PUMILACIDIN†, A COMPLEX OF NEW ANTIVIRAL ANTIBIOTICS

PRODUCTION, ISOLATION, CHEMICAL PROPERTIES, STRUCTURE AND BIOLOGICAL ACTIVITY

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New antibiotic pumilacidins A, B, C, D, E, F and G were isolated from the culture broth of a strain of *Bacillus pumilus*. They are cyclic acylheptapeptide composed of a β-hydroxy fatty acid, two L-leucine, two D-leucine, L-glutamic acid, L-aspartic acid and L-isoleucine (or L-valine). Pumilacidin components were inhibitory to herpes simplex virus type 1 and H^+, K^+-ATPase and demonstrated antiulcer activity in rat.

In a systematic search for microbial metabolites effective against herpes simplex virus type 1 (HSV-1), a bacterial strain No. M937-B1 isolated from a soil sample collected near Lake Yamanaka, was found to produce a complex of new acylpeptide antibiotics, pumilacidin. The producing strain was identified as *Bacillus pumilus* by taxonomical studies. The antibiotic complex was extracted from the fermentation liquor with butanol and separated by chromatography into seven components, pumilacidins A, B, C, D, E, F and G. Their structures were determined by chemical and spectral studies to be cyclic acylheptapeptides differing from each other in their acyl side chain and/or amino acid composition. Pumilacidin exhibited antiviral activity against HSV-1-KOS strain and also inhibitory activity against hog gastric, H^+, K^+-ATPase. Antiulcer activity of pumilacidin B was demonstrated experimentally in rats.

Taxonomy

The producing organism, strain No. M937-B1, was isolated from *Leucobrynum neigherrense*, moss which grew on a rotten tree trunk near Lake Yamanaka, Yamanashi Prefecture, Japan. Strain M937-B1 is an aerobic, Gram-positive, spore-forming oblong bacterium, classified as belonging to the genus *Bacillus*. Strain M937-B1 was characterized by the methods of GORDON *et al.*,[1] and GIBSON and GORDON.[2] The taxonomic position was determined according to the descriptions of NORRIS *et al.*[3] The morphological, cultural and physiological characteristics (Table 1) indicated that strain No. M937-B1 is classified as *B. pumilus* Meyer and Gottheil, 1901.

Fermentation

The stock culture of *B. pumilus* strain No. M937-B1 was propagated on an agar slant at 28°C for 2 days. The agar slant medium consisted of soluble starch 0.5%, glucose 0.5%, fish meat extract 0.1%, yeast extract 0.1%, NZ-case 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.6% (pH 7.0).

† Pumilacidin was first called BU-3392V.

Table 1. Taxonomic characteristics of strain M937-B1.

Morphology:	Physiological characteristics[b]	Strain M937-B1	*Bacillus subtilis* PCI 219
Vegetative cells;			
Oblong or short straight rods			
$0.6 \sim 0.7 \times 1.0 \sim 3.1\ \mu m$			
Motile, Gram-positive and not acid-fast	Production of:		
Spores[a];	Acid from glucose	+	+
Elliptical, $0.6 \sim 0.8 \times 1.0 \sim 1.2\ \mu m$	mannitol	+	−
Not swollen sporangia	xylose	+(w)	−
Central position	arabinose	+(w)	+
Cultural characteristics:	Gas from glucose	−	−
Gross colonial morphology;	Acetoin (VP-reaction)	+	+
Nutrient agar: incubated at 28°C for 3 days	Catalase	+	+
Circular and umbonate, 2 to 3 mm i.d.,	Nitrate reductase	−	+
entire or somewhat irregular margin,	Hydrolysis of:		
opaque density and wrinkled surface	Starch	−	+
Scant viscosity, pale yellow color, no swarming	Casein	+	+(w)
	Growth in:		
	Anaerobic agar	−	−
	7% NaCl	+	+
	50°C	+	+
	65°C	−	−
	0.001% lysozyme	−	+(w)
	pH 6 in V-P medium	+	+

[a] The endospores were not born in Difco nutrient agar but born when $MnSO_4 \cdot H_2O$ was supplemented at 0.0005% (w/v) as described by NORRIS *et al.*[3]

[b] +: Positive, −: negative, +(w): weakly positive.

A well-matured agar slant was used to inoculate five 500-ml Erlenmeyer flasks each containing 100 ml of vegetative medium composed of soybean meal 3%, glucose 3%, Pharmamedia 0.5%, yeast extract 0.1% and $CaCO_3$ 0.3%, the pH being adjusted to 7.0 before sterilization. The flasks were incubated at 28°C for 24 hours on a rotary shaker with shaking at 200 rpm and the resultant culture was transferred into a 20-liter stir jar fermentor containing 12 liters of production medium having the same composition as the vegetative medium.

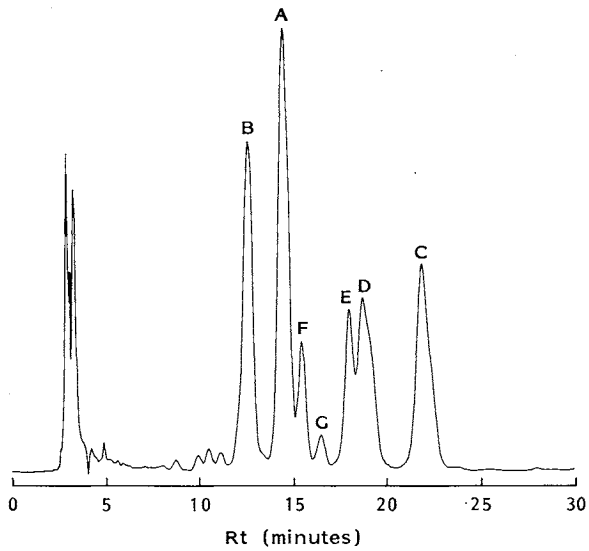
The fermentation was carried out at 28°C for 93 hours with stirring at 250 rpm and aeration at 12 liters per minute. The antiviral activity in the fermentation broth was determined by the conventional plaque reduction assay against HSV-1 (KOS strain).

Isolation and Purification of Pumilacidin Complex

Extraction and Purification of Pumilacidin Complex

The harvested broth (245 liters) was adjusted to pH 4.0 with 6 N HCl and extracted with butanol (145 liters). The organic layer was separated with a Sharpless centrifuge (Kokusan Seiko Co., No. 4A) and concentrated to 6 liters under reduced pressure. This was added dropwise to 15 liters of ethyl acetate and the precipitate formed was filtered off. The filtrate was evaporated *in vacuo* to an oil (*ca.* 660 g) which was adsorbed batchwise on non-ionic porous polymer resin, Diaion HP-20 (Mitsubishi Chemical Industries Limited, 3 liters). The resin was packed in a column (70 i.d. × 1,000 mm) and washed with water and 50% aqueous methanol (5 liters each). Subsequent elution with 80% aqueous acetone (20 liters) gave, after evaporation *in vacuo*, an active viscous oil (185 g). One third of the crude oil was charged on a column of Silica gel 60 (E. Merck, Darmstadt, No. 9385, 40 i.d. × 250 mm) which was developed with methylene chloride - methanol - 28% ammonia (first 90 : 10 : 1 and then 80 : 20 : 1). The eluate was collected in fractions

Fig. 1. HPLC of pumilacidins A to G.



Column: YMC-Pack R-ODS-5 (4.6 i.d. × 250 mm, 5 μm), mobile phase: 2-propanol - acetonitrile - H_2O (14 : 6 : 5) containing 0.1% TFA, flow rate: 1 ml/minute, detection: UV absorption at 210 nm.

and examined by TLC (chloroform - methanol - acetic acid, 18 : 2 : 1, Rf 0.68). The appropriate fractions were pooled, concentrated *in vacuo* and freeze-dried to yield a crude yellow powder (6.9 g). The remaining crude oil was chromatographed in a similar manner and the combined active solid (total 20.7 g) was applied on a column of Wakogel C-300 (Wako Pure Chemical Industries, 40 i.d. × 650 mm), which was washed with methylene chloride (3 liters) and then eluted stepwise with methylene chloride - methanol (49 : 1, 19 : 1 and 9:1). After TLC examination, the fractions containing pumilacidin were pooled, concentrated *in vacuo* to give a pale yellow powder of semi-pure pumilacidin (16.8 g). This powder was subjected to column chromatography on Silica gel 60 (40 i.d. × 300 mm). After washing with methylene chloride - acetic acid (100 : 1), elution was carried out with methylene chloride - methanol - acetic acid (99 : 1 : 1 and 98 : 2 : 1). The fractions which showed a single spot by TLC were pooled, concentrated *in vacuo* and freeze-dried to yield a white powder of pumilacidin complex (12.5 g). This solid was shown to be a mixture of seven components by HPLC analysis (Fig. 1).

Separation of Pumilacidin Components

The pumilacidin complex obtained above was applied to a column of LiChroprep RP-18 (E. Merck, 22 i.d. × 700 mm) which had been equilibrated with acetonitrile - 0.01 M phosphate buffer pH 7.0 (3 : 7). Elution was carried out with the same solvent mixture with a ratio at first of 2 : 3 and then 1 : 1. Pumilacidin B was eluted first followed by pumilacidins A, F, G, E, D and C in that order. By HPLC examination, the relevant fractions were pooled and evaporated to remove acetonitrile and the resultant aqueous solution was desalted by use of Diaion HP-20 chromatography. Repetition of the chromatographic separation three times starting with 3.7 g each of the pumilacidin complex yielded pumilacidins B (1.5 g), A and B mixture (2.14 g), A (2.5 g), A and F mixture (84 mg), A, F, G, E and D mixture (1.66 g), E and D mixture (346 mg), E, D and C mixture (2.36 g) and C (159 mg). Isolation of pumilacidins D, E, F and G was achieved by preparative HPLC (Gilson Model 303 system with semi-preparative D-ODS-5 column, mobile phase;

2-propanol - acetonitrile - water (14 : 6 : 5) containing 0.1% trifluoroacetic acid) of the appropriate samples obtained above. The appropriate eluates were pooled, concentrated *in vacuo*, and chromatographed on Sephadex LH-20 (Pharmacia Fine Chemicals, 22 i.d. × 700 mm, methanol as solvent). From the pumilacidins D and E mixture (136 mg), a homogeneous amorphous powder of pumilacidin D (49 mg) was obtained. The complex of pumilacidins A, F, G, E and D (509 mg) was repeatedly purified by preparative HPLC to yield an amorphous solid of pure pumilacidins E (54 mg), F (27 mg) and G (20 mg). As will be discussed in the structure determination, pumilacidins E, F and G are homogeneous compounds, while pumilacidins A, B, C and D are all inseparable mixtures of two subcomponents.

Physico-chemical Properties

Pumilacidin components were isolated as amorphous white powders. As shown in Table 2, they showed similar physico-chemical properties. All seven components are soluble in lower alcohols, chloroform, acetone, ethyl acetate and dimethyl sulfoxide, but insoluble in hexane and water. They gave positive response to iodine vapor and H_2SO_4, but negative response to ninhydrin and anthrone reagents. The molecular formula of each component was assigned based on its mass spectrum and elemental analysis. Pumilacidin components showed only end absorption in the UV spectra. The IR spectra of pumilacidins A and B (Figs. 2 and 3) showed intense amide carbonyl bands at 1655 and 1540 cm^{-1}, and an ester carbonyl band at 1730 cm^{-1}. A peptide structure was indicated by the ^1H NMR spectrum of pumilacidin A in DMSO-d_6 (Fig. 4), which showed the presence of amide NH signals between δ 7.6 and 8.3. The spectrum also showed a broad signal at δ 12.2 assignable to two carboxylic acid protons and several aliphatic methyl and methylene signals at around δ 0.7 and 2.0. The amino acid composition of pumilacidin components was determined by amino acid analysis as shown in Table 2.

Structural Studies

The constituent amino acids of pumilacidin were isolated by acid hydrolysis followed by cation exchange resin chromatography on Dowex 50WX8 (Dow Chemical Co.). Their configurations were determined as shown in Table 2 from their specific optical rotational values coupled with HPLC analysis

Fig. 2. IR spectrum of pumilacidin A (in KBr).

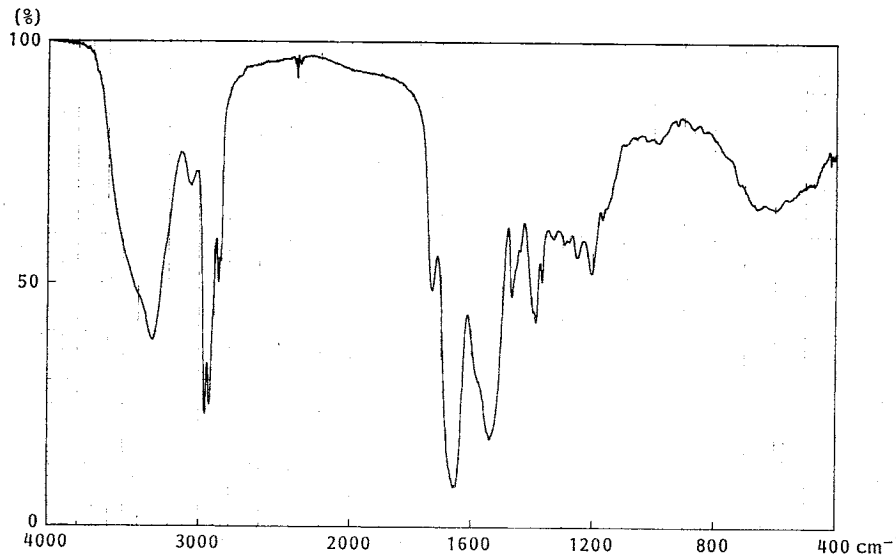


Fig. 3. IR spectrum of pumilacidin B (in KBr).

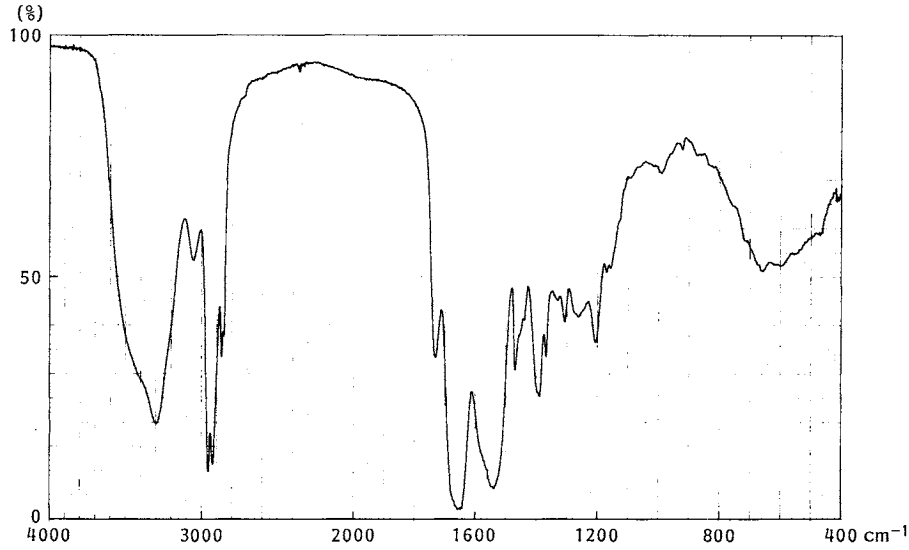
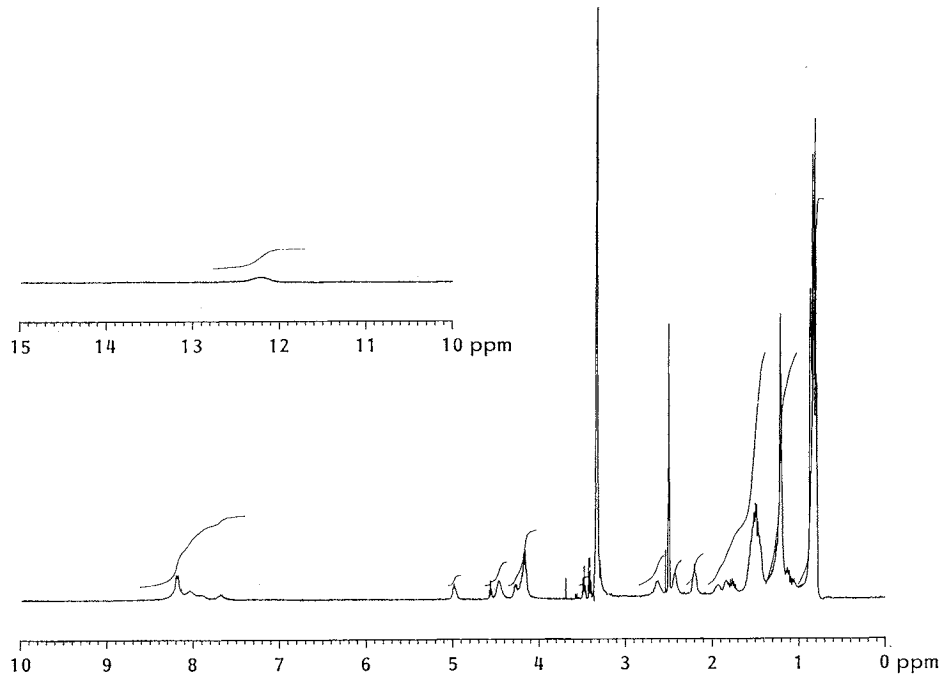


Fig. 4. ^{1}H NMR spectrum of pumilacidin A (400 MHz, in DMSO-d_6).



using chiral recognition column (Enantio L1, Tosoh Manufacturing Co., Ltd., 4.6 i.d. × 150 mm, 1 mM $CuSO_4$ as mobile phase). The fatty acids of pumilacidin components were characterized as their methyl esters obtained by acid methanolysis of each component. The GC mass and ^{1}H and ^{13}C NMR spectral analysis revealed that both pumilacidins A and B contained 3-hydroxy-13-methyltetradecanoic acid or

Table 2. Physico-chemical properties of pumilacidin components.

	A	B	C	D
Nature	White powder	White powder	White powder	White powder
MP (°C)	146~150	160~163	134.5~138	137~142
$[\alpha]_D^{23}$ (MeOH)	−12.2°(*c* 0.5)	−16.9° (*c* 1)	−14.0° (*c* 1)	−16.6° (*c* 0.5)
SI-MS (*m/z*)	1,073 $(M+Na)^+$, 1,089 $(M+K)^+$	1,059 $(M+Na)^+$, 1,075 $(M+K)^+$	1,101 $(M+Na)^+$, 1,117 $(M+K)^+$	1,087 $(M+Na)^+$, 1,103 $(M+K)^+$
Molecular formula	$C_{54}H_{95}N_7O_{13}\cdot H_2O$	$C_{53}H_{93}N_7O_{13}\cdot 3H_2O$	$C_{56}H_{99}N_7O_{13}\cdot H_2O$	$C_{55}H_{97}N_7O_{13}\cdot 2H_2O$
Calcd C	60.71	58.38	61.34	60.03
H	9.15	9.15	9.28	9.25
N	9.18	8.99	8.94	8.91
Found C	60.68	58.34	61.30	60.48
H	9.03	8.67	9.16	9.04
N	9.14	8.79	8.87	8.82
Amino acid composition	L-Asp (1), L-Glu (1), L-Leu (2), D-Leu (2), L-Ile (1)	L-Asp (1), L-Glu (1), L-Leu (2), D-Leu (2), L-Val (1)	L-Asp (1), L-Glu (1), L-Leu (2), D-Leu (2), L-Ile (1)	L-Asp (1), L-Glu (1), L-Leu (2), D-Leu (2), L-Val (1)

	E	F	G
Nature	White powder	White powder	White powder
MP (°C)	137~140.5	139~143	134~138
$[\alpha]_D^{23}$ (MeOH)	−15.2° (*c* 0.5)	−20.1° (*c* 0.38)	−19.5° (*c* 0.3)
SI-MS (*m/z*)	1,087 $(M+Na)^+$, 1,103 $(M+K)^+$	1,073 $(M+Na)^+$, 1,089 $(M+K)^+$	1,073 $(M+Na)^+$, 1,089 $(M+K)^+$
Molecular formula	$C_{55}H_{97}N_7O_{13}\cdot 2H_2O$	$C_{54}H_{95}N_7O_{13}\cdot 2H_2O$	$C_{54}H_{95}N_7O_{13}\cdot H_2O$
Calcd C	60.03	59.70	60.71
H	9.25	9.18	9.15
N	8.91	9.02	9.18
Found C	60.44	59.98	60.65
H	9.04	8.97	9.07
N	8.79	8.85	8.73
Amino acid composition	L-Asp (1), L-Glu (1), L-Leu (2), D-Leu (2), L-Ile (1)	L-Asp (1), L-Glu (1), L-Leu (2), D-Leu (2), L-Val (1)	L-Asp (1), L-Glu (1), L-Leu (2), D-Leu (2), L-Val (1)

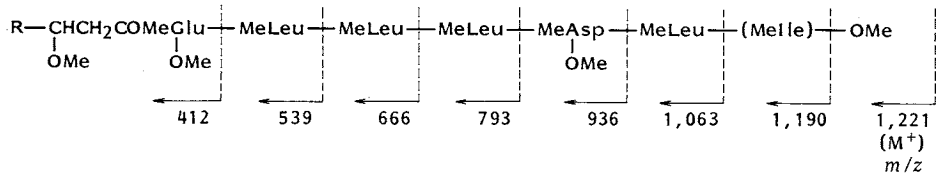
3-hydroxy-12-methyltetradecanoic acid (ratio=*ca.* 3:7), pumilacidins C and D 3-hydroxy-15-methylhexadecanoic acid or 3-hydroxy-14-methylhexadecanoic acid (ratio=*ca.* 3:7), pumilacidins E and F 3-hydroxy-14-methylpentadecanoic acid, and pumilacidin G 3-hydroxyhexadecanoic acid.

Upon alkaline hydrolysis with a mixture of 0.5 N NaOH and methanol (1:1) at room temperature for 18 hours, pumilacidins A and B were cleaved at the lactone linkage to yield the bio-inactive linear hydroxyacid peptide and its dehydrated α,β-unsaturated acid peptide derivatives. Cleavage with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in dry toluene[4] gave only the latter derivative. These derivatives were permethylated by HAKOMORI's method,[5] and the resulting volatile permethylates were analyzed by in-beam EI-MS. Successive fragmentations due to successive loss of the fatty acid and amino acids revealed the order of all the linkages of pumilacidin B but the position of isoleucine in pumilacidin A was not clarified (Fig. 5).

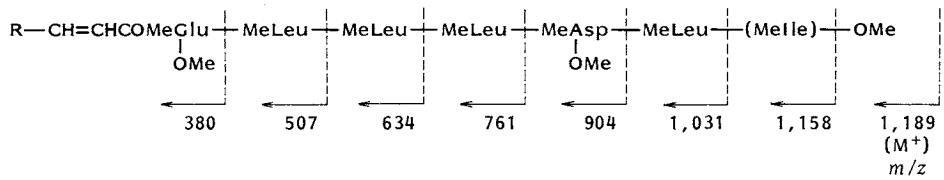
The α,β-unsaturated acid derivative obtained from pumilacidin A was digested with papain to give a hexapeptide and an acylglutamic acid. Upon stepwise Edman degradation, the amino acid sequence of the hexapeptide was determined to be H-Leu-Leu-Leu-Asp-Leu-Ile-OH. In each degradation step, the peptide fragments were acid-hydrolyzed and treated with D-amino acid oxidase for determination of the

Fig. 5. Mass fragmentation of permethylated derivatives.

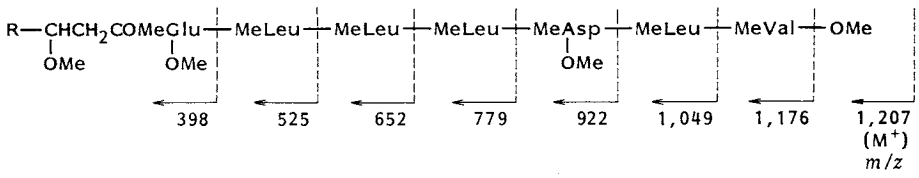
Hydroxyacid peptide of pumilacidin A:



Dehydrated acid peptide of pumilacidin A:



Hydroxyacid peptide of pumilacidin B:



Dehydrated acid peptide of pumilacidin B:

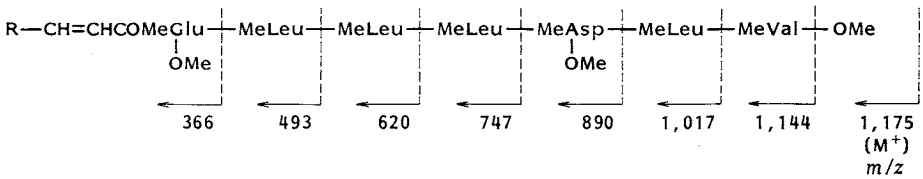


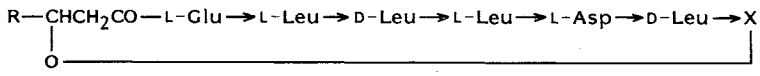
Table 3. Amino acid analysis after digestion by D-amino acid oxidase.[a]

Peptides	Analyzed ratio					Theoretical ratio				
	Asp	Glu	Val	Ile	Leu	Asp	Glu	Val	Ile	Leu
Pumilacidin A:	0.70	1.11		1	2.36	1	1		1	2
L-Leu-D-Leu-L-Leu-Asp-D-Leu-Ile	0.59			1	1.94	1			1	2
D-Leu-L-Leu-Asp-D-Leu-Ile	0.72			1	1.07	1			1	1
L-Leu-Asp-D-Leu-Ile	0.59			1	1.12	1			1	1
Asp-D-Leu-Ile	0.84			1	0.13	1			1	0
D-Leu-Ile			NT						1	0
Pumilacidin B:	0.76	1.21	1		2.34	1	1	1		2
L-Leu-D-Leu-L-Leu-Asp-D-Leu-Val	0.91		1		2.11	1		1		2
D-Leu-L-Leu-Asp-D-Leu-Val	0.76		1		1.21	1		1		1
L-Leu-Asp-D-Leu-Val	0.82		1		1.16	1		1		1
Asp-D-Leu-Val	1.01		1		0.05	1		1		0
D-Leu-Val			1		0.04			1		0

[a] Sigma Type X, in 0.05 M phosphate buffer (pH 8.0), 37°C, 2 days in a reaction tube for PICO TAG amino acid analysis.

NT: Not tested.

Fig. 6. Total structures of pumilacidin components.



A R=$CH_3CH(CH_3)(CH_2)_9$- or $CH_3CH_2CH(CH_3)(CH_2)_8$-
X=L-Ile

B R=$CH_3CH(CH_3)(CH_2)_9$- or $CH_3CH_2CH(CH_3)(CH_2)_8$-
X=L-Val

C R=$CH_3CH(CH_3)(CH_2)_{11}$- or $CH_3CH_2CH(CH_3)(CH_2)_{10}$-
X=L-Ile

D R=$CH_3CH(CH_3)(CH_2)_{11}$- or $CH_3CH_2CH(CH_3)(CH_2)_{10}$-
X=L-Val

E R=$CH_3CH(CH_3)(CH_2)_{10}$-
X=L-Ile

F R=$CH_3CH(CH_3)(CH_2)_{10}$-
X=L-Val

G R=$CH_3(CH_2)_{12}$-
X=L-Val

position of the D-leucine.[6] The degradation results were analyzed as shown in Table 3, which allowed us to assign the complete amino acid sequence of pumilacidin A including two enantiomeric sets of leucines. The amino acid and fatty acid sequence of pumilacidin B was similarly elucidated (Fig. 5 and Table 3). Participation of the α-carboxyl groups of aspartic acid and glutamic acid in peptide linkage was also demonstrated through these studies.

To clarify the location of the lactone linkage, intact pumilacidins A and B and their alkaline hydrolysis products were reduced with $LiBH_4$ in THF and the amino acid composition of the products was analyzed. The reduction products of pumilacidins A and B were found to lack isoleucine and valine residues, respectively, by amino acid analysis. The linear peptides of pumilacidins A and B afforded the same amino acid composition as the original antibiotics. Based on these results, it was concluded that the lactone linkage was between carboxyl group of *C*-terminal isoleucine (pumilacidin A) or valine (pumilacidin B) and the β-hydroxy group of the fatty acid moiety. The total structures of pumilacidins A and B were thus established as shown in Fig. 6 and those of other components were determined in analogous fashion.

Biological Activity

Antiviral Activity

The antiviral activity of pumilacidins A and B was assessed by plaque reduction and dye uptake assays[7] using HSV-1-Vero cell system. Aliquots (50 μl each) of medium containing various concentrations of the test compounds were poured into wells of a 96-well microplate and 200 μl of the cell suspension containing 2×10^4 cells was added. To each well, 50 μl of medium containing approximately $30 \times 50\%$

Table 4. Antiviral activity against herpes simplex virus type 1.

	Dye uptake assay		Plaque reduction assay	
	ID_{50} (μg/ml)	TD_{50}	ID_{50} (μg/ml)	TD_{50}
Pumilacidin A	6.7	16.5	3.8	25
Pumilacidin B	5.2	17.0	4.0	25
Acyclovir	0.2	>100	1.1	>100

Virus: HSV-1-KOS strain, cells: Vero cells, medium: EAGLE's minimum essential medium containing 5% fetal bovine serum.

Table 5. Inhibitory effect of pumilacidins A and B and reference compounds on the hog gastric H^+, K^+-ATPase.

Compound	IC_{50} (μg/ml)
Pumilacidin A	2.7
Pumilacidin B	3.2
Colistin	>200
Amphomycin	>200
Aspartocin	147
Polypeptin	26.7
Omeprazole	12.3
SCH 28080	11.5

tissue culture infectious dose of virus was added. For cytotoxicity test, a similar set of wells without added virus solution was prepared. After 72 hours incubation, the degree of inhibition of viral-induced cytopathic effect and drug-induced cytotoxicity were determined. The ID_{50} was expressed as the concentration showing 50% inhibition of cytopathic effect of the control and 50% toxic dose (TD_{50}) was the concentration inhibiting 50% of the control growth of Vero cells without virus. Acyclovir was used as the reference compound in the assay; the results are shown in Table 4. Pumilacidins A and B exhibited antiviral activity against HSV-1 with ID_{50} values of 6.7 and 5.2 μg/ml in the dye uptake assay, respectively. By plaque reduction assay, ID_{50} values of pumilacidins A and B were 3.8 and 4.0 μg/ml, respectively.

Antimicrobial Activity

Pumilacidin did not show inhibitory activity against bacteria and fungi at 1,000 μg/ml by paper-disk assay.

H^+, K^+-ATPase Inhibitory Activity

Gastric H^+, K^+-ATPase was prepared from hog stomachs according to the method of G. SACCOMANI *et al.*[8] with a minor modification. Since the gastric H^+, K^+-ATPase is known to hydrolyze *p*-nitrophenylphosphate (*p*NPP) as well as ATP, *p*NPP was used as a substrate in this experiment. A solution (215 μl) of the enzyme and test compound in 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM $MgCl_2$ and 100 mM KCl was added into a well of a 96-well microplate and pre-incubated for 3 hours at 37°C. The reaction was initiated by addition of 10 μl of 100 mM *p*NPP. After 30 minutes-incubation at 37°C, 20 μl of 1 M NaH_2PO_4 - 200 mM EDTA solution was added and the amount of released *p*-nitrophenol was determined colorimetrically with a microplate autoreader (Titertek Multiskan MCC) at 405 nm. Four known acylpeptide antibiotics and two known H^+, K^+-ATPase inhibitors, omeprazole and SCH 28080, were comparatively tested with the results shown in Table 5. Both pumilacidins A and B inhibited hog gastric H^+, K^+-ATPase in a concentration-dependent manner with IC_{50} values of 2.7 and 3.2 μg/ml, respectively. They showed much more potent inhibitory activity than the known acylpeptide antibiotics tested. It should be noted that pumilacidins A and B were approximately 4 times more potent than either omeprazole and SCH 28080 in our test.

Protection against Gastric Ulcer Formation

The anti-gastric ulcer activity of pumilacidin B was evaluated in the Shay rat model. Male Wistar

rats weighing approximately 200 g were fasted for 24 hours before the experiment. Under ether anesthesia the abdomen of the rat was incised and the pylorus ligated. Ten hours later, the animals were killed and the forestomach was examined for ulcer formation. The test compound was given subcutaneously in a volume of 0.1 ml/100 g body weight soon after pylorus ligation. One ml of 1% Brilliant Blue 6B was given intravenously 10 minutes before killing the animal to stain the ulcer area. The number of ulcers was counted and arbitrarily classified into an ulcer index from 0 to 5. All control rats subjected to pylorus ligation had gastric ulcers of ulcer index 5 in the forestomach. As shown in Table 6, Shay ulcers were significantly inhibited (68%) when 100 mg/kg of pumilacidin B was administered.

Table 6. Protective effect of pumilacidin B on Shay ulcers in rats.

Compound	Subcutaneous dose (mg/kg)	Ulcer index (mean ± SE)	inhibition (%)
Control	—	5.0 ± 0	—
Pumilacidin B	100	1.6 ± 0.93[a]	68
	50	4.4 ± 0.24	12

[a] $P<0.01$.

Discussion

A complex of novel acylpeptide antibiotic pumilacidin was isolated from *B. pumilus* No. M937-B1. They are cyclic depsipeptides with a heptapeptide cyclized through a β-hydroxy fatty acid. Separation efforts led to the isolation of seven components although the structural studies revealed that pumilacidins A, B, C and D were still mixtures of two subcomponents with isomeric fatty acids. Pumilacidins A, C and E contain L-isoleucine at the *C*-terminal, while pumilacidins B, D, F and G have L-valine at this position.

Three structurally related antibiotics, surfactin,[9,10] acylpeptides[11] and esperin[12] have been reported as the metabolites of *Bacillus* strains. Surfactin was shown to possess potent surfactant activity, inhibitory effect on the thrombin-fibrinogen system and cytolytic activity against Ehrlich carcinoma cells. Esperin was reported to be an antibacterial antibiotic, and acylpeptides an inhibitor of adenosine-3′,5′-monophosphate diesterase. Pumilacidin screened as an antiviral antibiotic active against herpes simplex virus was found to have inhibitory activity against gastric H^+, K^+-ATPase and to be protective against gastric ulcers *in vivo*. The above observations show that this type of acylpeptide has various biological activities. It should be noted that the linear peptides prepared by lactone ring opening are biologically inactive.

Experimental

General

TLC was carried out on precoated Silica gel 60 plates (E. Merck, Darmstadt, No. 5715) and HPLC on Gilson Model 303 system under the conditions as described. The IR spectra were determined on a Jasco IR-810 and the UV spectra on a UVIDEC-610C spectrometer. The ^1H and ^{13}C NMR spectra were recorded on a Jeol JNM-GX 400. The ordinary mass spectra were obtained with a Hitachi RMU-6MG mass spectrometer modified with an in-beam electron impact system and the SI-MS with Hitachi M-80B (Xenon, 8KeV). The GC and the GC-MS were run with a Shimadzu GC-4BP-T and with a Jeol JMS-DX-300 (EI 70 eV), respectively. Optical rotations were determined with a Jasco model DIP 140. Amino acid analysis were carried out with a Hitachi 034-2U amino acid analyzer on PICO TAG amino acid analysis system (Waters Associates, Inc.).

Complete Acid Hydrolysis of Pumilacidins A and B Mixture

A mixture of pumilacidins A and B (1.24 g) was hydrolyzed with 6 N HCl (50 ml) at 110°C for 16 hours in a sealed tube. The hydrolysate was washed with diethyl ether and concentrated to dryness *in vacuo*. The sticky residue was dissolved in water and applied on a column of Dowex 50WX8 (H^+) which was developed with aqueous HCl. The eluate was monitored by the TLC and the fractions containing homogeneous amino acid were pooled and concentrated. Aspartic acid (125 mg) and glutamic acid (85 mg)

Table 7. GC and GC-MS analysis of fatty acid methyl esters from pumilacidins.

Fatty acid methyl esters isolated from	GC[a]	GC-MS (m/z)[b]		
	Rt (minutes)	M^+	Base peak	Diagnostic peaks
Pumilacidin A	12.9	272	103	254, 241, 199, 74
Pumilacidin B	12.9	272	103	254, 241, 199, 74
Pumilacidin C	16.6	300	103	282, 269, 227, 170, 74
Pumilacidin D	16.6	300	103	282, 269, 227, 170, 74
Pumilacidin E	14.5	286	103	268, 213, 74
Pumilacidin F	14.5	286	103	268, 213, 74
Pumilacidin G	15.3	286	103	268, 213, 74

[a] OV-17.3%, He, temperature programing 5°C/minute from 140°C.
[b] OV-17.3%, He, temperature programing 8°C/minute from 140°C.

were eluted with 0.2 N HCl, valine (61 mg) with 0.3 N HCl, and a mixture of isoleucine and leucine (550 mg) with 0.4 N HCl.

Aspartic acid, $[\alpha]_D^{27}$ +20.7° (*c* 2.2, 5 N HCl); glutamic acid, $[\alpha]_D^{27}$ +25.6° (*c* 1.5, 5 N HCl); valine, $[\alpha]_D^{26}$ +24.3° (*c* 1.15, 5 N HCl).

The chirality of isoleucine and leucine was determined by HPLC using a chiral recognition column, TSKgel Enantio L1 (Tosoh Manufacturing Co., Ltd., 4.6 i.d. × 150 mm, 1 mM $CuSO_4$ as mobile phase, flow rate 1 ml/minute, detection 254 nm, temperature 50°C). The leucine showed two equimoler ratio peaks corresponding to L-leucine (Rt, 6.6 minutes) and D-leucine (7.0 minutes). Isoleucine showed a single peak at 7.1 minutes which was attributed to L-enantiomer (*cf.* D-isoleucine, Rt 6.2 minutes).

Isolation of Fatty Acids from Pumilacidin Components

Each of pumilacidin components (5 mg) was heated with 2.5 N anhydrous methanolic HCl (1 ml) at 90°C for 16 hours in a sealed tube. After evaporation of MeOH in argon stream, the oily residue was dissolved in diethyl ether (2 ml), washed with water (1 ml) and dried over anhydrous sodium sulfate. Evaporation of the solvent in argon stream gave the methyl ester of the constituent fatty acid which was analyzed by GC and GC-MS (Table 7).

By a similar hydrolysis, larger amounts of the fatty acid methyl esters of pumilacidins A (9 mg), C (5 mg), E (2 mg) and G (1 mg) were prepared starting from pumilacidins A (194 mg), C (100 mg), E (30 mg) and G (10 mg), respectively. The ^{1}H NMR of the fatty acid methyl esters of pumilacidin A; methyl 3-hydroxy-13-methyltetradecanoate, δ 0.86 (6H, d, J=6.9 Hz), 2.41 (1H, dd, J=15.0 and 9.3 Hz), 2.52 (1H, dd, J=15.0 and 3.2 Hz), 3.71 (3H, s), 3.99 (1H, m) and methyl 3-hydroxy-12-methyltetradecanoate, δ 0.84 (3H, t, J=6.9 Hz), 0.86 (3H, d, J=6.9 Hz), 2.41 (1H, dd, J=15.0 and 9.3 Hz), 2.52 (1H, dd, J=15.0 and 3.2 Hz), 3.71 (3H, s), 3.99 (1H, m).

The ^{1}H NMR of the fatty acid methyl esters of pumilacidin C; methyl 3-hydroxy-15-methylhexadecanoate, δ 0.86 (6H, d, J=6.9 Hz), 2.41 (1H, dd, J=15.0 and 8.9 Hz), 2.52 (1H, dd, J=15.0 and 3.2 Hz), 3.71 (3H, s), 4.00 (1H, m) and methyl 3-hydroxy-14-methylhexadecanoate, δ 0.84 (3H, t, J=6.9 Hz), 0.86 (3H, d, J=6.9 Hz), 2.41(1H, dd, J=15.0 and 8.9 Hz), 2.52 (1H, dd, J=15.0 and 3.2 Hz), 3.71 (3H, s), 4.00 (1H, m).

The ^{1}H NMR of the fatty acid methyl ester of pumilacidin E; methyl 3-hydroxy-14-methylpentadecanoate, δ 0.86 (6H, d, J=6.6 Hz), 2.40 (1H, dd, J=15.0 and 9.2 Hz), 2.52 (1H, dd, J=15.0 and 2.9 Hz), 3.71 (3H, s), 3.99 (1H, m).

The ^{1}H NMR of the fatty acid methyl ester of pumilacidin G; methyl 3-hydroxyhexadecanoate, δ 0.88 (3H, t, J=7.0 Hz), 2.41 (1H, dd, J=15.0 and 9.2 Hz), 2.52 (1H, dd, J=15.0 and 2.9 Hz), 3.71 (3H, s), 4.00 (1H, m).

Cleavage of Lactone Linkage of Pumilacidins A and B

Pumilacidin A (100 mg) was dissolved in a mixture of 0.5 N NaOH (6 ml) and MeOH (6 ml) and the solution was stirred for 18 hours at room temperature. The mixture was then poured into water (100 ml),

neutralized with 1 N HCl, and chromatographed on Diaion HP-20 for desalting. The hydrolysis products which were eluted with 80% aqueous acetone were purified by silica gel chromatography (Wakogel C-300 20 i.d. × 300 mm). Upon a stepwise elution with a mixture of $CHCl_3$ - MeOH - AcOH (98 : 2 : 1 and 90 : 10 : 1), the linear hydroxyacid peptide (35 mg) and its dehydrated α,β-unsaturated acid peptide (48 mg) were obtained as white amorphous powder after evaporation of the appropriate fractions. The hydroxyacid derivative of pumilacidin A, UV λ_{max}^{MeOH} nm 204 (ε 11,700); the dehydrated acid derivative, UV λ_{max}^{MeOH} nm 207 (ε 32,700). In a similar way, the linear hydroxyacid peptide (93 mg) and its dehydrated derivative (96 mg) of pumilacidin B were obtained from pumilacidin B (200 mg). Pumilacidin A (150 mg) was heated at 100°C with DBU, 3 ml in dry toluene (15 ml) for 3 hours. After cooling to room temperature, EtOAc (100 ml) was added to the reaction mixture and the solution was washed with 1 N HCl and water. The solution was then concentrated *in vacuo* and the residue chromatographed on silica gel by a similar solvent system as above. The homogeneous dehydrated acid peptide (79 mg) and some starting material were obtained from the chromatography.

Permethylation of the Linear Peptide Derivatives of Pumilacidins A and B

To a solution of sodium dimethylsulfinyl anion which was prepared from NaH (100 mg of 50% oil suspension) and DMSO (3 ml) the linear peptide derivative of pumilacidin A (11 mg) and CH_3I (1 ml) were added and stirred in N_2 stream for 1 hour at room temperature. The reaction mixture was poured into water (50 ml) and the permethylated product was extracted with $CHCl_3$. After the extract was concentrated *in vacuo*, the residue was subjected to preparative TLC ($CHCl_3$ - MeOH - AcOH, 18 : 2 : 1). Single product (3 mg) was obtained by elution of around Rf 0.85 and analyzed by the in-beam EI-MS. The dehydrated linear peptide of pumilacidin A and two peptides of pumilacidin B were permethylated in a similar manner.

Enzyme Hydrolysis of the Dehydrated Linear Peptide of Pumilacidins A and B

The dehydrated form of the linear peptide of pumilacidin A (75 mg) was suspended in a mixture of DMSO (1.2 ml) and water (33 ml). Papain (Sigma Type IV, No. P-4762, 70 mg) was added to the suspension and the suspension stored at 37°C for 9 days. The reaction mixture was then concentrated and the residue was chromatographed on silica gel. By elution with a mixture of $CHCl_3$ - MeOH - AcOH (98 : 2 : 1), a mixture of the starting material and acylglutamic acid (45 mg) was obtained. Following elution with a 90 : 10 : 1 mixture afforded a ninhydrin-positive hexapeptide (22 mg). Separation of acylglutamic acid and the starting material was performed by preparative TLC ($CHCl_3$ - MeOH - AcOH, 18 : 2 : 1) and the dehydrated linear peptide (18 mg, Rf 0.59) and the acylglutamic acid (23 mg, Rf 0.32) were obtained. The corresponding linear peptide derivative of pumilacidin B (75 mg) was hydrolyzed in a similar manner to afford the hexapeptide (18 mg), the acylglutamic acid (23 mg) and the starting material (17 mg).

Edman Degradation of Hexapeptides of Pumilacidins A and B

Phenyl isothiocyanate (0.2 ml) was added to a solution of the hexapeptide (10 mg) dissolved in a mixture of EtOH (1.4 ml), water (0.2 ml) and triethylamine (0.2 ml). The reaction mixture was kept at 40°C for 1 hour under N_2 atmosphere and then the solvent was removed *in vacuo*. After addition of water (1 ml) to the residue, excess phenyl isothiocyanate was removed by extraction with benzene (1 ml × 3). The aqueous layer was concentrated to a dried residue which was taken up in 0.2 ml of TFA. The solution was warmed at 40°C for 40 minutes under N_2 atmosphere and evaporated to remove TFA. The residue was dissolved in 1 ml of 0.1 N HCl and the solution extracted with three 1 ml-portions of diethyl ether. The ethereal extract was concentrated and the residue was treated with 1 N HCl at 80°C for 10 minutes to afford the phenylthiohydantoin (PTH) amino acid. The aqueous layer was lyophilized and the resulting peptide was used for the next cycle of Edman degradation and for digestion with D-amino acid oxidase. A part of the peptide fragment was hydrolyzed in a reaction tube of PICO TAG system (150°C, 1 hour). After washing with triethylamine, D-amino acid oxidase (Sigma Type X, No. A-5418, 5 mg/ml in 0.05 M phosphate buffer, pH 8.0) was added to the residue and kept 37°C for 2 days. The amino acid composition of this reaction products was also analyzed by the above PICO TAG method.

The PTH amino acid obtained in each cycle of the Edman degradation was analyzed by HPLC. Column: YMC-Pack R-ODS-5 (4.6 i.d. × 250 mm, 5 μm), mobile phase: CH_3CN - 0.01 M sodium acetate

Table 8. Amino acid analysis of pumilacidins and their derivatives.

Component	Treatment	Analyzed ratio					Theoretical ratio				
		Asp	Glu	Val	Ile	Leu	Asp	Glu	Val	Ile	Leu
Pumilacidin A	—	1.27	1.34		0.85	4	1	1		1	4
	$LiBH_4$	0.88	1.15		0	4	1	1		0	4
Linear peptide of pumilacidin A	—	0.93	0.99		0.72	4	1	1		1	4
	$LiBH_4$	0.91	0.93		0.64	4	1	1		1	4
Pumilacidin B	—	1.07	1.28	1.01		4	1	1	1		4
	$LiBH_4$	1.05	1.35	0		4	1	1	0		4
Linear peptide of pumilacidin B	—	0.93	0.98	0.79		4	1	1	1		4
	$LiBH_4$	1.09	1.09	0.74		4	1	1	1		4

buffer pH 4.5 (79 : 121) containing 0.02% sodium lauryl sulfate, flow rate: 1 ml/minute, detection: UV absorption at 269 nm. Authentic sample of the PTH amino acid (Rt in minutes): PTH-Asp (1.6), PTH-Glu (2.1), PTH-Val (8.3), PTH-Ile (12.9), PTH-Leu (14.2).

Determination of Lactone Linkage

Pumilacidins A and B (10 mg) were treated with $LiBH_4$ (50 mg) in THF (5 ml) under reflux for 6 hours. After addition of 60% aqueous methanol (10 ml), the mixture was concentrated to dryness. The residue was extracted with ethyl acetate at pH 2.0 and the extract was concentrated under reduced pressure. The concentrate was then hydrolyzed with 6 N HCl at 110°C for 16 hours. The linear peptides of pumilacidins A and B obtained by alkaline hydrolysis as described before were also reduced with $LiBH_4$ and hydrolyzed with 6 N HCl. The acid hydrolysates were analyzed on the Hitachi amino acid analyzer (Table 8).

Addendum in Proof

Recently, a complex of new acylpeptide daitocidin was disclosed in Japan Kokai as an inhibitor of H^+, K^+-ATPase and phospholipase A2.[13] Although the chirality of their amino acids and the structures of their fatty acids were not described, daitocidins A_1, A_2, B_1, B_2 and B_3 may be identical with pumilacidins B, D, A, F and C, respectively, from the reported physico-chemical data.

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