Signal Transduction Inhibitors, Hibarimicins A, B, C, D and G Produced by *Microbispora*

I. Taxonomy, Fermentation, Isolation and Physico-chemical and Biological Properties

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Strain TP-A0121 which produces a complex of novel tyrosine kinase inhibitors designated hibarimicins A, B, C, D and G was considered to be a new subspecies of *Microbispora rosea*, and the name, *Microbispora rosea* subsp. *hibaria*, was proposed. Hibarimicins A, B, C and D specifically inhibited the *src* tyrosine kinase activity without affecting protein kinase A or protein kinase C. They also showed *in vitro* anti-Gram-positive bacterial and antitumor activities. The molecular formulae of hibarimicins A, B, C, D and G were assigned to be $C_{85}H_{112}O_{37}$, $C_{85}H_{112}O_{37}$, $C_{83}H_{110}O_{36}$, $C_{85}H_{112}O_{38}$, and $C_{85}H_{112}O_{39}$ respectively.

During the screening of microbial products for tyrosine kinase inhibitors, we found strain TP-A0121 producing a complex of novel antibiotics. The producing strain was isolated from a soil sample collected at Hibari, Toyama Prefecture, Japan on June 2, 1995, was characterized by the formation of longitudinal pair spores on aerial mycelium and was identified as *Microbispora rosea* subsp. *hibaria* TP-A0121 based on the DNA-DNA homological comparison to the type species of the genus. This complex collectively named hibarimicins consisted of more than 10 components. Hibarimicins were extracted from the whole broth with ethyl acetate and were separated into the individual components by chromatographic techniques.

In this paper, we describe the taxonomy of the producing strain, fermentation, isolation, physico-chemical and biological properties of hibarimicins A, B, C, D and G. Biological properties of hibarimicins G will be reported comparatively with those of novel metabolites in this culture elsewhere. Further structural studies on hibarimicins A, B, C, D and G will be reported in the accompanying paper[1].

Materials and Methods

Instrumental Analysis

MPs were determined with a YANACO MP J-3 and are uncorrected. UV-visible and IR spectra were recorded on a HITACHI U-3210 and a SHIMADZU FTIR-8100, respectively. TMS was used as an internal standard. FAB-MS were measured on a JEOL JMS-HX1100 spectrometer using 3-nitrobenzyl alcohol as a matrix.

Taxonomy

Taxonomic characteristics of strain TP-A0121 were determined by cultivation on various media described by SHIRLING and GOTTLIEB[2], WAKSMAN[3] and ARAI[4]. Morphological characteristics were observed after incubation of the culture at 30°C for 14 days on oatmeal-yeast extract agar. Cultural and physiological characteristics were determined after growth at 30°C for 14 days. The color names and hue numbers were assigned using the

Manual of Color Names (Japan Color Enterprise Co., Ltd., 1987). Temperature range for growth was determined using a temperature gradient incubator TN-2148 (Advantec Toyo Co.). The carbon utilization was determined by the method of SHIRLING and GOTTLIEB[2]. Cell wall composition was analyzed by the method of LECHEVALIER *et al.*[5], using thin layer chromatography sheets as described by STANECK *et al.*[6]. Phospholipids, menaquinones and fatty acids analyses were determined by the methods of LECHEVALIER[7], COLLINS *et al.*[8] and SUZUKI *et al.*[9], respectively. DNA was isolated by the procedure of FURUMAI *et al.*[10], and the G+C content was determined with HPLC[11,12]. DNA-DNA hybridization was performed according to the method of EZAKI *et al.*[13].

Biological Activities

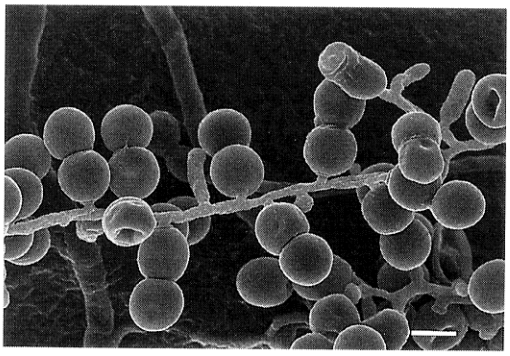
The inhibitory activity of hibarimicins on protein kinases including protein kinase A, protein kinase C, protein tyrosine kinase and calmodulin-dependent protein kinase III was examined according to the procedure previously reported[14,15]. In brief, a postnuclear supernatant of v-src transformed NIH3T3 cells was incubated with activators of protein kinases and [γ-^{32}P]ATP, and the phosphorylated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

In vitro antimicrobial activity was determined by the serial 2-fold agar dilution method using Nutrient Agar (Difco laboratories), pH 7.0. A 5-ml suspension containing 10^5 cells per ml was used as inoculum of the test organisms. The MIC values were determined after the incubation for 18 hours at 32°C.

In vitro cytotoxicity against B16-F10 and HCT-116 cells were determined as follows. B16-F10 cells were grown in Eagle's minimum essential medium (Nissui) supplemented with fetal calf serum (FCS, 10%) and kanamycin (60 μg/ml), and HCT-116 cells were grown in McCoy's 5A Medium (Gibco) supplemented with FCS (10%), benzylpenicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified CO_2 incubator. The above exponentially growing cells were harvested, counted and suspended in the culture media at 1.5×10^4 (B16-F10) and 3.0×10^4 (HCT-116) cells/ml. The cells were plating into 96-well tissue culture plates with the test materials and incubated for 72 hours. The cytotoxic activities were colorimetrically determined at 540 nm after staining viable cells with neutral red solution.

Fig. 1. Scanning electron micrograph of *Microbispora rosea* subsp. *hibaria* TP-A0121.

Bar represents 1 μm.



Strain TP-A0121 grown on oatmeal-yeast extract agar for 1 week at 30°C, showing longitudinal pair spores with smooth surface.

Results and Discussion

Taxonomy of the Producing Strain

On observation with light microscope, spores in characteristic longitudinal pair spores were formed on aerial mycelium, but not formed on substrate mycelium. By scanning electron microscope, the spore was oval in shape, 1.2×1.3 μm in size, with smooth surface (Fig. 1). Strain TP-A0121 grew relatively well on various media. The aerial mycelium was visible on ISP med. Nos. 2, 3 and 7, and Bennett's agar. Table 1 shows the cultural characteristics. Aerial mass color was pale pink. Vegetative mycelium and reverse side of colony was yellowish white to dark yellowish brown. Light yellow or bright yellow diffusible pigments were formed in ISP-7 and Bennett's agar or ISP-2 and -5 agar media, respectively. As summarized in Table 2, starch hydrolysis, nitrate reduction, milk peptonization and gelatin liquefaction gave a positive reaction. The temperature range for growth was 18~44°C, and the optimum temperature for growth was 28~39°C. L-Arabinose, D-xylose, D-glucose, D-fructose, sucrose, D-mannitol, D-mannose, D-galactose, maltose, glycerol, lactose and D-sorbitol were utilized by strain TP-0121 for growth. L-rhamnose, raffinose and inositol were not utilized.

Whole cell hydrolysates contained *meso*-diaminopimelic acid, glucose and madurose, therefore, the cell wall type was IIIB. The strain TP-A0121 has type PIV phospholipid (presence of phosphatidylethanolamine and unknown glucosamine containing phospholipids). 44.3% MK-9 (H_2), 30.4% MK-9 (H_4) and 23.0% MK-9

Table 1. Cultural characteristics of strain TP-A0121.

Medium	Vegetative mycelium	Reverse side	Aerial mycelium	Diffusible pigment
Sucrose nitrate agar (Waksman med. No. 1)	White (389)	White (393)	None	None
Glycerol nitrate agar	White (389)	White (393)	None	None
Glucose asparagine agar (Waksman med. No. 2)	Pale beige (85)	Pale beige (85)	None	None
Yeast extract-malt extract agar (ISP med. 2)	Dark grayish brown (121), good	Dark grayish brown (123)	Pale pink (4), powdery	Bright yellow (138)
Oatmeal agar (ISP med. 3)	Brown (95)	Brown (95)	Pale pink (4), powdery	None
Inorganic salts-starch agar (ISP med. 4)	Pale beige (84)	Pale beige (84)	None	None
Glycerol asparagine agar (ISP med. 5)	Pale beige (85)	Pale beige (85)	None	None
Tyrosine agar (ISP med. 7)	Dark grayish brown (123)	Dark grayish brown (123)	White (389)	Light yellow (133)
Nutrient agar (Waksman med. 14)	Yellowish brown (101)	Yellowish brown (101)	None	None
Bennett's agar (Waksman med. 30)	Dark yellowish brown (106), good	Dark yellowish brown (106)	Pale pink (4), powdery	Light yellow (133)

Table 2. Physiological characteristics of strain TP-A0121.

Test	Results
Starch hydrolysis (On ISP med. 4)	Positive
Nitrate reduction (Difco, nitrate broth)	Positive
Milk (Difco, 10% skimmed milk)	
Coagulation	Negative
Peptonization	Positive
Cellulose decomposition (sucrose nitrate solution with a paper strip as the sole carbon source)	Negative (Growth: good)
Gelatin liquefaction	
on plain gelatin	Positive
on glucose peptone gelatin	Positive
Melanin formation (on ISP med. 7)	Negative
Temperature range for growth (on Yeast starch agar)	18～44°C
Optimum temperature (on Yeast starch agar)	28～39°C
pH range for growth (on Trypticase soy broth, BBL)	5～9
Optimum pH (on Trypticase soy broth, BBL)	6～7

(H_0) were the major menaquinones detected. Fatty acids consisted of 20% 14-methyl-pentadecanoic acid (*i*-16), 17% hexadecanoic acid (16:0), 8% 10-methylhexadecanoic acid (10Me-16:0), 7% heptadecanoic acid (17:0), 7% 10-methylheptadecanoic acid (10Me-17:0), and other minor fatty acids (Table 3).

The morphological characteristics and chemotaxonomic information were consistent with the assignment of strain TP-A0121 to the genus *Microbispora* Nonomura and Ohara 1957[16]. From the known species of *Microbispora*, strain TP-A0121 was related to *Microbispora rosea* subsp. *rosea* Nonomura and Ohara 1957[17]. However, the DNA-DNA hybridization between strain TP-A0121 and *M. rosea* subsp. *rosea* IFO14044^T indicated that these two strains are not identical (Table 4). Thus, we consider strain TP-A0121 to be a novel subspecies of *M. rosea* subsp. *rosea*, and the name *Microbispora rosea* subsp. *hibaria* Furumai, Kajiura and Oki subsp. nov. is proposed, referring to the place of origin.

Table 3. Cellular fatty acid composition of strain TP-A0121.

Fatty acid		Composition %
Normal acid	14:0	1
	15:0	4
	16:0	17
	$16:1^9$	5
	17:0	7
	18:0	3
Iso acid	14:0	1
	15:0	3
	16:0	20
	17:0	1
Anteiso acid	15:0	3
	17:0	5
10-Methyl acid	16:0	8
	17:0	7
	18:0	3

Table 4. DNA-DNA homology between strain TP-0121 and *Microbispora rosea* subsp. *rosea* IFO14044^T.

Strain	G+C (%) contents	DNA-DNA hybridization (%)	
		TP-A0121	IFO14044^T
TP-A0121	74.3	100	62
IFO14044^T	73.5	68	100

Fermentation

A loopful of a mature slant culture of *Microbispora* subsp. *hibaria* TP-A0121 was inoculated into a 500-ml K-1 flask containing 100 ml of the seed medium consisting of soluble starch (Wako Chemical Co.) 1%, glucose 0.5%, NZ-case (Humco Scheffield Chemical Co.) 0.3%, yeast extract (Difco Laboratories) 0.2%, tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The flask was incubated at 30°C for 4 days on a rotary shaker (200 rpm). Two-ml aliquots of the seed culture were transferred into thirty 500-ml K-1 flask each containing 100 ml of the production medium consisting of mannose 4%, Pharmamedia (Trader's Protein) 2% and $CaCO_3$ 0.3%. The pH of the medium was adjusted to 7.0 before autoclaving. Fermentation was carried out for 10 days under the same conditions described for the seed culture. The production of hibarimicins in the fermentation was monitored by UV-vis spectroscopy and HPLC using a C-18 reverse-phase column (Cosmosil 5C18-AR, 4.6×250 mm, Nacalai Tesque Inc.) and acetonitrile-0.15% phosphate buffer, pH 3.5 (1:1) at a flow rate of 0.7 ml/minute with 254 nm detection. The retention times of hibarimicins A, B, C, D and G were 24.0, 18.2, 13.8, 12.5 and 8.2 minutes, respectively. For preparation of the assay sample, a 5-ml portion of the whole broth was extracted with the same volume of ethyl acetate at pH 5.0. This ethyl acetate layer was diluted (×10 fold) with a solution consisting of 0.01 N NaOH : MeOH (1:10) and 50 μl of the diluted extract was used for HPLC analysis. The production of hibarimicins A, B, C, D and G in shake flask culture reached a maximum of 39, 153, 172, 306 and 45 μg/ml, respectively, at 10 days.

Isolation

The fermented whole broth (3 liters) was extracted with ethyl acetate (3 liters) and the extract was concentrated *in vacuo* to dryness (3.67 g). The extract was dissolved in methanol (50 ml) and charged on a column of Diaion HP-20 (40 mm i.d. × 300 mm, Mitsubishi Chemical Industries Ltd.). The resin was washed with 50% aqueous methanol (1.0 liter) and eluted with 80% aqueous acetone (1.0 liter). Evaporation of the eluate yielded a crude hibarimicin complex (1.68 g). This complex (500 mg) was applied onto a column of YMC GEL ODS (40 mm i.d. × 450 mm, ODS-AM 120-S50, YMC Co., Ltd.), and developed with a mixture of methanol-0.15% KH_2PO_4 adjusted to pH 3.5 (70:30). The eluate was fractionated and the fractions were examined by TLC and HPLC. A mixture of hibarimicins G was eluted first, followed by hibarimicins C and D then hibarimicin B, hibarimicin A. The appropriate fractions of the eluate were pooled and concentrated to aqueous solution. Each solution was extracted with ethyl acetate and the extract was evaporated *in vacuo* to give a mixture of hibarimicins C and D (169.0 mg) and semi-pure hibarimicins A, B and G. Each of the hibarimicins A, B and G were rechromatographed on a column of Sephadex LH-20 (22 mm i.d. × 450 mm, Pharmacia Fine Chemicals AB) and eluted with CH_2Cl_2-methanol (1:1) to give pure hibarimicin A (23.0 mg), hibarimicin B (68.0 mg) and hibarimicin G (25.5 mg), respectively. Isolation of hibarimicins C and D from the mixture (169 mg) was achieved by preparative TLC (RP-18 F254S, Art. 15389, E. Merck, Darmstadt) using methanol-water (87.5:12.5) as the developing solvent. Two red bands (hibarimicin C, Rf 0.49; hibarimicin D, Rf 0.43) were scraped off, and each band was

Table 5. Physico-chemical properties of hibarimicins A, B, C, D and G.

	A	B	C	D	G
Nature	Red powder	Red powder	Red powder	Red powder	Red powder
M.P. (°C, dec)	>200	>200	>200	>200	>200
UV λ_{max} nm (ε) in MeOH	239 (43,100)	240 (41,200)	240 (43,900)	244 (38,700)	240 (46,300)
	281 (47,800)	278 (43,800)	279 (46,500)	278 (46,500)	277 (50,100)
	433 (13,200)	432 (13,800)	432 (14,500)	433 (16,000)	431 (15,700)
	511 (11,900)	511 (10,700)	510 (11,400)	509 (10,100)	512 (12,500)
in 0.1 N HCl-MeOH (1:9)	239 (46,900)	239 (44,900)	239 (46,300)	239 (48,300)	239 (49,400)
	281 (49,100)	281 (46,800)	280 (48,500)	281 (50,700)	280 (52,600)
	430 (13,000)	431 (12,300)	432 (12,800)	433 (13,600)	430 (13,300)
	510 (12,100)	511 (11,600)	509 (11,800)	510 (11,300)	513 (13,000)
in 0.1 N NaOH-MeOH (1:9)	235 (36,900)	235 (36,400)	235 (36,000)	236 (37,800)	236 (39,000)
	277 (49,300)	278 (46,900)	278 (47,000)	278 (48,400)	277 (51,800)
	435 (20,200)	436 (19,200)	436 (19,000)	437 (20,700)	437 (21,600)
	613 (9,200)	614 (8,800)	615 (9,100)	613 (8,900)	614 (9,500)
	648 (9,200)	647 (8,800)	647 (9,100)	648 (8,700)	645 (9,400)
IR ν_{max} cm^{-1} (KBr)	3450	3450	3450	3450	3450
	2940	2940	2940	2940	2940
	1700	1705	1700	1700	1700
	1620	1620	1620	1620	1620
	1455	1460	1455	1455	1455
	1410	1405	1405	1405	1405
	1120	1125	1120	1120	1120
	1055	1060	1055	1055	1055
FAB-MS *m/z* positive	1725 $[M]^+$	1725 $[M]^+$	1683 $[M]^+$	1741 $[M]^+$	1757 $[M]^+$
	1748 $[M+Na]^+$	1748 $[M+Na]^+$	1706 $[M+Na]^+$	1764 $[M+Na]^+$	1780 $[M+Na]^+$
Molecular formula	$C_{85}H_{112}O_{37}$	$C_{85}H_{112}O_{37}$	$C_{83}H_{110}O_{36}$	$C_{85}H_{112}O_{38}$	$C_{85}H_{112}O_{39}$
HPLC[a] Rt (min.)	24.5	18.9	13.7	12.7	8.2
TLC[b] Rf	0.19	0.21	0.25	0.30	0.39

[a] Column: Cosmosil 5C18AR (4.6 i.d. × 250 mm).
Mobile phase: CH_3CN-0.15% potassium phosphate buffer (pH 3.5) (1:1).
Flow rate: 0.7 ml/minute. Detection: UV absorption at 254 nm.
[b] Plate: TLC RP-18F_{254s} (Merck, Art 15389). Solvent: CH_3CN-H_2O (55:45).

extracted with CH_2Cl_2-methanol (1:1). Each extract was concentrated and charged on a column of Sephadex LH-20 (20 mm i.d. × 450 mm) and eluted with CH_2Cl_2-methanol (1:1). Evaporation of the eluate gave pure hibarimicin C (42.1 mg) and hibarimicin D (81.7 mg).

Physico-chemical Properties

The physico-chemical properties of hibarimicins A, B, C, D and G are described in Table 5. The ultraviolet absorption spectrum of hibarimicin A is shown in Fig. 2. They were soluble in dimethylsulfoxide, methanol, acetonitrile, acetone and dichloromethane, but insoluble in hexane and water. The molecular formulae of hibarimicins A, B, C, D and G were assigned to be $C_{85}H_{112}O_{37}$, $C_{85}H_{112}O_{37}$, $C_{83}H_{110}O_{36}$, $C_{85}H_{112}O_{38}$, and $C_{85}H_{112}O_{39}$, respectively, based on their FAB-MS and NMR spectra. The physico-chemical properties of hibarimicins B and D were identical to those of angelmicins B and A[17].

Biological Properties

As previously described[14], the protein kinase assay system that we employed allowed detection of protein kinase A (PKA), protein kinase C (PKC), protein tyrosine kinase (PTK) and calmodulin-dependent protein kinase III (CAMKIII) activities in one single assay. As shown in Fig. 3-A, hibarimicins A, B, C and D specifically inhibited the PTK activity, while they had little effect on PKA or PKC activity. The inhibition of PTK was even more evident on the alkali-treated gel (Fig. 3-B). IC_{50} values inhibiting PTK activity were within a range of 10~40 μg/ml in which hibarimicin A appeared to be most potent among the hibarimicins (Fig. 4). It was noted that they also inhibited CAMKIII activity. Several other tyrosine kinase inhibitors such as herbimycin A and tyrphostins also showed a similar effect on CAMKIII[15].

Fig. 2. UV spectra of hibarimicin A (25 μg/ml).

—— MeOH, – – –, 0.1 N HCl - MeOH (1 : 9), — — — 0.1 N NaOH - MeOH (1 : 9).

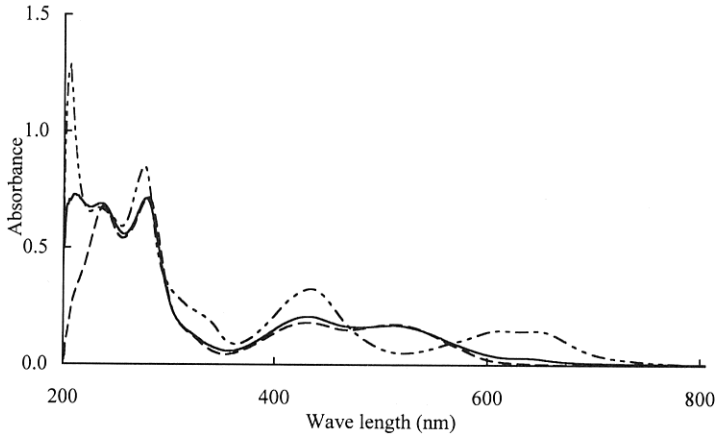
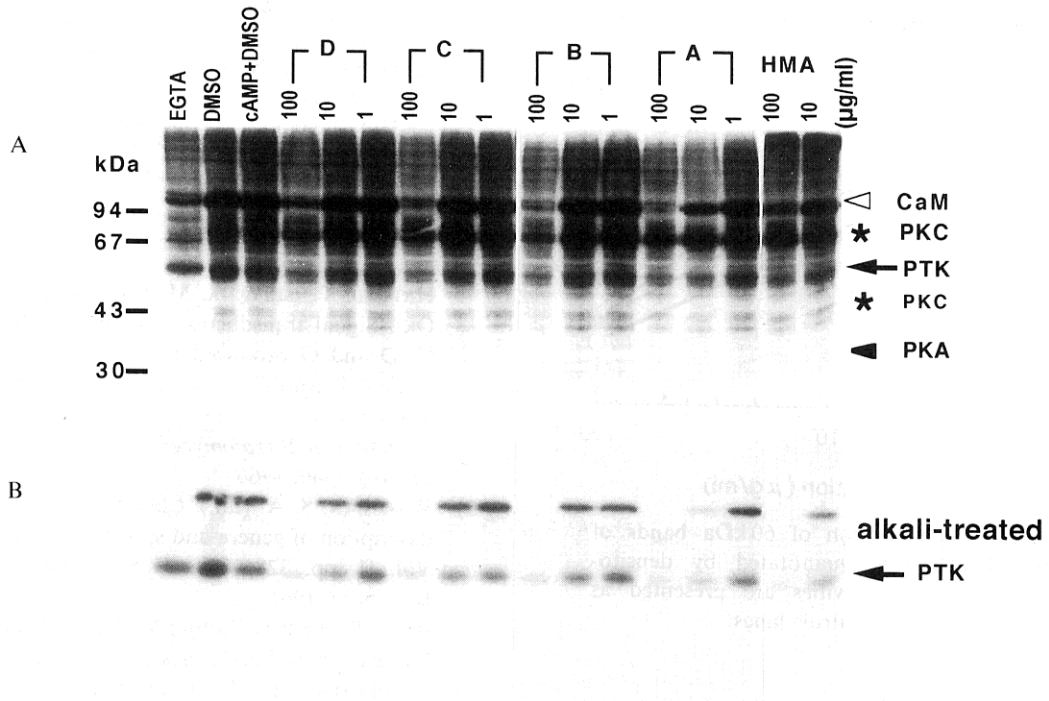


Fig. 3. Effect of hibarimicins A, B, C and D on multiple protein kinase activities.



Protein kinase reaction was performed as described in Materials and Methods in the presence of indicated additions. The final concentrations were: EGTA, 0.5 mM; cAMP, 20 μM; and DMSO, 10%. The phosphorylated proteins were analyzed using SDS-PAGE (9% gel) and visualized by autoradiography. Shown are results before (A) and after (B) 1 N KOH treatment at 55°C for 2 hours to enrich phosphotyrosine. Arrowheads, stars, arrows, and open triangles represent PKA, PKC, PTK and CAMKIII activities, respectively. Positions and sizes of markers are shown at left. Herbimycin A (HMA) was used as a positive control.

Thus, hibarimicins are specific inhibitors of PTK activity. However, further studies both *in vitro* and *in vivo* are required to distinguish the characteristics of hibarimicins from other PTK inhibitors.

In vitro antimicrobial activities of hibarimicins A, B, C and D are shown in Table 6. Each component showed moderate inhibitory activity against Gram-positive bacteria with MICs of 0.8 ~ 12.5 μg/ml.

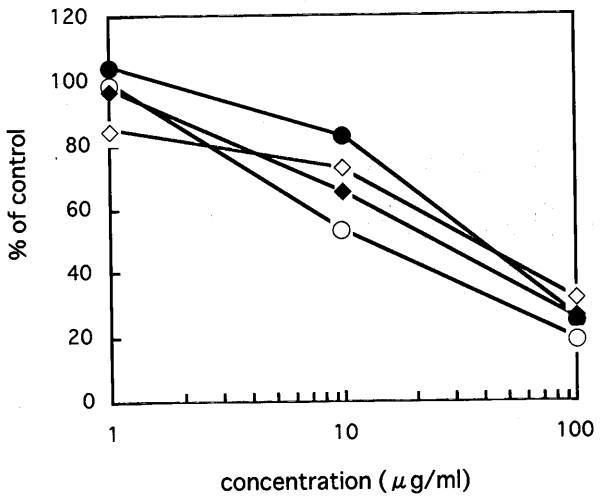
Hibarimicins A, B, C and D were tested for their *in vitro* cytotoxicity against B16-F10 (murine melanoma)

Table 6. *In vitro* antimicrobial activity of hibarimicins A, B, C and D.

Test organism	Minimum inhibitory concentration (μg/ml)			
	A	B	C	D
Staphylococcus aureus FDA 209P JC-1	0.8	0.8	1.6	0.8
Staphylococcus aureus Smith	12.5	12.5	12.5	6.3
Micrococcus luteus ATCC 9341	3.2	1.6	1.6	1.6
Bacillus subtilis ATCC 6633	3.2	3.2	3.2	3.2
Escherichia coli NIHJ JC-2	>100	>100	>100	>100
Escherichia coli K12	>100	>100	>100	>100
Klebsiella pneumoniae ATCC 10031	>100	>100	>100	>100
Citrobacter freundii GN7391	>100	>100	>100	>100
Salmonella typhi 901	>100	>100	>100	>100
Pseudomonas aeruginosa A9843A	>100	>100	>100	>100

Fig. 4. Effect of hibarimicins A, B, C and D on *src* tyrosine kinase activity.

○ A, ● B, ◇ C, ◆ D.



Tyrosine phosphorylation of 60 kDa bands of alkali-treated gels was quantitated by densitographycally and the activities are presented as percentages of those in controls lanes.

and HCT-116 (human colon carcinoma) cells. They showed potent cytotoxicity as shown in Table 7.

Table 7. *In vitro* cytotoxicity.

	IC_{50} (μg/ml)	
	B16-F10	HCT-116
Hibarimicin A	1.2	2.5
Hibarimicin B	0.7	1.9
Hibarimicin C	0.8	2.7
Hibarimicin D	2.0	3.6

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