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Pyruvoyl, a novel amino protecting group on the solid phase peptide synthesis and the peptide condensation reaction

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ABSTRACT

In one of the peptide condensation methods termed thioester method, an amino protecting group is required in the lysine side chain. In this study, to investigate the efficiency of the pyruvoyl group as an amino protecting group, we synthesized N^{α}-fluorenylmethoxycarbonyl (Fmoc)-N^{ε}-pyruvoyl-lysine and introduced it into peptides and glycopeptides by the ordinary Fmoc-based solid phase peptide synthesis. The pyruvoyl peptide could be condensed with a peptide thioester by the thioester method, and this protecting group was easily removed by *o*-phenylenediamine treatment without significant side reactions.

The thioester method is one of the peptide condensation methods for synthesizing long peptide chains.[1] In this method, since a peptide thioester is activated by a silver ion and is allowed to react with a free amino group, protecting groups at amino and thiol groups are required in the peptide side chains. Until now, the *tert*-butoxycarbonyl (Boc) group was usually used for protection of amino groups in this method.[2] However, this group is labile under acidic conditions, and it is difficult to synthesize a Boc-protected peptide segment directly by the ordinary fluorenylmethoxycarbonyl (Fmoc)-based solid phase peptide synthesis (SPPS). In our previous report, we demonstrated that azido group could be used as an alternative amino protecting group, which is stable under both acidic and basic conditions.[3] In this study, we developed another amino protecting group.

In 1965, Dixon and Moret reported the removal method for an N-terminal amino acid residue from proteins.[4] In the initial step of this method, the N-terminal amino group was specifically converted into a carbonyl group via a metal ion-mediated transamination reaction. The residual α-ketoacyl group was then cleaved by the *o*-phenylenediamine treatment. To date, this transamination reaction has been widely used for an N-terminus-specific modification such as fluorescent labeling and dinitrophenyl group attachment through the hydrazido linkage.[5] Recently, Kawakami et al. reported that the α-ketoacyl group of the intermediate could be used as an N-terminus-specific protecting group.[6] Since this protecting group is stable under both acidic and basic conditions, it might be a good candidate as an alternative protecting group for lysine side chains in the thioester method.

To investigate the feasibility of the pyruvoyl group, a prototype of α-ketoacyl group, as an amino protecting group on Fmoc-SPPS and the peptide condensation reaction by the thioester method, we synthesized N^{α}-Fmoc-N^{ε}-pyruvoyl-lysine (Fmoc-Lys(Pyv)-OH, **1**), and used it for synthesizing model peptide and glycopeptide.

At first, we attempted to introduce the pyruvoyl group directly to the amino group of Fmoc-Lys-OAll (**2**) using pyruvic acid and condensation reagents such as dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt) and HBTU, but the desired product was not obtained due to the decomposition of the pyruvic acid during the activation step. To avoid the decomposition, the carbonyl group of pyruvic acid was protected by 1,1-dimethylhydrazine, yielding dimethylhydrazonopropionic acid (**3**).[7] Then, the carboxyl group of **3** was activated by DCC/HOBt and condensed with **2** to obtain Fmoc-Lys(dimethylhydrazonopropanoyl)-OAll (**4**) (Scheme 1). Unexpectedly, the hydrazono group was hydrolyzed during separation in a separatory funnel and purification by silica gel column chromatography, and only Fmoc-Lys(Pyv)-OAll (**5**) was obtained in 78% yield.[8] The allyl group of **5** was then deprotected by 5,5-dimethylcyclohexane-1,3-dione (Dimedone) and tetrakis(triphenylphosphine)Pd(0) in dimethoxyethane, and the desired product **1** was obtained in 87% yield.[9]

Using this protected amino acid **1**, we attempted to synthesize the model peptide, pigment dispersing hormone (PDH)-I (**6**), by the thioester method. PDH-I is a crustacean neuropeptide isolated from the prawn, *Marsupenaeus japonicus*, and consists of 18 amino acid residues in H-Asn-Ser-Glu-Leu-Ile-Asn-Ser-Leu-Leu-Gly-Ile-Pro-Lys-Val-Met-Thr-Asp-Ala-NH_2 sequence.[10]

As described previously, Fmoc-PDH-I(1-10) thioester (**7**) was synthesized by the *N*-alkylcysteine (NAC)-assisted thioesterification method.[3,11] The C-terminal segment (**8**), PDH-I(11-18), whose lysine side chains were protected by the pyruvoyl group, was also synthesized by ordinary Fmoc-SPPS. When the pyruvoyl peptide

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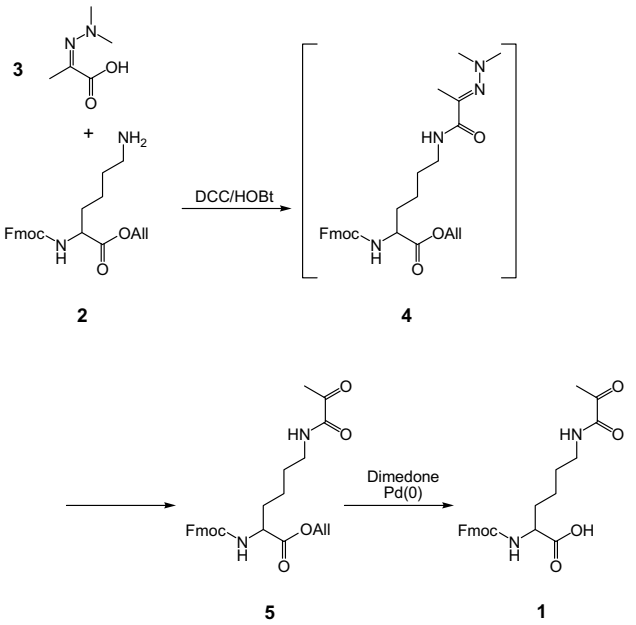


Scheme 1.

was cleaved from a solid support by Reagent K,[12] which is a TFA cocktail containing ethanedithiol as a scavenger of the trityl group, the α-carbonyl group on the pyruvoyl moiety was partially converted to a thioketal structure. On the other hand, triisopropylsilane could not be utilized as a scavenger due to its ability to reduce the carbonyl group to a hydroxyl group. To avoid the decomposition of the pyruvoyl group, we decided to add ethanedithiol to the TFA cocktail just before the cleavage reaction is complete to catch the trityl cation. Using this method, thioketal formation was completely suppressed, and no significant side reaction was observed (see Supplementary figure). In reversed-phase (RP)-HPLC analysis of the crude peptide, the desired product gave a broad, sometimes split peak due to the equilibrium between keto- and hydrate-forms or the intramolecular hemiacetal at the pyruvoyl group. The yield of **8** after RP-HPLC was 34%.

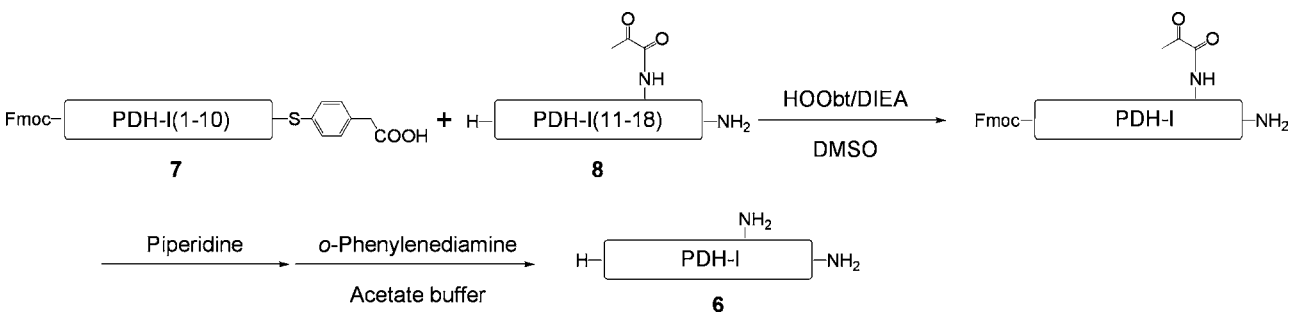
The peptide segments were condensed by the Ag^+-free thioester method (Scheme 2).[13] Segments **7** and **8** were dissolved in dimethylsulfoxide (DMSO) containing 5% 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOObt), and the coupling reaction was initiated by the addition of diisopropylethylamine (DIEA) at a concentration of 5%. The reaction was almost complete within 2 h, and the N-terminal Fmoc group was cleaved by the piperidine treatment. Then, the crude peptide was precipitated by diethyl ether. When the pyruvoyl group was deprotected by the method reported by Kawakami et al.,[6] methionine residue in the product was almost completely oxidized to methionine sulfoxide during the deprotecting reaction. To inhibit the oxidation, dimethylsulfide was added to the deprotecting reagent at the concentration of 10%. By this modification, the desired product **6** was obtained in 30% yield, and the oxidized product was also obtained in 11% yield.[14]

Having established the condensation and deprotection protocol, we then synthesized Contulakin-G (**9**), which is a toxic glycopeptide isolated from *Conus geographus* venom. It consists of 16 amino acid residues in pGlu-Ser-Glu-Glu-Gly-Gly-Ser-Asn-Ala-Thr-Lys-Lys-Pro-Tyr-Ile-Leu sequence and an O-linked glycan on the threonine side chain.[15] The N-terminal peptide thioester segment (**10**), Contulakin-G(1-6)-$SC_6H_4CH_2COOH$, was synthesized by the NAC-assisted thioesterification reaction.[11] Fmoc-*N*-ethyl-*S*-trityl-cysteine (Fmoc-(Et)Cys(Trt)-OH) was introduced to CLEAR-Amide resin and the peptide chain was elongated by ordinary Fmoc-SPPS. After cleavage from the solid support, the peptide was thioesterified by the addition of 4-mercaptophenylacetic acid (MPAA) under the acidic conditions. The reaction was almost complete within 24 h giving the desired peptide thioester **10**. The yield of **10** after purification by RP-HPLC was 5.8%.

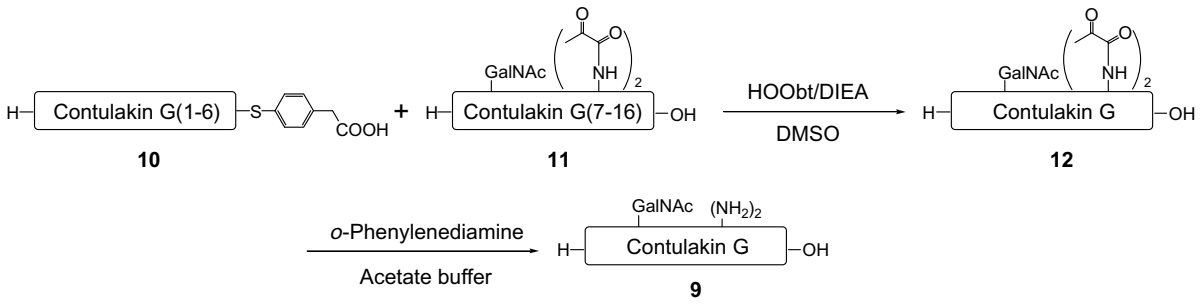
The Contulakin-G(7-16) segment having *N*-acetylgalactosamine on the threonine side chain (**11**) was synthesized by Fmoc-SPPS using Fmoc-Leu-CLEAR-Acid resin as a starting material. The peptide chain was elongated manually, and the peptide was cleaved from the resin by the TFA cocktail treatment. Benzyl groups on the glycan moiety were deprotected by the low-acidity TfOH treatment.[2b,16] The yield of **11** after RP-HPLC purification was 6.5%.

Peptide segments **10** and **11** were dissolved in 5% HOObt/DMSO and then DIEA was added to this solution (Scheme 3, Fig. 1). The condensation reaction was almost complete within 2 h, and the crude peptide was purified by gel filtration HPLC. After lyophilization, the protecting group was cleaved in the same manner as for the PDH-I synthesis. However, the yield of **9** after RP-HPLC purification was only 17%, and two by-products with molecular masses larger by 28 and one with molecular mass larger by 56 were obtained. These results suggested that the pyruvoyl groups were partially converted to formyl groups. This side reaction was suppressed when the pH of the deprotection conditions was changed from pH 5.0 to pH 4.0, and the yield increased to 32%.[17] As shown in Figure 1c, no formylation was observed in these conditions.

To test the stability of pyruvoyl group under the conditions of NAC-assisted thioesterification reaction, peptide **8** was treated with the solution, 2% MPAA/6 M urea dissolved in 0.1% TFA/50% CH_3CN. However, a part of pyruvoyl group (10–20%) was converted into thioketal within 24 h, which might be promoted by the acidic conditions used in the NAC method. If other post-SPPS thioesterification methods, which use basic or neutral conditions, such as safety-catch linker,[18] cysteinylproryl ester,[19] cysteine derived oxazolidinone,[20] and *N*-acylurea forming linker,[21] are used, a pyruvoyl peptide thioester would be obtained in better yield.



Scheme 2.



Scheme 3.

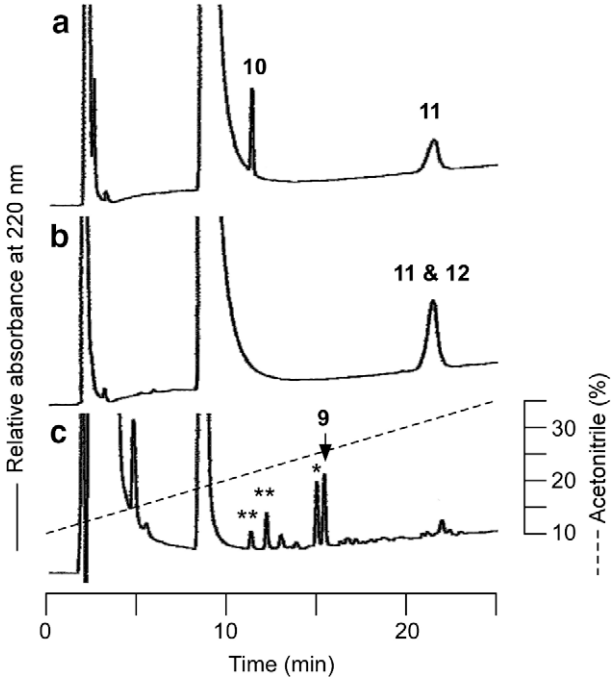


Figure 1. RP-HPLC elution profiles. (a) Coupling reaction mixture of **10** and **11** (0 h). (b) Two hours after the reaction. (c) Reaction mixture after deprotection. *, pyruvoyl-deprotected compound of the remaining C-terminal segment **11**. **, non-peptidic components. Elution conditions: column, Mightysil RP-18 GP (4.6 × 150 mm, Kanto, Japan) at a flow rate of 1 ml/min; eluent, A, 0.1% TFA, B, acetonitrile containing 0.1% TFA.

In our preliminary experiments, pyruvoyl group could be specifically removed from a peptide containing pyruvoyl, Fmoc and azido groups without undesirable side reaction (data not shown). These results indicated that a site-specific amino group modification after peptide condensation reaction came to be possible by this protecting group.

In conclusion, we have synthesized Fmoc-Lys(Pyv)-OH and have introduced it into the peptide and the glycopeptide by Fmoc-SPPS. The pyruvoyl peptide could be used for the peptide condensation reaction by the thioester method, and the pyruvoyl groups were easily cleaved without significant side reactions. This protecting group was stable under both acidic and basic conditions, and was compatible with acid-labile protecting groups such as benzyl groups used for the protection of carbohydrate moieties as shown in this study.

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Supplementary data

Supplementary data (experimental procedures and the HPLC chromatograms after cleavage of peptide **8** from the resin) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.12.005.

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8. Analytical data of compound **5**: $[\alpha]_D$ −1.4 (*c*, 1.0). R_f 0.25 (75:25 toluene/EtOAc). ^{1}H NMR ($CDCl_3$): δ 7.76 (d, 2H, *J* = 7.6 Hz, Ar), δ 7.60 (d, 2H, *J* = 7.2, Ar), δ 7.39 (t, 2H, *J* = 7.6, Ar), δ 7.31 (t, 2H, *J* = 7.6, Ar), δ 7.03 (br, 1H, N$^{\varepsilon}H$), δ 5.90 (m, 1H, –C*H*=CH$_2$), δ 5.45 (d, 1H, *J* = 8.4, N$^{\alpha}H$), δ 5.33 (d, 1H, *J* = 16.8, –CH=C*H*$_2$), δ 5.26 (d, 1H, *J* = 10.4, –CH=C*H*$_2$), δ 4.64 (d, 2H, *J* = 5.6, –OC*H*$_2$–), δ 4.41 (m, 3H, C$^{\alpha}H$, C*H*$_2$(Fmoc)), δ 4.22 (t, 1H, *J* = 6.8, C*H*(Fmoc)), δ 3.28 (m, 2H, C$^{\varepsilon}H_2$), δ 2.45 (s, 3H, Pyv), δ 1.87 (m, 1H, C$^{\beta}H_2$), δ 1.70 (m, 1H, C$^{\beta}H_2$), δ 1.56 (m, 2H, C$^{\delta}H_2$), δ 1.39 (m, 2H, C$^{\gamma}H_2$).
9. Analytical data of compound **1**: $[\alpha]_D$ +10.7 (*c*, 0.5). R_f 0.11 (50:50:1 toluene/EtOAc/AcOH). ^{1}H NMR (DMSO-d_6): δ 7.93 (d, 2H, *J* = 7.2 Hz, Ar), δ 7.77 (d, 2H, *J* = 7.2, Ar), δ 7.59 (s, 1H, N$^{\varepsilon}H$), δ 7.46 (t, 2H, *J* = 7.6, Ar), δ 7.37 (t, 2H, *J* = 7.6, Ar), δ 4.33 (d, 2H, *J* = 6.4, C*H*$_2$(Fmoc)), δ 4.27 (m, 1H, C*H*(Fmoc)), δ 3.97 (m, 1H, C$^{\alpha}H$), δ 3.15 (m, 2H, C$^{\varepsilon}H_2$), δ 2.37 (s, 3H, Pyv), δ 1.74 (m, 1H, C$^{\beta}H_2$), δ 1.66 (m, 1H, C$^{\beta}H_2$), δ 1.50 (m, 2H, C$^{\delta}H_2$), δ 1.36 (m, 2H, C$^{\gamma}H_2$).
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14. Analytical data of peptide **6**: MALDI-TOF mass, found: *m/z* 1914.3, calcd: 1914.0 (M+H)$^+$. Amino acid analysis: $Asp_{2.92}Thr_{0.96}Ser_{1.61}Glu_{0.99}Pro_{0.99}Gly_1Ala_{0.98}Val_{0.98}Met_{0.79}Ile_{1.95}Leu_{2.95}Lys_{0.99}$.
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