Surfactin-like structures of five cyclic depsipeptides from the marine isolate of *Bacillus pumilus*

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Five cyclic depsipeptides with molecular masses of 1007, 1021, 1021, 1035, and 1035 were obtained from *Bacillus pumilus* KMM 150 associated with Australian marine sponge *Ircinia* sp. Their structures were assigned by mass spectrometric techniques (high-resolution fast atom bombardment and electron impact mass spectrometry), chemical modification, and extensive spectroscopic analysis, including several types of two-dimensional NMR.

Key words: lipopeptides, fatty acids, cyclic depsipeptides; surfactin; *Bacillus pumilus*; marine sponge *Ircinia* sp.

We studied the cytotoxic compounds, bacircines **1**—**5**, produced by the bacteria *B. pumilus*. The microorganisms were isolated from marine sponge *Ircina* sp. The bacircines were found to have structures somewhat similar to those of surfactins, the cyclic depsipeptides produced by *B. subtilis* and *B. subtilis natto*,[1—5] which contain fatty β-hydroxy acids and seven amino acids. The surfactins possess cytolytic activity against fungi and yeasts,[6—8] and they inhibit formation of blood clots,[1] manifest an antitumor effect toward Ehrlich's carcinoma,[9,10] and have a hypocholesterinemic action.[11] They also suppress adenosin-3′,5′-cyclomonophosphate diesterase[12] and are the most active biosurfactants.[1,13] We have established that at concentrations of more than 2.5—10 μg mL^{-1} bacircines cause anomalies in the development of ova of echinus and stop blastomere fission. Detailed investigation of the chemical structure of individual bacircines allowed us to conclude that there were some differences in the compositions of the surfactin and the starting mixture that we have isolated from the marine bacteria *B. pumilus*. The goal of this work was the isolation of individual bacircines and the investigation of their structure.

An ethyl acetate extract of microbial cells was concentrated *in vacuo* and subjected to gradual chromatography on a silica gel column using a hexane—ethyl acetate—methanol mixture as the eluent. The combined fractions F_8—F_{12} (see Experimental) were separated by HPLC to afford bacircines **1** (14.5 % based on the total peptide fraction), **2** (11 %), **3** (8 %), **4** (33.5 %), and **5** (33 %). In the UV spectra of compounds **1**—**5** there are no absorption maxima in the region of $\lambda > 225$ nm. In the IR spectra there are absorption bands at 3312, 3071, 1733, 1727, 1720, 1666, and 1228 cm^{-1}, which are characteristic of the carbonyl groups of esters and amides. The band at 3312 cm^{-1} corresponds to oscillation of the bonded NH groups. The above data, along with the NMR spectra and the lipophilic character of the compounds obtained, enabled us to assume that bacircines are the cyclic depsipeptides.

Acid hydrolysis of compounds **4** and **5** afforded five reaction products. Four of them were identified (using an automatic amino acid analyzer) as Leu, Val, Asp, and Glu in 4 : 1 : 1 : 1 ratio. The chromatomass-spectrometric (GLC-MS) analysis of the butyl esters of *N*-trifluoroacetyl derivatives of these compounds confirmed this amino acid composition. In the secondary ions' mass spectrum (SI MS) of bacircines **4** and **5** there are the peaks of $[M+H]^+$ (m/z 1036) and $[M+Na]^+$

† Deceased.

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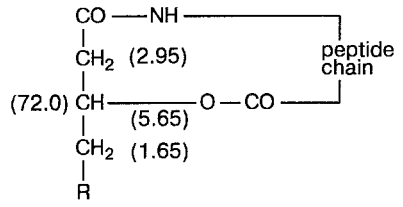
(*m/z* 1058) ions. In the electron impact mass spectra (EI MS) there are the peaks of $[M-H_2O]^+$ ions with *m/z* 1017. In the EI MS of **4** and **5** there are also intense peaks of ions containing the lipophilic fragment of the molecule with *m/z* 222 [$C_{15}H_{26}O$: (lipophilic part − 2H)$^+$] and 223 [$C_{15}H_{27}O$: (lipophilic part − H)$^+$]. Taking into account the data on the amino acid composition, depsipeptides **4** and **5** can be described by the empirical formula $C_{53}H_{93}N_7O_{13}$ (M = 1035).

In the molecules of compounds **4** and **5**, the lipophilic part is bound to the N-end of the peptide part by an amide bond, since in the NMR spectra of these compounds there are seven signals for the amide protons and ten signals for the carbonyl carbon atoms, seven of the latter referring to the amide carbonyl groups. The spectra also point out the interaction of seven amide protons with seven methine protons. The eighth methine proton (δ 5.65) does not interact with the amide proton and is attached to the carbon atom with the signal at δ 72.0. Moreover, this methine proton interacts with two CH_2 groups (δ 2.95 and 1.65). The first one is not connected to other protons, while the second interacts with the protons with δ 1.4; the latter, in turn, interact with the proton whose signal lies at δ 1.24. All the above indicates that the structure of the lipophilic part of the molecule is as follows ($\delta^{13}C$ and δ^1H are given in parentheses):



Combination of GLC-MS analysis and NMR spectroscopic data allows the fifth component of the hydrolysate of compounds **4** and **5** to be identified as 3β-hydroxypentadecanoic acid. The NMR spectra of compounds **4** and **5** point out the presence of twelve methyl groups, ten of them belonging to the amino acids mentioned above (all of the doublets in the ^1H NMR spectra) in their structures. Hence, the fatty acid moiety must contain two Me groups. In the HMOC spectra of compound **4** there is only one methyl triplet, which corresponds to the signal of the carbon atom at δ 11.6. This makes it possible to conclude[14] that the end part (R) of the molecule of **4** has an *anteiso*-structure, *i.e.*, R = $-(CH_2)_7-(CH_3)CH-CH_2CH_3$ (δ 11.6). The EI mass spectra of compounds **4** and **5** show that the ion with *m/z* 223 is actually the acylium cation of the dehydrated C_{15}-β-hydroxy acid, and the ion with *m/z* 334 [223+Glu(−H_2O)]$^+$ is connected with the cation with *m/z* 223 by a metastable bond. The subsequent fragments with *m/z* 447, 560, and 659 point to the following chain: lipophilic part—Glu—Leu—Leu—Val... Besides, the presence of the ion with *m/z* 659 that is metastably connected with the ion with *m/z* 334 confirms that the Glu-residue can not be dependent on the Leu—Leu—Val chain. This is proved by the compositions of the ions in the metastable defocusing spectra. This amino acid sequence is confirmed by the EI spectra of permethylated derivatives **4b** and **5b**, where the peaks with *m/z* 779, 922, and 1049 point out the sequence ...Val—Asp—Leu—Leu-residues (Scheme 1).

The EI mass spectra of the methyl esters of compounds **4** and **5** (**4a** and **5a**, respectively) contain the peaks of the molecular ions with *m/z* 1063, which indicate the presence of two free COOH groups in the starting molecules. Taking into consideration that one of them belongs to Glu (*m/z* 334 [223+Glu(−H_2O)]$^+$), the second may belong either to Asp-residue or to the terminal Leu-residue. In order to solve this problem, compound **4** was reduced with $LiBH_4$ with subsequent complete acid hydrolysis and preparation of the butyl esters of *N*-trifluoroacetylated derivatives of the hydrolysis mixture. GLC-MS analysis of these derivatives demonstrated the presence of Asp among other amino acids, however leucine alcohol was present in the mixture. In addition, linear peptide **4c** (obtained by alkaline hydrolysis of **4** in methanol) was hydrolyzed by carboxypeptidase Y, which eliminated the C-terminal amino acid, and leucine was then detected in the hydrolysate. It was proved that the lactone cycle was formed by β-hydroxy acid and the carboxyl group of the seventh Leu-residue. The reported amino acid sequence for **4** and **5** is in complete agreement with the data of the two-dimensional NMR spectroscopy of these compounds.

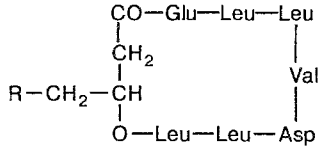
In the SI MS of bacircines **1**, **2**, and **3** there are peaks of $[M+H]^+$ ions (*m/z* 1008, 1022, and 1022, respectively). This indicates that the amino acid composition of **1**, **2**, and **3** is similar to that of **4** and **5**, and only the fragment ions of the lipophilic parts of these groups of compounds, which appear under electron impact, are different. Thus, instead of the ions with *m/z* 222—223 (for compounds **4** and **5**), the ions with *m/z* 208—209 (for compounds **2** and **3**) or 194—195 (for compound **1**) are observed. The EI mass spectra of permethylated derivatives **1b**, **2b**, and **3b** demonstrate an amino acid sequence similar to that of peptides **4** and **5**. Consequently, bacircines **1**, **2**, and **3** are homologs of **4** and **5**, but contain C_{13}—C_{14}-β-hydroxy acids instead of C_{15}-β-hydroxy acid as do **4** and **5**. In addition, in

Scheme 1

$RCH_2CH(OMe)CH_2COMeGlu(OMe)MeLeuMeLeuMeVal$	MeAsp(OMe)	MeLeu	MeLeu(OMe)
m/z: 779	922	1049	

the NMR spectra of all five depsipeptides there are differences in the structures of R fragments of fatty acids. The ^{13}C NMR spectra of compounds **1** and **4** contain one signal of an Me group at δ 11.6, corresponding to the *anteiso*-structure of R. In the spectra of compounds **2** and **5**, there are two signals of Me groups at δ 18.7 and 19.4, which, according to the literature data,[14] confirm the *iso*-structure of R, whereas in the spectrum of compound **3** there is signal for the carbon atom of the Me group at δ 13.9 typical of the normal fragment R.

The structures of the bacircines appeared to have much in common with those of esperin produced by *B. mesentericus*[15,16] and surfactin isolated by cultivation of *B. subtilis* and *B. subtilis natto*.[1–5] The composition and the sequence of the amino acid residues in esperin coincide with those established for bacircines, except that esperin has a smaller peptide ring formed by the fifth Asp-, and not by the seventh Leu-residue. Earlier surfactin was believed to be a mixture of cyclic lipopeptides constructed, like bacircines, from one and the same heptapeptide and β-hydroxy acids of different chain length (from 13 to 15 carbon atoms), among which 3β-hydroxy-13-methyltetradecanoic acid is the main one. Using two-dimensional COSY, TOCSY, and ROESY NMR[17] along with FAB-MS,[18] it was established that *B. subtilis* can produce surfactin as a mixture of peptidolipids that differ not only in the homology of the lipid parts, but also in the terminal amino acid residues. New variants of surfactin are [Val7] and [Ile7] surfactins. Such a substitution of the C-terminal amino acid residue affects the whole molecular structure of the lipopeptide. Production of these new analogs probably depends on the composition of the culture medium and on the differences in the bio-organisms themselves. We did not find these surfactin variants under the cultivating conditions used, and we have demonstrated that the strain *B. pumilus* associated with the sponge *Ircina* sp. produces a mixture of at least five peptidolipids, which have the same peptide ring with the traditional C-Leu-end, but differ in the homology and isomerism of their fatty acid parts. Peptides **1** and **4** are the new compounds. Determination of the stereochemistry of their amino acids is in progress.



1: R = —$(CH_2)_5$—(Me)CH—CH_2Me

2: R = —$(CH_2)_7$—$CHMe_2$

3: R = —$(CH_2)_8$—CH_2Me

4: R = —$(CH_2)_7$—(Me)CH—CH_2Me

5: R = —$(CH_2)_8$—$CHMe_2$

Detection of the normal, *iso*-, and *anteiso*-isomers of C_{13}—C_{15}-β-hydroxy acids in the side chain of bacircines complements the data presented in the literature.[19,20] These subtle differences in the structure of bacircines probably affects their properties and functions. These are now under study.

Experimental

Animals were collected in Australian waters (Hautman Reef) at a depth of 0.5 m (Expeditionary voyage No. 4, the ship "Academition Oparin," July 1987).

The starting mixture was analyzed by TLC on silica gel 5/40 μ (SFR) in a chloroform—methanol—water mixture (65 : 25 : 4). Chromatograms were developed using toluidinyl chloride. Melting points were measured on an electrically heated block. EI MS and SI MS (low resolution), as well as data on the elemental composition of the ions, were obtained on an MKh 1310 instrument. EI MS were recorded at an ionizing voltage of 50 V, collector current of 60 μA, and vaporizer temperature of 200—220 °C; SI MS: ionization by a Cs^+ beam with the energy of 7 kV using a matrix of glycerol with an admixture of AcOH (0.1 mol L^{-1}). NMR spectra were recorded on a Bruker AM 300 spectrometer (300 and 75 MHz for ^1H and ^{13}C, respectively). $[\alpha]_D$ Values were determined using a Perkin-Elmer 141 polarimeter with a lamp with λ = 578 nm (Sweden). UV spectra were recorded on a Specord UV-VIS M 40 spectrophotometer (Germany) in methanol.

Amino acid analysis was carried out on a Biotronik LC 2000 amino acid analyzer (Sweden) using a DS-6A resin column (220×6 mm). HPLC was performed on a Waters instrument (USA) using an UZDN-2T ultrasonic disintegrator. GL chromatograms were obtained on a Pye Unicam 104 chromatograph.

Fermentation of the strain *B. pumilus* (KMM 150). Cultivating medium (*C*/g L^{-1}): K_2HPO_4 (0.07), NH_4Cl (1.0), yeast extract (5.0), $FeSO_4$ (0.025), 1 *M* Tris buffer (pH 7.5) (20.0), artificial seawater (200.0). Fermentation temperature was 24—26 °C. The duration of cultivation was 17 h.

Isolation of depsipeptides 1—5. Culture fluid (10 L) was centrifuged at 500 *g* for 40 min to give 44 g of cell mass. The cells were suspended in distilled water (70 mL), frozen, defrosted, and destroyed with ultrasonics for 2 min at 18-second intervals. The suspension of destroyed cells was extracted three times with ethyl acetate with stirring with a magnetic stirrer (20 min). The combined ethyl acetate extracts were dried over Na_2SO_4, filtered, evaporated to dryness, and separated on a silica gel (40/100, Chemapol, CzSFR) column with gradual elution with: hexane—ethyl acetate mixture, 3 : 1 (40 mL, F_1 and F_2); 2 : 1 (42 mL, F_3 and F_4); 1 : 1 (40 mL, F_5 and F_6); 1 : 3 (40 mL, F_7 and F_8); ethyl acetate (40 mL, F_9 and F_{10}); ethyl acetate—methanol (95 : 5, 40 mL, F_{11} and F_{12}). The combined fractions F_8—F_{12} were separated by HPLC (SGX×C_{18}, 5μ; 4×250 mm, v = 2 mL min^{-1}), λ = 226 nm; an acetonitrile—trifluoroacetic acid (0.01 %) mixture (70 : 30) was used as the eluent, yielding the following individual depsipeptides: **1**, $[\alpha]_D$ −36.47° (*c* 0.5, methanol); **2**, $[\alpha]_D$ −31.48° (*c* 0.5, methanol); **3**, $[\alpha]_D$ −32.4° (*c* 0.5, methanol); **4**, $[\alpha]_D$ −28.6° (*c* 0.5, methanol), m.p. 143.5—144.5 °C; **5**, $[\alpha]_D$ −33.6° (*c* 0.5, methanol), m.p. 142.5—143.5 °C.

Scheme 2

Peptide	RCH(OMe)CH_2MeGlu(OMe)	MeLeu	MeLeu	MeVal	MeAsp(OMe)	MeLeu	MeLeu(OMe)
1	384	511	638	751	894	1021	
2	398	525	652	765	908	1035	
3							
4	412	539	666	779	922	1049	
5							

IR ($CHCl_3$), ν/cm^{-1}: **1**, **2**, and **3** — 3312, 3071, 2960, 2930, 1733, 1727, 1720, 1666; **4** and **5** — 3312, 3071, 3014, 3010, 2960, 2930, 1733, 1727, 1720, 1666. SIMS (**1**, **2**, **3**, **4**, and **5**, respectively), *m/z*: 1008, 1022, 1022, 1036, 1036 $[M+H]^+$; 1030, 1044, 1044, 1058, 1058 $[M+Na]^+$; 1046, 1060, 1060, 1074, 1074 $[M+K]^+$. EI MS for **4** and **5**, *m/z* (I_{rel} (%)): 1017 $[M-H_2O]$ (0.8), 222 $[C_{15}H_{26}O]$ (42), 223 $[C_{15}H_{27}O]$ (45), 334 [223+(Glu$-H_2O$), $C_{20}H_{32}NO_3$] (42), 351 $[C_{20}H_{33}NO_4]$ (39), 447 [334+Leu, $C_{26}H_{43}N_2O_4$] (16), 560 [447+Leu, $C_{32}H_{54}N_3O_5$] (14), 659 [560+Val, $C_{37}H_{63}N_4O_6$] (5.5), 774 [659+Asp] (0.5).

Complete acid hydrolysis of depsipeptides 1—5. A compound (1 mg) was dissolved in 5.6 *N* HCl (1 mL) in a glass ampule, and the ampule was evacuated and kept for 24 h at 105 °C. The cooled reaction mixture was diluted with bidistilled water, concentrated *in vacuo*, and analyzed using an amino acid analyzer.

Preparation of butyl esters of *N*-trifluoroacetyl derivatives of the components of 4 for GLC-MS analysis. Gaseous HCl was passed through a solution of compound **4** (1 mg) in *n*-butanol (1 mL) until the concentration of HCl became equal to 3 mol L^{-1} (saturation reached). The reaction mixture was refluxed for 1 h and then cooled, and the solvent was removed *in vacuo*. The residue was suspended in CH_2Cl_2 (1 mL), and the anhydride of trifluoroacetic acid (1 mL) was added. The reaction mixture was heated to 150 °C and kept for 10 min at this temperature. The cooled reaction mixture was evaporated in a nitrogen flow in order to prevent decomposition of the products obtained. A CH_2Cl_2 solution of the residue was subjected to GLC-MS analysis (3 % QF-1, 90—220 °C, $v = 4$ °C min^{-1}). Peak *1* — Val, peak *2* — Leu, peak *3* — Asp, peak *4* — Glu (all identified with the known samples), peak *5* — C_{15}-β-hydroxy acid. EI MS of peak *5*, *m/z* (I_{rel} (%)): 57 (50), 69—70 (100), 151 (30), 180 (25), 193 (50), 198 (15), 222 $[M^+-CF_3COOH-C_4H_9OH]$, 241 $[CF_3COOCHCH_2COOC_4H_9]$, 254 $[M^+-CF_3COO-43]$, 281 $[296-CH_3]$, 296 $[M^+-CF_3COOH]$.

Preparation of methyl esters 4a and 5a. An ether solution of diazomethane (1 mL) was added to a solution of compound **4** or **5** (1 mg) in methanol (0.5 mL), and the reaction mixture was allowed to stand for 30 min at ~20 °C. The solvent was evaporated. EI MS, *m/z*: 1063 $[M^+]$, 366 [334+32], 479 [447+32], 592 [560+32], 691 [659+32], 820 [774+32+14], 933 [820+Leu].

Preparation of compounds 1b—5b *via* Hakomori's methylation of depsipeptides. A solution of methylsulfinyl carbanion (200 μL) was added to a solution of a compound (2 mg) in DMSO (500 μL), and the reaction mixture was stirred for 7 h at 24 °C. Afterwards, MeI (200 μL) was added, and the reaction mixture was left overnight at ~20 °C. The solvent was evaporated, and the residue was subsequently washed with water and methanol on Sep-pak-C_{18}. The methanolic solution was concentrated, and the residue was analyzed by EI mass spectrometry (Scheme 2, *m/z*).

Preparation of linear peptide 4c. Compound **4** (1 mg) was dissolved in 0.1 *N* methanolic NaOH (1.5 mL) and left overnight at ~20 °C. The reaction mixture was evaporated *in vacuo* to dryness, and the residue was dissolved in water, acidified with HCl, and extracted with ethyl acetate. The solvent was removed.

Preparation of reduced peptide 4d. A small amount of $LiBH_4$ (on the edge of a spatula) was added to a solution of compound **4** in methanol (3 mL). The reaction mixture was left overnight at ~20 °C. The reaction was terminated by addition of 1 *M* HCl, and the solvent was concentrated. The residue was dissolved in water (2 mL) and extracted three times with butanol. The organic layer was evaporated *in vacuo*. In the SI MS there was a peak with *m/z* 1046 $[M+Li]^+$. The reduced material was hydrolyzed with 5.6 *N* HCl for 24 h at 105 °C. After the usual work-up, butyl esters of *N*-trifluoroacetyl derivatives were prepared for GLC-MS analysis as described above. EI MS of the reduced amino acid, *m/z*: 309, 294, 266, 252, 240, 196, 182, 154, 153, 140, 139, 126, 114, 86, 69.

Hydrolysis of linear peptide 4c by carboxypeptidase Y. Acetate buffer (71 μL, 0.05 *M*, pH 5.6) and a solution of carboxypeptidase Y (100 μg) in the same buffer (100 μL) were added to a solution of linear depsipeptide **4c** (80 μg) in DMSO (9 μL). 47-μL Aliquots were taken after 15 min, 30 min, and 2 h, and then the reaction was stopped by addition of acetic acid (5 μL). The reaction mixture was concentrated and analyzed using an amino acid analyzer.

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