STUDIES ON NEW ANTIFUNGAL ANTIBIOTICS, GUANIDYLFUNGINS A AND B

I. TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

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Guanidylfungin A, $C_{58}H_{103}N_3O_{18}$, and guanidylfungin B, $C_{57}H_{101}N_3O_{18}$, were isolated from the mycelia of *Streptomyces hygroscopicus* No. 662 by means of silica gel absorption and reversed phase liquid chromatographies. The guanidylfungins were active against fungi and Gram-negative bacteria.

In the course of our screening for antifungal substances from soil microorganisms, two new antifungal antibiotics, named guanidylfungins A and B, together with the known antibiotics, nigericin[1] and elaiophylin[2] have been isolated from the mycelia of an actinomycete strain. The content of the guanidylfungins in the mycelia was as much as 10% (weight/dry weight mycelia). The producing organism, strain No. 662, was isolated from a soil sample collected at Sapporo-city, Hokkaido, Japan.

In this paper we describe the taxonomy of the producing organism, and the production, isolation, physico-chemical and biological properties of the guanidylfungins.

The structures of guanidylfungins A and B are presented in the accompanying paper[3].

Materials and Methods

General

Most of the taxonomic studies were carried out in accordance with the methods recommended by International Streptomyces Project (ISP)[4].

Melting points were determined by a Yazawa micro apparatus and are uncorrected. UV spectra were measured using a Uvidec 610 spectrometer. IR spectra were recorded with a Jasco A-102 infrared spectrometer. ^1H and ^{13}C NMR spectra were measured by a FX-100 (^1H 99.6 MHz, ^{13}C 25.1 MHz) and a FX-400 (^1H 400.5 MHz, ^{13}C 100.7 MHz).

Analytical high performance liquid chromatography (HPLC) was carried out on a reversed phase column (Nucleosil $5C_{18}$) with a Tri Rotar pump, employing a Uvidec spectrometer and a Shodex SE-30 differential refractometer as detectors. Preparative reversed phase liquid chromatography was carried out in a Waters Associated 500 instrument, using a PrepPak $500/C_{18}$ column.

Detection of Guanidylfungins A and B

Bioassay of guanidylfungins A and B was carried out by the paper disc diffusion method based on their antifungal activity against *Candida albicans* IAM 4888, *Aspergillus fumigatus* IAM 2046 and *Mucor racemosus christianus*, using a medium composed of malt extract 1.0%, glucose 2.0%, yeast extract 0.4% and agar 1.0% (pH 6.0).

The guanidylfungins were chemically detected by TLC (Kieselgel 60, Merck) with a solvent system of chloroform - methanol - water (65: 25: 4) or 2-butanol - water (4: 1) using the color reaction to vanil-

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Fig. 1. Electron micrographs of spore chains (a) and spores (b) of strain No. 662 on oat meal agar. Bar represents 1 μm.

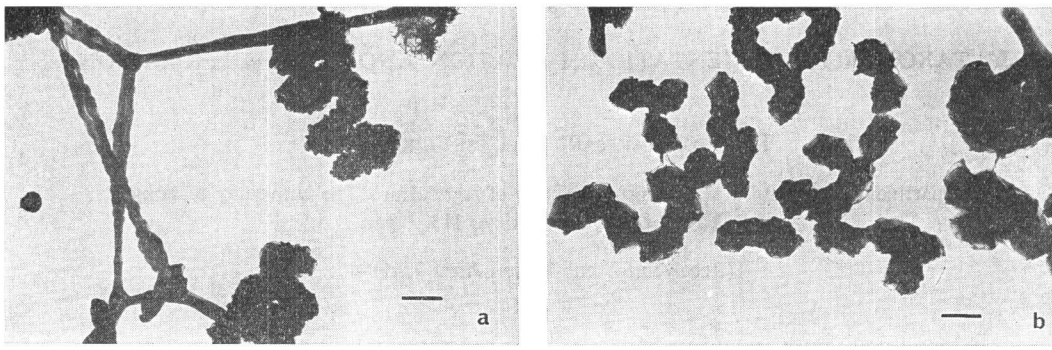


Table 1. Cultural characteristics of strain No. 662.

Medium	Growth and reverse	Aerial mycelium	Soluble pigment
Sucrose - nitrate agar	Moderate, colorless	Gray to brownish gray	None
Glucose - asparagine agar	Moderate, pale yellow	Scant, white	None
Glycerol - asparagine agar	Moderate, pale yellow to pale brownish yellow	Scant, white	Pale brownish yellow
Starch - yeast extract agar	Good, pale yellow to brownish yellow	Abundant, gray to brownish gray	Pale brownish yellow
Tyrosine agar	Good, brownish yellow	White to pale reddish white	None
Nutrient agar	Moderate, pale yellow	Scant, white	None
Yeast extract - malt extract agar	Good, yellowish brown	Abundant, gray to brownish gray	Pale yellowish brown
Oat meal agar	Good, pale yellowish gray	Gray to brownish gray	None

lin-sulfuric acid spray and/or HPLC with a solvent of methanol - 0.01 M aqueous ammonium acetate (4: 1) using the UV absorption at 210~220 nm.

Results

Taxonomic Studies

Strain No. 662 was cultivated at 27°C for 14 days on various media such as yeast extract - malt extract agar, inorganic salts - starch agar, glucose - asparagine agar and tyrosine agar shown in Table 1.

Its morphological characteristics were examined with both optical and electron microscopes. The substrate mycelium did not form fragments. The aerial mycelium branched monopodially and formed spiral chains of spores with 10~30 spores per chain (Fig. 1a). Spores had a warty surface and were cylindrical to ellipsoidal in shape (Fig. 1b) with a size of 0.7~0.8×1.0~1.1 μm. No sclerotic granules, sporangia or zoospores were observed.

The growth characteristics of strain No. 662 are summarized in Table 1. The physiological properties and utilization of carbon sources of the strain are shown in Tables 2 and 3, respectively. According to these results, the main characteristics of strain No. 662 are summarized as follows: Cylindrical to ellipsoidal spores with warty surface in spirals at the top of the aerial mycelium, aerial mass color gray to brownish gray, reverse color pale yellow to yellowish brown, melanoid pigments are not produced. The strain showed moist, black and hygroscopic patches on the aerial mycelial mat at the beginning of the isolation, but this property was lost after several generations of cultivation.

Table 2. Physiological characteristics of strain No. 662.

Temperature range for growth	15~37°C
Liquefaction of gelatin	+
Hydrolysis of starch	+
Coagulation of skim milk	−
Peptonization of skim milk	+
Melanin production	−
Reduction of nitrate	−

+ Positive, − negative.

Table 3. Carbon utilization of strain No. 662.

Positive	D-Glucose, D-fructose, L-rhamnose, D-mannitol, L-arabinose, D-xylose, *i*-inositol, raffinose.
Negative	Sucrose

Among the known species of *Streptomyces* described in "BERGEY's Manual of Determinative Bacteriology" 8th Ed.[5], the ISP reports[6], WAKSMAN's "The Actinomycetes" vol. II[7] and other references, strain No. 662 resembles *Streptomyces hygroscopicus* except for the utilization of raffinose. We propose *Streptomyces hygroscopicus* No. 662 for strain No. 662 in view of the production of guanidylfungins A and B.

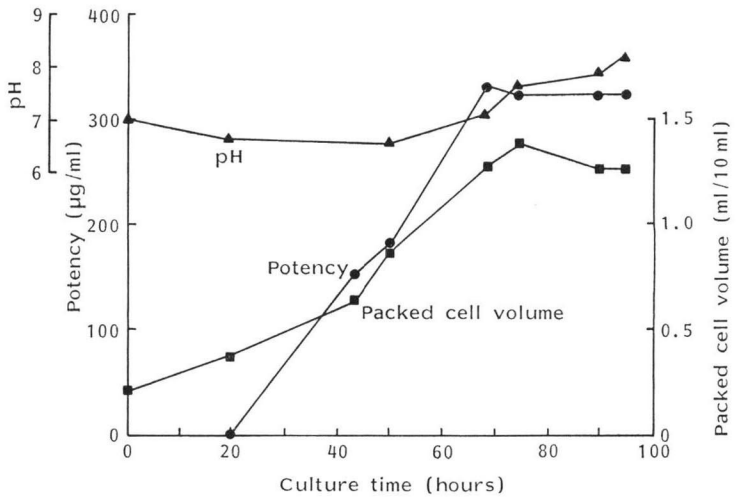
Fermentation

A spore suspension from a slant of the strain was inoculated in 100 ml of a medium consisting of glucose 1.0%, Polypeptone 0.2%, beef extract 0.1% and yeast extract 0.1% in tap water (pH 7.2) in a 500-ml Erlenmeyer flask and cultured with shaking for 26 hours at 27°C. The culture was transferred into a 30-liter fermentor containing 20 liters of the medium composed of soluble starch 1.0%, glucose 1.0%, corn steep liquor 1.5%, yeast extract 0.1%, *N*-acetylglucosamine 0.025%, KH_2PO_4 0.05%, NH_4Cl 0.03%, $FeSO_4$ 0.01% and $CaCO_3$ 0.3% (pH 7.2). Fermentation was carried out at 27°C for 4 days under aeration (10 liters/minute) and agitation (300 rpm).

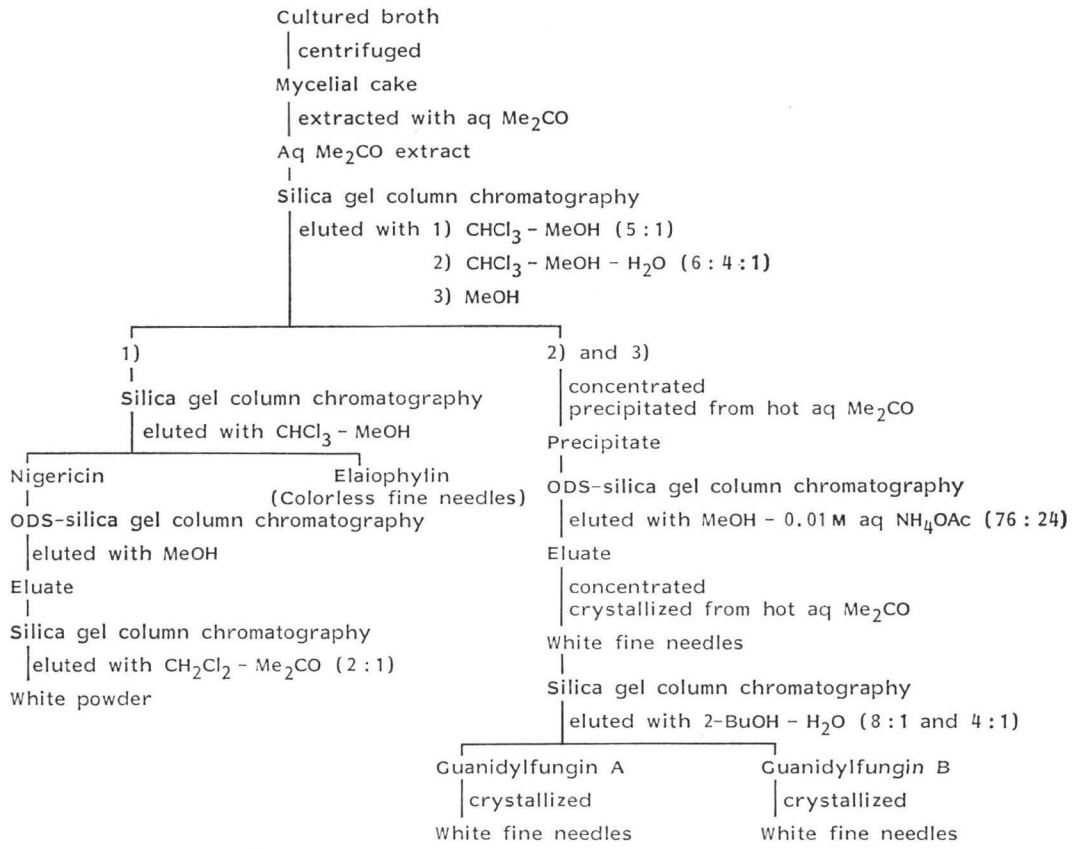
The antibiotics were assayed by the antifungal activity and chemical detection by TLC or HPLC in an aqueous acetone extract of the mycelia. The extract was found to contain two novel antifungal antibiotics, guanidylfungins A and B, in addition to the known antibiotics, nigericin and elaiophylin.

The production of the guanidylfungins was almost parallel to the increase of mycelial growth (Fig. 2) and the content of the antibiotics in the mycelia was 8 to 10% (weight/dry weight mycelia).

Fig. 2. Time course of guanidylfungins A and B production.



Scheme 1. Isolation procedure of guanidylfungins A and B, nigericin and elaiophylin.



Isolation and Purification

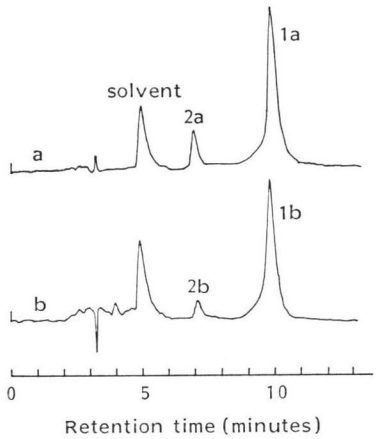
The isolation procedure for guanidylfungins A and B, nigericin and elaiophylin is summarized in Scheme 1. The mycelial cake separated by centrifugation from a cultured broth (12 liters) was extracted with acetone - water (7: 3). The aqueous acetone extract was concentrated *in vacuo* to a brownish paste. The paste was chromatographed on a silica gel column with chloroform - methanol (5: 1), chloroform - methanol - water (65: 25: 4 and 6: 4: 1) and methanol.

The fractions containing guanidylfungins A and B, which were eluted with chloroform - methanol - water (6: 4: 1) and methanol, were collected and concentrated to dryness *in vacuo* to obtain a crude powder. The crude powder was dissolved in hot aqueous acetone, and the solution was left overnight at room temperature yielding a yellowish powder precipitate (4 g). Further purification of the yellowish powder was carried out by preparative liquid chromatography on PrepPak 500/C_{18} with methanol - 0.01 M aqueous ammonium acetate (76: 24). The fractions containing the guanidylfungins were collected, concentrated and crystallized from hot aqueous acetone to obtain white fine needles (2 g). The crystals, which were a mixture of guanidylfungins A and B, were separated into guanidylfungin A and guanidylfungin B by silica gel column chromatography with 2-butanol - water (8: 1 and 4: 1). The fractions containing guanidylfungin A and guanidylfungin B respectively were collected and crystallized from hot aqueous acetone to obtain white fine needles (guanidylfungin A: 1,080 mg, guanidylfungin B: 45 mg).

HPLC analysis of the pure guanidylfungin A (Fig. 3a) showed that it contained a minor component (2a) besides a major one (1a), but the two were found to be interconvertible. Similarly guanidylfungin B (Fig. 3b) contained a minor component (2b) besides a major one (1b), and the two were interconvertible.

The fractions containing nigericin and elaiophylin, which were eluted with chloroform - methanol (5: 1), were collected and purified by preparative liquid chromatography on PrepPak 500/C_{18} with methanol and by silica gel column chromatography with chloroform - methanol or dichloromethane - acetone to obtain pure nigericin (white powder, mp 255~265°C (dec)) and pure elaiophylin (colorless needles, mp 170~175°C).

Fig. 3. HPLC of guanidylfungins A (a) and B (b).
Column: Nucleosil 5C_{18} (6 mm×150 mm)
Mobile phase: MeOH - 0.01 M aq NH_4OAc (76: 24), 1.0 ml/minute.
Detection: UV 215 nm.



Physico-chemical Properties

Some properties of guanidylfungins A and B are summarized in Table 4. The antibiotics were stable at alkaline pH, but very unstable at acidic pH. They showed no characteristic absorption in the UV spectra. Their IR spectra (Fig. 4) exhibited broad strong bands at 3600~3000 cm^{-1} due to multiple hydroxyl groups and at 1750~1600 cm^{-1} due to carboxyl groups. The ^1H NMR spectrum of guanidylfungin A (Fig. 5) revealed the presence of four olefinic protons at 5.72, 5.44, 5.33 and 5.17 ppm, two acyloxy methine protons at 5.17 and 4.73 ppm, two methylene protons at 3.22 and 3.16 ppm, one *N*-methyl protons at 2.83 ppm, seven doublet methyl protons at 0.6~1.2 ppm and two singlet methyl protons at 1.6 ppm. The ^{13}C NMR spectrum of guanidylfungin A (Fig. 6a) showed more than fifty carbons,

Table 4. Physico-chemical properties of guanidylfungins A and B.

	Guanidylfungin A	Guanidylfungin B
Appearance	White fine needles	White fine needles
MP	>155°C (dec)	138~142°C (dec)
SIMS	1,130 (M+H), 374, 224	1,116 (M+H), 360, 210
Molecular formula	$C_{55}H_{103}N_3O_{18}$ (MW 1,129)	$C_{57}H_{101}N_3O_{18}$ (MW 1,115)
Anal Calcd	C 61.65, H 9.12, N 3.72, O 25.51	C 61.34, H 9.06, N 3.77, O 25.83
Found	C 61.55, H 9.27, N 3.65, O 25.65	C 60.87, H 9.30, N 3.55
UV (MeOH)	End absorption	End absorption
IR (KBr) cm^{-1}	3400, 2980, 2940, 1730, 1640, 1600, 1460, 1380, 1160, 1060	3350, 2930, 2900, 1710, 1660, 1580, 1450, 1370, 1080, 1060
Solubility: Soluble	$(CH_3)_2SO$, pyridine, aq acetone	$(CH_3)_2SO$, pyridine, aq acetone
Insoluble	$CHCl_3$, EtOAc, acetone, H_2O	$CHCl_3$, EtOAc, acetone, H_2O
Color reaction: Positive	$KMnO_4$, vanillin-H_2SO_4, I_2 vapour, Dragendorff	$KMnO_4$, vanillin-H_2SO_4, I_2 vapour, Dragendorff, Sakaguchi
Negative	2,4-Dinitrophenylhydrazine, $FeCl_3$	2,4-Dinitrophenylhydrazine, $FeCl_3$
Silica gel TLC (Rf value)		
$CHCl_3$ - MeOH - H_2O (65: 25: 4)	0.17	0.17
2-BuOH - H_2O (4: 1)	0.25	0.30

Fig. 4. IR spectra of guanidylfungins A and B in KBr.

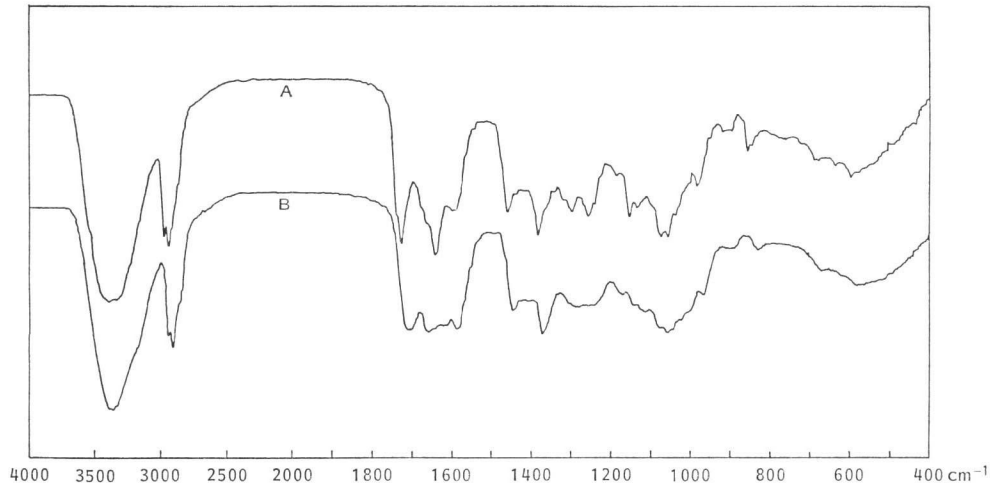
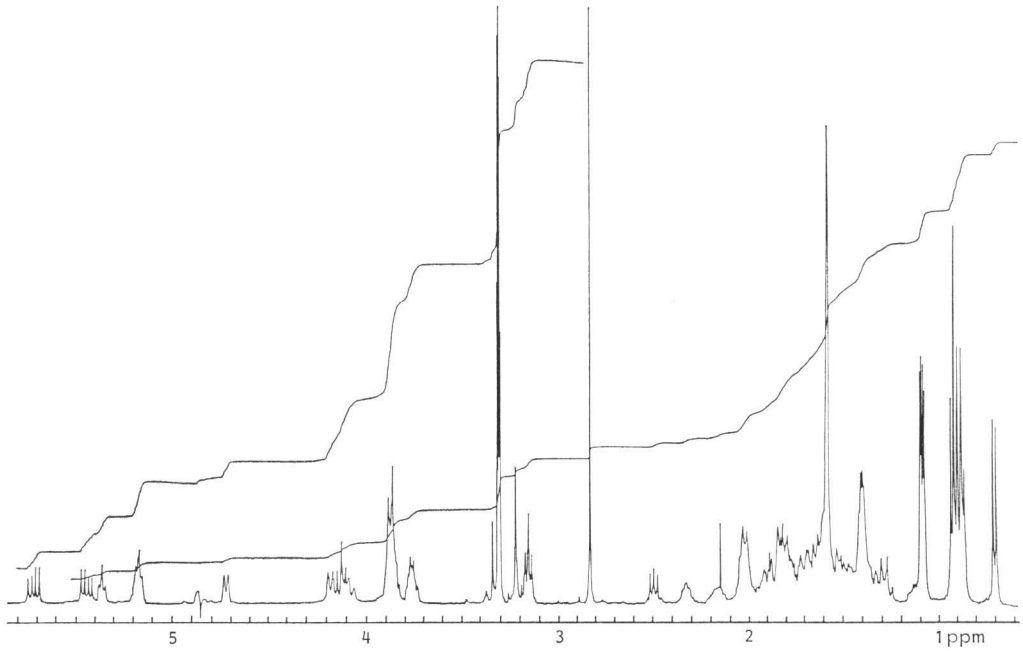


Fig. 5. ^{1}H NMR spectrum of guanidylfungin A (400 MHz in CD_3OD, HDO was decoupled by homogated decoupling).



which contained three carbonyl carbons at 174.5, 170.2 and 170.0 ppm, one guanidyl carbon at 156.4 ppm, six olefinic carbons at 120~140 ppm, one hemiketal carbon at 98.3 ppm, more than ten carbons bearing hydroxyl or acyloxy groups at 60~80 ppm, nine methyl carbons at 8~17 ppm and others. The ^{1}H NMR (100 MHz in CD_3OD, data not shown) and ^{13}C NMR (Fig. 6b) of guanidylfungin B were very similar to those of guanidylfungin A except the absence of an *N*-methyl group (δ_H 2.83, δ_C 27.6). The secondary ion mass spectra (SIMS) of guanidylfungins A and B exhibited their [M+H] ion peaks at *m/z* 1,130 and 1,116, respectively.

Fig. 6. ^{13}C NMR spectra of guanidylfungins A (a) and B (b) (25 MHz in DMSO-d_6).

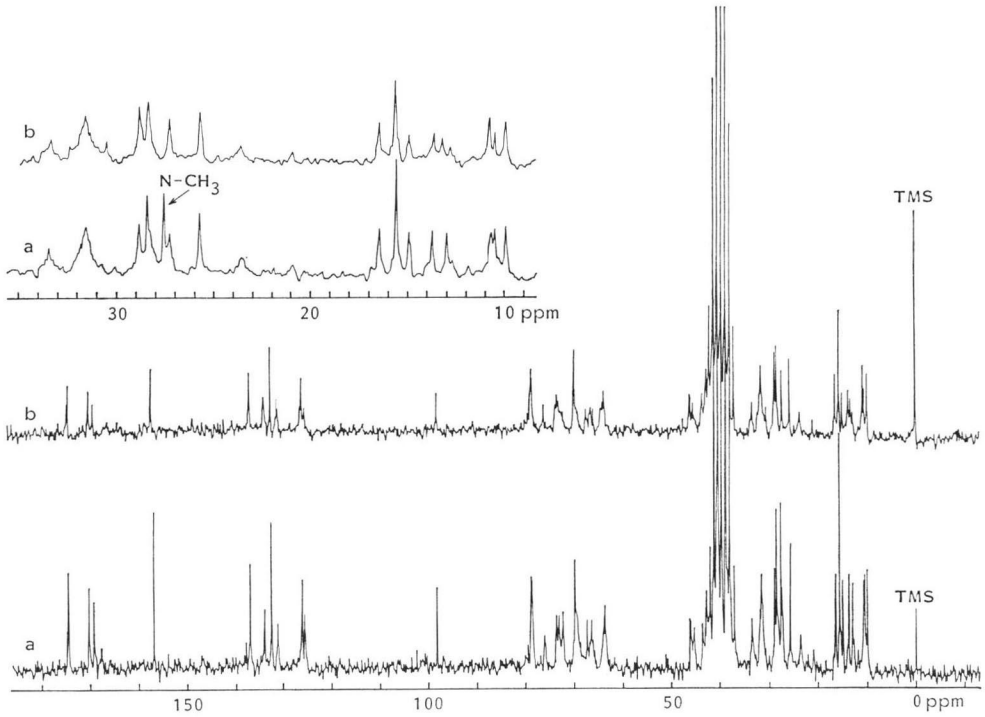


Table 5. Antimicrobial spectra of guanidylfungins A and B.

Test organism	Incubation temp (°C)	Medium*	MIC (μg/ml)	
			A	B
Staphylococcus aureus FDA 209P	37	1	12.5	50
Bacillus subtilis PCI 219	37	1	25	50
Escherichia coli K-12	37	1	>100	>100
Pseudomonas aeruginosa IAM 1180	37	1	>100	
Vibrio metschnikovii IAM 1039	37	1	>100	
Candida albicans IAM 4888	30	2	25	100
Saccharomyces cerevisiae IAM 4020	30	2	50	
Aspergillus fumigatus IAM 2153	30	2	50	
A. oryzae	30	2		25
Trichophyton mentagrophytes	30	2	12.5	6.25
Sporotrichum schenckii	30	2	25	
Paecilomyces varioti IAM 5001	30	2	6.25	

* 1: Heart infusion agar, 2: glucose 20 g, malt extract 10 g, yeast extract 4 g, agar 15 g/liter H_2O (pH 6.0).

From these results of ^1H, ^{13}C NMR, SIMS and elemental analysis, the molecular formulas of guanidylfungins A and B were determined to be $C_{58}H_{103}N_3O_{18}$ and $C_{57}H_{101}N_3O_{18}$, respectively. The result that guanidylfungin B was positive to the color reaction with Sakaguchi reagent and other physicochemical properties of the guanidylfungins revealed the presence of a di-substituted guanidine in guanidylfungin A and of a mono-substituted one in guanidylfungin B.

Nigericin and elaiophylin were identified by comparison with their published data[8~10] of the TLC, UV, IR, ^1H and ^{13}C NMR spectra.

Biological Properties

The antimicrobial spectra determined by agar dilution method are shown in Table 5. Guanidylfungins A and B were active against fungi and Gram-positive bacteria. The acute toxicity (LD_{50} in mice) of guanidylfungin A was 12.5 to 25 mg/kg when administered intraperitoneally.

Discussion

Guanidylfungins A and B were compared with other known antibiotics having some points of similarlity in physico-chemical and biological properties. Among several known antibiotics azalomycins F_{3a}, F_{4a} and F_{5a}[11~13], scopafungin[14,15], copiamycin[16,17], neocopiamycin A[18], niphithricins A and B[19], tendomycin[20], niphimycin[21,22] and primycin[23,24] were similar to guanidylfungins A and B in their analytical properties as well as in their biological properties.

In the UV spectra, however, the guanidylfungins were clearly different from azalomycin Fs, scopafungin, copiamycin, neocopiamycin A, niphithricins A and B, niphimycin and tendomycin. In the SIMS and ^{13}C NMR, the guanidylfungins were also distinguished from those similar antibiotics.

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