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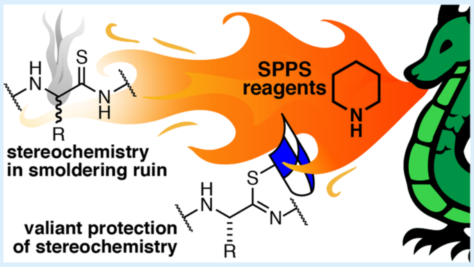
A Bottom-Up Approach To Preserve Thioamide Residue Stereochemistry during Fmoc Solid-Phase Peptide Synthesis

Luis A. Camacho, III, Bryan J. Lampkin, and Brett VanVeller*

Department of Chemistry, Iowa State University, Ames, Iowa 50011, United States

Supporting Information

ABSTRACT: Thioamides are useful biophysical probes for the study of peptide structure and folding. The α-C stereochemistry of thioamide amino acids, however, is easily epimerized during solid-phase peptide synthesis (SPPS), which limits the sequence space that is available to thioamide incorporation. This work demonstrates that the α-C stereochemistry of thioamides can be reversibly protected in a manner that is compatible with the standard methodology of SPPS to enable the facile implementation of thioamide probes.



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→π^* interactions between adjacent peptide bonds (i to i + 1).[6−8] Thioamides can also be employed in photochemical schemes as photoswitches[9,10] or as fluorescent quenchers to study conformational changes in peptides.[11−15]

In view of the importance of thioamides in peptide chemistry,[16] methods for their incorporation into peptides with robust stereochemical integrity are highly desirable. Activated thioamide precursors can be prepared from Fmoc-protected amino acids[17,18] and introduced 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 thioamide residue is prone to racemization under conditions required for Fmoc deprotection.[19−22] 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 the yield.[20−22] Indeed, the lack of literature precedent for a C-terminal thioamide, we surmise, is due to its current synthetic inaccessibility, where a thioamide at the C-terminus is necessarily exposed to Fmoc SPPS reagents for longer periods.[23] Accordingly, thioamides are often incorporated only within a few residues of the end of the sequence to limit such side reactivity. This approach, however, leaves a considerable amount of sequence space potentially off-limits to these important biophysical probes. Moreover, the growth in the number of thioamide-containing natural products, which continue to be discovered, further motivates the development of improved methods for thioamide synthesis.[1b]

The goal of this study was to develop a general strategy for the robust synthesis of thioamide-containing peptides with high stereochemical integrity.

The acidity of the α-proton next to a thioamide (**2**) moiety is ~3 pK_a units lower than that of the corresponding oxoamide (**1**) (Figure 1).[20,24] This difference arises from the ability of the lower-energy π^*(C═S) antibonding orbital relative to the π^*(C═O) counterpart to more favorably stabilize the anion of the conjugate base through resonance. Thus, we reasoned that raising the energy of the orbital responsible for stabilizing the

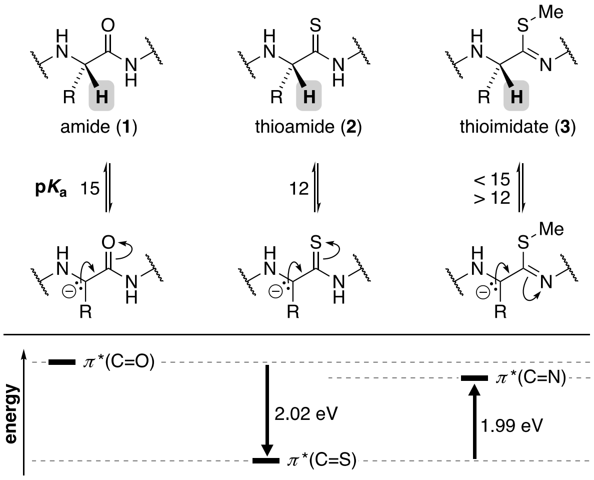


Figure 1. Rationale for the acidities of the α-CH moieties in **1**−**3**. Orbital energies were estimated by DFT B3LYP/6-311+g(d,p) NBO analysis.

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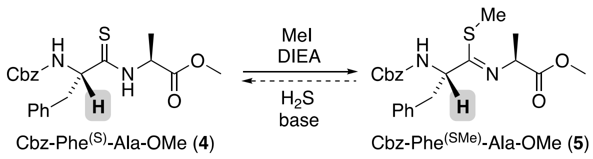
conjugate base would confer resistance to epimerization at the α-C stereocenter. We hypothesized that converting the thioamide into a thioimidate (**3**) would present a higher-energy π^*(C=N) orbital that does not stabilize the conjugate base as well and, in turn, decreases the acidity of the α-CH moiety.

The protection of a thioamide as a thioimidate has been employed previously in the synthesis of natural product derivatives,[25] but the α-CH pK_a has not been determined. Thus, to test our hypothesis, we synthesized thioimidate **5** from thioamide **4** (Scheme 1) to assess how **5** might resist epimerization.

Scheme 1. Protection of a Thioamide as a Thioimidate



We chose to investigate the stability of the model dimer Cbz-Phe$^{(X)}$-Ala-OMe (where X = S or SMe) because phenylalanine thioamides have been shown to be especially prone to epimerization at the α-C.[20] Notably, the conditions to make **5** are compatible with SPPS techniques (MeI/DIEA) and could conceivably be applied directly on resin following installation of the thioamide using standard thioacylating agents.[17,18]

We next exposed each dimer (**4** and **5**) to bases that are commonly employed during the Fmoc deprotection phase of SPPS and monitored the extent of epimerization by LCMS (Figure 2). Both dimers were also tested against trialkylamine bases commonly used for the peptide coupling steps (e.g., DIEA and NMM). Both **4** and **5** showed no epimerization with DIEA or NMM over the times measured (Figure S1), which agrees with previous observations.[20,26]

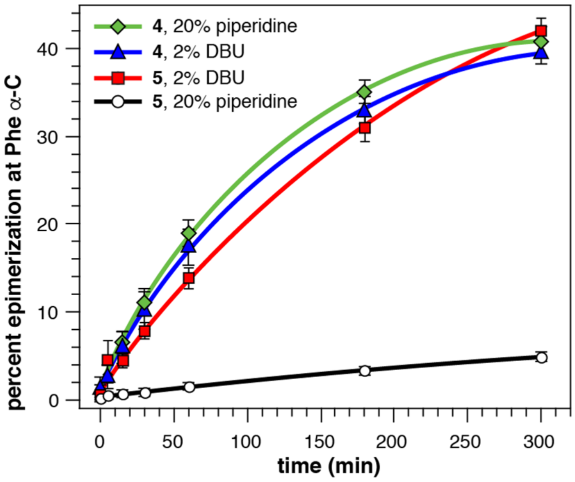


Figure 2. Epimeric stability of **4** and **5** with typical reagents used in SPPS. Solutions of **4** and **5** (0.1 M) in DMF with the indicated bases (v/v) relative to 1,3,5-trimethoxybenzene as internal standard.

The thioamide dimer **4** showed significant epimerization in under an hour in the presence of piperidine and DBU. Traditional time frames for Fmoc deprotection using 20% piperidine can be as long as 10 min per coupling. Thus, it is reasonable to conclude that significant epimerization of the thioamide α-C would occur if peptide elongation were carried out beyond even a few couplings after coupling of the thioamide residue. Moreover, such stereochemical impurities may be impossible to separate or even detect with longer sequences. While modified conditions for Fmoc deprotection with abbreviated deprotection times and lower concentrations of the base have been reported in an attempt to reduce the epimerization reaction shown here,[20,21] a drawback of shorter and more dilute Fmoc deprotection conditions is the potential for incomplete deprotection, leading to amino acid deletions.[27] Ultimately, the results in Figure 2 suggest that any attempts to modify the conditions will be stymied by the fact that the α-CH of the thioamide residue is simply too acidic to tolerate the basic conditions of Fmoc SPPS.

In contrast to thioamide **4**, the thioimidate protection in **5** withstood epimerization to a far greater extent in the presence of 20% piperidine in DMF (Figure 2). Alternatively, because **5** epimerizes at similar rates as **4** in the presence of DBU, it appears that the thioimidate is acidic enough to be deprotonated by bases that are stronger than piperidine (pK_{aH} = 11). The significantly diminished rate of epimerization of **5** over 300 min led us to conclude that the pK_a of the thioimidate must be greater than those of thioamides (>12) but ultimately lower than those of amides (<15) (Figure 1). While the rate of epimerization is likely sequence-specific, the results in Figure 2 confirm our design hypothesis that thioimidates will be far more resistant to epimerization than their thioamide analogues during SPPS. In addition, the higher-energy π^*(C=N) orbital is less electrophilic, protecting the thioamide site against nucleophilic degradation, which is a possible side reaction of thioamides during lengthy SPPS procedures. This result represents a significant advancement over the current status quo for thioamide stability during synthesis.

We next sought to evaluate the sensitivity of thioimidates to acidic conditions that are commonly used to cleave the peptide from the supporting solid resin and/or to cleave side-chain protecting groups (Figure 3). We observed that thioimidate dimer **5** decomposed rapidly within minutes upon exposure to even dilute (2%) TFA. Alternatively, **5** displayed excellent stability toward strong proton-donor solvents like TFE and

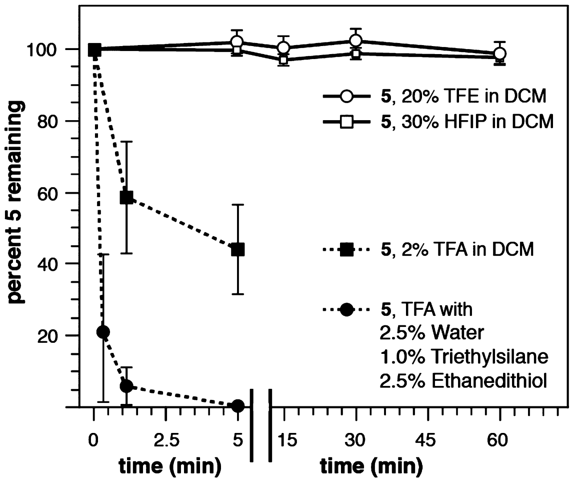


Figure 3. Stability of **5** (0.1 M) with typical solvents/reagents used for peptide cleavage from SPPS resin.

HFIP that are employed to cleave protected peptides from so-called super-acid-sensitive resins (e.g., 2-chlorotrityl resin).

The sensitivity of thioimidates to acid was not unexpected given the known basicity of imidates—the oxygen congeners of **3** (imidate $pK_{aH} \sim 6-7$).[28−30] This sensitivity, however, must be taken into account 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.[14,26,31] Thus, we propose that the thioimidate must be converted back to a thioamide prior to any treatment with TFA. Alternatively, the peptide containing the thioimidate can be cleaved intact from fast-cleaving resins (e.g., 2-chlorotrityl resin) with TFE and HFIP solutions. Thus, thioimidate protection functions in analogy to the protecting groups of the sensitive functional groups of amino acid side chains during SPPS, in which fully protected peptides can be cleaved under similar conditions of TFE or HFIP solutions. With this proof-of-concept reactivity established, we next sought to demonstrate conditions for the interconversion between thioamide and thioimidate that could be easily implemented within the SPPS workflow.

To assess the viability of thioimidate protection during SPPS, we synthesized the short sequence $F^{(S)}$AKFG (**6**) on 2-chlorotrityl resin following established procedures for the introduction of thioamide residues on-resin.[26] The thioamide was subsequently protected as the thioimidate with MeI and DIEA in DMF, conditions that are compatible with SPPS resins (**6** → **7**; Figure 4).[32] After installation of the thioimidate protection, SPPS could continue normally from **7** with Fmoc deprotection and amino acid coupling cycles to elongate the peptide. At the conclusion of the sequence, conversion back to the thioamide can then be accomplished with H_2S gas in the presence of a weak base such as collidine (**7** → **6**; Figure 4).

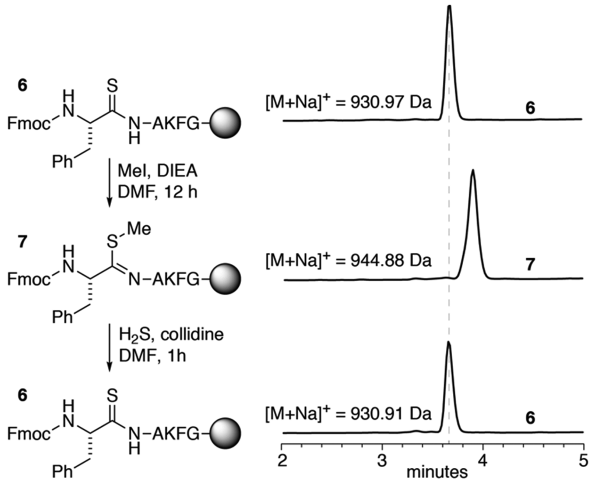


Figure 4. On-resin interconversion of thioamide to thioimidate and back to thioamide. The reactions can be performed using a standard peptide synthesis reaction vessel and were tracked with UPLC and MALDI-TOF.

The procedure for protection and deprotection of the thioamide described in Figure 4 can be easily implemented within the standard SPPS workflow to improve the stereochemical purity of thioamide-containing peptides (Table 1). The synthesis of **8** and **10** was carried out using conventional thioamide amino acid incorporation and peptide elongation. Unsurprisingly, significant epimerization of the thioamide residue occurs during subsequent Fmoc deprotection of additional amino acids. Alternatively, peptides **9** and **11** were synthesized by immediately protecting the thioamide as a thioimidate after installation and subsequently converting the thioimidate back to the thioamide once the final sequence was complete. In this manner, epimerization of the peptide was curtailed.

Table 1. Protection of α-C Stereochemistry during SPPS

peptide	sequence	thioimidate protection	epimerization of F^S (%)[a]
6	Fmoc-F^SAKFG-OH	–	<0.01
8	H-KAF^SAKFG-OH	no	12.8
9	H-KAF^SAKFG-OH	yes	<0.01
10	H-AAKAF^SAKFG-OH	no	22.1
11	H-AAKAF^SAKFG-OH	yes	<0.01

[a] Determined from peak areas of HPLC traces and control peptides containing authentic D-F^s. Identity confirmed by MALDI-TOF.

Because thioamides can be introduced into sequences using reliable thioacylating reagents,[17,18] there is a perception that current methods to incorporate thioamides into peptides via SPPS are sufficient. However, that perception is belied by the poor stability that thioamides display once they are incorporated and subsequent amino acids are coupled onto the peptide. This drawback may explain the limited sequence space in which these important biophysical probes have been employed.

We have developed a strategy to reversibly protect the thioamide as a thioimidate, which dramatically reduces epimerization of the α-C stereocenter. Our strategy for thioamides operationally mimics the current use of protecting groups for the sensitive functional groups of amino acid side chains and can be easily implemented within current Fmoc SPPS workflows. This work therefore provides immediate and enabling tools that allow peptide chemistry laboratories to address questions in the folding and stability of proteins by opening up previously uncharted sequence space to the incorporation of thioamide biophysical probes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b02598.

Experimental details, NMR spectra, and LC traces (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*bvv@iastate.edu

ORCID

Bryan J. Lampkin: 0000-0002-8633-1227
Brett VanVeller: 0000-0002-3792-0308

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) (a) Choudhary, A.; Raines, R. T. An Evaluation of Peptide-Bond Isosteres. *ChemBioChem* **2011**, *12*, 1801−1807. (b) 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) Mundlapati, V. R.; Gautam, S.; Sahoo, D. K.; Ghosh, A.; Biswal, H. S. Thioamide, a Hydrogen Bond Acceptor in Proteins and Nucleic Acids. *J. Phys. Chem. Lett.* **2017**, *8*, 4573−4579.

(3) Miwa, J. H.; Pallivathucal, L.; Gowda, S.; Lee, K. E. Conformational Stability of Helical Peptides Containing a Thioamide Linkage. *Org. Lett.* **2002**, *4*, 4655−7.

(4) Newberry, R. W.; VanVeller, B.; Raines, R. T. Thioamides in the Collagen Triple Helix. *Chem. Commun.* **2015**, *51*, 9624−7.

(5) 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 α-Helical, β-Sheet, and Polyproline II Helical Contexts. *Chem. Sci.* **2017**, *8*, 2868−2877.

(6) Newberry, R. W.; Raines, R. T. The $n \to \pi^*$ Interaction. *Acc. Chem. Res.* **2017**, *50*, 1838−1846.

(7) Newberry, R. W.; VanVeller, B.; Guzei, I. A.; Raines, R. T. $n \to \pi^*$ Interactions of Amides and Thioamides: Implications for Protein Stability. *J. Am. Chem. Soc.* **2013**, *135*, 7843−6.

(8) Choudhary, A.; Gandla, D.; Krow, G. R.; Raines, R. T. Nature of Amide Carbonyl−Carbonyl Interactions in Proteins. *J. Am. Chem. Soc.* **2009**, *131*, 7244−6.

(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*, 4910−8.

(10) Huang, Y.; Cong, Z.; Yang, L.; Dong, S. A Photoswitchable Thioxopeptide Bond Facilitates the Conformation-Activity Correlation Study of Insect Kinin. *J. Pept. Sci.* **2008**, *14*, 1062−8.

(11) 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*, 14718−20.

(12) 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*, 6827−37.

(13) Goldberg, J. M.; Wissner, R. F.; Klein, A. M.; Petersson, E. J. Thioamide Quenching of Intrinsic Protein Fluorescence. *Chem. Commun.* **2012**, *48*, 1550−2.

(14) 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*, 18651−8.

(15) 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. (Cambridge, U. K.)* **2018**, *54*, 1766−1769.

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

(17) Shalaby, M. A.; Grote, C. W.; Rapoport, H. Thiopeptide Synthesis. α-Amino Thionoacid Derivatives of Nitrobenzotriazole as Thioacylating Agents. *J. Org. Chem.* **1996**, *61*, 9045−9048.

(18) Yang, J.; Wang, C.; Xu, S.; Zhao, J. Ynamide-Mediated Thiopeptide Synthesis. *Angew. Chem., Int. Ed.* **2019**, *58*, 1382−1386.

(19) Bordwell, F. G. Equilibrium Acidities in Dimethyl-Sulfoxide Solution. *Acc. Chem. Res.* **1988**, *21*, 456−463.

(20) 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.

(21) 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.

(22) 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.

(23) Reiner, A.; Wildemann, D.; Fischer, G.; Kiefhaber, T. Effect of Thioxopeptide Bonds on α-Helix Structure and Stability. *J. Am. Chem. Soc.* **2008**, *130*, 8079−84.

(24) Bordwell, F. G. Equilibrium Acidities in Dimethyl Sulfoxide Solution. *Acc. Chem. Res.* **1988**, *21*, 456−463.

(25) Xie, J.; Okano, A.; Pierce, J. G.; James, R. C.; Stamm, S.; Crane, C. M.; Boger, D. L. Total Synthesis of [Ψ[C(=S)NH]Tpg^4]-Vancomycin Aglycon, [Ψ[C(=NH)NH]Tpg^4]Vancomycin Aglycon, and Related Key Compounds: Reengineering Vancomycin for Dual D-Ala-D-Ala and D-Ala-D-Lac Binding. *J. Am. Chem. Soc.* **2012**, *134*, 1284−97.

(26) Mukherjee, S.; Verma, H.; Chatterjee, J. Efficient Site-Specific Incorporation of Thioamides into Peptides on a Solid Support. *Org. Lett.* **2015**, *17*, 3150−3.

(27) Zinieris, N.; Leondiadis, L.; Ferderigos, N. Nα-Fmoc Removal from Resin-Bound Amino Acids by 5% Piperidine Solution. *J. Comb. Chem.* **2005**, *7*, 4−6.

(28) Hand, E. S.; Jencks, W. P. Mechanism of Reaction of Imido Esters with Amines. *J. Am. Chem. Soc.* **1962**, *84*, 3505−3514.

(29) Schmir, G. L.; Cunningham, B. A. Iminolactones. 1. The Mechanism of Hydrolysis. *J. Am. Chem. Soc.* **1965**, *87*, 5692−5701.

(30) Cunningham, B. A.; Schmir, G. L. Iminolactones 0.2. Catalytic Effects on Nature of Products of Hydrolysis. *J. Am. Chem. Soc.* **1966**, *88*, 551−558.

(31) Stolowitz, M. L.; Paape, B. A.; Dixit, V. M. Thioacetylation Method of Protein Sequencing: Derivatization of 2-Methyl-5(4*H*)-thiazolones for High-Performance Liquid Chromatographic Detection. *Anal. Biochem.* **1989**, *181*, 113−9.

(32) de Araujo, A. D.; Hoang, H. N.; Kok, W. M.; Diness, F.; Gupta, P.; Hill, T. A.; Driver, R. W.; Price, D. A.; Liras, S.; Fairlie, D. P. Comparative α-Helicity of Cyclic Pentapeptides in Water. *Angew. Chem., Int. Ed.* **2014**, *53*, 6965−9.

(33) Camacho, L., III; Lampkin, B. J.; VanVeller, B. A Strategy for Thioamide Incorporation during Fmoc Solid-Phase Peptide Synthesis with Robust Stereochemical Integrity. ChemRxiv Preprint, 2019; DOI: 10.26434/chemrxiv.8206247.v1.