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N-Methylsansalvamide, a cytotoxic cyclic depsipeptide from a marine fungus of the genus *Fusarium*

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Abstract

N-Methylsansalvamide (**1**), a new cyclic depsipeptide, was isolated from extracts of a cultured marine fungus, strain CNL-619, identified as a member of the genus *Fusarium*. *N*-Methylsansalvamide exhibits weak in vitro cytotoxicity in the NCI human tumor cell line screen (GI_{50} 8.3 μM). The structure of **1** was determined by combined spectral and chemical methods.

Keywords: *Fusarium* sp.; Marine fungi; Cyclic depsipeptide

1. Introduction

In contrast to how little is known of the distributions of higher fungi within diverse marine habitats, the associations of fungi with marine plants have been well documented (Kohlmeyer and Kohlmeyer, 1979). As part of a comprehensive examination of marine plant-associated fungi, we have focused considerable attention on fungi associated with the siphonaceous green algae, which are abundant in tropical, shallow-water environments. Although fungi isolated from these plants can be related at the generic level to terrestrial plant pathogens, the secondary metabolites they produce can be diverse and unique. Our studies of siphonaceous algae have included the Caribbean green alga *Avrainvillea* sp. from which we have consistently obtained marine fungi. In this paper, we report the isolation and chemical investigation of a *Fusarium* species (strain CNL-619) which produces *N*-methylsansalvamide (**1**), a weakly cytotoxic, cyclic depsipeptide. The new depsipeptide is related to sansalvamide (**2**), its demethyl analog, which we isolated earlier from an unrelated fungal strain, and reported as possessing cytotoxic and antiviral properties (Belofsky et al., 1999). Sansalvamide was of particular interest since it was found to be a selective inhibitor of MCV topoisomerase (Hwang et al., 1999).

2. Results and discussion

Fusarium sp., strain CNL-619, collected in the US Virgin Islands, was cultured in a seawater-based medium. The mycelium and broth were extracted with ethyl acetate and the solvents concentrated to provide a crude extract that showed in vitro cytotoxicity toward human colon adenocarcinoma (HCT-116). Bioassay-guided fractionation using C-18 column chromatography, size exclusion column chromatography (Sephadex LH-20) and C-18 reversed-phase HPLC provided purified *N*-methylsansalvamide (**1**) as a viscous oil (yield 3.1 mg/l).

N-Methylsansalvamide was analyzed for $C_{33}H_{52}N_4O_6$ by high resolution mass spectrometry and combined spectral data. The ^1H and ^{13}C NMR spectral data acquired for **1** illustrated typical resonances for a cyclic peptide (Table 1). The presence of five carbonyl resonances at δ 171.6, 172.1, 172.3, 172.9 and 173.3 suggested that the new molecule was a pentapeptide. The presence of a methine carbon at δ 76.4, which was clearly not of amino acid origin, together with presence of three NH protons and an N-Me functionality, led to the proposal that *N*-methylsansalvamide (**1**) was a monocyclic depsipeptide containing a hydroxy acid component.

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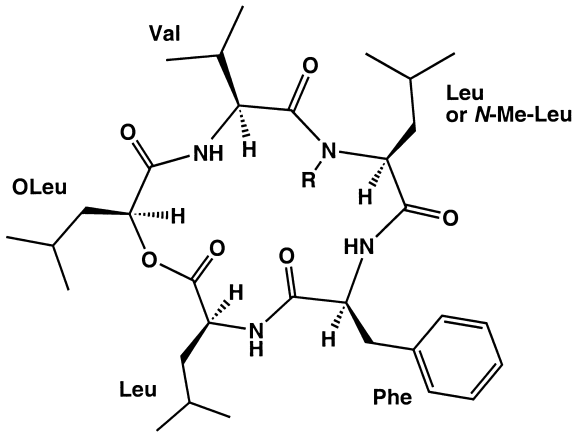
The structure of *N*-methylsansalvamide (**1**) was assigned using a combination of one and two-dimensional NMR spectroscopic methods. Analysis of 1D-TOCSY data established the presence of valine (Val), leucine (Leu), and phenylalanine (Phe) (Table 1). Analysis of 1D-TOCSY, HMQC and HMBC data further established the presence of one *N*-methylleucine (*N*-Me-Leu) and one leucic acid component (*O*-Leu). The sequence of the cyclic peptide was established by analysis of HMBC data, which generally showed correlations of the carbonyl carbons of each amino acid with the corresponding NH proton of the adjacent residue. Connection of the N-methylleucine residue with valine was accomplished in the HMBC experiment by interpretation of correlations from the valine carbonyl resonance (δ 173.3) with the *N*-methyl protons of *N*-Me-Leu. The valine residue was connected to the leucic acid component through correlations of the *O*-Leu carbonyl resonance (δ 171.6) to the α proton of the valine unit.

The absolute stereochemistries of the amino acids in *N*-methylsansalvamide were determined by acid hydrolysis followed by derivatization of the amino acids with Marfeys' Reagent (Marfey, 1984) and HPLC analysis. By co-injection with appropriate standards, the amino acids in **1** were all shown to possess *L* configurations. To determine the stereochemistry of leucic acid, the depsipeptide was subjected to hydrolysis and methylation (Abou-Masour et al., 1995) resulting in the isolation of a linear peptide, **3**, a derivative which was used to prepare the *R*- and *S*-Mosher esters (Dale et al., 1969; Ohtani et al., 1991) of the free alcohol at C-2. Subsequent NMR spectroscopic analysis of the $\Delta\delta$ values for the *R*- and *S*-MTPA esters enabled assignment of the absolute stereochemistry at C-2 as *S*, for *L*-leucic acid (Fig. 1).



N-Methylsansalvamide (**1**) is closely related to sansalvamide (**2**), a cyclic depsipeptide previously isolated from cultures of an unrelated marine *Fusarium* sp. found in association with the seagrass *Halodule wrightii* collected in the Bahamas (Belofsky et al., 1999). Both **1** and **2** are moderate cytotoxins with mean GI_{50} values of 8.3 and 3.6 μM, respectively, in the National Cancer Institute's human tumor cell line screen.

Table 1
NMR spectral data for **1** in pyridine-d_5[a]

Position	^{13}C	^{1}H *J* in brackets	HMBC Correlations
O-Leu			
1	171.6 C	–	
2	76.4 CH	5.36 *dd* (3.9, 9.3)	171.6, 41.5, 25.7
3	41.5 CH_2	1.90 *m*	
		2.10 *m*	76.4
4	25.7 CH	1.90 *m*	
5	21.8 CH_3	0.95 *d* (5.1)	
6	22.1 CH_3	0.90 *d* (7.5)	
Val			
1	173.3 C	–	
2	55.6 CH	4.98 *dd* (6.3, 8.9)	173.3, 31.2, 17.7, 20.7, 171.6
3	31.2 CH	2.31 *m*	17.7, 20.7
4	17.7 CH_3	1.12 *d* (6.6)	20.7
5	20.7 CH_3	1.01 *d* (6.9)	17.7
NH	–	7.76 *d* (9.0)	173.3, 171.6
N-Me-Leu			
1	172.3 C	–	
2	68.4 CH	3.72 *dd* (6.6, 8.4)	172.3, 38.3, 26.1, 40.0
3	38.3 CH_2	2.30 *m*	
		2.00 *m*	
4	26.1 CH	1.60 *m*	38.3, 23.4
5	24.1 CH_3[b]	0.83 *d* (6.3)	
6	23.4 CH_3[b]	0.82 *d* (6.6)	
N-Me	40.0 CH_3	3.18 *s*	68.4, 173.3
Phe			
1	172.9 C	–	
2	56.7 CH	5.11 *dd* (8.7, 15.2)	172.9, 139.1, 172.1
3	38.0 CH_2	3.55 *dd* (6.3, 13.8)	172.9, 56.7, 139.1, 129.4
		3.41 *dd* (9.0, 13.7)	172.9, 56.7, 139.1, 129.4
4	139.1 C	–	
5	129.4×2 CH	7.30 *m*	
6	130.0×2 CH	7.32 *m*	
7	127.5 C	7.28 *m*	
NH	–	8.68 *d* (9.0)	172.3
Leu			
1	172.1 C	–	
2	52.6 CH	4.89 *m*	38.2
3	38.2 CH_2	2.20 *m*	
		1.96 *m*	52.6
4	25.1 CH	1.51 *m*	
5	22.5 CH_3	0.94 *d* (6.3)	
6	23.7 CH_3	0.87 *d* (6.9)	
NH	–	9.43 *d* (8.7)	52.6, 172.9

[a] ^{1}H NMR data were recorded at 300 MHz and ^{13}C NMR data were recorded at 75 MHz.

[b] Resonances may be interchanged.

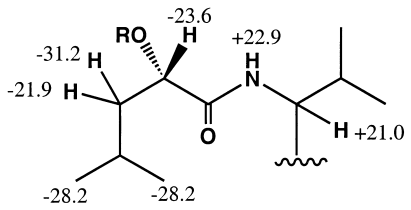


Fig. 1. **3a** R = S-MTPA; **3b**, R = R-MTPA; $\Delta\delta$ values expressed in Hz (300 MHz).

3. Experimental

3.1. Producing organism isolation and identification

Fungal strain CNL-619 was obtained from a sample of the green alga *Avrainvillea* sp. collected from a mangrove at a depth of 2 m along the southeast end of St. Thomas at Bovoni Cay, United States Virgin Islands, in 1996. *Fusarium* strain CNL-619 was isolated on an agar medium YPG (0.1% glucose, 0.05% peptone, 0.01% yeast extract, 0.01% penicillin G/streptomycin sulfate, 100% seawater). Strain CNL-619 showed a good genus level match with *Fusarium* based on fatty acid methyl ester analysis (similarity index 0.772, Microbial ID Inc., Newark, DE, USA).

3.2. Cultivation of Fusarium strain CNL-619

Fusarium sp., strain CNL-619, was cultivated under static conditions in 2.8 L Fernbach flasks (20×1 l) at 25°C in Marine Broth 2216 (DIFCO Labs., Detroit, MI, USA) prepared with 100% seawater. After 21 days, the mycelium was collected by filtration, freeze-dried, and extracted with 2.5 l of 1:1 methanol:dichloromethane. The culture broth was extracted with 25 l of ethyl acetate, and dried with anhydrous sodium sulfate. Both extracts were concentrated by rotary evaporation and combined.

3.3. Purification and properties of N-methylsansalvamide (*1*)

The crude extract was fractionated by monitoring in vitro cytotoxicity against HCT-116 colon adenocarcinoma as a guide. The crude extract was first fractionated by reversed-phase, C-18 flash column chromatography using gradient elution (100% H_2O to 100% MeOH). Cytotoxic fractions were combined and re-fractionated by size exclusion chromatography (Sephadex LH-20) using isooctane: toluene: MeOH (3:1:1) as the eluting solvent mixture. Cytotoxic fractions from the latter fractionation were also combined and purified by C-18, reversed-phase HPLC (Dynamax semi-preparative column, 60 Å, 10×250 mm) with MeOH:H_2O (9:1), to yield *N*-methylsansalvamide (**1**, 61.7 mg) as the sole cytotoxic component of the mixture. *N*-Methylsansalvamide (**1**) showed the following spectral characteristics: Colorless oil, $[\alpha]_D$ −132° (*c*, 0.415, CH_2Cl_2); UV λ_{max} ($CHCl_3$): 269 (ϵ 1200), 256 (ϵ 1320), 220 (ϵ 10,120) nm; IR ν_{max} (film, NaCl) (cm^{-1}) 3307, 2950, 1737, 1666, 1631, 1519; HRFABMS $[M+Cs]^+$ m/z 733.2968 calculated for $C_{33}H_{52}N_4O_6$: 733.2941); ^1H and ^{13}C NMR data see Table 1.

3.4. Acid hydrolysis of N-methylsansalvamide (*1*)

N-Methylsansalvamide (2 mg) was dissolved in 6 N HCl (0.5 ml) and heated at 110°C for 16 h. The solvents and volatile residual acid were then removed under high vacuum to yield a reaction mixture that was used without further purification.

3.5. Preparation of amino acid Marfey derivatives

1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA), (50 μl, 10 mg/mL) was dissolved in acetone and 1 M $NaHCO_3$ (100 μl) was added to the acid hydrolysate from *N*-methylsansalvamide. The mixture was maintained at 80°C for 3 min, then 2 N HCl (50 μl) and 50% aqueous MeCN (300 μL) were added. An aliquot of the resulting mixture was analyzed by C-18 reversed-phase HPLC (Hewlett Packard 1090 Diode Array) eluting with a linear gradient from 10 to 50% aqueous MeCN (0.1% TFA) over 60 min; UV (340 nm). The amino acids were identified by co-injection with authentic amino acid standards. Retention times (min) in parentheses are as follows: L-*N*-Me-Leu (45.03), D-*N*-Me-Leu (46.70); L-Leu (44.98), D-Leu (49.31); L-Phe (44.51), D-Phe (48.48); L-Val (38.80), D-Val (45.22).

3.6. Base hydrolysis of N-methylsansalvamide (*1*)

Compound **1** (10.3 mg) was dissolved in MeOH (0.2 ml) and 0.27 N NaOH (0.5 ml). The mixture was stirred at room temperature for 1 h. The reaction mixture was then neutralized with HCl (0.27 N) and the solvents were removed under N_2. Methylation was accomplished by the addition of CH_2N_2 in ether until a yellow color persisted. The solvent was evaporated under N_2 and the product purified by reversed-phase C-18 HPLC using MeOH:H_2O (9:1) to obtain the linear peptide **3** (6.3 mg). The peptide was isolated as a colorless oil, which showed the following spectral properties: ^1H NMR (300 MHz, Pyridine-d_5) δ: 0.74 (3H, *d*, *J* = 6.6 Hz); 0.79 (3H, *d*, *J* = 6.6 Hz); 0.82 (3H, *d*, *J* = 6.9 Hz); 0.91 (3H, *d*, *J* = 6.3 Hz); 0.96 (3H, *d*, *J* = 6.6 Hz); 0.98 (3H, *d*, *J* = 6.6 Hz); 1.05 (3H, *d*, *J* = 6.3 Hz); 1.09 (3H, *d*, *J* = 6.6 Hz); 2.02–1.85 (5H, *m*); 1.73 (1H, *m*); 1.68 (1H, *t*, *J* = 9.6 Hz); 1.55 (1H, *m*); 3.17 (1H, *dd*, *J* = 7.8, 14.4 Hz); 3.45 (1H, *dd*, *J* = 6.3, 13.8 Hz); 3.20 (3H, *s*); 3.69 (3H, *s*); 4.54 (1H, *dd*, *J* = 3.3, 3.9, 9.6 Hz); 5.06 (1H, *dd*, *J* = 8.1, 15.6 Hz); 5.22 (1H, *dd*, *J* = 6.3, 6.6, 9.2 Hz); 5.37 (1H, *dd*, *J* = 7.8, 8.4, 14.4 Hz); 5.71 (1H, *dd*, *J* = 6.3, 9.0 Hz); 8.56 (1H, *d*,

$J = 9.0$ Hz); 9.25 (1H, *d*, $J = 8.1$ Hz); 9.65 (1H, *d*, $J = 8.4$ Hz). EIMS *m*/*z* 632.70 (M^+) (11).

3.7. Preparation of Mosher ester derivatives (**3a**, **3b**) of linear peptide **3**

A mixture of the linear peptide **3** (4.4 mg, 6.9 μM), (*S*)-MTPA (20.7 mg, 87 μM), dicyclohexylcarbodiimide (19.8 mg, 96 μM), and 4-(dimethylamino)pyridine (5.4 mg, 45 μM) in CH_2Cl_2 (0.5 ml) was stirred at room temperature for 72 h. The solvent was removed under N_2 and the residue was purified by reversed-phase, C-18 HPLC using MeOH (100%) to give the (*S*)-MTPA ester **3a** (3.0 mg). The same experimental procedure was followed for the production of the corresponding (*R*)-MTPA ester **3b**.

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