



Purification and characterization of Pseudomycin, a phytotoxin and antimycotic produced by *Pseudomonas syringae*
by Leslie Ann Harrison

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology
Montana State University
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Abstract:

Many microorganisms produce secondary metabolites which affect other organisms sharing their ecological niche. Such may be the case when a plant pathogen invades its host. *Pseudomonas syringae*, a plant pathogenic bacterium, produces a number of such metabolites one of which has not been previously purified and identified.

This thesis describes the isolation and characterization of a small peptide from *Pseudomonas syringae* called pseudomycin. The peptide was purified using acetone precipitation, standard column chromatography, and high performance liquid chromatography. Final characterization was by means of fast atom bombardment (FAB) mass spectroscopy and amino acid analysis.

Once purified, interest lies in the biological activity of the compound. Pseudomycin was tested on a number of monocots and dicots and found to be selectively phytotoxic. In addition this peptide formed zones of inhibition when oversprayed with suspensions of fungal mycelia or bacteria, demonstrating antimicrobial activity against both plant and human pathogens.

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PURIFICATION AND CHARACTERIZATION OF PSEUDOMYCIN,
A PHYTOTOXIN AND ANTIMYCOTIC PRODUCED BY
PSEUDOMONAS SYRINGAE

by

Leslie Ann Harrison

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

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ABSTRACT

Many microorganisms produce secondary metabolites which affect other organisms sharing their ecological niche. Such may be the case when a plant pathogen invades its host. Pseudomonas syringae, a plant pathogenic bacterium, produces a number of such metabolites one of which has not been previously purified and identified.

This thesis describes the isolation and characterization of a small peptide from Pseudomonas syringae called pseudomycin. The peptide was purified using acetone precipitation, standard column chromatography, and high performance liquid chromatography. Final characterization was by means of fast atom bombardment (FAB) mass spectroscopy and amino acid analysis.

Once purified, interest lies in the biological activity of the compound. Pseudomycin was tested on a number of monocots and dicots and found to be selectively phytotoxic. In addition this peptide formed zones of inhibition when oversprayed with suspensions of fungal mycelia or bacteria, demonstrating antimicrobial activity against both plant and human pathogens.

Microbial metabolites may or may not play a role in plant disease. By studying bacterial metabolites we may gain a greater understanding of host/pathogen interactions in plant disease and therefore evaluate effective treatment. Applications may also include use as selective natural product herbicides or fungicides for the treatment of plant or human disease.

CHAPTER I

INTRODUCTION

The pseudomonad group of bacteria are found ubiquitously as free living saprophytes in soils, water, and in association with both plants and animals. These organisms play roles which can be at opposite ends of the biological spectrum. Some pseudomonad strains actively mineralize organic material, a great benefit to the economy of nature (25). At the same time many are causative agents of disease in man, animals, and plants.

Numerous diseases with a wide range of symptoms may be caused by phytopathogenic pseudomonads. These include necrotic lesions and spots on fruit, stems, and leaves; hyperplasias (galls, scabs); tissue maceration (rots); cankers; blights; and vascular infections (wilts). Pseudomonad incited plant diseases can be found worldwide and affect most major groups of plants (25).

Pseudomonas syringae is principally known as a group of unspecialized foliar pathogens which survive in association with the host plant and propagative material of the host plant. Bacterial blight caused by P. syringae van Hall is a foliar disease of wheat (Triticum aestivum L.), in the northern United States (43). This disease affects spring

and winter wheat in Minnesota, South Dakota, and Montana (32,34). Yield losses from bacterial leaf blight are incompletely assessed, but foliage destruction may exceed 50% under ideal environmental conditions (43). The symptoms include grey green necrosis and bleaching of leaves, glumes, sheaths, and culms. This disease is more often found in wheat, but is occasionally found in barley (16).

P. syringae in stone and pome fruits exists in lesions, cankers, or tumors. One of the more serious canker diseases, particularly in California, is caused by P. syringae. This bacterium is most active in the fall and winter months, producing cankers which originate primarily in nodal areas. After infection, the bacterial cells multiply and move quickly through cortex tissues producing cankers which appear as depressed, water soaked areas, with a brown color and sour smell (36).

There are at least two features which enhance the pathogenicity of P. syringae (36). First, in culture and in infected plant tissue it may produce a toxin that destroys host cell membranes and thus contributes to symptom development (5,7). The site of action of one of these toxins, syringomycin, has been investigated. Bidwai, et al.(2), demonstrated preferential stimulation of vanadate-sensitive ATPase activity of the plasma membrane of red beet storage tissue. The second feature is the capability of some strains to act as nuclei for formation of ice crystals.

At temperatures just below 0° C, most plants that grow in temperate zones escape significant frost damage because water in their tissues remains in liquid form, supercooled. If ice nucleating bacteria are present, ice crystals form and disrupt plant tissue, resulting in typical disease symptoms. *P. syringae* in combination with freezing conditions causes severe damage to plants that would not be harmed by either agent alone (36).

Symptoms produced as a result of bacterial toxins have been documented since the early portion of the twentieth century. Identification of the chemical nature of such toxins, however, has been very limited. Prior to 1970 no correct toxin structure had been published (21). One of the first investigations was that by Braun and Woolley of the wildfire toxin (tabtoxin) and its involvement in the wildfire disease of tobacco (21). In recent years chemical structures of toxins from a number of bacterial pathogens have been elucidated.

There has been a number of definitive studies concerning toxin production as a part of the disease process in *Pseudomonas* infections. For example, halo blight of beans, caused by *P. phaseolicola*, is mediated by a toxin (12). Several researchers characterized phaseolotoxin from *P. phaseolicola*, as a tripeptide composed of ornithine, alanine, and homoarginine with a phosphosulfamyl group attached to the ornithine (20,38,39). A number of other

toxins are elaborated by members of the P. syringae group; including tabtoxin (28,29,37), coronatine (19,23), syringomycin, syringotoxin (10), tagetitoxin (18,22), and several that are still uncharacterized. The strains that produce these toxins and the structural characteristics are summarized in a review by Mitchell (21).

Those toxins that are of particular interest in relation to this study are syringomycin and syringotoxin. These two compounds are peptidyl, broad spectrum antibiotics and phytotoxins isolated from ecotypic strains of P. syringae. Preparations of syringomycin yielded amino acids tentatively identified as serine, phenylalanine, an unidentified basic amino acid and arginine in a 2:1:2:1 molar ratio (10). The original structure was confirmed and the unidentified amino acid was identified as 2,4-diaminobutyric acid in studies reported in a recent paper by Ballio et al. (1). Syringotoxin, isolated from a strain of P. syringae from a citrus host, contained substances tentatively identified as threonine, serine, glycine, ornithine, and the again originally unidentified amino acid, 2,4-diaminobutyric acid (21).

Dr. Bruce Hemming, a former researcher in our laboratory, demonstrated the production of a toxin by an isolate of P. syringae, obtained from barley (Hordeum vulgare), which showed many similarities to syringomycin and syringotoxin. The partially purified toxin was found to be

ninhydrin positive, have a small molecular weight, and demonstrated antimycotic activity (11).

Thus, the first goal of this research project was to develop a method of purification for this toxin. Previous attempts at thin layer chromatography and high performance liquid chromatography separation had failed. Initial toxin preparations seemed to be fairly unstable which confounded the interpretation of data. The method of choice for purification must separate the toxin or unknown toxin from all other components without drastically altering its bioactivity.

The second goal was to biologically characterize this toxin. This toxin and several previously described toxins, showed antimycotic activity. It is necessary to know which of the plant pathogenic fungi are sensitive to the toxin to consider application. The same is true for human fungal pathogens.

CHAPTER II

PURIFICATION AND CHEMICAL CHARACTERIZATION OF PSEUDOMYCIN

Introduction

Before any biological activity can be studied in depth a certain line of cause and effect must be established. Is the effect due to the compound of interest or due to a small contaminant carried along in the procedure? If an unknown can be isolated to purity, then identified, this cause and effect relationship can be established.

Purification of the toxin, tentatively called pseudomycin, has been a challenge due to loss of biological activity at various stages of purification. Conditions such as pH, heat stability, and solvent solubilities must be considered. In addition, there are problems such as ionic exchange or nonspecific irreversible binding to glass, silica, or even column packing material.

During the experimental stages of purification, a variety of methods were attempted for the separation of pseudomycin. For example, a common step in separation science before high performance liquid chromatography, is preparative thin layer chromatography. This method (Appendix), was attempted with some success, but proved

inefficient when compared to later techniques. The main problem seemed to be lack of elution of the sample from the silica. Successful elution was accomplished eventually by using 3N hydrochloric acid or 3N trifluoroacetic acid as the eluant. Surprisingly, this did not appreciably decrease the activity of the compound.

Alternate methods for toxin purification were used with varying results. Some of these techniques may be useful in future research. For this reason several are briefly presented in the Appendix.

Presented in this chapter is a purification scheme which is fairly uncomplicated and results in several milligrams of purified toxin per liter of culture medium extracted. Following the description of the purification are the chemical analyses that led to the identification of a small peptidyl phytotoxin.

Materials and Methods

Bacterial Strains

Pseudomonas syringae MSU 174, isolated from a Montana barley field, was obtained from Dr. David Sands, Montana State University. In an attempt to increase toxin production, MSU 174 was mutagenized by transposon mutagenesis using TN 905 (13). A "super producer" was isolated and was called strain 206. Strain 206 was used by Dr. Rudy Scheffer to test the biocontrol aspects of

P. syringae against Dutch Elm Disease. One year after the initiation of the experiment, samples were taken from the tree branches for the reisolation of strain 206. Strain 206 was reisolated and positively identified by Southern blot techniques. The strain now produced a larger zone of inhibition with the bioassay and was labeled P. syringae MSU 16H. This is the strain that was used in the purification of pseudomycin.

P. syringae MSU 16H was stored in 15% glycerol at -70° C. Transfers were made every two weeks to Kings B media (Appendix) and grown at room temperature.

Butanol Extraction

P. syringae 16H was grown in shake culture (180 rpm), for six days at 23° C in Dyes media supplemented with FeCl_3 , ornithine, and histidine (Appendix). Cultures were centrifuged at 5000 X g for 10 min. to pellet the cells. The supernatant liquid was flash evaporated to 200 ml at 38° C, then extracted three times with an equal volume of 1-butanol. The butanol fraction was extracted with water, then the butanol phase was flash evaporated to dryness at 38° C. The dried concentrate was resuspended in methanol for transfer, then dried under nitrogen for storage at -20° C (Appendix, Figure 8).

Acetone precipitation

Cells were grown in PDB as a still culture at 23° C for

6 days. Cultures were mixed 1:1 (v/v) with acetone then centrifuged at 5000 X g for 10 minutes. The liquid supernatant was flash evaporated to 200 ml then brought to a final concentration of 60% acetone. This mixture was allowed to precipitate overnight at 4° C with gentle stirring. The precipitate was removed by centrifugation at 5000 X g for 10 minutes. The liquid supernatant was flash evaporated to dryness then resuspended in one liter of 0.1% TFA (Appendix, Figure 9).

Amberlite column

Amberlite XAD-2 (mesh size 20-60) was washed with 0.1% TFA, then packed in a 1.6 X 40 cm column. The column was equilibrated with 0.1% TFA. The sample that was resuspended in one liter 0.1% TFA (butanol or acetone procedure), was loaded onto the column at 1 ml/min. with a Waters M25 solvent delivery system. Pseudomycin was eluted with a nonlinear gradient of 0 to 100% 1-propanol with 0.1% TFA according to Bidwai, et al. (2) (Appendix, Figure 10). The gradient former was a Kratos Spectroflow 430. Fractions were collected with a Gilson microfractionator and tested for bioactivity. Active fractions were pooled, dried with the rotary evaporator, and resuspended in 50% 1-propanol containing 0.1% TFA. Final drying of small quantities, before storage at -20° C, was accomplished by placing the sample under a flow of nitrogen gas.

Geotrichum candidum bioassay

The sample (10 μ g) was applied to a PDA plate (Appendix) and allowed to dry. The plate was oversprayed with a sterile water suspension of Geotrichum candidum, sealed with parafilm, and incubated overnight at room temperature. Zones of complete inhibition were noted.

Geotrichum candidum was kindly provided by Dr. Don Mathre, Montana State University.

HPLC Purification

Active fractions from the Amberlite column were filtered through a 0.2 micron filter (Gelman Sciences) then subjected to reverse phase high performance liquid chromatography (RP-HPLC) on a 4.6 X 100 mm C8 column (Amicon MC-250) with a 1-propanol, nonlinear gradient (0 to 30% 1-propanol, 0.1% TFA, Waters #2 gradient over 35 min.). The flow rate of the mobile phase was 1 ml/min. Each peak was collected, evaporated, and resuspended in 50% 1-propanol with 0.1% TFA before being bioassayed. Single active peaks were combined then reinjected for further purification.

Collections from a single peak were again subjected to RP-HPLC utilizing a second solvent system. The sample was eluted with a linear, acetonitrile, 0.1% TFA gradient (Waters #6, 0 to 80% acetonitrile, 0.1% TFA) over a 20 minute period.

Purification of larger quantities of pseudomycin was done on a 10 X 250 mm Amicon MC-250 C8 preparative column with a flow rate of 2 ml/min.

With both columns, a Waters system was used including a model 440 absorbance detector monitoring at a wavelength of 254, and a model 660 solvent programmer. Two solvent delivery systems were required, a model M45 and a model A6000.

Collections from each peak to be used for biological testing were flash evaporated and resuspended in 50% 1-propanol containing 0.1% TFA. Samples for mass spectrometry were flash evaporated then concentrated, but not dried, under nitrogen. Samples for amino acid analysis were collected from the HPLC directly into polypropylene cryovials, and assayed directly without further concentration.

Syringomycin

Purified syringomycin was kindly provided by Dr. John Takemoto at Utah State University.

FAB Mass Spectrometry

Fast atom bombardment (FAB) mass spectrometry was performed by Joe Sears, department of chemistry, Montana State University. Analysis was with a VG MassLab Trio2 automated mass spectrometer connected to a Hewlett Packard model 5890 gas chromatograph. The column was a 30 meter P-5 microcolumn. The sample was run in both a glycerol and

thioglycerol matrix.

Nuclear Magnetic Resonance

The proton nuclear magnetic resonance (NMR) spectrum was recorded on a 500 MHz Bruker spectrometer. Chemical shifts were recorded in ppm units relative to trimethylsilane (0 ppm) with D₂O. NMR spectrometry analysis was kindly provided by Dr. Ed Dratz, department of chemistry, Montana State University.

Absorbance Characteristics

Pseudomycin, dissolved in 0.1% TFA, was scanned on a Beckman DU-50 Spectrophotometer. The solution (100 μ l) was weighed on a Cahn model G electrobalance to obtain a value for the calculation of the molar concentration. The molar extinction coefficient was calculated using the maximum absorbance and the molar concentration. The scan and weighing were repeated three times and the average result was recorded.

Thin Layer Chromatography

The RP-HPLC sample of pseudomycin (3 μ g) was spotted onto a silica gel 60 F254 thin layer plate (E. Merck Science). The same amount of syringomycin was spotted adjacent to the pseudomycin for a comparative standard. TLC plates were run in three separate solvent systems; 1) 1-butanol:pyridine:acetic acid:water, 2) 1-butanol:2-

picoline:acetic acid:water, and 3) 1-butanol:2,6-lutidine:acetic acid:water. The ratio was the same for each system, 15:10:3:12, (v/v). TLC plates were sprayed with ninhydrin reagent; 0.5% ninhydrin in 95% ethanol (v/v). The compounds appeared as light purple spots.

Amino Acid Analysis

Samples for amino acid analysis and sequencing were sent to Dr. David Teplow at the California Institute of Technology. All of the instrumentation for both amino acid analysis and sequencing was by Applied Biosystems Inc. For the amino acid analysis, a model 420 derivatizer coupled to a model 130 HPLC system was utilized. Data was acquired and analyzed by a model 920 software system. Amino acid sequencing was done with a model 477 sequencer with on line PTH amino polythiohydantoin (PTH) amino acid analysis. Peak quantitation was performed by software supplied with the model 477 sequencer. Peak identification was done manually by comparison to standard chromatograms.

Heat Stability

A 1-butanol crude extract (75 μ l) of pseudomycin, at a concentration of 33 μ g/ μ l, was placed into each of eight 1.5 ml Eppendorf tubes (or ReactivialsTM for the 100° C test). Tubes were then incubated at each of four temperatures (15, 30, 60, and 100° C) for six days. Samples (5 μ l) were removed from each tube at 0, 0.5, 1, 2, 4, 8, 24, 48, 72,

and 96 hours and spotted on a PDA plate. After drying, the plates were oversprayed with G. candidum, then incubated at room temperature overnight. Inhibition was recorded as plus or minus at each time and temperature.

pH Sensitivity

A 1-butanol crude extract (5 μ l), at a concentration of 100 mg/ml, was dissolved in 200 μ l of the treatment solution (Table 1). The concentration of each buffer was 0.01 M. Each buffer was adjusted to a pH equal to its pK_a with 1 M sodium hydroxide or 1 M hydrochloric acid. The samples were incubated at room temperature for four days. At 0, 1, 2, 3, and 4 days, each solution (10 μ l) was spotted onto PDA and bioassayed. All treatments were run in duplicate. The positive control was the 1-butanol extract dissolved in water and methanol. The negative controls were the buffers without the extract.

Table 1. Buffers for testing pH sensitivity of the toxin.

Buffers	pH
1. Citric Acid	3.06
2. Citric Acid	4.75
3. Ammonium Acetate	4.75
4. Citric Acid	5.40
5. Pipes	6.80
6. Tris	8.00
7. Tris	9.00
8. Boric Acid	10.00

Pseudomycin Stability in Various Solvents

A 1-butanol crude extract (5 μ l at 33 μ g/ μ l) was added to 100 μ l of solvent. Each solvent (100 μ l) alone was used for the negative control. The solutions were incubated at room temperature for 24 hours, at which time they were dried under nitrogen then resuspended in 15 μ l of methanol and 10 μ l were removed for bioassay.

The solvents tested were: 1-propanol, (50% and 100%); TFA, (1, 2, and 3 M); HCl, (0.5, 1, 2, and 4 M); 0.1% TFA, 10% acetonitrile; 0.1% TFA, 90% acetonitrile; 100% 1-butanol; 100% methanol; 100% water; and 0.1% TFA.

Results and Discussion

Bioassay

Before a protocol for toxin purification can be adopted, a good bioassay must be developed which is preferably both sensitive and quantitative. At least two methods are described in the literature for testing syringomycin; 1) antimycotic activity against Rodotorula pilimanae, and 2) antimycotic activity against Geotrichum candidum. Both organisms are fast growing and show good sensitivity to the phytotoxin. The overspray method utilizing G. candidum was the method of choice because of the availability of the organism. Fractions resulting from the various purification steps were spotted onto PDA (Appendix) and allowed to dry. The plate was oversprayed

with a sterile water suspension of the fungus then incubated overnight at room temperature. Presence of toxin was indicated by a clear zone of inhibition. Weak zones of inhibition, that eventually overgrew with mycelia, were considered negative.

Known amounts of crude extract were spotted for assay on separate PDA plates. Great variation in zone size was noted depending on the age of the plate and the amount the solution spread on the plate. In an attempt to reduce variation, the sample was applied to antibiotic filter discs which were then laid on the agar plate before overspraying. The toxin adsorbed to the disc, preventing diffusion into the agar plate. This method was not suitable for the assay. It was therefore inaccurate to quantitate the amount of toxin present according to zone size. Results in the bioassays were recorded as plus or minus.

Purification

Solvents used during the purification procedures could affect the activity of the toxin. To check for possible changes in activity, 165 μg of 1-butanol crude extract was added to 10 μl of the solvent to be tested. Each vial was incubated for 24 hours at room temperature then dried under nitrogen and resuspended in 15 μl of methanol. An aliquot (10 μl) was removed from each vial and bioassayed. Each solvent was tested without the crude extract as a negative

control. Pseudomycin remained active in all solvents tested except the 4 M HCl (Table 2). This may be due to either a low pH value or a high salt concentration resulting from the evaporation step. TFA, at 3 M, and the remaining concentrations of HCl, also decreased the zones of inhibition.

Table 2. Sensitivity of toxin to different solvents. (-) = no zone of inhibition (+) = a zone of inhibition < 1 cm. dia. ++ = a zone of inhibition > or = 1 cm. dia.

Solvent	Treatment	Control
100% 1-propanol	++	-
50% 1-propanol	++	-
3 M TFA	+	-
2 M TFA	++	-
1 M TFA	++	-
0.5 M TFA	++	-
0.1% TFA, 10% acetonitrile	++	-
0.1% TFA, 90% acetonitrile	++	-
0.5 M HCl	+	-
1 M HCl	+	-
2 M HCl	+	-
4 M HCl	-	-
100% 1-butanol	++	-
100% methanol	++	-
100% water	++	-
100% water, 0.1% TFA	++	-

Heat stability was tested at four different temperatures; 15, 30, 60, and 100° C (Table 3). Butanol crude extract (75 μ l), at a concentration of 33 μ g/ μ l was incubated at each temperature for six days. Throughout the incubation period, 5 μ l aliquots were removed and bioassayed. The toxin showed heat stability for up to four days when incubated at 15 or 30° C. When incubated at 60° C

there was total loss of activity by the fourth day. When the extract was incubated at 100° C there was a large decrease in the zone of inhibition for toxin heated 30 minutes and a complete loss of activity for toxin heated four hours. This toxin demonstrates considerable heat stability.

Table 3. Heat stability of the toxin. (+) = zones of inhibition < 2 cm. (++) = zones of inhibition > or = 2 cm. (-) = no apparent zone of inhibition.

Temp. ° C	Time (hours)									
	0	0.5	1	2	4	8	24	48	72	96
15	++	+	++	++	+	+	++	+	+	+
30	++	++	++	++	++	+	++	+	+	+
60	+	+	+	+	+	+	+	+	+	+
100	++	+	+	+	-	-	-	-	-	-

Previous observations indicated the loss of toxin activity at pH values less than 6.5 (Dr. Avi Nachmias, unpublished). Partially purified pseudomycin (5 μ l), at a concentration of 100 mg/ml, was dissolved in 200 μ l of water, methanol (positive control), and each of eight different buffers (0.01 M). In addition, one tube of each treatment without the extract was prepared as a negative control. Each buffer was adjusted to a pH equal to its pK_a . Each solution was incubated at room temperature. After 0, 1, and 2 days, 10 μ l of each solution was spotted onto PDA and bioassayed. Pseudomycin remained active in the water, methanol, and the buffers at or below a pH of 4.75 (Table 4). Activity in buffers at or above pH 5.4 was apparent

initially but the zones of inhibition were overgrown in two days. The static inhibition was also noted in the same control treatments and may be due to high pH values. These were recorded as negative results.

Table 4. Sensitivity of activity to pH changes.

Treatments		pH
1. Citric Acid		3.06
2. Citric Acid		4.75
3. Ammonium Acetate		4.75
4. Citric Acid		5.40
5. Pipes		6.80
6. Tris		8.00
7. Tris		9.00
8. Boric Acid		10.00

Results								
Day	1	2	3	4	5	6	7	8
0	+	+	+	+	+	+	+	+
1	+	+	+	+	+	-	-	-
2	+	+	+	+	+	-	-	-
3	+	+	+	+	+	-	-	-
4	+	+	+	+	+	-	-	-

Cultures of *P. syringae* MSU 16H were initially grown for six days in shake culture in modified Dyes media supplemented with ferric chloride, ornithine, and histidine (Appendix). Previous research indicated that ferric chloride at concentrations of 2 μ M increased production of the toxin from *P. syringae* MSU 174 (11) and of syringomycin (9). Hemming (11) examined toxin production after the addition of individual amino acids. Both histidine and ornithine significantly increased toxin production.

Cells were removed from culture by centrifugation. To

determine if the majority of the toxin was extruded from the cells, pelleted cells were checked for toxin content. The cells were washed twice by suspending them in water, then centrifuging. Lysis was accomplished by first freezing at -70°C , then thawing, repeating the action twice. Second, the suspension was sonicated in a Kontes sonicator for 10 minutes. Cellular debris was resuspended in 5 ml of water, then 10 μl was spotted onto PDA and bioassayed as described above. No zones of inhibition were noted. All of the toxin appeared to have been extruded from the cells.

The culture supernatant liquid was extracted with 1-butanol. The toxin partitioned into the butanol phase which was subsequently flash evaporated to dryness then resuspended in methanol (Appendix, Figure 8). An average of 0.227 grams of crude extract was recovered per liter of culture (Table 5).

Table 5. Purification table for the butanol extraction procedure.

Stage of Purification	grams dry weight per liter	purification fold
Centrifuge supernatant	1.657	1
Butanol extract	0.227	7
Amberlite XAD-2	0.0066	251
RP-HPLC	0.0002	8285

At a later date a more efficient purification protocol was adopted. This protocol was a slight modification of that published by Bidwai, et al. (2), for the purification

of syringomycin.

Cells were grown for six days in still culture in PDB at room temperature. The culture was mixed 1:1 (v/v) with acetone then centrifuged to remove cellular debris. The supernatant was first flash evaporated to 200 ml, then acetone was added to 60% (v/v). The mixture was allowed to precipitate overnight. Precipitate was removed by centrifugation. Toxin activity remained in the supernatant liquid. The supernatant liquid was concentrated, then resuspended in one liter of 0.1% trifluoroacetic acid (TFA), and loaded onto an Amberlite XAD-2 column (Appendix, Figure 9). After elution with a propanol gradient, the fractions were bioassayed.

Positive fractions were combined, filtered, and subjected to reverse phase high performance liquid chromatography (RP-HPLC). Two different solvent systems were utilized; 1) a 1-propanol nonlinear gradient, and 2) an acetonitrile linear gradient. Propanol has been recommended as the solvent of choice for the separation of peptides and proteins because the concentrations needed for elution are lower than with other organic modifiers, reducing the chance of activity loss. Acetonitrile is a good choice for the same reasons. TFA (0.1%) is necessary for adjustment of the pH (see Chapter III) and acts as an ion pairing agent. TFA modifies the polarity of the peptide through ion-pair formation which leads to an increase in retention time and

therefore, better separation (26,42). Volatility of TFA is also a positive aspect when considering amino acid analysis and sequencing.

The propanol gradient was a nonlinear (Waters #2) gradient of 0 to 30% 1-propanol, 0.1% TFA over 35 minutes. Each peak from the propanol gradient was collected, concentrated by flash evaporation, then tested for bioactivity. Peaks 3, 4, and 6 were found to be active (Figure 1). The eluate collected from 16 to 20 minutes (labeled #5 in Figure 1) was also active but no definite peak was seen on the elution profile. The retention times are listed in Table 6. These peaks were reinjected for further purification.

Table 6. Retention times of peaks from the HPLC after elution with a nonlinear propanol gradient.

Peak number	retention time
3	13 min. 30 sec.
4	14 min. 45 sec.
5	-----
6	22 min. 0 sec.

The final step was to subject the eluate from peak #4 to the RP-HPLC with the linear acetonitrile gradient, 0 to 80% acetonitrile over 20 minutes. The retention time was 14 minutes and 45 seconds (Figure 2). This procedure yielded an average of 0.0015 grams of purified toxin as compared to 0.0002 grams with the butanol procedure (Tables 5 and 7).

Figure 1. RP-HPLC elution profile of partially purified pseudomycin from the Amberlite XAD-2 column.

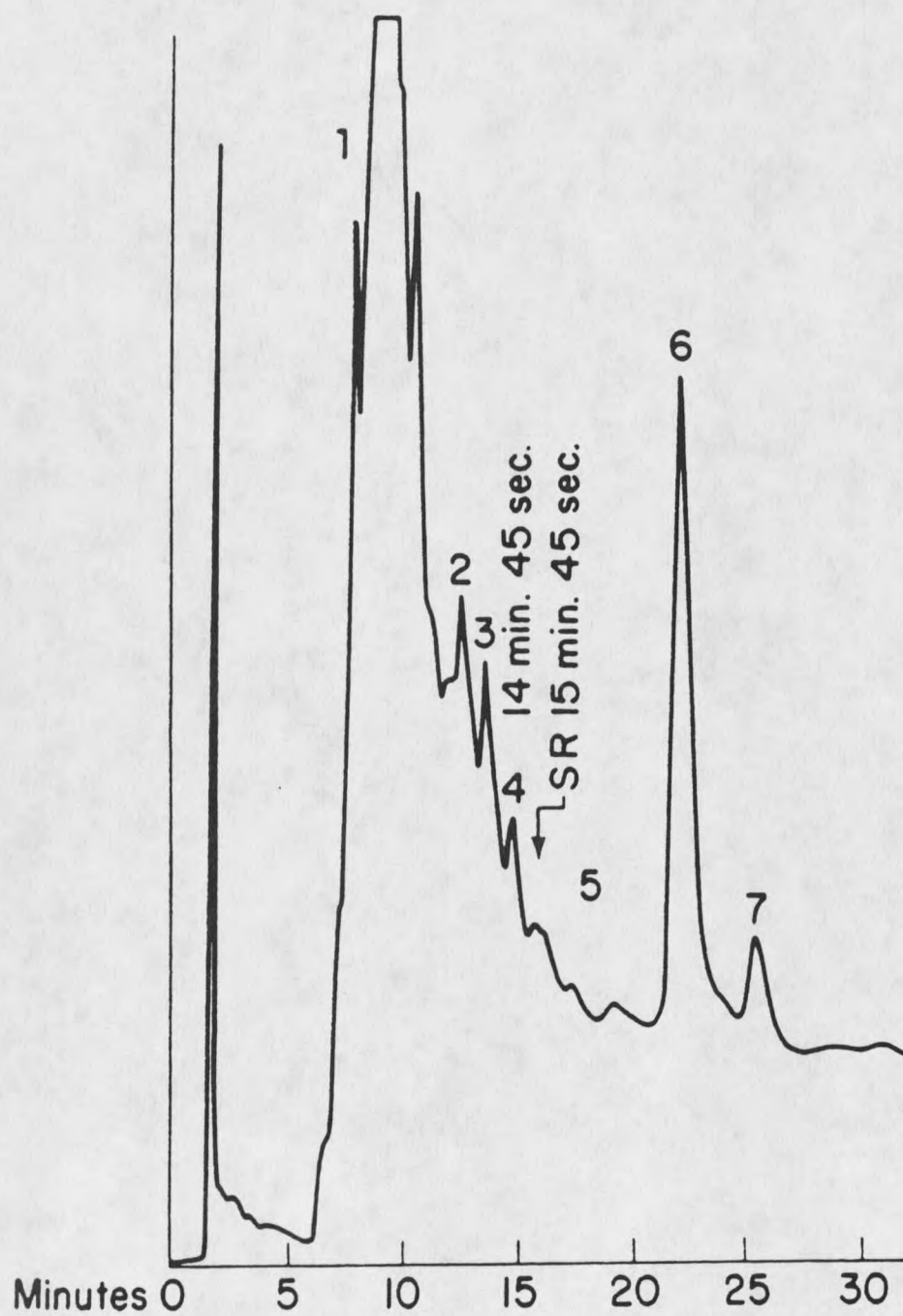
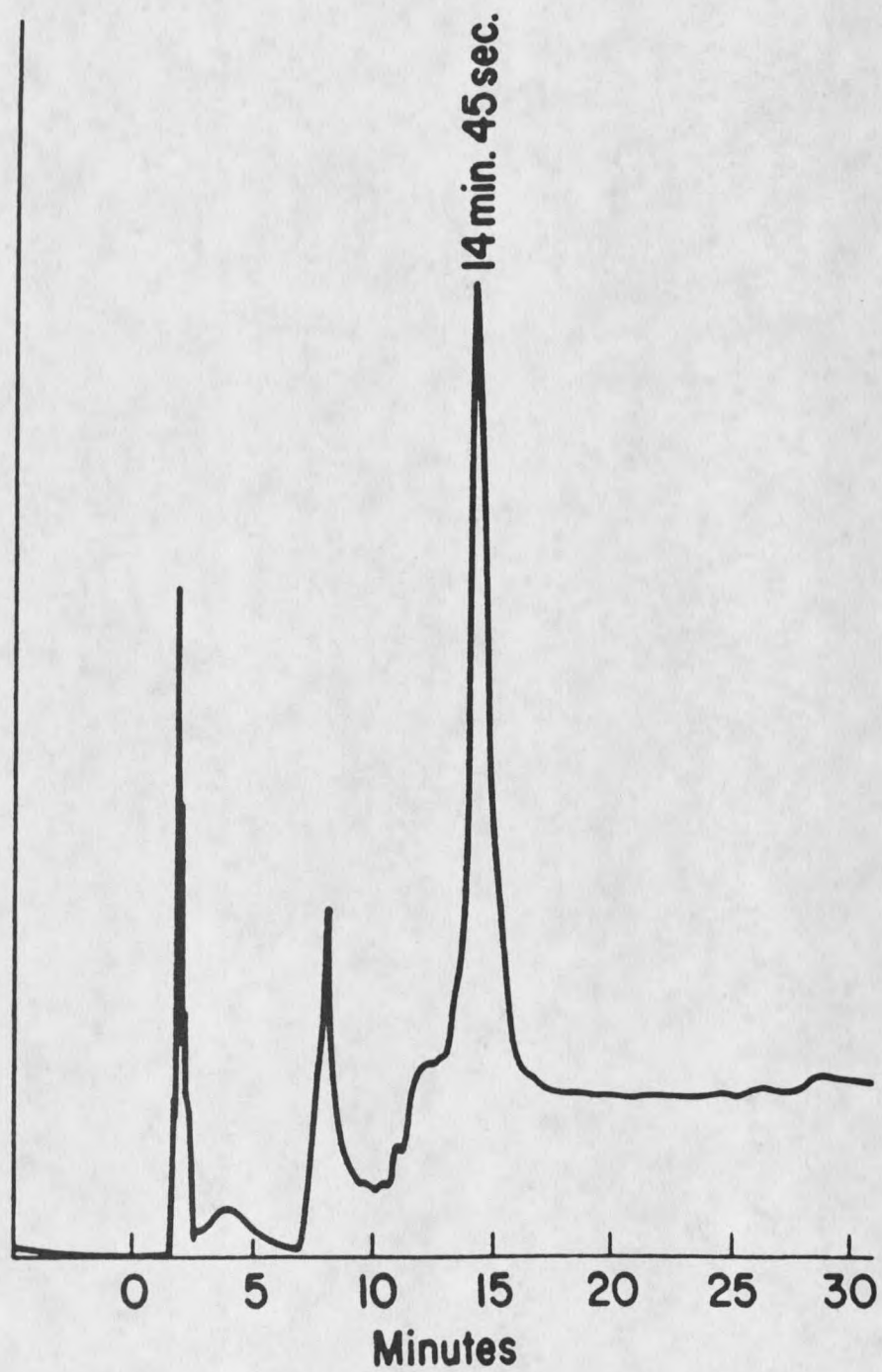


Figure 2. RP-HPLC elution profile of pseudomycin run on a linear gradient of 0-80% acetonitrile over 20 minutes.



Both the 1-butanol extraction method and the acetone precipitation method resulted in the isolation of the same compound as confirmed by thin layer chromatography and mass spectrometry data.

Table 7. Purification table for the acetone precipitation procedure.

Stage of Purification	Grams of Dry Weight per liter	Purification Fold
PDA culture	27.6	1
1 st acetone ppt.	15.0	1.8
2 nd acetone ppt.	12.8	2.2
Amberlite XAD-2	0.016	1,725
RP-HPLC	0.0015	18,400

As a preliminary check, the eluate collected at the time of each peak from the propanol gradient, was subjected to FAB mass spectrometry. HPLC peak number 4 was the only peak that appeared to be pure, having a single mass ion peak. Mass spectra data from 3 and 5 had more than one peak, one of which had the same mass ion assignment as the peak from HPLC peak number 4. Spectral data for HPLC peak number 6 also showed several peaks but none at the same mass as HPLC peak number 4. This may suggest a second antimycotic compound in these preparations.

PDB alone was carried through the same purification steps as the *P. syringae* culture. There were no detectable toxins present in PDB alone.

Confirmation of Purity

Purity is mandatory for the proper identification of an unknown compound. A single peak upon elution from the HPLC column is suggestive of purity but in itself is not enough. Thin layer chromatography (TLC) is one method for examining purity. Peptides are markedly hydrophilic compounds and are only slightly soluble in nonaqueous solvents. Solvent systems used in their separation must generally contain water. Butanol: formic acid: water (75:15:10) was first utilized as suggested by Brenner (3). This solvent system was inadequate because it did not substantially move the compound away from the origin. Butanol:pyridine:acetic acid:water (15:10:3:12), was the second solvent of choice. The RP-HPLC sample (3 μ g) was spotted on to a silica gel TLC plate. Syringomycin was also spotted on the plate for a comparative standard. Both compounds migrated as single spots with R_f 's of .59 and .37, respectively (Figure 3). Not only did pseudomycin appear pure, but it migrated differently than syringomycin. To further establish purity, pseudomycin was run in two additional solvent systems; picoline or lutidine was substituted for pyridine. These solvents are methylated analogs of pyridine. Again single spots were apparent with R_f values as indicated in Figure 3.

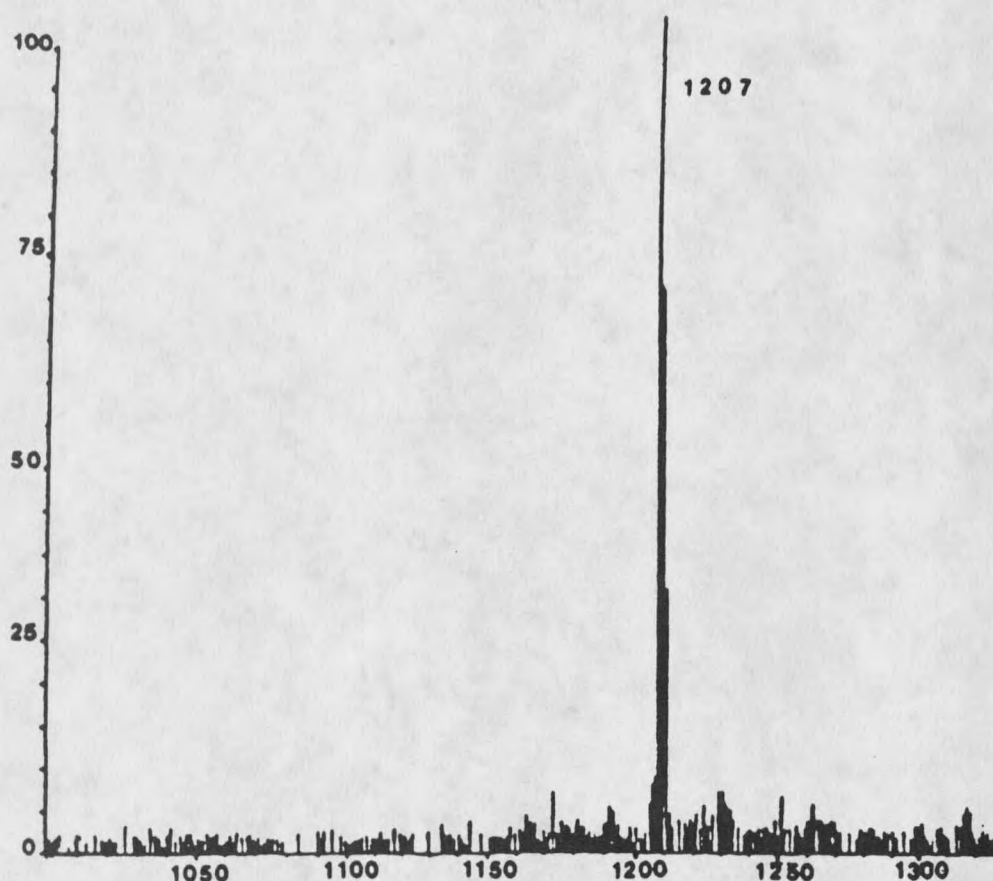
Figure 3. Thin layer chromatography of pseudomycin.



Chemical Characterization

The purified sample was subjected to Fast Atom Bombardment (FAB) mass spectrometry. Mass spectrometry utilizing electron bombardment for peptides requires chemical degradation and derivatization, while FAB mass spectrometry makes it possible to analyze underivatized large polar biomolecules (12). The sample was run in two different matrices, glycerol and thioglycerol. The mass H^+ was found to be 1207 (Figure 4). The same sample was used for amino acid analysis and sequencing. Preliminary results for pseudomycin indicate the presence of seven amino acids; aspartate, serine, lysine, phenylalanine, and arginine (1:1:1:3:1) and one unknown amino acid. The amino acid analysis of syringomycin confirmed published data; arginine, phenylalanine, serine, and 2,4-diaminobutyric acid (1:1:2:2).

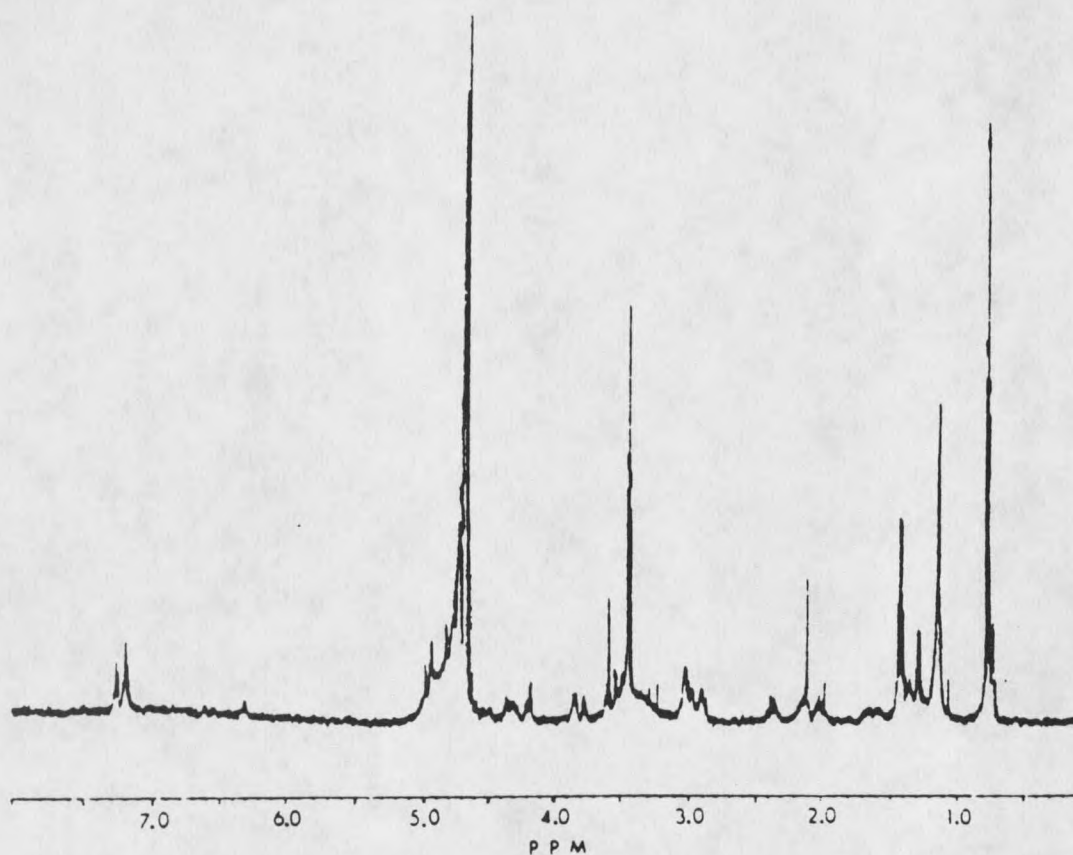
Figure 4. FAB mass spectra of purified pseudomycin.



Using post column derivatization, with the Applied Biosystems instrumentation described in the materials and methods, the fourth amino acid in syringomycin, 2,4-diaminobutyric acid, is indistinguishable from phenylalanine. The ^1H -NMR spectrum (Figure 5) indicates the presence of protons on an aromatic ring in the range of 7.1-7.4 ppm which may be from phenylalanine. This suggests that phenylalanine is one of the amino acids but does not rule out the presence of 2,4-diaminobutyric acid. The sum of the molecular weights of the suspected amino acids is considerably lower than the mass obtained from the FAB mass

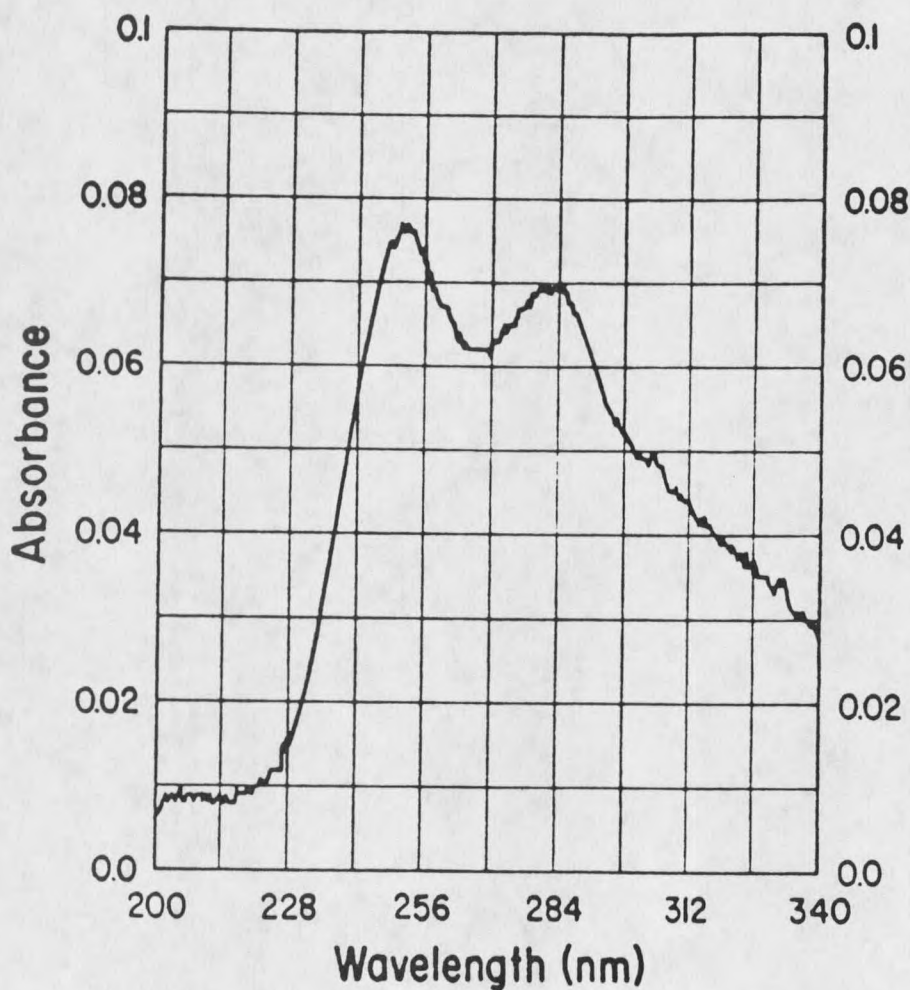
spectrometry data. This may be due to the presence of a long chain fatty acid attached to the peptide, similar to the one found in syringomycin (1)

Figure 5. ^1H -NMR spectrum of pseudomycin.



Pseudomycin, dissolved in 0.1% TFA, was scanned using a spectrophotometer. There were two peaks of absorbance (Figure 6); one at 254 nm and one at 280 nm. The solution (100 μ l) was weighed on an electrobalance. The molar extinction coefficient was calculated for each wavelength; $A_{m254} = 167$, $A_{m280} = 146$.

Figure 6. Spectrophotometric scan of pseudomycin dissolved in 0.1% TFA.



CHAPTER III

BIOLOGICAL STUDIES

Introduction

Biological systems, unlike controlled chemical environments, are constantly changing entities; not always predictable by the investigator. Plant disease can involve two such systems, the host and the parasite, each with its own set of variables. To isolate and study a single variable may be impossible. To minimize this difficulty one could first study each component individually.

The first step was to study growth characteristics of *P. syringae* and its toxin production in the absence of its host. This included a growth curve, the time of maximum toxin production and possible induction of toxin production by compounds from plants or fungi affected by the toxin.

The second step was to study the antagonism of the toxin towards other organisms. Similar toxins such as syringomycin have demonstrated both antimycotic and phytotoxic affects (8). These aspects were also studied with pseudomycin and are discussed in this chapter.

Materials and Methods

Growth Curve

Data for a growth curve was gathered for modified Dyes media (mDyes, Appendix). Two tubes, each with 3 ml of mDyes was inoculated with a single colony of P. syringae MSU 16H then grown in shake culture (180 rpm) at room temperature, overnight. After 24 hours, these cultures contained $\approx 6 \times 10^7$ org./ml and were used to inoculate two side arm flasks containing 250 ml of mDyes. The bacteria were grown in shake culture, at room temperature, for a total of six days. At timed intervals (0.5, 1, 2, 4, 8, 24, 48, 72, and 96 hours) Klett readings were taken on a Klett-Summerson model 800-3, photoelectric colorimeter. Klett readings were averaged then recorded as Klett units versus time (hours). The instrument was zeroed with uninoculated media.

The same procedure was repeated to obtain a growth curve with PDB in still culture.

Production Over Time

Fifteen 100 ml aliquots of PDB, in 300 ml flasks, were inoculated with a 3 ml overnight PDB culture. Still cultures were grown in triplicate, at room temperature, for 0, 3, 6, 9, or 12 days before fractionation by acetone precipitation. The dry weight of each fraction was recorded. The preparation was resuspended in 50% 1-propanol with 0.1 % TFA at concentrations of 500, 50, 5, 0.5 and 0.05

ug/ul. Each solution (10 μ l) was spotted onto a PDA plate then bioassayed. Activity was recorded as plus or minus at each concentration and time.

Induction by Geotrichum

Four of six one liter flasks containing 200 ml of mDyes media, were inoculated with G. candidum and allowed to grow overnight on the shaker at 23° C. The other two were not inoculated, but otherwise were handled in the same manner. After turbid growth of the cultures (24 hr.), all six flasks were autoclaved for 20 min. at 120° C. After cooling, two uninoculated and two inoculated flasks were secondarily inoculated with P. syringae 16H. The flasks were incubated on the shaker at room temperature for 6 days before extraction with 1-butanol. Each extract was resuspended in methanol at a concentration of 100 μ g/ μ l then spotted onto a PDA plate for bioassay.

Induction by Arbutin

Nine one liter flasks, containing 500 ml of media, were prepared as follows: three flasks of PDB, three flasks of PDB with 1 mM arbutin, and three flasks with 0.001 mM arbutin. All flasks were inoculated and allowed to grow as previously described. After six days, each culture was harvested and pseudomycin was isolated by the acetone precipitation procedure. The partially purified pseudomycin was dissolved in 50% propanol with 0.1% TFA at

concentrations of 500, 50, 5, 0.5, 0.05, and 0.005 $\mu\text{g}/\mu\text{l}$. Each (10 μl) was spotted onto PDA and bioassayed.

Plant Material

The sources of the plant material used for testing phytotoxic activity of the toxin are listed in Table 8. All plants were grown under controlled conditions with 8 hr. of darkness at 28° C and 16 hr. of light at 32° C per day.

Table 8. Sources of Plant Materials.

Common Name	Genera	Plant Material	Source
Corn	<u>Zea</u>	Seed	Burpee's
Rice	<u>Oryza</u>	Seed	Gary Strobel, MSU*
Wheat	<u>Triticum</u>	Seed	MSU seed lab
Sugarcane	<u>Saccharum</u>	Whole Plant	Gary Strobel, MSU
Timothy	<u>Phleum</u>	Seed	Doug Kenfield, MSU
Tall fescue	<u>Festuca</u>	Seed	Doug Kenfield, MSU
Crabgrass	<u>Digataria</u>	Seed	Doug Kenfield, MSU
Sunflower	<u>Helianthus</u>	Seed	Burpee's
Tomato	<u>Lycopersicon</u>	Seed	Burpee's
Cucumber	<u>Cucumis</u>	Seed	Burpee's
Geranium	<u>Geranium</u>	Whole Plant	Ernst's Home Center
Carob	<u>Cerantonia</u>	Seed	Tunisia
Crown of Thorns	<u>Euphorbia</u>	Whole Plant	Gary Strobel, MSU
Sicklepod	<u>Cassia</u>	Seed	Doug Kenfield, MSU

*MSU-Montana State University

Assay for Phytotoxic Activity

Individual leaves were placed in a sterile petri dish and kept at high humidity by placing moist filter paper in the bottom of the dish. The solution to be assayed was dissolved in 0.05% TFA and applied to the leaf using a leaf puncture droplet overlay technique. An excised leaf was

punctured using a Hamilton syringe, then a drop of the test sample was applied to the puncture wound. When testing partially purified pseudomycin from the acetone precipitation procedure, 1, 10, and 100 μg of the solution was applied. For purified pseudomycin, 1, 5, and 10 μg were applied. The control was 0.05% TFA and was applied in the same manner. Each petri dish was sealed with parafilm and incubated for a maximum of 4 days at room temperature, under light.

Fungal Strain Source

The source of the fungal strains used to test antimycotic activity are listed in Table 9.

Table 9. Fungal strain sources.

<u>Fungus</u>	<u>Source</u>
<u>Cephalosporium gramineum</u>	Dr. Donald Mathre, MSU*, MT
<u>Pyrenophora teres</u>	Dr. Mike Bjarko, MSU, MT
<u>Pyrenophora graminea</u>	Dr. Mike Bjarko, MSU, MT
<u>Rynchosporium secalis</u>	Alfredo Martinez, MSU, MT
<u>Ceratocystis ulmi</u>	Dr. Gary Strobel, MSU
<u>Rhizoctonia solani</u>	ATCC 28268
<u>Botrytis alli</u>	UCD 1159
<u>Sclerotinia sclerotiorum</u>	Dr. David Sands, MSU
<u>Verticillium dahliae</u>	Dr. Donald Mathre, MSU, T-9
<u>Verticillium dahliae</u>	Dr. Donald Mathre, MSU, SS-4
<u>Thielaviopsis basiola</u>	Dr. Donald Mathre, MSU, CA
<u>Fusarium oxysporum</u>	ATCC E16322
<u>Fusarium culmorum</u>	Bill Grey, MSU, MT
<u>Fusarium graminearum</u>	Bill Grey, MSU, MT

*MSU-Montana State University

Assay for Antimycotic Activity Against Plant Pathogenic Fungi

Partially purified pseudomycin (after Amberlite XAD-2 column) was dissolved in 50% 1-propanol with 0.1% TFA at a concentration of 10 $\mu\text{g}/\mu\text{l}$. The solution (10 μl) was spotted onto the appropriate solid medium (Table 10) and allowed to dry. The solvent (10 μl) alone was spotted adjacent to the pseudomycin as a negative control. Each plate was oversprayed with a sterile water suspension of the fungus, sealed with parafilm, and incubated accordingly (Table 10).

Table 10. Growth conditions used for plant pathogenic fungi in the assay for antimycotic activity.

<u>Organism</u>	<u>Media</u>	<u>Temperature</u>
<u>Rynchosporium secalis</u>	Lima Bean	15°
<u>Ceratocystis ulmi</u>	PDA	23°
<u>Cephalosporium</u> sp.	PDA	23°
<u>Pyrenophora teres</u>	PDA-V8	15°
<u>Pyrenophora graminea</u>	PDA-V8	15°
<u>Rhizoctonia solani</u>	PDA	23°
<u>Botrytis alli</u>	PDA	23°
<u>Sclerotinia sclerotiorum</u>	modified CD	23°
<u>Verticillium albo-atrum</u>	PDA	23°
<u>Verticillium dahliae</u>	PDA	23°
<u>Thielaviopsis basicola</u>	PDA	23°
<u>Fusarium oxysporum</u>	Corn Meal	23°
<u>Fusarium graminearum</u>	Corn Meal	23°
<u>Fusarium culmorum</u>	Corn Meal	23°

Assay for Antibacterial Activity

Three genera of bacteria were tested for their sensitivity to pseudomycin; 1) Pseudomonas syringae MSU 16H, 2) Xanthomonas campestris, and 3) Corynebacterium michigenense. The media used for testing was Kings B for P.

syringae and X. campestris, and Cory medium for C. michigenense (Appendix). Pseudomycin preparation (10 μ l) from Amberlite XAD-2 column, at concentrations of 250, 25, 2.5, 0.25, and 0.025 μ g/ μ l, were spotted onto the solid media and allowed to dry. The plates were oversprayed with a bacterial suspension in sterile water ($\approx 2 \times 10^{11}$ org./ml), then incubated at room temperature for 48 hours.

Stability of Pseudomycin in Human Serum

Human whole blood was allowed to clot, then centrifuged at 5000 X G to separate the serum from the cells. Partially purified pseudomycin was suspended in the serum, in duplicate tubes, at concentrations of 50, 5, 0.5, 0.05, and 0.005 μ g/ μ l. The toxin was dissolved in water, at the same concentrations, for the control. One set of tubes was incubated for two days at room temperature and one set at 37° C. The toxin was tested for activity at 24 and 48 hours using Geotrichum as the assay organism.

Endotoxin Assay

E-toxate (Limulus polyphemus amoebocyte lysate, Sigma) was used for the detection of endotoxin.

All glassware was soaked in a 4% solution of dimethyldichlorosilane, then rinsed thoroughly with double distilled water. The glassware was then heat cured at 100° C overnight.

Twelve of the silanized tubes were prepared as

indicated in Table 11. The pseudomycin, water, and endotoxin standard dilutions were added first, followed by the E-toxate working solution. Each tube was mixed gently, then incubated for one hour undisturbed at 37° C. After incubation, the tubes were removed, one at a time, and slowly inverted 180 degrees while observing for evidence of gelation. A positive test is the formation of a hard gel which permits complete inversion of the tube or vial without disruption of the gel. All other results were considered negative.

Minimum Inhibitory Concentrations for Human Fungal Pathogens

One milligram of purified pseudomycin was sent to Dr. Mike Rinaldi at the Audie L. Murphy Memorial Veterans' Hospital at San Antonio, Texas, to determine the minimum inhibitory concentration (MIC) towards human fungal pathogens. A serial dilution tube test was used in which the toxin was diluted out in tubes containing equal amounts of the fungus to be tested which were suspended in the appropriate medium. The tubes were incubated at room temperature or 30° C for the mould fungi or yeast fungi, respectively. After incubation, the last tube in the dilution series which inhibited the growth of the fungi, was recorded as the minimum inhibitory concentration.

Table 11. Contents of tubes for the endotoxin assay. All samples were dissolved in endotoxin freewater and adjusted to pH 6.5 with endotoxin free NaOH.

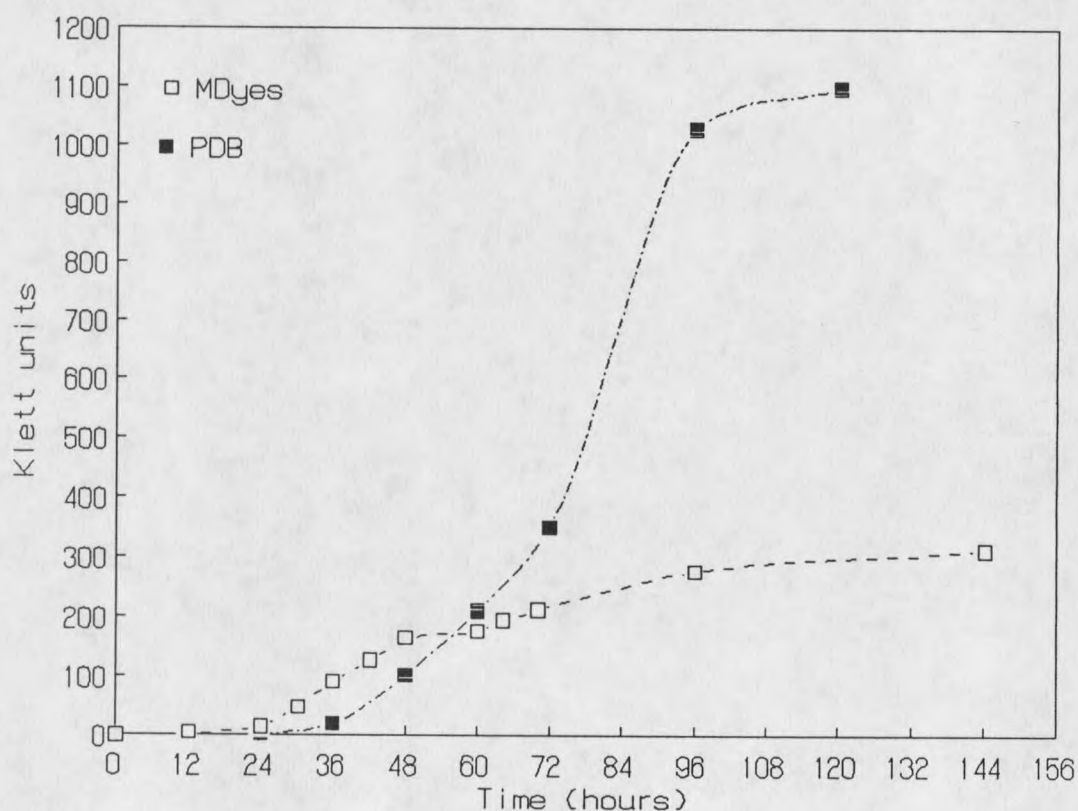
	<u>Tubes</u>						<u>Sample</u>					
	A	B	C	D-H			1	2	3			
	Tests for endotoxin in sample	Tests for etoxate inhibitor in sample	Negative control	Standard dillutions			BuOH crude extract	eluted from TLC plate	Amberlite XAD-2 column			
Contents	A1	A2	A3	B1	B2	Tubes B3	C	D	E	F	G	H
Sample	.1 ml	.1 ml	.1ml	.1ml	.1ml	.1 ml						
Water							.1 ml					
Endotoxin Standard				.004 μ g	.004 μ g	.004 μ g		.008 μ g	.004 μ g	.002 μ g	.001 μ g	.0005 μ g
Etoxate	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml

Results and Discussion

Growth of *P. syringae* Correlated with Toxin Production

Growth of *P. syringae* MSU 16H followed the "normal" sigmoidal growth pattern when grown in mDyes medium (Figure 7), (Appendix).

Figure 7. Growth curve for *P. syringae* 16H grown as still culture in PDB and shake culture in mDyes medium.



mDyes is a minimal medium so growth may be slower than in a high nutrient medium. The bacteria remained in lag phase for up to 24 hours at which time they entered logarithmic growth. Growth tapered off and entered stationary phase at

about 96 hours. A similar growth pattern resulted with PDB, however the bacteria reached log phase and stationary phase later, 36 hours and 144 hours respectively (Figure 7). The PDB culture also reached a higher density than the mDyes culture. This is most likely because it is a still culture rather than a shake culture. Sometimes it is advantageous to use a minimal media when growing cultures for natural product isolation, to minimize extraneous compounds requiring separation from the natural product. In this case, the yield of pseudomycin from cultures of P. syringae grown in PDB, as compared to the yield from P. syringae grown in mDyes, is so much greater that the advantage of simplicity is lost (Tables 5 and 7).

To examine toxin production over time, fifteen, 100 ml aliquots of PDB, were inoculated with a 3 ml overnight culture of P. syringae. The cultures were grown in triplicate as still cultures at room temperature for 0, 3, 6, 9, and 12 days before fractionation by acetone precipitation. Each fraction was dried under nitrogen and weighed. The preparation was resuspended in 50% 1-propanol with 0.1% TFA at concentrations of 500, 50, 5, 0.5 and 0.05 $\mu\text{g}/\mu\text{l}$. Each of these (10 μl) was spotted onto PDA and bioassayed (Table 12). The presence of toxin was detected as early as 6 days when a total of 500 μg was spotted (10 μl of 50 $\mu\text{g}/\mu\text{l}$). The activity seemed to remain the same until the termination of the experiment at 12 days. The bacterial

culture is entering the stationary phase of growth at 6 days (Figure 7) so the toxin may be a secondary metabolite.

Table 12. Production of pseudomycin over time. (+) = zone of inhibition, (-) = no zone of inhibition.

Day	Dry Wt. (g/100ml)	Concentration ($\mu\text{g}/\mu\text{l}$)				
		500	50	5	0.5	0.05
0	2.32 +/- .01	-	-	-	-	-
3	1.67 +/- .09	-	-	-	-	-
6	1.36 +/- .47	+	+	-	-	-
9	1.10 +/- .18	+	+	-	-	-
12	1.01 +/- .30	+	+	-	-	-

Growth of P. syringae along with toxin production was checked in three other types of media: Dyes salts with either 1.5% glucose, 0.5% yeast extract (YE), or 2% proteose peptone #3 (PP) (wt/v). P. syringae did not grow in Dyes salts plus 1.5% glucose. There was good growth in the Dyes salts plus YE, but the zone of inhibition seemed static (slowly overgrown). With the Dyes salts plus PP, there was no clear zone of inhibition. No further experimentation followed.

Induction of Toxin Production

Some toxin producing plant pathogenic fungi lose their ability to produce their toxin in the absence of the host plant or constituents of the host plant (15). In other cases there may be some constitutive production of the toxin, but the amount produced is increased in the presence of plant material (4). This phenomena has also been

observed with some, but not all, strains of P. syringae (27, personal communication, Dr. Dennis Gross). Production of syringomycin has been increased up to 1000 fold. The host compound which stimulates production of syringomycin is arbutin (4-hydroxyphenyl- β -D-glucopyranoside) which can be isolated from the leaves of blueberry, cranberry and pear trees (44).

Pseudomycin was grown as previously described in nine flasks with 500 ml of PDB. Four of the flasks were supplemented with arbutin; two at a final concentration of 1 mM and two at 0.001 mM. The cultures were carried through the acetone precipitation procedure up to the second precipitation step. The preparations were then dried under nitrogen and resuspended in 50% 1-propanol, 0.1% TFA at concentrations of 500, 50, 5, 0.5, and 0.05 mg/ml. Each preparation was bioassayed. Arbutin did not stimulate toxin production in P. syringae MSU 16H. This may be related to the fact that this strain was isolated from barley rather than pear leaves and that syringomycin and pseudomycin are not chemically identical.

P. syringae may produce this toxin as a microbial antagonist toward other microorganisms, and this may be induced by the challenging organisms. Four of six flasks with 200 ml of PDB were inoculated with G. candidum and allowed to grow overnight at room temperature. They were then autoclaved and allowed to cool. Two of the flasks with

G. candidum and two without were inoculated with P. syringae. The cultures were allowed to grow for 6 days, as previously described, then extracted with 1-butanol. The extracts were resuspended in methanol at a concentration of 100 $\mu\text{g}/\mu\text{l}$ and 10 μl of each was spotted onto PDA and bioassayed. There was no difference in activity noted. Conceivably, better tests may be to use supernatant from a G. candidum that has been filtered or to allow both organisms to grow simultaneously before extraction.

Antibiotic Activity

Recorded observations of antagonistic interactions between different bacteria probably date from those of Pasteur and Joubert who noted the inhibitory effect of bacteria from urine on Bacillus anthracis (41). Many of these studies explored possibilities of controlling diseases with nonpathogenic antagonistic microorganisms. This idea can be applied to both plant pathogens and/or human pathogens.

Pseudomycin was first tested for antimycotic activity against a selection of plant pathogenic fungi. Partially purified pseudomycin (10 μl) in 1-propanol, 0.1% TFA (10 $\mu\text{g}/\mu\text{l}$) was spotted onto the appropriate solid media (Table 10). The plate was then oversprayed with a sterile water suspension of the fungus, then incubated. Incubation time and temperature varied according to the fungus to be tested

(Table 10). Pseudomycin, at this concentration did not inhibit all the genera of fungi tested (Table 13); Cephalosporium sp., both Pyrenophora spp., Botrytis alli and Fusarium graminearum tested negative. The antimycotic sensitivity is not necessarily genus specific, as can be seen in the Fusarium group. This suggests that sensitivity to pseudomycin may have some usefulness as a tool for Fusarium classification.

Table 13. Pseudomycin antimycotic activity towards plant pathogenic fungi.

Fungus	Activity
<u>Rynchosporium</u> <u>secalis</u>	+
<u>Ceratocystis</u> <u>ulmi</u>	+
<u>Cephalosporium</u> sp.	-
<u>Pyrenophora</u> <u>teres</u>	-
<u>Pyrenophora</u> <u>graminea</u>	-
<u>Rhizoctonia</u> <u>solani</u>	+
<u>Botrytis</u> <u>alli</u>	-
<u>Sclerotinia</u> <u>sclerotiorum</u>	+
<u>Verticillium</u> <u>albo-atrum</u>	+
<u>Verticillium</u> <u>dahliae</u>	+
<u>Thielaviopsis</u> <u>basicola</u>	+
<u>Fusarium</u> <u>oxysporum</u>	+
<u>Fusarium</u> <u>graminearum</u>	-
<u>Fusarium</u> <u>culmorum</u>	+

The same screen as described for the fungi was used to test the toxin sensitivity of three plant bacterial pathogens; 1) Corynebacterium michigenense, a gram positive organism, 2) Xanthomonas campestris, a gram negative organism, and 3) P. syringae MSU 16H, the organism that produces the toxin. Pseudomycin (10 μ l) was spotted onto Cory media (Appendix) for C. michigenense and King's B for

X. campestris and P. syringae, at concentrations of 250, 25, 2.5, 0.25, and 0.025 $\mu\text{g}/\mu\text{l}$. They were then oversprayed with $\approx 2 \times 10^{11}$ org./ml and incubated at room temperature for 48 hours. Both X. campestris and C. michigenense were inhibited by a total of 2.5 mg and weakly inhibited by a total of 0.25 mg. These are relatively high concentrations required for inhibition. P. syringae was not inhibited by the toxin.

Phytotoxic Activity

Partially purified pseudomycin (from Amberlite XAD-2 column) was used to test phytotoxicity in a total of six different monocots and seven different dicots. One hundred, 10, and 1 μg of pseudomycin, dissolved in 0.05% TFA was applied to a puncture wound on the leaf surface. After incubation for four days at room temperature in a moist petri dish, the leaves generally developed zones of necrosis surrounded by an area of chlorosis. This is similar to the symptoms that develop with leaf blight. In addition the monocots displayed brown runners and the dicots veinal necrosis. All of the monocots were affected by the toxin. Three of the dicots were not affected at these concentrations; carob (Cerantonia), cucumber (Cucumis), and geranium (Geranium) (Table 14).

Table 14. Phytotoxicity of partially purified pseudomycin. (N) = necrosis, (C) = chlorosis, (V) = vein necrosis, (R) = brown runners, (W) = water soaking, (G) = green island. The response was estimated to be weak (+), moderate (++), or severe (+++). (-) = no difference relative to the control.

Monocots	Amount Applied (μ g)			Dicots	Amount Applied (μ g)		
	100	10	1		100	10	1
Timothy	NCR +++	NCR +++	NCR ++	Sunflower	NC ++	NC ++	NC +
Corn	NCW +++	NCW +++	NCW ++	Carob	--	--	--
Tall Fescue	GCR +++	GCR +++	GCR ++	Cucumber	--	--	--
Crabgrass	WNC +++	WNC +++	WNC +++	Tomato	NV ++	NV ++	--
Sugarcane	NR +++	NR +++	NR +	Geranium	--	--	--
Wheat	NC +++	NC +++	NC +	Crown of Thorns	NV +++	NV ++	NV +
				Sicklepod	NV +++	NV ++	NV +

Due to the limited availability of purified toxin, only four monocots and four dicots were tested with the purified sample in the same manner. Three of the four dicots tested were affected at all concentrations (Table 15). The fourth, timothy (Phleum), showed no adverse effects at any of the concentrations. This is contradictory to the test with partially purified extract. This may be due to concentration or a second toxin. All dicots tested developed zones of necrosis. Cucumber, which did not show zones of necrosis before, now showed zones of necrosis. This is hard to explain unless there is some kind of inhibitor specific for cucumber in the partially purified extract.

Table 15. Phytotoxic activity of purified pseudomycin. Pseudomycin ($0.58 \mu\text{g}/\mu\text{l}$) was applied to the leaf surface in 17, 8.5, and $1.7 \mu\text{l}$ quantities. (+) = reaction to toxin, (-) = no reaction to toxin.

Monocots	$1\mu\text{g}$	$5\mu\text{g}$	$10\mu\text{g}$	Dicots	$1\mu\text{g}$	$5\mu\text{g}$	$10\mu\text{g}$
Rice	+	+	+	Sicklepod	-	+	+
Wheat	+	+	+	Sunflower	+	+	+
Corn	+	+	+	Cucumber	+	+	+
Timothy	-	-	-	Tomato	-	+	+

Use of Toxin Against Human Fungal Pathogens

Medically important fungi include about 50 species out of a total of 40,000-50,000 different fungi (33). Infections include superficial mycoses and systemic mycoses. In many cases systemic mycoses lead to death.

Antifungal chemotherapy is limited both in the number of available agents and in therapeutic applications. Many of the antifungal agents are toxic even at low concentrations. For example, Amphotericin B, used for the treatment of superficial Candida infections, is active in vitro at concentrations of 0.01-2 $\mu\text{g/ml}$. It is also highly toxic, and some impairment of renal function is seen in all patients regardless of dosage given (33). New chemicals are needed for human antifungal agents.

One of the requirements of a human antifungal agent is stability in blood serum. Pseudomycin was mixed with human serum at concentrations of 50, 5, 0.5, and 0.05 $\mu\text{g}/\mu\text{l}$. Duplicate tubes were incubated at both 37° C and room temperature. Controls of the pseudomycin dissolved in water were also incubated at the same temperatures. The serum mixtures and the controls were bioassayed at 24 and 48 hours. The pseudomycin remained active in both the serum mixtures and the control for the full 48 hours.

If a partially purified preparation of pseudomycin were to be used for intravenous treatment, it must be tested for the presence of endotoxins. These are lipopolysacchrides from the cell walls of gram negative bacteria. When minute amounts of endotoxins are exposed to Linulus amoebocyte lysate, the lysate increases in viscosity and eventually gels (35). This is the basis of the Etoxate test. Three different samples were tested for the presence of endotoxin;

1) BuOH crude extract, 2) TLC eluate and, 3) Amberlite XAD-2 eluate. Each sample was mixed with amoebocyte lysate alone (Table 16, tubes A) to test for the presence of endotoxin. The samples were also mixed with lysate and a known amount of endotoxin standard (tubes B). These tubes test for the presence of an inhibitor in the sample. The BuOH crude extract and the eluate from the XAD-2 Amberlite column tested negative for endotoxin (tube A), but the corresponding tube B was negative. This means there is an inhibitor in the sample and the test is invalid. The sample from TLC tested negative for endotoxin and the inhibitor. This test indicates a certain level of purity required for applied use of this toxin. The minimum inhibitory concentration of the toxin against seven human fungal pathogens was estimated using a serial tube dilution technique. The toxin was most effective at inhibiting the yeast fungi, Candida and Cryptococcus (Table 17).

Table 16. Results of Etodate test for endotoxins. All samples were dissolved in endotoxin freewater and adjusted to pH 6.5 with endotoxin free NaOH.

<u>Tubes</u>						<u>Sample</u>						
A	Tests for endotoxin in sample					1	BuOH crude extract					
B	Tests for etoxate inhibitor in sample					2	eluted from TLC plate					
C	Negative control					3	Amberlite XAD-2 column					
D-H	Standard dillutions											
Contents	A1	A2	A3	B1	B2	Tubes B3	C	D	E	F	G	H
Sample	.1 ml	.1 ml	.1ml	.1ml	.1ml	.1 ml						
Water							.1 ml					
Endotoxin Standard				.004 μ g	.004 μ g	.004 μ g		.008 μ g	.004 μ g	.002 μ g	.001 μ g	.0005 μ g
Etoxate	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml	.1ml
Results	-	-	-	-	+	-	-	+	+	+	+	+

Table 17. Minimum inhibitory concentration of pseudomycin against human fungal pathogens. A tube serial dilution technique was used.

Organism	MIC (ug/ml)	
	24 hr	48 hr
<u>Candida albicans</u> 89-80	3.12	25
<u>Candida albicans</u> 89-96	3.12	12.5
<u>Candida tropicalis</u>	3.12	6.25
<u>Cryptococcus neoformans</u>	1.56	3.1
	<u>48 hr</u>	<u>72 hr</u>
<u>Bipolaris spicifera</u>	12.5	>50
<u>Aspergillus fumigatus</u>	12.5	50

CHAPTER IV

SUMMARY

The first goal of this research, to develop a method of pseudomycin purification, was fulfilled. The previous problems with instability were corrected by using compatible solvents and maintaining a low pH by adding TFA to all the solvents. Acetone precipitation, followed by column chromatography with the nonionic adsorbent, Amberlite XAD-2 column, seemed to be an adequate method of purification. A typical problem with the separation of peptides by RP-HPLC is exceedingly long retention times. This problem is solved by the addition of the ion pairing agent TFA, and the use of a gradient rather than an isocratic system. The efficiency of this procedure was not estimated due to the inability to effectively quantitate the amount of toxin present at each step in purification. An attempt at quantification was made by Gross (9). Arbitrary units were assigned, by serial diluting the sample and assigning one unit to the quantity in the last dilution, resulting in a complete zone of inhibition. In reality this method should not be used before the compound is purified.

The purification of this toxin allowed the acquisition of accurate mass spectrometry data, amino acid analysis, and

amino acid sequencing. The result is the identification of a novel peptidyl toxin produced by Pseudomonas syringae MSU 16H.

Why study toxins produced by plant pathogens? Whether or not toxins are involved in plant disease is a controversial question. In the case of P. syringae, the toxins seem to play a role in disease (5,7). However, it is also stated, that the toxin in itself is not enough for the development of disease (36). These theories can be studied further with the purified toxin.

There are other applications of the toxin beside the basic understanding of host/pathogen interactions. The taxonomy of the fluorescent group of phytopathogenic pseudomonads has been debated for years. Few, if any, properties except for phytopathogenicity, separate the fluorescent phytopathogens from P. fluorescens. These include presence of cytochrome c (30), carbon source utilization tests (17,31), and even DNA-DNA homologies (24). Toxin production and which toxin is produced, may be used as an identification tool.

The fact that pseudomycin is phytotoxic may also be of benefit. P. syringae could be used as a biocontrol agent or the toxin alone could be applied. The interest of weed scientists and agricultural chemists in naturally occurring compounds as herbicides has recently increased greatly. Registration of such naturally produced chemicals may be

less time consuming and expensive than synthetic compounds. Purified natural compounds may have at least two potential advantages; 1) as weed control agents they have longer shelf life, wider range of storage conditions, a broader environmental window for application, less storage space, and a greater ease of application than with living organisms and 2) new microbial phytotoxins may be useful in expanding the number of sites of action of herbicides, in that there is little overlap between the known sites of action of commercial herbicides and of microbially-produced phytotoxins (6).

Fungi adversely affect the health and well being of mankind in numerous ways. The most direct of these ways include a variety of disease processes affecting humans. Others, maybe less direct but in some ways more deleterious include adverse economic and social effects of plant disease. Pseudomycin may be used as an antimycotic for the control of human pathogens or as a broad range antifungal agent for plant pathogenic fungi.

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APPENDICES

APPENDIX A

MEDIA

MEDIA

Potato Dextrose Agar (PDA)

Difco brand dehydrated

Potato Dextrose Broth (PDB)

Difco brand dehydrated

King's B

38 g. Difco brand Pseudomonas agar F
18 ml. glycerol
100 ml. 1000

Potato Dextrose Agar V-8

39 g. Difco brand Potato Dextrose Agar
18 ml. V-8 juice
1000 ml. water

Lima Bean Agar

Difco brand dehydrated

Cornmeal Agar

Difco brand dehydrated

Modified Czapek Dox

Czapek Dox broth	17.5 g.
Noble Agar	7.5 g.
Casamino Acids	0.5 g.
Yeast Extract	0.5 g.
Water	500 ml.

Cory Media

Glucose	3.7 g.
Agar	5.0 g.
Yeast Extract	2.5 g.
Calcium carbonate	1.2 g.
Water	250 ml.

Modified Dyes

Stock A	100 g/l	$(\text{NH}_4)_2\text{HPO}_4$
Stock B	20 g/l	KCl
Stock C	20 g/l	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

per liter:

13 ml. stock A
13 ml. stock B
13 ml. stock C

Autoclave for 20 min. at 120° C.
After autoclaving sterily add:

25 ml. 40% glucose
5 ml. 0.01 M FeCl_3
5 ml. 0.5 M ornithine
10 ml. 0.2 M histidine

APPENDIX B
PURIFICATION PROCEDURES

Figure 8. Butanol extraction schedule

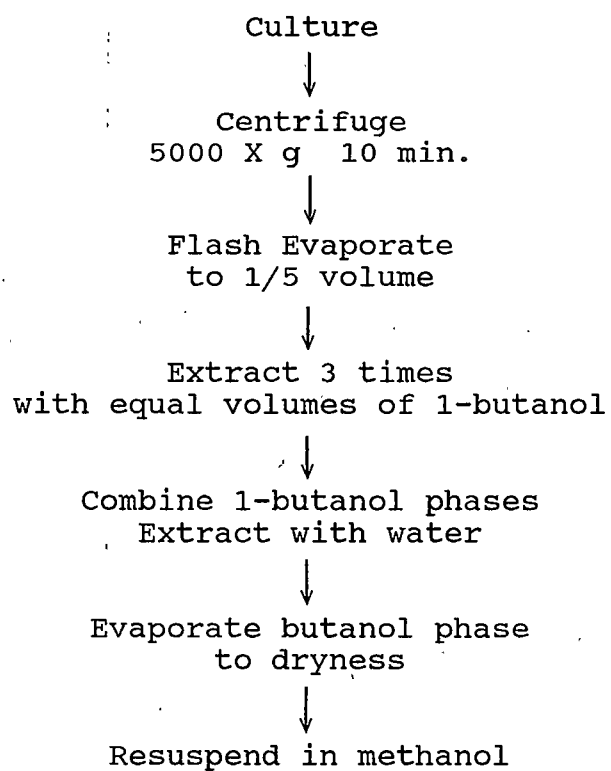


Figure 9. Acetone precipitation and purification schedule.

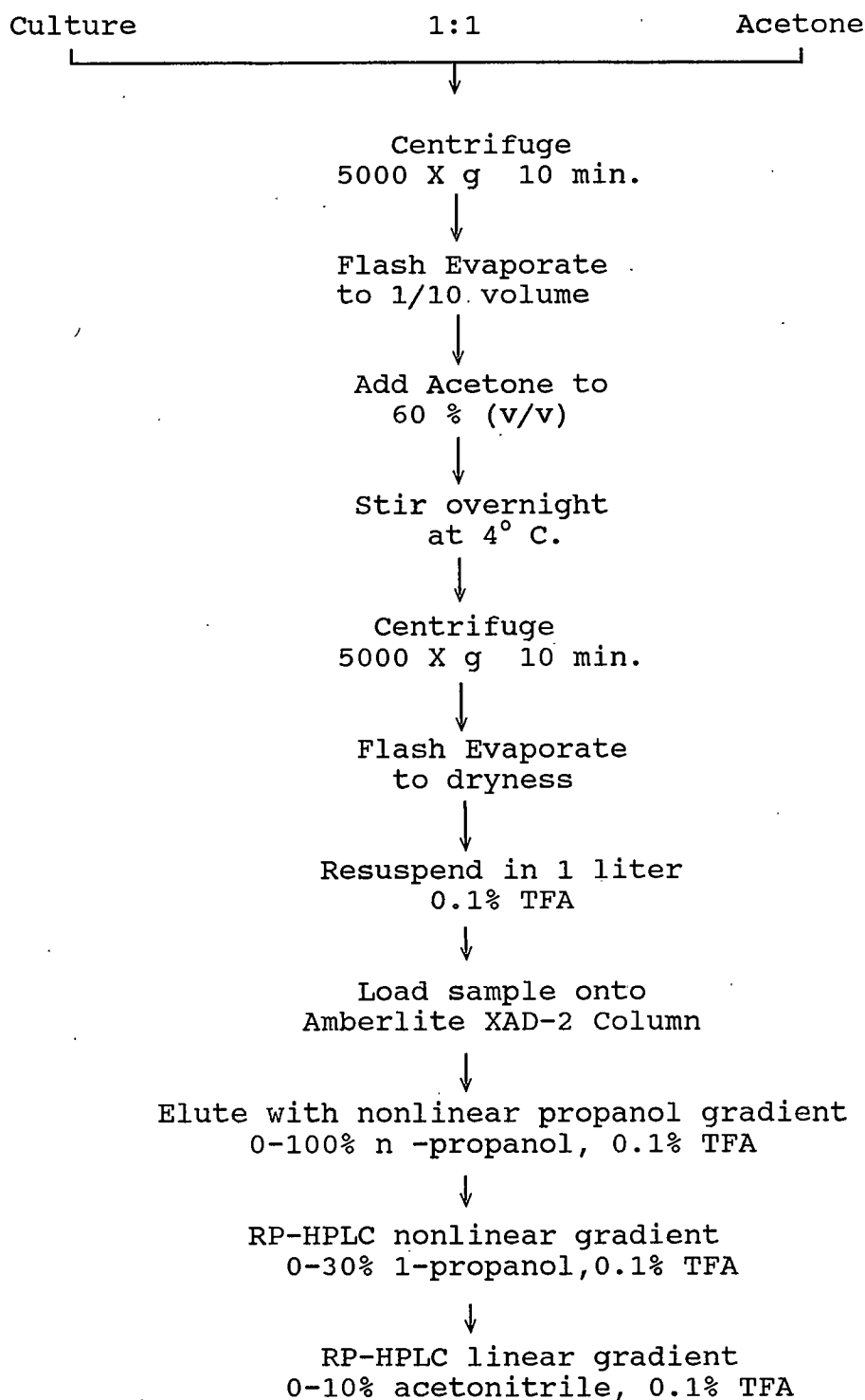
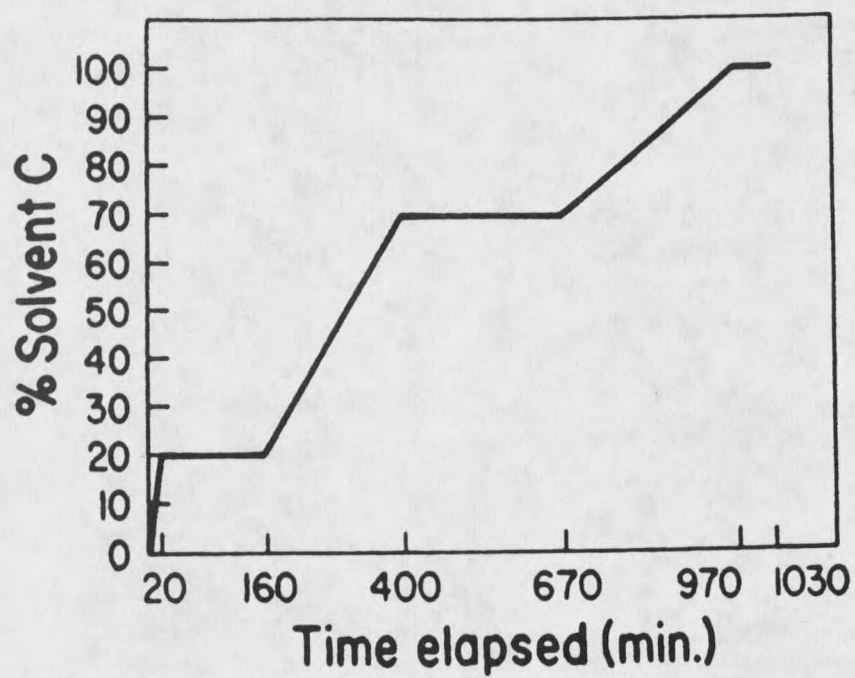


Figure 10. Nonlinear 1-propanol gradient for eluting the Amberlite XAD-2 column.



APPENDIX C
CHROMATOGRAPHY

Preparative Thin Layer Chromatography

1-butanol crude extract (40 mg) was spotted as a line, one inch from the bottom of a preparative TLC plate (E. Merck Sciences, silica gel F-254, 20 X 20 cm, 0.5 mm thick). The plate was developed for approximately three hours in 1-butanol:formic acid:water (75:15:10). A one inch strip, on the side of the plate, was sprayed with ninhydrin spray reagent (0.5% ninhydrin in 95% ethanol) to identify the bands. Each band was scraped, poured into a 3 ml SPE brand extraction column, then eluted with 1 ml of 3 M HCl in methanol. Each sample was dried under a stream of nitrogen and resuspended in methanol before it was bioassayed.

The band at the origin was bioactive. A second band at R_f 0.22 showed static bioactivity.

After eluting with HCl the compound may form chloride salts which are not conducive for further analysis. TFA and lower concentrations of HCl were tested as an alternative eluant.

The 1-butanol crude extract was applied to the TLC plate and developed as described above. The band at the origin was divided into eight separate sections and then scraped. Four different concentrations of TFA and four different concentrations of HCl were used to elute the sample: 0.5, 1.0, 2.0, and 3.0 M TFA; 0.5, 1.0, 2.0, and 3.0 M HCL. After elution, the samples were dried under a

stream of nitrogen. TFA at a concentration of 1.0 M seemed to be the best choice (Table 18).

Table 18. Solvents used to elute active bands from the preparative TLC plates. In each case the acid was mixed with methanol.

Solvent	Zone of Inhibition (cm)
0.5 M TFA	1.1
1.0 M TFA	1.2
2.0 M TFA	1.0
3.0 M TFA	0.9
0.5 M HCl	0.8
1.0 M HCl	0.4
2.0 M HCl	0.6
3.0 M HCl	0.6

Column chromatography

Biogel P2 (200-400 mesh) was washed with double distilled water then packed in a 1.5 X 60 cm column. 1-butanol crude extract (0.46 ml of 20 mg/ml) was applied to the column then eluted with water. The water was pumped at a rate of 0.7 ml per minute by a model RP-SYK FMI lab pump (Fluid Metering Inc.). Fractions were collected (2 ml), lyophilized, then tested for bioactivity. All fraction were negative. When 100 ul of 1 mM FeCl_3 in methanol was added to each fraction, then again bioassayed, activity was observed in fractions 6 through 9.

Biogel P2 was saturated with 1 mM FeCl_3 then washed with water before packing. Two small samples of the packing material were dried, weighed, then analyzed for total iron per gram of packing material. There was an average of 457

ug of iron per gram of packing material in the FeCl_3 saturated Biogel P2 as compared to less than 0.01 ug/gm in the nonsaturated Biogel P2.

1-butanol crude extract was loaded onto the packed column then washed with water (3 times the void volume). The sample was then eluted with 1 mM FeCl_3 . Fractions were collected, lyophilized, then resuspended in 100 ul of methanol. Each fraction (10 ul) was spotted onto PDA and bioassayed. Active fractions eluted at approximately one column void volume (30 mls).

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