Ashour, M. et al. Kahalalide derivatives from the Indian Sacoglossan mollusk Elysia grandifolia. J. Nat. Prod. 69, 1547–1553

ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · DECEMBER 2006

Impact Factor: 3.8 · DOI: 10.1021/np060172v · Source: PubMed

Kahalalide Derivatives from the Indian Sacoglossan Mollusk *Elysia grandifolia*

Mohamed Ashour,[†,‡] RuAngelie Edrada,[‡] Rainer Ebel,[‡] Victor Wray,[§] Wim Wätjen,[||] K. Padmakumar,[⊥] Werner E. G. Müller,[Δ] Wen Han Lin,[#] and Peter Proksch*[,‡]

Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, Geb. 26.23, 40225 Düsseldorf, Germany, Department of Structural Biology, Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany, Institut für Toxikologie, Heinrich-Heine-Universiät Düsseldorf, Universitätsstrasse 1, Geb. 22.21, 40225 Düsseldorf, Germany, Centre for Marine Biodiversity, Department of Aquatic Biology and Fisheries, University of Kerala, Trivandrum-695581, India, Institut für Physiologische Chemie und Pathobiochemie, Johannes-Gutenberg-Universität, Duesbergweg 6, 55128 Mainz, Germany, and National Research Laboratory of Natural and Biomimetic Drugs, Peking University, 100083 Beijing, People's Republic of China

Received April 18, 2006

Two new cyclic depsipeptide derivatives, kahalalides R (**1**) and S (**2**), together with two known congeners, kahalalides F (**3**) and D (**4**), were isolated from the Indian sacoglossan mollusk *Elysia grandifolia.* The structures of the new compounds were unambiguously established on the basis of NMR spectroscopic (^{1}H, ^{13}C, COSY, HMBC) and mass spectrometric (FABMS, ESIMS, MALDI-TOF/PSD) data, which also included Marfey amino acid analyses. The new derivative kahalalide R was found to exert comparable or even higher cytotoxicity than the potential drug candidate kahalalide F toward the MCF7 human mammary carcinoma cell line.

Sacoglossan mollusks of the genus *Elysia* have been intensively investigated for their biologically active natural products, which include diterpenoids,[1] polypropionates,[2] and depsipeptides.[3−11] *Elysia*-derived natural products show significant biological and pharmacological activities. For example, the polypropionates exhibited ichthyotoxicity,[2] while the diterpenoids and depsipeptides were shown to have fish deterrent activity and are used as a chemical defense by the sacoglossan.[1,9] The peptide derivatives, which carry the trivial name kahalalides, were further described to have antimalarial,[8a] anticancer,[4] antipsoriatic,[12a] antituberculosis,[8b] and antifungal activity.[12b] The depsipeptides were reported to be active against *Herpes simplex* type II (HSV II).[12c] The name kahalalide was derived from the mollusk's collection site at Black Point near the shores of Kahala District of Oahu Island in Hawaii. Kahalalides have been isolated from the sacoglossans *E. rufescens*[3−5] and *E. ornata*[6] (Plakobranchidae) as well as from their green algal diet, *Bryopsis* sp.[4−6,9−11] (Bryopsidaceae). The green algal diet, *Bryopsis* sp., has been found to yield kahalalides A,[4] B,[4] F,[4,6,9] G,[5] K,[10] O,[6] P,[11] and Q.[11] The kahalalide peptides consist of 3 to 13 amino acid units, ranging from a C_{31} tripeptide to a C_{75} tridecapeptide, with cyclic and linear components, the latter terminating in a saturated fatty acid moiety. Ten of these derivatives are cyclic depsipeptides, kahalalides A−F, K, and O−Q, while three analogues, kahalalides G, H, and J, are linear peptides. Kahalalide F (**3**) and its linear analogue kahalalide G are the only congeners that feature the atypical amino acid *Z*-dehydroaminobutyric acid (*Z*-Dhb).

Due to their biological importance, kahalalides A, B, and F have been chemically synthesized.[7,13] To date, kahalalide F is the only derivative reported to have significant activity toward solid tumor cell lines, including human colon and lung cancers, and against some pathogenic microorganisms that cause opportunistic infections in HIV/AIDS patients.[3] Kahalalide F has attracted the most attention and has been the subject of a patent application.[12a,c] Its mode of action and preclinical toxicity have also been studied,[14−17] and the compound is currently in phase II clinical trial as a potential anticancer agent.[18−20] It has been revealed that kahalalide F preferentially induces cell death via oncosis in the tumor cell.[17] Kahalalide F displays both in vitro and in vivo antitumor activities in various solid tumor models, including colon, breast, non-small-cell lung, and, in particular, prostate carcinoma. In vitro antiproliferative studies showed activity among certain prostate cancer cell lines (PC-3, DU-145, T-10, DHM, and RB), but no activity was found against the hormone-sensitive LnCAP prostate cancer cell line. In vivo models also confirmed selectivity and sensitivity of the prostate tumor xenograft derived from hormone-independent prostate cancer cell lines, PC-3 and DU-145. Furthermore, in vitro evaluation exhibited that this activity is selective but not restricted to prostate tumor cells.[20]

Chemical investigation of the Indian sacoglossan mollusk *Elysia grandifolia* Kelaart 1858[21] yielded two known analogues, kahalalides F (**3**) and D (**4**), along with two other new kahalalide derivatives, herein designated as kahalalides R (**1**) and S (**2**). This paper describes the isolation, structure elucidation, and biological activity of these isolated depsipeptides. Their structures were unambiguously elucidated by extensive NMR spectroscopic analyses and a combination of different mass spectrometric measurements, as well as through chemical derivatization and Marfey analysis. Bioassays showed that the novel compound **1** exerted equal or greater cytotoxic activity than kahalalide F (**3**).

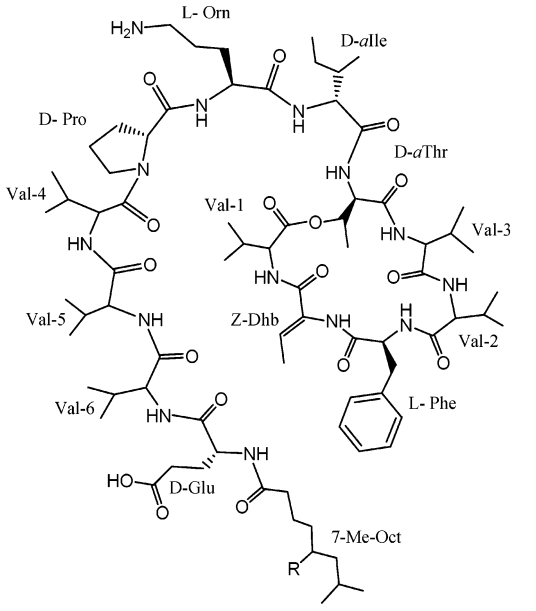
Results and Discussion

Sacoglossan mollusks *E. grandifolia*, which were observed to be feeding on the algae *Bryopsis*, were collected from the Gulf of Mannar and Palk Bay in India. Our interest in this mollusk was due to the strong biological activity of its crude MeOH extract. Organic extracts of the specialist herbivore *E. grandifolia* and its dietary green alga *B. plumosa* exhibited antimicrobial activity, cytotoxicity, feeding deterrence, and ichthyotoxicity.[22] Inspection of the LCMS chromatograms of the crude methanol extract suggested the presence of kahalalide derivatives, with positive pseudomolecular ion peaks at *m*/*z* 879.8, 914.9, 596.7, 835.5, 1478.8, 1494.3, and 1240.0 corresponding to the known kahalalides B, C, D, E, F, G, and J, respectively. The presence of two unidentified ion peaks at *m*/*z* 1520.2 and 1536.0 was noted. Subsequent to solvent partitioning of the crude organic extract

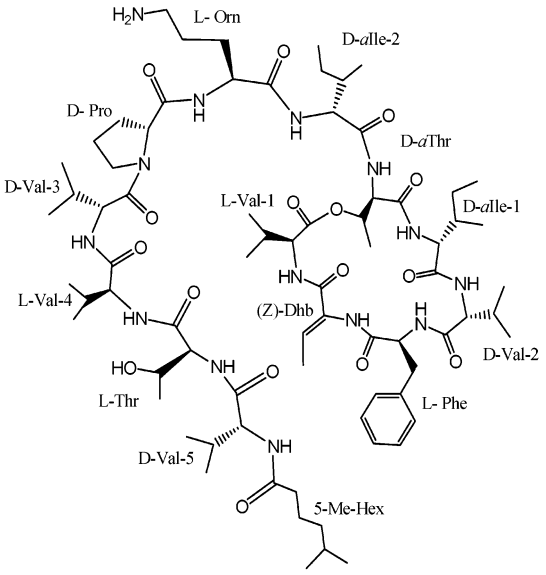
* Corresponding author. Tel: 0049/211-8114163. Fax: 0049/211-8111923. E-mail: proksch@uni-duesseldorf.de.
† Permanent address: Al-Azhar University, Faculty of Pharmacy, Nasr City, Cairo, Egypt.
‡ Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität Düsseldorf.
§ Helmholtz Centre for Infection Research.
|| Institut für Toxikologie, Heinrich-Heine-Universität Düsseldorf.
⊥ University of Kerala.
Δ Johannes-Gutenberg-Universität.
Peking University.

10.1021/np060172v CCC: $33.50
Published on Web 11/10/2006

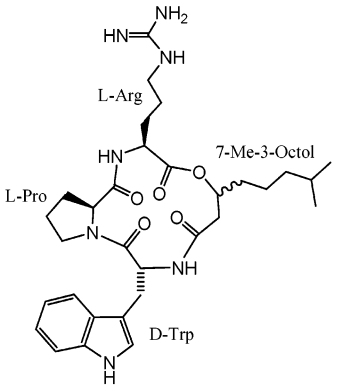
Chart 1



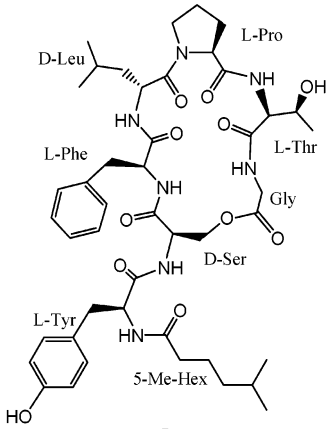
1: R = H
2: R = OH



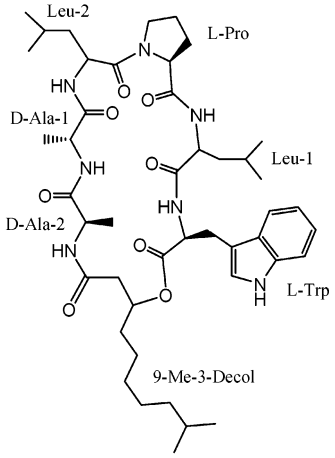
3



4



5



6

between hexane and aqueous methanol, kahalalide analogues were obtained from the polar methanol fraction. The presence of novel versus known congeners was determined by LC-DAD-ESIMS, and the obtained total ion chromatogram was compared to that of the methanol extract of the authentic Hawaiian sacoglossan *E. rufescens*, which had been previously shown to contain kahalalides B (**5**), D (**4**), E (**6**), and F (**3**) as its major compounds.[23] MS/MS spectra of the kahalalide derivatives present in the Hawaiian sacoglossan extract were particularly useful as references for the analysis of both known and new congeners in the Indian sacoglossan *E. grandifolia*. Structure elucidation of the known compounds was also confirmed by comparison of their ^{1}H and ^{13}C NMR data with those published in the literature.[3,4]

Kahalalide R (**1**) was obtained as a white, amorphous powder. The reflector mode MALDI-TOF mass spectrum of **1** performed with delayed extraction showed positive monoisotopic ion peaks at *m*/*z* 1520.2 $[M + H]^+$, 1542.2 $[M + Na]^+$, and 1557.2 $[M + K]^+$. In (+)-ESIMS, a pseudomolecular ion was detected at *m*/*z* 1520.8 $[M + H]^+$ that was compatible with the molecular formula $C_{77}H_{127}N_{14}O_{17}$. Inspection of the ^{1}H and ^{13}C NMR spectra of **1** revealed structural features similar to kahalalide F (**3**). The region between 6.50 and 9.70 ppm of the ^{1}H spectrum of **1** accounted for a similar number of 14 deshielded amide N*H* resonances. One of those is a singlet at δ 9.69 for the α,β-unsaturated amino acid *Z*-Dhb, and another is a broad 2H singlet at δ 7.69 for the terminal NH_2 of Orn. Compound **1** was also ninhydrin-positive, thus supporting the presence of the free amino group. The aromatic region indicated the presence of Phe. An apparent difference observed between the ^{1}H spectrum of **1** and **3** was the absence of a second methyl triplet at δ 1.02 that suggested the disappearance of one Ile unit in the structure of **1**. An additional carbonyl signal was observed in the ^{13}C NMR spectrum between 163.5 and 174.1 ppm. The DEPT spectrum also revealed the presence of 12 regular amino acid residues in the new analogue **1**, as indicated by the 12 α-carbon methine signals between 51.0 and 60.0 ppm. The DEPT spectrum showed 15 instead of 12 methylene carbons and the absence of an oxygenated methine carbon signal in the 65 to 75 ppm region, which indicated a deficit of one Thr unit that was replaced by another amino acid compared to kahalalide F. Similar to kahalalide F (**3**), the aromatic region of the ^{13}C NMR spectrum of **1** showed evidence for the presence of Phe and Dhb. The ^{13}C NMR and DEPT spectral data were in agreement with the molecular formula as determined by HRMS.

The COSY spectrum of **1** revealed 14 spin systems, 12 of which contained an amide N*H* doublet. The remaining two spin systems showed no correlations to any amide N*H* proton and were assigned

Table 1. ^{1}H and ^{13}C NMR Data of Kahalalide R (**1**) in DMSO-d_6

amino acid	no.	δ_C	δ_H		amino acid	no.	δ_C	δ_H	
Val-1	1	169.7	(N*H*) 6.73	(d, *J*=8.5 Hz)	Pro	1	172.6		
	2	60.2	3.85	(t, *J*= 9.0 Hz)		2	55.6	4.37	(m)
	3	30.1	1.38	(m)		3	29.7	2.01, 1.98	(m, m)
	4	16.5	0.62	(d, *J*=6.3 Hz)		4	27.2	1.78, 1.89	(m, m)
	5	19.2	0.60	(d, *J* = 6.3 Hz)		5	47.0	3.78, 3.52	(m, m)
(*Z*)-Dhb	1	163.5	(N*H*) 9.69	(s)	Val-4	1	172.6	(N*H*) 8.11	(d, *J*=8.5 Hz)
	2	130.4				2	55.9	4.26	(m)
	3	130.2	6.36	(q, *J*=7.0 Hz)		3	31.0	1.94	(m)
	4	12.5	1.28	(d, *J*=7.5 Hz)		4	22.8	0.84	(m)
						5	19.0	0.83	(m)
Phe	1	171.3	(N*H*) 8.78	(d, *J*=5.5 Hz)	Val-5	1	171.2	(N*H*) 7.89	(d, *J*=8.5 Hz)
	2	55.65	4.43	(q, *J*=6.5 Hz)		2	59.5	4.38	(m)
	3	36.1	2.94, 2.92	(m, m)		3	31.6	1.88	(m)
	4	137.0				4	19.3	0.82	(m)
	5,5'	128.5	7.28	(m)		5	19.6	0.83	(m)
	6,6'	129.5	7.29	(m)					
	7	126.7	7.25	(m)					
Val-2	1	172.6	7.61 (N*H*)	(d, *J*=8.5 Hz)	Val-6	1	171.2	(N*H*) 7.86	(d, *J*=8.5 Hz)
	2	55.4	4.45	(m)		2	51.89	4.37	(m)
	3	31.5	2.18	(m)		3	30.7	1.95	(m)
	4	19.5	0.80	(d, *J*=7.0 Hz)		4	22.2	0.84	(m)
	5	18.5	0.62	(d, *J*=6.5 Hz)		5	22.9	0.86	(m)
Val-3	1	170.0	(N*H*) 8.82	(d, *J*=8.5 Hz)	Glu	1	170.9	(N*H*) 7.93	(d, *J*=7.5 Hz)
	2	59.3	4.13	(m)		2	56.0	4.41	(m)
	3	31.5	1.94	(m)		3	28.0	1.59, 1.50	(m)
	4	18.6	0.81	(d, *J*=7.0 Hz)		4	38.5	2.75	(m, m)
	5	19.5	0.82	(d, *J*=6.5 Hz)		5	169.4	(O*H*) 7.71	(bs)
*a*Thr	1	168.7	(N*H*) 8.55	(d, *J*=8.0 Hz)	7-Me-Oct	1	172.5		
	2	56.4	4.50	(t, *J*=7.8 Hz)		2	35.1	2.11	(m)
	3	70.0	4.96	(m)		3	23.6	1.51	(m)
	4	17.3	1.08	(d, *J*=6.5 Hz)		4	29.5	1.28	(m)
*a*Ile	1	171.3	(N*H*) 7.87	(d, *J*=8.2 Hz)		5	29.5	1.25	(m)
	2	57.2	4.34	(m)		6	38.3	1.15, 1.10	(m)
	3	30.2	1.73	(m)		7	27.3	1.52	(m)
	4	14.6	1.21	(m)		8	22.5	0.80	*a*
	5	26.0	1.02	(t, *J*=6.5 Hz)		9	22.5	0.81	*a*
	6	11.8	0.82	(d, *J*=6.5 Hz)					
Orn	1	171.5	(N*H*) 7.93	(d, *J*=8.5 Hz)					
	2	51.1	4.49	(m)					
	3	30.9	1.69, 1.82	(m, m)					
	4	28.5	1.52	(m)					
	5	38.3	2.74	(m)					
			(NH_2) 7.72	(bs)					

[a] Resonance is underneath the methyl signals of Val and *a*Ile.

to Pro and a saturated fatty acid. The COSY spectrum indicated the presence of 7-methyloctanoic acid (7-Me-Oct). Sequential correlations of 7-Me-Oct were observed from the α-methylene signal at δ 2.11 to the subsequent methylenes at δ 1.51 (H_2-3), 1.28 (H_2-4), 1.25 (H_2-5), and 1.10/1.15 (H_2-6), terminating with an aliphatic methine proton at δ 1.52 (H-7) and two methyl functions at δ 0.80 and 0.81. In the ^{13}C and DEPT spectra of the latter, characteristic signals[23] appeared for C-8/9 at δ 22.5 (q), C-7 at δ 27.3 (d), and C-6 at δ 38.3 (t). This was unequivocally assigned by HMQC (see Table 1).

A TOCSY experiment corroborated the assignments obtained from the COSY spectrum. The TOCSY spectrum unambiguously resolved the amine- and α-proton resonances of each of the different 13 amino acid residues in the structure of the new depsipeptide congener **1**. Kahalalide R (**1**) was thus shown to contain six units of Val, one unit each of Phe, *a*Ile (*allo*-Ile), *a*Thr (*allo*-Thr), Orn, Pro, Glu, Dhb, and 7-Me-Oct. In **1**, Val and Glu units replaced Thr and Ile previously found in kahalalide F. The TOCSY spectrum allowed the overlapping 12 methyl doublets (J = 6.5 Hz) that belong to the six Val units to be explicitly assigned to their respective spin systems. The presence of Glu further explains the additional carbonyl signal observed in the ^{13}C NMR spectrum, while the extra two methylene carbons could be accounted for by the replacement of the 5-methylhexanoic acid (5-Me-Hex) in kahalalide F with 7-methyloctanoic acid (7-Me-Oct) in compound **1**.

HMBC and ROESY experiments established the connectivity and sequence of the amino acids in the peptide structure of **1** (Figure 1). The sequence 7-Me-Oct−Glu−Val-6−Val-5−Val-4, elucidated as fragment I, was established through the HMBC correlations of N*H* signals at δ 7.93, 7.86, 7.89, and 8.11 for Glu, Val-6, Val-5, and Val-4, respectively, to their neighboring vicinal (^2J) carbonyls of 7-Me-Oct, Glu, Val-6, and Val-5 at δ 172.5, 170.9, and 171.2, respectively. Connectivities for fragment II, Pro−Orn−*a*Ile−*a*Thr−Val-3−Val-2−Phe−*Z*-Dhb−Val-1, were similarly determined through HMBC correlations of the N*H* signals to their neighboring vicinal carbonyls. The cyclization of Val-1 to *a*Thr was confirmed by an HMBC cross-peak between the carboxyl signal at δ 169.7 for Val-1 and the characteristically deshielded β-proton of *a*Thr at δ 4.96. This ring closure was corroborated by a ROESY correlation of the β-proton of *a*Thr with the α-proton of Val-1 at δ 3.85. The connectivity of fragment I with II was established through the HMBC correlation of the δ-proton of Pro at δ 3.52 with the carbonyl of Val-4 at 172.6 ppm. This was in agreement with a ROESY cross-peak between the δ-proton of Pro at 3.78 ppm and the α-proton of Val-4 at 4.26 ppm.

The ROESY spectrum of compound **1** also supported the *Z* stereochemistry of the Dhb unit as shown by a correlation between the N*H* singlet at δ 9.69 and the methyl doublet at δ 1.28 (J = 6.93 Hz). Together with kahalalides F and G, the new analogues kahalalides R (**1**) and S (**2**) are the only derivatives that contain the relatively uncommon amino acid *Z*-Dhb. *Z*-Dhb was reported

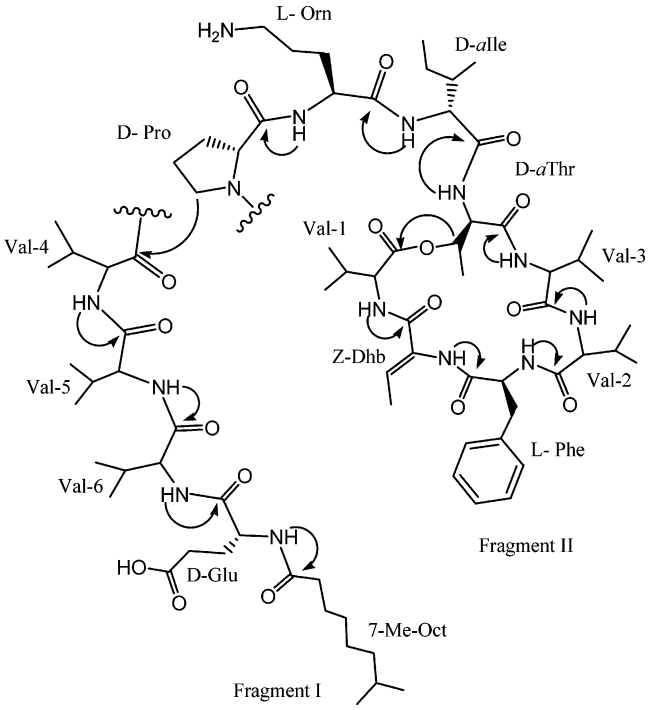


Figure 1. Key HMBC correlations of kahalalide R (**1**).

as a constituent of peptides isolated from the terrestrial blue-green algae[25] and from a herbivorous marine mollusk.[26]

The amino acid sequence of kahalalide R (**1**) was further confirmed by mass spectrometric methods involving both ESI[27] and MALDI-TOF-PSD[28−30] experiments. Similar results were obtained from the (+)ESIMS/MS spectrum, where fragment ion peaks were observed at *m*/*z* 1251.6, 1151.6, 1052.6, 952.9, 742.7, and 629.5 corresponding to [M − (7-Me-Oct−Glu)]$^+$, [M − (7-Me-Oct−Glu−Val-6)]$^+$, [M − (7-Me-Oct−Glu−Val-6−Val-5)]$^+$, [M − (7-Me-Oct−Glu−Val-6−Val-5−Val-4)]$^+$, [M − (7-Me-Oct−Glu−Val-6−Val-5−Val-4−Pro−Orn)]$^+$, and [M − (7-Me-Oct−Glu−Val-6−Val-5−Val-4−Pro−Orn−*a*Ile)]$^+$, respectively. However, the utility of the MS/MS fragmentation of **1** was limited to confirmation of the linear peptide side chain. In contrast, the amino acid sequence of the cyclic component of **1** was indicated in the MALDI-TOF-PSD spectrum (Table 3) by ion composition peaks at *m*/*z* 628.4 [Thr(-H)−Val-1−Dhb−Phe−Val-2−Val-3]$^+$, 330.2 [Val-1−Dhb−Phe]$^+$ or [Dhb−Phe−Val-2]$^+$, 247.1 [Val-2−Phe]$^+$, and 183.1 [Val-1−Dhb]$^+$. The terminal functional units 7-Me-Oct−Glu were also shown by peaks at *m*/*z* 270.1 [7-Me-Oct−Glu]$^+$ and 142.1 [7-Me-Oct]$^+$. The stereochemistry of the amino acids was determined by Marfey analysis,[31] which identified one mole unit each of D-Glu, D-Pro, L-Orn, D-*a*Ile, D-*a*Thr, and L-Phe. For the six Val units, Marfey analysis suggested the presence of both D and L isomers in compound **1**. The stereochemistry of the individual Val units could not, however, be unambiguously determined, as there are 3 mol each of Val in both the cyclic and linear fragments. This is not surprising, as the stereochemistry of Val-3 and Val-4 in kahalalide F has been long debated. The overall structure of kahalalide F was initially elucidated by Scheuer's group in 1993,[3] where Val-3 and Val-4 were respectively assigned the L and D stereochemistry.[27] The originally proposed structure was then synthesized by the groups of Albericio and Giralt[13] and showed differences in chromatographic and spectroscopic behavior between the synthesized and the natural peptide, suggesting that the stereochemistry of Val-3 and Val-4 should be reversed. Later, Rinehart's group[32,33] proved that the stereochemistry of Val-3 and Val-4 plays an important role in the activity of kahalalide F, since the depsipeptide with L-Val-3 and D-Val-4 in its structure was not active, while the molecule with D-Val-3 and L-Val-4 was active.

Kahalalide S (**2**) was obtained as a white, amorphous powder. The reflector mode MALDI-TOF mass spectrum of **2** performed with delayed extraction showed positive monoisotopic ion peaks at *m*/*z* 1536.0 [M + H]$^+$, 1558.0 [M + Na]$^+$, and 1574.0 [M + K]$^+$. In (+)-ESIMS a pseudomolecular ion was detected at *m*/*z* 1535.9 [M + H]$^+$, which was compatible with the molecular formula $C_{77}H_{127}N_{14}O_{18}$ as established by HRESIMS. Inspection of the ^{1}H and ^{13}C NMR spectra of **2** revealed that kahalalide S shared very similar structural features with **1** (Table 2). The amino acid residues of depsipeptide **2** were identical to those of **1**. An additional oxygenated methine carbon at δ 67.5 and the subsequent loss of one methylene carbon in the 20 to 25 ppm region were observed in **2**. COSY and TOCSY spectra of **2** revealed that the fatty acid residue in **2** was 5-hydroxy-7-methyloctanoic acid (7-Me-5-Octol). HMBC and ROESY spectra of **2** indicated an amino acid sequence identical to **1**. The ROESY spectra of **2** also verified the configuration of *Z*-Dhb in the depsipeptide. The connectivity of 7-Me-5-Octol to Glu was confirmed by the HMBC correlation of the Glu-N*H* resonance at δ 7.92 with the C-1 carbonyl signal at δ 172.5 of the 7-Me-5-Octol unit.

The amino acid sequence of depsipeptide **2** was further confirmed by ESIMS/MS fragmentation and MALDI-TOF-PSD (Table 4) experiments. The terminal unit, 7-Me-5-Octol-Glu, was evident from the peak at *m*/*z* 286.2 in the MALDI-TOF-PSD spectrum. Amino acid analysis using Marfey's method[31] revealed the same stereochemistry for each of the amino acids as determined in **1**. The same problem was encountered in **2** as in **1** regarding the assignment of the stereochemistry of each of the six Val units.

Except for the presence of glutamic acid (Glu) in kahalalides R (**1**) and S (**2**), the new derivatives have a set of amino acids similar to those of kahalalide F (**3**). The new analogues further differ from kahalalide F with regard to the occurrence of 7-Me-Oct or 7-Me-5-Octol instead of hexanoic acid.

To date, kahalalide R is the only congener with a significant bioactivity that is comparable to kahalalide F. This is also the first report of kahalalide congeners isolated from an *Elysia* species collected beyond the geographical area of the Pacific. *Elysia* species from the Caribbean have yielded only diterpenoids[1] and polypropionates,[2,3] although both species feed on a similar green algal diet, *Bryopsis*. Recently, the group of Hamann and Hill[34] described a kahalalide F-producing *Vibrio* associated with both the algae *Bryopsis* sp. and the sacoglossan mollusk *E. rufescens*. It was hypothesized that *E. rufescens* acquires kahalalide F-producing *Vibrio* from the surface of *Bryopsis* and maintains these microbes as symbionts.

The known derivatives, kahalalides B (**5**), D (**4**), E (**6**), and F (**3**), together with the new congeners, kahalalides R (**1**) and S (**2**), were assayed for their cytoxicity toward L1578Y, HeLa, PC12, H4IIE, and MCF7 cancer cell lines. Kahalalides F and R were found to be comparably cytotoxic toward MCF7 cells (Figure 2), with IC_{50} values of 0.22 ± 0.05 and 0.14 ± 0.04 μmol/L, respectively. Kahalalides S and E were less cytotoxic in MCF7 cells, with IC_{50} values of 3.55 ± 0.7 and 4.5 ± 0.49 μmol/L, respectively. Kahalalide R was cytotoxic toward the mouse lymphoma L1578Y cell line at an IC_{50} of 4.28 ± 0.03 nmol/mL, nearly identical to that of kahalalide F, with an IC_{50} of 4.26 ± 0.04 nmol/mL. The kahalalides including kahalalides F and R were found to be inactive toward HeLa, H4IIE, and PC12 cancer cell lines. These results suggest some selectivity and specificity of kahalalides F and R.

Furthermore, in an agar diffusion assay, kahalalide R at 5 μg/disk, showed strong antifungal activity against the plant pathogens *Cladosporium herbarum* and *C. cucumerinum*, with inhibition zones of 16 and 24 mm, respectively. These results were almost identical to kahalalide F (17 and 24 mm, respectively) and nystatin (19 and 39 mm, respectively). Kahalalides F and R were inactive toward Gram + and − bacteria. Kahalalide S was neither antibacterial nor antifungal.

Table 2. ^{1}H and ^{13}C NMR Data of Kahalalide S (**2**) in DMSO-d_6

amino acid	no.	δ_C	δ_H		amino acid	no.	δ_C	δ_H	
Val-1	1	167.9	(N*H*) 6.73	(d, *J*=9.0 Hz)	Pro	1	172.0		
	2	60.2	3.85	(t, *J*=9.0 Hz)		2	55.6	4.37	(m)
	3	30.1	1.38	(m)		3	29.7	2.01, 1.98	(m, m)
	4	16.5	0.62	(d, *J*=7.0 Hz)		4	24.8	1.78, 1.89	(m, m)
	5	19.2	0.60	(d, *J*=6.0 Hz)		5	47.2	3.78, 3.52	(m, m)
(*Z*)-Dhb	1	163.0	(N*H*) 9.69	(s)	Val-4	1	170.2	(N*H*) 8.11	(d, *J*=8.5 Hz)
	2	131.0				2	59.5	4.26	(m)
	3	130.1	6.36	(q, *J*=7.0 Hz)		3	31.0	1.94	(m)
	4	12.5	1.28	(d, *J*=7.5 Hz)		4	19.4	0.84	(m)
						5	19.0	0.83	(m)
Phe	1	171.3	(N*H*) 8.78	(d, *J*=5.5 Hz)	Val-5	1	171.3	(N*H*) 7.88	(d, *J*=8.5 Hz)
	2	55.6	4.43	(q, *J*=6.5 Hz)		2	59.5	4.32	(m)
	3	36.1	2.94	(m)		3	31.6	1.88	(m)
	4	137.0				4	19.3	0.82	(m)
	5,5′	128.5	7.28	(m)		5	19.6	0.83	(m)
	6,6′	129.5	7.29	(m)					
	7	126.7	7.25	(m)					
Val-2	1	172.8	(N*H*) 7.61	(d, *J*=8.5 Hz)	Val-6	1	171.2	(N*H*) 7.89	(d, *J*=8.5 Hz)
	2	55.4	4.45	(m)		2	51.9	4.24	(m)
	3	32.5	2.18	(m)		3	30.7	1.95	(m)
	4	19.5	0.62	(d, *J*=7.0 Hz)		4	22.2	0.84	(m)
	5	18.5	0.80	(d, *J*=6.5 Hz)		5	22.9	0.86	(m)
Val-3	1	170.0	(N*H*) 8.82	(d, *J* = 8.5 Hz)	Glu	1	170.9	(N*H*) 7.92	(d, *J*=7.5 Hz)
	2	59.3	4.13	(m)		2	56.0	4.21	(m)
	3	31.5	1.94	(m)		3	28.0	1.59, 1.50	(m)
	4	18.9	0.81	(d, *J*=7.0 Hz)		4	38.5	2.75, 3.29	(m)
	5	19.1	0.82	(d, *J*=6.5 Hz)		5	169.4	(O*H*) 7.72	(bs)
*a*Thr	1	168.5	(N*H*) 8.55	(d, *J*=8.0 Hz)	7-Me-	1	172.5		
	2	57.1	4.50	(t, *J*=7.8 Hz)	5-Octol	2	35.4	2.11	(m)
	3	70.0	4.96	(m)		3	23.6	1.61,1.52	(m)
	4	17.3	1.08	(d, *J*=6.5 Hz)		4	22.6	1.25	(m)
*a*Ile	1	171.3	(N*H*) 7.87	(d, *J*=8.2.Hz)		5	67.5	3.41	(m)
	2	57.27	4.34	(m)				(O*H*) 4.22	(d)
	3	30.2	1.73	(m)		6	38.3	1.12	(m)
	4	14.6	1.21	(m)		7	27.3	1.71	(m)
	5	26.0	1.02	(t, *J*=6.5 Hz)		8	23.4	0.80	*a*
	6	11.9	0.82	(d, *J*=6.5 Hz)		9	23.4	0.81	*a*
Orn	1	171.5	(N*H*) 7.93	(d, *J* = 8.5 Hz)					
	2	51.1	4.49	(m)					
	3	30.9	1.69, 1.82	(m, m)					
	4	28.5	1.5	(m)					
	5	38.3	2.74	(m)					
			(NH_2) 7.72	(bs)					

[a] Resonance is underneath the methyl signals of Val and *a*Ile.

Table 3. Important MALDI-TOF-PSD Fragment Ions of Kahalalide R (**1**)

ion composition	*m*/*z*
M−[7-Me-Oct−Glu]$^+$	1250.6
M−[7-Me-Oct−Glu−Val-6]$^+$	1150.7
M−[7-Me-Oct−Glu−Val-6−Val-5]$^+$	1051.6
M−[7-Me-Oct−Glu−Val-6−Val-5−Val-4]$^+$	952.6
M−[7-Me-Oct−Glu−Val-6−Val-5−Val-4−Pro]$^+$	855.5
M−[7-Me-Oct−Glu−Val-6−Val-5−Val-4−Pro−Orn]$^+$	741.4
Cyclo[Thr(-H)−Val-1−Dhb−Phe−Val-2−Val−3]$^+$	628.4
[Val-1−Dhb−Phe]$^+$ or [Dhb−Phe−Val-2]$^+$	330.2
[Val-2−Phe]$^+$	247.1
[Val-1−Dhb]$^+$	183.1
[Pro−Orn]$^+$	212.1
[7-Me-Oct−Glu]$^+$	270.1
[7-Me-Oct]$^+$	142.1

Table 4. Important Maldi-TOF-PSD Fragment Ions of Kahalalide S (**2**)

ion composition	*m*/*z*
M−[7-Me-5-Octol−Glu]$^+$	1250.6
M−[7-Me-5-Octol−Glu−Val-6]$^+$	1150.7
M−[7-Me-5-Octol−Glu−Val-6−Val-5]$^+$	1051.6
M−[7-Me-5-Octol−Glu−Val-6−Val-5−Val-4]$^+$	952.6
M−[7-Me-5-Octol−Glu−Val-6−Val-5−Val-4−Pro]$^+$	855.5
Ile + Cyclo[Thr(-H)−Val-1−Dhb−Phe−Val-2−Val-3]$^+$	741.4
Cyclo[Thr(-H)−Val-1−Dhb−Phe−Val-2−Val-3]$^+$	628.4
[Val-1−Dhb−Phe]$^+$ or [Dhb−Phe−Val-2]$^+$	330.2
[Val-2−Phe]$^+$	247.1
[Val-1−Dhb]$^+$	183.1
[Pro−Orn]$^+$	212.1
[7-Me-5-Octol−Glu]$^+$	286.2

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer-241 MC polarimeter. For HPLC analysis, samples were injected into an HPLC system with a photodiode-array detector (Dionex, Munich, Germany). Routine detection was at 254 nm in aqueous MeOH. The separation column (125 × 4 mm, i.d.) was prefilled with C_{18} (Knauer, Berlin, Germany). Semipreparative HPLC was performed on Merck-Hitachi Eurospher-100-C18, L-7100 pump, and L-7400 UV detector. TLC was performed on TLC plates precoated with Si 60 F_{254} (Merck, Darmstadt, Germany). The compounds were detected from their UV absorbance and with ninhydrin spray.

1D and 2D NMR spectra (chemical shifts in ppm) were recorded on Bruker Unity 500, Bruker ARX 400, and/or DMX 600 NMR spectrometers using standard Bruker software, and DMSO-d_6 was used as a solvent. ESI mass spectra were obtained on a Thermofinnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system equipped with a photodiode array detector. HRESIMS were determined on a Micromass S-Tof 2 mass spectrometer. MALDI-TOF-PSD spectra were obtained on a PerSeptive Biosystems Voyager-DE PRO MALDI-TOF mass spectrometer and on a Bruker Esquire 2000 LC-MS system equipped with an electrospray source. Sample application for MALDI-TOF-PSD MS was carried out directly on sample plates with a mixture of 1 μL of matrix (saturated 2,5-dihydroxybenzoic acid in 50%

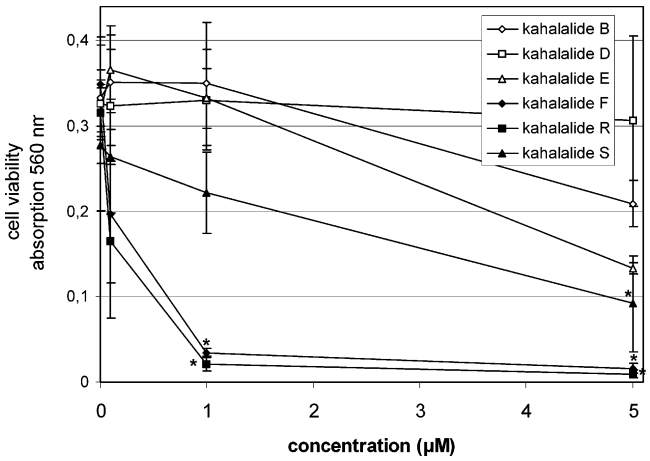


Figure 2. Cytotoxicity of kahalalides in MCF7 cells. MCF7 cells were incubated with different kahalalides for 24 h, then MTT reduction as marker of cell viability was measured. Results are expressed as absorption of reduced MTT (560 nm) ± SEM (n = 3), $p < 0.05$ vs control (DMSO).

acetontrile, 0.3% TFA) and 1 μL of a 50% MeOH solution containing about 0.2 μg of the sample.

Animal Material. *Elysia grandifolia* (family Elysiidae, order Sacoglossan) were collected by snorkeling from the Gulf of Mannar and Palk Bay, Rameswaram, India (9°15′ N; 79°15′ E) at 1 to 2 m depth. They were found feeding on the green alga *Bryopsis plumosa* (Hudson). Collected sacoglossans (17 mollusks; 77 g wet wt) were immediately preserved in MeOH and kept frozen at −80 °C until extraction work. The animal, analyzed for taxonomic characteristics, was less than 4 cm in length and had a translucent green color with a large parapodial margin. The parapodial margin had a very thin, black line and a submarginal yellow or orange band. The tip of the rhinophores had a black color. The body is covered with numerous black and white dots. It had a long renopericardial ridge with seven pairs of dorsal vessels as described by Eliot (1906). The figure of the teeth showed blunt tips and rather broad, denticulate blades as reported by O'Donoughe (1932) for specimens from the Gulf of Mannar. The sacoglossan was identified by Dr. Kathe Rose Jensen, National Natural History Museum, Copenhagen. Voucher specimens (EG-TMMP-3) were deposited in the Centre for Marine Diversity, University of Kerala, India.

Extraction and Isolation. Samples of *E. grandifolia* were extracted with methanol by maceration. The dried crude extract was then sent to our laboratory in May 2003 for further chemical investigation. The total MeOH extract (2.5 g) of *E. grandifolia* was partitioned between *n*-hexane and 90% aqueous MeOH, and the MeOH fraction was then concentrated under vacuum. The sea salt was precipitated, and the resulting mother liquor was concentrated and dried. The dried extract (550 mg) was chromatographed over Sephadex LH 20 with MeOH to give 17 fractions. The fractions were then subjected to HPLC-DAD and LC-MS analysis. Fractions 2 to 4 showed pseudomolecular ion peaks for kahalalides F (**3**), R (**1**), and S (**2**). Fractions 5 and 6 exhibited only the pseudomolecular ion peak for the major compound, kahalalide F (**3**). Fractions 7 to 12 indicated the presence of other kahalalide derivatives, which exhibited the positive pseudomolecular ion peaks *m*/*z* 879.8, 914.9, 1494.3, and 1240.0, corresponding to known kahalalides B, C, G, and J, respectively. The amounts of these respective fractions were too small to allow further isolation. Fraction 13 yielded kahalalide D (4, 3.4 mg).

The fractions that contained kahalalides F, R, and S were further purified by semipreparative HPLC. The separation was carried out by utilizing a gradient elution with MeOH/H_2O to yield pure compounds: kahalalide R (**1**, 20 mg), S (**2**, 8 mg), and F (**3**, 103 mg).

Kahalalide R (1): white, amorphous solid; $[\alpha]^{25}_D$ −18 (*c* 0.35 MeOH); UV (MeOH) λ_{max} 204 nm; ^{1}H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 1519.9460 $[M + H]^+$ (calcd for $C_{77}H_{127}N_{14}O_{17}$ 1519.9504).

Kahalalide S (2): white, amorphous solid; $[\alpha]^{25}_D$ −15 (*c* 0.33 MeOH); UV (MeOH) λ_{max} 203 nm; ^{1}H and ^{13}C NMR, see Table 2; HRESIMS *m*/*z* 1535.9450 $[M + H]^+$ (calcd for $C_{77}H_{127}N_{14}O_{18}$ 1535.9453).

Marfey Analysis.[30] A solution of 0.5 to 1.0 mg of peptide was hydrolyzed by treatment with 2 mL of 6 N HCl and left at 112 °C for 24 h in a sealed ampule. The solution was then concentrated using N_2 gas. Aqueous solutions of 50 mM of each of the amino acids were used. In an Eppendorf tube, 3.6 μmol of a 1% acetone solution of FDAA (*N*-(5-flouro-2,4-dinitrophenyl)-L-alaninamide) and 20 μmol of a 1 M solution of $NaHCO_3$ were added to 2.5 μmol of amino acid. The reaction mixture was heated with frequent shaking over a hot plate at 40 °C for 1 h and then cooled to RT. Then, 20 μmol of 2 M HCl and 1 mL of MeOH were added to the reaction mixture. The samples were analyzed by LC-MS, by which molecular weights and retention times were compared with those of standard amino acids.

Cytotoxicity Assay. Antiproliferative activity was examined against several cell lines, including MCF-7, PC12, HeLa, L1578Y, and H4IIE, and was determined by an MTT assay as described earlier.[34,35]

Antimicrobial Activity. The crude extracts and the pure compounds were tested for activity against the following standard strains: bacteria *Bacillus subtilis* DSM2109 and *Escherichia coli* DSM10290; the yeast *Sacharomyces cerevisiae* DSM1333; and two fungal strains, *Cladosporium herbarum* DSM62121 and *C. cucumerinum* DSM62122. The agar diffusion assay was performed according to the Bauer-Kirby test (DIN 58940). Prior to the assay, a few colonies (3 to 10) of the organism were subcultured in 4 mL of tryptose-soy broth (Sigma, FRG) and incubated for 2 to 5 h to produce a bacterial suspension of moderate cloudiness. The yeast and fungal strains were suspended in universal yeast broth and potato dextrose medium, respectively. The suspension was diluted with sterile saline solution to a density visually equivalent to that of a $BaSO_4$ standard. The standards were prepared by adding 0.5 mL of 1% $BaCl_2$ to 99.5 mL of 1% H_2SO_4 (0.36 N). The prepared bacterial broth was inoculated onto Müller-Hinton agar plates (Difco). *S. cerevisiae* and the pathogenic fungal strains was inoculated on YM and PD agar plates. For screening, aliquots of the test solution were applied to sterile filter-paper disks (5 mm diameter, Oxoid Ltd.) using a final disk loading concentration of 500 μg for the crude extract and 50 and 100 μg for the pure compounds. The impregnated disks were placed on agar plates previously seeded with the selected test organisms. The plates were incubated at 37 °C for 24 h, and antimicrobial activity was recorded as the clear zones of inhibition surrounding the disks. The diameter, which included the disk itself, was measured in mm.

Acknowledgment. This study was supported by a grant of the BMBF awarded to P.P. This work was possible through the Indo-German (DST-DAAD) bilateral research project (INT/DST-DAAD-P-61/2002) on "Chemical ecology of marine algae and specialist opisthobranchs" funded to K.P. and P.P. A scholarship granted and financed by the Egyptian Government (Ministry of Higher Education) to M.A. is gratefully acknowledged. We thank C. Kakoschke and B. Jaschok-Kentner for NMR data (HZI, Braunschweig), M. Nimtz for the HRMS (HZI, Braunschweig), and Anagnostec Gmbh for the MALDI-TOF-PSD measurements.

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

References and Notes

(1) Paul, V. J.; Van Alstyne, K. L. *J. Exp. Mar. Biol. Ecol.* **1988**, *119*, 15−29.
(2) (a) Gavagnin, M.; Spinella, A.; Castelluccio, F.; Cimino, G.; Marin, A. *J. Nat. Prod.* **1994**, *57*, *298*−304. (b) Cueto, M.; D'Croz, L.; Mate, J. L.; San-Martin, A.; Darias, J. *Org. Lett.* **2005**, *7*, 415−418.
(3) Hamann, M. T.; Scheuer, P. J. *J. Am. Chem. Soc.* **1993**, *115*, 5825−5826.
(4) Hamann, M. T.; Otto, C. S.; Scheuer, P. J.; Dunbar, D. C. *J. Org. Chem.* **1996**, *61*, 6594−6600.
(5) Goetz, G.; Nakao, Y.; Scheuer, P. J. *J. Nat. Prod.* **1997**, *60*, 562−567.
(6) Horgen, F. D.; de los Santos, D. B.; Goetz, G.; Sakamoto, B.; Kan, Y.; Nagai, H.; Scheuer, P. J. *J. Nat. Prod.* **2000**, *63*, 152−154.
(7) Bourel-Bonnet, L.; Rao, K. V.; Hamann, M. T.; Ganesan, A. *J. Med. Chem.* **2005**, *48*, 1330−1335.

(8) (a) El-Sayed, K. A.; Dunbar, D. C.; Goins, D. K.; Cordova, C. R.; Perry, T. L.; Wesson, K. J.; Sanders, S. C.; Janus, S. A.; Hamann, M. T. *J. Nat. Toxins* **1996**, *5*, 261−285. (b) El-Sayed, K. A.; Bartyzel, P.; Shen, X.; Perry, T. L.; Zjawiony, J. K.; Hamann, M. T. *Tetrahedron* **2000**, *56*, 949−953.
(9) Becerro, M. A.; Goetz, G.; Paul, V. J., Scheuer, P. J. *J. Chem. Ecol.* **2001**, *27*, 2287−2299.
(10) Kan, Y.; Fujita, T.; Sakamoto, B.; Hokama, Y.; Nagai, H. *J. Nat. Prod.* **1999**, *62*, 1169−1172.
(11) Dmitrenok, A.; Iwashita, T.; Nakajima, Terumi; Sakamoto, B.; Namikoshi, M.; Nagai, H. *Tetrahedron* **2006**, *62*, 1301−1308.
(12) (a) Izquierdo D.; Miguel A. Use of kahalalide compounds for the manufacture of a medicament for the treatment of psoriasis. PCT Int. Appl. 2004, 23 pp. CODEN: PIXXD2 WO 2004075910. (b) Donia M.; Hamann M. T. *Lancet Infect. Dis*. **2003**, *3*, 338−348. (c) Scheuer, P. J.; Hamann, M. T.; Gravalos, D. G. Kahalalide F or salts of this sacoglossan peptide in treatment of tumors and viral infections in mammals. 2000, 5 pp. CODEN: USXXAM US 6011010.
(13) (a) López-Maciá, A.; Jiménez, J. C.; Royo, M.; Giralt, E.; Albericio, F. *J. Am. Chem. Soc.* **2001**, *123*, *11398*−11401. (b) López-Maciá, A.; Jiménez, J. C.; Royo, M.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **2001**, *41*, 9765−9769.
(14) García-Rocha, M.; Bonay, P.; Avila, J. *Cancer Lett.* **1996**, 43−50.
(15) Sewell, J. M.; Mayer, I.; Langdon, S. P; Smyth, J. F.; Jodrell, D. I.; Guichard, S. M. *Eur. J. Cancer* **2005**, *41*, 1637−1644.
(16) Janmaat, M. L; Rodriguez, J. A; Jimeno, J.; Kruty, F. A.; Giaccone, G. *Mol. Pharmacol.* **2005**, *68*, 502−510.
(17) Suarez, Y.; Gonzalez, L.; Cuadrado, A.; Berciano, M.; Lafarga, M.; Munoz, A. *Mol. Cancer Ther*. **2003**, *2*, 863−872.
(18) Brown, A. P.; Morrissy, R. L.; Faircloth, G. T.; Levine, B. S. *Cancer Chemother. Pharmacol.* **2002**, *50*, 333−340.
(19) Ciruelos, C.; Trigo, T.; Pardo, J.; Paz-Ares, L.; Estaun, N.; Cuadra, C.; Domínguez, M.; Marín, A.; Jimeno, J.; Izquierdo, M. *Eur. J. Cancer* **2002**, *38*, Suppl. 7, S33.
(20) Rademaker-Lakhai, J. M.; Horenblas, S.; Meinhardt, W.; Stokvis, E.; de Reijke, T. M.; Jimeno, J. M.; Lopez-Lazaro, L.; Lopez-Martin, J, A.; Beijnen, J. H; Schellens, J, H. *Clin. Cancer Res.* **2005**, *11*, 1854−1862.
(21) (a) Kelaart, E. F. *J. Ceylon Branch R. Asiatic Soc., Columbo* **1858**, *3*, 84−139. (b) Jensen, K. R. [Message in] Sea Slug Forum; 2001 (Nov 26), Australian Museum, Sydney. Available from http://www.seaslugforum.net/find.cfm?id=5727. (c) Rudman, W. B. [Message in] Sea Slug Forum; 2001 (November 19), Australian Museum, Sydney. Available from http://www.seaslugforum.net/factsheet.cfm?base=elysgran.
(22) Padmakumar, K. *Phuket Marine Biological Center Special Publication.* **1998**, *18*, 187.
(23) Authentic samples of Hawaiian sacoglossan *E. rufescens* collected from Black Point, Kahala, Oahu Island, were provided by D. Horgen (University of Hawaii at Manoa).
(24) Wang, C. Y.; Wang, B. G.; Wiryowidagdo, S.; Wray, V.; van Soest, R.; Steube, K. G.; Guan, H. S.; Proksch, P.; Ebel, R. *J. Nat. Prod.* **2003**, *66*, 51−56.
(25) Moore, R. E.; Bornemann, V.; Niemczura, W. P.; Gregson, J. M.; Chen, J.; Norton, T. R.; Patterson, G. M. L.; Helms, G. L. J. *J. Am. Chem. Soc.* **1989**, *111*, 6128−6132.
(26) Pettit, G. R.; Kamano, Y.; Herald, C. L.; Dufresne, C.; Cerny, R. L.; Herald, D. L.; Schmidt, T. M.; Kizu, H. J. *J. Am. Chem. Soc.* **1989**, *111*, 5015−5017.
(27) Goetz, G.; Yoshida, W. Y.; Scheuer, P. J. *Tetrahedron* **1999**, *55*, 7739−7746.
(28) (a) Giorgiani, F.; Cappiello, A.; Beranova-Giorgiani, S.; Palma, P.; Trufelli, H.; Desiderio, D. M. *Anal. Chem.* **2004**, *76*, 7028−7038. (b) Loo, J. A.; Loo R. R. O. In *Electrospray Ionization Mass Spectrometry of Peptides and Proteins*; Cole, R. B., Ed.; John Wiley & Sons, Inc.: New York, 1997.
(29) Spengler, B. *J. Mass Spectrom.* **1997**, *32*, 1019−1036.
(30) Puapaiboon, U.; Tylor, R. T.; Jainhuknan, J. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 516−520.
(31) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(32) Jimeno, J. M.; Lopez-Martin, J. A.; Casado, A. R.; Izquierdo, M. A.; Scheuer, P. J.; Rinehart, K. *Anti-Cancer Drugs* **2004**, *15*, 321−329.
(33) Bonnard, I.; Manzanares, I.; Rinehart, K. L. *J. Nat. Prod.* **2003**, *66*, 1466−1470.
(34) Hill, R. T.; Hamann, M. T.; Enticknap, J.; Rao, K. V. Kahalalide-producing bacteria and methods of identifying kahalalide-producing bacteria and preparing kahalalides. PCT Int. Appl. 2005, 41 pp. CODEN: PIXXD2 WO 2005042720.
(35) Kreuter, M. H.; Robitzki, A.; Chang, S.; Steffen, R.; Michaelis, M.; Kljajic, Z.; Bachmann, M.; Schröder, H. C.; Müller, W. E. G. *Comp. Biochem. Physiol.* **1992**, *101C*, 183−187.
(36) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55−63.

NP060172V