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

**G. K. Oleinikova, A. S. Dmitrenok, V. G. Voinov, E. L. Chaikina, L. S. Shevchenko, and T. A. Kuznetsova**

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Marine bacteria of the genus *Bacillus* produce biologically active low-molecular-weight cyclic peptides [1-3]. In continuation of the search for these compounds, we investigated the strain *Bacillus subtilis* KMM 1922, which was isolated from a specimen of the sponge *Stelletta validissima* (Ituruts Island, Kuril Islands, 1986, Research Vessel "Akademik Oparin"), and found that the bacterium cultivated in liquid medium synthesizes a compound with anticandidal and cytotoxic activities.

Column chromatography over Polychrom-1 and silica gel isolated from a culture of the fungus (5 L) a white powder (**1**, 375 mg) that had a melting point of 295-305°C after reprecipitation from methanol. It did not dissolve in nonpolar solvents and was poorly soluble in alcohols, water, acids, and bases. The UV spectrum (50% ethanol) had an absorption at 280-285 nm, characteristic of tyrosine. The IR spectrum (KBr) contained absoprtion bands for peptide (1678, 1662, 1643, 1546, 1514 $cm^{-1}$) and carboxyl (1710 $cm^{-1}$) and lacked absorption bands for ether and ester.

An amino-acid analysis (6 N HCl, 100°C, 24 h) found six $\alpha$-amino acids Asp, Tyr, Glu, Pro, Thr, and Ser in a 2:1:1:1:1:1 ratio. The $CHCl_3$ extract of the hydrolysate contained an aliphatic amino acid (AA) that gave a positive reaction with ninhydrin. Matrix activated laser desorption/ionization (MALDI) and electro-spray mass spectrometry of the fraction 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 spectrum were identical to those obtained for the mixture of homologous peptides bacillomycin D1 and D2 that were previously isolated [4]. The ratio of strengths of the molecular peaks (1:5) showed that the peptide with MW 1044 Da predominated.

The PMR and $^{13}C$ NMR spectra of **1** and two-dimensional correlation COSY and HSQC spectra enabled signals in the spectra to be unambiguously assigned. The PMR spectrum (500 MHz, $C_5D_5N$, $\delta$, ppm, J/Hz, 0 = TMS) of **1** contained signals for amide and carbamide protons: 9.61 (1H, d, J = 8, NH, Tyr), 8.91 (1H, br.s, NH Asn2), 8.81 (1H, d, J = 5.5, NH Asn1), 8.61 (1H, d, J = 5, NH Ser), 8.43 (1H, br.s, $NH_2$ Asn2), 8.23 (1H, br.s, $NH_2$, Asn1), 8.18 (1H, br.s, NH Glu), 8.04 (1H, d, J = 8.4, NH Thr), 8.01 (1H, d, J = 9.3, NH AA), 7.75 (1H, br.s, $NH_2$ Asn2), 7.63 (1H, br.s, $NH_2$ Asn1), 7.48 (d, J = 8.4), and 7.05 (4H, d, J = 8.4, aromatic group of Tyr); seven $\alpha$- or $\beta$-methine protons: 5.36 (1H, m, C-2, Asn2), 5.32 (1H, m, C-2 Tyr), 5.23 (1H, m, C-2, Asn1), 4.96 (1H, dd, J = 6.4, J = 2.2, C-3 Thr), 4.94 (1H, dd, J = 6.4, J = 2.4, C-3 Thr), 4.85 (m, 1H, C-2 Glu, 1H C-2 Ser), 4.83 (1H, m, C-2 Thr), 4.60 (1H, m, C-3 AA); a threonine methyl at 1.34 (3H, d, J = 6.6); an AA *iso*-propyl at 0.84 (6H, d, J = 6.6), and a weak multiplet at 0.85 for the n-AA methyl. The $^{13}C$ NMR spectrum (125 MHz, $C_5D_5N$, $\delta$, ppm, 0 = TMS) confirmed the presence of carbamide and carboxyl C atoms in the peptide: 173.84 (C-1 Tyr), 173.79 (C-1 Glu), 173.31 (C-1 Asn1), 172.89 (C-1 Asn2, C-1 AA), 172.61 (C-4 Asn2), 172.38 (C-1 Pro), 172.32 (C-4 Asn1), 171.74 (C-1 Ser), 171.21 (C-1 Thr); a triose (signals at 116.18, 128.65, 131.31, and 157.53); in the $\alpha$- and $\beta$-positions to the carbamide: 62.34 (C-2 Pro), 59.31 (C-2 Ser), 58.16 (C-2 Thr), 55.87 (C-2 Tyr), 55.48 (C-2 Glu), 52.89 (C-2 Asn1), 50.38 (C-2 Asn2), 47.36 (C-2 AA), 66.11 (C-3 Thr), 63.67 (C-3 Ser), 42.06 (C-3 AA), 37.89 (C-3 Asn2), 37.13 (C-3 Asn1), 36.55 (C-3 Tyr), 29.6 (C-3 Pro), 27.84 (C-3 Glu); Thr methyl (20.88), and AA *iso*- (22.76) and *n*-methyls (14.24). These data indicate that the aliphatic amino acid is the sum of homologous $\beta$-amino acids of the *n*- and *iso*-series. The HMBC spectra of **1** indicate the presence of couplings between the AA amino proton and Thr carbonyl C; between the AA carbonyl C and the asparagine amino proton; and between the Glu carbonyl C and the Ser amino proton (Fig. 1). The NOESY spectrum of **1** showed coupling between the following protons: Tyr amino and Asn C-2; amino of the other asparagine and Tyr C-2; amino groups: Glu and Pro C-2, Thr and Ser C-2,

Pacific Institute of Bioorganic Chemistry, Far-East Division, Russian Academy of Sciences, 690022, Vladivostok, pr. 100-Letiya Vladivostoka, 159, Russian Federation, fax (4232) 31 40 50, e-mail: kuzta@piboc.dvo.ru. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 191-192, March-April, 2005. Original article submitted December 7, 2004.

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Ser and Glu C-2, AA and Thr C-2; and asparagine and AA C-2. Based on this, the following sequence of amino acids in **1** can be proposed:

→ Pro-CO→Glu-CO→Ser-CO→Thr-CO→AA-CO→Asn1-CO→Tyr-CO→

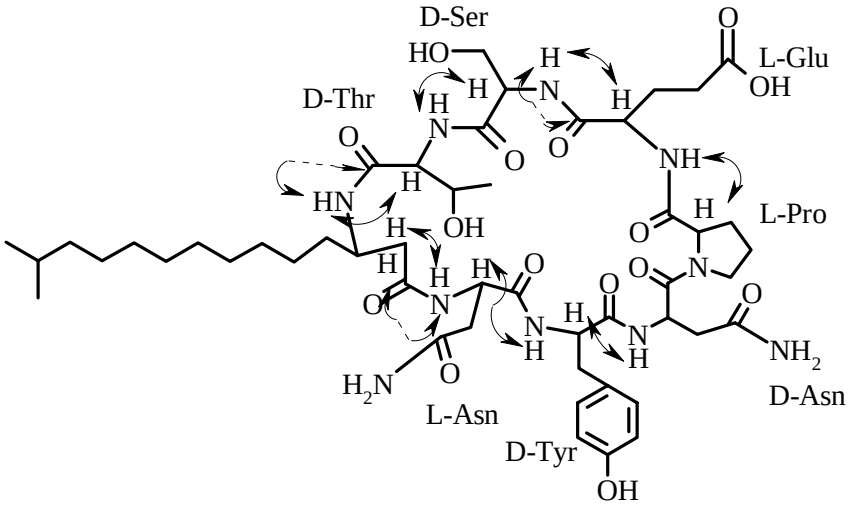


Fig. 1. Bacillomycin D2 [arrows show the correlations in HMBC (← – – →) and NOESY (↔) spectra of the peptide].

The proposed amino-acid sequence in the peptide was confirmed by tandem electrospray mass spectrometry. It was demonstrated that the amino-acid fragment split as follows if the peptide chain is cleaved between the proline and asparagine units:

$$1045^{+}\ (M + \xrightarrow{-Asn} 93 \xrightarrow{-Tyr} 76 \xrightarrow{-Asn} 65 \xrightarrow{-AA} 41 \xrightarrow{-Thr} 31 \xrightarrow{-Ser} 227 \xrightarrow{-Glu} 98 \quad (Pro + H)$$

Thus, it was proved that the amino-acid sequence of the peptide with MW 1044 corresponds with that of bacillomycin D2. Figure 1 shows the structure of the peptide and the correlations in the HMBC and NOESY spectra of it.

It can be seen that the peptide exhibits cytotoxic activity toward Ehrlich's carcinoma cells ($IC_{50}$ = 6 μg/mL) and murine erythrocytes.

It was established that its membranotropic action depends on the pH. Thus, the concentrations causing erythrocyte destruction after 30 min at pH values 7.0, 6.0, and 5.0 were 25, 5.0, and 2.5 μg/mL, respectively. The cytotoxic activity of the peptide was determined by the literature method [5, 6].

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