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Efficient Site-Specific Incorporation of Thioamides into Peptides on a Solid Support

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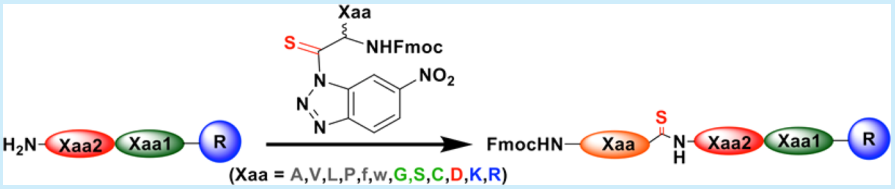
Supporting Information



ABSTRACT: Designing bioactive peptides containing "thioamide" functionality to modulate their pharmacological properties has been thwarted so far because of various synthetic challenges. The fast, efficient, and inexpensive synthesis and incorporation of a wide range of thionated amino acids into a growing peptide chain on a solid support is reported using standard Fmoc-based chemistry. The commonly employed methodology is comprehensively investigated and optimized with significant improvements regarding the quantity of reagents and reaction conditions. The utility of the protocol is further demonstrated in the synthesis of dithionated linear and monothionated cyclic peptides, which has been a daunting task.

Replacement of an amide bond in a peptide by structurally analogous functionalities has opened up a whole new area of peptide bond surrogates. Such replacement of a peptide bond consequently alters the spectroscopic, physicochemical, structural, and biological properties of the native peptide, giving rise to enhanced biological activities and intriguing structural features. Of the various peptide bond surrogates known, a "thioamide", where the peptide bond "O" atom is replaced with "S" (Figure 1),

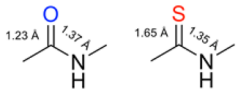


Figure 1. Comparison of bond lengths between amide and thioamide.

is perhaps the closest and simplest one.[1] Although a thioamide is an isosteric replacement of an amide bond, it manifests markedly different structural and biological properties.[2] The C═S bond in thioamide is significantly longer (~1.65 Å) than a C═O bond in the peptide (~1.23 Å) due to the increased size of sulfur.[3] Thioamide N−H acts as a stronger H-bond donor than the "oxo" amide N−H, and S acts as a weaker H-bond acceptor than O. This aspect has, in fact, been utilized to site-specifically study the dynamics of hydrogen bond formation in β-sheets and α-helices.[4,5] Thiopeptides have also demonstrated enhanced in vivo activity by virtue of their enzymatic stability as compared to their "oxo" congeners.[6]

One of the major hurdles toward the study of thionated peptides has been the installation of thioamide linkage into peptides in a site-specific and controlled manner. Available literature on incorporation of the thioamide moiety into a peptide chain has been limited to either using thionating agents such as Lawesson's reagent (LR)[7] or using thioacylating agents such as thiobenzotriazolides,[8] thioacylfluorobenzimidazolinone,[6] and the azirine/oxazolone method.[9] Thionating agents are often required in excess under long refluxing conditions in anhydrous solvents.[7,10] Such treatment usually furnishes an inseparable mixture of products, which restricts their usage for the site-specific installation of the thioamide unit. Their utility is mostly limited to peptides with hydrophobic side chains.[7] Usually, thioacylating reagents are superior to thionating reagents in terms of achieving site-specific incorporation of the thioamide moiety. However, use of thioacylating reagents can suffer from oxo-amide formation and their epimerization during formation under basic conditions.[11]

Following the work of Rapoport et al.,[8] we sought to optimize the reaction conditions used to incorporate thioamide moieties into a growing peptide on a solid support. Herein, we present the systematic and comprehensive optimization study of such site-specific thioamide incorporation using Fmoc chemistry. We have made significant improvements over the conventional methodology (Table 2) regarding the amount of reagents used, time required, conversion, purity, substrate scope, etc. Additionally, we have elaborated our optimized protocol to incorporate two consecutive thioamide linkages into a growing peptide chain.

To obtain the thioamide precursors of the commercially available orthogonally protected Fmoc amino acids (**2a−l**), we modified the protocols reported by Degrado[4b] and Rapoport et al.[8] We chose the HCTU/DIPEA method over the conventional IBCF/NMM method as it furnished **2a−l** in higher yields. It is worth noting that in all the cases except the Arg-aminoanilide precursor (**11**) (Table 1) only a single purification step after the

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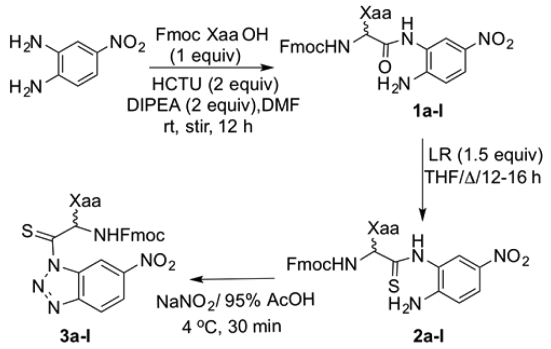
Table 1. Synthesis of Benzotriazolide Precursors from Fmoc Amino Acids



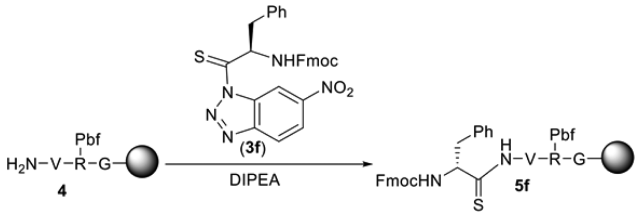
entry	compd	Xaa	yield (%)[a]
1	**2a**	Ala	53
2	**2b**	Val	76
3	**2c**	Leu	60
4	**2d**	Pro	83
5	**2e**	Gly	80
6	**2f**	D-Phe	81
7	**2g**	D-Trp(Boc)	66
8	**2h**	Ser(*t*-Bu)	53
9	**2i**	Cys(Trt)	34
10	**2j**	Asp(*t*-Bu)	63
11	**2k**	Lys(Boc)	25
12	**2l**	Arg(Pbf)	92

[a]Yields of **2a**–**l** after chromatographic purification; **1l** was purified prior to thionation.

thionation with Lawesson's reagent was sufficient to obtain **2a**–**k** with high purity (for details, refer to Supporting Information). Furthermore, the thionation of the aminoanilide precursor of Fmoc-Lys(Boc)-OH (**1k**) into the corresponding thioacyl precursor **2k** with Lawesson's reagent led to partial deprotection of the Boc-protecting group. Thus, we reprotected the free primary amine side chain of lysine using $(Boc)_2O/NaHCO_3$ (Figure S11). The benzotriazolides (**3a**–**l**) were generated prior to the coupling onto the resin-bound peptide and were used directly without purification due to their instability.

To identify the suitable solvent and reaction conditions for the incorporation of thioamides into a peptide on a solid support, we chose the tripeptide sequence VRG loaded on 2Cl-TCP resin to react with D-phenylalanine benzotriazolide (**3f**) in the presence of a non-nucleophilic base, DIPEA, as our model system. **3f** was chosen because of its ease of synthesis and handling. We carried out our experiments on 0.03 mmol scale (with respect to 2Cl-TCP resin). We observed that the solvent had a significant impact on the conversion and on the undesired formation of the oxo-amide **4a** via a water-mediated pathway (Table 2a). Our results indicated anhydrous dichloromethane (DCM) to be the solvent of choice, but the comparable efficacy of regular DCM prompted us to use it throughout the entire study, circumventing the laborious process of solvent drying. Next, we optimized the quantity of the benzotriazolide, base, and time necessary to achieve a quantitative conversion of the N-term deprotected tripeptide (**4**) to the corresponding thionated tetrapeptide (**5f**) (Table 2b). It was surprising that the reduction in the amount of the benzotriazolide and the base from 5 to 1 equiv and from 10 equiv to none, respectively, had almost no effect on the conversion of **4** to **5f**.

Table 2. Optimization of Reaction Conditions for Thioamide Incorporation on a Solid Support



a)	entry[a]	solvent	**4** (%)	**4a** (%)	**5f** (%)
	1	THF	trace	24	76
	2	THF (anhyd)	trace	6	94
	3	DMF	43	18	39
	4	NMP	42	33	25
	5	DCE	21	ND	79
	6	DCM	2	ND	98
	7	**DCM (anhyd)**	1	ND	99

b)	entry[b]	**3f** (equiv × times)	DIPEA (equiv × times)	time (min)	**4**/**5f** (%)
	1	5 × 2	10 × 2	90 × 2	2:98
	2	3.5 × 2	7 × 2	90 × 2	3:97
	3	2 × 2	4 × 2	90 × 2	7:93
	4	2 × 2	4 × 2	45 × 2	2:98
	5	2 × 2	2 × 2	45 × 2	1:99
	6	2 × 2	1 × 2	45 × 2	1:99
	7	1.5 × 2	3 × 2	45 × 2	2:98
	8	**1.5 × 2**	**1.5 × 2**	**45 × 2**	**1:99**
	9	1.5 × 2	0.75 × 2	45 × 2	1:99
	10	1.5 × 2	NIL	45 × 2	2:98
	11	1 × 2	2 × 2	45 × 2	2:98

[a]Optimization done with 5 equiv × 2 of **3f**. **4a**: Fmoc-fVR(Pbf)G-OH. [b]Optimization done with DCM; % conversion calculated by HPLC.

Thus, conventional use of 5 equiv of benzotriazolide and 10 equiv of DIPEA twice[12] was found to be unnecessary, as suggested by our data (Table 2b). In fact, the excessive use of thioacylating agents along with base led to further complications, such as degradation and conversion into the oxo-amide peptide, while attempting the insertion of two consecutive thioamide moieties in a peptide sequence. Although the importance of the base was unclear at small-scale (0.03 mmol) couplings, we observed its necessity while scaling up (0.18 mmol) the benzotriazolide coupling for preparative purposes, to neutralize the residual acetic acid (Figure S32). It is important to note here that, although for our particular model scheme, we could achieve comparable conversions using even lesser amounts of reagents (entry 11, Table 2b), the substrate generality was lacking, as we obtained lower yields with other sequences.

Subsequently, the optimized condition (entry 8, Table 2b) was utilized to synthesize the monothionated peptides **5a**–**l** using **3a**–**l** with the common peptide sequence VRG loaded onto 2Cl-TCP resin to demonstrate the substrate scope with a wide variety of orthogonally protected benzotriazolides (Table 3b). Most of the conversions, except for **5b**, **5e**, and **5i**, were quantitative with high purity (Table 3a). We speculated that the lower conversion of **5b** and **5i** was presumably due to the *β*-branch in Val (**3b**) and the bulky trityl side chain present in Cys (**3i**). An increase in the amount of **3i** (2.5 equiv × 2) also did not improve the conversion to **5i**. While we tried to install **3b** and **3i** on a different sequence, fVRG (henceforth, the lower case letter would refer to a D-amino acid, while the symbol "prime" would refer to the thioamide moiety), there was hardly any improvement on the conversion

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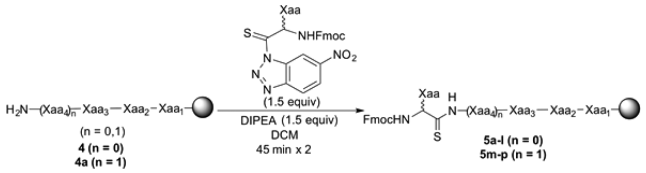
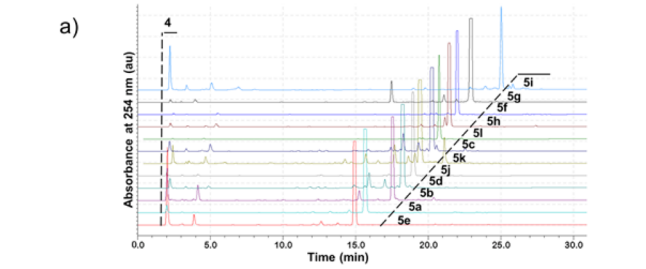
Table 3. Synthesis of Monothionated Peptides Using the Optimized Protocol





b)

entry	compd	sequence[a]	conv (%)
1	**5a**	A′VRG	96
2	**5b**	V′VRG	83
3	**5c**	L′VRG	95
4	**5d**	P′VRG	95
5	**5e**	G′VRG[b]	69
6	**5f**	fVRG	99
7	**5g**	w′VRG	87
8	**5h**	S′VRG	92
9	**5i**	C′VRG	72
10	**5j**	D′VRG	97
11	**5k**	K′VRG	92
12	**5l**	R′VRG	99
13	**5m**	V′fVRG	80
14	**5n**	G′fVRG[b]	80
15	**5o**	G′DfVR[b]	81
16	**5p**	C′fVRG	74

[a]Fmoc and orthogonally protected; % conversion calculated by HPLC. [b]Reaction with 5 equiv of benzotriazolide (1×), 30 min without base.

(Table 3b and Figures S48 and S55). This suggests that, for **3b** and **3i**, the steric demand of the side chains dictates the final conversion into the thionated peptides.

Coupling of glycine benzotriazolide (**3e**) onto **4** to yield **5e** turned out to be rather challenging. The optimized condition, as discussed above, led to only 56% conversion into **5e**. We discovered that **3e** was unstable in the presence of DIPEA, leading into its slow degradation, consequently lowering the product conversion. Thus, we performed a series of reactions to optimize the coupling efficiency of **3e** onto **4** (Table S2). However, we could achieve a maximum of 69% conversion into **5e** with 5 equiv of **3e** and a shorter reaction time of 30 min. Nevertheless, when this optimized condition was tested against two other sequences, **5n** and **5o**, we could achieve higher conversions of 80 and 81%, respectively (Figures S50 and S53), suggesting that the coupling efficiency of **3e** onto peptides is sequence-dependent. The HPLC chromatogram of **5h** revealed the presence of two peaks of identical mass in the ratio of 2:1, suggesting an epimerization during the incorporation of **3h** (Figure S36). To circumvent this problem, we performed the reaction in the absence of DIPEA, which slightly improved the ratio (2.5:1) but significantly reduced the conversion to **5h** (78% for both diastereomers), re-emphasizing the robustness of our optimized protocol (Figure S37). These results suggested that the epimerization of **3h** took place during the formation of **1h** using HCTU and DIPEA. Therefore, we resynthesized **1h** using the IBCF and NMM method[12] and performed the coupling of **3h** with **4**, which significantly suppressed the epimerization, yielding **5h** (entry 8, Table 3b) in an epimeric ratio 15:1 (Figure S38).

We further applied our optimized protocol to incorporate two consecutive thioamide linkages (Table 4) into a peptide sequence, which is rather difficult to introduce via solution-phase thionation. With the exception of the V′f′VRG sequence (entry 4), we obtained reasonably good conversion and crude purity (Figures S57–S82) of the synthesized dithionated peptides.[7b] However, V′f′VRG and a few others displayed the formation of a compound with 34 Da lower mass than the expected peptide. We envisioned that this sequence-dependent phenomenon could be attributed to the formation of a thiazole moiety via an acid-catalyzed "Edman degradation"[11a] type transformation with the elimination of H_2S (M = 34) (see Supporting Information for details). Note that the synthesized dithionated peptides with our optimized protocol were absolutely free of any oxo-amide counterpart.[13]

Table 4. Synthesized Dithionated Peptides with the Optimized Protocol

entry	compd	sequence[a]	conv (%)	M − 34 (%)
1	**6**	A′V′VRG	89	0
2	**7**	f′V′VRG	77	23
3	**8**	A′f′VRG	80	18
4	**9**	V′f′VRG	0	76
5	**10**	G′f′VRG	60	0
6	**11**	f′f′VRG	95	0
7	**12**	w′f′VRG	73	0
8	**13**	D′f′VRG	95	0
9	**14**	R′f′VRG	98	0
10	**15**	f′D′VRG	90	10
11	**16**	D′D′VRG	99	0
12	**17**	f′R′VRG	80	16
13	**18**	D′R′VRG	100	0

[a]Fmoc and orthogonally protected; % conversion calculated by HPLC.

To further broaden the scope of this protocol, we attempted the synthesis of a monothionated cyclic peptide (Scheme 1) as peptide macrocycles are gaining attention over the past few years.[14] Synthesis of the linear peptide **21** with an internal thioamide was accomplished using the optimized protocol, and even after repetitive Fmoc deprotections and amino acid couplings, the crude peptide showed a remarkably clean profile with no traces of epimerization or oxo-amide formation (Scheme 1a and Figure S83). Finally, **21** was cleaved off of the resin and cyclized via HOBt/DIC (Scheme 1b) and DPPA/$NaHCO_3$ (Scheme 1c) methods in solution to form **22** with significant purity and conversion, suggesting the compatibility of thioamides with the cyclization reagents. Unfortunately, the final global deprotection of **22** led to spontaneous degradation, and we failed to characterize the degradation products. Therefore, we further synthesized cyclo(-RGD′fV-) using DPPA/$NaHCO_3$-mediated cyclization (Figures S88–S91) and deprotected the orthogonal protecting groups with a milder cleavage cocktail of TFA/DCM/TIPS/H_2O (20:75:2.5:2.5) in contrast to the reported one (Figure S92).[15]

However, it is important to note that in our hands multiple orthogonally protected thiopeptides yielded the final deprotected peptide with a cleavage cocktail of TFA/DCM/TIPS/H_2O (47.5:47.5:2.5:2.5) without substantial loss due to cleavage

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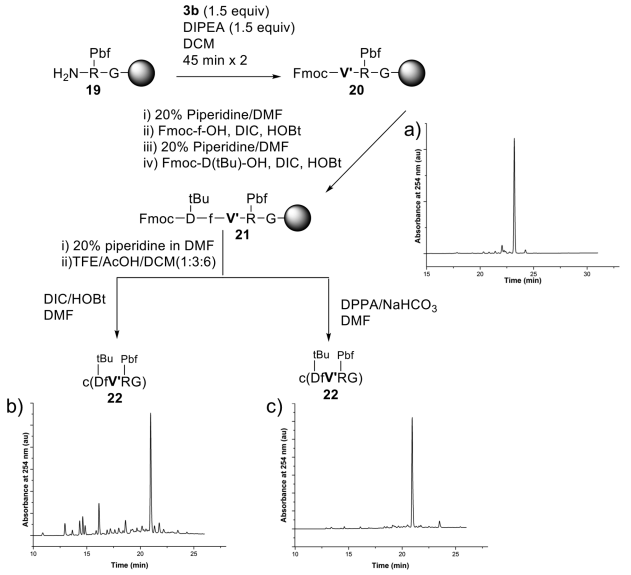
Scheme 1. Synthesis of Monothionated Cyclic Peptide 22



of the peptide bond following thioamide linkage. We observed that these thionated cyclic peptides are stable over a period of months without any traceable degradation or oxidation. To the best of our knowledge, this is the first report synthesizing a cyclic thioamide-containing peptide on a solid support in a controlled, regiospecific way using thioacylating agent under mild conditions (Figure S94).

In summary, we have successfully incorporated one and two consecutive thioamide moieties into a growing peptide chain on a solid support in a site-specific manner, avoiding any side reactions that complicate the synthesis. Additionally, we have shown the broad substrate scope of our optimized protocol by manifesting examples of aliphatic, aromatic, hydrophilic, and hydrophobic amino acids with diverse orthogonal protecting groups. Finally, we applied our protocol for the synthesis of cyclic peptides containing a thioamide linkage and demonstrated the compatibility of thioamide linkage with base, two major cyclization agents, and acidic conditions necessary for global deprotection. We strongly believe that, with this thoroughly optimized synthetic strategy, mono- to multiply thionated linear and cyclic peptides would be accessible and can pave the way toward the long sought use of thioamides as a tool to modulate pharmacological properties of peptides.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, HPLC traces, and characterization data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01484.

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Notes

The authors declare no competing financial interest.

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