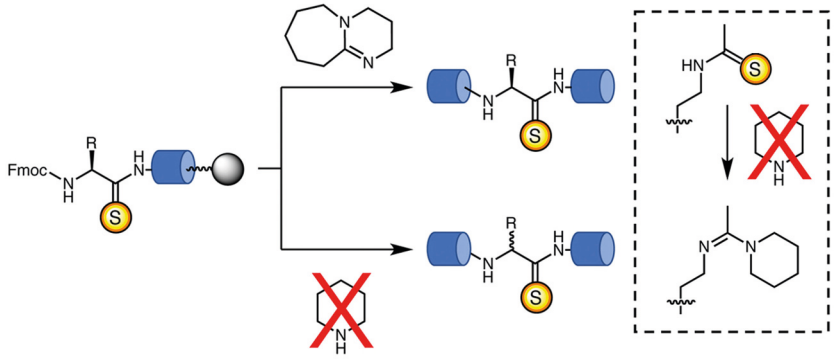
Synlett D. M. Szantai-Kis et al. Letter

Thieme Chemistry Journals Awardees – Where Are They Now? Improved Fmoc Deprotection Methods for the Synthesis of Thioamide-Containing Peptides and Proteins

D. Miklos Szantai-Kis[a‡]
Christopher R. Walters[b‡]
Taylor M. Barrett[b]
Eileen M. Hoang[b,c]
E. James Petersson[*a,b]

[a] Department of Biochemistry and Molecular Biophysics, Perelman School of Medicine, University of Pennsylvania, 3700 Hamilton Walk, Philadelphia, PA 19104, USA
ejpetersson@sas.upenn.edu
[b] Department of Chemistry, University of Pennsylvania, 231 S. 34th Street, Philadelphia, PA 19104, USA
[c] Swarthmore College, 500 College Ave, Swarthmore, PA 19081, USA

‡ These authors contributed equally to this work



Received: 12.03.2017
Accepted after revision: 10.04.2017
Published online: 10.04.2017
DOI: 10.1055/s-0036-1589027; Art ID: st2017-r0178-l

Abstract Site-selective incorporation of thioamides into peptides and proteins provides a useful tool for a wide range of applications. Current incorporation methods suffer from low yields as well as epimerization. Here, we describe how the use of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) rather than piperidine in fluorenylmethyloxycarbonyl (Fmoc) deprotection reduces epimerization and increases yields of thioamide-containing peptides. Furthermore, we demonstrate that the use of DBU avoids byproduct formation when synthesizing peptides containing side-chain thioamides.

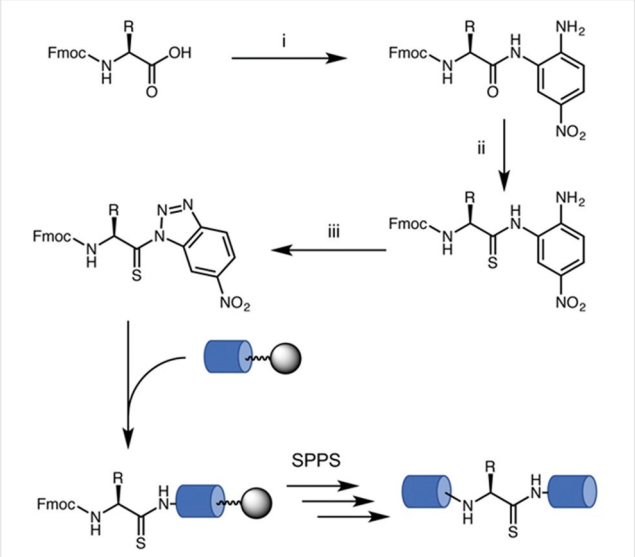
Key words peptides, sulfur, solid-phase synthesis, protecting groups, proteins

Thioamides are single-atom substitutions of the canonical amide bond in which the carbonyl oxygen is replaced by a sulfur atom. This substitution lends thioamides distinct physiochemical properties compared to their oxygen counterparts (herein referred to as 'oxoamides' for clarity), while being similar in size and structure.[1] Thioamides exhibit increased nucleophilicity and electrophilicity,[2] greater metal-binding affinity,[3] altered hydrogen-bonding propensities,[4] and unique spectroscopic features.[5,6] These properties have been exploited in numerous applications wherein thioamide-containing peptides have been used as metal-binding substrates,[7] photoswitches,[8] probes to monitor peptide or protein folding,[6,9] protease inhibitors,[10] and fluorescence quenchers.[5,11,12] Improved methods for the synthesis of thioamide-containing peptides will enable such biophysical experiments, as well as studies of thioamide-containing natural products.[13]

For these and other types of applications, site-specific incorporation of thioamides into peptides is required. This

E. James Petersson was educated at Dartmouth College, where he worked in the laboratory of David Lemal. He then studied under Dennis Dougherty at the California Institute of Technology as an NIH Predoctoral Fellow. After obtaining his PhD in 2005, he worked as an NIH Postdoctoral Fellow at Yale University with Alanna Schepartz. He was appointed as assistant professor in the Department of Chemistry at the University of Pennsylvania in 2008 and in the Biochemistry and Molecular Biophysics group in the Perelman School of Medicine in 2013. In 2015, he was promoted to the rank of associate professor. Prof. Petersson's research focuses on the development of new methods for protein labeling with synthetic amino acids and their application to studying protein folding and stability. He has been the recipient of several awards, including a Sloan Fellowship, an NSF CAREER award, recognition as a Searle Scholar, and a 2017 Thieme Chemistry Journals Award.

is usually achieved by synthesizing thioamide precursors which can then be incorporated into peptides using fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS). The activated precursors can be synthesized from Fmoc-protected amino acids in three steps (Scheme 1) based on a nitrobenzyltriazolide (Nbt) route developed by Rapoport et al.[14] Thioamides are generally compatible with the reagents used in SPPS including bases, activators, cleavage additives, and trifluoroacetic acid (TFA).



Scheme 1 Synthesis of activated nitrobenzotriazolide (Nbt) thioamide precursors for SPPS and their use to synthesize thioamide containing peptides. *Reagents and conditions*: i) Isobutyl chloroformate (IBCF), 4-nitro-*o*-phenylenediamine, *N*-methylmorpholine, THF, –20 °C → 25 °C, Ar;[15] ii) P_4S_{10}, Na_2CO_3, THF, Ar;[16] iii) $NaNO_2$, 95% $AcOH_{(aq)}$, 0 °C.[17]

There are three side reactions that can often occur with thioamide-containing peptides. First, prolonged exposure to high TFA concentrations during cleavage of the peptide from the resin can lead to an Edman degradation type reaction resulting in backbone cleavage at the n+1 position[18] (see Supporting Information, Figure S2). Therefore, especially for longer peptides, one must consider a tradeoff between complete removal of acid-labile protecting groups and degradation when determining the duration of TFA exposure. Second, our laboratory as well as Chatterjee et al. reported that thioamide precursors can react with residual amounts of water during coupling, leading to the exchange of sulfur for oxygen.[12,19] The use of anhydrous CH_2Cl_2 greatly reduced the formation of the undesired oxoamide. Last, epimerization can occur at the α-carbon of the thioamino acid (pK_a = ca. 13, see our rationale for estimating this value in Supporting Information[20]). Prolonged exposure to base during Fmoc deprotection can catalyze this epimerization reaction. Recently, it was reported that lowering the concentration of piperidine and shortening Fmoc deprotection times to one minute resulted in less epimerization of thioamides.[21] However, since the half-life of the Fmoc group under these conditions is 20–45 seconds, this approach significantly decreases the yield of isolated protein.[22]

A 20% (v/v) solution of piperidine in dimethylformamide (DMF) is the most commonly used solution for Fmoc deprotection in SPPS, but there are alternatives: A 50% (v/v) solution of morpholine in DMF is considered a milder condition and is often used for sensitive glycopeptides.[23] In contrast, a 2% (v/v) solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) is considered a harsher deprotection condition.[24] Unlike piperidine or morpholine, DBU is a non-nucleophilic base and is not able to scavenge the reactive dibenzofulvene group resulting from the Fmoc deprotection.[24] Therefore, either a nucleophile needs to be added as a scavenger, or reaction times must be kept short and the resin washed extensively after deprotection.

Here, we demonstrate that for the synthesis of thioamide-containing peptides (herein referred to as 'thiopeptides') the use of 2% (v/v) DBU in DMF as an Fmoc-deprotection solution is superior compared to 20% (v/v) piperidine in DMF. We synthesized several thiopeptides with either of the aforementioned deprotection solutions. Throughout this series, peptides synthesized with 2% DBU typically exhibited a lower percentage of epimerization than those synthesized with 20% piperidine, as well as higher isolated yield. Additionally, we encountered the formation of a side product when using piperidine or morpholine in the synthesis of N_ε-thioacetyl lysine (Lys(Ac^S)) containing peptides. This side product was not separable from the desired product via high-performance liquid chromatography (HPLC). However, the use of 2% DBU avoided the formation of this side product altogether. Thus, DBU deprotections are generally superior to piperidine deprotections for both backbone and side-chain thioamides.

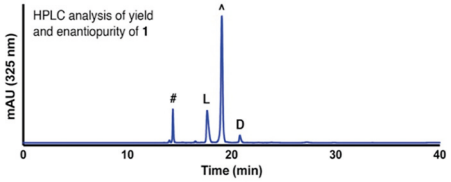
We designed a set of model peptides containing a mixture of nonpolar (Phe and Ala) and polar (Lys and Glu) amino acids (Table 1). For each sequence, 7-methoxycoumarin-4-ylalanine (Mcm or µ) was installed as the first amino acid on 2-chlorotrityl resin. This chromophore has an absorption maximum at 325 nm and allows for facile tracking of the peptides by analytical HPLC. Thioamides also have a shifted absorption maximum (272 nm) relative to oxoamides (215 nm), which can be used to characterize the thiopeptides. To discriminate between genuine L-thiaomino acid peptides and their D-epimers, we synthesized both L- and D-thionitrobenzotriazolide monomers (Fmoc-ala^S-NBt and Fmoc-phe^S-NBt, using the lower-case letter convention for D-amino acids) for inclusion into the model peptides.[15–17] The retention times for the authentic D-thioamino acid peptides were used as standards for each of the first four sets of thiopeptides. Additionally, a separate assay was performed to assess how each condition affected yield. In these experiments, 7-methoxycoumarin-4-acetic acid (Mca) was added to each peptide sample to a final concentration of 500 µM, representing the maximum theoretical yield of

peptide in each HPLC sample. The integration value of this standard peak was compared to the integration of the product peak in each chromatogram (at 325 nm) to calculate the yield.

We began our analysis with peptide **1**, which contained a thioamide linkage at the central Phe. Treatment with 20% piperidine (v/v) in DMF for 20 minutes led to epimerization of 16%, as calculated by the ratio of peak integrations (at 325 nm) for the L- and D-epimers. The identical masses of the L- and D-product peaks were confirmed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). To test DBU deprotection conditions, the same peptide was synthesized and elongated up to the thioamino acid position using standard SPPS with 20% piperidine deprotections. After the thioacylation reaction, the peptide was treated with 2% v/v DBU in DMF (2 mL) for two minutes before draining and washing the resin and then repeating the process twice more. Each subsequent deprotection was done using these conditions. It should be noted that a scavenger was not used in the deprotection solution, but no characterizable byproducts containing the mass of a dibenzofulvene adduct were observed. The aforementioned deprotection conditions lead to only 3% epimerization, significantly decreasing the amount of D-byproduct observed in comparison to the piperidine deprotections. In addition, the yield was increased from 30% to 36% when DBU was used (Table 1). Oxoamide controls for the L- and D-peptides were also synthesized using piperidine as the deprotection reagent and no epimerization was observed for these peptides (Figure S10).

Encouraged by these results, we synthesized four additional peptide series (**2**–**5**) with L- and D-thioamino acids. Series **2** changes the sequence to put the Phe^S linkage at the N-terminus of the peptide, allowing us to quantify the epimerization effects from a single deprotection reaction. Only 1% epimerization was detected with piperidine; epimerization was undetectable with DBU conditions. This result indicates that the initial deprotection step may not have a significant effect and implies that the thioamino acid is vulnerable to epimerization at each subsequent deprotection step. The epimerization of thiopeptides may also depend upon local sequence or the identity of the thioamino acid. Therefore, we synthesized peptides **3** and **4** with Ala^S at each Ala position within the same sequence as peptide **2**. We detected 3% epimerization with piperidine and 1% epimerization with DBU for peptide **3**. This is in agreement with the expected additive effects of multiple piperidine treatments. Unfortunately, the L- and D-epimers of peptide **4** were not separable by analytical HPLC. This highlights the need for Fmoc removal conditions that minimize epimerization of thioamino acid positions, as epimerization byproducts may not always be chromatographically distinguishable from the desired product.

Table 1 Comparison of Yield and Epimerization between Peptides Synthesized with either DBU or Piperidine[a]



Peptide[b]	Epimerization (%)	Purity (%)[c]	Yield (%)
KAF^{S}AKμ (**1**) – piperidine	16.1	56.2	29.9
KAF^{S}AKμ (**1**) – DBU	5.0[d]	61.1	35.9
F^{S}AKAKμ (**2**) – piperidine	1.0	89.0	44.3
F^{S}AKAKμ (**2**) – DBU	0.0	88.5	51.3
FA^{S}KAKμ (**3**) – piperidine	3.2	68.5	44.0
FA^{S}KAKμ (**3**) – DBU	1.0	67.3	44.7
FAE^{S}AKμ (**5**) – piperidine	5.6	71.5	18.2
FAE^{S}AKμ (**5**) – DBU	1.0	71.6	22.7
CVNY^{S}EEFVQMMTAK (**6**) – piperidine	49.9	8.6	n/d[d]
CVNY^{S}EEFVQMMTAK (**6**) – DBU	9.3	12.3	n/d[d]
CVNYEEF^{S}VQMMTAK (**7**) – piperidine	28.1	25.0	n/d[d]
CVNYEEF^{S}VQMMTAK (**7**) – DBU	4.5[e]	22.0[e]	n/d[d]

[a] Graph: Example chromatogram for epimer quantification and yield determination. L and D denote epimers of **1**, KAF^{S}AKμ and KAf^{S}AKμ, respectively, # denotes KAAKμ, the product of failed F^S coupling. ^ denotes Mca standard used to quantify yield.
[b] Peptides in one-letter code. Superscript S denotes thioamino acids; μ = 7-methoxycoumarin-4-yl-alanine.
[c] Purity calculation based on HPLC peak area of desired product.
[d] No external standard added for yield quantification.
[e] Average of two experiments.

The peptides synthesized thus far contained nonpolar thioamino acids, therefore we synthesized peptide **5** containing a Glu^S linkage to verify that epimerization can be suppressed independent of side-chain identity (although % epimerization may vary depending on amino acid identity). In agreement with the effects discussed in peptides **1**–**3**, DBU treatments for peptide **5** decreased epimerization from 6% to 1%. The peak-area percentage was calculated for peptides **1**–**3** and **5** in order to quantify the effects of DBU deprotections on the purity of the products. In each case, the purity of the desired L-peptide was similar across methods, with the exception of peptide **1**, where purity was increased using the DBU method (Figure S2). In samples containing the Mca standard discussed above, the yield of desired L-product was either maintained or increased through use of DBU, with the most significant increases in yield observed in peptides **1** and **2** (30% to 36% and 44% to 51%, respectively). Taken together, these results indicate that in sequences where there are several deprotections after the thioamide installation, it is beneficial to use DBU as a

deprotection method to reduce epimerization and even increase yield and purity of the desired L-thiopeptide product.

To extend these results to a longer, more sequence diverse system, we synthesized a peptide derived from the C-terminus of the calcium-binding protein calmodulin (CaM).[25] We have described the synthesis and characterization of $CaM_{135\text{-}148}$-Cys_{135} including many thioamide substitutions recently,[26] and we wished to see what improvements could be made by using DBU deprotection conditions. We established two target sites at N-terminal and central positions within the 14-mer peptide ($CaM_{135\text{-}148}$-$Cys_{135}Tyr^{S}_{138}$ and $CaM_{135\text{-}148}$-$Cys_{135}Phe^{S}_{141}$, **6-L** and **7-L**, respectively). Each peptide was synthesized on a 50 µmol scale on 2-chlorotrityl resin with either piperidine or DBU deprotection conditions. Thiopeptide products were detected by their characteristic absorbance at 272 nm and percent epimerization calculated for each peptide based on the retention times of an authentic D-thioamino acid peptide ($CaM_{135\text{-}148}$-$Cys_{135}phe^{S}_{141}$, **7-D**) and MALDI-MS analysis of the peaks from analytical HPLC chromatograms (**6** and **7**). In each of the CaM thiopeptides, the epimerization with piperidine is more exaggerated than with any of the model peptides discussed thus far (50% and 28% for **6** and **7**, respectively). As expected, DBU deprotection decreased the observed epimerization percentages for peptides **6** and **7** to 9% and 5%, respectively. These results highlight the positional and sequence variability in the amount of epimerization observed with piperidine-based Fmoc removal, as well as the net positive effects that DBU deprotections can have in all cases observed here. However, by chromatographic peak area percentage analysis, DBU does appear to decrease the overall purity in **7** as it may lead to additional byproducts that we were unable to assign by MALDI-MS analysis. We hope to further improve our methodology by using an additive during Fmoc deprotection in order to decrease the formation of undesired byproducts. Nevertheless, DBU deprotections did lead to a 7% increase in peak area percentage for **6**.

Finally, we compared our DBU protocol to the protocol used in Mukherjee et al., which also reported reduced epimerization during thiopeptide synthesis.[21] Peptide **1** was synthesized up to the coupling of Phe^{S}_{3}, and then the resin was split. For one half of the resin, the peptide was elongated using 2 × 1 minute Fmoc deprotections with 10% piperidine, as in Mukherjee et al. For the other half of the resin, we elongated the peptide using our procedure of 3 × 2 minute deprotections with 2% DBU. We observed similar purity (63% vs. 61% with DBU), but a higher degree of epimerization (7% vs. 1% with DBU) in the synthesis using 10% piperidine. We also synthesized the longer peptide **7** using the same split-resin approach for direct comparison of the two methods. Here, we observed somewhat lower purity (14% vs. 17% with DBU) with significantly higher epimerization (23% vs. 2% with DBU) in the synthesis using 10% piperidine. Thus, we find that although the two methods give peptides of comparable overall purity, the lower levels of epimerization seen with DBU represent a significant advantage for our method, as peptide epimers may not always be separable by HPLC (as seen for peptide **4**).

We are also interested in incorporating side-chain thioamides as probes for fluorescence-quenching studies or protein interactions and as mimics of post-translational modifications. Thus, we attempted to synthesize peptide **9** (Figure 1) containing the side-chain thioamide $Lys(Ac^{S})$. However, during this synthesis, we encountered a +51 mass product. This product was not observed while synthesizing the corresponding oxopeptide **8** (Figure 1), which contained N_{ε}-(acetyl)-lysine. This implied that the modification must have been part of the thioamide motif. To investigate this, we tested several different cleavage solutions to cleave the peptide from the resin (Table S1). However, all tested cleavage conditions still resulted in the +51 mass adduct, leading us to believe that this side product was not formed during peptide cleavage.

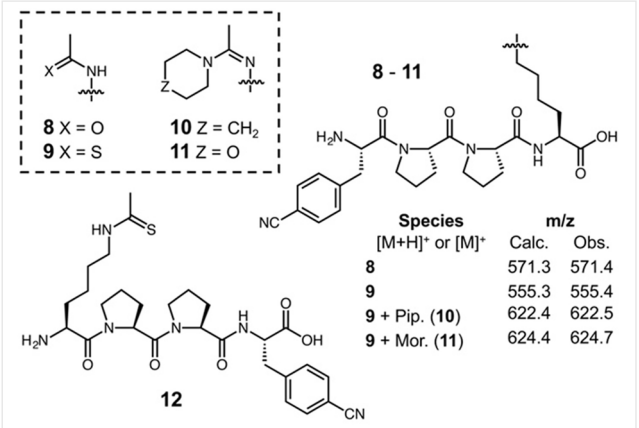
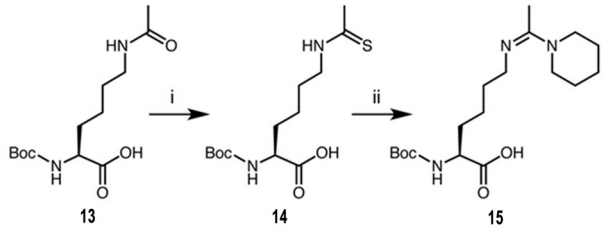


Figure 1 $Lys(Ac^{S})$-containing peptides used to study byproduct formation under piperidine deprotection conditions. MALDI-MS masses of **8**, **9** (using DBU deprotection), **10** (byproduct of **9** synthesis using piperidine deprotection), and **11** (byproduct of **9** synthesis using morpholine deprotection); Pip. = piperidine, Mor. = morpholine.

We hypothesized that the +51 mass corresponded to piperidine adduct **10**. Indeed, thioamides have been used previously to prepare amidines, as in Boger's syntheses of vancomycin derivatives.[27] This was confirmed with several experiments. We synthesized peptide **9** using either 50% (v/v) morpholine in DMF or 2% (v/v) DBU in DMF as deprotection solutions. We observed a +53 mass when Fmoc deprotections were performed with morpholine, which we believe corresponds to peptide **11**. No side product was observed in the peptide synthesized with DBU. We also synthesized peptide **12** (Figure 1), where N_{α}-(Boc)-N_{ε}-(thioacetyl)lysine [Boc-$Lys(Ac^{S})$] was installed at the N-terminus of the peptide and no +51 side product was observed after cleavage (TFA only to cleave Boc group, no base required).

To further verify adduct formation, we dissolved Boc-Lys(Ac^S) in 50% (v/v) piperidine in DMF and isolated lysyl amidine **15** by HPLC (Scheme 2).[28,29] NMR, UV/vis spectroscopy, and high-resolution mass spectrometry confirmed the identity of **15**. Specifically, we observed cross peaks in a nuclear Overhauser effect spectroscopy (NOESY) experiment between the CH_3 group on the side chain with the piperidinyl ring as well as the protons attached to the δ-carbon of the Lys side chain. Additionally, we observed correlations of that same methyl group with two nitrogen atoms using proton–nitrogen heteronuclear multibond correlation (HMBC) spectroscopy. A more detailed illustration of the observed correlations can be found in the Supporting Information (Figure S7). These amino acid and peptide studies confirm the necessity of using DBU in Fmoc deprotections when synthesizing Lys(Ac^S)-containing peptides. We are currently investigating the syntheses of peptides containing other side-chain thioamide groups.



Scheme 2 Synthesis of **15**. *Reagents and conditions*: i) ethyl dithioacetate, Na_2CO_3, EtOH–H_2O;[28] ii) 50% piperidine in DMF.[29]

With the preparation of each backbone thiopeptide system, we have demonstrated reduction of epimerization at the thioamino acid α-carbon by performing Fmoc-deprotection reactions with DBU in the place of piperidine. Although only four different amino acids were tested (Ala^S, Phe^S, Glu^S, and Tyr^S), the positional and sequence variability with which this effect is demonstrated provides strong evidence that this result is general. Furthermore, we demonstrated that in at least one side-chain thioamide case, the use of DBU as the deprotection reagent was superior to piperidine or morpholine. While we have not yet observed a piperidine adduct in a backbone thioamide, it may occur at sterically accessible sites such as Gly^S residues. Such adducts would presumably not be stable, but could lead to Edman-type degradation, as well as hydrolysis of the peptide backbone or oxoamide formation by reaction with water under the acidic resin cleavage conditions. All of these side reactions would be avoided by using DBU for Fmoc deprotections during peptide synthesis. We expect that these synthetic developments will help to improve the accessibility of thioamide-containing peptides and proteins, further enabling the broad spectrum of biophysical and biochemistry experiments made possible by this versatile functional group.

Funding Information

This work was supported by funding from the National Science Foundation (NSF CHE-1150351 to E.J.P.). Instruments supported by the NSF and National Institutes of Health include: HRMS (NIH RR-023444) and MALDI-TOF MS (NSF MRI-0820996). C.R.W. thanks the NIH for funding through the Structural Biology and Molecular Biophysics Training Program (T32 GM008275). T.M.B. thanks the NIH for funding through the Chemistry-Biology Interface Training Program (T32 GM071399).

Acknowledgment

We thank Dr. George Furst and Dr. Jun Gu for assistance with NMR spectroscopy and Dr. Charles Ross III for assistance with HRMS.

Supporting Information

Supporting information for this article is available online at https://doi.org /10.1055/s-0036-1589027. A detailed description of materials and experimental methods used in this manuscript can be found in the Supporting Information.

References and Notes

(1) (a) Bondi, A. *J. Phys. Chem.* **1964**, *68*, 441. (b) La Cour, T. F. M.; Hansen, H. A. S.; Clausen, K. I. M.; Lawesson, S. O. *Int. J. Peptide Protein Res.* **1983**, *22*, 509. (c) Bardi, R.; Piazzesi, A. M.; Toniolo, C.; Jensen, O. E.; Omar, R. S.; Senning, A. *Biopolymers* **1988**, *27*, 747.
(2) (a) Wipf, P.; Hayes, G. B. *Tetrahedron* **1998**, *54*, 6987. (b) Jagodziński, T. S. *Chem. Rev.* **2003**, *103*, 197.
(3) Misra, S. K.; Tewari, U. C. *Transition Met. Chem.* **2002**, *27*, 120.
(4) Dudek, E. P.; Dudek, G. O. *J. Org. Chem.* **1967**, *32*, 823.
(5) Goldberg, J. M.; Batjargal, S.; Petersson, E. J. *J. Am. Chem. Soc.* **2010**, *132*, 14718.
(6) Miwa, J. H.; Pallivathucal, L.; Gowda, S.; Lee, K. E. *Org. Lett.* **2002**, *4*, 4655.
(7) Bond, M. D.; Holmquist, B.; Vallee, B. L. *J. Inorg. Biochem.* **1986**, *28*, 97.
(8) Wildemann, D.; Schiene-Fischer, C.; Aumüller, T.; Bachmann, A.; Kiefhaber, T.; Lücke, C.; Fischer, G. *J. Am. Chem. Soc.* **2007**, *129*, 4910.
(9) (a) Culik, R. M.; Jo, H.; DeGrado, W. F.; Gai, F. *J. Am. Chem. Soc.* **2012**, *134*, 8026. (b) Miwa, J. H.; Patel, A. K.; Vivatrat, N.; Popek, S. M.; Meyer, A. M. *Org. Lett.* **2001**, *3*, 3373.
(10) Goldberg, J. M.; Chen, X.; Meinhardt, N.; Greenbaum, D. C.; Petersson, E. J. *J. Am. Chem. Soc.* **2014**, *136*, 2086.
(11) Goldberg, J. M.; Wissner, R. F.; Klein, A. M.; Petersson, E. J. *Chem. Commun.* **2012**, *48*, 1550.
(12) Goldberg, J. M.; Batjargal, S.; Chen, B. S.; Petersson, E. J. *J. Am. Chem. Soc.* **2013**, *135*, 18651.
(13) (a) Hayakawa, Y.; Sasaki, K.; Adachi, H.; Furihata, K.; Nagai, K.; Shin-ya, K. *J. Antibiot.* **2006**, *59*, 1. (b) Lincke, T.; Behnken, S.; Ishida, K.; Roth, M.; Hertweck, C. *Angew. Chem. Int. Ed.* **2010**, *49*, 2011.
(14) Shalaby, M. A.; Grote, C. W.; Rapoport, H. *J. Org. Chem.* **1996**, *61*, 9045.
(15) Thioamino acid precursors for SPPS were synthesized as Fmoc-thionitrobenzotriazolide monomers. The synthesis follows a standard route shown in Scheme 1. The synthesis of Fmoc-Phe^S-

Nbt serves as an example. See the Supporting Information for structures of compounds **S1**–**S3**.

(9*H*-fluoren-9-yl)methyl(*R*)-{1-[(2-amino-5-nitrophenyl)amino]-1-oxo-3-phenylpropan-2-yl}carbamate (S1)

Fmoc-Phe-OH (1.29 mmol, 500 mg) was dissolved in THF (15 mL) and stirred under argon. The solution was then cooled to –10 °C. NMM (2.58 mmol, 0.284 mL) was slowly added. Next, isobutyl chloroformate (1.42 mmol, 0.186 mL) was carefully added dropwise. The reaction was then stirred at –10 °C for 15 min, after which 4-nitro-*o*-phenylenediamine (1.42 mmol, 218 mg) was added. The reaction was stirred at –10 °C for 2 h and continued stirring at r.t. overnight under argon. The next day the solvent was removed in vacuo. The yellow solid was dissolved in DMF (20 mL), and the product was precipitated by addition of sat. KCl solution (100 mL). This precipitate was filtered and washed with cold water and Et_2O. The product was dried under vacuum overnight. Compound **S1** (1.13 mmol, 589 mg) was obtained in a crude yield of 87.4% and used without further purification

(16) **(9*H*-Fluoren-9-yl)methyl-(*R*)-(1-[(2-amino-5-nitrophenyl)amino]-3-phenyl-1-thioxopropan-2-yl)carbamate (S2)**

Na_2CO_3 (1.13 mmol, 120 mg) and P_4S_{10} (1.13 mmol, 502 mg) were suspended in THF (20 mL) and was stirred under argon atmosphere at r.t. for 30 min. The reaction was cooled to 0 °C. Compound **S1** (1.13 mmol, 589 mg) was dissolved in THF (10 mL) and added to the reaction. After 1.5 h, the reaction was complete and condensed to dark yellow oil by rotary evaporation. The crude product was run over a short silica plug column to remove insoluble P_4S_{10} aggregates. The crude product was purified by flash using EtOAc–hexanes (1:1). The desired product **S2** was obtained as an orange powder in 54.2% yield (0.612 mmol, 330 mg). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.07 (s, 1 H), 7.90 (dd, *J* = 9.0, 2.6 Hz, 1 H), 7.73 (dd, *J* = 10.6, 7.5 Hz, 2 H), 7.58 (s, 1 H), 7.48 (d, *J* = 7.5 Hz, 1 H), 7.41 – 7.32 (m, 3 H), 7.30–7.17 (m, 9 H), 6.48 (d, *J* = 9.0 Hz, 1 H), 5.78 (s, 1 H), 4.86 (s, 1 H), 4.10 (dt, *J* = 16.7, 8.3 Hz, 5 H), 3.21 (d, *J* = 9.2 Hz, 0 H), 1.59 (s, 1 H), 1.36–1.04 (m, 1 H).

(17) **(9*H*-Fluoren-9-yl)methyl-(*R*)-(1-{6-nitro-1*H*-benzo[*d*][1,2,3]triazol-1-yl}-3-phenyl-1-thioxopropan-2-yl)carbamate (S3)**

Compound **S2** (0.612 mmol, 330 mg) was dissolved in 95% $AcOH_{(aq)}$ (10 mL) and cooled to 0 °C. After 5 min, $NaNO_2$ (0.765 mmol, 52.8 mg) was added slowly to the reaction. After 30 min, ice-cold Milli-Q water (100 mL) was added to the reaction. The resulting orange precipitate was filtered and washed with additional cold Milli-Q water. After drying the product was obtained as an orange powder in 84.0% yield (0.514 mmol, 256 mg) and was used directly in SPPS without further purification. ^{1}H NMR (500 MHz, $CDCl_3$, major rotamer only): δ = 9.61 (s, 1 H), 8.44 (d, *J* = 8.9 Hz, 1 H), 8.29 (d, *J* = 8.9 Hz, 1 H), 7.74 (d, *J* = 7.6 Hz, 2 H), 7.52 (t, *J* = 6.9 Hz, 2 H), 7.38 (t, *J* = 7.6 Hz, 2 H), 7.28 (q, *J* = 8.0 Hz, 2 H), 7.23–7.13 (m, 5 H), 6.56 (d, *J* = 7.4 Hz, 1 H), 5.66 (d, *J* = 9.3 Hz, 1 H), 4.46–4.35 (m, 1 H), 4.33 (t, *J* = 9.0 Hz, 1 H), 3.40 (dd, *J* = 14.0, 5.4 Hz, 1 H), 3.10 (dd, *J* = 13.8, 8.0 Hz, 1 H). ESI$^+$-HRMS: *m/z* calcd for $C_{30}H_{23}N_5O_4S^+$: 550.1549; found $[M + H]^+$: 550.1550.

(18) Stolowitz, M. L.; Paape, B. A.; Dixit, V. M. *Anal. Biochem.* **1989**, *181*, 113.

(19) Mukherjee, S.; Verma, H.; Chatterjee, J. *Org. Lett.* **2015**, *17*, 3150.

(20) (a) Bordwell, F. G.; Bartmess, J. E.; Hautala, J. A. *J. Org. Chem.* **1978**, *43*, 3095. (b) Bordwell, F. G.; Fried, H. E. *J. Org. Chem.* **1991**, *56*, 4218. (c) Bordwell, F. G.; Ji, G. Z. *J. Am. Chem. Soc.* **1991**, *113*, 8398. (d) Huang, Y.; Jahreis, G.; Lucke, C.; Wildemann, D.; Fischer, G. *J. Am. Chem. Soc.* **2010**, *132*, 7578. (e) Stroud, E. D.; Fife, D. J.; Smith, G. G. *J. Org. Chem.* **1983**, *48*, 5368.

(21) Mukherjee, S.; Chatterjee, J. *J. Pept. Sci.* **2016**, *22*, 664.

(22) Zinieris, N.; Leondiadis, L.; Ferderigos, N. *J. Comb. Chem.* **2005**, *7*, 4.

(23) Liebe, B.; Kunz, H. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 618.

(24) Wade, J. D.; Bedford, J.; Sheppard, R. C.; Tregear, G. W. *Pept. Res.* **1991**, *4*, 194.

(25) Chin, D.; Means, A. R. *Trends Cell Biol.* **2000**, *10*, 322.

(26) Walters, C. R.; Szantai-Kis, D. M.; Zhang, Y. T.; Reinert, Z.; Horne, W. S.; Chenoweth, D. M.; Petersson, E. J. *Chem. Sci.* **2017**, *8*, 2868.

(27) Okano, A.; James, R. C.; Pierce, J. G.; Xie, J.; Boger, D. L. *J. Am. Chem. Soc.* **2012**, *134*, 8790.

(28) **N^2-(*tert*-Butoxycarbonyl)-N^6-ethanethioyl-L-lysine (Boc-Lys(Ac^S)-OH, 14)**

Boc-Lys-OH (**13**, 369 mg, 1.50 mmol, 1.0 equiv) was suspended in EtOH (4.4 mL) and 10% (w/v) Na_2CO_3 solution (4.0 mL) added. Ethyl dithioacetate (189 µL, 1.65 mmol, 1.1 equiv) added and stirred overnight. The solvent was removed in vacuo and the solid redissolved in H_2O (10 mL). The reaction mixture was acidified with 3 M HCl until the solution became milky white (ca. pH 2). The aqueous phase was extracted three times with $CHCl_3$ (10 mL). The combined organic phase was dried over Na_2SO_4, and the solvent was removed in vacuo. The product was obtained as yellow foam in high yield (423 mg, 1.39 mmol, 92.4%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 10.88 (s, 1 H), 8.37 (s, 1 H), 5.35 (d, *J* = 7.7 Hz, 1 H), 4.07 (d, *J* = 75.4 Hz, 1 H), 3.52 (s, 2 H), 2.50–2.34 (m, 3 H), 1.77 (s, 1 H), 1.69–1.49 (m, 3 H), 1.42–1.23 (m, 11 H). ESI$^+$-HRMS: *m/z* calcd for $C_{13}H_{25}N_2O_4S^+$: 305.1535; found $[M + H]^+$: 305.1556.

(29) **N^2-(*tert*-Butoxycarbonyl)-N^6-[1-(piperidin-1-yl)ethylidene]-L-lysine (15)**

Boc-Lys(Ac^S)-OH (**14**, 133 mg, 0.438 mmol) was dissolved in 50% (v/v) piperidine in DMF (2 mL). After stirring for 5 h, the reaction mixture was diluted with 0.1% TFA in H_2O and purified by reverse phase HPLC. Fractions containing product **15** or unreacted starting material **14** were collected separately and lyophilized. After lyophilization starting material **14** was dissolved in 50% (v/v) piperidine in DMF and, after 5 h the reaction was purified as before. This procedure was repeated one more time until enough product was collected for NMR analysis (1.90 mg, 4.05 µmol, 0.9%). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 12.58 (s, 1 H), 8.68 (s, 1 H), 7.05 (d, *J* = 7.8 Hz, 1 H), 3.84 (td, *J* = 8.8, 4.6 Hz, 1 H), 3.57 (s, 4 H), 3.32 (s, 2 H), 2.28 (s, 3 H), 1.67–1.54 (m, 8 H), 1.49 (dt, *J* = 13.3, 7.0 Hz, 2 H), 1.37 (s, 9 H), 1.36–1.28 (m, 2 H). ^{13}C NMR (126 MHz, DMSO): δ = 174.28, 162.44, 155.65, 78.02, 53.36 (+), 49.45 (–), 46.48 (–), 43.89 (–), 30.38 (–), 28.86 (–), 28.25 (+), 25.64 (–), 24.74 (–), 23.06 (–), 22.65 (–), 14.35 (+). ESI$^+$-HRMS: *m/z* calcd for $C_{23}H_{27}N_2O_4S^+$: 356.2549; found $[M + H]^+$: 356.2558. Additional 2D NMR correlations are given in the Supporting Information.