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Peptide thioester preparation based on an *N*-*S* acyl shift reaction mediated by a thiol ligation auxiliary

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Abstract—Formation of peptide thioesters, based on an *N* to *S* acyl shift mediated by an auxiliary, *N*-4,5-dimethoxy-2-mercaptobenzyl (Dmmb) group, under acidic conditions, is described. The protected peptide was assembled on a hydroxymethylphenylacetamidomethyl resin via an *N*-Dmmb-amino acid residue according to standard Fmoc solid-phase peptide synthesis following treatment with trifluoroacetic acid. The peptide α-thioester was released from the resin by reaction with 2-mercaptoethanesulfonic acid in the presence of *N*,*N*-diisopropylethylamine.

Peptide thioesters are key building blocks in contemporary ligation chemistry for polypeptide synthesis such as the thioester method[1,2] and native chemical ligation.[3,4] During the course of our studies related to new ligation methods, we developed the *N*-4,5-dimethoxy-2-mercaptobenzyl (Dmmb) group as an auxiliary for extended chemical ligation.[5] After the ligation of the peptide segments, the Dmmb group can be removed by acidic treatment such as trifluoromethanesulfonic acid in trifluoroacetic acid (TFA), although unexpected *N* to *S* acyl shift occurred, in part, under the acid conditions.[6] The *N*-*S* acyl shift represents the first step in protein splicing reactions[7,8] and in the case of peptide thioester production this intramolecular reaction is used to accomplish thioester synthesis by an intermolecular thiol exchange reaction.[9,10] Similarly to protein splicing reactions, this *N*-*S* acyl shift reaction, which is mediated by the thiol auxiliary residue, is utilized for the manufacture of peptide thioesters. In this scenario, thioester synthesis can be realized with no thioester bond present during peptide chain elongation. As a result, this alternative for thioester production is amenable to standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. In other words, this synthetic strategy would avoid the drawbacks associated with the decomposition of thioester bonds by piperidine treatment and racemization of the amino acid residue involved in the thioester bond during peptide chain elongation. Here, we describe preliminary results of peptide thioester formation via the *N*-*S* acyl shift reaction on the solid support.

The strategy for the synthesis of a peptide thioester via an *N*-*S* acyl shift is shown in Scheme 1. Peptide **1**, with the Dmmb group attached to the backbone as a thioester-producing auxiliary, is treated with an acid, such as TFA, resulting in the peptide thioester intermediate **2** via an intramolecular *N*-*S* acyl shift. To avoid an *S* to *N* reverse acyl shift, an external thiol must be added to afford the thiol-exchanged peptide thioester **3** and aminothiol **4**.

The first model peptide thioester, Fmoc-His-Pro-Ile-Arg-Gly-$SCH_2CH_2SO_3H$ (**8a**), was synthesized according to Scheme 2. The protected peptide, Fmoc-His(Trt)-Pro-Ile-Arg(Pbf)-Gly, was assembled by Fmoc-based solid-phase peptide synthesis (SPPS) starting from **5**, which contains a Dmmb auxiliary group attached to the Gly-Ala-OCH_2-Pam resin (Pam: phenylacetamidomethyl). The peptide resin **6a** was treated with a mixture of 88% TFA/5% water/5% phenol/2% triisopropylsilane (v/v/v/v) for 1 h to remove the protecting groups as well as to initiate the *N*-*S* acyl shift, and to provide the resin-bound thioester intermediate **7a**.

Keywords: Fmoc solid-phase peptide synthesis; *N*-*S* Acyl shift; Peptide thioester; Thioester-producing auxiliary.
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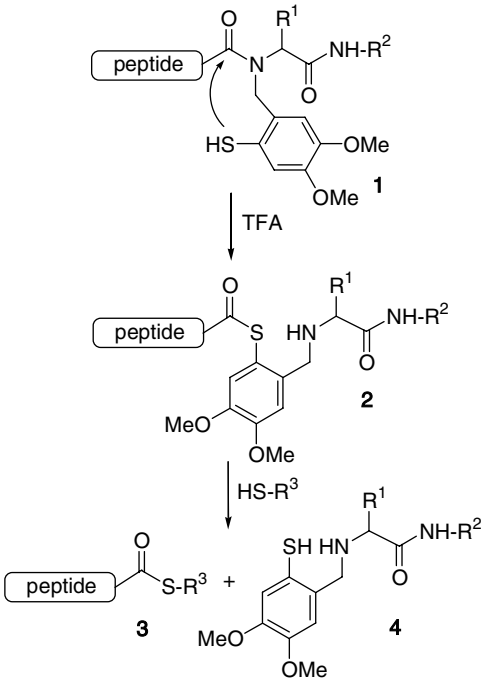
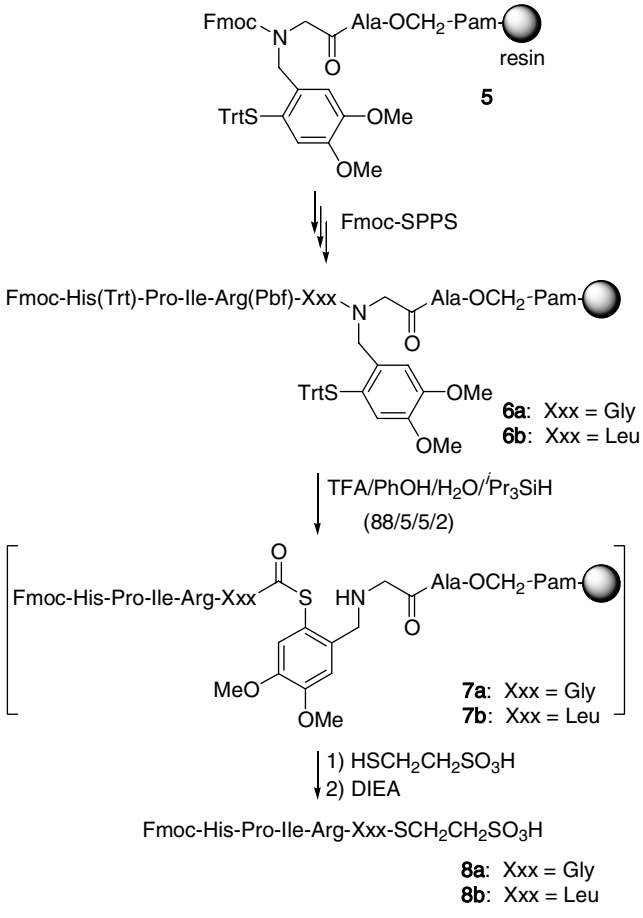


Scheme 1. 4,5-Dimethoxy-2-mercaptobenzyl group mediated peptide thioester synthesis.

After washing, first with dichloromethane, and then with 1-methylpyrolidin-2-one, the resin was treated with 0.1 M (final concentration) 2-mercaptoethanesulfonic acid in DMF, prepared in situ from sodium mercaptoethanesulfonate and 4 M hydrogen chloride in 1,4-dioxane, followed by 0.2 M (final concentration) *N*,*N*-diisopropylethylamine (DIEA) to release the peptide as peptide thioester **8a**[11] (Fig. 1A). The crude thioester was purified by reversed-phase (RP) HPLC to give **8a** in 8.2% yield, based on the Ala residue on the resin. After the cleavage reaction by thiol, the resin was treated with a mixture of 12 M HCl and propionic acid (1:1, v/v) to hydrolyze the peptide, and applied for amino acid analysis, showing that the peptide still remained on the resin.

In peptide ligation reactions, *S*-*N* acyl shift is affected by the side chains of the amino acids at the ligation site.[12] When an alanine residue is attached to the Dmmb auxiliary, *S*-*N* acyl shift proceeds more slowly than in the case of a glycine residue. Therefore alanine was examined as the site of attachment for the Dmmb auxiliary, instead of glycine, to examine suppression of the *S*-*N* reverse acyl shift. In this case, from the protected peptide resin **9** (Fig. 2), peptide thioester **8a** was obtained in an increased yield of 16%. Furthermore, when peptide resin **9** was treated for 12 h under acidic conditions followed by the thiol, the yield of peptide thioester **8a** increased



Scheme 2. Synthesis of peptide thioester **8** via an *N*-*S* acyl shift on resin.

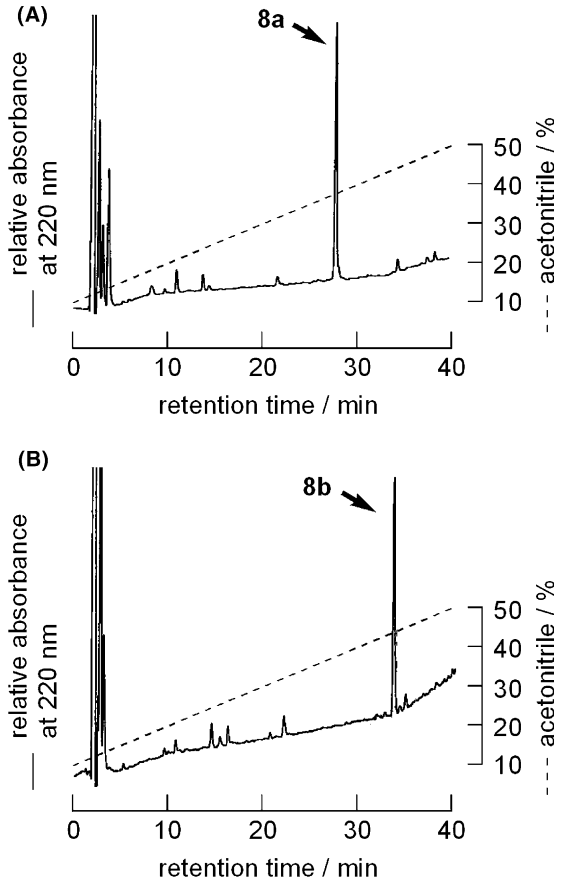


Figure 1. RP-HPLC elution profiles of the peptide thioesters **8** cleaved from the resin **6** (4 h). (A) **8a**, (B) **8b**. Column: Cosmosil 5C18-AR-II (4.6 × 150 mm), eluent: 0.1% TFA in aq acetonitrile, 1.0 mL/min.

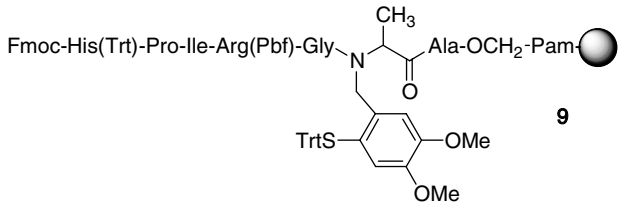


Figure 2. Structure of protected peptide resin **9**.

to 31%. These results indicate that both suppression of the *S*-*N* reverse acyl shift and the time of acid treatment are effective in increasing the yield.

To investigate epimerization at the thioester position, the peptide leucyl thioester **8b**[11] was synthesized from resin **6b** accordingly (yield 5.2%). The distinct peak of a D-Leu residue-containing peptide thioester was not detected in the RP-HPLC profile of the crude material (Fig. 1B), when the chromatogram was compared with that of an authentic D-Leu residue-containing peptide sample prepared via Boc SPPS.

Several methods for the preparation of peptide thioesters by the Fmoc-SPPS have been reported. However, the direct preparation of peptide thioester is accompanied by epimerization at the amino acid residue adjacent to the thioester moiety, when published procedures are applied.[13] Another indirect method, based on a safety catch linker, results in modifications such as alkylation at the mandatory activation step for the sulfonamide,[14,15] and suspicion of loss of peptide chain by the activation of the linker by acetylation at the capping step.[16] The new method described here represents a promising approach for the preparation of peptide thioesters without epimerization or other side reactions.

In conclusion, we have shown that the *N*-*S* acyl shift can be mediated by attaching a thiol auxiliary residue to the peptide backbone. This method can be applied for peptide thioester syntheses following standard Fmoc-SPPS protocols. Although the efficiency of peptide thioester production achieved by thiol treatment of the deprotected peptidyl-resin is not currently sufficient in terms of the yield, the observation of the *N*-*S* acyl shift reaction provides an important lead to open a new methodology for the preparation of peptide thioester.

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