Supporting Information

Construction of a novel aminofluorene-based ratiometric near-infrared fluorescence probe for monitoring the activity of carboxylesterase in living cells

Jun-Mei Li,[#] Yan-Zhao Liu,[#] Xiao-Fang Lv, Ding-Heng Zhou, Hong Zhang, Yu-Jin Chen and Kun Li*

Key Laboratory of Green Chemistry and Technology of Ministry of Education, College of Chemistry, Sichuan University, 29, Wangjiang Road, Chengdu 610064, P. R. China.

Correspondence Author:
Email: *kli@scu.edu.cn (Kun Li)*

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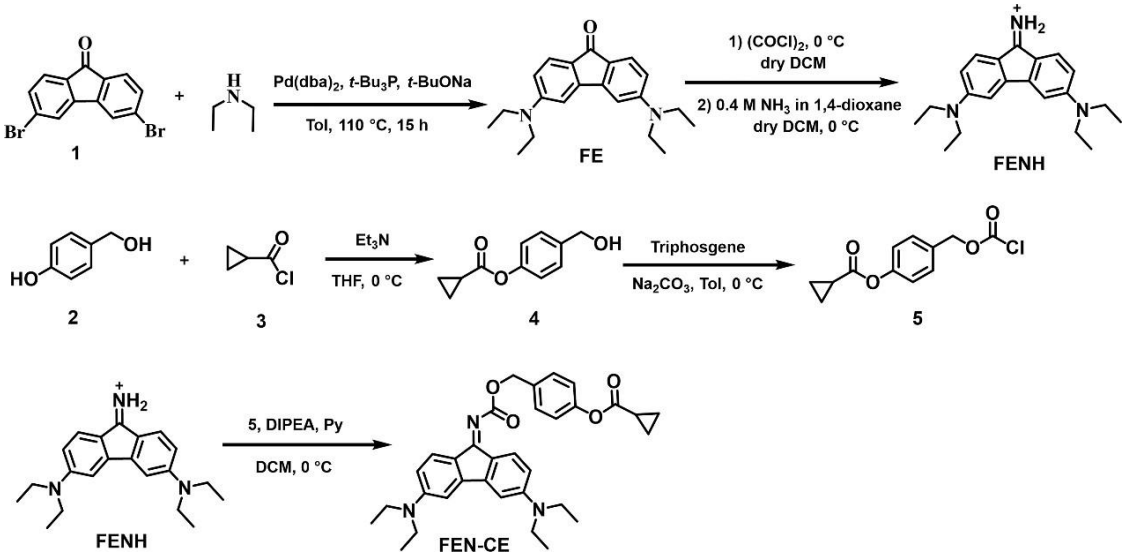
1. General Information

Unless otherwise noted, all materials were purchased from commercial suppliers and used without further purification. All solvents were dried according to the standard methods prior to use. In the optical spectroscopic studies, all of the solvents were either HPLC or spectroscopic grade. Except for the specially mentioned, all the reactions were monitored by thin layer chromatography (TLC) and were visualized under UV light. Column chromatography was carried out using 200-300 mesh silica gel (Qingdao Ocean Chemicals). NMR spectra were recorded on a Bruker AMX-400 spectrometer at 25 ℃ (^{1}H NMR: 400 MHz, ^{13}C NMR: 100 MHz) and chemical shifts (λ) are expressed in parts per million (ppm) using the internal standard tertramethylsilane or the deuterated solvent ($CDCl_3$, CD3OD, DMSO-d_6) as reference. Spin multiplicities in 1H NMR are reported as singlet (s), doublet (d), double doublet (dd), triplet (t), quartet (q), multiplet (m), or broad (br). The High-resolution mass spectra (HRMS) were obtained on a Finnigan LCQDECA. The pH values were determined by a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. UV absorption spectra were measured on a Horiba Duetta fluorescence and absorbance spectrometer. Fluorescence spectra were measured on a Hitachi F-7000. Cell imaging was performed on a Zeiss LSM 780 confocal laser scanning microscope. In addition, all cells (HepG2, PC12, B16 and HeLa) used for confocal imaging were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd.

2. Experimental Section

2.1 Synthesis and characterization



Scheme S1. Synthesis of **FENH** and **FEN-CE**.

Synthesis of **FE**:

The $Pd(dba)_2$ (86 mg, 5 mol %) and *t*-Bu_3P (10% w/v in Tol, 486 μL, 0.08 equiv.) were suspended in deoxygenated toluene (40 mL), and the 3,6-dibromo-*9H*-fluoren-9-one (**1**, 1.01 g, 3.0 mmol, 1.0 equiv.), diethylamine (483 mg, 6.6 mmol, 2.2 equiv.) and *t*- BuONa (0.86 g, 9.0 mmol, 3.0 equiv.) were added to the mixture under N_2 flow. Then the mixture was refluxed for 12 h under N_2. After the reaction was completed, the mixture was filtered on Celite and the residue was washed with DCM. The filtrate was removed under vacuum. The residue was purified by column chromatography on silica gel (PE: EA = 3: 1) to afford **FE** as a tan solid in 72% yield.

FE: ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.47 (d, *J* = 8.4 Hz, 2H), 6.72 (d, *J* = 2.3 Hz, 2H), 6.42 (dd, *J* = 8.5, 2.3 Hz, 2H), 3.47 (q, *J* = 7.1 Hz, 8H), 1.24 (t, *J* = 7.1 Hz, 12H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 191.2, 152.0, 146.2, 125.4, 123.6, 109.6, 102.3, 44.7, 12.8; HRMS (ESI): calcd for $C_{21}H_{27}N_2O$ $[M + H]^+$ 323.2118, found 323.2110.

Synthesis of **FENH**:

The compound **FE** (128.8 mg, 0.4 mmol, 1.0 equiv.) was dissolved in 15 ml dry

DCM and stirred at rt, and then the oxalyl chloride (20.0 equiv.) was added dropwise to the solution. After stirred for 30 min at rt, the mixture was concentrated under vacuum to afford the residue, which was dissolved in 12 mL dry DCM, and added to the dry DCM solution of NH_3 (0.4 M in 1,4-dioxane, 10 mL, 4.0 mmol, 10.0 equiv.). The mixture was stirred at room temperature for 1 h. After the reaction was completed, the solution was removed under vacuum. The residue was purified by column chromatography on silica gel (DCM: MeOH: Et_3N = 200: 2: 1) to afford **FENH** as a purple solid in 84% yield.

FENH: ^{1}H NMR (400 MHz, DMSO-d6) δ 10.87 (s, 2H), 7.82 (d, J = 8.8 Hz, 2H), 7.23 (d, J = 2.3 Hz, 2H), 6.47 (dd, J = 8.9, 2.2 Hz, 2H), 3.51 (q, J = 7.0 Hz, 8H), 1.13 (t, J = 7.0 Hz, 12H). 13C NMR (101 MHz, Methanol-d4) δ 154.3, 146.6, 127.2, 116.7, 109.4, 105.0, 44.7, 11.6. ESI-MS: [M]+ Calcd for 322.2278, Found: 322.2273.

Synthesis of **4**:

The compound **2** (3.1 g, 25.0 mmol, 1.0 equiv.) and triethylamine (10.5 mL, 75 mmol, 3.0 equiv.) were dissolved in 80 mL dry THF under an N_2 atmosphere. The mixture was stirred for 5 min at 0 °C, and then compound **3** (3.14 g, 30 mmol, 1.2 equiv.) was dropped into the mixture slowly at the temperature. After the reaction was completed, brine was added to the mixture. The product was extracted with ethyl acetate, and the organic layer was removed under vacuum. The residue was purified by column chromatography on silica gel (petroleum ether: ethyl acetate = 5: 1) to afford **4** as a colorless liquid in 91% yield.

4: ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.31 (dd, *J* = 8.5, 3.8 Hz, 2H), 7.03 (dd, *J* = 8.5, 2.8 Hz, 2H), 4.59 (d, *J* = 6.9 Hz, 2H), 2.25 (br, 1H), 1.84 - 1.78 (m, 1H), 1.20 - 1.07 (m, 2H), 1.02 -0.98 (m, 2H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 173.7, 150.0, 138.4, 128.0, 121.6, 64.6, 13.0, 9.3.

Synthesis of **FEN-CE**:

Triphosgene (441.0 mg, 1.5 mmol) and Na_2CO_3 (720.0 mg, 6.8 mmol) were added in 10 mL toluene under an N_2 atmosphere. The mixture was stirred at 0 ℃for 30 min , and then the compound **4** (288.0 mg, 1.5 mmol) in 2 mL toluene was dropped into the mixture. After stirring for 6 h at room temperature, the solution was slowly added into the dry DCM solution of **FENH** (48.0 mg, 0.15 mmol), DIPEA (130 μL, 0.75 mmol),

pyridine (121 μL, 1.5 mmol). The mixture was stirred at room temperature for 12 h. After the reaction was completed, the solution was removed under vacuum. The residue was purified by column chromatography on silica gel (PE: DCM: Et_3N = 200: 40: 1) to afford **FEN-CE** as an orange solid in 68% yield.

FEN-CE: ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.50 (d, *J* = 8.2 Hz, 2H), 7.27 - 7.22 (m, 2H), 7.09 (d, *J* = 8.0 Hz, 2H), 6.70 (s, 2H), 6.33 (d, *J* = 8.3 Hz, 2H), 5.32 (s, 2H), 3.44 (q, *J* = 7.0 Hz, 8H), 1.87 - 1.80 (m, 1H), 1.21 (t, *J* = 7.0 Hz, 12H), 1.16 – 1.13 (m, 2H), 1.04 – 0.99 (m, 2H). ^{13}C NMR (101 MHz, Chloroform-d) δ 173.3, 164.8, 163.9, 151.2, 150.7, 145.0, 133.4, 130.1, 126.2, 121.6, 109.8, 102.2, 67.3, 44.6, 12.9, 12.7, 9.2. ESI-MS: $[M + H]^+$ Calcd for 540.2857, Found: 540.2841.

2.2 Absorption and fluorescence spectra

The stock solutions of **FEN-CE** and **FENH** were prepared for 5 mM by DMSO. The excited wavelengths of **FEN-CE** and **FENH** were 480 nm and 580 nm, respectively.

2.3 Theoretical calculations

The optimal structure of **FENH** was based on its single crystal (by the slow solvent evaporation of the mixed chloroform and hexane). The optimal structure of **FEN-CE** was optimized by Gaussian 09 package[1] with a DFT PBE0/6-31g (d,p) basis set at the SMD water model. Their frontier orbital energies were calculated by the same methods, and the results were processed by Multifwn[2] and VMD[3].

2.4 Determination of the detection of limit

The detection of limit (LOD) was determined by the fluorescence titration of carboxylesterase (CE) in the presence of **FEN-CE**. The stock solution of **FEN-CE** was diluted to 10 μM in PBS buffer (pH 7.4), and incubated with various of concentrations of CE (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.6, 1, 2, 3, 4, 5, 6, 8, 10 U/mL) separately for 3 h at 37 °C. Then, the fluorescence spectra of **FEN-CE** and **FENH** were measured by Hitachi F-7000. The ratio of the fluorescence intensities of **FEN-CE** at 650 nm and **FENH** at 680 nm (I_{680}/I_{650}) was fitted to a linear relationship with enzyme concentration. The LOD was measured by the equation: LOD = 3σ/k, where σ is the standard deviation of the blank fluorescence of probe without CE, k is the slope of the fluorescence signal versus CE concentrations.

2.5 Enzymatic Kinetics Assays

Various concentrations (0, 1, 2, 4, 6, 8, 10 U/mL) of CE were separately added to PBS buffer containing 10 μM **FEN-CE**, and then the I_{680}/I_{650} was detected at various incubation times (0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 180 min).

2.6 Michaelis constant:

arious concentrations of **FEN-CE** (2, 4, 6, 8, 10, 12, 14 μM) in PBS buffer were added with CE (30 U/mL) separately, and then the I_{680}/I_{650} was recorded every 30 seconds for 7 times. Relative initial reaction velocity (V) of every concentration was counted according to the consumption of **FEN-CE** (here is expressed as **[FEN-CE]**) and the reaction time. Then the relative velocity was plotted against the concentrations of the probe and fitted to the Michaelis-Menten curve by origin. The kinetic parameters were calculated by the following formula (1)[4]:

$$V = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1)$$

Among them, [S] represents the concentration of **FEN-CE**, K_m represents the Michaelis constant, and V_{max} represents the maximum rate of the enzymatic reaction. V could be calculated through the linear relationship between **[FEN-CE]** and response time, and the formula (2) was as follows:

$$V = [\boldsymbol{FEN} - \boldsymbol{CE}]/t \quad (1)$$

2.7 Selectivity assay

Each species (10 U/mL CE, AChE, and BChE; 100 μg/mL lipase, chymotrypsin, trypsin, cellulase, elastase, lysozyme, and BSA; 100 μM H_2O_2, ClO^-, $\cdot OH$, $O_2^{\bullet-}$, $ONOO^-$, *t*- BuOOH, GSH, Hcy, Cys, Fe^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, and Ca^{2+}) were administered to 10 μM **FEN-CE** in PBS buffer. After 3-hour incubation at 37 °C, the I_{680}/I_{650} was measured by Hitachi F-7000.

2.8 Molecular docking

Protein preparation: the crystal structure of CE was downloaded from Protein Data Bank (PDB code: 1mx5). The original ligand and water were removed by Discovery Studio for docking studies.

Ligand (probes) preparation: The structures of **FEN-CE** and **FENH** for docking studies were based on the optimized structure by Gaussian 09 and the crystal structure,

respectively.

The docking calculations were performed by the AutoDock Vina suite of programs[5]. The displaying images were rendered with PyMOL[6].

2.9 Analysis of stability

The stock solution of **FEN-CE** and **FENH** were diluted to 10 μM with different pH (3.0~9.0) of PBS buffer solutions, and then the fluorescence spectra of **FEN-CE** and **FENH** were measured and normalized under various of PBS solutions to explore the properties of **FEN-CE** and **FENH** under different pH conditions. Besides, the working solution of **FEN-CE** and **FENH** (10 μM) was incubated at 37 ℃ for 0-5 h and different temperatures (25 ℃-60 ℃) for 5 h to explore the thermo-stability of **FEN-CE** and **FENH,** respectively.

2.10 Cell culture

HepG2 and HeLa cells were cultured in DMEM containing 10% fetal bovine serum and 1% Antibiotic-antimycotic at 37 °C in a 5% CO_2/95% air incubator. PC12 and B16 cells were cultured in RPMI Medium 1640 basic containing 10% fetal bovine serum and 1% Antibiotic-antimycotic at 37 °C in a 5% CO_2/95% air incubator.

2.11 Cell viability assay

Toxicity toward HepG2 and PC12 cells was determined by CCK-8 assay. About 10000 cells per well were seeded in 96-well plates and cultured overnight. The medium was replaced with 100 μL of fresh medium with different concentrations of probes. 24 hours later, 100 μL serum-free medium containing 10% CCK-8 solution replaced the old medium in each well for additional about 30 min incubation. The absorbance at 450 nm was measured using a microplate reader (Spark). The metabolic activity of the probes- treated cells was expressed as a relative to untreated cell controls taken as 100% metabolic activity.

2.12 Cell imaging

Cells were grown on a coverslip overnight in a 35-mm petri dish. The cells were stained with a certain dye (1 mM stock solution) at a certain concentration for a certain time. The cells were imaged under CLSM using proper excitation and emission filters for **FEN-CE**: λ_{ex} = 488 nm, λ_{em} = 500-630 nm; and for **FENH**: λ_{ex} = 630 nm, λ_{em} = 680-

740 nm.

HepG2 cells were first fixed with 99% cold methanol for 1 minute, and then washing twice with PBS, further treated with working solutions **FEN-CE** (4 μM) and the different concemtions exogenous of CE (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 6.0 U/mL), which had been cultured in PBS for 3 hours, for 10 min to perform confocal imging.

3. Supplementary figures

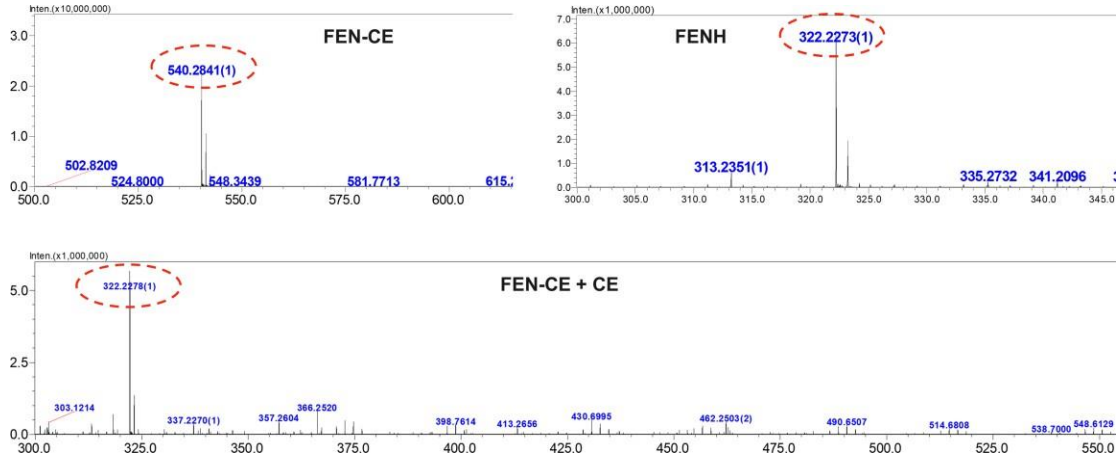


Fig. S1 HRMS of **FEN-CE**, **FENH**, and **FEN-CE** incubated with CE.

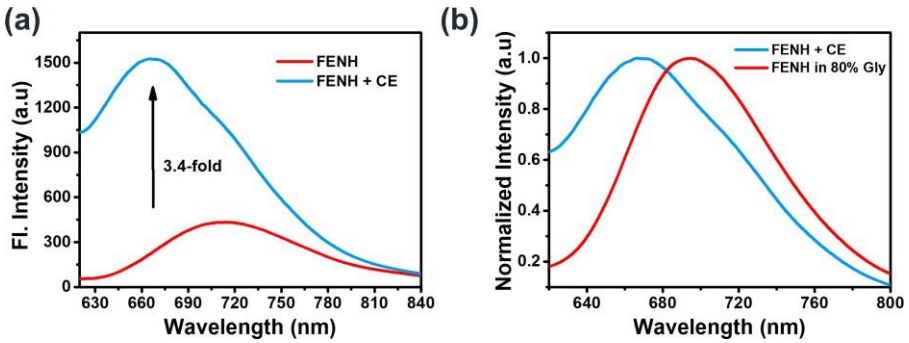


Fig. S2 (a) Fluorescence spectra of **FENH** (10 μM) incubated with or without CE (10 U/mL) in PBS buffer (pH 7.4). (b) Normalized fluorescence spectra of **FENH** (10 μM) incubated with CE (10 U/mL) or 80% glycerin (Gly) in PBS buffer (pH 7.4).

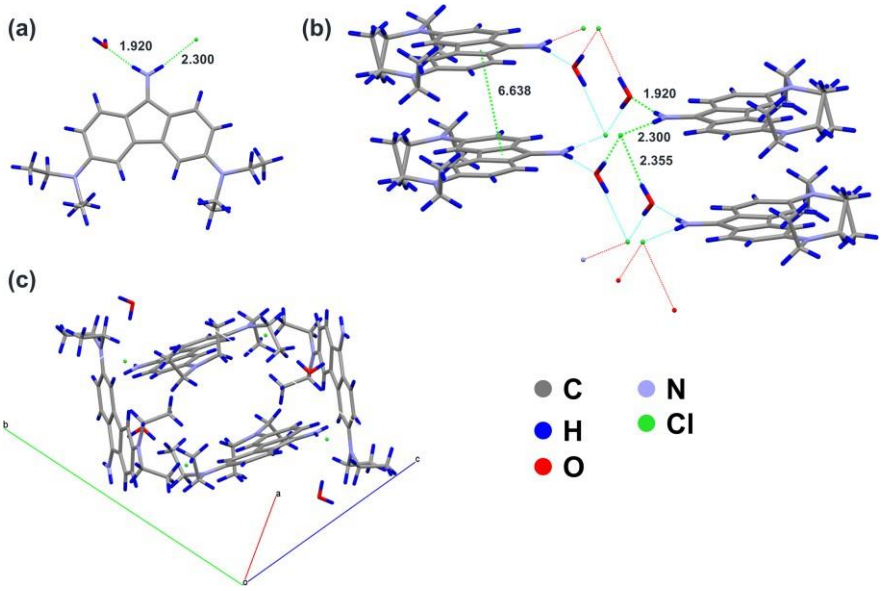


Fig. S3 The crystal structure of **FENH** and the non-covalent intermolecular interaction among them.

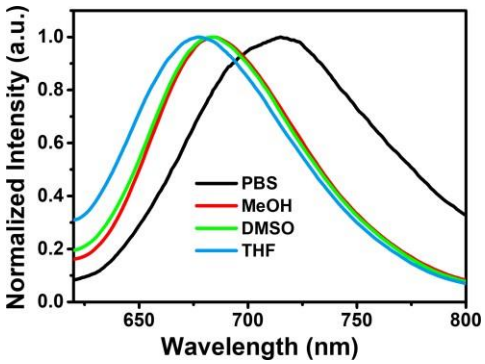


Fig. S4 Normalized fluorescence spectra of **FENH** (10 μM) in varied solvents (MeOH, DMSO, PBS, and THF).

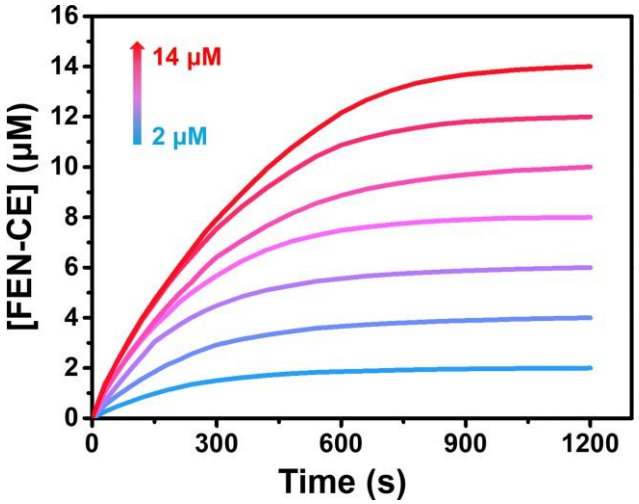


Fig. S5 Plot of **FEN-CE** (2 μM, 4 μM, 6 μM, 8 μM, 10 μM, 12 μM, 14 μM) consumption (**[FEN-CE]**) incubated with 10 U/mL CE vs the response time.

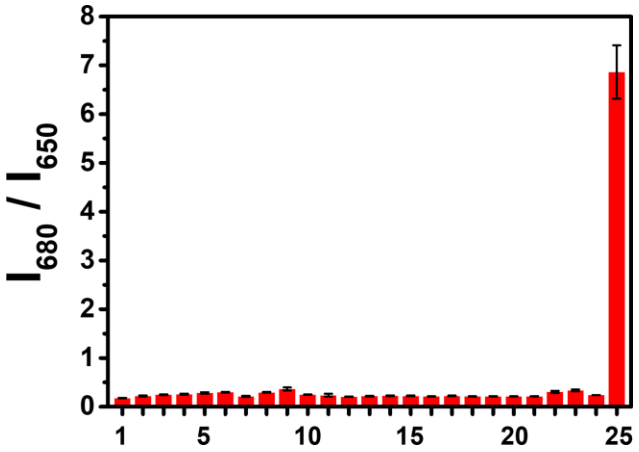


Fig. S6 Fluorescence intensity ratio (I_{680}/I_{650}) of **FEN-CE** (10 μM) response to numerous analytes including: (1) Blank, (2) AChE, (3) BChE, (4) lipase, (5) chymotrypsin, (6) trypsin, (7) cellulase, (8) elastase, (9) lysozyme, (10) BSA, (11) H_2O_2, (12) ClO^-, (13) •OH, (14) $O_2^{\bullet-}$, (15) $ONOO^-$, (16) *t*-BuOOH, (17) GSH, (18) Hcy, (19) Cys, (20) Fe^{2+}, (21) Mg^{2+}, (22) Cu^{2+}, (23) Zn^{2+}, (24) Ca^{2+}, and (25) CE.

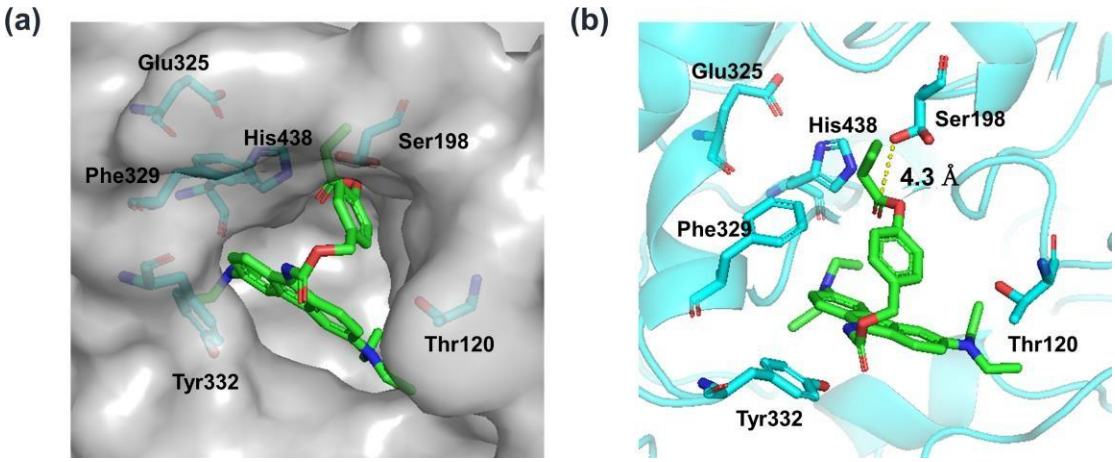


Fig. S7 Simulated binding models of **FEN-CE** with BChE (PDB code: 1p0m) in surface type (a) and in cartoon type (b).

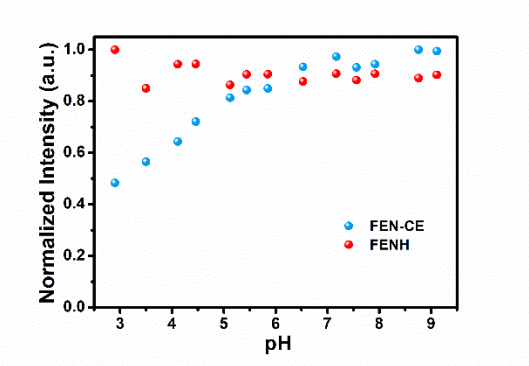


Fig. S8 Normalized fluorescence intensity of **FEN-CE** and **FENH** in PBS solution with different pH values.

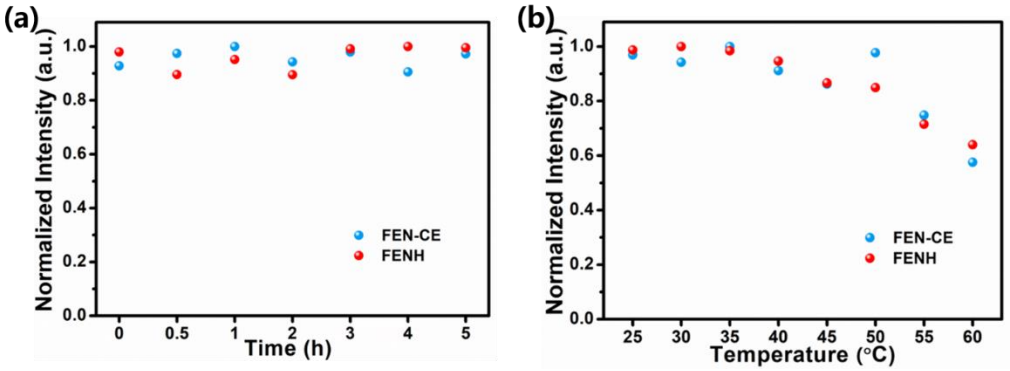


Fig. S9 (a) Normalized fluorescence intensity of **FEN-CE** and **FENH** in PBS solution of pH 7.4 with different times at 37 ℃. (b) Normalized fluorescence intensity of **FEN-CE** and **FENH** in PBS solution of pH7.4 with different temperatures.

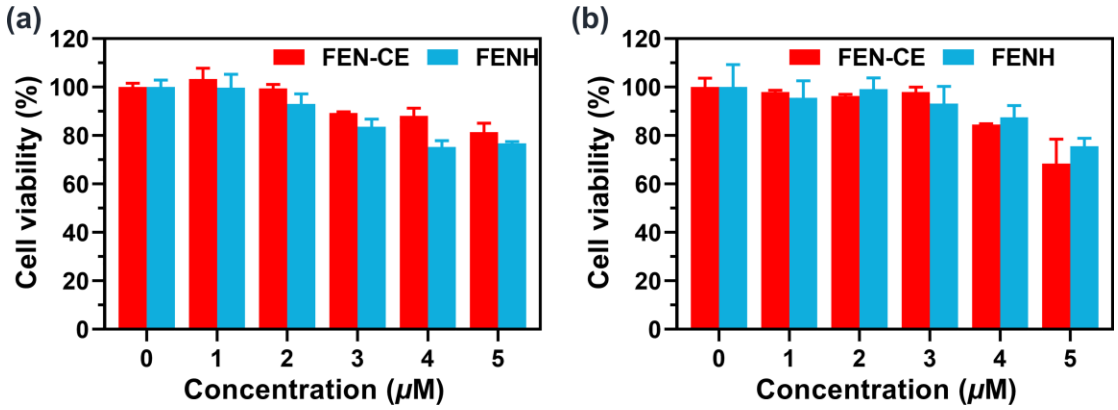


Fig. S10 Cell viability of PC12 cells (a) and HepG2 cells (b) incubated with **FEN-CE** or **FENH** for 24 h.

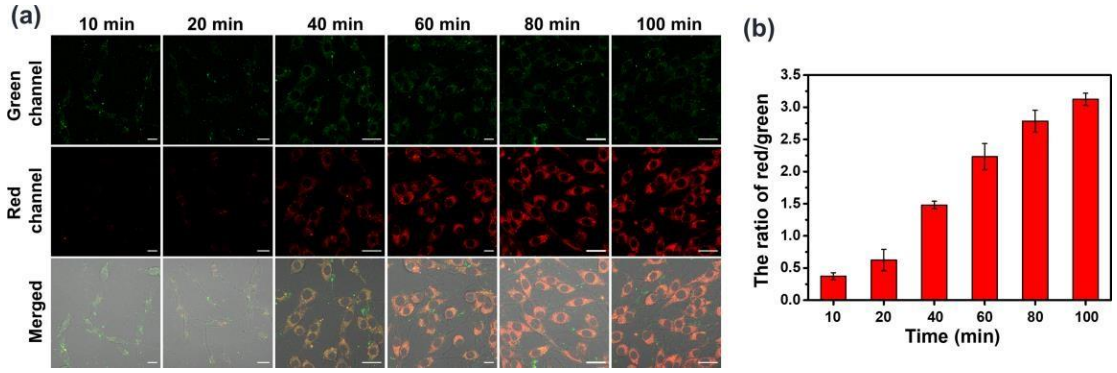


Fig. S11 (a) Confocal images of PC12 cells incubated with **FEN-CE** (4 μM) at 37 °C for different time in 1640 culture medium. (b) The fluorescence intensity ratio of red to green at different incubation time in a, n = 6. Scale bar: 20 μm.

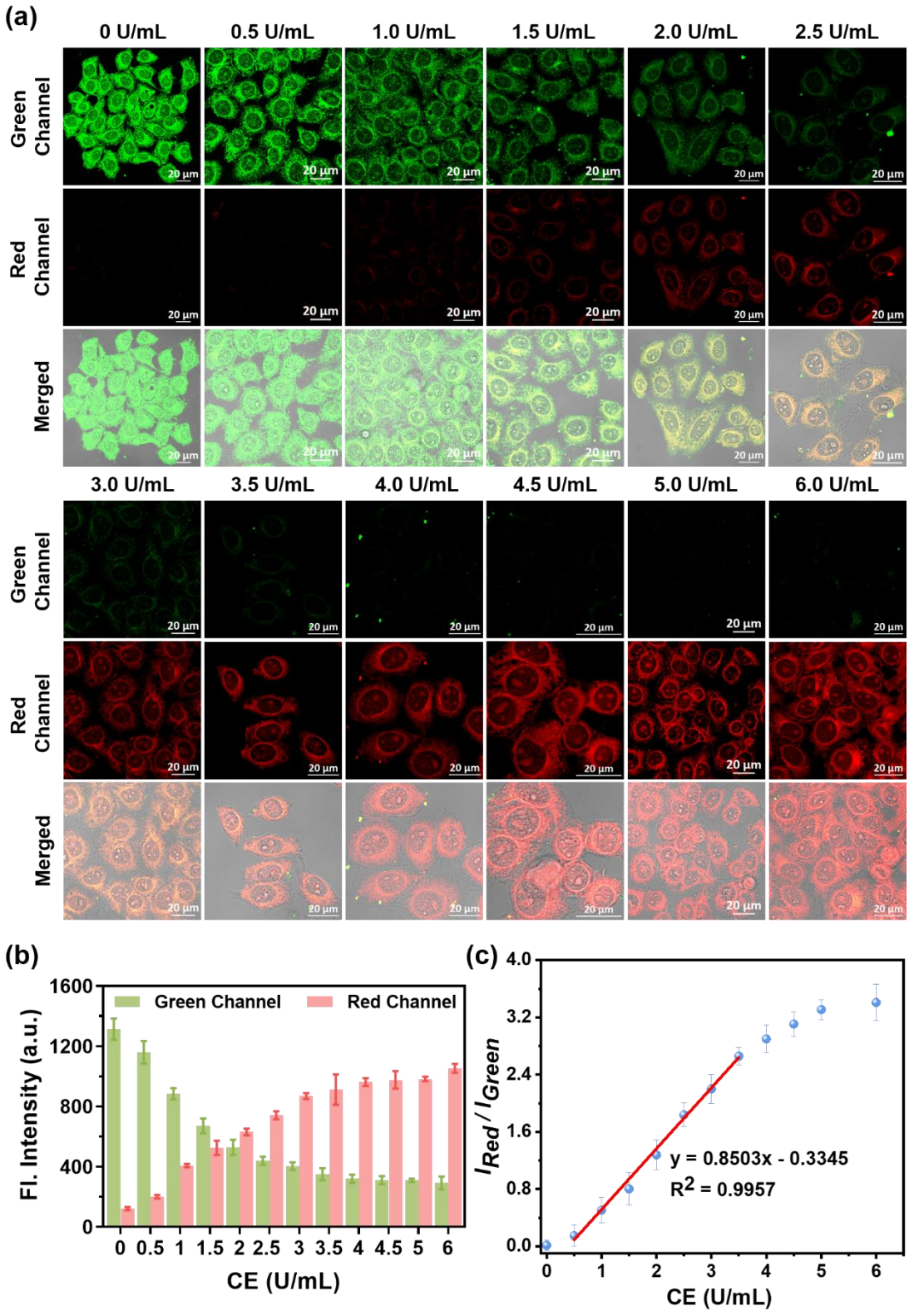


Fig. S12 (a) The confocal images of MeOH-fixed cells (HepG2) being further incubated with **FEN-CE** (4 μM) and diffenert concentions exogenous CE (after pretreated in PBS solution for 3 h at 37 °C) for 10 min . Green channel: λex = 488 nm, λem = 500-630 nm; Red channel: λex = 633 nm, λem = 680-740 nm. (b) The fluorescence intensity of green and red channels in a, n=6. (c) The fluorescence intensity ratio of red to green in a, n = 6. Scale bar: 20 μm.

Table 1 Crystal data and structure refinements of **FENH**

Empirical formula	$C_{21}H_{30}ClN_3O$
Formula weight	375.93
Temperature/K	191.0
Crystal system	monoclinic
Space group	$P2_1/c$
a/Å	6.6375(5)
b/Å	16.2977(10)
c/Å	18.8687(14)
α/°	90
β/°	95.082(3)
γ/°	90
Volume/Å^3	2033.1(2)
Z	4
ρ_{calc}g/cm^3	1.228
μ/mm^{-1}	0.203
F(000)	808.0
Crystal size/mm^3	0.43 × 0.22 × 0.18
Radiation	MoKα (λ = 0.71073)
2Θ range for data collection/°	5.004 to 49.994
Index ranges	$-7 \le h \le 7$, $-19 \le k \le 19$, $-22 \le l \le 22$
Reflections collected	28653
Independent reflections	3563 [R_{int} = 0.1115, R_{sigma} = 0.0567]
Data/restraints/parameters	3563/0/242
Goodness-of-fit on F^2	1.039
Final R indexes [I>=2σ (I)]	R_1 = 0.0449, wR_2 = 0.0967
Final R indexes [all data]	R_1 = 0.0699, wR_2 = 0.1121
Largest diff. peak/hole / e Å^{-3}	0.22/-0.25

CCDC number: 2250441

4. ^{1}H and ^{13}C NMR Spectra

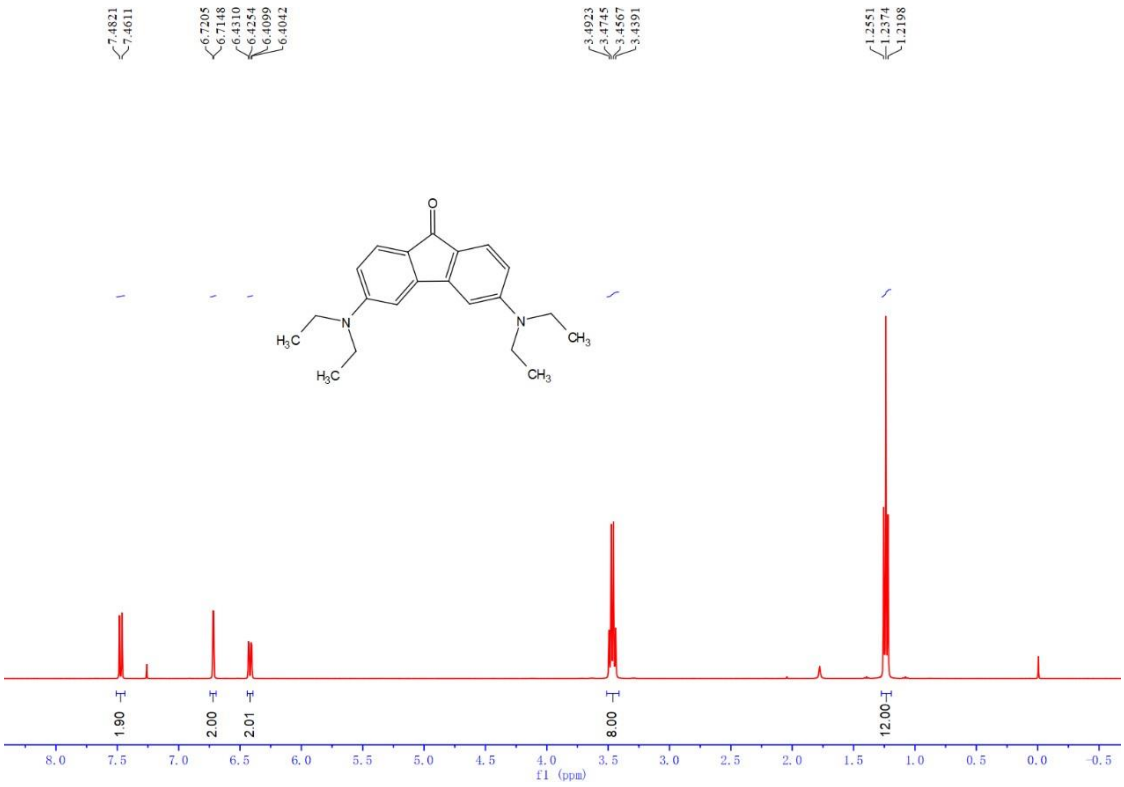


Fig. S13 ^{1}H NMR of Compound **FE** in $CDCl_3$.

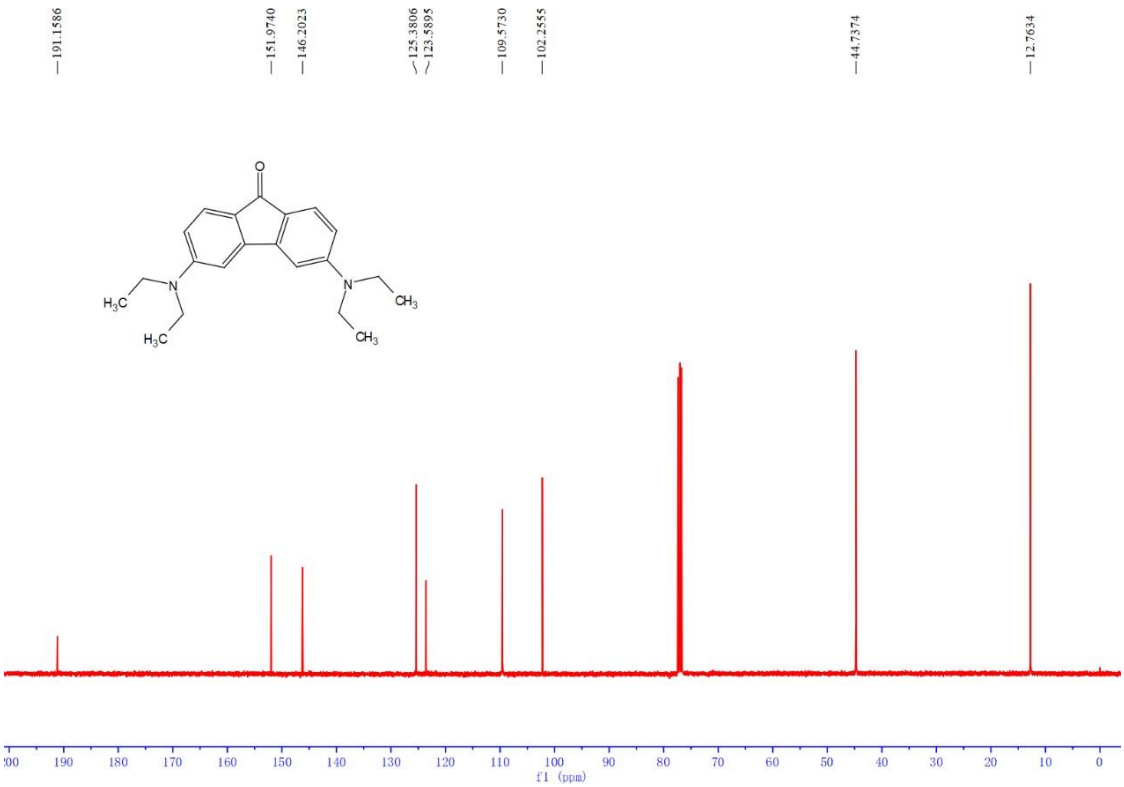


Fig. S14 ^{13}C NMR of Compound **FE** in $CDCl_3$.

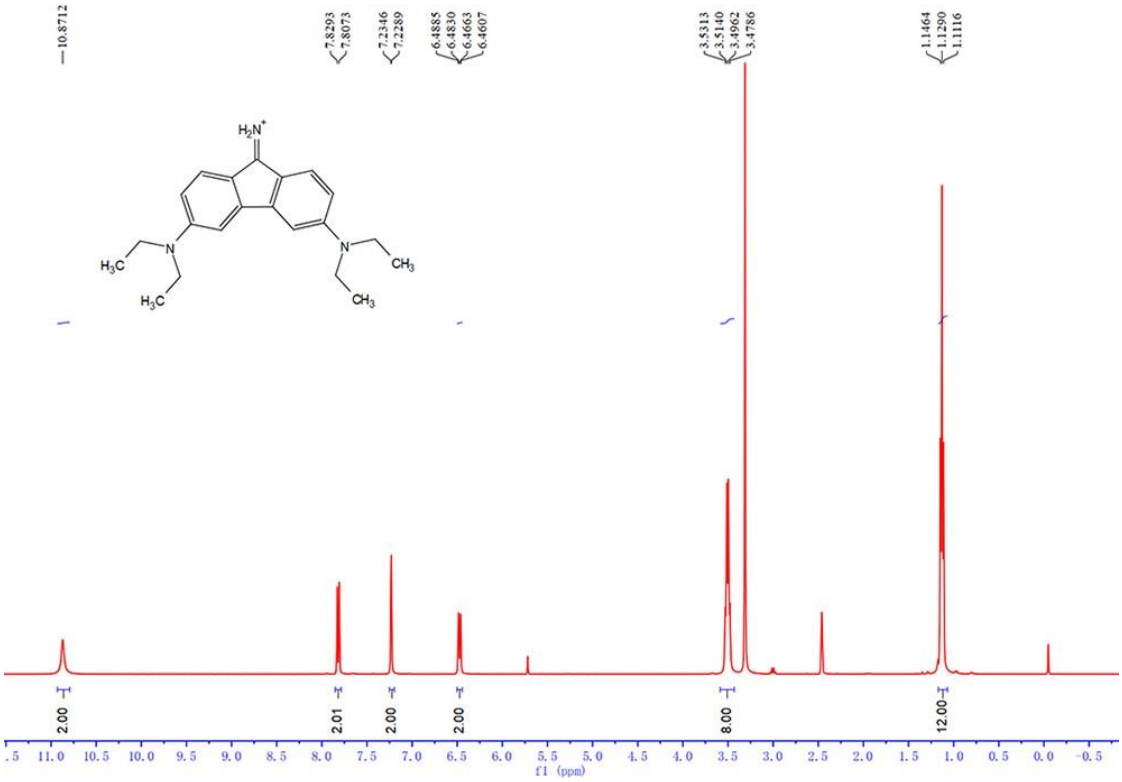


Fig. S15 ^{1}H NMR of Compound **FENH** in DMSO-d_6.

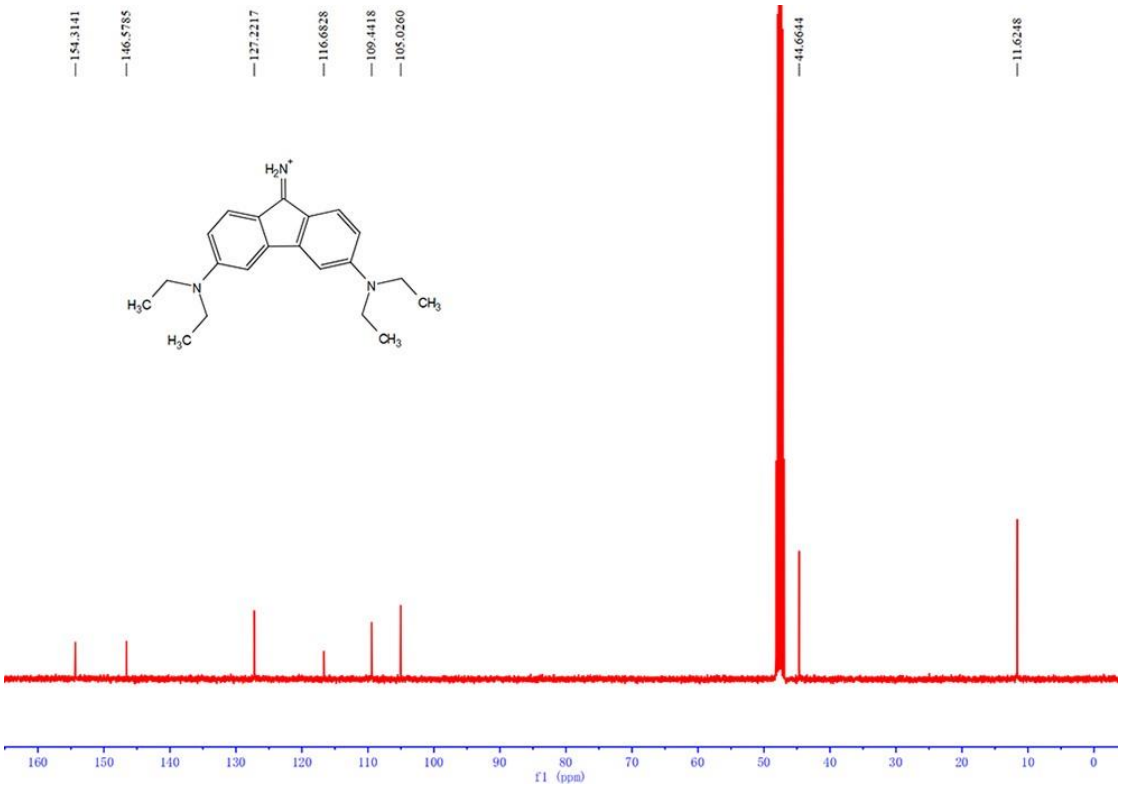


Fig. S16 ^{13}C NMR of Compound **FENH** in CD_3OD.

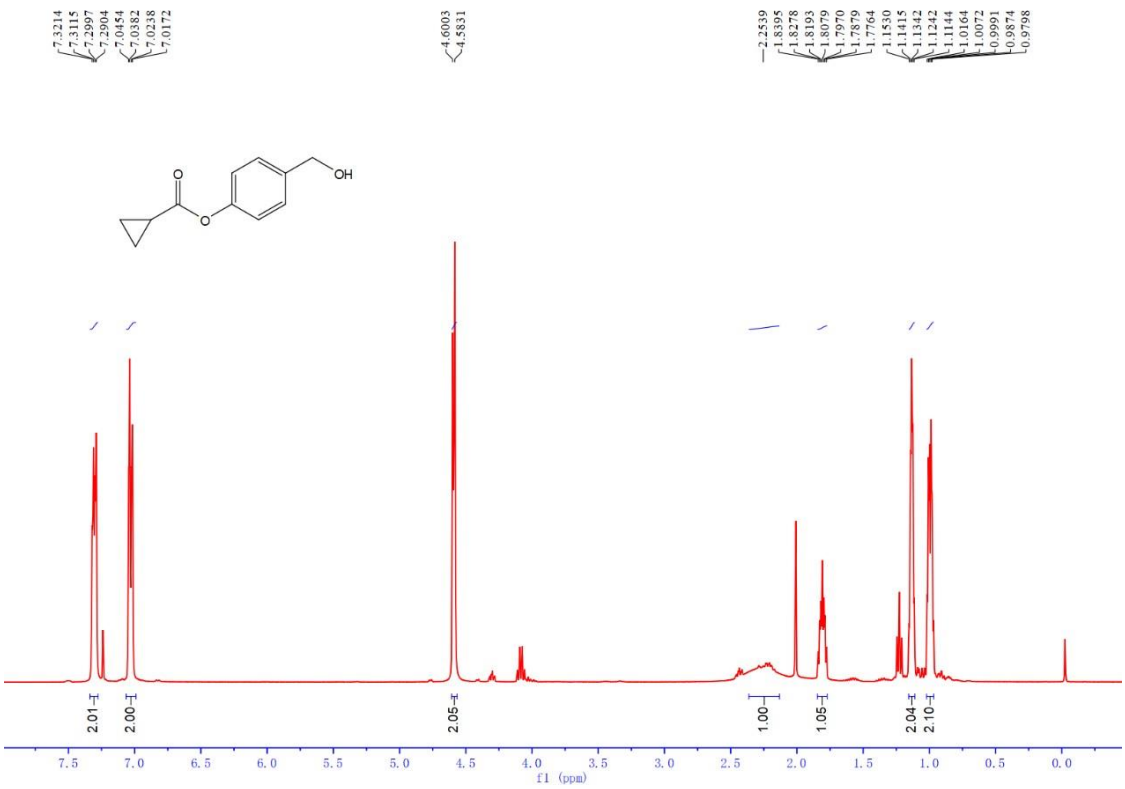


Fig. S17 ^{1}H NMR of Compound **4** in $CDCl_3$.

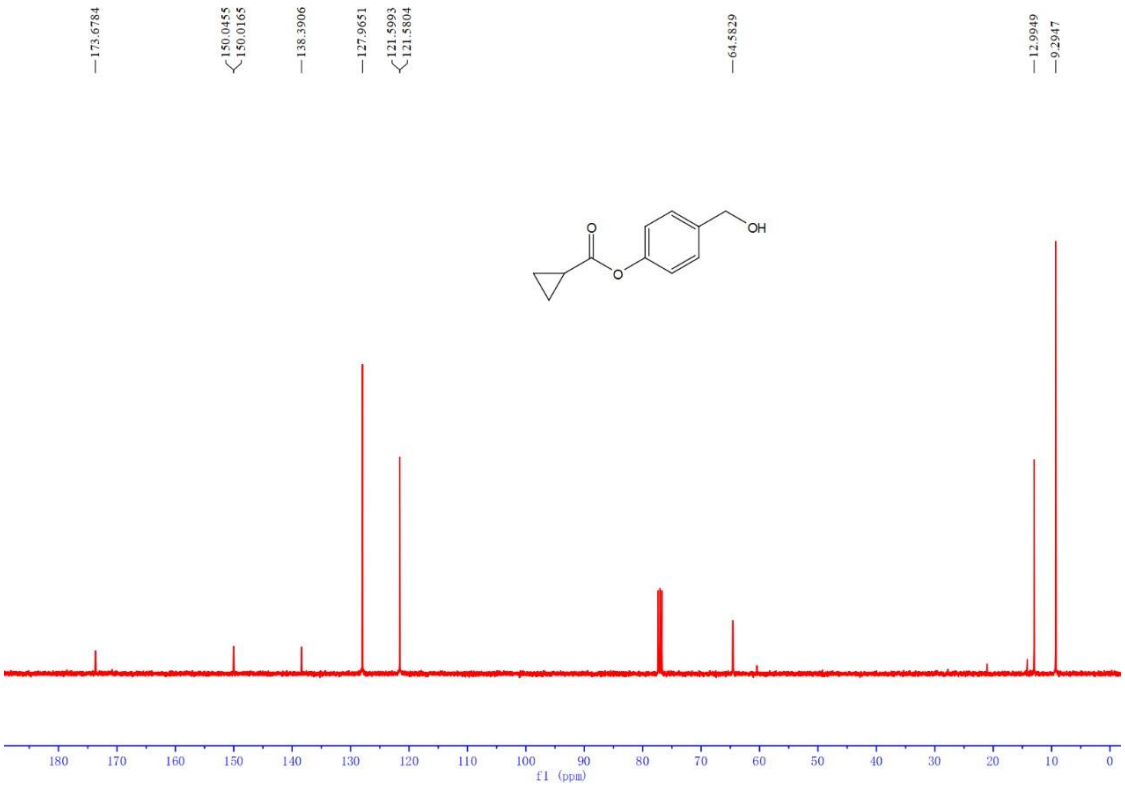


Fig. S18 ^{13}C NMR of Compound **4** in $CDCl_3$.

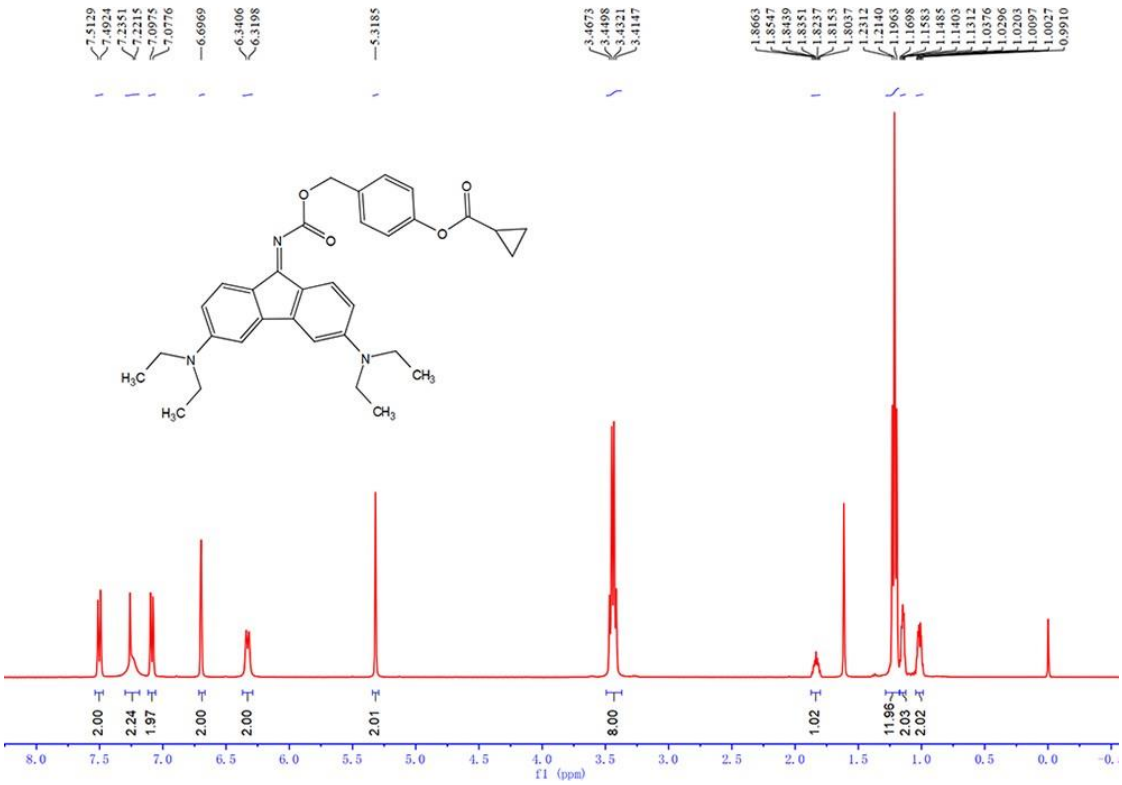


Fig. S19 ^{1}H NMR of Compound **FEN-CE** in $CDCl_3$.

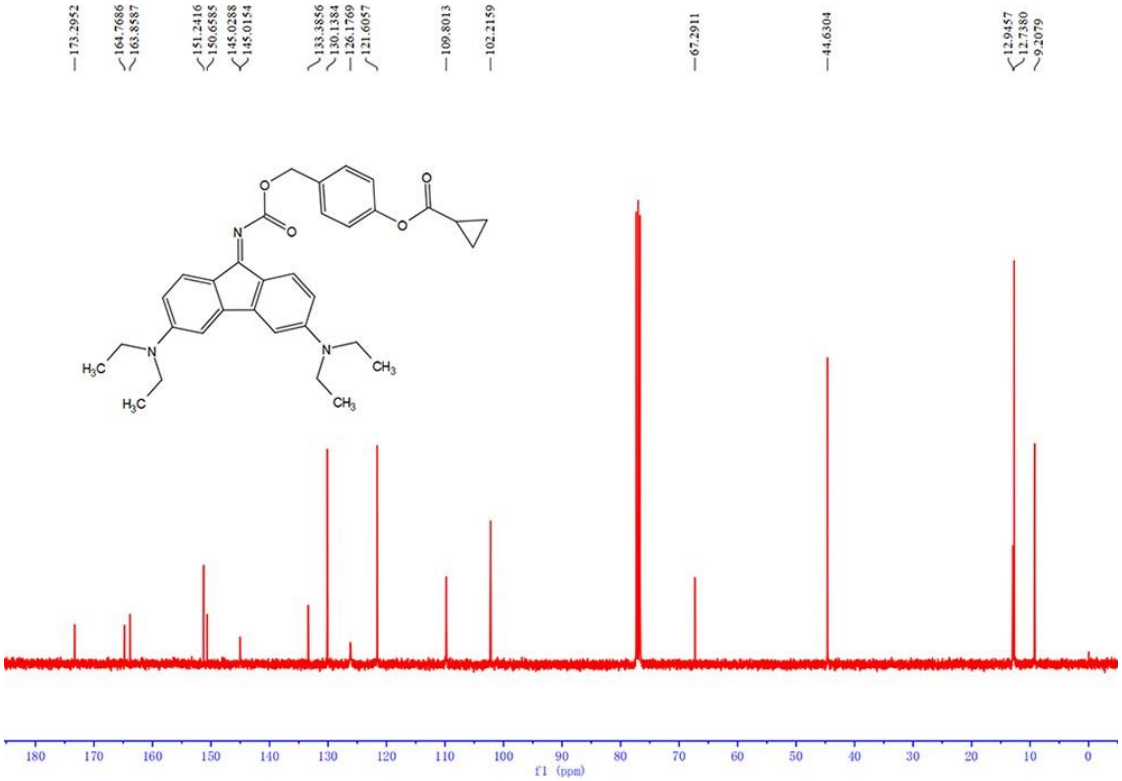


Fig. S20 ^{13}C NMR of Compound **FEN-CE** in CDCl3.

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