Protocol

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Efficient method of circumventing insolubility problems with fully protected peptide carboxylates via *in situ* direct thioesterification reactions

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A straightforward and convenient protocol is presented for the direct thioesterification of fully protected peptide *C*-terminal carboxylates synthesized by Fmoc strategy. This methodology specifically serves to overcome the frequent insolubility problem of these fully protected carboxolate isolates during the thioesterification process by carrying out the reaction as an *in situ* procedure on the freshly cleaved 1% TFA/DCM solution of carboxylate. The direct thioesterification of a number of insolubility prone peptide systems is explored and compared with some control systems for ease of conversion to the corresponding thioesters. It is shown that although the fully protected carboxylates are indeed insoluble to varying degrees in the thioesterification reactions carried out using the classical approach, full dissolution is maintained and complete conversion is evident using the *in situ* methodology. This protocol serves to remove a frequent stumbling block in the preparation of peptide thioesters via the direct approach, allowing for facile entry into previously difficult systems traditionally unapproachable through this method. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

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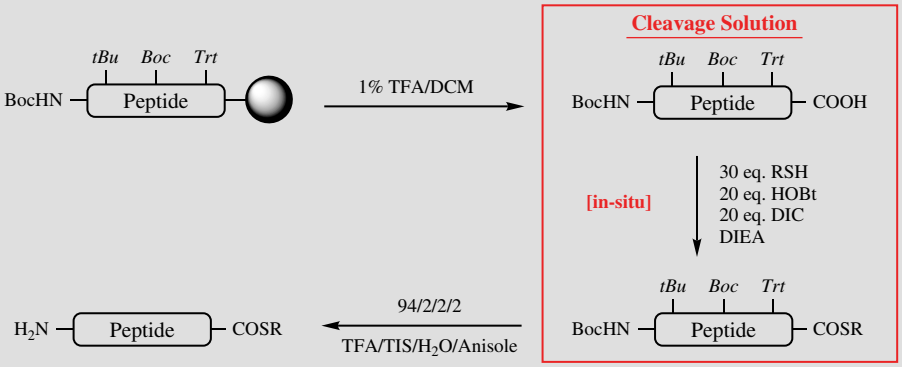
Keywords: thioester; thioesterification; native chemical ligation; insoluble; carboxylate

REACTION SCHEME



GENERAL OPTIMIZED PROCEDURE

Thioesterification of freshly-cleaved, fully-protected peptide carboxylate. The α-Boc-protected, fully constructed peptide sequence on 2-Cl-Trt solid support (40 µmol w.r.t. resin loading) is treated with1% *v*/*v* TFA/DCM (3 x 3 mL) for 5 minutes each, the supernatants being combined into a 50 mL reaction vessel containing 1.1 Eq. DIEA (w.r.t. total amount of TFA in cleavage fractions). To this solution is added 30 eq. thiol and 20 eq. each of HOBt and DIC. The solution is stirred overnight at room temperature and then concentrated, followed by trituration with diethyl ether. The thioester residue was then deprotected via treatment with cleavage cocktail 94:2:2:2 TFA/TIS/H_2O/Anisole for 2 hours. The crude product was purified by preparative RP-HPLC on a Waters SymmetryPrep C18 column, eluted with a linear gradient of 0-55% of ACN in H_2O, both phases containing 0.1% TFA.

Scope and Comments

Peptide thioesters represent a very important class of compounds with a wide variety of applications. Techniques such as native chemical ligation [1] and expressed protein ligation [2] have allowed entry into the design and chemical synthesis of small pro-

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teins over 50 residues in length, typically out of the reach of conventional SPPS. These enhanced synthetic methodologies dependent upon peptide thioester precursors have to a certain degree displaced other techniques such as convergent synthesis [3] and biological expression vectors [4] in ease of operation and architectural scope. Because a crucial component in these reactions is the *C*-terminal thioester module, it becomes important to develop a dependable and convenient approach toward its manufacture. In peptide thioester production based upon the Boc strategy, these intermediates can simply be constructed directly on the solid support [5], because the conditions of peptide synthesis do not degrade the fragile thioester functionality. However, for peptide thioester synthesis based upon the Fmoc strategy, conditions of piperidine-mediated α-deprotection easily cleave this delicate anchoring connectivity, and alternative means of thioester construction must be utilized. Traditionally, this has occurred as a post-synthetic modification in the last step of the synthesis. There are many elegant and clever techniques reported in the literature for Fmoc-compatible thioester construction such as use of the Kenner Safety-Catch Linker [6], the Aryl Hydrazide approach [7], side-chain anchoring strategies with a masked thioester component [8], and thioester-compatible Fmoc-deprotection protocol [9] to name a few.

Although there exist a diversity of methods for Fmoc-compatible peptide thioester formation, in our hands we found these techniques to be overly complex, to give substandard syntheses, or to involve tedious purification procedures. The technique we have found which offers the most convenient and thorough approach toward peptide thioester synthesis is the direct conversion of fully protected peptide carboxylates into their corresponding thioesters [10,11]. Overall, it is a very dependable, high-yielding and uncomplicated synthetic design toward the direct construction of these important intermediates involving the simple incubation of the fully protected *C*-terminal carboxylate with an excess of thiol under standard carbodiimide coupling conditions. Although this methodology typically allows for the smooth conversion of a previously isolated peptide carboxylate into its corresponding thioester, in a significant number of cases the carboxylate residue is insoluble to varying degrees in the thioesterification milieu. In many of these instances using this classic approach, we have found that no amount of agitation or solvent manipulation will compel the insoluble residue completely back into solution. The result is, at best, a mixture of desired thioester and starting carboxylate. We have found that this problem has plagued not only us, but also other research groups with whom we have similar interests (Nilsson B, personal communication).

The protocol which we report on here illustrates a dependable and reproducible method which can circumvent the challenges presented in the cases wherein the starting peptide carboxylate exhibits this insolubility problem during the thioesterification step. We have found it to be quite elegant in its simplicity and breadth of application. Indeed, every problem sequence to which we have applied this process has resulted in full conversion to the desired *C*-terminal thioester product (Figure 1). Our approach is, in essence, an *in situ* reaction in which the solution containing the fully protected carboxylate precursor (a 1% TFA/DCM cleavage solution from 2-Cl-Trt resin) is, following neutralization, subjected directly and without isolation to thioesterification using the epimerization-suppressing conditions of Quibell & coworkers [12]. Although there have been many different studies which show that different solvent/reagent conditions can be effective in *C*-terminal functionalization of different peptide systems [11,13,14], we have found that this simple and convenient approach only requires the addition of the proper reagents and incubation of the solution overnight. Removal of the solvent *in vacuo* and trituration with cold Et_2O affords the fully protected *C*-terminal thioester which then undergoes routine TFA-mediated deprotection to give the desired crude product with full conversion to the desired thioester (purity dependent upon the initial purity of the corresponding *C*-terminal carboxylate, which can be easily determined through a simple resin test-cleave prior to thioesterification). Peptide thioester yields were typically in the range of 50–65%.

We present here an overview of this process carried out on three peptide sequences which we have found to be insoluble under the conditions of thioesterification, comparing their manufacture using the 'classic' technique (derived from reaction of *previously isolated*, fully protected carboxylate under identical conditions of thioesterification) with the *in situ* approach (Table 1). We contrast these 'problem sequences' with the thioesterification of two peptides, which do not present issues with their solubility, illustrating nicely this method's ability to yield thioester sequences on insolubility prone systems which compare favorably with the products of routine sequences. Where appropriate, we show representative HPLC comparisons of each of these approaches, graphically illustrating the utility of this method (Figure 1; see also Supporting Information).

Finally, this methodology is probed as to the issue of potential *C*-terminal racemization during thioesterification [13] through the use of the epimerization-prone sequence Ac-HAAF [14] as the carboxylate substrate in this *in situ* process (Figure 2). Indeed, there is virtually no observed epimerization of the test sequence following workup and cleavage, further illustrating the utility of this method. This is in sharp contrast to the results of the classic thioesterification approach, in which there was very little conversion of the carboxylate to the thioester, with significant epimerization apparent in the HPLC product profile.

Experimental Procedure

SPPS elongation of peptide sequences

All peptides used in this study were synthesized on a Symphony multiple peptide synthesizer (Protein Technologies Inc.) on a 40 µmol scale via Fmoc protocol, utilizing 2-chlorotrityl chloride resin. The first residue was attached to the 2-Cl-Trt resin using an optimized methodology as previously described [15]. Double coupling using standard HBTU activation was employed for peptide elongation (4 eq. Fmoc amino acid and HBTU in 0.4 M NMM/DMF; 2 × 45 min). Peptides I and III required the insertion of a Fmoc-Thr-Pro-OH Dimer and a Fmoc-Ser-Ser($\Psi^{Me,Me}$pro)-OH Pseudoproline Dipeptide sequence at certain points in their respective chains to overcome intrastrand aggregation to produce deletion-free isolates [16,17]. As a final step in peptide elongation, terminal α-Fmoc protection was converted to Boc as previously described [15]. To assure proper construction of the linear sequences, test-cleaves of the resins were carried out on small portions of each of the resins in the following fashion: approximately 20 post-synthetic resin beads were incubated with 1 ml TFA, 50 µl H_2O, and 50 µl triisopropylsilane (TIS) for 2 h. After filtering away the resin beads, the supernatant was concentrated and triturated with Et_2O to yield, following centrifugation, a crude pellet which was immediately subjected to analytical HPLC and MALDI analysis (see Supporting Information).

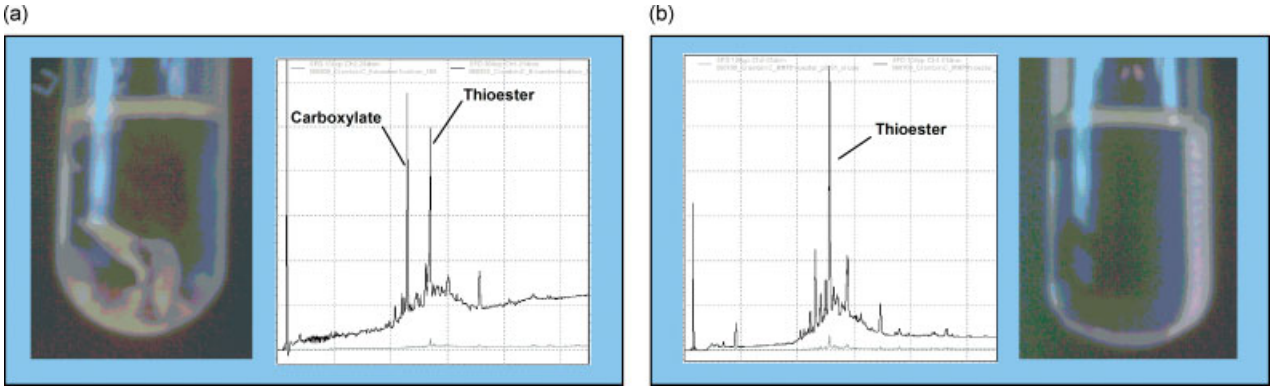

Figure 1. Representative example of the direct thioesterification of an insolubility prone peptide sequence (Peptide II) using (a) classic conditions and (b) the '*in situ*' approach. Photographic visualization and crude HPLC chromatograms illustrate the stark differences in the effectiveness of each approach.

Table 1. Peptide sequences used in this exercise. Entries I, II and III exhibited varying degrees of insolubility using the classic direct thioesterification approach. Entries IV and V, sequences which did not exhibit insolubility in the thioesterification milieu, were used as control systems

		Thioesterification Method		
Entry	Peptide Sequence	Classic	*In situ*	Yield
I	C(Acm)RLPG[TP]EALCATYTG	Insoluble	Soluble	59%
II	TTCCPSIVARSNFNA	Insoluble	Soluble	51%
III	EKRPRTAF[SS]EQLARL	Insoluble	Soluble	65%
IV	QKEKEKKEQEIKKKFKLTGPIQVIHLAKA	Soluble	Soluble	26%
V	MALAVRVVYCGAUGYKPKYLQLKEKLEHEFPG	Soluble	Soluble	55%

[XX] = Dimer dipeptide or Pseudoproline Dipeptide insertion sequences (necessary to prevent intrastrand aggregation during synthesis).

In situ direct thioesterification of fully protected carboxylate fragments

Peptide resin (40 μmol w.r.t. loading) was washed with DCM (3 × 3 ml) and shaken with aliquots of 1% TFA/DCM (3 × 3 ml) for 3 min each. These acidic supernatants were combined in a 50-ml reaction vessel containing 232 μl (1.33 mmol, 1.1 eq. w.r.t. total TFA in mixture) DIEA to yield a final solution concentration of 4.44 mM in peptide. To this solution was added 133 μl (1.20 mmol, 30 eq.) methyl 3-mercaptopropionate, 108 mg (0.8 mmol, 20 eq.) HOBt, 124 μl (0.8 mmol, 20 eq.) DIC, and 230 μl (1.33 mmol) additional DIEA. The mixture was then stirred overnight and concentrated. The crude protected thioester was precipitated by multiple triturations with cold diethyl ether followed by centrifugation. Deprotection was carried out by treatment of the crude product with 94 : 2 : 2 : 2 TFA/TIS/H_2O/anisole for 2 h at room temperature.

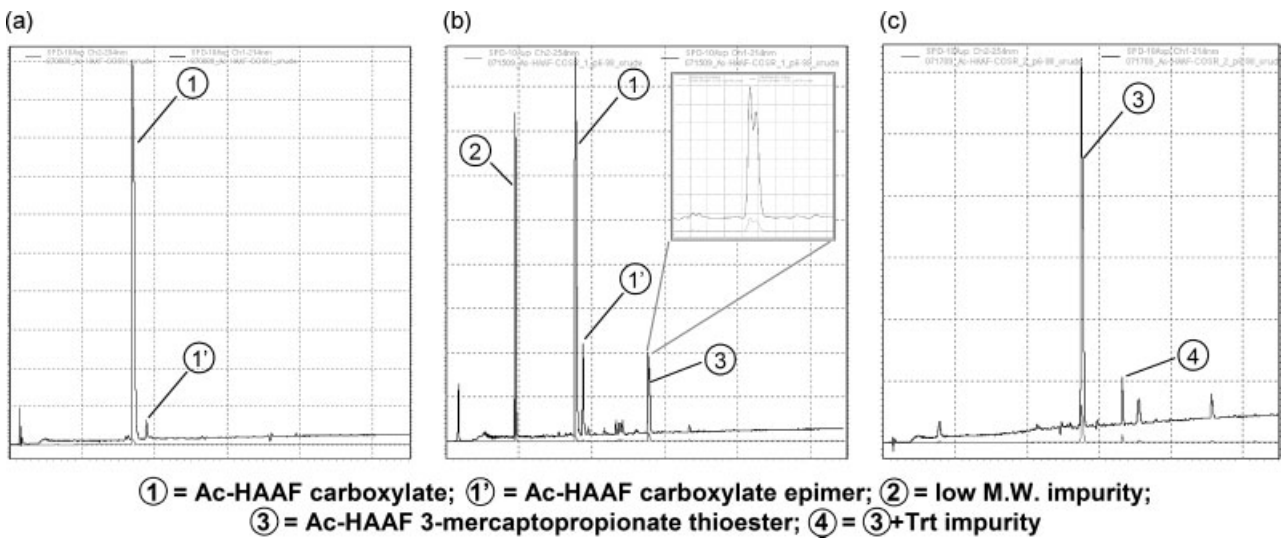

Figure 2. Crude HPLC chromatograms of epimerization study on Ac-HAAF test sequence [14] subjected to various thioesterification conditions. (a) Direct cleavage of Ac-HAAF carboxylate, (b) thioesterification using the classic approach (note split peak on thioester, signifying epimerization) and (c) thioesterification using the '*in situ*' approach.

Following concentration of the deprotection solvent to one fifth its original volume in a stream of nitrogen, the crude deprotected peptide thioester was isolated by precipitation into cold diethyl ether. The crude product was purified by preparative RP-HPLC on a Waters SymmetryPrep C18 column, eluted with a linear gradient of 0–55% of Buffer B (in Buffer A) for 55 min (Buffer A: 0.1% TFA/H_2O, Buffer B: 0.1% TFA/ACN). Subsequent freezing of the preparatory HPLC fractions to $-80\,^{\circ}C$ and lyophilization afforded the desired purified peptide thioester as a colorless fluffy solid.

Supporting information

Supporting information may be found in the online version of this article.

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