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Synthesis of thioester peptides for the incorporation of thioamides into proteins by native chemical ligation‡

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Abstract

Thioamides can be used as photoswitches, as reporters of local environment, as inhibitors of enzymes, and as fluorescence quenchers. We have recently demonstrated the incorporation of thioamides into polypeptides and proteins using native chemical ligation (NCL). In this protocol, we describe procedures for the synthesis of a thioamide precursor and an NCL-ready thioamide-containing peptide using Dawson's *N*-acyl-benzimidazolinone (Nbz) process. We include a description of the synthesis by NCL of a thioamide-labeled fragment of the neuronal protein α-synuclein.

Keywords

Thioamide; Native Chemical Ligation; Thioester; Fmoc SPPS

Scope and Comments

The thioamide is a nearly isosteric substitution of the carbonyl oxygen in the peptide bond with a sulfur atom [1]. Conservative backbone modification with a thioamide has been used to analyze the importance of specific hydrogen bonds to the folding of secondary structure motifs and to coupled protein folding and binding processes [2,3]. Because of its enhanced proteolytic stability and modulated activity, thioamide substitution has also been applied in several biologically active oligopeptides [4-6]. For example, Fischer and coworkers showed that a thioamide-containing peptide substrate resisted hydrolysis by prolyl oligopeptidase [5]. The red-shifted absorption band of the thioamide peptide bond can be used in several different ways to study structural changes in the backbone with high spatial and temporal control. The π-to-π^* transition at 270 nm and the *n*-to-π^* transition at 340 nm give rise to thioamide-specific CD signatures that can be identified against a background of oxoamide signal at shorter wavelengths [7,8]. Wildemann *et al.* assembled ribonuclease S (RNAse S) through noncovalent association of a proteolytic fragment of RNAse S and a synthetic

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Supporting Information

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thiopeptide and turned its enzymatic activity on and off through *cis/trans* photoisomerization of the thioamide [9]. Our laboratory expanded the utility of thioamides by demonstrating that a thioamide can act as a quencher of various fluorophores such as endogenous Tyr or Trp, and the unnatural amino acids *p*-cyanophenylalanine, 7-methoxycoumarin-4-ylalanine, and acridon-2-ylalanine [10-12]. Quenching can occur through either Förster resonance energy transfer or photo-induced electron transfer mechanisms, depending on the identity of the chromophore. The placement of thioamides is expected to be tolerated in almost any position in a protein because of its small size, although the sulfur substitution may prove disruptive to hydrogen bonding in some cases [7,13-15]. Despite these attractive applications, the incorporation of thioamides has traditionally been limited to peptides made through solid phase peptide synthesis (SPPS).

Recently, we have shown that a thioamide can be incorporated into a full-length protein by exploiting the native chemical ligation (NCL) reaction [16,17]. NCL requires two fragments: one bearing an *N*-terminal Cys and the other bearing a *C*-terminal thioester [18]. The synthesis of the *N*-terminal Cys fragment is straightforward, but the development of an efficient strategy for synthesizing a thioamide-containing *C*-terminal thioester using Fmoc-based SPPS has required considerable study. Boc-based SPPS, commonly used in the NCL community, is not viable as the acidic cleavage conditions degrade the thioamide peptide. We have previously published syntheses using solution-phase thioesterification with PyBOP activation as well as on-resin thioesterification using an Aimoto-type Cys-Pro-Gly linker where the Cys is protected as a disulfide and Gly is replaced by glycolic acid (C^bPG_o) (Figure 1) [16,19]. However, the former method could lead to epimerization at the α-carbon of the *C*-terminal residue and often suffers from poor solubility of the protected peptides [20]. Although the latter method alleviates the epimerization problem, it requires one to synthesize a dipeptide with the *C*-terminal residue of the thioester fragment coupled to Cys, which tends to couple to the Pro on the resin in a very low yield. In unpublished work, we have used an α-hydroxycysteine (Cya) strategy developed by Muir and Botti to generate thiopeptide thioesters (Figure 1) [21,22]. While the latent thioester of Cya did ligate rapidly, the synthesis of Cya is non-trivial (11% yield over five steps) and may be a barrier for some users. In contrast to these three methods, Dawson's *N*-acyl-benzimidazolinone (Nbz) strategy could be used to generate thioamide-containing thioester peptides in relatively high yields and involves a simple synthesis using commercially available components [23]. After elongation of the peptide chain on 3,4-diaminobenzoyl (Dbz) resin, the introduction of 4-nitrophenylchloroformate closes the ring to form the *N*-acylurea moiety, Nbz.

In this protocol, we describe our exploration of the compatibility of thioamides with the acylating conditions and report an optimized procedure for the synthesis of Nbz-thiopeptides. In addition, we describe the synthesis of the Fmoc-based SPPS thioamide precursor, thiocarboxybenzotriazole **4a**, using 4-nitro-1,2-phenylendiamine and P_4S_{10} (Scheme 1) [24]. The use of 4-nitro-1,2-phenylenediamine presents several advantages over *N*-Boc-phenylenediamine, which was used in our previous reports: (i) 4-nitro-1,2-phenylenediamine is generally more affordable than *N*-Boc-phenylenediamine, (ii) the removal of the Boc protecting group with TFA is omitted, simplifying the procedure slightly, and (iii) the nitro route is compatible with amino acids bearing acid-labile

protecting groups such Glu or Asp. There are some advantages to the Boc route; Boc-protection suppresses the formation of a benzimidazole (BzIm) side product during the thionation step (conversion of **2b** to **3b**), and the lack of the nitro group in the benzotriazole products (e.g. **4b**) makes them more stable. Nonetheless, the nitro route (i.e. **2a–4a**) is generally preferable. The most common thionation reagents are Lawesson's reagent (LR) and P_4S_{10} [25,26]. Although LR is reported to have higher thionation yields, this was not observed for certain amino acids in our hands. For Ala, thionation with LR was higher yielding (89% vs 10%). However, the yield for the rest of the amino acids tested was limited to 10–35%, and recycling of the unreacted oxoanilide (**2a**) was necessary to obtain a reasonable thionation yield. In contrast, thionation with P_4S_{10} proved to be more efficient and higher yielding, particularly for β-branched amino acids such as valine.

Here, we describe the preferred synthetic strategy for the thioamide precursor, an efficient method to obtain thiopeptide thioesters using Dawson's resin, and the use of a thiopeptide thioester in an NCL reaction to synthesize a fragment of the Parkinson's disease protein α-synuclein, Ac-$\alpha S_{1\text{-}19}$ V'_3 (the prime symbol denotes a backbone thiocarbonyl at the indicated residue) [27,28]. Recently, Lee and coworkers have characterized the structure and binding affinity of this fragment to the calcium signaling protein calmodulin (CaM) [29]. Our laboratory is currently exploring the αS/CaM interaction using thioamide fluorescence quenching.

Experimental Procedure

Optimized Thiovaline Precursor Synthesis

The synthesis of thiovaline precursor **4a** is presented as a general method. We chose to compare methods using Fmoc-Val-OH (**1**) because the thionation yield of this amino acid was particularly low using previously published methods. As a first step, the carboxyl group of Fmoc-Val-OH was amidated with 4-nitro-1, 2-phenylenediamine, a latent benzotriazole. To a solution of Fmoc-Val-OH in THF stirred in an NaCl/ice bath at −10 °C, NMM and isobutylchloroformate were added to generate Fmoc-Val-anhydride *in situ*, and then, 4-nitro-1,2-phenylenediamine was added. After stirring overnight at room temperature (r.t.), the reaction solvent THF was removed by rotary evaporation. The crude mixture was dissolved in DMF, and a saturated KCl solution was added, resulting in the precipitation of a yellow solid. This precipitation removes the NMM salts effectively, although organic side products precipitate too. Because of the poor solubility of compound **2a** in DCM, complete purification via silica chromatography was difficult. Thus, compound **2a** was isolated in 90% yield with 73% purity after KCl precipitation.

Thionation of Fmoc-Val-nitroanilide **2a** with LR resulted in either low conversion at r.t. or formation of significant amounts of a benzimidazole side product at 70 °C. On the other hand, thionation with P_4S_{10} was high yielding with minimal side product formation. To a solution of P_4S_{10} and anhydrous Na_2CO_3 in THF, compound **2a** was added and stirred at r.t under slow argon flow. The reaction was monitored by TLC to track the consumption of **2a**. Upon completion, the reaction solution was filtered through Celite, washed with 5% $NaHCO_3$, and purified on a silica column to afford pure compound **3a** as yellow solid in

86% yield. Purification of P_4S_{10} by Soxhlet extraction is often recommended. We find this to be unnecessary and obtain high yields with commercial P_4S_{10} stored in a dessicator at r.t. and used directly.

To form the benzotriazole **4a**, $NaNO_2$ was added to compound **3a** in glacial acetic acid diluted with 5% water. Nitrous acid generated *in situ* reacts with the primary amine of compound **3a**, forming a benzotriazole through intramolecular diazonium cyclization. This reaction completed in 30 min, and compound **4a** precipitated upon the addition of cold water to the reaction solution. Purification via silica chromatography afforded very pure **4a** as an orange solid in a 90% yield. However, Ala and other amino acids with protected side chains such as Asp degraded in the process of chromatographic purification. Thus, we recommend minimal handling of the benzotriazoles to prevent hydrolysis and degradation through cyclization. As long as the aminothioacylanilide (e.g. **3a**) is pure going into the cyclization reaction, precipitation provides sufficiently pure material for peptide coupling. After precipitation followed by filtration, the nitrobenzotriazole **4a** was dried in the presence of P_2O_5 under vacuum at r.t. overnight and was used for peptide synthesis. The detailed procedures and 1H and 13C NMR spectra for compound 2a, 3a, and 4a are reported in the Supporting Information.

Thiopeptide-Nbz Synthesis

The thiopeptide Ac-MDV′FMKGL-Nbz (**7**) was synthesized on commercially available Dawson Dbz AM resin (Novabiochem®, San Diego, CA, USA). After removal of the Fmoc protecting group, the first amino acid was loaded by HATU/DIPEA activation. The peptide was elongated by standard SPPS procedures with HBTU/DIPEA activation. Thiovaline was introduced by adding the preactivated derivative Fmoc-thioval-nitrobenzotriazole (**4a**) with DIPEA but without HBTU. The last amino acid was loaded as Ac-Met to avoid undesired acetylation on the Dbz group when an acetylating reagent such as Ac_2O is used. After assembly of peptide **5**, the resin was treated with *p*-nitrophenyl chloroformate to form the peptidyl carbamate **6**, which was then converted to peptide–Nbz resin by a subsequent treatment of DIPEA. The Nbz peptide **7** was cleaved from resin by TFA treatment and purified by preparative HPLC. The detailed procedures for peptide synthesis are provided in the Supporting Information.

Native Chemical Ligation

To a solution of 6M guanidinium hydrochloride (Gdn•HCl) and 200 mM sodium phosphate, triscarboxyethyl phosphine and thiophenol were added such that their final concentrations were 20 mM and 1% (v/v), respectively. It is crucial to adjust the pH of the solution to 7.0 and to degas it with Ar before use in ligation. Ac-$\alpha S_{1\text{-}8}V'_3$-Nbz (**7**, 0.35 μmol) and $\alpha S_{9\text{-}19}C_9$ (**8**, 0.51 μmol) were dissolved in NCL buffer, and the reaction was initiated upon combining the two peptides. After purging with Ar briefly, the reaction solution was placed in an incubator and shaken at 1000 rpm at 37 °C overnight. An aliquot (25 μl) was taken out periodically, quenched by the addition of 975 μl of 0.1% TFA in water, and stored at −20 °C until analysis by analytical reversed phase HPLC. Conversion to the product, Ac-$\alpha S_{1\text{-}19}$ V'_3 (**9**), was greater than 95% by peak area (relative to unreacted **7** and hydrolyzed **7**) after 30 min (Figure 2). The thiopeptide reactant and products are sufficiently stable that reactions

can be allowed to stand overnight. The product was isolated in 15% yield as determined by UV/Vis spectroscopy.

It is worthwhile to note that the same procedures can be used to obtain a full-length protein labeled with a thioamide by expressing a protein fragment (e.g. $\alpha S_{9\text{-}140}C_9$) with an *N*-terminal Cys, revealed by treatment with Factor Xa protease or methionine aminopeptidase [17]. With either N-terminal proteolysis method, the presence of the thioamide in the product should be verified by MALDI MS, for full-length proteins trypsin-digestion and MALDI MS is recommended.

Limitations

Native chemical ligation enables the incorporation of thioamides into peptides and proteins but also leaves Cys at the ligation site, which may be undesirable. The common strategies for the removal of Cys must be evaluated for compatibility with thioamides. Radical desulfurization of Cys to form Ala can compete with desulfurization of thioamides [30,31]. Alternatively, selective masking of Cys with alkylating reagents is possible, but the alkylation reaction must be monitored closely to avoid thioamide alkylation [32].

To circumvent the branching of Dbz resin, Mahto *et al.* employed allyloxycarbonate (Alloc) as a protecting group on the second amino group [33]. The deprotection of the Alloc group requires the use of catalytic Pd^0, which can desulfurize thioamides (unpublished results) [34]. Thus, thioamide reactivity must be considered in the use of alternative protecting groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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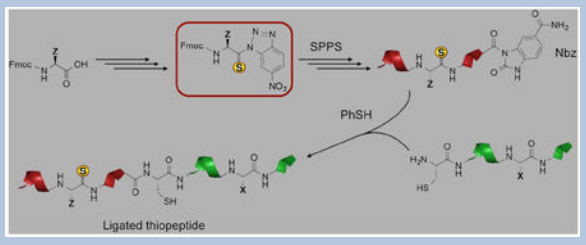
References

1. Hoeg-jensen T. Review: endothiopeptides alias peptide thioamides. Phosphorus Sulfur Silicon Relat Elem. 1996; 108:257–278.
2. Culik RM, Jo H, DeGrado WF, Gai F. Using thioamides to site-specifically interrogate the dynamics of hydrogen bond formation in β-sheet folding. J Am Chem Soc. 2012; 134:8026–8029. [PubMed: 22540162]
3. Bachmann A, Wildemann D, Praetorius F, Fischer G, Kiefhaber T. Mapping backbone and side-chain interactions in the transition state of a coupled protein folding and binding reaction. Proc Natl Acad Sci U S A. 2011; 108:3952–3957. [PubMed: 21325613]
4. Kessler H, Matter H, Geyer A, Diehl H-J, Köck M, Kurz G, Opperdoes FR, Callens M, Wierenga RK. Selective inhibition of trypanosomal triosephosphate isomerase by a thiopeptide. Angew Chem Int Ed. 1992; 31:328–330.
5. Schutkowski M, Jakob M, Landgraf G, Born I, Neubert K, Fischer G. Probing substrate backbone function in prolyl oligopeptidase catalysis. Eur J Biochem. 1997; 245:381–385. [PubMed: 9151967]

6. Zhang W, Li J, Liu L-W, Wang K-R, Song J-J, Yan J-X, Li Z-Y, Zhang B-Z, Wang R. A novel analog of antimicrobial peptide polybia-MPI, with thioamide bond substitution, exhibits increased therapeutic efficacy against cancer and diminished toxicity in mice. Peptides. 2010; 31:1832–1838. [PubMed: 20600424]
7. Miwa JH, Pallivathucal L, Gowda S, Lee KE. Conformational stability of helical peptides containing a thioamide linkage. Org Lett. 2002; 4:4655–4657. [PubMed: 12489953]
8. Hollósi M, Kollát E, Kajtár J, Kajtár M, Fasman GD. Chiroptical labeling of folded polypeptide conformations: the thioamide probe. Biopolymers. 1990; 30:1061–1072.
9. Wildemann D, Schiene-Fischer C, Aumuller T, Bachmann A, Kiefhaber T, Lucke C, Fischer G. A nearly isosteric photosensitive amide-backbone substitution allows enzyme activity switching in ribonuclease S. J Am Chem Soc. 2007; 129:4910–4918. [PubMed: 17397159]
10. Goldberg JM, Batjargal S, Petersson EJ. Thioamides as fluorescence quenching probes: minimalist chromophores to monitor protein dynamics. J Am Chem Soc. 2010; 132:14718–14720. [PubMed: 20886849]
11. Goldberg JM, Speight LC, Fegley MW, Petersson EJ. Minimalist probes for studying protein dynamics: thioamide quenching of selectively excitable fluorescent amino acids. J Am Chem Soc. 2012; 134:6088–6091. [PubMed: 22471784]
12. Goldberg JM, Wissner RF, Klein AM, Petersson EJ. Thioamide quenching of intrinsic protein fluorescence. Chem Commun. 2012; 48:1550–1552.
13. Miwa JH, Patel AK, Vivatrat N, Popek SM, Meyer AM. Compatibility of the thioamide functional group with beta-sheet secondary structure: incorporation of a thioamide linkage into a beta-hairpin peptide. Org Lett. 2001; 3:3373–3375. [PubMed: 11594837]
14. Reiner A, Wildemann D, Fischer G, Kiefhaber T. Effect of thioxopeptide bonds on alpha-helix structure and stability. J Am Chem Soc. 2008; 130:8079–8084. [PubMed: 18512914]
15. Choudhary A, Raines RT. An evaluation of peptide-bond isosteres. ChemBioChem. 2012; 12:1801–1807. [PubMed: 21751326]
16. Batjargal S, Wang YJ, Goldberg JM, Wissner RF, Petersson EJ. Native chemical ligation of thioamide-containing peptides: development and application to the synthesis of labeled alpha-synuclein for misfolding studies. J Am Chem Soc. 2012; 134:9172–9182. [PubMed: 22468862]
17. Wissner RF, Batjargal S, Fadzen CM, Petersson EJ. Labeling proteins with fluorophore/thioamide Förster resonant energy transfer pairs by combining unnatural amino acid mutagenesis and native chemical ligation. J Am Chem Soc. 2013; 135:6529–6540. [PubMed: 23594264]
18. Dawson PE, Muir TW, Clarklewis I, Kent SBH. Synthesis of proteins by native chemical ligation. Science. 1994; 266:776–779. [PubMed: 7973629]
19. Kawakami T, Aimoto S. The use of a cysteinyl prolyl ester (CPE) autoactivating unit in peptide ligation reactions. Tetrahedron. 2009; 65:3871–3877.
20. Nagalingam AC, Radford SE, Warriner SL. Avoidance of epimerization in the synthesis of peptide thioesters using Fmoc protection. Synlett. 2007:2517–2520.
21. Botti P, Villain M, Manganiello S, Gaertner H. Native chemical ligation through *in situ* O to S acyl shift. Org Lett. 2004; 6:4861–4864. [PubMed: 15606085]
22. George EA, Novick RP, Muir TW. Cyclic peptide inhibitors of staphylococcal virulence prepared by Fmoc-based thiolactone peptide synthesis. J Am Chem Soc. 2008; 130:4914–4924. [PubMed: 18335939]
23. Blanco-Canosa JB, Dawson PE. An efficient Fmoc-SPPS approach for the generation of thioester peptide precursors for use in native chemical ligation. Angew Chem Int Ed. 2008; 47:6851–6855.
24. Shalaby MA, Grote CW, Rapoport H. Thiopeptide synthesis. Alpha-amino thionoacid derivatives of nitrobenzotriazole as thioacylating agents. J Org Chem. 1996; 61:9045–9048. [PubMed: 11667896]
25. Cava MP, Levinson MI. Thionation reactions of Lawesson reagents. Tetrahedron. 1985; 41:5061–5087.
26. Scheeren JW, Ooms PHJ, Nivard RJF. General procedure for conversion of a carbonyl group into a thione group with tetraphosporous decasulfide. Synthesis. 1973:149–151.
27. Lashuel HA, Overk CR, Oueslati A, Masliah E. The many faces of alpha-synuclein: from structure and toxicity to therapeutic target. Nat Rev Neurosci. 2013; 14:38–48. [PubMed: 23254192]

28. Spillantini MG, Schmidt ML, Lee VMY, Trojanowski JQ, Jakes R, Goedert M. [alpha]-Synuclein in Lewy bodies. Nature. 1997; 388:839–840. [PubMed: 9278044]
29. Gruschus JM, Yap TL, Pistolesi S, Maltsev AS, Lee JC. NMR structure of calmodulin complexed to an N-terminally acetylated alpha-synuclein peptide. Biochemistry. 2013; 52:3436–3445. [PubMed: 23607618]
30. Yan LZ, Dawson PE. Synthesis of peptides and proteins without cysteine residues by native chemical ligation combined with desulfurization. J Am Chem Soc. 2001; 123:526–533. [PubMed: 11456564]
31. Hendrickson TL, Imperiali B. Metal-ion dependence of oligosaccharyl transferase – implications for catalysis. Biochemistry. 1995; 34:9444–9450. [PubMed: 7626614]
32. Hopkins CE, Hernandez G, Lee JP, Tolan DR. Aminoethylation in model peptides reveals conditions for maximizing thiol specificity. Arch Biochem Biophys. 2005; 443:1–10. [PubMed: 16229814]
33. Mahto SK, Howard CJ, Shimko JC, Ottesen JJ. A reversible protection strategy to improve Fmoc-SPPS of peptide thioesters by the N-acylurea approach. ChemBioChem. 2011; 12:2488–2494. [PubMed: 21910203]
34. Grieco P, Gitu PM, Hruby VJ. Preparation of ‘side-chain-to-side-chain’ cyclic peptides by allyl and Alloc strategy: potential for library synthesis. J Peptide Res. 2001; 57:250–256. [PubMed: 11298927]

REACTION SCHEME



GENERAL OPTIMIZED PROCEDURE

Thioamide precursors can be synthesized by a general procedure as follows (using thiovaline as an example): Fmoc-Val-OH was coupled to 4-nitro-1,2-phenylenediamine to form an aminoacyl anilide, which was treated with P_4S_{10} to thionate the carbonyl. $NaNO_2$ treatment was used to form the benzotriazole for peptide coupling. The thiopeptide was then synthesized on 3,4-diaminobenzoyl (Dbz) resin, which was treated with *p*-nitrophenyl chloroformate to form a C-terminal *N*-acyl-benzimidazolinone (Nbz), activating the thiopeptide for native chemical ligation (NCL). NCL reactions were carried out under standard conditions in denaturing buffer (6 M guanidinium hydrochloride).

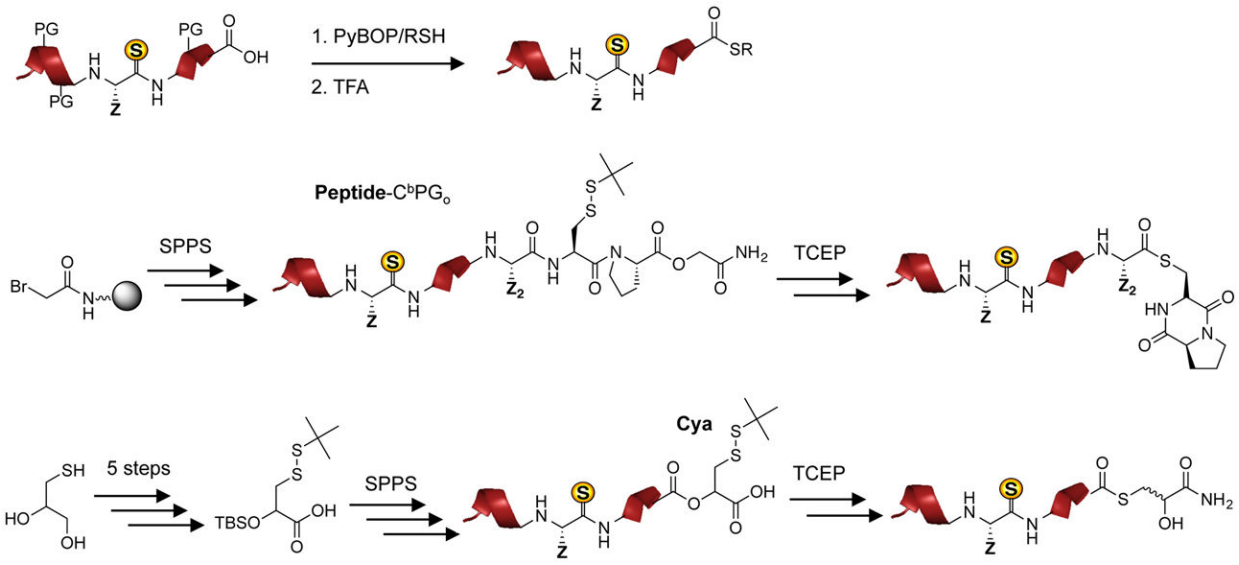


Figure 1.

Other Fmoc-based thioesterification methods tested. Top: off-resin activation of protected peptides. Middle: on-resin synthesis of protected Cys-Pro-glycolic acid (C^bPG_o) linker, with off-resin activation by reduction of *t*-Bu disulfide. Bottom: on-resin incorporation of *α*-hydroxycysteine (Cya), with off-resin activation by reduction of *t*-Bu disulfide.

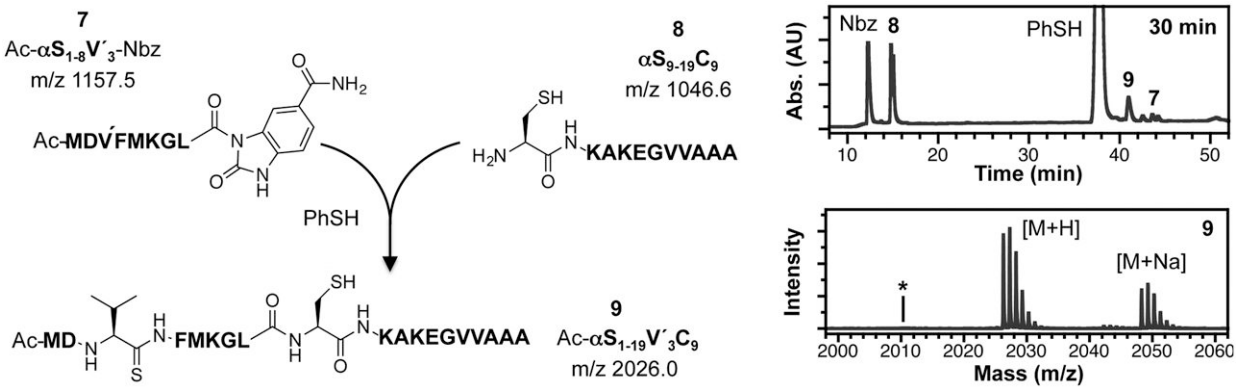
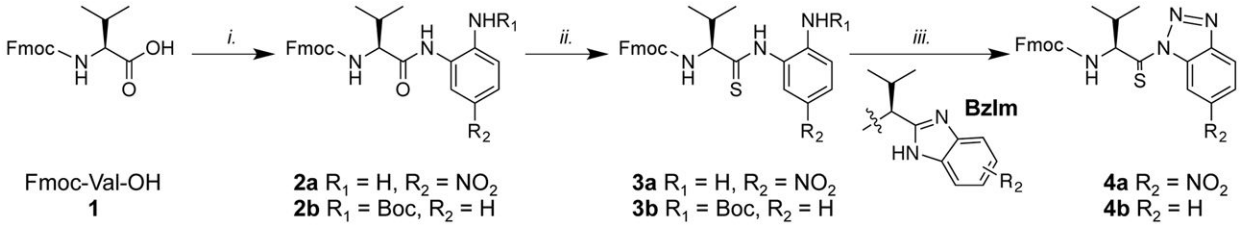


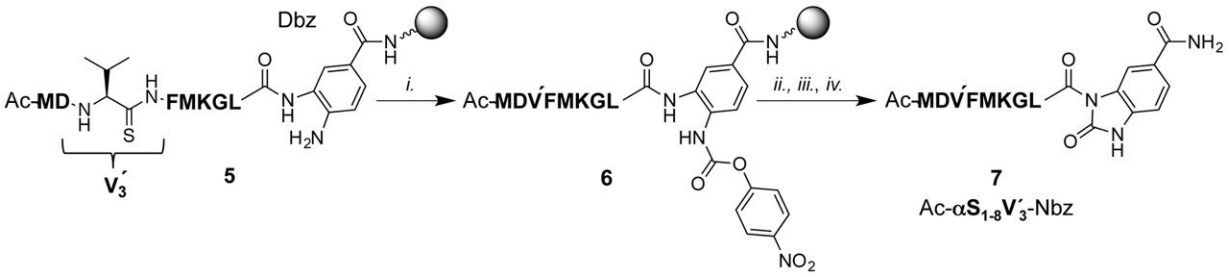
Figure 2.

Native chemical ligation to form Ac-αS$_{1\text{-}19}$ V′$_3$ (**9**). Left: reaction scheme. Right: reaction monitored by HPLC (monitored at 215 nm) and MALDI MS of product (**9**) peak (*m/z* Calcd: 2026.0, Found: 2026.3). Asterisk indicates the expected mass of Ac-αS$_{1\text{-}19}$ (oxoamide at position 3).



Scheme 1.

Synthesis of Fmoc-thiovaline-benzotriazole derivatives **4a**. Reagents and conditions: (i) NMM, isobutylchloroformate, 4-nitro-1,2-phenylenediamine, THF, overnight, r.t. (90%), (ii), P_4S_{10}, Na_2CO_3, THF, r.t. (86%), and (iii) $NaNO_2$, AcOH, H_2O, r.t. (90%). Compounds **2b**, **3b**, and **4b** are shown for discussion purposes.



Scheme 2.

Synthesis of Ac-$\alpha S_{1\text{-}18}$ V'_3-Nbz (**7**). Reagents and conditions: (i) SPPS on Dbz AM resin, r.t., (ii) *p*-nitrophenyl chloroformate, r.t., (iii) DIPEA/DMF, r.t., and (iv) TFA/TIPS/thioanisole/DCM (80 : 5 : 2.5 : 12.5), r.t.