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Application of D,L-FDLA Derivatization to Determination of Absolute Configuration of Constituent Amino Acids in Peptide by Advanced Marfey's Method

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Abstract: A derivatization procedure including D,L-FDLA was developed for the advanced Marfey's method which is an unempirical method for the determination of the absolute configuration of an amino acid. The procedure was successfully applied to the determination of the absolute configuration of Ahp, one of constituent amino acids in aeruginopeptin 228-A (**1**).

In a previous report, we proposed a method using LC/MS for the determination of the absolute configuration of the constituent amino acids in natural products[1]. Since this method does not necessarily require an amino acid standard, it is applicable to unusual amino acids for which a standard is not available. We applied the method to the characterization of the constituent amino acids in anabaenopeptins[2] which contain homotyrosine and *N*-methylalanine as unusual amino acids, and satisfactory results were obtained without their standards. This procedure has been designated as "advanced Marfey's method" because it is based on Marfey's method[3]. In this method, the absolute configuration of a target amino acid is deduced from its HPLC retention times of the 1-fluoro-2,4-dinitrophenyl-5-L-leucine-amide (L-FDLA) derivative of the original amino acid and its enantiomer formed by racemization. Therefore, the racemization is essential for this method and the following characteristics are required for the racemization:

1. Applicable to any amino acids including unusual ones.
2. Complete racemization is achievable even though the sample amount is very small.
3. Simple and rapid.
4. Resulting racemized mixture should not contain any impurities such as by-products, reaction reagents and/or inorganic salts.

Taking into account all these points, we applied the following procedure to racemize the amino acids presented in a previous report; a hydrolysate is heated with acetic anhydride and triethylamine to racemize each amino acid through an oxazolone intermediate, and the resulting racemized *N*-acetyl amino acids are subsequently hydrolyzed again. Although this procedure is simple and applicable to small amounts of amino acids from a peptide, it is time-consuming and it is difficult to complete the racemization. Furthermore, the procedure has an additional drawback that it does not give an enantiomeric mixture, but a diastereomeric mixture in the case of amino acids with two asymmetric carbons such as threonine and isoleucine.

We considered that the above racemization process is not necessary if 1-fluoro-2,4-dinitrophenyl-5-D-leucine-amide (D-FDLA) is available as an additional derivatization reagent. As shown in Fig. 1, the relationship between L-FDLA derivatives of the D-amino acids (D-L type derivative) and those of the L-amino acids (L-L type) are diastereomeric, usually giving different retention times on HPLC. On the other hand, the relationships between the L-D and D-L types and between the D-D and L-L types are enantiomeric and each pair will show the same retention times. Namely, the D-FDLA derivative of a target amino acid and the L-FDLA derivative of its enantiomer of the target amino acid have the same retention time on HPLC. Accordingly, it is expected that in order to obtain the same effect as the racemization mentioned above, an equal mixture of D- and L-FDLA is used as the derivatization reagent instead of L-FDLA alone. In order to confirm this consideration, D-FDLA was prepared in the same manner as L-FDLA and 19 commercially available amino acids were derivatized with D- and L-FDLA. The derivatives were separated by HPLC and the retention time of each amino acid derivative was compared. These experiments clearly showed that two enantiomeric pairs, the D-D and L-L type and the D-L and L-D type derivatives, each has the same retention time. Particularly, when this method is applied to amino acids with two asymmetric centers such as threonine and isoleucine, the resulting two peaks correspond to the L-derivatives of the original amino acid and its enantiomeric isomer. These results indicate that a derivatization with D,L-FDLA is applicable to the racemization step in the advanced Marfey's method in place of the conventional method.

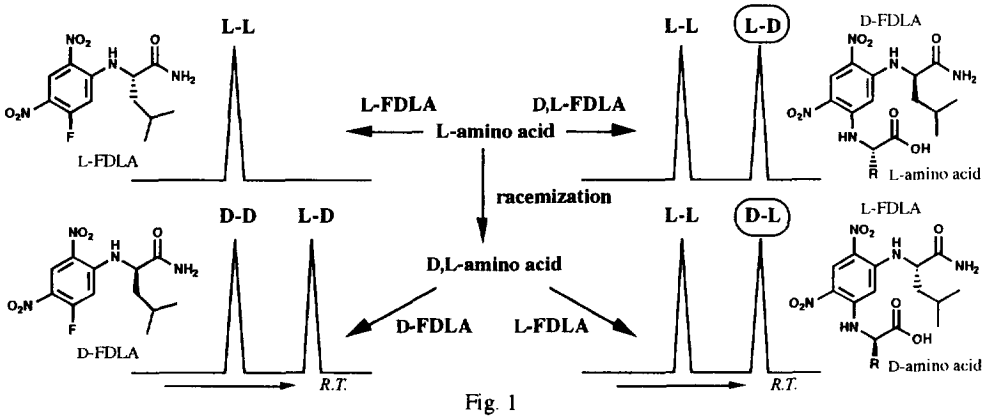

Fig. 1

In order to demonstrate the applicability of the advanced Marfey's method with this derivatization procedure, we tried to confirm the absolute configuration of the constituent amino acids in aeruginopeptin 228-A and microcystin LR produced by cyanobacteria. Aeruginopeptin 228-A[4] (**1**) was produced by a cyanobacterium, *Microcystis aeruginosa* M228 and contained 3-amino-6-hydroxy-2-piperidone (Ahp) composed of Glu-γ-carbonyl-γ-aldehyde as the unusual amino acid as shown in Fig. 2. Although cyclic depsipeptides containing an Ahp unit as a bioactive compound like **1** have frequently been found in cyanobacteria[5-8], the absolute configuration of Ahp in all of them has not yet been chemically elucidated due to its labile property upon acid hydrolysis. The reduction product (**2**) of **1** with $NaBH_4$ in MeOH gave pentahomoserine (not commercially available) and Pro^9 together with other constituent amino acids upon hydrolysis for 2 hrs[10]. The hydrolysate of **2** was divided into two portions and each portion was derivatized with L- or D-FDLA. The L-FDLA derivatives alone and the equal mixture of the D- and L-FDLA derivatives were analyzed using ESI LC/MS in the negative ion mode[11].

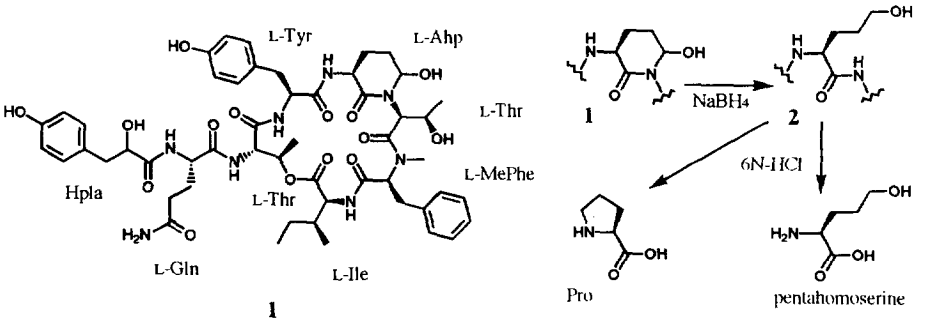

Fig. 2

Figs. 3 (a) and (b) shows the mass chromatograms monitored at *m/z* of the deprotonated ion of the constituent amino acids derivatized with L- and D,L-FDLA, respectively. The D- and L-FDLA derivatives of pentahomoserine and Pro were detected on the mass chromatograms monitored at *m/z* 426 and 408, respectively. The D-FDLA derivatives of these amino acids have longer retention times than those of their L-FDLA derivatives on each mass chromatogram (Fig. 3 (b)), indicating that they have L-configurations. Therefore, we can conclude that all constituent amino acids including Ahp in **1** have the L-configuration as shown in Fig. 2. Recently, an Ahp-containing cyclic depsipeptide, A90720A, was also isolated from a cyanobacterium and the absolute configuration of Ahp was determined as the L-configuration based on an X-ray crystallographic method[12].

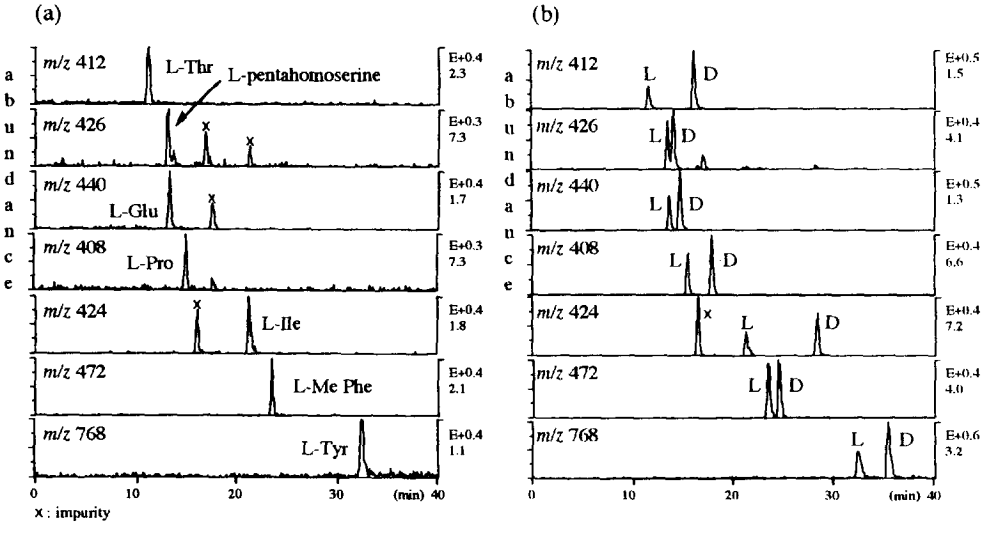

Fig. 3

Microcystin LR is composed of 7 amino residues, of which ß-methylaspartic acid (ß-MeAsp) has two asymmetric carbons in this molecule and it has already been determined to be the D-*erythro* configuration[13,14]. The hydrolysate of microcystin LR was divided into two portions and each portion was derivatized with L- or D-FDLA. The L-FDLA derivatives alone and the equal mixture of the D- and L-FDLA derivatives were analyzed using ESI LC/MS in the negative ion mode. A comparison of the retention times of both peaks on the mass chromatograms monitored at *m/z* 440 showed that the L-FDLA derivative is eluted after its D-FDLA derivative, indicating that the α-carbon atom has a D-configuration. Because the relative configuration of ß-MeAsp from the toxin had already been elucidated to be *erythro*, it was determined to have the D-*erythro* configuration.

The established procedure not only satisfies all points required for the racemization described above, but also has the following additional advantages: according to the separation mechanism previously reported[1], the initial advanced Marfey's method using conventional racemization is applicable to the separation of optical isomers of not only amino acids but also compounds with a primary amino group. However, it is difficult to determine the absolute configuration of such a compound for the case when the compound is labile during racemization and/or it is difficult to be racemized. On the other hand, the present procedure with D,L-FDLA allows one to determine the absolute configuration of such amino compounds, because it does not require the racemization process. Due to this D,L-FDLA system, the advanced Marfey's method has been improved to be simple more, rapid and reliable, and its applicability has been significantly extended.

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11. The derivatized hydrolysate of **2** with D- and L-FDLA was analyzed by ESI LC/MS under the following conditions: LC, column, Develosil ODS-HG-5 (150 x 2.0 mm I.D., Nomura Chemical, Seto Japan); mobile phase, A) H_2O containing 0.01 M TFA, B) CH_3CN; gradient rate, A) : B) = 7:3 --> 3:7 (40 min), linear; flow rate, 0.2 ml/min : MS, TSQ7000 (Finnigan MAT, California, U.S.A.).
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