FENGYCIN - A NOVEL ANTIFUNGAL LIPOPEPTIDE ANTIBIOTIC PRODUCED BY *BACILLUS SUBTILIS* F-29-3

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Fengycin is an antifungal lipopeptide complex produced by *Bacillus subtilis* strain F-29-3. It inhibits filamentous fungi but is ineffective against yeast and bacteria. The inhibition is antagonized by sterols, phospholipids and oleic acid, whereas two other unsaturated fatty acids increase the antifungal effect. Fengycin consists of two main components differing by one amino acid exchange. Fengycin A is composed of 1 D-Ala, 1 L-Ile, 1 L-Pro, 1 D-allo-Thr, 3 L-Glx, 1 D-Tyr, 1 L-Tyr, 1 D-Orn, whereas in fengycin B the D-Ala is replaced by D-Val. The lipid moiety of both analogs is more variable, as fatty acids have been identified as *anteiso*-pentadecanoic acid (*ai*-C_{15}), *iso*-hexadecanoic acid (*i*-C_{16}), *n*-hexadecanoic acid (*n*-C_{16}), and there is evidence for further saturated and unsaturated residues up to C_{18}.

Strain F-29-3, originally described as *Nocardia* sp.[1,2] before it was correctly determined as *Bacillus subtilis*, is known for its antifungal effects especially against *Rhizoctonia solani* in laboratory and greenhouse experiments with infected test plants[3,4]. In our attempts to identify the antifungal activity, we first isolated bacilysin (bacillin, tetaine[5~10]), an antibiotic inhibitory for budding fungi and bacteria. *Rhizoctonia solani*, *Paecilomyces varioti* and other filamentous fungi proved to be insensitive for bacilysin. These organisms, however, were inhibited by a different active compound of the same bacterial strain (F-29-3). This antibiotic was formerly unknown, it is now described under the name fengycin. Its production, purification, antimicrobial properties, chemical and other data are reported in the present paper.

Materials and Methods

Production of Fengycin by Fermentation

Pure chemicals of analytical grade, Difco (Bacto) preparations of microbial nutrients and deionized water were used, and pH values were adjusted with KOH prior to sterilization (autoclave, 120°C, 20 minutes), unless otherwise specified.

Fengycin was produced in shake cultures using 500-ml baffled Erlenmeyer flasks with 100 ml liquid medium each or in a 25-liter fermenter. Two nutrient media were alternatively used (concentrations in g/liter): SMU (soybean meal, Henselwerk, Magstadt, 20, mannitol 20, urea 20, pH 7.5) and ACS (sucrose 100, citric acid 11.7, Na_2SO_4 4, yeast extract 5, $(NH_4)_2HPO_4$ 4.2, KCl 0.76, $MgCl_2 \cdot 6H_2O$ 0.420, $ZnCl_2$ 0.0104, $FeCl_3 \cdot 6H_2O$ 0.0245, $MnCl_2 \cdot 4H_2O$ 0.0181, adjusted to pH 6.9 with NH_4OH).

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The antibiotic producing bacterium, *Bacillus subtilis* (Ehrenberg) Cohn strain F-29-3, was subcultured on nHA medium (yeast extract 4, malt extract 10, D-glucose 4, agar 20, H_2O 1 liter, pH 7.5) incubated for at least 2 days at 37°C and stored at room temperature (22~26°C) for 2~8 weeks.

The bacterial growth of one slant was suspended in H_2O (8 ml). 100 ml culture media (SMU, ACS) were inoculated with the bacterial (spore) suspension (1 ml) and incubated on a shaker (120 rpm, 25 mm radius of the circular movement) at 27°C for 24 hours. The culture was used as inoculum for fermentation cultures (fermentor type b 25, Giovanola Frères SA, Monthey, Switzerland; 12.5 liters air/minute, 800 rpm, 28°C). The fermenter cultures were harvested after 7 days (SMU) or 5 days (ACS).

Isolation of Fengycin

Step 1*: The fermentation broth (10 liters) was adjusted with 5 N HCl to pH 2.5. The precipitate was separated by centrifugation (8,000 rpm, 10 minutes (supernatant discarded)) and extracted with 1 liter of EtOH 95%. The extraction was repeated with aq EtOH 70% (1 liter).

Step 2: The pooled extracts were mixed with 10% charcoal (w/v), stirred for 30 minutes and filtered through a suction funnel. The charcoal was eluted with 2 liters of $CHCl_3$ - MeOH - H_2O (65: 25: 4). The eluate was concentrated and dissolved in 10 ml of $CHCl_3$ - MeOH (1: 3).

Step 3: The solution was dropped slowly in cold ether (1 liter). The floccose precipitates were collected by filtration.

Step 4: The dried precipitate was subjected to countercurrent distribution (Craig apparatus, type Boy 505, Labortec, Buchs, Switzerland) in $CHCl_3$ - MeOH - H_2O (2: 2: 1) under the following conditions: Shaking speed 70 movements/minute, shaking time 1 minute, waiting time for phase separation 12 minutes. Fractions containing fengycin were collected, concentrated and lyophilized.

Step 5: The preparation was chromatographed on Sephadex LH-20 (400 ml, 60 cm×2.9 cm) with MeOH at a flow rate of 15 ml/hour. The active fractions were pooled, evaporated *in vacuo* and lyophilized.

Step 6: The lyophilizate was applied on a column of silica gel (400 ml, 57 cm×3 cm, flow rate 30 ml/hour), which was equilibrated and eluted with butanol - EtOH - 0.1% CH_3COOH (1: 4: 1).

Characterization of Fengycin

Antimicrobial Activities: Antifungal activities in culture filtrates and liquid preparations during isolation experiments were determined by agar diffusion tests using *Paecilomyces varioti* Tü 137 as test organism.

MIC and ID_{50} were determined by a disc diffusion method. Microorganisms inhibited by at least 100 μg/ml were subjected to further tests using an agar dilution method. The MIC was determined after 24 hours of incubation at 37°C for bacteria and after 2~10 days at 28°C or 37°C for fungi.

The hyphal growth on test plates was microscopically observed for morphological changes and compared with controls.

Thin-layer Chromatography: For routine analyses Rf values of the fengycin mixture were determined in saturated glass chambers at 21°C on precoated glass plates with Silica gel 60F 254 (Merck, 5719) using the systems 1 and 2 (Table 2). Fengycin was detected with chlorine - 4,4′-bis(dimethylamino)diphenylmethane (TDM), water, iodine and Ninhydrin reagent.

Determination of Mutual Influences of Lipids and Fengycin on Fungi

Influences of lipids on the antifungal activity of fengycin were determined by cross-tests[11] using *Paecilomyces varioti* Tü 137 as test organism.

The fengycin antagonism of ergosterol was demonstrated by serial dilution tests with agar media. Paper discs with 10 μl of fengycin solutions (10.0, 3.16 and 1.0 mg/ml) were placed on the test plates composed of malt agar (15 ml), Tü 137 spore suspension (250 μl) and ergosterol solutions (750 μl) in acetone (1,000, 316, 100, 31.6 and 10.0 μg/ml). Inhibition zones were observed after 24 hours of incubation at 37°C.

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* See Table 1.

Fig. 1. Time course of production of bacilysin and fengycin in SMU.

■ Bacilysin production determined from the inhibition of *Candida albicans* Tü 565, ● fengycin production determined from the inhibition of *Paecilomyces varioti* Tü 137, △ absorbance at 578 nm (culture - dilution, 1 : 10), □ pH of the culture.

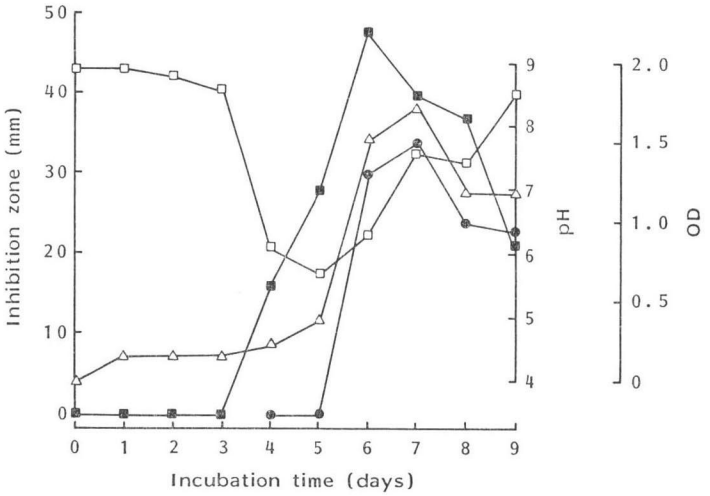
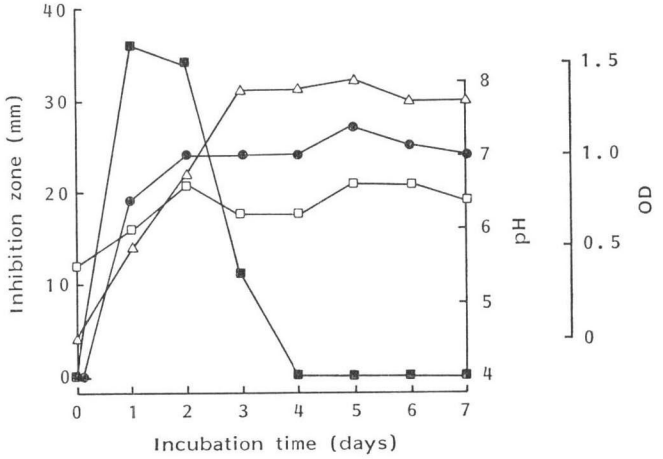


Fig. 2. Time course of production of bacilysin and fengycin in ACS.

■ Bacilysin production determined from the inhibition of *Candida albicans* Tü 565, ● fengycin production determined from the inhibition of *Paecilomyces varioti* Tü 137, △ absorbance at 578 nm (culture - dilution, 1 : 10), □ pH of the culture.



Hemolysis Test

Hemolytic activities of fengycin, alamethicin, iturin A_L and sodium dodecylsulfate (SDS) were assayed as described by HSUCHEN and FEINGOLD[12], IRMSCHER and JUNG[13].

Results

Production and Isolation

Two antibiotics were isolated from *Bacillus subtilis* F-29-3. One of them was identified as the known compound bacilysin. The other was a newly described lipopeptide named fengycin. The maximum concentration of fengycin during fermentation (Figs. 1 and 2) was reached after seven days

Table 1. Isolation of fengycin from 10 liters of culture medium (SMU) of *Bacillus subtilis*.

Purification steps	Dry weight (mg)	Antibiotic content calcd from antibiotic activity (mg)	Recovery of antibiotic activity (%)		Specific activity
			Previous step	Total recovery	
1. Ethanol extracts	65,000	15,247	100	100	0.23
2. Eluate from charcoal	27,000	14,180	93	93	0.52
3. Ether precipitate	8,500	2,836	20	18.6	0.33
4. Countercurrent distribution, active fractions	3,700	2,694	95	17.7	0.73
5. Gel chromatography, active fractions	3,300	2,694	100	17.7	0.82
6. Eluate from silica gel	1,940	1,940	72	12.7	1.0

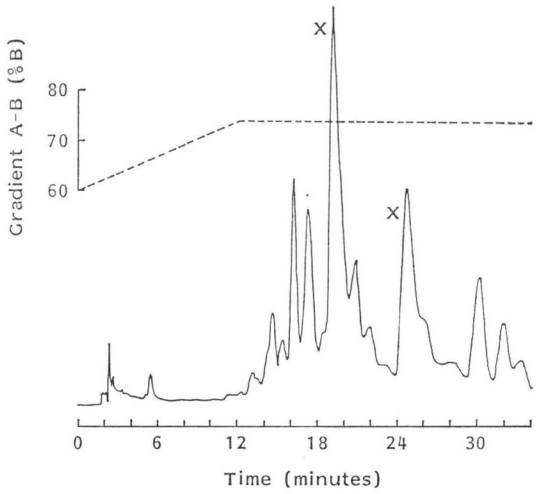
Table 2. Rf values of the fengycin mixture on silica gel TLC.

Solvent system	Rf*
1. $CHCl_3$ - MeOH - 70% EtOH (7: 3: 5)	0.24**
2. $CHCl_3$ - MeOH - 17% NH_4OH (2: 2: 1)	0.54~0.59
3. Phenol - H_2O (75: 25)	0.44
4. $CHCl_3$ - MeOH - H_2O (65: 25: 4)	0.07
5. Butanol - CH_3COOH - H_2O (4: 1: 1)	0.19
6. Butanol - CH_3COOH - H_2O (4: 3: 3)	0.79
7. Butanol - EtOH - 0.1% CH_3COOH (1: 4: 1)	0.43

* Determined by bioautography.

** Only in solvent system 1, the fengycin complex separates into four Ninhydrin positive peptides with Rf 0.20, 0.24, 0.26 and 0.30.

Fig. 3. HPLC chromatogram of fengycin on Nucleosil 7μC_{18} with a two system solvent gradient (------) of A=CH_3CN - H_2O (10:90)+0.1% TFA, B=CH_3CN - H_2O (70 : 30)+0.1% TFA.
X indicates isolated components.



in SMU (1,450 μg/ml) or five days in ACS (400 μg/ml). The isolation and purification of fengycin (Table 1) yielded about 2 g of a colorless powder representing 13% of the original activity present in 10 liters of culture medium (SMU).

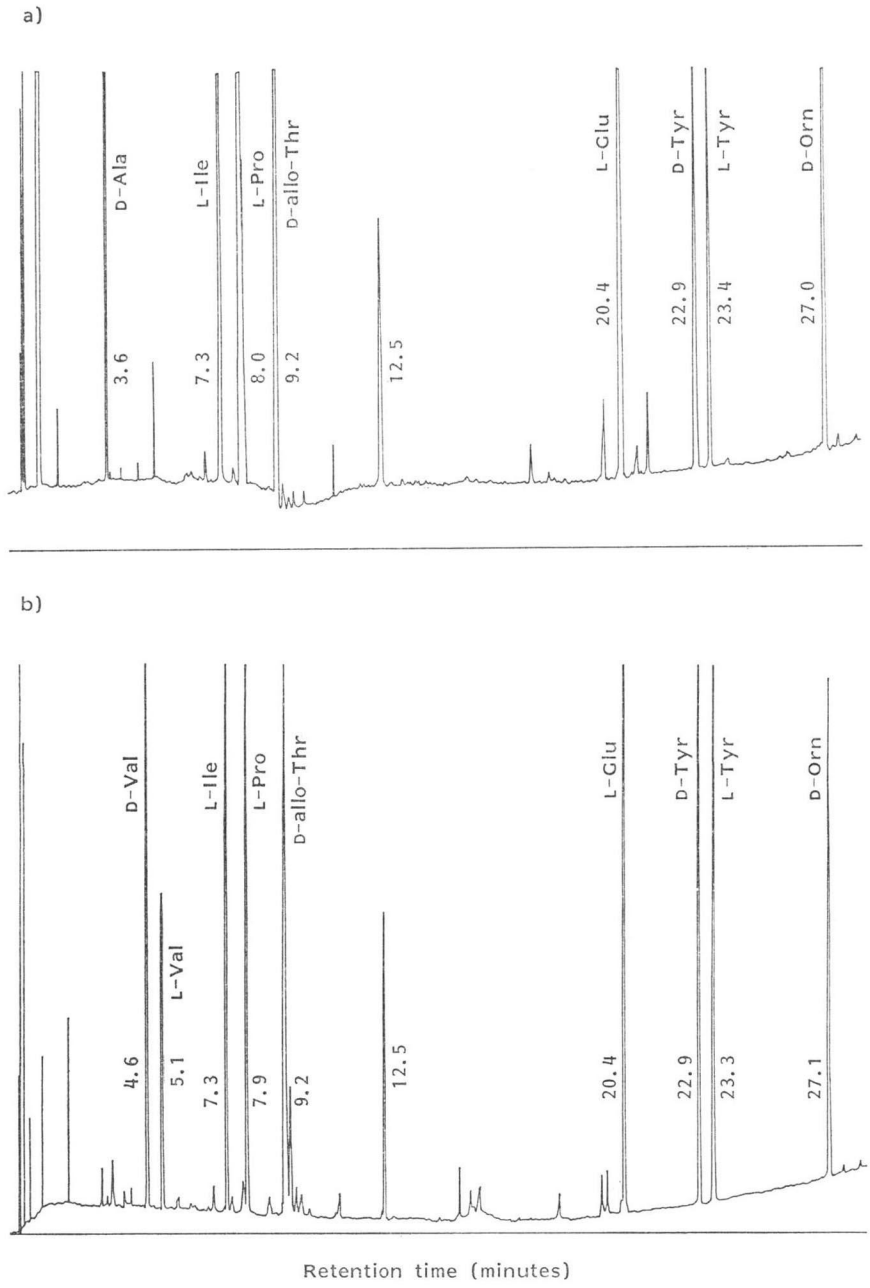
Physico-chemical Properties

Solubility and Stability

Fengycin is a colorless powder, soluble in polar organic solvents, *e.g.*, methanol, ethanol and dimethylformamide, slightly soluble in dichloromethane and *tert*-butanol, insoluble in water, acetone and diethylether. Fengycin starts melting and partially decomposing at 177°C, whereby its color is changing to red, complete melting at 188°C.

Fengycin was stable for several days in Britton-Robinson buffer[14] between pH 4 and 10 at room temperature, at −18°C no loss of the fengycin activity was observed within 5 months. However, the activity decreased slightly in buffers below pH 4 after 2 days. As a solid fengycin was stable for months at room temperature, in methanolic solution for weeks and in weakly acidic or alkaline

Fig. 4. Gas chromatogram of pentafluoropropionyl-amino acid methyl esters.
Glass capillary 20 m, stationary phase Chirasil-Val[17,18], carrier gas H_2, FID, 3 minutes 80°C, 4°C/minute to 200°C.



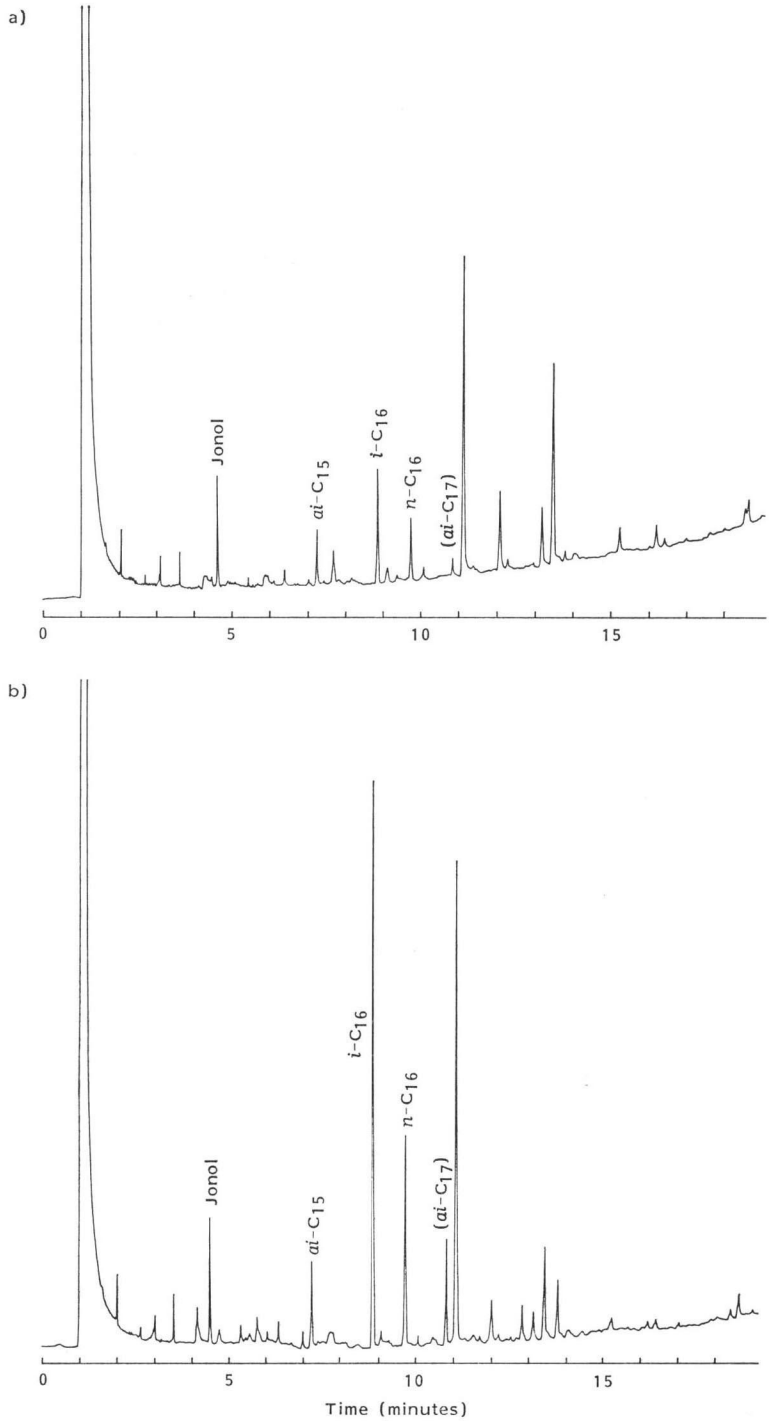
solutions for several days.

Diluted mineral acids decomposed fengycin at 60°C within one to several hours yielding different biologically inactive fragments of similar properties as fengycin according to their Rf values and countercurrent distribution coefficients.

Treatment with 5% $NaHCO_3$ solution (60°C, 4 hours) resulted in a fragment which showed after

Fig. 5. Gas chromatogram of the fatty acid methyl esters from fengycin.

a) Before hydrogenation. b) After hydrogenation. Glass capillary 20 m, Carbowax 20 M, carrier gas H_2, FID, 2 minutes 130°C, 4°C/minute to 240°C.



total hydrolysis (6 N HCl, 18 hours, 110°C) the same amino acid composition as fengycin. The slightly alkaline conditions for hydrolysis may lead to the cleavage of a lactone bond as shown for other lipopeptidic antibiotics, *e.g.* herbicolin[15,16].

Microheterogeneity of Fengycin

Fengycin preparations were composed of four active fractions, which were separable on TLC (system 1): Rf 0.20 (fengycin A_1), 0.24 (A_2), 0.26 (B_1), 0.30 (B_2) (Table 2). Fengycins A_1 and A_2 were too closely related to be separated in preparative scale on TLC. Fengycins B_1 (major product) and B_2 were isolated by silica gel column chromatography (butanol - ethanol - 0.1% acetic acid, 1:4:1).

The components of fengycin were also fractionated on HPLC (Fig. 3) to give a high number of peaks due to the different fatty acid residues. Again two B components indicated by X were isolated and analyzed; their amino acid compositions were identical and showed 0.75 equivalents of valine but only 0.16 equivalents of alanine, whereas the component A possessed a higher alanine content.

Chemical Composition

Fengycin components are lipopeptides. According to amino acid analysis (Biotronic, system LC 6000 E), two-dimensional TLC (system 2 followed by system 3, Table 2) and combined gas chromatography mass spectrometry (GC-MS) of total hydrolysates fengycin consists of 1 D-Val (respectively 1 D-Ala), 1 L-Ile, 1 L-Pro, 1 D-allo-Thr, 3 L-Glx, 1 D-Tyr, 1 L-Tyr and 1 D-Orn. According to their amino acid composition, fengycin A (with 1 D-Ala; A_1: Rf 0.20; A_2: Rf 0.24) and fengycin B (with 1 D-Val; B_1: Rf 0.26; B_2: Rf 0.30) are distinguished. The configuration of the amino acids was determined by gas chromatography on a chiral phase (Chirasil-Val[17,18], Fig. 4).

Analogous to the procedure described for bacillomycin F[10] the fatty acids were extracted from the acidic total hydrolysate with chloroform and analyzed as methyl esters by GC-MS. Three of the main peaks (Fig. 5a) could be correlated to the fatty acids *anteiso*-pentadecanoic acid (*ai*-C_{15}), *iso*-hexadecanoic acid (*i*-C_{16}) and *n*-hexadecanoic acid (*n*-C_{16}). Comparison with gas chromatograms of the hydrogenated fatty acid methyl esters showed that the mixture contained some unsaturated fatty acids (Fig. 5b), as indicated also in the ^{13}C NMR spectrum of fengycin.

Elemental analysis of the fengycin mixture

Anal Calcd for 3 Glu, 1 Val: C 60.19, H 7.71, N 10.43.
Found: C 59.87, H 7.78, N 10.66.

These values were calculated for hexadecanoic acid representative for all other fatty acid constituents and a molecular mass of 1,477 ($C_{74}H_{113}N_{11}O_{20}$).

From the elemental analysis it can be concluded that fengycin contains most probably three glutamic acid residues and no glutamine.

Spectra

The UV absorption maxima of the fengycin complex at 276 nm in methanol and at 293 nm in alkaline methanolic solution are indicative of tyrosyl peptides.

The IR spectrum of fengycin in KBr (Fig. 6) shows bands at 3400 cm^{-1} for amino- and hydroxyl groups of amino acids. The bands at 2860, 2930 and 2960 cm^{-1} reflect the aliphatic side chains and at 2060 cm^{-1} the phenolic ring of tyrosine. At 1650 and 1520 cm^{-1} strong bands appear due to the peptide bonds. The shoulder at 1760 cm^{-1} could be due to an ester linkage.

Fig. 6. IR spectrum of fengycin (pellet, 1.5 mg in 300 mg KBr).

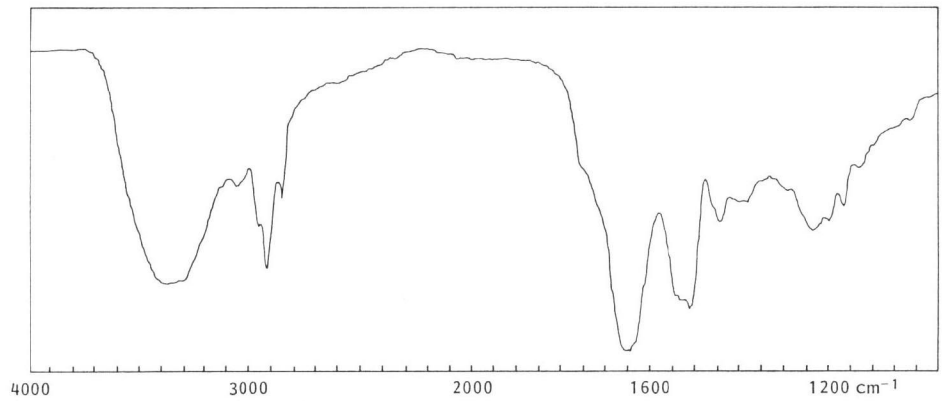
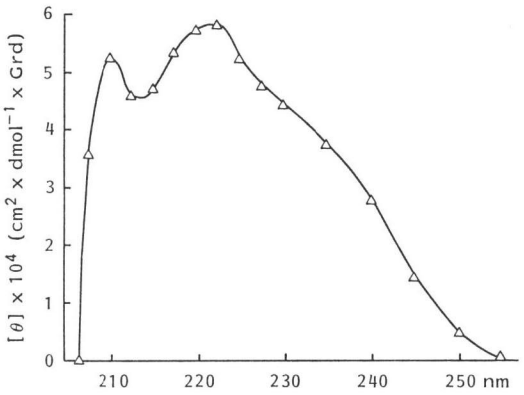


Fig. 7. Circular dichroism spectrum of fengycin (1.5 mg/ml, MeOH).



The FD mass spectrum of the fengycin mixture indicated a molecular mass of 1,541 dalton, whereas the molecular mass of the component B_1 was 1,492 as determined by FAB mass spectrometry. The FAB spectrum exhibited also the peaks of higher and lower homologs (m/z=1,506 and 1,478).

Contrary to the CD spectra of most other peptides and peptide antibiotics fengycin shows positive ellipticities from 260 to 206 nm (Fig. 7). This may be due to the high content of D-amino acids. The spectrum indicates at least three positive Cotton effects at about 240 (sh), 220 (maximum), and at about 207 nm.

The ^{13}C NMR spectrum (100 MHz, Fig. 8) exhibits carbonyl resonances between 170 and 181 ppm, all carbon signals of the different amino acids known from amino acid analysis and GC-MS were compatible with known data[20,21]. The resonances of the various fatty acid chains are found mainly between 10 and 40 ppm, most of them could be assigned by comparison with published data[22~24]. Some of the unsaturated carbon atoms showing resonances at 122.4, 131.4, 134, 135 and possibly 151.3 ppm can be attributed to olefinic fatty acid residues.

Biological Properties

Antimicrobial Spectrum

Table 3 summarizes the antimicrobial spectrum of fengycin and its MIC and ID values. The antibiotic activity against fungi ranges from the most sensitive *Pyricularia oryzae* (MIC 1.0 μg/ml) *via Conidiobolus coronatus* (MIC 3.16 μg/ml), *Curvularia lunata* (MIC 3.16 μg/ml), *Fusarium sp.* (MIC 10 μg/ml), *Rhizomucor miehei* (MIC 10 μg/ml), *Alternaria kikuchiana* (MIC 10 μg/ml) and *Rhizoctonia solani* (MIC 3.16 μg/ml) to organisms like some *Aspergillus*, *Trichophyton* species and yeast (not inhibited by 1,000 μg/ml).

Mycotypha africana in YMA at 27°C develops normal sporulating hypha and is inhibited by fengy-

Fig. 8. ^{13}C NMR spectrum (100 MHz, in MeOH-d_4).
a) Region of 0~80 ppm. b) Region of 100~185 ppm.

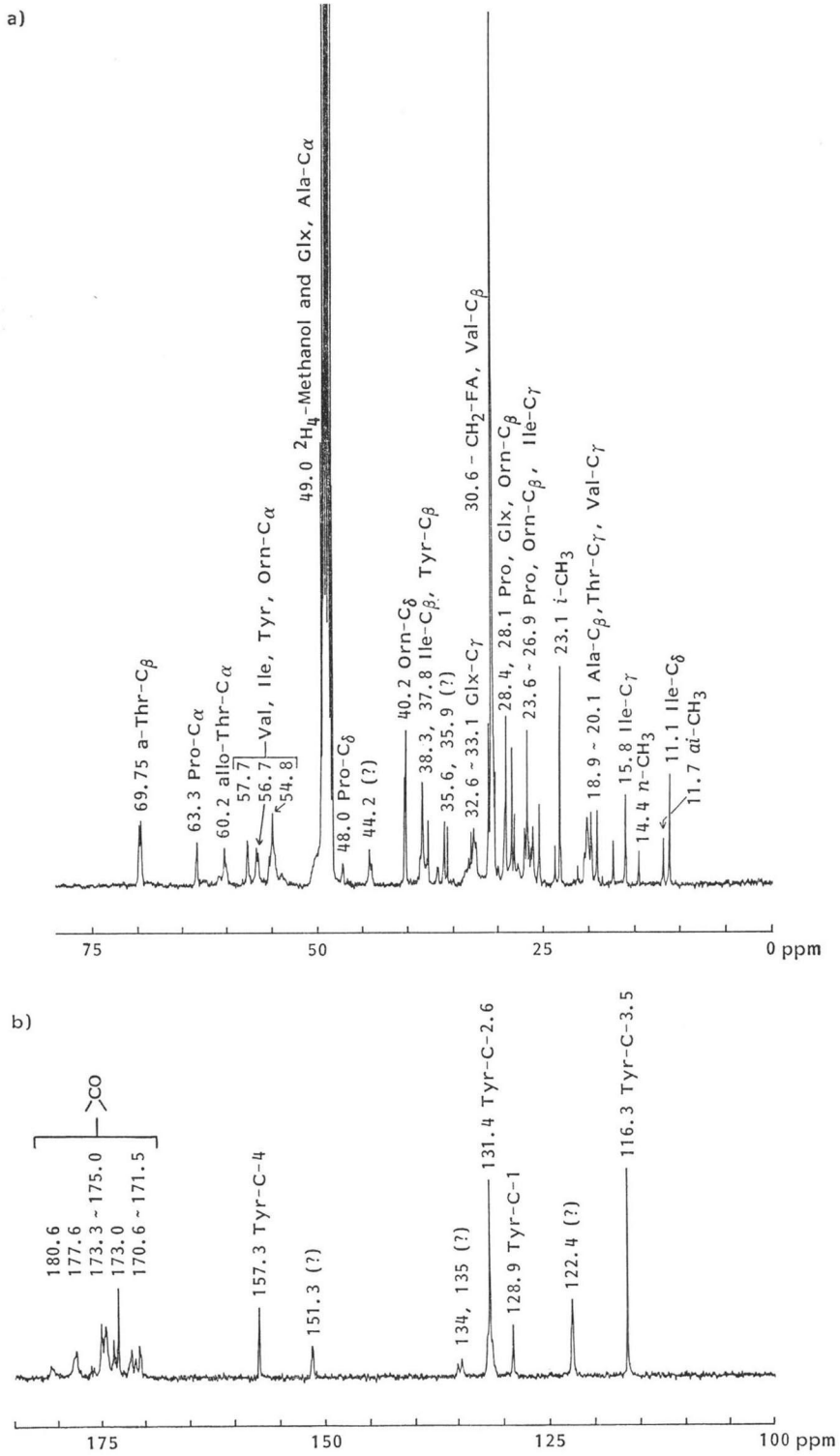


Table 3. Antimicrobial spectrum of fengycin.

Microorganisms	Test media*	Temperature (°C)	MIC (μg/ml)[a]	MIC (μg/ml)[b]	ID_{50} (μg/ml)
Bacteria					
Bacillus subtilis ATCC 6051	NA	37	>	—	—
Escherichia coli K 12	NA	37	>	—	—
Micrococcus luteus ATCC 381	NA	27	>	—	—
Staphylococcus aureus Tü 202	NA	37	>	—	—
Streptomyces viridochromogenes Tü 57	nYMA	27	>	—	—
Fungi					
Oomycota					
Phytophthora palmivora Tü 666	YMA	27	>100	>100	—
Pythium debaryanum DSM 62946	MA	27	—	>100	—
Saprolegnia sp. Tü 543	MA	27	—	>100	—
Zygomycota					
Basidiobolus microsporus Tü 174	YMA	27	25	3.16	0.4
Conidiobolus coronatus Tü 256	YMA	27	25	3.16	0.7
Mucor hiemalis (+) Tü 179	YMA	27	100	>100	6.0
M. hiemalis (−) Tü 180	YMA	27	1,000	—	—
M. hiemalis (+ −)	YMA	27	50	>100	6.0
Mycotypha africana Tü 260	YMA	27	25	—	—
	YMG	37	>100	—	—
Rhizomucor miehei Tü 284	YMA	37	25	10	5.2
Ascomycota					
Endomycetes and related Deuteromycota					
Candida albicans Tü 565	MA	27	>	—	—
Eremascus albus Tü 185	YMA	27	>	—	—
Nematospora coryli Tü 194	YMA	27	>	—	—
Saccharomyces cerevisiae Tü 125	MA	27	>	—	—
Schizosaccharomyces pombe Tü 501	YMA	27	>	—	—
Eurotiales and related Deuteromycota					
Aspergillus fumigatus Tü 149	YMA	37	>	—	—
A. terreus Tü 155	YMA	27	1,000	—	—
Paecilomyces varioti Tü 137	MA	37	10	>100	0.35
Scopulariopsis brevicaulis Tü 8058	YMA	27	250	—	—
Thielaviopsis basicola DSM 63050	PDA	RT	—	100	10.0
Onygenales and related Deuteromycota					
Epidermomyces floccosus Tü 576	YMA	27	100	—	—
Microsporum gypseum Tü 282	YMA	27	250	—	—
Trichophyton mentagrophytes Tü 295	YMA	27	>	—	—
T. rubrum Tü 299	YMA	27	>	—	—
Pezizales					
Ascodesmis sphaerospora Tü 147	YMA	27	25	>100	0.2
Sphaeriales and related Deuteromycota					
Colletotrichum acutatum Tü 8064	YMA	27	25	>100	—
Fusarium sp. Tü 638	YMA	27	25	10	0.9
F. sp. Tü 8014	YMA	27	>	—	—
*Neurospora crassa***	MA	27	>	—	—
Pyricularia oryzae Tü 692	YMA	27	12.5	1.0	0.05
Trichothecium roseum Tü 300	YMA	27	1,000	—	—
Verticillium lecanii Tü 264	YMA	27	200	—	—
Helotiales and related Deuteromycota					
Botrytis cinerea Tü 157	YMA	27	50	>100	12
Monilinia fructicola CBS 203.25	PDA	27	—	100	1.1
Sclerotinia sclerotiorum DSM 1946	YMA	27	—	>100	—

Table 3. (Continued)

Microorganisms	Test media*	Temperature (°C)	MIC (μg/ml)[a]	MIC (μg/ml)[b]	ID_{50} (μg/ml)
Dothideales and related Deuteromycota					
Alternaria kikuchiana Tü 169	CMA	27	1.56	10	0.1
Curvularia lunata Tü 627	YMA	27	0.78	3.16	0.04
Mycosphaerella pinodes DSM 62763	PDA	RT	—	100	0.01
Pleospora herbarum DSM 62928	PDA	RT	—	>100	—
Stemphylium sp. Tü 609	YMA	27	12.5	3.16	0.14
S. sarciniforme DSM 63045	PDA	RT	—	>100	0.4
Basidiomycota and related Deuteromycota					
Rhizoctonia solani Tü 8104	MA	27	>	3.16	4
Rhodotorula rubra Tü 8093	YMA	27	>	—	—
Schizophyllum commune Tü 294	YMA	27	—	>100	2
Sporobolomyces singularis Tü 8098	YMA	27	>	—	—

* MA: Malt extract 2%, agar 2%; YMA: yeast extract 0.4%, malt extract 1%, glucose 0.4%, agar 2%; YMG: as for YMA, but glucose 20%; PDA: potato dextrose agar (Difco) 3.9%; CMA: corn meal agar (Difco) 1.7%.

** Shaer and Dodge strain from Prof. G. WINKELMANN.

[a] Agar diffusion tests. >: No inhibition zones at 1,000 μg/ml and lower concentrations.

[b] Serial dilution tests (ZÄHNER and MAAS, 1972).

RT: Room temperature.

Table 4. Morphological effects of fengycin on test fungi and minimal antibiotic concentration inducing the morphological changes.

Test organisms	Test medium*	Temperature (°C)	Morphological effects			Minimal effective concentration (μg/ml)
			Bulging	Curling	Emptying	
Ascodesmis sphaerospora	YMA	27	+	—	—	0.3
Botrytis cinerea	YMA	27	+	—	—	3.16
Colletotrichum acutatum	YMA	27	+	—	—	0.1
Monilinia fructicola	PDA	27	—	—	+	0.3
Mucor hiemalis (+)	YMA	27	—	—	+	0.3
M. hiemalis (+ −)	YMA	27	+	—	+	1.0
Mycosphaerella pinodes	PDA	RT	—	—	+	0.01
Paecilomyces variotii	MA	37	+	—	—	1.0
Pleospora herbarum	PDA	RT	+	—	+	1.0
Pyricularia oryzae	YMA	27	+	—	—	0.1
Rhizoctonia solani	MA	27	+	—	—	1.0
Schizophyllum commune	YMA	27	+	—	—	1.0
Stemphylium sp.	YMA	27	+	+	+	0.03
Thielaviopsis basicola	PDA	RT	+	—	—	0.3

+: Changes observed, −: no changes observed.

* MA: Malt extract 2%, agar 2%; YMA: yeast extract 0.4%, malt extract 1%, glucose 0.4%, agar 2%; YMG: as for YMA, but glucose 20%; PDA: potato dextrose agar (Difco) 3.9%.

RT: Room temperature.

cin, however, the same fungus in YMG at 37°C grows with budding cells only and is then insensitive against fengycin. This was also observed for other budding fungi (all Endomycetes; budding Basidiomycetes: *Rhodotorula*, *Sporobolomyces*).

Morphological changes due to fengycin were obtained in several fungi as bulging, curling or emptying of the hyphae. The different types of morphological aberrations and the minimal concentrations of fengycin which induced them are summarized in Table 4.

Table 5. Influence of some lipids on the inhibition of *Paecilomyces varioti* Tü 137 by fengycin*.

Test substances (lipid compounds)	Influence on the inhibition zone by lipid concentrations (mg/ml)			
	0.5	1.0	2.0	10.0
Cholesterol	(+)	+	+	+
Cholesterol methyl ether	(−)	(−)	−	T
Ergosterol	+	+	+	+
Stigmasterol	+	+	+	+
Phosphatidic acid	○	○	○	+
Phosphatidyl choline	+	+	+	+
Phosphatidyl ethanolamine	+	+	+	+
Phosphatidyl glycerol	○	○	○	+
Phosphatidyl serine	(+)	+	+	T
Oleic acid (only 1 mg/ml tested)		+		
cis-Vaccenic acid	−	−	−, T	T
trans-Vaccenic acid	(−)	(−)	−	T
Petroselinic acid	−	−, T	−, T	T

* Concentration of fengycin=1 mg/ml.

+: Reduction of the inhibition zone (=antagonism), (+): weak antagonism, ○: no influence on the inhibition due to fengycin, −: inhibition, cumulating with the fengycin effect (additive or synergistic actions), (−): weak cumulative inhibitory effect, T: inhibition by the test substance alone.

Morphological changes have been observed (Table 4) for almost all fengycin-sensitive fungi (Table 3). For *Colletotrichum acutatum* and *Pleospora herbarum* no growth inhibition could be measured but hyphal aberrations were stated. On the other hand, in some sensitive fungi (*Basidiobolus*, *Fusarium*, *Alternaria* and others) no morphological changes were seen.

Hemolytic Activity

Hemolysis of fengycin was tested in comparison with alamethicin[13,25], iturin A_L[22,26] and sodium dodecylsulfate (SDS). The concentration which induced 50% hemolytic activity was 2.4×10^{-5} mol/liter for iturin A_L, 3.4×10^{-5} mol/liter for alamethicin, 7.3×10^{-5} mol/liter for SDS and 1.9×10^{-3} mol/liter for fengycin.

The relative hemolytic activities of fengycin and iturin A_L and their MIC values for two fungi can be estimated as follows:

Hemolytic activity	fengycin - iturin A_L, 1: 70
MIC for *Rhizomucor*	fengycin - iturin A_L, 1: 1
MIC for *Paecilomyces*	fengycin - iturin A_L, 1: 10

Thus, compared to iturin A_L, fengycin exhibits similar MIC values, but it is less toxic for erythrocytes.

Effect of Lipids on the Fengycin Activity

In a cross test, non-diluted egg yolk antagonized the antifungal activity of fengycin. In the same test system cholesterol, ergosterol, phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine antagonized the inhibition of *Paecilomyces* by fengycin (Table 5). Phosphatidic acid and phosphatidyl glycerol had weak antagonistic influence. In contrast, cholesterol methyl ether, *cis*- and *trans*-vaccenic acid and petroselinic acid increased the fengycin inhibition. Some lipids were toxic for *Paecilomyces* inhibited by fengycin.

In a serial dilution test, the antagonism was observed with 100 μg/ml or more ergosterol in the sub-

strate. For a fengycin concentration of 1 mg/ml the inhibition zone on Tü 137 test plates decreased from 17 mm to 7 mm with an increasing concentration of ergosterol from 10 to 1,000 μg/ml. In each case the inhibition zone increased regularly with log c (fengycin) indicating, that the diffusion of the antibiotic was not influenced by ergosterol in the test medium.

Fengycin was chromatographed on TLC in the presence of ergosterol (20 mg/ml) in system 1 (Table 2). TLC plates were developed with water and Ninhydrin. The Rf value of fengycin had changed from 0.24 to 0.20 with ergosterol indicating the formation of fengycin-ergosterol complex.

Discussion

Of the possible types of antifungal actions (disturbance of macromolecule biosyntheses, inhibition of energy transfer, interference with membrane and cell wall functions) a primary effect of fengycin on the cytoplasmic membrane appears most probable. Evidence for this assumption was contributed by the results of experiments with lipid fengycin antagonists (Table 5) and by the demonstration of a fengycin-ergosterol complex. The different behavior of filamentous and budding colonies of the same fungus towards fengycin (*Mycotypha*, Table 3) may also be explained by a different sterol content of the respective cell membranes.

A certain similarity of fengycin and its sterol-, fatty acid- and phospholipid-antagonism exists with the cyclic lipopeptides of *Bacillus* species[27], the cyclic peptide mycobacillin[28,29], and the abnormal polyene compound lipomycin[30] and their antagonists. For these antibiotics with similar antagonists, the membrane of the target cell is considered the primary site of action, however, there are individual differences: Mycobacillin is not antagonized by fatty acids, and the cyclic lipopeptides bacillomycin L and iturin A cause hemoglobin release from erythrocytes, whereas fengycin is less toxic for erythrocytes.

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