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Efficient Microwave-Assisted Tandem *N*- to *S*-Acyl Transfer and Thioester Exchange for the Preparation of a Glycosylated Peptide Thioester

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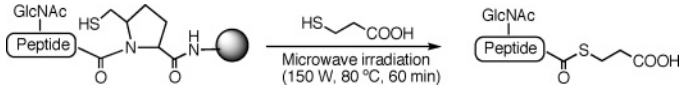
ABSTRACT



A peptide carrying a mercaptomethylated proline derivative at the C-terminus was prepared by solid-phase peptide synthesis (SPPS) and converted to the thioester of 3-mercaptopropionic acid (MPA) by aqueous MPA under microwave irradiation conditions. This post-SPPS thioesterification reaction was successfully applied to the synthesis of a glycopeptide thioester composed of 25 amino acid (AA) residues, which was then used for the preparation of a 61-AA glycopeptide by the thioester condensation method.

Segment condensation methods, such as the thioester method,[1,2] and native chemical ligation[3,4] have been highly optimized for the chemical synthesis of proteins. The key intermediates for these strategies are the peptide thioesters, which have been mainly prepared by the *tert*-butoxycarbonyl (Boc) mode solid-phase method. Because of the increasing interest in the posttranslational modifications of protein, such as glycosylation, the preparation of a peptide thioester by the 9-fluorenylmethoxycarbonyl (Fmoc) strategy, which does not use harsh acidic conditions, has been reported.[5−20] These methods include the use of thioester-compatible Fmoc cleavage cocktails with a preassembled thioester linkage on resin,[6,10] post solid-phase peptide synthesis (post-SPPS) thioesterification using sulfonamide linkers,[7,9] aryl hydrazine support,[15] protected peptide segments,[5,8,11−13] and post-SPPS thioesterification by an *O*- to *S*- or *N*- to *S*-acyl transfer reaction.[16−20] Some of these methods were actually applied to the preparation of glycosylated peptide thioesters, which led to the successful synthesis of glycoproteins.[7,16,21−27] However, the

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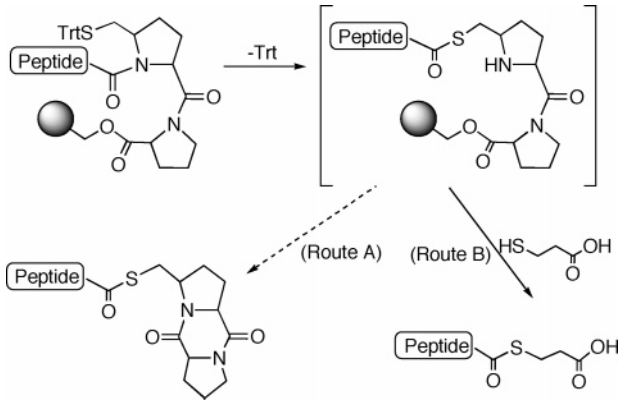
preparation of glycosylated peptide thioesters is still a major problem in glycoprotein synthesis. The use of the sulfonamide linker requires alkylation at the final activation step, but the reagent also alkylates the free hydroxyl groups at the carbohydrate portion, unless they are protected.[28] We previously prepared a peptide thioester representing the sequence of an extracellular matrix metalloproteinase inducer, emmprin (34−58), carrying N-linked pentasaccharide by the thioester-compatible Fmoc deblocking reagent, but the overall yield was 1.8%. The low yield might be attributable to the partial cleavage of the preformed thioester linkage during Fmoc removal.[22]

Diketopiperazine formation is a well-known side reaction during SPPS, especially when the C-terminal's two residues contain Pro residue(s). This reaction usually occurs during the removal of the N-protecting group from the dipeptide. We speculated that this reaction might occur on resin even after the chain assembly has completed, if the peptide has Pro-Pro at its C-terminus and if anchimeric assistance by a thiol group promotes the cleavage of the dipeptide (Scheme 1, route A). Our initial attempt was to develop a post-SPPS

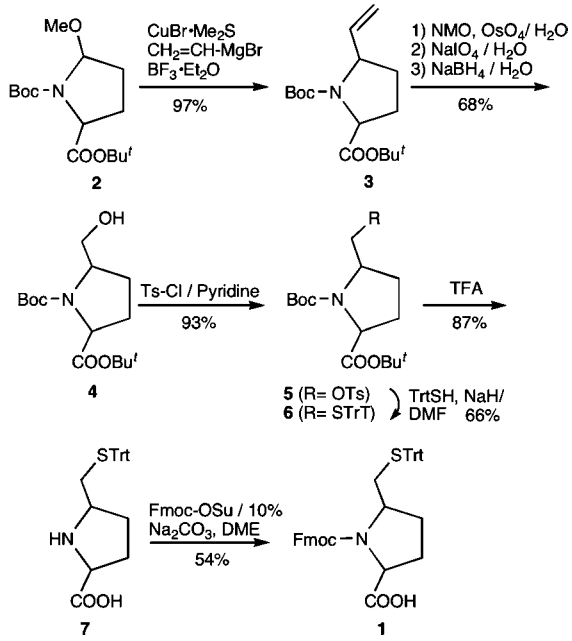
Scheme 1



thioesterification reaction based on route A. Unfortunately, the reaction did not proceed as described below. However, by the careful examination of the reaction, we found a novel thioesterification reaction using 3-mercaptopropionic acid (MPA) (route B). This procedure was further modified to develop an efficient microwave-assisted post-SPPS thioesterification reaction, which was applied to the synthesis of a glycosylated peptide thioester.

To examine the reaction in Scheme 1, a proline derivative carrying protected thiol group **1** was prepared according to Scheme 2. Starting from the known compound **2**,[29] a vinyl

Scheme 2



group was introduced to the C5 position using vinylmagnesium bromide. The obtained compound **3** was converted to glycol by osmium oxidation. The glycol moiety was oxidatively cleaved by KIO_4 and then reduced by $NaBH_4$ to obtain alcohol **4**. The hydroxyl group of compound **4** was tosylated and converted to an STrt group to obtain compound **6**. Then, the Boc and ^{t}Bu ester were removed by TFA. The amino group of the obtained compound **7** was protected by the Fmoc group to obtain key intermediate **1**. The overall yield of compound **1** was 19%. This unit was then used for the synthesis of protected peptide resin, Fmoc−Leu-Lys(Boc)-Gly-Pro(CH_2STrt)-Pro−OCH_2−Merrifield resin **9**. However, at the dipeptide stage, diketopiperazine derived from Pro-(CH_2STrt)-Pro was formed. Thus, the second and third amino acids were introduced using Fmoc−Gly-Pro(CH_2STrt) **8**, which was prepared by the coupling of compound **7** with Fmoc−Gly−OSu. The obtained resin **9** was treated with Reagent K[30] for 1 h to generate free thiol groups. Thiol-induced diketopiperazine formation (route A) was then attempted using this resin under acidic to basic conditions. However, we could not observe the progress of the reaction by HPLC analysis.

To analyze the factors responsible for preventing the progress of the reaction shown in route A, the peptide cleaved under the forced conditions (Reagent K treatment at 60 °C for 4 h) was examined by HPLC. The obtained peptide showed two peaks on HPLC, as shown in Figure 1a (0 h).

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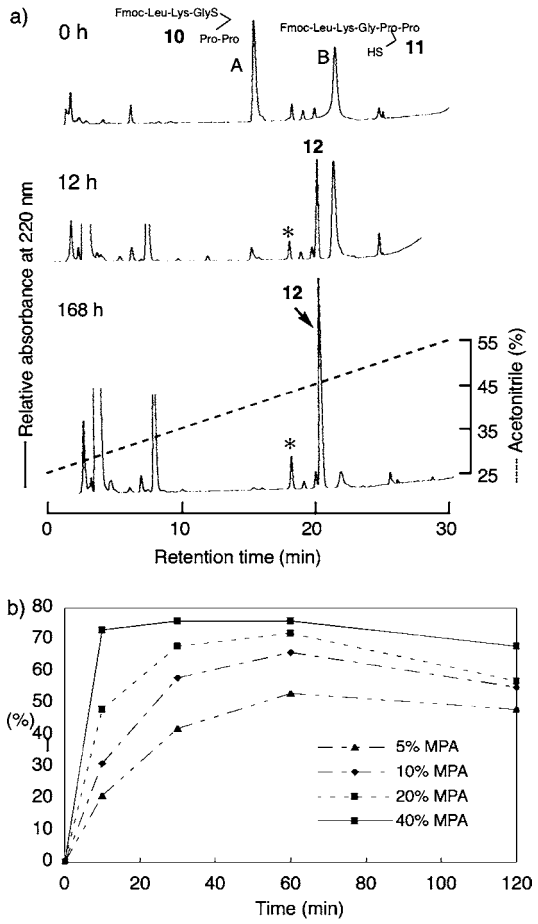

Figure 1. (a) HPLC profile of the thioesterification of a peptide mixture of **10** and **11**. HPLC elution conditions: eluent, aqueous acetonitrile containing 0.1% TFA; column, Mightysil RP-18 GP (4.6 × 150 mm) at a flow rate of 1 mL/min. * denotes the hydrolyzed product of **12**. (b) Time course of the yield of the peptide thioester **12** under microwave irradiation conditions in aq MPA.

Both peaks had the same mass number. When peak A collected by HPLC was redissolved in aq $NaHCO_3$ (pH 8) and immediately analyzed by HPLC, it was completely converted to peak B. In contrast, the conversion of peak B to peak A occurred slowly under acidic conditions (3% aq TFA, 2 days). Peak A was negative to Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid)), whereas peak B was positive. These data supported the possible structures of **10** and **11** shown in Figure 1a, which means that the thioesterification occurs under acidic conditions. However, the formation of diketopiperazine on resin might be slower than the reverse *S*- to *N*-acyl migration in a weakly acidic or basic condition, which prevents the reaction shown in route A.

On the basis of these results, we attempted the intermolecular thioester exchange reaction in the presence of an excess amount of MPA (Scheme 1, route B). The peptide mixture of **10** and **11** was dissolved in 40% aq MPA at room temperature, and the formation of Fmoc−Leu-Lys-Gly−SCH_2CH_2COOH **12** was monitored by HPLC. As shown in Figure 1a, the thioester **10** was converted to the tripeptide thioester of MPA **12** within 12 h without serious side reactions. By elongation of the reaction time (168 h, 7 days), the amide **11** was also converted to the desired thioester. It seems that under these conditions (pH = 1.3), the amide **11** is gradually converted to the thioester **10**, which is then transthioesterified by MPA. The yield, calculated by the area ratio of the product to all peptidic components eluted by HPLC, was 70%. Under these conditions, the hydrolyzed peptide was less than 15%. To enhance the reaction rate, the thioesterification reaction was performed under microwave irradiation. This technique is becoming popular even in peptide synthesis.[31,32] In 40% MPA, the reaction was almost completed within 30 min under microwave irradiation conditions (150 W, 80 °C) (Figure 1b). The reaction was slower in 20% and 10% MPA; nevertheless, it took only about 1 h for completion. Considering the easiness in the purification step, the reaction in these decreased concentrations of MPA might be favorable. On the basis of the successful clean and quick conversion to the thioester, we thought this reaction could become a novel method for obtaining peptide thioesters despite the failure in diketopiperazine formation.

To examine the general applicability of this method for peptide thioester preparation, an *N*-acetylglucosaminylated peptide thioester representing the sequence of emmprin (34−58) was prepared as shown in Figure 2a. In this synthesis, we intended to obtain a peptide thioester directly from the solid support. Thus, amino PEGA resin, which retains high-swelling ability in aqueous media, was used to achieve an efficient thioesterification reaction in aq MPA. The C-terminal proline that was unnecessary in route B was omitted. Thus, Fmoc-Pro(CH_2STrt) **1** was directly introduced to the amino groups in the resin using the 1,3-dicyclohexylcarbodiimide (DCC)−1-hydroxybenzotriazole (HOBt) method. Then, the sequence of emmprin (34−58) was synthesized using the ABI433A peptide synthesizer by the 0.1 mmol scale FastMoc protocol (0.1 Ω MonPrevPk), which uses *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU)−HOBt-*N*,*N*-diisopropylethylamine (DIEA) as a coupling reagent (the details of the protocol are described in the Supporting Information). Asn[44] was introduced using Fmoc−Asn($GlcNAcBn_3$) by the DCC−HOBt method. After the completion of the peptide chain assembly, the resin was treated by Reagent K, followed by the "low-acidity TfOH" mixture (TfOH, Me_2S, *m*-cresol, 1,2-ethanedithiol, TFA)[22,23,33,34] at −10 °C for 2 h to remove the benzyl groups of the carbohydrate moiety. Then, the resin was treated with aq MPA under microwave irradiation conditions. In contrast to the model experiment shown in Figure 1, the reaction in 40% MPA was too drastic to obtain the desired thioester in high yield. In MPA concentrations

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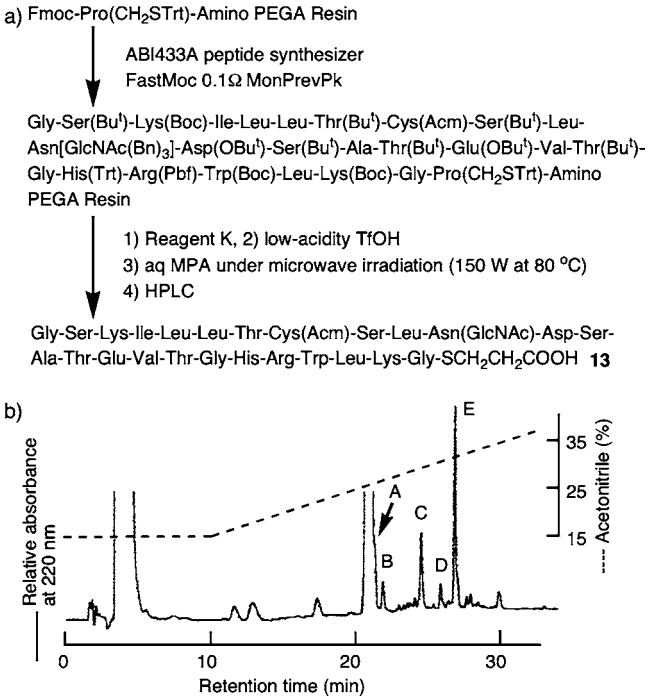
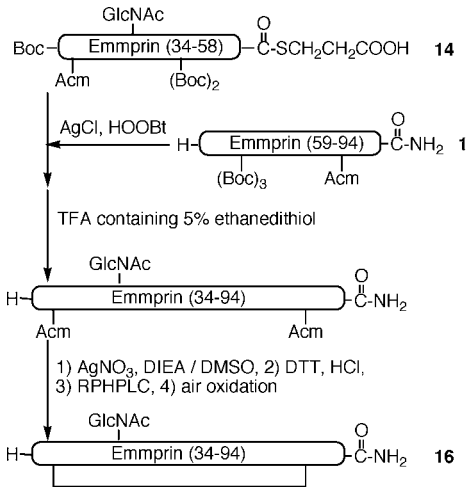


Figure 2. Synthesis of peptide thioester **13**: (a) synthetic route, (b) RPHPLC profile. Elution conditions: same as those in Figure 1a. Peaks A (eluted as a right-side shoulder of a large peak derived from MPA) and B: see text. C: Defective peptide thioester, which lacks C-terminal HRWLKG. D: Hydrolyzed product of **13**. E: Peptide **13**.

of 10% and 20%, the desired thioester **13** was obtained in good purity within 1 h as shown in Figure 2b. The isolated yield of the product was 4.2% and 3.7% in the case of 10% and 20% MPA, respectively, based on the amino group in the initial resin. These yields are over 2 times higher than that of the previous synthesis,[22] demonstrating the efficiency of the new method. The amino acid analysis of the residual resin showed that the peptide that remained on the resin was as low as 5%. However, it has to be noted that the peptide bond between Asp[45] and Ser[46] showed marked lability to 10−20% MPA treatment. The peptide bond was cleaved in about a 25% ratio to the desired product (peak A in Figure 2b, emmprin (48−58)-SCH_2CH_2COOH; peak B, [Asn-(GlcNac),[44] Cys(Acm)[41]]−emmprin (34−45)). At present, we have not searched for conditions to avoid this issue, but a further increase in the yield might be expected by solving this problem.

Then, the use of this thioester for segment coupling was demonstrated. The amino groups of the peptide thioester **13** were protected by the Boc groups using Boc−OSu. The obtained peptide **14** was condensed with C-terminal peptide emmprin (59−94) **15** by the thioester method following the previous synthesis (Scheme 3). The segment coupling

Scheme 3



proceeded well as in the previous synthesis.[22] The removal of Boc and Acm groups, followed by air oxidation, gave the final product, the extracellular first Ig domain of emmprin (34−94) carrying GlcNAc **16** (25% based on peptide **15**).

In conclusion, we have developed a novel post-SPPS thioesterification reaction under microwave irradiation conditions. This method is fully compatible with the conventional Fmoc solid-phase method and gives a peptide thioester in good yield. The obtained peptide thioester was successfully applied to the synthesis of the *N*-acetylglucosaminylated peptide composed of 61 amino acid residues by the thioester method, demonstrating the usefulness of the thioester obtained by this novel method. A series of potent N to S migratory devices for thioesterification are currently being investigated.

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Supporting Information Available: Experimental procedures for **1** and **3**−**16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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