Kahalalides V–Y Isolated from a Hawaiian Collection of the Sacoglossan Mollusk *Elysia rufescens*

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Four new kahalalides, V (**1**), W (**2**), X (**3**), and Y (**4**), as well as six previously characterized kahalalides have been isolated from a two-year collection of the sacoglossan mollusk *Elysia rufescens*. Curiously, kahalalide B, previously isolated in high yield from *E. rufescens*, was found to be essentially absent from these collections despite identical collection sites and times with previous collections. In addition, kahalalide K, which to date has only been reported from *Bryopsis* sp., was found in this collection of *E. rufescens*, suggesting that the production of these metabolites could potentially be from a microbial association with the mollusk and algae, and this relationship is continuously evolving in response to changes in the environment and predation. The structures of new peptides have been established on the basis of extensive 1D and 2D NMR spectroscopic data analysis. Kahalalide V (**1**) was ascertained to be an acyclic derivative of kahalalide D (**5**), while kahalalide W (**2**) was determined to have a 4-hydroxy-L-proline residue instead of the proline in **5**. The arginine residue of kahalalide X (**3**), an acyclic derivative of kahalalide C, was determined to have an L configuration. Kahalalide Y (**4**) was found to have an L-proline residue instead of the hydroxyproline in kahalalide K. It is clear from this collection of *E. rufescens* that the discovery of new kahalalide-related metabolites is still highly feasible.

Sacoglossan mollusks are a group of marine invertebrates found within the subclass Opisthobranchia and have been extensively studied for their notable production of bioactive secondary metabolites.[1] They are considered to be specialists both in the manner in which they feed and the organisms that they feed upon. Many species are herbivores feeding primarily on green algae, from which they are able to sequester functioning chloroplasts. In some cases, these organelles are used as a source of photosynthetic energy, as shown by ^{14}C experiments establishing the conversion of carbonate to a variety of carbohydrates, which can then in turn be used to biosynthesize secondary metabolites, with the majority of these being associated with ecological functions, including chemical defense.[1,2]

Elysia rufescens is an orange-fringed, blue-green, soft-bodied marine mollusk in the sacoglossan family Elysiidae that has been studied extensively due to the fact that it has been demonstrated to sequester the bioactive depsipeptide kahalalide F (KF, **6**). The mollusk can be observed feeding on *Bryopsis* sp., from which KF has also been isolated.[3] Through the algal diet, *E. rufescens* can apparently accumulate KF as well as other minor compounds.[1] To date, 12 cyclic depsipeptides including kahalalides A–F,[3,4] K,[5] O,[6] P, Q,[7] R, and S[8] as well as three acyclic peptides, kahalalides G, H, and J,[9] have been previously isolated from either *Bryopsis* sp. or *E. rufescens*.

In previous studies, KF exhibited significant in vitro cytotoxic activity with selectivity for solid tumor cell lines including lung (A-549) and colon (HT29 and LOVO) cancers. In addition, KF displayed potent in vivo activity against prostate tumor cell lines (PC3 and DU145) by inhibiting 40–52% of growth.[10] Recently, a phase I clinical trial was completed for patients with prostate cancer by PharmaMar in Madrid, Spain, and revealed no cumulative toxicities associated with the administration of KF. Reports suggest that KF can be continuously administered for up to 20 months in patients showing clinical benefits and is currently being evaluated in phase II clinical trials for liver carcinoma, non-small-cell lung cancer (NSCLC), and melanoma.[11,12] KF is considered to have a unique mechanism of action when compared to other forms of chemotherapy both in clinical development and in commercial use. Previous reports have shown that KF targets the lysosomal membrane of tumor cells, with studies demonstrating that the drug induces oncosis, ultimately leading to cell death.[13,14] A recent study has suggested that the specificity of KF may involve protein or membrane interaction, based on the differential effects observed with KF-exposed HepG2 and PLC/PRF/5C cells.[15] Janmaat et al. demonstrated that KF sensitivity is correlated with ErbB3 expression levels and KF treatment induces down-regulation of ErbB3.[16] In addition, KF and its semisynthetic derivatives were found to have in vitro antifungal activity against various AIDS opportunistic infectious strains, which expands the therapeutic indications for this novel drug lead.[8,17] Due to the potential therapeutic value of KF, we have renewed investigations for kahalalide derivatives from *E. rufescens*. To date, little is known about the life cycle of *E. rufescens*. It can be speculated that the life span and growth rate of the species is rapid and the mollusk must reproduce and grow quickly due to the fact that the mollusk is not found during the month of January but can be found easily in the tens to hundreds of thousands during the months of March, April, and May at this location. To evaluate the bioactivity of KF and to prepare semisynthetic analogues, we collected around 18 kg (dry weight) of *E. rufescens* over the period of two years. In the course of the isolation of KF, four new peptides (**1**–**4**) and six known kahalalides (A, C−F, K) were obtained. We report here the isolation and structure determination of the new kahalalides.

Results and Discussion

The animal material was extracted with methylene chloride and subsequently with EtOH. The EtOH extract was fractionated by VLC on a Si gel column using an EtOAc−MeOH gradient system. Several kahalalide-containing fractions were identified by LCMS and ^1H NMR. Separation and purification of these fractions by repeated reversed-phase HPLC resulted in the isolation of four new peptides with six previously characterized kahalalides as pure products. The known kahalalides (A, C−F, K) were identified by comparison of their spectroscopic data with those previously reported in the literature.

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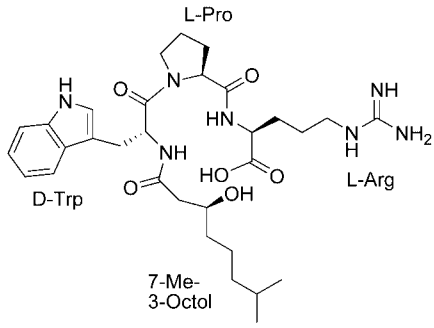

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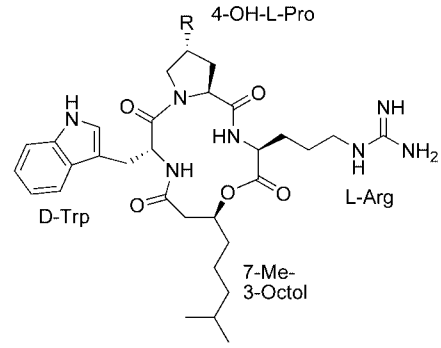

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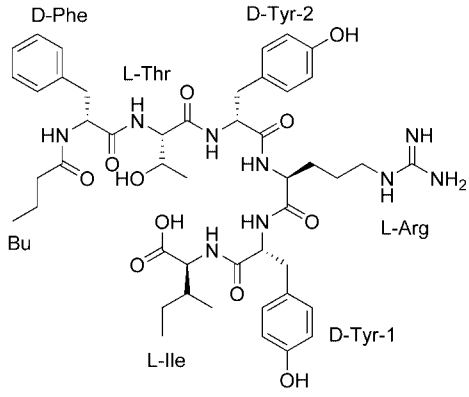

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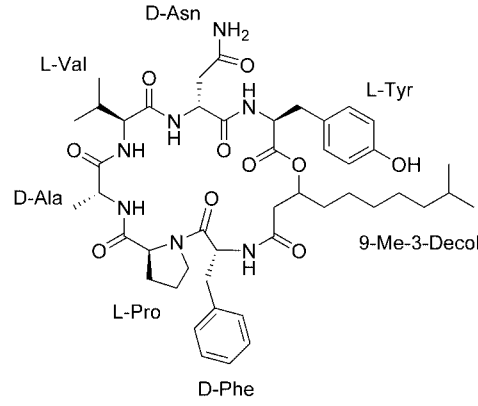

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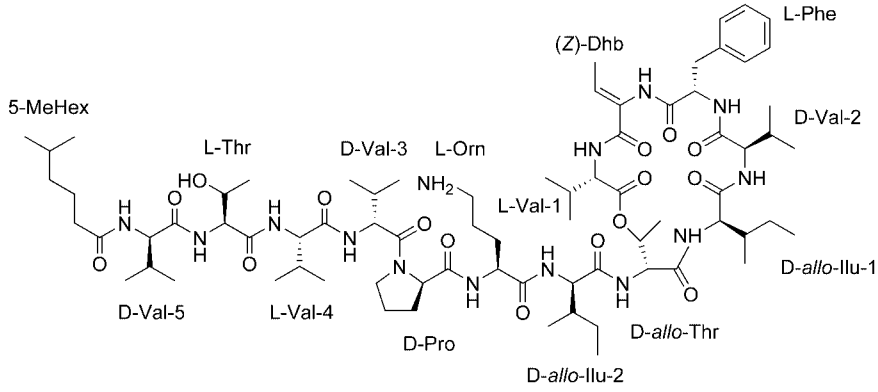

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Kahalalide V (**1**) was isolated as an amorphous solid and showed a quasimolecular ion peak at *m*/*z* 614.3563 $[M + H]^+$ in HRESIMS (positive mode), which suggested a molecular formula of $C_{31}H_{47}N_7O_6$ (calcd for $C_{31}H_{47}N_7O_6$, 614.3560), corroborated by ^{13}C NMR data that showed 31 carbons (see Table 1). The ^1H NMR spectrum of **1** showed a group of signals for α-protons at 4.1–4.6 ppm, and the ^{13}C NMR data showed the signals for four carbonyls, suggesting that **1** was a peptide possessing a fatty acid side chain. The ^1H and COSY NMR data of **1** indicated the presence of a 3-hydroxy-7-methyloctanoic acid moiety (7-Me-3-Octol), a common fatty acid component of the kahalalides,[4] which was validated by the interpretation of 2D NMR spectra. The absence of HMBC correlations between the hydroxyl methine of 7-Me-3-Octol and any of the carbonyl groups from the amino acids suggested that **1** was an acyclic structure. Compared to the molecular weight of kahalalide D (**5**), an increase of 18 mass units in **1** further supported that **1** could be a hydrolyzed (at the ester) acyclic derivative of **5**. Analysis of the NMR data including COSY, NOESY, HMQC, and HMBC spectra revealed that **1** is made up of three amino acids: arginine, proline, and tryptophan. The connectivity between vicinal amino acids could be demonstrated by a combination of ROESY and HMBC spectra. The HMBC experiment provided a correlation between the NH (δ 8.82) of Trp and the carbonyl (δ 172.4) of 7-Me-3-Octol, indicating the connectivity between Trp and the fatty acid. The HMBC correlations between the NH (δ 7.48) of Arg and the carbonyl (δ 171.1) of Pro were also observed. A ROESY experiment provided the Pro-Trp connection through the correlation of the α-proton (δ 4.55) of Trp and the protons (δ 3.70 and 2.70) of Pro. The ROESY experiment also showed a correlation between the NH (δ 8.82) of Trp and the methylene (δ 2.52 and 2.59) of 7-Me-3-Octol. The NH of Arg (δ 7.65) was correlated to the α-proton (δ 4.07) of Pro. The absolute configuration of the amino acids was determined by Marfey's analysis,[18] which indicated the presence of D-Trp, L-Pro, and L-Arg (see the Supporting Information). The absolute configuration at C-25 of the 7-Me-3-Octol residue of **1** was established by application of the modified Mosher's

Table 1. ^{1}H and ^{13}C NMR Data for Kahalalide V (**1**) in DMSO-d_6[a]

amino acid	no.	δ_C (mult.)[b]	δ_H (mult.)[b]	J (Hz)	key HMBC correlations	ROESY
Arg	1	172.5 (s)	NH 6.81, q	6.0	Arg-NH, Arg-2, 3	Arg-2, Pro-2
	2	52.0 (d)	4.10, q	6.0		Arg-NH, Arg-3, 4, 5
	3	28.0 (t)	1.71, m 1.59, m			Arg-2, 4
	4	24.5 (t)	1.45, m			H-3, H-5
	5	40.9 (t)	3.05, t NH 7.65, br s	5.5		Arg-2, 4, 5-NH
	6	157.5 (s)				
Pro	1	171.1 (s)			Pro-2, 3	
	2	61.2 (d)	4.07, dd	8.4, 4.2		Arg-NH, Pro-3
	3	29.5 (t)	1.86, m 1.64, m			Pro-2
	4	24.5 (t)	1.67, m			Pro-5
	5	46.6 (t)	3.70, m 2.70, m			Trp-2
Trp	1	171.9 (s)	NH 8.82, d	5.4	Trp-NH, Trp-2, 3	Trp-2, 3, 7-Me-3-Octol-2
	2	53.4 (d)	4.55, m		Trp-NH, Trp-3	Trp-NH, Trp-3, 5
	3	25.4 (t)	3.14, dd 2.98, dd	14.0, 9.2 14.0, 6.5	Trp-2, 5	Trp-NH, Trp-2, 5, 11
	4	109.2 (s)			Trp-5	
	5	124.5 (d)	7.17, s		Trp-3	Trp-3, 8
	6	136.8 (s)				
	7	127.5 (s)				
	8	111.9 (d)	7.31, d	7.6		Trp-5, 9
	9	121.5 (d)	7.05, t	7.6		Trp-8, 10
	10	118.9 (d)	6.96, t	7.6		Trp-9, 11
	11	118.6 (d)	7.46, d	7.6		Trp-3, 10
7-Me-3-Octol	1	172.4 (s)			Trp-NH, 7-Me-3-Octol-2	
	2	38.5 (t)	2.59, dd 2.52, dd	15.2, 4.8 15.2, 9.2	7-Me-3-Octol-3	Trp-NH, 7-Me-3-Octol-3
	3	72.5 (d)	5.03, m			7-Me-3-Octol-2, 4, 5
	4	34.1 (t)	1.66, m 1.57, m			
	5	22.9 (t)	1.26, m			
	6	38.0 (d)	1.18, m			7-Me-3-Octol-7, 8, 9
	7	27.8 (q)	1.50, m		7-Me-3-Octol-8, 9	7-Me-3-Octol-8, 9
	8, 9	22.9 (q)	0.83, d	6.4	7-Me-3-Octol-7	7-Me-3-Octol-8

[a] Assignments based on ^{1}H, ^{13}C, DEPT, COSY, HMQC, and HMBC NMR (100/400 MHz) experiments at room temperature. [b] Chemical shifts expressed in ppm referenced to the solvent peaks δ_H 2.48 and δ_C 40.99 for DMSO-d_6.

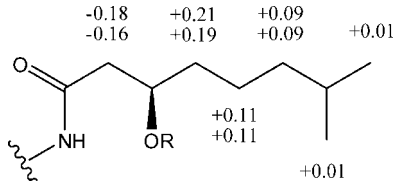

Figure 1. Selected $\Delta\delta$ values ($\delta_S - \delta_R$, ppm) for (*R*)- and (*S*)-MTPA esters of kahalalide V (**1**) (pyridine-d_5, 500 MHz).

method.[19] The secondary hydroxyl group of 7-Me-3-Octol was esterified with (*S*)- and (*R*)-isomers of 2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid chlorides (MTPACl, Figure 1). Analysis of the proton NMR signals from both the (*S*)- and (*R*)-MTPA esters [$\Delta\delta$ ($\delta_S - \delta_R$), H$_2$-2 $\Delta\delta$ –0.18 and –0.16, H$_2$-4 $\Delta\delta$ +0.21 and +0.19, H$_2$-5 $\Delta\delta$ +0.11, and H$_2$-6 $\Delta\delta$ +0.09] indicated that the absolute configuration at C-3 of 7-Me-3-Octol should be *R*.

Kahalalide W (**2**) was obtained as an amorphous solid, and the molecular formula of **2** was determined to be $C_{31}H_{45}N_7O_6$ by its positive HRESIMS signal measured at *m/z* 612.3452 $[M + H]^+$ (calcd for $C_{31}H_{45}N_7O_6H$, 612.3510). The ^{1}H and ^{13}C NMR data of **2** were similar to those of kahalalides V (**1**) and D (**5**). The presence of 7-Me-3-Octol was elucidated by the interpretation of 2D NMR data, which was shown to form an amide bond with the Trp residue. A difference of 16 mass units in **2** compared to **5** was attributed to the presence of a 4-hydroxyproline residue in **2** in place of Pro. The ^{1}H NMR spectrum of **2** reveals the OH signal at ca. δ 7.0 and the signals of the β- and γ-positions of Pro were shifted downfield relative to **5**. Analysis of HMQC, HMBC, ROESY, and DEPT NMR data, in combination with interpretation of MS experiments, was consistent with the presence of 4-hydroxyproline, and thus the amino acid sequence was assigned for **2**. Marfey's analysis showed that the amino acids of **2** have the following configuration: D-Trp, *trans*-4-hydroxy-L-Pro, and L-Arg. The relative configuration of the 4-hydroxyproline unit could be determined from the clear NOESY correlations between the α-proton H-2 and both H-3$_B$ and H-5$_A$, while proton H-4 shows correlations only to H-3$_A$ and H-5$_B$. This configuration was shown to be *trans* C-2 *S* and C-4 *R*.

Kahalalide X (**3**) was obtained as an amorphous powder, and a molecular formula was determined to be $C_{47}H_{66}N_9O_{11}$ by its positive HRFABMS measured for the peak at *m/z* 932.4863 $[M + H]^+$ (calcd for $C_{47}H_{66}N_9O_{11}$, 932.4882). The IR spectrum displayed absorptions at 1640 and 1540 cm^{-1} characteristic of amide carbonyl groups and showed a broad absorption band at 3290 cm^{-1} consistent with the presence of OH and NH functionalities. The primary structure of **3** was deduced by analysis of homo- and heteronuclear 2D NMR data. The ^{1}H NMR spectrum in DMSO-d_6 (see Table 3) displayed six signals between δ 7.72 and 8.72, which were assigned by 2D NMR spectroscopic data as amide NH protons. The ^{13}C NMR spectrum (see Table 3) contained five amide carbonyl signals near δ 170, confirming the peptide structure. Detailed analysis of COSY, TOCSY, HMBC, and HMQC data for compound **3** revealed the presence of six known amino acids, including two Tyr (Tyr-1 and -2), an Arg, an Ile, a Thr, and a Phe. Further analysis of the NMR data indicated that **3** contained a butyric acid (Bu) previously observed in kahalalide C. The Ile-Tyr-1 sequence was established with an HMBC correlation between the NH (δ 7.79) of Ile and the carbonyl (δ 170.6) of Tyr-1. Similarly, it was possible to establish the Tyr-1-Arg-Tyr-2-Thr sequence by an HMBC experiment utilizing the NH protons (δ 8.16, 7.38, and 7.62) and their connection to the carbonyl of the vicinal amino acid (δ 171.6, 171.6, and 166.1), respectively. The Thr-Phe sequence was established with an HMBC correlation between the α-proton (δ 4.29) of Thr and the carbonyl (δ 172.6) of Phe. The amino acid sequence of **3** was thus determined to be Bu-Phe-Thr-Tyr-Arg-Tyr-Ile. The absence of HMBC correlations between Thr and Ile revealed **3** as an acyclic derivative of kahalalide C, which was also consistent with the molecular formula. The absolute configuration of each amino acid was determined using Marfey's method[18] and determined to be D-Phe, L-Thr, D-Tyr, L-Arg, and L-Ile.

The molecular formula of kahalalide Y (**4**) was established as $C_{46}H_{66}N_7O_{10}$ on the basis of the HRESIMS data, *m/z* 876.4864.

Table 2. ^{1}H and ^{13}C NMR Data for Kahalalide W (**2**) in DMSO-d_6[a]

amino acid	no.	δ_C (mult.)[b]	δ_H (mult.)[b]	*J* (Hz)	key HMBC correlations	ROESY
Arg	1	171.8 (s)	NH 6.81, d	6.0	Arg-NH, Arg-2, 3	Arg-2, 3, Pro-2
	2	51.8 (d)	4.12, q	6.0		Arg-NH, 3, 4, 5
	3	27.9 (t)	1.69, m 1.59, m			Arg-2, 4
	4	24.5 (t)	1.45, m			Arg-3, 5
	5	40.6 (t)	3.04, q NH 7.65, br s	6.6		Arg-2, 4, 5-NH
	6	157.3 (s)				
4-OH-Pro	1	171.6 (s)			Pro-2, 3	
	2	61.4 (d)	4.08, dd	8.4, 4.2		Arg-NH, Pro-3, 4
	3	29.3 (t)	1.85, m 1.64, m			Pro-2, Pro-4
	4	71.9 (d)	4.48, m			Pro-3, 5
	5	47.1 (t)	3.69, m 2.75, m			Pro-4, Pro-2
Trp	1	172.2 (s)	NH 8.81		Trp-NH, Trp-1	Trp-2, 3, 7-Me-3-Octol-2
	2	54.2 (d)	4.57, m		Trp-NH	Trp-NH, Trp-3, 5
	3	25.3 (t)	3.14, dd 2.98, dd	14.0, 9.2 14.0, 6.4		Trp-NH, Trp-2, 5, 11
	4	109.2 (s)				
	5	124.3 (d)	7.18, s			Trp-3, 8
	6	136.6 (s)				
	7	126.7 (s)				
	8	111.3 (d)	7.29, d	7.6		Trp-5, 9
	9	120.9 (d)	7.05, t	7.6		Trp-8, 10
	10	118.3 (d)	6.97, t	7.6		Trp-9, 11
	11	118.4 (d)	7.47, d	7.6		Trp-2, 3, 10
7-Me-3-Octol	1	171.9 (s)			Trp-NH, 7-Me-3-Octol-2	
	2	38.4 (t)	2.60, dd 2.53, dd	15.6, 5.2 15.6, 9.2	7-Me-3-Octol-3	Trp-NH, 7-Me-3-Octol-3
	3	71.9 (d)	4.99, m			7-Me-3-Octol-4
	4	33.6 (t)	1.68, m 1.57, m			
	5	22.7 (t)	1.24, m			
	6	38.0 (d)	1.17, m			7-Me-3-Octol-7, 8, 9
	7	27.6 (q)	1.51, m		7-Me-3-Octol-8, 9	7-Me-3-Octol-8, 9
	8, 9	23.1 (q)	0.84, d	6.4	7-Me-3-Octol-7	7-Me-3-Octol-7

[a] Assignments based on ^{1}H, ^{13}C, DEPT, COSY, HMQC, and HMBC NMR (100/400 MHz) experiments at room temperature. [b] Chemical shifts expressed in ppm referenced to the solvent peaks δ_H 2.48 and δ_C 40.99 for DMSO-d_6.

The formula was corroborated by the ^{13}C NMR spectrum, which displayed signals for 46 carbons. Examination of the ^{1}H NMR spectrum suggested a peptide with aromatic and aliphatic residues. A group of signals for α-protons between δ 4.1 and 4.9 ppm implied a peptide of 5–6 amino acid residues. Another group of signals for methylene groups between δ 1.1 and 1.6 suggested the likely presence of a fatty acid. Examination of ^{1}H, COSY, and TOCSY NMR spectra suggested **4** consist of Ala [δ 7.69 (NH, 1H), 4.15 (Ala α, 1H), and 1.14 (Ala β, 3H)], Val [δ 7.30 (NH, 1H), 4.18 (Val α, 1H), 2.21 (Val β, 1H), 1.02 (Val γ, 3H), and 0.96 (Val γ, 3H)], and Asn [δ 8.97 (Asn NH, 1H), 8.41 and 7.89 (NH_2), 5.87 (Asn α, 1H), 3.06 (Asn β-Ha, 1H), and 3.36 (Asn β-Hb, 1H)]. In addition, the ^{1}H NMR spectrum showed a *p*-hydroxy phenyl moiety at δ 6.97 (2H, d, J = 8.0 Hz) and 6.59 (2H, d, J = 8.0 Hz) and a monosubstituted phenyl moiety at δ 7.21 (2H, d, J = 7.2 Hz), 7.18 (2H, t, J = 7.2 Hz), and 7.12 (1H, t, J = 7.2 Hz), indicating the presence of Tyr and Phe. The sequence of amino acids Phe-Pro-Ala-Val-Asn-Tyr was confirmed on the basis of correlations between the NH of each amino acid residue beginning at the acid terminus with the carbonyl carbon of the adjacent downstream (Table 4). The existence of a 3-hydroxy-9-methyldecanoate moiety was also determined by both the COSY and TOCSY NMR spectra. Connectivity between amino acids and 3-hydroxy-9-methyldecanoate was elucidated by the NOESY spectrum, in which correlations between the fatty acid and both Phe and Tyr were observed. These spectral features suggested that **4** retains similar structural features to kahalalide K[5] with the exception of a Pro residue replacing the 4-hydroxy-Pro of kahalalide K, which was in accordance with MS data showing 16 mass units less than kahalalide K. The absolute configuration of the amino acids in **4** was determined by Marfey's method.[18] However, determination of the absolute configuration of the 3-hydroxy-9-methyldecanoate moiety was hampered by a limited amount of sample.

Kahalalide D (**5**) did not elicit any other biological activities in our evaluations including cytotoxicity, antibacterial, antifungal, and antiparasitic assays. In preliminary tests using a rodent forced swim test model, **5** appeared to have some activity in the control of depression without noticeable toxicity. Further reevaluation of its antidepressant activity revealed that it does not yield consistent antidepressant-like actions in the forced swim test (see Supporting Information).

As shown in this study, the discovery of new metabolites related to the kahalalides is still possible. Kahalalide B was previously reported in high yield from *E. rufescens* a decade ago.[4] However, only trace amounts of kahalalide B were identified from these recent collections. In addition, kahalalide K, which to date has only been reported from *Bryopsis* sp., was identified from this collection of the snail. These findings suggest that the production of these metabolites could potentially be from an associated microorganism, and this relationship continuously evolves in response to changes in the environment and predation.

Experimental Section

General Experimental Procedures. Optical rotations were determined with a JASCO DIP 370 digital polarimeter. UV and IR spectra were respectively obtained using a Perkin-Elmer Lambda 3B UV/vis spectrophotometer and an AATI Mattson, Genesis Series FTIR. The ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 using a Varian NMR spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C nuclei. The HRMS spectra were measured using a Bruker micrOTOF fitted with an Agilent 1100 series HPLC and electrospray ionization source.

Biological Material. *E. rufescens* was collected from the waters of Kahala Bay near Black Point, Oahu, during the months of March, April, and May of 2003 and 2004. The seasonal growth of *E. rufescens* is highly dependent upon its algal diet of *Bryopsis pennata*, which in turn may be influenced by a variety of conditions including the amount of rainfall and the intensity of the surf around the island. *B. pennata* was found in significant quantities both attached and free-floating around the reef flat, mostly concentrated within the center and outer rocky area of the bay. Two different species of *Elysia* were observed grazing on the algae, *E. ornata* and *E. rufescens*, with the latter representing the more abundant animals (1:20). The mollusks were collected easily by hand and stored in plastic specimen bags. Both collections were

Table 3. ^{1}H and ^{13}C NMR Data for Kahalalide X (**3**) in DMSO-d_6[a]

amino acid	no.	δ_C (mult.)[b]	δ_H (mult.)[b]	J (Hz)	key HMBC correlations	ROESY
Ile	1	171.2 (s)	NH 7.79, q	7.2	Ile-2, 3	Ilu-2, Tyr-1–2
	2	56.8 (d)	4.15, t	6.9		
	3	36.9 (t)	1.79, m			Ilu-NH, Ilu-5
	4	15.1 (q)	0.65, d	6.8		
	5	24.6 (t)	1.18, m			Ilu-3
	6	11.3 (d)	0.81, d	7.2		
Tyr-1	1	170.6 (s)	NH 8.16, d	8.0	Tyr-1–2, 3	Tyr-1–2, Arg-2
	2	54.4 (d)	4.39, m			
	3	35.7 (t)	2.85, m 2.64, m			Tyr-1–5, 5′
	4	127.9 (s)			Tyr-1–2	
	5, 5′	130.1 (d)	7.02, d	8.4	Tyr-1–3	Tyr-1–3
	6, 6′	115.2 (d)	6.58, d	8.4	Tyr-1–5, 5′	
	7	156.3 (s)			Tyr-1–5, 5′	
Arg	1	171.6 (s)	NH 7.38, d	6.1	Tyr-1-NH, Arg-2	Tyr-2–2
	2	52.4 (d)	3.91, q	6.1		Arg-3, 4
	3	26.9 (t)	1.49, m 1.38, m			Arg-4, 5
	4	24.8 (t)	1.05, m			Arg-5-NH
	5	49.4 (t)	2.94, m NH 9.73, br s			
	6	157.1 (s)	NH 9.12, br s		Arg-5	Arg-5
Tyr-2	1	171.6 (s)	NH 7.62, d	7.1	Tyr-2–2, 3	Thr-2
	2	54.3 (d)	4.42, m			
	3	37.7 (t)	2.81, m 2.61, m			Tyr-2–5, 5′
	4	127.4 (s)				
	5, 5′	130.3 (d)	6.97, d	8.4		
	6, 6′	115.4 (d)	6.54, d	8.4		
	7	155.9 (s)			Tyr-2–5, 5′	
Thr	1	166.1 (s)	NH 8.41, d	8.1	Thr-2	Phe-NH
	2	56.9 (d)	4.29, d	10.2		Thr-4
	3	69.6 (d)	5.28, q	6.5		
	4	16.4 (q)	0.81, d	7.4		Thr-2
Phe	1	172.6 (s)	NH 8.45, d	7.8	Phe-2	Bu-2
	2	53.6(d)	4.69, m			
	3	33.1 (t)	2.84, d	6.5	Phe-5, 5′	Phe-5, 5′
	4	137.7 (s)				
	5, 5′	128.4 (d)	7.28, d	7.4		Phe-7
	6, 6′	128.8 (d)	7.21, t	7.4		
	7	125.9 (d)	7.14, t	7.4		Phe-5, 5′
Bu	1	172.9 (s)			Bu-2, 3	
	2	37.1 (t)	1.98, m			Bu-4
	3	18.6 (t)	1.47, sextet	6.2		
	4	13.6 (q)	0.81, t	7.1		Bu-2

[a] Assignments based on ^{1}H, ^{13}C, DEPT, COSY, HMQC, and HMBC NMR (100/400 MHz) experiments at room temperature. [b] Chemical shifts expressed in ppm referenced to the solvent peaks δ_H 2.48 and δ_C 40.99 for DMSO-d_6.

evaluated by comparing the total and individual mean weight of *E. rufescens* harvested each year in order to document the effect of harvesting on both the population biomass of the mollusk and the average size of individuals over time.

Extraction and Isolation. The freeze-dried *E. rufescens* samples (2003) were extracted three times with CH_2Cl_2 followed by EtOH. The CH_2Cl_2 extract was not processed further, as LCMS analysis indicated that it did not contain KF or related peptides. The EtOH extracts were combined and concentrated and then chromatographed using VLC on a silica column with a stepwise gradient of *n*-hexane, hexane−EtOAc (1:1), EtOAc, EtOAc−MeOH (9:1, 8:2, 7:3, 1:1), and MeOH. LCMS analysis of the fractions indicated that the EtOAc−MeOH (8:2 and 7:3) fractions contained kahalalide-related depsipeptides including KF. These two fractions were combined, concentrated, and further purified by column chromatography followed by HPLC using an RP C8 column and a gradient elution from H_2O−MeCN−TFA (80:20:0.1) and a NH_2 column using an EtOAc−MeOH gradient, yielding 10 peptides which were identified as kahalalide A (0.026 g, 6.5×10^{-6} % wet weight),[4] kahalalide C (0.120 g, 3.1×10^{-5} % wet weight),[4] kahalalide D (**5**, 0.400 g, 1.0×10^{-5} % wet weight),[4] kahalalide E (0.018 g, 4.5×10^{-6} % wet weight),[4] kahalalide F (**6**,1.8 g, 4.5×10^{-4} % wet weight),[3] kahalalide K (0.016 g, 4.0×10^{-6} % wet weight),[5] kahalalide V (**1**, 0.012 g, 3.1×10^{-6}% wet weight), kahalalide W (**2**, 0.008 g, 5.0×10^{-7}% wet weight), and kahalalide X (**3**, 0.005 g, 2.8×10^{-7}% wet weight). Final purification of each peptide was accomplished by HPLC on RP C8 MeCN−H_2O with 0–3% TFA.

Kahalalide V (1): Colorless, amorphous solid; $[\alpha]_D$ −19.5 (*c* 0.94, MeOH); UV (MeOH) λ_{max} nm (log ϵ) 208 (4.42), 218 (3.79), 285 (2.63) nm; IR 3314 (s, br), 2957 (s, br), 1739 (s), 1654 (s), 1535 (s), 1449 (s), 1185 (s), 1026 (s), 748 (s) cm^{-1}; for ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 614.3563 ([M + H]$^+$, calcd for $C_{31}H_{47}N_7O_6$ 614.3560).

Kahalalide W (2): Colorless, amorphous solid; $[\alpha]_D$ −48 (*c* 0.57, MeOH); UV (MeOH) λ_{max} nm (log ϵ) 226 (2.58); IR (film) 3284, 2920, 1643, 1537 cm^{-1}; for ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS *m*/*z*612.3452 ([M + H]$^+$, calcd for $C_{31}H_{45}N_7O_6$ 612.3510).

Kahalalide X (3): Colorless, amorphous solid; $[\alpha]_D$ +28 (*c* 0.39, MeOH); UV (MeOH) λ_{max} nm (log ϵ) 206 (3.89), 226sh (2.54) nm; IR 3261 (s, br), 2962 (s, br), 1672 (s), 1563 (s), 1456 (s), 1412 (s), 1251 (s) cm^{-1}; for ^{1}H and ^{13}C NMR data, see Table 3; HRESMS *m*/*z* 932.4863 ([M + H]$^+$ calcd for $C_{47}H_{66}N_9O_{11}$ 932.4882).

Kahalalide Y (4): Colorless, amorphous solid; $[\alpha]_D$ +39.0 (*c* 0.5, MeOH); UV (MeOH) λ_{max} nm (log ϵ) 206 (4.38), 226 (4.06), 277 (3.26); for ^{1}H and ^{13}C NMR data, see Table 4; HRESMS *m*/*z*876.4864 ([M + H]$^+$ calcd for $C_{46}H_{66}N_7O_{10}$ 876.4882).

(*R*)- and (*S*)-MTPA Esters of Kahalalide V (1). Compound **1** (2.5 mg) was dissolved in 1500 μL of pyridine and treated with 15 μL of (*R*)-(−)-MTPA chloride at room temperature for 24 h. After addition of MeOH (1800 μL), the solvent was removed in vacuo, and the residue was purified on Si gel (EtOAc−MeOH, 8:2) to afford the (*S*)-MTPA ester (1.5 mg) of **1a**. The (*R*)-MTPA ester (**1b**) of **1** was prepared with (*S*)-(−)-MTPA chloride according to the same procedure as described above. Selected signals (3-hydroxy-7-methyloctanoic acid) of the (*S*)-MTPA ester of **1**: ^{1}H NMR (C_5D_5N, 500 MHz) δ 2.63 (1H, m, H-2a), 2.57 (1H, m, H-2b), 1.71 (1H, m, H-4a), 1.61 (1H, m, H-4b), 1.31 (2H, m, H-5), 1.21 (2H, m, H-6), 1.29 (1H, m, H-7) and 0.86 (6H, d, J = 6.3 Hz, H-8 and 9). Selected signals (3-hydroxy-7-methyloctanoic acid) of the (*R*)-MTPA ester of **1**: ^{1}H NMR (C_5D_5N, 500 MHz) δ 2.45

Table 4. ^{1}H and ^{13}C NMR Data for Kahalalide Y (**4**) in DMSO-d_6[a]

amino acid	no.	δ_C (mult.)[b]	δ_H (mult.)[b]	J (Hz)	key HMBC correlations	ROESY
Phe	1	172.6 (s)	NH 7.61, d	9.4	Phe-2	9-Me-3-Decol-2
	2	55.6 (d)	5.04, m			
	3	39.4 (t)	2.96, dd	7.3, 3.0	Phe-5	Phe-5
	4	137.7 (s)				
	5, 5′	130.4 (d)	7.18, d	7.2		Phe-3, 7
	6, 6′	128.8 (d)	7.21, t	7.2		
	7	126.4 (d)	7.12, t	7.2		Phe-5
Pro	1	171.1 (s)			Pro-2, 3	
	2	61.2 (d)	4.07, dd	8.6, 4.1		Ala-NH, Pro-4
	3	29.5 (t)	1.86, m 1.64, m			Pro-5
	4	24.5 (t)	1.67, m			
	5	46.6 (t)	3.70, m 2.68, m			Pro-2
Ala	1	171.2 (s)	NH 7.69, d	7.4		
	2	47.5 (d)	4.15, d	7.4	Ala-3	
	3	16.3 (q)	1.14, d	6.8		
Val	1	173.2 (s)	NH 7.30, d	7.9		
	2	61.3 (d)	4.18, m		Val-4, 5	Asn-NH
	3	31.6 (d)	2.21, m			Val-4, 5
	4	19.7 (q)	1.02, m		Val-3, 5	
	5	18.2 (q)	0.96, m		Val-3, 4	
Asn	1	172.9 (s)	NH 8.97, d	6.9	Asn-2	
	2	51.9 (d)	5.87, m			Tyr-NH
	3	41.4 (d)	3.06, br d 3.36, dd	14.6 14.6, 6.4	Asn-2	Asn-2, 4-NH_2
	4	171.2 (s)	NH_2 8.41, s 7.89,s			Asn-2
Tyr	1	170.5 (s)	NH 7.81, br s			Asn-2
	2	58.4 (d)	4.39, m			Tyr-3, 5, 5′
	3	37.8 (t)	2.76, m		Tyr-2, 5, 5′	Tyr-5, 5′, 6, 6′
	4	126.9 (s)				
	5, 5′	126.2 (d)	6.97, d	8.0	Tyr-6, 6′	Tyr-3
	6, 6′	116.4 (d)	6.59, d	8.0	Tyr-5, 5′	
	7	156.2 (s)			Tyr-5, 5′	
9-Me-3-Decol	1	171.4 (s)			9-Me-3-Decol-2, 3	
	2	39.4 (t)	2.61, dd 2.43, dd	14.2, 2.0 14.2, 4.0	9-Me-3-Decol-3	Phe NH, 9-Me-3-Decol-3, 4, 5
	3	71.3 (d)	4.95, m			9-Me-3-Decol-4, 5, 6
	4	34.2 (t)	1.18, m 1.07, m		9-Me-3-Decol-3, 5	9-Me-3-Decol-2, 3, 5
	5	24.7 (t)	1.27, m		9-Me-3-Decol-3, 4	
	6	38.4 (t)	1.21, m			
	7	28.1 (t)	1.57, m			9-Me-3-Decol-9
	8	38.2 (t)	1.16, m			
	9	27.1 (d)	1.48, m		9-Me-3-Decol-10, 11	
	10, 11	22.1 (q)	0.84, d	6.6	9-Me-3-Decol-9	9-Me-3-Decol-8, 9

[a] Assignments based on ^{1}H, ^{13}C, DEPT, COSY, HMQC, and HMBC NMR (100/400 MHz) experiments at room temperature. [b] Chemical shifts expressed in ppm referenced to the solvent peaks δ_H 2.48 and δ_C 40.99 for DMSO-d_6.

(1H, m, H-2a), 2.41 (1H, m, H-2b), 1.93 (1H, m, H-4a), 1.82 (1H, m, H-4b), 1.42 (2H, m, H-5), 1.29 (2H, m, H-6), 1.28 (1H, m, H-7) and 0.86, 6H, d, J = 6.3 Hz, H-8 and 9).

Marfey's Analysis of 1–4. Kahalalides V−Y (**1**–**4**) (100 μg each) were respectively dissolved in 6 N HCl (500 μL) and degassed under vacuum for 1 min, then hydrolyzed at 105 °C for 20 h. The acid hydrolysate was dried under N_2, and 10 μL of a 0.1% FDAA solution in Me_2CO and 20 μL of 0.1 N $NaHCO_3$ were added, followed by heating at 40 °C for 1 h. After cooling to room temperature, the reaction mixture was neutralized with 20 μL of 0.2 M HCl. The mixture was dried with centrifugation in vacuo. The residue was dissolved in 1 mL of either DMSO or 50% aqueous MeCN, and the solution was analyzed by either HPLC with triethylammonium phosphate buffer, pH 3.0, and MeCN gradient solvent system or LCMS using a gradient from 20% to 60% aqueous MeCN (0.1% formic acid) over 50 min. Results were confirmed by co-injection with standard derivatives of the corresponding authentic amino acids.

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Note Added after ASAP Publication: The trivial names kahalalides T–W in the version posted on April 12, 2008, were changed to kahalalides V–Y in the version posted on April 22, 2008, to avoid duplication in the naming of this series.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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