NOVEL CYTOCIDAL ANTIBIOTICS, GLUCOPIERICIDINOLS A_1 AND A_2

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURE ELUCIDATION AND BIOLOGICAL CHARACTERISTICS

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(Received for publication June 23, 1989)

Two novel antibiotics, glucopiericidinols A_1 (**1**) and A_2 (**2**) were isolated from the cultured broth of *Streptomyces* sp. OM-5689. The structures of these two compounds were deduced employing spectroscopic analyses. These antibiotics showed potent cytocidal activities against HeLa S_3 cells *in vitro* (MIC **1**: 0.39 μg/ml, **2**: 0.10 μg/ml) when the cells were exposed to the antibiotics for 3 days. Although **1** and **2** showed no activity at 1,000 μg/ml against various Gram-positive and Gram-negative bacteria, yeast or fungi, they did have inhibitory activity against *Piricularia oryzae* (MIC of **1**: 125 μg/ml, of **2**: 31 μg/ml).

In the course of a screening program for novel antibiotics showing cytocidal activities against HeLa S_3 cells *in vitro*, a fraction of fermentation broth of *Streptomyces* sp. OM-5689, isolated from a soil sample collected in Shizuoka Prefecture, Japan showed potent cytocidal activity. Two active components designated as glucopiericidinols A_1 (**1**) and A_2 (**2**) were obtained from the cultured broth of this microorganism.

The present paper deals with the taxonomic studies of the producing strain, and the production, isolation and structure elucidation of the new antibiotics. The biological activities of glucopiericidinols against HeLa S_3 cells and *Piricularia oryzae* are also presented.

Materials and Methods

General Experimental Procedures

UV spectra were recorded on a Shimadzu model UV-200S spectrophotometer and IR spectra on a Jasco model A-102 interferometer. MS were obtained with a Jeol model DX-300 mass spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Varian XL-400 instrument. Kieselgel 60 (Merck) and Sephadex LH-20 (Pharmacia Fine Chemicals) were used for column chromatography and DC-Fertigplatten Kieselgel 60 (Merck) was used for TLC analysis and for preparative TLC. TRI Rotar-V (Jasco) and Uvidec-100-V (Jasco) instruments were used for HPLC.

Taxonomic Studies

Type of diaminopimelic acid (DAP) was determined by the method of HASEGAWA *et al.*[1].

To investigate the cultural and physiological characteristics, the International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[2] and those recommended by WAKSMAN[3] were used. Cultures were observed after incubation at 27°C for 2 weeks. Color names and hue numbers indicated in Table 1 are those of Color Harmony Manual (4th Ed.)[4]. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[5] containing 1% carbon source at 27°C.

Anti HeLa S_3 Tests

HeLa S_3 cells were maintained in monolayers in EAGLE's minimum essential medium (MEM)

supplemented with 10% bovine serum and an antibiotic (60 μg/ml of kanamycin) at 37°C.

To determine the cytotoxicity of the test materials, HeLa S_3 cells (5×10^4) in 2 ml of medium were plated in a 30-mm Petri dish and incubated for 48 hours at 37°C in a 5% CO_2 - 95% air atmosphere. Each culture dish was filled with fresh medium containing a different concentration of the antibiotic. After the incubation for 72 hours, the HeLa S_3 cells were trypsinized to form a single cell suspension, and cells were counted in hemocytometer.

Antimicrobial Activity Test

The antimicrobial activity of glucopiericidinols A_1 (**1**) and A_2 (**2**) was determined using 6 mm paper discs (Toyo Seisakusho Co., Ltd.) and Mueller-Hinton agar medium (Difco) for bacteria and potato broth agar medium for fungi or yeasts. Antimicrobial activity was observed after 24 hours incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

Results

Taxonomy of the Producing Strain OM-5689

The vegetative mycelia grow abundantly on both synthetic and complex agar media, and do not show fragmentation into coccoid or bacillary elements. The aerial mycelia grow abundantly on yeast extract - malt extract agar, oatmeal agar, inorganic salts - starch agar, glucose - asparagine agar and glycerol - asparagine agar. The mature sporophores were of the *Rectiflexibilis* type and had more than 20 spores per chain. The spores were cylindrical in shape, 1.6×0.7 μm in size and had a smooth surface (Fig. 1). Sclerotic granules, sporangia and flagellated spores were not observed.

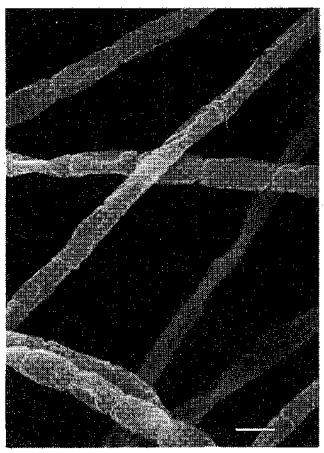
The cultural and physiological properties, and the utilization of carbon sources of OM-5689 are shown in Tables 1, 2 and 3, respectively.

The strain exhibits the following properties. Sporophore, *Rectiflexibilis*; spores, cylindrical and smooth surface; color of vegetative mycelia, ivory; color of aerial mycelia, gray or white; soluble pigment, not produced; DAP isomer in cell wall, LL-type.

Based on the taxonomic properties described above, strain OM-5689 is considered to belong to the genus *Streptomyces*; and to be a strain of the gray series of the PRIDHAM and TRESNER's system[6]. The strain was deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. OM-5689 and the accession No. of FERM P-10617.

Fig. 1. Scanning electron micrograph of spore chains of *Streptomyces* sp. OM-5689 grown on tyrosine agar for 14 days.

Bar represents 1.0 μm.



Fermentation and Isolation of the Active Components

A stock culture of the producing organism was inoculated into a 500-ml Sakaguchi flask containing 100 ml seed medium consisting of glucose 2.0%, meat extract 0.5%, peptone 0.5%, dry yeast 0.3%, NaCl 0.5% and $CaCO_3$ 0.3% (pH 7.0 before sterilization). The flasks were incubated at 27°C for 72 hours on a reciprocal shaker. Then 400 ml of the resulting culture was transferred to a 30-liter

Table 1. Cultural characteristics of strain OM-5689.

Medium	Cultural characteristics	Medium	Cultural characteristics
Yeast extract - malt extract agar[a]	G: Good, dull gold (2ng) R: Mustard brown (3ni) AM: Good, ashes (5fe) SP: None	Tyrosine agar[a]	G: Good, mustard tan (2lg) R: Covert tan (2nl) AM: Good, oyster white (b) SP: None
Oatmeal agar[a]	G: Good, putty (1dc) R: Pearl (3ba) AM: Good, silver gray (3fe) SP: None	Sucrose - nitrate agar[a]	G: Good, pearl (2ba) R: Ivory tint (2cb) AM: Moderate, natural (2dc) SP: None
Inorganic salts - starch agar[a]	G: Good, mustard gold (2ne) R: Nude tan (4ge) AM: Good, silver gray (3fe) SP: None	Glucose - nitrate agar[a]	G: Moderate, light ivory (2ca) R: Parchment (1cb) AM: Very poor, light mustard tan (2ie) SP: None
Glycerol - asparagine agar	G: Good, mustard (2le) R: Light brown (3lg) AM: Good, white - ivory tint (a - 2eb) SP: None	Glycerol - calcium malate agar[b]	G: Good, yellow maple (3le) R: Camel (3ie) Am: Moderate, white (a) SP: None
Glucose - asparagine agar	G: Good, ivory (2db) R: Mustard (2le) AM: Good, white - silver gray (a - 3fe) SP: None	Glucose - peptone agar[b]	G: Good, yellow maple (3le) R: Yellow maple (3ng) AM: Good, white - downpink (a - 7dc) SP: None
Peptone - yeast extract - iron agar[a]	G: Moderate, cream (1 1/2ca) R: Cream (1 1/2ca) AM: None SP: None	Nutrient agar[b]	G: Moderate, cream (1 1/2ca) R: Cream (1 1/2ca) AM: None SP: None

[a] Medium recommended by ISP.
[b] Medium recommended by S. A. WAKSMAN.
Abbreviations: G, growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.

Table 2. Physiological properties of strain OM-5689.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Liquefaction of gelatin (21~22°C)	+
Peptonization of milk (37°C)	−
Coagulation of milk (37°C)	−
Cellulolytic activity	−
Hydrolysis of starch	+
Temperature range for growth	15~36°C

+: Active, −: inactive.

Table 3. Utilization of carbon sources by strain OM-5689.

Utilized:	D-Glucose, D-fructose, D-mannitol, L-arabinose, D-xylose, sucrose
Weakly utilized:	L-Rhamnose
Not utilized:	*i*-Inositol, raffinose, melibiose

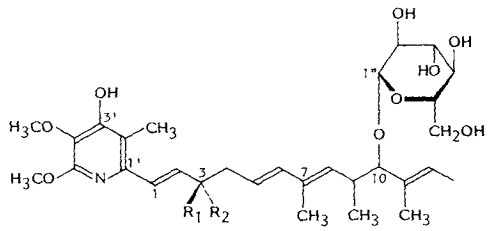
fermenter containing 20 liters of the same medium as above. The fermentation was carried out at 27°C for 96 hours using an agitation rate of 160 rpm and an aeration rate of 60 liters/minute.

The whole fermentation broth of *Streptomyces* sp. OM-5689 (20 liters) was extracted with EtOAc (18 liters) and the EtOAc layer was concentrated *in vacuo* to about 1 liter, washed with H_2O (0.5 liter) and dried over Na_2SO_4 (anhydrous). Concentration of the EtOAc layer resulted in a brown oil (3.3 g).

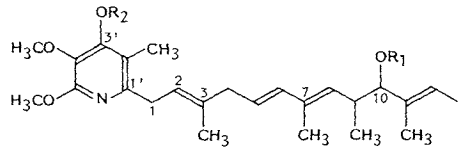
The brown oil was chromatographed over Silica gel 60 (Merck, 5.5 × 20 cm) using $CHCl_3$ - MeOH as solvent. Fractions exhibiting cytocidal activity against HeLa S_3 cells were collected and the active fractions (1.3 g) were rechromatographed over Sephadex LH-20 (3.0 × 40 cm). Elution with MeOH gave a mixture

Table 4. Physico-chemical properties of glucopiericidinols A_1 (**1**) and A_2 (**2**).

	1	2
Appearance	Yellow oil	Yellow oil
TLC (silica gel)		
$CHCl_3$-MeOH (9:1)	0.25	0.25
BuOH-AcOH-H_2O (4:1:2)	0.79	0.79
$[\alpha]_D^{18}$	+38° (*c* 0.08, $CHCl_3$)	−20° (*c* 0.06, $CHCl_3$)
Molecular formula	$C_{31}H_{47}NO_{10}$	$C_{31}H_{47}NO_{10}$
MW	593	593
UV λ_{max}^{MeOH} nm	222, 241 (sh), 296	216, 238 (sh), 290
IR ν_{max} (smear) cm^{-1}	3380, 1570, 1460, 1410, 1125	3380, 1570, 1460, 1410, 1125
Color reaction		
Positive	Iodine, 50% $H_2SO_4+\Delta$, phosphomolybdic acid, DRAGENDORFF's reagent	Iodine, 50% $H_2SO_4+\Delta$, phosphomolybdic acid, DRAGENDORFF's reagent
Negative	Ninhydrin reagent, $FeCl_3$	Ninhydrin reagent, $FeCl_3$



1, **2** R_1, R_2 = CH_3, OH

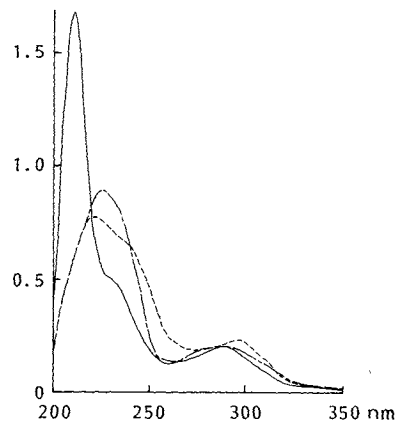


3 R_1 = Glu R_2 = H
4 R_1 = H R_2 = Glu

of glucopiericidinols A_1 (**1**) and A_2 (**2**) and glucopiericidin A (**3**). The mixture was further separated by a silica gel column (3.3 × 30 cm) eluted with $CHCl_3$-MeOH (9:1) to afford a mixture of **1** and **2** (25 mg). Final purification of the mixture was by HPLC (YMC D-ODS-5, Yamamura Chemical Labs., 2.2 × 27 cm, eluant: CH_3CN-H_2O-AcOH (40:60:0.1), flow rate: 9.0 ml/minute, detection: UV at 254 nm) to give glucopiericidinol A_1 (**1**, 3.3 mg, retention time (t_R) 57.2 minutes) and glucopiericidinol A_2 (**2**, 2.6 mg, t_R 65.1 minutes).

Fig. 2. UV spectrum of glucopiericidinol A_1 (**1**).

---- MeOH, ——— acidic MeOH, —— basic MeOH.



Physico-chemical Properties of Glucopiericidinols A_1 (**1**) and A_2 (**2**)

Physico-chemical properties of **1** and **2** are summarized in Table 4. UV absorption spectrum of **1** is shown in Fig. 2. Compounds **1** and **2** gave positive color reaction with iodine, 50% sulfuric acid, phosphomolybdic acid and DRAGENDORFF's reagent and were negative to ninhydrin and $FeCl_3$.

Structure Elucidation of Glucopiericidinols A_1 (**1**) and A_2 (**2**)

Physico-chemical properties of glucopiericidinols A_1 and A_2 are similar to those of piericidins[7~10]

Table 5. ^{1}H NMR spectra of glucopiericidinols A_1 (**1**) and A_2 (**2**) and glucopiericidin A (**3**) in $CDCl_3$: δ (*J*/Hz).

Position	1	2	3
1	6.87 d (15)	6.83 d (15)	3.36 d (7) (2H)
2	6.77 d (15)	6.73 d (15)	5.73 m
4	2.44 m (2H)	2.35 dd (13, 9.5)	2.77 d (7) (2H)
		2.54 dd (13, 6)	
5	5.67 m	5.61 m	5.61 dt (15.5, 7)
6	6.20 d (15.5)	6.20 d (15.5)	6.05 d (15.5)
8	5.31 d (10)	5.31 d (10)	5.23 d (9.5)
9	2.79 m	2.80 m	2.77 m
10	3.42 m[a]	3.41 m[a]	3.45 d (4)
12	5.43 m	5.43 m	5.40 m
13	1.63 d (6)	1.63 d (6)	1.62 d (6)
14 (11-CH_3)	1.62 s	1.62 s	1.61 s
15 (9-CH_3)	0.77 d (7)	0.77 d (7)	0.75 d (7)
16 (7-CH_3)	1.81 br s	1.77 br s	1.78 br s
17 (3-CH_3)	1.40 s	1.41 s	1.73 br s
6′ (2′-CH_3)	2.18 s	2.18 s	2.08 s
7′ (4′-OCH_3)	3.88 s	3.88 s	3.85 s
8′ (5′-OCH_3)	4.00 s	4.00 s	3.94 s
1″	4.14 d (8)	4.15 d (8)	4.14 d (8)
2″	3.22 t (8)	3.22 t (8)	3.23 t (8)
3″	3.42 m[a]	3.41 m[a]	3.48 t (8)
4″	3.42 m[a]	3.41 m[a]	3.42 m
5″	3.29 m	3.30 m	3.28 m
6″	3.64 m	3.64 m	3.64 dd (12, 6)
	3.81 m	3.81 m	3.81 br d (12)

[a] Signals overlapped.

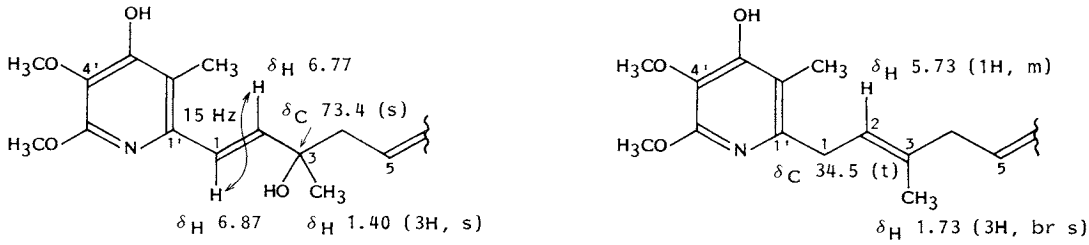
Table 6. ^{13}C NMR spectra of glucopiericidinols A_1 (**1**) and A_2 (**2**)[a] and glucopiericidin A (**3**)[11] in $CDCl_3$: δ.

Position	1	2	3	Position	1	2	3
1	140.1 d	140.0 d	34.5 t	17 (3-CH_3)	28.4 q	27.7 q	16.6 q
2	140.4 d	140.5 d	122.3 d	1′	144.3 s	144.3 s	150.8 s
3	73.4 s	73.1 s	134.7 s	2′	112.8 s	112.9 s	112.3 s
4	46.2 t	36.1 t	43.0 t	3′	153.7 s	153.6 s	154.2 s
5	123.7 d	123.6 d	126.7 d	4′	121.8 s	122.0 s	128.0 s
6	138.8 d	138.8 d	135.7 d	5′	154.1 s	154.1 s	153.6 s
7	135.3 s	135.2 s	134.4 s	6′ (2′-CH_3)	10.1 q	10.1 q	10.5 q
8	136.3 d	136.1 d	134.4 d	7′ (4′-OCH_3)	60.7 q	60.7 q	60.5 q
9	35.3 d	35.2 d	35.3 d	8′ (5′-OCH_3)	53.0 q	53.0 q	53.1 q
10	94.7 d	94.7 d	94.2 d	1″	104.8 d	103.9 d	103.7 d
11	134.5 s	134.4 s	135.4 s	2″	74.6 d	74.5 d	74.4 d
12	123.2 d	123.1 d	123.3 d	3″	76.2 d	76.2 d	76.4 d
13	13.1 q	13.1 q	13.2 q	4″	70.8 d	70.8 d	70.8 d
14 (11-CH_3)	10.9 q	10.9 q	11.1 q	5″	75.4 d	75.3 d	75.5 d
15 (9-CH_3)	16.6 q	16.7 q	17.0 q	6″	62.9 t	62.8 t	62.5 t
16 (7-CH_3)	13.4 q	13.4 q	13.0 q				

[a] Assignments were based on comparison with the literature[10,11].

and glucopiericidins[11]. In combination with the ^{1}H and ^{13}C NMR data (Tables 5 and 6), glucopiericidinols A_1 and A_2 were estimated to have the same molecular formula, $C_{31}H_{47}NO_{10}$ (MW 593), which was supported by fast atom bombardment (FAB)-MS (*m/z* 594 $(M+H)^+$) and high-resolution electron impact (HREI)-MS (*m/z* 593.3210, $\Delta+1.3$ mmu) using the mixture of **1** and **2**. Interestingly, after HPLC separation, a clear (*quasi-*)molecular ion peak for **1** and **2** could not be observed by FAB or EI-MS. The

Fig. 3. Partial structures of glucopiericidinol A_1 (**1**) and glucopiericidin A (**3**).



phenomenon was previously reported that the piericidin A group showed only a trace of molecular ion peak in the EI-MS[9]. The molecular formula suggested that glucopiericidinols A_1 and A_2 possess one more oxygen atom than glucopiericidins A (**3**) and B (**4**)[11].

The ^1H and ^{13}C NMR spectra of **1** and **2** (Tables 5 and 6) were quite similar to those of **3** and **4**. Through the comparison of the ^{13}C NMR data at C-10 position (**1** and **2**: δ 94.7, **3**: δ 94.2, and **4**: δ 82.8), the position of the sugar moiety was concluded to be the same with **3** and the structural differences of **1** and **2** from **3** were found for the signals of C-1 ~ C-3 part (Fig. 3). The signal for a methylene carbon due to C-1 (δ 34.5 (t)) observed in the ^{13}C NMR of **3** was absent in that of **1** or **2**, while a signal for an oxygenated quaternary carbon was observed (δ 73.4 (s) for **1**; δ 73.1 (s) for **2**). The ^1H NMR of **1** or **2** showed a characteristic AB quartet in the sp^2 region (δ 6.77 and 6.87 (J = 15 Hz) for **1**; δ 6.73 and 6.83 (J = 15 Hz) for **2**), those signals were not observed in the ^1H NMR of **3**. These NMR data along with the consideration of the presence of one additional oxygen atom suggested that **1** and **2** possess a methyl and a hydroxyl groups at C-3 and a disubstituted double bond at C-1 and C-2. UV absorption spectra of **1** (λ_{max}^{MeOH} nm 222, 241 (sh), 296) and **2** (λ_{max}^{MeOH} nm 216, 238 (sh), 290) showed little similarity to that of **3** (λ_{max}^{MeOH} nm 203, 233, 237, 267)[11] probably due to the extension of the conjugation of the pyridine chromophore with the Δ_1-double bond. The coupling constant of these two signals ($J_{1,2}$ = 15 Hz) indicated 1*E*-configuration for each of **1** and **2**.

Since all spectral data (UV, IR, ^1H and ^{13}C NMR) of **1** and **2** were quite similar to each other, **1** and **2** were deduced to be stereoisomers at C-3 position. The hypothesis is also verified by the polarimetric analysis data of these compounds (**1**: $[\alpha]_D^{18}$ +38° (*c* 0.08, $CHCl_3$), **2**: $[\alpha]_D^{18}$ −20° (*c* 0.06, $CHCl_3$)). From all of the observations described above, the structures of glucopiericidinols A_1 and A_2 were concluded to be **1** and **2**. The absolute stereochemistry at C-3 of **1** and **2** remains to be defined.

Biological Activity Tests of Glucopiericidinols A_1 (**1**) and A_2 (**2**)

Glucopiericidinols A_1 (**1**) and A_2 (**2**) showed no antimicrobial activities at the concentration of 1,000 μg/ml against *Bacillus subtilis* KB27 (PCI 219), *Staphylococcus aureus* KB34 (FDA 209P), *Micrococcus luteus* KB40 (PCI 1001), *Mycobacterium smegmatis* KB42 (ATCC 607), *Escherichia coli* KB8 (NIHJ), *E. coli* KB176 (NIHJ JC-2), *Pseudomonas aeruginosa* KB105 (P3), *Xanthomonas oryzae* KB88, *Bacteroides fragilis* KB169, *Acholeplasma laidlawii* PG8 KB174, *Aspergillus niger* KF103 (ATCC 6275),

Table 7. Biological activities of glucopiericidinols A_1 (**1**) and A_2 (**2**) and glucopiericidin A (**3**).

	MIC (μg/ml)		
	1	**2**	**3**
Anti-HeLa S_3 activities	0.39	0.10	0.25
Antimicrobial activities against *Piricularia oryzae*	125	31	31

Mucor racemosus KF223 (IFO 4581), *Candida albicans* KF1 and *Saccharomyces sake* KF26.

Anti-HeLa S_3 activity and inhibitory activity against *P. oryzae* KF180 of the antibiotics are shown in Table 7. These activities of glucopiericidinol A_2 (**2**) were slightly stronger than those of glucopiericidinol A_1 (**1**) and almost the same as those of glucopiericidin A (**3**).

Discussion

Two novel antibiotics, glucopiericidinols A_1 (**1**) and A_2 (**2**) were isolated from the cultured broth of *Streptomyces* sp. OM-5689.

Structures of glucopiericidinols A_1 and A_2 have been studied employing spectroscopic analyses and structures **1** and **2** are presented for these novel antibiotics.

Several piericidin antibiotics, piericidins A[7] and B[8], $A_1 \sim A_4$, $B_1 \sim B_4$, $C_1 \sim C_4$ and $D_1 \sim D_4$[9,10] and glucopiericidins A and B[11] have been isolated. All of these piericidin antibiotics formerly isolated possess a double bond between C-2 and C-3 position and have similar UV absorption spectra to each other. However, glucopiericidinols A_1 (**1**) and A_2 (**2**) possess a double bond conjugated to the pyridine ring namely between C-1 and C-2 and have quite a different UV spectral pattern compared to those of formerly isolated piericidins. In addition, it was reported that glucopiericidin A showed inhibitory activity against the growth of Gram-positive bacteria[11] whereas glucopiericidinols A_1 (**1**) and A_2 (**2**) do not.

We are now investigating the biological activities of glucopiericidinols A_1 (**1**) and A_2 (**2**) further and the results will be reported elsewhere.

Acknowledgment

This work was supported in part by Grants-in-Aid from the Ministry of Health and Welfare, and the Ministry of Education, Science and Culture, Japan, and by funds from Japan Keirin Association.

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