NEW PIERICIDIN GLUCOSIDES, GLUCOPIERICIDINS A AND B

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The new piericidin group antibiotics, glucopiericidins A and B were isolated from the culture broth of *Streptomyces pactum* S48727 (FERM P-8117) as co-metabolite of piericidin A_1.

The structures of glucopiericidins A and B were determined as piericidin A_1, 10-*O*-β-D-glucoside and piericidin A_1, 3'-*O*-D-glucoside on the basis of their spectral and chemical properties, respectively.

Glucopiericidins were more potent in inhibiting antibody formation than piericidin A_1 *in vitro*. In addition, these substances showed better antimicrobial activities than piericidin A_1. Acute toxicities of these substances in mice were lower than that of piericidin A_1.

This indicates that D-glucose in glucopiericidin molecules is important in modulating their physiological activities.

In the course of a screening for physiologically active substances, a strain of actinomycetes, S48727, was shown to produce new piericidin glucosides, glucopiericidins A and B in addition to the known antibiotic piericidin A_1[1~3].

They showed antimicrobial activity and *in vitro* inhibitory activity against antibody formation.

This paper reports the taxomony of the producing organism and the fermentation, the isolation, structures and biological properties of glucopiericidins A and B.

Taxonomy

Strain S48727 was isolated from a soil sample collected at Futaba-gun, Fukushima Prefecture, Japan.

The organism was identified as a strain of *Streptomyces pactum*[4,5].

It has the fundamental characteristics of the organism, namely, mature aerial mass color is in the gray color series on most media. No characteristic color is observed in reverse side of colony commonly used in taxonomic studies[6] (Table 1).

The sporophores are Spiral type and have more than ten spores per chain. The spores with hairy surface are cylindical or oval, $0.5 \sim 0.8 \times 0.7 \sim 1.4$ μm. Whole cell hydrolysates of strain S48727 showed that it contained LL-diaminopimelic acid.

Melanoid pigment are not formed in peptone - yeast extract - iron agar, tyrosine agar and Tryptone - yeast extract broth (Table 2). No soluble pigment is produced. D-Glucose and raffinose are utilized for growth (Table 3).

This strain also produced piericidin A_1, but not other piericidins such as piericidins B_1 and C_1.

Fermentation

A loopful of strain S48727 on agar slant was inoculated into a 500-ml Sakaguchi flask containing

Table 1. Cultural characteristics of strain S48727.

Medium	Aerial mycelium	Reverse side of color	Soluble pigment
Sucrose - nitrate agar	Light brownish gray	Colorless	None
Glucose - asparagine agar	Light gray	Pale yellow	None
Glycerol - asparagine agar	White	Pale yellow	None
Inorganic salts - starch agar	Light brownish gray to light gray	Light yellowish brown	None
Tyrosine agar	Light brownish gray	Yellowish brown	None
Nutrient agar	None	Light yellowish brown	None
Yeast extract - malt extract agar	White to light brownish gray	Yellowish brown	None
Oatmeal agar	Light brownish gray	Pale yellow to bright greenish yellow	None
Glycerol - nitrate agar	Light brownish gray	Pale yellow	None
Calcium - malate agar	Poor	Colorless	None

Table 2. Physiological properties of strain S48727.

Temperature range for growth	18~37°C
Optimum temperature	26~34°C
Starch hydrolysis	Positive
Gelatin liquefaction	Positive
Milk peptonization	Positive
Milk coagulation	Positive
Melanin production	Negative
Nitrate reduction	Negative

Table 3. Carbon sources utilization of strain S48727.

L-Arabinose	−
D-Xylose	−
D-Glucose	+
D-Fructose	−
Sucrose	−
Inositol	−
L-Rhamnose	−
Raffinose	+
D-Mannitol	−
D-Mannose	−
Salicin	±

+: Utilized, ±: weakly utilized, −: not utilized.

100 ml of seed medium (Table 4).

The seed culture was incubated at 28°C for 48 hours on reciprocal shaker with 8 cm-throw at 120 rpm, and 2 ml of the growth was transferred to 500-ml Sakaguchi flask containing 100 ml of production medium (Table 4). The fermentation was carried out for 70~100 hours under the same conditions described above.

A typical time course and HPLC profile of the fermentation are shown in Figs. 1 and 2, respectively.

Table 4. Media used for production of glucopiericidins A and B.

Seed medium (%)		Production medium (%)	
Glucose	1	Glucose	2
Soluble starch	1	Proteose peptone	1
Polypeptone	0.5	$CuSO_4 \cdot 5H_2O$	0.0007
Meat extract	0.5	$FeSO_4 \cdot 7H_2O$	0.0001
Yeast extract	0.3	$ZnSO_4 \cdot 7H_2O$	0.0002
NaCl	0.3	$MnSO_4 \cdot 4H_2O$	0.0008
$MgSO_4 \cdot 7H_2O$	0.1	adjust to pH 7.0	
$CaCO_3$	0.3		
$CuSO_4 \cdot 5H_2O$	0.0007		
$FeSO_4 \cdot 7H_2O$	0.0001		
$ZnSO_4 \cdot 7H_2O$	0.0002		
$MnSO_4 \cdot 4H_2O$	0.0008		
adjust to pH 7.0			

Fig. 1. Time course of fermentation of *Streptomyces pactum* S48727.
Glucose (□), potency (glucopiericidin A: ○, piericidin A_1: ●), packed cell volume (▲), pH (■).

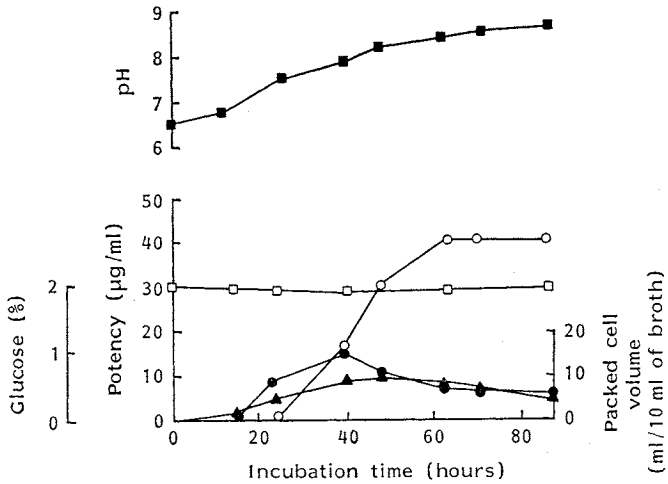
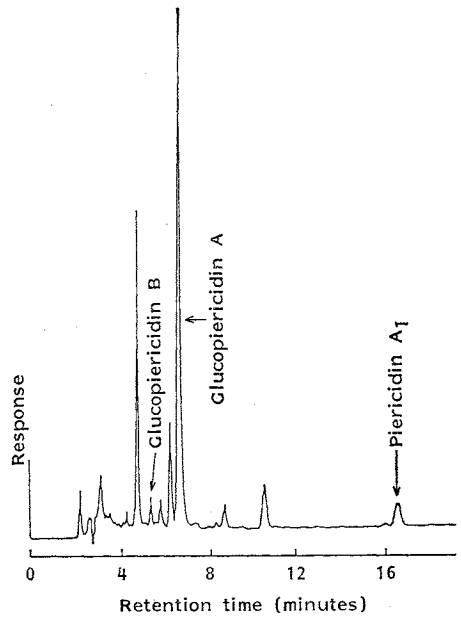


Fig. 2. HPLC profile of fermentation broth.
Column: Nucleosil 5C18, 4.6×250 mm, solvent: CH_3CN - H_2O - AcOH (70 : 30 : 1), detection: *A* 240 nm, flow rate: 1.0 ml/minute, chart speed: 5 mm/minute.



Though glucose is only carbon source, it is hardly consumed, and mycelium increased slightly. Glucopiericidin A is produced after piericidin A_1 as shown in Fig. 1. This indicates that piericidin A_1 has been converted to glucopiericidin A by glucosylation.

The sample for HPLC was prepared after 96 hours incubation by ethyl acetate extract. Glucopiericidin A is a main product, whereas piericidin A_1 and glucopiericidin B are minor products as shown in Fig. 2.

Isolation

Most of the antibiotics was found in the broth filtrate extract.

After fermentation was completed, the culture broth (10 liters) was centrifuged. The collected cake was extracted twice with 2 liters of methanol. The methanol extract was concentrated *in vacuo* and remaining aqueous solution was extracted twice with 2 liters of ethyl acetate. The broth filtrate was extracted twice with 15 liters of ethyl acetate.

The ethyl acetate extracts from cake and filtrate were combined and concentrated *in vacuo* to an oily residue. After washing with *n*-hexane, the oily residue was chromatographed on a silica gel column (3.0×45 cm) with chloroform - methanol (98: 2, 96: 4).

Piericidin A_1 fractions eluted before glucopiericidins A and B.

Glucopiericidin A fractions were concentrated *in vacuo* to dryness, dissolved in a small amount

Table 5. Physico-chemical properties of glucopiericidins A and B.

	Glucopiericidin A	Glucopiericidin B
Appearance	Amorphous powder	Amorphous powder
Nature	Weakly acidic	Neutral
$[\alpha]_D^{20}$	$-26.4°$ (c 1.0, $CHCl_3$)	$-10.0°$ (c 0.9, MeOH)
FAB-MS $(M+H)^+$	578	578
Elemental analysis (%)		
Found:	C 64.49, H 8.23, N 2.43	C 64.21, H 8.34, N 2.51
Calcd:	C 64.45, H 8.20, N 2.42	C 64.45, H 8.20, N 2.42
Molecular formula	$C_{31}H_{47}O_9N$	$C_{31}H_{47}O_9N$
UV λ_{max}^{MeOH} nm (ε)	203 (45,400), 233 (40,000), 237 (40,800), 267 (6,100)	203 (39,700), 233 (40,050), 237 (40,200), 275 (8,300)
IR ν_{max}^{KBr} cm^{-1}	3400, 2950, 1585, 1470, 1415, 1125	3400, 2950, 1585, 1470, 1405, 1070
Product by acid hydrolysis	D-Glucose	D-Glucose

FAB-MS: Fast atom bombardment mass spectra.

Fig. 3. ^1H NMR spectra of glucopiericidins A and B (90 MHz in $CDCl_3$).

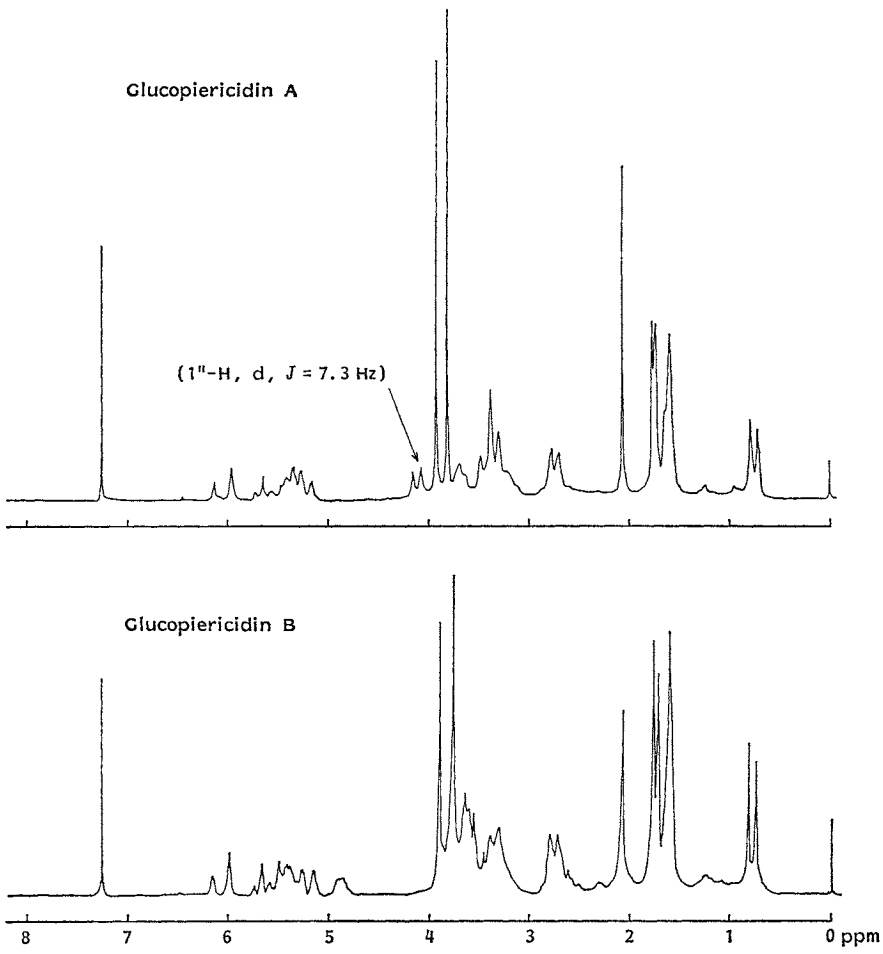


Table 6. Assignments of ^{13}C NMR spectra of piericidin A_1, glucopiericidins A and B (22.5 MHz in $CDCl_3$).

	Piericidin A_1	Gluco-piericidin A	Gluco-piericidin B		Piericidin A_1	Gluco-piericidin A	Gluco-piericidin B
C-1	34.5 t	34.5 t	34.5 t	C-17	13.1 q	13.0 q*	13.0 q*
C-2	122.4 d	122.3 d	122.0 d	C-1′	150.8 s	150.8 s	151.2 s
C-3	134.7 s	134.7 s	135.8 s	C-2′	112.2 s	112.3 s	118.6 s
C-4	43.2 t	43.0 t	43.1 t	C-3′	154.3 s	154.2 s	155.3 s
C-5	126.7 d	126.7 d	126.5 d	C-4′	128.0 s	128.0 s	133.2 s
C-6	135.9 d	135.7 d	135.8 d	C-5′	153.7 s	153.6 s	154.8 s
C-7	134.7 s	134.4 s	135.0 s	C-6′	10.5 q	10.5 q	11.6 q
C-8	133.2 d	134.4 d	133.2 d	C-7′	60.5 q	60.5 q	60.9 q
C-9	37.0 d	35.3 d	36.9 d	C-8′	53.0 q	53.1 q	53.2 q
C-10	82.9 d	94.2 d	82.8 d	C-1″		103.7 q	104.1 d
C-11	135.9 s	135.4 s	135.8 s	C-2″		74.4 d**	74.3 d**
C-12	123.3 d	123.3 d	123.2 d	C-3″		75.5 d	75.9 d
C-13	13.1 q	13.2 q*	13.1 q*	C-4″		70.8 d	70.0 d
C-14	10.7 q	11.1 q	10.7 q	C-5″		76.4 d**	76.5 d**
C-15	17.5 q	17.0 q	17.4 q	C-6″		62.5 t	61.9 t
C-16	16.6 q	16.6 q	16.6 q				

*,** Assignments may be interchanged.

of methanol and subjected to column chromatography on Sephadex LH-20 (2.0×55 cm) using methanol as the eluent. A 150-mg of glucopiericidin A was obtained as colorless amorphous powder. Fifteen mg of glucopiericidin B was obtained as colorless amorphous powder by the same method.

Structures

Physico-chemical properties of glucopiericidins A and B are summarized in Table 5.

They are amorphous powders. Though glucopiericidin A is weakly acidic, glucopiericidin B is neutral. They are soluble in methanol, ethyl acetate, acetone and chloroform, and insoluble in *n*-hexane and water.

The same molecular formula $C_{31}H_{47}O_9N$ was determined from the mass spectra (*m/z* 578, $(M+H)^+$) and elemental analyses of glucopiericidins A and B. They were hydrolyzed by 5 N HCl at 60°C for 30 minutes and analyzed with GC. D-Glucose was found in both compounds.

The structural determination of glucopiericidins A and B was performed by comparing the ^1H and ^{13}C NMR spectra with those of piericidin A_1. The ^1H NMR spectra of glucopiericidins A and B are shown in Fig. 3. The ^1H NMR spectrum of glucopiericidin A shows one characteristic anomeric proton as a doublet at δ 4.13 (J=7.3 Hz), whereas that of glucopiericidin B shows one anomeric proton as a broad signal near δ 4.8. This indicates that D-glucose is linked by a β-configulation in glucopiericidin A. But, it is vague in glucopiericidin B whether the configuration of D-glucose is an α or β-linkage. Assignments of ^{13}C NMR spectra of piericidin A_1, glucopiericidins A and B are shown in Table 6. In the ^{13}C NMR spectra of glucopiericidins A and B, new 6 signals derived from D-glucose (C-1″~C-6″) are observed in comparison with that of piericidin A_1. The ^{13}C NMR spectrum of glucopiericidin A, C-10 (94.2, d) shows a down-field shift (about 11 ppm) significantly and C-8 (134.4, d), C-9 (35.3, d), C-11 (135.4, s), C-14 (11.1, q) and C-15 (17.0, q) are changed in comparison with that of piericidin A_1. On the other hand, in that of glucopiericidin B, C-1′ (151.2, s)~C-7′ (60.9, q) derived from pyridine ring are changed in comparison with that of piericidin A_1.

From the results described above, the structures of glucopiericidins A and B are determined as

Fig. 4. Structures of glucopiericidins A and B.

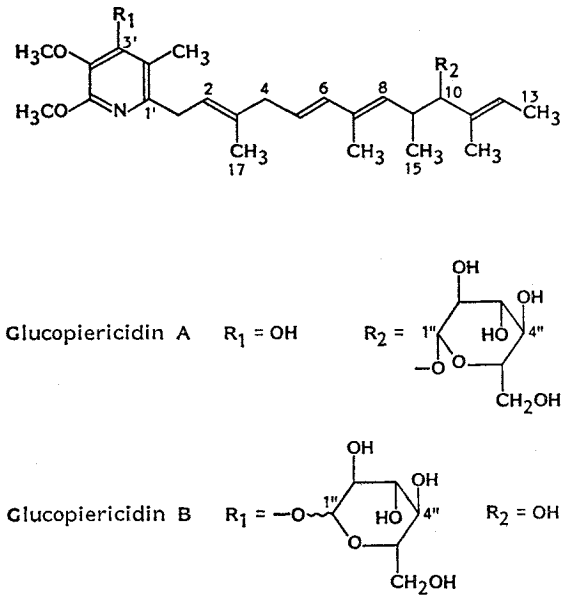


Table 7. Antimicrobial spectra of glucopiericidins A and B.

	MIC (μg/ml)	
	A	B
Bacillus subtilis ATCC 6633	50	25
Staphylococcus aureus ATCC 6538P	50	25
S. aureus Terajima	50	50
S. aureus Smith	50	50
S. epidermidis ATCC 12228	25	25
Streptococcus faecalis IFO 12964	12.5	25
Micrococcus luteus ATCC 9341	50	25
M. lysodeikticus IFO 3333	50	12.5
Escherichia coli O-1	>100	>100
Trichophyton mentagrophytes QM 248	>100	50
T. tonsurans IFO 5928	>100	25
T. rubrum J	>100	50
Microsporum gypseum IFO 8321	>100	50
M. audouinii IFO 6074	>100	25
M. cookei IFO 8303	>100	25
Epidermophyton floccosum IFO 9045	>100	50
Piricularia oryzae IAM 5016	50	25

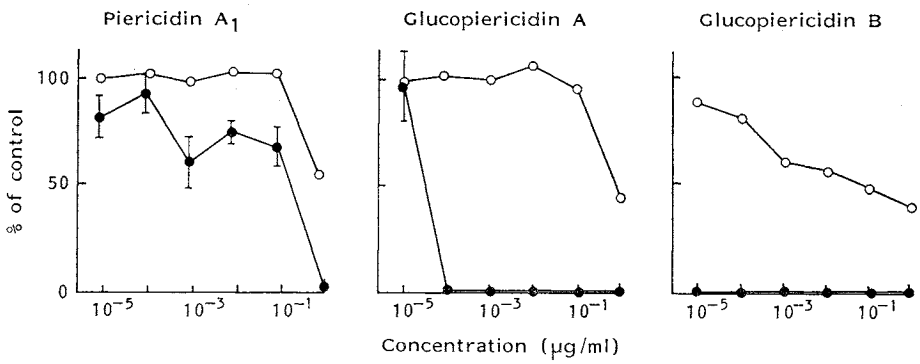
piericidin A_1, 10-*O*-β-D-glucoside and piericidin A_1, 3′-*O*-D-glucoside as shown in Fig. 4, respectively.

Biological Properties

The effects of piericidin A_1, glucopiericidins A and B on antibody formation to sheep red blood cells (SRBC) in mouse spleen cell cultures were examined by the procedure of MISHELL & DUTTON[7]. At the same time, the effects of these substances to L5178Y leukemia cells were examined as a marker of cytotoxicity.

Spleen cells from CDF_1 mouse in cold HANKS' balanced salts solution (HBSS) were washed and

Fig. 5. Effect of piericidin A_1, glucopiericidins A and B on antibody formation to SRBC by spleen cell cultures and cytotoxicity against L5178Y cell cultures.
○: L5178Y, ●: antibody formation.



suspended to 1.5×10^7 cells/ml in RPMI-1640 supplemented with sodium pyruvate, non-essential amino acids, glutamine and 10% fetal calf serum. In a 24-well micro-test-plate, the spleen cell suspension (0.5 ml) was added 0.75×10^6 cells of SRBC as the antigen and incubated in humidified 5% CO_2 - 95% air at 37°C. Each substance was added at the initiation of the culture. After 4 days, antibody formation was measured by counting plaque forming cells (PFC). L5178Y cells (4×10^4) were incubated with each substance in 200 μl of RPMI-1640 supplemented with 10% fetal calf serum at 37°C in humidified 5% CO_2 - 95% air and the cell number was counted by a Coulter counter after 48 hours incubation.

The results are shown in Fig. 5. In piericidin A_1, antibody formation is weakly suppressed among 20~30% at the concentration of $10^{-5}\sim10^{-1}$ μg/ml, whereas no effect is observed on cytotoxicity at the same concentration. Antibody formation is totally inhibited at the concentration of 10^{-4} μg/ml of glucopiericidin A, whereas no effect is observed on cytotoxicity at the concentration of $10^{-5}\sim10^{-1}$ μg/ml as same as piericidin A_1. Glucopiericidin B totally inhibited antibody formation at the concentration of 10^{-5} μg/ml, whereas dose related cytotoxicity was observed. The separation of inhibition of antibody formation and the cytotoxicity by glucopiericidins clearly indicate that the two effect are not related. D-Glucose in glucopiericidins molecule seemed to be necessary for inhibitory activity since piericidin A_1 was less effective than the glucosylated substances in tests.

The antimicrobial spectra of glucopiericidins were determined by the agar dilution method.

As shown in Table 7, glucopiericidin A inhibits the growth of Gram-positive bacteria and *Piricularia oryzae*, whereas glucopiericidin B is more active than glucopiericidin A against those of strains and inhibits the growth of fungi, more ever. Glucopiericidins in general are more active antimicrobial agents than piericidin A_1.

The acute toxicity of glucopiericidins A and B was determined in mice. An intravenous dosage of 30 mg/kg or more sacrificed all the mice, but at 10 mg/kg all survived. Then, in piericidin A_1, an intravenous dosage of 1.0 mg/kg or more sacrificed all the mice.

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