This is to certify that the

thesis entitled

BIOACTIVE CONSTITUENTS FROM NITROGEN-FIXING STREPTOMYCES SPP.

presented by

Di Zhang

has been accepted towards fulfillment
of the requirements for

Master degree in Horticulture

Major professor

Date 8/22/96

# BIOACTIVE CONSTITUENTS FROM NITROGEN-FIXING *STREPTOMYCES SPP.*

**By**

**Di Zhang**

**A THESIS**

**Submitted to**
**Michigan State University**
**in partial fulfillment of the requirements**
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**1996**

## ABSTRACT

### BIOACTIVE CONSTITUENTS FROM NITROGEN-FIXING *STREPTOMYCES SPP.*

**By**

**Di Zhang**

*Streptomyces spp.* are well known to produce antibiotics for pharmaceutical and agricultural applications. However, after years of research for novel antibiotics it is evident that isolation of additional novel compounds with biological activity has become more difficult. We have investigated a unique culture collection of 48 nitrogen-fixing *Streptomyces spp.* from China for the production of bioactive compounds.

To avoid replication of our screening effects, the 48 strains was evaluated using rep-PCR, a high resolution genomic fingerprinting technique. The rep-PCR analysis showed genomic diversity within the culture collection.

Preliminary bioassays indicated that one of the *S. griseofuscus* strains exhibited high mosquitocidal activity. Hence, it was further investigated for the chemical characterization of the compound. Bioassay-directed purification and identification of the active fraction from *S. griseofuscus* afforded compound 1 which gave 100% mortality of mosquito larvae (*Aedes*

*egyptii*) at 20 ppm within 24 h. Also, it inhibited the growth of tobacco hornworm (*Manduca sexta*), cornear worn (*Helicovarpa zea*) and gypsy moth (*Lymantria despar*) at 100 ppm.

## ACKNOWLEDGMENTS

I thank my major advisor, Dr. Muraleedharan Nair, for his advice and encouragement throughout most of the project. He brought me into natural products and opened a wonderful field to me. I thank Dr. Marcia Murry, who helped me come to this country and introduce me to the world of molecular biology which I had never touched before. This research work would not have been possible without her extremely patient help. I thank Dr. John Kelly for his guidance during these two years.

I also thank Dr. Yu-chen Chang, Dr. Amitabh Chandra and Dr. James Nitao who have been extremely helpful since the first day I came into the lab. Without their help, my research would have taken longer than it did.

I am glad to have so many good friends, especially the members of the Bioactive Natural Products Laboratory, Mark Kelm, Jennifer Miles, Geoff Roth, Haibo Wang and Andrew Erickson. They not only gave me great help during my research work, they also taught me English and American culture.

I would finally like to thank my parents, they are always there for me, I would not be here to get my degree without their encouragement.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACN | Acetonitrile |
| BNPL | Bioactive Natural Products Laboratory |
| bp | Base pair |
| $CHCl_3$ | Chloroform |
| CD | Circular dichroism |
| $CDCl_3$ | Deuterated chloroform |
| DEPT | Distortionless Enhancement by Polarization Transfer |
| DMSO | Dimethyl sulfoxide |
| DQFCOSY | Double quantum filtered correlated spectroscopy |
| dd | Doublet of doublet |
| EIMS | Electron impact ionization mass spectrometer |
| FABMS | Fast atom bombardment mass spectroscopy |
| HPLC | High performance liquid chromatography |
| HMQC | Hetornuclear multiple quantum correlated |
| MeOH | Methanol |
| MS | Mass spectroscopy |
| MW | Molecular weight |
| m/z | Mass-to-charge ratio |
| NMR | Nuclear magnetic resonance |
| PCR | Polymerase chain reaction |
| PDA | Potato dextrose agar |
| TLC | Thin layer chromatography |
| $^1$HNMR | Proton nuclear magnetic resonance |
| $^{13}$CNMR | $^{13}$Carbon nuclear magnetic resonance |
| YPDA | Yeast Potato Dextrose Agar |
| $\delta$ | Chemical shifts |
| J | Coupling constant |

## LIST OF APPENDICES

# Chapter I

# Literature review

## Introduction

The use of plants and plant extracts in human medicine is recorded in the most ancient archaeological finds. Even today, plant-derived compounds constitute a significant fraction of the pharmaceuticals employed in human medicine. However, the exploration of microorganisms as sources of therapeutically useful compounds has a much shorter history.

Many microorganisms are well known to produce bioactive primary and secondary metabolites. Secondary metabolites are naturally produced substances which do not play an explicit role in the internal energy mechanism of the organisms that produce them (Stone et al., 1992). Secondary metabolites are biosynthesised by bacteria, algae, lower animals such as corals, sponges and plants. It is proposed that the production of secondary metabolites increases an organism's fitness for survival by acting as an chemical defense mechanism. These compounds are produced in nature and are implicated in competition between bacteria, fungi and amoebae and between microorganisms and higher plants, insects or large animals (Vining, 1990).

About 75% of the bioactive secondary metabolites reported from microorganisms are produced by *Streptomyces spp* (Vining, 1990) . Early emphasis on screening had been on compounds with antibiotic activity for the fight against bacterial and fungal infections.

Unquestionably, the antibiotics, which are secondary metabolites produced by microorganisms, have been the basis of major advances in the practice of medicine. However, recent evidence indicates that the contribution of microbes will not be limited to antibiotic production alone. Current studies suggest that microbial metabolites show potential as therapeutic agents in a variety of human diseases (Monaghan et al., 1990).

After years of search for novel secondary metabolites from *Streptomyces spp.*, it is evident that isolation of additional novel metabolites from this genus. has become extremely tedious. Genes that encode the biosynthesis of specific antibiotics can be found in several *Streptomyces spp.* and the same compound can be "rediscovered" in new isolates. Therefore, it is necessary to develop the methodologies to allow better taxonomic identification of new isolates and to correlate the antibiotic production to specific type strains.

## Classification of *Streptomyces*

Early classification and identification methods for *Streptomyces* were based on phenotypic characteristics, such as the morphology of spores, color of aerial mycelium, utilization of carbon sources, sensitivity to actinophages, serological reactions, biochemical and other genetic traits (Kurylowica et al., 1975). The major limitations of these methods are that many traits are influenced by environmental factors and that the tests derived from one group of organisms were not always useful with other groups. To overcome these limitations, genotypic methods for the identification and classification of strains are now being used, because it is presumed that chromosomal DNA is unaffected by environmental conditions. Furthermore, since nucleic acids are universal, they provide a standard

molecule with which a wide range of organisms can be compared and classified. A variety of molecular typing techniques have been developed, each with utility at different taxonomic levels. For example, DNA-DNA hybridization methods are used to define bacterial species (Brenner, 1973); sequence comparisons of 16S rRNA are used to assign taxa at the genus level and above (Woese, 1987); rep-PCR fingerprinting is a facile and very sensitive method with high resolution which can distinguish strains at the subspecies level (de Bruijn, 1992).

Ribosomal RNAs are present in all living organisms and are essential elements in protein synthesis. The RNA molecule shows a high degree of functional constancy and has changed very little during evolution (Woese, 1987). Phylogenetic analyses based comparisons of 16S rRNA nucleotide sequences have the advantage of providing information for individual organisms that can be processed using estimates of similarity and clustering algorithms (Priest et al., 1993). 16S sequences data from all taxa have been accumulated into universal database (Priest et al., 1993). Before 1985, the 16S rRNA was too large to sequence in its entirety, so a cataloguing approach was adopted (Priest et al., 1993). Later studies involving complete 16S rRNA sequences showed that it did not change the major conclusion of the cataloguing work. A phylogenetic tree for the bacteria based on 16S rRNA nucleotide sequences data was published by Woese in 1992. These studies divide the eubacteria into 11 major divisions with *Streptomyces* clustering with other gram-positive bacteria. Fox et al.(1987) utilized 16S rRNA catalogues to analyze the actinomycete branch of the gram-positive bacteria. Four species classified within the genus *Kitasatospora* share many of the phenotypic characteristics typical of streptomycetes

and hybridized with a 16S rRNA gene probe specific to *Streptomyces spp.* indicating a close relationship between the two genera. The four species were renamed as members of *Streptomyces* genus (Wellington et al., 1992).

DNA-DNA hybridization compares the similarity between DNA from two organisms. One current definition of a bacterial species states that organisms with greater than 70% DNA-DNA hybridization are considered to be members of the same species (Wayne, 1987). There are two current methods to determine the similarity of organisms based on DNA reassociation: the free solution assay, and the immobilized DNA assay (Priest et al., 1993). Both methods use a large excess of single-stranded DNA sheared to a constant molecular weight and a radio labeled single-stranded DNA from the reference strain for hybridization. The advantage of the DNA reassociation method is that the estimate of relatedness between organisms is based on the complete genotype rather than a single component of the genome, such as the rRNA nucleotide sequence. The limitation of this technique is that it is time consuming. Full similarity matrices with estimates of DNA homology between each and every strain are seldom produced.

Labeda reported the DNA similarities among 15 *Streptomyces* strains, including nine strains identified as *S. violaceusniger*, three as *S. hygroscopicus*, and one each as *S. endus*,*S. sparsogenes* and *S. melanosporofaciens*. Reassociation kinetics showed that the strains identified as *S. violaceusniger* were genetically heterogeneous. These strains clustered into seven different DNA homology groups at a level of DNA relatedness of >70% (Labeda, 1991). In 1992, Labeda evaluated the DNA similarities among species of *S. cyaneus* and *S. lavendulae*. A total of 18 strains from the *S. cyaneus* cluster

exhibited at least approximately 50% DNA relatedness to each other. Among the eight strains of *S. lavendulae*, four of them showed more than 80% DNA similarity, but the other strains exhibited low similarity and, therefore, should be considered to be species other than *S. lavendulae*. In 1993, Labeda further reported the DNA relatedness among strains of *S. lavendulae*. He compared 10 strains of *S. lavendulae* with 11 other species. The results showed that the 21 studied strains segregated into 14 clusters when grouped at >70% DNA relatedness, including 10 single-member clusters. Four strains were found to be at >79% DNA relatedness and the others were considered to be valid species other than *S. lavendulae*. Witt et al.(1990) used both DNA-DNA hybridization and 16S rRNA to evaluate 40 strains of genera *Streptomyces* and *Streptoverticillium*. The results indicated that they can be classified into a single genus.

A rep-PCR (repetitive element sequence-based PCR) molecular fingerprinting method is one of the most sensitive typing methods that differentiate strains at the subspecies level. The term rep-PCR refers to the general methodology involving the use of oligonucleotide primers complimentary to short repetitive sequence elements that are dispersed throughout the prokaryotic kingdom (Tower et al., 1993). Repetitive extragenic palindromic (REP) (Stern et al., 1984), enterobacterial repetitive intergenic consensus (ERIC) (Hulton et al., 1991) and BOX (Martin et al., 1992) elements are used to amplify the DNA of the microorganisms by PCR and the products are separated by electrophoresis to establish the fingerprint patterns. The distribution of repetitive elements is thought to represent an intrinsic property of the structure of bacterial genomes (Martin et al., 1992). The BOX element was the most recent identified analogous repetitive sequence, it can be

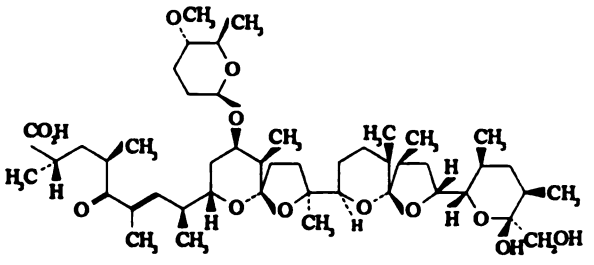
used to generate genomic fingerprints of both gram-positive and gram-negative bacteria (Versalovic et al., 1994). Rep-PCR had been used to develop phylogenetic relationships in *Citrobacter diversus* (Woods et al., 1992), *Rhizobium meliloti* (de Bruijn, 1992) and other bacterial species including the actinomycete, *Frankia* (Murry et al., 1995).

## Bioactive compounds from *Streptomyces spp.*

Penicillin's proven efficacy and its widespread use in the therapy of human infectious diseases led to the discovery of several microbial secondary metabolites to fight other infections in human. The use of these secondary metabolites have been extended to agricultural, pharmaceutical and animal health applications.

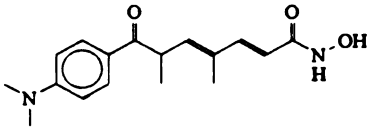
**Antimicrobial agents**: During the discovery of many antibiotics, bioassays were focused on use of fungi and bacteria as test species. Yazumycin was produced by *S.lavendulae* and only had activity against gram-negative bacteria (Akasaki et al., 1968; Neuss et al., 1970). Hygromycin B, flambamycin, A-130 and two salts were extracted from *S. hygroscopicus* (Ninet et al., 1974; Kubota et al., 1975; Tsuji et al., 1976). Flambamycin exhibited activity against both gram-positive and some gram-negative bacilli (Ninet et al., 1974). A-130 was reported to have gram-positive bactericidal and fungicidal activities (Kubota et al., 1975). The organic salts, indentified as $C_{46}H_{79}O_{15}Na$ and $C_{48}H_{81}O_{19}Na$ were active against gram-positive bacteria only (Tsuji et al., 1976).

Another *S. hygroscopicus* yielded a polyether antibiotic, Ro 21-6150, that was active against gram-positive bacteria including *Mycobacterium phlei* and showed modest activity against fungi and yeast ( Liu et al., 1976).



Ro 21-6150

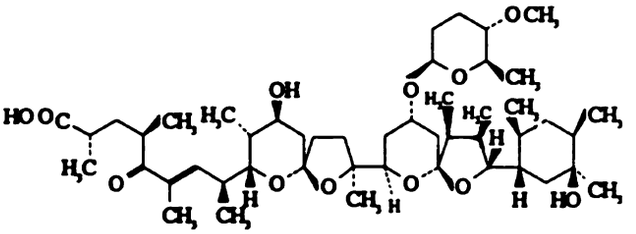
The metabolite trichostatin was isolated from several strains of *S.hygroscopicus*. It is an acidic compound with two nitrogens in its structure. This compound had activity against trichophytons and some fungi. Also, it is a typical example illustrating that hydroxamic acids can be used as excellent antibiotics against bacteria (Tsuji et al., 1976).



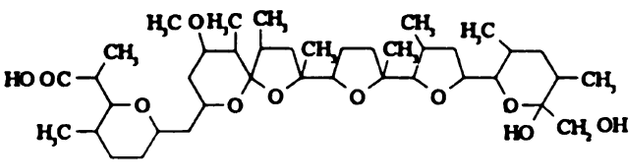
Trichostatin A

The antibiotic leuseramycin was produced by strain of *S. hygroscopicus*. It is active against a wide range of gram-positive bacteria and certain phytopathogenic fungi, but inactive against gram-negative bacteria (Mizutani et al., 1980). Another *S. hygroscopicus* strain produced nigericin, elaiophylin and niphimycin, antibiotics with activities against gram-positive bacteria and fungi (Grabley et al., 1990; Fiedler et al., 1981). Both leuseramycin and nigericin are polyethers. Their activities against phytopathogenic fungi demonstrated that polyether antibiotics have potential to combat

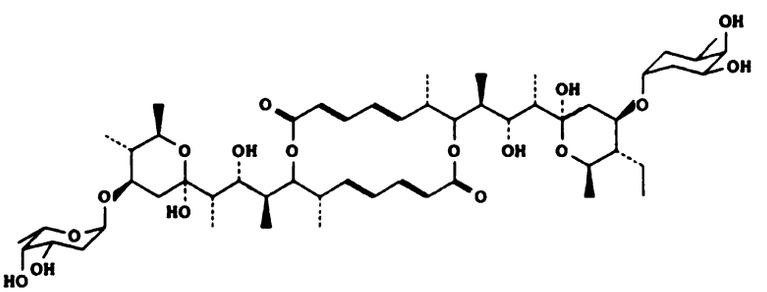
several agricultural pests. Elaiophylin, a macrodiolide with a polyether functionality, exhibited very good antifungal activity.



Lasalocid



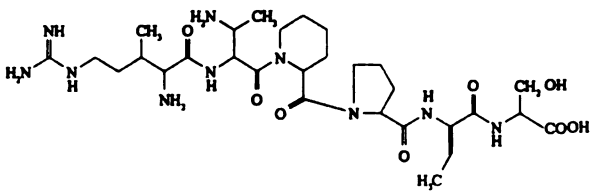
Nigericin



Elaiophylin

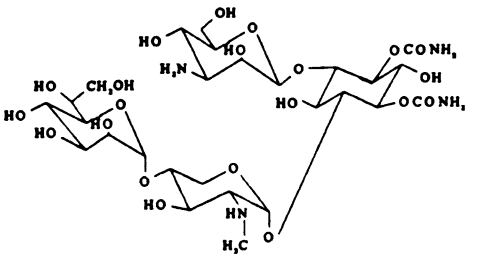
A basic peptide antibiotic, lavendomycin, isolated from *S. lavendulae* subspp. *brasilicus*, was active against gram-positive bacteria *in vitro* and *in vivo*. However, it was inactive against gram-negative bacteria and fungi (Kamori et al., 1985). The antibiotic A21978C, produced by *S. roseosporus*, is an acidic lipopeptide antibiotic complex that inhibited the growth of gram-positive bacteria (Debono et al., 1987, Boeck et al., 1988).

A novel aminoglycoside antibiotic, boholmycin, was produced by *S. hygroscopicus*. It has a pseudotetrasaccharide structure composed of a heptose, two aminosugars and a



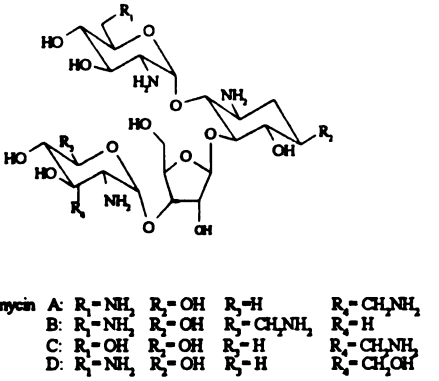
Lavendomycin

dicarbamoyl-*scyllo*-inositol. Antibacterial activity of boholmycin was weak, but it exhibited-broad spectrum activity against gram-positive and gram-negative bacteria, including aminoglyciside-resistant bacterial strains (Saitoh et al., 1988).

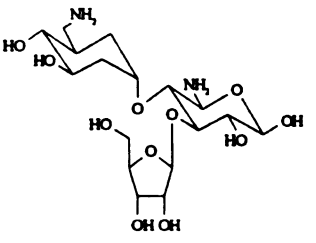


Boholmycin

Inosamycins A, B, C, D and E, an antibacterial complex with aminocyclitol moieties, were produced by *S. hygroscopicus*. Inosamycins A to D contain three sugar



Inosamycin A: $R_1$=$NH_2$ $R_2$=OH $R_3$=H $R_4$=$CH_2NH_2$
B: $R_1$=$NH_2$ $R_2$=OH $R_3$=$CH_2NH_2$ $R_4$=H
C: $R_1$=OH $R_2$=OH $R_3$=H $R_4$=$CH_2NH_2$
D: $R_1$=$NH_2$ $R_2$=OH $R_3$=H $R_4$=$CH_2OH$



Inosamycin E

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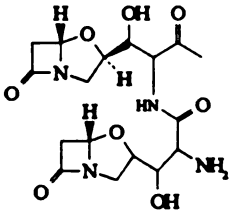
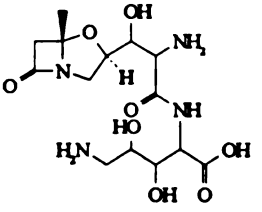
moieties and E contain two (Tsunakawa et al., 1985). Inosamycin A is the most active among the inosamycins against gram-positive, gram-negative and acid-fast bacteria. The structure function relationship showed that the activities are proportional to the number of the amino groups present and their orientation (Tsunakawa et al., 1985).

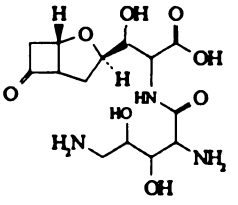
Clavamycins A-E were isolated from *S.hygroscopicus* strains. These antibiotics were active against *Candida spp.* Clavamycin A and D are cyclic amino compounds and are inhibitory to the yeast cell wall synthesis (King et al., 1986; Naegeli et al., 1986).



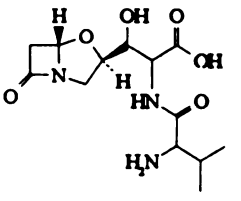
Clavamycin A



Clavamycin B

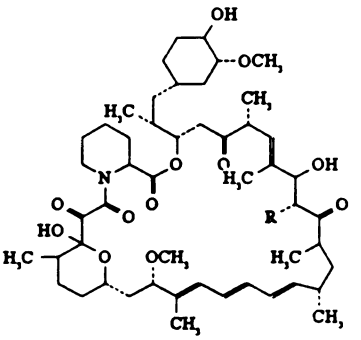


Clavamycin C



Clavamycin D

The macrocyclic lactone rapamycin and demethoxyrapamycin were produced by *S. hygroscopicus* and were active on *Candida albicans*, *Microsporum gypseum* and



Rapamycin R= $OCH_3$
AY-24,668 R= H

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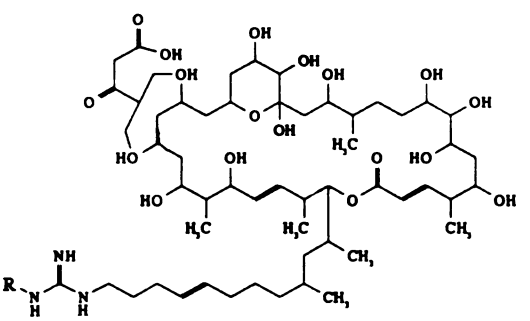
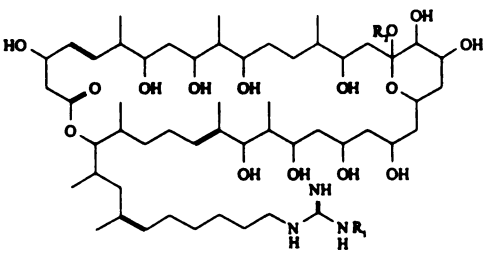
*Trichophyton granulosum* (Vezina et al., 1975; Sehgal et al., 1975, 1983). The antimicrobial activities of rapamycin were much higher than activities of its derivative. Later studies exhibited that rapamycin showed activity against several tumors, but demethoxyrapamycin had only slight activity against P388 lymphocytic leukemia (Sehgal et al., 1983). All these indicated that the methoxy group in rapamycin is important for its activities.

Copoamycin and neocopoamycin were isolated from *S. hygroscopicus* var. *crystallogenes* and were inactive against gram-positive and gram-negative bacteria, but inhibited the growth of a wide range of fungi. Neocopoamycin A proved to be more active against a number of fungi than copoamycin. The only difference between these two compounds was the guanidine moiety (Arai et al., 1965 and 1984).



Neocopiamycin A R= H
Copiamycin R= $CH_3$

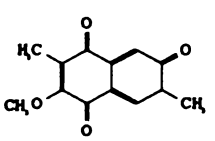
Two other guanidine antibiotics, guanidylfungin A and B, were extracted from the mycelia of *S. hygroscopicus*. These antibiotics are 36-carbon polyhydroxyl macrocyclic lactones with a guanidine and a monoester of malonic acid moieties. These compounds were active against fungi and gram-negative bacteria (Takesako et al., 1984).



Guanidylfungin A $R_1 = CH_3$, $R_2 = H$
Guanidylfungin B $R_1 = R_2 = H$

**Anticancer agents:** Further developments in mechamism of actor based bioassays has led to the discovery and the application of novel secondary metabolites for use in cancer therapy.

*S. lavendulae* is reported to produce mimosamycin and chlorocarcins A, B and C (Arai et al., 1976; Mikami et al., 1976). Mimosamycin, a low molecular weight compound, was active against mycobacteria, including streptomycin-sensitive and resistant strains of human Tubercle bacilli and other gram-positive bacteria (Fukumi et al., 1978; Kubo et al., 1988). Among chlorocarcins, chlorocarcin A proved to be the most active and inhibited the growth of a number of gram-positive bacteria. This compound was also highly active on murine tumors such as *Ehrlich carcinoma* (Arai et al., 1976; Mikami et al., 1976).



Mimosamycin

Saframycins are another class of antitumor agents produced by *S. lavendulae* (Ikeda et al., 1983). Their structures contain 1,3'-dimeric isoquinoline moieties. Saframycin A

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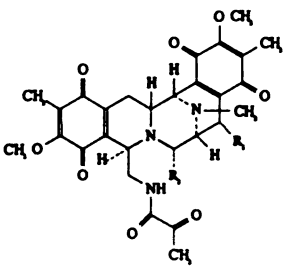
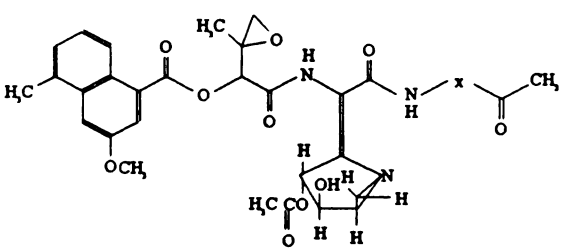
gave the highest activity, B and C were less inhibitory to various experimental tumors. The enhanced activity of saframycin A is probably due to the nitrile group present in its structure (Arai et al., 1977, 1980; Cooper et al., 1985; Kubo et al., 1987 and 1988).



Saframycin A : $R_1$=H, $R_2$=CN
Saframycin B : $R_1$=$R_2$=H
Saframycin C : $R_1$=$OCH_3$, $R_2$=H

Azinomycins A and B were extracted from *S. griseofuscus* (Nagaoka et al., 1986). Azinomycin B was highly effective against intraperitoneally inoculated tumors such as P338 *leukemia*, B-16 *melanoma* and *Ehrlich carcinoma*. Azinomycin A was somewhat less effective than azinomycin B. Both azinomycins were active against gram-positive and gram-negative bacteria, but inactive against yeast and fungi (Ishizeki et al., 1987). The active site of these compounds was proposed to be the 1-azabicyclo-[3.1.0]-hexane ring system (Yokoi et al., 1986).



Azinomycin A x=$CH_2$
B x=C=CHOH

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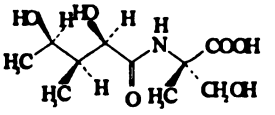
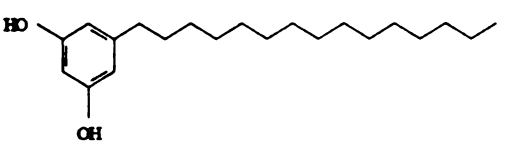
A low-molecular-weight immunomodulator, conagenin, was produced by *S. roseosporus* with inhibitory activity on the proliferation of rat splenic T cells (Yamashita

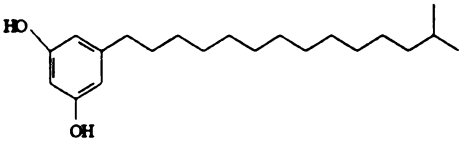


Conagenin

et al., 1991). *S. cyaneus* yielded adipostatin A and B which were effective in prevention of corpulence by inhibiting triglyceride accumulation (Tsuge et al., 1992). Adipostatin A and B were identified as 5-n-pentadecylresorcinol and 5-iso pentadecylresorcinol, respectively. Comparison of the activities of adipostatins to those of 3-pentadecylphenol, 5-methylresorcinol and stearic acid indicated that adipostatins were 10 times more active than the test compounds. This suggested that the length of the alkyl side chain and the number of hydroxy groups on the aromatic ring were important for their biological activity (Tsuge et al., 1992).



Adipostatin A



Adipostatin B

An antimicrobial acidic protein, with a molecular weight of 15,000 (Otani et al., 1991), was isolated from *S. globisporus*. It had potent cytotoxicity against KB *carcinoma* cells *in vitro*. Also, it inhibited transplanted tumors in mice (Hu et al., 1988; Zhen et al., 1989).

*S. hygroscopicus* afforded a novel antibiotic, eponemycin, and its analogues

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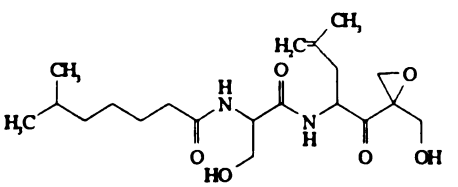
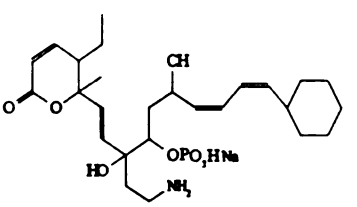
diacetyleponemycin, dihydroeponemycin and tetrahydroeponemycin, with specific *in vivo* antitumor activity against B16 *melanoma*. However, they did not exhibit inhibitory activity against gram-positive and gram-negative bacteria or fungi up to 100 $\mu g \cdot ml^{-1}$. Eponemycin, composed of isooctanoic acid, L-serine and an aminoepoxyketone, is more active than its analogues. The diacetyl and dihydro derivatives were less active compared to eponemycin, but the activity of a tetrahydro derivative without the epoxide in its structure was much less active than the others. That indicated that an epoxide in the structure is essential for antitumor activity (Sugawara et al., 1990).



Eponemycin

Phospholine, an antitumor antibiotic, was isolated from *S. hygroscopicus;* it showed strong activity against L1210,P388 murine leukemia cells and EL-4 murine lymphocytic cells. The active sites of this antibiotic were considered to be α, β-unsaturated δ-lactone and phosphoric acid ester functional groups (Ozasa et al., 1989).



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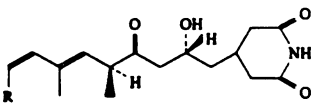
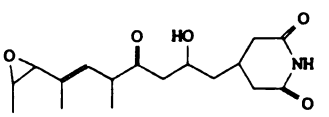
Another strain of *S. hygroscopicus* produced several glutarimide antibiotics S-632-A1, A2, B1 and B2. They had cytotoxic activity against KB tissue culture cells and also activity against *Sacchromyces spp.* However, they are inactive against filamentous fungi and bacteria. Glutarimide B1 was the most active among the isomers, and this may be due to its epoxide functonality and spatial orientation. The diastereoisomers of S-632-B1 and -B2 are still unknown (Otani et al., 1989).

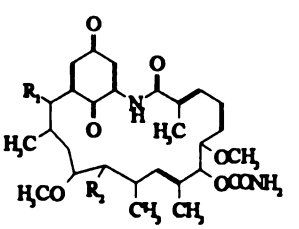



S-632-$A_1$ R=H
S-632-$A_2$ R=$CH_3$




S-632-$B_1$ and $B_2$

The benzoquinoid compounds ansamycin, herbimycin A and C and their dihydroderivatives produced by *S. hygroscopicus* exhibited activity against the P-388 and KB lymphocytic leukemia. Also, herbimycin A and C exhibited strong herbicidal and cytotoxic activities, but there were no reported biological activity for the dihydroderivatives (Lin et al., 1988). The replacement of the methoxy group with a hydroxy group in herbimycin resulted in a decrease in the activity when tested against P-388 and KB lymphocytic leukemia. Furthermore, the *p*-quinone type antibiotics had




Herbimycin A $R_1$=$OCH_3$, $R_2$=$OCH_3$
Herbimycin B $R_1$=H, $R_2$=OH
Herbimycin C $R_1$=$OCH_3$, $R_2$=OH

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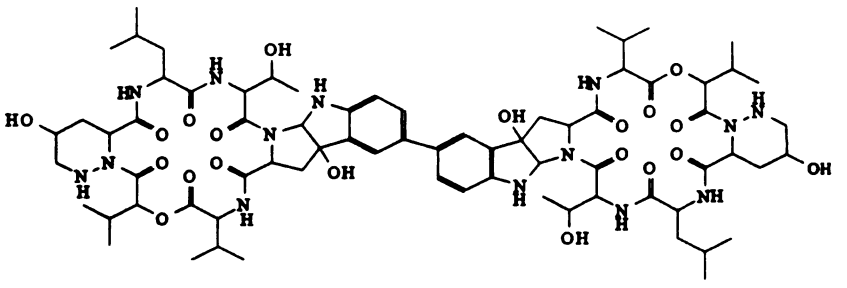
higher activity than that of their corresponding dihydroderivatives in assays with P-388 and KB lymphocytic leukemia (Lin et al., 1988).

A cyclohexadepsipeptide antibiotic, himastatin, isolated from *S. hygroscopicus* contained valine, leucine, threonine, α-hydroxyisovaleric acid, 5-hydroxypoperazic acid and a dimeric hexahydropyrroloindole in its structure (Leet, 1990; Mamber, 1994). Himastatin prolonged the life span of mice inoculated with P388 leukemia and B16 melanoma cells. Also, it exhibited antimicrobial activity against gram-positive bacteria but was inactive against gram-negative bacteria (Lam et al., 1990).



Himastatin

**Agricultural pest managing agents from *Streptomyces spp.***

**Activity against plant pathogens:** Ileumycin is an antifungal antibiotic isolated from the culture broth of *S. lavendulae*. Ileumycin exhibited activity against *Glomerella cingulata*, a pathogenic fungus of grapevines, but was inactive against other fungi, yeasts and bacteria (Kawakami et al., 1978). Validamycin and aabomycin A are produced by different strains of *S. hygroscopicus* and were used to protect rice plants from fungal infection (Uyeda et al.,1988; Iwasa et al., 1970). Validamycin is an aminoglycoside antibiotic that was effective in protecting rice plants against sheath blight disease caused

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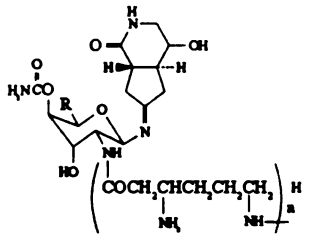
by *Rhizoctonia solani* (Uyeda et al.,1988; Iwasa et al., 1970; Aizawa et al., 1969). Aabomycin A exhibited inhibitory activity against many fungi, especially against *Piricularia oryzae*, the causative agent of the rice blast disease (Aizawa et al., 1969; Yamaguchi et al., 1969; Seino et al., 1970).

Racemomycin-B, the main antibiotic from *S. lavendulae*, exhibited antimicrobial activity against a variety of plant pathogenic microorganisms and root growth inhibitory activities against *Brassica rapa* L. at 50 ppm. Also, it strongly inhibited the growth of *Pseudomonas syringae pv. tabaci* IFO-3508 (MIC 0.4 $\mu g \cdot ml^{-1}$) and showed antifungal activity against six *Fusarium oxysporum* species (MIC 0.1-0.2 $\mu g \cdot ml^{-1}$). However, racemomycin-B was more active than racemomycin-A and C. Therefore, the biological activities of racemomycin were proportional to the increase in the number of β-lysine moieties in their molecule. Racemomycin-D has one more β-lysine moiety than racemomycin B, but, the activities of racemomycin-D have not been reported yet (Inamori et al., 1990). Curromycin A and B were extracted from *S. hygroscopicus* and had similar



R=$CH_2OH$

Racemomycin-B n=3
Racemomycin-C n=2
Racemomycin-A n=1
Racemomycin-D n=4

antibacterial activity when tested against *Bacillus subtilis*. The difference between currromycin A and B is the presence of a dimethyl ether in curromycin A rather than a

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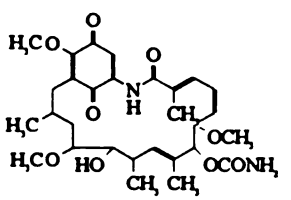
methyl group as in B (Ogura et al., 1985).

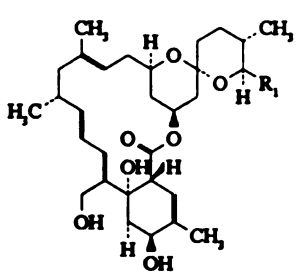
**Herbicidal activities:** The herbicide herbimycin was produced by *S. hygroscopicus* and showed herbicidal activities against most mono- and di-cotyledonous plants, especially against *Cyperus microiria* Steud. However, *Oryza sativa* showed strong resistance to herbimycin (Omura et al., 1979).

Geldanamycin and nigericin were produced by *S. hygroscopicus* and also inhibited the radicle elongation of garden cress ( *Lepidium sativum* L.) (Heisey et al., 1986). Both geldanamycin and herbimycin B have structures similar to that of ansamycin.

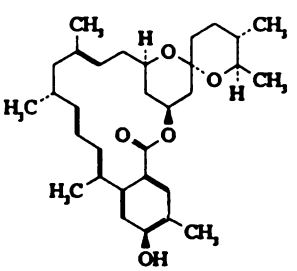


Geldanamycin

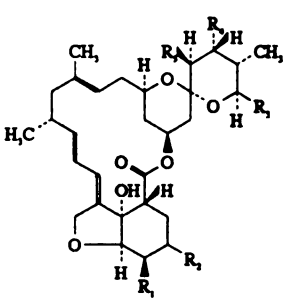
**Insecticidal activities:** Several macrotetrolides were isolated from *S. globisporus* and were identified as nonactin, dinactin and trinactin. The insecticidal activity of these compounds were confirmed by the larval mortality of the Colorado potato beetle (*Leptinotarsa decemlineata*) (Jizba et al., 1991). Milbemycins, a 16-numbered macrolide



Milbemycin $b_1$ series



Milbemycin $b_2$ series



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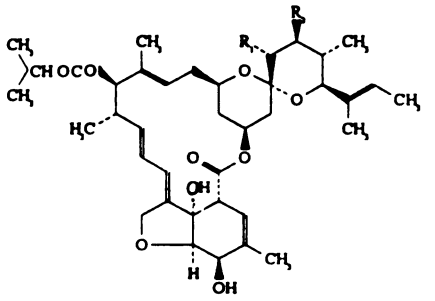
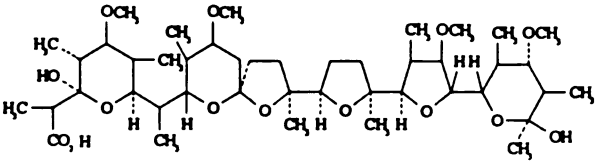
antibiotic from *S. hygroscopicus* exhibited activity against aphids and *Lepidoptera* larvae (Takihychi et al., 1980,1983; Ono et al., 1983; Mishima et al., 1983). Several substituted milbemycins UK-78,694, UK-80,694 and UK-80-695 also were reported to have excellent insecticidal activity (Haxell et al., 1992). These compounds also exhibited potent *in vitro* nematicidal activity effect 95% mortality at 0.01 ppm against *Caenorhabditis elegans* and exhibited 100% mortality at 1 mg against blowfly larva, *Lucilia cuprina*.



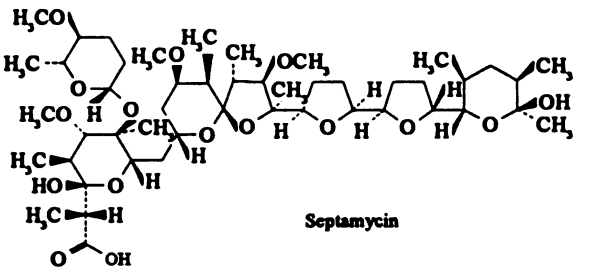
UK-78,624 $R_1$= OH, $R_2$= H
UK-80,694 $R_1$= OH, $R_2$= $OCOCHMe_2$
UK-80,695 $R_1$= $R_2$= H

**Veterinary use**: Several reports indicate that polyether antibiotics have potential applications in veterinary use (Ninet et al., 1976; Ohshima et al., 1976). Emericid protected chickens and rabbits against coccidiosis at 0.006-0.02% levels in the diet. It also

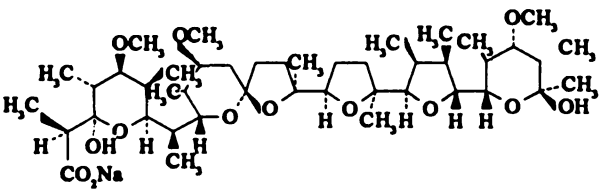


Emericid

had activity against gram-positive bacteria in vitro (Ninet et al., 1976). Septamycin, DE-3936 Na salt, emericid and carriomycin were produced by *S. hygroscopicus*. Steptamycin

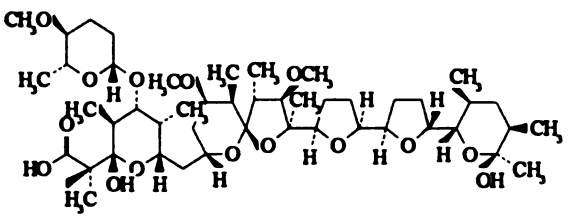


Septamycin

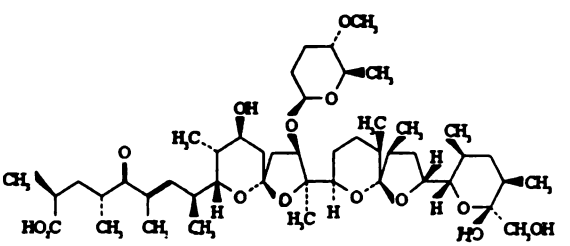


DE-3936 Na salt

possessed antibacterial activity as well as excellent activity against Newcastle Disease and herpes simplex viruses (Keller-Juslen et al., 1975). DE-3936 Na salt with a MF of $C_{44}H_{75}O_{14}Na$ inhibited the growth of gram-positive bacteria, mycobacteria, mycoplasma and protozoa, especially coccidia (Ohshima et al., 1976). Carriomycin was coccidiostatic, with antimicrobial activities against several fungi, yeasts and bacteria (Imada et al., 1978).



Carriomycin



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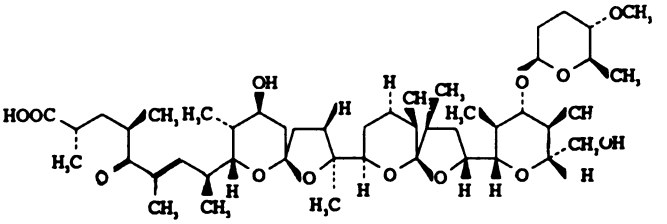
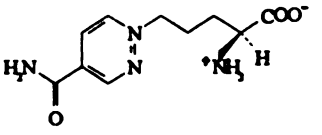
Endusamycin isolated from *S. aureus* was effective against coccidia in poultry and exhibited antibacterial activity against gram-positive and anaerobic bacteria (Oscarson et al., 1989).

Another polyether antibiotic produced by *S. hygroscopicus spp.* was CP-80 219. It afforded anticoccidial activity against *Eimeria tenella* in chickens between 30 and 120 $mg \cdot kg^{-1}$. It also exhibited good activity against a number of gram-positive bacteria and the spirochete, *Treponema hyodysenteriae* but not was not gram-negative aerobes, including *Escherichia coli.* (Dirlam et al., 1990).



CP-80,219

*S. violaceoniger sp. griseofuscus* yielded pyridazomycin with an amino acid side chain in its structure. It was the first naturally occurring antibiotic with a pyridazine moiety. It had significant antifungal activity. Also, it was slightly active against *Bacillus subitlis* (Grote et al., 1988).



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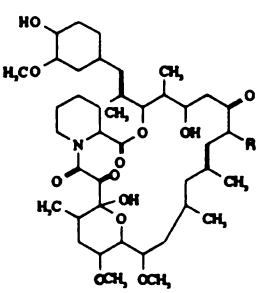
**Novel biological activities of known compounds:** Most of the antibiotics known today were discovered using antibacterial or antifungal assays. Throughout the development of effective and safe antibiotics, countless useful bioactive compounds were abandoned.

The rediscovery of known compounds with unknown activity has proven to be very useful (Monaghan et al., 1990). For example, ascomycin was an antifungal antibiotic isolated from the fungal body of a *Streptomyces* strain KK317 in 1963 (Arai et al., 1963). The producer strain was closely related to *S. hygroscopicus*. Ascomycin lacked activity against gram-positive and gram-negative bacteria except for *Mycobacterium* 607. Also, it was inactive against yeast-like fungi including *Candida albicans*, but it was very active against filamentous fungi *Rhizopus nigricas*, *Aspergillus orizae* and *Penicillium chrysogenum* in later studies (Arai et al., 1962). Recent studies revealed that macrolide antibiotics FR-900520 and FR-900523 isolated from *S.hygroscopicus*, with 23 carbons, were novel immunosuppressants and FR-900520 was identical in structure to ascomycin (Morisaki et al., 1992). These compounds were exhibited a prolonged skin allograft survival in rats (Hatanaka et al., 1988).




FR-900520 R=$CH_2CH_3$
FR-900523 R=$CH_3$

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**Hypothesis:** A wide range of bioactive compounds have been isolated from *Streptomyces spp.* (Monaghan and Tkacz, 1990). However, there is great need for additional novel bioactive compounds. Also, additional screening tools are available now to discover active compounds or templates for pharmaceutical and agricultural use. Gottlieb (1976) reported that 1 to 52% of isolates from soil inhibit microbial pathogens. Also, the number of inhibitory compounds identified from soil increased with the number of test organisms used (Maplestone et al., 1992). The Bioactive Natural Product Laboratory in the Depatment of Horticulture and Pesticide Research Center at Michigan State University acquired a culture collection of nitrogen-fixing *Streptomyces* from China (Zhang, 1994). These organisms are unique among *Streptomyces spp.* In that they can fix atmosphere nitrogen. Therefore, it is our hypothesis that many bioactive compounds could be isolated from this *Streptomyces* culture collection using bioassay-directed isolation and fractionations. Since it is unlikely that these strains have been screened before, these cultures could be a potential source for novel active compounds or known compounds with new activities.

## Chapter II

## High resolution rep-PCR genomic fingerprinting of *Streptomyces* strains


### Abstract

To establish the genetic diversity of our nitrogen-fixing strain collection, rep-PCR was used as a facile means to fingerprint the genome of each strain. All cultures were grown in YMG medium. The DNA of these strains were extracted from the cells by thermocycling, and the BOXA1R primer was used to amplify the DNA template in crude extracts using the polymerase chain reaction (PCR). The PCR products were separated electrophoretically on an agarose gel. The banding pattern is characteristic of each strain and provides an extremely high-resolution fingerprint that can be used to discriminate among closely related isolates within the same species. Genomic fingerprinting indicated that these strains are genetically diverse, and only two of the *S. griseofuscus* strains seemed to be closely related.

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## Introduction

Until recently, bacteria were classified largely according to their form and physiology. Taxonomic changes were frequent, and researchers had their favorite characters or taxonomic schemes. The history of *Streptomyces spp.* classification is no exception (Embley et al.,1994). In 1975, Kurylowicz et al. summarized the characteristics commonly used to classify the *Streptomyces spp.* These included morphological, physiological, ecological and biochemical characteristics, sensitivity to actinophages and serological reactions. These traits are phenotypic rather than genotypic characteristics and can show variation with different growth conditions.

Genotypic methods now are accepted tools in microbial systematics for strain identification and estimation of phylogenetic relationships. The use of PCR for amplification of specific nucleic acid sequences was first described by Saiki et al. in 1985. It is an enzymatic method employing a thermostable polymerase enzyme to produce multiple copies of specific nucleic acid regions quickly and exponentially allowing a million-fold or more amplifications of the target DNA. The reaction is achieved with a heat-stable DNA polymerase, a DNA template, oligonucleotide primers and the standard deoxyribonucleotide triphosphates (ATP, CTP, GTP, TTP). The amplification process can be divided in three steps which are repeated in cycles: first, the DNA sample is heated up to 94°C to disassociate the template DNA duplex, then the temperature is decreased to 40 - 55°C to allow the primer to anneal to the complimentary sequences of the template strands, and finally, the temperature is raised, typically to 72°C, for the extension reaction.

Repetitive element sequence-based PCR (rep-PCR) refers to the general methodology involving the use of oligonucleotide primers complimentary to short repetitive-sequence elements which occur on the genome, these elements are highly conserved and are present throughout the prokaryotic kingdom (Towner et al., 1993). Consensus sequence primers were designed for two such repetitive elements, the repetitive extragenic palindromic (REP) element (Stern et al., 1984) and the repetitive intergenic consensus (ERIC) elements (Hulton et al., 1991) and have been used extensively to fingerprint the genomes of taxonomically diverse prokaryotes. Outwardly directed primer sets based on REP and ERIC consensus sequences can be used to generate clearly resolvable DNA fragments by PCR amplification using template genomic DNA from species which contain these sequences (Woods et al., 1992). The DNA fragments represent amplification of DNA between adjacent repetitive elements (Woods et al., 1992). The differently sized fragments generated in a reaction produce a "fingerprint" pattern when separated electrophoretically. The specific banding pattern is reproducible and characteristic of each strain (de Bruijn, 1992; Murry et al., 1995) A third primer, the BOX element, first described in gram-positive bacteria, was introduced by Martin et al. (1994) and has found utility in fingerprinting the genomes in all major prokaryotic taxa. The term rep-PCR refers to the application of any of these three repetitive sequence primers to generate DNA fingerprints by the PCR reaction. The primer used in this experiment is the prokaryotic-specific BOXA1R primer (Versalovic et al., 1994).

A collection of soil *Streptomyces spp.* were isolated under diazotrophic conditions from a variety of sites in China (Zhang, personnel communication). These strains reduce acetylene under nitrogen-fixing culture conditions. This activity responds to ammonia inhibitor. These results were confirmed using the $^{15}N$ isotope assay for nitrogen activity

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(Zhang, personal communication). To date, nitrogen-fixation has been reported only in a *Streptomyces thermoautotrophicus* isolates (Gadkari, 1992). Thus, the capacity for nitrogen fixation suggest these strains may be an unusual group of *Streptomyces spp.*

The strains of this collection were classified according to "The Manual of Identification of *Streptomyces spp.*" (1975) by Dr. Zhang. These strains tentatively assigned to *Streptomyces globisporus, S. roseosporus, S. lavendulae, S. glaucus, S. cinereus, S. viridis, S. cyaneus, S. griseorubroviolaceus, S. griseofuscus, S. aureus* and *S. hygroscopicus*. BOX-PCR was adapted to characterize this collection at the sub-species level.

## Material and Methods

**Culture isolation:** About 30 g of soil samples were collected in triplicate by Dr. Zhong-ze Zhang (personal communication) at several locations in China (Table 2.1). Ten gram soil samples from each site were stirred with 100 ml of sterile water for 20 min. The supernatant (1 ml) was diluted with 9 ml of sterile water. Two drops from each of these dilutions were transferred onto a nitrogen-free, defined solid media containing (1.09 $g \cdot L^{-1}$ $KH_2PO_4$, 0.44 $g \cdot L^{-1}$ $KHPO_4$, 0.0014 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.34 $g \cdot L^{-1}$ NaCl, 0.34 $g \cdot L^{-1}$ $CaSO_4 \cdot 2H_2O$, 0.01 $gL^{-1}$ $FeSO_4 \cdot 7H_2O$, 0.0027 $g \cdot L^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, 9.1 $g \cdot L^{-1}$ sucrose, 34.2 $g \cdot L^{-1}$ KOH, 18 $g \cdot L^{-1}$ agar and 114 $g \cdot L^{-1}$ $H_3PO_4$). The plates were incubated for 5-7 days at 28°C. Individual colonies then were restreaked on plates containing this solid medium to obtain pure cultures derived from a single spore. The pure cultures were then transferred onto solid nitrogen-free medium slants and stored at 25°C.

Table 2.1. List of nitrogen-fixing *Streptomyces spp.* grown in YMG medium

| No. | Identification No. | Name | Appearance |
|---|---|---|---|
| 1 | MSU/ZD/001 | *S.globisporus* | yellow |
| 2 | MSU/ZD/002 | *S.globisporus* | white |
| 3 | MSU/ZD/003 | *S.globisporus* | yellow |
| 4 | MSU/ZD/004 | *S.roseosporus* | pink |
| 5 | MSU/ZD/005 | *S.roseosporus* | white |
| 6 | MSU/ZD/006 | *S.glaucus* | white |
| 7 | MSU/ZD/007 | *S.glaucus* | white brown |
| 8 | MSU/ZD/008 | *S.glaucus* | white |
| 9 | MSU/ZD/009 | *S.glaucus* | white |
| 10 | MSU/ZD/010 | *S.glaucus* | white |
| 11 | MSU/ZD/011 | *S.glaucus* | white brown |
| 12 | MSU/ZD/012 | *S.glaucus* | white&pink |
| 13 | MSU/ZD/013 | *S.cinereus* | white |
| 14 | MSU/ZD/014 | *S.cinereus* | white |
| 15 | MSU/ZD/015 | *S.cinereus* | yellow |
| 16 | MSU/ZD/016 | *S.cinereus* | white |
| 17 | MSU/ZD/017 | *S.cinereus* | white |
| 18 | MSU/ZD/018 | *S.cinereus* | yellow |
| 19 | MSU/ZD/019 | *S.viridis* | yellow |
| 20 | MSU/ZD/020 | *S.viridis* | yellow |
| 21 | MSU/ZD/021 | *S.viridis* | white brown |
| 22 | MSU/ZD/022 | *S.cyaneus* | white brown |
| 23 | MSU/ZD/023 | *S.griseorubroviolaceus* | white green |
| 24 | MSU/ZD/024 | *S.griseorubroviolaceus* | yellow |
| 25 | MSU/ZD/025 | *S.griseorubroviolaceus* | white |

| | | | |
|---|---|---|---|
| 26 | MSU/ZD/026 | *S.griseorubroviolaceus* | yellow |
| 27 | MSU/ZD/027 | *S.griseorubroviolaceus* | white |
| 28 | MSU/ZD/028 | *S.griseorubroviolaceus* | yellow |
| 29 | MSU/ZD/029 | *S.griseorubroviolaceus* | white |
| 30 | MSU/ZD/030 | *S.griseofuscus* | yellow |
| 31 | MSU/ZD/031 | *S.griseofuscus* | white |
| 32 | MSU/ZD/032 | *S.griseofuscus* | white slow |
| 33 | MSU/ZD/033 | *S.griseofuscus* | grey brow |
| 34 | MSU/ZD/034 | *S.griseofuscus* | yellow brown |
| 35 | MSU/ZD/035 | *S.griseofuscus* | white black |
| 36a | MSU/ZD/036a | *S.griseofuscus* | white green |
| 36b | MSU/ZD/036b | *S.griseofuscus* | white yellow |
| 37 | MSU/ZD/037 | *S.aureus* | white |
| 38 | MSU/ZD/038 | *S.aureus* | white |
| 39 | MSU/ZD/039 | *S.aureus* | yellow |
| 40 | MSU/ZD/040 | *S.aureus* | white |
| 41 | MSU/ZD/041 | *S.aureus* | yellow |
| 42 | MSU/ZD/042 | *S.aureus* | yellow |
| 43 | MSU/ZD/043 | *S.aureus* | yellow |
| 44 | MSU/ZD/044 | *S.aureus* | yellow |
| 45 | MSU/ZD/045 | *S.aureus* | white |
| 46 | MSU/ZD/046 | *S.aureus* | yellow |
| 47a | MSU/ZD/047a | *S.aureus* | orange |
| 47b | MSU/ZD/047b | *S.aureus* | bale yellow |
| 48 | MSU/ZD/048 | *S.hygroscopicus* | white |

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**Growth medium:** Strains were subcultured to YMG slants (yeast extract 4 $g \cdot L^{-1}$, malt extract 10 $g \cdot L^{-1}$, glucose 4 $g \cdot L^{-1}$ and agar 18 $g \cdot L^{-1}$) and incubated for 8 days at 26°C. After 8 days, a loop full of cells were transferred into a 20-ml test tube containing 5 ml of YMG liquid media (yeast extract 4 $g \cdot L^{-1}$, malt extract 10 $g \cdot L^{-1}$ and glucose 4 $g \cdot L^{-1}$). The inoculated test tubes then were placed on a rotary shaker at 110 rpm at 26°C for 14 days.

**Isolation of DNA:** 1 ml aliquots of *Streptomyces spp.* cultures were centrifuged (10,000 rpm at 4°C), and the cell pellet was washed twice with TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0). The pellet was resuspended in 0.1 ml of TE, and the suspension was frozen at -20°C overnight. An aliquot (15 µl) of the cell suspension was diluted in 15 µl Gitschier buffer (83 mM $(MH_4)_2SO_4$, 335 mM Tris HCl pH 8.8, 33.5 mM $MgCl_2$, 33.5 µm EDTA, 150 mM β-mercaptoethanol and 850 µl bovine serum albumin·$ml^{-1}$), and subjected to the following temperature regime in a thermocycler: 95°C for 7 min, 7 cycles of 4°C for 7 min, 40°C for 7 min, and 80°C for 7 min (Frank Spooner, personal communication).

**BOX-PCR:** BOX-PCR reactions were carried out as described by Murry et al. (1995) using 3µl of the cell preparations for each 25 µl of the reaction mixture. The BOXA1R primer (5'CTACGGCAAGGCGACGCTGACG3', Versalovic et al., 1994) was used at 15 ng per 25 µl of reaction mixture. The primers were synthesized by the Macromolecular Sructure, Sequence, and Synthesis Facility at Michigan State University using an Applied Biosystems DNA synthesizer (Model 380B, Foster City, California). The PCR reactions were performed in 5 µl Gitschier buffer, 0.2 µl bovine serum albumin (20 mg·$ml^{-1}$), 2.5 µl DMSO (10%, v:v), 1.25 µl 25 mM dNTP-Mix (1:1:1:1), 1.25 µl (50 pmol) Box primer, 0.4 µl (2 units) Taq

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polymerase (Perkin-Elmer, Cat # N808-0070) and $H_2O$ to adjust the final volume to 25 µl. PCR amplifications were performed in an automated DNA thermal cycler (Perkin-Elmer DNA Thermal Cycler). 6 to 10 µl of PCR products were separated electrophoretically on 1.5% agarose gels in 0.5X TAE buffer (50X TAE buffer: Tris base 108 $g \cdot L^{-1}$, 85% phosphoric acid 15.1 $ml \cdot L^{-1}$ and 1.5 M EDTA 40 $ml \cdot L^{-1}$) at 4.5-5$V \cdot cm^{-1}$, stained with ethidium bromide and photographed under UV light.

## Results and Discussion

Strain classification of this culture collection was carried out by Dr. Zhang using the criteria established in "The Manual for the Identification of *Streptomyces spp.*" (Table 2.1). The classification was based on cell morphology and culture characteristics such as color of hyphae in specific medium and carbon utilization patterns. Figures 2.1and 2.2 show ethidium-bromide stained gels of the genomic fingerprints of *Streptomyces spp.* generated from crude cell extracts of each strain using the BOX A1R primer. Box-PCR generated fingerprints revealed considerable genomic diversity within strains classified earlier as members at the same species. The fingerprint patterns characteristic of each strain were similar in the number and size range of the DNA banding patterns to those patterns reported in earlier work which utilized BOX-PCR to characterize *Frankia* (Actinomyces) strains (Murry et al., 1995)

*S. globisporus* strains MSU/ZD/001 and MSU/ZD/003 share a common DNA fragment of 1,018 bp while the rest of the bands were different in these two strains. *S. globisporus*, strain MSU/ZD/002, had a fingerprint pattern distinct from those patterns seen in strains 001 and 003. The two strains of *S. roseosporus*, MSU/ZD/004 and MSU/ZD/005, shared a common band of 800 bp, but otherwise the overall pattern was different. *S.*

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*globisporus* (MSU/ZD/003) and *S. roseosporus* (MSU/ZD/004) showed comigrating bands at 1,018, 900, and 850 bp, which indicates that these two strains are closely related to each other although they were not placed in the same group by the criteria used by Dr. Zhang. *S. glaucus*, MSU/ZD/007 and MSU/ZD/009, had comigrating bands at 1,100 and 970 bp. Each of the other strains grouped as *S. glaucus* showed unique patterns.

Among all strains in this culture collection, the BOX-PCR fingerprint patterns of two *S. cinereus* strains, MSU/ZD/013 and MSU/ZD/014, were the only two with very high-similarity to each other. Each of the other 4 strains grouped as *S. cinereus* showed a distinct fingerprint pattern. Similarly, the three strains of *S. viridis* each gave a unique pattern. *S. griseorubroviolaceus*, strains MSU/ZD/023 and MSU/ZD/025, shared one comigrating band of 1,200 bp, two bands at 850 bp and one band at 550 bp. *S. griseorubroviolaceus*, strains MSU/ZD/026 and MSU/ZD/027, showed fingerprint patterns that were distinct from those of *S. griseorubroviolaceus*, strains MSU/ZD/023 and MSU/ZD/025, but shared common fragments of 650 and 506 bp.

The fingerprint patterns of the seven strains of *S. griseofuscus* did not show any similarity to each other nor to any of the other strains. The two of the *S. aureus* strains, MSU/ZD/037 and MSU/ZD/038, exhibited a common fragment of 1,450 bp in size, two bands at about 900 bp and one bands at 550 bp. *S. cyaneus*, strain MSU/ZD/022, and *S. hygroscopicus*, strains MSU/ZD/048, also gave unique fingerprints.

In earlier work (Murry et al. 1995), strains of *Frankia* (Actinomycetales) known to belong to the same genomic species (based on DNA-DNA hybridization) showed similar Box-PCR fingerprint patterns with at least 2 prominent bands in common. Strains from different genomic species shared no common bands. Hence, strains MSU/ZD/013 and MSU/ZD/014,

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which display nearly identical fingerprints, are clearly close related and probably belong to the same genomic species. The occurrence of comigrating bands shared by some strains suggest that they may be the members of the same genomic species. Thus, the strains grouped according to phenotypic characteristics by Dr. Zhang appear to be heterogeneous than expected and may represent more than one genomic species. For example, *S. globisporus*, strains 001 and 003 are probably members of the same genomic species while strain 002, also classified as *S. globisporus* appears to more distantly related. These results in general indicate that the genetic diversity within this culture collection is large. However, note that the high resolution of the BOX-PCR technique does not by itself allow classification of strains into defined species. Further analysis utilizing DNA-DNA hybridization or 16S rRNA sequence comparisons will allow assignment of these strains to known genomic species.

Antibiotic production of *Streptomyces* is known to be strain-specific. However, strains within the same species are known to produce different antibiotics or different quantities of the same antibiotic, whereas strains currently classified as different *Streptomyces spp.* may produce the same antibiotics (Loria et al., 1995). Thus, we have concluded that because of the large degree of genetic variation among this culture collection, it was necessary to screen each strain to test the potential biological activities of their secondary metabolites.

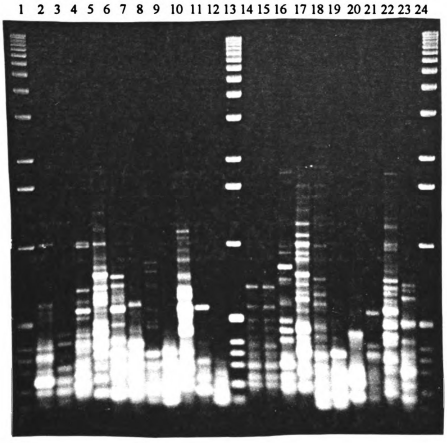


Figure 2.1: BOX-PCR fingerprint patterns of genomic DNA from *Streptomyces spp.* strains. A. Lanes: 1 ,13 and 24, 1Kb marker; 2, strain 001; 3, 002; 4, 003; 5, 004; 6, 005; 7, 006; 8, 007; 9, 008; 10, 009; 11, 010 ;12, 011; 14, 013; 15, 014; 16, 015; 17, 016; 18, 017; 19, 018; 20, 019; 21, 020; 22, 0211; 23, 022.

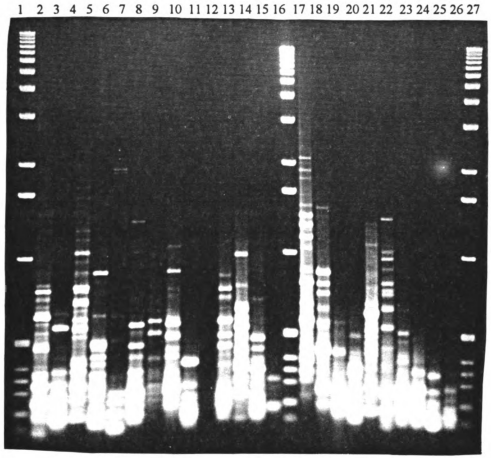


**Figure 2.2**: BOX-PCR fingerprint patterns of genomic DNA from *Streptomyces spp.* strains. **B. Lanes**: 1 ,16 and 27, 1Kb marker; 2, 023; 3, 024; 4, 025; 5, 026; 6, 027; 7, 028; 8, 029; 9, 030; 10, 031; 11, 032; 12, 033; 13, 034; 14, 035; 15, 036; 17, 037; 18; 038; 19, 039; 20, 040; 21, 041; 22, 043; 23, 044; 24, 045; 25, 046, 26, 048.

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# Chapter III

## Bioactive extracts from nitrogen-fixing *Streptomyces spp.*


### Abstract

Preliminary antimicrobial , mosquitocidal and anti-cancer bioassays were carried out on cell and cell-free culture extracts from 48 strains of *Streptomyces spp.* obtained from China. All cultures were grown in both A9 and YMG media. The solvent extracts of cells and lyophilized cell-free medium were bioassayed against several test strains of bacteria, fungi and the mosquito larvae, *Aedes egypti*. Anticancer bioassays were conducted on mutant *Saccharomyces cerevisae* strains to determine the presence of *top I* or *II* inhibitory activities. All antimicrobial bioassays were performed by spotting 250 µg of crude extracts on agar plates inoculated with the appropriate test strain. The mosquitocidal bioassay was conducted using 250 ppm in 1 ml of water. Crude extracts from both cell and cell-free medium of most strains showed some activity against the gram-positive bacteria *Streptococcus aureus* and *Staphylococcus epidermidis*. About half of the strains tested showed growth-inhibitory activity against the fungal plant pathogen, *Gleosporum spp.* Also, some strains produced compounds that are inhibitory to the growth of *Escherichia coli* and *Candida albicans*. Crude extracts from several strains showed significant mosquitocidal activity against *Aedes aegypti* larvae. Seven of the strains studied exhibited anticancer activity based on topisomerase assays.

## Introduction

Centuries of human use of medicinal plants and herbs guided scientists to isolate and purify multitudes of active principles for pharmacological use. Such historical data were not evident for the use of microorganisms in human medicine. The researchers first focused their attention on plants, because of the abundance of material and relative ease with which it could be collected (Vining, 1990).

At the beginning of this century, natural product chemists began to investigate the secondary metabolites from microorganisms. The metabolites usually were isolated from the cultures which were easy to grow and also gave high yields of active compounds. Decades ago, the study of secondary metabolites was focused primarily on the production of bioactive components. Since the discovery of penicillin, secondary metabolites isolated from microorganisms became another rich source for pharmaceutical and agricultural applications.

Secondary metabolites exhibit their antibiotic activity because of their ability to inhibit essential metabolic processes of other microorganisms (Vining, 1990). Throughout the development of effective and safe antiinfectives, countless antibiotics were abandoned (Monaghan et al., 1990). One of the reasons is that many of the antibiotics were directed against one of the universal primary metabolic pathway reactions, frequently involving energy transduction or gene expression. Thus, they are toxic to animals and have to be discarded (Vining, 1990). However, some of the antibiotics which show growth-inhibitory activity in a wide range of organisms have found use as anticancer drugs, because of their toxicity to rapidly proliferating cells (Vining, 1990). Anthracycline and bleomycin are the members of this group of antibiotics (Tsukagoshi et al., 1986).

The insecticidal activity of some compounds produced by microorganisms has been

demonstrated (Fabre et al., 1988). The existence of microbial epizootics among insect populations accounts for fluctuations in agricultural pest infestation (Vining,1990). Although the causes of insect mortality are often complex, the formation of toxic agents by an invading microorganism is sometimes a factor. Direct screening of microorganisms has uncovered a variety of secondary metabolites with insecticidal activity such as milbemycins (Takiguchi et al., 1980).

A high proportion of microorganisms isolated from soil showed various activities (Gottlieb, 1976). Among the soil microorganisms, *Streptomyces spp.* is known to produce about 75% of the antibiotics currently in use. Even today, many new active secondary metabolites are isolated from *Streptomyces spp.* with novel biological activities. Most of the bioactive secondary metabolites are discovered by using numerous screening methods. Hence, novel biological activity may be found for known compounds.

As research progresses, the screening for antibiotics became more selective. However, fermentation broths submitted for assays have remained largely from those of randomly chosen cultures (Monaghan, 1990). The Bioactive Natural Product Laboratory (BNPL) at Michigan State University recently has acquired a culture collection of nitrogen fixing *Streptomyces spp.* from China (Zhang, 1994). The production of secondary metabolites by these unique *Streptomyces spp.* have not been investigated until now. Since the *Streptomyces spp.* is a rich source for bioactive compounds, it is logical to investigate new groups of *Streptomyces spp.* (Monaghan, 1990). The collection from China was isolated from regions not yet extensively sampled and because these strains fix nitrogen, they present an unique group among *Streptomyces* spp. Thus, this strains collection may produce novel bioactive compounds.

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## Material and Methods

***Streptomyces spp* isolates**: More than 68 strains of *Streptomyces spp.* were isolated from soil samples at various locations in China (Zhang, 1994 personal communication). They were classified according to "The Manual for the Identification of *Streptomyces spp.*" (1975) and belong to *Streptomyces globisporus*, *S. roseosporus*, *S. lavendulae*, *S. glaucus*, *S. cinereus*, *S. viridis*, *S. cyaneus*, *S. griseorubroviolaceus*, *S. griseofuscus*, *S. aureus* and *S. hygroscopicus*.

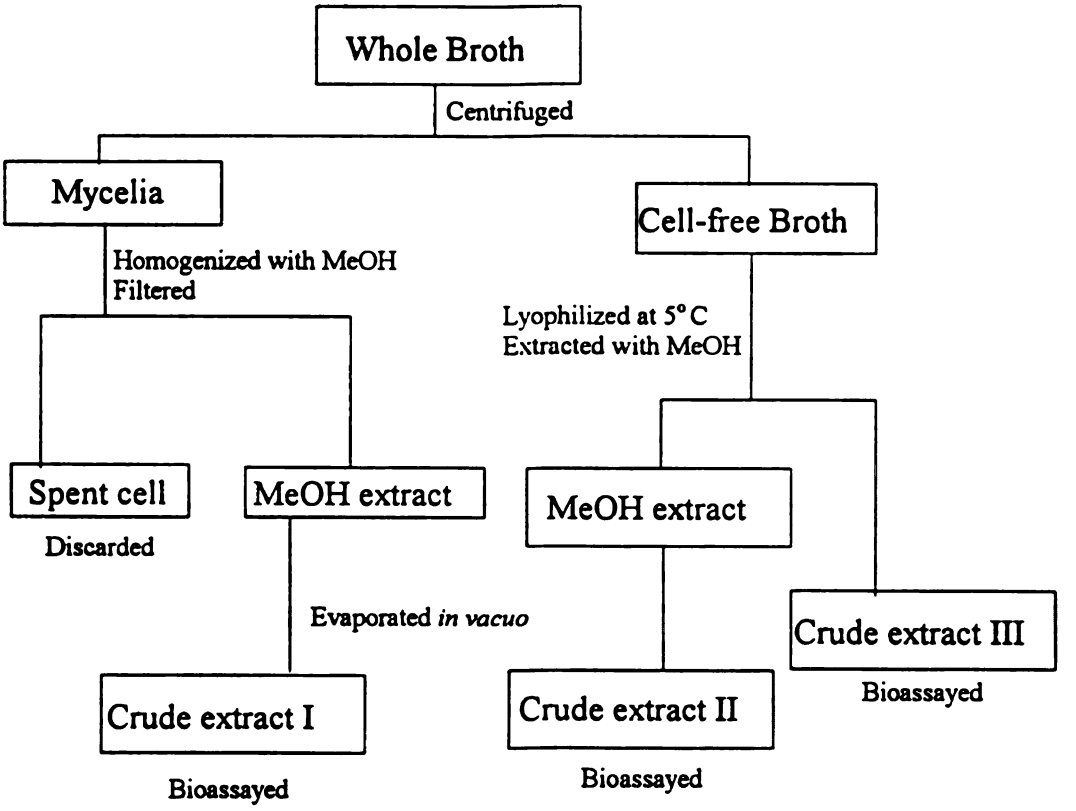
**Culturing the organism**: The cultures were initially grown in an inorganic medium (1.09 $g \cdot L^{-1}$ $KH_2PO_4$, 0.44 $g \cdot L^{-1}$ $KHPO_4$, 0.0014 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.34 $g \cdot L^{-1}$ NaCl, 0.34 $g \cdot L^{-1}$ $CaSO_4 \cdot 2H_2O$, 0.01 $g \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$, 0.0027 $g \cdot L^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, 9.1 $g \cdot L^{-1}$ sucrose, 34.2 $g \cdot L^{-1}$ KOH, 18 $g \cdot L^{-1}$ agar and 114 $g \cdot L^{-1}$ $H_3PO_4$) and then transferred to solid YMG (yeast extract 4 $g \cdot L^{-1}$, malt extract 10 $g \cdot L^{-1}$, glucose 4 $g \cdot L^{-1}$ and agar 18 $g \cdot L^{-1}$) medium. Twenty of the 68 cultures in the collection were unable to grow in YMG medium (Table 2.1).

**Production of bioactive metabolites**: The cultures were grown on YMG slants and incubated for 8 days at 26°C. After 8 days, the cells were transferred into 400 ml Erlenmeyer flasks containing either 100 ml YMG or A9 (peptone 4 $g \cdot L^{-1}$, glucose 10 $g \cdot L^{-1}$ and molasses 20 $g \cdot L^{-1}$) liquid media, separately. The inoculated flasks then were placed on a rotary shaker at 110 rpm at 26°C for 8 days.

The cells were harvested from YMG and A9 media by centrifugation at 10,000 rpm and 4°C. The wet cells were homogenized and extracted with 100 ml of MeOH (Crude I). The cell-free media was lyophilized, and the residue was extracted with 15 ml of MeOH (Scheme 3.1). The MeOH-soluble extract (Crude II) and the MeOH-insoluble portion (Crude III) were dissolved in DMSO and distilled water, respectively, and bioassayed at 250 ppm concentrations.

**Antimicrobial bioassays**: Cultures of *Gleosporum spp.* were grown on PDA medium (potato dextrose agar 39 $g \cdot L^{-1}$). Cultures of *Candida albicans* were grown on YMG medium, and cultures of *Staphylococcus epidermidis*, *Streptococcus aureus* and *Escherichia coli* were grown on Emmons medium (neopeptone 10 $g \cdot L^{-1}$, glucose 20 $g \cdot L^{-1}$ and agar 18 $g \cdot L^{-1}$). The test organisms were harvested after 8 days of growth by resuspending the fully grown culture from growth plates in 10 ml of sterile saline solution. The cell suspension was adjusted to $10^3$ colony forming units per milliliter (CFU/ml). Bioassay plates were made by spreading cell suspensions (100 µl) of the test organisms on the surface of solid agar containing the respective medium. The test sample (250 $\mu g \cdot 20 \mu l^{-1}$ of solvent, DMSO or $H_2O$) was spotted on plates inoculated with the test organism. The plates were incubated at 28°C for 72 h before the zone of inhibition, characterized by the absence of microorganism's growth, was measured. Pure solvent (20µl) served as controls.

**Mosquitocidal assay**: This bioassay was conducted on the fourth instar of the mosquito larvae, *Aedes aegypti*, reared from eggs (Courtesy of Dr. Alexander Raikhel, Department of Entomology, Michigan State University) (Nair et al., 1989). Fifteen larvae (3-4 days old)




Scheme 3.1 Processing of fermentation broth from *Streptomyces spp.*

were placed in 980 µl of distilled water in a test tube and the crude extracts, 250 $\mu g \cdot 20\ \mu l^{-1}$ of DMSO or $H_2O$, were added. Controls received 20 µl of pure solvent, either DMSO or $H_2O$. The test tubes were covered and kept at room temperature. The number of dead larvae was recorded at 2, 4, 6 and 24 h intervals. Each treatment was repeated in triplicate.

**Anticancer assay**: *Saccharomyces cerevisiae* mutant cell cultures, JN394, JN394$t_{-1}$ and JN394$t_{2-5}$ were used to test for anticancer activities (Nitiss et al., 1993). Strain JN394 is hypersensitive to topoisomerase *I* and *II* poisons due to the mutations that destroyed the *RAD52* repair pathway. Strain JN394$t_{-1}$ is isogeneic to JN394 except that the *top I* gene is deleted. The deletion of *top I* gene results in the lack of response to topoisomerase *I* poisons. JN394$t_{2-5}$ carries the *top 2-5* gene and is resistant to the topoisomerase *II* poisons but responds to the topoisomerase *I* poisons (Table 3.1).

Table 3.1. Topoisomerase sensitivity of yeast strains in the anticancer assay

| *S. cerevisae* strain | *Top I* | *Top II* |
|---|---|---|
| JN394 | + | + |
| JN394$t_{-1}$ | - | + |
| JN394$t_{2-5}$ | + | - |

The organisms were cultured for seven days on solid YPDA medium (yeast extract, 20 $g \cdot L^{-1}$; peptone, 10 $g \cdot L^{-1}$; dextrose, 20 $g \cdot L^{-1}$; adenine sulfate, 2 $ml \cdot L^{-1}$ from 0.5% stock solution and agar 17 $g \cdot L^{-1}$). Ten ml of sterile saline solution was added to the surface of a fully grown culture in a Petri dish to resuspend the cells, and then the cell concentration was adjusted to $5 \times 10^6$ CFU/ml. Bioassay plates were made by spreading cell suspension (100µl)

on the surface of solid agar YPDA medium. The test samples (250 $\mu g \cdot 20\ \mu l^{-1}$ of solvent) were spotted directly on the surface of the plates inoculated with the respective organisms and were incubated at 28°C. The zone of inhibition, characterized by the absence of microbial growth, was measured in mm after 72 h. The extracts which did not show a zone of inhibition was recorded as -.

## Results and Discussion

The anticancer assays showed that 7 of the 48 strains were potential candidates for the production of anticancer compounds (Table 3.2). One strain of *S. viridis* (MSU/ZD/021) and three strains of *S. aureus* (MSU/ZD/037, MSU/ZD/043 and MSU/ZD/044) showed activities against JN394 and JN394$t_{2\text{-}5}$. Strain 043 gave 20 and 30 mm of zones of inhibition when the crude extracts were tested against JN394 and JN394$t_{2\text{-}5}$, respectively. Strain 021 gave more than a 30-mm zone of inhibition when it was tested against JN394. These results indicated that the test extracts were active against topoisomerase *I* poisons but were not active against top *II* enzyme. Two strains of *S. cinereus* (MSU/ZD/013 and MSU/ZD/014) and one strain of *S. griseofuscus* (MSU/ZD/032) exhibited similar activity against JN394 and JN394$t_{-1}$. The crude extracts from these three strains gave excellent zones of inhibition and are potential sources for *top II* poisons.

Four strains, *S. roseosporus* (MSU/ZD/004), *S. glaucus* (MSU/ZD/007), *S. cinereus* (MSU/ZD/013) and *S. viridis* (MSU/ZD/019) showed activity against *C. albicans spp.* (Table 3.3). The strain *S. cinereus* produced an intracellular secondary metabolite when it was grown in YMG medium. The cell extract gave a 10 mm of zone of inhibition against *C. albicans*. *S. roseosporus*, *S. glaucus* and *S. viridis* produced compounds that were inhibitory

to the growth of *C. albicans*. Also, *S. roseosporus* and *S. viridis* gave 24 and 6 mm of zones of inhibition, respectively, when they were grown in A9 medium. These strains did not produce active metabolites when fermented in YMG. *S. glaucus* produced active extract from both A9 and YMG medium and gave 17 and 10 mm zones of inhibition against *C. albicans*, respectively. TLC of these extracts showed that *S. glaucus* yielded identical compounds.

There were only two strains, *S. glaucus* (MSU/ZD/012) and *S. cinereus* (MSU/ZD/017), which exhibited activity against *E. coli* (Table 3.4). The activity was found in the media but not cell extracts in both of these strains. The MeOH extract of the dried YMG medium from *S. glaucus* gave 6 mm of zones of inhibition against *E. coli*. The MeOH extracts of dried cell-free YMG and A9 broth from *S. cinereus* were active against *E. coli* and showed 8 and 7 mm of zones of inhibition, respectively. In addition, strain 017 released water-soluble secondary metabolites into the A9 growth medium and gave an 11-mm of zone of inhibition against *E. coli*.

Antifungal assays with *Gleosporum spp.* showed that 15 strains had marginal activity. All of the extracts gave of zones inhibition less than 10 mm. The cell extracts from *S. roseosporus* (MSU/ZD/005), three strains of *S. glaucus* (MSU/ZD/006, MSU/ZD/008 and MSU/ZD/012), *S. viridis* (MSU/ZD/021), *S. cyaneus* (MSU/ZD/022) and *S. griseorubroviolaceus* (MS/ZD/026) exhibited antifungal activity (Table 3.5). Two strains of *S. globisporus* (MS/ZD/002 and MSU/ZD/003), *S. roseosporus* (MS/ZD/004) and three *S. cinereus* (MSU/ZD/014, MSU/ZD/015 and MSU/ZD/016) released antifungal compounds to their growth media and inhibited the growth of *Gleosporum spp.* Both cell and media extracts of *S. glaucus* (MSU/ZD/007) and *S. cinereus* (MS/ZD/013) were active against

*Gleosporum spp.*

Extracts from a total of 20 strains showed activities against *Streptococcus aureus* and *Staphylococcus epidermidis* (Table 3.6). Most of these strains gave between 10 and 30 mm of zones of inhibition. The activities were found in both cell and media extracts. *S. griseofuscus* (MSU/ZD/033) was the most active among these strains and the zones of inhibition were 35 and 30 mm for *S. aureus* and *S. epidermidis,* respectively.

Crude extracts from strains of *S. cinereus* (MSU/ZD/013), *S. griseoubroviolaceus* (MS/ZD/026), *S. griseofuscus* (MS/ZD/032 and MSU/ZD/033) and *S. aureus* (MSU/ZD/044), exhibited significant mosquitocidal activity (Table 3.7). These extracts gave 100% mortality at 24h when tested at 250 ppm concentration. When strains *S. griseoubroviolaceus* and *S. aureus* (MSU/ZD/044) were grown in YMG medium, the extracts from these two strains gave 100% mortality at 6 and 4 h, respectively. However, the activity decreased significatly when both strains were grown in A9 medium. The TLC of the cell extract from *S. griseoubroviolaceus* showed that there were more compounds in YMG extract than that of A9. Also, *S. cinereus* and *S. aureus* (MSU/ZD/033) exhibited different activity on mosquito larvae when they were grown in YMG or A9 media. The crude extract from *S. cinereus* grown in A9 medium gave 100% mortality at 4 h but the YMG extract was active only at 24 h. Similarly, the YMG and A9 extracts from the strain 033 produced 100% mortality against mosquito larvae at 2 and 4 h, respectively. This result indicates that the growth environment of these cultures had a significant effect on the synthesis of secondary metabolites. However, comparison of the TLC of these crude extracts suggests that these strains produce the same antibiotic under different fermentation conditions.

The bioassays showed that results suggest that the crude extracts from more than half

of the culture collection were active against gram-positive bacteria, and some of them exhibited marginal activities against fungi and yeast. The mosquitocidal assay showed that five strains produced active extracts against mosquito, especially *S. griseofuscus* (MS/ZD/033). Therefore, this strain was studied further for the isolation and identification of the mosquitocidal compound. Chapter IV of this thesis describes the insecticidal compound 1 isolated from *S. griseofuscus*.

Table 3.2. The results of preliminary anticancer assays measured as zone of inhibition in mm

| Organism - Strain No. | *Saccharomyces cerevisae* | | |
|---|---|---|---|
| | JN394 | JN394$t_{-1}$ | JN394$t_{2-5}$ |
| *S. cinereus* - 13 | 20 | 20 | - |
| *S. cinereus* - 14 | 20 | 20 | - |
| *S. viridis* -21 | 30 | - | 10 |
| *S. griseofuscus* - 32 | 20 | 10 | - |
| *S. aureus* - 37 | 10 | - | 10 |
| *S. aureus* - 43 | 20 | - | 30 |
| *S. aureus* - 44 | 10 | - | 20 |

Table 3.3. The list of active extracts from *Streptomyces spp.* grown in YMG or A9 medium at 250 ppm against *Candida albicans*

| Organism - Strain No. | Growth Medium | Active portion | Zone of inhibition (mm) |
|---|---|---|---|
| *S. roseosporus* - 4 | A9 | Crude II | 24 |
| *S. glaucus* - 7 | YMG | Crude II | 10 |
| *S. glaucus* - 7 | A9 | Crude II | 17 |
| *S. cinereus* - 13 | YMG | Crude I | 10 |
| *S. viridis* - 19 | A9 | Crude II | 6 |

Table 3.4. The list of active extracts from *Streptomyces spp.* grown in YMG or A9 medium at 250 ppm against *E. coli*

| Organism - Strain No. | Growth Medium | Active portion | Zone of inhibition (mm) |
|---|---|---|---|
| *S. glaucus* - 12 | YMG | Crude II | 6 |
| *S. cinereus* - 12 | YMG | Crude II | 8 |
| *S. cinereus* - 17 | A9 | Crude II | 7 |
| *S. cinereus* - 17 | A9 | Crude III | 11 |

Table 3.5. The list of active extracts from *Streptomyces spp.* grown in YMG or A9 medium at 250 ppm against *Gleosporum spp*

| Organism - Strain No. | Growth Medium | Active portion | Zone of inhibition (mm) |
|---|---|---|---|
| *S. globisporus* - 2 | A9 | II | 5 |
| *S. globisporus* - 3 | Both | II | 7 |
| *S. roseosporus* - 4 | A9 | II | 7 |
| *S. roseosporus* - 5 | A9 | I | 7 |
| *S. glaucus* - 6 | YMG | I | 6 |
| *S. glaucus* - 7 | A9 | II | 5 |
| *S. glaucus* - 7 | Both | I | 6 |
| *S. glaucus* - 8 | YMG | I | 5 |
| *S. glaucus* - 12 | Both | I | 6 |
| *S. cinereus* - 13 | Both | I | 9 |
| *S. cinereus* - 13 | YMG | II | 6 |
| *S. cinereus* - 14 | YMG | II | 6 |
| *S. cinereus* - 15 | Both | II | 6 |
| *S. cinereus* - 16 | YMG | II | 6 |
| *S. viridis* - 21 | Both | I | 7 |
| *S. cyaneus* - 22 | Both | I | 10 |
| *S. griseorubroviolaceus* - 26 | A9 | I | 10 |

Both: YMG and A9

Table 3.6. The list of active extracts from *Streptomyces spp.* grown in YMG medium at 250 ppm against *Streptococcus aureus* and *Staphylococcus epidermidis*

| Organism - Strain No. | Growth medium | Active portion | Zone of inhibition (mm) *S. aureus* | *S. epidermidis* |
|---|---|---|---|---|
| *S. roseosporus* - 4 | YMG | I, II and III | 21, 25, 0 | 0, 20, 13 |
| *S. roseosporus* - 5 | YMG | II and III | 13, 13 | 15, 13 |
| *S. glaucus* - 7 | YMG | I, II and III | 24, 15, 12 | 24, 15, 11 |
| *S. glaucus* - 7 | A9 | I, II and III | 25, 12, 12 | 22, 12, 12 |
| *S. glaucus* - 8 | YMG | I, II and III | 16, 10, 0 | 0, 13, 10 |
| *S. cinereus* - 13 | YMG | I | 32 | 30 |
| *S. cinereus* - 13 | A9 | I | 30 | 30 |
| *S. cinereus* - 17 | YMG | III | 0 | 7 |
| *S. viridis* - 21 | YMG | I and II | 27, 13 | 17, 0 |
| *S. viridis* - 21 | A9 | I and II | 20, 10 | 17, 0 |
| *S. cyaneus* - 22 | YMG | I | 0 | 15 |
| *S. cyaneus* - 22 | A9 | I | 0 | 12 |
| *S. griseorubroviolaceus* - 24 | YMG | II | 13 | 0 |
| *S. griseorubroviolaceus* - 25 | YMG | I | 8 | 25 |
| *S. griseorubroviolaceus* - 25 | A9 | I | 10 | 0 |
| *S. griseorubroviolaceus* - 26 | YMG | I and III | 23, 0 | 15, 17 |
| *S. griseorubroviolaceus* - 26 | A9 | I | 10 | 10 |
| *S. griseofucus* - 30 | A9 | III | 0 | 12 |
| *S. griseofucus* - 31 | YMG | II | 0 | 10 |
| *S. griseofucus* - 32 | YMG | I and II | 26, 0 | 26, 10 |
| *S. griseofucus* - 33 | YMG | I and III | 35, 0 | 30, 5 |
| *S. griseofucus* - 33 | A9 | I and II | 30, 20 | 28, 12 |
| *S. griseofucus* - 34 | YMG | I, II and III | 35, 35, 0 | 28, 20, 30 |
| *S. griseofucus* - 34 | A9 | I, II and III | 30, 22, 0 | 30, 0, 10 |
| *S. aureus* - 38 | A9 | I | 21 | 20 |
| *S. aureus* - 44 | YMG | I | 15 | 23 |
| *S. aureus* - 45 | YMG | I | 13 | 15 |
| *S. aureus* - 46 | YMG | I, II and III | 20, 18, 14 | 18, 11, 14 |
| *S. aureus* - 46 | A9 | I and III | 15 | 17, 15 |

Table 3.7. The list of the active extracts produced by *Streptomyces spp.* that exhibited 100% mortality against mosquito larvae *Aedes aegypti* at 250 ppm.

| Organism - Strain No. | Growth Media | Time (h) |
|---|---|---|
| *S. cinereus* - 13 | YMG | 24 |
| *S. cinereus* - 13 | A9 | 4 |
| *S. griseorubroviolaceus* - 26 | YMG | 6 |
| *S. griseofuscus* - 32 | YMG | 4 |
| *S. griseofuscus* - 32 | A9 | 4 |
| *S. griseofuscus* - 33 | YMG | 2 |
| *S. griseofuscus* - 33 | A9 | 4 |
| *S. aureus* - 44 | YMG | 4 |

# Chapter IV

## An insecticidal metabolite from nitrogen fixing *Streptomyces griseofuscus*


### Abstract

*S. griseofuscus*, strain MSU/ZD/033, was grown in YMG media for 8 days at 26°C on a shaker, the cells were harvested and extracted with MeOH:$CHCl_3$ mixture. The insecticidal compound 1 was purified from the crude cell extract by MPLC, TLC and finally by a recycling preparative HPLC. The identification of this compound was achieved by NMR, MS and UV spectral methods on the natural product and its methylated and acetylated derivatives. It is identical to an antibacterial antibiotic, indanomycin, reported earlier. In our tests, compound 1 showed bactericidal and insecticidal activities. In feeding trials using artificial diet, compound 1 demonstrated a 50% weight reduction for gypsy moth (*Lymantria dispar*) and tobacco hornworm (*Manduca sexta*) neonate larvae at 100 ppm concentration at 6 days. Also, it reduced the weight of corn earworm (*Helicovarpa zea*) at 100 ppm by 33% after six days. Compound 1 gave 100% mortality on 4 th instar mosquito larvae, *Aedes aegypti*, at 20 ppm. This is the first report of insecticidal activity of compound 1.

## Introduction

More than half of the worldwide expenditure on agrochemicals was devoted to insecticides in an effort to defy the continuous onslaught of over half a million different herbivorous insect species (Ley et al., 1993). Despite this, 15% of the crops planted are lost to feeding insects and other pests (Ley et al., 1990). The cost of this damage and its effect on agriculture has increased the demand for more effective crop protection agents.

For many years, pest control has been based primarily on the utilization of synthetic compounds (Fabre et al., 1988). Dichlorodiphenyltrichloroethane (DDT) was the first major synthetic insecticide used widely, but it caused enormous environmental and human health problems. Since then, numerous other insecticides have been developed for insect control. Currently, the three groups of insecticides which show more environmentally acceptable properties are organophosphates, carbamates and pyrethroids (Pickett, 1988). However, these compounds are toxic toward a wide range of insects and, while effective against the target pest, often kill other insect species including natural enemies of pests (Ley et al., 1990). The continued use of these compounds has resulted in the development of resistance to these compounds in pest populations, environmental pollution and hazard to humans (Munakata, 1975). Therefore, new insecticidal compounds with less environmental impact are sought to manage insects. Natural products are considered to be an ideal source for these requirements and several natural products have been developed as insecticidal agents (Fabre, 1988).

The use of plants or plant extracts against insects has been known for a long time. Plants have evolved to produce chemical defenses against insect attack and thus, provide us with a rich source of biologically active metabolites including repellents, attractants and insect growth regulators (Ley et al., 1990). Plant-derived compounds such as alkaloids, terpenes,

fatty acids were the first shown to have insecticidal activity. The pyrethroids account for about one third of the world's insecticide use. They were developed by the structural modification or total synthesis based on insecticides isolated from pyrethrum flowers. (Pickett, 1988).

The microbially produced insecticidal metabolites are a relatively new discovery compared to the plant derived insecticides because the early screening of microbial metabolites was focused on antimicrobial activity alone. Since secondary metabolites from microorganisms were found to be effective against human diseases, their use was explored in agricultural pest control as well. About 75% of microbially produced antibiotics were isolated from the actinomycete family, especially members of *Streptomyces spp.* Screening of microorganisms for the production of antimicrobial activities resulted in the discovery of many secondary metabolites with insecticidal activity, such as milbemycins (Mishima et al., 1983) and prasinons (Box et al., 1973). Also, the use of additional novel bioassays facilitated the discovery of several secondary metabolites from fermentation products with novel biological activities.

The chapter III of this thesis describes the preliminary bioassays of extracts from a variety of nitrogen-fixing *Streptomyces spp.* These assays indicated that *S. griseofuscus* (MS/ZD/033) produced the most active metabolite against mosquito larvae *Aedes aegypti.* Hence, mosquitocidal-assay-directed purification of the cell extract from *S. griseofuscus* was carried out to elucidate the structure of the active metabolite. This chapter describes the fermentation, isolation, purification and structure elucidation of the mosquitocidal compound **1** (Fig. 4.1) from *S. griseofuscus.*

## Materials and methods

**General experimental methods:** The media used in the fermentation or growth of organisms were liquid YMG (yeast extract 4 $g \cdot L^{-1}$, malt extract 10 $g \cdot L^{-1}$ and glucose 4 $g \cdot L^{-1}$ ); solid YMG (yeast extract 4 $g \cdot L^{-1}$, malt extract 10 $g \cdot L^{-1}$, glucose 4 $g \cdot L^{-1}$ and agar 18 $g \cdot L^{-1}$ ); A9 (peptone 4 $g \cdot L^{-1}$, glucose 10 $g \cdot L^{-1}$ and molasses 20 $g \cdot L^{-1}$); PDA (potato dextrose agar 39 $g \cdot L^{-1}$) and Emmons (neopeptone 10 $g \cdot L^{-1}$, glucose 20 $g \cdot L^{-1}$ and agar 18 $g \cdot L^{-1}$).

The ingredients of dry diet for insects were: for gypsy moth; 36 g wheat, 7.5 g casein, 2.4 g Wesson's salt mix, 0.6 g sorbic acid, 0.3 g methylparaben (p-hydroxy-benzoic acid methyl ester), 3.0 g Hoffman-LaRoche #26862 vitamin mix (Bell et al., 1981); for corn earworm; 63.8% corn meal (gelatinized), 24.2% soy flour (defatted and toasted), 5% nonfat dry milk, 5% soy oil (refined and stabilized), 2% vitamin-mineral premix (Burton, 1969); for tobacco hornworm; 100 g wheat germ (pre-ground), 45 g casein (purified), 40 g sucrose, 30 g torula yeast, 15 g salt mixture, 4 g ascorbic acid, 1.5 g sorbic acid, 1.0 g methyl-P-hydroxy benzoate, 0.5 g cholesterol, 30 mg vitamins (Yanamoto, 1969). The insect diets were made as follows. The agar solution (1.8% agar for gypsy moth, 1.4% agar for corn earworm, 1.9% agar for tobacco hornworm) was held at 50°C and then added to the dry diet for gypsy moth (845 mg) (Bell et al., 1981), corn earworm (940 mg) (Joyner et al., 1985) and tobacco hornworm (950 mg) (Bell et al., 1976) until the total diet weighed 5 g. Compound **1**, dissolved in 25 µl of DMSO, was mixed with the diet. Controls received 25 µl of DMSO alone.

The NMR spectra of compound **1** in DMSO solution were recorded at 45°C on Varian VXR500 MHz spectrometers at Max T. Rogers NMR facility in the Department of Chemistry at Michigan State University. The spectra of methylated and acetylated derivatives

in $CDCl_3$ solution were recorded on Varian VXR300 MHz spectrometers at the same facility. The UV spectrum of compound **1** at 10 ppm in MeOH was recorded on a Shimadzu UV-260 spectrophotometer. CIMS and FABMS were obtained on JEOL JMS-AX505 and JEOL JMS-HX110 mass spectrometers at the MSU Mass Spectroscopy facility in the Department of Biochemistry, which is supported in part by a grant (DRR-00480) from the Biotechnology Research Technology Program, National Center for Research Resources, National Institutes of Health. Circular Dichroism analysis was conducted using a 1 $mg \cdot ml^{-1}$ solution of compound **1** in MeOH on a JASCO J-710 spectropolarimeter (Jasco Incorporated, Japan). The nitrogen gas (99.99%) was generated by a nitrogen gas generator model NG-150 (Peak Scientific) at a flow rate of 20 $L \cdot min^{-1}$. The melting point was recorded on a Thomas Model 40 micro hot-stage apparatus and was not corrected.

Silica gel TLC plates with organic binder (250μ) were used for purification (Analtech). Final purification of compound **1** was performed on a recycling preparative HPLC model LC-20 and connected with a fraction collector model AS-20 (Japan Analytical Industry Co). The columns used were Shodex GS 3-10 2F (Asahi Chemical Industry Co., Ltd) and Jaigel-ODS S-343-15 (Japan Analytical Industry Co., Ltd). The GS 3-10 column was 20 × 300 mm and its precolumn was 8 × 40 mm. The ODS column was 20 × 250 mm and it's precolumn was 8 × 40 mm. N-nitroso-N-methylurea, KOH, pyridine and acetic anhydride used in the methylation and acetylation reaction of compound **1** were purchased from Sigma Chemical Company, J.T.Baker Chemical Co., Mallinckrodt, Inc. and Columbus Chemical Industries,Inc., respectively.

**Fermentation of *S. griseofuscus* for the production of the insecticidal compound**: Cultures of *S. griseofuscus* stored on inorganic medium (1.09 $g \cdot L^{-1}$ $KH_2PO_4$, 0.44 $g \cdot L^{-1}$ $KHPO_4$, 0.0014 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.34 $g \cdot L^{-1}$ NaCl, 0.34 $g \cdot L^{-1}$ $CaSO_4 \cdot 2H_2O$, 0.01 $g \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$, 0.0027 $g \cdot L^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, 9.1 $g \cdot L^{-1}$ sucrose , 34.2 $g \cdot L^{-1}$ KOH, 18 $g \cdot L^{-1}$ agar and 114 $g \cdot L^{-1}$ $H_3PO_4$) were transferred onto YMG media slants and incubated for eight days at 26°C. These cultures then were transferred into 400-ml seed flasks containing 100 ml of YMG liquid medium. The inoculated flasks were kept on a rotary shaker at 110 rpm at 26°C for eight days, subcultured to 2 L seed flasks containing 400-ml of A9 medium and were incubated on a rotary shaker at 110 rpm and 26°C for eight days.

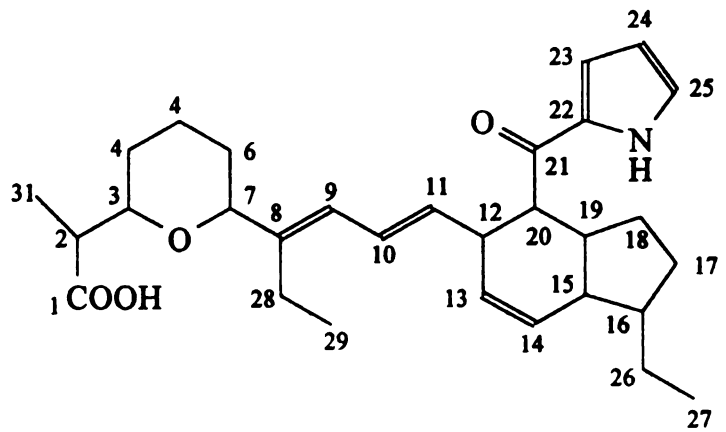
**Isolation and purification of the insecticidal compound 1**: The fermentation broth of *S. griseofuscus* (6 L) was centrifuged at 4°C and $10^4$ rpm for 10 min to separate the mycelia from the broth (Scheme 4.1). The wet cell-pellet (400 g) was extracted with MeOH:$CHCl_3$ (3:1, 800 ml) followed by 100% $CHCl_3$ (500 ml). The residual cell mass was discarded. The combined organic extracts were evaporated to dryness under vaccum and yielded a powdered product (5.8 g). This product (5.8 g) was partitioned with MeOH:$CHCl_3$ (1:10, 15 ml × 4) and the soluble portion was evaporated to dryness (4.5 g). This product (4.5 g) was extracted further with acetonitrile (3×15ml), and the soluble portion was evaported to dryness to yield an amorphous powder (1.6 g).

The acetonitrile extract (1.6 g) was fractionated on a GS 3-10 column using MeOH:$H_2O$ (85:15) as a mobile phase at a flow rate of 5 $ml \cdot min^{-1}$ and detected at 228 nm. The fraction with 42 min retention time (889 mg) was chromatographed further on silica

TLC plates with $CHCl_3$:Benzene:MeOH (25:15:3) as the mobile phase. The band at 0.75 Rf was collected, eluted with MeOH (15 ml), yielding a pale yellow powder (533 mg). This product was purified finally by preparative HPLC on an ODS column using MeOH:$H_2O$ (95:5) as the mobile phase at a flow rate of 3 $ml \cdot min^{-1}$ and detected at 228 nm. The peak at 76 min was collected and evaporated to dryness. The resulting product was a white crystalline powder, compound **1** (480 mg) (Scheme 4.1). The yield of compound **1** was 80 $mg \cdot L^{-1}$ of the fermentation broth.

**Compound 1**: A white crystalline solid, m.p. 98-101°C; MF $C_{31}H_{43}NO_4$; UV $\lambda_{max}$ (MeOH): 243 ($\epsilon$: 36630) and 289 nm ($\epsilon$: 17847); EIMS, m/z (relative abundance): 55 (15), 67 (18), 94 (100), 119 (18), 135 (15), 157 (14), 187 (10), 212 (10), 238 (10), 251 (65), 281 (9), 334 (6), 399 (7), 420 (5), 464 (80), 493 (19, $M^+$). $^1$HNMR and $^{13}$CNMR chemical shift values are shown in Table 4.1. Original spectra of compound **1** are presented in the Appendix. They are: $^1$HNMR, Appendix I; $^{13}$CNMR, Appendix II; DEPT (Distortionless enhancement by polarization transfer), Appendix III; DQFCOSY (double quantum filtered correlated spectroscopy), Appendix IV; HMQC (Heteronuclear multiple quantum correlated), Appendix V; EI mass spectrum, Appendix VI; UV, Appendix VII; CD, Appendix VIII.

**Methylation of compound 1**: The diazomethane, $CH_2N_2$, used for the methylation reaction of compound **1** was prepared as follows: KOH (25 g) was dissolved in 75 ml of distilled water and the solution was kept in an ice bath. Diethyl ether (75 ml) was poured into this solution, and the mixture was maintained on the ice bath for another 15 min. N-nitroso-N-methylurea (1.125 g) was added slowly to the above mixture and stirred until it was reacted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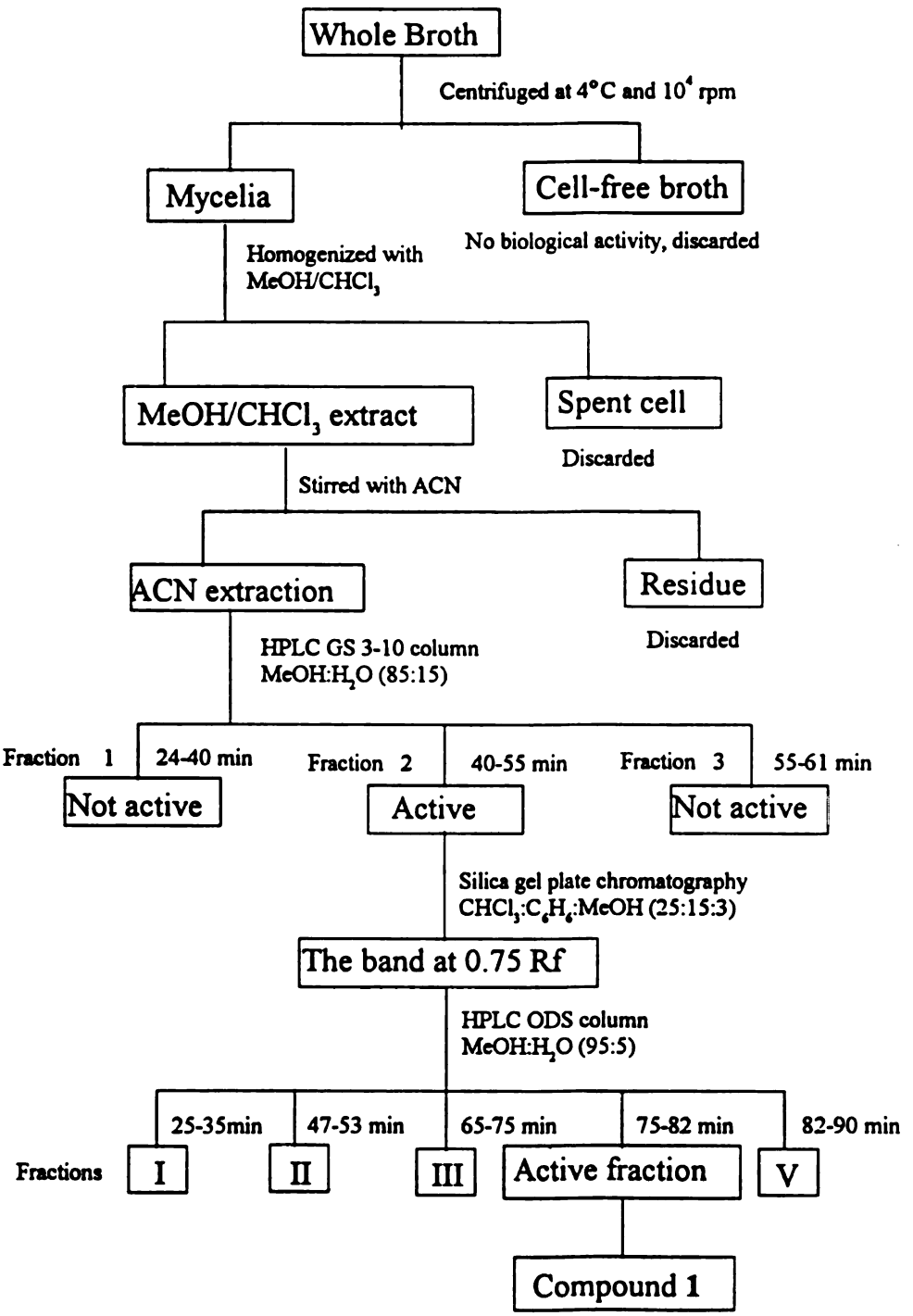
Compound **1**

completely. The yellow ether layer then was separated and washed with 100 ml of cold water to remove any trace quantity of KOH. The $CH_2N_2$ solution in ether was kept over KOH pellets until used.

Compound **1** (6.4 mg) was dissolved in ether (4 ml) and reacted with 6 ml of $CH_2N_2$ in ether. The reaction was maintained at room temperature for 45 min and evaporated to dryness. The methylated product, compound **2** (6.32 mg), gave $^1$H NMR signals (Appendix. IX) : δ 3.65 (1H, s, -$COOCH_3$), 2.76 (1H, m, H-2), 3.70 (1H, m, H-3), 1.24 (1H, m, H-4), 1.70 (1H, m, H-4), 1.41 (1H, m, H-5), 1.63 (1H, m, H-4), 1.95 (1H, m, H-6), 4.10 (1H, m, H-7), 5.82 (1H, d, J=11.5 Hz, H-9), 5.78 (1H, d, J=15 Hz, H-10), 5.40 (1H, dd, J=15.0, 9.0 Hz, H-11), 3.32 (1H, m, H-12), 5.49 (1H, d, J=9.5 Hz, H-13), 5.95 (1H, d, J=10 Hz, H-14), 1.58 (1H, m, H-15), 1.48 (1H, m, H-16), 1.28 (1H, m, H-17), 1.84 (1H, m, H-17), 1.01 (1H, m, H-18), 1.15 (1H, m, H-18), 1.93 (1H, m, H-19), 3.38 (1H, dd, J=6.5, 11 Hz, H-20), 6.86 (1H, m, H-23), 6.25 (1H, m, H-24), 7.00 (1H, m, H-25), 1.23 (1H, m, H-26), 1.64 (1H, m, H-26), 0.91 (3H, t, J=7.5 Hz, H-27), 1.73 (1H, m, H-28), 1.98 (1H, m, H-28), 0.76 (3H, t, J=7.5 Hz, H-29), 0.81 (3H, d, J=6.5 Hz, H-30), 1.08 (3H, d, J=7.0 Hz, H-31), 9.58 (1H, s, -NH). EIMS of this methylated product (Appendix. X) gave the molecular ion at m/z 507 with 20% relative abundance. The major fragments observed in the MS spectrum of compound **2** were at m/z (relative abundance): 55 (33), 69 (18), 94 (100), 109 (22), 135 (20), 159 (17), 226 (20), 252 (12), 265 (100), 281 (10), 306 (4), 334 (5), 359 (5), 413 (9) and 478 (10).

**Acetylation of compound 2**: Compound **2** (6.32 mg) was dissolved in pyridine (3 ml) and mixed with acetic anhydride (3 ml). The reaction mixture was kept in the dark at room




Scheme 4.1. Isolation and purification of insecticidal compound **1**

temperature for 12 h and evaporated to dryness. The crude mixture was purified by TLC using the mobile phase $CHCl_3$:benzene:MeOH (25:15:3) on silica gel plates and yielded 6.2 mg of the product. The $^1H$ NMR of the resulting product gave the signals as follows: δ 3.65 (1H, s, $-COOCH_3$), 2.76 (1H, m, H-2), 3.70 (1H, m, H-3), 1.24 (1H, m, H-4), 1.70 (1H, m, H-4), 1.41 (1H, m, H-5), 1.63 (1H, m, H-4), 1.95 (1H, m, H-6), 4.10 (1H, m, H-7), 5.82 (1H, d, J=11.5 Hz, H-9), 5.78 (1H, d, J=15 Hz, H-10), 5.40 (1H, dd, J=15.0, 9.0 Hz, H-11), 3.32 (1H, m, H-12), 5.49 (1H, d, J=9.5 Hz, H-13), 5.95 (1H, d, J=10 Hz, H-14), 1.58 (1H, m, H-15), 1.48 (1H, m, H-16), 1.28 (1H, m, H-17), 1.84 (1H, m, H-17), 1.01 (1H, m, H-18), 1.15 (1H, m, H-18), 1.93 (1H, m, H-19), 3.38 (1H, dd, J=6.5, 11 Hz, H-20), 6.86 (1H, m, H-23), 6.25 (1H, m, H-24), 7.00 (1H, m, H-25), 1.23 (1H, m, H-26), 1.64 (1H, m, H-26), 0.91 (3H, t, J=7.5 Hz, H-27), 1.73 (1H, m, H-28), 1.98 (1H, m, H-28), 0.76 (3H, t, J=7.5 Hz, H-29), 0.81 (3H, d, J=6.5 Hz, H-30), 1.08 (3H, d, J=7.0 Hz, H-31), 9.58 (1H, s, -NH). Thess results showed that the acetylation of compound **2** did not occur and indicated that hydroxy groups were absent in compound **1**.

**Circular Dichroism (CD) of compound 1**: Pure compound **1** was dissolved in MeOH (1 $mg \cdot ml^{-1}$) and the CD was determined under the following conditions: scan mode: wavelength; scan range: 200-500 nm, sensitivity 500 mdeg, response 64 msec, scan speed 500 $um \cdot min^{-1}$, band width 1 nm, accumulation 0.2 $nm \cdot data^{-1}$. Compound **1** exhibited an absorbance at 328.7 nm, and the CD value was $\Delta\epsilon = -58.883$ mdeg (Appendix. VIII).

**Antimicrobial activities of compound 1**: Cultures of *Gleosporum sp.* on PDA medium, *Candida albicans* on YMG medium, cultures of *Staphylococcus epidermides*, *Streptococcus*

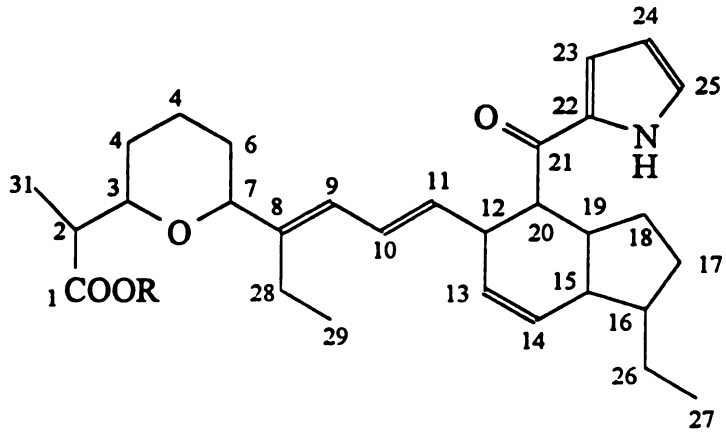



Figure. 4.1 Compound **1** R = H
Compound **2** R = $CH_3$

Table 4.1. $^{1}H$- and $^{13}C$NMR chemical shifts for compound 1.

| Carbon No. | $^{1}H$ ppm | | $J_{HH}$ Hz | | $^{13}C$ ppm | Carbon No. | $^{1}H$ ppm | | $J_{HH}$ Hz | | $^{13}C$ ppm |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 COOH | 3.40 | (1H) | - | | 175.9 | 17 | 1.28, 1.88 | (2 H) | m | | 29.1 |
| 2 | 2.92 | (1H) | dt | 17.5, 7.0 | 39.5 | 18 | 1.16, 0.96 | (2 H) | m | | 26.5 |
| 3 | 3.82 | (1H) | m | | 75.1 | 19 | 1.80 | (1H) | m | | 40.4 |
| 4 | 2.01, 1.70 | (2 H) | m | | 21.1 | 20 | 3.43 | (1H) | dd | 6.5, 11.0 | 51.1 |
| 5 | 1.40, 1.77 | (2 H) | m | | 25.3 | 21 | - | - | - | | 189.6 |
| 6 | 1.92 | (1H) | m | | 29.2 | 22 | - | - | - | | 131.9 |
| 7 | 4.19 | (1H) | m | | 72.6 | 23 | 6.98 | (1H) | d | 3.0 | 115.6 |
| 8 | - | - | - | | 141.3 | 24 | 6.16 | (1H) | t | 2.5 | 109.4 |
| 9 | 5.69 | (1H) | d | 11.5 | 122.6 | 25 | 6.99 | (1H) | d | 1.5 | 124.8 |
| 10 | 5.90 | (1H) | d | 15.0 | 126.0 | 26 | 1.64, 1.85 | (2 H) | m | | 26.5 |
| 11 | 5.33 | (1H) | dd | 15.0, 9.0 | 131.8 | 27 | 0.77 | (3 H) | t | 7.5 | 13.3 |
| 12 | 3.36 | (1H) | m | | 44.1 | 28 | 1.39, 1.81 | (2 H) | m | | 19.6 |
| 13 | 5.46 | (1H) | dt | 9.5, 3.5 | 129.9 | 29 | 0.90 | (3 H) | t | 7.5 | 12.1 |
| 14 | 5.92 | (1H) | d | 10.0 | 128.4 | 30 | 0.70 | (3 H) | d | 6.5 | 12.6 |
| 15 | 1.60 | (1H) | m | | 49.2 | 31 | 0.94 | (3 H) | d | 7.0 | 14.3 |
| 16 | 1.36 | (1H) | m | | 43.1 | NH | 11.63 | (1 H) | - | | |

*aureus* and *Escherichia coli* on Emmons medium were grown prior to the assays. The test organisms were harvested by suspending them in 10 ml of sterile saline solution. Cell suspension was adjusted to $10^3$ colony forming units per milliliter (CFU/ml). Bioassay plates were made by spreading cell suspension (100 µl) on the surface of the respective medium. Compound 1 (1, 25, 50 and 100 ppm in DMSO) was spotted on plates lawned with the test organism and were incubated at 28°C for 72 h. The zone of inhibition, characterized by the absence of the microorganism's growth, was measured. Compound 1 was active against *S. aureus* and *S. epidermidis* at 1 ppm concentration as indicated by 3.3- and 2.9-cm zones of inhibition, respectively. There was no activity against fungi, yeast or other test bacteria when tested at 100 ppm.

**Mosquitocidal activity of compound 1**: The mosquitocidal assay was conducted on the fourth instar mosquito larvae, *Aedes aegypti*, reared from eggs (Courtesy of Dr. Alexander Raikhel, Department of Entomology, MSU) (Nair et al., 1989). Fifteen larvae (3-4 days old) were placed in 980µl of distilled water in a test tube. Serial dilutions of compound 1 (1, 10, 20, 30, 40, 50 and100 $\mu g \cdot 20\mu l^{-1}$ in DMSO) were added to these test tubes containing mosquito larvae. Controls received 20 µl of pure DMSO. The test tubes were covered and maintained at room temperature. The number of dead larvae was recorded at 24 h. Each treatment was repeated in triplicates. Compound 1 was mosquitocidal at 20 ppm and resulted in 100% mortality.

**Insecticidal assays**: Gypsy moth eggs were obtained from The Forest Pest Management Institute, Sault Ste. Marie, Ontario, Canada. Corn earworm and tobacco hornworm eggs

were purchased from the insect rearing facility in the Department of Entomology, North Carolina State University, Raleigh, North Carolina. Compound 1 was dissolved in 25 µl of DMSO and mixed with diet mixtures. The diet was dispensed into 3.5 ml polystyrene vials (Sarstedt) and one larvae was placed in each vial. Gypsy moth larvae were used at two to three days of age, corn earworm and tobacco hornworm larvae were neonates. Each treatment had fifteen replicates. The larvae were weighed at six days. Dunnet's Test was used to determine the significance of weight reduction in these assays. The results (Fig. 4.4) indicate that compound 1 showed significant weight reduction at 100 ppm for tobacco hornworm and gypsy moth, but had only slight activity against cornear worm at this concentration.

**Results and Discussion:**

On YMG solid medium, *S. griseofuscus* grew as grey colonies. It produced antibiotic in both YMG and A9 media. The growth of the organism was rapid and yielded about 66 g of wet cells per liter after 8 days of fermentation.

The wet cells were extracted with $CHCl_3$:MeOH solvent mixture which extracted the active metabolite efficiently. The dried crude extract was fractionated with $CHCl_3$ and ACN to remove most of the insoluble material. A preliminary mosquitocidal assay and TLC proved that all of the active compound present in the extract was in the ACN-soluble portion. The ACN extract was purified further on a GS 3-10 polyvinyl alcohol column by preperative HPLC. The polyvinyl alcohol adsorbent separates the compounds by their molecular size and adsorption to the column matrix. Compounds with high molecular weight will not flow through the packing material pores and will have lower retention times. In order to obtain

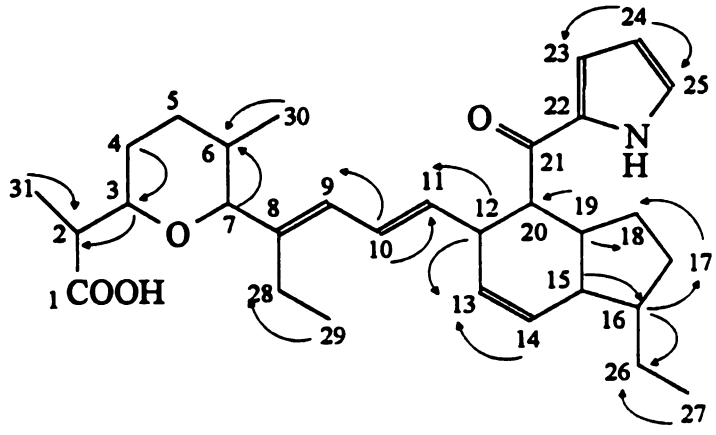
pure compound 1, the active fraction from GS 3-10 HPLC purification was chromatographed on silica gel TLC, followed by preperative HPLC.

The $^1$HNMR spectrum of compound 1 showed four methyl groups, two triplets at 0.77 and 0.90 ppm and two doublets at 0.70 and 0.94 ppm, respectively. This indicated that compound 1 contained two $CH_3CH_2$- and two $CH_3CH$- functionalities. The peaks at 2.92, 3.36, 3.43, 3.82 and 4.19 ppm were assigned to methine protons that are influenced by electrons withdrawing O or N functionalities. The peaks at δ 5.33, 5.46, 5.69, 5.90, 5.92 and 6.16 were indicative of vinylic protons. The assignment of H-10 and H-11 protons as *trans* to each other was derived from the 15 Hz coupling constant. Similarly, H-13 and H-14 protons were assigned as *cis*. Also, the signals at δ 6.16, 6.98 and 6.99 were indicative of vinylic protons. The broad peak, exchanged with $D_2O$ at 11.63 ppm suggested that compound 1 contained a carboxylic acid or an amino moiety in the molecule.

The peaks in the $^{13}$CNMR spectrum at 75.1 and 72.6 ppm were typical of oxygenated carbons. Also, 10 carbons appearing between 100 and 170 ppm were assigned to 8 vinylic and 2 quaternary carbons. The signals at 175.9 and 189.6 ppm were representative of a carboxylic and an α, β-unsaturated carbonyl, respectively. The DEPT spectrum of compound 1 confirmed a total of 31 carbons.

The HMQC spectrum of compound 1 provided the proton-carbon correlations and was very helpful in elucidating the structure of compound 1. The COSY of compound 1 showed the connectivities of $H_{28}$-$H_{29}$, $H_{31}$-$H_2$-$H_3$, $H_{30}$-$H_6$-$H_7$, $H_9$ - $H_{10}$ - $H_{11}$ - $H_{12}$-$H_{13}$-$H_{14}$, $H_{27}$-$H_{26}$-$H_{16}$, $H_{15}$-$H_{16}$-$H_{17}$-$H_{18}$-$H_{19}$-$H_{20}$ and $H_{23}$-$H_{24}$-$H_{25}$ (Fig. 4.2). However, the correlation for $H_{14}$-$H_{15}$, $H_4$-$H_5$-$H_6$ and $H_{12}$-$H_{20}$ was not detected in the spectrum.

The presence of carboxylic acid and amino groups in compound 1 were also



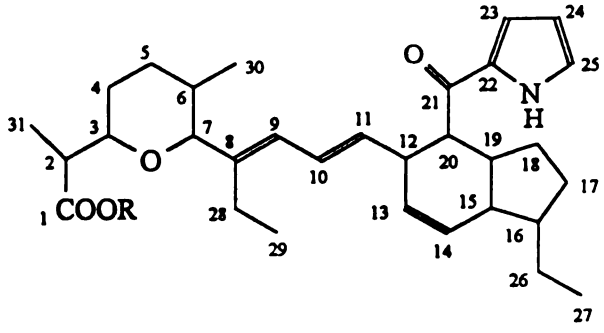

Compound 1

**Figure 4.2 The proton correlations in compound 1 from DQFCOSY spectrum**

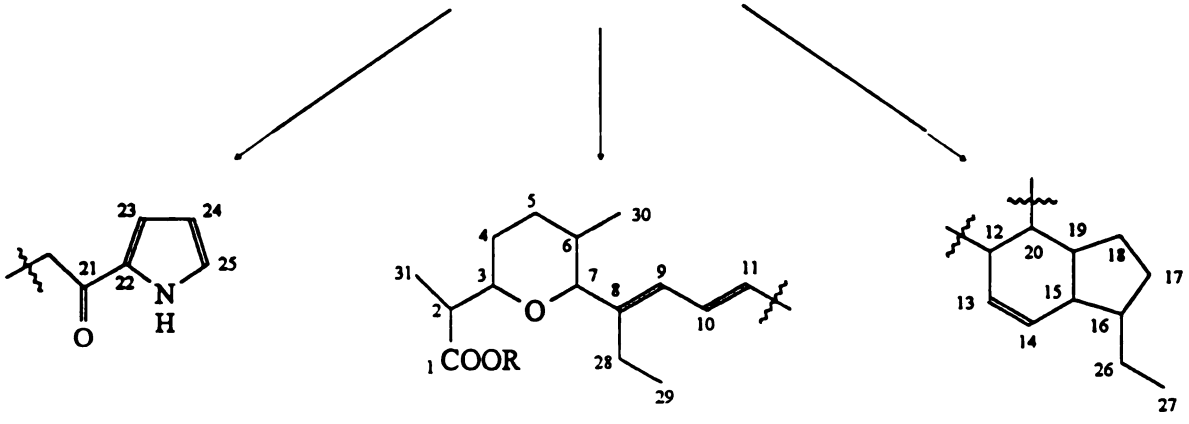
confirmed by methylation and acetylation reactions. The $^1$HNMR of the methylated product, compound **2**, showed a sharp singlet at 3.65 ppm and integrated for three protons. Also, there was a broad peak at 9.58 ppm which exchanged with $D_2O$. This suggested that an amino group was present in compound **1** in addition to a -COOH group. $^1$HNMR of the acetylated product from compound **2** did not show any change and confirmed the presence of a secondary NH group in the molecule. Also, it confirmed that both C-3 and C-7, appeared at 75.1 and 72.6ppm, respectively, had alkyl substituents.

MS data of compound **1** and its methyl ester supported the proposed structure for compound **1**. The MS of compound **1** and **2** gave molecular ions at m/z 493 and 507, respectively. The m/z peak at 94 was present in both compounds **1** and **2** and it indicated as fragment I (Fig. 4.3). The peak at m/z 251 in compound **1** and the peak at m/z 265 in compound **2** were due to fragment II (Fig. 4.3). The molecular formula of compound **1** suggested the presence of 11 double-bond equivalents (DBE). The MS fragments at m/z 94 and 251 accounted for 8 DBE and fragment III for 3 DBE (Fig. 4.3). The UV spectrum of compound **1** gave $\lambda_{max}$ at 244 and 289 nm, respectively and was in agreement with NMR and MS assignments for compound **1**.

The CD for an organic compound results from the difference in absorption of right and left circularly polarized light (Crabbe, 1972). CD techniques can be applied to any optically active compound with a chromophore (light absorbing group) including inherently dissymmetric chromophores such as nonplanar aromatic substances, coupled oscillators formed by two nonconjugated chromophores such as homoconjugated dienes, and perturbed symmetrical chromophores, like double bonds and carbonyl groups. The CD of compound **1** exhibited a strong negative absorption at 328.7 nm (Appendix VIII). The absorbance



Compound 1 R = H
Compound 2 R = $CH_3$



m/z 94 (100)

R = H m/z 251 (65)
R = $CH_3$ m/z 265 (100)

m/z 148

Figuer 4. 3. Major fragments observed in the MS spectra of compound 1 and 2.

$\Delta\epsilon = A_L - A_R$, where $A_L$ is left-polarized light and $A_R$ is right-polarized light. The negative result indicated that compound 1 absorbs more of the right-polarized light. Based on all the spectral data and chemical methods, the structure of compound 1 is proposed in Figure 4.1.

Compound 1 is identical to an antibacterial antibiotic, indanomycin, reported earlier (Beloeil et al., 1984; Liu et al. 1979; Westley et al. 1979).

Compound 1 resulted in 100% mortality of mosquito larvae at 20 ppm in 24 h and was active against gram-positve bacteria *Streptococcus aureus* and *Staphylococcus epidermidis*. Also, it showed 50% weight reduction for gypsy moth, tobacco hornworm and 33% for corn earworm (Fig. 4.4) at 100 ppm after six days of feeding. The mosquitocidal activity of compound 1 is probably not comparable to commercial insecticides. Therefore, the potential for compound 1 as a commercial product is slim, mainly because of its low efficacy. However, this compound might serve as a template for the synthesis of mosquitocidal and insecticidal compounds. This is the first report of the insecticidal activity for compounds of this nature.

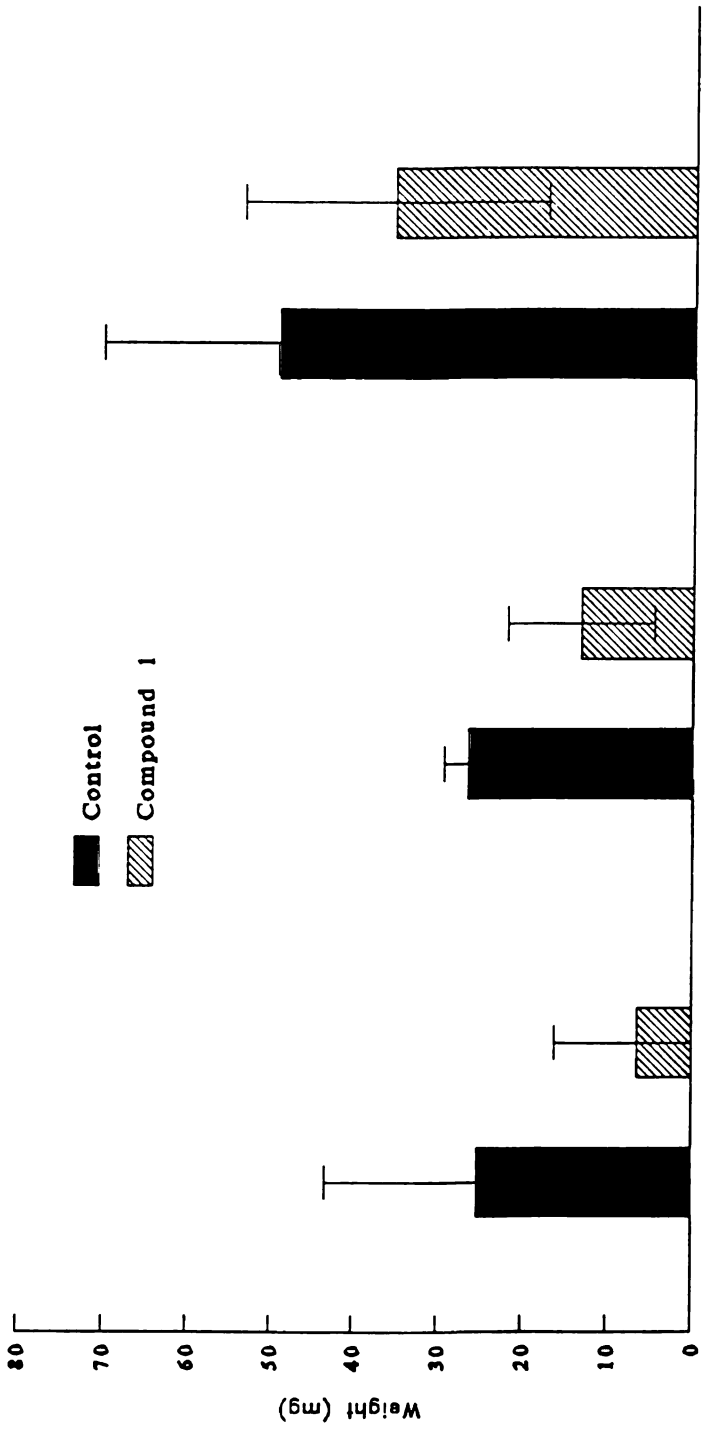



**Figure 4.4** Growth inhibitory assays of compound 1 against insects

## Chapter V

## Summary and Conclusions

Cultures of more than 68 strains of *Streptomyces spp.* isolated from soil samples collected from various location in China by Dr Zhang were grown initially in an nitrogen-free inorganic media. Only 48 strains were able to grow on YMG medium and were studied futher. To establish the genetic diversity of these cultures, rep-PCR was used as a facile means to fingerprint the genome of each strain. After the cultures were grown in YMG medium for fourteen days, the DNA of each strain was extracted from the cells by thermocycling, and the BOXA1R primer was used to amplify the DNA template in crude extracts using polymerase chain reaction. The products were separated electrophoretically on agarose gel. The banding pattern provided an extremely high resolution fingerprint. Genomic fingerprinting of this culture collection indicated that these strains are genetically diverse, and only two of the strains seemed to be closely related.

Preliminary antibacterial, antifungal, mosquitocidal and anti-cancer bioassays were performed on cell and cell-free broth extracts from 48 strains of *Streptomyces spp.* at 250 ppm. All cultures were grown in A9 and YMG media, separately. Anti-cancer bioassays were evaluated by using mutant *S. cerevisae* strains, seven strains showed zones of inhibition. The crude extracts of five strains were active against mosquito larvae, *Aedes aegypti*. Most of the extracts from both cell and cell-free broth exhibited activity against the gram-positive

bacteria *Streptococcus aureus* and *Staphylococcus epiderimidis*. About half of the strains tested showed growth-inhibitory activity against the plant pathogen, *Gleosporum spp*. Also, some strains produced compounds that were inhibitory to the growth of *Escherichia coli* and *Candida albicans*. However, none of the crude extracts of this culture collection showed any activities when tested on *Botrytis spp*., *Asperillus spp*., *Fusarium oxysporum*, *F. moniliforme* and *Rhizoctonia spp*.

Bioassay-directed fractionation of the crude extract afforded a mosquitocidal compound **1** from *S. griseofuscus*, strain MSU/ZD/033. Structure of compound **1** was determined by $^{1}$HNMR, $^{13}$CNMR, COSY, HMQC spectra and further confirmed by MS, methylation and acetylation reactions. Insecticidal and antibacterial activities were evaluated for compound **1**. Compound **1** resulted in 100% mortality of mosquito larvae at 20 ppm in 24 h. It also reduced 50% of the weight of gypsy moth and tobacco hornworm, 33% of the weight of corn earworn at 100 ppm. In addition, compound **1** was active against gram-positive bacteria *Streptococcus aureus* and *Staphylococcus epidermidis*,as shown by 3.3- and 2.9-cm zones of inhibitions, respectively. There was no activity against fungi, yeast and other test bacteria when tested at 100 ppm.

The experiments reported in this thesis yielded a known compound with new biological activities. Compound **1** was reported previously with antibacterial activities. This is the first report of its insecticidal activities.

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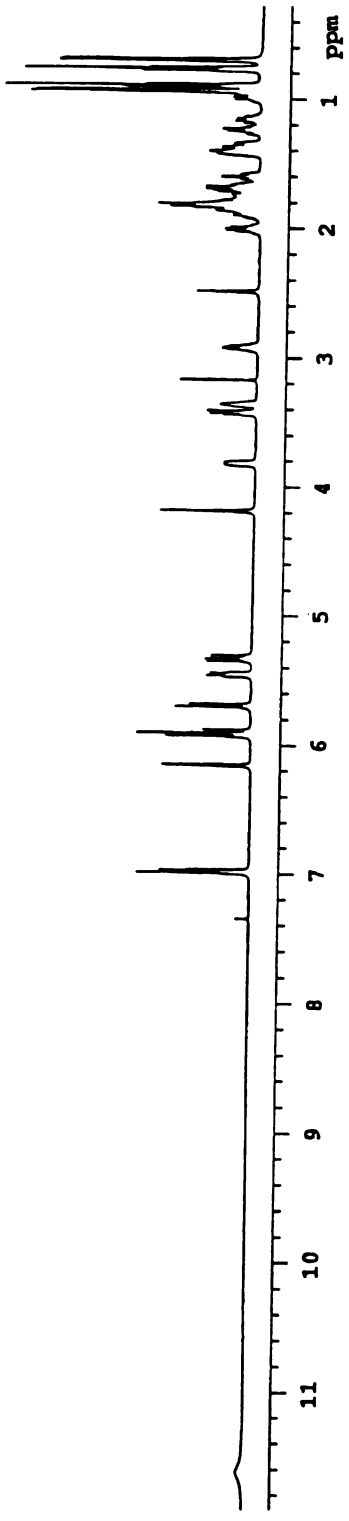
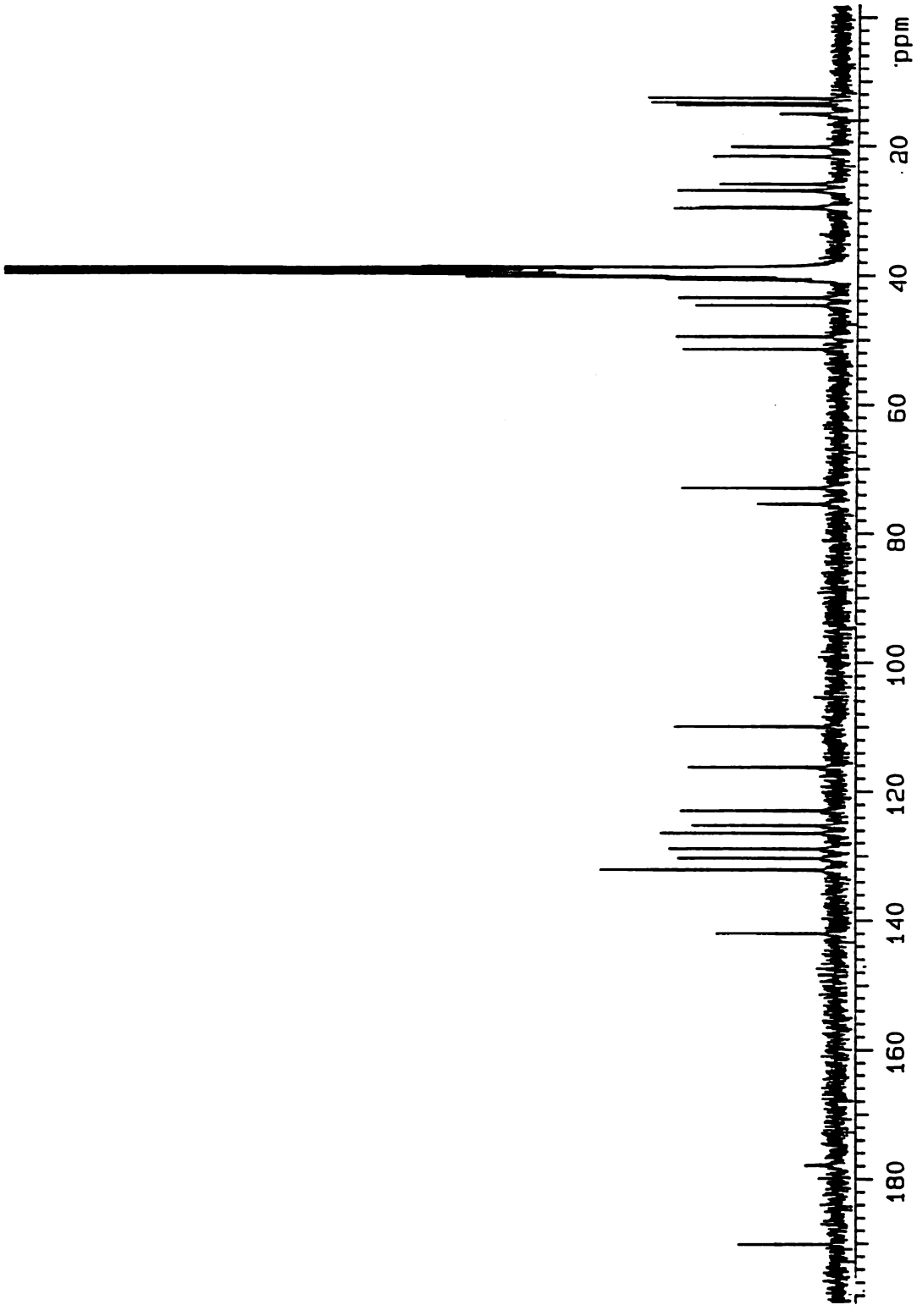
# APPENDICES

# APPENDIX I
## $^1$HNMR of compound 1

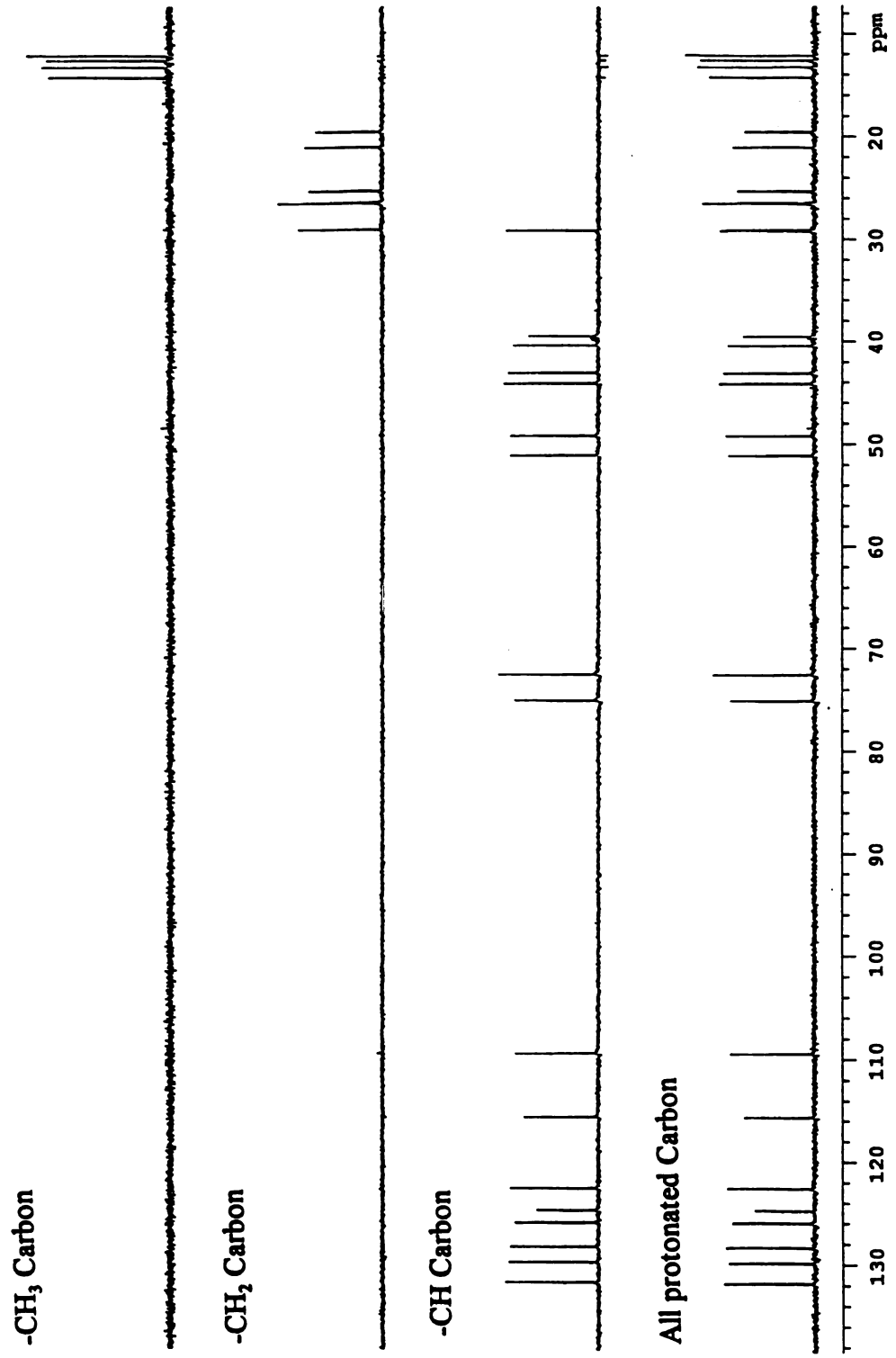


# APPENDIX II

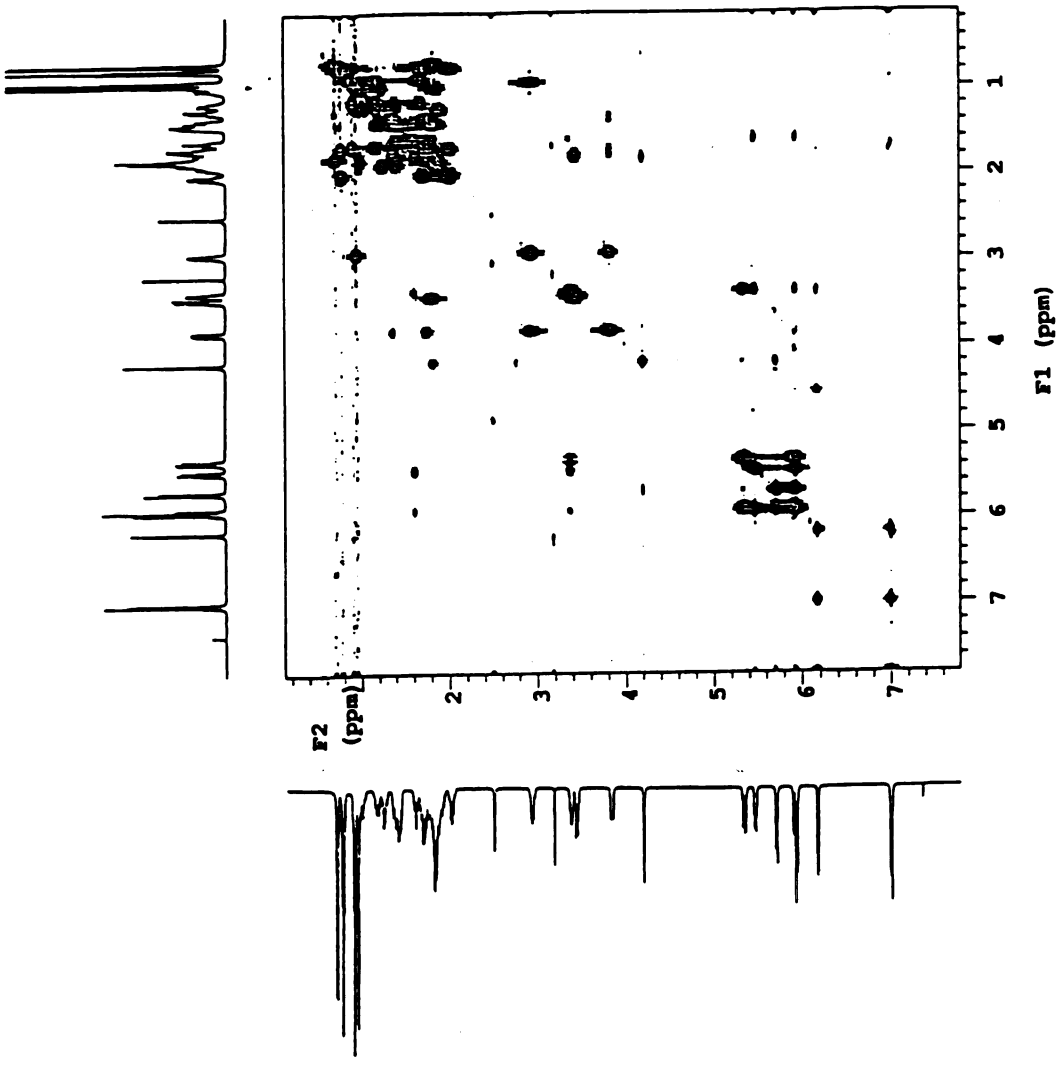
## $^{13}$CNMR of compound 1



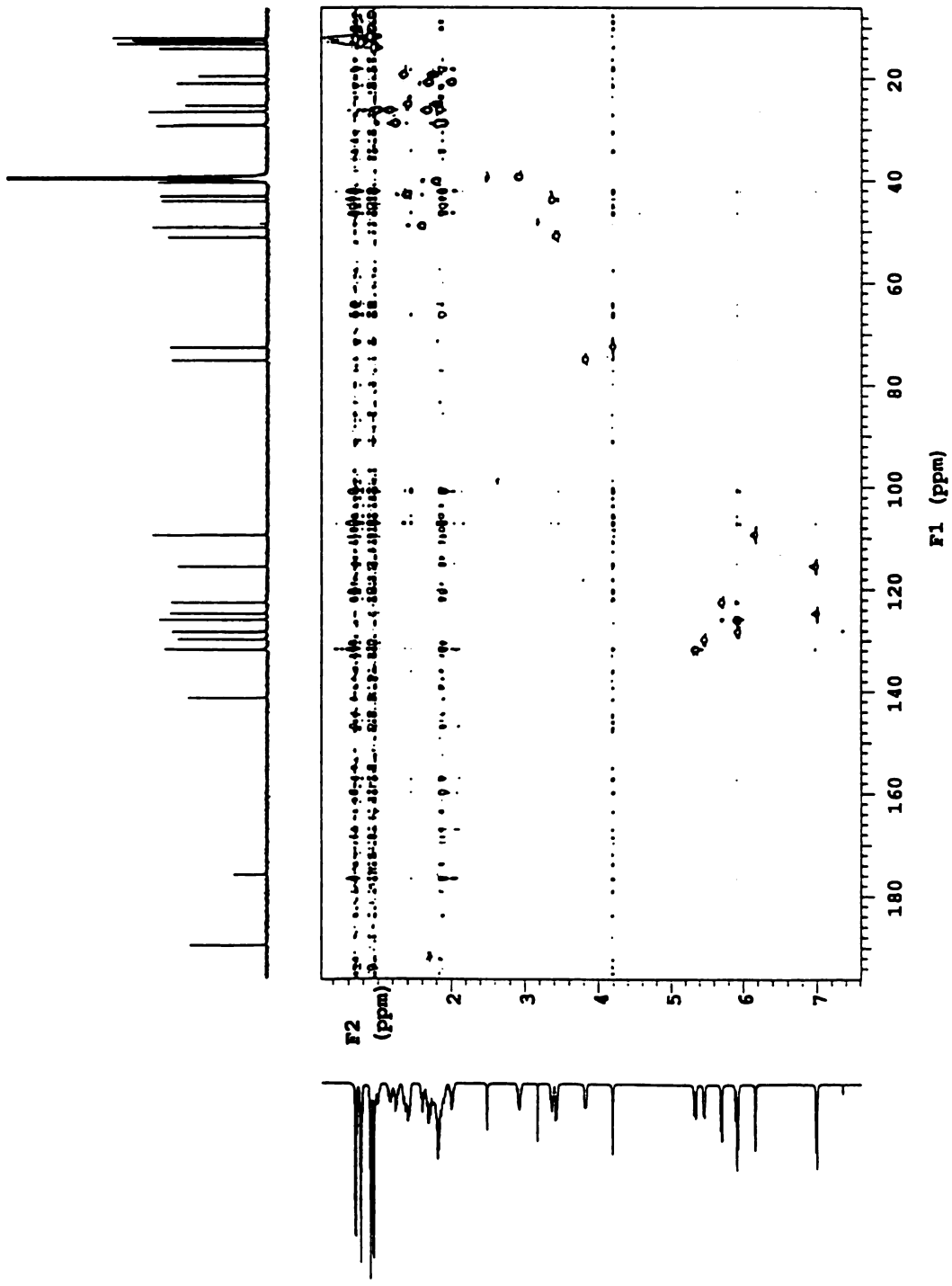
# APPENDIX III
## DEPT of compound 1



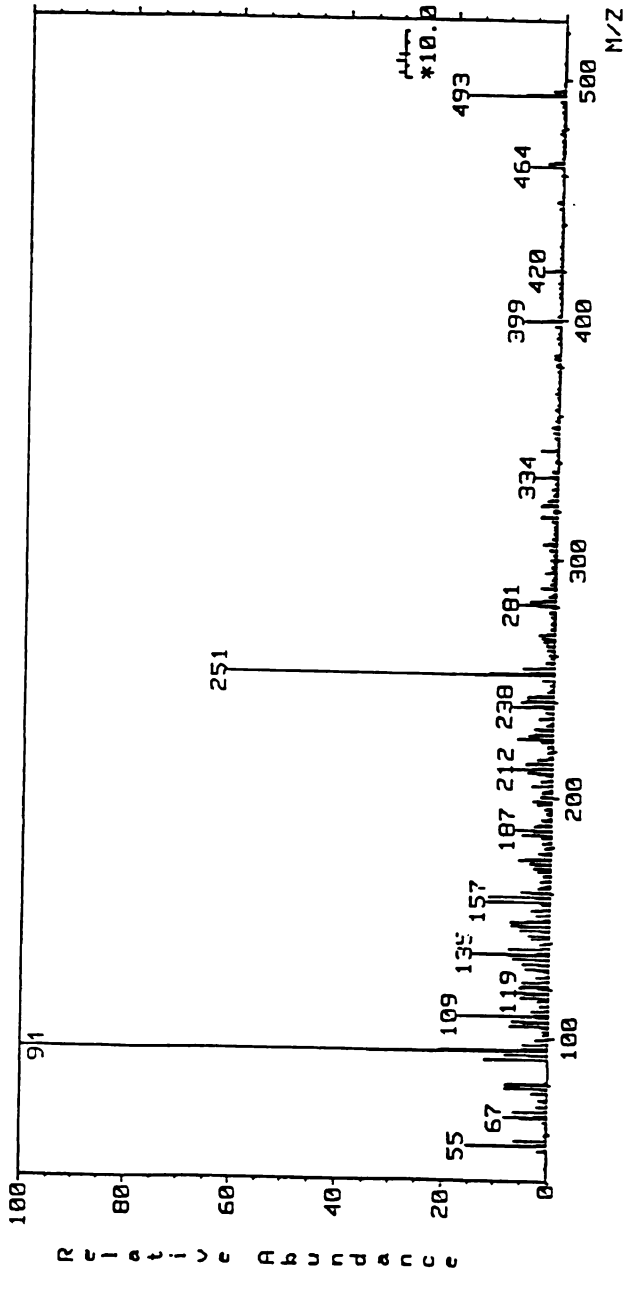
# APPENDIX IV
## COSY of compound 1



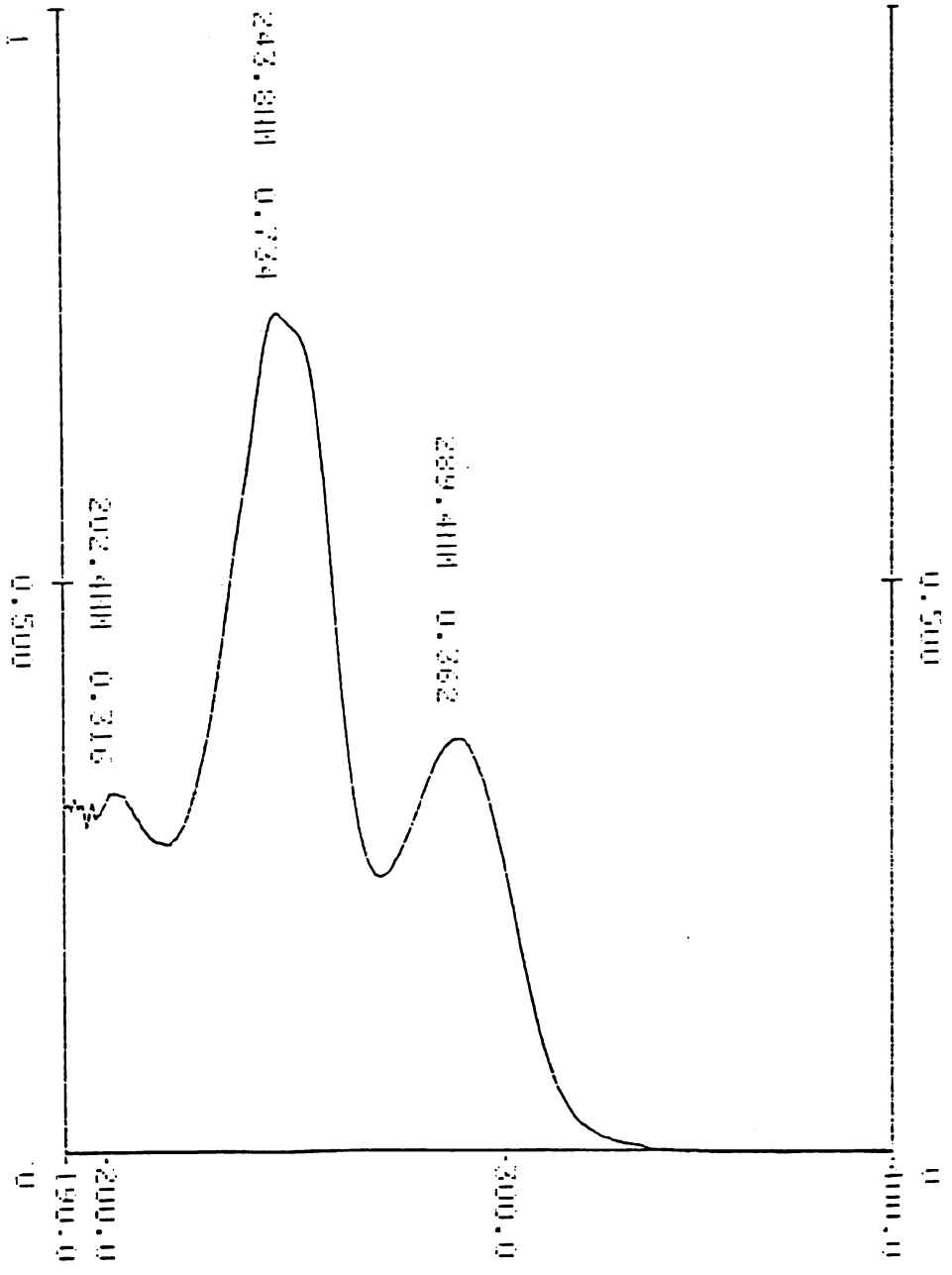
# APPENDIX V
## HMQC of compound 1



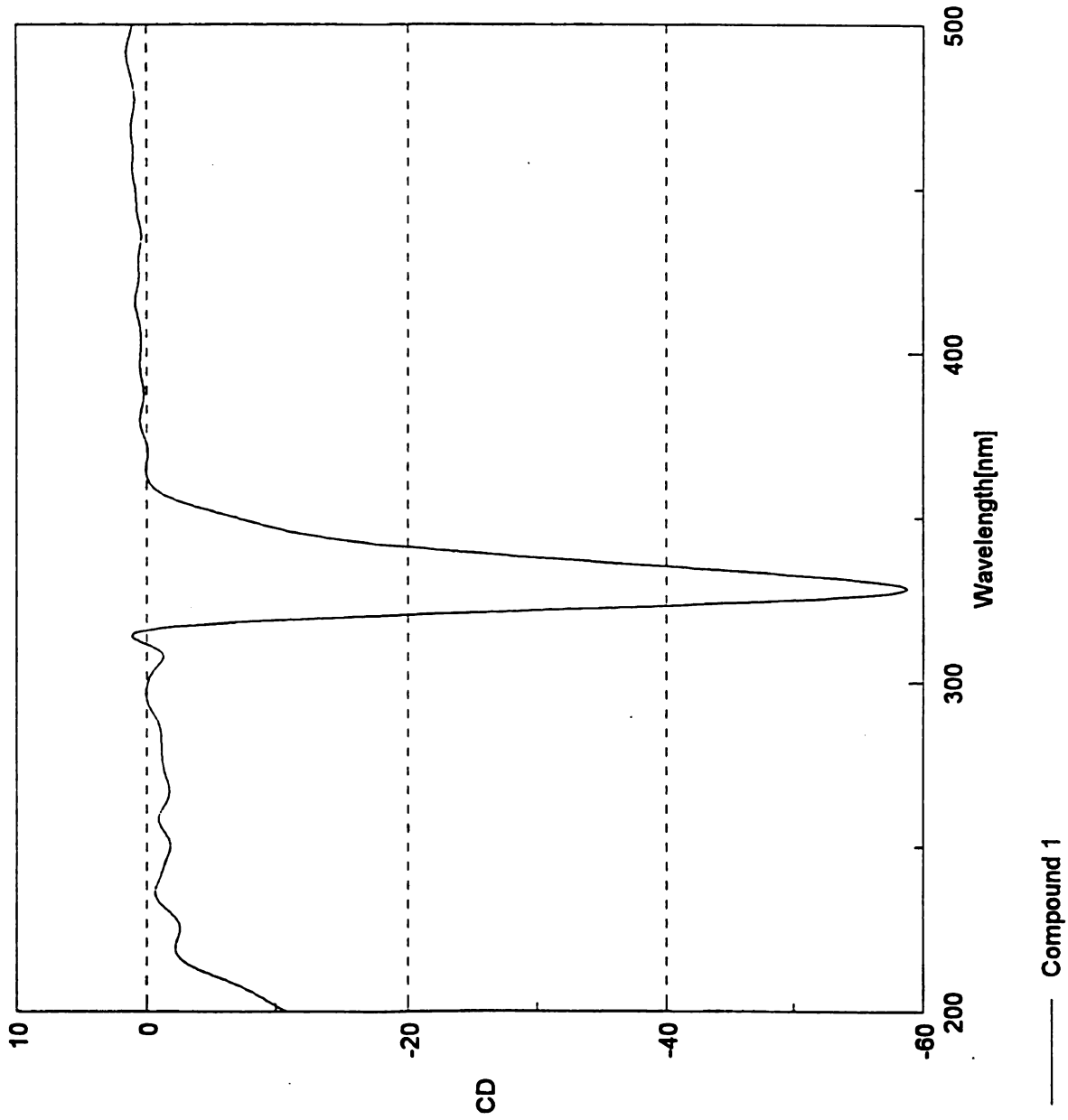
# APPENDIX VI
## EIMS spectrum of compound 1



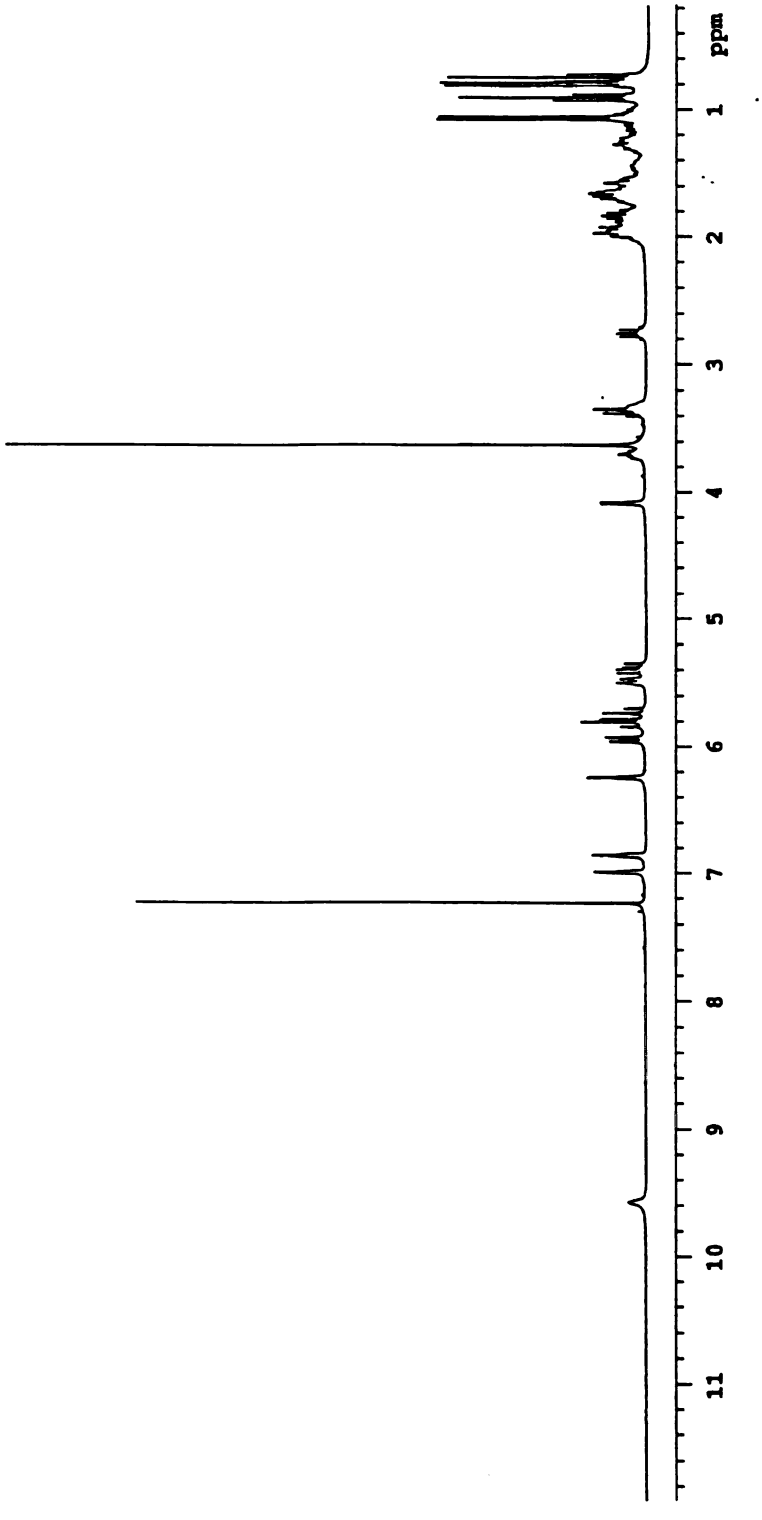
# APPENDIX VII
## UV spectrum of compound 1



# APPENDIX VIII
## CD of compound 1



# APPENDIX IX
## $^1$HNMR of compound 2



# APPENDIX X
## EIMS spectrum of compound 2