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Improved acidolytic deprotection conditions for the Fmoc-based solid-phase synthesis of thioxo peptides

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Abstract—The acid-mediated cleavage of a synthetic thioxo peptide was monitored using deprotection cocktails with varying concentrations of TFA. Thioxo peptides are acid labile, undergoing cleavage at the amide linkage immediately following the thioamide linkage in the sequence. This acid lability makes Fmoc-based synthesis the method of choice for thioxo peptide preparation. The extent of cleavage increases with increased TFA concentration and longer reaction time. Data presented herein indicate that deprotection protocols involving low TFA concentration (ca. 80%) and short reaction times (ca. 2 h) minimize the losses due to acidolytic cleavage.

Thioxo peptides, homologs of peptides in which one or more of the backbone amide linkages are replaced by thioamide linkages, have received considerable attention recently for several reasons. Thioxo peptide analogs of biologically active peptides have shown, in some cases, increased activity, increased receptor selectivity, and increased enzymatic stability.[1] The spectroscopic properties of the thioamide differ sufficiently from those of the amide to allow the thioamide to serve as a probe of local conformation even in a large peptide.[2] The thioamide NH is a stronger hydrogen bond donor than the amide NH,[3] so thioxo peptide analogs of peptides with well-defined conformations can provide information about the influence of backbone hydrogen bonding on conformation.

Most studies of thioxo peptides have been limited to small, flexible peptides, but advances in the synthesis of thioacylating agents should allow the site-specific incorporation of thioamides into synthetic peptides of any length.[4,5] Published strategies for inserting thioamides into synthetic peptides rely primarily on the Boc/benzyl protection strategy, in which the Boc group is used for temporary protection of the amino functionality and benzyl ethers and esters are used for protection of reactive side chains for the duration of the synthesis. Boc/benzyl syntheses require treatment of the peptide with TFA (usually 50%) following the addition of each amino acid to the peptide chain, and require a final deprotection step with strong acid (HF or TFMSA) to remove the peptide from the resin and simultaneously remove side-chain protecting groups. The repeated acidolytic Boc deprotection steps are problematic, leading to thiazolone formation and resultant cleavage of the peptide at the amide bond following the thioamide bond in the sequence. This reaction is mechanistically similar to the Edman degradation, and a version of the Edman reaction using an N-terminal thioacetyl group completely cleaves the N-terminal residue of a peptide in just 10 min in neat TFA.[6]

In our hands, the extent of TFA-induced cleavage at sites within the peptide chain appears to be sequence dependent, and can be as high as 5–10% of the peptide chains during a standard 30 min treatment with 50% TFA. Treatment with TFMSA or HF induces the same cleavage reaction to an even greater extent than TFA. In one example acidolytic cleavage during a TFMSA deprotection consumed more than 60% of the thioxo peptide. The literature offers few alternatives for the preparation of thioxo peptides, although Schutkowski and coworkers have had success with Lewis acid deprotection of thioxo peptides prepared using extremely

Abbreviations: Boc, *tert*-butoxycarbonyl; Bpoc, 2-(biphenyl-4-yl)-propan-2-yloxycarbonyl; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMF, dimethylformamide; EDT, ethanedithiol; ESI-MS, electrospray ionization-mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; RP-HPLC, reverse-phase high-performance liquid chromatography; *t*-Bu, *tert*-butyl; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; TIS, triisopropylsilane.

Keywords: thioxo peptide; thioamide; solid-phase peptide synthesis; thiazolone.

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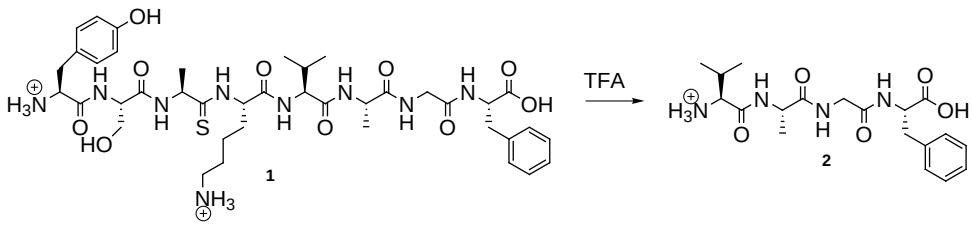
acid labile resins and the Bpoc amino protecting group.[7] The Fmoc/*t*-Bu protection strategy is an attractive alternative to the Boc/benzyl approach because it requires only a single acid treatment (typically 90–95% TFA for 2–4 h) at the end of the synthesis.

The long-term goal of our research is to prepare large thioxo peptides (12–50 residues) in order to study the influence of backbone hydrogen-bonding on peptide conformation. The Fmoc/*t*-Bu protection strategy offers the advantages of relatively low cost, commercially available amino acids and resins that are compatible with automated peptide synthesizers. Thus, we sought to modify this standard methodology to allow high yield synthesis of thioxo peptides. Optimum deprotection conditions for synthetic peptides vary depending on the amino acids present, their number and sequence, their side-chain protecting groups. For thioxo peptides, there is the additional consideration of the acid lability of the thioxo peptide. We present data on the cleavage of a thioxo peptide in several different deprotection cocktails to be used as a guide in developing optimum deprotection conditions for a particular thioxo peptide.

In designing our study, we sought to compare currently used deprotection strategies and to develop a strategy that will work for a wide range of synthetic peptides. Our test peptide was designed to include two of the most common protecting groups used in Fmoc-based peptide synthesis: the *tert*-butyl ether and the *tert*-butyl carbamate. A third common protecting group, the *tert*-butyl ester, is more labile in acid than both the ether and the carbamate and is expected to be easily cleaved under the conditions reported here.[8] The cocktails tested include scavengers typically used to trap reactive carbonium ions during cleavage.

This study was performed using an eight-residue thioxo peptide (**1**) and the corresponding C-terminal tetrapeptide (**2**) that is produced following acidolytic cleavage (Scheme 1). Both peptides were prepared by solid-phase synthesis on the Wang resin using Fmoc-protected amino acids. Amino acids were coupled using DIPCDI/HOBt activation in DMF, starting with commercially available Fmoc-phenylalanyl Wang resin. The thioxo peptide was prepared using Fmoc-Ala-thioxo-6-nitrobenzotriazolide for thioacylation.† Protection of reactive side chains was achieved with Boc (Lys) and tBu (Tyr and Ser). Synthesis was carried out using an Advanced ChemTech Model 90 benchtop peptide synthesizer. Peptides were purified by RP-HPLC and characterized by NMR and ESI-MS.‡ We developed an RP-HPLC protocol to cleanly separate the thioxo peptide from the tetrapeptide cleavage product and used this protocol to follow the acidolytic cleavage of the pure thioxo peptide.



Scheme 1.

Table 1. Extent of peptide backbone cleavage resulting from treatment of thioxo peptide with different cleavage cocktails and reaction times

Entry	% TFA	Cleavage cocktail	% of peptide cleaved[a]			
			0.5 h	1.0 h	2.0 h	3.0 h
1	95	A: TFA, water, TIS (95:2.5, 2.5)	10	19	29	36
2	92.5	B: TFA, EDT, water, TIS (92.5, 2.5, 2.5, 2.5)	10	17	27	33
3	90	C: TFA, thioanisole, EDT, anisole (90:5:3:2)	8	15	26	30
4	87.5	D: TFA, thioanisole, water, EDT (87.5:5:5:2.5)	8	14	25	30
5	83	E: TFA, water, TIS (83:12:5)	5	12	18	23
6	80	F: TFA, water, TIS (80:15:5)	3	7	10	12
7	80	G: TFA, water, TIS, EDT (80:7.5:5:2.5)	2	6	8	10

[a] % of peptide cleaved is calculated based on RP-HPLC at 225 nm as (area of peak **2**)/((area of peak **1**)+(area of peak **2**)).

† The Fmoc-Ala-thioxo-6-nitrobenzotriazolide was prepared according to a modified procedure from Ref. 5.

‡ Peptide **1**: ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.53 (1H, d), 8.44 (1H, d), 8.39 (1H, dd), 8.37 (1H, d), 8.33 (1H, d), 8.25 (1H, d), 8.21 (1H, d), 7.39 (2H, m), 7.34 (1H, m), 7.30 (2H, d), 7.15 (2H, d), 6.86 (2H, d), 4.97 (1H, m), 4.66 (1H, m), 4.6 (2H, m), 4.5 (1H, m), 4.35 (1H, m), 4.18 (1H, m), 3.97 (2H, m), 3.88 (2H, m), 3.15 (2H, m), 3.0 (3H, m), 2.92 (1H, m), 2.13 (1H, m), 2.02 (2H, m), 1.70 (2H, m), 1.45 (2H, m), 1.42 (3H, d), 1.39 (3H, d), 0.98 (3H, d), 0.95 (3H, d); ESI-MS 858.5 (calcd 857.4). Peptide **2**: ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.39 (1H, dd), 8.25 (1H, d), 8.21 (1H, d), 7.39 (2H, m), 7.32 (3H, m), 4.66 (1H, m), 4.35 (1H, m), 4.18 (1H, m), 3.97 (2H, m), 3.11 (2H, m), 2.13 (1H, m), 1.39 (3H, d), 0.98 (3H, d), 0.95 (3H, d); ESI-MS 393.4 (calcd 392.2).

Seven deprotection cocktails with varying concentrations of TFA were evaluated. Results are summarized in Table 1. In each experiment, 10 mg of thioxo peptide was suspended in 500 μL cocktail for 3 h at room temperature, with occasional swirling. Aliquots were removed and analyzed by RP-HPLC; integration of the peaks corresponding to the full-length thioxo peptide and the truncated peptide was used to calculate the percentage of thioxo peptide that was cleaved at the Lys–Val bond. Cleavage of the peptide continued throughout the length of the experiment, demonstrating the importance of keeping reaction times as short as possible. Acidolytic cleavage increased uniformly with increasing TFA concentration. The minor components of the cocktails (water, thiols, silanes) had little effect on the extent of cleavage observed.

To confirm that deprotection cocktails with lower concentrations of TFA can remove side-chain protecting groups and cleave peptide from the Wang resin, deprotection cocktails F and G were tested on resin-bound, protected thioxo peptide. Resin-bound thioxo peptide (250 mg, 0.092 mmol peptide/g based on quantitative analysis of Fmoc group) was treated with piperidine to remove the N-terminal Fmoc group and then suspended in the cocktail (5 mL) for 2 h at room temperature with occasional swirling. The crude peptide was immediately precipitated with ether, filtered, and dried in vacuo. Crude peptide was suspended in 2.5% acetic acid and purified by RP-HPLC. The yields of pure **1** obtained were 14.4 mg (73%) for cocktail F and 15.1 mg (76%) for cocktail G. RP-HPLC analysis of the crude peptide indicated that the truncated peptide accounted for approximately 12% of the peptide cleaved from the resin.

In summary, we present data on the acidolytic cleavage of a synthetic thioxo peptide. A deprotection cocktail with a TFA concentration of 80% is suitable for use with thioxo peptides prepared by solid-phase synthesis with the Fmoc/*t*-Bu protection strategy. While some thioxo peptide is inevitably lost to thiazolone-mediated acidolytic cleavage, this cleavage can be minimized by limiting the TFA concentration in the deprotection cocktail and keeping the reaction time short. Side-chain protecting groups can be selected for compatibility with these deprotection conditions, and thioxo peptides can be prepared in good yield.

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