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Synthesis of Peptides Containing *C*-Terminal Methyl Esters Using Trityl Side-Chain Anchoring: Application to the Synthesis of a-Factor and a-Factor Analogs

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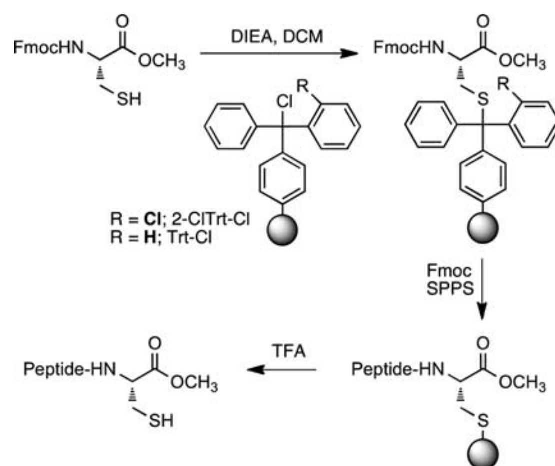
ABSTRACT



A new cysteine anchoring method was developed for the synthesis of peptides containing *C*-terminal cysteine methyl esters. This method consists of attachment of Fmoc-Cys-OCH_3 to either 2-ClTrt-Cl or Trt-Cl resins (via the side-chain thiol) followed by preparation of the desired peptide using Fmoc-based SPPS. We applied this method to the synthesis of the mating pheromone a-factor and a 5-FAM labeled a-factor analog. The peptides were obtained with high yield and purity and were shown to be bioactive in a growth arrest assay.

Due to the importance of peptides in studies of biological processes, new, more efficient, synthetic methods are needed.[1] Peptides containing methyl esters at the *C*-terminus can be used as tools for the study of protein prenylation due to the presence of this modification in fully processed farnesylated and geranylgeranylated proteins.[2] These peptides can also be useful for a variety of biological applications since the presence of the alkyl ester at the *C*-terminus increases the hydrophobicity of the peptide making them membrane permeable.[3,4] Although such ester-modified

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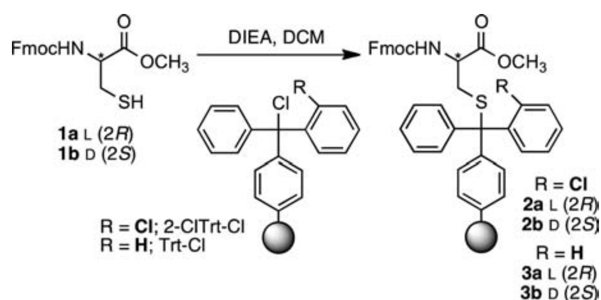
peptides can be useful in a variety of ways, only a handful of reports in the literature describe their synthesis.[3,5−9]

One previously used method to obtain peptides with *C*-terminal esters is the Merrifield Boc/Bzl protecting group strategy that involves attachment of the peptide to the resin through a benzyl ester linkage and requires the use of hydrofluoric acid (HF) for the release of the peptide from the solid support.[5] Another method, used by Waldmann and co-workers, utilizes a hydrazine containing resin and consists of producing prenylated peptides containing *C*-terminal methyl esters directly from the solid support.[8] This hydrazine procedure requires an oxidation step with copper with concomitant methanolysis in order to cleave the peptide from the resin. In general, the use of such strong acidic/oxidative conditions makes these methods less expeditious from an experimental point of view and less versatile in their scope.

Scheme 1. Attachment of Fmoc-Cys-OCH_3 onto Trityl-Based Resins[a]



[a] * = stereogenic center of the cysteine α carbon.

A different approach for solid phase peptide synthesis (SPPS) consists of anchoring the peptide to a solid support through the side chain of a trifunctional amino acid. Reports on the synthesis of peptides, using cysteine anchoring methods, have shown that they assist in reducing the risk of racemization and formation of byproducts commonly found in SPPS.[10,11]

The method described herein consists of the attachment of Fmoc-Cys-OCH_3 to a trityl-based resin to form a thioether bond. An important feature of such resins is that they are commercially available; in contrast, the XAL handle developed by Barany and co-workers requires a multistep sequence for its synthesis.[12] Hence, the sole requirement necessary to implement this new method is accessibility to Fmoc-Cys-OCH_3 (or a related ester if desired), a compound obtainable in one step from Fmoc-Cys-OH. After growing the desired peptide using Fmoc-based SPPS, the peptide of interest is cleaved from the resin by deprotection of the Cys sulfur under acidic conditions. While a trityl-based resin has been used to synthesize peptides containing a *C*-terminal cysteamine linker (via resin linkage through the thiol), that work did not describe the use of such resin for the preparation of *C*-terminal cysteine esters.[13] Thus, to the best of our knowledge, this is the first report on the use of cysteine side-chain anchoring to trityl-based resins for the synthesis of peptides containing methyl esters at the *C*-terminus.

The first peptide prepared with this method was the yeast mating pheromone **a**-factor from *Saccharomyces cerevisiae*. This dodecapeptide has attracted considerable attention in the field of protein prenylation due to its similarity with the *C*-terminal portion of larger, farnesylated proteins. Moreover, in common with these proteins, the farnesyl moiety and the methyl ester group incorporated at the *C*-terminal Cys of the **a**-factor peptide have been shown to be critical for its bioactivity.[14,15] Fluorescently labeled analogs of **a**-factor would be particularly useful for studying their binding to cell-surface receptors.[16] Unfortunately, most of the existing methods described in the literature for peptide *C*-terminal methyl ester synthesis are not convenient or suitible due to their use of aforementioned acidic or oxidative conditions.

The method reported here began with the attachment of Fmoc-Cys-OCH_3 to commercially available 2-ClTrt-Cl resin via its thiol functionality in order to obtain **2a** (Scheme 1). The loading of the first amino acid was determined by Fmoc absorbance after deprotection with 20% piperidine in DMF. The **a**-factor sequence was then assembled using Fmoc-based SPPS to obtain **4a** (Scheme 2). Since the linkage between the cysteinyl thiol and 2-ClTrt resin is acid labile, the peptide was cleaved from the resin upon treatment with Reagent K (TFA/thioanisole/phenol/water/ethanedithiol, 82.5:5:5:5:2.5) along with simultaneous deprotection of the acid-labile amino acid side-chain protecting groups. Using this concise procedure, it was possible to obtain the **a**-factor precursor peptide **5** with a crude purity of 82% (Figure 1a). This peptide was then purified and obtained in 42% overall yield. Farnesylation of **5** was performed using conditions developed by Naider[17] and previously reported by Mullen et al. for **a**-factor synthesis to yield **6**.[18]

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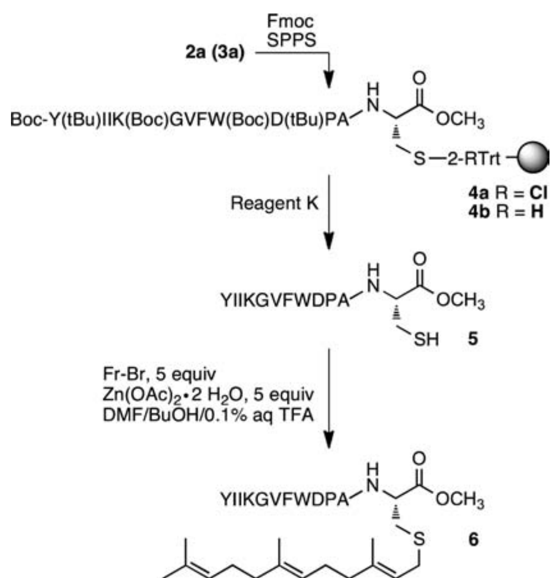
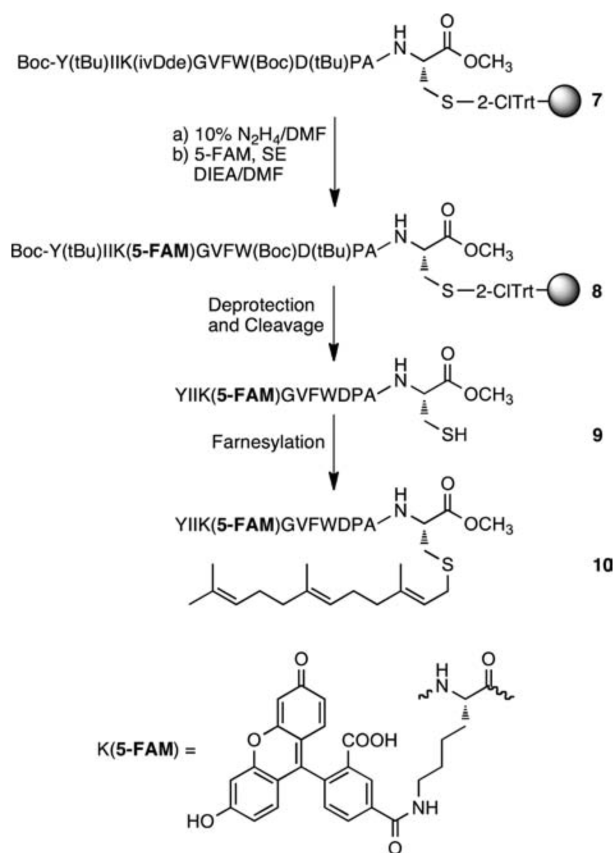
Scheme 2. Synthesis of the Mating Pheromone **a**-Factor Using the Cysteine Anchoring Method[a]



[a] The **a**-factor precursor peptide was synthesized on resin, and farnesylation of peptide **5** was performed in solution to obtain peptide **6**.

Scheme 3. Strategy Used for the Synthesis of 5-FAM-Labeled **a**-Factor Precursor Peptide **9** and 5-FAM Labeled **a**-Factor (**10**)



Having demonstrated the success of this simple method for the preparation of **a**-factor, we next undertook the synthesis of a fluorescently labeled analog of **a**-factor. The 5-FAM fluorophore was selected due to its high fluorescence, low cost, and ease of attachment to lysine side chains during SPPS.[19] Importantly, studies have shown that amino acid substitution[20] or truncation[21] of the **a**-factor lysine residue does not significantly affect the bioactivity of the pheromone; we envision that fluorescently labeled forms of **a**-factor will be useful for monitoring the binding of the pheromone to its cognate receptor as well as for photoaffinity labeling applications. It should be noted that earlier efforts to prepare such an analog using hydrazine resin were unsuccessful presumably due to overoxidation of the fluorophore in the Cu-catalyzed cleavage step.

The synthesis of 5-FAM labeled **a**-factor was analogous to that of the standard **a**-factor (Scheme 3) with slight modifications. After attachment of Fmoc-Cys-OCH_3 to 2-ClTrt-Cl resin, the peptide was extended using SPPS. However, the lysine residue incorporated an ivDde-protected ε-amino group resulting in the formation of intermediate **7**. This protecting group was orthogonally cleaved in the presence of hydrazine followed by coupling of the 5-FAM fluorophore to obtain **8**. Deprotection of side chains and cleavage of the peptide from the resin were achieved simultaneously in the presence of Reagent K to give an 81% crude purity for peptide **9** as shown in Figure 1b. Purification of the crude material produced the peptide in 36% yield which was then farnesylated to give peptide **10**.

Because the yields of these peptides were somewhat lower than anticipated, we chose to repeat the synthesis shown in Scheme 2 using a different trityl-based resin. Thus, peptide **5** was synthesized using a commercially available Trt-Cl resin in order to determine if the absence of the electon-withdrawing chloride would have an effect on the final yield (see Supporting Information (SI)). To our surprise, the yield of peptide **5** increased from 45% to 82% by simply changing the resin to Trt-Cl (Table 1). We hypothesized that the presence of the electron-withdrawing chloride in the 2-ClTrt containing resin increases the risk of β-elimination by increasing the leaving group ability of the protected thiol resulting in peptide loss from the resin during each piperidine deprotection cycle. Accordingly, we treated resin-bound model tripeptides **11a** and **12a** with piperidine for varying times and determined the amount of peptide remaining on the solid support via quantitative ninhydrin analysis; interestingly, no significant loss of peptide occurred except after very long (24 h) exposure (Table S1). In contrast, when resin bound **2a** or **3a** was treated with piperidine, some loss of peptide from the solid support was observed (Table S2). The loss was greater using the 2-ClTrt-Cl resin (26%) than with the

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Trt-Cl resin (9%), consistent with the hypothesis that β-elimination is responsible for the reduction in yield observed with the former. The observation that some loss occurs with **2a** and **3a** but not with **11a** and **12a** suggests that this β-elimination reaction is context dependent. Importantly, Trt-Cl resin appears to be the support of choice for this application.

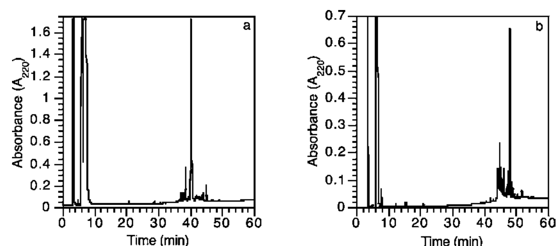


Figure 1. HPLC of (a) crude peptide **5** and (b) crude peptide **9** after cleavage from the 2-ClTrt resin.

Since the procedure reported here uses a side-chain cysteine anchoring method, and the Cys is not attached to the solid support via an ester linkage, the risk of racemization of the *C*-terminal Cys is minimal.[10] However, since it has been previously reported that repetitive treatment with piperidine can sometimes cause racemization,[22] a study was carried out to examine whether this method leads to a loss in chiral integrity of the Cys residue. Model tripeptides **13a** and **13b** containing a methyl ester at the *C*-terminus were synthesized using 2-ClTrt-Cl (Scheme 4) and Trt-Cl resin. The *C*-terminal acid forms of these tripeptides have been previously reported to manifest baseline resolution when separated by HPLC.[11] Peptides **13a** (R_t = 15.5 min) and **13b** (R_t = 14.9 min) were analyzed by HPLC individually and via coinjection, and the data showed negligible (< 1%) racemization product for both resins (see SI).

To verify that peptides **6** and **10**, synthesized with the cysteine anchoring method described herein, are biologically active, we tested their ability to cause growth arrest of cells expressing the Ste3p **a**-factor receptor. In that assay, previously characterized, **a**-factor with wild-type potency stimulated growth arrest with an end point of 0.12 ng;[5] the synthetic material produced in this study (**6**) yielded an identical end point. In contrast, the 5-FAM **a**-factor (**10**) manifested an end point of 8.0 ng indicating that while the fluorescent analog has somewhat lower activity, it still possesses significant (12.5%) bioactivity and hence should be useful for future studies.

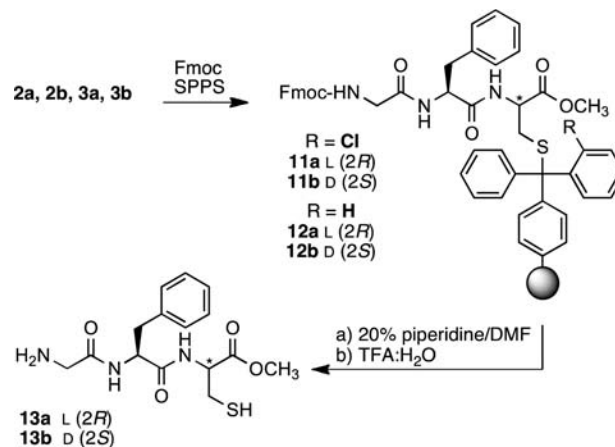
In summary, a new method has been developed for the synthesis of peptides containing methyl esters at the *C*-terminus. This method was applied to the synthesis of **a**-factor and a fluorescently labeled **a**-factor analog producing crude materials of high purity. A comparison between two trityl-based resins for the synthesis of the **a**-factor precursor peptide showed that the yield of the desired peptide almost doubled when using the Trt-Cl resin instead of the 2-ClTrt-Cl resin. Model tripeptides were synthesized using both resins, and HPLC analysis showed formation of the desired peptide without significant racemization. The bioactivities of the synthesized **a**-factor and 5-FAM labeled **a**-factor were confirmed by a growth arrest assay. The 5-FAM **a**-factor analog showed significant although somewhat reduced bioactivity. The success of this simple method for the synthesis of peptides containing *C*-terminal Cys methyl esters using commercially available resins opens the door to the synthesis of a wide variety of *C*-terminal ester modified peptides that can be used to study protein prenylation and other structurally related biological processes.

Table 1. Comparison of % Yield Obtained with 2-ClTrt-Cl and Trt-Cl Resins for the Synthesis of **5**

entry	resin	purity of crude[a] (%)	isolated yield[b] (%)
1	2-ClTrt-Cl	82	45
2	Trt-Cl	81	82

[a] Purity of peptide **5** in the crude mixture after deprotection and cleavage from the resins but prior to HPLC purification. [b] After purification.

Scheme 4. Synthesis of Model Peptide H-Gly-Phe-Cys-OCH_3[a]



[a] * = stereochemistry of the cysteine α carbon.

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Supporting Information Available. Experimental procedures, HPLC and MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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