GALACARDINS A AND B†, NEW GLYCOPEPTIDE ANTIBIOTICS

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A strain of actinomycetes identified as *Saccharothrix* sp. SANK 64289 was found to produce new antibiotics, galacardins A and B. Their physico-chemical properties showed that they were new members of glycopeptide antibiotics. They were structurally related to β-avoparcin but differed from it only in sugar composition. Though β-avoparcin does not contain galactose, galacardins A and B did contain two and one moles of galactose, respectively. They showed strong antimicrobial activity against Gram-positive bacteria and also showed excellent *in vivo* protective activity against *Staphylococcus aureus* infection in mice.

In the course of our intensive screening program for antibiotics with spheroplast forming activity, we found that a strain identified as *Saccharothrix* sp. SANK 64289 produced new glycopeptide antibiotics, galacardins A and B. In this paper, we report the taxonomy of the producing organism, the fermentation, isolation, physical and chemical properties, structural elucidation and biological properties of galacardins A and B.

Materials and Methods

Taxonomic Studies

The producing organism, strain SANK 64289 was isolated from a soil sample collected in Ohmachi City, Nagano Prefecture, Japan.

Morphological, cultural and physiological characteristics of strain SANK 64289 were carried out by the methods described by the International Streptomyces Project (ISP)[1] and WAKSMAN[2]. Several other media were also used for morphological studies. The color names were assigned according to "Guide to Color Standard" (a manual published by Nippon Shikisai Kenkyusho, Tokyo, Japan). The culture was observed after incubation at 28°C for 14 days unless otherwise stated. Purified cell wall and whole cell hydrolysate were analyzed by using the methods of BECKER *et al.*[3] and LECHEVALIER[4]. Mycolic acid, muramic acid, menaquinones and phospholipids were identified by using the procedure of HECHT and CAUSEY[5], UCHIDA and AIDA[6], COLLINS *et al.*[7] and LECHEVALIER *et al.*[8], respectively.

Fermentation

A loopful amount of the culture of strain SANK 64289 was inoculated into a 500-ml Erlenmeyer flask that contained 80 ml of medium consisting of glucose 3%, $MgSO_4 \cdot 7H_2O$ 0.2%, soybean meal 3%, $CaCO_3$ 0.4%, pressed yeast 1% 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 5-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 jar fermenter containing 15 liters of the medium. Fermentation was carried out at 28°C for 144 hours with an air-flow rate of 15 liters per minute and an agitation rate of 150 rpm. Packed cell volume was determined after centrifugation of 5 g of the culture broth at 3,000 rpm for 10 minutes.

The production of galacardins was monitored by the paper-disc agar diffusion method using *Staphylococcus aureus* FDA 209P as a test organism.

Isolation of Galacardins

60 liters of fermentation broth were filtered with the aid of Celite 545. Fifty five liters of filtered broth were adsorbed on a Diaion HP-20 column (Mitsubishi Chemical Ind. Ltd., Japan, 5.5 liters) and after washing with 25 liters of water, the antibiotics were eluted with 33 liters of 60% aqueous methanol. This effluent was concentrated under reduced pressure and lyophilized to afford 95.1 g of the crude powder. This crude powder was dissolved in 10 liters of distilled water and applied on a column of Affi-gel 10 (Bio Rad Co., Ltd., 540 ml) liganded with D-alanyl-D-alanine. The column was washed with 15 liters of 30% aqueous CH_3CN and antibiotics were eluted with 18 liters of 30% aqueous CH_3CN with 0.35 M ammonium bicarbonate. Then, active fractions were collected, desalted and lyophilized to obtain 4.94 g of crude powder. This crude powder was then dissolved in a small amount of water and applied on a preparative HPLC (ODS H-5251, Senshu, solvent: 15% CH_3CN in 0.2% TFA, detection: 280 nm, flow rate: 9.9 ml/minute). The portions corresponding to galacardin A or B were collected, desalted and lyophilized to yield 1.18 g of galacardin A and 1.06 g of galacardin B.

Biological Activities

MICs of galacardins were determined by a serial dilution method. The medium used were Mueller-Hinton agar for aerobic bactria and GAM agar for anaerobic bacteria. The cultures were incubated at 37°C for 24 hours at inoculum size of 10^6 cfu/ml.

In Vivo Protection Test

S. aureus 56 was grown in 10 ml of Nutrient broth (Eiken) at 37°C overnight without shaking. After harvesting and washing with ice-cold 0.85% NaCl, cells were suspended in 20 ml of 0.85% NaCl containing 5% mucin. *ddY* Female mice were infected intraperitoneally with 1×10^8 cells and treated with antibiotics subcutaneously twice, immediately and again 4 hours after infection. ED_{50} was determined on day 7 after infection.

Amino Acid Analysis

1 mg of galacardin A or B was hydrolyzed with 1 ml of 55% hydroiodic acid containing a trace of red phosphorus[9] at 105°C for 20 hours in a sealed ampoule. After drying *in vacuo*, the residue was analyzed by HPLC. Actinoidic acid, *N*-methyl-4-hydroxyphenylglycine and 3-Cl-4-hydroxyphenylglycine were eluted at 2.41, 3.53 and 5.00 minutes (ODS H-2151, 5% CH_3CN in 0.2% TFA, 1.5 ml/minute), respectively and monodechloro-dideoxyvancomycinic acid was eluted at 4.8 minutes (ODS H-2151, 16% CH_3CN in 0.2% TFA, 1.5 ml/minute).

Sugar Analysis

1 mg of galacardin A or B was hydrolyzed with 0.5 ml of 5% HCl-methanol at 100°C for 16 hours. Resulting hydrolysates were converted to their TMS derivatives with *N-O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA, 30 μl) in pyridine (50 μl) at 60°C for 16 hours. The TMS derivatives were assigned to those of authentic sugars by gas chromatography (2% OV-17, 3 mm × 50 cm column, 100~230°C, 10°C/minute temperature programming, He gas 60 ml/minute).

Treatment of Galacardin A with α-Galactosidase

0.5 mg of galacardin A was dissolved in 1 ml of 0.05 M citrate buffer, pH 4.0, and 0.1 unit of α-galactosidase (Shigma Co., Ltd.) was added. The reaction mixture was allowed to stand at room temperature and applied directly to HPLC analysis.

Results

Taxonomy of Strain SANK 64289

Strain SANK 64289 grew well on the defined and the organic media with pasty growth. Aerial mycelium was moderate to abundant on the agar media tested and was white in color. In aged culture, aerial mycelium was fragmented into coccoid elements. The substrate mycelium was pale yellowish gray. Neither melanoid pigment nor soluble pigment was produced. D-Glucose was utilized and D-fructose or sucrose was weakly utilized, while L-arabinose, D-xylose, inositol, D-mannitol, L-rhamnose and raffinose were not. Cultural and physiological properties were summarized in Tables 1 and 2, respectively. In whole-cell hydrolysates of strain SANK 64289, *meso*-diaminopimelic acid was present and galactose, mannose and rhamnose were detected as major components. Nocardiomycolic acids were not present. The acyl type of cell-wall peptidoglycan was determined to be *N*-acetylmuramic acid. The strain contained MK-9 (H4) and MK-10 (H4) as the predominant menaquinones and phospholipids of type P-II. From the results mentioned above, strain SANK 64289 was considered to belong to genus *Saccharothrix*. The progeny of the strain SANK 64289 has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Ibaraki Prefecture, Japan, with the accession number of FERM P-10940.

Fermentation of a Strain SANK 64289

The time course of production of galacardins in a 30-liter jar fermenter is shown in Fig. 1. The antibiotic production started at 72 hours and reached maximum at 144 hours. The maximal potency in the fermentation was 70 μg/ml as galacardin A.

Isolation and Purification of Galacardins

The isolation and purification of galacardins A and B from 55 liters of filtered broth were carried

Table 1. Cultural characteristics of strain SANK 64289.

Yeast extract-malt extract agar (ISP 2)	G: Abundant, wrinkled, pasty, pale yellowish brown
	AM: Good, velvety, white
	R: Yellowish brown
	SP: None
Oatmeal agar (ISP 3)	G: Good, flat, pasty, pale yellowish brown
	AM: Scant, white
	R: Pale yellowish brown
	SP: None
Inorganic salts-starch agar (ISP 4)	G: Moderate, flat, pasty, pale yellowish brown
	AM: Moderate, velvety, white
	R: Pale yellowish orange
	SP: None
Glycerol-asparagine agar (ISP 5)	G: Abundant, flat, pasty, pale yellowish brown
	AM: Abundant, velvety, white
	R: Pale yellowish brown
	SP: None

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

Table 2. Physiological properties of strain SANK 64289.

Starch hydrolysis	−
Gelatin liquefaction	+
Nitrate reduction	+
Milk coagulation	+
Milk peptonization	+
Growth temperature	8~36°C
Optimum temperature	20~31°C
Sodium chloride torelance	3%
Substrate decomposition:	
Casein	+
Tyrosine	+
Xanthine	−
Lysozyme resistance	Resistant
Acid production from:	
Adonitol	−
Arabinose	−
Cellobiose	−
Erythritol	−
Lactose	−
Maltose	+
Mannose	+
Raffinose	−
Rhamnose	−
Sucrose	+

out according to the scheme shown in Fig. 2. One of the characteristic features of this procedure is the use of Affi-gel 10 liganded with D-alanyl-D-alanine which was known to be a very efficient method[10] for the purification of glycopeptide antibiotics. Further purification was mainly achieved by preparative HPLC because active fractions from affinity chromatography contained many minor substances corresponding to galacardins A and B.

Physico-chemical Properties of Galacardins A and B

The physico-chemical properties of galacardins A and B are summarized in Table 3. Galacardins are amorphous white powder, soluble in water, methanol, scarcely soluble in acetone, insoluble in ethyl acetate and chloroform. The antibiotics showed positive reaction for ninhydrin, Rydon-Smith reagent and ferric chloride. Their molecular weights and molecular formulae were determined to be 2,265 ($C_{101}H_{121}N_9O_{46}Cl_2$) and 2,103 ($C_{95}H_{111}N_9O_{41}Cl_2$), respectively from analyses of FAB-MS,

Fig. 1. Production of galacardins.

□ pH, ○ packed cell volume (PCV) (ml/10 g), △ potency (μg/ml).

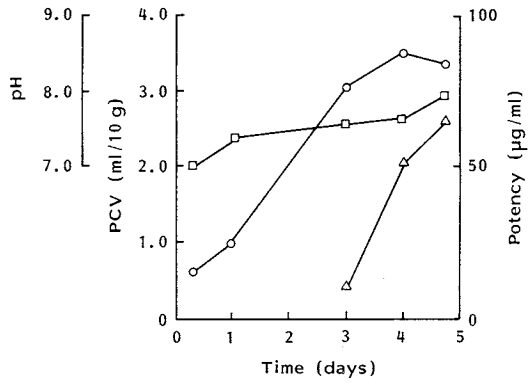


Fig. 2. Purification of galacardins A and B.

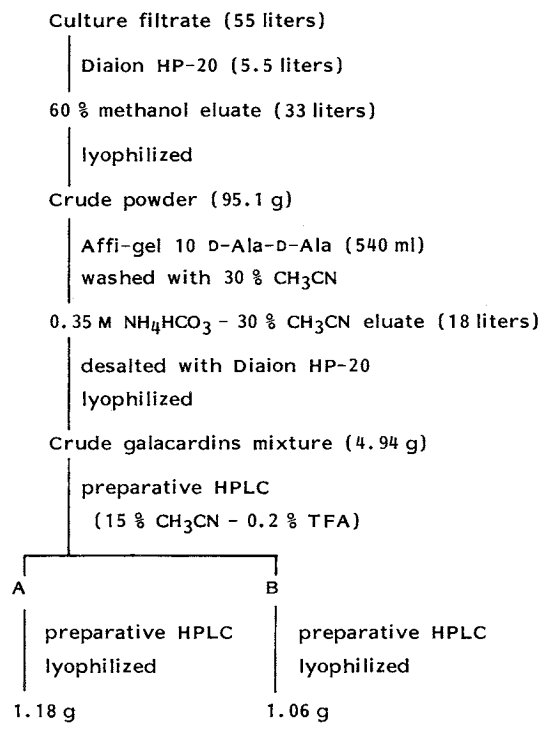
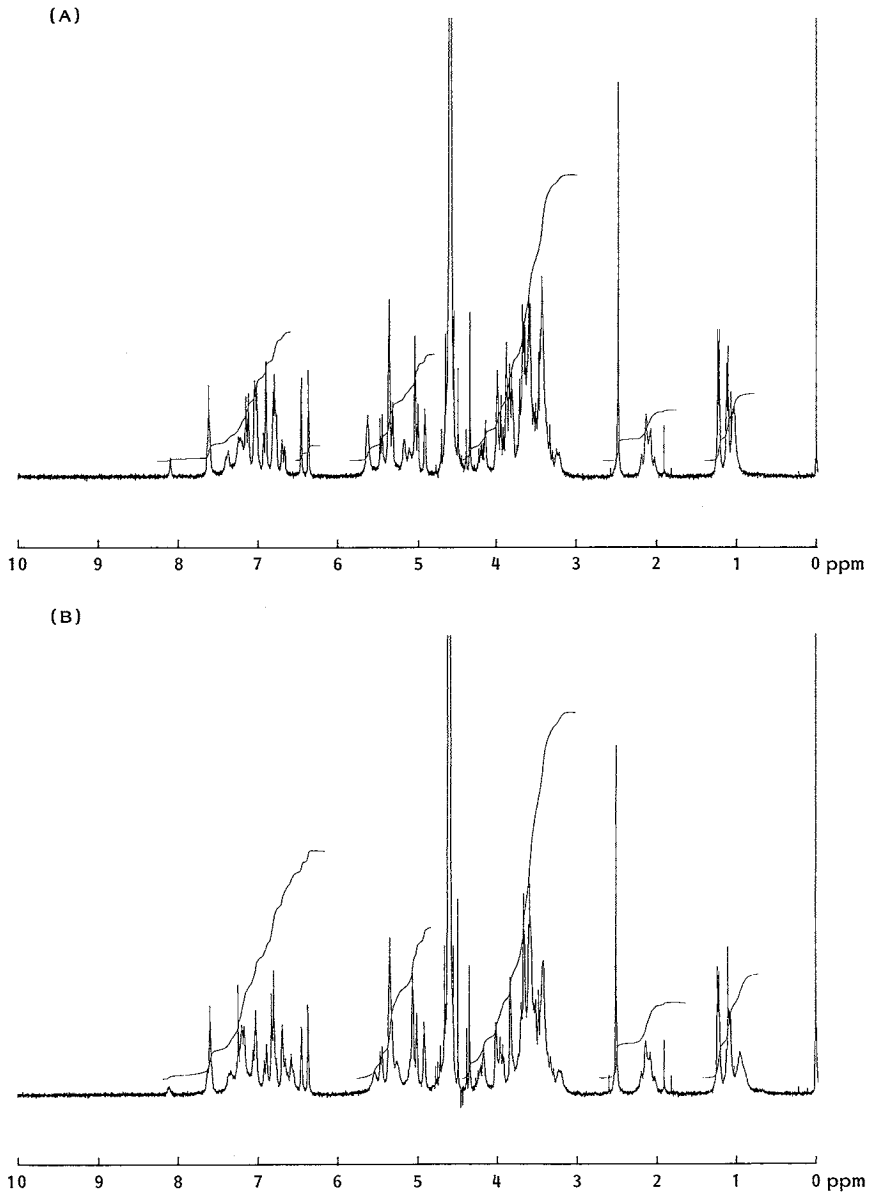


Table 3. Physico-chemical properties of galacardins A and B.

	A	B
Nature	Amphoteric white powder	Amphoteric white powder
$[\alpha]_D^{25}$	−38.5° (*c* 1.00, H_2O)	−52.4° (*c* 0.97, H_2O)
Elementary analysis (TFA salt, %)	C 50.55, H 5.62, N 5.06, Cl 2.39, F 1.16	C 48.30, H 5.20, N 5.08, Cl 2.75, F 1.43
Molecular formula	$C_{101}H_{121}N_9O_{46}Cl_2$	$C_{95}H_{111}N_9O_{41}Cl_2$
MW (FAB-MS $(M+H)^+$)	2,265 (2,266)	2,103 (2,104)
UV $\lambda_{max}^{H_2O}$ nm (ε)	280 (8,969)	279 (10,620)
IR (KBr) cm^{-1}	1656, 1597, 1504	1655, 1590, 1506
Color reaction	Ninhydrin, $FeCl_3$, Rydon-Smith	Ninhydrin, $FeCl_3$, Rydon-Smith
Solubility		
Soluble:	H_2O, MeOH	H_2O, MeOH
Insoluble:	EtOAc	EtOAc

Fig. 3. ^{1}H NMR spectra of galacardins A (A) and B (B).



elemental analyses and analyses of degradation products. The UV spectra of galacardins A and B were 280 nm (8,969), and 279 nm (10,620) in aqueous solution, respectively. The IR spectra of galacardins were very similar to those of glycopeptide antibiotics showing the characteristics bands of glycosides, phenols and amides. The NMR spectra of galacardins A and B were shown in Fig. 3.

Biological Properties of Galacardins A and B

The antimicrobial activities of galacardins A and B compared with those of β-avoparcin and vancomycin are shown in Table 4. Galacardins showed strong activity against Gram-positive bacteria including methicillin-resistant *S. aureus* (MRSA) as did vancomycin and β-avoparcin. The *in vitro*

Table 4. Antimicrobial spectra of galacardins A and B, AVP and VCM.

Test organism	Medium	MIC (μg/ml)			
		A	B	AVP	VCM
Staphylococcus aureus FDA 209P	A	3.12	1.56	1.56	1.56
S. aureus SANK 70175	A	6.25	3.12	3.12	1.56
S. aureus SANK 70775	A	6.25	3.12	3.12	1.56
S. aureus SANK 70589 (MRSA)	A	50	25	12.5	3.12
S. aureus SANK 70689 (MRSA)	A	12.5	12.5	12.5	1.56
S. aureus SANK 70789 (MRSA)	A	12.5	3.12	12.5	1.56
S. aureus SANK 70889 (MRSA)	A	6.25	3.12	3.12	1.56
S. aureus SANK 70989 (MRSA)	A	12.5	6.25	3.12	0.78
S. aureus SANK 71089 (MRSA)	A	6.25	6.25	6.25	1.56
Mycobacterium smegmatis ATCC 607	A	25	25	6.25	6.25
Enterococcus faecalis subsp. *liquefaciens* S-299	A	6.25	3.12	1.56	6.25
Escherichia coli NIHJ JC-2	A	>100	>100	>100	>100
E. coli SANK 79885	A	50	25	25	50
Klebsiella pneumoniae PCI 602	A	>100	>100	>100	>100
Serratia marcescens SANK 73060	A	>100	>100	>100	>100
Proteus vulgaris OX 19	A	>100	>100	>100	>100
P. mirabilis SANK 71873	A	>100	100	100	>100
Pseudomonas aeruginosa NCTC 19490	A	>100	>100	>100	>100
Bacteroides fragilis SANK 71176	B	>100	>100	>100	>100

Medium A: Mueller-Hinton agar. Medium B: GAM agar.
Inoculum size: 10^6 cells/ml.
A: Galacardin A, B: galacardin B. AVP: β-avoparcin, VCM: vancomycin.

antimicrobial activities of galacardins were quite similar to those of β-avoparcin.

In vivo efficacies (ED_{50}) of galacardin A and β-avoparcin against *S. aureus* 56 infection in mice were calculated to be 19.0 mg/kg and 17.0 mg/kg, respectively.

Structure Determination

From the results mentioned above galacardins A and B were suggested to belong to the group of glycopeptide antibiotics. Generally, glycopeptide antibiotics are composed of several sugars and amino acis. For the determination of amino acid components, galacardins A and B were reductively hydrolyzed with HI in the presence of red phosphorus. Monodechlorodideoxyvancomycinic acid, actinoidic acid, 3-Cl-4-hydroxyphenylglycine and *N*-methyl-4-hydroxyphenylglycine were detected commonly in galacardins A and B by HPLC analysis. It is well known that the allylic positions of the vancomycinic moiety, which is one of the characteristic components of glycopeptide antibiotics, are easily deoxygenated under reductive conditions[11].

The carbohydrate moieties of galacardins were derermined mainly from the analysis of acid hydrolysates. One mol each of glucose, mannose and rhamnose were identified. In addition, 2 and 1 mol of galactose in galacardins A and B, respectively, were detected by gas chromatography analysis followed by GC-MS analysis of the TMS derivatives of hydrochloric acid hydrolysates, using authentic sugars as standards. Ristosamine was not detectable by GC analysis, but the existence of 2 mol of ristosamine was deduced from the following results. 1) By MS/MS fragmentation analysis of galacardin A, the fragment ion peaks at *m/z* 292 and 130 were detected indicating the existence of ristosaminylglucose

Table 5. Hydrolysis products of galacardins A and B.

	A	B
Sugar	Ristosamine (2 mol), glucose (1 mol), rhamnose (1 mol), mannose (1 mol), galactose (2 mol)	Ristosamine (2 mol), glucose (1 mol), rhamnose (1 mol), mannose (1 mol), galactose (1 mol)
Amino acid	Monodechlorodideoxyvancomycinic acid, actinoidic acid, 3-chloro-4-hydroxyphenylglycine, *N*-methyl-4-hydroxyphenylglycine	

Table 6. HPLC retention times for galacardin B and D-galactosyl-β-avoparcin.

	Retention time (minutes)	
	System A	System B
Galacardin B	4.57	3.81
Galactosyl-β-avoparcin	3.24	3.20

HPLC system A: Column, ODS H-2151; solvent, 15% CH_3CN - 0.2% TFA; flow, 1.5 ml/minute; detection, 280 nm.

HPLC system B: Column, ODS H-2151; solvent, 15% CH_3CN in 0.01 M sodium heptane sulfonic acid with 2.5% AcOH; pH 4.0 NH_4OH; flow, 1.5 ml/minute; detection, 280 nm.

Fig. 4. Enzymatic degradation of galacardin A by α-galactosidase.

(A) 0 hour, (B) 3 hours, (C) 7 hours.

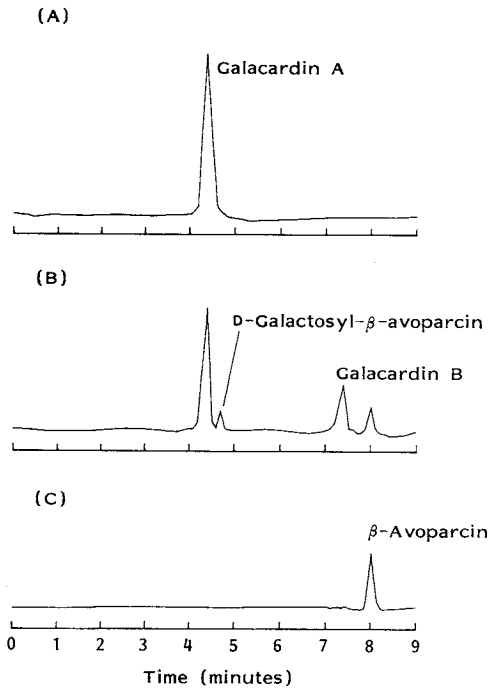
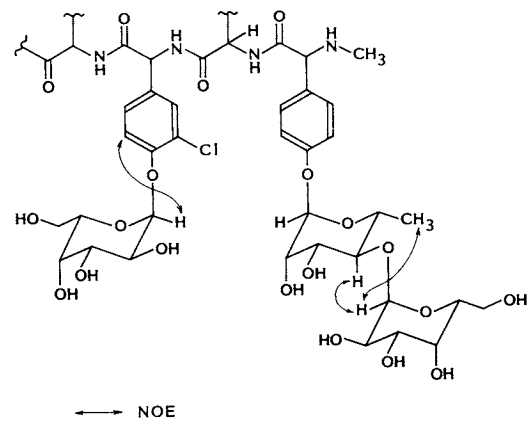


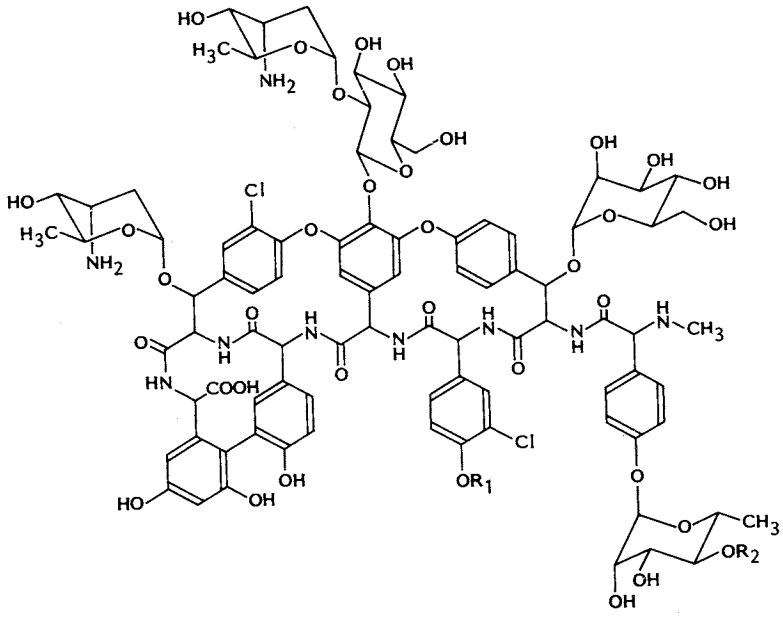
Fig. 5. NOEs of galacardin A.



and ristosamine, respectively, 2) pseudoaglycone obtained by mild hydrolysis of galacardin A (5% HCl - MeOH, 1 hour, 60°C) showed same retention time in HPLC analysis at that of β-avoparcin which is characterized to have both 1 mol of ristosamine and mannose which are attached to monodechlorovancomycinic acid (see Fig. 6), indicating that one of ristosamine was bound to the peptide nucleus and another was bound to glucose as ristosaminylglucose. Two methyl peaks at 1.16 and 1.36 ppm in ^1H NMR also showed the existence of 2 mol of ristosamine in galacardins as well as β-avoparcin, while the third methyl peak at 1.22 ppm was assigned as that of rhamnose. These data indicated that neutral and amino sugar compositions except galactose were the same as that of β-avoparcin[12] as well as amino acid composition. The results of the analysis of acid hydrolysates of galacardins are summarized in Table 5. Since D-galactosyl-β-avoparcin has been already reported by McGahren *et al.*[13] as a minor component of the avoparcin complex, we compared galacardin B directly with D-galactosyl-β-avoparcin. Galacardin B was clearly differed from D-galactosyl-β-avoparcin in two different solvent systems of HPLC analyses (Table 6).

For further understanding of the structure galacardin A was treated with α-galactosidase at 25°C in

Fig. 6. Structures of galacardins A and B.



Galacardin A	R_1 = Galactose	R_2 = Galactose
Galacardin B	R_1 = H	R_2 = Galactose
β-Avoparcin	R_1 = H	R_2 = H
D-Galactosyl-β-avoparcin	R_1 = Galactose	R_2 = H

0.05 M citrate buffer pH 4.0. Two kinds of degradation products, (D-galactosyl-β-avoparcin and galacardin B) were detected by HPLC analysis after 3 hours of incubation, and after 7 hours galacardin A was converted to β-avoparcin (Fig. 4). As galacardins were not hydrolyzed by β-galactosidase, two galactoses in galacardins were supposed to be attached to β-avoparcin by α-glycosidic linkage.

From these results the structure of galacardins A and B were determined to be di-galactosyl-β-avoparcin and mono-galactosyl-β-avoparcin, respectively. The accurate binding positions of galactose were determined by 2D NMR spectroscopy. As shown in Fig. 5, the NOEs were observed between the proton at C1-position of one galactose and the proton at C5-position of 3-Cl-4-hydroxyphenylglycine, and the proton at C1-position of another galactose and the protons at *C*-methyl and C4-position of rhamnose. Thus the total structures of galacardins are established as shown in Fig. 6. The details of structural studies will be reported elsewhere.

Discussion

A strain of *Saccharothrix* sp. SANK 64289 was found to produce new glycopeptide antibiotics, galacardins A and B. The species of *Saccharothrix* was amended by LABEDA *et al.* in 1984[14]. This genus has been reported to produce many kinds of antibiotics, such as BM 782 complex[15], LL-C19004[16], dopsisamine[17] and polynitroxin[18], but galacardins were the first report of glycopeptide antibiotics from *Saccharothrix*. Galacardins were very closely related to β-avoparcin but clearly differed in sugar components. The structural studies revealed that galacarcin A is di-galactosyl-β-avoparcin and B, mono-galactosyl-β-avoparcin. Their antimicrobial activities are also very similar to that of β-avoparcin.

Biosynthetic studies have been carried out on vancomycin[19], ristocetin[20] and aridicins[21,22], suggesting that attachment of sugar moieties follows the formation of the aglycone. The production level of galacar-

dins A and B through the fermentation from 5 to 10 days was not changed. D-Galactosyl-β-avoparcin was also produced at the same level as galacardin B, but the production of β-avoparcin was negligible in the fermentation broth of SANK 64289. The study of biosynthesis of galacardins also may give us a good example for understanding the biosynthetic pathway of glycopeptide antibiotics.

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