Supporting Information

Small Molecule Diselenide Additives for *In Vitro* Oxidative Protein Folding

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General Methods

^{1}H NMR spectra were recorded on a Bruker 400 or 500 MHz instruments. ^{13}C NMR spectra were recorded at 100 or 125 MHz, and ^{77}Se NMR spectra were recorded at 95 MHz spectrometers. NMR spectra (^{1}H, ^{13}C and ^{77}Se) were recorded in $CDCl_3$ and D_2O, and chemical shifts are reported with respect to solvent signal as a reference (if TMS is there in $CDCl_3$, then with respect to TMS). Mass spectra (MS) were recorded with Thermo Fisher Scientific LCQ Fleet Ion-Trap mass spectrometer in the positive mode. HR-MS were recorded on Agilent 6550 iFunnel Q-TOF LC/MS system. Preparative HPLC was performed using Waters 150LC system (Xselect® Peptide CSH™ C18 OBD™ 130 Å, 5 μm, 30x250 mm) to purify diselenides **1-3**. Oxidative folding was followed on Waters Acquity UPLC system using Waters Acquity UPLC® HSS T3 (1.8 μm, 2.1x100 mm column), and the absorbance of the different species was recorded at 214 nm.

Supporting Materials

Deuterated oxide (D_2O) and $CDCl_3$ were purchased from Sigma-Aldrich (Rehovot, Israel). All solvents: *N,N*-dimethylformamide (DMF), dichloromethane, and acetonitrile (ACN), tetrahydrofuran (THF), methanol (MeOH), ethanol (EtOH) were purchased from Bio-Lab (Jerusalem, Israel) and were peptide synthesis, HPLC or ULC-grade. Trifluoroacetic acid (TFA) was a generous gift from Halocarbon Products (River Edge, NJ). All other reagents including elemental selenium and *L*-malic acid were purchased from Sigma-Aldrich (Rehovot, Israel). Bovine pancreatic trypsin inhibitor (BPTI) was a generous gift of Bachem, and was reduced with excess DTT for two hours, followed by purification using preparative HPLC. Reduced BPTI was used in folding studies as previously described.[Refs. 7, 21, 23, 41 in the main text]

Experimental Section

1,2-*bis*(2-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)ethyl)diselane (5): Triethylamine (0.83 mL, 5.99 mmol) was added to alcohol **4**[1] (0.46 g, 3.15 mmol) in CH_2Cl_2 (5 mL) at 0 ^oC under N_2 atmosphere. Then Ms-Cl (0.29 mL, 3.78 mmol) was added dropwise and stirred at 0 ^oC for 2 h. Then water was added to the reaction mixture, extracted twice with CH_2Cl_2, dried over Na_2SO_4 and evaporated. The crude mesylate **4a** obtained was directly used in the next step.

$N_2H_4{\cdot}H_2O$ (0.17 mL, 3.47 mmol) was added dropwise under argon to elemental Se (0.28 g, 3.47 mmol), DMF (8 mL) and NaOH (0.19 g, 4.73 mmol) at room temperature, and the mixture was stirred at 60 ^oC for 2.5 h.[2] The reaction mixture was cooled to room temperature, mesylate **4a** in DMF (6 mL) was added dropwise and stirred for 4 h at room temperature. Water was added to the reaction mixture and extracted twice with ether. Combined organic layers were washed with water, brine, dried (Na_2SO_4), evaporated and the resulting crude was purified by flash column chromatography (60-120 mesh Silica gel, 6% EtOAc in petroleum ether) to afford **5** (0.50 g, 76% yield) as a yellow oil (which solidified under freeze cooling); ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.26-4.15 (m, 2H, OCH), 4.14-4.05 (m, 2H, OCH_2), 3.63-3.54 (m, 2H, OCH_2), 3.34-3.08 (m, 1.2H, CH_2Se), 3.06-2.88 (m, 2.8H, CH_2Se), 2.15-1.92 (m, 4H, CH_2), 1.42 (s, 1.8H, CH_3), 1.41 (s, 4.2H, CH_3), 1.35 (s, 6H, CH_3).

(2*S*,2'*S*)-4,4'-diselanediyl*bis*(butane-1,2-diol) (1): TFA (3.5 mL) was added to compound **5** (0.35 g, 0.84 mmol) in MeOH (3.5 mL) at room temperature and stirred for 1.5 h. Then volatiles were removed under reduced pressure at 40 ^oC. Another MeOH (2.5 mL) and TFA (2.5 mL) were added sequentially at room temperature and stirred for 1 h, then removed under reduced pressure. Further addition of ether and evaporation removes volatiles to give crude **1** (quantitative yield-0.28 g) as a yellow-grey solid. Then the crude material was purified by preparative RP-HPLC (C18-Column) to give pure **1** (0.17 g, 61%) as a pale yellow solid; ^{1}H NMR (500 MHz, D_2O): δ 3.86-3.80 (m, 2H, C*H*OH), 3.62 (dd, 2H, *J* = 11.8, 4.0 Hz, CH_2OH), 3.52 (dd, 2H, *J* = 11.8, 6.7 Hz, CH_2OH), 3.12-3.06 (m, 2H, CH_2Se), 3.04-2.97 (m, 2H, CH_2Se), 2.02-1.94 (m, 2H, CH_2), 1.91-1.82 (m, 2H, CH_2); ^{13}C NMR (125 MHz, D_2O): δ 71.1, 65.2, 33.7, 25.0; ^{77}Se NMR (95 MHz, D_2O): δ 293.4; HR-MS (ESI+): *m/z* calculated for $C_8H_{18}O_4Se_2$ $(M+H)^+$ 338.9614, found 338.9611; calculated for $C_8H_{18}O_4Se_2$ $(M+Na)^+$ 360.9433, found 360.9428.

1,2-*bis*((2,2-dimethyl-1,3-dioxolan-4-yl)methyl)diselane (7): Triethylamine (0.83 mL, 5.99 mmol) was added to the alcohol **6** (3.0 g, 22.73 mmol) in CH_2Cl_2 (30 mL) at 0 ^oC under N_2 atmosphere. Then Ms-Cl (1.93 mL, 25.0 mmol) was added dropwise and stirred at 0 ^oC for 2-3 h. Water was added to reaction mixture, extracted twice with CH_2Cl_2, dried (Na_2SO_4), and evaporated giving crude mesylate **6a**,[3,4] which was used in next step without further purification. ^1H NMR (400 MHz, $CDCl_3$): δ 4.41-4.35 (m, 1H, OCH), 4.23 (d, 2H, *J* = 5.4 Hz, CH_2OMs), 4.11 (dd, 1H, *J* = 8.8, 6.5 Hz, OCH_2), 3.83 (dd, 1H, *J* = 8.8, 5.5 Hz, OCH_2), 3.07 (s, 3H, OMs), 1.45 (s, 3H, CH_3), 1.37 (s, 3H, CH_3).

DMF (45 mL) was added to the Se (2.0 g, 25.0 mmol) and NaOH (1.45 g, 36.36 mmol) at room temperature, followed by dropwise addition of $N_2H_4{\cdot}H_2O$ (1.21 mL, 25.0 mmol), at the end the mixture was stirred at 60 ^oC for 2-3 h under argon.[2] The reaction mixture was cooled to room temperature, and mesylate **6a** in DMF (20 mL) was added dropwise and stirred for 3 h. Water was added to the reaction mixture and the mixture was extracted twice with ether. Combined organic layers were washed with water, brine, dried (Na_2SO_4), and evaporated and the resulting crude was purified by flash chromatography (60-120 mesh Silica gel, 6% EtOAc in pet. ether) to afford **7** (1.75 g, 40% yield) as a yellow oil; ^1H NMR (400 MHz, $CDCl_3$): δ 4.52-4.29 (m, 2H, OCH), 4.20-4.06 (m, 2H, OCH_2), 3.82-3.67 (m, 2H, OCH_2), 3.47-3.14 (m, 2H, CH_2Se), 3.14-2.67 (m, 2H, CH_2Se), 1.46-1.42 (m, 6H, CH_3) (1.44-s, 1.43-s), 1.38-1.34 (m, 6H, CH_3) (1.369-s, 1.368-s, 1.363-s, 1.362-s).

3,3'-diselanediyl*bis*(propane-1,2-diol) (2): TFA (15 mL) was added to compound **7** (1.58 g, 4.05 mmol) in MeOH (15 mL) at room temperature and stirred for 1.5 h. Volatiles were removed under reduced pressure at 40 ^oC. Again MeOH (10 mL) and TFA (10 mL) were added sequentially at room temperature and stirred for 1 h. Volatiles were removed under reduced pressure. Finally ether addition and evaporation completed volatiles removal to give crude **2** (quantitative yield-1.26 g) as a yellow-orange syrup. The crude material (0.16 g) was purified by preparative RP-HPLC (C18-Column) to give pure **2** (0.063 g, 40%) as a pale yellow oil; ^1H NMR (400 MHz, D_2O): δ 4.03-3.96 (m, 2H, C*H*OH), 3.74 (dd, 1H, *J* = 11.8, 4.1 Hz, CH_2OH), 3.73 (dd, 1H, *J* = 11.8, 4.1 Hz, CH_2OH), 3.63 (dd, 2H, *J* = 11.8, 6.3 Hz, CH_2OH), 3.22 (dd, 2H, *J* = 12.9, 4.8 Hz, CH_2Se), 3.11-3.04 (m, 2H, CH_2Se); ^{13}C NMR (100 MHz, D_2O): δ 71.43, 71.40, 64.5, 32.6, 32.5; ^{77}Se NMR (95 MHz, D_2O): δ 273.8, 273.5; HR-MS (ESI+): *m/z* calculated for $C_6H_{14}O_4Se_2$ $(M+Na)^+$ 332.9120, found 332.9103.

(*R*)-thiazolidine-4-carboxylic acid (8): *L*-Cysteine (15.2 g, 125.46 mmol) was dissolved in water (40 mL), then 35% HCHO (11.4 mL) was added at room temperature and stirred for 4-6 h.[5] Then 40 mL of EtOH was added to the white turbidity reaction mixture and kept at 0 ^{o}C for 1-2 h. Then filtered, washed with 1:1 ratio of H_2O and EtOH mixture (40 mL), then with some amount of EtOH and dried to give **8** (16.02 g, 96%) as a white solid, which was directly used in next step without further purification; ^{1}H NMR (400 MHz, D_2O): δ 4.49-4.42 (m, 2H, SCH_2N, CαH), 4.35 (d, 1H, *J* = 10.3 Hz, SCH_2N), 3.43 (dd, 1H, *J* = 12.1, 7.4 Hz, CβH), 3.33 (dd, 1H, *J* = 12.1, 5.7 Hz, CβH).

(*R*)-3-(*tert*-butoxycarbonyl)thiazolidine-4-carboxylic acid (9): $(Boc)_2O$ (6.56 g, 30.08 mmol) in dioxane (30 mL) was added to compound **8** (4.0 g, 30.08 mmol) in cold 1 N NaOH (30 mL, 30.08 mmol) at 0 ^{o}C and stirred for 2 h, and later at room temperature for 3 h. Unreacted $(Boc)_2O$ was removed by washing twice with hexane, acidified the aqueous layer with 1N HCl, extracted twice with EtOAc, dried (Na_2SO_4) and evaporated to give **9** (5.10 g, 73% yield) as a white solid, which was directly used in next step without further purification; ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.18-9.72 (brs, 1H, COOH), 4.94-4.37 (m, 3H, SCH_2N, CαH), 3.44-3.21 (m, 2H, $C\beta H_2$), 1.49, 1.45 (2 x s, 9H, CMe_3).

***tert*-butyl (*R*)-4-(hydroxymethyl)thiazolidine-3-carboxylate (10)**: DCC (3.89 g, 18.88 mmol) was added to the mixture of compound **9** (4.0 g, 17.17 mmol) and HONSu (2.17 g, 18.88 mmol) in dry THF (~40 mL) at 0 ^{o}C and stirred at same temperature for 1 h. Then filtered using THF (3 x 4 mL) to remove the precipitated urea by-product.[6]

$NaBH_4$ (1.30 g, 34.33 mmol) in water (8 mL) was added to activated -ONSu ester in THF at 0 ^{o}C. After 10 min, 0.5 N HCl (40 mL) was added to quench the unreacted $NaBH_4$. Reaction mixture was extracted with EtOAc (3 x 80 mL), combined organic layers were washed with 5% Na_2CO_3 (8 x 20 mL), brine (3 x 80 mL), dried (Na_2SO_4) and evaporated to give crude alcohol **10** (2.60 g, 69% yield) as a white gummy solid, which was directly used in next step without further purification.

di-*tert*-butyl 4,4'-(diselanediyl*bis*(methylene))(4*S*,4'*S*)-*bis*(thiazolidine-3-carboxylate) (11): Triethylamine (3.30 mL, 23.74 mmol) was added to the crude alcohol **10** (2.60 g, 11.87 mmol) in CH_2Cl_2 (26 mL) at 0 ^{o}C under N_2 atmosphere. Then Ms-Cl (0.29 mL, 3.78 mmol) was added dropwise and stirred to room temperature for 2 h. Water was added at 0 ^{o}C to the

reaction mixture, extracted twice with CH_2Cl_2, dried (Na_2SO_4), evaporated and the crude mesylate **10a** obtained was directly used in next step.

Elemental selenium (1.47 g, 18.42 mmol) and $NaBH_4$ (0.50 g, 12.48 mmol) were placed under argon, and absolute EtOH (1.5 mL) was added dropwise while stirring at -15 °C.[7] After the initial vigorous reaction had subsided, additional absolute EtOH (35 mL) was added. After 10 min the cooling bath was removed, and the reaction mixture was heated under reflux for 90 min. It was cooled to room temperature again, and excess hydrogen selenide (H_2Se) was expelled by passing argon through the brownish-red mixture. The expelled gas was passed through a 5% aqueous solution of lead(II) acetate (w/v) to trap any hydrogen selenide (**Caution**: hydrogen selenide is highly toxic!). Then mesylate **10a** was dissolved in absolute EtOH (35 mL) and added dropwise at room temperature and stirred for overnight. Then the reaction mixture was quenched by addition of acetic acid (1.5 mL) and concentrated to a volume of ~10 mL *in vacuo*. Water was added to the resulting suspension and extracted twice with ether. Combined organic layers were washed with water, brine (twice), dried (Na_2SO_4), evaporated and the resulting crude was purified by column chromatography (60-120 mesh Silica gel, 10% EtOAc in pet. ether) to afford **11** (1.20 g, 36% yield) as a yellow syrup (which solidified under freezing); ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.71-4.38 (brs, 4H, SCH_2N), 4.33-4.23 (m, 2H, CαH), 3.37-3.05 (m, 8H, $C\beta H_2$, CH_2Se), 1.47 (s, 18H, CMe_3); ^{13}C NMR (100 MHz, D_2O): δ 153.1, 80.9, 60.5, 48.5, 31.6, 28.5, 28.3.

(*4S,4'S*)-4,4'-(diselanediylbis(methylene))bis(thiazolidin-3-ium) 2,2,2-trifluoroacetate (3): 1:1 ratio of TFA (2.5 mL), CH_2Cl_2 (2.5 mL) were added to compound **11** (1.14 g, 2.02 mmol) at room temperature and stirred for 30 min. TLC showed the disappearance of starting material. Then volatiles were removed under vacuum at room temperature, triturated with ether, filtered to give the crude **3** (1.06 g, 91% yield) as an orange solid. Then the crude material (0.20 g) was purified by preparative-RP-HPLC (C18-Column) to give pure **3** (0.14 g, 70%) as a yellow solid (TFA salt); ^{1}H NMR (400 MHz, D_2O): δ 4.49 (d, 2H, J = 10.2 Hz, SCH_2N), 4.43 (d, 2H, J = 10.2 Hz, SCH_2N), 4.37-4.28 (m, 2H, C*H*N), 3.54-3.32 (m, 6H, 2 x SCH_2, $SeCH_2$), 3.16 (dd, 2H, J = 12.0, 7.6 Hz, $SeCH_2$); ^{13}C NMR (100 MHz, D_2O): δ 63.4, 47.7, 33.6, 26.7; ^{77}Se NMR (95 MHz, D_2O): δ 380.4, 303.2; HR-MS (ESI+): *m/z* calculated for $C_8H_{16}N_2S_2Se_2$ $(M+H)^+$ 364.9164, found 364.9148.

References

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SI Figures:

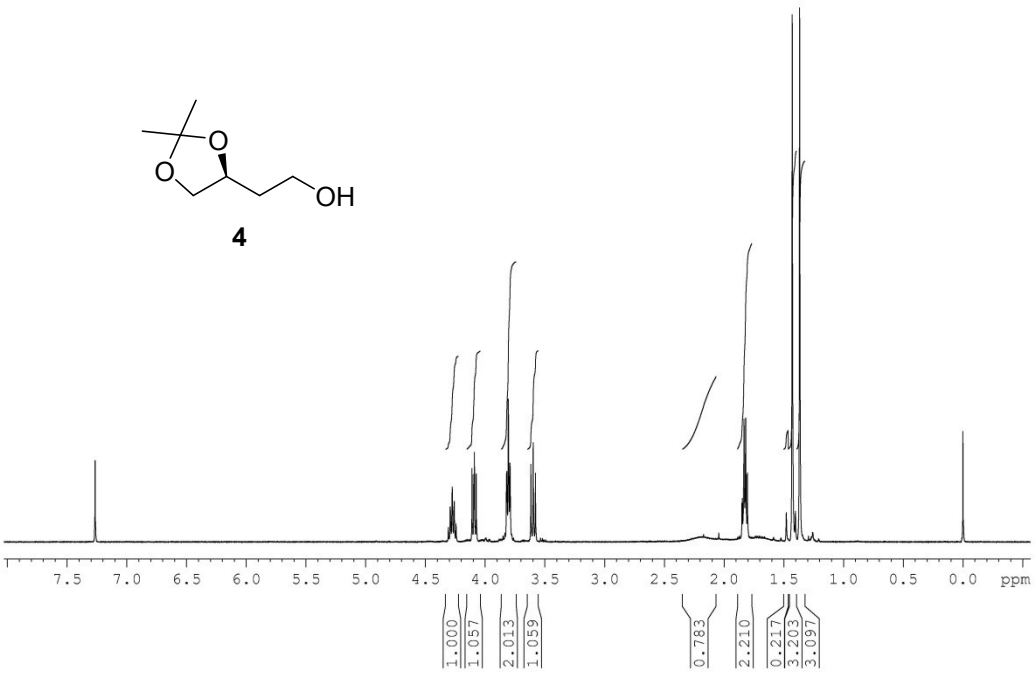

Figure S1. ^{1}H NMR Spectrum of **4** (400 MHz, $CDCl_3$)

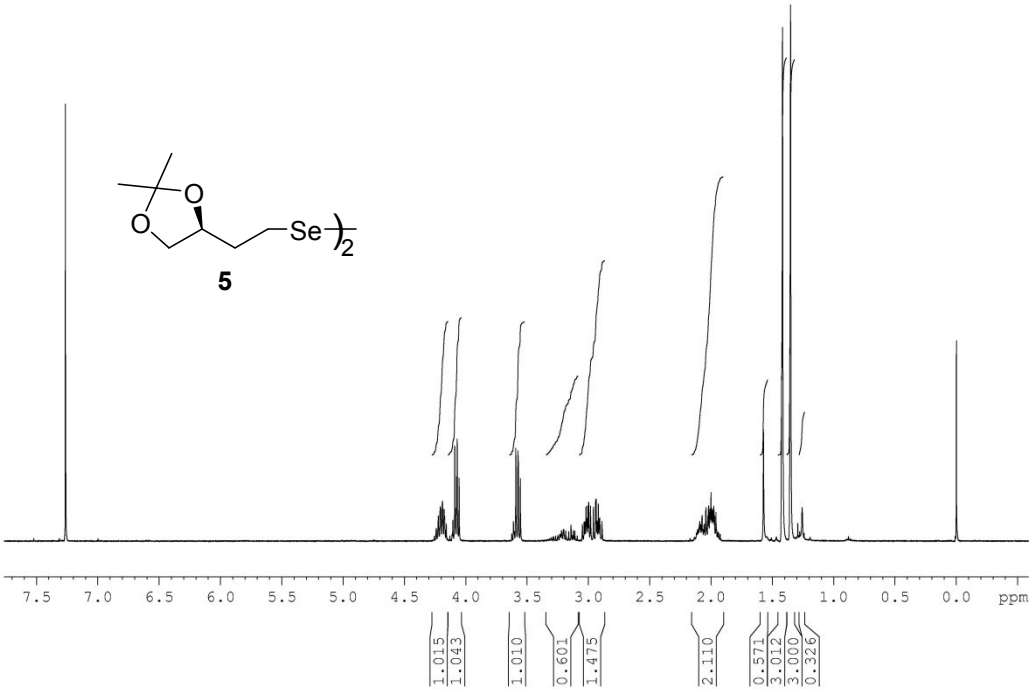

Figure S2. ^{1}H NMR Spectrum of **5** (400 MHz, $CDCl_3$)

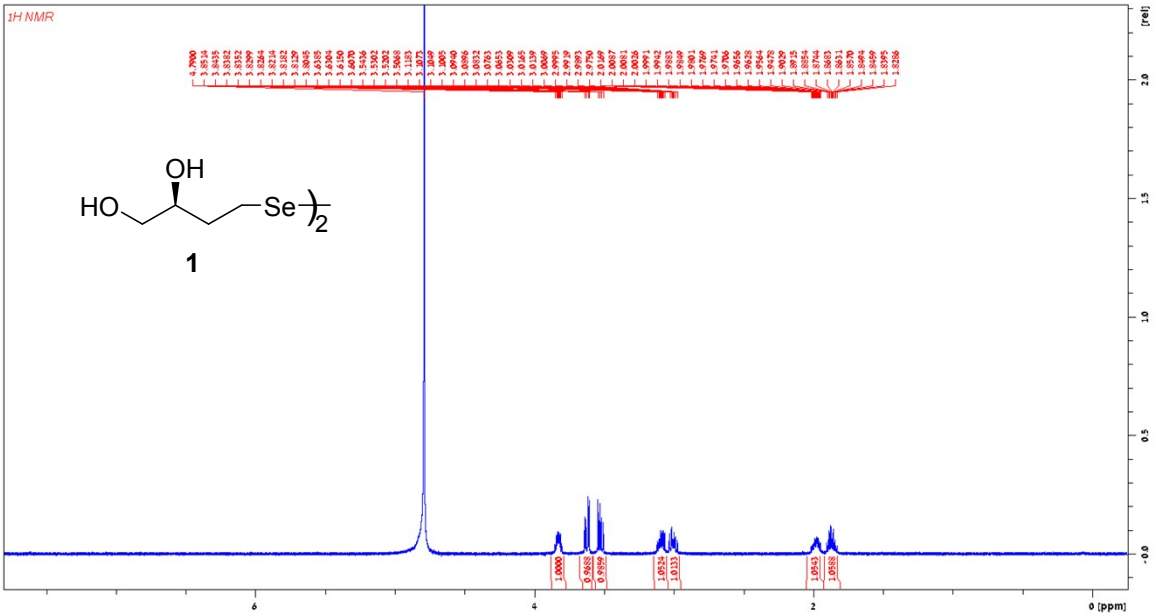

Figure S3. ^{1}H NMR Spectrum of **1** (500 MHz, D_2O)

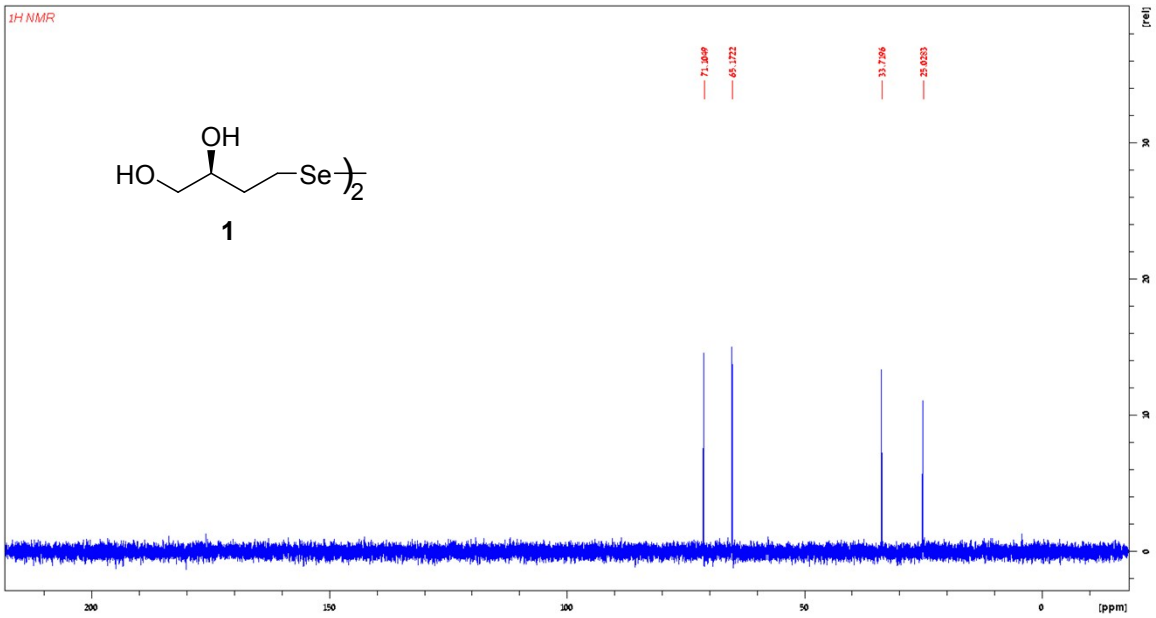

Figure S4. ^{13}C NMR Spectrum of **1** (125 MHz, D_2O)

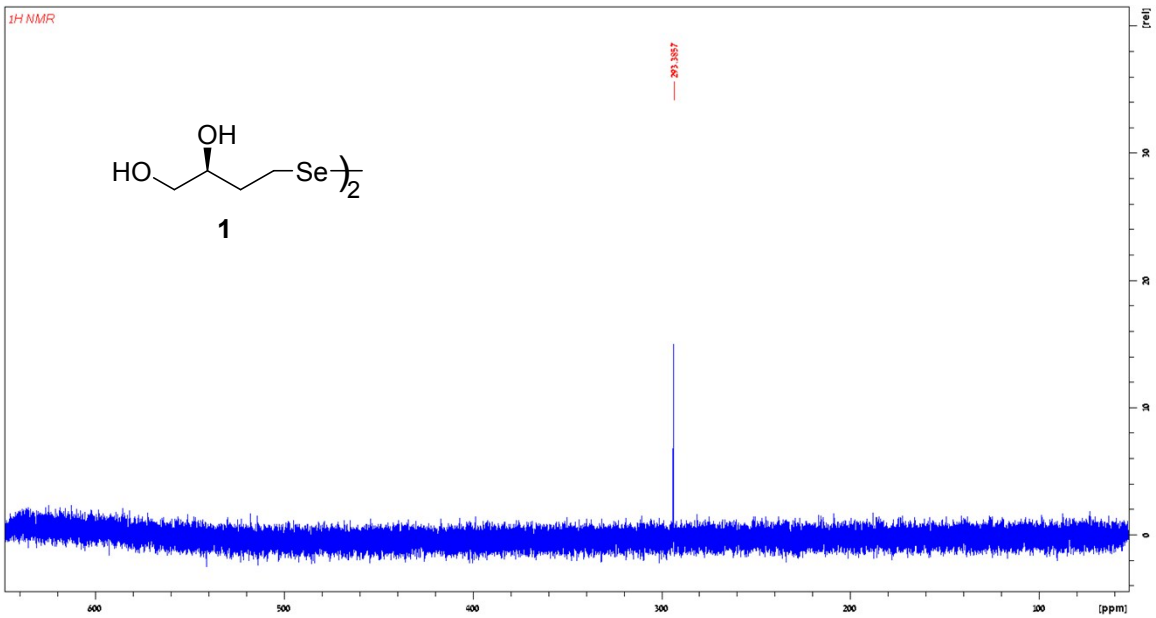

Figure S5. ^{77}Se NMR Spectrum of **1** (95 MHz, D_2O)

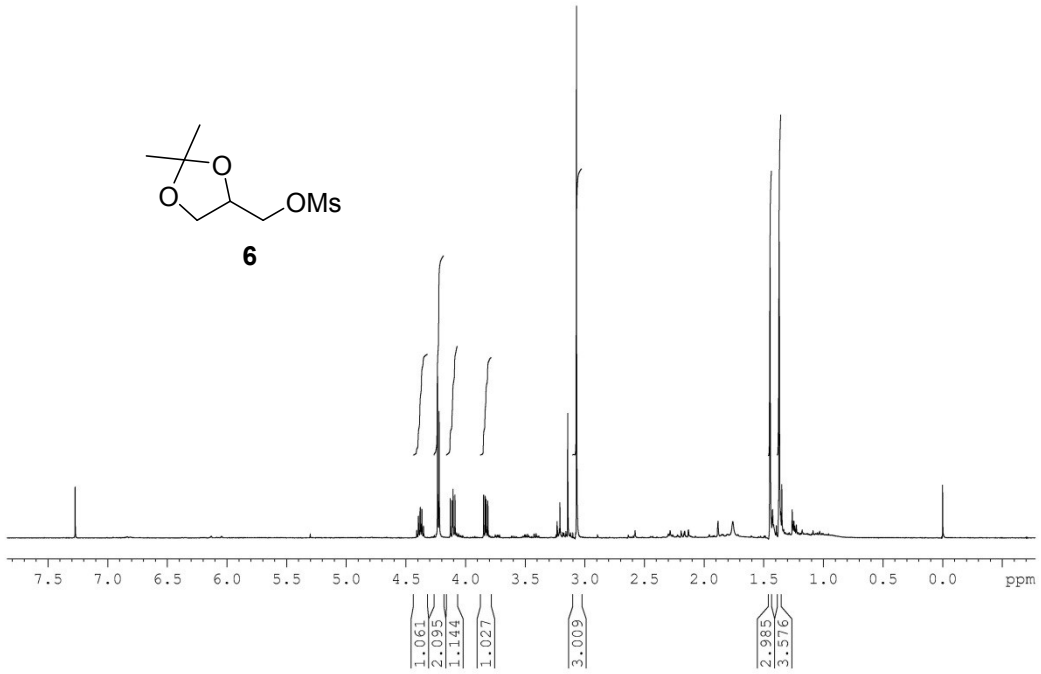

Figure S6. ^{1}H NMR Spectrum of **6** (400 MHz, $CDCl_3$)

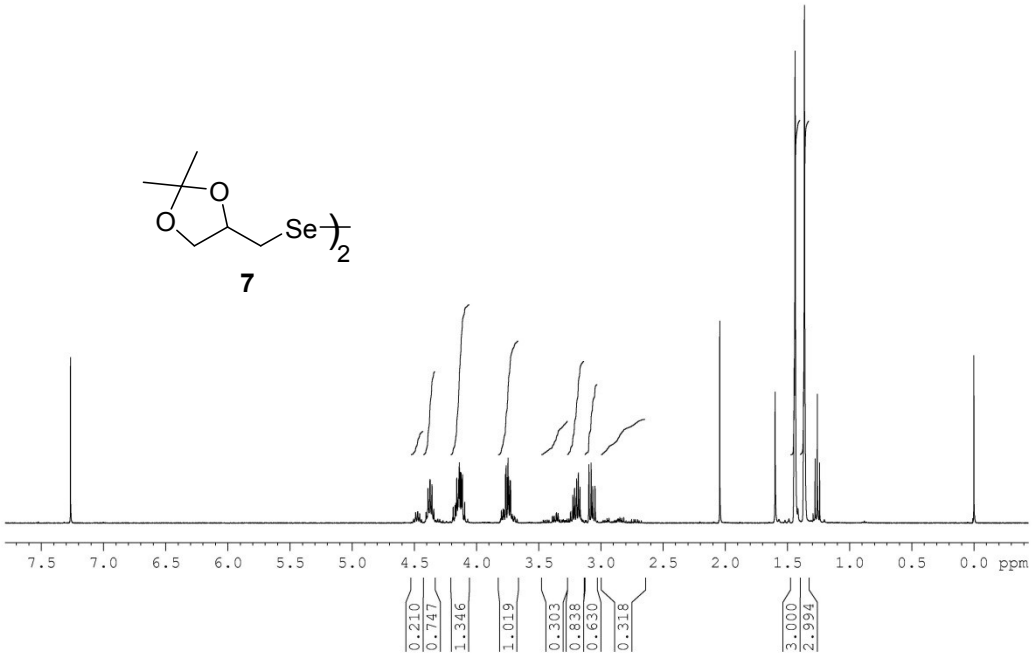

Figure S7. ^{1}H NMR Spectrum of **7** (400 MHz, $CDCl_3$)

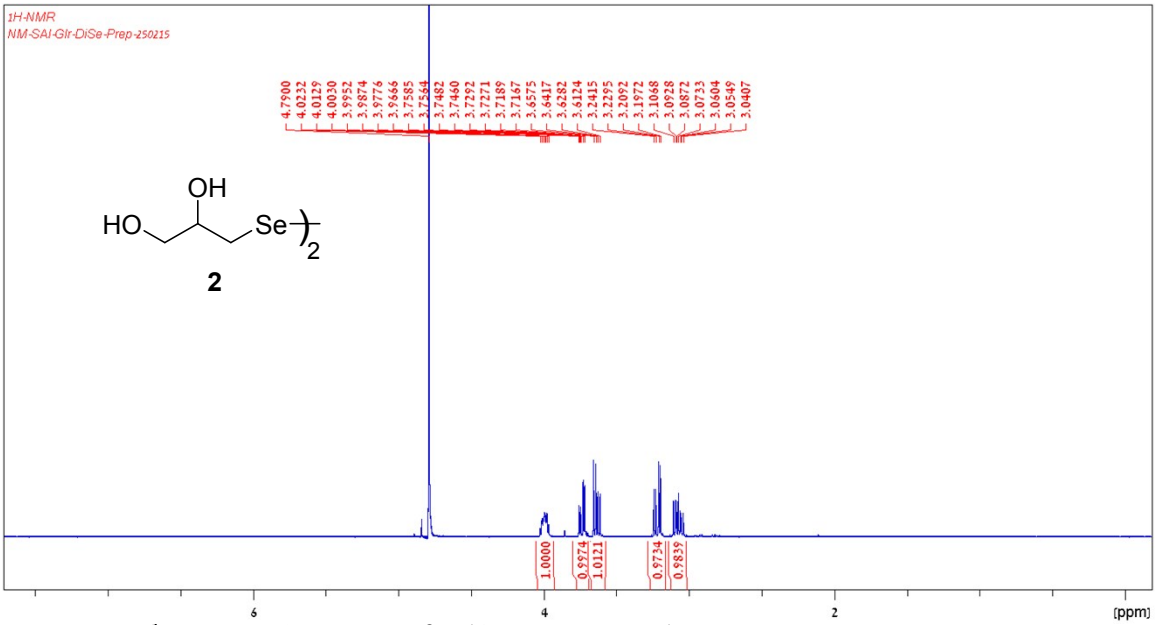

Figure S8. ^{1}H NMR Spectrum of **2** (400 MHz, D_2O)

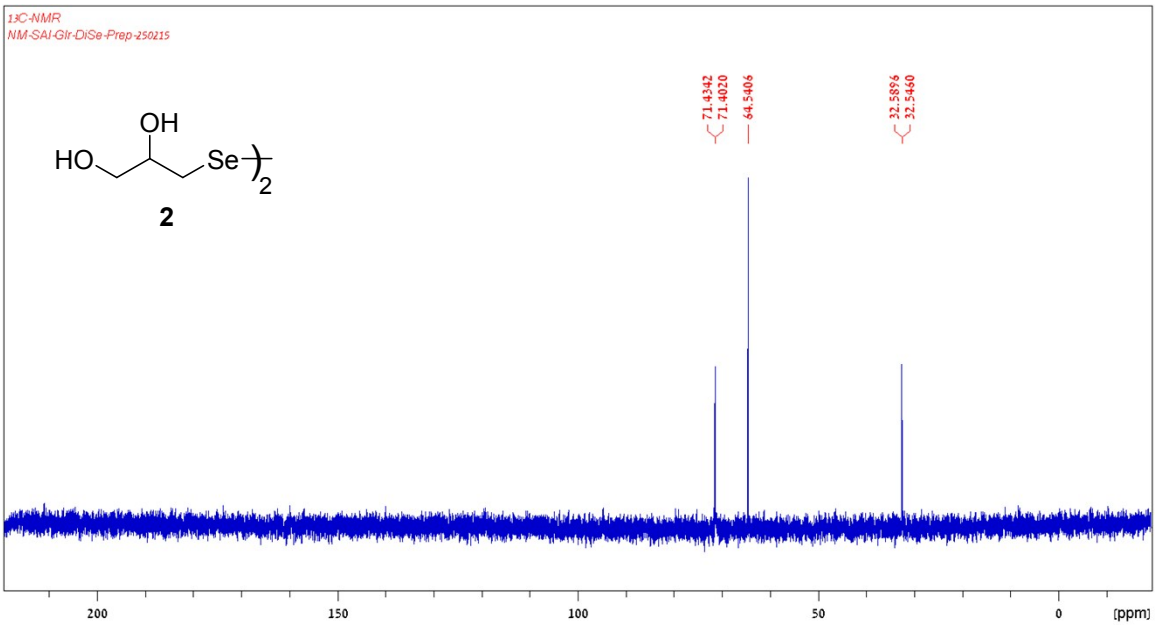

Figure S9. ^{13}C NMR Spectrum of **2** (100 MHz, D_2O)

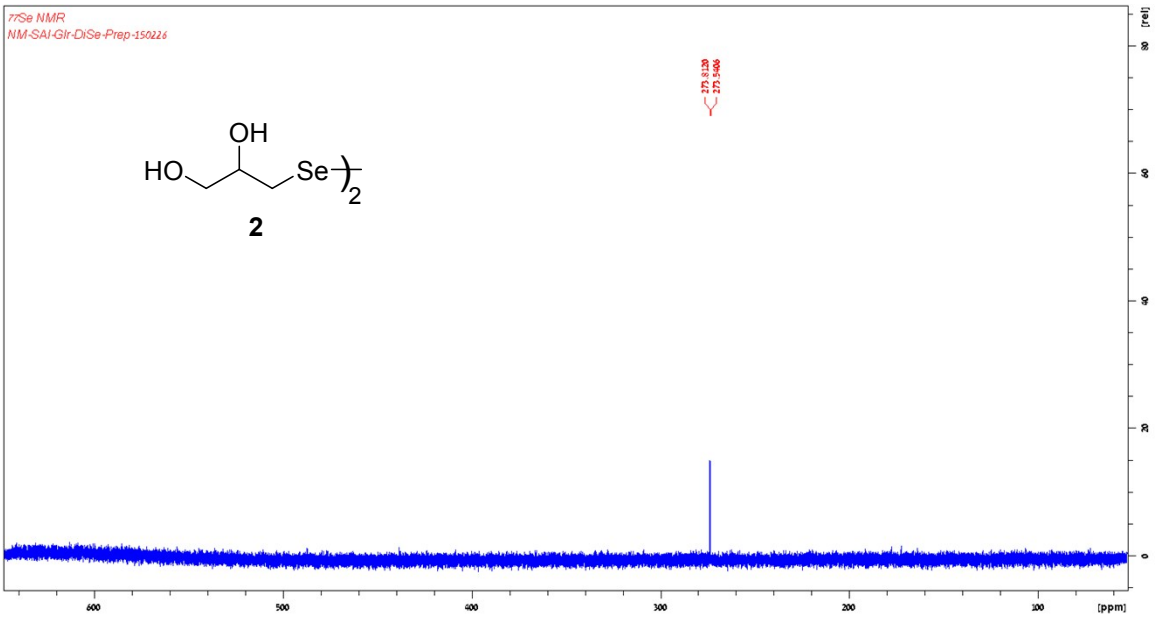

Figure S10. ^{77}Se NMR Spectrum of **2** (95 MHz, D_2O)

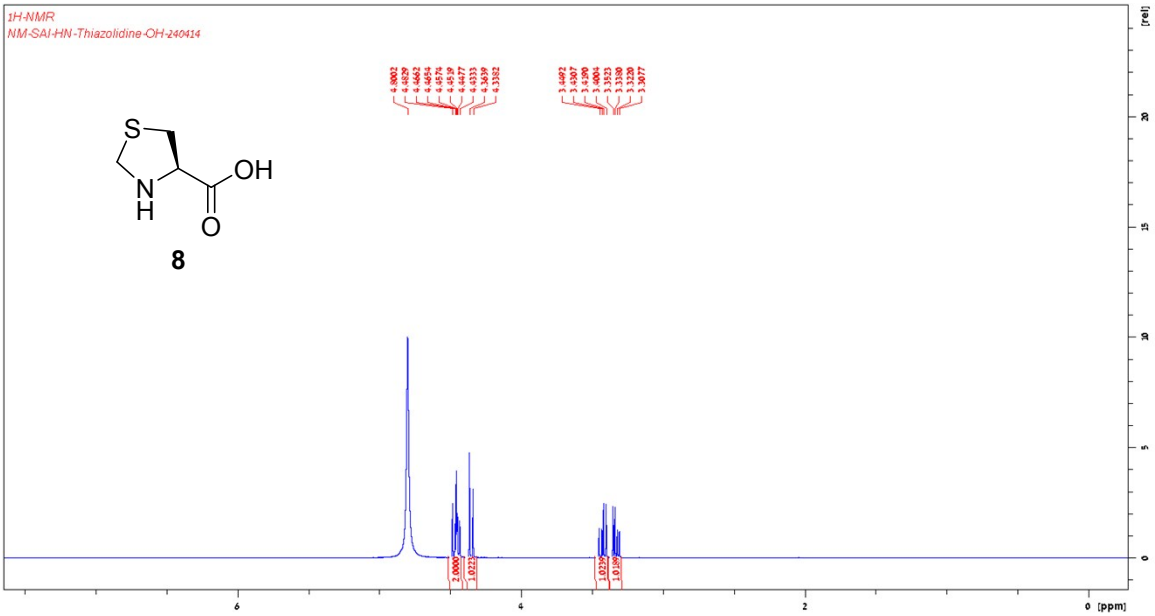

Figure S11. ^{1}H NMR Spectrum of **8** (400 MHz, D_2O)

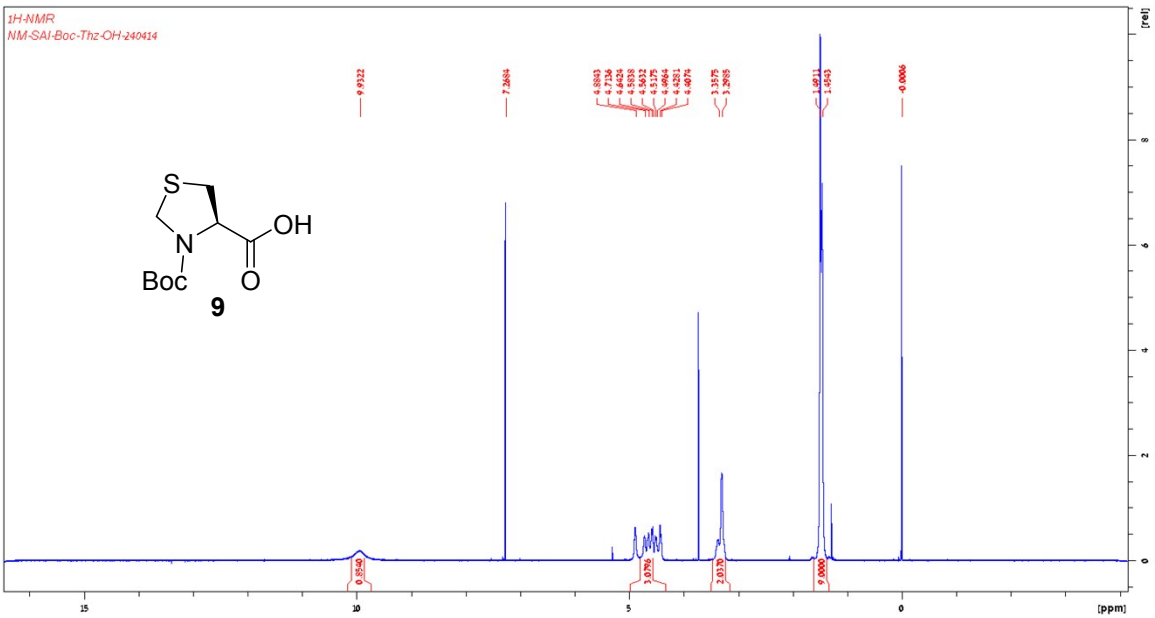

Figure S12. ^{1}H NMR Spectrum of **9** (400 MHz, $CDCl_3$)

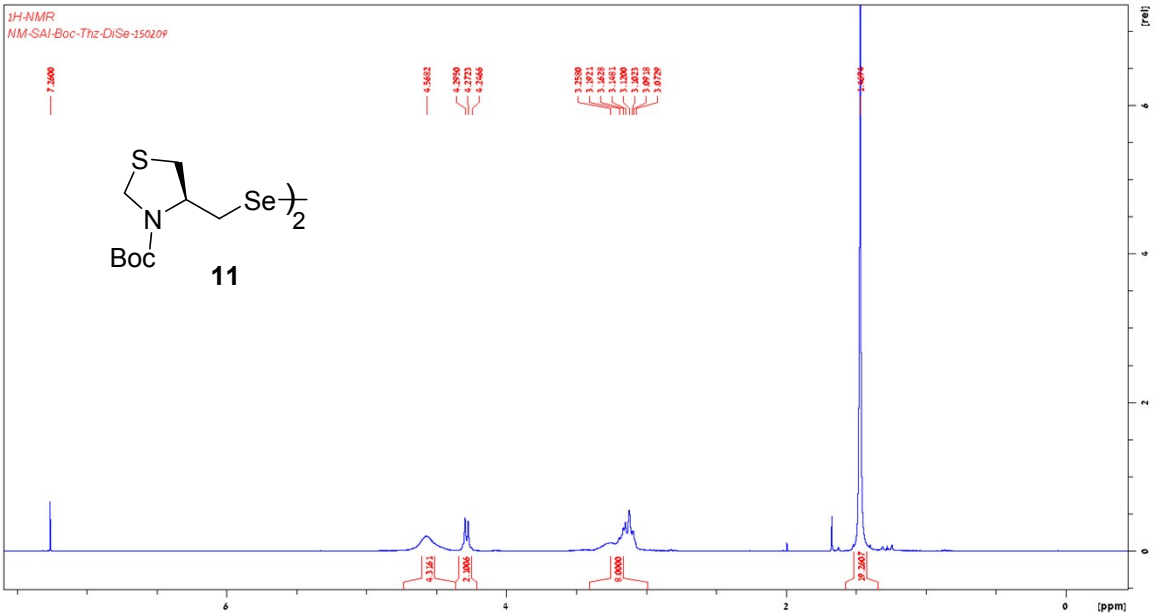

Figure S13. ^{1}H NMR Spectrum of **11** (400 MHz, $CDCl_3$)

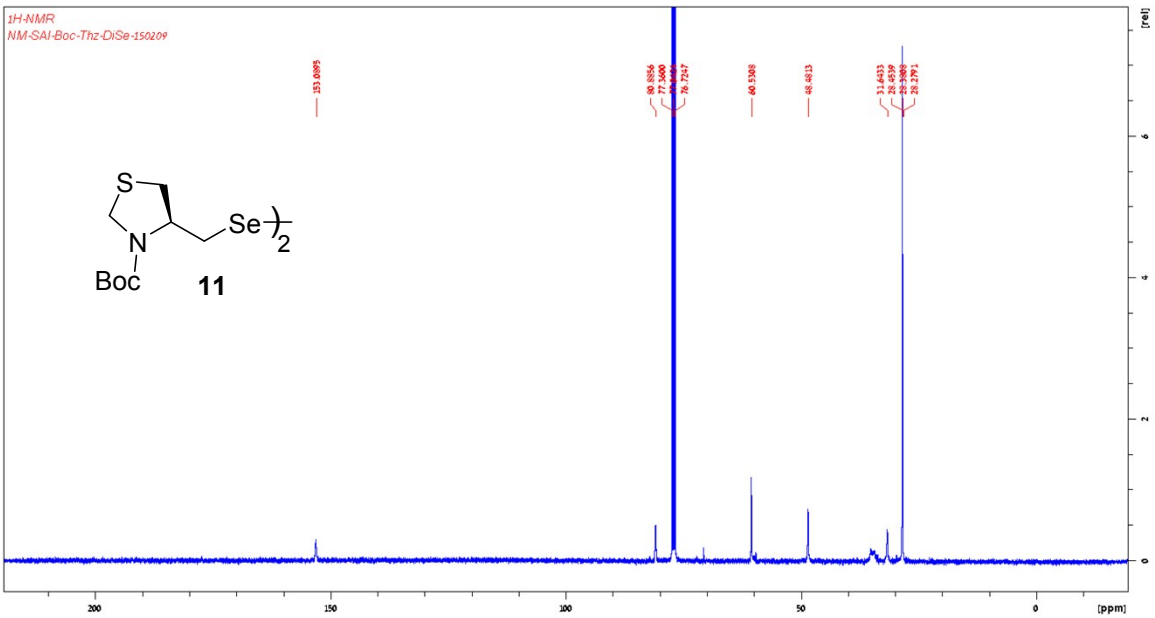

Figure S14. ^{13}C NMR Spectrum of **11** (100 MHz, $CDCl_3$)

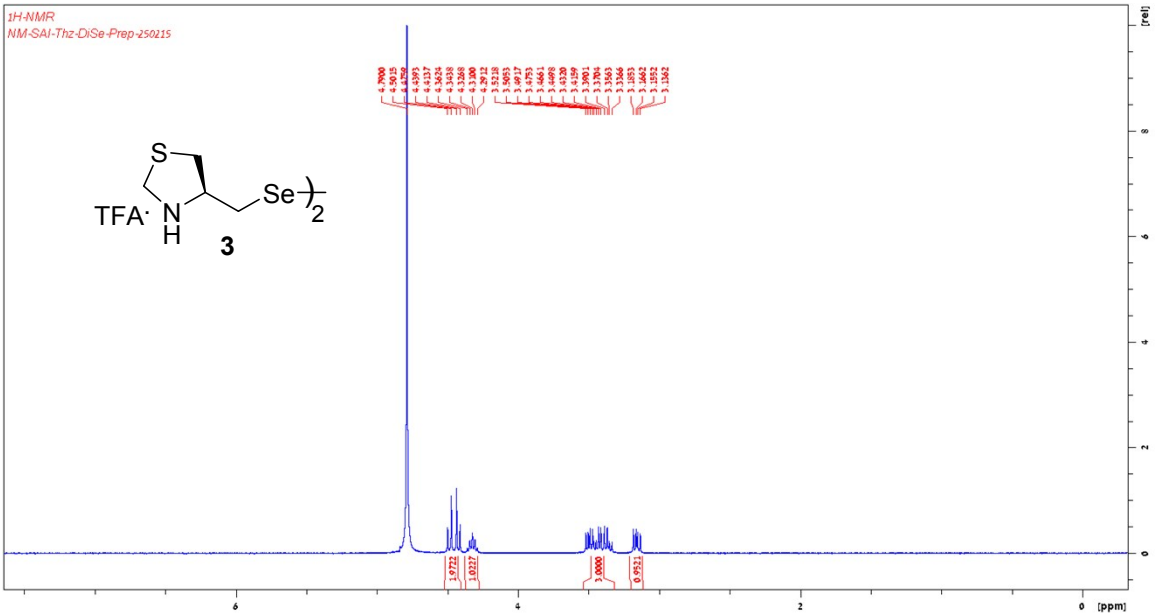

Figure S15. ^{1}H NMR Spectrum of **3** (400 MHz, D_2O)

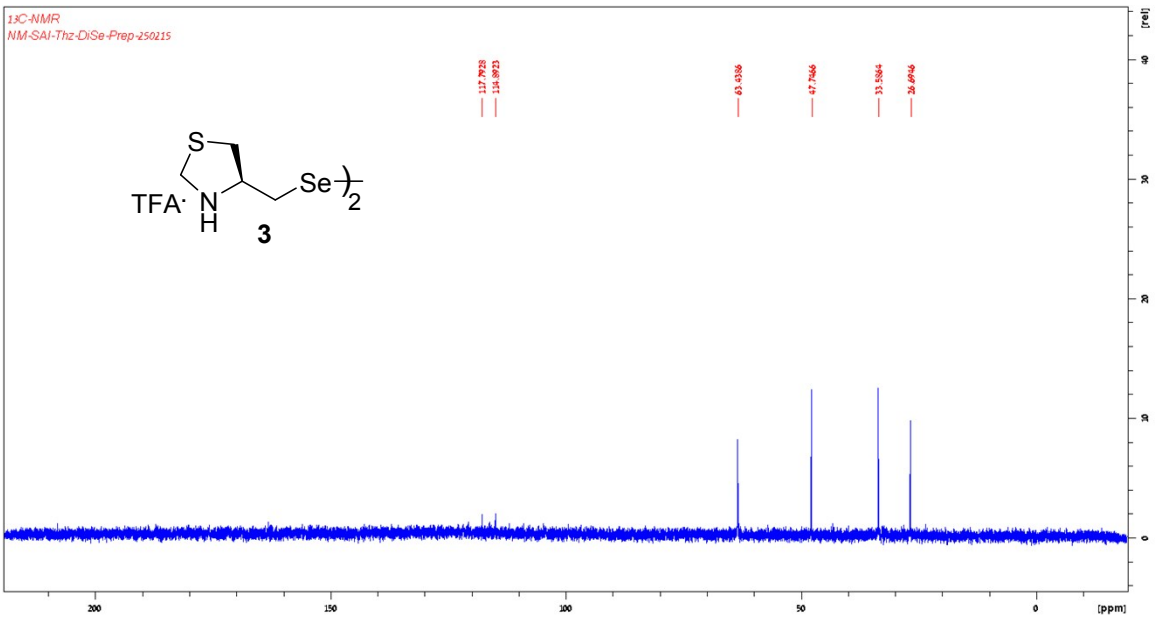

Figure S16. ^{13}C NMR Spectrum of **3** (100 MHz, D_2O)

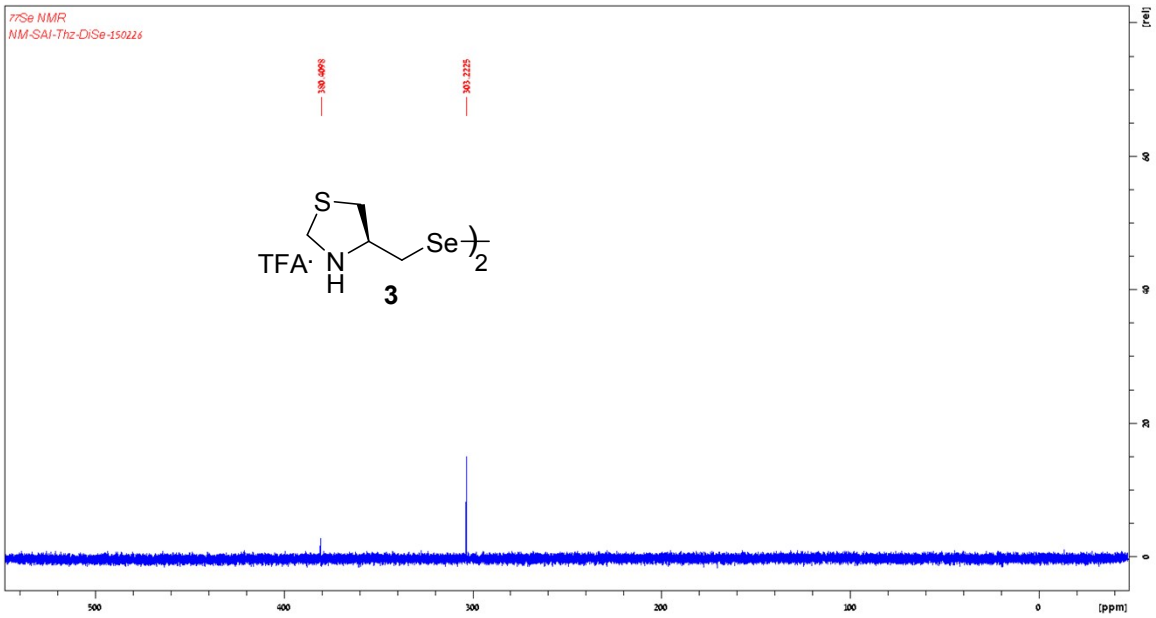

Figure S17. ^{77}Se NMR Spectrum of **3** (95 MHz, D_2O)

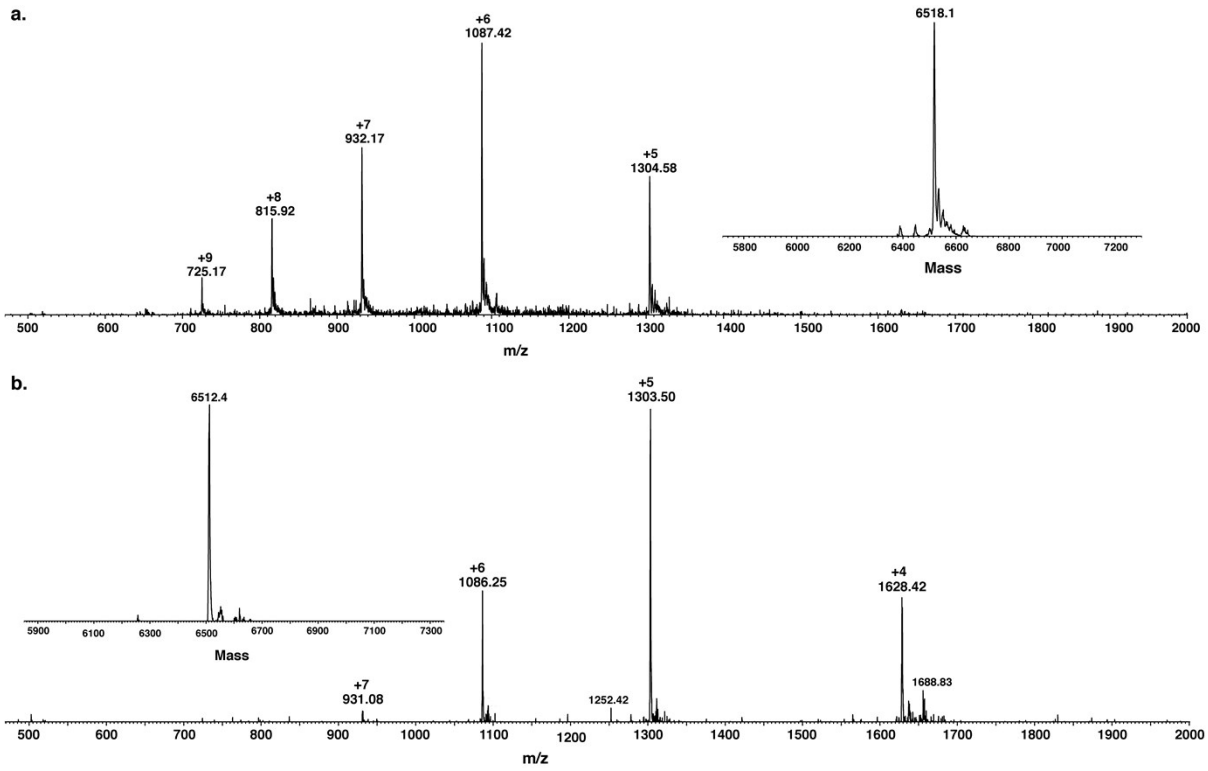

Figure S18. Mass spectrum of **a**. reduced BPTI and **b.** native BPTI. Deconvolution of the MS data was performed with the help of MagTran v1.03 software.