A NEW ANTIFUNGAL ANTIBIOTIC, CYSTARGIN†: FERMENTATION, ISOLATION, AND CHARACTERIZATION

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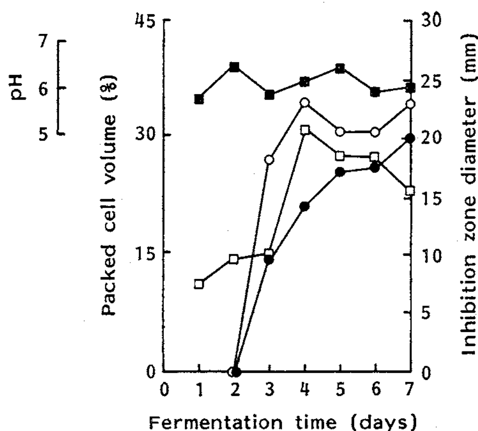
A new sulfur-containing peptide antifungal antibiotic, cystargin, was isolated from the fermentation broth of a new species of genus *Kitasatosporia*, designated as *Kitasatosporia cystarginea*. On acid hydrolysis, cystargin ($C_{60}H_{77}N_{19}O_{17}S_6$) gave equimolar glycine, proline, aspartic acid and arginine. By performic acid oxidation, cysteic acid was detected after hydrolysis. It showed a growth inhibitory activity against various phytopathogenic fungi and inhibition of β-1,3-glucan synthetase from *Saccharomyces cerevisiae*.

An antibiotic which has a primary action site against microbial cell wall, is expected to have selective toxicity. Several antibiotics are known to inhibit fungal cell wall synthesis. Polyoxins[1] and neopolyoxins[2] produced by *Streptomyces* are inhibitors of chitin synthetase. Lipopeptins[3] and neopeptins[4] from *Streptomyces* were shown to inhibit glucan and mannan synthesis. Fungal metabolite, aculeacins[5], echinocandins[6] and papulacandins[7] are known to inhibit fungal glucan synthetase.

In the course of our screening program for inhibitors of fungal cell wall biosynthesis, a strain of Actinomycete was found to produce a substance which inhibits yeast glucan synthetase. The strain was isolated from a soil sample collected in Yamaguchi Prefecture, Japan. Taxonomic studies showed that it was a new species of genus *Kitasatosporia* and designated as *Kitasatosporia cystarginea* sp. nov.[8]. The active substance exhibited growth inhibitory activity against various phytopathogenic fungi accompanied by swelling of mycelia. The antibiotic was isolated from the culture broth of the strain in pure form. It was concluded to be a novel peptide antibiotic from the physico-chemical and biological properties. This paper is concerned with fermentation, isolation and characterization of the new antibiotic, which is designated as cystargin.

Fig. 1. Profile of cystargin fermentation.

□ Packed cell volume, ■ pH value, ○ antifungal activity of mycelial extract against *Pyricularia oryzae*, ● antifungal activity of culture filtrate.



Fermentation

Seed culture was carried out by inoculating spores from an agar slant culture of *K. cystarginea* into a 500-ml cylindrical flask containing 70 ml

† Cystargin is the identical compound with RK-419 which appeared in Jpn. Kokai 146188 ('86), July 3, 1986.

Fig. 2. Isolation procedure of cystargin.

Culture broth

Filtrate

Mycelial cake

extract with acetone - H_2O (7 : 3)
concentrate to remove acetone

Diaion HP-10

elute with aceton - H_2O (1 : 1)
concentrate *in vacuo*
extract with *n*-butanol
concentrate to remove *n*-butanol
dissolve in acetone - EtOH - H_2O

MCI gel CHP20P

develop with acetone - H_2O (1 : 1)
concentrate and lyophilize

Sephadex LH-20

develop with MeOH
concentrate *in vacuo*

HPLC (Reverse phase column)

elute with MeOH - H_2O (85 : 15)
concentrate and lyophilize

Cystargin

Table 1. Physico-chemical properties of cystargin.

MP	>260°C (dec)
$[\alpha]_D^{22}$ (*c* 0.5, 50% aq EtOH)	+43.6°
Molecular formula	$C_{60}H_{77}N_{19}O_{17}S_6$
MW	1,527
Positive (FAB-MS)	*m/z* 1,528
Negative (FAB-MS)	*m/z* 1,526
Anal Calcd for $C_{60}H_{77}N_{19}O_{17}S_6$:	C 47.15, H 5.04, N 17.41, S 12.57.
Found:	C 47.21, H 4.87, N 17.02, S 12.17.
IR ν_{max}^{KBr} cm^{-1}	3350, 1660, 1515

of medium composed of glucose 2%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4%, soy bean flour 2.5%, sodium chloride 0.2%, and dipotassium hydrogen phosphate 0.005%. The flask was cultivated for 48 hours at 28°C on a rotary shaker. Each 2 ml-portion of the culture broth was transfered into flasks containing 70 ml of the same medium and they were incubated at 28°C. The time course of growth and antibiotic production in a flask culture are shown in Fig. 1. In case of jar fermentation, the culture broth of two flasks at 48 hours was added into a 30-liter jar fermentor containing 18 liters of the same medium as inoculum for large scale fermentation. The cultivation was continued at 28°C for 72 hours under aeration of 12.5 liters/minute and agitation of 350 rpm.

Isolation

The isolation procedure of cystargin is outlined in Fig. 2. The activity was monitored by a conventional paper disc method using *Pyricularia oryzae* as a test organism. Cystargin was extracted

from both culture filtrate and mycelial extract. Finally it was obtained as an amorphous powder by lyophilization after purification with HPLC. The overall yield from fermentation broth was 3.5%.

Characterization

Cystargin is a colorless powder which is soluble in dimethyl sulfoxide, dimethylformamide and methylcellosolve, moderately soluble in methanol, ethanol and acetone, hardly soluble in water and butanol, and insoluble in other organic solvents. Physico-chemical properties are listed in Table 1. The molecular formula was determined as $C_{60}H_{77}N_{19}O_{17}S_6$ based on the elemental analysis and fast atom bombardment (FAB)-MS. It showed a positive color reaction to Rydon-Smith, potassium permanganate and Lemieux reagents and negative to ninhydrin and anisaldehyde - sulfuric acid reagents. It exhibited absorption maxima at 222 nm (ε 61,800), 270 (28,400) and 300 (21,000) in the UV spectrum (Fig. 3). The IR and ^{1}H NMR spectra are shown in Figs. 4 and 5, respectively. Formation of the molecular aggregates may be the cause of broadening of signals in the ^{1}H NMR spectrum.

Cystargin was hydrolyzed with 6 N hydrochloric acid at 110°C for 18 hours and the hydrolysate was applied to amino acid analyzer. Glycine (1.00), aspartic acid (0.93), proline (0.88), arginine (1.03), and cystine (0.26) were detected. These amino acids were also observed on TLC. Cystargin was oxidized with performic acid, and the product was hydrolyzed with 6 N hydrochloric acid in the same manner. Cysteic acid was detected by amino acid analyzer in 2.5 molar ratio, indicating the presence of a cystein equivalent.

Biological Properties

Cystargin showed antifungal activity against various phytopathogenic fungi, but no activity against bacteria and yeast. The antifungal spectrum is shown in Table 2. Swelling of mycelium in tested fungi was observed microscopically after agar dilution assay (Fig. 6). It inhibited β-1,3-glucan synthetase prepared from *Saccharomyces*

Fig. 3. UV spectrum of cystargin in MeOH.

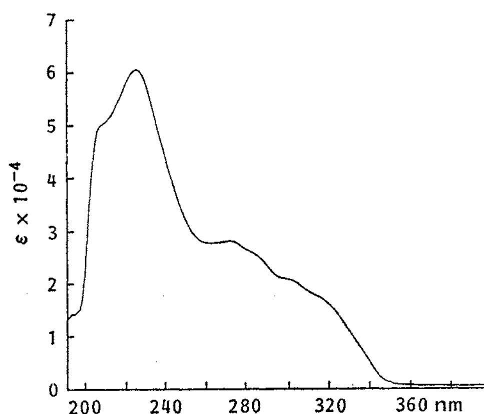


Fig. 4. IR spectrum of cystargin (KBr).

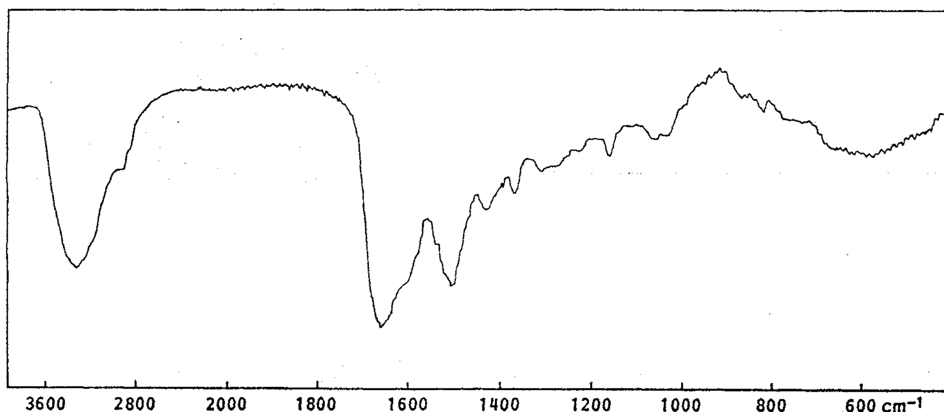


Fig. 5. ^1H NMR spectrum of cystargin (400 MHz, CD_3OD, WEFT, 2.3s).

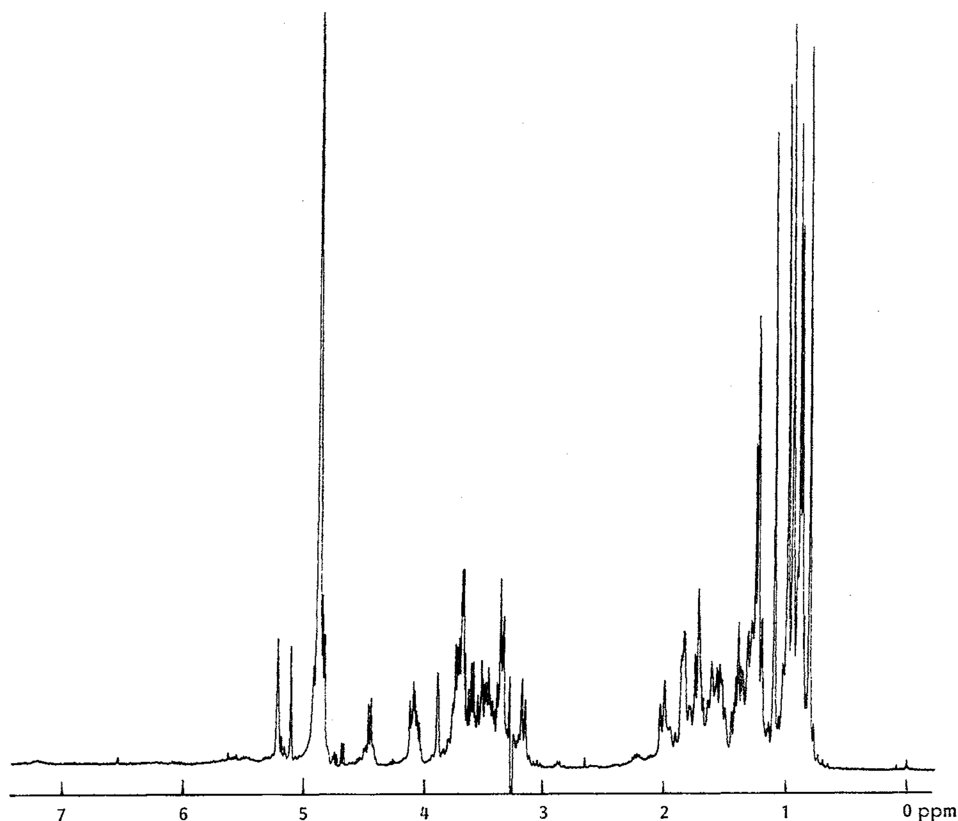


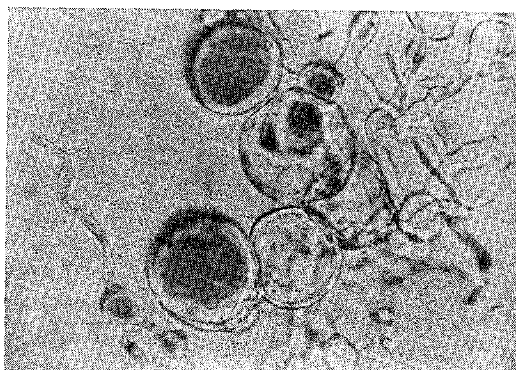
Table 2. Antifungal activity of cystargin.

Test organisms	MIC (μg/ml)
Aspergillus oryzae IFO 5239	100
Penicillium chrysogenum	>100
Trichophyton mentagrophytes IFO 6202	12.5
Cochliobolus miyabeanus IFO 5277	12.5
Pyricularia oryzae IFO 5994	1.6
Glomerella cingulata IFO 9767	100
Colletotrichum lagenarium IFO 7513	12.5
Botrytis cinerea IFO 5365	6.2
Alternaria mali IFO 8984	>100
Fusarium oxysporum IFO 9761	>100
Rhizoctonia solani IFO 6258	6.2

MICs were determined at 27°C by dilution method on potato - sucrose agar and the incubation time was 72 hours.

Fig. 6. Microscopic photograph of swelling of mycelium of *Pyricularia oryzae* induced by cystargin.

The bar represents 100 μm.



cerevisiae with the ID_{50} of 200 μg/ml. The acute toxicity of cystargin in mice was low. Oral administration of 200 mg/kg caused no toxic sign to mice.

Discussion

Based on the physico-chemical properties described above, cystargin belongs to the class of antifungal sulfur-containing peptide antibiotics. Characteristic UV spectrum suggests that the chromophore of the antibiotic is similar to those of antifungal peptide antibiotics, saramycetin[9] and globopeptin[10]. These antibiotics gave glycine, aspartic acid, proline, and threonine after acid hydrolysis. However, arginine, instead of threonine, was obtained by acid hydrolysis of cystargin. In spite of the presence of arginine in the molecule, cystargin did not exhibit basicity on high voltage paper electrophoresis with a buffer of pH 2.0 (0.1 M pyridine - formic acid). It is suggested that the guanidyl group of arginine is masked in the cystargin molecule.

Although cystargin inhibited β-1,3-glucan synthetase from *S. cerevisiae*, the ID_{50} value was much higher than the MIC against phytopathogenic fungi (Table 2). Therefore, the mechanism of action other than glucan synthesis is considered to be involved.

Experimental

General

The mp was taken on a Yanagimoto micro melting point apparatus and was uncorrected. The UV spectrum was measured on a Hitachi 220A spectrophotometer and IR spectrum on a Shimadzu IR-27G recording IR spectrophotometer. Optical rotation was determined on a Perkin-Elmer 241MC polarimeter. ^{1}H NMR spectrum was obtained on a Jeol GX-400 FT NMR spectrometer. Positive and negative FAB-MS were recorded on a Jeol JMS-HX110 mass spectrometer. Amino acid analysis was carried out by Hitachi high speed amino acid analyzer model 835. HPLC was done by using Hitachi 635A liquid chromatograph.

Isolation

Culture broth (75 liters) was filtered with Celite to separate culture filtrate and mycelial cake. The latter was soaked in acetone - water (7 : 3) overnight and the extract was filtered and concentrated *in vacuo* to remove acetone. Resulting aqueous suspension was combined with the culture filtrate and applied to Diaion HP-10 (8 liters). After washing with water and methanol - water (3 : 7), the antifungal activity was eluted with 35 liters of acetone - water (1 : 1). The eluate was evaporated *in vacuo* to 10 liters and extracted with butanol. The organic layer was separated and concentrated to dryness. The residual material (77 g) was dissolved in a mixed solution of acetone - ethanol - water and loaded on a column of MCI gel CHP20P (150~300 μm) packed with acetone - water (1 : 1), which was developed with the same solvent and the active fractions were collected and concentrated *in vacuo*. The residual solution was lyophilized to give 13 g of crude powder. It was dissolved in a small amount of methanol and loaded on a column of Sephadex LH-20 packed with methanol. It was developed with methanol and active fractions were combined. After evaporation and lyophilization, 220 mg of powder was obtained. For further purification, preparative HPLC was carried out by using a reverse phase column (Nucleosil $5C_{18}$, 20×250 mm, monitored by 220 nm) with methanol - water (85 : 15) as a solvent system. The eluate obtained was concentrated to a small volume and lyophilized to yield 35 mg of pure cystargin as an amorphous powder.

Acid Hydrolysis

Into a glass tube containing 0.5 mg of cystargin, 1 ml of 6 N hydrochloric acid was added and air was degassed by vacuum pump. After the tube was sealed, it was heated at 110°C for 18 hours in a hydrolysis furnace. The solution was evaporated to dryness with addition of water several times. After dried in a vacuum desiccator, half of the hydrolysate was provided for amino acid analysis and the rest was used for TLC.

Into a 30-ml round bottom flask containing 0.5 mg of cystargin, 0.45 ml of formic acid and 0.5 ml of 30% hydrogen peroxide were added. The reaction mixture stood at room temperature for 40 minutes. Then 10 ml of water was added to the solution and the solution lyophilized. The procedure was repeated three times. The oxidation product was subjected to acid hydrolysis in the same manner

described above.

β-1,3-Glucan Synthetase Assay

The method described by CABIB *et al.* was used with some modification[11]. A particulate enzyme prepared from *S. cerevisiae* GS-1-36, a mutant strain that does not accumulate glycogen, was used. The reaction mixture contained 2.5 mM UDP-[*U*-^{14}C]glucose (0.1 μCi/μmol), 1 mM ATP, 0.2% bovine serum albumin, 0.25 mM EDTA, 80 mM Tris - chloride, pH 8, and a particulate enzyme in a total volume of 50 μl, which was incubated for 2 hours at 30°C. The reaction was stopped by heating at 100°C for 3 minutes. All of the reaction mixture was spotted on a paper (Whatman 3MM) and chromatography was carried out by using ethanol - 1 N acetic acid (7 : 3) as a developing solvent. An origin portion of the paper was cut off and transferred to a scintillation vial, which was counted in a toluene scintillator.

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