

Supplementary information

**Fluorescence Probe for Butyrylcholinesterase Activity in Human Serum Based on
Fluorophore with Specific Binding affinity for Human Serum Albumin**

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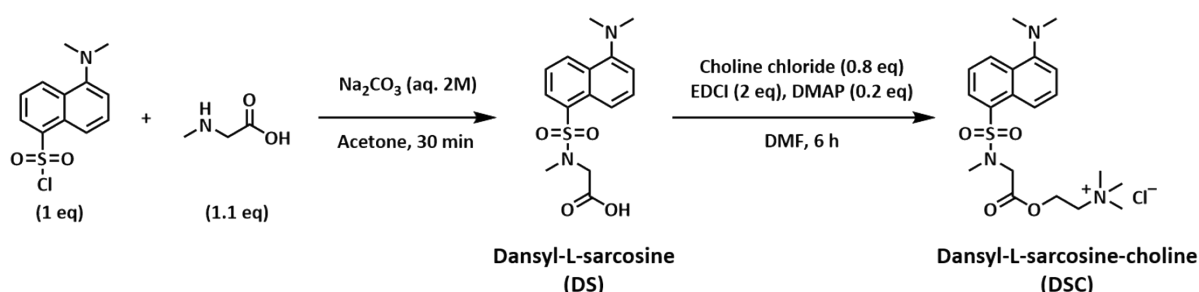
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Experimental

Materials and instrumentation

Chemical reagents were purchased from commercial sources (Sigma-Aldrich, Tokyo Chemical Industry, and Alfa Aesar) and were used without further purification. Fluorescence spectra were recorded on an Agilent Cary Eclipse fluorescence spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a JEOL (400 MHz) NMR spectrometer. Mass spectra were recorded on an Agilent 6520 accurate-mass quadrupole time-of-flight mass spectrometer (MS) with an electrospray ionization (ESI) source. Liquid chromatography MS (LC-MS) spectra were recorded on Micromass Quattro Micro.

Synthesis of dansyl-L-sarcosine-choline as caged-fluorophore



Scheme S1 Synthesis scheme for dansyl-L-sarcosine-choline.

Synthesis of dansyl-L-sarcosine (DS)

Dansyl chloride (2.89 mmol, 0.78 g) was dissolved in 10 mL of acetone, and filtered to remove the insoluble white solid impurities. L-sarcosine (3.18 mmol, 0.28 g) in 4 mL of aq. Na_2CO_3 solution (2 M) was added to the above solution and stirred at room temperature for 30 min. After the addition of dichloromethane (DCM) and water, the pH was adjusted to 5–6 using HCl solution (a.q. 2 N). The organic layer was collected, washed with water and brine, dried on sodium sulfate, and concentrated under reduced pressure. The mixture was purified via column chromatography (DCM:methanol = 10:1) to yield a pale yellow solid (0.60 g, yield 64.36%). ^1H -NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.42 (d, J = 8.5 Hz, 1H), 8.24–8.29 (m, 2H), 7.55–7.60 (m, 2H), 7.23 (d, J = 7.3 Hz, 1H), 3.67 (s, 2H), 2.87 (s, 3H), 2.81 (s, 6H). ^{13}C -NMR (101 MHz, $\text{DMSO}-d_6$) δ 151.27, 135.85, 129.51, 129.21, 129.11, 127.73, 127.71, 123.71, 119.34, 115.06, 52.56, 45.04, 35.13. HR-MS (ESI): $[\text{M}+\text{H}]^+$ m/z calculated for $\text{C}_{20}\text{H}_{30}\text{N}_3\text{O}_4\text{S}^+$: 323.1060; found: 323.1051.

Synthesis of dansyl-L-sarcosine-choline (DSC)

DS (0.31 mmol, 0.10 g), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI, 0.62 mmol, 0.12 g), and 4-(dimethylamino)pyridine (DMAP, 0.06 mmol, 0.01 g) were dissolved in 5 mL of dimethylformamide. Choline chloride (0.25 mmol, 0.03 g) was added to the above mixture. After stirring for 6 h at room temperature, the mixture was diluted with water and washed with DCM. NaCl was added to the mixture, and the mixture was extracted with excess DCM. Then, the organic layer was concentrated under reduced pressure to yield a pale-

yellow oil (0.06 g, yield 56.00%). $^1\text{H-NMR}$ (400 MHz, D_2O) δ 8.33 (d, J = 8.7 Hz, 1H), 8.02-8.06 (m, 2H), 7.51 (dd, J = 8.7, 7.6 Hz, 2H), 7.24 (d, J = 7.6 Hz, 1H), 4.34 (t, J = 2.3 Hz, 2H), 4.16 (s, 2H), 3.44-3.46 (m, 2H), 2.97 (s, 9H), 2.81 (s, 3H), 2.69 (s, 6H). $^{13}\text{C-NMR}$ (101 MHz, D_2O) δ 169.87, 150.68, 132.70, 130.49, 130.20, 129.25, 129.04, 128.83, 124.07, 119.35, 116.12, 64.29, 59.11, 53.68, 50.61, 44.93, 35.54. HR-MS (ESI): $[\text{M}]^+$ m/z calculated for $\text{C}_{20}\text{H}_{30}\text{N}_3\text{O}_4\text{S}^+$: 408.1952; found: 408.1946.

Mechanism study

DSC (10 μM) was added to buffer solutions (phosphate, 20 mM, pH 7.0) containing various concentrations of HSA (0, 20 μM) and BChE (0, 2 U/mL). Fluorescence spectra of the solutions were recorded at 2 min.

Next, **DS** (10 μM) or **DSC** (10 μM) was added to buffer solutions (phosphate, 20 mM, pH 7.0) containing the various concentrations of HSA (0, 20 μM) and BChE (0, 2 U/mL). After the solutions were incubated for 2 min, acetone was added to each solution to inactivate the BChE. The solutions were centrifuged at 14,000 $\times g$ for 25 min, and the supernatants were measured via high-resolution MS (HRMS) and LC-MS.

Also, various albumins (human, bovine, rat, and rabbit; 20 μM) were added to buffer solutions (phosphate, 20 mM, pH 7.0) containing **DSC** (10 μM) in the presence or absence of BChE (2 U/mL). Fluorescence intensities of the solutions were recorded at 30-s intervals for 5 min.

Assay conditions for BChE assay

DSC (10 μM) was added to various buffer solutions (phosphate, 20 mM, pH 6.0–8.0) containing HSA (10 μM) in the presence or absence of BChE (2 U/mL). Fluorescence spectra of the solutions were recorded at 485 nm at 30-s intervals for 5 min.

Next, various concentrations of HSA (from 0 to 50 μM) were added to buffer solutions (phosphate, 20 mM, pH 7.0) containing **DSC** (10 μM) in the presence or absence of BChE (2 U/mL). Fluorescence spectra of the solutions were recorded at 30-s intervals for 5 min.

Quantitative assay of BChE activity

Various concentrations of BChE (from 0 to 2 U/mL) were added to buffer solutions (phosphate, 20 mM, pH 7.0) containing HSA (20 μM) and **DSC** (10 μM). Fluorescence spectra of the solutions were recorded at 485 nm at 10-s intervals for 5 min. The observed rate (k_{obs}) for the enzymatic reaction was obtained from the initial linear fluorescence change between 0 and 30 s.

Selectivity test for BChE activity over other enzymes

AChE (20 U/mL) was added to buffer solutions (phosphate, 20 mM, pH 7.0) containing HSA (20 μ M) and **DSC** (10 μ M) in the presence or absence of BChE (2 U/mL). Fluorescence spectra of the solutions were recorded at 30-s intervals for 5 min.

Next, other enzymes (2 U/mL) were added to buffer solutions (phosphate, 20 mM, pH 7.0) containing HSA (20 μ M) and **DSC** (10 μ M). Fluorescence spectra of the solutions were recorded at 30-s intervals for 5 min.

Selectivity test for BChE activity over biothiols and oxidant

A biothiol (20 μ M of glutathione(GSH), cysteine(Cys), homocysteine(Hcy), and methionine(Met)) or an oxidant (20 μ M of H₂O₂) was added to a buffer solution (phosphate, 20 mM, pH 7.0) containing HSA (20 μ M) and **DSC** (10 μ M) in the presence or absence of BChE (2 U/mL). Fluorescence spectra of the solutions were recorded at 30-s intervals for 5 min.

Practical application for real human serum

A serum (10 μ L) was directly added to a buffer solution (190 μ L) containing only **DSC**. Fluorescence spectrum of the solution was recorded at 485 nm at 10-s intervals for 2 min. k_{obs} for the enzymatic reaction was obtained from the initial linear fluorescence change between 0 and 30 s.

Based on the Ellman method, various concentrations of BChE (from 0 to 0.12 U/mL) were added to buffer solutions (phosphate, 20 mM, pH 7.0) containing 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB, 0.3 mM) and butyrylthiocholine iodide (BTCh, 0.475 mM). Absorbance spectra of the solutions were recorded at 412 nm at 10-s intervals for 2 min. k_{obs} for the enzymatic reaction was obtained from the initial linear fluorescence change between 0 and 30 s. The plot of k_{obs} shows linearity in the range from 0 to 0.12 U/mL for the BChE activity (Fig. S7). Next, a 100-fold diluted serum was added to a buffer solution containing DTNB (0.3 mM) and BTCh (0.475 mM). Absorbance spectra of the solutions were recorded at 412 nm at 10-s intervals for 2 min. k_{obs} for the enzymatic reaction was obtained from the initial linear fluorescence change between 0 and 30 s.

Screening of BChE inhibitors

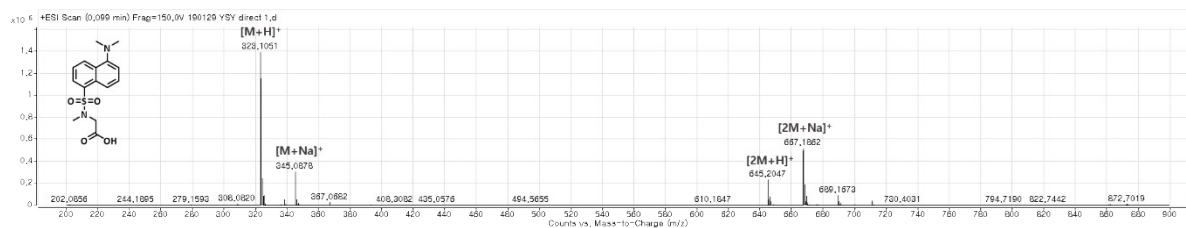
Various concentrations of tacrine (from 0 to 50 nM) were added to buffer solutions (phosphate, 20 mM, pH 7.0) containing BChE (0.5 U/mL), and the solutions were incubated for 15 min. After HSA (20 μ M) and **DSC** (10 μ M) were added to the solutions, the fluorescence intensities of the solutions were recorded at 485 nm at 10-s intervals for 5 min. The inhibition efficiency (IE) was calculated for the half-maximal inhibitory concentration

(IC₅₀) value of the inhibitor: $(IE) = [(F.I.)_{(no\ inhibitor)} - (F.I.)_{(inhibitor)}] / [(F.I.)_{(no\ inhibitor)} - (F.I.)_{(no\ inhibitor\ and\ BChE)}]$, where F.I. refers to the fluorescence intensity at 480 nm after 5 min of incubation. Galantamine (from 0 to 50 μM), chlorpromazine (from 0 to 30 μM), huperzine A (from 0 to 400 μM), and rivastigmine (from 0 to 10 μM) were also tested by the same method.

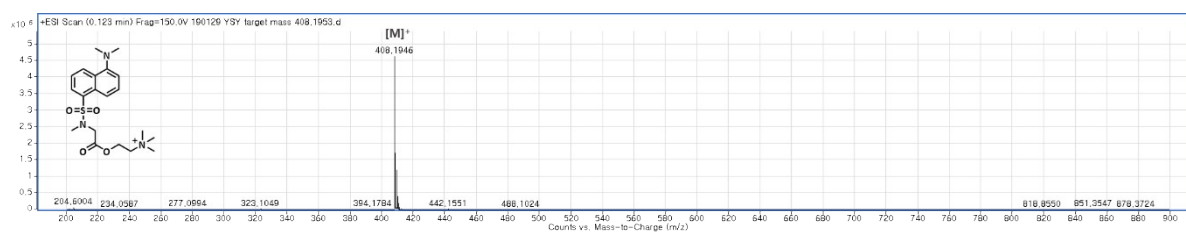
Supplementary result

Mechanism study via HR-MS

(a)



(b)



(c)

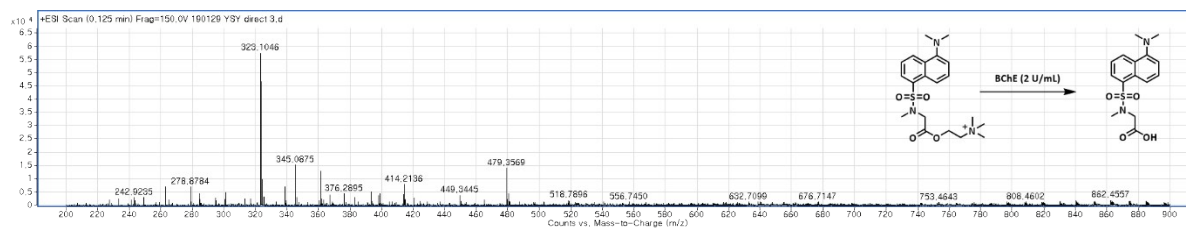
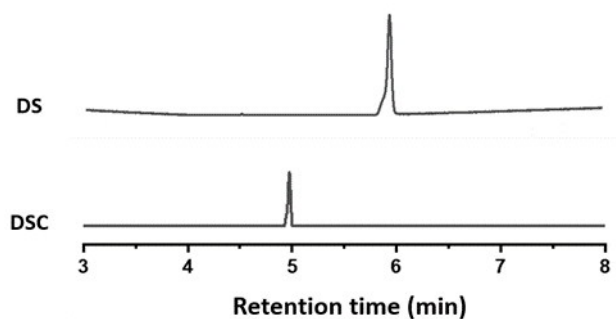


Fig. S1 HR-MS spectra of (a) DS, (b) DSC, and (c) DSC incubated with BChE (2 U/mL) for 2 min in a buffer solution (Phosphate, pH 7.0).

Mechanism study via LC-MS

(a)



(b)

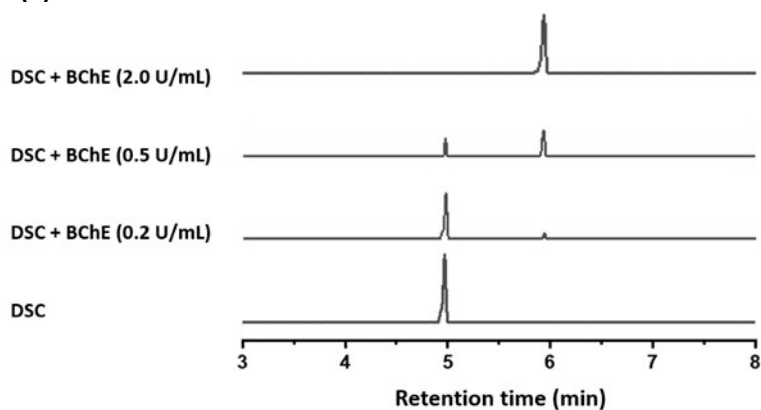


Fig. S2 (a) HR-MS spectra for **DS** and **DSC** in a buffer solution (Phosphate, pH 7.0). (b) LC-MS spectra for **DSC** incubated with BChE (2 U/mL) for 2 min in a buffer solution (Phosphate, pH 7.0).

Effect of various albumin in the assay system

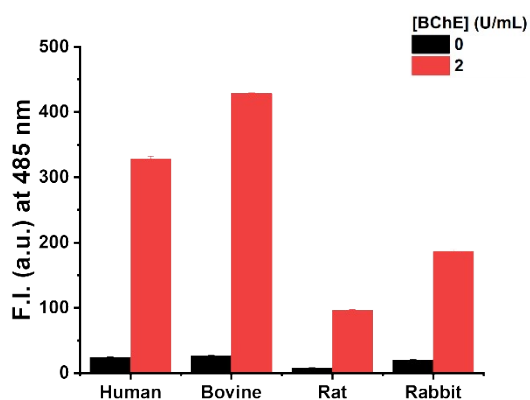


Fig. S3 Fluorescence intensities of **DSC** with various serum albumin (obtained from human, bovine, rat, and rabbit) in buffer solutions (Phosphate, 20 mM, pH 7.0) in the absence (black bar) or presence (red bar) of BChE. [DSC] = 10 μ M, [HSA] = 20 μ M, [BChE] = 2 U/mL, λ_{ex} = 340 nm.

pH test

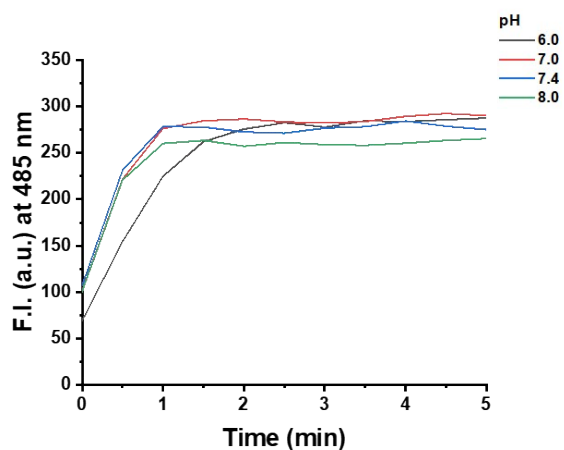


Fig. S4 Fluorescence intensity *versus* time for **DSC** containing HSA and BChE in buffer solutions of different pH (Phosphate, 20 mM, pH 6.0–8.0). [**DSC**] = 10 μ M, [HSA] = 10 μ M, [BChE] = 2 U/mL, λ_{ex} = 340 nm.

Titration for BChE activity

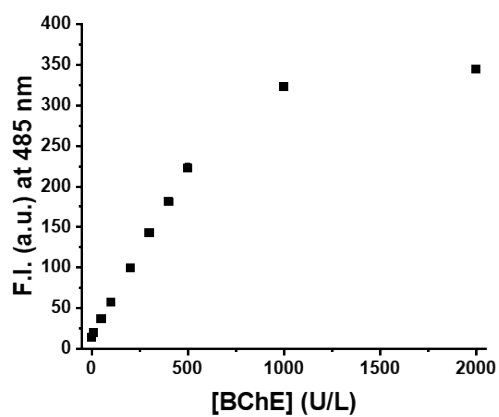


Fig. S5 Fluorescence intensity of **DSC** containing HSA and BChE of various concentrations (0–2 U/mL) in the buffer solutions (Phosphate, 20 mM, pH 7.0) at 2 min. [**DSC**] = 10 μ M, [HSA] = 20 μ M, λ_{ex} = 340 nm.

Selectivity test

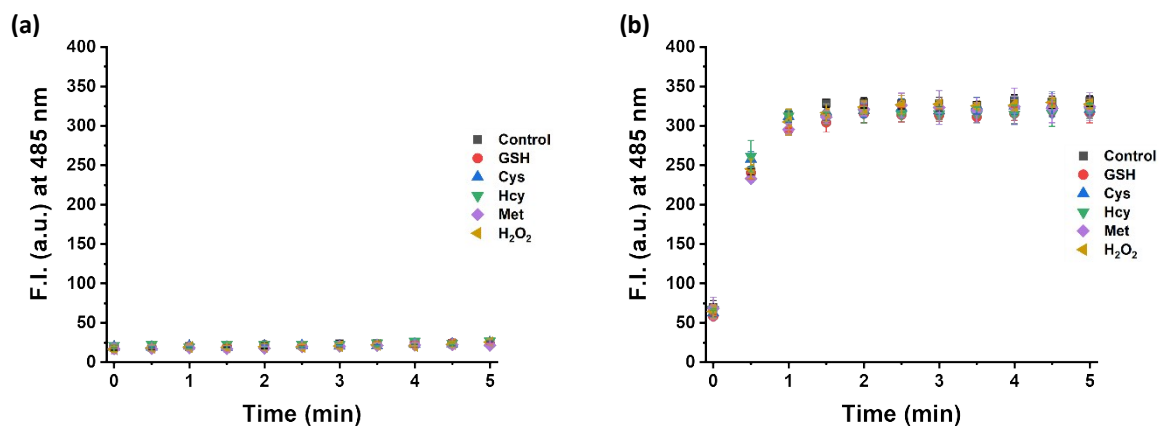


Fig. S6 Fluorescence spectra of **DSC** with various biothiols or H₂O₂ in buffer solutions (Phosphate, 20 mM, pH 7.0) containing HSA in the (a) absence or (b) presence of BChE. [**DSC**] = 10 μ M, [HSA] = 20 μ M, [BChE] = 2 U/mL, [biothiols] = [H₂O₂] = 20 μ M, λ_{ex} = 340 nm.

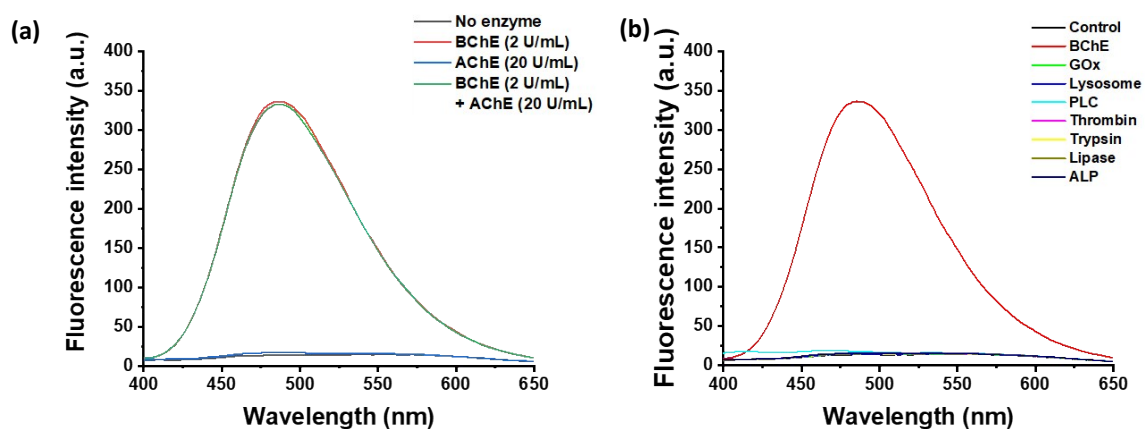


Fig. S7 (a) Fluorescence spectra of **DSC** with BChE, AChE, and BChE+AChE in buffer solutions (Phosphate, 20 mM, pH 7.0) containing HSA at 2 min. (b) Fluorescence spectra of **DSC** with BChE or other enzymes in buffer solutions (Phosphate, 20 mM, pH 7.0) containing HSA at 2 min. [**DSC**] = 10 μ M, [HSA] = 20 μ M, [BChE] = [other enzymes] = 2 U/mL, [AChE] = 20 U/mL, λ_{ex} = 340 nm.

Ellman method

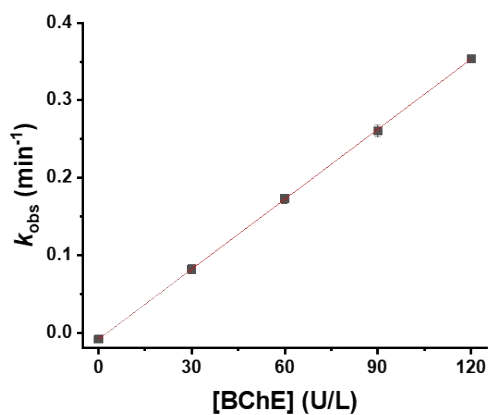


Fig. S8 Plot of k_{obs} for the assay solutions *versus* concentration of BChE by the Ellman method.

Inhibition efficiency of ChE inhibitors

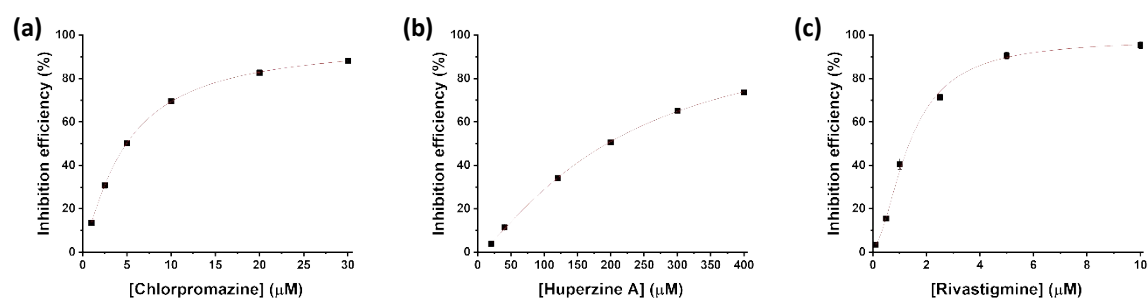


Fig. S9 BChE inhibition efficiency of (a) chlorpromazine, (b) huperzine A, and (c) rivastigmine in the buffer solution (Phosphate, 20 mM, pH 7.0) containing DSC, HSA, and BChE. [DSC] = 10 μM , [HSA] = 20 μM , [BChE] = 0.5 U/mL, λ_{ex} = 340 nm.

NMR spectra

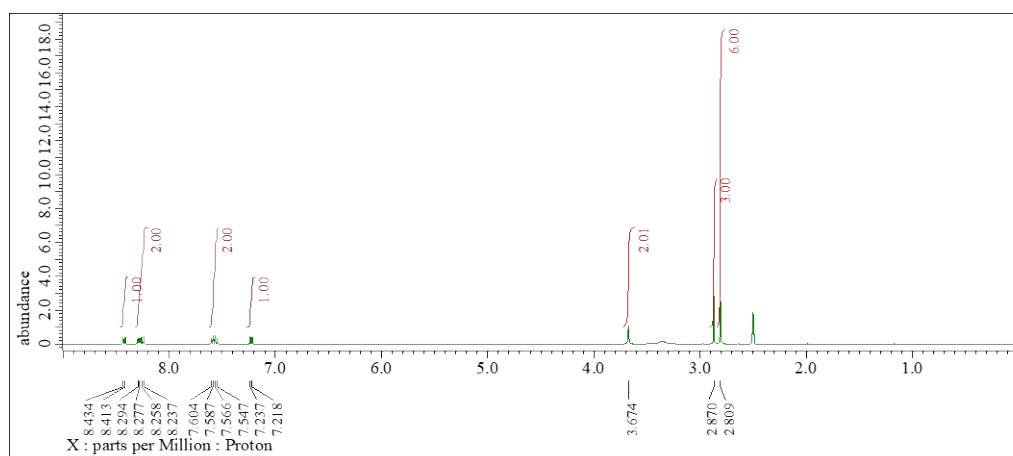


Fig. S10 ¹H NMR spectrum of DS.

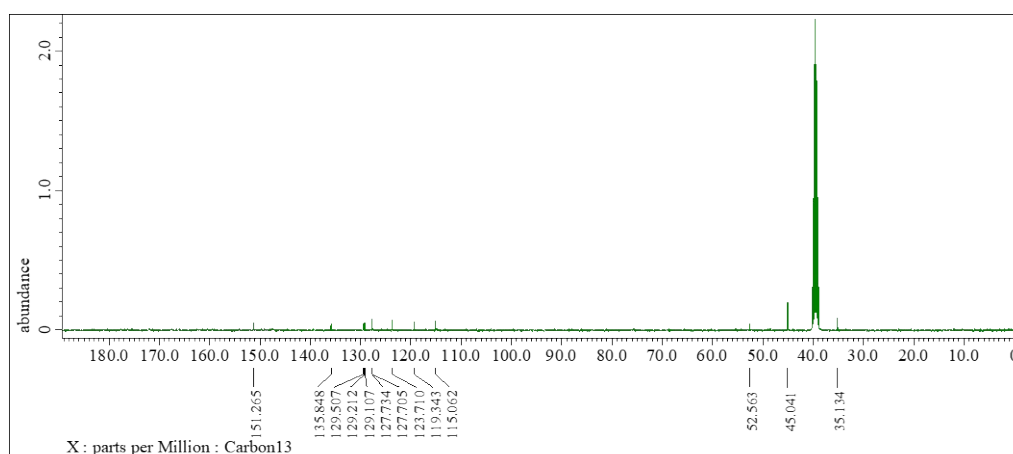


Fig. S11 ¹³C NMR spectrum of DS.

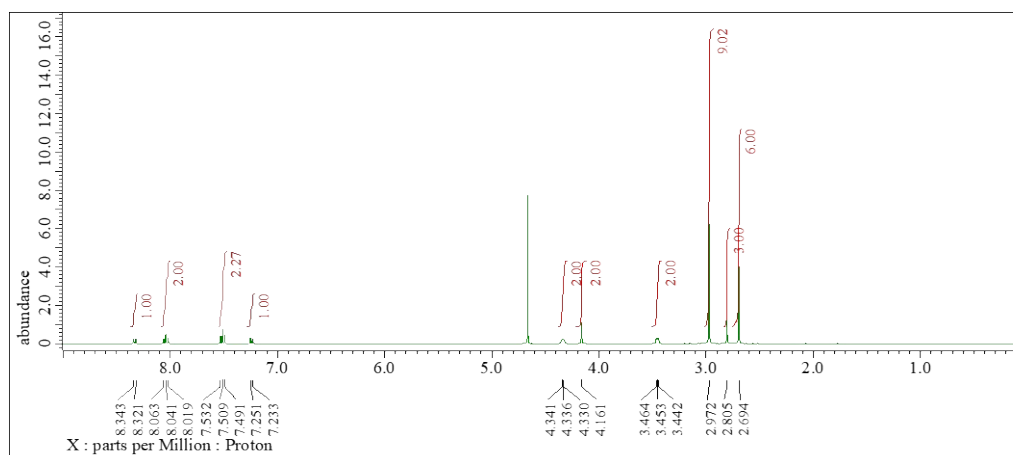


Fig. S12 ¹H NMR spectrum of DSC.

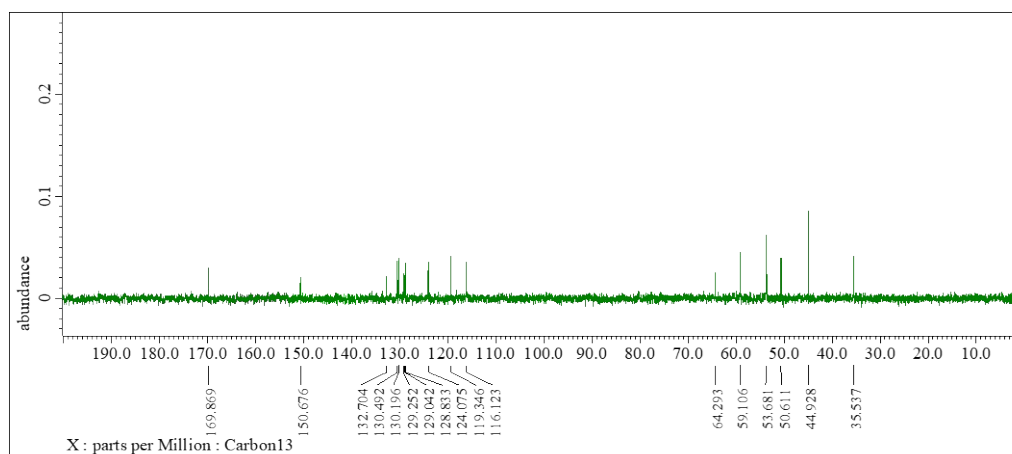


Fig. S13 ^{13}C NMR spectrum of DSC.