

Incorporating Thioamides into Proteins by Native Chemical Ligation

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Abstract

The thioamide is a versatile replacement of the peptide backbone with altered hydrogen bonding and conformational preferences, as well the ability participate in energy and electron transfer processes. Semi-synthetic incorporation of a thioamide into a protein can be used to study protein folding or protein/protein interactions using these properties. Semi-synthesis also provides the opportunity to study the role of thioamides in natural proteins. Here we outline the semi-synthesis of a model protein, the B1 domain of protein G (GB1) with a thioamide at the N-terminus or the C-terminus. The thioamide is synthetically incorporated into a fragment by solid-phase peptide synthesis, whereas the remainder of the protein is recombinantly expressed. Then, the two fragments are joined by native chemical ligation. The explicit protocol for GB1 synthesis is accompanied by examples of applications with GB1 and other proteins in structural biology and protein misfolding studies.

Keywords: Thioamide, native chemical ligation, semi-synthesis, GB1, calmodulin, α -synuclein

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1. Introduction

The thioamide is a single atom, O-to-S, substitution of the canonical amide bond, with altered chemical and physical properties that can have subtle or profound effects on the surrounding molecule. These effects underlie the use of thioamides as synthetic probes and help to explain the existence of thioamides in a small, but growing, number of known natural peptides and proteins. With a lower N-H pK_a the thioamide is more acidic than the amide, and therefore is a stronger hydrogen bond donor (Jensen, 1981, Dudek, & Dudek, 1967). Conversely, the less electronegative sulfur makes the thioamide a weaker hydrogen bond acceptor (Lee et al., 2002). The red-shifted thioamide π - π^* absorption (Sifferlen et al., 1999) has spectral overlap with certain fluorophores allowing for Förster resonance energy transfer (FRET) (Petersson, Goldberg, & Wissner, 2014). The lower oxidation potential of the thioamide suggests that it could be involved in electron transfer processes (Bordwell, Algrim, & Harrelson, 1988). This can be exploited by using the thioamide for photoinduced electron transfer (PeT) based quenching of appropriately matched fluorophores (Petersson, Goldberg, & Wissner, 2014). These altered characteristics can be utilized to study protein or protein-substrate dynamics (Batjargal et al., 2012, Goldberg, Speight, Fegley, & Petersson, 2012, Goldberg, Wissner, Klein, & Petersson, 2012, Goldberg, Batjargal, & Petersson, 2010), as well as to modify peptides or proteins for improved stability or to serve as sensors (Barrett et al., 2020, Chen et al., 2017, Goldberg et al., 2014, Huang et al., 2016, Liu et al., 2019).

Examples of natural thioamide peptides include methanobactin (Kim, 2004, Kim et al., 2004) and thioviridamide (Hayakawa et al., 2006, Hayakawa et al., 2006), which are ribosomally synthesized and post-translationally modified peptides. At the time of publication, two proteins with thioamides have been identified. One is methyl coenzyme M reductase (MCR) from methanogenic and methanotrophic archaea, which has a thioglycine residue in the active site (Kahnt et al., 2007). Recently, a cryo-EM structure and follow-up mass spectrometry studies identified a backbone thioamide in the uL16 protein of the *E. coli* 70S ribosome (Watson et al., 2020). Although research has identified the enzymes responsible for thioamidation of MCR (Nayak, Mahanta, Mitchell, & Metcalf, 2017), the function of the thioamide remains elusive in the biomolecules. Descriptions of the recombinant expression of thioamide biosynthesis enzymes can be found in [Chapter 7](#) of this volume by Mahanta, Mitchell, and coworkers. Understanding how and why nature utilizes the thioamide will help to improve the potential of this modification for research purposes. To accomplish all of the above, methods are required to incorporate synthetic thioamides into proteins. Given the sequence limitations of biosynthetic incorporation of thioamides, chemical methods are needed for the general exploration of their properties.

Thioamide incorporation into peptides via solid-phase peptide synthesis (SPPS) has largely followed methods by Rapoport (Shalaby, Grote, & Rapoport, 1996), with recent updates by Chatterjee (Mukherjee, Verma, & Chatterjee, 2015, Mukherjee, & Chatterjee, 2016, Khatri, Bhat, & Chatterjee, 2020), Petersson (Szantai-Kis et al., 2017) and VanVeller (Camacho III, Nguyen, Turner, & VanVeller, 2019, Camacho III, Lampkin, & VanVeller, 2019). Review [Chapter 2](#) for more detailed information. To incorporate thioamides into biological substrates with more than ~20 amino acids, native chemical ligation (NCL) is most commonly used, where under aqueous and denaturing conditions at neutral pH, a peptide with a C-terminal thioester and a peptide with a N-terminal cysteine reversibly undergo a trans-thioesterification reaction, followed by an irreversible S $\rightarrow$ N acyl shift to form the ligated product (Dawson, Muir, Clark-Lewis, & Kent, 1994) (**Fig. 1**). We have shown that these methods are tolerant of a thioamide in either the C-terminal thioester or N-terminal cysteine fragments.

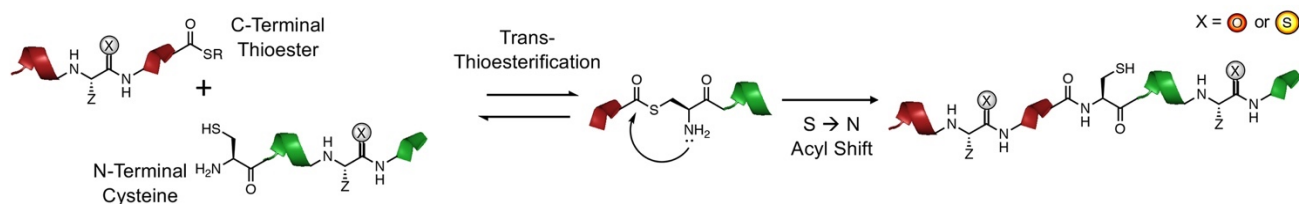
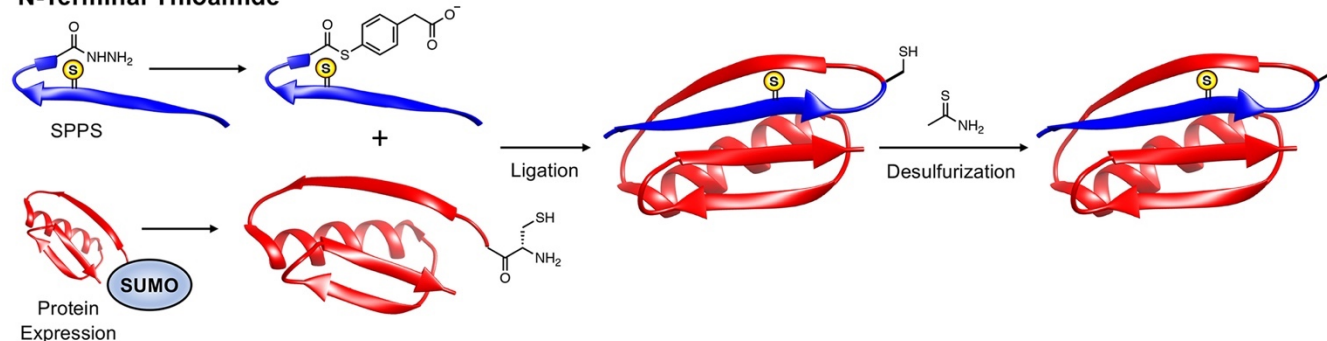


Fig. 1 Native chemical ligation (NCL). A peptide with an N-terminal cysteine side-chain undergoes reversible trans-thioesterification with a peptide with a C-terminal thioester. An irreversible S→N acyl shift generates the ligated product with a native peptide bond.

NCL methods circumvent many of the liabilities of thioamide incorporation by SPPS, which are more significant for longer peptide sequences. Firstly, the acidic conditions typically used for removal of sidechain protecting groups and resin cleavage in fluorenylmethoxycarbonyl (Fmoc) peptide synthesis can lead to cleavages near the thioamide residue through mechanisms similar to Edman degradation. Secondly, elongating the peptide sequence following thioamide incorporation risks epimerization with each basic deprotection step due to the lower thioamide C- α pK_a. Using NCL methods allows one to keep the SPPS segment containing the thioamide short, reducing the time needed for acidic deprotection and reducing the number of basic deprotection steps. While both fragments of the protein can be produced by SPPS, it is most convenient to express the segment containing only natural amino acids. In this chapter, we describe how to incorporate thioamides into the N- and C-termini of a representative protein, GB1, the B1 domain of protein G, using combinations of protein expression and SPPS (Gronenborn et al., 1991). A ligation with the thioamide at the N-terminus is outlined in Section 4, whereas a ligation with the thioamide at the C-terminus is outlined in Section 5 (**Fig. 2**). The small size of GB1 (56 amino acids) makes it accessible by direct SPPS as well as NCL using two SPPS fragments, so it is a useful model protein for comparing yields and serves as a good benchmark system for biophysical studies.

N-Terminal Thioamide



C-Terminal Thioamide

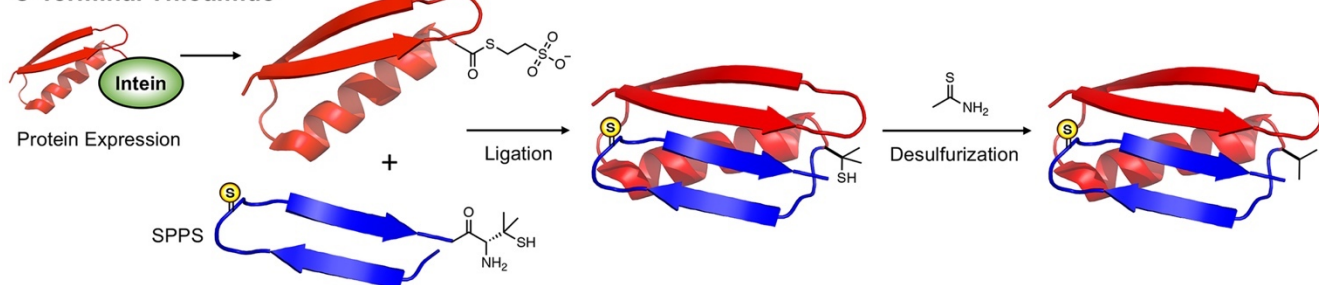


Fig. 2 N-terminal ligation (Section 4) and C-terminal ligation (Section 5) for thioamide incorporation into GB1 (PDB 2QMT).

2. Ligation strategy and design

2.1. Thioamide incorporation site

The choice of thioamide incorporation site will be dictated by the desired application, depending on whether the goal is to quench a fluorophore to monitor a specific conformational change, modulate a specific protein interaction, or alter the stability of a protein fold. While these considerations may broadly guide the location of the thioamide, choosing the specific residue to replace may be further informed by a desire to maximally stabilize or destabilize a fold, or to have the thioamide be a neutral modification for applications as a non-perturbing reporter chromophore. While no general computational method for predicting thioamide effects has yet been achieved (we have had success in rationally modulating protease recognition (Barrett, Chen, Liu, Giannakoulis, Phan, Wang, Keenan, Karpowicz, & Petersson, 2020) (Giannakoulis et al., 2020)), a series of studies have provided some insights on the effects of thioamides in model peptides and proteins. In an alanine-based model α -helix, central incorporation was strongly destabilizing, but N-terminal incorporation resulted in partial α -helix formation (Reiner, Wildemann, Fischer, & Kiefhaber, 2008). This was in contradiction to thioamide incorporation in a parallel two stranded coiled coil where a central thiomide did not disrupt helical character (Miwa, Pallivathucal, Gowda, & Lee, 2002). Incorporation of thioamides into a collagen mimetic peptide demonstrated an increased thermostability at Yaa in the canonical Xaa-Yaa-Gly repeat, where the thioamide is a hydrogen bond donor and positioned to strengthen an $n \rightarrow \pi^*$ interaction (Newberry, VanVeller, & Raines, 2015). Chatterjee and coworkers observed similar stabilizing effects in turn motifs in model proteins (Khatri et al., 2020). Additionally, Miwa reported that thioamide incorporation in the turn of an antiparallel β -hairpin, did not disturb the structure, even at high temperatures (Miwa et al., 2001). These studies demonstrate that the effect of the thioamide on protein stability depends on specific local interactions, rather on general rules regarding secondary structure. This was further supported by the findings of Walters and co-workers, where incorporation of a thioamide into full-length proteins with of varying secondary structure demonstrated differing thermostability depending on the position, even in neighboring positions within the same secondary structure (Walters et al., 2017).

Based on the findings listed above, we advise researchers to carefully consider where they choose to incorporate a thioamide. Placing a thioamide in the position of an important hydrogen bond acceptor might be interesting to pursue as a biophysical probe. However, synthetically it could negatively impact the folding of the protein and thereby make ligation and purification difficult. We recommend that researchers refer to the wild-type structure (if one exists), and (if possible) pick positions where the would-be thioamide is not acting as a hydrogen bond acceptor, and where the additional length of the C=S bond can be accepted, i.e. where the carbonyl is solvent-exposed in the wild-type structure.

2.2. Ligation site

The choice of ligation site will be guided by the location of the thioamide since it must be placed in the SPPS fragment. One further wishes to minimize the length of the SPPS segment and then connect the N- and C-terminal fragments at a site that will provide an optimal NCL yield. The kinetics of NCL are affected by the sterics of the C-terminal thioester residue (Hackeng, Griffin, & Dawson, 1999). Ligation with β -branched amino acids such as leucine, valine, and isoleucine will proceed more slowly than C-terminal glycine or alanine residues. Additionally, C-terminal residues such as lysine, aspartic acid, and glutamic acid are not recommended since they can cyclize and displace the thioester.

For the N-terminal residue, unless the sequence of the protein contains a favorably placed native cysteine, the ligation site must be erased to regenerate the native sequence. The two major methods for doing so are desulfurization and alkylation. Since these methods globally affect thiols, additional cysteines in the protein must

be protected or more complex and selective methods using selenol-based ligations must be used (Wang, Szantai-Kis, & Petersson, 2016) (Mitchell et al., 2015). Alkylation methods to generate Lys, Gln, and Glu analogs as well as authentic Met have been demonstrated in the presence of thioamides (Walters, Szantai-Kis, Zhang, Reinert, Horne, Chenoweth, & Petersson, 2017, Wissner, Wagner, Warner, & Petersson, 2013, Walters, Ferrie, & Petersson, 2018). However, since alkylation does not produce an authentic residue (with the exception of conversion of homocysteine to methionine), we here describe methods for desulfurization to disguise the ligation site.

2.3. Desulfurization

Native residues such as alanine and valine can be achieved via desulfurization of an N-terminal cysteine or penicillamine respectively. Radical initiated mechanisms such as that with water soluble 2,2'-azobis[2-(2-imidazolin-2-yl)propane] (VA-044), tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl), and *tert*-butylthiol as a hydrogen source will desulfurize the ligation site thiol (Wan, & Danishefsky, 2007). Although this proceeds with some thioamide to oxoamide conversion, conditions have been optimized for thioamide-containing substrates by adding sacrificial thioacetamide (Wang, Szantai-Kis, & Petersson, 2016). Desulfurization with RANEY[®] nickel results in cleavage at the thioamide position and is not recommended (Wang, Szantai-Kis, & Petersson, 2016).

Besides penicillamine, which is commercially available as an Fmoc-protected amino acid, the synthetic strategies for protected β - or γ -thiol analogs of various amino acids have been published (Table in (Malins, & Payne, 2015)). This allows for an increased scope of traceless ligation site positions. In 2020, a facile method for the synthesis of β -thiol derivatives via a photoredox-catalyzed asymmetric Giese reaction was published that allows for synthesis on a gram scale (Yin et al., 2020). Ligation with these analogs is expected to be slower than those with cysteine.

2.4. Expression of deletion construct

NCL was originally pioneered to ligate two peptides together, but for larger proteins, expression of one of the fragments eases synthetic difficulties (Muir, Sondhi, & Cole, 1998). To produce C-terminal thioesters, deletion fragments of the protein of interest can be recombinantly expressed with C-terminal intein tags (**Fig. 2**). These tags derive from engineered inteins that can be trapped as C-terminal protein thioesters rather than self-splicing. Addition of a nucleophilic thiol, such as 2-mercaptoethanesulfonic acid sodium salt (MESNa), results in cleavage of the intein and a C-terminal small molecule thioester ready for reaction via NCL (Shah, & Muir, 2014).

Expression of the N-terminal cysteine fragment can also be achieved. Constructs can be designed to express the C-terminal protein fragment with Met-Cys at the N-terminus, so that the endogenous methionine aminopeptidase in *E.coli* will cleave the initiating Met to reveal the N-terminal Cys (Gentle, De Souza, & Baca, 2004). Reaction with pyruvate or acetaldehyde *in vivo* results in the purified protein having an N-terminal thiazolidine ring, which can be opened by treatment with methoxylamine. However, in some cases, obtaining protein with pure N-terminal cysteine following methoxylamine treatment has proven difficult. Therefore, we here illustrate using an N-terminal small ubiquitin-like modifier (SUMO) protein tag, which can be site-specifically cleaved by the Ulp-1 protease (**Fig. 2**). Ulp-1 cleaves after the C-terminal Gly-Gly residues of SUMO and therefore tracelessly cleaves the tag to reveal the N-terminal Cys (Malakhov et al., 2004) (Komarov, Linn, Devereaux, & Valiyaveetil, 2009).

3. Chemical synthesis of thioamide precursor

A thorough description of the synthesis of thioamide precursors for SPPS is provided in [Chapter 2](#).

4. N-terminal Ligation: Thioamide-containing N-terminal fragment and expressed C-terminal fragment

This section outlines the synthesis of a thioamide-containing peptide with a C-terminal acyl hydrazide (GB1₁₋₁₂ L^S₇ NHNH₂) and the recombinant expression of a deletion fragment with an N-terminal cysteine (GB1₁₃₋₅₆ K₁₃C). Purification of the fragments, ligation, and desulfurization of the ligation site cysteine to alanine are described. Activation of the acyl hydrazide to the thioester is achieved using the method developed by Liu (sodium nitrite

oxidation to the acyl azide intermediate) (Zheng et al., 2013). Although not detailed below, this can also be achieved with method recently published by Dawson and coworkers (formation of an acyl pyrazole intermediate with acetyl acetone) (Flood et al., 2018).

4.1. Reagents and equipment

The supplies and instruments listed below are used for the preparation of constructs.

PCR reagents and equipment

T100 thermocycler (BioRad)
DNA Gel box (Fisher)
SYBR Safe DNA gel stain (Invitrogen)
Gel Loading Dye, Purple (6X), no SDS (New England BioLabs, # B7025S)
Agarose (Invitrogen)
Tris-acetate-EDTA (TAE) buffer
Q5 High-Fidelity DNA polymerase and Master Mix (New England Biolabs)
HiFi DNA Assembly Master Mix (New England Biolabs)
NEB 10 β High Efficiency competent *E.coli* cells (New England Biolabs)
Monarch Gel Extraction Kit (New England Biolabs)
Nanoquant plate (TECAN)
Infinite[®] M1000 Pro plate reader (TECAN)
SmartBlue transilluminator (Southern Labware)
DNase/RNase free water for molecular biology

Protein expression equipment

RC-5 centrifuge with SS-34 and GS3 rotors (Sorvall)
Q700 sonicator (QSonica)
Genesys 150 UV-Vis Spectrometer (Thermo Scientific)
SDS-PAGE gel box (Bio-Rad)
ÄKTA Explorer FPLC system (GE Healthcare Bio-Sciences)
HiTrap Q HP column (GE Healthcare Bio-Sciences)
HiLoad Superdex 75pg 16/600 column (GE Healthcare Bio-Sciences)
12 mL 2k MWCO Slide-A-Lyzer dialysis cassette (Thermo Scientific)
3.5 kDa MWCO dialysis tubing (Fisher)
500-1000 Da MWCO dialysis tubing (Spectrum)
Lyophilizer (Labconco)
Amicon Ultra-4 10k MWCO Spin Filter (EMD Millipore)
0.22 μ m PES Millex-GP syringe filter (EMD Millipore)
pH meter
micro pH electrode (Mettler Toledo LE422)

Protein expression reagents

E.coli BL21-DE3 competent cells (New England Biolabs)
Milli-Q filtered (18M Ω) water (Millipore)
Ampicillin Sodium salt
Kanamycin
Isopropyl β -D-1-thiogalactopyranoside (IPTG) (LabScientific Inc.)
Protease inhibitor cocktail cOmplete mini tablets, EDTA-Free, Easy Pack (Roche)
Nickel agarose resin (High Density) (GoldBio, #H-320-5)
Bond-Breaker TCEP solution, Neutral pH (ThermoFisher, #77720)
tris(2-carboxyethyl)phosphine (TCEP) HCl
1,4-dithiothreitol (DTT)
HEPES
Imidazole

Tris base
NaCl (Sigma, #S6191)

Peptide synthesis reagents

7-Azabenzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate (PyAOP) (ChemImpex, #30381)
2-chlorotrityl (Cl-Trt) resin (EMD Millipore)
9-Fluorenylmethyl carbazate (ChemImpex, #33069)

Peptide synthesis equipment

Luna Omega PS C18 preparative column (5 μ m particle size, 250 mm length, 21.2 mm diameter, 100 Å pore size) (Phenomenex)
Luna Omega PS C18 semi-preparative column (5 μ m particle size, 250 mm length, 10 mm diameter, 100 Å pore size) (Phenomenex)
Jupiter C4 semi-preparative column (10 μ m particle size, 250 mm length, 10 mm diameter, 300 Å pore size) (Phenomenex)
Agilent 1260 Infinity II Preparative HPLC
Luna C8(2) column (5 μ m particle size, 150 mm length, 4.6 mm diameter, 100 Å pore size) (Phenomenex)
Agilent 1260 Infinity II series Analytical HPLC
0.45 μ m PVDF syringe filter
Fritted syringe

NCL and desulfurization reagents

Sodium nitrite (NaNO_2)
Sodium phosphate dibasic (Na_2HPO_4)
Sodium phosphate monobasic monohydrate (NaH_2PO_4)
4-mecaptophenylacetic acid (MPAA)
Argon gas
VA-044 (Wako Pure Chemical Industries)
tert-butylthiol (t-BuSH)

Mass spectrometry instrument

Ultraflex III Matrix assisted laser desorption/ionization instrument (Bruker)
 α -cyano-4-hydroxycinnamic acid (CHCA) matrix

4.1.1. Recipes

LB media

Tryptone, 5 g
Sodium chloride, 10 g
Yeast extract, 5 g
Dissolve in 1 L Milli-Q water

SOC media

Tryptone, 5 g
Sodium chloride, 1.25 g
Yeast extract, 1.25 g
250 mM potassium chloride 2.5 mL
Milli-Q water, fill to 250 mL final volume
Autoclave the solution. Once the solution is cooled, add:
1 M MgCl_2 , 2.5 mL
40% (w/v) Glucose, 2.5 mL

14% Tris-Tricine SDS-PAGE gel

3x Gel buffer
3 M Tris
Adjust pH to 8.45

0.3% sodium dodecyl sulfate (SDS)
 10x Cathode buffer
 1 M Tris
 1 M Tricine
 1% SDS
 10x Anode buffer
 2 M Tris
 Adjust pH to 8.9 (with HCl)
 14% Separation gel
 1.75 mL 40% acrylamide
 1.67 mL 3x gel buffer
 0.80 mL 50% (v/v) glycerol
 0.74 mL Milli-Q water
 50 μ L APS
 5 μ L TEMED
 4% Stacking gel
 0.15 mL 40% acrylamide
 0.50 mL 3x gel buffer
 0.80 mL Milli-Q water
 25 μ L APS
 2.5 μ L TEMED

Coomassie sensitive stain

800 mL Milli-Q water & 50 g aluminum sulfate
 100 mL Ethanol
 200 mg Coomassie Brilliant Blue G-250
 23.5 mL O-phosphoric acid
 Add Milli-Q water up to 1000 mL

4.2. Preparation of GB1 constructs with N-terminal cysteine

4.2.1. Cloning of pET His₆-SUMO-GB1₁₃₋₅₆ K₁₃C via Gibson assembly

4.2.1.1. *PCR to prepare insert and vector plasmids*

Before the plasmid (pET His₆-SUMO-GB1₁₃₋₅₆ K₁₃C) can be formed via Gibson assembly, the insert and vector plasmids need to be linearized and extended with overhangs that corresponds to the other DNA fragment. This is done via simultaneous PCR of the insert and donor plasmid with a ‘Touchdown’ method. The primers are designed to amplify the insert fragment (GB1₁₃₋₅₆ K₁₃C), linearize the pET His₆-SUMO donor plasmid, and extend both with the necessary overhangs (underlined). The donor plasmid was generated by Scott Gradia (Addgene plasmid #29711).

- Insert donor plasmid: pTXB1 GB1₁₃₋₅₆ K₁₃C-MxeGyrA(Δ)-His₆
 - Vector donor plasmid: pET His₆-SUMO-TEV- EcoR1
 - Forward primer for insert: 5’ – GATTGGTGGGTGCGGGGAAA CAACA – 3’
 - Reverse complement primer for insert: 5’ – GGATCCGTTACTCGGTAACG GTGAACG – 3’
 - Forward primer for vector: 5’ – CGTTACCGAGTAACGGATCC GCGATCG – 3’
 - Reverse complement primer for vector: 5’ – TTTCCCCGCACCCACCAATCTGTTCTCT – 3’
- a. Dissolve primers in DNase/RNase free water to a final concentration of 50 μ M. (Dissolved primers can be stored at -20 °C). Do not vortex to dissolve.
 - b. Dilute plasmids to ≤ 1 ng/ μ L with DNase/RNase free water.

- c. Combine and store on ice: 21.0 μL DNase/RNase free water, 1.0 μL of each plasmid, 0.5 μL of each forward primer and 0.5 μL of each reverse complement primer. Add 25.0 μL 2x Q5 PCR Master Mix and mix by pipetting (final volume of 50.0 μL).
- d. Use a thermocycler to amplify the plasmid and simultaneously insert GB1 fragment with 'Touchdown' protocol.
 - i. With the 'Touchdown' protocol, start with an annealing temperature significantly above the calculated annealing temperature for all primers. Then successively decrease the temperature to allow for different annealing temperatures/ multiple PCR reactions to occur simultaneously. This also ensures that once the annealing temperature is below the ideal, then the desired template should be in greater excess, making it more likely to continue to be transcribed.

Touchdown PCR reaction

Step	Temperature	Duration
1	98 °C	00:30 min (30 sec)
2	98 °C	00:10 min
3	65 °C (-0.5 °C / cycle)	00:20 min
4	72 °C	03:00 min
5	Go to step 2	9 times
6	98 °C	00:10 min
7	60 °C	00:20 min
8	72 °C	03:00 min
9	Go to step 6	24 times
10	72 °C	02:00 min
11	4 °C	∞

- e. Purify the vector with a 1% agarose gel and the insert with a 3% agarose gel. Prepare gels directly in SYBR Safe dye. Use TAE buffer.
 - i. Add 10.0 μL of 6x DNA gel loading buffer (no SDS) to the PCR reaction. Load 30.0 μL onto the gel. Run gel at 200 V for 45 minutes.
 - ii. Visualize bands with a blue light transilluminator.
- f. Excise bands of expected size and extract using a DNA gel extraction kit according to the manufacturer's protocol.
- g. Quantify isolated DNA using Nanoquant plate and M1000 Pro plate reader.

4.2.1.2. Gibson assembly

- a. Assemble the PCR amplified vector DNA (100 ng) and insert DNA (5 eq) with NEBuilder HiFi DNA Assembly Mix according to manufacturer's instructions.
 - i. Use NEBio Calculator to calculate amount of DNA needed (<https://nebiocalculator.neb.com/#!/ligation>).
 - ii. Combine vector and insert in a PCR tube and add sterile Milli-Q water up to 10.0 μL .
 - iii. Add 10.0 μL 2x NEBuilder HiFi Assembly Master Mix.
 - iv. For positive control: Mix 10.0 μL of the control with 10.0 μL 2x HiFi Assembly Master Mix.
 - v. Set the thermocycler to incubate at 50 °C for 60 minutes
- b. Put reaction on ice and transform 2.0 μL into NEB10 β competent cells following manufacturer's protocol. Plate 50 μL and 100 μL onto LB plate supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin. Store upside down in incubator overnight at 37 °C.
- c. With a sterilized toothpick, streak an individual colony and inoculate 5 mL LB with 100 $\mu\text{g}/\text{mL}$ ampicillin. Repeat this twice. Let grow overnight at 37 °C, shaking at 250 rpm.
- d. Extract the DNA with a plasmid miniprep kit according to manufacturer's protocol. Elute plasmid with provided elution buffer.

- e. Quantify the DNA with a Nanoquant plate and M1000 Pro plate reader, then submit for Sanger-sequencing to verify that the assembly was successful (T7 promotor).

4.2.2. Expression of GB1₁₃₋₅₆ K₁₃C

4.2.2.1. Transformation

- a. Thaw BL21-DE3 *E.coli* aliquot on ice.
- b. Add 2.0 μ L of desired plasmid (pET His₆-SUMO-GB1₁₃₋₅₆ K₁₃C) to 50 μ L BL21-DE3 *E.coli*. Flick the tube to mix cells and DNA. Incubate on ice for 30 minutes.
- c. Heat shock at 42 °C for 45 seconds and then incubate on ice for 2 minutes.
- d. Add 450 μ L SOC media and incubate at 37 °C for 1 hour, shaking at 250 rpm.
- e. Plate 200 μ L on an LB plate supplemented with 100 μ g/mL ampicillin. Store upside down in an incubator overnight at 37 °C.

4.2.2.2. Primary culture

- a. Pick a single colony using a sterilized toothpick and inoculate 5 mL of LB media containing 100 μ g/mL ampicillin. Repeat this twice.
 - i. Set up three primaries per L of expression.
 - ii. Wrap plate with parafilm and store upside down at 4 °C. You can continue to pick colonies from plate for 1 week following transformation.
 - i. Instead of transforming again, a glycerol stock can be made at this step. After step b, mix 500 μ L of a saturated overnight primary with 500 μ L 50% (v/v) sterile glycerol. Store glycerol stock at -80 °C. Instead of picking a single colony, the glycerol stock can be scraped with a sterile pipette tip. Do not allow glycerol stock to thaw.
- b. Incubate primaries at 37 °C, shaking at 250 rpm until saturation (5-6 hours).
 - i. Primaries can also be incubated overnight.
 - ii. The cultures should be cloudy with an OD₆₀₀ = 0.6-0.7 measured on a UV-Vis instrument.
- c. Prepare 1 L of LB media.

4.2.2.3. Inoculation and induction of secondary culture

- a. Add ampicillin to LB media for final concentration of 100 μ g/mL.
- b. Inoculate 1 L of LB/ampicillin with two primary cultures. Incubate at 37 °C with 250 rpm shaking.
- c. Upon reaching an OD₆₀₀ = 0.6-1.0 measured on a UV-Vis instrument, move to an 18 °C incubator with 250 rpm shaking.
- d. After allowing the culture to cool (10-15 minutes), add isopropyl- β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and leave overnight (16-20 hours).

4.2.3. Purification of GB1₁₃₋₅₆ K₁₃C

4.2.3.1. Cell pelleting and lysis

- a. Pellet cells through centrifugation at 4,000 rpm (2,704 x g) with a GS-3 rotor for 20 minutes at 4 °C. Ensure tubes are balanced.
- b. Pour off media and leave bottles upside-down to dry for 30 seconds.
- c. Resuspend pellet with 30 mL lysis buffer (40 mM Tris, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 8.3) containing a Roche protease inhibitor cocktail tablet (EDTA-free).
 - i. PMSF has a half-life of ~1.5 hours at neutral pH. Add inhibitor tablet and PMSF immediately before use.
- d. Lyse resuspended cells on ice by sonication (30 amp power, one second pulse, two second rest, 5 minutes total).
- e. Pellet insoluble cell debris by centrifugation at 14,000 rpm (23,426 x g) with a SS-34 rotor for 30 minutes at 4 °C. Ensure tubes are balanced.

4.2.3.2. *Nickel purification and SUMO cleavage*

- a. Add nickel agarose slurry to a fritted column and drain. Add enough resin for 5 mL settled volume.
- b. Wash nickel agarose beads with 25 mL of equilibration buffer (50 mM HEPES pH 7.5).
 - i. In all following steps, do not let the resin run dry.
- c. Pour off supernatant from insoluble cell debris. Add supernatant to nickel resin and let incubate for 1 hour at 4 °C with mixing.
- d. Pour cell lysate and nickel resin mixture into the fritted column and allow to drain.
- e. Wash resin with 25 mL of equilibration buffer.
- f. Wash resin with 25 mL of wash buffer (50 mM HEPES, 5 mM imidazole, pH 7.5).
- g. Elute protein with 12 mL of elution buffer (50 mM HEPES, 300 mM imidazole, pH 7.5).
- h. Prepare 2.5 mL of 1 M 1,4-dithiothreitol (DTT) in Milli-Q water.
- i. Add to eluted protein: 24 µL of 1000x Ulp-1 stock (see [Section 4.2.5.3](#)) and DTT for a final concentration of 5 mM (for 12.5 mL eluted protein, 62.8 µL of 1 M DTT).
- j. Dialyze eluted protein, 5 mM DTT and Ulp-1 into 10 mM ammonium bicarbonate, 1 mM DTT (2 L) for 24 hours at 4 °C using 500-1000 Da MWCO dialysis tubing.
- k. Dialyze into 10 mM ammonium bicarbonate (2 L) overnight.
- l. Dialyze into 10 mM ammonium bicarbonate (2 L) for 3 hours.
- m. Add nickel agarose slurry to a fritted column and drain. Add enough resin for 4 mL settled volume.
- n. Wash nickel agarose beads with 25 mL dialysis buffer (10 mM ammonium bicarbonate).
- o. Add sample to nickel resin and let incubate for 1 hour at 4 °C with mixing.
- p. Collect the desired protein, GB1₁₃₋₅₆ K₁₃C in the flow-through. Wash with additional 5 mL of dialysis buffer.
- q. Elute Ulp-1, SUMO tag, and uncleaved complex with 10 mL elution buffer (50 mM HEPES, 300 mM imidazole, pH 7.5).
- r. Dialyze GB1₁₃₋₅₆ K₁₃C into 20 mM Tris pH 8.0 (2 L) overnight at 4 °C with 500-1000 Da MWCO dialysis tubing. Change dialysis twice (2 L for 3 hours each).
- s. Analyze expression and purification on a 14% Tris-tricine SDS-PAGE gel with Coomassie sensitive stain (**Fig. 3**).
 - i. The deletion fragment of GB1₁₃₋₅₆ K₁₃C will not stain with normal Coomassie.
 - ii. Although the flow-through appears pure by SDS-PAGE gel and MALDI, two species will elute at different % B on an anion-exchange column. Therefore, FPLC purification is required.

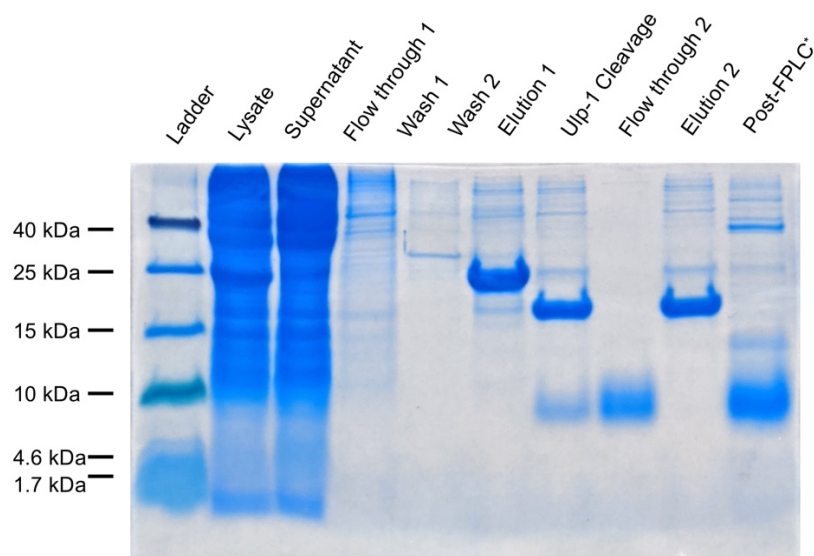


Fig. 3 Expression of GB1₁₃₋₅₆ K₁₃C and cleavage of SUMO tag visualized on a 14% Tris-tricine SDS-PAGE gel with Coomassie sensitive stain. *The aggregates observed in the Post-FPLC lane are an artifact of lyophilization. GB1 self-association has been previously reported (Li et al., 2011).

4.2.3.3. *FPLC purification via anion-exchange chromatography*

- Prepare, sterile filter, and degas FPLC buffer A (20 mM Tris pH 8.0) and buffer B (20 mM Tris, 1M NaCl, pH 8.0).
- Remove particulates from the sample with a 0.22 µm PES Millex-GP syringe filter.
- Add 20 µL 0.5 M TCEP Bond Breaker™ to reduce disulfide bonds. Let incubate for 15 minutes prior to run.
- Load half of the sample onto HiTrap Q HP column (5 mL). Using an ÄKTA FPLC, purify the GB1 construct using the gradient listed in **Table 1**. Confirm which fractions contain the desired product by MALDI-TOF MS ($[M+H]^+ = 4,795.10$).
- Combine fractions and dialyze into Milli-Q water (2 L) overnight at 4 °C with 500-1,000 Da MWCO dialysis tubing. Change dialysis twice (2 L for 3 hours each).
- Quantify the protein by UV-Vis ($\epsilon_{280} = 8,480 \text{ M}^{-1} \text{ cm}^{-1}$), make 0.5 or 1.0 µmol aliquots in pre-weighed tubes. Flash freeze in liquid N₂ and lyophilize.
- Weigh and record the mass of the dried aliquots.

4.2.4. **Expression of His₆-Ulp-1**

Expression and purification of His₆-Ulp-1 is based on the previously published procedure (Guerrero, Ciragan, & Iwai, 2015). Plasmid pFGET19_Ulp was generated by Hideo Iwai (Addgene plasmid #64697) (Guerrero, Ciragan, & Iwai, 2015).

- Using the same procedure as Section 4.2.2.1, transform the plasmid (pFGET19_Ulp1) into BL21-DE3 cells and grow on an LB plate supplemented with 50 µg/mL kanamycin.
- Set up primary cultures using the same procedure as Section 4.2.2.2, but with 50 µg/mL kanamycin.
- Inoculate 1 L LB with 50 µg/mL kanamycin as described in Section 4.2.2.3. Once an OD₆₀₀ = 0.6-0.8 is reached, keep culture at 37 °C and add 1 mM IPTG. Leave culture shaking at 37 °C for 5 hours.

4.2.5. **Purification of His₆-Ulp1**

- Harvest the cells by centrifugation as described in Section 4.2.3.1. Resuspend with 40 mL lysis buffer (40 mM Tris, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 8.3) containing a Roche protease inhibitor cocktail tablet (EDTA-free). Lyse and pellet as described.
- Set-up the nickel column as described in Section 4.2.3.2. Equilibrate nickel agarose beads with 25 mL of equilibration buffer (50 mM HEPES, 20 mM imidazole, pH 7.5). After incubation of the supernatant and nickel resin, wash with 30 mL equilibration buffer. Elute with 12 mL of elution buffer (50 mM HEPES, 300 mM imidazole, pH 7.5) and dialyze eluted protein into 20 mM Tris, 150 mM NaCl, pH 8.0 (2 L) overnight at 4 °C with 3.5 kDa MWCO dialysis tubing. Change dialysis twice (2 L for 3 hours each). Analyze expression and purification on a 14% Tris-tricine SDS-PAGE gel with Coomassie stain (**Fig. 4**).

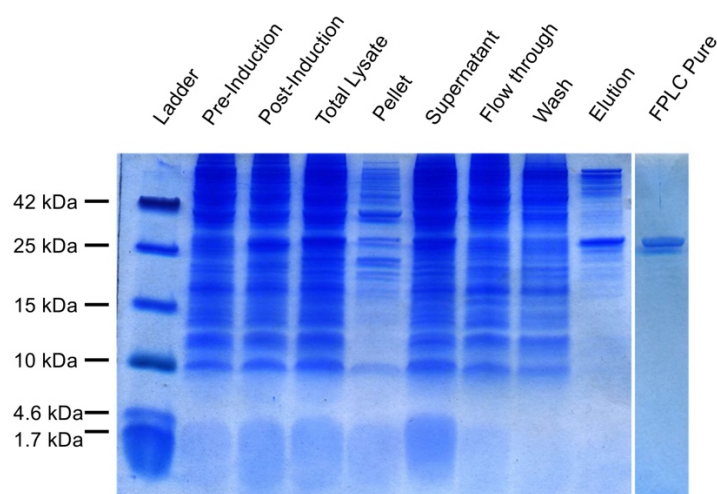


Fig. 4 Expression and purification of His₆-Ulp-1 visualized on a 14% Tris-Tricine SDS-PAGE gel with Coomassie stain.

- 4.2.5.3.** Purify according to Section 4.2.3.3, but with size-exclusion chromatography on a HiLoad Superdex 75pg 16/600 column with the isocratic gradient listed in **Table 1**. Confirm which fractions contain the desired product by SDS-PAGE. Combine fractions and concentration using an Amicon Ultra-4 10K MWCO Spin Filter to 1 mL. Mix 1 mL of protein solution with 20 μ L of 1 M DTT (10 mM final concentration) and 980 μ L 50% (v/v) glycerol. Make 50 μ L aliquots and store at -80°C.

4.3. Preparation of thioamide-containing peptide with C-terminal acyl hydrazide

4.3.1. Solid-Phase Peptide Synthesis (SPPS)

- Weigh out enough solid Cl-Trt resin for 100 μ mol scale synthesis based on resin loading (107.53 mg for 0.93 mmol/g loading). Add to fritted syringe outside of the hood. Rinse with dimethylformamide (DMF) and drain to bring resin to the bottom of the vessel. Add stir bar.
- Stir resin in 4 mL DMF for 30-40 minutes to swell.
- Weigh out 5 equivalences of solid Fmoc-NHNH₂ (MW = 254.28 g/mol, 127.14 mg) and dissolve in 4 mL DMF. Add 3 equivalences of *N,N*-Diisopropylethylamine (DIPEA) (52.2 μ L). Add to drained resin. Stir for 30 minutes.
- Wash resin with DMF and repeat coupling (step c).
- Drain and wash with DMF, methylene chloride (DCM), and DMF. Allow each wash to stir for 5 seconds before draining.
- Deprotect the Fmoc group with 4 mL 20% piperidine in DMF stirring for 10 minutes.
- Wash resin with DMF x 2, DCM, and DMF.
- Repeat deprotection with 4 mL 20% piperidine in DMF stirring for 10 minutes.
- Wash resin with (DMF, DCM) x 3, DMF.
- Continue as described in Chapter 2 for thioamide peptide synthesis.
- If you plan to leave the resin overnight, do not dry. Leave stirring slowly in DMF.
- After the last Fmoc group is deprotected, dry the resin. Slowly add cleavage solution (5 mL of 50% DCM (2.5 mL), 35% trifluoroacetic acid (TFA) (1.75 mL), 5% 1,2-ethanedithiol (EDT) (250 μ L), 5% thioanisole (250 μ L), 2.5% triisopropylsilane (TIPS) (125 μ L), 2.5% H₂O (125 μ L)). Leave rotating on a rotisserie for 1 hour at room temperature.
- Drain with N₂ into a scintillation vial and rinse with DCM.

- n. Remove cleavage solution by rotary evaporation. Rinse dried material with acetonitrile and remove solvent by rotary evaporation to remove excess TFA.
- o. Slowly add cold anhydrous diethyl ether to precipitate the peptide. Transfer material to 50 mL falcon tube. Use total of 30 mL diethyl ether. Store on dry ice for 5-10 minutes.
- p. Pellet peptide by spinning at 4,000 rpm (3,313 x g) for 5 minutes.
- q. Wash pellet with additional 30 mL of cold anhydrous diethyl ether (briefly sonicate to suspend pellet). Spin at 4,000 rpm (3,313 x g) for 5 minutes. Pour off ether layers (store ether layer at 4 °C until finish first-pass purification). Leave pellet open in hood overnight to allow residual ether to evaporate.

4.3.2. **RP-HPLC Purification**

- a. Dissolve peptide pellet in 5 mL H₂O + 0.1% TFA and 1 mL acetonitrile (ACN) + 0.1% TFA.
- b. Remove particulates from sample with 0.45 µm PVDF syringe filter.
- c. Purify with a Phenomenex Luna Omega PS C18 preparative column on an Agilent 1260 Infinity II Preparative HPLC using the gradient listed in **Table 1**. Identify which fractions contain the desired peptide with MALDI-TOF MS ($[M+H]^+ = 1,451.82$). Combine, flash freeze with liquid N₂ and lyophilize.
- d. If needed, perform second-pass purification.
- e. Re-dissolve the peptide in minimal 50% (v/v) ACN in Milli-Q water and quantify the peptide by UV-Vis ($\epsilon_{274} = 11,569 \text{ M}^{-1} \text{ cm}^{-1}$). Divide into 0.5 or 1 µmol aliquots in pre-weighed Eppendorf tubes. Flash freeze with liquid N₂ and speed-vac.
 - i. Lyophilization of the peptide dissolved in 50% (v/v) ACN in Milli-Q water will help to remove residual TFA.
- f. Weigh and record the mass of the dried aliquots.

4.4. **Native chemical ligation (NCL) of thioamide-containing N-terminal fragment and expressed C-terminal fragment**

4.4.1. **Activation with sodium nitrite (Liu method) (Zheng, Tang, Qi, Wang, & Liu, 2013)**

- a. Prepare and sterile filter activation buffer (6 M guanidine (Gdn) HCl, 0.2M NaH₂PO₄ pH 3.0) and NCL buffer (6 M Gdn HCl, 0.2 M phosphate, 0.2 M 4-mecaptophenylacetic acid (MPAA), pH 7.0).
 - i. For 10 mL of 0.2 M phosphate: 171 mg of sodium phosphate monobasic monohydrate (137.99 g/mol) and 108 mg of sodium phosphate dibasic (141.96 g/mol).
 - ii. The MPAA will not dissolve until base (5 M NaOH) is added.
- b. Before starting the NCL, purge buffers with argon (Ar) gas for 15 minutes.
 - i. Purge to avoid oxidation of thiols.
- c. Prepare 1 M sodium nitrite (NaNO₂) in sterile Milli-Q water.
 - i. Make this immediately before use.
- d. Prepare 2 mL of 0.5 M tris(2-carboxyethyl)phosphine (TCEP) HCl with neutral pH.
 - i. Dissolve TCEP salt with 1 and 5 M NaOH and check that pH is neutral with micro-pH meter.
 - ii. Purge with Ar to ensure that the solution does not become oxidized. Purge after each use.
 - iii. Solution is good to use for two days.
 - iv. Store solid TCEP HCl under Ar at 4 °C.
 - v. Do not use TCEP Bond BreakerTM, as adducts on the N-terminal cysteine fragment have been observed following use.
- e. Prepare a -15 °C NaCl/ice bath in a Dewar bowl.
- f. Dissolve the acyl hydrazide peptide (0.48 µmol) in activation buffer (96 µL) to 5 mM concentration.
- g. Let the peptide equilibrate to the -15 °C ice bath. Add stir bar and 10 equivalences of sodium nitrite from 1 M stock (4.8 µL). Leave peptide stirring in ice bath for 15 minutes. Monitor that the temperature stays at -15 °C.
- h. During the 15 minutes, dissolve the N-terminal cysteine protein (less than equimolar amount, 0.3 µmol) in enough NCL buffer to achieve a ratio of 16:1 MPAA/NaNO₂ (384 µL).

- i. Excess MPAA is necessary to deactivate remaining NaNO_2 in order to avoid nitroso formation on the N-terminal cysteine.
- i. Add the N-terminal cysteine protein (384 μL) to the peptide while still at -15°C . Remove from ice bath and check that the pH is ~ 7 . If needed adjust with 5 M NaOH. If the pH is close, avoid adjusting.
 - i. You cannot monitor the acyl hydrazide to acyl azide formation with MALDI-TOF MS. All attempts to get the acyl azide species to fly were unsuccessful. In our experience, the thioester forms immediately upon addition of the NCL buffer containing MPAA.
 - ii. Add the N-terminal cysteine protein while the peptide is still cold to avoid thermal degradation and Curtius rearrangement of acyl azide (Vinogradov, Simon, & Pentelute, 2016).
 - iii. Do not add TCEP as it will reduce the acyl azide to the amide.
 - iv. When adding NaOH, add to the side of the reaction vessel and spin down to add.
 - v. If the pH $> \sim 8$, the C-terminal thioester will hydrolyze. If you overshoot the pH, it cannot be saved by the addition of acid. Start again with fresh material.
 - vi. At pH ~ 7.5 , MPAA disulfide adducts are more likely to form on the N-terminal cysteine protein fragment.
 - vii. The final reaction volume will have ~ 1 mM of both fragments.
- j. Briefly purge the reaction with Ar and let the reaction stir at room temperature. Monitor by MALDI-TOF MS and analytical HPLC (**Fig. 5**). Periodically monitor pH.
 - i. For MALDI analysis, spot 1 μL of a 1:100 dilution of the NCL sample in $\text{H}_2\text{O} + 0.1\%$ TFA. Use α -cyano-4-hydroxycinnamic acid (CHCA) matrix.
 - ii. Using a 20-35% gradient over 40 minutes, perform analytical monitoring on a Phenomenex Luna C8(2) column with an Agilent 1260 Infinity II series Analytical HPLC system. Take 6 μL of the reaction and store on ice until the instrument is ready to run. Dilute the reaction with 174 μL $\text{H}_2\text{O} + 0.1\%$ TFA and add 20 μL 0.5 M TCEP neutral. Let the sample incubate for 10-15 minutes. Inject 100 μL (goal is to inject 3 nmoles of sample, i.e. 100 μL of 30 μM).
 - i. Using acidified water ensures that the samples are charged to assist with flying for MALDI-TOF MS and separation on the analytical. Note that this comes at a cost, since the transition to the acidic pH can go through the sample's PI, potentially resulting in it crashing out.
- k. After 5 hours, add enough 0.5 M TCEP neutral (50 μL) to have a final concentration of ~ 50 mM, check and adjust pH as needed.
 - i. TCEP will break the disulfides between MPAA molecules, as well as dimers of the N-terminal cysteine protein allowing for the NCL to proceed.
- t. After 24 hours, place the NCL on ice, dilute to 7 mL with sterile Milli-Q water and add 1 mL 0.5 M TCEP neutral. Dialyze into 20 mM Tris pH 8.0 (5 L) overnight at 4°C with a 12 mL 2 kDa MWCO Slide-A-Lyzer dialysis cassette. Change dialysis twice (5 L for 3 hours each).
 - i. The NCL is complete once there is no more C-terminal thioester peptide.
 - ii. Dialyze into buffer because the sample will crash out if dialyzed directly into Milli-Q water.
- l. Either flash freeze in liquid N_2 and lyophilize or add 100 μL 0.5 M TCEP neutral and dialyze into Milli-Q water (2 x 5 L for 3 hours each).
 - i. Only dialyze if able to purify the NCL that day, otherwise lyophilize.

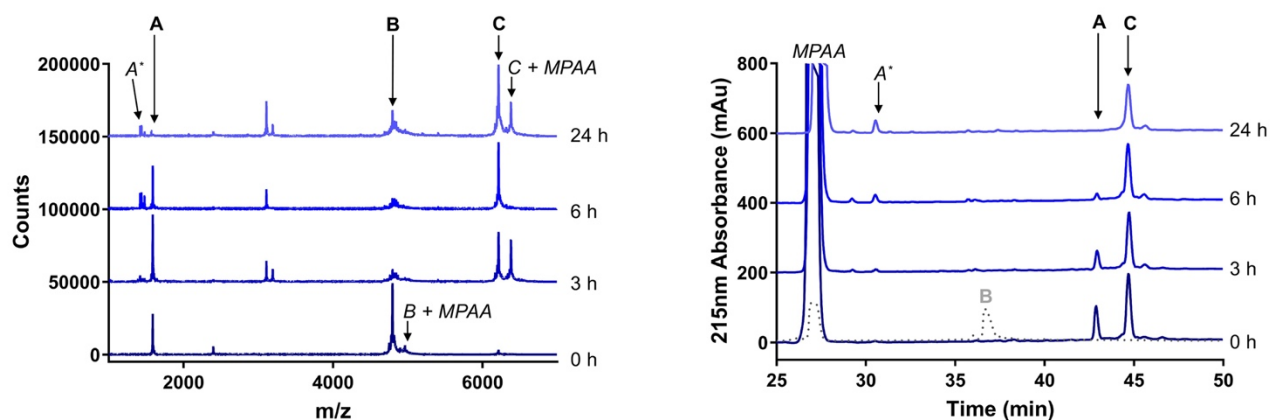


Fig. 5 MALDI and RP-HPLC analytical monitoring of the ligation between GB1₁₋₁₂ L^S₇- NHNH₂ (**A**) and GB1₁₃₋₅₆ K₁₃C (**B**). Although **B** is observed in the MALDI, it is consumed by the t = 0 hour analytical timepoint (MALDI is measured immediately, whereas the analytical RP-HPLC samples require incubation with TCEP before injection). A trace of purified **B** is displayed to show where it elutes.

4.4.2. RP-HPLC Purification

- After dialysis into Milli-Q water, store sample on ice. Add 100 μ L of 0.5 M TCEP neutral to 5 mL of sample and let incubate on ice for 15 minutes.
- Spin at 4,000 rpm (3,313 x g) for 5 minutes to pellet particulates.
- Inject 5 mL onto a Phenomenex Luna Omega PS C18 semi-preparative column. Purify on an Agilent 1260 Infinity II Preparative HPLC using the gradient listed in **Table 1**.
- Identify fractions with desired NCL product by MALDI-TOF MS ($[M+H]^+ = 6,214.84$). Combine, flash freeze with liquid N₂, and lyophilize.
- Re-dissolve in minimal 50% (v/v) ACN in Milli-Q water and quantify by UV-Vis ($\epsilon_{274} = 19,736 \text{ M}^{-1} \text{ cm}^{-1}$). Divide into pre-weighed aliquots, flash freeze with liquid N₂ and speed-vac.
 - Lyophilization of the NCL dissolved in 50% (v/v) ACN in Milli-Q water will help to remove residual TFA.
 - Isolated yields in the range of 20-30 %.

4.5. Desulfurization of ligation site cysteine to alanine

4.5.1. Desulfurization

- Prepare and sterile filter basic desulfurization buffer (6 M Gdn HCl, 0.2 M Na₂HPO₄, pH 7.0).
- Prepare the following solutions the day of desulfurization.
 - Add TCEP to basic desulfurization buffer. Dissolve 287 mg TCEP HCl in 1.5 mL basic desulfurization buffer. Adjust pH back to 7 with 5 M NaOH (buffer: ~2 mL of 6 M Gdn HCl, 0.2 M Na₂HPO₄, 0.5 M TCEP, pH 7.0). Purge with Ar for 15 minutes.
 - Add thioacetamide to desulfurization buffer with TCEP. Make 1 M thioacetamide stock by dissolving 37.5 mg thioacetamide in 250 μ L Milli-Q water and 250 μ L desulfurization buffer with TCEP. Add the entire 1 M thioacetamide stock to the desulfurization buffer with TCEP. Briefly purge final desulfurization buffer with Ar.
 - Final desulfurization buffer: 2.5 mL 6 M Gdn HCl, 0.2 M Na₂HPO₄, 0.5 M TCEP, 0.2 M thioacetamide, pH 7.0.
 - Prepare 0.5 M VA-044 (radical initiator) by dissolving 48 mg VA-044 in 300 μ L Ar purged Milli-Q water.
- Dissolve the purified NCL product (0.152 μ mol) in 1.22 mL of the final desulfurization buffer.
 - Want final NCL product concentration to be 0.1 mM (final reaction volume is 1.52 mL).

- d. Add 10% (v/v) *tert*-butylthiol (*t*-BuSH).
 - i. 10 % of final reaction volume (1.52 mL) is 152 μ L.
- e. Add 152 μ L 0.5 M VA-044 to achieve a final concentration of 50 mM VA-044.
- f. Store reaction under Ar and seal with parafilm.
- g. Incubate at 37 °C and monitor with MALDI-TOF MS (**Fig. 6**).
 - i. For MALDI analysis, spot 1 μ L of a 1:100 or 1:200 dilution of the sample in H₂O + 0.1% TFA. Use α -cyano-4-hydroxycinnamic acid (CHCA) matrix
 - ii. Avoid opening reaction. Store under Ar each time you open the reaction vessel.
- h. After 4-5 hours, purge reaction with Ar for 15 minutes to remove excess *t*-BuSH.
- i. Dilute reaction up to 10 mL with Milli-Q water. Spin down particulates at 4,000 rpm (3,313 x g) for 5 minutes.
- j. Store reaction on ice and purify immediately.
 - i. Reaction is not stable overnight. Plan to purify immediately following desulfurization.

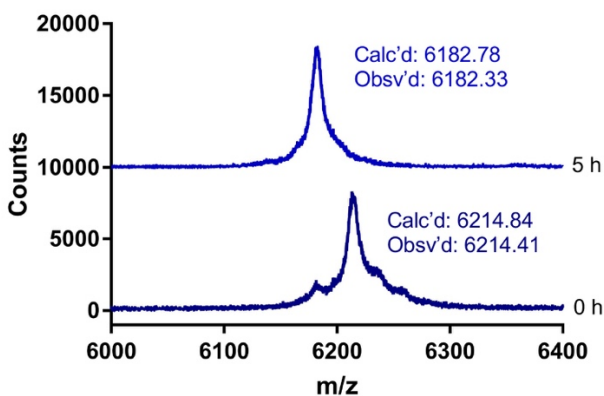


Fig. 6 MALDI monitoring of the desulfurization of GB1 L^S₇ K₁₃C to GB1 L^S₇ K₁₃A.

4.5.2. RP-HPLC Purification

- a. Inject 5 mL onto a Phenomenex Luna Omega PS C18 semi-preparative column. Purify on an Agilent 1260 Infinity II Preparative HPLC using the gradient listed in **Table 1**.
- b. Identify fractions with desired product by MALDI-TOF MS ($[M+H]^+ = 6,182.78$). Combine, flash freeze with liquid N₂, and lyophilize.
- c. Re-dissolve in minimal 50% (v/v) ACN in Milli-Q water and quantify by UV-Vis ($\epsilon_{274} = 19,736 \text{ M}^{-1} \text{ cm}^{-1}$). Divide into pre-weighed aliquots, flash freeze with liquid N₂ and speed-vac.
 - i. Lyophilization of the product dissolved in 50/50 ACN/ H₂O will help to remove residual TFA.
 - ii. Representative isolated yield of 61.8% (0.580 mg, 0.094 μ mol).

Table 1.

Construct	Calculated MW (Da)	Gradient	Elution %B
GB1 ₁₃₋₅₆ K ₁₃ C	4794.06	0-40% (30 CV)	26
His ₆ -Ulp1	27394.29	15% B (1.2 CV)*	70 min*
GB1 ₁₋₁₂ L ^S ₇ – NHNH ₂	1451.86	20-40% (20 min)	28.3
GB1 L ^S ₇ K ₁₃ C	6213.86	20-35% (30 min)	33.9

GB1 L ^S ₇ K ₁₃ A	6181.80	20-40% (40 min)	32.8
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*Elution time, rather than %B, listed for isocratic gradient.

5. C-terminal Ligation: Expressed N-terminal fragment and thioamide-containing C-terminal fragment

This section outlines the synthesis of a thioamide-containing peptide with a N-terminal ligation handle (GB1₃₉₋₅₆ V₃₉Pen T^S₄₉) and the recombinant expression of a deletion fragment with a C-terminal thioester (GB1₁₋₃₈-MESNa). Purification of the fragments, ligation and desulfurization of the ligation site penicillamine to valine are described.

5.1. Reagents and equipment

Refer to section 4.1 for the supplies and instruments used for the preparation of constructs. Additional reagents include the following.

PCR reagents

Phusion High Fidelity polymerase (New England Biolabs)

- Although this PCR was originally performed with a Phusion polymerase, it will also work with the Q5 polymerase.

T4 DNA ligase (New England Biolabs)

T4 polynucleotide kinase (New England Biolabs)

DNA Clean & Concentrator Kit, Plasmid Miniprep Kit (Zymo Research)

dNTPs (Invitrogen)

DH5α competent cells (New England BioLabs)

Protein expression reagents

Pierce Silver Staining kit (Thermo Fisher)

Sodium 2-mercaptoethanesulfonate (MESNa) (TCI)

SPPS reagents

Fmoc-L-Pen(Trt)-OH (ChemImpex #03915)

5.2. Preparation of GB1 constructs with C-terminal thioester

5.2.1. Preparation of plasmid for expression

5.2.1.1. Cloning of GB1₁₋₃₈-MxeGyrA-His₆

Deletion of the last 17 residues of GB1 to allow for expression of the N-terminal fragment is achieved with deletion PCR.

- Plasmid: pTXB1 GB1-MxeGyrA-His₆
 - Forward primer: 5' – TGCATCACGGGAGATGCA – 3'
 - Reverse primer: 5' – CCCATTATCATTGGCATACTGC – 3'
- Dissolve primers in DNase/RNase free water to a final concentration of 50 μM (dissolved primers can be stored at -20 °C). Do not vortex to dissolve.
 - Dilute plasmid to ≤ 1 ng/μL with DNase/RNase free water.
 - To generate the PCR master mix, combine in the following order: 35.5 μL DNase/RNase free water, 1.0 μL dNTPs, and 10.0 μL 5x HF buffer. Store on ice.
 - Combine in the following order: 46.5 μL PCR master mix, 1.0 μL forward primer, 1.0 μL reverse primer, 1.0 μL plasmid, and 0.5 μL Phusion polymerase.
 - Mix well by pipetting.
 - Use a thermocycler to perform the deletion PCR with the following protocol.

Thermocycler Protocol

Step	Temperature	Duration
1	98 °C	00:30 min (30 sec)
2	98 °C	00:10 min
3	66 °C	00:30 min
4	72 °C	03:00 min
5	Go to step 2	29 times
6	72 °C	10:00 min
7	4 °C	∞

- f. Clean up PCR with DNA Clean & Concentrator kit according to manufacturer's protocol (elute with 10.0 μ L).
- g. Add the following to 2.0 μ L PCR product: 5.0 μ L DNase/RNase free water, 1.0 μ L 10x T4 DNA ligase buffer, 0.5 μ L T4 DNA ligase, 0.5 μ L Dpn1 and 1.0 μ L T4 polynucleotide kinase (PNK). Incubate for 1 hour at 37 °C to circularize the DNA.
- h. Transform into DH5 α competent cells by adding entire circularization mixture to 70 μ L cells. Incubate on ice for 30 minutes. Heat shock at 42 °C for 45 seconds and leave on ice for 2 minutes. Add 930 μ L SOC media and incubate at 37 °C for 1 hour, shaking at 250 rpm. Plate 200 μ L on an LB plate supplemented with 100 μ g/mL ampicillin. Store upside down in an incubator overnight at 37 °C.

5.2.2. Expression of GB1₁₋₃₈-MES

- 5.2.2.1. Using the same procedure as Section 4.2.2.1 transform the plasmid (pTXB1 GB1₁₋₃₈-MxeGyrA-His₆) into BL21-DE3 cells and grow on an LB plate supplemented with 100 μ g/mL ampicillin.
- 5.2.2.2. Follow the protocol in Section 4.2.2.2 for primary culture set-up.
- 5.2.2.3. Follow the protocol in Section 4.2.2.3 for inoculation and induction of secondary culture.

5.2.3. Purification of GB1₁₋₃₈-MES

- 5.2.3.1. Follow the protocol in Section 4.2.3.1 for pelleting and lysing the cells.
- 5.2.3.2. Follow the protocol in Section 4.2.3.2 for preparation and running of the nickel column with the following modifications.
 - a. Collect the elution on ice and add solid 2-mercaptoethanesulfonic acid sodium salt (MESNa) for final concentration of 0.5 M MESNa (for 13 mL elution, 1.067 g MESNa). Vortex to dissolve. Let rotate for 60 hours at 4 °C.
 - b. Dialyze into 20 mM Tris pH 8.0 (2 L) for 3 hours at 4 °C with 500-1000 kDa MWCO dialysis tubing. Change dialysis twice (2 L) and let final dialysis proceed overnight.
 - c. Add nickel agarose slurry to a fritted column and drain. Add enough resin for 4 mL settled volume. Wash nickel agarose beads with 25 mL dialysis buffer (20 mM Tris pH 8.0).
 - d. Add sample to nickel resin and let incubate for 1 hour at 4 °C with mixing.
 - e. Collect the desired protein, GB1₁₋₃₈-MES in the flow-through. Wash with additional 5 mL of dialysis buffer. Keep collected sample on ice.
 - f. Elute intein and uncleaved complex with 10 mL elution buffer (50 mM HEPES, 300 mM imidazole, pH 7.5).
 - g. Spin down particulates at 4,000 rpm (3,313 x g) for 5 minutes and prepare to purify immediately.
 - i. The C-terminal MES thioester will hydrolyze if not purified quickly.
 - h. Analyze expression and purification with a 14% Tris-Tricine SDS-PAGE gel. Visualize with silver staining (Fig. 7).

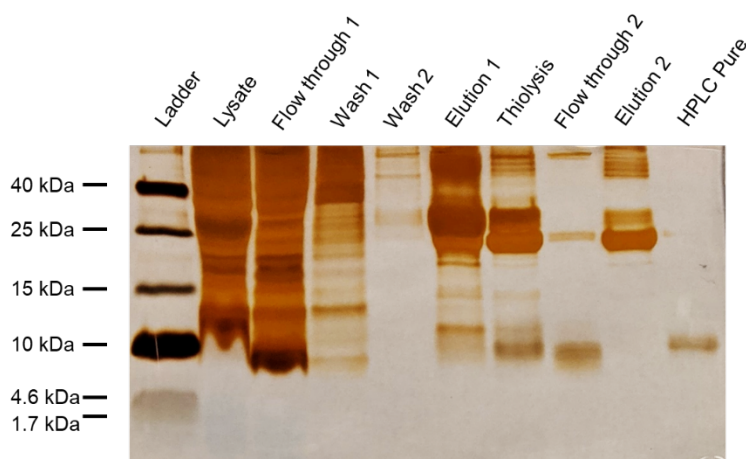


Fig. 7 Expression of GB1₁₋₃₈-MES and intein thiolysis visualized on a 14% Tris-tricine SDS-PAGE gel with silver staining.

- 5.2.3.3.** Purify on a Phenomenex Jupiter C4 semi-preparative column with the gradient listed in **Table 2**. Identify which fractions contain the desired peptide with MALDI-TOF MS ($[M+H]^+ = 4,287.69$). Combine, flash freeze with liquid N₂ and lyophilize. Re-dissolve in minimal 50% (v/v) ACN in Milli-Q water, divide into pre-weighed Eppendorf tubes, flash freeze with liquid N₂ and speed-vac. Weigh and record the mass of the dried aliquots
- Since the peptide does not have enough aromatic residues for UV quantification, quantify and aliquot based on mass.

5.3. Preparation of thioamide-containing peptide with N-terminal penicillamine

5.3.1. SPPS

- Weigh out enough solid Cl-Trt resin for 50 μ mol scale synthesis based on resin loading (53.76 mg of resin with a loading capacity of 0.93 mmol/g). Add to fritted syringe outside of the hood. Rinse with dimethylformamide (DMF) and drain to bring resin to the bottom of the vessel. Add stir bar.
- Stir resin in 4 mL DMF for 30-40 minutes to swell.
- Follow thioamide-peptide synthesis as described in [Chapter 2](#), but with the following modifications:
- For coupling of Fmoc-Pen(Trt)-OH (MW: 613.80 g/mol), dissolve 153.45 mg (5 equivalent) amino acid, 85.34 mg (4.5 equivalent) HBTU, and 87.03 μ L (10 equivalent) DIPEA in 2 mL of 50% (v/v) DMF/anhydrous DCM.
 - Let the reaction run for 1 hour at room temperature.
 - After the reaction is complete, drain the solution then rinse with DCM/DMF/DCM.
 - Perform a second 1 hour coupling to ensure efficiency.
 - Drain the solution and rinse with DCM/DMF/DCM
- Upon completion of the synthesis, rinse the resin with extensive DMF and DCM, then dry under vacuum prior to cleavage. Cleave the peptide from the resin by treatment with a fresh cleavage cocktail (2 mL of 90% TFA (1.8 mL), 5% EDT (100 μ L), 2.5% TIPS (50 μ L), and 2.5% Milli-Q H₂O (50 μ L)) for 45 minutes with stirring.
- After treatment, expel the cocktail solution from the reaction vessel with N₂ into a scintillation vial and reduce to a minimal volume by rotary evaporation.
- Add 10 mL cold ethyl ether to the peptide vial to precipitate out the peptide and sonicate if needed.
- Transfer the resulting solution into a tube for centrifugation at 4,000 rpm (3,313 x g) for 10 minutes and decant the ether layer.

- i. Flash freeze the peptide with liquid N₂ and lyophilize.

5.3.2. Purify on a Phenomenex Luna Omega PS C18 preparative column following Section 4.3.2 with the gradient listed in **Table 2**. Identify fractions with desired product by MALDI-TOF MS ($[M+H]^+ = 2,125.87$). Combine, flash freeze with liquid N₂, and lyophilize. If needed, perform second-pass purification. Quantify by UV-Vis ($\epsilon_{274} = 16,931 \text{ M}^{-1} \text{ cm}^{-1}$).

5.4. Native chemical ligation of expressed N-terminal fragment and thioamide-containing C-terminal fragment

5.4.1. NCL

- a. Prepare 10.0 mL of NCL buffer and 2.0 mL of 0.5 M TCEP neutral as described in Section 4.4.1.
- b. Dissolve the C-terminal thioester protein (0.5 μmol) in NCL buffer to 2.0 mM concentration (250 μL).
 - i. Once dissolved in the NCL buffer, the C-terminal alkyl thioester (MESNa) will exchange with the MPAA in the buffer, resulting in a MPAA thioester.
- c. Add C-terminal thioester protein to equimolar N-terminal penicillamine peptide (0.5 μmol). Check that the pH is ~ 7 , if needed adjust with 5 M NaOH. If the pH is close, avoid adjusting.
 - i. When adding NaOH, add to side of reaction vessel and spin down to add.
 - ii. If the pH $> \sim 8$, the C-terminal thioester will hydrolyze. If you overshoot the pH, it cannot be saved by the addition of acid. Start again with fresh material.
- d. Briefly purge reaction with Ar and let the reaction stir at room temperature. Monitor by MALDI-TOF MS and analytical HPLC (**Fig. 8**) as described in Section 4.4.1, but with a 25-35% gradient over 30 minutes. Periodically monitor pH.
- e. After 6 hours, add enough 0.5 M TCEP neutral to have a final concentration of 50 mM (28.0 μL), check and adjust pH as needed.
- f. After 24 hours, place the NCL on ice, dilute to 2.5 mL with sterile Milli-Q water and add 250 μL 0.5 M TCEP neutral. Dialyze into 20 mM Tris pH 8.0 (5 L) overnight at 4°C with a 3 mL 2 kDa MWCO Slide-A-Lyzer dialysis cassette. Change dialysis twice (5 L for 3 hours each).
 - i. The NCL is complete once there is no more C-terminal thioester protein.
 - ii. Dialyze into buffer because the sample will crash out if dialyzed directly into Milli-Q water.
- g. Either flash freeze in liquid N₂ and lyophilize or dialyze into Milli-Q water (2 x 5 L for 3 hours each).
- h. Only dialyze if able to purify the NCL that day, otherwise lyophilize.

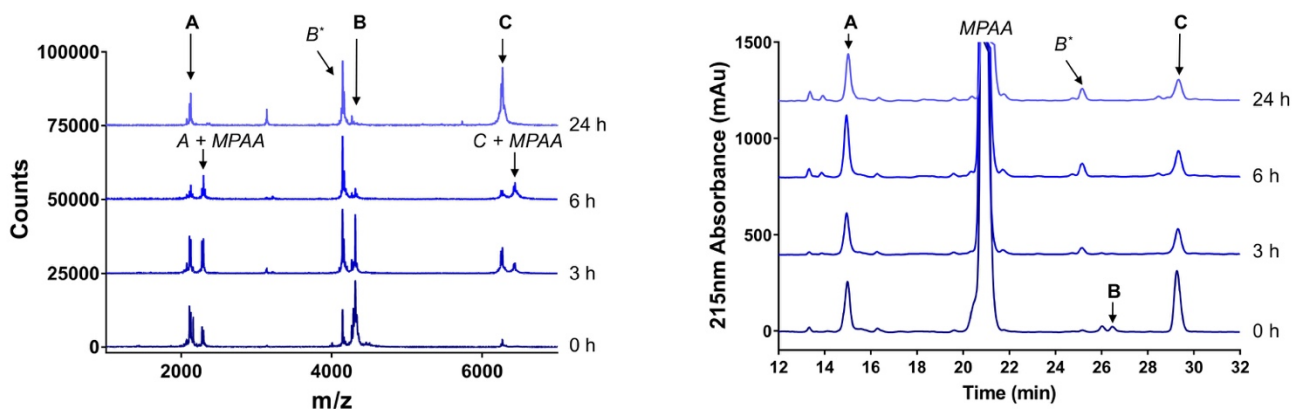


Fig. 8 MALDI and RP-HPLC analytical monitoring of the ligation with GB1₃₈₋₅₆ V₃₉Pen T^S₄₉ (A) and GB1₁₋₃₈ - MES (B).

5.4.2. For purification of the NCL product, follow the procedure in Section 4.4.2. Add 100 μL of 0.5 M TCEP neutral and dilute up to 5 mL with Milli-Q water. Purify on a Phenomenex Luna Omega PS C18 semi-preparative column using the gradient listed in **Table 2**. Identify fractions with desired product by MALDI-TOF MS ($[M+H]^+ = 6,271.84$). Quantify by UV-Vis ($\epsilon_{274} = 19,736 \text{ M}^{-1} \text{ cm}^{-1}$). Representative isolated yield of 28.7 % (0.9 mg, 0.143 μmol).

5.5. Desulfurization of penicillamine to native valine

5.5.1. Following the procedure in Section 4.5.1, for desulfurization of the penicillamine. For 0.143 μmol purified NCL, dissolve in 1.148 mL, add 143.5 μL *t*-BuSH, and 143.5 μL 0.5 M VA-044 (final volume is 1.435 mL). The reaction is complete after ~20 hours of incubation at 37 °C (**Fig. 9**). Dilute up to 5 mL with Milli-Q water and purify immediately.

- Reaction is not stable overnight. Plan to purify immediately following desulfurization.

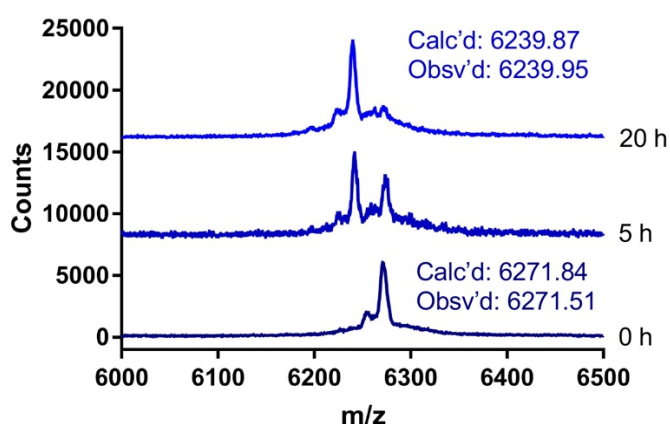


Fig. 9 MALDI monitoring of the desulfurization of GB1 V₃₉Pen T^S₄₉ to GB1 T^S₄₉.

5.5.2. For purification of GB1 T^S₃₉, follow the procedure in Section 4.5.2 using the gradient listed in **Table 2**. Identify fractions with desired product by MALDI-TOF MS ($[M+H]^+ = 6,239.87$). Quantify by UV-Vis ($\epsilon_{274} = 19,736 \text{ M}^{-1} \text{ cm}^{-1}$). Representative isolated yield of 89.3 % (0.8 mg, 0.128 μmol).

Table 2.

Construct	Calculated MW (Da)	Gradient	% B
GB1 ₁₋₃₈ -MES	4286.86	20-40% (30 min)	30.2
GB1 ₃₉₋₅₆ V ₃₉ Pen T ^S ₄₉	2126.29	20-40% (21 min)	31.1
GB1 V ₃₉ Pen T ^S ₄₉	6270.96	20-40% (40 min)	32.9
GB1 T ^S ₄₉	6238.90	20-40% (40 min)	34.8

6. Applications of thioamide proteins

Synthesized proteins may be used in a wide variety of applications, as we and others have described in detail elsewhere (Walters, 2017, Petersson, Goldberg, & Wissner, 2014, Barrett, Fiore, Liu, & James Petersson, 2019, Mahanta, Szantai-Kis, Petersson, & Mitchell, 2019). Here, we illustrate three of the most common types of applications: studying the role of thioamides in natural proteins, probing backbone interactions in protein folding, and monitoring protein folding or protein/protein interactions. In all cases, preparation of a wild-type (WT) control

protein that is identical except for the O-to-S substitution is essential to demonstrating that the effect derives exclusively from the thioamide.

A better understanding is desired of the role the thioamide serves in natural proteins. For MCR, it has been suggested that the thioamide is involved in electron transfer within the protein system, possibly by coupling conformational with reduction, and the mystery of its role persists in spite of structural and mechanistic studies with and without the thioamide (Goenrich, Duin, Mahlert, & Thauer, 2005, Nayak et al., 2020, Thauer, 2019). The size and complexity of MCR prohibit chemical synthesis of authentic MCR to probe this process, but smaller proteins can serve as model systems to understand thioamide electron transfer. For example, GB1 has a single C-terminal Trp and this residue's intrinsic fluorescence is quenched by PeT with the thioamide (**Fig. 10**). Calibration of Trp/thioamide PeT using data from a polyproline “ruler” experiment can help to elucidate the impact of the electron transfer pathway on rates and efficiencies (Goldberg, Wissner, Klein, & Petersson, 2012). GB1 presents a model system from which to study electron transfer by varying the position of the thioamide as well as the identity of the redox partner through unnatural amino acid mutagenesis and/or post-translational attachment of various chromophores (Goldberg, Batjargal, Chen, & Petersson, 2013, Wissner, Batjargal, Fadzen, & Petersson, 2013).

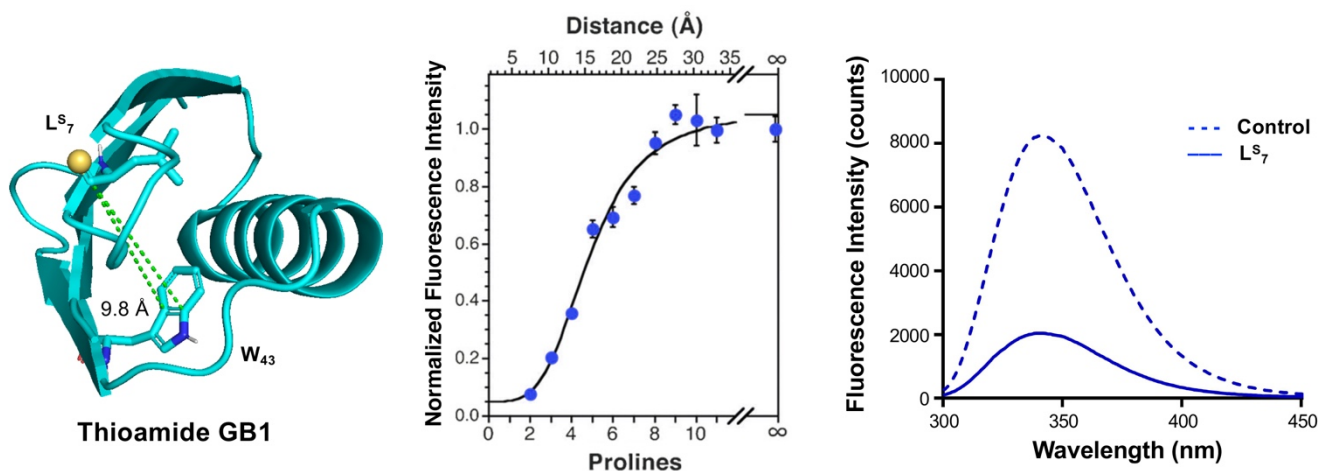


Fig. 10 Thioamide PeT in a model protein. From Left: In GB1 (PDB: 2QMT), Leu₇ and Trp₄₃ are located 9 Å apart (Frericks Schmidt et al., 2007). The distance dependence of thioamide PeT-based quenching of Trp was measured using a spectroscopic “ruler” of Pro repeats (Goldberg, Wissner, Klein, & Petersson, 2012). The level of quenching by L^{S7} in GB1 can be measured by comparing the fluorescence of concentration-matched samples of GB1-K₁₃A (Control) and GB1-L^{S7}K₁₃A (L^{S7}). Middle figure adapted with permission from Goldberg *et al.* *Chem. Commun.* 2012 (Goldberg, Wissner, Klein, & Petersson, 2012). Copyright (2012) Royal Society of Chemistry.

Studying the effects of thioamide substitution on protein folding is informative both on the role of backbone interactions in general and on the impact of thioamides in natural or engineered proteins. Although multiple efforts (outlined in Section 2.1) have been made to understand the structural impact of the thioamide in biomacromolecules, the models for their effects on protein folding is still crude. The protocols outlined in this chapter can be used to synthesize multi-mg quantities of material for structural biology studies, specifically X-ray crystallography and NMR. GB1 represents a model β -sheet protein, and our lab has also investigated a model α -helical protein, calmodulin (CaM) (**Fig. 11**). For ¹⁵N or ¹³C NMR, the expressed portion of the protein can be labeled by standard protocols for incorporation of isotopes through cell growth with labeled metabolites (Marley, Lu, & Bracken, 2001). Labeling the SPPS fragment is possible through the use of commercially available isotopically labeled Fmoc amino acid building blocks, but the cost of these experiments may be prohibitive. Otherwise, the expressed portion of the protein can be isotopically labelled, and global conclusions can be made as to the effect of the thioamide on the

overall structure. For example, several variants of CaM were synthesized by C-terminal ligation at a Gln₁₃₅-to-Cys mutant. Unfolding studies using circular dichroism showed that the V^S₁₃₆ variant was substantially stabilized, so it was selected for further study by NMR. Segmentally-labeled CaM was prepared by NCL using an expressed, ¹⁵N labeled C-terminal fragment and a thioamide-containing N-terminal portion prepared by SPPS.

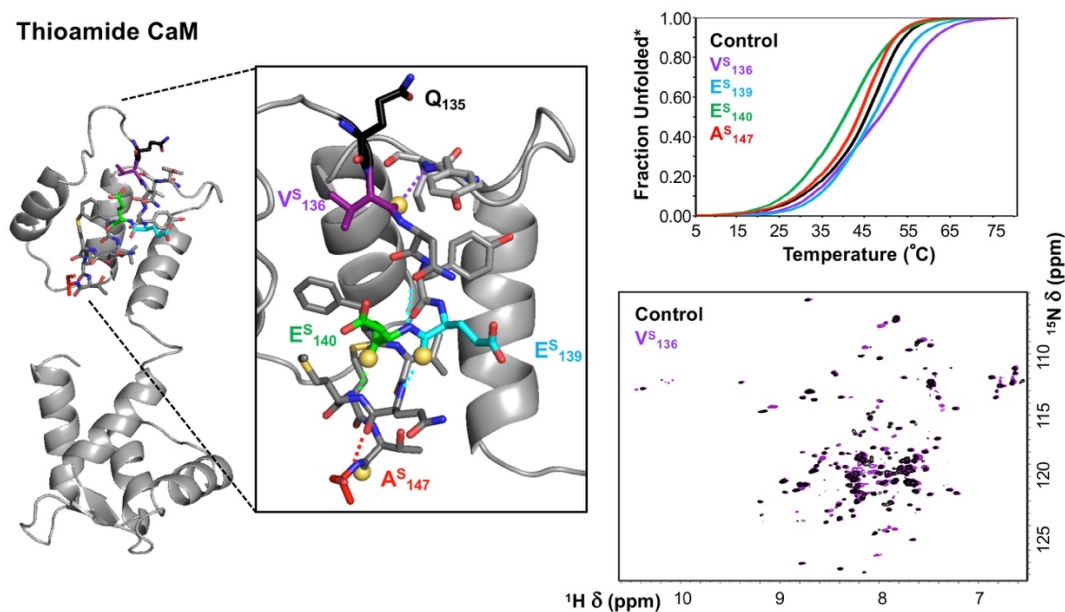


Fig. 11 Thioamide incorporation into calmodulin (CaM). Left: Sites of thioamide incorporation shown in a structure of apo CaM (PDB: 1CFD) (Kuboniwa et al., 1995). Top Right: The thermal stability of the thioamide-containing proteins varied depending upon position (Left-to-right: E^S₁₄₀, A^S₁₄₇, Control, E^S₁₃₉, V^S₁₃₆. Bottom Right: ¹H-¹⁵N HSQC of CaM-V^S₁₃₆ in comparison to the all-amide control suggests no global conformational changes upon thioamide incorporation.

In non-perturbing locations, the thioamide can be used as a FRET or PeT partner to monitor protein misfolding. Previously our lab utilized the ability of the thioamide to quench Trp fluorescence in order to track the misfolding of a protein (α -synuclein, α S) that has been associated with Parkinson's Disease (Batjargal et al., 2012). Since thioamide PeT-based quenching of Trp is distance-dependent (up to 30 Å) (Goldberg, Wissner, Klein, & Petersson, 2012) (**Fig. 10**), comparison of Trp fluorescence against the all-amide control allowed for monitoring of intramolecular misfolding events (**Fig. 12**). The protein was synthesized by ligation at a Ser₉-to-Cys mutant, and then mixed 1:10 with WT α S for aggregation so that fluorescence changes would arise only from intramolecular conformational changes upon formation of oligomers or fibrils. Subsequent studies have used unnatural amino acid mutagenesis to incorporate other fluorescent donors to pair with the thioamide (Wissner, Wagner, Warner, & Petersson, 2013, Wissner, Batjargal, Fadzen, & Petersson, 2013).

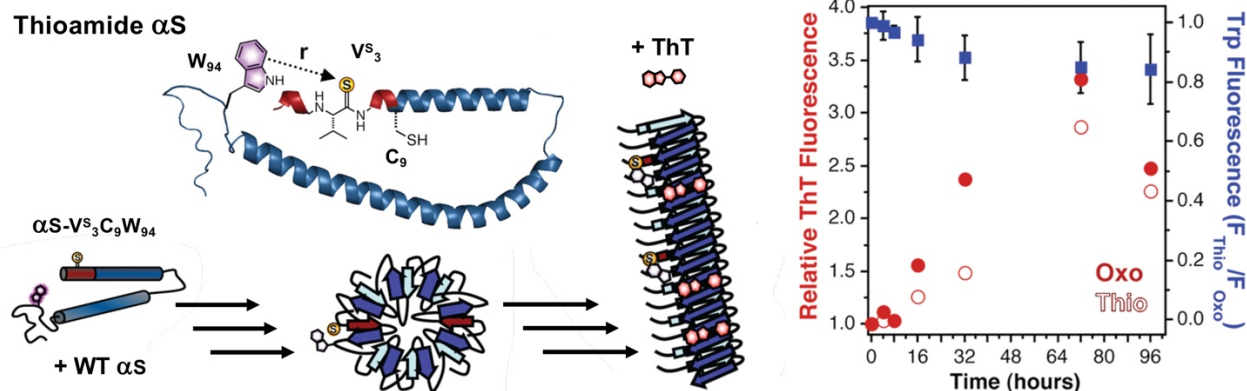


Fig. 12 Studying α -synuclein (α S) misfolding with a thioamide probe pair. Incorporation of a terminal thioamide acceptor (V^S_3) and Trp donor (W_{94}) into α S was accomplished by expressing the C-terminal portion and ligating a thioamide-labeled N-terminal portion. Tracking the change in Trp fluorescence (circles) relative to an all-amide (Oxo) control experiment and comparing to the overall rate of fibrillization (ThT fluorescence, squares), demonstrated that the termini were closer together in the fibrillar and oligomeric states than as a monomer. Adapted with permission from Batjargal *et al.* *J. Am. Chem. Soc.* 2012 (Batjargal, Wang, Goldberg, Wissner, & Petersson, 2012). Copyright (2012) American Chemical Society.

7. Conclusion

In this chapter, we have outlined how to generate a full-length protein with an unnatural thioamide at the N- or C-termini. The thioamide is incorporated by SPPS, and the full-length protein is generated by NCL. The thioamide-containing protein can be used to interrogate important backbone hydrogen bonds (if positioned as an acceptor), or to generate a more stable secondary structure (if positioned as a hydrogen bond donor). As previously demonstrated, the thioamide can quench fluorescence in a PeT or FRET based mechanism depending upon the fluorophore pair (PeT: 5-15 Å, FRET: 8-30 Å). Since the thioamide is a single-atom substitution, this can provide atomistic understanding of protein dynamics or a protein-substrate interaction.

Although this protocol outlines the semi-synthesis of a protein with an N- or C-terminal thioamide, the techniques are transferable to incorporation into the middle of a larger protein. For example, accessing a central thioamide is possible by recombinant expression of the N- and C-terminal fragments and SPPS of the thioamide-containing central fragment (**Fig. 13**). The N-terminal fragment can be generated with a thioester using an intein fusion (as described for GB1 in section 4). The C-terminal fragment can be generated with a Cys (using a SUMO tag (as described for GB1 in section 5)). The central, thioamide-containing fragment can be prepared with a thioester and a protected Cys. Ligation at the C-terminus followed by deprotection permits the second ligation to generate the full-length protein, which can be desulfurized to give the final product. Acyl hydrazide strategies can also be used to ligate segments in the N-to-C direction with activation of the acyl hydrazide after the first ligation. Care must be taken when deciding the order of ligations since the middle fragment with both ligation handles can ligate with itself unless protected. Using these strategies, and protein that is synthetically accessible can conceivably be generated as a thioamide variant.

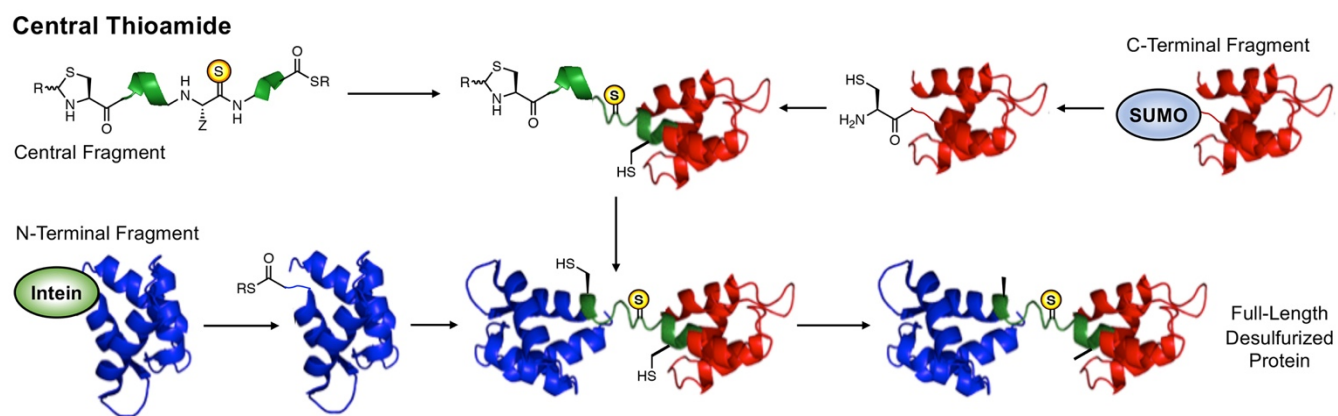


Fig. 13 Scheme for a three-part ligation to incorporate a thioamide into the center of a protein.

Acknowledgement

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