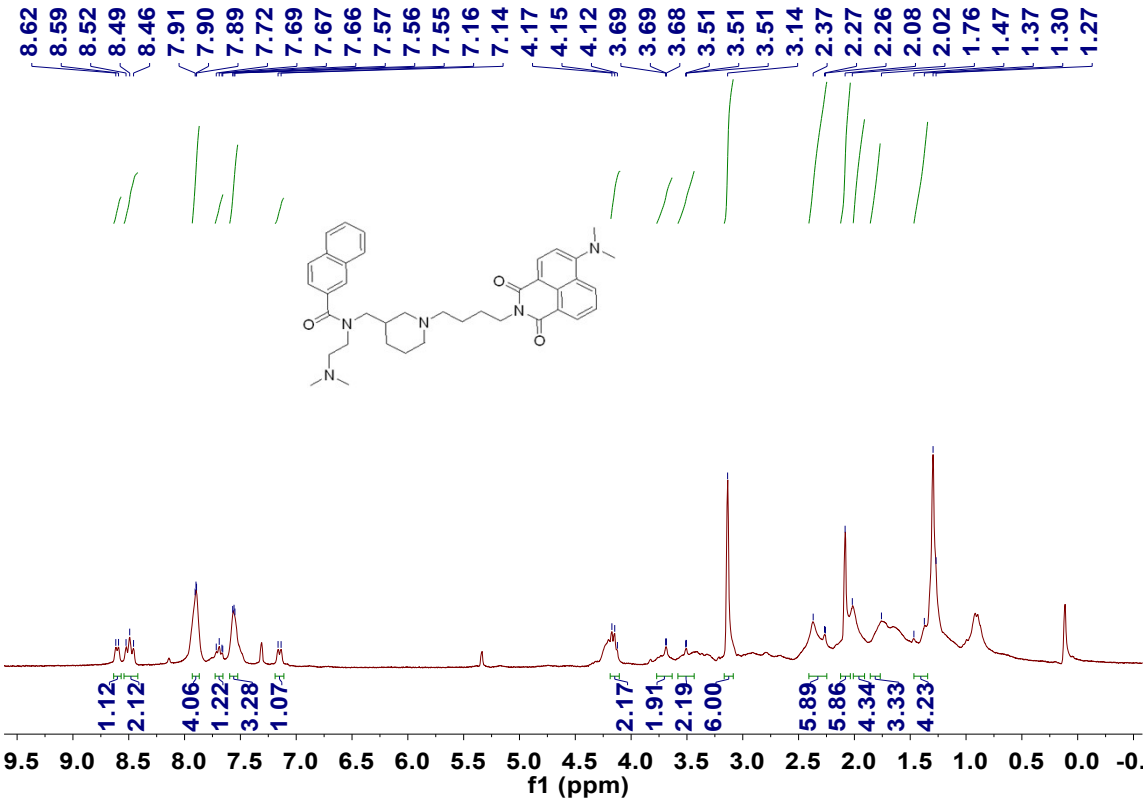

Fig. S20 ^{1}H NMR of **ESP4**

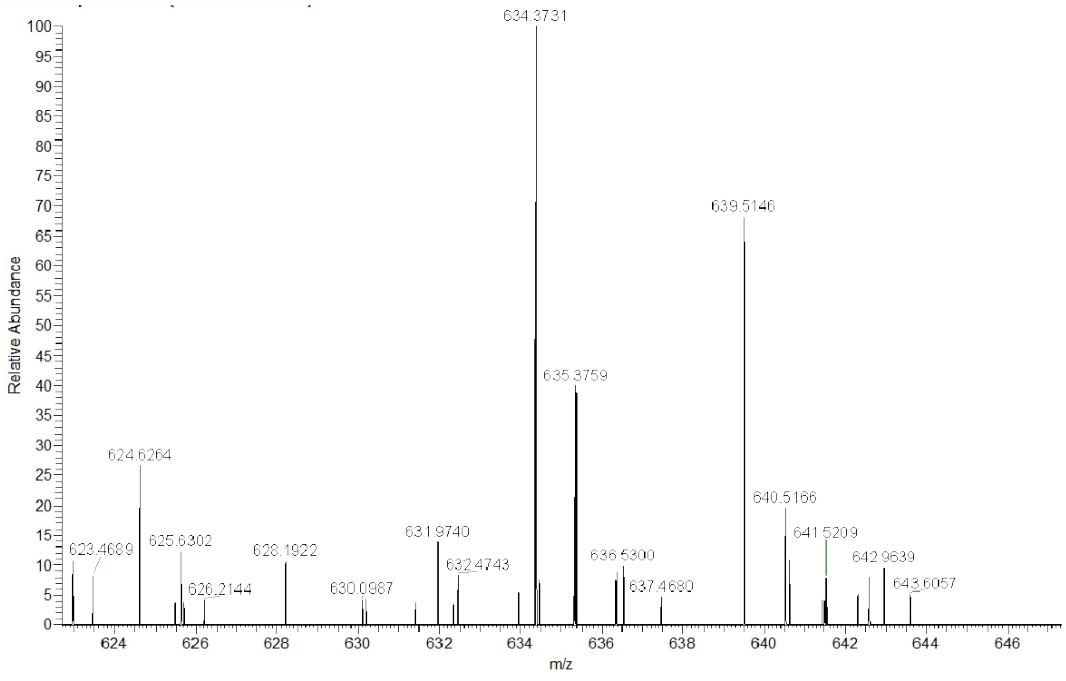

Fig. S21 ESI-HRMS of **ESP4**

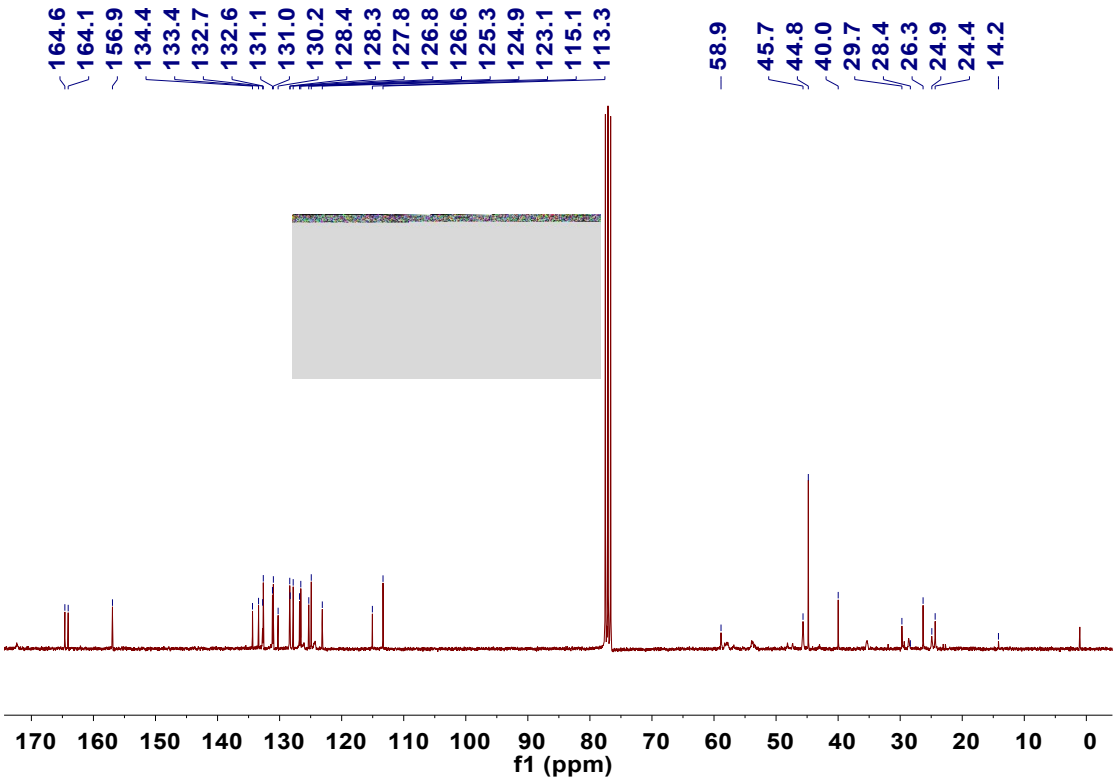

Fig. S22 ^{13}C NMR of **ESP4**

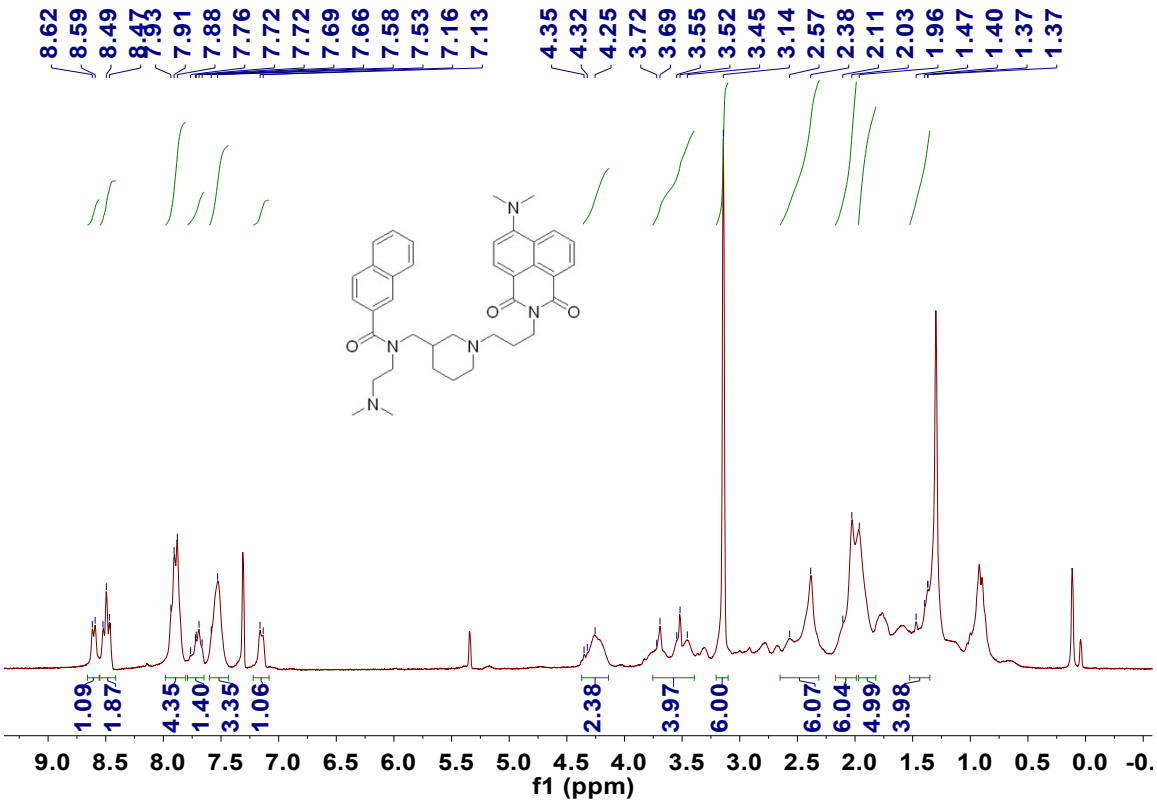

Fig. S23 ^{1}H NMR of **ESP5**

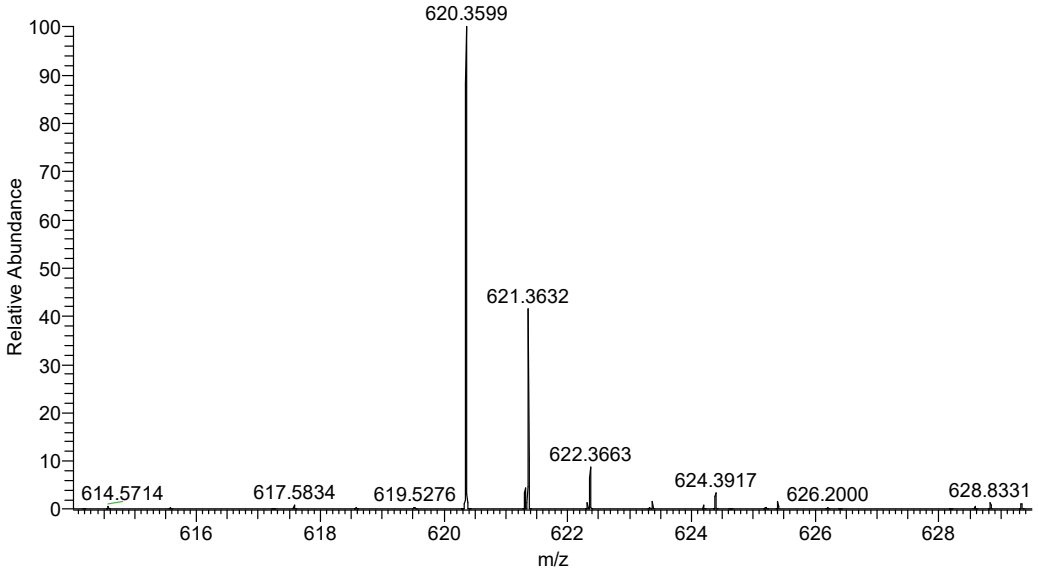

Fig. S24 ESI-HRMS of **ESP5**

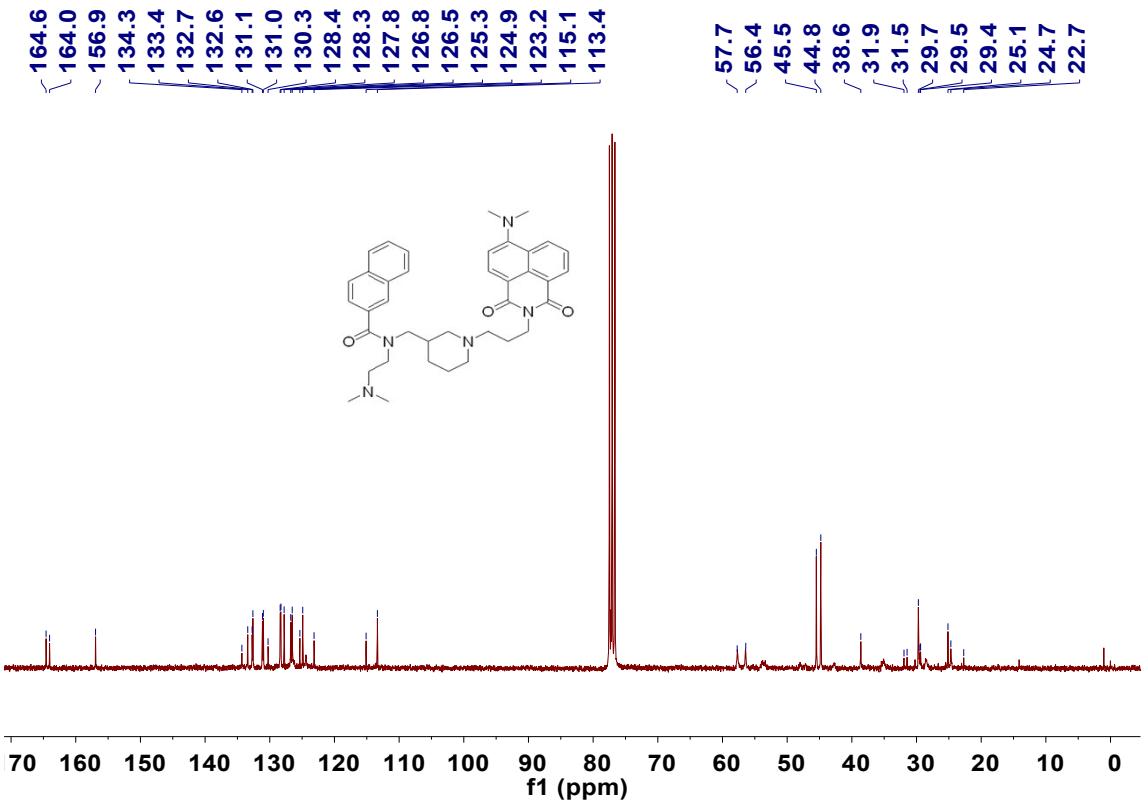

Fig. S25 ^{13}C NMR of **ESP5**

13. Reference

1. U. Kosak, B. Brus, D. Knez, S. Zakelj, J. Trontelj, A. Pislar, R. Sink, M. Jukic, M. Zivin, A. Podkowa, F. Nachon, X. Brazzolotto, J. Stojan, J. Kos, N. Coquelle, K. Salat, J. P. Colletier and S. Gobec, *J. Med. Chem.*, 2018, **61**, 119-139.
2. W. Cheng, Y. Xie, Z. Yang, Y. Sung, M.-Z. Zhang, Y. Ding and W. Zhang, *Anal. Chem.*, 2019, **91**, 5817-5823.
3. D. Knez, N. Coquelle, A. Pislar, S. Zakelj, M. Jukic, M. Sova, J. Mravljak, F. Nachon, X. Brazzolotto, J. Kos, J. P. Colletier and S. Gobec, *Eur J Med Chem*, 2018, **156**, 598-617.
4. D. Song, T. Ding, W. Zhai, L. Shao, N. Guo, L. Jiang, W. Zhang, F. Zhao, J. Wang, J. Wang, J. Ma and L. Yan, *Analyst*, 2023, **148**, 6325-6333.
5. M. Eugenio Vázquez, D. M. Rothman and B. Imperiali, *Org. Biomol. Chem.*, 2004, **2**, 1965-1966.
6. B. Wang, J. Shi, N. Guo, L. Shao, W. Zhai, L. Jiang, F. Zhao, J. Wang, J. Wang, L. Du, X. Pang and L. Yan, *Anal. Chim. Acta*, 2023, **1281**, 341900.