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Koshikamide B, a Cytotoxic Peptide Lactone from a Marine Sponge *Theonella* sp

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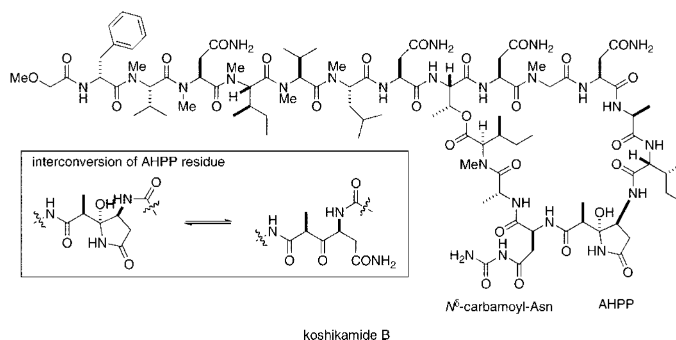

Koshikamide B (**1**) has been isolated from two separate collections of the marine sponge *Theonella* sp. as the major cytotoxic constituent. Koshikamide B is a 17-residue peptide lactone composed of six proteinogenic amino acids, two D-isomers of proteinogenic amino acids, seven *N*-methylated amino acids, and two unusual amino acid residues. The unusual amino acids are N^{δ}-carbamoylasparagine and 2-(3-amino-2-hydroxy-5-oxopyrrolidin-2-yl)propionic acid (AHPP); the former is first found as the constituent of peptides, whereas the latter is a new amino acid residue. The N-terminus of koshikamide B is blocked by a methoxyacetyl group. The structure of koshikamide B (**1**) has been determined by interpretation of spectral data and analysis of chemical degradation products. Koshikamide B (**1**) exhibits cytotoxicity against P388 murine leukemia cells and the human colon tumor (HCT-116) cell line with an IC_{50} value of 0.45 and 7.5 μg/mL, respectively.

Introduction

Marine sponges of the genus *Theonella* frequently contain nonribosomal peptides with interesting biological activities,[1,2] viz. theonellamides[3] and microsclerodermins (antifungal),[4] cyclotheonamides (serine protease inhibition),[5] polytheonamides (cytotoxic),[6] and papuamides (antiviral).[7] Most of these peptides contain amino acid residue(s) reminiscent of mixed nonribosomal peptide synthetase (NRPS)−polyketide synthase (PKS)

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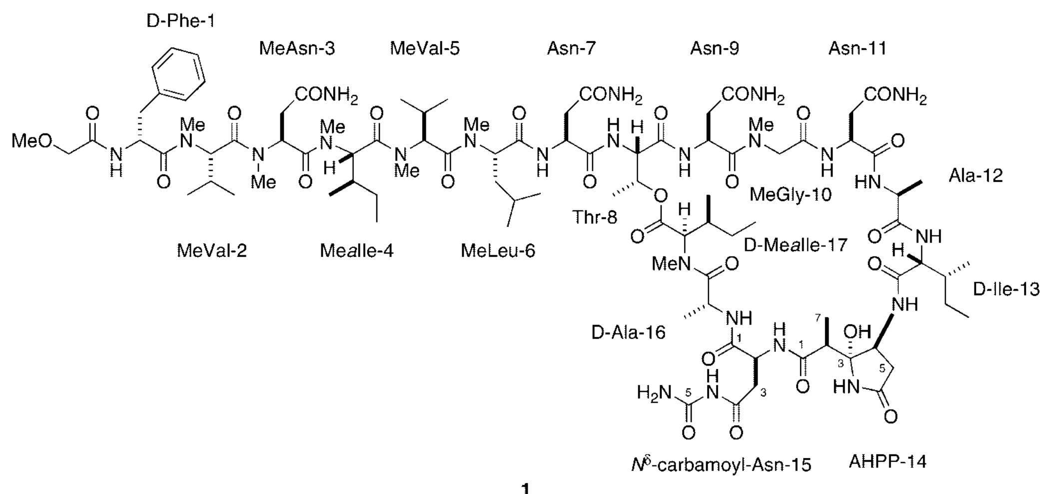
CHART 1



pathways of microorganisms,[8,9] suggesting the involvement of symbiotic microorganisms in their production.[10] Indeed, a biosynthetic gene cluster likely to be involved in the biosynthesis of onnamides, metabolites of a mixed NRPS-PKS pathway, was isolated from the metagenome of *T. swinhoei*.[11] In the course of our search for novel cytotoxic metabolites from marine invertebrates, we have isolated koshikamides A1 and A2[12,13] from a sponge of the genus *Theonella*. The extract of the same sponge contained a more abundant and more potent cytotoxic constituent named koshikamide B. In an independent investigation by the Scripps Institution of Oceanography group (SIO), a collection of *Theonella* sp. from Palau also yielded koshikamide B from the methanolic extract of the sponge. The isolation and structure elucidation of koshikamide B is presented below.

Results and Discussion

The EtOH extract of the sponge was partitioned between $CHCl_3$ and H_2O, and the organic layer was evaporated and further partitioned between 90% MeOH and *n*-hexane. The aqueous MeOH fraction was subjected to ODS flash chromatography, and the 90% MeOH eluate was separated by ODS HPLC with 1-PrOH/H_2O/TFA (30:70:0.05) to afford three cytotoxic peaks. Because the three fractions gave indistinguishable NMR and mass spectral data, they were judged as identical and named koshikamide B (**1**, 330 mg, Chart 1). A second collection of the sponge *Theonella* also yielded **1** from the methanolic extract of this sponge which was fractionated on HP-20 followed by HPLC using a poly(styrene−divinylbenzene) column (PRP-1).

Koshikamide B (**1**) exhibited the $[M - H]^-$ ion at *m/z* 2050 in the negative FAB-MS. The peptidic nature of **1** was inferred from the ^1H NMR spectrum, which exhibited signals for α-protons between δ 4.0 and 5.6 and those for amide protons between δ 7.8 and 8.9. The constituent amino acids of **1** were assigned by interpretation of the COSY, TOCSY, HMQC, and HMBC spectra. From the analysis, eight standard amino acid residues, Ala (×2), Thr, Phe, Asn (×3), and Ile, were identified. Their presence was confirmed by the amino acid analysis. Seven *N*-methylated amino acid residues, Sar (MeGly), MeVal (×2), MeLeu, MeIle (×2), MeAsn, and a methoxyacetyl group were also identified from 2D NMR data.

There were two unusual residues (residues X and Y, Figure 1). Residue X contained a pyrrolidone ring as identified by the COSY, HMQC, HMBC, and ^{15}N HMQC data. The C-3 quaternary carbon (δ 91.0) showed HMBC correlations with exchangeable protons at δ 6.65 and 7.98. The ^{15}N HMQC spectrum revealed that the proton at δ 7.98 was attached to a nitrogen at δ 137.0, wheras the proton at δ 6.65 was not attached to a nitrogen, indicating that C-3 was connected to an *N*-acyl and a hydroxyl group. A pair of methylene protons (δ 1.82, 2.81; H_2-5) were coupled to an amide proton at δ 8.88 and gave HMBC correlations with C-3 and a carbonyl carbon (δ 177.3). A methine proton at δ 2.32 (H-2) was substituted by a methyl group (δ 1.18, 3H, d) and correlated to C-3 in the HMBC spectrum, allowing us to define the structure of residue X as 2-(3-amino-2-hydroxy-5-oxopyrrolidin-2-yl)propionic acid (AHPP).

Residue Y contained NMR signals for an N^{δ}-substituted asparagine. Only one proton (δ 9.74) was attached to N^{δ}, and a pair of additional exchangeable protons (δ 7.48, 7.88) were observed. There was an unassigned carbon at δ 154.1, which should be incorporated in this residue. The structural assignment by NMR was hampered by the absence of HMBC correlations from the carbon at δ 154.1. The ^{15}N HMQC spectrum of **1** revealed that the protons at δ 7.48 and 7.88 were both connected to a nitrogen at δ 89.0, while the proton at δ 9.74 was connected to a nitrogen at δ 149.0, suggesting the presence of a ureido moiety. In the COSY and TOCSY spectra protons at δ 7.48, 7.88, and 9.74 were correlated to each other. In order to compare NMR data we prepared an N^{δ}-carbamoyl Asn derivative (**2**). The COSY and TOCSY spectra of the synthetic compound (**2**)

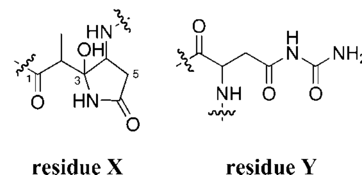


FIGURE 1. Gross structures of residue X and residue Y

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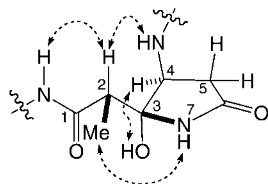
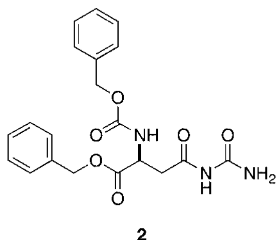


FIGURE 2. Selected NOESY correlations of the AHPP residue in **1**.

displayed the correlations among the ureido protons as observed in koshikamide B (**1**), and the ^{15}N chemical shift values for the ureido nitrogens in both compounds were almost identical. Therefore, residue Y was assigned as N^{δ}-carbamoyl Asn.



The amino acid sequence of **1** was determined by interpretation of the constant time HMBC (CT-HMBC)[14] and NOESY data. The resolution of the ^{13}C-axis of the HMBC spectrum is reduced due to the f_1-modulation caused by ^{1}H−^{1}H couplings. The CT-HMBC spectrum is free of f_1-modulation, which facilitates the signal assignments in crowded regions. Inter-residual HMBC cross peaks were observed across almost all of the amide bonds, allowing us to determine the amino acid sequence of **1**. The methoxyacetyl group was placed at the N-terminus, while the carboxyl group of the C-terminal Me*a*Ile was lactonized with Thr-8.

The stereochemistry of Phe, Thr, MeVal, MeAsn, and MeLeu residues was determined to be D, L, L, L, and L, respectively, by the Marfey method.[15] The stereochemistry of Ile and Asp in the hydrolysate was determined as D and L, respectively, by chiral GC analysis with Chirasil-Val. Therefore, three Asn and N^{δ}-carbamoylasparaginyl residues were all in the L-form. D- and L-Ala were detected in a 1:1 ratio by chiral GC analysis. Because it was not possible to completely separate the four isomers of MeIle and Me*a*Ile by chiral GC or Marfey's method, the stereochemical assignment of these residues was established in two steps. First, the hydrolysate of koshikamide B (**1**) was subjected to ODS-HPLC, and the peak eluting at the retention time of MeIle and Me*a*Ile, which coeluted, was isolated. The isolated amino acid was identified as Me*a*Ile by the ^{1}H NMR spectrum. The Marfey analysis of the isolated Me*a*Ile revealed that it was a 1:1 mixture of D and L isomers. Consequently, **1** contained one residue each of D- and L-Me*a*Ile.

Residue-specific stereochemical assignment of Ala and Me*a*Ile residues in **1** was accomplished by the fortuitous isolation of fragment peptides containing only one of these residues. Hydrolysis of **1** with 6 N HCl at room temperature for 25 h followed by HPLC separation afforded fragment peptides **3**−**5** (Figure 3), whose structures were assigned by a combination of FABMS and amino acid analysis. Compounds **3** and **4** contained Ala-16 and Me*a*Ile-17, whereas **5** contained Ala-12. The Marfey analysis of the hydrolysates revealed the presence of D-Me*a*Ile and D-Ala in both **3** and **4**. Therefore, Ala-16 and Me*a*Ile-17 were both shown to be D. Compound **5**

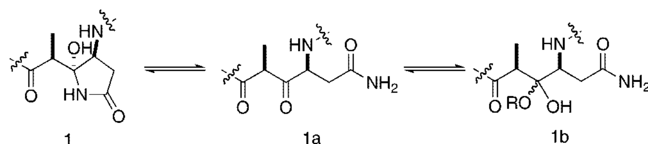
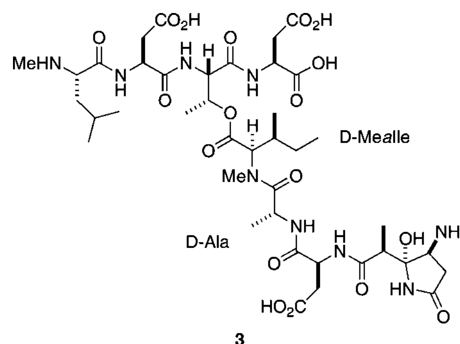
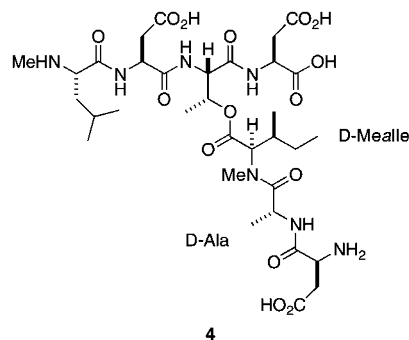
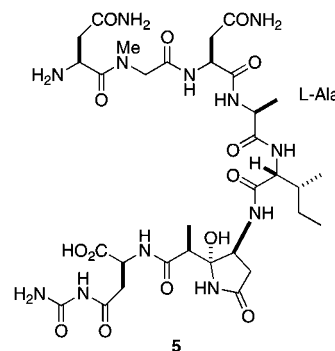


FIGURE 3. Equilibrium of the AHPP residue in **1**.

liberated L-Ala, as expected. Therefore, by default, Me*a*Ile-4 was assigned as L.







Stereochemistry of the AHPP residue was determined by a combination of spectral and chemical methods. A NOESY cross peak between OH-3 and H-4 showed that these protons were on the same face of the five-membered ring. Cross peaks between H-2 and 4-NH and between 2-CH_3 and 7-NH allowed us to define the relative stereochemistry of C-2 with respect to the five-membered ring (Figure 2). Therefore the stereochemistry of the AHPP residue was assigned as 2*S**, 3*R**, and 4*S**.

Koshikamide B was susceptible to reduction with $NaBH_4$ and gave the alcohol **6**. Interpretation of the NMR data of **6** demonstrated that the AHPP residue was converted to the 4-amino-5-carbamoyl-3-hydroxy-2-methylpentanoic acid residue, indicating that C-3 in the AHPP residue was transiently present as the ketone form (Figure 3). It is likely the reason for the multiplication of HPLC peaks which was caused by an

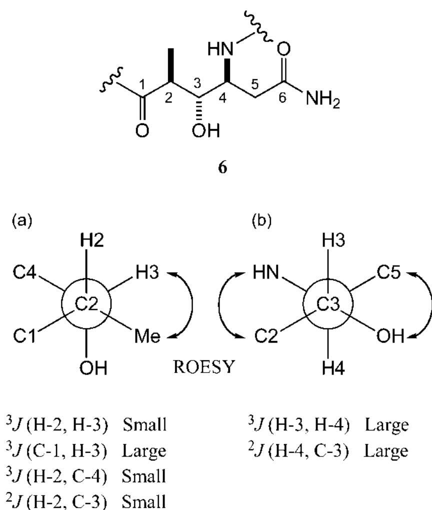


FIGURE 4. *J*-based configurational analysis of **6**.

equilibrium among the ketone, hemiacetal, and cyclized aminal forms in this residue (Figure 3). Compound **6** was used for further analysis of the stereochemistry of the AHPP residue by the application of *J*-based configuration analysis (Figure 4).[16] $^3J_{H,H}$ and $^{2,3}J_{C,H}$ values were measured by 1D TOCSY and *J*-resolved HMBC spectra,[17,18] respectively. A small coupling constant between H-2 and H-3 (0.8 Hz) suggested that they were gauche. H-2 exhibited a small ^3J coupling constant with C-4, and H-3 displayed a large ^3J coupling constant with C-1, indicating that H-2 and C-4 were gauche, while H-3 and C-1 were antiperiplanar. An intense ROESY cross peak between H-3 and 2-CH_3 was consistent with 2,3-anti stereochemistry with C-1 and C-4 in the gauche relationship (Figure 4a). On the other hand, the large coupling constant between H-3 and H-4 (10.4 Hz) and an intense ROESY cross peak between H-2 and 4-NH indicated 3,4-anti stereochemistry (Figure 4b). Therefore, the relative stereochemistry of C-2, C-3, and C-4 in **6** was assigned as 2*S**,3*R**,4*S**.

It was reported that the pyrrolidone-containing amino acid residue in microsclerodermin A gave a dehydration product by treatment with acid, and the product was used to determine the absolute stereochemistry of this unit.[4] The aminal hydroxyl group did not dehydrate either under acidic conditions or in the presence of dehydrating agents such as $POCl_3$. Under these reaction conditions, the products were always a complex mixture. Eventually, we found that **1** underwent dehydration on heating at 75 °C in EtOH and the product was the desired olefin **7**. Ozonolysis of **7** followed by a reductive workup furnished imide **8** which was hydrolyzed with 6 N HCl and the acid hydrolysate was subjected to the amino acid analysis and GC analysis, revealing that **8** afforded 5 equiv of L-Asp. The additional equivalent of L-Asp arose from the imide moiety in **8** which should have the 4*S*-stereochemistry (Scheme 1). Considering the relative stereochemistry mentioned above, the absolute configuration of the AHPP residue was concluded to be 2*S*,3*R*,4*S*.

Koshikamide B (**1**) exhibited cytotoxicity against P388 murine leukemia cells and human colon tumor (HCT-116) cell line with

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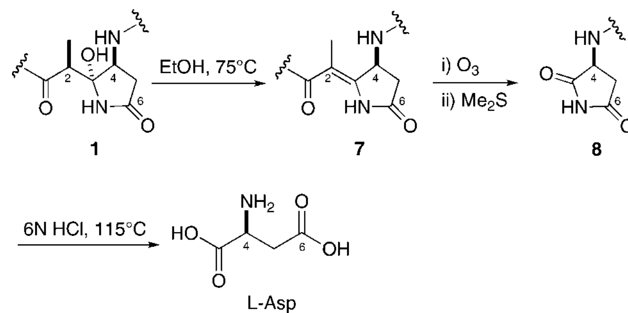
SCHEME 1



IC_{50} values of 0.45 and 7.5 μg/mL, respectively. Koshikamide B (**1**) represents a new class of the peptide lactone possessing two unique moieties: the carbamoylated Asn and AHPP residues. A ureido moiety is present in several peptides from the sponge *Theonella swinhoei*, e.g., konbamide, keramamide A, and mozamides, where the ureido moiety participates in the linkage between the *N*-terminal residue and another amino acid.[1,2] Although N^{δ}-carbamoyl Asn itself was discovered in urine of rats fed ozonated casein,[18] **1** is the first example of N^{δ}-carbamoyl Asn to be present as a constituent of a natural peptide. The AHPP unit is a new amino acid residue. Pyrrolidone-containing amino acid residues have only been found in microsclerodermins.[4] The AHPP residue in **1** may be biosynthesized by Claisen condensation of a propionate and Asn, followed by spontaneous cyclization. The present study demonstrated the transient presence of the ketone form **1a**. It is interesting to note that the structures of koshikamides A_1, A_2, and B are identical from the N-terminus until residue-7.[12,13]

Experimental Section

Animal Material. The sponge was collected by scuba at a depth of 15 m off Shimokoshiki Island, Kagoshima prefecture (129°244'N, 31°244'E), immediately frozen, and kept at −20 °C until processed. A voucher specimen (ZMA POR. 13011) was deposited at the Zoological Museum of the University of Amsterdam. The SIO collection of *Theonella* was hand-collected by Pat Colin from a depth of 85 m at Palau using a mixed gas rebreathing apparatus. The specimen was immediately frozen and kept at −20 °C until extraction. A voucher specimen has been deposited in the SIO Benthic Invertebrate Collection (nos. 99−411).

Extraction and Purifcation. The sponge (280 g; wet weight) was chopped into small pieces and extracted with EtOH (3 × 200 mL). The combined extracts were concentrated and partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was evaporated and partitioned between *n*-hexane and 90% MeOH. The 90% MeOH layer was fractionated by flash chromatography on ODS with MeOH/H_2O system; the active fraction eluted with 90% MeOH was further separated by reversed-phase HPLC on an ODS column with 30% 1-PrOH containing 0.05% TFA to furnish koshikamide B (**1**, 330 mg) as a white powder. The extract also afforded the known koshikamides A_1 and A_2. The SIO sponge (372 g; wet weight) was extracted with MeOH (2 × 750 mL) for 24 h. The second and then the first extracts were passed through a column of HP-20 (5 × 20 cm). The combined eluents was repassed through the column. Finally, the eluent was concentrated to 500 mL and diluted with H_2O (1.5 L) and passed again through the column. The column was eluted with 600 mL fractions of (1) H_2O, (2) 25% Me_2CO/H_2O, (3) 50% Me_2CO/H_2O, (4) 75% Me_2CO/H_2O, and (5) Me_2CO. Fraction 3 (500 mg) was then subjected to reversed-phase HPLC (Hamilton PRP-1; 21.5 × 250 mm; 10 mL/min; 30−50% CH_3CN/H_2O over 50 min) to give koshikamide B (**1**, 383 mg).

Koshikamide B (1): $[\alpha]_D$ −120 (*c* 0.1, 1-PrOH−H_2O, 4:6); UV (1-PrOH−H_2O, 4:6) 266 nm (ϵ 5.9 × 10^3); IR (film) ν_{max} 3850,

TABLE 1. ^{1}H and ^{13}C NMR Data of Koshikamide B (1) in DMSO-d_6

assignment		δ_H	δ_C	HMBC (C no.)[a]
methoxyacetyl	1		168.8	
	2	3.75	71.0	OMe, 1
	OMe	3.22	58.2	2
Phe-1	1		171.2	
	2	4.95 (m)	50.0	1, 3, (methoxyacetyl)-1
	3	2.89 (m)	36.7	1, 2, 1′, 3′, 5′
		3.02 (dd, 13.5, 5.8)		1, 2, 1′, 3′, 5′
	1′		137.0	
	2′, 6′	7.25 (m)	128.0	1′, 2′, 6′
	3′, 5′	7.28 (m)	129.1	3′, 4′, 5′
	4′	7.18 (m)	126.4	3′, 5′
	NH	7.91 (d, 8.1)		2, 3, (methoxyacetyl)-1
MeVal-2	1		168.8	
	2	4.90 (m)	58.0	1, 3, 4, 5, N-Me, (Phe-1)-1
	3	2.17 (m)	26.5	2, 5
	4	0.58 (d, 6.9)	17.8	2, 3, 5
	5	0.78 (m)	19.2	2, 3
	N-Me	2.88 (s)	29.8	2, (Phe-1)-1
MeAsn-3			169.3	
		5.68 (dd, 10.0, 4.6)	50.0	1, 3, N-Me, 4
		2.05 (dd, 15.4, 4.2)	34.5	1, 2, 4
		2.82 (m)		1, 2, 4
	4-CONH$_2$	6.77 (s)	170.9	3
		7.28 (s)		
	N-Me	2.76 (s)	30.3	2, (MeVal-2)-1
MeIle-4	1		169.0	
	2	5.08 (m)	56.2	1, 3, 4, 6, N-Me
	3	2.10 (m)	32.2	
	4	0.98 (m)	25.0	3, 6
		1.22 (m)		
	5	0.81 (m)	10.5	3, 4
	6	0.68 (d, 6.9)	13.2	2, 3, 4
	N-Me	2.81 (s)	29.2	2, (MeAsn-3)-1
MeVal-5	1		170.1	
	2	5.09 (m)	57.8	1, 3, 4, 5, N-Me
	3	2.17 (m)	26.5	2, 5
	4	0.74 (m)	17.6	2, 3, 5
	5	0.86 (m)	19.3	2, 3, 4
	N-Me	2.76 (s)	29.2	2, 1, (MeIle-4)-1
MeLeu-6	1		170.0	
	2	5.11 (m)	53.5	1, 3, N-Me
	3	1.50 (m)	36.2	2, 5, 6
		1.67 (m)		
	4	1.20 (m)	24.2	
	5	0.80 (m)	21.0	4, 6
	6	0.88 (m)	23.1	4, 5
	N-Me	2.85 (s)	30.0	2, (MeVal-5)-1
Asn-7	1		170.0	
	2	4.47 (m)	49.7	1, 3, 4
	3	2.38 (m)	36.2	1, 2, 4
		2.38 (m)		
	4-CONH$_2$	6.75 (s)	171.8	
		7.25 (s)		
	NH	7.72 (d, 6.9)		(MeLeu-6)-1
Thr-8	1		167.6	
	2	4.32 (t, 9.2)	56.4	1, 3, NH
	3	4.60 (m)	72.0	1, 2
	4	0.61 (d, 6.5)	16.7	2, 3
	NH	7.67 (d, 8.8)		(Asn-7)-1
Asn-9	1		171.8	
	2	5.18 (m)	44.6	1, 3, 4, (Thr-8)-1
	3	2.28 (m)	37.5	1, 2, 4
		2.58 (m)		1, 2, 4
	4-CONH$_2$	6.82 (s)	170.6	3
		7.26 (s)		
	NH	8.42 (d, 8.8)		(Thr-8)-1
MeGly-10	1		168.1	
	2	3.31 (m)	50.4	1, N-Me, (Asn-9)-1
		4.41 (m)		1, N-Me
	N-Me	3.18 (s)	37.3	2, (Asn-9)-1
Asn-11	1		172.1	
	2	4.43 (m)	50.5	1, 3, 4, (MeGly-10)-1
	3	2.28 (m)	36.7	1, 4
		2.28 (m)		
	4-CONH$_2$	6.90 (s)	170.4	3
		6.98 (s)		
	NH	8.10 (d, 6.2)		(MeGly-10)-1
Ala-12	1		172.1	
	2	4.05 (m)	48.5	1, 3
	3	1.28 (d, 6.9)	17.8	2
	NH	8.64 (m)		(Asn-11)-1
Ile-13	1		172.1	
	2	4.19 (m)	56.0	1, 3, 4
	3	1.57 (m)	35.0	
	4	1.05 (m)	24.2	3, 5
		1.38 (m)		
	5	0.72 (m)	9.5	3, 4
	6	0.72 (m)	14.8	2, 3, 4
	NH	8.38 (d, 9.6)		(Ala-12)-1
AHPP-14	1		177.3	
	2	2.32 (m)	38.8	1, 3, 7
	3		91.0	
	4	4.16 (m)	53.5	5, 6
	5	1.82 (d, 6.5)	35.8	3, 4, 6
		2.81 (m)		3, 4, 6
	6		176.5	
	7	1.18 (d, 7.3)	14.0	1, 2, 3
	6-NH	7.98 (s)		3, 4, 5, 6
	OH	6.65 (s)		2, 3
	4-NH	8.88 (d, 8.1)		(Ile-13)-1
carbamoyl Asn-15	1		169.2	
	2	4.98 (m)	49.0	1, 3, 4, (AHPP-14)-1
	3	2.45 (m)	38.8	1, 2, 4
		3.21 (m)		1, 2, 4
	4-CONH	9.74 (s)	172.8	4
	5-CONH$_2$	7.48 (s)	155.0	
	NH	7.88 (s)		(AHPP-14)-1
Ala-16	1	7.85 (d, 8.9)	174.2	
	2	5.0 (m)	44.7	1, 3
	3	1.34 (d, 7.3)	16.0	1, 2
	NH	8.61 (m)		(carbamoyl Asn-15)-1
MeIle-17	1		168.8	
	2	3.38 (m)	67.5	1, 3, 4, N-Me
	3	2.31 (m)	33.8	2, 4, 6
	4	1.05 (m)	27.2	3, 6
		1.78 (m)		
	5	0.85 (m)	11.5	3, 4
	6	0.73 (m)	14.8	2, 3, 4
	N-Me	3.18 (s)	39.2	2, (Ala-16)-1

[a] For intraresidual correlations, residue numbers are not specified.

3287, 2964, 2876, 1681, 1633, 1556, 1538, 1504, 1415, 1283, 1100, 1002, 755, 665 cm^{-1}; HRFABMS *m/z* 2074.0837 [M + Na]$^+$ (calcd for $C_{93}H_{150}N_{24}O_{28}Na$ 2074.0725); ^{1}H, ^{13}C, and ^{15}N NMR data (DMSO-d_6), see Table 1.

Amino Acid Analysis of Koshikamide B (1). A 1 mg portion of koshikamide B (**1**) was dissolved in 200 μL of 6 N HCl and heated at 110 °C for 15 h. The solvent was evaporated under a stream of N_2, and the residue was redissolved in 0.02 N HCl and subjected to amino acid analysis.

Synthesis of Benzyl N^{α}-Benzyloxycarbonyl-N^{δ}-carbamoyl-L-asparagine (2). To a solution of N^{α}-benzyloxycarbonyl-L-asparagine (100 mg, 0.376 mmol) in DMF (300 μL) was added benzyl bromide (500 μL, 4.21 mmol) in the presence of a small amount of K_2CO_3. After being stirred for 3 h at room temperature, the reaction mixture was quenched with water. The mixture was extracted with ethyl acetate (100 mL) to give crude benzyl N^{α}-benzyloxycarbonyl-L-asparagine, which was dissolved in dioxane (50 mL) and treated with monochloroacetyl isocyanate (600 μL, 6 mmol). The reaction mixture was stirred for 3 h at room temperature. After additions of methanol (4 mL) and Zn dust (1.0 g), the suspension was stirred for 3 h at room temperature. Zn dust was then removed by filtration, and the filtrate was concentrated. The product was purified by column chromatography on silica gel with $CHCl_3$/MeOH (98:2) to give benzyl N^{α}-benzyloxycarbonyl-N^{δ}-carbamoyl-L-asparagine (**2**, 122.3 mg, 82%). **2**: ^{1}H NMR (DMSO-d_6) δ 10.15 (s, 1H), 7.83 (d, 1H, J = 8.1 Hz), 7.59 (s, 1H), 7.35−7.32 (m, 10H, ArH), 7.15 (s, 1H), 5.12 (s, 2H, CH_2Ph), 5.03 (s, 2H, CH_2Ph), 4.54 (m, 1H, H-2), 2.88 (dd, H-3), 2.71 (dd, H-3).

$NaBH_4$ Reduction of Koshikamide B (1). To a solution of koshikamide B (**1**, 10 mg) in MeOH (0.5 mL) was added $NaBH_4$ (5 mg), and the mixture was stirred at room temperature for 30 min. The reaction was quenched by addition of water, the MeOH

was removed under high vacuum, and the residue was dissolved in aqueous 1-PrOH (1 mL) and purified by ODS HPLC [10 × 250 mm; 30% 1-PrOH containing 0.05% TFA (2 mL/min) detection with absorption at 210 nm] to obtain **6** (7.2 mg).

HPLC Analysis of the Marfey Derivatives. To the acid hydrolysate of koshikamide B (**1**, 1 mg) were added 50 μL of 1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide in acetone (10 mg/mL) and 100 μL of 1 M $NaHCO_3$, and the mixture was kept at 80 °C for 3 min. To the reaction mixture were added 50 μL of 2 N HCl and 100 μL of 50% MeCN containing 0.05% TFA and the mixture analyzed by ODS HPLC [10 × 250 mm: linear gradient elution from MeCN/H_2O/TFA (25:75:0.05) to MeCN/H_2O/TFA (55:45:0.05) in 60 min; at a flow rate of 1.0 mL/min] peaks were detected with UV absorption at 340 nm. Each peak was identified by comparing the retention time with that of the FDAA derivative of standard amino acids. The amino acid standards showed the following retention times: D-MeAsp 18.0 min, L-Thr 19.8 min, L-*allo*-Thr 20.6 min, L-MeAsp 22.2 min, D-*allo*-Thr 26.6 min, D-Thr 27.2 min, L-MeVal 38.1 min, L-Phe 38.8 min, D-MeVal 40.8 min, L-MeLeu 41.2 min, D-Phe 43.4 min, D-MeLeu 43.4 min.

Chiral GC Analysis of the Acid Hydrolysate. Koshikamide B (**1**, 500 μg) was heated with 6 N HCl (200 μL) at 105 °C for 15 h. After evaporation of the solvent under a stream of nitrogen, 10% HCl−MeOH (100 μL) was added, and the sealed vial was heated at 105 °C for 45 min. After evaporation of the reagent under a stream of nitrogen, TFAA (30 μL) in CH_2Cl_2 (50 μL) was added to the residue, and the sealed vial was heated at 105 °C for 10 min. The product was again dried under a stream of nitrogen and redissolved in CH_2Cl_2 (100 μL), and the solution was subjected to GC analysis. The retention time of the standard amino acids were as follows: D-Ala 9.6 min, L-Ala 11.0 min, L-Ile 14.6 min, D-Ile 15.3 min, D-*allo*-Ile 15.8 min, L-*allo*-Ile 16.2 min, D-Asp 21.0 min, L-Asp 21.5 min.

Isolation of Me*a*Ile. Koshikamide B (**1**, 9.2 mg) was heated with 6 N HCl (150 μL) at 105 °C for 24 h, and the hydrolysates were purified by ODS-HPLC [10 × 250 mm; elution with H_2O containing 0.05% TFA (2 mL/min); detection with absorption at 210 nm]. The peak eluted at the retention time of authentic samples of MeIle and Me*a*Ile, which were coeluted, was collected. Isolated Me*a*Ile: ^{1}H NMR (D_2O) δ 3.70 (br s), 2.72 (3H, s), 2.08 (m), 1.56 (sep, $J = 6.5$ Hz), 1.28 (m), 1.06 (3H, d, $J = 6.9$ Hz), 0.98 (3H, t, $J = 7.3$ Hz).

Peptide Fragments 3−5. Koshikamide B (**1**, 10 mg) was hydrolyzed with 6 N HCl (1 mL) at room temperature for 25 h, and the hydrolysate was neutralized with 5 N NaOH and purified by ODS HPLC [10 × 250 mm × 2; elution with a linear gradient from 15% to 60% MeCN containing 0.05% TFA (2 mL/min); detection with absorption at 220 nm]; peaks between t_R 35 and 65 min were further purified by ODS HPLC [10 × 250 mm × 2; elution with a linear gradient from 3% to 35% MeCN containing 0.05% TFA (2 mL/min); detection with absorption at 220 nm], to afford the peptide fragments **3**, **4**, and **5**. **3**: FAB-MS/MS [*m/z* 942 $(M + H)^+$, 789 (M − pyrrolidone unit)$^+$, 675 (M − pyrrolidone unit − Asp)$^+$, 604 (M − pyrrolidone unit − Asp-Ala)$^+$]. **4**: FABMS [*m/z* 790 $(M + H)^+$]. **5**: FABMS [*m/z* 829 $(M + H)^+$]. Constituent amino acids of **3**−**5** were analyzed by amino acid analysis, Marfey's analysis, and chiral GC analysis.

Dehydration of Koshikamide B (1) Followed by Ozonolysis. A solution of **1** (5 mg) in ethanol (1 mL) was heated at 75 °C for 17 h. The reaction mixture was purified by HPLC [10 × 250 mm; 30% 1-PrOH containing 0.05% TFA (2 mL/min) detection with absorption at 270 nm] to afford the olefin **7** (2.8 mg). Compound **7** gave the $(M - H)^-$ ion peak at *m/z* 2032 in the FABMS. A stream of ozone was bubbled into a solution of **7** (2 mg) in MeOH (1 mL) at room temperature for 30 min. The solution was dried under a stream of nitrogen, and the residue was reduced by addition of Me_2S (100 μL). The solution was kept at room temperature for 1 h, and the excess reagent was removed under high vacuum. The reaction product was hydrolyzed and subjected to GC analysis as described above.

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Supporting Information Available: Spectral data of **1**, **2** and **6**−**8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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