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Sansalvamide: A New Cytotoxic Cyclic Depsipeptide Produced by a Marine Fungus of the Genus *Fusarium*.

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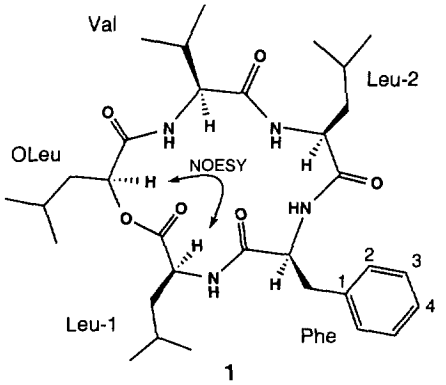
Abstract: A new cyclic pentadepsipeptide, sansalvamide (**1**), has been isolated from organic extracts of the mycelium of a fungus of the genus *Fusarium* collected from the surface of the seagrass *Halodule wrightii*. The structure of **1** was determined through extensive analysis of 1D and 2D NMR data. Sansalvamide exhibited selective *in vitro* cytotoxicity toward COLO 205 colon and SK-MEL-2 melanoma cancer cell lines.

Marine microorganisms have proven to be a promising source for the production of novel antibiotic, antitumor, and antiinflammatory agents.[2] The marine fungi, particularly those associated with marine animals[3] and plants,[4] appear to be an unusually rich resource for secondary metabolites. In this paper, we extend previous studies and report the isolation of a new cytotoxic cyclic depsipeptide, sansalvamide A (**1**), produced by a fungus associated with a marine higher plant. The fungal strain, culture # CNL 292, was obtained from the surface of the seagrass *Halodule wrightii* collected in the inner lagoon of Little San Salvador Island, Bahamas in 1996.



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The fungus, determined to be a *Fusarium* sp. by fatty acid methyl ester (FAME) analysis,[5] was cultured (17 L) in a seawater-based medium.[6] The resulting broth and mycelium were extracted separately to afford crude extracts of 0.5 g and 19.2 g, respectively. The isolation of sansalvamide (**1**) from the mycelium extract was accomplished using bioassay-guided fractionation monitoring *in vitro* cytotoxicity against a human colon carcinoma cell line (HCT-116). The crude mycelium extract was fractionated over silica gel and active fractions of similar composition were pooled and subsequently repurified over silica gel. Final purification was accomplished by precipitation of **1** (642 mg) from Et_2O solution by dropwise addition of hexane. Sansalvamide (**1**) analyzed for $C_{32}H_{50}N_4O_6$ (10 unsaturations) by HRFABMS and ^{13}C NMR data.[7] In the ^{13}C NMR spectrum, five signals were observed between δ171-175, values typically assigned to the amide carbonyls of a peptide. Distinctive IR absorptions indicated the presence of at least one ester (1744 cm^{-1}) in addition to the amide (1661 cm^{-1}) functionalities. Four ^{13}C NMR signals observed between δ 52-61 were characteristic of the α-carbons of amino

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acid residues. One additional signal at δ 76.2 was highly suggestive of the α-carbon of an α-hydroxy acid moiety. The assignment of one ester and four amide groups accounted for all of the nitrogens, oxygens, and five of the unsaturations required by the molecular formula. Four signals in the aromatic region of the ^{13}C NMR spectrum between δ 128-139 were characteristic of a mono-substituted phenyl group, and suggested the presence of a phenylalanine (Phe) residue, which accounted for an additional four unsaturations, and left one remaining unsaturation, requiring that **1** possess one ring. Application of 2D NMR experiments (HMQC, HMBC, COSY, TOCSY), which allowed for the identification of all of the amino- and hydroxy-acid residues of **1**, revealed the presence of valine (Val), phenylalanine (Phe), two leucines (Leu-1 and Leu-2), and leucic acid (OLeu). Two- and three-bond HMBC correlations (Table) from the α- and/or β-protons of each residue to their neighboring carbonyl carbons were useful in assigning the carbonyl signals and in establishing the amino acid sequence of **1**.

Table. NMR Spectral Data for Sansalvamide (1) in CD_3OD

position	^1H	^{13}C	Key HMBC correlations	position	^1H	^{13}C	Key HMBC correlations
Leu-1				**Leu-2**			
C=O	—	171.3		C=O	—	174.1	
α-CH	4.73 (dd; 9.8, 5.9)	52.6	173.71, 171.3	α-CH	3.83 (br dd; 8.8, 5.4)	56.2	174.1
β-CH_2	1.64-1.86 (m)	41.6	171.3, 52.6, 26.0	β-CH_2	1.72 (m)	40.1	26.2
γ-CH	1.62 (m)	26.0[a]			1.38 (m)		26.2
γ-CH_3	0.99 (d;6.4)	23.5[b]	41.6	γ-CH	1.41 (m)	26.2[a]	
	0.96 (d;6.4)	22.4[c]	41.6	γ-CH_3	0.85 (d; 6.4)	23.2[b]	40.1
					0.81 (d; 5.9)	22.2[c]	40.1
OLeu							
C=O	—	172.9		**Phe**			
α-CH	5.06 (dd; 8.8, 4.9)	76.2	172.9, 171.3, 42.1, 26.1	C=O	—	173.7[d]	
β-CH_2	1.76-1.88 (m)	42.1	172.9, 76.2, 26.1	α-CH	4.60 (dd; 10.8, 4.4)	58.1	174.1, 173.71,
	1.58-1.64 (m)		172.9, 26.1				138.8, 38.4
γ-CH	1.66-1.78 (m)	26.1[a]		β-CH_2	3.23 (dd; 13.9, 4.6)	38.4	173.71, 58.1
γ-CH_3	0.99 (d; 6.4)	23.4[b]	42.1		3.06 (dd; 13.7, 10.7)		173.71, 58.1
	0.96 (d; 6.4)	22.2[c]	42.1	1	—	138.8	
				2/6	7.25-7.32 (m)	130.4	38.4
Val				3/5	7.25-7.32 (m)	129.7	38.4
C=O	—	173.8[d]		4	7.22 (m)	128.0	130.4, 129.7
α-CH	4.09 (d; 8.8)	60.9	173.79, 172.9, 32.2				
β-CH	2.10 (m)	32.2					
γ-CH_3	0.96 (d; 6.4)	20.0	60.9				
	0.92 (d; 6.8)	19.0	60.9				

[a, b, c, d] Values for these assignments may be interchanged. ^1H NMR data recorded at 300 MHz; ^{13}C NMR data recorded at 75 MHz.

Correlations of both the Leu-1 and OLeu α-protons, at δ 4.73 and 5.06 respectively, to the carbonyl carbon at δ 171.3 established the partial sequence Leu-1 → OLeu. Correlations of both OLeu and Val (δ 4.09) α-protons, to the carbonyl carbon at δ 172.9 further extended the sequence to Leu-1 → OLeu → Val. Three-bond HMBC correlations from the β-protons of both Leu-1 and OLeu assigned the carbonyl carbons belonging to these two residues as δ 171.3 and δ 172.9 respectively, and necessitated that OLeu is acylated by Leu-1, and Val is acylated by OLeu. Correlations of both Leu-2 and Phe α-protons, at δ 3.83 and δ 4.60, to the carbonyl carbon at δ 174.1, established the partial sequence Leu-2 → Phe. Both β-protons of Phe (δ 3.23 and 3.06) showed three-bond HMBC correlations to one of the two overlapping carbonyl carbons at δ 173.7, which indicated that Phe is acylated by Leu-2, since Leu-2 and Phe were already shown to be connected by the carbon at δ 174.1.

Correlations from the α-protons of Phe, Leu-1, and Val to the two carbonyl carbons at δ 173.7 connected the two partial sequences to form the overall sequence for sansalvamide in the only way possible, where Leu-2 is acylated by Val, and Leu-1 is acylated by Phe as shown for **1**.

Further support for the peptide sequence was provided following isolation of the linear peptide **2**, which was produced via base hydrolysis of **1**.[8] Electrospray MS-MS analysis revealed product ions corresponding to sequential losses expected from both termini of **2** as shown in the Figure.

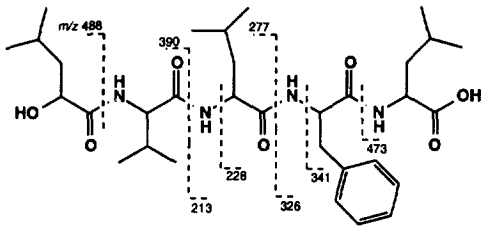

Figure. Electrospray MS-MS Analysis of Sansalvamide Hydrolysis Product **2**.

A sample of **1** was hydrolyzed in 6N HCl (110 °C, 24h) and the resulting amino acids were converted to pentafluoropropyl isopropyl ester derivatives[9] which were analyzed by chiral capillary GC. Retention times of the derivatives, and coinjection with standards, revealed that the four amino acid residues in **1** possess L-configurations. The NOESY NMR spectrum of **1** in $CDCl_3$[10] revealed a key correlation between the α-proton of Leu-1 and that of Oleu, suggesting that both hydrogens are on the same (α) face of the molecule, and implying that the OLeu residue also possesses the L-configuration. In order to confirm this result, a second sample of **1** (50 mg) was hydrolyzed in a similar manner and the hydrolysate evaporated to dryness. This material was redissolved in H_2O (20 mL) and extracted twice with equal portions of EtOAc to provide, upon evaporation, an oily material which proved to be pure leucic acid on the basis of ^1H NMR data.[11] The optical rotation of this material showed it to possess the L-configuration when compared to an authentic standard of L-leucic acid.[12]

Sansalvamide (**1**) was found to be responsible for the majority of the cancer cell cytotoxicity present in the crude extract, exhibiting an *in vitro* IC_{50} value of 9.8 μg/mL toward HCT-116 colon carcinoma. Interestingly, the linear peptide **2** was found to be inactive in this assay, suggesting that the cyclization is required for cytotoxicity. Sansalvamide, when further evaluated for cancer cell cytotoxicity in the National Cancer Institute's 60 cell-line panel,[12] showed a mean IC_{50} of 27.4 μg/mL. More importantly, sansalvamide showed greater potency toward the colon cancer cell-line COLO 205, and melanoma cell-line SK-MEL-2 with IC_{50} values of 3.5 and 5.9 μg/mL, respectively. These values are comparable to those recorded for some FDA-approved antitumor agents, e.g. mitomycin C, which exhibits an IC_{50} value of 5.3 μg/mL toward these same two cell-lines.[14] In contrast, the highly selective antitumor agent paclitaxel (mean IC_{50} = 17.0 μg/mL over all cell-lines) exhibits an IC_{50} value of 0.02 μg/mL toward the breast cancer cell-line MAXF 401, suggesting that the potency of compound **1** is well below the useful range for compounds of this type.

ACKNOWLEDGMENTS

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5. FAME (Fatty Acid Methyl Ester) analysis (Microbial ID Inc., Newark, DE), similarity index 0.845. The identification was also confirmed by Professor E. B. Gareth Jones.

6. The fungus was cultured for 20 days (static) at 28 °C in YPG+C medium: yeast extract (0.5%), peptone (0.5%), glucose (1.0%), crab meal (0.2%), and seawater (100%).

7. Sansalvamide (**1**) was isolated as a white powder; mp 143-152 °C; $[\alpha]_D$ -115° (*c* 0.001, MeOH); UV (MeOH) 202 nm (ε 11690), 214 (6180), 254 (211); IR (NaCl) 3275 (br), 2959, 2927, 1744, 1661, 1538, 1469, 1247 cm^{-1}; HRFABMS obsd. *m/z* 587.3869 $(M+H)^+$, calcd. for $C_{32}H_{51}N_4O_6$, 587.3808 (Δ -6.0 mDa).

8. Sansalvamide (**1**, 43 mg) was dissolved in 1 mL of 0.5M NaOMe in MeOH and stirred at rt for 24h. The reaction mixture was neutralized with 0.5M HCl and the product was purified by C_{18} chromatography (MeOH elution) to afford the linear peptide **2** [40 mg; LRMS obsd. *m/z* 603.3 $(M-H)^-$, calcd. for $C_{32}H_{52}N_4O_7$, 604.4].

9. Alltech Kit cat# 18093. See: Ibrahim, K. E.; Couch, M. W.; William, C. M.; Budd, M.; Yost, R. A.; Midgley, J. M. *Anal. Chem.* **1984**, *56*, 1695.

10. ^1H NMR spectra in $CDCl_3$ were generally poorly resolved. Clear signals for N-H and α-protons were seen only at high concentrations (e.g. >50 mg/mL) suggesting increased order in solution due to inter- or intramolecular hydrogen bonding. All observed NOESY correlations agreed with the proposed sequence for **1**.

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12. Leucic acid from **1** $[\alpha]_D$ = -28.0°; leucic acid standard (Aldrich) $[\alpha]_D$ = -29.0° (*c* 0.002 g/mL, 1N NaOH, for both samples).

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14. Data for known anticancer agents are available on the World Wide Web: http://ctep.info.nih.gov/ and http://epnws1.ncifcrf.gov/.