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Broad Spectrum and Mode of Action of an Antibiotic Produced by *Scytonema* sp. TISTR 8208 in a Seaweed-Type Bioreactor

APARAT CHETSUMON, FUSAKO UMEDA, ISAMU MAEDA, KIYOHITO YAGI,* TADASHI MIZOGUCHI AND YOSHIHARU MIURA

Faculty of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita, Osaka 565, Japan

ABSTRACT

A photobioreactor was constructed using anchored polyurethane foam strips (1 × 1 × 40 cm) fixed onto a stainless-steel ring to prevent flotation, as a biomass support material (BSM). This type of reactor was named a seaweed-type bioreactor. A filamentous cyanobacterium, *Scytonema* sp. TISTR 8208, which produces a novel cyclic dodecapeptide antibiotic, was immobilized in seaweed-type photobioreactor and cultivated with air containing 5% CO_2 sparged at a gas flow rate of 250 mL/min under illumination at a light intensity of 200 μmol photon $m^{-2}s^{-1}$. The antibiotic produced in the seaweed-type photobioreactor was purified by HPLC and examined regarding its spectrum and mode of action. The antibiotic effectively inhibited the growth of Gram-positive bacteria, pathogenic yeasts, and filamentous fungi, but it had only a weak effect on Gram-negative bacteria. Scanning electron micrograph analysis showed that the most characteristic change was swelling of the cells after exposure to the antibiotic. The antibiotic seems to alter the conformation of the microbial cell membrane, thereby changing its permeability, leading to osmotic shock.

Index Entries: Cyanobacteria; *Scytonema;* photobioreactor, antibiotic; polyurethane foam.

INTRODUCTION

Prokaryotic cyanobacteria can be grown photoautotrophically using light energy and CO_2, which is a major greenhouse gas partially responsible for global warming. Recently developed technology enables CO_2 to be

* Author to whom all correspondence and reprint requests should be addressed.

recovered from the emission gases of steam power plants. It is environmentally and economically important to produce valuable substances photoautotrophically from CO_2 by cyanobacteria. Various strains of cyanobacteria are known to produce intracellular and extracellular metabolites, with diverse biological activities, which are classified into two groups *(1)*. The first group contains lactones, phenols, and acids such as cyanobacterin, a γ-lactone antialgal from *Scytonema hofmanni (2)*, an antibacterial, brominated phenol from *Calothrix brevissima (3)*, and an antimicrobial, *O*-methyl acid from various shallow-water varieties *(4)*. The second group, which is the major one, consists of nitrogen-containing substances, such as a cytotoxin, malyngamide D from *Lyngbya majuscula (5)*, and an antialgal and antimycotic, hapalindole A from *Hapalosiphon fontinalis (6)*. The authors previously screened nine strains and five genera of cyanobacteria for antibiotic production, and a filamentous cyanobacterium, *Scytonema* sp. TISTR 8208, had the strongest activity against the bacteria tested *(7)*. Stable immobilization of the cyanobacterium, which was shown to produce a cyclic peptide antibiotic, could be established by utilizing a fibrous biomass support material. The optimal medium composition for the production of the antibiotic was determined *(8)*, and a seaweed-type photobioreactor was then constructed for the continuous cultivation of the cyanobacterium *(9)*. Stable production of the antibiotic was achieved in the bioreactor for 16 d.

The aim of the present study was to characterize the antibiotic produced by immobilized *Scytonema* sp. TISTR 8208, to determine its spectrum and mode of action toward susceptible microorganisms.

MATERIALS AND METHODS

Cultivation of cyanobacterium

Scytonema sp. TISTR 8208, which was obtained from the culture collection of the Thailand Institute of Scientific and Technological Research Center (TISTR), was cultivated in modified BGA medium (MBGA) *(7)*. The seaweed-type photobioreactor used is illustrated in Fig. 1. Details of the bioreactor dimensions were given previously *(9)*. Cyanobacterial cells of about 1.6 g dry wt were inoculated into the 2.3-L bioreactor containing 2.0 L of MBGA medium. The bioreactor was incubated in a $30 \pm 1°C$ incubation room. The basal conditions of light illumination and gas (air containing 5% CO_2) flow rate were 200 μmol photon $m^{-2}s^{-1}$ and 250 mL/min, respectively, unless otherwise stated. A linear bank of fluorescent lamps was used on one side of the bioreactor.

Purification of Antibiotic

The culture supernatant was concentrated under reduced pressure at 30°C, and the antibiotic was extracted with methanol. After vacuum drying, the sample was then partitioned into a solvent system of $CHCl_3$:

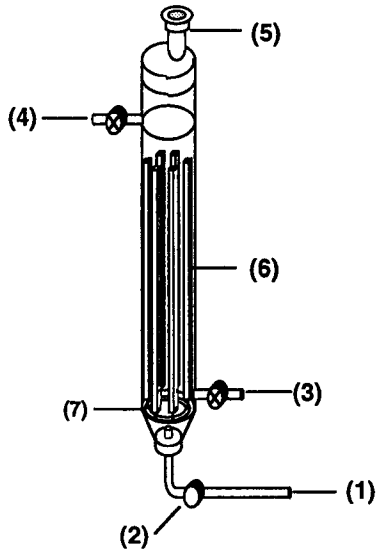

Fig. 1. Structure of seaweed-type photobioreactor. (1), air inlet; (2), air filter; (3) and (4), sampling ports; (5), air outlet; (6), polyurethane foam strips; (7), stainless-steel ring.

methanol:H_2O at a ratio of 2:1:1. The lower phase was applied onto a silica gel 60 column and eluted with $CHCl_3$:methanol:H_2O at a ratio of 10:3:1. Fractions of 2 mL were collected, and this aliquot volume was used for antibiotic assay against *Bacillus subtilis* ATCC6633. The active fractions were collected and further purified by reverse-phase HPLC, using a YMC ODS AM-302 column (10 × 250 mm, Yamamura Chemical Laboratories, Japan). The elution was carried out with H_2O:CH_3CN:TFA in a ratio of 650:350:1, at a flow rate of 4 mL/min. The concentration of the purified sample was 0.001% (w/v).

Determination of Minimum Inhibitory Concentrations (MICs)

The MIC test against bacterial strains was done by the microtube broth-dilution technique *(10)*, and the MICs against yeasts and filamentous fungi were determined by the tube broth-dilution method *(11)*.

Analysis of Mode of Antibiotic Action

The mode of action of the antibiotic was observed by a scanning electron micrograph (SEM) (JEOL, JEM-2000 EX). Microbial cells were treated and observed with the SEM according to the methods described by Mallie et al. *(12)*.

RESULTS AND DISCUSSION

The filamentous cyanobacterium *Scytonema* sp. TISTR 8208 was cultivated photoautotrophically in the seaweed-type bioreactor for 2 wk. Secreted antibiotic was purified as described in Materials and Methods. As

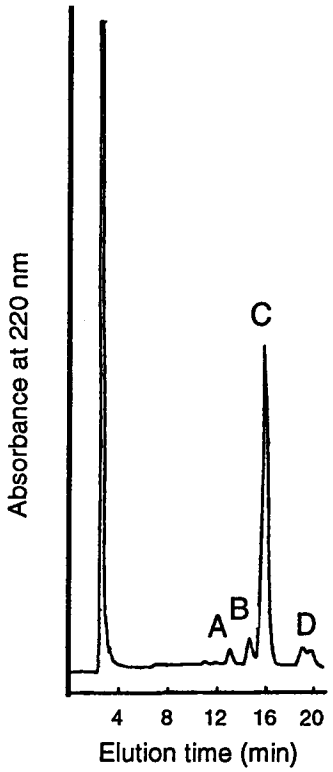

Fig. 2. Purification of the antibiotic secreted from *Scytonema* sp. TISTR 8208 by reverse-phase HPLC. A partially purified sample was injected into a YMC ODS AM-302 column, and the antibiotic was eluted as described in Materials and Methods.

shown in Fig. 2, four peaks were obtained in reverse-phase HPLC, and the antibiotic activity was found in peaks A, B, and C. The major fraction, C, eluted at a retention time of 16 min, was used for the subsequent characterization. NMR and FAB mass analysis revealed that the antibiotic was a novel cyclic dodecapeptide with a mol wt of 1490 (data not shown).

The MICs of the antibiotic against various microorganisms were determined and compared with those of representative antibiotics. The antibiotic was shown to have strong activity toward Gram-positive bacteria, such as *B. subtilis*, at the same level as streptomycin and tetracycline (Table 1). However, the action was weak toward Gram-negative bacteria, especially when compared with gentamicin. Although the antibiotic activity against pathogenic yeasts varied, depending on the strain tested, it was stronger than that of imidazole, which is the nucleus of antifungal imidazole derivatives (Table 2). The antibiotic was also shown to have stronger activity against filamentous fungi than cycloheximide (Table 3).

The mode of action of the antibiotic against a bacterium (*B. subtilis* ATCC 6633), a yeast (*Pichia membranaefaciens* TISTR 5107), and a dermatophyte (*Microsporum audouini* IFO 8147), which were the strains most sus-

Table 1
MICs of Antibiotics Against Bacteria

Test organism	Antibiotic (μg/mL)					
	Sample	Ampicillin	Gentamicin	Kanamycin	Streptomycin	Tetracycline
Bacteria						
B. subtilis ATCC 6633	4.0	0.13	0.25	1.0	4.0	4.0
E. coli ATCC 8739	64.0	2.0	8.0	32.0	32.0	0.5
P. aeruginosa ATCC 9027	128.0	128.0	1.0	64.0	32.0	8.0
P. alkanolyticum IFO 12319	16.0	16.0	0.5	16.0	2.0	0.5
P. putida ATCC 15175	64.0	64.0	0.13	32.0	1.0	32.0
P. reptilivora IFO 3461	128.0	64.0	0.06	32.0	1.0	16.0

Table 2
MICs of Antibiotics Against Yeasts

Test organism	Antibiotic (μg/mL)	
	Sample	Imidazole
Yeasts		
C. albicans IFO 0579	32.0	64.0
C. albicans TISTR 5239	32.0	64.0
C. krusei TISTR 5099	8.0	32.0
C. tropicalis TISTR 5045	16.0	16.0
P. kluyveri TISTR 5150	16.0	64.0
P. membranaefaciens TISTR 5107	4.0	32.0

Table 3
MICs of Antibiotics Against Fungi

Test organism	Antibiotic (μg/mL)	
	Sample	Cycloheximide
Filamentous fungi		
A. fumigatus IFO 31952	16.0	32.0
A. fumigatus TISTR 3108	64.0	32.0
A. niger TISTR 3390	16.0	16.0
F. solani IFO 31093	16.0	16.0
M. audouini IFO 8147	4.0	4.0
T. mentagrophytes IFO 32412	16.0	64.0

ceptible to the antibiotic, was investigated. Cells were treated with 40 μg/mL antibiotic for 2 h in the case of *B. subtilis*, and for 18 h in the case of *P. membranaefaciens* and *M. audouini*. Figure 3 shows photographs of treated and nontreated cells taken under SEM observation. Swelling occurred in *B. subtilis* within 2 h of the commencement of antibiotic treatment, as a result of cell membrane damage. In *P. membranaefaciens* TISTR 5107, swollen cells with wrinkled, knob-like portions were observed within 24 h prior to cell lysis. Cell membrane damage was also evident in *M. audouini* as a result of antibiotic treatment. These changes in cell conformation were dose- and time-dependent (data not shown). The findings clearly showed that the antibiotic altered the microbial cell membrane, thereby changing its permeability. This led to osmotic shock, thus allowing leakage of the intracellular cell contents. Well-known antifungal antibiotics, such as polyene antibiotics and imidazoles, have the same kind of actions to the susceptible microorganisms *(11)*. The polyene antibiotics interact with sterols in the cell membrane, to give either membrane fragmentation or lipid rearrangement *(13)*. Imidazole and its derivatives interact with unsaturated

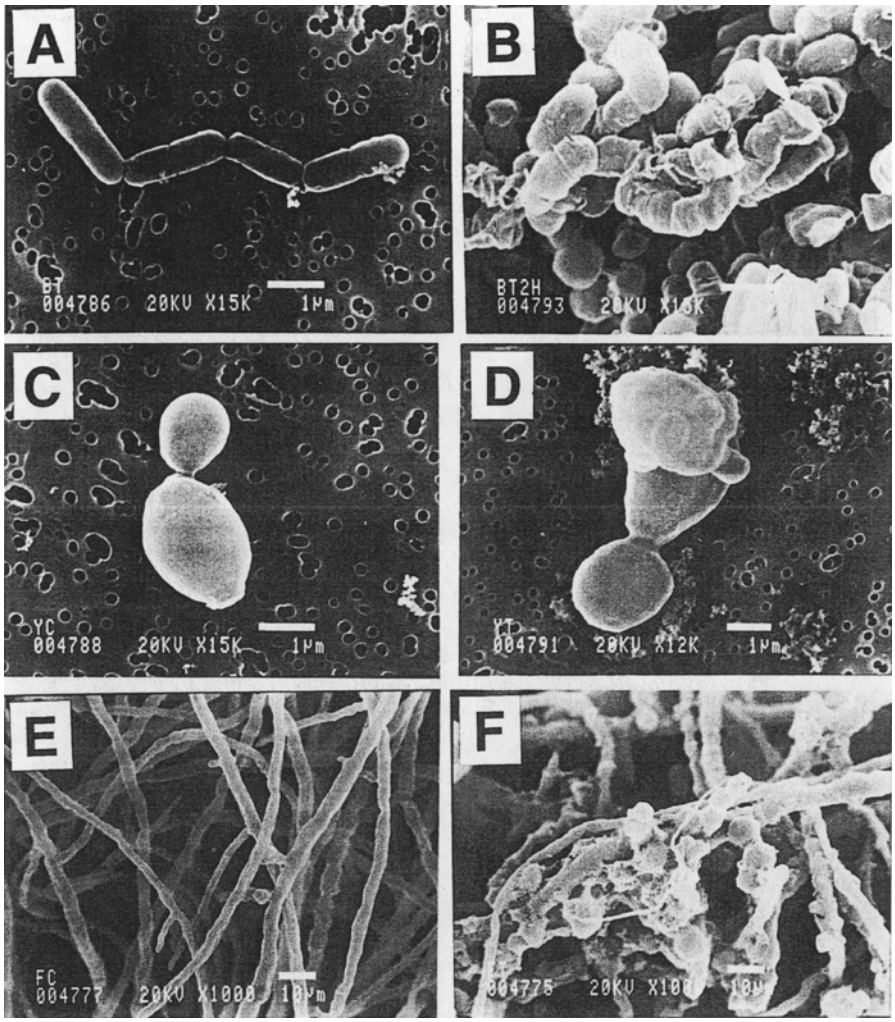

Fig. 3. Scanning electron micrographs of microorganisms before and after treatment by the antibiotic. The bacterium *B. subtilis* ATCC 6633 (**A** and **B**), yeast *P. membranaefaciens* TISTR 5107 (**C** and **D**), and filamentous fungus *M. audouini* TISTR 8147 (**E** and **F**) were used to analyze the mode of action of the antibiotic. A, C, and E are nontreated cells. B, D, and E are cells treated as described in the text. Bars represent 1 μm.

phospholipids in the cell membrane of susceptible organisms, thereby altering its permeability *(14)*. Further investigation is now under way to elucidate the exact mechanism of the action of the cyclic peptide antibiotic produced by *Scytonema* sp. TISTR 8208. *Scytonema* sp. strain U-3–3, which appears to be similar to TISTR 8208, has been reported to produce an cyclic peptide named scytonemin A *(15)*, the structure of which is different from the antibiotic purified in this work. Since scytonemin A has calcium-antagonistic properties, the substance purified from *Scytonema* sp. TISTR 8208 might have some pharmacological activity. The continuous production of

such a valuable product in a photobioreactor, using solar energy, appears to be a very promising means of recycling CO_2.

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