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TETRAHEDRON

Keramamides K and L, New Cyclic Peptides Containing Unusual Tryptophan Residue from *Theonella* Sponge

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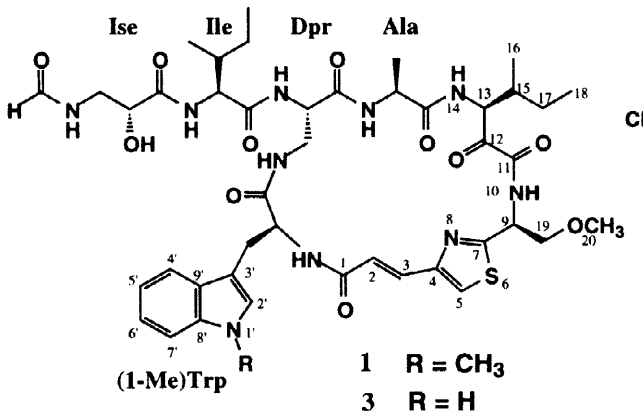
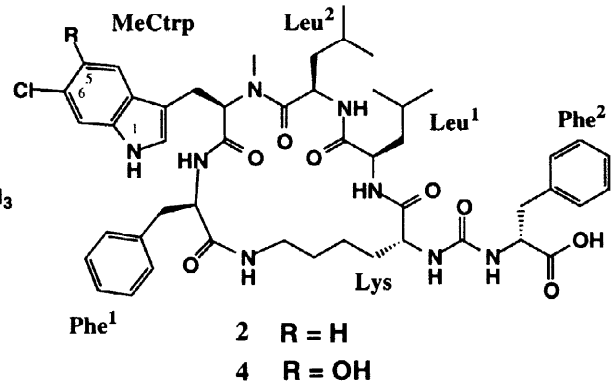
Abstract: Two new cyclic peptides, keramamides K (**1**) and L (**2**), with an unusual tryptophan residue have been isolated from an Okinawan marine sponge *Theonella* sp., and the structures including absolute stereochemistry were elucidated on the basis of 2D NMR and FABMS/MS data and degradation experiments.

Recently many cyclic peptides and depsipeptides possessing unique structures and interesting biological activities have been isolated from marine sponges.[1] In our continuing search for bioactive substances from marine organisms,[2,3] we previously isolated a series of novel cyclic peptides, keramamides A ~ H and J, from Okinawan marine sponges *Theonella* sp.[4-7] Further investigation on extracts of the other *Theonella* sponge resulted in the isolation of two new cyclic peptides, keramamides K (**1**) and L (**2**). Keramamide K (**1**) was a thiazole-containing cyclic peptide possessing a 1-methyltryptophan residue, while keramamide L (**2**) was a cyclic peptide having an ureido bond and a 6-chloro-*N*-methyltryptophan residue. Here we describe the isolation and structure elucidation of **1** and **2**.

The MeOH extract of the sponge *Theonella* sp. (SS-342) collected off Kerama Islands, Okinawa, was partitioned between EtOAc and water. The EtOAc soluble materials were subjected to a silica gel and a LH-20 columns followed by reversed-phase HPLC on ODS to afford keramamides K (**1**, 3 x 10^{-4} %, wet weight) and L (**2**, 1 x 10^{-4} %) as colorless amorphous solids together with keramamides A (**4**) ~ H and J (**3**).



1 R = CH_3
3 R = H



2 R = H
4 R = OH

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Table 1. ^{1}H and ^{13}C NMR Data of Keramamide K (1) in DMSO-d_6

positn.		δ_H		δ_C		positn.		δ_H		δ_C	
CHO		7.99	s	161.5	d		5	7.89	s	123.4	d
Ise	CO			171.3	s		7			167.8	s
	α	3.97	m	70.1	d		9	5.26	m	51.7	d
	β	3.51	m	41.3	t		10	9.03	d, 9.0		
		3.09	m				11			162.1	s
	OH	5.90	d, 5.4				12			196.7	s
	β-NH	7.90	m				13	5.26	m	60.0	d
Ile	CO			170.4	s		14	8.62	d, 7.6		
	NH	7.62	d, 9.2				15	2.35	m	36.2	d
	α	4.21	m	56.2	d		16	0.96[a]	q, 6.8	16.1	q
	β	1.70	m	37.2	d		17	1.28	m	23.3	t
	γ-CH_3	0.84[a]	m	15.3	q		18	0.86[a]	m	11.8	q
	γ-CH_2	1.42	m	24.3	t		19	3.70[b]	m	72.5	t
		1.02	m				20	3.49[a]	s	58.4	q
	δ-CH_3	0.83[a]	m	11.2	q	(1-Me)Trp	CO			167.0	s
Dpr	CO			170.1	s		NH	8.20	m		
	α-NH	8.31	d, 7.5				α	4.44	m	58.3	d
	α	4.28	m	51.0	d		β	3.38	m	28.7	t
	β	3.66	m	40.6	t			3.09	m		
		2.75	m				1'-CH_3	3.29[a]	s	31.3	q
	β-NH	7.98	m				2'	8.46	s	129.6	d
Ala	CO			175.3	s		3'			114.2	s
	NH	7.82	m				4'	8.15	d, 7.5	121.2	d
	α	4.56	m	48.4	d		5'	7.21	t, 7.5	122.4	d
	β	1.41[a]	d, 7.1	16.9	q		6'	7.23	t, 7.5	123.0	d
	1			164.4	s		7'	7.50	d, 7.5	112.6	d
	2	6.81	d, 15.1	123.6	d		8'			137.2	s
	3	7.42	d, 15.1	132.8	d		9'			125.6	s
	4			149.2	s						

[a]3H. [b]2H.

The molecular formula of keramamide K {**1**, $[\alpha]_D^{28}$ -25° (*c* 0.1, MeOH)} was determined as $C_{44}H_{60}N_{10}O_{11}S$ by HRFABMS [*m/z* 937.4217 $(M+H)^+$, Δ -2.5 mmu]. Amino acid analyses of the hydrolysate of **1** revealed 1 mol each of alanine (Ala), isoleucine (Ile), isoserine (Ise), and 2,3-diaminopropionic acid (Dpr). Comparison of ^{1}H and ^{13}C NMR data (Table 1) of **1** with those of keramamide J (**3**) suggested that **1** possessed an (*O*-methylseryl)thiazole (C-1–N-10, C-19, and C-20) and a 3-amino-4-methyl-2-oxohexanoic acid moieties (C-11–C-18). Signals due to a tryptophan residue [αC; δ 58.3 (d), βC; δ 28.7 (t), C-2'; δ 129.6 (d), C-3'; δ 114.2 (s), C-4'; δ 121.2 (d), C-5'; δ 122.4 (d), C-6'; δ 123.0 (d), C-7'; δ 112.6 (d), C-8'; δ 137.2 (s), C-9'; δ 125.6 (s)] were observed in the ^{13}C NMR spectrum, and the ^{1}H and ^{13}C NMR data indicated the presence of an *N*-methyl group (δ_H 3.29; δ_C 31.3). The *N*-methyl proton showed the NOESY cross-peaks for H-2' and H-7' on the tryptophan residue, indicating the presence of a 1-methyltryptophan [(1-Me)Trp] residue. C_{18} HPLC analyses of the hydrolysate of **1** with 6 N HCl in the presence of phenol and mercaptoethanol revealed that **1** had 1 mol of a (1-Me)Trp residue.

Evidences for amino acid sequence of keramamide K (**1**) were provided by NOESY and FABMS/MS data. The presence of a segment, Ise–Ile–Dpr–Ala, was deduced from NOESY cross-peaks for αH-

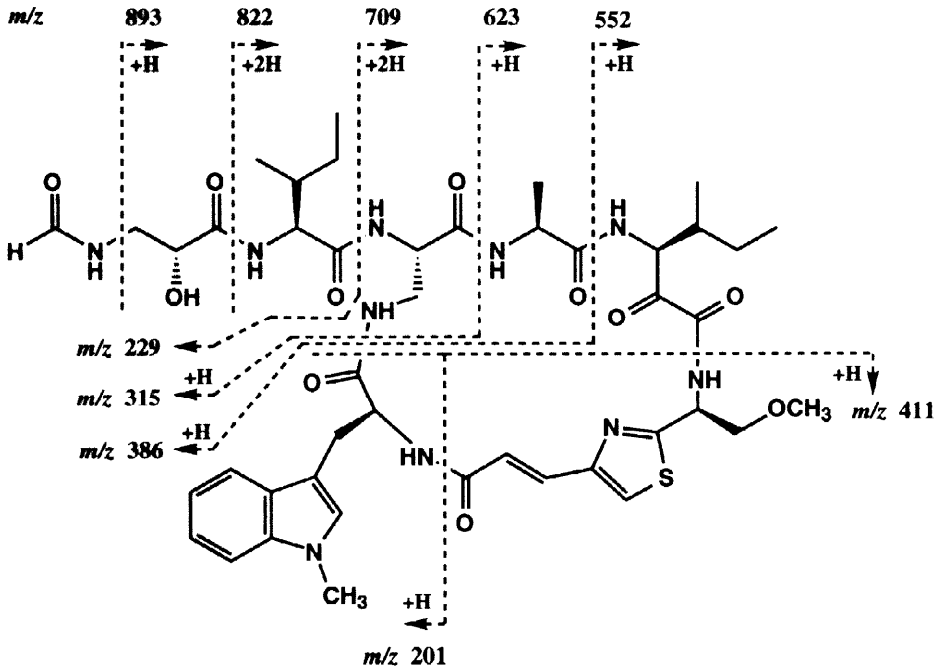

Figure 1. FABMS/MS Fragmentations of Keramamide K (**1**) (Precursor ion, *m/z* 937)

Ise/NH-Ile, αH-Ile/αNH-Dpr, and αH-Dpr/NH-Ala. The NOESY correlation for αH-Ala/NH-14 indicated that the Ala residue was attached to the NH-14 of the 3-amino-4-methyl-2-oxohexanoic acid residue. Furthermore, the FABMS/MS spectrum of **1** [precursor ion, *m/z* 937 $(M+H)^+$] showed product ions corroborating the amino acid sequence as shown in Figure 1.

Absolute configurations of Ile, Dpr, and Ala residues were determined as all L-configurations by chiral HPLC analyses of the hydrolysate, while Ise was D-form. The α-keto-β-amino acid portion (C-11 ~ C-18) was transformed into Ile by treatment of **1** with H_2O_2/NaOH followed by acid hydrolysis. Chiral HPLC analyses of Ile in the degradation product revealed to be L-form. The stereochemistry at C-9 was deduced to be L-configuration from chiral HPLC analyses of *O*-methylserine [(*O*-Me)Ser] generated by ozonolysis of **1** followed by acid hydrolysis. The (1-Me)Trp residue was assigned as L-configuration by C_{18} HPLC analyses of (1-fluoro-2,4-dinitrophen-5-yl)-L-alaninamide (FDAA) derivatives[8] of the hydrolysate obtained as described above. Therefore the structure of keramamide K was determined to be **1**.

HRESIMS data [*m/z* 927.4541 $(M+H)^+$, Δ +0.6 mmu] of keramamide L (**2**) indicated the molecular formula to be $C_{49}H_{63}N_8O_8Cl$. Amino acid analysis of the acid hydrolysate of **2** showed the presence of 1 mol of lysine (Lys) and 2 mol each of leucine (Leu) and phenylalanine (Phe). The ^1H and ^{13}C NMR data (Table 2) of **2** were very close to those of keramamide A (**4**) except for lack of the phenolic hydroxy proton signal (δ_H 9.13 brs) of 6-chloro-5-hydroxy-*N*-methyltryptophan observed for **4**.[4] ^1H-^1H COSY, HOHAHA, HMQC, and ROESY data suggested that the *N*-methyltryptophan residue in **2** was substituted by chloline atom at C-6. The gross structure of keramamide L (**2**) was elucidated mainly by analysis of FABMS/MS product ions obtained from *m/z* 927 as a precursor ion (Figure 2). The characteristic product ion peaks at *m/z* 762 and 736 were attributed to fissions at the ureido bond between Phe^2 and Lys residues. Chiral HPLC analyses of the acid hydrolysate of **2** revealed that Phe, Leu, and Lys were L-forms.

Table 2. ^{1}H and ^{13}C NMR Data of Keramamide L (2) in DMSO-d_6

positn.		δ_H		δ_C		positn.		δ_H		δ_C	
Phe^1	CO			170.8	s	Leu^2	δ-CH_3	0.36[b]	d, 6.6	22.6	q
	NH	6.13	m				δ-CH_3	0.20[b]	d, 6.6	19.9	q
	α	4.51	m	54.7	d	Leu^1	CO			173.1	s
	β	3.22	m	38.0	t		NH	8.50	d, 5.1		
		2.73	m				α	4.23	m	50.8	d
	1			138.2	s		β	1.54[a]	m	39.9	t
	2,6	7.05[a]	m	128.8[c]	d		γ	1.73	m	22.9	d
	3,5	7.30–7.15	m	128.3[c]	d		δ-CH_3	0.89[b]	d, 6.6	22.8	q
	4	7.30–7.15	m	126.8	d		δ-CH_3	0.85[b]	d, 6.6	21.8	q
MeCtrp	CO			170.3	s	Lys	CO			172.0	s
	N-CH_3	1.90[b]	s	27.5	q		αNH	6.40	m		
	α	4.73	m	61.5	d		α	3.88	m	54.5	d
	β	3.12	m	22.4	t		β	1.40[a]	m	32.0	t
		2.81	m				γ	1.53[a]	m	20.2	t
	1	11.02	brs				δ	1.40[a]	m	29.0	t
	2	6.99	brs	123.5	d		ε	3.58	m	38.4	t
	3			114.5	s			2.84	m		
	4	7.57	d, 8.4	122.3	d		εNH	7.38	m		
	5	6.98	d, 8.4	120.3	d	CO(ureido)				156.8	s
	6			128.3	s	Phe^2	CO_2H			173.5	s
	7	7.35	brs	113.2	d		NH	8.79	d, 9.1		
	8			137.4	s		α	4.19	m	53.5	d
	9			125.3	s		β	2.97	m	37.3	d
Leu^2	CO			172.3	s			2.88	m		
	NH	8.50	d, 5.1				1			137.3	s
	α	4.04	m	47.0	d		2,6	7.30–7.15	m	129.2[c]	d
	β	0.92	m	37.6	t		3,5	7.30–7.15	m	128.1[c]	d
		-0.61	m				4	7.30–7.15	m	126.5	d
	γ	1.25	m	22.2	d						

[a]2H. [b]3H. [c]2C.

The 6-chloro-*N*-methyltryptophan (MeCtrp) residue was deduced to be L-configuration from chiral HPLC analyses of *N*-methylaspartic acid [(*N*-Me)Asp], which was obtained by ozonolysis of **2** followed by acid hydrolysis.[9] Thus the structure of keramamide L was assigned as **2**.

Keramamide K (**1**) is a new thiazole-containing cyclic peptide possessing a (1-Me)Trp residue, while keramamide L (**2**) is a new cyclic peptide having an ureido bond and a MeCtrp residue. The peptide containing a (1-Me)Trp residue like keramamide K (**1**) is very rare from natural sources, although only one peptide, chetomin[10] from the fungi *Chaetominium cochliodes*, has been reported. On the other hand, keramamide L (**2**) is the first peptide with a MeCtrp residue from natural origin. Keramamides K (**1**) and L (**2**) exhibited cytotoxicity against L1210 murine leukemia cells (IC_{50}, 0.72 and 0.46 μg/mL, respectively) and KB epidermoid carcinoma cells (IC_{50}, 0.42 and 0.9 μg/mL, respectively) in vitro.

Experimental Section

General Procedures. Optical rotations were observed using a JASCO DIP-370 polarimeter. UV and IR spectra were taken on JASCO Ubest-35 and JASCO FT/IR-5300 spectrometers, respectively. ^{1}H and ^{13}C NMR spectra were recorded on Bruker ARX-500 and JEOL EX-400 spectrometers, respectively. FABMS/MS spectra were obtained on a JEOL JMS-HX/HX 110A tandem mass spectrometer by using dithiothreitol/dithioerythritol as a matrix. ESI mass spectra were measured on a JEOL SX-102A spectrometer at 40 V as a focus voltage.

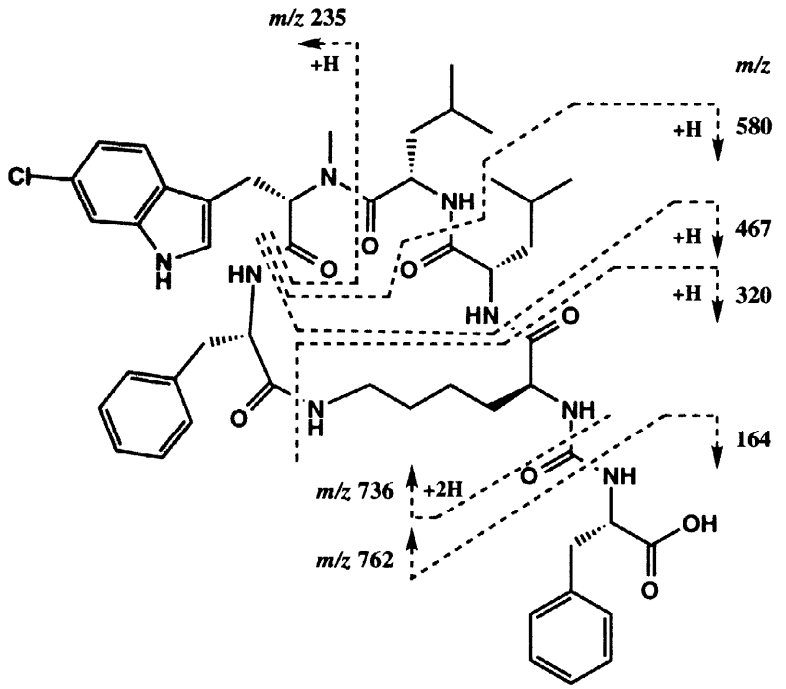

Figure 2. FABMS/MS Fragmentations of Keramamide L (**2**) (Precursor ion, *m/z* 927)

Isolation. The sponge *Theonella* sp. (SS-342) was collected off Kerama Islands, Okinawa, and kept frozen until used. The sponge (5.8 kg, wet weight) was extracted with methanol (2 L x 2). The methanolic extract (360 g) was patitioned between 1 M NaCl aq. (800 mL) and ethyl acetate (800 mL x 3). The EtOAc soluble material (11.6 g) was subjected to a silica gel ($CHCl_3$/MeOH, 85:15), and Sephadex LH-20 columns (MeOH), and then C_{18} HPLC [Develosil ODS-HG-5, Nomura Chemical, 10 x 250 mm; eluent, CH_3CN/H_2O (40:60); flow rate, 2.5 mL/min; UV detection at 270 nm] to afford keramamides K (**1**, 3 x 10^{-4} %, wet weight, t_R 32 min) and L (**2**, 1 x 10^{-4} %, t_R 15 min).

Keramamide K (1). Colorless amorphous solid; $[\alpha]_D^{28}$ -25° (*c* 0.1, MeOH); IR (film) ν_{max} 3300, 1650, and 1520 cm^{-1}; UV (MeOH) λ_{max} 269 (ε 20000) nm; ^1H and ^{13}C NMR (Table 1); FABMS (positive) *m/z* 937 $(M+H)^+$; HRFABMS *m/z* 937.4217 $(M+H)^+$, calcd for $C_{44}H_{61}N_{10}O_{11}S$, 937.4242.

Keramamide L (2). Colorless amorphous solid; $[\alpha]_D^{22}$ -60° (*c* 0.1, MeOH); IR (film) ν_{max} 3250 (br), 2980, 1720, and 1650 cm^{-1}; UV (MeOH) λ_{max} 213 (ε 18000), 285 (2300), 297 (1800), and 305 (sh) nm; ^1H NMR (Table 1); ESIMS *m/z* 927 and 929 [$(M+H)^+$, ca. 3:1]; HRESIMS *m/z* 927.4541 $(M+H)^+$, calcd for $C_{49}H_{63}N_8O_8{}^{35}Cl$, 927.4535.

Amino Acid Analysis by Chiral HPLC. Keramamide K or L (**1** or **2**, each 100 μg) was dissolved in 6N HCl (100 μL) and heated in a sealed tube at 110 °C for 24 h. Chiral HPLC analyses were carried out using a SUMICHIRAL OA-5000 column [Sumitomo Chemical Industry; 4 x 150 mm; 40 °C, detection at 254 nm]. Retention times (min) of authentic amino acids were as follows: L-Lys (12.0) and D-Lys (13.6) [eluent: H_2O containing 1.0 mM $CuSO_4$, flow rate 0.2 mL/min]; L-Ala (6.6), D-Ala (9.6), L-Dpr (9.4), and D-Dpr (10.0) [eluent: H_2O containing 1.0 mM $CuSO_4$, flow rate 1.0 mL/min]; L-Leu (4.8), D-Leu (6.4), L-Phe (8.8), D-Phe (11.4), L-Ile (11.0), allo-L-Ile (10.0), D-Ile (18.4), and D-allo-Ile (15.2) [eluent: MeOH/H_2O (15:85) containing 2.0 mM $CuSO_4$, flow rate 1.0 mL/min]; L-Ise (39.2) and D-Ise (64.8) [eluent: MeOH/H_2O (25:75) containing 2.0 mM $CuSO_4$, flow rate 1.0 mL/min]. Retention times of the hydrolysate of **1** were as follows: L-Ala (6.6), L-Dpr (9.4), L-Ile (11.0), and D-Ise (64.8). Retention time of the hydrolysate of **2** were as follows: L-Lys (12.0), L-Leu (4.8), and L-Phe (8.8).

Determination of the Stereochemistry at C-13 of 1. Keramamide K (**1**, 100 μg) was treated with 5 % NaOH (0.5 mL) and 30% H_2O_2 aqueous solutions at 65 °C for 45 min. The reaction mixture was hydrolyzed with 6N HCl at 110 °C for 24 h. The hydrolysate was subjected to chiral HPLC analyses as described above, and the retention time of Ile in the hydrolysate was 11.0 min (L-Ile).

Determination of the Stereochemistry at C-9 in 1. To a MeOH solution (0.5 mL) of keramamide K (**1**, 200 μg) were bubled a stream of ozone at room temperature for 1 min. The reaction mixture was treated with 6N HCl at 110 °C at 24 h, and the hydrolysate was subjected to chiral HPLC anlyses [SUMICHIRAL OA-5000, 4 x 150 mm; 40 °C, flow rate, 1.0 mL/min; eluent: H_2O containing 1.0 mM $CuSO_4$]. The retention times of authentic L- and D-(*O*-Me)Ser were found to be 8.2 and 11.6 min, respectively. The retention time of (*O*-Me)Ser in the degradation product was found to be 8.2 min [L-(*O*-Me)Ser].

Determination of the Stereochemistry of (1-Me)Trp. Keramamide K (**1**, 200 μg) was treated with 6N HCl (100 μL) containing 0.05% phenol and 2% mercaptoethanol at 150 °C for 1 h. The hydrolysate was reacted with 1% FDAA solution in acetone (5 μL) and 1M $NaHCO_3$ aq. (10 μL) at room temperature for 15 h. After addition of 2N HCl (5 μL) and then evaporation of the solvent, the reaction mixture was dissolved in DMSO (50 μL) and MeOH (150 μL). The FDAA derivatives of L- and D-(1-Me)Trp[11] were prepared by the same procedure as described above. The FDAA derivative of the hydrolysate of **1** was subjected to C_{18} HPLC analyses using Inatosil ODS-2 column (GI Science Inc., 4.6 x 150 mm) and the following gradient program; solvent A, acetonitrile; solvent B, 50 mM triethylammonium phosphate (pH 3.0); time (min)/A(%)/B(%), 0/10/90, 40/60/40; flow rate, 1.0 mL/min; detection at 340 nm. Retention times of the FDAA derivatives of authentic L- and D-(1-Me)Trp were 28.7 and 30.9 min, respectively. The retention time of FDAA derivative of (1-Me)Trp in the hydrolysate of **1** was found to be 28.7 min [L-(1-Me)Trp].

Determination of the Stereochemistry of MeCtrp. Keramamide L (**2**, 300 μg) in AcOH (300 μL) was treated with ozone at -78 °C for 1 min. After remove of excess ozone by a stream of nitrogen, the mixture was treated with 30% H_2O_2 (300 μL) at room temperature for 3 h. The reaction mixture was hydrolyzed with 6N HCl (100 μL) at 110 °C for 5 h. The hydrolysate was subjected to chiral HPLC analyses [SUMICHIRAL OA-5000, 4 x 150 mm; 40 °C, flow rate, 1.0 mL/min; eluent: H_2O containing 2.0 mM $CuSO_4$]. The retention times of authentic L- and D-(*N*-Me)Asp were found to be 23.5 and 18.6 min, respectively. The retention time of MeAsp in the degradation product of **2** was found to be 23.5 min [L-(*N*-Me)Asp].

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