NEW DITERPENOID ANTIBIOTICS, SPIROCARDINS A AND B

MUTSUO NAKAJIMA, TAKAO OKAZAKI, SEIGO IWADO,
TAKESHI KINOSHITA[†] and TATSUO HANEISHI[††]

Fermentation Research Laboratories,
[†]Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd.,
1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan
[††]Technical Licensing Department, Sankyo Co., Ltd.,
2-7-12 Ginza, Chuo-ku, Tokyo 104, Japan

(Received for publication April 25, 1989)

New antibiotics spirocardins A and B[†††] were isolated from the culture broth of an actinomycete isolated from a soil sample collected near Lake Hibara, Fukushima Prefecture, Japan. The producing strain was classified as *Nocardia* sp. SANK 64282.

The antibiotics were isolated from the culture filtrate by solvent extraction and purified further by silica gel and preparative reverse phase column chromatography. They were primarily active against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* and limited species of Gram-negative bacteria such as *Bacteroides fragilis* and *Klebsiella pneumoniae*. They were also moderately active against several species of *Mycoplasma*. The molecular formulae of spirocardins A and B were $C_{20}H_{30}O_6$ and $C_{20}H_{32}O_6$, respectively.

From their physico-chemical characteristics they were revealed to be diterpenoid antibiotics with closely related structures and the former was easily converted to the latter by the reduction with $NaBH_4$.

During the course of antibiotic screening, novel antibiotics were obtained from the culture filtrate of an actinomycete isolated from a soil sample collected near Lake Hibara, Fukushima Prefecture, Japan. The producing organism, strain SANK 64282, was found to have the macroscopic, microscopic and whole-cell hydrolysis properties characteristic of the genus *Nocardia*.

The present paper deals with fermentation, isolation, physico-chemical and biological properties of spirocardins A and B. The structure elucidation of spirocardins A and B and detailed taxonomy of the producing organism will be reported elsewhere.

Production of Spirocardins A and B

One loopful growth of the strain SANK 64282 on an agar slant was inoculated into a 500-ml Erlenmeyer flask containing 80 ml of a medium composed of glucose 1%, glycerol 1%, sucrose 1%, pressed yeast 1%, soybean meal 2%, oatmeal 0.5%, Casamino acids 0.5%, $CaCO_3$ 0.1%, and Nissan disfoam CB-442 (Nissan Chemical Co., Japan) 0.01%. The pH of the medium was adjusted to 7.0 before sterilization. The flasks were incubated on a rotary shaker at 28°C for 96 hours.

The 35-ml aliquots of the culture from the Erlenmeyer flask were inoculated into 2 liter Erlenmeyer flasks each containing 700 ml of the medium described above and incubated at 28°C for 48 hours. The 2.5-liter aliquots of the culture from the 2 liter Erlenmeyer flasks were further inoculated into a 30-liter fermenter containing 15 liters of the same medium and incubated at 28°C for 24 hours. After inoculation of 15 liters of the seed culture into a 600-liter fermenter containing 300 liters of the medium composed of glucose 2%, Lustergen FK (Nippon Starch Chemical Co., Ltd., Japan) 1%, pressed yeast 0.9%, meat

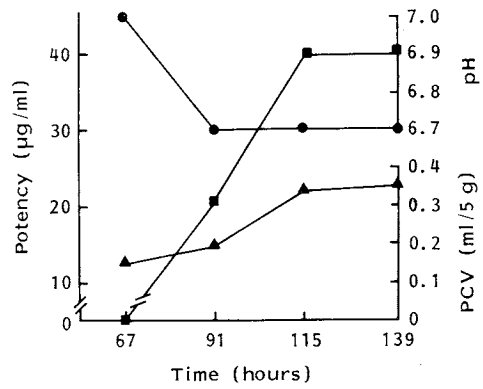
[†††] Jpn. Kokai 126,089 ('85), July 5, 1985.

extract 0.5%, Polypepton 0.5%, $CaCO_3$ 0.3%, NaCl 0.5%, and Nissan disfoam CB-442 0.01%, fermentation was carried out for 115 hours with 100 rpm agitation and 150 liters/minute aeration at 28°C.

Mycelial growth was expressed as packed cell volume (PCV) (ml) obtained after centrifugation of 5 g of the culture broth at 3,000 rpm for 15 minutes. Antibiotic production during fermentation was monitored by the paper disc-agar diffusion assay using *Staphylococcus aureus* FDA 209P JC-1 as a test organism.

An example of typical time course of the fermentation in the 600-liter fermenter is shown in Fig. 1.

Fig. 1. Fermentation of spirocardins A and B.
■ Potency, ▲ PCV, ● pH.



The maximal potency of the antibiotics, approximately 40 μg/ml, was obtained after 115 hours of fermentation.

Isolation

The 300-liter of the culture broth from a 600-liter fermenter were filtered with the aid of 15 kg of diatomaceous earth (Celite 545, Johns Manville Products Co., U.S.A.). The filtrate was extracted twice with half the volume of ethyl acetate, which was then concentrated to 2 liters under reduced pressure. The concentrate was washed once with 2 liters of saturated sodium chloride solution. After drying the washed concentrate over anhydrous sodium sulfate, it was evaporated under reduced pressure to give 43 g of oily product. The oily material dissolved in a minimal volume of ethyl acetate was applied to a column of silica gel (Mallinkrodt Co., U.S.A.) packed with a mixture of *n*-hexane and acetone (20:1), and the components were separated into two active fractions containing spirocardins A and B as the major constituents.

These two fractions were further purified independently by column chromatography on silica gel, followed by preparative reverse phase column chromatography (LiChroprep RP-8). Finally pure spirocardins A and B (170 and 190 mg, respectively) were obtained as amorphous powders.

Physico-chemical Properties

Spirocardins A and B were obtained as neutral, lipophilic, colorless powders soluble in alcohol, ethyl acetate, and chloroform, but insoluble in water. They reacted positively on TLC to iodine, potassium permanganate and sulfuric acid, and showed no characteristic absorption maximum at more than 220 nm in the UV region. Their IR spectra indicated characteristic absorption due to hydroxyl and carbonyl functions at 3400 and 1705 cm^{-1} (Figs. 3 and 4). The molecular formula of spirocardins A and B were established as $C_{20}H_{30}O_6$, MW 366 ($(M+H)^+$ 367) and $C_{20}H_{32}O_6$, MW 368 ($(M+H)^+$ 369), respectively, from high-resolution fast atom bombardment (HRFAB)-MS together with the data obtained by spectral analyses.

Their physico-chemical data suggested both substances were closely related diterpenoid antibiotics and spirocardin B was assumed to be a reduced form of spirocardin A. Treatment of spirocardin A with $NaBH_4$ gave spirocardin B but the oxidation of spirocardin B to spirocardin A was not successful. In the ^1H NMR spectrum of spirocardin A in $CDCl_3$ at 400 MHz, three singlet methyl groups at 0.98, 1.36 and

Fig. 2. Isolation and purification of spirocardins A and B.

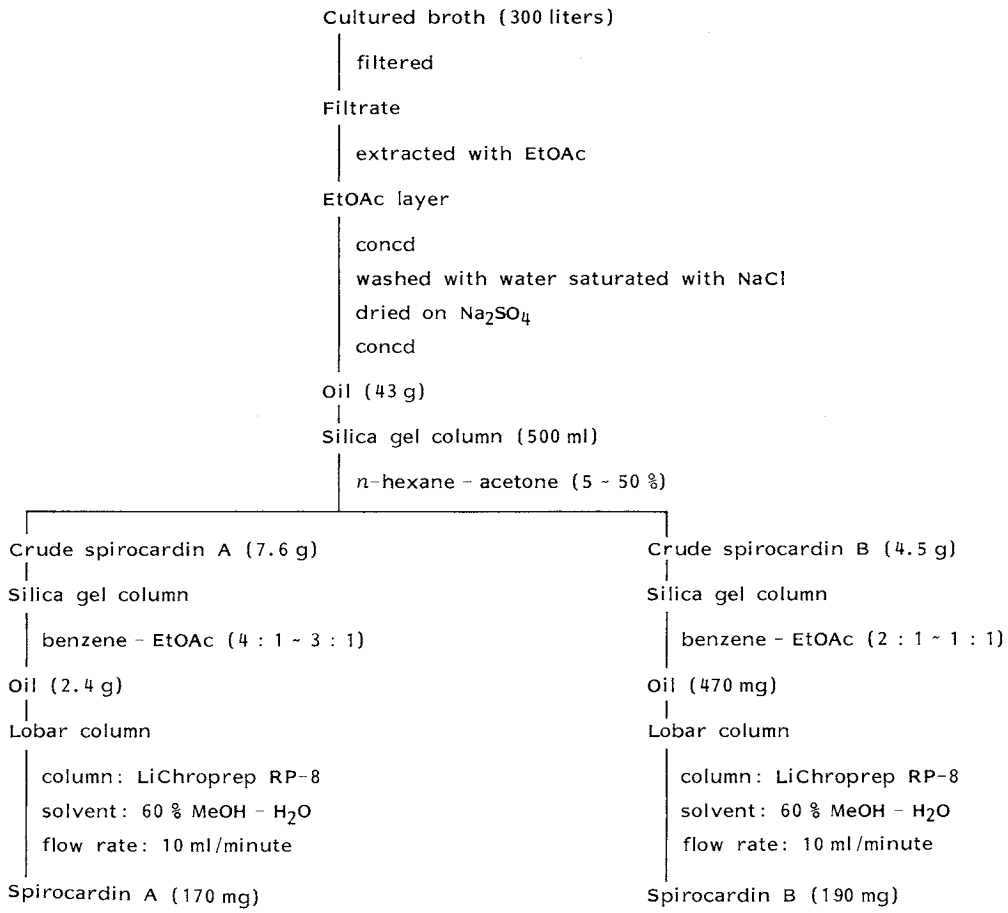


Fig. 3. IR spectrum of spirocardin A in $CHCl_3$.

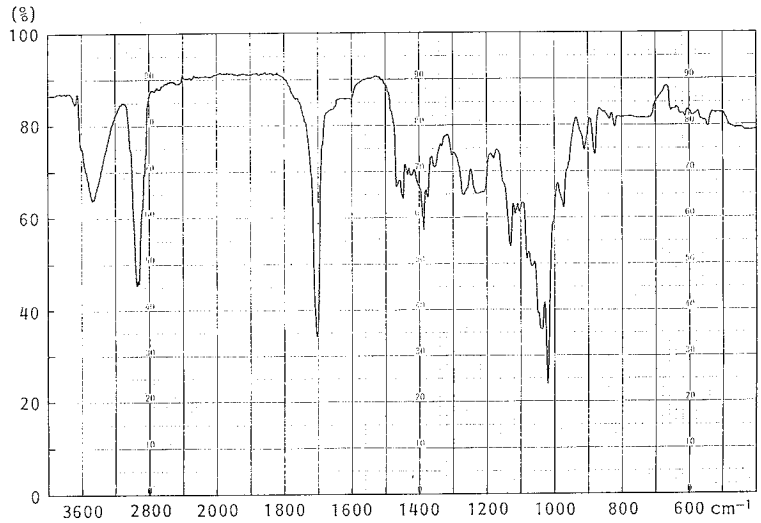


Fig. 4. IR spectrum of spirocardin B in $CHCl_3$.

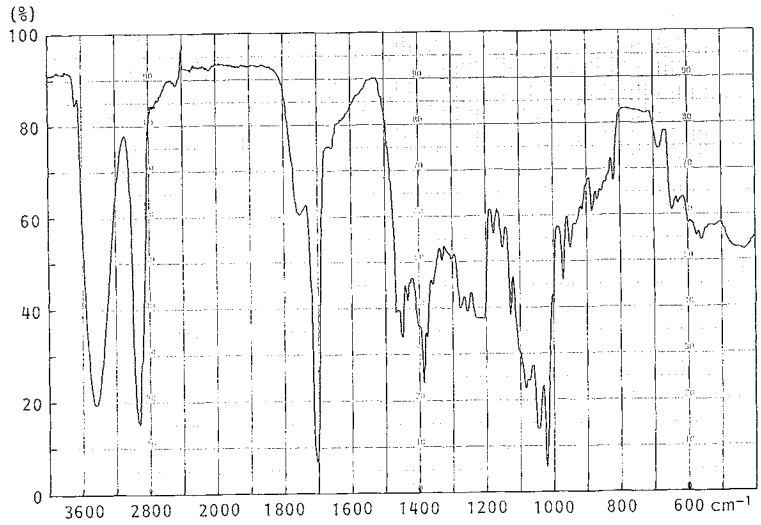


Table 1. Physico-chemical properties of spirocardins A and B.

	Spirocardin A	Spirocardin B
Nature	Colorless powder	Colorless powder
$[\alpha]_D^{25°}$	$-52.7°$ (c 0.48, $CHCl_3$)	$-35.5°$ (c 0.62, $CHCl_3$)
Molecular formula	$C_{20}H_{30}O_6$	$C_{20}H_{32}O_6$
MW (FAB-MS)($(M+H)^+$)	366 (367)	368 (369)
Solubility: Soluble	MeOH, EtOAc, $CHCl_3$	MeOH, EtOAc, $CHCl_3$
Insoluble	H_2O	H_2O
Rf (Merck Art. No. 5715)	0.3 (EtOAc-Benzene, 1:1)	0.15 (EtOAc-Benzene, 1:1)
Color reaction	$KMnO_4$, H_2SO_4 (positive)	$KMnO_4$, H_2SO_4 (positive)
UV (MeOH)	End absorption	End absorption

Fig. 5. ^1H NMR spectrum of spirocardin A in $CDCl_3$ (400 MHz).

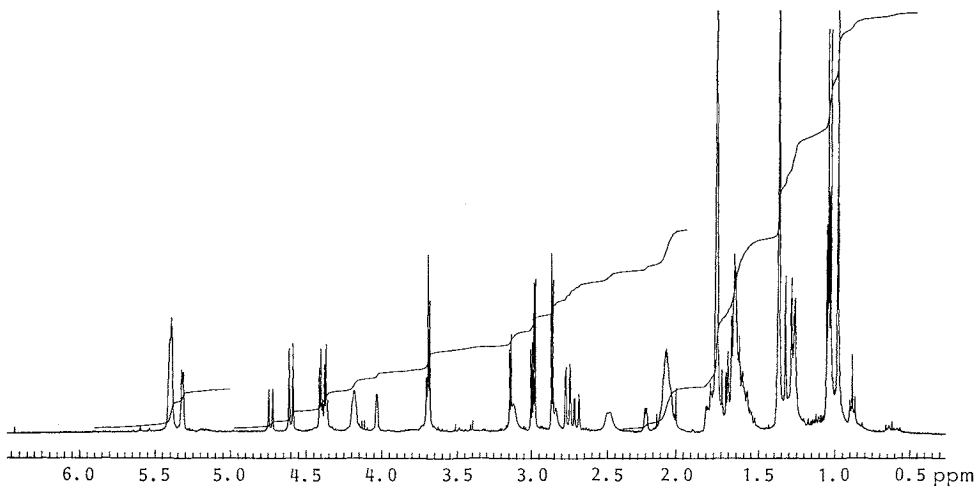


Fig. 6. ^{1}H NMR spectrum of spirocardin B in $CDCl_3$ (400 MHz).

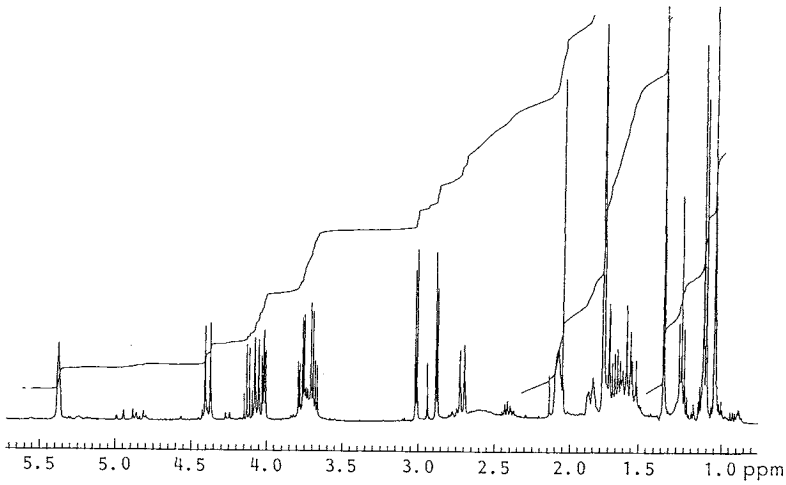
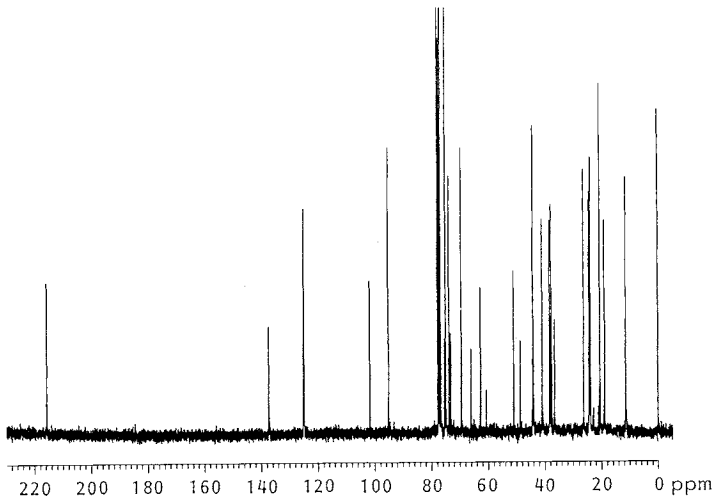


Fig. 7. ^{13}C NMR spectrum of spirocardin A in $CDCl_3$.



1.78 ppm, a doublet methyl group at 1.05 ppm and an olefinic proton at 5.39 ppm were observed. The spectrum of spirocardin B demonstrated its similarity to spirocardin A by the presence of three singlet and one doublet methyl groups, and an olefinic proton, but differed markedly in the region 3.7~4.7 ppm, as shown in Figs. 5 and 6.

^{13}C NMR spectra of spirocardins A and B are shown in Figs. 7 and 8.

The spectrum of spirocardin B showed all twenty carbons of its molecular formula, including four methyl, five methylene and five methine, two olefinic carbons and one carbonyl carbon as well as three quaternary carbons.

The spectrum of spirocardin A differed mainly from that of spirocardin B in the signals corresponding to carbons bearing oxygen atoms; new paired signals due to cyclic acetal carbons were observed around 100 ppm instead of a signal ascribed to hydroxymethyl at 63 ppm in spirocardin B. There are also some other paired signals in spirocardin A. These findings suggested that there is an extra cyclic structure in

Fig. 8. ^{13}C NMR spectrum of spirocardin B in $CDCl_3$.

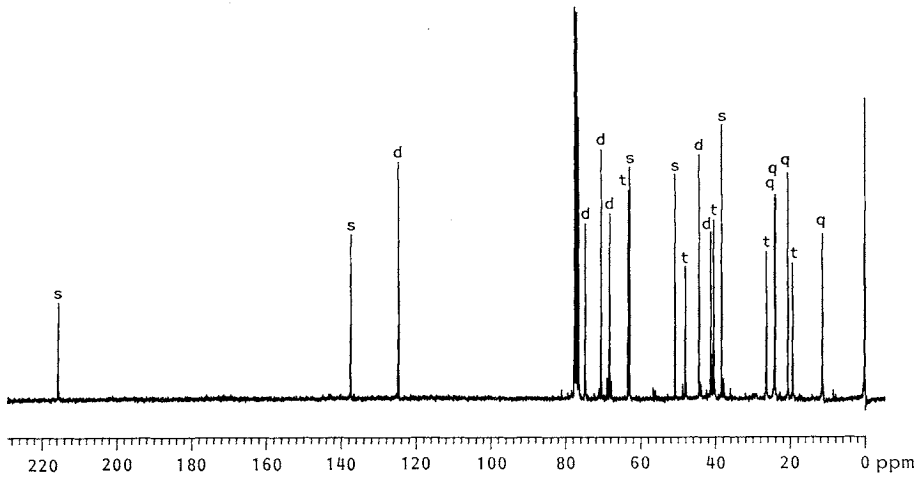
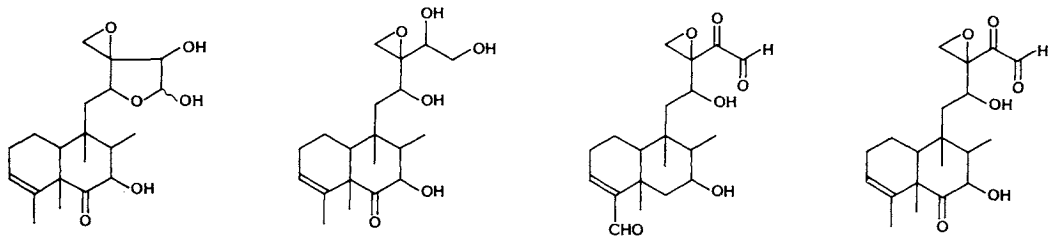


Chart 1.



Spirocardin A Spirocardin B Clerocidin Terpentecin

spirocardin A and that the paired signals are due to stereoisomerization of the ring.

From the analyses of these spectral data, their tentative structures are proposed to be diterpenoid antibiotics as shown in Chart 1.

Structural studies of these two antibiotics will be reported in the near future.

Biological Properties

The MICs of spirocardins A and B against bacteria, yeasts and fungi were determined by a serial 2-fold agar dilution method. The media used were sensitivity disc agar (Nissui Co., Ltd., Japan) supplemented with 2% glycerol for bacteria, GAM Agar (Nissui Co., Ltd., Japan) for anaerobic bacteria, PPLO Agar (Eiken Co., Ltd., Japan) for mycoplasma, and Sabouraud-dextrose Agar (Eiken Co., Ltd., Japan) for yeasts and fungi. The MICs were determined after incubation for 24 or 48 hours at 37°C for bacteria, and for 2 to 14 days at 26°C for yeasts and fungi.

As shown in Table 2, spirocardin A was active against Gram-positive bacteria, including mycobacteria, and moderately active against Gram-negative bacteria, particularly *Klebsiella pneumoniae* PCI 602, but inactive against yeasts and fungi.

The antimicrobial activity of spirocardin B was generally one-fourth that of spirocardin A. Spirocardins A and B were also tested against anaerobic bacteria using the GasPak anaerobic system (BBL division of Becton, Dickinson and Co., U.S.A.). They were moderately active against *Bacteroides fragilis*,

Table 2. Antimicrobial spectra of spirocardins A and B.

Test organism	Medium	MIC (μg/ml)	
		Spirocardin A	Spirocardin B
Staphylococcus aureus FDA 209P JC-1	A	6.25	25
S. aureus SANK 71183 (MRSA)	A	6.25	25
S. aureus SANK 71283 (MRSA)	A	6.25	25
S. aureus Smith	A	3.13	12.5
S. epidermidis SANK 71575	A	1.56	6.25
Enterococcus faecalis SANK 71478	A	6.25	25
Bacillus subtilis PCI 219	A	0.78	6.25
Micrococcus luteus PCI 1001	A	0.78	3.13
Mycobacterium smegmatis ATCC 607	A	3.13	12.5
Escherichia coli NIHJ JC-2	A	>100	>100
Klebsiella pneumoniae PCI 602	A	0.39	1.56
Proteus vulgaris OX-19	A	25	50~100
P. mirabilis SANK 70461	A	100	>100
Pseudomonas aeruginosa NCTC 10490	A	100	100
Serratia marcescens SANK 73060	A	50	>100
Candida albicans YU 1200	B	>100	>100
Piricularia oryzae SANK 14758	B	>100	>100

Inoculum size: 10^6 cells/ml.

Table 3. Antimicrobial spectra of spirocardins A and B.

Test organism	MIC (μg/ml)	
	Spirocardin A	Spirocardin B
Bacteroides fragilis SANK 71176	6.25	50
B. fragilis subsp. *fragilis* SANK 71376	6.25	50
Eubacterium aerofaciens SANK 72276	6.25	50
Fusobacterium necrophorum SANK 71676	100	>100
Peptostreptococcus micros SANK 71876	6.25	25
P. parvulus SANK 72376	25	>100
Propionibacterium acnes SANK 71976	12.5	100
Veillonella alcalescens SANK 72476	>100	>100
Clostridium difficile ATCC 9689	12.5	50
C. perfringens ATCC 13124	50	>100

Inoculum size: 10^6 cells/ml.

Peptostreptococcus micros, *Peptostreptococcus parvulus* and *Clostridium difficile* (Table 3).

The antibiotics were also active against *Ureaplasma urealyticum* and other mycoplasma species such as *Mycoplasma pulmonis*, *Mycoplasma canis*, *Mycoplasma felis* and *Mycoplasma arthritidis* (Table 4). The relative activities of spirocardins A and B against anaerobic bacteria and mycoplasma species were similar to the results in aerobic bacteria. The *in vitro* cytocidal activity of spirocardin A for animal cell-lines such as P388 cells expressed in terms of 50% inhibitory concentration (IC_{50}) (μg/ml) was determined to be 0.52.

The *in vivo* antimicrobial efficacy of spirocardin A in mice was examined using intravenous infection of *S. aureus* Smith, but no protective effects of subcutaneous antibiotic injection could be observed.

The antitumor activity of spirocardin A in mice is now under investigation.

All mice received intraperitoneally 100 mg/kg of spirocardin A or B, and tolerated it well without any toxic symptoms.

Table 4. Antimicrobial spectra of spirocardins A and B.

Test organism	MIC (μg/ml)		Test organism	MIC (μg/ml)	
	Spirocardin A	Spirocardin B		Spirocardin A	Spirocardin B
Mycoplasma mycoides PG1	12.5	50	*M. hyorhinis* PG29	50	>100
M. agalactiae PG2	50	>100	*M. gallisepticum* PG31	50	>100
M. arthritidis PG6	6.25	50	*M. gallisepticum* S6	6.25	50
M. bovigenitalium PG11	50	>100	*M. felis* CO	6.25	50
M. canis PG14	6.25	25	*Acholeplasma laidlawii* PG10	50	>100
M. hominis PG21	25	100	*Ureaplasma urealyticum* (Human)	1.56	6.25
M. pulmonis PG22	3.13	50			

Inoculum size: 10^6 cells/ml.

Discussion

These physico-chemical and biological properties of spirocardins A and B, in addition to the information on their producing organism as belonging to the genus *Nocardia*, were compared with those of known substances isolated from microorganisms. Both antibiotics spirocardins A and B were revealed to be new antibiotics categorized as diterpenoids. Structurally related compounds (Chart 1) include clerocidin[1,2] derived from fungi, and terpentecin[3,4] as the sole diterpene compound in the antibiotics from actinomycetes except for the spirocardins. Clerocidin possesses antitumor activity along with antimicrobial activities and terpentecin is reported to possess antitumor activity *in vivo* as well as antimicrobial activities against Gram-positive and Gram-negative bacteria.

Discussion on the structural relationships among closely related diterpenoid antibiotics such as the spirocardins, terpentecin and clerocidin will be reported in a subsequent paper.

References

1) ANDERSEN, N. R.; H. O. B. LORCK & P. R. RASMUSSEN: Fermentation, isolation and characterization of antibiotic PR-1350. J. Antibiotics 36: 753~760, 1983
2) ANDERSEN, N. R. & P. R. RASMUSSEN: The constitution of clerocidin: a new antibiotic isolated from *Oidiodendron truncatum*. Tetrahedron Lett. 25: 465~468, 1984
3) TAMAMURA, T.; T. SAWA, K. ISSHIKI, T. MASUDA, Y. HOMMA, H. IINUMA, H. NAGANAWA, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: Isolation and characterization of terpentecin, a new antitumor antibiotic. J. Antibiotics 38: 1664~1669, 1985
4) ISSHIKI, K.; T. TAMAMURA, Y. TAKAHASHI, T. SAWA, H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: The structure of a new antibiotic, terpentecin. J. Antibiotics 38: 1819~1821, 1985