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Zygosporamide, a cytotoxic cyclic depsipeptide from the marine-derived fungus *Zygosporium masonii*

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Abstract—Zygosporamide (**1**), a new cyclic pentadepsipeptide, was isolated from the seawater-based fermentation broth of a marine-derived fungus identified as *Zygosporium masonii*. The structure of **1**, which is composed of α-hydroxyleucic acid and both D- and L-amino acids, was determined by combined spectral and chemical methods. Despite a simple structure, zygosporamide illustrated significant cytotoxicity in the NCI's 60 cell line panel (median $GI_{50} = 9.1$ μM), with highly enhanced selectivity against the CNS cancer cell line SF-268 ($GI_{50} = 6.5$ nM) and the renal cancer cell line RXF 393 ($GI_{50} \leqslant 5.0$ nM).

Studies of marine-derived fungi during the past 15 years have demonstrated that these organisms produce diverse, bioactive secondary metabolites.[1] The most recent review of secondary metabolites from marine-derived fungi revealed that strains belonging to the common terrestrial genera *Aspergillus* and *Penicillus* produced most of the reported metabolites.[2] *Zygosporium masonii* is a terrestrial fungus known for the production of cytochalasins.[3] As is the case with most marine-derived *Aspergillus* and *Penicillium* species, it is not clear if marine-derived *Z. masonii* is metabolically active in the marine environment or how adaptations to marine conditions may influence the production of unique secondary metabolites. As a part of our interest in the discovery of drug leads for the treatment of cancer, we isolated a strain (CNK458) of the fungus *Z. masonii* from a marine cyanobacterium collected off the island of Maui, Hawaii.[4] Cultivation of this isolate in a seawater-based medium resulted in the production of a new cytotoxic cyclic depsipeptide, zygosporamide (**1**). Here, we report the isolation, structure elucidation, and biological activity of zygosporamide.

Zygosporamide (**1**),[5] $[\alpha]_D$ −112 (*c* 0.26, CH_3CN), was isolated as a white powder and analyzed for the molecular formula $C_{36}H_{50}N_4O_6$ (14 units of unsaturation) by high resolution mass spectral data, $[M+H]^+$ $m/z = 635.3806$ (calcd for $C_{36}H_{51}N_4O_6$ 635.3809).

This formula was fully supported by ^1H and ^{13}C NMR data, comprehensive analysis of which (Table 1) showed distinctive characteristics of a depsipeptide. In the ^1H NMR spectrum, four D_2O exchangeable doublet resonances at δ_H 7.66, 7.16, 7.11, and 6.97, were assigned as four amide protons, while five protons at δ_H 4.83, 4.74, 4.69, 4.12, and 4.08 were assigned as α-amino acid methine protons. Four double doublet proton signals at δ_H 3.34, 3.12, 3.03, and 2.88 were indicative of two phenylalanine units. Five carbonyl signals at δ_C 173.7, 173.1, 172.7, 171.4, and 170.9 were observed in the ^{13}C NMR spectrum and four out of the five carbonyls were assigned as amides. The infrared spectrum of **1** showed a absorption band at 1743 cm^{-1} indicative of an ester carbonyl. This, coupled with the presence of one oxygen-bearing carbon (δ_C 76.6) in ^{13}C NMR spectrum of **1**, suggested that one of the carbonyl signals was from an ester functionality.

Further analysis of DEPT and 2D NMR spectral data (COSY, TOCSY, HMQC, and HMBC) allowed five subunits to be established: two leucines (Leu), two phenylalanines (Phe), and one α-hydroxyleucic acid (*O*-Leu). Since the two aromatic rings and five carbonyls

Keywords: Zygosporamide; Marine-derived fungus; *Zygosporium masonii*; Cancer cell cytotoxicity; Central nervous system cancer; Renal cancer.
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Table 1. NMR spectral data for zygosporamide (**1**) in CD_3CN

	C/H #	δ_H (J in Hz)[a]	δ_C[b]	
L-Phe-1	1		171.4	C
	2	4.83, ddd (11.0, 7.5, 5.0)	53.5	CH
	3a	3.12, dd (14.0, 11.0)	37.3	CH_2
	3b	3.34, dd (14.0, 11.0)		
	4		138.7	C
	5/9	7.31, m	130.5	2CH
	6/8	7.28, m	129.2	2CH
	7	7.23, m	127.6	CH
	2-NH	7.66, d (7.5)		
L-Leu (Leu-1)	10		172.7	C
	11	4.08, m	53.8	CH
	12	1.26, dd (7.5, 7.0)	40.8	CH_2
	13	1.54, m	25.5	CH
	14	0.86, d (6.5)	22.7	CH_3
	15	0.90, d (6.5)	22.7	CH_3
	11-NH	7.16, d (8.5)		
D-Leu (Leu-2)	16		173.1	C
	17	4.12, m	54.0	CH
	18a	1.41, m	39.6	CH_2
	18b	1.55, m		
	19	1.52, m	25.4	CH
	20	0.77, d (6.5)	21.2	CH_3
	21	0.85, d (6.5)	22.3	CH_3
	17-NH	7.11, d (6.5)		
L-Phe-2	22		173.7	C
	23	4.59, ddd (9.5, 7.5, 6.0)	53.6	CH
	24a	2.88, dd (13.5, 9.5)	39.7	CH_2
	24b	3.03, dd (13.5, 6.0)		
	25		138.3	C
	26/30	7.31, m	130.4	2CH
	27/29	7.28, m	129.2	2CH
	28	7.23, m	127.5	CH
	23-NH	6.97, d (7.5)		
O-Leu	31		170.9	C
	32	4.74, dd (10.0, 5.5)	76.6	CH
	33a	1.50, m	40.8	CH_2
	33b	1.72, m		
	34	1.42, m	25.2	CH
	35	0.86, d (6.5)	23.0	CH_3
	36	0.92, d (6.5)	23.2	CH_3

Assignment by DEPT and gHMQC.
[a] 500 MHz.
[b] 125 MHz.

accounted for 13 of the 14 unsaturations in **1**, zygosporamide was deduced to be a monocyclic depsipeptide.

The sequence of the five fragments in **1** was determined by analysis of HMBC NMR correlation data. HMBC correlations from H-3a and H-3b (β-protons of Phe-1) to C-1 (δ_C 171.4) revealed that this carbonyl carbon was positioned in the Phe-1 unit. Long range correlations observed from H-2 (α-proton of Phe-1) and H-11 (α-proton of Leu-1) to C-10 (carbonyl carbon of Leu-1) established the Phe-1 to have Leu-1 linkage. Both 11-NH (Leu-1) and H-17 (α-proton of Leu-2) showed correlations with the C-16 carbonyl carbon of Leu-2 defining the Leu-1 to Leu-2 sequence. Similarly, correlations from 17-NH (Leu-2) and H-23 (α-proton of Phe-2) to C-22 (carbonyl carbon of Phe-2) allowed the sequence of Leu-2 to Phe-2 to be established. A three bond correlation from H-23 (α-proton of Phe-2) and H-32 (α-proton of *O*-Leu) to C-31 (carbonyl carbon of *O*-Leu) established the linkage Phe-2 to *O*-Leu. Lastly, the ring closure linkage was secured by a four bond HMBC correlation between H-32 (α-proton of *O*-Leu) and C-1 (carbonyl carbon of Phe-1), which allowed the structure of zygosporamide to be completed (Fig. 1).

Treatment of **1** with NaOMe in methanol resulted in a smooth conversion to the linear peptide, methyl ester **2**. The ESI positive mode mass spectrum showed clear serial loss of the predefined subunits through cleavage across the amide bonds (Fig. 2), thus confirming the sequence assignments made by interpretation of NMR data.

The absolute configurations of the amino acid units were partly established by acid hydrolysis, subsequent Marfey's derivatization of the hydrolysate, and LC/MS analysis.[6] The hydrolysate was identified to possess two L-Phe, one L-Leu, and one D-Leu. The absolute stereochemistries of Leu-1 and Leu-2 were proposed by cautious analysis of 2D ROESY, 1D NOE spectra (Fig. 3). The cross ring NOE/ROE correlation between one β-proton (δ_H 3.12) of L-Phe-1 and the amide proton (δ_H 6.97) of L-Phe-2 was clearly observed. In addition, correlations between the other β-proton (δ_H 3.34) and one of the β-protons (δ_H 1.50) of *O*-Leu, and between the other β-proton of *O*-Leu and the amide proton (δ_H 6.97) of L-Phe-2, revealed that the lipophilic parts of L-Phe-1 and *O*-Leu and the amide proton of L-Phe-2 are on the same side. The NH proton (δ_H 7.66) of L-Phe-1 showed a NOE/ROE correlation with the aromatic protons ($\delta_H \sim 7.3$) of L-Phe-1, indicating that this amide proton is oriented toward the aromatic ring. The NOE/ROE correlation between the β-protons (δ_H

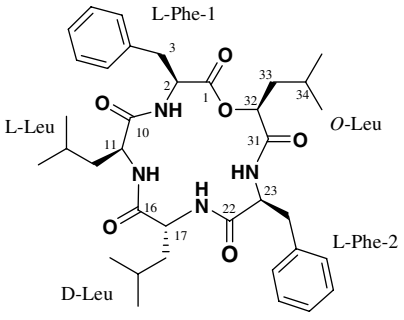

Figure 1. The structure of zygosporamide.

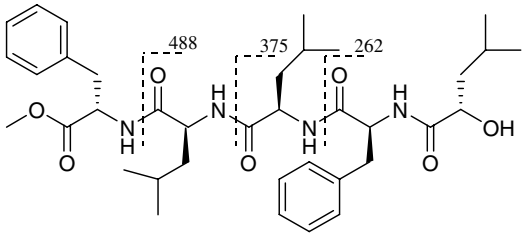

Figure 2. Mass spectral cleavage (ESI-MS) of methyl ester **2**.

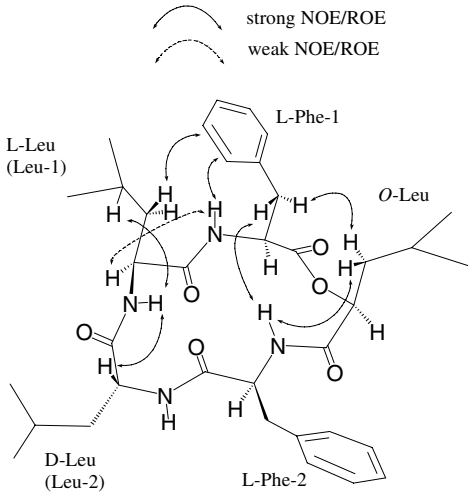

Figure 3. The key NOE/ROE correlations of **1**.

1.54) of Leu-1 and the aromatic protons ($\delta_H \sim 7.3$) of L-Phe-1 provides evidence that the side chains of Leu-1, L-Phe-1, and *O*-Leu, and the amide proton of L-Phe-2, are on the same side of the ring. The α-proton (δ_H 4.08) of Leu-1 displayed a very weak correlation with the amide proton of L-Phe-1. These correlations support the assignment of an L-configuration of Leu-1. The NOE/ROE correlation between NH (δ_H 7.16) and the λ-proton (δ_H 1.26) of Leu-1 indicates that the amide proton is oriented toward the side chain of Leu-1. The strong correlation between the amide proton of Leu-1 and the α-proton (δ_H 4.12) of Leu-2 suggests that Leu-2 has D-configuration. On this basis, the absolute configurations of Leu-1 and Leu-2 were proposed as L and D, respectively.

In order to determine the absolute stereochemistry at C-32 of *O*-Leu, the *S*- and *R*-Mosher esters (**3a** and **3b**) of the linear peptide **2** were prepared.[7] Interpretation of ^{1}H chemical shift differences, based on the ^{1}H NMR and TOCSY spectra of the *S*- and *R*-MTPA esters, revealed the absolute configuration of C-32 as *S* (Fig. 4).

The structural features of **1** are similar to those of the exumolides,[8] sansalvamide,[9] and *N*-methyl sansalvamide[10] as the amino acids are hydrophobic and the ring is closed by an ester linkage. However, none of those cyclic peptides involves a D-amino acid.

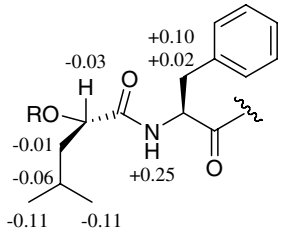

Figure 4. 3a: R = *S*-MTPA; **3b**: R = *R*-MTPA, $\Delta\delta_{S-R}$ values are displayed in ppm.

Zygosporamide is one of the first examples of a cyclic depsipeptide containing a D-amino acid residue. In the extensive structure–activity studies reported with sansalvamide analogs, the substitution of one D-amino acid residue increased the anti-tumor activity against colon cancer cell line (HT-29) more significantly than the introduction of an *N*-methyl group, indicating an important role of a D-amino acid in this class of depsipeptides.[11]

The biological activity of zygosporamide (**1**) was evaluated against various cancer cell lines and drug-resistant

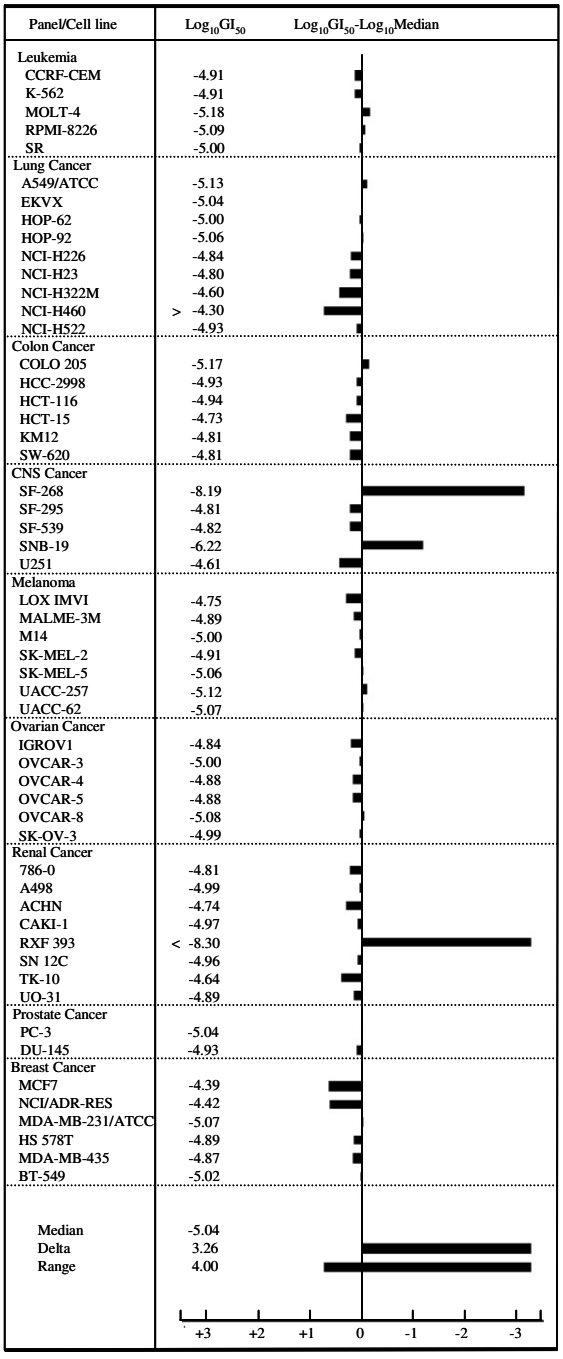

Figure 5. Mean graph illustrating 50% growth inhibition of NCI cell lines.

pathogens. Zygosporamide showed no significant inhibition of methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, and amphotericin-resistant *Candida albicans*. However, this cyclic peptide displayed highly selective cytotoxicity in the National Cancer Institute cell line assay (Fig. 5). Zygosporamide showed selective inhibition of the CNS (central nervous system) cancer cell line SF-268 and renal cancer cell line RXF 393, with GI_{50} values of 6.5 nM and less than 5.0 nM, respectively. These values are at least 1000 times more selective than most of the 54 tested cancer cell lines considering that the median $GI_{50} = 9.1$ μM.

Zygosporamide is the first cyclic D-amino acid bearing depsipeptide to be isolated from *Z. masonii*. This new peptide displayed significant cell inhibition selectivity against a variety of cancer cell lines. The isolation of zygosporamide further supports previous observations that marine isolates of terrestrial fungal species are a rich source of new secondary metabolites.

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Supplementary data

General experimental procedures, detailed cultivation, extraction, and isolation methods, methanolysis of **1**, preparation of MTPA derivatives of **2**, HR-MS, 1D and 2D NMR spectra of **1**, and ^{1}H NMR spectra of **2**, **3a**, and **3b**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.08.113.

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4. The fungal strain CNK458 was isolated from a cyanobacterium collected off Maui, Hawaii in 1997. The isolate was identified as a *Zygosporium masonii* based on morphological characteristics by the Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl).
5. White powder; $[\alpha]_D$ −112 (*c* 0.26, CH_3CN); UV (CH_3CN) λ_{max} (log ε) 206 (4.55), 219 (4.06), 259 (2.82) nm; IR (neat, $CHCl_3$) ν_{max} 3295, 2955, 1743, 1661, 1537, 1243, 756 cm^{-1}; HRESI-TOF $[M+H]^+$ at *m*/*z* 635.3806 (calcd for $C_{36}H_{51}N_4O_6$ $[M+H]^+$ 635.3809).
6. (a) Marfey, P. *Carlsberg. Res. Comm.* **1984**, *49*, 591–596; (b) Compound **1** (0.5 mg) was hydrolyzed with 6 N HCl (500 μL) at 110 °C for 18 h. The solution was concentrated under a stream of N_2, the residue was resuspended twice in 1 mL of water and dried under a stream of N_2 to completely remove the acid. Next, 1 N $NaHCO_3$ (100 μL) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-Marfey reagent) in acetone (10 mg/mL, 50 μL) were added to the hydrolysate. The mixture was incubated at 80 °C for 3 min. The reaction was then quenched by adding 2 N HCl (50 μL) and diluted with 50% aqueous CH_3CN (300 μL). The retention times of the Marfey derivatives were analyzed by LC/MS (Hewlett-Packard Series 1100, C_{18}, Agilent, 4.6 mm × 100 mm, 5 μm) with a linear gradient from 10% to 50% aqueous CH_3CN (0.1% TFA) over 45 min and compared with those of the authentic standards (L-Leu: 36.5 min, D-Leu: 40.8 min, L-Phe: 36.4 min, and D-Phe: 39.8 min).
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