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HALOBACILLIN: A CYTOTOXIC CYCLIC ACYLPEPTIDE OF THE ITURIN CLASS PRODUCED BY A MARINE *BACILLUS*

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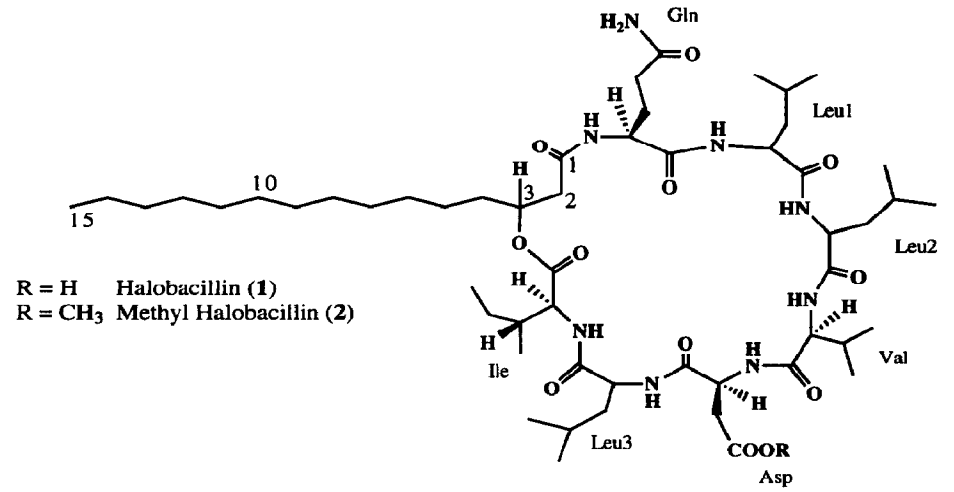
Summary - Halobacillin (**1**), a new cyclic acylpeptide of the iturin class, has been isolated from cultures of a *Bacillus* species (culture # CND-914) obtained from a deep-sea sediment core. The structure of the new compound was assigned on the basis of comprehensive NMR studies of **1** and methyl halobacillin (**2**), and evaluation of the amino acids obtained by acid hydrolysis.

The iturins, a class of cyclic acylpeptides produced exclusively by several *Bacillus* species,[2] are characterized as polar cyclic heptapeptides with lipophilic β-acyloxy or β-amino fatty acid components. Numerous pharmacological properties have been reported for various iturins, including potent antifungal,[2a,2c] antibiotic,[2e] and antitumor[2a] activities. In this paper, we report the isolation and structure elucidation of halobacillin, a novel acylpeptide similar to surfactin, one of the most effective biosurfactants known.[3] Halobacillin is produced by a *Bacillus* species, culture CND-914, isolated from a marine sediment core taken at -124 m near the Guaymas Basin, Mexico. Halobacillin represents the first acylpeptide of the iturin class produced by a marine isolate. Halobacillin showed moderate human cancer cell cytotoxicity, but in contrast to the iturins, no antifungal or antibiotic activity.



R = H Halobacillin (**1**)
R = CH_3 Methyl Halobacillin (**2**)

The ethyl acetate extract of a 10L seawater-based fermentation[4] was subjected to LH-20 size-exclusion chromatography, reversed-phase vacuum flash chromatography, then reversed-phase HPLC in MeOH:H_2O mixtures to yield 153mg (7.2% of crude) halobacillin (**1**).[5] Broad NMR signals and poor chromatographic characteristics due to the free acid and amide functionalities necessitated methylation of **1** for the majority of the NMR experiments performed.[6] The ^1H and ^{13}C NMR spectra of the methylated product were very clear in DMSO-d_6 with the exception of the overlapping methylene signals (see Table below). Using TOCSY data, the seven amino acid residues were identified, although glutamine and aspartic acid could not be distinguished from glutamic acid and asparagine, respectively, at this point. These ambiguities were eliminated using HMBC heterocorrelation NMR experiments. Correlations showed that methylation occured only on the aspartic acid residue and that the amide protons correlated to carbon atoms from the glutamine residue. Combining HMBC and TOCSY information yielded the secondary structure of the cyclic heptapeptide as -Gln-Leu-Leu-Val-Asp-Leu-Ile-. Acid hydrolysis, followed by derivatization to the pentafluoropropyl isopropyl (PFP-IPA) esters and GCMS analysis with a chiral capillary column, indicated the absolute stereochemistries were *l*-Gln, *l*-Val, *l*-Asp, *l*-Ile, and *d*:*l*-Leu in a 2:1 ratio.[6] Although these data confirmed the absolute stereochemistries of the amino acids, the positions of the *d*-leucine residues could not be assigned.

Methyl halobacillin (**2**), produced by diazomethane methylation,[7] analyzed for $C_{54}H_{97}N_8O_{12}$ [$(M+1)^+$] by HRFABMS methods. Accounting for the amino acid residues, a formula of $C_{15}H_{28}O_2$ remained for the fatty acid portion of the molecule. The broad doublet and the chemical shift of the C2 protons indicated a β-acyloxy fatty acid. The cyclic nature of the peptide, and the amide and ester linkages of the β-acyloxy acid to the peptide, were determined by HMBC correlations within the Gln and Ile residues (Table). This left one methyl and 11 methylene carbons which could only be assigned as an alkyl chain at C-3. No data could be obtained, however, to assign the stereochemistry at this chiral carbon.

Table. NMR Data for Halobacillin (1) and Methyl halobacillin (2)

Residue/ Position		[a]δ ^{13}C	DEPT	δ ^1H	TOCSY	HMBC (6Hz)	ROESY[b]
Gln	Cα	52.9	CH	4.13	NH, ß, γ	CO, 1, ß, γ	Leu1NH, Nε1, ß
	Cβ	31.7	CH2	2.06	NH, α, γ	∂	α, Nε1,2, NH, Chain
				2.01	NH, α, γ	∂	α, Nε1,2, NH, Chain
	Cγ	28.3	CH2	1.84	NH, α, ß	∂, α, ß	NH
				1.73	NH, α, ß	∂, α, ß	NH
	Cδ	174.1	C	-	-	-	-
	Nε1			7.30	Nε2	∂	α, ß
	Nε2			6.82	Nε1		ß
	NH			8.17	α, ß, γ	α, 1	3, 2, ß, γ
	CO	171.2	C	-	-	-	-
Leu1	Cα	51.6	CH	4.24	NH, ß	CO	NH, ValNH, ß
	Cβ	41.3	CH2	1.46	NH, α	CO	α, NH
	Cγ	24.0	CH	1.52	NH		
	Cδ1	23.0	CH3	0.84		∂2, ß	
	Cδ2	22.7	CH3	0.85		∂2, ß	
	NH			8.08	α, ß, ∂2	GlnCO	α, Glnα, ß
	CO	172.2	C	-	-	-	-
Leu2	Cα	52.1	CH	4.22	NH, ß	CO, ß, γ	ValNH, NH, ß
	Cβ	39.9	CH2	1.51	NH, α		α, NH
	Cγ	24.3	CH	1.35			
	Cδ1	23.0	CH3	0.88		∂2, ß	

Residue/ Position		[a]δ ^{13}C	DEPT	δ ^{1}H	TOCSY	HMBC (6Hz)	ROESY[b]
	Cδ2	21.4	CH3	0.79		∂1, γ	
	NH			8.44	α, ß	α, ß, 3	α, Leu1α, ß
	CO	172.5	C	-	-	-	-
Val	Cα	58.4	CH	4.04	NH, ß	CO, Leu2CO, ß, ∂1, ∂2	AspNH, NH, ß
	Cβ	30.2	CH	2.04	NH, α	α, ∂1, ∂2	α, NH, AspNH
	Cγ1	19.1	CH3	0.81		α, ß, ∂2	
	Cγ2	17.8	CH3	0.77		α, ß, ∂1	
	NH			8.02	α, ß	∂1, Leu2CO	Leu2α, α, ß
	CO	170.8	C	-	-	-	-
Asp	Cα	49.7	CH	4.61	NH, ß	ValCO, CO, ß	Leu3NH, NH, ß
	Cβ	36.05	CH2	2.75	NH, α	γ, α	α, NH, Leu3NH
	Cγ	170.5	C	-	-	-	-
	OMe	51.5	CH3	3.56	-	γ	----------
	NH			8.27	α, ß	ß, ValCO	α, Valα, ß, Valß
	CO	169.8	C	-	-	-	-
Leu3	Cα	50.6	CH	4.47	NH, ß	CO, AspCO, ß	IleNH, ß
	Cβ	41.6	CH2	1.38	NH, α		α
				1.32	NH, α		α
	Cγ	33.8	CH	1.26			
	Cδ1	22.0	CH3	0.82		γ	
	Cδ2	11.2	CH3	0.80		ß, γ	
	NH			7.93	α, ß	AspCO	Aspα, Aspß
	CO	171.8	C	-	-	-	-
Ile	Cα	56.5	CH	4.16	NH	CO, Leu3CO, ß, γ1, γ2	NH, ß, Chain
	Cβ	36.0	CH	1.84	NH		α, NH
	Cγ1	24.5	CH2	1.29			
	Cγ2	15.5	CH3	0.82		ß, γ1, α	
	Cδ	21.6	CH3	0.80		ß	
	NH			8.28	α, ß	α, Leu3CO	Leu3α, α, ß, Chain
	CO	170.7	C	-	-	-	-
Alkyl	1	169.3	C	-	-	-	-
Chain	2	40.7	CH2	2.36	3, 1.19	1, 4	3, GlnNH, Chain
	3	71.7	CH	4.97	2, 1.19	IleCO, 1, 2	GlnNH, 2, 4, Chain
	4	33.0	CH2	1.52			3
	5	40.4	CH2	1.46			
	5	36.1	CH2	1.21			
	7	31.4	CH2	1.19			
	8	29.4	CH2	1.19			
	9	28.9	CH2	1.19			
	10	28.9	CH2	1.19			
	11	28.6	CH2	1.19			
	12	26.5	CH2	1.19			
	13	38.6	CH2	1.08			
	14	26.8	CH2	1.19			
	15	22.6	CH3	0.84		13, 14	

[a] All ^{1}H experiments performed at 500MHz; all ^{13}C experiments performed at 50 MHz. All NMR experiments (except ROESY) on **2** were in DMSO-d_6 at room temperature. [b] ROESY experiment performed with halobacillin (**1**).

From these data, we propose structure **1** for halobacillin, a cytotoxic acylpeptide similar to surfactin. Halobacillin inhibits the growth of human colon tumor cells (HCT-116) with an IC_{50} of 0.98 µg/ml, but thus far it shows none of the antibacterial activity exhibited by surfactin. The major difference between halobacillin and

surfactin is the replacement of the glutamic acid of surfactin with a glutamine in halobacillin. Dramatic differences in biological activity have been demonstrated among the closely related iturin acylpeptides.[8] In a consistent fashion, the small differences between surfactin and halobacillin produce profound differences in biological activity. It should also be emphasized that halobacillin is produced only in seawater-based media. Marine *Bacillus* species have been investigated for their secondary metabolites in few instances, with only one unique enzyme[9] and one novel carbohydrate[10] reported in the literature. This work adds to the observations that deep-sea sediments may be a rich source of microorganisms producing novel compounds. Prior to this report, a group of cytotoxic and antiviral macrolides, the macrolactins,[11] and bisucaberin, a unique bacterial siderophore[12] are the only compounds reported from these sources.

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4. Isolate CND-914 was cultured in 10L of a medium consisting of 1% starch, 0.4% yeast extract, 0.2% peptone, 75% seawater, 25% deionized water, buffered at pH 8.0 with 10 ml 1.0M tris buffer.

5. For halobacillin (**1**): a white, non-crystalline solid, $[\alpha]_D$ -10.6° (c 2.82, MeOH); IR (film) 3420, 3288, 3200, 3065, 2922, 1730, 1639, 1537 cm^{-1}; HRFABMS $(M+1)^+$ m/z 1035.7076 for $C_{53}H_{95}N_8O_{12}$, Δ -0.6ppm

6. Halobacillin (**1**) (50 mg) was dissolved in 3 ml 7:3 acetone:ether and treated with freshly prepared diazomethane. The product, methyl halobacillin (**2**), was obtained after solvent evaporation and used without further purification. Compound **2** showed: IR (film) 3429, 3299, 3210, 3060, 2921, 1734, 1644, 1539 cm^{-1}; HRFABMS $(M+1)^+$ m/z 1049.7206 for $C_{54}H_{97}N_8O_{12}$, Δ -1.6ppm.

7. The acid hydrolysis mixture (6N HCl, 110°, 8 hr) from halobacillin (**1**) was analyzed by GCMS using a Chirasil-Val capillary column after conversion of the AAs to their corresponding PFP-IPA esters.

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