KARNAMICIN†, A COMPLEX OF NEW ANTIFUNGAL ANTIBIOTICS

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

MAKI NISHIO, KOZO TOMATSU††, MASATAKA KONISHI, KOJI TOMITA, TOSHIKAZU OKI and HIROSHI KAWAGUCHI

Bristol-Myers Research Institute, Ltd., Tokyo Research Center, 2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

JON CLARDY

Department of Chemistry, Cornell University, Ithaca, New York 14853-1301, U.S.A.

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A complex of new antifungal antibiotics designated karnamicin was isolated from the cultured broth of *Saccharothrix aerocolonigenes* No. N806-4. Fifteen components have so far been isolated from the complex; the major component karnamicin B_2 was identified by X-ray crystallography to be a novel molecule unrelated to known antibiotics.

All components of karnamicin exhibited a rather broad spectrum of activity against fungi and yeasts with MICs ranging from 3.1 to 50 μg/ml.

In a systematic search for microbial metabolites effective against fungi and yeasts, an actinomycete strain No. N806-4 isolated from an Indian soil sample was found to produce a complex of new antifungal antibiotics, karnamicin. The producing strain was identified as *Saccharothrix aerocolonigenes* by our taxonomical studies. The antibiotic complex was extracted from the fermentation liquor using non-ionic porous polymer resin and was separated into the individual components by various chromatographic techniques. Fifteen components (karnamicins A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3, C_4, C_5, D_1, D_2, D_3 and D_4) have been isolated, and their chemical properties and biological activity examined. This paper describes the taxonomy and fermentation of the producing strain, and the isolation and physico-chemical and biological properties of karnamicin components. The structure studies will be reported elsewhere.

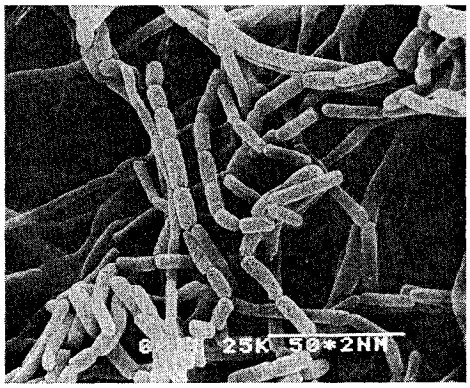
Producing Organism

An actinomycete, strain No. N806-4, was isolated from a soil sample collected in Sankeshwar, Karnataka State, India. Strain No. N806-4 forms branched hyphae which develop into substrate and aerial mycelia. Typical nocardioform fragmentation of substrate mycelium, which affords rod or coccoid elements, is not observed, but partial fragmentation occurs. Long straight chains of cylindrical segments ($0.4\sim0.6\times0.8\sim1.8$ μm) (Fig. 1) are formed in all parts of the aerial mycelium. After further incubation, spores are formed discontinuously with intercalary empty hyphae in the segmented aerial mycelium. The mature spores are oval ($0.6\sim0.8\times0.8\sim1.2$ μm), non-motile, and have smooth surface. The aerial mycelia often fuse into thick fascicle (Fig. 2) which occurs also in the sporulated hyphae (Fig. 3). Constricted zigzag hyphae and sclerotic granule are occasionally observed on the

† Originally called BU-3557.

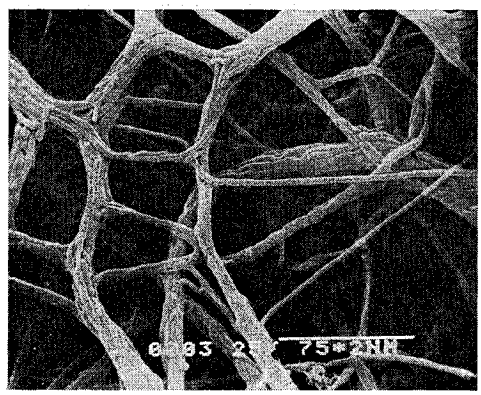
†† Deceased.

Fig. 1. Chains of cylindrical spores on the aerial mycelium.



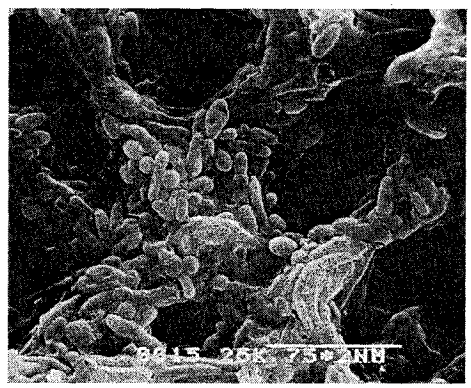
Scanning electron micrograph of *Saccharothrix aerocolonigens* strain N806-4.
Medium: Tyrosine agar (ISP medium No. 7). Cultivation: 28°C for 14 days.

Fig. 2. Thick fascicles of aerial hyphae.



Scanning electron micrograph of *Saccharothrix aerocolonigens* strain N806-4.
Medium: Tyrosine agar (ISP medium No. 7). Cultivation: 28°C for 14 days.

Fig. 3. Fused coccoid fragments of aerial mycelium.



Scanning electron micrograph of *Saccharothrix aerocolonigens* strain N806-4.
Medium: Tyrosine agar (ISP medium No. 7). Cultivation: 28°C for 14 days.

aerial mycelium. The cultural characteristics are shown in Table 1, and the physiological characteristics which include the system of GORDON *et al.*,[1] are given in Table 2. Asporogenic variants, which lack the ability to form aerial mycelium, occur after repeated transfers or during long storage. An asporogenic variant, No. N806-4-V7 maintains the ability to produce all components of antibiotic karnamicin. The presence of *meso*-diaminopimelic acid, ribose, glucose, galactose, rhamnose and mannose in the whole cell hydrolysate of strain No. N806-4 indicates that the strain belongs to cell wall Type III and sugar pattern C. The strain has Type PII phospholipid (presence of phosphatidylethanolamine and phosphatidylinositol), and contains MK-9(H_4) as the major menaquinone. Glycolate test is negative. Mycolic acid is absent.

The morphology, cultural characteristics and cell chemistry of strain No. N806-4 place it in the genus *Saccharothrix*,[2] in which two species, *Saccharothrix australiensis*[2] and *S. aerocolonigenes*[3,4] have been described. *S. australiensis* is different from strain No. N806-4 in the following characteristics; the formation of light brownish gray aerial mycelium and dark brown vegetative mycelium in ISP medium No. 2, and clear difference in GORDON's physiological tests. Among the known strains of *S. aerocolonigens*, only strain C38383 (ATCC 39243) which is a rebeccamycin-producing organism, forms spore chains on the aerial mycelium.[5] Morphologically, strain No. N806-4 is related to *S. aerocolonigenes* strain C38383. In addition, strain No. N806-4 is similar to strain C38383 and other strains of *S. aerocolonigenes* in the physiological characteristics of GORDON's tests.[1] Thus, strain

Table 1. Cultural characteristics of strain N806-4.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose - nitrate agar (CZAPEK - DOX agar)	Moderate	Scant; white	Moderate orange yellow (71)	Pale yellow (89)
Tryptone - yeast extract broth (ISP No. 1)	Moderate, floccose and not turbid	None	None	None
Yeast extract - malt extract agar (ISP No. 2)	Good	Poor; white	Dark orange yellow (72)	None
Oatmeal agar (ISP No. 3)	Moderate	Poor; white	Moderate yellow (87) to colorless	None
Inorganic salts - starch agar (ISP No. 4)	Moderate	Poor; white	Moderate orange yellow (71)	None
Glycerol - asparagine agar (ISP No. 5)	Moderate	Moderate; white	Pale yellow (89)	None
Peptone - yeast extract - iron agar (ISP No. 6)	Moderate	Scant; white	Colorless	None
Tyrosine agar (ISP No. 7)	Moderate	Moderate; white	Moderate olive brown (95)	Grayish yellow (90)

Observation after incubation at 28°C for 3 weeks.
Color name used: ISCC-NBS Color-Name Charts.

Table 2. Physiological characteristics of strain N806-4.

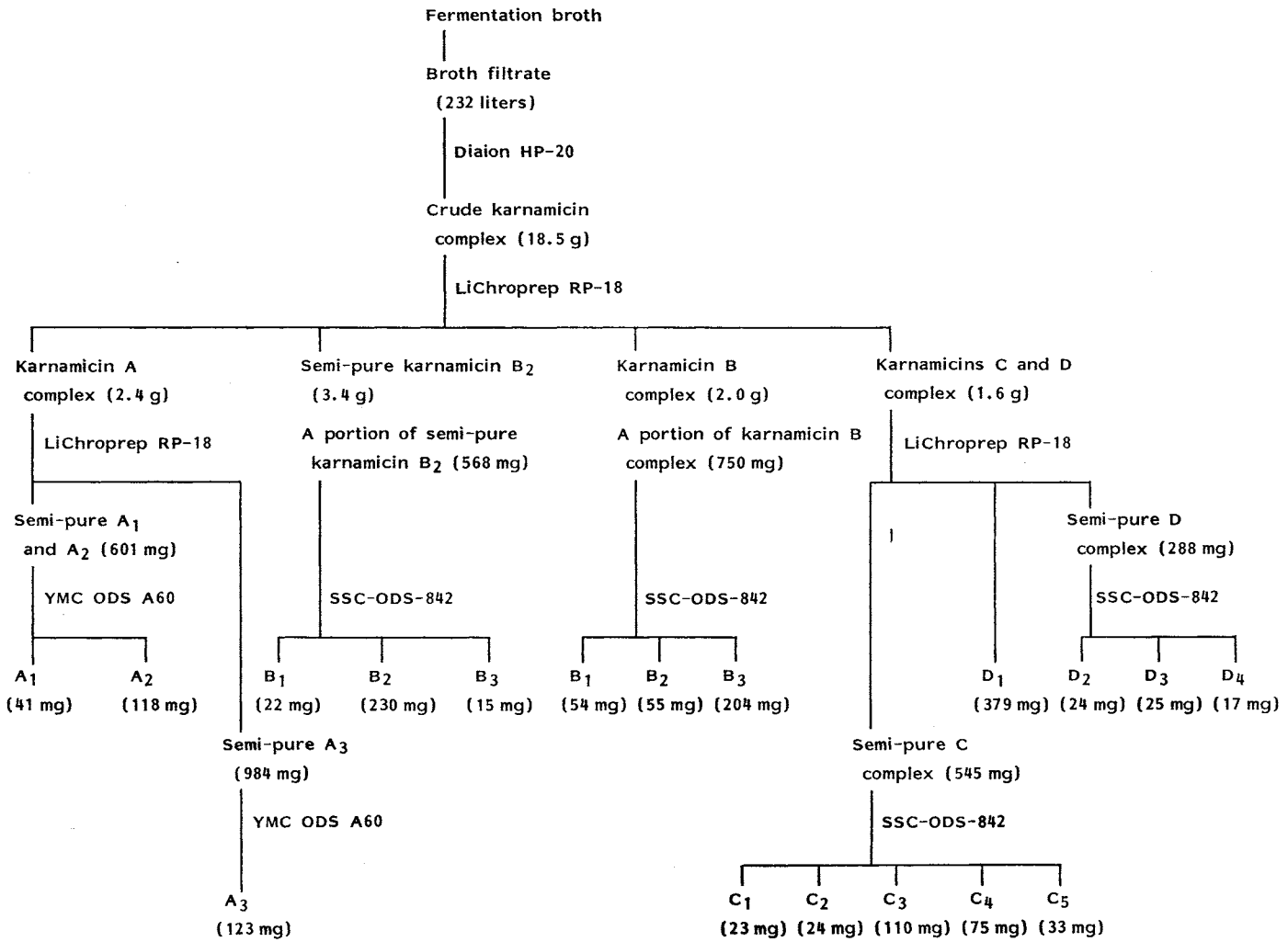
Production of:		Utilization of:	
Gelatinase	+	Benzoate	−
Amylase	+	Citrate	+
Nitrate reductase	+	Mucate	−
Tyrosinase	−	Succinate	+
Tolerance to:		Tartrate	−
Lysozyme, 0.01% (w/v)	+	Acid from:	
NaCl, 1~4% (w/v)	+	Glycerol	+
5% (w/v)	−	D(−)-Arabinose	+
Temperature:		L(+)-Arabinose	+
Growth range	22~42°C	L-Rhamnose	+
No growth	18 and 45°C	D-Glucose	+
Hydrolysis of:		D-Mannose	+
Adenine	−	Lactose	+
Hypoxanthine	+	Cellobiose	+
Tyrosine	+	Melibiose	+
Xanthine	−	Trehalose	+
Casein	+	Raffinose	+
Urea	+	D(+)-Melezitose	−
Esculin	+	Inositol	+
Hippuric acid	+	D-Mannitol	+
Survival at 50°C, 8 hours	−	D-Sorbitol	−
		Erythritol	−
		Methyl α-glucoside	−
		Adonitol	−

No. N806-4 was classified as *S. aerocolonigenes* (ATCC 53712).

Fermentation

A piece of the slant culture of *S. aerocolonigenes* No. N806-4 was inoculated in a 500-ml Erlen-

Fig. 4. Isolation procedure of karnamicin components.



meyer flask containing 100 ml of the seed medium consisting of soluble starch 0.5%, glucose 0.5%, fish meat extract (Mikuni) 0.1%, yeast extract (Oriental) 0.1%, NZ-case (Sheffield) 0.2%, NaCl 0.2% and $CaCO_3$ 0.1%. Shake cultivation was carried out at 28°C for 4 days on a rotary shaker (200 rpm). The seed culture (2 ml) was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium composed of mashed potato (Snow Milk Products) 4%, corn steep liquor 2%, NaCl 0.2% and $CaCO_3$ 0.3% (pH 7.2 before autoclaving). The fermentation was carried out on a rotary shaker (200 rpm) at 28°C for 8 days. The antifungal activity was determined by the conventional broth dilution method in Sabouraud dextrose medium with a 96-well microtiter plate using *Candida albicans* A9540 as the indicator organism. The turbidity was determined by Titertek Multiskan MCC (Flow Laboratories) at 540 nm and HPLC was also used in parallel with the bioassay. The antifungal activity of the flask fermentation reached a maximum on the 6th day at a concentration of 200 μg/ml. Tank fermentation was also carried out. The seed culture was prepared by the same procedure as the flask fermentation using the following seed medium, glucose 2%, soybean meal 1% and $CaCO_3$ 0.5%. After cultivation on a rotary shaker at 28°C for 4 days, the seed culture (2 liters) was inoculated to a 200-liter tank fermentator which contained 120 liters of the fermentation medium having the same composition as the flask fermentation. The tank fermentor was operated at 28°C with agitation at 250 rpm and aeration rate of 120 liters/minute. The broth pH gradually rose with the progress of fermentation and reached 7.8 after 90 hours when the peak antibiotic potency of 112 μg/ml (HPLC assay) was obtained.

Isolation

The isolation procedure for the karnamicin components is summarized in Fig. 4. Active fractions were examined by bioassay and/or HPLC analysis. The HPLC mobility of each component is shown in Table 3.

Extraction and Primary Separation

The activity in the broth filtrate (232 liters) was adsorbed on Diaion HP-20 (Mitsubishi Chemical Industries Limited, 23 liters). The resin was washed with 50% aqueous methanol, and eluted with 80% aqueous acetone. The eluate was concentrated *in vacuo* to an aqueous solution which was chromatographed on a column of Diaion HP-20 (3.4 liters) using 60% aqueous acetone. Evaporation of the active eluate yielded a crude karnamicin complex (18.5 g). The solid (2.4 g) was applied on a column of LiChroprep RP-18 (E. Merck, 22×450

Table 3. Analytical HPLC of karnamicin components.

Karnamicin	Retention time (minutes)		
	Condition A[a]	Condition B[b]	Condition C[b]
A_1	7.14	5.11	
A_2	7.46	7.21	
A_3	7.89	10.97	
B_1	9.44	10.12	
B_2	8.87	14.59	
B_3	9.57	22.63	
C_1	10.34		11.50
C_2	10.63		15.02
C_3	10.99		14.95
C_4	11.53		9.59
C_5	12.48		15.09
D_1	11.72		14.99
D_2	11.17		9.22
D_3	11.85		10.49
D_4	12.24		17.44

[a] Column: Microsorb Short One C_{18} (4.6 i.d.×100 mm, Rainin Instrument). Mobile phase: Acetonitrile - 0.15% KH_2PO_4 adjusted to pH 3.5 with H_3PO_4. Gradient: 15:85~40:60, from 0~3 minutes, 40:60, from 3~9 minutes and 40:60~55:45, from 9~12 minutes, 55:45, from 12~15 minutes. Flow rate: 1.2 ml/minute. Detection: UV absorption at 254 nm.

[b] Column: MCI GEL ODS 1HU (4.6 i.d.×150 mm, Mitsubishi Chemical Industries Limited). Mobile phase: Methanol - 0.03 M phosphate buffer, pH 7.0, condition B; 25:23, condition C; 27:21. Flow rate: 1.2 ml/minute. Detection: UV absorption at 254 nm.

mm), and developed with a mixture of acetonitrile - 0.15% KH_2PO_4, pH 3.5 (36:64). Karnamicin A complex was eluted first followed by karnamicin B_2, a mixture of karnamicins B_1, B_2 and B_3 and then karnamicins C and D complex. Each fraction was concentrated *in vacuo* and extracted with ethyl acetate for desalting. Repetition of the chromatography for the remaining crude karnamicin complex yielded karnamicin A complex (2.4 g), semi-pure karnamicin B_2 (3.4 g), karnamicin B complex (2.0 g) and karnamicins C and D complex (1.6 g) from the crude karnamicin complex (16.8 g).

Purification of Karnamicin A Complex

Karnamicin A complex (2.3 g) was chromatographed on a column of LiChroprep RP-18 (22×450 mm) eluting with acetonitrile - 0.15% KH_2PO_4, pH 3.5 (33.3:66.7). Upon monitoring the eluate by HPLC, a mixture of karnamicins A_1 and A_2 was eluted first followed by nearly homogeneous karnamicin A_3. The relevant fractions were concentrated and extracted with ethyl acetate. Evaporation of the extracts gave semi-pure karnamicins A_1 and A_2 mixture (601 mg) and karnamicin A_3 (984 mg). The karnamicins A_1 and A_2 mixture (550 mg) was re-chromatographed on a column of YMC GEL ODS A60 (Yamamura Chemical Lab., 22×450 mm). Elution was carried out with methanol - 0.03 M phosphate buffer, pH 7.0 (48:52). The first active fractions containing homogeneous karnamicin A_1 were pooled, concentrated and then extracted with ethyl acetate. Evaporation of the extract yielded a white solid of karnamicin A_1 (41 mg). The second active fractions were similarly worked up to give pure karnamicin A_2 (118 mg). The solid of karnamicin A_3 (705 mg) was further purified by a column of YMC GEL ODS A60 (22×450 mm) using methanol - 0.03 M phosphate buffer, pH 7.0 (50:50). After examination of the eluates by HPLC, the heart-cuts of karnamicin A_3 fractions were concentrated *in vacuo* to remove methanol. Extraction of the aqueous concentrate with ethyl acetate followed by concentration of the extract afforded pure karnamicin A_3 (123 mg).

Purification of Karnamicin B Fraction

The semi-pure karnamicin B_2 (47 mg) was purified by preparative HPLC with a SSC-ODS-842 column (Senshu Kagaku, 30×250 mm). The elution was carried out with a mixture of methanol and 0.03 M phosphate buffer, pH 7.0, 54:46 from 0~15 minutes, a linear gradient 54:46~65:35 from 15 to 80 minutes, 65:35 from 80 to 100 minutes at flow rate of 12.0 ml/minute. The eluates were monitored by UV absorption at 280 nm. Karnamicin B_1 was eluted at the retention time of 47 minutes followed by karnamicin B_2 at 58 minutes and then karnamicin B_3 at 72 minutes. The appropriate eluates were pooled, concentrated and desalted by Diaion HP-20 chromatography. Repetition of the preparative HPLC for the remaining semi-pure karnamicin B_2 yielded pure karnamicin B_1 (22 mg), karnamicin B_2 (230 mg) and karnamicin B_3 (15 mg) from the semi-pure solid (568 mg). Karnamicin B complex (750 mg) was purified by preparative HPLC as described above to yield pure karnamicin B_1 (54 mg), karnamicin B_2 (55 mg) and karnamicin B_3 (204 mg).

Karnamicin B_1 (44 mg) was crystallized from hexane - chloroform to give colorless needles (9 mg), while colorless rods (22 mg of karnamicin B_2 and 30 mg of karnamicin B_3) were obtained from chloroform by crystallization of karnamicin B_2 (52 mg) and karnamicin B_3 (48 mg), respectively.

Purification of Karnamicins C and D Complex

Separation of karnamicins C and D complex (1.6 g) was performed by LiChroprep RP-18 column (22×450 mm) with methanol - 0.03 M phosphate buffer (pH 7.0) as eluent (52:48, 1.0 liter and a linear gradient from 52:48 to 60:40, 1.0 liter). Fractionation of the eluate was guided by the HPLC and

Table 4. Physico-chemical properties of karnamicin components.

	A_1	A_2	A_3	B_1	B_2
Nature	White amorphous powder	White amorphous powder	White amorphous powder	Colorless needles	Colorless rods
MP (°C)	56.5~58.5	60~63	69~72	151~153	171~172.5
$[\alpha]_D^{25}$ ($CHCl_3$)	−11° (*c* 0.5)	−14° (*c* 0.5)	−19° (*c* 0.25)	0° (*c* 0.5)	0° (*c* 0.5)
UV λ_{max} nm (ε)					
in MeOH	219 (25,000), 242 (22,700), 315 (6,700)	219 (25,500), 242 (23,500), 316 (6,800)	218 (26,400), 242 (23,700), 317 (6,800)	219 (25,900), 242 (23,600), 316 (6,900)	219 (26,100), 242 (23,500), 317 (6,800)
in 0.1 N HCl - MeOH (1:9)	220 (25,700), 243 (25,700), 318 (7,500)	220 (23,900), 243 (23,900), 318 (6,900)	220 (25,000), 243 (25,000), 317 (7,300)	220 (sh, 23,900), 243 (26,400), 318 (8,000)	220 (sh, 23,500), 243 (26,900), 318 (8,100)
in 0.1 N NaOH - MeOH (1:9)	220 (23,400), 251 (21,600), 281 (17,200), 342 (9,000)	220 (23,400), 251 (21,500), 282 (17,000), 342 (9,000)	220 (24,200), 251 (22,200), 282 (17,400), 342 (9,200)	220 (23,300), 251 (21,300), 292 (16,600), 341 (9,100)	221 (23,300), 252 (21,100), 293 (16,600), 342 (9,000)
IR ν_{max}^{KBr} cm^{-1}	3400, 1665, 1590	3300, 1665, 1580	3350, 1665, 1580	3385, 1705, 1665, 1600	3300, 1675, 1600
EI-MS (*m/z*)	383 (M^+), 368, 311	397 (M^+), 382, 311	411 (M^+), 393, 311	365 (M^+), 295	367 (M^+), 352, 295
Molecular formula	$C_{16}H_{21}N_3SO_6$	$C_{17}H_{23}N_3SO_6$	$C_{18}H_{25}N_3SO_6$	$C_{16}H_{19}N_3SO_5$	$C_{16}H_{21}N_3SO_5$
Elemental analysis					
Calcd for			$C_{18}H_{25}N_3SO_6 \cdot \frac{1}{2}H_2O$:	$C_{16}H_{19}N_3SO_5$:	$C_{16}H_{21}N_3SO_5$:
			C 51.42	C 52.59	C 52.30
			H 6.23	H 5.24	H 5.76
			N 9.99	N 11.50	N 11.44
			S 7.62	S 8.77	S 8.73
Found:			C 51.03	C 52.89	C 51.89
			H 5.95	H 5.21	H 5.64
			N 9.57	N 11.26	N 11.23
			S 7.42	S 8.75	S 8.82

EI-MS: Electron impact MS.

Table 4. (Continued)

	B_3	C_1	C_2	C_3	C_4
Nature	Colorless rods	White amorphous powder	White amorphous powder	White amorphous powder	White amorphous powder
MP (°C)	187.5~189.5	152.5~154	130.5~133	165.5~167.5	124.5~126.5
$[\alpha]_D^{25}$ ($CHCl_3$)	0° (*c* 0.5)	+14° (*c* 0.25)	−8° (*c* 0.25)	+20° (*c* 1.0)	0° (*c* 1.0)
UV λ_{max} nm (ε)					
in MeOH	220 (25,400), 242 (23,200), 317 (6,800)	220 (25,000), 243 (22,200), 317 (6,600)	220 (26,000), 243 (24,000), 318 (7,100)	220 (25,800), 242 (23,200), 317 (6,800)	220 (25,900), 243 (23,900), 317 (7,100)
in 0.1 N HCl - MeOH (1 : 9)	220 (sh, 22,100), 243 (26,500), 318 (7,900)	222 (sh, 23,100), 242 (23,500), 317 (7,500)	222 (sh, 22,500), 243 (26,600), 318 (8,200)	222 (sh, 25,000), 244 (28,300), 318 (8,400)	223 (sh, 23,200), 243 (26,100), 318 (8,000)
in 0.1 N NaOH - MeOH (1 : 9)	218 (21,400), 252 (20,200), 294 (16,200), 342 (8,700)	220 (22,100), 252 (19,800), 294 (15,700), 342 (8,400)	217 (22,100), 253 (20,600), 294 (16,800), 342 (9,100)	220 (17,300), 251 (21,000), 294 (16,600), 341 (8,800)	217 (22,300), 252 (20,600), 294 (16,700), 342 (9,100)
IR ν_{max}^{KBr} cm^{-1}	3240, 1680, 1610	3330, 1670, 1600	3200, 1670, 1600	3200, 1670, 1605	3390, 1705, 1660, 1590
EI-MS (*m/z*)	381 (M^+), 366, 295	367 (M^+), 338, 295	381 (M^+), 295	381 (M^+), 336, 308, 295	379 (M^+), 308, 295
Molecular formula	$C_{17}H_{23}N_3SO_5$	$C_{16}H_{21}N_3SO_5$	$C_{17}H_{23}N_3SO_5$	$C_{17}H_{23}N_3SO_5$	$C_{17}H_{21}N_3SO_5$
Elemental analysis					
Calcd for	$C_{17}H_{23}N_3SO_5$: C 53.53 H 6.08 N 11.02 S 8.40				
Found:	C 54.11 H 6.00 N 10.70 S 8.24				

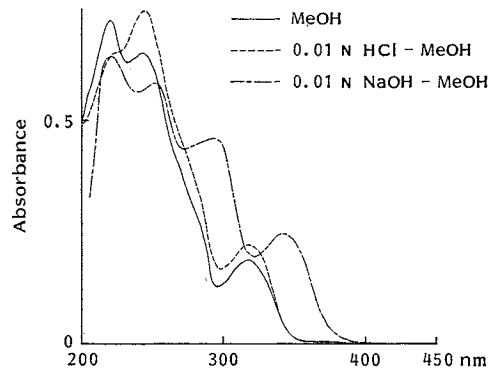
Table 4. (Continued)

	C_5	D_1	D_2	D_3	D_4
Nature	White amorphous powder	White amorphous powder	White amorphous powder	White amorphous powder	White amorphous powder
MP (°C)	138~140	146.5~148	139~140	153~154	164~165.5
$[\alpha]_D^{25}$ ($CHCl_3$)	+19° (*c* 1.0)	0° (*c* 1.0)	0° (*c* 0.2)	+4° (*c* 0.18)	0° (*c* 0.2)
UV λ_{max} nm (ε)					
in MeOH	220 (25,700), 243 (23,900), 317 (7,100)	219 (26,300), 242 (23,500), 317 (6,800)	220 (25,800), 244 (23,900), 315 (7,900)	220 (25,200), 244 (23,200), 316 (7,300)	220 (25,400), 244 (24,000), 315 (7,500)
in 0.1 N HCl - MeOH (1:9)	220 (24,600), 243 (24,700), 317 (7,400)	220 (sh, 23,300), 243 (26,400), 317 (8,000)	219 (sh, 21,400), 244 (25,400), 319 (7,900)	220 (sh, 21,000), 244 (25,700), 318 (7,800)	219 (sh, 22,000), 244 (26,200), 319 (7,900)
in 0.1 N NaOH - MeOH (1:9)	217 (22,200), 252 (20,700), 283 (16,700), 343 (8,900)	221 (22,900), 252 (20,800), 292 (16,400), 342 (8,800)	220 (21,500). 254 (20,900), 294 (16,800), 342 (9,400)	221 (23,300), 254 (22,200), 294 (17,900), 344 (9,800)	221 (22,400), 254 (21,600), 293 (17,200), 344 (9,700)
IR ν_{max}^{KBr} cm^{-1}	3300, 1670, 1600	3250, 1675, 1610	3400, 1710, 1665, 1600	3340, 1675, 1600	3390, 1705, 1665, 1600
EI-MS (*m/z*)	367 (M^+), 352, 311	395 (M^+), 308, 295	379 (M^+), 308, 295	381 (M^+), 352, 295	379 (M^+), 308, 295
Molecular formula	$C_{16}H_{21}N_3SO_5$	$C_{18}H_{25}N_3SO_5$	$C_{17}H_{21}N_3SO_5$	$C_{17}H_{23}N_3SO_5$	$C_{17}H_{21}N_3SO_5$
Elemental analysis					
Calcd for					
Found:					

the three separated fractions were worked up to yield karnamicin C complex (545 mg), pure karnamicin D_1 (379 mg) and karnamicin D complex (288 mg).

Karnamicin C complex was purified by preparative HPLC with SSC-ODS-842. Elution was carried out with acetonitrile - 0.15% KH_2PO_4, pH 3.5, 25 : 75 ~ 41.5 : 58.5 from 0 ~ 15 minutes, 41.5 : 58.5 from 15 ~ 70 minutes and 41.5 : 58.5 ~ 55 : 45 from 70 ~ 90 minutes at flow rate of 12.0 ml/minute. The eluate was monitored by UV absorption at 300 nm, and the relevant fractions (retention time: karnamicin C_1, 57 minutes, karnamicin C_2, 59 minutes, karnamicin C_3, 63 minutes, karnamicin C_4, 70 minutes and karnamicin C_5, 81 minutes) were pooled. This preparative HPLC was repeated and similar fractions were combined. The fractions were concentrated and extracted with ethyl acetate to yield pure karnamicin C_1 (23 mg), karnamicin C_2 (24 mg), karnamicin C_3 (110 mg), karnamicin C_4 (75 mg) and karnamicin C_5 (33 mg). Karnamicin D complex was similarly purified on SSC-ODS-842 column with methanol - 0.03 M phosphate buffer, pH 7.0 (a linear gradient: 60 : 40 ~ 70 : 30 from 0 ~ 100 minutes. Retention time: karnamicin D_2, 30 minutes, karnamicin D_3, 47 minutes and karnamicin D_4, 68 minutes) to yield pure karnamicin D_2 (24 mg), karnamicin D_3 (25 mg) and karnamicin D_4 (17 mg).

Fig. 5. UV spectrum of karnamicin B_2 (10 μg/ml).



Physico-chemical Properties

The physico-chemical properties of 15 components of karnamicin are described in Table 4. They are soluble in benzene, chloroform, dimethyl sulfoxide, ethyl acetate, methanol and ethanol, but insoluble in *n*-hexane and water. They gave positive response to Dragendorff and

Fig. 6. IR spectrum of karnamicin B_1.

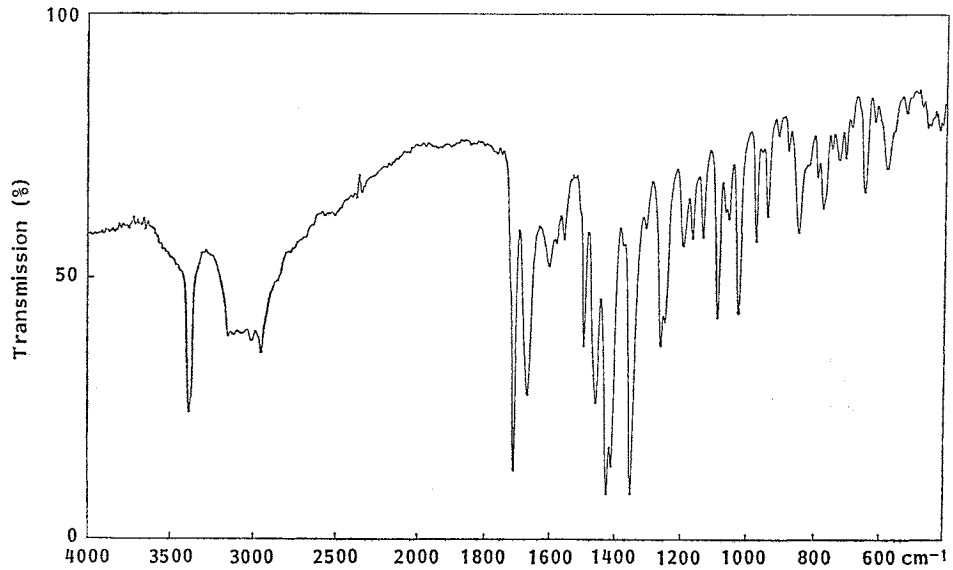


Fig. 7. IR spectrum of karnamicin B_2.

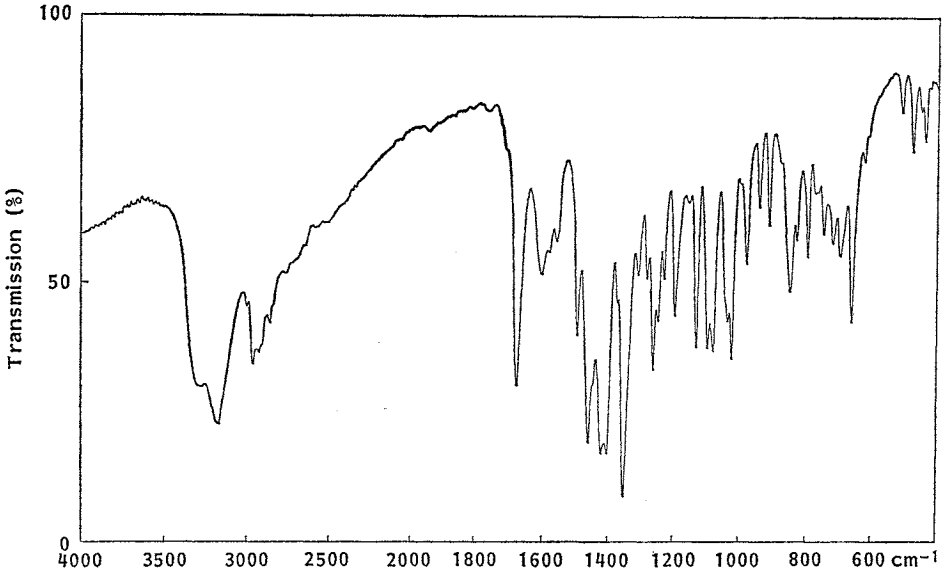
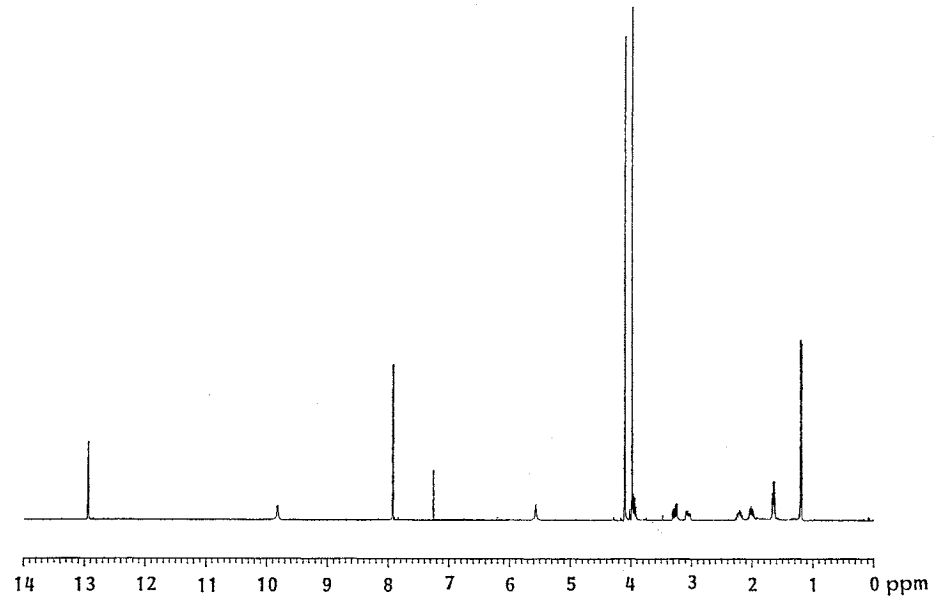


Fig. 8. ^1H NMR spectrum of karnamicin B_2 (400 MHz, in $CDCl_3$).



Rydon-Smith reagents, but negative response to Erhlich, Sakaguchi and ninhydrin reagents. Their UV absorption maxima are located at 219, 242 and 317 nm in methanol and acidic methanol and shifted to 220, 252, 293 and 342 nm in alkaline methanolic solution. Karnamicins A_1, A_2, A_3 and C_5, which have α-hydroxyalkyl side chain on the thiazole ring, exhibited absorption maxima at 220, 251, 281 and 342 nm in alkaline solution. The UV spectrum of karnamicin B_2 is shown in Fig. 5. Their IR spectra exhibited in common the presence of amide at 1660~1680 cm^{-1}. The spectra of

Fig. 9. ^{13}C NMR spectrum of karnamicin B_2 (100 MHz, in $CDCl_3$).

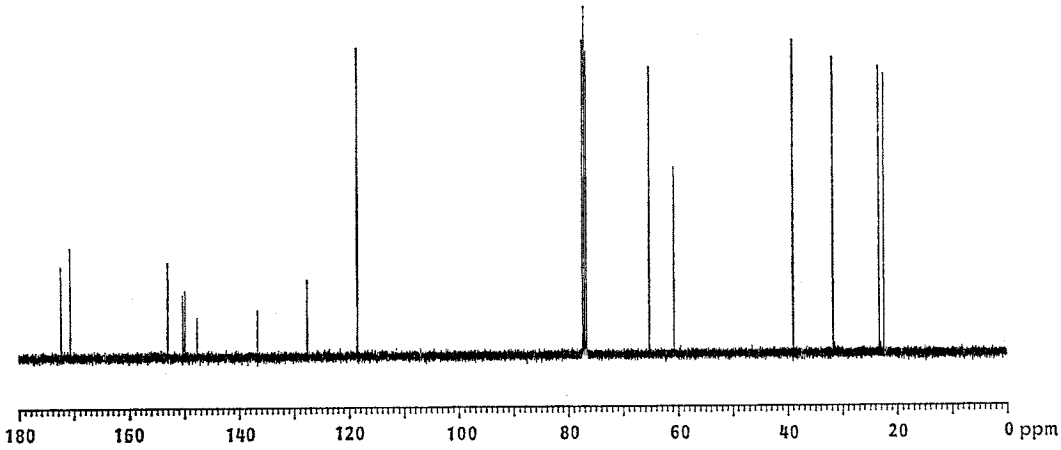
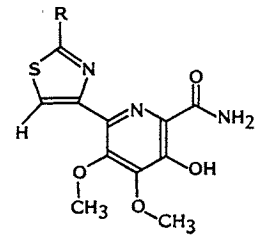
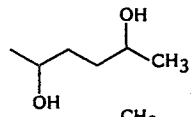
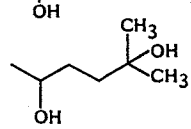
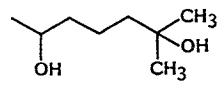


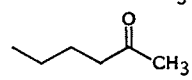
Fig. 10. The structures of karnamicin components.

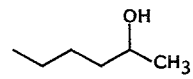


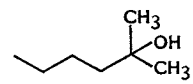
Karnamicin A_1 R= 

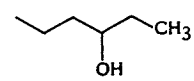
Karnamicin A_2 R= 

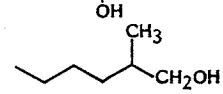
Karnamicin A_3 R= 

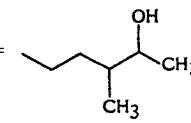
Karnamicin B_1 R= 

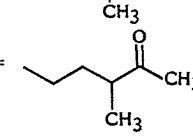
Karnamicin B_2 R= 

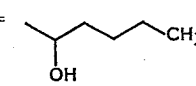
Karnamicin B_3 R= 

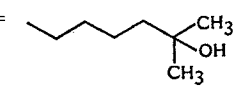
Karnamicin C_1 R= 

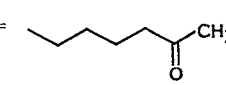
Karnamicin C_2 R= 

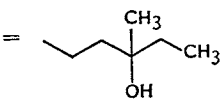
Karnamicin C_3 R= 

Karnamicin C_4 R= 

Karnamicin C_5 R= 

Karnamicin D_1 R= 

Karnamicin D_2 R= 

Karnamicin D_3 R= 

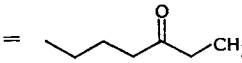
Karnamicin D_4 R= 

Table 5. *In vitro* antifungal activity of karnamicins in Sabouraud dextrose broth.

Test organism	MIC (μg/ml)														
	A_1	A_2	A_3	B_1	B_2	B_3	C_1	C_2	C_3	C_4	C_5	D_1	D_2	D_3	D_4
C. a. A9540	50 (1.6)[a]	>100 (3.1)	>100 (3.1)	12.5	3.1	6.3	3.1 (1.6)	3.1 (1.6)	6.3 (3.1)	6.3 (3.1)	6.3 (3.1)	3.1	3.1 (1.6)	3.1 (1.6)	3.1 (1.6)
A. f. IAM 2034	6.3 (3.1)	12.5 (6.3)	6.3 (3.1)	6.3	6.3	12.5	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1
T. m. No. 4329	>100 (25)	>100 (50)	>100 (50)	6.3	6.3	6.3	6.3 (3.1)	6.3 (3.1)	6.3 (3.1)	6.3 (3.1)	6.3	6.3 (3.1)	3.1	3.1	6.3 (3.1)

[a] Values in parenthesis indicate partial inhibition.

C. a.; *Candida albicans*, *A. f.*; *Aspergillus fumigatus*, *T. m.*; *Trichophyton mentagrophytes*.

Table 6. *In vitro* antifungal activity of karnamicin in Sabouraud dextrose agar.

Test organism	MIC (μg/ml)														
	A_1	A_2	A_3	B_1	B_2	B_3	C_1	C_2	C_3	C_4	C_5	D_1	D_2	D_3	D_4
C. a. IAM 4888	25 (12.5)[a]	100 (12.5)	>100 (<6.3)	50	25	50	50 (12.5)	50 (<6.3)	50 (<6.3)	50 (12.5)	50 (12.5)	50 (12.5)	25	25	50 (25)
C. a. A9540	25 (12.5)	>100 (12.5)	>100 (<6.3)	25	25	50	50 (<6.3)	50 (<6.3)	50 (<6.3)	50 (12.5)	50 (<6.3)	50 (12.5)	25	25	50 (25)
C. n. D49	25	>100	>100	50	25	50	50	50	50	50	50	50	25	25	25
C. n. IAM 4519	25	>100	>100	25	25	50	50	50	50	50	50	50	25	25	25
A. f. IAM 2530	25	25	25	50	25	50	25	25	25	25	25	25	25	25	25
A. f. IAM 2034	25	25	25	50	25	50	25	25	25	25	25	25	25	25	25
P. o. D91	50	50	25	NT	NT	NT	25	25	25	25	25	25	25	25	25
T. m. D155	100	50	50	NT	25	NT	25	25	25	25	25	25	12.5	25	25
T. m. No. 4329	100	50	50	50	25	50	25	12.5	25	25	25	25	25	25	25
B. d. D40	NT	25	25	12.5	25	25	12.5	12.5	12.5	25	12.5	25	6.3	3.1	6.3
S. s. IFO 8158	12.5	>100	>100	50	50	50	25	25	25	25	25	25	25	25	25
M. s. IFO 5317	6.3	12.5	12.5	25	12.5	25	<6.3	<6.3	<6.3	12.5	<6.3	<6.3	25	12.5	25

[a] Values in parenthesis indicate partial inhibition.

NT: Not tested.

C. a.; *Candida albicans*, *C. n.*; *Cryptococcus neoformans*, *A. f.*; *Aspergillus fumigatus*, *P. o.*; *Piricularia oryzae*, *T. m.*; *Trichophyton mentagrophytes*, *B. d.*; *Blastomyces dermatitidis*, *S. s.*; *Sporothrix schenckii*, *M. s.*; *Mucor spinosus*.

karnamicins B_1, C_4, D_2 and D_4 had an additional carbonyl absorption at 1705~1710 cm^{-1} which is attributed to the ketone carbonyl group in the alkyl chain. The IR spectra of karnamicins B_1 and B_2 are shown in Figs. 6 and 7, respectively. Figs. 8 and 9 show the ^1H and ^{13}C NMR spectra of karnamicin B_2, respectively. Karnamicin components have the same thiazolylpyridinecarboxamide nucleus and differ from each other in the alkyl side chain moiety. As reported in a separate paper, the structures of all 15 components of karnamicin have been determined by X-ray crystallography and spectral data (Fig. 10).

Biological Activity

The *in vitro* antifungal activity of the components of karnamicin was determined against various fungi by serial broth and agar dilution methods. Sabouraud dextrose broth and agar were used for the above experiments. The inoculum size was adjusted to 10^4~10^6 and 10^5~10^9 cfu/ml for broth and agar methods, respectively. After incubation at 28°C for 48 or 72 hours, the MIC, the lowest concentration of antibiotic causing virtually complete inhibition of growth, was determined. As shown in Tables 5 and 6, all components of karnamicin exhibited significant *in vitro* antifungal activity against *C. albicans*, *Aspergillus fumigatus* and *Trichophyton mentagrophytes* in the broth dilution method, and also showed moderate antifungal activity against various clinically important pathogenic fungi in Sabouraud dextrose agar. The morphological change-inducing activity of karnamicin components was examined with a mutant of *C. albicans* A9540. The mutant, *C. albicans* A9540-2 does not form pseudomycelium when serum is added to the culture media, while the parent strain forms pseudomycelium in the culture condition. No pseudomycelium formation is observed for both strains cultivated in the media without serum. The suspension of the mutant strain (10^4 cfu/ml) and each component of karnamicin was incubated for 72 hours at 37°C in Eagle's minimum essential medium supplemented with 10% calf serum. The inhibitory activity of karnamicin components against fungal growth was determined by the turbidity described above. Furthermore, morphological change of the mutant strain was obserbed microscopically. The results are shown in Table 7. All components of karnamicin demonstrated an obvious pseudomycelium-inducing effect against a yeast-

Table 7. Inhibitory and pseudomycelium-inducing activities against a yeast-form mutant of *Candida albicans*, A9540-2.

Karnamicin	MIC (μg/ml)	Pseudomycelium induction (μg/ml)
A_1	>100 (6.3)[a]	100~6.3
A_2	>100 (6.3)	100~6.3
A_3	>100 (6.3)	100~6.3
B_1	12.5 (3.1)	6.3~3.1
B_2	12.5 (3.1)	3.1~1.6
B_3	25 (6.3)	6.3~3.1
C_1	12.5 (3.1)	3.1
C_2	12.5 (3.1)	3.1
C_3	6.3 (3.1)	3.1
C_4	12.5 (6.3)	6.3
C_5	12.5 (6.3)	6.3~3.1
D_1	12.5 (6.3)	6.3
D_2	12.5 (6.3)	6.3
D_3	12.5 (6.3)	6.3
D_4	12.5 (6.3)	6.3

[a] Values in parenthesis indicate partial inhibition.

Table 8. *In vivo* antifungal activity against *Candida albicans* A9540 vaginal infection in mice.

Concentration (%)[a]	Inhibition of growth (%)		
	Karnamicin B_2	Amphotericin B	Nystatin
2.0	100	99	
1.0	99	80	
0.50	97	81	
0.25	14	81	99
0.13	13	55	99
0.063	0	33	68
0.031		0	0
EC_{50} (%)	0.34	0.11	0.042

[a] 0.02 ml of the compound solution in Solbase was instilled intravaginally once a day from days 0 to 4.

Table 9. *In vitro* antibacterial activity of karnamicins in nutrient agar.

Test organism	MIC (μg/ml)											
	A_1	A_2	A_3	B_1	B_2	B_3	C_1	C_2	C_3	C_4	C_5	D_1
Staphylococcus aureus FDA 209P	50	50	50	25	25	50	25	50	100	25	25	50
S. aureus No. 52-34[a]	50	100	100	50	50	100	100	100	>100	100	100	100
S. aureus A20239[b]	50	100	100	50	50	100	100	100	>100	100	50	100
S. aureus BX1633-2[c]	50	100	100	50	50	100	100	100	>100	100	50	100
S. aureus A15097[d]	50	50	50	25	50	50	50	100	>100	50	50	50
S. epidermides D153	50	100	50	50	25	50	25	50	25	50	25	50
Enterococcus faecalis A9612	100	100	100	100	100	>100	50	100	>100	100	50	50
Bacillus subtilis PCI 219	50	25	50	25	25	25	25	25	50	25	25	25
Escherichia coli NIHJ	100	>100	>100	>100	>100	>100	100	>100	>100	100	100	100
Klebsiella pneumoniae D11	50	>100	>100	50	50	100	>100	>100	>100	>100	100	>100
Proteus mirabilis A9554	100	>100	>100	>100	>100	>100	100	>100	>100	100	100	100
P. vulgaris A9436	100	100	100	>100	>100	>100	100	100	>100	100	100	100
Serratia marcescens A20222	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Pseudomonas aeruginosa A9930	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

[a] Erythromycin-resistant strain. [b] Aminoglycoside-resistant strain. [c] Penicillinase-producing strain. [d] Methicillin-resistant strain.

form mutant of *C. albicans*, A9540-2, whereas the known antifungal agents tested did not induce any morphological change of the yeast.

Topical application of karnamicin B_2 against *C. albicans* A9540 vaginal infection was examined in comparison with amphotericin B and nystatin. Groups of 5 female mice (ICR, 18~21 g) were treated subcutaneously with 0.5 mg of estradiol benzoate 3 days before and 4 days after *C. albicans* A9540 vaginal infection. A cell suspension (0.01 ml) of *C. albicans* containing 10^8 cfu/ml was inoculated intravaginally on day-0. Then, 0.02 ml of the compound dissolved in Solbase (1:1 mixture of polyethyleneglycol 400 and polyethyleneglycol 4000, Dainippon Pharmaceutical Co., Ltd.) was instilled intravaginally once a day on days 0 to 4. On day-7, vaginal exudate was taken by a thin glass rod and spread on the YGP agar plate containing yeast extract 0.2%, glucose 1.5%, peptone 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% and agar 1.5%. Viable cell count was made after incubation for 2 days at 28°C. The EC_{50} value, which is the drug concentration (%) giving 50% inhibition of the control, was calculated by the method of least squares. As shown in Table 8, karnamicin B_2 exhibited activity against *C. albicans* vaginal infection by topical application. The activity of karnamicin B_2 was slightly weaker than that of amphotericin B and about one-eighth that of nystatin.

Table 10. *In vitro* activity against *Trichomonas vaginalis* in thioglycolate broth.

Test organism	MIC (μg/ml)	
	Karnamicin B_2	Metronidazole
T. vaginalis Tv-2	25	0.8

Inoculum size: 10^5 cells/ml.

The *in vitro* antibacterial activity of the components of karnamicin was determined by the serial dilution method in nutrient agar against Gram-positive and Gram-negative aerobic bacteria. The inoculum size was adjusted to $10^5 \sim 10^6$ cfu/ml. The MIC was defined as the lowest concentration of test compound completely inhibiting bacterial growth after 18-hour incubation at 37°C. As shown in Table 9, all components of karnamicin exhibited weak *in vitro* antibacterial activity against Gram-positive bacteria, but were almost inactive against Gram-negative organisms. Karnamicin B_2 was tested for *in vitro* anti-trichomonal activity compared with metronidazole. Table 10 shows MIC values determined after 24-hour incubation at 37°C. Karnamicin B_2 exhibited appreciable activity against the clinical isolate of *Trichomonas vaginalis*.

Discussion

Karnamicin, a complex of antifungal antibiotics with a novel chemotype, was isolated from the cultured broth of *S. aerocolonigenes* No. N806-4. Chromatographic separation of the complex resulted in the isolation of 15 components, karnamicins A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_2, C_3, C_4, C_5, D_1, D_2, D_3 and D_4, which have similar biological and chemical properties. Karnamicin components exhibited a wide spectrum of antifungal activity and weak antibacterial activity against Gram-positive bacteria. Karnamicin B_2 showed *in vivo* efficacy against *C. albicans* vaginal infection by topical application. It is interesting to note that karnamicin induced morphological change of a certain mutant of *C. albicans* A9540. A mutant which grows only in yeast form in the presence of serum, was induced to form pseudomycelia by karnamicin components. No other antifungal and antibacterial antibiotics we tested so far caused the pseudomycelium induction of this mutant strain. This suggests that karnamicin has a unique mechanism of activity against fungi and yeasts.

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