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Loloatin B, A Cyclic Decapeptide Antibiotic Produced in Culture by a Tropical Marine Bacterium

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Abstrtact: *Loloatin B (1), a novel cyclic decapeptide antibiotic with potent gram positive activity, is produced in culture by a Bacillus sp. isolated from the tissues of a marine worm collected in Papua New Guinea.*
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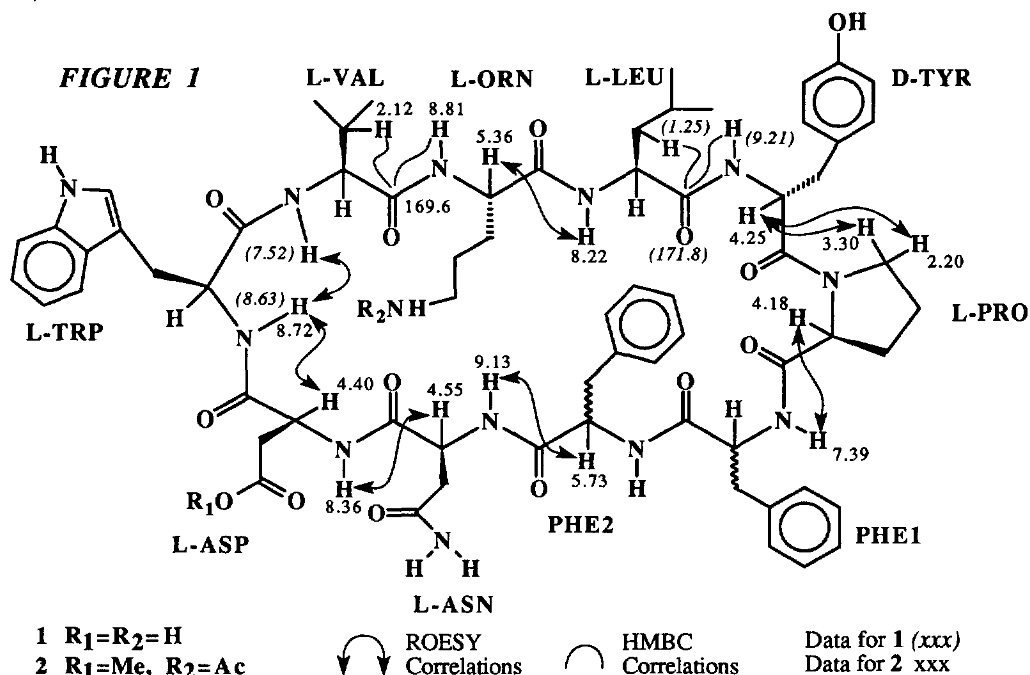
Microorganisms isolated from marine habitats have emerged as a promising source of new bioactive metabolites with potential for development into drugs for treating human diseases.[1] One area where new drugs are desperately needed is in the treatment of antibiotic resistant strains of gram positive human pathogens. Methicillin resistant strains of *Staphylococcus aureus* (MRSA) cause infections that are refractory to standard anti-staphylococci antibiotics and in many cases vancomycin is the antibiotic of last resort. Consequently, it is of great concern that vancomycin resistant strains of MRSA may develop. Infections due to enterococci have been difficult to treat for many years because these organisms are intrinsically resistant to many antibiotics. Ampicillin has been the mainstay for treatment of uncomplicated enterococcal infections, but many strains have now become resistant to ampicillin. Vancomycin is again the only effective treatment for these ampicillin resistant enterococcal infections. In the past few years, vancomycin resistant enterococcal strains (VRE) have begun to appear and they are rapidly spreading across North America. There are no effective antibiotics currently available for such organisms and the recent report of an outbreak of VRE with a 73% mortality rate has highlighted the seriousness of the situation.[2]

As part of an ongoing program designed to screen microorganisms isolated from the oceans for the production of new antibacterial agents,[3,4] we have found that a marine *Bacillus* sp. collected in Papua New Guinea produces loloatin B (1) when grown on solid agar. Loloatin B (1) shows *in vitro* antibacterial activity against a number of gram positive antibiotic resistant human pathogens.

The marine bacterial isolate MK-PNG-276A, identified as a *Bacillus* sp. by MIDI analysis of cellular fatty acids, was obtained from the tissues of an unidentified tube worm collected at -15 m off of Loloata Island, Papua New Guinea. MK-PNG-276A was cultured on trays of solid tryptic soy agar supplemented with NaCl to a concentration of 1%. Twenty six 400 mL trays (9" x 15" x 1/4" deep agar) were cultured for five days after which the cells were gently scraped from the agar surface. Lyophilized cells (61.5g dry weight) were extracted with three 600 mL portions of methanol that were combined, filtered, and reduced *in vacuo* to give a brown/gray tar. The tar was dissolved in 750 mL of MeOH/H_2O (1:4) and sequentially extracted with hexanes (3 x 250 mL) and EtOAc (3 x 250 mL). The combined EtOAc extracts were reduced *in vacuo* to give a taupe/brown crystalline solid (5.5 g). Fractionation of the taupe/brown solid via Sephadex LH-20 chromatography using MeOH as an eluent gave an early eluting fraction that showed antibiotic activity against

MRSA and enterococci species. Further purification of this fraction by preparative reversed-phase column chromatography and reversed-phase HPLC chromatography using an eluent comprised of MeOH/H_2O (9:1) containing 0.1% TFA gave pure loloatin B (**1**) as a tan/white amorphous solid (1.87 g, 3% dry weight of cells).[5]



Loloatin B (**1**) gave a (M + H) ion in the HRFABMS at m/z 1296.64232 appropriate for a molecular formula of $C_{67}H_{85}N_{13}O_{14}$ (ΔM + 0.46 ppm). Detailed analysis of the ^{1}H, ^{13}C, COSY, HOHAHA, HMQC, HMBC and ROESY data for loloatin B (**1**) and its N-acetyl methyl ester derivative **2**, identified the ten amino acids residues indicated in Table 1. Hydrolysis of **1** at 100 °C with 6N HCl containing thioglycolic acid and examination of the pentafluropropionamide isopropyl ester derivatives of the liberated amino acids via chiral GC analysis confirmed the presence of L-valine, L-ornithine, L-leucine, D-tyrosine, L-proline, L-phenylalanine, D-phenylalanine, L-tryptophan and L-aspartic acid (from ASP and ASN). The ten identified amino acid residues accounted for all of the atoms in the molecular formula of **1**, and 31 of the 32 sites of unsaturation demanded by the molecular formula. Thus, loloatin B (**1**) had to be a monocyclic decapeptide.

The amino acid sequence in **1** was determined by analysis of the HMBC and ROESY data for both **1** and **2**, as shown in Figure 1. ROESY correlations observed in **2** between amino acid α-methine protons and adjacent residue NH protons unambiguously identified the following five amide bonds: ORN-CO/LEU-NH (δ 5.36/8.22), ASP-CO/TRP-NH (δ 4.40/8.72), ASN-CO/ASP-NH (δ 4.55/8.36), PHE2-CO/ASN-NH (δ 5.73/9.13) and PRO-CO/PHE1-NH (δ 4.18/7.39). A ROESY correlation observed between the TYR α-methine resonance at δ 4.25 and the PRO δ-proton resonance at δ 3.30 in **2** identified the TYR-CO/PRO-N

Table 1: NMR data (500MHz) for loloatin B (1) (DMSO-D_6) and N-acetyl loloatin B methyl ester (2) (DMSO-D_6/Benzene-D_6 95:5).

		loloatin B (1)	methyl ester (2)				loloatin B (1)	methyl ester (2)	
Res.		δ ^{1}H	δ ^{13}C	δ ^{1}H	Res.		δ ^{1}H	δ ^{13}C	δ ^{1}H
VAL	NH	7.52 (d,J=8.0)	-	7.58 (d,J=8.0)	PHE1	CH_2	2.25 (m)	37.2	2.25 (m)
	αCH	4.56 (m)	57.0	4.65 (m)		*i*C	-	138.6	-
	βCH	2.01 (sept,J=7.0)	31.2	2.12 (sept,J=6.7)		*o*CH	7.18	128.8	7.18
	γCH_3	0.93 (d)	18.6	0.97 (d)		*m*CH		127.4	
	γCH_3	0.93 (d)	17.8	0.97 (d)		*p*CH		125.8	
	CO	-	169.6	-		CO	-	171.8	-
ORN	NH	8.88 (d,J=8.9)	-	8.81 (d,J=8.8)	PHE2	NH	9.05 (d,J=9.0)	-	8.97 (d,J=9.4)
	αCH	5.27 (bm)	51.1	5.36 (bq,J=8.2)		αCH	5.57 (bt)	52.5	5.73 (bt,J=9.1)
	βCH_2	1.8 (m)	31.6	1.76 (m)		βCH_2	2.75 (m)	40.3	2.75 (m)
	γCH_2	1.7 (m)	24.4	1.57 (m)			3.02 (m)		3.1 (m)
	δCH_2	2.8,2.9 (m)	38.5	3.03 (m)		*i*C	-	137.4	-
	δNH	7.45 (bs)	-	7.45 (bt)		*o*CH	7.18	129.2	7.28
	CO	-	168.5	-		*m*CH		127.4	7.09
	CH_3		22.5	1.79 (s)		*p*CH		125.8	
	CO		170.5	-		CO	-	170.8	-
LEU	NH	7.92 (bs)	-	8.22 (d,J=8.2)	ASN	NH	9.03 (d,J=6.0)	-	9.13 (d,J=6.7)
	αCH	4.55 (m)	50.2	4.65 (m)		αCH	4.49 (m)	49.0	4.55 (m)
	βCH_2	1.35 (m)	41.7	1.39 (m)		βCH_2	3.37 (m)	35.2	3.37 (m)
		1.25 (m)		1.46 (m)			3.0 (m)		3.10 (m)
	γCH	1.5 (m)	24.4	1.55 (m)		CO	-	173.0	-
	δCH_3	0.93 (d)	22.1	0.97 (d)		NH_2	8.04 (bs)	-	8.05 (bs)
	δCH_3	0.93 (d)	22.8	0.97 (d)			7.45	-	7.45
	CO	-	171.8	-		CO	-	170.3	-
TYR	NH	9.21 (s)	-	9.24 (s)	ASP	NH	8.32 (d,J=4.2)	-	8.36 (d,J=4.6)
	αCH	4.22 (m)	54.4	4.25 (m)		αCH	4.26 (m)	51.8	4.40 (m)
	βCH_2	2.70 (m)	34.9	2.88 (m)		βCH_2	2.35 (m)	34.7	2.5 (m)
		2.81 (m)		2.82 (m)			2.25 (m)		2.6 (m)
	*i*C	-	126.2	-		γCO	-	170.5	-
	*o*CH	6.98 (d,J=8.6)	130.0	7.01 (J=8.2)		CH_3		51.3	3.56 (s)
	*m*CH	6.61 (d,J=8.6)	114.8	6.69 (J=8.2)		CO	-	169.9	-
	*p*COH	not observed	156.2	not observed	TRP	NH	8.63 (d,J=9.8)	-	8.72 (d,J=9.8)
	CO	-	170.6	-		αCH	4.5 (m)	55.4	4.60(m)
PRO	N	-	-	-		βCH_2	3.15 (m)	28.4	3.24 (m)
	αCH	4.07 (d,J=7.6)	59.4	4.18 (d,J=7.6)		C	-	110.6	-
	βCH_2	1.43 (m)	28.2	1.45 (m)		C	-	127.0	-
		1.25 (m)		1.24 (m)		CH	7.5	118.0	7.54
	γCH_2	1.07 (m)	21.8	1.0 (m)		CH	7.0	118.2	7.01
		0.411 (m)		0.41 (m)		CH	7.03 (m)	120.7	7.06
	δCH_2	2.20 (m)	45.5	2.20 (m)		CH	7.32 (J=8.0)	111.1	7.37
		3.30 (m)		3.30 (m)		C	-	136.1	-
	CO	-	169.3	-		NH	10.81 (bs)	-	10.84 (bs)
PHE1	NH	7.23 (d,J=9.5)	-	7.39 (d)		CH	7.02 (d)	122.9	7.09 (d)
	αCH	4.5 (m)	53.2	4.65 (m)		CO	-	171.0	-

amide bond, and a strong ROESY correlation between the VAL-NH resonance at δ 7.52 and the TRP-NH resonance at δ 8.63 in **1** identified the TRP-CO/VAL-NH amide linkage. The latter ROESY correlation suggests the possibility of a β-bulge in the TRP/VAL region of the cyclic peptide as shown in Figure 1.[6] HMBC correlations from both the VAL β-methine proton resonance at δ 2.12 and the ORN-NH resonance at δ 8.81 to the well resolved carbonyl resonance at δ 169.6 in derivative **2** identified the VAL-CO/ORN-NH amide bond, and HMBC correlations from both the LEU β methylene proton resonance at δ 1.25 and the TYR-NH resonance at δ 9.21 to the carbonyl resonance at δ 171.8 in **1** identified the LEU-CO/TYR-NH amide bond. The final PHE1-CO/PHE2-NH amide bond was required to complete the macrocyclic ring.

HRFABMS and MS/MS studies supported the amino acid sequence derived from the NMR data. The MS/MS data was consistent with initial cleavage of the ring at the TYR-CO/PRO-N bond to give a linear decapeptide that sequentially loses LEU-TYR (m/z 1019), ORN-LEU-TYR (m/z 905) and TRP-VAL-ORN-LEU-TYR (m/z 621). FABMS peaks at m/z 245 and 377 could be assigned to the protonated fragments PRO-PHE1 and PHE2-ASN-ASP, respectively.

Loloatin B (**1**) is a novel cyclic decapeptide antibiotic that has some structural features in common with the tyrocidines.[7] In preliminary tests for antimicrobial activity, loloatin B (**1**) inhibited the growth of methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Enterococcus* sp., and penicillin resistant *Streptococcus pneumoniae* with MICs of 1-2 μg/mL. Studies of the spectrum of antimicrobial activity and MIC values as well as *in vivo* toxicity and antimicrobial activity of loloatin B (**1**) are in progress and will be reported elsewhere.

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