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An efficient peptide ligation using azido-protected peptides via the thioester method

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ABSTRACT

Azido-protected Fmoc–Lys–OH (Fmoc–Lys(N_3)–OH) was synthesized from Fmoc–Lys–OH by the copper(II)-catalyzed diazo transfer method, and introduced to a peptide by the ordinary Fmoc-based solid-phase peptide synthesis. This azido peptide could be condensed with a peptide thioester by the Ag^+-free thioester method without any significant side reactions. The azido group was easily reduced to an amino group by Zn powder after peptide condensation.

The solid-phase peptide synthesis (SPPS) is usually limited to a length of approximately less than 50 residues long. To overcome this problem, condensation methods involving two or more peptide segments are employed for the synthesis of longer peptide sequences. To date, two methods have been developed: native chemical ligation[1] and the thioester method.[2,3] In the native chemical ligation reaction, no protecting group is needed for amino or thiol groups, although a cysteine residue is required at the ligation point. On the other hand, although any residue at the ligation point can be used in the thioester method, protecting groups are required for amino and thiol groups.

For protection of thiols in the ordinary fluorenylmethoxycarbonyl (Fmoc)-based SPPS, the trityl group is usually used. This group is removed by the trifluoroacetic acid (TFA) treatment commonly used for cleavage from the solid support. However, the released peptide having free thiol(s) cannot be used directly for the thioester method condensation reaction. To avoid this inconvenience, acetamidomethyl group is used for the synthesis of peptide segment in the thioester method because of its stability under both basic and acidic conditions.[3] For the protection of lysine side chains, *tert*-butoxycarbonyl (Boc) groups are usually introduced to the peptide segments used for this method after the cleavage from the resins and the purification steps.[3] To overcome this inconvenience, benzyloxycarbonyl (Z) groups have been used for protection of the amines. Z groups are stable under TFA acidic condition, and the peptide segments having Z groups can be used directly for the thioester method without additional protection of amines. However, the strong hydrophobicity of the Z groups sometimes gives difficulty in the purification of the peptide segment. In addition, this protecting group is removed during the deprotection of the benzyl groups used for the hydroxyl groups of the carbohydrate moiety in our glycopeptide synthesis.[4,5] Recently, Chen et al. reported that the ivDde group could be used for the protection of the lysine side chains.[6] This protecting group is stable under both acidic and basic conditions, and can be specifically removed by hydrazine treatment;[7] however, it is also hydrophobic and may cause the problem stated above when many lysine residues exist in the peptide. Hence, an alternative less-hydrophobic protecting group for amines stable under both acidic and basic conditions was desired.

The azide moiety might be a good candidate for this purpose. In a few experiments, azido-protected Fmoc–Lys–OH (Fmoc–Lys(N_3)–OH) was introduced to peptides. These azido peptides were used for synthesizing dendrimers or the activity-based protein profiling method via 'click chemistry' reaction.[8,9] However, the methods for deprotection (reduction) of azide moieties at the peptide side chains have not yet been developed. We found that the azide-protected peptides could be reduced by Zn/AcOH. This useful protocol enabled us to synthesize the physiologically active peptides by utilizing azido-protected Fmoc–Lys–OH (Fmoc–Lys(N_3)–OH) into the ordinary Fmoc-based SPPS and subsequent segment condensation by the thioester method. In this study, we synthesized pigment dispersing hormones (PDHs) of the kuruma prawn, *Marsupenaeus japonicus*, as model peptides. It was reported that there were two

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PDH-I NSELINSLLGIPKVMTDA-NH_2
PDH-II NSELINSLLGLPKFMIDA-NH_2

Figure 1. Amino acid sequences of pigment dispersing hormones. An arrow indicates the peptide coupling site.

PDHs in the sinus gland of *M. japonicus*; PDH-I and -II.[10] As shown in Figure 1, both consist of 18 amino acid residues and an amidated C-terminus. Since the N-terminal 10 residues of PDHs are identical and only 3 residues in the C-terminal half are different, we synthesized an N-terminal 10-residue peptide thioester as the common peptide segment and two C-terminal 8-residue peptides having an azide moiety at Lys.[13]

Fmoc–Lys(N_3)–OH **1** was synthesized from Fmoc–Lys–OH **2** by the copper(II)-catalyzed diazo transfer method (Scheme 1).[11,12] Although K_2CO_3 is generally used as a base for the introduction of the azido group to the amino acid,[13–15] Fmoc group is not stable in the K_2CO_3 solution. Therefore, to avoid the cleavage of the Fmoc group, $NaHCO_3$ was used as the base. Azido-protected lysine **1** was introduced to the peptide segments, **3a** and **b**, by the Fmoc-based SPPS. As reported previously, treatment of the TFA cocktail containing triisopropylsilane as a scavenger of the trityl group for 1.5 h did not decompose the azide group,[15] although about 20% of azide group was converted to the amino group within 2 h when 1,2-ethanedithiol was used instead of triisopropylsilane due to the reducing ability of the thiol compound (data not shown). The segments **3a** and **b** were purified by reversed-phase (RP)-HPLC. The isolated yields were 56% and 37%, respectively.

The N-terminal peptide thioester **4** was synthesized by the *N*-alkylcysteine (NAC)-assisted thioesterification method developed in our previous study.[16,17] Fmoc-*N*-ethyl-*S*-trityl-cysteine (Fmoc–(Et)Cys(Trt)–OH) was introduced to Rink Amide MBHA resin and the peptide chain was elongated by ordinary Fmoc-based SPPS. After cleavage from the resin, the peptide was thioesterified by the addition of 4-mercaptophenylacetic acid under the acidic condition. This reaction was almost completed within 24 h giving the desired peptide thioester. The yield of **4** after purification by RP-HPLC was 4.8%. In our preliminary experiment, a peptide thioester containing azide groups could be prepared by this NAC-mediated method without significant decomposition of the azide groups.

The peptide segments were condensed by the Ag^+-free thioester method (Scheme 2).[6,17] Segments **3a** and **4** were dissolved in dimethylsulfoxide (DMSO) containing 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HOObt), and the reaction was initiated by addition of *N*,*N*-diisopropylethylamine (DIEA) (Fig. 2a).[18] In this reaction, the azide group did not produce any by-products. The coupling reaction was completed within 3 h and the desired peptide **5a** was obtained (Fig. 2b). After condensation, the Fmoc group at the amino terminus was removed by piperidine treatment. The azide group was converted to amino group by Zn/AcOH treatment

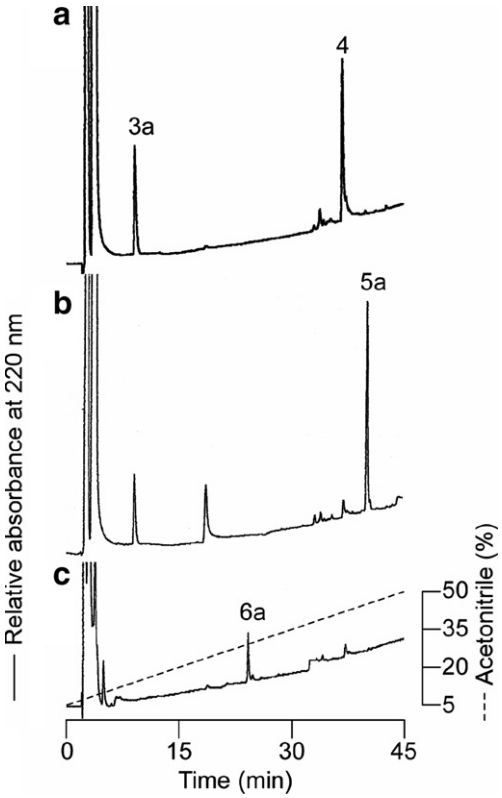
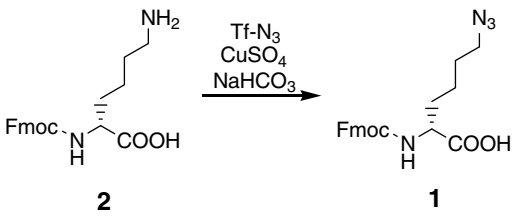
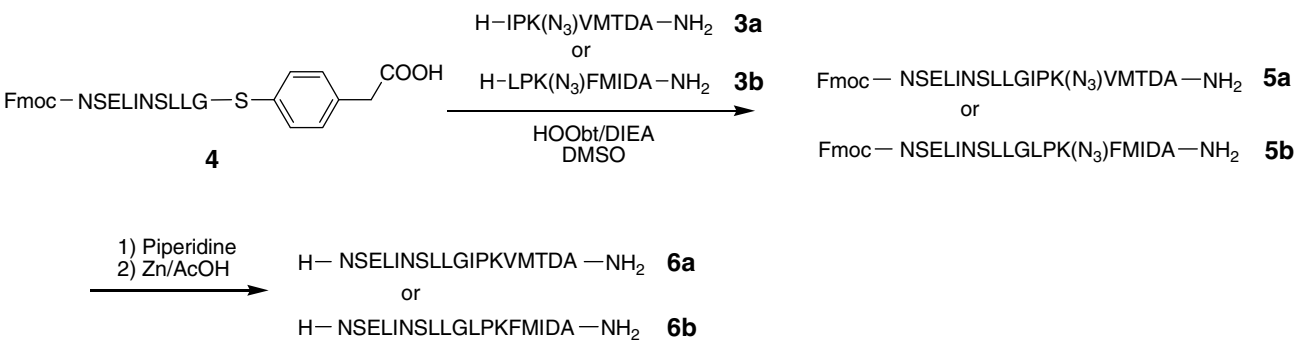


Figure 2. RP-HPLC elution profiles of peptide condensation and deprotection. (a) Coupling reaction mixture of **3a** and **4** (0 h). (b) Three hours after the coupling reaction. (c) Reaction mixture after deprotection of the Fmoc group and the reduction of azide group. Elution condition: column, YMC-Pack PROTEIN-RP (4.6ϕ × 150 mm, YMC, Japan) at a flow rate of 1 ml/min.



Scheme 1. Azido transfer to lysine side chain.



Scheme 2. Coupling reaction of peptide segments and deprotection.

within 30 min, and the desired product **6a** was obtained without any significant side reactions (Fig. 2c). The isolated yield calculated from the amount of the N-terminal segment **4** used for the coupling reaction was 47%. The peptide segments **3b** and **4** were coupled and deprotected in the same manner as described above, and the desired product **6b** was obtained with 57% yield.

To examine the biological activity of the synthetic PDHs, **6a** and **b** (1 μg each/individual) were injected into the eyestalk-ablated prawns. The melanophore index was used to assess the pigment dispersing activity.[19] After injection of **6a** and **b**, the melanophores indices were changed from 1 or 2 to 5 within 15 min, and the dispersion was maintained for 60 min. On the other hand, the change of indices was not observed in negative control. These results indicated that the synthetic peptides were fully active.

In conclusion, we have synthesized Fmoc–Lys(N_3)–OH and introduced it to the peptide by the Fmoc-based SPPS. The azido peptides could be used for the peptide condensation by the Ag^+-free thioester method, and the azide groups were easily converted to amino groups without any undesirable reactions. Azide groups were compatible with acid-labile protecting groups such as the benzyl groups used for the protection of carbohydrate and phosphate moieties. It is, therefore, likely that this method is a good tool for synthesizing glyco- and/or phosphoproteins. Application of this method for the synthesis of a large glycoprotein is now in progress.

Acknowledgments

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Supplementary data

The photographs of melanophores before and after the synthetic peptide injections are available free of charge via the internet. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.07.037.

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12. Sodium azide (1.45 g, 22.3 mmol) was dissolved in distilled H_2O (4.3 ml) with CH_2Cl_2 (7.2 ml). Triflic anhydride (0.73 ml, 4.46 mmol) was added slowly, and stirred for 2 h. The CH_2Cl_2 phase was removed and aqueous phase was extracted with CH_2Cl_2 (3.6 ml × 2). The organic fractions containing triflyl azide were combined, washed with 5% aq $NaHCO_3$, and used without further purification. Fmoc–Lys–OH hydrochloride (900 mg, 2.23 mmol), $NaHCO_3$ (1.87 g, 22.3 mmol), and $CuSO_4$ pentahydrate (5.5 mg, 22.3 μmol) were dissolved in distilled H_2O (8 ml) and methanol (16 ml). Triflyl azide in CH_2Cl_2 (14.4 ml) was added with stirring at room temperature and the reaction continued overnight. The organic solvents were removed under high vacuum, and the remained solution was acidified at pH 2 by the addition of aq HCl. After extraction with ethyl acetate (EtOAc, 2 × 20 ml), the organic fractions were combined, washed with saturated NaCl aq solution, and dried over Na_2SO_4. After concentration, the crude product was chromatographed on silica gel with toluene/EtOAc (3:1) containing 1% AcOH to give Fmoc–Lys(N_3)–OH **1** as white powder quantitatively.
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18. Peptide segments **3a** (450 μg, 0.50 μmol) and **4** (390 μg, 0.27 μmol) were dissolved in DMSO (50 μl) containing 10% 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HOObt) and 5% DIEA, and incubated at room temperature for 3 h. Piperidine (12.5 μl) was then added to this solution, and kept at room temperature for 20 min. The crude peptide was precipitated by the addition of 10 times volume of diethyl ether, washed twice with ether, and dried under vacuum. The precipitant was dissolved in 50% aq AcOH (300 μl) containing excess amount of Zn powder, and vortexed for 30 min. Zn powder was removed by filtration, and the desired product **6a** was purified by RP-HPLC.
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