# A highly selective near infrared fluorescent probe for carboxylesterase 2 and its biological applications

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## 1. Materials and methods

### *1.1 General Experimental Procedures*

Bis-p-nitrophenylphosphate (BNPP) and loperamide (LPA) were purchased from *J&K* Chemicals. Lysozyme (LS), N-acetyl-glucosaminidase (NAG), human serum albumin (HSA), pancreatin (PAN), acetyl coenzyme A (CoA), peroxidase (POX), bovine serum albumin (BSA), carboxylesterases 1 (CES 1) and 2 (CES 2), *β*-galactosidase (GLA), carbonic anhydrase (CAS), *β*-glucosidase (*β*-GLC), and *β*-glucuronidase (GLU) were all obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Glutamate (Glu), myristic acid serine (Ser), glutathione (GSH), bilirubin, glycine (Gly), arginine (Arg), tryptophan (Trp), tyrosine (Tyr), glucose and glutamine (Gln) were purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China).

### *1.2. Synthesis of DSAB*

Benzoyl chloride (0.12 mmol, mixed with 1 mL of $CH_2Cl_2$) was added dropwise to a solution of **DSAO** (35.7 mg, 0.1 mmol) and $Et_3N$ (0.15 mmol) in 5 mL of $CH_2Cl_2$ at 0 °C in 30 min (**Scheme 1**). After stirring at this temperature for 1 h, the mixture was warmed to room temperature and stirred overnight. The residue obtained after removing the solvent was separated by a silica gel column and eluted with EtOAc–hexane (1: 5) to obtain 10.2 mg (22.1%) of **DSAB** as a yellow solid.

**DSAB**: $^1$H NMR (600 MHz, DMSO-*d6*) δH 8.11 (dd, *J* = 8.3, 1.1 Hz, 2H), 8.02 (s, 1H), 7.85 (d, *J* = 8.3 Hz, 1H), 7.75 (tt, *J* = 7.5, 1.2 Hz, 1H), 7.60 (t, *J* = 7.5 Hz, 2H), 7.49 (dd, *J* = 8.5, 2.3 Hz, 1H), 7.22 (dd, *J* = 2.3 Hz, 1H), 7.04 (d, *J* = 9.9 Hz, 2H), 6.53 (d, *J* = 9.9 Hz, 2H); $^{13}$C NMR (150 MHz, DMSO-*d6*) δC 184.9, 172.3, 164.1, 153.0, 150.0, 145.6, 139.7, 139.6, 138.1, 135.1, 134.3, 133.4, 131.8, 130.0, 129.7, 128.9, 128.5, 125.1, 123.8, and 122.3; HRESIMS *m/z* 484.0100 $[M + Na]^+$ (calcd. for $C_{25}H_{13}Cl_2NO_4Na$, 484.0114). (**Fig. S1-S4**)

### *1.3. The assay system for detecting CES 2 activity*

The assay was conducted in the 200 μL standard incubation system as the previous description which including potassium phosphate buffer (100 mM, pH = 7.4), human recombinant CES 2.[1] The reaction was initialed by adding the **DSAB** (final concentration of 10 μM) after a pre-incubation for 3 min at 37 °C. And then, the reaction was terminated by adding ice-cold acetonitrile (100 μL) after incubation for different times at 37 °C. The supernatant was obtained after centrifuging at 20000 × g for 20 min at 4 °C, and analyzed on Synergy H1 Microplate Reader (Bio-Tek) and HPLC.

### *1.4. Selectivity assay*

In order to investigate the selectivity of **DSAB** for CES 2, **DSAB** was incubated with various enzymes, including LS, PAN, CoA, NAG, CAS, POX, CES 1 and 2, GLA, *β*-GLC, GLU, HSA, and BSA, in our standard incubation system for 30 min, and the enzyme concentration was set at 5 μg/mL, respectively. Additionally, the stability of **DSAB** in several amino acids, endogenous substances (including: Glu, GSH, Gln, Ser, Arg, myristic acid, Gly, Trp, bilirubin, glucose, Tyr,) and metal ions (including: $Mn^{2+}$, $Ca^{2+}$, $Zn^{2+}$, $K^+$, $Ba^{2+}$, $Mg^{2+}$, $Sn^{4+}$, $Fe^{3+}$, $Ni^{2+}$, $Na^+$) were also evaluated according to the previous reported method.[2]

Next, the selectivity of **DSAB** toward CES2 was confirmed by chemical inhibition in pooled human liver microsomes (HLM); briefly, different inhibitors bis-p-nitrophenyl phosphate (BNPP), loperamide (LPA), Baicalin, and EDTA were co-incubated with **DSAB** in our incubation system, respectively. The inhibition activity was calculated by comparing with the control group.

***1.5. Fluorescence imaging of DSAB in living cells***

Firstly, the cytotoxicity of **DSAB** in living cells was also evaluated by the CCK8 assay (Roche Diagnosis, Indianapolis, IN). In briefly, HepG2 cells were cultured in a DME/F-12 medium with 10% (*v/v*) fetal bovine serum (FBS) and 1% (*v/v*) antibiotics (penicillin/streptomycin) at 37 °C with 5% $CO_2$, before assay, HepG2 cells ($6 \times 10^3$ cells/mL) were seeded in a 96-well plate overnight. Next day, the fresh medium which contained different concentration of **DSAB** (0, 1, 10, 50, or 100 μM) was added in the cell, respectively and cultured for another 24 h. At last, the culture medium was discarded, and free culture medium containing 10% (*v/v*) CCK-8 reagent was added and reacted for1 h then the absorbance was recorded at 450 nm on Synergy H1 Reader (Bio-Tek). The cell viability was calculated by compared with the control group.

Next, the fluorescence imaging of **DSAB** was performed in living HepG2 cells. Briefly, HepG2 cells were seeded on 20 mm glass polylysine-coated confocal cell culture dishes and attached overnight. Next, the fresh medium containing 20 μM **DSAB** was added and incubated for another 1h, the inhibition group was pre-incubated with the CEs general inhibitor BNPP (50 μM) for 30 min and then added **DSAB** for the fluorescence imaging, control group was replaced by blank solvent. At last, the culture medium was discarded and washing the living cells three times with free medium and imaged on the confocal microscope (Leica TCS SP8).

***1.6 The activity of CES2 in different tissues S9***

In our present work, the activity of CES2 in different tissues S9 including: Liver, Intestine, Kidney was measured by **DSAB**. The assay was performed in our standard system mentioned above. The protein concentration of different tissues S9 was set at 0.1 mg/mL, the **DSAB** was 10 μM. After incubated at 37 ℃ for 30 mins, the supernatant was centrifuged at 20000 × g for 20 min at 4 °C, and analyzed the fluorescence properties on Synergy H1 Microplate Reader (Bio-Tek). The catalytic velocity was calculated according the standard curve of **DSAO** the metabolite of **DSAB** catalyzed by CES2.

***1.7. The discovery of CES 2 inhibitors via a high-throughput screening method***

Herein, in order to discovery the novel inhibitors from herbal medicines which was widely used all over the word, the inhibitory effect of 68 kinds herbal medicines on CES 2 were assayed based on the probe reaction of **DSAB** medicated by CES 2. In the assay system, we used pooled human liver microsomes (HLM) as the enzyme source and the concentration of HLM was set at 4 μg/mL and reaction time was 30 mins. The control group was added blank solvent.

*Schisandra chinensis* was proved to have a potent inhibition on CES 2 among various herbal medicines, in order to elucidate the key inhibition components from *Schisandra chinensis*, *S. chinensis* (200 g) was extracted by 95% EtOH (5 L/2 h for 3 times). After then, ten fractions (Fr. 1-Fr. 10) were obtained by preparative HPLC. The mobile phases

including A: containing 10% MeOH 0.03% trifluoroacetic acid water; B:100% MeOH. The preparation method was as followed: 0−15 min 66% B; 15−35 min 66-100% B; 35−40 min 100% B; 40−45 min 100-66% B; 45−55 min 66% B and the flow rate was set at 10 mL/min. Then, the inhibitory effect of Fractions toward CES 2 were assayed as mentioned above. According to the result of bioassay-guided performance,[3] compounds 1 (5.0 mg), 2 (3.5 mg), 3 (3.4 mg), and 4 (4.2 mg) were purified from Fr. 5-Fr. 10 by preparative HPLC eluted with MeOH-$H_2O$ (from 40% to 70%). The structures of Compounds were analyzed by NMR analysis and their inhibition curves were also performed and obtained their inhibition $IC_{50}$ values.

***1.8 Molecular docking***

Amino acid sequence, O00748, of human CES 2 was downloaded from UniProt database, and was used to build its homology structure on a basis of human CES 1 (PDB: 1YAH) by Discovery Studio 3.5 (Accelrys, SanDiego, CA, USA) as previously described.[4] The 3D structure of compound 2 was optimized at the CHARMm force field, and CDOCKER was used to analyze its interactions with human CES 2.

**Reference**


1. X. Tian, F. Yan, J. Zheng, X. Cui, L. Feng, S. Li, L. Jin, T. D. James and X. Ma, *Analytical chemistry*, 2019, **91**, 15840-15845.
2. X. Tian, Z. Tian, Y. Wang, J. Hou, L. Feng, L. Song, J. Cui, X. Huo, X. Zhao, D. Zhou, C. Wang, C. Sun, J. Ning and X. Ma, *Sensors and Actuators B: Chemical*, 2018, **277**, 423-430.
3. X. Tian, T. Liu, L. Li, B. Shao, D. Yao, L. Feng, J. Cui, T. D. James and X. Ma, *Analytical chemistry*, 2020, **92**, 9493-9500.
4. J. Yi, R. Bai, Y. An, T. T. Liu, J. H. Liang, X. G. Tian, X. K. Huo, L. Feng, J. Ning, C. P. Sun, X. C. Ma and H. L. Zhang, *International journal of biological macromolecules*, 2019, **133**, 184-189.

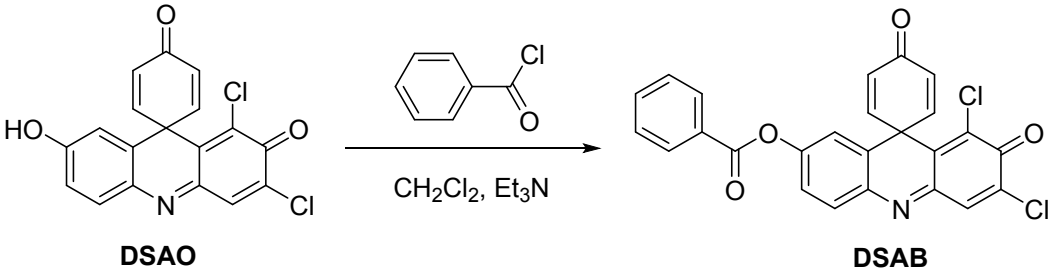


**Fig. S1** The synthesis pathway of **DSAB**.

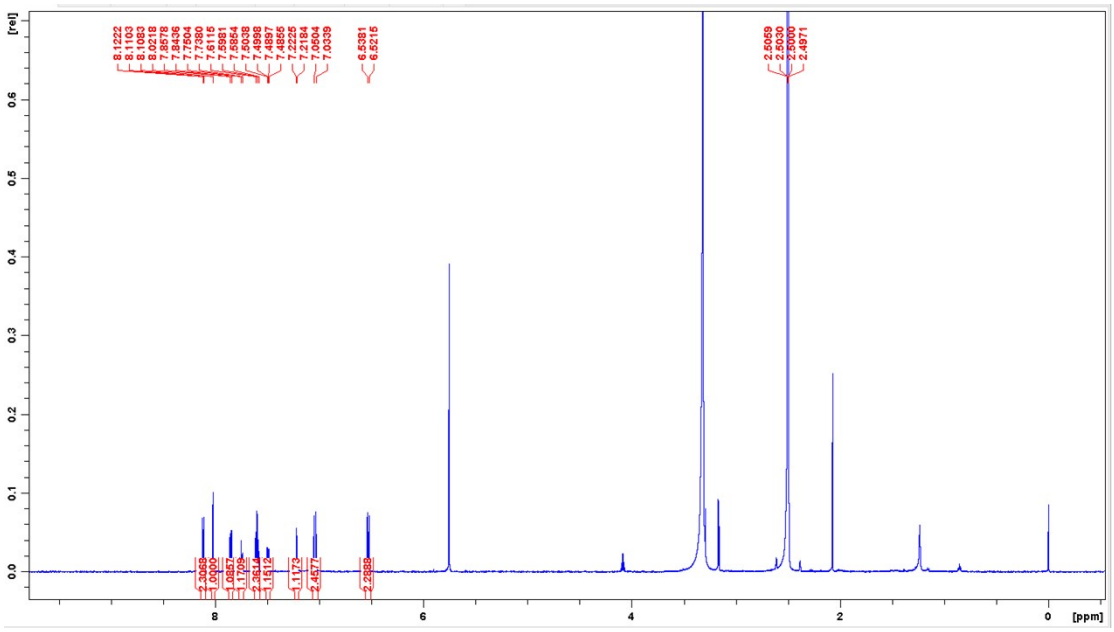


**Fig. S2** $^{1}$H NMR (600 MHz, DMSO-$d_6$) spectrum of **DSAB**

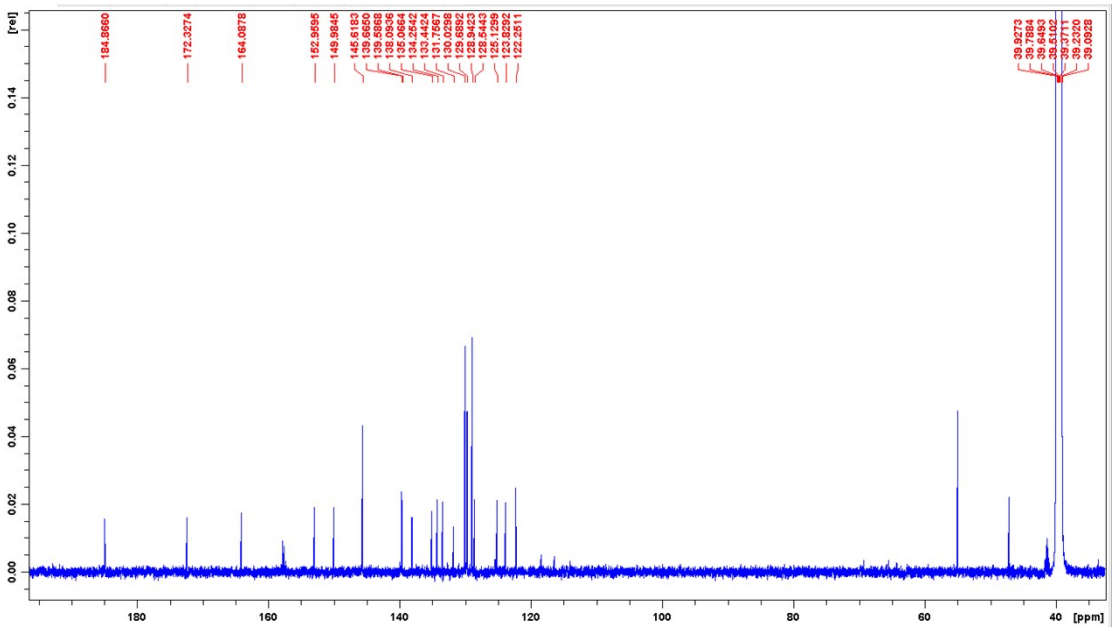


**Fig. S3** $^{13}$C NMR (150 MHz, DMSO-$d_6$) spectrum of **DSAB**

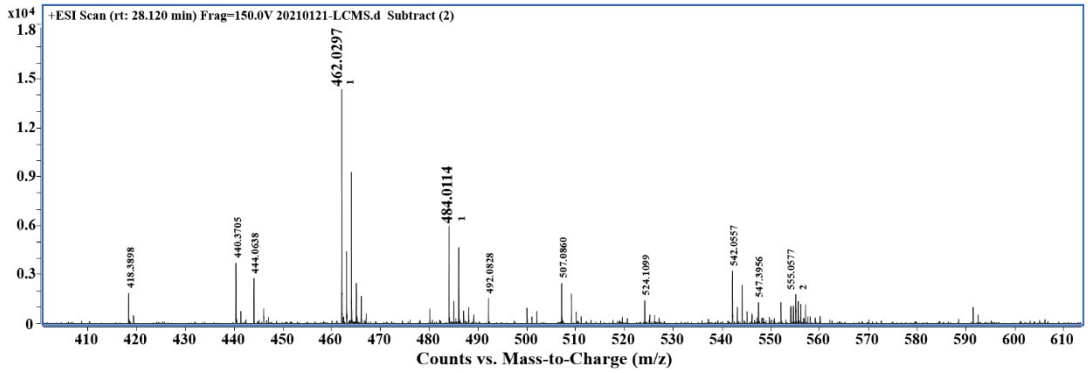


**Fig. S4** HRMS spectrum of **DSAB** in positive model.

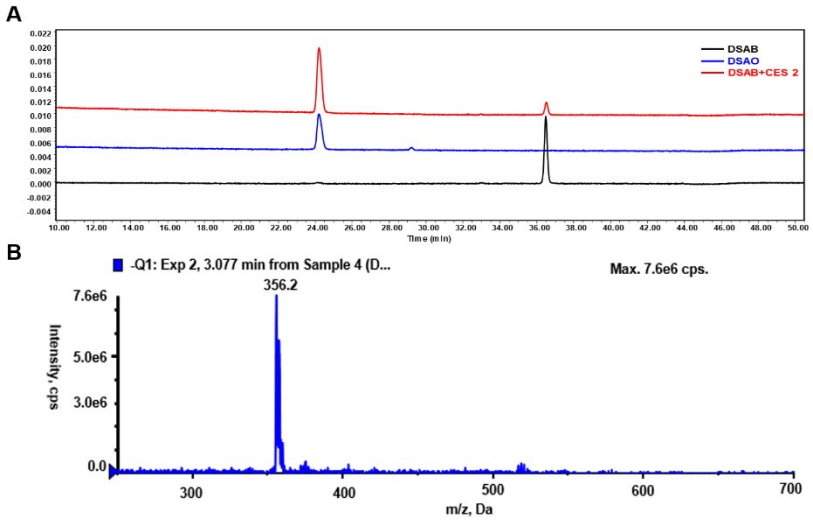


**Fig. S5** (A) HPLC spectra of **DSAB** or the reaction solution of **DSAB** (100 μM) and CES 2 (10 μg/mL) for 30 min; (B) the MS/MS analysis for the metabolite of **DSAB** catalyzed by CES 2.

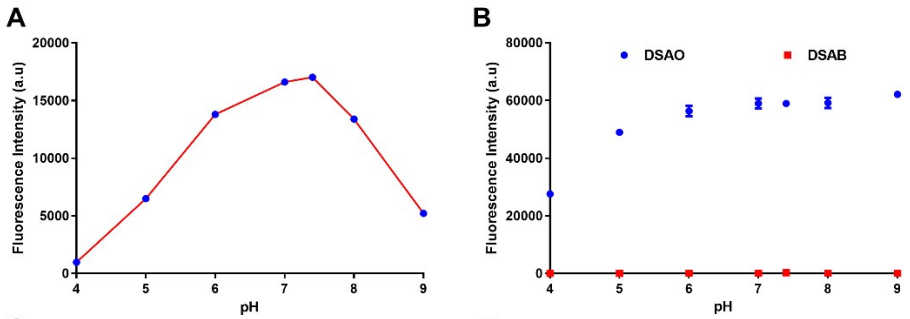


**Fig. S6** (A) Effects of pH on the fluorescence intensity of the reaction system of **DSAB** (10 μM) and **CES 2** (5 μg/mL). (B) Effects of pH on the fluorescence intensities of **DSAB** (10 μM) and **DSAO** in the assay system (Buffer-acetonitrile *v:v* = 2:1).

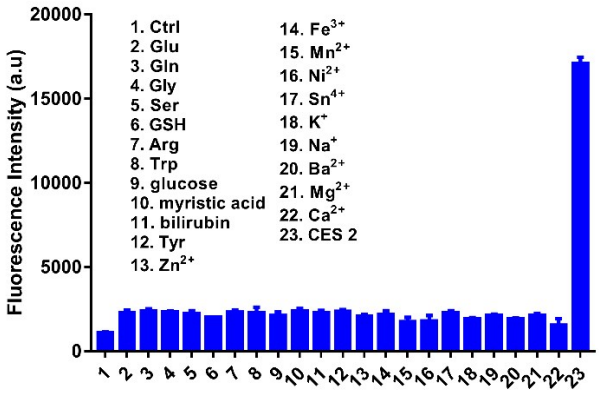


**Fig. S7** Stability of **DSAB** (10 μM) toward various analytes in in vitro assay system.

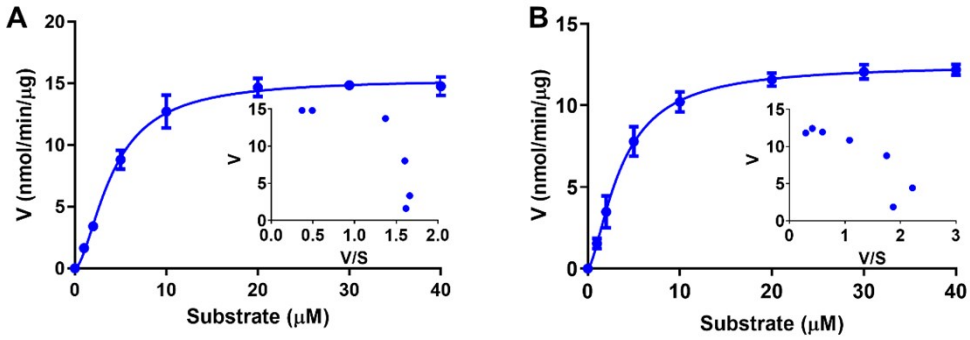


**Fig. S8** Kinetic plots of **DSAB** hydrolysis in pooled human kidney microsome (HKM) (A) and pooled human intestine microsome (HIM) (B), respectively.

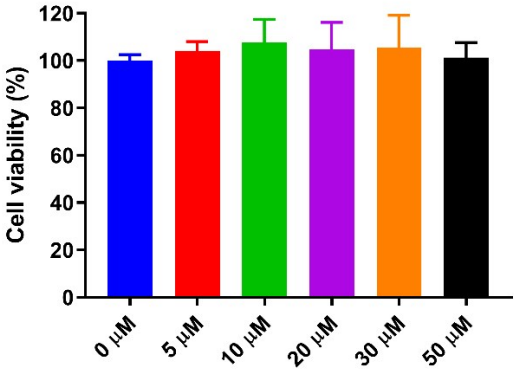


**Fig. S9** The cytotoxicity of **DSAB** toward HepG2 cells.

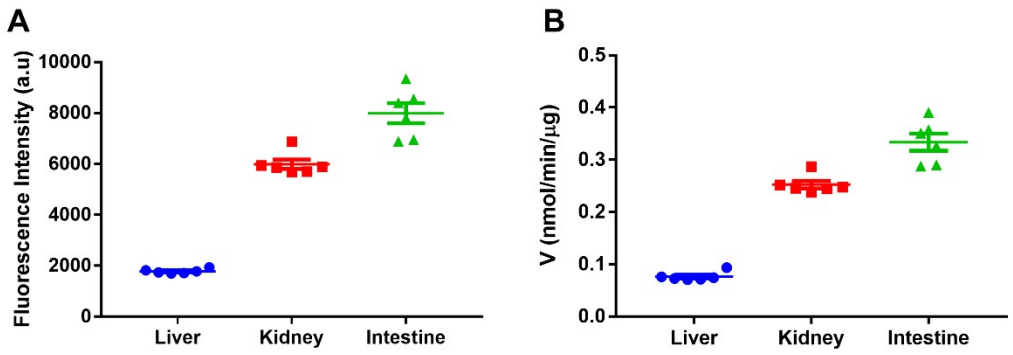


**Fig. S10.** (A) Fluorescence intensities response of **DSAB** after incubating with liver, kidney, and intestine S9 for 30 min, $\lambda_{ex}$ 630 nm. Data were shown as the mean ± SEM, n=6. (B) Catalytic velocity of liver, kidney, and intestine S9 for the hydrolysis progress of **DSAB**.

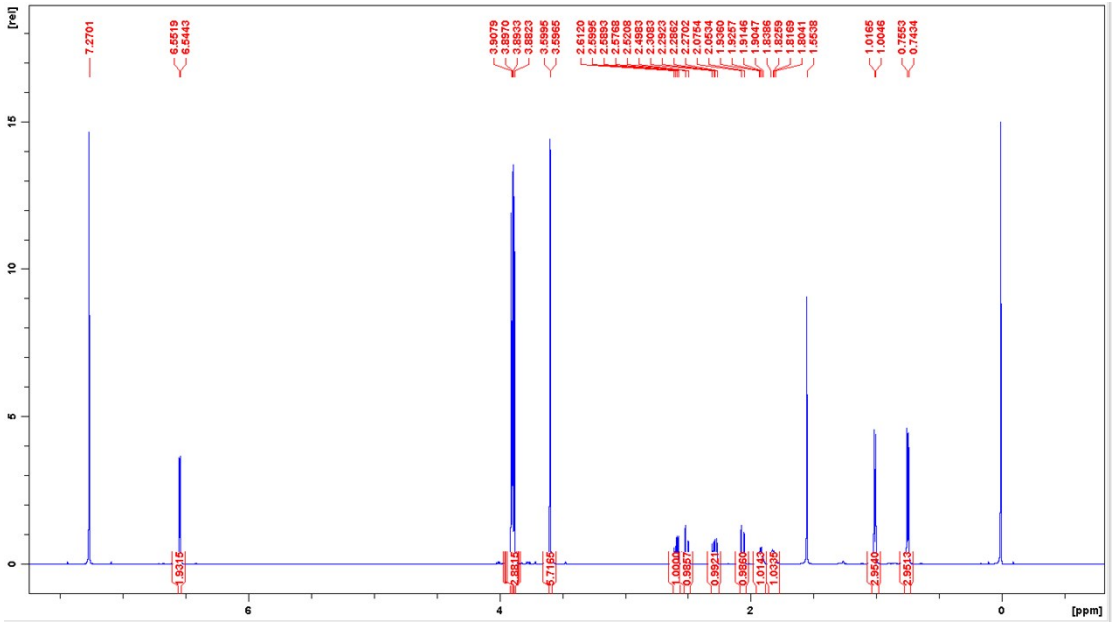


**Fig. S11** $^1$H NMR (600 MHz, $CDCl_3$) spectrum of 1

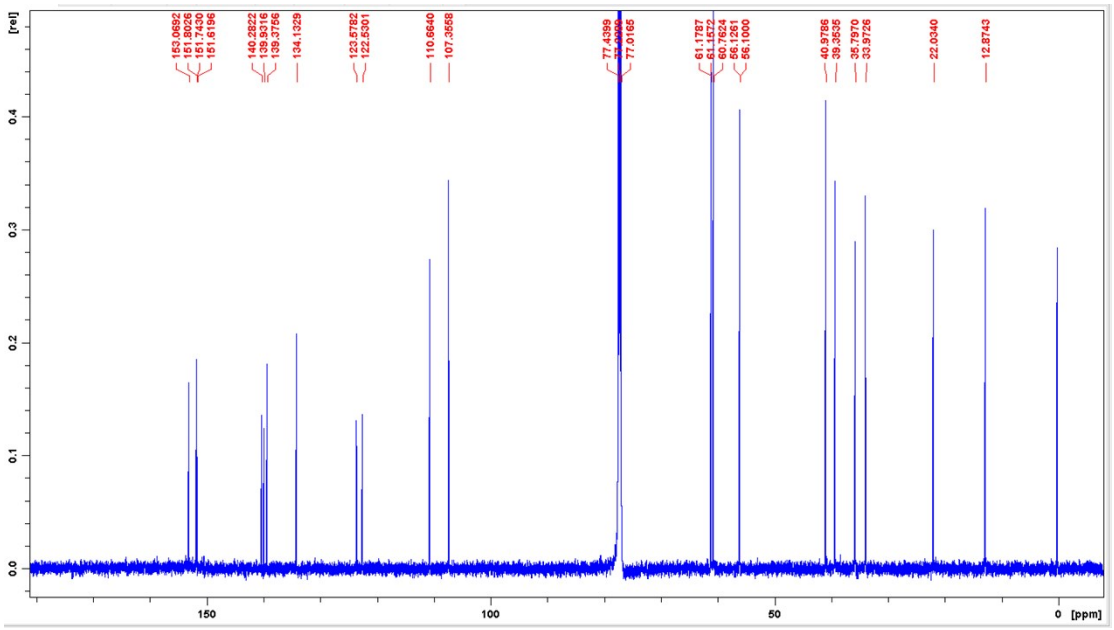

**Fig. S12** $^{13}C$ NMR (150 MHz, $CDCl_3$) spectrum of 1

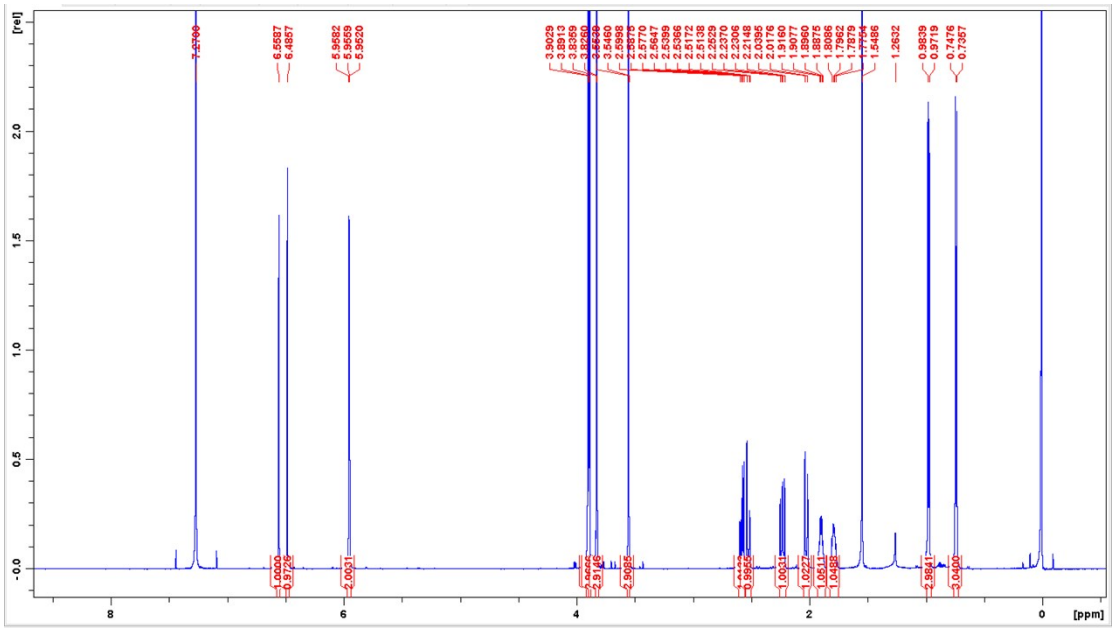

**Fig. S13** $^{1}H$ NMR (600 MHz, $CDCl_3$) spectrum of 2

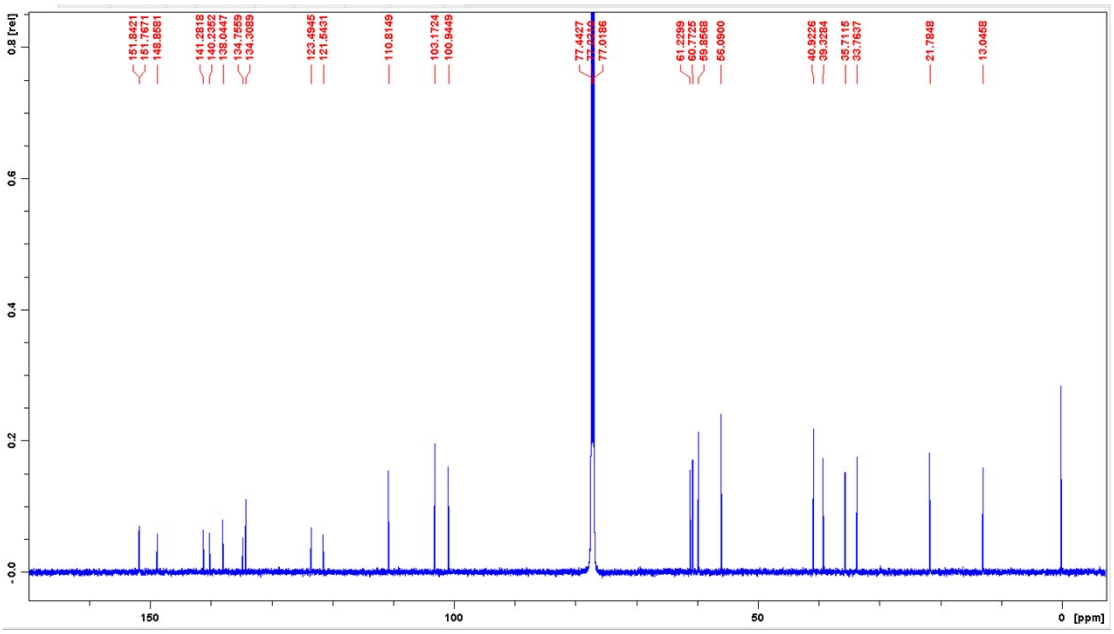


**Fig. S14** $^{13}C$ NMR (150 MHz, $CDCl_3$) spectrum of 2

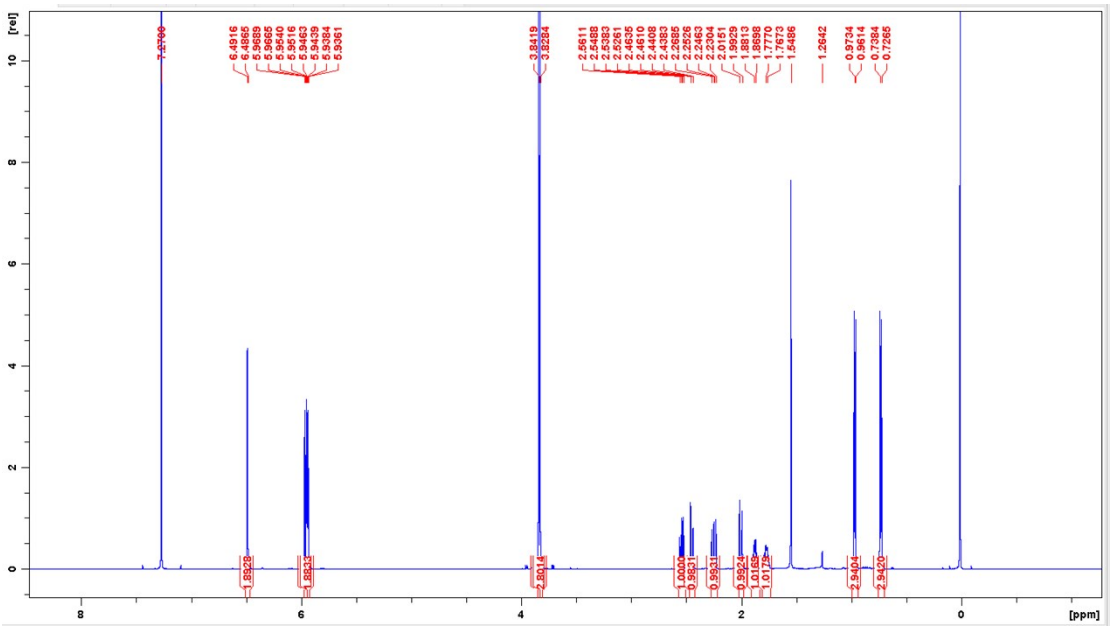


**Fig. S15** $^{1}H$ NMR (600 MHz, $CDCl_3$) spectrum of 3

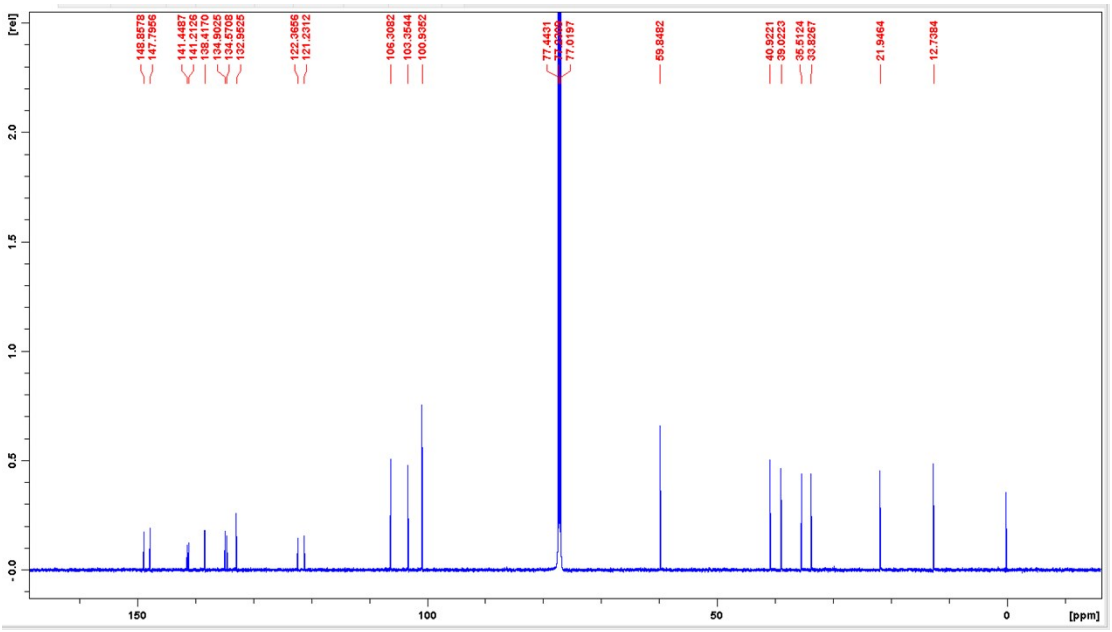


**Fig. S16** $^{13}C$ NMR (150 MHz, $CDCl_3$) spectrum of 3

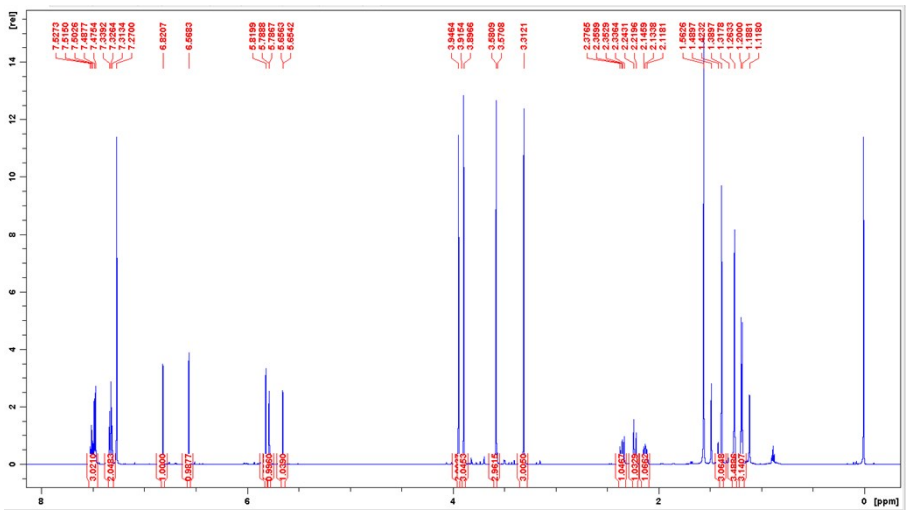


**Fig. S17** $^{1}H$ NMR (600 MHz, $CDCl_3$) spectrum of 4

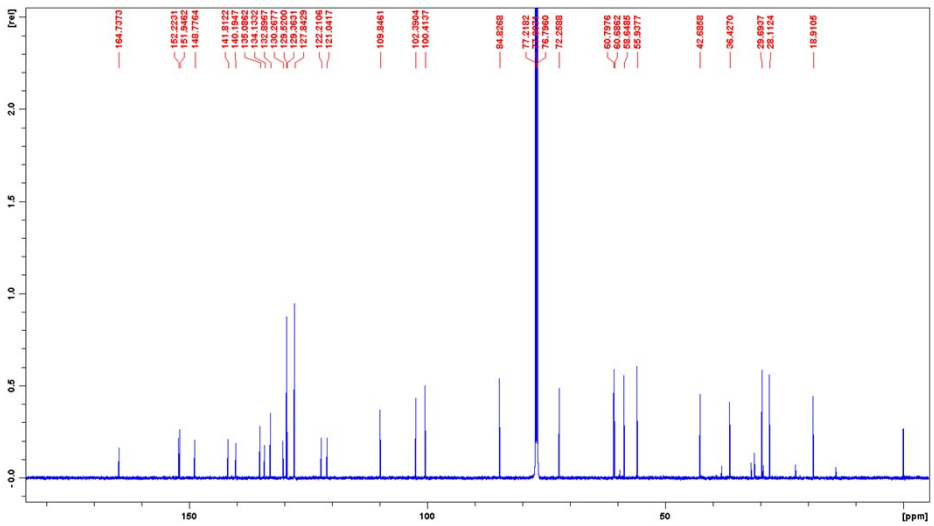


**Fig. S18** $^{13}C$ NMR (150 MHz, $CDCl_3$) spectrum of 4

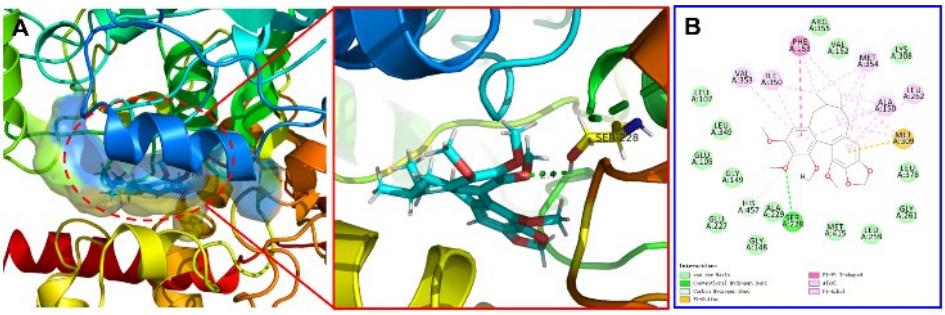


**Fig. S19** (A) Schisandrin B (inhibitor 2) could enter into the cavity of CES 2 *via* a hydrogen bond interaction with Ser228. (B) 2D interactions of Schisandrin B (inhibitor 2) with CES 2.

**Table S1**. Kinetic parameters for **DSAB** hydrolysis in different enzyme sources

| Enzyme sources | $V_{max}$ （nmol/min/μg） | $K_m$ (μM) | $CL_{int}$ (mL/min/μg) |
|---|---|---|---|
| CES 2 | 29.74 ± 1.01 | 6.55 ± 0.49 | 4.54 |
| HLM | 34.78 ± 4.05 | 7.66 ± 6.97 | 4.54 |
| HKM | 15.36 ± 0.28 | 4.10 ± 0.20 | 3.74 |
| HIM | 12.49 ± 0.25 | 3.66 ± 0.20 | 3.41 |

**Table S2. Information of TCMs**

| No. | Name |
|---|---|
| 1e | *Houttuynia cordata* |
| 1f | *Acacia catechu* |
| 1g | *Pseudostellaria heterophylla* (Miq.) Pax |
| 1h | *Scaphium wallichii* Schott & Endl. |
| 1i | *Codonopsis pilosula* (Franch.) Nannf. |
| 2a | *Euryale ferox* Salisb. ex DC |
| 2b | *Aster tataricus* L. f. |
| 2c | *Areca catechu* L. |
| 2d | *Citrus reticulata* Blanco |
| 2e | *Curcuma longa* L. |
| 2f | *Corydalis bungeana* Turcz. |
| 2g | *Polygonum aviculare* L. |
| 2h | *Illicium verum* Hook. f. |
| 2i | *Dioscorea polystachya* Turczaninow |
| 3a | *Physalis alkekengil*.var.francheti(Mast.)Makino |
| 3b | *Zanthoxylum nitidum* (Roxb.) DC. |
| 3c | *Canavalia gladiata* (Jacq.) DC |
| 3d | *Fallopia multiflora* (Thunb.) Harald. |
| 3e | *Celosia cristata* L. |
| 3f | *Foeniculum vulgare* Mill. |
| 3g | *Dendranthema indicum* |
| 3h | *Stemona japonica* (Bl.) Miq |
| 3i | *Dimocarpus longan* Lour. |
| 4a | *Aconitum carmichaelii* |
| 4b | *Coptis chinensis* Franch. |
| 4c | *Trachelospermum jasminoides* (Lindl.) Lem. |
| 4d | *Cucumis satiuus* L. |
| 4e | *Schisandra chinensis* |
| 4f | *Poria cocos* Schw.Wolf |

| | |
|---|---|
| 4g | *Dictamnus dasycarpus* Turcz. |
| 4h | *Sparganium stoloniferum* (Graebn.) Buch.-Ham. ex Juz. |
| 4i | *Leonurus japonicus* Houtt. |
| 5a | *Hibiscus sabdariffa* L. |
| 5b | *Atractylodes macrocephala* Koidz. |
| 5c | *Litchi chinensis* Sonn. |
| 5d | *Alpinia officinarum* Hance |
| 5e | *Aconitum carmichaeli* Debx. |
| 5f | *Pyrola calliantha* H. Andres |
| 5g | *Aucklandia costus* Falc. |
| 5h | *Desmodium styracifolium* (Osbeck.) Merr. |
| 5i | *Boehmeria nivea (*L.*)* Gaudich. |
| 6a | *Geranium wilfordii* Maxim. |
| 6b | *Stellaria dichotoma* var. lanceolata Bge. |
| 6c | *Clematis chinensis* Osbeck. |
| 6d | *Monascus purpureus* Went. |
| 6e | *Lobelia chinensis* Lour. |
| 6f | *Fibraurea recisa* Pierre. |
| 6g | *Lilium brownii* var. viridulum. |
| 6h | *Morinda officinalis* How. |
| 6i | *Root of Isatis indigotica* Fortune. |
| 7a | *Eucommia ulmoides* Oliver. |
| 7b | *Pteris multifida* Poir. |
| 7c | *Allium fistulosum* L. |
| 7d | Massa Medicata Fermentata |
| 7e | *Cynomorium songaricum* Rupr. |
| 7f | *Scutellaria barbata* D. Don |
| 7g | *Hordeurn vulgare* L. |
| 7h | *Hydnocarpus anthelmintica* Pierre. |
| 7i | *Polygonatum sibiricum* Red. |
| 8a | *Ophiopogon japonicus* (Linn. f.) Ker-Gawl. |
| 8b | *Abrus cantoniensis* Hance. |
| 8c | *Isatis indigotica* Fortune. |
| 8d | *Citrus medica* L. var. *sarcodactylis* Swingle. |
| 8e | *Cinnamomum cassia* Presl. |
| 8f | *Semen sinapis* L. |
| 8g | *Bambusa tuldoides* Munro. |
| 8h | *Leonurus japonicus* Houtt. |
| 8i | *Morus alba* L. |