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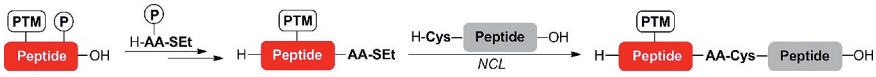
SHORT COMMUNICATION

Peptide Thioesters



PTM = post-translational modification, **AA-SEt** = amino thioester, **P** = Fmoc SPPS side-chain protection, NCL = native chemical ligation

The synthesis of peptide alkyl thioesters by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) is developed. The free C terminus of a fully protected peptide is coupled in solution with the free amino group of an amino thioester. This furnishes the fully protected peptide thioester, which can be globally deprotected to afford the desired unprotected peptide thioester.

Keywords: Amino acids / Peptides / Protein modifications / Glycosylation / Peptide thioesters

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Preparation of Peptide Thioesters through Fmoc-Based Solid-Phase Peptide Synthesis by Using Amino Thioesters

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Keywords: Amino acids / Peptides / Protein modifications / Glycosylation / Peptide thioesters

An effective procedure for the synthesis of peptide alkyl thioesters by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis was developed. The free C terminus of a fully protected peptide was coupled in solution with the free amino group of an amino thioester. This furnished the fully protected peptide thioester, which was globally deprotected to afford the desired unprotected peptide thioester. The method is compatible with labile groups such as phosphoryl and glycosyl moieties.

Introduction

Peptide and protein ligation strategies, such as native chemical ligation (NCL)[1] and expressed protein ligation (EPL),[2] are versatile methods for the construction of larger peptides and proteins from peptide fragments. Central to these methodologies is the formation of an amide bond between unprotected peptides and proteins with a C-terminal peptide thioester and an N-terminal cysteine, respectively.[1a,3] Sequential ligation of peptide fragments can then facilitate fully chemically synthesized proteins that are neither restricted by the general limitations of recombinant expression nor to accumulation of impurities during solid-phase peptide synthesis (SPPS).[1,2] Prominent examples of the application of NCL includes the total synthesis and subsequent determination of the X-ray crystal structure of the 203 amino acid HIV-1 protease,[4] as well as the 166 amino acid polypeptide erythropoietin (EPO) including a very recent synthesis of its glycosylated version.[5] EPL has been used in extensive studies of complex proteins such as K^+ channels[6] and histones.[7]

Peptide thioesters are typically prepared by *tert*-butoxycarbonyl (Boc) SPPS, in which the thioester functionality is released upon cleavage from the resin by treatment with HF.[8] Owing to the strong acidic cleavage conditions, thioester peptides containing acid-labile groups, including important post-translational modifications (PTMs) such as phosphates and glycosylates, cannot generally be prepared by Boc-SPPS. This, combined with the challenges in handling HF, has spurred substantial interest in developing alternative methods that are based upon the milder 9-fluorenylmethoxycarbonyl (Fmoc) SPPS methodology.[9]

The current Fmoc-SPPS methods for the preparation of peptide thioesters were recently reviewed by Mende and Seitz[9] and include side-chain or backbone anchoring and subsequent C-terminal functionalization,[10] application of tris(thioorthoesters) generated by treatment of the peptide with ethanethiol and $AlMe_3$,[11] thiolysis of peptides bound to safety-catch sulfonamide linkers,[12] and, finally, several versions of intramolecular O→S or N→S acyl shifts.[13] These methodologies each contain inherent challenges such as epimerization and hydrolysis or formation of diketopiperazines,[9] and a general method based on Fmoc-SPPS seems yet to be established. Peptide thioesters can also be synthesized directly by adding a C-terminal amino acid surrogate thioester or indirectly through a masked surrogate thioester;[5b,9] the latter was introduced by Danishefsky and co-workers.[14] This can be achieved by coupling the free C terminus of a fully protected peptide with amino thioesters followed by global deprotection to afford the peptide thioester. However, this principle has so far been restricted to the introduction of, for example, Gly- and Ala-terminated peptide thioesters[10a,15] or side-chain anchoring of Thr.[16] Herein, we explore this methodology for the synthesis of peptide alkyl thioesters by Fmoc SPPS, which allows the introduction of any proteinogenic amino acid as the C-terminal thioester. Importantly, we demonstrate that this method can be used in the preparation of peptide thioesters containing PTMs such as phosphorylations and glycosylations and that these are conveniently used in NCL.

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Results and Discussion

To develop a general method based on Fmoc SPPS for the synthesis of peptide thioesters, we envisioned that an amino acid and a thiol would first be combined to form the

C-terminal amino thioester building block, which could then be coupled to a protected peptide in solution. This would generate a protected peptide thioester, which subsequently could be deprotected to provide the desired peptide thioester (Figure 1).

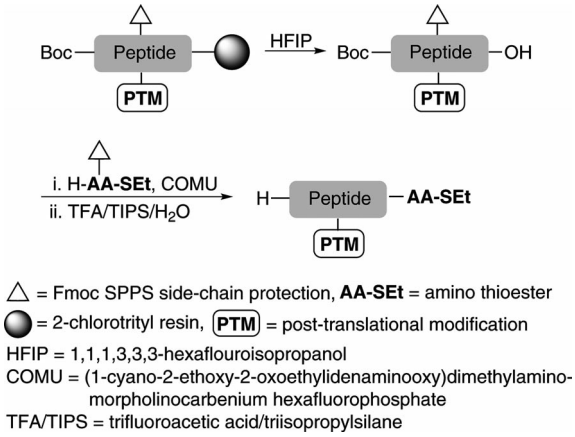
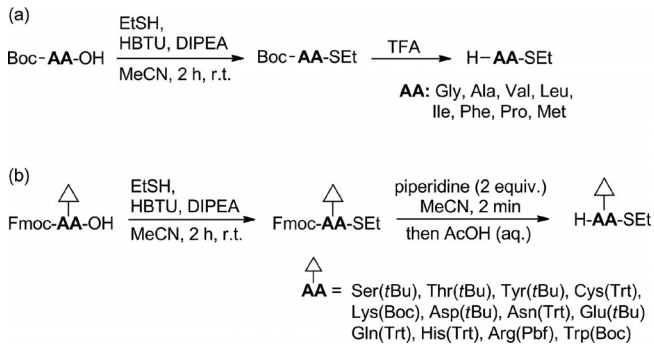


Figure 1. Outline of the principle for the generation of peptide thioesters containing acid-labile PTMs. The peptide is prepared by standard Fmoc SPPS and released as a fully protected peptide, which is subsequently coupled with an amino thioester. Global deprotection provides the desired peptide thioester.

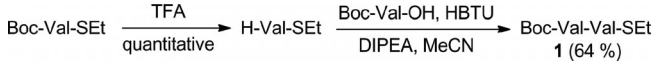
A central requirement was the generation of the C-terminal amino thioester building blocks for all 20 proteinogenic amino acids, which would be the amino thioesters with a free N terminus and appropriate side-chain protecting groups. The free carboxy termini of the protected amino acids were treated with ethanethiol in the presence of *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIPEA)[17] to provide fully protected amino thioesters (see the Supporting Information).

For the eight amino acids without side-chain protecting groups (Gly, Ala, Val, Leu, Ile, Phe, Pro, Met), *N*-Boc-protected amino thioesters were smoothly converted into the corresponding free amino thioesters with trifluoroacetic acid (TFA) in quantitative yields (Scheme 1a). For the amino acids with acid-labile side-chain protecting groups used in Fmoc SPPS [Ser(*t*Bu), Thr(*t*Bu), Tyr(*t*Bu), Cys(Trt), Lys(Boc), Asp(*t*Bu), Asn(Trt), Glu(*t*Bu), Gln(Trt), His(Trt), Arg(Pbf), Trp(Boc)], another strategy was required, and the corresponding Fmoc-protected amino thioesters were therefore generated. Knowing that thioesters are generally prone to degradation upon treatment with secondary amines, we found that treating Fmoc-protected amino thioesters with piperidine (2 equiv.) for 2 min followed by quenching in aqueous acetic acid smoothly afforded the desired side-chain-protected amino thioesters (Scheme 1b), analogously to a previous report.[18] Notably, the reaction was free of any amide side products, and the amino thioesters were shelf-stable, as no byproducts were observed upon storage at room temperature for several days. We also verified that the chirality was maintained during thioester synthesis and subsequent removal of the N-protecting groups by chiral HPLC analysis, which showed no sign of racemization (>99% *ee*, see the Supporting Information).



Scheme 1. Ethyl amino thioesters from (a) standard *N*-Boc-protected amino acids and (b) appropriately protected standard *N*-Fmoc-protected amino acids.

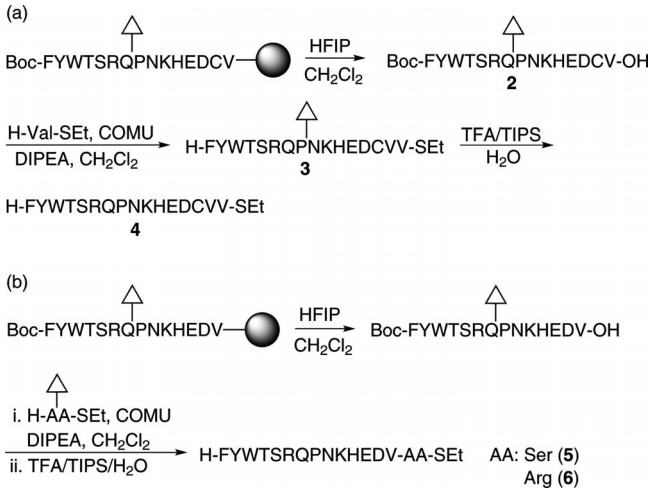
As initial proof-of-principle of introducing the amino thioester, we prepared a dipeptide thioester. The amino thioester building block H-Val-SEt was coupled with Boc-Val-OH in solution by using HBTU. The crude dipeptide thioester was purified by normal-phase chromatography to provide Boc-Val-Val-SEt (**1**, Scheme 2) in high yield.



Scheme 2. Synthesis of the Boc-Val-Val-SEt dipeptide.

Having all the C-terminal amino thioester building blocks available and having demonstrated the feasibility of the coupling of these to a free C terminus, we wanted to apply the methodology to the synthesis of peptide thioesters. We prepared a model 15 amino acid peptide, FYWTSRQPNKHEDCV, by standard Fmoc SPPS that included all side-chain protecting groups and a bulky C terminus. A Boc-protected amino acid was introduced as the N-terminal amino acid, which could be deprotected by TFA treatment concomitant with the side-chain protecting groups. All proteinogenic amino acids fulfilling these requirements are commercially available. The peptide was cleaved from the 2-chlorotrityl resin by using 1,1,1,3,3,3-hexaflouroisopropanol (HFIP),[19] which furnished the fully protected peptide with a free carboxy terminus (i.e., **2**; Scheme 3a). The crude peptide was then treated with the free amino thioesters by using different coupling reagents, knowing that epimerization of the C-terminal residue of **2** (i.e., Val) might be a challenge (Table S2, Supporting Information). Indeed, under the standard coupling conditions with the use of HBTU, a mixture of isomers was obtained in which the D-Val isomer was present in 35%, and this indicated that a large degree of epimerization had occurred. The same degree of epimerization was observed by using bromotris(dimethylamino)phosphonium hexafluorophosphate (BroP)[20] (34% D isomer), whereas the use of the pentafluorophenyl ester reduced the extent of epimerization (11% D isomer). Gratifyingly, if the coupling was carried out with 4-(1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylaminomorpholinocarbenium hexafluorophosphate

(COMU)[21] in dichloromethane, the D isomer content was only approximately 1%, and these conditions were therefore used throughout this work.



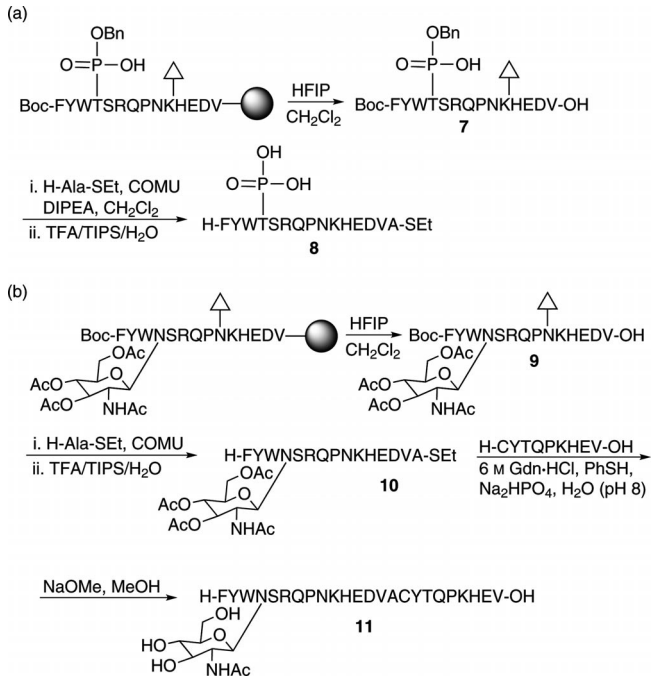
Scheme 3. Synthesis of peptide thioesters. (a) Coupling of a protected peptide in solution with an amino thioester building block. (b) Coupling of a protected peptide in solution with a side-chain-protected amino thioester.

By using these conditions, peptide **2** was coupled with H-Val-SEt to afford fully protected peptide thioester **3**. After normal-phase purification of **3**, treatment with TFA provided the desired unprotected 16 amino acid peptide thioester **4** (Scheme 3a). Normal-phase chromatography, that is, standard silica purification, of fully protected peptide intermediate thioester **3** is crucial to the success of this approach.[22] Direct deprotection of crude **3** did not provide the peptide thioester, and, likewise, coupling of fully protected peptide **3** directly with ethanethiol provided a complex reaction mixture.

To examine the feasibility of introducing a C-terminal amino thioester with a side-chain protecting group, we prepared a protected 14 amino acid peptide, FYWTSRQPNKHEDV, analogously to peptide **2**. H-Ser(*t*Bu)-SEt and H-Arg(Pbf)-SEt were used as amino thioester building blocks, and their respective thioester peptides, **5** and **6**, respectively, were conveniently obtained (Scheme 3b).

One of the advantages of both NCL and EPL is the ability for site-specific introduction of unnatural moieties and PTMs,[23] and ligation-based strategies have been used to generate proteins endowed with PTMs at specific sites that are not attainable by conventional recombinant methods.[24] One of the primary shortcomings of Boc SPPS is that the preparation of phosphorylated and glycosylated peptide thioesters is generally not possible owing to the strong acidic (HF) treatment. Thus, a particular challenge for any general Fmoc SPPS method is to allow easy access to peptide thioesters decorated with phosphorylations and glycosylations, and we therefore examined our methodology for generating such peptide thioesters.

First, we investigated the compatibility for generating phosphorylated peptide thioesters and synthesized a model 14 amino acid peptide with a phosphorylated threonine, Fmoc-*O*-(benzylphospho)-L-threonine [Fmoc-Thr-(PO_3BnH)-OH],[25] by standard Fmoc SPPS. The protected phosphorylated peptide was cleaved with HFIP and coupled to H-Ala-SEt to give the corresponding protected thioester **7**. Global deprotection including debenzylation of the phosphoryl group gratifyingly provided the phosphorylated peptide thioester **8** (Scheme 4a).



Scheme 4. Synthesis of peptide thioesters with PTMs. (a) Synthesis of a phosphorylated peptide thioester. (b) Synthesis and ligation of a glycosylated peptide thioester.

Analogously, a glycosylated model 14 amino acid peptide was synthesized by Fmoc SPPS to examine if glycosyl moieties are compatible with the developed methodology. A protected, glycosylated asparagine, *N2*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-*N*-[3,4,6-tri-*O*-acetyl-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl]-L-asparagine [Fmoc-Asn-(Ac_3AcNH-β-Glc)-OH],[26] was introduced, and the fully protected glycopeptide was cleaved from the resin to provide **9** (Scheme 4b). Then, H-Ala-SEt was coupled to the free C terminus of **9**, and TFA cleavage allowed deprotection of the amino acid side chains while keeping the protecting groups of the glycosyl moiety. This provided the desired glycosylated peptide thioester **10** (Scheme 4b). To demonstrate that peptide **10** could be conveniently used in NCL, it was ligated to an N-terminal Cys peptide, CYTQPKHEV, by using standard NCL conditions. The ligated product was purified and subsequently treated with sodium methoxide to remove the *O*-acetyl protecting groups of the glycol moiety to furnish unprotected, glycosylated 24 amino acid peptide **11**. Thus, the methodology conveniently provides access to both phosphorylated and glycosylated peptide thioesters on the basis of conventional Fmoc SPPS.

Conclusions

A general Fmoc SPPS based method for the synthesis of peptide thioesters was developed, and the applied amino thioester building blocks were synthesized for all the corresponding 20 proteinogenic amino acids. An advantage of using amino ethyl thioester building blocks in the synthesis of peptide thioesters is that direct handling of foul-smelling thiols is avoided. It is believed that an important feature of the described method is that the fully protected peptide thioesters are purified by normal-phase chromatography to remove the byproducts from the coupling reagents, as these significantly hamper the generation of the subsequent unprotected peptide thioester. We also attempted direct coupling of free thiols with the C termini of the peptides, as previously reported,[17b,27] but this did not provide the desired peptide thioesters.

A primary objective was to generate peptide thioesters with acid-labile PTMs such as glycosylations and phosphorylations, which are generally not attainable even by Boc SPPS. This was demonstrated by the straightforward synthesis of two peptide thioesters containing these important PTMs. The glycopeptide thioesters were subsequently used in a NCL to provide a 24-mer glycopeptide. Fully protected peptide thioesters can easily be solubilized in organic solvents, which allows easy handling and purification of the peptide derivatives in organic media.

Experimental Section

General Procedure for the Synthesis of Peptide Thioesters: A fully protected peptide with a free carboxy terminus (0.25 mmol) was dissolved in a solution of a free amino thioester (H-AA-Set, 0.3 mmol) and DIPEA (0.105 mL, 0.6 mmol) in dichloromethane (0.7 mL), and the reaction vessel was placed in a freezer at –18 °C. COMU (0.128 g, 0.3 mmol) was added, and vigorous shaking was maintained at room temperature for 1 h. The reaction mixture was separated directly on silica by means of ethyl acetate. An inhomogeneous mixture of TFA/TIPS/H_2O (18:1:1, 4 mL) was added, and shaking was maintained for 1 h. After concentration in a gentle stream of nitrogen, the crude peptide thioester was washed with cold diethyl ether (3 × 5 mL) to give the crude product, which was successively separated by HPLC to afford the peptide thioester.

Supporting Information (see footnote on the first page of this article): Experimental procedures, characterization data, and ^1H and ^{13}C NMR spectra of the products.

Acknowledgments

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