ELSEVIER

Journal of Chromatography A, 921 (2001) 187–195

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Abnormal elution behavior of ornitine derivatized with 1-fluoro-2,4-dinitrophenyl-5-leucinamide in advanced Marfey's method

Ken-ichi Harada*, Akiko Matsui, Yohei Shimizu, Ryoko Ikemoto, Kiyonaga Fujii

Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468-8503, Japan

Received 12 March 2001; accepted 20 April 2001

Abstract

During the course of establishing the advanced Marfey's method that has been developed to non-empirically determine the absolute configuration of constituent amino acids in a peptide using LC–MS, we encountered the ''ornitine mystery'' in the di-DLA (2,4-dinitrophenyl-5-leucinamide) derivative such that the elution order of ornitine (Orn) was opposite (D→L) in spite of their relatively long retention time. In order to resolve this problem, the elution behavior of several mixed DLA and DPEA (2,4-dinitrophenyl-5-phenylethylamine) derivatives with different absolute configurations was carefully observed during HPLC. The length of the methylene chain in basic amino acids was obviously critical for this behavior, because Dab (2,4-diamino-*n*-butyric acid) and lysine (Lys) did not exhibit this abnormality. The presence of the carboxyamide moiety at the ω position was also essential for this phenomenon, because it was never observed in the DPEA derivatives at the ω position. Furthermore, it was found that the following combination of absolute configurations of Orn and DLA at the ω position only induced this abnormality: D-Orn and L-DLA, and L-Orn and D-DLA. This suggested that the structural interaction such as hydrogen bonding between the carboxyamide of DLA at the ω position and carboxylic acid at the α position in these derivatives reduced their retention power on the reversed-phase column.

Keywords: Marfey's method, advanced; Derivatization, LC; Amino acids; Ornitine; Fluorodinitrophenylleucinamide; Peptides

1. Introduction

The ''advanced Marfey's method'' has been developed to non-empirically determine the absolute configuration of constituent amino acids in a peptide using LC–MS [1,2]. This method consists of Marfey's method [3] as a chromatographic technique for the separation of amino acids into each enantiomer, the detection of the amino acid by mass spectrometry and a procedure for obtaining the corresponding enantiomer from either the L- or D-amino acid. In this method, the resolution between the L- or D-amino acids derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA) is due to the difference in their hydrophobicity, which is derived from the *cis* (*Z*)- or *trans* (*E*)-type arrangement of the two more hydrophobic substituents. Therefore, the L-amino acid is usually eluted before the corresponding D-amino acid [4]. The separation mechanism for the resolution of both resulting diastereomers is based on

*Corresponding author. Tel.: +81-52-832-1781; fax: +81-52-834-8780.

E-mail address: kiharada@meijo-u.ac.jp (K.-i. Harada).

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00884-6

a rigidly fixed conformation assisted by the intramolecular hydrogen bonding between the nitro groups and α-amino groups of the amino acid and leucinamide moiety [4].

Although Marfey's method was combined with usual mass spectrometry such as electrospray ionization (ESI) and frit-fast atom bombardment (FAB) mass spectrometry, the resulting sensitivity of the amino acids derivatized with FDAA was not sufficient for the practical use. Therefore, the derivatizing reagent was changed to 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) instead of FDAA and the applicability of Marfey's method including LC–MS was improved [2]. In addition to these improvements, DL-FDLA derivatization was introduced for obtaining the corresponding enantiomer on the chromatogram [5] instead of the conventional chemical racemization and its utility has been successfully extended from amino acids to primary amines [6]. Thus, we have established a nonempirical method using LC–MS, the ''advanced Marfey's method'', which includes HPLC with a rational guideline, a sensitive derivatizing reagent, FDLA, and a racemization procedure using DL-FDLA [2]. The wide utility of this method has been confirmed in several naturally occurring peptides [7,8]. Furthermore, this methodology is being extended to the determination of the absolute configuration of secondary alcohols [9,10].

To elucidate the elution order for DL-amino acid derivatives, a reasonable separation mechanism was proposed for Marfey's method mentioned above. The elution order of a desired amino acid can be elucidated from the comparison of the hydrophobicity between the α-carboxyl group and the side chain of the amino acid on the basis of our proposed mechanism. However, it is difficult to evaluate definitely the hydrophobicity of the two functional groups. We considered that the retention time of the amino acid derivatives is usable for the elution order, because the α-carboxyl group is common to amino acids, and the hydrophobicity of the DAA (2,4-dinitrophenyl-5-L-alaninamide) derivatives of an amino acid is dependent on that of the side chain of the derivatized amino acid. Therefore, the relationship between the retention time and the elution order of the DL-amino acid derivatives is closely related. In other words, an amino acid derivative with a longer retention time has the usual elution order (L→D), whereas another amino acid derivative with a shorter retention time may have the opposite elution order. The serine and asparagine derivatives are regarded as critical samples and their average retention times are situated at the turning points for the elution order [4].

However, the di-DAA and -DLA (2,4-dinitrophenyl-5-L-leucinamide) derivatives of ornitine (Orn) showed a very curious separation behavior such that the elution order was opposite in spite of their relatively long average retention time [4], which has been called the ''ornitine mystery''. Unless the problem of this abnormal elution behavior of the di-DAA and DLA derivatives of Orn can be resolved, the advanced Marfey's method could not be a reliable and nonempirical method. In this paper, we investigate why the di-DAA and -DLA derivatives of Orn show the abnormal elution behavior and propose another method for Orn based on the obtained results.

2. Experimental

2.1. Reagents

The preparation of FDAA, FDLA and FDPEA (1-fluoro-2,4-dinitrophenyl-5-(*R*,*S*)-phenylethylamine) were previously described [4]. The amino acids were purchased from Sigma (St. Louis, MO, USA). Reagent-grade acetonitrile from Nacalai tesque (Kyoto, Japan) was distilled and filtered for HPLC, and water was purified using a purification system (Barnsted, Dubuque, IA, USA). All other chemicals and solvents were of analytical grade.

2.2. HPLC analysis

HPLC was performed using a Tosoh (Tokyo, Japan) dual-pump delivery system composed of two Model CCPS pumps, a Model SD-8022 degasser, a Model MX-8010 mixer, a Model CO-8020 column oven, a Model UV-8020 UV–Visible detector, a Model PX-8020 system controller, a Model 991J photodiode array detector from Waters (Milford, MA, USA) and a Model C-R6A integrator from Shimadzu (Kyoto, Japan). Separations were carried out on a TSK gel ODS-80Ts (150×4.6 mm I.D.,

Tosoh) column heated at 40°C. For gradient elution in HPLC, mobile phase A was prepared from 0.01 *M* trifluoroacetic acid (TEA) in aqueous solution and mobile phase B was acetonitrile. Linear gradients started with 30% B and finished with 70% B in 40 min for the DLA derivatives and started with 40% B and finished with 80% B in 40 min for the DPEA derivatives. The flow-rate was 1 ml/min with UV detection at an absorbance of 340 nm and 250–500 nm by photodiode array detection.

2.3. Derivatization of amino acids with FDLA or FDPEA

To 50 μl of a 50-m*M* aqueous solutions of the amino acids, 20 μl of 1 *M* sodium bicarbonate and then 100 μ1 of 1% FDLA or FDPEA in acetone were added. The solution was vortexed and incubated at 40°C for 1 h. Reactions were quenched by the addition of 20 μl of 1 *M* HCl. Samples were diluted with 810 μl of acetonitrile and 1 μl of this solution was analyzed by HPLC.

2.4. Preparation of Orn- and Lys-α-(D,L)-DLA-ω-(R,S)-DPEA, Orn- and Lys-α-(R,S)-DPEA-ω-(D,L)-DLA and Orn- and Lys-α-(D,L)-DLA-ω-(D,L)-DLA derivatives

To 250 μl of a 50-m*M* aqueous solution of D-Orn or -Lys 100 μl of 1 *M* sodium bicarbonate and then 500 μl of 0.5% FDLA in acetone were added. The solution was vortexed and incubated at 40°C for 1 h. The reactions were quenched by the addition of 100 μl of 1 *M* HCl and the reaction mixture was evaporated to dryness. The residue was subjected to the following preparative HPLC: column, TSK gel ODS-80Ts (150×4.6 mm I.D., Tosoh); mobile phase A, 0.01 *M* TFA (aq.); mobile phase B, acetonitrile; gradient condition, mobile phase of 30→40% B (10 min)→90% (15 min) (linier gradient mode); flow-rate, 1 ml/min; detection, 410 nm (UV) and 250–500 nm (photodiode array detection); column temperature, 40°C. This preparative separation gave D-Orn-α-mono-DLA (1.2 mg) and D-Orn-ω-mono-DLA (1.2 mg), and D-Lys-α-mono-DLA (1.1 mg) and D-Orn-ω-mono-DLA (1.4 mg). These derivatives were dissolved in 100 μl of acetonitrile and divided into two portions. To 50 μl of each solution was added 40 μl of 1 *M* sodium bicarbonate and then 100 μl of 1% FDLA or FDPEA in acetone. The solution was vortexed and incubated at 40°C for 1 h. The reactions were quenched by the addition of 40 μl of 1 *M* HCl and 770 μl of acetonitrile was added to this solution. One microlitre of this solution was subjected to HPLC analysis. This operation was repeated for the derivatives of DL-Orn and DL-Lys.

3. Results

3.1. High-performance liquid chromatographic behavior of DAA, DLA and DPEA derivatives of three basic amino acids

For the basic amino acids such as Orn and lysine (Lys), three derivatives, the α-mono (a), ω-mono (b) and di- (c) derivatives, are formed during the derivatization with FDAA or FDLA (Fig. 1) [4]. The ω-mono derivatives cannot be separated and the resolution of the α-mono derivatives is dependent on the pH of mobile phase used. Therefore, the di-DAA or -DLA derivatives are usually used for the determination of the absolute configuration in this

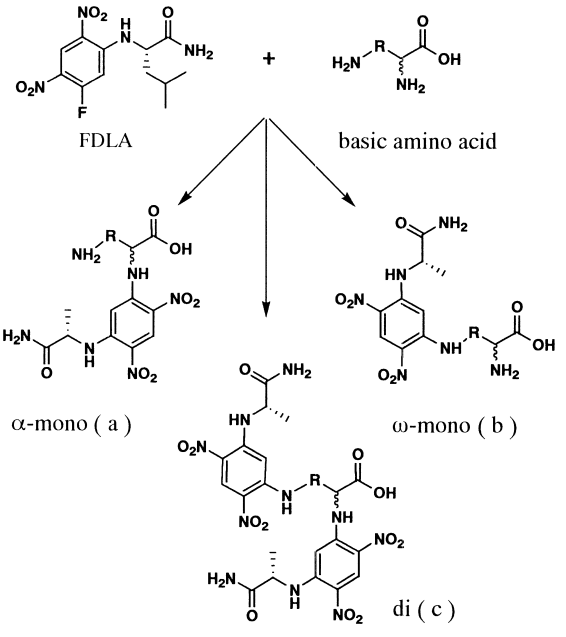

Fig. 1. Derivatization of basic amino acids with FDLA.

method. Fig. 2 shows the high-performance liquid chromatograms of the three basic amino acids, 2,4-diamino-*n*-butyric acid (Dab), Orn and Lys derivatized with FDAA. While the L-isomers of the di-DAA derivatives of Dab and Lys are eluted prior to the corresponding D-isomers, the opposite elution is found in the case of Orn. The same behavior was also observed for the DLA derivatives (data not shown), which is called the ''ornitine mystery''. On the other hand, DPEA (2,4-dinitrophenyl-5-(*R*,*S*)-phenylethylamine), which has been developed as an anisotropic reagent for the resolution of the absolute configuration of amino acids using NMR spectroscopy [11], derivatives (Fig. 3) of the three basic amino acids show the normal elution order (L→D) (Fig. 4). Furthermore, there was another abnormal point in the chromatograms of the Orn-DAA and -DLA derivatives. The retention times of the three basic acids-DAA and -DPEA deitivatives became longer with an increase in the length of their alkyl side chains, whereas that of D-Orn-DAA only became shorter in comparison with those of the corresponding D-Dab and D-Lys derivatives (Figs. 2 and 4). These results indicated that the elution behavior of the L-Orn-DAA and -DLA derivatives is normal but the retention times of the D-Orn-DAA and -DLA derivatives are abnormally shorter. Namely, the retention power of the D-Orn-DAA and -DLA was significantly reduced. This chromatographic behavior also suggested that the carboxyamide at the α or ω position of D-Orn-DAA and -DLA participate in this phenomenon.

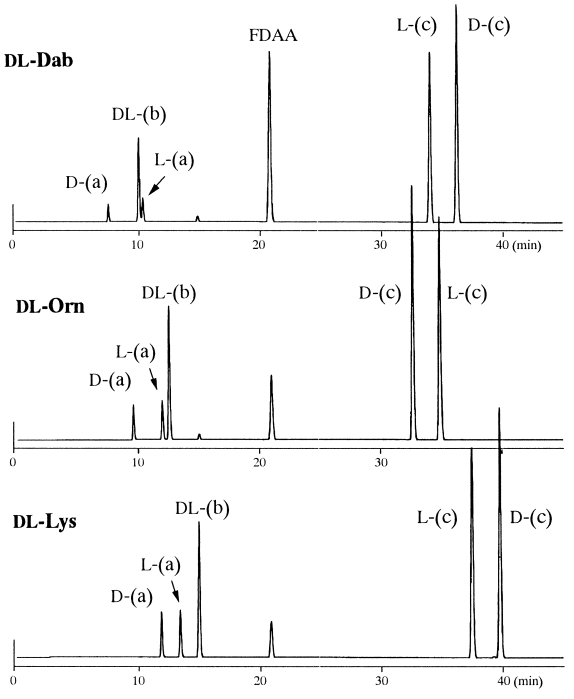

Fig. 2. High performance liquid chromatograms of three basic amino acids derivatized with L-FDAA. HPLC conditions: column, TSK gel ODS-80Ts (150×4.6 mm I.D.); mobile phase, 0.01 *M* aq. TFA in CH_3CN; gradient rate, CH_3CN 15–45% (45 min); flow-rate, 1.0 ml/min; column temperature, 40°C.

3.2. High performance liquid chromatographic behavior of mixed DLA and DPEA derivatives of ornitine and lysine

In order to investigate which caroboxyamide group at the α or ω position plays a critical role in the abnormal elution behavior, mixed derivatives, the α-(D,L)-DLA-ω(*R*,*S*)-DPEA (a), α-(*R*,*S*)-DPEA-ω-(D,L)-DLA (b) and α-(D,L)-DLA-ω-(D,L)-DLA (c) derivatives were prepared according to the procedure shown in the Experimental section (Fig. 3). The HPLC analysis data of the four derivatives of Orn- and Lys-α-DLA-ω-DPEA (Fig. 3**a**) are shown in Table 1. The elution behavior of all the derivatives of both amino acids are quite similar apart from their retention times, indicating that the elution order is dependent on the absolute configuration of DLA at the α position and the carboxyamide moiety at the α position do not influence the elution behavior.

Table 2 shows the HPLC analysis data of the Orn- and Lys-α-DPEA-ω-DLA (Fig. 3**b**) derivatives. The Lys derivatives show the normal elution behavior including elution order, retention time and retention time difference (Δt_R). Although the Orn derivatives are eluted according to the separation mechanism, the retention times and Δt_R are significantly different from those of the Lys derivatives. In particular, the difference in the Δt_R between derivatives **1** and **4** and derivatives **2** and **3** is significant, suggesting that the carboxyamides at the ω position influence the elution behavior. This can be explained using the working hypothesis shown in Fig. 5. The four derivatives of Lys (**1**–**4**) show the normal elution behavior according to our separation mechanism [4]. For derivative **1**, the L-Orn derivative is normally

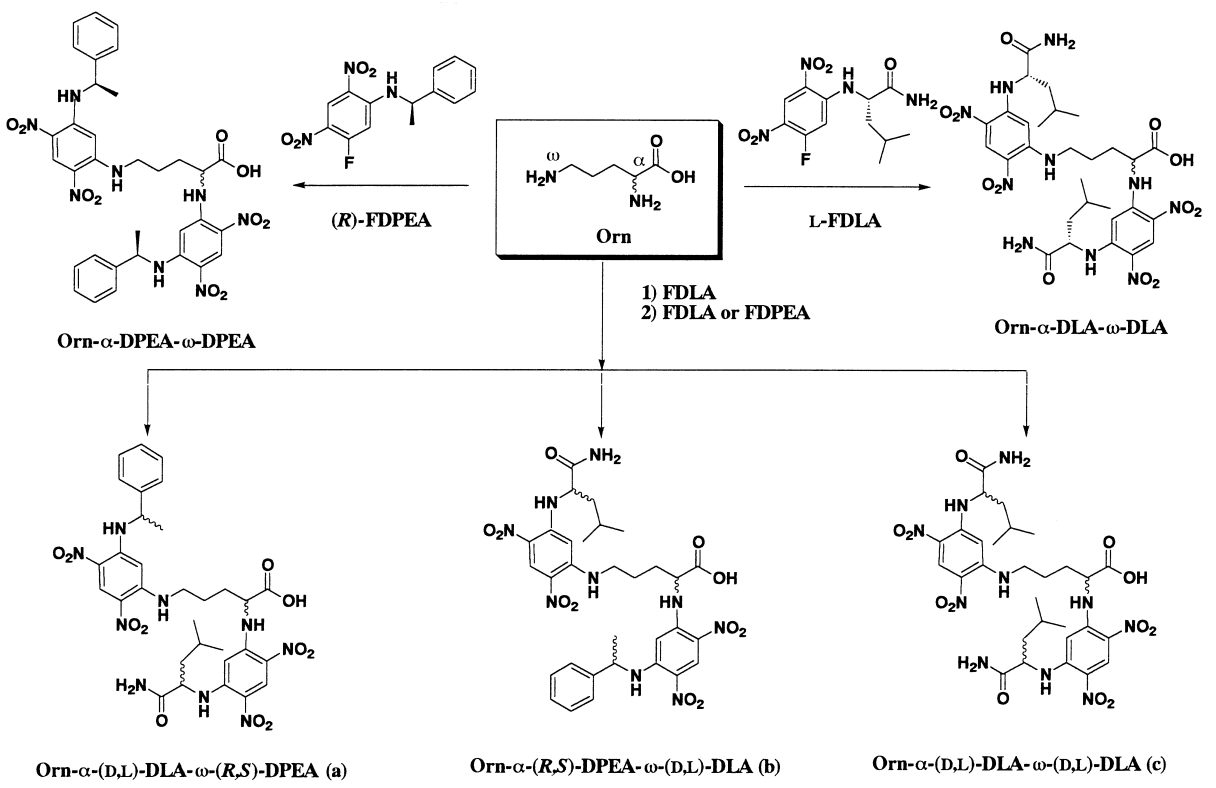

Fig. 3. Reaction scheme of various DLA and DPEA derivatives of basic amino acids.

eluted, whereas the retention time of the D-derivatives becomes extremely shorter probably due to the participation of the carboxyamide moiety at the ω position. Conversely, while the elution of the D-Orn derivative (**2**) is normal, the L-derivative is abnormally eluted earlier. The elution behavior of the remaining two derivatives (**3** and **4**) can be also elucidated using Fig. 5. These experiments clearly suggested that this abnormal elution behavior was significantly related to the absolute configurations of the Orn tested and DLA at the ω position. In other words, when DLA is present at the ω position, Orn with the opposite absolute configuration is strongly influenced to have the shorter retention time.

3.3. High performance liquid chromatographic behavior of mixed DLA derivatives of ornitine and lysine

From the experiments mentioned above, it was demonstrated that the abnormal elution behavior of the Orn-di-DLA derivative was caused by the structural relationship between the absolute configurations of the Orn tested and the carboxyamide of DLA at the ω position. Table 3 summarizes the HPLC analysis data of four Orn- and Lys-di-DLA derivatives with different configurations (Fig. 3**c**). The elution order of the Lys-DLA derivatives is dependent on the absolute configuration of DLA at the α position and not related to that of DLA at the ω position. As mentioned earlier, the elution order of the Orn-di-DLA derivatives was opposite to that of Lys, which can be explained based on the results mentioned above as follows: the elution of L-Orn-α,ω-di-L-DLA (**1**) is normal, whereas the peak of D-Orn-α,ω-L-DLA (**1**) was moved from the normal position corresponding to that of D-Orn-α-L-DLA-ω-D-DLA (**3**) to give a significantly shorter retention time (Fig. 6). The relationship between derivatives **1** and **2** is enantiomeric, so that the elution order of **2** is opposite. Although the elution order of D-Orn-α-L-DLA-ω-D-DLA (**3**) is normal, the retention time of

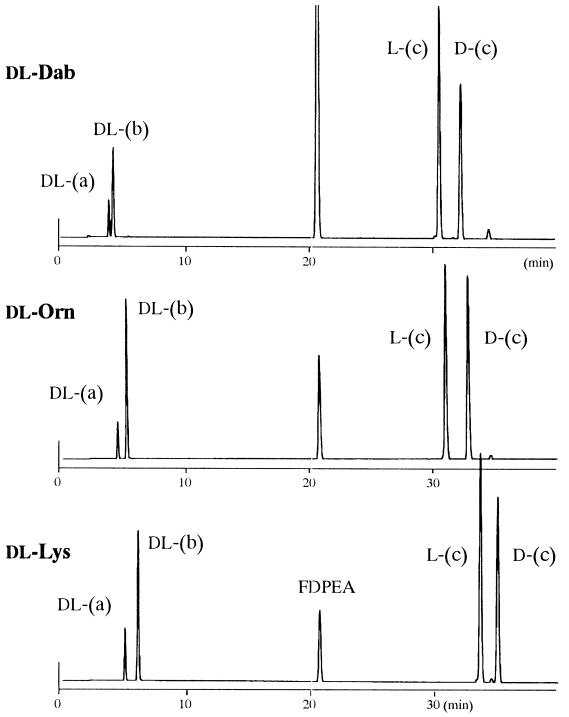

Fig. 4. High performance liquid chromatograms of three basic amino acids derivatized with (*R*)-FDPEA. HPLC conditions: column, TSK gel CDS-80Ts (150×4.6 mm I.D.); mobile phase, 0.01 *M* aq. TFA in CH_3CN; gradient rate, CH_3CN 40–80% (40 min); flow-rate, 1.0 ml/min; column temperature, 40°C.

L-Orn-α-L-DLA-ω-D-DLA became shorter from the normal retention time corresponding to that of L-Orn-α-L-DLA-ω-L-DLA (Fig. 6). The relationship between derivatives **3** and **4** is enantiomeric, so that the elution order of **4** is opposite. These experiments confirmed that this abnormal elution behavior was derived from the relationship between the absolute configuration of the Orn tested and the carboxyamide of DLA at the ω position. In particular, the D-Orn-α,ω-di-L-DLA was signigicantly influenced by this abnormal effect to induce the reversed elution. The L-Orn-α,ω-di-D-DLA derivative was also affected by this abnormal effect, but the opposite elution order was not found.

4. Discussion

In order to clarify the ''ornitine mystery'' of the di-DLA derivatives such that the elution order is opposite (D→L) in spite of their relatively long retention times, we investigated carefully the elution behavior of several Orn-DLA and DPEA derivatives in comparison with those of Lys that always shows the normal elution behavior. The obtained experimental results are shown in Fig. 7 and are summarized as follows:

1. no di-DPEA derivatives show the abnormality;
2. the DLA moiety at the α position does not contribute to this abnormal elution behavior;
3. the DLA moiety at the ω position participates significantly in this phenomenon;
4. this abnormal elution behavior is only induced by the interaction between D-Orn and L-DLA at the ω position, and between L-Orn and D-DLA at the ω position;
5. the former case described above is more seriously affected than that of the latter case, so that the former derivative is only eluted in the opposite order.

Thus, it is found that the ''ornitine mystery'' is caused by the abnormal reduction of the retention

Table 1
HPLC analysis data of Orn- and Lys- α-(D,L)-DLA-ω-(*R*,*S*)-DPEA derivatives

	Position		Lys				Orn			
	α	ω	Elution order	$t_{R,L}$	$t_{R,D}$	Δt_R	Elution order	$t_{R,L}$	$t_{R,D}$	Δt_R
1	L-DLA	*R*-DPEA	L→D	39.9	43.1	3.2	L→D	36.8	39.4	2.6
2	D-DLA	*R*-DPEA	D→L	43.2	39.5	−3.7	D→L	39.7	36.5	−3.2
3	L-DLA	*S*-DPEA	L→D	39.5	43.1	3.6	L→D	36.5	39.9	3.4
4	D-DLA	*S*-DPEA	D→L	43.1	39.8	−3.3	D→L	39.3	36.7	−2.6

t_R: retention time (min), Δt_R: $t_{R,D} - t_{R,L}$(min). HPLC conditions: Jasco dual-pump system; column, TSK gel ODS-80Ts (150×4.6 mm I.D.); mobile phase, 0.01 *M* aq. TFA in CH_3CN; gradient rate, CH_3CN 30–90% (60 min); flow-rate, 1.0 ml/min; detection, UV 340 nm; column temperature 40°C.

Table 2
HPLC analysis data of Orn- and Lys-α-(*R*,*S*)-DPEA-ω-(D,L)-DLA derivatives

	Position		Lys				Orn			
	α	ω	Elution order	$t_{R,L}$	$t_{R,D}$	Δt_R	Elution order	$t_{R,L}$	$t_{R,D}$	Δt_R
1	*R*-DPEA	L-DLA	L→D	39.9	41.9	2.0	L→D	38.0	38.3	0.3
2	*R*-DPEA	D-DLA	L→D	39.9	41.0	1.0	L→D	36.2	39.7	3.5
3	*S*-DPEA	L-DLA	D→L	41.0	39.3	−1.1	D→L	39.9	36.2	−3.7
4	*S*-DPEA	D-DLA	D→L	41.9	39.8	−2.1	D→L	38.2	37.9	−0.3

t_R: retention time (min), Δt_R: $t_{R,D} - t_{R,L}$ (min). HPLC conditions: Jasco dual-pump system; column, TSK gel ODS-80Ts (150×4.6 mm I.D.); mobile phase, 0.01 *M* aq. TFA in CH_3CN; gradient rate, CH_3CN 30–90% (60 min); flow-rate, 1.0 ml/min; detection, UV 340 nm; column temperature 40°C.

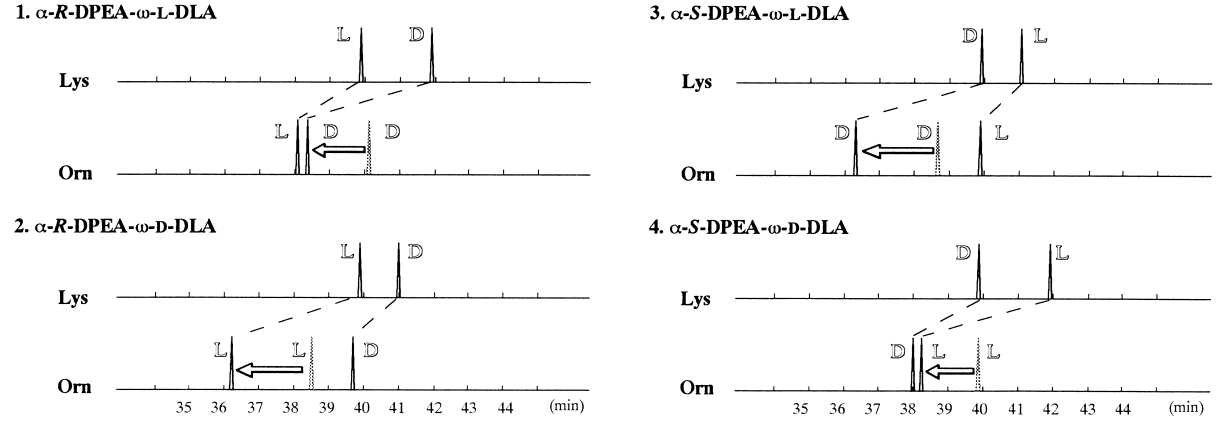

Fig. 5. Elution behavior of Orn- and Lys-α-(*R*,*S*)-DPEA-ω-(D,L)-DLA derivatives in HPLC.

power of the D-Orn-α,ω-di-L-DLA derivative. This phenomenon may be concerned with the following three points:

1. the number of methylene units in the basic acids;
2. the carboxyamide moiety at the ω position;
3. the relationship of the absolute configurations between Orn and DLA at the ω position.

The length of the methylene chain is obviously critical for this behavior, because Dab and Lys do not exhibit this abnormality. The presence of a

Table 3
HPLC analysis data of Orn- and Lys-α-(D,L)-DLA-ω-(D,L)-DLA derivatives

	Position		Lys				Orn			
	α	ω	Elution order	$t_{R,L}$	$t_{R,D}$	Δt_R	Elution order	$t_{R,L}$	$t_{R,D}$	Δt_R
1	L-DLA	L-DLA	L→D	33.1	35.0	1.9	D→L	31.4	30.1	−1.3
2	D-DLA	D-DLA	D→L	35.0	33.1	−1.9	L→D	30.1	31.4	1.3
3	L-DLA	D-DLA	L→D	33.2	35.3	2.1	L→D	30.8	33.0	2.2
4	D-DLA	L-DLA	D→L	35.3	33.2	−2.1	D→L	33.0	30.8	−2.2

t_R: retention time (min), Δt_R: $t_{R,D} - t_{R,L}$ (min). HPLC conditions: Jasco dual-pump system; column, TSK gel ODS-80Ts (150×4.6 mm I.D.); mobile phase, 0.01 *M* aq. TFA in CH_3CN; gradient rate, CH_3CN 30–90% (60 min); flow-rate, 1.0 ml/min; detection, UV 340 nm; column temperature 40°C.

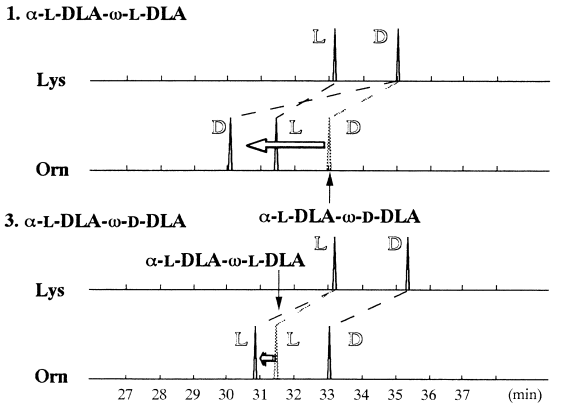

Fig. 6. Elution behavior of Orn- and Lys-α-(D,L)-DLA-ω-(D,L)-DLA derivatives in HPLC.

carboxyamide moiety at the ω position is also essential for this phenomenon, because it is never observed in the DPEA derivatives at the ω position. Interestingly, the following combination of the absolute configurations of Orn and DLA at the ω position induces only this abnormality: D-Orn and L-DLA, and L-Orn and D-DLA. These results suggest that a structural interaction such as hydrogen bonding between the carboxyamide of DLA at the ω position and the carboxylic acid at the α position reduces their retention power on the reversed-phase column and the elution order is reversed in some cases.

In order to elucidate the conformation of the D-Orn-α,ω-di-L-DLA derivative by X-ray crystallography, we have tried crystallization, but no suitable crystalline has been obtained. Although the NMR and UV spectral analysis of this compound has been carried out, no structural information of high quality has been obtained so far. In this study, it is further confirmed that the ''ornitine mystery'' of the di-DLA derivative always occurs. Therefore, we recommend the use of DPEA derivatives for the analysis of Orn instead of the DLA derivatives in spite of the lower resolution power.

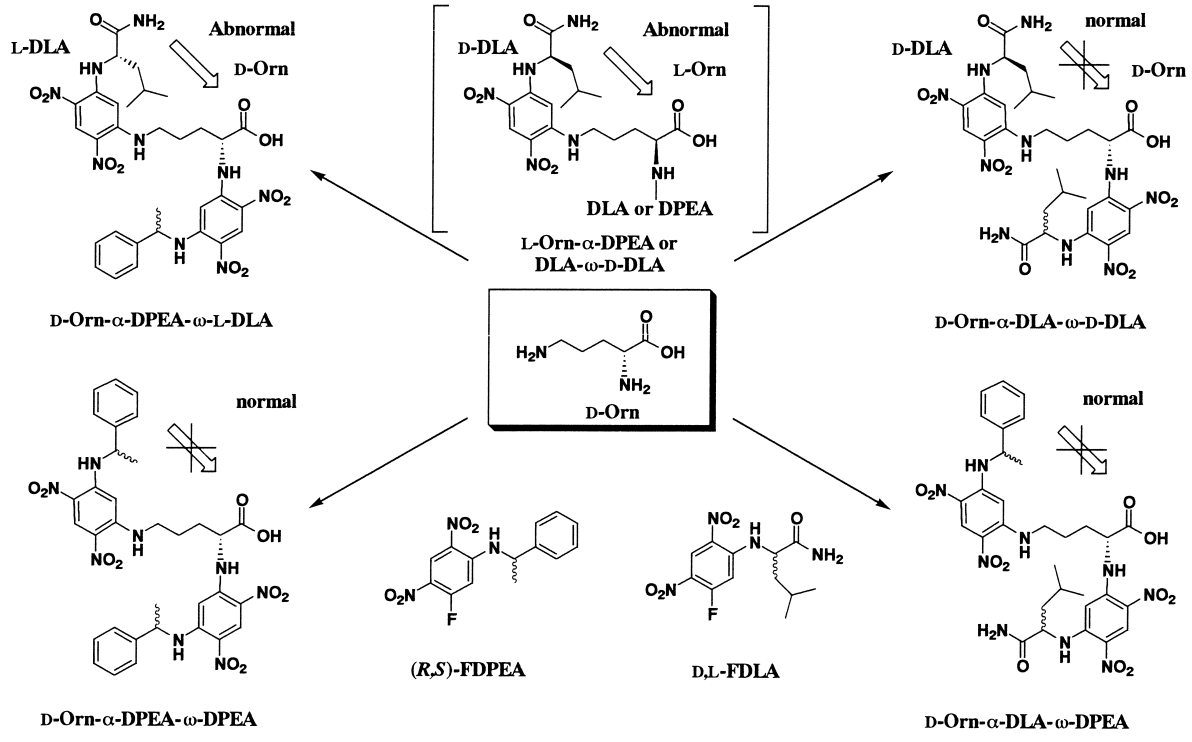

Fig. 7. Abnormal elution behavior of Orn-di-DLA and Orn-α-DPEA-ω-DLA derivatives.

References

[1] K.-I. Harada, K. Fujii, T. Mayumi, Y. Hibino, M. Suzuki, Y. Ikai, H. Oka, Tetrahedron Lett. 36 (1995) 1515.
[2] K. Fujii, Y. Kai, H. Cka, M. Suzuki, K.-I. Harada, Anal. Chem. 69 (1997) 5146.
[3] P. Marfey, Carlsberg Res. Commun. 49 (1984) 591.
[4] K. Fujii, Y. Ikai, T. Mayumi, H. Oka, M. Suzuki, K.-I. Harada, Anal. Chem. 69 (1997) 3346.
[5] K.-I. Harada, K. Fujii, K. Hayashi, M. Suzuki, Y. Ikai, H. Cka, Tetrahedron Lett. 37 (1996) 3001.
[6] K. Fujii, T. Shimoya, Y. Ikai, H. Oka, K.-I. Harada, Tetrahedron Lett. 39 (1998) 2579.
[7] K. Fujii, K. Sivonen, T. Kashiwagi, K. Hirayama, K.-I. Harada, J. Org. Chem. 64 (1999) 5777.
[8] Y. Suzuki, M. Ojika, Y. Sakagami, K. Kaida, R. Fudou, T. Kameyama, J. Antibiotics 54 (2001) 22.
[9] K.-I. Harada, Y. Shimizu, A. Kawakami, K. Fujii, Tetrahedron Lett. 40 (1999) 9081.
[10] K.-I. Harada, Y. Shimizu, A. Kawakami, M. Norimoto, K. Fujii, Anal. Chem. 72 (2000) 4142.
[11] K.-I. Harada, Y. Shimizu, K. Fujii, Tetrahedron Lett. 39 (1998) 6245.