Scleritodermin A, a Cytotoxic Cyclic Peptide from the Lithistid Sponge *Scleritoderma nodosum*

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The lithistid sponge *Scleritoderma nodosum* contains a new cyclic peptide, scleritodermin A (**1**), the structure of which incorporates L-proline, L-serine, and keto-*allo*-isoleucine units as well as a novel conjugated thiazole moiety (ACT) and *O*-methyl-*N*-sulfoserine. Scleritodermin A (**1**) inhibited tubulin polymerization and showed significant in vitro cytotoxicity against human tumor cell lines.

Marine sponges belonging to the polyphyletic order Lithistida are an excellent source of bioactive metabolites with novel chemical architectures.[1] Along with unusual sterols, alkaloids, and macrolides, they produce a diverse array of highly modified peptides, especially cyclic peptides with nonproteinogenic amino acids and polyketide-derived moieties. Lithistid peptides such as cyclotheonamides A and B and keramamide F exemplify these features.[2,3] Since the relationships between species in this order continue to challenge taxonomists,[4] chemotaxonomic studies may be of great importance, despite the fact that in one case the cyclic peptide attributed to the sponge was located in symbiotic filamentous bacteria.[5] Among the many bioactivities reported for metabolites from lithistid sponges, cytotoxicity is the most commonly reported,[1] which led us to investigate samples from the National Cancer Institute's Active Repository.[6]

Ten extracts were selected from the NCI Active Repository on the basis of taxonomy. The 10 extracts, all of which were cytotoxic in the NCI 60-cell-line panel, were partitioned between methanol and hexanes, and the methanolic extracts were fractionated using reversed-phase chromatography. On the basis of ^{1}H NMR data and electrospray ionization mass spectrometry (ESIMS), known cytotoxins and their close structural relatives could be identified in nine of the extracts.[7] In this paper we report the structural elucidation of a new peptide, scleritodermin A (**1**), from the sponge *Scleritoderma nodosum* Thiele 1900 (Scleritodermidae). Scleritodermin A (**1**) has significant in vitro cytotoxicity against a panel of human tumor cells lines and acts through inhibition of tubulin polymerization and the resulting disruption of microtubules. Because of their importance in mitotic spindle function, microtubules are the target of a number of clinically useful natural product anticancer drugs that include paclitaxel, a tubulin polymerization inducer, and vinblastine, a tubulin polymerization inhibitor.[9]

Scleritodermin A (**1**) was isolated as an off-yellow powder. HRFABMS gave an ion at m/z = 872.3740 [M − SO$_3$ + H]$^+$, indicating a formula of C$_{42}$H$_{54}$N$_7$O$_{10}$SNa (Δ = 12.7 ppm). In both ESIMS and FABMS experiments

molecular ions were observed at m/z = 930 [M − Na + H$_2$]$^+$, 952 [M + H]$^+$, and 974 [M + Na]$^+$, and a sharp IR band was observed at 1240 cm^{-1}, typical of sulfated compounds. The IR spectrum contained several peaks between 1630 and 1700 cm^{-1}, and the ^{1}H and ^{13}C NMR spectra (Table 1) exhibited standard proton chemical shifts for amino acids. In particular, several exchangeable NH signals between 7 and 9 ppm were coupled to signals between 4 and 5.5 ppm that were assigned to the α-protons of a peptide. Amino acid side chains corresponding to tyrosine, isoleucine, serine, serine methyl ether (SerOMe), and two units of proline were identified on the basis of ^{1}H, ^{13}C, GCOSY, TOCSY, GHMQC, and GHMBC NMR data and comparison with literature values for these residues.

1, Scleritodermin A

2, Oriamide homologous fragment
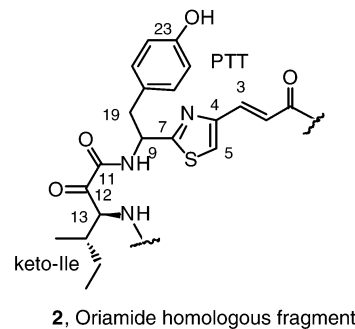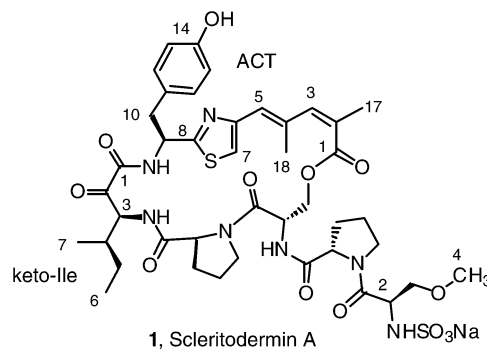
Scleritodermin A (**1**) incorporated several features not usually present in ribosomally transcribed peptides. Atypical chemical shift data together with GHMBC (Table 1) and NOESY NMR experiments revealed that both the Ile and Tyr residues were modified at their carboxyl termini.

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Table 1. ^{1}H and ^{13}C NMR Data for Scleritodermin A (**1**) in DMSO-d_6

amino acid	C no.	δ_C	δ_H	mult., J (Hz)	HMBC
ACT	1	167.9			
	2	124.6			
	3	144.5	7.54	s	C-1, C-2, C-5, C-17, C-18
	4	135.1			
	5	134.2	7.41	s	C-3, C-6, C-7, C-18
	6	149.9			
	7	119.0	7.71	s	C-6, C-8
	8	167.2			
	9	55.0	5.25	ddd, 9.5, 9, 6	C-8, C-10
	10	37.7	3.14	m, 2 H	C-8, C-11
	11	127.8			
	12/16	130.5	7.20	d, 8.5	C-10, C-12/16, C-13/15, C-14
	13/15	115.4	6.70	d, 8.5	C-11, C-12/16, C-13/15, C-14
	14	156.2			
	17	17.3	2.22	s, 3 H	C-1, C-2, C-3
	18	14.0	2.05	s, 3 H	C-3, C-4, C-5, C-6
	NH		8.76	d, 9.5	
	OH		9.28	s	C-13/15, C-14
keto-Ile	1	164.2			
	2	198.2			
	3	58.7	5.13	dd, 8, 4.5	C-2
	4	36.0	2.08	m	
	5	23.8	1.26	m, 2 H	C-4, C-6, C-7
	6	16.3	0.91	t, 3 H, 7.5	C-3, C-4, C-5
	7	11.8	0.79	d, 3 H, 6.5	C-4, C-5
	NH		7.99	d, 8	C-1(Pro)
Pro	1	171.7			
	2	59.1	4.55	dd, 9, 3	C-4
	3	30.4	1.80	m	
			2.00	m	
	4	24.2	1.80	m, 2 H	
	5	47.1	3.46	m	
			3.67	q, 8.5	
Ser	1	167.2			
	2	49.4	4.52	m	C-1
	3	62.5	3.96	t, 11	
			4.41	dd, 11, 4.5	
	NH		8.26	d, 6.5	C-1(Pro 2)
Pro 2	1	171.8			
	2	59.8	4.26	dd, 9, 3	
	3	29.6	1.84	m, 2 H	
	4	24.0	1.80	m, 2 H	
	5	46.9	3.45	m	
			3.88		
Ser-OMe	1	171.9			
	2	53.5	4.10	dt, 7.5, 5	C-1, C-3
	3	73.0	3.40	m, 2 H	
	4	58.6	3.20	s, 3 H	C-3
	NH		4.30	br	C-2

Table 2. Comparison of Chemical Shifts with Those of Oriamide (**2**), Supporting the ACT−keto-Ile Linkage (stereochemistry is not identical in these compounds)

	1				**2**		
amino acid	C no.	δ_C	δ_H	amino acid	C no.	δ_C	δ_H
ACT	4	135.1		PTT	2	123.7	6.76
	5	134.2	7.41		3	132.8	7.39
	6	149.9			4	150.0	
	7	119.0	7.71		5	123.4	7.83
	8	167.2			7	170.7	
	9	55.0	5.25		9	54.7	5.21
	10	37.7	3.14		19	39.5	3.10
							3.02
	11	127.8			20	127.7	
	12/16	130.5	7.20		21	130.7	7.16
	13/15	115.4	6.70		22	115.5	6.69
	14	156.2			23	156.5	
	NH		8.76		NH		9.37
	OH		9.28		OH		9.27
keto-Ile	1	164.2		keto-Ile	11	164.0	
	2	198.2			12	197.0	
	3	58.7	5.13		13	60.4	5.04

mamides and konbamides,[3,10] and comparison of chemical shifts for **1** with those associated with the thiazole moieties in known compounds aided in structure elucidation (Table 2). For instance, a proton singlet at δ 7.71 with a GHMQC correlation to a carbon signal at δ 119.0 is characteristic of a thiazole rather than an oxazole ring.[10] The proposed structure of ACT was supported by the observation of 14 out of 15 expected GHMBC correlations (Table 1).

The amino acid sequence of scleritodermin A (**1**) was determined by interpretation of GHMBC and NOESY correlations and was confirmed by observation of the appropriate series of mass spectral fragments and peptide sequencing. GHMBC and NOESY data led to the identification of two major fragments: one consisted of the keto-Ile-Pro-Ser-Pro-SerOMe portion and the other comprised the ACT portion. The former was defined by NOESY correlations from keto-Ile(NH) to Pro(H-2), from Pro(H-5) to Ser(H-2 and H-3), from Ser(NH) to Pro(H-2), and from Pro(H-4) to SerOMe(H-2) and by the inter-residue GHMBC correlations listed in Table 1.

Connection of the two fragments and placement of the sulfite group were accomplished by comparison of NMR data, ESIMS, and chemical sequence analysis. In particular, ESIMS fragment patterns played a role in limiting the possible structures. The following fragments were observed: 928 [M]$^-$, 848 [M − SO$_3$]$^-$, 818 [M − SO$_3$ − CH$_2$O]$^-$, 650 [M − SO$_3$ − SerOMe − Pro]$^-$, 633/632 [M − SO$_3$ − SerOMe − Pro − H$_2$O]$^-$, 581 [M − SO$_3$ − SerOMe − Pro − Ser]$^-$, 563 [M − SO$_3$ − SerOMe − Pro − Ser − H$_2$O]$^-$, 484 [M − SO$_3$ − SerOMe − Pro − Ser − Pro]$^-$, 326 [M − SO$_3$ − SerOMe − Pro − Ser − Pro − keto-Ile − H$_2$O]$^-$. These fragments unambiguously demonstrated the connectivity of keto-Ile and ACT. The connection was further supported by chemical sequencing and a comparison of chemical shift data with those from the keramamides[3] and oriamide[11] (Table 2).

The molecular formula required the peptide to be cyclic, and the NMR data alone could not be used to distinguish between cyclization via Ser or SerOMe. The Ser OH was not observed in several different NMR solvents, and the SerOMe NH was the only other heteroaromatic subunit in the molecule for which all bonds had not been defined. Thus, the two remaining modifications were required to involve the Ser β-oxygen and the SerOMe NH. Evidence for connectivity between the C-terminus of ACT and the β-oxygen of Ser was obtained using N-terminal peptide

The presence of keto-Ile was suggested by a weak GHMBC correlation between the keto-Ile(H-3) and a carbon at δ 198.2 ppm. Additionally, the unusual downfield C-3 signal at δ 58.7 and comparison with chemical shifts of known peptides containing α-keto-amide moieties[3,10] suggested the presence of a keto-Ile moiety. Ile could be observed by GC−MS following the chemical degradation method of Kobayashi et al.,[10] but not following acid hydrolysis, further substantiating the occurrence of keto-Ile in **1**. The modified tyrosine was clearly attached to an unsaturated moiety, on the basis of GHMBC correlations between C-8 and H-7, H-9, and H-10. A broad UV maximum at λ = 305 nm indicated a conjugated system, and unusual NMR chemical shifts and GHMBC correlations led to the proposal that a conjugated thiazole moiety, 2-(1-amino-2-*p*-hydroxyphenylethane)-4-(4-carboxy-2,4-dimethyl-2*Z*,4*E*-propadiene)-thiazole (ACT), was present. Conjugated thiazoles have precedents in marine cyclic peptides, notably in the kera-

sequencing, chemical shift data, and ESIMS. From the N-terminus, the peptide sequence consisted of SerOMe-Pro-X-Pro-X. No amino acid residue was observed in the third or fifth round of sequencing reactions, consistent with the presence of a modification on the Ser residue and the keto-Ile residue. A standard of Ser-SO$_3$ gave both Ser and dehydroalanine under conditions identical to those used for sequencing, indicating that the first X residue was not likely to be sulfated serine. The Ser β-protons were anomalously downfield (δ 3.96 and 4.41), indicating that Ser was likely to be an ester. The large chemical shift difference between these protons was also indicative of a constrained, chiral environment. By contrast, the SerOMe residue contained β-protons that were coincident (δ 3.40), indicating likely free rotation. The SerOMe was also not likely to be amide linked to the C-terminus of ACT because of its behavior in sequencing reactions, in which it was observed at the N-terminus, and the upfield shift of the NH signal (δ 4.30 ppm). Finally, the ESIMS peak at m/z = 650 amu was consistent with this linkage. Therefore, all available data indicated that Ser and ACT were linked by an ester bond.

The sulfite was shown to be present as SerOMe sulfamate. Sulfamates induce an upfield shift in β-protons, and this was observed in SerOMe (δ 3.40 ppm).[12] The NH chemical shift (δ 4.30 ppm) was also consistent with literature values for sulfamates, and the NH proton was strongly COSY correlated to SerOMe(H-2). By comparison, sulfamate NH protons from modified Tyr residues in ianthesine derivatives exhibited chemical shifts of δ 4.91 and 4.52.[12] β-Protons in the same system were shifted from δ 3.20 and 2.98 to δ 2.98 and 2.86, approximately $\Delta\delta$ 0.2 ppm upfield. Similar β-proton shifts ($\Delta\delta \approx 0.2$ ppm) were observed in the minalemines upon sulfation.[12]

The absolute configurations of the Ile, Ser, Tyr, and Pro residues were determined using chemical degradation, derivatization, and analysis by chiral GC−MS. Ozonolysis and hydrolysis converts the ACT to aspartic acid,[13] and hydrolysis degrades the peptide to produce the free amino acids. Comparison with authentic standards of DL-Asp, DL-Pro, and DL-Ser showed that the Tyr, Pro, and Ser were all in the L configuration. The stereochemistry of Ile was determined using H$_2$O$_2$/NaOH degradation followed by acid hydrolysis, derivatization, and chiral GC−MS analysis to obtain an additional peak that corresponded to L-*allo*-Ile. Rigorous hydrolysis appeared to lead to decomposition of SerOMe. Therefore, Marfey's method was employed following mild hydrolysis to determine the configuration of SerOMe, using authentic DL-SerOMe standards.[14] A peak coeluted with D-SerOMe under two different chromatographic conditions, indicating that this residue was in the D-configuration.

The configuration of the double bonds in ACT was evident from the NOESY spectrum. The strong NOESY correlations between CH$_3$-17 and CH-3, CH-3 and CH-5, and CH$_3$-18 and CH-7 require the ACT residue to have the 2Z,4E configuration.

Numerous minor peaks could be observed in the ^{1}H NMR spectrum of **1**, although these peaks did not interfere with ^{13}C or 2D NMR and appeared to comprise <10% of the sample. Since **1** was isolated as a single, broad peak under a range of C$_{18}$ HPLC conditions, it was proposed that these peaks comprised conformational isomers of the parent compound, a phenomenon that is well known in peptide chemistry.[15] To further investigate the purity of scleritodermin A (**1**), the compound was injected onto a phenyl-hexyl HPLC column and chromatographed using ammo-

Table 3. in Vitro Cytotoxicity of Scleritodermin A (**1**) against Human Tumor Cell Lines

	IC$_{50}$ (μM)^a			
	HCT116	HCT116/VM46	A2780	SKBR3
scleritodermin A (**1**)	1.9	5.6 (2.9)^b	0.940	0.670
vinblastine	0.003	0.135 (45)	0.005	0.072

^{a} Cytotoxicity assessed by MTS assay after 72 h drug exposure. ^{b} Value in parentheses is fold resistance relative to parental HCT116 cell line.
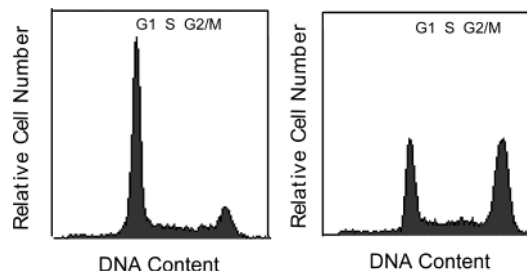

Figure 1. Flow cytometry analysis of A2780 cells after 24 h treatment with scleritodermin A (**1**).

nium bicarbonate buffer and acetonitrile. Under these conditions, three sharp peaks were observed and collected. However, ^{1}H NMR spectra of these peaks were identical to the initial spectrum of **1**. When the purified samples were reinjected onto the phenyl-hexyl column, the same pattern of three sharp peaks was observed, indicating that the peaks represent transiently stable conformational isomers of **1**.

Scleritodermin A (**1**) was evaluated for in vitro biological activity. The compound demonstrated significant in vitro cytotoxicity against a panel of human tumor cell lines (IC$_{50}$ < 2 μM), including colon carcinoma HCT116, ovarian carcinoma A2780, and breast carcinoma SKBR3 (Table 3). The cell line HCT116/VM46 overexpresses the drug efflux pump P-glycoprotein and exhibits the multidrug-resistant phenotype, including resistance to lipophilic natural products such as vinblastine.[16] Scleritodermin A (**1**), however, demonstrated very little cross-resistance against this particular cell line, suggesting that it is a weak substrate for P-glycoprotein.

To gain insight into the mechanism of action of this marine natural product, we examined its ability to affect the progression of A2780 cells through the cell cycle. Treatment of A2780 cells with scleritodermin A (**1**) for 24 h induced a G2-M block at a concentration of 1.3 μM (Figure 1). In addition, the ability of this compound to produce programmed cell death in ovarian cells was examined. Preliminary results, using the TUNEL assay[17] by flow cytometry, suggest that scleritodermin A (**1**) can induce apoptosis 5.5-fold after 24 h drug exposure at a concentration close to its cytotoxic IC$_{50}$ (1.3 μM).

Arrest of cells in G2/M is characteristic of compounds that target tubulin.[9] Therefore, the ability of scleritodermin A (**1**) to promote or inhibit calf brain tubulin polymerization in vitro was tested. Although this compound did not promote tubulin polymerization, it did inhibit GTP-induced tubulin polymerization 50% at a concentration of 10 μM, a level of potency that was about one-tenth of that observed for vinblastine in the same assay.[9] In recent years, other natural products from the marine environment and cyanobacteria, such as the halichondrins,[18] dolastatins,[19] and cryptophycins,[20] have been discovered that also disrupt tubulin polymerization, demonstrating that marine organisms are a rich source of this class of antitumor compounds.

This study demonstrates the potential value of the NCI Active Repository to supply extracts of less common species for the identification of new, bioactive molecules from marine sponges. In particular, we have demonstrated that selection of extracts based on taxonomic identification, together with a dereplication scheme involving NMR and mass spectrometry to identify known compounds, can increase the probability of discovering novel cytotoxic compounds such as scleritodermin A (**1**).

Experimental Section

General Experimental Procedures. ^{1}H, DQCOSY, GCOSY, TOCSY, GHMQC, GHMBC, and NOESY NMR spectra were recorded on a 300 or 500 MHz spectrometer. ^{13}C and DEPT NMR spectra were recorded on a 400 MHz spectrometer. All NMR data for **1** are reported in DMSO-d_6. Other experimental procedures were described previously.[21]

Animal Material. The sponge *Scleritoderma nodosum* was collected by divers from the Coral Reef Research Foundation from a rock face at a depth of 50 m on the northwest side of Olango Island, Cebu, Philippines (10°16.25′ N, 124°02.19′ E). The sponge was identified by Dr. Michelle Kelly, and a voucher specimen has been deposited at the Smithsonian Museum (0CDN2121). A second sample of *S. nodosum* (0CDN5908) was collected in Milne Bay, Papua New Guinea.

Isolation of Scleritodermin A (1). The organic extract (1.46 g) was partitioned between hexanes (3 × 100 mL) and methanol (100 mL) to give a MeOH fraction (950 mg) and a hexanes fraction (600 mg). The MeOH fraction was chromatographed on a C_{18} Sep Pak using a solvent gradient from 10 to 100% CH_3CN in 10% steps. Fractions eluting in 30−40% CH_3-CN were pooled and further purified by C_{18} HPLC (220 nm detection) using 28% CH_3CN in H_2O to obtain scleritodermin A (**1**, 6 mg). Fractions eluting between 40 and 50% CH_3CN were combined and chromatographed on a Sephadex LH-20 column in MeOH. High-molecular-weight fractions were pooled and purified on a C_{18} preparative plate with 4:1 CH_3CN/H_2O to obtain additional scleritodermin A (**1**, 6 mg). In an improved isolation procedure, the MeOH fraction was partitioned between 15% MeOH in H_2O and CH_2Cl_2, leading to a much cleaner sample that did not require chromatography on Sephadex LH-20.

Scleritodermin A (1): off-yellow powder; $[\alpha]_D$ −41.0° (c 0.1, methanol); UV (MeOH) 200 nm (ϵ 26 500), 305 nm (ϵ 11 300); IR (film) 3470, 3290, 2660, 1630−1700, 1520, 1450, 1240, 1210, 1155, 1125, 1040 cm^{-1}; ^{1}H NMR (DMSO-d_6), see Table 1; ^{13}C NMR (DMSO-d_6), see Table 1; HRFABMS m/z 872.3740 (calcd for $C_{42}H_{55}N_7O_{10}SNa$ 872.3628).

Determination of in Vitro Cytotoxicity. Parental HCT116 (human colon carcinoma) cells and a subline, HCT116/VM46 expressing the multidrug phenotype,[16] A2780 (human ovarian carcinoma) cells, and SKBR3 (human breast carcinoma) cells were maintained in RPMI 1640 media, 5 mM HEPES buffer, and 10% fetal bovine serum. In vitro cytotoxicity was assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-2H-tetrazolium, inner salt) assay.[22] Cells were seeded in microtiter plates, and 24 h later drugs were added and serially diluted. The cells were incubated at 37 °C for 72 h, at which time MTS and phenazine methosulfate were added. The absorbance was read at 492 nm, and the resulting optical density was proportional to the number of viable cells. The results are expressed as an IC_{50} value.

In Vitro Tubulin Polymerization. Inhibition of tubulin polymerization was assessed as follows. Twice cycled tubulin, in tubulin polymerization buffer (100 mM MES pH 6.6, 1 mM EGTA, 0.5 mM $MgCl_2$), was incubated with scleritodermin A (**1**) or vinblastine. Tubulin polymerization was initiated by the addition of 500 μM GTP. The absorbance was read at 350 nm every 36 s with water warming the cuvettes at 37 °C in a Beckman 7400 UV spectrophotometer. Tubulin polymerization was determined using a procedure based on that of Swindell et al.[23]

FACS Analysis. Cell Cycle Progression and Apoptosis. A2780 cells were seeded in six-well plates, and scleritodermin A (**1**) was added the following day. After a 24 h drug incubation, cells were collected by trypsinization and fixed in 80% ethanol/water overnight at −20 °C. The cells were then washed with phosphate-buffered saline (PBS), stained with propidium iodide (50 μg/mL) and Rnase (10 μg/mL) for 1 h at RT, and analyzed by a FACScalibur flow cytometer (Beckton and Dickinson). The proportions of cells in G0/G1, S, and G2-M phases were calculated from their histograms using ModFit software. Apoptosis analysis was performed by TUNEL assay[17] (Pharmingen Apo-Direct Kit) after 24 h drug treatment. Data acquisition and analysis was carried out by FACS using doublet discrimination gates.

Supporting Information Available: Experimental details for the stereostructure elucidation, as well as NMR and ESIMS spectra used to establish the structure of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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