Discobahamins A and B, New Peptides from the Bahamian Deep Water Marine Sponge Discodermia sp.

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DISCOBAHAMINS A AND B, NEW PEPTIDES FROM THE BAHAMIAN DEEP WATER MARINE SPONGE *DISCODERMIA* SP.

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ABSTRACT.—Discobahamin A [**1**] and discobahamin B [**2**] are two bioactive peptides isolated from a new species of the Bahamian deep water marine sponge *Discodermia*. The discobahamins are inhibitors of the growth of *Candida albicans*, and the isolation and structure elucidation of **1** and **2** by nmr and chemical methods is described.

A number of interesting biologically active compounds have been reported from the marine sponge genus *Discodermia*. The examples reported from this genus include: discodermins (1–3), antimicrobial peptides reported from *Discodermia kiiensis*; calyculins (4), potent inhibitors of protein phosphatases 1 and 2A (5) isolated from *Discodermia calyx*; discodermindole (6), an antitumor compound reported from *Discodermia polydiscus*; discodermolide (7), an immunosuppressive polyhydroxylated lactone (8,9) and discodermide (10), a *Candida albicans*-active macrocyclic lactam reported from *Discodermia dissoluta*; and polydiscamide A (11), an A549 human lung cancer-active compound reported from a new *Discodermia* sp.

In our search for biologically active substances from marine organisms, an extract from a deep water marine sponge *Discodermia* sp. inhibited the growth of *C. albicans*. This new species of *Discodermia* was collected by manned submersible at Goulding Cay, Bahamas at a depth of 180 m. Ethanol extraction of the frozen sponge yielded an extract that was partitioned between EtOAc and water. The EtOAc-soluble fraction on bioassay-guided Si gel chromatography followed by reversed-phase hplc furnished two new antifungal constituents, designated discobahamin A [**1**] and discobahamin B [**2**]. The discobahamins are related in structure to the reported cyclic peptides orbiculamide A (12) and keramamides B–D (13) isolated from two *Theonella* sp. collected from Hachijo-jima Island and Kerama Island, respectively. The structural characteristics of the discobahamins are the substitution of a 5-hydroxytryptophan residue in place of a 2-bromo-5-hydroxytryptophan residue in the cyclic system and mixed substitution of amino acid residues in the side-chain. The N-terminus of the discobahamins is protected by 2-hydroxy-3-methylpentanoic acid (Hmp) as in keramamides. However, the discobahamins have one less amino acid residue than the keramamides in the side-chain.

The hr fab mass spectrum of discobahamin A [**1**] calculated to a molecular formula of $C_{47}H_{65}N_9O_{11}$. Although it was negative to ninhydrin, the ^{1}H- and ^{13}C-nmr data for **1** listed in Table 1 suggested a peptide structure. The presence of a tryptophan residue in **1** was suggested by the uv spectrum and also by the formation of a precipitate with Fast Red B salt (14). A standard amino acid analysis of the hydrolysate indicated the presence of ornithine, proline, alanine and 2-aminopentanoic acid (nVal). Analysis of ^{1}H- and ^{13}C-nmr data (Table 1) and ^{1}H-^{1}H COSY, TOCSY, DEPT, HMQC and HMBC nmr data revealed the presence of 2-hydroxy-3-methylpentanoic acid (Hmp), theonalanine (Thl) (12), leucine (12) and 5-hydroxytryptophan in addition to the amino acid residues detected in the amino acid analysis. The uv absorption spectrum of **1** was consistent for the presence of 5-hydroxytryptophan and theonalanine residues (12) and the results from an HMBC nmr experiment confirmed the position of the hydroxy group in the tryptophan residue. A comparison of ^{1}H- and ^{13}C-nmr data corresponding to theonalanine residue in **1** with those reported for the same residue in orbiculamide (12) confirmed the

TABLE 1. ^{13}C- and ^{1}H-nmr Data for Discobahamin A [**1**] and B [**2**] in DMSO-d_6.[a]

	Discobahamin A		Discobahamin B	
	^{13}C[b]	^{1}H[c]	^{13}C	^{1}H
Hmp				
1	172.5 s		172.6 s	
2	74.9 d	3.72 (d, 5.4)	74.9 d	3.72 (d, 5.4)
3	38.1 d	1.70 (m)	38.1 d	1.70 (m)
4	23.1 t	1.11, 1.35 (m)	23.1 t	1.11, 1.35 (m)
5	11.6 q	0.82 (t, 6.6)	11.6 q	0.82 (t, 6.6)
6	15.4 q	0.86 (d, 6.5)	15.4 q	0.86 (d, 6.5)
nVal				
1	171.1 s			
2	51.1 d	4.32 (m)		
3	34.8 t	1.45, 1.55 (m)		
4	18.1 t	1.21 (m)		
5	13.5 q	0.80 (t, 6.6)		
NH		7.58 (d, 8.5)		
Orn				
1	169.3 s		164.1 s	
2	49.7 d	4.43 (m)	49.8 d	4.45 (m)
3	28.7 t	1.70, 1.40 (m)	28.7 t	1.70, 1.40 (m)
4	25.2 t	1.40 (m)	25.2 t	1.40 (m)
5	37.7 t	2.62, 3.45 (m)	37.7 t	2.62, 3.45 (m)
αNH		8.10 (d, 7.5)		8.06 (d, 7.5)
δNH		7.50 (m)		7.50 (m)
Htrp				
1	171.1 s		171.1 s	
2	53.4 d	4.57 (m)	53.4 d	4.58 (m)
3	27.6 t	3.10 (dd, 4.2, 14.3)	27.6 t	3.10 (dd, 4.2, 14.3)
		2.83 (dd, 8.2, 14.3)		2.83 (dd, 8.2, 14.3)
αNH		8.06 (d, 9.5)		8.03 (d, 9.5)
NH		10.41 (s)		10.40 (s)
2′	123.6 d	6.94 (s)	123.7 d	6.94 (s)
3′	109.2 s		109.2 s	
4′	102.1 d	6.81 (d, 1.5)	102.1 d	6.81 (d, 1.5)
5′	150.1 s		150.1 s	
6′	111.1 d	6.54 (dd, 8.5, 1.5)	111.1 d	6.54 (dd, 8.5, 1.5)
7′	111.4 d	7.07 (d, 8.5)	111.4 d	7.07 (d, 8.5)
8′	130.5 s		130.5 s	
9′	128.3 s		128.3 s	
Thl				
1	164.2 s		164.2 s	
2	123.7 d	7.17 (d, 15.5)	123.8 d	7.16 (d, 21.2)
3	127.1 d	6.61 (d, 15.5)	127.1 d	6.61 (d, 21.2)
4	136.9 s		136.9 s	
5	139.3 d	8.20 (s)	139.3 d	8.20 (s)
6	165.2 s		165.2 s	
7	43.9 d	4.84 (dq, 6.0, 7.1)	43.9 d	4.84 (dq, 6.0, 7.1)
8	17.9 q	1.45 (q, 7.1)	17.9 q	1.45 (q, 7.1)
NH		9.13 (d, 6.0)		9.12 (d, 8.2)
Tle				
1	159.6 s		159.6 s	
2	195.8 s		195.8 s	
3	52.2 d	4.92 (m)	52.2 d	4.92 (m)
4	38.4 t	1.30, 1.50 (m)	38.4 t	1.30, 1.50 (m)
5	24.7 d	1.80 (m)	24.7 d	1.80 (m)
6	23.0 q	0.85 (d, 6.5)	23.0 q	0.85 (d, 6.5)
7	20.7 q	0.85 (d, 6.5)	20.7 q	0.85 (d, 6.5)
NH		8.16 (d, 5.0)		8.15 (d, 7.0)

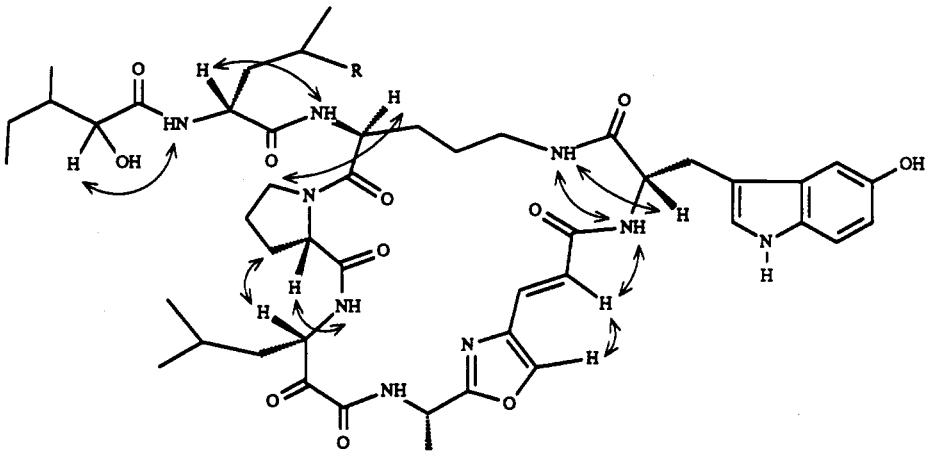
TABLE 1. Continued.

	Discobahamin A		Discobahamin B	
	$^{13}C^{b}$	$^{1}H^{c}$	^{13}C	^{1}H
Pro				
1	170.9 s		170.8 s	
2	58.3 d	4.35 (m)	58.3 d	4.37 (m)
3	29.3 t	1.85, 2.15 (m)	29.3 t	1.85, 2.15 (m)
4	24.4 t	1.85 (m)	24.4 t	1.85 (m)
5	46.7 t	3.45, 3.73 (m)	46.7 t	3.45, 3.73 (m)
Leu				
1			171.5 s	
2			50.0 d	4.32 (m)
3			39.0 t	1.40 (m)
4			24.0 d	1.20 (m)
5			21.5 q	0.80 (d, 6.5)
6			23.0 q	0.80 (d, 6.5)
NH				7.57 (d, 12.2)

[a]Table entries are chemical shift, ppm from solvent (multiplicity, J in Hz).
[b]Assignments based on APT, DEPT and HMQC experiments.
[c]Assignments based on COSY and TOCSY experiments.

presence of theonalanine in **1**. The presence of one methyl doublet at 1.45 ppm, characteristic for alanine, indicated that the alanine detected in the amino acid analysis had been liberated from the theonalanine residue upon acid hydrolysis (15). The HMBC nmr results indicated that in leucine the nitrogen-bearing methine proton at 4.92 ppm was correlated to the carbonyl signal at 195.8 ppm. This carbon could be placed next to an amide carbon at 159.6 ppm, which is reminiscent of an α-keto amide and thus confirmed the presence of theoleucine (Tle) as in orbiculamide. Selective irradiation of the theonalanine NH (9.13 ppm) in a selective INEPT nmr experiment (16) indicated correlations to both carbonyl groups (159.6, 195.8 ppm) of theoleucine, and thus established the connectivity between Thl and Tle residues. Analysis of results from the HMBC nmr experiment provided the sequences of two peptide fragments, Hmp-nVal-Orn, and Htrp-Thl-Tle, in **1**.

The results from the HMBC nmr experiment were not sufficient to connect the remaining proline residue to either of the fragments. A phase-sensitive ROESY nmr experiment (17) gave nOe connectivities (Figure 1) between δCH_2 of Proline and αH of ornithine, and αH of proline and NH of theoleucine. These results placed the proline residue between ornithine and theoleucine. The terminal NH of ornithine indicated nOe connectivities to αH and NH of 5-hydroxytryptophan and these correlations established the sequence of all seven amino acid residues. These data further indicated that discobahamin A was composed of a cyclic moiety and a side-chain. The cyclic moiety consisted of Pro, Orn, Htrp, Thl and Tle units connected in sequence. The side-chain was attached to αNH of the Orn residue and consisted of nVal whose N-terminus was shown to be protected by Hmp. The chiral tlc analysis of the hydrolysate of **1** with authentic samples revealed that the amino acid residues nVal, Ala (Thl) and Pro bore L configurations. Similarly, the chiral tlc analysis of the N-acetylmethylester derivatives of the hydrolysate of **1** revealed that Orn bore the L configuration. Oxidation of **1** with 35% H_2O_2/0.1 N aqueous NaOH followed by acid hydrolysis furnished L-leucine (18) which was detected by chiral tlc analysis with an authentic sample. Similarly, Lemieux-Rudloff oxidation (19) of **1** with $NaIO_4/KMnO_4$ followed by acid hydrolysis yielded L-aspartic acid, as detected by chiral tlc analysis. These data established the structure of discobahamin A [**1**].



1 R=H
2 R=CH_3

FIGURE 1. Structures and nOe correlations.

The molecular formula of discobahamin B [**2**] was determined to be $C_{48}H_{67}N_9O_{11}$ by hrfabms data. Amino acid analysis revealed that discobahamin B contained L-leucine in place of an nVal residue in discobahamin A. Further, the ^{13}C nmr comparison of **1** and **2** indicated that the carbon signals of the terminal ethyl group (18.1 ppm, γ-CH_2; 13.5 ppm, CH_3) of nVal in the spectrum of **1** was substituted by an isopropyl group (21.5, 23.0 ppm, two CH_3; 24.0 ppm, CH) in the spectrum of **2**. These data established the structure of discobahamin B [**2**].

Both discobahamin A and B exhibited weak antifungal activity against the yeast form of *C. albicans*.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Uv spectra were taken with a Perkin-Elmer Lambda 3 B uv/visible spectrophotometer. Ir spectra were obtained on a Perkin Elmer 1310 spectrophotometer. ^{13}C-nmr spectra were measured on a Bruker AM-360 at 360.13 MHz. ^1H-nmr and all 2D nmr spectra were measured on a Bruker AM-500 at 500.13 MHz for ^1H and 125.76 MHz for ^{13}C. All nmr spectra were measured in DMSO-d_6. Chemical shifts were referenced to solvent DMSO-d_6 signal at 2.49 ppm for ^1H and 39.5 ppm for ^{13}C. All 2D and nOe difference nmr spectra were run non-spinning mode. The hrms were obtained on a Kratos MS-80RFA mass spectrometer at the Chemical Instrumentation Center, Yale University. Optical rotations were measured with a Jasco DIP 360 digital polarimeter.

COLLECTION AND TAXONOMY.—The sponge is a new species of *Discodermia* (Phylum Porifera, Class Demospongia, Order Lithistida, Family Discodermiidae, Genus *Discodermia*, DBMR no. 9-XI-90-1-001), and was collected by the Johnson-Sea-Link manned submersible at a depth of 180 m at Goulding Cay, Bahamas (latitude 24° 58.56′ N, longitude 77° 30.57′ W). The sponge consists of several compressible, basally coalescent tubes, the surface of which are smooth and slightly fleshy, but which are microscopically roughened. The sponge is cream color in life. The skeleton of the sponge is consists of a light reticulation of fine desmas through which tracts of oxeas radiate towards the surface. The sponge surface is lined with irregular cup-shaped discotriaenes. A voucher specimen is deposited in the Harbor Branch Oceanographic Museum (catalog no. 003:00847).

EXTRACTION AND ISOLATION.—The freshly thawed sponge (1580 g, wet wt) was extracted three times with EtOH. The concentrated extract was then partitioned between EtOAc and H_2O. The EtOAc-soluble fraction (1.9 g) showed activity against *Candida albicans* (MIC=62.5 μg/ml; RPMI-1640 growth medium). This active fraction was chromatographed on Si gel (Kieselgel 60 H) using a CH_2Cl_2/MeOH step gradient. The antifungal active fraction (34 mg, *C. albicans* MIC=0.8 μg/ml; RPMI-1640) that eluted with 4% MeOH/CH_2Cl_2 on reversed-phase hplc (C_{18}, 5 μm, 250×10 mm) with 35% H_2O/MeOH containing 0.1% TFA, gave discobahamin-A [**1**] and discobahamin-B [**2**] as the active components.

Discobahamin A [**1**].—Pale yellow gum (7.2 mg, 0.0004% from frozen sponge); $[\alpha]^{24}D$ −29° (*c*=0.5 MeOH); ir (neat) 3300, 1641 cm^{-1}; uv λ max (MeOH) 203 nm (ε 1400), 220 (10200), 267 (6800), 305 (1500); nmr see Table 1; hrfabms (nitrobenzyl alcohol) *m/z* 932.4856, Δ 1 mmu for $C_{47}H_{66}N_9O_{11}$ (M+H).

Discobahamin B [**2**].—Pale yellow gum (3.0 mg, 0.0002%); $[\alpha]^{24}D$ −31° (*c*=0.1 MeOH); ir (neat) 3300, 1614 cm^{-1}; uv λ max (MeOH) 203 nm (ε 1400), 220 (10200), 267 (6800), 305 (1500); nmr see Table 1; hrfabms (nitrobenzyl alcohol) *m/z* 946.5022, Δ 2 mmu for $C_{48}H_{68}N_9O_{11}$ (M+H).

Amino acid analysis and determination of stereochemistry: (a) Peptides **1** and **2** (1.0 mg each) were hydrolyzed separately in 6 N HCl (110°, 24 h), and the hydrolysates were diluted with H_2O and lyophilized. Analysis by tlc against authentic samples [Si gel, *n*-BuOH-AcOH-H_2O (5:1:1), ninhydrin] indicated the presence of ornithine, proline, alanine and norvaline in **1** and ornithine, proline, alanine and leucine in **2**. (b) Peptides **1** and **2** (1.0 mg each) were treated separately with aqueous solutions of $NaIO_4$ (1 ml, 0.02 M) and $KMnO_4$ (1 ml, 0.003 M) at room temperature overnight and hydrolyzed with 6N HCl (110°, 24 h). Tlc analysis with an authentic sample indicated the presence of aspartic acid in **1** and **2**. (c) Peptide **1** (1.0 mg) was treated with 35% H_2O_2 and 0.1 N NaOH at room temperature overnight and hydrolyzed with 6N HCl (110°, 24 h). The presence of leucine in the mixture was detected by the tlc analysis with an authentic sample. (d) A portion of the hydrolysate of the peptide **1** from (a) was first methylated with CH_2N_2 in Et_2O/MeOH and then acetylated with $(CH_3CO)_2O$/pyridine. The removal of the solvents afforded the amino acid derivatives.

Chiral tlc analysis (Chiralplates, Macherey-Nagel) with Me_2CO-H_2O-MeOH (5:1:1) or H_2O-MeOH (4:1) against authentic D and L standards (Sigma) showed all amino acids to have the L configurations.

BIOLOGICAL ACTIVITY.—Both discobahamins-A and -B showed weak activity with MIC of 25 μg/ml against the yeast form of *C. albicans* (Sabouraud dextrose broth). The mycelial form of *C. albicans* was inhibited at equivalent levels (MIC=50 μg/ml) when tested in RPMI-1640 growth medium (20).

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