

## INSTRUCTIONS FOR PREPARING MANUSCRIPTS/EXPANDED ABSTRACTS

All presenters are expected to submit either an expanded abstract or a full-length manuscript based on their talk or poster at the time of the meeting. (The expanded abstract or manuscripts are distinct from the brief abstract to be submitted previously.) These will be published in the Online Proceedings of the meeting. Detailed instructions for preparation of expanded abstracts and manuscripts follow.

### General Instructions

Manuscripts should be submitted during registration at the meeting on cd or diskette with one hardcopy. Please identify your cd/disk with last name, session time & date. Microsoft Word is the preferred format. In the event that MS Word is not available, Word Perfect is acceptable. All manuscripts should be single-spaced and “camera ready.” Please use the font Times New Roman and a pitch size of 12 points. Use American English spelling throughout the manuscript.

All full-length manuscripts should include the following: Abstract (brief, no more than 10% of the text), Introduction, Materials and Methods, Results and Discussion, Conclusions, and Literature Cited. If an Acknowledgement section is included it should follow the Results and Discussion. These major headings should be in bold type and all caps, on a separate line, and left justified. Secondary headings should be title case, bold, and left justified. If further subheadings are needed for clarification, they should be in italics and within the paragraph, followed with a period, and 2 spaces before the text begins.

Title of paper should be all caps, bold type, and flush left, same size font. List authors on separate line, using footnotes for complete addresses. Manuscripts and expanded abstracts should have 1-inch margins (top, bottom, left and right), 5 space indentation on all paragraphs. Manuscripts and abstracts are left justified only. Extended abstracts would follow the same format.

*Tables.* Each table should start with a title that describes the contents. Tables should appear within text, where first cited.

*Figures.* Figures should appear within text, where first cited. Figure captions are placed below the figure.

*Photographs.* Photos are acceptable since this will be an online proceedings. Please be sure they are of good quality.

# SCREENING OF POLLEN TUBE GROWTH INHIBITORS: IDENTIFICATION OF CLETHRAMYCIN FROM A PLANT-ASSOCIATED ACTINOMYCETE

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## ABSTRACT

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 *Streptomyces hygroscopicus* TP-A0623. The producing strain was isolated from a root of *Clethra barbinervis* collected in Toyama, Japan. Clethramycin showed *in vitro* antifungal activity against yeast such as *Candida albicans* and *C. glabrata* with the MIC of 0.5~8 µg/ml.

## INTRODUCTION

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 (Mascharenhas, 1993). This process is inhibited by cytochalasin or latrunculin B, an inhibitor of actin polymerization, and thus pollen tube growth is also inhibited (Gibbon, 1999). 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).

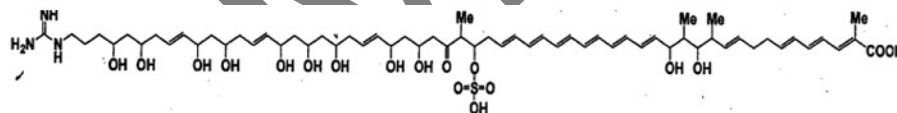


Fig. 1. Structure of clethramycin

## 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 min. 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.

**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

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soluble starch 1%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, tryptone 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 1.5%, yeast extract 0.3% and HP-20 resin 1.0%. Fermentation was carried out for 5 days at 30°C on a rotary shaker (200 rpm).

*Isolation.* The fermented whole broth was extracted with 50% aqueous methanol and the filtrate was evaporated *in vacuo*. The residual aqueous solution was adjusted to pH 7.0 and loaded on a column of HP-20. The column was eluted with aqueous acetone and the acetone was removed by evaporation. The aqueous layer was lyophilized to afford a brown powder. A portion of the powder was dissolved in DMSO and applied onto an ODS gel column. The column was eluted with 20~80% acetonitrile in 0.15%  $KH_2PO_4$  buffer (pH 3.5). The combined fraction was adjusted to pH 6, 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. By repeating the chromatography, 112 mg of clethramycin was obtained from 10 liters of culture broth.

*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).

## 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. Pollen tube growth was inhibited by protein kinase and actin polymerization inhibitors, but not by the inhibitors of tubulin 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 not. Kinase inhibition activity of staurosporine and herbimycin A is accountable for their effect on pollen tube growth because the pollen germination is controlled by MAP kinases (Wilson, 2000). 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.

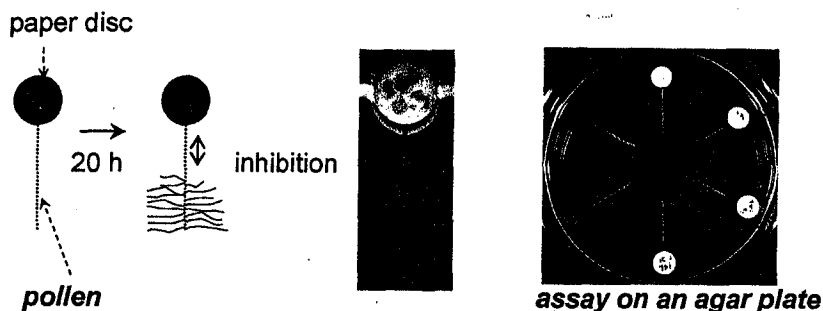


Fig. 2. Assay of pollen tube growth inhibition

**Structure Determination.** Clethramycin was obtained as a yellow powder, soluble in methanol and insoluble in chloroform and ethyl acetate. Its UV-vis spectrum showed a typical pattern of polyene antibiotics with the absorption maxima at 337, 356 and 378 nm, indicating the presence of a conjugated hexaene moiety. The FAB-MS measurement of clethramycin gave the parent ion peak  $[M-H]$  at  $m/z$  1216.7 in negative mode and  $[M+Li]^+$  at  $m/z$  1224.6 in the presence of lithium chloride in positive mode. In the negative mode FAB-MS/MS spectrum, distinctive fragment ions were observed at  $m/z$  97 and 80, indicating the presence of a sulfate group.

The  $^1H$  and  $^{13}C$  NMR spectra of clethramycin showed a characteristic feature of the reduced-type polyolefinic polyketide. Olefinic carbons and protons were observed in the narrow spectral regions and the peaks for more than twenty carbons were detected. In addition, fifteen methylene and twelve hydroxylated methine groups were detected. One singlet and three doublet methyl groups, presumably derived from the methylmalonate, were easily recognized in the spectrum. Since it was considered that most of the remaining carbons were derived from the malonate,  $^{13}C$ -labeled clethramycin was prepared by the fermentation fed with 1,2- $^{13}C_2$ -acetate. The 2D-INADEQUATE experiments in combination with HMQC and DQF-COSY confirmed the incorporation pattern of the acetate units. By precisely analyzing DQF-COSY, TOCSY, HMBC and ROESY, the overall carbon skeleton of clethramycin was determined as shown in Fig. 1.

Perhaps linearmycins (Sakuda, 1996) are the closest known compounds. The major differences between clethramycin and linearmycins are the presence of *o*-sulfate and guanidino functionalities in clethramycin. These structural differences seem to reflect on the biological properties. Linearmycin A shows more potent antimicrobial activity against bacteria than yeasts, whereas the trend in clethramycin is adverse.

**Biological Properties.** In addition to the pollen tube growth inhibition, 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  $\mu g/ml$  against HeLa cells and 120  $\mu g/ml$  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*. Although the site of action of clethramycin is not elucidated, it might inhibit the biosynthesis or transport of the cell wall precursors both in the pollen and yeast, considering its potent antifungal activity and structural resemblance to linearmycin.

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# DOES PREHARVEST APPLICATION OF NAA INDUCE RIPENING ON CLIMACTERIC TREE FRUIT?

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## ABSTRACT

1-Naphthaleneacetic acid (NAA) has been an effective bioregulator for the control of preharvest fruit abscission on apples and pears for more than 60 years. Although it has been reported NAA at super-optimal rates for control of preharvest drop may advance ripening, data reported herein suggest this may not be the case. Like ethylene, NAA promotes fruit abscission in the early spring, presumably by an ethylene-induction mechanism. Because ethylene also stimulates fruit ripening in the fall, is it tempting to assume NAA acts similarly on mature fruit tissue, even though ethylene promotes whereas NAA inhibits fruit abscission. When applied to individual 'Delicious' spurs 2 weeks before commercial harvest, fruit treated with ACC produced ethylene sooner than untreated fruit, whereas those treated with NAA produced less than controls (Fig 1.). These data indicate both leaves and fruit retain the ability to produce ethylene from

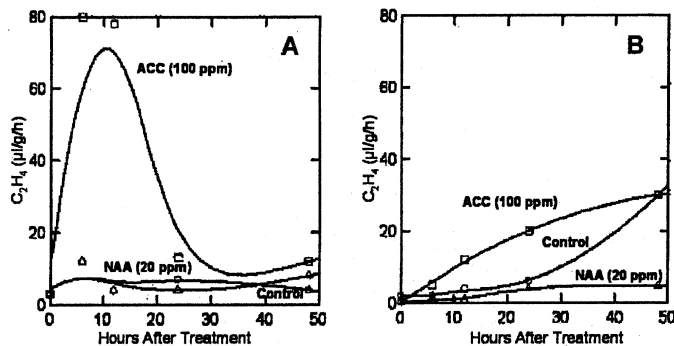


Fig. 1. Ethylene response of 'Delicious' spur leaves (A) and fruit (B) to NAA or ACC applied 2 weeks before commercial harvest.

ACC. NAA does not appear to induce ethylene in these tissues, and in fact suppresses ethylene production in whole fruit. In a related trial, 'Delicious' trees receiving a 4X treatment of NAA (80 ppm) 2 weeks before commercial harvest were examined for changes in quality. Treated fruit had similar soluble solids, firmness, and rate of starch conversion, but a marked reduction in ethylene increase two weeks after initial treatment (Data not shown).

Opposing fruit abscission responses to preharvest ethylene or NAA, therefore, may be due to: 1) differential tissue sensitivity or response to NAA; 2) differential auxin status across the abscission zone created by the changing absorptive surface areas of leaves and fruit; or 3) NAA-induced inhibition of ethylene in the stem abscission zone as is evidenced in fruit tissue (Fig. 1). As seen in the figure to the right, when all 'Delicious' fruit were removed 2 weeks after treatment, mean starch rating was similar between those from treated and control trees. Apparent is the absence from the untreated controls of fruit with more advanced starch clearing, i.e., those fruit which probably dropped prematurely (Fig. 2). Indeed, that NAA advances ripening may be a perception based on the artificial retention of fruit of advanced maturity.

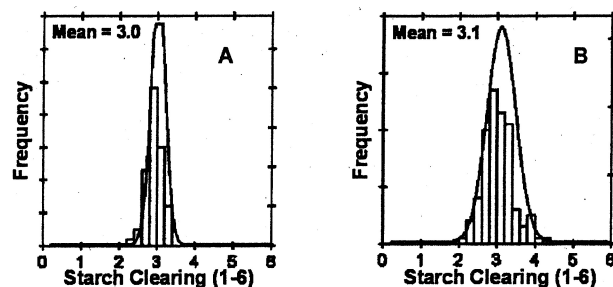


Fig. 2. Starch clearing ratings of control (A) and NAA-treated (80 ppm) (B) fruit showing similar means but different distributions.

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