

Kahalalide F Analogues from the Mucous Secretion of Indian Sacoglossan Mollusk *Elysia ornata*

M. Letizia Ciavatta,^{*}†Prabha Devi,[†] ‡Marianna Carbone,[†] Véronique Mathieu,[§] Robert Kiss,[§] Agostino Casapullo[⊥] and Margherita Gavagnin[†]

[†]*Consiglio Nazionale delle Ricerche (CNR), Istituto di Chimica Biomolecolare (ICB), Via Campi Flegrei, 34, 80078 Pozzuoli (Na), Italy,* [§]*Laboratoire de Cancérologie et de Toxicologie Expérimentale, Faculté de Pharmacie, Université Libre de Bruxelles (ULB), Campus de la Plaine, Boulevard du Triomphe, 1050, Brussels, Belgium,* [⊥]*Dipartimento di Farmacia, Università degli Studi di Salerno, Via Giovanni Paolo II 132, 84084 Fisciano (Sa), Italy*

* To whom correspondence should be addressed.

Tel: +39-081-8675243. Fax: +39-081-8041770. E-mail: lciaavatta@icb.cnr.it

‡ Postdoc, CSIR - National Institute of Oceanography, Dona Paula, Goa, 403 004 India.

ABSTRACT

Bioassay-guided fractionation of the CHCl₃-MeOH extract of *Elysia ornata* mucus showing antifungal activity led us to isolate kahalalide F (KF) along with two unprecedented related compounds. The structures of KF congeners, kahalalide Z₁ (**1**) and kahalalide Z₂ (**2**), were established by combination of NMR spectroscopic and mass spectrometry data, also including Marfey amino acid analysis. Compounds **1** and **2** displayed significant antimicrobial properties against several fungal pathogens infesting economically important plants, food and fish, and were also found to inhibit the *in vitro* growth of a series of cancer cell lines in a comparable fashion with KF.

Kahalalides are a large family of peptides isolated from both the herbivorous marine mollusks of the genus *Elysia* and their algal prey of the genus *Bryopsis*.¹ Among them, some selected cyclodepsipeptide members, which exhibit the uncommon “head-to-side-chain” structural arrangement ring-closed through an ester bond, show therapeutic profiles of interest with highly diverse biological activities including antiviral, antifungal, antileishmanial, as well as immunosuppressive properties.¹⁻³ The most representative compound is kahalalide F (KF), which is the largest and the most biologically active cyclic peptide of the group, originally isolated from the sacoglossan mollusk *Elysia rufescens* and subsequently from the alga *Bryopsis pennata* upon which it feeds.⁴ Preclinical studies showed strong activity of KF against cell lines and tumor specimens derived from different human solid tumors, including non-small cell lung cancer (NSCLC), prostate, breast, ovarian, and colon carcinomas. Interestingly, the activity of KF was correlated with ErbB3 (HER3) expression and was associated with the inhibition of the downstream phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway^{5,6} in sensitive cell lines. Licensed to PharmaMar, KF entered clinical trials against prostate cancer, malignant melanoma and non-small cell lung cancer, with a mechanism of action that involved oncosis (ischemic cell death).⁷ But, recently, the lead was dropped from phase II clinical trials due to a lack of efficacy that was ascribed to pharmacokinetic issues.¹ Considering the high potential for a cytotoxic drug candidate from this class, several attempts have been made to modify the molecule to produce analogues with a higher potency, better delivery, or longer half-life. More than 150 analogues were obtained by solid phase peptide synthesis^{8,9} and more recently a semi-synthetic approach to produce KF analogues starting from natural KF has been also performed.¹⁰ In parallel, the search of new naturally-occurring KF congeners continues to be of a certain interest.

In the course of a screening for new antifungal compounds from marine mollusks, we have selected the extract of the mucous secretion of an Indian population of *Elysia ornata* (Swainson, 1840). The bioassay-guided fractionation of this extract resulted in the identification of two new antimicrobial KF analogues, kahalalides Z₁ (**1**) and Z₂ (**2**), co-occurring with the main metabolite kahalalide F. The isolated compounds were also subjected to an *in vitro* growth inhibition assay, showing a bioactivity profile comparable with that of KF.

We report here the isolation and the chemical characterization of kahalalides **1** and **2** and the evaluation of their bioactivity in term of *in-vitro* growth inhibition of various cancer cell lines. Since fungal diseases are second to bacterial infection that causes immense economic loss in agriculture and

aquaculture, we also report here the *in vitro* antifungal activity of these two kahalalides (**1** & **2**) against several plant, food and fish infesting fungal pathogens.

Results and Discussion

The sacoglossan mollusk *E. ornata* (300 individuals) was sampled by snorkeling along the coasts of Okha (India) and the mucus released by the animals was also collected. The biological material (mollusk and mucus) was initially lyophilized and then extracted with a mixture of CHCl₃/MeOH (1:1). The resulted extracts were screened for antifungal properties against a series of plant, food, and fish pathogens (see Experimental). Comparison of their activity was made with standard antibiotic, ketoconazole, to explain the significantly strong antifungal activity of the crude extracts. Since, the most interesting activity profile was exhibited by the mucus extract (600 mg) it was subsequently submitted to bioassay-guided fractionation. This procedure led us to isolate a bioactive sub-fraction containing a mixture of kahalalides, as suggested by preliminary ¹H NMR and LCMS analysis. In particular, inspection of the LCMS chromatogram revealed the presence of three main peaks corresponding to the ion species at m/z 1478.8 [M+H]⁺, 1449.9 [M+H]⁺, and 1461.7 [M+H]⁺, the first of which isobaric with kahalalide F whereas the other two possibly related to unprecedented kahalalide derivatives. Reversed phase HPLC chromatography of a portion of this mixture afforded KF (9.2mg), along with the two analogues, named kahalalide Z₁ (**1**, 4.5mg) and kahalalide Z₂ (**2**, 5.0mg).

Kahalalide F was identified by comparison of its spectroscopic data with those reported in the literature.^{4,11,12} The structures of the new molecules were elucidated by extensive 1D and 2D NMR experiments, performed in DMSO and by tandem-MS analysis (ESI-MS/MS). A preliminary analysis of NMR spectra (Tables 1 and 3) of compounds **1** and **2** revealed structural features common to KF. In addition, it was evident that both analogues **1** and **2** shared the same amino acid sequence, differing only in the nature of the *N*-terminal acid moiety. Comparison of the NMR spectra of **1** and **2** with respect to KF suggested the presence of different residues in the macrocyclic region and in the linear portion of the metabolites. This evidence was also confirmed by the different ESI-MS/MS fragmentation pattern observed for **1** and **2** with respect to KF, as shown in details below.

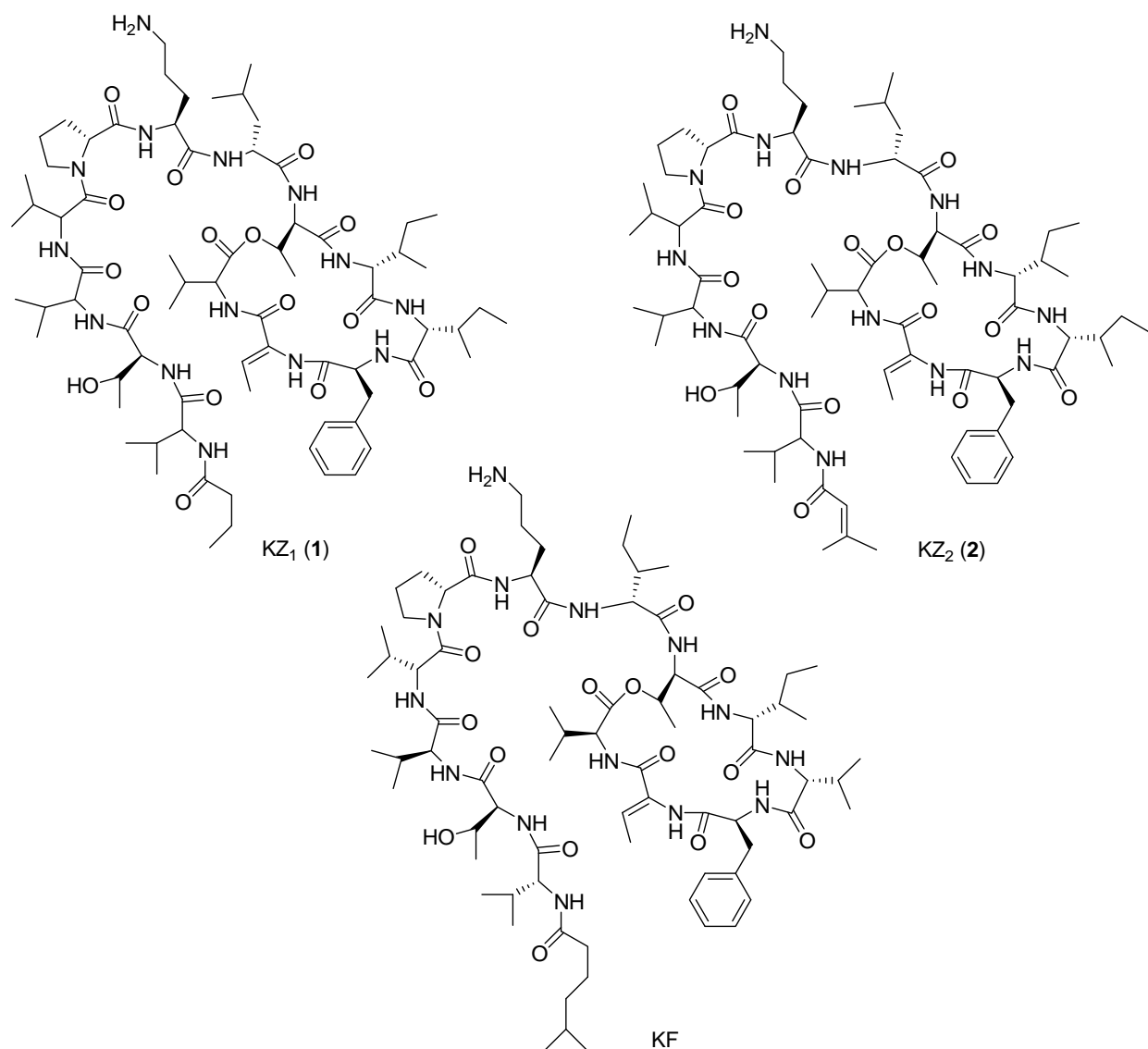
Kahalalide Z₁ (**1**) was obtained as a white amorphous powder. The positive HR-ESI mass spectrum showed a sodiated molecular ion peak at m/z 1471.8887[M+Na]⁺, which was consistent with the molecular formula C₇₃H₁₂₀N₁₄O₁₆. Inspection of its ¹H NMR spectrum showed a region between

6.79 and 9.67 ppm containing 13 deshielded NH resonances, including a broad 2H singlet at δ_{H} 7.64 assignable to the Orn residue terminal amino group, common with KF. Moreover, in the low-field region of the proton spectrum were present, similarly to KF, signals consistent with the presence of Phe and Dhb residues, whereas in the high-field region the methyl doublets and triplets pattern was quite different. The occurrence of 12 regular amino acid residues in **1** was indicated by the 12 α -carbon methine signals, recorded between δ 50.9 and 60.0 in the ^{13}C NMR spectrum, which also contained two carbinolic resonances at δ 66.2 and 69.7 according to the presence of two Thr residues as in KF. The COSY spectrum of **1** showed 14 isolated proton sequences, including 12 spin systems with an amide NH connection assigned to regular amino acid moieties and two additional sequences with no correlations with any amide NH proton, that were attributed to a Pro and to the terminal butanoic acid residue. In particular, in the COSY experiment, spin systems attributable to Val, Leu, and Ile along with those consistent with the presence of Thr, Orn, Phe and Dhb residues were recognized. The following TOCSY analysis allowed the full assignment of the NH and α -proton resonances of 12 regular amino acid units as well as most methyl groups of different Val, Leu and Ile residues (Table 1). Analysis of both HMBC and NOESY spectra of **1** led us to assess the entire sequence of the depsipeptide. A series of key HMBC correlations (Figure 1) indicated the connection of the single amino acid units in the chain fragment starting from the N-terminal acid to Val-2 residue as well as in the other portion including the cycle from Val-1 to Pro. In particular, the sequence was inferred by significant long-range correlations observed between the NH of each amino acid and the vicinal carbonyl (2J) of the corresponding consecutive fragment (Figure 1).

Table 1. ^1H and ^{13}C NMR Data (600 MHz) of kahalalide Z_1 (**1**) in $\text{DMSO-}d_6$

Amino acid	no.	δ_{C} (mult) ^a	δ_{H} (mult)	Amino acid	no.	δ_{C} (mult)	δ_{H} (mult)	
Val-1	1	169.4, C	(NH) 6.79, d 9.0	L-Orn	1	169.8, C	(NH) 7.99, ^g d	
	2	60.0, CH	3.85, app t 9.0		2	51.2, CH	4.35, m	
	3	29.4, CH	1.37, m		3	23.4, CH ₂	1.46, m	
	4	18.5, CH ₃	0.59, d 6.4		4	29.7, CH ₂	1.68/1.45, m	
	5	19.2, CH ₃	0.64, d 6.4		5	38.5, CH ₂	2.73, m	
(Z)-Dhb	1	163.1, C	(NH) 9.67, s	D-Pro	1	170.6, C	(NH ₂) 7.64 br s	
	2	130.1, C	-		2	59.4, CH	4.34, m	
	3	129.8, CH	6.33, q 7.0		3	29.4, CH ₂	2.00, m	
L-Phe	4	12.2, CH ₃	1.25, d 7.0	4	24.4, CH ₂	1.78, m		
	1	171.3, C	(NH) 8.77, ^g	5	47.1, CH ₂	3.77/3.52, m		
	2	55.86, ^b CH	4.39, m	Val-2	1	171.3, C	(NH) 8.07 d 8.0	
	3	35.96, CH ₂	2.93, br d 8.4		2	57.8, ^d CH	4.23, m	
	4	136.8, C			3	30.8, ^e CH	1.95, m	
	5,5'	128.2, CH	7.25, m		4	18.5, ^f CH ₃	0.86, ^g d	
	6,6'	129.2, CH	7.24, m		5	19.2, ^f CH ₃	0.86, ^g d	
7	126.5, CH	7.19, m	Val-3	1	170.5, C	(NH) 7.57, d 8.3		
D-alloIle-1	1	172.6, C		(NH) 7.54, d 8.8	2	57.3, ^c CH	4.26, m	
	2	53.8, CH		4.55, br d 8.4	3	30.1, ^e CH	1.98, m	
	3	40.0, CH		1.89, m	4	18.2, ^f CH ₃	0.81, ^g d	
	4	14.1, CH ₃		0.61, d 6.8	5	19.4, ^f CH ₃	0.81, ^g d	
	5	25.6, CH ₂	1.25/0.96, m	L-Thr	1	169.6, C	(NH) 7.79, d 8.3	
	6	11.8, CH ₃	0.89, t 7.0		2	55.88, ^b CH	4.249, m	
D-alloIle-2	1	170.0, C	(NH) 8.75, ^g		3	66.2, CH	3.96, m; 4.86, m	
	2	57.3, ^c CH	4.26, m		4	19.4, CH ₃	0.98, d 6.1	
	3	37.9, CH	1.68, m		Val-4	1	171.5, C	(NH) 7.86, d 8.6
	4	14.5, CH ₃	0.75, d 7.0			2	57.9, ^d CH	4.22, m
	5	25.6, CH ₂	1.28/1.00, m	3		29.9, ^e CH	1.97, m	
	6	11.3, CH ₃	0.82, ^g t	5		18.6, ^f CH ₃	0.84, ^g d	
D-alloThr	1	168.4, C	(NH) 8.55, d 8.8	5		19.1, ^f CH ₃	0.84, ^g d	
	2	56.3, CH	4.48, app t 9.0	D-Leu	1	172.3, C		
	3	69.7, CH	4.98, m		2	37.0, CH ₂	2.13, m	
	4	16.8, CH ₃	1.08, d 6.3		3	18.7, CH ₂	1.50, m	
D-Leu	1	172.0, C	(NH) 7.97, ^g d		4	13.6, CH ₃	0.83, ^g t	
	2	50.9, CH	4.27, m		Butanoyl	1	172.3, C	
	3	41.1, CH ₂	1.39, m			2	37.0, CH ₂	2.13, m
	4	24.1, CH	1.53, m	3		18.7, CH ₂	1.50, m	
	5	22.7, CH ₃	0.83, ^g d	4		13.6, CH ₃	0.83, ^g t	
	6	21.8, CH ₃	0.80, ^g d					

^a determined by edited HSQC; ^{b-c} assignments with the same superscript may be interchanged; ^f Methyl signals of Val-3, Val-4 and Val-5 were not assigned to each residue; ^g J value was not determined due to overlapped signals



The Pro residue was located between Val-2 and Orn on the basis of both NOESY interactions between the α proton of Val-2 (δ_{H} 4.23) and the δ protons of Pro (δ_{H} 3.77/3.52) and the HMBC cross-peak observed between the Pro carbonyl (δ_{C} 170.6) and the Orn NH (δ_{H} 7.97). Similarly to KF, the Val-1 carboxyl was esterified to the $-\text{OH}$ of a Thr unit as suggested by a diagnostic HMBC correlation between the Val-1 carbonyl signal (δ_{C} 169.4) and the deshielded Thr β -proton (δ_{H} 4.98). The NOESY spectrum supported the *Z* double bond geometry in the Dhb unit by the cross-peak between the NH singlet at δ_{H} 9.67 and the methyl doublet at δ_{H} 1.25 ($J = 7$ Hz). By these data, the amino acid composition of kahalalide Z_1 (1) was determined to include four Val units, two residues of both Thr and

Ile, and one unit of each Phe, Z-Dhb, Orn, Leu, and Pro, thus differing from KF for the presence of a Leu residue rather than a Val. According to the molecular formula, the structure of **1** was completed by the terminal butanoyl moiety replacing the 5-methyl-hexanoic acyl residue in KF.

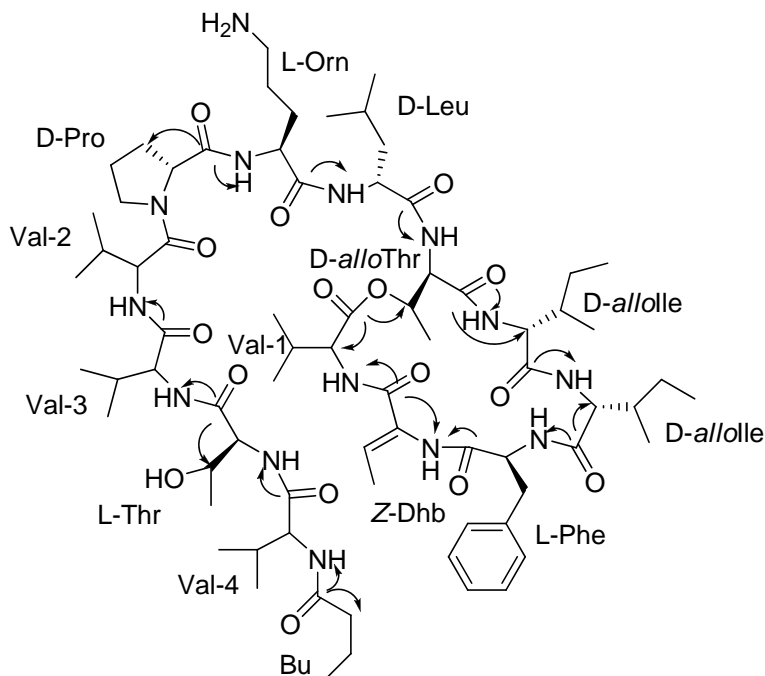


Figure 1.Key HMBC correlations of kahalalide Z₁ (**1**)

The sequence of the single amino acid units of kahalalide Z₁ (**1**) was further supported by analysis of its ESI-MS/MS spectrum (positive ion mode), where the observed diagnostic fragment ion peaks (see Table 2) were consistent with the proposed structure.

Table 2.Significant ESI/MS/MS fragment ions of kahalalide Z₁ (**1**)

Ion composition	<i>m/z</i>
[MH] ⁺	1449.9
[MH – Bu – Val 4] ⁺	1280.6
[MH – Bu – Val 4 – Thr] ⁺	1179.5
[MH – Bu – Val 4 – Thr – Val 3] ⁺	1080.6
[MH – Bu – Val 4 – Thr – Val 3 – Val 2] ⁺	981.5
[MH – Bu – Val 4 – Thr – Val 3 – Val 2 – Pro – Orn] ⁺	770.4

The absolute configuration of the single aminoacids was determined by Marfey's analysis, which indicated L-Thr, D-*allo*-Thr, D-Pro, L-Orn, D-*allo*-Ile (x 2), D-Leu, L-Phe. The position of L-Thr and D-*allo*-Thr was suggested equivalent to that of KF by comparison of their NMR signals. The absolute configuration of Val individual residues remained unassigned due to the presence of both D and L enantiomers of Val (2 mol/units for each enantiomer). However, attempts to define at least the stereochemistry of the final Val residue (Val-4), which was attached to the butanoic moiety, were made, according to the procedure reported in the literature.¹³ The partial hydrolysis of aliquots of KZ₁ was conducted in selected conditions with the aim to recover the terminal moiety containing Val-4 but, unfortunately, no defined reaction product was obtained, most likely due to the small amount of available material.

Kahalalide Z₂ (**2**) exhibited a sodium adduct ion at m/z 1483.8948, 12 units higher than KZ₁, indicating the molecular formula C₇₄H₁₂₀N₁₄O₁₆. Analysis of its NMR data revealed an extensive structural similarity with kahalalide Z₁ (**1**), in particular the same amino acid sequence (Table 3). Compound **2** differed from **1** only in the nature of the terminal acyl residue, which was found to contain five instead of four carbons (C₅H₇O rather than C₄H₇O). This fragment was easily identified as derived by 3-methyl-2-butenic acid (or senecioic acid) on the basis of NMR analysis. In fact, the ¹H NMR spectrum of KZ₂(**2**) displayed signals due to an olefinic proton at δ_H 5.83 (1H), and two vinyl methyls at δ_H 2.05 (3H) and 1.77 (3H) whereas the carbon spectrum contained signals at δ_C 166.0 (CO), 148.9 (C), 118.9 (CH), 26.8 (CH₃), and 19.3 (CH₃). An expected HMBC correlation between the carbonyl signal and the proton resonances due to the tri-substituted double bond system confirmed the structure of the terminal acid moiety. Senecioic acid has never been reported in natural or synthetic kahalalides, even though it has been found in other marine depsipeptides (i.e. largamides).¹⁴

Table 3. ^1H and ^{13}C NMR Data (600 MHz) of kahalalide Z_2 (**2**) in $\text{DMSO-}d_6$

Amino acid	no.	δ_{C} (mult) ^a	δ_{H} (mult)	Amino acid	no.	δ_{C} (mult) ^a	δ_{H} (mult)
Val-1	1	169.4, C	(NH) 6.79, d 9.0	L-Orn	1	169.8, C	(NH) 7.99, ^g d
	2	60.0, CH	3.85, app t 9.0		2	51.2, CH	4.35, m
	3	29.4, CH	1.37, m		3	23.4, CH ₂	1.46, m
	4	18.2, CH ₃	0.59, d 6.4		4	29.7, CH ₂	1.68/1.45, m
	5	19.2, CH ₃	0.64, d 6.4		5	38.5, CH ₂	2.73, m
(Z)-Dhb	1	163.1, C	(NH) 9.67, s	D-Pro	1	170.6, C	
	2	130.1, C	-		2	59.4, CH	4.34, m
	3	129.8, CH	6.33, q 7.0		3	29.4, CH ₂	2.00, m
L-Phe	4	12.2, CH ₃	1.25, d 7.0	4	24.4, CH ₂	1.78, m	
	1	171.3, C	(NH)	5	47.1, CH ₂	3.77/3.52, m	
	2	55.86, ^b CH	4.39, m	Val-2	1	171.3, C	(NH) 8.07, d 8.0
	3	35.96, CH ₂	2.93, br d 8.4		2	57.8, ^d CH	4.23, m
	4	136.8, C			3	30.8, ^e CH	1.95, m
	5,5'	128.2, CH	7.25, m		4	18.5, ^f CH ₃	0.86, ^g d
	5,6'	129.2, CH	7.24, m		5	19.2, ^f CH ₃	0.86, ^g d
7	126.5, CH	7.19, m	Val-3	1	170.5, C	(NH) 7.57, d 8.3	
D-allolle-1	1	172.6, C		(NH) 7.54, d 8.8	2	57.3, ^c CH	4.26, m
	2	53.8, CH		4.55, br d 8.4	3	30.1, ^e CH	1.98, m
	3	40.0, CH		1.89, m	4	18.2, ^f CH ₃	0.81, ^g d
	4	14.1, CH ₃		0.61, d 6.8	5	19.2, ^f CH ₃	0.81, ^g d
	5	25.6, CH ₂	1.25/0.96, m	L-Thr	1	169.6, C	(NH) 7.79, d 8.3
5	11.8, CH ₃	0.89, t 7.0	2		55.88, CH	4.249, m	
D-allolle-2	1	170.0, C	(NH) 8.75, ^g d		3	66.2, CH	3.96, m; 4.86, m
	2	57.3, ^c CH	4.26, m		4	19.4, CH ₃	0.98, d 6.1
	3	37.9, CH	1.68, m		Val-4	1	171.5, C
	4	14.5, CH ₃	0.75, d 7.0	2		57.9, ^d CH	4.256, m
	5	25.6, CH ₂	1.28/1.00, m	3		29.9, ^e CH	1.97, m
5	11.3, CH ₃	0.80, ^g t	5	18.6, ^f CH ₃		0.84, ^g d	
5	19.1, ^f CH ₃	0.84, ^g d	D-alloThr	5		19.1, ^f CH ₃	0.84, ^g d
1	168.4, C	(NH) 8.55, d 8.8		D-Leu	1	166.0, C	
2	56.3, CH	4.48, app t 9.0			2	118.9, CH	5.83, br s
3	69.7, CH	4.98, m			3	148.9, C	-
4	16.8, CH ₃	1.08, d 6.3			4	19.3, CH ₃	2.05, s
5	22.7, CH ₃	0.83, ^g d	5		26.8, CH ₃	1.77, s	
D-Leu	1	172.0, C	(NH) 7.97, ^g d	Sen	1	166.0, C	
	2	50.9, CH	4.29, m		2	118.9, CH	5.83, br s
	3	41.1, CH ₂	1.39, m		3	148.9, C	-
	4	24.1, CH	1.53, m		4	19.3, CH ₃	2.05, s
	5	22.7, CH ₃	0.83, ^g d		5	26.8, CH ₃	1.77, s
	5	21.8, CH ₃	0.80, ^g d				

^a determined by edited HSQC; ^{b-c} assignments with the same superscript may be interchanged; ^f methyl signals of Val-3, Val-4 and Val-5 were not assigned to each residue; ^g*J* value was not determined due to overlapped signals

The linkage of the terminal acyl moiety with the peptide chain was supported by the diagnostic HMBC correlation of the senecioic acid carbonyl (δ_C 166.0) with Val-1 NH (δ_H 7.75), which was the only signal with different value, among the entire sequence of amino acid residues, with respect to KZ₁ (**1**). Indeed, the COSY and TOCSY experiments of compound **2** indicated the same amino acid spin systems as in **1**. The NOESY interactions between α -proton at δ_H 4.23 and δ -protons at δ_H 3.77/3.52 aided us to link the residue of Val-3 to Pro, as well as to confirm the *Z* geometry of the Dhb residue. Finally, HMBC correlations allowed the connection of all the amino acid units from Val-1 to Pro and from N-terminal acid to Val-3 (Figure 2). The amino acid sequence of KZ₂ was further confirmed by the ESI/MS/MS fragmentation (Table 4). The absolute configuration of the single amino acids in **2** was determined to be the same as **1** by using Marfey's analysis. Analogous with KZ₁, the absolute configuration of the four Val residues in KZ₂ remained undetermined.

Table 4. Significant ESI/MS/MS fragment ions of kahalalide Z₂ (**2**)

Ion composition	<i>m/z</i>
[MH] ⁺	1461.7
[MH – Sen – Val 4] ⁺	1280.6
[MH – Sen – Val 4 – Thr] ⁺	1179.5
[MH – Sen – Val 4 – Thr – Val 3] ⁺	1080.6
[MH – Sen – Val 4 – Thr – Val 3 – Val 2] ⁺	981.5
[MH – Sen – Val 4 – Thr – Val 3 – Val 2 – Pro – Orn] ⁺	770.4

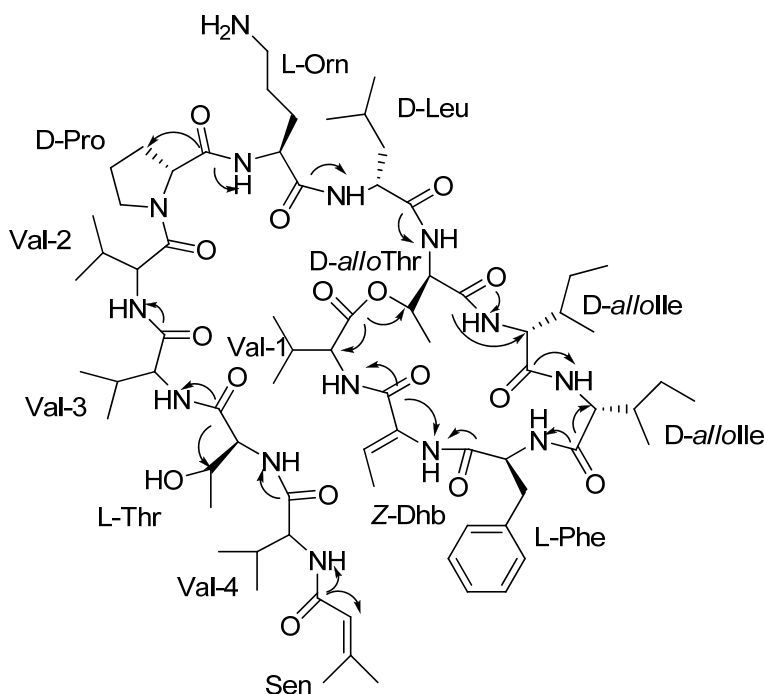


Figure 2. Key HMBC correlations of kahalalide Z₂ (**2**)

Pure kahalalides KZ₁, KZ₂ and KF were tested for the antifungal activity by disc diffusion assay against a number of plant, food and fish infesting fungal pathogens, as already done with the crude extract. The antifungal activity was evaluated by measuring in millimeters the distinct zone of growth inhibition formed around the active compound. In several cases the growth of fungal pathogen which was not inhibited by the standard was significantly inhibited by KZ₁/KZ₂ and KF. The results are reported in Table 5 according to the legend.¹⁵

Table 5. *In vitro* antifungal activity of pure compounds (KZ₁, KZ₂ & KF) against plant, food and fish pathogens. Std = Ketoconazole, (-) inactive, (+) mild activity, (++) moderate activity, (+++) significant activity and (+++++) strong activity.

Plant pathogens	Disease caused	Std	KZ₁	KZ₂	KF
<i>Sclerotium rolfsii</i> (<i>Solanum tuberosum</i>)	Stem and leaf rot	++	++	++	+
<i>Aspergillus niger</i> gr (<i>Archus sapodilla</i>)	Rot disease	-	++	++	+
<i>Aspergillus niger</i> (<i>Psidium guajava</i>)	Black rot disease	++	-	-	-
<i>Penicillium piceum</i> (<i>Archus sapodilla</i>)	Leaf rot disease	-	+++	+++	++
<i>Aspergillus niger</i> (<i>Mangifera indica</i>)	Black mold rot	+++	++	-	++
<i>Trichoderma koningii</i> Oudemans (<i>M. indica</i>)	Fruit rot	+++	++	++	++
<i>Trichoderma atroviride</i> (<i>Anacardium occidentale</i>)	Rot disease	-	++	++	+
<i>Rhizoctonia solani</i> (<i>Amaranthus</i>)	Leaf blight	-	++	+	-
<i>Colletotrichum gloeosporioides</i> (<i>Cinnamomum verum</i>)	Leaf spot	-	+++	++	+++
<i>Helminthosporium oryzae</i> (<i>Oryza sativa</i>)	Spot blotch	-	+++	++	+
<i>Pestalotia</i> sp. (<i>Cocos nucifera</i>)	Gray leaf blight	-	++	+++++	++
<i>Fusarium</i> sp. (<i>Solenostemon scutellarioides</i>)	Fusarium wilt	-	+	+++	-
Food pathogens (Source)					
<i>Penicillium griseofulvum</i> Dierckx (Indian bread)	Infests food	+	-	-	+
<i>Aspergillus flavus</i> (<i>Daucus carota</i>)	Infests food	+++	+++	++	+
<i>Aspergillus niger</i> (Indian bread)	Infests food	-	+	+	-
Fish pathogens (Source)					
<i>Exophiala salmonis</i> (<i>O. mossambicus</i>)	Systemic mycosis	-	+	-	-
<i>Branchiomyces demigrans</i> (<i>O. mossambicus</i>)	Branchiomycosis	-	-	++	++
<i>Saprolegnia</i> sp. (<i>O. mossambicus</i>)	Saprolegniasis	+	-	-	++

The in vitro growth inhibitory activity of the isolated kahalalides was also investigated on four human cancer cell lines (Table 6). KZ₁ exhibited a selective inhibitory in vitro growth effect on A549 and Hs683 cancer cell lines comparable to that of KF, whereas KZ₂ showed to be active only on A549.

Table 6. Determination of the IC₅₀ in vitro growth inhibitory concentrations of KZ₁, KZ₂ and KF in four human cancer cell lines after 72 h of cell culture with the compounds of interest

Compounds	Human cancer cell lines ^a				
	A549	Hs683	SKMEL28	U373N	Mean + SEM
KZ ₁ (1)	1	3	14	31	12 + 7
KZ ₂ (2)	3	15	44	84	37 + 18
KF	1	2	6	20	7 + 15

^aThe origin and histological type of each human cell line analyzed are as follows. Carcinoma models included the A549 NSCLC (DSMZ code ACC107) cancer cell line. Glioma models included the Hs683 oligodendroglioma (ATCC code HTB-138) and the U373 (ECACC code 08061901) cell lines. One melanoma model included the SKMEL-28 (ATCC code HTB-72) cell line. DSMZ means Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), ATCC means American Type Culture Collection (Rockville, MD, USA) and ECACC means European Collection of Cell Cultures (Salisbury, UK).

In conclusion, two unprecedented KF analogues, kahalalide Z₁ and kahalalide Z₂, with a bioactivity profile comparable with KF, have been isolated from the Indian sacoglossan *E. ornata*. A previous study on a Hawaiian population of this mollusk had resulted in the characterization of an inactive member of kahalalide family, kahalalide O, which was later detected in the alga *Bryopsis* from the same collection area.¹⁶ Analogous to this, KZ₁ and KZ₂ should have an algal dietary origin.

Experimental section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP 370 digital polarimeter. The UV spectra were recorded on an Agilent 8453 spectrophotometer. ¹H and ¹³C NMR spectra were recorded on DRX 600, Avance 400, and DPX 300 MHz Bruker spectrometers in *d*₆-DMSO, with chemical shifts reported in ppm referred to DMSO (δ 2.50 for proton and δ 40.5 for carbon) as internal standard. ESIMS and HRESIMS were obtained on a Micromass Q-TOF Micro spectrometer coupled with a HPLC Waters Alliance 2695. The instrument was calibrated by using a PEG mixture from 200 to 1000 MW. The ESI-MS/MS analysis were performed on a Thermo LTQ-Orbitrap XL spectrometer, calibrated with ProteoMass™ LTQ/FT-Hybrid ESI Pos. Mode CalMix (Sigma-Aldrich). Sephadex LH-20 (GE Healthcare Bio-Sciences, Uppsala) was carried out using an open pre-packed column. Silica gel chromatography was performed using precoated Merck F254 plates and

Merck Kieselgel 60 powder. HPLC purification was carried out on a ThermoSpectraSystem SCM 1000 liquid chromatograph equipped with a UV wavelength detector Spectra System UV 2000. Human cancer cell lines were obtained from the American Type Culture Collection (ATCC), the European Collection of Cell Culture (ECACC), and the Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ). The code number and histological type of each of the cell lines used in the current study are detailed in Table 6. The cell lines were cultured as detailed previously.¹⁷⁻¹⁹

Biological material. *E. ornata* (300 individuals) was picked up by snorkeling along the coasts of Okha (India) during October 2010. The live animals and the mucus (150 mL) released from them, were immediately frozen at -20°C and then lyophilized before extraction. A voucher specimen (code ElyOkha) is available for inspection at the National Institute of Oceanography.

Extraction of biological material. The freeze-dried animals (160 mg dry weight) and mucus (700 mg dry weight) of *E. Ornata* were exhaustively extracted with a 1:1 CHCl₃/MeOH mixture (5 x 150 mL and 5 x 300 mL) with repeated sonication. The combined extracts were concentrated *in vacuo*, to give 150.0 mg and 600.0 mg extracts from the animals and the mucus, respectively. The two extracts were subjected to biological screening against several pathogens wherein the mucus extract displayed a more significant activity.

Bioassay guided fractionation of KZ₁, KZ₂ and KF (1-3). In order to isolate the compounds responsible for the antifungal activity, the mucus extract of *E. ornata* was subjected to a series of bioassay guided purifications. Firstly, the extract (600 mg) was fractionated on a Sephadex LH-20 in MeOH to yield an active kahalalides-containing sub-fraction (300 mg). An aliquot of this fraction (100 mg) was further purified on silica gel column by eluting with a gradient system of CHCl₃, CHCl₃/MeOH and finally MeOH to give ten sub-fractions (1-10), finding activity only in sub-fraction 7 (35 mg). Preliminary ¹H NMR spectra of this fraction revealed the presence of a mixture of peptides that was further purified on RP Amide HPLC column (MeCN/H₂O/TFA 0.01%; gradient from 70% to 100% in 40 min.; flow 1.5 mL/min) to afford in order of increasing polarity KF (9.2 mg, Rt 31.7), KZ₂ (2, 4.5 mg, Rt 26.4), and KZ₁ (1, 5.0 mg, Rt 25.3).

kahalalide Z₁ (1): amorphous white powder; $[\alpha]_D^{25}$ -3.9 (c 0.15, MeOH); UV (MeOH) λ_{\max} (log ϵ) 204 (4.35), 235 (3.73); ¹H and ¹³C NMR see Table 1; ESIMS m/z 1472 [M+Na]⁺ and HRESIMS m/z 1471.8887 [M+Na]⁺, (calcd for C₇₃H₁₂₀N₁₄O₁₆Na 1471.8904).

kahalalide Z₂ (2): amorphous white powder; $[\alpha]_D^{25}$ -3.5 (c 0.21, MeOH); UV (MeOH) λ_{\max} (log ϵ) 204 (4.35), 235 (3.73); ¹H and ¹³C NMR see Table 2 and 2; ESIMS m/z 1484; HRESIMS m/z 1483.8948 [M+Na]⁺, (calcd for C₇₄H₁₂₀N₁₄O₁₆Na 1483.8904).

Marfey's analysis. Compounds **1–3** (250 μ g each) were hydrolyzed by heating in HCl (6 N, 500 μ L) at 110 °C for 24 h. Upon reaching room temperature (25 °C), the solutions were evaporated to dryness and dissolved in H₂O (50 μ L). To each peptide acid hydrolysate solution (or to 50 μ L of a 50 mM solution of the respective amino acid standard) was added NaHCO₃ (1 M; 20 μ L) and then a 1% (w/v) solution (100 μ L) of FDVA (N α -(2,4-dinitro-5-fluorophenyl)-L-valinamide, a variant of Marfey's reagent) in acetone. The mixtures were heated for 1 h at 40 °C. The reactions were stopped by addition of HCl (1 N, 20 μ L). The derivatized peptide hydrolysate solutions were diluted by addition of 300 μ L of MeCN, and the derivatized amino acid standards solutions were diluted by addition of 770 μ L of MeCN. A small amount of these solutions (4 μ L of **1**, **2**, and **3** and 2 μ L of the standards) was analyzed by LC-MS (Ascentis RP-Amide column, 25 \times 0.46 cm, 5 μ m; 0 min 35% CH₃CN, 65 % H₂O + TFA 0.1% to 40 min 50% CH₃CN, 50 % H₂O + TFA 0.1%, detection at 340 nm). Retention times and molecular weights of the FDVA amino acid derivatives were compared with those obtained from standard amino acids.

Antifungal activity. Eighteen fungal pathogens (12 phyto, 3 food-infesting and 3 fish pathogens) were used for biological screening. Crude organic extracts, fractions obtained from the different purification steps and the pure compounds were tested for antifungal activity following the standard disc diffusion assay as described earlier.¹⁵ Briefly, Whatman (GF/C) filter paper discs of 6 mm diameter were prepared and sterilized for 15 minutes under 15 lb/m² pressure. Known concentration (20 μ g/disc) of the crude extract and 10 μ g/disc of the pure compound was loaded onto sterile paper discs and allowed to air-dry on the laminar airflow. Positive and negative control discs were prepared using ketoconazole (10 μ g/disc) and methanol respectively. The discs, in duplicates, were placed on mycological agar plates seeded with the different test fungal pathogens and incubated for 24–48 hours at 27 °C. The zone of growth inhibition formed around each disc was measured in millimeters (Table 5) and the activity was scored using symbols expressed as inactive (-), mild activity (+), moderate activity (++), significant activity (+++) and strong activity (++++) respectively.

Determination of the IC₅₀ Growth Inhibitory Concentrations in vitro. The MTT colorimetric assay was used as detailed previously. Each experimental condition was assessed in six replicates. The origin of each cell line is detailed in the legend to Table 6.

Acknowledgements The authors thank for technical assistance Mrs D. Melck of ICB-NMR service, Mr. M. Zampa of “Servizio di Spettrometria di Massa” for HRESIMS. This research work was financially supported by POR”. One of the authors, P.D. acknowledges EMBO, Postfac Germany and Dept of Biotechnology, Govt of India (DBT-CREST) for the award of post doctoral fellowships during which period this work was undertaken. RK is a director of research with the Fonds National de la Recherche Scientifique (FRS-FNRS, Belgium). The in vitro growth inhibitory effects of the compounds under study on cancer cells were determined thanks to grants awarded by the Belgian Brain Tumor Support (BBTS, Belgium).

Supporting Information Available: 1D and 2D NMR spectra of compounds 1 and 2, and KF. This material is available free of charge via the Internet at <http://?????>

References and Notes

1. Gao, J.; Hamann, M.T. *Chem. Rev.*, **2011**, *111*, 3208-3235 and references therein cited.
2. Pelay-Gimeno, M.; Tulla-Puche, J.; Albericio, F. *Mar. Drugs*, **2013**, *11*, 1693-1717.
3. Shilabin, A. G.; Hamann, M. T. *Bioorg. Med. Chem.* **2011**, *19*, 6628–6632.
4. Hamann, M.T.; Scheuer, P.J. *J. Am. Chem. Soc.* **1993**, *115*, 5825-5826.
5. Faircloth, G.T.; Smith, B.; Grant, W.; Jimeno, J.M.; Garcia-Gravalos, L.; Scotto, K.; Shtil, A. *Proc. Am. Assoc. Cancer Res.* **2001**, *42*, 213.
6. Janmaat, M.; Rodriguez, J.; Jimeno, J.; Kruyt, F.; Giaccone, G. *Mol. Pharmacol.* **2005**, *68*, 502–510.
7. Von Schwarzenberg, K.; Vollmar, A.M. *Cancer Lett.* **2013**, *332*, 295–303.
8. Shilabin, A. G.; Kasanah, N.; Wedge, D. E.; Hamann, M. T. *J. Med. Chem.* **2007**, *50*, 4340.
9. Jimenez, J. C.; López-Macià, A.; Gracia, C.; Varon, S.; Carrascal, M.; Caba, J. M.; Royo, M.; Francese, A. M.; Cuevas, C.; Giralte, E.; Albericio, F. *J. Med. Chem.* **2008**, *51*, 4920.
10. Wang, B.; Waters, A. L.; Valeriote, F. A.; Hamann, M. T. *Biochim. Biophys. Acta* **2015**, *1850*, 1849–1854.
11. ¹H and ¹³C NMR spectra of KF recorded in DMSO-d₆ were included in the Supporting Information of ref. 4. Full assignment of proton and carbon values, not previously published, has been made in this work and reported in Supplementary Material.
12. Tilvi, S.; Naik, G. C. *J. Mass Spectrom.* **2007**, *42*, 70-80.
13. Goetz, G.; Yoshida, W. Y.; Scheuer, P. J. *Tetrahedron* **1999**, *55*, 7739–7746.
14. Plaza, A. and Bewley, C. A. *J. Org. Chem.*, **2006**, *71*, 6898-6907.
15. Devi, P.; Wahidulla, S.; Tonima, K.; D’Souza, L. *J. Geo. Mar. Sci.* **2011**, *40*, 338-346.
16. Horgen, F. D.; delos Santos, D. B.; Goetz, G.; Sakamoto, B.; Kan, Y.; Nagai, H.; Scheuer, P. J. *J. Nat. Prod.* **2010**, *63*, 152-154.
17. Carbone, M.; Ciavatta, M.L.; Wang, J.R.; Cirillo, I.; Mathieu, V.; Kiss, R.; Mollo, E.; Guo, Y.W.; Gavagnin, M. *J. Nat. Prod.* **2013**, *76*, 2065-2073.
18. Berger, G.; Gasper, R.; Lamoral-Theys, D.; Wellner, A.; Gelbcke, M.; Gust, R.; Nève, J.; Kiss, R.; Goormaghtigh, E.; Dufresne, F. *Int. J. Oncol.* **2010**, *37*, 679-686.
19. Eamvijarn, A.; Kijjoa, A.; Bruyère, C.; Mathieu, V.; Manoch, L.; Lefranc, F.; Silva, A.; Kiss, R.; Herz, W. *Planta Med.* **2012**, *78*, 1767-1776.

