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Keramamides E, G, H, and J, New Cyclic Peptides Containing an Oxazole or a Thiazole Ring from a *Theonella* Sponge

Jun'ichi Kobayashi*, Fumio Itagaki, Hideyuki Shigemori, Toshifumi Takao[a], and Yasutsugu Shimonishi[a]

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan, and [a]Institute for Protein Research, Osaka University, Osaka 565, Japan

Abstract. Four new cyclic peptides, keramamides E (**1**), G (**2**), H (**3**), and J (**4**), containing an oxazole or a thiazole ring have been isolated from the Okinawan marine sponge *Theonella* sp. and the structures elucidated by 2D NMR data and degradation experiments. The sequence of amino acid residues in **1** ~ **4** was determined on the basis of FAB MS/MS data.

Marine sponges of the genus *Theonella* have been shown to be a rich source of unique secondary metabolites with intriguing structures and interesting biological activities.[1] In our continuing studies on bioactive substances from marine organisms,[2] we previously isolated some cyclic peptides, keramamides A[3], B ~ D[4], and F[5], from an Okinawan marine sponge *Theonella* sp. Further investigation on extracts of this sponge led to isolation of four new cyclic peptides, keramamides E (**1**), G (**2**), H (**3**), and J (**4**), containing an oxazole or a thiazole ring and many unusual amino acids. This paper describes the isolation and structure elucidation of **1** ~ **4** on the basis of spectral data, especially FAB MS/MS data, and chemical means.

The methanol/toluene (3:1) extract of the sponge collected off Kerama Islands, Okinawa, was partitioned between toluene and water. The chloroform extract of the aqueous phase was subjected to silica gel and Sephadex LH-20 columns, and HPLC on ODS to yield keramamides E (**1**, 4.6 x 10^{-5} %, wet weight of the sponge), G (**2**, 5.3 x 10^{-5} %), H (**3**, 2.8 x 10^{-5} %), and J (**4**, 5.0 x 10^{-5} %) together with known cyclic peptides, keramamides B (**5**)[4], C (**6**)[4], D (**7**)[4], and F (**8**)[5].

The molecular formula of keramamide E (**1**) was determined as $C_{53}H_{75}N_{10}O_{12}Br$ by HRFABMS [*m/z* 1123.4818, $(M+H)^+$, Δ +1.0 mmu].[6] Amino acid analyses of the acid hydrolysate of **1** showed the presence of 1 mol each of proline (Pro), ornithine (Orn), isoleucine (Ile), alanine (Ala), and norvaline (nVal). Extensive analyses of the ^1H (Table 1) and ^{13}C NMR data of **1** including ^1H-^1H COSY, HMQC, and NOESY spectra by comparison with those of keramamide B (**5**) suggested the gross structure of **1** containing a 2-hydroxy-3-methylpentanoic acid (Hmp), a 2-bromo-5-hydroxytryptophan (BhTrp), and segments **a** and **b**. The presence of an Hmp group at Ile-NH was revealed by the NOESY correlation between Ile-NH and Hmp-α. The ^1H and ^{13}C NMR spectral data of **1** [δ_H 8.22 (1H, s); δ_C 136.9 s, 139.5 d, and 165.2 s] indicated the presence of an oxazole ring in segment **b**. The sequence of amino acid residues in **1** was determined by FAB MS/MS experiments. The MS/MS product ions observed in the FAB MS/MS spectra from the protonated molecular ions (*m/z* 1123 and 1125) are shown in Table 2 and Fig. 1. Chiral HPLC analysis (SUMICHIRAL OA-5000) of the acid hydrolysate of **1** clarified that Ala, Ile, Orn, Pro, and nVal were L-form. The BhTrp residue was converted into Asp by treatment of **1** with ozone followed by

CH_3CO_3H,[4] while the α-keto-β-amino acid (a) was transformed into Leu by treatment of **1** with H_2O_2/NaOH.[5] Both Asp and Leu in the degradation products were determined to be L by the chiral HPLC analysis. The absolute stereochemistry of Hmp generated by alkaline hydrolysis of **1** was determined to be 2*S*,3*S* by comparison of retention times in chiral HPLC with those of (2*S*,3*S*)-, (2*R*,3*R*)-, (2*S*,3*R*)-, and (2*R*,3*S*)-Hmp derived from L-Ile, D-Ile, L-allo-Ile, and D-allo-Ile through deamination with $NaNO_2$, respectively.[7] Thus the structure of keramamide E was concluded to be **1**.

Similarly the absolute configurations of Hmp in keramamides B ~ D (**5** ~ **7**)[4] which had remained to be determined were concluded to be 2*S*,3*S* by the same method as described above.

The HRFABMS [*m/z* 921.3886 $(M+H)^+$, Δ -3.7 mmu] of keramamide G (**2**) established the molecular formula to be $C_{43}H_{56}N_{10}O_{11}S$, which was the same as that of keramamide F (**8**). Amino acid analyses of the acid hydrolysate of **2** revealed 1 mol each of Ala, isoserine (Ise), 2,3-diaminopropionic acid (Dpr), and Ile. Extensive analyses of the ^1H (Table 1) and ^{13}C NMR data of **2** including ^1H-^1H COSY, HMQC, and

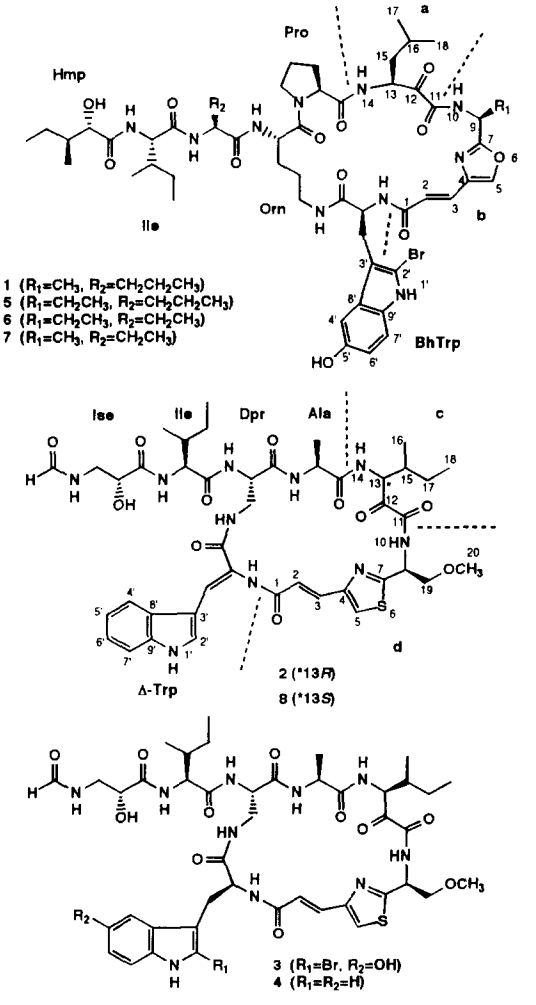

Table 1. ^{1}H NMR Data of Keramamides E (1), G (2), H (3), and J (4) in DMSO-d_6

position		1 J(Hz)			2 J(Hz)	3 J(Hz)	4 J(Hz)
Hmp	OH	5.50[a] (brd) 5.4	CHO		8.03(s)	8.03 (s)	8.03 (s)
	α	3.74 (m)					
	β	1.70 (m)	Ise	NH	7.97 (m)	7.97 (m)	7.98 (m)
	γ-CH_2	1.34, 1.14 (m)		α	4.04 (m)	4.01 (m)	4.02 (m)
	γ-CH_3	0.82 (m)		β	3.14 (m)	3.14 (m)	3.13 (m)
	δ-CH_3	0.81 (m)			3.50 (m)	3.48 (m)	3.43 (m)
				OH		5.96 (d) 5.9	5.98 (d) 5.4
Ile	NH	7.52 (d) 9.3					
	α	4.26 (m)	Ile	NH	7.67 (d) 8.8	7.67 (d) 9.8	7.68 (d) 8.8
	β	1.70 (m)		α	4.24 (m)	4.25 (m)	4.25 (dd) 8.8, 6.8
	γ-CH_2	1.40, 0.98 (m)		β	1.74 (m)	1.75 (m)	1.74 (m)
	γ-CH_3	0.82 (m)		γ-CH3	0.85 (m)	0.83 (m)	0.85 (m)
	δ-CH_3	0.81 (m)		γ-CH2	1.43 (m)	1.40 (m)	1.42 (m)
					1.05 (m)	1.00 (m)	1.05 (m)
nVal	NH	8.09 (d) 7.8		δ-CH3	0.85 (m)	0.85 (m)	0.84 (m)
	α	4.23 (m)					
	β	1.43, 1.53 (m)	Dpr	α-NH	8.08 (d) 7.8	8.12 (d) 7.8	8.14 (d) 6.8
	γ	1.30 (m)		α	4.42 (m)	4.47 (m)	4.46 (m)
	δ	0.86 (m)		β	2.92 (m)	2.85 (m)	2.83 (m)
					3.60 (m)	3.57 (m)	3.46 (m)
Orn	α-NH	7.90 (d) 6.8		β-NH	8.04 (m)	7.67 (m)	7.82 (m)
	α	4.45 (m)					
	β	1.63,1.36 (m)	Ala	NH	8.02 (m)	8.06 (d) 5.9	8.09 (d) 7.3
	γ	1.37 (m)		α	4.72 (m)	4.63 (m)	4.67 (m)
	δ	3.44, 2.64 (m)		β	1.24 (d) 6.8	1.36 (d) 6.8	1.34 (d) 7.3
	δ-NH	7.49 (m)					
			d	2	6.91 (d) 15.1	6.69 (d) 15.1	6.61 (d) 14.9
Pro	α	4.34 (m)		3	7.42 (d) 15.1	7.15 (d) 15.1	7.15 (d) 14.9
	β	2.17, 1.85 (m)		5	7.90 (s)	7.76 (s)	7.75 (s)
	γ	1.86, 1.47 (m)		9	4.81 (m)	4.76 (m)	4.75 (dd) 10.0, 6.8
	δ	3.70, 3.51 (m)		10	9.31 (d) 6.8	9.25 (d) 7.3	9.28 (d) 6.8
			c	13	5.49 (m)	5.40 (dd) 9.5, 3.4	5.46 (dd) 9.8, 3.4
b	2	6.58 (d) 15.1		14	8.53 (d) 9.3	8.45 (d) 9.5	8.61 (d) 9.8
	3	7.14 (d) 15.1		15	2.46 (m)	2.45 (m)	2.46 (m)
	5	8.22 (s)		16	0.75 (d) 6.8	0.79 (d) 7.3	0.77 (d) 7.3
	9	4.84 (m)		17	1.18 (m)	1.22 (m)	1.22 (m)
	10	9.20 (d) 5.4		18	0.84 (m)	0.89 (t) 7.3	0.82 (m)
a	13	4.89 (m)		19	3.96 (m)	3.93 (dd) 9.0, 5.6	3.92 (m)
	14	8.18 (d) 5.0			4.02 (m)	4.02 (m)	3.99 (m)
	15	1.37 (m)		20	3.39 (s)	3.39 (s)	3.38 (s)
	16	1.46 (m)					
	17	0.83 (m)		α'		4.66 (m)	4.64 (m)
	18	0.83 (m)		β'	7.86 (s)	2.89 (m)	3.13 (m)
	19	1.49 (d) 6.8				3.35 (m)	3.35 (m)
				1'-NH	11.51 (s)	11.20 (s)	10.77 (s)
BhTrp	NH	8.12 (d) 11.2		2'	7.33 (d) 2.4		6.98 (d) 2.0
	α	4.58 (m)		4'	7.71 (d) 8.3	6.91 (d) 2.4	7.56 (d) 7.8
	β	3.06, 2.83 (m)		5'	7.15 (t) 7.3		6.93 (t) 7.8
	1'	11.23 (s)		6'	7.10 (t) 7.3	6.59 (dd) 8.5, 2.4	7.02 (t) 7.8
	4'	6.88 (d) 2.0		7'	7.41 (d) 7.8	7.04 (d) 8.5	7.28 (d) 7.8
	6'	6.58 (dd) 8.8, 2.0		α'-NH	9.07 (s)	8.08 (d) 9.0	8.05 (d) 7.0
	7'	7.02 (d) 8.8		5'-OH		8.76 (s)	
	OH	8.69 (s)					

a) δ in ppm

NOESY spectra by comparison with those of keramamide F (8) showed the presence of a formyl group, an α,β-dehydrotryptophan (Δ-Trp), and segments **c** and **d**. The presence of a thiazole ring in segment **d** was

Table 2. FAB MS/MS Data of Keramamide E (1)

m/z	*m/z*	Assignment of MS/MS product ions[c]
1123[a]	1125[b]	M + H (parent ion)
1105	1107	M - H_2O + H
1067	1069	M - C_4H_9 + 2H
1037	1039	M - $C_5H_{11}O$ + 2H
1009	1011	Ile-nVal-cyclo(Orn-Pro-a-b-BhTrp) + 2H
896	898	nVal-cyclo(Orn-Pro-a-b-BhTrp) + 2H
797	799	cyclo(Orn-Pro-a-b-BhTrp) + 2H
683	685	Pro-a-b-BhTrp + 2H
655	657	Pro-a-b-BhTrp - CO + 2H
403	403	Pro-a-b + H
327	327	Hmp-Ile-nVal + H
306	306	a-b + H
228	228	Hmp-Ile
200	200	Hmp-Ile - CO
165	165	b + H
122	122	b - C_2H_5N
115	115	Hmp

a)^{79}Br parent ion. b)^{81}Br parent ion. c)The amide bond cleavages are assumed to occur between NH and CO (the B-type fragmentation).[8]

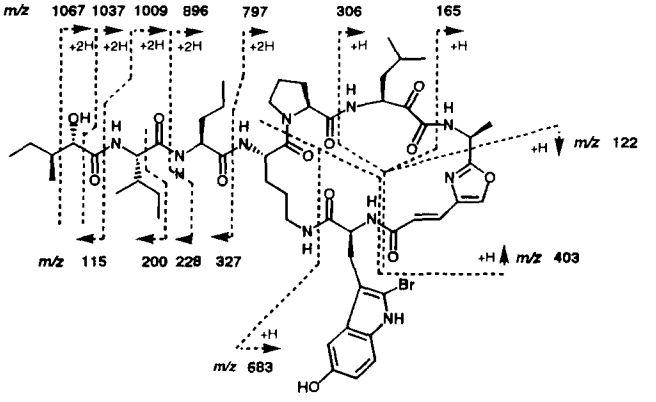

Fig. 1 FAB MS/MS Fragmentations of Keramamide E (1)

indicated by the ^{1}H and ^{13}C NMR spectral data of 2 [δ_H 7.90 (1H, s); δ_C 149.4 s, 123.1 d, and 165.9 s]. Further substantial evidences for the structure of 2 were obtained from the FAB MS/MS spectrum from the $(M + H)^+$ ion (*m/z* 921), which afforded MS/MS product ions corroborating well the amino acid sequence as shown in Fig. 2. Thus the gross structure of keramamide G (2) was elucidated to be the same as that of keramamide F (8). The differences between 2 and 8 were found only for carbon chemical shifts at C-13 (2, δ_C 56.7; 8, δ_C 59.7), C-16 (2, δ_C 14.3; 8, δ_C 16.0), and C-17 (2, δ_C 26.7; 8, δ_C 23.4), suggesting that the stereochemistry at C-13 of the α-keto-β-amino acid (c) in 2 was different from that of 8. Oxidation of 2 with H_2O_2/NaOH[5] followed by acid hydrolysis furnished Ile of which the absolute configuration was determined to be D by chiral HPLC analysis. Chiral HPLC analysis of the acid hydrolysate of 2 clarified that Ala, Ile, and Dpr were L-form, while Ise was D-form. Treatment of 1 with ozone for degradation of the (*O*-methylserine)thiazole (d) yielded *O*-methylserine, which was L-form from chiral HPLC analysis. Thus the structure of keramamide G was assigned to be 2.

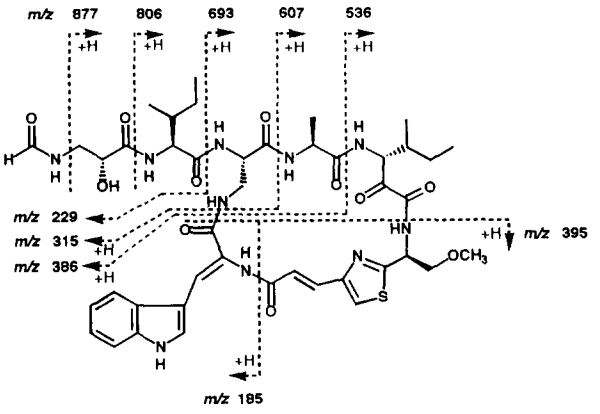

Fig. 2 FAB MS/MS Fragmentations of Keramamide G (2)

The HRFABMS [*m/z* 1018.3190, M^+, Δ -2.9 mmu] of keramamide H (**3**) established its molecular formula to be $C_{43}H_{57}N_{10}O_{12}BrS$. Amino acid analyses of the acid hydrolysate of **3** revealed 1 mol each of Ala, Ise, Dpr, and Ile. The ^1H NMR (Table 1) spectrum of **3** was almost the same as that of **2** except for its tryptophan moiety. Comparison of the ^1H-^1H COSY spectrum of **3** with those of **1** and **2** revealed the presence of BhTrp [δ_H 11.20 (s), 6.91 (d, J = 2.4 Hz), 6.59 (dd, J = 8.5 and 2.4 Hz), 7.04 (d, J = 8.5 Hz), 8.08 (d, J = 9.0 Hz), and 8.76 (s)] for **3** in place of Δ-Trp for **2**. Furthermore, the FAB MS/MS spectra from the protonated molecular ions (*m/z* 1017 and 1019) of **3** supported the gross structure proposed for keramamide H. Ala, Ile, and Dpr in the acid hydrolysate of **3** were L-form by chiral HPLC analysis, while Ise was D-form. The BhTrp residue in **3** was converted into Asp by treatment of **3** with ozone followed by CH_3CO_3H, while the α-keto-β-amino acid (**c**) was transformed into Ile by treatment of **3** with H_2O_2/NaOH. Degradation of the (*O*-methylserine)thiazole (**d**) with ozone yielded *O*-methylserine. Asp, Ile, and *O*-methylserine in the degradation products were determined to be L by chiral HPLC analysis. Thus the structure of keramamide H was assigned to be **3**.

The molecular formula of keramamide J (**4**) was determined as $C_{43}H_{58}N_{10}O_{11}S$ by HRFABMS [*m/z* 923.4099 $(M + H)^+$, Δ +1.3 mmu] and the molecular weight was larger than that of keramamide G (**2**) by 2 Daltons. The ^1H NMR data (Table 1) of **4** were similar to those of **2** except for resonances of tryptophan moiety. Aromatic proton signals [δ_H 6.98 (d, J = 2.0 Hz), 7.56 (d, J = 7.8 Hz), 6.93 (t, J = 7.8 Hz), 7.02 (t, J = 7.8 Hz), 7.28 (d, J = 7.8 Hz), and 8.05 (d, J = 7.0 Hz)] for **4** were assigned as those of tryptophan by the ^1H-^1H COSY data of **4** and comparison with the chemical shifts reported for a tryptophan residue in polydiscamide A.[9] Hydrolysis of **4** under mild acidic condition followed by amino acid analysis of the products revealed 1 mol each of Ala, Ise, Dpr, Ile, and Trp. The amino acid sequence of **4** was deduced from the FAB MS/MS data. Chiral HPLC analysis of the acid hydrolysate of **4** clarified that Ala, Ile, Dpr, and Trp were L-form, while Ise was D-form. The α-keto-β-amino acid (**c**) was transformed into Ile by treatment of **4** with H_2O_2/NaOH, while the (*O*-methylserine)thiazole (**d**) was converted into *O*-methylserine by the same method as described above. Both Ile and *O*-methylserine were determined to be L by chiral HPLC analysis. Thus the structure of keramamide J was concluded to be **4**.

Keramamides E (**1**), G (**2**), H (**3**), and J (**4**) are new cyclic peptides containing an oxazole or a thiazole ring in addition to various unusual amino acids. Although there are many reports on cyclic peptides having oxazole and/or thiazole ring(s) from tunicates[10] and terrestrial microorganisms,[11] very few peptides containing conjugated oxazole or thiazole ring(s) have been isolated from natural origin. Such α-keto-β-amino acids contained in **1** ~ **4** have been reported for poststatin, a peptide from *Streptomyces viridochromogenes*[12], while 2-hydroxy-3-methylpentanoic acid moiety has been found in peptides from a black yeast *Aureobasidium pullulans*.[13] Keramamide E (**1**) exhibited cytotoxicity against L1210 murine leukemia cells and KB human epidermoid carcinoma cells with IC_{50} values of 1.60 and 1.55 μg/mL, respectively, while keramamides G (**2**), H (**3**), and J (**4**) showed weak cytotoxicity (IC_{50} ~10 μg/mL).

Experimental Section

General Methods. Optical rotations were measured on a JASCO DIP-370 polarimeter. UV and IR spectra were obtained on JASCO Ubest-35 and JASCO IR report-100 spectrometers, respectively. ^1H and ^{13}C NMR spectra were recorded on a JEOL EX-400 spectrometer in DMSO-d_6. The 2.50 ppm resonance of residual CD_3SOCHD_2 and 39.5 ppm of $(CD_3)_2SO$ were used for ^1H and ^{13}C NMR spectra as internal references, respectively. Mass spectra were obtained on a JEOL JMS-HX/HX 110A tandem mass spectrometer by using glycerol as a matrix.

Collection, Extraction, and Isolation. The sponge *Theonella* sp. was collected off Kerama Island, Okinawa and was kept frozen until used. The toluene/MeOH (1:3, 2 L x 2) extract of the sponge (4 kg, wet weight) was suspended with 1M NaCl (1 L) and was extracted with toluene (600 mL x 2). The aqueous layer was extracted with $CHCl_3$ (800 mL x 2). The $CHCl_3$-soluble material (2.1 g) was subjected to a silica gel column with gradient elution of MeOH (2-50%) in $CHCl_3$. The fraction eluted with 15% MeOH in $CHCl_3$ was separated by a silica gel column with toluene/MeOH (98:2 to 70:30) to afford crude peptide fractions **I** and **II**. The fraction **I** was further purified by a Sephadex LH-20 column (MeOH) followed by reversed-phase HPLC [YMC-Pack AM-324 ODS, Yamamura Chemical, 10 x 250 mm; flow rate: 2.0 mL/min; eluent: MeOH/H_2O/CF_3CO_2H, 70:30:0.1; UV detection at 254 nm] to give keramamides B (**5**, 7.4 mg, t_R 48.0 min), C (**6**, 11.8 mg, t_R 40.1 min), and D (**7**, 10.1 mg, t_R 31.5 min) and a fraction (12.3 mg, t_R 37.1 min), which was purified by the same reversed-phase HPLC column with CH_3CN/H_2O/CF_3CO_2H (42:58:0.1) to afford keramamide E (**1**, 1.8 mg, t_R 63 min). The fraction **II** was separated by a Sephadex LH-20 column (MeOH) followed by the same HPLC column with CH_3CN/H_2O (40:60) to give keramamide F (**8**, 5.1 mg, t_R 22.0 min) and the fractions **III** (t_R 23 ~ 26 min) and **IV** (t_R 26 ~ 37 min). Fraction **III** was subjected to the same HPLC column with MeOH/H_2O/CF_3CO_2H (70:30:0.1) to afford keramamides G (**2**, 2.1 mg, t_R 16 min) and H (**3**, 1.1 mg, t_R 18 min). Fraction **IV** was purified under the same HPLC condition to yield keramamide J (**4**, 2.0 mg, t_R 36 min).

Keramamide E (1): colorless solid; $[\alpha]^{22}_D$ -39° (*c* 0.1, MeOH); IR (KBr) ν_{max} 3400, 1670, 1640, and 1520 cm^{-1}; UV(MeOH) λ_{max} 269 (ε 30900) and 310 (sh) nm; ^1H NMR (Table 1); ^{13}C NMR (DMSO-d_6) δ_C 172.6 (Hmp-CO), 74.9 (Hmp-α), 38.1 (Hmp-β), 23.2 (Hmp-γ-CH_2), 15.3 (Hmp-γ-CH_3), 11.7 (Hmp-δ-CH_3), 170.6 (Ile-CO), 55.8 (Ile-α), 37.3 (Ile-β), 24.2 (Ile-γ-CH_2), 15.4 (Ile-γ-CH_3), 10.9 (Ile-δ-CH_3), 171.2 (nVal-CO), 52.0 (nVal-α), 34.0 (nVal-β), 18.5 (nVal-γ), 13.6 (nVal-δ), 169.4 (Orn-CO), 49.7 (Orn-α), 37.8 (Orn-β), 25.1 (Orn-γ), 38.5 (Orn-δ), 171.2 (Pro-CO), 58.4 (Pro-α), 29.5 (Pro-β), 24.4 (Pro-γ), 46.9 (Pro-δ), 163.9 (C-1), 123.5 (C-2), 127.4 (C-3), 136.9 (C-4), 139.5 (C-5), 165.2 (C-7), 43.9 (C-9), 159.6 (C-11), 195.8 (C-12), 52.4 (C-13), 29.1 (C-15), 24.8 (C-16), 20.8 (C-17), 23.1 (C-18), 18.0 (C-19), 170.7 (BhTrp-CO), 53.4 (BhTrp-α), 27.9 (BhTrp-β), 109.5 (BhTrp-2'), 109.0 (BhTrp-3'), 102.3 (BhTrp-4'), 150.7 (BhTrp-5'), 111.5 (BhTrp-6'), 110.9 (BhTrp-7'), 128.2 (BhTrp-8'), and 130.5 (BhTrp-9'); FABMS (positive) *m/z* 1123 and 1125 (1:1) $(M+H)^+$; exact mass found *m/z* 1123.4818, calcd for $C_{53}H_{76}N_{10}O_{12}Br$ 1123.4808; FAB MS/MS (Table 2).

Keramamide G (2): colorless solid; $[\alpha]^{21}_D$ +10° (*c* 0.12, MeOH); IR (KBr) ν_{max} 3400, 1660, and 1520 cm^{-1}; UV(MeOH) λ_{max} 224 (ε 26500), 279 (21200), and 340 (11700) nm; ^1H NMR (Table 1); ^{13}C NMR (DMSO-d_6) δ_C 161.5 (CHO), 171.4 (Ise-CO), 70.2 (Ise-α), 41.6 (Ise-β), 170.1 (Ile-CO), 56.2 (Ile-α), 37.1 (Ile-β), 15.3 (Ile-γ-CH_3), 24.3 (Ile-γ-CH_2), 11.0 (Ile-δ-CH_3), 169.3 (Dpr-CO), 51.1 (Dpr-α), 41.6 (Dpr-β), 173.8 (Ala-CO), 47.6 (Ala-α), 20.0 (Ala-β), 164.7 (Δ-Trp-CO), 121.9 (Δ-Trp-α), 124.0 (Δ-Trp-

β), 127.1 (Δ-Trp-2'), 109.6 (Δ-Trp-3'), 118.0 (Δ-Trp-4'), 121.9 (Δ-Trp-5'), 120.0 (Δ-Trp-6'), 111.9 (Δ-Trp-7'), 135.4 (Δ-Trp-8'), 127.3 (Δ-Trp-9'), 163.3 (C-1), 124.0 (C-2), 132.1 (C-3), 149.4 (C-4), 123.1 (C-5), 165.9 (C-7), 53.7 (C-9), 161.0 (C-11), 197.2 (C-12), 56.7 (C-13), 37.0 (C-15), 14.3 (C-16), 26.7 (C-17), 11.6 (C-18), 72.6 (C-19), and 58.3 (C-20); FABMS (positive) *m/z* 921 $(M+H)^+$; exact mass found *m/z* 921.3886, calcd for $C_{43}H_{57}N_{10}O_{11}S$ 921.3923; FAB MS/MS (positive) *m/z* 921 $[M + H]^+$, 903 $[M - H_2O + H]^+$, 877 $[M - CH_2NO + H]^+$, 833 $[M - C_3H_6NO_2 + H]^+$, 806 [Ile-cyclo(Dpr-Ala-**c**-**d**-ΔTrp) + $2H]^+$, 790 [Ile-cyclo(Dpr-Ala-**c**-**d**-ΔTrp) - NH $+H]^+$, 693 [cyclo(Dpr-Ala-**c**-**d**-ΔTrp) + $2H]^+$, 677 [cyclo(Dpr-Ala-**c**-**d**-ΔTrp) - NH + $H]^+$, 607 [Ala-**c**-**d**-ΔTrp + $H]^+$, 536 [**c**-**d**-ΔTrp + $H]^+$, 395 [**d**-ΔTrp + $H]^+$, 386 [Ise(CHO)-Ile-Dpr-Ala + $H]^+$, 315 [Ise(CHO)-Ile-Dpr + $H]^+$, 229 [Ise(CHO)-Ile]$^+$, 185 [ΔTrp + $H]^+$, and 117 [Ise(CHO) + $H]^+$.

Keramamide H (3): colorless solid; $[\alpha]^{20}_D$ -42° (*c* 0.055, MeOH); IR (KBr) ν_{max} 3400, 1660, and 1520 cm^{-1}; UV (MeOH) λ_{max} 223 (sh) and 277 (ε 23100) nm; ^1H NMR (Table 1); FABMS (positive) *m/z* 1017 and 1019 (1:1, $M+H)^+$; exact mass found *m/z* 1018.3190 (M^+), calcd for $C_{43}H_{57}N_{10}O_{12}{}^{81}BrS$ 1018.3219; FAB MS/MS (positive) *m/z* (for ^{79}Br parent ion) and *m/z** (for ^{81}Br parent ion) 1017 and 1019* $[M + H]^+$, 999 and 1001* $[M - H_2O + H]^+$, 973 and 975* $[M - CH_2NO + H]^+$, 929 and 931* $[M - C_3H_6NO_2 + H]^+$, 902 and 904* [Ile-cyclo(Dpr-Ala-**c**-**d**-BhTrp) + $2H]^+$, 886 and 888* [Ile-cyclo(Dpr-Ala-**c**-**d**-BhTrp) - NH $+H]^+$, 789 and 791* [cyclo(Dpr-Ala-**c**-**d**-BhTrp) + $2H]^+$, 774 and 776* [cyclo(Dpr-Ala-**c**-**d**-BhTrp) - NH + $H]^+$, 703 and 705* [Ala-**c**-**d**-BhTrp + $H]^+$, 632 and 634* [**c**-**d**-BhTrp + $H]^+$, 491 and 493* [**d**-BhTrp + $H]^+$, 386 and 386* [Ise(CHO)-Ile-Dpr-Ala + $H]^+$, 315 and 315* [Ise(CHO)-Ile-Dpr + $H]^+$, 282 and 284* [BhTrp + $2H]^+$, 229 and 229* [Ise(CHO)-Ile]$^+$, and 117 and 117* [Ise(CHO) + $H]^+$.

Keramamide J (4): colorless solid; $[\alpha]^{18}_D$ +8.4° (*c* 0.1, MeOH); IR (KBr) ν_{max} 3410, 1680, and 1540 cm^{-1}; UV (MeOH) λ_{max} 222 (ε 45200) and 278 (27200) nm; ^1H NMR (Table 1); ^{13}C NMR (DMSO-d_6) δ_C 161.4 (CHO), 171.4 (Ise-CO), 70.0 (Ise-α), 41.3 (Ise-β), 169.2 (Ile-CO), 58.3 (Ile-α), 37.1 (Ile-β), 15.3 (Ile-γ-CH_3), 24.2 (Ile-γ-CH_2), 11.0 (Ile-δ-CH_3), 169.2 (Dpr-CO), 51.3 (Dpr-α), 41.9 (Dpr-β), 174.1 (Ala-CO), 48.1 (Ala-α), 19.7 (Ala-β), 171.6 (Trp-CO), 58.3 (Trp-α), 28.3 (Trp-β), 127.1 (Trp-2'), 111.3 (Trp-3'), 122.5 (Trp-4'), 118.1 (Trp-5'), 120.6 (Trp-6'), 111.5 (Trp-7'), 137.5 (Trp-8'), 127.3 (Trp-9'), 164.4 (C-1), 124.4 (C-2), 131.2 (C-3), 149.2 (C-4), 122.6 (C-5), 165.5 (C-7), 53.7 (C-9), 161.4 (C-11), 197.0 (C-12), 56.1 (C-13), 37.0 (C-15), 14.2 (C-16), 26.7 (C-17), 11.5 (C-18), 72.5 (C-19), and 58.3 (C-20); FABMS (positive) *m/z* 923 $(M+H)^+$; exact mass found *m/z* 923.4099 calcd for $C_{43}H_{59}N_{10}O_{11}S$ 923.4086; FAB MS/MS (positive) *m/z* 923 $[M + H]^+$, 905 $[M - H_2O + H]^+$, 879 $[M - CH_2NO + H]^+$, 835 $[M - C_3H_6NO_2 + H]^+$, 808 [Ile-cyclo(Dpr-Ala-**c**-**d**-Trp) + $2H]^+$, 792 [Ile-cyclo(Dpr-Ala-**c**-**d**-Trp) - NH $+H]^+$, 695 [cyclo(Dpr-Ala-**c**-**d**-Trp) + $2H]^+$, 679 [cyclo(Dpr-Ala-**c**-**d**-Trp) - NH + $H]^+$, 609 [Ala-**c**-**d**-Trp + $H]^+$, 538 [**c**-**d**-Trp + $H]^+$, 397 [**d**-Trp + $H]^+$, 386 [Ise(CHO)-Ile-Dpr-Ala + $H]^+$, 315 [Ise(CHO)-Ile-Dpr + $H]^+$, 229 [Ise(CHO)-Ile]$^+$, 188 [Trp + $2H]^+$, and 117 [Ise(CHO) + $H]^+$.

Amino Acid Analysis by Chiral HPLC. Keramamides E, G, or H (**1**~**3**, each 0.1 mg) was hydrolyzed with 6N HCl (1.0 mL) at 110°C for 24 h and keramamide J (**4**, 0.1 mg) was hydrolyzed with 4N methanesulfonic acid (0.1 mL) at 115°C for 24h. Chiral HPLC analyses were carried out using a SUMICHIRAL OA-5000 [Sumitomo Chemical Industry, 4 x 150 mm, 40 °C, UV detection at 254 nm]. Retention times (min) of standard amino acids were as follows: L-Orn (13.1) and D-Orn (14.2) [eluent: H_2O containing 1.0 mmol/L of $CuSO_4$, flow rate: 0.2 mL/min]; L-Ala (5.7), D-Ala (8.1), L-(*O*-Me)Ser (9.7), D-(*O*-Me)Ser (14.0), L-Pro (12.7), D-Pro (27.2), L-Dpr (19.7), D-Dpr (22.9), L-nVal (23.8), and D-nVal (44.1) [eluent: H_2O containing 1.0 mmol/L of $CuSO_4$, flow rate: 1.0 mL/min]; L-Ile (13.3), allo-L-Ile (11.3), D-Ile (21.3), and allo-D-Ile (17.4) [eluent: MeOH/H_2O (15:85) containing 2.0 mmol/L of $CuSO_4$, flow rate: 1.0 mL/min]; L-Ise (25.7), D-Ise (41.4), L-Trp (34.2), and D-Trp (37.0) [eluent: MeOH/H_2O (30:70) containing 2.0 mmol/L of $CuSO_4$, flow rate: 1.0 mL/min]. The retention times (min) of hydrolysates of **1** ~ **4** were as follows; L-Orn (13.1), L-Ala (5.7), L-Pro (12.7), L-nVal (23.8), and L-Ile (13.3) in **1**; L-Ala (5.7), L-Dpr (19.7), L-Ile (13.3), and D-Ise (41.4) in **2** and **3**; L-Dpr (19.7), L-Ile (13.3), L-Trp (34.2), and D-Ise (41.4) in **4**.

Preparation of 2-Hydroxy-3-methylpentanoic Acid. A solution of $NaNO_2$ (50 mg) in water (0.5 mL) was added to a stirred and ice-salt-cooled solution of L-Ile (50 mg) in 1N H_2SO_4 (1 mL). The mixture was stirred for 12 h and then extracted with THF (10 mL). The THF solution was washed with brine, dried ($MgSO_4$) and concentrated under reduced pressure to give (2*S*,3*S*)-Hmp (30 mg, 60%): ^1H NMR ($CDCl_3$) δ 0.94 (3H, t, J = 7.5 Hz), 1.03 (3H, d, J = 6.9 Hz), 1.29 (2H, m), 1.90 (1H, m), and 4.18 (1H,

d, J = 3.3 Hz). According to essentially the same procedure as described above, DL-Ile, allo-L-Ile, and allo-D-Ile afforded (2*S*,3*S*)- and (2*R*,3*R*)-Hmp, (2*S*,3*R*)-Hmp, and (2*R*,3*S*)-Hmp, respectively. (2*S*,3*R*)-Hmp: ^{1}H NMR ($CDCl_3$) δ 0.89 (3H, d, J = 7.1 Hz), 0.97 (3H, t, J = 7.5 Hz), 1.38 (2H, m), 1.90 (1H, m), and 4.29 (1H, d, J = 2.7 Hz).

Determination of the Stereochemistry of 2-Hydroxy-3-methylpentanoic Acid in Keramamides E (1) and B ~ D (5 ~ 7). Keramamide E (**1**, 0.2 mg) was hydrolyzed with 1N NaOH/MeOH (1:4, 0.5 mL) at 50 °C for 8 h. The reaction mixture acidified by 1N HCl (0.3 mL) was then extracted with THF (5 mL x 3) and the residue was subjected to chiral HPLC analysis using a SUMICHIRAL OA-5000 [4 x 150 mm, 30 °C, flow rate: 1.0 mL/min; eluent: CH_3CN/H_2O (20:80) containing 2.0 mmol/L of $CuSO_4$; detection: UV at 254 nm]. Retention times (min) of synthetic 2-hydroxy-3-methylpentanoic acid (Hmp) isomers were as follows; 2*S*,3*R*-Hmp (26.0), 2*S*,3*S*-Hmp (28.9), 2*R*,3*S*-Hmp (38.4), and 2*R*,3*R*-Hmp (43.4). The retention time of Hmp in the hydrolysate of **1** was found to be 28.9 min. Keramamides B, C, or D (**5** ~ **7**, each 0.2 mg) was hydrolyzed with 1N NaOH/MeOH (1:4, 0.5 mL) at 50 °C for 8 h. The reaction mixture acidified by 1N HCl (0.3 mL) was then extracted with THF (5 mL x 3) and the residue was subjected to chiral HPLC analysis as described above. The retention times of Hmp in the hydrolysates of **5**, **6**, and **7** were found to be all 28.9 min.

Determination of the Stereochemistry of the (*O*-Methylserine)thiazole in Keramamides G (2), H (3), and J (4). A stream of O_3 was bubled into a 1 mL MeOH solution of keramamides G, H, or J (**2**~**4**, each 0.2 mg) at room temperature for 8 min. The reaction mixture was subjected to hydrolysis and the chiral HPLC analysis using a SUMICHIRAL OA-5000 [4 x 150 mm, 40 °C, flow rate: 1.0 mL/min; eluent: H_2O containing 1.0 mmol/L of $CuSO_4$]. Retention times of authentic L-(*O*-Me)Ser and D-(*O*-Me)Ser were 9.7 and 14.0 min, respectively. The retention times of (*O*-Me)Ser in the oxidation product of **2**, **3**, and **4** were found to be all 9.7 min.

Determination of the Stereochemistry of the α-Keto-β-amino Acid in Keramamides E (1), G (2), H (3), and J (4). To a stirred solution of keramamide E (**1**, 0.2 mg) in 5% NaOH (0.5 mL) was added dropwise 30% H_2O_2 (0.1 mL). After stirring at 65 °C for 40 min the reaction mixture was hydrolyzed with 6N HCl (1.0 mL) at 110 °C for 24h and subjected to chiral HPLC analysis using SUMICHIRAL OA-5000 [40°C; flow rate: 1.0 mL/min; detection: UV at 254 nm]. Retention times of authentic L-Leu, D-Leu, L-Ile, D-Ile, allo-L-Ile, and allo-D-Ile [eluent: MeOH/H_2O (15:85) containing 2.0 mmol/L of $CuSO_4$] were 14.4, 21.9, 13.3, 21.3, 11.3, and 17.4 min, respectively. The retention time of Leu in the degradation product of **1** was found to be 14.4 min. Keramamides G, H, or J (**2** ~ **4**, each 0.2 mg) was treated with 30% H_2O_2 (0.1 mL) in 5% NaOH (0.5 mL) at 65 °C for 40 min. The reaction mixture was hydrolyzed with 6N HCl (1.0 mL) at 110 °C for 24 h. The hydrolysate was applied to the chiral HPLC analysis as described above. The retention times of Ile in the degradation products of **2**, **3**, and **4** were found to be 21.3, 13.3, and 13.3 min , respectively.

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