



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 10 μ m. 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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Andrea Anne Stierle

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August 1988

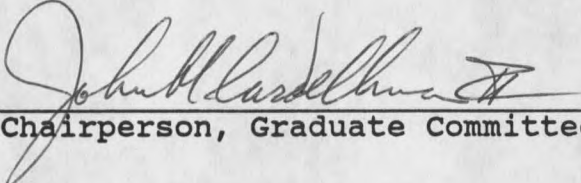
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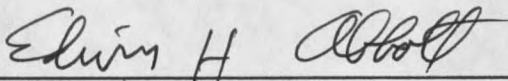
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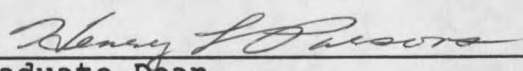
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ABSTRACT

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.

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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.

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 Staphylococcus aureus, 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

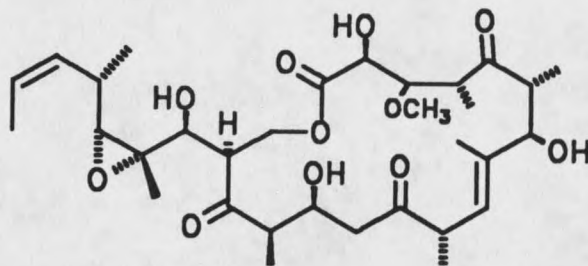
conditions (5). Any suspected instance of symbiosis must be carefully examined to verify both the existence and the nature of such a relationship.

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 ($1 \times 10^{-4}\%$ 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.



1

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 Drechslera maydis, 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 Cercospora

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 devastating 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,

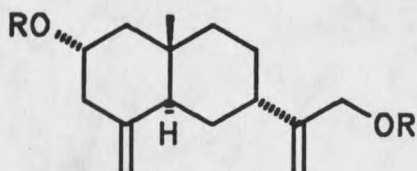
was responsible for the occurrence of the rust epidemics. They endeavored to appease the anger of the god by holding an annual festival. The faithful gathered in a sacred grove: red wine was poured over an altar and a red dog was sacrificed (18). Other than the obvious effects on the red dog, no mention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

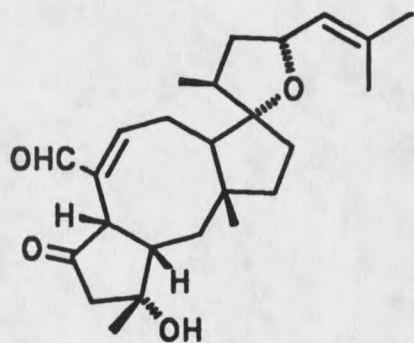
Orsenigo isolated ophiobolin A, 3, from cultures of Cochliobolus miyakanus, the perfect stage of Helminthosporium oryzae, the cause of brown spot of rice seedlings. The toxin inhibits the growth of rice roots and coleoptiles, and induces chlorosis in the shoots of treated rice seedlings. Activity studies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

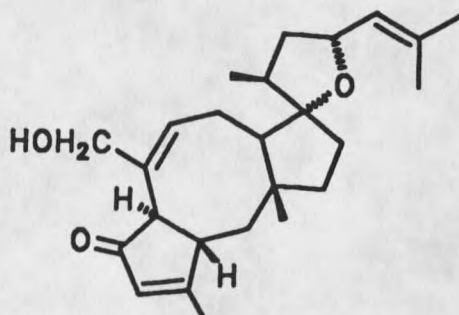
The Southern corn leaf blight of 1970 (1) is an example of a devastating epiphytotic event orchestrated by human intervention. Corn plants were developed with Texas-male-sterile cytoplasm (Tms) and disseminated to the agricultural community. Corn with Tms cytoplasm (most of the corn planted) proved to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

epidemic that caused the greatest crop damage in the shortest period of time on record.

Drechslera maydis, the causal agent of Southern corn leaf blight produces, a series of phytotoxic sester-terpenes. The identity of these toxins has been the center of much controversy. Earlier studies on the nature of the phytotoxins responsible for the devastating corn crop losses of the summer of 1970 have indicated that the compound was either a terpene (28) or a linear polyketopolyalcohol (29). Sugawara et al. used X-ray crystallography to confirm the identity of ophiobolin I, 4, which was an important reference compound for the characterization of the other four ophiobolins (30).



3



4

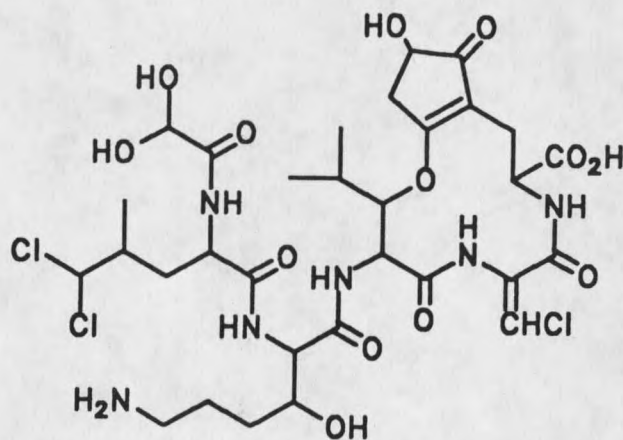
During their work on the D. maydis phytotoxins, Sugawara et al. became interested in examining fungal pathogens of weeds. They studied leaf spot of johnsongrass

(Sorghum halapense L.), a serious weed in tropical and subtropical areas of the world. The disease is caused by D. sorghicola whose perfect stage was found to be genetically compatible with the perfect stage of D. maydis (R.R. Nelson, personal communication to Sugawara et al.). They examined the phytotoxins of both fungi in their activity towards corn bearing Tms cytoplasm. Both of these fungi induced disease symptoms on corn, and both produced ophiobolins, although the concentrations of the various members of this class of compounds differed. This study emphasizes the importance of a thorough investigation of phytotoxins produced by weeds, because the toxic secondary metabolites of weed pathogens may be injurious to crop plants as well (30).

Cochliobolus victoriae Nelson, the perfect stage of Helminthosporium victoriae, was recognized as the causal agent of victoria blight of oats over 40 years (31). This disease was actually the unfortunate result of an attempt to render oat plants resistant to crown rust, Puccinia coronata. It was determined that susceptibility to crown rust was a function of a single gene locus; commercial varieties of oats were therefore introduced carrying the Pc gene for resistance to this disease. Unfortunately, it was discovered that crown rust resistance and victoria oat blight susceptibility were either closely linked or controlled by the same genetic locus. Subsequent studies

have shown that sensitivity to *H. victoriae* is controlled by a single dominant gene with the homozygous dominant genotype conferring sensitivity and the homozygous recessive genotype conferring insensitivity (32).

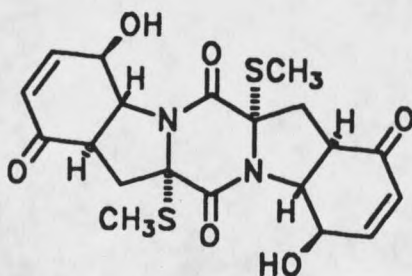
What is perhaps most interesting about the studies centered on victoria blight of oats was the early recognition by Meehan and Murphy in 1947 (11) of a remarkable substance called a host specific toxin, responsible for the induction of disease symptoms. It was not until ten years later, however, that this somewhat unstable metabolite was isolated from cultures of *H. victoriae* (33), and almost forty years elapsed before the structure of the toxin, victorin, 5, could be determined (31).



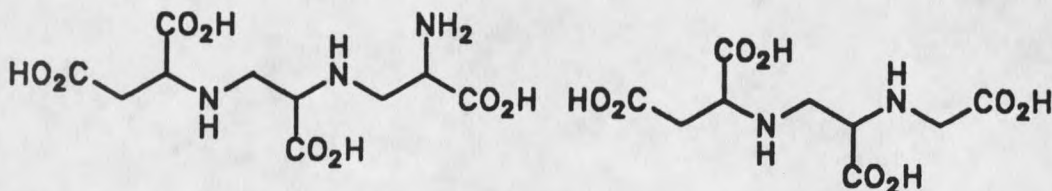
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Several other phytotoxins which are derived from amino acids have been isolated and characterized. Exserohilone,

6, was isolated from Exserohilum holmii, a pathogen of the weed Dactyloctenium aegyptium (crowfoot grass), a serious grassy weed in all major tropical and subtropical parts of the world (34). The toxin is a diketopiperazine with nonspecific activity towards crowfoot grass and several other plants against which it was tested. Other amino acid-derived phytotoxins include lycomarasmine, a non-specific wilt toxin produced by the wilt fungus Fusarium oxysporum (20), and the related compounds aspergillomarasmine A, 7, and B, 8 (20).



6



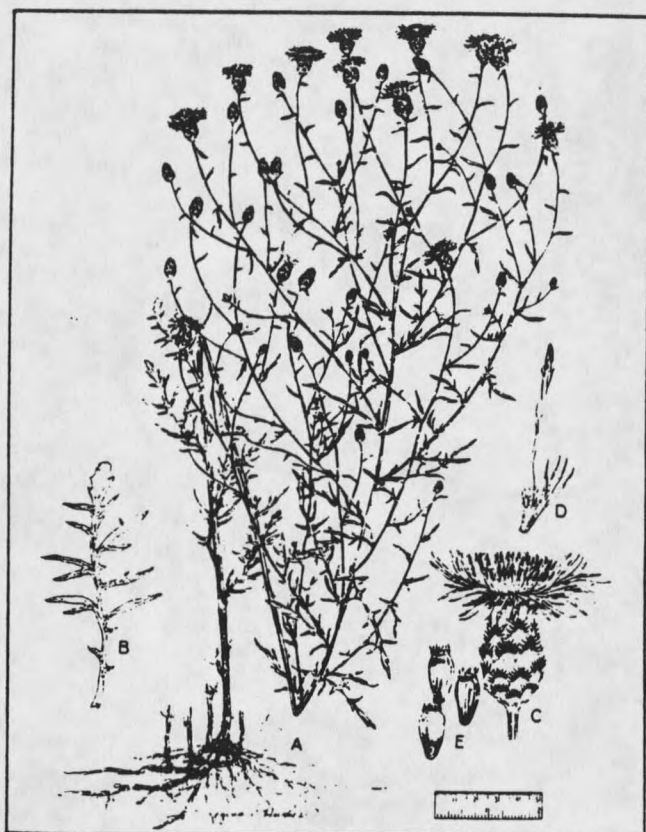
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Selection of a Suitable Target Plant

The choice of a target plant is an important one. The discovery of a pathogen that can produce compounds deleterious to lettuce might be an interesting academic exercise, but would have limited importance to the plant pathology community. Of course, pathogens that elaborate toxins to crop plants have an important place in the screening of disease-resistant cultivars for dissemination to the agricultural community. The discovery of a weed pathogen, however, has more immediate significance as a potential biocontrol tool. And the more economically important the weed, the greater the significance of a biocontrol agent.

In Montana, public enemy number one, at least in plant pathological parlance, is spotted knapweed (Centaurea maculosa). Knapweed, a member of the family Compositae, is a hardy perennial with purple flowers characterized by dark-tipped bracts (Figure 1) (35). Knapweed itself is a plant with low forage value, and is generally ignored by grazing animals. Its rapid spread has been aided by the lack of natural predators in the areas to which it was introduced and by its ability to compete successfully with native grasses (13).

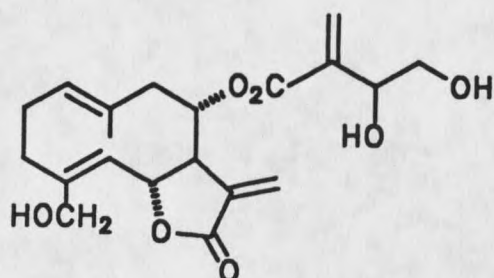


Regina Hughes, from Selected Weeds of the United States, USDA, Ag Handbook 366.

Figure 1. Spotted knapweed: a) habit; b) enlarged leaf; c) flower head; d) disk flower; e) achenes.

Knapweed plants actually produce their early season rosettes in the fall rather than the spring, unlike most plants. This hardy rosette overwinters and revives rapidly with the first spring thaw, a distinct advantage in the competition for space, moisture, and soil nutrients. The production of certain allelochemicals, in particular the sesquiterpene lactone cnicin, 9, has been attributed to various knapweed species (13). The weed is tolerant of

wide fluctuations in temperature and total precipitation, and competes particularly well in dry areas.



9

Knapweed reproduces in typical composite fashion by seed dispersal in late summer and early fall. A single plant can produce 900-1800 seeds per season (36). Mechanical agents such as the wind can disseminate the seeds over fairly large distances (10-20 m). Animals (including humans) may serve as unwitting carriers of the seeds into previously uninfected areas. In a study on diffuse knapweed (*Centaurea diffusa*), a close relative of spotted knapweed, the front of distribution in a particular area advanced 120 m over a three year period (13). The low forage value of this plant, its extreme hardiness, and its ability to replace economically important plants make spotted knapweed an ideal target for attempted biocontrol.

Spotted knapweed poses a significant threat as a weed species, as a result of its extensive incursion into rangelands of northwestern United States and southwestern Canada. It ranks as the number one weed problem on

rangeland in western Montana. Since its introduction to the United States in the early 1900's this hardy composite has invaded millions of acres of prime rangeland with an estimated 70% decline in forage production (36). Records at Montana State University indicate that spotted knapweed was first collected in Ravalli county in the mid-1920's. It has since spread in an alarming fashion (Fig. 2) (35) across the entire western half of the state, with reports of its occurrence in every county in Montana. Spotted knapweed infests over two million acres in this state alone.

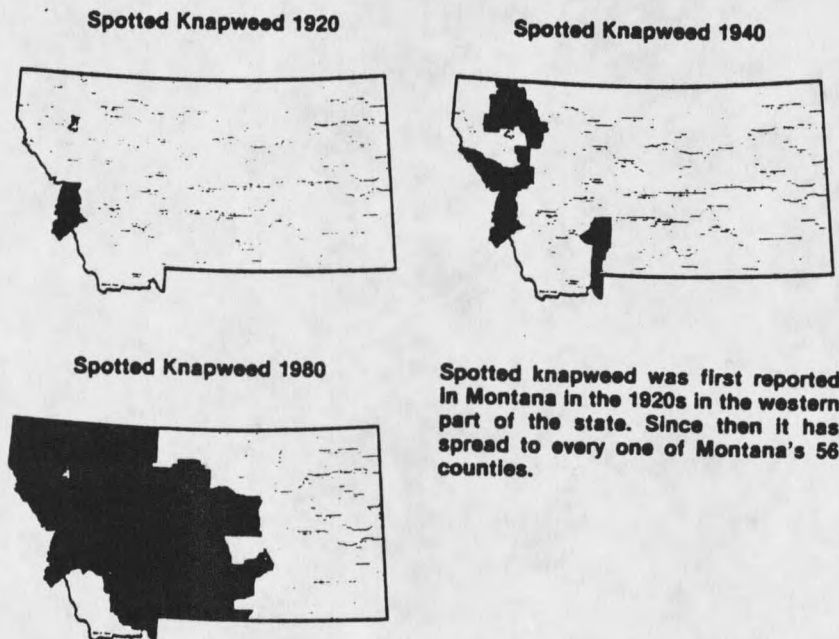


Figure 2. Spread of knapweed in Montana since mid-1920's.

Discovery of a Pathogen

Locating spotted knapweed plants posed no difficulties as the range of this regionally ubiquitous plant includes most of southwestern Montana. Locating a diseased knapweed plant was considerably more challenging. Knapweed is a hardy plant, not particularly susceptible to blights or pathogen induced diseases. The discovery of an obviously diseased plant required several weeks of exploring various regions in southwestern Montana.

A two month search for an appropriately infected plant culminated in the discovery of black leaf blighted Centaurea maculosa in Silver Bow County, Montana, in July, 1984. A seriously compromised individual was found on the northern slope of Big Butte, a five hundred foot cinder cone in Butte, Montana. Approximately thirty percent of the aerial leaves and ninety percent of the flowers were covered either by black fungal growth or by dark brown, weeping lesions. The plant was carefully collected, placed in a sterile plastic bag, and returned to the laboratory for study.

After returning to the laboratory, we endeavored to isolate the disease causal agent in pure culture. Diseased pieces of stems, leaves, and flowers were excised from the plant using sterile technique and placed on DIFCO bacto-agar plates. No additional nutrients were added to the

plates. It was hoped that by limiting the available nutrients to the remnants of the plant itself and bacto-agar we could select for microorganisms capable of utilizing knapweed for food.

Several fungi grew on the plates and great care was taken to isolate the individual organisms as pure cultures. Single spore transfers were used whenever possible to facilitate this process. Once the fungi were established as demonstrably pure cultures, we attempted to determine which, if any, of these isolates were responsible for the induction of black-leaf blight disease of knapweed.

Following the mandate of Köch's Postulates (12), the nature of the pathogen was determined by challenging healthy knapweed plants with pure isolates of the fungi obtained from the lesions. Healthy knapweed plants were nicked with a sterile razor blade on their stems and leaves. Each plant was nicked in ten locations, with no two nicks on the same branch. A piece of mycelia impregnated agar $\sim 27\text{mm}^3$ was applied to eight of the nicked sites on each plant. One plant was not infected with any of the fungi. The plants were allowed to grow undisturbed after they had been inoculated with the fungi, with daily checks to determine if the fungi were actually growing. After five days it was apparent that the fungi were not impacting the plants in any significant fashion. Fungi require fairly humid conditions (90%-95%) for growth and

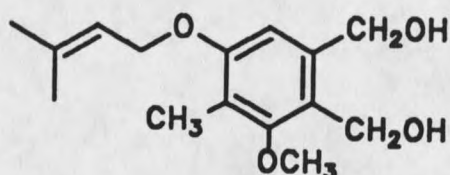
sporulation (16), and the laboratory did not provide adequate moisture for the fungi to develop. The inoculation procedure was repeated with new plants which were encased in sterile, clear plastic bags to facilitate the need for increased humidity. Only one of the fungal isolates induced the formation of weeping necrotic lesions within a five day period. The pathogen was reisolated from the diseased plants and was morphologically identical to the applied fungus. The inoculation procedure was repeated with five healthy plants and in each case the fungus induced the formation of necrotic lesions at the site of inoculation. The organism was identified as Alternaria alternata Lam. by the CBS (Mycological Laboratory), Baarn, Netherlands.

Alternaria sp. are members of the deuteromycetes or imperfect fungi: no sexual stage has yet been isolated for this genus. The chronic asexual condition results in the production of large numbers of spores, the usual infective agents in most fungal plant diseases. Asexual reproduction and spore formation are usually highly repetitive processes, and are instrumental in the development of plant disease epidemics (37).

Alternaria sp. have been isolated as causative organisms in a variety of plant diseases and have been shown to produce a wide variety of phytotoxic compounds. The fungus is capable of elaborating toxins with broad-

spectrum activity and toxins with extremely limited host-range activity. Some of the toxins have only been isolated from single species while others appear to be quite widespread throughout the genus.

Alternaria produce several aromatic phytotoxins. A number of species produce zinniol, 10, an aromatic compound first described as a major phytotoxic metabolite of Alternaria zinniae (38). Of ten species of Alternaria tested for in vitro zinniol production, five were found to produce the toxin (39). The aromatic pentacyclic perylenquinones, which will be discussed in detail in a later section, have also been isolated from this genus.



10

It is not the ability to produce generic phytotoxins that distinguishes Alternaria sp., but rather the ability to produce phytotoxins with a high degree of host specificity. More than any other genus of fungi or bacteria, the Alternaria, and in particular the various form species of A. alternata, have produced host-specific toxins that exhibit remarkable selectivity. As of 1982, seven such toxins (Table 1 and Figure 3) had been isolated

and of these, six have been fully characterized (5). What is remarkable about the host-specific toxins isolated to date is that only one (or a few) cultivars of a given host species are susceptible to either the live pathogen or its toxins; all other cultivars are resistant.

Results

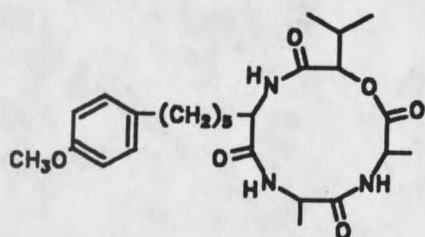
The various form species of A. alternata are credited with the production of a variety of host specific toxins on crop plants. We hoped that this propensity for phytotoxin production by A. alternata could be exploited against spotted knapweed.

In the course of this study we isolated a series of compounds that expressed varying degrees of phytotoxicity to knapweed. The phytotoxins represent three distinct classes of organic compounds: diketopiperazines, perylenequinones, and tetramic acids. Following the isolation and elucidation of the various compounds a detailed bioactivity profile of each class was developed with respect to the degree of phytotoxicity and the degree of host selectivity towards knapweed.

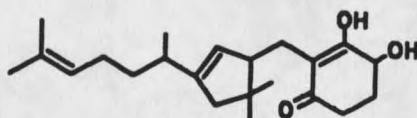
Table 1. Host-specific toxins of *Alternaria alternata*

Pathotype	Disease	Host	Toxin
Apple	Alternaria blotch of apple	apple, pear	AM-toxin 11
Tangerine	Brown spot of citrus	Dancy tangerine, Emperor mandarin	ACT-toxin 12
Pear	Black spot of Japanese pear	Japanese pear cv. Nijisseiki	AK-toxin 13
Strawberry	Black spot of strawberry	strawberry	AF-toxin 14
Tomato	Stem canker	Tomato cv. First, cv. Early pak 7	AL-toxin 15
Lemon	Brown spot of Rough lemon	Rough lemon	ACR-toxin 16
Tobacco	Brown spot of tobacco	Tobacco	AT-toxin *

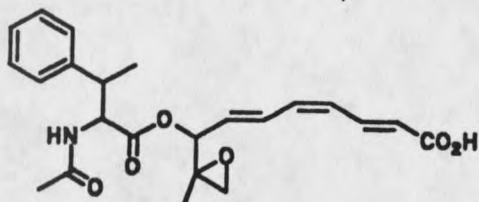
* not characterized.



11



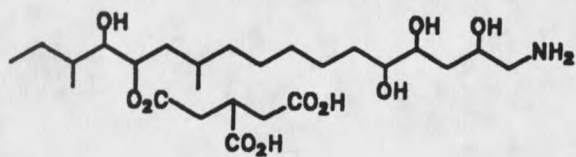
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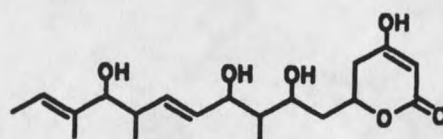
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14



15



16

Figure 3. Structures of host-specific toxins isolated from various form-species of *Alternaria alternata*.

Isolation of Diketopiperazines

A series of diketopiperazines of varying toxicity to knapweed was consistently isolated from 24 day cultures of A. alternata. The EtOAc soluble extract of the culture filtrate was chromatographed on Bio-Beads S-X4 (hexane- CH_2Cl_2 -EtOAc, 4:3:1). This chromatography yielded twelve fractions; only fractions 4, 5, and 6 induced necrotic lesions on knapweed tissues. Size exclusion chromatography of fraction 5 on Sephadex LH-20 (CH_2Cl_2 -MeOtBu-iPrOH, 1:1:1) yielded maculosin, 17. Similar treatment of fraction 6 gave a mixture that was readily resolved by centrifugal counter-current chromatography (CHCl_3 -MeOH- H_2O , 25:34:20, lower phase mobile) into two compounds, 18 and 19. Fraction 4 yielded diketopiperazine 20 upon further size exclusion chromatography on Bio-Beads S-X8 (CH_2Cl_2 -cyclohexane, 3:2).

The CH_2Cl_2 soluble extract of the culture filtrate was applied to a Sephadex LH-20 column (CH_2Cl_2 -MeOH, 1:1). The fourth eluant yielded 21, 22, and 23, using centrifugal countercurrent chromatography as described above. Table 2 outlines the separation techniques used to isolate compounds 17-23, their percent yield relative to the total organic extract, and the molecular weight of each compound.

Table 2. Diketopiperazines isolated, fraction of origin, separation technique, % yield, and molecular weight.

INITIAL FRACTION	CHROMATOGRAPHY TECHNIQUE	COMPOUND	R _t (hr)	%YIELD ^a	MOLECULAR WEIGHT
<u>EtOAc Extract</u>					
E	IH-20 ^b	17	2.10	0.74%	260
F	IH-20 ^b				
	ccc ^c	18	0.38	0.39%	244
		19	0.45	0.44%	244
D	S-X8 ^d	20	1.33	0.23%	224
<u>CH₂Cl₂ Extract</u>					
J	ccc ^c	21	0.70	0.10%	196
I	ccc ^c	22	0.63	0.23%	210
H	ccc ^c	23	0.56	0.08%	168

^a %Yield = weight of compound/total organic weight x 100

^b The solvent system used was CH₂Cl₂-t-butylmethyl ether-isopropanol, 1/1/1.

^c The solvent system used was CH₂Cl₂-cyclohexane, 3/2.

^d The solvent system used CH₂Cl₂-MeOH-H₂O, 25/34/20.

Structure Elucidation of Diketopiperazines

The first compound to be characterized was the most active of the compounds isolated. The molecular formula $C_{14}H_{16}N_2O_3$, which required eight sites of unsaturation, was assigned to compound 17 by high-resolution mass spectrometry. Phenolic and amide functional groups were indicated by infrared absorptions at 3590, 3380, and 1670 cm^{-1} respectively (42). These assignments were supported by a detailed analysis of 1H NMR and mass spectrometry.

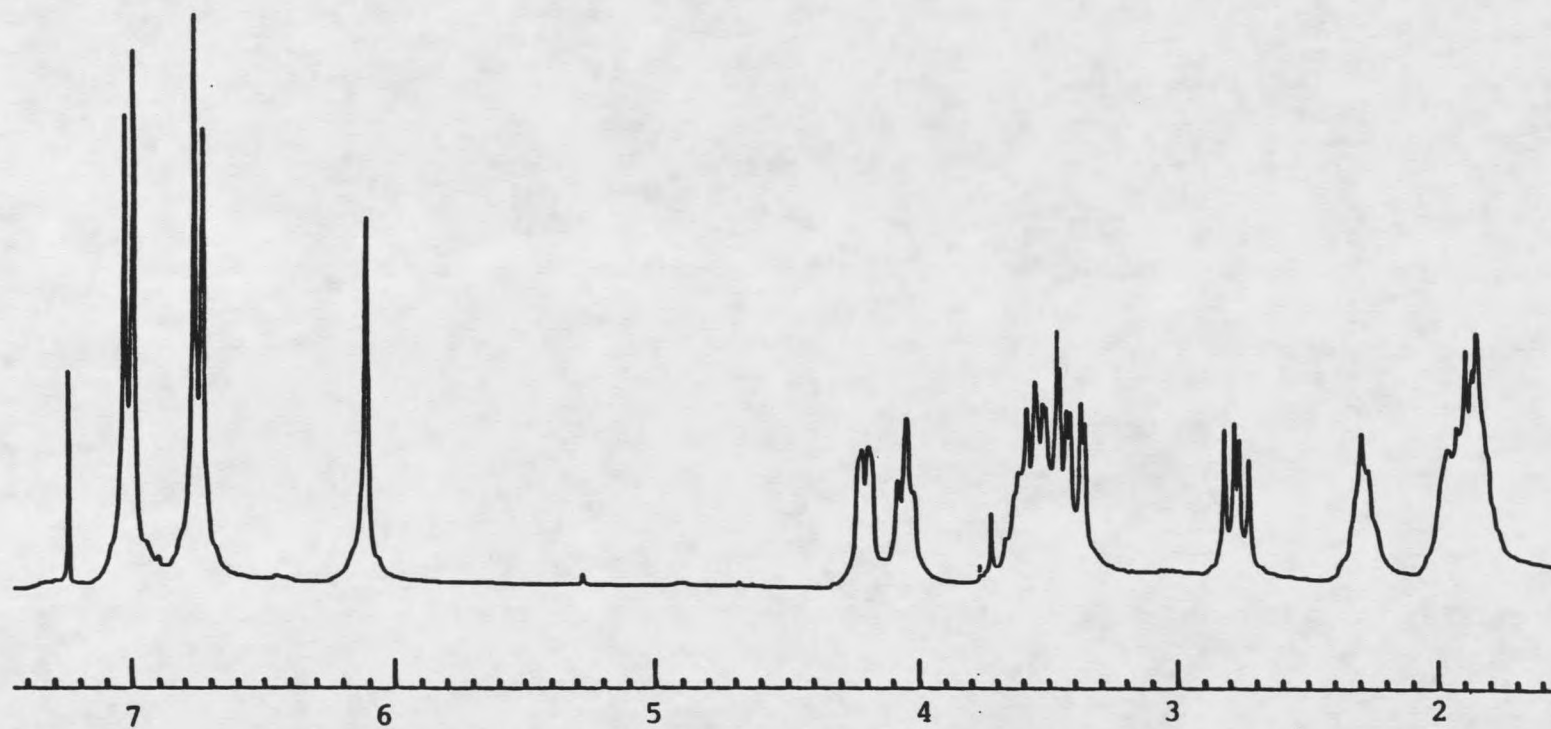
1H NMR analysis (Figure 4) began with examination of the aromatic region of the spectrum. The aromatic doublets at $\delta 6.76$ and $\delta 7.03$ were mutually coupled by 8.2 Hz, indicative of a para disubstituted benzene ring (41). The 0.51 ppm upfield shift of the doublet at $\delta 6.76$, relative to classic benzene absorption of $\delta 7.27$, is typical of aromatic protons adjacent to a hydroxyl group. The 0.24 ppm upfield shift of the companion doublet is typical of the ortho effect of an adjacent alkyl substituent. With the supporting phenolic infrared absorption at 3590 cm^{-1} , the data presented a strong case for the presence of a phenolic moiety.

Mass spectral analysis revealed two major fragments resulting from electron impact: a m/z 107 fragment and a m/z 154 fragment (Figure 5). The m/z 107 fragment is typical of a hydroxytropylium ion, $C_7H_7O^+$. The m/z 154

fragment must be $C_7H_9N_2O_2$, accommodating the four remaining sites of unsaturation. This second fragment must be responsible for the amide absorptions at 3380 and 1670 cm^{-1} in the infrared spectrum.

The coexistence of a hydroxy benzylic moiety and amide functionality together in the same molecule suggested the presence of a tyrosyl derivative. The benzylic methylene should be coupled to a methine with an approximate absorbance of $\delta 3.8$ (41). Extensive decoupling experiments indicated that the methine at $\delta 4.20$ was coupled to two mutually coupled doublets of doublets at $\delta 3.44$ and $\delta 2.76$. These two multiplets displayed typical geminal coupling (15 Hz), indicative of methylene protons, with reasonable benzylic methylene proton chemical shifts. The disparity of their chemical shifts indicated proximity to a chiral center (42).

The region between $\delta 3.00$ and $\delta 4.50$ of the 1H NMR proved to be quite diagnostic in identifying this phytotoxin. It also established an excellent means of identifying other members of the same class of compounds. The chemical shifts of the two methines at $\delta 4.20$ and $\delta 4.07$ were amenable to adjacent oxygen functionalities, although somewhat downfield of protons in typical ether systems ($\delta 3.6$) (43).



36

Figure 4. ^1H NMR Spectrum of Maculosin

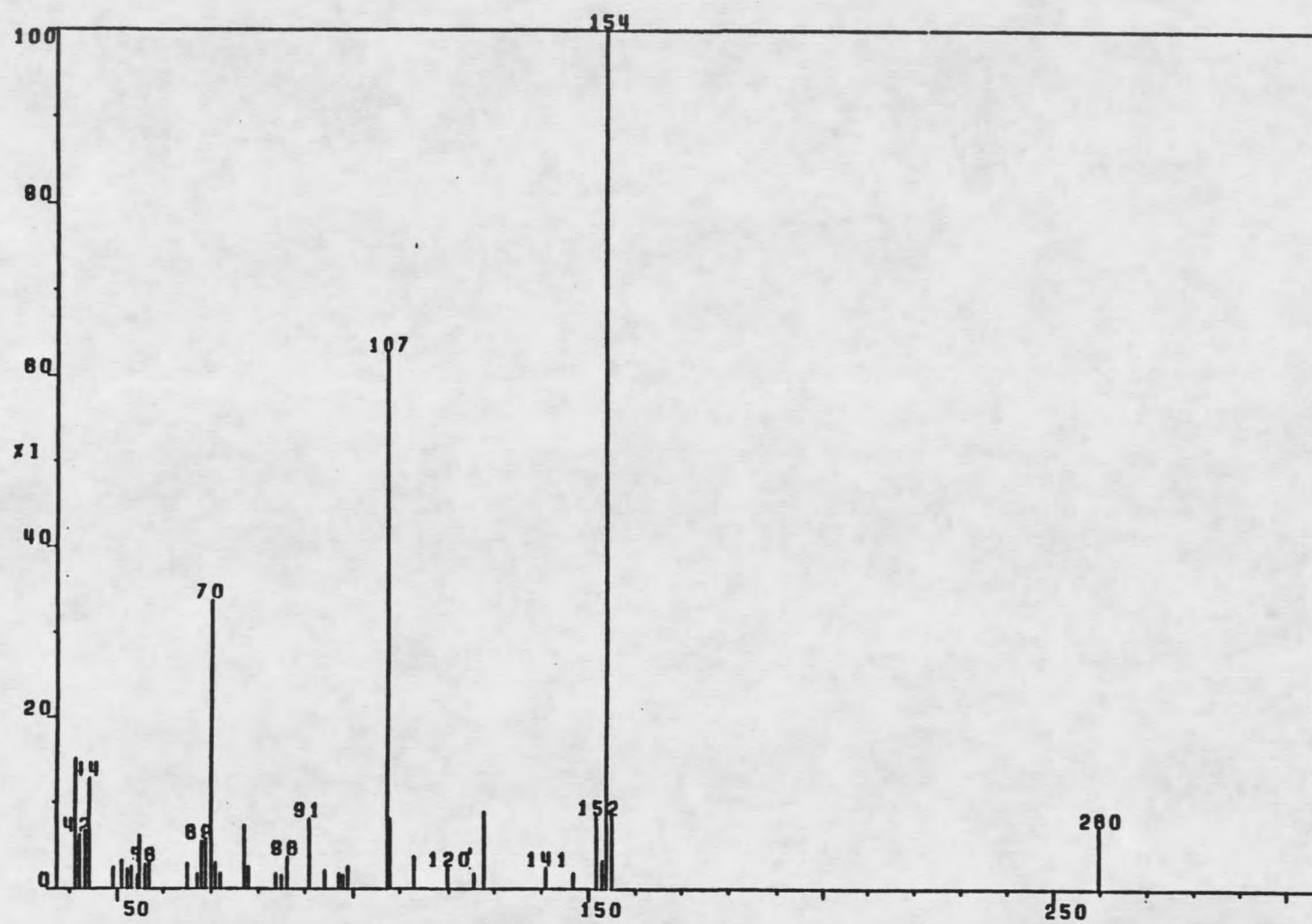


Figure 5. Mass Spectrum of Maculosin

Infrared analysis precluded the presence of ester functionality; therefore it was necessary to invoke two electron-withdrawing groups to explain the chemical shifts of the methine protons. The tentative assignment of the methine at $\delta 4.20$ as part of a tyrosyl moiety concurred with this supposition. At this point, the proposed part structure consisted of a tyrosyl moiety (Figure 6), which accounted for $C_9H_9NO_2$ and five sites of unsaturation. The remaining fragment was C_5H_7NO and accommodated three sites of unsaturation.

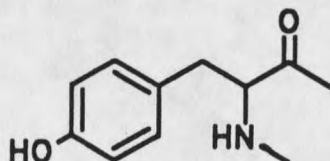
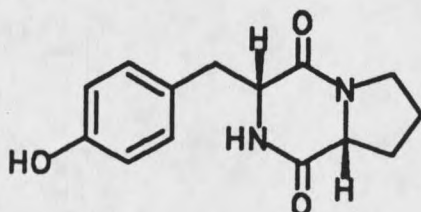


Figure 6. Tyrosyl fragment

The remaining nitrogen and oxygen atoms could represent an additional amide functionality, which suggested a dipeptide. If this were indeed the case then the carbonyl would account for one additional site of unsaturation, and the fragment CON. Two sites of unsaturation must be provided by the remaining C_4H_7 , which, in the absence of olefinic or acetylenic signals, required the presence of two rings. The methine at $\delta 4.07$ also required proximity to both a carbonyl and a nitrogen for

its chemical shift, supporting the presence of an additional amide moiety. Adjacent to the only remaining nitrogen, the methine was also coupled to the multiplets at $\delta 2.31$ (1H, 7.1 Hz) and $\delta 1.93$ (3H, 1.0Hz). The higher field multiplet was also coupled to the two proton multiplet at $\delta 3.57$, a typical absorption for a ring methylene adjacent to a nitrogen. This created a five-membered ring, suggestive of proline. The need for an additional ring and the presence of two amides suggested a diketopiperazine skeleton, which completed the preliminary structural characterization. Compound 17 was designated maculosin in recognition of its phytotoxicity towards Centaurea maculosa.



17

The structure assignment did not address stereochemical determination of the two chiral centers. Although relative stereochemistry can be determined in a variety of ways, synthesis of the compound following a protocol that proceeds without racemization provides the absolute assignment. It also provides more material, which was an

important consideration. We were interested in developing a more complete activity profile of the compound, and additional material would be beneficial. The synthesis of cyclo(L-Pro-L-Tyr) followed the method of Nitecki et al., which is known to proceed without racemization (44). The synthetic and natural products were virtually identical by ^1H NMR, mass spectrometry, optical rotation, and phytotoxic behavior.

Careful inspection of ^1H NMR (Figures 7 and 8) and mass spectrometry suggested that 17 and 18 were closely related. The molecular formula $\text{C}_{14}\text{H}_{16}\text{N}_2\text{O}_2$ was assigned by mass spectrometry to 18, indicating eight sites of unsaturation. Inspection of infrared analysis again indicated the presence of an amide moiety. The ^1H NMR of compound 18 was particularly reminiscent of 17, although the aromatic regions were different: 18 was clearly not a para-disubstituted benzene derivative. The higher field absorbances were particularly coincident. Mass spectral analysis demonstrated that the two compounds differed by a hydroxyl moiety, which is supported by the absence of phenolic stretch in the infrared analysis of 18. Electron impact mass spectrometry of 18 resulted in two major fragments: m/z 153 and m/z 91. The m/z 91 moiety is typically tropylium ion, suggesting that 18 consists of phenylalanine and proline, instead of tyrosine and proline. Repeated decoupling experiments verified this possibility.

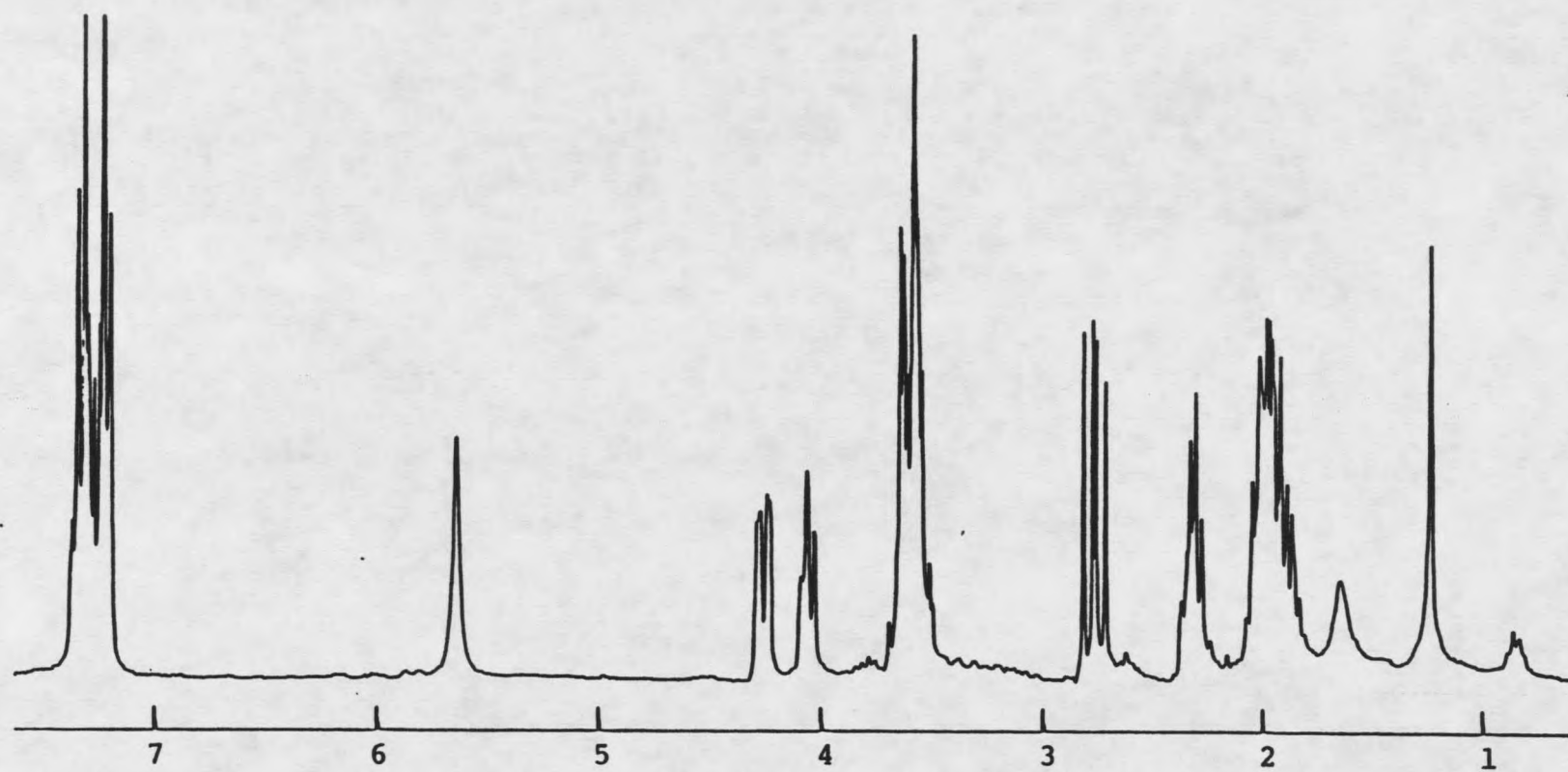
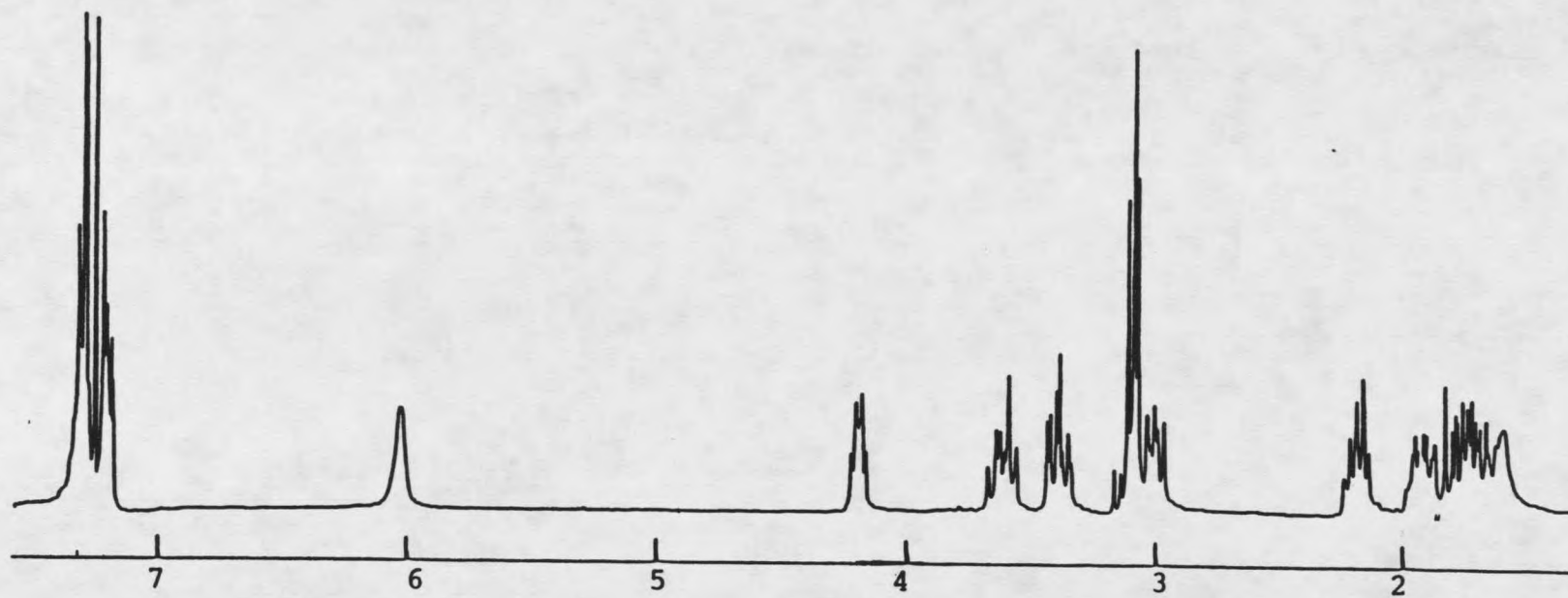


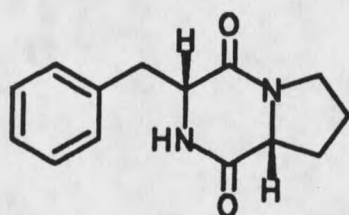
Figure 7. ^1H NMR Spectrum of 18



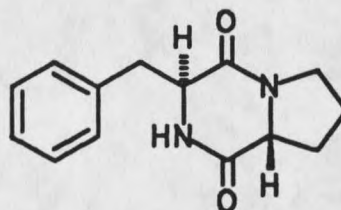
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Figure 8. ^1H NMR Spectrum of 19

The spectral data of 18 and 19 also exhibited striking similarities. Careful inspection of the data indicated that the two compounds were diastereomers; comparison of the ^1H NMR with that of synthetic compounds (45) verified the identity of the two isomers as cyclo(L-Pro-L-Phe), 18, and cyclo(L-Pro-D-Phe), 19. This was the first report of the DL isomer from a natural source.



18



19

Comparison of the spectral data of the first three compounds resulted in the following observations:

1) Electron impact mass spectrometry of each diketopiperazine exhibited a base peak of m/z 153.0 or 154.0, which represented the bicyclic diamido-proline moiety of the compounds.

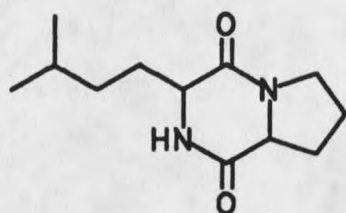
2) The ^1H NMR of each compound exhibited two absorbances between δ 3.80 and δ 4.20, which represented the methine protons adjacent to the carbonyls and α to the amide nitrogen (Table 3).

3) Infrared analysis exhibited characteristic amide absorption ca. 3380 and 1670 cm^{-1} .

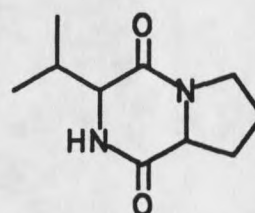
Table 3. Comparison of ^1H NMR data for diketopiperazines, maculosin, 17, (L-Pro-L-Phe), 18, and (L-Pro-D-Phe), 19, listing chemical shifts (δ), multiplicities, and coupling constants (Hz).

	<u>Maculosin</u>	<u>L-Pro-L-Phe</u>	<u>L-Pro-D-Phe</u>
H3	4.20 dd 3.2,10.3	4.20 dd 3.2,10.3	4.20 dd 3.3,10.3
H4	5.89 s	5.70 s	6.20 s
H6	4.07 t 7.1	4.07 t 7.1	3.01 t 7.4
H7b	2.31 m	2.31 m	2.21 m
H7a,8	1.93 m	1.93 m	1.80 m
H9	3.57 m	3.59 m	3.38 m, 3.21 m
H10a	2.76 dd 10.3,14.7	2.76 dd 10.3,15.1	3.08 m
H10b	3.44 dd 3.2,14.7	3.64 dd 3.2,15.1	3.08 m
H12,16	7.03 d 8.2	7.22 m	7.22 m
H13,15	6.76 d 8.2	7.22 m	7.22 m

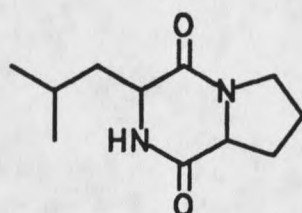
Compounds 20, 21, 22, and 23 exhibited these same properties and were readily characterized by examination of their spectral data. ^1H NMR and mass spectral analysis identified the four compounds as cyclo(Pro-Hleu), 20, cyclo(Pro-Val), 21, cyclo(Pro-Leu), 22, and cyclo(Pro-Ala), 23.



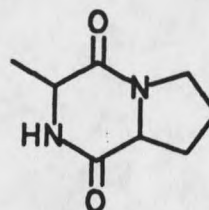
20



21



22



23

Isolation of Perylenequinones 24 - 27

The CH_2Cl_2 soluble extract exhibited moderate phytotoxicity towards knapweed. Size exclusion chromatography on Sephadex LH-20 with CH_2Cl_2 -MeOH, (1:1) yielded four fractions. Fraction 3, which induced necrotic lesions on knapweed leaves but not on johnsongrass at 100 ppm, was permeated through Sephadex LH-20 column using MeOH- CH_3CN , (1:1). The second fraction of this chromatography exhibited phytotoxic behavior but, although an obvious mixture of aromatic metabolites by ^1H NMR, resisted attempts at further separation by size exclusion chromatography. Separation of these closely related compounds proved challenging, but centrifugal

countercurrent chromatography provided baseline resolution of 24 - 27 using a solvent system of CHCl_3 -MeOH- H_2O (25:34:20, lower phase mobile).

We were able to afford baseline resolution of several complex mixtures with great facility through the use of the CCC. Two such mixtures, one containing the diastereomers cyclo(L-Pro-L-Phe) and cyclo(L-Pro-D-Phe) and the other a mixture of structurally similar perylenequinones, were each resolved in less than one hour from time of injection (Figure 9). Separation in the CCC is primarily a partition phenomenon and conformational analysis of these compounds suggested a mechanism responsible for their resolution.

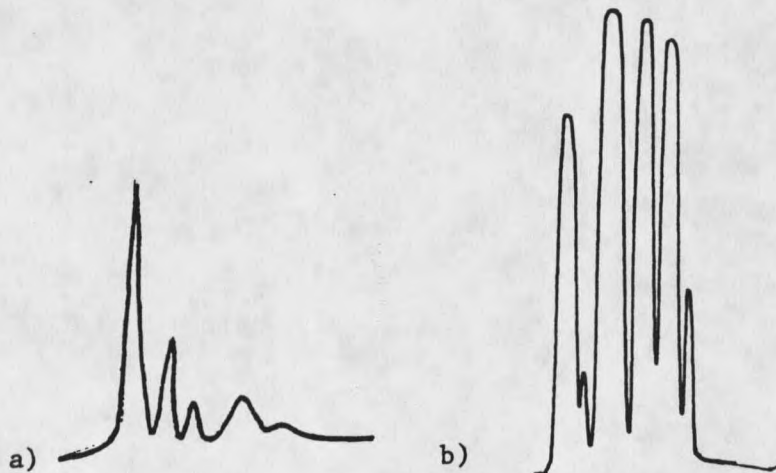


Figure 9. a) CCC trace of separation of diastereomers of cyclo(Pro-Phe); b) CCC trace of separation of perylenequinones.

Diketopiperazines usually assume one of three rotamers: folded, extended towards nitrogen, or extended towards

oxygen. Comparisons of the diastereomers of cyclo(Pro-Phe) by lanthanide-assisted ^{13}C NMR and ^1H NMR, and circular dichroism indicated that they assume different, often solvent related conformations (45, 46). The preferred rotamer for cyclo(L-Pro-L-Phe) in chloroform is extended towards nitrogen, which may be stabilized by induced polarization of the electronegative aromatic ring by the proximity of the electropositive amide hydrogen. Cyclo(L-Pro-D-Phe) assumes a folded conformation in a variety of solvents, including chloroform, consistent with observations derived from calculations of the energy differences of various rotamers (Figure 10) (45, 46).

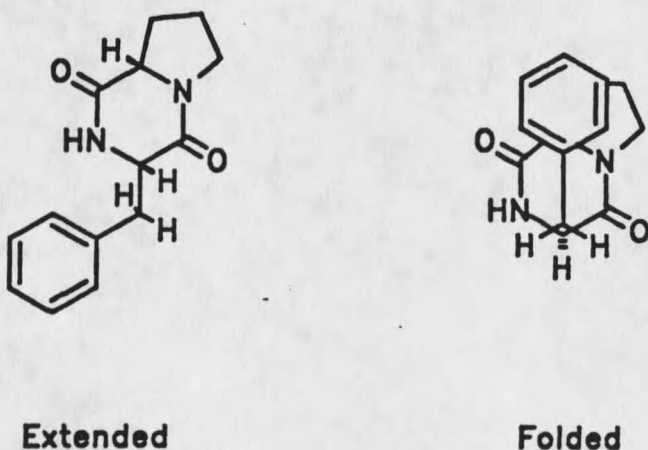


Figure 10. Conformational rotamers of cyclo(Pro-Phe).

These conformational differences are also responsible for the disparity in ^1H NMR that was observed between the two diastereomers of cyclo(Pro-Phe). Under identical conditions, the amide proton of the L-proline moiety of

cyclo(L-Pro-D-Phe) is shifted approximately 1 ppm upfield of the amide proton of the L-proline moiety of its diastereomer. The folded conformation preferred by the LD isomer situates the amide proton in the shielding zone of the aromatic phenylalanine ring, creating this upfield shift.

Facile separation of the four perylenequinones by CCC was based on subtle differences in partition coefficients of the compounds. These differences were the result of enhanced polarity due to variation in the number of polar substituents or in sites of unsaturation.

Structure Elucidation of Perylenequinones

Several apparently related aromatic compounds were isolated from the methylene chloride soluble extract. Compound 24 had a molecular formula of $C_{20}H_{16}O_6$, requiring thirteen sites of unsaturation, based on high-resolution mass spectrometry. A strong infrared absorption near 1645 cm^{-1} indicated several conjugated, carbonyl systems: extended quinone, hydrogen bonded $o\text{-CO-C}_6\text{H}_4\text{-OH}$, or chelated, enolic, β -keto esters (40). A phenolic absorption at 3580 cm^{-1} supported the second possibility, but did not preclude the coexistence of the other functionalities. The ^1H NMR contained four doublets typical of para-disubstituted benzene; the size of the molecule and the large degree of unsaturation suggested a

polyaromatic system. ^1H NMR also suggested some degree of symmetry. Combining data derived from proton decoupling experiments and mass spectrometry with the systems suggested by infrared analysis resulted in one skeletal possibility (Figure 11). Only this system accommodated intramolecular hydrogen bonding between phenolic $-\text{OH}$ and an ortho-carbonyl, as well as an extended quinone system.

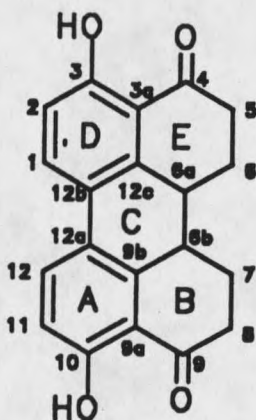
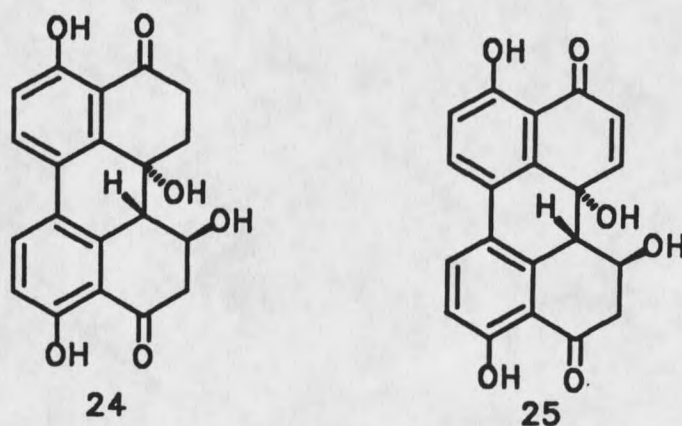


Figure 11. Perylenequinone skeleton of 24.

Fitting the data to this skeleton was straightforward, and resulted in a structure that corresponded to the perylenequinone altertoxin I, reported by Stack et al. (47) as a mutagen (Ames test) from *A. alternata*. Okuno et al. (48) also found this compound in cultures of *A. alternata* and reported it to be phytotoxic to lettuce.

Perylenequinone 25 was assigned a molecular formula of $\text{C}_{20}\text{H}_{14}\text{O}_6$ based on high-resolution mass spectrometry, indicating one more site of unsaturation than in 24. A

comparison of the ^1H NMR spectrum with that of 24 suggested that the two compounds differed by the presence of a double bond in the E ring. Careful examination of the spectral data showed that 25 was identical to alteichin, reported by Robeson et al. as a phytotoxin towards water hyacinth produced by Alternaria eichorniae (49). Okuno et al. also found this compound in an earlier study of A. alternata (48).



Perylenequinone 26, had a molecular weight of m/z 366.0752 which required a molecular formula of $\text{C}_{20}\text{H}_{14}\text{O}_7$ (Figure 12). Inspection of ^1H NMR data (Table 4 and Figure 13) indicated a double bond in the E ring, similar to 25, but with an unusual 1,2 diol system in the B ring. Detailed comparisons of the data collected for this compound, as well as 27, with data available for known perylenequinones suggested that they were both novel

compounds. Mass spectral, infrared and ^1H NMR analyses, however, suggested inclusion in the perylenequinone compound class.

Table 4. Comparison of ^1H NMR data (in CDCl_3) for alterlosin I, 26 and II, 27.

ALTERLOSIN I, 26		ALTERLOSIN II, 27	
H-1,12	7.85, d (8.4)	H-1 ax	2.46, td (13.9, 4)
	7.92, d (8.4)	H-1 eq	3.10, m
H-2,11	7.07, d (8.4)	H-2 ax	3.10, m
	7.12, d (8.4)	H-2 eq	2.67, dt (15.4, 4)
H-5	6.39, d (10.1)	H-5	7.09, dd (8.4, 1.2)
H-6	7.84, d (10.1)	H-6,11	7.82, d (8.4)
H-6b	3.37, d (10.1)		7.84, d (8.4)
H-7	4.48, dd (10.8,10.1)	H-12	7.11, d (8.4)
H-8	4.36, d (10.8)	H-6b	3.18, dd (9.1, 1.2)
		H-7	4.40, dd (9.1, 8.7)
		H-8	4.43, d (8.7)

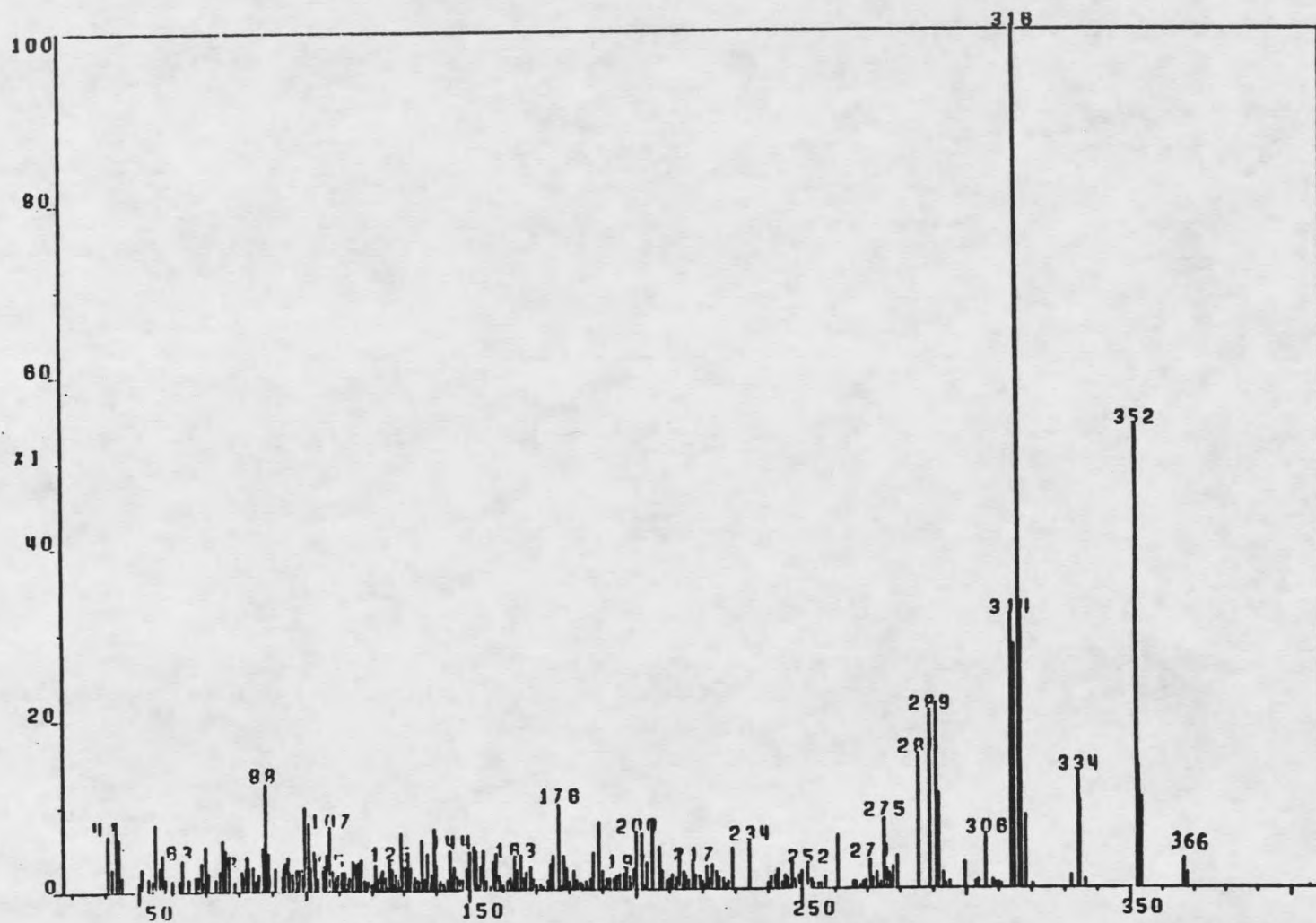


Figure 12. Mass Spectrum of 26

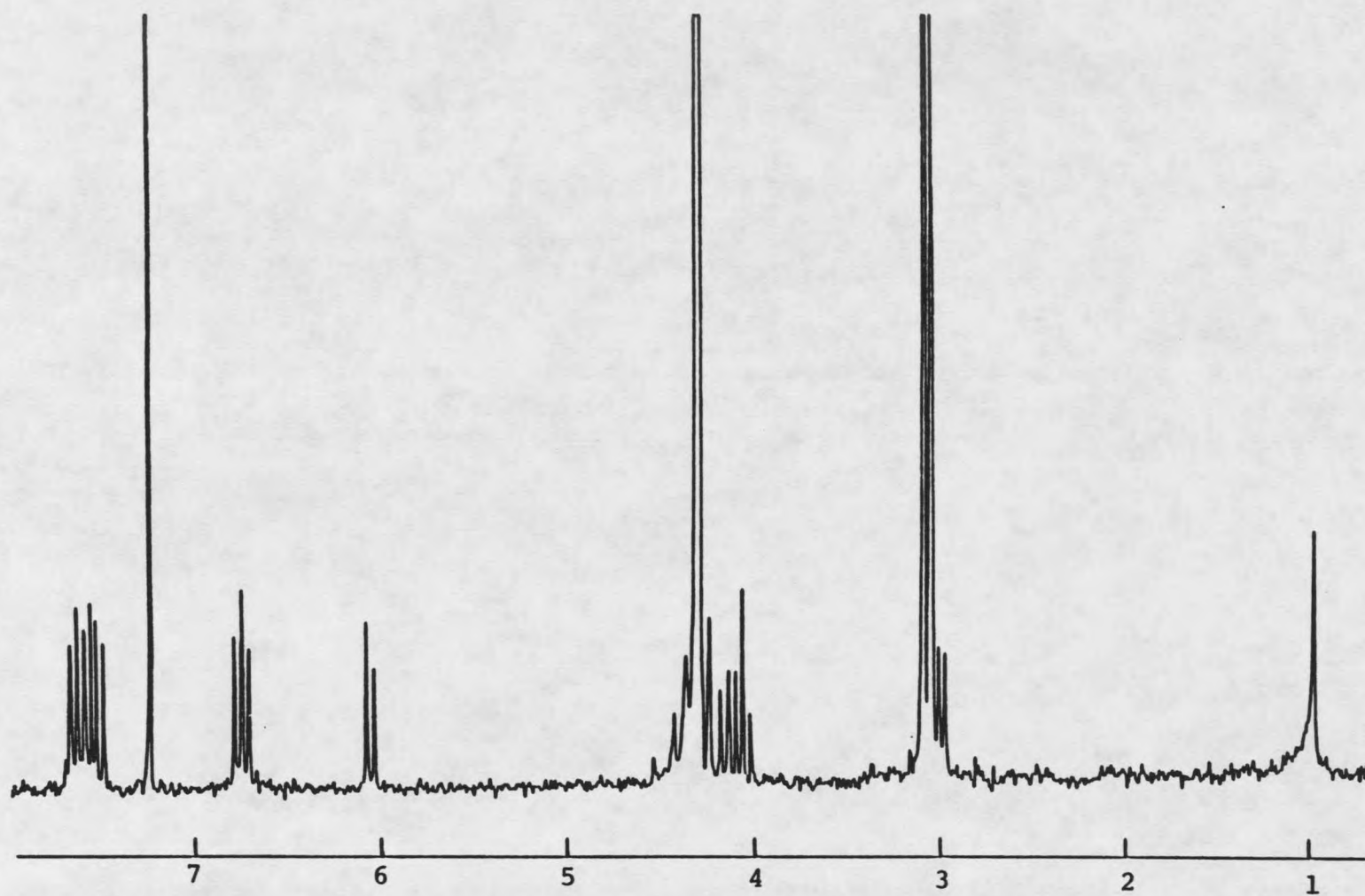
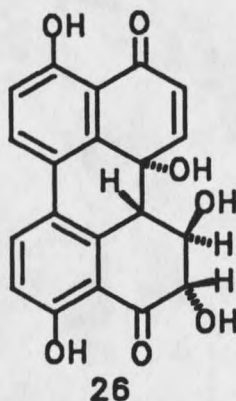


Figure 13. ^1H NMR Spectrum of 26

The molecular formula indicated fourteen sites of unsaturation in 26, reminiscent of 25, with an additional oxygen functionality. Coupling constants associated with the B ring methines indicated that the three protons were mutually trans with typical axial-axial interactions ($J \approx 9\text{hz}$). Difference nOe experiments also supported this axial-axial interaction: irradiation of H-6b induced no enhancement in H-7, but enhanced H-8 by 11%. This was the first report of 26, and the first member of this series with a 1,2 trans diol. When tested for phytotoxicity against knapweed 26 induced the formation of a 1mm necrotic fleck at the inoculation site (10^{-4}M), and was therefore designated alterlosin I.



Perylenequinone 27 represented a distinct departure from the system type established by the other three members of this series. Although high-resolution mass spectrometry determined a molecular formula $\text{C}_{20}\text{H}_{16}\text{O}_7$ (Figure 14) with

fragmentation patterns typical of perylenequinones, spectral anomalies indicated that the structure was clearly not the dihydro-analog of 26. Infrared analysis (liquid film) showed the carbonyl absorption of this molecule to be at a somewhat lower energy than was found in the rest of the series (1630 cm^{-1} compared to 1645 cm^{-1}), which is more typical of an isolated, hydrogen-bonded ortho-OH-C₆H₄-CO than of an extended quinone system (40). Compounds 24-26 were run both in chloroform and neat, as liquid films, and no difference was observed in the carbonyl absorbance at 1645 cm^{-1} . This constancy obviated solvent effects as the cause of variation in infrared absorbance between the first three compounds and 27.

The ultraviolet absorption of 27 appeared at 348 nm, as opposed to the more typical 358-380 nm (47). This hypsochromic shift suggested that the usual extensive conjugation was disrupted. The deviations from typical values observed in both infrared and ultraviolet spectroscopies suggested that 27 represented a departure from the usual parallel array.

Examination of the ¹H NMR (Table 4 and Figure 15) and of data from repeated decoupling experiments indicated that aromatic H-5 and H-6b were coupled to each other by 1.0 Hz. This deviated from the usual data which we observed, as perylenequinones 24-26 did not exhibit this type of coupling.

Examination of ^1H NMR also indicated some interesting second order coupling in the B ring of the molecule. Chemical shifts and decoupling data suggested a 1,2 diol system. Using an ^1H NMR simulation program, NMRCALC, (50) we implemented the coupling constants determined by Karplus correlations of Dreiding models of our proposed structure, and the chemical shifts indicated by ^1H NMR for the methines involved in the 1,2 diol. The program created an excellent facsimile of the actual spectrum, which supported our structure for ring B. The coupling constants indicated by Karplus correlations were in excellent accord with measurable values. The dihedral angle between H-6b and H-7 was 175° , and generated a coupling constant of 9.1 Hz, certainly supportive of axial-axial interaction. The dihedral angle between H-7 and H-8 was 170° , also indicative of vicinal diaxial protons (Figure 16).

Difference nOe experiments were important in defining the structure and relative stereochemistry of 27. Irradiation of OH proton at $\delta 3.76$ induced a 7% enhancement of H-6b as well as a 6% enhancement of H-2 ax. Irradiation of H-6b did not enhance H-6 axial, a striking anomaly when compared to the enhancements induced in the similar difference nOe experiments with 24 and 25 (20%). The lack of an nOe response generally receives scant attention, as negative results indicate either the absence of interaction or the failure of the experiment. In this case, however,

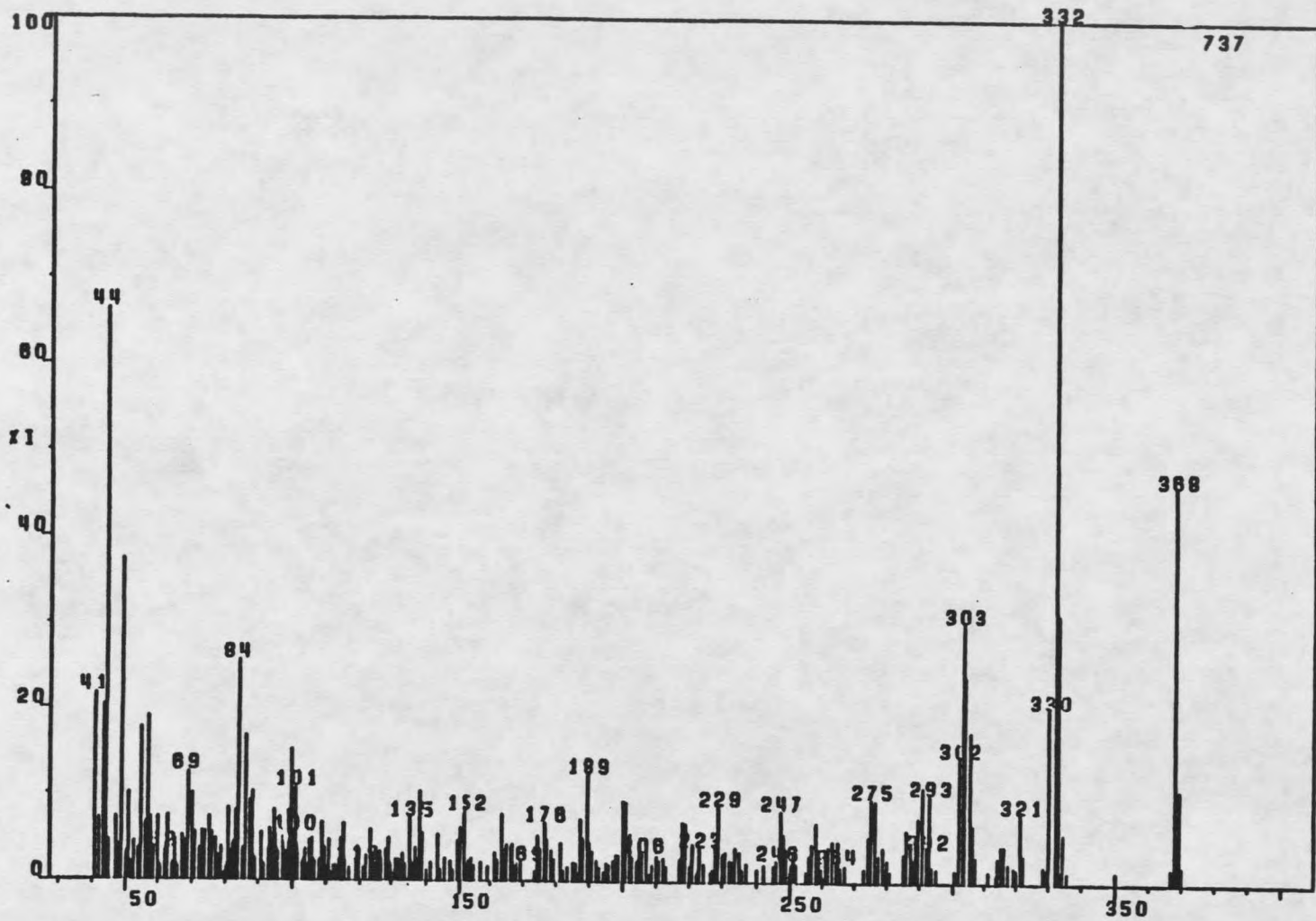
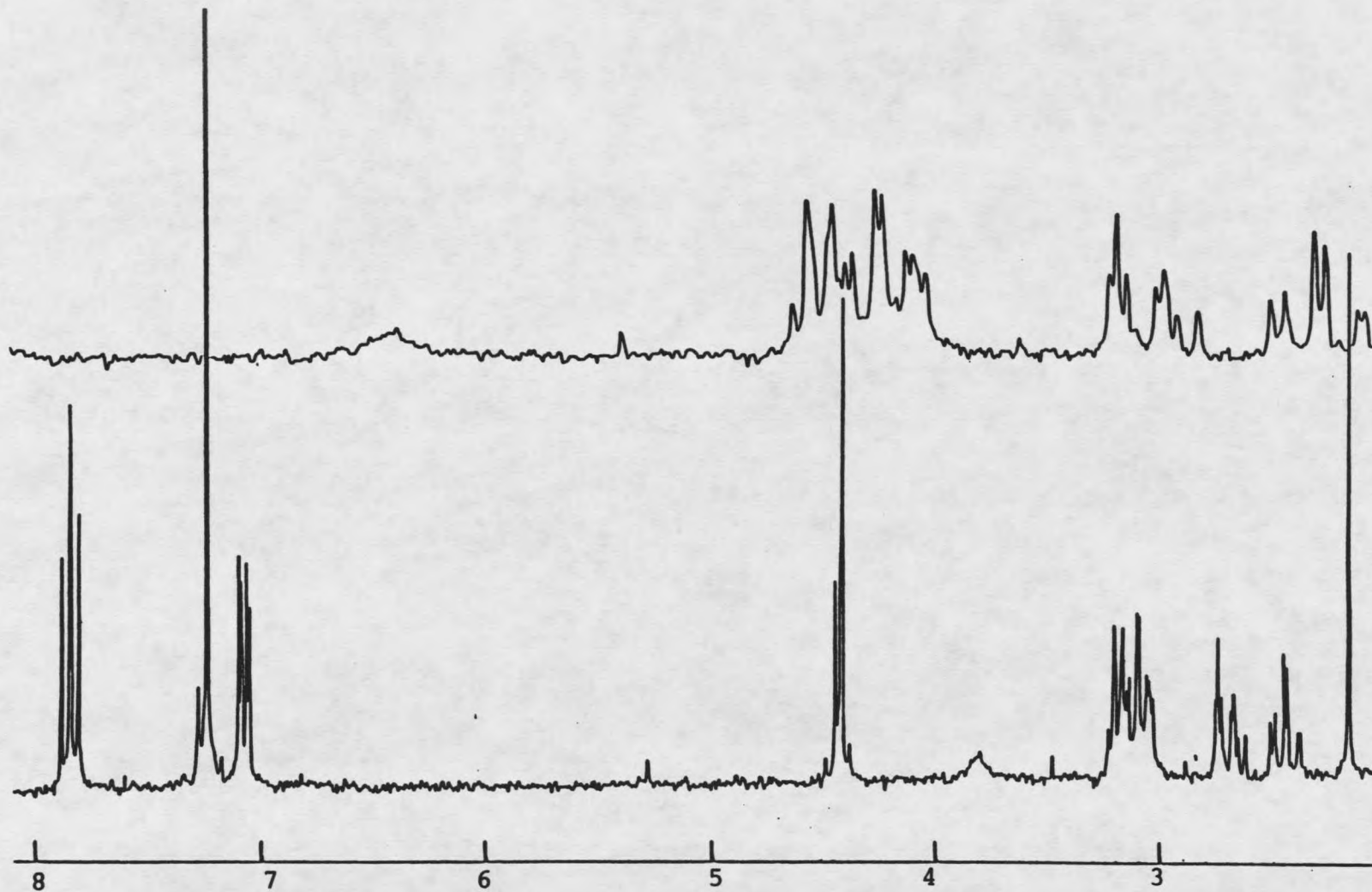


Figure 14. Mass Spectrum of 27



58

Figure 15. ^1H NMR Spectrum of 27

the lack of nOe enhancement between H-6 axial and H-6b suggested a deviation from the normal parallel skeleton, a possibility that required further examination and corroboration.

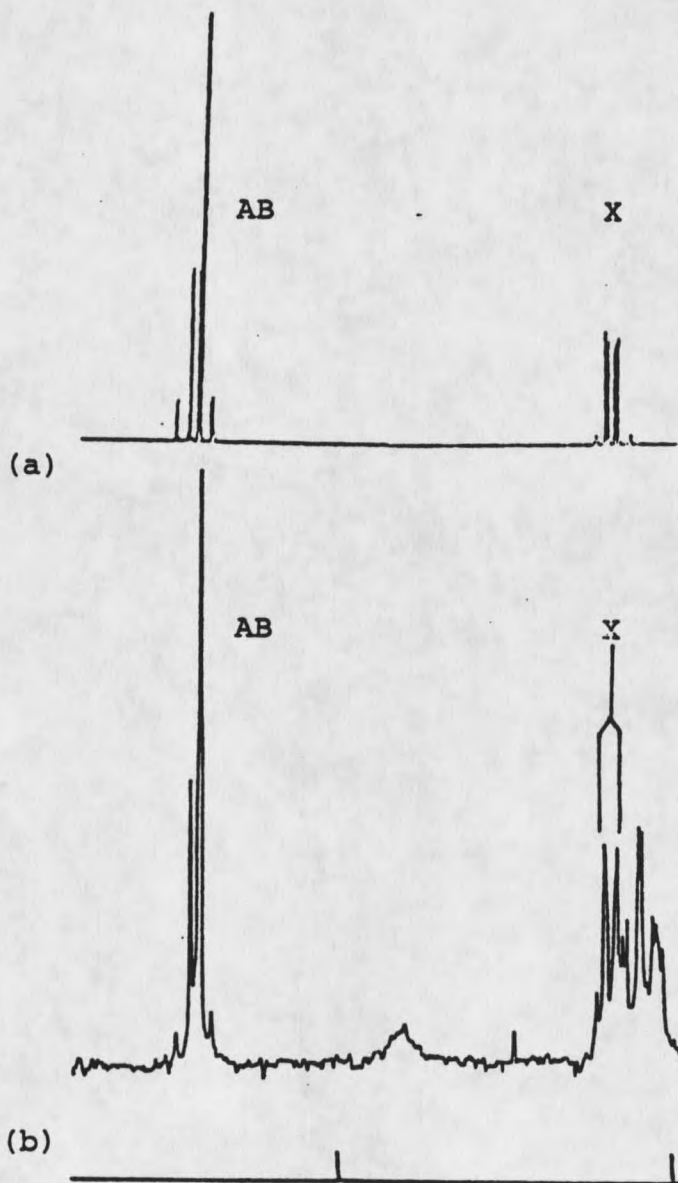
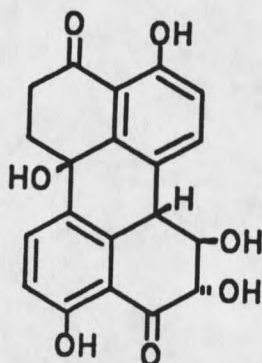


Figure 16. a) NMR simulation of ABX system using NMRCALC; b) Actual ^1H NMR spectrum.

At this point we had several pieces of data that suggested that the structure of 27 represented a distinct departure from the normal parallel skeleton of the perylenequinones: 1) shift to longer wavelenghts in infrared absorbance; 2) hypsochromic shift in ultraviolet absorbance; 3) deviation from typical nOe enhancements; 4) atypical coupling between H-6b and aromatic proton. These spectral anomalies lead to the proposal of a diagonal array for 27. This structure would include the necessary disruption of conjugation to create the spectral anomalies observed.



27

Variations in infrared and ultraviolet absorbances are unfortunately not always given as much credibility as they perhaps deserve. A new structure cannot be proven based on these deviations or on negative nOe data. If the absorbance anomalies were dismissed as trivial, the nOe data could actually be ascribed to two different

