Investigation of biologically active metabolites from symbiotic microorganisms
by Andrea Anne Stierle

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Chemistry
Montana State University
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Abstract:
The investigation of symbiotic relationships between macroorganisms and microorganisms is a complex, fascinating research endeavor. Throughout the course of this project we explored two such interactions: the relationship between a terrestrial weed and a plant pathogenic fungus, and the relationship between a marine sponge and a gram positive bacterium. Each of these interactions represents a different aspect of symbiosis.

Centaurea maculosa Lam, spotted knapweed, was the focus of a study concerning a parasitic fungus-weed interaction. Alternaria alternate was isolated from a black leaf blighted specimen of spotted knapweed and was determined to be the causative organism of the observed plant disease. A series of diketopiperazines were consistently isolated from liquid cultures of Alternaria alternate. The most active of these compounds, maculosin, cyclo(L-Pro-L-Tyr), caused black necrotic lesions in a nicked leaf bioassay at 10μm. In tests against nineteen plant species, both monocots and dicots, maculosin was phytotoxic only to spotted knapweed. Several other diketopiperazines isolated from the fungus exhibited either reduced phytotoxicity or none at all. This is the first report of a host specific toxin from a weed pathogen.

Additional phytotoxic fractions from the organic soluble extracts of the fungus yielded a series of perylenequinones. Two of these compounds were novel and phytotoxic, and two were known but inactive. The organic extracts also yielded the known toxin tenuazonic acid. All three classes of compounds were compared individually and in combination with respect to phytotoxicity and host specificity.

Not all symbioses are as well-defined as the host-parasite relationship. A Micrococcus sp. was consistently isolated from tissues excised from the marine sponge Tedania ignis, despite the collection locale, suggesting some form of symbiosis, but not clarifying the degree of interaction. A relationship which we initially considered ambiguous between the sponge and the bacterium evolved into a true case of mutualism. Of particular interest was the isolation of three compounds from the liquid culture of the bacterium that were previously attributed to the sponge. In addition to these three compounds, several aromatic metabolites not usually associated with living organisms were also isolated from the liquid culture, characterized, and tested for biological activity.

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FROM SYMBIOTIC MICROORGANISMS

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APPROVAL

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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.

23 Aug 88
Date

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Chairperson, Graduate Committee

Approved for the Major Department

23 Aug 88
Date

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Head, Major Department

Approved for the College of Graduate Studies

September 16, 1988
Date

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Graduate Dean

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Date _August 22, 1988_

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ABSTRACT

The investigation of symbiotic relationships between macroorganisms and microorganisms is a complex, fascinating research endeavor. Throughout the course of this project we explored two such interactions: the relationship between a terrestrial weed and a plant pathogenic fungus, and the relationship between a marine sponge and a gram positive bacterium. Each of these interactions represents a different aspect of symbiosis.

Centaurea maculosa Lam, spotted knapweed, was the focus of a study concerning a parasitic fungus-weed interaction. Alternaria alternata was isolated from a black leaf blighted specimen of spotted knapweed and was determined to be the causative organism of the observed plant disease. A series of diketopiperazines were consistently isolated from liquid cultures of Alternaria alternata. The most active of these compounds, maculosin, cyclo(L-Pro-L-Tyr), caused black necrotic lesions in a nicked leaf bioassay at 10 μM. In tests against nineteen plant species, both monocots and dicots, maculosin was phytotoxic only to spotted knapweed. Several other diketopiperazines isolated from the fungus exhibited either reduced phytotoxicity or none at all. This is the first report of a host specific toxin from a weed pathogen.

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INTRODUCTION

The investigation of symbiotic relationships requires an understanding of the scope and variability of possible interactions between organisms. In a literal sense, symbiosis simply means "living together"; in the true biological sense, however, it involves a close association between two members of different species (1). There are three extremes of symbiotic relationships. If the relationship is beneficial to both species it is called mutualism. A classic example of mutualism is the lichen, which is part fungus and part alga. The two unrelated organisms together form a closely integrated unit capable of growth under conditions that neither the fungus nor the alga could survive alone (1).

A relationship that benefits one species while neither harming nor benefiting the other species is called commensalism. The remora enjoys such a relationship with the shark. An indifferent swimmer, the remora attaches itself to the shark by means of an adhesive organ on its head. The "guest" is not only transported courtesy of its host, but also appropriates a portion of the shark's kill when it feeds (2).

If one species is harmed and the other species benefits it is called parasitism. Parasitism is considered to be a

special form of predation in which the predator is
considerably smaller than the prey (3). Familiar examples
of parasites are mosquitoes, fleas, and ticks.

An intriguing aspect of symbiosis is the interaction of
macroorganisms and microorganisms in intimate contact with
one another. Just as in interactions between two
macroorganisms, such relationships can be mutualistic,
commensal, or parasitic. In most cases, it is the
microorganism that derives consistent benefit from such
associations, and any harm is usually derived by the
macroorganism (4). The exploration of such relationships
and, in particular, the chemical manifestations of such
interactions, provide a rich arena for natural products
research.

It is sometimes difficult to determine the nature of a
particular symbiotic association. All of the details of a
relationship may not be known or fully understood, and
relationships are subject to change with time or various
circumstances. This is especially true in ascertaining the
nature of macroorganism-microorganism interactions. Human
beings play host to a rich population of skin bacteria,
such as <u>Staphylococcus aureus</u>, which generally coexist as
harmless commensals on the surface of the epidermis. When
aspirated into the lungs or introduced into a wound, these
harmless commensals may become parasitic (4). Saprophytic
fungi may parasitize live plants under the proper

conditions (5). Any suspected instance of symbiosis must be carefully examined to verify both the existence and the nature of such a relationship.

In general, a relationship is considered symbiotic if two organisms are consistently found together even if the scope of their interaction is unknown or undefined (1). In the course of this study we chose to examine such relationships in both marine and terrestrial ecosystems. The target macroorganisms, _Centaurea_ _maculosa_ and _Tedania_ _ignis_, were chosen because both possess intrinsic biological significance.

Spotted knapweed is the most important weed pest in Montana, and we were most interested in discovering a bacterium or fungus capable of producing phytotoxins deleterious to this plant. The first part of this study centers on the discovery of such a pathogen, an examination of its disease-inductive propensity towards knapweed, and the determination of its ability to produce substances injurious to the plant.

Organic extracts of the marine sponge _Tedania_ _ignis_ exhibit both cytotoxicity and in vivo tumor inhibition, and have yielded the potent cytotoxic macrolide designated tedanolide, 1 (6). The extremely low yields of tedanolide (1×10^{-4}% dry weight) suggested to Schmitz that this metabolite might actually prove to be of microbial origin, as was found with tetrodotoxin (7). It was our intention

to culture the microbial flora from this sponge, as part of a larger culture effort. We could then determine if these microscopic organisms are capable of producing interesting, pharmacologically compelling compounds.
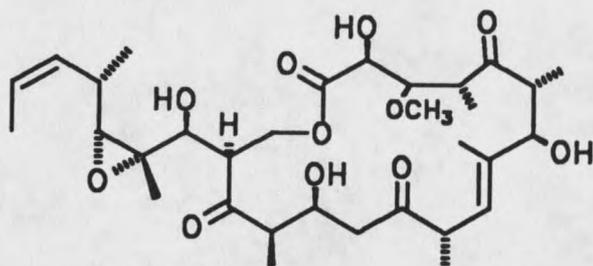
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As natural products chemists we emphasized the chemical ramifications of interactions between organisms. In exploring the parasitism of the plant-fungus relationship we were primarily interested in toxin production, particularly host-specific toxin production, by the parasite. In exploring the marine organisms our goals were somewhat less specific, if not more ambitious. The particular bacterium which we studied was constantly isolated from collections of the sponge, regardless of the collection site. This constancy of association defines the interaction as symbiotic, but does not establish the nature of the symbiosis.

TERRESTRIAL PARASITISM

Background

The examination of the parasitism of plants by fungi is supported by extensive plant pathological lore. Fungi have long been recognized as causal agents of plant diseases. Many disease symptoms are often associated with the elaboration of one or more phytotoxins by a pathogenic fungus. To date, phytotoxins capable of expressing host specificity at the species or cultivar level are known only as metabolites of pathogens of crop plants. This is probably because crop plants are typically agro-ecosystems with a strictly homogeneous genetic base, which serves as a huge reservoir of effectively identical plant material. This homogeneity renders these plants susceptible to widespread devastation by one or more pathogens. The 1970 Southern corn leaf blight epidemic in the US-Canada, attributed to the pathogenic fungus <u>Drechslera maydis</u>, supports this supposition (8). This epidemic caused the greatest crop loss in the shortest time span of any plant disease on record. Corn plants with Texas-male-sterile (Tms) cytoplasm, which comprised most of the corn planted, were particularly vulnerable. A pathogen has the potential to develop and spread quickly under such conditions and can be easily observed and isolated.

This does not appear to be the case with weeds, which can be defined as opportunistic plants that compete economically or aesthetically with whatever plants are intentionally cultivated (9). Common weedy plants usually exist in a population having a heterogeneous genetic base which tends to preclude the development of such an epidemic, making discovery of the pathogen difficult, if not impossible.

The isolation and investigation of weed pathogens is important not only because of their intrinsic ability to serve as biocontrol agents, but also because of their propensity for producing novel bioactive substances. These substances may act as herbicides or provide important chemical leads to the herbicide industry (10). It was our intention to harness this propensity for phytotoxin production exhibited by a number of pathogenic fungi and direct it towards an important weed pest.

Phytotoxins vary greatly in their disease inductive abilities and in their degree of host-selectivity. We were most interested in isolating a pathogen specific to a particular weed capable of producing toxins that were also host-specific for that weed. R. K. S. Wood (5) defined host-specific toxins as follows:

> Microorganism X (but not others) produces substance Y which damages plant or plant group A but not others, and only A is parasitized.

The concept of a host specific toxin is certainly not new. Over 40 years ago Meehan and Murphy (11) recognized the influence of such a compound in the disease symptomology of victoria blight of oats, which will be discussed in more detail later. Several compounds with apparent true host specificity have been isolated and characterized. It is interesting to note that no host specific toxins have ever been found to weed plants.

The focus of this study was the discovery of a weed pathogen capable of producing compounds with phytotoxicity only towards a particular weed. Once such a weed pathogen was discovered, the chemistry responsible for its virulence and host-specificity would be thoroughly investigated.

Biorationale for Research Design

There are two approaches to the utilization of plant pathogenic microorganisms as biocontrol agents. The first approach involves the inoculation of the target organism, in this case a weed, with the live pathogen, in an attempt to induce disease symptoms in the plant. The second approach involves application of selected, bioactive chemicals (phytotoxins) produced by the pathogen to the target weed to produce disease symptoms. In both cases it is necessary to follow the dictates of Köch's postulates (12) to establish absolutely an isolated pathogen as the instigator of plant disease:

1) The pathogen must be isolated from the tissues of a diseased host and established in a pure culture.

2) The pure cultural isolate must be capable of consistently inducing disease symptoms in a healthy host.

3) The pathogen must then be reisolated from the disease-induced host and re-established as a pure culture.

The deliberate use of live plant pathogenic bacteria, fungi, and viruses to achieve economic control of weeds is a relatively new endeavor. Throughout the world, the past twenty-five years have witnessed numerous attempts to control important weed pests through the introduction of selected microorganisms. Plants that have been transported into new regions either by accident or by design often assume the status of weeds due to a lack of natural predators (13). Pathogens are sought from the original geographic location of the plant for introduction into its new environs. Ideally, the pathogen will become established, reach epiphytotic levels, and eventually stabilize as an endemic population once the weed is reduced to subeconomic levels. Skeletonweed (_Chondrilla juncea_ L.) has been successfully controlled in Australia by the introduction of the rust fungus _Puccinia chondrillina_ from the Mediterranean region where the weed originated (14). Attempts to duplicate this success in the United States include the control of pamakani weed (_Ageratina riperia_) in Hawaii by introduction of the deuteromycete _Cercosporella_

ageratina from the Caribbean area, where the weed
originated.

A variant of this tactic involves the importation of
pathogenic strains from the same or related host species to
infest indigenous weeds which have evolved in the absence
of the pathogen. Historically crucial diseases such as
white pine blister rust and Dutch Elm disease in the United
States, and grape powdery mildew rust in Europe, all
involved the accidental introduction of pathogens to
indigenous, vulnerable plant populations with devasting
results (15). The intentional introduction of pathogenic
organisms to weed populations may prove similarly
devastating to economically undesirable plants. The
deployment of fungal pathogens to indigenous weed
populations is approaching commercial use in at least three
cases. Water hyacinth (Eichhornia crassipes Sohms) control
by application of Cercospora rodmanii is currently being
tested by the U.S. Army Corps of Engineers. The fungus is
expected to be marketed by Abbott Laboratories in the near
future (14). The fungus Phytophthora palmivora has
recently been registered, also by Abbott Laboratories, as a
mycoherbicide for the control of strangler vine (Morrenia
odorata Lindl.) in Florida citrus groves (14). The
indigenous fungus Colletotrichum gloeosporoides f. sp.
aeschynomene is being field-tested in Arkansas as a control
agent for northern jointvetch in rice and soybeans, and

should be available commercially pending approval from the
Environmental Protection Agency (14). Such successful
applications of plant pathogens as control agents in the
fight against weeds should inspire further investigation of
this area of biocontrol.

The utilization of live pathogens as bioherbicidal
agents is not without risk. The pathogen may actually
prove more virulent to important crop plants in the area
than to the desired weedy target, with predictable dire
results. The pathogen may be spread beyond the target
environs by the natural but somewhat unpredictable
dispersal mechanisms of wind, water, and unwitting animal
vectors (16).

The uncontrolled spread of an unnaturally enriched
microorganism unleashed on an unsuspecting public is, of
course, the stuff of nightmares and Hollywood horror
movies. The more realistic concern is actually the
inability of a chosen pathogen to propagate disease in a
target area. There are many factors that govern the
ability of a pathogen to enter the host plant and produce
disease, an ability that can be termed the inoculum
potential of a particular plant pathogen (17).

Inoculum potential is a function of inoculum density,
nutrient availability to the propagules of infection,
environmental factors, virulence of the pathogen, and host
susceptibility (17). A successful biocontrol program which

involves the direct application of a pathogen to a target
weed must consider all of these factors, and manipulate
them as much as possible to insure infection and disease
production.

Inoculum density refers to the number of viable
propagules per unit area of a leaf or stem, or per unit
volume of soil or water (17). The inoculum density
requisite to the induction of disease in a plant community
is variable, and many investigators have attempted to
quantify the effects of varying densities. Researchers at
the Boyce Thompson Institute have studied the dynamics of
inoculum density for fungal pathogens that attack the
aerial portions of plants. They found that numerous spores
were required to produce one lesion on a leaf. For
instance, four hundred uredospores of the wheat stem rust
fungus are required to produce one lesion, while only
fifteen sporangiospores of the potato late-blight fungus
are needed per lesion. Spores of many obligate parasites
such as the rusts produce a volatile chemical that inhibits
the germination of neighboring spores. In this case, as
inoculum density increases, the percentage of spores that
germinate decreases. One cannot simply conclude that by
doubling or tripling the inoculum density, the inoculum
potential is linearly affected (17).

Nutrient availability would not be a limiting factor
in disease induction as the direct interaction of the

pathogen with the weed provides a ready source of simple sugars, fats and complex carbohydrates usually vital for spore germination (17). Of much greater importance in determining whether a propagule will germinate, grow, and infect a host are environmental factors such as humidity and temperature, factors which are unpredictable at best. Most plant pathogens germinate and grow at temperatures similar to those which are optimum for the growth and germination of higher plants. Unexpected frosts can severely retard the growth and development of most plants and, therefore, of most pathogens. Moisture also affects germination of fungal spores and establishment of bacterial pathogens in the host plant. Most fungal spores require high moisture conditions for germination. Alternaria sp. require a relative humidity of 90-95% for germination, while the uredospores of rust fungi require free water for germination. It is important to note that in the cases involving successful control of weeds using live pathogens cited above, all of the weeds were found in areas with warm, moist climates. Since it was our intention to isolate a plant pathogen against a Montana weed we would not enjoy either optimum temperatures or humidity for pathogen growth and establishment. Indeed, once an appropriate pathogenic fungus was isolated from our target weed we could not successfully inoculate healthy host

plants with the fungus unless artificially high humidity was provided (17).

After considering our options, we decided to utilize the chemical approach to weed control rather than the live pathogen approach. This method also involves a search for an appropriately diseased plant and the isolation of the responsible pathogen. The pathogen would be established in pure culture and determined to be the true cause of deleterious symptoms in our target weed. It would then be grown in culture and its organic and aqueous extracts analyzed for phytotoxicity. The extracts would be subjected to a variety of separation techniques including gel permeation, centrifugal countercurrent and high performance liquid chromatographies, following a bioassay guided fractionation scheme. This process should culminate in the isolation of one or more phytotoxic metabolites. These metabolites would then be characterized and further tested to establish their host range and relative phytotoxicity profiles.

Phytotoxins: Historical Perspective

Man has been aware of the link between plant disease and blights for many centuries. The Romans were aware of the periodic plagues of rust on their wheat and actually attempted to control its ravages with "scientific" methods. They believed that a particularly capricious god, Robigus,

was responsible for the occurrence of the rust epidemics.
They endeavored to appease the anger of the god by holding
an annual festival. The faithful gathered in a sacred
grove: red wine was poured over an altar and a red dog was
sacrificed (18). Other than the obvious effects on the red
dog, no mention is made of the success or failure of this
endeavor.

Since Roman times, attempts to identify and control
the causes of plant disease have met with much greater
success. The earliest authenticated bacterium induced
plant disease was fire blight of pear (19). This was the
first of many diseases proven to be of microbial origin.
The causative agent is often found to be a bacterium or
fungus capable of elaborating a variety of phytotoxic
compounds deleterious to the host plant. Phytotoxins vary
in their degree of disease induction as well as in their
degree of host-specificity. Toxins may be quite
cosmopolitan in their effects or they may target a single
cultivar of a given plant species, with disastrous results
for that particular plant, but no other (5).

A variety of phytotoxic compounds have been isolated
and identified to date, with activity expressed almost
exclusively towards important crop plants such as corn,
oats, and sugar cane. A disproportionately high percentage
of the phytotoxins characterized to date are metabolites of
the saprophytic fungal genera _Alternaria_, _Fusarium_, and

Helminthosporium (also called Drechslera) and the perfect
stage of Helminthosporium, Cochliobolus (20). No attempt
will be made to enumerate all of the phytotoxins isolated
and characterized to date, but several representative toxin
types will be presented.

In 1971 Steiner and Byther (21) reported that a host-
specific toxin was produced by Helminthosporium sacchari,
the causal agent of eye spot disease of sugar cane. The
fungus caused eye-shaped lesions on leaves followed by the
development of reddish brown runners extending from the
lesion towards the leaf tip. Since the fungus could only
be isolated from the lesion and not from the runners the
investigators concluded that a toxin was involved in the
symptomology. An unidentified toxin was isolated from the
fungus which produced runners only on susceptible cultivars
of sugar cane. Steiner and Strobel isolated the same toxin
from H. sacchari and designated it helminthosporoside (22).
The original structure proposed for the toxin was revised
by Beier et al. (23); however, Macko et al. proposed the
definitive structure for helminthosporoside, 2 (24).
Intensive investigation into the mechanism of toxicity as
well as the determinant for the demonstrated host-
specificity of helminthosporoside indicated the involvement
of a membrane-binding effect. An interesting result of
this work is the use of helminthosporoside as a screening
tool for disease resistance in sugar cane varieties (20).
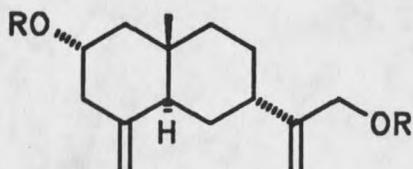
2 R=-O-(β-GALACTOFURANOSYL)-β-GALACTOFURANOSIDE

Orsenigo isolated ophiobolin A, 3, from cultures of
Cochliobolus miyakanus, the perfect stage of
Helminthosporium oryzae, the cause of brown spot of rice
seedlings. The toxin inhibits the growth of rice roots and
coleoptiles, and induces chlorosis in the shoots of treated
rice seedlings. Activity studies suggest that the toxin
irreversibly damages plant membranes (20).

The structure of the sesterterpene ophiobolin A, 3, was
deduced by Nozoe et al. (25) and by Canonica et al. (26).
Ophiobolin A was the first representative of a new family
of sesterterpenoid phytotoxins; several other members have
been discovered and much attention has been paid to their
biosynthesis (27).

The Southern corn leaf blight of 1970 (1) is an example
of a devastating epiphytotic event orchestrated by human
intervention. Corn plants were developed with Texas-male-
sterile cytoplasm (Tms) and disseminated to the
agricultural community. Corn with Tms cytoplasm (most of
the corn planted) proved to be extremely vulnerable to the
pathogenic fungus Drechslera maydis, which resulted in an