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BACILLOMYCIN D FROM THE MARINE ISOLATE OF *Bacillus subtilis* KMM 1922

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Strain KMM 1922 of Bacillus subtilis*, a producer of the antifungal iturin peptide antibiotic bacillomycin D, was isolated from a specimen of the Kuril sponge* Stelletta validissima*. The structure of the compound was proved using two-dimensional NMR spectroscopy, tandem electrospray mass spectrometry, and literature data. The peptide was shown to exhibit a pH-dependent cytotoxic activity.*

Key words: facultative marine bacteria *Bacillus subtilis*, iturins, bacillomycins, NMR spectroscopy, mass spectrometry, sponge *Stelletta validissima*.

Marine bacteria of the genus *Bacillus* are producers of biologically active low-molecular-weight cyclic peptides [1-3]. In continuation of the search for such compounds, we investigated the strain *B. subtilis* KMM 1922 isolated from a specimen of the sponge *Stelletta validissima* (Iturup island, Kuril Islands, 1986, SRS Academician Oparin). It was demonstrated that the bacterium cultivated in liquid medium synthesizes compounds with anticandidal and cycotoxic activities.

Column chromatography over Polychrom-1 and silica gel of bacterium culture (5 L) isolated a white powder (**1**, 375 mg) that had mp 295-305°C after reprecipitation from CH_3OH, was insoluble in nonpolar solvents, and poorly soluble in alcohols, water, acids, and bases. The UV spectrum of **1** had absorption characteristic of tyrosine at 280-285 nm. The IR spectrum of **1** exhibited absorption bands for peptide (1678, 1662, 1643, 1546, 1514 cm^{-1}) and carboxyl (1710 cm^{-1}) and lacked absorption bands for ethers and esters.

Amino-acid analysis indicated the presence of six α-amino acids Asp, Tyr, Glu, Pro, Thr, and Ser in the ratio 2:1:1:1:1:1. An aliphatic amino acid (AA) that gave a positive rection with ninhydrin was observed in the $CHCl_3$ extract of the hydrolysate.

Matrix-assisted laser-desorption/ionization (MALDI MS) and electrospray (ESMS) mass spectrometry of **1** gave two values for the molecular weight (MW) (1030 and 1044 Da). The amino-acid composition of **1** and the MWs in the mass spectra were identical to those obtained for a mixture of homologous peptides that was isolated previously, bacillomycins D1 and D2 [4]. The ratio of intensities of the molecular ions (1:5) showed that D2 with MW 1044 Da predominated.

The PMR and ^{13}C NMR data (Table 1) also agreed completely with those obtained earlier [4] for bacillomycins D1 and D2. Two-dimensional (2D) correlation NMR spectroscopy (COSY, TOCSY, NOESY, HSQC, and HMBC) enabled signals in the PMR and ^{13}C NMR spectra of **1** to be unambiguously assigned. In addition, previously published [1] assignments for the signals from Asp1, Tyr, Asp2, and the β-amino acid were corrected. The results confirmed that all α-amino acids listed above were present, that the aspargic acids were present as the amides, and that the glutamic acid was not the amide. The results showed that the aliphatic β-amino acids in the side chain of the peptides were the sum of homologous β-amino acids of the *n*- and *iso*-series.

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TABLE 1. ^{1}H and ^{13}C NMR Spectra of **1** (δ, ppm)

Atom	δ ^{13}C NMR	^{1}H NMR (J/Hz)	Atom	δ ^{13}C NMR	^{1}H NMR (J/Hz)
β-AA			Asn2		
NH	-	8.01 (d, J = 9.3)	NH	-	8.91 br.s
1	172.9	-	α	50.4	5.36 m
2	42.1	H_A 2.62 (dd, J = 3.6, 14.9)	β	37.9	H_A 3.55 (dd, J = 9.1, 15.1)
		H_B 2.41 (dd, J = 10.5, 14.9)			H_B 3.19 (dd, J = 5.3, 15.1)
3	47.4	4.60 (m)	γ	172.6	-
4	35.8	1.48, (2H, m)	δ (NH_2)	-	H_A 8.43 br.s; H_B 7.75 br.s
5-10, 11a	29.6-30.2	1.25 m	C=O	172.9	-
12a	32.1	1.25 (2H, m)	Pro		
13a	22.9	1.25 (2H, m)	α	62.3	4.72 (dd, J = 5.1, 8.4)
14a	14.2	0.85 (3H, t, J = 6.6)	β	29.6	2.11 (2H, m)
11b	27.7	1.25 (2H, m)	γ	25.0	H_A 1.89 m; H_B 1.70 m
12b	39.3	1.13 (2H, m)	δ	48.6	H_A 1.70 m; H_B 4.07 m
13b	28.2	1.46 m	C=O	172.4	-
14b, 15b	22.8	0.84 (6H, d, J = 6.6)	Glu		
Asn1			NH	-	8.18 br.s
NH	-	8.81 (d, J = 5.5)	α	55.5	4.85 m
α	52.9	5.23 m	β	27.8	H_A 2.79 (dd, J = 10.6, 16.2)
β	37.1	3.00 (2H, m)			H_B 2.65 (dd, J = 4.0, 16.2)
γ	172.3	-	γ	31.8	3.00 (2H, m)
δ (NH_2)	-	H_A 8.23 br.s; H_B 7.26 br.s	C=O	169.3	-
C=O	173.3	-	COOH	173.8	-
Tyr			Ser		
NH	-	9.61 (d, J = 8.0)	NH	-	8.61 (d, J = 5.0)
α	55.9	5.32 (dt, J = 9.5, 4.5)	α	53.9	4.85 m
β	36.6	H_A 3.71 (dd, J = 5.0, 14.0)	β	63.7	H_A 4.38 (dd, J = 11.6, 3.7)
		H_B 3.38 (dd, J = 9.6, 14.0)			H_B 4.36 (dd, J = 11.6, 3.9)
1′	128.7	-	C=O	171.7	-
2′, 6′	131.3, 2C	7.48 (2H, d, J = 8.4)	Thr		
3′, 5′	116.2, 2C	7.05 (2H, d, J = 8.4)	NH	-	8.04 (d, J = 8.4)
4′	157.5	-	α	58.2	4.83 m
C=O	173.8	-	β	66.1	4.95 (qd, J = 6.5, 2.3)
			γ	20.9	1.34 (3H, d, J = 6.5)
			C=O	171.2	-

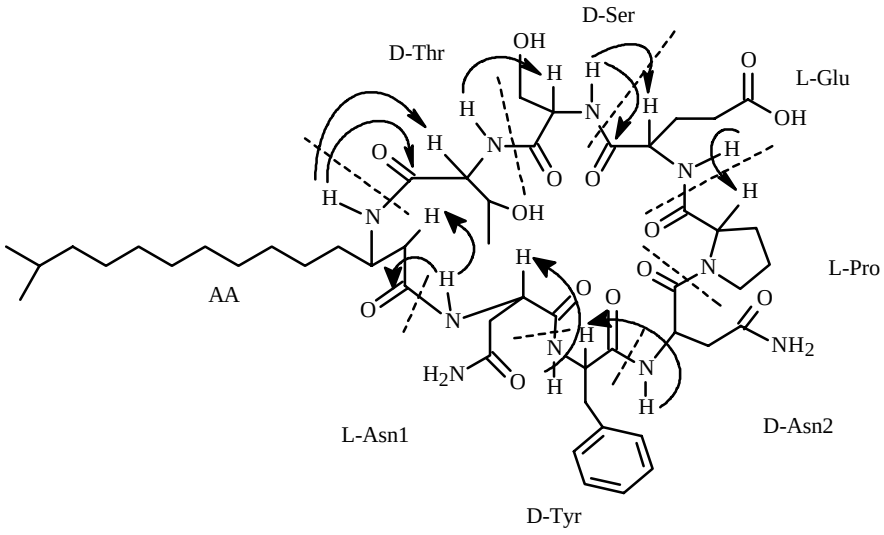
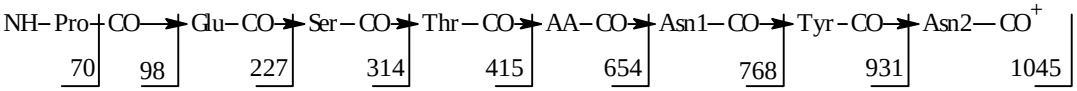


Fig. 1. HMBC and NOESY correlations in spectra of bacillomycin D2.

The HMBC spectrum of **1** revealed coupling between the carbamide proton of the AA and the carbonyl C of Thr, between the carbamide proton of Asn1 and the carbonyl C of the AA, and between the carbamide proton of Ser and the carbonyl C of Glu (Fig. 1). The NOESY spectrum of **1** established couplings between the following protons: N(H) of Tyr and HC(2) of Asp1, N(H) of Asp2 and HC(2) of Tyr, N(H) of Glu and HC(2) of Pro, N(H) of Thr and HC(2) of Ser, N(H) of Ser and HC(2) of Glu, N(H) of AA and HC(2) of Thr, and N(H) of Asp and HC(2) of AA (Fig. 1). The HMBC and NOESY correlations given above suggested the following amino-acid sequence in **1**: →Pro–CO→Glu–CO→Ser–CO→Thr–CO→AA–CO→Asn1–CO→Tyr–CO→Asn2–CO→.

Tandem ESMS also confirmed the proposed amino-acid sequence for bacillomycin D2. It is known that the fragmentation of cyclic peptides begins with the protonated native peptide in two types of mass spectrometry, fast-atom bombardment (FABMS) and MALDIMS [5]. For proline-containing peptides, protonation occurs mainly at the amide N of proline [6]. Then, the neighboring N-acyl bond breaks and the resulting linear acyl ion is cleaved to form acyl ions containing proline at the N-terminus. In our instance (ESMS), the cyclic peptide bacillomycin D2 is also protonated to form the 1045 ion, $[M + H]^+$. Then, the acyl bond between Asn2 and Pro breaks to form the linear acyl ion "A" (Scheme 1).



Scheme 1

Scheme 1 shows the fragmentation of the linear acyl ion "A" in the tandem ESMS.

The results presented above proved that the amino-acid sequence in the peptide with MW 1044 that was isolated by us corresponds completely with that in bacillomycin D2 [1]. Figure 1 shows the peptide structure and the correlations observed in the HMBC and NOESY spectra.

The fraction of **1** exhibited cytotoxic activity for tumor cells (IC_{50} 6.0 μg/mL). Experiments with erythrocytes showed that the effectiveness of bacillomycin D membrane action, like that of cyclic acyldepsipeptides [7], depends significantly on the pH of the medium. Thus, the peptide induces erythrocyte hemolysis at pH 7.2, 6.0, and 5.0 at concentrations of 25.0, 5.0, and 2.5 μg/mL, respectively.

EXPERIMENTAL

Cultivation of microorganisms and isolation of peptides were performed as previously described [8].

UV spectra were recorded on a Specord M40 UV-VIS spectrometer (Germany) in ethanol (50%).

IR spectra were obtained on a Perkin—Elmer 983 instrument (USA) in KBr disks.

Melting points were determined on a Boetius stage (Germany).

^1H and ^{13}C NMR spectra were recorded on Bruker DMX 750 (Germany) (750 MHz and 188 MHz, 310 K) and Bruker DMX 500 (Germany) (500 MHz and 125 MHz, 310 K) in C_5D_5N with Me_4Si internal standard. All 2D correlation experiments were performed using standard methods. ESMS mass spectra were measured in an Auto Spec "Micro Mass" instrument (England) (4 kV, 50% He, 5 μL/min). MALDI mass spectra were recorded in a Perceptive Biosystem Voyager Elite (USA) instrument.

Total acid hydrolysis of the fraction of **1** used HCl (6 N) at 100°C for 24 h. Qualitative and quantitative analyses of α-amino acids were carried out in a Biotronic KC 200 amino-acid analyzer (Sweden) using DC-6A resin (220×6 mm column) by the method of Moore and Stein [9].

Determination of Cytotoxic Activity. Alcohol solutions of bacillomycin D (0.001 mL) at various concentrations were added to a suspension (0.1 mL) of Ehrlich carcinoma tumor cells in medium 199 (1×10^6 cells/mL). The vitality of the tumor cells was determined after incubation at 37°C for 2 h with staining by an isotonic solution (0.17%) of tripan blue under a microscope. The concentration causing lysis of 50% of the tumor cells (IC_{50}) was determined [10].

Determination of Hemolytic Activity. Hemolytic activity was determined using a suspension of blood erythrocytes from white mongrel mice with optical density 1.0 at 700 nm in phosphate buffer (66 mM) at pH 7.2, 6.5, 6.0, 5.5, and 5.0 containing NaCl (120 mM) and KCl (4 mM). An alcohol solution of bacillomycin D (0.02 mL) was mixed with the erythrocyte

suspension (1.98 mL) and incubated at 37°C for 30 min. After incubation, the optical density of the suspension was recorded on a spectrophotometer at 700 nm and the concentration of the substance at which 50% of the erythrocytes was lysed (GC_{50}) was determined [11].

Fraction 1. Mass spectrum (MALDI, *m/z*, I_{rel}, %): 1083 (31) $[M_2 + K]^+$, 1069 (53) $[M_1 + K]^+$, 1067 (100) $[M_2 + Na]^+$, 1053 (40) $[M_1 + Na]^+$, 1045 (50) $[M_2 + H]^+$, 1031 (28) $[M_1 + H]^+$; [(+)-ESMS, *m/z*, I_{rel}, %]: 1045 (100) $[M + H]^+$, 1028 (68) $[M + H - OH]^+$, 944 (24) $[M + H - Thr]^+$, 931 (46) $[M + H - Asn]^+$, 882 (25) $[M + H - Tyr]^+$, 768 (80) $[M + H - Tyr - Asn]^+$, 705 (30) $[M + H - Thr - AA]^+$, 654 (60) $[M + H - Asn - Tyr - Asn]^+$, 631 (95), 618 (85), 591 (35) $[M + H - Thr - AA - Asn]^+$, 542 (50), 529 (30) $[M + H - Tyr - Asn - AA]^+$, 455 (85) $[542 - Ser]^+$, 428 (45), $[M + H - Tyr - Asn - AA - Thr]^+$, 415 (45) $[M + H - Asn - Tyr - Asn - AA]^+$, 341 (34) $[M + H - Tyr - Asn - AA - Thr - Ser]^+$, 314 (56) $[M + H - Asn - Tyr - Asn - AA - Thr]^+$, 278 (40), 227 (50) $[M + H - Asn - Tyr - Asn - AA - Thr - Ser]^+$, 198 (64).

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