Tandem Driven Dynamic Self-Inhibition of Acetylcholinesterase

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General methods

All commercially available starting materials and solvents were of reagent grade and used as received. Acetylcholinesterase (AChE, EC 3.1.1.7) was from *Electrophorus electricus*, purchased from Sigma-Aldrich, type VI-S lyophilized powder (690 U/mg). ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Bruker Avance 400 spectrometer at 400 (100) MHz or Bruker DMX 500 spectrometer at 500 (125) MHz respectively. Chemical shifts are reported as δ values (ppm) with D_2O (^{1}H-NMR δ 4.79) as an internal standard. *J* values are given in Hertz (Hz). d-Buffer solutions were prepared from D_3PO_4 (85% w/w in D_2O) and NaOD (40% w/w in D_2O) using a WinLab Data Line pH meter. UV-Vis measurements were performed using a Perkin Elmer, Lambda 759 UV-Vis spectrometer. Elemental analysis was performed by H. Kolbe Mikroanalytisches Laboratorium, Mülheim an der Ruhr, Germany. High resolution mass spectroscopy (HRMS) was performed by the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. Capillary electrophoresis (CE) was performed using an Agilent HP^{3D}CE instrumentation equipped with a UV diode array detector and ChemStation software (Agilent Technologies Inc., Palo Alto, CA, USA). Thin layer chromatography (TLC) was performed on Polygram® SIL G/UV_{254} silica plates (0.20 mm, Macherey-Nagel) and was visualized with UV-detection. Flash column chromatography was performed on silica gel 60, 0.040-0.063 mm (SDS).

Sodium *S*,*S*-bis(2-sulfoethyl)benzene-1,4-bis(carbothioate) (1)

To a mixture of sodium 2-mercaptoethanesulfonate (184 mg, 1.12 mmol), TEA (100 μL, 0.717 mmol) in diethyl ether (8 mL), terephthaloyl dichloride (111 mg, 0.545 mmol) was added at 0 ºC and stirred for 15 minutes, then warmed to rt and stirred for 7 h. Additional TEA (50 μL, 0.359 mmol) was added three times: 2, 4 and 6 h after the reaction start (total of 250 μL, 1.79 mmol). The mixture was extracted with H_2O (4×3 mL) and the combined aqueous phases were washed with EtOAc (3 mL). After evaporation of H_2O *in vacuo*, the residue was recrystallized from H_2O/EtOH and a white solid was obtained (58 mg, 23%). ^{1}H-NMR (400 MHz, D_2O) δ 8.10 (4H, s), 3.45-3.48 (4H, m), 3.25-3.28 (4H, m); ^{13}C-NMR (125 MHz, D_2O) δ 194.2, 140.4, 127.6, 50.2, 23.8; Elemental analysis (%): found: C 31.54, H 2.70, S 27.83; calc. for $C_{12}H_{12}Na_2O_8S_4$: C 31.44, H 2.64, S 27.98.

Sodium *S*,*S*-bis(2-sulfoethyl)benzene-1,3-bis(carbothioate) (2)

To a mixture of sodium 2-mercaptoethanesulfonate (412 mg, 2.51 mmol) and TEA (300 μL, 2.15 mmol) in diethyl ether (30 mL), isoterephthaloyl chloride (250 mg, 1.23 mmol), was added at 0 °C and stirred for 15 minutes. Additional TEA (250 μL, 1.79 mmol) was added and the reaction mixture was then allowed to warm to rt and stirred for 21 h. The mixture was extracted with H_2O (4x8 mL) and the combined aqueous phases were washed with EtOAc (6 mL). After evaporation of H_2O *in vacuo*, the solid was recrystallized from H_2O/EtOH and a light yellow solid was obtained (171 mg, 30%). ^{1}H NMR (400 MHz, D_2O); δ 8.48 (1H, s), 8.25 (2H, dd, J_1=1.8, J_2=7.9), 7.70

(1H, t, *J* = 7.9 Hz), 3.45-3.49 (4H, m), 3.25-3.29 (4H, m); ^{13}C NMR (125 MHz, D_2O); 193.6, 136.7, 132.2, 129.6, 125.3, 50.2, 23.7; Elemental analysis (%): found: C 31.28, H 2.67, S 28.01; calc. for $C_{12}H_{12}Na_2O_8S_4$: C 31.44, H 2.64, S 27.98.

Sodium 2-(benzoylthio) ethanesulfonate (3)

To a mixture of sodium 2-mercaptoethanesulfonate (138 mg, 0.84 mmol) and TEA (150 μL, 1.07 mmol) in diethyl ether (12 mL), benzoyl chloride (354 mg, 2.52 mmol), was added at 0 °C and stirred for 15 minutes. Additional TEA (150 μL, 1.07 mmol) was added and the reaction mixture was allowed to warm to rt and stirred for 20 h. The mixture was extracted with H_2O (2x6 mL) and the combined aqueous phases were washed with EtOAc (6 mL). After evaporation of H_2O *in vacuo*, the residue was washed with CH_3CN, and then filtered to yield a white solid (100 mg, 44%). m. p. 318.2-318.9 ^{o}C; ^{1}H NMR (500 MHz, D_2O); δ 7.98 (2H, d, *J*=7.4), 7.70 (1H, t, *J*=7.4), 7.55 (2H, t, *J*=7.9), 3.40-3.43 (2H, m), 3.22-3.25 (2H, m); ^{13}C NMR (125 MHz, D_2O); 195.1, 136.3 134.3, 129.0, 127.1, 50.3, 23.5; ^{13}C NMR (125 MHz, D_2O); 195.1, 136.3 134.3, 129.0, 127.1, 50.3, 23.5; HRMS: found 290.9729; calc. for $C_9H_9Na_2O_4S_2$ [M+Na^+] 290.9738.

2-(4-(carbonyl)benzoylthio)-N,N,N-trimethylethanaminium chloride (4)

Thiocholine (200 mg, 1.26 mmol), TEA (229 μL, 1.63 mmol) and terephthaloyl chloride (100 mg, 0.50 mmol) was added to acetonitrile (6.75 mL) under stirring at 0 °C. The mixture was stirred for 15 min and then overnight at rt. The resulting mixture was filtered and washed with acetonitrile and ether. The solid was partly dissolved in water at 95 °C and filtered while hot. The water was evaporated *in vacuo*, and the crude product was purified by column chromatography (acetonitrile) to yield a pale yellow solid. (48 mg, 22%). m. p. 277.9-278.3 ^{o}C; ^{1}H NMR (400 MHz, D_2O); δ 8.02 (4H, s), 3.50-3.54 (4H, m), 3.44-3.48 (4H, m), 3.18 (18H, s); ^{13}C NMR (125 MHz, D_2O); 192.9, 140.0, 127.8, 64.4, 52.9, 21.8. *Cf.* Figure S10 for CE electropherogram.

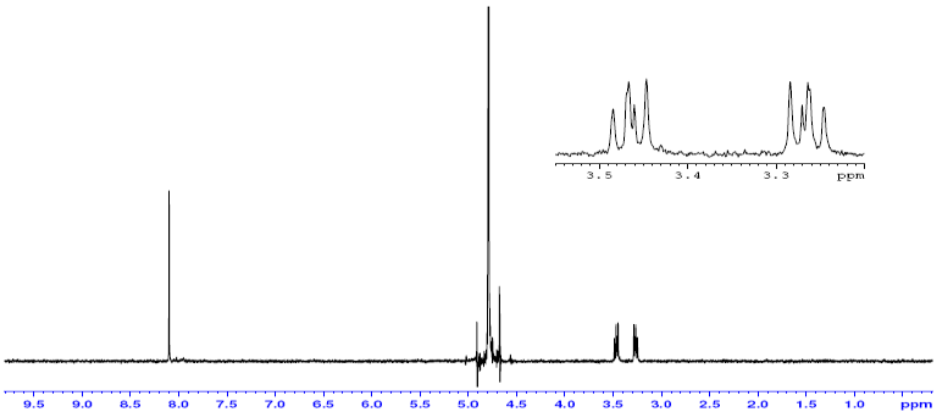

Figure S1: ^{1}H-NMR spectra of compound **1** in D_2O.

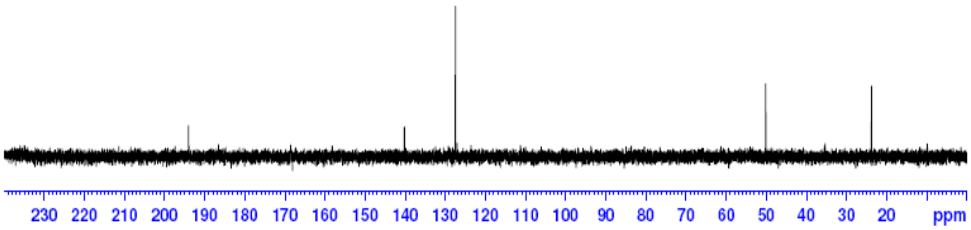

Figure S2: ^{13}C-NMR spectra of compound **1** in D_2O.

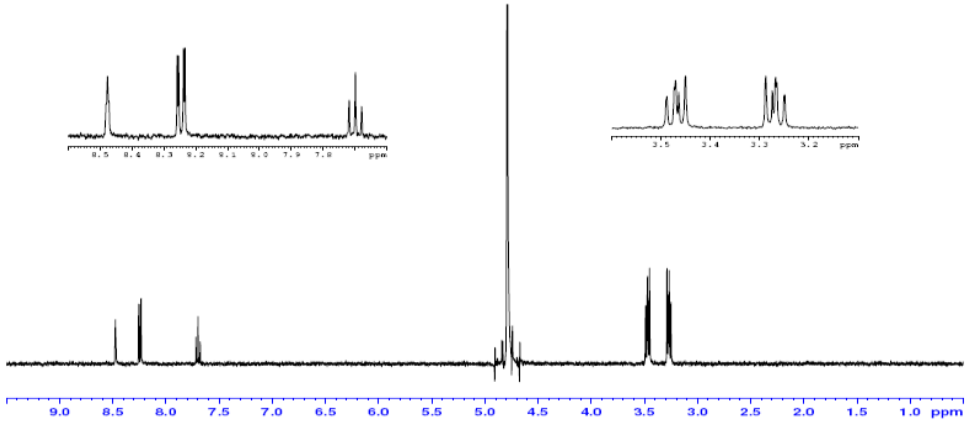

Figure S3: ^{1}H-NMR spectra of compound **2** in D_2O.

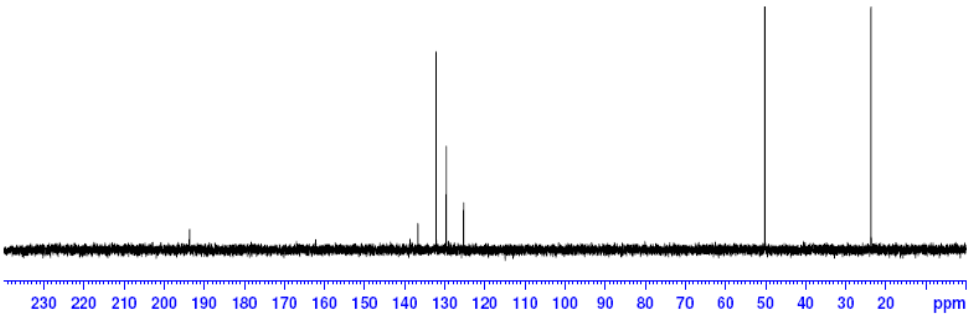

Figure S4: ^{13}C-NMR spectra of compound **2** in D_2O.

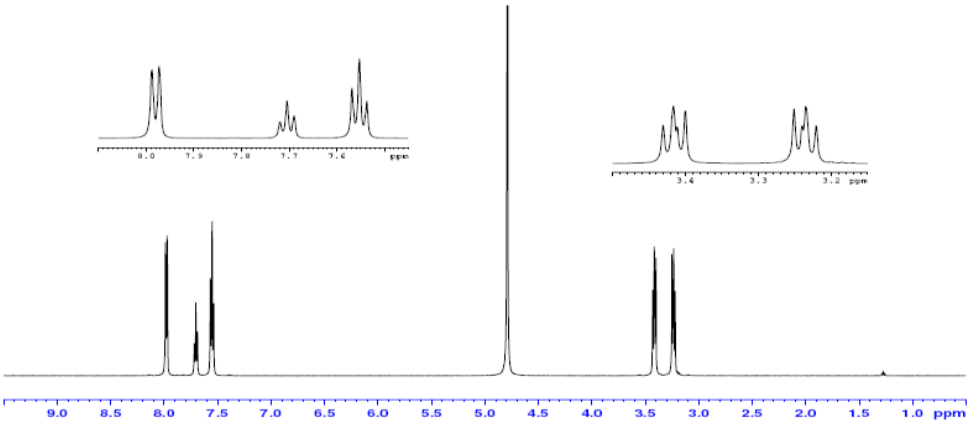

Figure S5: ^{1}H-NMR spectra of compound **3** in D_2O.

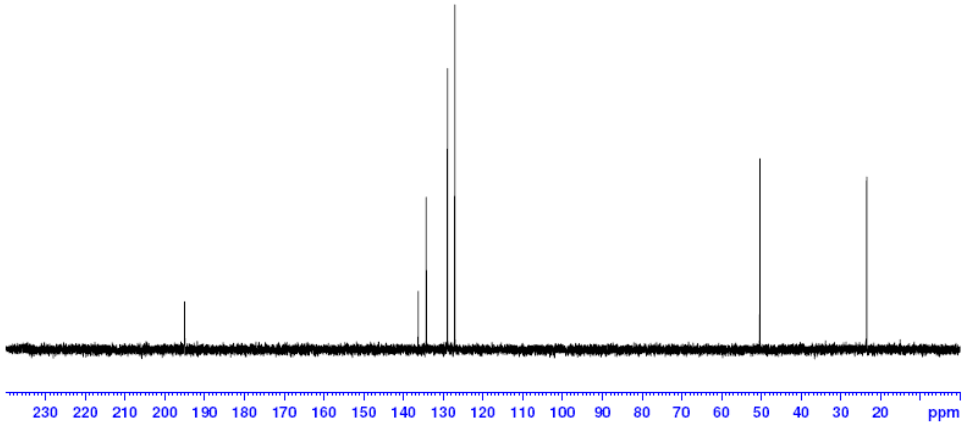

Figure S6: ^{13}C-NMR spectra of compound **3** in D_2O.

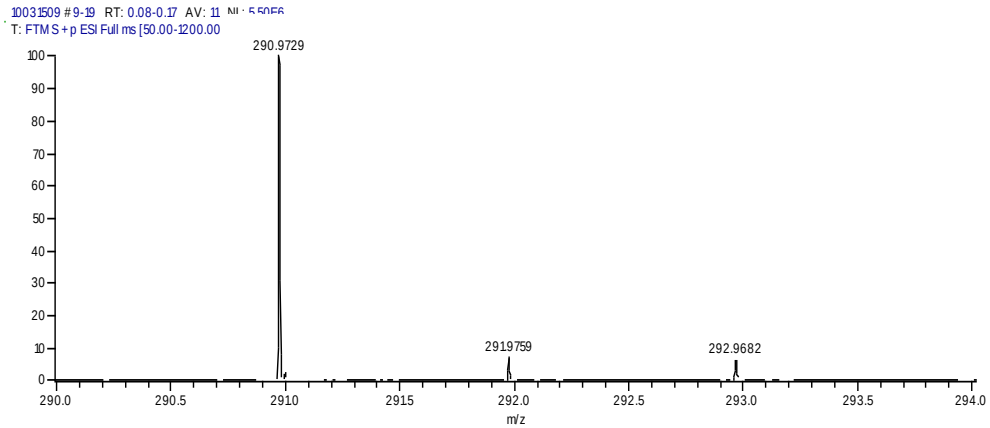

Figure S7: HRMS spectra of compound **3**.

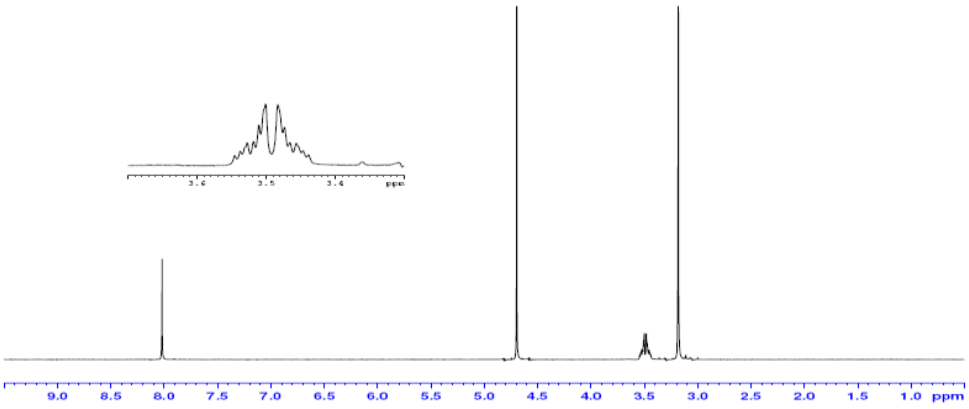

Figure S8: ^{1}H-NMR spectra of compound **4** in D_2O.

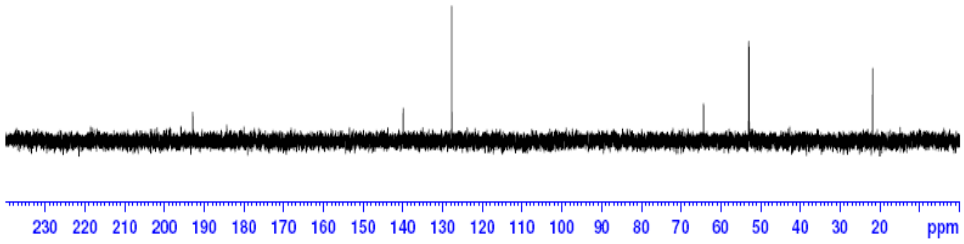

Figure S9: ^{13}C-NMR spectra of compound **4** in D_2O.

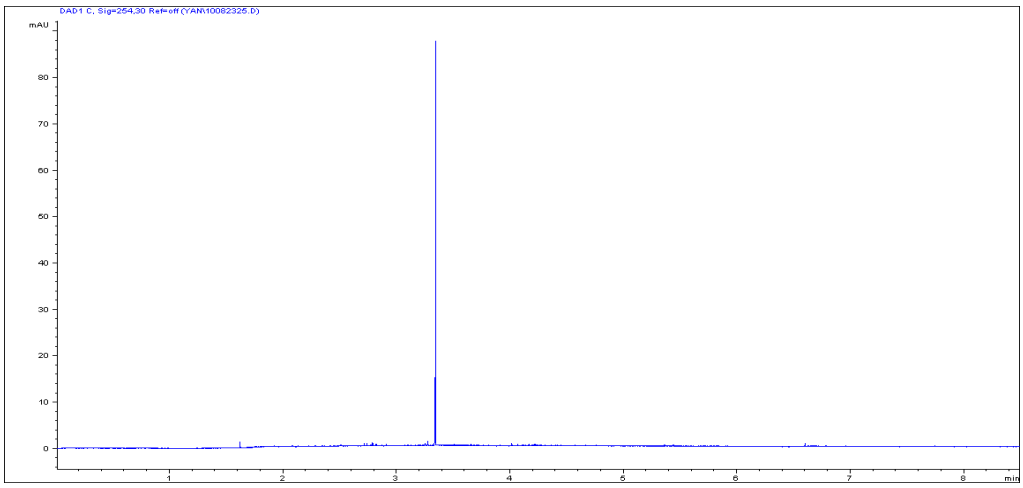

Figure S10: CE electropherogram of compound **4**. Untreated fused-silica capillary (50 µm ID, 375 µm OD, Polymicro Technologies) was used while applying a voltage of 20 kV. Sodium dihydrogenphosphate monohydrate (100 mM, pH 4.4) was used as carrier electrolyte. Compound **4** (200 µM) was injected for 10 seconds under a pressure of 34 mbar. The measurements were performed at 25 °C at 254 nm.

Generation of self-inhibition systems

Stock solutions of each of the compounds/constituents were mixed together to give a final concentration of 2 mM of acetylthiocholine (ATCh), 0.2 mM each of sodium *S*,*S*-bis(2-sulfoethyl)benzene-1,4-bis(carbothioate), sodium *S*,*S*-bis(2-sulfoethyl) benzene-1,3-bis(carbothioate) and sodium 2-(benzoylthio)ethanesulfonate in a 50 mM D_3PO_4-NaOD buffer solution at pD 7.4. For the hydrolysis of ATCh, 0.2 mU of AChE was further added. The formation of acetate was followed by ^{1}H-NMR at different time intervals.

Control experiments

Control experiments were carried out to confirm the self-inhibitory effects (Figure S11). As expected, sodium 2-mercaptoethanesulphonate reduced the hydrolysis rate to a minor extent since it can undergo transthiolesterification with ATCh, lowering the concentration of available substrate. The combination of compound **1** with ACh resulted in no visible inhibition.

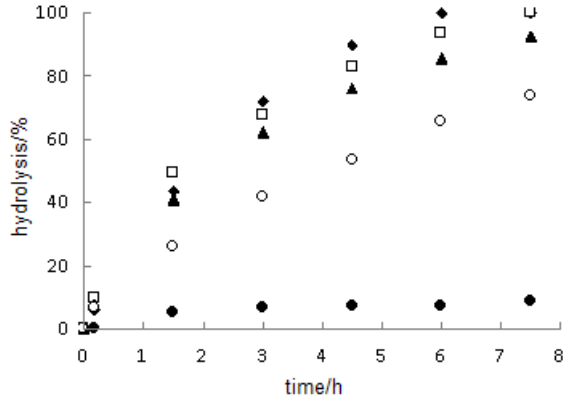

Figure S11. Control experiments carried out using 2 mM of ATCh or ACh, 0 or 0.2 mM of compound **1** or 2-mercaptoethanesulfonate, and 0.2 mU of AChE: ◆ ACh and AChE; □ ACh, AChE and compound **1**; ▲ ATCh and AChE; ○ ACh, AChE and 2-mercaptoethanesulfonate; ● ATCh, AChE and compound **1**.

Typical NMR spectra of the self-inhibition process

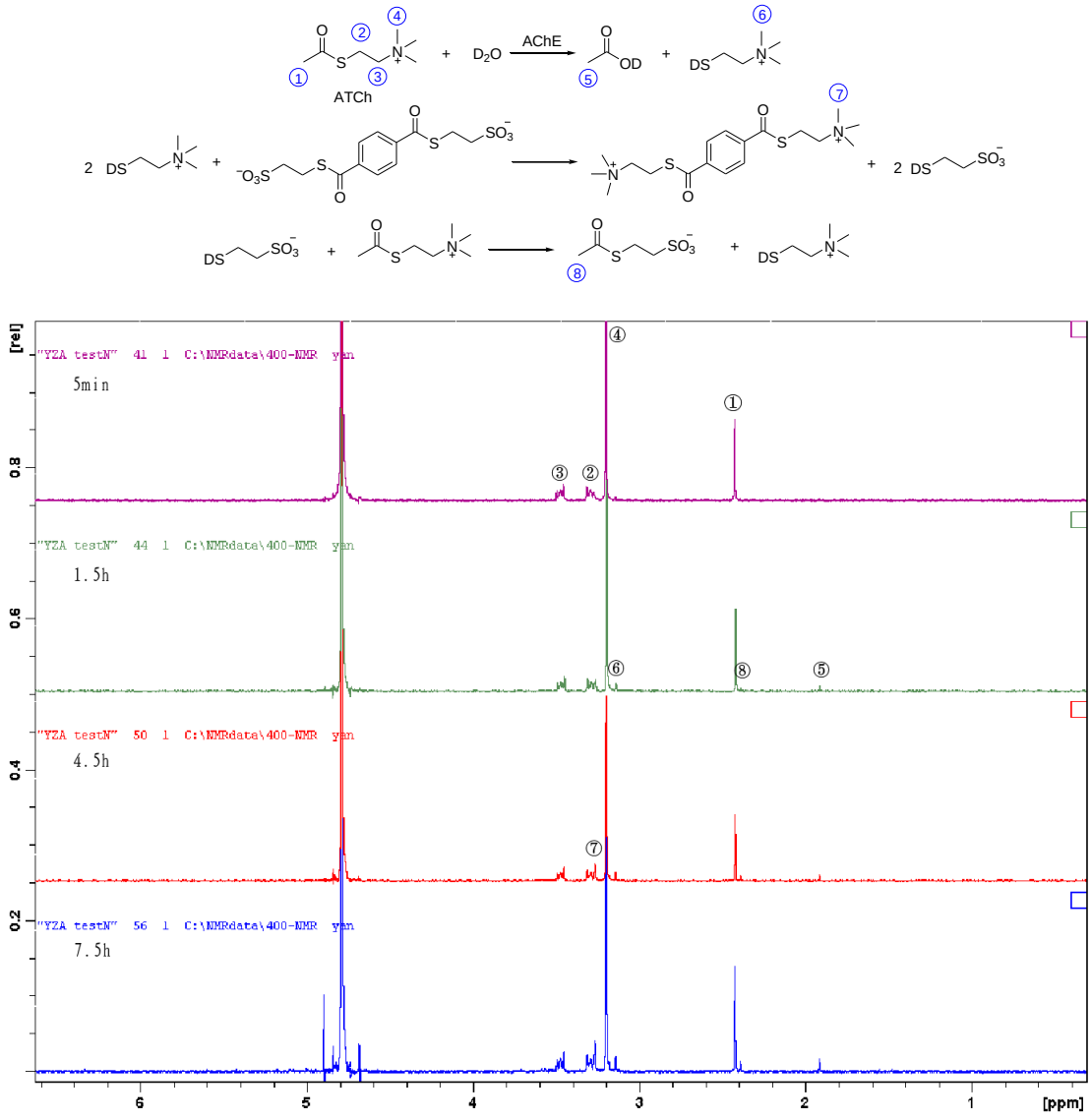

Figure S12. ^{1}H-NMR spectra for self-inhibition reactions in system containing ATCh, AChE and compound **1** in buffer solution at different times. Labeled peaks correspond to protons in the reaction scheme.

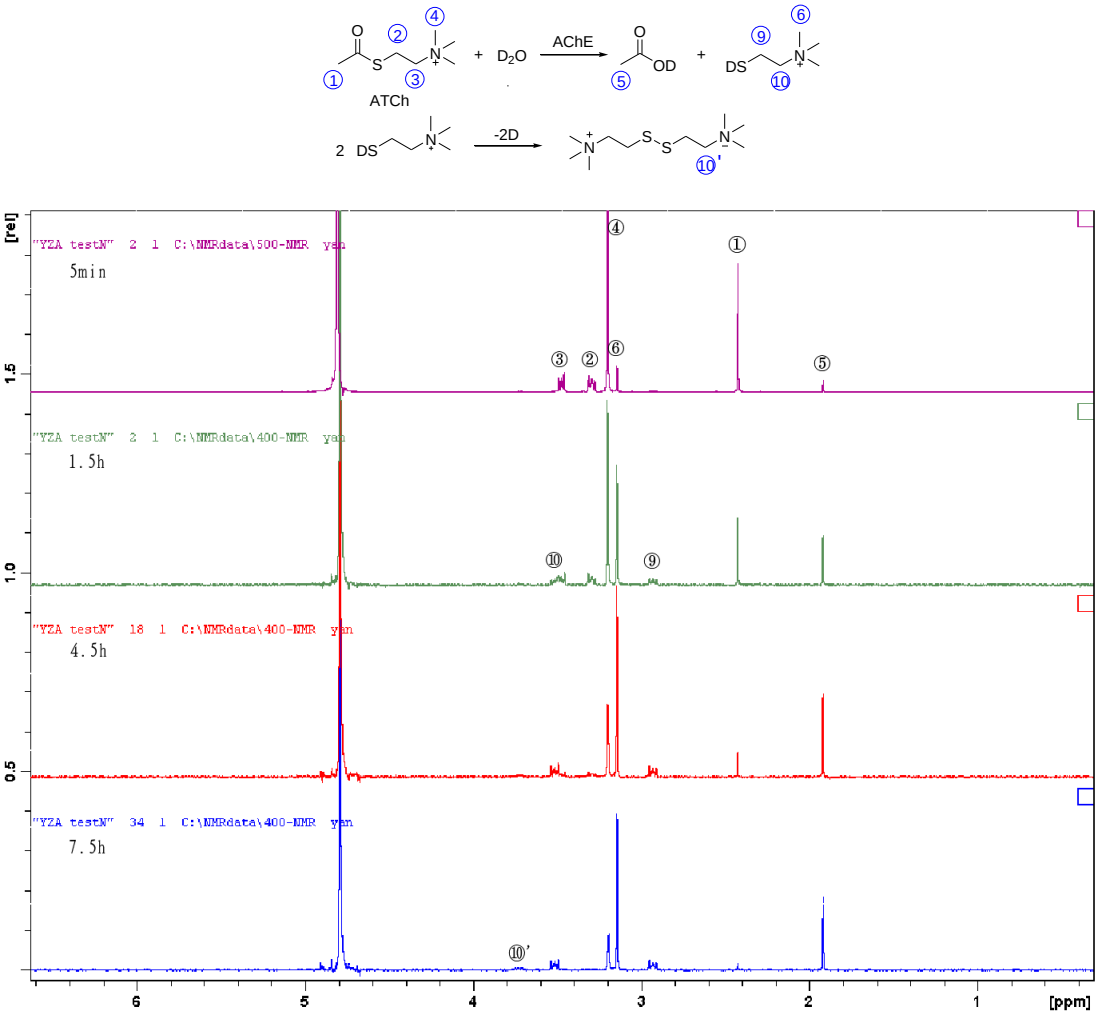

Figure S13. ^{1}H-NMR spectra for reactions in system containing ATCh and AChE in buffer solution at different times. Labeled peaks correspond to protons in the reaction scheme.

General kinetics studies and determination of K_M

The Ellman method was used to determine AChE activity in the absence or presence of inhibitor (compound **4**).[1] To a 50 mM phosphate buffer solution (pH 7.4), was added 5,5'-dithiobis(2-nitrobenzoate) (DTNB, 250 μM) with different concentrations of ATCh to make a total volume of 2 mL. AChE (0.5 U in 1 mL buffer) was added to initiate the hydrolysis process. The formation of 5-thio-2-nitrobenzoate (TNB) was recorded by UV-Vis spectroscopy at 412 nm using a time-drive analysis method over the first 3 min. Blank reactions were performed using buffer solution. K_M was obtained using non-linear regression analysis (Figure S14) (GraphPad Software, San Diego, CA, USA).

Determination of inhibition constants

K_i and αK_i were determined using the Ellman method by adding the inhibitor (compound **4)** into the solution. The concentration of ATCh was varied from 8.3 μM to 233.3 μM, while the concentration of compound **4** was fixed to 0 nM, 33 nM, and 100 nM, respectively. The inhibition constants were estimated using non-linear regression analysis (GraphPad).

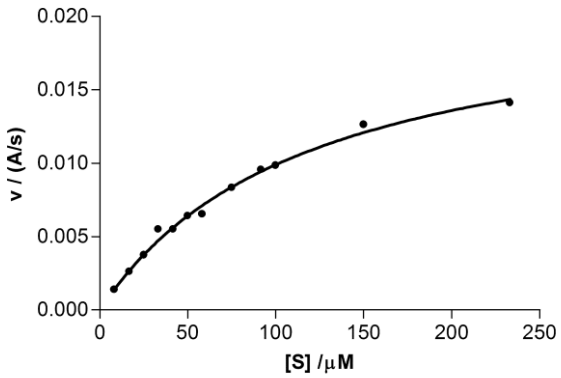

Figure S14. UV-Vis analysis of AChE hydrolysis. K_M = 114 μM.

References

1. C. L. Ellman, K. D. Courtney, V. Andres, Jr., R. M. Featherstone, *Biochem. Pharmacol.* 1961, **7**, 88.