New C_{14}-surfactin methyl ester from the marine bacterium *Bacillus pumilus* KMM 456

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New C_{14}-surfactin methyl ester and eight earlier described surfactins were isolated from the marine bacterium *Bacillus pumilus* associated with zoantharia *Palythoa* sp. The structure of the compounds isolated was established based on the data from amino acid analysis, one- and two-dimensional NMR spectroscopy, and tandem nanoelectrospray mass spectrometry. The absolute configurations of amino acids were determined by Marfey's method. Cytotoxic activity of a number of surfactins with respect to the tumor cells HCT-116 and MDA-MB-231 was studied.

Key words: marine isolate of bacterium *Bacillus pumilus*, surfactins, tandem nanoelectrospray mass spectrometry.

Bacteria of the family *Bacillus* produce a wide range of biologically active lipopeptides, which are highly promising for the use in medicine. Surfactins, cyclic depsipeptides, containing a β-hydroxy acid and several amino acids, are of special interest among them. Compounds of this large class are mixtures of isoforms differing from each other in both the amino acid composition of the peptide core and the length (C_{12}—C_{15}) and branching (*iso*-, *anteiso*-, *n*-) of their hydrophobic fatty-acid moiety.

The history of surfactins began from the report[1] on isolation of new lipopeptide with exceptional surfactant properties from the culture medium of the bacterium *Bacillus subtillus*. Then, a large body of works were devoted to the study of the structures and biological action of this class of compounds. It was shown that they possess hemolytic,[2] antiviral,[3,4] and antitumor[5,6] activity, exhibit hypocholesterinemic effect,[7] and are strong biosurfactants.[1,8] High biological activity of surfactins opens wide prospects for their use in medicine and biotechnology, while the presence of a large amount of isoforms allows one to study dependence of the biological activity of these compounds on their structures.

Continuing search for the producents of biologically active compounds among marine bacteria isolates, we found that the strain *Bacillus pumilus* KMM 456 synthesizes compounds with high hemolytic activity. The individual peptides **1**—**9** (in the order of elution in reversed-phase chromatography) were isolated by chromatography from the ethyl acetate extract of bacterial cells.

The structures of compounds isolated were established based on the data from amino acid analysis, one- and two-dimensional NMR spectroscopy, tandem nanoelectrospray mass spectrometry, as well as on the comparison of their spectra with those reported earlier. The absolute configurations of amino acids were determined by Marfey´s method. One of the depsipeptides, compound **7**, was isolated from bacteria of the family *Bacillus* for the first time, the rest are identical to the those described earlier.

The ESI-MS of compound **7** contains peaks of the cationized molecules at *m/z* 1058 $[M + Na]^+$ and 1080 $[M + 2\,Na - H]^+$ (Fig. 1). The molecular formula of **7** was

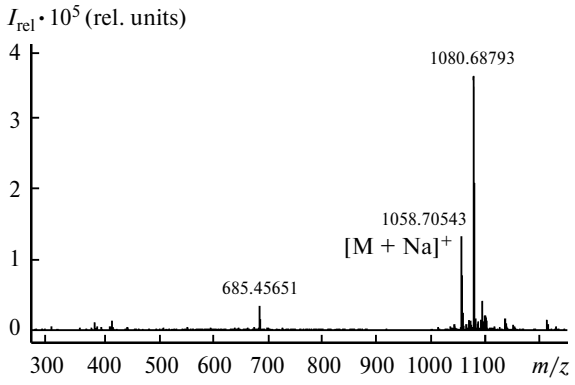

Fig. 1. The ESI mass spectrum of surfactin **7**.

† Deceased.

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determined as $C_{53}H_{93}N_7O_{13}$ based on the analysis of the ESI-TOF high resolution mass spectrum (found: m/z 1058.6719 $[M + Na]^+$, calculated 1058.6729).

Amino acid analysis of hydrolyzate of compound **7** indicates the presence of four amino acids: Asp, Glu, Val, and Leu in the ratio 1 : 1 : 1 : 4.

The ^1H NMR spectrum of compound **7** exhibits signals for seven amide protons in the region δ 7.57—8.40, seven α-methine protons at δ 4.04—4.54, and an intensive signal for the methylene groups of the fatty-acid chain at δ 1.24. A singlet signal for the methyl protons at δ 3.58 indicates the presence of the —COOMe group in the molecule, while the resonance at δ 5.07 evidences the presence of the —CHOC(O)— group. The ^{13}C NMR spectrum of compound **7** contains ten signals for the carbonyl carbon atoms, seven signals for C_α, and a signal for the methoxy group at δ 51.3. The correlations observed in the COSY-45 spectrum indicate the presence of the isolated spin system $—CH_2—CH(O)—CH_2—CH_2—$ in the structure of **7**.

The amino acid sequence, position of the methoxy group, and the length of the fatty-acid chain in **7** were established based on the tandem mass spectrometric data for the ion with m/z 1058.69 $[M + Na]^+$ (Fig. 2). A series of fragment ions obtained by simple cleavage of the lipopeptide in the ESI-MS/MS (1058→945→832→717→618→505→392) determines the C-terminal sequence of amino acids Leu—Leu—Asp—Val—Leu—Leu (Fig. 3, *a*). Cleavage of the peptide by the mechanism of double hydrogen transfer leads to the formation of two series of fragment ions:[9] 707→594→481→382→267 (Leu—Leu—Val—Asp) (Fig. 3, *b*) and 1058→927→814→671→572 (Fig. 3, *c*). The difference in 18 mass units between the fragment ions 832 (Leu—Leu) and 814 (Leu—Leu—OH_2) (Fig. 3, *a* and *c*) indicates that leucine is the C-terminal amino acid, while the ester bond in the lipopeptide is formed between the carbonyl group of leucine and the hydroxy group of the aliphatic moiety. These data allowed us to establish the amino acid sequence in compound **7**: N—Leu—Leu—Val—Asp—Leu—Leu—C. The ion fragmentation unambiguously shows that aspartic acid is not methylated and, therefore, compound **7** contains methyl ester of glutamic acid.

The spectrum contains an ion with m/z 392.27 corresponding to the residue of the fatty acid and GluOMe (see Fig. 3, *a*). The difference between this ion and GluOMe is 249 molecular mass units, which corresponds to the residue of a β-hydroxy acid with 14 carbon atoms ($C_{13}H_{26}ONaCO^-$). The ^{13}C NMR spectrum of compound **7** (DMSO-d_6) exhibits characteristic signals at δ 13.9 and 31.3, which indicate the linear structure of the acid.[10]

The absolute configuration of amino acids in compound **7** was determined by a modified Marfey's method using their L-FDAA-derivatives[11] (Table 1).

The ESI-MS spectra of compounds **1** and **3**—**5** exhibit peaks of cationized molecules at m/z 1030, 1044, 1044, and 1058 ($[M + Na]^+$) which correspond to the molecular masses 1007 (**1**), 1021 (**3**), 1021 (**4**), and 1035 (**5**), respectively. Amino acid analysis of hydrolyzates of these com-

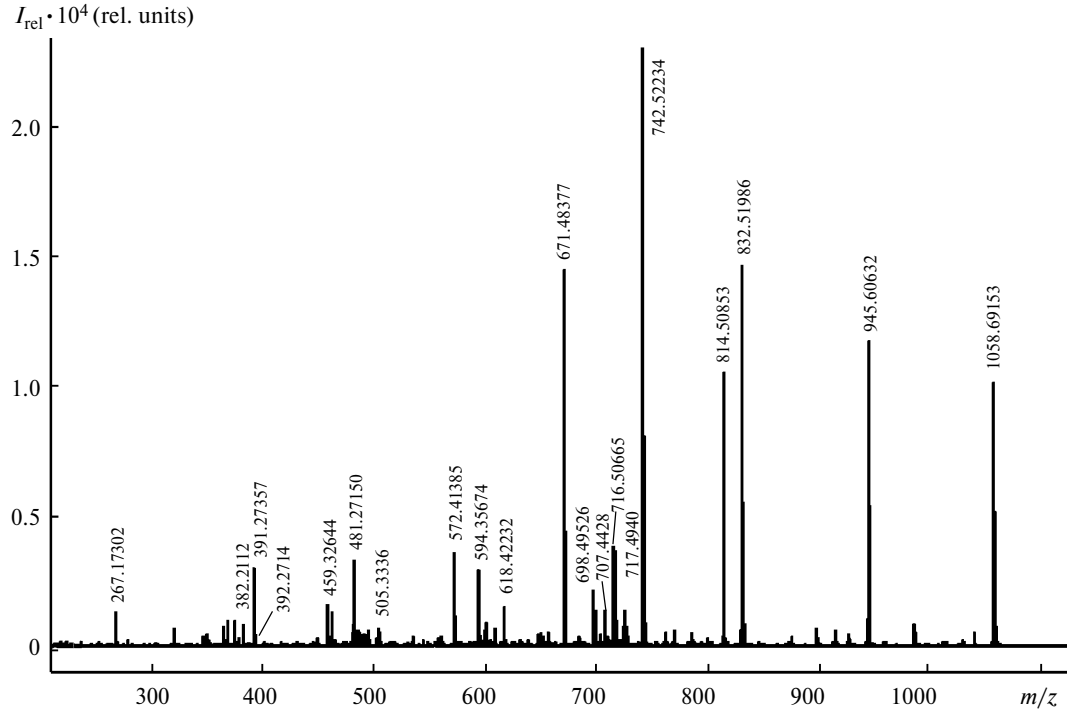
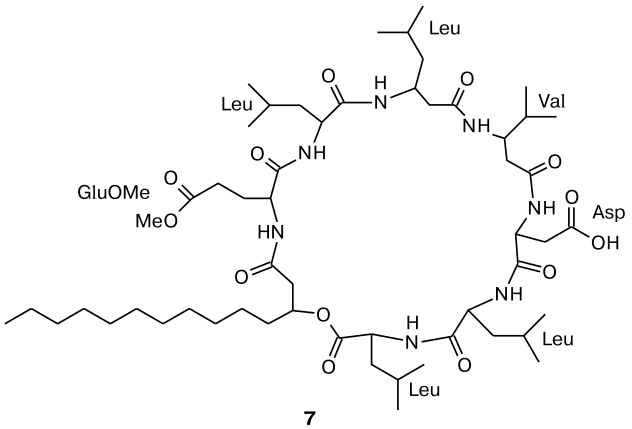


Fig. 2. The tandem nanoelectrospray mass spectrum (ESI-MS) of surfactin **7**.



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pounds indicates the presence of four amino acid residues: Asp, Glu, Val, and Leu (1 : 1 : 1 : 4). The ^1H and ^{13}C NMR spectra of **1** and **3**—**5** (in C_5D_5N) agree with the spectral data for [Leu7]-surfactin,[12] while the fragmentation in the ESI-MS/MS on simple cleavage and cleavage with the double hydrogen transfer corresponds to the literature data.[9] The length and type of the fatty-acid chains were

Table 1. Stereochemistry of amino acids of surfactin **7**

Amino acid	t_R[a]/min (configuration)	
	I	II
Glu	5.17 (L), 6.25 (D)	5.19 (L)
Asp	8.74 (L), 9.55 (D)	8.65 (L)
Val	20.30 (L), 25.27 (D)	20.23 (L)
Leu	23.71 (L), 28.04 (D)	23.70 (L)[b], 28.02 (D)[b]

[a] Retention times of derivatives of the individual amino acids (I) and derivatives of the amino acides in peptide **7** (II) are given.
[b] The ratio L : D = 1 : 1.

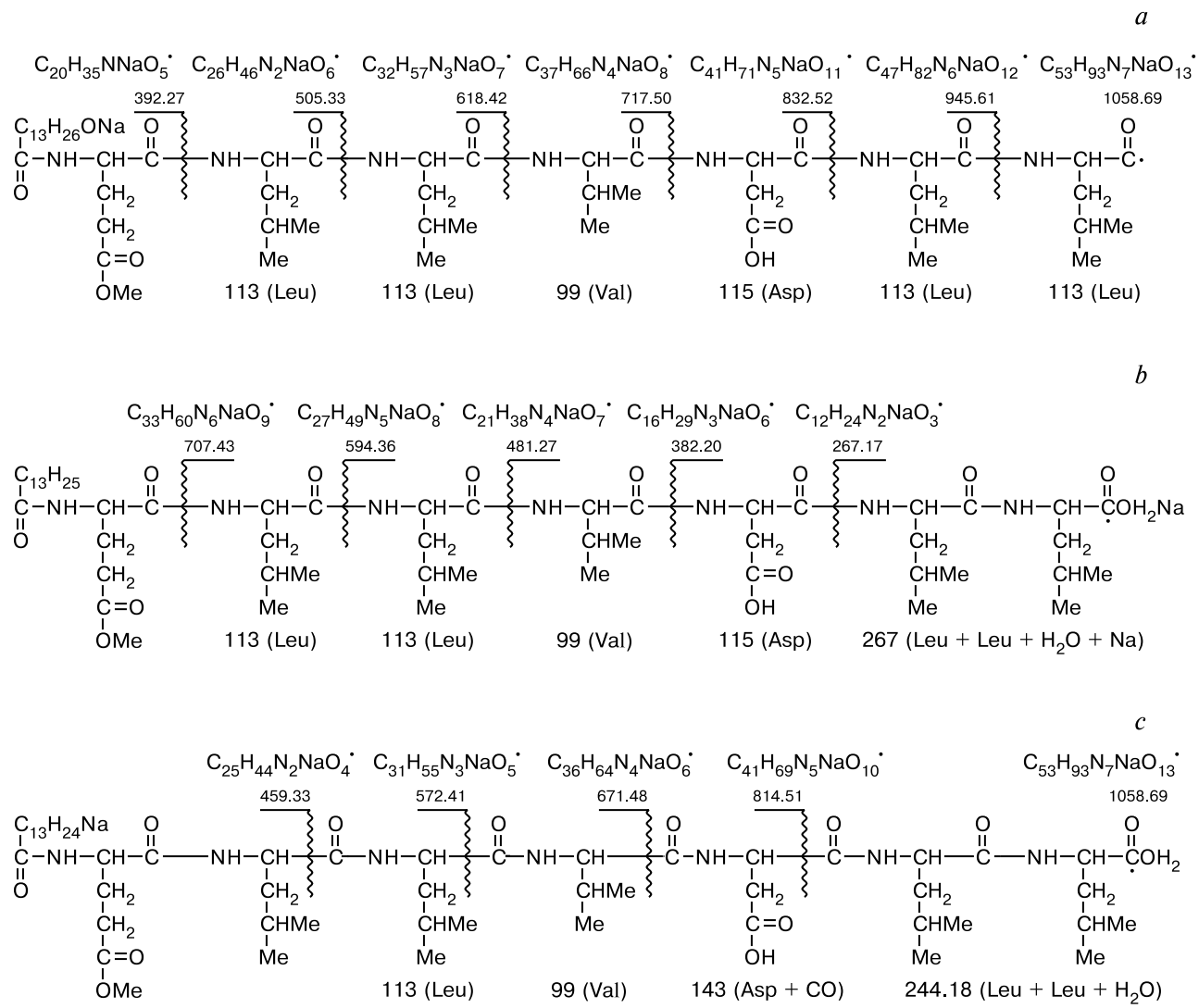

Fig. 3. Fragmentation of the ion with *m/z* 1058 on simple cleavage (*a*) and cleavage with the double hydrogen transfer (*b*, *c*).

determined based on the molecular mass values and chemical shifts for the terminal methyl groups. As a result, the structures of depsipeptides **1**, **3**, **4**, and **5** were established as [Leu7]-surfactins with the fatty-acid chains of C_{13}, C_{14}, C_{14}, and C_{15} in length and *anteiso*-, *iso*-, *n*-, and *anteiso*-structure, respectively.

In the ESI-MS of compound **2**, there are peaks of the protonated and cationized molecules at *m/z* 1008 $[M + H]^+$ and 1030 $[M + Na]^+$. Amino acid analysis of compound **2** shows the presence of the same four amino acid residues, *viz.*, Asp, Glu, Val, and Leu (1 : 1 : 1 : 4). The series of fragment ions 1030→917→804→689→590→477, 707→594→481→382→267, 786→643→544→431 obtained in the ESI-MS/MS allowed us to establish the sequence N—Glu—Leu—Leu—Val—Asp—Leu—Leu—C in peptide **2**. They differ by 14 mass units from the corresponding series of peaks for C_{14}-surfactin.[9] The ^{13}C NMR spectrum exhibits signals at δ 14.0 and 31.3 indicating the linear structure of the fatty acid.[10] Thus, peptide **2** was identified as [Leu7]-surfactin with the C_{13}-(*n*)-fatty-acid chain. Earlier,[13] this surfactin was isolated from the culture medium of *Bacillus natto*.

In the ESI-MS spectra of compound **6**, there are peaks of the protonated and cationized molecules at *m/z* 1022 $[M + H]^+$ and 1044 $[M + Na]^+$. Amino acid analysis shows the presence of four amino acid residues: Asp, Glu, Val, and Leu (1 : 1 : 2 : 3). The series of fragment ions obtained on the cleavage of the ion with *m/z* 1022 $[M + H]^+$ in ESI-MS/MS (1022→923→810→695→596→483→370) evidences the sequence C—Val—Leu—Asp—Val—Leu—Leu—Glu—N in compound **6**. The ^{13}C NMR spectrum of compound **6** (in C_5D_5N) exhibits two additional (as compared to the spectrum of peptide **5**) signals for C_γ atoms of valine at δ 19.3 and 19.6 and a signal for C_α at δ 59.1 The signal for the methyl group at δ 11.5 indicates the *anteiso*-structure of the fatty acid. Thus, compound **6** was identified as [Val7]-surfactin with the C_{15}-*anteiso*-fatty-acid chain.

The ESI-MS spectra of compound **8** contain peaks of the protonated and cationized molecules at *m/z* 1036 $[M + H]^+$ and 1058 $[M + Na]^+$. Amino acid analysis shows the presence of five amino acid residues: Asp, Glu, Val, Leu, and Ile (1 : 1 : 1 : 3 : 1). Fragmentation of the ion with *m/z* 1036 $[M + H]^+$ in ESI-MS/MS corresponds to the fragmentation of [Leu7]-surfactin with the C_{15}-*iso*-fatty-acid chain.[14] The ^{13}C NMR spectrum of compound **8** (in C_5D_5N) exhibits signal for the C_α atom at δ 58.1 and signal for the γ-methyl group at δ 16.2, which are characteristic of the isoleucine residue.[12] The signal for the methyl group at δ 11.5 indicates the *anteiso*-structure of the fatty acid. Thus, compound **8** was identified as [Ile7]-surfactin with the C_{15}-*anteiso*-fatty-acid chain.

The ESI-MS spectra of compound **9** exhibit peak of the cationized molecule at *m/z* 1072 $[M + Na]^+$. Amino acid analysis shows the presence of four amino acid residues: Asp, Glu, Val, Leu (1 : 1 : 1 : 4). The ^{13}C NMR spectrum of compound **9** (in C_5D_5N) shows a signal at δ 51.4, while the ^1H NMR spectrum exhibits a three-proton singlet, which indicates the presence of a methoxy group in the molecule. The ESI-MS/MS-fragmentation of the ion with *m/z* 1072 on the simple cleavage and cleavage with the double hydrogen transfer corresponds to the fragmentation of the C_{15}-surfactin methyl ester, recently[15] isolated from the strain *Bacillus subtilis*. The signal for the methyl group at δ 11.5 indicates the *anteiso*-structure of the fatty acid. Thus, peptide **9** was identified as the surfactin [Leu7]-*O*-methyl ester with the C_{15}-*anteiso*-fatty-acid chain.

The structure of all surfactins obtained are given in Table 2.

Determination of cytotoxic activity of the isolated compounds

Cytotoxic activity of surfactins **4**—**6** and **8** in the concentration 100 μmol L^{-1} was studied with respect to two lines of tumor cells: the human intestine cancer HCT-116 (ATCC # CCL-247) and the human breast cancer MDA-MB-231 (ATCC # HTB-26) using MTS-reagent. Surfactins **4**—**6** exhibit weak effect on the HCT-116 cells growth after 72 h. At the same time, surfactin **8**, differing

Table 2. Structures of surfactins from the bacterium *B. pumilus* KMM 456

Surfactin	β-Hydroxy acid	Amino acid sequence	$[M + H]^+$
1	*anteiso*-C_{13}	N—Glu—Leu—Leu—Val—Asp—Leu—Leu—C	1008
2	*n*-C_{13}	N—Glu—Leu—Leu—Val—Asp—Leu—Leu—C	1008
3	*iso*-C_{14}	N—Glu—Leu—Leu—Val—Asp—Leu—Leu—C	1022
4	*n*-C_{14}	N—Glu—Leu—Leu—Val—Asp—Leu—Leu—C	1022
5	*anteiso*-C_{15}	N—Glu—Leu—Leu—Val—Asp—Leu—Leu—C	1036
6	*anteiso*-C_{15}	N—Glu—Leu—Leu—Val—Asp—Leu—Val—C	1022
7	*n*-C_{14}	N—Me—O—Glu—Leu—Leu—Val—Asp—Leu—Leu—C	1036
8	*anteiso*-C_{15}	N—Glu—Leu—Leu—Val—Asp—Leu—Ile—C	1036
9	*anteiso*-C_{15}	N—Me—O—Glu—Leu—Leu—Val—Asp—Leu—Leu—C	1050

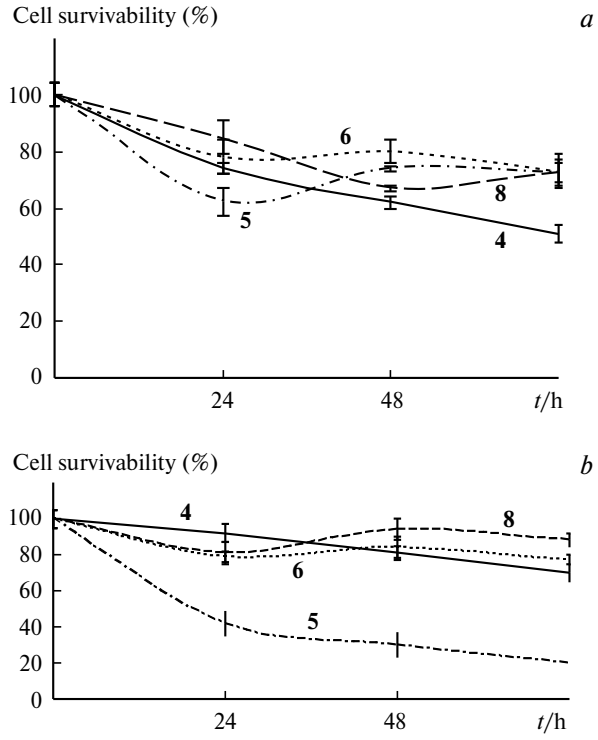

Fig. 4. Effect of surfactins **4**—**6** and **8** on the survival of cells MDA-MB-231 (*a*) and HST-116 (*b*).

from **5** and **6** by the replacement of the Leu7 and Val7 residues by Ile7, inhibits growth of these cells by 80%. Surfactins **5**, **6**, and **8** shows weak effect with respect to the MDA-MB-231 cells. Surfactin **4**, after 72 h showed inhibition of growth of these cells by 49% (Fig. 4).

The activity of surfactins **4**—**8** in the concentration 100 μmol L^{-1} with respect to formation and growth of the cells colonies MDA-MB-231 was studied also using the soft agar method (the cell neoplastic transformations). It was shown that surfactins **4**, **5**, **6**, **7**, and **8** inhibit growth of colonies of these cells by 20, 79, 57, 41, and 69%, respectively, as compared to the untreated cells (Fig. 5).

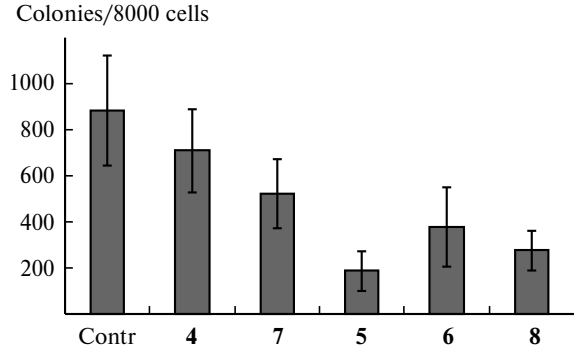

Fig. 5. Effect of surfactins **4**—**8** on the formation and growth of colonies of cells MDA-MB-231; Contr is control.

Experimental

Optical rotation was measured on a Perkin—Elmer 343 polarimeter (Germany). ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-700 spectrometer (700 and 175.6 MHz, respectively) using Me_4Si as an internal standard. Mass spectra ESI-TOF were obtained on an Agilent 6510 LC Q-TOF spectrometer (USA). The voltage on the capillary, nozzle, skimmer was 3500, 215, and 65 V, respectively, the impact energy was 35 V. Column chromatography was performed on silica gel L (40/100 μm, Chemapol, Czechoslovakia), HPLC was performed on an Agilent 1100 Series chromatograph (USA) using a Supelco Discovery C18 column (25 cm × 4.6 mm, 5 μm). Amino acid analysis was performed on a Biochrom-30 analyzer (England) using a Vetropac 8 μm resin (200 × 4.6 mm column) and the reaction with a ninhydrin reagent at 135 °C.

Cultivation of bacterium *B. pumilus* (KMM 456). The bacterium was isolated from the zoantharia *Palythoa* sp. gathered in the Vietnam waters (Ku-Lao-Re lake) at the depth of 2 m. Fermentation was performed on a shaker at 25 °C for 24 h in the medium of the following composition (*C*/g L^{-1}): K_2HPO_4, 0.07; NH_4Cl, 1.0; yeast extract, 5.0; tripton, 5.0; sodium citrate, 0.01; glucose, 5.0; 1 *M* Tris-buffer (pH 7.5), 20.0; artificial sea water, 200.0.

Isolation of compounds 1—9. The culture media (1.8 L) were centrifuged for 30 min at 5000 *g*. The cells obtained were suspended in DI water and destroyed by sonication. The suspension of the destroyed cells were thrice extracted with ethyl acetate. The combined extract was dried with Na_2SO_4, filtered, concentrated to dryness, and the residue (440 mg) was subjected to chromatography on a column with silica gel in the gradient system hexane—ethyl acetate (5 : 1, 3 : 1, 1 : 1, 1 : 4). The fraction eluted with the system 1 : 4 (215 mg) was subjected to HPLC on a Supelco Discovery C18 column (25 cm × 4.6 mm, 5 μm) in the system acetonitrile—0.01% aq. CF_3COOH (80 : 20) to obtain individual surfactins **1** (8 mg), **2** (2 mg), **3** (8 mg), **4** (10 mg), **5** (12 mg), **6** (3 mg), **7** (2 mg), **8** (2 mg), and **9** (2 mg) with the retention times 11.6, 12.5, 14.5, 15.7, 18.4, 20.2, 21.8, 24.0, and 25.4 min, respectively.

C_{14}-Surfactin methyl ester (7). Molecular formula $C_{53}H_{93}N_7O_{13}$, white powder, $[\alpha]^{20}_D$ −32.5 (*c* 0.5, MeOH).

ESI-HRMS, found: *m/z* 1058.6719 $[M + Na]^+$, calculated 1058.6729.

Total acid hydrolysis of surfactins. A depsipeptide (0.4 mg) in a glass tube was dissolved in HCl (5.6 *M*, 0.8 mL), evacuated, and heated for 24 h at 105 °C. The cooled reaction mixture was diluted with biDI water, concentrated *in vacuo*, and analyzed on the amino acid analyzer.

The absolute configurations of amino acids were determined by Marfey´s method.[11]

Culturing of cancer cells. The human intestine cancer cells HCT-116 (ATCC # CCL-247) and human breast cancer cells MDA-MB-231 (ATCC # HTB-26) were cultured in the McCoy 5a medium and DMEM, respectively, 10% FBS with addition of penicillin (100 units L^{-1}) and streptomycin (100 μg L^{-1}) in an MCO-18AIC incubator (Sanyo, Japan) at 37 °C, the content of CO_2 was 5%.

Determination of cytotoxicity of surfactins. The cells HCT-116 and MDA-MB-231 ($1.5 \cdot 10^5$ mL^{-1}) were dispersed in 96-well plates and cultured in 200 μL of 10% aq. McCoy 5a and DMEM, respectively in a CO_2-incubator at 37 °C for 24 h. Then the cells

were treated with surfactins (100 μmol L^{-1}) in the fresh medium and incubated for 24 h. After incubation, 5-(3-carboxymethoxyphenyl)-3-(4,5-dimethylthiazol-2-yl)-2-(4-sulfophenyl)-2*H*-tetrazolium (15 μL) was added into each well and incubated for 4 h (37 °C, 5% CO_2). Optical density was measured on a Bio-Tek Instruments spectrophotometer (USA) at two wavelengths (490 and 630 nm) and the ratio $A_{490/630}$ was determined (see Ref. 16).

Neoplastic transformation of cells (the soft agar method). The action of surfactins on the formation and growth of colonies of human breast cancer cells MDA-MB-231 were determined using method of soft agar. The cells MDA-MB-231 ($2.4 \cdot 10^4$ mL^{-1}) were treated with surfactins (100 μmol L^{-1}) in 1 mL of 0.33% aq. BME-agar containing 10% FBS over 3.5 mL of 0.5% aq. BME-agar containing 10% FBS and 100 μ*M* of surfactin. The cells were cultivated for 21 days at 37 °C, 5% CO_2. Colonies of human intestine cancer cells were evaluated using a Motic AE 20 reversed microscope (China) and the MoticImagePlus program.

Statistical processing of the data were performed using the Student *t*-criterion under conditions of a given confidence level equal to 95% (the SigmaPlot 2000 program, version 6 (SPSS Inc., USA)).

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