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Microsclerodermins C - E, Antifungal Cyclic Peptides from the Lithistid Marine Sponges *Theonella* sp. and *Microscleroderma* sp.

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Abstract: Three new cyclic peptides, microsclerodermins C - E (**3-5**), were isolated from two species of lithistid sponges from the Visayan Islands, Philippines. The sponge *Theonella* sp. contained peptides **3** and **4**, while *Microscleroderma* sp. contained peptides **4** and **5**. Their structures, which feature three unprecedented amino acids, were elucidated using spectroscopic methods and chemical degradation.

Sponges of the order Lithistida are unparalleled as sources of unusual natural products in the marine environment.[1] Bioactive compounds isolated from these sponges include discodermolide,[2] swinholides,[3] and calyculins;[4] the sponges also contain an arsenal of modified peptides with close structural affinities to cyanobacterial natural products.[1,5] In addition to chemical riches, lithistid sponges often harbor a large number of symbionts, that in *Theonella swinhoei* include a filamentous bacterium which has been shown to contain the peptide theopalauamide[6] and a unicellular bacterium containing swinholide A.[7] We have been studying the peptide chemistry of lithistid sponges because of the bioactivity of many of their metabolites and our interest in understanding the association between the sponges and their bacterial flora.

During field collections in the Philippines in 1996, we encountered several lithistid sponges, including a *Theonella* sp. containing microsclerodermins C (**3**) and D (**4**) and a *Microscleroderma* sp. containing microsclerodermins D (**4**) and E (**5**), which are related to microsclerodermins A (**1**) and B (**2**) from *Microscleroderma* sp.[8] Since these compounds are thought to be synthesized by microbes, the presence of peptide **4** in both genera of sponges brings into question the specificity of symbiosis among lithistid sponges. Filamentous, non-photosynthetic microorganisms were found only in the *Theonella* sp. In this paper, we report the isolation and characterization of microsclerodermins C - E (**3-5**).

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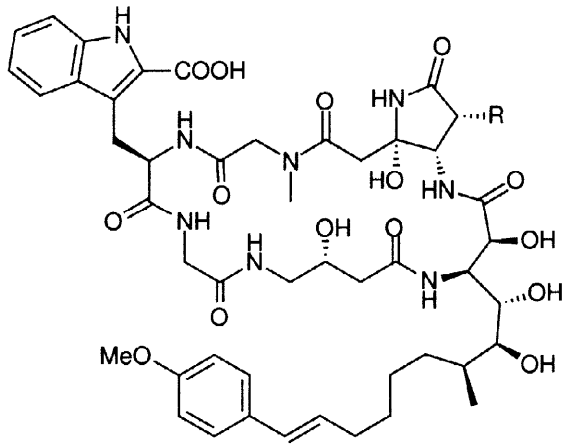

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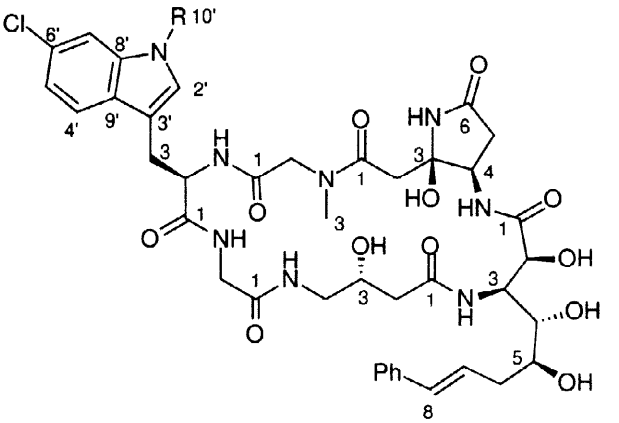

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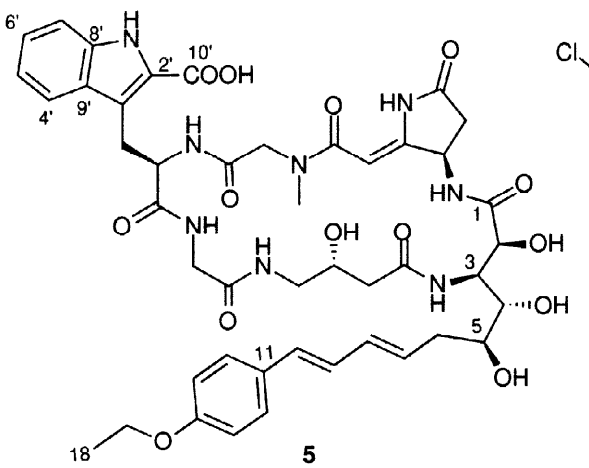
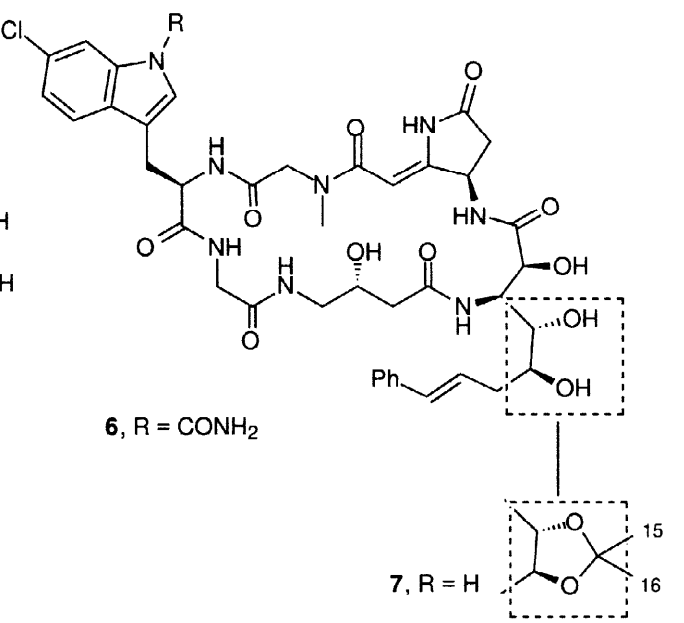


5



6, R = $CONH_2$

7, R = H

Isolation and Structural Determination of Microsclerodermins C and D

A lithistid sponge, *Theonella* sp., was collected using SCUBA off Olango Island in the Philippines at a depth of about 20 meters. This organism was selected for chemical investigation because light microscopy revealed that the sponge contained an abundance of filamentous bacteria, and the aqueous extract was active against the yeast *Candida albicans*. Microsclerodermins C (**3**) and D (**4**) were obtained by sequential extraction of the lyophilized sponge with solvents of increasing polarity. Rotary evaporation of the 1:1 acetonitrile/water extract gave a white precipitate that was subsequently centrifuged and subjected to reversed-phase chromatography. Peptide-containing fractions were lyophilized and resuspended in acetonitrile-water mixtures, which readily dissolved microsclerodermin D (**4**) but left a precipitate of pure microsclerodermin C

(**3**). Microsclerodermin D (**4**) was further purified and separated from dissolved microsclerodermin C (**3**) by reversed-phase HPLC.

Microsclerodermin C (**3**) was isolated as a white powder. The molecular formula, $C_{41}H_{50}ClN_9O_{13}$, was determined by high resolution mass measurement. In the ^1H NMR spectrum, a series of NH signals between δ 7.48 and 8.70 coupled to signals in the correct region for α-protons of amino acids suggested that the compound was a peptide. Despite its similarities with microsclerodermins A (**1**) and B (**2**), an affinity with those peptides was not immediately obvious from NMR spectral data (see Table 1), and thus the two-dimensional structure was elucidated by NMR methods. The ^1H, ^{13}C, and GHMQC NMR data indicated that there were six amino acids, including one that was *N*-methylated, and five hydroxyl groups. These experiments also suggested the presence of a monosubstituted phenyl group and an indole substituted at the N' and 6' positions. A DQCOSY spectrum was used to construct the major features of the constituent amino acids, and the resulting assignments were confirmed using the GHMBC spectrum. From these data, glycine, *N*-methyl glycine, and 4-amino-3-hydroxybutyric acid (GABOB) were readily identified. In addition, these experiments showed that the indole unit was part of tryptophan and that the phenyl group was conjugated to a double bond. GHMBC (see Figure 1) and ROESY (see Figure 3) experiments were used to determine the connectivity between the *N*-Me-Gly, disubstituted tryptophan, Gly, and GABOB residues. However, the pyrrolidone and 3-amino-8-phenyl-2,4,5-trihydroxyoct-7-enoic acid (APTO) groups could not be completely constructed from these data alone.

Assignment of the APTO structure was complicated by overlapping OH and CH protons in the NMR spectrum. For example, H-2 of this unit could not be distinguished from OH-5 in any two-dimensional NMR experiment, and APTO OH-4 was difficult to differentiate from pyrrolidone H-4. To resolve these overlapping signals, a complete NMR data set was acquired at 40 °C, at which temperature the exchangeable OH signals were overlapping with different CH signals in the ^1H NMR spectrum. Comparison of the two data sets were used to unambiguously construct two halves of the APTO unit, C-12 to C-5 and C-4 to C-1, and to assign resonances belonging to the pyrrolidone group. Unfortunately, a lack of observable coupling between H-4 and H-5 and the absence of any GHMBC correlations between the two parts of the molecule made the assignment of the APTO structure difficult. The APTO unit was postulated on the basis of ROE correlations between the two halves of the amino acid, but was not confirmed until the acetonide **7** was synthesized.

In the pyrrolidone residue, the cyclic portion was established using the GHMBC spectrum, which included correlations from OH-3 to C-2 and C-4, and from NH-3 to C-2, C-3, C-4, C-5 and C-6. The relative stereochemistry of the pyrrolidone was established using the ROESY spectrum. After defining the amino acid

Table 1. NMR data for microsclerodermin C (**3**) and dehydromicrosclerodermin C (**6**) in DMSO-d_6

		3		**6**	
amino acid	assignment	δ_C	δ_H	δ_C	δ_H
APTO	1	172.6		173.5	
	2	69.6	4.38 m	69.5	4.40 m
	3	53.3	4.13 t, 10.0	53.5	4.18 m
	4	70.1	3.29 m	70.2	3.29 m
	5	69.0	3.59 q, 4.5	69.0	3.61 m
	6	36.6	2.35 m	36.6	2.35 m
	7	128.1	6.27 m	128.2	6.32 dt, 16.0, 7.0
	8	130.8	6.40 d, 16.0	130.8	6.40 d, 16.0
	9	137.4		137.4	
	10,14	128.4	7.29 m	128.5	7.30 m
	11,13	125.7	7.33 m	125.8	7.34 m
	12	126.9	7.19 m	126.9	7.20 m
	NH3		7.48 m		7.12 m
	OH2		6.20 d, 6.0		
	OH4		4.49 d, 9.5		
	OH5		4.38 d, 5.0		
GABOB	1	172.9		172.4	
	2	41.1	2.16 m	41.5	2.13 t, 12.0
			2.43 m		2.30 m
	3	67.3	3.71 m	66.8	3.73 m
	4	45.0	2.57 m	45.2	2.60 m
			3.39 m		3.38 m
	NH4		7.48 m		7.38 m
	OH3		4.90 d, 5.5		
Gly	1	168.8		168.8	
	2	42.7	3.37 m	42.8	3.45 m
			3.77 m		3.72 m
	NH2		8.55 d, 6.0		8.40 m
6'-Cl-N'-form.-Trp	1	171.5		170.8	
	2	54.6	4.23 m	54.8	4.09 m
	3	25.8	3.03 m	25.1	2.95 m
					3.22 m
	2'	124.2	7.75 s	124.5	7.75 s
	3'	114.9		115.4	
	4'	120.2	7.60 m	120.2	7.60 m
	5'	121.8	7.23 m	121.9	7.24 m
	6'	not obsd		not obsd	
	7'	114.9	8.27 d, 2.0	114.9	8.27 d, 2.0
	8'	135.9		135.9	
	9'	128.5		128.5	
	10'	152.3		152.4	
	NH2		8.70 d, 3.5		8.71 d, 5.0
N-Me-Gly	1	170.4		170.9	
	2	49.7	3.97 m	50.6	3.45 m
			4.00 m		4.50 m
	3	36.4	2.91 s	37.0	2.98 s
pyrrolidone	1	170.4		168.1	
	2	38.8	2.65 d, 17.0	87.8	5.25 s
			2.89 d, 17.0		
	3	85.6		157.5	
	4	50.5	4.47 m	46.0	5.23 t, 9.5
	5	35.2	2.27 m	34.1	2.48 m
					2.68 m
	6	173.1		174.8	
	NH3		7.98 s		10.38 s
	OH3		6.02 s		—
	NH4		7.56 m		8.38 m

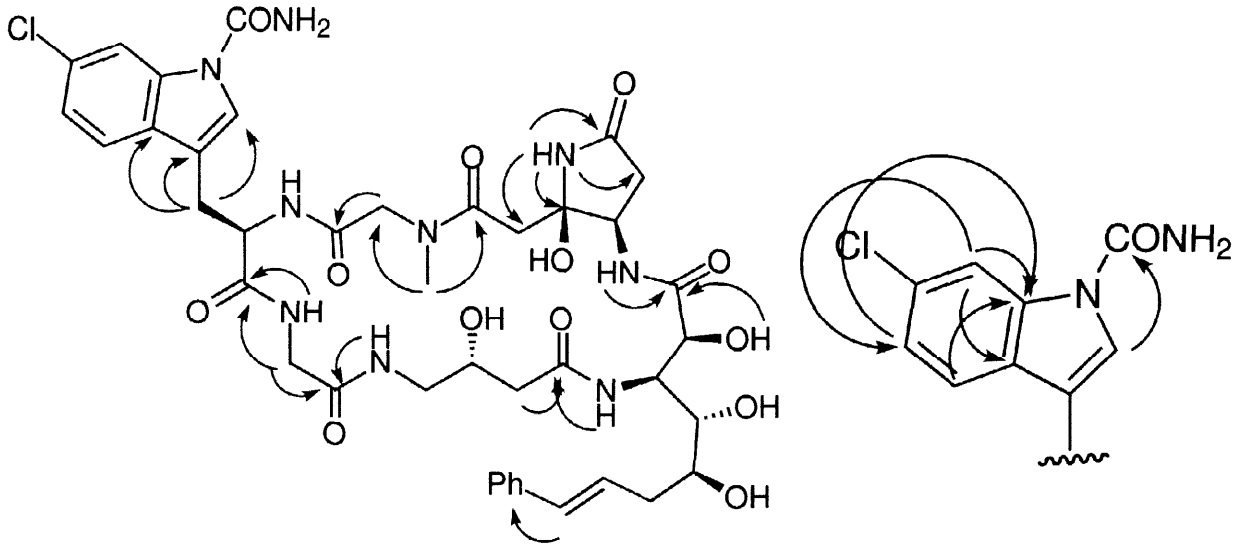


Figure 1. Key GHMBC correlations used to determine the structure of microsclerodermin C (**3**). The indole portion is shown on the right.

structures, the peptide sequence was fully defined on the basis of GHMBC and ROESY correlations, and the similarity to microsclerodermins A (**1**) and B (**2**) was recognized. The substituents on the tryptophan unit were not identified until the structure of microsclerodermin D (**4**) was established.

Microsclerodermin C (**3**) was readily dehydrated by addition of a trace amount of TFA to the NMR solvent. The structure of anhydromicrosclerodermin C (**6**, $C_{41}H_{48}N_9O_{12}Cl$) was elucidated using a full NMR data set to confirm the proposed structure of **3** (see Table 1). Interestingly, several peaks in the ^1H NMR spectrum of **3** appeared to be doubled (~5:1 ratio of peaks), but no signal doubling was visible in the ^1H NMR spectrum of the dehydrated compound **6**. Since no evidence could be obtained to indicate that OH-3 of the pyrrolidone residue had two different configurations or that **3** was contaminated by **6**, we conclude that the signal doubling must have resulted from different conformers of **3**.

Microsclerodermin D (**4**, $C_{40}H_{47}ClN_8O_{12}$) was isolated as a white powder. Full NMR spectral data (see Table 2), including DQCOSY, GHMQC, GHMBC, and NOESY indicated that **4** was identical to **3** except for the tryptophan unit, which was no longer *N*-substituted. The molecular formula indicated that a chlorine atom was present in **4** and NMR data established that the chlorine was located on the 6' position of the indole unit.

The only question remaining was the identity of the functional group on the indole nitrogen in **3**. The molecular mass, established by FABMS measurements, showed that **3** was 43 AMU heavier than **4**. Microsclerodermin C (**3**) also had one more ^{13}C NMR signal than **2**, at δ 152.3 ppm. This ^{13}C resonance

Table 2. NMR data for microsclerodermin D (**4**) in DMSO-d_6

		4	
amino acid	assignment	δ_C	δ_H
APTO	1	172.6	
	2	69.5	4.41 d, 6.0
	3	53.2	4.14 m
	4	70.0	3.32 m
	5	69.0	3.61 m
	6	36.4	2.35 m
	7	128.2	6.35 m
	8	130.8	6.42 d, 16.0
	9	136.5	
	10,14	128.5	7.30 m
	11,13	125.8	7.36 m
	12	126.8	7.20 t, 7.5
	NH3		7.44 m
	OH2		6.25 d, 6.0
GABOB	1	172.7	
	2	41.0	2.16 m
			2.43 m
	3	67.1	3.73 m
	4	45.0	2.64 m
			3.39 m
	NH4		7.48 m
	OH3		4.85 d, 4.0
Gly	1	168.8	
	2	42.7	3.35 m
			3.75 m
	NH2		8.54 br t, 4.5
6'-Cl-Trp	1	171.9	
	2	55.5	4.18 m
	3	26.0	2.98 m
	2'	124.9	7.28 s
	3'	110.0	
	4'	119.6	7.55 d, 8.5
	5'	118.6	7.01 d, 8.5
	6'	not obsd.	
	7'	111.0	7.39 m
	8'	134.4	
	9'	125.9	
	NH2		8.65 brd, 3.0
	NH1'		11.05 s
N-Me-Gly	1	170.2	
	2	49.8	3.84 m
			4.10 m
	3	36.7	2.92 s
pyrrolidone	1	170.5	
	2	38.0	2.70 m
			2.86 m
	3	85.6	
	4	50.6	4.47 m
	5	35.1	2.28 m
	6	173.2	
	NH3		7.98 m
	OH3		6.10 s
	NH4		7.54 s

showed a weak correlation to H-2' in the GHMBC spectrum, confirming the placement of this unit on the aromatic nitrogen. Unfortunately, two possible structures could be proposed based on these data: the functional group in question could be either CH_4N_2 (guanidinium salt) or CH_2NO (urea). FABMS measurements could be used to support either answer. While the exact mass of microsclerodermin C (**3**) was closer to the guanidinium salt, the FABMS spectrum of anhydromicrosclerodermin C (**6**) matched the urea formula more closely. A ^1H NMR experiment on **3** was employed to discriminate between the two possibilities. Triethylamine (1 μL increments up to 3 μL) was added to an NMR tube containing 6 mg of **6** in DMSO-d_6. The ^1H NMR spectrum of the basic solution, especially in the aromatic region, was identical to that of **6** in neutral or slightly acidic solution, indicating that the N'-substituting group was unaffected by the pH change. Thus, **3** is a urea rather than a guanidinium salt. To the best of our knowledge, 6'-chloro-N'-formamidotryptophan and APTO are unprecedented amino acids.

Anhydromicrosclerodermin C (**6**) was used in chemical degradation experiments to establish the absolute stereochemistry of **3**. Treatment of peptide **4** with periodate, followed by ozonolysis using an oxidative workup and acid-catalyzed hydrolysis gave a mixture of amino acids that were derivatized and analyzed by chiral GC-MS. Comparison with derivatives of amino acid standards allowed the identification of (3*R*)-3-hydroxy-4-aminobutyric acid, (2*S*,3*S*)-3-hydroxyaspartic acid, and (*R*)-aspartic acid. Although we were unable to cleanly form an acetonide from **6**, treatment of anhydromicrosclerodermin D with 2,2-dimethoxypropane using pyridinium *p*-toluenesulfonate as catalyst gave the acetonide **7** in quantitative yield. A NOESY experiment performed on **7** gave results similar to those found for the acetonide of microsclerodermin A (**1**),[8] indicating that the stereochemistry of the APTO group was (2*S*,3*R*,4*S*,5*S*,7*E*).

Isolation and Structural Determination of Microsclerodermin E

The sponge *Microscleroderma* sp. was collected at a depth of 15 m near Panglao Island, Visayas, Philippines. The sponge, while much different in appearance than the *Theonella* sp., also had antifungal activity but did not appear to contain filamentous microorganisms. Initial extraction and separation procedures were identical to those used to isolate **3** and **4**. Following purification through a C_{18} Sep Pak, it was found that the resulting peptide mixture (approximately 70% one compound) was completely insoluble in many solvents and was only moderately soluble in DMSO. The peptide mixture was found to be soluble in mixtures containing acetonitrile and 0.1 N NH_4HCO_3, so this system was used for a reversed-phase HPLC separation that yielded microsclerodermins D (**4**) and E (**5**). The identity of microsclerodermin D (**4**) was readily established by comparing ^1H and ^{13}C NMR and HRMS data with those determined previously. It is possible that microsclerodermin C (**3**) was also present in the sponge before separation, since mild aqueous

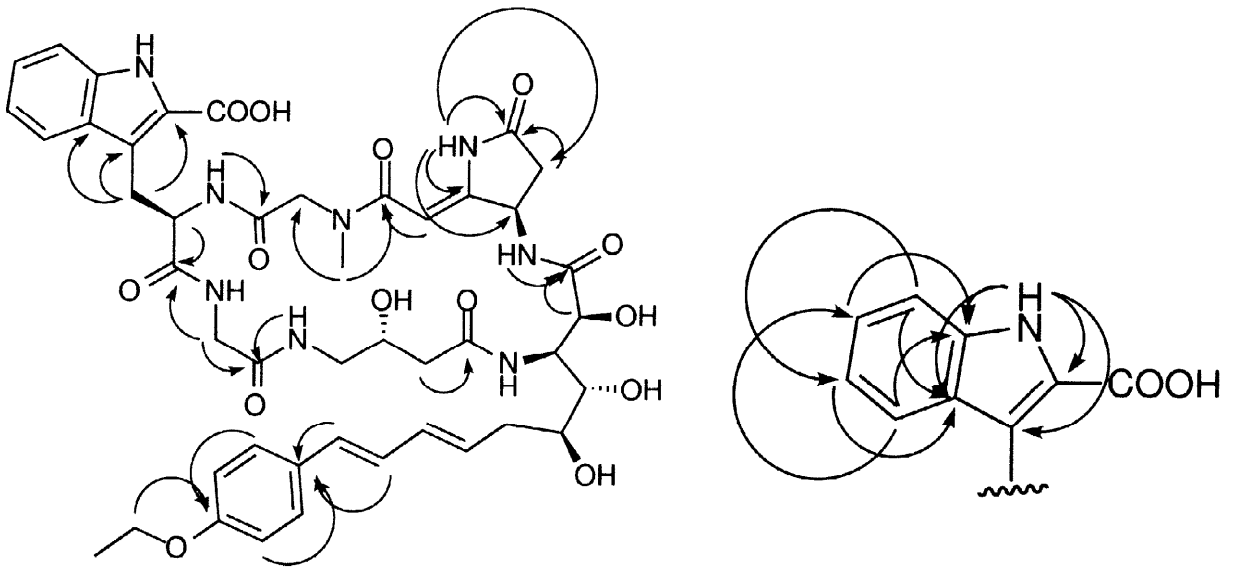


Figure 2. Key GHMBC correlations used to determine the structure of microsclerodermin E (**5**). The indole portion is shown on the right.

base hydrolysis rapidly converts **3** to **4**.

Microsclerodermin E (**5**) was isolated as a white powder. Its molecular formula ($C_{45}H_{54}N_8O_{14}$) suggested that it had a close structural affinity to microsclerodermin C (**3**), with the lack of the chlorine atom and four additional carbon atoms being the major differences. The NMR data, including ^1H, ^{13}C, DEPT, DQCOSY, TOCSY, NOESY (see Figure 3), GHMQC and GHMBC (see Figure 2), established the relationship between the two compounds (see Table 3). From these data, the presence of GABOB, *N*-Me-Gly, and Gly were readily established and confirmed by comparison with the spectral data of **3**. The pyrrolidone residue was established by NMR data, especially GHMBC data from NH-5, and confirmed by comparison with the spectral data of anhydromicrosclerodermin C (**6**). Interestingly, the pyrrolidone residue in **5** did not appear to dehydrate during separation, since key signals were present in the NMR spectra of the crude mixture of peptides before acid or base treatment. In addition, **4** was isolated from this sponge with its pyrrolidone OH intact, even after HPLC separation.

The two residues which appeared unique to microsclerodermin E (**5**) were the substituted tryptophan and the amino acid identified as 3-amino-10-(*p*-ethoxyphenyl)-2,4,5-trihydroxydeca-7,9-dienoic acid (AETD). The AETD carbons bound to oxygen and nitrogen had nearly identical NMR chemical shifts and 2-D NMR data to those of the APTO group in **3** and **4**, leading to the proposed structure for C-1 to C-7. The ^1H and DQCOSY NMR spectra indicated that this unit was connected to a diene, rather than the single double bond found in **3**. GHMBC correlations were used to connect the diene to the aromatic unit, which was identified as

Table 3. NMR data for microsclerodermin E (**5**) in DMF-d_7 with trace TFA[9]

		5[b]	
amino acid	assignment	δ_C	δ_H
AETD	1	174.9	
	2	71.1	4.69 s
	3	55.4	4.34 t, 10.0
	4	71.8	3.46 m
	5	70.6	3.72 m
	6	37.7	2.39 m
	7	132.9	5.83 dt, 15.0, 8.0
	8	133.4	6.25 dd, 15.0, 10.5
	9	128.5	6.73 dd, 16.0, 10.5
	10	130.8	6.45 d, 16.0
	11	131.3	
	12,16	128.5	7.41 d, 8.5
	13,15	115.7	6.91 d, 8.5
	14	159.6	
	17	64.3	4.04 q, 5.0
	18	15.4	1.35 t, 5.0
	NH3		7.57 d, 10.5
GABOB	1	174.2	
	2	43.1	2.19 dd, 14.0, 9.0
			2.49 dd, 14.0, 1.0
	3	68.7	3.93 br
	4	46.7	2.92 m
			3.45 m
	NH4		7.35 brt, 9.0
Gly	1	170.5	
	2	44.2	3.62 m
			3.70 m
	NH2		8.45 t, 6.5
Trp-2'-COOH[a]	1	172.4	
	2	58.1	4.24 dt, 9.0, 2.0
	3	26.4	3.60 m
			3.70 m
	2'	126.4	
	3'	119.7	
	4'	121.5	7.72 d, 8.5
	5'	120.9	7.16 t, 8.5
	6'	125.8	7.30 t, 8.5
	7'	113.5	7.54 d, 8.5
	8'	137.5	
	9'	129.3	
	10'	164.9[c]	
	NH2		8.88 d, 2.0[d]
	NH1'		11.58 br s
N-Me-Gly	1	172.4	
	2	51.8	3.47 d, 16.0
			4.64 d, 16.0
	3	37.9	3.09 s
pyrrolidone	1	169.9	
	2	89.0	5.45 s
	3	159.5	
	4	47.4	5.42 dt, 5.0, 9.0
	5	35.0	2.58 dd, 18.0, 5.0
			2.89 dd, 18.0, 9.0
	6	176.1	
	NH3		8.59 d, 9.0
	NH4		10.60 s

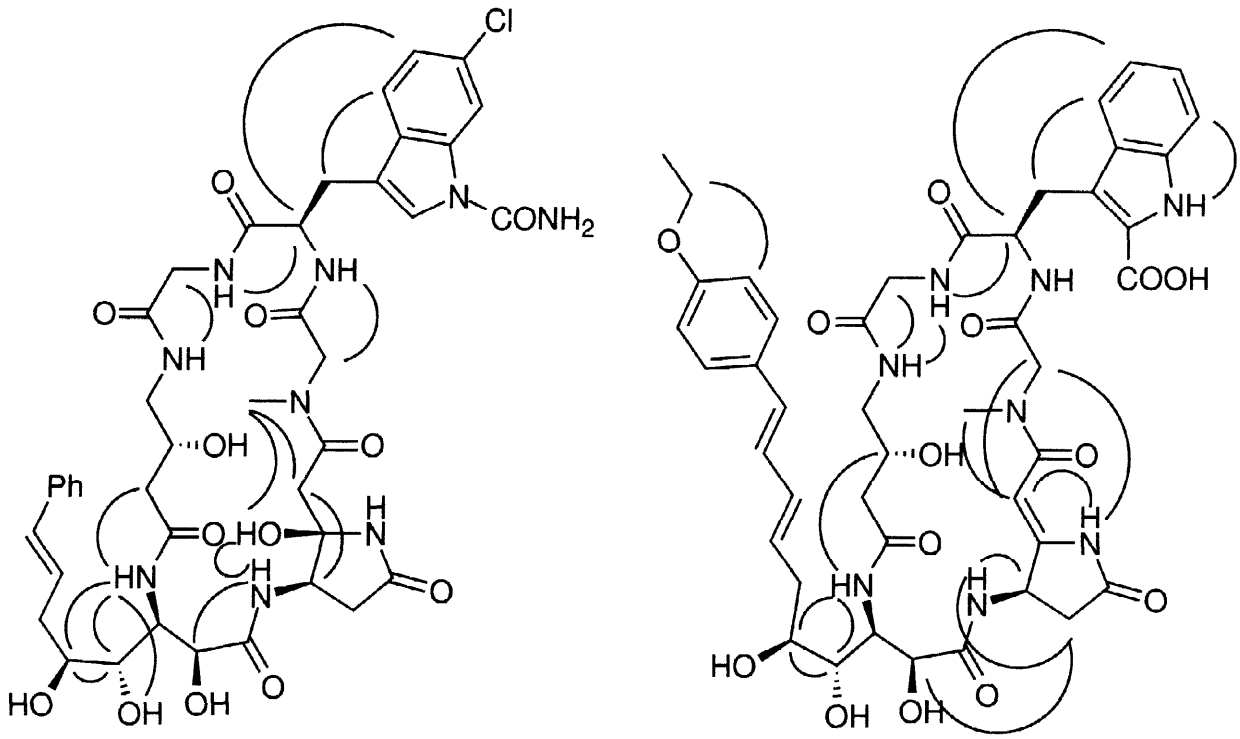


Figure 3. Summary of key NOESY/ROESY correlations used to determine the structure of microsclerodermins C (**3**, left) and E (**5**, right).

a *p*-ethoxy phenyl ring[10] based on ^{1}H and ^{13}C NMR chemical shift data. The ethyl group was not produced during the purification of **5**, since the O-ethyl signals were visible in the ^{1}H and ^{13}C NMR spectra of the crude peptides. Thus, the AETD residue accounted for the extra C_4H_7 in **5** compared with **3**.

Following the identification of all other residues, it was apparent from the molecular formula that the tryptophan residue was substituted with a carboxylate group. The identification of this residue was complicated by the fact that the α-NH proton was not visible in the NMR spectra of either the original extract or the purified peptide (**5**). Upon acidification of the NMR solvent with TFA vapor, however, the Trp NH proton signal was revealed at δ 8.84 ppm (d, $J = 2$ Hz). From the DQCOSY and GHMBC data, the tryptophan residue was substituted adjacent to the aromatic nitrogen. These data confirmed the core structure of the tryptophan unit, but there were no NMR correlations to the substituent adjacent to the aromatic nitrogen to confirm placement of the carboxyl group.

The only remaining unassigned NMR signal, at δ 164.9 in the ^{13}C spectrum, was consistent with a COOH at C-2' based on comparison to simulated NMR spectra. The presence of a carboxylate anion was also indicated by the sensitivity of many chemical shifts of the amino acids, especially tryptophan, to pH changes. In basic or neutral solution, the carboxylate ^{13}C NMR signal resonated at δ 167.9 ppm, but addition of trace

acid significantly shifted the resonance. Low resolution positive ESIMS gave a molecular ion at 953, corresponding to $(M+Na)^+$, while negative ESIMS gave 929 (M^-). In addition, methylation of **5** with diazomethane afforded a mono-methylated compound with a high resolution mass spectrum that further corroborated the proposed structure of **5**. Finally, comparison of ^{13}C NMR data from **5** with those of anhydromicrosclerodermin A[8] served to confirm the structure.

The absolute configuration of microsclerodermin E (**5**) was determined to be identical to that of **3** and **4** by employing the same sequence of reactions used for **6** and comparing GC-MS peaks with those resulting from authentic standards. Only two stereocenters, C-4 and C-5 in the AETD residue, were not determined by chemical means. Because their ^1H and ^{13}C NMR chemical shift data were close to those of known microsclerodermins, C-4 and C-5 were assumed to have the same absolute configurations in all compounds.

Bioactivity

Microsclerodermins C (**3**), D (**4**), E (**5**), and anhydromicrosclerodermin C (**6**) were active against *Candida albicans* in a paper disk assay. Peptide **3** was most active at 5 μg per disk, followed by **5** at 10 μg, **6** at 50 μg and **4** at 100 μg. Reported concentrations are the minimum at which inhibition was observed.

EXPERIMENTAL SECTION

General Experimental Procedures: ^1H, DQCOSY, GHMQC, and GHMBC, TOCSY and ROESY NMR spectra were recorded on a Varian Inova 300 MHz spectrometer. ^{13}C and DEPT spectra were recorded on a Varian Gemini 2000 400 MHz spectrometer. A NOESY spectrum was recorded on a Varian Unity 500 MHz spectrometer. All NMR data are reported in DMSO-d_6 except for those of peptide **5**, which were reported in DMF-d_7 with trace TFA. TOCSY spectra were obtained with 80 msec mixing times, and ROESY spin locks were established by continuous pulsing for 300 msec. High-resolution FABMS data were obtained from the Mass Spectrometry Facility, University of California at Riverside. Optical rotations were measured on an Autopol III polarimeter. UV and IR spectra were recorded on Perkin Elmer Lambda 3B and 1600 FT-IR instruments, respectively. Absolute configurations were determined using an Alltech Chirasil-Val column with a Hewlett Packard 5890 GC-MS system. The temperature was ramped from 50 °C to 210 °C over 45 minutes in all GC-MS experiments. GC-MS retention times were similar to previously reported results.[8]

Isolation of Microsclerodermins C (3) and D (4): A sample of *Theonella* sp. (NCI 2218) was collected at a depth of 20 m near Olango Island, Visayas, Philippines (N 10° 16' 53.2", E 124° 2' 57.6"). The sponge was lyophilized (39.4 g dry weight) and sequentially extracted with solvents of increasing polarity: 2 x DCM; 2 x 1:1 EtOAc/acetone; 5 x 1:1 CH_3CN/H_2O. The CH_3CN was removed from the aqueous extract by rotary

evaporation, and the resulting solution was centrifuged to yield a white precipitate. The solid material was dry loaded on a C_{18} Sep Pak (Waters) and subjected to a gradient of 0-100% CH_3CN (aq). Fractions eluting between 50 and 60% acetonitrile were combined and resuspended in 10 mL of 1:1 CH_3CN/H_2O. The suspension was centrifuged for 10 minutes, yielding a white powder of pure microsclerodermin C (**3**, 30 mg). The eluant was subjected to HPLC on a C_{18} preparative column using 37% CH_3CN (aq) to yield an additional 43 mg of **3** (total: 73 mg, 0.19% dry weight) and microsclerodermin D (**4**, 30 mg, 0.077% dry weight).

Isolation of Microsclerodermin E (5): A sample of *Microscleroderma* sp. (NCI 2309) was collected at a depth of 15 m near Panglao Island, Visayas, Philippines (N 9° 23' 37.3", E 123° 43' 80.1"). The sponge was lyophilized (127.5 g dry weight), then extracted with 4 x EtOAc and 5 x 1:1 H_2O/CH_3CN. The aqueous extract was dried by rotary evaporation until the formation of a white precipitate, which was removed by centrifugation. The white powder was dry loaded on a C_{18} Sep Pak and subjected to a gradient of 20-100% CH_3CN (aq). Fractions between 30-40% CH_3CN were pooled and repurified on a C_{18} HPLC column using 73:27 0.1 N NH_4HCO_3/CH_3CN at 3 mL/min. Two major fractions eluted after approximately 1 hour: microsclerodermins D (**4**, 8.6 mg, 0.007% dry weight) and E (**5**, 15 mg, 0.012% dry weight).

Microsclerodermin C (3): white powder; $[\alpha]_D$ -24° (c 0.063, 1:1 MeOH/DMSO); UV (MeOH) 202 (ε 46,500), 239 (28,000), 250 (27,100), 256 (28,000); IR (AgCl) 3390, 1650-1660, 1540, 1435, 1410 cm^{-1}; ^1H NMR (DMSO-d_6) see Table 1; ^{13}C NMR (DMSO-d_6) see Table 1; HRFABMS obsd *m/z* 934.3270 $(M+Na)^+$, $C_{41}H_{50}ClN_9O_{13}Na$ requires *m/z* 934.3114.

Microsclerodermin D (4): white powder; $[\alpha]_D$ -56° (c 0.07, 1:1 MeOH/H_2O); UV (MeOH) 202 (ε 56,000), 224 (34,000), 242 (16,100), 269 (9600), 277 (9000), 287 (3700); IR (AgCl) 3310, 1660, 1545, 1405 cm^{-1}; ^1H NMR (DMSO-d_6) see Table 2; ^{13}C NMR (DMSO-d_6) see Table 2; HRFABMS obsd *m/z* 891.3090 $(M+Na)^+$, $C_{40}H_{49}ClN_8O_{12}Na$ requires *m/z* 891.3056.

Microsclerodermin E (5): white powder; $[\alpha]_D$ -24° (c 0.19, 1:1 MeOH/0.1 N NH_4HCO_3 (aq)); UV (MeOH) 203 (ε 33,100), 206 (34,700), 218 (33,000), 284 (37,200); IR (AgCl) 3310, 2930, 1740, 1660, 1540 cm^{-1}; ^1H NMR (DMF-d_7) see Table 3; ^{13}C NMR (DMF-d_7) see Table 3; HRFABMS obsd *m/z* 931.4026 $(M+H)^+$, $C_{45}H_{55}N_8O_{14}$ requires *m/z* 931.3838.

Preparation of anhydromicrosclerodermin C (6): A solution of peptide **3** (15 mg) in DMSO-d_6 (750 μL) in an NMR tube was acidified with a trace amount of TFA and immediately observed by ^1H NMR. Quantitative dehydration occurred in less than 5 minutes to yield **6** (15 mg, 100% yield): white powder; ^1H NMR (DMSO-d_6) see Table 1; ^{13}C NMR (DMSO-d_6) see Table 1; HRFABMS obsd *m/z* 894.3276 $(M+H)^+$, $C_{41}H_{49}ClN_9O_{12}$ requires *m/z* 894.3189.

Preparation of acetonide 7: Vapors of TFA were added to a solution of peptide **4** (7 mg) in dry DMF (100 μL) in a conical vial. The vial was left for 5 minutes, then dried *in vacuo* to yield anhydromicrosclerodermin D. The product was added to a solution of dry DMF (100 μL) containing dimethoxypropane (400 μL) and a catalytic amount of pyridinium *p*-toluensulfonate. The solution was stirred overnight, then dried under a stream of nitrogen and *in vacuo* to give the pure acetonide **7** (7 mg, 100% yield): white powder; ^{1}H NMR (DMSO-d_6) APTO: 1.37 (s, 6H, H-15,16), 2.29 (m, 1H, H-6), 2.40 (m, 1H, H-6'), 3.77 (dd, J = 8,10 Hz, 1H, H-4), 4.06 (m, 1H, H-5), 4.22 (s, 1H, H-2), 4.35 (t, J= 10 Hz, 1H, H-3), 6.26 (dt, J = 16,7.5 Hz, 1H, H-7), 6.45 (d, J = 16 Hz, 1H, H-8), 7.12 (m, 1H, NH-3), 7.20 (t, J = 6 Hz, 1H, H-12), 7.30 (m, 2H, H-10,14), 7.42 (m, 2H, H-11,13); GABOB: 2.06 (m, 1H, H-2), 2.25 (m, 1H, H-2'), 2.99 (m, 1H, H-4), 3.19 (m, 1H, H-4'), 3.94 (m, 1H, H-3), 7.38 (m, 1H, NH-4); Gly: 3.62 (d, J = 4 Hz, 1H, H-2), 3.64 (d, J = 4 Hz, 1H, H-2'), 8.37 (t, J = 4 Hz, 1H, NH-2); Trp-6'-Cl: 3.10 (m, 1H, H-3), 4.14 (m, 1H, H-2), 7.02 (d, J = 8.5 Hz, 1H, H-5'), 7.32 (s, 1H, H-2'), 7.33 (m, 1H, H-7'), 7.55 (d, J = 8.5 Hz, 1H, H-4'), 8.79 (d, J = 4.5 Hz, 1H, NH-2), 11.01 (s, 1H, NH-1'); NMeGly: 3.10 (s, 3H, H-3), 3.46 (d, J = 16 Hz, 1H, H-2), 4.53 (d, J= 16 Hz, 1H, H-2'); pyrrolidone: 2.48 (m, 1H, H-5), 2.75 (m, 1H, H-5'), 5.24 (m, 1H, H-4), 5.29 (s, 1H, H-2), 8.36 (d, J = 7.5 Hz, 1H, NH-4), 10.40 (s, 1H, NH-3); HRFABMS obsd *m/z* 891.3534 $(M+H)^+$, $C_{43}H_{52}ClN_8O_{11}$ requires *m/z* 891.3444.

Methylation of 5: Peptide **5** (0.6 mg) was suspended in EtOH (1.5 mL) and cooled in an ice bath. Diazomethane was distilled into the solution, which was then brought to room temperature and allowed to react overnight. The solvent was evaporated under nitrogen, and the sample was loaded onto a C_{18} analytical HPLC column using 40% CH_3CN (aq) as eluant. A single major fraction (0.5 mg) was recovered. The ^{1}H NMR spectrum (DMSO-d_6) contained a new peak at δ 3.87; HRFABMS obsd *m/z* 945.3999 $(M+H)^+$, $C_{46}H_{57}N_8O_{14}$ requires *m/z* 945.3994.

Degradation and derivatization of 5 and 6 for GCMS: Peptide **6** (300 μg) was dissolved in water (100 μL, acidified to pH = 4 with AcOH), to which was added $NaIO_4$ (300 μg). The reaction was stirred overnight, then lyophilized. The periodate cleavage product was dissolved in MeOH (200 μL) and ozonized at -78 °C for 50 minutes. The reaction was quenched with 50% H_2O_2 (5 drops), brought to room temperature, and allowed to stand for 1 hour. The reaction mixture was dried under a stream of N_2 followed by lyophilization, then hydrolyzed and derivatized as described previously.[8] After drying under nitrogen, the residue was dissolved in 100 μL EtOAc (GC-MS grade) and subjected to GC-MS using a chiral column. Comparison of retention times and MS fragmentation patterns with amino acids that had been derivatized in the same manner gave (3*R*)-3-hydroxy-4-aminobutyric acid, (2*S*,3*S*)-3-hydroxyaspartic acid, and (*R*)-aspartic acid. Microsclerodermin E (**5**) was analyzed in an identical manner.

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9. a) Most ^{13}C and ^{1}H NMR signals were highly pH dependent. b) Observed with trace TFA. c) In basic solution, δ 167.9 ppm. d) Not observed in basic or neutral solution.

10. We found no record of natural products containing the *p*-ethoxy phenyl moiety in computer databases.