Keramamide A, a Novel Peptide from the Okinawan Marine Sponge *Theonella* sp.

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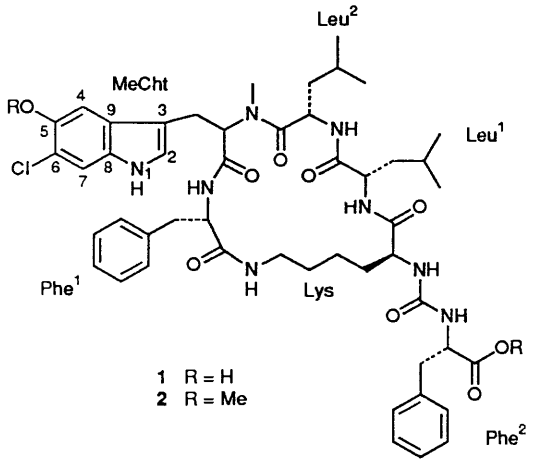
A novel peptide, keramamide A **1**, has been isolated from the Okinawan marine sponge *Theonella* sp. and the structure established as a unique hexapeptide containing a hitherto unknown amino acid 6-chloro-5-hydroxy-*N*-methyltryptophan, and possessing an unusual ureido bond. The structural assignment was made on the basis of spectroscopic results (two-dimensional NMR: ^{1}H–^{1}H COSY, NOESY, ROESY, COLOC, HMQC, HMBC and HOHAHA; and FAB MS/MS).

Marine sponges of the genus *Theonella* have been shown to be a rich source of unique secondary metabolites with intriguing structures and interesting biological activities.[1–3] During our continuing studies on bioactive substances from Okinawan marine organisms,[4] we recently investigated extracts of a sponge belonging to the genus *Theonella* and isolated the novel peptide, keramamide A **1**. Here we describe the isolation and structure elucidation of **1**. Keramamide A **1** consists of six amino acid residues, one of which, 6-chloro-5-hydroxy-*N*-methyltryptophan (MeCht), was hitherto unknown; it also contains an unusual ureido bond consisting of the α-NH of the lysine and the α-NH of the phenylalanine residues. The structure was fully established on the basis of extensive spectroscopic analyses including several types of two-dimensional NMR studies as well as FAB MS/MS experiments.



1 R = H
2 R = Me

The sponge,† collected off Kerama Islands, Okinawa, was extracted with methanol–toluene (3:1). The toluene- and chloroform-soluble fractions of the extract were subjected to flash chromatography on a silica gel column with methanol–chloroform (50:50) followed by gel filtration on Sephadex LH-20 with methanol and reversed-phase HPLC on ODS (methanol–water–trifluoroacetic acid, 80:20:0.1) to give keramamide A‡ **1** (0.001% yield, wet weight).

Keramamide A **1** was negative to ninhydrin but positive to Fast Red B salt,[5] indicating the absence of an *N*-terminus and the presence of a pyrrole chromophore. The molecular formula was determined as $C_{49}H_{63}ClN_8O_9$ by HRFABMS [positive, *m/z* 943.4485 $(M + H)^+$ for $C_{49}H_{64}ClN_8O_9$, Δ 0.0 mmu]. A standard amino acid analysis of the acid hydrolysate of **1** suggested the presence of leucine (Leu), phenylalanine (Phe), and lysine (Lys) residues. The presence of a carboxy group was inferred from the broad IR absorption at 3600–2400 cm^{-1} and a broad proton signal at δ_H 12.7 in the ^{1}H NMR spectrum of **1**. Treatment of **1** with diazomethane afforded a methyl ester **2** in which the 5-hydroxy group in MeCht had been methylated.§ An extensive NMR analysis on **1** (see Table 1) including ^{1}H–^{1}H COSY, HOHAHA,[6] HMQC,[7] COLOC,[8] and HMBC[9] spectra recorded in [$^2H_6$]-DMSO showed the presence of the spin systems of six amino acid residues, namely, two Leu, two Phe, one Lys and previously unknown MeCht. For the MeCht residue, the 2,5,6-trisubstituted indole portion was clearly supported by the HMBC correlations and the ^{13}C chemical shifts of the indole ring carbons were consistent with calculated values.[10] Fairly highfield resonances for one of the β-protons (δ_H–0.45) and methyl protons (δ_H 0.27 and 0.44) of one leucine residue (Leu^2) were observed. These large shieldings were accounted for by diamagnetic anisotropy due to the ring current effects[11] of the indole nucleus and this suggested that the Leu^2 residue adjoined the MeCht residue. Evidence for the amino acid sequence of **1** was provided by NOESY, ROESY,[12] COLOC and HMBC correlations and established that the sequence for the cyclic pentapeptide moiety was cyclo-(Phe^1–MeCht–Leu^2–Leu^1–Lys).¶ The remaining Phe^2 residue was shown to be attached to the α-NH of Lys through an unusual ureido linkage by the NOESY correlation of NH(Phe^2)/α-NH(Lys) as well as the HMBC cross peaks for NH(Phe^2)/CO(ureido; δ_C 156.8), α-H(Phe^2)/CO(ureido), and α-NH(Lys)/CO(ureido). It was established that the free carboxy group was present in the branched Phe^2 group. The proposed structure **1** for keramamide A based on the above NMR data was wholly supported by FAB MS/MS[13] evidence. The daughter ions obtained by the collisionally activated dissociation (CAD) spectra of the molecular protonated ions (*m/z* 943 and 945) are presented in Table 2. The presence or not of a chlorine atom in a particular daughter ion was established

† The brown sponge *Theonella* sp. used in this study was characterized by a yellow inner body.

‡ **1**: $[\alpha]_D^{20}$ −190 (*c* 0.03, MeOH); ν_{max}/(KBr)/cm^{-1} 3250, 3050, 1715, 1640, 1540 and 1020; λ_{max}(MeOH)/nm 213 (ε 26 000), 287 (5600), 303 (5200) and 315sh.

§ **2**: $\delta_H(CDCl_3)$ 3.48 and 3.93 (each 3 H, s, 2 × MeO); FABMS *m/z* 939 ($M^+ + H - MeOH$; hydantoin ion analogous to **a**).

¶ The following sequential cross peaks were observed: [NOESY and/or ROESY (H/H)] NH(Phe^1)/α-H(MeCht), α-H(Phe^1)/α-H(MeCht), α-H(MeCht)/α-H(Leu^2), NH(Leu^2)/α-H(Leu^1), NH(Leu^1)/β-H$_2$(Lys), NH(Leu^1)/α-NH(Lys), ε-NH(Lys)/α-NH(Phe^1) and ε-NH(Lys)/ α-H(Phe^1); [HMBC and/or COLOC (H/C)] NH(Phe^1)/CO(MeCht), NMe(MeCht)/CO(Leu^2), NH(Leu^2)/CO(Leu^1), NH(Leu^1)/CO(Lys) and ε-NH(Lys)/CO(Phe^1).

Table 1 ^1H and ^{13}C NMR spectral data for keramamide A **1** recorded in [$^2H_6$]-DMSO

Position		^1H		J/Hz	^{13}C	
Phe[1]	CO				170.8	s
	NH	8.69	d	8.8		
	α	4.52	m		54.7	d
	β	2.73	dd	14.2, 5.5	37.8	t
		3.36	dd	14.2, 3.2		
	1				138.2	s
	2,6	7.08 (2 H)	d	6.8	128.8	d
	3,5	7.15–7.30	m		128.2	d
	4	7.15–7.30	m		126.0	s
MeCht	CO				169.5	s
	NMe	1.94 (3 H)	s		27.5	q
	α	4.69	m		60.9	d
	β	2.76	dd	14.5, 5.5	22.2	t
		3.07	dd	14.5, 2.8		
	1-NH	10.65	d	2.0		
	2	6.89	d	2.0	125.0	d
	3				108.9	s
	4	6.99	s		103.7	d
	5				145.8	s
	6				115.9	s
	7	7.28	s		111.7	d
	8				130.3	s
	9				126.7	s
	5-OH	9.13	br s			
Leu[2]	CO				172.2	s
	NH	8.49	d	5.4		
	α	4.25	m		47.1	d
	β	−0.45	t	11.2	37.3	t
		0.97	m			
	γ	1.40	m		23.2	d
	Me	0.44 (3 H)	d	6.6	22.4	q
	Me′	0.27 (3 H)	d	6.6	19.7	q
Leu[1]	CO				173.2	s
	NH	6.89	d	2.4		
	α	4.07	m		50.8	d
	β	1.51 (2 H)	m		39.5	t
	γ	1.71	m		23.8	d
	Me	0.89 (3 H)	d	6.6	22.8	q
	Me′	0.84 (3 H)	d	6.6	21.7	q
Lys	CO				172.0	s
	α-NH	6.39	d	7.6		
	α	3.88	m		54.5	d
	β	1.40 (2 H)	m		31.8	t
	γ	1.51 (2 H)	m		20.2	t
	δ	1.40 (2 H)	m		28.2	t
	ε	2.85	m		38.2	t
		3.60	m			
	ε-NH	7.40	dd	8.1, 2.7		
Phe[2]	CO_2H				173.5	s
	CO_2H	12.7	br s			
	NH	6.22	d	8.3		
	α	4.36	m		53.7	d
	β	2.87	dd	13.9, 7.6	37.6	t
		3.00	dd	13.9, 5.4		
	1				137.3	s
	2,6	7.15–7.30	m		129.1	d
	3,5	7.15–7.30	m		128.1	d
	4	7.15–7.30	m		126.3	s
	CO(ureido)				156.8	s

by comparison of the CAD spectra of the two parent ions (m/z 943 with ^{35}Cl and m/z 945 with ^{37}Cl). In this way the presence of the ureido bond (m/z 778/780 **b** and 752/754 **c**)* and the amino acid sequence in keramamide A **1** were firmly established. The

* The hydantoin ion **a** also corroborated the presence of the ureido bond.[14]

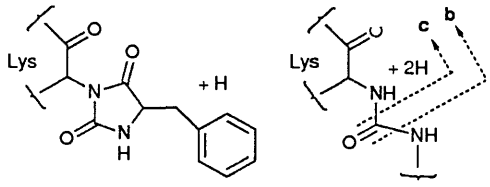
† The absolute configuration of the MeCht residue remains undefined.

‡ Keramamide A **1** exhibited no cytotoxicity against murine lymphoma L1210 and human epidermoid carcinoma KB cells in vitro at 10 μg/mL.

Table 2 FAB MS/MS data for keramamide A **1**

m/z[a]	m/z[b]	Assignment of daughter ions[c,d]
943	945	M + H (parent ion)
925	927	M + H-H_2O (hydantoin ion); **a**
830	832	MeCht-Phe-Lys(-urPhe)-Leu + H
778	780	Leu-Leu-MeCht-Phe-Lys-CO(ureido); **b**
752	754	Leu-Leu-MeCht-Phe-Lys + 2 H; **c**
580	580	Phe-Lys(-urPhe)-Leu + H
477	479	Leu-Leu-MeCht + H
467	467	Phe-Lys(-urPhe) + H
439	439	Phe-Lys(-urPhe) − CO + H
433	433	Lys(-urPhe)-Leu + H
398	400	MeCht-Phe + H
320	320	Lys(-urPhe) + H
251	253	MeCht + H
223	225	MeCht − CO + H
180	182	MeCht − COCHNMe

[a] For ^{35}Cl parent ion. [b] For ^{37}Cl parent ion. [c] The amide bond cleavages are assumed to occur between NH (or NMe) and CO (the B-type fragmentation).[17] [d] 'urPhe' denotes phenylalanine attached through an ureido bond.



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chiral GC analysis (Chirasil-Val®, Alltech) of the *N*-trifluoroacetyl/methyl ester derivatives of the hydrolysate of **1** clarified that all of the Leu, Phe and Lys residues in **1** were L-forms.† The structure of keramamide A was thus established as **1**. Of the six amide-NH protons, NH(Phe[1]), ε-NH(Lys) and NH(Leu[2]) showed a very slow deuterium-exchange rate. It appears likely from model considerations that the NH(Phe[1]) and ε-NH(Lys) are hydrogen-bonded with oxygen atoms of the amide carbonyls of Leu[2] and Lys, respectively, and the NH(Leu[2]) is sterically hindered by hydrophobic alkyl side chains of the two Leu (Leu[1] and Leu[2]) residues.

Keramamide A **1** is a unique peptide with a modified tryptophan residue (MeCht) and an ureido bond first isolated from marine organisms. This peptide may be produced by any symbiotic microorganism in the sponge *Theonella* sp.[14] Keramamide A **1**‡ exhibited inhibitory activity against sarcoplasmic reticulum Ca^{2+}-ATPase[15,16] (IC_{50} 3 × 10^{-4} mol dm^{-3}).

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