HELVECARDINS A AND B, NOVEL GLYCOPEPTIDE ANTIBIOTICS

I. TAXONOMY, FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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A strain of actinomycetes identified as *Pseudonocardia compacta* subsp. *helvetica* produced new glycopeptide antibiotics, helvecardins A and B. They were isolated from culture broth mainly by affinity chromatography of D-alanyl-D-alanine and preparative HPLC. The physico-chemical properties of helvecardins A and B showed that they resemble each other. Though helvecardin A was structurally related to β-avoparcin, it clearly differed in the presence of an *O*-methyl moiety in its NMR spectrum.

In the course of our screening program for new antibiotics that inhibit bacterial cell wall peptidoglycan synthesis, one strain of actinomycetes, identified as *Pseudonocardia compacta* subsp. *helvetica* SANK 65185, was found to produce new glycopeptide antibiotics active against Gram-positive bacteria.

In this paper we report the taxonomy of the producing organism, and the fermentation, isolation, and physico-chemical properties of helvecardins A and B[1]. The structural elucidation and biological properties of the antibiotics are reported in accompanying papers[2,3].

Materials and Methods

Taxonomic Studies

The producing organism, strain SANK 65185, was isolated from a soil sample collected in Zürich, Switzerland.

Methods and media described by the International Streptomyces Project (ISP)[4], WAKSMAN[5] and GORDON *et al.*[6], were used to determine most of the cultural and physiological characteristics. For morphological observation, a light microscope and an S-510 scanning electron microscope (Hitachi Co., Ltd.) were used. Purified cell wall and whole-cell hydrolysates were analyzed by the methods of BECKER *et al.*[7], LECHEVALIER and LECHEVALIER[8], and HASEGAWA *et al.*[9]. Phospholipid, acyl type in the cell wall, menaquinones, and mycolic acids were respectively analyzed by the methods of LECHEVALIER and LECHEVALIER[10], UCHIDA and AIDA[11], COLLINS *et al.*[12], and HECHT and CAUSEY[13].

Fermentation

A loopful amount of a culture of strain SANK 65185 was inoculated into a 500-ml Erlenmeyer flask that contained 80 ml of medium consisting of glucose 3%, pressed yeast 1%, soybean meal 3%, $CaCO_3$ 0.4%, $MgSO_4 \cdot 7H_2O$ 0.2%, and Nissan Disfoam CB-442 0.01%. The pH of the medium was adjusted to 7.2 before sterilization. The inoculated flasks were incubated on a rotary shaker (220 rpm) at 28°C for 84 hours. Then a 25-ml aliquot of the culture was transferred into a 2-liter flask containing 500 ml of the

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same medium. After 24 hours of incubation on a rotary shaker, a 750-ml aliquot of this second seed culture was added to a 30-liter fermenter containing 15 liters of the medium. Fermentation was carried out at 28°C for 96 hours with an air-flow rate of 15 liters/minute and an agitation rate of 150 rpm. The antibacterial activity was determined by paper-disc agar diffusion assay using *Staphylococcus aureus* FDA 209P as the test organism. Packed cell volume was determined after centrifugation of 5 g of the culture broth at 3,000 rpm for 10 minutes.

Results

Taxonomy of Strain SANK 65185

Strain SANK 65185 grew relatively well and formed lumpy colonies on both organic and synthetic media. It formed straight to flexuous aerial mycelium on most media (Plate 1). Both the aerial and the vegetative hyphae elongated by acropetal budding, and side branches arose from lateral buds (Plate 2). In an aged culture those hyphae became fragmented into irregular size or formed spores. The spores were cylindrical with a smooth surface, as revealed by scanning electron microscopy.

The culture characteristics on various agar media are shown in Table 1. The vegetative growth color was pale yellowish orange. Aerial hyphae were produced relatively well on most media. Soluble pigment was not detected.

In Table 2 the physiological properties are shown. It was demonstrated that the organism was Gram-positive and non-acid-fast. It did not grow under anaerobic conditions. Starch hydrolysis and melanin formation could not be demonstrated, however gelatin liquefaction, milk coagulation, and peptonization were detected.

meso-Diaminopimelic acid, arabinose and galactose were found in the cell wall, indicating that the cell wall type was type IV and the whole-cell sugar pattern was A. Mycolic acid was not detected. The types of the acyl group, the phospholipid, and the major menaquinones of the cell wall were respectively acetyl type, PIII, and MK-9 (H4). From these results, strain SANK 65185 was presumed to belong to genus *Pseudonocardia* and among known species[14,15], *P. compacta* was selected as the most closely related species. A direct comparison of strain SANK 65185 and the type strain of *P. compacta* showed that they were very closely related to each other but different in three points:

i) Strain SANK 65185 utilized citrate but not sucrose, whereas *P. compacta* did only sucrose.

ii) Strain SANK 65185 formed acid from various kinds of carbohydrates, whereas *P. compacta* did so only from glucose.

Plate 1. Light micrograph of strain SANK 65185 (on potato extract-carrot extract agar, 28°C, 7 days).

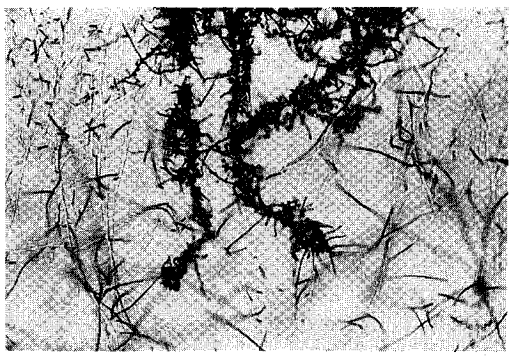


Plate 2. Scanning electron micrograph of strain SANK 65185 (in yeast extract-glucose broth, 28°C, 1 day).

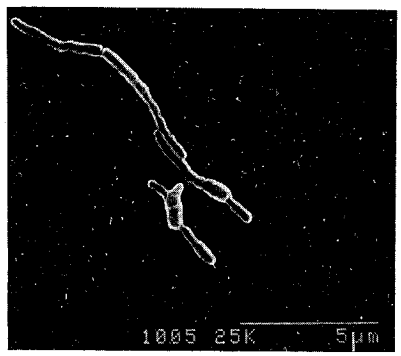


Table 1. Cultural characteristics of Strain SANK 65185.

Yeast extract-malt extract agar (ISP 2)	G:	Abundant, wrinkled, pale yellowish orange
	AM:	Good, velvety, white
	R:	Dull yellowish orange
	SP:	None
Oatmeal agar (ISP 3)	G:	Good, flat, pale yellowish orange
	AM:	Moderate, velvety, white
	R:	Pale yellowish orange
	SP:	None
Inorganic salts-starch agar (ISP 4)	G:	Good, flat, pale yellowish orange
	AM:	Good, velvety, white
	R:	Pale yellowish brown
	SP:	None
Glycerol-asparagine agar (ISP 5)	G:	Abundant, wrinkled, pale yellowish orange
	AM:	Moderate, velvety, white
	R:	Pale yellowish brown
	SP:	None
Peptone-yeast extract-iron agar (ISP 6)	G:	Good, wrinkled, pale yellowish orange
	AM:	None
	R:	Pale yellowish brown
	SP:	None
Tyrosine agar (ISP 7)	G:	Abundant, wrinkled, pale yellowish orange
	AM:	Good, velvety, white
	R:	Yellowish brown
	SP:	None
Sucrose-nitrate agar	G:	Abundant, flat, pale yellowish orange
	AM:	Good, velvety, white
	R:	Pale yellow
	SP:	None
Glucose-asparagine agar	G:	Abundant, flat, pale yellowish orange
	AM:	Good, velvety, white
	R:	Pale yellowish brown
	SP:	None
Nutrient agar (Difco)	G:	Good, wrinkled, pale yellowish orange
	AM:	None
	R:	Pale yellowish brown
	SP:	None
Potato extract-carrot extract agar	G:	Moderate, flat, pale yellowish orange
	AM:	Good, velvety, white
	R:	Pale yellowish orange
	SP:	None
Water agar	G:	Moderate, flat, pale yellowish orange
	AM:	Poor, white
	R:	Yellowish gray
	SP:	None

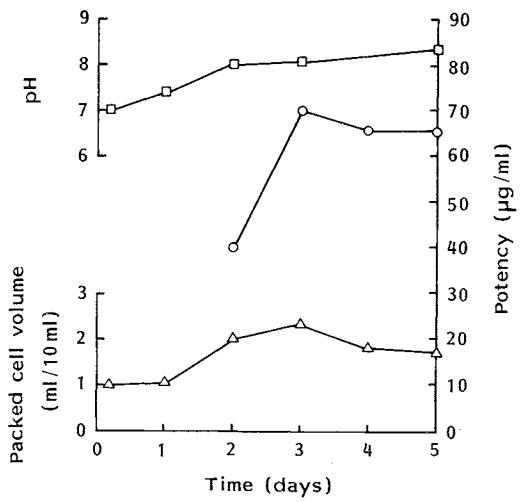
G: Growth, AM: aerial mycelium, R: reverse, SP: soluble pigment.

iii) Strain SANK 65185 was sensitive to erythromycin, benzylpenicillin, cephaloridine, novobiocin, nalidixic acid and nitrofuran, whereas *P. compacta* was resistant to these antibiotics.

These differences are insufficient to conclude that these two strains are different species, so strain SANK 65185 was considered to represent a new subspecies of *P. compacta*, for which the name *P. compacta* subsp. *helvetica*, subsp. nov. (L. adj. fem., pertaining to swiss) is proposed. Progeny of this type strain have been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ibaragi, Japan, with an accession number of FERM P-8634.

Fig. 1. Fermentation of strain SANK 65185.

○ Potency (μg/ml), □ pH, △ packed cell volume.



Fermentation of Strain SANK 65185

The typical time course of fermentation of helvecardins in a 30-liter fermenter is shown in Fig. 1. The growth of SANK 65185, gradually increased in the first 48 hours, and antibiotic production started at 48 hours, reaching its maximum at 72 hours

Table 2. Physiological properties of strain SANK 65185.

Acid fastness	−	Acid production:	
Gram stain	−	Pyruvate	−
Decomposition:		Arabinose	+
Adenine	NG	Dulcitol	−
Casein	+	Erythritol	+
Xanthine	−	Glucose	+
Hypoxanthine	+	Inositol	+
Tyrosine	+	Lactose	+
Starch hydrolysis	−	Mannitol	+
Gelatin liquefaction	+	Mannose	+
Milk coagulation	+	Melibiose	+
Milk peptonization	+	Raffinose	−
Melanoid pigment formation:		Rhamnose	−
ISP 1	−	Sorbitol	−
ISP 6	−	Trehalose	+
ISP 7	−	Adonitol	+
Resistant to:		Cellobiose	+
Benzylpenicillin	R	Maltose	+
Lysozyme	R	Melezitose	−
Carbohydrate utilization:		Growth temperature	7~36°C
D-Glucose	+	(optimum temperature)	(14~27°C)
L-Arabinose	−	Survival at 50°C	−
D-Xylose	+	Growth under anaerobic condition	−
Inositol	+	NaCl torelance:	
D-Mannitol	+	1%	+
D-Fructose	+	2%	+
L-Rhamnose	−	3%	+
Sucrose	−	5%	+
Raffinose	−	7%	±
D-Galactose	+	10%	NG
D-Mannose	+	Antibiotic resistance:	
Cellobiose	+	Chloramphenicol	S
Lactose	±	Tetracycline	S
Maltose	−	Doxycycline	S
Trehalose	+	Streptomycin	S
Dulcitol	−	Kanamycin	S
Inulin	−	Gentamicin	S
Dextrin	−	Erythromycin	S
Soluble starch	±	Benzylpenicillin	S
Salicin	±	Cephaloridine	S
Sodium acetate	±	Cephalothin	R
Sodium succinate	±	Mikamycin	S
Glycerol	+	Clindamycin	S
Cellulose	−	Polymyxin B	R
Control	−	Novobiocin	S
Organic acid utilization:		Nalidixic acid	S
Citrate	+	Fusidic acid	S
Lactate	−	Sulfisoxazole	R
Oxalate	+	Nitrofurantoin	S
Succinate	+	Furazolidon	R
Benzoate	+		

NG: No growth, R: resistant, S: sensitive.

after inoculation.

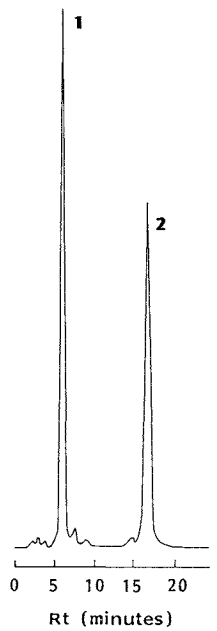
Isolation of Helvecardins A and B

Thirty liters of the fermentation broth were filtered with the aid of Celite 545, and the cake was

washed with water. Thirty liters of the filtrate thus obtained was adsorbed on a column of Diaion HP-20 (Mitsubishi Chemical Industries Limited, Japan, 3 liters), and after washing with 9 liters of water, the antibiotics were eluted out with 18 liters of aqueous acetone. After the active eluate was concentrated *in vacuo* to remove the acetone, the resulting liquid was washed with an equal volume of butanol, concentrated *in vacuo*, and lyophilized to obtain 18.3 g of crude powder. Then 18 g of this crude powder were dissolved in 500 ml of distilled water and adsorbed on a column of Affi-gel 10 (Bio Rad Co., Ltd., 100 ml) coupled with D-alanyl-D-alanine, which is known to be an efficient method for the purification of glycopeptide antibiotics[16]. The column was washed with 300 ml of 30% aqueous CH_3CN, and the antibiotics were eluted with 3 liters of 30% aqueous CH_3CN with 0.35 M ammonium bicarbonate. The active eluate was concentrated, desalted with Diaion HP-20 (100 ml), and lyophilized to obtain 784.4 mg of crude powder. Then this powder was dissolved in a small amount of distilled water and applied on a preparative HPLC, in which ODS-H-5251 (Senshu Kagaku Co.) was developed with 15% aqueous CH_3CN with 0.01 M phosphate buffer, pH 6.5. The HPLC profile of helvecardins A and B is shown in Fig. 2. The portion corresponding to A or B was collected, desalted, and lyophilized as mentioned before, to obtain 78.1 mg of A and 222.7 mg of B, respectively.

Fig. 2. HPLC profile of helvecardins A and B.

1 Helvecardin A, **2** helvecardin B.



Column: ODS-H-2151 (6 mm-d × 150 mm), solvent: 1.7% (w/v) sodium heptane sulfonate, 2.0% AcOH, 0.4% NH_4OH and 16% CH_3CN in distilled water, flow rate: 1.5 ml/minute, detection: 280 nm.

Physico-chemical Properties of Helvecardins A and B

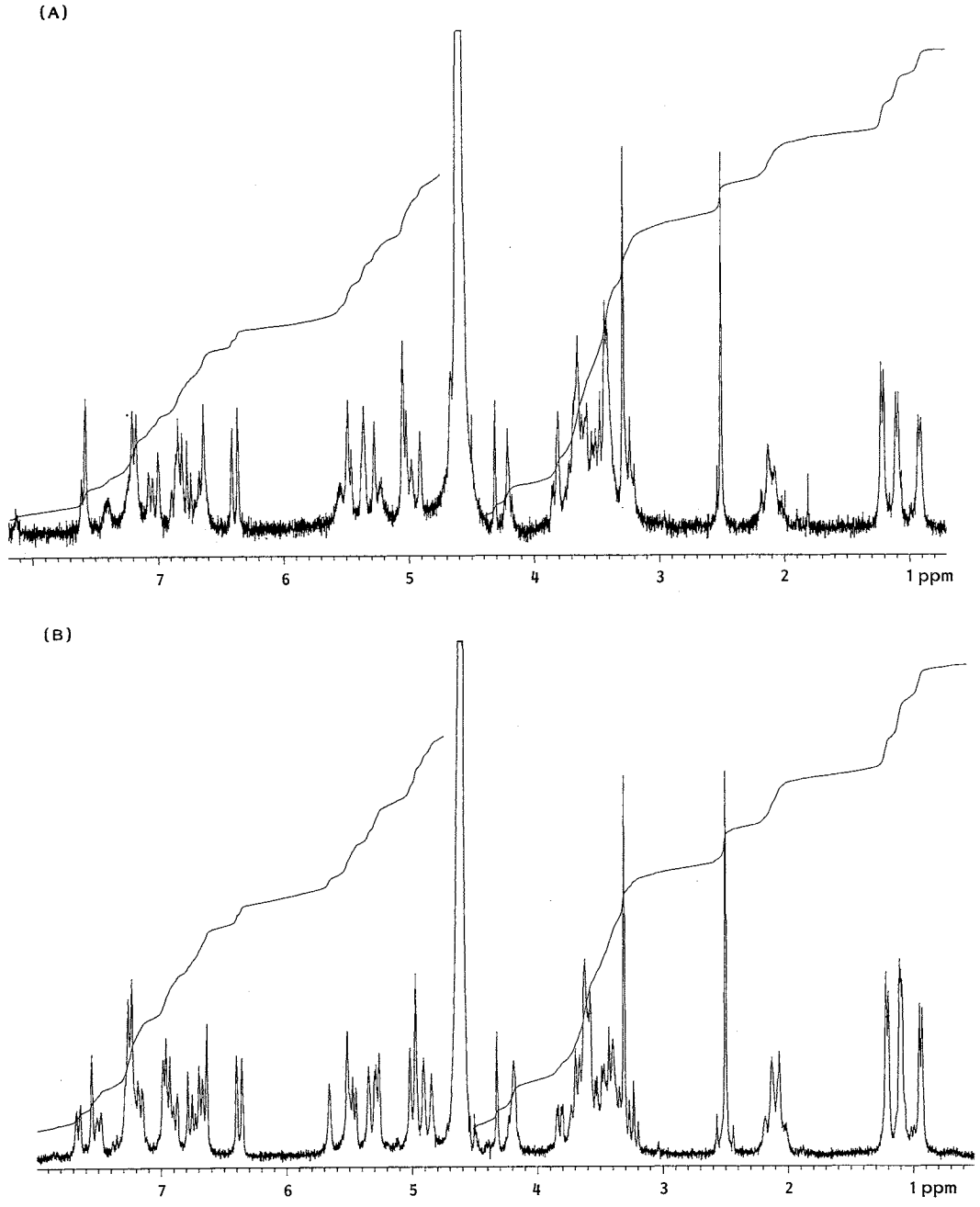
The physico-chemical properties of helvecardins A and B are summarized in Table 3. They are amphoteric, amorphous white powder, and soluble in water and methanol but not in ethyl acetate and chloroform. The antibiotics showed positive reac-

Table 3. Physico-chemical properties of helvecardins A and B.

	A	B
Nature	Amphoteric white powder	Amphoteric white powder
$[\alpha]_D^{25}$	−73.6° (*c* 0.99, 0.1 N HCl)	−95° (*c* 1.08, 0.1 N HCl)
Elementary analysis	C 50.50, H 5.60, N 5.60, Cl 3.25	C 50.65, H 5.23, N 5.75, Cl 3.30
Molecular formula	$C_{90}H_{103}N_9O_{36}Cl_2$	$C_{84}H_{93}N_9O_{31}Cl_2$
FAB-MS *m/z* $(M+H)^+$	1,956	1,794
UV $\lambda_{max}^{H_2O}$ nm (ε)	280 (8,016)	281 (7,440)
IR ν (cm^{-1})	3400, 1670, 1585, 1500	3400, 2920, 1650, 1595, 1500
Color reaction	Ninhydrin, $FeCl_3$, Rydon-Smith	Ninhydrin, $FeCl_3$, Rydon-Smith
Solubility		
Soluble:	H_2O	H_2O
Insoluble:	EtOAc	EtOAc

Fig. 3. NMR spectra of helvecardins A and B.

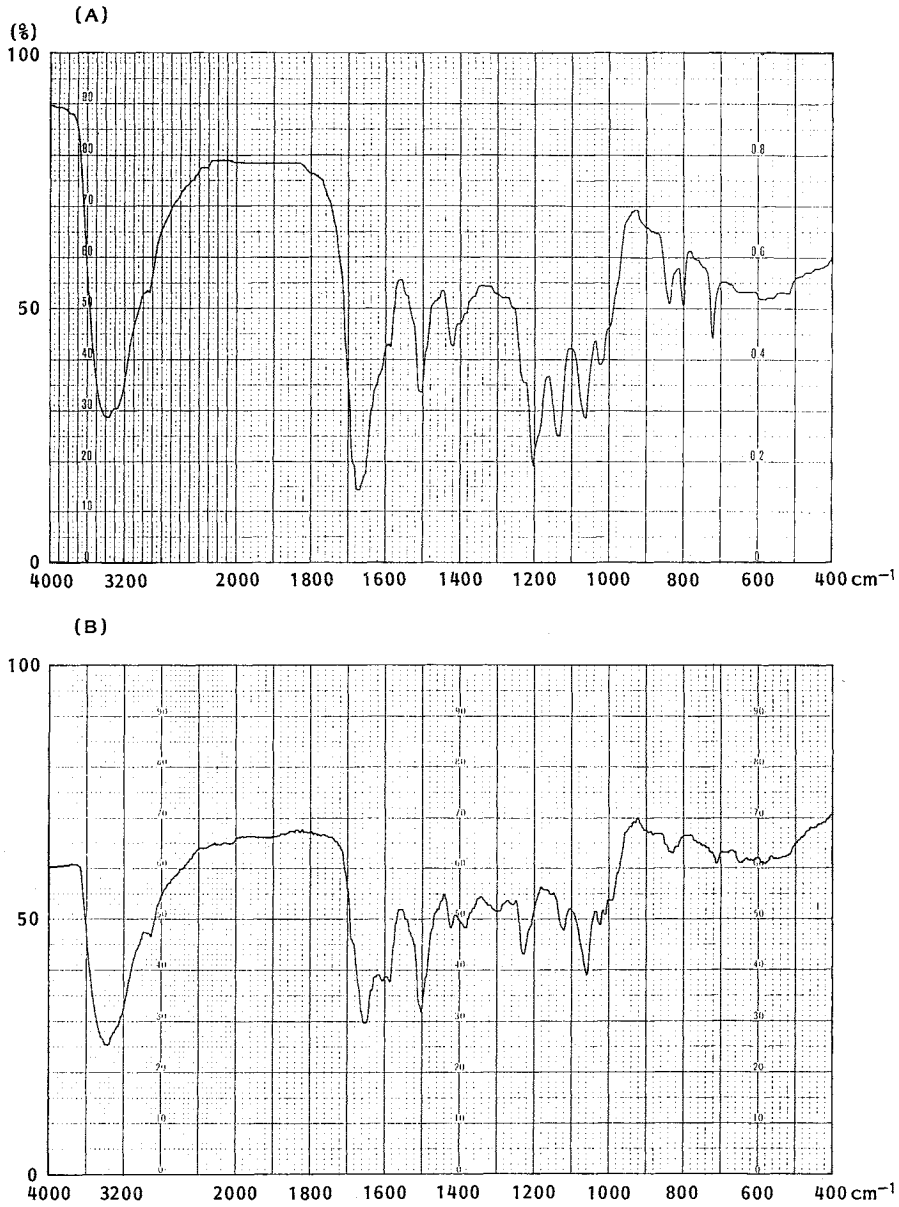
(A) Helvecardin A, (B) helvecardin B.



tions for ferric chloride, iodine, Rydon-Smith reagent, and ninhydrin. Their MW's and molecular formulae were determined to be *m/z* 1,955 ($C_{90}H_{103}N_9O_{36}Cl_2$) and 1,793 ($C_{84}H_{93}N_9O_{31}Cl_2$), respectively, from analyses of FAB-MS, elemental analyses, and analyses of their degradation products, as mentioned in the accompanying paper[2]. The UV spectra of helvecardins A and B in aqueous solution were 280 nm (ε 8,016) and 281 nm (ε 7,440), respectively. Their NMR and IR spectra are shown in Figs. 3 and 4. These

Fig. 4. IR spectra of helvecardins A and B (KBr).

(A) Helvecardin A, (B) helvecardin B.



data showed strong similarity with β-avoparcin but clear difference in several points, such as the presence of an *O*-methyl group at 3.27 and 3.30 ppm in ^{1}H NMR spectra, and their quasimolecular ion peaks $(M+H)^+$ at *m/z* 1,956 and 1,794, respectively.

Discussion

A strain SANK 65185 was considered to be a member of *P. compacta* from its taxonomic properties, but a direct comparison of it with the type strain of *P. compacta* showed some differences between them.

As these differences are insufficient to determine it as a new species, it was finally concluded to be a new subspecies of *P. compacta*, and it was designated *P. compacta* subsp. *helvetica* SANK 65185. Azureomycin[17] was the first example of glycopeptide antibiotics isolated from the genus *Pseudonocardia*, and helvecardins A and B were the second ones.

Helvecardins A and B are very similarly related to β-avoparcin in nature, but they clearly differed in the presence of an *O*-methyl group. From the difference in MW's among A, B and β-avoparcin, A is proposed to be *O*-methyl-β-avoparcin, and B is a dehexose derivative of A. Indeed, in the fermentation broth of *P. compacta* subsp. *helvetica*, β-avoparcin was also detected based on HPLC analysis.

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