The Structure of Two Fengycins from *Bacillus subtilis* S499 §

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The structures of the two fengycins, lipopeptides from *Bacillus subtilis*, were elucidated by spectroscopic methods and chemical degradation. They show a close structural relationship to the plipastatins from *Bacillus cereus* differing only in the stereochemistry of the Tyr residues.

Introduction

Lipopeptides consist of a hydrophilic peptide and a hydrophobic long-chain carboxylic acid. Due to their amphiphilic character they are surface active. This enables them to form micellae in aqueous media and monolayers at phase bounds. Thus, bacteria producing lipopeptides can grow on apolar substrates. Lipopeptides may also possess antibiotic activities: If they can penetrate cell membranes they form ion channels which increase the permeability resulting in an uncontrolled loss of dissolved materials and subsequently the death of the cell (Besson *et al.*, 1984). Some of those peptide antibiotics are in clinical use (Katz and Demain, 1977; Simon and Stille, 1997).

Bacillus subtilis is one of the most versatile producer of cyclic lipopeptides. The **iturin** group – bacillomycines (e.g., Peypoux *et* al., 1985), bacillopeptines (e.g., Kajimura *et al.*, 1995), and mycosubtilines (e.g., Besson and Michel, 1990) comprises lactams containing a C_{14}-C_{17} 3-amino-fatty acid and seven amino acids, the 2nd and 3rd one being always D-Tyr-D-Asn. The **surfactins** are lactones incorporating a C_{13}-C_{15} 3-hydroxy fatty acid and seven amino acids two of which are variable (L-Glu-L-Leu-D-Leu-L-X^4-L-Asp-D-Leu-L-X^7) (e.g., Peypoux *et al.*, 1994). For the **fengycins** (Vanittanakom *et al.*, 1986) a lactonic structure consisting of a C_{16}-C_{18} 3-hydroxy-fatty acid followed by L-Glu-D-Orn-(D or L)-Tyr-D-aThr-L-Glu-D-(Ala or Val)-L-Pro-L-Glu-(L or D)-Tyr-L-Ileu was suggested (Koch, 1988). In contrast to the surfactins where the ester bond is formed between the C-terminal amino acid and the hydroxyl group of the fatty acid in the fengycins it connects the C-terminal amino acid and Tyr third in the sequence. Several questions regarding the proposed structure remained open: The enantiomeric Tyr were not located, the sequence aThr-Glu-Ala/Val-Pro-Glu could be reversed, and it was assumed only that the ester bond was located between the C-terminal Ile and the first Tyr. In addition, the ion at *m/z* 1505 in the FAB mass spectrum was mistaken for M^+ instead of $[M + H]^+$ as it is typical for peptides. One of the three Glu could, therefore, actually be Gln.

In this contribution proof of the correct structure by NMR and MS techniques combined with degradation studies will be presented.

Abbreviations: Common amino acids, 3-letter code; Dab, 2,4-diaminobutyric acid; TAP-derivatives, N/O-trifluoroacetyl-(amino acid)-isopropyl esters; DMSO, dimethylsulfoxid; GC, gas chromatography; RP-HPLC, reversed phase high performance liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; FAB, fast atom bombardment; NMR-techniques: COSY, correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; NOE, nuclear Overhauser enhancement effect; NOESY nuclear Overhauser enhancement and exchange spectroscopy.

§ Part LXXXIII of the series "Bacterial Constituents". For Part LXXXII see Georgias *et al.* (1999).

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Materials and Methods

Instruments

Mass spectrometry: Finnigan-MAT HSQ-30 (FAB, matrix thioglycerol/dithiodiethanol) with FAB gun 11 NF (IonTech Ltd., Teddington, GB, 8 kV) FAB gas Xe; Finnigan 900 ST (ESI; 50 mM solutions in CH_3OH/H_2O 4:1 v/v); GC/MS Incos 500 (all Finnigan-MAT, Bremen) with Varian (Sunnyvale CA, USA) GC 3400.

NMR: DPX 300 (^1H 300, ^{13}C 75.5 MHz) and DRX 500 (^1H 500, ^{13}C 125.8 MHz) (both Bruker, Karlsruhe). Samples: 15 mg fengycin IX in 0.6 ml CD_3OH or DMSO-d_6, degassed for 2 min. in an ultrasonic bath. Chemical shifts relative to methanol or DMSO (^1H: δ(methanol) = 3.30, δ(DMSO) = 2.49 ppm; ^{13}C: δ(methanol) = 29.0, δ(DMSO) = 39.5 ppm).

IR: Spectrophotometer 283 (Perkin-Elmer, Überlingen): to 1.7 mg fengycin dissolved in 0.3 ml CH_3OH 100 mg KBr were added. After bringing to dryness i. v. a tablet was prepared.

UV/Vis: Lambda 7 (Perkin-Elmer, Überlingen): 1 mg fengycin in 2 ml CH_3OH.

Chromatography: RP-HPLC, Knauer (Berlin), column Nucleosil 100–7 C_{18}, 7 μm (Macherey & Nagel, Düren); column chromatography, silicagel 60M (Macherey & Nagel, Düren); GC, HRGC 4160 (Carlo Erba, Mailand, I), column Chirasil-L-Val (Chrompack, Frankfurt/M).

Chemicals

Desalted water was distilled twice in a quartz apparatus, passed through a XAD-4 resin column and a sterile filter (4 μm) (Millipore, Bedford MA, USA). All solvents and chemicals were p. a. quality.

Bacterial culture and isolation of the fengycins

The strain *Bacillus subtilis* S499 was grown in a liquid medium containing in 1 l water 30 g peptone, 20 g saccharose, 7 g yeast extract, 1.9 g KH_2PO_4, 1 μg $CuSO_4$, 5 μg $FeCl_3{\cdot}5H_2O$, 4 μg $NaMoO_4$, 2 μg KI, 3.6 mg $MnSO_4{\cdot}H_2O$, 450 mg $MgSO_4$, 14 mg $ZnSO_4{\cdot}7H_2O$, 10 μg H_3BO_3 and 10 mg citric acid. The solution was brought to pH 7.0 with 5 N NaOH and sterilized.

The bacteria were grown in 12 l medium contained in a 20 l fermenter stirred with 200 rpm at 30 °C and aerated actively with 3.6 l/min. sterile air. The pH was regulated to 7.0 with 3 N NaOH and 3 N H_3PO_4, respectively. For inocculation 500 ml of a preculture was used grown under the same conditions. The culture was stopped after 3 days and the cell material was removed by centrifugation. The supernatant was adsorbed on RP C_{18} resin and washed successively with 0.8 l H_2O and 1.6 l CH_3OH/H_2O 1:1 (v/v). The lipopeptides were then eluted with 0.8 l CH_3OH. The eluate was brought to dryness, the residue was redissolved in $CHCl_3/CH_3OH/H_2O$ 65:25:4 (v/v) and chromatographed on silicagel 60 with the same solvent. In this way surfactins and iturins were eluted. Subsequently the fengycins were eluted with $CHCl_3/C_2H_5OH/CH_3OH/H_2O$ 7:3.5:3:1.5 (v/v).

The fengycin mixture was separated by RP-HPLC on nucleosil 100–7 C_{18} with a gradient of 0.1% trifluoroacetic acid in H_2O and CH_3OH (23:77 for 33 min, 10:90 for 10 min, 23:77 for 7 min) and rechromatographed with the same system (20:80 for 33 min, 10:90 for 5 min, 20:80 for 5 min) for fengycin IX and 10:90 for fengycin XII (all v/v).

Amino acid and fatty acid analysis

Total hydrolysis and TAP-derivatisation was effected as described earlier (Michalke *et al.*, 1996).

Reactions proving the lactone structure

Hydrolysis. A solution of 2 mg of fengycin IX and XII, respectively, in 0.2 ml CH_3OH and 0.1 ml 1 N NaOH were kept for 2 hrs. at room temp. After addition of 1 ml 0.1 N HCl the reaction mixture was brought to dryness i. v. The fengycinic acids thus obtained were purified by HPLC as described above for the rechromatographing of the fengycins.

Reduction with $LiBH_4$. 2 mg fengycin IX were added to a solution of 0.6 mg $LiBH_4$ in 1 ml tetrahydrofuran and stirred for 5 hrs. at 75 °C, then a mixture of 5 ml 6 N HCl and 15 ml CH_3OH was added in portions. The product was brought to dryness, several times dissolved in water and again brought to dryness, and finally without further purification subjected to total hydrolysis (see above).

Reduction with $NaBH_4$. 2 mg fengycin IX in 0.5 ml CH_3OH were added to a solution of 6 mg $NaBH_4$ in 0.3 ml CH_3OH/H_2O (1:1, v/v). After 4

days several drops of glacial acetic acid were added. The mixture was brought to dryness and without further purification subjected to total hydrolysis (see above).

Oxidation. 2 mg fengycin IX dissolved in 0.1 ml of a freshly prepared solution of 100 mg CrO_3 and 0.1 ml pyridin in 3 ml glacial acetic acid were kept for 24 hrs. at room temp. The sample was diluted with H_2O and concentrated i.v.; this procedure was repeated several times, the residue was dissolved in 0.6 ml H_2O and extracted 4 times with *n*-butanol. After 4 days the yellow color of the united organic phases had changed to pale green. The reaction mixture was brought to dryness i.v., dissolved in H_2O and brought to dryness again. The residue was subjected to total hydrolysis (see above).

Results

From the fengycin mixture obtained by RP-HPLC two fractions were isolated which from their molecular masses seemed to correspond to components described by Koch (1988), viz. fengycin IX and XII. Fengycin IX is a major constituent, XII could be obtained only in small amounts.

The structure of fengycin IX

In the UV spectrum only the Tyr absorptions at 220 and 274 nm can be seen. The IR spectrum shows strong amide I (1663 cm^{-1}) and II (1542 cm^{-1}) bands. A weak shoulder at 1750 cm^{-1} can be interpreted as an ester C=O-band; it is corroborated by the C-O-band at 1204 cm^{-1} typical for phenol esters. The substitution band for a *p*-substituted benzene ring occurs at 801 cm^{-1} (Günzler and Böck, 1983).

Analysis of the amino acid TAP derivatives showed the presence of D-Ala, L-Glu, L-Ile, D-Orn, L-Pro, D-aThr, D- and L-Tyr. In the GC two additional peaks of almost equal (ca. 3:4) abundance can be found which show the same MS fragmentation pattern. The only characteristic peak of high abundance in addition to the common hydrocarbon ions is *m/z* 255 which could be $[M - C_3H_6 - CF_3COO]^+$ of the TAP derivative of 3-hydroxyhexadecanoic acid. This is puzzeling since whereever the chirality of the 3-hydroxy fatty acids of bacterial lipopeptides had been determined it had been found to be R. Whether racemisation occured during the analysis or the original TAP derivative and a decomposition product give the same mass spectra is a moot point.

The nominal molecular mass of fengycin IX as determined by FAB-MS is 1462 u ($[M+H]^+$ at *m/z* 1463; cf. also the ESI data below). Taking into account the amino and fatty acid analysis the composition of fengycin IX should be 1 Ala, 1 Gln, 2 Glu, 1 Ile, 1 Orn, 1 Pro, 1 aThr, 2 Tyr and $C_{15}H_{30}(OH)COOH$ minus 1 H_2O due to the lactone structure suggested by the IR spectrum. The lactone bond could be confirmed chemically: Mild alkaline hydrolysis that does not cleave amide bonds lead to the ring opened fengycinic acid IX (nominal molecular mass by ESI-MS 1480 u). Treatment with $NaBH_4$ or $LiAlH_4$ resulted in the reduction of Ile to the corresponding alcohol isoleucinol (after total hydrolysis and TAP derivatisation Ile was missing among the products; instead the presence of bis-trifluoroacetyl isoleucinol could be demonstrated by GC-MS) showing that Ile was involved in the ester bond. The alcohol component of the ester bond could be aThr, one of the Tyr or the hydroxy fatty acid. CrO_3 oxidizes compounds with free hydroxyl groups while esterified hydroxyls are not affected. Amino acid analysis after oxidation showed that aThr, L-Thr and the hydroxy fatty acid were missing. Hence the hydroxyl group of D-Thr is esterified.

NMR-analyses of fengycin IX confirmed the composition mentioned above and allowed to establish the sequence of the amino acids (for a detailled discussion of the NMR techniques see Evans, 1995; the proton resonances within one amino acid residue can be detected by COSY and HOHAHA experiments, direct ^1J C,H-connections follow from HMQC, while ^2J up to ^4J couplings can be determined by HMBC experiments allowing *i. a.* to establish connections between protons and the CO of the preceding amino acid, while NOESY connects NH- with CH-protons of the preceding amino acid). The ^1H- and ^{13}C NMR spectra were determined both in CD_3OH and in DMSO-d_6 (see Tables I/II and III/IV). Overlapping patterns can be separated in this way.

The signals of the 3-hydroxyhexadecanoic acid correspond to the literature data (Nishikiori *et al,* 1986a) establishing the sequence -CH_2-CHOH-CH_2-CONH-. Only the triplet for the terminal

Table I. ^{1}H-NMR signals of fengycin IX in CD_3OH at 298 K. (FA: 3-Hydroxy-*n*-hexadecanoic acid residue)

Fatty acid	H_2	H_3	H_4	H_{5-15}	H_{16}	
FA	2.39	4.01	1.45	1.24	0.88	
Amino acid	**NH**	**H_α**	**H_β**	**H_γ**	**H_δ**	**NH_2**
Glu[1]	8.32	4.31	2.42	1.98 2.08		
Orn	8.65	4.30	1.72 1.93	1.72	2.94	–
aThr	6.88	3.98	3.35	1.08		
Glu[2]	7.79	4.04	2.02	2.30 2.61		
Ala	8.60	4.98	1.32			
Pro		4.32	1.92 2.31	2.01	3.64	
Gln	7.56	4.39	1.97	2.41 2.53		6.80 7.59
Ile	8.62	3.84	1.79	CH_2 1.27 CH_3 0.95	0.84	
Amino acid	**NH**	**H_α**	**H_β**	**$H_{2/6}$**	**$H_{3/5}$**	
Tyr[1]	7.92	4.31	2.86 3.27	7.16	7.12	
Tyr[2]	7.89	4.66	3.01 3.09	7.09	6.70	

CH_3-group can be seen which demonstrates the absence of any branching.

For two Glu the shift of the γ-CH_2-groups indicates a free γ-carboxylic acid (Wüthrich, 1976). The third glutamic acid is derivatized as Gln: Due to the restricted rotation of an amide bond 2 signals for the γ-$CONH_2$-group and 2 signals for the γ-CH_2-group coupling with each other can be discerned (cf. Voss *et al.*, 1999). The free δ-NH_2-group of Orn follows from a comparison with literature data especially for the δ-CH_2-group (e.g., Gwose and Taraz, 1992) and by the ^3J-coupling between NH and CH_α. In contrast to amide protons the exchange for a free NH_2-group is too fast for a detection of the corresponding signal. That the OH-group of aThr is free can be deduced from the shift of the ß-CH proton (Wüthrich, 1976); in the case of esterification it would be expected at a lower field by ca. 1 ppm (Poppe *et al.*, 1987). For the two Tyr especially the aromatic resonances differ due to the electron withdrawing ester group in one case.

After the amidic NH protons had been identified by correlations with the CH protons of the respective amino acids the sequence of the amino acids could be determined by NOESY interactions between the NH and the α-protons of the preceding amino acid (full arrows in Fig. 1). The Pro/Ala sequence was established by a NOE effect between the δ-CH_2 of Pro and the α-CH of Ala. Additional sequence information and thus confirmation was gained from HMBC correlations between CO signals and the NH or CH signals of the following amino acid (half arrows in Fig. 1).

The amino acid sequence as derived from the NMR data could be confirmed by ESI-MS.

Table II. ^{13}C-NMR signals of fengycin IX in CD_3OH at 298 K. (FA: 3-Hydroxy-*n*-hexadecanoic acid residue)

Fatty acid	CO	C_2	C_3	C_4	C_{5-13}	C_{14}	C_{15}	C_{16}
FA	174.8	44.2	69.7	38.4	30.7	32.9	23.6	14.3
Amino acid	**CO**	**C_α**	**C_β**	**C_γ**	**C_δ**	**CO**		
Glu[1]	174.6	55.0	27.1	27.9		176.6		
Orn	173.4	54.9	28.9	25.1	40.4			
aThr	172.2	60.5	69.7	20.5				
Glu[2]	174.6	55.0	27.5	31.2		177.6		
Ala	173.1	47.4	17.1					
Pro	174.9	63.3	30.3	26.0	52.2			
Gln	173.3	54.6	26.2	32.5		175.2		
Ile	170.6	60.9	35.9	CH_2 26.6 CH_3 15.6	11.0			
Amino acid	**CO**	**C_α**	**C_β**	**C_1**	**$C_{2/6}$**	**$C_{3/5}$**	**C_4**	
Tyr[1]	171.8	57.7	37.6	134.9	131.1	122.7	151.3	
Tyr[2]	175.0	56.5	38.5	128.8	131.4	116.3	157.4	

Table III. ^{1}H-NMR signals of fengycin IX in DMSO-d_6 at 298 K. (FA: 3-Hydroxy-*n*-hexadecanoic acid residue)

Fatty acid	H_2	H_3	H_4	H_{5-15}	H_{16}	
FA	2.22	3.79	1.31	1.16	0.84	
Amino acid	**NH**	**H_α**	**H_β**	**H_γ**	**H_δ**	**NH_2**
Glu[1]	8.06	4.24	1.73 1.85	2.20 2.27		
Orn	8.23	4.30	1.53 1.72	1.72	2.76	9.83
aThr	7.71	3.91	3.48	0.74		
Glu[2]	7.89	3.95	1.78 1.97	2.17 2.35		
Ala	8.15	4.60	1.11			
Pro		4.22	1.75 2.06	1.72 1.90	3.39 3.46	
Gln	7.95	4.18	1.68 1.97	2.15		6.77 7.31
Ile	8.63	4.06	1.78	CH_2 1.05 1.28 CH_3 0.85	0.78	
Amino acid	**HN**	**H_α**	**H_β**	**$H_{2/6}$**	**$H_{3/5}$**	
Tyr[1]	8.14	4.42	2.82 2.90	7.15	6.92	
Tyr[2]	7.97	4.69	2.77 2.90	6.99	6.62	

ESI is a so-called soft ionization method yielding mainly $[M + H]^+$ and hardly any fragment ions. The formation of structure specific fragments can, however, be induced by collision with an auxilliary gas (collision induced decomposition, CID). With the Finnigan 900 ST mass spectrometer this is possible either in the octapole before the ion trap or in the ion trap. In the latter case ions of a single mass can be selected and brought to decomposition. In this way sequences of fragmentation processes can be determined (MS^n) (Lehmann, 1996). The two techniques give partially identical, but to some extent also complementary results.

The most important fragments are formed by the cleavage of the amide bonds. Those containing the N-terminus (NH_2-CHR-CO^+ etc.) are termed B_n-fragments the index giving the number of amino acids contained, those comprising the C-terminus (HOOC-CHR-NH_3^+ etc.) Y_m+2-fragments (the number indicating the 2 additional H). Internal fragments formed by further decomposition of B- or Y-fragments and containing neither the N- nor the C-terminus are described as Y_mB_n-fragments (Papayannopoulos, 1995).

Cyclopeptides can be sequenced by CID-MS, but since in the first step any of the peptide bonds may be opened the mass spectra can be rather complex (Eckart, 1994). For the present investigation, therefore, the ring-opened fengycinic acid IX was subjected to CID. The results are assembled in Fig. 2.

Summarizing it can be stated that except for the ester bond between L-Ile and D-Tyr only peptidic bonds (CO-NH_α) are present in fengycin IX. The δ-NH_2-group of Orn, the OH-group of D-aThr and L-Tyr and the γ-COOH-

Table IV. ^{13}C-NMR signals of fengycin IX in DMSO-d_6 at 298 K. (FA: 3-Hydroxy-*n*-hexadecanoic acid residue)

Fatty acid	CO	C_2	C_3	C4	C_{5-13}	C_{14}	C_{15}	C_{16}
FA	171,6	43,3	67,5	37,1	29,1	31,2	23,6	14,0
Amino acid	**CO**	**C_α**	**C_β**	**C_γ**	**C_δ**	**CO**		
Glu[1]	171,7	52,4	27,1	30,3		174,3		
Orn	170,3	52,1	28,8	24,8	44,1			
aThr	169,9	58,8	67,5	20,2				
Glu[2]	171,3	52,7	26,0	30,0		174,0		
Ala	169,4	46,4	17,2					
Pro	171,9	60,3	29,4	27,1	47,0			
Gln	170,3	52,1	26,5	31,5		172,1*		
Ile	170,1	57,5	35,0	CH_2 24,8 CH_3 15,3	10,7			
Amino acid	**CO**	**C_α**	**C_β**	**C_1**	**$C_{2/6}$**	**$C_{3/5}$**	**C_4**	
Tyr[1]	171,0	54,8	37,5	134,5	130,3	121,2	149,1	
Tyr[2]	171,9	53,8	37,9	127,2	130,2	114,9	155,9	

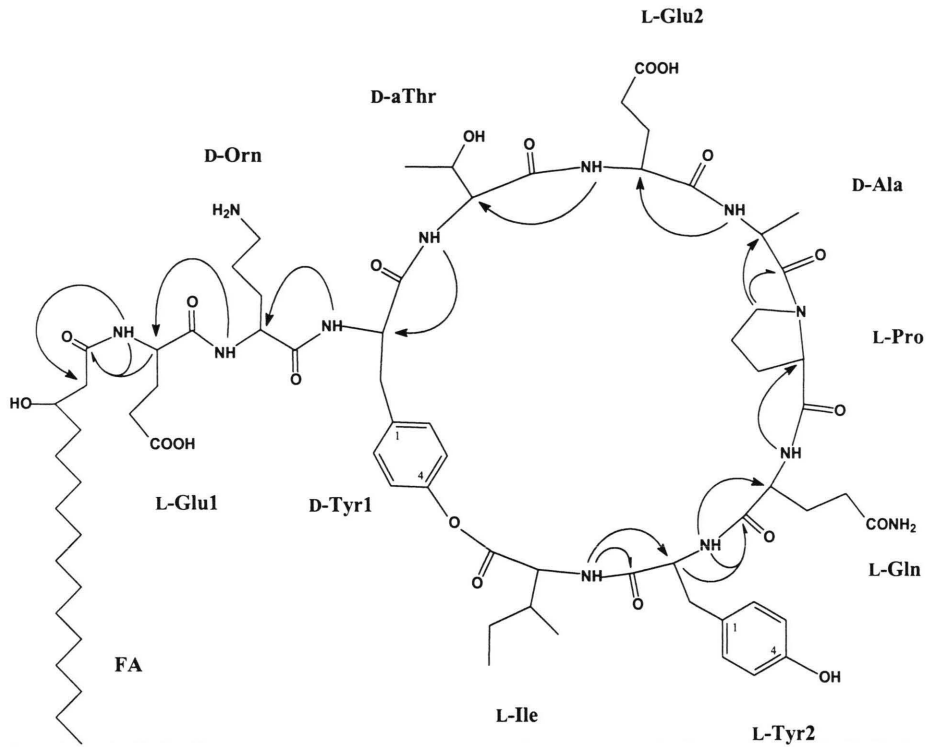


Fig. 1. Sequence specific correlations in the NMR spectra of fengycin IX (**1**): full arrows NOE effects (NOESY), half arrows long range coupling (HMBC).

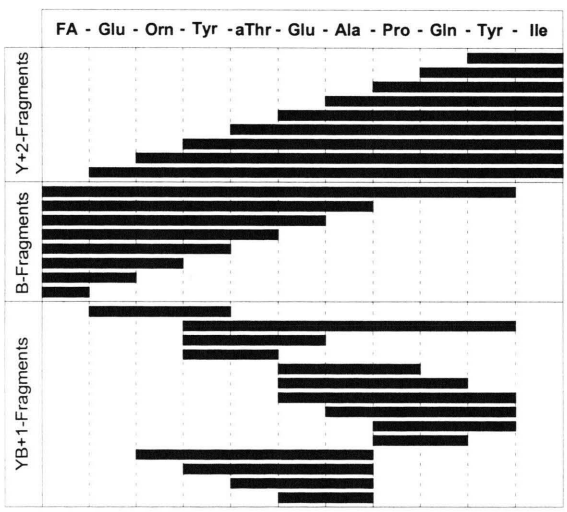


Fig. 2. Sequence specific ESI MS^n-fragments of fengycinic acid IX.

groups of two L-Glu are free, the third one is present as L-Gln. The structure of fengycin IX is **1** as depicted in Fig. 4.

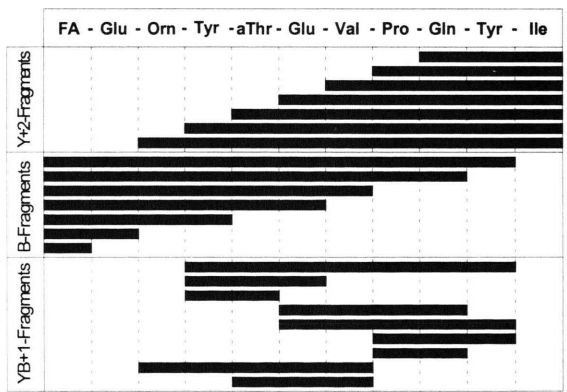
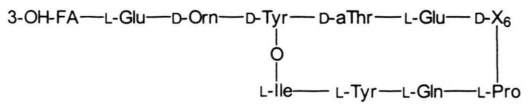


Fig. 3. Sequence specific ESI MS^n-fragments of fengycinic acid XII.

The structure of fengycin XII

Fengycin XII was available only in small amounts. The structure elucidation rests, therefore, mainly on a total hydrolysis and a MS sequence analysis. As stated in the introduction, the structures of the lipopeptides within the same class



Fengycin	3-OH-Fatty acid	D-X_6
Fengycin IX (**1**)	n-C_{16}	Ala
Fengycin XII (**2**)	C_{17}	Val

Fig. 4. Structure of fengycin IX (**1**) and of fengycin XII (**2**).

are highly conserved; thus, a close similarity between fengycin IX and XII can be assumed.

The UV and IR spectra of the two fengycins are practically identical. The amino acid analysis after total hydrolysis showed the presence of D-Val instead of D-Ala. Again two peaks in the GC-MS analysis (about 3:4 in intensity) came from the TAP-derivatized hydroxy fatty acid giving identical mass spectra, the main fragment (*m/z* 269) being by 14 u heavier than in the case of fengycin IX. This indicates the presence of a 3-hydroxyheptadecanoic acid residue. These results are in agreement with the molecular mass of 1504 u as determined by FAB- and ESI-MS (42 u heavier than fengycin IX, viz. C_{17}- vs. C_{16}-acid and Val vs. Ala). Mild hydrolysis resulted in the formation of fengycinic acid XII (addition of 18 u as determined by ESI-MS) in agreement with the presence of an ester bond. Under the same experimental conditions as above fengycinic acid XII was subjected to octapole and ion trap CID. The fragments obtained are assembled in Fig. 3. All the data are in agreement with analogous structures for fengycin IX (**1**) and XII (**2**) as depicted in Fig. 4. In the absence of NMR data it cannot be decided whether the C_{17}-acid is straight or branched.

Discussion

The structural suggestions of Koch (1988) for her fengycins A (C_{16}-to C_{18}-3-hydroxy fatty acids, X_6 = Ala) and B (*i*-C_{16} and a C_{17} acid, X_6 = Val) could be confirmed and completed. Of interest is the following observation: From *Bacillus cereus* lipopeptides named **plipastatins** had been isolated. They differ in their structures from the **fengycins** described here only insofar, that the D- and the L-Tyr are interchanged. However, plipastatins and fengycins differ drastically in their biological activities: Fengycins stimulate the enzyme phospholipase A_2 (Vanittanakom *et al.*, 1986), plipastatins act as inhibitors (Nishikiori *et al.*, 1986 a and b). Also the antibiotic activity seems to be different.

Acknowledgement

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Note added in proof: In a recent publication (Steller *et al.*, 1999) a fengycin structure is proposed which locates correctly Glu and Gln, but places D- and L-Tyr in the wrong way.

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