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Supporting information

A near-infrared fluorescent probe based on hemi-cyanine skeleton for detecting CES1 activity and evaluating pesticide toxicity

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Experimental section methods

Synthesis

The synthetic route of **CHC-COOH** and **CHC-CES1** was showed in **Scheme S2**.

Compound 1: 15.22 g (100 mmol) 4-hydrazinobenzoic acid and 12.93 g (150 mmol) 3-methyl-2-butanone were added to a 500 mL flask, then 250 mL ethanol was added to that flask. With string, 2.5 mL sulfuric acid was added dropwise to the flask and then the flask was kept at 90 ℃ overnight. After finishing of the reaction confirmed by TLC, the mixture was cooled to room temperature and then was added to cool water. Precipitate was obtained by filtration and washed with EA and water for several times to afford **compound 1** as drab yellow solid (11.38 g, yield: 56%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.97 (s, 1H), 7.90 (d, J = 8.0 Hz, 1H), 7.49 (d, J = 8.0 Hz, 1H), 2.24 (s, 3H), 1.26 (s, 6H). ^{13}C NMR (126 MHz, DMSO) δ 191.59 (s), 167.48 (s), 157.37 (s), 146.05 (s), 129.60 (s), 127.29 (s), 122.68 (s), 119.07 (s), 53.42 (s), 22.22 (s), 15.27 (s). HRMS (ESI positive) calcd for $[M+H]^+$ 204.1019, found 204.1016.

Compound 2: 4.06 g (20 mmol) **compound 1** and 12.55 g (100 mmol) 2-bromoethanol were added to a 250 mL flask with 100 mL acetonitrile as solvent. The mixture was kept refluxed at 90 ℃ for a week. After cooling to room temperature, 250 mL diethyl ether was added to the mixture and precipitate was obtained by filtration and washed with EA and water for several times to afford **compound 2** as light pink power (2.95 g, yield: 45%) and **compound 2** was used in next step without further purification. ^{1}H NMR (500 MHz, MeOD) δ 8.38 (s, 1H), 8.28 (d, J = 8.4 Hz, 1H), 8.04 (d, J = 8.5 Hz, 1H), 4.92 (s, 3H), 4.80 – 4.72 (m, 2H), 4.15 – 4.02 (m, 2H), 1.69 (s, 6H). ^{13}C NMR (126 MHz, MeOD) δ 202.64 (s), 168.09 (s), 145.73 (s), 143.66 (s), 133.65 (s), 132.23 (s), 125.72 (s), 117.02 (s), 59.56 (s), 56.41 (s), 52.22 (s), 49.96 (s), 22.90 (s). HRMS (ESI positive) calcd for $[M]^+$ 248.1281, found 248.1278.

Compound 3: 10.6 g 4-(diethylamino) salicylaldehyde (55 mmol) and 17.6 g diethyl malonate (110 mmol) were dissolved in 110 mL ethanol, and 2 mL piperidine was added and refluxed at 90 ℃ for 3 h. After the reaction completion, the solvent was removed under reduced pressure. Then the reactant was dissolved in 50 mL acetic acid mixed with 50 mL of concentrated hydrochloric acid and refluxed at 130 ℃ for 3 h. At the end of the reaction, the reaction solution was added into ice water, and the pH was adjusted with 40% sodium hydroxide solution until a large amount of solid were precipitated. Then, precipitate was obtained by filtration to afford **compound 3** as yellow solid (10.2 g, yield :85%).^{1}H NMR (600 MHz, $CDCl_3$) δ 7.53 (d, J = 9.3 Hz, 1H), 7.28 – 7.20 (m, 1H), 6.56 (dd, J = 8.8, 2.4 Hz, 1H), 6.49 (d, J = 2.3 Hz, 1H), 6.03 (d, J = 9.3 Hz, 1H), 3.41 (q, J = 7.1 Hz, 4H), 1.21 (t, J = 7.1 Hz, 6H). ^{13}C NMR (126 MHz, CDCl3) δ 161.49 (s), 156.41 (s), 150.35 (s), 143.63 (s), 128.74 (s), 108.41 (s), 107.88 (s), 96.72 (s), 44.36 (s), 12.14 (s). HRMS (ESI positive) calcd for $[M+H]^+$ 218.1176, found 218.1174.

Compound 4:10 ml phosphorus trichloride was mixed with 10 ml DMF and

heated at 50 °C for 0.5 h. Then 4.4 g **compound 3** (20 mmol) was dissolved in 20 mL DMF and added to the reaction solution dropwise under nitrogen protection and heated at 50 °C for 5 h. At the end of the reaction, the reaction solution was added into ice water, and the pH was adjusted with 20% sodium hydroxide solution until a large amount of solid was precipitated. Then the solution was filtered and dried to obtain **compound 4** as a yellow solid (3.3 g, yield: 67%).^{1}H NMR (600 MHz, $CDCl_3$) δ 10.13 (s, 1H), 8.26 (s, 1H), 7.41 (d, J = 9.0 Hz, 1H), 6.64 (d, J = 9.0 Hz, 1H), 6.49 (s,1H), 3.48 (q, J = 7.1 Hz, 5H), 1.26 (t, J = 7.1 Hz, 7H). ^{13}C NMR (126 MHz, CDCl3) δ 187.63 (s), 161.70 (s), 158.81 (s), 153.49 (s), 145.30 (s), 132.50 (s), 114.00 (s), 110.26 (s), 108.10 (s), 96.97 (s), 45.25 (s), 12.42 (s). HRMS (ESI positive) calcd for $[M+H]^+$ 246.1125, found 246.1122.

Compound CHC-COOH:8.5 g **compound 2** (26 mmol) and 3.2 g **compound 4** (13 mmol) were dissolved in 10 ml tetrahydrofuran and 40 ml ethanol, then 7.7 g ammonium acetate (100 mmol) was added, and the mixture was heated at 50 ℃ for 24 h. At the end of the reaction, the solvent was removed under reduced pressure and the mixture was purified by silica gel chromatography (dichloromethane: methanol = 20:1), to obtain compound **CHC-COOH** as blue solid (1.2 g, yield: 17%). HRMS (ESI positive) calcd for $[M]^+$ 475.2227, found 475.2216.

Compound CHC-CES1:110 mg **compound 5** (0.2 mmol) was dissolved in 20 mL methanol, then 1 mL sulfuric acid was added and the mixture was refluxed for 5 h. At the end of the reaction, the solvent was removed under reduced pressure and the mixture was purified by silica gel chromatography (dichloromethane: methanol = 50:1), to afford compound **CHC-CES1** as blue solid (33 mg, yield: 29%). ^{1}H NMR (400 MHz, MeOD) δ 8.58 (s, 1H), 8.43 (d, J = 15.6 Hz, 1H), 8.35 (s, 1H), 8.26 (d, J = 8.5 Hz, 1H), 8.10 (d, J = 15.6 Hz, 1H), 7.84 (d, J = 8.5 Hz, 1H), 7.62 (d, J = 9.1 Hz, 1H), 6.96 (dd, J = 9.3, 1.6 Hz, 1H), 6.69 (s, 1H), 4.69 – 4.63 (m, 2H), 4.10 (t, J = 4.5 Hz, 2H), 3.99 (s, 3H), 3.66 (dd, J = 14.1, 7.0 Hz, 4H), 1.91 (s, 6H), 1.31 (t, J = 7.0 Hz, 6H).^{13}C NMR (101 MHz, MeOD) δ 184.24 (s), 165.93 (s), 160.00 (s), 158.46 (s), 155.20 (s), 152.21 (s), 150.55 (s), 145.12 (s), 143.28 (s), 132.69 (s), 130.56 (s), 129.93 (s), 123.44 (s), 114.25 (s), 112.51 (s), 111.54 (s), 110.48 (s), 109.94 (s), 96.58 (s), 58.64 (s), 51.71 (s), 51.65 (s), 45.22 (s), 39.01 (s), 25.83 (s), 11.43 (s). HRMS (ESI positive) calcd for $[M+H]^+$ 489.2384, found 489.2374.

Materials. Various hydrolases including: Carboxylesterase 1B (CES1B), Carboxylesterase 1C (CES1C), Carboxylesterase 2 (CES2), Dipeptidyl peptidase 4 (DPP4), Human Serum Albumin (HSA), β-Glucuronidase (β-Glu), β-galactosidase (β-GAL), Acetylcholinesterase (AchE), Bovine Serum Albumin (BSA), Carbonic Anhydrase (CAS), α-Glucosidase (α-Glc) were purchased from Sigma Aldrich. Amino acids: serine (Ser), tryptophan (Try), tyrosine(Tyr),glutamine (Glu), glycine (Gly), arginine (Arg), cysteine(Cys), glutathione(GSH), lysine (Lys), and glutamic acid (Gln) were obtained from Shanghai Yuanye. Inhibitors including Ketoconazole (TKZ), Bis(4-nitrophenyl) phosphate (BNPP), 27-Hydroxycholesterol (27-OH), Loperamide

(LPA), Alogliptin, Irosutat were purchased from Shanghai Yuanye. HepG2 and LoVo cells were obtained from ATCC. The fluorescence tests were analyzed on a Synergy H1 multimode microplate reader (BioTek). HRMS detection was measured on AB Sciex X500R. NMR spectra analysis were acquired in Bruker advance 600 and 400.

Fluorescence response of CHC-CES1 toward CES1. The activity assay of CES1 was performed in the standard incubation system containing 100 mM potassium phosphate buffer (pH 7.4), CES1 (40 μg/mL) and **CHC-CES1** (10 μM), with a final incubation volume of 200 μL. After incubating at 37 °C for 30 min, 100 μL acetonitrile was added to terminate the reaction and followed by centrifugation, the supernatant was obtained to analyze using microplate reader and HPLC. Next, the standard curve of CES1 enzyme activity toward **CHC-CES1** was constructed. Brifly, **CHC-CES1** (10 μM) were incubated in the presence of different concentrations of CES1B (1, 2, 3, 4, 5, 6, 7, 8, 9 μg/mL) and CES1C (1, 2, 3, 4, 5, 6, 7 μg/mL) at 37 ℃ for 30 min, respectively. At last, the fluorescence signal response of **CHC-CES1** toward CES1B and 1C was obtained, then the regression curve was obtained.

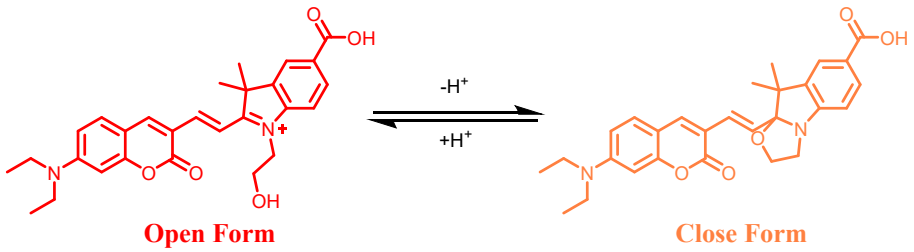
Selectivity and stability of CHC-CES1 toward CES1. For the selectivity assaying, **CHC-CES1** was incubated separately with different hydrolases: CES1B, CES1C, DPP4, HSA, *β*-Glu, *β*-GAL, AchE, BSA, CAS, CES2, *α*-Glc. For the chemical inhibition, different inhibitors including TKZ (1 μM), BNPP (10 μM), 27-OH (10 μM), LPA (50 μM), alogliptin (1 μM), irosutat (1 μM) were co-incubated with **CHC-CES1** in HLM incubation system. At last, the remaining activity was calculated by comparing with the control group (blank solvent instead of inhibitors).

Additionally, to examine the stability of **CHC-CES1**, the following common metal ions (final concentrations were 10 μM): Mg^{2+}, Ni^{2+}, Fe^{3+}, Ca^{2+}, K^{+}, Mn^{2+}, Zn^{2+}, Sn^{4+}, Cu^{2+}, Ba^{2+}, CO_3^{2-}, SO_4^{2-} , Na^{+} and endogenous amino acids (final concentrations were 200 μM): serine (Ser), tryptophan (Try), tyrosine(Tyr),glutamine (Glu), glycine (Gly), arginine (Arg), cysteine(Cys), glutathione(GSH), lysine (Lys), and glutamic acid (Gln) were co-incubated with **CHC-CES1** at 37 °C for 30 min and analyzed the fluorescence change to evaluate the stability of the probe.

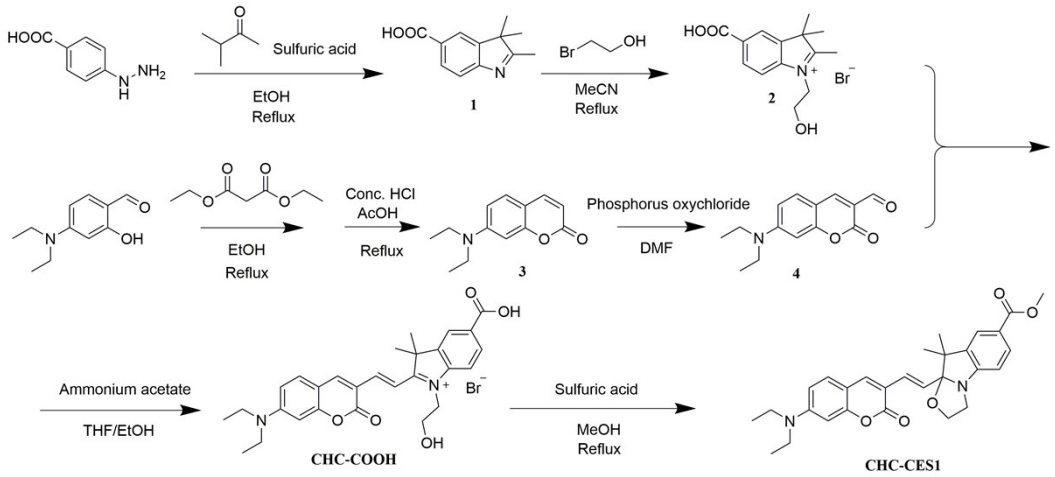
Fluorescence imaging application. In brief, HepG2 cells were seeded on glass polylysine-coated confocal dishes. After attached for over night, Fresh culture medium containing **CHC-CES1** was added to cells with the final concentration at 10 μM and incubated at 37 °C for 30 min. Meanwhile, for inhibition group, **BNPP** (CES inhibitor, 20 μM) was pretreated for 30 min and added the probe as description avove. After incubation, the cells was washed using blank culture medium three times to remove the residual **CHC-CES1** and imaged on the confocal microscope (Leica TCS SP8). The imaging condition was as follow: excitation: 630 nm, collection: 645 − 700 nm.

Pesticides inhibition assay. Single, binary and ternary pesticides were prepared (final concentrations according **Table.S1**). For CES1 inhibition measurement, HLM (40 μg/mL) was incubated separately with different pesticide group at 37 ℃ for 3 min.

Next, **CHC-CES1** (10 μM) was added to pesticides pre-incubated solution and co-incubated at 37 ℃ for another 30 min, at last, 100 μL cold acetonitrile was added to stop reaction, and fluorescence intensity was measured, and remaining activity was calculated by comparing with the control group (without pesticides).



Scheme S1. Intramolecular spirocyclization behavior of **CHC-COOH** with pH change.



Scheme S2. The synthetic route of **CHC-COOH** and **CHC-CES1**.

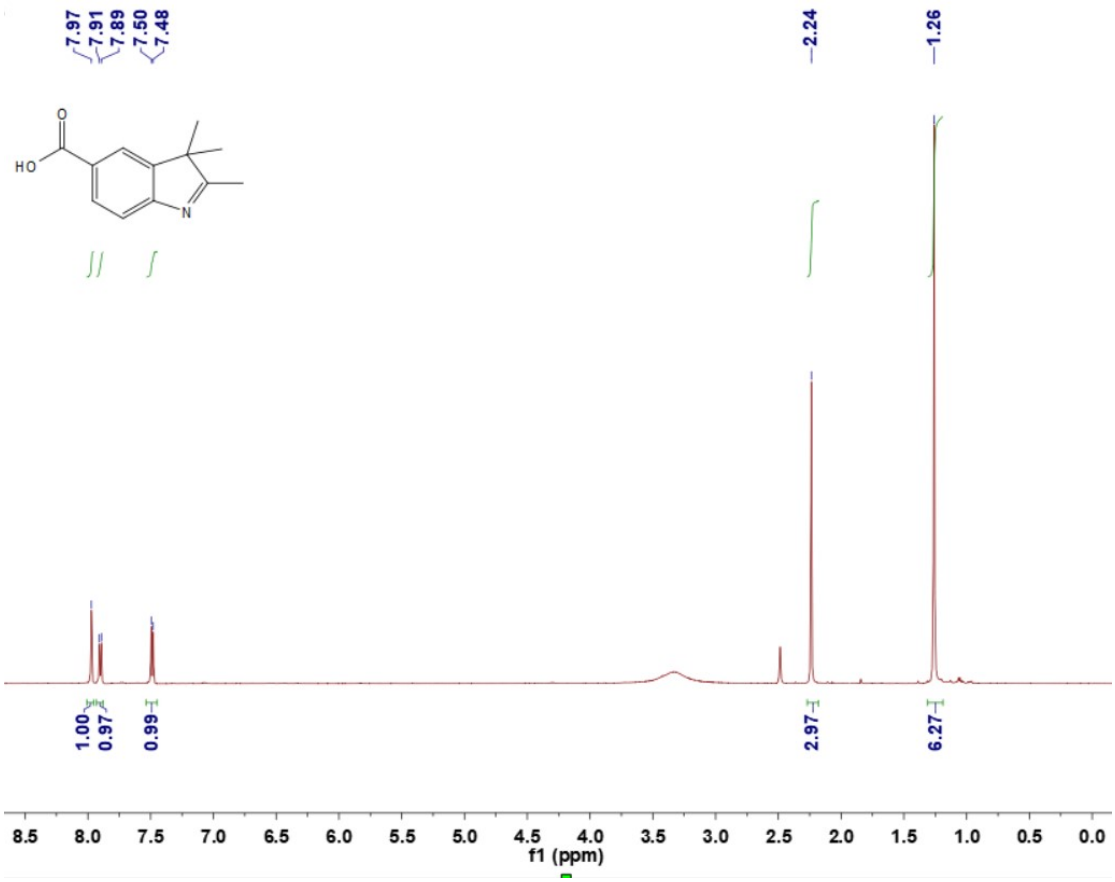


Fig. S1. [1]H NMR spectrum of **1** in DMSO-*d6*.

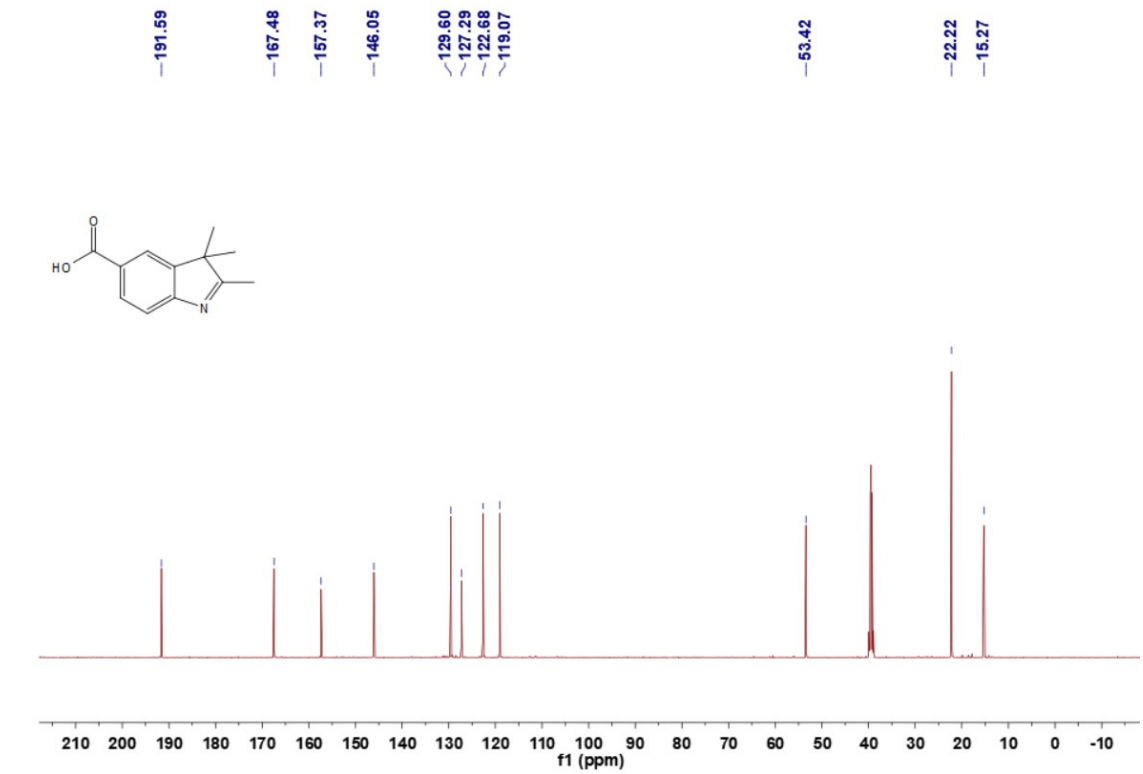


Fig. S2. [13]C NMR spectrum of **1** in DMSO-*d6*.

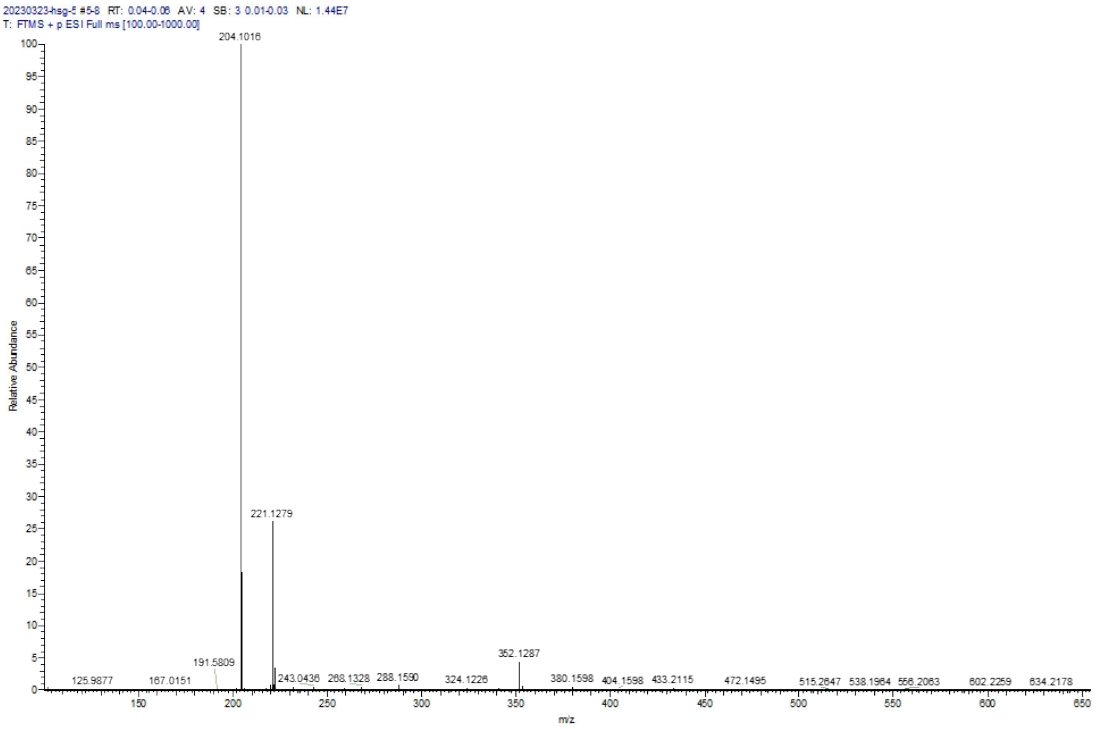


Fig. S3. HRMS of **1**.

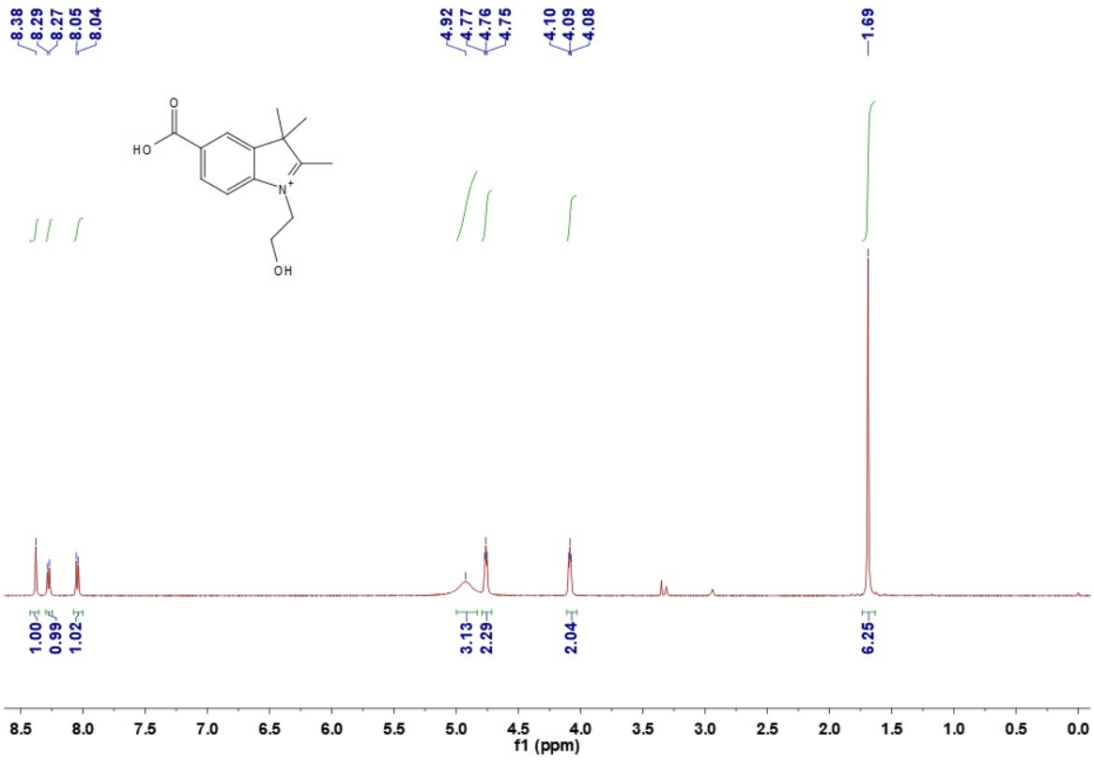


Fig. S4. ^{1}H NMR spectrum of **2** in DMSO-*d6*.

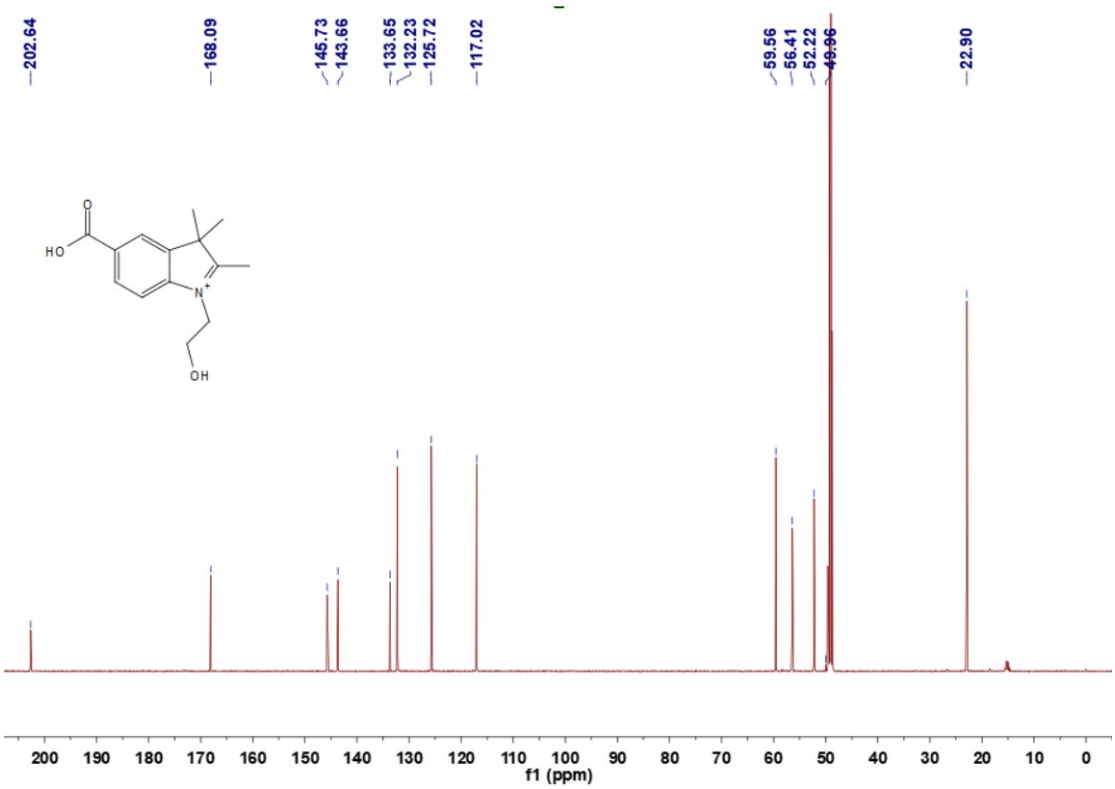


Fig. S5. ^{13}C NMR spectrum of **2** in DMSO-*d6*.

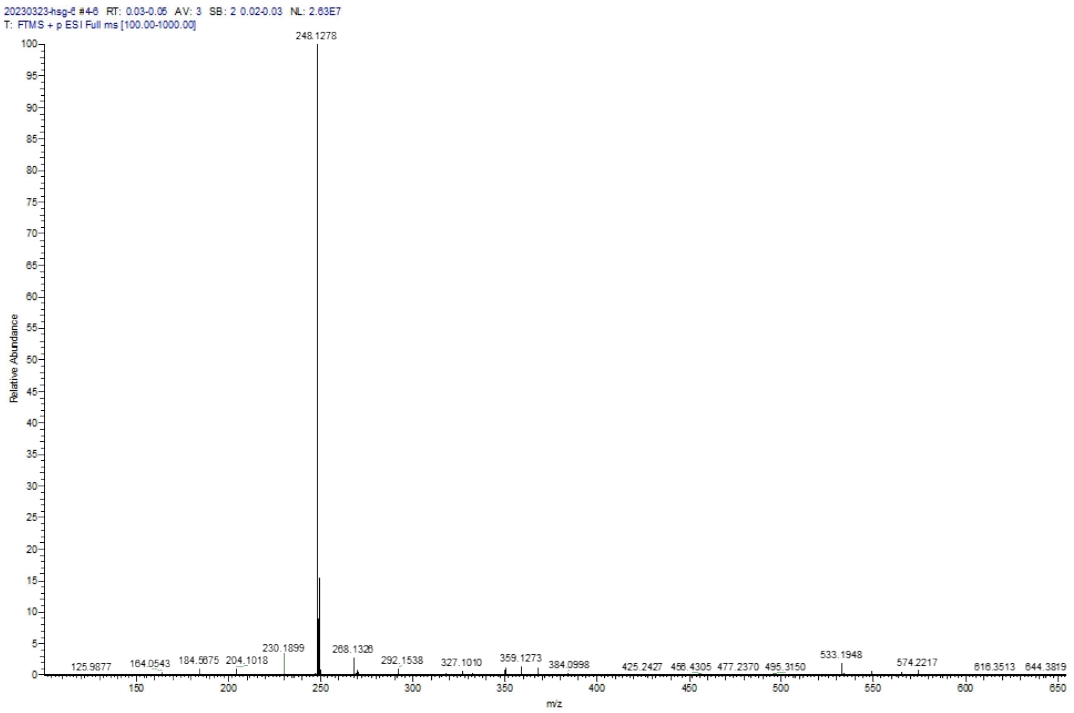


Fig. S6. HRMS of **2**.

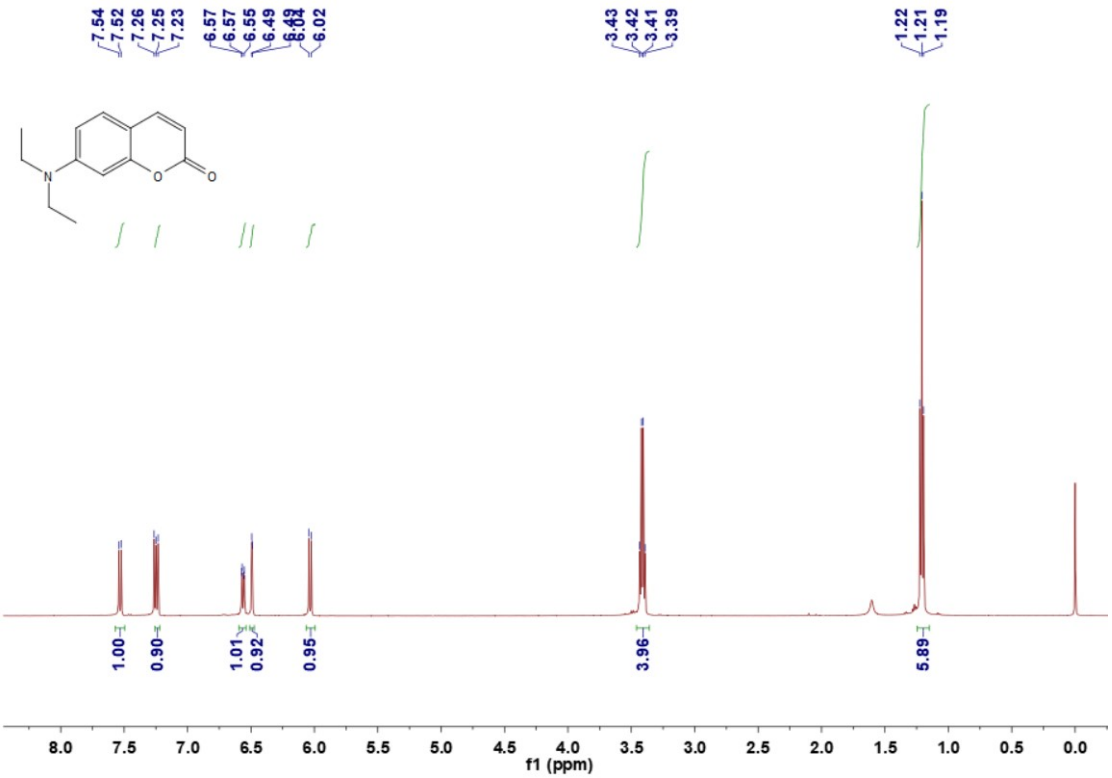


Fig. S7. ^{1}H NMR spectrum of **3** in $CDCl_3$.

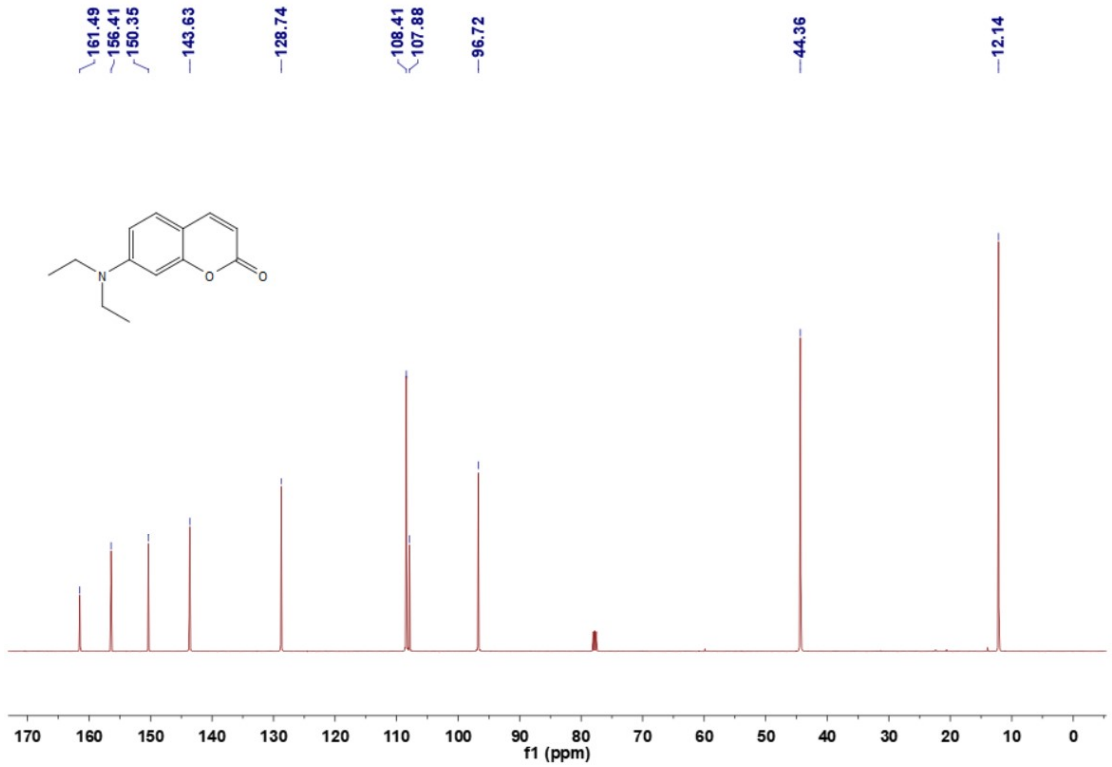


Fig. S8. ^{13}C NMR spectrum of **3** in $CDCl_3$.

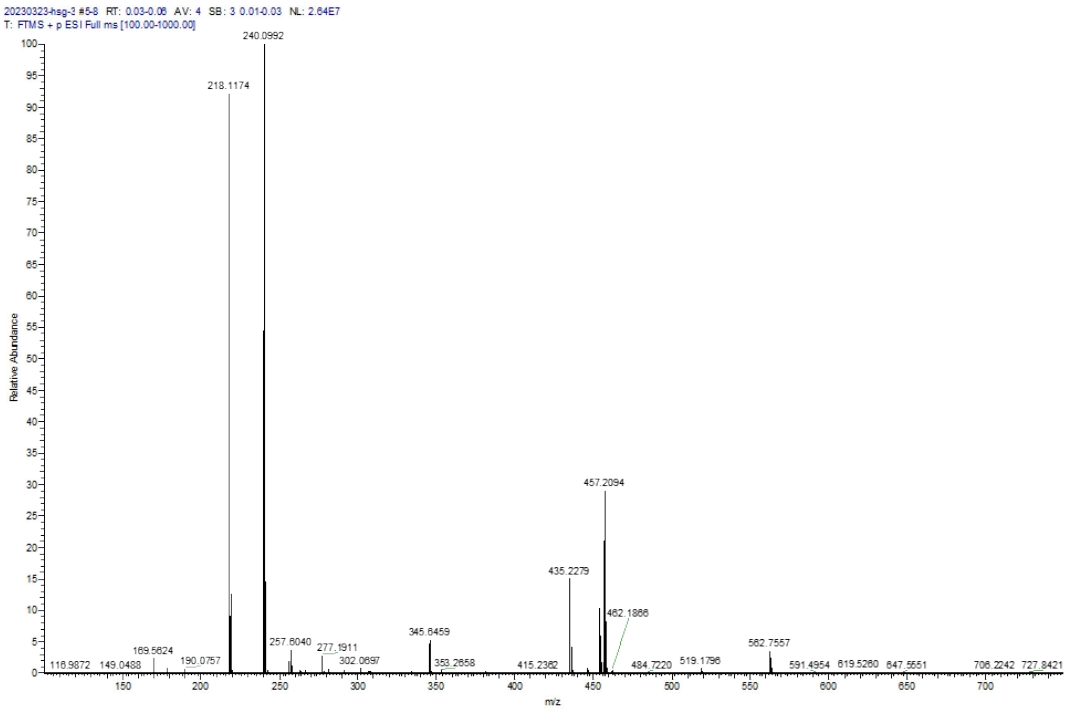


Fig. S9. HRMS of **3**.

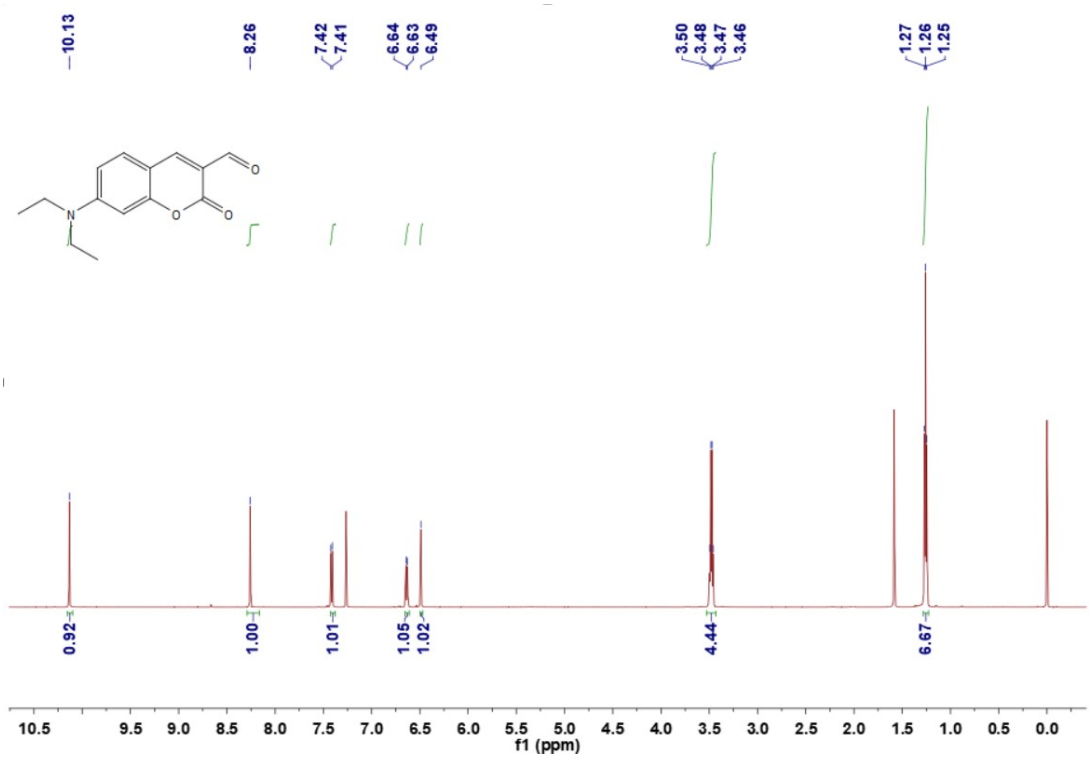


Fig. S10. ^{1}H NMR spectrum of **4** in $CDCl_3$.

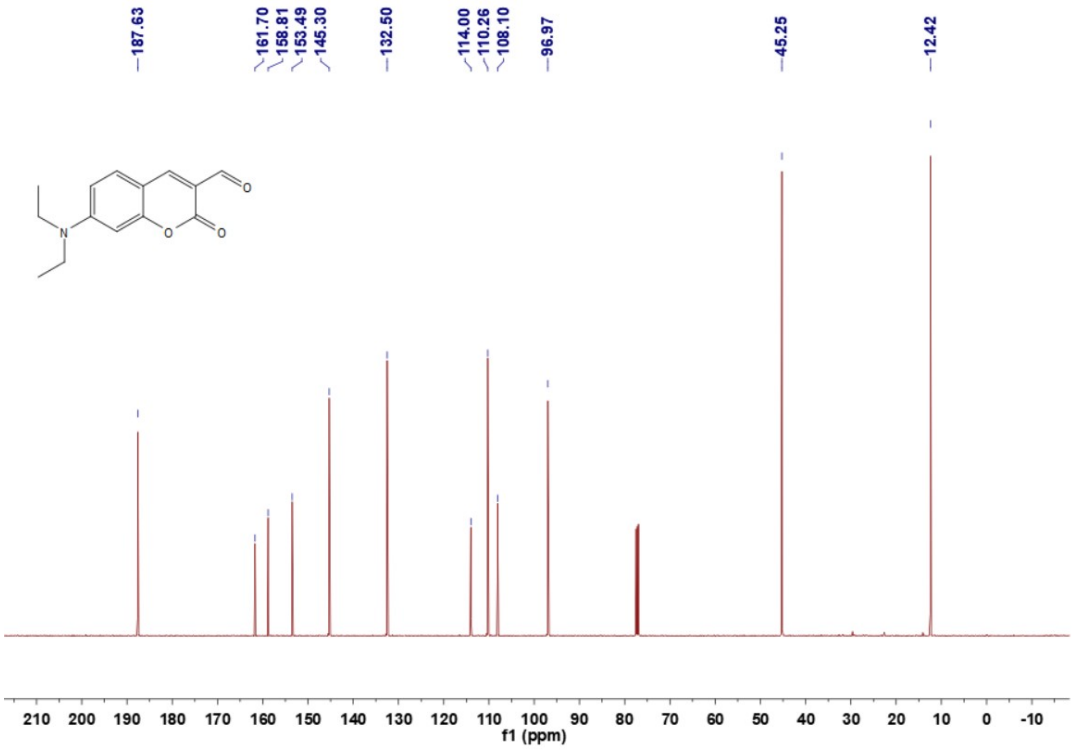


Fig. S11. ^{13}C NMR spectrum of **4** in $CDCl_3$.

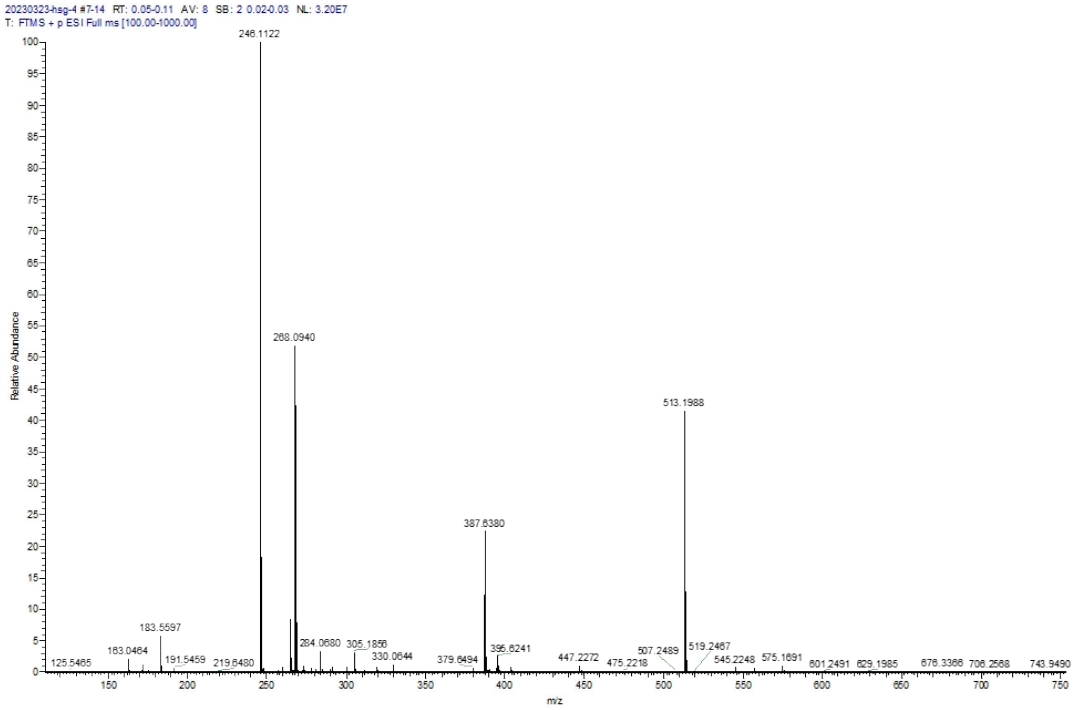


Fig. S12. HRMS of **4**.

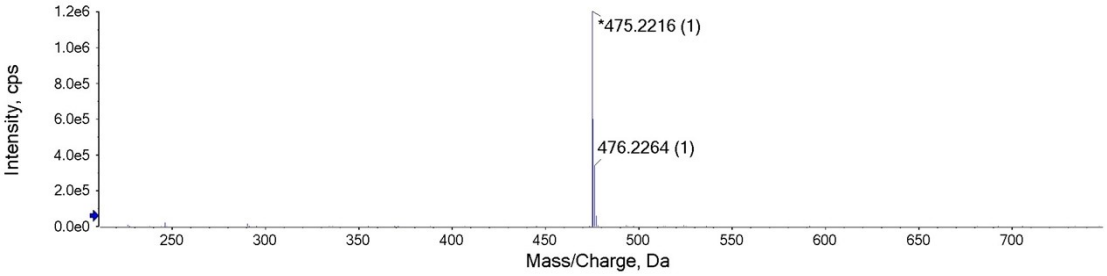


Fig. S13. HRMS of **CHC-COOH**.

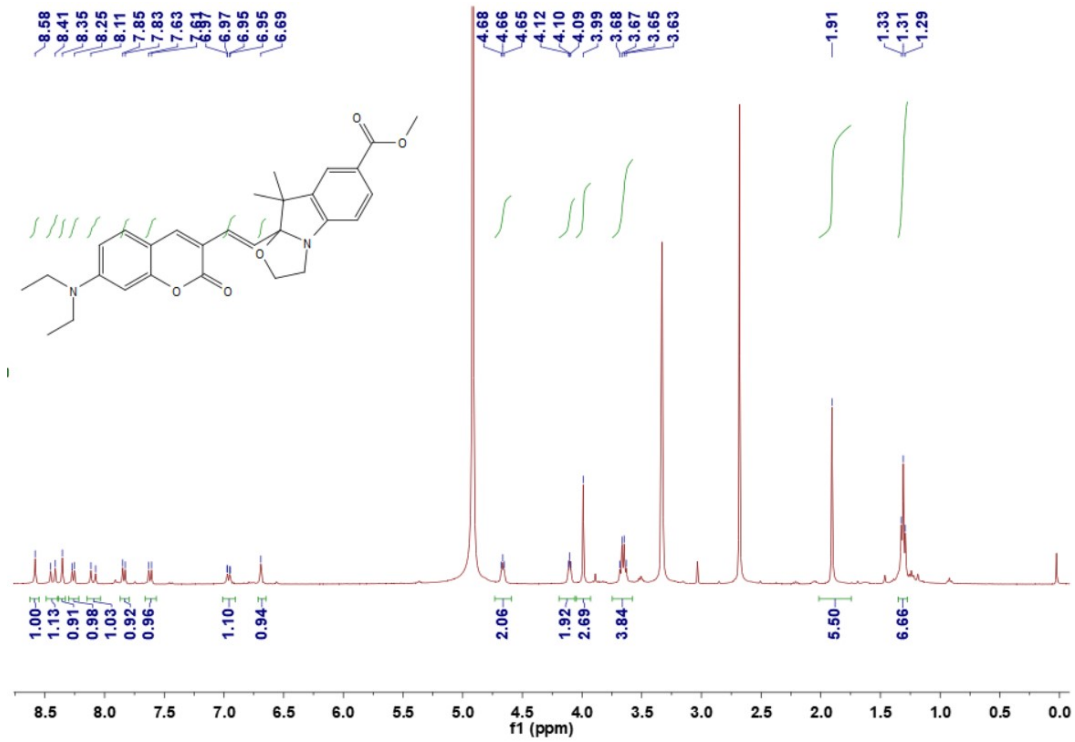


Fig. S14. ^{1}H NMR spectrum of **CHC-CES1** in Methol-d_4.

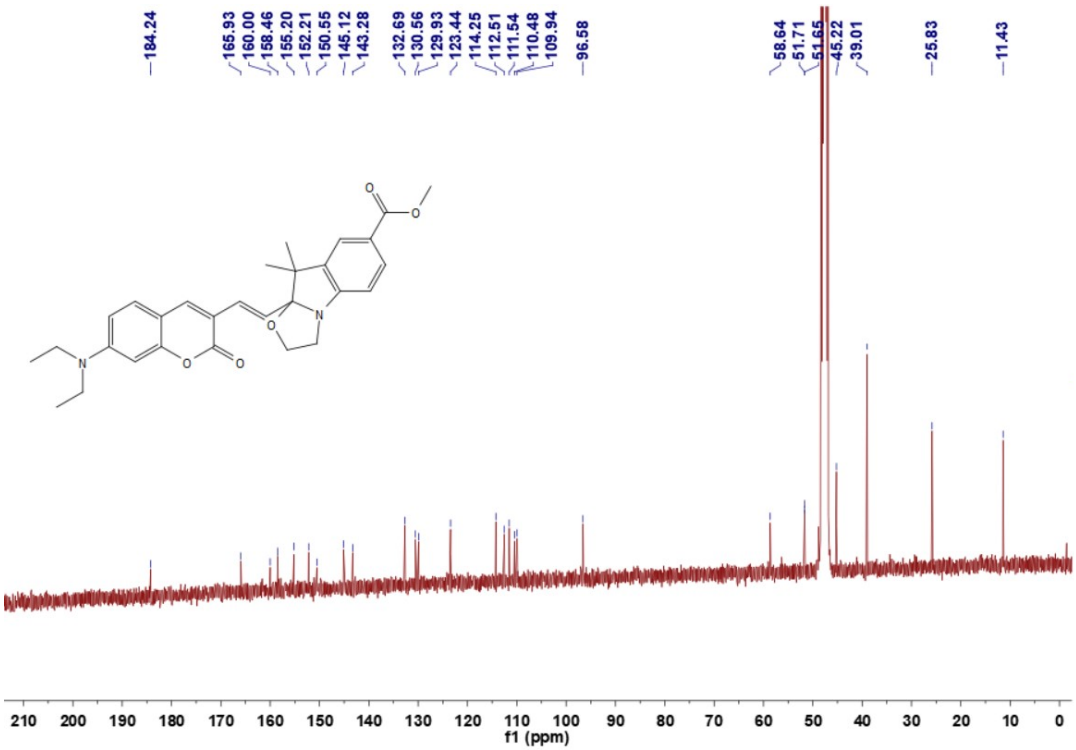


Fig. S15. ^{13}C NMR spectrum of **CHC-CES1** in Methol-d_4.

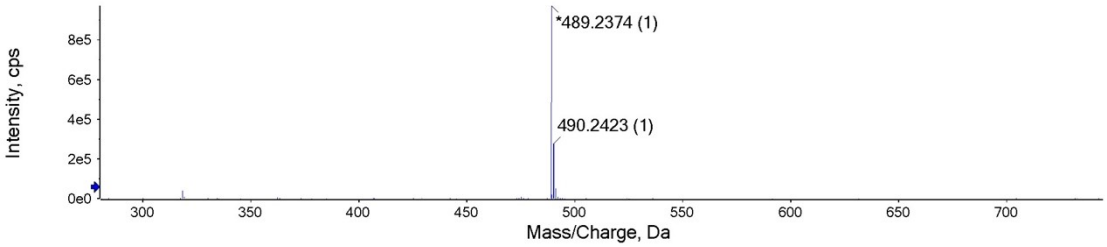


Fig. S16. HRMS of **CHC-CES1**.

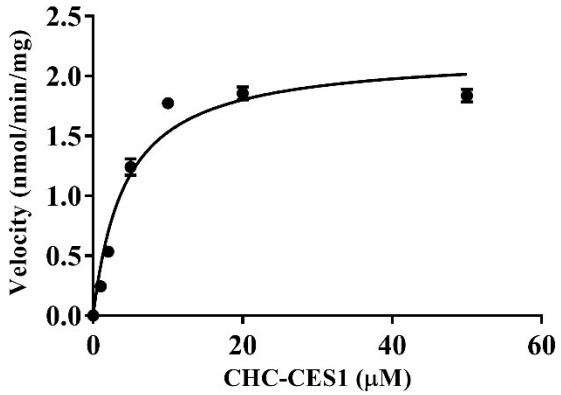


Fig. S17 The kinetic of **CHC-CES1** in CES1B.

Table. S1 Pesticide number and pesticide final concentration in inhibition measurement.

	Pesticide name	Final concentration[a] (mg/mL)
1	Chlorpyrifos methyl	0.25
2	Chlorpyrifos	10
3	Dimethoate	15
4	Bifenthrin	8
5	Cypermethrin	2
6	Beta cypermethrin	1
7	Pyridaben	4
8	Chlorfenapyr	4
9	Carbofuran	0.5
10	Cholrothalonil	10
11	Iprodione	25
12	Acetamiprid	15
13	Imidacloprid	15
14	Carbendazim	15
15	Prochloraz	0.5
16	Dimethomorph	0.02
17	Difenoconazole	3

Note: (a) The final concentration was set according to the maximum residue of pesticide in food stipulated in standardization administration of the People's Republic of China of food safety (GB 2763~2019).