A New Depsipeptide from the Sacoglossan Mollusk *Elysia ornata* and the Green Alga *Bryopsis* Species[1]

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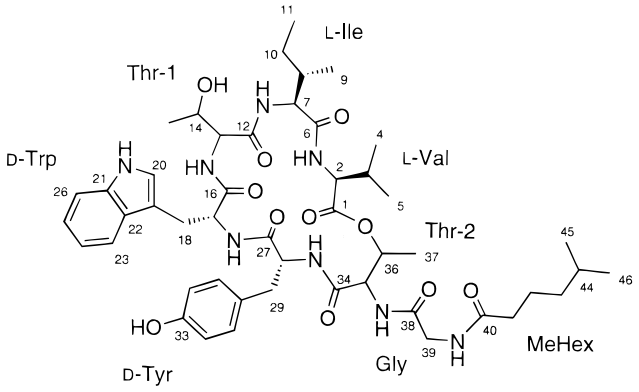
A new cyclic depsipeptide, kahalalide O (**1**), was isolated from the sacoglossan *Elysia ornata* and its algal diet *Bryopsis* sp. The structure was elucidated primarily by NMR and MS spectral methods, and the stereochemistry of the amino acid residues was determined by chiral HPLC and Marfey analyses. Unlike the related metabolite kahalalide F, which is in development as a potential anticancer agent, kahalalide O (**1**) was inactive in arresting the growth of P-388, A549, HT29, and MEL28 cancer cell lines in vitro.

Seven species of the sacoglossan molluscan genus *Elysia* (family Plakobranchidae) have been described from Hawaiian waters.[3] The observation that *E. degeneri* Ostergaard, 1955 feeds on the green algal genus *Udotea* prompted us to collect *Elysia* because diterpene aldehydes possessing feeding deterrent properties had been isolated from *Udotea* sp.[4] However, we failed to locate *E. degeneri*. Instead, while snorkeling off Diamond Head Beach in the Kahala district of Oahu, we found an abundant supply of *E. rufescens* (Pease, 1871) feeding on the green alga *Bryopsis* sp. This research has resulted in the characterization of six cyclic and two acyclic depsipeptides from the mollusk and the alga.[5−7] The largest cyclic peptide, kahalalide F, with 13 amino acids and one fatty acid (mol wt 1478), proved to be the only member with significant bioactivity.[8] It is currently in preclinical evaluation for its selective activity against lung and colon cancers.[9] It is worth noting that the acyclic analogue, kahalalide G, is biologically inactive.

While collecting *E. rufescens* for reisolation of kahalalide F, we observed another *Elysia* species, *E. ornata* Pease, 1860, which also was associated with the green alga *Bryopsis* sp. Investigation of *E. ornata* led to the isolation of one new compound, kahalalide O (**1**), which is the subject of this report. Subsequently, this compound was isolated, in substantially lower yield, from a population of *Bryopsis* sp. collected off the west coast of Oahu. With seven amino acids and one fatty acid, kahalalide O (**1**) is the second largest cyclic kahalalide so far and the only one with two aromatic amino acids, Trp and Tyr.

About 280 animals of *E. ornata* (900 g wet wt) were collected by snorkeling in February and March 1997, at low tide near Black Point, Oahu. Sequential extraction of the freeze-dried animals (122 g dry wt) with MeOH and MeOH−CH_2Cl_2 (1:1), partitioning of the extracts between CH_2Cl_2 and water, extracting the aqueous phase with 1-butanol, renewed partitioning of the combined organic phases between hexane and aqueous MeOH, and re-extraction of the aqueous MeOH phase with CH_2Cl_2 yielded 6.3 g of residue. Reversed-phase vacuum−liquid chromatography (VLC) of the yellow-brown CH_2Cl_2 residue furnished 353 mg of an aqueous MeOH fraction, which was subjected to two stages of reversed-phase HPLC procedures, resulting in the isolation of kahalalide O (**1**, 5.5 mg, 6.1×10^{-4}% based on wet wt) as a pale yellow amorphous powder.



1 Kahalalide O

Isolation of **1** from *Bryopsis* sp. proceeded by combining both the MeOH and acetone extracts of 2.5 kg of fresh alga. The aqueous MeOH-phase residue, which resulted from partitioning of the dried extract between hexane and MeOH−H_2O (8:2), was fractionated over silica. The fractions eluting with CH_2Cl_2−MeOH (7:3 and 1:1) were further fractionated by two steps of reversed-phase HPLC leading to the isolation of 0.8 mg of kahalalide O (**1**, 3.2×10^{-5}% based on wet wt).

The molecular formula on the basis of HRFABMS data is $C_{48}H_{68}N_8O_{11}$. It was corroborated by the ^{13}C NMR spectrum, which displayed signals for 48 carbons. The ^1H NMR spectrum in deuterated MeCN revealed seven NH proton signals between δ 6.63 and 8.42, pointing to seven amino acid residues. They were identified as Gly, Ile, Thr (2), Trp, Tyr, and Val by NMR spectral analyses.

The presence of 5-methylhexanoic acid (MeHex) was also indicated by the NMR data. Sequential COSY correlations were observed between the methylene signals at δ 2.23 (H-41), 1.55 (H-42), and 1.16 (H-43) and between the methine resonance at δ 1.38 (H-55) and two methyl doublets at δ 0.73 and 0.74 (H-45 and H-46). Although no clear COSY cross-peak was observed between H-43 and H-44 of MeHex, the connection between C-43 and C-44 was confirmed by HMBC correlations between protons at H-45 and/or H-46

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Table 1. NMR Assignments of Kahalalide O (**1**)[a]

residue	position	^{13}C	mult	^{1}H	mult (*J* value)	key HMBC correlations to C
Val	1	170.4	s			H-2, H-36, Val-NH[b]
	2	60.5	d	3.79	t (4.9)	
	NH			6.63	d (4.9)	
	3	30.3	d	1.88	m	
	4	18.8	q	0.65	d (6.7)	
	5	18.7	q	0.72	d (6.4)	
Ile	6	171.3	s			H-7, Val-NH
	7	60.9	d	4.02	br t (4.6)	
	NH			7.37	d (5.8)	
	8	36.6	d	2.00	m	
	9	16.4	q	0.95	d (7.0)	
	10	25.4	t	1.37	m	
				1.25	m	
	11	12.2	q	0.86	t (7.3)	
Thr-1	12	173.2	s			H-7, H-13, H-14, Ile-NH
	13	59.4	d	4.81	m	
	NH			7.40	d (8)[c]	
	14	70.3	d	3.77	m	
	15	20.2	q	1.11	d (6.1)	
Trp	16	173.2	s			H-17, H-18, Thr-1-NH[d]
	17	55.1	d	4.76	m	
	NH			8.24	d (9.2)	
	18	30.2	t	3.37	dd (15.0, 4.0)	
				3.07	dd (15.0, 10.4)	
	19	111.4	s			
	20	124.2	d	7.09	d (2.1)	
	NH			9.27	br s	
	21	137.6	s			
	22	128.3	s			
	23	119.3	d	7.58	d (7.9)	
	24	120.0	d	7.04	td (7.5, 0.9)	
	25	122.7	d	7.14	td (7.5, 0.9)	
	26	112.5	d	7.40	d (8)[c]	
Tyr	27	171.2	s			H-17, H-28, H-29, Trp-NH
	28	58.2	d	4.33	td (9.6, 6.7)	
	NH			7.81	d (10.1)	
	29	39.5	t	2.55	dd (13.6, 6.3)	
				2.50	dd (13.5, 9.1)	
	30	128.8	s			
	31,31′	131.3	d	6.67	d (8.2)	
	32,32′	115.9	d	6.48	d (8.5)	
	33	156.6	s			
Thr-2	34	168.9	s			H-35, Tyr-NH
	35	57.2	d	4.42	dd (9.1, 2.1)	
	NH			8.42	d (9.5)	
	36	70.6	d	5.48	qd (6.4, 2.1)	
	37	16.3	q	1.09	d (6.4)	
Gly	38	172.0	s			H-39, Thr-2-NH
	39	44.9	t	4.45	dd (15.0, 4.0)	
				3.52	dd (14.7, 7.0)	
	NH			7.72	dd (6.4, 3.7)	
5-MeHex	40	177.9	s			H-39, H-42, H-43, Gly-NH
	41	37.2	t	2.22	t (7.5)	
	42	25.2	t	1.55	m	
	43	38.9	t	1.16	m	H-45, H-46
	44	28.9	d	1.38	m	H-43, H-45, H-46
	45	22.7	q	0.73	d (4.9)	H-43
	46	22.6	q	0.74	d (6.4)	H-43

[a] In CD_3CN; proton spectrum referenced to residual $C\underline{H}D_2CN$ (δ 1.93); carbon spectrum referenced to $\underline{C}D_3CN$ (δ 1.3); spectra taken at 125 and 500 MHz for carbon and proton, respectively. Assignments based on ^{1}H, ^{13}C, DEPT, COSY, HMQC, and HMBC experiments. HMBC optimized for 7 Hz. Coupling values given in Hertz; chemical shift values given in parts per million. [b] Weak signal. [c] Signals overlap; coupling constant is approximate. [d] Cross peak observed may reflect 3-bond coupling to C-1 of Thr-1; however, 2-bond correlations are the only or the prominent correlations observed for all other NH protons in the molecule.

(δ 0.73−0.74) and carbons resonating at δ 38.9 (C-43) and 28.9 (C-44), while protons on C-43 correlated with carbons at δ 28.9 (C-44) and 22.7−22.8 (C-45 and/or C-46).

The sequence of the amino acids was determined by an HMBC experiment. The sequences Trp−Tyr−Thr-2−Gly−MeHex and Val−Ile were assigned based on correlations between the NH of each amino acid residue (beginning at the acid terminus) with the carbonyl carbon of the adjacent downstream residue (Table 1). The ester linkage between Val and Thr-2 was inferred by the cross-peak between H-36 (δ 5.48) and C-1 (δ 170.4). The carbonyl carbons of both Thr-1 and Trp resonate at δ 173.2, a signal that shows cross-peaks with the NH of both Ile (δ 7.37) and Thr-1 (δ 7.40). With the position of Trp firmly established, the only sequence consistent with these data is Ile−Thr-1−Trp.

The stereochemistry of the Ile, Thr (2), Tyr, and Val residues was determined by chiral HPLC analysis; the stereochemistry of Trp was determined by Marfey analysis.[10] These analyses indicated the presence of L-Ile, both L-Thr and D-*allo*-Thr, D-Trp, D-Tyr, and L-Val.

Unlike the related kahalalide F, which is in development as a potential anticancer drug, kahalalide O (**1**) is not significantly cytotoxic; it fails to inhibit the growth of P-388 (murine lymphoma), A549 (human lung carcinoma), HT29 (human colon carcinoma), and MEL28 (human melanoma) cancer cell lines in vitro at a concentration of 10 μg/mL.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a General Electric GN Omega 500 MHz spectrometer. The IR spectra were measured on a Perkin−Elmer 1600 FTIR; UV spectra were determined with a Hewlett−Packard 8452A diode array instrument. Optical rotation was determined with a JASCO DIP-370 digital polarimeter. FABMS data were acquired on a ZAB-SE4F mass spectrometer using NBA matrix with and without NaCl.

Animal Material. About 278 animals (994 g wet wt) were collected by snorkeling (−1 m) at low tide near Black Point, Oahu during February and March 1997. The freshly collected organisms were frozen and freeze-dried (122 g dry wt).

Plant Material. A sample (2.5 kg, wet wt) of a species belonging to the genus *Bryopsis* was collected at the Waianae Boat Harbor, Oahu, Hawaii, during July 1995. The collected sample is identical to voucher specimen #9507019 deposited at the Suntory Institute for Bioorganic Research.

Extraction and Isolation of Kahalalide O (1) from *E. ornata*. The freeze-dried animals were extracted successively with MeOH (750 mL, then 5 × 500 mL) and MeOH−CH_2Cl_2 (1:1, 7 × 500 mL). The extracts were combined (43.7 g) and partitioned between CH_2Cl_2 and water, followed by further partitioning of the aqueous layer with 1-BuOH. The two organic phases were combined, concentrated, and partitioned between hexane and MeOH−H_2O (9:1). The water concentration of the aqueous phase was adjusted to 60% prior to repartitioning with CH_2Cl_2, yielding 6.3 g of a CH_2Cl_2 phase residue, which was subjected to VLC over ODS using MeOH−H_2O mixtures. The fraction eluting with MeOH−H_2O (70:30, 353 mg) was fractionated by sequential reversed-phase HPLC (COSMOSIL 5C18-AR) using MeOH−H_2O−TFA (80:20:0.05) followed by MeCN−H_2O−TFA (43:57:0.05) to yield kahalalide O (**1**, 5.5 mg).

Extraction and Isolation of Kahalalide O (1) from *Bryopsis* sp. The fresh plant material was promptly extracted with acetone and MeOH. The combined extracts were partitioned between hexane and MeOH−H_2O (8:2). The aqueous MeOH phase was subjected to Si gel chromatography [mobile phases: CH_2Cl_2−MeOH (9:1, 7:3, 1:1, and 3:7) and MeOH]. The fractions eluting with CH_2Cl_2−MeOH (7:3 and 1:1) contained an unidentified peptide. Sequential reversed-phase HPLC (CAPCELL PAK C_{18} UG80, 20 × 250 mm; TSK-GEL ODS 120-T column, 7.8 × 300 mm) with a CH_3CN−H_2O gradient (30:70 to 80:20) yielded kahalalide O (**1**, 0.8 mg).

Kahalalide O (1): pale yellow amorphous solid; $[\alpha]^{28}_D$ −8.0° (*c* 0.26, MeOH); UV λ_{max} (MeOH) 222, 281 nm; IR ν_{max} (film on NaCl) 3363 br, 1731, 1681, 1667, 1660, 1651, 1643, 1633, 1620, 1614 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 933.5099 (MH^+, $C_{48}H_{69}N_8O_{11}$ requires 933.5085, Δ −1.4 mmu).

Chiral HPLC Analysis of Hydrolysate of 1. After hydrolysis of kahalalide O (**1**, 200 μg) in 6 N HCl at 109 °C for 18 h, the reaction mixture was dried under N_2 followed by further drying under vacuum. The hydrolyzed residues were eluted from a C_{18} Sep-Pak (Waters 20805) using MeOH−H_2O (20:80), dried under vacuum, and reconstituted with 100 μL of H_2O prior to HPLC analysis using a Chirex (D) Penicillamine column [Phenomenex 00G-3126-E0, 4.6 × 250 mm; detection: UV 245 nm; injected amount 7 nmol; mobile phase I, 1.9 mM $CuSO_4$ in MeCN−H_2O (5:95), flow rate 1.0 mL/min; mobile phase II, 2.0 mM $CuSO_4$ in H_2O, flow rate 1.2 mL/min]. The following standards coeluted with peaks from the hydrolysate of **1**: Gly, L-Ile, L-Thr, D-*allo*-Thr, d-Tyr, and L-Val. Mobile phase I elution times were as follows: Gly (6.0 min), L-*allo*-Ile (33.3 min), l-Ile (39.7 min), D-*allo*-Ile (42.7 min), D-Ile (51.3 min), L-Tyr (53.5 min), D-Tyr (63.1 min). Mobile phase II elution times were as follows: L-Thr (7.7 min), D-Thr (9.4 min), L-*allo*-Thr (10.8 min), d-*allo*-Thr (12.2 min), L-Val (22.7 min), and D-Val (38.5 min). Due to inconveniently long retention times for D- and L-Trp with the strongest recommended solvent systems, the stereochemistry of this residue was determined by Marfey analysis.

Marfey Analysis of Hydrolysate of 1. Compound **1** (100 μg) was dissolved in 6 N HCl (500 μL), hydrolyzed at 105 °C for 17 h, and dried under N_2. To DL- and L-Trp and the hydrolysate of **1** were added 50 μL of 0.1% FDAA solution in acetone and 100 μL of 0.1 N $NaHCO_3$. After heating at 80 °C for 3 min, the reaction mixtures were cooled to room temperature and neutralized with 50 μL of 0.2 N HCl. Prior to analysis, the reaction products were diluted with 100 μL of MeCN−H_2O−TFA (50:50:0.05).

The reaction products were analyzed by reversed-phased HPLC [COSMOSIL 5C_{18}-AR, MeCN−H_2O−TFA (37.5:62.5:0.05)]. The derivative of D-Trp coeluted with a derivative peak of the hydrolysate of **1**. Elution times were as follows: L-Trp (33.3 min) and D-Trp (42.7 min).

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