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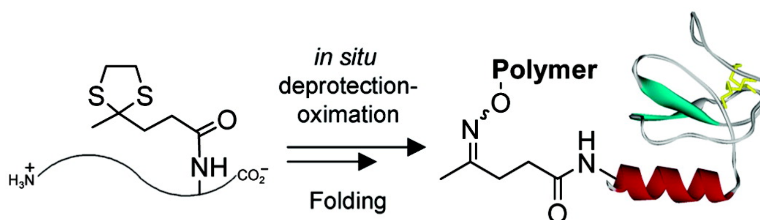

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A New Approach to the Chemical Synthesis of Keto-Proteins

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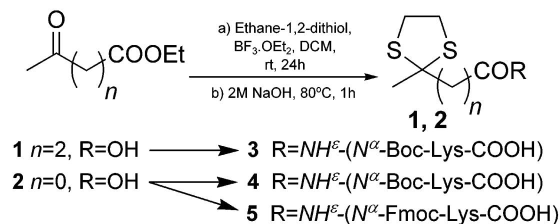
The conjugation of polymers to chemically synthesized or recombinant proteins has valuable research and therapeutic applications. Two recent examples include synthetic erythropoiesis protein (SEP),[1] a monodisperse 51-kilodalton protein-polymer construct, and pegylated interferon alpha-2b.[2] With the firm establishment of native chemical ligation as a practical method for the total synthesis of proteins,[3] we have sought to develop synthetic strategies that can be used to engineer or tune the biological or physiochemical properties of synthetic proteins in a site-specific manner.[1,4] Moreover, we have focused on simplifying and improving synthetic methods for the preparation of peptides with tailored chemoselective functionality to facilitate the selective chemical modification of proteins, for instance, with monodisperse polymers.[5]

Keto-peptides are important derivatives for participating in chemoselective conjugation processes with aminooxy compounds in aqueous solution.[6] The introduction of keto groups into peptides in the form of levulinic,[1] pyruvic,[7] or keto-containing amino acids[8] can be carried out during or after solid-phase chain assembly at an orthogonally protected amine, such as the N^{ϵ}-amino group of a given lysine residue. Transamination of *N*-terminal peptide residues with sodium glyoxylate and Cu^{2+} catalysis is another route to keto-peptides.[9] Although these chemistries can indeed give the desired keto-peptide, our experiences are that their synthesis, cleavage, analysis, and isolation have been significantly more complex, variable and lower yielding than that of the parent peptide. This difference is by and large more prominent with increasing peptide size and functionality and becomes an important consideration for protein synthesis which typically employs relatively large peptides (>30 residues). In addition, the shelf life of the keto-peptides after purification is generally reduced.[10]

We explored the feasibility of developing a protecting group strategy primarily for keto groups (e.g., levulinyl[1]) that would be compatible with contemporary chemical protein synthesis strategies. This goal was based upon the rationale that the release of reactive keto groups during acidolytic (TFA or HF) deprotection, resin cleavage, and purification processes is generally more problematic and ideally should be avoided. To meet our needs, a reversible protection strategy that would allow for the complete protection of the keto group throughout peptide assembly, peptide side-chain deprotection, cleavage from resin, purification, and native chemical ligation processes was necessary. After considering the suitability of different classes of carbonyl protecting groups,[11] we examined cyclic ketals, specifically 1,3-dioxolanes, 1,3-oxothiolanes, and 1,3-dithiolanes. Initial experiments with simple model peptide systems coupled with various cyclic ketal-protected forms of levulinic (Lev) and pyruvic (Pyr) acids demonstrated that only the 1,3-dithiolane group had the required acid stability for our needs. The 1,3-dioxolane group was readily hydrolyzed in 97% aqueous TFA (1 h), whereas the 1,3-oxothiolane group was only partially stable to these conditions. Upon further examination of the 1,3-dithiolane derivatives of levulinyl and pyruvyl groups, we found that they were both indeed stable to conditions typically employed in

Scheme 1



chemical protein synthesis, for example: (1) HBTU/DMF/DIEA in DMF coupling cycles; (2) >30 × 2 min neat TFA deprotection cycles; (3) >30 × 20 min 30% piperidine/DMF deprotection cycles; (4) 97% aqueous TFA at room temperature for 1 h; (5) HF/*p*-cresol (9:1) at 0 °C for 1 h; (6) 0.1% aqueous TFA/acetonitrile HPLC purification buffers; and (7) 6 M Gn·HCl, 0.3 M sodium phosphate, pH 7 containing thiophenol, TCEP, and β-mercaptoethanol.

The 1,3-dithiolane-protected forms of levulinic acid **1** (DTL) and pyruvic acid **2** (DTP) were easily synthesized in two steps from the corresponding ethyl esters (Scheme 1). To bypass the need for postsynthesis modification processes for the introduction of these groups during solid-phase synthesis, we prepared Boc-Lys(DTL)-OH, **3**, Boc-Lys(DTP)-OH, **4**, and Fmoc-Lys(DTP)-OH, **5** derivatives. The Boc-Lys(DTL)-OH, **3**, and Boc-Lys(DTP)-OH, **4**, were synthesized via a copper-complexed lysine intermediate, followed by coupling of the acids **1** and **2**, decomplexation, and N^{α}-protection with Boc_2O. Fmoc-Lys(DTP)-OH, **5**, was synthesized in solution by direct coupling of the acid **2** to Fmoc-Lys-OH followed by flash purification. We have subsequently shown the utility of these residues in stepwise Boc synthesis of α-endorphin analogues (**6**, **7**) and Fmoc synthesis of model peptide **8** (Table 1).

An important aspect of this strategy was the ability to efficiently hydrolyze the 1,3-dithiolane to regenerate the keto group at the peptide level, especially in the presence of unprotected cysteine residues, which are present at ligation sites following native chemical ligation. Even though methods have been reported for unmasking the 1,3-dithiolane group in small organic molecules,[11] few appeared immediately compatible with peptides, as they generally involved refluxing conditions in nonpolar solvents. Nevertheless, we found the use of heavy metal salts (Ag^{+} and Hg^{2+}) in aqueous solution were quite effective for dithiolane hydrolysis. The use of these heavy metals has precedence in peptide chemistry for the deprotection of Acm groups.[12] In practice, for peptides lacking cysteine, such as peptides **6** and **7**, both DTL and DTP groups were rapidly unmasked with Hg^{2+} to regenerate the corresponding free keto-peptides (**9** and **10**) at room temperature in 0.5−2h (10−15 equiv of Hg^{2+} in 50% aqueous AcOH), respectively. Interestingly, for cysteine-containing peptides, 4-picolyl *S*-protection was required for Hg^{2+} deprotection to proceed cleanly, albeit at a slower rate (*results not shown*). After further investigation, we found that AgOTf[13] (100 equiv, room temperature, 24 h) in 50% aqueous ACN was able to cleanly remove the dithiolane group from cysteine-free DTL-peptides (**6**) (Figure 1)

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Table 1. Peptides Synthesized with Compounds **3**, **4**, and **5**

compd	peptide sequence	HPLC purity	[M + H]+
6	YGGFMTSEK(DTL)SQTPLVT	>90%	1921 Da
7	YGGFMTSEK(DTP)SQTPLVT	>90%	1893 Da
8	AEK(DTP)ITKA	>95%	906 Da
9	YGGFMTSEK(Lev)SQTPLVT	>90%	1845 Da
10	YGGFMTSEK(Pyr)SQTPLVT	>90%	1769 Da

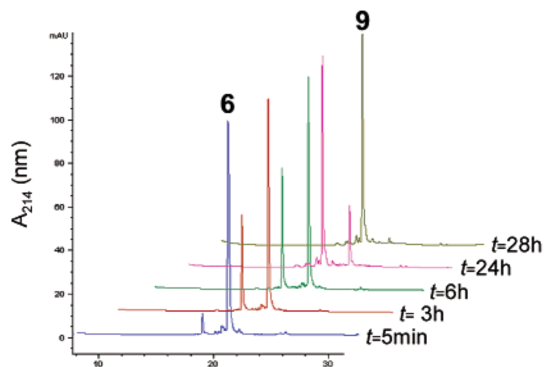
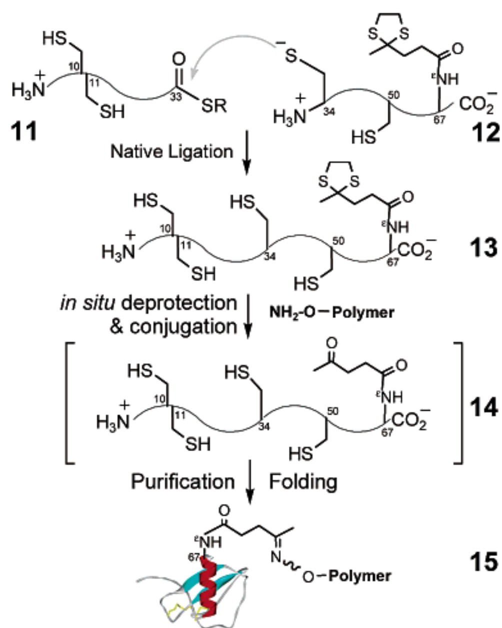


Figure 1. HPLC analysis of the 1,3-dithiolane deprotection process of peptide **6** with AgOTf in 50% aqueous ACN to give levulinic-peptide **9**.

Scheme 2



and also from unprotected cysteine-containing DTL-peptides (**12**) in high yield (with increased AgOTf equivalents), thereby providing additional synthetic flexibility.

To determine the practicality of this new method for chemical protein synthesis, we undertook the synthesis of a monodisperse polymer−RANTES conjugate (**15**) using the strategy outlined in Scheme 2. First, the RANTES(1−33) thioester-peptide, SPYSS-DTTPCCFAYIARPLPRAHIKEYFYTSGK (**11**), and RANTES-(34−68)[M67Lys$^{(DTL)}$] N-terminal cysteine-peptide, CSNPAV-VFVTRKNRQVCANPEKKWVREYINSLEK$^{(DTL)}$S (**12**), were prepared with Boc-SPPS in good yield. The full-length protein backbone (**13**) was obtained by native chemical ligation[3] of peptides **11** and **12** in pH 7 guanidine/phosphate buffer in 54% yield after purification. The 1,3-dithiolane group in the full-length peptide **13** was in situ deprotected (**14**, not isolated) and oximated with a 16 kDa aminooxy-containing monodisperse polymer[1] in overall 62% yield (by HPLC). The workup involved the addition of β-mercaptoethanol to both complex the excess silver salts (which were then removed by simple filtration) and reduce back to the free thiols. The isolated oximated product was then successfully folded in pH 8 guanidine/Tris-buffer with cysteine/cystine, purified to give final conjugate **15**, and characterized (Figure 2). This new protection methodology for keto-proteins should be broadly applicable with other nucleophilic derivatives,[8,14] peptide aldehydes, and in increasingly larger protein systems thereby improving the engineering of novel biomolecules.

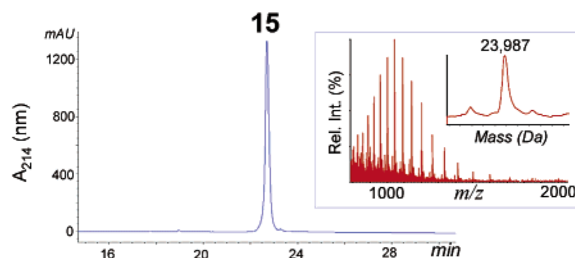


Figure 2. HPLC analysis of the folded RANTES-polymer conjugate, **14**. Inset: The electrospray mass spectrum and its parent mass reconstruct of **14**. Experimental mass: 23 987 Da; calculated mass (avg), 23 986 Da.

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Supporting Information Available: Experimental details for the preparation of compounds and general procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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