ISOLATION AND CHARACTERIZATION OF NEW PEPTIDE ANTIBIOTICS, PLUSBACINS $A_1 \sim A_4$ AND $B_1 \sim B_4$

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New antibiotics, plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$, were isolated from the culture broth of a strain of *Pseudomonas* sp. These antibiotics were isolated as a complex by column chromatographies on Diaion HP-20 and silica gel, and then separated by HPLC. They are amphoteric in nature. The hydrochlorides are obtained as colorless powders, soluble in methanol and alkaline water. From their physico-chemical properties, these antibiotics are presumed to be acyloctapeptides containing a lactone linkage, and their differences occur in amino acid and fatty acid residues. The antibiotics are active against Gram-positive bacteria *in vitro* and *in vivo*.

In the course of our screening work for new antibiotics from bacterial strains, a strain numbered PB-6250 related to the genus *Pseudomonas* was found to produce an antibiotic principle, which showed inhibitory activity against methicillin-resistant *Staphylococcus aureus* and proved to be a cell wall synthesis-inhibitor. It was isolated as a complex of peptide antibiotics, which was then separated by HPLC into eight components. They were shown to be new antibiotics and named plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$. Their structures will be presented in the succeeding paper[1].

In this paper, the taxonomy of the producing strain, the production and isolation of the antibiotics as well as the physico-chemical and biological properties are presented.

Taxonomy

The producing organism numbered PB-6250 was isolated from a soil sample collected in Okinawa-honto, Okinawa Prefecture, Japan.

The organism is an aerobic Gram-negative, non-sporulating rod ($0.5 \sim 0.7 \times 2.0 \sim 5.0\ \mu m$) with rounded ends. It is weakly motile by one or several polar flagella. It exhibits good growth at 28°C and on nutrient agar it forms circular, convex, opaque, entire, glistening and wet colonies with brownish cream color. Soluble, brown pigment is diffused around colonies. Poly-β-hydroxybutyrate is not accumulated as an intracellular carbon reserve. Other physiological characteristics are shown in Table 1.

Acid formation was observed from D-glucose, D-fructose, maltose and trehalose, but not from D,L-arabinose, D-xylose, D-mannitol, lactose and sucrose. No gas formation was observed from the above carbohydrates.

From comparison of these characteristics with those of bacteria registered in the Volume 1 of BERGEY's Manual of Systematic Bacteriology[2], the organism should be ascribed to the genus *Pseudomonas*. According to further comparison with the registered species of the genus, the organism is most related to *Pseudomonas paucimobilis*, especially in the feeble motility, but differed in the inability of starch hydrolysis and mole% G+C of DNA.

Table 1. Physiological characteristics of strain PB-6250.

Properties observed	Results	Properties observed	Results
Catalase test	+	β-Galactosidase test	+
Oxidase test	+	Urease test	−
OF-test	Oxidative	Deoxyribonuclease test	−
Peptonization of milk	+	Acylamidase test	−
Coagulation of milk	+	Phenylalanine deaminase test	−
Gelatin liquefaction	+	Tween 80 esterase test	+
Starch hydrolysis	−	Voges-Proscauer test	−
Esculin hydrolysis	+	Methyl red test	−
Indole production	−	Nitrate reduction	−
H_2S production	−	Denitrification	−
Arginine dihydrolase test	−	Citrate utilization	+
Lysine decarboxylase test	−	Fluorescent pigment	−
Ornithine decarboxylase test	−	Mole% G+C of DNA	69.4

From the above, the organism was designated as *Pseudomonas* sp. PB-6250, and deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the accession No. FERM BP-2938.

Fermentation

The cell suspension of the strain PB-6250 was inoculated into 1 liter of a medium consisting of glucose 1.0% and yeast extract 0.5% (pH not adjusted) in a 2-liter Erlenmeyer flask, which was cultivated at 28°C for 18 hours on a rotary shaker. A 7-liter portion of the cultured broth was then inoculated into 200 liters of a medium consisting of starch 2.0% and CA-1 diet (Clea Japan, Inc.) 2.0% (pH not adjusted) in a 500-liter fermenter. Fermentation was carried out at 28°C for 72 hours under aeration of 200 liters per minute and agitation of 250 rpm.

Isolation of Plusbacin Complex

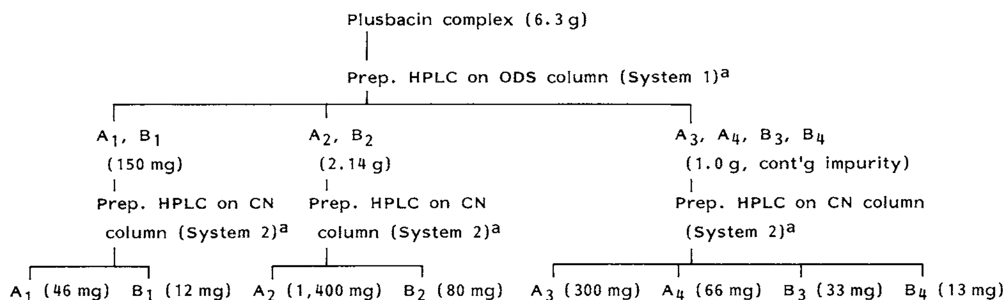
NaCl (25 kg) was added to the culture broth (200 liters), which was adjusted to pH 3.0 with HCl and centrifuged. The cell mass obtained was extracted twice with *ca.* 50 liters of 70% aq acetone. The extract was evaporated under reduced pressure to a nearly aqueous solution (*ca.* 15 liters). The aqueous solution, after addition of water (10 liters) and adjustment to pH 8.0 with dil NaOH, was passed through a column (14 x 70 cm) of Diaion HP-20 (Mitsubishi Kasei Co., Ltd.). The column was washed with water and eluted in a gradient manner with 30% aq acetone to 80% acetone. The active eluate (*ca.* 10 liters) was evaporated under reduced pressure to a nearly aqueous solution, adjusted to pH 2.5 and extracted with BuOH (5 liters). The BuOH extract was concentrated and a crude powder (23 g) was precipitated by addition of acetone.

The crude powder was applied on a column of silica gel (Merck, 70 ~ 230 mesh, 1,000 g) packed with $CHCl_3$ - EtOH - 10% AcOH (4 : 7 : 2) and eluted with the same solvent. The active eluate from the column was concentrated and extracted with BuOH at pH 2.0 (HCl). The extract was water-washed, concentrated, and the plusbacin complex was precipitated by addition of acetone as a colorless powder (6.3 g).

Separation of Plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$ by HPLC

The complex obtained as above was separated by preparative HPLC on a ODS column (System 1. Column; YMC AP-324 ODS (50 x 500 mm) (Yamamura Chemicals Co., Ltd.). Mobile phase; CH_3CN - 50 mM phosphate buffer, pH 7.5 containing 50 mM Na_2SO_4 (36 : 64). Flow rate; 100 ml/minute.

Fig. 1. Separation of plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$.



[a] Detailed conditions are described in the text.

Table 2. HPLC of plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$.

	Retention volume (ml)							
	A_1	B_1	A_2	B_2	A_3	B_3	A_4	B_4
HPLC system 1	6.0	6.5	9.0	9.6	15.6	16.4	17.2	18.7
HPLC system 2	14.5	15.4	18.9	21.4	26.6	30.5	28.2	32.3

System 1. Column; Nucleosil $5C_{18}$ (4.6 × 150 mm), mobile phase; CH_3CN - 50 mM phosphate buffer, pH 7.5, containing 50 mM Na_2SO_4 (34 : 66), flow rate; 1.575 ml/minute, detection; OD at 220 nm.

System 2. Column; Ultron 7 CN (4.6 × 250 mm), mobile phase; CH_3CN - 50 mM phosphate buffer, pH 2.2, containing 50 mM Na_2SO_4 (25 : 75), flow rate; 1.575 ml/minute, detection; OD at 220 nm.

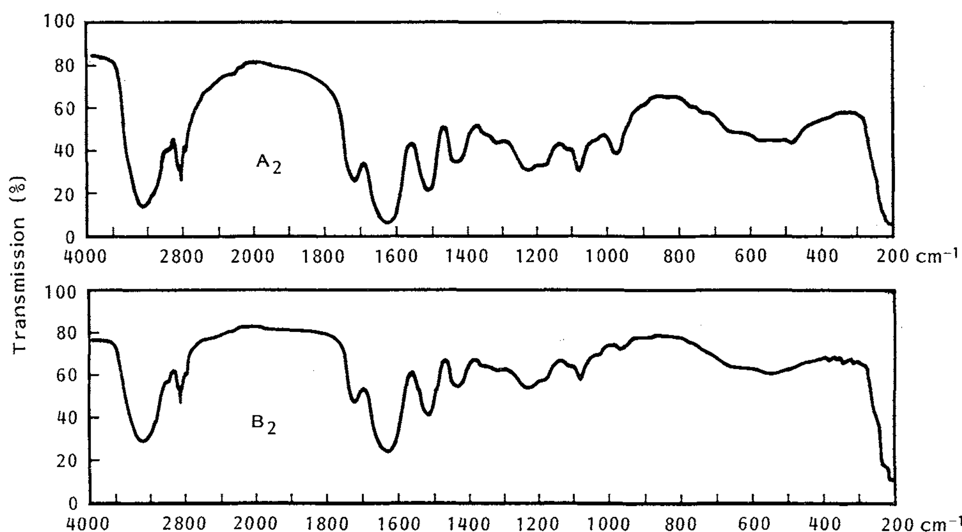
Detection; OD at 220 nm. Sample application: 500 mg sample in aq solution (pH 8.0) was injected for a run) into three fractions containing components A_1 and B_1; A_2 and B_2; and A_3, A_4, B_3 and B_4, respectively. Each of the fractions was concentrated and extracted with BuOH at pH 2.5 (HCl). The BuOH extract was washed with 0.1 N HCl and then with water and concentrated. The addition of acetone to the concentrate gave the hydrochloride of the fraction. These fractions were further separated by preparative HPLC on a CN column (System 2. Column; Ultron 70 CN (20 × 250 mm) (Shinwa Chemicals Co., Ltd.). Mobile phase; CH_3CN - 50 mM phosphate buffer, pH 2.2 containing 50 mM Na_2SO_4 (25 : 75). Flow rate; 11.25 ml/minute. Detection; OD at 220 nm. Sample application; 5 mg sample in 50% aq MeOH solution was injected for a run), resulting in fractions containing single components. Each of the fractions was processed in the same manner as above, affording the hydrochloride of each the component as a colorless powder. An example of the separation is illustrated in Fig. 1.

Physico-chemical Properties

Plusbacin complex is not separable by TLC on a silica gel plates (Merck, GF_{254}) using any of the following systems: *n*-BuOH - AcOH - H_2O (4 : 2 : 1) Rf of 0.27; *n*-BuOH - *n*-PrOH - AcOH - H_2O (10 : 6 : 1 : 4) Rf of 0.34 and $CHCl_3$ - EtOH - H_2O (4 : 7 : 2) Rf of 0.27. Plusbacins $A_{1\sim4}$ and $B_{1\sim4}$ can be distinguished by HPLC. The retention volumes in two systems of HPLC are given in Table 2.

Plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$ are quite similar to each other in physico-chemical properties. They are amphoteric in nature and their hydrochlorides are obtained as colorless powders. The hydrochlorides are soluble in methanol and dimethyl sulfoxide, sparlingly soluble in ethanol, and substantially insoluble in acetone, ethyl acetate and chloroform. They are slightly soluble in water and easily soluble in alkaline

Fig. 2. IR spectra of plusbacins A_2 and B_2 hydrochlorides (KBr).



water. They are positive to Sakaguchi reaction, but negative to ninhydrin reaction.

Plusbacins A_2 and B_2 hydrochlorides exhibited an end absorption in the UV spectra measured in water. In the IR spectra, they showed dominant absorptions at 1640 and 1535 cm^{-1}, due to peptide bonds, and an absorption at 1735 cm^{-1} probably due to a lactone linkage (Fig. 2).

Molecular formulas of plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$ were determined by high resolution mass spectrometry measured with their hydrochlorides. The results are shown in Table 3. Elemental analysis of the main component plusbacin A_2 sodium salt was shown below.

Table 3. High resolution mass spectrometry of plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$.

Plusbacin	*m/z* $(M+H)^+$	Δ mmu	Molecular formula
A_1	1,130.5569	−0.5	$C_{48}H_{79}N_{11}O_{20}$
A_2	1,144.5735	0.2	$C_{49}H_{81}N_{11}O_{20}$
A_3	1,158.5883	−0.5	$C_{50}H_{83}N_{11}O_{20}$
A_4	1,158.5893	0.4	$C_{50}H_{83}N_{11}O_{20}$
B_1	1,114.5613	−1.3	$C_{48}H_{79}N_{11}O_{19}$
B_2	1,128.5796	1.3	$C_{49}H_{81}N_{11}O_{19}$
B_3	1,142.5945	0.5	$C_{50}H_{83}N_{11}O_{19}$
B_4	1,142.5934	−0.5	$C_{50}H_{83}N_{11}O_{19}$

Anal Calcd for $C_{49}H_{80}N_{11}O_{20}Na \cdot H_2O$: C 49.70, H 6.98, N 13.01, Na 1.94.
Found: C 49.54, H 7.09, N 12.76, Na 1.91.

Constituent amino acids of plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$ were examined by acid hydrolysis followed by automatic amino acid analysis. Two moles of β-hydroxyaspartic acid, one mole each of threonine, serine, alanine, arginine and an unknown amino acid were commonly found in the hydrolysates of plusbacins $A_1 \sim A_4$. One mole of proline was found in addition to the above amino acids in the hydrolysates of plusbacins $B_1 \sim B_4$. The intensity of the peak of the unknown amino acid was stronger in the hydrolysates of A series antibiotics than in B series. In paper chromatography, the ninhydrin coloration of the unknown amino acid was yellow just like that of proline.

The unknown amino acid was assumed to be hydroxyproline from the fact that the difference of the molecular weights between the components of A series and B series was 16 (one oxygen atom) as shown in Table 3. The assumption was confirmed by further experiments presented in the succeeding paper[1].

Table 4. Antimicrobial activities of plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$.

Test organism	MIC (μg/ml)							
	A_1	A_2	A_3	A_4	B_1	B_2	B_3	B_4
S. aureus 209P JC-1	0.05	0.1	0.1	0.1	0.1	0.1	0.2	0.4
S. aureus Smith	1.6	0.8	0.4	0.4	1.6	0.8	1.6	1.6
S. aureus SR5597*	1.6	0.8	0.4	0.4	1.6	0.8	0.8	0.8
S. aureus SR5598*	1.6	0.8	0.8	0.4	1.6	0.8	1.6	0.8
S. aureus SR5580*	1.6	0.8	0.8	0.4	1.6	0.8	1.6	1.6
S. aureus SR5584*	1.6	1.6	0.8	0.4	3.1	1.6	1.6	1.6
S. epidermidis A14990	0.8	0.2	0.2	0.4	0.8	0.4	0.4	0.8
E. faecalis SR1004	6.3	3.1	1.6	1.6	6.3	1.6	1.6	3.1
E. faecium SR4512	6.3	3.1	1.6	1.6	12.5	3.1	3.1	6.3

* Methicillin-resistant strains.
Medium: Sensitivity test agar (Nissui).
Inoculum size: 10^6 cells/ml.

Thus, it was suggested that two moles of hydroxyproline were contained in the components of A series, whereas one mole of hydroxyproline was replaced by proline in the components of B series.

The constituent fatty acids were tentatively examined by gas-liquid chromatography with their methyl esters. From graphical comparison of retention times with reference fatty acid methyl esters, the fatty acid residues of plusbacins A_1 and B_1 were the same one and assumed to be 3-hydroxy-tetradecanoic acid; A_2 and B_2 3-hydroxy-isopentadecanoic acid; A_3 and B_3 3-hydroxy-isohexadecanoic acid; and A_4 and B_4 3-hydroxy-hexadecanoic acid.

Table 5. *In vivo* efficacy of plusbacins A_2 and A_3 against bacterial infection in mice.

Antibiotic	*Staphylococcus aureus* Smith ED_{50} (mg/kg)	*Streptococcus pyogenes* C-203 ED_{50} (mg/kg)
Plusbacin A_2	2.04	1.93
Plusbacin A_3	0.47	0.73

Challenge: ip, treatment: sc (0, 5 hours).

Biological Properties

Plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$ are active against Gram-positive bacteria including strains of methicillin-resistant *Staphylococcus aureus* in the usual agar dilution method (Table 4). These antibiotics were not active against Gram-negative bacteria tested, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Proteus mirabilis* and *Serratia marcescens* (MICs >100 μg/ml).

Curative effects in mice infected with *S. aureus* Smith and *Streptococcus pyogenes* C-203 were observed with plusbacins A_2 and A_3. The experimental conditions and ED_{50} values are shown in Table 5.

Discussion

Previously we have reported the isolation of katanosins A and B, undecapeptides containing a lactone linkage, from a strain of *Cytophaga* sp., which are cell wall synthesis-inhibitors[3,4]. Here, we reported the isolation of plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$ from a strain of *Pseudomonas* sp., which are also cell wall synthesis-inhibitors indicated by the fact that they inhibit incorporation of radioisotopic diaminopimeric acid into the cell wall peptidoglycan of a *Bacillus* strain[5] (data not shown). Plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$ are presumed to be acyloctapeptides containing a lactone linkage. The structures of these antibiotics are presented in the succeeding paper[1].

Among the known antibiotics of bacterial origin, empedopeptin (BMY-28117) isolated from *Empedobacter halodium* nov. sp.[6,7] is somewhat similar to plusbacins. The antibiotic is also an

acyloctapeptide containing a lactone linkage. However, plusbacins are clearly differentiated by constituent amino acids and fatty acids.

Experimental

The UV absorption spectra were measured with a Hitachi 323 spectrometer, IR absorption spectra with a Jasco DS-403G spectrometer and high resolution mass spectrometry with a Hitachi M-90 mass spectrometer. Amino acid analyses were carried out with a Hitachi amino acid autoanalyzer 835 and gas-liquid chromatography with a Shimadzu Gas Chromatography GC-7AG.

Constituent Amino Acids

Hydrochlorides of all plusbacins (1~2 mg) were hydrolyzed with constant boiling HCl at 110°C for 20 hours. The hydrolysates were subjected to amino acid analysis. A synthesized specimen of β-hydroxyaspartic acid was used as a reference. The hydrolysates were also examined by paper chromatography on a paper (Toyo Roshi No. 51) with *n*-BuOH - AcOH - H_2O (4 : 1 : 2) visualized by ninhydrin coloration. An unknown amino acid was observed commonly in all the components. The unknown amino acid produced yellow coloration with ninhydrin reaction on paper chromatography similar to that noted with proline. It was subsequently identified as L-*trans*-β-hydroxyproline. The identification experiment and the results of amino acid analyses using the amino acid as a reference are presented in the succeeding paper[1].

Constituent Fatty Acids

Hydrochlorides of all plusbacins (1~2 mg) were hydrolyzed with constant boiling HCl at 110°C for one hour. Fatty acids contained in the hydrolysates were extracted with ethyl ether, and methylated with dimethylsilyldiazomethane. The methyl esters were subjected to gas-liquid chromatography using a SPB-1 fused silica capillary column (0.25 mm × 30 m), film thickness 0.25 μm (Supelco Inc.) under the following conditions: Carrier gas; He, temperature programing; 4°C/minute from 150°C to 250°C, detection; FID at 250°C.

The fatty acid methyl esters derived from plusbacins A_1 and B_1 showed the same retention time, indicating these components contained the same fatty acid residue. The same retention time was also observed with the fatty acid methyl esters from A_2 and B_2; A_3 and B_3; and A_4 and B_4.

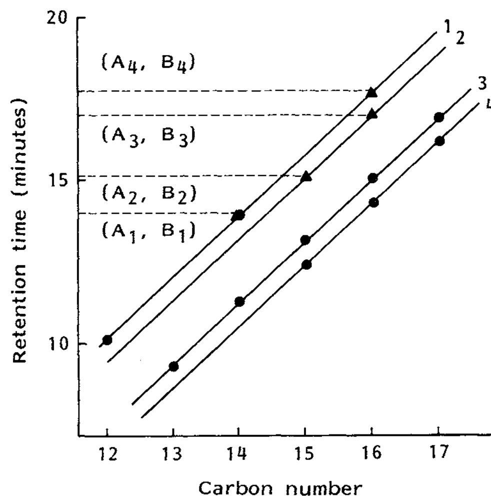
Reference compounds used are methyl esters of tridecanoic acid, tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, heptadecanoic acid, isopentadecanoic acid, isohexadecanoic acid, isoheptadecanoic acid, 3-hydroxy-dodecanoic acid and 3-hydroxy-tetradecanoic acid. When the retention times were plotted for their carbon number on a section paper, parallel linear relationships were obtained with *normal*-fatty acid methyl esters, *iso*-fatty acid methyl esters and 3-hydroxy-fatty acid methyl esters as shown in Fig. 3.

The retention time of the fatty acid methyl esters derived from plusbacins A_1 and B_1 coincided with that of 3-hydroxy-tetradecanoic acid. The fatty acid methyl esters from A_2 and B_2 was assumed to be 3-hydroxy-isopentadecanoic acid, those from A_3 and B_3 to be 3-hydroxy-isohexadecanoic acid and those from A_4 and B_4 to be 3-hydroxy-hexadecanoic acid from the above relationships.

Fig. 3. Retention time and carbon number in gas-liquid chromatographies of fatty acid methyl esters.

● Reference sample, ▲ test sample.

1: *Normal*-3-hydroxy-fatty acid methyl esters. 2: *Iso*-3-hydroxy-fatty acid methyl esters. 3: *Normal*-fatty acid methyl esters. 4: *Iso*-fatty acid methyl esters.



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References

1) SHOJI, J.; H. HINOO, T. KATAYAMA, Y. NAKAGAWA, Y. IKENISHI, K. IWATANI & T. YOSHIDA: Structures of new peptide antibiotics, plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$. J. Antibiotics 45: 824~831, 1992
2) PALLERONI, N. J.: *Genus* I. *Pseudomonas Migula* 1894. *In* BERGEY's Manual of Systematic Bacteriology. Volume 1. *Eds*., N. R. KRIEG & J. G. HOLT, pp. 141~199, Williams & Wilkins Co., 1984
3) SHOJI, J.; H. HINOO, K. MATSUMOTO, T. HATTORI, T. YOSHIDA, S. MATSUURA & E. KONDO: Isolation and characterization of katanosins A and B. J. Antibiotics 41: 713~718, 1988
4) KATO, T.; H. HINOO, Y. TERUI, J. KIKUCHI & J. SHOJI: The structures of katanosins A and B. J. Antibiotics 41: 719~725, 1988
5) ŌMURA, S.; H. TANAKA, R. ŌIWA, T. NAGAI, Y. KOYAMA & Y. TAKAHASHI: Studies on bacterial cell wall inhibitors. VI. Screening method for the specific inhibitors of peptidoglycan synthesis. J. Antibiotics 32: 978~984, 1979
6) KONISHI, M.; K. SUGAWARA, M. HANADA, K. TOMITA, K. TOMATSU, T. MIYAKI, H. KAWAGUCHI, R. E. BUCK, C. MORE & V. Z. ROSSOMANO: Empedopeptin (BMY-28117), a new depsipeptide antibiotic. I. Production, isolation and properties. J. Antibiotics 37: 949~957, 1984
7) SUGAWARA, K.; K. NUMATA, M. KONISHI & H. KAWAGUCHI: Empedopeptin (BMY-28117), a new depsipeptide antibiotic. II. Structure determination. J. Antibiotics 37: 958~964, 1984