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Chemical investigations of endophytitc and fungicolous fungi

Annalisa Marie Jordan
University of Iowa

CHEMICAL INVESTIGATIONS OF ENDOPHYTIC AND FUNGICOLOUS FUNGI

by

Annalisa Marie Jordan

An Abstract

Of a thesis submitted in partial fulfillment of the
requirements for the Doctor of
Philosophy degree in Chemistry in
the Graduate College of
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May 2012

Thesis Supervisor: Professor James B. Gloer

ABSTRACT

Fungal infections of crops and humans are widespread, and the costs associated with their prevention and treatment highlight a need for new antifungal agents. In addition, increased antimicrobial resistance enhances the need for new classes of antibiotics. Fortunately, fungi themselves are a rich source of bioactive secondary metabolites, including some clinically useful antifungal agents.

The role of fungi as decomposers sometimes results in these organisms being parasitic, mutualistic, or commensal with other organisms in their ecological communities. Such interspecies relationships are commonly associated with the production of biologically active compounds. The research described in this thesis involves studies of fungi from different ecological niches as part of an ongoing search for new bioactive natural products.

Chapters 2–4 in this thesis describe investigations of secondary metabolites produced by selected fungal endophytes of corn. Endophytic fungi are those that grow in association with a plant host. Our group has previously reported the occurrence of antifungal pyrrocidines and phytotoxic dihydroresorcylide analogues from the maize–protective endophyte *Acremonium zeae*. The further studies discussed in this thesis describe bioactive secondary metabolites produced by isolates of three other endophytic fungal species. While

no new natural products were discovered through these studies, antifungal and antiinsectan activities of the known metabolites encountered were determined for the first time. In addition, LCMS methods were developed for two of these endophytes in efforts to detect fungal metabolites in infected plant tissues as a key step towards exploring the protective and/or infectious roles these fungi and their metabolites may have in their plant hosts.

The second group of studies presented focus on the secondary metabolites produced by three Hawaiian fungicolous fungal isolates. Previous studies of such fungi in our group have resulted in the identification of many new antifungal metabolites. The work presented here describes five new antifungal and/or antiinsectan natural products, as well as nine previously reported metabolites and a new compound generated during the isolation process.

A variety of chromatographic techniques were used to isolate the compounds described in these studies, with the final step in most cases being HPLC. Structure elucidation of metabolites was accomplished primarily through analysis of 1D– and 2D–NMR and HRMS data.

Abstract Approved: _____

Thesis Supervisor

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Title and Department

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Date

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Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Annalisa Marie Jordan

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Chemistry at the May 2012 graduation.

Thesis Committee:

James B. Gloer, Thesis Supervisor

Daniel M. Quinn

Gregory K. Friestad

Claudio J. Margulis

Jonathan A. Doorn

Para mi tía, Heidi
This one is for the both of us.

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To say that I am grateful for the support of my family would be an understatement. Few people are blessed to have as supportive a family as mine. Their encouragement gave me the courage to try anything that would make me happy, be that the performing arts, softball, or chemistry and teaching. Mom and Dad, you have supported me through *every* possible situation. You listened when I needed and advised when necessary. This has strengthened my character and I would not have completed this degree without you. Though you were not in Iowa, your support has given me more strength than you will ever know. A special thank you goes to my mom for being my conference travel buddy and for helping to edit this thesis. I treasure our trips and feel lucky that you would want to participate in the chemistry side of my life. Hermana Nici, you are the most creative person I know and that you for helping me to develop creatively as well. This is an essential aspect of my job and I know that I am a better teach because of you. Thank you also for being my best friend growing up and (sort of) putting up with me copying everything you did. At least I grew out of that phase and found chemistry. Most importantly, thank you for keeping me grounded by being an absolute nut about living life to its fullest.

The hardest thing I did 7 years ago was leave my family so I could continue down a path that would lead me to a career of my dreams. In that time I lost two uncles who have supported me in ways I may never know. Uncle Randy and Uncle Kevin, I miss you every day and wish I hadn't missed out on those last few years while in graduate school. Just know that your passion for life, the outdoors, and ridiculous fun are alive in me and is being passed on to my students. To all my family, Grandma and Grandpa Johnson, Joyce, Craig, Kari, Sam, Sophie, Scott, Susie, Brian, LaVonne, Amanda, Abby, Allie, Emily, Jon, Heidi, Perry, Shelly, Ella, and Pierce: I love you all and can't express how happy I am to be back home so that I can spend my weekends with you again around a bonfire or at the farm. I'll see you at Dairy Queen and the waterslide!

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When I left Minnesota, I did not think I would find my own family in Iowa. Chuck, you are my best friend and I love you with my whole heart. You make me laugh and challenge me intellectually. You have been with me every step of the way in graduate school and some of my fondest memories are from our trip to Europe. Being with you in Iowa City has been an amazing adventure and I look forward to our future in Minnesota. I asked a lot of you in this drawn out process and now I'm done! Thank you for sticking it out, sweetheart.

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LIST OF ABBREVIATIONS

Akt	protein kinase B, PKB
APCI	atmospheric pressure chemical ionization
ARS	Agricultural Research Service
CBS	Centraalbureau voor Schimmelcultures
cz	clear zone
DEPT	Distrotionless enhancement by polarization transfer
dpi	days post–inoculation
DPYA	dextrose–peptone–yeast extract agar
EtOAc	ethyl acetate
EI	electron impact (ionization)
ESI	electrospray ionization
GenBank	National Center for Biotechnology Information
GGPP	geranylgeranyl–pyrophosphate
GI_{50}	growth inhibition of 50%
HIF–1	hypoxia–inducible factor 1
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum correlation
HPLC	high performance liquid chromatography
Hsp90	heat shock protein 90

ITS	internal transcribed spacer region
LC–HRESITOFMS	liquid chromatography–high resolution electrospray ionization time-of-flight mass spectrometry
LC–HRAPCITOFMS	liquid chromatography–high resolution atmospheric pressure chemical ionization time-of-flight mass spectrometry
MeCN	acetonitrile
MeOH	methanol
MIC	minimum inhibitory concentration
mz	mottled zone
NCAUR	National Center for Agricultural Utilization Research
NMR	nuclear magnetic resonance
NRRL	USDA Agricultural Research Service Culture Collection/Northern Regional Research Laboratory
PAF	platelet activating factor
PI3K	phosphatidylinositol 3–kinase
PDA	potato dextrose agar
PDB	potato dextrose broth
PMA	phosphomolybdic acid
rg	reduced growth
RG	receiver gain
TFA	trifluoroacetic acid

TIC	total ion current
USDA	United States Department of Agriculture
UV	ultraviolet
YMG	yeast–malt–glucose

CHAPTER 1

INTRODUCTION

Combating fungal disease continues to be a concern in medicine and agriculture. In medicine, the number of patients diagnosed with fungal infections has increased significantly in the past two decades.[1] A large number of these cases represent immunocompromised patients, making treatment extremely difficult and contributing to higher mortality rates.[1-8] Even though there are numerous antifungal compounds currently used for treating fungal infections (e.g., nystatin, amphotericin B, fluconazole, and caspofungin), there is a need for new antifungal therapies[1]. This is due in part to an increase in drug resistance among fungal pathogens.[1,2,8] There are even fewer antifungal treatments available for immunocompromised patients due to dose–limiting side-effects, and these treatments have shown effectiveness only against a limited range of fungi.[1,3,6]

There is also a need for new fungicides in agriculture. Fungal pathogens infect and/or contaminate a wide variety of grains and other crops both pre-harvest and during the storage process.[9-13] These pathogens attack and/or colonize commonly consumed human and animal food products, such as peanuts, coffee beans, corn, and wheat.[9-16] Crops that have been damaged or tainted by fungi generate large economic losses in agriculture, destroying crops

and/or causing disease in both humans and livestock that consume the contaminated products.[14-17]

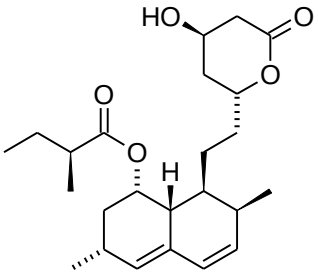
A few of the most commonly encountered fungal pathogens in medicine and agriculture are *Aspergillus* and *Fusarium* spp.[1, 5-7,10-13,15-17] These fungi are known to produce mycotoxins (secondary metabolites that are toxic to some degree to mammals). Such compounds have been linked to many different health problems, and individual agents are known to be carcinogenic, immunotoxic, mutagenic, nephrotoxic, neurotoxic, and teratogenic.[18-24] Aflatoxins and fumonisins are mycotoxins produced by *Aspergillus* and *Fusarium* spp., respectively.[16,18,25-27] Other examples of commonly encountered mycotoxins are ochratoxins and trichothecenes.[28-34] Mycotoxin-producing fungi not only contaminate crops in the field, but the toxins they generate have been detected at appreciable levels in harvested products.[9-11,13,14,16,26,35] These toxins are linked to toxicosis in sheep, cattle, and horses that consume the feed products.[10,14,16,18,22,24] Fungal toxicosis has also caused global health problems, such as human primary liver cancer in Africa and southeast Asia, ergotism in Europe, alimentary toxic aleukia in Russia, and acute aflatoxicoses in south and east Asia.[18]

Approximately 20 mycotoxins are currently monitored for their occurrence in food stuffs such as cereal grains, oil seeds, and tree nuts.[18] Unfortunately, mycotoxins do not readily degrade through digestion, cooking, or

freezing.[9,17] Therefore, the only way to prevent mycotoxicosis is to prevent the growth of mycotoxin-producing fungi. Fungal pathogens have a worldwide catastrophic impact in agriculture and medicine. The effort to combat fungal infections through the discovery of new antifungal agents is an ongoing and difficult process.

Plants, bacteria, and fungi are all examples of organisms being investigated as sources for new antifungal compounds. Of these, fungi are the most biologically diverse and abundant. It is estimated that there are over 1.5 million different species of fungi on earth.[36] In total, only about 5% of these have been characterized by mycologists, and a only subset of these have been investigated for the chemistry they produce.[36,37] Fungi serve an important role as decomposers in the nutrient cycles of a variety of ecosystems. As a result, fungi are found in many different environments, including aquatic and terrestrial ecosystems, and commonly live in association with and/or encounter other organisms such as plants, cyanobacteria, algae, insects, worms, and other fungi.[19,22,35,37-47] Fungal species are not limited to one fungal niche, but may grow more abundantly in certain areas. The diversity of fungal habitats likely contributes to the wide variety of biologically active secondary metabolites produced by fungi.

Fungi are in constant competition for nutrients and survival with other organisms within their communities. It is thought that fungi have evolved the ability to produce antimicrobial agents as a form of defense and/or offense in such competitive environments. More specifically, fungi sometimes produce antifungal compounds that can prevent the growth of other, competing fungal species.[37] Some of the antimicrobial compounds produced by fungi now serve as vital medicinal agents. Examples are the statins (*e.g.*, lovastatin; **1**), which are a multibillion dollar class of compounds used as cholesterol-lowering agents.[48]



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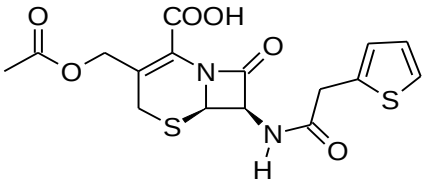

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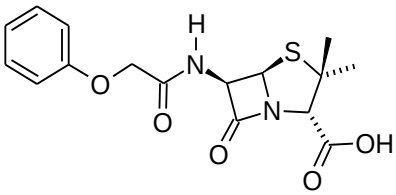

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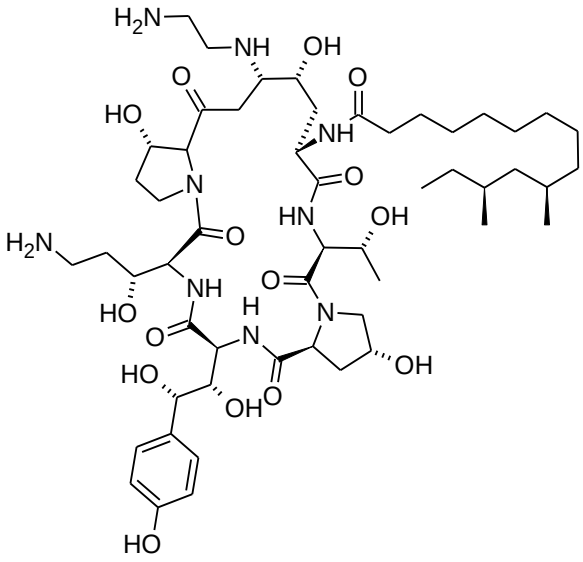
Cephalosporins (*e.g.,* cefalotin; **2**) and penicillins (*e.g.*, phenoxymethylpenicillin; **3**) are classic examples of important antibacterial agents produced by fungi.[49-51] Fungi are also known to produce a wide variety of antifungal metabolites. Cancidas (**4**) is one such antifungal agent that is now in clinical use.[52] Because of their abundance, species diversity, relatively simple culturing conditions, and ability to produce many types of antimicrobial secondary metabolites, fungi comprise an exceptional source for the prospective discovery of new antifungal agents.



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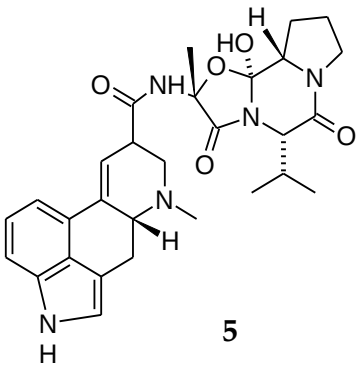
When considering what types of fungi to investigate as sources for new antimicrobial agents, fungal endophytes are strong candidates. Endophytic

fungi inhabit plant tissues for all or part of the plant's life cycle; causing no apparent disease.[20,39,53-55] All plant species are known to harbor fungal endophytes in their natural environments.[36,56-58] A delicate balance of fungal virulence and plant defense exists within the fungal endophyte–plant host relationship. However, in most cases it is unclear whether the endophyte is acting as a mutualist, a phytopathogen, or neither.[20, 59] Because of their biodiversity and unique relationships with plants, there has been much focus on fungal endophytes and their metabolites over the past few decades. This has resulted in the discovery of many new bioactive natural products.[19-21,39,60-67]

In certain cases, it has been demonstrated that endophytes have the ability to protect their host plants against environmental stresses, whether abiotic (*e.g.*, drought and extreme temperatures) or biotic (*e.g.*, insect or mammal herbivory, or microbial pathogens).[54,57,58,67-73] For example, *Dichanthelium lanuginosum* plants inhabited by a *Curvularia* sp. showed enhanced thermotolerance when exposed to constant soil temperatures of 50°C for 3 days and intermittent temperatures of 65°C for 10 days. By contrast, endophyte-free plants became shriveled and chlorotic at 50°C.[68] Similar results were observed for tomato and watermelon plants colonized by certain fungal endophytes. In another important crop plant, wheat, enhanced thermotolerance was not observed with endophyte colonization. However, endophyte-infected wheat was able to withstand 18 days

of drought compared to uninfected controls, which only withstood 10 days of drought.[69]

As noted above, some fungal endophytes are also known to produce compounds that deter both mammalian and insect herbivory, as well as produce antimicrobials that defend against pathogens.[21,22,57,60,63,74,75] As decomposers, endophytic fungi must obtain nutrients from their plant hosts. Plants produce these nutrients through photosynthesis and subsequent metabolic processes. The fungus can then use these nutrients to biosynthesize antimicrobial agents that might serve to defend against other organisms in the ecosystem. Bioactivities proposed for such systems include antiinsectan, antiherbivory, herbicidal, antiviral, antialgal, antibacterial, and antifungal effects.[57,58,70-73] Some examples of these biologically active compounds include ergot (*e.g.*, ergovaline; **5**) and loline (*e.g.*, *N*–formylloline; **6**) alkaloids known to display antiherbivory activity.[22,39,57,76]



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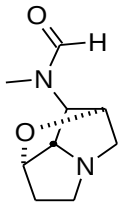

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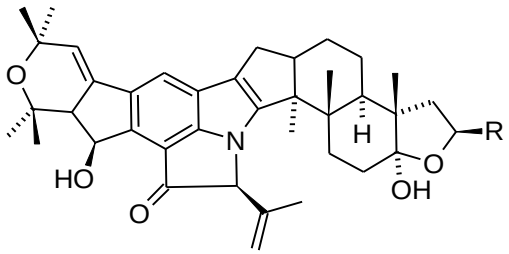
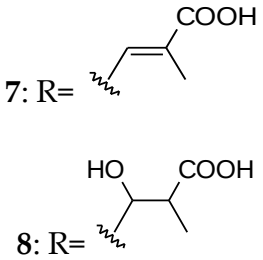
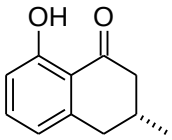
Nodulisporic acids A_1 (**7**) and A_2 (**8**) are potent insecticides,[19] and mellein (**9**) displays a broad spectrum of antimicrobial activity as phytotoxicity.[19] Nitronaphthalene (**10**)[66] and cerebrosides (**11**)[77] are examples of antibacterial endophytic fungal metabolites, and brefeldin A (**12**) is an antiviral and antifungal agent.[19]







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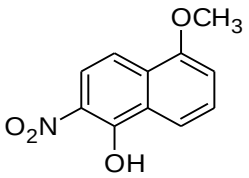

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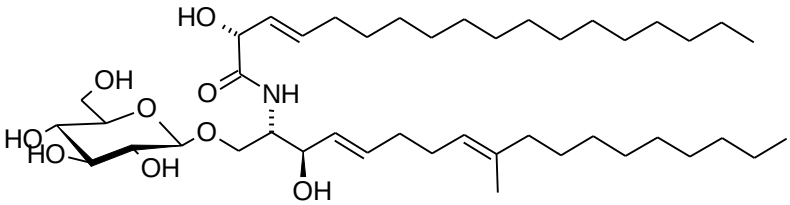

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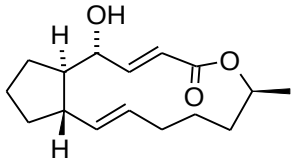

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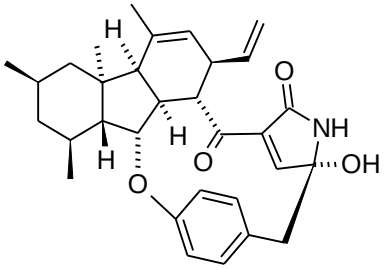
A recent review of endophytic fungal natural products reveals that a considerable number of these metabolites display antifungal activity.[19] Pyrrocidines A (**13**) and B (**14**) and preussomerins (*e.g.,* preussomerin EG_1; **15**) are just a few examples of new antifungal metabolites from endophytic fungi.[60,63,78,79]



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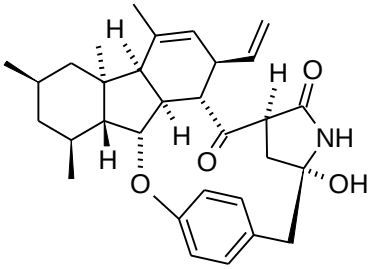

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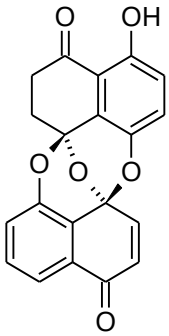

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Though a variety of antimicrobial agents have been reported from endophytic fungi, very little research has been conducted to support the claims that certain endophytic fungal metabolites contribute to host plant defenses. One of the most thoroughly studied examples of a fungal endophyte providing enhanced defense for a plant host is that of *Neotyphodium* spp. deterring

herbivory in tall fescue grasses.[21,22,57,74,76] These grasses were first studied because of widespread illness observed in animals consuming the grass. It was later determined that an endophytic *Neotyphodium* sp. and its metabolites were the source of toxicosis among livestock that had grazed the *Neotyphodium*–infected grasses. Consumption of the infected plant material resulted in weight reduction, difficulty reproducing, and a decrease in milk production.[21,76] While the presence of *Neotyphodium* spp. was undesirable for grazing mammals, the production of fungal metabolites that deter feeding in both mammals and insects, is presumed to benefit both the endophytic fungus and its plant host.[22,76]

Studies from a number of different research groups have worked to identify, detect, and quantify the *Neotiphodium* metabolites responsible for the antiherbivory effects displayed by *Neotiphodium*–infected plants.[21,22,57] Ergot and pyrrolizidine alkaloids are two classes of fungal metabolites produced by *Neotiphodium* spp. that are thought to contribute to decreased herbivory of grasses, with ergovaline (**5**) and *N*–formylloline (**6**) displaying the most potent antiherbivory effects. Ergovaline (**5**) deters grass grazing by mammals and other vertebrates and *N*–formylloline (**6**) deters insect herbivory.[22,76] Detection of these metabolites in infected plant tissues was accomplished through a series of cultivation, separation, chromatographic, and spectroscopic techniques that have been improved over time. For example, *Neotyphodium*–infected grasses, plants,

and/or seeds were ground and then sequentially extracted with hexanes and a 1:1 mixture of EtOH and water. The ethanolic extract was then subjected to cation exchange chromatography, resulting in a mixture containing gram–quantities of compound **6**.[21,22] Hydrochloric acid was added to this mixture to precipitate the amine salt of *N*–formylloline (**6**).[21] Wide–bore gas chromatography was used to detect and quantify compounds **5** and **6** from organic extracts of *Neotyphodium*–infected grasses.[21,22] Ergovaline (**5**) has been reported at concentrations of approximately 5 mg per kg of plant tissue (dry weight), while loline alkaloids have been detected at levels as high as 10 g per kg of dry plant matter.[22] Biosynthetic studies have helped to identify fungal gene clusters responsible for *N*–formylloline synthesis and resulted in the use of ELISA assays to detect these genes as proof of *Neotiphodium* infection in plant tissues. Fungal endophytes are the only microorganisms known to enhance antiherbivory defense in a plant host.[74] Multiple studies of *Neotyphodium* spp. and their plant hosts spanning the past half century provide a rare look into the chemistry behind the delicate relationship that can exist between a fungal endophyte and its plant host.

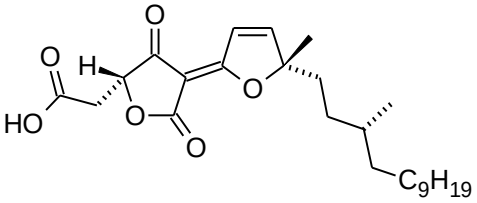
Research in our group continues to focus on investigation of fungi from different ecological niche groups as sources for new antifungal chemistry, and fungal endophytes are among the groups under current investigation. Recently,

members of our research group and our collaborators at the USDA in Peoria, IL, were able to demonstrate that *Acremonium zeae* is a protective endophyte of maize via production of antimicrobial metabolites.[60,63,78] Pyrrocidines A (**13**) and B (**14**) , produced by *A. zeae*, display antibacterial and antifungal properties and were detected in the extracts of *A. zeae*–infected maize kernels.[60,63,78] These compounds show antifungal effects toward two common corn pathogens, *A. flavus* and *F. verticillioides*, and may protect maize from other microbial pathogens as well. In a continuation of this project, a new fungal metabolite, dihydroresorcylide, and two analogues were isolated from *A. zeae* and showed phytotoxic effects towards maize plants in leaf–puncture wound assays.[60] While these resorcylide compounds were inactive in antifungal assays, it is possible that these compounds aid in *A. zeae* infestation of the maize hosts, while their pyrrocidine co–metabolites deter the growth of other microbial pathogens.[60] Ultimately, these antifungal and phytotoxic fungal metabolites were detected in *A. zeae*-infested corn kernels using LC–APCIMS.[60] Though this is a step toward proving roles that endophytic fungal metabolites might play in the disease cycle and/or defense of maize plants, most endophytic fungal metabolite research focuses mainly on the identification of new biologically active metabolites with little concern for any role the fungal metabolites might play in the endophytic relationship.

In the course of our ongoing studies of endophytic fungi, a number of different endophytic fungal isolates were investigated for their abilities to produce antifungal metabolites. A significant portion of the research described in this thesis is dedicated to the isolation and identification of antifungal metabolites produced by some of these fungi. While these efforts ultimately did not afford new natural products, numerous antimicrobial agents were identified, including compounds with previously unreported antimicrobial effects, furthering the hypothesis that endophytic fungi produce antimicrobial agents that can act as defenses for both the producing fungus and plant host. Details of these projects are described in Chapters 2–4 of this thesis.

The remainder of the research described in this thesis focused on the isolation and identification of antifungal metabolites produced by mycoparasitic and fungicolous fungi. Fungicolous fungi are those that colonize a fungal host.[37] A specific subset of these are considered mycoparasites because they have been demonstrated as true parasites of their fungal hosts.[40,46] Proving a parasitic relationship is somewhat difficult and requires detailed studies of a given host-colonist relationship. For this reason, many fungi that colonize others are classified simply as fungicolous, even though they may be truly mycoparasitic. Regardless of their classification, both mycoparasitic and fungicolous fungi are known to produce a wide variety of antimicrobial agents, including antifungal

compounds.[41-43,80,81] Lowdenic acid (**16**)[82], penifulvin A (**17**)[81], and acreseptin C (**18**)[83] are examples of new natural products produced by fungicolous fungi and discovered by members of our research group. Lowdenic acid is an antifungal, antibacterial agent and penifulvin A has antiinsectan effects.[81,82] Acreseptin C is inactive in assays employed thus far, but contains a rare, naturally occurring sulfone group, and further assays may yet prove it to be bioactive in some way.[83]



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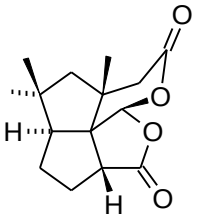

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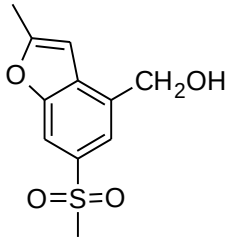

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As part of the research described in this thesis, a number of different fungicolous fungal isolates were investigated for their abilities to produce new antifungal metabolites. This research resulted in the identification of a variety of new secondary metabolites, including compounds active against the fungal pathogen *A. flavus*. These compounds were of both terpenoid and polyketide biosynthetic origins. The isolation, structure elucidation, and biological activities of metabolites identified from fungicolous fungal isolates will be discussed in Chapters 4–6 of this thesis.

CHAPTER 2

ANTIFUNGAL METABOLITES PRODUCED BY *COLLETOTRICHUM GRAMINICOLA*, A FUNGAL ENDOPHYTE OF MAIZE, AND POSSIBLE ROLES OF FUNGAL CHEMISTRY WITHIN THE DISEASE CYCLE OF MAIZE PLANTS

In a continuation of our chemical investigations of agricultural fungal endophytes, several isolates from maize seeds were identified as *Colletotrichum graminicola* (Holomorph: *Glomerella graminicola*). Extracts from cultures of these fungi displayed antifungal activity in assays versus *A. flavus* and *F. verticillioides*, as well as antiinsectan activity against the corn pest *Spodoptera frugiperda* (fall armyworm). It is possible that *C. graminicola* produces antifungal metabolites to exclude other fungal phytopathogens from colonizing the maize plant host. It may also produce antiinsectan chemicals that could serve as defenses for both the fungal endophyte and the plant host against fungivory and herbivory. For these reasons, we became interested in the chemicals produced by this fungal species.

The seeds from which *C. graminicola* fungal isolates were collected originated from a variety of corn plants grown in fields throughout the Midwest. *C. graminicola* is a commonly encountered fungal endophyte in the U.S. and is

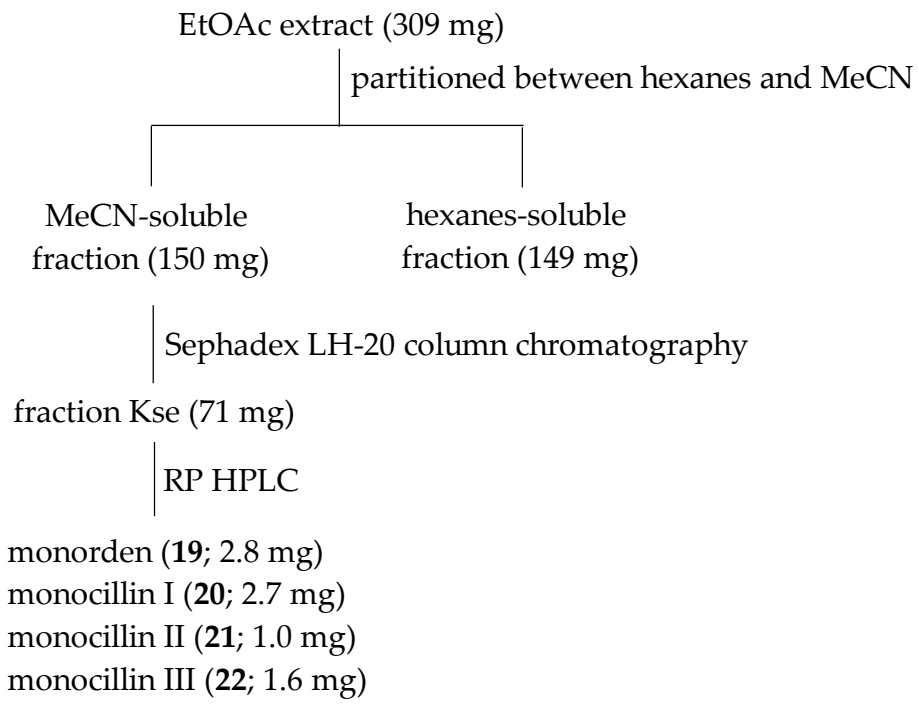
best known as a vascular pathogen of maize (*Zea mays*).[84] As an endophyte, *C. graminicola* causes anthracnose stalk rot and leaf blight of its maize plant host, resulting in significant economic losses.[84-86] Even though this is a very common fungal pathogen of cereal crops, there have been no reports of chemistry from *C. graminicola*, furthering our interest in the chemistry produced by this fungal endophyte.

Secondary Metabolites from *C. graminicola*

An isolate of *C. graminicola* (NRRL 47511) was obtained from a maize seed and identified on the basis of micromorphology[87] and partial DNA sequence analysis.[88,89] Its sequence was deposited in GenBank (GQ221856). This isolate was cultured by solid–substrate fermentation on rice for 30 days at 25°C. The fermented rice substrate was then fragmented and extracted with EtOAc to yield 309 mg of a crude extract. This extract was subjected to bioassay–guided fractionation by Sephadex LH–20 and reversed phase HPLC (Scheme 1), resulting in the identification of four known antifungal metabolites: monorden (a.k.a., radicicol; **19**), and monocillins I, II, and III (**20**–**22**).

Monorden and Monocillins I–III

The most abundant metabolite obtained, monorden (**19**), was identified on the basis of its ^{1}H NMR (Table 1) and HRMS data (obsd *m*/*z* 363.0628 (rel int



Scheme 1. Isolation of monorden (**19**) and monocillins I–III (**20**–**22**) from *C. graminicola* (NRRL 47511)

100%), $[M–H]^-$, 365.0624 (rel int 33%), calcd for $C_{18}H_{16}ClO_6$, 363.0635 and 365.0606) and by comparison with literature values.[86,93,96-99] The ^{1}H NMR spectrum displayed an H–bonded phenolic OH signal, a methyl doublet, four olefinic multiplets, three oxymethine multiplets, an aromatic singlet, and four resonances representing the protons of two methylene units. The only inconsistencies with previous reports of data for **19** were signals representing the H_2–11 methylene protons. These signals appeared as a broad singlet and doublet when data were collected at 18°C, while published reports list both signals as doublets with a 16.6 Hz geminal coupling.[40,97-100] In an effort to explore this issue,

^{1}H NMR data were collected at 18, 25, and 45°C (Figure 1). Only when temperatures exceeded those of standard conditions did the H_2–11 methylene signals sharpen to doublets with the expected *J*–value of 16.6 Hz. This effect may be associated with the reported presence of three different conformers of monorden (**19**) in solution.[101]

Table 1. ^{1}H NMR data for monorden (**19**) and monocillins I–III (**20–22**) (400 MHz; $CDCl_3$)

pos.	**19**	**20**	**21**	**22**
1	1.52 (d, 6.8)	1.60 (d, 6.9)	1.31 (d, 6.7)	1.43 (d, 6.6)
2	5.49 (m)	5.56 (m)	5.33 (m)	5.24 (m)
3	1.96 (ddd, 3.8, 8.9, 15)	1.98 (ddd ,3.8, 9.4, 15)	2.19 (br dt, 4.4, 14)	1.85 (br dt, 4.3, 16)
	2.34 (ddd, 3.2, 3.6, 15)	2.40 (br dt, 3.1, 15)	2.68 (ddd, 3.9, 8.3, 14)	2.07 (ddd, 3.2, 5.3, 16)
4	3.16 (br s)	3.25 (m)[b]	5.12-5.27 (m)	2.72 (m)
5	2.92 (br dt, 2.8, 8.8)	3.08 (br dt, 2.5, 9.4)[b]	5.12-5.27 (m)	2.52 (br dt, 2.6, 9.6)
6	5.83 (dd, 2.8, 11)	5.94 (dd, 3.1, 10)	1.99 – 2.13 (m)[c]	2.24 (m)
			2.04 (m)[c]	–[d]
7	6.16 (dddd, 0.9, 1.7, 9.2, 11)	6.24 (ddd, 1.4, 10, 11)	2.23 – 2.37 (m)[c]	2.38 (ddd, 3.6, 7.3, 14)
			2.28 (dd, 5.0, 9.2)[c]	2.50 (m)
8	7.44 (dd, 9.2, 16)	7.87 (dd, 11, 16)	6.65 (ddd, 6.6, 8.6, 16)	6.82 (ddd, 4.5, 11, 16)
9	6.10 (d, 16)	6.01 (d, 16)	5.78 (d, 16)	6.07 (dd, 1.0, 16)
10				
11	3.91 (br d, 16)[a]	3.62 (d, 14)	3.75 (d, 17)	3.58 (d, 18)
	4.78 (br d, 16)[a]	5.22 (d, 14)	4.15 (d, 17)	4.48 (d, 18)
12				
13		6.36 (s)	6.21 (d, 2.6)	6.18 (d, 2.5)
14-O*H*	6.05 (s)	7.08 (br s)		
15	6.62 (s)	6.36 (s)	6.37 (d, 2.6)	6.38 (d, 2.5)
16-O*H*	11.08 (br s)	11.36 (s)	11.69 (s)	11.93 (s)

[a]These signals appeared as a broad doublet and a singlet that sharpened significantly upon collection of data at increasing temperatures (up to 45°C). [b]These methine signals may be interchanged. [c]These methylene signals may be interchanged. [d]A resonance representing one of the six methylene protons in monocillin III (**22**) could not be definitively located due to severe overlap in the corresponding region of the spectrum.

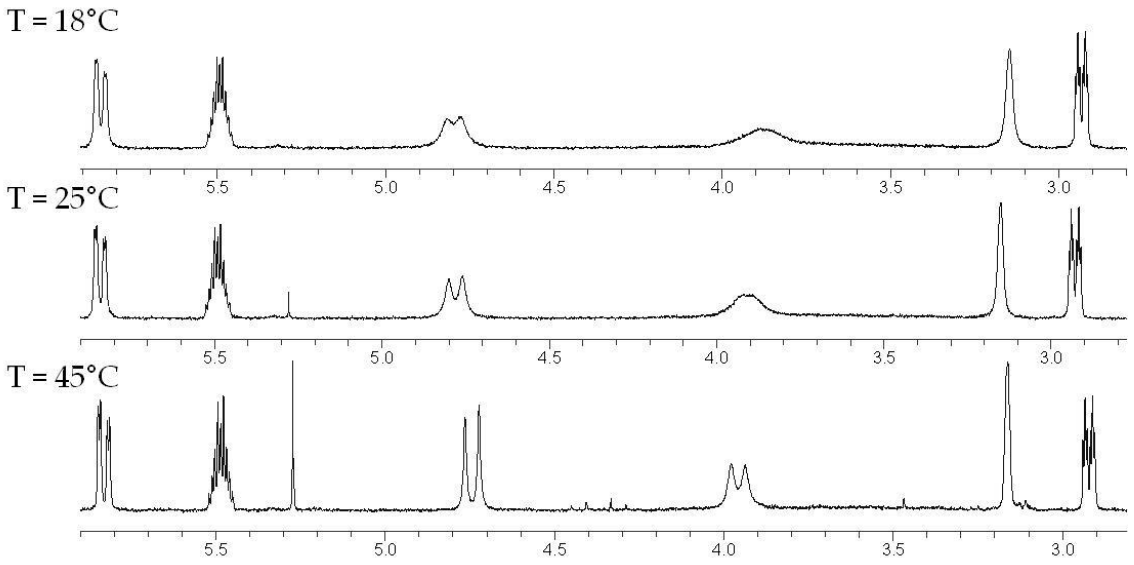
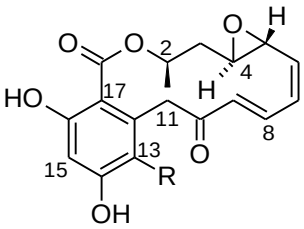


Figure 1. ^{1}H NMR spectra of monorden (**19**) at different temperatures (400 MHz; $CDCl_3$)

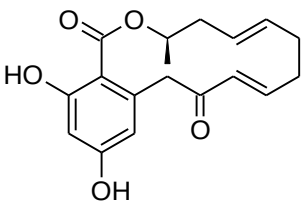
Monorden (**19**) was first isolated from a culture of *Monosporium bonorden* in 1953 and displayed antibiotic activities, including antifungal effects.[102,103] Its chemical structure, however, was not reported until 1964, when it was independently and simultaneously described by two research groups, leading to the two different common names of **19**: monorden and radicicol (from *Neonectria radicicola*).[97,100] The absolute configuration of **19** was described in 1987 by analysis of X–ray diffraction data, which resulted in assignment of *R*–configurations for monorden's three stereogenic sp^3 carbons.[86] Monorden (**19**) has since been described from a number of different fungi, including *Pochonia* and *Humicola* spp., an isolate of *Penicillium luteo–aurantium* from New Zealand, and a marine

isolate of *Neocosmospora tenuicristata* from Japan, as well as isolates of *Didymosporium radicicola* and *Chaetomium chiversii*.[40,86,104-109]



19: R= Cl

20: R= H



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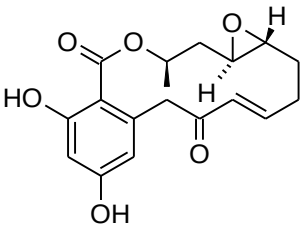

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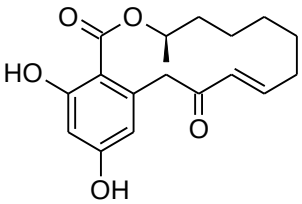

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The other metabolites identified from the fermentation extracts of *C. graminicola* isolate NRRL 47511 were monocillins I–III (**20–22**). These compounds were also identified on the basis of ^{1}H NMR (Table 1) and MS data, and by comparison with literature values.[93,96,101] These compounds were previously reported in 1980 as antifungal metabolites produced by another fungal isolate, *Monocillium nordinii*, along with monorden (**19**), and monocillins IV and V.[96] Monocillin I (**20**) is the dechloro–analog of monorden (**19**) and has antibiotic

activity similar to that of **19**.[96,110] Monocillins II and III are less frequently encountered and are not as biologically active as their analogs **19** and **20**.[96]

Several metabolites similar in structure to **19–22** have been previously reported from fungal isolates of corn including resorcylides, curvularin, and the mycotoxin zearalenone.[60,90,91] Pochonins are perhaps the closest analogues of **19–22**, differing only in oxidation and/or chlorination at certain positions.[92,93] However, their fungal source (*Pochonia chlamydosporia*) was not a corn isolate and was described as being derived simply from plant debris.[93] Compounds **19–22** are polyketide–derived 14–membered resorcylic acid lactones that vary in oxidation and contain one more acetate unit in their macrocycle than does resorcylide (**23**).[60] Their biosynthesis involves the condensation of nine acetate units that are selectively reduced or undergo cyclization/aromatization and halogenation.[94,95]

Monorden (**19**) displays an array of biological activity, including antifungal, antimalarial, and antibiotic effects.[40,86,96,102,110] Compound **19** has also been investigated for its abilities to regenerate muscle tissue damaged by snake venom and for use as a hair–growth stimulant.[92,111] More recently, the majority of research involving monorden (**19**) has focused on its abilities to inhibit Hsp90.[101,109,112-117] The term Hsp90 is used to refer to members of a family of heat shock proteins found abundantly in all eukaryotic organisms and aids in

protection of cells when exposed to higher temperatures and other cellular stresses.[118] These proteins function as molecular chaperones of various cellular proteins, including those associated with cell cycle progression and survival.[119,120] In cancer cells, Hsp90 aids in stabilization of over–expressed PI3K and Akt, two proteins involved in signal transduction and prevention of apoptosis.[120] Inhibition of these two proteins therefore interferes with cell proliferation.[118,120] As a result, inhibition of Hsp90 may lead to apoptosis of cancer cells.[118] Compounds such as monorden (**19**), which competitively binds the ATP binding site of Hsp90 with nanomolar affinity, disable Hsp90 ATPase activities required for its role as a molecular chaperone, and are therefore viewed as potential candidates for anticancer drugs.[112] Geldanamycin, novobiocin, and their analogues are also Hsp90 inhibitors, though monorden (**19**) is orders of magnitudes more potent.[101,112,121] While these natural products have been pivotal in advancing the discovery of Hsp90 inhibitor anticancer agents, the compounds are not viable therapeutic agents because of toxicity (geldanamycin) and instability within the cell (monorden).[115,121] Synthetic analogues of monorden (**19**), including several oximes and 14, 16–dipalmitoyl–radicicol, have displayed antitumor effects *in vivo*, though toxicity has limited the efficacy of the radicicol oximes.[101,109,113,115,117] Because of the wide variety of biological activities displayed by monorden (**19**) and its potential use in cancer treatment, some have attempted

its total synthesis, and successful outcomes have been reported by Lampilas, *et al.* in 1992 and Garbaccio, *et al.* in 2001.[98,122]

As eukaryotic organisms, maize plants also have Hsp90 proteins. Recently, monorden (**19**) and monocillin I (**20**) were shown to inhibit plant Hsp90 as well.[123] We hypothesize that monorden (**19**) and/or its analogs inhibit Hsp90 chaperones of R–proteins in maize plants, resulting in suppression of plant defenses and thereby fostering colonization by the monorden–producing species (*C. graminicola*). We also hypothesize that, as antifungal agents, monorden (**19**) and monocillins may help to exclude other competing maize fungal phytopathogens and endophytes from growth in the host plant.

Potential Role of *C. graminicola* Metabolites within the Disease Cycle of Maize

In order to determine whether compounds **19**–**22** could play a significant role within the *C. graminicola* disease cycle of maize plants, we first needed to determine if *C. graminicola* isolates other than NRRL 47511 would show a pattern of monorden (**19**) and/or monocillin production. Two more *C. graminicola* isolates (NRRL 47509 = GQ221855 and ENDO–3137) were analyzed. NRRL 47509 was isolated from a maize seed collected from a commercial field plot near Cerro Gordo, IL, while ENDO–3137 (ARS NRRL Culture Collection number has not yet been assigned) is from the same type of seed and from the same location as

NRRL 47511. Both of these isolates were cultured in a manner similar to that described for NRRL 47511 and then extracted with EtOAc. The organic extracts were then evaporated to dryness and partitioned between hexanes and acetonitrile. ^{1}H NMR analysis of the corresponding acetonitrile–soluble partitions again resulted in the detection of monorden (**19**) as a major metabolite produced by both isolates. Monocillins I–III (**20–22**) may also be produced by NRRL 47509 and ENDO–3137 as minor components. However, their presence could not be confirmed merely by analysis of ^{1}H NMR spectra of their crude acetonitrile–soluble fractions due to sample complexity. Further sample purification would be required to determine whether these compounds are present, but because monorden (**19**) is by far the major metabolite present, and because its activity is much more widely recognized, we did not pursue efforts to detect **20–22** in these extracts.

Having demonstrated that multiple isolates of *C. graminicola* produce monorden (**19**) and in at least one instance its analogs (**20–22**), we next wanted to determine if these compounds could serve defensive purposes. It seemed likely that compounds **19–22** were responsible for the original antifungal and antiinsectan activity displayed by *C. graminicola* EtOAc extracts. For this reason, the most abundant metabolite produced, monorden (**19**), and the acetonitrile–soluble fraction of NRRL 47509 (which was composed mostly of **19**) were

subjected to standard disc assays against *A. flavus* and *F. verticillioides*, as well as antiinsectan feeding assays vs. the fall armyworm. At 500 μg/disc, both the pure sample of monorden (**19**) and sample composed mostly of monorden (**19**) afforded large clear inhibitory zones in standard disc assays against *A. flavus*; a measure of fungistatic activity. However, there was no appreciable inhibition of fungal growth in similar assays versus *F. verticillioides*. When fall armyworms were fed a pinto bean diet containing 2000 ppm monorden (**19**) or an extract composed mostly of **19** (i.e., that of NRRL 47509), the growth rate of the worms were reduced by 60 and 54%, respectively in relation to controls fed only the pinto bean diet. While these values do not necessarily represent potent effects, the high concentration of monorden (**19**) in these *C. graminicola* extracts could account for the antifungal and antiinsectan activity originally displayed by the extracts.

We next wanted to determine if monorden (**19**) or monocillins I–III (**20–22**) have antifungal activity against other fungal endophytes and pathogens of maize. MIC and GI_{50} values were determined for monorden (**19**) and monocillins I–III (**20–22**) against a panel of fungi known to colonize maize (Table 2). Assays were performed using 96–well plates containing fungal pathogen/endophyte inoculum and either 1, 2, 3, 5, 10, 25, or 50 μg mL^{-1} of compounds **19**, **20**, **21**, or **22**. Plates were then incubated for up to 64 h at 25°C. Every 8 or 16 hours, the

plates were examined using a plate reader at 550 nm to measure the fungistatic activity of these compounds against the various fungal pathogens and endophytes of maize. Nystatin was used as a positive antifungal control for these assays and methanol was used as a negative control. Monorden (**19**) and monocillin I (**20**) were the most active compounds (Table 2), showing significant fungistatic activity against *Stenocarpella maydis*, a fungal pathogen known to cause stalk rot in maize plants. These compounds also demonstrated antifungal effects against *Alternaria alternata*, *Bipolaris zeicola*, and *Curvularia lunata*, which are foliar pathogens of maize. Monorden (**19**) was the only compound that showed antifungal activity against *A. flavus*, and this activity was only observed when the inoculum used in the assay consisted of *A. flavus* conidia. Monocillins II (**21**) and III (**22**) were inactive at levels tested against all of the fungal pathogens and endophytes listed in Table 2. None of the compounds tested were as potent in these assays as the nystatin control. Nystatin is a well known broad–spectrum antifungal agent. Even though the most abundant antifungal metabolite, **19**, is not viewed as a potent inhibitor of fungal growth for some of the organisms listed, the sheer amounts of the compound produced by *C. graminicola* could still have a significant effect.

Table 2. Antimicrobial activity of monorden (**19**) and monocillin I (**20**) against fungal endophytes and pathogens of maize[124]

Maize endophyte or pathogen[a]	Monorden [b]		Monicillin I [b]		Nystatin[b]	
	MIC[c]	GI_{50}[d]	MIC[c]	GI_{50}[d]	MIC[c]	GI_{50}[d]
Fusarium graminearum NRRL 31250	>50	>50	>50	>50	≤5	1-50
Nigrospora oryzae NRRL 6414	>50	>50	>50	>50	≤5	2-50
Stenocarpella maydis NRRL 31249	≤25	3-50	≤50	5-50	≤1	1-50
Trichoderma viride NRRL 6418	>50	>50	>50	>50	>50	10-50
Alternaria alternata NRRL 6410	≤50	10-50	>50	>50	≤5	1-50
Bipolaris zeicola NRRL 47238	≤50	25-50	>50	>50	–	–
Curvularia lunata NRRL 6409	>50	25-50	>50	>50	≤5	2-50
Acremonium zeae NRRL 13540	> 50	>50	>50	>50	≤3	1-50
Fusarium verticillioides NRRL 25457	>50	>50	>50	>50	≤10	3-50
Aspergillus flavus NRRL 6541 (conidia)	>50	50	>50	>50	≤10	3-50
Aspergillus flavus NRRL 6541 (conidia and hyphal cells)	>50	>50	>50	>50	≤10	3-50

[a]Inoculum consisted of a suspension of conidia and/or hyphal cells from PDA slant cultures that had been grown for 6 days at 25°C. [b]Concentrations tested: 1, 2, 3, 5, 10, 25, and 50 μg mL^{-1}. [c]MIC = minimum inhibitory concentration. [d]GI_{50} = growth inhibition ≥ 50% relative to controls.

Monorden (**19**) has been reported to inhibit the growth of other organisms that colonize maize plants.[110] *Debaryomyces hansenii* (syn. *Debaryomyces nicotinae*) is a yeast known to colonize Illinois maize plants and its growth is inhibited by **19**.[45, 110] The broad spectrum antimicrobial activity demonstrated by monorden (**19**) may ultimately result in the exclusion of other competing fungal species from maize plants, thereby fostering colonization of the plant by *C. graminicola*.

We have thus far demonstrated that *C. graminicola* produces compounds **19**–**22**, and that monorden (**19**) and monocillin I (**20**) are antifungal and antiinsectan agents that show effects against numerous species of maize endophytes and pathogens. To further explore the possible effects of *C. graminicola* metabolites on the disease cycle of maize plants, we needed to test the phytotoxic effects of **19**–**22** against the maize plants themselves. In addition, detection of these fungal metabolites in *C. graminicola*–inoculated and *C. graminicola*–infected maize plant tissues would be an important step in evaluating their possible significance.

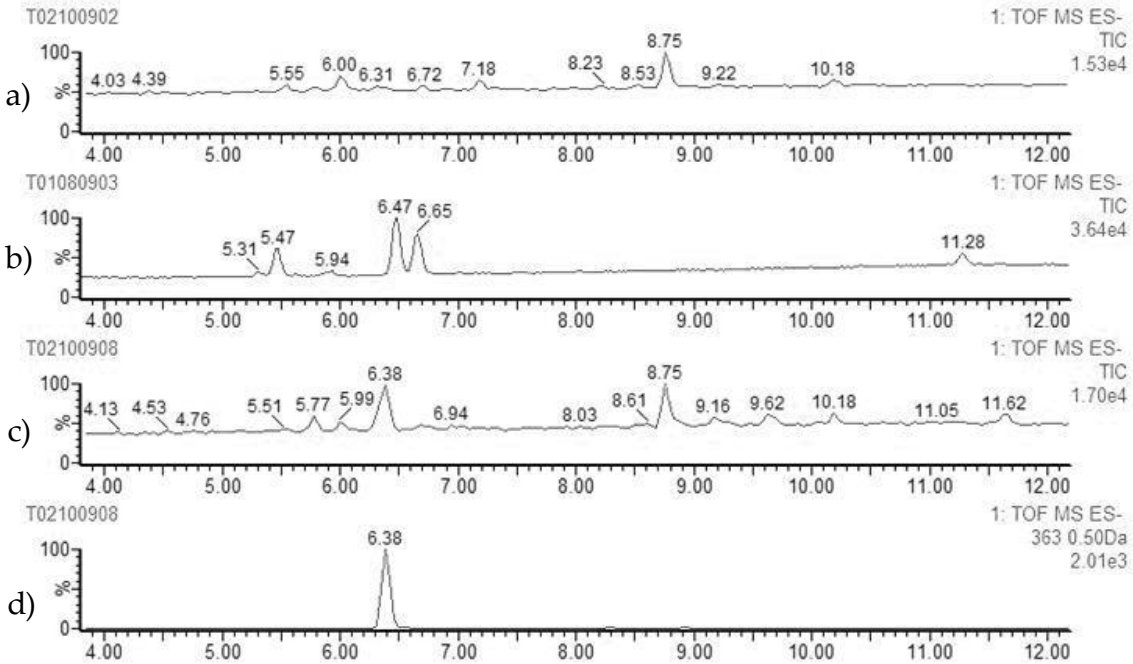
In leaf–puncture wound assays, monorden (**19**), monocillin I (**20**), and oxalic acid (positive control) produced lesions averaging 3.5, 2.5, and 3.0 mm in length, respectively, as opposed to negative (solvent) controls which only generated lesions averaging 0.25 mm in length. Monorden (**19**) and monocillin I (**20**) have previously shown some minor phytotoxic effects in maize plants and

chestnut leaves.[86,108] Phytotoxicity caused by *C. graminicola* metabolites is significant in that this may aid in the process of fungal invasion and colonization of the plant host.

In an effort to determine if compounds **19–22** could be detected in *C. graminicola* infected maize plants, steam–sterilized green maize stalks (pre–harvest) and stalk residues (post–harvest) from varying field locations were toothpick wound–inoculated with either of two isolates of *C. graminicola* (NRRL 47511 or 47509). After incubation for 7 (green stalks) or 14 (stalk residues) days, maize stalk segments were cut open and extracted with EtOAc. The resulting organic extracts were then partitioned between hexanes and acetonitrile. The acetonitrile–soluble partition was then subjected to LC–HRESITOFMS analysis for detection of monorden (**19**) and/or its monocillin analogs (Figure 2).

To detect these compounds in living maize stalks, maize plants were cultured in a growth chamber at the United States Department of Agriculture (USDA) National Center for Agricultural Utilization Research (NCAUR) labs in Peoria, IL. After plant maturation (37 days after planting of maize seeds), stalks were toothpick– wound–inoculated with either NRRL 47511 or 47509. A subset of the stalks displaying anthracnose lesions were harvested 31 days after inoculation. Others displaying such effects were allowed to grow until the stalks wilted (56 days post–inoculation) and were then harvested. Harvested stalks

were extracted with EtOAc to afford organic extracts which were subsequently partitioned between hexanes and acetonitrile. Acetonitrile–soluble fractions were subjected to analysis using the same LC–HRESITOFMS method as above for green maize stalks and stalk residues inoculated with *C. graminicola* isolates.



[a]Total ion current (TIC) chromatogram of (–) ions for the MeCN–soluble partition from the EtOAc extract from an uninoculated corn stalk standard. [b]TIC standard chromatogram of (–) ions for a chromatographic fraction containing monorden (**19**) and monocillins I–III (**20**–**22**). [c]TIC chromatogram of (–) ions for the MeCN–soluble partition from the EtOAc extract from a steam–sterilized maize stalk residue from Peoria, IL inoculated with *C. graminicola* isolate NRRL 47511. [d]Negative ion current chromatogram for the mass range 362.5–363.5 Da for the MeCN–soluble fraction from the EtOAc extracts from a steam–sterilized maize stalk residue from Peoria, IL inoculated with *C. graminicola* isolate NRRL 47511.

Figure 2. Negative ion LC–HRESITOFMS chromatograms of the MeCN partitions from extracts from maize stalks inoculated with *C. graminicola*

Monorden (**19**; t_R = 6.38 min; Figures 2 and 3) was detected upon LC–HRESITOFMS analysis of MeCN–soluble fractions from extracts of stalk residues collected from a cornfield plot in Dunlap, IL that had been inoculated with either NRRL 47511 or 47509. LC–HRESITOFMS analysis of a standard of **19** resulted in ions at *m*/*z* 363.0621 (100%) and 365.0638 (33%) which correspond to the $[M–H]^-$ pseudomolecular ion isotopic pattern expected for a chlorinated compound of this type (Figure 3). Similar analysis of extracts from steam–sterilized stalk residues and green stalks from an NCAUR field plot inoculated with NRRL 47511 showed a peak at the proper retention time that had the same accurate mass and $[M–H]^-$ pattern. LC–HRAPCITOFMS analysis was also attempted because of prior success with this technique detecting metabolites from another fungal endophyte of corn (*Acremonium zeae*) in a similar project.[60,63,78] ESIMS, however, proved to be a better ionization technique for the monorden/monocillin types of compounds and gave molecular ions for monorden (**19**) in samples that lacked molecular ions when using APCIMS for detection.

^{1}H NMR data were also collected for these extracts. However, due to small sample amounts, relatively high limits of detection associated with NMR analysis, and the presence of complex mixtures of other metabolites found in the acetonitrile partitions (some of which come from the plant substrate), monorden (**19**) could only be directly detected by ^{1}H NMR from the stalk residues collected

from the NCAUR plot, as it was most abundant in that extract. Using the HRESITOFMS method, monorden (**19**) was not detected in control, uninoculated steam sterilized stalks from the Dunlap and NCAUR cornfields, and monocillins I–III (**20**–**22**) were not detected in extracts from green stalks and stalk residues.

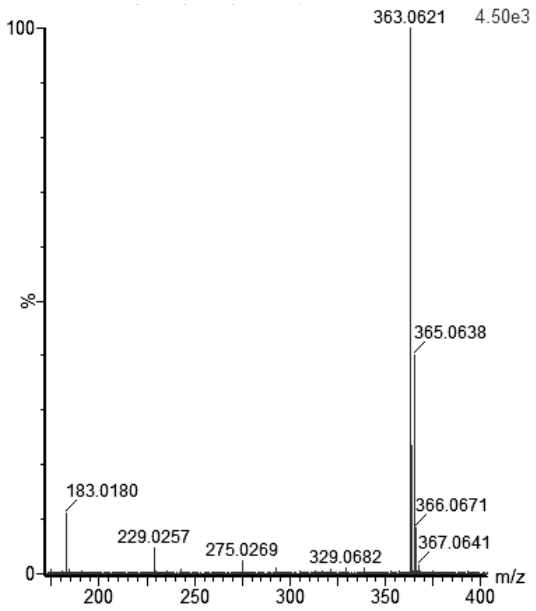


Figure 3. Representative negative ion LC–HRESITOFMS spectrum of monorden (**19**; peak at 6.38 min from plot *d* in Figure 2).

The LCMS method used in these analyses allowed qualitative detection of compounds **19**–**22** in organic extracts. It did not, however, allow determination of the concentrations of these compounds in the tissues. While this type of

LCMS method could result in detection of small organic compounds when employing sample concentrations as low as the pg/μL range in some cases (depending on the type and size of the molecule being investigated), the samples investigated in this project were complex mixtures of numerous plant and fungal metabolites, requiring sample concentrations of 50 ng/μL (200 ng/injection) in order to detect individual components. Sample complexity rendered identification of other fungal metabolites difficult with the LCMS methodology used. Further method development would be needed if such analyses prove to be warranted in the future.

Monorden (**19**) was not detected in extracts from anthracnose lesions of living maize plant stalks that had been inoculated with *C. graminicola* isolates using this method. However, such compounds could be present at levels below those which could be detected by the method used. As with the stalk residue and green stalk extracts in which we were unable to detect monorden or monocillins, small sample size and high concentrations of plant metabolites in these extracts may have played a role in not being able to detect these compounds. Also, the functioning vascular system of living corn plants may disperse monorden (**19**) being produced by *C. graminicola* throughout the entire plant, making its concentration too low to be detected with this method.

Furthermore, binding of monorden (**19**) by maize plant Hsp90 could also render this compound undetectable by the NMR and MS techniques used.

C. graminicola Metabolite Summary

C. graminicola is a widespread vascular pathogen of cereal crops, yet no chemistry had been previously reported for this species. Monorden (**19**) and monocillins I–III (**20–22**) were determined to be the major metabolites produced by isolates of *C. graminicola* on the basis of NMR and MS analysis of their extracts. Monorden (**19**) is responsible for the antifungal and antiinsectan activity displayed by these EtOAc extracts. As an antifungal antibiotic with potent Hsp90–inhibitory activity, we hypothesize that monorden (**19**) production by *C. graminicola* may not only help to exclude other endophytes and pathogens from its plant host, but may also inhibit Hsp90 chaperones of R–proteins in maize, thus resulting in suppression of host plant defenses. Both of these effects would foster colonization of the host corn plant by *C. graminicola*. In antifungal assays versus a variety of maize pathogens and endophytes, monorden (**19**) and monocillin I (**20**) displayed activity against a number of fungal species, most notably the maize stalk rot pathogen *S. maydis*. To determine if these compounds were produced by *C. graminicola* within dead and/or living maize plant tissues, pre–harvest stalk residues, post–harvest stalk residues, and living maize stalks were inoculated with either of two isolates of *C. graminicola* (NRRL 47511 or

47509). Monorden (**19**) was detected in the acetonitrile–soluble partitions from a number of EtOAc extracts from maize stalk residues and green stalks that had been inoculated with either of the two isolates through MS and/or NMR analysis. These data demonstrated that *C. graminicola* has the ability to produce these compounds when using corn plant material as a substrate. We were unable, however, to detect monorden (**19**) from the acetonitrile–soluble partitions from extracts from living maize plants that had been inoculated with either of the same two *C. graminicola* isolates. This could be due in part to small sample sizes, the presence of abundant plant metabolites that would interfere with the analysis, and the potential for monorden (**19**) to be dispersed throughout the entire living corn plant or bound by maize Hsp90, thereby rendering the amount present undetectable. With the methodology employed, monocillins I–III (**20–22**) were not detected in any of the maize stalk extracts discussed. Even though we were unable to prove the production of monorden (**19**) by *C. graminicola* within the living tissues of maize plants or a resulting crippling of plant defenses, we were able to demonstrate a pattern of monorden (**19**) production by *C. graminicola*.[124] Considering the nanomolar–scale binding affinity of monorden (**19**) for Hsp90, it may only require sub–microgram levels of monorden (**19**) per liter of plant vascular fluid to interfere to some extent with plant cellular processes. It seems likely that monorden (**19**) is produced by *C. graminicola*

within the living tissues of its maize plant host at some level. Even at very low concentrations, it could play a role in the delicate balance between fungal virulence and plant defense for this fungal endophyte. Further analysis of extracts using more sensitive detection techniques or alternative approaches may result in the identification of monorden (**19**) from the extracts of living maize plants that have been inoculated with *C. graminicola*.

CHAPTER 3

ISOLATION AND IDENTIFICATION OF METABOLITES PRODUCED BY THE COMMON CORN ENDOPHYTE *BIPOLARIS ZEICOLA*

During the course of our investigations into the chemistry produced by fungal endophytes, multiple isolates obtained from maize seeds and sorghum were later identified as the common corn pathogen *Bipolaris zeicola* (teleomorph = *Cochliobolus carbonum*[125]; synanamorph = *Helminthosporium carbonum*[126]). Fermentation extracts from these isolates displayed antifungal activity against *A. flavus* and *F. verticillioides*, as well as antiinsectan activity against fall armyworm. While several biologically active secondary metabolites have been reported from *B. zeicola*, none of these metabolites are known to cause the antifungal and antiinsectan effects displayed by these *B. zeicola* extracts.

The Biological Significance of *B. zeicola*

B. zeicola is a problematic pathogen of gramineous plants, including all cereal plants such as maize.[126-128] This fungus is the cause of Northern corn leaf spot and charred ear mold.[127,128] There are three races of *B. zeicola* that have been described and most research has focused on race 1, which is particularly virulent to corn hybrids that are homozygous and recessive at the nuclear *Hm* locus.[128] *B.*

zeicola race 2 is the most commonly encountered of the three races and is found colonizing maize throughout the Midwestern U.S. It is considerably less virulent than race 1. However, it is still a cause of disease for some hybrids.[129] Race 3 of *B. zeicola* has been proven to be a pathogen of rice leaves, but only in a laboratory setting.[130]

While *B. zeicola* has never caused an agricultural disease epidemic in the US, it is closely related to two species that have caused epidemics, *Helminthosporium maydis* (cause of Southern corn leaf blight in 1970) and *Bipolaris victoriae* (1940s oat cultivars epidemic).[128,131,132] Pathologists and breeders closely monitor *B. zeicola* because it has already demonstrated the ability to evolve new races that can cause disease in formerly resistant maize lines.[128] In cases where maize crops were infested with *B. zeicola*, yield losses were as high as 80%.[133] In 2000, 14% of field crops sampled in Iowa were found to host *B. zeicola*, with some of these stalks displaying pith disintegration and stalk discoloration.[134]

There is some confusion regarding the nomenclature of *Bipolaris* spp. These organisms were formerly classified within the genus *Helminthosporium.* However, the *Helminthosporium* (= *Helmisporium*[127]) genus became rather large and encompassed fungal species with significantly differing morphologies. This led to the subdividing and renaming of all *Helminthosporium* spp. into two separate genera: *Bipolaris* and *Drechslera*.[127] Many of the earliest metabolites

reported from what is now known as *B. zeicola* are named for its synanamorph, *Helminthosporium carbonum*.

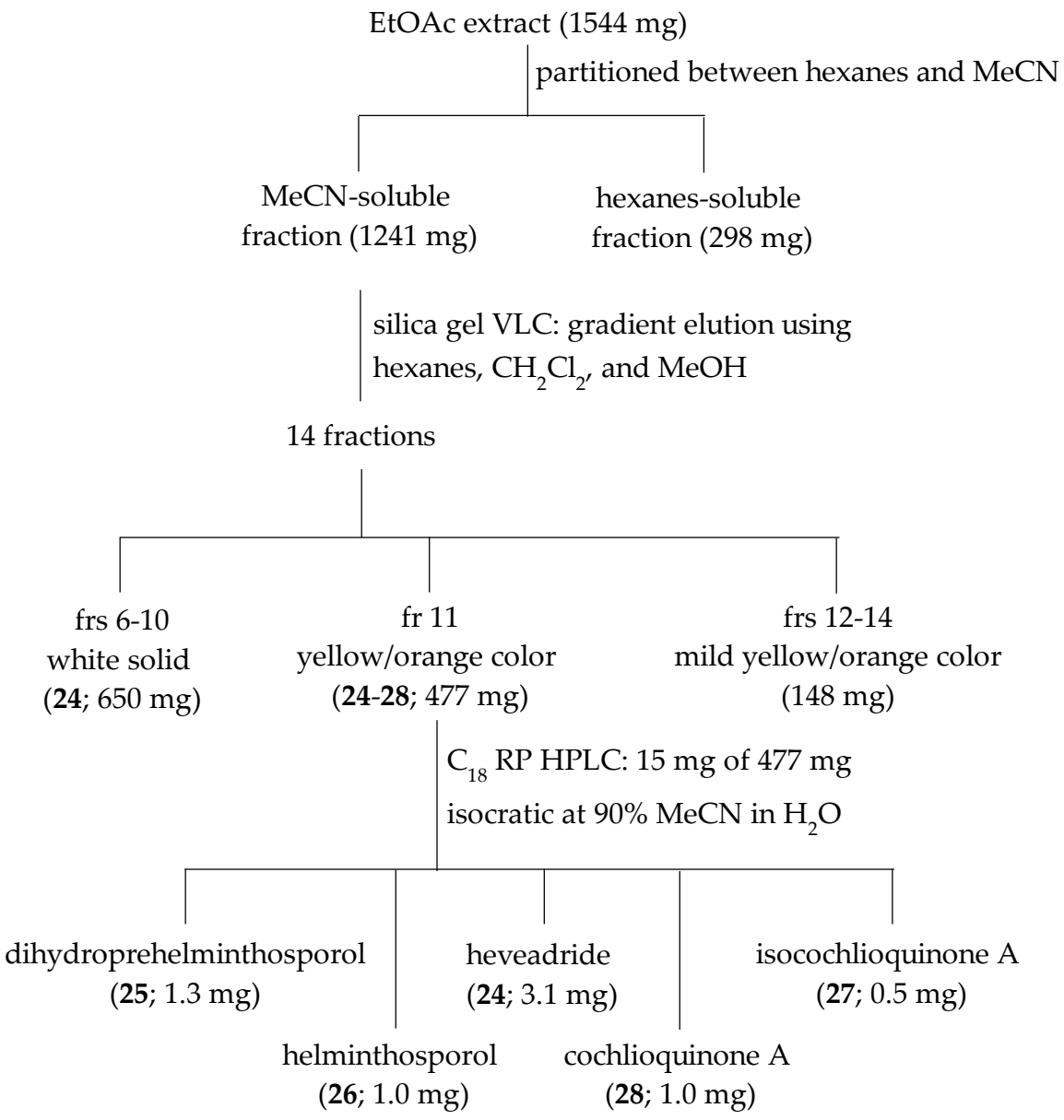
Secondary Metabolites from *B. zeicola* Isolates

NRRL 47238, 47500, and 47503

The antifungal and antiinsectan effects displayed by *B. zeicola* extracts led us to investigate the chemistry produced by a variety of *B. zeicola* isolates from both maize and sorghum hosts. Three *B. zeicola* isolates were identified on the basis of micromorphology[87] and partial DNA sequence analysis.[88,89] One of these isolates, NRRL 47503 (ENDO–3130), was isolated from a 'Mandan Bride' corn seed. EtOAc extracts from a rice fermentation of NRRL 47503 showed significant antifungal effects against *A. flavus* (cz 40 mm) and *F. verticillioides* (mz/rg 32 mm) after 24 hrs, though it was less effective after 48 hrs (*A. flavus* mz = 32 mm; *F. verticillioides*, no growth inhibition zone). NRRL 47503 extracts also reduced the growth rate of fall armyworms by 50% related to controls. To determine which secondary metabolites were responsible for these antifungal and antiinsectan effects, the EtOAc extract from NRRL 47503 was subjected to an isolation process involving silica gel VLC and RP–HPLC. ^{1}H NMR data were collected for all VLC fractions. Unfortunately, chemical structures could not be elucidated from these ^{1}H NMR data alone. Fractions were subjected to antifungal assays prior to further data collection and isolation processes.

At approximately the same time, the EtOAc extract from cultures of another fungal strain (ENDO–3090, unidentified at the time) was being chemically investigated as a result of its antifungal and antiinsectan activities. This isolate originated from sorghum rather than corn and its crude extract (1544 mg) was subjected to an isolation process (Scheme 2) similar to that used for the EtOAc extract of NRRL 47503. ^{1}H NMR data for the resulting chromatographic fractions revealed that the crude extracts from NRRL 47503 and ENDO–3090 were composed of similar secondary metabolites. For this reason, unidentified fungus ENDO–3090 was also thought to be *B. zeicola*. This was confirmed via a partial DNA sequence analysis.[88,89] ENDO–3090 was deposited with the USDA NCAUR NRRL collection and assigned the culture number NRRL 47500, and its partial DNA sequence was deposited in GenBank (GQ 253957). As shown in Scheme 3, the fractions from this isolate were chosen for further purification, affording five metabolites (**24**–**28**) described in detail below. The same metabolites were detected in the fractions for NRRL 47503.

The major component isolated from *B. zeicola* NRRL 47500 accounted for almost one third of the crude extract mass and consisted of a white powder. ^{1}H NMR data (Table 3) for this compound displayed many broad resonances, making it difficult to construct partial structures to aid in a literature search. ^{13}C and DEPT NMR data were collected, revealing that the structure contained



Scheme 2. Isolation of heveadride, dihydroprehelminthosporol, helminthosporol, isocochlioquinone A, and cochlioquinone A (**24**–**28**) from *B. zeicola* NRRL 47500

18 carbons, with signals at δ 165 and 22.4 representing two carbons each. Four ester, amide, and/or acid carbonyl carbons and four olefinic carbons were

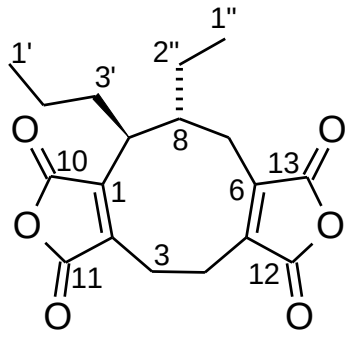
evident. The complete absence of oxygenated sp^3 carbon signals in the ^{13}C NMR data, and the absence of the acid (H–O) or amide (H–N) proton resonances in the ^1H NMR spectrum suggested that the compound contained a pair of maleic anhydride–type units. DEPT data were used to assign the remaining carbons as two methine, six methylene, and two methyl units. Incorporating the two proposed maleic anhydride units into a literature search for a molecule containing 18 carbons and 6 oxygens resulted in the identification of this major metabolite as heveadride (**24**).[159] Epiheveadride, the diastereomer of compound **24**, was also identified as a minor component of the 7th VLC fraction, which was composed almost entirely of heveadride (**24**).[159]

Heveadride (**24**) is a so–called nonadride that was previously reported as a metabolite of *H. hevea*.[135] Nonadrides are a class of compounds containing a nine–membered carbocyclic ring that is fused with two maleic anhydride units. The central ring also contains two *n*–alkyl chain substituents with varying levels of oxidation and unsaturation.[136] The structure of heveadride (**24**) was originally elucidated by analysis of ^1H NMR, ^{13}C NMR, and MS data.[135,137] The NMR data obtained for the sample isolated from *B. zeicola* were consistent with published data for heveadride (**24**).[159] The absolute configuration of heveadride (**24**) was originally determined by X–ray crystallographic analysis of its *p*–bromoanilide derivative.[138]

Table 3. ^{1}H and ^{13}C NMR data for heveadride (**24**)

Position	δ_C [a]	δ_H [b]
1	148.2	-
2	145.1	-
3	21.1	2.33 (br t, 13)
		3.08 (m)
4	22.4	2.20 (m)
		3.23 (m)
5	144.0	-
6	144.5	-
7	26.0	2.12 (br d, 13)
		2.88 (br d, 13)
8	40.6	2.22 (m)
9	46.1	2.24 (m)
10	163.5	-
11	164.8	-
12	165.7	-
13	165.7	-
1'	13.9	0.86 (t, 7.0)
2'	21.6	1.04 (m)
		1.15 (m)
3'	30.7	1.62 (m)
		1.94 (m)
1''	12.7	1.18 (br s)
2''	22.4	1.20 (m)
		1.82 (m)

[a]100 MHz; $CDCl_3$. [b]400 MHz; $CDCl_3$.



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Fractions 10 and 11 from silica gel column chromatography of both NRRL 47503 and 47500 contained similar mixtures of metabolites based on analysis of their ^{1}H NMR data. Fraction 11 from NRRL 47500 (15 of 477 mg) was further

separated over a C_{18} RP–HPLC column using a gradient of acetonitrile in water. Five metabolites were isolated and identified, including a further sample of the previously described heveadride (**24**).

The second metabolite collected from RP–HPLC fractionation (**25**) displayed a methyl singlet and two methyl doublets in its ^{1}H NMR spectrum (Table 4). Signals at δ 4.81 and 4.92 contained a 2.5–Hz geminal coupling characteristic of protons of a terminal olefin. There were also four doublet–of–doublet proton signals between δ 3.20 and 4.70. These were assigned to two oxymethylene units based on geminal coupling constants and chemical shift. Two methine signals were situated at δ 2.35 and 2.52. Resonances representing eight to ten other aliphatic protons were not distinguishable due to overlap and signal complexity. From the ^{1}H NMR profile, it was estimated that there were 15 carbons, 25–27 protons, and 2 oxygens in the molecular formula. A literature search utilizing information obtained from the ^{1}H NMR data was not sufficient to enable identification of the compound, resulting in further data collection. 2D–NMR data were collected for **25**, as MS instruments were not available at this point in time due to building renovations. Both HMQC and HMBC experiments were performed. HMQC analysis confirmed the presence of the subunits already assigned on the basis of ^{1}H NMR data, and also enabled assignment of

overlapping aliphatic proton signals for two methylene and four methine units.

HMBC data are provided in Table 4, with key correlations displayed in Figure 4.

Table 4. NMR data for dihydroprehelminthosporol (**25**) and helminthosporol (**26**) and HMBC correlations observed for **25**

	Dihydroprehelminthosporol (**25**)			Helminthosporol (**26**)
Pos.	δ_H (mult., J_{HH} in Hz)[a]	δ_C[b]	HMBC (H → C#)	δ_H (mult., J_{HH} in Hz)[c]
1		47.7		
2	1.34 (m)	42.9	1, 3, 4, 7, 8, 13, 15	1.36-1.42 (m)
	1.51 (m)		1, 3, 7, 8, 15	1.36-1.42 (m)
3	1.08 (m)	25.3	1, 2, 4, 9	0.86 (m)
	1.70 (m)		1, 2, 4, 5	1.75 (m)
4	1.16 (m)	49.4		1.01-1.08 (m)
5	2.35 (s)	38.8	1, 3, 4, 6, 7, 8, 9, 13, 14	3.17 (s)
6	2.52 (m)	45.9	4, 5, 7, 8, 14	
7		159.0		
8	1.58 (m)	57.6	1, 2, 4, 5, 6, 7, 13	1.66 (dd, 5.2, 8.6)
9	1.34 (m)	30.8	3, 4	1.01-1.08 (m)
10	0.85 (d, 6.6)	20.9	4, 9, 11	0.75 (d, 5.7)
11	0.96 (d, 6.6)	21.6	4, 9, 10	1.07 (d, 4.7)
12	4.81 (d, 2.5)	104.9	1, 2, 6, 7, 14	2.00 (s)
	4.92 (d, 2.5)	104.9	1, 2, 6, 7, 14	
13	3.26 (dd, 8.6, 11)	63.8	1, 5, 8	3.32 (dd, 8.6, 11)
	3.65 (dd, 7.2, 11)		1, 5, 8	3.63 (dd, 5.2, 11)
14	3.48 (dd, 8.6, 11)	67.0	5, 6, 7	10.02 (s)
	3.69 (dd, 6.1, 11)		5, 6, 7	
15	1.01 (s)	20.3	1, 2, 3, 7, 8	1.02 (s)

[a]600 MHz; $CDCl_3$. [b]Carbon chemical shifts were determined from HMQC and HMBC data.
[c]400MHz; $CDCl_3$

Both methyl doublets showed HMBC correlations to methine carbons at δ 30.8 and δ 49.4, demonstrating the presence of an isopropyl unit linked to a CH. Other HMBC correlations summarized in Figure 4 indicated the presence of a cyclohexane ring, with substituents at the positions shown in partial structure (a). An HMBC correlation from $H_{1.58}$ to $C_{49.5}$ seemed improbable for the partial structure shown in Figure 4a and could only be explained by the linkage shown in Figure 4b which became clear after identification of **25**. A literature search using this partial structure in Figure 4a led to recognition of this metabolite as dihydroprehelminthosporol (**25**).[139]

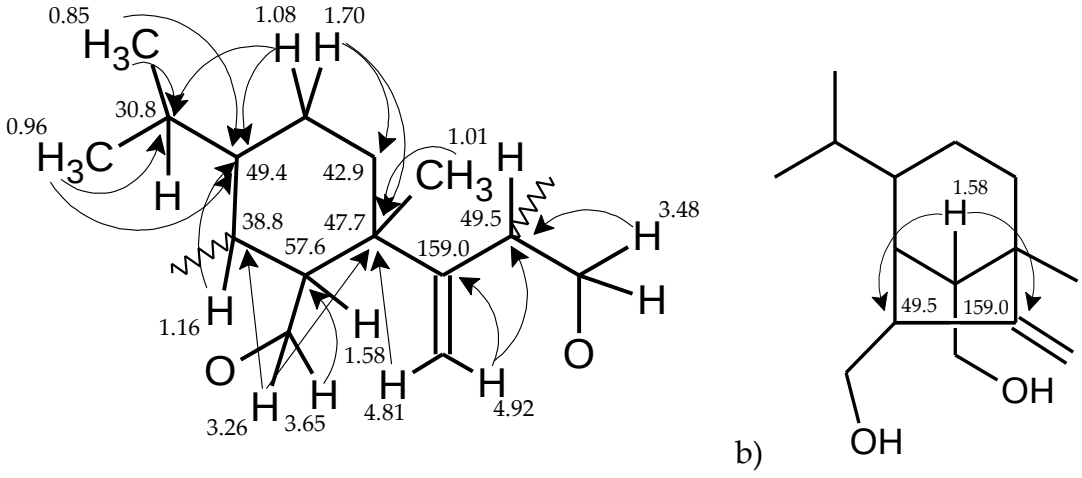
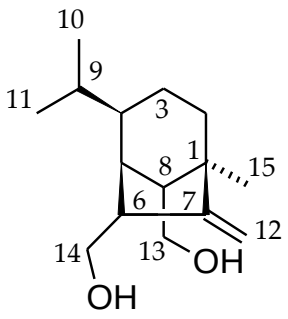


Figure 4. Selected HMBC correlations with partial chemical shift data for dihydroprehelminthosporol (**25**)

Dihydroprehelminthosporol (**25**) was first reported from *Bipolaris victoriae* and later identified as a metabolite of an unidentified *Bipolaris* sp. (ATCC 64838) pathogenic to Johnson grass.[139-141] It was co–isolated with several closely related compounds, including prehelminthosporol, helminthosporol, helminthosporic acid, helminthosporol acid, isosativenediol, victoxinine, and prehelminthosporolactone.[139-141] Dorn and Arigoni first reported the structure of dihydroprehelminthosporol (**25**) in 1972. However, its specific rotation was the only spectroscopic datum reported. Its ^{1}H and ^{13}C NMR data were not reported until 1988, and many signals were left unassigned.[139] Data listed for compound **25** were consistent with those provided in previous reports.[139] This compound is very similar to prehelminthosporol, which contains a hemiacetal at C–14 involving the oxygen at position 13. It is possible that prehelminthosporol degraded to helminthosporol (**26**). Reduction of **26** with $NaBH_4$ resulted in the formation of compound **25**.[139]



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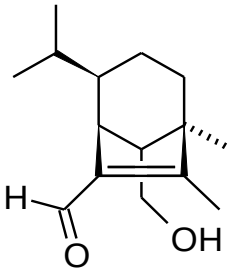
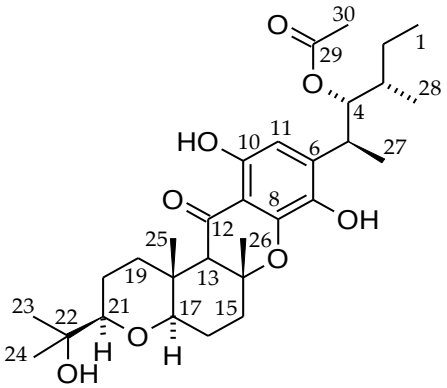


26

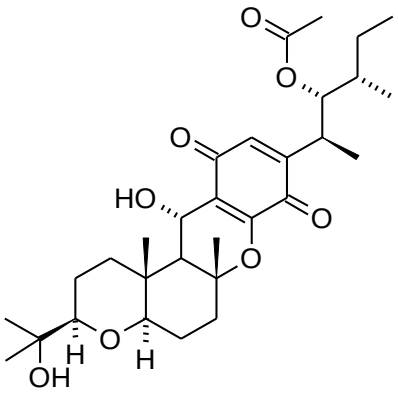
^{1}H NMR data for the second compound collected from the RP–HPLC separation of the NRRL 47500 extract (**26**) were strikingly similar to the data for dihydroprehelminthosporol (**25**).[139] There were signals representing an isopropyl unit, as well as a methyl singlet. However, there was also a second methyl singlet at δ 2.00, indicative of an olefinic methyl unit not found in **25**. The terminal olefin proton signals were also not observed in data for compound **26**, so it was thought that the olefin unit in **26** might be rearranged relative to that of **25**, with C–12 being a methyl carbon attached to a C6–C7 olefin unit in **26**. In addition, two oxymethylene signals observed in the spectrum of **25** were replaced with an aldehyde proton resonance at δ 10.02 in that of **26**. This could be envisioned as a result of oxidation at either position 13 or 14. A review of compounds similar in structure to dihydroprehelminthosporol (**25**), but containing an aldehyde and olefinic methyl unit led to identification of this compound as helminthosporol (**26**).

Helminthosporol (**26**), as previously mentioned, was isolated along with a number of similar compounds from the extracts of an unidentified *Bipolaris* isolate.[139] It was first described in 1963 as a metabolite of *B. sorokiniana*.[142-144] Compound **26** has also been isolated from a marine algicolous isolate of *Drechslera dematioidea*, along with the metabolite isocochlioquinone A (**27**), as well as from *B. victoriae*.[145, 146] The structure of helminthosporol (**26**) was

elucidated from elemental, IR, UV, optical rotation, and NMR analysis, as well as results from chemical derivatization, culminating in a report of its relative configuration that was extrapolated from that of its co–isolated metabolites.[139,142-144] Its absolute stereostructure was recently confirmed via 1D and 2D NMR experiments in conjunction with NOE difference and specific rotation data, and comparison with the absolute configuration of co–metabolites.[145] Since these initial reports, over 20 compounds related to helminthosporol (**26**) have been isolated and characterized.[146] Helminthosporol and its analogs are sesquiterpenoids thought to be biosynthesized from a non–head–to–tail linking of three isoprene units.[147] While helminthosporol (**26**) has been encountered on more than one occasion, it is likely that this compound is a decomposition product of the acetal formed during the isolation of prehelminthosporol.[148]



27



28

The fourth component collected from the RP–HPLC separation of extracts from NRRL 47500 had a significantly different ^{1}H NMR profile when compared with compounds **24**–**26** (Table 5). This compound (**27**) displayed resonances for eight methyl units: overlapping methyl doublet and triplet resonances upfield of δ 0.85, two methyl singlets at δ 1.11 and 1.46, two singlets and a doublet overlapping at approximately δ 1.17, and a methyl singlet located at δ 2.00. The ^{1}H NMR profile of this fraction was not clearly characteristic of polyketide or terpenoid biosynthetic origins, and it was not immediately clear whether the signal at δ 2.00 represented an olefinic or acetyl methyl unit. Another signal of note was a hydrogen–bonded phenolic OH resonance located at δ 10.76. A literature search was conducted for known metabolites from *Helminthosporium* spp. that contain eight methyl units and a hydrogen–bonded phenol moiety. This process suggested that compound **27** was likely to be a member of a class of over a dozen known compounds called cochlioquinones. After comparison with published data, compound **27** was determined to be the previously reported metabolite isocochlioquinone A (**27**). The structure of isocochlioquinone A was elucidated in 1994 by Miyagawa and coworkers using NMR and MS techniques. X–ray crystallographic analysis of the *p*–iodobenzenesulfonyl derivative of **27** enabled determination of its absolute configuration.[151] ^{1}H NMR data for the

Table 5. ^{1}H NMR data for isocochlioquinone A (**27**) and cochlioquinone A (**28**)

	Isocochlioquinone A (**27**)	Cochlioquinone A (**28**)
Position	δ_H (mult., J_{HH} in Hz)[a]	δ_H (mult., J_{HH} in Hz)[a]
1	0.81 (t, 7.4)	0.83 (t, 7.5)
2	1.2-1.3 (m)	1.36-1.50 (m)
	1.0-1.1[b] (m)	1.36-1.50 (m)
3	1.5-1.85 (m)	1.56-1.70 (m)
4	5.20 (dd, 5.3, 7.5)	4.90 (dd, 5.1, 7.5)
5	3.54 (m)	3.35 (m)
10 (OH)	10.76 (s)	
11	6.50 (s)	6.61 (s)
12		4.92 (dd, 1.6, 11)
12 (OH)		3.77 (d, 1.6)
13	2.76 (s)	1.72 (d, 11)
15	2.06 (m)	1.90 (dt, 3.7, 13)
	1.0-1.1[b] (m)	2.07 (dt, 3.3, 13)
16	1.5-1.85 (m)	1.56-1.70 (m)
	1.5-1.85 (m)	1.75-1.83 (m)
17	3.14 (dd, 3.6, 12)	3.15 (dd, 3.9, 12)
19	1.2-1.3 (m)	1.36-1.50 (m)
	2.73 (m)	2.46 (m)
20	1.5-1.85 (m)	1.36-1.50 (m)
	1.45[b] (m)	1.75-1.83 (m)
21	3.24 (dd, 2.9, 11)	3.23 (2.7, 12)
22 (OH)	2.49 (br s)	2.48 (br s)
23	1.17 (s)	1.15 (s)
24	1.17 (s)	1.17 (s)
25	1.11 (s)	1.00 (s)
26	1.46 (s)	1.30 (s)
27	1.17 (d. 6.9)	1.10 (d, 7.4)
28	0.85 (d, 6.7)	0.82 (d, 6.9)
30	2.03 (s)	2.03 (s)

[a]400 MHz; $CDCl_3$

sample of isocochlioquinone A (**27**) isolated from *B. zeicola* matched closely with previously published values.[150,151]

The cochlioquinones vary slightly in levels of oxidation and unsaturation at various positions, mostly within the quinone moiety. Isocochlioquinone A (**27**) is an intramolecular redox isomer of its quinone analog, cochlioquinone A (**28**). The last fraction collected from HPLC separation of the NRRL 47500 extract was a yellow pigment and appeared to contain a mixture of compounds **27** and **28**, with the major component being cochlioquinone A (**28**). Compounds **27** and **28** had significantly differing HPLC retention times, suggesting that any isocochlioquinone A (**27**) present in the cochlioquinone A (**28**) fraction was a result of cochlioquinone A decomposition. The structure of cochlioquinone A was originally assigned by analysis of NMR and X–ray crystallographic data.[152] Data for both compounds **27** and **28** were consistent with those previously reported for these compounds.[151-153]

Cochlioquinones are products of mixed terpenoid and polyketide biosynthesis. In cochlioquinone A (**28**), a sesquiterpene is linked to a *p*–benzoquinone ring containing a C_7 sidechain.[152] Acetate units were attached to the C_7 side-chain in both compounds **27** and **28**. The biosynthetic origin of cochlioquinone A (**28**) has been confirmed by [1,2–$^{13}C_2$]acetate feeding experiments in cultures of *Cochliobolus miyabeanus* and reflects prenylation,

followed by decarboxylation and hydroxylation of the aromatic nucleus, and then cyclization of the farnesyl chain.[154] Isocochlioquinone A (**27**) and cochlioquinone A (**28**) have been reported as co–metabolites from a number of different fungal species (*B. bicolor* El–1, *B. cynodotis* cynA, *B. brizae*, *B. oryzae*, *Cochliobolus* sp. UFMGCB–555, and *Drechslera dematioidea*), and are also sometimes isolated with other cochlioquinone analogs.[64,145,149,151-153,155-158] In only one case has isocochlioquinone A (**27**) been reported as a co–metabolite of helminthosporol (**26**).[145] While it is not unusual for compounds from within the same family to be isolated from the same fungal extracts, compounds **24**–**28** have not previously been reported from the same fungal isolate.

A third *B. zeicola* isolate (NRRL 47238 = FJ213843 = ENDO–3039), was collected from an Illinois maize seed, cultured in a solid–substrate rice fermentation, and extracted with EtOAc. This extract showed similar antifungal and antiinsectan effects to those displayed by NRRL 47503 (from maize host) and 47500 (from sorghum host) isolates. ^{1}H NMR data of the acetonitrile–soluble partition from this extract displayed resonances consistent with heveadride (**24**) as its major component, with other signals possibly representing compounds **25**–**28**. Thus, it appears that compounds **24**–**28** are produced by *B. zeicola* isolates, regardless of the plant host from which the fungal sample was collected. While

compounds **24**–**28** have all been previously reported, this constitutes the first reports of these metabolites from *B. zeicola*.

Biological Activity of *B. zeicola* Metabolites from NRRL 47238, 47500, and 47503

All compounds (**24**–**28**) were subjected to antifungal and antiinsectan assays against *A. flavus*, *F. verticillioides*, and the fall armyworm to determine which, if any, of these components were responsible for the activity originally displayed by the EtOAc extracts from *B. zeicola* isolates. Dihydroprehelminthosporol (**25**) and cochlioquinone A (**28**) were moderately active in disc assays against *F. verticillioides*, while isocochlioquinone A (**27**) was inactive. It could be concluded that even though the fraction of cochlioquinone A (**28**) tested contained **27** as a minor component, any fungistatic effects exhibited against *F. verticillioides* were a result of cochlioquinone A's (**28**) presence. Unfortunately, none of the metabolites isolated were active against *A. flavus* at levels up to 200 µg/disc (Tables 6 and 7). It is not known which components were responsible for the activity originally observed against *A. flavus*, but it is possible that an as–yet unidentified component from the *B. zeicola* isolates was the source of this activity. Compounds **24**, **25**, and **27** all reduced the growth rate of *S. frugiperda* in feeding experiments, thus accounting for some, if

not all, of the antiinsectan activity displayed by *B. zeicola* isolates.

Helminthosporol (**26**) and cochlioquinone A (**28**) were inactive in these assays.

Table 6. Biological activity of heveadride (**24**) and dihydroprehelminthosporol (**25**)

	24	**25**
Phytotoxicity	[a]Caused lesions in maize leaves at 10 μg/droplet in leaf-puncture wound assays	Caused lesions in leaf spot assays towards maize at 2.5 μg/mL; sorghum at 5 μg/mL; sicklepod at 2.5 μg/mL; morning glory at 10 μg/mL; bentgrass at 2.5 μg/mL[140]; Johnson grass at 12 μg/5μL[139]
Antifungal	*Arthroderma benhamiae; Cryptococcus neoformans; Candida kefyr; C. albicans; Epidermophyton floccosum; F. solani; Microsporum canis; Scedosporium apiospermum; Trichophyton mentagrophytes; T. raubitschekii; T. rubrum; T. tonsurans; T. violaceum; Trichosporon asahii*[159]	[a]mz = 19 mm at 200 μg/disc in standard disc assays against *F. verticillioides* after 48 hours
Antiinsectan	[a]29 % reduction in growth rate of fall the armyworm at 400 ppm	[a]11% reduction in growth rate of the fall armyworm at 180 ppm

[a]Assays performed as a part of this research

Even though heveadride (**24**), the most abundant metabolite isolated from the *B. zeicola* extracts, was inactive in antifungal assays against *A. flavus* and *F.*

verticillioides, it has been reported as a broad spectrum antifungal agent against various filamentous fungi (Table 6).[159] This compound has also demonstrated mild antifungal effects against yeasts, such as *Trichosporon asahii* and *C. albicans*.[159]

Due to the large amounts of heveadride (**24**) produced by all of the *B. zeicola* isolates investigated, it was thought that heveadride (**24**) might be likely to facilitate fungal invasion of maize plants in some way. In an effort to explore this issue, maize leaves were subjected to leaf–puncture wound assays with heveadride (**24**). Ten micrograms of heveadride generated, on average, 4.9–mm–diameter lesions on maize leaves, demonstrating phytotoxicity towards the plant host of *B. zeicola*.

Heveadride (**24**) was not the only phytotoxin present in extracts from *B. zeicola* isolates. Dihydroprehelminthosporol (**25**) is a non–host specific phytotoxin (Table 6). It is known to display phytotoxic effects against the host plants from which its fungal source is commonly isolated, including maize and sorghum.[140] The diol unit of dihydroprehelminthosporol (**25**) appears to be required for these phytotoxic effects, since the diacetate derivative of dihydroprehelminthosporol (**25**) did not show phytotoxicity.[139] Helminthosporol (**26**) is also a known phytotoxin and studies have been conducted to determine its mode of action (Table 7). This compound inhibits ion and electron transport in various plant cell systems.[150] For example, the H^+ gradient is an essential

Table 7. Biological activities of helminthosporol (**26**), isocochlioquinone A (**27**) and cochlioquinone A (**28**).

	[a]Phytotoxic, [b]Antifungal, [c]Antiinsectan, and [d]Antibacterial effects	Other Activities
26	[a]Disrupts the membrane permeability to protons and substrate anions of the electron and ion transport systems in mitochondria, chloroplasts and microsomes; inhibits 1,3-β-glucan synthase activity[150] [b]*M. violaceum*[145]; [d]*B. megaterium*[145]	Promotes growth of rice seedlings[143]; stimulates sugar release from deembryonated barley seeds[161]; inhibits Acyl-CoA cholesterol transferase[162]; inhibits *Plasmodium falciparum*[145]
27	[a]Inhibits root growth of Italian rye grass, finger millet, and rice[149, 151, 153] [b]*M. violaceum*[145]; [d]*B. megaterium*[145]	Antiparasitic vs. *Leishmania amazonensis* (EC_{50} = 4.1 μM)[64]; inhibits mitochondrial NADH-ubiquinone reductase complex I[153]; inhibits *P. falciparum*[145]; competitively binds human CCR5 (IC_{50} =50 μM)[155]
28	Inhibits root growth of Italian rye grass, finger millet, and rice[149, 151, 153] [b,e,f] mz = 19 mm at 200 μg/disc in standard disc assays against *F. verticillioides*; [b]*A. niger*[157]; [c,e]14 % reduction in growth rate of the fall armyworm at 100 ppm; [d]*Staphylococcus aureus* and *B. subtilis*[157]	Antiparasitic vs. *L. amazonensis* (EC_{50} = 1.7 μM)[64]; inhibits mitochondrial NADH-ubiquinone reductase complex I[153]; competitively binds human CCR5 (IC_{50} = 11 μM)[155];inhibits diacylglycerol kinase[156]

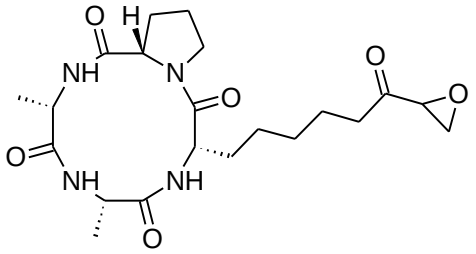
[e]Assays performed as a part of this research. [f]Assayed fraction was a mixture of **27** and **28**, with cochlioquinone A (**28**) as the major component.

pathway for nutrient uptake in plant cells. Helminthosporol (**26**) is shown to inhibit nutrient uptake in plants and may therefore contribute to the disease cycle of *B. zeicola* in maize plants.[150] The precursor to helminthosporol (**26**), prehelminthosporol, showed even more potent phytotoxicity in similar *in vivo*

assays of barley roots.[160] Because of this, one would likely expect to see phytotoxic activity in *B. zeicola* extracts regardless whether *B. zeicola* produces prehelminthosporol or helminthosporol. Further phytotoxic effects were reportedly displayed by helminthosporol (**26**) by preventing plant callous formation in damaged plant tissues through inhibition of 1,3–*β*–glucan synthase activity in plant conductive tissue cells, thus disabling a vital plant defense mechanism.[150] Various other biological activities reported for compounds **26**–**28** are listed in Table 7. These activities include plant regulatory, antibacterial, antimalarial, anticancer, and antiprotazoan effects.

Previously Described Secondary Metabolites from *B. zeicola*

The most notorious compound previously known from *B. zeicola* is HC–toxin I (**29**) which is produced exclusively by the race 1 subspecies.[163-165] This compound is one of a relatively small number of metabolites that have been identified as host selective toxins (HST). HSTs are compounds produced by fungi that selectively damage their hosts and cause few detrimental effects to nonhosts.[38,146,163,166,167] HC–toxin I (**29**) is a cyclic tetrapeptide (D–Pro→ L–Ala→ D–Ala→ L–Aeo where Aeo is an unusual 2–amino–9,10–epoxy–8–oxo–decanoic acid residue).[164,165,168-171]



29

Maize resistance to *B. zeicola* race 1 is attributed to a dominant gene known as *Hm*.[128] Plants are considered susceptible to *B. zeicola* race 1 infection if they are homozygous recessive at the *Hm* nuclear locus (*hm/hm).*[128] Resistant corn plants contain at least one dominant allele (*Hm/hm* or *Hm/Hm*).[128] Compound **29** is responsible for the fungal specificity of *B. zeicola* race 1 for maize plants that are homozygous recessive at the *Hm* nuclear locus (*hm/hm*).[128] This compound is able to inhibit root growth of susceptible maize (*hm/hm*; 0.5 μg/mL) and resistant maize root growth (*Hm/hm* and *Hm/Hm*; 50 μg/mL) (Table 8).[167, 172] The Aeo carbonyl and epoxide groups are necessary for HC–toxin I (**29**) activity in susceptible maize. Hybrids resistant to *B. zeicola* race 1 are able to deactivate HC–toxin I (**29**) via a toxin reductase (encoded for by the *Hm* nuclear locus).[128,166,173-175] Susceptible hybrids (*hm/hm*) have a transposon insertion and deletion within the genetic locus encoding for the toxin reductase.[176]

Table 8. Secondary metabolites previously reported from *B. zeicola* and published biological activity

Metabolite	Biological Activity
HC–toxin I (**29**)[a]	Inhibits root growth of susceptible seedlings (ED_{50} = 0.2 μg/mL)[174]; Inhibit histone deacetylase from maize, *Physarum*, and chicken[177]
HC–toxin II[a]	Inhibits root growth of susceptible seedlings (ED_{50} = 0.4 μg/mL)[174]
HC–toxin III[a]	Inhibits root growth of susceptible seedlings (ED_{50} = 2.0 μg/mL)[174]
Bz–cmp	Modifies maize chitinase (ChitA) proteins[178]
Cochlioquinone A1	Anti–angiogenic agent[179]
BZR cotoxins I–IV[b]	Rice leaf sheath susceptibility inducing factors: fungal non–pathogens of rice are able to colonize rice in the presence of BZR co–toxins. Any combination of co–toxins is more active than an individual component.[130,180]
Prehelminthosporol	Non–host specific phytotoxin[181]

[a]Reportedly produced exclusively by *B. zeicola* race 1. [b]Reportedly produced exclusively by *B. zeicola* race 3.

Two other HC–toxins have been described; HC–toxins II (glycine replaces D–alanine residue of **29**) and III (trans–3–hydroxyproline replaces D–proline residue of **29**).[174,182,183] These compounds are less potent than **29** as root growth inhibitors.[174]

Besides the small organic molecules previously described, *B. zeicola* has recently been shown to secrete a chitinase modifying protein termed Bz–cmp (Table 8).[178] This protein was extracted from the cultures of *B. zeicola* NRRL 47238 (FJ213843; ENDO–3039), one of the strains investigated in this work. Bz–

cmp has an approximate molecular mass of 54 kDa.[178] In denaturing chitinase zymograms of protein extracts from *B. zeicola*–diseased maize seeds, two maize chitinase activity bands were absent that were present in the protein extracts of asymptomatic, healthy maize seeds.[178] Plants are presumed to produce chitinases (proteins that cleave β–1,4–glucosidic bonds in chitin) at least in part to act as defense against organisms with chitin in their cells walls, such as insects and fungi. The production of Bz–cmp by *B. zeicola* renders this plant defense useless, potentially aiding in colonization of the maize plant by *B. zeicola*.[178]

The Potential Role of *B. zeicola* Metabolites in the Disease Cycle of Maize Plants

As discussed, *B. zeicola* produces a number of phytotoxic and antimicrobial compounds. After analysis of the metabolites produced by *B. zeicola* isolates NRRL 47503, 47500, and 47238, and subsequent evaluation of their biological activities, we hypothesized that these metabolites may play a significant role in the *B. zeicola* disease cycle in maize plants.

To examine the ability of these metabolites to exclude the growth of other maize fungal pathogens, the EtOAc extract of NRRL 47238 was subjected to antifungal assays against a series of fungi that are known pathogens of corn. The extract inhibited fungal growth in disc assays against eight of the ten fungal species examined: *Alternaria alternata*, *Stenocarpella maydis*, *Nigrospora oryzae*,

Fusarium graminearum, *Colletotrichum graminicola, Curvularia lunata*, *Acremonium zeae*, and *F. verticillioides*. The extracts were inactive against *A. flavus* and *Trichoderma viride*. It is interesting that, in the first round of antifungal testing against *A. flavus*, the extracts from all three *B. zeicola* isolates displayed antifungal effects against this pathogen, yet no metabolites were isolated that displayed this activity and a second round of testing resulted in the EtOAc extract of NRRL 47238 being inactive against this organism. It could be that an anti–*Aspergillus* metabolite present in the original EtOAc extracts decomposes or is not consistently produced.

When researching the activities reported for known metabolites **24**–**28**, it was noted that the major metabolite heveadride (**24**) is from a family of compounds called nonadrides. Members of this family are known to strongly inhibit the effects of Ras farnesyl transferase.[136] The Ras family of proteins is involved in eukaryotic cellular signal transduction and Ras farnesyltransferase is an enzyme that post–translationally modifies Ras proteins. Inhibition of Ras farnesyltransferase ultimately interferes with cell signal transduction by disrupting the functions of Ras proteins. Compounds that inhibit Ras farnesyltransferase are being investigated as cancer therapeutics.[184] As eukaryotic organisms, maize plants use Ras farnesyltransferase to post–translationally modify R–proteins that are involved in immune response.[185-187] It

is quite possible that heveadride (**24**) produced by *B. zeicola* inhibits maize Ras farnesyltransferase proteins, subsequently shutting down cellular signaling processes involved in plant immune response.

It appears that *B. zeicola* counters plant defenses with both small (heveadride; **24**) and large molecules (Bz–cmp[178]). As previously mentioned, the *B. zeicola* isolate NRRL 47238 studied in this work was recently reported to produce the maize chitinase–modifying protein Bz–cmp.[178] This protein shuts down the maize plant's ability to break down fungal chitin, disabling another plant defense.[178] The other phytotoxins identified from *B. zeicola* extracts may also play a role in the phytotoxicity of *B. zeicola* towards maize. Once fungal colonization has occurred, the antifungal, antibacterial, and antiinsectan effects displayed by *B. zeicola* metabolites may deter insect herbivory and colonization by other fungal and bacterial spp. Further exploration of antifungal effects of specific *B. zeicola* metabolites against maize fungal pathogens, and efforts to detection these compounds in the necrotic tissues of maize stalks could help shed further light on the intricacies of plant–fungal interactions.

CHAPTER 4

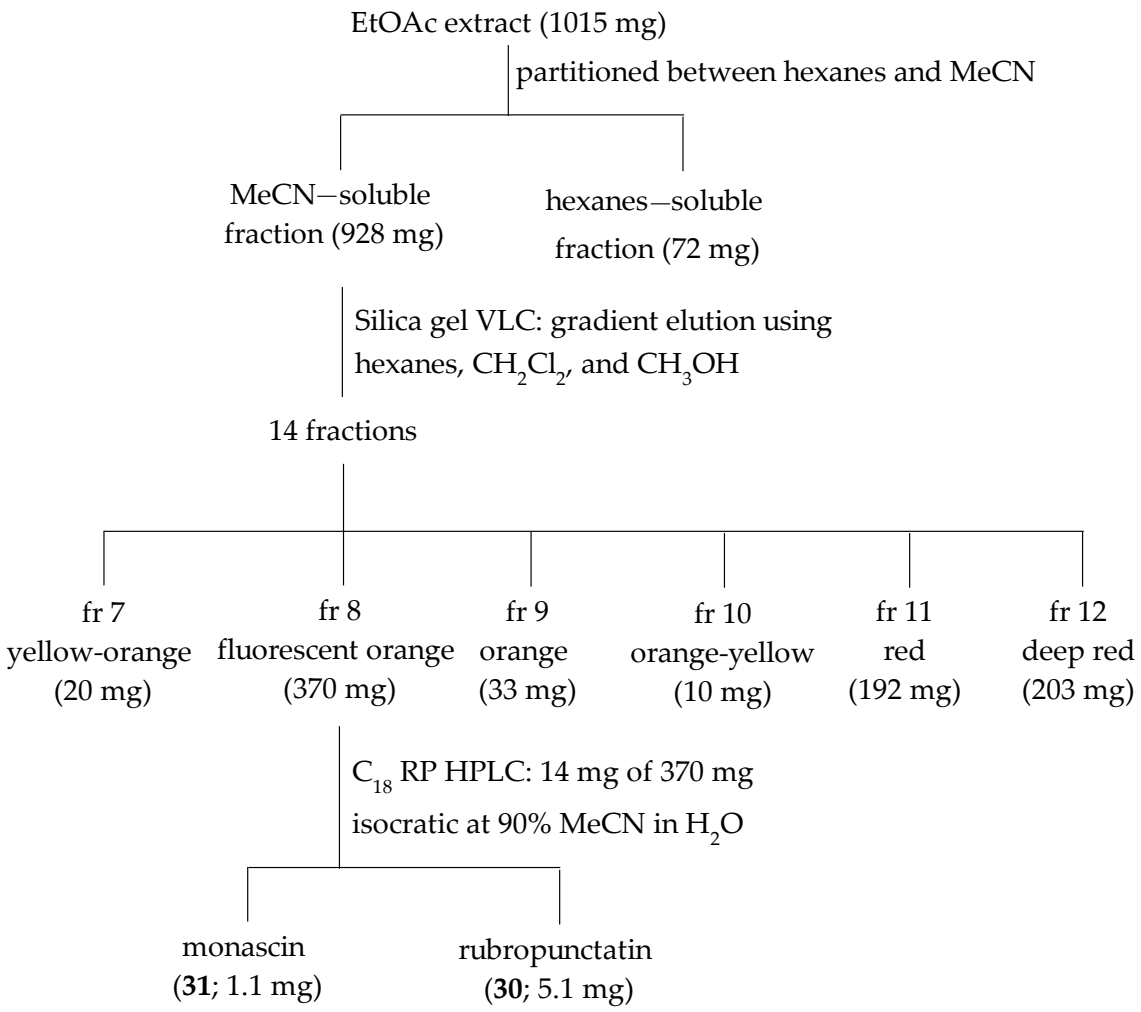
SELECTED FUNGAL METABOLITES PRODUCED BY ENDOPHYTIC AND FUNGICOLOUS FUNGAL ISOLATES

Chemical Investigations of An Isolate of *Monascus ruber*

Climate–controlled storage conditions of maize results in the colonization of harvested maize by a variety of fungal pathogens. Maize stored in a constant temperature and humidity chamber of 30°C and 30% relative humidity led to bright red fungal growth on some of the corn seeds.[188] The causative agent was isolated and cultured. Prior to its identification as *Monascus ruber*, the isolate was cultured in a solid–substrate rice fermentation and subjected to chemical investigation.

Secondary Metabolites from M. ruber

Fungal endophyte ENDO–3131 was collected from rehydrated corn seeds that were sealed in a jar and incubated in the dark for 21 days at 30°C and 30% moisture content.[188] The fungus was grown in solid–substrate rice fermentation for 30 days at 25°C. Extraction of the resulting culture with EtOAc yielded 1015 mg of a crude extract. This extract showed antifungal and antiinsectan effects and was subsequently subjected to chemical investigation in order to identify the compounds responsible for these activities. The isolation of metabolites from ENDO–3131 is summarized in Scheme 3.



Scheme 3. Chromatographic separation of the EtOAc extract from *M. ruber* ENDO–3131

A number of the initial resulting silica VLC fractions displayed bright yellow, orange, and red colors. Natural products that emit intense colors tend to be highly conjugated compounds. ^{1}H NMR data for several of the silica VLC

fractions displayed signals for olefinic or aromatic protons that were consistent with the presence of such metabolites.

The eighth VLC fraction was fluorescent orange, and its ^{1}H NMR spectrum showed signals representing a number of olefinic multiplets, as well as an alkyl side–chain. However, the sample required further purification in order to carry out detailed spectroscopic analysis. Two colorful components were collected upon C_{18} RP–HPLC separation of this VLC fraction (Scheme 3). One component retained the same bright orange color displayed by the parent VLC fraction. The ^{1}H NMR spectrum of this component displayed signals for both unsaturated and saturated carbon chains (Table 9). Signals implying the presence of olefins included a doublet of quartets at δ 6.57 and a doublet of doublets at δ 1.93 for a methyl group to which it was coupled. Another, similar doublet of quartets was found at δ 6.02, along with three other signals representing either aromatic or olefinic protons. Quite a bit of signal overlap existed in the upfield region of the spectrum where signals representing the saturated aliphatic portion of the molecule were found, however, signals for two more methyl units (vinyl methyl doublet at δ 1.69 and a triplet at 0.87) and a methylene unit (doublets of triplets at δ 2.89 and 2.95) were discernable. The methyl triplet at δ 0.87 most likely represented the terminal methyl protons of a

Table 9. ^{1}H and ^{13}C NMR data for rubropunctatin (**30**) and ^{1}H NMR data for monascin (**31**)

	30		**31**
Position	δ_C[a]	δ_H[b] (mult., *J* in Hz)	δ_H[b] (mult., *J* in Hz)
1	18.7 (q)	1.93 (dd, 1.6, 7.0)	1.85 (dd, 1.5, 7.0)
2	136.4 (d)	6.57 (dq, 16,7.0)	6.49 (dq, 16, 7.0)
3	109.5 (d)	6.02 (br dq, 16, 1.7)	5.88 (br dq, 16, 1.6)
4	156.4 (s)	-	-
5	122.4 (d)	6.87 (d, 1.1)	5.25 (s)
6	113.3 (s)	-	-
7	104.2 (d)	6.12 (s)	2.59 (ddd, 7.2, 7.2, 18)
			2.99 (ddd, 7.5, 7.5, 18)
8	116.3 (s)	-	3.21 (ddd, 4.2, 12, 13)
9	85.8 (s)	-	-
10	190.8 (s)	-	-
11	141.6 (s)	-	-
12	152.8 (d)	7.84 (m)	4.70 (br d, 13)
			5.05 (dd, 1.2, 13)
13	28.3 (q)	1.69 (s)	1.43 (s)
14	171.5 (s)	-	-
15	169.2 (s)	-	3.63 (d, 13)
16	197.4 (s)	-	-
17	41.6 (t)	2.89 (dt, 17, 7.3)	2.41 (br dd, 12, 18)
		2.95 (dt, 17, 7.3)	2.66 (ddd, 1.1, 4.2, 18)
18	31.4 (t)	1.60 (m)	1.60 (m)
19	28.3 (t)	1.30 (m)	1.30 (m)
20	23.4 (t)	1.30 (m)	1.30 (m)
21	13.9 (q)	0.87 (t, 7.0)	0.88 (t, 7.0)

[a]100 MHz; $CDCl_3$. Carbon multiplicities were determined from a DEPT experiment. [b]400 MHz; $CDCl_3$.

saturated hydrocarbon chain, while the methylene protons were tentatively placed alpha to a carbonyl unit. Four other methylene signals were found at

approximately δ 1.30 and two more at δ 1.60. These signals, along with the previously described methyl and methylene units could comprise an acyl chain six carbons in length. Further information was needed to completely elucidate the unsaturated portion of this compound and to confirm the identity of the aliphatic side chain.

Because MS capability was temporarily unavailable, ^{13}C NMR and DEPT data were collected (Table 9). ^{13}C NMR data displayed resonances for 21 carbons, consisting of three methyls, four methylenes, five methines, and nine non-protonated carbons based on DEPT data. Two of the non–protonated carbons were determined to be the carbonyl carbons of α,β–unsaturated ketones based on their chemical shifts at δ 197.4 and 190.8. Signals at δ 171.6 and 169.2 were thought likely to be acyl carbons of esters. A literature search incorporating 21 carbons, 22 hydrogens, and 5 or 6 oxygens, as well as a vinyl methyl and α,β–unsaturated ketone group helped to identify the orange compound as the known fungal metabolite rubropunctatin (**30**).[189,190] The absolute configuration of rubropunctatin was originally determined as shown from circular dichroism experiments.[191]

Rubropunctatin (**30**) contains three conjugated carbonyls, two of which correspond to α,β unsaturated ketones and one that corresponds to an ester. The carbon signal at δ 169.2 originally thought to be that of a second ester carbon was

determined to be an oxygenated olefinic carbon. The NMR data collected were similar to data previously reported for rubropunctatin (**30**).[192,193] However, Table 9 provides more accurate and detailed ^{13}C and ^{1}H NMR data than previous reports, and also includes signal assignments that were not provided in prior literature accounts.

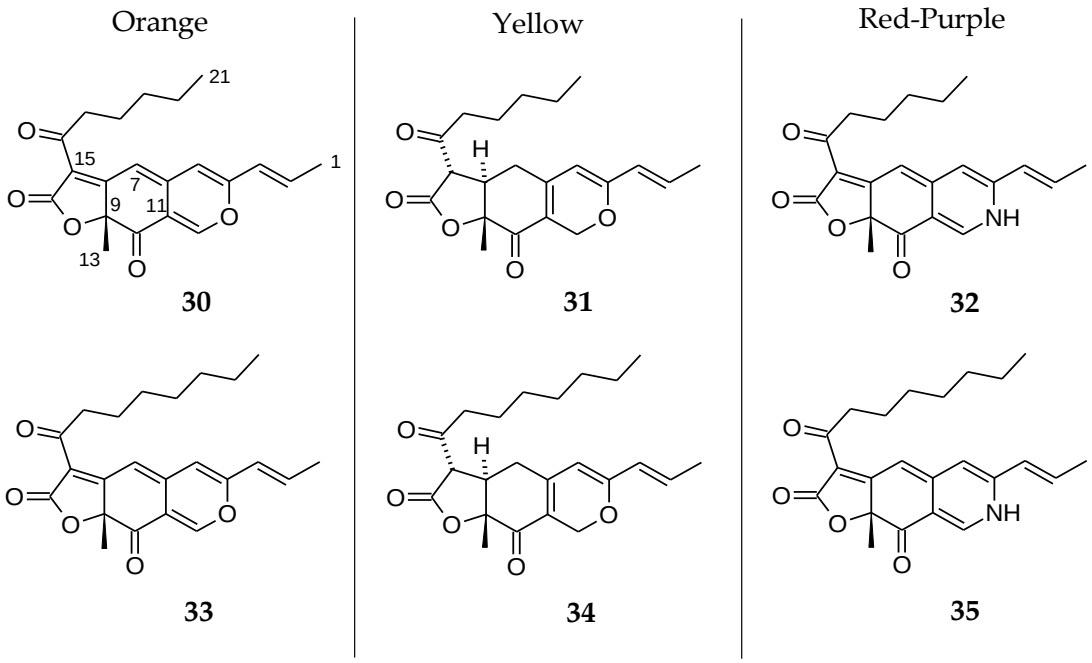


Figure 5. Common *Monascus* spp. pigments: rubropunctatin (**30**), monascin (**31**), rubropunctamine (**32**), monascorubrin (**33**), ankaflavin (**34**), and monascorubramine (**35**)

Rubropunctatin (**30**) was first described in 1959 by Haws and coworkers through chemical derivatization, UV, IR, and elemental analysis.[190] Compound **30** has since been reported from a number of *Monascus* spp., including *M. ruber*,

M. anka, *M. pilosus*, and *M. purpureus*.[193-198] Rubropunctatin (**30**) is related to a number of azaphilone–type pigments (Figure 5). The only structural difference between compounds of the same color is the length of the alkyl chains. The main differences between the orange and yellow pigments were the reduction of the azaphilone core to a pyran ring system (**30** and **31**; **33** and **34**) while the difference between orange and purple pigments was the replacement of oxygen with nitrogen via Schiff base formation (**30** and **32**; **33** and **35**).

A common occurrence with azaphilone type compounds is the formation of a Schiff base in the presence of ammonia.[199] For example, reaction of the orange pigment rubropunctatin (**30**) with ammonia yields the red/purple product rubropunctamine (**32**; Figure 6).[190]

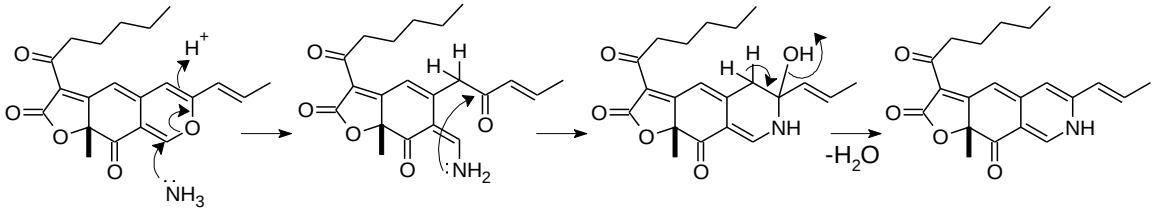


Figure 6. Schiff base formation of rubropunctamine (**32**) from rubropunctatin (**30**)[199]

The second compound collected from HPLC fractionation was a brilliant yellow color. Having already identified the orange compound as rubropunctatin

(**30**), we thought it probable that the yellow pigment was either monascin (**31**) or ankaflavin (**34**). ^{1}H NMR data for the yellow pigment displayed two fewer olefinic proton signals than did rubropunctatin (**29**). However, two oxymethylene protons with a 13–Hz geminal coupling were present at δ 5.05 and 4.70, consistent with reduction of compound **30** at C–12. Another pair of geminal methylene multiplets was found at δ 2.59 and 2.99, representing another possible point of reduction in the azaphilone system. The presence of another methine signal at δ 3.21 was consistent with reduction of the olefin between C–15 and C–8 of **30**. As with rubropunctatin (**30**), the yellow pigment (**31**) was found to have a six–membered acyl chain upon analysis of signal integration in the ^{1}H NMR spectrum. These ^{1}H NMR data were consistent with those published for the yellow pigment monascin (**31**; a.k.a. monascoflavin).[192,193] However, the data reported in the literature contain some minor errors that are corrected in the listing presented here in Table 9.

Monascin (**31**) was first isolated in 1926 along with monascorubrin (**33**) from *M. purpureus*, but spectroscopic data were not reported until much later.[200-205] It has since been reported as a metabolite of other *Monascus* spp., including *M. ruber*.[193,196,204] Assignment of the six–membered acyl chain was originally accomplished through oxidation of monascin (**31**), which afforded hexanoic acid.[203] Ozonolysis of **31** produced acetaldehyde, among other

products.[204] Unlike rubropunctatin (**30**), monascin (**31**) does not undergo a reaction with ammonia, presumably due to its saturation at C–12.[204] The absolute configuration of monascin (**31**) was eventually determined by X–ray crystallographic analysis.[206]

Rubropunctatin (**30**) and monascin (**31**) have been co–isolated several times.[193,196] These compounds have also been isolated alongside other pigments and azaphilone derivatives commonly produced by *Monascus* spp.[193,194,196,198,207-210]

Monascus spp. have been extensively investigated for their abilities to produce pigments for use as natural food colorants.[211-215] In the course of these studies, it was discovered through sodium [1–^{14}C]–acetate and sodium [1–^{14}C]–formate incorporation experiments that both **30** and **31** are products of the polyketide pathway (Figure 7).[190,204,216-218] Their syntheses are accomplished in the cytosol by polyketide synthase I.[219]

After identification of compounds **30** and **31** from ENDO–3131, we thought it probable that the as–yet unidentified source organism was from the genus *Monascus*. Partial DNA sequence analysis and studies of the organism's micromorphology confirmed the identity of ENDO–3131 as an isolate of *M. ruber.*

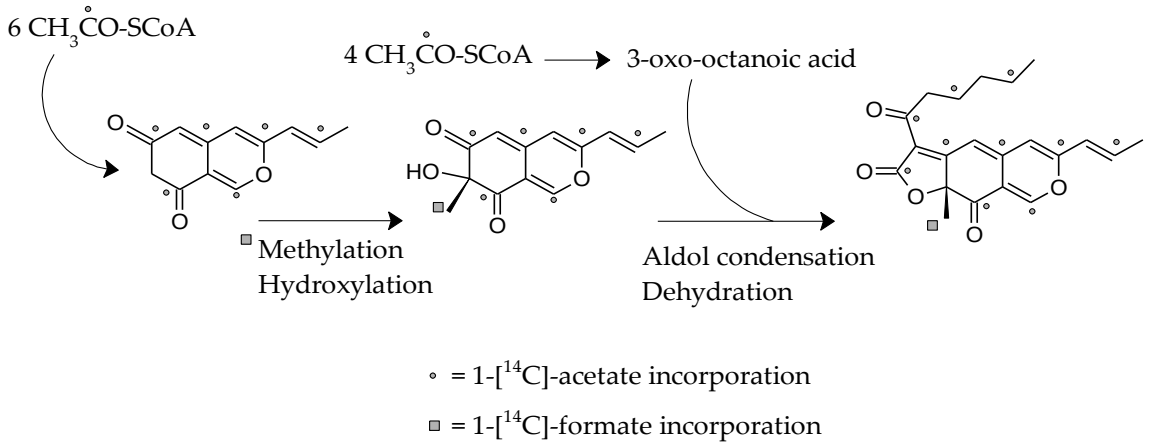


Figure 7. Biosynthetic scheme of rubropunctatin (**30**)[216,217,220]

Monascus spp. are best known for the production of lovastatin (= monacolin K, = mevinolin, **1**), a commercially available cholesterol–lowering agent that has generated billions of dollars for the pharmaceutical industry.[195,223] However, *Monascus* spp. (including *M. ruber*) have been used for hundreds of years in East Asian countries in the production of a number of food products including red rice wine and soybean products.[197,211,221] These fungi have also been used to preserve meat and fish and are also the source of the red pigment in a traditional medicinal product called red mold rice.[197,204,211,222] *Monascus* spp. (including *M. ruber*) have long been fermented with rice to make red mold rice.[195,207-210,223,224] This product is sold worldwide as a supplement, and is now known to contain lovastatin (**1**), reflecting an intriguing correlation between traditional and modern medicine. However, red mold rice is not a legally

approved medicament in the US for lowering cholesterol and cannot be marketed as such.[197] Considerable efforts have been invested in determining culture conditions for various *Monascus* spp. in efforts to maximize the production of lovastatin (**1**) while minimizing the production of toxins[197] or other fungal products.[48,209]

*Biological Activity of Rubropunctatin (**30**) and Monascin (**31**)*

Of the two metabolites described above from *M. ruber* ENDO–3131, only rubropunctatin (**30**) displayed antifungal effects in disk assays against *A. flavus* (200 μg sample/disc: cz 23 mm after 24 hr; mz 17 mm after 48 hr) and *F. verticillioides* (200 μg sample/disc: mz 21 mm after 24 hr; mz 17 mm after 48 hr). Monascin (**31**) was inactive in these assays at 200 μg sample/disc. Both **30** and **31** reduced the growth rate of fall armyworm (*S. frugiperda*). In this case, however, monascin (**31**) was the more potent compound, reducing the growth rate of the fall armyworm by 33% at a dietary level of 220 ppm. Rubropunctatin (**30**) reduced the growth rate by 26% at 740 ppm. These antifungal and antiinsectan effects are comparable to those displayed by the original EtOAc extract of ENDO–3131, and since these compounds were the most abundant metabolites isolated from the extracts of ENDO–3131, it appears that they are likely responsible for most of the antifungal and antiinsectan effects originally observed for the extract.

Several studies have reported on the biological activities of rubropunctatin (**30**) and monascin (**31**), as well as related azaphilone analogs and pigments. Rubropunctatin (**30**) has been reported to display antibacterial effects against *B. subtilis* and *E. coli*[192,194,225] while monascin (**31**) was only mildly active against *B. subtilis*.[192] Both compounds show embryotoxic and teratogenic effects, as well as anti-inflammatory and antitumor activity.[192-194,225] There is only one prior report of antifungal activity of rubropunctatin (**30**), and that was against *Candida pseudotropicalis*.[192] In general, rubropunctatin (**30**) is more active in bioassays than its Schiff base analog, rubropunctamine (**32**).[190] This could be because some of the effects of rubropunctatin (**30**) arise from Schiff base formation with amino groups from various enzymes.

Recently, rubropunctatin (**30**) and monascin (**31**) were tested for their abilities to inhibit and bind Hsp90. Monascin (**31**) was essentially inactive (IC_{50} >100 μM), but rubropunctatin (**30**) was relatively potent (IC_{50} of 0.040 μM).[226] The binding affinity of rubropunctatin is on the same order of magnitude as the aforementioned monorden (**19**; 0.020 μM), obtained from another endophytic fungus as described in Chapter 3.[119] It is possible that rubropunctatin's (**30**) potent binding of the protein is a result of its ability to readily react with amino groups (Figure 6) which monascin (**31**) does not possess. Ultimately it is

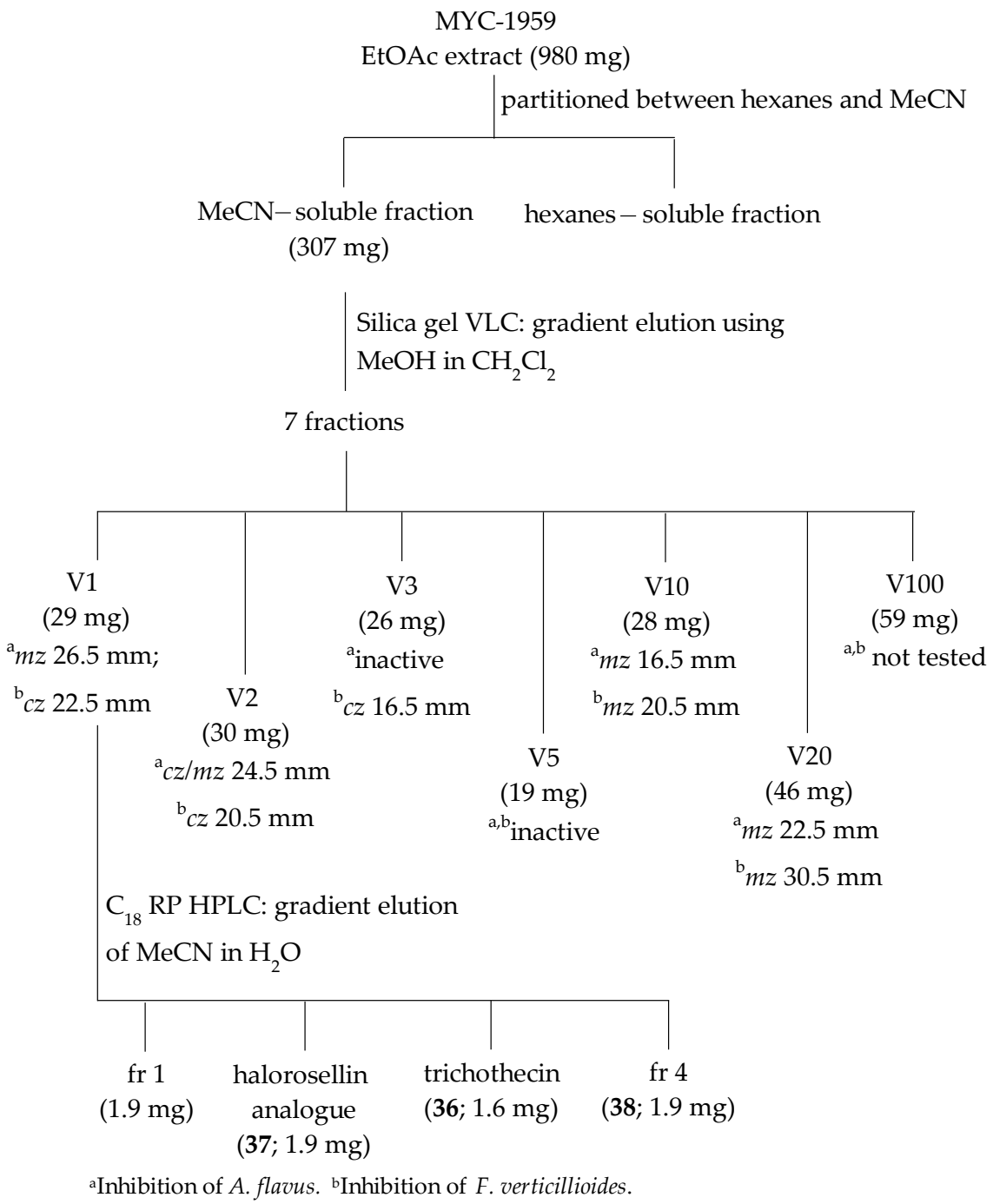
intriguing, and potentially significant, that these studies of another endophytic isolate have afforded yet another potent inhibitor of Hsp90.

A New Bioactive Metabolite Produced by an Isolate of *Acremonium crotocinigenum*

An organic extract from fermentations of a Hawaiian fungicolous isolate (MYC–1959) displayed moderate antifungal effects against *F. verticillioides* and 100% mortality against fall armyworm. The extract was also active in HIF–1 (hypoxia–inducible factor 1) binding assays conducted at the National Cancer Institute. For these reasons, this extract was subjected to a fractionation process resulting in identification of two known fungal metabolites, trichothecin (**36**) and a halorosellin analogue (**37**), as well as a new natural product containing an unusual 7–membered lactone with two exomethylene units (**38**). The following isolation and identification of these metabolites is described here, along with details of their biological activities.

Secondary Metabolites from Acremonium crotocinigenum MYC–1959

Partitioning of the ethyl acetate extract of fermented rice cultures of MYC–1959 (980 mg) resulted in approximately one third of the extract dissolving into an acetonitrile–soluble fraction (307 mg). This fraction was then chromatographed over silica gel VLC using a gradient elution of methanol in dichloromethane (Scheme 4). The seven resulting subfractions were tested



[a]Inhibition of *A. flavus*. [b]Inhibition of *F. verticillioides*.

Scheme 4. Chromatographic separations and antifungal activity of subfractions from the fermented rice extracts of MYC–1959.

against *A. flavus* and *F. verticillioides*. Several fractions showed activity against *A. flavus*. This activity was unexpected because the EtOAc extract of MYC–1959 was inactive in initial *A. flavus* antifungal screenings. It is possible that the abundance of fat–soluble metabolites (approximately two thirds of the EtOAc extract) that are commonly inactive in antifungal assays masked the activity of these materials. Most subfractions also inhibited the growth of *F. verticillioides*. Subfraction V1 inhibited the growth of both *A. flavus* and *F. verticillioides* more than any other subfraction and was therefore the first fraction subjected to further chromatographic analysis.

Reversed–phase HPLC of subfraction V1 resulted in isolation of these pure compounds. ^{1}H NMR data for one of these compounds (V1.3; 1.6 mg) showed signals diagnostic for a pair of isolated methylene epoxide protons having a geminal 4–Hz *J*–value (δ 2.83 and 3.12). Such signals are somewhat characteristic of the trichothecene family of mycotoxins and a literature search led to the identification of this compound as the known metabolite trichothecin (**36**).[29,33] Though trichothecin (**36**) has been thoroughly studied in part due to its toxicity, most of the spectroscopic data available in the literature is restricted to ^{13}C NMR spectra from [1,2–^{13}C]–acetate biosynthetic labeling studies.[31,227-229] Combrinck *et al.* reported the first complete ^{1}H and ^{13}C NMR data analysis with

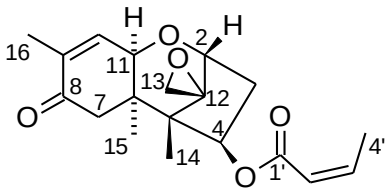


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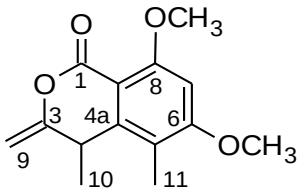
Table 10. ^{1}H NMR data for trichothecin (**36**)

position	^{a} δ_H (mult., J_{HH} in Hz)
1	–
2	3.92 (d, 5.3)
3	2.62 (dd, 7.9, 16)
	2.08 (m)
4	5.56 (dd, 3.7, 7.9)
5	–
6	–
7	2.28 (dd, 1.5, 15)
	2.87 (m)
8	–
9	–
10	6.47 (m)
11	3.94 (br d, 5.7)
12	–
13	2.83 (d, 4.0)
	3.12 (d, 4.0)
14	1.06 (d, 0.9)
15	0.70 (s)
16	1.82 (br s)
1'	–
2'	5.81 (dq, 12, 1.8)
3'	6.32 (dq, 12, 7.4)
4'	2.14 (dd, 1.8, 7.4)

[a]400 MHz; $CDCl_3$

spectral assignments confirmed through ^{1}H–^{1}H COSY and ^{1}H–^{13}C HETCOR spectroscopy.[29]

^{1}H NMR profiles for the two other metabolites isolated from subfraction V1 (**37** and **38**) were significantly less complex than that of trichothecin (**36**). The spectrum of compound **37** contained only eight proton resonances, four of which were for methyl groups (two methoxy singlets, one aryl methyl singlet, and a methyl doublet; Table 11). Two remaining mutually coupled doublets (J = 1.5 Hz) at δ 4.41 and 4.63 represented geminal protons of an olefinic methylene unit. It was recognized at this time that another member of our group had recently elucidated the structure of a compound having the same ^{1}H NMR profile as compound **37**.[41] This metabolite, 6,8-dimethoxy-4,5-dimethyl-3-methyleneisochroman-1-one (**37**) is an analogue of a compound called halorosellin A which has a glucose substituent attached to the C–8 oxygen rather than a methyl group, as in **37**.[41,230]



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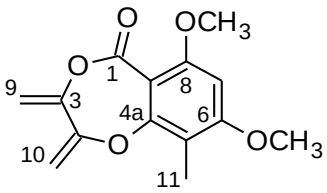

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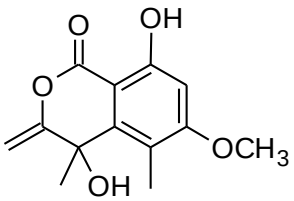

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^{1}H NMR data for the third compound (**38**) isolated from the reversed–phase HPLC separation of subfraction V1 were very similar to those of **37**. While

Table 11. 1D– and 2D–NMR data for 6,8-dimethoxy-4,5-dimethyl-3-methyleneisochroman-1-one (**37**) and lactone **38**

	37	**38**		
position	[a] δ_H (mult., J_{HH} in Hz)	[b] δ_H (mult., J_{HH} in Hz)	[c] δ_C	[d] H→C
1	–	–	[e] –	
3	–	–	156	
4	3.75 (q, 7.0)	–	149	
4a	–	–	153	
5	–	–	111	
6	–	–	162	
7	6.37 (s)	6.29 (s)	92.5	5, 6, 8, 8a, 11
8	–	–	158	
8a	–	–	104	
9	4.41 (d, 1.5)	[f] 4.85 (d, 1.7)	[f] 97.5	3, 4
	4.63 (d, 1.5)	[f] 4.93 (d, 1.7)		
10	1.35 (d, 7.0)	[g] 5.09 (d, 1.8)	[g] 107	3, 4
		[g] 5.14 (d, 1.8)		
11	2.09 (s)	2.08 (s)	8.0	4a, 5, 6
6–OCH_3	3.89 (s)	3.87 (s)	56.5	6
8–OCH_3	3.93 (s)	3.86 (s)	55.8	8

[a] 300 MHz; $CDCl_3$. [b] 600 MHz; $CDCl_3$. [c] ^{13}C–NMR data for **38** (150 MHz; $CDCl_3$). ^{13}C resonances and one–bond ^1H–^{13}C correlations were determined from HMQC data. [d] ^1H–^{13}C HMBC correlations for **38** (600 MHz; $CDCl_3$). [e] Carbon resonance was not observed in 2D data. [f,g] Terminal methylene proton and carbon unit assignments may be interchanged.

there were also eight proton resonances in the spectrum of **38**, signals for a $CHCH_3$ unit were absent, and two additional terminal olefinic methylene doublets were present instead (δ 5.09 and 5.14; Table 11). An unsaturation between C–4 and C–10 in **38** would account for the difference in these ^1H NMR spectra. A literature search did not result in identification of a known metabolite

matching this proposed structure. Therefore, HMQC, HMBC, and MS data were collected to confirm the assignment (Table 11).

HMQC data enabled determination of one–bond proton–carbon correlations, while HMBC data were used to assign the location of substituents on the aromatic ring as well as the two terminal olefin units (Figure 8). ^{13}C NMR

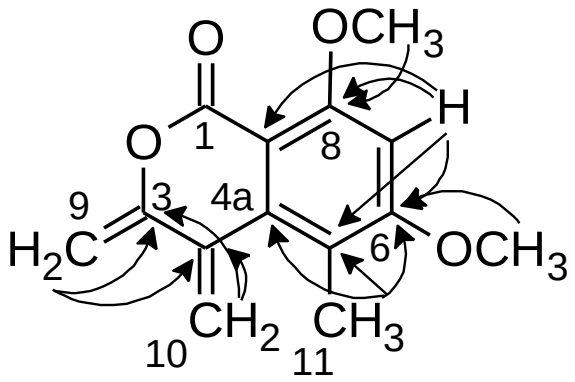


Figure 8. HMBC correlations for the initial structure proposed for **38**

assignments were determined from these 2D–NMR data, although no correlations were observed to the C–1 carbonyl carbon. $H_{6.29}$ showed four HMBC correlations to non–protonated olefinic carbons at δ 162, 158, 111, and 104, likening the presence of an aromatic ring in the structure of **38** (Figure 8). Protons of the aryl methyl singlet at δ 2.08 showed similar correlations to olefinic carbons at δ 153, 111, and 162. A correlation from the signal of one of the

methoxy groups (δ 3.87) to this same carbon at δ 162 positioned it ortho to both the aromatic proton and aryl methyl unit. The other C–8 methoxy unit was placed at this position based on an HMBC correlation from its protons to C_{158}. Concrete assignment of the two terminal methyl units was not possible because the proton signals for both of these units showed correlations to carbons at δ 149 and 156. All of these 2D–NMR data were consistent with a structure similar to **37**, but also having a C4–C10 terminal olefin (Figure 8). This structure would have a molecular formula of $C_{14}H_{14}O_4$ and a molecular mass of 246.26 Da. However, EIMS data produced an apparent molecular ion of 262.16 *m*/*z*, which would correspond to a molecular formula of $C_{14}H_{14}O_5$. Placement of an additional oxygen atom between C–4 and C–4a as shown in structure **38** would account for the downfield shifts of C–4 and C–4a. This would also explain the lack of HMBC correlations between the H_2–10 methylene protons and C–4a, though this is viewed as negative evidence. The proposed structure for **38** is somewhat similar to depsidone metabolites that have been described from lichen, fungal, and plant sources.[61,62,231-235] Depsidones are 7–membered lactones flanked by two highly functionalized aromatic rings joined between carbons C4a–8 and C3–C4 of the lactone ring. Though compound **38** is not a depsidone, it is presumably polyketide–derived, as is the co–occurring analog **37**, and could undergo an oxidation similar to that which leads to depsidone structures.

Another relative of **37** and **38**, 4,8–dihydroxy–6–methoxy–4,5–dimethyl–3–methyleneisochroman–1–one (**39**), has the same carbon skeleton as compound **37**, and is oxidized at C–4, though it does not contain a C4–C10 olefin.[236]

The biological activities of compounds **36** and **37** have been described in previous reports. Trichothecin (**36**) is a broad spectrum antifungal agent showing activity against *C. albicans*, *A. fumigatus*, *A. niger*, and *F. graminearum*, among others species.[30-32,34] Though it is a potent antifungal agent, trichothecin (**36**) was inactive in antibacterial assays against *S. aureus*, *B. subtilis*, and *E. coli*.[31] Thoughts of using trichothecin (**36**) as a treatment for fungal infections were quickly abandoned due to its toxicity in animal testing.[30] 6 ,8-Dimethoxy-4,5-dimethyl-3-methyleneisochroman-1-one (**37**) was reportedly inactive in antibacterial (*B. subtilis* and *S. aureus*) and antifungal (*C. albicans* and *A. flavus*) assays, though it did generate a 30–mm inhibition zone of fungal growth at 100 μg/disc in assays against *F. verticillioides*.[41] Compound **38** was inactive against *F. verticillioides* at this level, but was mildly active against *A. flavus*, causing a growth inhibition zone of 16.5 mm in assays at 100 μg/disc. Compound **38** likely contributes to antiinsectan activity of the MYC–1959 extract as it caused a 33% reduction in the fall armyworm's growth rate in comparison with controls at a dietary level of 148 ppm.

Compound **37** was the most abundant metabolite from the acetonitrile–soluble partition from the EtOAc extract of MYC–1959 while compound **38** and several other unidentified simple aromatic compounds comprised the majority of the rest of the extract on the basis of ^{1}H NMR analysis of chromatographic fractions. Trichothecin (**36**) was only detected in the first two VLC subfractions as a minor metabolite and therefore may not play a major role in the biological activity of this extract. It is likely that compounds **37** and **38** are the main source of antifungal and antiinsectan activity displayed by MYC–1959 crude extracts.

It was later discovered after partial DNA sequence analysis of MYC–1959 that it is an isolate of *Acremonium crotocinigenum* (NRRL 45419, CBS 120950).[237] It is therefore an isolate of the same species (MYC–1590; NRRL 40192) that produced compound **37** in the previously described work, though this isolate was obtained from a basidioma of an *Earliella scabrosa* host, and MYC–1959 was collected from a basidioma of a *Phellinus gilvus* host.[41] Though both MYC–1590 and MYC–1959 were shown to produce compound **37**, MYC–1959 had a wider variety of observed biological activity, presumably because of the presence and abundance of compound **38**.[41]

CHAPTER 5

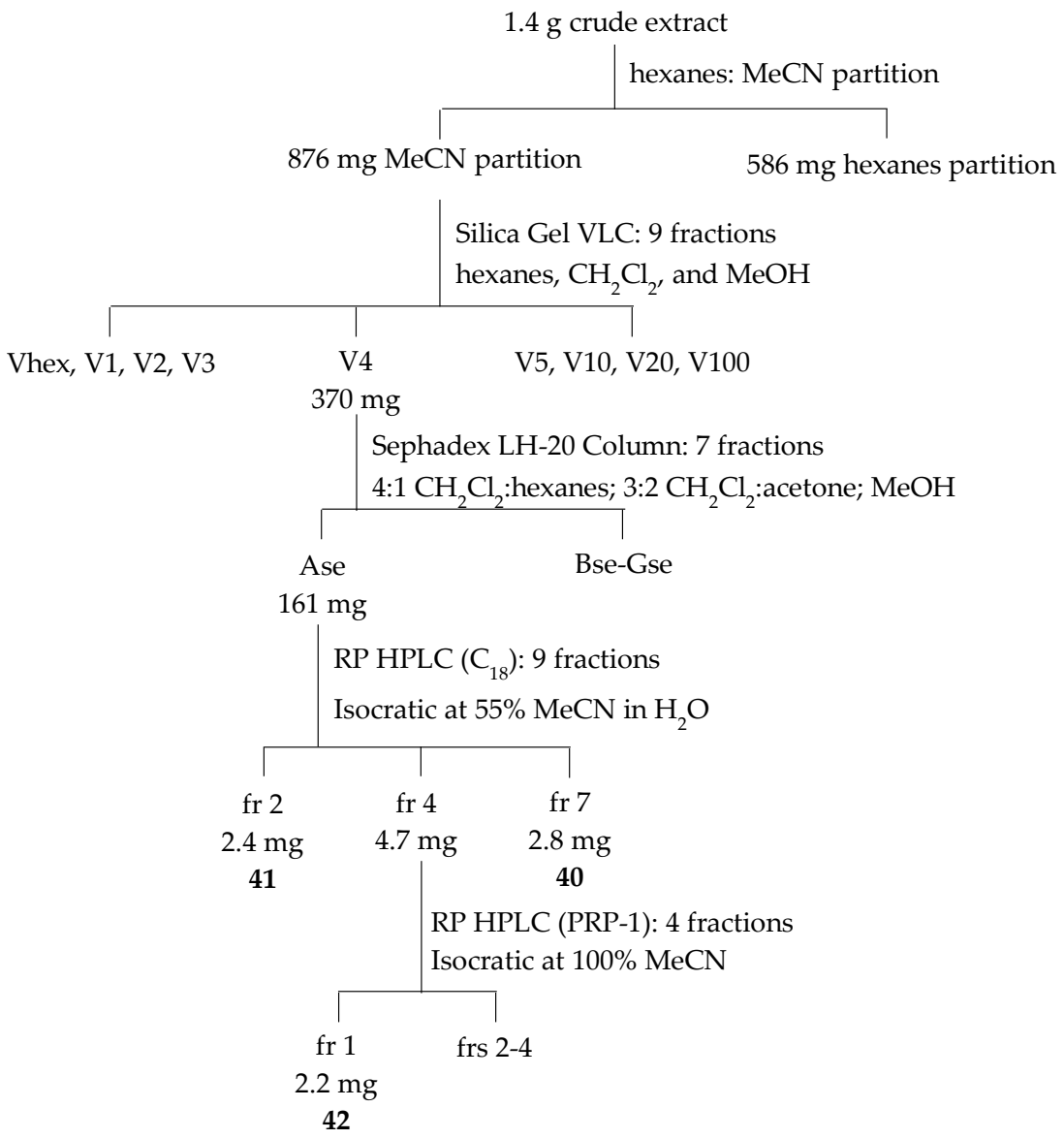
PHOMACTIN ANALOGUES FROM A HAWAIIAN FUNGICOLOUS FUNGAL ISOLATE

The Hawaiian Islands play host to a wide variety of biologically diverse organisms in part due to their tropical location and volcanic climate.[238] Ongoing studies of Hawaii's fungal ecology have resulted in the identification of over 3000 fungal species.[238] Many of these fungi compete with each other for survival and growth. This competition often results in the colonization of a fungal host by other parasitic fungal species, some of which may be parasitic. The host–parasite relationship can either be described as mycoparasitic or fungicolous. Mycoparasitic fungi are proven parasites to their fungal hosts while fungicolous fungi are only known to colonize fungal hosts. Regardless of the label applied, either the fungal host or the colonist may produce antifungal compounds in a process of "chemical warfare" to ward off the attacking species or to facilitate invasion and colonization. With these concepts in mind, chemical investigations of fungicolous and mycoparasitic fungi from Hawaii were undertaken by our research group, and have resulted in the identification of many antifungal compounds, some of which have new chemical structures.[37,41-44,81] Thus, the research described in this Chapter is part of an ongoing investigation of secondary metabolites from fungi that grow in association with fungal hosts.

Several fungicolous fungal specimens were found as colonists of a white mycelial host growing on the undersurface of a dead hardwood branch in a Hawaiian coastal forest. One of these fungicolous isolates, MYC–1969, was grayish–black in color and did not sporulate when cultured. For this reason, the yet unidentified MYC–1969 specimen was characterized as *Mycelia sterilia*. Ethyl acetate extracts from solid–substrate fermentation cultures of MYC–1969 showed antifungal activity against *A. flavus* and *F. verticillioides* and antiinsectan effects against *Spodoptera frugiperda*. The isolation and identification of antifungal and antibacterial metabolites from these MYC–1969 extracts is presented here.

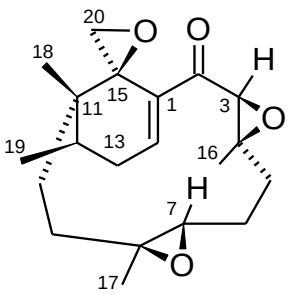
Secondary Metabolites from an Unidentified Fungicolous Isolate: MYC–1969

The solid–substrate fermentation mixture of MYC–1969 was extracted with EtOAc to afford 1.4 g of crude extract. In an effort to isolate and identify the metabolites responsible for the antifungal and antiinsectan activity displayed by these extracts, several chromatographic techniques were employed, resulting in the discovery and structure determination of three new phomactin analogues (**40**–**42**; Scheme 5). We propose the names phomactins K, L, and M (**40**–**42**) for these metabolites, following in sequence with previously described compounds from this family.



Scheme 5. Chromatographic separation of the EtOAc extracts from the fermentation of MYC–1969

Compound **40** was determined to have the molecular formula of $C_{20}H_{28}O_4$ (7 unsaturations) based on ESIMS and NMR data. The ^{1}H NMR spectrum displayed a trisubstituted olefin signal, an oxymethine singlet, a doublet between



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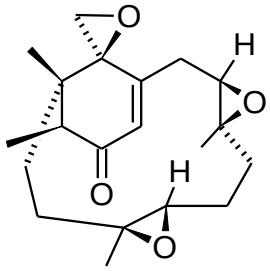

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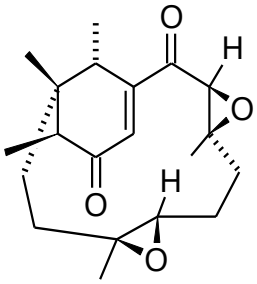

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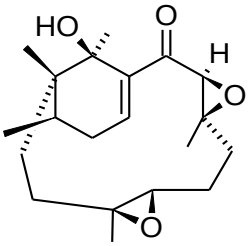

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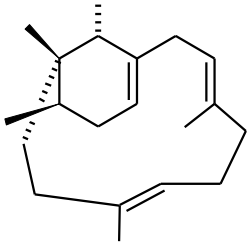

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δ 3 and 4, geminal protons of an oxymethylene unit, three methyl singlets, and a methyl doublet. There were also numerous diastereotopic methylene protons and a methine multiplet that were assignable only after HMQC and HMBC analysis (Table 12). ^{13}C NMR assignments were established on the basis of HMQC and HMBC data and revealed the presence of four quaternary carbons and an α,β-unsaturated ketone unit (Table 12). The planar structure of compound **40** was constructed by detailed analysis of HMBC data, as summarized in Figure 9, and was found to contain the same skeletal structure as the phomactin family of compounds,[240,241] with a composition particularly similar

Table 12. ^{1}H and ^{13}C NMR data for phomactins K (**40**) and L (**41**)

	Phomactin K (**40**)		Phomactin L (**41**)	
position	δ_C [a]	δ_H (mult., *J* in Hz) [b]	δ_C [a]	δ_H (mult., *J* in Hz) [b]
1	140.6		151.3	
2	196.6		32.2	2.08, m
				2.58, dd (2.9, 14)
3	65.0	3.57, s	61.8	2.62, dd (2.9, 11)
4	63.3		60.4	
5	36.1	1.12, m	36.2	0.97, m
		2.29, ddd (3.0, 4.4, 13)		2.19, m
6	24.3	1.50, m	24.3	1.44, m
		2.17, m		2.10, m
7	56.0	3.01, dd (4.0, 11)	55.8	2.80, dd (4.1, 11)
8	59.0		58.4	
9	28.8	1.76, dd (8.2, 16)	28.1	1.64, m
		2.09, m		1.99, m
10	27.8	1.17, m	30.7	1.14, m
		1.32, m		1.28, m
11	38.2		40.2	
12	38.8	1.68, m	55.1	2.19, m
13	32.2	2.11, dd (4.3, 21)	202.8	
		2.68, ddd (3.0, 6.8, 21)		
14	138.5	6.22, m	132.9	6.06, s
15	56.4		56.9	
16	13.8	1.29, s	15.4	1.44, s
17	19.2	1.30, s	19.2	1.23, s
18	16.5	0.83, s	17.0	0.87, s
19	17.6	1.10, d (7.3)	14.5	1.20, d (7.6)
20	48.3	3.05, d (3.5)	47.7	3.10, d (3.6)
		3.07, d (3.5)		3.24, d (3.6)

[a]150 MHz; $CDCl_3$. [b]600 MHz; $CDCl_3$.

to that of the known fungal metabolite phomactin F (**43**).[239] However, **40** was determined to be a new analogue of this family.

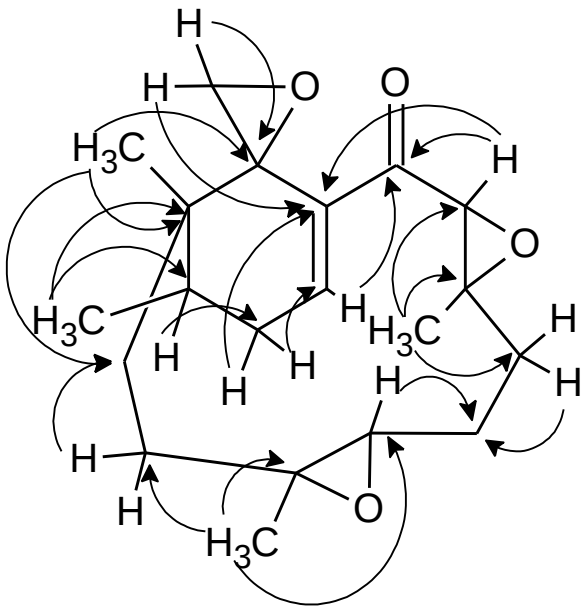


Figure 9. Key HMBC correlations for phomactin K (**40**)

Key distinguishing features of the ^{1}H NMR data for **40** include an AB pattern centered at δ 3.05 which is characteristic of isolated geminal epoxide protons (J = 3.5 Hz). This readily distinguishes compound **40** from all other members of the phomactin family. While 14 of the 16 previously published phomactins contain epoxides, none contain the geminal epoxide protons at C–20 of compound **40**.[240,241] Another feature of the NMR data for **40** is a 21-Hz geminal coupling constant displayed by the H_2-13 methylene signals. The ^{13}C NMR data

for **40** were consistent with those observed for similarly substituted phomactins, except for the somewhat unusual upfield chemical shift of C–14, which could be attributed to the $C_{1\text{-}14}$ enone system being in a twisted state.[239] Compound **40** was named phomactin K (**40**) after its previously described fungal metabolite relatives.

The relative configuration of phomactin K (**40**) was assigned based on NOESY data (Figure 10), and was found to be analogous to that of phomactin F (**43**) and other closely related phomactins.[239] The $C_{3\text{–}4}$ and $C_{5\text{–}6}$ epoxides of **40** were determined to be *trans* substituted based on the H–3 to H–$5_{1.12}$ and H_3–17 to H–$6_{1.50}$ correlations. A correlation between the H_3-18 and H_3-19 proton signals indicated that these methyls have a *cis* relative orientation on the cyclohexene ring. A correlation between H–12 and H_2–10 confirmed the relative configuration assigned to the stereocenter at C–12.

The $C_{15\text{-}20}$ geminal epoxide is new to the phomactin family and its relative configuration was also determined via NOESY analysis. A NOESY correlation between H-$20_{3.05}$ and H-$9_{1.76}$ placed C-20 on the opposite face of the cyclohexene ring system as the C-18 and C-19 methyl groups. A correlation was also observed between epoxide protons H–$20_{3.06}$ and H–$3_{3.57}$, further supporting the relative configuration assigned to **40** in Figure 10. Because phomactin K (**40**) was found to possess a relative configuration as phomactin F (**43**), the absolute

configuration was inferred by analogy as shown,[239] although this was not independently verified.

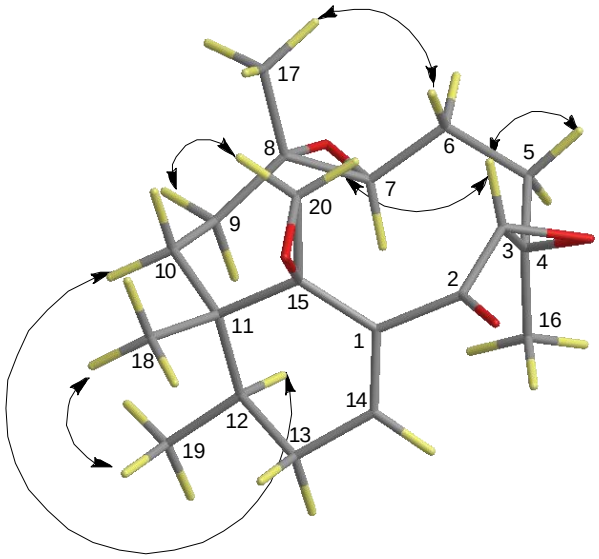


Figure 10. Selected NOESY correlations for phomactin K (**40**)

Compound **41** was found to be an isomer of **40** ($C_{20}H_{28}O_4$; 7 unsaturations) based on HRESIMS data and the ^{1}H NMR data for **41** closely resembled those of **40** (Table 12). A notable difference was the absence of the C–13 methylene protons and the presence of a pair of methylene protons that seemed likely to be at C–2 based on their chemical shifts and *J*–values. In addition, chemical shift differences for the cyclohexene ring atoms of compounds **40** and **41** are consistent with the presence of a ketone carbonyl at C–13 of compound **41**. These

structural changes were confirmed by HMBC data, including correlations of H_2–2 with C–1 and C–3, as well as H–12 with C–13. Relative configuration for compound **41** was assigned by analogy to that of compound **40**. Compound **41** is also a new fungal metabolite for which we propose the name phomactin L (**41**).

Compound **42** also had a molecular formula of $C_{20}H_{28}O_4$ (7 unsaturations), based on HRESIMS data. Like phomactins K (**40**) and L (**41**), compound (**42**) contains C7–C8 and C3–C4 epoxides. However, it lacks the geminal epoxide protons at C–20. Instead, a $CHCH_3$ unit is present. In addition, the signal for H-12 in **42** is a doublet of quartets arising from a vicinal 7.9 Hz coupling to H_3-19 and a four-bond *w*-coupling of 3.1 Hz to olefinic H-14; both of which are not observed in the spectra of **40** or **41**. These couplings were confirmed through ^{1}H NMR decoupling experiments. Irradiation at δ 3.11 simplified the signals at δ 5.78 to a doublet and 1.45 ppm to a singlet, while irradiation of the methyl doublet signal at δ 1.05 simplified a multiplet δ 2.10 which corresponded to the methine proton of the $CHCH_3$ unit. The ^{13}C NMR and DEPT spectra reflect the presence of a fifth methyl unit, a new methine carbon, and a second ketone carbonyl, while lacking signals for a 1,1-disubstituted epoxide (Table 13). HMBC data were used to assign the planar structure of **42**. Location of carbonyls C–2 and C–13 were confirmed by HMBC correlations between H–3 and C–2 and

Table 13. NMR spectroscopic data for phomactin M (**42**)

position	δ_C, mult.[a]	δ_H (mult., *J* in Hz)[b]
1	162.6, qC	
2	198.4, qC	
3	66.7,CH	3.71, s
4	63.6, qC	
5	35.5, CH_2	1.25, m
		2.30, ddd (3.5, 5.0, 14)
6	24.6, CH_2	1.49, m
		2.15, m
7	57.4, CH	2.91, dd (3.9, 10)
8	60.1, qC	
9	30.4, CH_2	1.77, m
		2.05, m
10	30.2, CH_2	1.22, m
		1.50, m
11	42.7, qC	
12	36.4, CH	3.11, dq (3.1, 7.9)
13	201.6, qC	
14	126.1, CH	5.78, dd (0.8, 3.1)
15	54.8, CH	2.10, m (7.5)
16	15.1, CH_3	1.36, s
17	18.5, CH_3	1.25, s
18	23.6, CH_3	1.09, s
19	14.0, CH_3	1.45, d (7.9)
20	11.8, CH_3	1.05, d (7.5)

[a]150 MHz; $CDCl_3$. Carbon multiplicity determined from DEPT experiments (100 MHz; $CDCl_3$). [b]600 MHz; $CDCl_3$.

between H_3–19 and C–13. The presence of the new $CHCH_3$ unit was supported by a correlation from H_3–20 to C–15. The configuration at C–15 was assigned as shown on the basis of a NOESY correlation between H–12 and H_3–20. Correlations between H_3–18 and H_3–19 and H–15 placed these units on the opposite face of the cyclohexene ring, as shown. Orientation of the C3–C4 and C7–C8 epoxides was determined from NOESY correlations between H_3–17 and H_2–6 and between H–3 and H_2–5. This relative configuration matches that of the corresponding stereocenters in the related known phomactin–type compound Sch 49026 (**44**).[242] The name phomactin M is proposed for **42**. A fourth HPLC fraction appeared to contain the known compound phomactin F (**43**) based on analysis of its ^{1}H NMR data. However, this was a sub–milligram fraction existing as a mixture of components, so efforts at further purification were not undertaken.

Phomactins K–M (**40**–**42**) were tested in disc assays against *A. flavus*, *Candida albicans*, *E. coli*, and *S. aureus* (100 μg/disc), and the results are summarized in Table 14. Phomactin K (**40**) showed activity against *A. flavus*, but did not significantly reduce fungal growth in more quantitative MIC assays against this organism at levels up to 50 μg/mL. Though phomactin K (**40**) was not as potent an antifungal agent as the antifungal standards, it appears to be a contributing factor in the activity displayed by the original MYC-1969 extract.

Table 14. Antifungal and antibacterial effects of phomactins K–M (**40**–**42**)

Sample	*A. flavus*	*C. albicans*	*E. coli*	*S. aureus*
Phomactin K (**40**)	rg 32	(-)	rg 12	cz 9
Phomactin L (**41**)	(-)	(-)	rg 20	(-)
Phomactin M (**42**)	(-)	(-)	(-)	(-)
Nystatin	cz 18 + rg 20	nt	nt	nt
Filipin	nt	cz 19	nt	nt
Gentamicin	nt	nt	cz 40	cz 35

cz = clear zone; rg = reduced growth; nt = not tested

Compound **40** was also mildly active against the bacterial pathogens *E. coli* and *S. aureus*, but was not as potent as the antibacterial standard gentamicin. Phomactin M (**42**) showed no activity in these assays at the levels tested, and phomactin L (**41**) displayed only mild activity against *E. coli*. Phomactins K– M (**40**–**42**) were not tested for their activity against *F. verticillioides* and the fall armyworm because the fraction from which these compounds were isolated was not active in these assays.

Phomactins are diterpenes that were originally reported as PAF (platelet activating factor) antagonists, with phomactin D being the most potent metabolite (inhibition of platelet aggregation IC_{50} 0.80 μM; inhibition of binding IC_{50} 0.12 μM).[243-245] Derivatization at C–2, C–7/8, and C–20 revealed that a β–hydroxy group at C–2 increases PAF antagonism. Epoxidation at C7–C8

decreased the activity, and acetylation of the hydroxyl group at C–20 increased PAF antagonism.[245] Phomactins K–M (**40–42**) all contain C7–C8 epoxide units and would presumably be less potent PAF antagonists. However, it is not known how epoxidation at C-20 would affect this activity.

All but four of the published phomactins have been reported from a marine *Phoma* sp. (SANK 11486) that was collected from the shell of a crab (*Chionoecetes opilio*) from the waters of Fukui Prefecture, Japan.[240,241] The other four analogues were derived from an unidentified marine fungus (MPUC 046) collected from the surface of the marine brown alga *Ishige okamurae* collected from the Tateishi Kanagawa Prefecture of Japan.[241] Genomic analysis of the MPUC 046 isolate in comparison with the SANK 11486 isolate revealed that the two organisms are not closely related phylogenetically.[241] Though all other phomactins originated from marine waters in Japan, MYC–1969 is of a Hawaiian terrestrial origin. Unfortunately, cultures of the source organism of phomactins K–M (**40–42**; MYC–1969) did not retain viability, and could not be identified.

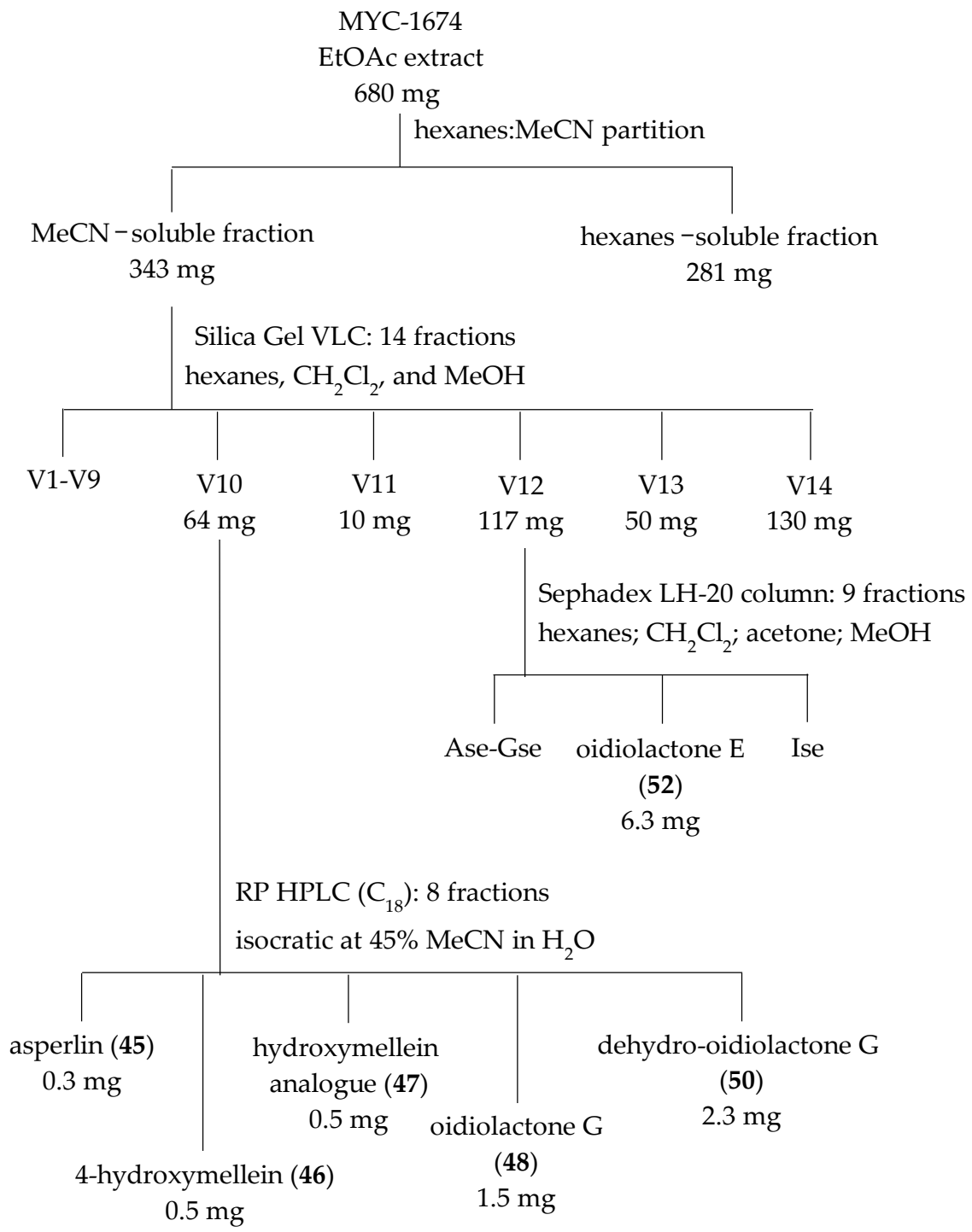
CHAPTER 6

ANTIFUNGAL DITERPENOID AND OTHER METABOLITES FROM A HAWAIIAN FUNGICOLOUS ISOLATE OF *NEOFUSICOCCUM PARVUM*

Continuing our studies of Hawaiian mycoparasitic and fungicolous fungi, several isolates were found colonizing the basidioma of a *Rigidoporus microsporus* host that was growing on a dead hardwood branch found in an Alien Wet Forest at the Hilo Zoo in Hawaii. Organic extracts from one isolate obtained from this sampling (MYC–1674 = NRRL 46122) inhibited the growth of *F. verticillioides,* thereby leading to chemical investigation of this organism. These studies led to the isolation and characterization of eight fungal metabolites and a new compound generated as an artifact of the isolation process. Details of this work are presented in this chapter.

Secondary Metabolites from *Neofusicoccum parva* MYC–1674

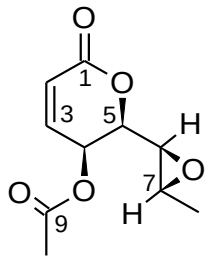
The evaporated EtOAc extracts from fermented rice cultures of MYC–1674 (680 mg) were subjected to several types of chromatography in an effort to purify the metabolites present (Scheme 6). The crude extract was partitioned between hexanes and acetonitrile, and the acetonitrile-soluble portion was then subjected



Scheme 6. Chromatographic separation of the fermentation extract from MYC–1674 (NRRL 46122).

to silica gel VLC. Three of the VLC fractions comprised a majority of the original sample's mass and were found to be complex mixtures upon analysis of their ^{1}H NMR data.[246]

One of these fractions (V10; 64 mg) was further separated by C_{18} RP–HPLC, eluting isocratically with 45% acetonitrile in water, to afford five metabolites which were identified by analysis of NMR and MS data. Three of these metabolites were readily identified as the known, simple fungal metabolites: asperlin (**45**; 0.3 mg), 4-hydroxymellein (**46**; 0.5 mg), and 5,8–dihydroxy–3–methyl–3,4–dihydroisocoumarin (**47**; 0.5 mg) based on comparison of their ^{1}H NMR spectra with published data (Tables 15– 16).[246-250]



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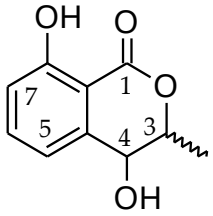

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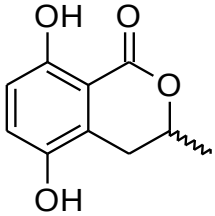

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Table 15. ^{1}H NMR data for asperlin (**45**)

Position	[a] δ_H (mult., J_{HH} in Hz)
1	–
2	6.20 (dd, 0.6, 9.8)
3	6.84 (dd, 5.2, 9.8)
4	5.49 (ddd, 0.6, 3.6, 5.2)
5	4.34 (dd, 3.6, 4.9)
6	2.99 (dd, 2.2, 4.9)
7	3.03 (dq, 2.2, 5.2)
8	1.32 (d, 5.2)
9	–
10	2.13 (s)

[a]400 MHz; $CDCl_3$

Table 16. ^{1}H NMR data for 4–hydroxymellein (**46**) and 5,8–dihydroxy–3–methyl–3,4–dihydroisocoumarin (**47**)

	46	**47**
Position	[a] δ_H (mult., J_{HH} in Hz)	[a] δ_H (mult., J_{HH} in Hz)
1	–	–
2	–	–
3–Me	1.51 (d, 6.2)	1.54 (d, 6.4)
3	4.55 – 4.63 (m)	4.68 (ddq, 3.5, 12, 6.4)
4	2.02 (br d, 6.0; OH)	2.67 (ddd, 0.8, 12, 17)
	4.55 – 4.63 (m)	3.12 (dd, 3.5, 17)
5	7.01 (m)[b]	4.45 (br s)
6	7.53 (dd, 7.6, 8.4)	6.78 (dd, 0.8, 9.0)
7	6.99 (m)[b]	6.96 (d, 9.0)
8	10.99 (s)	10.59 (s)

[a]400 MHz; $CDCl_3$ [b]These signals may be interchanged.

The other two compounds isolated by RP–HPLC of fraction V10 (**48** and **50**) appeared to be significantly more complex than compounds **45–47** upon analysis of their ^{1}H NMR data. In order to elucidate the structure or the first of these compounds (**48**), MS and 1D and 2D–NMR data were collected.

^{1}H NMR data for compound **48** displayed signals for an oxymethine, two oxymethylene protons with a large geminal coupling, a trisubstituted olefin, and two methyl groups bound to quaternary carbons. The upfield region of the spectrum also displayed six diastereotopic methylene multiplets between δ1 and 2.15 that were only assignable after HMQC and HMBC analyses (Table 17). ^{13}C NMR data displayed 16 resonances, including signals for two carboxy-type carbonyl carbons and two olefinic carbons. Comparison with DEPT data assisted in the assignment of two quaternary sp^{3} carbons and confirmed the trisubstituted nature of the olefinic unit (Table 17).

The ^{1}H NMR data for compound **48** suggested this metabolite to be of terpenoid origin. However, sixteen–carbon molecules are more typically encountered as products of polyketide biosynthesis. These conflicting concepts led to further ambiguity when trying to determine the structure of compound **48**. To confirm that all sixteen resonances observed in the ^{13}C NMR spectrum were those of compound **48**, MS data were collected. ESIMS data displayed an $(M+H)^{+}$ ion at *m*/*z* 277, which could correspond to a molecular formula of $C_{16}H_{20}O_{4}$. This

formula would be consistent with seven degrees of unsaturation, accommodating the two carbonyls and the olefin unit, and requiring four rings. Eventually, HMQC and HMBC data were used to assign the planar structure of compound **48**. Key HMBC correlations are shown in Figure 11. These data permitted assignment of the structure of **48**, though they did not provide direct evidence for the connection of C–5 and C–6. However, irradiation of H–6 (δ 4.94) in a ^{1}H NMR decoupling experiment simplified the 5.1-Hz doublet at δ 1.82 (H–5) to a singlet, thereby confirming this connection of C5 and C6 and completing the gross structure of compound **48** shown in Figure 11.

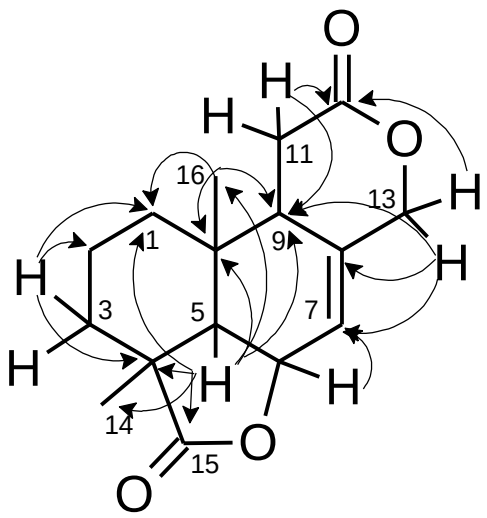


Figure 11. Selected HMBC correlations for oidiolactone G (**48**)

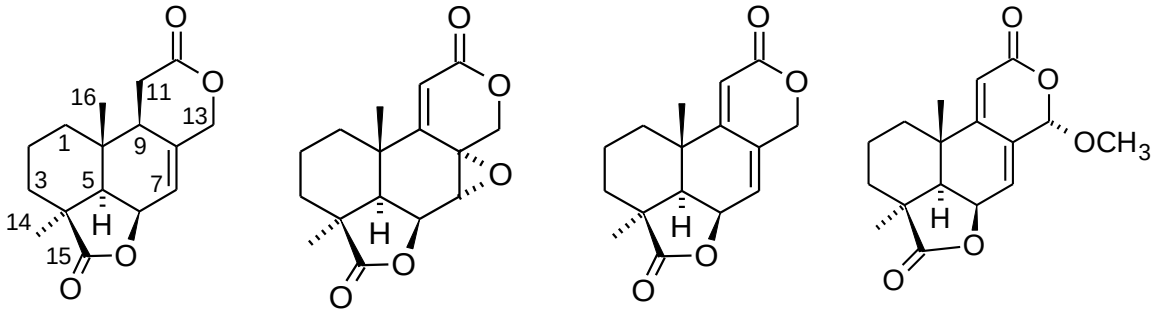
Table 17. NMR data for oidiolactone G (**48**) and dehydro–oidiolactone G (**50**)

	Oidiolactone G (**48**)				dehydro–oidiolactone G (**50**)
Position	[a,b] δ_C	[b,c] δ_H (mult., J_{HH} in Hz)	[d] $^1H–^1H$ decoupling results	[c] $^1H–^{13}C$ HMBC correlations (H → C)	[e] δ_H (mult., J_{HH} in Hz)
1	32.4	1.27 (m)	H_2–1b	2, 3, 5, 9, 10, 16	1.65 (m)
		1.59 (m)			1.72 (m)
2	17.7	1.58 (m)			1.68 (1H, m)
		1.75 (m)	H_2–1a, H_2–2a, H_2–3b, H–6	1, 3, 4, 10	1.85 (m)
3	27.9	1.52 (m)			1.54 (m)
		2.15 (m)	H_2–1b, H_2–2b, H_2–3a	1, 2, 4, 5, 14, 15	2.25 (m)
4	42.6	–			–
5	51.3	1.82 (d, 5.1)	H–6	1, 4, 9, 10, 14, 15, 16	1.93 (d, 4.7)
6	72.4	4.94 (m)	H–7, H–5	7, 8, 10	5.01 (m)
7	119.3	6.04 (m)	H–6, H_2–13a, H_2–13b		6.19 (m)
8	138.5	–			–
9	45.0	2.36 (m)	H–6, H_2–11b, H_2–13a, H_2–13b		–
10	33.7	–			–
11	29.3	2.39 (dd, 10, 15)	H_2–11b	8, 9, 10, 12 ,13 [f]	5.76 (d, 1.7)
		2.61 (dd, 5.5, 15)	H_2–11a	8, 9, 10, 12 ,13 [f]	–
12	172.5	–			–
13	69.9	4.73 (ddd, 1.8, 3.5, 14)	H_2–13b	7, 8, 9, 12	4.87 (m)
		4.81 (ddd, 1.2, 2.3, 14)	H_2–13a	7, 8, 9, 12	4.97 (d, 14)
14	24.3	1.32 (s)		3, 4, 5, 15	1.17 (s)
15	181.4	–			–
16	17.6	0.86 (s)		1, 5, 9, 10	1.32 (s)

[a] 100 MHz; $CDCl_3$. Carbon multiplicities were determined from DEPT experiments (100 MHz; $CDCl_3$), and are consistent with the assignments. [b] One–bond $^1H–^{13}C$ correlations were determined from HMQC data (600 MHz; $CDCl_3$). [c] 600 MHz; $CDCl_3$. [d] $^1H–^1H$ decoupling data were collected at 400 MHz ($CDCl_3$). Signals listed were simplified upon irradiation at corresponding positions. [e] 400 MHz; $CDCl_3$. [f] weak signal.

A literature search for the structure shown in Figure 11 identified compound **48** as a synthetic intermediate in a process used for the preparation of oidiolactone C (**49**).[251] Although compound **48** has been previously described,

this is the first report of its occurrence as a natural product. We propose the name oidiolactone G for **48**, following in the name sequence of previously described members of this family of fungal metabolites.



48 **49** **50** **51**

The final compound characterized from the RP–HPLC of fraction V10 (**50**) had a ^{1}H NMR profile similar to that of oidiolactone G (**48**). The spectrum of compound **50** differed only in the appearance of an additional olefinic proton resonance and the absence of two diastereotopic methylene multiplets (Table 17). Consideration of other previously described oidiolactones aided in the identification of compound **50** as 3a,10b–dimethyl–1,2,3,3a,5a7,10b,10c–octahyhdro–5,8–dioxa–acephenanthrylene–4,9–dione.[252] We propose the name dehydro–oidiolactone G for **50** due to the similarity between this compound and oidiolactone G (**48**). These compounds differ due to the presence of a C_9–C_{11} olefin unit in dehydro–oidiolactone G (**50**). Compound **50** is structurally similar

to the known metabolites LL–Z1271α (**51**) and LL–Z1271γ in that it only lacks either a methoxy (LL–Z1271α) or hydroxyl (LL–Z1271γ) functional group at C–13.[253,254]

Analysis of ^{1}H NMR data for other fractions from the chromatographic separations of extracts from NRRL 46122 led to the identification of four more oidiolactone metabolites. One fraction thought to contain oidiolactones was the 12th VLC fraction. Further separation of this fraction over Sephadex LH–20 resulted in the eighth fraction (Hse; 6.3 mg) being composed almost entirely of the known fungal metabolite oidiolactone E (**52**; Scheme 6).[255] Identification of oidiolactone E (**52**) was achieved through comparison of this fraction's ^{1}H NMR spectrum with published data (Table 18).[255]

Spectroscopic data for the methanol wash (V14; 130 mg) of the initial chromatographic separation of MYC–1674 were also suggestive of the presence of oidiolactones. However, fraction V14 was a mixture of several compounds and needed further purification prior to complete characterization of its components. Attempts to purify the components of fraction V14 using C_{18} RP–HPLC were unsuccessful as a result of poor peak resolution, despite the employment of multiple elution methods. Therefore, the remaining portion of V14 (~130 mg) was subjected to another silica gel VLC column eluting with a gradient of methanol in acetone.

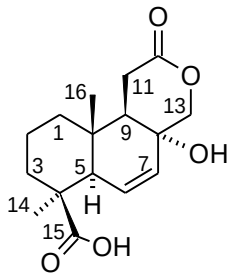


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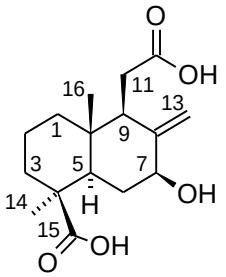
Table 18. ^{1}H NMR data for oidiolactone E (**52**)

position	^{a} δ_H (mult., J_{HH} in Hz)
1	1.03 (m)
	1.53 (m)
2	1.03 (m)
	1.69 (br d, 13)
3	1.80 (m)
	2.27 (br d, 13)
4	–
5	1.87 (m)
6	6.61 (dd, 1.9, 10)
7	5.76 (dd, 3.1, 10)
8	–
9	2.20 (br d, 9.5)
10	–
11	2.40 (d, 18)
	2.90 (dd, 18, 9.5)
12	–
13	3.45 (d, 12)
	3.64 (d, 12)
14	1.32 (s)
15	–
16	0.72 (s)

^{a}400 MHz; $CDCl_3$

LL-Z1271β (**53**) was encountered as one of a number of components present in several fractions from further separation of fraction V14 (Scheme 7). However, definitive identification of this major component was not possible using the ^{1}H NMR data from any of these complex fractions.



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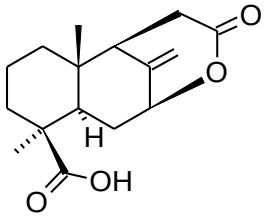

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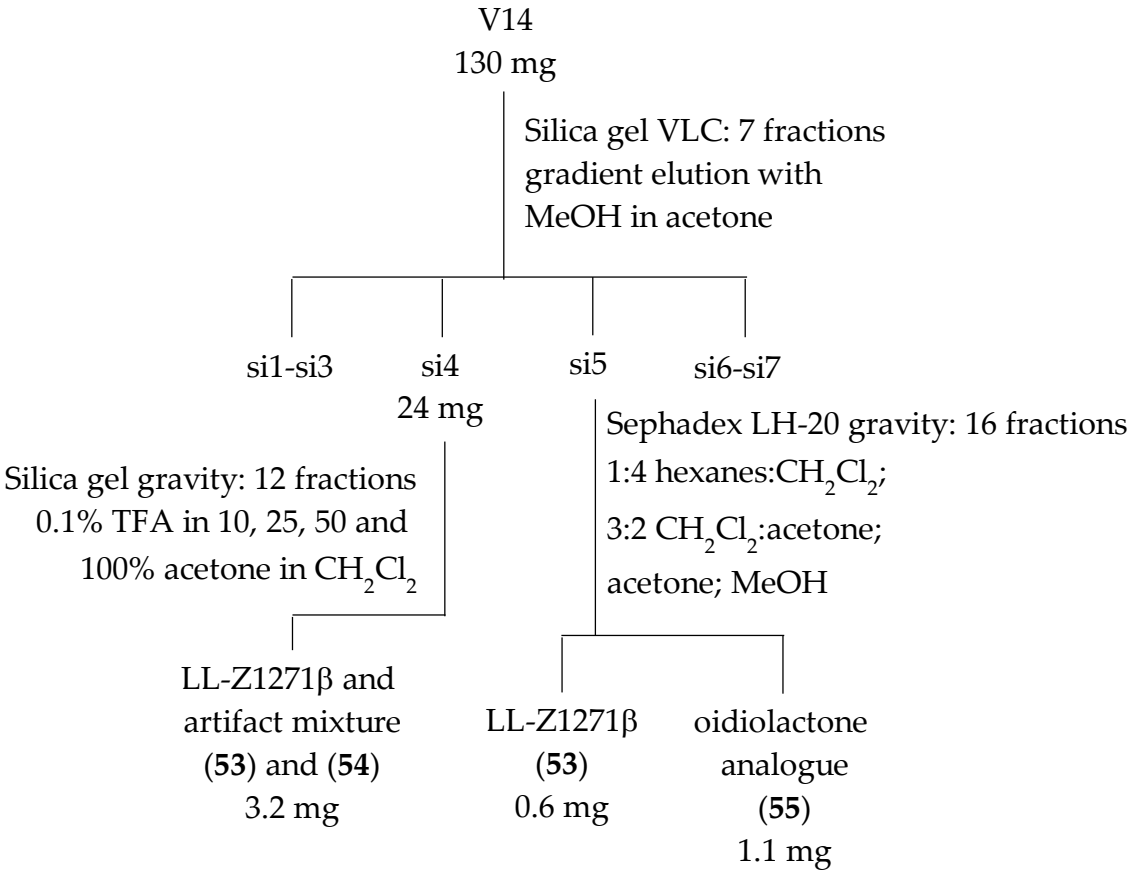


Scheme 7. Chromatographic separations of the 14th VLC fraction from the MYC–1674 extract

Further chromatography over silica gel was performed using a step gradient of acetone in CH_2Cl_2 containing 0.1% TFA for one of the largest samples containing compound **53** (si4; 24 mg). ^{1}H NMR data for the resulting subfractions did not indicate isolation of the major component. Instead, data for one of these subfractions showed that an analogue (**54**) not present in the original fraction (si4) was co–eluting with the major component (Figure 12). ^{1}H–^{1}H decoupling, ^{13}C NMR, DEPT, and 2D–NMR data were collected in an effort to identify both the major and minor components (**53** and **54**, respectively). Ultimately, however, conclusive identification was only possible after complete separation of these compounds.

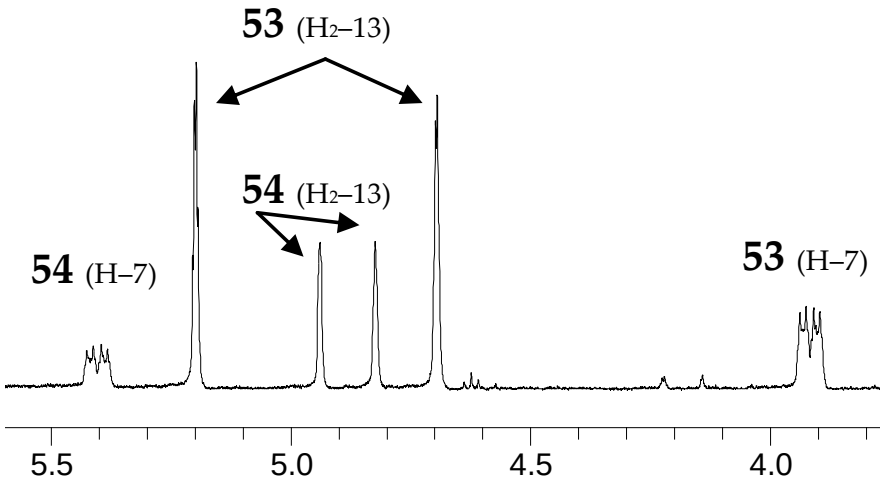


Figure 12. Partial ^{1}H NMR spectrum of LL–Z1271β (**53**) and artifact **54** (acetone–d_6; 400 MHz)

Isolation of compound **53** was only successful when less acidic conditions were used. This was evident when fraction si5, which contained similar components, was separated over Sephadex LH–20 (Scheme 7). This chromatography step resulted in the isolation and subsequent identification of the known compound LL-Z1271β (**53**) after thorough NMR analysis (Table 19).[254]

After isolation and identification of LL-Z1271β (**53**), compound **54** was identified as an artifact of the isolation process that had co-eluted with LL-Z1271β (**53**) upon fractionation of si4 under acidic conditions as noted in Scheme 7. Re–exposure of these components to 0.1% TFA in acetone and CH_2Cl_2 for three hours did not significantly alter the ratio of **53** to **54** in the sample, suggesting that the mixture may already have been in thermodynamic equilibrium. Exposing a pure sample of **53** to acidic solution might generate **54**, thus proving lactone **54** to be an isolation artifact. However, due to limited quantities of sample, this experiment was not conducted, and isolation of **54** was not accomplished.

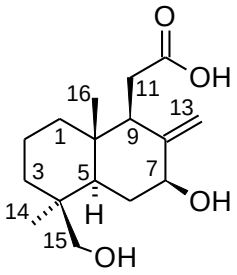
Analysis of NMR data for both the mixture and pure LL–Z1271β (**53**) helped to distinguish the key artifact and fungal metabolite ^{1}H NMR resonances from one another. One difference in the ^{1}H NMR data was the downfield shift of H–7 from δ 3.90 in **48** to δ 5.40 in artifact **54**. This was indicative of acylation at this position in the artifact (**54**; Figure 12). Unfortunately, no HMBC correlation

Table 19. NMR data for LL–1271β (**53**) and artifact (**54**)

		LL-Z1271β (**53**)			**54**
Position	[a,b] δ_C	[b,c] δ_H (mult., J_{HH} in Hz)	[d] decoupling results	[e] ^{1}H-^{13}C HMBC Correlations (H → C)	[c,g] δ_H (mult., J_{HH} in Hz)
1	39.7	1.18 (m)			
		1.70 (m)	H–9		
2	20.7	1.49 (m)			
		1.91 (m)			
3	38.9	1.10 (m)		1, 2, 4, 14, 15	
		2.25 (m)		1	
4	44.3	–			–
5	54.0	1.51 (m)		4, 6, 7, 9, 10, 15, 16	
6	36.3	1.86 (m)		5, 7, 10	
		2.28 (m)		7, 8, 10	
7	73.7	3.90 (m)	H–6a, H–6b, H–13a, H–13b		5.40 (m)
8	152.7	–			–
9	51.1	2.15 (m)		11	
10	39.9	–			
11	30.5	2.38 (dd, 11, 16)		8, 9, 10, 12	
		2.49 (dd, 3.7, 16)	H–11a	8, 9, 10, 12	
12	174.9	–			–
13	103.3	4.70 (m)	H–7, H–13b	7, 9	4.83 (m)
		5.18 (m)	H–7, H–13a	7, 8 [f], 9	4.94 (m)
14	31.3	1.23 (s)		2 [f], 3, 4, 5, 15	1.26 (s)
15	178.9	–			–
16	13.3	0.64 (s)		5, 9, 10	0.70 (s)

[a] 100 MHz; acetone–d_6. Carbon multiplicity determined from DEPT experiments (100 MHz; CD_3OD) and are consistent with the assignments. [b] One–bond ^{1}H–^{13}C correlations were determined from HMQC data (600 MHz; acetone–d_6). [c] 600 MHz; acetone–d_6. [d] ^{1}H–^{1}H decoupling data collected at 400 MHz (acetone–d_6). Protons listed were simplified upon irradiation at corresponding positions. [e] ^{1}H–^{13}C HMBC correlations (600 MHz; acetone–d_6). [f] weak signal [g] Signals listed were the only ones distinguishable for artifact **54** upon analysis of the ^{1}H NMR data of LL–Z1271β (**53**) and its artifact mixture (**54**; acetone–d_6).

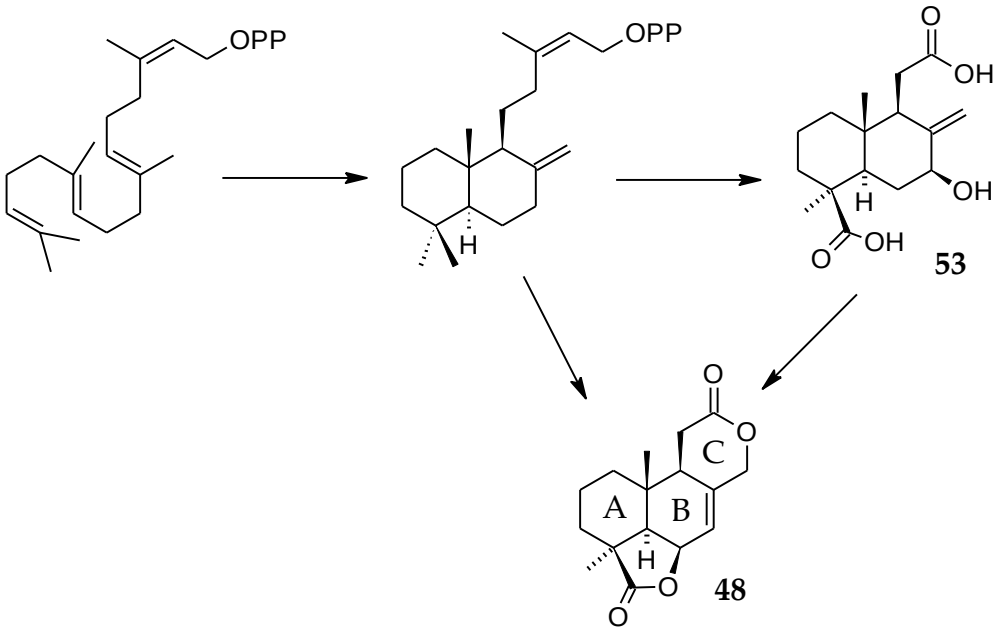
between H–5 and the acylating carbonyl was observed. However, this shift difference together with the dramatic difference in coupling values for the H_2-11 protons in compound **54** compared to the values in **53** enabled proposal of the lactonization product as shown. These signals appear as doublets of doublets showing vicinal 3.7– and 10.8–Hz *J*–values to H–9 in LL–Z1271β (**53**). However, in artifact **54**, the vicinal coupling to H–9 for one of the H_2–11 protons was reduced to nearly 0 Hz, which would be consistent with its incorporation into a bridged lactone structure of this general type due to the expected change in vicinal angle which could approach 90°.[256] The structure of artifact **54** is therefore proposed as shown based on its few discernable ^{1}H NMR signals. It is possible that there are more differences in the structures of **53** and **54** than the ones shown, but this seems unlikely.



55

In the process of isolating LL–Z1271β (**53**), trace amounts of another natural product (**55**) were obtained. The ^{1}H NMR data for **55** were very similar to those of **53**. The most notable difference in the spectrum of **55** compared to that of LL–Z1271β (**53**) was the presence of a pair of new geminal proton doublet resonances at δ 3.61 and 3.76 with a 10–Hz *J*–value. It is possible that these oxymethylene signals could represent a reduction at C–15 in compound **55** relative to **53**. While there are scenarios that could result in the formation of an oxymethylene unit elsewhere in the structure, oxidation or reduction anywhere else in the skeletal framework of a compound similar to LL–Z1271β (**53**) would result in other changes in the ^{1}H NMR spectrum that were not observed. The presence of a C–15 oxymethylene unit could also account for the somewhat upfield shift of the H_3–14 signal from δ 1.23 in **53** to δ 1.19. There were no other noticeable differences in the ^{1}H NMR profiles of compounds **53** and **55**. However, due to sample limitations, isolation of greater quantities that would enable complete characterization and biological testing of compound **55** was not carried out.

Oidiolactones are norditerpenoid metabolites that display a variety of plant growth regulatory and antifungal activities.[252,255,257,258] LL–Z1271α (**51**) was the first of this class of compounds to be described and was originally reported from an *Acrostalagmus* sp. (syn. *Verticillium*).[259] Further studies of this organism,



Scheme 8. Proposed biosynthesis of labdene– and norditerpenoid–type metabolites from GGPP (geranylgeranyl–pyrophosphate)[253,260]

Oidiodendron truncata, and a *Holwaya* sp. have resulted in the identification of almost a dozen oidiolactone analogues.[252] Though LL–Z1271β (**53**) does not contain a δ–lactone, as do compounds **48**–**52**, compound **53** does share a similar carbon framework. Biosynthetic studies have demonstrated that the C_{16} skeleton of the oidiolactones is derived from a labdene diterpenoid precursor which undergoes oxidative cleavage resulting in a loss of four carbons (Scheme 8).[253,260] Several other fungal metabolites have the same carbon skeleton as the oidiolactones, such as the podolactones from *Podocarpus neriifolius*, which contain epoxidation on the structure's A–ring.[253,257,261,262] Other examples include nagilactones and inumakilactones.[255]

The A–B ring juncture in the oidiolactones was assigned a *trans* relative orientation based on ^{1}H NMR *J*–values coupling constant, chemical shifts, NOE data, and X–ray crystallographic data for similar compounds in the literature.[252-255,258] The five–membered fused lactone found in oidiolactone G (**48**) and dehydro–oidiolactone G (**50**) is assigned as *cis* based on the 5.1–Hz $J_{5,6}$–value. For LL–Z1271β (**53**), Ellestad attributed the high field C–16 chemical shift (δ 0.64) to its *cis*–1,3–diaxial relationship with the C–15 carbonyl and further shielding from the C8–C13 exocyclic methylene unit.[254] This would result in a *trans* relative orientation between C14 and C16. Ozonolysis of the dimethyl ester acetate of compound **53** generated a C8–ketone derivative which allowed for determination of the absolute configuration of compound **53**.[254] A strong, negative cotton effect at 283 nm (in methanol) was consistent with the absolute configuration of LL–Z1271β (**53**) as shown.[254] X–ray analysis of compound **51** showed this molecule to contain the same relative configuration as LL–Z1271β (**53**) further confirming the close biosynthetic relationship between compounds **48–52** and compound **53**.[251, 252, 255] Absolute configurations for these compounds have been determined through various approaches, including stereoselective synthesis, derivatization, analysis of CD and crystal data, and comparison with the known absolute configurations of co–metabolites.[251-255]

The fungicolous isolate chemically investigated in this study, MYC 1674 (= NRRL 46122), was identified as an isolate of *Neofusicoccum parvum* (anamorph = *Botryosphaeria parva*) after comparison of partial sequence analysis data with those in the GenBank database (www.ncbi.nlm.nih.gov/BLAST; 100% match with *N. parvum*).[89,263] *N. parvum* was only distinguishable from other cryptic *Neofusicoccum* spp., such as *N. ribis*, after analysis of multiple gene sequencing data and phenotypic characteristics.[263,264] *N. parvum* has been reported on a number of occasions to appear as an asymptomatic endophyte of a variety of taxonomically distinct perennial plants such as grapes, kiwi, red mahogany, and olives, though this is the first report of this fungus colonizing a fungal host.[264-266] The biological activities of some other *N. parvum* culture filtrates have been described in the literature and these extracts are reported to be phytotoxic towards tobacco leaves, grape vines, and tomatoes, though there are no reports of metabolites from any of these cultures.[265]

Our studies focused on the identification of antifungal metabolites produced by this fungicolous isolate of *N. parvum.* Of the eight natural products identified, only two metabolites were tested for their antifungal effects due to difficult isolation procedures and very complex mixtures resulting in low yields of purified metabolites suitable for testing. Compounds **48** and **52** were subjected to antifungal standard disc assays against *F. verticillioides* (NRRL 25457;

500 μg sample/disc). Dehydro–oidiolactone G (**50**) displayed moderate effects against *F. verticillioides* after 48 hours, causing a clear zone of 28 mm in diameter. A nystatin standard afforded a comparable zone (27 mm), albeit at a much lower concentration (25 μg/disc). Oidiolactone E (**52**) was inactive in this assay at the same level. Both dehydro–oidiolactone G (**50**) and oidiolactone E (**52**) were previously known as antifungal agents effective against other fungi. Dehydro–oidiolactone G (**50**) was reported to inhibit the growth of *C. neoformans* and *C. albicans* at 16 and 64 μg/mL respectively.[252] Oidiolactone E (**52**) was reportedly antifungal towards *Ustilago violacea* and *Eurotium repens*.[255] Compounds **45**–**48**, and **53**–**55** were not tested in our assays against *F. verticillioides* due to limited quantities of pure samples, although asperlin (**45**) and the hydroxymellein analogues (**46**–**47**) are known antifungal agents.[250,267] The oidiolactone metabolites not tested in our antifungal assays were purportedly inactive in antifungal, herbicidal, and antibacterial assays described in previous studies. However, dehydro–oidiolactone G (**50**) is a widespread phytotoxin and is also known to be cytotoxic toward the murine P388 lymphocytic leukemia cell line (GI_{50} 0.31 μg/mL).[252,257] Dehydro–oidiolactone G (**50**) strongly inhibits the growth of lettuce, watercress, tomatoes, and onions.[257] Oidiolactone E (**52**) is herbicidal, but was reportedly inactive in antibacterial assays against *E. coli* and *B. megaterium*, and in antifungal assays against *Mycotypha microspora*.[255]

The original antifungal activity displayed by extracts from NRRL 46122 can be partially attributed to the presence of dehydro–oidiolactone G (**50**). However, a combination of all identified metabolites from this *N. parvum* extract, including the known antifungal agent asperlin (**45**) and the hydroxymellein analogues (**46**–**47**), were the likely cause of the fungistatic effects originally observed against *F. verticillioides*. Though biological testing of compounds **45**–**48**, and **53**–**55** could not be performed due to sample limitations, it is important to note that the oidiolactone–type compounds (including LL–Z1271β; **53**) were by far the most abundant secondary metabolites produced by this organism. Of these, oidiolactone E (**52**), dehydro–oidiolactone G (**50**), and LL–Z1271β (**53**) were found as major components in a variety of chromatographic fractions and are therefore the likely source of any antifungal activity displayed by *N. parvum* extracts.

Previous studies of *N. parvum* were of endophytic isolates and focused only on the phytotoxic effects of this organism. These prior studies did not lead to identification of fungal metabolites. The studies described here resulted in the isolation and identification of eight fungal metabolites from the extracts of a fungicolous isolate of *N. parvum*, one of which was found to significantly inhibit the growth of *F. verticillioides*.

CHAPTER 7

EXPERIMENTAL

General Procedures for Fungal Specimens and Isolates

Endophytic Fungi Collection, Isolation, and Fermentation

Plant hosts containing fungal endophytes were collected by Dr. Donald T. Wicklow of the U.S. Department of Agriculture (USDA), National Center for Agricultural Utilization Research (NCAUR), Peoria, IL. Shavings of plant material were plated onto PDA containing streptomycin and stored at 25°C. After 6 days, individual fungal colonies were isolated from one another. These isolates were stored on PDA slants at –20°C until fermentations were performed.

Scale–up fermentation was employed in order to provide enough fungal extract to identify secondary metabolites produced by a given fungus, and was accomplished by solid–substrate fermentation on rice. Fermentations were carried out in two 500–mL Erlenmeyer flasks, each containing 50 g of rice that had been soaked in 50 mL distilled water. Flasks were then autoclaved at 1.055 kg f cm^{-2} for 30 min and cooled to room temperature. Upon cooling, flasks were inoculated with a suspension of conidia and hyphal cells collected from the PDA slant cultures. Solid–substrate rice fermentations lasted approximately 30 days at 25°C.

Fungicolous Fungi Collection, Isolation, and Fermentation

Fungal hosts containing various fungicolous fungal species were collected by Dr. Donald T. Wicklow of the USDA NCAUR, Peoria, IL. Individual fungal colonies were isolated from one another, stored, and cultured in the same manner described above for fungal endophytes.

Extraction of Solid–substrate Rice Fermentations

Solid–substrate rice fermentations were mechanically fragmented with a spatula and then soaked in EtOAc (3 x 100 mL). Organic extracts for a given culture were combined and evaporated to dryness. In cases where more crude extract was required, additional solid–substrate rice fermentation cultures were produced and extracted in the same way.

Identification of Fungal Species

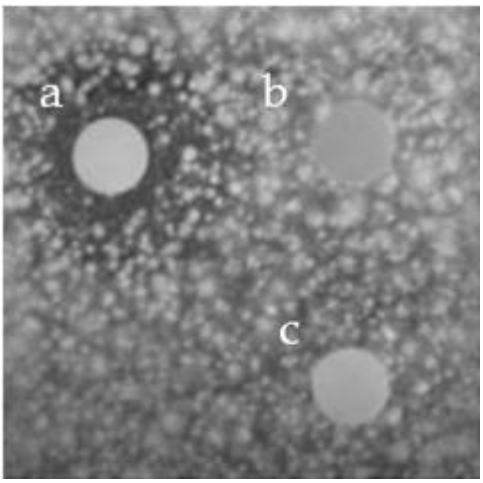
Fungal isolates were identified by Dr. D.T. Wicklow, initially on the basis of micromorphology.[87] All cultures of interest were deposited in the NRRL collection of the USDA NCAUR and assigned accession (NRRL) numbers. Selected isolates of interest were further characterized via partial sequence analysis of the internal transcribed spacer region (ITS) and domains D1 and D2 of the nuclear large subunit (28S) rDNA gene using ITS5 and NL4 as polymerase chain reaction and sequencing primers.[88,89] Sequences were subsequently deposited in GenBank (National Center for Biotechnology Information).

General Procedures for Biological Assays

Antifungal Assays

Antifungal assays against *Aspergillus flavus* (NRRL 6541) and *Fusarium verticillioides* (NRRL 25457) were performed by Dr. D.T. Wicklow. In certain cases, antifungal assays were performed by Dr. Wicklow using other fungal species upon request. Assays against *Candida albicans* (ATCC 14053) were conducted in our labs at The University of Iowa. Antifungal assays were conducted for all crude extracts, as well as column fractions, and pure compounds when appropriate as a part of the bioassay–guided fractionation process. For crude extracts, 1 mg of extract was dissolved in MeOH and pipetted onto an analytical grade paper disc (12.5–mm diameter). Five hundred μg of material were used for testing column fractions and 100 μg for assays of pure compounds. Paper discs containing samples for antifungal testing were then dried for 30 min in a laminar flow hood to remove solvent. After drying, a maximum of four discs were placed equidistant from one another yeast–malt–glucose (YMG) that had been inoculated with a spore suspension of *A. flavus* (NRRL 6541) or *F. verticillioides* (NRRL 25457), giving a final spore suspension of 1×10^2 cells mL^{-1}. Plates were incubated at 25°C for up to four days and samples were evaluated for their ability to inhibit fungal growth after 24, 48, and in some cases, 96 hours. Evaluation of antifungal activity was completed by measuring a

zone of inhibition of fungal growth encompassing analytical discs (diameter in mm). Three descriptions were used to characterize inhibition of fungal growth: cz (clear zone), mz (mottled zone), and rg (reduced growth). Any of these would constitute a positive result in antifungal assays and would be indicative of fungistatic activity (e.g., Figure 13).



[a]Crude extract exhibiting both cz and mz inhibition of fungal growth. [b]Crude extract with no inhibition of fungal growth. [c]MeOH control also lacking inhibition of fungal growth

Figure 13. Standard Disc Assay of Crude Extracts[a,b] and MeOH Control[c] Against *A. flavus*

Antifungal assays against *C. albicans* were carried out in our labs as needed. One hundred mL of Tryptic soy agar (Difco) were prepared according to product instructions, sterilized by autoclaving, and then cooled to 45°C. This

solution was aerated on an orbital shaker for 36 to 48 hr (150 rpm at room temperature). A 1 mL suspension of *C. albicans* inoculum that was prepared by dissolving one *C. albicans* bactrol disk in 50 mL of sterile yeast maintenance broth was transferred to the agar (Difco). The resulting culture was poured into Petri dishes (100 x 15 mm), allowed to set, and then stored at 4°C. Samples to be tested (100–200 μg/disk) were impregnated on paper disks (6.25 mm), the solvent allowed to evaporate, the disks placed on the agar surface, and incubated at room temperature for 1 to 3 days. The broad–spectrum antifungal agent nystatin was used as a positive control in antifungal assays while MeOH was used as a negative control to demonstrate that any observed antifungal activity was not caused by organic solvent used for dissolving fungal metabolites.

Antiinsectan Assays

Dr. Patrick F. Dowd of the NCAUR designed and carried out antiinsectan assays for fungal crude extracts, column fractions, and pure compounds involved in this research. Antiinsectan activity of metabolites and other mixtures were tested in a standard pinto bean dietary assay against the fall armyworm *Spodoptera frugiperda*. The diet consisted of 120 g dried pinto beans mixed with the following ingredients: 43 g wheat germ, 28 g brewer's yeast, 12 g sugar, 12 g formaldehyde (39%), 8 g Vanderzant's vitamin mix, 2.8 g ascorbic acid, 1.75 g methylparaben, 0.9 g sorbic acid, 1.5 mL propionic–phosphoric acid solution

(42% propionic acid, 4.2 % phosphoric acid), and 550 mL of water. Five–mL aliquots of the molten diet solution (60°C) were pipetted into test tubes (100 x 16 mm). Half of these test tubes were used for control group feeding studies, and the other half mixed with fungal metabolites and fed to the larvae worms for observation of weight gain/reduction and insect mortality.

Fungal metabolites to be subjected to antiinsectan assays were dissolved in 125 μL of acetone and then added to test tubes containing the molten diet mixture (crude extracts, 2000 ppm; column fractions and pure compounds varied in amount, but were typically tested at approximately 100 ppm wet weight). For control group diets, only 125 μL of acetone were added to the molten diet test tube mixture. Test tubes were vortexed for 20 seconds in order to mix the pinto bean dietary components with fungal compounds and/or organic solvents. After mixing, the test tube contents were emptied into a Petri dish, cooled to room temperature, and placed in a fume hood for 20 min to allow time for any residual solvent to evaporate. Upon cooling, the dietary mixture was cut into 250–mg segments and one segment placed in each well of a 24–well immunoassay plate. A single neonate *S. frugiperda* larva was also placed in each well. Forty neonate larvae were tested per sample. Immunoassay plates were covered in Parafilm, a sheet of cardboard, and a plastic lid to prevent dietary components from drying out. Bioassays lasted 7 days (14:10 light:dark photoperiod; 40% humidity; 27°C).

Neonates were inspected at 2, 4, and 7 days. Those surviving 7 days were weighed and control group larvae compared with test group larvae. Antiinsectan activity was reported as percent reduction in weight gain of test larvae relative to control larvae. Percentage mortality was reported for those bioassays in which mortality was observed.

Antibacterial Assays

All antibacterial assays were conducted in our laboratory at The University of Iowa. *Staphylococcus aureus* (ATCC 29213) bioassay plates were generated as needed. *S. aureus* inoculum was prepared by dissolving one *S. aureus* bactrol disc (Difco) in 50 mL of sterile Difco nutrient broth, then aerating the mixture at 200 rpm for 36–48 hr at room temperature. Two mL of this inoculum were added to 100 mL sterile tryptic soy agar (Difco) that had been prepared according to product directions and cooled to 45–50°C. The inoculum and media were gently swirled to homogenize the mixture without causing bubbles to form. Five mL of the inoculated medium were poured into Petri dishes, solidified by cooling, and stored in a refrigerator at 4°C.

Escherichia coli (ATCC 25922) bioassay plates were generated as needed. *E. coli* inoculum was prepared by dissolving one *E. coli* bactrol disc (Difco) in 50 mL of sterile Difco nutrient broth, then aerating the mixture at 150 rpm for 36–48 hr at room temperature. Two mL of this inoculum were added to 100–mL sterile

tryptic soy agar (Difco) that had been prepared according to product directions and cooled to 45–50°C. The inoculum and media were gently swirled to homogenize the mixture without causing bubbles to form. Five mL of the inoculated medium were poured into Petri dishes, solidified by cooling, and stored in a refrigerator at 4°C.

Samples for antibacterial testing were dissolved in organic solvent and impregnated on filter paper discs (6.25 mm in diameter). Solvent was allowed to evaporate resulting in 25 μg/disc for the control agent and 200 μg/disc for test compounds. These discs were placed on the surface of either *S. aureus* or *E. coli* agar plates. Assays were performed in triplicate and up to four discs were placed equidistantly on an agar plate. Plates were incubated at room temperature and evaluated for inhibition of bacterial growth at 24 and 48 hours. Antibacterial activity was reported as a diameter (in mm) of inhibition of bacterial growth around the filter paper discs. Gentamycin (25 μg/disc) was used as an antibacterial standard in *S. aureus* and *E. coli* assays. A paper disc that had been exposed to methanol was used as a negative control.

General Analytical Procedures

Solvents and Reagents

Reagent grade solvents were used for chromatographic separations and extractions, and were purchased from Sigma–Aldrich or Fisher Scientific.

Organic solvents used in HPLC were purchased from the same distributors, but were of analytical grade. Distilled H_2O was purified using a SYBRON/Barnstead NANOpure system with a pre–treatment cartridge CO1801 (catalog number D0835), two MACROpure cartridges MB1801 (D0809), and a 0.2–μm Barnstead gamma–irradiated hollow–fiber filter (D3750). All solvents used for HPLC were degassed for 20 min i*n vacuo* and sonicated prior to use for HPLC. Deuterated solvents used for NMR spectroscopy were purchased from Sigma–Aldrich.

Evaporation

Samples dissolved in organic solvents were most frequently dried under airflow in a chemical ventilation hood. Some fractions collected from silica VLC were dried under vacuum using a Buchi RE–111 rotary evaporator with a precision model S35 vacuum pump.

Weight Measurements

A Mettler AE 160 analytical balance was used to weigh crude extracts, partition and chromatographic fractions, and pure compounds.

Silica Gel Column Chromatography (Gravity)

A solvent elution scheme for the separation of the MeCN soluble partition of a crude extract was developed by use of thin–layer chromatography (TLC) analysis. Samples were dissolved in MeCN and then spotted on a polyester–backed TLC plate (Sorbent Technologies, 200–μm with UV254, 4x8 cm). The TLC

plate was then placed in a beaker containing solvent mixtures of varying polarities (as well as filter paper to encourage solvent evaporation) and then covered with a watch glass. This served as a developing chamber. TLC analysis was also used to determine whether or not a sample would likely elute from silica or remain on the stationary phase. TLC plates were developed using UV detection (254 nm), exposure to iodine vapor, and/or phosphomolybdic acid (PMA) staining.

Science–grade silica gel (63–200 μm; Scientific Adsorbents Incorporated) was used for silica gel gravity column chromatography. Silica was initially soaked overnight in the first eluent to be used in the separation process, e.g. hexanes. A glass column was fitted with a cotton plug, followed by approximately 1 cm of sand, and then the prepared silica gel. Next, the MeCN–soluble partition of a crude extract was dissolved in a minimum amount of MeCN and pipetted on top of the freshly poured silica gel column. The column was then topped with approximately 3 cm of sand. Samples were eluted according to a methodology determined from the previously preformed TLC analysis.

Silica VLC Column Chromatography (Flash):

The same grade of silica was used for silica vacuum liquid chromatography (VLC) as was used for silica gel gravity column

chromatography. TLC analysis was performed prior to chromatographic separation to determine if fractions would likely elute from a silica stationary phase. Samples to be separated by VLC were prepared for chromatography by dissolving the material in various organic solvents (usually hexanes, CH_2Cl_2, and/or MeOH) and then adding the solution to approximately 20 mL of dry silica in a 50–mL Büchner funnel. The flask was then fitted with a rubber septum, which was then punctured with two hypodermic needles. An airflow tube was then connected to one of the hypodermic needles, using the second needle for ventilation. The silica–sample mixture was then dried under airflow until the silica flowed dryly and freely in the funnel.

The VLC apparatus was assembled by fitting a sintered glass column with a vacuum funnel to a 500–mL round bottom flask. One cm of sand was then poured over the frit of the column. This was followed by dry silica, and then the sample prepared for separation as above (i.e., the sample on dried silica). Finally, enough sand was added to the top of the column to prevent the column surface from being disturbed by addition of eluents. During the separation process, a partial vacuum was formed in the VLC apparatus through water aspiration. Samples were eluted according to a predetermined methodology that was based on sample polarity and TLC results. Between 150 mL and 400 mL of

eluate were collected for each fraction. Both TLC analysis and observation of color band movement were used to guide fraction collection.

Sephadex LH–20

Sephadex LH–20 was purchased from Sigma–Aldrich and was used for gel filtration chromatography of samples with limited quantity or those displaying poor separation over silica. Columns were prepared in a manner similar to that used for gravity silica gel chromatography. A standard elution scheme was employed for Sephadex LH–20 column chromatography.[268] Fractions were collected on the basis of color band movement, solvent volume, and TLC analysis.

Semi–preparative HPLC

Method development for HPLC separation of fractions was performed on a System Gold 125 Beckman Instruments solvent delivery module. A model 168 photodiode array detector was used for UV detection. Both solvent delivery module and detector were controlled by system Gold 32–Karat software using an IBM 300PL PC. For sample injection, a Rheodyne model 7725 injector was attached to the solvent delivery module.

After determining a solvent elution method and suitable wavelength for UV detection, HPLC separation and fraction collection were performed on a Beckman 110B solvent delivery module with a system Gold 166 variable

wavelength detector. A Beckman Altex 210A injector was used as the injection port for HPLC separations on this apparatus. The HPLC system was controlled by a NEC PC–8300 microcomputer. This computer was interfaced with a Beckman System Gold Analog Interface module 406 using NEC PC–8201 Basic Version 1.1 Microsoft software.

Apollo and Grace C_{18} (5–μm particle size, 10 mm x 250 mm) HPLC columns were used for most reversed–phase separations. A Hamilton PRP–1 reversed–phase column (10–μm particle size, 10 mm x 250 mm) was used where specified. HPLC chromatograms generated from separations on this solvent delivery system were recorded using a model LR93025 Linear chart recorder, and were monitored at specified wavelengths.

Spectroscopic Instrumentation

For all NMR experiments, samples were dissolved in no more than 700 μL of deuterated solvent, transferred to a 5–mm glass NMR tube, and capped with a plastic top. The solvents used were $CDCl_3$, acetone–d_6, and CD_3OD. NMR data were collected using Bruker AVANCE–300, AVANCE–400, DRX–400, or AVANCE–600 spectrometers. These instruments used XWINNMR 3.5 and TOPSPIN 1.3 software. Spectra from NMR experiments display chemical shifts (δ) in ppm downfield of tetramethylsilane (TMS). Residual protiated solvent peaks were used as internal reference standards and were assigned the following

chemical shifts: $CDCl_3$ (δ_H = 7.24, δ_C = 77.0); acetone–d_6 (δ_H = 2.05, δ_C = 29.9); CD_3OD (δ_H = 3.31, δ_C = 49.0).

^{1}H NMR Spectroscopy

^{1}H NMR data were collected on the following Bruker spectrometers with 5–mm probes operating at the following frequencies: AVANCE–300 (300.132 MHz), AVANCE–400 (400.132 MHz), DRX–400 (400.133 MHz), and AVANCE–600 (600.252 MHz). The AVANCE–300 spectrometer used a Quadra Nuclei probe (QNP) with capabilities of detecting ^{1}H, ^{13}C, ^{19}F, and ^{31}P nuclei. The AVANCE–400 instrument used a broadband fluorine observe (BBFO) gradient–enabled automatic tuning and matching probe, which could be used to observe ^{15}N–^{31}P, as well as ^{13}C and ^{1}H nuclei. The DRX–400 spectrometer employed a multinuclear broadband (BBO) probe and the AVANCE–600 spectrometer uses a multinuclear broadband inverse probe (BBI). Variable temperature ^{1}H NMR and homonuclear decoupling experiments were performed on the DRX–400 instrument. All data were collected at room temperature unless otherwise indicated. ^{1}H NMR data were processed using NMR Utility Transfer Software (NUTS) 2D Version– 20060331.

^{13}C NMR Spectroscopy

^{13}C NMR and DEPT data were recorded on either the DRX–400 or AVANCE–400 spectrometers at room temperature, operating at a frequency of

100.623 MHz. The probes used for collection of ^{13}C and DEPT data were the same as those listed for the collection of ^{1}H NMR data. Data were processed using NUTS 2D Version–20060331.

2D–NMR Spectroscopy

2D NMR experiments (HMBC, HMQC, and NOESY) were recorded using the AVANCE–600 spectrometer with a 5–mm inverse probe (BBI). This instrument operated at a ^{1}H frequency of 600.252 MHz and ^{13}C frequency of 150.948 MHz. Data were collected at room temperature and processed using XWINNMR 3.2 software on a Silicon Graphics (SGI O2) computer.

Mass Spectrometry

Mass spectra were obtained and analyzed to determine the molecular weights and/or elemental composition of compounds. Low–resolution mass spectra were obtained for all samples for which mass spectra were needed. These data were used in some cases to determine the best ionization technique for high–resolution mass spectrometry. Samples were ionized using EI, ESI, or APCI methods. Low–resolution EI mass spectra were obtained on a Thermo Voyager mass spectrometer employing either a direct inlet solids–probe (DIP) or gas chromatograph (GC) for sample introduction. Low–resolution ESI mass spectra were recorded on a Thermo LCQ Deca quadrupole ion–trap instrument. High–resolution mass spectra were obtained using either ESI or EI techniques on

a Micromass Autospec double–focusing magnetic sector mass spectrometer. HRMS samples were also sometimes analyzed by ESI or APCI methods on a Waters Q–Tof Premier hybrid quadrupole time–of–flight mass spectrometer.

Other Spectroscopic Instrumentation

Optical rotations were recorded using a Rudolph Research Autopol III automatic polarimeter and UV spectra were obtained using a Varian Cary 100 Bio UV–visible spectrometer.

General Procedures for NMR Experiments

Variable Temperature Experiment

Variable temperature ^{1}H NMR data were collected for samples in which broadness of certain signals made assigning *J*–values difficult. Parameters for this experiment were the same as for a standard ^{1}H NMR experiment with the exception of the probe temperature being adjusted to 20, 25, or 45°C. The instrument was allowed to heat or cool to the specified temperature prior to collection of data. Enough scans were recorded to achieve a suitable signal-to-noise ratio (*s*/*n*). Data were processed using NUTS software.

Homonuclear Decoupling Experiment

Data from these experiments were used to determine which protons of a molecule are coupled to one another. These data also helped to elucidate the coupling constants between protons of a molecule whose chemical shifts were

found in regions of signal overlap. XWINNMR 3.5 software was used to set the parameters for homonuclear decoupling experiments. After inserting the NMR sample into the probe, locking the magnet on the appropriate deuterated solvent frequency, and tuning and matching the spectrometer, the sample spinner was turned on and instrument shimmed. A new general experimental file was created by typing "datasetname". Parameters for the first experiment's file (EXPO 1) were those of a standard ^{1}H NMR experiment. This file was used for determining the proper number of scans for a suitable *s*/*n* and the numerical value of the receiver gain (RG), to shim the spectrum, and to calibrate the residual solvent peak. From this first ^{1}H NMR spectrum, signals were chosen for irradiation and their frequencies (O2 parameter value in Hz) recorded. Information from EXPO 1 was applied to subsequent homonuclear decoupling experimental files. These files were created as EXPO 2, EXPO 3, etc. Parameters for homonuclear decoupling experimental files were chosen by typing "rpar" and choosing "AA_HomoDecouple" from the dropdown menu. The same number of scans and RG as EXPO 1 were applied to homonuclear decoupling experiments. To irradiate a given signal, a homonuclear decoupling experimental file was created and the O2 value of that proton frequency entered into the "SFO2" cell of the "F2–acquisition parameters" menu. Within this same menu, the decoupling power (P24) value was set between 50 and 75 dB,

depending on the magnitude of the *J*–values for a given signal. Signals with large coupling constants to be irradiated would employ a P24 value closer to 50 dB, while those with smaller coupling constants would have a lower P24 value set closer to 75 dB. Subsequent experimental files were created for each signal position to be irradiated.

After creation of all the homonuclear decoupling experimental files, the second file (EXPO 2; first homonuclear decoupling file) was re–opened in the queue window. Experimental data collection was initiated by typing "xau", a command which first requests the number of experiments to be run and then initiates these experiments beginning with the file that is currently open in the XWINNMR queue. Spectra recorded from these experiments were pre–calibrated as a result of EXPO 1. Data resulting from these experiments were processed using NUTS software.

^{13}C and DEPT Experiments

Both XWINNMR and TOPSPIN software were used to record ^{13}C NMR DEPT data. Data from a DEPT experiment were used to elucidate the presence of CH, CH_2, and CH_3 units. These data resulted in positive signals for methyl and methine carbons (signals point upward), and negative signals for methylene carbons (point downward). A sample for DEPT data collection was inserted into the spectrometer's probe, the magnet tuned and matched, the solvent signal

locked, sample spun, and the instrument shimmed. To generate a ^{13}C or DEPT spectrum, a standardized ^{1}H NMR file was opened in the queue window from either of the two software programs listed. As with most NMR experiments, the first experiment (EXPO 1) was reserved for optimization of shimming and determination of a suitable number of experimental scans. In most cases, ^{13}C NMR data were collected as EXPO 2. This was accomplished by choosing the "13C_BBO" file parameters from the NMR data browser window. The third experiment (EXPO 3) would then contain parameters for recording ^{13}C NMR DEPT data by choosing the "13C_DEPT–135" program file. The first file to be recorded was opened in the queue window and initiated by typing "xau" or clicking the "N*ZG" button. In general, these files were designed so that DEPT data collection took half as long as standard broadband ^{13}C NMR data collection. Recorded data were processed using NUTS software.

HMQC[269] and HMBC[270] Experiments

HMQC is an inverse detection technique used to assign one–bond carbon–hydrogen correlations. This type of analysis is an inverse technique because the less sensitive (and less relatively abundant) ^{13}C is being indirectly detected by the more sensitive (and more relatively abundant) ^{1}H nucleus to which it is attached and coupled. Therefore, non–protonated carbons are not detected in HMQC analysis. HMBC analysis similarly was used to identify long–range two– and

three–bond $^{1}H–^{13}C$ correlations, but experiments were optimized for much smaller ^{2}J and ^{3}J values, rather than the large ^{1}J values encountered in HMQC experiments.

TOPSPIN 1.3 software was used for recording HMQC and HMBC data. An NMR sample was inserted into the probe, the spectrometer tuned and matched, the solvent locked, sample spun, and magnet shimmed. A standard ^{1}H NMR file was opened and a general file name created. The first experiment (EXPO 1) was recorded to optimize the spectrometer's shimming and to calibrate the residual solvent peak. Once a satisfactory ^{1}H NMR spectrum was obtained, the spectral window of the experiment was adjusted so that it was only as wide as the peaks displayed. This spectrum width was to be applied to the two–dimensional experiments. In general, a smaller spectral window resulted in a shorter experimental running time. EXPO 1 was re–recorded with the smaller spectral window. Once this was accomplished, the spectrometer's spinning was shut off for HMQC and HMBC analysis.

After obtaining a good ^{1}H NMR spectrum, EXPO 2 was created for HMQC analysis. A pulse calibration was performed to determine the 90° and 180° pulses. To accomplish this, the following parameters were assigned in the pulse program experiment: ns = 1; ds = 0; PULPROG = zg; P1 = 25. A ^{1}H NMR spectrum was then obtained and usually displayed negative peaks for all

chemical shifts. The value for P1 was gradually increased and data recorded until chemical shifts had reached a 360° null (spectrum resembles a flat line). The P1 value for this spectrum was noted. The 90° pulse was equivalent to one quarter of the 360° null P1 value. This calculated P1 value was entered into the P0 and P1 parameters for EXPO 2. The 180° pulse was equivalent to half of the 360° null P1 value and this number was inserted to the P2 parameter for EXPO 2.

The program file for EXPO 2 was next changed to a 1D HMQC file (PULPROG = hmqcgpnd1d) to determine the number of scans necessary to achieve a good *s*/*n* (signal to noise ratio) for the HMQC 2D data. The following parameters were assigned: TD = 2k; ns = 16; ds = 4; rg = 16k; and D1 = 4 sec. One–dimensional data were recorded and the number of scans (ns) increased until the preferred *s*/*n* was reached. A 1D–HMQC spectrum looks very similar to a standard ^{1}H NMR spectrum. However only ^{13}C–^{1}H signals were represented and all signals were doubled (*e.g.*, they appear twice and are centered around the δ value at which they appear in the ^{1}H NMR spectrum). Having determined the number of scans required to run the 2D HMQC experiment, the program file of EXPO 2 was once again changed (PULPROG = hmqcgpqf). Upon changing the program, the experimental file was changed from 1D to 2D. This experiment used the same parameters as those for the 1D HMQC experiment with the following exceptions: FnMODE = QF; F1 nucleus = 13C; TD = 256; ND_010 = 2; si

= 1024; ds = 96; ns = the number of scans determined from the 1D HMQC experiments.

A third file (EXPO 3) was created for HMBC analysis. Defining the parameters for HMBC analysis was very similar to the protocol used for HMQC. This experiment used the same values for the 90° and 180° pulse that were determined from the HMQC experiment. It also used the same number of scans that were calculated for HMQC analysis as well as all other parameters, with the exception of the following: PULPROG = hmbcgplpndqf; O2P = 100 to 105.

To initiate the HMQC and HMBC experiments, EXPO 2 was read into the queue window and the "N*ZG" button selected. Data were processed using XWINNMR 3.2 software on a Silicon Graphics (SGI O2) computer.

NOESY Experiment

NOESY (nuclear Overhause effect spectroscopy) data were used to help elucidate the relative configurations of individual compounds. These data provide through space ^{1}H–^{1}H correlations based on the nuclear Overhauser effect. Before collection of NOESY data, a well–shimmed ^{1}H NMR spectrum (EXPO 1) with a minimal spectral window was obtained in the same manner as was done for HMQC and HMBC data analysis. A second file was then generated for the NOESY experiment in EXPO 2. Pulse calibrations were completed using

the same procedures listed for HMQC analysis; where the 90° pulse was the P0 and P1 parameters and 180° pulse was the P2 parameter.

To determine the mixing time for the NOESY experiment, the pulse program file (PULPROG) was changed to "t1ir1d" using the following parameters: ns = 1; ds = 0; D1 = 4 sec; D7 = 10 msec. A ^{1}H NMR spectrum was obtained using these conditions and the D7 domain changed until the best null of signals was observed in the ^{1}H NMR spectrum. Once the D7 null time value was determined, it was divided by 0.7 (D7 in msec/0.7), resulting in the NOESY mixing time (D8). This calculated value for D8 was entered into the program conditions. Another variable domain, D1, represents the relaxation delay parameter and was calculated to be 1.5 times longer than D8.

After mixing time calibrations, the experimental program file (PULPROG) was changed to "noesygpph" with the following fields entered as listed: TD = 4k; ns = 16; ds = 16; TDF1 = 512; ND_010 = 1; GPZ1[%] = +40; GPZ2[%] = –40; si = 1024. The IN_010 value was calculated to be two times the value of the DW parameter. The NOESY experiment was initiated by typing zg.

Fungal Endophyte Experimental Procedures

Procedures for the Isolation and Characterization of Colletotrichum graminicola Metabolites[124]

Fungal Material

Three isolates of *C. graminicola* were collected from maize seeds obtained by Dr. Donald T. Wicklow of the USDA NCAUR. One of these isolates, NRRL 47511 (GQ221856; ENDO–3144) was obtained from a 'Mandan Bride' variety of maize seed that was grown at an undisclosed field location in Michigan. The seed was obtained from Seed Saver's Exchange in Decorah, IA. A second isolate, NRRL 47509 (GQ221855; ENDO–3120), was obtained from a white corn seed that was grown commercially near Cerro Gordo, IL. The third isolate (ENDO–3137) was from the same variety of maize seed from Michigan as NRRL 47511, but an ARS (NRRL) Culture Collection number has not yet been assigned.

NRRL 47511 was cultured on PDA slants for 6 days at 25°C, and these slants were used to provide inoculum for solid–substrate rice fermentations. A suspension of conidia and hyphal cells with a propagule density of approximately 4 x 10^4/mL was prepared from the slants and used as inoculum for solid–substrate rice fermentation, which consisted of two 500–mL Erlenmeyer flasks each with 50 g rice (Botan Brand; J.F.C. International, Los Angeles, CA) and 1 mL fungal inoculum. The rice was soaked in distilled water overnight and then autoclaved at 1.055 kgf cm^{-2} for 30 min prior to inoculation with NRRL

47511. After inoculation, fermented rice flasks were incubated at 25°C for 30 days. Fungal isolates NRRL 47509 and ENDO–3137 were also subjected to similar solid–substrate rice fermentations.

Extraction and Isolation

After one month, the fermented rice substrates were mechanically fragmented using a large spatula and then extracted with 100 mL EtOAc three times each. The EtOAc extracts from cultures of a given isolate were then combined and evaporated to dryness resulting in a crude extract (NRRL 47511 = 309 mg; NRRL 47509 = 190 mg; ENDO–3137 = 138 mg). A 5–mg portion of each sample was set aside for testing against a panel of pathogenic fungi, including *A. flavus* and *F. verticillioides*. The remainder of the crude extract was then stored at –20°C prior to chemical fractionation.

To remove biologically inactive fats, all three crude extracts from the fermentations of *C. graminicola* isolates were dissolved in 10 mL MeCN and then washed three times with hexanes (5 mL each time). The resulting MeCN–soluble fraction was evaporated to dryness, yielding 150 mg of material for NRRL 47511, 105 mg for NRRL 47509, and 58 mg for ENDO–3137. Only the MeCN–soluble fraction of NRRL 47511 was subjected to column chromatography for the purposes of identification of natural products produced by *C. graminicola*. ^{1}H NMR data for the MeCN–soluble fractions of NRRL 47509 and ENDO–3137 were

used for comparison of metabolites with NRRL 47511 in an effort to determine if there was a pattern of specific metabolite production by *C. graminicola* isolates.

The MeCN–soluble fraction from NRRL 47511 (150 mg) was chromatographed on a Sephadex LH–20 column (1.5 x 30 cm), eluting progressively with 4:1 CH_2Cl_2:hexanes (solvent A); 3:2 CH_2Cl_2:acetone (solvent B); 1:4 CH_2Cl_2:acetone (solvent C); and methanol (solvent D).[268] 15 fractions were collected partly on the basis of color band elution and the fractions evaporated to dryness: fraction 1 (20 mL solvent A, 9.5 mg); fr 2 (10 mL solvent A, 5.8 mg); fr 3 (20 mL solvent A, red band, 8.8 mg); fr 4 (20 mL solvent A, 3.3 mg); fr 5 (10 mL solvent A, red band, 2.8 mg); fr 6 (15 mL solvent A, red and yellow band, 4.1 mg); fr 7 (20 mL solvent A, yellow band, 12.5 mg); fr 8 (20 mL solvent A, yellow band, 8.2 mg); fr 9 (20 mL solvent B, 2.6 mg); fr 10 (15 mL solvent B, light yellow band, 13.7 mg); fr 11 (40 mL solvent B, 71.1 mg); fr 12 (45 mL solvent B, gold band, 17.0 mg); fr 13 (40 mL solvent C, 7.8 mg); fr 14 (50 mL solvent C, 4.7 mg); fr 15 (100 mL solvent D).

A portion of the eleventh fraction (11 of 71 mg) was subjected to reversed–phase HPLC using an Alltech Apollo C_{18} column (10 x 250 mm; 5–μ particle size) eluting at a flow rate of 2 mL min^{-1} with UV detection at 214 nm. Fr 11 was dissolved in MeCN and 1–mg portions were injected onto the HPLC column and eluted as follows: isocratic at 40% MeCN in H_2O over 30 min; ramp from 40% to

100% MeCN in H_2O over 5 min; isocratic at 100% MeCN over 15 min. This protocol afforded monorden (**19**; t_R = 29 min; 2.8 mg), monocillin I (**20**; t_R = 32 min; 2.7 mg), monocillin II (**21**; t_R = 39 min; 1.0 mg), and monocillin III (**22**; t_R = 22 min; 1.6 mg). ^{1}H NMR data for fr 10 from the Sephadex LH–20 column displayed resonances consistent with the presence of monorden (**19**) in this fraction as well, though the fraction was not further purified.

Monorden (**19**; also known as radicicol) was obtained as a colorless solid; ^{1}H NMR see Table 1; LC–HRESITOFMS obsd *m/z* 363.0628 ([M–H] $^-$; rel int (100), 365.0624 (33)), calcd for $C_{18}H_{16}ClO_6$, 363.0635 (100), 365.0606 (33).

Monocillin I (**20**) was obtained as a colorless solid; ^{1}H NMR see Table 1; LC–HRESTOFIMS obsd *m/z* 329.1014 ([M–H]$^-$), calc for $C_{18}H_{17}O_6$, 329.1025.

Monocillin II (**21**) was obtained as a colorless solid; ^{1}H NMR see Table 1; LC–HRESITOFMS obsd *m/z* 315.1236 ([M–H]$^-$), calc for $C_{18}H_{19}O_5$, 315.1233.

Monocillin III (**22**) was obtained as a white solid; ^{1}H NMR see Table 1; LC–HRESITOFMS obsd *m/z* 331.1178 ([M–H]$^-$), calc for $C_{18}H_{19}O_6$, 331.1182.

*Antiinsectan Activity of Monorden (**19**)*

Monorden (**19**) was tested in antiinsectan assays to determine if it was responsible for the antiinsectan activity originally displayed by the EtOAc extract. At 2000 ppm, monorden (**19**) caused a 60% rgr in an assay against the fall armyworm. The MeCN partition of the EtOAc extract from NRRL 47509 was

also subjected to antiinsectan assays. This sample generated an rgr of 54% against the fall armyworm when tested at 2000 ppm. ^{1}H NMR analysis of this partition fraction demonstrated monorden (**19**) to be the major metabolite of this extract.

In Vitro Activity Determination for Compounds ***19–22***

Monorden (**19**) and monocillins I–III (**20–22**) were tested for fungistatic activity against a panel of maize fungal endophytes and pathogens. Nystatin (Sigma–Aldrich, St. Louis, MO) was used as a positive antifungal control, and MeOH as a negative control. Test compounds were dissolved in MeOH such that suitable aliquots of MeOH solution could be dispensed to add 1, 2, 3, 5, 10, 25, and 50 μg of the sample to each of eight replicate wells of a 96–well plate (0.32 cm^2 growth area; 370 μL volume; BD Primaria clear 96–well Microtest Plate No. 353872, Becton Dickinson, Franklin Lakes, NJ), and then evaporated to dryness. A 200–μL aliquot of PDB seeded with a fungal endophyte/pathogen was added to each plate well, along with 10 μL of MeOH to redissolve test compounds. Plates were placed on a shelf at room temperature (~25 °C) for up to 64 hours. Every 8 to 16 hours, plates were examined using a plate reader at a wavelength of 550 nm (Dynatech MR 5000 with BioLinx Version 2.0 Assay Management Software; Dynatech Laboratories Inc., Chantilly, VA) to measure inhibition of fungal growth. MIC values represent the minimum amount of test

compound associated with a lack of pathogen growth. GI_{50} values represent the amount of test compound associated with growth inhibition of 50% in comparison with negative MeOH controls. MIC values were first determined for all four compounds against *A. flavus* (NRRL 6541), *F. verticilliodes* (NRRL 25457), *Alternaria alternata* (NRRL 6410), *Nigrospora oryzae* (NRRL 6414), and *Diplodia maydis* (NRRL 31249). Monorden (**19**) and monocillin (**20**) were the only compounds displaying significant fungistatic activty. For this reason, MIC and GI_{50} values were determined only for these two metabolites against the full panel of maize fungal pathogens and endophytes listed in Table 2. The test strains used in these antifungal assays were also isolated from maize seeds (*A. zeae* NRRL 13540; *A. alternata* NRRL 6410; *A. flavus* NRRL 6541; *Curvularia lunata* NRRL 6409; *N. oryzae* NRRL 6414; *Trichoderma viride* NRRL 6418 from North Carolina; *Fusarium graminearum* NRRL 31250; *S. maydis* NRRL 31249 from Indiana; *Bipolaris zeicola* NRRL 47238 from Cerro Gordo, IL; *F. verticillioides* NRRL 25457 from South Carolina).

Leaf–puncture Wound Assay [271]

Maize leaf blades were cut from 4–week old maize seedlings (Burrus 794sRR) that were growing in a greenhouse. Six needle punctures (~0.25 mm in length) were made per blade. Monorden (**19**) and monocillin I (**20**) were evaluated for their phytotoxic effects against these maize leaves, using oxalic

acid as a positive control and a 1:1 solution of MeOH:H_2O as a negative control.[272] Compounds were dissolved in a 1:1 MeOH:H_2O solution to a concentration of 2 μg/μL. One drop of this solution (5 μL) was added to each of the six wound puncture sites on a maize blade. Leaves were then incubated for 3 days (21–23°C) on moistened filter paper in a Petri dish. A stereomicroscope was used after incubation to measure the length of nectroctic leasions formed by phytotoxic compounds.

Inoculation of Non–living and Living Maize Stalks

Stalk Residues (non–living maize tissue):

Maize stalk residues (stalks one week post–harvest in 2008) not displaying symptoms of stalk rot were collected from two locations: an NCAUR field plot and a commercial plot near Dunlap, IL. Stalks were removed of leaves, rinsed in tap water, and then cut into 7–cm segments. Stalk residue segments were then placed among six humidity chambers (Pyrex storage dishes, 100 x 80 mm with lids, Corning No. 3250, Corning Glassworks, Corning, NY), keeping residues from different locations in separate chambers. Water was added to chambers in order to increase moisture of dry stalks to ~ 300 % Md $\left(\frac{wet\ weight - dry\ weight}{dry\ weight} x\ 100\right)$ and then autoclaved at 1.055 kg fp cm^{-2} for 30 min, followed by cooling to room temperature. Steam–sterilized stalks were then toothpick wound–inoculated with either of two isolates of *C. graminicola* (NRRL 47511 or 47509) by insertion of

toothpicks containing a fungal isolate into both ends of a stalk segment. Toothpick wound–inoculated stalks were then placed back into humidity chambers, sealed with parafilm, and incubated for 14 days at 25°C. Following incubation, toothpicks were removed and the puncture sites revealed severe mold formation within maize stalk residue segments. These samples were transfered to pre–weighed freezer bags, weighed, freezed–dried, and weighed again. Uninoculated stalk segments served as controls.

Green Stalks (non–living maize tissue):

A maize stalk in the dent stage of kernel maturity was harvested from an NCAUR field plot and its leaves removed. The stalk was then cut into 7–cm segments (24 pieces), and four segments placed in each of six humidity chambers. Stalks were then autoclaved at 1.055 kg fp cm^{-2} for 30 min. Upon cooling of steam–sterilized segments, green stalks were toothpick wound–inoculated in the same manner as stalk residues. After 7 days of incubation at 25°C, toothpicks were removed to reveal heavy mold formation growing from the ends of green stalk stegments. These molded segments were weighed in pre–weighed freezer bags, freeze–dried, and weighed again. Uninoculated green stalk segments served as controls.

Growth Chamber Maize Stalks (living maize tissue):

Eighteen pots (12.5 cm) were filled half way with pasteurized SB–300 bark. Pasteurized growing mix (Redi–earth, Sun Gro Horticulture, Canada) was placed on top of this, filling the rest of the pots, and contained a slow–release fertilizer (Osmocote Classic, Scotts International, Marysville, OH) and trace elements (Micromax, Scotts International). Maize seeds (F–2 seeds produced by a commercial hybrid Burrus 794sRR) were planted in each of these 18 pots (12.5 cm) and grown in an ecological chamber (Model GC–16; Enconair, Winnipeg, MB, Canada). Daylight simulation consisted of a 16–hour photoperiod and temperatures ranging from 25–27°C. Nighttime temperatures were fixed between 20 and 22°C. Plants were watered daily with deionized water. After 30 days of growth, plants were given a calcium and magnesium supplement (Cal–Mag Special 15–5–15, Peters Excel, Scotts International) and then allowed to grow for one more week prior to inoculation with *C. graminicola* isolates. After 37 days, a toothpick was inserted 5 mm deep into each of the first four internodes above the root crown of a maize plant. These toothpicks were removed and replaced with toothpicks containing either NRRL 47511 or 47509. Living maize stalks began to display symptoms of stalk anthracnose 31 dpi (31 days post–inoculation, 63 days after planting), and some of these stalks were harvested. Other stalks were allowed to grow until both stalk anthracnose and wilting were

observed (56 dpi; 93 days after planting) before harvesting. Internodal sections (1st, 2nd, and 3rd) of 31 dpi– and 56 dpi– harvested stalks were split longitudinally and revealed discolored pith. A portion of the discolored pith (2–3 mm^2) was removed from each stalk section approximately 3–cm from the puncture site. This sampling was cultured on PDA containing streptomycin (25 mg L^{-1}) to confirm *C. graminicola* as the fungus causing the stalk anthracnose and wilting in these maize plants. Stalk sections were freeze–dried and weighed in the same manner as *C. graminicola*–inoculated non–living maize stalk segments and residues.

LC–HRESITOFMS Detection of C. graminicola Metabolites

C. graminicola–inoculated stalk residues, green stalk segments, and infected–necrotic stalk lesions from growth chamber plants were cut into 1–2 cm pieces and then soaked in 150 mL of EtOAc for 30 min. This was done three times and the EtOAc extracts from a given stalk segment were combined with those from stalk segments in the same incubation chamber and evaporated to dryness. These organic extracts were next partitioned between hexanes and MeCN. The resulting MeCN fractions were then subjected to LC–HRESITOFMS and ^{1}H NMR in an effort to determine whether monorden (**19**) or monocillins I–III (**20**–**22**) were present in sample extracts. ^{1}H NMR data were obtained on a Bruker DRX–400 MHz instrument using $CDCl_3$ as solvent. Mass spectrometric

data were acquired on a Waters Q–Tof Premier mass spectrometer interfaced with a Waters Acquity UPLC system. Samples were analyzed using negative ion ESI. Data were acquired at high resolution over the mass range 100–1000 Da with parallel UV detection at 254 nm. The eluting solvents used were 5 % MeCN in H_2O with 0.1% formic acid (solvent A) and MeCN with 0.1% formic acid (solvent B). All solvents used for LC–HRESITOFMS were Optima LC–MS grade (Fisher Scientific, Pittsburgh, PA). Samples were chromatographed over a Waters BEH reversed–phase C_{18} column (2.1mm x 10 cm; 1.7–μ particle size) at a flow rate of 0.25 mL min^{-1} with a sample injection volume of 4 μL using the following method: ramp from 20% to 60% solvent B over 10 min; isocratic at 60% solvent B over 15 min; ramp to 100% solvent B over 10 min. A fraction containing monorden (**19**) and monocillins I–III (**20**–**22**) was used as a standard for determination of LC–HRESITOFMS retention times (**19**: t_R = 6.38 min; **20**: t_R = 6.60 min; **21**: t_R = 11.23 min; **22**: t_R = 5.42 min).

Procedures for the Isolation and Characterization of Bipolaris zeicola Metabolites

Fungal Material

Three isolates of *Bipolaris zeicola* were obtained by Dr. Donald T. Wicklow from the USDA Agricultural Research Service (NRRL) Culture Collection in Peoria, IL. Two of these samples were collected from corn seeds and one from sorghum seed. NRRL 47503 (GQ 253958; ENDO–3130) was isolated from a

'Mandan Bride' variety corn seed produced at an unreported field in Michigan in 2004. The seed was later obtained from Seed Savers Exchanged in Decorah, IA in 2005. The second isolate, NRRL 47238 (FJ213843; ENDO–3039) also originated from corn seed, i.e. field corn from Cerro Gordo, IL. The third isolate, NRRL 47500 (GQ 253957; ENDO–3090), differs from the other two in that it was isolated from sorghum rather than corn. The sorghum was produced in Patagonia, AZ in 2004. This grain was later obtained from Native Seeds Search in Tucson, AZ in 2005.

Fungal isolates NRRL 47503, 47238, and 47500 were individually cultured on potato dextrose agar (PDA) slants for six days at 25 °C. A suspension of conidia and hyphal cells prepared from these cultures served as inoculum. Fermentations were carried out in two 500–mL Erlenmeyer flasks, each containing 50 g of rice (Botan Brand; J.F.C. International, Los Angeles, CA) that were soaked overnight in distilled water (50 mL) before being autoclaved at 1.055 kg f cm^{-2} for 30 min. After the flasks had cooled to room temperature, they were inoculated with 1.0 mL of the hyphal fragment spore suspension from one of the isolates (NRRL 47503, 47238, or 47500). Fermentation flasks were allowed to incubate for 30 days at 25°C.

Extraction and Isolation

After one month of incubation, the fermented rice substrates were first mechanically fragmented using a spatula and then extracted three times with EtOAc (100 mL each time). The EtOAc extracts of a given *B. zeicola* culture were then combined and evaporated to dryness. This process afforded crude extracts amounting to 857 mg for NRRL 47503, 1544 mg for NRRL 47500, and 1321 mg for NRRL 47238. A portion of the EtOAc extract from each of the rice fermentations (5 mg) was set aside for biological testing. The remainder of the extracts were stored at –20°C until chemically investigated. The extracts from NRRL 47503 and 47500 were chemically investigated at the same time because it was not known (at the time) that these extracts both originated from *B. zeicola* isolates. Large clumps of a white solid were seen in the brown EtOAc extract of NRRL 47500. A tweezers was used to remove a portion (3.7 mg) of this material and ^{1}H NMR data were collected. It was not immediately clear from the resulting data as to the chemical composition of the white solid. The remainder of the EtOAc extracts were partitioned between hexanes and MeCN. The EtOAc extract was dissolved in 10 mL of MeCN and then washed three times with 5 mL of hexanes. More MeCN and/or hexanes were added to further dissolve the EtOAc extract if necessary. Both fractions from the partitioning were evaporated to dryness, weighed, and ^{1}H NMR data were collected. Partitioning resulted in MeCN–

soluble fractions of 1241 mg for NRRL 47500, 709 mg for NRRL 47503, and 1072 mg for NRRL 47238.

The MeCN–soluble fraction of NRRL 47503 (709 mg) was subjected to silica gel VLC (8.5 x 12 cm) using a gradient elution of hexanes, CH_2Cl_2, and MeOH with increasing polarity. Fifteen fractions were collected on the basis of color band elution and then evaporated to dryness: fr 1 (200 mL hexanes); fr 2 (200 mL of 7.5% CH_2Cl_2 in hexanes); fr 3 (200 mL of 15% of CH_2Cl_2 in hexanes); fr 4 (200 mL of 25% CH_2Cl_2 in hexanes); fr 5 (200 mL of 45% CH_2Cl_2 in hexanes); fr 6 (200 mL of 80% CH_2Cl_2 in hexanes, 52 mg of mostly white solid); fr 7 (200 mL CH_2Cl_2, 177 mg of a white solid); fr 8 (200 mL of 0.75% MeOH in CH_2Cl_2, 31 mg of mostly white solid); fr 9 (200 mL of 1.5% MeOH in CH_2Cl_2, 25 mg of mostly white solid); fr 10 (250 mL of 3% MeOH in CH_2Cl_2, yellow–orange color band, 119 mg); fr 11 (200 mL of 7% MeOH in CH_2Cl_2, 107 mg); fr 12 (200 mL of 12% MeOH in CH_2Cl_2, 43 mg); fr 13 (200 mL of 25% MeOH in CH_2Cl_2, 43 mg); fr 14 (200 mL acetone, 27 mg); fr 15 (350 mL MeOH, 68 mg).

A similar VLC elution scheme was employed for the separations of the MeCN–soluble fraction from NRRL 47500 (1241 mg), except that acetone was not employed to elute the column prior to the final MeOH wash. Fourteen fractions were collected from this chromatography step on the basis of color band elution and each was evaporated to dryness to give: fr 1 (200 mL hexanes); fr 2 (200 mL

of 7.5% CH_2Cl_2 in hexanes); fr 3 (200 mL of 15% of CH_2Cl_2 in hexanes); fr 4 (200 mL of 25% CH_2Cl_2 in hexanes); fr 5 (200 mL of 45% CH_2Cl_2 in hexanes); fr 6 (200 mL of 80% CH_2Cl_2 in hexanes, 171 mg of mostly white solid); fr 7 (200 mL CH_2Cl_2, 379 mg of white solid); fr 8 (200 mL of 0.75% MeOH in CH_2Cl_2, 40 mg of mostly white solid); fr 9 (200 mL of 1.5% MeOH in CH_2Cl_2, 32 mg of mostly white solid); fr 10 (200 mL of 3% MeOH in CH_2Cl_2, 27 mg of mostly white solid); fr 11 (200 mL of 7% MeOH in CH_2Cl_2, yellow–orange color band, 477 mg); fr 12 (200 mL of 12% MeOH in CH_2Cl_2, slight yellow color band, 82 mg); fr 13 (200 mL of 25% MeOH in CH_2Cl_2, slight yellow color band, 56 mg); fr 14 (350 mL MeOH, slight yellow color band, 10 mg). These masses total 1274 mg, for which the positive mass discrepancy may be attributed to solvent trapping in some of the larger samples. The physical properties of chromatographic fractions from both NRRL 47503 and 47500 were very similar and ^{1}H NMR data of these fractions proved these extracts to be composed of similar secondary metabolites.

In an effort to determine the chemical components of these isolates, further separations and spectroscopic data collection were conducted for NRRL 47500. ^{1}H NMR data for VLC fractions 6–10 matched the ^{1}H NMR profile for the white compound removed from the MeCN–soluble partition as noted above. Fraction 11, which displayed a yellow–orange color band during elution, was further purified over reversed–phase HPLC according to the following method:

1 mg of sample (dissolved in MeCN) per injection; mobile phase: 90% MeCN in H_2O (flow rate of 2 mL/min for 40 min); stationary phase: Alltech Apollo C_{18} column (10 x 250 mm; 5–μ particle size); UV detection at 215 nm. This resulted in 5 fractions containing the following known compounds: fr 1 (dihydroprehelminthosporol, **25**; t_R = 7.7 min; 1.3 mg)[139-141], fr 2 (helminthosporol, **26**; t_R = 8.7 min; 1.0 mg)[139,142-146] , fr 7 (heveadride, **24**; t_R = 25.2 min; 3.1 mg)[135-138], fr 8 (major component: cochlioquinone A, **28**; minor component: isocochlioquinone A, **27**; t_R = 27.4 min; 1.0 mg)[150-153], and fr 9 (isocochlioquinone A, **27**; t_R = 30.5 min; 0.5 mg)[150-153]. All of these compounds were identified by comparison of ^{1}H and ^{13}C NMR spectra with literature values and in some cases, the assignments were confirmed by analysis of 2D–NMR data.

Heveadride (**24**) was obtained as a white solid; ^{1}H NMR data see Table 3.

Dihydroprehelminthosporol (**25**) was obtained as a colorless solid; 1D– and 2D–NMR data see Table 4.

Helminthosporol (**26**) was obtained as a colorless solid; ^{1}H NMR data see Table 4.

Isocochlioquinone A (**27**) was obtained as a yellow oil; ^{1}H NMR data see Table 5.

Cochlioquinone A (**28**) was obtained as a major component in a mixture with isocochlioquinone A (**27**). The mixture was a yellow oil; ^{1}H NMR data see Table 5.

The antiinsectan effects of compounds **24**-**28** are summarized in Tables 6 and 7.

Antifungal Activity of B. zeicola Metabolites and the NRRL 47238 Crude Extract

The crude extract from *B. zeicola* isolate NRRL 47238 was tested against a panel of maize fungal endophytes and pathogens in disc assays using methods similar to those described for *C. graminicola* crude extracts. The test strains used were also isolated from maize seeds (*A. zeae* NRRL 13540; *A. alternata* NRRL 6410; *A. flavus* NRRL 6541; *Colletotrichum graminicola* NRRL 47511; *Curvularia lunata* NRRL 6409; *F. graminearum* NRRL 31250; *F. verticillioides* NRRL 25457; *Nigrospora oryzae* NRRL 6414; *S. maydis* NRRL 31249; *Trichoderma viride* NRRL 6418). The crude extract from NRRL 47238 inhibited the following corn pathogens: *A. zeae* (17 mm); *A. alternata* (37 mm); *A. flavus* (filtered conidial inoculum; 27 mm); *C. graminicola* (23 mm); *C. lunata* (23 mm); *F. graminearum* (33 mm); *F. verticillioides* (33 mm); *F. verticillioides* (filtered conidial inoculum; 37 mm); *N. oryzae* (33 mm); *S. maydis* (43 mm).

Compounds **24**–**28** were tested for fungistatic activity in standard disc assays against *A. flavus* (NRRL 6541) and *F. verticillioides* (NRRL 25457), in

experiments using a filtered conidial inoculum. Dihydroprehelminthosporol (**25**) and a fraction consisting almost entirely of cochlioquinone A (**28**) were both active in preventing the growth of *F. verticillioides* (mz = 18.5 mm at 200 µg sample/disc; Tables 6 and 7). Isocochlioquinone A (**27**), heveadride (**24**) and helminthosporol (**26**) were inactive in these assays when tested at levels of 200 µg sample/disc. Isocochlioquinone A (**27**) was a contaminant in the antifungal sample of cochlioquinone A (**28**) and therefore the fungistatic activity displayed by the fraction containing their mixture is presumably a result of cochlioquinone A's (**28**) presence alone. None of the isolated compounds was active against *A. flavus* at levels tested (200 µg sample/disc).

Leaf–puncture Wound Assay

Maize leaf blades were cut from 4–week old maize seedlings (Burrus 794sRR) that were growing in a greenhouse. Six needle punctures (~0.25 mm in length) were made per blade. Heveadride (**24**) was evaluated for its phytotoxic effects against these maize leaves, using oxalic acid as a positive control and a 1:1 solution of MeOH:H_2O as a negative control. Compound **24** was dissolved in a 1:1 MeOH:H_2O solution to a concentration of 2 µg μL^{-1}. One drop of this solution (5 µL) was added to each of the six wound puncture sites on a maize blade. Leaves were then incubated for 3 days (21–23°C) on moistened filter paper in a Petri dish. A stereomicroscope was used after incubation to measure

the length of necrotic lesions formed by phytotoxic compounds (Table 6). Four of the six of the wound puncture sites displayed lesions averaging 4.9 mm in length. The two puncture sites without lesions were a result of these wounds penetrating through the leaf, allowing the heveadride (**24**) to pass through the leaf unabsorbed.

Procedures for the Isolation and Characterization of Monascus ruber Metabolites

Fungal Material

Corn seeds were stored at 30°C in sealed jars having controlled moisture content to investigate the post–harvest contamination of grain in the storage process.[188] A bright red fungal growth was noted on rehydrated corn seeds (produced in 2005 near Decatur, IL; shelled grain purchased from Kelly Seeds, Peoria, IL) that had been incubated in the dark with 30% moisture content for 21 days. Select corn seeds containing the red fungal growth were subcultured resulting in the isolation of ENDO–3131. This isolate was later determined to be an isolate of *M. ruber* based on micromorphology.

ENDO–3131 was grown on 100 g of rice (2 x 50 g) for 30 days at 25°C. The resulting fermentation mixture was then extracted with EtOAc to afford 1.0 g of crude extract. This extract showed activity against *A. flavus* (cz 18 mm), *F. verticillioides* (cz 14 mm), and the fall armyworm (50% rgr).

Extraction and Isolation

The ENDO–3131 extract (1.0 g) was partitioned between hexanes and MeCN to yield 72 mg and 928 mg fractions respectively. The MeCN partition was subjected to silica gel VLC using a step gradient of hexanes, CH_2Cl_2, and MeOH (100% hexanes; 7.5, 15, 25, 45 and 80% CH_2Cl_2 in hexanes; 100% CH_2Cl_2; 0.75, 1.5, 3, 7, 12, and 25% MeOH in CH_2Cl_2; 100% MeOH) to afford 14 fractions: fr 1– fr 4 (negligible mass); fr 5 (1.1 mg); fr 6 (2.4 mg); fr 7 (yellow–orange; 19.5 mg); fr 8 (fluorescent orange; 370 mg); fr 9 (orange; 32.9 mg); fr 10 (orange–yellow; 9.6 mg); fr 11 (red; 192 mg); fr 12 (deep red; 203 mg); fr 13 (100 mg); fr 14 (263 mg). These masses total 1.2 g, implying that solvent was trapped in some of these fractions. Fraction V8 (370 mg) was further separated over reversed-phase HPLC (isocratic at 90% MeCN in H_2O over 20 minutes) using a Grace Apollo C_{18} column (10 x 250 mm; 5µ particle size) with injection volumes of 100 µL (1 mg/100 µL; 14 injections) and eluting at a flow rate of 2 mL/min with UV detection at 230 nm. This process afforded rubropunctatin (**30**; 5.1 mg, t_R 11.2 min)[189-193] and monascin (**31**; 1.1 mg, t_R 10.1 min).[192,193] These compounds were identified by comparison of ^{1}H and ^{13}C NMR data with literature values.

Rubropunctatin (**30**) was obtained as orange crystals from MeOH; ^{1}H and ^{13}C NMR data see Table 9.

Monascin (**29**) was obtained as yellow crystals from MeOH; ^{1}H NMR data see Table 9.

*Antifungal and Antiinsectan Activity of Rubropunctatin (**30**) and Monascin (**31**)*

Compounds **30** and **31** were tested in antifungal disc assays against *A. Flavus* and *F. verticillioides* (200 μg sample per disc). Rubropunctatin (**30**) inhibited the growth of both pathogens, causing 17–mm mottled zones in each assay after 48 hours. Monascin (**31**) was inactive in these assays at levels tested. Both compounds also reduced the growth rate of the fall armyworm. When the insects were fed a diet containing 740 ppm of rubropunctatin (**30**), their growth rate was reduced by 26% relative to controls. A similar experiment with 220 ppm monascin (**31**) reduced the growth rate of the fall armyworm by 33%.

Fungicolous Fungal Experimental Procedures

Procedures for the Isolation and Characterization of Metabolites from an Isolate of Acremonium crotocinigenum

Fungal Material

A fungal specimen was collected from the surface of a polypore host (*Phellinus gilvus*) that was growing on a dead hardwood branch in Hawaii on Nov. 5th, 2002 (Casuarina forest, Mackenzie State Park, Puna district, Hawaii, HI). From this host, a white, velvety fungicolous fungus was isolated (MYC–1959). Fungicolous isolate MYC–1959 was fermented on rice (2 x 50 g) for 30 days at

25°C. The resulting fermentation culture was then mechanically fragmented and extracted with EtOAc, yielding 979 mg of crude extract. This extract showed antifungal effects against *F. verticillioides* (mz 10) and caused 100% mortality in a dietary assay against *S. frugiperda*. MYC–1959 was classified as an isolate of *Acremonium crotocinigenum* (NRRL 45419) on the basis of micromorphology and DNA analysis. It was deposited in the USDA NRRL collection and assigned the accession number NRRL 45419.

Extraction and Isolation

The organic extract from NRRL 45419 was subjected to a bioassay–guided fractionation process. The extract (979 mg) was first partitioned between hexanes and MeCN and dried under airflow. The MeCN–soluble fraction (307 mg) was then redissolved in 5 mL of a 50:50 acetone:MeOH solution and then subjected to silica gel VLC. The column was eluted according to the following method and fractions were then evaporated to dryness: V1 (200 mL of 1% MeOH in CH_2Cl_2; 29 mg); V2 (200 mL of 2% MeOH in CH_2Cl_2; 30 mg); V3 (200 mL of 3% MeOH in CH_2Cl_2; 26 mg); V5 (200 mL of 5% MeOH in CH_2Cl_2; 19.3 mg); V10 (200 mL of 10% MeOH in CH_2Cl_2; 28 mg); V20 (200 mL of 20% MeOH in CH_2Cl_2; 46 mg); V100 (300 mL of 100% MeOH; 59 mg). These fractions, excluding the MeOH wash (V100), were tested against *A. flavus* and *F. verticillioides*, affording the following results: V1 (mz 26 mm; cz 22 mm); V2 (cz

24 mm; cz 20 mm); V3 (inactive; cz 16 mm); V5 (inactive; inactive); V10 (mz 16 mm; mz 20 mm); V20 (mz 5 mm *weak*; mz 9 mm). It is not clear why some of these fractions displayed antifungal activity against *A. flavus*, given that the crude extract was inactive against this pathogen, but it is possible that active constituents too dilute in the original extract were sufficiently concentrated in these fractions to show such effects.

Fraction V1 was selected for further purification based on its biological activity and ^{1}H NMR profile. A portion of this fraction was separated over RP–HPLC using an Alltech Apollo C_{18} column (10 x 250 mm; 5–μ particle size) eluting at a flow rate of 2 mL min^{-1} with UV detection at 215 nm. Fr V1 was dissolved in MeCN and 1–mg portions were injected onto the HPLC column and eluted as follows: ramp from 50% to 65% MeCN in H_2O over 40 min; isocratic at 65% MeCN over 5 min; ramp from 65% to 80% MeCN in H_2O over 2 min; ramp from 80% to 50% MeCN in H_2O over 2 min. This chromatography afforded trichothecin (**36**; 1.6 mg; t_R = 24 min),[29,33] 6,8-dimethoxy-4,5-dimethyl-3-methyleneisochroman-1-one (**37**; 1.9 mg; t_R = 23 min),[41] and (**38**; 1.9 mg; t_R = 30 min). Compounds **36** and **37** were identified by comparison of their ^{1}H NMR and MS data to literature values.

Trichothecin (**36**) was obtained as a white solid; ^{1}H NMR data see Table 10.

6,8-dimethoxy-4,5-dimethyl-3-methyleneisochroman-1-one (**37**) was obtained as a colorless solid; ^{1}H NMR data see Table 11.

Lactone **38** was obtained as a colorless solid; 1D– and 2D– NMR data see Table 11. EIMS obsd *m/z* 262, HREIMS data were not attainable due to degradation of sample prior to the analysis.

*Antifungal and Antiinsectan Assays for Lactone **38***

In antifungal assays against *A. flavus*, compound **38** inhibited fungal growth (17 mm) at 200 µg–sample per disc. It was inactive, however, when tested at the same levels against *F. verticillioides*. This compound was moderately active at reducing the growth of the fall armyworm (33% rgr) when insects were fed a diet containing 150 ppm of compound **38**.

Procedures for the Isolation and Characterization of Metabolites from Unidentified Fungicolous Isolate MYC–1969

Fungal Material

A white mycelial growth was collected from the undersurface of a dead hardwood branch in the Casuarina forests of Hawaii's MacKenzie State Park in November of 2002. From this mycelial growth, a smooth black cotton culture with brown hyphae (MYC-1969) was isolated. MYC-1969 was grown on 100 g of rice for 30 days at 25°C. The resulting fermentation mixture was then extracted with EtOAc to afford 1.4 g of crude extract. This extract showed activity against

A. flavus, *F. verticillioides*, and the fall armyworm. Thus far, the culture has been designated *Mycelia sterilia* due to a lack of sporulation. Attempts to reisolate MYC-1969 from the original white mycelial specimen were unsuccessful, presumably due to fungal cells not surviving the freezer and refrigeration processes.

Extraction and Isolation

MYC-1969 (1.4 g) was partitioned between hexanes and MeCN to yield fractions weighing 586 mg and 876 mg fractions respectively. The MeCN–soluble partition was subjected to a silica gel VLC process employing a step gradient of hexanes, CH_2Cl_2, and MeOH (100% hexanes; 1, 2, 3, 4, 5, 10 and 20% MeOH in CH_2Cl_2; 100% MeOH) to afford 9 fractions. Fraction V4 (370 mg) was then chromatographed over a Sephadex LH-20 column using a gradient elution of hexanes, CH_2Cl_2, acetone, and MeOH (4:1 CH_2Cl_2:hexanes, 3:2 CH_2Cl_2:acetone, 1:4 CH_2Cl_2:acetone, and 100% MeOH)[268] to afford 7 fractions (A-G). Fraction A (161 mg) appeared to contain a class of related molecules based on ^{1}H NMR data and was further separated by reversed-phase HPLC (isocratic at 55% MeCN in H_2O over 25 min) using an Alltech Apollo C_{18} column (10 x 250 mm) eluting at a flow rate of 2 mL/min with UV detection at 215 nm. This process afforded phomactin K (**40**; 2.8 mg, t_R 20.4 min), phomactin L (**41**; 2.4 mg, t_R 14.3 min) and another fraction (4.7 mg, t_R 16.5 min) whose major component was later

identified as phomactin M (**42**). The mixture containing phomactin M (**42**) was further purified by reversed-phase HPLC (isocratic at 100% MeCN for 15 min) using a PRP-1 column with a flow rate of 2 mL/min with UV detection at 215 nm to afford phomactin M (**42**; 2.2 mg; t_R 6.35 min).

Phomactin K (**40**) was obtained as a colorless oil; $[\alpha]^{20}_D$ + 55 (*c* 0.14, $CHCL_3$). ^1H and ^{13}C NMR data see Table 12; HMBC data: H-3 → C-1, 2, 4, 5; H_2-5 → C-4, 6 16; H_2-6 → C-5, 7; H-7 → C-6; H_2-9 → C-8, 10, 11; H_2-10 → 9, 11, 15, 18; H-12 → C-10, 11, 13, 14, 15, 19; H_2-13 → C-1, 11, 14, 18; H-14 → C-2, 13; H_3-16 → C-3, 4, 5; H_3-17 → C-7, 8, 9; H_3-18 → C-10, 11, 12, 15; H_3-19 → C-11, 12, 13; H_2-20 → C-1, 15; ESIMS obsd *m*/*z* 333, calcd for $C_{20}H_{29}O_4$ ($[M + H]^+$), 333.4451; efforts to acquire HRMS data on this sample using various techniques were unsuccessful.

Phomactin L (**41**): colorless oil; $[\alpha]^{20}_D$ +44 (*c* 0.12, $CHCl_3$); ^1H and ^{13}C NMR data, see Table 12; HMBC data: H_2-2 → C-1, 3, 4, 14, 15; H-3 → C-2; H_2-5 → C-3, 4, 6, 16; H_2-6 → C-5; H-7 → C-6; H_2-9 → C-7, 8, 10, 11; H_2-10 → 8, 9, 11, 15, 18; H-12 → C-10, 11, 13, 14, 15, 19; H-14 → C-2, 3, 12; H_3-16 → C-3, 4, 5; H_3-17 → C-7, 8, 9; H_3-18 → C-10, 11, 12, 13, 15; H_3-19 → C-11, 12, 13; H_2-20 → C-1, 15; HRESIMS obsd *m*/*z* 333.2042, calcd for $C_{20}H_{29}O_4$ $[(M + H)^+]$, 333.4451.

Phomactin M (**42**) was obtained as a colorless oil; $[\alpha]^{20}_D$ +43 (*c* 0.14, $CHCl_3$); ^1H NMR, ^{13}C NMR, DEPT and decoupling data, see Table 13; HMBC data: H-3 → C-1, 2, 4, 5; H_2-5 → C-3, 4, 6, 7, 16; H_2-6 → C-4, 5, 7, 8; H-7 → C-6, 8, 9;

H_2-9 → C-7, 8, 10, 11, 17; H_2-10 → C-8, 9, 11, 12, 15, 18; H-12 → C-1, 2, 10, 11, 13, 14, 15, 19; H-14 → C-2, 12, 15; H-15 → C-10, 11, 12, 13, 14, 20; H_3-16 → C-3, 4, 5; H_3-17 → C-7, 8, 9; H_3-18 → C-10, 11, 12, 13, 15; H_3-19 → C-1, 11, 12; H_3-20 → C-11, 13, 15; HRESIMS obsd *m/z* 333.2058, calcd for $C_{20}H_{29}O_4$ [$(M + H)^+$], 333.4451.

*Antifungal and Antibacterial Assays for phomactins K–M (**40**–**42**)*

Phomactins K–M (**40**–**42**) were tested in antifungal disc assays against *A. flavus* and *C. albicans*, and antibacterial assays against *E. coli* and *S. aureus*. All compounds were tested at levels of 100 μg sample/disc and the results are summarized in Table 14.

Procedures for the Isolation and Characterization of Metabolites from a Fungicolous Isolate of Neofusicoccum parvum

Fungal Material

A *Rigidosporus microsporus* specimen that had been colonized by fungicolous fungi was collected from a dead, hardwood branch rotting in a Hawaiian Alien Wet Forest at the Hilo Zoo in November of 2002. The host basidioma was placed in a paper container, air dried, and then stored in a 5°C refrigerator until isolation of fungicolous fungal isolates was performed. A surface–sterilized fingernail file was used to generate filings of host tissues. Basidioma filings (100–200 mg) were then plated on dextrose–peptone–yeast extract agar (DPYA) containing the antibacterial agents streptomycin (23 mg/L)

and tetracycline (1.25 mg/L).[273] Culture plates were incubated in the dark for 5 days at 25˚C which generated many fungicolous fungal colonies containing distinctive morphological features. Each distinctive colony was isolated from others and PDA slant–cultured for 7 to 12 days (25˚C). One of these isolates (MYC–1674 = NRRL 46122) was later subjected to fermentation as described above (100 g rice; 30 d; 25 ˚C) and extracted with EtOAc to yield 678 mg of a dried crude extract which showed antifungal activity against *F. verticillioides*.

Extraction and Isolation

The fermented rice extracts of NRRL 46122 were first partitioned between hexanes and MeCN resulting in 343 mg of MeCN–soluble fraction (Scheme 6). This fraction was separated over a silica gel VLC column (8.5 x 12 cm), eluting with solvent mixtures of increasing polarity comprised of hexanes, CH_2Cl_2, and MeOH. Fourteen fractions were collected on a volumetric basis and evaporated to dryness: V1 (300 mL of hexanes, 2 mg); V2 (200 mL of 3.75% CH_2Cl_2 in hexanes, 1 mg); V3 (200 mL of 15% of CH_2Cl_2 in hexanes, 1 mg); V4 (200 mL of 25% CH_2Cl_2 in hexanes, 1 mg); V5 (200 mL of 45% CH_2Cl_2 in hexanes, 1 mg); V6 (200 mL of 80% CH_2Cl_2 in hexanes, 6 mg); V7 (300 mL of CH_2Cl_2, 6 mg); V8 (300 mL of 0.75% MeOH in CH_2Cl_2, 1 mg); V9 (700 mL of 1.5% MeOH in CH_2Cl_2, 12 mg); V10 (300 mL of 3% MeOH in CH_2Cl_2, 64 mg); V11 (200 mL of 7% MeOH in CH_2Cl_2, 10 mg); V12 (200 mL of 12% MeOH in CH_2Cl_2, 117 mg); V13 (200 mL of

25% MeOH in CH_2Cl_2, 50 mg); V14 (500 mL MeOH, 130 mg). These masses total 402 mg and the positive mass discrepancy is likely due to solvent trapped in some of the larger samples.

^{1}H NMR data analysis for fraction V10 (64 mg) revealed the presence of members of a class of distinctive metabolites, thus leading to further purification by reversed–phase HPLC (isocratic at 45% MeCN in H_2O over 38 min) using an Alltech Apollo C_{18} column (10 x 250 mm; 5–μ) and eluting at a flow rate of 2 mL/min with UV detection at 214 nm. This afforded five metabolites: asperlin (**45**; 0.3 mg, t_R 14.0 min),[246-248] 4–hydroxymellein (**46**; 0.5 mg, t_R 17.2 min),[250] 5,8–dihydroxy–3–methyl–3,4–dihydroisocoumarin (**47**; 0.5 mg, t_R 19.6 min),[249] oidiolactone G (**48**; 1.5 mg, , t_R 21.2 min),[251] and dehydro–oidiolactone G (**50**; 2.3 mg, t_R 24.4 min).[252]

^{1}H NMR data for another fraction (V12, 117 mg) revealed that this fraction was also composed of a mixture containing oidiolactone analogues. Fraction V12 was further separated over a Sephadex LH–20 column using a gradient elution of hexanes, CH_2Cl_2, acetone, and MeOH (4:1 CH_2Cl_2:hexanes, 3:2 CH_2Cl_2:acetone, 1:4 CH_2Cl_2:acetone, and 100% MeOH)[268] which afforded 9 fractions (A-I). The eighth fraction (Hse; 6.3 mg) was composed almost entirely of the known metabolite oidiolactone E (**52**).

Another fraction (V14; 130 mg) appeared to contain an oidiolactone metabolite as its major component from analysis of its ^{1}H NMR data. Several different chromatographic techniques were used to purify the as–yet unidentified major metabolite from this fraction. Although the metabolite (LL–Z1271β; **53**) ultimately proved to be known, sample impurities and residual NMR solvent peaks prevented identification of it from the ^{1}H NMR data at this stage. Attempts were made to purify V14 by reversed–phase HPLC. However, long retention times coupled with fraction complexity and peak broadness prevented purification of the major component using this approach. Efforts were next made to purify compounds from V14 over a silica gel gravity column (2.5 x 30 cm; 75 mL dry silica) using increasingly polar solvent mixtures of MeOH in acetone (Scheme 7). This resulted in 25 column fractions that were combined on the basis of TLC analysis, ultimately giving seven fractions: si1 (8 mg); si 2 (1.1 mg); si 3 (11.4 mg); si4 (23.8 mg); si5 (25 mg); si 6 (15.2 mg); si7 (13.9 mg). VLC fractions si4 and si5 contained the most material and were almost entirely composed of compound **53**. Several other fractions contained this compound, suggesting the presence of one or more polar (possibly carboxylic acid) functional groups in the structure that could contribute to tailing on silica gel.

To remedy this, fraction si4 was further separated over another silica gel gravity column (1.5 x 30 cm; 30 mL silica) using the following solvents in the

ratios listed: solvent A = TFA; solvent B = acetone; solvent C = CH_2Cl_2; solvent D = MeOH. This step (Scheme 7) resulted in the collection of 25 fractions based on elution volume, though not all fractions contained significant mass: siA (40 mL of 0.1% A and 10% B in C; 7.7 mg); siB (30 mL of 0.1% A and 10% B in C; 0.7 mg); siC (15 mL of 0.1% A and 25% B in C; 1.7 mg); siD (20 mL of 0.1% A and 25% B in C; 3.2 mg); siE (15 mL of 0.1% A and 25% B in C; 0.9 mg); siF (15 mL of 0.1% A and 25% B in C; 0.6 mg); siX (80 mL of 0.1% A and 100% B; 29.8 mg); siY (100 mL D; 16.9 mg). Fractions siX and siY trapped solvent and collected silica runoff from the gravity column contributing to their larger–than–expected masses. ^{1}H NMR data for fractions siC and siD showed that an artifact had formed from compound **53** during the isolation process, presumably due to the incorporation of the acid (TFA) in the eluent (Figure 12). Compound **53** and its artifact (**54**) were obtained as an inseparable mixture that remained unchanged in component ratio after re–dissolving in acidic solution (0.1% A and 25% B in C) for 3 hours. No further separations were performed on these samples due to limited sample amounts and continued chromatographic complexity.

A second attempt was made to isolate compound **53** by chromatography of subfraction si5 under more benign conditions (Scheme 7). A Sephadex LH–20 column (30 mL dry column material) was used as the stationary phase and a standard elution scheme was employed for the mobile phase.[268] Sixteen fractions

were collected on the basis of volume and dried under airflow: se1 (100 mL of 4:1 hexanes:CH_2Cl_2; 0.2 mg); se2 (50 mL of 4:1 hexanes:CH_2Cl_2; 0.3 mg); se3 (30 mL of 4:1 CH_2Cl_2:acetone; 1.9 mg); se4 (30 mL of 4:1 CH_2Cl_2:acetone; 1.2 mg); se5 (30 mL of 4:1 CH_2Cl_2:acetone; 0.3 mg); se6 (15 mL of 3:2 CH_2Cl_2:acetone; 0.6 mg); se7 (15 mL of 3:2 CH_2Cl_2:acetone; 1.3 mg); se8 (15 mL of 3:2 CH_2Cl_2:acetone; 0.2 mg); se9 (20 mL of 3:2 CH_2Cl_2:acetone; 1.5 mg); se10 (20 mL of 3:2 CH_2Cl_2:acetone; 0.6 mg); se11 (20 mL of 3:2 CH_2Cl_2:acetone; 0.6 mg); se12 (35 mL of 1:4 CH_2Cl_2:acetone; 1.3 mg); se13 (20 mL of 1:4 CH_2Cl_2:acetone; 1.1 mg); se14 (25 mL of 1:4 CH_2Cl_2:acetone; 1.3 mg); se15 (30 mL of 1:4 CH_2Cl_2:acetone; 1.1 mg); se16 (150 mL of MeOH; 3.6 mg). ^{1}H NMR data for fraction se10 showed this fraction to consist of a purified sample of compound **53**. More extensive NMR analysis at this stage helped to identify this compound as LL–Z1271β (**53**).[254] Another oidiolactone was isolated from this chromatography step (**55**; se13; 1.1 mg) though complete structure elucidation could not be accomplished by NMR due to sample limitations and extensive overlap of methylene signals in the ^{1}H NMR spectrum. Analysis of NMR data for other fractions did not show the presence of artifact **54**. Known compounds encountered in these fractions (**45–48**, **50**, **52**, and **53**) were identified by comparison of their 1D– and 2D–NMR and MS data with literature values.

Asperlin[246-248] (**45**; 0.3 mg): colorless oil; ^{1}H NMR data see Table 15.

4–Hydroxymellein[250] (**46**; 0.5 mg) and 5,8–dihydroxy–3–methyl–3,4–dihydroisocoumarin[249] (**47**; 0.5 mg): colorless oils; ^{1}H NMR data see Table 16.

Oidiolactone G[251] (**48**; 1.5 mg): colorless oil; ESIMS obsd *m/z* 277; ^{1}H NMR, DEPT, ^{13}C NMR, ^{1}H –^{1}H decoupling, HMQC, and HMBC data, see Table 17.

Dehydro–oidiolactone G[252] (**50**; 2.3 mg): colorless oil; ^{1}H NMR data, see Table 17.

Oidiolactone E[255] (**52**; major component of fraction Hse = 6.3–mg): colorless oil; ^{1}H NMR data, see Table 18.

LL–Z1271β[254] (**53**; 0.6 mg): colorless oil; ^{1}H NMR, DEPT, ^{13}C NMR, ^{1}H –^{1}H decoupling, HMQC, and HMBC data, see Table 19.

LL–Z1271β artifact (**54**; minor component of 3.2–mg fraction siD); partial ^{1}H NMR data, see Table 19.

*Antifungal Assays for Dehydro–oidiolactone G (**50**) and Oidiolactone E (**52**)*

The tedious isolation processes required for the isolation of oidiolactone metabolites from these complex mixtures limited the amount of purified sample that was collected for each of the nine compounds encountered in this extract. Oidiolactone E (**52**) and dehydro–oidiolactone G (**50**) were thus the only metabolites subjected to antifungal assays. Both compounds were tested in disc assays against *F. verticillioides* at concentrations of 500 μg per disc. Dehydro–

oidiolactone G (**50**) generated a fungistatic region with a cz of 29 mm in diameter. The nystatin standard afforded a similar clear zone at 25 μg/disc. Oidiolactone E (**52**) was inactive in the assay at this level.

APPENDIX

SELECTED SPECTRA

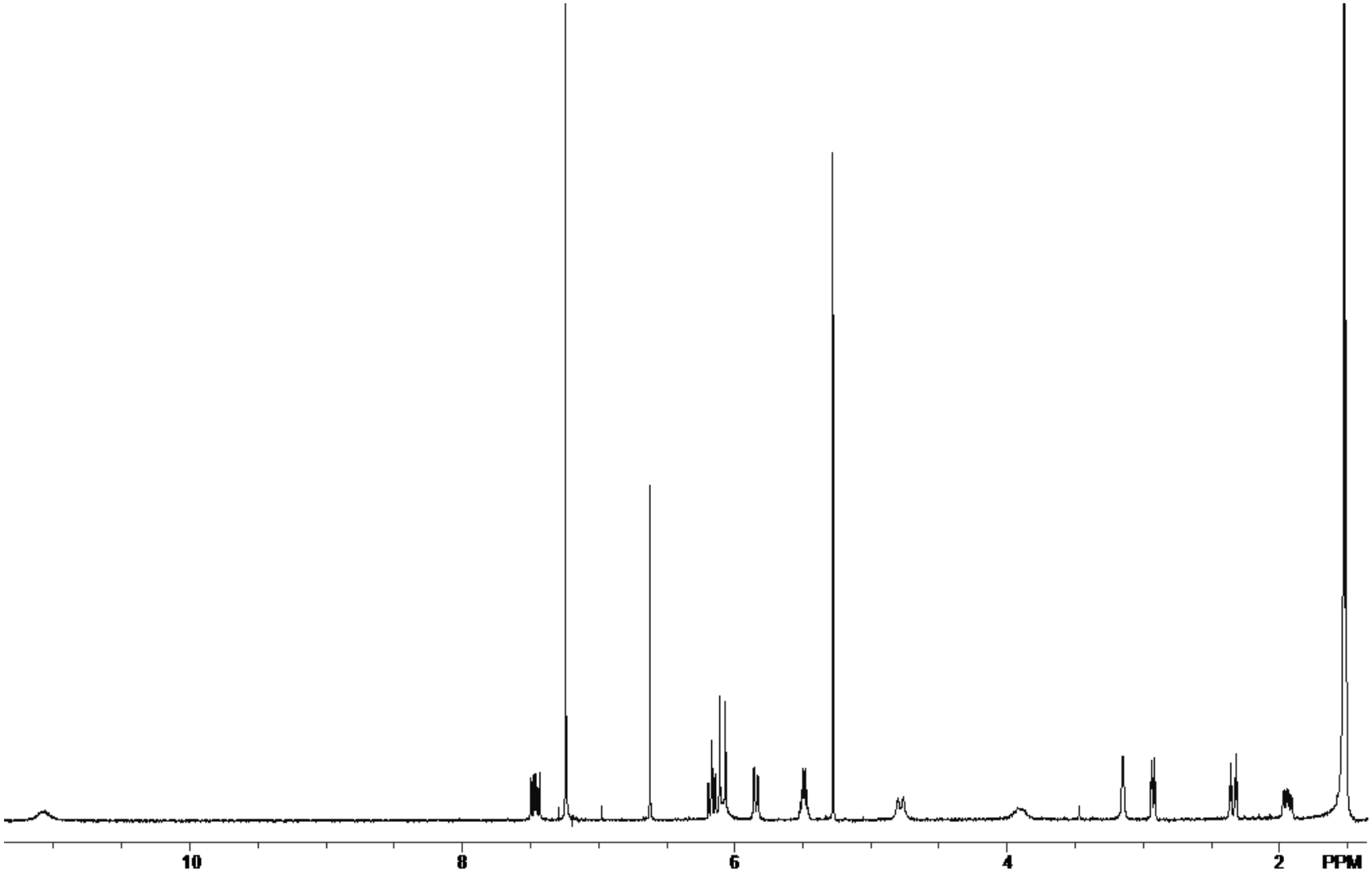


Figure A1. ^{1}H NMR spectrum of monorden (**19**, 18°C) ($CDCl_3$; 400 MHz).

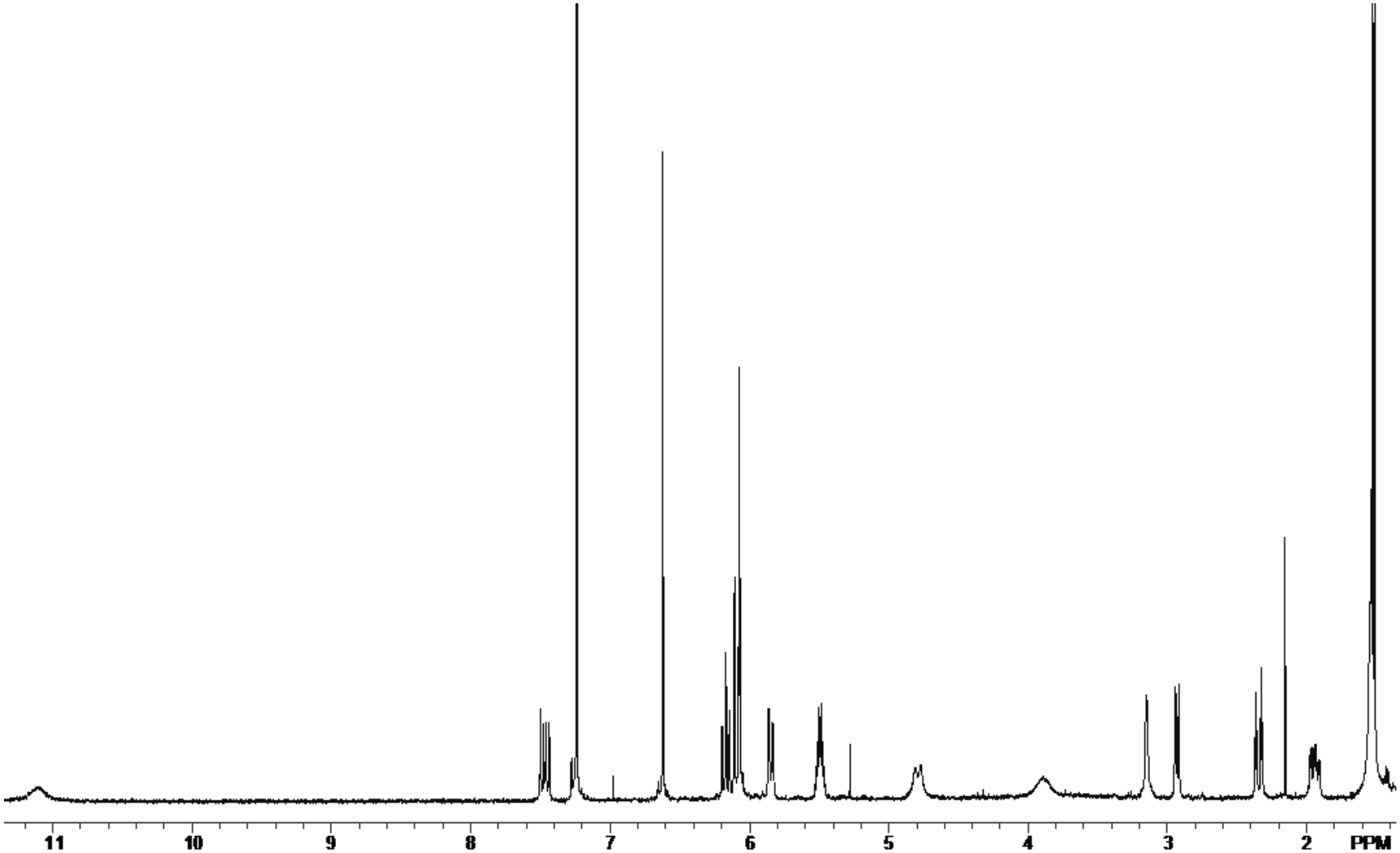


Figure A2. ^{1}H NMR spectrum of monorden (**19**, 20°C) ($CDCl_3$; 400 MHz).

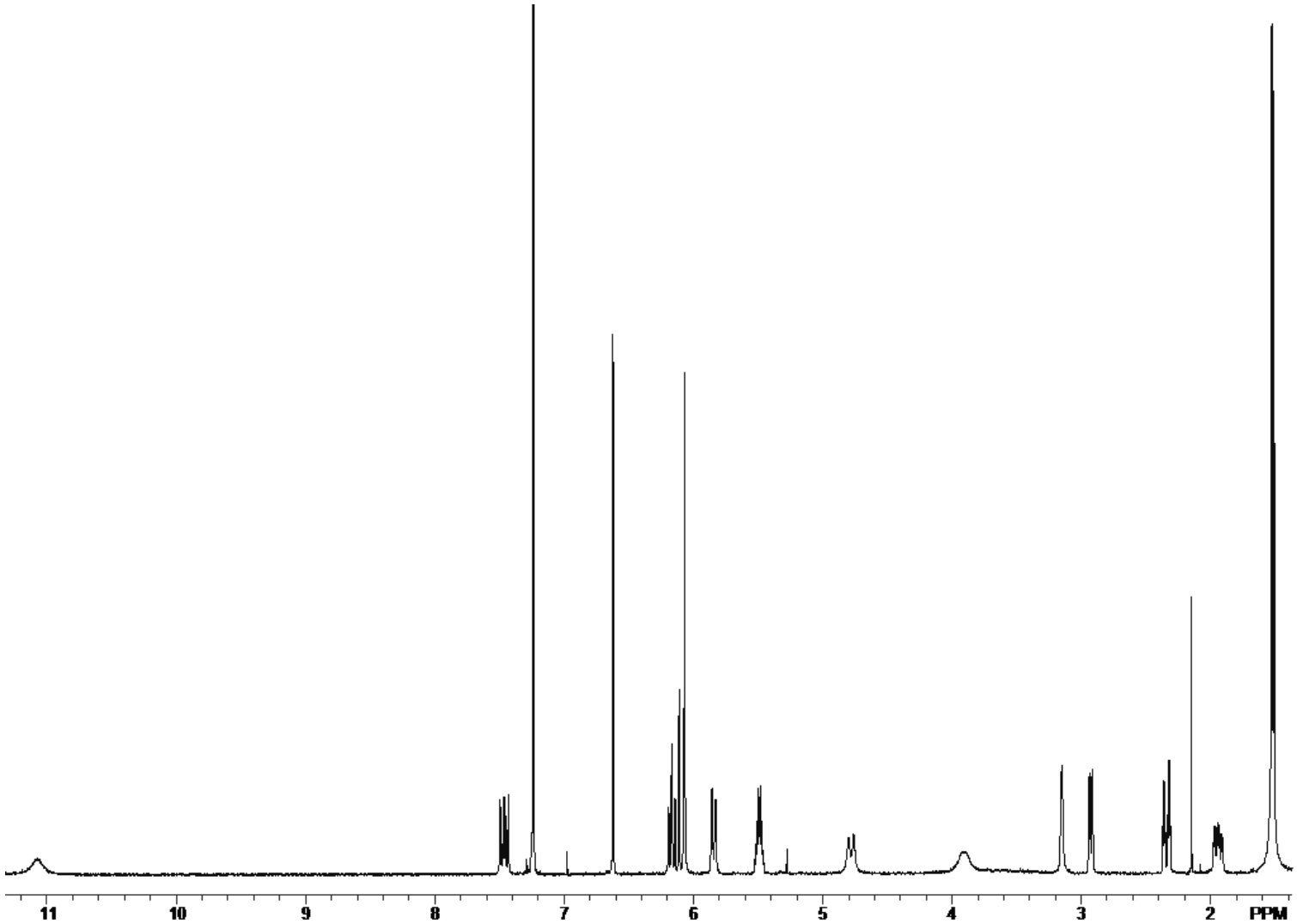


Figure A3. ^{1}H NMR spectrum of monorden (**19**, 25°C) ($CDCl_3$; 400 MHz).

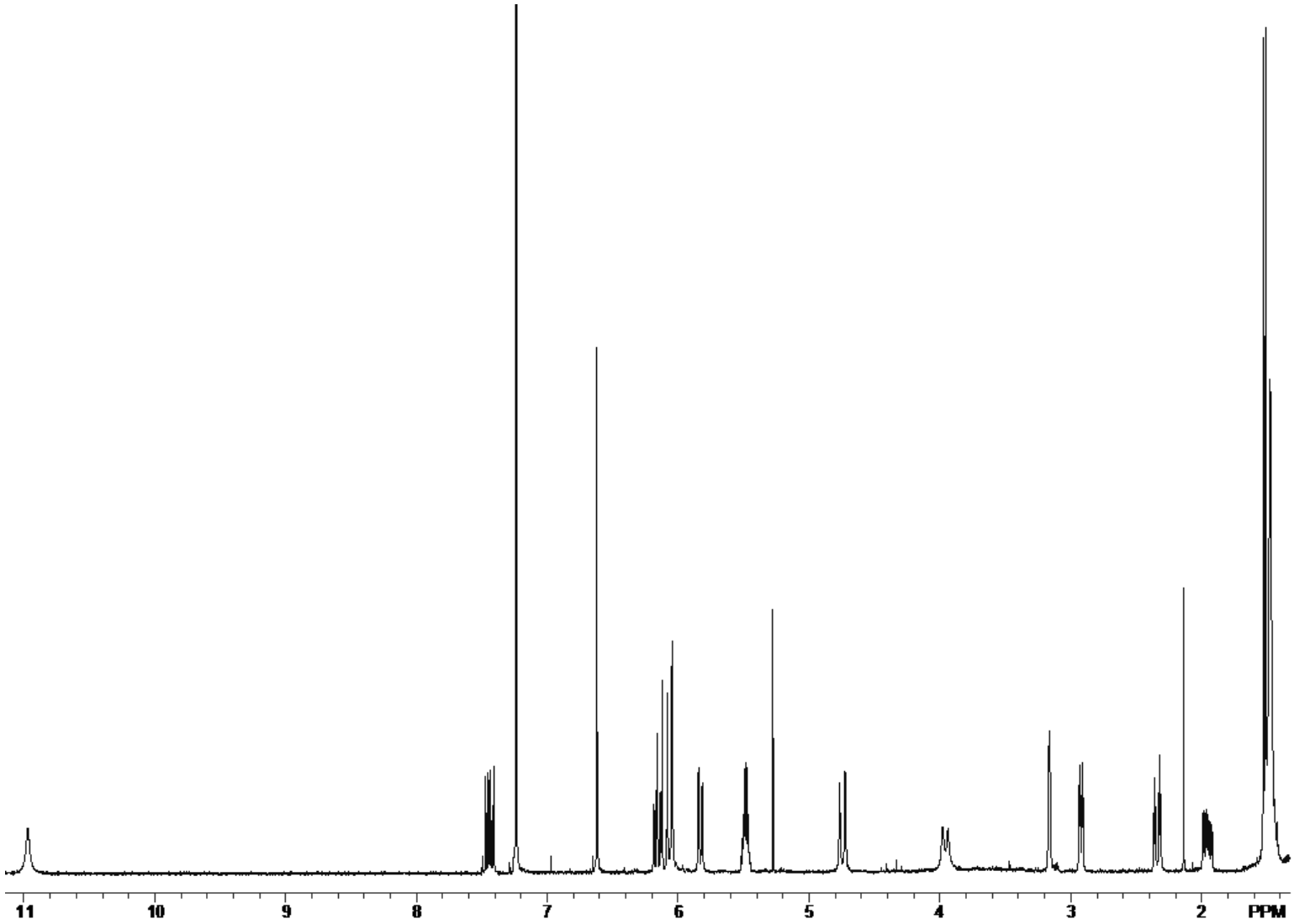


Figure A4. ^{1}H NMR spectrum of monorden (**19**, 45°C) ($CDCl_3$; 400 MHz).

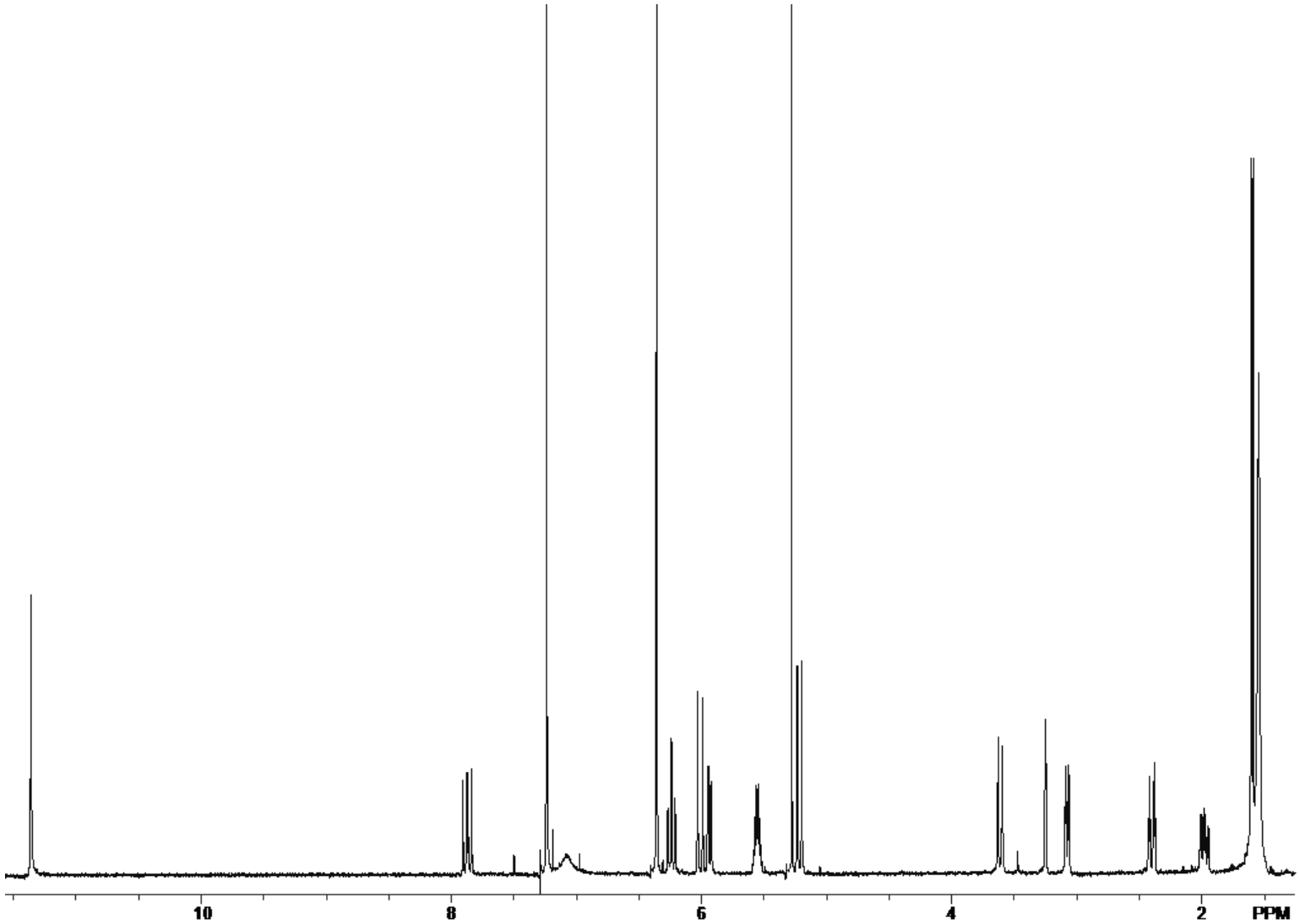


Figure A5. ^{1}H NMR spectrum of monocillin I (**20**) ($CDCl_3$; 400 MHz).

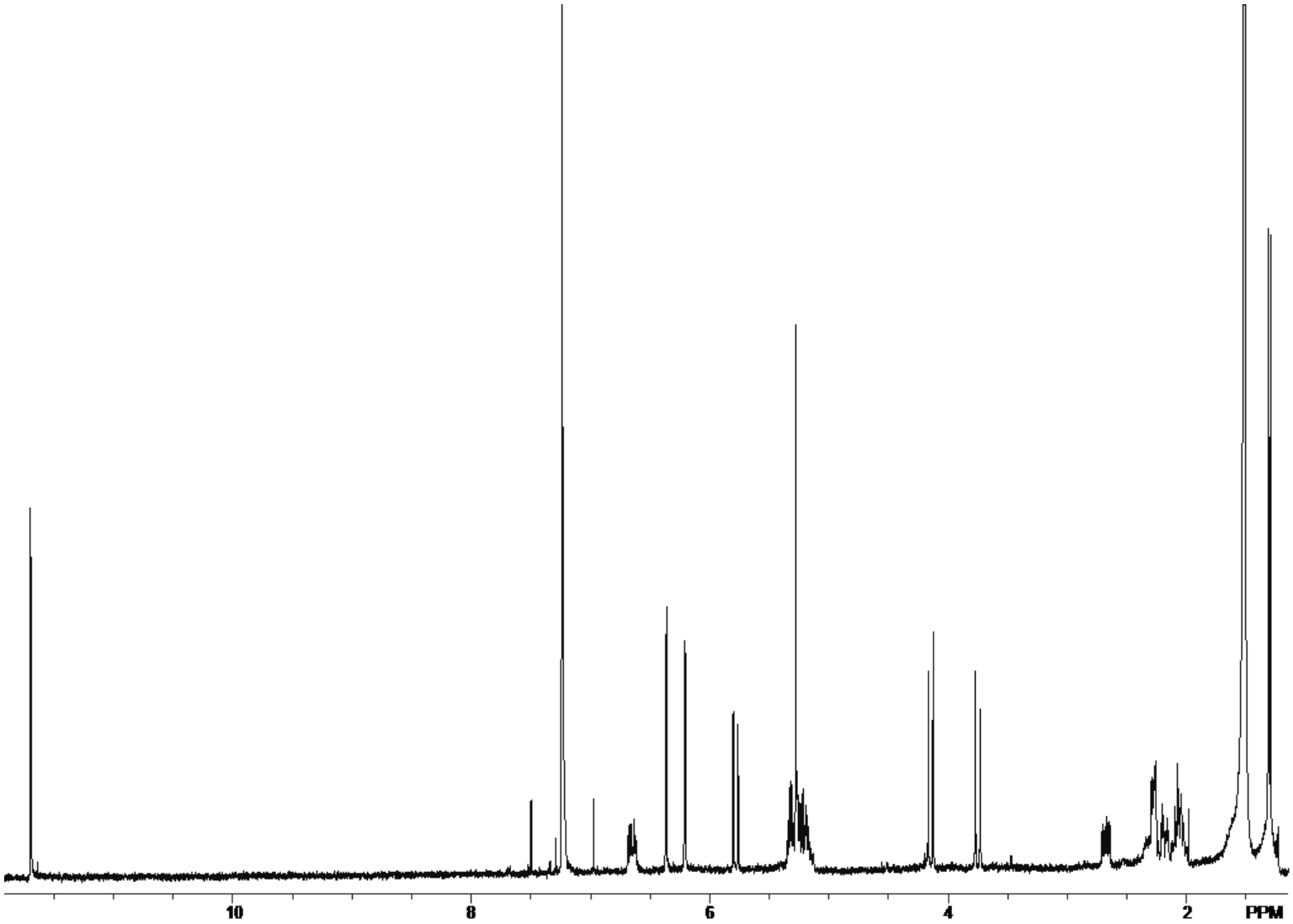


Figure A6. ^{1}H NMR spectrum of monocillin II (**21**) ($CDCl_3$; 400 MHz).

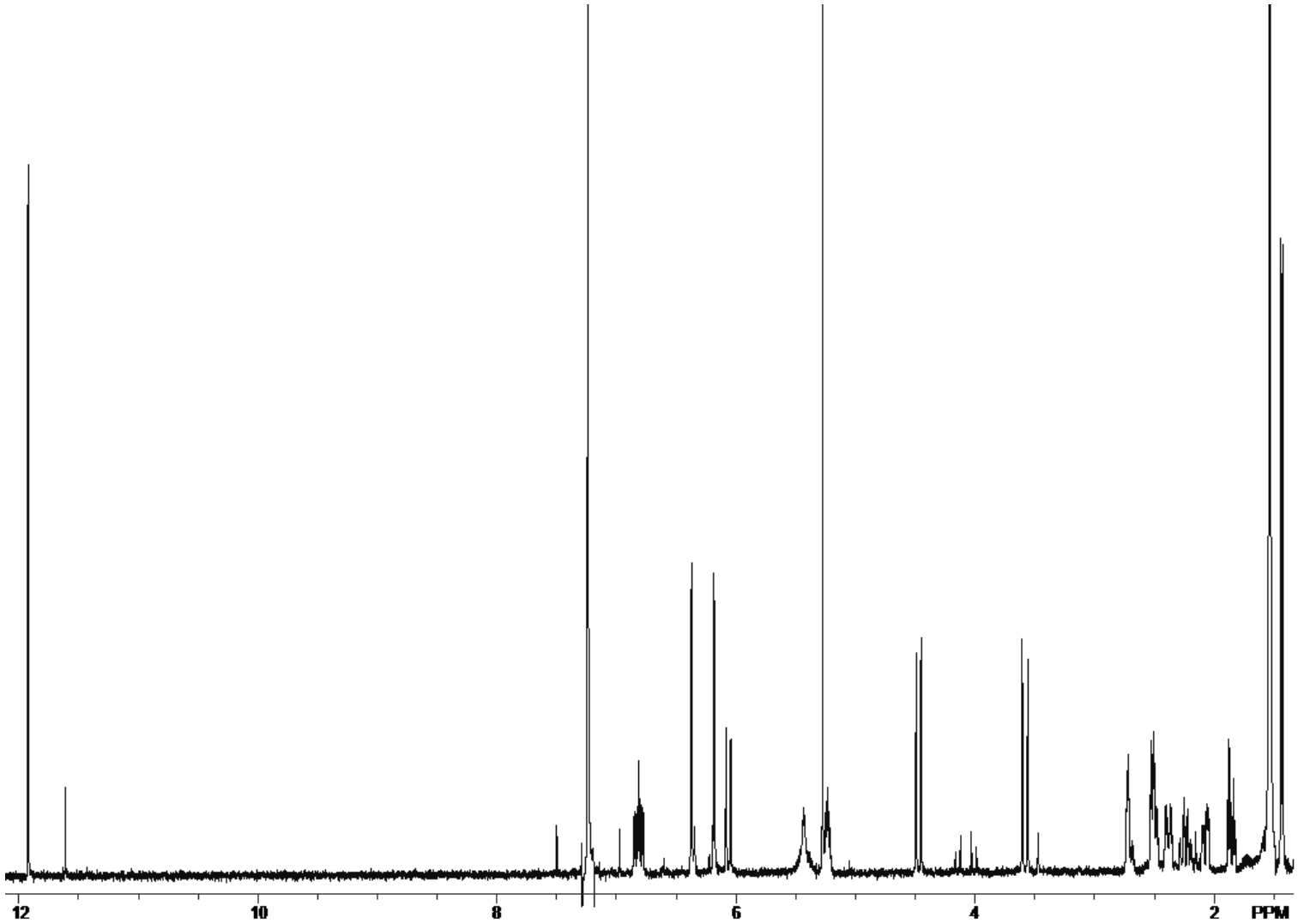


Figure A7. ^{1}H NMR spectrum of monocillin III (**22**) ($CDCl_3$; 400 MHz).

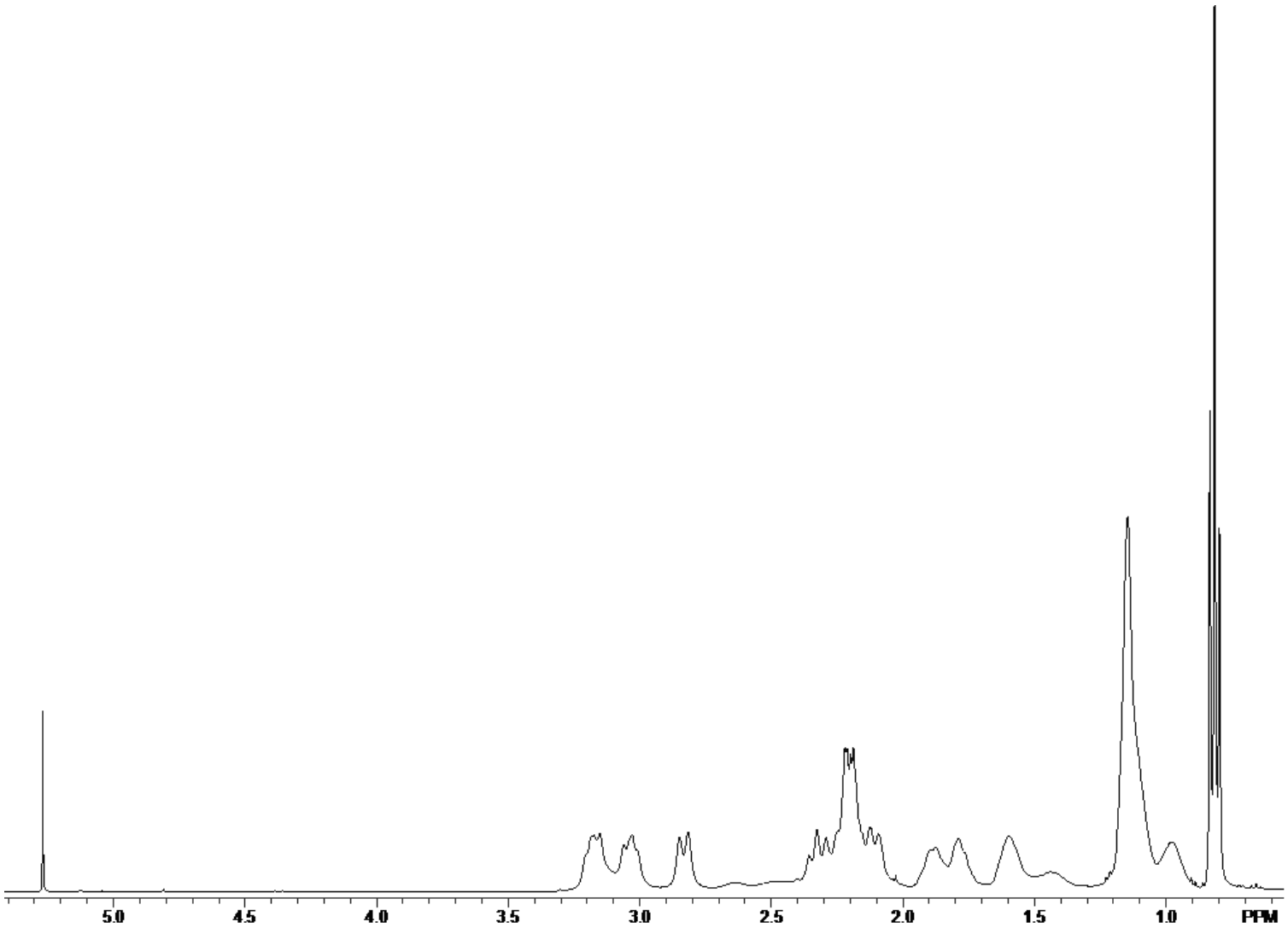


Figure A8. ^{1}H NMR spectrum of heveadride (**24**) ($CDCl_3$; 400 MHz).

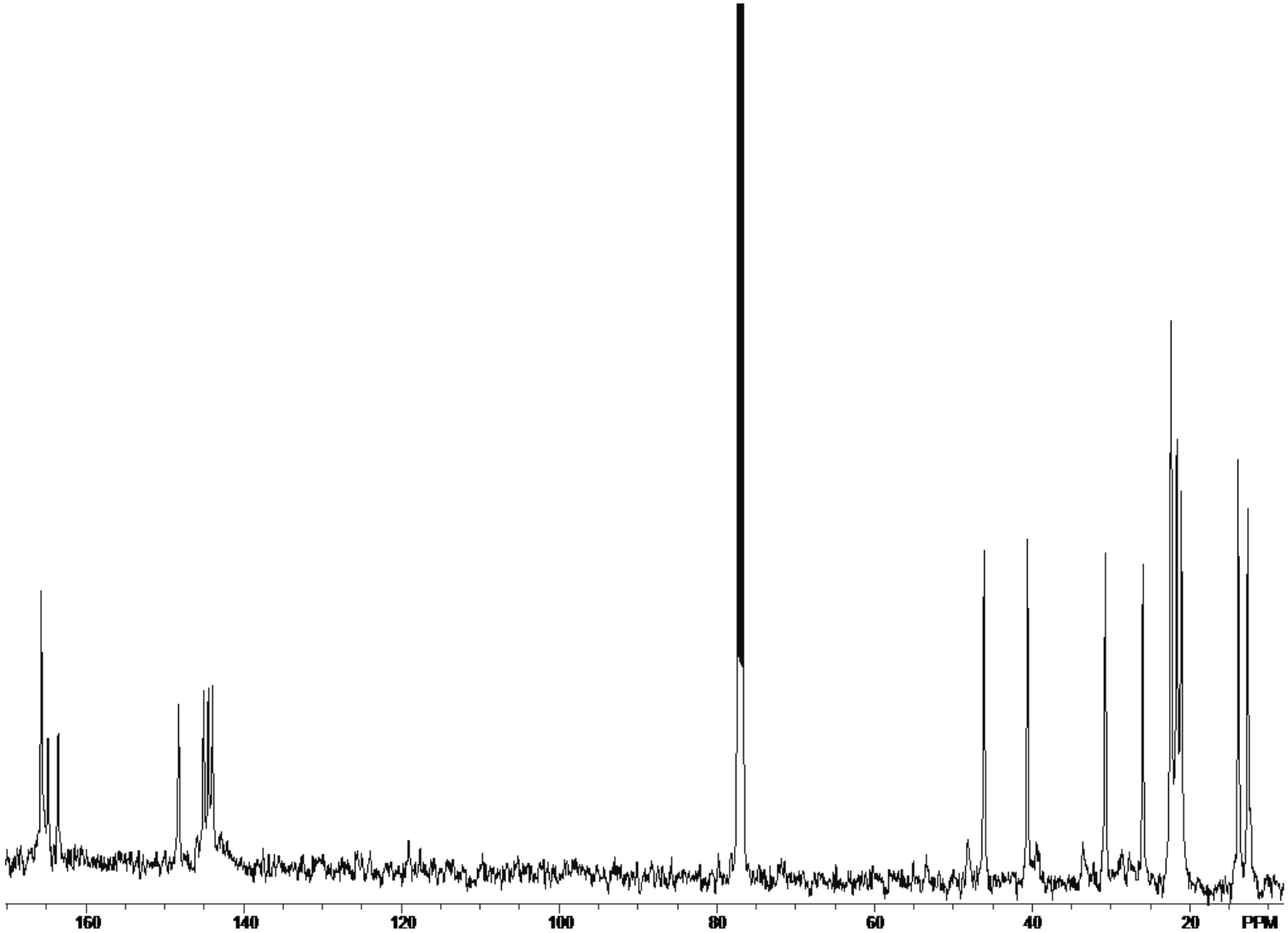


Figure A9. ^{13}C NMR spectrum of heveadride (**24**) ($CDCl_3$; 100 MHz).

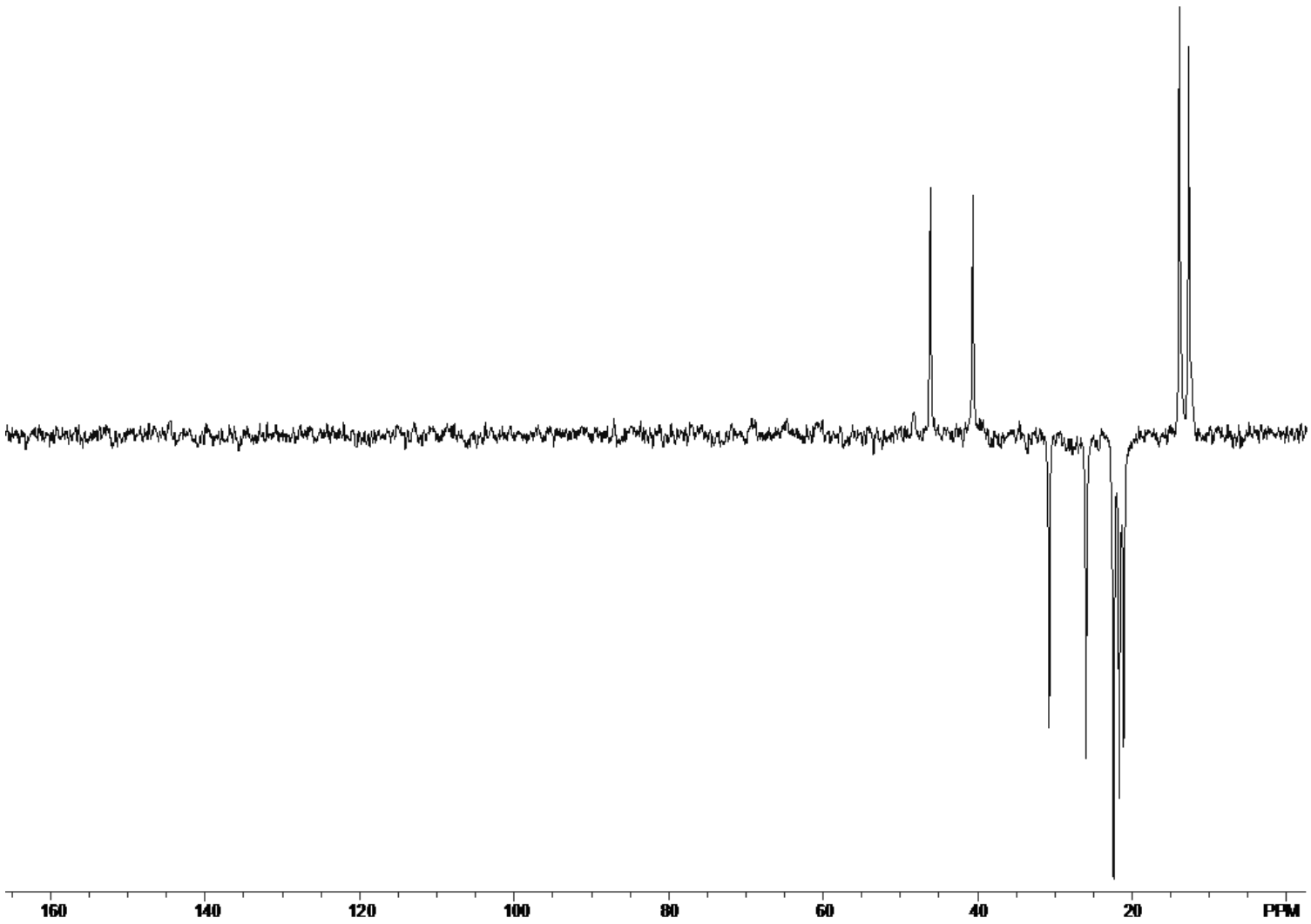


Figure A10. ^{13}C DEPT spectrum of heveadride (**24**) ($CDCl_3$; 100 MHz).

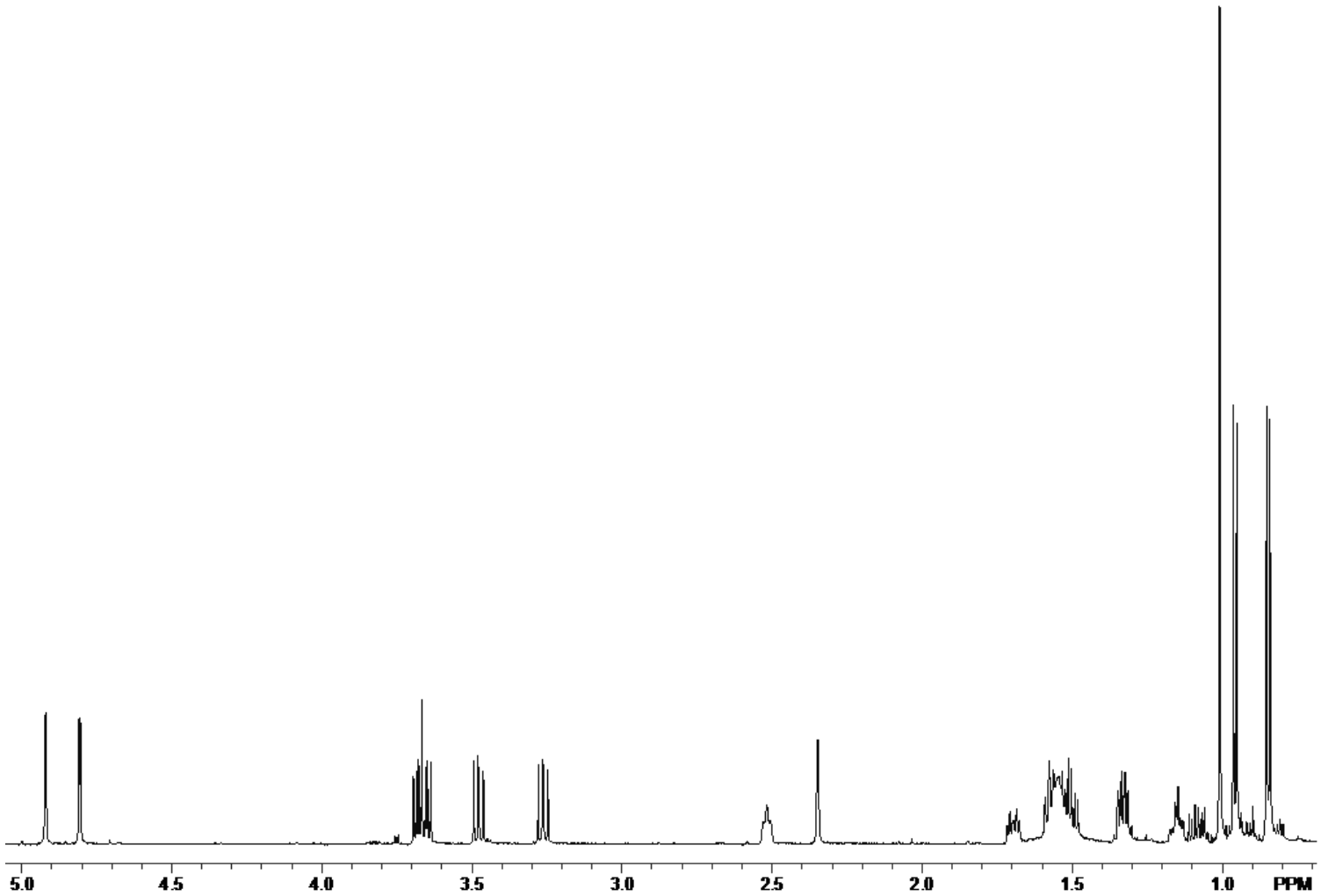


Figure A11. ^{1}H NMR spectrum of dihydroprehelminthosporol (**25**) ($CDCl_3$; 600 MHz).

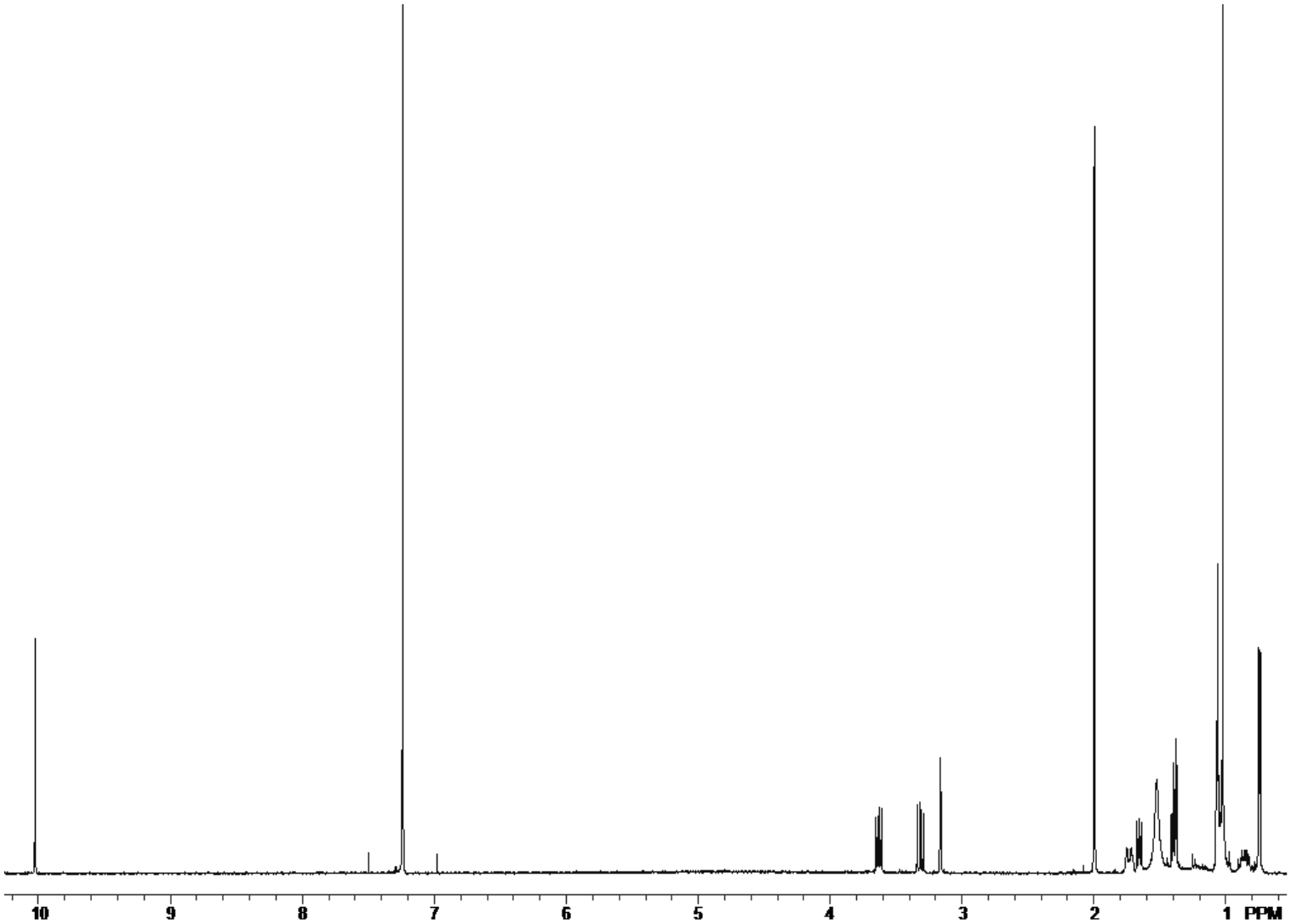


Figure A12. ^{1}H NMR spectra of helminthosporol (**26**) ($CDCl_3$; 400 MHz).

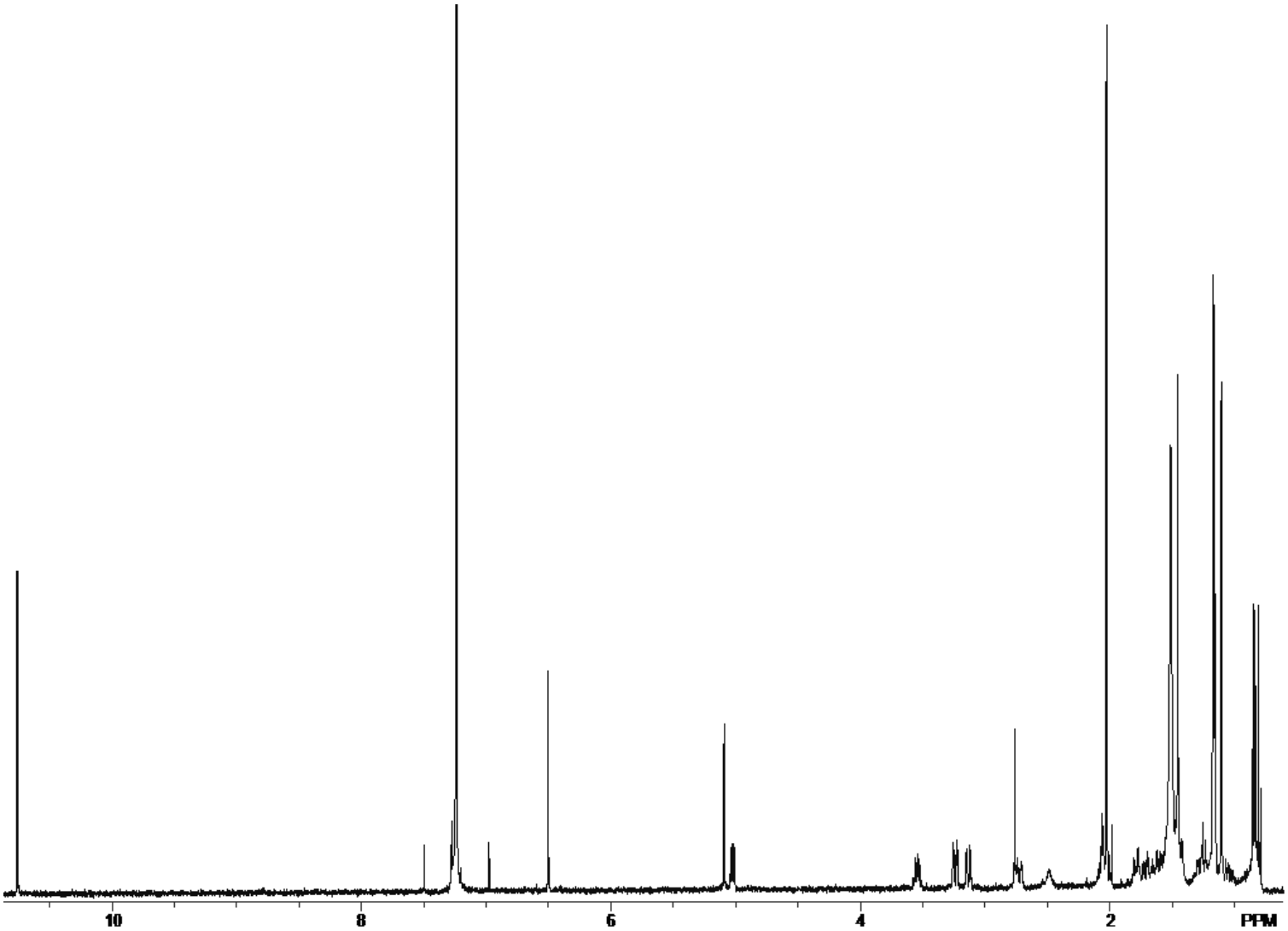


Figure A13. ^{1}H NMR spectrum of isocochlioquinone A (**27**) ($CDCl_3$; 400 MHz).

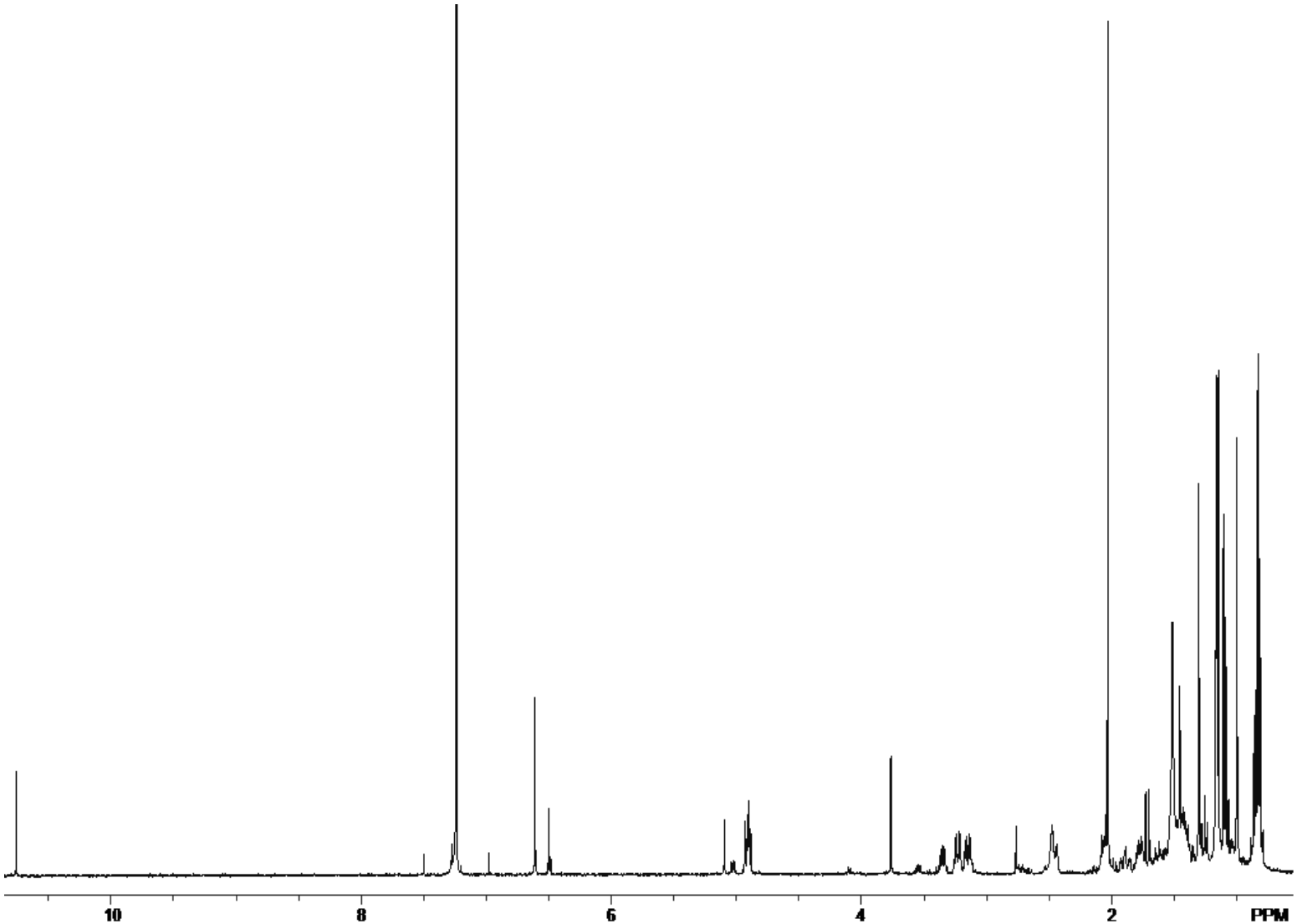


Figure A14. ^{1}H NMR spectrum of cochlioquinone A (**28**) (major) and isocochlioquinone A (**27**) (minor)($CDCl_3$; 400 MHz).

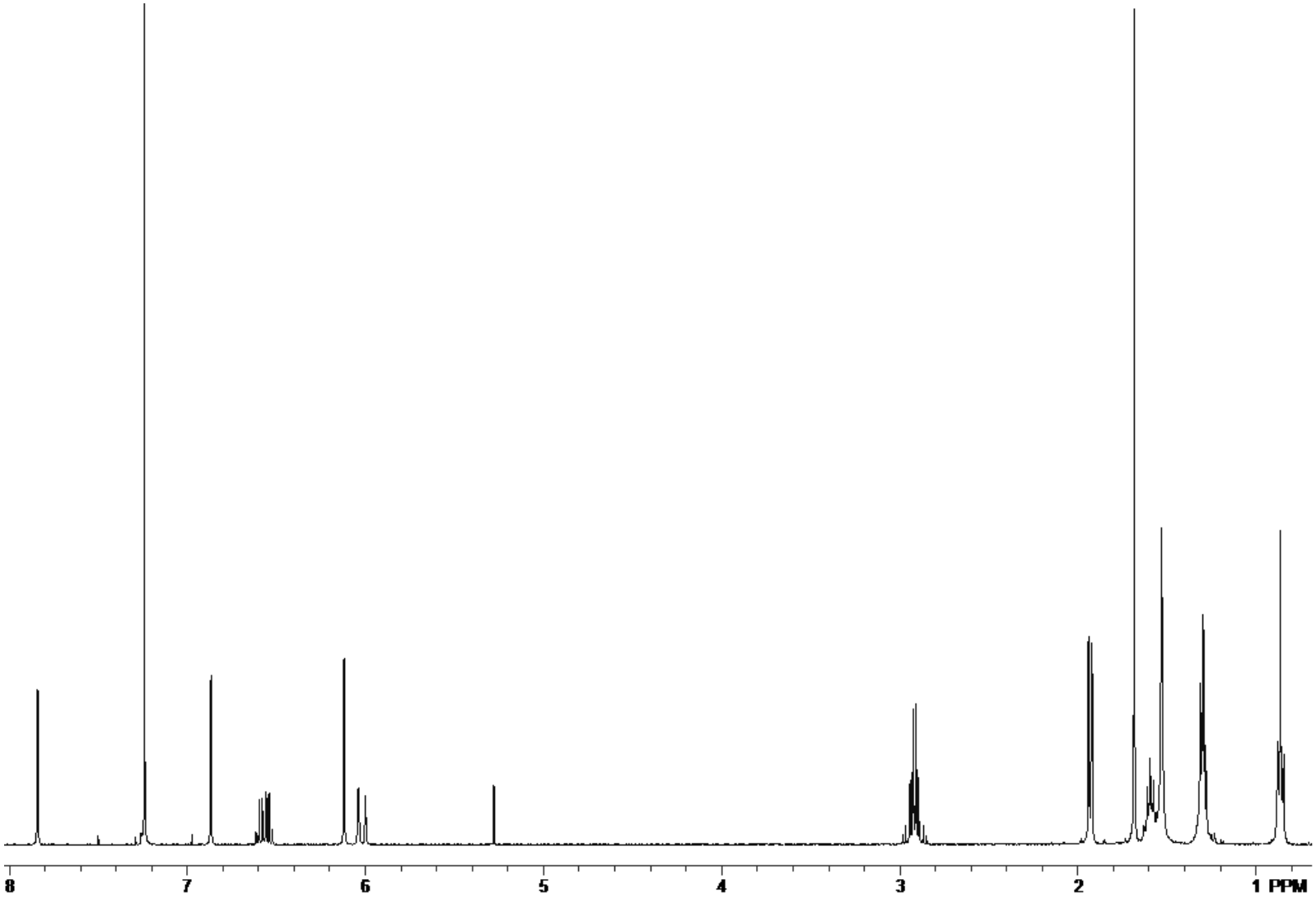


Figure A15. ^{1}H NMR spectrum of rubropunctatin (**30**) ($CDCl_3$; 400 MHz).

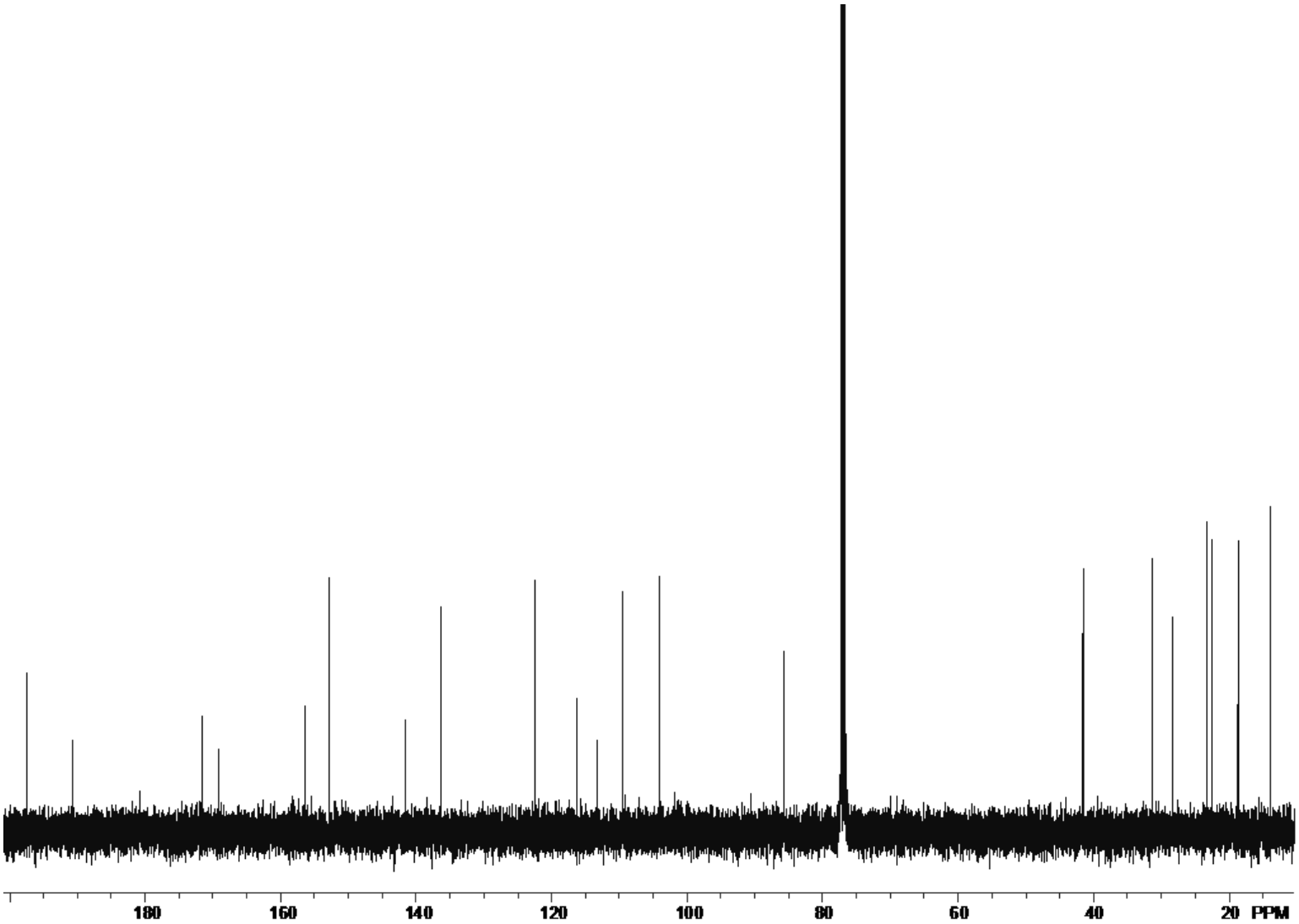


Figure A16. ^{13}C NMR spectrum of rubropunctatin (**30**) ($CDCl_3$; 100 MHz).

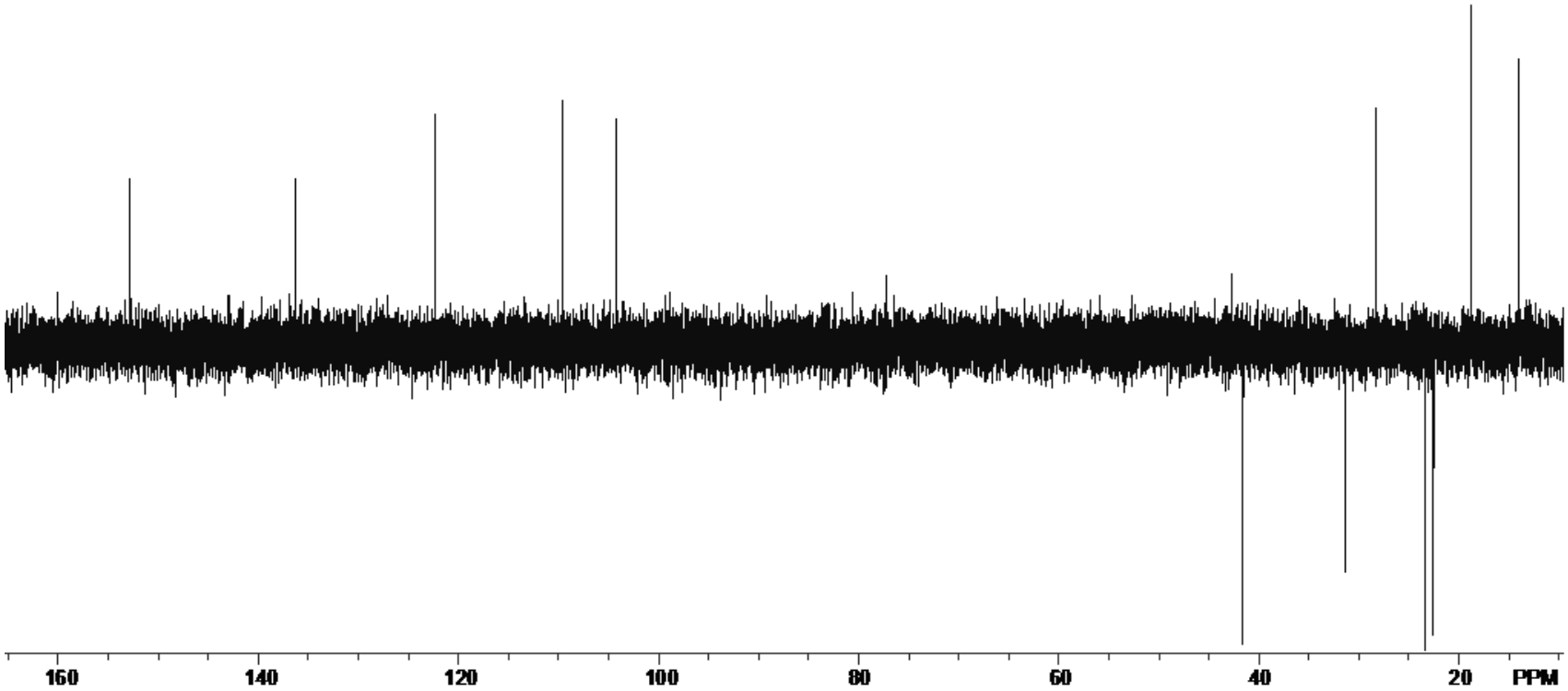


Figure A17. ^{13}C DEPT spectrum of rubropunctatin (**30**) ($CDCl_3$; 100 MHz).

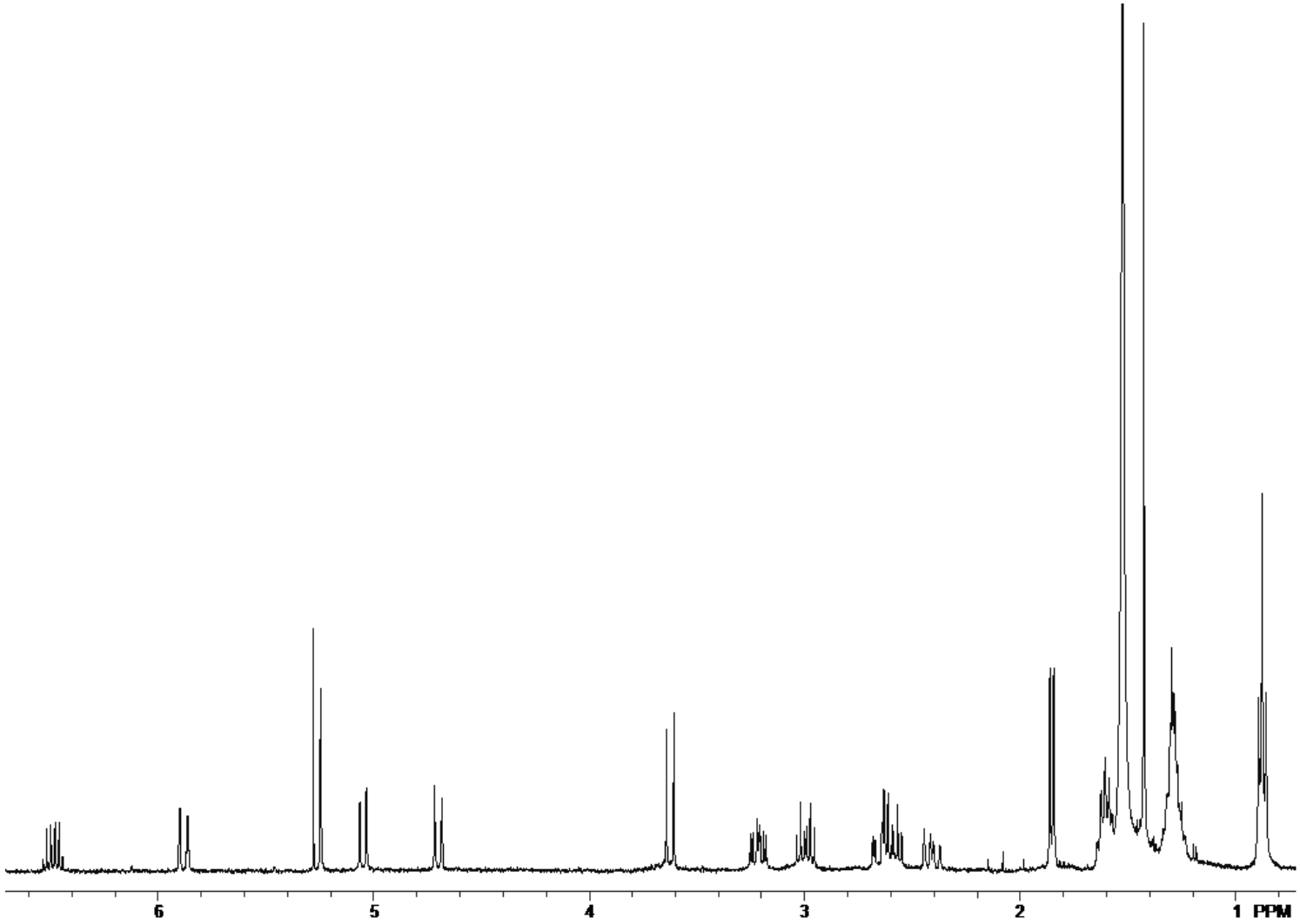


Figure A18. ^{1}H NMR spectrum of monascin (**31**) ($CDCl_3$; 400 MHz).

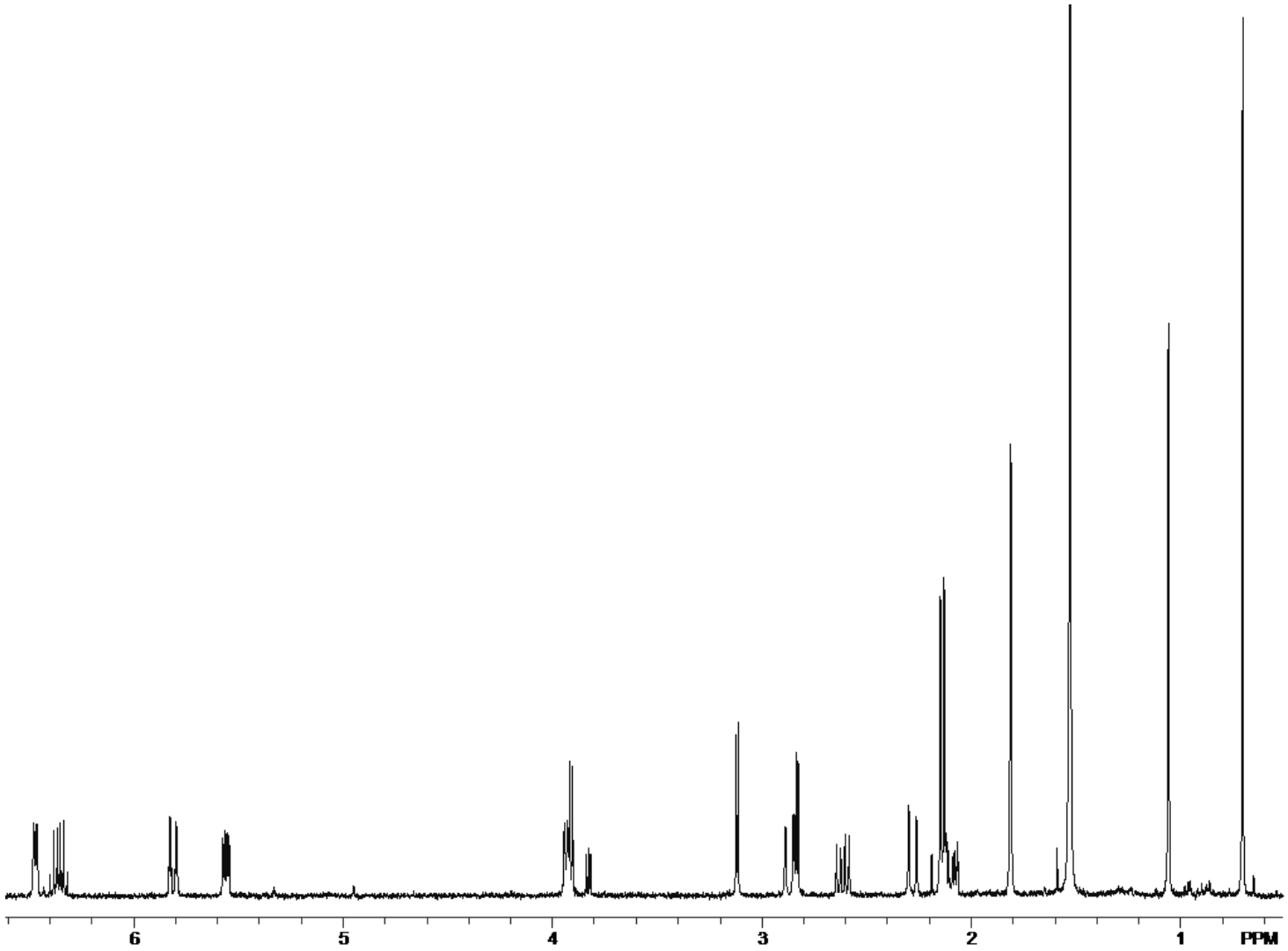


Figure A19. ^{1}H NMR spectrum of trichothecin (**36**) ($CDCl_3$; 400 MHz).

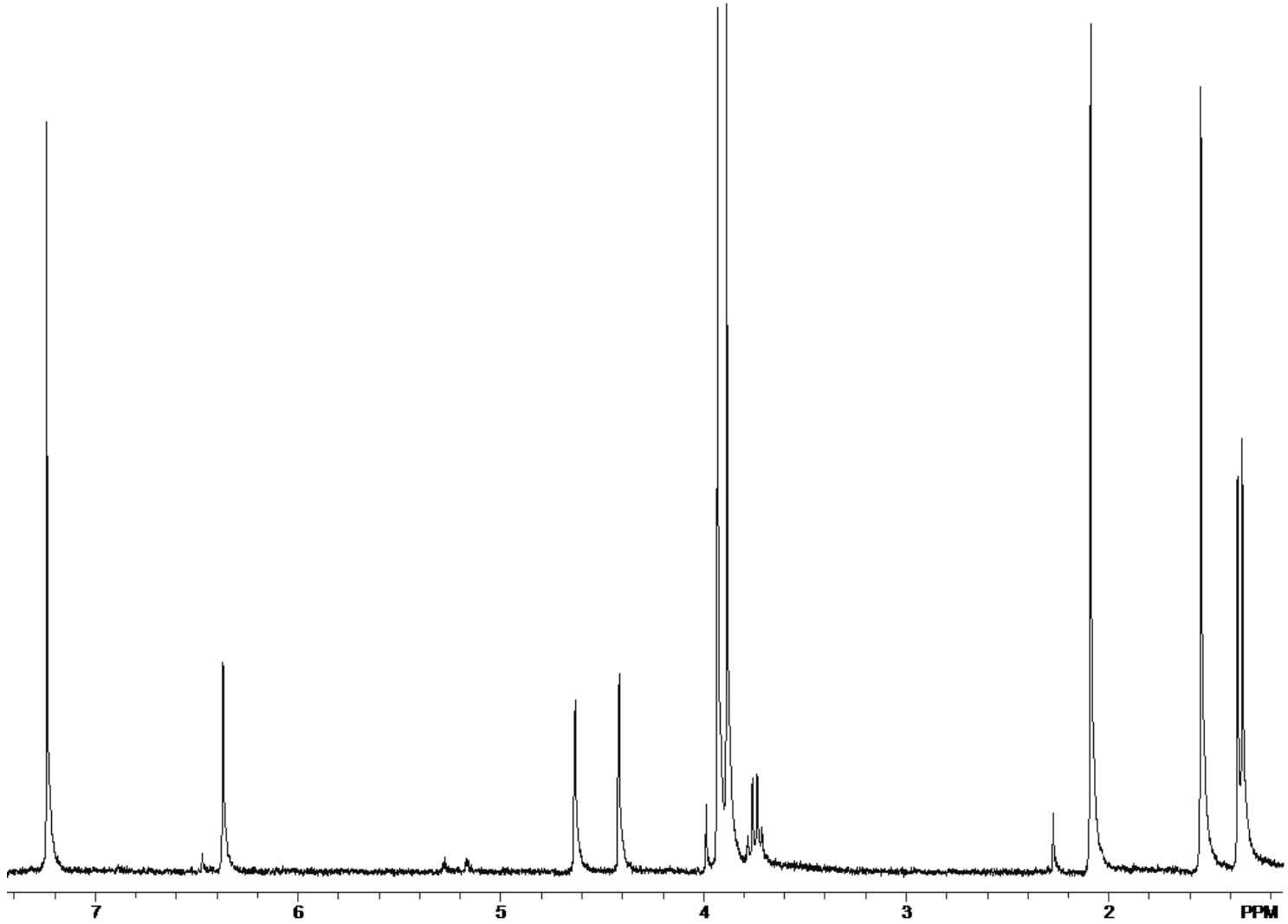


Figure A20. ^{1}H NMR spectrum of 6,8-dimethoxy-4,5-dimethyl-3-methyleneisochroman-1-one (**37**) ($CDCl_3$; 300 MHz).

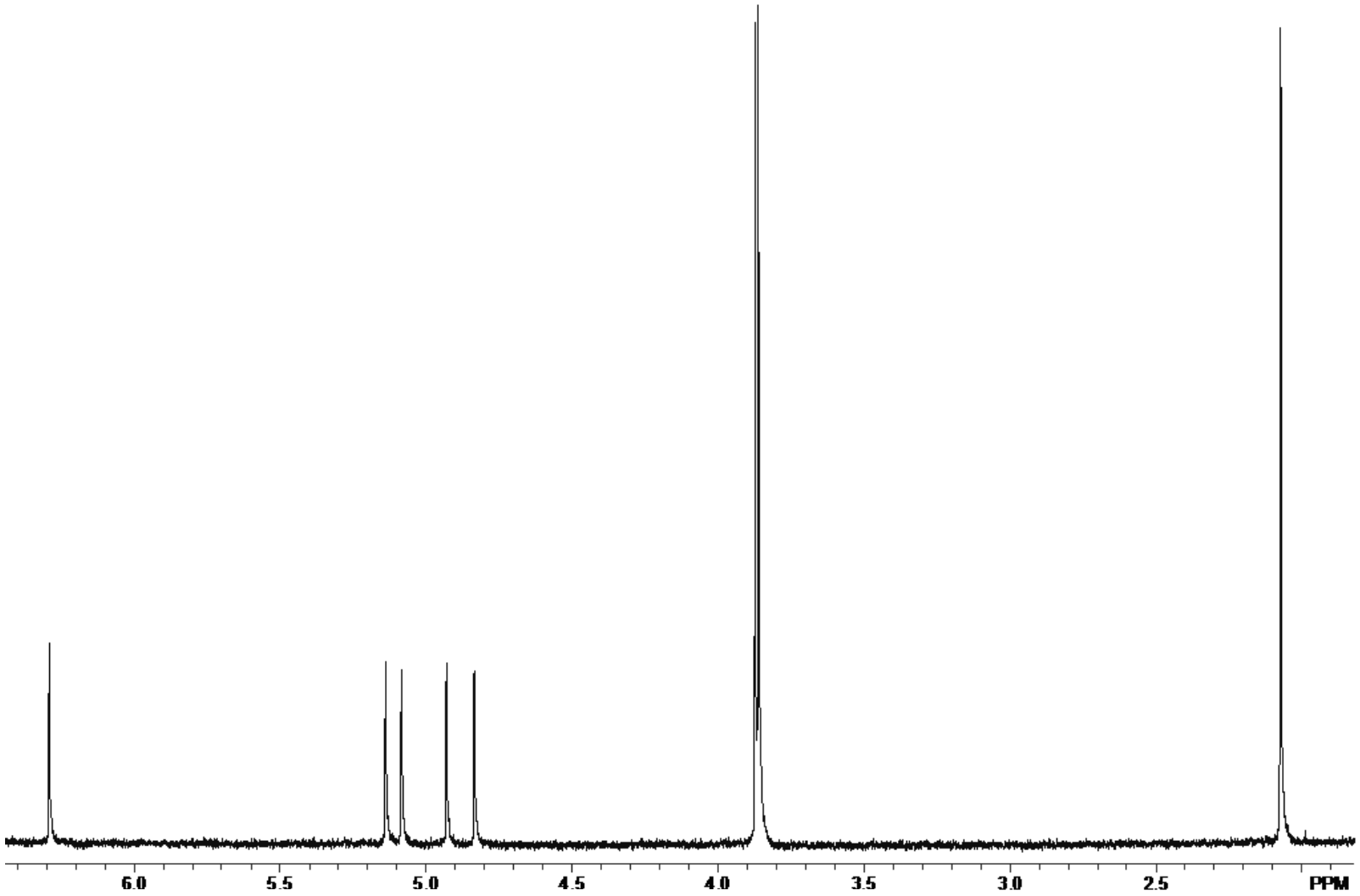


Figure A21. ^{1}H NMR spectrum of compound **38** ($CDCl_3$; 600 MHz).

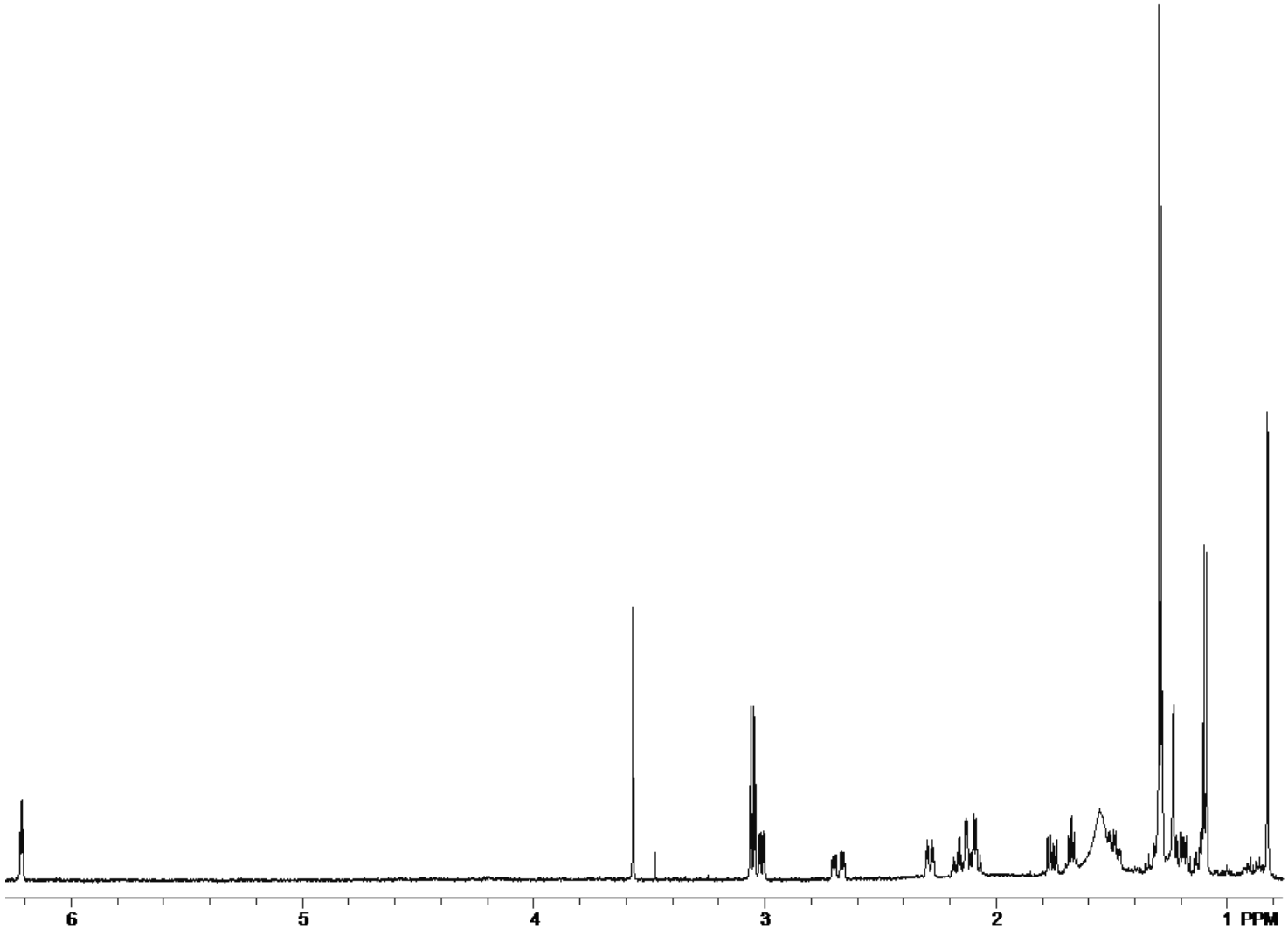


Figure A22. ^{1}H NMR spectrum of phomactin K (**40**) ($CDCl_3$; 600 MHz).

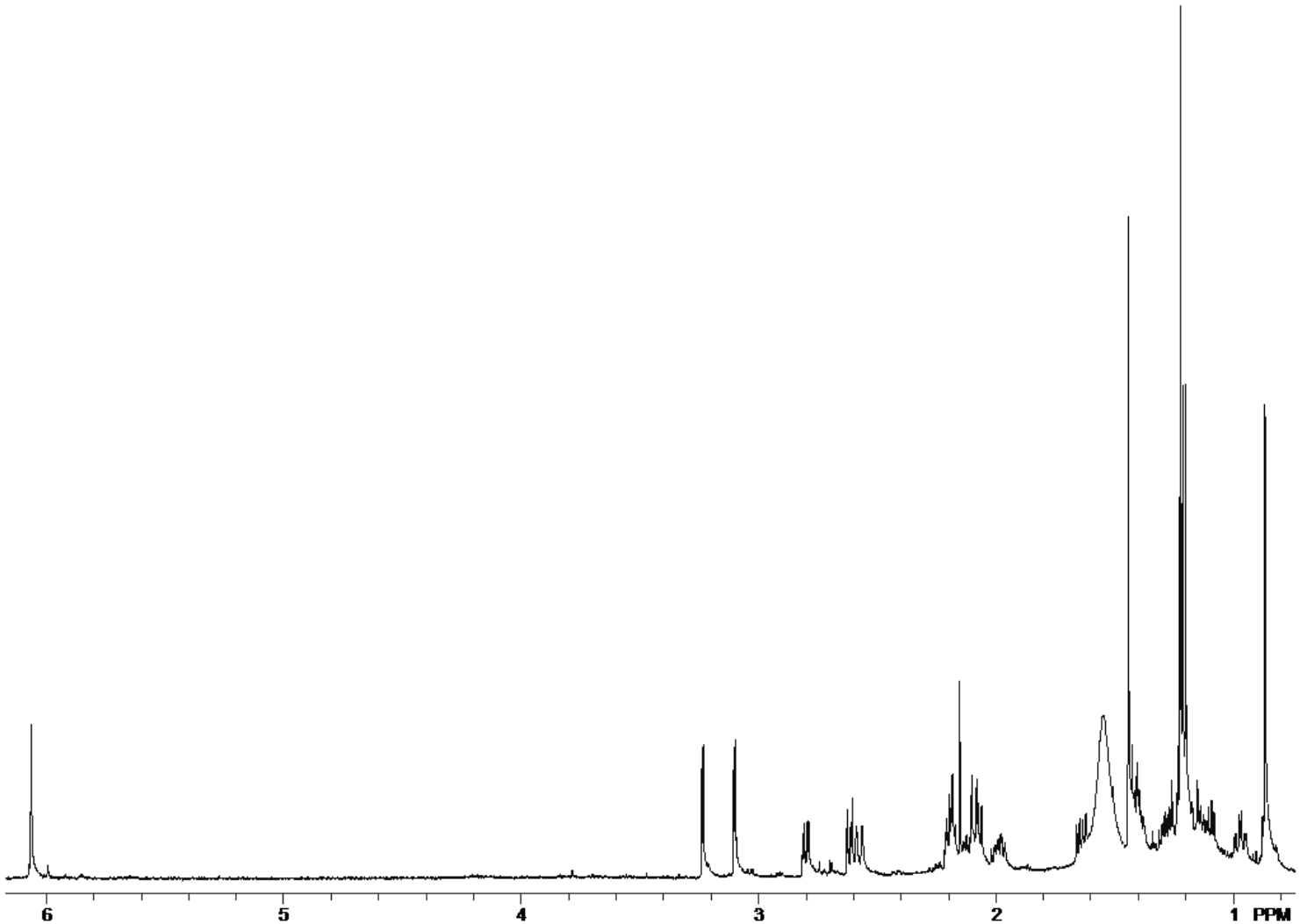


Figure A23. ^{1}H NMR spectrum of phomactin L (**41**) ($CDCl_3$; 600 MHz).

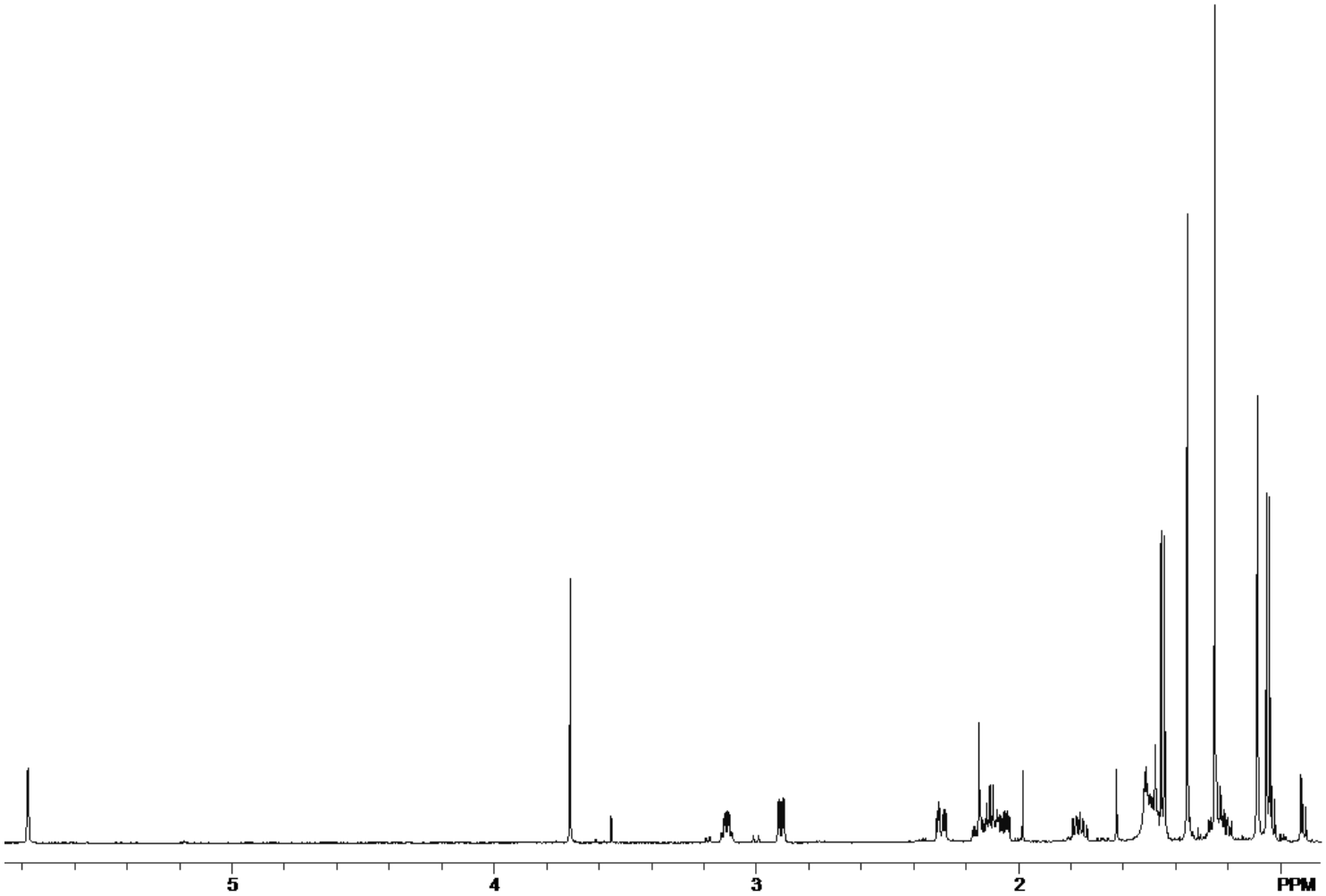


Figure A24. ^{1}H NMR spectrum of phomactin M (**42**) ($CDCl_3$; 600 MHz).

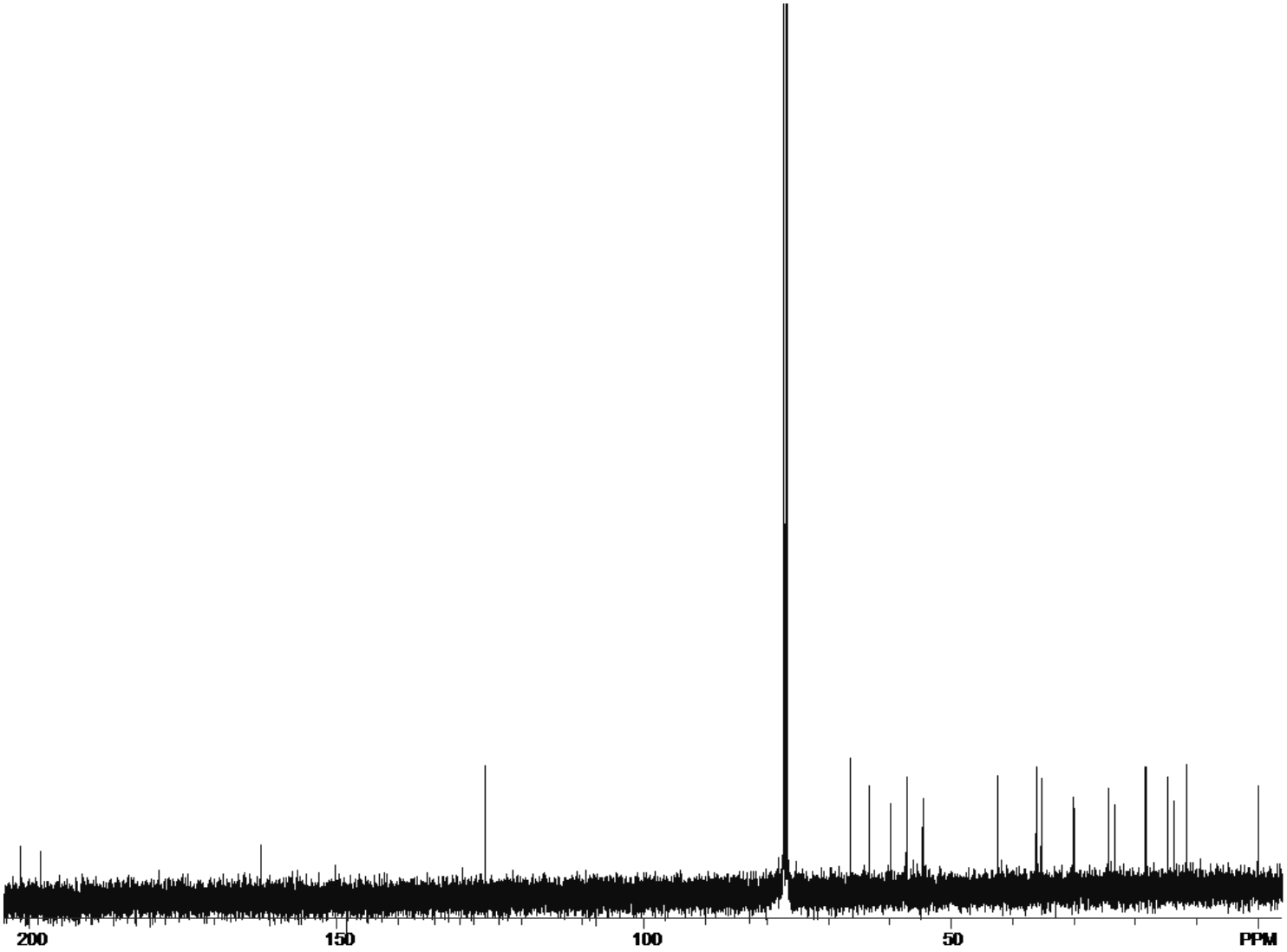


Figure A25. ^{13}C NMR spectrum of phomactin M (**42**) ($CDCl_3$; 100 MHz).

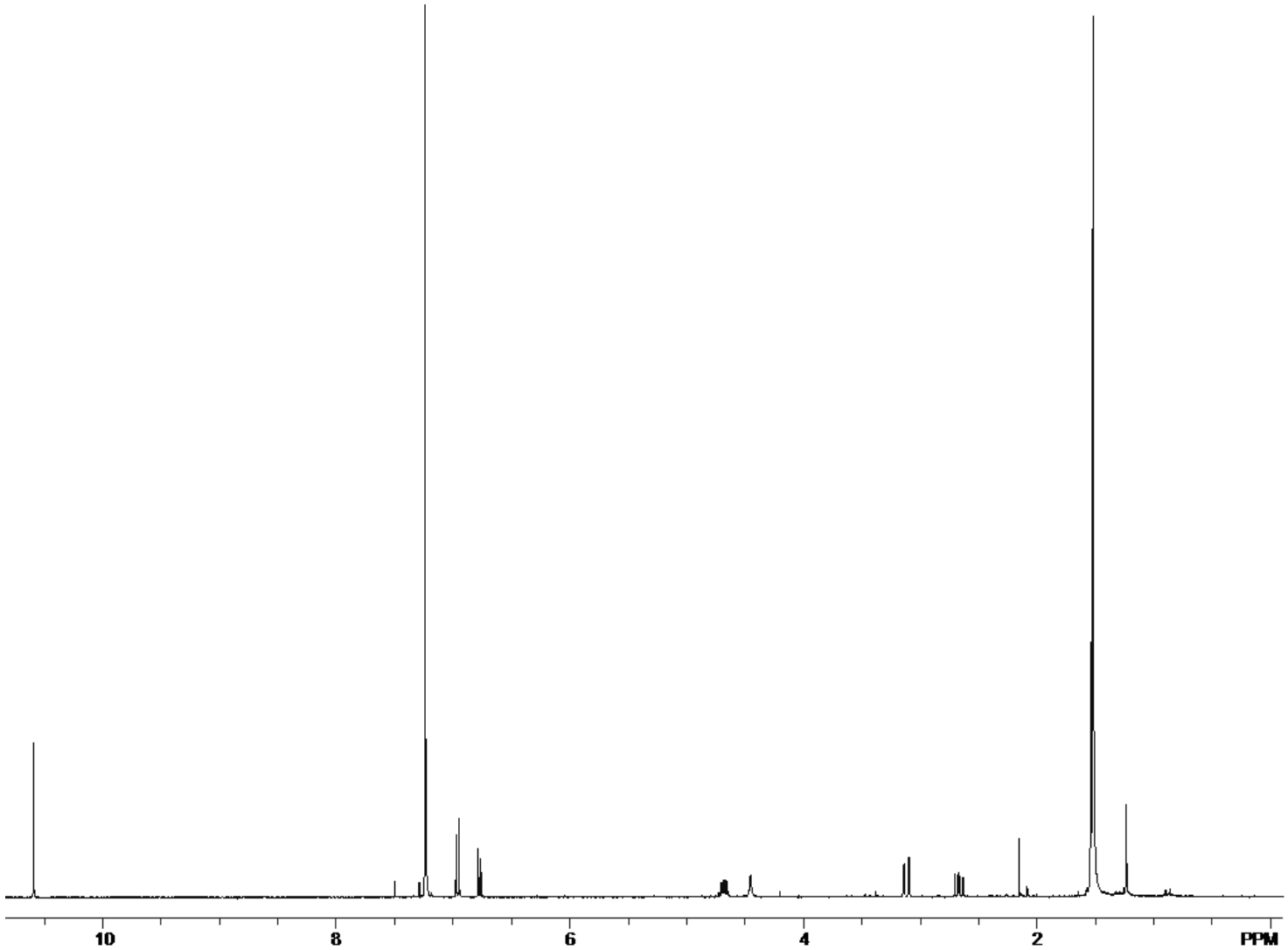


Figure A26. ^{1}H NMR spectrum of aspirlin (**45**) ($CDCl_3$; 400 MHz).

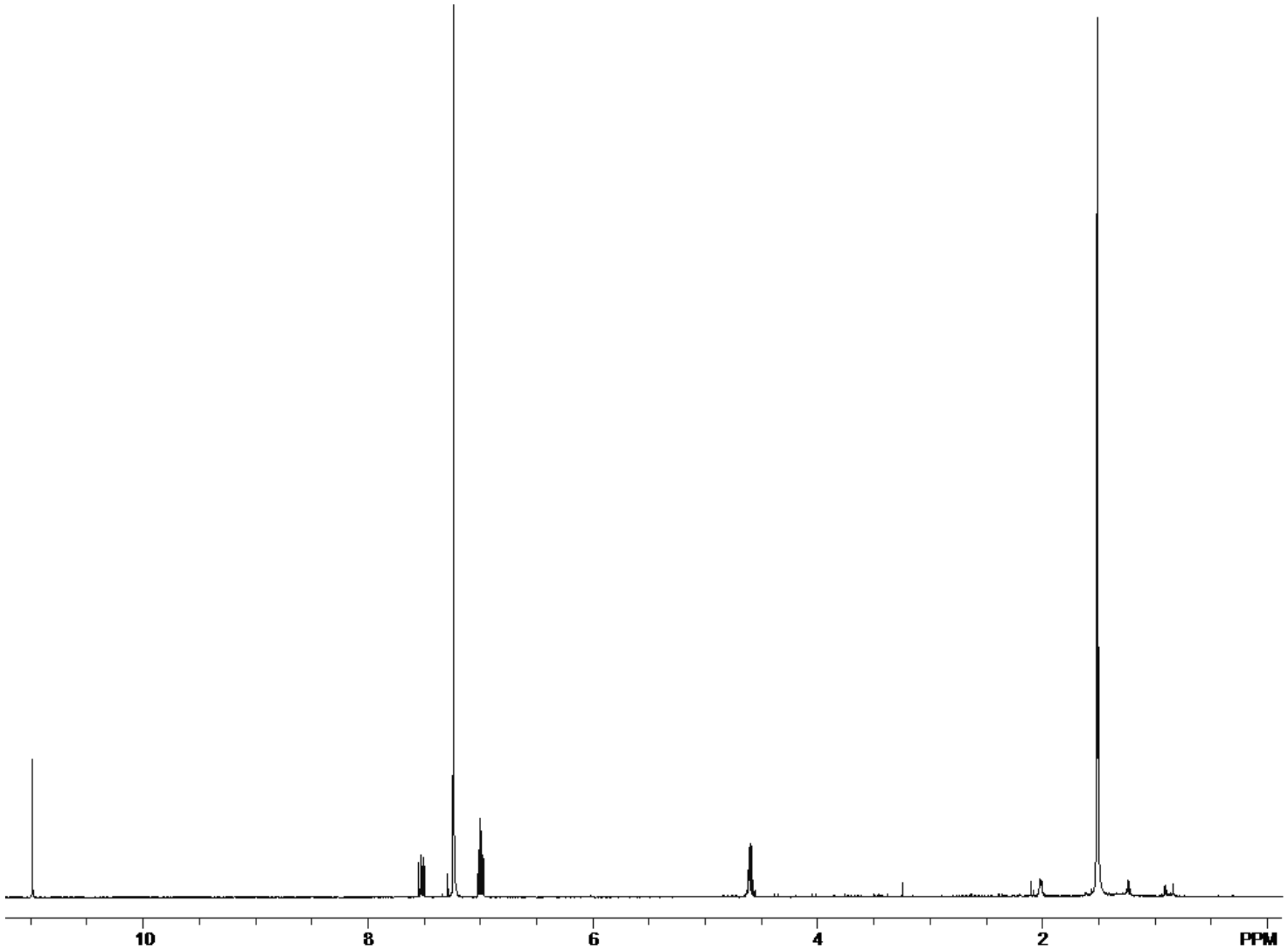


Figure A27. ^{1}H NMR spectrum of 4-hydroxymellein (**46**) ($CDCl_3$; 400 MHz).

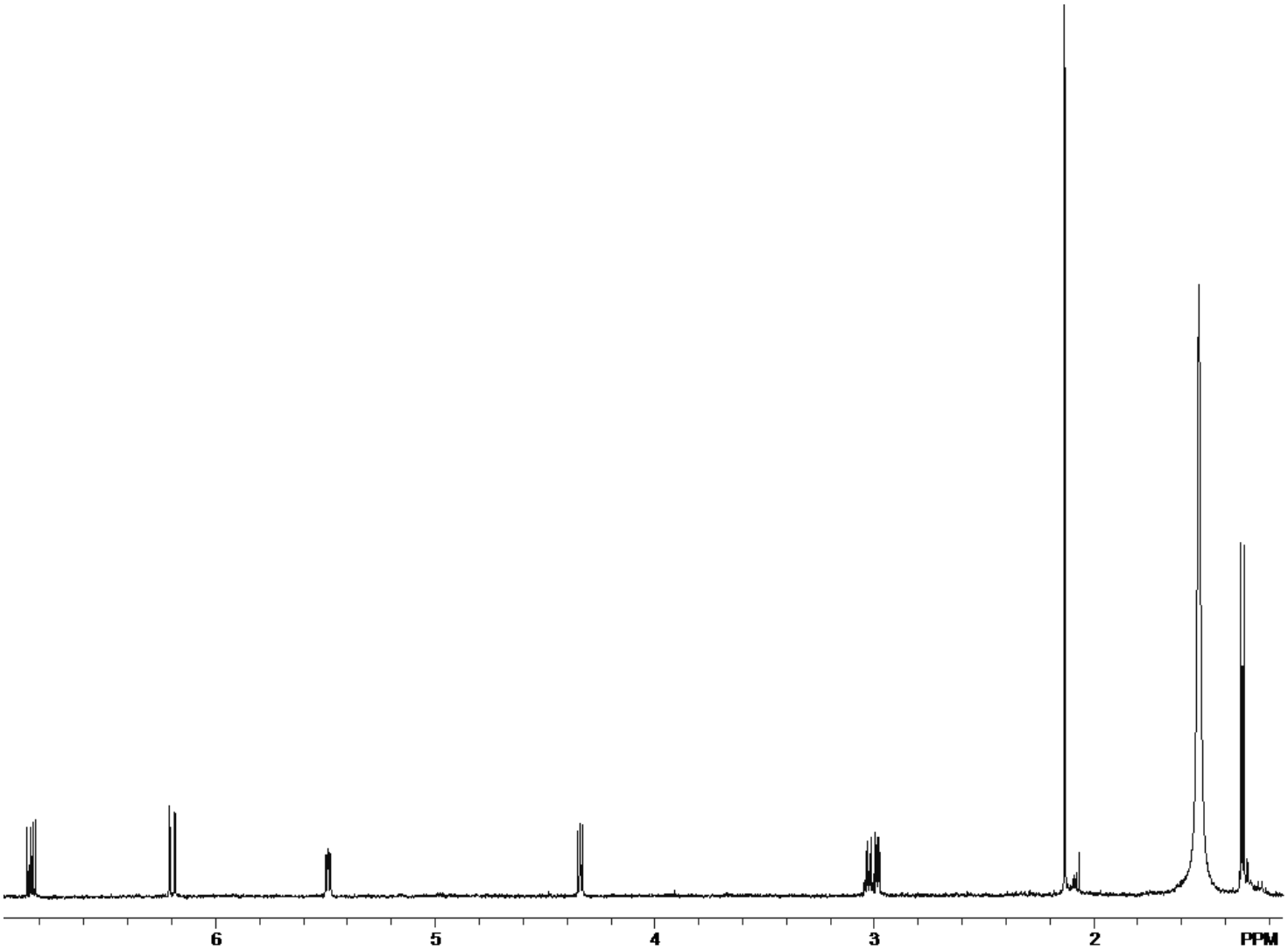


Figure A28. ^{1}H NMR spectrum of 5,8–dihydroxy–3–methyl–3,4–dihydroisocoumarin(**47**) ($CDCl_3$; 400 MHz).

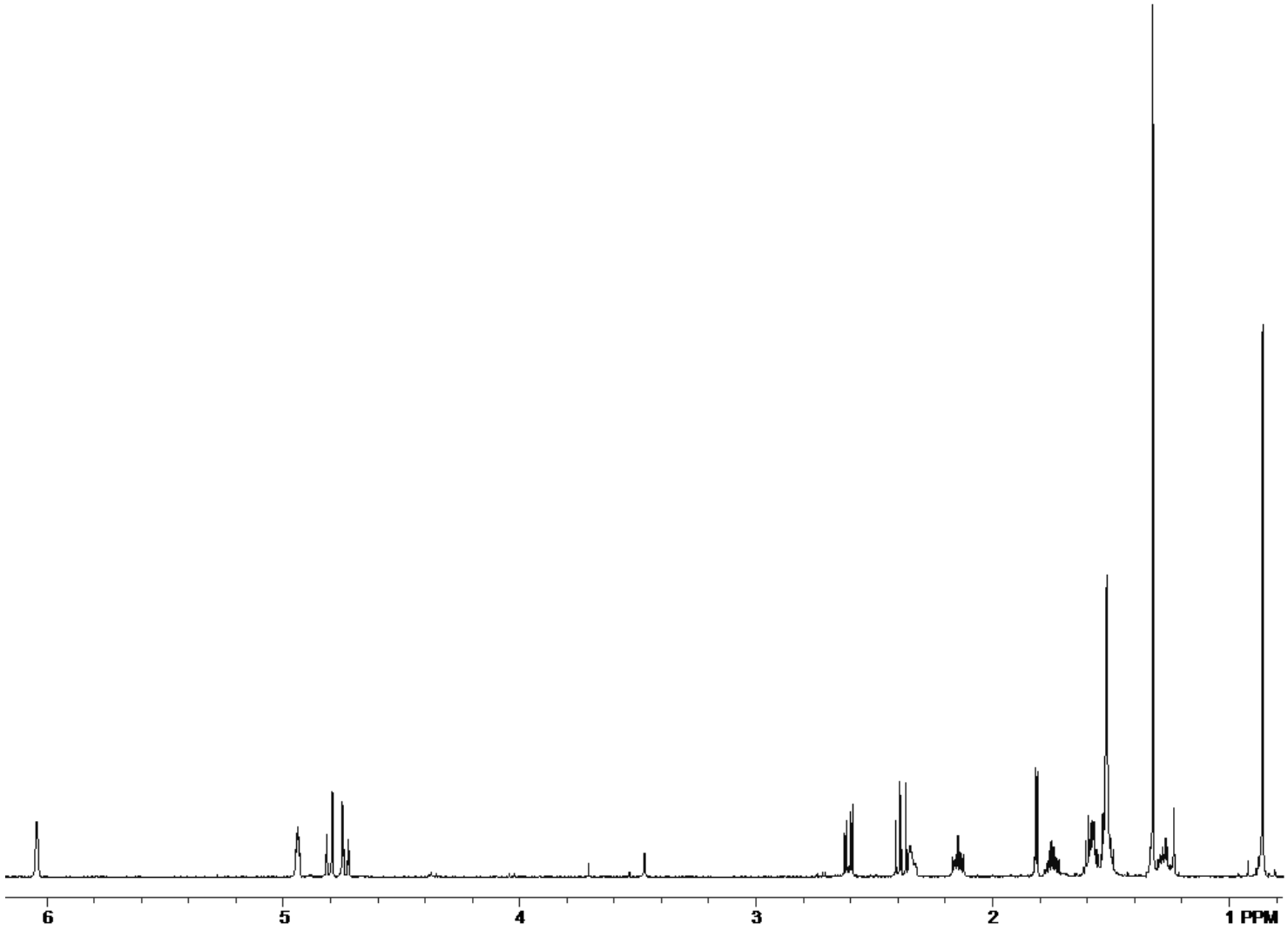


Figure A29. ^{1}H NMR spectrum of oidiolactone G (**48**) ($CDCl_3$; 600 MHz).

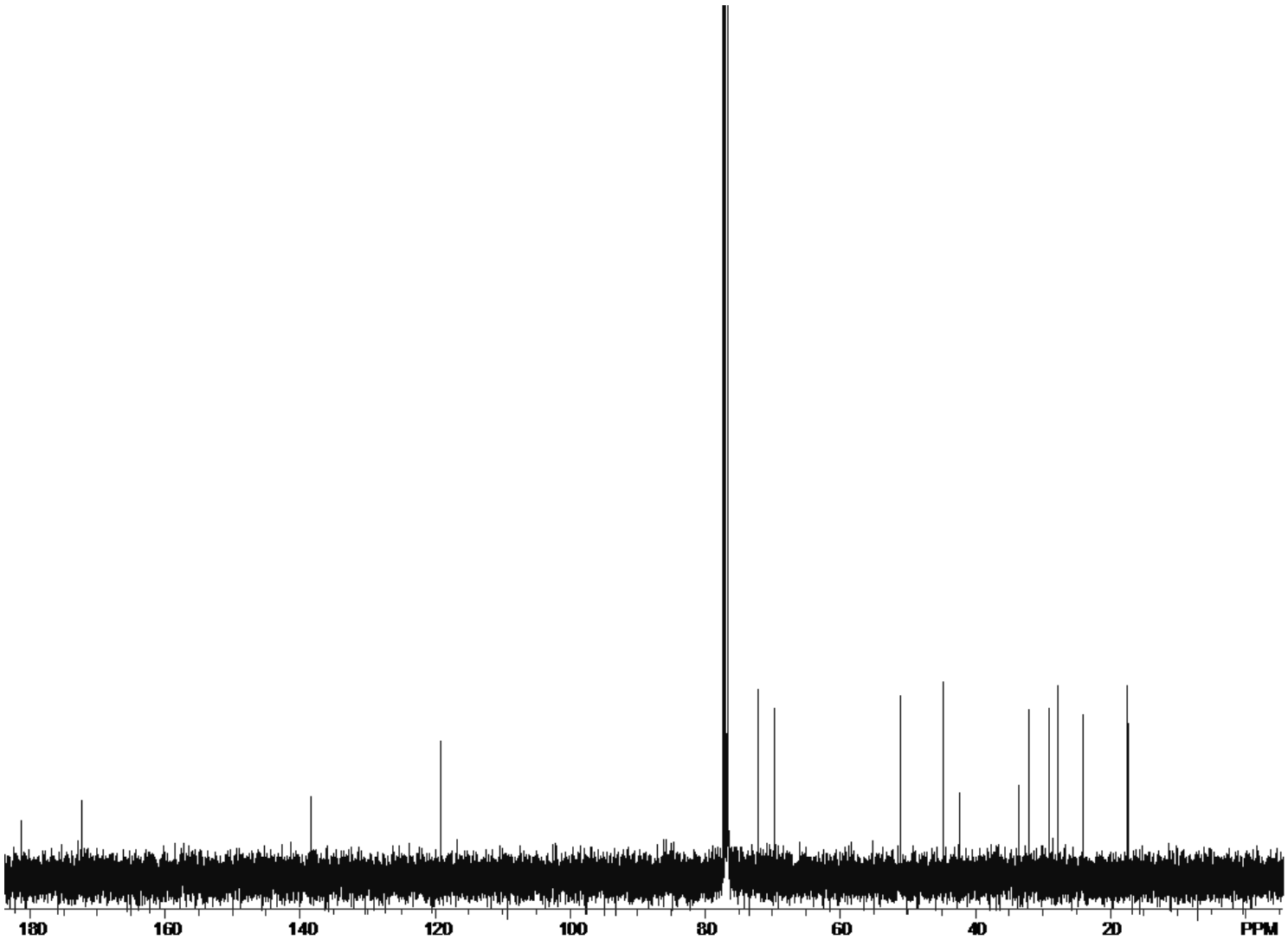


Figure A30. ^{13}C NMR spectrum of oidiolactone G (**48**) ($CDCl_3$; 100 MHz).

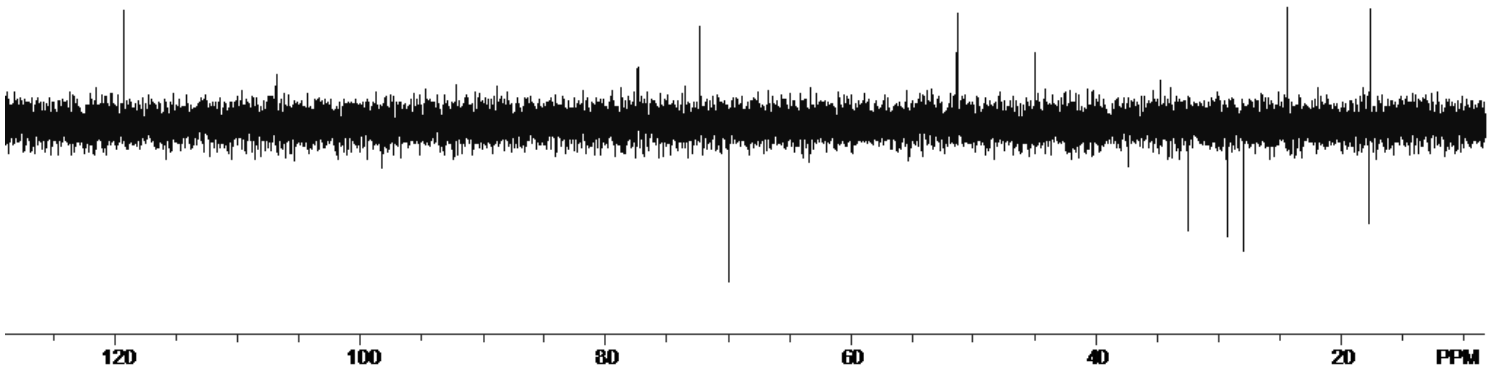


Figure A31. ^{13}C DEPT spectrum of oidiolactone G (**48**) ($CDCl_3$; 100 MHz).

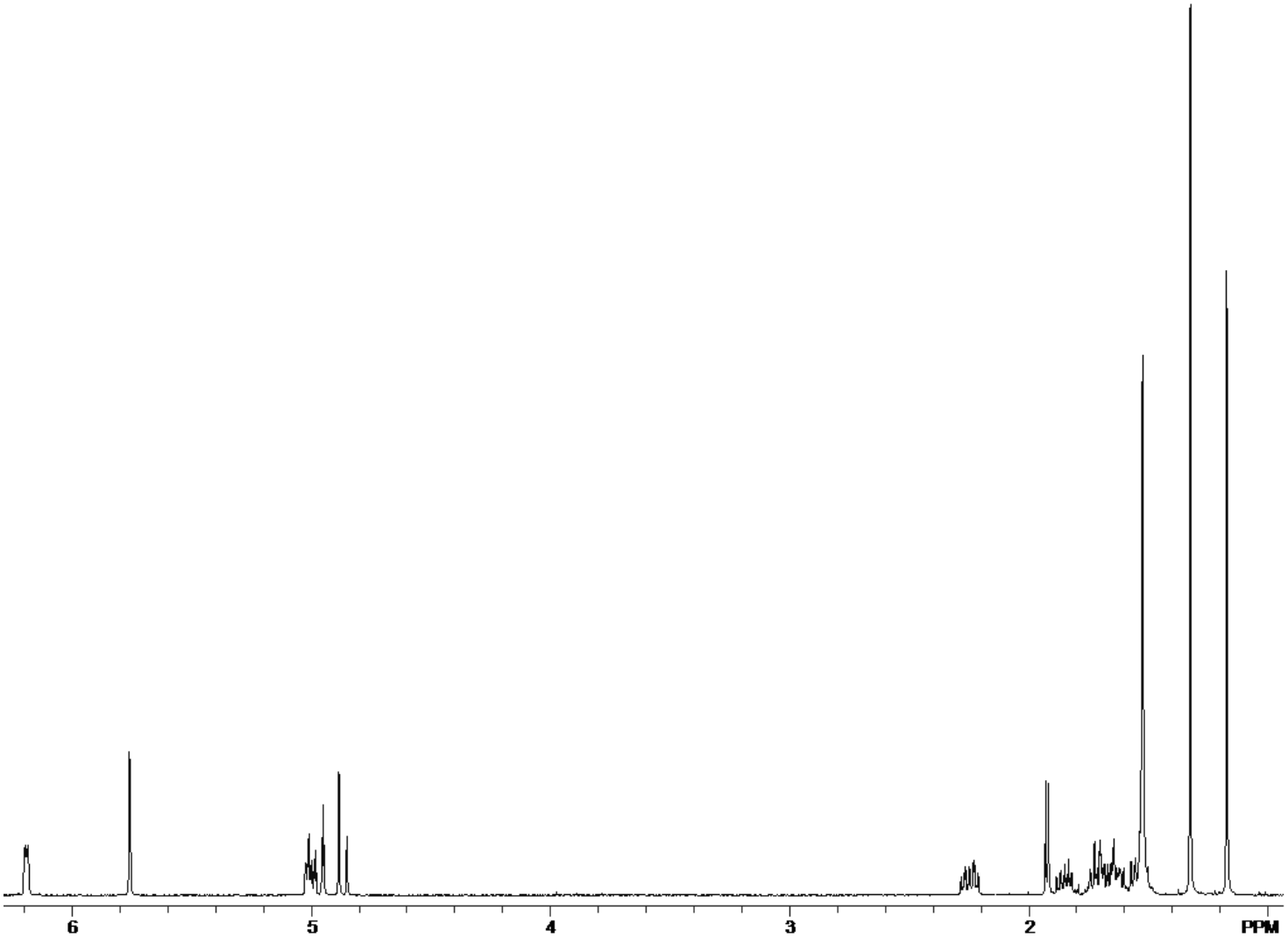


Figure A32. ^{1}H NMR spectrum of dehydro–oidiolactone G (**50**) ($CDCl_3$; 400 MHz).

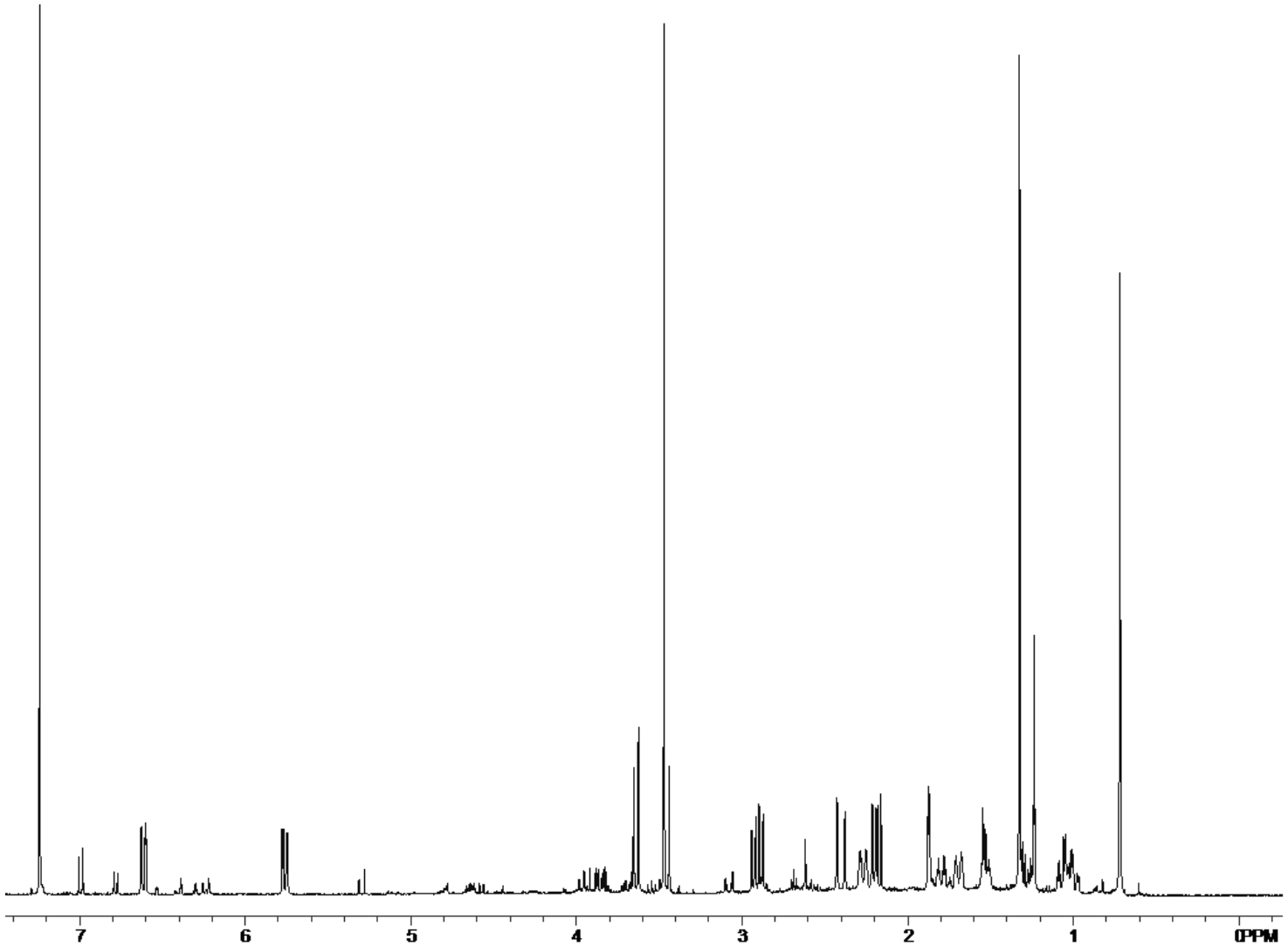


Figure A33. ^{1}H NMR spectrum of oidiolactone E (**52**) ($CDCl_3$; 400 MHz).

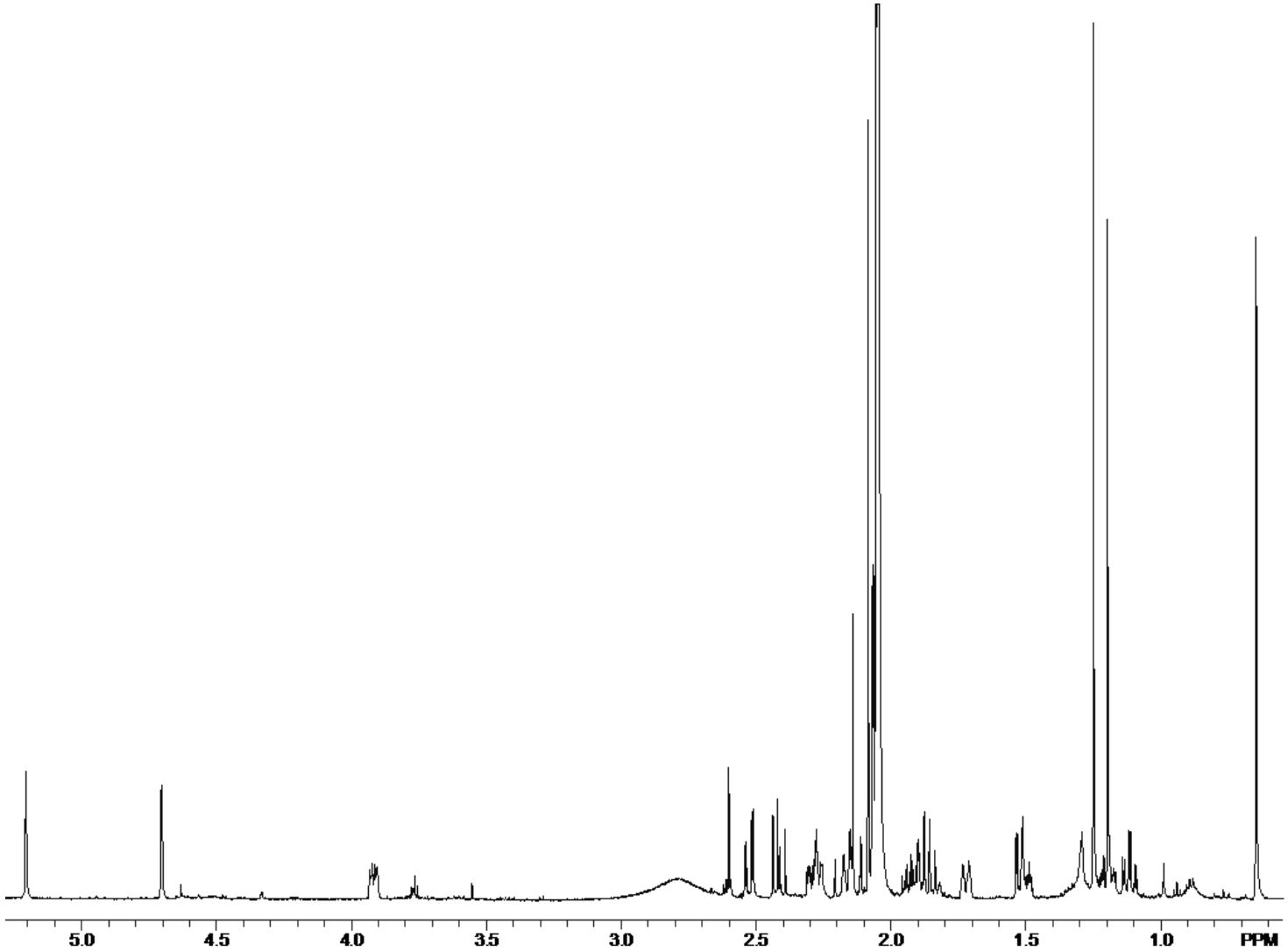


Figure A34. ^{1}H NMR spectrum of LL–Z1271β (**53**) (acetone–d_6; 600 MHz).

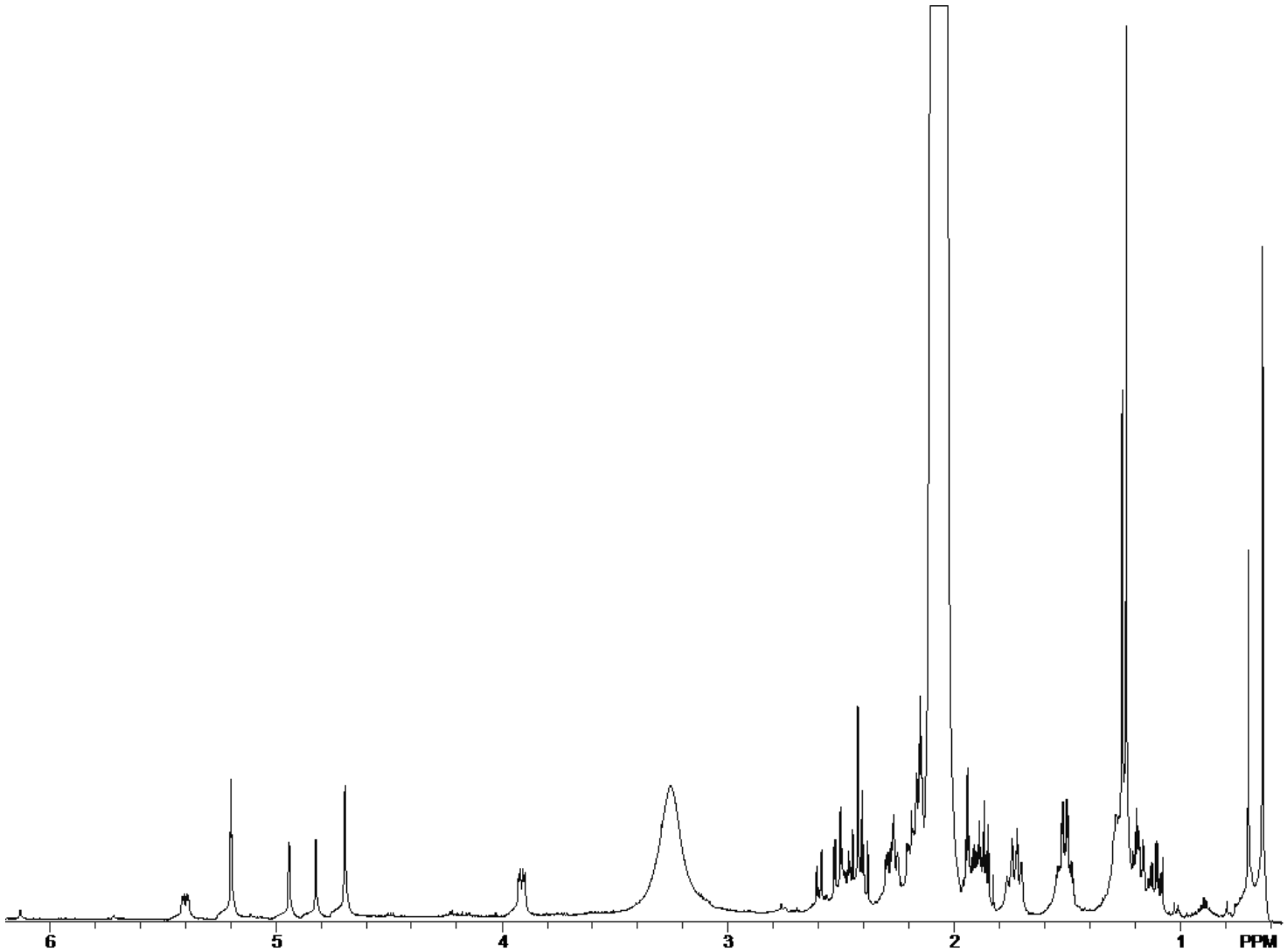


Figure A35. ^{1}H NMR spectrum of LL–Z1271β (**53**) and dehydro–LL–Z1271β artifact (**54**)mixture (acetone–d_6; 400 MHz).

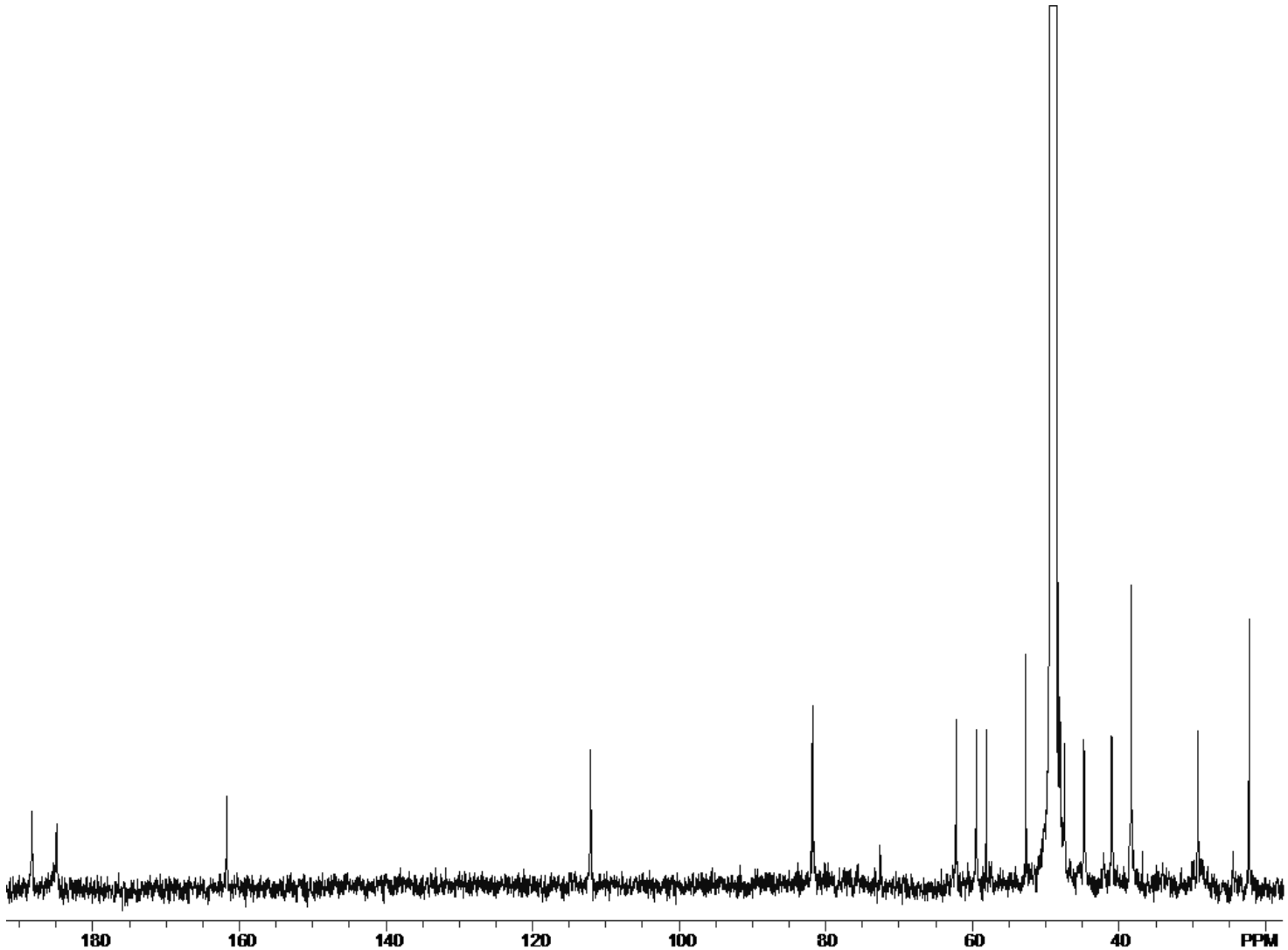


Figure A36. ^{13}C NMR spectrum of LL–Z1271β (**53**) and dehydro–LL–Z1271β artifact (**54**)mixture (CD_3OD; 150 MHz).

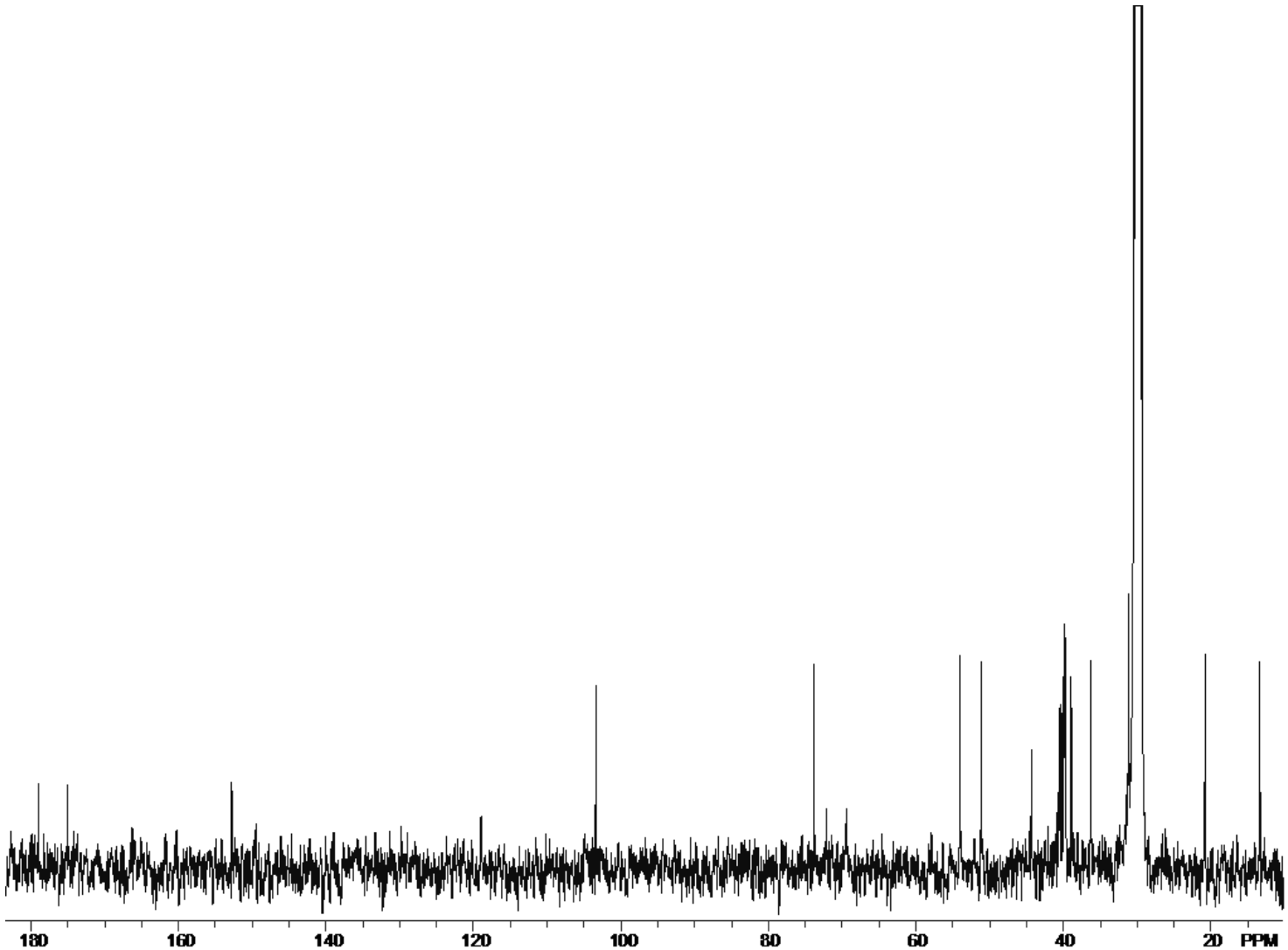


Figure A37. ^{13}C NMR spectrum of LL–Z1271β (**53**) and dehydro–LL–Z1271β artifact (**54**)mixture (acetone–d_6; 100 MHz).

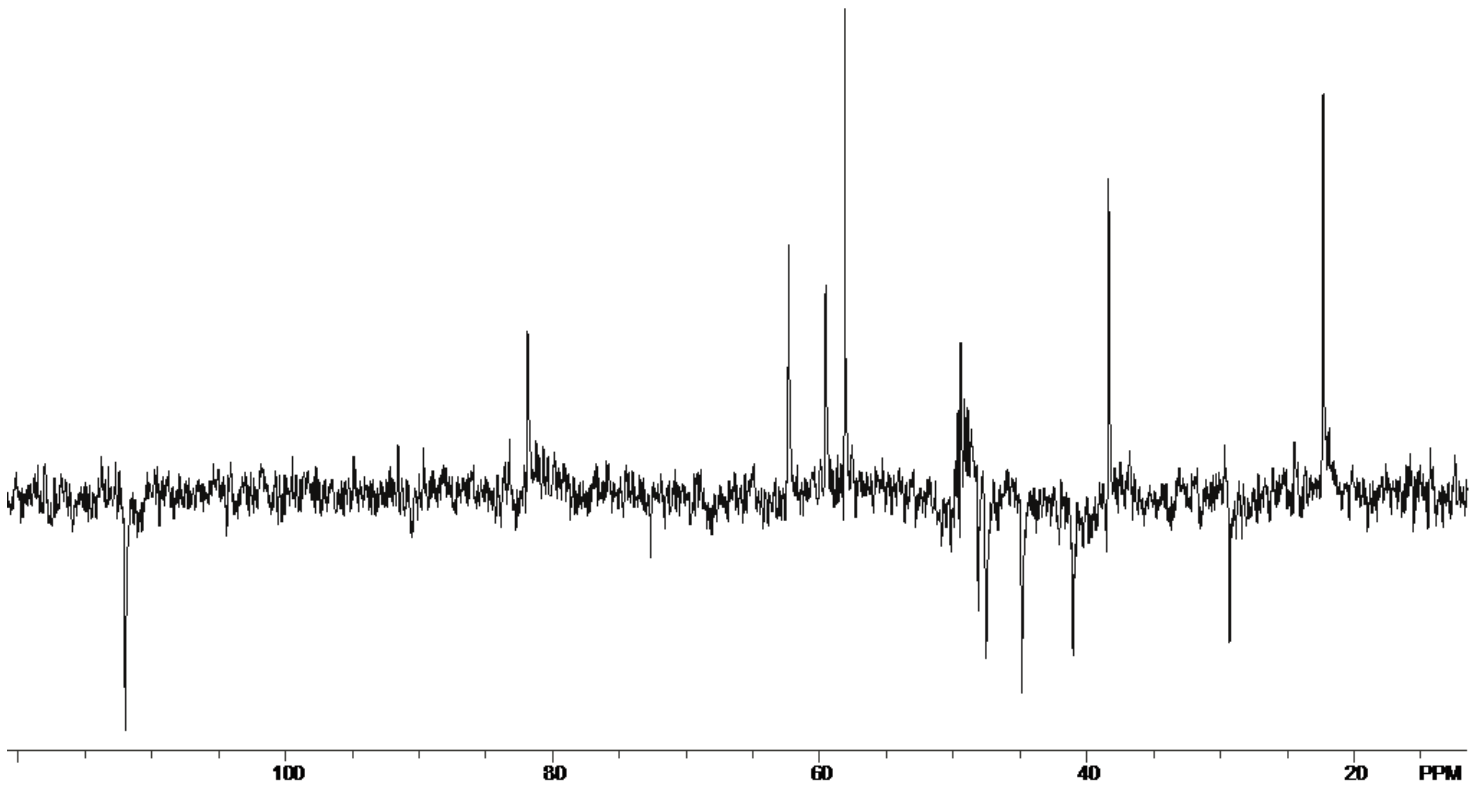


Figure A38. ^{13}C DEPT spectrum of LL–Z1271β (**53**) and dehydro–LL–Z1271β artifact (**54**)mixture (CD_3OD; 100 MHz).

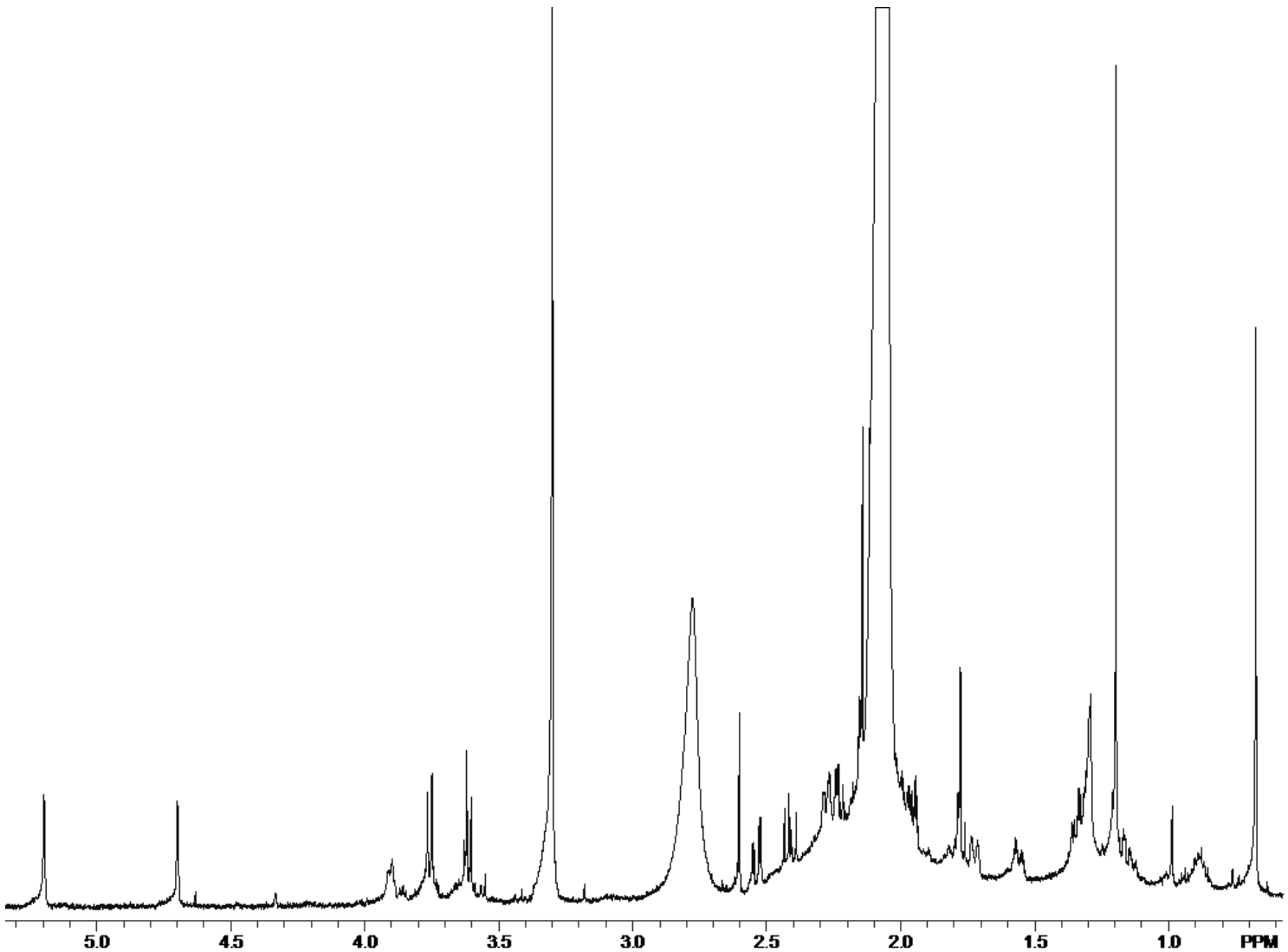


Figure A39. ^{1}H NMR spectrum of proposed compound **55** (acetone–d_6; 600 MHz).

REFERENCES

(1) Mathew, B. P.; Nath, M. *Chem. Med. Chem.* **2009**, *4*, 310-323.

(2) Bagg, J.; Sweeney, M. P.; Lewis, M.; Jackson, M. S.; Coleman, D.; Mosaid, A. A.; Baxter, W.; McEndrick, S.; McHugh, S. *Palliat. Med.* **2003**, *17*, 477-481.

(3) Flanagan, P. G.; Barnes, R. A. *J. Hosp. Infect.* **1998**, *38*, 163-177.

(4) Gloor, M.; Müller, C. A.; Womi, M.; Stahel, P. F.; Redaelli, C.; Uhl, W.; Büchler, M. W. *Arch. Surg.* **2001**, *136*, 592-596.

(5) Morrison, V. A.; Haake, R. J.; Weisdorf, D. J. *Am. J. Med.* **1994**, *96*, 497-503.

(6) Martino, P.; Girmenia, C. *Support Care Cancer* **1993**, *1*, 240-244.

(7) Cornely, O. A.; Maertens, J.; Winston, D. J.; Perfect, J.; Ullmann, A. J.; Walsh, T. J.; Helfgott, D.; Holowiecki, J.; Stockelberg, D.; Goh, Y.; Petrini, M.; Hardalo, C.; Suresh, R.; Angulo-Gonzalez, D. *N. Engl. J. Med.* **2007**, *356*, 348-359.

(8) Bastert, J.; Schaller, M.; Korting, H. C.; Evans, E. G. V. *Int. J. Antimicrob. Agents* **2001**, *17*, 81-91.

(9) Bokhari, F. M.; Aly, M. M. *Adv. Biol. Res.* **2009**, *3*, 71-78.

(10) Julian, A. M.; Wareing, P. W.; Phillips, S. I.; Medlock, V. F. P.; MacDonald, M. V.; del Río, L. E. *Mycopathologia* **1995**, *129*, 5-16.

(11) Dutton, M. F.; Kinsey, A. *Mycopathologia* **1995**, *131*, 31-36.

(12) L-Baeckström, G.; Lundegårdh, B.; Hanell, U. *J. Sci. Food Agric.* **2006**, *86*, 2560-2578.

(13) Duran, R. M.; Cary, J. W.; Calvo, A. M. *Open Mycology Journal* **2009**, *3*, 27-36.

(14) Rabie, C. J.; Kellerman, T. S.; Kriek, N. P. J.; Van Der Westhuizen, G. C. A.; De Wit, P. J. *Food Chem. Toxicol.* **1985**, *23*, 349-353.

(15) Marasas, W. F. O.; Wehner, F. C.; van Rensburg, S. J.; van Schalkwyk, D. J. *Phytopathology* **1981**, *71*, 792-796.

(16) Thiel, P. G.; Marasas, W. F. O.; Sydenham, E. W.; Shephard, G. S.; Gelderblom, W. C. A. *Mycopathologia* **1992**, *117*, 3-9.

(17) Turner, P. C.; Moore, S. E.; Hall, A. J.; Prentice, A. M.; Wild, C. P. *Environ. Health Perspect.* **2003**, *111*, 217-220.

(18) Steyn, P. S. *Toxicol. Lett.* **1995**, *82/83*, 843-851.

(19) Gunatilaka, A. A. L. *J. Nat. Prod.* **2006**, *69*, 509-526.

(20) Tan, R. X.; Zou, W. X. *Nat. Prod. Rep.* **2001**, *18*, 448-459.

(21) Bush, L. P.; Fannin, F. F.; Siegel, M. R.; Dahlman, D. L.; Burton, H. R. *Agric. Ecosystems, Environ.* **1993**, *44*, 81-102.

(22) Bush, L. P.; Wilkinson, H. H.; Schardl, C. L. *Plant Physiol.* **1997**, *114*, 1-7.

(23) Davis, R. A.; Longden, J.; Avery, V. M.; Healy, P. C. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2836-2839.

(24) Squire, R. A. *Science* **1981**, *214*, 877-880.

(25) Sargeant, K.; Sheridan, A.; O'Kelly, J.; Carnaghan, R. B. A. *Nature* **1961**, *192*, 1096-1097.

(26) Codner, R. C.; Sargeant, K.; Yeo, R. *Biotechnol. Bioeng.* **1963**, *5*, 185-192.

(27) Gelderblom, W. C. A.; Jaskiewicz, K.; Marasas, W. F. O.; Thiel, P. G.; Horak, R. M.; Vleggaar, R.; Kriek, N. P. J. *Appl. Environ. Microbiol.* **1988**, *54*, 1806-1811.

(28) Amezqueta, S.; Gonzalez-Penas, E.; Murillo-Arbizu, M. *Food Control* **2009**, *20*, 326-333.

(29) Combrinck, S.; Gelderblom, W. C. A.; Spies, H. S. C.; Burger, B. V.; Thiel, P. G.; Marasas, W. F. O. *Appl. Environ. Microbiol.* **1988**, *54*, 1700-1702.

(30) Freeman, G. G. *J. Gen. Microbiol.* **1955**, *12*, 213-221.

(31) Freeman, G. G.; Morrison, R. I. *Biochem J.* **1949**, *44*, 1-5.

(32) Freeman, G. G.; Morrison, R. I. *Microbiol.* **1949**, *3*, 60-68.

(33) Loukaci, A.; Kayser, O.; Bindseil, K. -.; Siems, K.; Frevert, J.; Abreu, P. M. *J. Nat. Prod.* **2000**, *63*, 52-56.

(34) Sorenson, W. G.; Sneller, M. R.; Larsh, H. W. *Appl. Microbiol.* **1975**, *29*, 653-657.

(35) Cole, R. J.; Cox, R. H. In *Handbook of Toxic Fungal Metabolites;* 1981; pp 1-66.

(36) Hawksworth, D. L. *Mycol. Res.* **2001**, *105*, 1422-1432.

(37) Gloer, J. B. In *Applications of Fungal Ecology in the Search for New Bioactive Natural Products;* Kubicek, C. P., Druzhinina, I. S., Eds.; The Mycota; Springer-Verlag: New York, 2007; pp 251-277.

(38) Scheffer, R. P.; Livingston, R. S. *Science* **1984**, *223*, 17-21.

(39) Schulz, B.; Bolye, C.; Draeger, S.; Rommert, A.; Krohn, K. *Mycol. Res.* **2002**, *106*, 996-1004.

(40) Wicklow, D. T.; Joshi, B. K.; Gamble, W. R.; Gloer, J. B.; Dowd, P. F. *Appl. Environ. Microbiol.* **1998**, *64*, 4482-4484.

(41) Shim, S. H.; Sy, A. A.; Gloer, J. B.; Wicklow, D. T. *Bull. Korean Chem. Soc.* **2008**, *29*, 863-865.

(42) Deyrup, S. T.; Gloer, J. B.; O'Donnell, K.; Wicklow, D. T. *J. Nat. Prod.* **2007**, *70*, 378-382.

(43) Schmidt, L. E.; Gloer, J. B.; Wicklow, D. T. *J. Nat. Prod.* **2007**, *70*, 1317-1320.

(44) Mudur, S. V.; Gloer, J. B.; Wicklow, D. T. *J. Antibiot.* **2006**, *59*, 500-506.

(45) Nout, M. J. R.; Platis, C. E.; Wicklow, D. T. *Can. J. Microbiol.* **1997**, *43*, 362-367.

(46) Joshi, B. K.; Gloer, J. B.; Wicklow, D. T. *J. Nat. Prod.* **1999**, *62*, 730-733.

(47) Kirk, P. M.; Cannon, P. F.; Minter, D. W.; Stalpers, J. A., Eds.; In *Ainsworth and Bisby's Dictionary of the Fungi;* Cromwell Press: Trowbridge, 2008; Vol. 10, pp 771.

(48) Panda, B. P.; Javed, S.; Ali, M. *Czech. J. Food Sci.* **2009**, *27*, 352-360.

(49) Burton, H. S.; Abraham, E. P. *Biochem. J.* **1951**, *50*, 168-174.

(50) Fleming, A. *Br. J. Exp. Pathol.* **1929**, *10*, 226-236.

(51) Arnstein, H. R. V.; Grant, P. T. *Biochem. J.* **1954**, *57*, 353-359.

(52) Letscher-Bru, V.; Herbrecht, R. *J. Antimicrob. Chemoth.* **2003**, *51*, 513-521.

(53) Bacon, C. W.; White, J. F. J., Eds.; In *Microbial Endophytes;* 2000; , pp 487.

(54) Sieber, T. N.; Sieber-Canavesi, F.; Dorworth, C. E. *Mycologia* **1990**, *82*, 569-575.

(55) Wilson, D. *Oikos* **1995**, *73*, 274-276.

(56) Arnold, A. E.; Maynard, Z.; Gilbert, G. S.; Coley, P. D.; Kursar, T. A. *Ecol. Lett.* **2000**, *3*, 267-274.

(57) Clay, K. *Ecology* **1988**, *69*, 10-16.

(58) Petrini, L. E.; Petrini, O.; Laflamme, G. *Phytoprotection* **1989**, *70*, 97-103.

(59) Freeman, S.; Rodriguez, R. J. *Science* **1993**, *260*, 75.

(60) Poling, S. M.; Wicklow, D. T.; Rogers, K. D.; Gloer, J. B. *J. Agric. Food Chem.* **2008**, *56*, 9-3009.

(61) Abdou, R.; Scherlach, K.; Dahse, H. -.; Sattler, I.; Hertweck, C. *Phytochemistry* **2010**, *71*, 110-116.

(62) Pittayakhajonwut, P.; Dramae, A.; Madla, S.; Lartpornmatulee, N.; Boonyuen, N.; Tanticharoen, M. *J. Nat. Prod.* **2006**, *69*, 1361-1363.

(63) Wicklow, D. T. *Phytopathology* **2006**, *99*, 109-115.

(64) Campos, F. F.; Rosa, L. H.; Cota, B. B.; Caligiorne, R. B.; Rabello, A. L. T.; Alves, T. M. A.; Rosa, C. A.; Zani, C. L. *PLoS Neglected Tropical Diseases* **2008**, *2*, e348.

(65) Huang, Z.; Cai, X.; Shao, C.; She, Z.; Xia, X.; Chen, Y.; Yang, J.; Zhou, S.; Lin, Y. *Phytochemistry* **2008**, *69*, 1604-1608.

(66) Krohn, K.; Kouam, S. F.; Cludius-Brandt, S.; Draeger, S.; Schulz, B. *Eur. J. Org. Chem.* **2008**, , 3615-3618.

(67) Simons, L.; Bultman, T. L.; Sullivan, T. J. *J. Chem. Ecol.* **2008**, *34*, 1511-1517.

(68) Redman, R. S.; Sheehan, K. B.; Stout, R. G.; Rodriguez, R. J.; Henson, J. M. *Science* **2002**, *298*, 1581.

(69) Anonymous *Science* **2003**, *301*, 1466.

(70) Carroll, G. *Ecology* **1988**, *69*, 2-9.

(71) Funk, C. R.; Halisky, P. M.; Johnson, M. C.; Siegel, M. R.; Stewart, A. V.; Ahmed, S.; Hurley, R. H.; Harvey, I. C. *Bio/Technology* **1983**, *1*, 189-191.

(72) Webber, J. *Nature* **1981**, *292*, 449-451.

(73) Webber, J.; Gibbs, J. N. *Trans. Brit. Mycol. Soc.* **1984**, *82*, 348-352.

(74) Bultman, T. L.; Bell, G.; Martin, W. D. *Ecology* **2004**, *85*, 679-685.

(75) Zhang, D.; Nagabhyru, P.; Schardl, C. L. *Plant Physiol.* **2009**, *150*, 1072-1082.

(76) Siegel, M. R.; Bush, L. P. *Recent Adv. Phytochem.* **1996**, *30*, 81-120.

(77) Shu, R. G.; wang, F. W.; Yang, Y. M.; Liu, Y. X.; Tan, R. X. *Lipids* **2004**, *39*, 667-673.

(78) Wicklow, D. T.; Roth, S.; Deyrup, S. T.; Gloer, J. B. *Mycol. Res.* **2005**, *109*, 610-618.

(79) Macias-Rubalcava, M. L.; Hernandez-Bautista, B. E.; Jimenez-Estrada, M.; Gonzalez, M. C.; Glenn, A. E.; Hanlin, R. T.; Hernandez-Ortega, S.; Saucedo-Garcia, A.; Muria-Gonzalez, J. M.; Anaya, A. L. *Phytochemistry* **2008**, *69*, 1185-1196.

(80) Mudur, S. V.; Swenson, D. C.; Gloer, J. B.; Campbell, J.; Shearer, C. A. *Org. Lett.* **2006**, *8*, 3191-3194.

(81) Shim, S. H.; Swenson, D. C.; Gloer, J. B.; Wicklow, D. T. *Org. Lett.* **2006**, *8*, 1225-1228.

(82) Angawi, R. F.; Swenson, D. C.; Gloer, J. B.; Wicklow, D. T. *J. Nat. Prod.* **2003**, *66*, 1259-1262.

(83) Schmidt, L. E. Chemical Investigations of fungicolous/mycoparasitic fungi from Hawaii, The University of Iowa, Iowa City, IA, 2007.

(84) Bergstrom, G. C.; Nicholson, R. L. *Plant Dis.* **1999**, *83*, 596-608.

(85) White, D. G., Ed.; In *Compendium of Corn Diseases;* American Phytopathological Society Press: St. Paul, MN, 1999; .

(86) Cutler, H. G.; Arrendale, R. F.; Springer, J. P.; Cole, P. D.; Roberts, R. G.; Hanlin, R. T. *Agric. Biol. Chem.* **1987**, *51*, 3331-3338.

(87) Sutton, B. C. In *The Coelmycetes: Fungi Imperfecti with Pycnidia, Acervuli and Stromata;* Commonwealth Mycological Institute: Kew, England, 1980; .

(88) O'Donnell, K. *Sydowia* **1996**, *48*, 57-70.

(89) White, T. J.; Bruns, T.; Lee, S.; Taylor, J. W. In *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics;* Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J., Eds.; PCR Protocols: a Guide to Methods and Applications; Academic Press: New York, 1990; pp 315-322.

(90) Mirocha, C. J.; Schauerhamer, B.; Christensen, C. M.; Kommedahl, *Appl. Environ. Microbiol.* **1979**, *38*, 557-558.

(91) Stob, M.; Baldwin, R. S.; Tuite, J.; Andrews, F. N.; Gillette, K. G. *Nature* **1962**, *196*, 1318.

(92) Shinonaga, H.; Kawamura, Y.; Ikeda, A.; Aoki, M.; Sakai, N.; Fujimoto, N.; Kawashima, A. *Tetrahedron Lett.* **2009**, *50*, 108-110.

(93) Hellwig, V.; Mayer-Bartschmid, A.; Müller, H.; Greif, G.; Kleymann, G.; Zitzmann, W.; Tichy, H. -.; Stadler, M. *J. Nat. Prod.* **2003**, *66*, 829-837.

(94) Winssinger, N.; Barluenga, S. *Chem. Commun.* **2007**, *43*, 22-36.

(95) Reeves, C. D.; Hu, Z.; Reid, R.; Kealey, J. T. *Appl. Environ. Microbiol.* **2008**, *74*, 5121-5129.

(96) Ayer, W. A.; Lee, S. P.; Tsuneda, A.; Hiratsuka, Y. *Can. J. Microbiol.* **1980**, *26*, 766-773.

(97) McCapra, F.; Scott, A. I.; Delmotte, P.; Delmotte–Plaquée, J.; Bhacca, N. S. *Tetrahedron Lett.* **1964**, *5*, 869-875.

(98) Garbaccio, R. M.; Stachel, S. J.; Baeschlin, D. K.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2001**, *123*, 10903-10908.

(99) Mirrington, R. N.; Ritchie, E.; Shoppee, C. W.; Sternhell, S.; Taylor, W. C. *Aust. J. Chem.* **1966**, *19*, 1265-1284.

(100) Mirrington, R. N.; Ritchie, E.; Shoppee, C. W.; Taylor, W. C.; Sternhell, S. *Tetrahedron Lett.* **1964**, *5*, 365-370.

(101) Moulin, E.; Zoete, V.; Barluenga, S.; Karplus, M.; Winssinger, N. *J. Am. Chem. Soc.* **2005**, *127*, 6999-7004.

(102) Delmotte, P.; Delmotte–Plaquée, J. *Nature* **1953**, *171*, 344.

(103) Hughes, S. J. *Can. J. Botany* **1958**, *36*, 727-836.

(104) Tanaka, Y.; Shiomi, K.; Kamei, K.; Sugoh-Hagino, M.; Enomoto, Y.; Fang, F.; Yamaguchi, Y.; Masuma, R.; Zhang, C. G.; Zhang, X. W.; Omura, S. *J. Antibiot.* **1998**, *51*, 153-160.

(105) Arai, M.; Yamamoto, T. *J. Antibiot.* **2003**, *56*, 526-532.

(106) Lam, K. S.; Mamber, S. W.; Pack, E. J.; Forenza, S.; Fernandes, P. B.; Klaus, D. M. *Appl. Microbiol. and Biotechnol.* **1998**, *49*, 579-583.

(107) Nozawa, K. *J. Nat. Prod.* **1979**, *42*, 374-377.

(108) Arai, M. *Chem. Pharm. Bull.* **1989**, *37*, 2870-2872.

(109) Turbyville, T. J.; Kithsiri Wijeratne, E. M.; Liu, M. X.; Burns, A. M.; Seliga, C. J.; Luevano, L. A.; David, C. L.; Faeth, S. H.; Whitesell, L.; Gunatilaka, A. A. L. *J. Nat. Prod.* **2006**, *69*, 178-184.

(110) Evans, G.; White, N. H. *Trans. Br. Mycol. Soc.* **1966**, *49*, 563-576.

(111) Conte, T. C.; Franco, D. V.; Baptista, I. L.; Bueno Jr., C. R.; Selistre-de-Ara *Toxicon* **2008**, *52*, 146-155.

(112) Roe, S. M.; Prodromou, C.; O'Brien, R.; Ladbury, J. E.; Piper, P. W.; Pearl, L. H. *J. Med. Chem.* **1999**, *42*, 260-266.

(113) Oikawa, T.; Onozawa, C.; Kuranuki, S.; Igarashi, Y.; Sato, M.; Ashino, H.; Shimamura, M.; Toi, M.; Kurakata, S. *Cancer Sci* **2007**, *98*, 219-225.

(114) Workman, P.; Burrows, F.; Neckers, L.; Rosen, N. *Ann. N. Y. Acad. Sci.* **2007**, *1113*, 202-216.

(115) Chiosis, G.; Lucas, B.; Huezo, H.; Solit, D.; Basso, A.; Rosen, N. *Curr. Cancer Drug Targets* **2003**, *3*, 371-376.

(116) Neckers, L. *Curr. Med. Chem.* **2003**, *10*, 733-739.

(117) Soga, S.; Shiotsu, Y.; Akinaga, S.; Sharma, S. V. *Curr. Cancer Drug Targets* **2003**, *3*, 359-369.

(118) Csermely, P.; Schnaider, T.; Soti, C.; Prohászka, ; Nardai, G. *Pharmacol. Ther.* **1998**, *79*, 129-168.

(119) Schulte, T. W.; Akinaga, S.; Soga, S.; Sullivan, W.; Stensgard, B.; Toft, D.; Neckers, L. M. *Cell Stress Chaperon.* **1998**, *3*, 100-108.

(120) Chang, F.; Lee, J. T.; Navolanic, P. M.; Steelman, L. S.; Shelton, J. G.; Blalock, W. L.; Franklin, R. A.; McCubrey, J. A. *Leukemia* **2003**, *17*, 590-603.

(121) Guarnieri, M. T.; Zhang, L.; Shen, J.; Zhao, R. *J. Mol. Biol.* **2008**, *379*, 82-93.

(122) Lampilas, M.; Lett, R. *Tetrahedron Lett.* **1992**, *33*, 777-780.

(123) McLellan, C. A.; Turbyville, T. J.; Wijeratine, E. M. K.; Kerschen, A.; Vierling, E.; Queitsch, C.; Whitesell, L.; Gunatilaka, A. A. L. *Plant Physiol.* **2007**, *145*, 174-182.

(124) Wicklow, D. T.; Jordan, A. M.; Gloer, J. B. *Mycol. Res.* **2009**, *113*, 1433-1442.

(125) Alcorn, J. L. *Mycotaxon* **1983**, *17*, 1-86.

(126) Ullstrup, A. J. *Phytopathology* **1944**, *34*, 214-222.

(127) Hesseltine, C. W.; Ellis, J. J.; Shotwell, O. L. *J. Agr. Food Chem.* **1971**, *19*, 707-717.

(128) Walton, J. D.; Ransom, R.; Pitkin, J. W. In *Northern Corn Leaf Spot: Chemistry, Enzymology, and Molecular Genetics of a Host-Selective Phytotoxin;* Macmillan Pub. Co.: New York, 1997; Vol. 3, pp 94-123.

(129) Fisher, D. E.; Hooker, A. L.; Lim, S. M.; Smith, D. R. *Phytopathology* **1976**, *66*, 942-944.

(130) Xiao, J. Z.; Tsuge, T.; Doke, N.; Nakatsuka, S.; Tsuda, M.; Nishimura, S. *Physiol. Mol. Plant Pathol.* **1991**, *38*, 67-82.

(131) Pringle, R. B. *Plant Physiol.* **1971**, *48*, 756-759.

(132) Walton, J. D. *Phytochemistry* **2006**, *67*, 1406-1413.

(133) Wegulo, S. N.; Rivera-C, J. M.; Martinson, C. A.; Nutter, F. W., Jr. *Plant Dis.* **1998**, *82*, 547-554.

(134) Gatch, E. W.; Munkvold, G. P. *Plant Dis.* **2002**, *86*, 1156-1162.

(135) Crane, R. I.; Hedden, P.; MacMillan, J.; Turner, W. B. *J. Chem. Soc. , Perkin Trans. 1* **1973**, , 194-200.

(136) White, J. D.; Kim, J.; Drapela, N. E. *J. Am. Chem. Soc.* **2000**, *122*, 8665-8671.

(137) Nieminen, S.; Tamm, C. *Helv. Chim. Acta* **1981**, *64*, 2971-2801.

(138) Hosoe, T.; Fukushima, K.; Itabashi, T.; Nozawa, K.; Takizawa, K.; Kawai, K. *Heterocycles* **2004**, *63*, 2581-2589.

(139) Pena-Rodriguez, L. M.; Armingeon, N. A.; Chilton, W. S. *J. Nat. Prod.* **1988**, *51*, 821-828.

(140) Pena-Rodriguez, L. M.; Chilton, W. S. *J. Nat. Prod.* **1989**, *52*, 899-901.

(141) Dorn, F.; Arigoni, D. *Chem. Commun.* **1972**, , 1342-1343.

(142) Tamura, S.; Sakurai, A. *Agr. Biol. Chem.* **1964**, *28*, 337-338.

(143) Tamura, S.; Sakurai, A.; Kainuma, K.; Takai, M. *Agr. Biol. Chem.* **1965**, *29*, 216-221.

(144) Tamura, S.; Sakurai, A.; Kainuma, K.; Takai, M. *Agr. Biol. Chem.* **1963**, *7*, 738.

(145) Osterhage, C.; König, G. M.; Höller, U.; Wright, A. D. *J. Nat. Prod.* **2002**, *65*, 306-313.

(146) Chelkowski, J., Ed.; In *Helminthosporia Metabolites, Biology, Plant Diseases: Bipolaris, Drechslera, Exserohilum;* 1995, pp 220.

(147) Spencer, E. Y.; Ludwig, R. A.; De Mayo, P.; White, R. W.; Williams, R. E. *Advan. Chem. Ser.* **1966**, *53*, 106.

(148) De Mayo, P.; Williams, R. E.; Spencer, E. Y. *Can. J. Chem.* **1965**, *43*, 1357-1365.

(149) Miyagawa, H.; Nagai, S.; Tsurushima, T.; Sato, M.; Ueno, T.; Fukami, H. *Biosci. Biotech. Biochem.* **1994**, *58*, 1143-1145.

(150) Briquet, M.; Vilret, D.; Goblet, P.; Mesa, M.; Eloy, M. *J. Bioenerg. Biomembr.* **1998**, *30*, 285-295.

(151) Lim, C.; Miyagawa, H.; Akamatsu, M.; Nakagawa, Y. *J. Pesticide Sci.* **1998**, *23*, 281-288.

(152) Carruthers, J. R.; Cerrini, S.; Fedeli, W.; Casinovi, C. G.; Galeffi, C.; Torracca Vacarro, A. M.; Scala, A. *Chem. Commun.* **1971**, , 164-166.

(153) Lim, C.; Ueno, H.; Miyoshi, H.; Miyagawa, H.; Iwamura, H.; Ueno, T. *J. Pesticide Sci.* **1996**, *21*, 213-215.

(154) Canonica, L.; Beretta, M. G.; Colombo, L.; Gennari, C.; Ranzi, B. M.; Scolastico, C. *J. Chem. Soc. , Perkin Trans. I* **1980**, , 2686-2690.

(155) Yoganathan, K.; Yang, L.; Rossant, C.; Huang, Y.; Ng, S.; Butler, M. S.; Buss, A. D. *J. Antibiot.* **2004**, *57*, 59-63.

(156) Phuwapraisirisan, P.; Sawang, K.; Siripong, P.; Tip-pyang, S. *Tetrahedron Lett.* **2007**, *48*, 5193-5195.

(157) Bicalho, B.; Goncalves, R. A. C.; Zibordi, A. P. M.; Manfio, G. P.; Marsaioli, A. J. *Z. Naturforsch.* **2003**, *58c*, 746-751.

(158) Barrrow, K. D.; Murphy, W. S. *J. Chem. Soc. , Perkin Trans. I* **1972**, , 2837-2839.

(159) Hosoe, T.; Fukushima, K.; Itabashi, T.; Nozawa, K.; Takizawa, K.; Okada, K.; Takaki, G.; Kawai, K. *J. Antibiot.* **2004**, *57*, 573-578.

(160) Olbe, M.; Sommarin, M.; Gustafsson, M.; Lundborg, T. *Plant Pathol.* **1995**, *44*, 625-635.

(161) Briggs, D. E. *Nature* **1966**, *210*, 418-419.

(162) Park, J. K.; Hasumi, K.; Endo, A. *J. Antibiot.* **1993**, *46*, 1303-1305.

(163) Walton, J. D.; Panaccione, D. G. *Annu. Rev. Phytopathology* **1993**, *31*, 275-303.

(164) Pringle, R. B.; Scheffer, R. P. *Phytopathology* **1967**, *57*, 1169-1172.

(165) Scheffer, R. P.; Ullstrup, A. J. *Phytopathology* **1965**, *55*, 1037-1038.

(166) Dunkle, L. D.; Cantone, F. A.; Ciuffetti, L. M. *Physiol. Mol. Plant Pathol.* **1991**, *38*, 265-273.

(167) Kuo, M.; Yoder, O. C.; Scheffer, R. P. *Phytopathology* **1970**, *60*, 365-368.

(168) Pope, M. R.; Ciuffetti, L. M.; Knoche, H. W.; McCrery, D.; Daly, J. M.; Dunkle, L. D. *Biochem.* **1983**, *22*, 3502-3506.

(169) Gross, M. L.; McCrery, D.; Crow, F.; Tomer, K. B.; Pope, M. R.; Ciuffetti, L. M.; Knoche, H. W.; Daly, J. M.; Dunkle, L. D. *Tetrahedron Lett.* **1982**, *23*, 5381-5384.

(170) Walton, J. D.; Earle, E. D.; Gibson, B. W. *Biochem. Biophys. Res. Commun.* **1982**, *107*, 785-794.

(171) Kawai, M.; Rich, D. H.; Walton, J. D. *Biochem. Biophys. Res. Commun.* **1983**, *111*, 398-403.

(172) Walton, J. D. *Curr. Top. Plant Physiol.* **1993**, *11*, 116-124.

(173) Weiergang, I.; Wood, K. V.; Dunkle, L. D.; Nicholson, R. L. *Physiol. Mol. Plant Pathol.* **2004**, *64*, 273-279.

(174) Rasmussen, J. B.; Scheffer, R. P. *Plant Physiol.* **1988**, *86*, 187-191.

(175) Walton, J. D.; earle, E. D. *Physiol. Plant Pathol.* **1983**, *22*, 371-376.

(176) Multani, D. S.; Meeley, R. B.; Paterson, A. H.; Gray, J.; Briggs, S. P.; Johal, G. S. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 1686-1691.

(177) Brosch, G.; Ransom, R.; Lechner, T.; Walton, J. D.; Loidl, P. *Plant Cell* **1995**, *7*, 1941-1950.

(178) Naumann, T. A.; Wicklow, D. T.; Kendra, D. F. *Physiol. Mol. Plant Pathol.* **2009**, *74*, 134-141.

(179) Jung, H. J.; Lee, H. B.; Lim, C.; Kim, C.; Kwon, H. J. *Bioorg. Med. Chem.* **2003**, *11*, 4743-4747.

(180) Xiao, J.; Tsuge, T.; Doke, N. *Physiol. Mol. Plant Pathol.* **1992**, *40*, 359-370.

(181) Apoga, D.; Åkesson, H.; Jansson, H.; Odham, G. *Eur. J. Plant. Pathol.* **2002**, *108*, 519-526.

(182) Tanis, S. P.; Horenstein, B. A.; Scheffer, R. P.; Rasmussen, J. B. *Heterocycles* **1986**, *24*, 3423-3431.

(183) Kim, S.; Knoche, H. W. *Tetrahedron Lett.* **1985**, *26*, 969-972.

(184) Leonard, D. M. *J. Med. Chem.* **1997**, *40*, 2971-2990.

(185) Goritschnig, S.; Weihmann, T.; Zhang, Y.; Fobert, P.; McCourt, P.; Lin, X. *Plant Physiol.* **2008**, *148*, 348-357.

(186) Yalovsky, S.; Kulukian, A.; Rodriguez-Concepcion, M.; Young, C. A.; Gruissem, W. *Plant Cell* **2000**, *12*, 1267-1278.

(187) Qian, D.; Zhou, D.; Ju, R.; Cramer, C. L.; Yang, Z. *Plant Cell* **1996**, *8*, 2381-2394.

(188) Wicklow, D. T.; Weaver, D. K.; Throne, J. E. *J. Stored Prod. Res.* **1998**, *34*, 355-361.

(189) Haws, E. J.; Holker, J. S. E. *J. Chem. Soc.* **1961**, , 3820-3822.

(190) Haws, E. J.; Holker, J. S. E.; Kelly, A.; Powell, A. D. G.; Robertson, A. *J. Chem. Soc.* **1959**, *70*, 3610.

(191) Steyn, P. S.; Vleggaar, R. *J. C. S. Perkin I* **1976**, , 204-206.

(192) Martinkova, L.; Patakova-Juzlova, P.; Kren, V.; Kucerova, Z.; Havlicek, V.; Olsovky, P.; Hovorka, O.; Rihova, B.; Vesely, D.; Vesela, D.; Ulrichova, J.; Prikrylova, V. *Food Additives and Contaminants* **1999**, *16*, 15-24.

(193) Akihisa, T.; Tokuda, H.; Yasukawa, K.; Ukiya, M.; Kiyota, A.; Sakamoto, N.; Suzuki, T.; Tanabe, N.; Nishino, H. *J. Agric. Food Chem.* **2005**, *53*, 562-565.

(194) Nozaki, H.; Date, S.; Kondo, H.; Kiyohara, H.; Takaoka, D.; Tada, T.; Nakayama, M. *Agric. Biol. Chem.* **1991**, *55*, 899-900.

(195) Endo, A. *J. Antibiot.* **1979**, *32*, 852-854.

(196) Wild, D.; Toth, G.; Humpf, H. *J. Agric. Food Chem.* **2002**, *50*, 4002.

(197) Elsenbrand, G. *Mol. Nutr. Food Res.* **2006**, *50*, 322-327.

(198) Loret, M.; Morel, S. *J. Agric. Food Chem.* **2010**, *58*, 1800-1803.

(199) Lin, T. F.; Yakushijin, K.; Buchi, G. H.; Demain, A. L. *J. Ind. Microbiol.* **1992**, *9*, 173-179.

(200) Nishikawa, E. *J. Agric. Chem. Soc. Japan* **1932**, *8*, 1007.

(201) Nishikawa, E. *J. Agric. Chem. Soc. Japan* **1926**, *2*, 688.

(202) Ohashi, M.; Terahara, A.; Nakanishi, K.; Yamaguchi, I.; Hayakawa, N. *Bull. Chem. Soc. Jpn.* **1969**, *33*, 1312-1313.

(203) Ohashi, M.; Yamamura, S.; Terahara, A.; Nakanishi, K. *Bull. Chem. Soc. Jpn.* **1969**, *33*, 1630-1632.

(204) Whalley, W. B. *Pure Appl. Chem.* **1963**, *7*, 565-587.

(205) Inouye, Y.; Nakanishi, K.; Nishikawa, H.; Terahara, A.; Yamamura, S. *Tetrahedron* **1962**, *18*, 1195-1203.

(206) Whalley, W. B.; Ferguson, G.; Marsh, W. C.; Restivo, R. J. *J. Chem. Soc. Perkin I* **1976**, , 1366-1369.

(207) Miyake, T.; Mori, A.; Kii, T.; Okuno, T.; Usui, Y.; Sato, F.; Sammoto, H.; Watanabe, A.; Kariyama, M. *J. Ind. Microbiol. Biotechnol.* **2005**, *32*, 103-108.

(208) Chiu, C. H.; Ni, K. H.; Guu, Y. K.; Pan, T. M. *Appl. Microbiol. Biotechnol.* **2006**, *73*, 297-304.

(209) Lee, C. L.; Wang, J. J.; Kuo, S. L.; Pan, T. M. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 1254-1262.

(210) Lin, Y. L.; Wang, T. H.; Lee, M. H.; Su, N. W. *Appl. Microbiol. Biotechnol.* **2008**, *77*, 965-973.

(211) Pisareva, E. I.; Kujumdzieva, A. V. *Biotechnol. Biotechnol. Eq.* **2010**, *24*, 501-506.

(212) Campoy, S.; Rumbero, A.; Martin, J. F.; Liras, P. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 488-496.

(213) Teng, S. S.; Feldheim, W. *Chromatographia* **1998**, *47*, 529-536.

(214) Miyake, T.; Kono, I.; Nozaki, N.; Sammoto, H. *Food Sci. Technol. Res.* **2008**, *14*, 194-197.

(215) Sweeny, J. G.; Estrada-Valdes, M. C.; Iacobucci, G. A.; Sato, H.; Sakamura, S. *J. Agric. Food Chem.* **1981**, *29*, 1189-1193.

(216) Hadfield, J. R.; Holker, J. S. E.; Stanway, D. N. *J. Chem. Soc. (C)* **1967**, , 751-755.

(217) Birch, A. J.; Cassera, A.; Fitton, P.; Holker, J. S. E.; Smith, H.; Thompson, G. A.; Whalley, W. B. *J. Chem. Soc.* **1962**, 3583-3586.

(218) Kurono, M.; Nakanishi, K.; Shindo, K.; Tada, M. *J. Pharm. Soc. Japan* **1963**, *11*, 359-362.

(219) Juzlova, P.; Martinkova, L.; Kren, V. *J. Ind. Microbiol.* **1996**, *16*, 170.

(220) Hawksworth, D. L.; Pitt, J. I. *Aust. J. Bot.* **1983**, *31*, 51-61.

(221) Carvalho, J.; Oishi, B.; Pandey, A.; Soccol, C. *Braz. Arch. Biol. Technol.* **2005**, *48*, 885-894.

(222) Ma, J.; Li, Y.; Ye, Q.; Li, J.; Hua, Y.; Ju, D.; Zhang, D.; Cooper, R.; Chang, M. *J. Agric. Food Chem.* **2000**, *48*, 5220-5225.

(223) Alberts, A. W., et al *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 3957-3961.

(224) Serizawa, N.; Nakagawa, K.; Hamano, K.; Tsujita, Y.; Terahara, A.; Kuwano, H. *J. Antibiot.* **1983**, *36*, 604-607.

(225) Martinkova, L.; Juzlova, P.; Vesely, D. *J. Appl. Bacteriol.* **1995**, *79*, 609-616.

(226) Musso, L.; Dallavalle, S.; Merlini, L.; Bava, A.; Nasini, G.; Penco, S.; Giannini, G.; Giommarelli, C.; De Cesare, A.; Zuco, V.; Vesci, L.; Pisano, C.; Dal Piaz, F.; De Tommasi, N.; Zunino, F. *Bioorg. Med. Chem.* **2010**, *18*, 6031-6043.

(227) Dockerill, B.; Hanson, J. R.; Siverns, M. *Phytochemistry* **1978**, *17*, 427-430.

(228) Hanson, J. R.; Marten, T.; Siverns, M. *J. Chem. Soc. Perkin I* **1974**, , 1033-1036.

(229) Tidd, B. K. *J. Chem. Soc. (C)* **1967**, , 218-220.

(230) Chinworrungsee, M.; Kittakoop, P.; Isaka, M.; Chanphen, R.; Tanticharoen, M.; Thebtaranonth, Y. *J. Chem. Soc. , Perkin Trans. I* **2002**, , 2473-2476.

(231) Poch, G. K.; Gloer, J. B. *J. Nat. Prod.* **1991**, *54*, 213-217.

(232) Millot, M.; Tomasi, S.; Articus, K.; Rouaud, I.; Bernard, A.; Boustie, J. *J. Nat. Prod.* **2007**, *70*, 316-318.

(233) Chomcheon, P.; Wiyakrutta, S.; Sriubolmas, N.; Ngamrojanavanich, N.; Kengtong, S.; Mahidol, C.; Ruchirawat, S.; Kittakoop, P. *Phytochemistry* **2009**, *70*, 407-413.

(234) Lang, G.; Cole, A. L. J.; Blunt, J. W.; Robinson, W. T.; Munro, H. G. *J. Nat. Prod.* **2007**, *70*, 310-311.

(235) Ngoupayo, J.; Tabopda, T. K.; Ali, M. S.; Tsamo, E. *Chem. Pharm. Bull.* **2008**, *56*, 1466-1469.

(236) Pongcharoen, W.; Rukachaisirikul, V.; Phongpaichit, S.; Rungindamai, N.; Sakayaroi, J. *J. Nat. Prod.* **2006**, *69*, 856-858.

(237) Schol-Schwarz, M. B. *Brit. Mycol. Soc.* **1965**, *48*, 51-53.

(238) Eldredge, L. G.; Evenhuis, N. L. *Bishop Museum Occasional Papers* **2003**, *76*, 1-30.

(239) Sugano, M.; Sato, A.; Iijima, Y.; Furuya, K.; Kuwano, H.; Hata, T. *J. Antibiot.* **1995**, *48*, 1188-1190.

(240) Goldring, W. P. D.; Pattenden, G. *Acc. Chem. Res.* **2006**, *39*, 354-361.

(241) Ishino, M.; Kiyomichi, N.; Takatori, K.; Sugita, T.; Shiro, M.; Kinoshita, K.; Takahashi, K.; Koyama, K. *Tetrahedron* **2010**, *66*, 2594-2597.

(242) Tokiwano, T.; Fukushi, E.; Endo, T.; Oikawa, H. *Chem. Commun.* **2004**, , 1324-1325.

(243) Sugano, M.; Sato, A.; Iijima, Y.; Furuya, K.; Haruyama, H.; Yoda, K.; Hata, T. *J. Org. Chem.* **1994**, *59*, 564-569.

(244) Sugano, M.; Sato, A.; Iijima, Y.; Oshima, T.; Furuya, K.; Kuwano, H.; Hata, T.; Hanzawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 5463-5464.

(245) Sugano, M.; Sato, A.; Saito, K.; Takaishi, S.; Matsushita, Y.; Iijima, Y. *J. Med. Chem.* **1996**, *39*, 5281-5284.

(246) Valverde, S.; Herradon, B.; Rabanal, R. M.; Martin-Lomas, M. *Can. J. Chem.* **1987**, *65*, 339-342.

(247) Argoudelis, A. D.; Zieserl, J. F. *Tetrahedron Lett.* **1966**, *18*, 1969-1973.

(248) Ramesh, S.; Franck, R. W. *Tetrahedron: Asymmetry* **1990**, *1*, 137-140.

(249) deAlvarenga, M.A.; Fo, R.B.; Gottlieb, O.R.; Dias, J.P.deP.; Magalhaes, A.F.; Magalhaes, E.G.; deMagalhaes, G.C.; Magalhaes, M.T.; Maia, J.G.S.; Marques, R.; Marasaioli, A.J.; Mesquita, A.A.L.; deMoraes, A.A.; deOliveira, A.B.; deOliveira, G.G.; Pedreira, G.; Pereira, S.K.; Pinho, S.L.V.; Sant'ana, A.E.G.; Santos, C.C. *Phytochemistry* **1978**, *17*, 511-516.

(250) Höller, U.; König, G. M.; Wright, A. D. *J. Nat. Prod.* **1999**, *62*, 114-118.

(251) Barrero, A. F.; Quílez Del Moral, J. F.; Cuerva, J. M.; Cabrera, E.; Jiménez-González, D. *Tetrahedron Lett.* **2000**, *41*, 5203-5206.

(252) Pettit, G. R.; Tan, R.; Herald, D. L.; Hamblin, J.; Pettit, R. K. *J. Nat. Prod.* **2003**, *66*, 276-278.

(253) Sato, M.; Kakisawa, H. *J. Chem. Soc. Perkin I* **1976**, 2407-2413.

(254) Ellestad, G. A.; Evans Jr., R. H.; Kunstmann, M. P. *Tetrahedron Lett.* **1971**, *12*, 497-500.

(255) Markus, J.; Krohn, K.; Flörke, U.; Aust, H.; Draeger, S.; Schulz, B. *J. Nat. Prod.* **1999**, *62*, 1218-1221.

(256) Ashenhurst, J. A.; Gleason, J. L. *Tetrahedron Lett.* **2008**, *49*, 504-507.

(257) Macías, F. A.; Simonet, A. M.; Pacheco, P. C.; Barrero, A. F.; Cabrera, E.; Jiménez-González, D. *J. Agric. Food Chem.* **2000**, *48*, 3003-3007.

(258) Sato, M.; Ruo, T.; Hayashi, T.; Kakisawa, H.; Miyaki, T.; Yamamoto, H.; Fujisawa, K. *Tetrahedron Lett.* **1974**, *15*, 2183-2186.

(259) Ellestad, G. A.; Evans Jr., R. H.; Kunstmann, M. P.; Lancaster, J. E.; Morton, G. O. *J. Am. Chem. Soc.* **1970**, *92*, 5483-5489.

(260) Kakisawa, H.; Sato, M.; Ruo, T.; Hayashi, T. *Chem. Commun.* **1973**, , 802-803.

(261) Galbraith, M. N.; Horn, D. H. S. *Chem. Commun.* **1970**, , 170-171.

(262) Macías, F. A.; Simonet, A. M.; Pacheco, P. C.; Barrero, A. F.; Cabrera, E.; Jiménez-González, D. *J. Agric. Food Chem.* **2000**, *48*, 3003-3007.

(263) Crous, P. W.; Slippers, B.; Wingfield, M. J.; Rheeder, J.; Marasas, W. F. O.; Phillips, A. J. L.; Alves, A.; Burgess, T.; Barber, P.; Groenewald, J. Z. *Studies in Mycology* **2006**, *55*, 235-253.

(264) Pavlic, D.; Slippers, B.; Coutinho, T. A.; Wingfield, M. J. *Mol. Phylogenet. Evol.* **2009**, *51*, 259-268.

(265) Martos, S.; Andolfi, A.; Luque, J.; Mugnai, L.; Surico, G.; Evidente, A. *Eur. J. Plant Pathol.* **2008**, *121*, 451-461.

(266) Lazzizera, C.; Frisullo, S.; Alves, A.; Phillips, A. J. L. *Plant Pathol.* **2008**, *57*, 948-956.

(267) Komai, S.; Hosoe, T.; Nozawa, K.; Okada, K.; Campos Takaki, G. M.; Fukushima, K.; Miyaji, M.; Horie, Y.; Kawai, K. *Mycotoxins* **2003**, *53*, 11-18.

(268) Cardellina II, J. H. *J. Nat. Prod.* **1983**, *46*, 196-199.

(269) Bax, A.; Subramanian, S. J. *J. Mag. Reson.* **1986**, *67*, 565-569.

(270) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093-2094.

(271) Sugawara, F.; Strobel, G.; Fisher, L. E.; Van Duyne, G. D.; Clardy, J. In *In Bipolaroxin, a selective phytotoxin produced by Bipolaris cynodontis;* 82; , pp 8291-8294.

(272) Godoy, G.; Steadman, J. R.; Dickman, M. B.; Dam, R. *Physiol. Mol. Plant Pathol.* **1990**, *37*, 179-191.

(273) Papavizas, G. C.; Davey, C. B. *Soil Sci.* **1959**, *88*, 112-117.