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Sequential peptide chemical ligation by the thioester method and extended chemical ligation

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Abstract—The sequential chemical ligation of peptide thioesters by a combination of the thioester method and extended chemical ligation using a photoremovable auxiliary, 2-mercapto-1-(2-nitrophenyl)ethyl group, is described. The thiazolidine ring was used as a protecting group for the N-terminal 1,2-aminoethanethiol moiety of the auxiliary in the middle peptide thioester. After the first thioester coupling, the thiazolidine ring was opened by treatment with *O*-methylhydroxylamine. Second coupling by extended chemical ligation followed by UV irradiation gave the target polypeptide.

The chemical ligation of peptide segments, prepared by either synthetic or biological methods, is a widely used procedure in polypeptide synthesis.[1] Since the introduction of peptide thioesters as building blocks in the thioester method,[2,3] ligation chemistry such as the native chemical ligation[4,5] has also been developed. The thioester method can be performed at any ligation site, while it requires some protecting groups, an activator such as silver salts, and organic solvents. On the other hand, native chemical ligation can be carried out under neutral aqueous conditions without the need for protecting groups, although a cysteine residue is required at the condensation site. Several groups have introduced auxiliaries that permit ligation to proceed in the absence of a cysteine residue.[6–9] The combination of these ligation methods offers flexibility in the choice of the condensation site in the multi-step polypeptide synthesis. We previously proposed the possibility of sequential chemical ligations in which native chemical ligation and the thioester method are combined by the introduction of *S*-thiosulfonate as a protecting group for the thiol group.[10] Here, we describe another strategy, in which the thioester method and extended chemical ligation are combined, using a photoremovable auxiliary in a sequential manner.

The use of a thiazolidine skeleton as a protection for 2-aminoethanethiol moiety was previously reported to be useful for an N-terminal cysteine residue in sequential native chemical ligation reactions.[11] This protection would also be promising for the auxiliary in extended chemical ligation as well. Based on this protection, we designed a sequential coupling strategy for polypeptide synthesis by a combination of the thioester method and extended chemical ligation using a photoremovable ligation auxiliary, a 2-mercapto-1-(2-nitrophenyl)ethyl (Mnpe) group,[9] as shown in Scheme 1. Challenges are whether the thiazolidine is stable under the conditions used in the thioester method, in the presence of silver salts, and whether it would be opened under the mild conditions used.

As a model peptide, Leu-Lys-Asn-Thr-Ser-Val-Leu-Gly-Ala-Gly-Gly-Gln-Thr-Gln-Asp-His-Phe-Lys-Leu-Thr-Ser-Leu-Pro-Val-Leu-Ile-Arg-Leu-NH_2 (**7**) was synthesized (In Scheme 1, peptide 1: Leu-Lys-Asn-Thr-Ser-Val-Leu-Gly-Ala-Gly, peptide 2: Gln-Thr-Gln-Asp-His-Phe-Lys-Leu, peptide 3: Thr-Ser-Leu-Pro-Val-Leu-Ile-Arg-Leu). An N-terminal peptide thioester, Leu-Lys-Asn-Thr-Ser-Val-Leu-Gly-Ala-Gly-SCH_2CH_2CO-Leu-NH_2 (**1**),[12] and a C-terminal peptide, Thr-Ser-Leu-Pro-Val-Leu-Ile-Arg-Leu-NH_2 (**3**),[12] were prepared by conventional Boc and Fmoc solid-phase peptide synthesis, respectively.[13]

Keywords: Extended chemical ligation; Peptide thioester; Photoremovable ligation auxiliary; Sequential chemical ligation; Thiazolidine; Thioester method.
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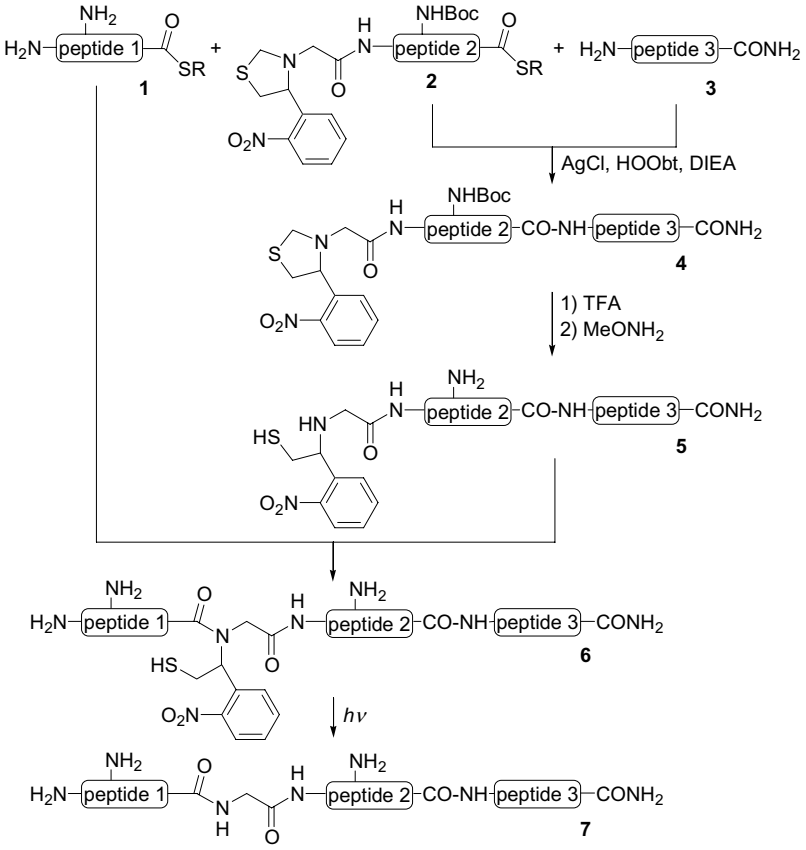
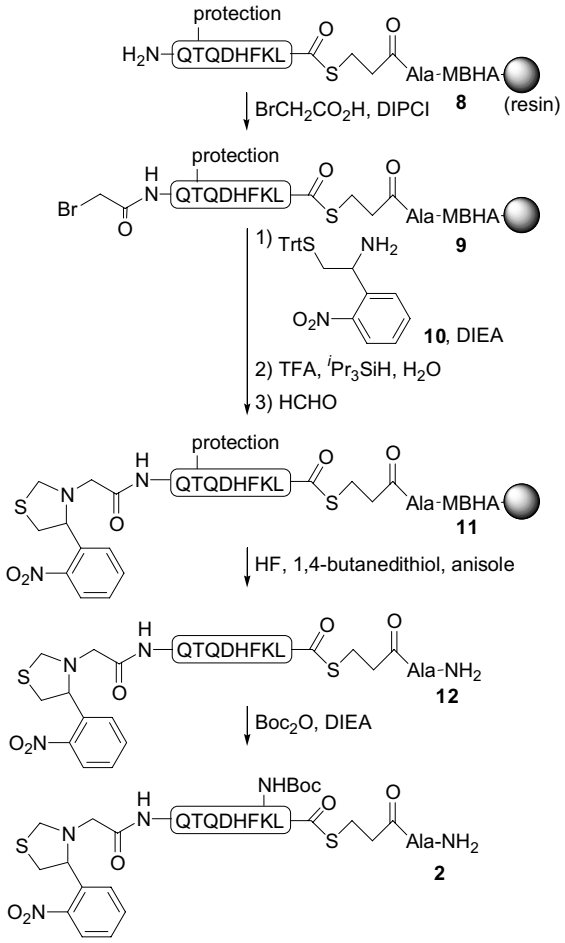


Scheme 1. A sequential coupling strategy for polypeptide synthesis by a combination of the thioester method and extended chemical ligation.

A middle peptide thioester **2** having both the ligation auxiliary and thioester moieties, corresponding to the sequence, Gly-Gln-Thr-Gln-Asp-His-Phe-Lys-Leu was synthesized as shown in Scheme 2. The peptide thioester was constructed on a *p*-methylbenzhydrylamine resin based on Boc chemistry,[13] and the final Gly residue, containing an *N*-Mnpe group, was introduced in two steps; in the first step, bromoacetic acid (6 equiv) was attached to the N-terminus of the protected peptide resin **8** using diisopropylcarbodiimide (DIPCI) (3 equiv) in DMF for 1 h to give **9**, followed by the treatment with the auxiliary amine **10**[14] (8 equiv) in DMSO for 24 h and further 24 h after the addition of *N*,*N*-diisopropylethylamine (DIEA). After removing the trityl (Trt) group by treatment with trifluoroacetic acid (TFA) containing 2% triisopropylsilane and 2% water, the thiazolidine ring was constructed by treatment with 5% formalin in *N*-methylpyrrolidinone for 4 h to give the protected peptide thioester resin **11**. After treatment with hydrogen fluoride containing 7.5% 1,4-butanedithiol and 7.5% anisole, followed by reversed-phase (RP-) HPLC, peptide thioester **12**[12] was obtained in 10% yield based on the Leu residue attached to the thioester. The introduction of a Boc group, followed by RP-HPLC gave building block **2**[12] in 75% yield.

The first ligation of peptide blocks **2** and **3** (1.1 equiv) was carried out under the conventional conditions of the thioester method.[13] Thus, the peptides were reacted in the presence of silver chloride (3 equiv), 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOObt) (30 equiv), DIEA (20 equiv) in DMF for 48 h to give peptide **4**[12] (Fig. 1(A)). After the addition of dithiothreitol (DTT), followed by washing with ether, the residue was treated with TFA containing 5% water for 0.5 h and again washed with ether. The residue was dissolved in 0.1 M sodium phosphate buffer (pH 6.2), and *O*-methylhydoxylamine hydrochloride (final concentration: 0.4 M) was added to the solution.[11a] The mixture was stirred for 4 h and DTT was added (Fig. 1(B)), and RP-HPLC gave the peptide building block **5**,[12] ready for the next extended chemical ligation, in 47% yield based on peptide **2** in three steps. The next extended chemical ligation of peptide **5** with the peptide thioester **1** (1.8 equiv) was carried out in 0.1 M sodium phosphate buffer containing 6 M guanidine hydrochloride and 2% thiophenol (v/v) for 24 h. After the addition of DTT (Fig. 1(C)), RP-HPLC purification gave peptide **6**[12] in 77% yield. Finally, the Mnpe group was removed by UV irradiation at 365 nm (Handheld Lamp UVL-56, 6 W, UVP, Upland, CA) in sodium phosphate buffer (pH 5.3) containing 6 M guanidine hydrochloride for 1 h to give peptide **7**[12] (Fig. 1(D)) in 56% yield after RP-HPLC purification.

In conclusion, the thioester method and extended chemical ligation can be combined in a sequential manner using more than three building blocks for polypeptide synthesis. A thiazolidine ring can be used as a temporal protecting group for the ligation auxiliary. Because this



Scheme 2. Preparation of middle peptide building block **2**. Protecting groups for the side chains of amino acid residues were benzyl for Thr, cyclohexyl for Asp, benzyloxymethyl for His, and 2-chlorobenzyloxycarbonyl for Lys.

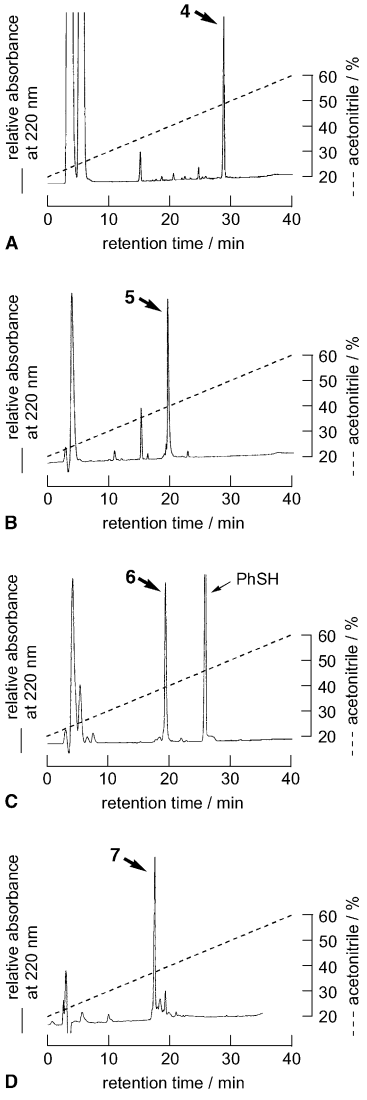


Figure 1. RP-HPLC elution profiles of the reaction mixtures. (A) First coupling by the thioester method (48 h and DTT); (B) removal of the protecting groups after the first ligation (4 h and DTT); (C) second coupling by the extended chemical ligation (24 h and DTT); (D) removal of the Mnpe group (1 h). Column: YMC-Pack Pro C18 (4.6 × 150 mm), eluent: 0.1% TFA in aq acetonitrile; 1.0 mL/min.

protection has been shown to be useful in sequential native ligation reactions,[11] it would also be applicable for a combination of the thioester method and native chemical ligation. These combinations of ligation methods provide a flexible choice of condensation sites in polypeptide synthesis using multi-component peptide building blocks.

Acknowledgements

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12. **1**: MALDI-TOF MS found *m/z* 1160.1, calcd 1159.7 ($M+H^+$); **2**: ESI MS found *m/z* 1524.4, calcd 1524.7 ($M+H^+$); **3**: ESI MS found *m/z* 1010.8, calcd 1010.7

($M+H^+$); **4**: MALDI-TOF MS found *m*/*z* 2359.0, calcd 2358.3 ($M+H^+$); **5**: MALDI-TOF MS found *m*/*z* 2247.3, calcd 2246.2 ($M+H^+$); **6**: MALDI-TOF MS found *m*/*z* 3189.9, calcd 3188.7 ($M+H^+$); **7**: MALDI-TOF MS found *m*/*z* 3007.5, calcd 3008.2 ($M+H^+$); **12**: ESI MS found *m*/*z* 1424.4, calcd 1424.6 ($M+H^+$).

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14. The photoremovable ligation auxiliary amine **10** was previously prepared starting from 2-nitrobenzaldehyde in Ref. 9. As an alternate and simple route, 2-bromo-2′-nitroacetophenone was reacted with triphenylmethanethiol to give a protected thiol compound, and was then reacted with *O*-methylhydroxylamine to give the oxime derivative, followed by reduction to give amine **10**. Details of this will be reported elsewhere.