A Potent, Versatile Disulfide-Reducing Agent from Aspartic Acid

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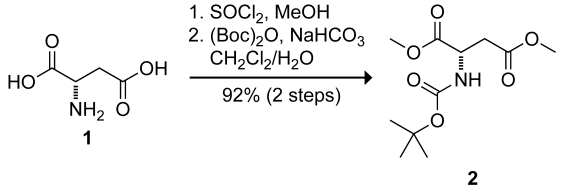
I. General

Commercial reagents were used without further purification. Dithiothreitol (DTT) was from Research Products International (Mt. Prospect, IL). Bis(2-mercaptoethyl)sulfone (BMS) was from Santa Cruz Biotechnology (Santa Cruz, CA). Papain (lyophilized powder from papaya latex), creatine kinase (lyophilized powder from rabbit muscle), hexokinase (lyophilized powder from *Saccharomyces cerevisiae*), glucose-6-phosphate dehydrogenase (ammonium sulfate suspension from baker's yeast), N_α-benzoyl-L-arginine-4-nitroanilide hydrochloride, (*S*)-methyl methanethiosulfonate (Kenyon's reagent), *trans*-4,5-dihydroxy-1,2-dithiane (oxidized DTT), oxidized L-glutathione, oxidized 2-mercaptoethanol, and DOWEX 50WX4-400 ion-exchange resin were from Sigma Chemical (St. Louis, MO). Bis(2-mercaptoethyl) sulfone disulfide (oxidized BMS) was synthesized as reported previously.[1]

All glassware was oven or flame-dried, and reactions were performed under N_2(g) unless stated otherwise. Dichloromethane, diethyl ether, and tetrahydrofuran were dried over a column of alumina. Dimethylformamide and triethylamine were dried over a column of alumina and purified further by passage through an isocyanate scrubbing column. Flash chromatography was performed with columns of 40–63 Å silica, 230–400 mesh (Silicycle, Québec City, Canada). Thin-layer chromatography (TLC) was performed on plates of EMD 250-μm silica 60-F_{254}. The term "concentrated under reduced pressure" refers to the removal of solvents and other volatile materials using a rotary evaporator at water aspirator pressure (<20 torr) while maintaining the water-bath temperature below 40 °C. Residual solvent was removed from samples at high vacuum (<0.1 torr). The term "high vacuum" refers to vacuum achieved by a mechanical belt-

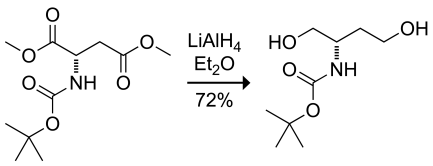
drive oil pump. ^{1}H NMR spectra were acquired at ambient temperature with a Bruker DMX-400 Avance spectrometer at the National Magnetic Resonance Facility at Madison (NMRFAM) and referenced to TMS or residual protic solvent. ^{13}C NMR spectra were acquired with a Varian MercuryPlus 300 and referenced to residual protic solvent. Electrospray ionization (ESI) mass spectrometry was performed with a Micromass LCT at the Mass Spectrometry Facility in the Department of Chemistry at the University of Wisconsin–Madison. Ellman's assay for sulfhydryl groups was performed with a Varian Cary 50 Bio UV–Vis spectrophotometer. UV absorbance spectra of oxidized DTBA and oxidized DTT were acquired with a Varian Cary 300 Bio UV–Vis spectrophotometer. Thiol pK_a values were determined by using a Varian Cary 50 Bio UV–Vis spectrophotometer. Equilibrium, reduction potential, and kinetic studies on peptides and small molecules were performed on an analytical HPLC (Waters system equipped with a Waters 996 photodiode array detector, Empower 2 software and a Varian C18 reverse phase column). Kinetic studies on proteins were carried out using a Varian Cary 300 Bio UV–Vis spectrometer with a Cary temperature controller.

II. Chemical Syntheses



L-Aspartic acid (**1**; 5.002 g, 37.58 mmol) was added to an oven-dried round-bottom flask and placed under an atmosphere of dry N_2(g). The starting material was then dissolved partially with 60 mL of anhydrous methanol, and the mixture was cooled to 0 °C. Once the desired temperature was reached, thionyl chloride (8.2 mL, 110 mmol) was added drop-wise. After the addition was complete, the reaction mixture became homogenous, and was warmed slowly to room temperature and left to stir for 14 h. The reaction mixture was then concentrated under reduced pressure, and the resulting diester was dissolved in 150 mL of DCM and 100 mL of water. To this biphasic solution was added sodium bicarbonate (4.212 g, 50.14 mmol) and di-*t*-butyl dicarbonate (9.841 g, 45.09 mmol), and the reaction mixture was heated at reflux for 4 h. After the reaction was confirmed to be complete by TLC, the reaction mixture was allowed to cool to room temperature. The organic layer was separated, and the aqueous layer was extracted three times with 150 mL of DCM. The organic extracts were combined, washed with 250 mL of saturated NaCl(aq), dried over $MgSO_4$(s), and concentrated under reduced pressure. Flash chromatography (35% v/v ethyl acetate in hexanes) was used to isolate **2** as a white solid (9.080 g, 92%, 2 steps).

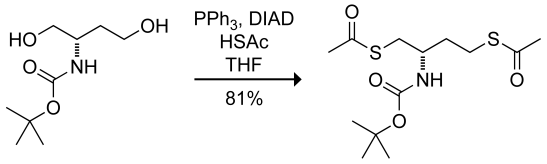
^{1}H NMR (400 MHz, $CDCl_3$) δ = 5.49 (d, J = 8.3 Hz, 1H), 4.60–4.57 (m, 1H), 3.76 (s, 3H), 3.70 (s, 3H), 3.01 (dd, J = 17, 4.4 Hz, 1H), 2.83 (dd, J = 17.0, 4.7), 1.45 (s, 9H); **^{13}C NMR (75 MHz, $CDCl_3$)** δ = 171.6, 171.5, 155.5, 80.3, 52.8, 52.1, 50.0, 36.8, 28.4; **HRMS** (ESI) calculated for $[C_{11}H_{19}NO_6Na]^+$ ($M+Na^+$) requires m/z = 284.1105, found 284.1113.



2 **3**

An oven-dried round-bottom flask was charged with lithium aluminum hydride (0.870 g, 22.9 mmol) and placed under an atmosphere of dry N_2(g). The flask was cooled to 0 °C in an ice bath, and 100 mL of anhydrous diethyl ether was added. In a separate dry round-bottom flask, compound **2** (2.021 g, 7.735 mmol) was dissolved in 50 mL of anhydrous diethyl ether. Sonication was required to make the solution completely homogenous. The ester was then added drop-wise to the reaction mixture. Once the addition was complete, the reaction mixture was stirred at 0 °C for an additional 30 min, warmed to room temperature, and allowed to react for an additional 2 h. Subsequently, the reaction mixture was quenched at 0 °C by the slow, sequential addition of 0.87 mL of water, 0.87 mL of 15% w/w NaOH, and 2.6 mL of water. The mixture was left to stir at room temperature for 1 h. The aluminum salts were collected by vacuum filtration, and subjected to continuous solid–liquid extractions with dichloromethane using a Soxhlet apparatus. The organic extracts and the original organic filtrate were combined and concentrated under reduced pressure. Flash chromatography (ethyl acetate) was used to isolate **3** as a white solid (1.310 g, 82%). Compound **3** had been prepared from L-aspartic acid by a different route.[2]

^{1}H NMR (400 MHz, DMSO-d_6) δ = 6.46 (d, J = 8.8 Hz, 1H), 4.56 (t, J = 5.7 Hz, 1H), 4.34 (t, J = 5.1 Hz, 1H), 3.46–3.37 (m, 3H), 3.32 (dt, J = 10.6, 5.4 Hz, 1H), 3.23 (dt, J = 10.6, 5.9 Hz, 1H), 1.69–1.61 (m, 1H), 1.45–1.37 (m, 1H), 1.37 (s, 9H); **^{13}C NMR (75 MHz, $CDCl_3$)** δ = 157.2, 80.1, 65.4, 58.9, 49.5, 35.0, 28.5; **HRMS** (ESI) calculated for $[C_9H_{19}NO_4Na]^+$ ($M+Na^+$) requires m/z = 228.1207, found 228.1201.

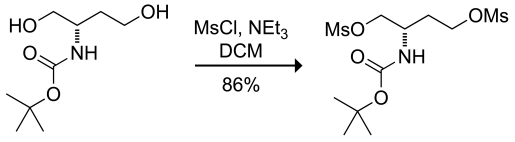


3 **4**

A dry round-bottom flask was charged with triphenylphosphine (1.711 g, 6.523 mmol) and placed under an atmosphere of dry N_2(g). Anhydrous THF (27 mL) was then added, and the solution was placed in an ice bath and cooled to 0 °C. Diisopropyl azodicarboxylate (1.3 mL, 6.6 mmol) was added drop-wise to the flask. Once the addition was complete, the reaction mixture was allowed to stir for an additional 20 min. Compound **3** (0.559 g, 2.72 mmol) in 10 mL of dry THF and thioacetic acid (0.47 mL, 6.6 mmol) was then added with stirring. The reaction mixture was stirred at 0 °C for 1 h, and then at room temperature for 16 h. (Longer reaction times resulted in lower yields.) The mixture was concentrated under reduced pressure. Flash chromatography (30% v/v ethyl acetate in hexanes) was used to isolate **4** as a white solid (0.711 g, 81%). Compound **4** had been prepared from L-aspartic acid by a different route.[2]

^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.59 (d, J = 7.9 Hz, 1H), 3.85–3.76 (m, 1H), 3.12–2.95 (m, 3H), 2.82 (ddd, J = 13.7, 8.5, 7.1 Hz, 1H), 2.36 (s, 3H), 2.33 (s, 3H), 1.84–1.75 (m, 1H), 1.74–1.64 (m, 1H), 1.44 (s, 9H); **^{13}C NMR (75 MHz, $CDCl_3$)** δ = 195.9, 195.6, 155.6, 79.7, 50.1,

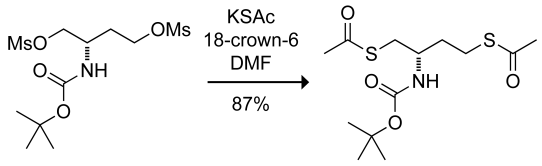
34.5, 33.8, 30.73, 30.71, 28.5, 25.9; **HRMS** (ESI) calculated for $[C_{13}H_{23}NO_4S_2Na]^+$ ($M+Na^+$) requires m/z = 344.0961, found 344.0962.



3 **6**

A dry round-bottom flask was charged with **3** (1.178 g, 5.739 mmol) and placed under dry N_2(g), Anhydrous DCM (125 mL) was then added, and the solution was cooled to 0 °C. Triethylamine (4.0 mL, 29 mmol) was added, followed by slow drop-wise addition of methanesulfonyl chloride (MsCl) (1.0 mL, 13 mmol). After stirring at 0 °C for 30 min, the reaction mixture was allowed to warm slowly to room temperature and left to react for an additional 30 min. The reaction mixture was quenched by the addition 100 mL of water, and extracted with DCM. The combined organic extracts were washed with brine, dried over $MgSO_4$(s), and concentrated under reduced pressure. Flash chromatography (60% v/v ethyl acetate in hexanes) was used to isolate **6** as a white solid (1.782 g, 86%).

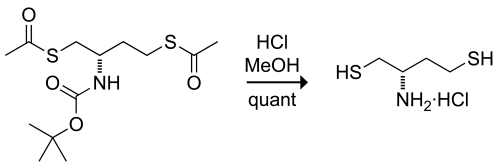
^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.81 (d, J = 9.7 Hz, 1H), 4.39–4.26 (m, 4H), 4.10–4.05 (m, 1H), 3.06 (s, 3H), 3.05 (s, 3H), 2.13–1.96 (m, 2H), 1.48 (s, 9H); **^{13}C NMR (75 MHz, $CDCl_3$)** δ = 155.4, 80.6, 71.0, 66.3, 47.0, 37.7, 37.6, 31.2, 28.5; **HRMS** (ESI) calculated for $[C_{11}H_{23}NO_8S_2Na]^+$ ($M+Na^+$) requires m/z = 384.0758, found 384.0775.



6 **4**

Compound **6** (0.610 g, 1.688 mmol), potassium thioacetate (0.482 g, 4.22 mmol), and 18-crown-6 (1.351 g, 5.111 mmol) were added to a dry round-bottom flask and dissolved with 150 mL of anhydrous DMF. The reaction mixture was stirred under under dry N_2(g) for 24 h. The DMF was removed under reduced pressure. Flash chromatography (30% v/v ethyl acetate in hexanes) was used to isolate **4** as a white solid (0.475 g, 87%). Compound **4** had been prepared from L-aspartic acid by a different route.[2]

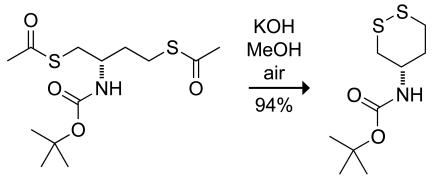
^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.59 (d, J = 7.9 Hz, 1H), 3.85–3.76 (m, 1H), 3.12–2.95 (m, 3H), 2.82 (ddd, J = 13.7, 8.5, 7.1 Hz, 1H), 2.36 (s, 3H), 2.33 (s, 3H), 1.84–1.75 (m, 1H), 1.74–1.64 (m, 1H), 1.44 (s, 9H); **^{13}C NMR (75 MHz, $CDCl_3$)** δ = 195.9, 195.6, 155.6, 79.7, 50.1, 34.5, 33.8, 30.73, 30.71, 28.5, 25.9; **HRMS** (ESI) calculated for $[C_{13}H_{23}NO_4S_2Na]^+$ ($M+Na^+$) requires m/z = 344.0961, found 344.0962.



4 **5** (DTBA·HCl)

Compound **4** (0.601 g, 1.87 mmol) was added to a flame-dried round-bottom flask under dry N_2(g). Anhydrous methanol (20 mL) was added, followed by 10 mL of 3 N HCl in methanol. The reaction mixture was heated at reflux for 4 h, concentrated under reduced pressure, and stored *in vacuo* with P_2O_5 and KOH for 48 h.[3] (Scratching the bottom of the flask facilitated crystal formation.) Compound **5** (DTBA·HCl) was rinsed with cold toluene, and isolated by vacuum filtration as a white solid (0.320 g, quant). DTBA made in this manner was determined to be 99% pure according to Ellman's assay for sulfhydryl groups (*vide infra*).[4]

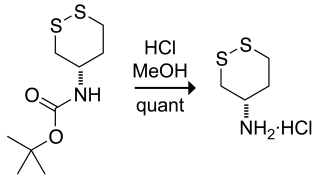
^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.29 (s, 3H), 3.34–3.32 (m, 1 H), 2.96 (t, J = 8.7 Hz, 1H), 2.81–2.75 (m, 2H), 2.60–2.56 (m, 3H), 1.95–1.86 (m, 2H); **^{13}C NMR (75 MHz, DMSO-d_6)** δ = 51.2, 35.0, 26.0, 19.6; **HRMS** (ESI) calculated for $[C_4H_{12}NS_2]^+$ (M^+) requires m/z = 138.0406, found 138.0405.



4 **7**

Compound **4** (0.482 g, 1.50 mmol) and potassium hydroxide (0.340 g, 6.06 mmol) were dissolved in 50 mL of methanol, and the resulting solution was stirred for 16 h while bubbling a light stream of air through the solution. The methanol was removed under reduced pressure, and the mixture was extracted with DCM, washed with brine, and dried over $MgSO_4$(s). Flash chromatography (20% v/v ethyl acetate in hexanes) was used to isolate **7** as a white solid (0.331 g, 94%).

^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.08 (d, J = 7.9 Hz, 1H), 3.53–3.41 (m, 1H), 3.07–3.01 (m, 1H), 2.91–2.85 (m, 2H), 2.60 (dd, J = 13.0, 10.5 Hz, 1 H), 2.08–2.03 (m, 1H), 1.67–1.57 (m, 1H), 1.38 (s, 9H); **^{13}C NMR (75 MHz, DMSO-d_6)** δ = 155.2, 78.7, 49.3, 37.9, 34.9, 34.5, 28.9; **HRMS** (ESI) calculated for $[C_9H_{17}NO_2S_2]^+$ (M^+) requires m/z = 258.0593, found 258.0602.



7 **8** (oxidized DTBA·HCl)

Compound **7** (0.402 g, 1.71 mmol) was added to a round-bottom flask. Anhydrous methanol (20 mL) was added, followed by 10 mL of 3 N HCl in methanol. The reaction mixture was heated at reflux for 4 h under N_2(g), concentrated under reduced pressure, and stored *in vacuo* with P_2O_5 and KOH for 24 h. (Scratching the bottom of the flask facilitated crystal formation.) Compound **8**, oxidized DTBA·HCl, was isolated as a white solid (0.289 g, quant).

^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.29 (s, 3H), 3.43–3.37 (m, 1H), 3.15–3.08 (m, 2H), 3.02–2.96 (m, 1H), 2.88 (dd, J = 13.1, 10.6 Hz, 1H), 2.32–2.28 (m, 1H), 1.85–1.77 (m, 1H); **^{13}C NMR (75 MHz, DMSO-d_6)** δ = 48.7, 34.6, 32.8, 31.5; **HRMS** (ESI) calculated for $[C_4H_{10}NS_2]^+$ (M^+) requires m/z = 136.0250, found 136.0249.

III. Purity of DTBA assessed by Ellman's assay for sulfhydryl groups

A reaction buffer (0.10 M sodium phosphate buffer, pH 8.0, containing 1 mM EDTA) was prepared by the Pierce protocol. Ellman's reagent solution was primed by adding Ellman's reagent (4 mg) to 1 mL of the reaction buffer. A 2.50×10^{-4} M solution of DTBA was then prepared using the reaction buffer. Ellman's reagent solution (50 µL) was added to each of two vials containing 2.5 mL of reaction buffer. Reaction buffer (250 µL) was added to one of these vials, and its absorbance at 412 nm was used as a blank. DTBA solution (250 µL) was added to the other vial. After 10 min, its absorbance at 412 nm was recorded. Using Beer's law ($c = A/(\varepsilon \cdot l)$ with A = 0.623, l = 1 cm, and ε = 14,150 $M^{-1}cm^{-1}$) gave a thiol concentration of 4.40×10^{-5} M. Because DTBA contains two thiol groups, the assay solution had a DTBA concentration of 2.20×10^{-5} M. Accounting for dilution and using the equation $M_1 \cdot V_1 = M_2 \cdot V_2$, where $V_1 = 2.50 \times 10^{-4}$ L, $M_2 = 2.20 \times 10^{-5}$ M, and $V_2 = 2.8 \times 10^{-3}$ L, yielded $M_1 = 2.46 \times 10^{-4}$ M and thus a DTBA purity of $(2.46 \times 10^{-4}$ M$)/(2.50 \times 10^{-4}$ M$) \times 100\% = 98.4\%$. Three repetitions of this assay gave (99 ± 1)% purity. This assay revealed that commercial DTT and BMS had >98% purity.

IV. Determination of thiol pK_a values

The thiol pK_a values of DTBA were determined by measuring its absorbance at 238 nm in solutions of various pH. The deprotonated thiolate absorbs much more strongly at 238 nm than does its protonated counterpart.[5] This attribute was exploited for determining thiol pK_a values as described previously.[6] Buffered stock solutions of K_3PO_4, K_2HPO_4, and KH_2PO_4 (100 mM) were degassed and flushed with N_2(g) for 1 h immediately prior to use. A stock solution of DTBA (1.5 mM) in KH_2PO_4 was then prepared. Various combinations of the buffered stock solutions were combined in duplicate to give two identical sets of 1-mL solutions of pH 5.5–11. KH_2PO_4 stock solution (70 µL) was added to each replicate pair of solutions and used to set the A_{238} to zero. Dithiol solution (70 µL) was then added to its complementary 1-mL vial, and its absorbance at 238 nm was recorded. The pH of the solution was then immediately measured using a Beckman pH meter, which had been calibrated prior to use with pH 7 and pH 10 standard solutions from Fisher Scientific. This process was repeated multiple times to obtain a plot of A_{238} *vs* pH (**Figure S1**).

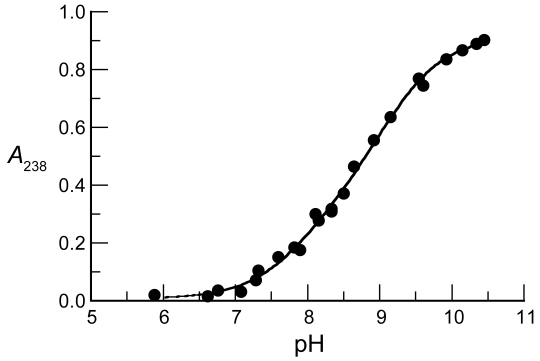


Figure S1. Effect of pH on absorbance at 238 nm of DTBA (0.10 mM) in 0.10 M potassium phosphate buffer. Fitting the data to eq 1 yielded pK_a values of 8.2 ± 0.2 and 9.3 ± 0.1, and extinction coefficients of ε_{SH}^{SH} = 83.27 $M^{-1}cm^{-1}$, $\varepsilon_{SH}^{S^-}$ = 3436 $M^{-1}cm^{-1}$, and $\varepsilon_{S^-}^{S^-}$ = 9169 $M^{-1}cm^{-1}$ with $r^2 > 0.99$.

pK_a values were determined by fitting the data in **Figure S1** to eq 1,[6] which is derived from Beer's law and the definition of the acid dissociation constant.[6] In eq 1, C_T is total thiol concentration, ε_{SH}^{SH} is the extinction coefficient of the doubly protonated form, $\varepsilon_{SH}^{S^-}$ is the extinction coefficient of the singly protonated form, and $\varepsilon_{S^-}^{S^-}$ is the extinction coefficient of the unprotonated form. Both pK_a values and extinction coefficients were determined from the curve fit with the program Prism 5.0 (GraphPad Software, La Jolla, CA).

$$A_{238} = C_T \left(\frac{\varepsilon_{S^-}^{S^-} 10^{pH-pK_{a2}} + \varepsilon_{SH}^{S^-} + \varepsilon_{SH}^{SH} 10^{pK_{a1}-pH}}{10^{pH-pK_{a2}} + 1 + 10^{pK_{a1}-pH}} \right) \quad (1)$$

V. Reduction potential of DTBA

The reduction potential ($E°'$) of DTBA was determined by using HPLC to determine the equilibrium constant for its reaction with oxidized DTT (eq 2), and then inserting this value into a variation of the Nernst equation (eq 3).[1] Data were obtained by a procedure similar to that described previously.[1,6] DTBA (10.5 mg, 0.06 mmol) and oxidized DTT (9.2 mg, 0.06 mmol) were added to a 25-mL round-bottom flask. The flask was then flushed with N_2(g) for 30 min.

$$K_{eq} = \frac{[DTT][\text{oxidized DTBA}]}{[DTBA][\text{oxidized DTT}]} = \frac{[DTT]^2}{[\text{oxidized DTT}]^2} \quad (2)$$

$$E_{DTBA}{}^{o'} = E_{DTT}{}^{o'} - \frac{RT}{nF} \ln \frac{[DTT]^2}{[\text{oxidized DTT}]^2} \quad (3)$$

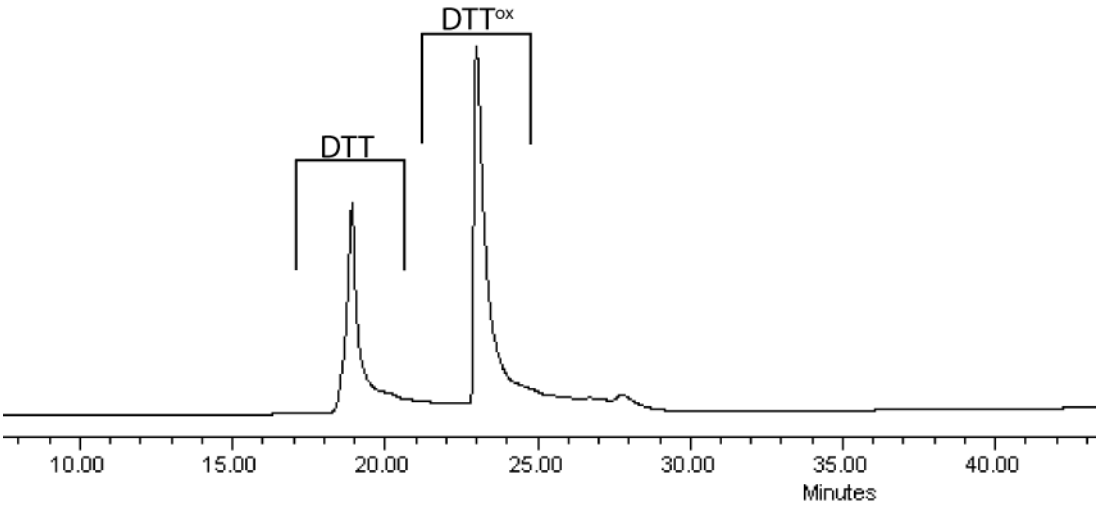


Figure S2. Representative HPLC chromatogram of the redox equilibrium between DTBA and DTT. Compounds were detected by their absorbance at 205 nm.

A 50 mM stock solution of potassium phosphate buffer (pH 7) was degassed and purged with N_2(g) for 30 min immediately prior to use. Buffer (15 mL) was added, and the reaction mixture was stirred under N_2(g) for 24 h at room temperature. The reaction mixture was then quenched by the addition of 3 N HCl (1:100 dilution). The reaction mixture was passed through a 4.5-µm filter, and 100 µL of the reaction mixture was analyzed immediately by HPLC using a Waters system equipped with a Waters 996 photodiode array detector, Empower 2 software, and a Varian C18 reverse-phase column. The column was eluted at 1.0 mL/min with water (5.0 mL), followed by a linear gradient (0–40% v/v) of acetonitrile/water over 40 min. Compounds were detected by their absorbance at 205 nm. Reduced and oxidized DTBA are highly polar and elute from the column immediately (as confirmed by LC–MS). Two peaks, however, were clearly visible in the chromatogram (**Figure S2**). HPLC analysis of standards revealed that the two peaks were reduced DTT (retention time: 19 min) and oxidized DTT (retention time: 23 min). Calibration curves were generated and found to be linear over the used concentration range. From these curves, the equilibrium concentrations of reduced and oxidized DTT were determined, and a $K_{eq} = 0.469 \pm 0.131$ for the reaction was found. Assuming that DTT has $E^{\circ\prime} = -0.327$ V,[3] eq 3 (which is a variation of the Nernst equation) was used to calculate that DTBA has $E^{\circ\prime} = -(0.317 \pm 0.002)$ V. This value is the mean ± SE from seven experiments. The reverse reaction between oxidized DTBA and reduced DTT revealed that equilibrium had been established under the experimental conditions.

VI. Reduction potential of BMS

The procedure described in Section V was also performed with BMS. With $K_{eq} = 0.0517 \pm 0.0194$ and assuming $E^{\circ\prime} = -0.327$ V for DTT,[3] BMS was found to have $E^{\circ\prime} = (-0.291 \pm 0.002)$ V, which was again the mean ± SE from seven experiments. The reduction potential for BMS was reported previously to be $E^{\circ\prime} = -0.31$ V.[1]

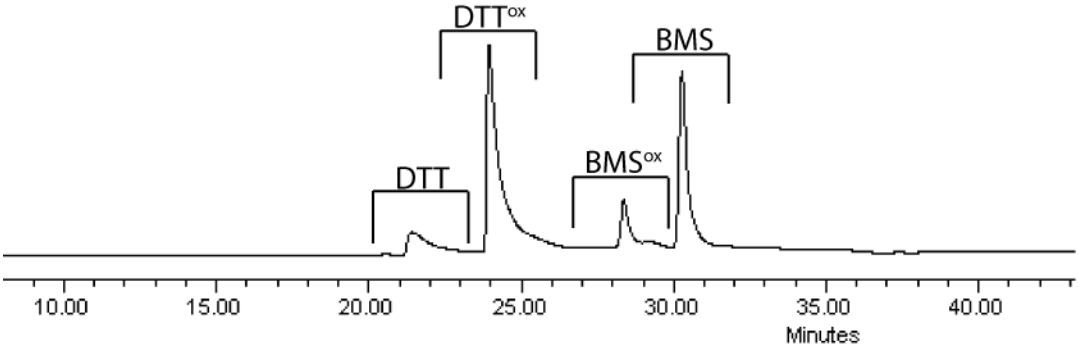


Figure S3. Representative HPLC chromatogram of the redox equilibrium between BMS and DTT. Compounds were detected by their absorbance at 205 nm.

VII. Kinetic studies on the reduction of oxidized βME

$$-\frac{\partial[\text{disulfide}]_{\text{total}}}{\partial t} = k_{\text{obs}}[\text{disulfide}]_{\text{total}}[\text{thiol}]_{\text{total}}$$

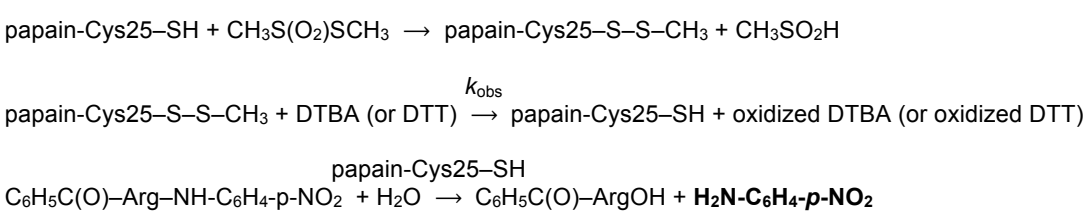
The observed second-order rate constant (k_{obs}) for a thiol–disulfide interchange reaction was determined by adapting a procedure describe previously.[7] For disulfide = oxidized βME, a 50 mM stock solution of potassium phosphate buffer was degassed and purged with N_2(g) for 30 min immediately prior to use. A stock solution of oxidized βME (10 mM) in 50 mM potassium phosphate buffer, pH 7.0, was purged with N_2(g) for 30 min immediately prior to use. A 25-mL round-bottom flask was charged with DTBA (4.3 mg, 0.025 mmol) or DTT (3.9 mg, 0.025 mmol), and placed under N_2(g). Phosphate buffer (2.5 mL) was added to the round-bottom flask containing the dithiol. Oxidized βME stock solution (2.5 mL) was then added, and the reaction mixture was stirred at room temperature under N_2(g) for 1 min. The reaction mixture was quenched by the addition of 0.10 mL of 3 N HCl. The reaction mixture was passed through a 4.5-μm filter, and 100 μL of the reaction mixture was analyzed immediately by HPLC using a Varian C18 reverse-phase column. The column was eluted at 1.0 mL/min with water (5.0 mL), followed by a linear gradient (0–40% v/v) of acetonitrile/water over 40 min. The extent of reduction was determined by integrating the newly formed peak corresponding to βME at 205 nm (retention time: 8 min). This process was repeated for reaction times of 2 and 4 min. Calibration curves were generated and found to be linear over the used concentration range. The amount of residual oxidized βME was calculated, and second-order rate constants were calculated from a linear fit of the data in **Figure 1A** (that is, $k_{obs} = [(1/c_{final}) - (1/c_{initial})]/t$). The initial values of concentration in the reaction mixture were [DTBA or DTT] = [oxidized βME] = $c_{initial}$ = 5 mM. Rate constants were the mean ± SE from three experiments. DTBA: k_{obs} = (0.29 ± 0.02) $M^{-1}s^{-1}$ and DTT: k_{obs} = (0.084 ± 0.004) $M^{-1}s^{-1}$. The same procedure was performed for reactions at pH 5.5, giving DTBA: k_{obs} = (0.0093 ± 0.0003) $M^{-1}s^{-1}$ and DTT: k_{obs} = (0.0021 ± 0.0002) $M^{-1}s^{-1}$ (**Figure 1A**).

VIII. Kinetic studies on the reduction of oxidized L-glutathione

An experiment similar to that in Section VII was conducted with disulfide = oxidized L-glutathione. Reactions were quenched at various time points (2, 4, 6, and 8 min) and 100 μL was

analyzed by HPLC (1.0 mL/min with water (5.0 mL) in 0.1% v/v TFA, followed by a linear gradient (0–40% v/v) of acetonitrile in 0.1% v/v TFA over 40 min). The extent of reduction was determined by integrating the newly formed L-glutathione reduced peak at 220 nm (retention time of 7 min). Second-order rate constants were calculated from a linear fit of the data in **Figure 1B** (that is, $k_{obs} = [(1/c_{final}) - (1/c_{initial})]/t$). Rate constants were the mean ± SE from three experiments. DTBA: $k_{obs} = (0.83 \pm 0.04)\ M^{-1}s^{-1}$ and DTT: $k_{obs} = (0.16 \pm 0.02)\ M^{-1}s^{-1}$.

IX. Kinetic studies on the reactivation of papain



Cys25 near the active site of papaya latex papain was oxidized as a mixed disulfide by a procedure described previously.[8] Briefly, a stock solution of methyl methanethiosulfonate (3.5 mM) was prepared by dilution of 5 µL of methyl methanethiosulfonate with 15 mL of 0.10 M potassium phosphate buffer, pH 7.0, containing EDTA (2 mM). KCl (0.011 g, 0.15 mmol) was added to 1.5 mL of this stock solution. The solution was deoxygenated by bubbling N_2(g) through it for 15 min. Next, papain (5 mg, 150 units) was added, and the resulting solution was incubated at room temperature under N_2(g) for 12 h. Excess methyl methanethiosulfonate was removed by size-exclusion chromatography using a Sephadex G-25 column. The final concentration of papain was determined by A_{280} using $\varepsilon_{280} = 5.60 \times 10^4\ M^{-1}cm^{-1}$.[9] A solution (0.26 mL) of the chromatographed protein was diluted with 4.94 mL of deoxygenated aqueous buffer (0.10 M imidazole–HCl buffer, pH 7.0, containing 2 mM EDTA). Enzyme solution (1.25 mL) was then added to four separate vials. DTBA or DTT (10 µL of a 1 mM solution) was added to one of the vials, and a timer was started. The initial concentrations in the reaction mixture were dithiol reducing agent: 7.9×10^{-6} M and inactive protein: 4.9×10^{-6} M. At various times, an 0.20-mL aliquot was removed from the reaction mixture and added to a cuvette of 0.8 mL of substrate solution (1.25 mM *N*-benzoyl-L-arginyl-*p*-nitroanilide in 0.10 M imidazole–HCl buffer, pH 6.0, containing 2 mM EDTA). The rate of change in absorbance at 410 nm was recorded at 25 °C. A unit of protein is defined by the amount of enzyme required to produce 1 µmol/min of 4-nitroaniline. Using an extinction coefficient for 4-nitroaniline of ε = 8,800 $M^{-1}cm^{-1}$ at 410 nm,[10] the number of units of active papain in solution at each time point was calculated. To determine the possible number of units of active papain in the reaction mixture, a large excess of DTT (~10^3-fold) was added to one vial and the activity was assessed. As a control, it was determined that the concentrations of DTT used had no bearing on the assay data other than activating the protein. y = enzymatic activity (%) at particular times was calculated by dividing the number of active units of enzyme by the possible number of units in the solution, and was plotted in **Figure 2A**. To determine the value of the second-order rate constant k_{obs} for the reducing agents, the second-order rate equation (eq 4) was transformed into eq 5, which was fitted to the data with the program Prism 5.0. In eq 4 and 5, A_o = [inactive protein]$_{t=0}$, A = [inactive protein]$_t = A_o - A_o y$, B_o = [reducing agent]$_{t=0}$, and B = [reducing agent]$_t = B_o - A_o y$. Values of k_{obs} were the mean ± SE from three experiments. DTBA: $k_{obs} = (1275 \pm 69)\ M^{-1}s^{-1}$ and DTT: $k_{obs} = (90.4 \pm 5.2)\ M^{-1}s^{-1}$.

$$\frac{1}{B_o - A_o}\ln\frac{A_o B}{A B_o} = k_{obs} t \quad (4)$$

$$y = \frac{B_o - B_o e^{k_{obs} t (A_o - B_o)}}{B_o - A_o e^{k_{obs} t (A_o - B_o)}} \times 100\% \quad (5)$$

X. Kinetic studies on the reactivation of creatine kinase

creatine kinase-Cys283–SH + GSSG ⟶ creatine kinase-Cys283–S–SG + GSH

creatine kinase-Cys283–S–SG + DTBA (or DTT) $\xrightarrow{k_{obs}}$ creatine kinase-Cys283–SH + oxidized DTBA (or DTT)

creatine phosphate + ADP $\xrightarrow{\text{creatine kinase-Cys283–SH}}$ creatine + ATP

ATP + D-glucose $\xrightarrow{\text{hexokinase}}$ ADP + D-glucose-6-phosphate

D-glucose-6-phosphate + $NADP^+$ $\xrightarrow{\text{glucose-6-phosphate dehydrogenase}}$ D-gluconate-6-phosphate + H^+ + **NADPH**

Cys283 in the active site of rabbit muscle creatine kinase was oxidized as a mixed disulfide by a procedure described previously,[11] but with a slight modification in the measurement of active enzyme. A unit of enzyme was defined as the amount required to produce 1 μmol/min of NADPH. Using an extinction coefficient for NADPH of ε = 6.22 $mM^{-1}cm^{-1}$ at 340 nm, the units of active creatine kinase in solution at a particular time were calculated. To determine the possible number of units of active creatine kinase in the reaction mixture, a large excess of DTT (~10^3-fold) was added to one vial and the activity was assessed. As a control, it was determined that the concentrations of DTT used had no bearing on the assay data other than activating the protein. Enzymatic activity (%) at particular times was calculated by dividing the number of active units of enzyme by the possible number of units in the solution, and was plotted in **Figure 2B**. Values of the second-order rate constant k_{obs} were determined by using eq 5 as described in Section IX, and were the mean ± SE from three experiments. DTBA: k_{obs} = (16.2 ± 0.7) $M^{-1}s^{-1}$ and DTT: k_{obs} = (14.7 ± 0.4) $M^{-1}s^{-1}$.

XI. Separation of DTBA using an ion-exchange resin

A reaction buffer (0.10 M sodium phosphate, pH 8.0, 1 mM EDTA) was prepared. Ellman's reagent solution was prepared by adding Ellman's reagent (4 mg) to 1 mL of the reaction buffer. Next, to 25 mL of reaction buffer (0.10 M sodium phosphate, pH 8.0, 1 mM EDTA) was added DTBA (2.2 mg, 1.27 × 10^{-5} mol) and 1.7 g of DOWEX 50WX4-400 ion-exchange resin. The mixture was swirled for several minutes and filtered through a fritted syringe. Ellman's reagent solution (50 μL) was added to two separate vials containing 2.5 mL of reaction buffer. As a blank, 250 μL of reaction buffer was added to one of the vials, and the absorbance at 412 nm was set to zero. Filtrate (250 μL) was then added to the other vial and its absorbance was recorded. With A_{412} = 0.012 and using an extinction coefficient of ε = 14,150 $M^{-1}cm^{-1}$,[4b,c] it was calculated that >99% of DTBA was retained by the resin and thus removed from solution. The same assay

was repeated with DTT, resulting in <1% being removed from solution. See **Section III** for a more detailed explanation of similar calculations using Ellman's assay.

XII. Ultraviolet spectra of oxidized DTBA and oxidized DTT

Solutions of oxidized DTBA and DTT (1.0 mM) were prepared in Dulbecco's phosphate buffered saline (DPBS), and their ultraviolet spectra were recorded (**Figure S4**).

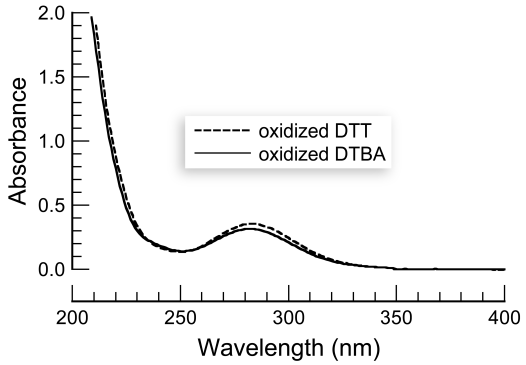
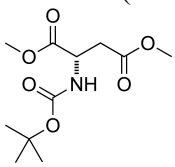
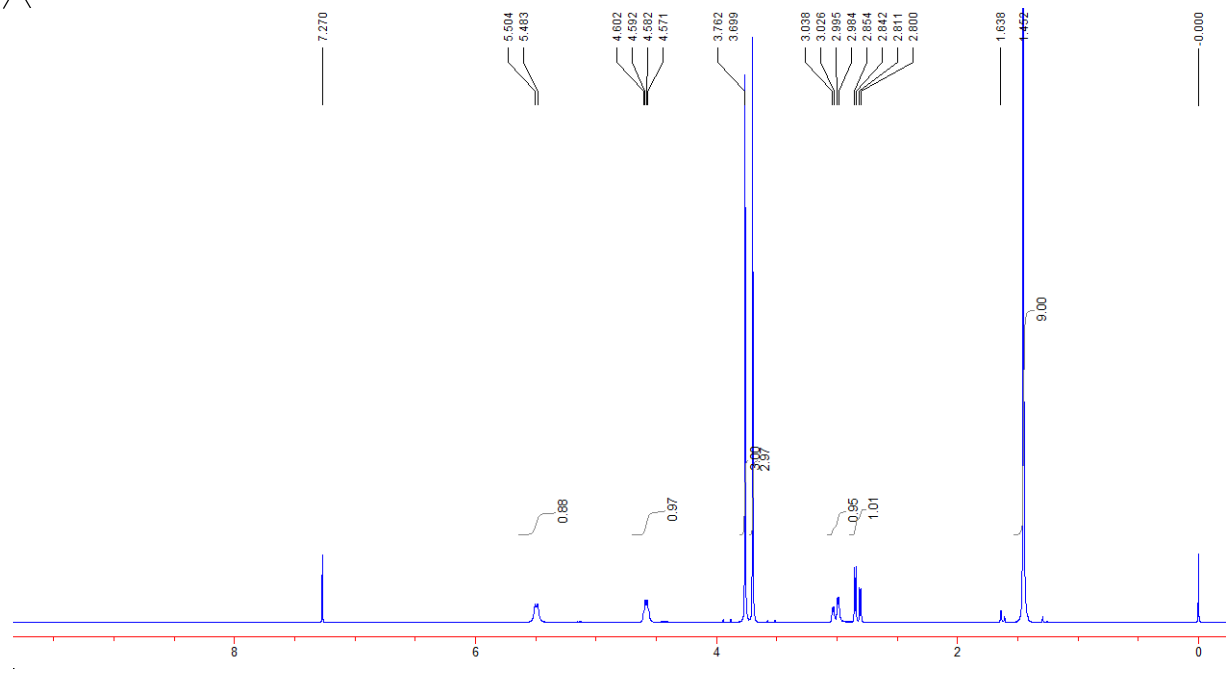


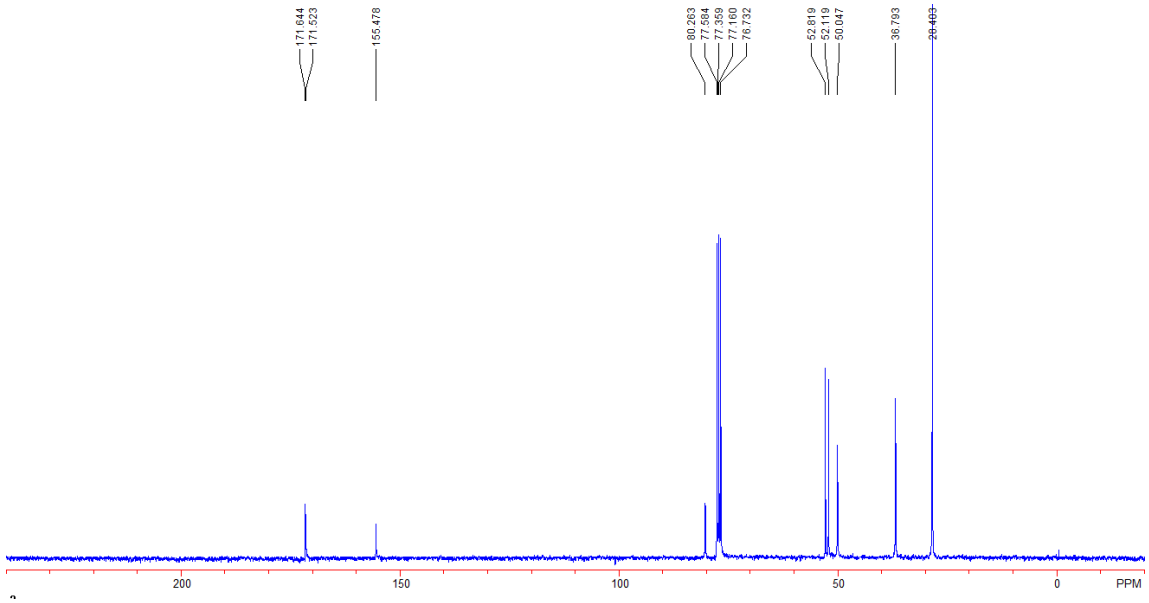
Figure S4. Ultraviolet spectrum of oxidized DTBA and oxidized DTT in DPBS.

XIII. NMR Spectra

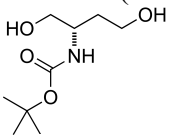
^{1}H NMR($CDCl_3$) and ^{13}C NMR($CDCl_3$) of **2**

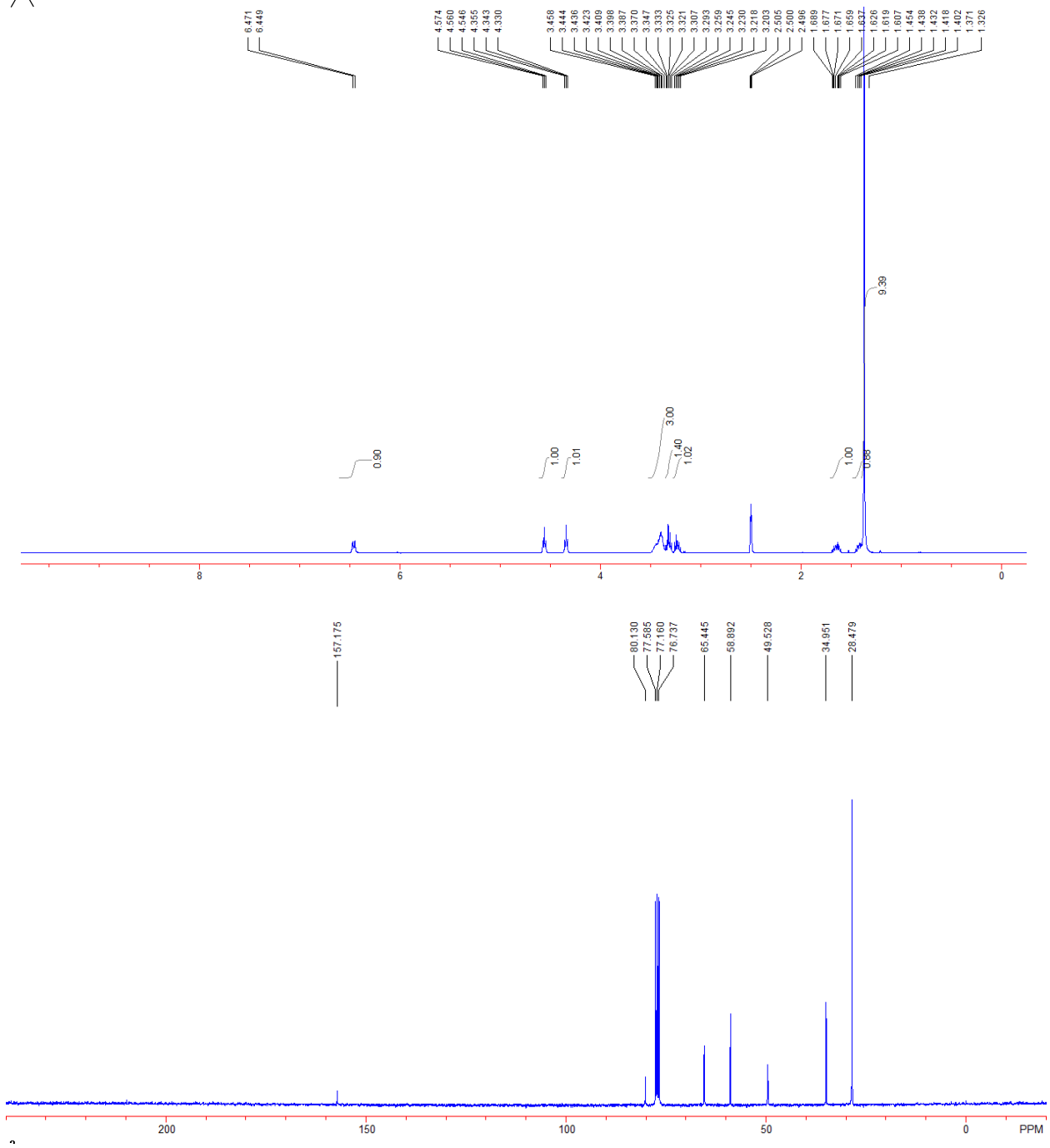




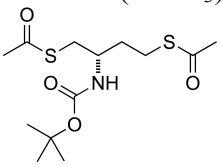


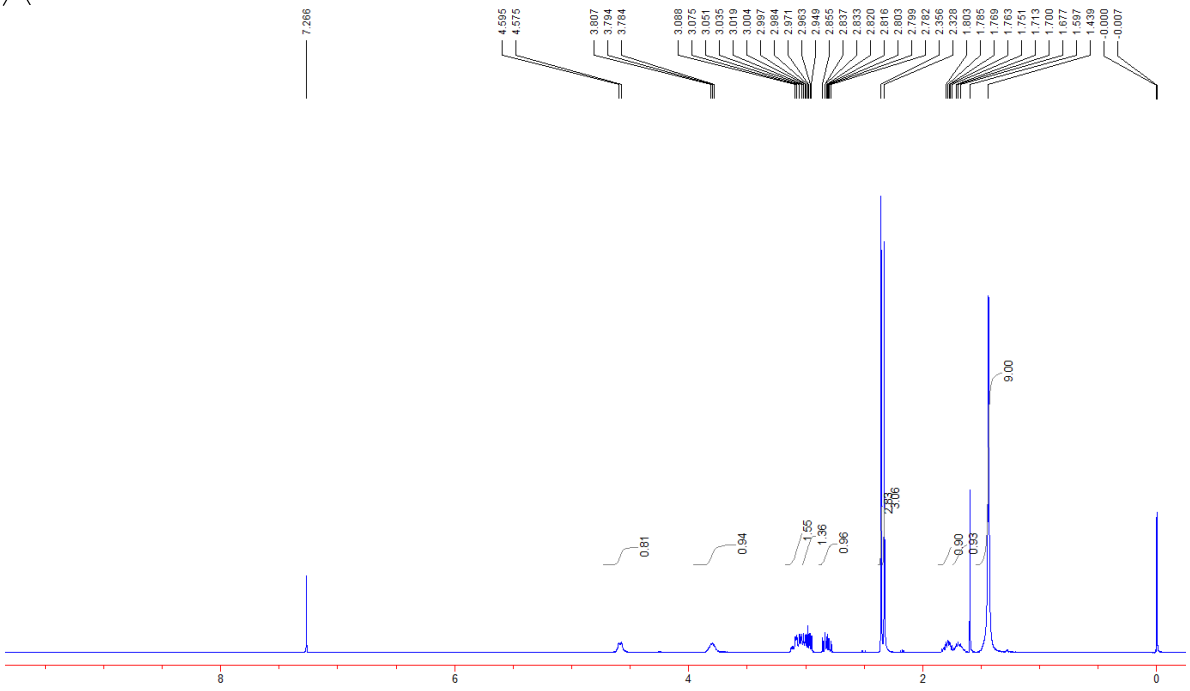
^{1}H NMR(DMSO-d_6) and ^{13}C NMR($CDCl_3$) of **3**

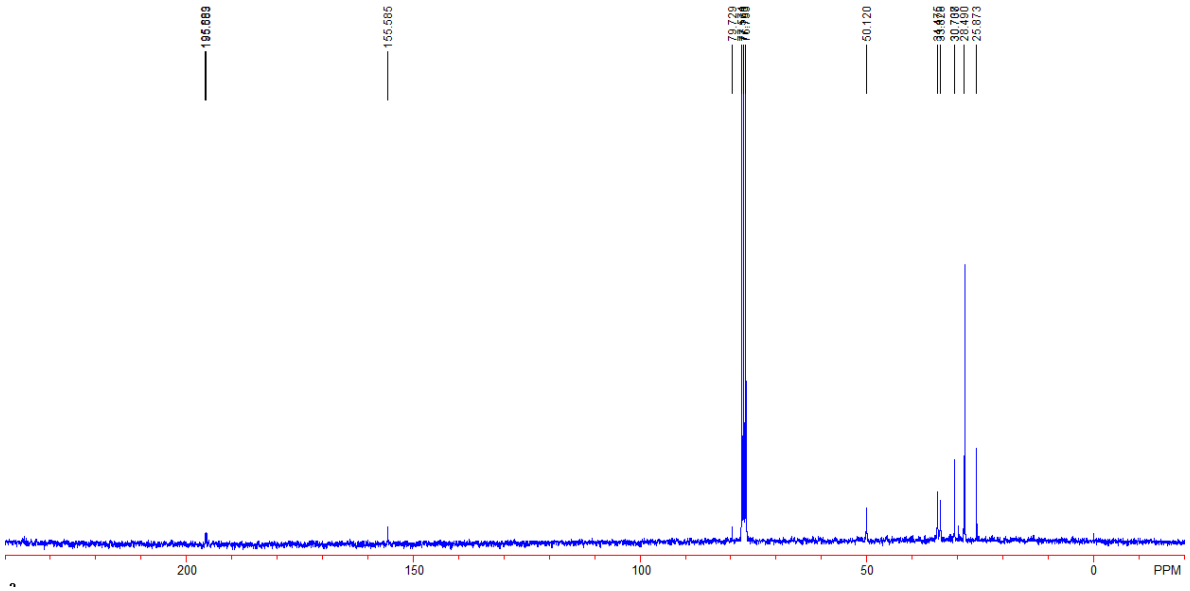




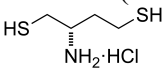
^{1}H NMR($CDCl_3$) and ^{13}C NMR($CDCl_3$) of **4**

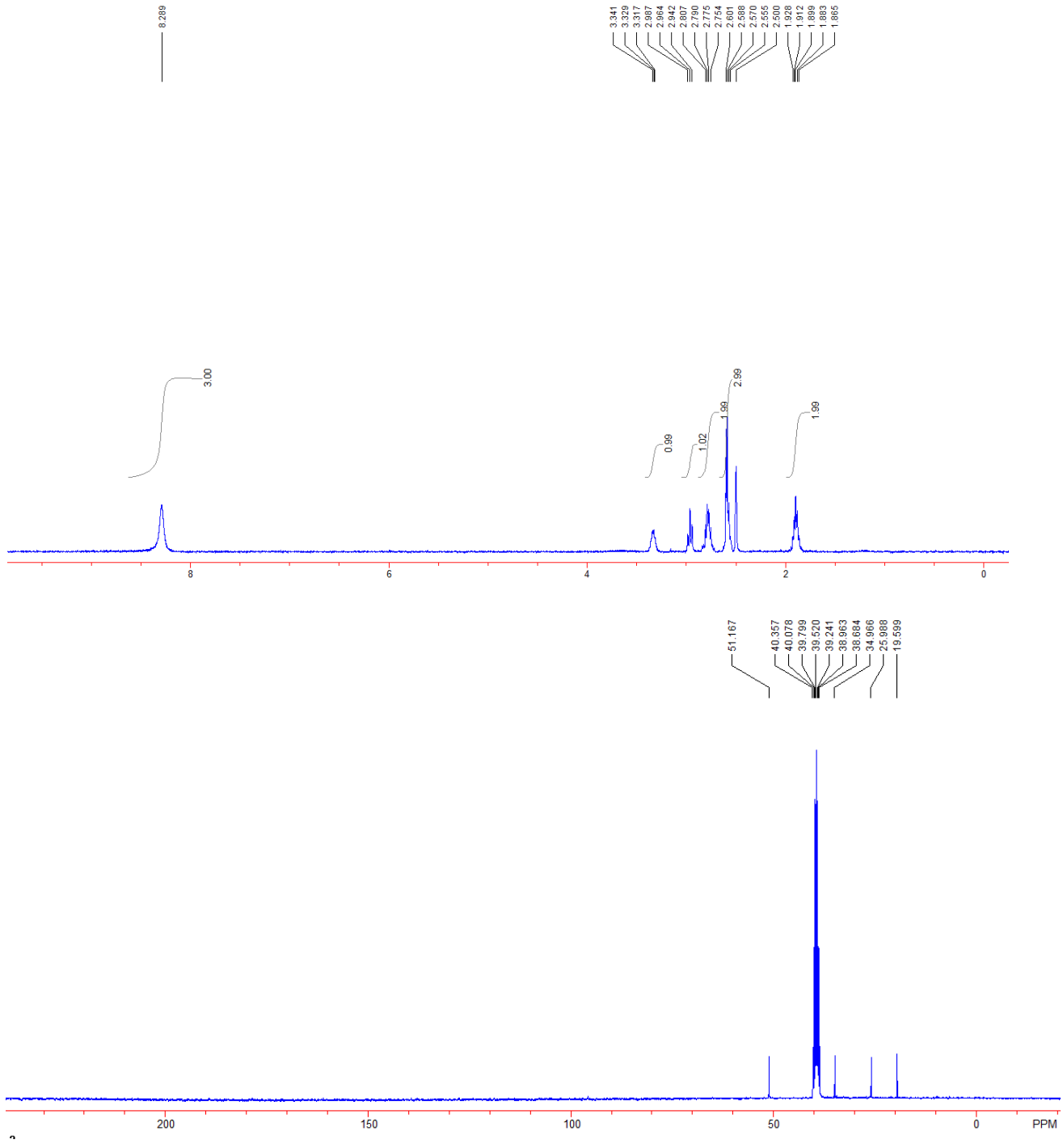




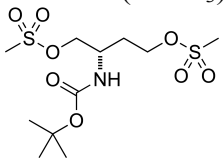


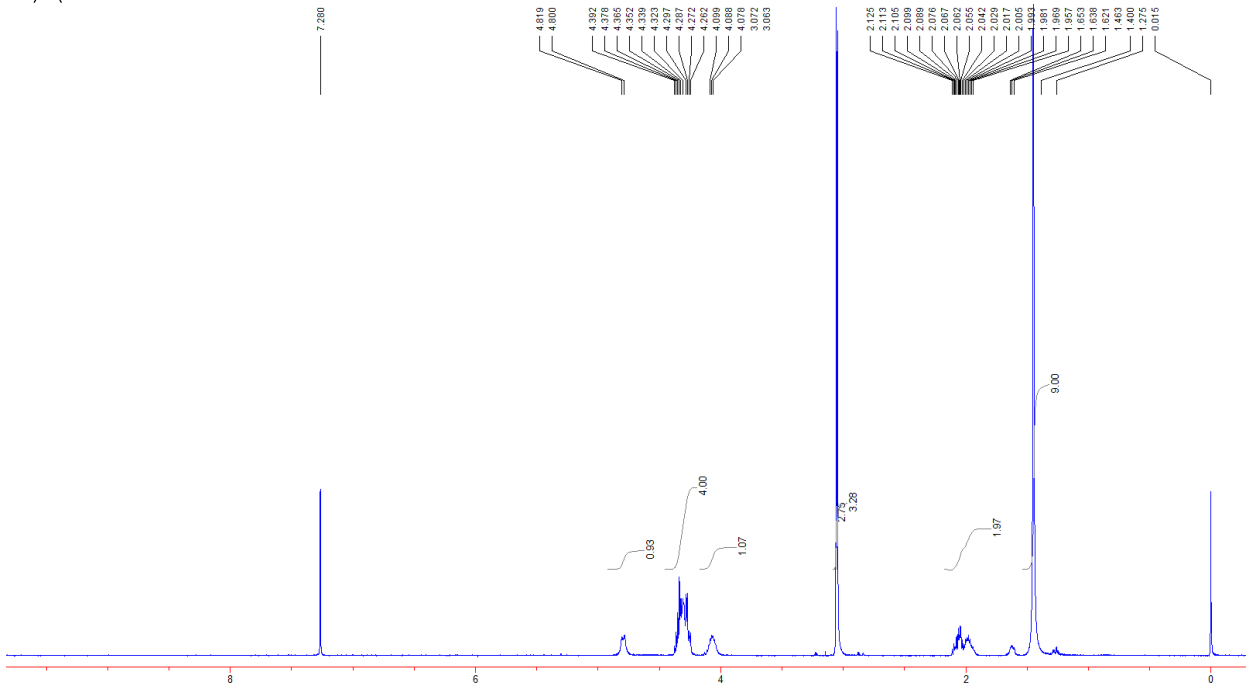
^{1}H NMR(DMSO-d_6) and ^{13}C NMR(DMSO-d_6) of **5**

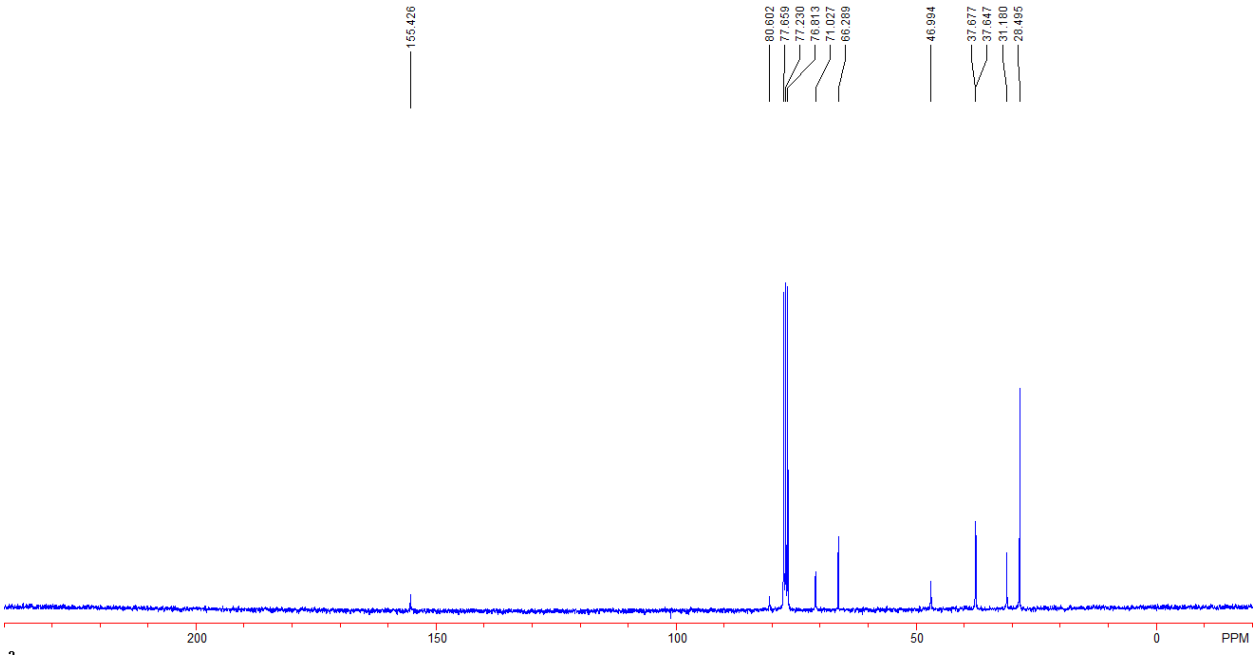




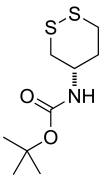
^{1}H NMR($CDCl_3$) and ^{13}C NMR($CDCl_3$) of **6**

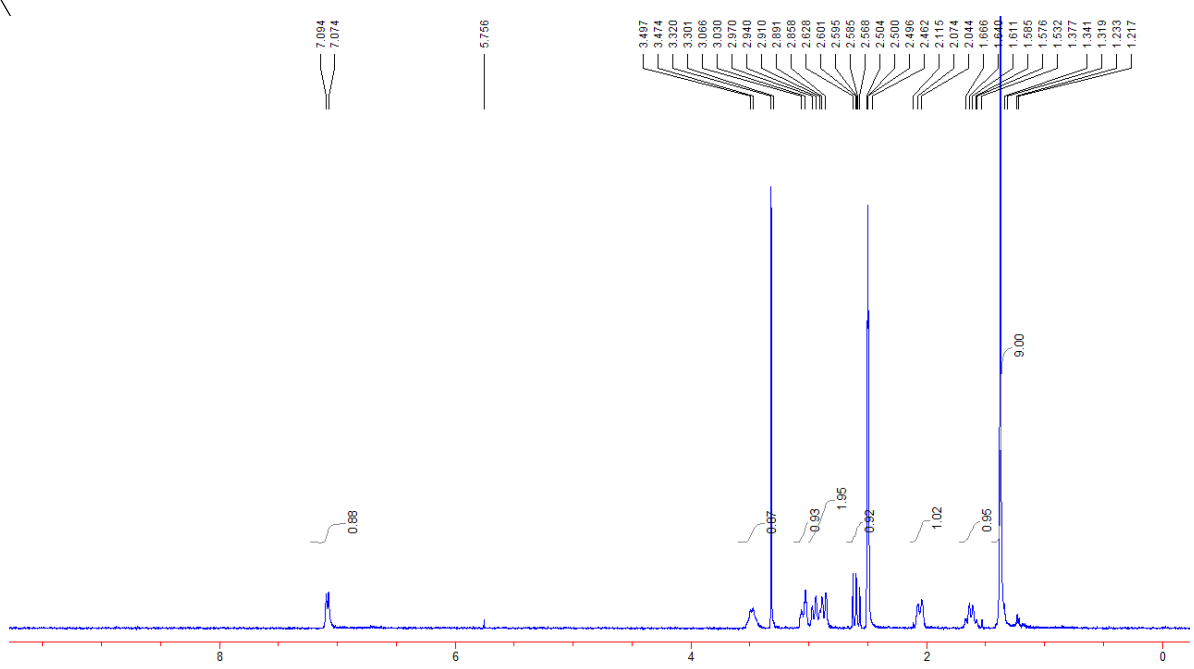


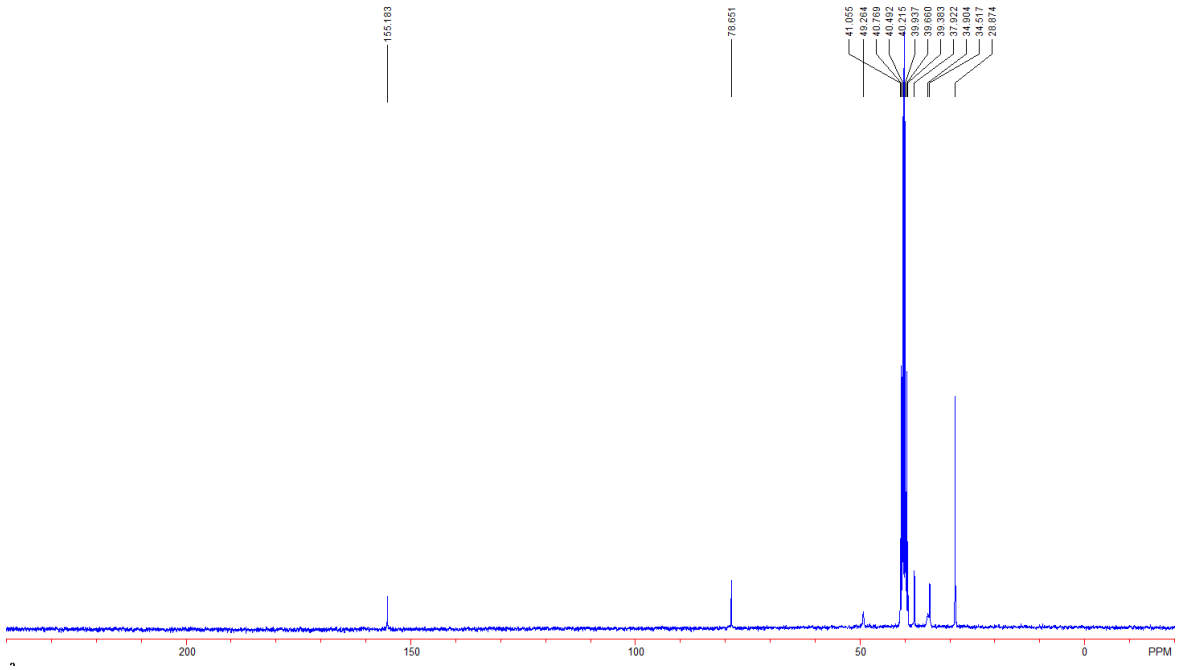




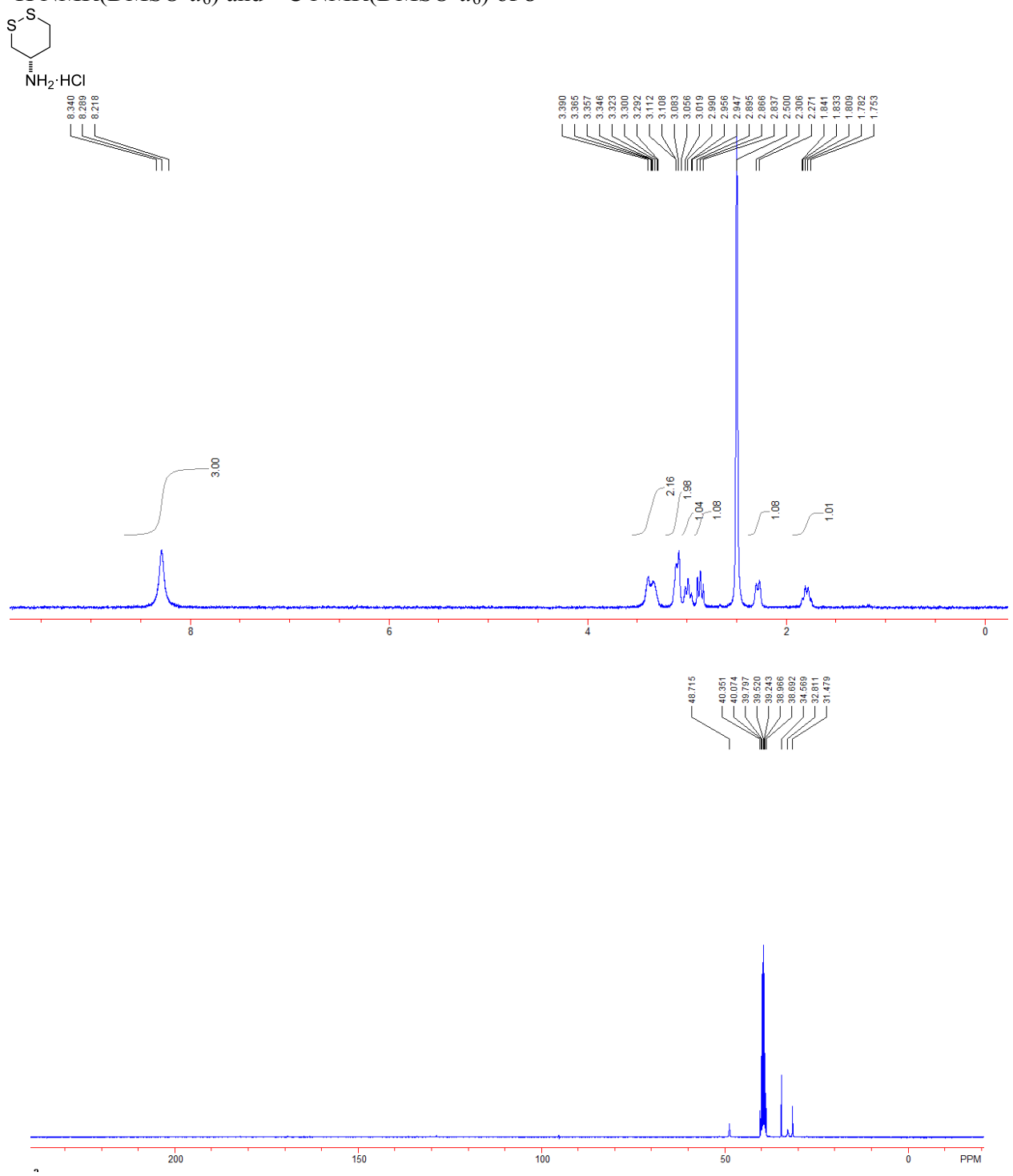
^{1}H NMR(DMSO-d_6) and ^{13}C NMR(DMSO-d_6) of **7**







^{1}H NMR(DMSO-d_6) and ^{13}C NMR(DMSO-d_6) of **8**



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