

A Strategy for Thioamide Incorporation during Fmoc Solid-Phase Peptide Synthesis with Robust Stereochemical Integrity

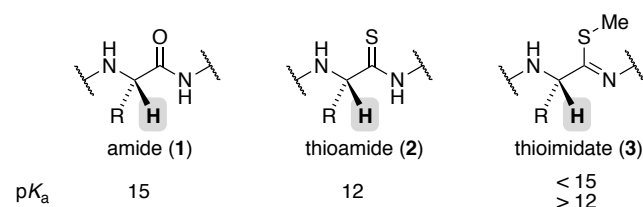
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Thioamides are versatile chemical probes for studying the function, behavior and stability of protein structure.^{1,2} Because thioamides display different strengths of hydrogen bond-donating and -accepting ability relative to amides (**1**), they have been used to probe the role of hydrogen bonding in protein secondary structure.³⁻⁵ Alternatively, the greater Lewis basicity of sulfur in thioamides (**2**) has been used to interrogate possible $n \rightarrow \pi^*$ interactions between adjacent peptide bonds (i to $i + 1$).⁶⁻⁸ Thioamides can also be employed in photochemical schemes as photoswitches⁹ or as fluorescent quenchers to study conformational changes within peptides.¹⁰⁻¹³

Given the importance of thioamides in peptide chemistry, methods for their incorporation into peptides with robust stereochemical integrity are highly desirable. Activated thioamide precursors can be prepared from Fmoc-protected amino acids¹⁵ and incorporated into peptides following standard solid-phase peptide synthesis (SPPS) protocols. While this approach provides for site-selective insertion of a thioamide into the sequence, the α -C of the thioamides residue is prone to racemization under conditions required for Fmoc deprotection (Scheme 1).¹⁶⁻¹⁹ Subsequent coupling and Fmoc-deprotection of additional residues following the thioamide leads to further deterioration of the stereochemical integrity of the thioamide residue—in addition to other side reactions that can compromise yield.¹⁷⁻¹⁹ For this reason, thioamides are often only incorporated within a few residues of the end of the sequence to limit such side reactivity. Thus, the goal of this study was to develop a strategy for robust synthesis of thioamide-containing peptides with high stereochemical integrity.

Scheme 1: Acidity of the α -C in 1–3.



Given the prominence of protecting groups in SPPS to cap and prevent unwanted reactivity of amino acid side chains, we reasoned that racemization of the thioamide could be attenuated via protection as a thioimidate (**3**). Thioimidates have been employed as protecting groups for thioamides in the synthesis of natural product

derivatives.²⁰ Unfortunately, the α -C pK_a in thioimidates has not been determined. We hypothesized, however, that the pK_a of the α -C of **3** was higher than **2** for the following reasons: The lone pair of electrons on the α -C of the conjugate base can delocalize into the $\pi^*_{C=N}$ bond in **3** or the $\pi^*_{C=S}$ bond in **2**. Because the $\pi^*_{C=S}$ bond is generally lower in energy relative to a $\pi^*_{C=N}$ bond, the lone pair of electrons can more favorably delocalize into the $\pi^*_{C=S}$ bond in **2**. The α -C conjugate base of **2** is therefore lower in energy than the conjugate base of **3** and, by extension, **2** would have a lower α -C pK_a than **3**. Thus, the hypothesized higher pK_a of the α -C of **3** relative to thioamide **2** could confer greater stability against racemization during SPPS conditions.

To test this hypothesis, we investigated the stability of the model dimer Cbz-Phe^(X)-Ala-OMe, where thioamides on phenylalanine have been shown to be especially prone to epimerization at the α -C.¹⁷ Dimers **4** (thioamide) and **5** (thioimide) were subjected to basic conditions common to Fmoc-deprotection during SPPS (Figure 1A). The thioamide dimer (**4**) shows significant epimerization in under an hour in the presence of piperidine and DBU—standard organic bases for Fmoc-deprotection. This result aligns with what has been observed previously.^{17, 18} Earlier reports focused on abbreviated deprotection times and lower concentrations of the base in an attempt to reduce the epimerization behavior shown here. A drawback of these approaches is the potential for decreased yields of deprotection and, ultimately, the final peptide.²¹

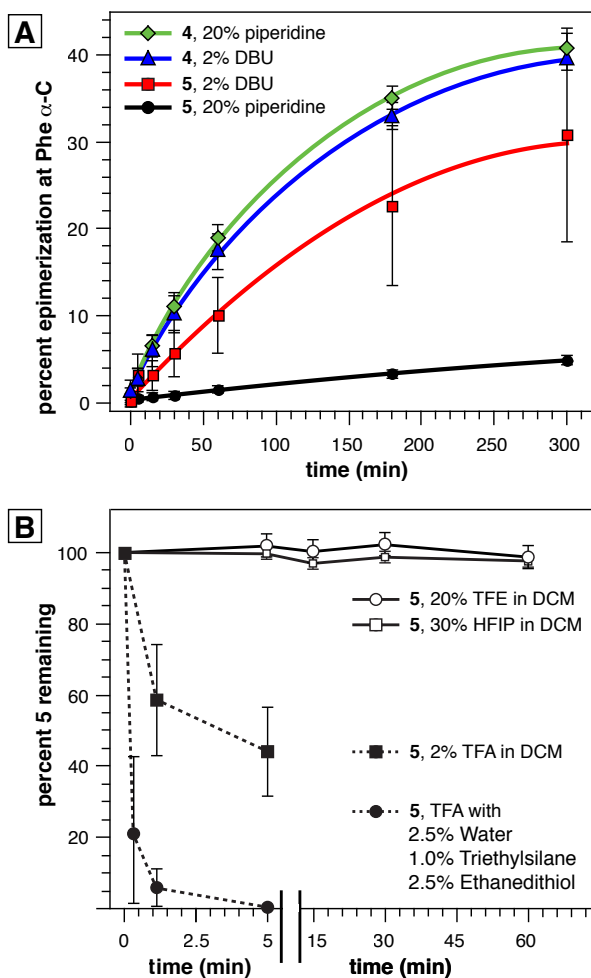
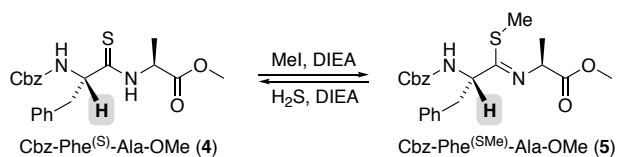


Figure 1. Stability test with typical reagents used in SPPS. **A.** Solutions of **4** and **5** (0.1 M in DMF) with bases indicated. **B.** Solutions of **4** and **5** (0.1 M) in solvents typically used in peptide cleavage from SPPS resin. Solutions were analyzed by UPLC-MS using 1,3,5-trimethoxybenzene as an internal standard. Solution percentages are based on v/v.

In contrast to thioamide dimer **4**, thioimide dimer **5** was less sensitive than **4** to strong bases such as DBU and withstood epimerization against piperidine far better than **4** during the same time frame. Thioimide **5** showed a slow but measurable epimerization over time. This result led us to propose that the pK_a of the α -C in thioimides must be greater than thioamides (> 12) but ultimately lower than amides (< 15 , Scheme 1)

Both dimers (**4** and **5**) were also tested against trialkylamines bases used commonly for the peptide coupling steps (e.g., DIEA and NMM). Both **4** and **5** showed no epimerization over the times measured (Figure S#).

Finally, while the rate of epimerization is likely to be sequence dependent, the results in Figure 1A confirm our hypothesis that thioimidates will be far more resistant to epimerization than their thioamide analogues during SPPS.

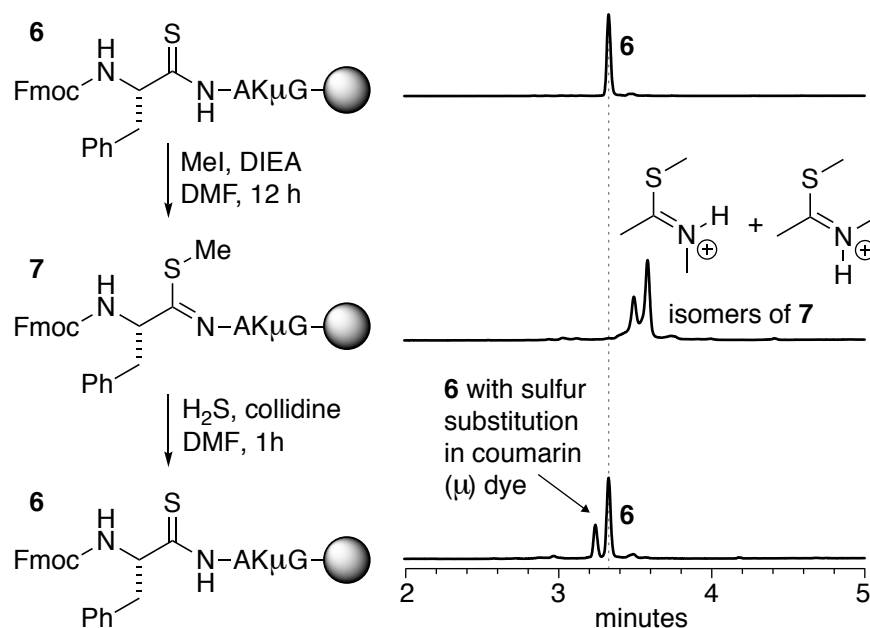
We next sought to evaluate the sensitivity of thioimidates to acidic conditions that are used commonly to cleave the peptide from the supporting solid resin and/or to cleave side chain protecting groups (Figure 1B). We observed that thioimide dimer **5** decomposed rapidly within 1 minute upon exposure to even dilute (2%) TFA. Alternatively, **5** displayed excellent stability to strong proton-donor solvents like TFE and HFIP that are employed to cleave protected peptides from so-called fast-cleaving resins (e.g., 2-Chlorotrityl resins).

The sensitivity of thioimidates to acid was not unexpected given the known basicity of imidates—the oxygen congener of **3** (imide $pK_{aH} \sim 6-7$).²²⁻²⁴ This sensitivity must be accounted for during synthetic planning. Thioamides are also prone to side reactivity during TFA cleavage from the resin, but strategies to mitigate unwanted reactivity have been reported.^{13, 25, 26} Thus, we propose that the thioimide must be converted back to a thioamide prior to any treatment with TFA, but the thioimide can be cleaved intact from fast-cleaving resins (e.g., 2-Chlorotrityl resins) with TFE and HFIP solutions. We this proof-of-concept reactivity established, we next sought to evaluate if thioamide/thioimide interconversion was feasible on solid-phase resin.

We synthesized the short oligomeric peptide **6** (Scheme 2). The thioamide was subsequently protected as the thioimide with MeI and the compatible trialkyl base, DIEA in DMF. The progress of the reaction (**6** → **7**) can be monitored via cleavage from a small amount of the resin followed by analysis with MS. The presence of two peaks for **7** was initially alarming, but we ascribe these two peaks to the cis and trans isomers shown in Scheme 2. Indeed, the NMR spectra for **5** shows two interconverting isomeric compounds which we ascribe to isomerism about the C=N bond in the thioimide.

Conversion back to the thioamide was accomplished by bubbling H₂S gas in the presence of a weak organic base such as collidine. We noticed a small amount of sulfur incorporation into the coumarin-tagged amino acid (denoted as μ in Scheme 2). In any event, thioamide **6** was restored.

Scheme 2: Interconversion of thioimideate “on-resin”.



CONCLUSION

We report a facile method to protect the stereochemical integrity of thioamides during synthesis. Thioamides represent important biophysical probes, but maintaining native chirality has been challenging until now. The reagents and procedures can be readily included into standard SPPS work-flows to enable easy implementation.

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Notes

Authors declare no competing financial interest.

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