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Tetrahedron Letters 44 (2003) 8085–8087

TETRAHEDRON LETTERS

Use of thiosulfonate for the protection of thiol groups in peptide ligation by the thioester method

Takeshi Sato and Saburo Aimoto*

Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Received 15 August 2003; revised 3 September 2003; accepted 5 September 2003

Abstract—Use of thiosulfonate for protecting thiol (-SH) groups in peptide ligation by the thioester method was examined. Thiosulfonate was introduced and was stable in the presence of silver ion, 4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine, and diisopropylethylamine. Based on these results, a strategy for using the thioester method and the native chemical ligation method in the synthesis of a single polypeptide is described.

The chemical synthesis of a large protein often requires multiple ligation steps. Moreover, in many cases, different ligation methodologies must be employed for a synthesis of a single protein. In this report, the use of thiosulfate for the protection of a thiol (-SH) group on a peptide under conditions of peptide ligation by means of the thioester method was examined. A strategy for a simple procedure that realizes the combination of two ligation chemistries, the thioester method[1] and the native chemical ligation (NCL) method,[2] for the synthesis of a single polypeptide is proposed.

The thioester method is an established methodology that is applied in the synthesis of various types of polypeptides.[3] The use of the thioester method requires a partially protected peptide thioester and a peptide in which side chain amino groups and -SH groups are protected with a *t*-butyloxycarbonyl (Boc) group and acetamidomethyl (Acm) group,[4] respectively. While the introduction of Boc groups is a simple technique by using a reagent such as *N*-*t*-butyloxycarbonyloxysuccinimide, it is difficult to introduce Acm groups or methylbenzyl groups to free -SH groups on a peptide. In the preparation of a building block for the thioester method, Cys is introduced into the peptide using Cys(Acm) at the peptide chain elongation reaction on a resin. There is, however, no established method for masking the free -SH group on a peptide. The application of NCL to prepare a building block for the thioester method is problematic. When a polypeptide is synthesized using NCL, the product contains at least one free -SH group of Cys due to the reaction mechanism involved. To overcome this problem, thiosulfonate ($S_2O_3^-$) was chosen as an -SH protecting group. Thiosulfonate was utilized in a chemical modification of a protein[5] and introduced for quantitative recovery of

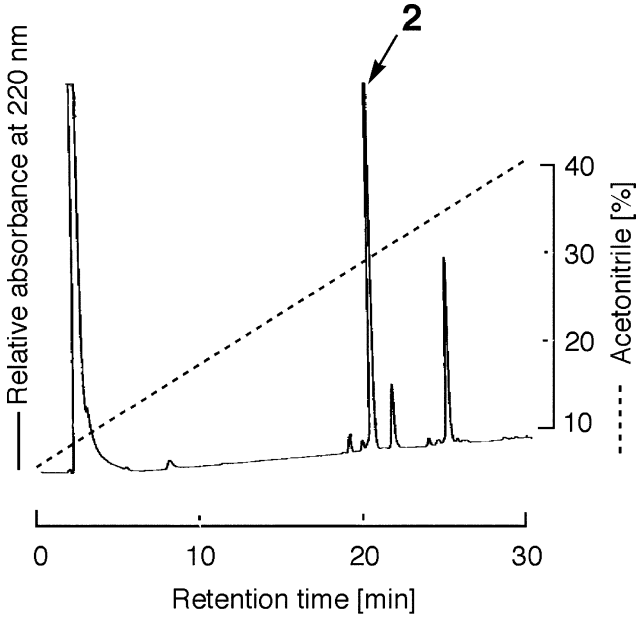

Figure 1. RP-HPLC elution profile of a reaction mixture of the introduction of thiosulfate to peptide (**1**). The peak indicated by an arrow contained the desired peptide component (**2**). Column: Cosmosil 5C18 AR-300 (4.6×150 mm), eluent: 0.1% TFA in aq. Acetonitrile, 1.0 ml/min.

* Corresponding author. Tel.: +81-6-6879-8601; fax: +81-6-6879-8601; e-mail: aimoto@protein.osaka-u.ac.jp

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doi:10.1016/j.tetlet.2003.09.039

cysteine in the amino acid analysis of proteins.[6] This thiosulfonate can be introduced to free -SH on a peptide by simply mixing the peptide and sodium tetrathionate ($Na_2S_4O_6$) in a solvent, which can then be removed by treatment with dithiothreitol (DTT). In this experiment, we examined the stability of thiosulfonate in the protection of -SH on a building block during a condensation reaction, in the presence of silver chloride, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine-(HOObt), and diisopropylethylamine(DIEA).

For this purpose, we prepared a cysteine containing peptide, representing a peptide synthesized by means of NCL, the amino acid sequence of which was Met-Ala-Glu-Asp-Trp-Leu-Asp-Cys-Pro-Ala-NH_2 (**1**). The thiosulfonate was introduced to the free -SH group on peptide **1** by mixing the peptide and sodium tetrathionate in DMSO in the presence of DIEA.[7a] Figure 1 shows the RP-HPLC elution profile of the reaction mixture (120 min after the addition of sodium tetrathionate). The peak corresponding to the desired peptide component, Met-Ala-Glu-Asp-Trp-Leu-Asp-Cys($S_2O_3^-$)-Pro-Ala-NH_2 (**2**) appeared at 20 min (shown by an arrow).[7b] The content of the 25 min peak was determined to be a dimer of peptide **1**.[7c] Since dimerization occurred in this system, the maximum isolated yield was 44%.

For the condensation reaction with peptide building block **2**, a peptide thioester, Fmoc-Arg-Lys(Boc)-Lys-(Boc)-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Gly-SCH_2CH_2-CO-Ala-OH (**3**) was prepared as an N-terminal building block using a procedure originally reported by Kawakami et al.[8] The coupling reaction of building blocks **2** and **3** by the thioester method was examined.[9] Figure 2 shows RP-HPLC elution profiles of the reaction mixtures at the reaction times of 0 min and 120 min. The retention of the desired product, Fmoc-Arg-Lys(Boc)-Lys(Boc)-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Gly-Met-Ala-Glu-Asp-Trp-Leu-Asp-Cys($S_2O_3^-$)-Pro-Ala-NH_2 (**4**), was 35 min,[10] and the coupling yield, based on the amount of building block **2**, was 60%. As shown in the elution profile, the coupling reaction proceeded without any significant side reactions, indicating that thiosulfonate is stable under the conditions used in the thioester method. The removal of the N-terminal Fmoc from peptide **4** for further synthesis was also examined. The deprotection was successfully performed to give the product (**5**) by treatment with 3% piperidine/DMSO (v/v) for 30 min (Fig. 3).[11]

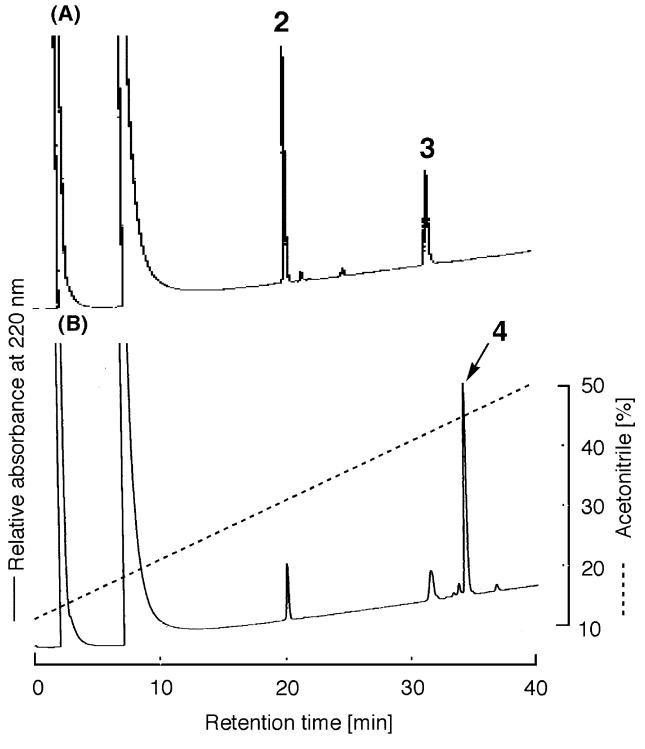


Figure 2. RP-HPLC elution profiles of the coupling reaction mixtures. (A) Elution profile of the starting materials (B) Elution profile of the coupling mixture at a reaction time of 120 min. The desired product is indicated by an arrow. Column: Cosmosil 5C18 AR-300 (4.6×150 mm), eluent: 0.1% TFA in aq. acetonitrile, 1.0 ml/min.

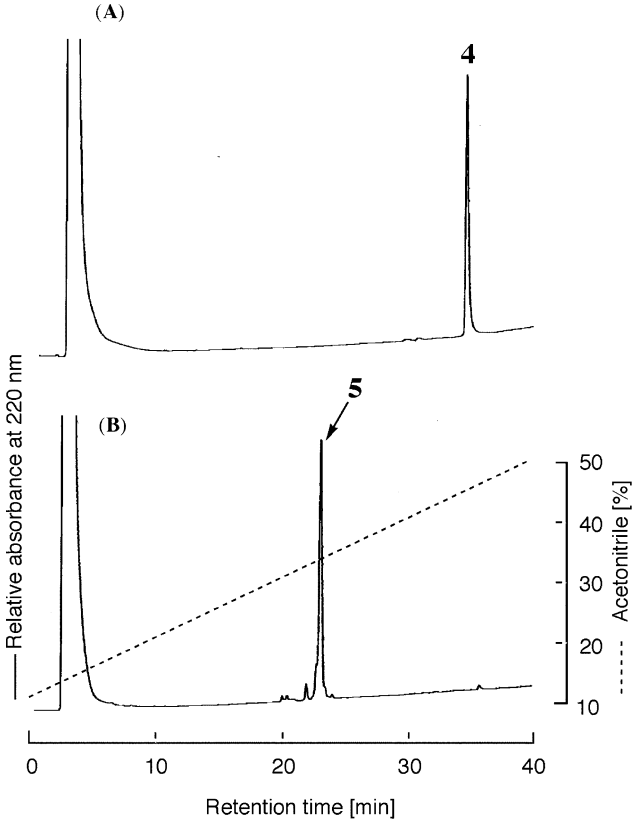
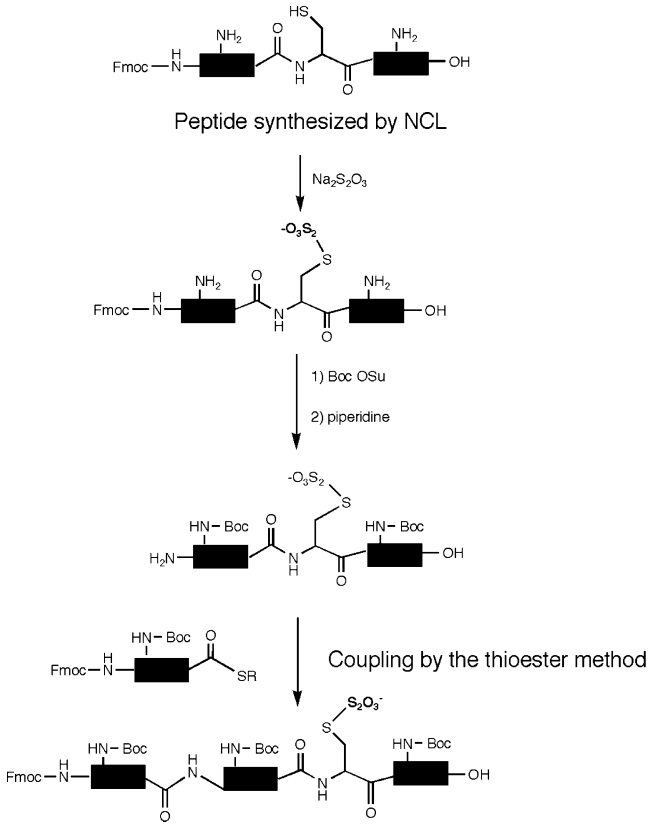


Figure 3. RP-HPLC elution profiles of the removal of Fmoc from **4**. (A) Elution profile of the starting material (**4**). (B) Elution profile of the reaction mixture at a reaction time of 30 min. The desired product **5** is indicated by an arrow. Column: Cosmosil 5C18 AR-300 (4.6×150 mm), eluent: 0.1% TFA in aq. acetonitrile, 1.0 ml/min



Scheme 1. Strategy for the synthesis of a single polypeptide by a combination of NCL and the thioester method.

As a result of this experiment, we conclude that thiosulfonate can be reliably used as a protecting group for an -SH group in peptide ligation using the thioester method. Furthermore, a strategy for the synthesis of a single polypeptide by a combination of NCL and the thioester method is proposed and shown in Scheme 1. The combination of these two techniques eliminates the restriction of amino acid sequence specificity at a site of peptide ligation for a synthesis of a larger polypeptide.

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7. (a) Peptide **1** (1.4 μmol) and $Na_2S_4O_6$ (2.8 μmol) were mixed in 3% DIEA in DMSO; (b) Peptide **2**: MS (MALDI-TOF) found 1260.0, calcd 1261.3 $(M+H)^+$; (c) The dimerization of peptide **1** can be avoided by carrying out the reaction without DIEA.
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9. Peptide **2** (0.51 μmol), peptide **3** (0.56 μmol), AgCl (1.7 μmol), HOOBt (16.8 μmol), and DIEA (11 μmol) was mixed and stirred in DMSO (400 μl).
10. Peptide **4**: MS (MALDI-TOF) found 3116.1, calcd 3119.0 $(M+H)^+$; Amino acid analysis $Asp_{2.2}Glu_{2.5}Pro_{1.0}Gly_{2.0}Ala_2Cys_{0.6}Met_{0.9}Leu_{1.0}Trp_{nd}Arg_{5.3}$.
11. Peptide **5**: MS (MALDI-TOF) found 2897.9, calcd 2896.8 $(M+H)^+$.