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STRUCTURAL AND MEMBRANE MODIFYING PROPERTIES OF SUZUKACILLIN, A PEPTIDE ANTIBIOTIC RELATED TO ALAMETHICIN

PART A. SEQUENCE AND CONFORMATION

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SUMMARY

The primary structure and conformation of the polypeptide antibiotic suzukacillin A are investigated. Suzukacillin A is isolated from the *Trichoderma viride* strain 1037 and exhibits membrane modifying and lysing properties similar to those of alamethicin.

A combined gas chromatographic mass spectrometric analysis of the trifluoroacetylated peptide methyl esters of partial hydrolysates revealed a tentative sequence of 23 residues including 10 2-methylalanines and one phenylalaninol, which shows many fragments known from alamethicin: Ac-Aib-Pro-Val-Aib-Val-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Leu-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu(Pheol)-Gln-OH. All chiral amino acids and phenylalaninol have L-configuration. Ultraviolet and infrared spectroscopy, circular dichroism in various solvents and in particular ^{13}C nuclear magnetic resonance have been used for a comparative study of suzukacillin with alamethicin. Suzukacillin has a partially α-helical structure and the helix content increases largely from polar to lipophilic solvents. Suzukacillin aggregates more strongly than alamethicin in aqueous media due to a longer α-helical part and higher number of aliphatic residues. A part of the α-helix is exceptionally stabilized due to 2-methylalanine residues shielding the peptide bonds from interactions with polar solvents. In lipophilic solvents and lecithin vesicles particularly large temperature induced reductions of the high α-helix content are found for alamethicin. Suzukacillin shows similar temperature coefficients in lipophilic media, however, in contrast to alamethicin a more linear change in intensity of the Cotton effects is observed.

INTRODUCTION

The polypeptide antibiotic suzukacillin A isolated in 1965 from the *Trichoderma viride* strains No. 63 Cl [1, 2] and No. 1037 were found to exhibit membrane

modifying properties similar to those of alamethicin [3]. Alamethicin has been isolated in 1966 from the culture medium of another *T. viride* strain [4, 5]. This peptide gained considerable interest because it induces a voltage-dependent ionic conductance in lipid membranes [6] caused by formation of pores with variable diameter [7].

The structural features of alamethicin have been investigated by us in detail using ^{13}C nuclear magnetic resonance and circular dichroism in order to obtain experimental access to the molecular mechanism of the pore forming process [8]. Some time ago it came to our attention that crude alamethicin, isolated from the culture broth, shows heterogeneity in its composition. Thin-layer chromatography on silicagel revealed several components [9]. Furthermore there are at least two other strains of *T. viride* which are known to produce different antibiotics containing several 2-methylalanine residues and other amino acids constituting alamethicin. One of these peptides is the antibiotic suzukacillin A [1, 2]. With respect to the different molecular mechanisms of other membrane modifying peptides, e.g. gramicidin A and valinomycin, the structural and conformational prerequisites for the peculiar pore forming action of alamethicin are of major interest.

The availability of membrane-active natural analogs of alamethicin and of peptides related to alamethicin such as suzukacillin facilitates the necessary structure-activity studies. Therefore we report in the following a sequential and spectroscopic investigation on suzukacillin A, which is based mainly on combined gas-liquid chromatography-mass spectrometric studies on partial hydrolysates, on ultraviolet and infrared absorption spectra and in particular on solvent and temperature dependent studies of the circular dichroism and the ^{13}C nuclear magnetic resonance. The results are discussed with respect to the data and properties found for alamethicin. Part B of our studies reports on the membrane modifying properties of suzukacillin on black lipid films [3]. In a separate communication a comparative study of the unusual membrane lysing properties of alamethicin [10] will be reported which have recently also been found for suzukacillin on erythrocytes and Ehrlich ascites tumor cells [11].

MATERIALS AND METHODS

Substances

Suzukacillin is isolated from the culture medium of *T. viride* 1037 as described [1, 2]. The crude antibiotic consists mainly of two peptide components, A and B, which are separated by preparative thin-layer chromatography on silicagel G or alumina column chromatography. All experiments described in the following are performed with the major component suzukacillin A, which is obtained in colourless hexagonal crystals from chloroform/acetone. The alamethicin used for comparison is purified and characterized by various methods as described in detail elsewhere [8].

In order to check the suzukacillin and alamethicin preparations for the presence of atoms other than C, H, O and N, X-ray photoelectron spectra were taken in all important regions of binding energies. The experimental procedure and spectrometer system used has been described previously [12]. Except for very small traces of Na^+, Cl^- and SO_4^{2-}, commonly present in biological materials, nothing else is detected.

All solvents used for the ultraviolet absorption and circular dichroism in-

vestigations are Uvasols® or p.a. grade. For the ^{13}C nuclear magnetic resonance measurements ^{12}C-enriched deuterated methanol and chloroform were used containing less than 0.01 % ^{13}C. All solvents and reagents are products of Merck Co., Darmstadt.

Gas-liquid chromatography-mass spectrometry

For the determination of the amino acid composition 1 mg of suzukacillin A is hydrolysed in 6 M HCl at 110 °C for 72 h in a sealed glass tube. After evaporation of the HCl the residue is esterified with a solution of 1.5 M HCl in isopropanol at 100 °C for 1 h. The reagent is removed in nitrogen stream and the amino acid isopropyl esters are acylated with a solution of trifluoroacetic anhydride in dichloromethane (50 : 200 µl) at room temperature. After removal of the reagent the mixture of trifluoroacetylated amino acid isopropyl esters is dissolved in 50 µl of chloroform and subjected to gas-liquid chromatographic and mass spectrometric investigations.

The absolute configuration of the amino acids is determined by gas-liquid chromatography of the trifluoroacetylated amino acid isopropyl esters on a 20 m glass capillary coated with *N*-trifluoroacetyl-L-valyl-L-valine cyclohexyl ester at 110 °C (Carlo Erba gas chromatograph model 2101) [13, 14].

For a partial hydrolysis 2.5 mg suzukacillin A crystals are hydrolysed at 37 °C for 38 h in 1 ml concentrated hydrochloric acid in a sealed vial. The hydrochloric acid is removed at reduced pressure, and the residue is taken up in 1 ml of 1.5 M methanolic HCl. After 1 h at 20 °C the esterifying reagent is removed in a stream of nitrogen. The residual mixture of peptide methyl ester hydrochlorides is trifluoroacetylated in 200 µl dichloromethane and 50 µl trifluoroacetic acid anhydride at 20 °C for 1 h. After removal of the reagent with a stream of nitrogen the residue is dissolved in 50 µl chloroform and investigated by gas-liquid chromatography and mass spectrometry. The trifluoroacetylated peptide esters are chromatographed on a 2 m packed glass column, packed with 3 % OV-17 on Chromosorb WAW. The instrument used is a LKB 9000 gas chromatograph-mass spectrometer combination. The mass spectra are recorded at 70 eV ionisation energy at an ion source temperature of 250 °C. Essentially the same procedure is used for a reinvestigation of the alamethicin sequence (see ref. 8).

Circular dichroism and ultraviolet absorption spectra

A Roussel-Jouan Dichrograph CD 185 (Roussel-Jouan, Paris) is used for the circular dichroism investigations and the measurements are standardized using epiandrosterone in dioxane solution with $[\Theta]_{304\ nm}$ of 10 925 degree · cm^2 · dmol^{-1}. The circular dichroism data are calculated from the spectra as mean circular dichroic absorption per amino acid residue $\Delta\varepsilon_M$, assuming a molecular weight of 1964.3 for alamethicin and a weight of 98 for the average amino acid residue (20 residues). These data include the corrections (*N*-acetyl-2-methylalanine and phenylalaninol residues) made on the primary structure by Martin and Williams [15] and by us [8]. The corresponding calculated data for suzukacillin A are: M_r 2346.8, weight of average amino acid residue 98 (24 residues). All circular dichroism spectra are recorded at different cell path lengths at least twice and the temperature of the cell holder was thermostated (±0.5 °C). The error of the molar ellipticities is about 10 % at 223 nm and somewhat larger at 207 nm. Ultraviolet absorption spectra are recorded on a

spectrometer Cary-15 (Varian GmbH, Darmstadt). Circular dichroism and ultraviolet absorption spectra are taken with peptide solutions of about 0.5 mg/ml.

^{13}C NMR spectra

Solutions of 150 mg suzukacillin per 1 ml deuterated ^{12}C-enriched solvent (methanol, chloroform and mixtures of both) are used for ^{13}C nuclear magnetic resonance measurements. The Fourier-transform spectra are taken with a Bruker HFX-90 spectrometer (38 cm magnet, 22.63 MHz for ^{13}C) with proton broad-band decoupling. 30 k-pulse interferograms are accumulated on an 8 k storage with an analog-digital resolution of 7 bits (computer FT-1083, Nicolet Co.). The pulse angle used is 60° because of the short relaxation times and the pulse interval is 0.82 s. The ^{13}C chemical shifts are automatically read and printed as δ values in ppm with reference to internal tetramethylsilane ($\delta = 0$ ppm). The off-resonance spectra are taken with different reduced coupling constants. The temperature of the samples is 30 °C.

Synthesis and characterization of 2-amino-1-hydroxy-3-phenylpropane

16.52 g (0.1 mol) phenylalanine and 4.92 g (0.13 mol) $NaBH_4$ are finely ground and suspended in 100 ml anhydrous tetrahydrofuran. 20 ml boron trifluoride diethyl ether complex are gradually added to the stirred mixture [16]. After 8 h the same amounts of $NaBH_4$ and BF_3 etherate are again added and the stirring continued for 8 h. 20 ml ethanol are added to destroy the excess $NaBH_4$. The solvent is evaporated to dryness on a rotatory evaporator. The residue is dissolved in cold aqueous 0.1 M NaOH and extracted three times with chloroform. The extract is dried over Na_2SO_4 and the solvent evaporated. The residual colourless oil crystallized spontaneously. Phenylalaninol is recrystallized from $CHCl_3$ light petroleum or ether to yield long thin needles (m.p. 90–91 °C, reported 91–93 °C [17]; $R_F = 0.45$ silicagel thin-layer chromatography plates Merck, solvent system 1-butanol/acetic acid/water, 3 : 1 : 1).

Elemental analysis. $C_9H_{13}NO$ (151.21) calculated: C, 71.49; H, 8.67; N, 9.26; found: C, 71.23; H, 8.70; N, 9.53.

^{13}C NMR in C^2HCl_3 (in ppm from $\delta_{TMS} = 0$ ppm): C_α, 54.2; C_β, 40.6; CH_2OH, 65.9; phenyl: C-1, 138.3; C-2, 6,129.2; C-3, 5,128.6; C-4, 126.3.

Mass spectrum. N,O-Di-trifluoroacetyl derivatives of both L- and D,L-phenylalaninol were prepared by reaction of trifluoroacetic anhydride in methylene chloride (50 : 200 µl) and the mass spectra were recorded on a gas chromatograph-mass spectrometer (LKB 9000, LKB Producter, Stockholm). Fragmentation (*m/e*): $M^+ = 343$, $M-F = 324$, $M-CF_3 = 274$, $M-CF_3CONH_2 = 230$, $M-CH_2OCOCF_3 = 216$, $CF_3CONH{=}C{=}CH_2 = 138$, $C_7H_7 = 91$, $CF_3 = 69$.

Resolution of D,L-*phenylalaninol*

The gas chromatographic resolution of *N,O*-bis-trifluoroacetyl-D,L-phenylalaninol was performed on glass capillary columns [14] coated with the chiral stationary dipeptidic phase *N*-trifluoroacetyl-L-valyl-valine-cyclohexylester as described above. Contrary to the usual order of emergence of trifluoroacetylated D- and L-amino acid esters on this and various other L,L-phases [13] the L-enantiomer of the amino alcohol phenylalaninol ((*S*)-2-amino-1-hydroxy-3-phenyl-propane) is eluted before the D-isomer.

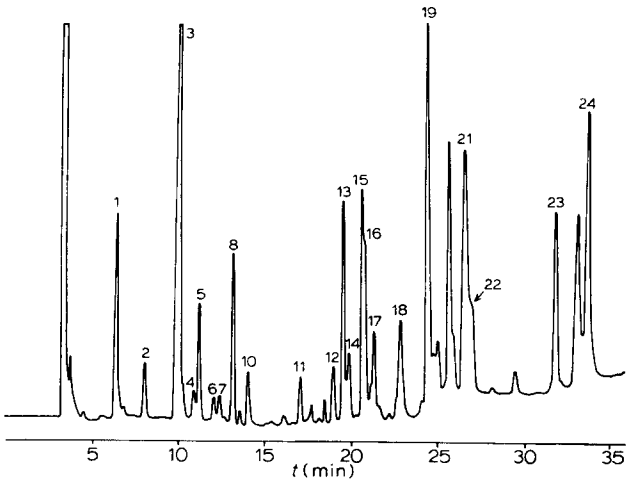

Fig. 1. Partial hydrolysate of suzukacillin A: example of a gas chromatogram of the trifluoroacetylated (TFA) peptide methyl esters, hydrolysis in concentrated hydrochloric acid at 37 °C for 38 h. (1), H-Aib-OMe (free NH_2); (2), TFA-Pro-OMe; (3), *N,O*-$(TFA)_2$-L-Phenylalaninol; (4), TFA-Aib-Ala-OMe; (5), TFA-Ala-Aib-OMe; (6), TFA-Aib-Gly-OMe; (7) TFA-Val-Aib-OMe and TFA-Aib-Val-OMe; (8), TFA-Leu-Aib-OMe; (9), TFA-Aib-Leu-OMe; (10), *N*-TFA-phenylalaninol (free OH); (11), TFA-Glu(OMe)-Aib-OMe; (12), TFA-Ala-Aib-Aib-OMe; (13), TFA-Ala-Aib-Ala-OMe; (14), TFA-Val-Aib-Aib-OMe; (15), TFA Gly-Aib-Aib-OMe; (16), TFA-Aib-Leu-Aib-OMe; (17), TFA-Val-Aib-Val-OMe; (18), TFA-Gly-Leu-Aib-OMe; (19), TFA-Glu(OMe)-Glu(OMe)-OMe; (20), TFA-Pro-Val-Aib-OMe; (21), TFA-Ala-Aib-Ala-Aib-OMe; (22), TFA-Glu-(OMe)Aib-Leu-OMe; (23), TFA-Pro-Val-Aib-Aib-OMe; (24), TFA-Pro-Val-Aib-Val-OMe.

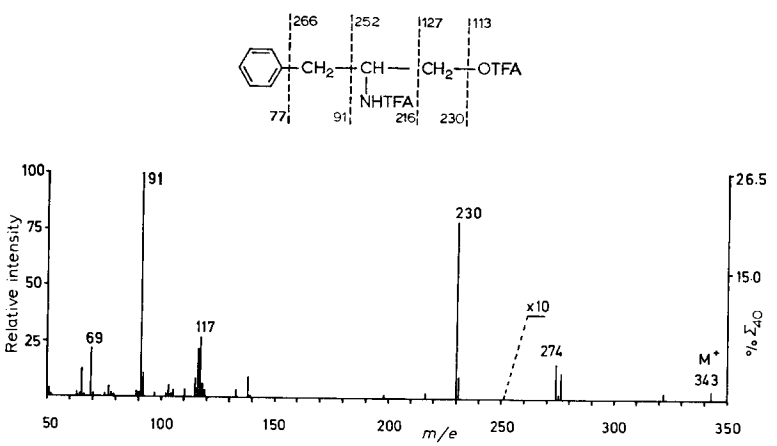

Fig. 2. Mass spectrum and fragmentation of trifluoroacetylated (TFA) phenylalaninol (peak No. 3 in Fig. 1, gas liquid chromatography-mass spectrometry analysis of a partial hydrolysate of suzukacillin A).

Ultraviolet absorption and circular dichroism of L-*phenylalaninol*

Concentration 25 mM in 0.1 M HCl, Temp. 23 °C: λ [nm] (ε): 251 (145), 257 (168), 263 (124); λ [nm] ([Θ] degree · cm^2 · dmol^{-1}): 251 (−53), 253 (−56), 259 (−73), 266 (−50).

RESULTS AND DISCUSSIONS

Sequence analysis

As in the case of alamethicin, routine analyses of total hydrolysates of suzukacillin A in the amino acid analyser give varying molar ratios of amino acids due to incomplete hydrolyses. Furthermore for the 2-methylalanine residues asymmetric peak shapes and an unusually low ninhydrin colour yield are found, which is about eight times lower compared to that of protein amino acids. However, there is a reproducible linearity between colour yield and amount of 2-methylalanine (Mayr and Jung, unpublished results). Therefore the amino acid ratio reported in the earlier study [1] is revised. Using the molar ratios obtained from gas chromatograms of esterified and trifluoroacetylated total and partial hydrolysates and those of several routine amino acid analysis on the ion-exchange analyser combined with results of the ^{13}C nuclear magnetic resonance study, we determined the amino acid composition given in the following for suzukacillin A and the major component of the alamethicins.

	Aib	Glx	Val	Ala	Pro	Gly	Leu	Pheol
Suzukacillin A	9–10	3	3	2	2	1–2	2	1
Alamethicin	7–8	3	2	2	2	2	1	1

Suzukacillin A consists of 23–24 and alamethicin of 19–20 residues.

In order to determine the sequence a partial hydrolysate is esterified, trifluoroacetylated and subjected to combined gas liquid chromatography-mass spectrometry. Fig. 1 shows a typical elution pattern and the assignments of the peaks of a number of well separated di-, tri- and tetrapeptides, which allow the construction of larger pieces of the suzukacillin A sequence. Furthermore this analysis revealed phenylalaninol to be a structural component. The existence of such a benzyl substituted residue was shown already in our earlier ^{13}C nuclear magnetic resonance spectra [8, 10] of alamethicin.

Fig. 2 shows the mass spectrum of the intense peak No. 3 of the gas chromatogram of the partial hydrolysate (Fig. 1) and the assignments of the fragment ions leading to the structure of phenylalaninol. This assignment is confirmed by comparison with synthetic phenylalaninol.

A further sample is totally hydrolysed and the resulting amino acids are esterified with isopropanol, trifluoroacetylated and subjected to gas-liquid chromatography on glass capillary columns coated with a chiral stationary dipeptidic phase for the determination of the absolute configuration. All chiral amino acids are found to have L configuration, including the amino alcohol phenylalaninol. With respect to the mechanism of the resolution of enantiomers by this method [13] it is of interest that the enantiomers of the D,L-amino alcohol show the reversed order of emergence than those of D,L-amino acids. According to models of solvent-solute

TABLE I

PEPTIDE FRAGMENTS OF SUZUKACILLIN FOUND BY GAS-LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS OF THE TRIFLUOROACETYLATED PEPTIDE METHYL ESTERS OF PARTIAL HYDROLYSATES AND THEIR ARRANGEMENT TO A POSSIBLE SEQUENCE

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                                                      Glu-Aib
                                                      Glu-Aib-Leu
                                                          Aib-Leu
      Pro-Val          Ala-Aib                            Aib-Leu-Aib              Pro-Val
      Pro-Val-Aib      Ala-Aib-Ala                            Leu-Aib              Pro-Val-Aib
      Pro-Val-Aib-Val  Ala-Aib-Ala-Aib                            Aib-Gly          Pro-Val-Aib-Aib
          Val-Aib          Aib-Ala                                    Gly-Leu          Val-Aib
          Val-Aib-Val          Ala-Aib                                Gly-Leu-Aib      Val-Aib-Aib Pheol
              Aib-Val          Ala-Aib-Aib                                Leu-Aib      Gly-Aib-Aib*Glu-Glu
Ac-Aib-Pro-Val-Aib-Val-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Leu-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu(Pheol)-Gln-OH
                                                                          (Gly)
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Tentative sequence of suzukacillin

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Ac-Aib-Pro- - - - - - - -  - - - - Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu(Pheol)-Gln-OH
                                            (Aib)**
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Sequence of alamethicin

* From a possible analog.

** Alteration of sequence [29].

interactions developed by Gil-Av and Feibush [18] the *N,O*-bis-trifluoroacetyl-phenylalaninol would belong to the so-called "C_7" type conformers. The D and L enantiomers form diastereomeric hydrogen-bridged complexes "D/LL" and "L/LL" with the chiral solvent "LL". Since the "D/LL" complex is the more stable one, the L-phenylalaninol emerges before the D-enantiomer. Correspondingly the same order of emergence has been observed earlier for the enantiomers of leucinol and valinol (*O*-pivaloyl-*N*-trifluoroacetyl derivatives) on the chiral phase *N*-lauroyl-L-valyl-*t*-butylamide [19]. L-Phenylalaninol has been detected before as a natural constituent of the antibiotic antiamoebin, a peptide isolated from the mycelium of the organism *Emericellopsis poonensis* [20].

Table I summarizes the results of the combined gas chromatography-mass spectrometry analysis and shows the overlapping of the fragments to yield the tentative total sequence of suzukacillin A in comparison to that of alamethicin. However, there are several other possibilities for an arrangement of the fragments. We were not able so far to detect by gas-liquid chromatography-mass spectrometry peptides possessing N-terminal acetylated 2-methylalanine residues, e.g. Ac-Aib-Pro-Val for suzukacillin or Ac-Aib-Pro-Aib for alamethicin. The Ac-Aib terminus is proposed by Martin and Williams according to proton nuclear magnetic resonance spectra for alamethicin [15]. A further ambiguity is the linkage of L-phenylalaninol to the C-terminus. We cannot judge, as yet, whether phenylalaninol is bound as -Glu(Pheol)-Gln-OH or as -Glu(OH)-Gln-Pheol. The latter possibility seems also likely, taking into account a common enzymic mechanism for the biosynthesis of alamethicin, suzukacillin and the well investigated gramicidin A. Gramicidin A possesses ethanolamine as α-carboxamide C-terminus. In order to evaluate these and some other questions open at present, larger fragments of partial hydrolysates are separated by ion-exchange chromatography. There is also no definite proof for the assignments of the Glx residues as Gln and Glu respectively as yet.

Infrared and ultraviolet absorption spectra

The infrared spectra of suzukacillin A and alamethicin are taken under the same conditions in KBr pellets and they coincide exactly. The amide I band is relatively broad extending from 1630 to 1690 cm^{-1} with a center at 1655 cm^{-1}. The amide II region extends from 1520 to 1550 with a center at 1530 cm^{-1}. The NH region has a narrow peak at 3300 cm^{-1} with a shoulder at 3450 cm^{-1}. Infrared absorption spectroscopy is certainly not sensitive enough to detect minor structural differences between two peptide analogs of molecular weights around 2000. However, the infrared spectra demonstrate at least that suzukacillin A and alamethicin must have very similar structures.

Due to the presence of phenylalaninol, the ultraviolet absorption spectra show the typical vibrational structure of a phenyl chromophore around 260 nm (Fig. 3). It is rather astonishing that this fact has not been recognized in earlier investigations on alamethicin. In the short-wave length region the absorption of the phenyl ring is partly superimposed on the fading end absorption of the peptide backbone chromophores (Fig. 4). The molar extinction coefficients around 260 nm are of the same order of magnitude for suzukacillin A, alamethicin, phenylalaninol and phenylalanine (Figs. 3 and 4). Independently of ^{13}C nuclear magnetic resonance these values prove the presence of 1 mol phenylalaninol. With the exception of the phenyl

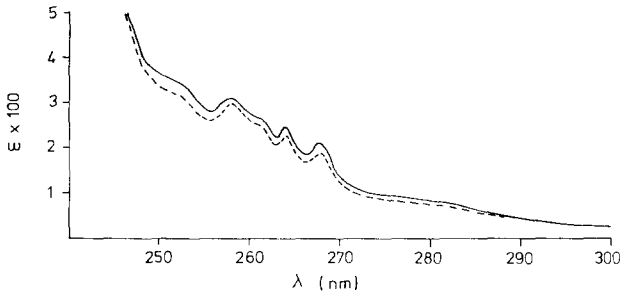

Fig. 3. Ultraviolet spectra of suzukacillin A (—) and alamethicin(– – –) in methanol at 20 °C, 0.995 mg/ml:

λ_{max}	(nm)	251 (sh)	258	261 (sh)	264	268	280 (sh)
ε_{max}	(suzukacillin)	357	312	268	245	201	85
ε_{max}	(alamethicin)	324	296	250	226	184	74

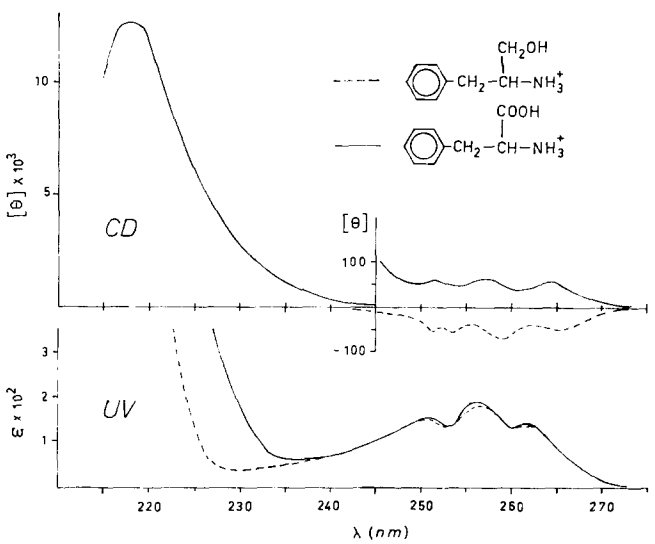

Fig. 4. Circular dichroism (CD) and ultraviolet (UV) absorption spectra of L phenylalaninol (– – –) and L phenylalanine (—) in 0.1 M HCl at 20 °C, concentrations as in Table II.

residue there are no side chain contributions to be considered either in the ultraviolet absorption or circular dichroism spectra.

Circular dichroism

The circular dichroism of suzukacillin A exhibits two pronounced negative Cotton effects at 223 and 207 nm, indicating a right-handed α-helical segment (Fig. 5). The solvent dependence of the circular dichroism of suzukacillin shows a strong increase of the intensities of the two negative Cotton effects from aqueous or strongly polar solvents to lipophilic solvents like 1-butanol or 1-octanol (Table II). As in the case of alamethicin [8], this is explained by an increase of the α-helix content in more lipophilic environments. The third, positive Cotton effect at 190 nm shown

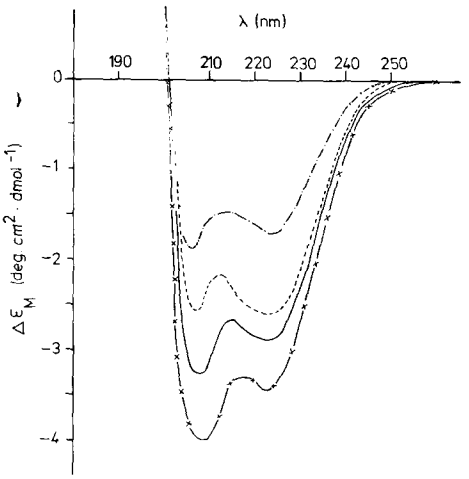

Fig. 5. Circular dichroism spectra of suzukacillin A in 1 butanol (×), ethanol 96 % (—) and 50 % (– – –), and in hexafluoroacetone trihydrate (– · –) (20 °C, 0.2 mM).

in the alamethicin spectra, which were taken in hexafluoroacetone trihydrate and trifluoroethanol [8], could not be recorded for the suzukacillin solutions. The ratio of intensities of the two negative Cotton effects $\Delta\varepsilon_{M223}/\Delta\varepsilon_{M207}$ reveals a more important difference between suzukacillin and alamethicin. This ratio remains constant at 0.8 for alamethicin in nonaqueous media and is above 1.0 in aqueous solutions. This behaviour has been attributed to the aggregation of alamethicin molecules to oligomers [8] because of the results of many concentration-dependent measurements of the circular dichroism that were compatible with reported results of surface tension measurements. Suzukacillin shows in all solvents a somewhat higher ratio of Cotton

TABLE II

SOLVENT DEPENDENCE OF THE CIRCULAR DICHROISM OF SUZUKACILLIN AND ALAMETHICIN (CONCENTRATION 0.2 mM, TEMPERATURE 23 °C)

Solvent	$\Delta\varepsilon_{M(207)}$		$\Delta\varepsilon_{M(223)}$		$\Delta\varepsilon_{M(223)}/\Delta\varepsilon_{M(207)}$		α helix estimated (%)	
	Suzuk-acillin	Alam-ethicin	Suzuk-acillin	Alam-ethicin	Suzuk-acillin	Alam-ethicin	Suzuk-acillin	Alam-ethicin
1-Octanol	−3.80	−4.50	−3.33	−3.53	0.88	0.78	36	37
1-Butanol	−3.93	−4.74	−3.47	−3.95	0.88	0.83	37	40
Ethanol 96 %	−3.23	−4.57	−2.88	−3.63	0.89	0.79	32	38
Ethanol 50 %	−2.53	−2.68	−2.59	−2.36	1.02	0.88	29	28
Acetonitrile	−3.77	−4.28	−3.09	−3.40	0.82	0.79	34	36
1,1,1-Trifluoro-ethanol	−2.81	−3.48	−2.28	−2.59	0.81	0.74	28	30
Hexafluoro-acetone-trihydrate	−1.90	−2.32	−1.68	−1.84	0.88	0.79	22	23

effect intensities than alamethicin. The values for aqueous alcoholic solutions with increasing water content indicate that the intermolecular interactions occur already at lower percentages of water than in the case of alamethicin. Suzukacillin is almost insoluble in water at pH 8 (phosphate buffer). And the solvent dependence of the circular dichroism reveals that the suzukacillin molecules also aggregate more strongly in aqueous environment than alamethicin molecules. This is also seen by the concentration dependence of the circular dichroism of a suzukacillin solution in aqueous ethanol. An increase of the ratio $[\Theta]_{223}/[\Theta]_{208}$ is also reported for polyglutamic acid in suspensions of aggregates of increasing average particle size [21]. Goodman et al. studied homologous series of linear oligopeptides in organic solvents and aqueous organic systems and they found aggregated species upon addition of water to organic solutions of methionine, leucine, isoleucine and valine homo-oligopeptides. Some of these hydrophobic peptides undergo conformational changes from partly α-helical to β-structures on aggregation [22]. In contrast to these small model peptides the stable hydrophobic N-terminal α-helical part of suzukacillin and alamethicin cannot change to a β-conformation on aggregation because of the 2-methylalanine residues and some sort of micellar aggregation must occur via interactions of helical parts in aqueous systems.

In order to obtain more information on the solvent-solute interaction of the two peptide antibiotics, measurements of the temperature dependence of the circular dichroism are made in various solvents. As shown earlier for alamethicin solutions the temperature dependence of the ellipticities deviates largely from linearity within the temperature ranges studied (−25–100 °C) [8]. A strong increase of the temperature coefficients of the 222 nm Cotton effect is found with decreasing solvent polarity. The smallest temperature-induced conformational changes are observed in the strongly hydrogen bonding hexafluoroacetone trihydrate [8]. As an example, the temperature dependence of the two negative Cotton effects of suzukacillin A in 1-octanol solution is shown in Fig. 6. As with alamethicin in lecithin vesicles and in this lipophilic solvent, we observe considerable variations of the α-helix content with temperature. In contrast to alamethicin [8] in the case of suzukacillin (Fig. 6) the

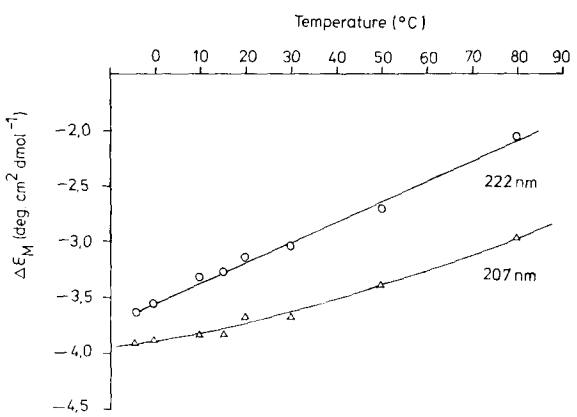

Fig. 6. Temperature dependence of the circular dichroism of suzukacillin A in 1-octanol, concentration 0.2 mM.

decrease in α-helix content with increasing temperature is more linear even at low temperatures in the α-helix promoting solvent 1-octanol. This behaviour may be due firstly to the relatively higher number of aliphatic residues between the two α-helix limiting proline residues. Secondly the more pronounced lipophilic behaviour of the suzukacillin molecules exhibited in the temperature dependence may also be explained by the existence of hydrogen bridged dimers. An experimental indication for this assumption may be given by the experiments on lipid bi-layers described in Part B [3]. However, there is an additional hint, since the ratios of the two negative Cotton effects show values around 0.9 in the temperature range up to 25 °C decreasing only to 0.8 at +50 °C. On the other hand the temperature dependence of the circular dichroism of alamethicin reveals in the same solvent and temperature range ratios of 0.8 and lower. We attributed values around 0.8 as indicative for the existence of monomers and values above 0.8 as originating from aggregated oligomers [8]. Therefore in some of the organic solvents suzukacillin may exist in equilibrium with a dimeric form in the investigated concentration range. Larger aggregates seem not to be found in water-free organic solvents even at relatively high concentrations. However, the assumption of dimers needs support by further concentration dependent circular dichroism measurements in various organic solvents. It is interesting in this context that a recent investigation of the concentration dependence of the circular dichroism of gramicidin A led also to the assumption of dimers in ethanolic solution [23]. However, it should be understood that we are dealing here with a system that is completely different from that of gramicidin A in both conformational and membrane-modifying aspects.

The percentages of α-helix content per mean residue given in Table III should be considered only as relative values with relatively large errors. The determination of more precise values by circular dichroism is restricted for small polypeptides with low helix content for several reasons. The values are calculated on the basis of commonly accepted standards of α-helical polyamino acids, which have average values of $[\Theta]_{222\,nm} = -36\,000$ degree · cm^2 · dmol^{-1} and $[\Theta]_{208\,nm} = -33\,000$ degree · cm^2 · dmol^{-1} [24]. The range of α-helix content given in Table III is independently confirmed at least for solutions in methanol, chloroform and dimethylsulfoxide by results of the ^{13}C investigation described in the following section. A complete destruction of the α-helical part of either suzukacillin or alamethicin is not possible by means of changes of pH or salt concentration, solvent changes (see also ref. 8), or thermal changes up to 100 °C in various solvents.

It should be pointed out that almost one half of the residues of suzukacillin and alamethicin consists of amino acids without intrinsic optical activity, 2-methylalanine and glycine. There is no doubt that in particular the 2-methylalanine methyl groups exert considerable steric and electronic influences to the environment of neighbouring peptide bonds. The sequential dependence of these contributions to the energies and rotational strengths of the peptide transitions is investigated quantitatively on synthetic 2-methylalanine peptides (Mayr, W., Oekonomopulos, R. and Jung, G., unpublished results).

In the circular dichroism spectra of both peptide antibiotics the presence of an intrinsically optically active phenyl chromophore cannot be detected even at high concentrations for the following reasons. Our ^{13}C nuclear magnetic resonance experiments indicate free mobility for the phenyl rings in alamethicin and suzukacillin.

TABLE III

ASSIGNMENTS OF THE ^{13}C NMR RESONANCES IN THE MEDIUM AND HIGH FIELD REGION OF SUZUKACILLIN A

ppm	Carbon	Amino acid residue
138.5	C-aromatic	C-1
129.6	C-aromatic	C-2,6
128.3	C-aromatic	C-3,5
126.3	C-aromatic	C-4
		Phenyl residue of phenylalaninol
63.9	CH_2	Phenylalaninol CH_2OH
63.5	CH(2)	Val-, Pro-C_α
55.6	C-quaternary	
57.1	C-quaternary	
56.6	C-quaternary, CH_2	Aib-C_α and phenylalaninol-C_α
56.2	C-quaternary	
55.5	CH	Glu-, Gln-C_α
54.3	CH	
53.0	CH(2–3)	Ala-C_α, Leu-C_α
49.1	(CH_2)	Pro-C_δ
45.1	CH_2	
44.8	CH_2	Gly-C_α
44.3	CH_2	
40.5	CH_2	Leu-C_β
39.7	CH_2	
37.2	CH_2	Phenylalaninol-C_β
32.3	CH_2	Gln-, Glu-C_γ
31.9	CH_2	
29.2	CH_2	Pro-C_β, Val-C_β
26.9	CH_3, CH_2	Gln-, Glu-C_β
26.5		Aib-C_β
26.1		
25.3	CH	Leu-C_γ
25.0	CH	Leu-C_γ
23.5	CH_2	Pro-C_γ
23.1	CH_3	Aib-C_β
22.7	CH_3	
22.0	CH_3	Leu-C_δ
21.3	CH_3	
20.2	CH_3	Val-C_β
19.9	CH_3	
18.9	CH_3	Val-C_γ
16.6	CH_3	Ala-C_β
7.2	CH_3	Unknown methyl group

However, free rotation cannot account alone for the non-observance of the Cotton effects of the phenyl chromophores, since the aromatic bands exist even in the very mobile low molecular weight chiral phenylalanine derivatives.

It is known that the contribution of the peptide bonds is dominant over side chain contributions because of the higher number of CONH groups and higher inherent optical activity. Secondly the sign and magnitude of the contribution of the π-π^* transitions above 220 nm of the phenyl residue may vary considerably. Thus the molar ellipticities $[\Theta]$ of the 250–270 nm Cotton effects observed in 0.1 M HCl so-

lutions of L-phenylalanine are positive (Fig. 3) and around 50 degree · cm^2 · $dmol^{-1}$, those observed for L-phenylalanine in peptides are negative and around 150 units [25–28]. The corresponding Cotton effects of free protonated L-phenylalaninol are negative and the magnitudes of molar ellipticity are very similar (Fig. 3). The molar ellipticities of alamethicin and suzukacillin are four orders of magnitude higher (Fig. 5).

^{13}C NMR spectra of suzukacillin

Suzukacillin shows a well resolved ^{13}C nuclear magnetic resonance spectrum (Fig. 7) which exhibits distinct differences when compared to that of alamethicin [8]. In particular the signals of the carbonyl, the quaternary and the methyl carbons and of the phenylalaninol carbons are very sharp. The secondary and tertiary carbons on the other hand are relatively broad, which is explained by extremely short T_2 relaxation times. The line-widths of these carbons are significantly broader than the shapes of corresponding alamethicin signals obtained under identical conditions. It can be concluded that this reflects the longer amino acid sequence of suzukacillin compared

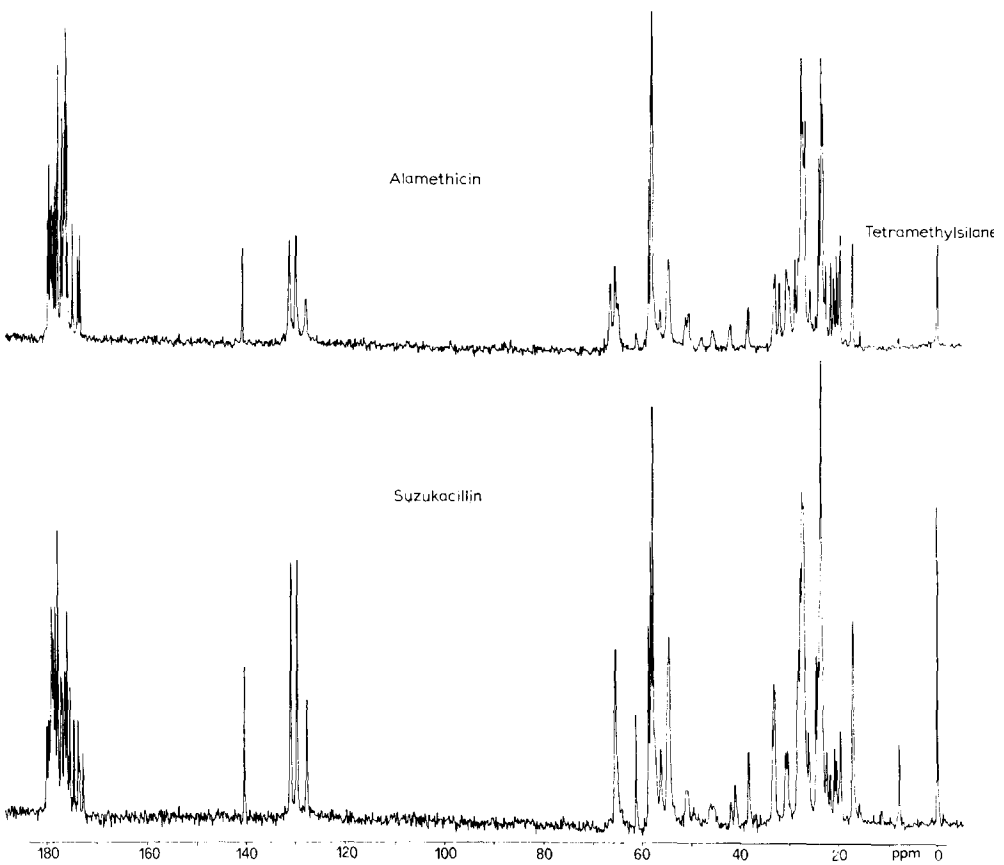

Fig. 7. 22.63 MHz ^{13}C NMR spectra of suzukacillin A (below) and alamethicin (above) in deuterated ^{12}C-enriched methanol solution (30 °C, 150 mg/ml).

to alamethicin. However, the existence of dimers and even higher aggregates in the concentrated solutions used for recording the ^{13}C nuclear magnetic resonance spectra can also cause this line broadening. Although the lines in the spectrum cannot all be assigned unequivocally at present, we are able to derive some important structural characteristics of suzukacillin, which are valuable for the investigation of the primary and secondary structure of this polypeptide.

Suzukacillin contains the amino acids Aib, Val, Ala, Pro, Leu, Gly, Gln, Glu and the amino alcohol phenylalaninol. The chemical shifts of the phenylalaninol residue (Table III) correspond to those of the same residue found in alamethicin [8]. Since the same is true for the carbons of the Glx residues we can safely assume that the C-terminal structural element, e.g. -Glu(Pheol)-Gln-OH, is also present in suzukacillin.

The signals of the β carbons of the Aib residues (Fig. 8) are divided into two distinct groups which show a shift difference of about 4 ppm. This value is much higher than that observed for the magnetic nonequivalence of the Aib methyl groups in small model peptides (0.4–0.8 ppm). This finding is characteristic for helical and non-helical 2-methylalanine residues within a peptide chain and corresponds to the spectroscopic behaviour experienced with alamethicin [8].

In the region of the glycine C_α carbons two absorptions at -45.1 and -44.8 ppm are found, which correspond to two glycines in different environments in unequal molar ratio. The analysis by combined gas-liquid chromatography-mass spectrometry revealed also a second glycine residue in the fragment Gly-Aib-Aib, which we attribute at present to an analog of suzukacillin A that could not be separated by thin-

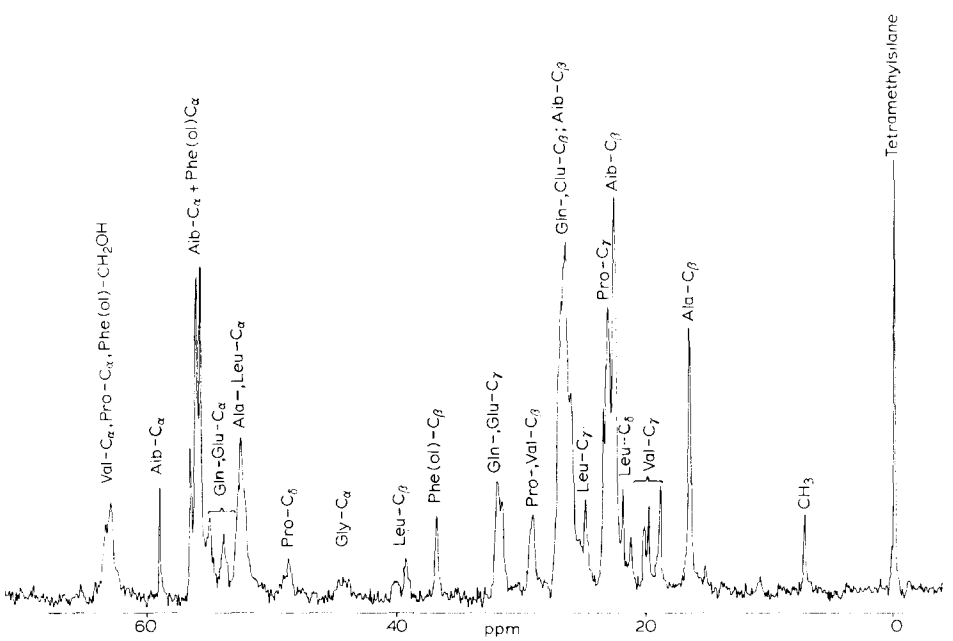

Fig. 8. Assignments of the resonances in the aliphatic region of the suzukacillin A ^{13}C NMR spectrum (expanded plot, 30 °C, 100 mg/ml, chloroform/methanol 4 : 1).

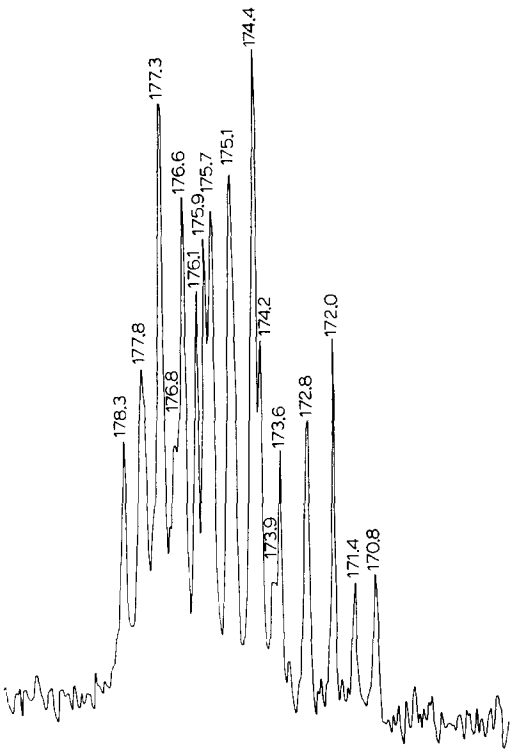

Fig. 9. Carbonyl part of the ^{13}C NMR spectrum of suzukacillin A (expanded plot, recording conditions as in Fig. 8).

layer chromatography. In the tentative sequence given in Table I the exchange of the valine by glycine in the Pro-Val-Aib-Aib part would be more likely than an exchange in the helical region. The incorporation of the complete fragment Gly-Aib-Aib would result in a sequence which seems to be too long to be compatible with various other experimental results.

Two differently linked leucines should exist because of the clearly separated Leu-C_β signals at 41.7 and 40.7 ppm. In the region of the C_β carbons of Gln and Glu residues three separated signals at 30.3, 32.8 and 32.6 ppm are found. This corresponds to three Glx residues as revealed by combined gas chromatography-mass spectrometry. One Aib-C_α appears 2 ppm down field from the C_α carbons of the remaining Aib residues and may belong to an N-terminal acetylated 2-methylalanine residue, which is proposed by Martin and Williams [15]. The carbonyl region (Fig. 9) results from about 25 amino acids and confirms also the longer primary structure of suzukacillin compared to that of alamethicin.

We cannot judge the origin of the signal of a methyl group at 7.2 ppm as yet (Figs. 7 and 8). In this region there is no absorption of the carbons of amino acids known so far and the methyl carbon of an acetyl group absorbs usually at 22 ppm. Investigations on the nature of this extremely high-field shifted methyl group are made at present. This particular methyl signal and two peaks at about 60 ppm are

also found in ^{13}C NMR spectra of fresh alamethicin preparations isolated from our own culture of *T. viride*. These three peaks are almost not detectable in our earlier spectra [8] and seem to disappear on prolonged acidic treatment or heating the sample. One could attribute this methyl signal to an unusually bonded group, such as the proposed positively charged 5-membered azacyclol [15]. However, this structure should be very labile under physiological conditions and also in the solvents used for extraction, purification and recording the spectra of the peptide antibiotics. Such a cyclol is probably converted by water elimination to an imidazolinium ion (Rothe, M., personal communication), which should exhibit an ultraviolet absorption around 235–240 nm. Unfortunately such a chromophore cannot be detected in the ultraviolet and circular dichroism spectra because of the peptide absorption. Furthermore such a permanently positively charged [15] heterocyclic N-terminus should give rise to electrophoretic mobility in acidic media. Electrophoretic migration is not pronounced for the main components of the suzukacillin and alamethicin. In this context it should be mentioned that the titration behaviour of our own alamethicin, and that of Upjohn, and of suzukacillin is very similar, although simple titration curves corresponding to the single carboxylic group with a pK of 5.5 reported earlier could not be recorded (Irmscher, G., Mayr, W. and Jung, G., unpublished results).

CONCLUSION

The new membrane-active peptide antibiotic suzukacillin A was found to have a primary structure closely related to that of alamethicin. However, the sequence is somewhat larger and the N-terminal part capable of forming a lipophilic α-helix extends longer. Therefore the main differencies between alamethicin and suzukacillin are exhibited by their different behaviours in mixed hydrophilic and lipophilic solvent systems. Suzukacillin A monomers are more strongly aggregated in the more aqueous systems via intermolecular interactions of the lipophilic N-terminal α-helices. Correspondingly in more lipophilic solvents eventually dimers are formed via hydrogen-bridged C-termini.

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