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Clethramycin, a New Inhibitor of Pollen Tube Growth with Antifungal Activity from *Streptomyces hygroscopicus* TP-A0623

I. Screening, Taxonomy, Fermentation, Isolation and Biological Properties

TAMOTSU FURUMAI*, TAKASHI YAMAKAWA, RYUJI YOSHIDA† and YASUHIRO IGARASHI

Biotechnology Research Center, Toyama Prefectural University,
5180, Kurokawa, Kosugi, Toyama 939-0398, Japan
† College of Technology, Toyama Prefectural University,
5180, Kurokawa, Kosugi, Toyama 939-0398, Japan

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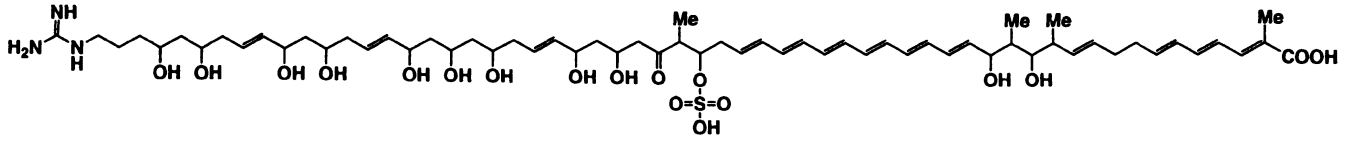
The cytoskeletal proteins, actin and myosin, play a central role in pollen tube growth. The pollen tube growth is inhibited by cytochalasin, which interferes with actin polymerization. In the screening of pollen tube growth inhibitors, clethramycin was found from the fermentation broth of an actinomycete strain TP-A0623. The producing strain was isolated from a root of *Clethra barbinervis* collected in Toyama, Japan and identified as *Streptomyces hygroscopicus* based on the taxonomic study. Clethramycin showed *in vitro* antifungal activity against yeast such as *Candida albicans* and *C. glabrata* with the MIC of 0.5~8 μg/ml, but weak activity against Gram-positive and negative bacteria (MIC $\geq$64 μg/ml). Cytotoxicity of clethramycin was moderate and the IC_{50} was 57 μg/ml against HeLa cells and 120 μg/ml against WI-38 cells.

In pollen tube growth, actin/myosin cytoskeleton plays an important role in the transport of the vesicles containing precursors for cell wall biosynthesis from the sites of their synthesis to the growing pollen tube tip[1]. This process is inhibited by cytochalasin or latrunculin B, an inhibitor of actin polymerization, and thus pollen tube growth is also inhibited[2]. An inhibitor of cytoskeletal function is expected to be a tool to probe the cell function and further to be a lead for therapeutic agents. In addition, in this process, organelles in which the cell wall precursors are synthesized such as the endoplasmic reticulum and Golgi apparatus are involved, and therefore the discovery of bioactive molecules with an unknown mode of action is expected. In this study, we screened for the inhibitors of pollen tube growth and found a new compound, clethramycin (Fig. 1). We herein report on the screening, taxonomy and fermentation of the producing strain, and the isolation and biological properties of clethramycin. Structure determination of clethramycin will be described in the accompanying paper[3].

Fig. 1. Structure of clethramycin.



* Corresponding author: furumai@pu-toyama.ac.jp

Materials and Methods

Microorganism

Strain TP-A0623, the clethramycin-producer, was isolated from a wild plant of *Clethra barbinervis* collected in Toyama prefecture, Japan. The root of the plant was cut into pieces of *ca.* 3 cm in length. They were successively immersed in 70% ethanol and 1% NaClO solution for 3 minutes. Then, they were rinsed with sterilized water and incubated on an agar plate consisting of agar 1.5%, amphotericin B 0.005% and methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate 0.02% at 32°C for 30 days. A colony of the strain TP-A0623 that grew out of a piece of the root was isolated and purified on an agar plate. A pure culture of strain TP-A0623 was preserved in 20% glycerol at −80°C. It was also maintained at 10°C for laboratory use as a slant on Bennett's agar.

Taxonomy

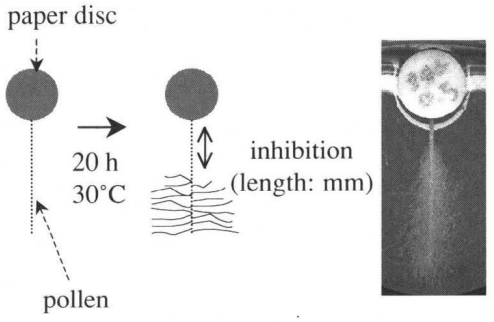
Taxonomic characteristics of strain TP-A0623 were determined by cultivation on various media described by SHIRLING and GOTTLIEB[4], WAKSMAN[5] and ARAI[6]. Morphological characteristics were observed after incubation of the culture at 30°C for 14 days on oatmeal agar (ISP med. 3) supplemented with 0.2% yeast extract. Cultural and physiological characteristics were determined after growth at 30°C for 14 days. The color names and hue numbers were assigned using the Manual of Color Names (Japan Color Enterprises Co., Ltd., 1987). The carbon utilization was determined by the method of SHIRLING and GOTTLIEB[4]. Cell wall composition was analyzed by the method of LECHEVALIER and LECHEVALIER[7], using thin layer chromatography plates as described by STANECK *et al.*[8].

Biological Assay

Pollen of the Japanese pear (*Pyrus* spp. cultivar Imagawaaki) was used for the assay. An agar plate containing sucrose 10%, $Na_2B_4O_7$ 0.01% and agar 1% (pH 6.3) was prepared and the pollen was placed in a line of 18 mm in length on the agar plate by using one edge of an 18 mm square cover glass. At the one end of the line of pollen, an 8-mm paper disc containing the test sample was placed. A number of pollen tubes grow almost perpendicularly to the line during incubation at 30°C for 20 hours in the dark. After the incubation, the pollen tube growth inhibition was measured under a light microscope. The strength of the inhibition was defined as the length (mm) of the zone in which the pollen tube growth was completely inhibited (Fig. 2).

In vitro antifungal activity was determined by the serial two-fold agar dilution method using RPMI 1640 medium containing 2% glucose (pH 7.0) with the inoculum size of 10^4 CFU/ml. MIC values were determined after the incubation at 32°C for 24 hours (*Candida albicans*) or 72 hours (other yeasts and fungus). *In vitro* antibacterial activity was determined using MUELLER-HINTON broth (Difco Laboratories) with the inoculum size of 10^5 CFU/ml. MIC values were determined after incubation at 37°C for 18 hours.

Fig. 2. Assay of pollen tube growth inhibition.



Results and Discussion

Screening

About one hundred fermentation extracts of actinomycetes isolated from the plant were subjected to the screening of the pollen tube growth inhibition assay. Among the six hits, strain TP-A0623 was chosen for its strongest activity. In the HPLC analysis of the extract from the strain, two classes of antibiotics were dereplicated based on the UV-vis spectrum and molecular mass matching. One includes antibiotics TAN 420C and TAN 420E and herbimycin A, and the other azalomycin B and its 11-*O*-methyl derivative.

Among these, herbimycin A and azalomycin B, and several commercially available antibiotics with the known mode of action were tested in this assay (Table 1). Pollen tube growth was inhibited by protein kinase and actin polymerization inhibitors, but not by the inhibitors of tublin depolymerization (paclitaxel), DNA polymerase (daunomycin) and protein synthesis (cycloheximide). In addition, two macrolides, filipin and azalomycin B inhibited the pollen tube growth whereas amphotericin B did not. Kinase inhibition activity of staurosporine and herbimycin

A accounts for their effect on pollen tube growth because the pollen germination is controlled by MAP kinases[9]. Therefore this assay system can be used for the detection of protein kinase inhibitors.

In addition to the dereplicated metabolites, we noticed the production of a hexaene antibiotic which showed pollen tube growth inhibition and antifungal activity against *C. albicans* in the fermentation extract. Although the production of hexaene antifungal antibiotics has been reported several times so far, few of them were characterized structurally. Therefore we attempted the isolation and structure determination of the hexaene antibiotic produced by strain TP-A0623.

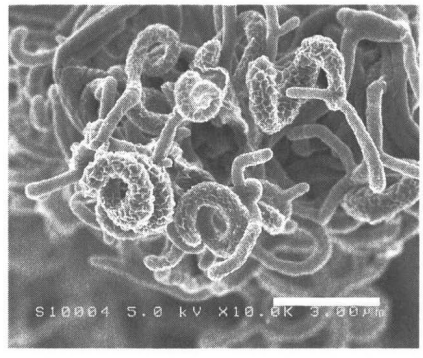
Table 1. Pollen tube growth inhibition by known antibiotics and clethramycin. (length: mm)

Compound	Amount (μg/disc) 50	5
Staurosporine	5.86	2.58
Herbimycin A	1.42	0.48
Geldanamycin	0.36	0
Cytochalasin D	2.38	0.22
Paclitaxel	0	0
Filipin	1.51	0.12
Amphotericin B	0	0
Azalomycin B	0.16	0
Daunomycin	0	0
Cycloheximide	0	0
Clethramycin	2.91	0.36

Taxonomy of the Producing Strain

By scanning electron microscope, spore chains are *Spirales*, and the spore surface is rugose (Fig. 3). The cultural characteristics are summarized in Table 2. The mass spore is gray and the color of the reverse side is dark brownish gray. Diffusible pigments were not formed. Inositol, L-rhamnose, D-mannitol, D-raffinose, D-fructose, D-xylose and L-arabinose were utilized by strain TP-A0623 for growth. Cellulose and sucrose were not utilized. Whole cell hydrolysates contained LL-diaminopimelic acid and glycine, and galactose, ribose and glucose as constituent amino acids and sugars, respectively. On the basis of these morphological and chemical characteristics, the strain TP-A0623 was identified as *Streptomyces hygroscopicus*.

Fig. 3. Scanning electron micrograph of *Streptomyces hygroscopicus* TP-A0623.



Bar represents 3 μm.

Table 2. Cultural characteristics of strain TP-A0623.

Medium	Aerial mycelium	Reverse side	Diffusible pigment	Growth
Yeast extract - malt extract agar (ISP med. 2)	Dark gray (417)	Dull yellow (150)	None	Good
Oatmeal agar (ISP med. 3)	Olive (164)	Dark brownish gray (127)	None	Good
Inorganic salts - starch agar (ISP med. 4)	Soft reddish yellow (146)	Dark reddish gray (418)	None	Good
Glycerol asparagine agar (ISP med. 5)	Light yellow (133)	Dark reddish gray (418)	None	Good
Peptone - yeast extract - iron agar (ISP med. 6)	Light reddish yellow (131)	Light reddish yellow (131)	None	Good
Tyrosine agar (ISP med. 7)	Pale yellow (128)	Reddish gray (408)	None	Good

Fermentation

A loopful of a mature slant culture of *S. hygroscopicus* TP-A0623 was inoculated into four 500-ml K-1 flasks containing 100 ml of the seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case (Wako Chemical USA, Inc.) 0.3%, yeast extract (Difco Laboratories) 0.2%, tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The flask was incubated at 30°C for 4 days on a rotary shaker (200 rpm). Three-ml aliquots of the seed culture were transferred into one hundred 500-ml K-1 flasks each containing 100 ml of the production medium consisting of soluble starch 2%, glucose 0.5%, glycerol 2%, Pharmamedia (Trader's Protein) 1.5%, yeast extract 0.3% and HP-20 (Mitsubishi Chemical Co.) 1.0%. Fermentation was carried out for 5 days at 30°C on a rotary shaker (200 rpm).

Isolation

The fermented whole broth (10 liters) was centrifuged (8,000 rpm, 10 minutes) to separate into the mycelium and supernatant. The mycelial cake was extracted with 50% aqueous methanol (5 liters) twice and the combined filtrates were evaporated *in vacuo*. The residual aqueous solution was combined with the supernatant, adjusted to pH 7 and loaded on a column of HP-20 (270×100 mm, i.d.). The column was eluted with a gradient of aqueous acetone (20, 40, 60, 80 and 100% acetone, 2 liters each). The activity was found in the fractions eluted with 40~100% acetone. The active fractions were combined and the acetone was removed by evaporation. The resultant aqueous solution was extracted with ethyl acetate. The aqueous layer was lyophilized to afford a brown powder (4.03 g) containing clethramycin. The crude powder (400 mg) was dissolved in DMSO and applied onto a column of ODS gel (200×40 mm, i.d., ODS-AM 120-S50, YMC Co., Ltd.). The column was eluted with 20~80% acetonitrile in 0.15% KH_2PO_4 buffer (pH 3.5) and the purity of active fractions was checked by HPLC. The combined fraction was adjusted to pH 6 with $NaHCO_3$ solution, evaporated and resultant aqueous solution was lyophilized. The resultant powdery material was extracted with a small amount of methanol and the extract was concentrated *in vacuo* to give pure clethramycin. The chromatography was repeatedly carried out and 112 mg of clethramycin was obtained from 10 liters of culture broth.

Table 3. Antimicrobial activity of clethramycin.

Organism	MIC (μg/ml)
Candida albicans TIMM1623	0.5
C. glabrata IFO0622	1
C. krusei IFO1395	1
C. tropicalis IFO1400	8
Cryptococcus neoformans ATCC90113	1
Aspergillus fumigatus TIMM0063	8
Staphylococcus aureus FDA209P	64
Enterococcus faecalis ATCC29212	>256
Escherichia coli NIHJ JC-2	256
Pseudomonas aeruginosa 3445	>256

Biological Properties

The antimicrobial activity of clethramycin is summarized in Table 3. Clethramycin showed strong activity against yeasts *Candida albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *Cryptococcus neoformans* and a fungus *Aspergillus fumigatus*, but very weak against Gram-positive and negative bacteria. Cytotoxic effect was observed at the rather higher concentrations. The IC_{50} was 57 μg/ml against HeLa cells and 120 μg/ml against WI-38 cells. Clethramycin showed no toxicity for male ICR mice (4 weeks old) by intraperitoneal administration at a dose of 10 mg/kg, but no therapeutic effect was observed with the experimental intravenous infection with *C. albicans* at the same dose.

Linearmycins, structural analogs of clethramycin, are reported to inhibit the spheroplast regeneration, namely the cell wall biosynthesis, of *C. albicans*[10]. Although the site of action of clethramycin is not elucidated, it might inhibit the biosynthesis or transport of the cell wall precursors in the pollen and yeast, considering its potent antifungal activity and structural resemblance to linearmycin.

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