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Exumolides A and B: Antimicroalgal Cyclic Depsipeptides Produced by a Marine Fungus of the Genus *Scytalidium*.

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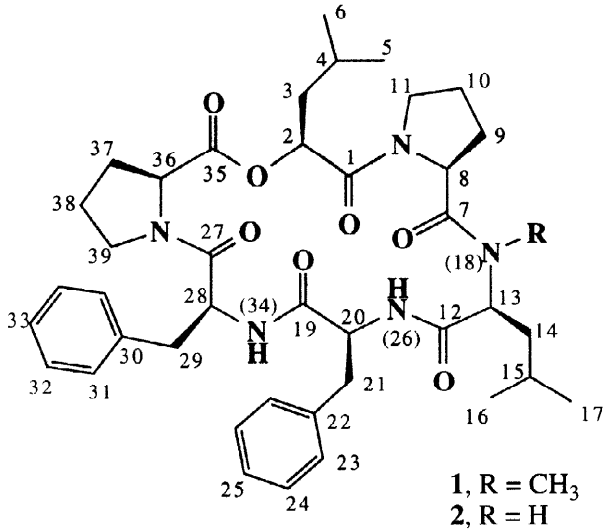
Abstract: Two new cyclic hexadepsipeptides, exumolides A (**1**) and B (**2**) were produced by a marine fungus, identified as a *Scytalidium* sp., collected in the Bahamas. Two dimensional NMR methods, coupled with tandem mass spectrometry (MS-MS), were used to establish the structures of the new compounds. The exumolides exhibit antimicroalgal activity against the unicellular chlorophyte *Dunaliella* sp. at 20 μg ml^{-1} in liquid media cell growth assays.

A great deal is known about the chemical role of terrestrial fungi in the pathogenicity of plants,[1] yet we are only now beginning to recognize the importance of the secondary metabolites produced by fungi associated with plants and algae in the marine environment. Although an increasing number of marine fungi are being examined for biologically active secondary metabolites,[2] very few of these studies have been aimed at the discovery of ecologically relevant natural products. In order to address questions concerning the natural role of fungal secondary metabolites, we have begun to investigate the chemical role of plant opportunistic fungi in the marine environment. As part of this program, we isolated a marine fungus which produced two new cyclic depsipeptides, exumolides A and B (**1, 2**) which exhibited antibiotic activity against the marine microalga *Dunaliella* sp. (Chlorophyta).



1, R = CH_3
2, R = H

The fungal isolate, designated CNC-389, was obtained from decaying plant material collected subtidally (-3 m) in the Exuma Islands, Bahamas. The fungal strain was identified as a member of the genus *Scytalidium* by fatty acid methyl ester (FAME) analysis.[3] The isolate was cultured in ten x 1L volumes using a marine nutrient medium.[4] The mycelium and broth were separated and the broth extracted with ethyl acetate. The broth extract was then concentrated and subjected to repeated size-exclusion chromatography (Sephadex LH-20; hexane:toluene:methanol, 3:1:1) to yield pure exumolides A (**1**) and B (**2**).[5]

Exumolide A (**1**) analyzed for $C_{41}H_{55}N_5O_7$ by HRFABMS ($[M+H]^+$ *m/z* 730.4220; calc. 730.4180), and by NMR spectral methods. The ^1H and ^{13}C-NMR spectra for **1** indicated resonances typical of a peptide

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(Table). The presence of 6 carbonyls (167.4 - 172.1 ppm) suggested the presence of a hexapeptide. The peptide nature of the molecule was further supported by the presence of 2 NH protons in the ^{1}H NMR spectrum and an *N*-Me carbon (28.8 ppm). However, the presence of a carbon at 70.8 ppm led to the proposal that the molecule contained a hydroxy acid moiety and therefore was a depsipeptide.

Table. Spectral Data for Exumolides A & B

	Exumolide A (1)		Exumolide B (2)	
C#	^{13}C[a]	^{1}H(δ)[b] Mult. (*J* (Hz))	^{13}C[c]	^{1}H(δ)[d] Mult. (*J* (Hz))
1	167.4		170.4	
2	70.8	5.11 (dd) 2, 12.5	73.4	4.78 (dd) 1.8, 10.8
3	38.9	1.80 (m); 1.42 (m)	39.7	1.33 (ddd) 1.8, 9.6, 15.0; 1.80 (m)
4	24.5	1.58 (m)	25.4	2.00 m
5	23.3	0.91 (d) 6.5	21.8	1.04 (d) 6.6
6	21.3	0.83 (d) 7.0	23.7	1.05 (d) 6.6
7	172.1		173.1	
8	56.4	4.72 (dd) 7.5	59.4	3.96 (dd) 3.6, 9.0
9	29.0	1.82 (m); 2.12 (m)	31.1	1.97 (m); 2.18 (m)
10	25.4	2.16 (m); 1.96 (m)	22.8	1.75 (m)
11	47.2	3.54 (m); 3.78 (ddd) 14.0, 8.5, 7.0	46.6	3.32 (ddd) 7.8, 7.8, 12.0; 3.59 (ddd) 4.8, 8.4, 12.0
12	169.6		172.6	
13	59.0	4.35 (m)	51.4	4.42 (ddd) 6, 9.6, 9.6
14	36.5	1.40 (m); 1.97 (m)	38.7	1.62 (m); 1.69 (m)
15	24.5	1.37 (m)	25.5	1.60 (m)
16	21.8	0.85 (d) 7.5	21.7	0.91 (d) 6.6
17	23.3	0.86 (d) 7.0	23.4	0.98 (d) 6.6
18	28.8 (NMe)	2.38 (s)	NH	6.95 (d) 9.0
19	169.6		170.5	
20	53.7	5.03 (dd) 9.0, 16.5	60.8	3.82 (ddd) 6.6, 6.6, 9.6
21	37.2	3.16 (dd) 13.5; 2.97 (dd) 9.0, 13.5	36.2	3.21 (dd) 6.6, 13.8; 3.38 (dd) 9.6, 13.8
22	136.6		137.8	
23	129.1 x2	7.14 (m)	129.6 x2	7.35 (m)
24	128.6 x2	7.24 (m)	129.2 x2	7.35 (m)
25	126.8	7.26 (m)	127.4	7.27 (m)
26	NH	8.17 (d) 9.0	NH	6.64 (d) 6.6
27	170.3		172.3	
28	54.5	4.37 (m)	54.1	4.52 (ddd) 4.8, 6.6, 9.6
29	40.3	2.73 (dd) 12.0; 3.17 (dd) 12.5	40.2	3.02 (dd) 9.6, 12.6; 3.07 (dd) 4.8, 12.6
30	136.6		137.8	
31	129.4 x2	7.14 (m)	129.8 x2	7.24 (m)
32	128.6 x2	7.24 (m)	128.9 x2	7.30 (m)
33	127.1	7.26 (m)	127.2	7.27
34	NH	6.92 (d) 6.0	NH	7.25
35	171.3		169.6	
36	59.4	3.28 (d) 7.5	61.3	4.24 (br d) 7.8
37	31.2	1.36 (m); 1.72 (m)	32.6	2.20 (m); 2.37 (br dd) 6.0, 12.6
38	22.2	1.66 (m)	22.5	1.70 (m)
39	46.0	3.37 (ddd) 8.5, 11.5, 8.5; 3.55 (m)	47.4	3.52 (m)

a) Recorded at 100 MHz in $CDCl_3$ b) Recorded at 500 MHz in $CDCl_3$ c) Recorded at 100 MHz in CD_2Cl_2 d) Recorded at 600 MHz in CD_2Cl_2 (assignments by DEPT, HMQC, HMBC, COSY 2D methods)

The structure of exumolide A was then assigned using a combination of 1- and 2-dimensional NMR methods complimented with tandem mass spectrometry (MS-MS). Analysis of the ^{1}H-^{1}H COSY spectrum indicated the presence of two units each of proline and phenylalanine. The methyl protons at 3.28 ppm were assigned to *N*-methylleucine (*N*-Me-Leu), and the hydroxy acid indicated by the carbon resonance at 70.8 ppm was established as leucic acid (O-Leu) by heteronuclear 2D NMR (HMQC and HMBC) experiments. The sequence of the peptide could not be established through analysis of the HMBC experiment. Instead, the cyclic

depsipeptide **1** was opened by methanolysis[6] resulting in the isolation of a linear peptide **3**, which was subsequently analyzed by electrospray MS-MS to determine its amino acid sequence (Figure 1).

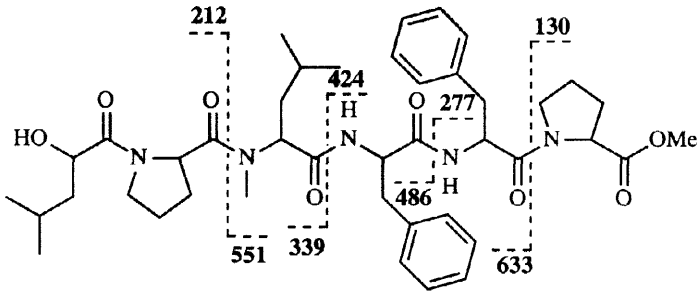
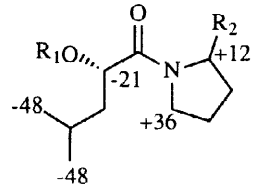


Figure 1. ESI MS/MS Analysis of Exumolide A Methanolysis Product (3)



3a R_1=(***R***)-MTPA, R_2=Peptide Chain
3b R_1=(***S***)-MTPA, R_2=Peptide Chain
$\Delta\delta$ values expressed in Hz (300 MHz)

Figure 2. Mosher Analysis of (3)

Acid hydrolysis of **1** followed by chiral GC-MS analysis of the TFA methyl ester derivatives,[7] including coinjection of the appropriate standards, confirmed the presence of L-proline and L-phenylalanine. Our attempts to resolve the TFA methyl ester derivative of *N*-Me-Leu by chiral GC-MS were unsuccessful, thus the absolute stereochemistry was solved by derivatization of the amino acid with Marfey's Reagent[8] and analysis by HPLC. The amino acid was determined to be in the L configuration based on retention time[8] and was further confirmed by coinjection with the appropriate standards.

The stereochemistry of O-Leu presented a challenge since it could not be readily obtained from the hydrolysate. Therefore, the methanolysis product (**3**) was used to prepare Mosher esters[9] of the free alcohol at C-2. Subsequent analysis of the ***R*** and ***S*** MTPA esters indicated that the absolute stereochemistry at C-2 was ***S*** (Figure 2).

Exumolide B (**2**) differed from **1** only by the loss of a methyl group, as indicated by the peak $[M+H]^+$ at *m/z* 716.4065 by HRFABMS indicating a molecular formula of $C_{40}H_{53}N_5O_7$ (calc. 716.4023). Examination of 2D NMR data showed the presence of leucine and not *N*-Me-Leu in **2**. COSY, HMQC, and HMBC NMR experiments allowed for the full assignment of the chemical shifts of exumolide B (**2**) (Table). Hydrolysis of **2**, followed by chiral GC-MS analysis, confirmed the presence of L-leucine and two units each of L-proline and L-phenylalanine. The absolute stereochemistry of O-Leu in (**2**) is predicted to be ***S*** by analogy to exumolide A.

Antimicroalgal activity of the exumolides was observed against the unicellular marine chlorophyte *Dunaliella* sp. using a liquid medium cell growth assay.[10] Exumolides A (**1**) and B (**2**) exhibited cytostatic effects resulting in a reduction in growth of 27% and 33%, respectively, at a concentration of 20 μg ml^{-1}. This study is the first to show that a *Scytalidium* sp. produces metabolites that inhibit the growth of marine algae. On the basis of this observation, it appears feasible that secondary metabolites may be involved in the fungal pathogenesis of marine plants. Other fungal isolates in this genus have been implicated in terrestrial plant diseases including leaf spot in banana (*Musa* sp.)[11] and root black rot in cassava, *Manihot esculenta*.[12] There is emerging evidence that marine fungi can significantly affect marine plants through the production of growth-inhibiting secondary metabolites.[10,13] However, additional studies are warranted, especially those targeting fungi that are known to be associated with diseases in marine plants and algae. The major difficulty in performing studies of this type is that many of these fungi are not readily amenable to laboratory culture and are hence difficult to study chemically. Nevertheless, continued studies that investigate the ecological functions of fungal phytotoxins will aid in the understanding of the roles marine fungi have in structuring marine plant communities.

Acknowledgments

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3. FAME (Fatty Acid Methyl Ester) analysis (Microbial ID Inc. Newark, DE), similarity index 0.882.

4. The fungal strain was cultured without shaking for 7 days at 28°C in marine medium YPG+C (yeast extract (0.5%), peptone (0.5%), glucose (1.0%), crab meal (0.2%) and seawater (100%)).

5. Exumolide A (**1**): white solid, $[\alpha]_D$ -278° (c 1.32, $CHCl_3$); UV λ_{max} (MeOH): 203 nm (log ε 4.54); IR ν_{max} (film, NaCl) 3292, 2957, 2872, 1736, 1647, 1450 and 1278 cm^{-1}. Exumolide B (**2**): white solid, $[\alpha]_D$ -288° (c 2.13, $CHCl_3$); UV λ_{max} (MeOH): 203 nm (log ε 4.52); IR ν_{max} (film, NaCl): 3361, 2957, 1736, 1654, 1648, 1522, 1448 and 1198 cm^{-1}.

6. To 17.0 mg of 1 was added 1 ml of 0.5 N NaOMe, and the mixture was allowed to stir at room temp. for 72 h. The reaction was quenched with dilute HCl and exhaustively extracted with methylene chloride. Concentration under reduced pressure yielded 15.6 mg of a crude product which was purified by Si Sep-Pak (95% $CHCl_3$/MeOH) and RP-C18 HPLC (80% aqueous MeOH) to give 3.0 mg of the linear peptide (**3**).

7. Hydrolysis of **1** was achieved using degassed 5.0 N HCl at 110° C for 12 h. The samples were dried and the methyl esters were prepared by adding 2 ml of a 1:1 mixture of acetyl chloride/methanol and allowing the mixture to stir at 100° C for 45 minutes. The solvent was then removed under a stream of nitrogen, and the methyl esters were dissolved in 200 µl of dichloromethane. Trifluoroacetyl anhydride (200 µl) was then added and the reaction mixture was heated to 100° C for 15 minutes. The solvent was again removed under nitrogen and the corresponding TFA-methyl ester derivatives were dissolved in dichloromethane and analyzed by GC-MS using an Alltech Chirasil-Val capillary column (0.32 mm x 25 m).

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