Bioactive metabolites of endophytic fungi of the Mexican yew (Taxus globosa) and the isolation and chemical modification of sphaeric acid
by Royce Allen Wilkinson

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry
Montana State University

Abstract:
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Epicorazine A, a previously known isolate of the fungus, Epicoccum nigrum, with wide spectrum antibiotic activity, was isolated and characterized. Additionally, a novel succinic acid derivative, sphaeric acid, was isolated from a fungus identified as a Sphaeriopsis sp.

Several synthetic modifications of the acid functionalities of sphaeric acid were carried out to explore any structure/function relationships. These modifications included esterifications, amidifications, a reduction, and a subsequent oxidation. A variety of activities were observed among the derivatives in each of the bioassays. However, few consistent trends Were observed throughout the entire range of assays.

Finally, the effect of various culture media on bioactive metabolite production was studied. Several possible bioactive fractions were observed. Succinic acid was isolated as one of the active fractions, and a mixture of triacylglycerols were isolated due to their physical and spectroscopic similarities to sphaeric acid. Several active fractions remain that have the potential of yielding novel bioactive metabolites.

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APPROVAL

of a thesis submitted by

Royce Allen Wilkinson

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Over the past decade there has been an increase in the need for novel pharmaceuticals. The development of microbial resistance to current clinical antibiotics, and the increasing number of immunocompromised hosts (AIDS patients, patients on chemotherapy, etc.) are two major reasons new antibiotics are required. New antitumor compounds with greater efficacy and fewer side effects are also in demand.

Endophytic microbes, fungi and bacteria living within the intercellular spaces of higher plants, are one possible source of novel bioactive compounds which are mostly unexplored. The endophytic microbes of yew trees were studied as a novel source of the antitumor drug, taxol, and the collection of microbes isolated in the search for taxol are also being screened for novel bioactive metabolites.

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Chapter 1

INTRODUCTION

The Antibiotic Paradox

The "antibiotic paradox" is a phrase coined by Stuart Levy, M.D. describing the fact that antibiotics have sown the seeds of their own downfall by selecting for strains of bacteria that can resist their activity. These "miracle drugs" achieved their great acclaim by rapidly curing previously fatal infections. However, bacteria responded to the widespread use of antibiotics by finding ways of becoming insensitive to the killing effects of these drugs. Beyond this, many of these resistant traits have been found to be transferable from one resistant bacteria to another; even bacteria of different types.[1]

This has become such a concern that even the popular media has covered this phenomenon. *Time* magazine ran a story in 1994 entitled "The Killers All Around" which listed recent outbreaks by new or mutated bacteria and viruses. These included the "flesh eating" streptococcus-A infections in England, a flare-up of tuberculosis in southern California, an epidemic of pertussis (whooping cough) in Cincinnati, and several unknown viral and bacterial illnesses including the Hantavirus outbreak in the southwestern US. As little as a generation ago, medical researchers had begun to believe the end of infectious diseases was

only a matter of time. As *Time* put it, "the question ceased to be, When will diseases be gone? and became, Where will the next deadly virus appear?"[2]

The leading causes of the "antibiotic paradox" have been the widespread use and misuse of these drugs. The use of antibiotics for ailments for which they have no value[3], the sustained use of a single antibiotic[4], and incomplete treatment, that is, stopping the antibiotic treatment before destroying all infectious bacteria[5], have all been factors in decreasing the susceptibility of these pathogens to the current drugs.

The answer to this problem lies not in abolishing the use of antibiotics, but in the wise use of them, as well as the discovery and development of new antibiotics. A new metabolite with a specific mode of action could have a big impact in antibiotic treatments. Even if the activity is insufficient for use as a therapeutic agent, the natural product could be useful as a biochemical tool as either a starting material or as a template for chemical synthesis. A large number of antibacterial compounds have been discovered, but there is still a need for new types of therapeutic agents. The natural mutation of both pathogenic and antibiotic producing organisms will continue to supply both the need and a source for these agents.[6]

Taxol is a classic example of a compound whose novel mode of action inspired tremendous interest. Unlike other antimitotic agents which inhibit microtubule assembly, taxol actually promotes the assembly of tubulin and stabilizes the microtubules so formed.[7] This unique mode of action, coupled

with taxol's particular efficacy against refractory breast and ovarian cancers, has made this one of the most important antitumor agents of the past two decades.[8]

In addition to the need for new antibacterial compounds, antifungal agents are becoming more important as the occurrence of fungal infections increases among immunocompromised hosts. Aging, acquired immunodeficiency syndrome (AIDS), and treatment with immunosuppressive drugs such as anticancer agents are current factors increasing the frequency of opportunistic fungal infections. In many of these cases, the actual cause of death is fungal growth in the organs and blood, rather than tumors or AIDS itself.[9]

Clinically useful antifungal compounds are less common than antibacterial agents for several reasons. First and foremost, fungi are eucaryotes similar to human and animal cells. Therefore, selective toxicity is less likely to occur with antifungal agents. In addition, potent fungicidal effects are desired, but almost all the antifungal drugs now available for use are fungistatic. Those compounds which show strong fungicidal activity are unfortunately also highly cytotoxic. Finally, the host defense system is still a major factor in successful antimicrobial chemotherapy. However, fungal infections are mostly associated with a depression in host immune action making antifungal therapy less effective.[10]

Why Fungi?

The fungi form one of the largest kingdoms of living organisms with a conservatively estimated total of about 1.5 million species. Based on this

estimated figure, only 4.6% of the world's fungi have been recognized, and secondary metabolites have only been documented in about 5000 (or 7%) of the known species.[11] Therefore, a large pool of unknown fungi exists which will likely be found to exhibit novel chemicals among their secondary metabolites.

Two major areas of bioactivity of fungal produced compounds which have precedence in the literature are antibiotic (antifungal and antibacterial) substances and antitumor agents. Amphotericin B is currently the only antifungal compound of microbial origin which is now available for systemic use.[12] It was isolated from a soil actinomycete, *Streptococcus nodosus*, by W. Gold in 1955. Despite its adverse side effects, it remains the standard treatment for most serious mycoses.[13]

Antibacterial agents from microorganisms are a little more common. Penicillin is probably the most celebrated antibiotic. Vancomycin, a fungal derived glycopeptide, is another commonly used antibiotic isolated from a strain of *Streptomyces* (now *Amycolatopsis*) *orientalis* in 1956.[14] There are also several synthetic analogs of microbial compounds used clinically as antibiotics. Included in these are analogs of the monocyclic β-lactams isolated from various soil bacteria and analogs of cephalosporin produced by the fungus *Cephalosporium acremonium*.[15,16] There have been several new antibacterial compounds isolated from microbes over the last few years. Omura's book, *The Search for Bioactive Compounds from Microorganisms*, contains a table of recently isolated compounds which inhibit bacterial cell wall formation. The

table lists 47 compounds (some previously identified) which were published since 1980.[17]

Actinomycin D, mitomycin C, doxorubicin, and bleomycin are among the microbe-derived compounds that are currently being used clinically as antitumor agents.[18] A major discovery in this area was the isolation of the enediyne class of antitumor compounds including calicheamicin and esperamicin by May Lee at the Lederle Laboratories of the American Cyanamid Company. These are some of the most potent agents ever discovered, with calicheamicin being over 1000 times more potent than clinically useful antitumor antibiotics. The calicheamicins are produced by the fermentation of *Micromonospora echinospora* ssp *calichensis*, a bacterium isolated from a soil sample collected in Texas.[19] Omura's book also contains a list of 58 novel antitumor antibiotics isolated from microorganisms since 1984.[20]

This study was concerned with the isolation of novel bioactive compounds from endophytic fungi of the mexican yew tree (*Taxus globosa*). Endophytes are microbes which commonly live in the intercellular spaces of stems, petioles, roots, and leaves of plants causing no outward manifestation of their presence. The associations of these microbes to their hosts range from mutualistic, to symbiotic, to commensal.[21] The microbes may mimic the plant and make the same secondary metabolites as its host, or it may contribute by producing one or more compounds that may protect the plant (and thus itself) from attack by insects, fungi, bacteria, or other predators. It is probable that some of these

bioactive compounds may also have pharmaceutical applications.[22] Plant endophytes are a particularly unexplored niche of fungi, and of the work done, there is limited knowledge of the metabolites of these particular fungi. Together this makes endophytic fungi possibly one of the largest potential areas for discovery in the plant/microbe world.[23]

The choice of yew trees as a source of endophytic fungi was driven by the high cost and limited supply of taxol. The work done in the Stierle and Strobel laboratories to isolate a fungal source of taxol[24] resulted in a large collection of fungi which could also be examined for other bioactive metabolites.

Preliminary Results

Six fungi isolated from the mexican yew (labled as MA17, MC1, ME1, ME7, ME19, MF1) were chosen for this study. The fungi were purified and grown in 300 ml of sterilized medium containing 10.0 g sucrose, 1.0 g Bacto-Soytone, and 1 ml of a 1.0 M phosphate buffer (pH = 6.8) per liter. The cultures were incubated at room temperature without shaking for twenty-one days.

The mycelia were removed by filtration through eight layers of cheesecloth. The mycelia were then ground and extracted with 1:1 chloroform:methanol for the mycelial/organic (M/O) fraction and then with water for the mycelial/aqueous (M/H_2O) fraction. The filtrate medium was also extracted with three successive portions of chloroform and two portions of ethyl acetate. The organic extracts

were combined for the filtrate/organic (F/O) fraction and the remaining aqueous solution was lyophilized and labeled as the filtrate/aqueous (F/H_2O) fraction.

The crude extracts were subjected to a brine shrimp toxicity assay and to agar-diffusion assays to test for bioactivities (See Chapter 2 for details). The agar-diffusion assays were carried out on 0.5 mg of each extract dissolved in 20 µL of methanol. Positive results are recorded on a scale (+ to +++++) according to the completeness and size of the zone of inhibition. Negative results are recorded as a negative (-) and those with multiple negatives (-- and ---) were zones of apparent growth promotion. The brine shrimp toxicity assay was performed on 1.5 mg of extract in 60 µL of methanol per 3 mL of brine water for the organic extracts. The aqueous extracts were 1.5 to 5 mg of sample in 30 µL of water per 3 mL of brine water. Results are given as the number of dead brine shrimp at each time interval. The results of these bioassays are given in Tables 1.1 and 1.2.

There was very little activity in any of the aqueous fractions. However, several of the organic fractions were active in at least one of the bioassays. The severe brine shrimp activity of fraction MA17 F/O and the antifungal activity of the ME19 F/O fractions led to the isolation of sphaeric acid (Chapter 3) and epicorazine A (Chapter 6) respectively.

Extract	G. cand.	C. alb.	B. sub.	P. aerug.	E. coli
MA17 F/O	-	+	+++	++++	+++
MA17 M/O	-	-	+	-	-
MA17 F/H_2O	-	-	-	-	-
MA17 M/H_2O	-	-	-	-	-
MC1 F/O	-	+	+	-	-
MC1 M/O	+	++	+	-	-
MC1 F/H_2O	-	-	-	-	-
MC1 M/H_2O	+	---	-	-	-
ME1 F/O	-	-	++	-	-
ME1 M/O	-	-	+	-	-
ME1 F/H_2O	-	---	-	-	-
ME1 M/H_2O	+	-	+	-	-
ME7 F/O	+	+	++++	-	-
ME7 M/O	+	+	+	-	-
ME7 F/H_2O	-	--	-	-	-
ME7 M/H_2O	+	-	+	-	-
ME19 F/O	+++++	+++++	+++++	-	-
ME19 M/O	++	++	++	-	-
ME19 F/H_2O	-	+	-	-	-
ME19 M/H_2O	-	+	+	-	-
MF1 F/O	-	+	++++	-	-
MF1 M/O	+	+	+	-	-
MF1 F/H_2O	-	-	-	-	-
MF1 M/H_2O	-	-	+	-	-

Table 1.1 Antibiotic bioassays of crude extracts

Extract	t=0h	t=1h	t=3h	t=6h	t=12h	t=24h	total
MA17 F/O	13	26	45	-	-	-	45
MA17 M/O	0	0	0	0	0	0	44
MA17 F/H_2O	0	0	0	0	1	2	15
MA17 M/H_2O	0	0	0	0	0	0	23
MC1 F/O	0	0	1	11	29	32	32
MC1 M/O	0	0	0	0	1	2	22
MC1 F/H_2O	0	0	0	0	2	2	25
MC1 M/H_2O	0	0	0	0	0	1	16
ME1 F/O	0	0	1	1	1	4	42
ME1 M/O	0	0	0	0	0	13	42
ME1 F/H_2O	0	0	0	0	0	1	23
ME1 M/H_2O	0	0	0	0	5	5	11
ME7 F/O	0	0	2	2	3	14	30
ME7 M/O	0	0	0	0	0	1	37
ME7 F/H_2O	0	0	0	0	0	0	25
ME7 M/H_2O	0	0	0	0	0	1	20
ME19 F/O	0	0	0	0	2	14	24
ME19 M/O	0	0	0	0	0	0	27
ME19 F/H_2O	0	0	0	0	2	2	22
ME19 M/H_2O	0	0	0	0	0	3	22
MF1 F/O	0	0	0	0	4	18	22
MF1 M/O	2	0	0	1	2	2	48
MF1 F/H_2O	0	0	0	0	5	4	34
MF1 M/H_2O	0	0	0	0	0	10	25

Table 1.2 Brine shrimp bioassays of crude extracts

Fungal Identification

The identification of the fungi involved in this study was performed by Dr. Gene Ford. ME19 was identified as an *Epicoccum* sp. based on its spore characteristics. However, the identification of MA17 was not so straightforward. By the time Dr. Ford examined the fungus, it had lost its ability to produce spores in culture under the same conditions that it had when originally isolated. His identification was then based on the initial observations of spores produced in early cultures and their documentation by photomicrographs.

Several authorities on mycological taxonomy examined cultures of this isolate. None of them were able to make a positive identification because of the inability to obtain adequate sporulation of the fungus. However, the fungus produces sterile fruiting structures (dark colored pycnidia with curled setae adorning the area around the ostiole) on many media. All the authorities agreed that the fungus belongs to the *Sphaeropsidales*. Brian C. Sutton believed the fungus is possibly a *Phyllosticta*, but without spores to examine, admitted this is an educated guess based only on hyphal and cultural appearance. R. A. Samson (Centraalbureau voor Schimmelcultures) considered it to possibly be a *Diplodia*, even though he did not observe any two-celled spores in culture. Likewise, Jeffery Stone at Oregon State University cultured the fungus and got a microconidial stage (possibly *Leptodothiorella*) and a pycnidial stage which produced dark single-celled spores which he speculated might be an immature

Diplodia stage. However, he did not observe any two-celled spores. A fourth mycologist from the University of Toronto, David Mallock, speculated that the fungus is *Chaetodiplodia* based on the setae found on the pycnidia. However, this ID also requires the spores to be two-celled.

In light of the inability of these experts to agree on an identification and using our own observations on fruiting body and spore formation, Dr. Ford believes this fungus to be a *Sphaeriopsis* sp. The reasons for this are as follows:

1) Only dark colored, single-celled pycnidiospores were observed, which rules out *Diplodia* which has two-celled spores.

2) Spore measurements showed their size to average 27.1 µm X 14.2 µm, which is slightly smaller than that reported for other members of this genus (30-45 µm X 10-22 µm).

3) No microconidial production was ever observed in our cultures.

Moreover, the setae adorning the pycnidia are produced under all cultural conditions (including culture on sterilized *Taxus* leaves), thus providing a constant character to be used in classifying this fungus. The small spore size and slightly coiled setae with their ornamentations could provide justification for the establishment of a new species.

Figure 1.1 shows the photomicrographs of the *Spaeriopsis* sp. From upper left to lower right the photos are as follows:

1) UL: Setae of pycnidium on a yew twig from above (Approximately X75).

2) UR: Pycnidium emerging from a yew twig (X200).

3) CL: Setae and pycnidium on a yew twig from the side (X400).

4) CR: Ornamentations on setae (X3000).

5) LL: Cross-section of seta and ornamentations (X14,500).

6) LR: Pycnidiospores (X170).

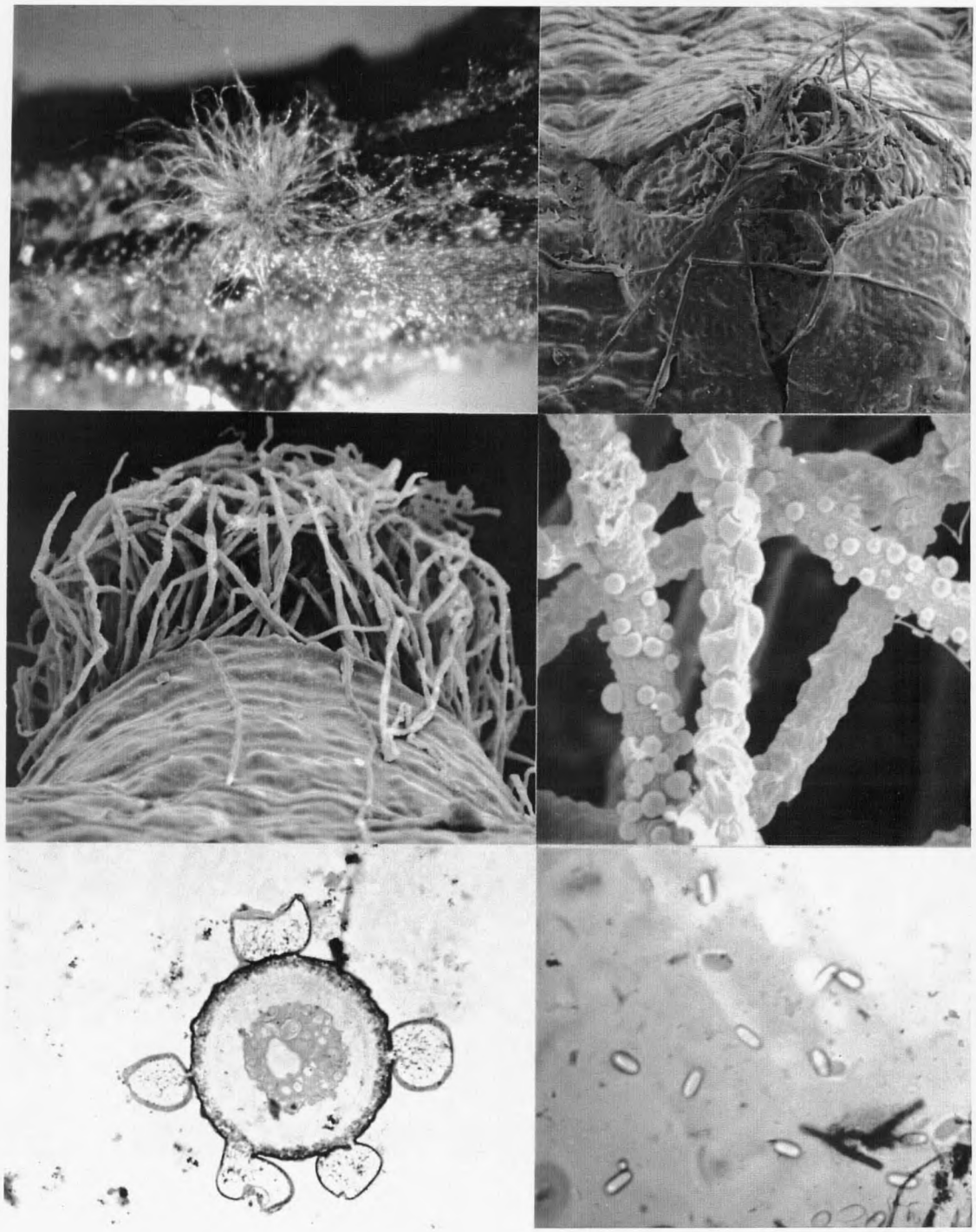

Figure 1.1 Photomicrographs of *Sphaeriopsis* sp.

Chapter 2

GENERAL EXPERIMENTAL METHODS

Throughout the scope of this work, there were many experimental methods which remained constant. This chapter covers the experimental portions for these processes. These are involved throughout the work from the fungal isolation, maintenance, and culturing, to the isolation and structure elucidation of the natural products, and to the determination of the biological activities of the compounds.

Fungal Isolation

The initial fungal isolation was performed by Dr. Bret Niedens. Twig samples of *Taxus globosus*, mexican yew, were surface sterilized with 95% ethanol. After evaporation of the ethanol in a sterile laminar flow hood, the outer bark of the twigs were removed. Small (~1 cm) pieces of inner bark (phloem-cambium and xylem tissues) were removed and placed on water agar plates. After a period of initial growth, hyphal tip transfers of the developing fungi were grown on potato dextrose agar and visually checked for purity.

Fungal Maintenance and Culturing

Microbial samples were maintained on a variety of agars, including Tryptic Soy Agar, Potato Dextrose Agar, M-1-D agar. Agars were prepared from Difco Microbiological agars, or from the corresponding Difco broths and Difco Bacto agar, except M-1-D (shown below). Fungi isolated from yew trees were maintained on the above agars containing 1% yew broth (discussed below).

Fungal culturing was carried out in a variety of synthetic media labled R-1, R-2, medium A, medium B, and medium C. Medium R-1 was the standard medium used in this study and medium R-2 was a high sugar/high nutrient version of medium R-1. Media A, B, and C were a series of trapping media used to limit the amounts of certain nutrients during culturing. The culturing was done in four liter and two liter erlenmeyer flasks, and one liter roux flasks. The volume of medium in each flask was generally chosen to give the largest surface area for growth. Fungi were grown for twenty-one days in still culture, with occasional swirling in some instances.

Yew Broth

Five grams of yew needles and small stems were placed in a beaker with 500 ml of water. The water was boiled for 5 minutes and then allowed to simmer for one hour without heating. The broth was passed through cheesecloth to remove the yew debris and the broth was frozen in ten milliliter portions.

M-1-D Agar

Major salts: $Ca(NO_3)_2$ - (0.28 g/L), KNO_3 - (0.08 g/L), KCl - (0.06 g/L); Minor salts: $FeCl_3 \cdot 6H_2O$ - (2.0 mg/L), $MnSO_4$ - (5.0 mg/L), $ZnSO_4 \cdot 7H_2O$ - (2.5 mg/L), H_3BO_3 - (1.4 mg/L), KI - (0.7 mg/L); $MgSO_4$ anhyd. - (0.36 g/L), $NaH_2PO_4 \cdot H_2O$ - (0.02 g/L), ammonium tartrate - (5.0 g/L), sucrose - (30 g/L), yeast extract (Difco) (0.25 g/L), agar (15 g/L).

R-1 Medium

Bacto soytone (Difco) - (1.0 g/L), sucrose - (10 g/L), 1.0 M $KHPO_4$ buffer (1.0 mL/L).

R-2 Medium

Bacto soytone (Difco) - (5.0 g/L), sucrose - (60 g/L), yeast extract (Difco) - (1.0 g/L), 1.0 M $KHPO_4$ buffer (1.0 mL/L).

Medium A

Bacto soytone (Difco) - (10 g/L), glucose - (40 g/L), $Mg_3(PO_4)_2 \cdot 12H_2O$ - (10 g/L).

Medium B

Bacto soytone (Difco) - (10 g/L), glucose - (40 g/L), $MgCO_3$ - (10 g/L).

Medium C

Bacto soytone (Difco) - (10 g/L), glucose - (40 g/L), $MgSO_4 \cdot 7H_2O$ - (20 g/L).

Extraction and Purifications

All initial mycelia and media extractions were conducted with Fisher Chemical HPLC grade solvents, EM Science HPLC grade solvents or Fisher Chemical ACS grade solvents. Water used for extractions was distilled water from a Wheaton Autostill 1.5 water still.

Low pressure chromatography was performed with various solid supports including Lipophilic Sephadex LH-20 (25-100 µm beads), Toyopearl HW-40F (45 µm beads), BioRad Chelex 100 (50-100 mesh), and J.T. Baker silical gel 40 µm flash chromatography packing. Solvents used were Fisher Chemical HPLC grade solvents and EM Science HPLC grade solvents. Water was distilled with a Wheaton Autostill 1.5 water still. Fractions were collected on a Gilson FC203B fraction collector, and fractions were determined by use of a Gilson 115 variable wavelength UV/VIS detector.

The HPLC system consisted of Waters 510 HPLC pumps, a Waters model 660 Solvent Programmer, a Waters model U6K Injector, and a Waters model 440 Absorbance Detector. Columns used were purchased from Rainin including:

Analytical:

Dynamax 60Å silica column, 4.6 mm ID X 25 cm L, 8 µm packing.

Microsorb silica column, 4.6 mm ID X 25 cm L, 5 µm packing.

Semi-preparative:

Dynamax 60Å silica column, 10 mm ID X 25 cm L, 8 µm packing.

Dynamax 60Å octadecyl column, 10 mm ID X 25 cm L, 8 µm packing.

Preparative:

Dynamax 60Å octadecyl column, 21.4 mm ID X 25 cm L, 8 µm packing.

All columns were protected by Rainin guard columns (5 cm L) of matching diameter and packing material. Solvents used were Fisher Chemical HPLC grade solvents and EM Science HPLC grade solvents. Water was distiilled by a Wheaton Autostill 1.5 water still. All solvents were filtered and degassed with Kontes Brand Ultra-Ware HPLC Resevoir systems through Gelman 5 µm PTFE membrane filters.

Bioassays

In order to establish the presence of bioactive compounds, to follow them through the isolation procedure, and as a final characterization of the purified compounds, bioassay screening methods were used. The screening methods included simple agar-diffusion assays for the antibacterial and antifungal activities, and brine shrimp toxicity bioassays for antitumor bioactivity. Final characterization assays included a paper disc diffusion assay, a mouse thymocyte proliferation assay, an inhibitory concentration against breast adenocarcinoma BT-20 assay, a minimum inhibitory concentration spot test, and a brine shrimp toxicity assay.

Agar-diffusion Assay

In this assay, the fractions to be tested were dissolved in a small amount of solvent and then spotted (10 µL) on an agar medium [Difco Antibiotic Medium 1 (Penassay Seed Agar) for bacteria, and Difco Potato Dextrose Agar for fungi]. The solvent was allowed to evaporate and then the test microbe was inoculated on the plate. The inoculation was accomplished by spraying the plate with an aqueous suspension of the microbe. A positive assay was inhibition of the growth of the microbe in the area where the sample was applied. This method was used to test for antifungal compounds against *Geotrichum candidum* and *Candida albicans* and antibacterial compounds against *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli*.

Brine Shrimp Bioassay

This assay was established as a general bioassay for a broad spectrum of pharmacological activities.[25] It is a convenient tool for isolating antitumor agents. Ferrigni and McLaughlin[26] used this assay to follow activity through fractionations in the isolation of piceatannol, an antileukemic compound, from the seeds of *Euphorbia lagascae*.

In this assay, the sample was dissolved in a small amount of solvent and added (30 µL of methanol) to a test vial of artificial sea water (3.0 ml). Approximately 20 brine shrimp, *Artemia salina*, were added to the vial. The brine shrimp were observed periodically over a twenty-four hour period and the number of dead brine shrimp in each vial were recorded. A positive assay was

the death of all or most of the brine shrimp by the end of the twenty-four hour period.

Paper Disc Diffusion Assay

This assay was a variation of the agar-diffusion assay[27] and was performed in the Stierle's laboratory at Montana Tech in Butte. The difference was the method of application of the sample. A known amount of sample (100 µg, 50µg, or 10 µg) dissolved in solvent was applied to a Difco Bacto Sterile Blank concentration disc, and the solvent was allowed to evaporate. The paper disc wais then placed on the agar and the test microbe was applied. The microbes tested against in this assay were *B. subtillis*, *Staphylococcus aureus*, *E. coli*, *P. aeruginosa*, *Vibrio harveyii*, *C. albicans*, *G. candidum*, *Aspergillus niger*, and *Fusarium oxysporum*.

Mouse Thymocyte Proliferation Assay

The interleukins are a group of glycoproteins which are involved in the regulation of the immune system. Interleukin-1 (IL-1) plays a central role in T-cell activation and has been found to be involved in a number of aspects of inflammation.[28] This assay was used as a general screen for agents that affect IL-1 action.

This assay was performed by Pharmagenesis Pharmaceuticals in Palo Alto, CA and the inhibition of IL-1 mediated thymocyte proliferation was reported.

The procedures followed were the standard, published procedures reported in Laboratory Methods in Immunology.[29]

Minimum Inhibitory Concentration (MIC) Against Breast Adenocarcinoma BT-20

This assay was performed by Cytoclonal Pharmaceuticals, Inc. in Dallas, Texas. The human breast cell cancer lines were exposed to serial dilutions of each derivative. After three days of exposure, the cells were stained with neutral red and the absorbance was measured at 540 nanometers. Samples were dissolved in either methanol or dimethylsulfoxide at negligible levels. The results were reported as inhibitory concentration (IC_{50}) values.

Minimum Inhibitory Concentration (MIC) Spot Test

Extracts were dissolved in methanol or chloroform to final concentrations of 10 µg/µL. Serial dilutions of each sample were made giving final test concentrations of 10, 5 and 2.5 µg/µL. Five microliters of each solution were spotted onto a half-strength YM agar plate (10.5 g/L YM broth (Difco), 15 g/L agar), and allowed to dry. An agar solution, containing *C. albicans,* was prepared to have an $O.D._{590}$ of 0.3. Three millilters of this agar was overlayed on the spotted agar plates and allowed to harden. The plates were incubated for 24 hours at 37 C and observed to determine the MIC as the last dilution where activity was observable.

Organisms for Antibiotic Assays

The microorganisms chosen for the antibiotic assays were chosen to give a broad basis to determine activity. These included the fungi and bacteria (Gram-positive and Gram-negative) indentified below.

Geotrichum Candidum: A member of Fungi Imperfecti that is ubiquitous in soil and dairy products, it is sometimes pathogenic in human respiratory and gastrointestinal tracts.[30]

Candida albicans: A yeast-like fungus, that is found in human and animal digestive tracts which can invade other tissues under certain conditions.[31] Invasive candidiasis is the most common fungal infection in patients with HIV infection, and is associated with increased mortality in bone marrow transplant recipients.[32]

Bacillus subtilis: It is a Gram-positive bacterium that is one of the most common non-pathogenic aerobic spore formers. It is related to *B. anthracis* which is the agent responsible for anthrax in cattle, sheep, and other agriculturally important animals.[33]

Pseudomonas aeruginosa: This is a Gram-negative bacterium and is the only Pseudomonas species that is pathogenic for man and animals.[34] It remains a problem pathogen, becoming resistant to virtually all the clinical antibacterial agents, and is a major challenge in the field of antibiotics.[35]

Escherichia coli: *E. coli*, a Gram-negative bacterium, is the predominant organism in the intestinal canal of man and animals. It can become pathogenic

and may invade the appendix, gall-bladder, peritoneal cavity, kidneys, and the urinary bladder.[36]

Staphylococcus aureus: A Gram-positive, pathogenic, bacterium which man and animals live with from birth until death, it only becomes infectious when their susceptibility is appreciably affected.[37] Its resistance has increased recently and currently vancomycin is the only clinical antibiotic that provides reliable activity against multiply resistant strains.[38]

Vibrio harveyii: This is a Gram-negative marine bacterium which is the primary colonizer in marine fouling. It is related to disease causing Vibrio including *V. choleræ* responsible for cholera.[39]

Aspergillus niger: Aspergillus is a pathogenic filamentous fungus which has a propensity for invasion of blood vessels.[40]

Fusarium oxysporum: The Fusarium are members of the Fungi Imperfecti which include saprophytes and many plant parasites.[41] It is emerging as a human pathogen in immunocompromised patients and often does not repsond to conventional doses of amphotericin B.[42]

Instrumentation for Structure Elucidation

Ultraviolet/Visible Spectrometer: Beckman DU-50 UV/VIS Spectrophotometer.

Polarimeter: Optical rotations were collected on a Perkin-Elmer model 241 MC polarimeter.

Infrared Spectrometer: IR spectra were collected on a Perkin-Elmer model 1600 FTIR.

Mass Spectrometry: Mass spectra were recorded on a VG 10E-HF Mass Spectrometer or a TRIO-2 Electrospray Mass Spectrometer.

TMS derivatives were prepared by reacting the sample with BSA [*N,O*-Bis(trimethylsilyl)acetamide].

Nuclear Magnetic Resonance: NMR spectra were recorded on a Bruker AC300 spectrometer, a Bruker DPX300 spectrometer, a Bruker DRX250 spectrometer, or a Bruker DRX500 spectrometer. Carbon assignments (methyl, methylene, methine, quaternary) were determined via DEPT 90 and DEPT 135 experiments.