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

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Abstract

Thioamides are single-atom substitutions of canonical amide bonds, and have been proven to be versatile and minimally perturbing probes in protein folding studies. Previously, our group showed that thioamides can be incorporated into proteins by native chemical ligation (NCL) with Cys as a ligation handle. In this study, we report the expansion of this strategy into non-Cys ligation sites, utilizing radical initiated desulfurization to “erase” the side chain thiol after ligation. The reaction exhibited high chemoselectivity against thioamides, which can be further enhanced with thioacetamide as a sacrificial scavenger. As a proof-of-concept example, we demonstrated the incorporation of a thioamide probe into a 56 amino acid protein, the B1 domain of Protein G (GB1). Finally, we showed that the method can be extended to β -thiol amino acid analogs and selenocysteine.

Graphical Abstract

Introduction

A thioamide is an amide bond isostere, where the carbonyl oxygen is substituted with sulfur. While similar in size, these two groups exhibit different chemical and physical properties. The C=S bond is weaker (average bond energy 130 kcal/mol vs. 170 kcal/mol) and more

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polar (5.07 D vs. 3.79 D) than the C=O bond. The thioamide N-H is more acidic (pK_a 18.5 vs. 25.5) than that of an oxoamide, making it a stronger hydrogen bond donor; it is a weaker acceptor due to the more delocalized partial negative charge on sulfur.⁻ The presence of the sulfur atom also gives a thioamide higher nucleophilicity and greater affinity for soft metals. Most importantly for our research efforts, the thioamide exhibits unique spectral features ($\pi-\pi^*$ absorption maximum 270 nm vs. 200 nm) and redox properties (E_{ox} 1.21 V vs. 3.29 V), allowing selective experiments using a thioamide label in a background of oxoamides.

Given the abundance of peptide bonds and side chain amides in proteins, site-specific oxoamide-to-thioamide substitution has proven to be a particularly useful tool in protein structural and functional studies. In an early example, Bond *et al.* introduced a thioamide at the scissile bond of a peptide substrate of carboxypeptidase A, and used it to delineate the role of the metal cofactor in the catalytic cycle. More recently, several groups incorporated thioamides into model peptides of different secondary structures, and found that judiciously placed thioamides could exert minimal structural perturbation,⁻ while exhibiting distinct circular dichroism signatures and photoswitch properties. In our previous work, we showed that thioamides can also quench the fluorescence of a variety of fluorophores in a distance-dependent manner. The quenching takes place either through a Förster resonance energy transfer (FRET) mechanism for UV range fluorophores,⁺ or through a photoinduced electron transfer (PET) mechanism for red-shifted fluorophores.⁺ We further demonstrated the utility of various fluorophore/thioamide pairs in monitoring protein thermal unfolding, protein-substrate binding and protease activities. The details of thioamide photophysics and biophysical applications have been recently reviewed.

To apply the fluorophore/thioamide dual labeling method to full-length proteins, we developed a semi-synthesis strategy to incorporate thioamides into proteins. As a backbone modification, the thioamide functional group can only be installed onto small molecules through solution phase thionation, or short peptides by fluorenylmethyloxycarbonyl (Fmoc) based solid phase peptide synthesis (SPPS).⁺ We extended this synthetic approach to full-length proteins by adopting native chemical ligation (NCL), where a small thioamide-containing fragment was first prepared by SPPS and then conjugated to an expressed protein fragment. NCL, as pioneered by Kent *et al.*,⁺ joins a C-terminal thioester and an N-terminal Cys through transthioesterification, after which an *S*-to-*N*-acyl shift takes place to form a native amide bond at the junction. We showed that the thioamide can be placed in either the thioester or N-terminal Cys fragment, and successfully prepared several constructs of thioamide-labeled α -synuclein (αS), a 140 aa protein implicated in Parkinson's disease.⁻ The detailed synthetic methodology has also been reviewed. We note that while an *in vitro* translation strategy incorporating a thioamide-containing dipeptide was recently demonstrated, the NCL method is more general in terms of sequence and produces higher protein yields.

One inherent limitation of the conventional NCL reactions used in our initial studies is the necessity of a Cys at the ligation site. Cys is among the least abundant amino acids (2.4% in the human proteome), and readily forms disulfide bonds under ambient conditions. For protein targets without a native Cys in their sequences (or at the desired ligation sites), NCL would leave an artifact of residual Cys in the ligated product, undermining the utility of the

thioamide as a minimalist probe and introducing complications during biophysical studies. To circumvent the Cys limitation, various groups have developed traceless NCL methods by either masking or “erasing” the side chain thiol. Masking techniques utilize alkyl halides to convert Cys into Lys/Glu/Gln analogs, or homocysteine (Hcs) into Met after ligation.[–] Alternatively, the side chain thiol can be “erased” by Raney nickel or radical initiated desulfurization, where either a Cys or a thiol analog of another amino acid is converted into the native residue (Ala in the case of Cys).[–] Thus far, synthetic β - or γ -thiol analogs of 12 native amino acids have been reported.[–] The radical initiated method may also be extended to selenol-containing residues such as selenocysteine (Sec) or β -selenophenylalanine, where the mild tris(2-carboxyethyl)phosphine (TCEP) initiated deselenization could preserve Cys residues in the sequence.[–] Having briefly explored the compatibility of thioamides with Hcs masking, we sought to further expand the scope of thioamide incorporation with the desulfurization/deselenization approach.

Results and Discussion

We started with a systematic evaluation of the compatibility of thioamides with various desulfurization/deselenization methods, namely TCEP assisted radical deselenization, 2,2'-Azobis[2-(2-imidazolin-2-yl)propane] (VA-044) initiated radical desulfurization and Raney nickel desulfurization. We chose to begin with TCEP based deselenization to take advantage of our prior knowledge that the thioamide is stable towards TCEP. We synthesized thioamide-containing peptide **S6** as a pro-thioester, subjected it to ligation with selenocystine (Sec₂), and then treated the reaction mixture with TCEP for deselenization. We were delighted to find that both the ligation and deselenization proceeded smoothly and chemoselectively, where the desired Ala peptide **S8** was generated as the final product (see ESI, Fig. S3). **S8** exhibited the characteristic thioamide π - π^* absorption around 270 nm. The positive results prompted us to further investigate selective desulfurization with VA-044 as radical initiator. When the Cys/thioamide-containing model peptide **1** was treated with VA-044, the desired Ala peptide **3** was generated as the major product, which also preserved the characteristic absorption of the thioamide. In contrast, when the same peptide was treated with Raney nickel, a cleavage product **2** was observed instead (Fig. 1).

These results suggest that the radical initiated mechanisms are key to their thioamide compatibility. Consistent with our prior knowledge that a thioamide can function as an acceptor for PET (where a single electron is transferred from thioamide to a paired fluorophore, generating a transient thioamide radical cation in the process),[–] thioamides appeared to be stable in the radical-rich environment of the desulfurization/deselenization mixture. On the contrary, Raney nickel is well-established for its broad substrate scope where thiols, thioethers and thionoesters (thiocarbonyl esters) are all effectively desulfurized; although the cleavage side reaction we observed in aqueous solution was somewhat different from the reductive desulfurization to an amine reported in organic solvent,[–] it was not surprising that this metal-sulfur affinity based reaction resulted in desulfurization of the thioamide. Given that Cys and β -thiol analogs are much easier to synthesize and handle than Sec or β -selenol compounds, we decided to pursue VA-044 initiated desulfurization as the primary method for our traceless NCL incorporation of

thioamides, and reserve TCEP assisted deselenization for applications where additional selectivity against Cys is needed.

Cysteine Desulfurization

Selective Desulfurization Using Thioacetamide Scavenger—To further characterize the VA-044 initiated desulfurization reaction, we systematically varied key parameters to identify the optimal conditions. We found that a high TCEP concentration (empirically above 40 mM) was necessary to avoid disulfide bond formation, which likely resulted from the collisional quenching of thiol radicals and was reversed by TCEP reduction (see ESI, Fig. S4). We also observed that the reaction was tolerant to a wide range of VA-044 concentrations; above a critical amount (empirically 10 equivalents), the desulfurization reaction proceeded smoothly with no complications for as high as 1000 equivalents of VA-044 (see ESI, Fig. S5), which is consistent with our current understanding that VA-044 merely served as a radical initiator and that propagation primarily occurred *via* the thiol additive *t*-BuSH. Under optimal conditions, the reaction completed in less than 10 min, with no major complications for up to 4 h. In addition, we showed that dissolved oxygen – which may function as collisional radical quencher – was well-tolerated, with only minor alterations in reaction kinetics (see ESI, Fig. S6). Denaturants such as guanidinium (Gdn) and urea (which may become necessary for proteins or long peptides) were also shown to be compatible with the radical initiated desulfurization (see ESI, Fig. S7).

When investigating the robustness of the chemoselectivity, we observed a small amount of desulfurization of the thioamide, resulting in the accumulation of the thioamide-to-oxoamide conversion side product **6** after prolonged treatment with VA-044 (see ESI, Fig. S8). Although **6** only represented a 6% side reaction after 2 h, it became significant after 18 h (15–20% side product formation). Although the thioamide C=S bond is much stronger than the Cys C-S bond, increased stability of the thioamide allylic-type radical may contribute to its susceptibility to desulfurization. We therefore turned to thioacetamide as a “suicide scavenger” to prevent desulfurization of the thioamide in the peptide. When the model peptide **5'** (prime symbols denote genuine peptide standards to distinguish them from species identified in reactions) was treated with VA-044 in the absence of thioacetamide, side product **6** was generated at 16% by 18 h; in the presence of thioacetamide, **5'** was fully stable over the same time period (see ESI, Fig. S9). When we applied this additive to the Cys/thioamide peptide **4**, the same suppression was observed, improving the yield of desired product **5** from 58% to 88% after an 18 h reaction (Fig. 2). As a further validation, we demonstrated that thioacetamide was effective over a wide range of concentrations as a scavenger (Fig. S10), and that the oxygen and denaturant tolerance was unaltered by its addition (Fig. S11). It is worth noting that the residual peak at 21.8 min represents a 2~3% Cys-to-Ser conversion (see ESI).

One Pot Ligation/Desulfurization—Having established a robust method for selective Cys desulfurization in the presence of thioamides, we next sought to apply this method in traceless NCL, where two peptide fragments are first joined through a Cys, which is subsequently “erased” to form an Ala peptide. In particular, we were interested in achieving one-pot ligation-desulfurization without an intermediate purification step to isolate the

ligated Cys product. The major hurdle was aromatic thiol additives such as thiophenol, which was used to accelerate the NCL reaction, but may also function as a radical quencher to sequester subsequent desulfurization. For large proteins, one may simply remove the residual PhSH by dialysis after ligation; for small proteins and peptides, however, a different strategy must be devised.

Knowing that the pK_a of PhSH is 6.5, we hypothesized that we could remove PhSH by lyophilization after NCL if the pH was kept sufficiently low to maintain its protonated form (vapor pressure 1.8 mBar). In contrast, if the ligation mixture (at neutral or basic pH) was lyophilized directly, the majority of the PhSH would exist as non-volatile thiophenolate, and interfere with the desulfurization. Indeed, when we acidified a neutral PhSH solution to pH 1.6 and subjected it to lyophilization, we observed a significant reduction in residual PhSH concentration (see ESI). Using thioester **7a** and Cys-containing peptide **7b**, we demonstrated that one-pot ligation-desulfurization can be successfully performed in the presence of PhSH as an aromatic thiol additive, by adopting the simple procedure of acidification and lyophilization (Fig. 3). As a further validation, we compared the desulfurization product **5** to the authentic peptide standard **5'**, and found them to be perfect matches in both mass and UV-Vis absorption profiles. With thioacetamide present as a scavenger, no thioamide-to-oxoamide conversion was observed.

Synthesis of GB1 Protein by Ligation/Desulfurization—We then applied the ligation-desulfurization method to the synthesis of a thioamide-containing version of the B1 domain of *Streptococcal* protein (GB1). GB1 is small protein, but has been studied extensively as a model biophysical system and has been used in protein engineering efforts involving both natural and unnatural amino acids.²⁷ Therefore, we are interested in synthesizing thioamide analogs of GB1 as part of a longer term effort to study the impact of thioamides on the folding and function of proteins. To synthesize GB1 using NCL and desulfurization, we chose a central Ala residue as a point of disconnection. The GB1 N-terminus was synthesized as an acyl hydrazide (GB1₁₋₂₃Leu^S₅-N₂H₃, **8a**) and converted to the phenyl thioester *in situ* using Liu's method (GB1₁₋₂₃Leu^S₅-SPh, **8b**). The GB1 C-terminus was synthesized by conventional SPPS with an Ala-to-Cys mutation at its N-terminus (Cys-GB1₂₅₋₅₆-OH, **9**). Ligation under standard conditions lead to quantitative conversion to form full length GB1₁₋₅₆Leu^S₅Cys₂₄-OH (**10a**), which was isolated by HPLC purification and desulfurized using VA-044 to give GB1₁₋₅₆Leu^S₅-OH (**10b**) in 71% yield. (See ESI for descriptions of the synthesis of **8a** and **9**, conversion of **8a** to **8b**, and desulfurization reactions)

We note that attempts to acidify the reaction and desulfurize after lyophilizing gave a number of byproducts which we attribute to the reactivity of residual NaNO₂ (from acyl hydrazide activation) in highly acidic media (see ESI, Fig. S18). Therefore, the one pot lyophilization/desulfurization procedure may not be applicable to the products of ligations using the Liu method, and other additives such as trifluoroethanethiol should be considered.

Desulfurization of Cys Analogs—To further expand the scope of traceless NCL for thioamide incorporation, we also investigated the desulfurization of β -thiol amino acid analogs, selective deselenization in the presence of both thioamide and Cys residues, as well

as the incorporation of Sec into expressed proteins. Similar to the conversion of Cys into Ala, synthetic β - or γ -thiol analogs of other amino acids may also be used as ligation handles and then “erased” to form the native residues. As a proof-of-concept example, we performed the VA-044 initiated desulfurization reaction on Pen-containing peptide **11** (Pen = penicillamine). Pen is the β -thiol analog of Val; we chose it because its desulfurization involves the formation of a tertiary carbon radical close to the peptide backbone, and is thus the most sterically demanding of all β -thiol analogs. Upon treatment with VA-044 in the presence of thioacetamide, **11** was converted to desired **5** in 83% yield, with its thioamide intact (Fig. 5). The reaction was somewhat slower than Cys desulfurization, presumably due to the steric constraints near the reaction center (see ESI, Fig. S19). The successful expansion of the VA-044 initiated radical desulfurization to include Pen clearly demonstrated the versatility of this method for thioamide incorporation, providing a gateway to other synthetic β - or γ -thiol analogs.

Selenocysteine Ligations

Deselenization in the Presence of Cysteine—While VA-044 initiated desulfurization indiscriminately desulfurizes all Cys in the target sequence, TCEP assisted deselenization only “erases” the selenol side chains of Sec or β -selenol amino acid analogs, and preserves the Cys residues. For proteins with endogenous Cys, selective deselenization would be the only method to truly achieve traceless thioamide incorporation. To ascertain the chemoselectivity for selenols in the presence of both Cys and thioamides, we synthesized a model peptide **12** with all three moieties, and subjected it to the standard TCEP deselenization procedure. Much to our surprise, while the thioamide was unaffected throughout the reaction, both deselenization of Sec₁ and desulfurization of Cys₅ took place, giving a mixture of 55% Ala₁/Cys₅ product **13** and 12% Ala₁/Ala₅ product **14** (Fig. 6). After examining the reaction mechanism, we realized that the side reaction originated from homolytic cleavage of Se–S bond as the first step of the deselenization reaction, which also generates a sulfur radical that can proceed with desulfurization (see ESI, Fig. S4).

Knowing that an aromatic thiol is tolerated in one-pot ligation-deselenization but not in desulfurization (see ESI, Fig. S3) · we hypothesized that an aromatic thiol may be able to selectively quench R–S· radicals but not R–Se· radicals, consistent with theoretical predictions based on a comparison of the bond dissociation energies of Se–H bonds (66 kcal mol^{–1}) alkyl S–H bonds (87 kcal mol^{–1}), and aromatic S–H bonds (79 – 81 kcal mol^{–1}). Indeed, using 4-mercaptophenylacetic acid (MPAA) as an aromatic thiol additive, we were able to effectively suppress desulfurization, giving the desired product **13** in 62% yield (Fig. 6). While in-depth mechanistic studies are necessary to fully characterize the reactivity of the various radicals involved, we have provided an effective empirical method for the synthesis of thioamide-containing proteins with endogenous Cys residues.

Selenocysteine Attachment to Expressed Proteins—Finally, to address the issue that ligation handles other than Cys (including Sec and synthetic β - or γ -thiol/selenol analogs) are not readily incorporated through cellular expression, we explored a post-translational chemoenzymatic modification approach with *E. coli* aminoacyl transferase (AaT) to install the ligation handle onto the N-termini of expressed protein fragments for

NCL. AaT is a component of the *E. coli* N-end degradation pathway; it recognizes an N-terminal Lys or Arg on an expressed protein, and then attaches a Leu or Phe onto its N-terminus from a tRNA donor. In protein engineering, AaT has been used to transfer a variety of unnatural amino acids, such as acridone and fluorinated leucine, for protein N-terminal modifications from either full-length or truncated tRNA donors. Our group was able to further reduce the amino acid donor to a minimal adenosine analog that resembles the 3'-end of the tRNA. Using this approach, we were able to identify novel AaT substrates such as benzophenone or disulfide protected amino acids.

By examining the crystal structure of AaT and its substrate scope, we hypothesized that wildtype AaT should be able to incorporate hemiselenide protected Sec. We note that while Sec can be naturally incorporated using specialized cellular machinery, it requires a Sec-Xaa_n-Cys motif (n = 2 ~ 4) to sequester the reactive selenol side chain as an intramolecular hemiselenide during expression and handling, which prevents general introduction of Sec. To screen hemiselenide protected Sec derivatives for activity as AaT substrates, we first devised a 6-step synthesis to generate the corresponding H-Sec(SR)-Ade donors **16a-e** from *L*-selenocystine **15** (See ESI, Fig. S22). Taking advantage of the thiol exchange properties of the Se-S bond, we can readily derivatize the side chain protecting group.

We then subjected these donors to an *in vitro* transfer assay using a model peptide LysAlaAcm **17**. The formation of Sec(SR)-LysAlaAcm products **18a-e** was clearly observed for all Sec-Ade donors tested (Fig. 7), with the isopropyl protected Sec(*S-i*-Pr) exhibiting the highest transfer efficiency. As a further validation, we also synthesized the Cys counterparts H-Cys(*S-i*-Pr)-Ade and H-Cys(*S-t*-Bu)-Ade of these donors, and observed nearly identical transfer efficiencies as the Sec substrates (data not shown), giving us confidence that the selenium in the hemiselenide side chains did not interfere with AaT enzyme function. Lastly, we demonstrated the application of this chemoenzymatic strategy on expressed protein fragments, where we generated αS₆₋₁₄₀ **S18** (a truncated version of αS with an N-terminal Lys) through cellular expression and successfully transferred Sec(*S-i*-Pr) onto its N-terminus from adenosine donor **16b** (see ESI, Fig. S27). While additional optimization is still needed to improve transfer efficiency, this chemoenzymatic approach proves to be a viable strategy for the incorporation of Sec as a ligation handle, and can conceivably be extended to other β- or γ-thiol/selenol analogs with directed evolution of AaT.

Conclusions

We have devised a method for the traceless incorporation of thioamides into peptides and proteins using chemoselective desulfurization/deselenization in combination with NCL. We demonstrated that Cys can be selectively converted into Ala using VA-044 as a radical initiator in the presence of a backbone thioamide, and that thioacetamide can be used as an additive to improve the robustness of this chemoselectivity. We also showed that by acidifying the ligation mixture, PhSH can be effectively removed by lyophilization, enabling one-pot ligation-desulfurization that had not previously been achieved. As a proof-of-concept example, we synthesized thioamide-containing GB1 through traceless NCL at a Leu site. We will use the methods developed here to synthesize other thioamide variants of GB1 in order to better understand the impact of thioamide incorporation on this small, well-

folded protein. We note that Liu's acyl hydrazide method gave high yields of the thioester protein fragment with no degradation of the thioamide and we will explore this method in the context of other thioamide ligations. To expand the scope of ligation sites, we also showed the selective desulfurization of β -thiol analogs using conversion of Pen into Val as an example, demonstrated selective deselenization in the presence of both Cys and a thioamide, and devised a chemoenzymatic approach to incorporate ligation handles other than Cys onto the N-termini of expressed proteins. With these methods, we are now able to perform traceless ligation at sites other than Cys for thioamide-containing peptides and proteins, making thioamide incorporation by semi-synthesis fully general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Thioamide Compatibility with Different Desulfurization Methods. Top: reaction scheme; Bottom: HPLC chromatogram monitored at 325 nm, and UV-Vis absorption profiles for major peak in each chromatogram. MALDI-TOF MS: [**1** + H]⁺: expected 1262.96, found 1262.56; [**2** + H]⁺: expected 988.53, found 988.46; [**3** + H]⁺: expected 1230.63, found 1230.48; [**3'** + H]⁺: expected 1230.63, found 1230.62. Asterisks indicate thioamide absorption at 272 nm; **3'** is a genuine product standard synthesized by SPPS for comparison to **3** formed in the reaction. Conditions: 0.1 mM peptide 1, 0.1% w/v Raney nickel, 100 mM Na₂HPO₄, 10 mM TCEP, pH 5.8, 12 h, for the Raney nickel method; 0.1 mM peptide 1, 10 mM VA-044, 40 mM TCEP, 200 mM Na₂HPO₄, 10% *t*-BuSH (v/v) pH 7.0, 2 h, for the VA-044 method. VA-044 = 2,2'-azobis[2-(2-imidazolin-2-yl)propane]; TCEP = tris(2-carboxyethyl)phosphine; Mcm = 7-methoxycoumarinylalanine.

Fig. 2.

Thioacetamide as a Small Molecule Scavenger. Top: reaction scheme; Bottom: HPLC chromatogram monitored at 325 nm, and UV-Vis absorption profiles for selected peaks in each chromatogram. MALDI-TOF MS: [**5** + H]⁺, expected 1450.64, found 1450.46; [**6** + H]⁺, expected 1434.67, found 1434.44. Asterisks indicate thioamide absorption at 272 nm; prime symbols denote genuine product standards synthesized by SPPS to distinguish them from species isolated in reactions. Conditions: 0.1 mM peptide **4**, 10 mM VA-044, 40 mM TCEP, with or without 100 mM thioacetamide, 200 mM Na₂HPO₄, 10% *t*-BuSH (v/v) pH 7.0, 18 h.

Fig. 3.

One-Pot Ligation-Desulfurization. Top: reaction scheme; Bottom: HPLC chromatogram monitored at 325 nm, and UV-Vis absorption profiles for major peaks in each chromatogram. MALDI-TOF MS: [**4** + H]⁺, expected 1482.62, found 1482.47; [**5** + H]⁺, expected 1450.64, found 1450.82; [**5'** + H]⁺, expected 1450.64, found 1450.80. Asterisks indicate thioamide absorption at 272 nm; **5'** is a genuine product standard synthesized by SPPS for comparison to **3** formed in the reaction. Ligation: 1 mM thioester **7a**, 1 mM peptide **7b**, 40 mM TCEP, 200 mM Na₂HPO₄, pH 7.0, overnight. Desulfurization: 0.1 mM reaction crude **4**, 10 mM A-044, 40 mM TCEP, 200 mM Na₂HPO₄, 10% *t*-BuSH (v/v) pH 7.0, 2 h.

Fig. 4.

Synthesis of Thioamide GB1 by Ligation and Desulfurization. Top: reaction scheme; Bottom: HPLC chromatograms monitored at 272 nm, and MALDI-TOF MS profiles for selected peaks in each chromatogram. MALDI-TOF MS: [**8b** + H]⁺, expected 2591.01, found 2590.90; [**9** + H]⁺, expected 3749.01, found 3748.58. [**10a** + H]⁺, expected 6228.84, found 6229.73; [**10b** + H]⁺, expected 6196.78, found 6196.39. Ligation: 0.5 mM thioester (quantitative from **8a**) **8b**, 0.5 mM peptide **9**, 6 M Gdn•HCl, 200 mM Na₂HPO₄, pH 7.0, overnight. Desulfurization: 0.1 mM purified **10a**, 50 mM VA-044, 400 mM TCEP, 6M GnHCl, 200 mM Na₂HPO₄, 10% *t*-BuSH (v/v) pH 7.0, overnight.

Fig. 5.

Extension of Desulfurization Method to Penicillamine (Pen). Top: reaction scheme; Bottom: HPLC chromatogram monitored at 325 nm, and UV-Vis absorption profiles for major peaks in each chromatogram. MALDI-TOF MS: [**11** + H]⁺, expected 1482.62, found 1482.56; [**5** + H]⁺, expected 1450.64, found 1450.82. Retention time: 16.0 min for **11**, and 16.2 min for **5**; the difference is small but reproducible. Asterisks indicate thioamide absorption at 272 nm. Conditions: 0.1 mM peptide **11**, 10 mM VA-044, 40 mM TCEP, 200 mM Na₂HPO₄, 10% *t*-BuSH (v/v), pH 7.0, 18 h.

Fig. 6.

Selective Deselenization of Sec in the Presence of both Cys and Thioamides. Top: reaction scheme; Bottom: HPLC chromatogram monitored at 325 nm, and UV-Vis absorption profiles for selected peaks in each chromatogram. MALDI-TOF MS: [**12** + H]⁺, expected 1148.37, found 1148.56; [**13** + H]⁺, expected 1070.48, found 1070.74; [**14** + H]⁺, expected 1038.50, found 1038.73. Asterisks indicate thioamide absorption at 272 nm. Conditions: 0.1 mM peptide **5**, 40 mM TCEP, 40 mM DTT, with or without 10 mM MPAA, 200 mM Na₂HPO₄, pH 7.0, 18 h.

Fig. 7. Chemoenzymatic Sec Incorporation by Aminoacyl Transferase (AaT). Top: synthesis and activity assay scheme; Bottom: HPLC chromatogram monitored at 325 nm, and MALDI-TOF MS characterization for product peaks. The selenium isotopic pattern was clearly shown. See ESI for a complete description of syntheses and AaT activities for all donors tested. Acm = 7-amino-3-methylcoumarin.