Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Chemoselective Modifications for the Traceless Ligation of Thioamide-Containing Peptides and Proteins

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General Information VA-044 was purchased from Wako Pure Chemical Industries (Osaka, Japan). Fmoc protected amino acids and peptide synthesis reagents were purchased from EMD Millipore (Billerica, MA, USA). *L*-Selenocystine was purchased from Sigma-Aldrich (St. Louis, MO, USA). N_{α}-Fmoc-L-Thioleucine-nitrobenztriazolide (Fmoc-Leu^{S}-Bt(NO_2)) was synthesized as previously described.[1] Hydrazine Hydrate solution was purchase from Oakwood Chemical (Estill, SC, USA). 5′-*O*-(4,4′-Dimethoxytrityl) adenosine ((DMT)-A) was purchased as a custom order from ChemGenes Corporation (Wilmington, MA, USA). Lysylalanylamino-methylcoumarin (LysAlaAcm) was purchased from Bachem (Torrence, CA, USA). $GB1_{24\text{-}56}$ A24C was purchased from Genscript (Piscataway, NJ, USA). The pEG6 plasmid, containing His_{10}-tagged *E. coli* AaT, was a gift from Alexander Varshavsky (California Institute of Technology). Bradford reagent assay kits were purchased from BioRAD (Hercules, CA, USA). Amicon Ultra centrifugal filter units (3k Da MWCO) were purchased from EMD Millipore. All other reagents and solvents were purchased from Fisher Scientific or Sigma-Aldrich unless otherwise specified.

High resolution electrospray ionization mass spectra (ESI-HRMS) were collected with a Waters LCT Premier XE liquid chromatograph/mass spectrometer (Milford, MA, USA). Low resolution electrospray ionization mass spectra (ESI-LRMS) were obtained on a Waters Acquity Ultra Performance LC connected to a single quadrupole detector (SQD) mass spectrometer. UV-Vis absorption spectra were acquired on a Hewlett-Packard 8452A diode array spectrophotometer (currently Agilent Technologies; Santa Clara, CA, USA). Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker DRX 500 MHz instrument (Billerica, MA, USA). Matrix assisted laser desorption/ionization with time-of-flight detector (MALDI-TOF) mass spectra were

acquired on a Bruker Ultraflex III instrument. Analytical HPLC was performed on an Agilent 1100 Series HPLC system. Preparative HPLC was performed on a Varian Prostar HPLC system (currently Agilent Technologies). HPLC columns were purchased from W. R. Grace & Compnay (Columbia, MD, USA).

Synthesis of Thioamide Precursors

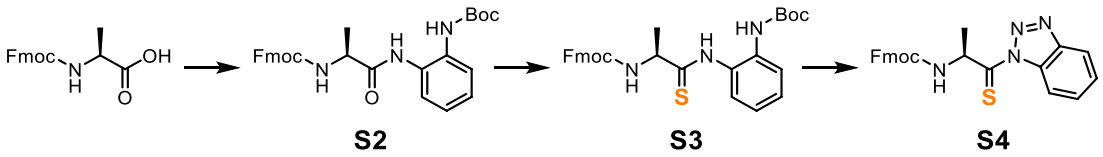

Fig. S1. Synthesis Scheme of Thioalanine Precursors for Thioamide Incorporation.

Synthesis of *N*-Boc-1,2-phenylenediamine (S1) 1,2-Phenylenediamine (5.9478 g, 55 mmol, 1.1 equiv) was dissolved in anhydrous tetrahydrofuran (THF, 50 mL). A solution of of di-*tert*-butyldicarbonate (Boc_2O, 10.9125 g, 50 mmol, 1 equiv) in anhydrous THF (50 mL) was added dropwise over 4 h on ice. Upon completion, the reaction was quenched by slow addition of H_2O (100 mL), and extracted with ethyl acetate (100 mL×3). The organic layers were combined, dried by rotary evaporation, and then purified by flash chromatography with 2:8 ethyl acetate/petroleum ether. A white flake was isolated as the final product (6.9823 g, 33.5 mmol, 67% yield). R_f 0.18 in 2:8 ethyl acetate/petroleum ether. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.27 (d, J = 7.6 Hz, 1H), 6.99 (t, J = 8.3 Hz, 1H), 6.77 (t, J = 7.6 Hz, 1H), 6.71 (d, J = 8.1 Hz, 1H), 6.60 (s, 1H), 3.76 (s, 2H), 1.54 (s, 9H). ^{13}C-NMR (500 MHz, $CDCl_3$): δ 154.16, 140.23, 126.17, 124.82, 119.44, 117.53, 80.48, 28.48. ESI$^+$-HRMS: calculated for $C_{11}H_{17}N_2O_2^+$: 209.12; found $[M + H]^+$: 209.14.

Synthesis of α-*N*-Fmoc-L-alanine-(*N*-Boc)-2-aminoanilide (S2). In an argon atmosphere, Fmoc-Ala-OH (1.5567 g, 5mmol, 1 equiv) was dissolved in 50 mL of dry tetrahydrofuran (THF)

and chilled in a 1:3 NaCl : ice bath (-10 °C). With stirring, N-methylmorpholine (NMM, 1.10 mL, 10 mmol, 2 equiv) and isobutylchloroformate (IBCF, 0.65 mL, 5 mmol, 1 equiv) were slowly added. The reaction was stirred at -10°C for 15 min, and *N*-Boc-1,2-phenylenediamine (**S1**, 1.0413g, 5 mmol, 1 equiv) was added. The reaction was allowed to proceed at -10°C for 2 h, and then at room temperature overnight (≥ 14 h). Upon completion, the solvent was removed by rotary evaporation. The residue was brought up in 40 mL of ethyl acetate, and extracted against 40 mL each of 1 M Na_2HPO_4, brine, 5% $NaHCO_3$, and brine. The aqueous layers were combined and extracted against 100 mL of ethyl acetate. The organic layers were combined, dried by rotary evaporation, and purified by flash chromatography in ethyl acetate/petroleum ether (35:65 v/v). A pale yellow solid was yielded as the final product (1.7944 g, 3.6 mmol, 77% yield). R_f 0.23 in 35:65 ethyl acetate/petroleum ether. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.73 (s, 1H), 7.70 (d, J = 7.6 Hz, 2H), 7.52 (t, J = 8.0 Hz, 2H), 7.48 (d, J = 7.4 Hz, 1H), 7.33 (t, J = 8.0 Hz, 2H), 7.31 (m, 1H), 7.22 (m, 2H), 7.12 (s, 1H), 7.10 (t, J = 7.5 Hz, 1H), 7.01 (t, J = 7.6 Hz, 1H), 5.87 (d, J = 7.1 Hz, 1H), 4.38 (m, 1H), 4.35 (t, J = 6.4 Hz, 2H), 4.14 (t, J = 6.7 Hz, 1H), 1.44 (s, 9H), 1.41 (d, J = 7.1 Hz, 3H). ^{13}C-NMR (500 MHz, $CDCl_3$): δ 171.83, 156.25, 154.11, 143.72, 141.28, 131.39, 128.81, 127.79, 127.11, 126.57, 125.40, 125.08, 124.20, 80.75, 68.62, 51.08, 47.09, 28.32, 22.11. ESI^+-HRMS: calculated for $C_{29}H_{31}N_3O_5Na^+$: 524.2161; found $[M + Na]^+$: 524.2148.

Synthesis of α-*N*-Fmoc-L-thioalanine-(*N*-Boc)-2-aminoanilide (**S3**). In an argon atmosphere, **S2** (1.6593 g, 3.3 mmol, 1 equiv) and Lawesson's reagent (1.0036 g, 2.5 mmol, 0.75 equiv) were refluxed in 30 mL of CH_2Cl_2 for 18 h. Upon completion, the solvent was removed by rotary evaporation. The crude product was purified by flash chromatography in ethyl acetate/petroleum ether (35:65 v/v) to yield a pale yellow foam (1.5214 g, 2.9 mmol, 89% yield).

R_f 0.43 in 35:65 ethyl acetate/petroleum ether. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 10.09 (s, 1H), 7.69 (d, *J* = 7.3 Hz, 2H), 7.61 (d, *J* = 7.4 Hz, 1H), 7.51 (d, *J* = 7.6 Hz,1H), 7.49 (d, *J* = 7.1 Hz,1H), 7.33 (t, *J* = 7.4 Hz, 2H), 7.30 (m, 1H), 7.23 (m, 2H), 7.22 (m, 1H), 7.05 (t, *J* = 7.6 Hz, 1H), 6.91 (s, 1H), 6.04 (d, *J* = 6.4 Hz, 1H), 4.67 (m, 1H), 4.31 (d, *J* = 6.1 Hz, 2H), 4.14 (t, *J* = 7.0 Hz, 1H), 1.50 (d, *J* = 6.6 Hz, 3H), 1.42 (s, 9H). ^{13}C-NMR (500 MHz, $CDCl_3$): δ 205.90, 155.94, 153.83, 143.61, 143.56, 141,22, 141.20, 132.95, 129.64, 128.49, 127.78, 127.14, 125.11, 125.07, 124.65, 123.59, 120.01, 81.07, 67.27, 55.58, 46.99, 28.26, 22.42. ESI$^+$-HRMS: calculated for $C_{29}H_{31}N_3O_4SNa^+$: 540.1933; found $[M + Na]^+$: 540.1914.

Synthesis of α-*N*-Fmoc-L-thioalanine-benzotriazolide (S4) In argon atmosphere, **S3** (1.0861 g, 2.1 mmol, 1 equiv) was dissolved in 10 mL of CH_2Cl_2 and chilled to 0 ˚C on ice. 10 mL of an ice cold TFA/CH_2Cl_2 (50:50 v/v) solution was slowly added, and the reaction was allowed to proceed at 0 ˚C for 2 h. Upon completion, the solvent was removed by rotary evaporation. The residue was dissolved in glacial AcOH (9.5 mL) and water (0.5 mL), and then chilled to 0 ˚C on ice. With stirring, $NaNO_2$ (0.2202 g, 3.2 mmol, 1.5 equiv) was added in small portions over 5 min. The reaction was allowed to proceed at 0 ˚C for 30 min, and then quenched with 30 mL of ice cold water. The precipitate was collected by filtration, dried on vacuum for 30 min, and then purified by flash chromatography in ethyl acetate/petroleum ether (35:65 v/v). A yellow foam was yielded as the final product (0.6286 g, 1.5 mmol, 69% yield). R_f 0.54 in 35:65 ethyl acetate/petroleum ether. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.75 (d, *J* = 8.3 Hz, 1H), 8.12 (d, *J* = 8.0 Hz, 1H), 7.74 (d, *J* = 7.1 Hz, 2H), 7.64 (m, 2H), 7.61 (m, 1H), 7.52 (t, *J* = 7.4 Hz, 1H), 7.38 (t, *J* = 6.7 Hz, 1H), 7.30 (t, *J* = 6.8 Hz, 1H), 6.31 (m, 1H), 5.91 (d, *J* = 8.8 Hz, 1H), 4.47 (dd, *J* = 10.3, 7.0 Hz, 1H), 4.34 (dd, *J* = 10.3, 7.0 Hz, 1H), 4.22 (t, *J* = 6.6 Hz, 1H), 1.66 (d, *J* = 6.6 Hz,

3H). ^{13}C-NMR (500 MHz, $CDCl_3$): δ 210.01, 155.61, 147.18, 144.03, 143.88, 141.41, 132.46, 131.68, 127.83, 127.32, 127.20, 125.32, 125.22, 120.92, 120.11, 116.31, 67.16, 57.27, 47.33, 23.10. ESI$^+$-HRMS: calculated for $C_{24}H_{20}N_4O_2SNa^+$: 451.1205; found $[M + Na]^+$: 451.1195.

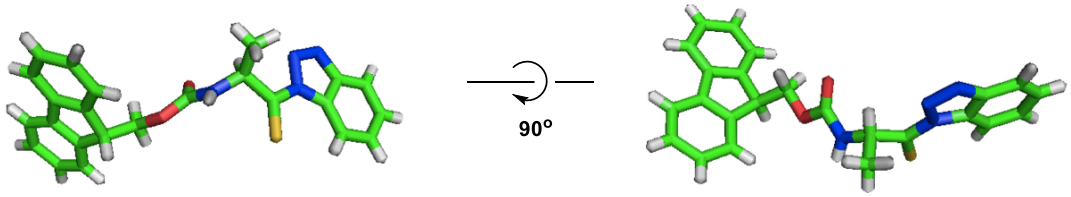

Fig. S2. Crystal Structure of Thioalanine Precursor. Crystal was grown in 35:65 ethyl acetate/petroleum ether; data were acquired on a Bruker Kappa APEX II Duo CCD diffractometer (Billerica, MA, USA). Graphics were generated using Schrödinger PyMOL (Cambridge, MA, USA). C=S bond length observed: 1.6 Å. Structure coordinates deposited with Cambridge Crystallographic Data Centre (1472443).

Peptide Synthesis and Purification Peptides were synthesized using standard Fmoc solid phase peptide synthesis (SPPS) procedure on either Rink Amide or 2-chlorotrityl chloride resin (100 - 200 mesh; 0.6 mmol substitution/g). For coupling, 5 equiv of amino acid and 5 equiv of HBTU were dissolved in dimethylforamide (DMF), pre-activated for 1 min in the presence of 10 equiv of *N*,*N*-diisopropylethylamine (DIPEA), and then stirred with the resin for 30 min at room temperature. For deprotection, 20% piperidine in DMF was stirred with the resin for 20 min. Thioalanine (denoted Ala^S or A^S) was introduced through activated benzotriazole precursors (**S4**); 2 equiv of the precursor was dissolved in dry CH_2Cl_2, and stirred with the resin for 45 min in the presence of 2 equiv DIPEA. For N-terminally acetylated peptides, an acetylation cocktail of 1:1:8 acetic anhydride/N-methylmorpholine/DMF was stirred with the resin twice, each for 10 min, after the last deprotection. Upon completion of SPPS, resins were rinsed thoroughly with CH_2Cl_2 and dried under vacuum.

For cleavage, resins were treated with a cleavage cocktail (12:1:1:26 trifluoroacetic acid/triisopropylsilane/H_2O/CH_2Cl_2) for 30 min. The solution was then collected by filtration, and dried by rotary evaporation. For purification, the crude residues were brought up in 1:10 CH_3CN/H_2O, and then purified by reverse phase HPLC with acidified (with 0.1% trifluoroacetic acid) CH_3CN/H_2O gradients. Individual fractions were charac-terized by MALDI-TOF MS, and dried by lyophilization. When necessary, the isolated products were subjected to multiple rounds of purification until 99.5% pure by MALDI-TOF MS and analytical HPLC. Solvent radients, retention times and MALDI-TOF MS results are listed in Table S1 through

Table ***S3***.

Table S1. Peptide Purification Methods and Retention Time.

Peptide	Gradient	Retention Time	Column
Ac-L**A**^{S}AKAG**C**AKXAG-NH_2 (**1**)	**1**	23.1 min	Vydac C18 Semi-prep
Ac-L**A**^{S}AKAG**A**AKXAG-NH_2 (**3'**)	**1**	22.3 min	Vydac C18 Semi-prep
Ac-G**L**^{S}KXAG-CPG_o (**S6**)	**4**	22.0 min	YMC-Pack Pro C8 Semi-prep
Ac-XTTEAVDA**C**T**A**^{S}AK-NH_2 (**4**)	**5**	16.0 min	Vydac C18 Semi-prep
Ac-XTTEAVDA**A**T**A**^{S}AK-NH_2 (**5'**)	**6**	21.6 min	Vydac C18 Semi-prep
Ac-XTTEAVDA**A**T**A**AK-NH_2 (**6'**)	**6**	19.9 min	Vydac C18 Semi-prep
Ac-XTTEAVDA**S**T**A**^{S}AK-NH_2 (**S10'**)	**6**	19.7 min	Vydac C18 Semi-prep
Ac-XTTEAVDA**C**T**A**AK-NH_2 (**S11'**)	**6**	21.2 min	Vydac C18 Semi-prep
Ac-XTTEAVDA-$SC_2H_4COOCH_3$ (**7a**)	**7**	17.6 min	YMC-Pack Pro C8 Semi-prep
CT**A**^{S}AK-NH_2 (**7b**)	**8**	12.8 min	Vydac C18 Semi-prep
UT**A**^{S}A**C**VFKX-NH_2^{Se-S} (**12**)**	**9**	24.5 min	YMC-Pack Pro C8 Semi-prep
Ac-XTTEA**Y**DAAT**A**^{S}AK-NH_2 (**11**)	**10**	34.6 min	Vydac C18 Prep
$GB1_{1-23}$ L^{S}5-N_2H_3 (**8a**)	**12**	21.9 min	Vydac C18 Prep
$GB1_{24-56}$ A24C-OH (**9**)	**13**	22.3 min	Vydac C18 Prep
GB1 L^{S}5 (**10b**)	**15**	21.3 min	Vydac C18 Semi-prep

* X = 7-methoxycoumarinylalanine; Y = penicillamine; U = selenocysteine; G_o = glycolic acid.

** The "Se-S" superscript denotes that **5** was isolated as an intramolecular hemiselenide species.

Table S2. HPLC Gradients for Peptide Purification and Characterization.

No.	Time (min)	%B	**No.**	Time (min)	**%B**	**No.**	Time (min)	**%B**
1	0:00	2	**2**	0:00	2	**3**	0:00	2
	5:00	2		5:00	2		8:00	2
	10:00	15		10:00	20		10:00	10
	25:00	30		25:00	50		30:00	50
	30:00	100		30:00	100		35:00	100
	35:00	100		35:00	100		40:00	100
	40:00	2		40:00	2		45:00	2
4	0:00	2	**5**	0:00	2	**6**	0:00	2
	10:00	2		5:00	2		5:00	2
	15:00	20		10:00	25		10:00	20
	30:00	50		25:00	40		25:00	35
	35:00	100		30:00	100		30:00	100
	40:00	100		35:00	100		35:00	100
	45:00	2		40:00	2		40:00	2
7	0:00	2	**8**	0:00	2	**9**	0:00	2
	5:00	2		5:00	2		5:00	2
	10:00	25		10:00	10		10:00	20
	30:00	45		20:00	20		40:00	50
	35:00	100		25:00	100		45:00	100
	40:00	100		30:00	100		50:00	100
	45:00	2		35:00	2		55:00	2
10	0:00	5	**11**	0:00	2	**12**	0:00	5
	5:00	5		5:00	2		5:00	5
	15:00	22		10:00	25		10:00	20
	45:00	28		40:00	40		25:00	30
	50:00	100		45:00	100		27:00	100
	55:00	100		50:00	100		30:00	100
	60:00	5		55:00	2		32:00	5
13	0:00	2	**14**	0:00	2	**15**	0:00	2
	5:00	2		15:00	2		10:00	2
	10:00	23		20:00	20		15:00	30
	45:00	35		50:00	50		35:00	50
	48:00	35		55:00	100		37:00	50
	50:00	100		60:00	100		39:00	100
	53:00	100		65:00	2		43:00	100
	55:00	2					45:00	2

* Solvent A: 0.1% trifluoroacetic acid in water; Solvent B: 0.1% trifluoroacetic acid in acetonitrile

Table S3. MALDI-TOF MS Characterization of Purified Peptides.

Peptide	$[M + H]^+$		$[M + Na]^+$	
	Calc'd	**Found**	**Calc'd**	**Found**
Ac-L**A**^{S}AKAG**C**AKXAG-NH_2 (**1**)	1262.59	1262.63	1282.58	1284.62
Ac-L**A**^{S}AKAG**A**AKXAG-NH_2 (**3'**)	1230.62	1230.78	1252.61	1252.77
Ac-G**L**^{S}KXAG-CPG_o (**S6**)	1005.41	1005.35	1027.40	1027.33
Ac-XTTEAVDA**C**T**A**^{S}AK-NH_2 (**4**)	1482.62	1482.42	1504.61	1504.41
Ac-XTTEAVDAA**T**A^{S}AK-NH_2 (**5'**)	1450.64	1450.80	1472.63	1472.79
Ac-XTTEAVDAA**T**A**A**K-NH_2 (**6'**)	1434.67	1434.81	1456.66	1456.80
Ac-XTTEAVDA**S**T**A**^{S}AK-NH_2 (**S10'**)	1466.64	1466.74	1488.63	1466.72
Ac-XTTEAVDA**C**T**A**AK-NH_2 (**S11'**)	1466.64	1466.80	1488.63	1466.81
Ac-XTTEAVDA-$SC_2H_4COOCH_3$ (**7a**)	1095.41	-	1117.40	1117.45
CTA^{S}AK-NH_2 (**7b**)	508.23	508.37	530.22	530.34
UT**A**^{S}A**C**VFKX-$NH_2^{Se–S}$ (**12**)**	1148.37	1148.46	1170.36	1170.44
Ac-XTTEA**Y**DAATA^{S}AK-NH_2 (**11**)	1482.62	1482.56	1504.61	1504.55
$GB1_{1-23}$ $L^{S}5$-N_2H_3 (**8a**)	2512.89	2512.25	2534.87	2534.24
$GB1_{1-23}$ $L^{S}5$-SPh (**8b**)	2591.01	2591.31	2613.00	-
$GB1_{24-56}$ A24C-OH (**9**)	3749.01	3748.85	3770.99	-
GB1 $L^{S}5$ A24C (**10a**)	6228.84	6229.73	6250.83	-
GB1 $L^{S}5$ (**10b**)	6196.78	6196.39	6218.77	-

* X = 7-methoxycoumarinylalanine; Y = penicillamine; U = selenocysteine; G_o = glycolic acid.
** **5** was isolated as an intramolecular hemiselenide species.

Synthesis of Reduced Ac-GL^{S}KXAG-CPG_o (S6) Ac-GL^{S}KXAG-C^{b}PG_o (**S5**) with a side chain tBuS− protecting group was synthesized as we described previously[2]. To generate the reduced **S6**, **S5** (200 nmol, ε_{325} = 12,000 M^{-1} cm^{-1}) was mixed with 100 μL of reduction buffer (40 mM TCEP, 200 mM Na_2HPO_4, pH 6.8) and 20 μL of CH_3CN. The reaction was allowed to proceed at 37 ˚C for 1 h. Upon completion, CH_3CN (80 μL) was added, and the supernatant was recovered by centrifugation at 13.2 krpm for 20 min. The crude was diluted into H_2O (800 μL), and then purified by reverse phase HPLC (Table S1). Isolated product was characterized by MALDI MS (

Table ***S3***), quantified by UV-Vis absorption, split into 20 nmol aliquots, and then lyophilized until further use. X = 7-methoxycoumarinylalanine; G_o = glycolic acid.

One-Pot Ligation Deselenization with Selenocystine A phosphate buffer stock (200 mM Na_2HPO_4, pH 8.0) and a 5 M NaOH solution were freshly degassed using the freeze-pump-thaw method. 2% (v/v) PhSH was added to the buffer stock, and pH was quickly adjusted back to 8.0 using the degassed 5 M NaOH solution under an argon atmosphere. A 1.1x *L*-selenocystine stock (2.2 mM) was prepared by dissolving *L*-seleno-cystine (0.0007 g, 2.2 μmol) in the above buffer (1 mL). To thioester **S6** (20 nmol, ε_{325} = 12,000 M^{-1} cm^{-1}), 10 μL of CH_3CN and 90 uL of the 1.1x *L*-selenocytine stock (final concentration 2 mM, 10 equiv) was added. The reaction was allowed to proceed at 37 °C for 12 h under an argon atmosphere.

For deselenization, a 50 μL aliquot of the ligation reaction was removed, and directly added to 50 μL of 2x deselenization buffer stock (40 mM TCEP, 40 mM DTT, 200 mM Na_2HPO_4, pH 8.0) that had been freshly degassed by freeze-pump-thaw method. The reaction was allowed to proceed at 37 °C overnight (≥ 14 h) under an argon atmosphere. Upon completion, both ligation and deselenization crudes were diluted with argon-purged H_2O, and then analyzed by reverse phase HPLC on a Luna C8 column using gradient **2**. Individual fractions were recovered, and analyzed by MALDI-TOF MS and UV-Vis absorption spectroscopy. (Fig. S3)

VA-044 Desulfurization for Thioamide Compatibility Studies Peptide **1** (10 nmol, ε_{325} = 12,000 M^{-1} cm^{-1}) was dissolved in 80 μL of argon-purged 1.25x buffer stock (50 mM TCEP, 125 mM Na_2HPO_4, pH 7.0). 10 μL of *t*-BuSH was added, followed by 10 μL of freshly prepared 10x

VA-044 stock (100 mM in argon-purged water). The reaction was allowed to proceed at 37 °C for 2 h under argon atmosphere, and then quickly quenched by chilling to 0 °C on ice. Excess *t*-BuSH was removed by a stream of argon, and then the crude mixture was diluted into H_2O for analysis by reverse phase HPLC on a Luna C8 analytical column using gradient **2**. Fractions were collected, and then analyzed by MALDI-TOF MS and UV-Vis absorption spectroscopy. Yield was quantified by integrating peak areas in HPLC chromatogram. See Fig. S4 for proposed mechanisms of radical initiated desulfurization and deselenization.

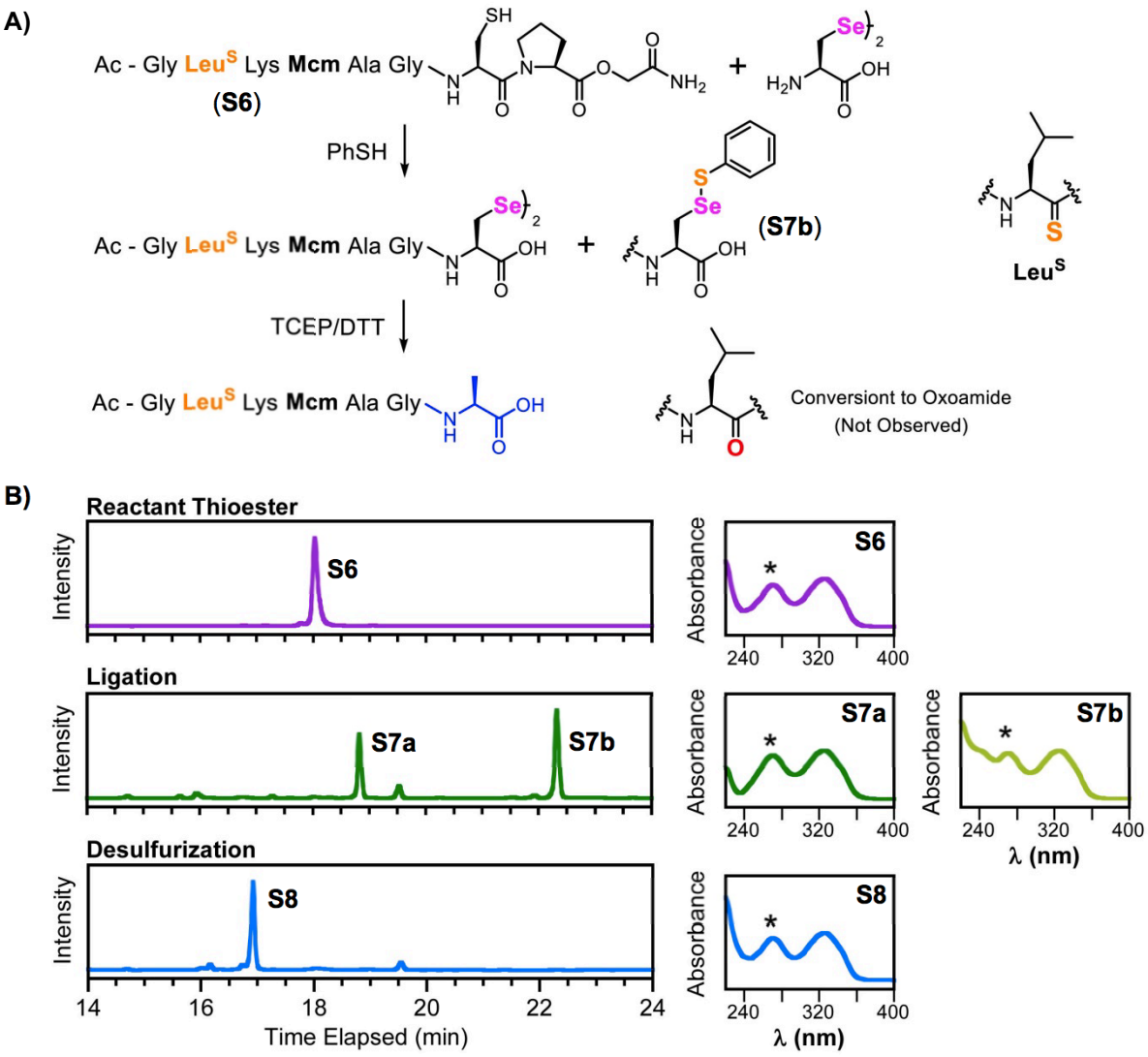

Fig. S3. Selective Deselenization of Sec in the Presence of Thioamide. A) Schematic representation of the test reaction; a reduced version of CPG_o instead of the tBuS-protected C^bPG_o was used to eliminate the need for TCEP as a reducing reagent in ligation. B) HPLC chromatogram monitored at 325 nm, and UV-Vis absorption spectra of isolated peptides. The star sign indicates thioamide absorption at 272 nm; **S7b** also contained a peak around 260 nm that is characteristic of −SPh group. Ligation Conditions: 0.2 mM peptide **S6**, 2 mM selenocystine, sat. PhSH, 200 mM Na_2HPO_4, pH 8.0, 12 h. Deselenization Conditions: 0.2 mM ligated peptide (without purification), 20 mM TCEP, 20 mM DTT, 200 mM Na_2HPO_4, pH 8.0, overnight. MALDI MS: [**S6** + H]$^+$, expected 1005.42, found 1005.35; [**S7a** + H]$^+$, expected 1795.55, found 1795.41; [**S7b** + H]$^+$, expected 1007.29, found 1007.25; [**S8** + H]$^+$, expected 819.37, found 819.19. TCEP = tris(2-carboxyethyl) phosphine; DTT = dithiothreitol; Mcm = 7-methoxycoumarinylalanine.

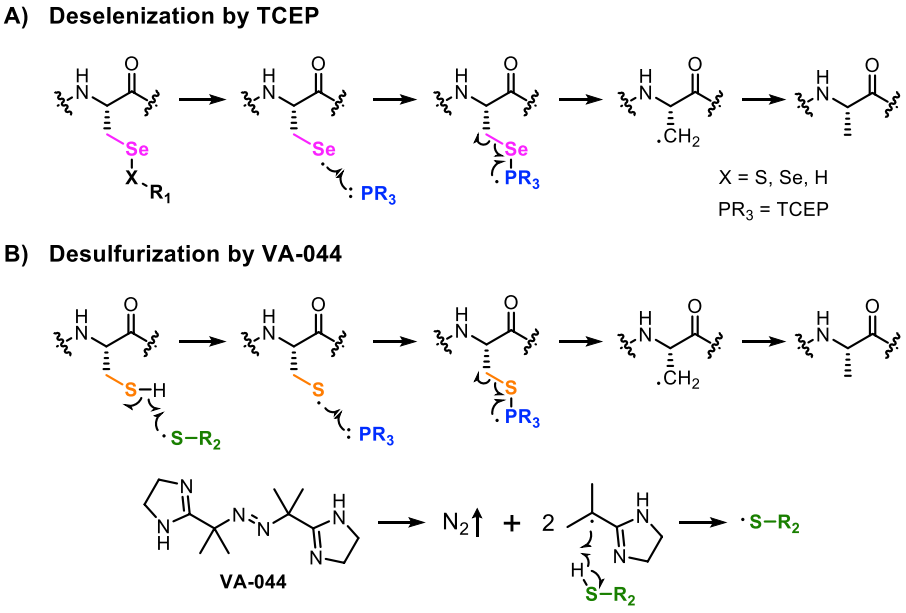

Fig. S4. Proposed Mechanisms for TCEP Deselenization and VA-044 Desulfurization.

Raney Nickel Desulfurization for Thioamide Compatibility Studies Peptide **1** (10 nmol, ε_{325} = 12,000 M^{-1} cm^{-1}) was dissolved in desulfurization buffer (100 µL, 100 mM Na_2HPO_4, 10 mM TCEP, pH 5.8), and then Raney nickel (0.1 mg) was added. The reaction was allowed to proceed at room temperature for 12 h, and then supernatant was recovered by centrifugation at 13.2 krpm for 20 min. The crude was analyzed by analytical RP-HPLC on a Luna C8 analytical column using gradient **2**. Individual fractions were collected manually, and then analyzed by MALDI-TOF and UV-Vis absorption spectroscopy.

VA-044 Desulfurization for Condition Optimization Peptide **4** (10 nmol, ε_{325} = 12,000 M^{-1} cm^{-1}) was dissolved in 80 µL of argon-purged 1.25x buffer stock (50 mM TCEP, 125 mM Na_2HPO_4, pH 7.0). 10 µL of *t*-BuSH was added, followed by 10 µL of freshly prepared 10x VA-044 stock (100 mM in argon-purged water). The reaction was allowed to proceed at 37 °C for 10 min under argon atmosphere, and then quickly quenched by chilling to 0 °C on ice. Excess *t*-BuSH

was removed by a stream of argon, and then the crude mixture was diluted into H_2O for analysis by reverse phase HPLC on a Luna C8 analytical column using gradient **3**. Fractions were collected, and then analyzed by MALDI-TOF MS and UV-Vis absorption spectroscopy. Yield was quantified by integrating peak areas in the HPLC chromatograms. All subsequent condition optimization experiments were conducted using permutations of this standard procedure.

TCEP Dosage Dependence The reactions were conducted similarly to the standard procedure, except that 1.25x buffer stocks of various TCEP concentration were used, and that a 2 h reaction time was adopted. At low TCEP concentrations, side product **S9** was observed, with a disulfide bond between Cys and ambient *t*-BuSH (Fig. S5). The side product was not observed at high TCEP concentrations.

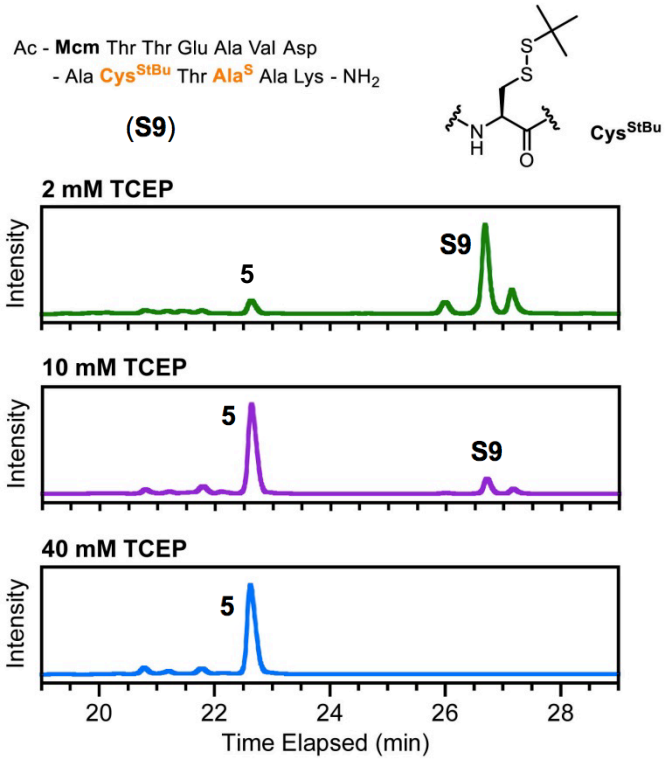

Fig. S5. TCEP Dosage Dependence and Generation of Disulfide Bonded Side Product. MALDI-TOF MS: [**S9** + H]$^+$, expected 1570.65, found 1570.74.

VA-044 Dosage Dependence Reactions were conducted similarly to the standard procedure, except that 10x VA-044 stocks at various concentrations were used for 2 h or 18 h (Fig. S6). Above 0.1 mM, no distinction was observed at the range of VA-044 concentrations tested. At very low VA-044 concentration, however, the reaction was significantly slower and messier − at 2 h, while all other conditions showed complete reaction, minimal product formation was observed at 0.01 mM VA-044. At 18 h, the reaction was still not complete, with additional side peaks in the chromagram.

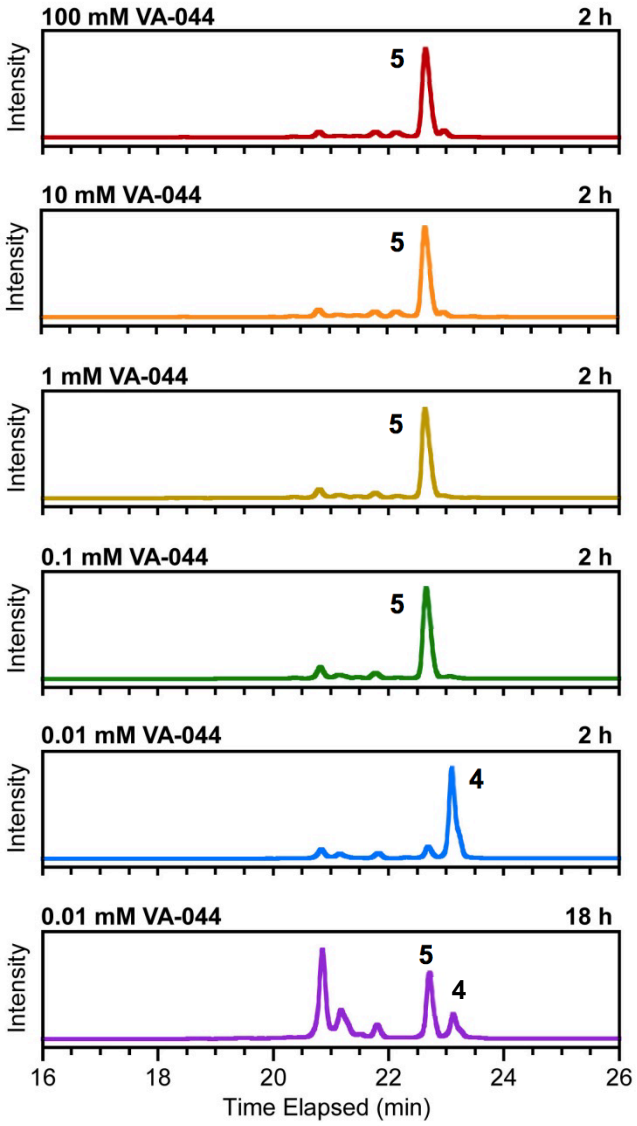

Fig. S6. VA-044 Dosage Dependence on Chemoselective Cys Desulfurization.

Oxygen Tolerance Reactions were conducted similarly to the standard procedure, except that an undegassed buffer stock was used with 5 min or 10 min reaction time. While both reactions completed within 10 min, the undegassed condition resulted in a slightly slower kinetics – 80% complete (as measured by reactant consumed) by 5 min as compared to 89% complete for the degassed condition (Fig. S7).

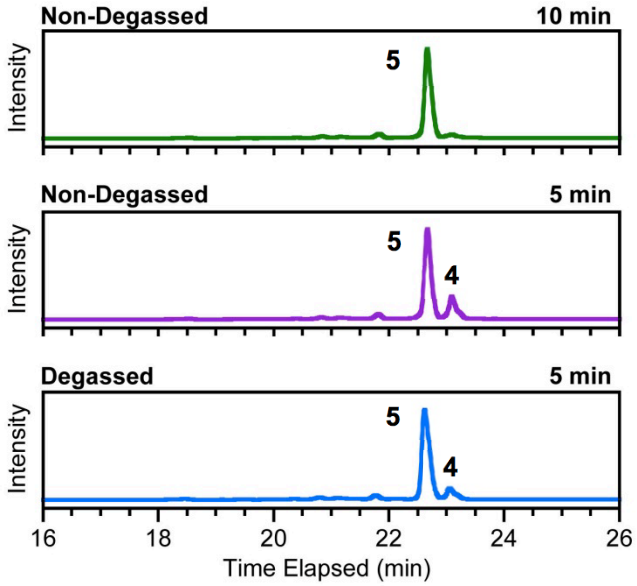

Fig. S7. Oxygen Tolerance on Chemoselective Cys Desulfurization.

Denaturant Tolerance Reactions were conducted similarly to the standard procedure, except that a 1.25x buffer stock with denaturant was used with 2 h reaction time. No difference was found as compared to denaturant free conditions (Fig. S8).

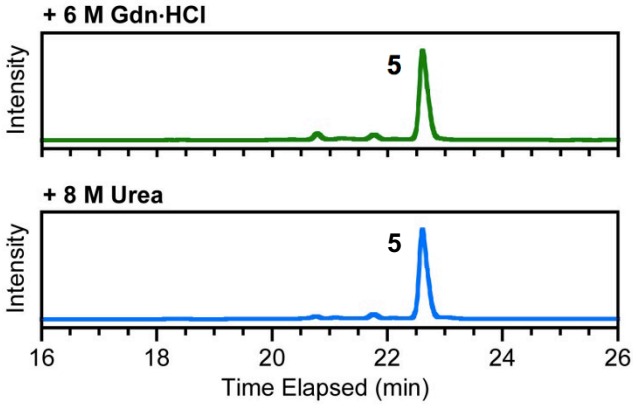

Fig. S8. Denaturant Tolerance on Chemoselective Cys Desulfurization.

Prolonged Reaction with Cys/Thioamide-Containing Peptide Reactions were conducted similarly to the standard procedure, except that longer reaction times were adopted. Side products, particularly **6**, accumulated over time (Fig. S9).

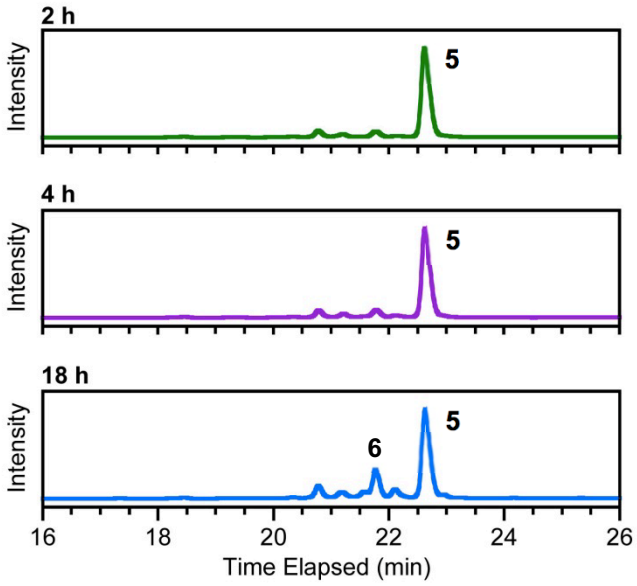

Fig. S9. Accumulation of Non-Selectively Desulfurized Side Product over Time.

Thioacetamide Suppression of Thioamide Degradation Reactions were conducted similarly to the standard procedure using Ala/thioamide peptide **5'**. Thioamide-to-oxoamide conversion was effectively suppressed after 18 h of exposure (Fig. S10).

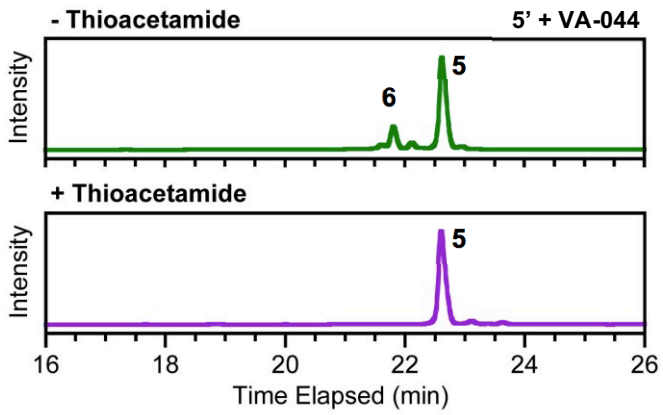

Fig. S10. Thioacetamide as Scavenger to Suppress Thioamide Side Reaction. MALDI-TOF MS: [**5'** + H]$^+$, expected 1450.64, found 1450.74; [**6** + H]$^+$, expected 1434.67, found 1434.87.

Thioacetamide Dosage Dependence Reactions were conducted similarly to the standard procedure, except that buffers with various concentrations of thioacetamide were used with 18 h reaction time. Suppression of thioamide-to-oxoamide conversion was effective over the wide range of concentrations tested; at very high concentration, thioacetamide may quench ambient radicals, leading to slower kinetics (Fig. S11).

Oxygen and Denaturant Tolerance in the Presence of Thioacetamide Reactions were conducted similarly to the control reactions without thioacetamide for 18 h. No distinction was found with the addition of thioacetamide (Fig. S12).

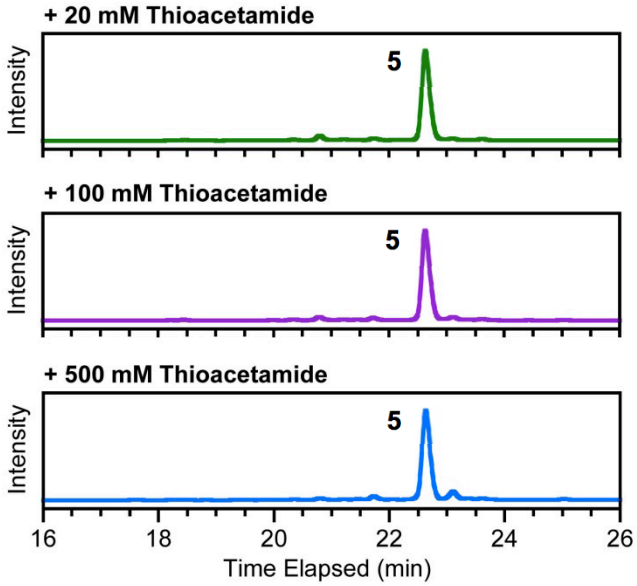

Fig. S11. Thioacetamide Dosage Dependence on Thioamide Protection.

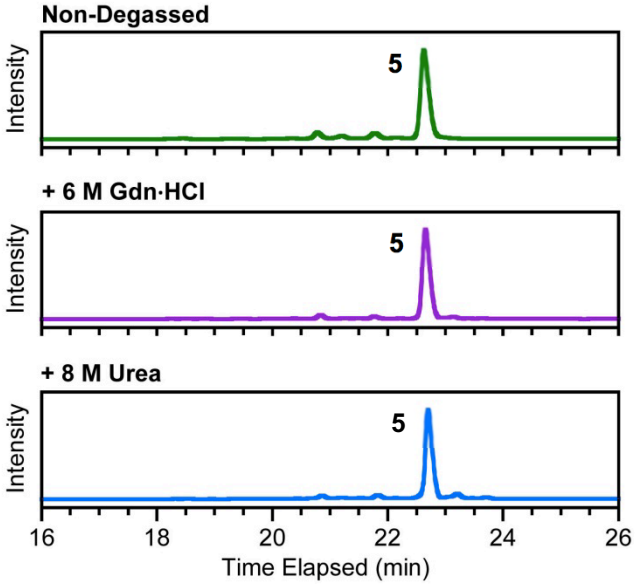

Fig. S12. Oxygen and Denaturant Tolerance in the Presence of Thioacetamide.

Characterization of Cys-to-Ser Conversion Side Products We were surprised to find a residual peak at 21.8 min even in the presence of thioacetamide, which should suppress thioamide-to-oxoamide conversion and abolish side product **6**. Upon a closer examination of the MALDI-TOF MS and UV-Vis absorption spectra (Fig. S14), we realized that the residual peak represents a separate side product **S10** where a Cys-to-Ser conversion took place with thioamide intact. The conversion could result from quenching of alkyl radical by water or dissolved oxygen in the reaction solution (Fig. S13); in fact, a similar conversion had been reported for oxygen-related complications in Sec deselenization.[3,4] We believe both water and oxygen contribute to the Cys side reaction observed here – a 2% conversion still took place in argon purged buffer, as compared to 3% in non-degassed buffer.

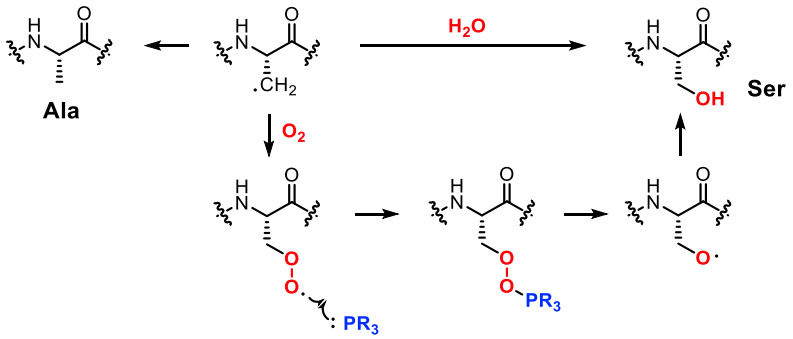

Fig. S13. Proposed Mechanism for the Cys-to-Ser Conversion Side Reaction.

To prove the identity of the residual peak, we synthesized three control peptides − Ala/oxoamide peptide **6'**, Ser/thioamide peptide **S10'**, and Cys/ oxoamide peptide **S11'** (prime symbol denotes the genuine peptide standards to distinguish them from species identified in desulfurization reactions). We showed that **6'** and **S10'** both eluted at 21.8 min under the gradient

used, but had vastly different UV-Vis absorption profiles. While **S11'** shares the same expected mass with **S10'**, it eluted at a different retention time, and of course, did not exhibit thioamide

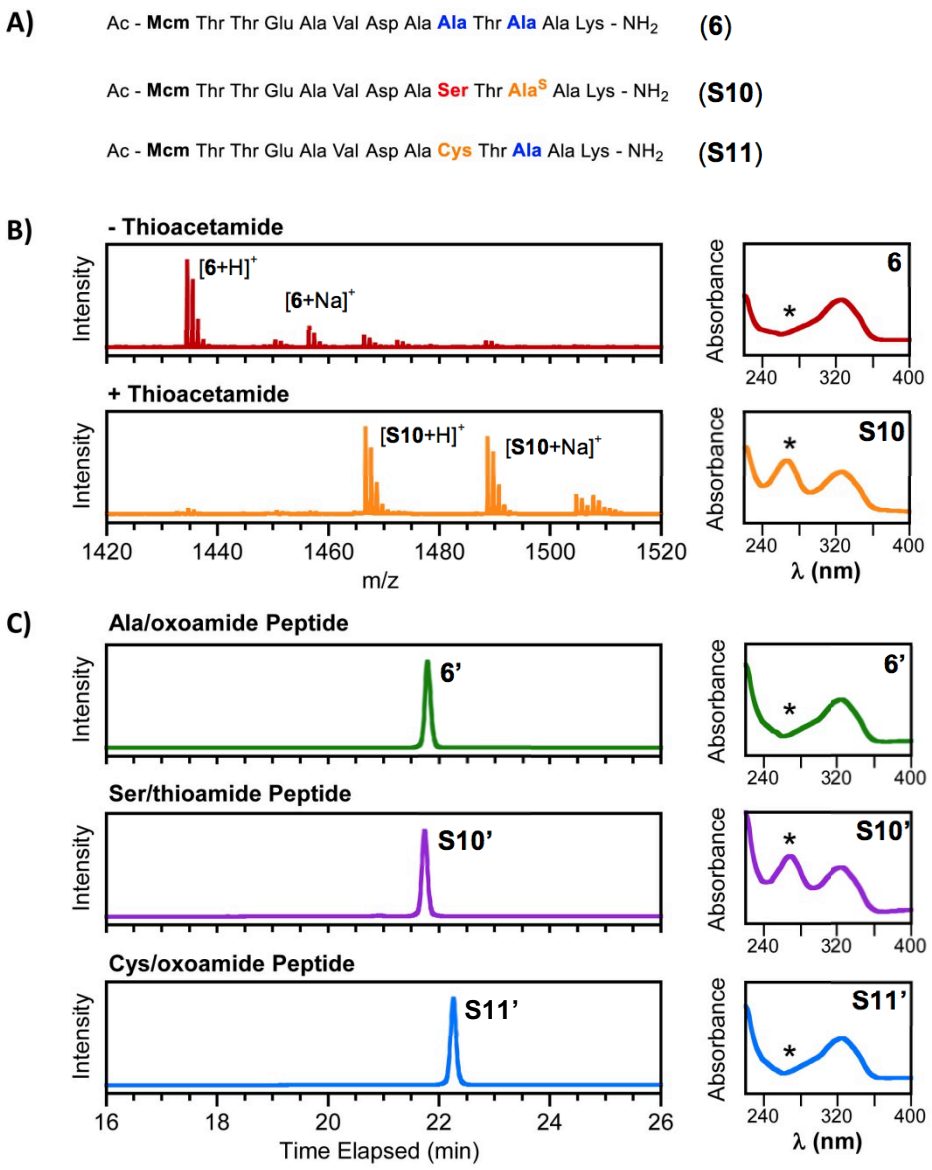

Fig. S14. Characterization of Cys-to-Ser Conversion Side Reaction. A) Sequence of all side products involved. B) MALDI-TOF mass spectra and UV-Vis absorption spectra for the 21.8 min peak isolated from reactions with or without thioacetamide. The retention times may be similar, but peak identities were vastly different. C) HPLC chromatrogram monitored at 325 nm and UV-Vis absorption spectra for genuine peptide standards synthesized by solid phase peptide synthesis (SPPS). Only **S10'** matches the profile observed in reaction with thioacetamide as additive. Mcm = 7-methoxycoumarinylalanine.

absorption at 272 nm (Fig. S14). Therefore, we conclude that the 2% residual peak corresponded to Cys-to-Ser conversion side product **S10**, and that thioamide-to-oxoamide conversion was fully suppressed by thioacetamide.

Synthesis of Ac-Mcm-TTEAVDA-$SC_2H_4COOCH_3$ Thioester (7a) Protected precursor Ac-Mcm-$T^{tBu}T^{tBu}E^{OtBu}AVD^{OtBu}$A-OH (**S12)** was synthesized on 2-chlorotrityl chloride resin using standard SPPS procedure, and cleaved with 1:1:8 acetic acid/trifluoroethanol/CH_2Cl_2. Lyophilized crude **S12** (20 μmol, 1 equiv) was mixed with dimethylformamide (DMF, 1 mL), tetrahydrofuran (THF, 1 mL), *N*,*N*-diisopropylethylamine (DIPEA, 34.8 μL, 200 μmol, 10 equiv) and methyl 3-mercapto-propionate (43.3 μL, 400 μmol, 20 equiv). PyBOP (0.0520 g, 100 μmol, 5 equiv) was added, and then the reaction was allowed to proceed at room temperature for 4 h. Upon completion, the reaction was quenched with 0.1% trifluoroacetic acid in water (10 mL). The precipitate was collected by centrifugation at 13.2 krpm for 20 min, and then cleaved with 3 mL of 1:1:38 triisopropylsilane/methyl 3-mercaptopropionate/trifluoroacetic acid for 30 min. The crude mixture was dried by rotary evaporation, and then brought up in 50:50 acetonitrile/water for purification by reverse phase HPLC. The purified product was quantified by UV-Vis absorption (ε_{325} = 12,000 M^{-1} cm^{-1}) and lyophilized. Mcm = 7-methoxycoumarinylalanine.

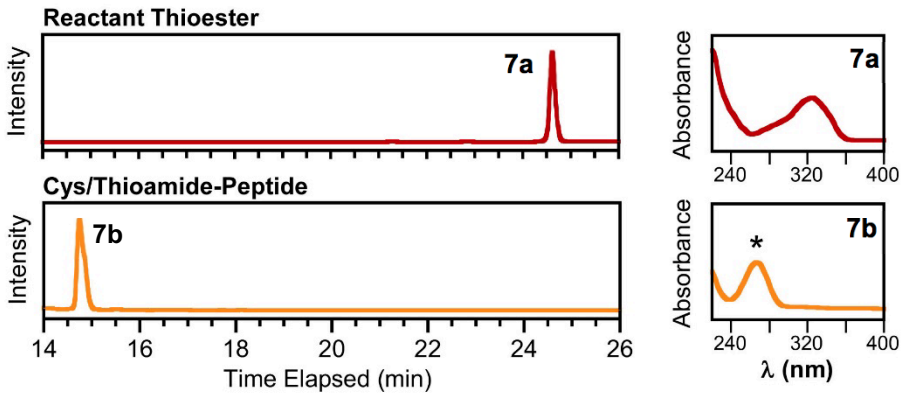

Fig. S15. Characterization of Reactant Thioester and Cys Peptide.

Theoretical Prediction of PhS^- Concentration at Various pH Knowing that the pK_a of thiophenol is 6.6 and that its solubility is 0.08% (7.3 mM), we can theoretically predict the concentration of PhS^- at any given pH by solving the acid-base equilibrium. Assuming that the protonated form PhSH can be fully removed by lyophilization (typical vacuum: 0.024 mBar << vapor pressure of PhSH: 1.8 mBar), the concentration of PhS^- would provide a good estimation for residual aromatic thiol/thiolate after lyophilization.

$$\mathrm{pKa} = -\log\frac{[\mathrm{H^+}]\cdot[\mathrm{PhS^-}]}{[\mathrm{PhSH}]} = 6.6$$

$$[\mathrm{PhS^-}] + [\mathrm{PhSH}] = 0.00726\ \mathrm{M}$$

$$\Rightarrow [\mathrm{PhS^-}] = \frac{0.00726\times10^{-6.6}}{[\mathrm{H^+}] + 10^{-6.6}}$$

When lyophilization is conducted at pH 7, as much as 5.2 mM of 7.3 mM total PhSH would remain in the PhS^- form, which would overwhelm the 0.1 mM peptide. When lyophilization is carried out at pH 1, however, the residual PhS- concentration is only 18 nM, an insignificant amount with respect to the 0.1 mM peptide.

Quantification of Residual PhSH/PhS$^-$ by Ellman's Reagent A saturated PhSH solution (sat. PhSH, 100 mM Na_2HPO_4, pH 7.0) and a 1% TFA stock (1% v/v trifluoroacetic acid in water) were freshly prepared. 1 mL of the PhSH solution was mixed with 10 mL of the 1% TFA stock; the pH of this solution was determined to be 1.6. The mixture was then lyophilized, and brought up in fresh Milli-Q water (1 mL). A 4x Ellman's reagent stock (5 mM Ellman's reagent, 50 mM sodium acetate) and a 2x Tris buffer (200 mM Tris, pH 8.0) were freshly prepared. A series of thiol standards were prepared from sodium 2-sulfanylethanesulfonate (MESNa) in water at 0, 5, 10, 20, 40, 60, 100, 150, 200, 300, and 500 μM. For each sample, the standard/sample (200 μL) were thoroughly mixed with the Ellman's reagent stock (200 μL) and 2x Trix buffer (400 μL). The mixtures were allowed to incubate at room temperature for 5 min, before aliquots (200 μL each) were transferred to a 96-well plate for absorbance measurements at 412 nm on a Tecan Infinite M1000 Pro plate reader (Tecan US Inc.; Morrisville, NC, USA). A standard curve were generated by linear regression of the absorbance values with respect to concentrations of standards (Fig. S16), from which the target sample concentration was extrapolated. The residual PhSH/PhS- after acidification-lyophilization of a saturated PhSH solution initially at pH 7.0 was determined to be (3.4 ± 1.9) μM. Althought this value is somewhat different from the theoretical value of 73 nM at pH 1.6, it represents a significant reduction from the 7.3 mM PhSH concentration at pH 7.0 and is negligible with respect to concentrations of all other reagents in the desulfurization reaction.

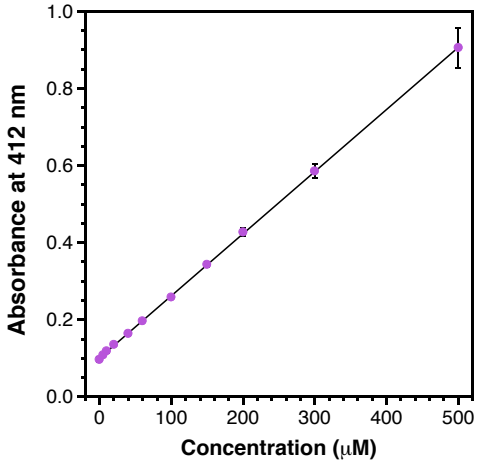

Fig. S16. Standard Curve for Quantification of Residual PhSH/PhS$^-$ by Ellman's Reagent Assay.

One-Pot Ligation-Desulfurization A phosphate buffer stock (40 mM TCEP, 100 mM Na_2HPO_4, pH 7.0) was freshly prepared and degassed by argon purging. 2% PhSH (v/v) was added, and then pH was quickly adjusted back to 7.0 under argon atmosphere. Thioester **7a** and peptide **7b** (50 nmol each) were each dissolved in 25 μL of the above buffer, and then combined. The reaction was allowed to proceed at 37 °C overnight.

For desulfurization, 10 μL of the ligation crude was removed, acidified with 100 μL of 1% trifluoroacetic acid in water, and then quickly frozen and lyophilized. The crude residue was brought up in 80 μL of freshly degassed 1.25x desulfurization buffer (50 mM TCEP, 125 mM Na_2HPO_4, pH 7.0), and then *t*-BuSH and VA-044 were added to proceed with the standard desulfurization procedure. Upon completion, all crude reaction samples were diluted into H_2O, and analyzed by reverse phase HPLC on a Luna C8 analytical column using gradient **3**. Individual fractions were collected, and then analyzed by MALDI-TOF MS and UV-Vis absorption spectroscopy.

Synthesis and Purification of $GB1_{1\text{-}23}Leu^{S}_{5}\text{-}N_2H_3$ (8a) Hydrazide resin for peptide synthesis was prepared according to Liu and coworkers[5]. For coupling, 5 equiv of amino acid and 5 equiv of HBTU were dissolved in DMF, pre-activated for 1 min in the presence of 10 equiv of DIPEA, and then stirred with the resin for 30 min at room temperature. For deprotection, 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF was stirred with the resin three times for 2 min each. Thioleucine (denoted Leu^{S} or L^{S}) was introduced through activated benztriazole precursor; 2 equiv of the precursor was dissolved in dry CH_2Cl_2, and stirred with the resin for 45 min in the presence of 2 equiv DIPEA. Upon completion of SPPS, resin was rinsed thoroughly with CH_2Cl_2 and dried under vacuum.

For cleavage, resin was treated with a cleavage cocktail (6:1:1:12 trifluoroacetic acid/triisopropylsilane/H_2O/CH_2Cl_2) for 30 min. The solution was then collected by filtration, and dried by rotary evaporation. For purification, the crude residues were brought up in 50:50 CH_3CN/H_2O, diluted with H_2O, and then purified by reverse phase HPLC using gradient **12**. Individual fractions were characterized by MALDI-TOF MS, and dried by lyophilization.

Synthesis and Purification of Cys-$GB1_{25\text{-}56}$-OH (9) Crude peptide was purchased from Genscript and received as a lyophilized white powder. Crude product was dissolved in 50:50 CH_3CN/H_2O and purified by reverse phase HPLC using gradient **13**.

Native Chemical Ligation to obtain $GB1_{1\text{-}56}Leu^{S}_{5}Cys_{24}$-OH (10a) Cys-$GB1_{25\text{-}56}$-OH **9** and $GB1_{1\text{-}23}Leu^{S}_{5}\text{-}N_2H_3$ **8a** were aliquoted in 50 nmol aliquots using ε_{280} = 8,480 M^{-1} cm^{-1} and ε_{274} = 11,569 M^{-1} cm^{-1}, the sum of contributions from the thioamide (ε_{274} = 10,169 M^{-1} cm^{-1}) and

tyrosine (ε_{274} = 1,400 M^{-1} cm^{-1}), respectively. Activation buffer (200 mM Na_2HPO_4, 6 M GnHCl, pH 3.0) and ligation buffer (200 mM Na_2HPO_4, 6 M GnHCl, pH 7.0) were degassed by purging with argon for 15 min. C-terminal fragment **9** dissolved in 48 µL ligation buffer and 2 µL thiophenol added. N-terminal hydrazide peptide **8a** was dissolved in 45 µL activation buffer and stirred at -15°C for 5 min. 5 µL of a 0.5 M $NaNO_2$ solution in activation buffer was added and stirred at -15°C. After 15 min reaction allowed to warm to room temperature for 1 min, C-terminal peptide added and pH adjusted to 7.0. Reaction stirred overnight at room temperature for 19 h. Analytical HPLC runs were performed on a Luna C8 column using gradient **14**.

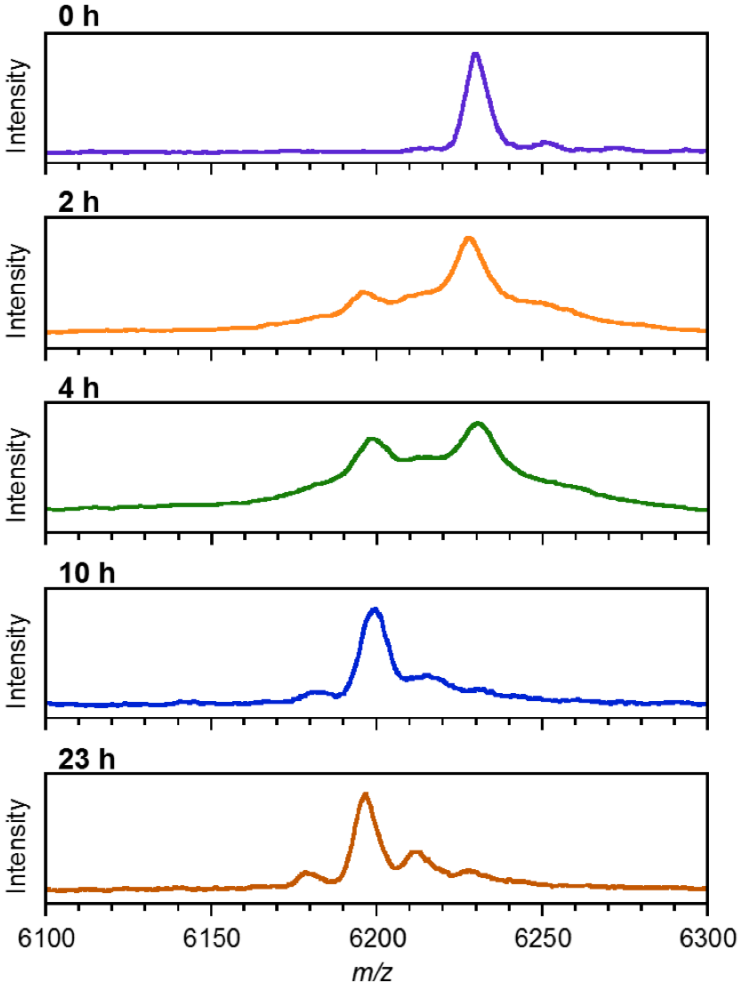

Fig. S17: Desulfurization time-course of $GB1_{1\text{-}56}Leu^{S}{}_{5}Cys_{24}$-OH.

Desulfurization to obtain GB1$_{1\text{-}56}$Leu$^{S}_{5}$–OH (10b) To the crude ligation reaction, 400 µL degased desulfurization buffer (200 mM Na_2HPO_4, 6 M GnHCl, 500 mM TCEP, 250 mM Thioacetamide, pH 7.0) were added and spin-concentrated to about 80 µL using a Millipore Amincon Ultracel 3K Spin Filter Unit at 13.2k rpm for 60 min at 4°C. This procedure was repeated one more time. To 80 µL of buffer exchanged ligation product **10a**, 10 µL *t*-BuSH and 10 µL of a 0.5 M VA-044 solution in desulfurization buffer were added. The reaction was allowed to proceed at 37°C and was monitored by MALDI-TOF MS (Fig. S17), since the desulfurized product **10b** has the same retention time as **10a**. After 23 h the reaction was complete and the crude reaction mixture was diluted to 3 mL with 0.1% TFA in H_2O and purified by reverse phase HPLC using gradient **15**. Analytical HPLC runs were performed on a Luna C8 column using gradient **14**. The product yield (71%) was determined from UV/Vis spectra of isolated products using ε_{274} = 19,736 M^{-1} cm^{-1}, the sum of contributions from the thioamide (ε_{274} = 10,169 M^{-1} cm^{-1}), tryptophan (ε_{274} = 5,357 M^{-1} cm^{-1}), tyrosine (ε_{274} = 1,400 M^{-1} cm^{-1}) and phenylalanine (ε_{274} = 5 M^{-1} cm^{-1}).

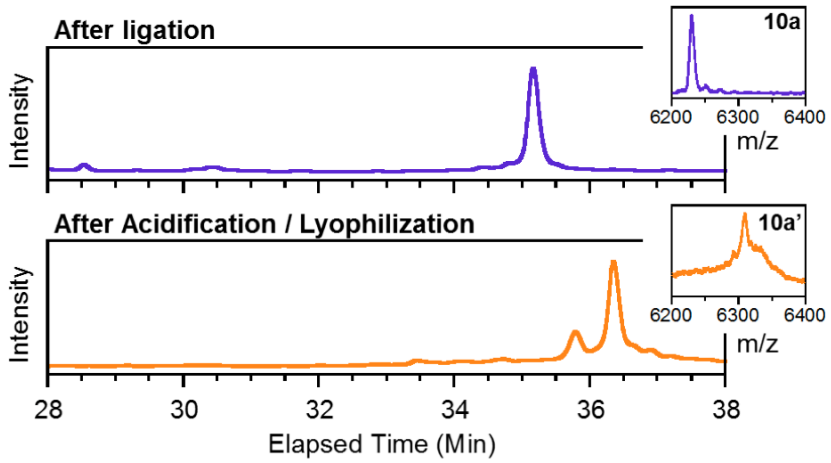

***Fig. S18*:** Attempted One-Pot Ligation Desulfurization is incompatible with hydrazide ligation chemistry.

One-Pot Ligation Desulfurization Attempts to obtain $GB1_{1\text{-}56}Leu^{S}_{5}$–OH (**10b**) via one-pot ligation desulfurization were not successful. Briefly, 10 µL of crude ligation product **10a** were mixed with 100 µL of a 1% TFA solution, frozen with liquid N_2 and lyophilized. The lyophilized product was dissolved in 600 µL water with 5 µL 0.5 M TCEP, incubated for 20 min and analyzed by analytical reverse phase HPLC on a Luna C8 column using gradient **14**. A retention time and mass shift was observed as shown in Fig. S18**.**

GB1 Sequences

Full Length Thioamide GB1 ($GB1_{1\text{-}56}$-Leu^{S}_{5}-OH, **10b**)

DTYKL^{S}ILNGK TLKGETTTEA VDAATAEKVF KQYANDNGVD GEWTYDDATK TFTVT E-OH

10 20 30 40 50 56

Thioamide GB1 N-Terminus Hydrazide ($GB1_{1\text{-}23}Leu^{S}_{5}$-$N_2H_3$, **8a**)

DTYKL^{S}ILNGK TLKGETTTEA VDA-CON_2H_3

Thioamide GB1 N-Terminus Thioester ($GB1_{1\text{-}23}Leu^{S}_{5}$-SR, **8b**)

DTYKL^{S}ILNGK TLKGETTTEA VDA-COSPh

GB1 C-Terminus (Cys-$GB1_{25\text{-}56}$-OH, **9**)

CTAEKVF KQYANDNGVD GEWTYDDATK TFTVT E-OH

Full Length Thioamide GB1 Cys_{24} ($GB1_{1\text{-}56}$-$Leu^{S}_{5}Cys_{24}$-OH, **10a**)

DTYKL^{S}ILNGK TLKGETTTEA VDACTAEKVF KQYANDNGVD GEWTYDDATK TFTVT E-OH

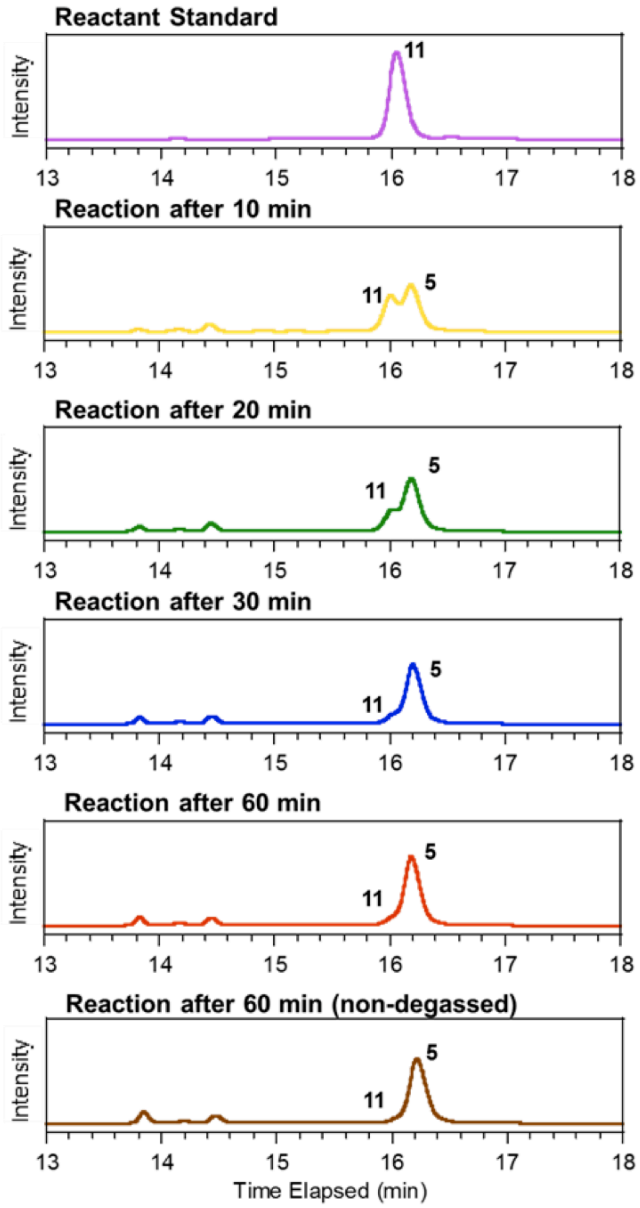

***Fig. S19*:** Desulfurization time-course of Pen containing peptides.

Synthesis of Pen-Containing Peptide Fmoc-Pen(Trt)-OH was purchased from Sigma-Aldrich, and incorporated into peptide **11** sequence using standard SPPS procedure.

Desulfurization of Pen/Thioamide-Containing Peptide Reaction was conducted using standard desulfurization procedure with thioacetamide. Briefly, Peptide **11** (10 nmol, ε_{325} = 12,000 M^{-1} cm^{-1}) was dissolved in 80 µL of argon-purged 1.25x buffer stock (50 mM TCEP, 125 mM thioacetamide, 125 mM Na_2HPO_4, pH 7.0). *t*-BuSH and VA-044 stock were then added, and the reaction was allowed to proceed at 37 °C for 10-60 min. The crude was analyzed by reverse phase HPLC on a Luna C8 analytical column using gradient **11**, and characterized by MALDI-TOF MS. After 60 minutes the reaction was complete as shown in ***Fig. S19***

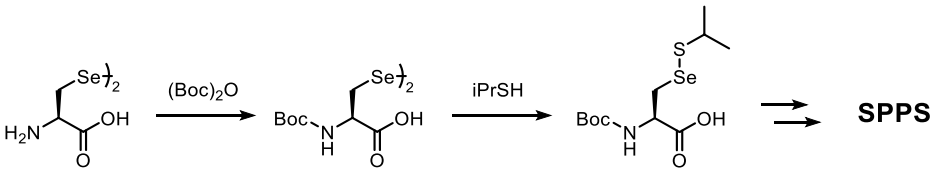

Fig. S20. Synthetic Scheme for Sec-Containing Peptides.

Synthesis of *N*,*N*'-di-Boc-*L*-selenocystine (S13) L-Selenocystine (0.1670 g, 0.5 mmol, 1 equiv) was dissolved in argon-purged H_2O (4 mL) and 1,4-dioxane (4 mL), and chilled to 0 °C on ice. Trimethylamine (900 µL, 6 mmol, 12 equiv) was added, followed by Boc anhydride (0.2728 g, 1 mmol, 2 equiv). The reaction was allowed to proceed on ice for 2 h, and then at room temperature overnight (≥ 12 h) under argon atmosphere. Upon completion, the reaction was diluted with H_2O (18 mL), acidified with 3 M hydrochloric acid (3 mL), and then quickly extracted with ethyl acetate (30 mL × 3). The organic layers were combined, concentrated by rotary evaporation, and then purified by flash chromatography in 0.1% acetic acid in ethyl acetate. A pale yellow foam was isolated as the product (0.2383 g, 0.45 mmol, 89% yield). R_f 0.15 in ethyl acetate with 0.1% acetic acid. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.93 (s, 2H), 5.65 (d, *J* = 6.9 Hz, 1H), 4.57 (m, 2H),

3.48 (m, 2H), 3.40 (dd, J = 12.7, 5.6 Hz, 2 H), 1.44 (s, broad, 18 H). ^{13}C-NMR (500 MHz, $CDCl_3$): δ 174.95, 155.84, 81.18, 54.01, 33.34, 28.66. ESI$^-$-HRMS: calculated for $C_{16}H_{27}N_2O_8Se_2^-$, 535.0098; found $[M - H]^-$ 535.0095.

Synthesis of Boc-Sec(S-*i*-Pr)-OH (S14) **S13** (0.1681 g, 0.31 mmol, 1 equiv) was dissolved in argon-purged tetrahydrofuran (THF, 5 mL). Triethylamine (439 μL, 3.2 mmol, 10 equiv) was added, followed by 2-propanethiol (599 μL, 6.3 mmol, 20 equiv). The reaction was allowed to proceed at room temperature for 3 h under argon atmosphere. Upon completion, the reaction mixture was dried by rotary evaporation. The residue was brought up in H_2O (10 mL), acidified with 3 M hydrochloric acid (1 mL), and quickly extracted with ethyl acetate (10 mL × 3). Organic layers were combined, concentrated by rotary evaporation, and then purified by flash chromatography in 5:5 ethyl acetate/ petroleum ether with 0.1% acetic acid. A white foam was isolated as the final product (0.2005 g, 0.59 mmol, 93% yield). R_f in 0.27 in 5:5 ethyl acetate/petroleum ether with 0.1% acetic acid. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.43 (d, J = 6.9 Hz, 1H), 4.63 (dd, J = 11.3, 6.9 Hz, 1H), 3.33 (dd, J = 12.5, 4.2 Hz, 1H), 3.24 (dd, J = 12.1, 6.5 Hz, 1H), 3.03 (septet, J = 6.7 Hz, 1H), 1.46 (s, 9H), 1.32 (d, J = 6.6 Hz, 6H). ESI$^+$-HRMS: calculated for $C_{11}H_{21}NO_4SSeNa$ 366.0254, found $[M + Na]^+$ 366.0252.

Synthesis of Sec-Containing Peptide Peptide **12** was synthesized using SPPS procedure on Rink amide resin. Sec was introduced as Boc-Sec(S-*i*-Pr)-OH (**S14**) using standard HBTU activation method. The peptide was cleaved with 12:1:1:26 trifluoroacetic acid/2-propanethiol/triisopropylsilane/CH_2Cl_2, and then purified by reverse phase HPLC. **12** was isolated as the primary product, with an intramolecular hemiselenide bond between Sec and Cys.

Deselenization of Sec/Cys/Thioamide-Containing Peptide Deselenization buffer (40 mM TCEP, 40 mM DTT, 200 mM phosphate, pH 7.0) was freshly prepared and degassed by the freeze-pump-thaw method. Peptide **12** (10 nmol, ε_{325} = 12,000 M^{-1} cm^{-1}) was dissolved in 100 µL of the above buffer, and then the reaction was allowed to proceed at 37 ˚C for 18 h. Upon completion, the reaction was chilled to 0 ˚C on ice, and then 100 µL of argon-purged CH_3CN was added. The supernatant was recovered by centrifugation at 13.2 krpm for 20 min, diluted into argon-purged H_2O (650 µL), and then analyzed by reserve phase HPLC on a Luna C8 analytical column using gradient **3**. Fractions were collected and analyzed for MALDI-TOF MS and UV-Vis absorption.

4-Mercaptophenylacetic Acid (MPAA) as Deselenization Additive Reaction was conducted similarly to the standard procedure, except that a buffer stock containing 10 mM MPAA was used. MPAA was chosen over PhSH for its low volatility.

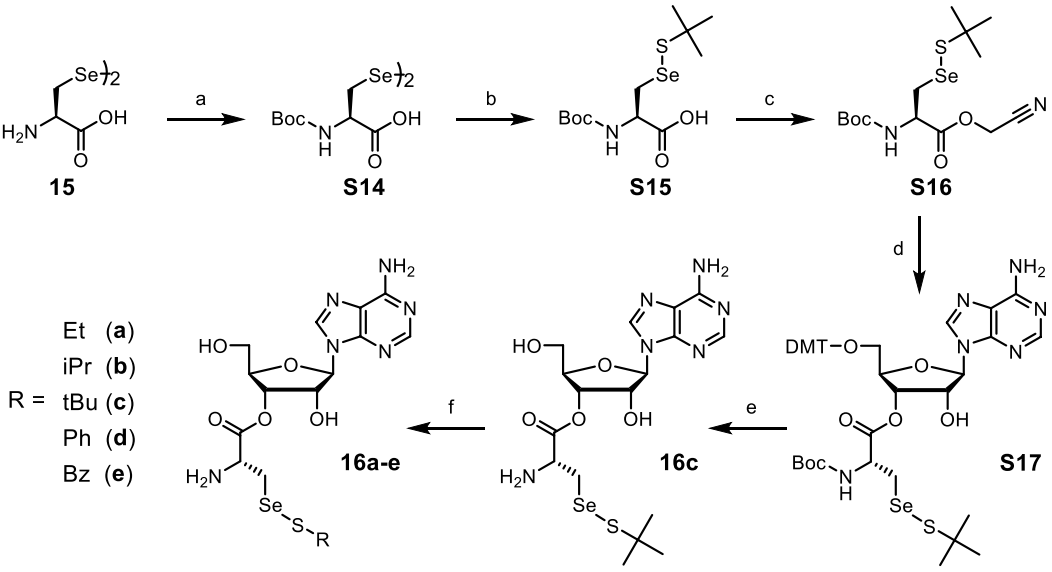

Fig. S21. Synthetic Scheme for H-Sec(SR)-Ade Donors.

Synthesis of Boc-Sec(*S*-*t*-Bu)-OH (S15) Tetrahydrofuran (THF) and triethylamine (Et_3N) were freshly degassed by the freeze-pump-thaw method. **S14** (0.2072 g, 0.4 mmol, 1 equiv) was dissolved in 6.5 mL of degassed THF, and then Et_3N (541 μL, 4 mmol, 10 equiv) and 2-methyl-2-propanethiol (*t*-BuSH, 1.1 mL, 10 mmol, 25 equiv) were added. The reaction was allowed to proceed at room temperature under an argon atmosphere for 4 h. Upon completion, the reaction mixture was concentrated by rotary evaporation. The residue was brought up in 10 mL of H_2O, acidified with 1 mL of 3 M HCl, and then extracted with ethyl acetate (10 mL × 3). The organic layers were combined and concen-trated by rotary evaporation, and then purified by flash chromatography in 5:5 ethyl acetate/petroleum ether with 0.1% acetic acid. A pale yellow crystalline solid was isolated as the final product (0.2523 g, 0.7 mmol, 91% yield). R_f = 0.25 in 5:5 ethyl acetate/ petroleum ether with 0.1% acetic acid. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 11.16 (s, 1H), 5.44 (d, *J* = 7.1 Hz, 1H), 4.64 (d, *J* = 3.7 Hz, 1H), 3.30 (dd, *J* = 11.9, 4.1 Hz, 1H), 3.22 (dd, *J* = 12.1, 5.8 Hz, 1H), 1.44 (s, 9H), 1.34 (s, 9H). ^{13}C-NMR (500 MHz, $CDCl_3$): δ 175.61, 155.69, 82.52, 53.78, 46.57, 34.39, 30.84, 28.61. ESI$^-$-HRMS: calculated for $C_{12}H_{22}NO_4SSe^-$: 356.0435; found $[M - H]^-$: 356.0442.

Synthesis of Boc-Sec(*S*-*t*-Bu)-OCH$_2$CN (S16) Tetrahydrofuran (THF) and diisopro-pylethylamine (DIPEA) were freshly degassed by the freeze-pump-thaw method. **S15** (0.2194 g, 0.64 mmol, 1 equiv) was dissolved in degassed THF (7.7 mL), and chilled to 0 ˚C on ice. DIPEA (335 μL, 1.9 mmol, 3 equiv) was added, followed by $ClCH_2CN$ (2.0 mL, 32 mmol, 50 equiv). The reaction was allowed to proceed on ice for 2 h, and then at room temperature overnight (≥ 12 h) under an argon atmosphere. Upon completion, the reaction mixture was concentrated by rotary evaporation, and then purified by flash chromatography in 5:5 ethyl acetate/petroleum ether. The

reactant was recovered in 5:5 ethyl acetate/petroleum ether with 0.1% acetic acid, and then subject to two additional rounds of $ClCH_2CN$ activation. A pale yellow solid was isolated as the product (0.1396 g, 0.35 mmol, 57% yield). R_f = 0.74 in 5:5 ethyl acetate/ petroleum ether. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.39 (d, *J* = 6.9 Hz, 1H), 4.79 (s, 2H), 4.72 (dd, *J* = 12.4, 5.7 Hz, 1H), 3.24 (dd, *J* = 13.2, 4.8 Hz, 1H), 3.20 (dd, *J* = 13.4, 5.7 Hz, 1H), 1.45 (s, 9H), 1.36 (s, 9H). ^{13}C-NMR (500 MHz, $CDCl_3$): δ 170.04, 155.29, 114.15, 81.01, 53.53, 49.48, 47.00, 33.55, 30.83, 28.59. ESI$^+$-HRMS: calculated for $C_{14}H_{24}N_2O_4SSeNa^+$: 419.0520; found $[M + Na]^+$: 419.0519.

Synthesis of Boc-Sec(*S*-*t*-Bu)-(5'-*O*-DMT)Adenosine (S17) Tetrahydrofuran (THF) was freshly degassed by the freeze-pump-thaw method. **S16** (0.1396 g, 0.35 mmol, 2 equiv) was dissolved in THF (4 mL). 5′-*O*-(4,4′-dimethoxytrityl) adenosine (0.1006 g, 0.18 mmol, 1 equiv) was added, followed by catalytic amount of tetrabutylammonium acetate (0.0027 g, 9 μmol, 0.05 equiv). The reaction was allowed to proceed overnight (≥ 12 h) under an argon atmosphere. Upon completion, the reaction was concentrated by rotary evaporation, and then purified by flash chromatography in 1:19 methanol/ethyl acetate. A white foam was isolated as the final product (0.1029 g, 0.11 mmol, 65% yield). R_f = 0.42 in 1:19 methanol/ethyl acetate. ^{1}H-NMR and ^{13}C-NMR sepctra (500 MHz, d^8-THF) are shown below (Fig. ***S22***). ESI$^+$-HRMS: calculated for $C_{43}H_{53}N_6O_9SSe^+$: 909.2760; found $[M + H]^+$: 909.2767.

Synthesis of H-Sec(*S*-*t*-Bu)-Ade (16c) Tetrahydrofuran (THF), CH_2Cl_2 and H_2O were freshly degassed by the freeze-pump-thaw method. **S17** (0.0200 g, 22 μmol, 1 equiv) was dissolved in degassed THF (1 mL) and chilled to 0 °C on ice. Triisopropylsilane (23 μL, 110 μmol, 5 equiv) was added, followed by *t*-BuSH (205 μL, 2.2 mmol, 100 equiv). Trifluoroacetic acid (1 mL) was

added dropwise, and then the reaction was allowed to proceed at room temperature for 2 h under an argon atmosphere. Upon completion, the reaction was dried by rotary evaporation. The residue was brought up in degassed CH_2Cl_2 (1 mL), and then extracted with degassed H_2O (1 mL × 3) to recover the product. The crude was directly purified by reverse phase HPLC using a binary solvent system of water/acetonitrile with 0.1% trifluoroacetic acid (Table S4 through Table S6).

Synthesis of Other H-Sec(SR)-Ade Donors (**16a-e**) All other adenosine donors were derivatized from **16c** through thiol exchange: H_2O was freshly degassed by the freeze-pump-thaw method. **16c** (0.0056 g, 11 μmol, 1 equiv) was dissolved in 1 mL of degassed H_2O. 100 μL of an appropriate thiol (ethanethiol for **16a**; 2-propanethiol for **16b**; thiophenol for **16d**; benzyl mercaptan for **16e**) was added, and the reaction was allowed to proceed at room temperature for 2 h under an argon atmosphere. Upon completion, excess thiol was evaporated by a stream of argon. The aqueous crude was directly purified by reverse phase HPLC (Table S4 through Table S6).

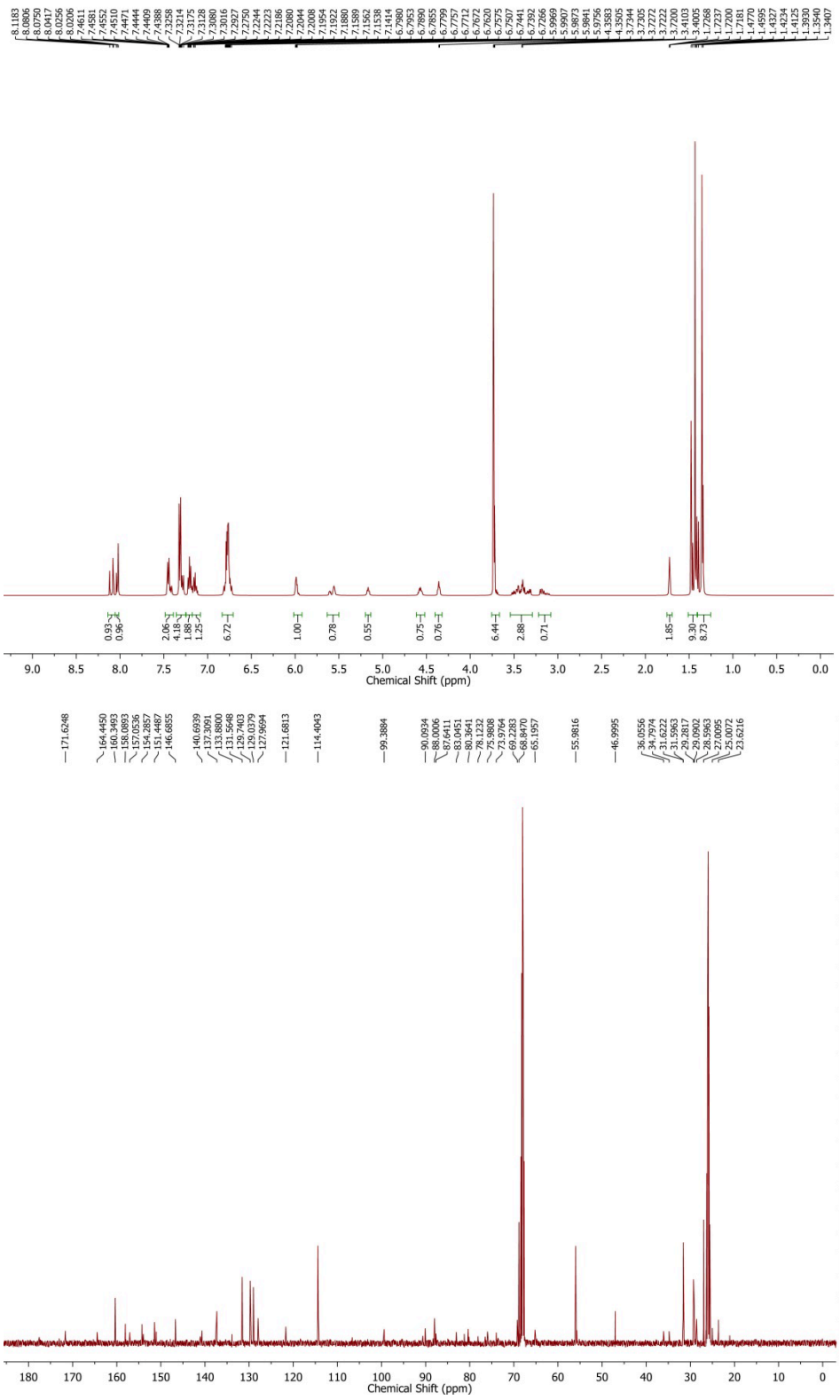

Fig. S22. ^{1}H and ^{13}C NMR Characterization of Boc-Sec(*S*-*t*-Bu)-(5'-*O*-DMT)Ade (**16c**)

Table S4. Adenosine Donor Purification Methods and Retention Time.

Compound	Gradient	Retention Time*	Column
H-Sec(*S*-Et)-Ade (**16a**)	**16**	16.2 / 18.2 min	YMC-Pack Pro C8 Semi-prep
H-Sec(*S*-*i*Pr)-Ade (**16b**)	**16**	19.2 / 20.8 min	YMC-Pack Pro C8 Semi-prep
H-Sec(*S*-*t*Bu)-Ade (**16c**)	**16**	21.1 / 23.4 min	YMC-Pack Pro C8 Semi-prep
H-Sec(*S*-Ph)-Ade (**16d**)	**16**	22.0 / 24.5 min	YMC-Pack Pro C8 Semi-prep
H-Sec(*S*-Bz)-Ade (**16e**)	**16**	22.7 / 25.0 min	YMC-Pack Pro C8 Semi-prep

* All adenosine donors are isolated as a mixture of interconverting 2'- and 3'-isomers.

Table S5. HPLC Gradients for Purification and Characterization.

No.	Time (min)	%B	**No.**	Time (min)	**%B**	**No.**	Time (min)	**%B**
16	0:00	2	**17**	0:00	1	**18**	0:00	2
	5:00	2		5:00	1		5:00	2
	10:00	10		10:00	30		25:00	20
	30:00	30		15:00	40		30:00	100
	35:00	100		20:00	100		35:00	100
	40:00	100		25:00	100		45:00	2
	45:00	2		27:00	1			
				30:00	1			

* Solvent A: 0.1% trifluoroacetic acid in water; Solvent B: 0.1% trifluoroacetic acid in acetonitrile

Table S6. MALDI-TOF MS Characterization of Purified Adenosine Donors.

Compound	$[M + H]^+$		$[M + Na]^+$	
	Calculated	**Found**	**Calculated**	**Found**
H-Sec(*S*-Et)-Ade (**16a**)	479.05	479.02	501.04	500.98
H-Sec(*S*-*i*Pr)-Ade (**16b**)	493.07	492.95	515.06	514.91
H-Sec(*S*-*t*Bu)-Ade (**16c**)	507.09	507.12	529.08	529.09
H-Sec(*S*-Ph)-Ade (**16d**)	527.05	527.12	-	-
H-Sec(*S*-Bz)-Ade (**16e**)	541.07	541.05	563.06	563.05

Characterization of 2'- and 3'-Isomers of Adenosine Donors As noted in our previous work[6], all adenosine donors were isolated as a mixture of 2'- and 3'-acylated isomers. To ascertain this observation in our hemiselenide protected Sec donors, we isolated the early and late peaks (assigned as 2'- and 3'-isomers based on the known thermodynamic preference for 3'-acylation of adenosine[7]) of H-Sec(*S*-*i*-Pr)-Ade, re-injected them separately, and observed interconversion of the two species (Fig. S23). Since these two isomers co-exist in rapid equilibrium, we reasoned that it would be unnecessary to obtain one pure isomer − as the 3'-isomer is consumed by AaT in chemo-enzymatic reaction, the 2'-isomer will convert into the 3'-isomer by equilibrating, thus supplementing substrates for the reaction.

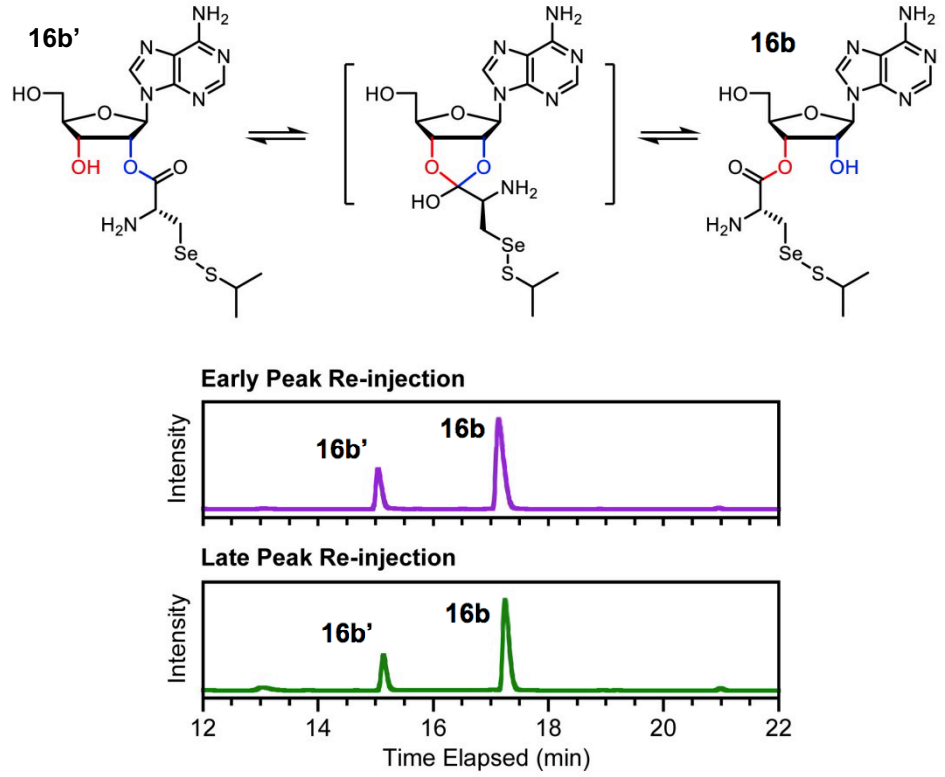

Fig. S23. Interconversion of 2'- and 3'-Isomers of H-Sec(S-*i*Pr)-Ade **16b**. Early peak (assigned as **16b'**) and late peak (assigned as **16b**) were isolated, brought up in H_2O, and re-injected onto reverse phase HPLC. Chromotogram monitored at 260 nm on a Jupiter C18 analytical column using gradient **11**. In both cases, the interconversion rapidly reached thermodynamic equilibrium of 1:3 **16b'**/**16b** under ambient conditions. MALDI-TOF MS: [**16b** + H]$^+$, expected 493.07, found 493.06; [**16b'** + H]$^+$, expected 493.07, found 493.05.

Versatility of Thiol Exchange on Hemiselenides To demonstrate the versatility of the thiol exchange reaction in derivatizing hemiselenides, we performed thiol exchange on H-Sec(*S*-*t*-Bu)-Ade **16c** and H-Sec(*S*-Bz)-Ade **16e**, and showed that the two species could be interconverted simply by adding different thiols in excess (Fig. S24).

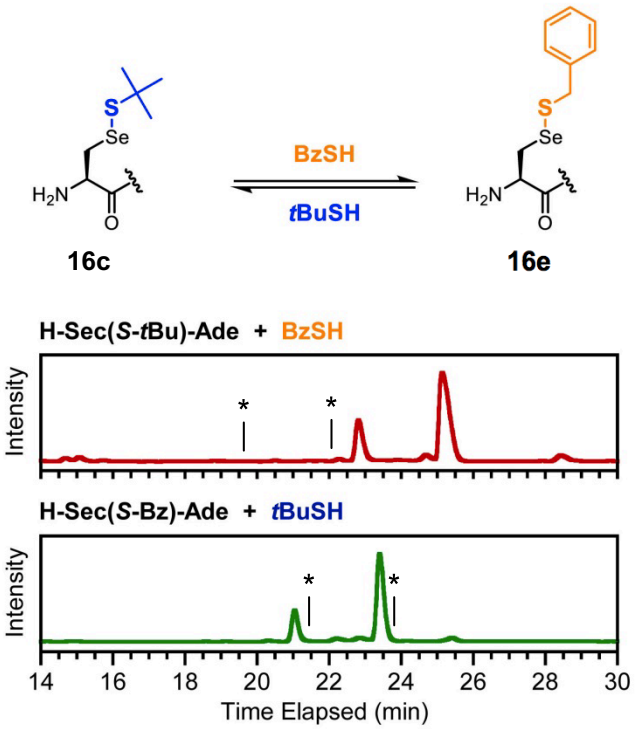

Fig. S24. Interconversion of Adenosine Analogs as Driven by Excess of Different Thiols. **16c** was readily converted into **16e** by treatment with excess BzSH; *vice versa*, **16e** could be generated from **6c** by treatment with excess *t*-BuSH. HPLC chromatograms of crude reaction mixtures monitored at 260 nm on a YMC-Pack Pro C8 semi-prep column using gradient **11**. MALDI-TOF MS: [**16c** + H]$^+$, expected 507.12, found 507.08; [**16e** + H]$^+$, expected 541.05, found 541.07. Star signs indicate the absence of the original adenosine donor added.

Expression and Purification of *E. coli* Aminoacyl Transferase (AaT) His_{10}-tagged *E. coli* amino acyl transferase (AaT) was expressed from a pEG6 plasmid (ampi-cillin resistant) in *E. coli* BL21-Gold (DE3) cells using a procedure adapted from Graciet *et al.*[8] In particular, no β-

mercaptoethanol (BME) was used in our purification, to avoid complications with the hemiselenide side chains. For protein expression, *E. coli* BL21-Gold (DE3) cells were transformed with the plasmid, and selected on an LB plate in the presence of ampicillin (Amp, 100 μg/mL) by overnight growth at 37 °C. 5 mL LB media was inoculated with a single colony, and then grown at 37 °C in the presence of Amp (100 μg/mL) until $OD_{600} \geq 0.5$. The primary culture was diluted into 1 L of LB media with Amp (100 mg/L), and grown at 37 °C until $OD_{600} = 0.6$. 100 mg of isopropyl *β*-D-thiogalactoside (IPTG, 0.1 mM final concentration) was added to induced AaT expre-ssion, and then the cells were grown at 25 °C for another 16 ~ 18 h.

For purification, cells were harvested at 6,000 rpm using a GS3 rotor on a Sorvall RC-5 centrifuge. Cell pellets were resuspended in Ni-NTA binding buffer (50 mM Tris, 10 mM imidazole, 300 mM KCl, pH 8.0 with protease inhibitor cocktail, 1 mM phenyl-methanesulfonyl fluoride and 10 units/mL DNAse1–Grade II), and then lysed by sonication. The crude was centrifuged at 13,200 rpm for 15 min, and then supernatant was recovered and incubated with Ni-NTA resin for 1 h on ice with gentle shaking. The resin was collected by filtration, rinsed with 4 volumes of wash buffer (50 mM Tris, 50 mM imidazole, 300 mM KCl, pH 8.0), and then eluted with 8 volumes of elution buffer (50 mM Tris, 250 mM imidazole, 300 mM KCl, pH 8.0). Pure fractions of AaT were identified by SDS-PAGE, and then collected and dialyzed into a storage buffer (50 mM Tris, 30 % glycerol, 120 mM $(NH_4)_2SO_4$, pH 8.0) at 4 °C overnight. After concentration determination by Bradford assay[9], the purified enzymes were stored at -80 °C until use.

AaT Activity Assay for Adenosine Donor Screening H_2O, acetone, a 10x buffer stock (500 mM Tris, 1.5 M KCl, 100 mM $MgCl_2$, pH 8.0), and a LysAlaAcm **17** stock (10 mM in water) were freshly degassed by the freeze-pump-thaw method. Sec adenosine donor was brought up in degassed H_2O, and adjusted to 10 mM based on UV-Vis absor-ption (ε_{260} = 15,400 M^{-1} cm^{-1}). Under an argon atmosphere, H_2O (41.6 μL), buffer stock (6.25 μL), LysAlaAcm stock (0.625 μL) and Sec adenosine donor stock (6.25 μL) were thoroughly mixed, and then AaT (7.81 μL, 0.8 mg/mL) was added to initiate the reaction. The reaction was allowed to proceed at 37 ˚C for 4 h under an argon atmosphere. Upon completion, the reaction was quenched with 1% acetic acid in degassed H_2O (187.5 μL). Degassed acetone (1 mL) was added, and then the mixture was kept at -20 °C for 1 h to precipitate the enzyme. Supernatant was recovered after centrifugation at 13,200 rpm and 4 ˚C for 20 min, and then excess acetone was removed by rotary evaporation. The residue was diluted into degassed H_2O (800 μL), and then analyzed by reverse phase HPLC on a Jupiter C18 analytical column using gradient **17**.

For quantification, peak identities were assigned based on MALDI-TOF MS (Table S7), and then transfer efficiencies were calculated from peak areas monitored at 325 nm. For each adenosine donor, the transfer efficiency was reported as the average value with standard deviation from three independent trials.

Table S7. Retention Time and MALDI-TOF MS Characterization of LysAlaAcm Peptides.

Peptide	Retention Time	$[M + H]^+$		$[M + Na]^+$	
		Calc'd	Found	Calc'd	Found
LysAlaAcm (**17**)	11.1 min	375.20	375.29	397.19	
Sec(*S*-Et)-LysAlaAcm (**18a**)	12.6 min	586.16	586.38	-	-
Sec(*S*-*i*Pr)-LysAlaAcm (**18b**)	13.1 min	600.17	600.14	622.16	622.11
Sec(*S*-*t*Bu)-LysAlaAcm (**18c**)	13.2 min	614.18	614.13	636.17	636.10
Sec(*S*-Ph)-LysAlaAcm (**18d**)	13.4 min	634.15	634.33	656.14	656.23
Sec(*S*-Bz)-LysAlaAcm (**18e**)	13.3 min	648.17	648.48	-	-
Phe-LysAlaAcm (**18f**)	12.4 min	522.26	522.33	544.25	544.31

* Retention time obtained on a Jupiter C18 analytical column using gradient **17**.

Oxygen Tolerance Reactions were conducted similarly as the standard procedure, except that non-degassed water and buffers were used (Figure S25). Transfer efficiencies were reported as the average value from three independent trials.

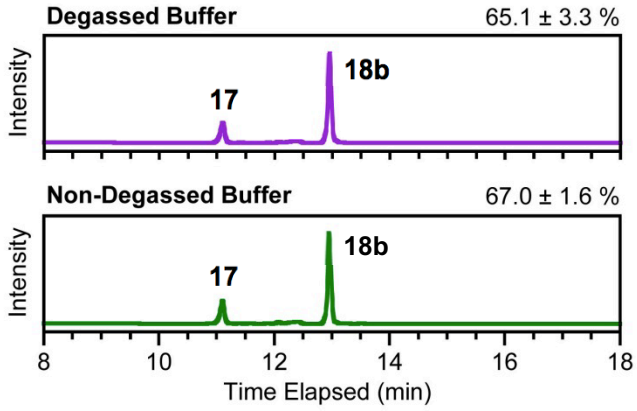

Figure S25. Oxygen Tolerance of Chemoenzymatic AaT Reaction with H-Sec(*S*-*i*-Pr)-Ade.

Expression and Purification of $\alpha S_{6\text{-}140}$ (S18) Expression and purification were performed as previously described.[6] Briefly, a pET-16b plasmid encoding for His_{Tag}-$\alpha S_{6\text{-}140}$ was transformed

into *E. coli* BL21 DE3 cells, and over-expressed with IPTG induction. After lysis of the cells, target protein was pulled down by Ni-NTA resin, and treated with Factor Xa to remove the His_{Tag}. The protein was dialyzed into storage buffer (20 mM Tris, 150 mM KCl, 10 mM Mg_2Cl_2, pH 8.0), quantified by BCA assay, and stored at -80 °C until use.

AaT-Mediated Chemoenzymatic Transfer of Sec(*S*-*i*-Pr) onto $\alpha S_{6\text{-}140}$ H-Sec(*S*-*i*-Pr)-Ade **16b** was brought up in H_2O, and adjusted to 10 mM based on UV-Vis absorption (ε_{260} = 15,400 M^{-1} cm^{-1}). H_2O (35.2 µL), 10x reaction buffer (6.25 µL, 500 mM HEPES, 1.5 M KCl, 100 M $MgCl_2$, pH 8.0), $\alpha S_{6\text{-}140}$ stock (6.94 µL, 0.9 mg/mL) and **16b** stock (6.25 µL) were thoroughly mixed. AaT stock (7.81 uL, 0.8 mg/mL) was added to initiate the reaction. The reaction was allowed to proceed at 37 ˚C for 4 h; an additional dose of **16b** stock (6.25 uL) was added each hour. Upon completion, the reaction was buffer-exchanged into H_2O using Amicon Ultra centrifugal filter units (3k Da MWCO). The resulting crude mixture was then directly analyzed by MALDI-TOF MS.

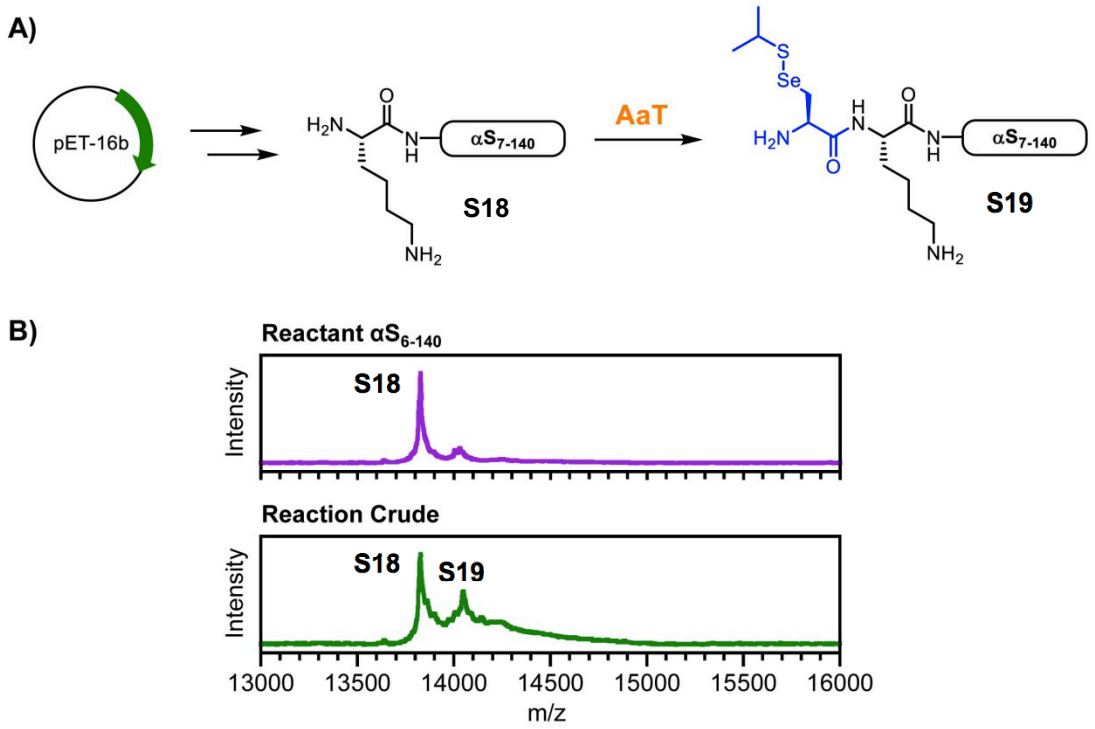

Fig. S26. Chemoenzymatic Incorporation of Sec(*S*-*i*-Pr) onto Express $\alpha S_{6\text{-}140}$. A) Schematic representation of the AaT chemoenzymatic modification reaction. $\alpha S_{6\text{-}140}$ was prepared through cellular expression, and then subject to AaT mediated N-terminal modification using **16b** as adenosine donor. B) MALDI-TOF MS characterization. [**S18** + H]$^+$, expected 13828.95, found 13824.14; [**S19** + H]$^+$, expected 14053.92, found 14049.13.

Deselenization of Sec(*S*-*i*-Pr)-$\alpha S_{6\text{-}140}$ To confirm the presence of Sec, 50 µL of the above reaction crude was mixed with 150 µL of a TCEP stock (50 mM Tris, 20 mM TCEP, 10 mM DTT, pH 8.0). The reaction was allowed to proceed at 37 ˚C for 6 h under an argon atmosphere. Upon completion, the reaction was buffer-exchanged into H_2O using Amicon Ultra centrifugal filter units (3k Da MWCO), and analyzed by MALDI-TOF MS.

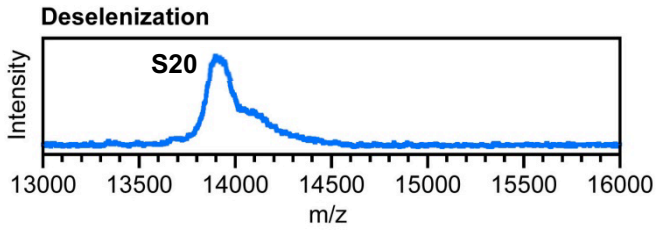

Fig. S27. Deselenization of Sec(*S*-*i*-Pr)-α$S_{6\text{-}140}$ into Ala- α$S_{6\text{-}140}$ **S20**. MALDI-TOF MS: [**S20** + H]$^+$, expected 13899.99, found 13893.67.

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