Kahalalide K: A New Cyclic Depsipeptide from the Hawaiian Green Alga *Bryopsis* Species

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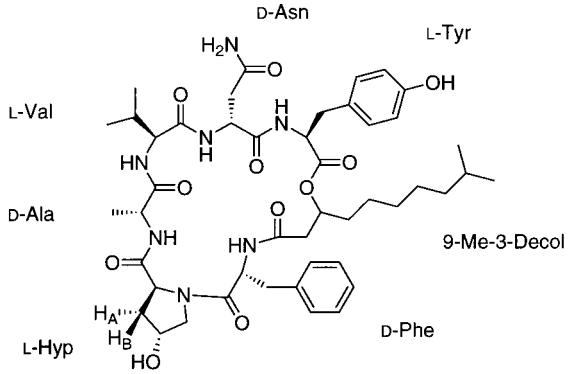
Kahalalide K (**1**), a new cyclic depsipeptide, was isolated from the Hawaiian green alga *Bryopsis* sp. Kahalalide K was determined to possess a new array of three L- and three D-amino acids, including a 3-hydroxy-9-methyldecanoic acid that had been previously reported in kahalalides E, H, and J.

Kahalalides A, B, F, and G, cyclic and acyclic depsipeptides, have been characterized from the green alga *Bryopsis* sp. In addition, kahalalides A−F, H, and J have been isolated from the sacoglossan mollusk *Elysia rufescens*.[1−3] Although most of the kahalalides lack significant bioactivity, kahalalide F shows potent bioactivity.[1,2] In this study, a new cyclic depsipeptide, kahalalide K (**1**) was isolated and characterized from the green alga, *Bryopsis* sp., along with the previously reported kahalalides A, B, and G. One of the characteristic properties of kahalalides is an isomethyl acyl group in their structures except kahalalide C. Kahalalide K (**1**) has a 3-hydroxy-9-methyldecanoic acid moiety that has also been identified in kahalalides E, H, and J.



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Kahalalide K was isolated from *Bryopsis* sp. (2.0 kg, wet wt), which was collected at Wai'anae Boat Harbor, O'ahu, Hawaii. The alga was extracted with acetone and methanol. The combined extracts were partitioned between hexane and MeOH−H_2O (8:2). The MeOH−H_2O (8:2) fraction was subjected to Si gel chromatography followed by reversed-phase HPLC, which yielded kahalalide K (**1**, 0.5 mg, 2.5 × 10^{-5}% based on wet wt).

NMR experiments were performed in deuterated pyridine solution to enhance peak separation; however, peak broadening was observed. Examination of ^{1}H NMR spectra suggested kahalalide K was a peptide with amide proton signals at 7.9−10.8 ppm. Kahalalide K contained Ala, which showed correlation in TOCSY spectra at 9.82 ppm (Ala NH, 1H), 4.95 ppm (Ala α, 1H), and 1.52 ppm (Ala β, 3H) and Val, whose signals were evident at 8.72 ppm (Val NH, 1H), 4.87 ppm (Val α, 1H), 2.26 ppm (Val β, 1H), 1.13 ppm (Val γ, 3H), and 1.16 ppm (Val γ, 3H). The ^{1}H NMR spectrum indicated the presence of a phenol moiety at 7.27 ppm (2H, d, J = 8.3 Hz) and 7.14 ppm (2H, d, J = 8.3 Hz) and a phenyl moiety at 7.62 ppm (2H, d, J = 7.5 Hz), 7.31 ppm (2H, t, J = 7.5 Hz), and 7.24 ppm (1H, t, J = 7.5 Hz). TOCSY spectra showed three AMX-type spin systems of amino acid residues. NOESY cross-peak between 7.62 and 3.70 ppm confirmed that the first AMX spin system [8.20 ppm (Phe NH, 1H), 5.56 ppm (Phe α, 1H), 3.28 ppm (Phe β-H_A, 1H), and 3.70 ppm (Phe β-H_B, 1H)] was phenylalanine. In the HMBC spectrum, correlation between the proton signal at 7.27 ppm (Tyr 2″ and 6″, 2H) and the carbon signal at 38.2 ppm (Tyr β-C) was observed. Conversely, HSQC-editing[4] spectrum showed the correlation between 38.2 ppm (Tyr β-C) and 3.27 ppm (Tyr β-H_A)/3.42 ppm (Tyr β-H_B). Thus, the second AMX spin system [10.61 ppm (Tyr NH, 1H), 5.29 ppm (Tyr α, 1H), 3.27 ppm (Tyr β-H_A, 1H), and 3.42 ppm (Tyr β-H_B, 1H)] was concluded to be tyrosine. Because the existence of an −NH_2 in the molecule was observed in the ^{1}H NMR spectrum at 8.43 and 7.92 ppm, the third AMX spin system [9.04 ppm (Asn NH, 1H), 5.86 ppm (Asn α, 1H), 3.04 ppm (Asn β-H_A, 1H), and 3.39 ppm (Asn β-H_B, 1H)] was suggested to be asparagine. However, no correlation between amino protons and Asn β-C or Asn β-$H_{A,B}$ could be observed by HMBC or NOESY. Finally, the existence of Asn was confirmed by MS/MS, resulting in acyclic kahalalide K due to hydrolysis (**2**, Figure 1). The last amino acid residue was 4-hydroxyproline (Hyp), which had a oxymethine at 4.82 ppm (Hyp γ, 1H), two methylenes, and no amide proton. The relative stereochemistry of Hyp could be determined from a NOESY spectrum. The cross-peaks 5.06 ppm (Hyp α, 1H)/2.46 ppm (Hyp β, H_A) and 4.82 ppm (Hyp γ, 1H)/2.51 ppm (Hyp β, H_B) appeared stronger than the cross-peaks Hyp α-H/Hyp β-H_B and Hyp γ-H/Hyp β-H_A. The NOESY results revealed that the stereochemistry between Hyp α-H and Hyp γ-H was trans.

The existence of a 3-hydroxy-9-methyldecanoic ester moiety[5,6] was mainly elucidated by the interpretation of FABMS and 2D NMR spectra. Connectivity between amino acids and 3-hydroxy-9-methyldecanoic acid was elucidated by the NOESY experiment. The correlation of a fatty acid with the amino acids was inconclusive from the HMBC spectrum, as the chemical shifts of the amide and ester carbonyl carbons were too crowded for identification. Sequential NOESY correlations from the NH protons to neighboring α protons were seen between 10.61 ppm (Tyr

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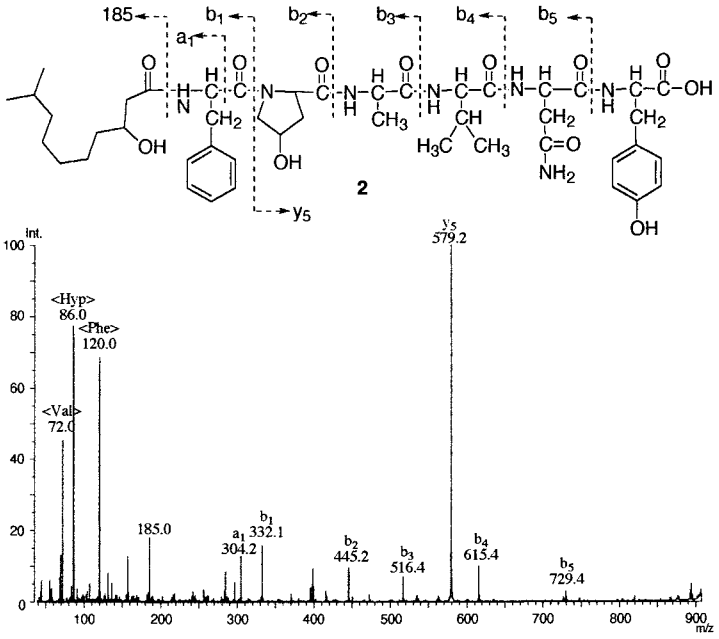

Figure 1. Positive FABMS/MS spectrum of acyclic kahalalide K (**2**), <amino acid> denotes the immonium ion.

NH, 1H)/5.86 ppm (Asn α, 1H), 9.82 ppm (Ala NH, 1H)/5.06 ppm (Hyp α, 1H), 9.04 ppm (Asn NH, 1H)/4.87 ppm (Val α, 1H), and 8.20 ppm (Phe NH, 1H)/2.91, 2.63 ppm (H-2, 2H). Other NOESY correlations were seen between 5.56 ppm (Phe α, 1H)/4.01, 4.18 ppm (Hyp δ, 2H) and 5.29 ppm (Tyr α, 1H)/5.16 ppm (H-3, 1H). The molecular formula of kahalalide K was determined from the positive mode HRFABMS, which showed $C_{46}H_{66}N_7O_{11}$ as $[M + H]^+$. In general, MS/MS is a powerful method to elucidate the sequence of amino acids in a peptide; however, the MS/MS spectrum of kahalalide K as a cyclic peptide was ambiguous. Thus, the compound was hydrolyzed under basic conditions to yield a linear product (**2**) for MS/MS measurement. In the MS/MS spectrum of the acyclic kahalalide K (**2**), b_{1-5} ions[7] clearly showed the sequence of amino acids and a fatty acid (Figure 1).

The absolute stereochemistry of the amino acids in kahalalide K was determined by Marfey's method[8] (see Experimental Section). Determination of the absolute stereochemistry of 3-hydroxy-9-methyldecanoic moiety was hampered by the limited amount of sample.

Kahalalide K (**1**), a new cyclic depsipeptide, along with kahalalides A, B, and G, was obtained as a constituent of *Bryopsis* sp. Kahalalide K (**1**) (100 μg/mL) showed no cytotoxic effects against mouse neuroblastoma (CCL131). In addition, *Gambierdiscus toxicus*, an epiphytic dinoflagellate, exposed to 100 μg/mL kahalalide K (**1**) displayed no changes in growth or morphology.[9]

Bryopsis sp. is a common green alga found in Hawaiian coastal water. In this study, *Bryopsis* sp. was collected at Wai'anae Boat Harbor, O'ahu, Hawaii. At Wai'anae Boat Harbor, we have observed that the epiphytic dinoflagellate, *G. toxicus* (a causative organism of ciguatera fish poisoning)[10] prefers to grow on *Bryopsis* sp. despite the existence of other macroalgae. Shimizu et al. reported at Kane'ohe Bay, O'ahu, Hawaii, that *G. toxicus* is also associated with *Bryopsis* sp., but is in limited numbers when compared with other host macroalgae.[11] In a recent study, we have isolated a sulfoquinovosyl monoacylglycerol as a constituent of *Bryopsis* sp., which affects the physiology of *G. toxicus*.[12] It is uncertain whether *Bryopsis* sp. produces attractant and/or growth stimulating factors for *G. toxicus*.

Because kahalalides A, B, and G were obtained from the mollusk *Elysia rufescens* and *Bryopsis* sp., Hamann et al. suggested that the compounds isolated from *E. rufescens* originated from its algal diet (*Bryopsis* sp.).[2] Hamann et al. also reported that *Bryopsis* sp. which contained kahalalides was not contaminated by blue-green algae.[1] However, extensive epiphytic growth, including dinoflagellates, blue-green algae, and diatoms, were observed associated with *Bryopsis* sp. collected at Wai'anae Boat Harbor.[13] Whether kahalalides are produced by *Bryopsis* sp. is still unclear. Kulolides, molluscan cyclic depsipeptides with unique fatty acid moieties analogous to kahalalides, are believed to originate from blue-green algae (i.e., *Lyngbya majuscula*).[14,15] This suggests that the true biological origin of kahalalides may be epiphytic, such as microalgae and/or bacteria associated with *Bryopsis* sp.

Experimental Section

Instruments. UV spectra were recorded on a Shimadzu UV-250 spectrometer. NMR data were measured on a Bruker DMX-750 spectrometer. FABMS were obtained on a JEOL JMS−HX/HX110A spectrometer. Optical rotations were determined on a JASCO DIP-1000 instrument.

Extraction and Isolation. *Bryopsis* sp. (2.0 kg, wet wt) was collected at Wai'anae Boat Harbor, O'ahu, Hawaii, during July 1996. The voucher specimen is on file (reference #9607008) at Suntory Institute for Bioorganic Research. The alga was washed with fresh seawater at the collection site. The alga was quickly transferred to the University of Hawaii and extracted successively with Me_2CO and MeOH. The combined extracts were partitioned between hexane and MeOH−H_2O (8:2). The MeOH−H_2O (8:2) fraction was subjected to Si gel chromatography [CH_2Cl_2−MeOH (9:1), CH_2Cl_2−MeOH (7:3), CH_2Cl_2−MeOH (1:1), CH_2Cl_2−MeOH (3:7), MeOH]. The kahalalides were found in the CH_2Cl_2−MeOH (7:3) and CH_2Cl_2−MeOH (1:1) fractions. ODS HPLC (TSK-GEL ODS 120-T column, 7.8 × 300 mm, TOSOH, Japan) with an aqueous CH_3-CN 30−70% gradient yielded kahalalides A (11 mg, 5.5 × 10^{-4}%), B (8 mg, 4.0 × 10^{-4}%), G (3 mg, 1.5 × 10^{-4}%), and K (**1**, 0.5 mg, 2.5 × 10^{-5}%). Kahalalides A, B, and G were identified by comparing the results of HRFABMS and ^{1}H NMR experiments of the isolated compounds with their reported values.[2]

Kahalalide K (1): $[\alpha]^{29.8}_D$ +36.0° (*c* 2.5, MeOH); HRFABMS $C_{46}H_{66}N_7O_{11}$ as $[M + H]^+$ *m/z* 892.4868 (Δ +4.8 mmu); UV

Table 1. ^{1}H NMR[a,b] and ^{13}C NMR[c] Data for Kahalalide K (**1**) in Pyridine-d_5

unit	position	^{13}C NMR (ppm)[d]	^{1}H NMR (ppm)[e]	mult.	J (Hz)	NOESY
9Me3Decol	1	[f]				
	2	43.2	H_A 2.63	br d	14.2	Phe NH
			H_B 2.91	br d	14.2	Phe NH, Val β
	3	74.1	5.16	br s		Tyr α
	4					
	5	[g, h, i, or j]				
	6					
	7					
	8	41.0	1.08			
	9	29.8	1.46			
	10	24.5	0.85	d	6.6	
	11	24.5	0.84	d	6.4	
phenylalanine (Phe)	C═O	[f]				
	NH		8.20	br s		H-2
	α	55.0	5.56	br t	12.8	Hyp δ-$H_{A,B}$
	β	39.4	H_A 3.28	[k]		
			H_B 3.70	t	12.8	Phe NH, Phe 2′, Phe 6′
	1′	140.0				
	2′, 6′	131.9	7.62	d	7.5	Phe β-H_B, Tyr α
	3′, 5′	130.2	7.31	t	7.5	
	4′	128.7	7.24	t	7.5	
hydroxyproline (Hyp)	C═O	[f]				
	α	62.4	5.06	t	8.0	Hyp β-$H_{A,B}$, Ala NH
	β	40.0	H_A 2.46	br dd	13.3, 8.0	Hyp α, Hyp γ
			H_B 2.51	br dd	13.3, 8.0	Hyp α, Hyp γ
	γ	71.5	4.82	br s		Hyp δ-$H_{A,B}$, Hyp β-$H_{A,B}$
	δ	58.5	H_A 4.01	br d	10.1	Hyp γ, Phe α, Phe β-H_A
			H_B 4.18	dd	10.1, 3.7	Hyp γ, Phe α
alanine (Ala)	C═O	[f]				
	NH		9.82	br d	6.1	Hyp α, Ala β
	α	51.3	4.95	br s		
	β	19.1	1.52	d	7.3	Ala NH
valine (Val)	C═O	[f]				
	NH		8.72	[l]		
	α	63.5	4.87	t	9.8	Asn NH
	β	33.9	2.26	br q	6.6	H-2, Asn NH
	γ	21.0	1.13	d	6.6	
	γ'	21.9	1.16	d	6.6	
asparagine (Asn)	C═O	[f]				
	NH		9.04	br d	6.0	Phe NH, Asn α, Val α, Val β
	α	52.3	5.86	q	6.0	Tyr NH, Asn NH, Asn β-$H_{A,B}$
	β	41.0	H_A 3.04	br dd	15.0, 6.0	Asn β-H_B, Asn α
			H_B 3.39	[m]		Asn β-H_A, Asn α
	γ	[f]				
	γ NH_2		H_A 8.43	s		γ NH_2-H_B
			H_B 7.92	s		γ NH_2-H_A
tyrosine (Tyr)	C═O	[f]				
	NH		10.61	br s		Asn α, Tyr α, Tyr β-$H_{A,B}$
	α	58.1	5.29	br q		H-3, Tyr β-$H_{A,B}$
	β	38.2	H_A 3.27	[k]		Tyr α, Tyr NH
			H_B 3.42	[m]		Tyr α, Tyr NH
	1″	129.1				
	2″, 6″	132.4	7.27	d	8.3	
	3″, 5″	117.8	7.14	d	8.3	
	4″	159.5				

[a] At 750 MHz. [b] Chemical shifts were determined using HSQC-editing and HMBC spectra. [c] At 188 MHz. [d] Reference of chemical shift was Py-d_5 as 149.8 ppm. [e] Reference of chemical shift was Py-d_5 as 8.72 ppm. [f] Chemical shifts were between 173−176.0 ppm. [g] ^{13}C = 27.9 ppm, ^{1}H = 0.98 ppm. [h] ^{13}C = 29.2 ppm, ^{1}H = 1.17 ppm. [i] ^{13}C = 31.4 ppm, ^{1}H = 1.03 ppm. [j] ^{13}C = 31.7 ppm, ^{1}H = 1.25 ppm. [k] These peaks were overlapped. [l] Peak was overlapped with Py-d_5. [m] Part of these peaks was overlapped.

(MeOH) λ_{max} (log ϵ) 206 (4.38), 226 (4.06), 277 (3.26); NMR data were shown in Table 1.

Acid Hydrolysis of Kahalalide K. Kahalalide K (0.1 mg) was hydrolyzed by heating the sample in a sealed vial at 120 °C for 22 h in 6 N HCl. The hydrolysate was dried with centrifugation in vacuo.

Base Hydrolysis of Kahalalide K. Kahalalide K (0.3 mg) was hydrolyzed with 0.1 N NaOH in aqueous MeOH (50 μL) at 37 °C for 90 min. After neutralization of the hydrolysate with 0.1 N HCl, ODS HPLC (Cosmosil 5C-18-AR−II, 10 × 250 mm) of the hydrolysate with an aqueous CH_3CN 0−60% gradient yielded hydrolyzed acyclic kahalalide K (**2**).

Marfey Analysis of Kahalalide K. Part of the acid hydrolysate was added to a 1% 1-fluoro-2,4-bis(nitrophenyl)-5-L-alanine amide (FDAA) solution in Me_2O (10 μL) and 2 N $NaHCO_3$ (20 μL). The sample was incubated for 60 min at 36 °C. The reaction mixture was neutralized with 1 N HCl (20 μL) after cooling to room temperature. The mixture was dried with centrifugation in vacuo. The residue was dissolved in 50 μL DMSO. The sample was analyzed by ODS HPLC with two different solvent systems (COSMOSIL 5C18-MS, solvent system I: CH_3CN 15−35% gradient in 18 mM triethylammonium phosphate buffer, pH 3.0; solvent system II: CH_3CN 30−35% gradient in 18 mM triethylammonium phosphate buffer, pH 3.0). Analysis with solvent system I showed that Hyp, Asn, and Phe possessed L-, D-, and D-configurations, respectively. The configuration of Ala, Val, and Tyr could not be determined with solvent system I due to poor peak resolution. Analysis with

solvent system II showed that Ala, Val, and Tyr possessed D-, L-, and L-configurations, respectively. Results were confirmed by co-injection with standard derivatives of the various authentic amino acids.

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Supporting Information Available: ^{1}H, HMBC, HSQC-editing, TOCSY, NOESY NMR spectra of **1** (6 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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