THE STRUCTURE OF PERMETIN A, A NEW POLYPEPTIN TYPE ANTIBIOTIC PRODUCED BY *BACILLUS CIRCULANS*

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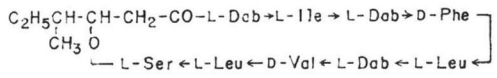
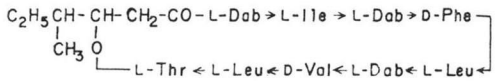
(Received for publication September 14, 1978)

The structure of permetin A(**I**), an antibiotic substance produced by *Bacillus circulans* AJ 3902, has been elucidated as a cyclic acyl peptide by means of the mass and nuclear magnetic resonance spectroscopic techniques.



I

The structure was found to be the same as polypeptin A(**II**) except that L-Thr in **II** is replaced by L-Ser in **I**. Details of the structural determination are given for the permetin A itself as well as for the hydrolyzed permetin A.



II

Permetin A, which has been isolated from *Bacillus circulans* AJ 3902, shows a broad-spectrum antibacterial activity against Gram-negative and Gram-positive bacteria. The isolation and some properties of permetin A was described in the preceding paper[1]. This preliminary structural study revealed permetin A to be a basic peptidolipid composed of Val, Leu, Ile, Phe, Ser and Dab* in the ratio of 1:2:1:1:1:1:3 together with an ether-extractable fatty acid. The constituents of permetin A have clearly indicated it to be a new antibiotic. As is well-known there has been two different classes of the Dab-containing antibiotics, namely polymyxins and polypeptins. Unlike the structural diversity found in polymyxin antibiotics, the latter class so far has only two members, both of which have very similar amino acid compositions to that of permetin A. In order to know if permetin A is in fact a similar type of antibiotic either to polymyxins or polypeptins, we have undertaken a detailed structural determination for permetin A and its derivatives. The description of the structural elucidation has been separated into several parts, although the order does not necessarily follow the actual time course of the structural studies.

Results and Discussion

Fatty Acid Moiety

Hydrolysis of permetin A with 6 N HCl at 110°C for 1 hour gave an oily material which separated

* 2,4-diaminobutyric acid.

from the solution, was extracted with ether and was then treated with N,N-dimethyl formamide dimethylacetal (Tokyo Kasei, Co., Ltd.) at 60°C for 20 minutes. The resulting methylated acid was subjected to the gas chromatography - mass spectrometric analysis. A symmetric single peak appeared in the chromatogram on an OV-101 column at 70°C, and its mass spectrum is shown in Fig. 1. The base peak at *m/e* 103 ($CHOHCH_2COOCH_3$) and a weak ion peak at *m/e* 159 (M−1) in the mass spectrum of the methyl ester suggest that the fatty acid to be β-hydroxy heptanoic acid.

By inspecting the ^{13}C-nmr spectrum of permetin A (Fig. 8), two high-field methyl signals, which appeared at the chemical shift range close to that typical for the isoleucine residue in peptides, were in fact to be attributable to the β-hydroxy heptanoic acid moiety in permetin A. This led us to conclude that the alkyl moiety of the acid is the *anteiso*-form and not the *iso*-form.

Fig. 1. Mass spectrum of methylester of the fatty acid component of permetin A.

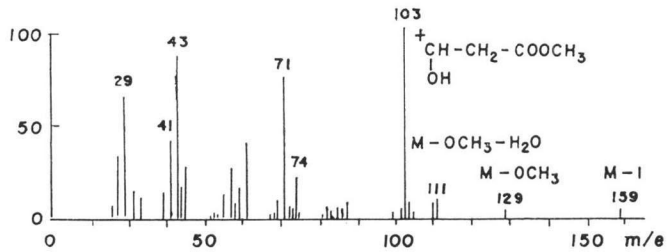
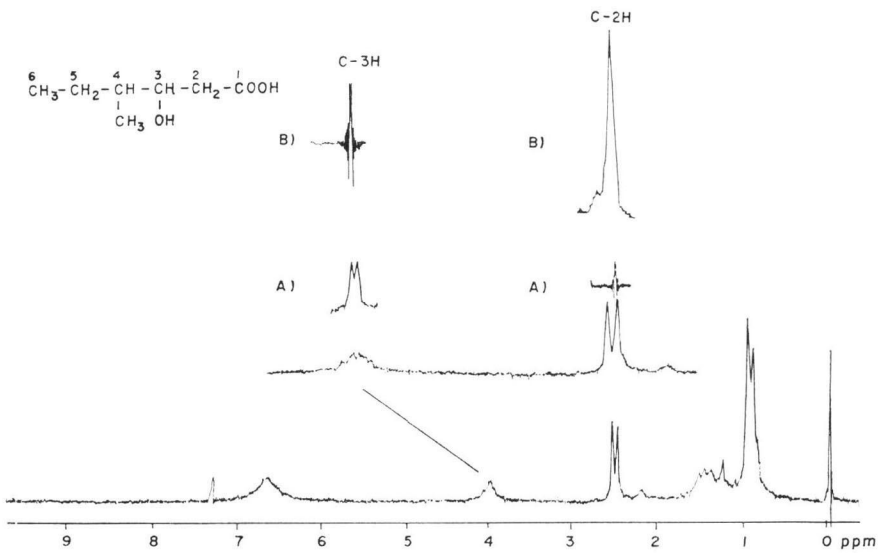
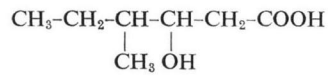


Fig. 2. 100 MHz ^1H-nmr spectrum of the fatty acid component of permetin A in $CDCl_3$. The inserted spectra are decoupled spectra described in the text.



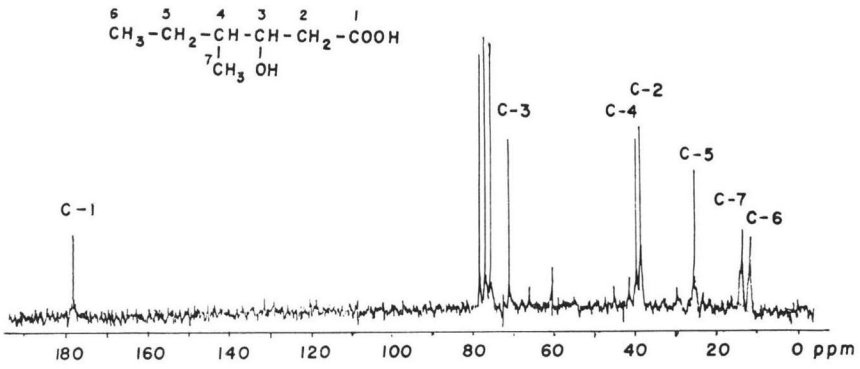
The 100 MHz ^1H-nmr spectrum of the isolated hydroxy acid (Fig. 2) further confirmed the structure of the acid. A doublet (2H) at 2.52 ppm was assigned to C–2H because it collapsed into a singlet on irradiating the proton signal at 4.0 ppm. The latter was unambiguously assigned to the single proton attached to the carbon bearing a hydroxyl group, namely C–3H. Irradiation of the C–2H signal turned the C–3H multiplet into a doublet. The C-4 therefore should have only one proton. These observa-

tion together with the ^{13}C-nmr spectrum of the isolated acid (Fig. 3), confirmed the structure of the acid to be 3-hydroxy-4-methylhexanoic acid (**III**), of which configuration is under investigation.



III

Fig. 3. ^{13}C-nmr spectrum of the fatty acid component of permetin A in $CDCl_3$.



Mass Spectral Analysis of Permetin A and the Hydrolyzed Permetin A

When permetin A was reacted with 2,4-dinitrofluorobenzene, the dinitrophenyl (DNP) derivative of permetin A was produced. By hydrolyzing the DNP-permetin A, only Dab was found to be modified, giving γ-DNP-Dab, and the other amino acids were all unchanged. This means that only Dab residues have free amino groups and the N-terminal amino group is blocked. Since a conventional sequence determination technique, such as EDMAN degradation, cannot be applied to the N-blocked peptides, we employed mass spectrometry as a principal tool to determine the amino acid sequence of permetin A. Fortunately the molecular size of this antibiotic was found to be within the limit where the mass spectrometric analysis could effectively be applied.

Prior to the analysis we modified permetin A according to the method of THOMAS[2], by which all peptide amino nitrogens were methylated and all free amine and hydroxyl groups, if any, were expected

Fig. 4. Mass spectrum of N-acetyl-permethylated permetin A.

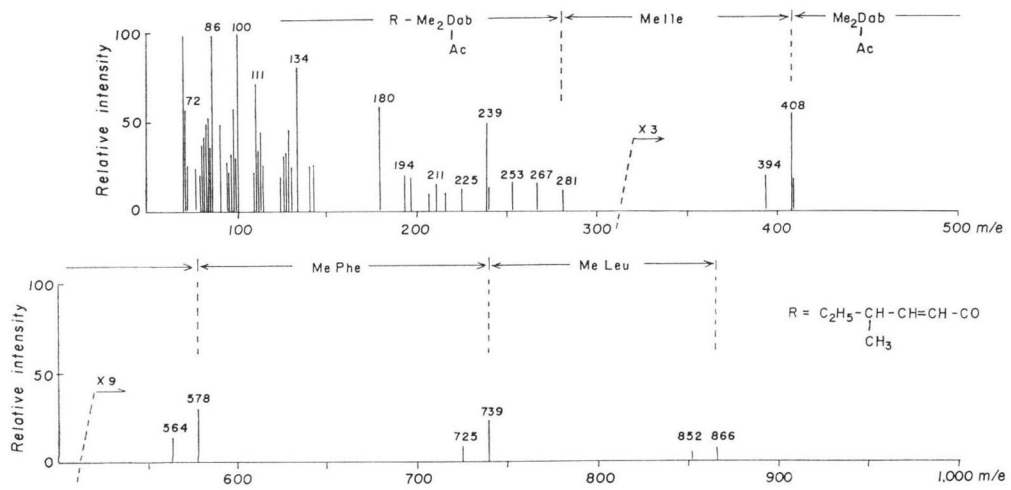
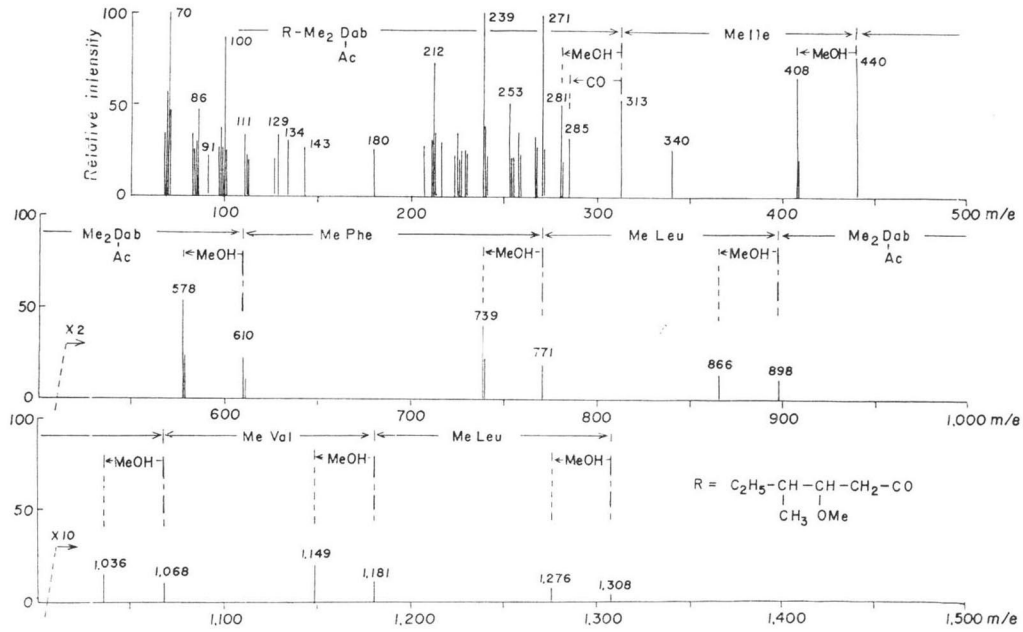


Fig. 5. Mass spectrum of N-acetyl-permethylated hydrolyzed permetin A (IV).



to be acetylated and methylated, respectively. The electron impact mass spectrum of this derivative of permetin A is shown in Fig. 4. The fragmentation profile was rather simple, as can be seen in Fig. 4, strongly suggesting permetin A to be either a linear N-acyl oligopeptide or a cyclic peptidolipid which has a fragile chemical bond being preferentially broken at an electron impact. Generally cyclic peptides should give rise to a more complex pattern due to the lack of preferential fission among various bonds in peptide rings.

The mass spectrum in Fig. 4 shows the sequence of the successive 5 amino acids, and the smaller peaks 14 mass units lower than the corresponding sequence determining peaks are probably unmethylated starting materials. The result suggested that the N-terminal amino acid was attached to the hydroxy fatty acid (**III**) in permetin A and the hydroxyl group of the fatty acid was bonded with some group in the peptide. On electron impact the bond with the hydroxyl group preferentially broke with a hydrogen rearrangement, resulting in an unsaturated acylated peptide ion. Successive fragmentation gave simple peptide peaks as shown in Fig. 4. The highest mass peak was observed at *m/e* 866, and therefore 4 amino acids, namely Dab, Val, Lx*, and Ser remained undetermined.

To confirm further the partial structure elucidated by the mass spectral analysis described above, we have derived a hydrolyzed permetin A (**IV**), which was obtained as a single product by alkaline hydrolysis.

$$C_2H_5\text{-}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{-}\underset{\displaystyle OH}{\underset{|}{CH}}\text{-}CH_2\text{-}CO\text{-Dab-Ile-Dab-Phe-Leu-Dab-Val-Leu-Ser-OH}$$

IV

The hydrolyzed antibiotic, after derivatization[2] similar to that for permetin A itself, gave also a rather straightforward mass spectrum (Fig. 5). By comparing the mass spectra of permetin A before and after alkaline hydrolysis, we could easily see that the hydroxyl group of **III** was methylated in the case of the

* Leu or Ile

hydrolyzed permetin A, which gave its sequence determining peaks 32 mass units(methanol) higher than the corresponding peaks of permetin A. This supports the previous view that the hydroxyl group in intact permetin A is chemically bonded, which is freed by alkaline hydrolysis and then methylated during the derivatization processes. The mass spectrometric analysis of **IV** also provided additional information about the remaining amino acid sequence, which is shown in Fig. 5. Note that Leu and Ile could not be discriminated by the mass spectra, and so the assignment of each fragment in 2 figures are based on the final structure of permetin A.

The sequence hereto obtained covers 8 out of the 9 amino acid residues in permetin A, determined by the quantitative amino acid analysis, and the remaining amino acid should therefore be Ser. The results from mass spectra were sufficient to assign the structure of permetin A as a cyclic peptidolipid, very similar to polypeptin A or B, and not to any of the polymyxins.

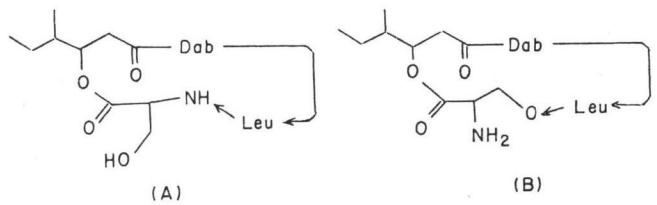
Chemical Structure around the Fatty Acid and Ser

In the preceding discussions we have shown the partial structure of permetin A except for the structure around Ser, which was missed in the mass spectra because of its very fragile nature toward fragmentation. Although we were quite certain from the amino acid analysis that permetin A has one Ser residue, we analyzed the C-terminal of the alkaline hydrolyzed permetin A (**IV**) by hydrazine method, which produced Ser with 70% of the theoretical value. Furthermore, the molecular weights of permetin A and **IV** were found to be 1,100 and 1,118, respectively by the field desorption mass spectrometry, which unambiguously showed both of these compounds have one Ser residue.

The ir spectrum[1] of permetin A showed an absorption at 1740 cm^{-1}, which is typical for the ester carbonyl stretching frequency. This absorption disappeared completely after permetin A was hydrolyzed with dilute alkaline solution to give **IV**. We therefore can be confident that the carboxyl group of Ser forms a lactone ring between the hydroxyl group of **III** attached to the terminal Dab residue.

The only remaining ambiguity in the back bone structure is now the linkage between Ser and Leu.

Scheme 1.



Both structures A and B (Scheme 1) are theoretically possible, although the latter structure looks very unusual. Structure B is also unlikely because it would introduce a second location where easy bond cleavage could occur. Discrimination between these 2 structures was done by the ^{13}C-nmr chemical shifts of the methylene carbon of Ser. The chemical shift of the methylene carbon in permetin A was found to be very similar to that of free Ser and 1.5 ppm higher field of that in Cbz-Ser-Gln. This shift difference was taken as an evidence of the free hydroxyl group in the Ser in permetin A, and the structure A should therefore be correct.

Configuration of the Amino Acid Constituents

The amino acid mixture obtained by vigorous hydrolysis of permetin A was converted to *n*-butyl-esters, which were then coupled with N-trifluoroacetyl-L-prolyl chloride to form diastereomeric di-

Scheme 2.

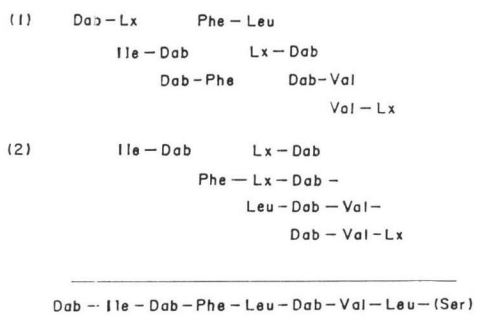
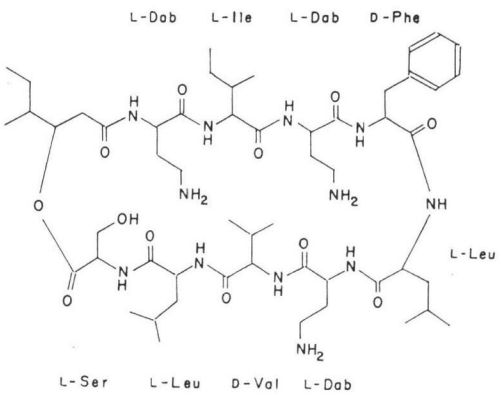


Fig. 6. The structure of permetin A.



peptides. Serine in the mixture was further O-trimethylsilylated in order to obtain a good separation. All of these derivatives of amino acid mixture were then analyzed by gas chromatography[3,4] on an OV-101 column, and their configurations were determined by comparing the retention times of each derivatives to those of the diastereomeric prolyl peptides derived from the authentic D- and L-amino acids. The amino acid components were found to be D-Val, L-Ile, L-Leu, L-Ser, D-Phe, and L-Dab.

Discrimination of Leu and Ile by the Mass Spectrometric Analysis of the Peptide Fragments of Permetin A Obtained by Partial Acid Hydrolysis

Further information, especially about the position of Ile and Leu was obtained by the mass spectral analysis of the peptide mixtures from permetin A after being hydrolyzed with following two different conditions: (1) 6 N HCl in H_2O at 110°C for 3 hours; (2) 6 N HCl in methanol at 70°C for 3 hours. Each of 2 peptide mixtures was converted to the N-TFA methylesters and was then analyzed by gas chromatography - mass spectrometry. Since Ile-Dab and Leu-Dab-Val were unambiguously identified by their fragmentation patterns of mass spectra[5], the 2 Lx at position 2 and 5 were determined to be Ile and Leu, respectively. The latter assignment was further confirmed by identifying the Phe-Leu fragment, whose structure was determined by comparison with the mass spectra of authentic Phe-Leu and Phe-Ile. The results automatically recommend the remaining uncharacterized Lx at position 8 be Leu.

The reconstructed amino acid sequence including Leu and Ile, by overlapping the identified peptide fragments, is shown in Scheme 2.

The results described above confirmed the structure of permetin A to be the structure shown in Fig. 6.

^{13}C-nmr Spectral Data

The final confirmation of the structure was done by a study of ^{13}C-nmr spectra. Fig. 7 shows the proton decoupled ^{13}C-nmr of permetin A. The partial spectrum of aliphatic carbon resonance region (Fig. 8) counts the signals of 37 carbons corresponding to all of the carbons due to the fatty acid and 9 amino acids. All of the signals were tentatively assigned, as being indicated in Fig. 8, by comparing the standard chemical shifts for respective amino acids[6] and by off-resonance experiment for the signals of fatty acid moiety. In the expanded carbonyl carbon region of the spectrum (Fig. 9) 10 carbons appeared between 171.1 and 175.3 ppm, which account for all of the carbonyl groups of permetin A; namely one from the acyl group and 9 from the amino acids. As all of the observed signals could be

Fig. 7. 25.2 MHz proton decoupled ^{13}C-nmr spectrum of permetin A in ^{13}C-depleted methanol-d_4.

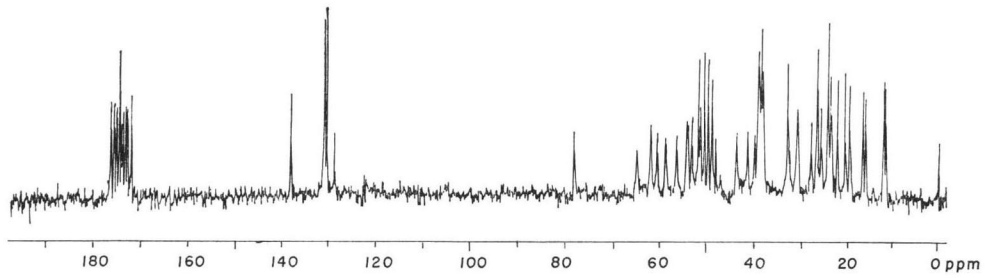


Fig. 8. Aliphatic carbon resonance region of the ^{13}C-nmr spectrum of permetin A, with tentative assignments. F. A. denotes the fatty acid (**III**) residue.

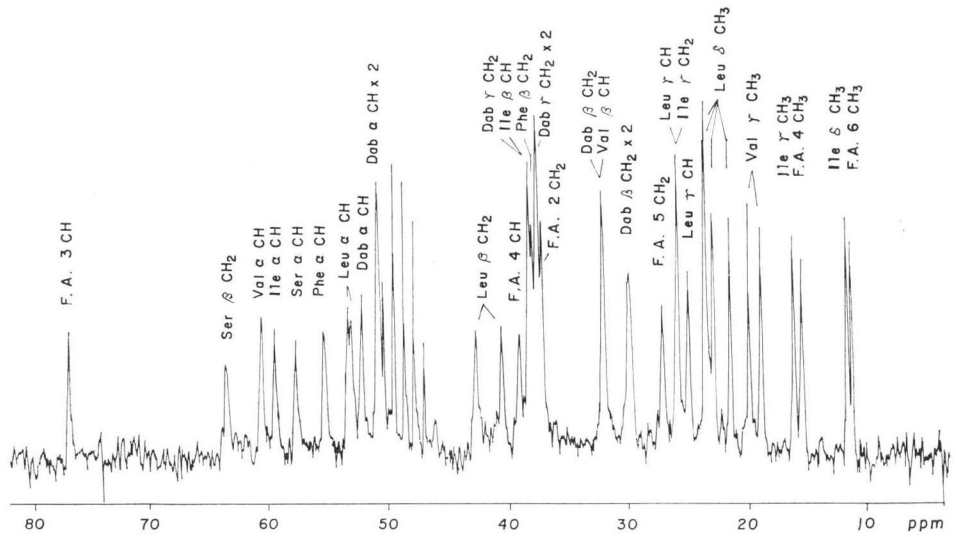
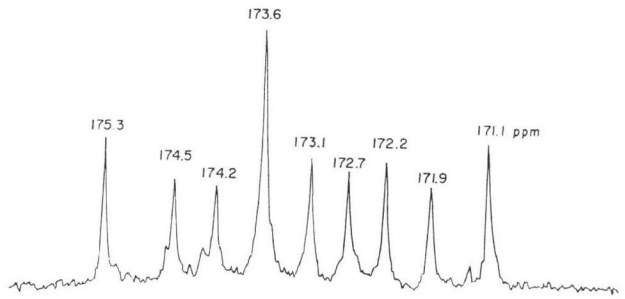


Fig. 9. Carbonyl carbon resonance region of the ^{13}C-nmr spectrum of permetin A.



assigned to the carbons of the proposed structure shown in Fig. 6, no other component is contained in permetin A.

Conclusion

The established structure of permetin A is found to be the same as polypeptin A[7], except that L-Ser in permetin A replaces L-Thr in polypeptin A. Therefore, permetin A should be considered as

the third member of polypeptin antibiotics. SOGN found polypeptin B[7] which differs from polypeptin A only for the structure of fatty acid side chain. We also found an isomeric fatty acid in the unfractionated permetin A preparation[8], however, we could not separate the permetin A containing this uncharacterized fatty acid from the other batches of fermentation. We thus assume the major component of permetin A has the fatty acid with the *anteiso*-form, and the isomer (permetin B), which might contain the *iso*-form of the acid, should be the minor component.

As we have mentioned already, the other class of Dab-containing cyclic antibiotics, polymyxins, has very diversified molecular structures, and we feel there might be many other polypeptins to be found in the cultured broths of similar kind of bacteria. It will be interesting to assess the activity difference between permetin A and polypeptins A and B, because all of them have antibacterial activity against some anaerobic bacteria, which resist most of the polymyxins.

Experimental

Spectral measurements

^{13}C (25.2 MHz) and ^{1}H (100.1 MHz) nmr spectra were measured on a XL-100 with an FT accessory. All chemical shifts were determined with reference to internal TMS. The spectra were measured in 5-mm sample tubes, employing $CDCl_3$ or ^{13}C-depleted methanol-d_4 as the solvent. Mass spectra were recorded on a Hitachi RMU-6MG mass spectrometer, employing the direct inlet system or gas chromatography - mass spectrometry. A glass column of 1 m×2 mm i.d. packed with 5% OV-101 on 80~100 mesh Diatoport S was used, using He as the carrier gas at a flow rate of 30 ml/min. Field desorption mass spectra were measured on a Hitachi RMU-7M mass spectrometer.

Alkaline hydrolysis of permetin A(I)

Two ml of 0.1 N NaOH was added to 30 mg of permetin A hydrochloride dissolved in methanol (2 ml). The methanolic alkali solution was allowed to stand at room temperature for 6 hours. The solution was then acidified to pH 2 with dilute HCl and was evaporated to dryness *in vacuo* at 40°C. After being completely dried, the hydrolysate was suspended in absolute ethanol, and the undissolved NaCl was removed by centrifugation. The supernatant of the suspension was evaporated, giving 25 mg of **IV** as colorless powder.

Partial acid hydrolysis of I

Two 0.5-mg portions of **I** hydrochloride were hydrolyzed: one portion was sealed with 0.5 ml of 6 N methanolic HCl into a Pyrex tube under nitrogen and was heated at 70°C for 3 hours; the other with 0.5 ml of 6 N HCl was heated also in a sealed tube at 110°C for 3 hours. The cooled partial hydrolysates of **I** were then evaporated to dryness. These mixtures of small peptide fragments were converted to their N-trifluoroacetyl methylester derivatives, as described.

N-Trifluoroacetyl methyl ester derivatives of the partial acid hydrolysates

The partial acid hydrolysates of **I** were dissolved in 1 ml of 6 N methanolic HCl and kept at room temperature for 1 hour. The reaction mixtures were dried *in vacuo* and the residues were taken up to methylene chloride (0.2 ml) and were treated with trifluoroacetic anhydride (0.2 ml) at room temperature for 30 minutes. After removing the solvent, each of the residues was dissolved in methanol and was subjected to gas chromatography - mass spectrometry analysis at the temperature range from 50°C to 250°C with the heating rate of 3°C/min.

N-Trifluoroacetyl-L-prolyl *n*-butyl ester derivatives of amino acids from the complete acid hydrolysate of I

After complete hydrolysis of permetin A hydrochloride (0.3 mg) by acid, the amino acid mixture was dissolved in 1 ml of 6 N *n*-butanolic HCl and was refluxed for 30 minutes. The reaction mixture was dried *in vacuo*, and was coupled with N-TFA-L-prolyl chloride (2 mol equivalent) in chloroform, in the presence of triethylamine (0.1 ml). After standing at room temperature for 15 minutes, the chloroform solution was washed twice with H_2O and then dried over Na_2SO_4. The reaction mixture

was dried *in vacuo* at 40°C, dissolved in *n*-butanol, and then subjected to gas chromatographic analysis. The column temperature program was set from 130°C to 220°C at a rate of 3°C/min. N-TFA-L-prolyl *n*-butyl ester of serine was further converted to O-trimethylsilyl derivative using N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA).

The hydrazinolysis of **I**

A solution of 1.3 mg of **I** in 1 ml of anhydrous hydrazine (freshly distilled from calcium oxide) was heated in a sealed tube at 100°C for 6 hours. After evaporating the unreacted hydrazine *in vacuo*, the residue was dissolved in 1 ml of H_2O and shaken for 2 hours at room temperature. The aqueous layer was separated and then evaporated to dryness, the amino acid composition of the acid hydrolysate of the residue was determined, showing Ser in 70% of the theoretical value.

	Detected Ser*
Permetin A (**I**)	23%
Hydrolyzed permetin A (**IV**)	70%
Glu-Ser (standard)	56%

* % to the theoretical values

DNP-Permetin A

To a solution of permetin A hydrochloride (4.8 mg) in 0.2 ml of 1% aqueous trimethylamine was added 0.4 ml of 5% 2, 4-dinitrofluorobenzene solution in ethanol. The mixture was kept overnight in a dark place at room temperature. The reaction mixture was diluted with H_2O and was extracted three times with ether. The aqueous layer was then evaporated to dryness and the residue was dissolved in methanol. The solution was applied to a thick-layered silica gel plate (20×20 cm, Merck) which was developed with methanol - chloroform (1: 9). The separated yellow zone (Rf value: 0.7~0.8) was extracted with methanol and were evaporated to dryness leaving 3.2 mg of DNP-permetin A. Amino acid analysis of the acid hydrolysate of the DNP-permetin A showed the following free amino acids: Phe, Ser, Val, Ile and Leu at the molecular ratio of 1.0: 1.1: 1.1: 0.8: 2.0. The acid hydrolysate of the DNP-permetin A showed an yellow spot with Rf 0.57 on a silica gel plate with *n*-propanol-28% aqueous ammonia (7: 3), which was identified to γ-DNP-Dab by comparing with the γ-DNP-Dab (Rf 0.57) derived from DNP-polymixin B. Rf values of α-DNP-Dab and α, γ-DNP-Dab were found to be 0.78 and 0.73 respectively, also indicating that the DNP-Dab from permetin A was the γ-isomer.

Acknowledgements

The authors express their deep thanks to Prof. S. MURAO of University of Osaka Prefecture for his continuous encouragement throughout this work. The authors also wish to thank Hitachi, Ltd. for the measurements of field desorption mass spectra.

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