cis,cis- and *trans,trans*-Ceratospongamide, New Bioactive Cyclic Heptapeptides from the Indonesian Red Alga *Ceratodictyon spongiosum* and Symbiotic Sponge *Sigmadocia symbiotica*

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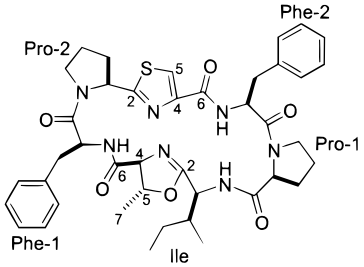
Chemical investigation of the marine red alga (Rhodophyta) *Ceratodictyon spongiosum* containing the symbiotic sponge *Sigmadocia symbiotica* collected from Biaro Island, Indonesia, yielded two isomers of a new and bioactive thiazole-containing cyclic heptapeptide, *cis,cis*-ceratospongamide (**1**) and *trans,trans*-ceratospongamide (**2**). Isolation of these peptides was assisted by bioassay-guided fractionation using a brine shrimp toxicity assay (*Artemia salina*). The structures of the ceratospongamides, which each consist of two L-phenylalanine residues, one (L-isoleucine)-L-methyloxazoline residue, one L-proline residue, and one (L-proline)thiazole residue, were established through extensive NMR spectroscopy, including $^{1}H-^{13}C$ HMQC−TOCSY, and $^{1}H-^{15}N$ HMBC experiments, as well as chemical degradation and chiral analysis. *cis,cis*- and *trans,trans*-ceratospongamide are stable conformational isomers of the two proline amide bonds. Molecular modeling of these two ceratospongamide isomers showed the *trans,trans* isomer to be quite planar, whereas the *cis,cis* isomer has a more puckered overall conformation. *trans,trans*-Ceratospongamide exhibits potent inhibition of $sPLA_2$ expression in a cell-based model for antiinflammation (ED_{50} 32 nM), whereas the *cis,cis* isomer is inactive. *trans,trans*-Ceratospongamide was also shown to inhibit the expression of a human-$sPLA_2$ promoter-based reporter by 90%.

Introduction

The marine red macroalga, *Ceratodictyon spongiosum* Zanardini (Rhodophyta), is a widely distributed tropical Indo-Pacific species occurring between southern Japan and eastern Australia. It is an intriguing organism in that the plant grows in nature with a sponge symbiont, *Sigmadocia symbiotica*.[1] The thallus of this algal species consists of a reticular meshwork of algal filaments that is surrounded and covered by the sponge symbiont. Despite its wide occurrence, information on its chemical constituents is lacking with the only report being a series of sphingosine-derived ceramides isolated from a Taiwanese collection of *C. spongiosum*.[2] As part of our continued search for bioactive secondary metabolites from marine sources, we have examined the organic extract of an Indonesian collection of this species and were able to isolate two stable conformers of a bioactive cyclic heptapeptide, *cis,cis*- (**1**) and *trans,trans*-ceratospongamide (**2**). These isolation efforts were guided by a brine shrimp toxicity assay.



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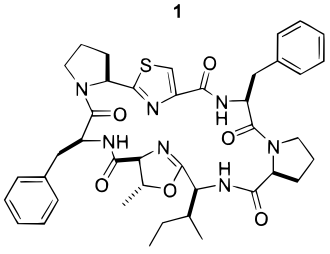

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Further evaluation of the bioactivity of these metabolites revealed the minor component, *trans,trans*-ceratospongamide (**2**), to be a potent inhibitor of the expression of a key enzyme in the inflammatory cascade, secreted phospholipase A_2 ($sPLA_2$), with an ED_{50} of 32 nM. The *cis,cis* conformer (**1**) was inactive. Additionally, the anti-$sPLA_2$ activity of *trans,trans*-ceratospongamide was observed in an sPLA2 promoter-based reporter assay.[3] During the inflammation process, the pro-inflammatory cytokine interleukin IL-1β induces the increased expression of $sPLA_2$ in various cell types, such as rheumatoid

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synoviocytes and hepatocytes.[4−7] The increased expression of $sPLA_2$ by IL-1β involves a complex signal transduction pathway involving proteins such as TRAF, MAP kinases, NFκ-β, and the *fos*/*jun* family of transcription factors.[8,9] Released $sPLA_2$ catalyzes the hydrolysis of arachidonic acid from the *sn*-2 position of membrane phospholipids, thus providing substrates for eicosanoid biosynthesis, including leukotrienes, prostaglandins, and thromboxanes, which are potent downstream mediators of inflammation implicated in several disease states including arthritis and sepsis. $sPLA_2$ has therefore become an important target for therapeutic agents in the control of inflammation.[10] Herein, we report the isolation, structural characterization, and biological profile of these unique cyclic peptides obtained from the Indonesian red alga *C. spongiosum* and its symbiotic sponge *S. symbiotica*.

Results and Discussion

Fresh samples of the symbiotic red alga *C. spongiosum* and sponge *S. symbiotica* were collected by hand in shallow reefs off the coast of Biaro Island, Indonesia. Upon collection, the specimens were steeped in isopropyl alcohol and kept frozen (−20 °C). The sample was subsequently thawed and repetitively extracted using CH_2Cl_2/MeOH to yield about 2 g of organic extract. A brine shrimp toxicity bioassay was used to direct the fractionation of the extract using a combination of normal-phase silica vacuum flash chromatography and repeated reversed-phase HPLC. This process afforded two stable conformers of a cyclic heptapeptide, *cis*,*cis*-ceratospongamide (**1**) as the major form and *trans*,*trans*-ceratospongamide (**2**) as the minor. Structural characterizations of these metabolites were carried out using a battery of 2D NMR experiments including $^1H-^{13}C$ HMQC−TOCSY and $^1H-^{15}N$ HMBC.

cis,*cis*-Ceratospongamide (**1**) was obtained as a white amorphous solid of molecular formula $C_{41}H_{49}N_7O_6S$ by HR-FABMS [positive ion, 3-nitrobenzyl alcohol, *m*/*z* 768.3545 $(M + H)^+$, (Δ +0.2 mmu)], yielding 21 degrees of unsaturation. Several features of the ^1H and ^{13}C NMR spectra suggested the peptidic nature of compound **1**; three amide-type protons were observed at δ 6.51, 6.58, and 8.12, and several ^{13}C NMR signals attributable to amide or ester carbonyls were located between δ 169−171. Moreover, the IR spectrum contained bands at 3295 and 1647 cm^{-1}, characteristic of peptide bonds. The cyclic nature of the compound was suggested from a positive ninhydrin test only following acidification (6 N HCl) and heating (100 °C) of the developed TLC plate. Confirmation of the peptidic nature of compound **1** came from routine amino acid analysis of the hydrolysate, which showed the presence of 1 molar equiv each of proline and isoleucine and 2 molar equiv of phenylalanine. However, further analysis by ^{13}C NMR and HMQC spectra revealed two units of proline and phenylalanine; the decreased molar equivalency of proline from amino acid analysis is likely due to this residue's acid lability.

The structure of **1** was determined by careful analysis of both one- and two-dimensional NMR spectral measurements, such as $^1H-^1H$ COSY, $^1H-^{13}C$ HMQC, $^1H-^{13}C$ HMQC−TOCSY, and HMBC, which revealed six independent spin systems. Particularly useful was HMQC−TOCSY, which unambiguously defined the spin system of individual amino acid units as well as resolved many overlapping proton signals. For instance, the α-protons of Pro-1 (δ 3.42) and Pro-2 (δ 5.14) showed correlations to carbon signals within the following proton spin systems: δ 61.1 (C^α), δ 30.9 (C^β), δ 21.8 (C^γ), and δ 46.6 (C^δ) for Pro-1 and δ 59.5 (C^α), δ 35.0 (C^β), δ 21.4 (C^γ), and δ 46.4 (C^δ) for Pro-2. Likewise, both α-protons of Phe-1 (δ 4.73) and Phe-2 (δ 4.74) have correlations to their respective C^α and C^β signals: δ 52.1 (C^α) and δ 39.0 (C^β) for Phe-1 and δ 53.7 (C^α) and δ 40.5 (C^β) for Phe-2. The β-protons in Phe-1 and Phe-2 in turn showed long-range HMBC correlations to carbon signals belonging to their respective phenyl groups. The presence of an Ile-like unit was confirmed from HMQC-TOCSY data which showed correlations between $C^{\gamma 2}H_3$-protons (δ 0.82) and carbon signals δ 11.6 ($C^{\delta 1}$), δ 24.6 ($C^{\gamma 1}$), δ 38.1 (C^β), and δ 51.4 (C^α).

Another spin system in **1** belonged to a cyclic unit, a methyl oxazoline ring, which was inferred from two characteristic mid-field carbon signals at δ 73.6 and 81.4 as well as an upfield methyl signal at δ 21.2. In addition, the HMQC−TOCSY showed correlations from the H-7 methyl protons to these midfield carbon signals. Together with the observation of H-4 (δ 3.17) and H-5 proton (δ 3.94) correlations to a carbon signal at δ 168.7 (C-2) by HMBC, a methyloxazoline moiety was firmly established to be part of compound **1**.

The final residue in **1** consisted of a broad singlet proton resonance at δ 8.01 with an associated carbon atom at δ 124.2, indicative of a thiazole ring. HMBC correlation of this proton signal to carbon signals δ 148.6 (C-4), δ 159.4 (C-6), and δ 169.8 (C-2) further supported the thiazole unit and correlated well with other marine derived thiazole-containing cyclic peptides in the literature.[11]

Determination of the sequence and connection of amino acid residues and other units (thiazole, methyl oxazoline) in **1** was achieved primarily by long range $^{13}C-^1H$ correlation experiments (HMBC) with different mixing times and a ROESY experiment. Characterization of a (proline)thiazole unit was established by the following HMBC correlations: H^α (Pro-2), $H^{\beta 1}$ (Pro-2), $H^{\beta 2}$ (Pro-2), and H-5 (thiazole) to C-2 (thiazole); H-5 (thiazole) to C-4 (thiazole). The (isoleucine)methyloxazoline segment was deduced in a similar manner by observing the following correlations: H^α (Ile), H^β (Ile), H-4 (Me-oxazoline), and H-5 (Me-oxazoline) to C-2 (Me-oxazoline). Two overlapping carbonyl signals at δ 169.6 (Me-oxazoline C-6 and Phe-1 C′) were assigned by the following HMBC correlations: H-4 (Me-oxazoline) and H^N (Phe-1) to C-6 (Me-oxazoline); H^α (Pro-2), $H^{\delta 1}$ (Pro-2), $H^{\delta 2}$ (Pro-2), and H^α (Phe-1) to C′ (Phe-1). Additional information from ROESY data confirmed the Phe-1-Pro-2 unit with a strong

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Table 1. ^{1}H and ^{13}C NMR Spectral Data (in ppm) for *cis,cis*-Ceratospongamide (1)[a] with HMBC and ROESY Correlations

amino acid	position[b]	^{1}H	m	*J* (Hz)	^{13}C	HMBC	ROESY
proline-1	H^{α}	3.42	d	7.9	61.1	21.8, 30.9, 169.7, 170.4	4.60, 4.74, 6.51
	H^{β}	1.09	m		30.9	21.8, 46.6, 61.1, 169.7	
	H^{β}	1.89	m			21.8, 46.6, 61.1, 169.7	
	H^{γ}	1.55	m		21.8	30.9, 46.6	1.89, 3.68
	H^{γ}	1.75	m			30.9, 61.1	
	H^{δ}	3.46	m		46.6	30.9, 61.1, 170.4	
	H^{δ}	3.68	m			30.9, 61.1	1.55, 1.75, 6.51
	C′				169.7		
isoleucine	H^{α}	4.60	dd	9.7, 1.7	51.4	15.5, 24.6, 38.1, 168.7, 169.7	0.82, 0.87, 1.73, 3.42, 6.51
	H^{β}	1.73	m		38.1	11.6, 15.5, 24.6, 51.4, 168.7	1.28
	$H^{\gamma 1}$	1.04	m		24.6	11.6, 15.5, 38.1, 51.4	
	$H^{\gamma 1}$	1.28	m			11.6, 15.5, 38.1, 51.4	4.60
	$(H^{\gamma 2})_3$	0.82	d	6.8	15.5		
	$(H^{\delta 1})_3$	0.87	t	7.4	11.6	24.6	
	H^{N}	6.51	d	9.7		51.4, 169.7	
Me-oxazoline	2				168.7		
	4	3.17	d	9.5	73.6	21.2, 81.4, 168.7, 169.6	1.28, 3.94, 4.74, 6.58
	5	3.94	m		81.4	73.6, 168.7, 169.6	
	6				169.6		
	7	1.24	d	6.2	21.2	73.6, 81.4	
phenylalanine-1	H^{α}	4.73	dt	8.4, 8.2, 7.3	52.1	39.0, 169.6	
	H^{β}	2.81	dd	13.2, 6.8	39.0	52.1, 136.9, 169.6	
	H^{β}	3.01	dd	13.2, 8.3		52.1, 136.9, 169.6	
	C^{γ}				136.9		
	$H^{\delta 1}$, $H^{\delta 2}$	7.23	m		129.4	128.1, 136.9	
	$H^{\epsilon 1}$, $H^{\epsilon 2}$	7.19	m		128.1	126.5, 129.4	
	H^{ζ}	7.19	m		126.5	128.1	
	C′				169.6		
	H^{N}	6.58	d	8.7		52.1, 169.6	2.81, 3.01, 4.73
proline-2	H^{α}	5.14	dd	7.4, 1.6	59.5	21.4, 46.4, 35.0, 169.8	2.04, 2.31, 3.95, 4.73
	H^{β}	2.04	m		35.0	21.4, 46.4, 169.8	1.89
	H^{β}	2.31	ddt	12.3, 7.6, 7.5		21.4, 59.5, 46.4, 169.8	
	H^{γ}	1.89	m		21.4	35.0, 46.4, 59.5	
	H^{γ}	1.89	m			35.0, 46.4, 59.5	
	H^{δ}	3.46	m		46.4	35.0, 59.5, 169.6	
	H^{δ}	3.95	m			59.5	
thiazole	2				169.8		
	4				148.6		
	5	8.01	s		124.2	59.5, 148.6, 159.4, 169.8	
	6				159.4		
phenylalanine-2	H^{α}	4.74	dt	10.7, 6.4, 4.9	53.7	40.5, 170.4	
	H^{β}	2.85	dd	12.8, 10.6	40.5	53.7, 136.0, 170.4	
	H^{β}	3.50	m				
	C^{γ}				136.0		
	$H^{\delta 1}$, $H^{\delta 2}$	7.34	m		129.4	128.9, 136.0	
	$H^{\epsilon 1}$, $H^{\epsilon 2}$	7.28	m		128.9	127.4, 129.4	
	H^{ζ}	7.34	m		127.4	128.9	
	C′				170.4		
	H^{N}	8.12	d	6.5		53.7, 159.4, 170.4	

[a] All spectra measured in $CDCl_3$ and referenced to residual solvent signal at δ_H 7.26 and δ_C 77.4. [b] Nomenclature in identifying amino acid residues in accordance with ref 24.

correlation between H^{α} (Pro-2) and H^{α} (Phe-1). Similarly, other residue connections were assigned from HMBC correlations: H^{α} (Pro-1) and H^{N} (Ile) to C′ (Pro-1); H-5 (thiazole) and H^{N} (Phe-2) to C-6 (thiazole); H^{α} (Phe-2), H^{α} (Pro-1), and H^{δ} (Pro-1) to C′ (Phe-2) (Table 1).

Information from a ^{1}H−^{15}N HMBC was used to confirm the sequence developed from the above HMBC data.[12−14] Such long-range ^{1}H−^{15}N coupling data are particularly useful since ^{15}N chemical shifts are more widely dispersed than those of ^{13}C or ^{1}H.[15] One of the two main residue sequences, Pro-2-thiazole-Phe-2, was confirmed from the following ^{1}H−^{15}N HMBC cross-peak data: N (thiazole)/H^{α} (Pro-2) and H^{N} (Phe-2); N (Phe-2)/H^{α} (Phe-2), $H^{\beta 1}$ (Phe-2), and $H^{\beta 2}$ (Phe-2). The second sequence, Pro-1-Ile-Me-oxazoline-Phe-1, was deduced as follows: N (Ile)/H^{α} (Pro-1) and H^{α} (Ile); N (Me-oxazoline)/H^{α} (Ile), H-4 (Me-oxazoline); N (Phe-1)/H-4 (Me-oxazoline) and H^{α} (Phe-1). Selected ^{1}H−^{15}N HMBC correlations are depicted in Figure 1. In addition, positive-ion FABMS of **1** gave fragmentation patterns supportive of the proposed amino acid sequence (Figure 2).

Absolute stereochemistry of *cis,cis*-ceratospongamide (**1**) was characterized by ozonolysis followed by acid hydrolysis and analysis by Marfey's method[16] as well as chiral GC−MS. Ozonolysis avoided racemization of Pro-2 by destroying the aromaticity of the thiazole.[17] Acid

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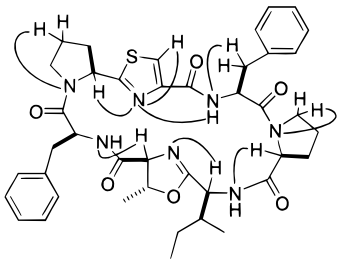

Figure 1. Selected correlations observed by $^1H-^{15}N$ HMBC of *cis,cis*-ceratospongamide (**1**).

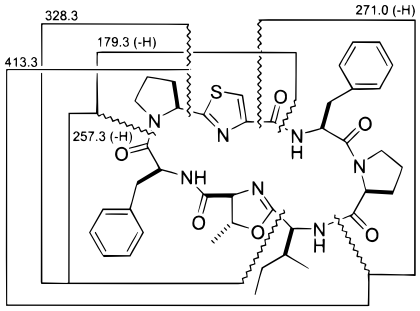

Figure 2. FAB mass spectral fragmentations observed for *cis,cis*-ceratospongamide (**1**) and *trans,trans*-ceratospongamide (**2**).

hydrolysis and derivatization with Marfey's reagent was followed by comparative HPLC analysis with derivatized standard D- and L-amino acids and established an L-configuration for each (L-isoleucine, L-phenylalanine, L-proline, and L-threonine). The all L-configuration for residues in **1** was also supported by chiral GC−MS analysis of the pentafluoropropyl isopropyl ester derivatives of the acid hydrolysate (see experimental).

The minor component, *trans,trans*-ceratospongamide (**2**), was also obtained as a white amorphous solid with IR and UV spectra identical to that of **1**. While metabolite **2** had an identical molecular formula as compound **1** as deduced by HR-FABMS [positive ion, 3-nitrobenzyl alcohol, *m/z* 768.3542 $(M + H)^+$ (Δ −0.1 mmu)], the magnitude of its optical rotation was different $[\alpha]_D$ −39.2° ($c = 0.52$). Analysis of COSY, HMQC, HMQC−TOCSY, and HMBC data showed that the planar structures of **1** and **2** were identical. Moreover, stereochemical analysis of **2** using Marfey's method[16] indicated that all amino acids were of the L-configuration. By NMR chemical shift analysis, the differences between **1** and **2** were confined to signals associated with the two proline units and, to some degree, the amino acids adjacent to these two proline units.

It is well documented that *cis*/*trans* conformational differences of proline amide bonds correlates with differential values between proline β and γ carbons ($\Delta\delta_{\beta\gamma}$).[18] In compound **2**, $\Delta\delta_{\beta\gamma}$ of Pro-1 and Pro-2 are 4.4 and 7.0 ppm, respectively. This is markedly different than the $\Delta\delta_{\beta\gamma}$ of Pro-1 and Pro-2 of **1** (9.1 and 13.6 ppm, respectively). Hence, both proline amide bonds in **1** are *cis* while those in **2** are *trans*. These conformational assignments are further supported from ROESY data which showed a strong correlation between the α-protons of Pro-2/Phe-1 as well as Pro-1/Phe-2 in the *cis* conformation (**1**), but no such correlations in the *trans* form (**2**). Therefore, the relationship of these two peptides is that of two stable proline peptide bond conformers.

Variable-temperature NMR experiments were performed on *cis,cis*- (**1**) and *trans,trans*-ceratospongamide (**2**) in order to probe (1) the interconvertability of these two proline amide conformers and (2) the degree and nature of intramolecular hydrogen bonding within these two molecules.[19] ^1H NMR spectra of the *cis,cis* isomer (**1**)- in DMSO over the range 25−90 °C showed no detectable conversion to the *trans,trans* isomer (**2**). However, when the cis,cis isomer (**1**) was heated to 175 °C in DMSO, the conversion to the *trans,trans* isomer (**2**) was detected using gradient HPLC under two different reversed-phase HPLC conditions [Phenomenex Phenosphere 10 C_8 90A and Phenomenex Sphereclone 5 μm ODS (2)] and was confirmed by co-injection with *trans,trans*-ceratospongamide (**2**) (see the Supporting Information). Because of the very small quantity of isolated *trans,trans* isomer (**2**), high-temperature interconversion experiments beginning with **2** were not conducted. Metabolites **1** and **2** are not the first stable cyclic peptide conformers to be reported from a marine source. Theopalauamide and isotheopalauamide are a pair of conformationally stable cyclic peptides that were recently reported from a sponge.[20] The major difference between these latter two peptides is the rotation of the bond between the α-carbon and the adjacent carbonyl group in the Phe residue.

Additional ^1H NMR spectra of compounds **1** and **2** were acquired at 5, 15, and 25 °C in $CDCl_3$. For **1**, $\Delta\delta/\Delta T$ (ppb/K) values for H^N-Ile, H^N-Phe-1, and H^N-Phe-2 were 4.05, 6.50, and 0.70, respectively. These suggest that the amide proton of Phe-2 participates in an intramolecular hydrogen bond while that of Ile and Phe-1 do not. A similar data set was obtained for compound **2** yielding values of 7.60 and 1.30 ppb/K for H^N-Ile and H^N-Phe-2, respectively. No data were obtained for H^N-Phe-1 as the amide proton was hidden within the envelope of aromatic protons.

The degree of antiinflammatory activity of compounds **1** and **2** was measured as the inhibition of secreted phospholipase A_2 by hepatocellular carcinoma cells stimulated with IL-1β. The *trans,trans* form (**2**) is a potent inhibitor of $sPLA_2$ expression with an ED_{50} of 32 nM. No inhibitory activity was recorded for the *cis,cis* form (**1**) up to a maximal concentration of 32 μM. Both compounds **1** and **2** showed only moderate potency in the brine shrimp toxicity assay (LD_{50} = *ca.*13−19 μM). In IL-1β stimulated, reporter-transfected HepG2 cells pretreated with *trans,trans*-ceratospongamide (**2**) (50 ng/mL), the expression of the reporter (−326 to +20) was reduced by 50% relative to control and 90% in the (−159 to +20) plasmid construct. These results taken together with the ELISA data suggest that *trans,trans*-ceratospongamide (**2**) inhibits $sPLA_2$ at the level of intracellular signaling by acting on cytoplasmic activation or transcription factor binding of specific IL-1β stimulated mediators.

The three-dimensional structures of *cis,cis*- (**1**) and *trans,trans*-ceratospongamide (**2**) were determined by molecular mechanics minimization. The 3-D models were constructed from distance geometry energy minimization calculations using additional distance restraints observed

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Table 2. ^{1}H and ^{13}C NMR Spectral Data (in ppm) for *trans,trans*-Ceratospongamide (2)[a] with HMBC and ROESY Correlations

amino acid	position[b]	^{1}H	m	*J* (Hz)	^{13}C	HMBC	ROESY
proline-1	H^{α}	4.32	t	7.3	61.4	25.5, 29.9, 169.9, 172.0	1.79, 3.49, 6.85
	H^{β}	1.98	m		29.9	25.5, 47.2, 61.4	
	H^{β}	2.19	m			61.4, 172.0	
	H^{γ}	1.79	ddq	14.3, 12.2, 7.4	25.5	29.9, 47.2, 61.4	4.32
	H^{γ}	2.04	m			29.9, 47.2, 61.4	
	H^{δ}	2.92	m		47.2	25.5, 29.9	4.98
	H^{δ}	3.49	m			25.5, 29.9, 61.4	2.89, 4.32, 4.98
	C′				172.0		
isoleucine	H^{α}	5.02	dd	8.8, 3.0	49.9	13.7, 26.2, 38.0, 168.2, 172.0	0.86, 1.01, 1.50, 8.53
	H^{β}	1.92	m		38.0	26.2	0.86, 1.01
	$H^{\gamma 1}$	1.16	ddq	13.7, 13.5, 7.3	26.2	11.9, 13.7, 38.0, 49.9	
	$H^{\gamma 1}$	1.50	ddq	13.7, 13.8, 7.6		11.9, 13.7, 38.0, 49.9	
	$(H^{\gamma 2})_3$	0.86	d	6.9	13.7	26.2, 38.0, 49.9	
	$(H^{\delta 1})_3$	1.01	t	7.3	11.9	26.2, 38.0	
	H^{N}	6.85	d	8.9		168.2, 172.0	4.32
Me-oxazoline	2				168.2		
	4	3.94	d	6.5	73.3	168.2, 170.2	1.27
	5	3.99	ddd	6.4, 6.2	80.5	21.9, 73.3	
	6				170.2		
	7	1.27	d	5.8	21.9	73.3, 80.5	
phenylalanine-1	H^{α}	5.09	dt	10.3, 3.2	52.6	37.8, 170.0	3.92
	H^{β}	3.20	dd	13.2, 3.0	37.8	52.6, 129.5, 136.8	3.92
	H^{β}	3.45	t	13.0		52.6, 129.5, 136.8	3.92
	C^{γ}				136.8		
	$H^{\delta 1}$, $H^{\delta 2}$	7.21	m		129.5	136.8, 126.6	
	$H^{\epsilon 1}$, $H^{\epsilon 2}$	7.30	t	7.4	128.0	129.5, 136.8	
	H^{ζ}	7.02	t	7.3	126.6	128.0, 129.5	
	C′				170.0		
	H^{N}	7.27	d	10.4		52.6, 170.2	
proline-2	H^{α}	5.57	dd	7.9, 2.6	57.9	24.8, 31.8, 46.4, 169.1	
	H^{β}	2.27	m		31.8	57.9	
	H^{β}	2.49	m			24.8, 57.9, 169.1	3.92
	H^{γ}	2.10	m		24.8	31.8, 46.4	
	H^{γ}	2.22	m			31.8, 46.4	
	H^{δ}	3.92	m		46.4	24.8	
	H^{δ}	3.92	m			24.8	
thiazole	2				169.1		
	4				148.7		
	5	7.98	s		122.9	148.7, 159.9, 169.1	
	6				159.9		
phenylalanine-2	H^{α}	4.98	dd	7.4, 7.3	51.9	39.6, 135.5, 159.9, 169.9	
	H^{β}	2.89	dd	13.8, 7.3	39.6	51.9, 129.5, 135.5, 169.9	
	H^{β}	2.99	dd	13.7, 6.2		51.9, 129.5, 135.5, 169.9	
	C^{γ}				135.5		
	$H^{\delta 1}$, $H^{\delta 2}$	7.13	d	7.1	129.5	126.5	
	$H^{\epsilon 1}$, $H^{\epsilon 2}$	7.16	t	7.5	128.2	129.5, 135.5	
	H^{ζ}	7.22	m		126.5	129.5	
	C′				169.9		
	H^{N}	8.53	d	8.4		51.9, 159.9, 169.9	

[a] All spectra measured in $CDCl_3$ and referenced to residual solvent signal at δ_H 7.26 and δ_C 77.4. [b] Nomenclature in identifying amino acid residues in accordance with ref 24.

from proton ROESY correlations (Tables 1 and 2) (Figure 3). The most striking difference between the two forms of ceratospongamide is the conformation of the macrocyclic ring. In the *trans,trans* isomer (**2**), the overall conformation of this ring is planar with amino acid side chains above and below the ring. Puckering of the macrocycle occurs in the *cis,cis* isomer (**1**), and the thiazole and Pro-2 rings are approximately orthogonal to their position in *trans,trans*-ceratospongamide (**2**). Moreover, Phe-1 as well as other amino acid residue side chains lie more in the plane of the macrocycle in the *cis,cis* isomer (**1**). As could be implied from the observed dramatic differences in biological activity of these two proline-amide isomers, the overall three-dimensional structures of these two substances are quite distinct (Figure 3).

Given the biological nature of this algal-sponge symbiotic pair, the true metabolic origin of these cyclic peptides is uncertain. However, recognizing the frequency of isolation of cyclic peptides from marine sponges, it would be logical that these two new cyclic peptides derive from the sponge symbiont. Further complication of this point is given by recent work by Bewley and Faulkner which provides evidence that similar cyclic peptides found in "sponges" actually derive from microorganisms living in association with their invertebrate hosts.[21] Therefore, it is uncertain if the sponge, one of the associated microorganisms, or the alga is the true source of metabolites **1** and **2**.

Experimental Section

Extraction and Isolation. *C. spongiosum* Zanardini containing symbiotic sponge *S. symbiotica* was collected on

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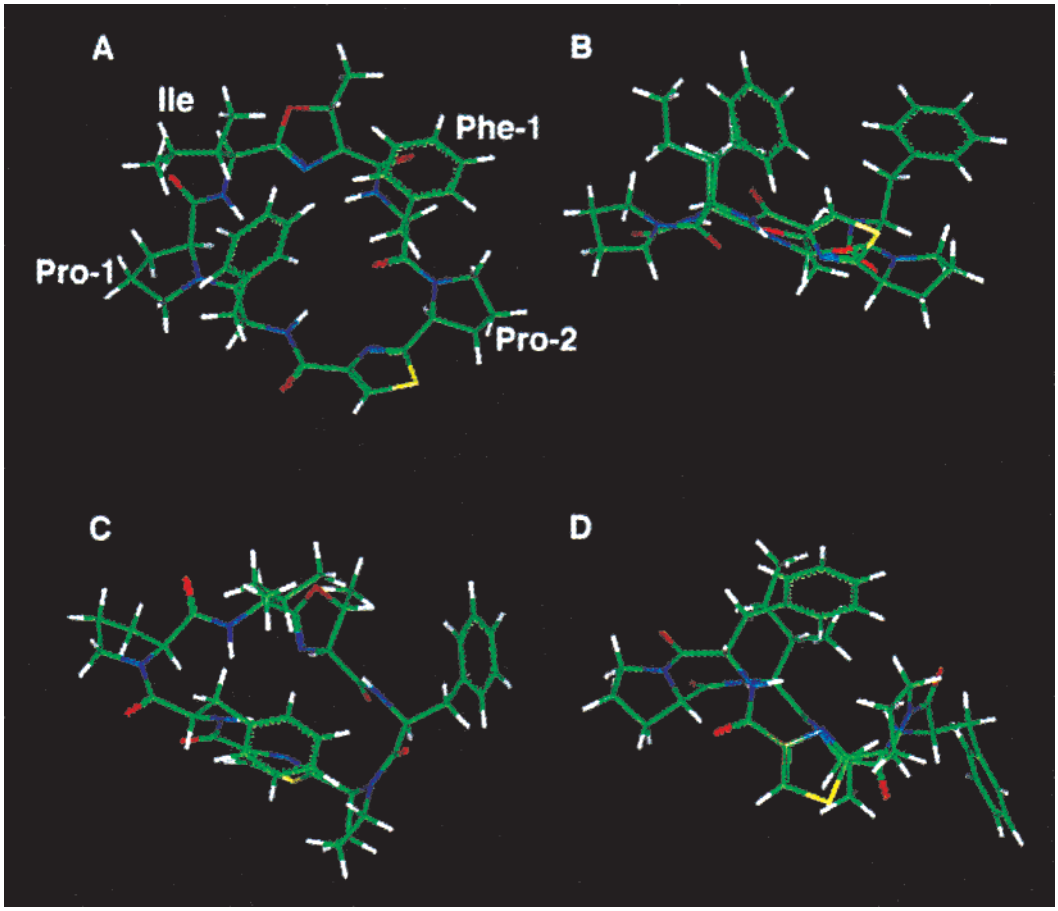

Figure 3. Molecular modeling of the ceratospongamides: (A) view of **2** looking down on macrocycle, (B) orthogonal view of **2**, (C) view of **1** looking down on macrocycle, (D) orthogonal view of **1**.

Nov 4, 1994 (IBI-4/Nov/94−15) in shallow waters by snorkeling at Biaro Island, Indonesia. The alga appeared as irregular clumps with multiple branching, commonly 10 cm or more in diameter and about 5 cm high.

The specimens were preserved in 2-propanol and maintained at −20 °C. After thawing, the sample was extracted three times using CH_2Cl_2/MeOH (2:1), and the crude extract (2 g) was initially fractionated using normal-phase Si vacuum flash chromatography (VLC) employing gradient solvent systems of hexanes/EtOAc/MeOH. Fractions eluted with EtOAc/MeOH (9:1) and EtOAc/MeOH (1:1) were combined based on their TLC profiles and refractionated over normal-phase Si VLC (gradient of $CHCl_3$/MeOH). Fractions eluting with 100% $CHCl_3$ to $CHCl_3$/MeOH (19:1) were enriched with peptides; they were combined and purified with repeated reversed-phase HPLC [Phenomenex Sphereclone 5μ ODS (2); MeOH/H_2O (78:22)] to yield pure *cis,cis*-ceratospongamide (**1**, 15.0 mg, 0.02%) and *trans,trans*-ceratospongamide (**2**, 4.0 mg, 0.005%).

***cis,cis*-Ceratospongamide (1):** white amorphous solid; $[\alpha]_D$ −190° (c = 0.13, $CHCl_3$); UV ($CHCl_3$) λ_{max} 246 nm (ϵ 11 827); IR (neat) 3385, 3295, 2967, 1647, 1534, 1444, 1200 cm^{-1}; LR−FABMS *m/z* 768 (100), 413 (7.8), 328 (5), 271 (7.1), 257 (5.7), 231 (12.8), 179 (11.5); ^1H NMR (600 MHz, $CDCl_3$) see Table 1; ^{13}C NMR (150 MHz, $CDCl_3$) see Table 1.

***trans,trans*-Ceratospongamide (2):** white amorphous solid; $[\alpha]_D$ −39.2° (c = 0.52, $CHCl_3$); UV ($CHCl_3$) λ_{max} 246 nm (ϵ 11 271); IR (neat) 3385, 3295, 2967, 1647, 1534, 1444, 1200 cm^{-1}; LR-FABMS *m/z* 768 (100), 413 (15), 328 (7.1), 271 (7.8), 257 (8.1), 231 (20), 179 (19.5); ^1H NMR (600 MHz, $CDCl_3$) see Table 1; ^{13}C NMR (150 MHz, $CDCl_3$) see Table 1.

Ozonolysis of 1 and 2. A stream of O_3 was carefully bubbled into a vial containing 1 mL of CH_2Cl_2 solution of **1** or **2** (200 μg; 0.260 μmol) at 25 °C for about 10 min. Solvent was removed under a stream of N_2, and the resulting residue was subjected to hydrolysis and derivatization as described below.

Stereochemical Determination of 1 and 2. Hydrolysis of **1** or **2** (400 μg; 0.520 μmol) was achieved in 1 mL of 6 N HCl in a sealed vial at 110 °C for 20 h. Traces of HCl were removed *in vacuo*. The resulting hydrolysate was resuspended in 30 μL of H_2O and derivatized with *N*α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA).[16] HPLC analyses of the FDAA-derivatized hydrolysate of **1** or **2** and standard FDAA-derivatized amino acids were carried out using Waters NOVAPAK C_{18} (3.9 × 150 mm column) with a linear gradient of triethylammonium phosphate (50 mM, pH 3.0)/MeCN 90:10 to 60:40 in 40 min at 1 mL/min (UV detection at λ 340 nm). The analysis established the presence of L-Thr (7.07 min) [D-Thr (11.97 min), L-allo-Thr (7.81 min), D-allo-Thr (8.50 min)], L-Pro (9.35 min), L-Ile (13.51 min) [D-Ile (15.49 min), L-allo-Ile (12.77 min), D-allo-Thr (14.77 min)], and L-Phe (13.94 min).

Amino Acid Analysis by Chiral GC. Compound **1** (200 μg) was hydrolyzed with 6 N HCl (1 mL) at 110 °C for 24 h. After the reaction mixture was dried under a stream of N_2, the amino acid hydrolysate was heated with acetyl chloride (150 μL) and ^{i}PrOH (500 μL) at 100 °C for 45 min. The mixture was dried under N_2, and the residue was treated with pentafluoropropionic anhydride (1 mL) in CH_2Cl_2 (2 mL) at 100 °C for 15 min. After 15 min, the excess reagent was evaporated with N_2 and the mixture of derivatized amino acids in CH_2Cl_2 was analyzed by GC−MS using an Alltech Capillary Chirasil-Val column (25 m × 0.25 mm; the program rate: 70−180 °C at 4 °C/min). The retention times for the *N*-pentafluoropropionyl isopropyl ester derivatives of the amino acids from compound **1** were at 6.26 min (L-Thr), 8.15 min (L-Ile), 11.02 min (L-Pro), 19.34 min (L-Phe).

Interconversion of *cis,cis*-Ceratospongamide (1) to *trans,trans*-Ceratospongamide (2). Compound **1** (1.5 mg, 1.95 μmol) was dissolved in 1.5 mL of DMSO in a vial and heated to 175 °C. After 30 min, the DMSO was removed *in*

vacuo and the residue dissolved in MeOH. This was analyzed for the presence of the *trans,trans* isomer (**2**) using gradient HPLC under two different reversed-phase HPLC conditions with a photodiode array detector: (a) Phenomenex Phenosphere 10 C_8 90A, 40% MeOH in water to 100% MeOH over 60 min, 1.5 mL/min [standard *cis,cis*-ceratospongamide (16.93 min), standard *trans,trans*-ceratospongamide (18.01 min), detected *trans,trans*-ceratospongamide (17.98 min)]; (b) Phenomenex Sphereclone 5μ ODS (2), 30% MeOH in water to 100% MeOH over 40 min, 2.4 mL/min [standard *cis,cis*-ceratospongamide (6.12 min), standard *trans,trans*-ceratospongamide (8.56 min), detected *trans,trans*-ceratospongamide (8.55 min); see the Supporting Information].

Drug Screening. Hepatocellular carcinoma cells (ATCC HB-8065) were prepared for drug screening by plating at 2×10^5/mL into 24-well tissue culture plates. After 24 h, the medium in each of the 24 wells was replaced with 1 mL of serum-free media. After a 1 h drug incubation (1% DMSO), the media in each of the drug treatment wells was replaced with minimal essential media containing 10% FBS, earle's salts, nonessential amino acids, and 400 pg/mL IL-1β (Genzyme, #80−3542−01). The cells were then incubated for 24 h. At the end of the IL-1β treatment, the cells were examined by light microscopy for signs of cytotoxicity. The conditioned media was frozen at −20 °C for further analysis by $sPLA_2$ ELISA.

Cellular Transfection. Cells and reporter plasmids were prepared as described elsewhere[3] (reporter plasmids graciously provided by Dr. Jean Luc Olivier, Laboratoire de Biochimie, Université Pierre et Marie Curie, Paris, France). IL-1β was used as the proinflammatory cytokine for these studies rather than IL-6.

CAT and $sPLA_2$ ELISA. Human-secreted-PLA_2 ELISA was completed as described elsewhere[22] (PLA_2 antibodies graciously provided by Dr. Lisa Marshall, SmithKline Beecham Pharmaceuticals, Department of Immunopharmacology, King of Prussia, PA). The expression level of reporter plasmids containing the chloramphenical acetyl transferase gene were quantified using a commercially available ELISA (Boehringer Mannheim, #1363727). Color development was measured at 490 nm on a Molecular Devices Vmax plate reader. The data were analyzed using Softmax software.

Computer Modeling. *cis,cis*- (**1**) and *trans,trans*-ceratospongamide (**2**) were refined by geometry optimization using the CVFF force field for peptides and proteins[23] as implemented in the Discover subroutine of Insight II (MSI/Biosym, San Diego, CA) molecular modeling package. The models were initially refined with 100 rounds of steepest gradient and then further refined with conjugate gradient. ROESY correlations between protons on separate residues (Tables 1 and 2) were used in energy minimization calculations by limiting their interatomic distances to within 5 Å. A Silicon Graphics Iris platform (OS Irix 6.3) was used to perform these calculations.

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Supporting Information Available: FABMS, ^{1}H NMR, ^{13}C NMR, 2D NMR spectra, and HPLC profiles of heating experiment confirming the thermal interconversion of **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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