Thioimidate Solutions to Thioamide Problems during Peptide Synthesis

Jacob Byerly-Duke, Aaron Donovan, Krishna K. Sharma, Rida Ibrahim, and Brett VanVeller*

Department of Chemistry, Iowa State University, Ames, IA 50011, USA

E-mail: bvv@iastate.edu

Abstract

Thioamides have structural and chemical similarity to peptide bonds, and therefore offer valuable insights when probing peptide backbone interactions, including hydrogen bonding, stereoelectronic, and hydrophobicity effects. There is a perception that methods to install thioamides within peptides are sufficient, yet anecdotal reports indicate that many labs have sought to employ thioamides in a variety of studies but the results of many synthetic campaigns do not yield the intended products, leading researchers to abandon such projects and any information these structural probes would provide. We catalogue and provide evidence for the major pitfalls associated with current methods to synthesize thioamide-containing peptides during each stage of solid-phase peptide synthesis (SPPS), including (A) thioamide coupling, (B) peptide elongation, and (C) peptide cleavage from resin. We then demonstrate the utility of thioimidate protecting groups as a means to side-step each of these problematic synthetic difficulties. Our approach is generally applicable to all peptides and ultimately permits access to an important benchmark α-helical peptide that had previously eluded synthesis and isolation. With the process of thionopeptide synthesis demystified, a broader range of

researchers should find it easier to employ thioamides in the study of peptide-based biomolecules.

Introduction

The prevalence of post-translational modification in natural protein synthesis suggests that expanding beyond the suite of functional groups found in the 20 canonical amino acids can impart specialized utility to peptide-based biomolecules. In particular, recently identified biosynthetic pathways that install thioamides in place of traditional oxoamides along the main-chain of peptides suggest an evolutionary benefit to the specific properties of thioamides.[1–8]

Thioamides have become intriguing probes of hydrogen-bonding,[9–12] hydrophobicity,[13,14] and stereoelectronic effects[15–20] along the peptide backbone. Thioamides can also serve as quenchers of fluorescent dyes in experiments designed to study peptide conformation and proteolysis.[21–29] Additionally, they can serve as reactive sites for further chemistry in peptide-based substrates.[30–35] Such examples create a perception that current methods to incorporate thioamides into peptides are sufficient. However, anecdotal reports indicate that many labs have sought to employ thioamides in a variety of peptide studies, but the results of many synthetic campaigns do not yield the intended peptide products, leading researchers to ultimately abandon such projects and any information these structural probes would provide regarding structure and function.

The stability of thioamides during the process of solid-phase peptide synthesis (SPPS) and subsequent cleavage from the solid-support is both sequence and position dependent.[36] A literature survey of previously synthesized thionopeptides illuminates the limitations and concerns described above (Figure 1). Examining the two residues (n and n+1) between which thioamides have been inserted reveals that only a few residues have been extensively investigated (Figure 1A). For example, valine frequently brackets the thioamide linkage,

regardless of whether the natural sequence contains valine at the specified position or not.[36] This observation suggests that the large, β-branched side chain of valine may confer beneficial effects to minimize troublesome side reactivity associated with thionopeptides during SPPS. Alternatively, the majority of reported thionopeptide sequences incorporate the thioamide close to the *N*-terminus (Figure 1B), near the end of the synthetic procedure where exposure to SPPS reagents is minimized.

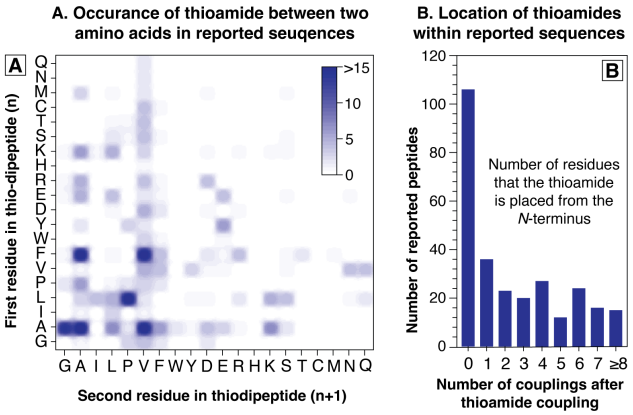
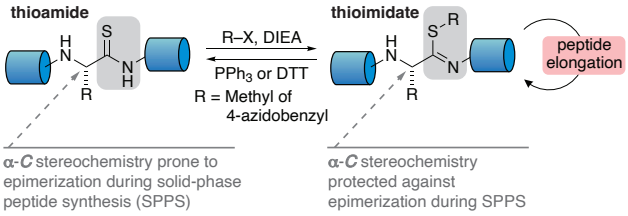


Figure 1: Data extracted from published thionopeptide sequences (as of Sep 2023): (A) A heat map showing the frequency of occurrence of a thioamide between two amino acids within the sequence. (B) Location of the thioamide along the backbone indicating most thioamides are placed very close to the *N*-terminus, near the end of the peptide synthesis campaign. (Data, sequence analysis, and references can be found in the SI).

Collectively, the literature surveys (Figure 1) indicated that thioamides are not a 'plug-and-play' peptide modification. This limitation restricts the sequence space for which thioamides can be used to probe structure and function. Thus, new and/or general synthetic strategies to arrive at thionopeptides are necessary to make thioamide modifications more accessible to the wider community of peptide scientists who may not be experts in the idiosyncrasies of thionopeptides.

Previously, we developed a strategy to circumvent the instability of thioamides during the Fmoc deprotection stage of SPPS (Scheme 1), enabling wider implementation of thioamides in more diverse sequence space.[37,38] The weaker C=S bond means that the α-CH of the residue bearing the thioamide is more acidic relative to the traditional oxoamide

(due to the lower energy $\pi^*_{C=S}$ orbital).[37] This increased acidity leads to deprotonation of the chiral α-CH position and subsequent loss of stereochemical integrity under the basic conditions of peptide elongation. The problem is particularly acute during treatment with the stronger piperidine base necessary to remove the Fmoc protecting group from the *N*-terminus in preparation for coupling of the next amino acid in the sequence.[37–40] Previous strategies to address these issues centered around lower concentrations of base and abbreviated deprotection times.[39,40] The drawback of such approaches is the risk of incomplete Fmoc deprotection, leading to amino acid deletions upon subsequent coupling cycles. For these reasons, we developed an alternative strategy that sought to directly address the acidity of the α-position.[37,38]

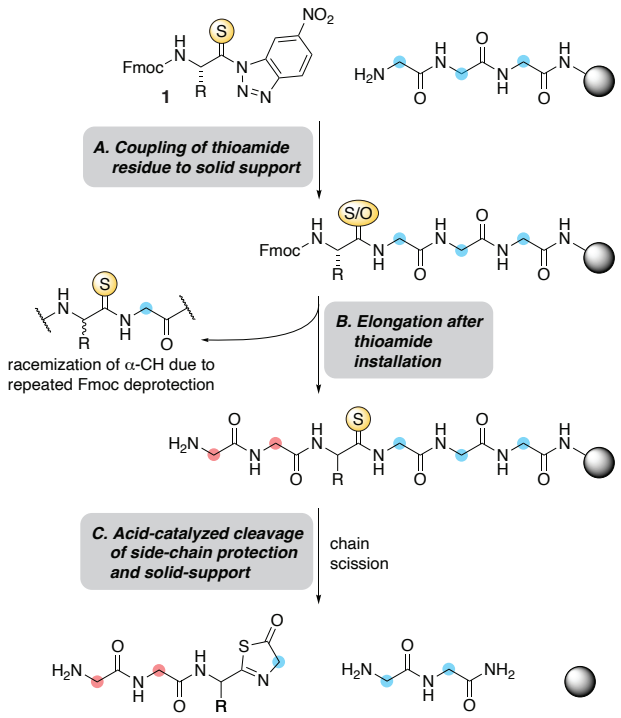


sequence	% epimerization at F(S)	
	unprotected thioamide	thioimidate protection
AAKAF(S)AKFG	22%	<0.01%

Scheme 1: Reversible Protection of Thioamide Residue Stereochemistry with Thioimidate[37]

Based on frontier orbital analysis, we identified the likely mechanism of epimerization of the thioamide and introduced the thioimidate as a potent and reversible strategy to protect the α-CH stereochemistry by raising the pK_a of the α proton.[37,38] This approach was easily implemented within the standard SPPS work flow to encourage use in other labs as it is analogous to the protection of the functional groups of amino-acid side chains. As an example, the thioimidate protection-deprotection strategy in Scheme 1 was used to synthesize the stereochemically pure peptide AAKAF^{S}AKFG, where significant epimerization (22%) was observed if the thioamide was left unprotected.

In addition to issues with the loss of stereochemical integrity during peptide elongation described above, thioamides pose problems during other stages of SPPS. This study identi-



Scheme 2: Overview of thioamide problems at each stage of SPPS

fies the potential issues of thioamides at each stage of SPPS: *(A)* thioamide coupling, *(B)* peptide elongation, *(C)* peptide cleavage from resin (Scheme 2), and outlines strategies to circumvent deleterious side reactivity. Ultimately, this study demonstrates best practices for implementing thioimidates as reversible protecting groups to permit thioamides to be installed into peptides that have eluded synthesis. We show how thioimidates are a general strategy to assist fragile thioamides through the demanding conditions of peptide synthesis and provide general access to these important peptide bond isosteres.

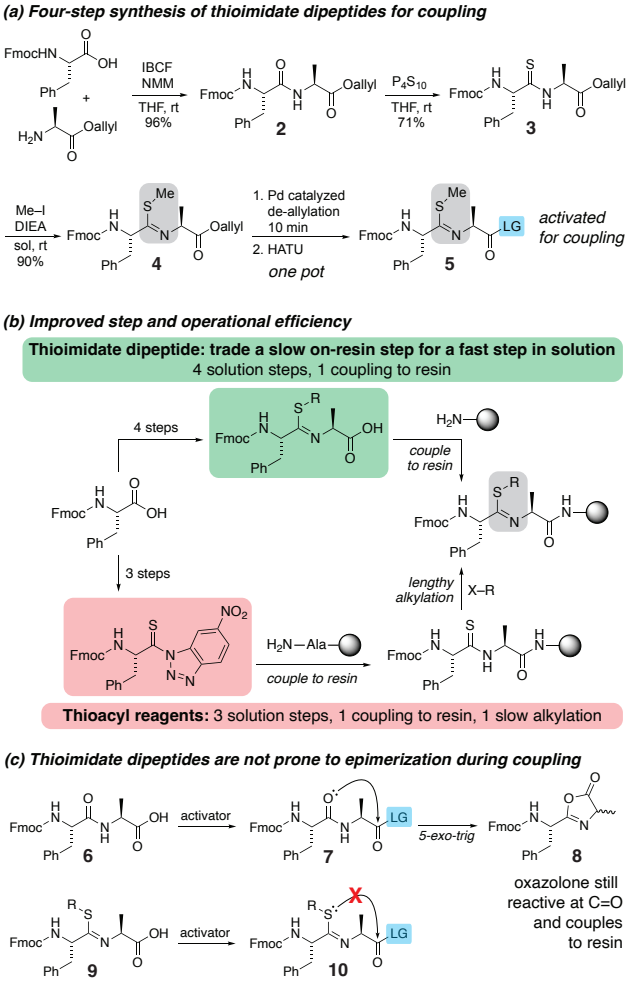
Results and Discussion

A. Coupling of thioamide residue to solid support.

The installation of thioamides into peptides during SPPS is most typically achieved using activated thioacylation reagents derived from commercially-available Fmoc amino acids.[41–44] The 4-nitrobenzotriazole reagents (**1**) were originally developed by Rapaport and cowork-

ers,[45] and later refined by Chatterjee and coworkers.[46] While These reagents allow thioamides to be added to the growing *N*-terminus of the resin-bound peptide, they are not without problems. The reagent is generated through an oxidation and used without purification in the coupling. The thioamide coupling step is not always quantitative, requiring Ac_2O capping in all cases to truncate unreacted chains. Additionally, inclusion of significant quantities of oxoamide can occur at these sites despite pure thioacyl reagent.[11,30] Thus, a more robust reagent that can be activated with conventional coupling agents (i.e. HATU, DIC, etc.) would improve the fidelity of thioamide insertion during SPPS.

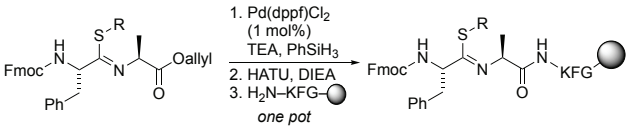
Our initial report of thioimidate protection of thioamides in peptides[37,38] outlined a procedure by which the thioamide was introduced into the peptide on solid phase using thioacyl reagents such as **1**. A lengthy alkylation step to convert the thioamide into the thioimidate then followed (protection reaction described in Scheme 1 and Scheme 3b bottom). A beneficial and more efficient alternative would be to couple the thioimidate into the peptide directly, ideally without introducing more steps or special considerations compared to existing methods. We settled on the synthesis and coupling of thioimidate dipeptides (Scheme 3a) with several notable features: (1) Synthesis and coupling of the dipeptide to resin requires the same number of steps (Scheme 3b top) to reach the final *on-resin* thioimidate as the thioacyl route (Scheme 3b bottom).[47] The operational efficiency of the process, however, is higher because a slow *on-resin* alkylation step is replaced with a faster and more easily monitored solution alkylation (**3**→**4**, Scheme 3a). (2) Coupling of the dipeptides to resin can be achieved with standard coupling reagents (such as HATU in this case). (3) dipeptide couplings can often lead to epimerization of the coupled residue because of a *5-exo-trig* cyclization of the adjacent amide onto the activated *C*-terminus (**7**→**8**, Scheme 3c).[48] The resulting oxazolone is also an electrophilic acylating agent and can still react with the resin-bound peptide, incorpating as a stereochemical defect. The enhanced nucleophilicity of a thioamide would exacerbate this side reaction even more. The sulfur atom of the thioimidate in **9**, however, is not nucleophilic and therefore this side reactivity is blocked.

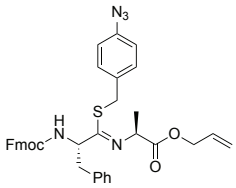
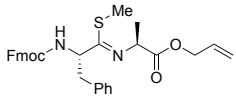
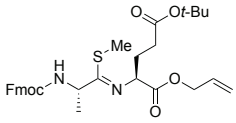
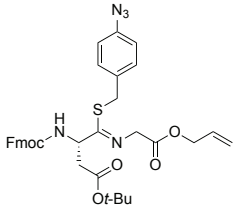


Scheme 3: Direct coupling of thioimidates into peptides

Using this dipeptide-based approach, we were able to incorporate protected thioamides directly on to resin (Table 1). Synthesis of the thioimidate dipeptide followed standard procedures (an example dipeptide synthesis is shown in Scheme 3a).[45,46] The allylic group was selected for *C*-terminal protection in the dipeptide because of the commercial availability of allyl ester amino acids, the orthogonality with which it can be deprotected using palladium catalysis, and the compatibility of those conditions with SPPS. This last point further improved on the efficiency of our strategy, as we found it most convenient to subject dipeptides (**4**) to de-allylation reagents, followed by direct addition of coupling reagent (HATU), and finally applying that mixture to resin for coupling and incorporation of the dipeptide (reaction in Table 1).

Table 1: Direct coupling of thioimidate dipeptides



entry	dipeptide	yield (%)
1		92
2		>95
3		>95
4		90

Yield determined by coupling 1-pyrenebutyric acid onto the *N*-terminus after dipeptide coupling and Fmoc removal. Peptides were then cleaved and analyzed by LCMS monitoring at 330 nm. Resin was 2-chlorotrityl Tentagel. Cleavage with 2% TFA in DCM.

Each dipeptide in Table 1 was coupled to the test sequence as described above and then cleaved for analysis. In general we observed excellent yields of coupled thioimidate with minimal observed oxoamide impurity (based on LCMS analysis of crude cleavage mixtures. See SI for further details). Unsurprisingly, the Pd-catalyzed de-allylation conditions were tolerant of a range of side-chain functional groups in the dipeptides investigated. Thus, because the progress of dipeptide construction and thioimidate protection can be readily monitored and characterized during small-molecule synthesis, this new method of directly coupling protected thioamides is both more efficient and highly preferable to existing methods.

B. Formation of thioimidate *on-resin* after thioamide installation and prior to elongation

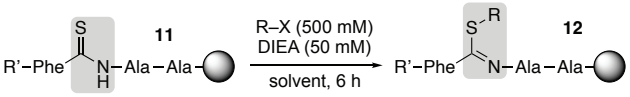
The dipeptide-coupling approach to install preformed thioimidate linkages into peptides (discussed above in section A.) provides protected thioamides without any subsequent steps needed before continuing conventional elongation of the peptide through SPPS. The drawback of this dipeptide approach, however, is that it decreases the sequence flexibility and diversity that is possible through coupling of a single thioacyl amino acid. For this reason, methods to efficiently convert thioamides into thioimidates *on-resin* are still valuable, yet a systematic investigation of the reaction conditions and potential pitfalls for this transformation has not been reported.

We therefore sought to probe the model reaction described in Table 2 to identify conditions to increase the efficiency with which thioamides within peptides could be alkylated to produce thioimidates. What was immediately clear, and not shown in Table 2, was that *N*,*N*-diisopropylethylamine (DIEA) was the most effective base for the transformation compared with weaker bases such as triethylamine and *N*-methylmorpholine (see SI for details). This result is unsurprising given DIEA is one of the strongest organic bases for SPPS that does not elicit deprotection of the *N*-terminal Fmoc protecting group. Accordingly, subsequent investigation was only carried out with DIEA.

With the base additive established, we next investigated the rate of thioimidate formation with several alkylating reagents. We identified MeI and the 4-azidobenzyl bromide as providing the most efficient thioimidate formation after 6 h (Table 2, entries 1 & 4). The increased efficiency of 4-azidobenzyl bromide (entry 4) relative to benzyl bromide (entry 2) is not immediately obvious as the electron-donating ability of the azide group is *chameleonic*,[49] but certainly appears to be beneficial in this context. Gratifyingly, the alkylation with 4-azidobenzyl bromide was greatly accelerated through an *in-situ* Finkelstein reaction with tetrabutylammonium iodide (entry 5).[50] Finally, DMF and DCM appeared to be the best solvents for the reaction (Table 2, entries 4 & 6–10).

The rapid reactivity of both MeI and 4-azidobenzyl bromide is certainly advantageous for thionopeptide synthesis, as deprotection conditions to convert the respective thioimidates back to the thioamide are well established. Restoration of the thioamide from 4-azidobenzyl thioimidates can be achieved through Sandmeyer reduction using mild reagents such as PPh_3 or DTT.[38] Methyl thioimidates must be deprotected using H_2S, a toxic gas,[37] but methyl thioimidates are also useful as reactive handles for the site-specific insertion of amidines into peptide backbones.[30]

Table 2: Efficiency of thioimidate formation *on-resin*



entry	R-X	solvent	yield (%)
1	Me–I	DMF	66
2	Bn–Br	DMF	24
3	Allyl–Br	DMF	16
4	4-N_3–BnBr	DMF	56
5	4-N_3–BnBr[a]	DMF	92
6	4-N_3–BnBr	DCM	54
7	4-N_3–BnBr	MeCN	46
8	4-N_3–BnBr	acetone	35
9	4-N_3–BnBr	MeOH	30
10	4-N_3–BnBr	THF	18

[a] Addition of TBAI at 5 mM. Percent yield calculated based on integration of LC peaks (330 nm, R' = 1-pyrenebutyric acid) corresponding to **11** and **12** (**12**/(**11**+**12**)). Resin was 2-chlorotrityl Tentagel. Cleavage with 2% TFA in DCM.

Risk of alkylation of side-chain functional groups

Extended exposure to alkylating reagents introduces the risk of alkylating reactive side-chain functional groups. For example, the imidazole of histidine is often protected with a Boc or trityl protecting group, but has been shown previously to alkylate with loss of the protecting group (Figure 2A).[51]

To investigate the potential for undesired alkylation elsewhere in the peptide we synthesized S-tag, derived from RNase A,[52,53] with a thioamide between Glu_9 and Arg_{10}.(**13**,

Figure 2B). Conversion to the thioimidate at this point in the synthesis allowed us to probe the alkylation potential of three specific amino acids of concern—Arg_{10}, His_{12}, and Met_{13}. Upon exposure to conditions to convert the thioamide to the thioimidate (**13**→**14**), we only observe alkylation of His_{12} (signified by concomitant loss of the Trt side chain protecting group as expected[51]). We did not observe any evidence of alkylation of Arg_{10} and Met_{13}. The undesired alkylation of His_{12} did not affect subsequent elongation of the remaining peptide (**14**→**15**). Moreover, the reduction with DTT to liberate the protected thioamide also returned the unalkylated His in the final cleavage and isolation (**15**→**16**). Thus, one must be cognizant of the potential for His to alkylated during conversion to thioimidate, but the overall process is invisible upon final work up. Of course, we also point out that any concerns regarding undesired alkylation of natural and unnatural amino acid side chains can be removed altogether by employing the thioimidate dipeptide strategy described in section A above.

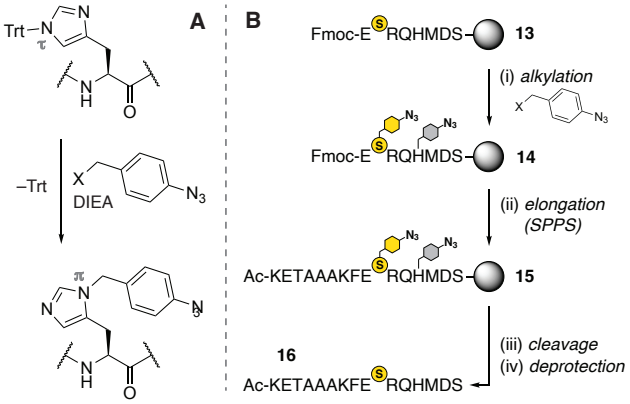


Figure 2: Synthesis of thiono S-Tag, displaying flexibility and functional group tolerance of thioimidate protection. Side products arising from methionine oxidation, overalkylation, and Edman-like degradation are all avoided. (i) 500 mM p-azidobenzyl bromide, 50 mM DIEA, 5 mM TBAI. (ii) Routine coupling and acetylation (see SI). (iii) 75/20/2.5/2.5 TFA/DCM/TIPSH/m-cresol, 1 h. (iv) 0.1 M DTT, 0.1 M DIEA, DMF, 2 h.

C. Chain scission during acid-catalyzed cleavage of side-chain protection and solid-support

The third and final prominent side reaction inherent to thionopeptide synthesis is arguably the most important and detrimental to productive yields. Strong acids (typically TFA) are employed to cleave the side-chain protecting groups and liberate the peptide from the resin. The nucleophilicity of sulfur and the presence of strong acid contrive to produce significant quantities of undesired chain scission at the thioamide residue (Scheme 2C).[36,54] In some cases, chain scission is the dominant product[36] unless significant tinkering with the cleavage cocktail is undertaken.[54] Indeed, if one is not aware of the potential for such products, one might analyze their crude cleavage mixture in bewilderment and conclude the entire synthesis was a failure. This side reactivity arises because of protonation of the amide carbonyl of the residue adjacent (n+1) to the thioamide residue (**17**→**18**, Figure 3). The activated carbonyl entices the nucleophilic sulfur into rapid *5-exo-trig* cyclization,[55] where Edman-like degradation leads to rapid chain scission and epimerization of the amino acid next to the thioamide residue.

We wondered if secondary thioamides might be more resistant to this reactivity, as protection of backbone amide nitrogen atoms with removable groups such as Hmb (*N*-(2-hydroxy-4-methoxybenzyl) is common to prevent aspartimide formation and reduce aggregation of peptides.[56] Unfortunately, secondary amides were similarly prone to *5-exo-trig* reactivity (**19**→**20**, Figure 3).

Intriguingly, acid-promoted cyclization was not observed when the adjacent, n+1 residue to the thioamide was a β-amino acid (**21**, Figure 3). This result suggests that β-thionopeptides may experience fewer synthetic hurdles than their α-thionopeptide cousins. Indeed, β-chiral amino acids would seemingly not require thioimidate protection to preserve stereochemistry during elongation. Consequently, β-thionopeptides appear be a productive and untapped area of exploration.

Ultimately, α-thionopeptide synthesis requires evaluation of both deprotection times and

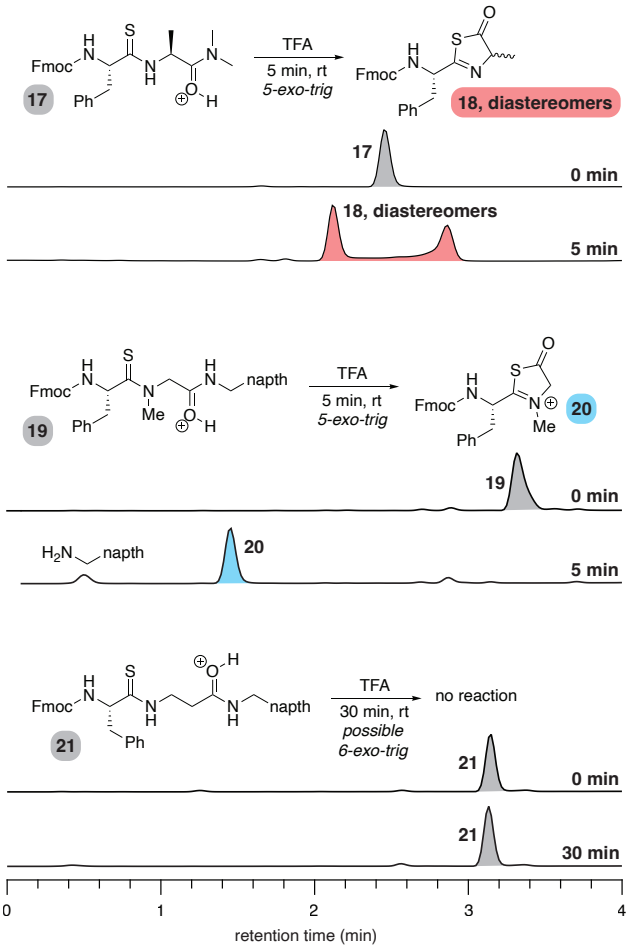


Figure 3: Stability tests of thioamide model dipeptides in the presence of TFA as solvent. Reactions were performed at room temperature and monitored by reverse-phase UPLC-MS. All starting materials and products were confirmed by detection of the molecular ion.

percentage of TFA in the cleavage cocktail, on case-by-case basis, to optimize for both the extent of side-chain deprotection and yield of the final, intact thionopeptide.[36,54] Nevertheless, there are simply sequences that cannot by isolated due to this detrimental side reactivity. Thus, a lack of generality significantly raises the barrier to entry for labs that seek to employ these important probes to address questions regarding protein conformation, interaction, and reactivity.

To address these drawbacks, we hypothesized that the reversible protection of a thioamide as a thioimidate might also hold the potential to circumvent the debilitating *5-exo-trig* cyclization behavior of the thioamide. By converting the thioamide into a thioimidate,

the nucleophilicity of the sulfur atom is attenuated, and, moreover, the =N– site of the thioimidate is highly basic[57] leading to protonation under the acidic conditions of cleavage, preventing any nucleophilic behavior. Indeed, model dipetide **22** displayed excellent stability upon exposure to TFA (Figure 4, where the minor peak arises due to isomerism about the C=N bond[38]), and provided an approach to further protect thioamides during peptide cleavage.

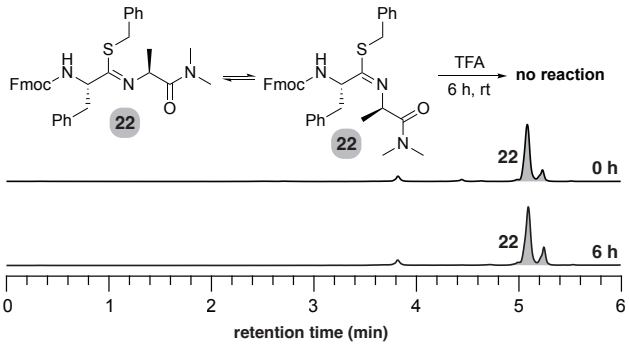
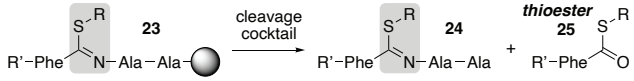


Figure 4: Stability tests of thioimidate model dipeptide **22** in the presence of TFA as solvent. Reactions were performed at room temperature and monitored by reverse-phase UPLC-MS. All starting materials and products were confirmed by detection of the molecular ion. The presence of two peaks for **22** is due to isomerism about the C=N bond as shown above.

While thioimidates appear to be a promising and unprecedented avenue to protect thioamides during acidic cleavage from resin, the approach is not without concern. Chiefly, in the context of solid-phase chemistry, thioimidates can hydrolyze to thioesters (**12**→**24**, another type of chain scission) if sufficient water is present in the cleavage cocktail (Table 3).[58,59] Hydrolysis of the thioimidate is dependent on the strength of the acid used for cleavage. Thus, weaker cleavage cocktails that only effect cleavage from acid sensitive resin (i.e. 2-chlorotrityl) without affecting side-chain protecting groups lead to very little thioester hydrolysis side product (entry 1). The stronger acid TFA is itself hygroscopic and extremely cumbersome to render anhydrous in laboratory settings. It is not surprising, therefore, that treatment with 'neat' TFA leads to more thioester but still provides serviceable yields of the liberated thioimidate peptide **23** (entry 2). Ultimately, we found TFA/DCM (75:25) to provide the highest percentage of **23** while still being acidic enough to cleave side-chain

protecting groups (entry 3). The addition of water to this cleavage mixture (entry 4) lead to significantly more thioester as anticipated. Entry 4 is an important result as water is often included in cleavage cocktails for the scavenging of reactive cations produced during deprotection and cleavage. While water is a benign additive during standard peptide cleavage, researchers must be cognizant of its side effects when utilizing thioimidates for thioamide protection.

Table 3: Stability of thioimidate to acidic cleavage cocktails



entry	cocktail	R	yield **24** (%)	yield **25** (%)
1	HFIP/DCM	Me	93	7
	(1:1)	Bn	98	2
2	TFA	Me	79	21
		Bn	75	25
3	TFA/DCM	Me	81	19
	(75:25)	Bn	94	6
		4-N_3-Bn	96	4
4	TFA/DCM/H_2O	Me	72	28
	(75:20:5)	Bn	64	36
		4-N_3-Bn	55	45

Percent yield calculated based integration of LC peaks (330 nm, R' = 1-pyrenebutyric acid). Resin was 2-chlorotrityl Tentagel.

New sequence space for thioamides

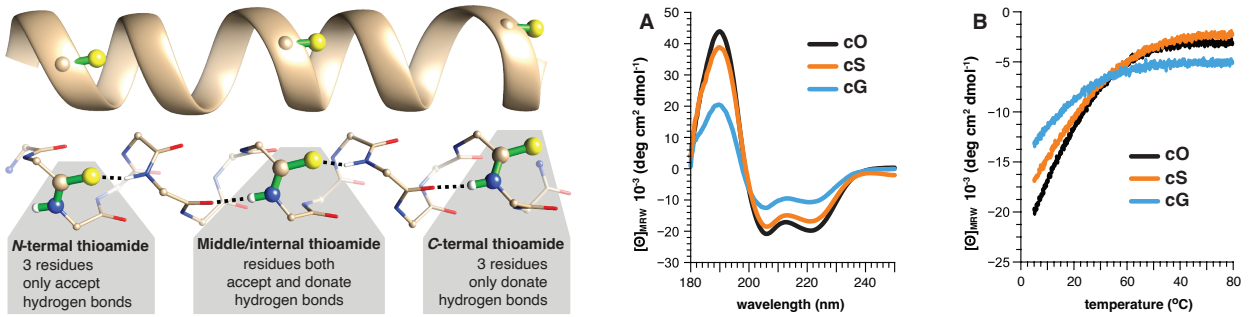
The previous sections document major pitfalls of thioamides during SPPS. We then described approaches for how thioimidate protection of thioamides can be implemented and exploited as a comprehensive strategy to assist reactive thioamides through the critical stages of SPPS. Armed with these improved methods to install and preserve thioamides during SPPS, we sought to boldly go where no thioamide has gone before.

Kiefhaber and coworkers investigated the placement of thioamides along a model α-helix (Table 4) with the intent of interrogating the impact that thioamide isosteres might have on helicity.[60] Briefly, within the linear hydrogen-bonding context of the α-helix (atoms in the C=S$\cdots$H–N interaction are roughly linear), thioamides are weaker hydrogen-bond acceptors and stronger hydrogen-bond donors than oxoamides.[9] Thus, it is not surprising that when placed within the final turn of the *N*-terminus, where backbone amides largely only accept hydrogen bonds,[61] that thioamides destabilize the helical fold (**nO** versus **nS**). Similarly, thioamides placed in the center of the peptide can destabilize the helical fold through a combination of poor hydrogen bond accepting and the longer C=S bond (**mO** versus **mS**), unless the thioamide can be sterically tolerated.[10,62] Interestingly, the extent of destabilization induced by the thioamide at both of these positions in the helix is greater (although within the margin of error of the measurement) than a glycine substitution, a known helix breaker (based on a lower $[\Theta]_{222}$ value, **nG** and **mG**).[63]

The most tempting place for a thioamide within an α-helix, therefore, is the last three residues of the *C*-terminus, where those three residues have the potential to only donate stronger hydrogen bonds and their poor hydrogen-bond accepting ability is not critical to the helical fold. While Kiefhaber and coworkers were able to study the effects of thioamides at the *N*-terminus and center of the peptide (Table 4, **nS** and **mS**), the *C*-terminal thioamide was noticeably absent leaving the set incomplete and the hypothesis untested! Indeed, our attempts to synthesize the *C*-terminal variant were completely unsuccessful because of the troublesome side reactivity of thioamides documented above. These results are further supported by the lack of *C*-terminal thioamides that have been reported in longer peptide sequences (Figure 1B).

Gratifyingly, the elusive *C*-terminal thioamide peptide (**cS**) was finally obtained through application of the synthetic strategies for thioimidate protection described in the previous sections (Scheme 4, see SI for complete synthetic details). Briefly, the thioimidate was used to protect the fragile thioamide during both peptide elongation and cleavage from resin

Table 4: Effect of *C*-terminal thioamide on helicity: (A) CD spectra and (B) thermal melting behavior of a *C*-terminal thioamide in an α-helix

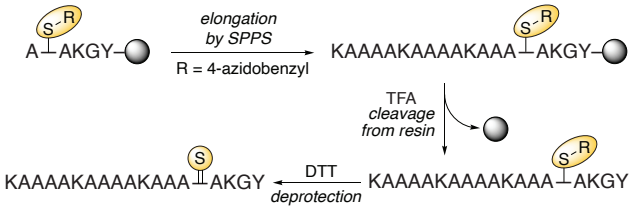


name	sequence	$[\Theta]_{222}$ [b] deg cm^2 dmol^{-1}	helicity (%)
nO[a]	Ac-DF^{O}AAAKAAAAKAAAAK-NH$_2$	–23 900	69 ± 5
nS[a]	Ac-DF^{S}AAAKAAAAKAAAAK-NH$_2$	–16 000	48 ± 8
nG[a]	Ac-DF^{O}GAAKAAAAKAAAAK-NH$_2$	–17 000	51 ± 5
mO[a]	Ac-KAAAAKAA^{O}AAKAAAAK-NH$_2$	–24 200	68 ± 5
mS[a]	Ac-KAAAAKAA^{S}AAKAAAAK-NH$_2$	–6 900	24 ± 8
mG[a]	Ac-KAAAAKAA^{O}GAKAAAAK-NH$_2$	–7 900	26 ± 5
cO	Ac-KAAAAKAAAAKAAA^{O}AKGY-NH$_2$	-19 700	58 ± 5
cS	Ac-KAAAAKAAAAKAAA^{S}AKGY-NH$_2$	-16 800	49 ± 8
cG	Ac-KAAAAKAAAAKAAA^{O}GKGY-NH$_2$	-10 600	33 ± 5

[a] Previously reported.[60] [b] Far-UV circular dichroism data measured at 5 °C in 100 mM pH 7.0 potassium phosphate buffer.

allowing, for the first time, isolation of the final thionopeptide (Table 4, **cS**).

With the previously elusive thionopeptide finally realized, we recorded far-UV circular dichroic (CD) spectra of both the thioamide (**cS**) and parent oxoamide (**cO**). Both have characteristic α-helical features, showing maxima at 190 nm and minima at 208 and 222 nm. The thionopeptide showed slightly reduced helicity, but similar melting behavior relative to the oxopeptide (Table 4A and B). The difference in helicity between the *C*-terminal oxoamide versus thioamide (**cO** versus **cS**) was also not as great as the difference for the *N*-terminal and middle peptides (Table 4). Perhaps the greatest evidence that thioamides are tolerated at



Scheme 4: Thioimidate protection preserves thioamides through elongation and resin cleavage

the *C*-termini of α-helical peptides is comparison to the glycine mutant (**cG**), which showed significantly reduced helicity relative to **cS**. This behavior is the opposite for *N*-terminal and middle variants in which the thioamide was more destabilizing than the glycine mutant. These observations confirm our hypothesis that the *C*-terminus of α-helical structures can tolerate thioamides probes with minimal perturbation relative to other positions.

Conclusion

Thioamides are the closest congener of oxoamides, making them a logical consideration when researchers seek to probe interactions along the peptide backbone. Accordingly, thioamides are valuable tools for studying hydrogen-bonding and hydrophobicity effects, and can serve as reactive sites for further chemistry. The synthesis of thioamides in peptides, however, is far from straightforward. This work sought to demystify the solid-phase peptide synthesis of thionopeptides by first outlining pitfalls that exist at every stage of SPPS (*(A)* thioamide coupling, *(B)* peptide elongation, *(C)* peptide cleavage from resin). We then demonstrated how reversible protection of the thioamide as a thioimidate resolved each pitfall, ultimately resulting in the first isolation of a benchmark α-helical peptide that had eluded synthesis until now. Thus, thioimidates are a general strategy to realize more productive yields of thionopeptides without significantly altering the normal SPPS work-flow. Side reactions related to the higher electrophilicity and acidity of the thioamide accumulate to degrade the target peptide, but the thioimidate protection strategy circumvents much of this side reactivity by altering the reactive C=S, α-CH, and NH altogether.

Author Information

Corresponding Author * bvv@iastate.edu

ORCID

Jacob Byerly-Duke: 0000-0003-4380-7747

Aaron Donovan: 0009-0009-4814-3485

Krishna K. Sharma: 0000-0003-4927-745X

Rida Ibrahim: 0000-0001-6470-3379

Brett VanVeller: 0000-0002-3792-0308

Acknowledgement

The authors acknowledge the National Institutes of Health, National Institutes of General Medical Sciences under award number R35 GM142883. We also acknowledge Dr. Luis Camacho III and Dr. Bryan J. Lampkin for enabling conversations regarding this work.

Supporting Information Available

The Supporting Information is available free of charge on the ACS Publications website. PDF file describing synthetic procedures and molecular characterization/purity (NMR, LC-MS traces). A CSV file comprising the data for Figure 1.

References

(1) Mahanta, N.; Szantai-Kis, D. M.; Petersson, E. J.; Mitchell, D. A. Biosynthesis and chemical applications of thioamides. *ACS Chem. Biol.* **2019**, *14*, 142–163.

(2) Dunbar, K. L.; Büttner, H.; Molloy, E. M.; Dell, M.; Kumpfmüller, J.; Hertweck, C.

Genome editing reveals novel thiotemplated assembly of polythioamide antibiotics in anaerobic bacteria. *Angew. Chem., Int. Ed.* **2018**, *57*, 14080–14084.

(3) Nayak, D. D.; Mahanta, N.; Mitchell, D. A.; Metcalf, W. W. Post-translational thioamidation of methyl-coenzyme M reductase, a key enzyme in methanogenic and methanotrophic Archaea. *Elife* **2017**, *6*.

(4) Mahanta, N.; Liu, A.; Dong, S.; Nair, S. K.; Mitchell, D. A. Enzymatic reconstitution of ribosomal peptide backbone thioamidation. *Proc. Natl. Acad. Sci.* **2018**, *115*, 3030–3035.

(5) Liu, A.; Si, Y.; Dong, S.-H.; Mahanta, N.; Penkala, H. N.; Nair, S. K.; Mitchell, D. A. Functional elucidation of (TfuA) in peptide backbone thioamidation. *Nat. Chem. Biol.* **2021**, *17*, 585–592.

(6) Steele, A. D.; Kiefer, A. F.; Shen, B. The many facets of sulfur incorporation in natural product biosynthesis. *Curr. Opin. Chem. Biol.* **2023**, *76*, 102366.

(7) Watson, Z. L.; Ward, F. R.; Méheust, R.; Ad, O.; Schepartz, A.; Banfield, J. F.; Cate, J. H. Structure of the bacterial ribosome at 2 Å resolution. *eLife* **2020**, *9*, e60482.

(8) Kahnt, J.; Buchenau, B.; Mahlert, F.; Krüger, M.; Shima, S.; Thauer, R. K. Post-translational modifications in the active site region of methyl-coenzyme M reductase from methanogenic and methanotrophic archaea. *The FEBS Journal* **2007**, *274*, 4913–4921.

(9) Lampkin, B. J.; VanVeller, B. Hydrogen Bond and Geometry Effects of Thioamide Backbone Modifications. *J. Org. Chem.* **2021**, *86*, 18287–18291, PMID: 34851645.

(10) Walters, C. R.; Szantai-Kis, D. M.; Zhang, Y.; Reinert, Z. E.; Horne, W. S.; Chenoweth, D. M.; Petersson, E. J. The effects of thioamide backbone substitution

on protein stability: a study in -helical, -sheet, and polyproline II helical contexts. *Chem. Sci.* **2017**, *8*, 2868–2877.

(11) Newberry, R. W.; VanVeller, B.; Raines, R. T. Thioamides in the collagen triple helix. *Chem. Commun.* **2015**, *51*, 9624–9627.

(12) Fiore, K. E.; Patist, M. J.; Giannakoulias, S.; Huang, C.-H.; Verma, H.; Khatri, B.; Cheng, R. P.; Chatterjee, J.; Petersson, E. J. Structural impact of thioamide incorporation into a -hairpin. *RSC Chem. Biol.* **2022**, *3*, 582–591.

(13) Verma, H.; Khatri, B.; Chakraborti, S.; Chatterjee, J. Increasing the bioactive space of peptide macrocycles by thioamide substitution. *Chem. Sci.* **2018**, *9*, 2443–2451.

(14) Ghosh, P.; Raj, N.; Verma, H.; Patel, M.; Chakraborti, S.; Khatri, B.; Doreswamy, C. M.; Anandakumar, S.; Seekallu, S.; Dinesh, M., et al. An amide to thioamide substitution improves the permeability and bioavailability of macrocyclic peptides. *Nat. Commun.* **2023**, *14*, 6050.

(15) Newberry, R. W.; Raines, R. T. The n→ * Interaction. *Acc. Chem. Res.* **2017**, *50*, 1838–1846, PMID: 28735540.

(16) Newberry, R. W.; VanVeller, B.; Guzei, I. A.; Raines, R. T. n→ * Interactions of Amides and Thioamides: Implications for Protein Stability. *J. Am. Chem. Soc.* **2013**, *135*, 7843–7846, PMID: 23663100.

(17) Bartlett, G. J.; Newberry, R. W.; VanVeller, B.; Raines, R. T.; Woolfson, D. N. Interplay of Hydrogen Bonds and n→ * Interactions in Proteins. *J. Am. Chem. Soc.* **2013**, *135*, 18682–18688, PMID: 24256417.

(18) Newberry, R. W.; Bartlett, G. J.; VanVeller, B.; Woolfson, D. N.; Raines, R. T. Signatures of n→ * interactions in proteins. *Protein Science* **2014**, *23*, 284–288.

(19) Khatri, B.; Majumder, P.; Nagesh, J.; Penmatsa, A.; Chatterjee, J. Increasing protein stability by engineering the n $\rightarrow$ * interaction at the -turn. *Chem. Sci.* **2020**, *11*, 9480–9487.

(20) Huang, Y.; Ferrie, J. J.; Chen, X.; Zhang, Y.; Szantai-Kis, D. M.; Chenoweth, D. M.; Petersson, E. J. Electronic interactions of i, i + 1 dithioamides: increased fluorescence quenching and evidence for n-to- * interactions. *Chem. Commun.* **2016**, *52*, 7798–7801.

(21) Robkis, D. M.; Hoang, E. M.; Po, P.; Deutsch, C. J.; Petersson, E. J. Side-chain thioamides as fluorescence quenching probes. *Biopolymers* **2021**, *112*, e23384.

(22) Walters, C. R.; Ferrie, J. J.; Petersson, E. J. Dithioamide substitutions in proteins: effects on thermostability, peptide binding, and fluorescence quenching in calmodulin. *Chem. Commun.* **2018**, *54*, 1766–1769.

(23) Petersson, E. J.; Goldberg, J. M.; Wissner, R. F. On the use of thioamides as fluorescence quenching probes for tracking protein folding and stability. *Phys. Chem. Chem. Phys.* **2014**, *16*, 6827–6837.

(24) Goldberg, J. M.; Batjargal, S.; Chen, B. S.; Petersson, E. J. Thioamide Quenching of Fluorescent Probes through Photoinduced Electron Transfer: Mechanistic Studies and Applications. *J. Am. Chem. Soc.* **2013**, *135*, 18651–18658.

(25) Culik, R. M.; Jo, H.; DeGrado, W. F.; Gai, F. Using Thioamides To Site-Specifically Interrogate the Dynamics of Hydrogen Bond Formation in -Sheet Folding. *J. Am. Chem. Soc.* **2012**, *134*, 8026–8029, PMID: 22540162.

(26) Goldberg, J. M.; Wissner, R. F.; Klein, A. M.; Petersson, E. J. Thioamide quenching of intrinsic protein fluorescence. *Chem. Commun.* **2012**, *48*, 1550–1552.

(27) Goldberg, J. M.; Batjargal, S.; Petersson, E. J. Thioamides as Fluorescence Quenching

Probes: Minimalist Chromophores To Monitor Protein Dynamics. *J. Am. Chem. Soc.* **2010**, *132*, 14718–14720, PMID: 20886849.

(28) Chen, X.; Mietlicki-Baase, E. G.; Barrett, T. M.; McGrath, L. E.; Koch-Laskowski, K.; Ferrie, J. J.; Hayes, M. R.; Petersson, E. J. Thioamide Substitution Selectively Modulates Proteolysis and Receptor Activity of Therapeutic Peptide Hormones. *J. Am. Chem. Soc.* **2017**, *139*, 16688–16695, PMID: 29130686.

(29) Barrett, T. M.; Chen, X. S.; Liu, C.; Giannakoulias, S.; Phan, H. A. T.; Wang, J.; Keenan, E. K.; Karpowicz, R. J. J.; Petersson, E. J. Studies of Thioamide Effects on Serine Protease Activity Enable Two-Site Stabilization of Cancer Imaging Peptides. *ACS Chem. Biol.* **2020**, *15*, 774–779, PMID: 32141733.

(30) O'Brien, E. A.; Sharma, K. K.; Byerly-Duke, J.; Camacho, L. A., 3rd; VanVeller, B. A general strategy to install amidine functional groups along the peptide backbone. *J. Am. Chem. Soc.* **2022**, *144*, 22397–22402.

(31) Thombare, V. J.; Hutton, C. A. Rapid, Traceless, AgI-Promoted Macrocyclization of Peptides Possessing an N-Terminal Thioamide. *Angew. Chem., Int. Ed.* **2019**, *58*, 4998–5002.

(32) Shabani, S.; Hutton, C. A. Depsipeptide synthesis using a late-stage Ag(i)-promoted macrolactonisation of peptide thioamides. *Chem. Commun.* **2021**, *57*, 2081–2084.

(33) Taresh, A. B.; Hutton, C. A. Backbone thioamide directed macrocyclisation: lactam stapling of peptides. *Org. Biomol. Chem.* **2022**, *20*, 1488–1492.

(34) Okano, A.; James, R. C.; Pierce, J. G.; Xie, J.; Boger, D. L. Silver(I)-Promoted Conversion of Thioamides to Amidines: Divergent Synthesis of a Key Series of Vancomycin Aglycon Residue 4 Amidines That Clarify Binding Behavior to Model Ligands. *J. Am. Chem. Soc.* **2012**, *134*, 8790–8793, PMID: 22568755.

(35) Huh, S.; Appavoo, S. D.; Yudin, A. K. Amidine Functionality As a Conformational Probe of Cyclic Peptides. *Org. Lett.* **2020**, *22*, 9210–9214, PMID: 33206539.

(36) Khatri, B.; Raj, N.; Chatterjee, J. In *Synthetic and Enzymatic Modifications of the Peptide Backbone*; Petersson, E. J., Ed.; Methods in Enzymology; Academic Press, 2021; Vol. 656; pp 27–57.

(37) Camacho, L. A.; Lampkin, B. J.; VanVeller, B. A Bottom-Up Approach To Preserve Thioamide Residue Stereochemistry during Fmoc Solid-Phase Peptide Synthesis. *Org. Lett.* **2019**, *21*, 7015–7018, PMID: 31403302.

(38) Camacho, L. A., 3rd; Nguyen, Y. H.; Turner, J.; VanVeller, B. Deprotection strategies for thioimidates during Fmoc solid-phase peptide synthesis: A safe route to thioamides. *J. Org. Chem.* **2019**, *84*, 15309–15314.

(39) Mukherjee, S.; Chatterjee, J. Suppressing the epimerization of endothioamide peptides during Fmoc/t-Bu-based solid phase peptide synthesis. *J. Pept. Sci.* **2016**, *22*, 664–672.

(40) Szantai-Kis, D. M.; Walters, C. R.; Barrett, T. M.; Hoang, E. M.; Petersson, E. J. Thieme Chemistry Journals Awardees–Where Are They Now? Improved Fmoc Deprotection Methods for the Synthesis of Thioamide-Containing Peptides and Proteins. *Synlett* **2017**, *28*, 1789–1794.

(41) Yang, J.; Wang, C.; Xu, S.; Zhao, J. Ynamide-mediated thiopeptide synthesis. *Angew. Chem., Int. Ed.* **2019**, *58*, 1382–1386.

(42) Wang, C.; Han, C.; Yang, J.; Zhang, Z.; Zhao, Y.; Zhao, J. Ynamide-mediated thioamide and primary thioamide syntheses. *J. Org. Chem.* **2022**, *87*, 5617–5629.

(43) Liao, Y.; Zhang, S.; Jiang, X. Construction of Thioamide Peptides from Chiral Amino Acids. *Angew. Chem., Int. Ed.* **2023**, *62*, e202303625.

(44) Zhang, Q.; Soulère, L.; Queneau, Y. Towards More Practical Methods for the Chemical Synthesis of Thioamides Using Sulfuration Agents: A Decade Update. *Molecules* **2023**, *28*.

(45) Shalaby, M. A.; Grote, C. W.; Rapoport, H. Thiopeptide synthesis. Alpha-amino thionoacid derivatives of nitrobenzotriazole as thioacylating agents. *J. Org. Chem.* **1996**, *61*, 9045–9048.

(46) Khatri, B.; Bhat, P.; Chatterjee, J. Convenient synthesis of thioamidated peptides and proteins. *J. Pept. Sci.* **2020**, *26*, e3248.

(47) In fact, because the thioacyl approach (bottom) requires coupling to of the alanine to the resin prior to couping with thioacyl reagent, this represents an additional step that is not accounted for in the comparison between both appraoches. Because coupling of amino acids to resin is considered routine and of little operational difficulty, this step was not included in the discussion.

(48) Goodman, M.; Levine, L. Peptide Synthesis via Active Esters. IV. Racemization and Ring-Opening Reactions of Opitcally Active Oxazolones. *J. Am. Chem. Soc.* **1964**, *86*, 2918–2922.

(49) Jawale, H.; Mistry, S.; Conder, C.; Wenthold, P. G. Investigation of the Substituent Effects of the Azide Functional Group Using the Gas-Phase Acidities of 3- and 4-Azidophenols. *J. Org. Chem.* **2022**, *87*, 985–992, PMID: 34965132.

(50) Finkelstein, H. Darstellung organischer Jodide aus den entsprechenden Bromiden und Chloriden. *Ber. Dtsch. Chem. Ges.* **1910**, *43*, 1528–1532.

(51) Qian, W.; Liu, F.; Burke, T. R. J. Investigation of Unanticipated Alkylation at the N( ) Position of a Histidyl Residue Under Mitsunobu Conditions and Synthesis of Orthogonally Protected Histidine Analogues. *J. Org. Chem.* **2011**, *76*, 8885–8890, PMID: 21950469.

(52) Raines, R. T. Ribonuclease A. *Chem. Rev.* **1998**, *98*, 1045–1066, PMID: 11848924.

(53) Raines, R. T.; McCormick, M.; Van Oosbree, T. R.; Mierendorf, R. C. *Applications of Chimeric Genes and Hybrid Proteins Part A: Gene Expression and Protein Purification*; Methods in Enzymology; Academic Press, 2000; Vol. 326; pp 362–376.

(54) Miwa, J. H.; Margarida, L. A.; Meyer, A. E. Improved acidolytic deprotection conditions for the Fmoc-based solid-phase synthesis of thioxo peptides. *Tetrahedron Lett.* **2001**, *42*, 7189–7191.

(55) Budzowski, A.; Linden, A.; Heimgartner, H. The 'Azirine/Oxazolone Approach' to the Synthesis of Aib-Pro Endothiopeptides. *Helv. Chim. Acta* **2008**, *91*, 1471–1488.

(56) Quibell, M.; Owen, D.; Packman, L. C.; Johnson, T. Suppression of piperidine-mediated side product formation for Asp(OBut)-containing peptides by the use of N-(2-hydroxy-4-methoxybenzyl)(Hmb) backbone amide protection. *J. Chem. Soc., Chem. Commun.* **1994**, 2343–2344.

(57) Chaturvedi, R. K.; Schmir, G. L. Hydrolysis of thioimidate esters. II. Evidence for the formation of three species of the tetrahedral intermediate. *J. Am. Chem. Soc.* **1969**, *91*, 737–746.

(58) Chaturvedi, R. K.; MacMahon, A. E.; Schmir, G. L. Hydrolysis of thioimidate esters. Tetrahedral intermediates and general acid catalysis. *J. Am. Chem. Soc.* **1967**, *89*, 6984–6993.

(59) Sharma, I.; Crich, D. Direct Fmoc-chemistry-based solid-phase synthesis of peptidyl thioesters. *J. Org. Chem.* **2011**, *76*, 6518–6524.

(60) Reiner, A.; Wildemann, D.; Fischer, G.; Kiefhaber, T. Effect of Thioxopeptide Bonds on -Helix Structure and Stability. *J. Am. Chem. Soc.* **2008**, *130*, 8079–8084.

(61) Wu, C.; Hoang, H. N.; Hill, T. A.; Lim, J.; Kok, W. M.; Akondi, K.; Liu, L.; Fairlie, D. P. Helical structure in cyclic peptides: effect of N-methyl amides versus esters. *Chem. Commun.* **2022**, *58*, 12475–12478.

(62) Miwa, J. H.; Pallivathucal, L.; Gowda, S.; Lee, K. E. Conformational Stability of Helical Peptides Containing a Thioamide Linkage. *Org. Lett.* **2002**, *4*, 4655–4657.

(63) Imai, K.; Mitaku, S. Mechanisms of secondary structure breakers in soluble proteins. *Biophys. (Nagoya-shi)* **2005**, *1*, 55–65.

TOC Graphic